Acetylcholinesterase and Butyrylcholinesterase-Based Biosensors for the Detection of Quaternary Ammonium Biocides in Food Industry

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Article

Acetylcholinesterase and Butyrylcholinesterase-Based Biosensors for the Detection of Quaternary Ammonium Biocides in Food Industry

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Abstract: A sensitive and robust electrochemical cholinesterase-based sensor was developed to detect the quaternary ammonium (QAs) biocides most frequently found in agri-food industry wash waters: benzalkonium chloride (BAC) and didecyldimethylamonium chloride (DDAC). To reach the maximum residue limit of 2.8 10^{-8} M imposed by the European Union (EU), two types of cholinesterases were tested, acetylcholinesterase (AChE, from Drosophila melanogaster) and butyrylcholinesterase (BChE, from horse serum). The sensors were designed by entrapping AChE or BChE on cobalt-phthalocyanine-modified screen-printed carbon electrodes. The limits of detection (LOD) of the resulting biosensors were 3.8 10^{-8} M for DDAC and 3.2 10^{-7} M for BAC, using respectively AChE and BChE. A simple solid phase extraction step was used to concentrate the samples before biosensor analysis, allowing the accurate determination of DDAC and BAC in tap water with limits of quantification (LOQ) as low as 1.3 10^{-9} M and 5.3 10^{-9} M, respectively. The biosensor was shown to be stable during 3-months storage at 4°C.

Keywords: quaternary ammoniums; biocides; cholinesterases; biosensor; screen-printed electrodes

1. Introduction

Disinfectant biocides are chemicals heavily used by the food industry to control microbiological contamination of surfaces in contact with food products dedicated to human consumption [1]. They were initially employed for food and water conservation, although there are early reports of their use in wound cleansing [2]. Biocides are also widely used in healthcare environments, particularly for disinfecting and sterilizing surfaces and medical equipment [3]. Different chemicals have been developed over the years, including phenols, chlorine-releasing agents (CRAs), alcohols, iodine, hydrogen peroxide, and silver compounds. In the 20th century, other chemical agents such as the quaternary ammonium (QA) compounds were introduced and used for various applications at suitable concentrations [4]. Owing to their low cost and broad biocidal spectrum towards bacteria, fungi, parasites, and viruses, benzalkonium chloride (BAC) and didecyldimethylamonium chloride (DDAC) are the most used QA-based biocides in agri-food industry, and more specifically for the disinfection of dairy industry equipment [1,5] (Figure 1). QA compounds are effective in eliminating microorganisms, but various studies have indicated that these surface agents adsorb on the equipment, resist washing, and some of their residues are transferred to the food, causing many health issues ranging from gastrointestinal problems to coma and death [6]. A study conducted by the European Food Safety Authority (EFSA) has demonstrated the presence of BAC and DDAC residues in 12 % of milk samples tested, 6 % in leafy vegetables, and 5 % in infant food [7]. Moreover, biocides are not biodegradable, posing a potential risk to the environment [8]. Although data on the detection of these biocides in the environment are scarce in the literature, BAC concentrations have been reported in milligrams per liter in hospital wastewater. In addition, wastewater plants do not

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have QAs contaminant treatment, resulting in their release as micropollutants into the environment [9]. For that reason, the regulatory framework EU regulation 1119/2014 for the presence of biocides in specific foods such as milk established a maximum residue limit for BAC and DDAC of 0.1 mg/Kg. A default standard biocide limit of 0.01 mg/kg was also established for wash water, equivalent to 0.028 μM [10].

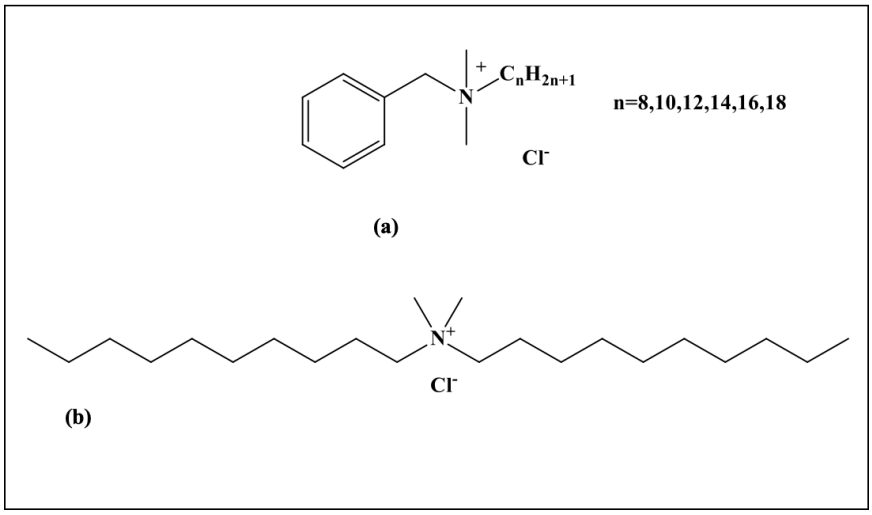

Figure 1. Chemical structures of BAC (a) and DDAC (b) biocides**.**

Several analytical techniques have been reported in the literature for the detection of biocides. Liquid chromatography combined with mass spectroscopy (LC-MS-MS) was used to detect BAC and DDAC with a very low LOD of 0.1 μg/L [11]. However these techniques present some disadvantages, as they cannot be used for on-site detection, they require trained personnel and sophisticated equipment requiring costly maintenance [12]. As EU policies impose routine testing on the agri-food industries for safety purposes, it appears necessary to develop a sensitive, cost-effective, and easy-to-use device for detecting these biocides in food and wastewater in real time. A study published in 1952 reported that acetylcholinesterase (AChE) from electric eel (Electrophorus electricus) was reversibly inhibited by certain quaternary ammonium salts [13]. This discovery opens the possibility of developing a cholinesterase (ChE) biosensor for the detection of these biocides based on the enzyme inhibition process. Cholinesterase biosensors are well known for their applications in pesticides detection, especially carbamate and organophosphate insecticides, but few studies have focused on biocide quantification. Compared to other types of biosensors, cholinesterase sensors are relatively inexpensive, making them promising and powerful tools for the detection of biocides in food and wash water [14].

The aim of the present study was to develop a novel cholinesterase biosensor capable of detecting BAC and DDAC biocides in tap water at low concentrations, based on cholinesterase inhibition. Preliminary tests were conducted by testing two types of cholinesterases, acetylcholinesterase and butyrylcholinesterase (BChE), to determine the most sensitive enzyme against the selected biocides. In a second step, amperometric enzyme biosensors were developed based on entrapment of the most suitable enzyme on the surface of cobalt-phtalocyanine modified screen-printed carbon electrodes. To the best of our knowledge, the described biosensor is the first device capable of detecting BAC and DDAC biocides in a convenient concentration range.

2. Materials and Methods

2.1. Materials

AChE from Drosophila melanogaster (wild type) was produced by Centre de Recherche de Biochimie Macromoléculaire (CRBM) (Montpellier, France). Butyrylcholinesterase from horse serum, and the substrate acetylthiocholine chloride and iodide were purchased from Sigma-Aldrich (St.

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Louis, MO, USA), butyrylthiocholine iodide was from Thermo Fisher Scientific (Kandle, Germany). The activity of cholinesterases was measured optically in the presence of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB-Ellman's reagent), purchased from Sigma-Aldrich. BAC (C12-C18) and DDAC quaternary ammoniums were purchased from Sigma-Aldrich. All the solutions were prepared daily in deionized water prior to each measurement. Biosurfine-MRH photopolymer (PVA) used for enzyme immobilization was kindly provided by Toyo Gosei Kogyo Co. (Chiba, Japan). Poly (vinyl) chloride (PVC) sheets (200 mm x 100 mm x 0.5 mm) (SKK, Denzlingen, Germany) were used as support for the screen-printed electrodes. Graphite (Electrodag 423SS) and silver/silver chloride (Electrodag 6037SS) screen-printing pastes were from Acheson (Plymouth, UK). Cobalt phthalocyanine (Co-PC) -modified carbon paste was purchased from Gwent Electronic Materials, Ltd. (Gwent, UK). A glycerophtalic paint (Akzonobel, France) was used as insulating layer. Oasis HLB solid-phase extraction cartridges used for samples pretreatment were purchased from Waters (Milford, MA, USA).

2.2. Methods

2.2.1. Determination of cholinesterases activity by colorimetry

Cholinesterase (ChEs) activity was measured before immobilization by spectrophotometry using a Shimadzu UV-1800 spectrophotometer. In the presence of the enzyme, the substrate acetylthiocholine (AtCh) or butyrylthiocholine (BtCh) (depending on the cholinesterase type) is hydrolyzed into thiocholine, which in turn reacts with Ellman's reagent 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), leading to the formation of a 5-thio-2-nitrobenzoic acid (TNB), which is responsible for the yellow coloration measured at 412 nm [15]. 1 enzyme unit was defined as the amount of enzyme allowing the transformation of 1 µmol of substrate per minute.

2.2.2. Biosensor implementation

The screen-printed electrodes used for the electrochemical measurements were fabricated in our laboratory using a DEK248 printing machine, according to a method already described [16]. The three-electrode system consisted of a Co-PC-modified carbon working electrode (4 mm diameter), a straight Ag/AgCl reference electrode (5 mm x 1.5 mm), and a curved carbon counter electrode (16 mm x 1.5 mm). Electrodes were cured at 60°C during 3 hours after each layer deposition.

Cholinesterase enzyme (AChE or BChE) was immobilized on the surface of the working electrode by entrapment in a polyvinyl alcohol photosensitive polymer (Biosurfine-MRH). The polymer was mixed in a 70%:30% ratio (v/v) with cholinesterase solution, and 3 µL of the mixture was spread on the surface of the working electrode. This process increases the stability of the enzyme by decreasing its denaturation and improving the lifespan of the biosensor [17]. The amount of enzyme immobilized was calculated to be 0.3 mU electrode. The modified electrode was placed under 2 white neon lights (Philips T5 short, 4000 K, 8 W, 380 lm) for 48 hours to allow the photopolymerization process, and then stored at 4°C before use.

Chronoamperometric measurements were carried out in a 10 mL cell using a PG581 Uniscan potentiostat (Uniscan Instruments, UK). The biosensor was immersed in 10 mL of PBS buffer at pH 7 and a potential of 0.1 V versus Ag/AgCl was applied, corresponding to the oxidation potential of CoPC. Upon addition of 100 µL of acetylthiocholine chloride at 1 mM, the oxidation current increased until reaching a plateau corresponding to the steady state response. Such measurement was repeated four times to confirm the stability of the biosensor response. For inhibition experiments, 1 mL of biocide solution was added to 9 mL of PBS buffer, the biosensor was incubated during 10 minutes in this mixture and its residual response was measured as described above. The cell was washed with PBS between measurements. The inhibition rate was then calculated using the following equation: $(I_0-I_{(biocide)})/I_0$,where I_0 and $I_{(biocide)}$ correspond to the intensity of the current measured in absence and in presence of biocide, respectively. Calibration curves were established using known concentrations of BAC and DDAC. To estimate the importance of matrix effect, inhibition experiments were carried out using biocides solutions prepared in deionized water or in tap water.

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2.2.3. Sample pretreatment by solid-phase extraction

An oasis HLB SPE cartridge was used due to its good extraction properties based on hydrophobic interaction with the apolar parts of both biocides. The cartridge was preconditioned with 5 mL of methanol, followed by 4 mL of distilled water. DDAC and BAC biocides were solubilized in either distilled or tap water, and each biocide solution (500 mL) was directly injected into the cartridge at a flow rate of 4 mL/min. Elution was performed using 5 mL methanol. Subsequently, methanol was evaporated using a rotary evaporator machine, and the biocides were finally diluted in 1 mL of water.

3. Results

3.1. Optimization of Operating Temperature

The effect of temperature on enzymes is well-known and has been widely described [18]. Therefore, in this study, we performed amperometric measurements at different temperatures, notably at 24°C (room temperature), 30°C, and 40°C to determine the optimal temperature of each enzyme used.

As shown in Figure 2, the biosensor response increased with temperature using both enzymes; a plateau was observed at 30°C for AChE-based biosensor while BChE-sensor response continued to increase between 30°C and 40°C. However, a better reproducibility of sensor responses was observed for both sensors at 30°C, so this temperature was chosen for further experiments.

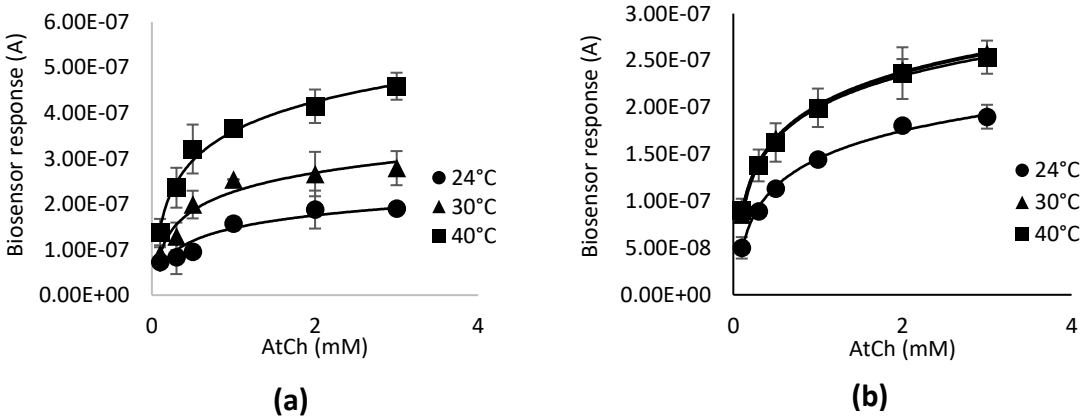

Figure 2. Response of the biosensor as a function of substrate (AtCh) concentration at different temperatures **(a)** BChE-based biosensor **(b)** AChE-based biosensor.

3.2. Biosensor detection of BAC and DDAC

3.2.1. Calibration curves

Biosensors calibrations were performed for standard biocide concentrations diluted in either distilled or tap water to determine the presence or absence of matrix effects. As shown in Figure 3 the percentage of inhibition was not affected by the matrix when tap water was used instead of distilled water. The limits of detection (LOD) of the biosensor, calculated as the biocide concentration inducing a 10 % decrease of the sensor response, were 1.30 10^{-6} M and 0.32 10^{-6} M for BAC biocide using AChE and BChE enzymes, respectively. The biosensors showed a better sensitivity to DDAC biocide, with LOD of 3.8 10^{-8} M and 2.2.10^{-7} M using AChE and BChE, respectively. It is interesting to notice that AChE sensor showed higher sensitivity to DDAC while BChE sensor was more sensitive to BAC. It is important here to stress that these concentrations correspond to the final concentration in the analytical cell, after a 10-fold dilution of the sample.

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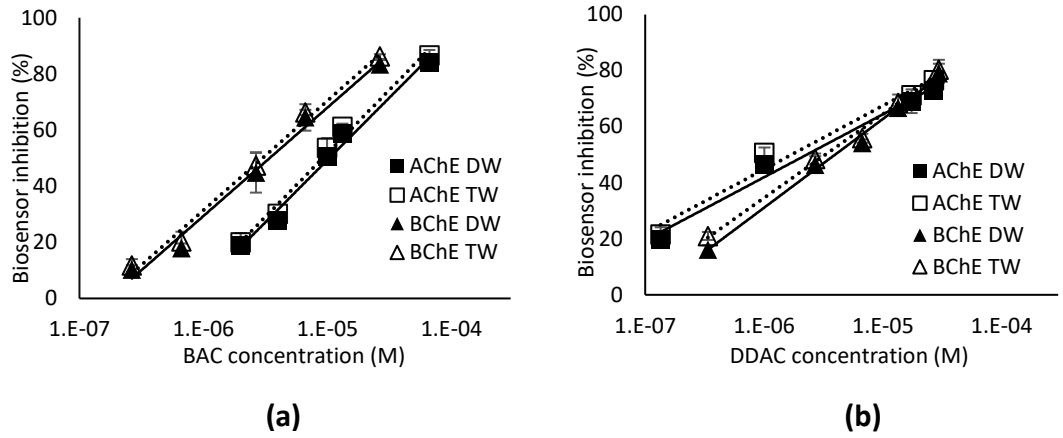

Figure 3. Inhibition effect of BAC (a) and DDAC (b) biocides on AChE and BChE-based biosensors. Biocides were either prepared in distilled water (DW) or tap water (TW). Equations of the obtained curves are the following: (a) AChE-DW: y = 19.273ln(x) + 271.23 (R^2 = 0.9864); AChE-TW: y = 19.625ln(x) + 277.62 (R^2 = 0.9863); BChE-DW: y = 16.775ln(x) + 261.12 (R^2 = 0.9883); BChE-TW: y = 16.952ln(x) + 265.6 (R^2 = 0.9913). (b) AChE-DW: y = 9.7538ln(x) + 176.67, R^2 = 0.9839); AChE-TW: y = 9.8656ln(x) + 181.03 (R^2 = 0.9736); BChE-DW: y = 13.757ln(x) + 221.11 (R^2 = 0.9935); BChE-TW: y = 12.983ln(x) + 213.91 (R^2 = 0.9920).

Although the detection limits of the developed sensors are in micromolar range, the sensitivity of these devices was not compatible with the limit of 0.028 µM imposed by European regulation. For this reason, a pre-concentration step was mandatory before biosensor analysis.

3.2.2. Sample pre-concentration and biosensor analysis

500 mL of tap water spiked with known concentrations of biocide were passed through a HLB SPE cartridge, eluted with 5 mL methanol, evaporated, and finally collected in 1 mL of tap water. The resulting concentrated extracts were then analyzed using the BChE and AChE-based biosensors, and the obtained inhibition percentage allowed calculating each biocide concentration, based on the corresponding calibration curve equation. The measured concentration was then compared to theoretical concentration, allowing calculation of recovery rates for each biocide tested. As shown in Table 1, BChE sensor was suitable for evaluating the efficiency of BAC preconcentration in a wide concentration range, showing recovery rates between 78 % and 109 %. Similarly, DDAC was determined using both biosensors with recovery rates ranging between 74.8 % and 99.3 % (Table 2). These results confirm the efficiency of HLB SPE concentration of BAC and DDAC compounds in a wide concentration range, suitable with targeted values.

Table 1. Recovery rates obtained for BAC after SPE preconcentration, determined either using AChE ou BChE-based biosensors (concentrations are those of biosensor measurement cell).

AChE biosensor y = 19.625ln(x) + 277.62			**BChE biosensor** y = 16.952ln(x) + 265.6		
$[BAC]_{theoretical}$ µM	$[BAC]_{measured}$ µM	Recovery (%)	$[BAC]_{theoretical}$ µM	$[BAC]_{measured}$ µM	Recovery (%)
0.27	-	-	0.27	0.29	109
0.67	-	-	0.67	0.61	91.7
2.65	2.30	86.8	2.65	2.18	82.2
26.5	22.5	86.5	26.5	20.3	78.1

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Table 2. Recovery rates obtained for DDAC after SPE preconcentration, determined either using AChE ou BChE-based biosensors (concentrations are those of biosensor measurement cell).

AChE biosensor y = 19.625ln(x) + 277.62			**BChE biosensor** y = 16.952ln(x) + 265.6		
$[DDAC]_{theoretical}$ µM	$[DDAC]_{measured}$ µM	Recovery (%)	$[DDAC]_{theoretical}$ µM	$[DDAC]_{measured}$ µM	Recovery (%)
0.14	0.11	81.5	0.14	-	-
0.34	0.25	75.8	0.34	0.27	74.8
0.75	0.62	80.0	0.75	0.74	99.3
2.70	2.2	81.5	2.70	2.60	96.3
29.7	23.7	89.4	29.7	27.0	90.9

Taking into account that the biocide samples were 500-fold concentrated through SPE extraction, and that the biosensor measurement involves a 10-fold dilution of the injected solution, the actual range of concentrations measurable by the developed method were calculated (Table 3). These results show that the wider range of biocide concentration is detected using the BChE sensor for BAC and using the AChE sensor for DDAC. Using these biosensors, the limits of quantification obtained for BAC and DDAC are respectively 5.3 10^{-9} M and 2.7 10^{-9} M. Keeping in mind that the maximum concentration tolerated in food industry wash waters is 2.8 10^{-8} M, the developed biosensors were sensitive enough to detect DDAC and BAC biocides, whatever the enzyme used.

Table 3. Range of actual concentrations of BAC and DDAC contained in tap water determined after SPE concentration and biosensor analysis using either AChE ou BChE-based sensors.

	BAC	**DDAC**
AChE-sensor	5.3-10^{-8} - 5.3 10^{-7} M	2.7-10^{-9} - 5.9 10^{-7} M
BChE sensor	5.3-10^{-9}- 5.3 10^{-7} M	6.7-10^{-9} -5.9 10^{-7} M

3.3. Biosensor Stability

Stability tests were performed using the developed biosensors. AChE or BChE-modified electrodes were fabricated and stored for a 5-month period at 4°C, and their response to 0.1 mM acetylthiocholine was measured every 30 days (n = 3 electrodes). As presented in Figure 4, the biosensors response remained stable during 3 months, whatever the enzyme used. A significative decrease of signal was noticed after 4 months storage, resulting in a 50 % loss after 5 months. These results show that both AChE and BChE-based biosensors can be stored at 4°C during at least 3 months without significative loss of activity.

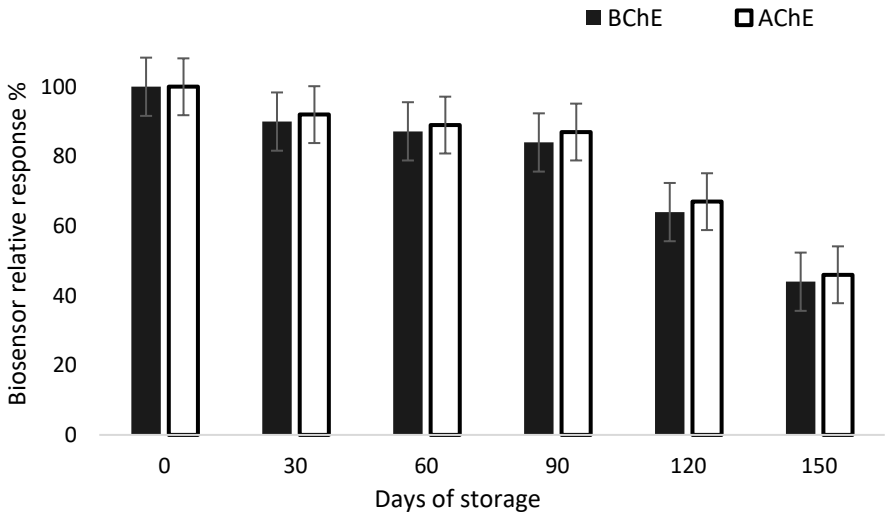

Figure 4. Relative response of AChE and BChE-based biosensors during storage at 4°C. The biosensor response was tested in presence of acetylthiocholine at 0.1 mM (b), as described in inhibition experiments.

4. Conclusions

A biosensor should acquire certain characteristics to be used in the real world. In this work, we developed cholinesterase-based biosensors capable of detecting BAC and DDAC biocides in tap water at very low concentrations. Calibrations experiments carried out either with distilled water or tap water samples showed the absence of matrix effects, whatever the enzyme and biocide used. A simple SPE preconcentration allowed attaining limits of quantification in the nanomolar range, compatible with regulations applied to food industry wash waters. The cholinesterase biosensors were also used to evaluate the efficiency of preconcentration step, and satisfying recovery rates were obtained, ranging from 78 % to 100 %. In addition, the developed biosensors exhibited good storage stability for over three months. The results presented in this paper show that cholinesterase-based biosensors are promising tools for the simple and sensitive detection of quaternary ammonium biocides in food industry wash waters. Future works will focus on the possibility of using artificial neural networks for analyzing complex biocide mixtures, as already described in our group for discriminating mixtures of organophosphate insecticides [19].

Author Contributions: Lynn Mouawad: Investigation, writing – original draft, Georges Istamboulie: Methodology, Gaëlle Catanante: Supervision, Funding acquisition, Writing - Review & Editing, Thierry Noguer: Supervision, Funding acquisition, Project administration, Writing - Review & Editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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