

1 **Electrochemical biosensor based on NAD(P)H-dependent Quinone Reductase**
2 **for rapid and efficient detection of vitamin K3**

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21 **Abstract**

22 Vitamin K refers to a group of vitamins that play an important role in blood coagulation and
23 regulation of bone and vascular metabolism. However, vitamin K3 may give severe side effects
24 in animal and humans when improperly added to food and feed due to its toxicity. Here, an
25 electrochemical biosensor, based on the YaiB NADPH-dependent quinone reductase from
26 *Lactococcus lactis* (YaiB), was developed to achieve rapid and redox probe-free detection of
27 vitamin K3. First, we demonstrated the ability of the carbon electrode to distinguish between 1,4-
28 benzoquinone and hydroquinone. Then, we engineered YaiB to work as a bioreceptor
29 immobilized at the electrode and we demonstrated its sensitivity and specificity to reduce
30 vitamin K3. Finally, to demonstrate the practical potential of the biosensor, we tested it directly
31 in spiked milk samples, achieving 15-minute quantification of the vitamin K3. The limit of
32 detection was 0.18 μ M and 0.86 μ M in buffer and milk, respectively.

33 **Key words:** Enzymatic sensor; Carbon Screen Printed Electrode; Voltammetry; Food quality;
34 Vitamin detection.

35

36 **1. Introduction**

37 Vitamin K, or naphthoquinone, is a family of fat-soluble vitamins that are essential nutrients
38 found in many foods. They are structurally related organic compounds containing the 2-methyl-
39 1,4-naphthoquinone group substituted with various hydrocarbon side chains at the C3 position.
40 Vitamin K is crucial in blood coagulation, bones calcification, brain and kidney function and is
41 involved in maintaining human health through its large spectrum of anticancer, antibacterial and
42 antiviral activities [1-4]. It is also involved in cellular respiration, photosynthetic mechanisms,
43 and oxidative phosphorylation [5]. In some cases, it is prescribed to elderly people as an anti-age,
44 anti-inflammatory, anti-oxidant factor and a promoter of cognition [1]. There are two main
45 forms of natural vitamin K, K₁ (phylloquinone) and K₂ (menaquinone), while vitamin K₃
46 (menadione) constitutes vitamin K₁ and K₂ catabolic product showing no hydrophobic tail (Fig.
47 1a). Human do not produce vitamin K. Green leafy vegetables are primarily source of vitamin
48 K₁, and vitamin K₂ is particularly found in eggs, meat, fermented food, such as cheese, and
49 additionally is synthesized by certain Gram-positive bacteria and archaea in microbiota of the
50 human intestine and digestive tract. Bacteria can produce different forms of vitamin K₂ (MK-4,
51 MK-7, MK-10). Vitamin K₃, being a non-active pro-vitamin, is a clotting drug and dietary
52 supplement, and is a common additive in animal feed. Vitamin K deficiency is rare in adult since
53 it is available from different sources. Moreover, there are still no reference ranges for vitamin K
54 levels in healthy people, in part because detection is difficult in low concentrations [1].
55 Nevertheless, newborns may suffer from vitamin K deficiency because they have low vitamin K
56 reserves in liver, and their immature intestinal flora cannot sufficiently synthesize it, especially
57 when newborns are exposed to antibiotics. Vitamin K deficiency may lead to bleeding in infancy
58 [6]. Still, vitamin K₃ in milk can cause allergic reactions, digestive problems, and even liver and
59 kidney damage [7]. Therefore, a sensitive method for detection and control of vitamin K in
60 foods, and particularly in milk, is needed.

61 The analytical methods of choice for vitamin K determination in foods is high-
62 performance liquid chromatography (HPLC) connected to spectrophotometric detection [8, 9].
63 HPLC might also be coupled with electrochemical [10, 11], or mass spectrometric detectors [12,
64 13]. These protocols need time-consuming sample preparation, are complex, of high cost, and

65 require large sample volume to be performed. Alternative methods have been proposed such as
66 flow injection [14], or using colorimetric [15, 16], spectrofluorimetric [17] and electrochemical
67 measurements [18-25]. Electrochemical detection provides the possibility of performing label-
68 free detection because vitamin K, like other quinones, (*i.e.*, oxidized derivatives of aromatic
69 compounds), is a redox-active molecule [5]. Quinones can be electrochemically reduced at
70 metal- or carbon-based electrodes by voltammetric methods at negative potentials [18, 22, 24-
71 26]. Reduction at electrodes is well-documented and involves transfer of two electrons and two
72 protons to generate hydroquinone [20]. Reported electrochemical sensors are mainly based on
73 nonspecific adsorption of quinones onto the electrode surface. However, these nonspecific
74 interactions can be affected by matrix compounds leading to low detection limit and poor
75 selectivity of the sensor when applied in food samples. Moreover, methods operating in a
76 cathodic mode require removing air oxygen dissolved in the sample to enable measurements.
77 Moreover, electrodes require polishing and cleaning steps to enable reproducible vitamin K
78 adsorption.

79 Electrochemical biosensors for detection of biomolecules have emerged as advantageous
80 analytical devices because they provide quantitative output, high sensitivity, low cost, and can be
81 easily scaled down to handheld format [27-29]. Since the first utilization of the glucose oxidase
82 enzyme as a sensitive component in a device that was able to convert a clinical concentration of
83 glucose into a digital signal, enzymatic electrochemical biosensors have undergone rapid
84 development [30-32]. Due to the natural specificity of an enzyme, enzymatic biosensors offer
85 excellent selectivity for their targets in practical applications ranging from medical diagnostics,
86 food safety, pharmaceutical production, fermentation, agricultural and environmental monitoring
87 [27, 33-36]. In addition, electrochemical biosensors are less perturbed by matrix effects than the
88 optical biosensors. To date, many enzymatic electrochemical biosensors have been proposed for
89 detection of water-soluble metabolites or biomarkers. However, they are still little explored as
90 analytical tools for quantification of fat-soluble molecules [37, 38].

91 Here we report, for the first time, the development of an enzymatic electrochemical
92 biosensor for vitamin K detection. The disposable carbon screen-printed electrode (C-SPE) was
93 functionalized with the quinone reductase enzyme from *Lactococcus lactis*, YaiB, which
94 specifically reduces vitamin K in the presence of NAD(P)H as electron acceptor and riboflavin

95 as electron donor (Fig. 1b). The product of the enzymatic reaction, hydroquinone, was directly
96 detected on the C-SPE operating in an anodic mode. The assay offers advantages of small sample
97 volume, good biocompatibility, affordability and rapidity, together with high sensitivity and
98 selectivity. Furthermore, different molecules of the vitamin K family were tested and the
99 efficiency of their detection correlated with the enzyme substrate specificity. Finally, the
100 biosensor was successfully applied for quantitative detection of vitamin K₃ in milk in a protocol
101 without requiring extraction or separation steps to eliminate matrix effects. The sensor may be
102 used as an alternative approach for routine analysis of food quality control for vitamin K₃.

103 **2. Experimental section**

104 **2.1. Reagents**

105 Riboflavin, NADPH tetrasodium salt, 1,4-benzoquinone, hydroquinone, vitamin K₃, MK-4, MK-
106 7, vitamin C, EDTA, β -mercaptoethanol, sodium dodecyl sulfate (SDS), glycerol, bromophenol
107 blue, Triton-X100, DL-Dithiothreitol (DTT), Isopropyl- β -D-thiogalactoside (IPTG), Tween 20,
108 glucose, dopamine, mesotrione and glutathione reduced were purchased from Sigma Aldrich
109 (France). Phosphate buffer saline was purchased from VWR Prolabo (France), Complete
110 protease inhibitor cocktail (Roche, Sigma, France), Glutathione-Sepharose 4B beads (GE
111 Healthcare, France), and mouse monoclonal anti-Glutathione-S-Transferase (anti-GST) antibody
112 and goat anti-mouse HRP-secondary antibody (Santa Cruz Biotechnology, France), were used
113 according to suppliers' instructions. Stock solutions of 1,4-benzoquinone and vitamins (2 mM)
114 were prepared in ethanol, and further dilutions starting from 10 μ M in water were made
115 extemporaneously. Phosphate buffer PBS (pH= 7.47) was purchased from VWR (Prolabo,
116 France).

117 **2.2. Plasmid construct**

118 DNA comprising sequence of the gene encoding *Lactococcus lactis* 1403 quinone reductase
119 (YaiB) enzyme was PCR-amplified and cloned in the pGEX.6P1 plasmid, at BamHI and XhoI
120 sites. The oligonucleotides used were: yaiB1403For 5'-
121 CGTGGATCCATGAAATCATATCAAGCAAATGAGC-3' (BamHI) and yaiB1403Rev 5'-
122 GATCCTCGAGTTATCTAGGTCGTAAAAGGCTG-3' (XhoI). Chromosome DNA from
123 *Lactococcus lactis* strain 1403 was used as a matrix. The PCR product was digested by BamHI
124 and XhoI enzymes and cloned into plasmid pGEX6P1 (Amersham, France) that was digested at

125 the same restriction sites. The final plasmid pGEX6P1-GST-YaiB bearing intact *yaiB* gene, was
126 confirmed by DNA sequencing and transformed into the *E. coli* strain BL21 for overproduction
127 of the GST-YaiB chimeric protein.

128 **2.3. Expression and purification of the recombinant quinone reductase**

129 *E. coli* BL21 (DE3) (Novagen, SigmaAldrich, France) transformed with pGEX-6P1 plasmids
130 (GST-YaiB or GST) was grown in 100 ml of Luria-Bertani (LB) medium containing
131 100 µg/ml ampicillin at 37°C overnight. The bacterial suspension was then diluted in 400 mL
132 LB supplemented with 2 mM riboflavin, to OD₆₀₀=0.01 (optical density at 600 nm) and allowed
133 to reach OD₆₀₀ 0.6 - 1. Protein expression was induced by adding 80 µg/ml IPTG to the
134 medium. After 15 h incubation at 37°C, bacteria were harvested by centrifugation. For
135 purification, the bacterial pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.8,
136 1 mM EDTA, 60 mM NaCl, 2 mM DTT, 0.2% Triton X-100) supplemented with 1 mg/ml
137 lysozyme and complete protease inhibitor cocktail. After 1 h incubation on ice, the suspension
138 was sonicated to break bacterial cells, and centrifuged at 10,000 × g, at 4°C for 30 min.
139 Glutathione-Sepharose beads were added to clarified supernatants and incubated at 4°C for 3 h.
140 Beads were then washed three times with lysis buffer and twice in elution buffer (50 mM Tris,
141 pH 8). Beads carrying bound proteins via their GST tag were incubated with 6.8 mg/ml
142 glutathione reduced in the elution buffer at 4°C, overnight. Eluted recombinant protein was
143 dialyzed against 20 mM Tris, pH 7.5. Protein concentration was measured using a Bradford
144 assay (Sigma France, Lezennes, France). For SDS-PAGE analysis of purified proteins, samples
145 were prepared in Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 5% β-mercaptoethanol, 2% SDS,
146 20% glycerol, 0.01% bromophenol blue), denatured at 95°C for 5 min, separated on a 12.5%
147 polyacrylamide gel, and stained with InstantBlue Coomassie protein stain (Abcam, France).
148 Alternatively, gels were electroblotted onto a nitrocellulose membrane for 1 h at 20 V.
149 Membranes were blocked in 10 mL of iBind flex solution (Life Technologies, France) for
150 30 min at room temperature. Primary anti-GST antibody (1:5,000 dilution) and peroxidase-
151 conjugated secondary antibody (1:10,000 dilution) were diluted in iBind flex solution and
152 incubated on membranes using the iBind™ Flex Western System (Thermo Fisher Scientific,
153 France) overnight at room temperature. Blots were rinsed in distilled water and antibody binding
154 was detected with chemiluminescent solution (Pierce, Fisher Scientific, France) and visualized

155 under a ChemiDoc MP imaging system (Biorad, France). PreScission protease (Invitrogen,
156 France) in a PreScission cleavage buffer was used to cleave the protein fusion and liberate YaiB.
157 The buffer containing YaiB was then dialyzed against PBS and concentrated using the Amicon
158 Ultra Centrifugal filters 10K (SigmaAldrich, France) to 2 mg/mL. Glycerol was added (5%–10%
159 v/v), and the protein preparation was aliquoted, flash-frozen in liquid nitrogen, and stored at - 20
160 °C.

161 **2.4. Quinone reductase modeling**

162 Models of quinone reductase protein YaiB from *Lactococcus lactis* were obtained with the tool
163 “Sequence Similarity Searching” on the FASTA website (
164 [https://www.ebi.ac.uk/Tools/services/web/toolresult.ebi?jobId=fasta-I20230308-121114-0892-](https://www.ebi.ac.uk/Tools/services/web/toolresult.ebi?jobId=fasta-I20230308-121114-0892-10169449-p1m)
165 [10169449-p1m](https://www.ebi.ac.uk/Tools/services/web/toolresult.ebi?jobId=fasta-I20230308-121114-0892-10169449-p1m)) based on Alpha-fold [39, 40]. The protein sequence is:

166 MKSYQANELDEKTVYKLLSGSIVPRPIAWVTSQNLEGLVNVAPFSFFNVASSNPPLLSISF
167 TGNKDSLNNLLTTKEAVVHLVNEDNVELMNQTAAPLAEHISEAEFFSLELVPSQKVQVP
168 SLKESNVRLETKLYHHLPLGESGHLVLEVVNFSFAEELLDEENFHVNLNKLKPVGRLA
169 GDDYSTLGNRFSLRPR. One of the 8 models obtained was further minimized and refined
170 using Discovery Studio version 2021 (Biovia, Dassault, France). The 3D coordinates of the
171 ligands were obtained at the PubChem website. Docking of various ligands, including
172 benzoquinone, dopamine, vitamin C, vitamin K₃, vitamine K₂ (MK-4, MK-7), glucose and
173 NADPH was performed using Libdock (blind docking using the whole protein) and then
174 CDOCKER at different cavities detected by the Receptor cavities finder tool. The ligands were
175 minimized *in situ* using the standard protocol.

176 **2.5. Spectrophotometric measurements**

177 The enzymatic activity of the purified quinone reductase was assessed using the UV/Vis
178 Spectrophotometer Libra S22 (Biochrom, France). The reaction mixture contained 10 µg/mL
179 YaiB in 20 mM Tris –HCl buffer, pH 7.5 in total volume of 500 µL. The mixture was preheated
180 at 37°C for 15 min, and the reaction was started by the addition of 0.2 mM NADPH, 0.02 mM
181 vitamin K₃, and 5 µM riboflavin, if not specified otherwise. Decrease in absorbance at 340 nm
182 due to NADPH oxidation was followed as described [16].

183 **2.6. Electrochemical measurements**

184 Cyclic voltammetry (CV) measurements and differential pulse voltammetry (DPP)
185 measurements were performed using a PalmSense4 electrochemical analyzer (PalmSens,
186 Netherlands) controlled by software PSTrace 5.9. Electrochemical experiments were done in
187 conventional three electrode glass cells (total volume of 5 ml) with a carbon paste electrode
188 (CPE) as working electrode, Ag/AgCl (3 M KCl) as reference electrode, and Pt wire as counter
189 electrode. The biosensors was developed using a commercial carbon screen-printed electrode (C-
190 SPE, model DRP-110) from DropSens (Metrohm, France) which contains carbon graphite as
191 working (4 mm diameter) and counter electrode, and a silver composite printed as a pseudo-
192 reference electrode over the same ceramic substrate. The working electrode was functionalized
193 by quinone reductase using drop-casting method. For this, 5 µl of the enzyme (0.5 mg/mL) was
194 deposited on the working electrode of the C-SPE for 10 min, and the resultant enzyme electrode
195 was placed in the refrigerator to dry at 4 °C overnight. Detection experiments were performed
196 in a PBS containing 0.2 mM NADPH and 5 µM riboflavin at a laboratory temperature of
197 25 ± 1 °C (Fig. 1b). PBS was used as a supporting electrolyte. CV was performed using 0.05
198 V/s scanning rate and 0.005 V step potential, in a potential range of – 1 V to + 1 V. DPV was
199 performed using 0.02 V/s scanning rate, 0.01 V step potential, 0.2 pulse amplitude, 0.02 s pulse
200 time, in a potential range from 0.5 V to -1 V.

201 **2.7. Scanning electron microscopy**

202 Surfaces of the working electrode of C-SPE were observed before and after YaiB immobilization
203 with electrodes mounted on aluminum stubs (32mm diameter) with carbon adhesive discs (EMS,
204 LFG-Distribution, Sainte Consorce, France). The surface was visualized by field emission gun
205 scanning electron microscopy (FEG SEM) as secondary electrons images (2 keV, spot size 30)
206 under high vacuum conditions with a Hitachi SU5000 instrument (Milexia, Saint-Aubin, France).
207 SEM analyses were performed at the Microscopy and Imaging Core Facility MIMA2 (INRAE,
208 Jouy-en-Josas, France) DOI : MIMA2, INRAE, 2018. Microscopy and Imaging Facility for
209 Microbes, Animals and Foods, <https://doi.org/10.15454/1.5572348210007727E12>.

210 **2.8. Size Measurements by Dynamic Light Scattering (DLS)**

211 YaiB size measurements were performed at 25 °C using the Zetasizer Pro (Malvern, France),
212 based on the principle of dynamic light scattering. Results were presented as size distribution
213 calculated from Malvern software ZS Xplorer. DLS measurements were carried out with 0.7
214 mg/mL YaiB and 10 µM vitamin K₃, in PBS. Each measurement was performed with three
215 successive runs, each involving 15 scans on the average.

216 **2.9. Milk application**

217 Powdered milk (Auchan, France) containing 36% of protein, 52% of lactose and 0.8% of fat
218 (w/w) was dissolved in PBS as a 1% solution and spiked with varying concentrations of vitamin
219 K₃, MK-4 or MK-7.

220

221 **3. Results and Discussion**

222 **3.1. Production and characterization of quinone reductase**

223 Quinone reductase, YaiB, is a flavoprotein that catalyzes the NAD(P)H-dependent two-electron
224 reduction of quinones to quinols (Schema S1). To maintain the apparent stoichiometry of one
225 flavin per YaiB and to co-purify quinone reductase with flavin, the induction of transformed *E.*
226 *coli* was performed in medium supplemented with riboflavin [16]. YaiB fused to glutathione S-
227 transferase (GST) was purified to > 95 % homogeneity using glutathione agarose affinity
228 chromatography. The purified enzyme was of yellow color, which is consistent with its affinity
229 for binding the flavin cofactor [41]. The absorption peak in the visible light range (412 nm)
230 confirmed co-purification of GST-YaiB with flavin (Fig. 2a). However, the low peak intensity
231 suggests that YaiB molecules were not saturated with this co-factor. When the purified complex
232 was analyzed by SDS-PAGE stained with Coomassie blue it was of expected molecular weight
233 of ~ 50 kDa (Fig. 2b). Homogeneity of the purified GST-YaiB was validated by Western blotting
234 (Fig. 2c). When GST was expressed under the same condition, Western blot suggested a high
235 propensity of GST to aggregate (Fig. 2c).

236 YaiB was then separated from GST by PreScission cleavage and characterized under
237 native conditions. For this, the enzyme solubilized in PBS was analyzed by DLS to determinate
238 its mean hydrodynamic radius (R_H). Fig. 2d shows that YaiB in PBS was strongly aggregated
239 with an R_H of several hundred nanometers. The sizes obtained from DLS measurements are

240 usually higher than real because protein particles in solution are solvated and dynamic. However,
241 an R_H ranging from 1 nm to 10 nm is expected for a spherical protein of ~ 20 kDa (molecular
242 weight of YaiB is ~ 21.8 kDa). Interestingly, the protein displayed different radii when the
243 enzyme was incubated with substrate and co-factors (Fig. 1d). DLS indicated that purified YaiB
244 was not monomeric but reorganized in the presence of the substrate vitamin K_3 .

245 The *L. lactis* YaiB enzyme was shown to reduce a variety of quinone derivatives *in vitro*
246 [42], but mainly highly toxic 1,4-benzoquinone *in vivo* [16]. To functionality characterize YaiB,
247 its enzymatic activity was followed using vitamin K_3 as a substrate and by spectrophotometric
248 measurements of the absorbance of NADPH at 340 nm. Upon reduction of vitamin K_3 , the
249 decrease in absorbance at 340 nm characterized NADPH oxidation to NADH. Although this
250 assay was previously employed for different NADPH-quinone reductases [41, 43], we observed
251 that it was nonspecific since decrease of NADPH absorbance occurred even in the absence of
252 vitamin K_3 (Fig. S2). We thus verified whether quinone reductase activity could be directly
253 evaluated by consumption of quinone or production of hydroquinone using cyclic voltammetry.

254 **3.2 Voltametric detection of 1,4-benzoquinone / hydroquinone**

255 First, electrochemical redox reactions of 1,4-benzoquinone and hydroquinone were investigated
256 because both forms are stable molecules. Benzoquinone was readily reduced at the CPE in a
257 potential range between -0.3 V and -0.7 V (vs. Ag/AgCl), with a current maximum at around -
258 0.55 V, producing a signal that overlapped with the background signal of PBS (Fig. 3a). Oxidase
259 hydroquinone produced a peak with a peak potential of + 0.08 V (vs. Ag/AgCl); in the reversal
260 sweep, the associated reduction peak appeared at about + 0.03 V (vs. Ag/AgCl) (Fig. 3b).

261 We next investigated whether CV can detect the product associated with the enzymatic
262 reduction of 1,4-benzoquinone or vitamin K_3 . The fast scan rate produced the PBS background
263 charging current for control solutions prepared without YaiB, NADPH, riboflavin, or the
264 substrates (Fig. 3c). The enzymatic reaction was then allowed to proceed in a PBS solution
265 containing 1,4-benzoquinone, NADPH as electron donor and riboflavin as co-factor, at 37°C for
266 10 min. The CV curve of the resulting solution showed one oxidation peak (Peak 1) near the
267 switching potential (Fig. 3c). Peak1 appeared irreversible with no detectable reduction peak.
268 Interestingly, although YaiB was co-purified with riboflavin, Peak1 was observed only with
269 riboflavin added to the solution. This may suggest that the co-factor is not tightly bound to YaiB

270 and/or that NADPH competed with riboflavin or 1,4-benzoquinone for the same binding site.
271 When the enzymatic reaction was performed using vitamin K₃ as substrate, a second peak
272 appeared corresponding to the oxidation at ~ -0.25 V (vs. Ag/AgCl, Peak2), as shown in Fig. 3d.
273 The reduction peak, Peak3, at the position similar to that of Peak2 was observable at -0.27 V (vs.
274 Ag/AgCl). Clearly, a pair of well-defined redox peaks, Peak2/Peak3, can be attributed to
275 menadiol produced by the enzymatic reduction of vitamin K₃. The intensity of the Peak 3 was
276 further used to quantify quinones in solutions.

277 **3.3 Detection of K₃**

278 The enzymatic biosensor was constructed using a disposable C-SPE, rather than the glassy
279 carbon electrode. This choice avoids polishing and cleaning of the working electrode, and
280 decreases sample volumes (Fig. 1b). YaiB was immobilized on the C-SPE working area by a
281 single drop-casting round. To assess the efficiency of electrode functionalization, the
282 morphology of its surface was characterized by SEM. The SEM micrograph in Fig. 4a shows
283 that the working area of the bare C-SPE was covered by randomly oriented micrometric carbon
284 flakes. Such a surface is characteristic for printed carbon electrode. SEM images of the
285 functionalized working electrode surface confirmed YaiB immobilization. The immobilized
286 enzyme is clearly differentiated as bright aggregated particles (Fig. 4b). The sizes of aggregates
287 varied from less than 100 nm to several μm . In comparison, the dimension of spheroidic
288 monomeric YaiB is expected to be less than 10 nm.

289 The differential pulse voltammograms of various concentrations of vitamin K₃ were
290 recorded in PBS containing NADPH and riboflavin (Fig. 4c). The peak at ~ -0.2 V (vs.
291 Ag/AgCl) corresponding to menadione reduction to menadiol increased linearly with increasing
292 vitamin K₃ concentrations ranging from 0.36 $\mu\text{g/mL}$ to 9 $\mu\text{g/mL}$. Calibration curves were
293 obtained by quantifying current intensity changes (ΔI) for each vitamin K₃ concentration, taken
294 from at least three independent electrodes per concentration (Fig. 4d). Sensor reproducibility was
295 estimated at 3 % according to the signal obtained upon addition of the same concentration of
296 vitamin K₃. ΔI plotted against the target concentrations was linear with a correlation coefficient
297 (R^2) of 99%. The regression equation obtained of the plot was $y(x) = 0.00572x + 0.00345$,
298 where x is the vitamin K₃ concentration (in $\mu\text{g/mL}$) and y is ΔI (in μA). The experimental limit
299 of detection (LoD) was 0.36 $\mu\text{g/mL}$ while the calculated LoD was 0.15 $\mu\text{g/mL}$ (calculated using

300 3 σ /m formula, where ‘ σ ’ is a standard deviation of the blank solution and ‘m’ is a slope of the
301 calibration curve). Taking into account the molecular weight of vitamin K₃ of 833, the calculated
302 LoD was 0.18 μ M. Comparison of obtained analytical parameters with those reported in the
303 literature (Table S1), indicates that the electrochemical biosensors based on YaiB enzyme allows
304 measuring vitamin K₃ concentration with a high sensitivity similar to previously published
305 electrochemical sensors. Significant added values of the present method come from replacing
306 nanoparticles and toxic materials, used in some previous works, with a natural enzyme enabled
307 to obtain environmentally friendly analytical tool, a shorter the time of electrode preparation, and
308 a lower cost of analysis.

309 **3.4. Selectivity, stability and interference study**

310 To examine the selectivity of the YaiB-C-SPE electrode, we challenged it with various organic
311 molecules, i.e., glucose, dopamine, vitamin C, mesotrione. No peak at -0.27 V (vs. Ag/AgCl)
312 was observed (Fig. 5a). Surprisingly, no oxidation peak was obtained with MK-4 or MK-7. This
313 may result from poor solubility of the vitamin K₂ molecules or steric hindrance that prevents
314 their access to the active site in YaiB. Interference of glucose (12 μ g/mL) was studied with 3.6
315 Mg/mL vitamin K₃. The response of vitamin K₃ was equivalent without and with glucose (Fig.
316 5a). The stability of the sensor was also tested by placing batches of functionalized electrode at
317 4°C. The intensity of vitamin K₃ reduction signal was stable for at least 30 days.

318 **3.5. Detection of other quinones and analytical applications**

319 Our enzymatic sensor provides specific and sensitive detection of vitamin K₃, indicating its
320 potential use for food screening. As signal generation is based on enzymatic production of the
321 reduced form of the molecule, menadiol, the sensors is expected to be specific when used
322 directly in complex matrices. We tested our sensor directly in milk as a model of food. In many
323 countries low-dose vitamin K₃ is used as an inexpensive nutritional supplement for livestock
324 [44]. When reconstructed milk was spiked with different concentrations of vitamin K₃, we
325 observe that signals were 4-fold amplified at -0.33 V (vs. Ag/AgCl) compared to those observed
326 in PBS (Fig. 5b). This may result from better solubility of vitamin K₃ in milk than in the buffer
327 solution taking into account its hydrophobic nature. Although in milk, the responses were higher
328 in intensity, the dynamic range was lower than in PBS, as saturation was obtained for
329 concentrations > 6 μ g/mL (Fig. 5c). The sensor was still able to detect the presence of the

330 vitamin K₃ in a sensitive way with the LoD of 0.72 µg/mL (0.86 µM), as calculated from 3σ/m
331 formula.

332 *In vivo*, the efficiency of YaiB to reduce quinone was shown only for 1,4-benzoquinone and
333 not to derivatives having an hydrophobic tail [16]. To verify whether our enzymatic biosensor
334 can detect quinone derivatives with side chains, we challenged it with MK-4 or MK-7 dissolved
335 in milk. No DPV signals was observed suggesting that quinones with side chains do not fit in the
336 active site of YaiB. This finding corroborates with the substrate specificity of YaiB observed *in*
337 *vivo* [16].

338 **3.6. Mechanism of action**

339 To investigate whether the differential recognition of vitamin K₃ by the YaiB quinone reductase
340 could have a structural basis, the structure of the YaiB monomer predicted by AlphaFold [39,
341 40] was minimized and further refined (Fig. 6a). The structure differed from those of other
342 flavin reductases reviewed recently [45], but presented some sequence homology as shown in
343 Fig. 6b-c. We identified in the protein 4 cavities able to bind ligands, the main ones are shown in
344 Figure 6d. Several ligands, including large ligands illustrated by vitamin K₂ (light blue in Figs.
345 6a, d, e) can be docked in a one site through multiple hydrophobic and stacking interactions of
346 the quinone ring with the protein. Specifically, stacking interactions were predicted between the
347 vitamin K₂ aromatic ring and YaiB residues P43, F44, and F46. Additionally a hydrogen bond
348 was predicted between one oxygen of the quinone and the OH moiety of S45, and another
349 quinone oxygen that interacts with K65. The latter binding site of vitamin K₂ was found in close
350 proximity with the NADPH binding site (depicted in yellow in Fig. 6a). Interestingly, vitamin K₃
351 was the only ligand bound at a distal site (Fig. 6a, d, f, shown in green). It was recognized *via*
352 multiple hydrophobic interactions of its aromatic ring with P24, P26, P174 while forming an H
353 bond between the quinone oxygen and the NH₂ side chain of I27. We suggest that the binding
354 site of vitamin K₂ could correspond to the binding site of the riboflavin) based on two criteria:
355 (1) the size of the isoalloxazine aromatic moiety of the flavin and vitamin K₂ are similar, (2)
356 NADPH and the vitamin K₂ quinone ring are in close proximity (~ 6 Å). Indeed, it is required for
357 electron transfer to take place between NADPH, the electron donor and the flavin, the electron
358 acceptor such a close proximity. The modeling suggests a possible competition of the ligands
359 with the flavin. Vitamin K₃ has a unique distal binding site in our model, which is in agreement

360 with experimental evidence. The model is designed for a YaiB monomer, and it is not yet
361 established whether the protein may adopt additional oligomeric forms, as some quinone
362 reductases can form dimers or tetramers [45]. The model, within this uncertainty of the actual
363 size of the protein, nevertheless predicts that vitamin K₃ is the only ligand to bind to the distal
364 site (right site in Fig. 6d) whereas other ligands, and likely the riboflavin bind to another site,
365 close to NADPH (left site in Fig. 6d).

366 **4. Conclusion**

367 In summary, we demonstrated that the NAD(P)H-dependent quinone reductase, YaiB, may be
368 used as a sensing element in the electrochemical biosensor for detection of vitamin K₃ in milk.
369 The overall protocol and the voltammetric method are simple; do not require expensive
370 equipment or toxic reagents. It uses small sample volumes (up to 40µL), and economizes on time
371 for sample preparation. Furthermore, the presented assay is performed in 15 min and the enzyme
372 retains its full activity for at least 1 month of storage. These characteristics place the present
373 biosensor among the most attractive for further improvements and optimization towards
374 commercialization. Electrochemical biosensors that detect fat-soluble molecules are rare in the
375 literature, especially those exploring enzymes as the sensing element. Despite the need for an
376 electron donor and acceptor, the biosensor is redox-free and label-free as the product of the
377 enzymatic reaction is an electroactive molecule that can be directly measured. Finally, the
378 propose method shows high potential as an alternative tool to expensive and time-consuming
379 high-performance liquid chromatography techniques presently in use for quantification of
380 vitamin K₃ in food samples.

381

382 **CRedit authorship contribution statement**

383 **Majd Khalife:** Formal analysis, Investigation. **Dalibor Stankovic:** Supervision,
384 Conceptualization, Writing - review & editing. **Vesna Stankovic:** Methodology, Writing -
385 review & editing. **Julia Danicka:** Formal analysis, Investigation. **Francesco Rizzotto:** Formal
386 analysis, Investigation. **Anny Slama-Schwok:** Methodology, Investigation, Writing - original
387 draft, Writing - review & editing. **Vlad Costache:** Investigation, Formal analysis. **Philippe**
388 **Gaudu:** Supervision, Conceptualization, Writing - review & editing. **Jasmina Vidic:**

389 Supervision, Conceptualization, Methodology, Writing - original draft, Writing - review &
390 editing.

391

392 **Declaration of competing interest**

393 The authors declare that they have no known competing financial interests or personal
394 relationships that could have appeared to influence the work reported in this paper.

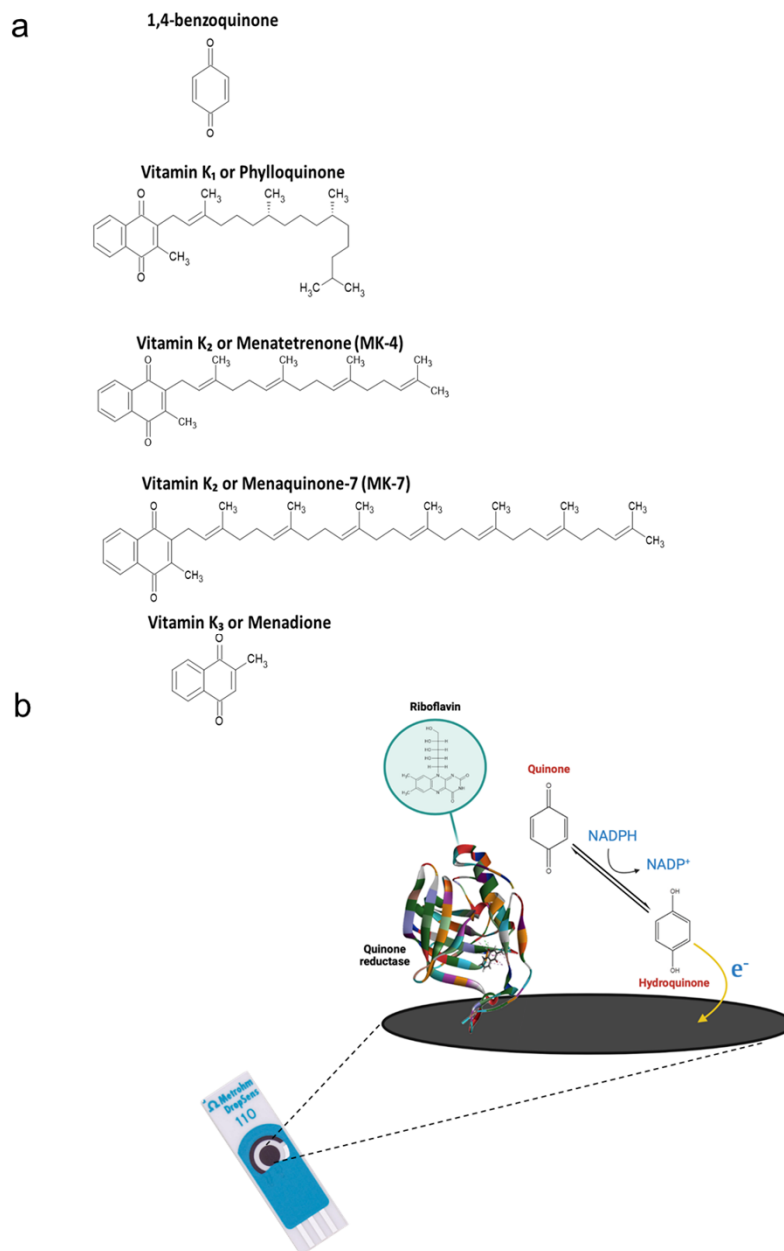
395

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409

410 **Figures:**



411

412 **Figure 1.** List of analytes and biosensor principle. **(a)** Chemical structure of 1,4-benzoquinone,

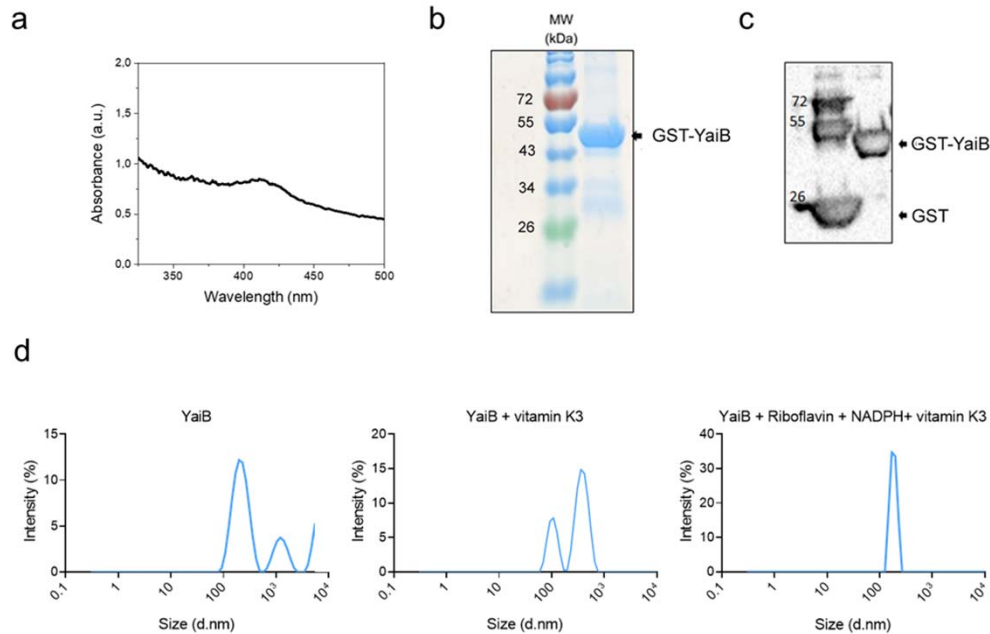
413 vitamin K₁, K₂, and K₃. **(b)** Scheme of the C-SPE modification and enzymatic sensor

414 construction. The working electrode of C-SPE was modified by drop casting with YaiB. The

415 detection was performed in solution containing NADPH as an electron donor, and riboflavin.

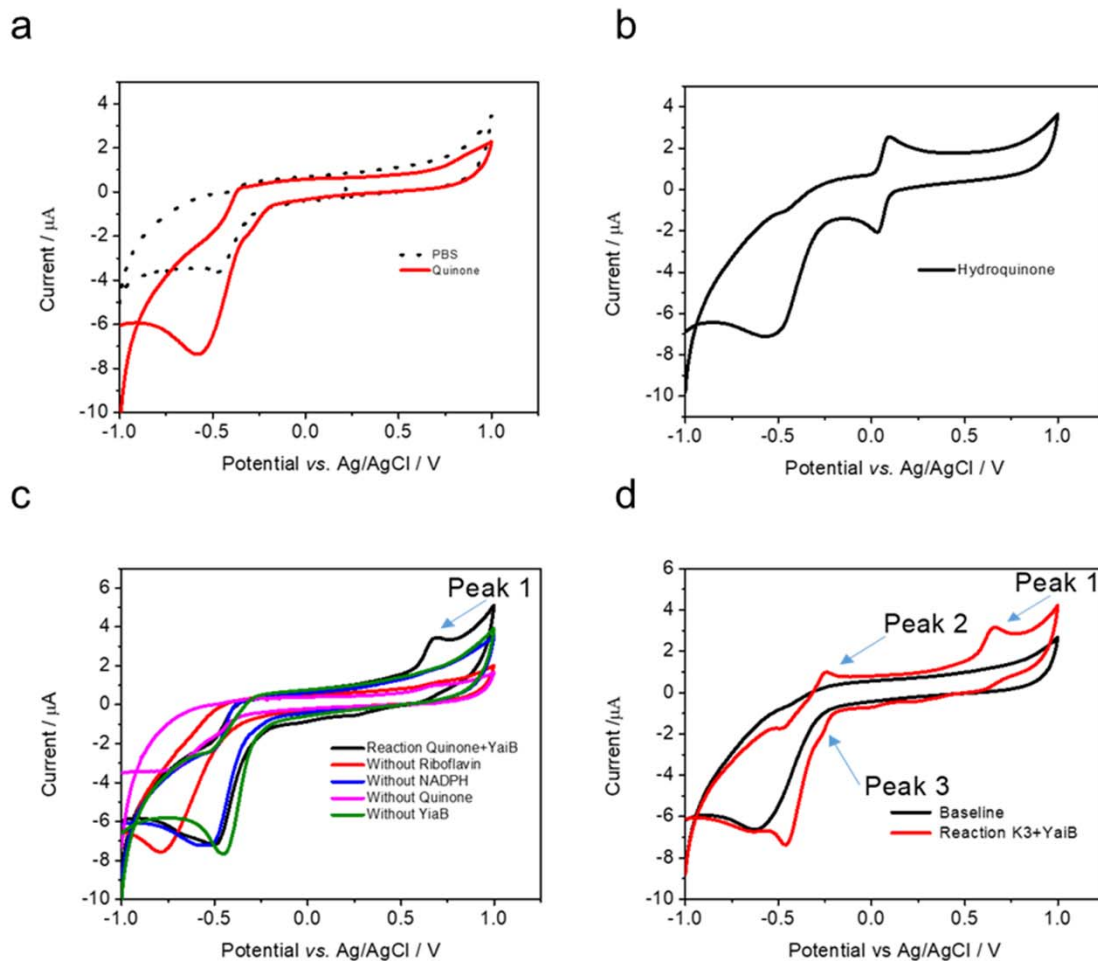
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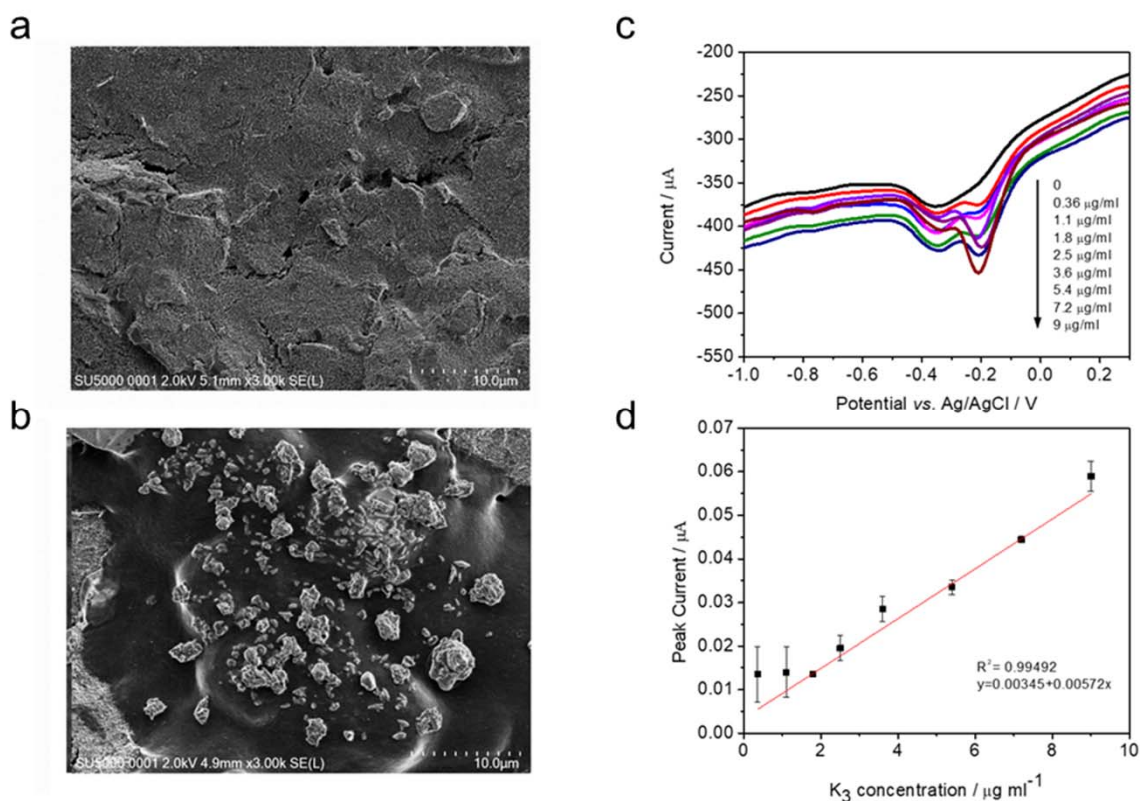
419 **Figure 2.** Purification and characterization of recombinant YaiB protein. (a) Absorption
420 spectrum of purified GST-YaiB protein (300-500 nm) indicates the presence of riboflavin. (b)
421 The protein fraction obtained by purification the GST tag was analyzed by SDS-PAGE and
422 InstantBleu Coomassie staining. Molecular masses (MW) corresponding to the ladder's bands
423 are indicated. (c), Western blot analysis of the purified GST-YaiB performed with an anti-GST
424 antibody. (d) Dynamic light scattering (DLS) analysis of the YaiB showing that in the absence of
425 vitamin K3, the average of three measurements showed polydisperse particles in solution with
426 high R_H indicating enzyme aggregation. In the presence of vitamin K3, the R_H shifted towards
427 lower values, showing poorer diffusion of the protein particles following substrate binding. Upon
428 addition of NADPH and riboflavin, the distribution turned to single peak indicating
429 monodisperse particles.



430

431 **Figure 3.** Cyclic voltammograms for 1,4-benzoquinone, hydroquinone and menadiol. (a) CV for
432 20 μM of 1,4-benzoquinone at unmodified glassy carbon electrode. (b) CV for 20 μM of
433 hydroquinone at unmodified glassy carbon electrode. (c) CV for enzymatic reduction of 1,4-
434 benzoquinone to hydroquinone at unmodified glassy carbon electrode using solution containing
435 YaiB, 1,4-benzoquinone, the electron donor NADPH and and electron acceptor Riboflavin
436 (black line). Control experiments were obtained in solution without riboflavin (red line), without
437 NADPH (blue line), without YaiB (green line), without 1,4-benzoquinone (pink line). (d) CV for
438 enzymatic reduction of vitamin K3 to menadiol at unmodified glassy carbon electrode.

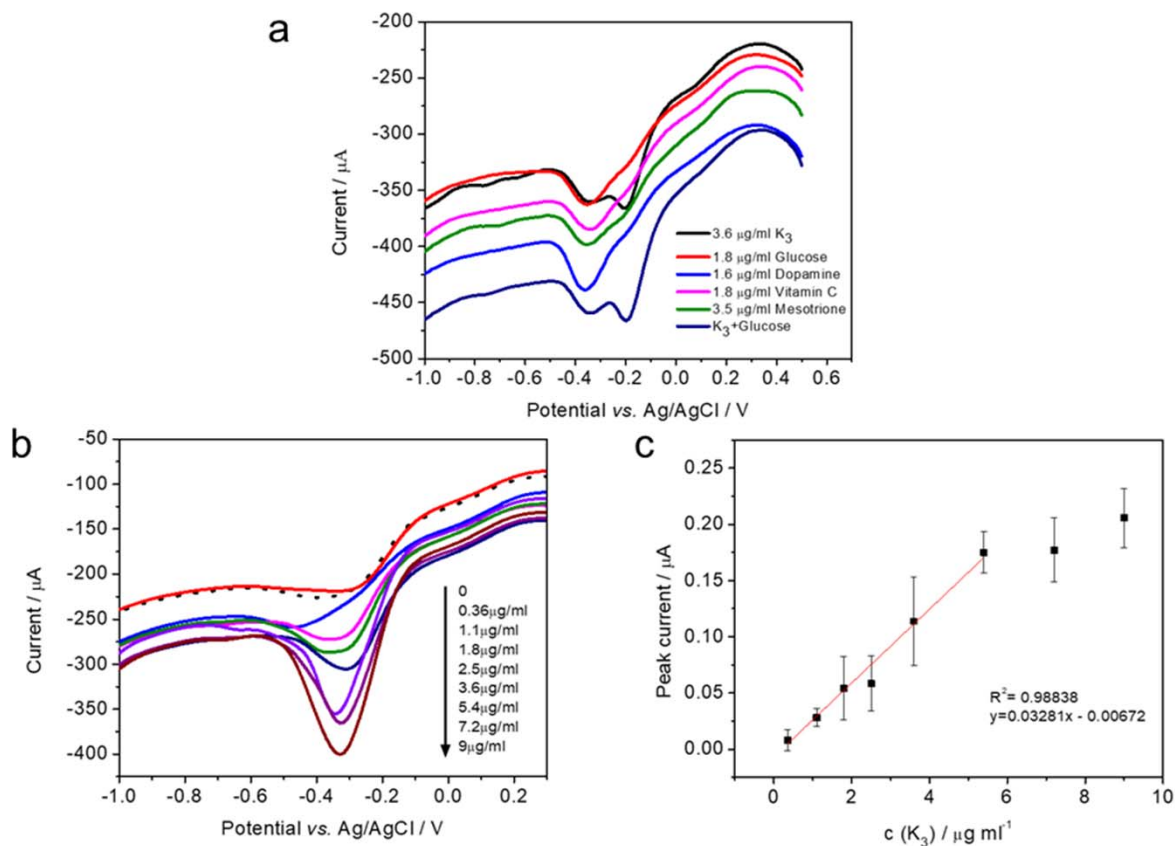
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440

441 **Figure 4.** C-SPE modification and vitamin K3 detection. (a) SEM image of the bare surface of
442 C-SPE. (b) SEM image of surface morphology of C-SPE after YaiB immobilization. (c) DPV
443 curves obtain for various concentration of vitamin K3 at C-SPE modified with YaiB in solution
444 containing NADPH and riboflavin. (d) Corresponding calibration was obtained by plotting the
445 current (μA) in a function of vitamin K3 concentrations (μg/mL). Data points are the mean
446 values obtained in 3 independent experiments ± SD.

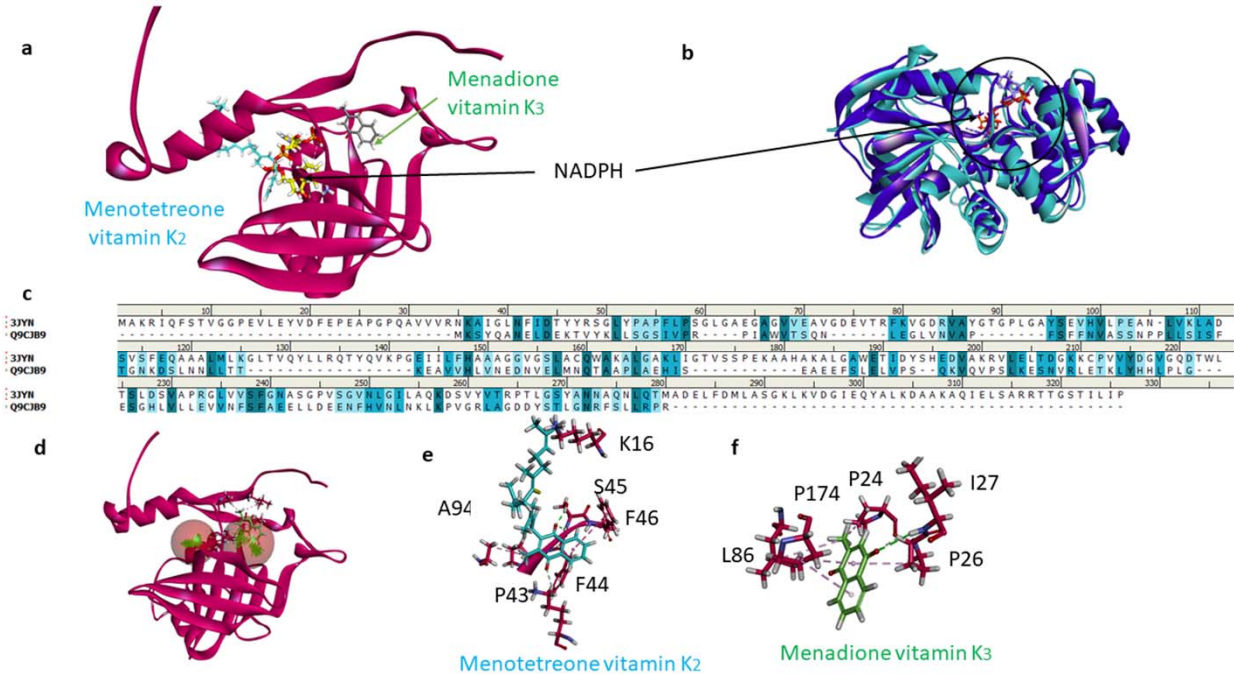
447



448

449 **Figure 5.**

450 DPV responses of developed enzymatic biosensor with control molecules and vitamin K3 at
451 different concentrations spiked in milk. (a) Control and interference studies of vitamin K3
452 detection using YaiB modified C-SPE. (b) Differential pulse voltammograms showing
453 dependence of the cathodic peak current at around -0.3V on vitamin K3 concentration. (c)
454 Corresponding calibration curve was obtained by plotting the current intensity of menadiol,
455 formed by the enzymatic reaction, in Vitamin K3 concentrations. Data points are the mean
456 values obtained in 3 independent experiments \pm SD.



457

458 **Figure 6:** Model of YaiB and its ligands. (a) Sequence alignment of YaiB and PDB 3JYN
 459 *Pseudomonas syringae* pv. Tomato DC3000 quinone oxidoreductase complexed with NADPH.
 460 (b) Overlay of two quinone oxidoreductase PDB 3JYN and 1IYZ, with emphasis on NADPH
 461 binding (arrow). (c) Modeled structure of YaiB with vitamin K2 (light blue) in vicinity to
 462 NADPH (yellow), only vitamin K3 (grey) is found at a distal site, at the other side from
 463 NADPH. (d) Detail of vitamin K3 binding to YaiB.

464

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