Quantitative Screening and Resolution of Carbamic and Organophosphate Pesticides Mixture in Extra Virgin Olive Oil by Acetylcholinesterase-Choline Oxidase Sensor

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Abstract: Acetylcholinesterase-choline oxidase amperometric bi-enzymatic biosensor in a flow injection configuration has been assembled in order to quantitatively detect carbamic and organophosphorus pesticides mixture in extra virgin olive oil samples. The recognition of the two different pesticide classes by this biosensor has been carried out by exploiting the well-known different inhibition mechanism of carbamic (reversible inhibition) and organophosphorus (irreversible inhibition) compounds versus acetylcholinesterase activity. In order to eliminate extraction clean-up steps for pesticides detection in extra virgin olive oil, inhibition calibration curves and analyses of spiked real samples were performed using hexan as the carrier in the flow injection apparatus. The results of the calibration curves showed a different inhibition power versus acetyl cholinesterase in function of the specific compound and found that a probable interaction exists between two or more compounds that affected the toxic power of the single pesticide. The limit of detection ($5 \mu$g·L$^{-1}$) obtained for the tested organophosphorus (Paraoxon; Fenitrothion) and carbamic (Methomyl; Carbaryl) pesticides was lower than residues established by European legislation, thus suggesting that the bi-enzymatic biosensor, extensively described in literature, can be useful and economically convenient for quantitative screening methods in food security monitoring.

Key words: Food analysis, extra virgin olive oil, pesticides, biosensor.

1. Introduction

Extra virgin olive oil (EV) is one of the most known and typical food products of the Mediterranean regions and Spain, Italy and Greece represent the main EV producers in the world. Its popularity depends on its appreciated sensorial properties besides positive effects for human health. EV total quality depends on multiple factors such as olive cultivars, production process, preservation methods and, last but not least, agronomic practices. Organophosphorous (OP) and Carbamic (CB) pesticides are normally used in olive fruit production to keep crops healthy and to prevent them being destroyed as a consequence of disease and infestation. A possible consequence of their use may be the presence of pesticide residues in the EV extracted from treated fruits. Maximum residue levels have been established by different institution (Food and Drug Administration, Codex Alimentarius Commission) in order to avoid possible risks for human health (ECs No. 396/2005; No. 178/2006 and No. 149/2008) [1-3]. A recent research [4] about pesticides contamination in commercial Italian EV olive oils shows 1.9% of irregular samples, 3.4% of regular samples with only one class of residues and 12% of regular olive oil samples with more than one residue, thus confirming the importance of the development of multi-residue analytical methods for the determination of pesticides in this important food product.

The most frequently used methods for detection of
OP and CB pesticides are based on gas and liquid chromatography in combination with mass spectrometry. While being sensitive and reliable, such methods require expensive laboratory equipments, trained personnel and complex procedures to treat and extract the real matrices.

The use of biosensors as an analytical method to determine the presence of neurotoxic pesticides represents an attractive alternative for research because of their high sensitivity, selectiveness, ease and rapidity of use. These analytical devices, based on the intimate contact between a biorecognition element that interacts with the analyte of interest and a transducer element that converts the biorecognition event into a measurable signal, are gaining momentum as complimentary screening assays together with classical analytical techniques due to their high selectivity and sensitivity, low instrumentation cost, easier procedures and rapidity of the assay. After the rapid screening step performed with biosensors on many real samples, only the positive ones can be performed with the classical analytical techniques with the aim of lowering the cost of field analysis. Among the different types of biosensors, the electrochemical ones are especially interesting due to the high sensitivity inherent to the electrochemical detection and the possibility to miniaturize the required instrumentation, providing compact and portable analysis devices. Electrochemical, mono or bi-enzyme, biosensors able to recognize CBs and OPs by specific inhibition of acetyl cholinesterase (AChE) as biorecognition element have been extensively reported [5-10]. In both categories OPs and CBs determination is performed by AChE activity measurements before and after exposure to a sample (incubation) and calculation of the inhibition due to OP and CB compounds. Performance of bi-enzymatic biosensor was based on two enzymatic reactions in series as reported in Eqs. (1) and (2).

\[
\text{Acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{acetate} + \text{choline} \quad (1) \\
\text{Choline} + 2O_2 \xrightarrow{\text{ChOx}} \text{betaine aldehyde} + 2 \text{H}_2\text{O}_2 \quad (2)
\]

OP and CB lower the enzyme activity of the first enzyme and therefore the production of choline and subsequently reduce the current produced by oxidation of hydrogen peroxide. In addition the enzyme (AChE) used in this biosensor is the same which determines neurotoxic effects in humans and animals and, for this reason, the measurements are directly related to the biological effect while the traditional chromatographic methods detect the concentration of the pesticides without any information about the neurotoxicity due to the mix of different compounds. For inhibition biosensors, the calibration curves and the measurements on real samples are conventionally expressed in concentration units of a pesticide which gives the same neurotoxic effect.

Research and development on biosensors for pesticides had many improvements in the last two decades and are still going for ever increasing complexity of the immobilization strategies of the enzymes [11], the use of novel biological mediators as well as bacteria [12] and miniaturization of the transducers [13]. Bachmann et al. [14, 15] proposed disposable screen-printed amperometric multielectrode biosensors based on inhibition of different types of native and recombinant AChEs and chemometric data analysis using Artificial Neural Networks (ANNs). Nevertheless these advanced studies of the applications to food industry requirements and real samples remain poor and not much explored, especially for screening of EV production. Moreover, the analysis of pesticide residues in EV is complicated, because of the inherent complexity of the matrix, mainly comprising triglycerides (98%-99%). Many pesticides are fat-soluble non-polar compounds and tend to concentrate and remain in the fat matrix. This behavior normally requires the use of additional extraction clean-up steps, to isolate or to extract the pesticide fraction from the whole fatty matrix, prior to conventional [16] or biosensor analysis [17]. The main strategies used for the isolation of pesticides in olive oil samples involve the application of techniques called
liquid-phase extraction (LPE) or liquid-solid extraction (LSE). In both cases the use of organic solvents such as hexan, acetonitrile was required [16]. These remarks highlight the advantage to develop pesticide biosensor working in organic solvent able to analyze food samples with relatively high fat content (i.e. > 15%), such as olive oil. Previous researches [8, 18-20] have shown the capability of acetylcholinesterase-choline oxidase bi-enzymatic biosensor working in non-aqueous solvents to obtain small or no inhibition effect concerning acetylcholinesterase activity in different organic solvents.

An important goal which could be obtained with amperometric biosensors based on inhibition of AChE is the capability to distinguish between pesticide classes (OP and CB) when both are present in a mixture. The aim of this research, where we used a basically assembled biosensor as extensively reported in literature, has been mainly focused on the development of a simple, inexpensive, and sensitive screening method based on bi-enzymatic (AChE-ChOx) amperometric biosensor able to obtain quantitative information with regard to the simultaneous presence of OPs and CBs in olive oil samples. The recognition of the two different pesticide classes by AChE-ChOx biosensor has been carried out by exploiting the well-known different inhibition mechanism of CB and OP compounds versus AChE activity. CB compounds form an unstable bond with esterasic site of AChE that spontaneously tends to break down in an interval time ranging from few minutes to one hour (reversible inhibition). OP compounds, on the contrary, form a highly stable covalent bond phosphate-enzyme, bringing about an irreversible inhibition [21].

Inhibition tests on AChE were carried out with OPs and CBs solutions at different concentration in hexan, whereas the tests on real samples were straight followed on spiked EV sample diluted with hexan solvent without no extraction step. To improve the speed and reproducibility of the analysis, the bi-enzymatic biosensor was connected to a Flow Injection Analysis (FIA) manifold.

2. Experiment

2.1 Reagents

Acetylcholinesterase (E.C. 3.1.1.7) Type V-S from electric eel (1000 units·mg⁻¹ protein), Choline oxidase (E.C. 1.1.3.17) from Alcaligenes species (10 units·mg⁻¹ solid), acetylcholine chloride, choline chloride, glutaraldehyde (50% aqueous solution), pyridine-2-aldoxime methiodide (PAM), D-aminopropyl CPG (200-400 mesh, average pore size of 500 Å, average amine content of 70 μmol·g⁻¹) Bovine Serum Albumin (BSA), Paraoxon, Fenitrothion, Methomyl, Carbaryl, were purchased from Sigma (St. Louis, USA). Hexane for the preparation of pesticide solutions and for the dilution of olive oil samples were HPLC grade. All other chemicals were of analytical grade or better. Residue-free virgin olive oil, certified by an accredited laboratory for the absence of pesticide residues, was provided by a local company.

2.2 Screen Printed Electrodes

Screen printed electrodes were produced in three steps, by screen printing different consecutive layers on transparent polyester films (Kemafoil® Coveme, Italy). A first layer of a graphite ink was deposed to define the conducting track and the working electrode, the second one was a silver/silver chloride ink used as reference electrode, while the third layer consisted in an insulating ink, UV polymerizable, resistant to organic solvents. Between the first and the second screen printing step, the strips were cured at 80 °C for 25 min. The insulating ink was polymerized by UV light (mod HB 311 Philips, Netherlands).

2.3 Preparation of Bi-enzymatic Biosensor

Well known procedures from the literature based on glutaraldehyde were used for immobilization of both enzymes on the electrode surface and on microreactor [22]. AChE immobilization was made on CPG. The linking ability of this reagent with two opposite
aldehydic groups was used to create alkyl bridges between the amino group of the glass surface and AChE. These bridges can be obtained with a two steps procedure and using a high concentration of glutaraldehyde.

300 µL of 2% solution of glutaraldehyde in phosphate buffer (PB) 0.1 M, pH 6.8 was added to a 65 mg of CPG. The mixture was shaken for 1 h and the activated glasses were rinsed with PB. Next, specific amounts of AChE in PB (24, 12, 6 and 3 units) were added to activated CPG glasses and the mixture was stored at 4 °C overnight. AChE immobilized was then inserted into a borosilicate glass column (L × I.D. 25 mm × 3 mm, Omnifit England) and connected to the FIA manifold.

The crosslinking ability of glutaraldehyde was used in a one step procedure where the enzyme (ChOx) is mixed with BSA and a lower concentration of the crosslinking agent to prevent its denaturation. ChOx immobilization was carried out directly on screen printed electrode by dropping on the working electrode 5 µL of a mixture of BSA (0.2 µg·µL⁻¹); Glutaraldehyde (2.5%) and ChOx (0.2 µg·µL⁻¹) (20:10:5). SPE was stored at room temperature for 1 h and successively at 4 °C overnight.

2.4 FIA System

FIA configuration used is shown in Fig. 1. The carrier stream was pumped through the FIA system by a peristaltic pump with four channels (Miniplus 3, Gilson, France), two of which were used for the fluxing PB and organic solvent pesticide solutions separately. Acetylcholinchloride solution was injected into bi-enzymatic biosensor by manual sample injection valve (six port loop inject valve, Omnifit, England) at 0.5 mL·min⁻¹. Acetylcholinchloride solution was injected into bi-enzymatic biosensor by manual sample injection valve (six port loop inject valve, Omnifit, England) at 0.5 mL·min⁻¹, and the current corresponding to the oxidation of hydrogen peroxide was measured by a potentiostat Palm Sens (Palm Instruments BV, Netherland).

A three way valve was inserted after AChE reactor and before the biosensor, in order to avoid contact between pesticides solution and ChOx immobilized on SPE. The components of the flow injection system were connected with 1.6 mm OD × 0.3 mm ID Polytetrafluoroethylene (PTFE) tubing.

2.5 Determination of OP and CB Pesticides by Inhibition Biosensor

The recognition and quantification of OP and CB compounds by bi-enzymatic biosensor is based on the inhibitory power of both pesticide classes versus the activity of acetylcholinesterase [23]. On the basis of two enzymatic (Eqs. (1) and (2)) in series it is possible to assay the AChE activity by hydrogen peroxide amount that is oxidized on the electrode surface at 0.7 V vs Ag/AgCl pseudo-reference electrode.

The inhibitory action of CB and OP compounds on AChE activity is performed by a minor choline amount resulting in a consequent reduction of the current produced by hydrogen peroxide. The recognition of the two different pesticide classes by ChE-ChOx biosensor has been carried out by exploiting the different inhibition mechanism of CB and OP compounds versus AChE activity. Previous research on the inhibition of AChE sensors by pesticides reported that incubation times between the immobilized enzyme and pesticides ranging from 10 min [8, 17] and 30 min [24, 25]. In our experiments inhibition tests were carried out by flowing at 0.1 mL·min⁻¹ for 45 min (flow and time experimentally optimized, data not shown), in FIA
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After 45 min of incubation in pure no significance variations in sensor response were detected if compared with the initial value. These data are in accordance with previous research [8]. Through a three way valve (V3, Fig. 1), positioned after AChE reactor, the pesticide solutions were discarded prior to converging on ChOx biosensor. The FIA manifold was then rinsed for 2 minutes with PB. The residue activity of AChE was measured by injection of Acetylcholine chloride (AChCl) 0.5 mM and on the basis of two enzymatic Eqs. (1) and (2) in series the heights of the amperometric peaks were collected. In the case of solutions with a single concentration of CB or OP compounds the percentage of inhibition I% was expressed by Eq. (3):

\[
I_{\text{CB}} = \left( \frac{i_0 - i_f}{i_0} \right) \times 100
\]

where \(i_0\) and \(i_f\) represent the height of the peak corresponding to oxidization of hydrogen peroxide before and after CB or OP pesticides respectively.

When a mix of both pesticide classes is introduced in flow system, the peak decrease depends on the inhibitory effect of CB and OP alike. The following procedure was then used to calculate the AChE inhibition of both pesticides classes:

- Injection of Acetylcholine 0.5 mM and measure of the biosensor response \(i_0\);
- Introduction of CB and OP in mix at given concentration;
- Waiting for 45 minutes;
- Injection of Acetylcholine 0.5 mM and measure of the biosensor response \(i_a\);
- Waiting for 30 minutes: this time was calculated on the basis of experimental tests for the recovery of 96.09% ± 3.44% of amperometric signal corresponding to Acetylcholine 0.5 mM before the inhibition with methomyl and carbaryl in range of 5-20 µg·L⁻¹;
- Injection of Acetylcholine 0.5 mM and measure of the biosensor response \(i_b\).

The percentage of inhibition I% of CB and OP was expressed by Eqs. (4) and (5) respectively:

\[
I_{\text{CB}} = \left( \frac{i_0 - i_a}{i_0} \right) \times 100
\]

\[
I_{\text{OP}} = \left( \frac{i_0 - i_b}{i_0} \right) \times 100
\]

where \(i_0\) represents the biosensor response in absences of pesticides, \(i_a\) is the biosensor response after the interaction between AChE and the pesticides in mix, \(i_b\) is the biosensor response after the recovery of the AChE inhibition caused by OP compounds. A typical biosensor response obtained through the procedure described above was reported in Fig. 2.

2.6 Reactivation of Inhibited AChE

The reactivation ranging from 98.5% and 100% of inhibited AChE after contact with solutions at different amounts of OP pesticide was carried out flowing PAM 0.1 M at 0.1 mL·min⁻¹ for 45 min followed by the PB at 0.5 mL·min⁻¹ for 20 min into the AChE reactor.

3. Results and Discussion

3.1 Inhibition Tests versus Different Amounts of AChE

The optimization of enzymatic biosensor is closely

![Fig. 2 Biosensor response after inhibition by CB and OP compounds in mix: \(i_0\)-Biosensor response in absences of pesticides; \(i_a\)-biosensor response after the interaction between AChE and the pesticides in mix; \(i_b\)-biosensor response after the recovery of the AChE inhibition caused by OP compounds.](image-url)
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linked to amperometric signal read by electrochemical transducer. Although a high enzyme activity is required for reproducible and long-term measurements with suitable electrode response on acetylcholine, sensitive inhibitor determinations are favoured at low enzyme loading. Pesticide detection with an optimized bi-enzyme sequence electrode involves low esterase activity but high choline oxidase activity allowing the signal amplitude to be affected by influencing this enzyme. Choline biosensor was then tested in order to evaluate the detection limit, linearity change and reproducibility. The calibration curve for choline measurements (Fig. 3) showed linearity ($R^2 = 0.999$) equal to 0.999 in the range between 0.5 and 1 mM with a slope of 112.47 nA/mM. The reproducibility of the choline biosensor was good being equal to 2.42% ($n = 3$ measurements). These results in particular as regards the range of sensibility of the biosensor were lower than other findings [26, 27], because the different amount of enzyme immobilized on the surface of the electrode in this study was thirty time lower than those used in the cited paper. The performances of AChE biosensors depend on their sensitivity to recognize small amount of pesticides ranging from 5-20 μg·L⁻¹ according to Codex Alimentarius MRLs established by European legislation (Reg. EC No. 149/2008) or quality control ones used in private industries.

The results presented in Table 1 showed the detection limits for Paraoxon and Methomyl using different unit of AChE. The amount of both pesticides is able to cause a perceptible inhibition of esterase activity decrease if a lower enzyme loading is immobilized on CPG glasses.

The calibration curve of Paraoxon on the base of the AChE inhibition percentage obtained with 2U of AChE immobilized on the CPG microreactor was reported in Fig. 4. The linear range (figure insert) is linear between 5-40 μg·L⁻¹ corresponding to $1.28 \times 10^8$-$1.45 \times 10^3$ mol·L⁻¹ with a degree of inhibition in the range 6%-35%. By use of bi-enzymatic AChE/Tyr SPE sensor with Phenylacetate as substrate Andreescu et al. [8] obtained a detection limit of $5 \times 10^{-3}$ mg·L⁻¹ ($1.82 \times 10^{-8}$ mol·L⁻¹). By use of mono enzymatic SPE sensor (AChE immobilized by cross-linking with GA on working electrode together with a mediator) Li et al. [28] obtained a detection limit of $4.95 \times 10^{-5}$ mg·L⁻¹ ($1.80 \times 10^{-7}$ mol·L⁻¹).

3.2 Calibration Curves of Pesticides Using a 3U AChE Microreactor

The stability of the bi-enzymatic biosensor was

<table>
<thead>
<tr>
<th>AChE U</th>
<th>CB Metomyl (µg·L⁻¹)</th>
<th>OP Paraoxon (µg·L⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>12</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>50</td>
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<tr>
<td>3</td>
<td>5</td>
<td>20</td>
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<td>2</td>
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<td>5</td>
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</table>

Table 1 Detection limits for Paraoxon and Methomyl using different amount of AChE immobilized on CPC glasses.
tested by measuring the analytical response of 9 acetylcholine chloride (AChCl) injections at 0.5 mM. The amperometric average signal was 85.04 nA ± 0.87 (n = 9) with a R.S.D relative standard deviation = 1.03%. The calibration plots for Methomyl-Paraoxon in mix (1:1) and Carbaryl-Fenitrothion in mix, obtained on the base of different inhibition mechanism of CB and OP compounds versus AChE activity, were shown in Figs. 5 and 6 respectively. In the same figures, the calibration curves of single pesticides were displayed too. No inhibitions were detected at Paraoxon amounts lower than 20 μg·L⁻¹ for solutions with single concentrations of OP. The data obtained for the different calibration curves highlighted that Paraoxon inhibition power increased when this was injected in mix with Methomyl compound: in fact, while no inhibitions were detected for single Paraoxon amounts lower than 20 μg·L⁻¹, inhibitions on AChE activity were recorded from 5 μg·L⁻¹ when two pesticide classes were injected in FIA system. A different response was observed for Methomyl compounds, that showed a higher inhibition power when injected as single pesticide versus AChE activity. As regards the sensibility to detect small amounts of Paraoxon, Carbaryl and Methomyl the AChE/ChOx biosensor proposed in this study showed results according to detection limits detected by Acetylcholinesterases sensor reported in previous papers [8, 24, 29]. No studies were found in literature for inhibition power of Fenitrothion versus AChE electrochemical sensor.

Power inhibition versus AChE activity changes when different molecules belonging to OP and CB pesticides get in contact with AChE. The calibration curves obtained with Carbaryl and Fenitrothion (Fig. 6) showed higher inhibition percentages than Methomyl and Paraoxon ones. Moreover, in this case of Fenitrothion power inhibition versus AChE increased when injected in mix, unlike Carbaryl, that seems to be suffering from the simultaneous presence of Fenitrothion compound. The calibration curves obtained for the tested pesticides highlighted a different toxic power versus AChE in function of the specific pesticide compound and found that a probable effect exists between two or more pesticide compounds that increases or reduces the toxic power observed when the single pesticide compound getting in contact with AChE. The toxicity of four different binary pesticides (OP and CB) mixture versus AChE was recently investigated in vivo by juvenile salmon survival [30]. The results obtained in this research showed that the binary pesticides mixture caused a synergistic AChE inhibition that are in contrast with those detected in our study. The latter may be probably explained by the different carrying out of the AChE inhibition tests, hence from different absorption rate of the different pesticides in blood.

Our method based on sequential discrimination of OP and CB pesticides, was able to show that a mix of these inhibitors, with different inhibition power and mechanisms (reversible or irreversible), affects the
final enzyme activity which is not due to the linear combination of inhibitory effects of the single pesticide alone. Further investigation is needed in order to understand these mechanisms and effects, which are strictly related to the toxicological effects of pesticide content in real samples on insects or humans.

3.3 Biosensor Evaluation with Extra Virgin Olive Oil Spiked Samples

The bi-enzimatic (AchE-ChOx) amperometric biosensor was used to analyze spiked (Methomyl and Paraoxon, 10 and 20 μg·kg⁻¹) EV sample. The effect of the EV matrix on the AChE activity was calculated measuring the enzyme activity as affected by the matrix with respect to that in pure hexane. The matrix effect was calculated according to Del Carlo et al. [17] by means of the following formula:

\[ E_m\% = 100 \times \frac{P_m}{P_e} \]  

where \( P_m \) and \( P_e \) represent the inhibition percentage after the injection of free residue EV diluted with hexane (1:1) and after the injection of pure hexane respectively.

Influence of organic matrix on the final activity of AChE was not negligible since the mean matrix effect 10% (\( E_m\% = 110 \)) was higher than the standard deviation (± 3) of measurement. Since the EV matrix showed an inhibition effect versus AChE equal to 10% (\( E_m\% = 110 \)) of pure hexan, the matrix effect must be subtracted from the value registered during the analysis on real matrices. The data on Methomyl and Paraoxon recovery present in spiked EV samples obtained using the % I value in the calibration equation of the corresponding pesticide in mix showed a good correlation between expected and obtained data (Table 2). These results highlighted that the bi-enzymatic biosensor proposed was able to detect lower concentration than the accepted maximum residue levels proposed by the European legislation, thus representing an applicable screening method in food security monitoring.

4. Conclusions

A screen printed ChOx biosensor coupled with an AChE CPG microreactor was assembled in a FIA manifold as extensively described in literature to obtain a simple and cheap device devoted to quantitative screening of pesticide multiresidues in EV. Results suggest that the use of AChE biosensor can be suitable as an early warning system in screening analysis for food security monitoring.

The obtained data on Methomyl and Paraoxon recovery present in spiked EV samples showed to be in good correlation with expected data. The results highlighted that the bi-enzymatic biosensor proposed was able to detect lower concentration than the accepted maximum residue levels proposed by the European legislation, thus representing an applicable screening method in food security monitoring.

Preliminary results showed a probable effect of competition between different classes of pesticides (OP and CB) and their different mechanisms of inhibition for the active site of AChE. For this reason further studies are in progress on this competition effect and on validation and quality control procedures stated from European regulations for pesticide residues analysis in food and feed.

References


<p>| Table 2  Biosensor data registered with spiked EV samples. |</p>
<table>
<thead>
<tr>
<th>Expected value (μg·kg⁻¹)</th>
<th>Biosensor result</th>
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<tbody>
<tr>
<td>Methomyl</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10.8</td>
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<tr>
<td>20</td>
<td>21.7</td>
</tr>
<tr>
<td>Paraoxon</td>
<td></td>
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<tr>
<td>10</td>
<td>10.4</td>
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<tr>
<td>20</td>
<td>20.6</td>
</tr>
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[26] F. Ricci, A. Amine, G. Palleschi, D. Moscone, Prussain Blue based screen printed biosensors with improved...


