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Assessment of analytical methods to determine pyrethroids content of bednets

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Abstract

Objective: To present and evaluate simple, cost effective tests to determine the amount of insecticide on treated materials.

Methods: We developed and evaluated a competitive immunoassay on two different platforms: a label-free impedimetric biosensor (EIS biosensor) and a lateral flow. Both approaches were validated by gas chromatography (GC) and ELISA, gold standards for analytical methods and immunoassays, respectively. Finally, commercially available pyrethroid treated ITN samples were analysed. Different extraction methods were evaluated.

Results: Insecticide extraction by direct infusion of the ITN samples with dichloromethane and dioxane showed recovery efficiencies around 100% for insecticide-coated bednets, and >70% for insecticide-incorporated bednets. These results were comparable to those obtained with standard sonication methods. The competitive immunoassay characterisation with ELISA presented a dynamic range between 12 nM – 1.5 µM (coefficient of variation (CV) below 5%), with an IC_{50} at 138 nM, and a limit of detection (LOD) of 3.2 nM. EIS biosensor had a linear range between 1.7nM – 61 nM (CV around 14%), with an IC_{50} at 10.4 nM, and a LOD of 0.6 nM. Finally, the lateral flow approach showed a dynamic range between 150 nM – 1.5 µM, an IC_{50} at 505 nM, and a LOD of 67 nM.

Conclusions: ELISA can replace chromatography as an accurate laboratory technique to determine insecticide concentration in bednets. The lateral flow approach developed can be used to estimate ITN insecticide concentration in the field. This new technology, coupled to the new extraction methods, should provide reliable guidelines for ITN use and replacement in the field.

Keywords: ITN, insecticide quantification, immunoassay, biosensor, chromatography, insecticide extraction

Introduction

Insecticides are the cornerstone of most vector control programmes designed to reduce the incidence of human and animal diseases. Indoor Residual Spraying (IRS) and insecticide treated nets (ITN) have been used to control major vector borne diseases i.e. dengue, malaria, leishmaniasis or Chagas disease [1]. IRS is the coating of walls and roofs of houses with long-lasting insecticides to kill adult vectors (e.g. mosquitoes) that get in contact with the treated surfaces. ITN requires treating nets with insecticide, and adult vectors that land on the treated nets will be killed. People are protected by sleeping under those nets. Two main techniques are used to treat nets with insecticide and ensure they are effective for several years under field conditions. The insecticide is either coated on the surface of the net or incorporated in the matrix (polyethylene or polypropylene) then used to make the net. Pyrethroids, i.e., permethrin, deltamethrin, cypermethrin, cyalothrin, are the most widely used insecticides in public health because of their relative safety for humans, high insecticidal power at low doses and rapid knock-down effects. The safety and efficiency of pyrethroids for different applications in vector control have been assessed by WHO [2]. Most of the commercial ITN therefore contain pyrethroids as the main active ingredient.

The efficacy of insecticide treated materials is reduced over time because of insecticide loss. In most cases, the recommended useful lifetimes of IRS and ITN are estimated based on laboratory tests. Field studies to determine the performance of these devices in real-use conditions are rare [3-7]. Performance may also be influenced by geographical and environmental conditions, and user handling [3, 5]. Thus, measuring insecticide concentration in ITN and treated walls is a priority. Regular evaluation of the insecticide concentrations would allow a rational insecticide use avoiding dangerous human exposure and ensure safe and efficient vector control measures. Usually, information on insecticide concentration is provided by gas chromatography (GC) or high performance liquid chromatography (HPLC) methods, which are not available to the general population and cannot be used in the field [8, 5]. Hence, there is a need for simple, cost effective field tests for nonspecialists to determine insecticide concentrations on treated nets and surfaces.

Many efforts have been made to develop new analytical techniques as an alternative to costly and complex laboratory tests to quantify insecticides on treated materials. For instance, several research initiatives developed colorimetric assays based on enzymatic detection such as glutathione transferase (GST), a detoxifying enzyme that catalyses the biotransformation of halogenated compounds such as pyrethroids [9-11]. Alternatively to the GST enzyme, an amperometric assay based on acetylcholinesterase (AChE) inactivation was developed for the monitoring of permethrin using a screen-printed three-electrode system [12]. Other research explored alternatives to enzymatic assays, for example an impedimetric test based on a layer-by-layer poly(allylamine chloride) (PAH) and poly[1-[4-(3-carboxy-4-hydroxyphenylazo)-benzenesulfonamide)-1,2-ethanediyl]sodium salt] (PAZO) polyelectrolyte [13], which can detect pyrethroids by direct physical interaction through stacking of benzene groups (present both in PAZO and pyrethroids). The Innovative Vector Control Consortium (IVCC) developed, and tested in the field, different colorimetric kit assays for indoor residual spraying (IRS) operations control, which are based on the detection of specific ions released from a targeted insecticide by alkaline hydrolysis (e.g., chloride ions from DDT hydrolysis [14]; or free cyanide obtained by hydrolysis of cyanopyrethroids - Type II pyrethroids [15]). The Centers for Disease Control and Prevention (CDC) applied the same principle to implement a colorimetric test to evaluate ITN [16]. All those previous examples are quite simple in operation and with analysis time around or below 30 minutes (i.e., sample extraction, preparation and reaction). In most cases, the result of the assay is semi-quantitative, allowing a binary decision. However, the specificity in some of these assays for univocally detection of pyrethroids is unclear or limited by the reaction used for detection, since other families of insecticides and other chemical compounds presents in the field can interfere with the reactions.

Analytical approaches based on the use of selective receptors may improve the selectivity of the above mentioned methods. Thus, fluorescence and chemiluminescence methods for deltamethrin detection based on molecular imprinted polymers (MIPs) and CdTe quantum dots as labels [17, 18] have shown accuracy and specificity, however the detectability achieved is low. Alternatively to those approaches, analytical systems with antibodies (Ab) as selective receptors elements have long been the most popular affinity-based recognition assays. Among selective assays, immunoassays have fascinating features, such as the possibility to respond selectively to biological or bioactive substances and the capability to respond in a physiological manner. This great affinity and specificity between the specific Ab and the analyte have turned these techniques into powerful analytical tools to detect and quantify insecticides at low concentrations and trace levels. The versatility and adaptability of the Ab allows the implementation of selective assays for a single family of insecticides, or spe-

cific members of the family even respect others in complex matrices. In terms of frequency of use, Enzyme-Linked Immuno Sorbent Assay (ELISA) is the gold standard for immunoassays. Regarding the detection of pyrethroids and pyrethroid metabolites, several assays to quantify residues in samples of different nature have been developed [19-21]. However, ELISA requires laboratory equipment, experienced personnel to run the analyses and is time-consuming (e.g., typically 1.5h to 2h including the multiple incubation times and washing steps). However, immunoassays can also be integrated in biosensing platforms to decrease both total analysis time and cost allowing automation and portability for in-field applications. These devices, called immunosensors, are analytical devices consisting of Ab in intimate contact with a physical transducer that converts the biorecognition process into a measurable signal (electrical, gravimetric, optical). A few such immunosenors for pyrethroid detection are available. Surface Plasmon Resonance (SPR), based on antibody-antigen recognition with/without enhancement with nanoparticles [22, 23], shows good sensitivity and specificity but operates in flow conditions, which can be a limitation in terms of cost and usability in field tests. The analyses are completed in less than 10 minutes, much faster than ELISA.

In the present study, alternatives to these analytical approaches were evaluated with the aim of using them in the field (e.g., to estimate the amount of active ingredient on bednets). Main requirements are low pyrethroid detectability levels, specificity, assay simplicity, short analysis time and low total cost per assay. With these objectives, the immunochemical determination of pyrethroids was investigated using two different platforms: (i) a label-free impedimetric immunosensorfor quantitative detection (Electrical Impedance Spectroscopy based biosensor – EIS biosensor); and (ii) a lateral flow immunocromatografic (LFIA) for semi-quantitative detection. Impedance spectroscopy is a powerful technique since it allows direct measurement of the resistance of a system. Surface phenomena taking place at the surface of an electrode can directly be recorded using this physical property. Thus, EIS biosensors provide the possibility to directly record specific biomolecular binding events when the electrode transducer has been previously biofunctionalized with a particular bioreceptor, providing quantitative data [24-28]. In contrast LFIAs are much more simple devices addressed to just detect the presence (or absence) of a particular target analyte. LFIAs are usually performed on porous paper or nitrocellulose membranes, able to allow the sample and the immunoreagents flow by capillarity, partially separating the components of the matrix and the selective capture of the analyte at particular sites of the membrane, where the bioreceptors have been immobilized. Usually, colloidal gold labelled immunoreagetns reagents are the species responsible for providing a colorimetric signal that can be visualized by the eye [29-31]. Both approaches have been validated by GC and ELISA. Finally, commercially available pyrethroid-treated ITN samples have been

analysed (i.e., pyrethroid-coated and pyrethroid-incorporated nets), for which purpose different extraction methods were tested in order to find better ways for in-field sampling while minimizing the impact on the ITN integrity. Thus we evaluated the extraction efficiency of a simple direct infusion of the bednets in extraction solvent, compared to standard sonication assisted extraction, as a reference method [32].

Materials and Methods

Net sampling and gas chromatography (GC-ECD)

The assays were conducted on two different types of ITN treated with pyrehtroids: (i) Interceptor bednets (BASF) use multifilament polyester surface coated with α-cypermethrin at a nominal concentration of 200 mg a.i./m^2; (ii) LifeNet bednets (BAYER) use mutifilament polypropylene with deltamethrin incorporated into every fibre, at nominal concentration of 340 mg a.i./m^2. Several square patches of 2X2 cm^2 were cut from the different nets provided by the manufacturers and used to test the different extraction methods.

Permethrin, α-cypermethrin, cypermethrin and deltamethrin standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). The solvents used in this study were all pesticide grade. n-Hexane, dichloromethane, methanol, ethanol and dioxane were from Merck (KGaA, Darmstadt, Germany)

The extraction capacity of n-hexane, dichloromethane, methanol, ethanol and dioxane was evaluated. Samples were introduced in screw cap 10 mL glass tubes, covered with solvents and stirred in a sonication bath with water, at room temperature (RT), during 15 minutes. The solvent was removed after extraction. This operation was repeated three times and all extraction solvents were combined and concentrated to 1 mL by vacuum rotary evaporation. These extracts were stored in 2 mL screw capped amber glass vials.

In field conditions, no sonication baths are available. The extraction capacities of dioxane and dichloromethane were therefore evaluated by infusion at RT. For this purpose, 2x2 cm net sections were introduced in screw cap glass tubes of 10 mL, filled with solvent to cover the net (4 mL) and left at RT for periods of 5-15 minutes. This method does not include a stirring step which involves lower potential extraction efficiency in comparison with sonication in which extraction is very intense.

For gas chromatography, aliquots of the above-mentioned sample extracts were evaporated to nearly dryness and redissolved in iso-octane for GC analysis. A GC equipped with a micro-electron capture detector (Agilent Technologies 6890 N) was used because the studied pyrethroids contain halogen atoms. Helium was the carrier gas (flow of 1.2 mL/min). Samples were injected in splitless (280°C) with a split time of 1.5 min. Separation was achieved with a HP-5MS fused silica capillary column (Agilent 19091S-433; 5% phenyl methyl siloxane; length 30 m, internal diameter 250 µm, phase thickness 0.25 µm). The oven was programmed from 150 °C (holding time 1 min) increasing to 250 °C at 15 °C/min, then to 280 °C at 2 °C/min and to 300 °C at 4 °C/min. The final holding time was 5 min. Detector temperature was 320°C. Nitrogen was used as make up gas at a flow of 60 mL/min.

Each compound was quantified by the sum of all isomeric peaks. Quantification was performed by the external standard method. One standard mixture was prepared with permethrin, deltamethrin and α-cypermethrin. Another standard mixture was prepared with cypermethrin. Linearity ranged between 10 and 100 pg/uL.

Competitive immunosassay

ELISA

The pH and the conductivity of all buffers and solutions were measured with a pH meter pH 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, DK). Washing steps were performed on ELX405 microplate washer (Biotek, Winooski, VT, USA). Absorbances were read on a Multiskan™ G spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA USA). The competitive immunoassay curves from the calibration with analytical standards were analyzed with a four parameter logistic equation using the software GraphPad Prism (GraphPad Sofware Inc., San Diego, CA, USA).

Immunochemicals and chemical reagents used to prepare buffers and ELISA assay were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Most of the chemicals used for cross-reactivity studies were acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Immunoreagents were kindly supplied by the Laboratory of Dr Bruce D. Hammock (Department of Entomology University of California, Davis). The preparation of the protein conjugates and the antisera is described elsewhere [19]. Stock solutions of the pyrethroid standards were prepared in DMSO at 10 mM

Unless otherwise indicated, phosphate-buffered saline (PBS) was a 0.01 M phosphate buffer on a 0.8% saline solution (pH 7.5). PBST was PBS with 0.05% Tween 20. Coating buffer was 0.05 M carbonate-bicarbonate buffer (pH 9.6). Citrate buffer was a 0.04 M solution of sodium citrate (pH 5.5). The substrate solution contained 0.01% TMB (3,3´,5,5´-tetramethylbenzidine) and 0.004% H_2O_2 in citrate buffer.

Microtiter plates were coated with coating antigen in coating buffer (0.5 μg mL^{-1}, 100 μL/well) overnight at 4 ^{o}C and covered with adhesive plate sealers. The following day, the plates were washed with PBST (four times, 300 μL/well) and deltamethrin standards (0.64 nM - 100 μM, in PBST) were added to the microtiter plates (50 μL/well), followed by the antisera (1/2000 in PBST, 50 μL/well). After 30 min of incubation time at RT, the plates were washed again as before and a solution of anti-rabbit IgG-HRP (1/6000 in PBST) was added (100 μL/well) and incubated for 30 min more at RT. The plates were washed again, and the substrate solution was added (100 μL/well). Colour development was stopped after 30 min at RT with 4 N H_2SO_4 (50 μL/well), and the absorbances were read at 450 nm. The standard curves were fitted to a four parameter equation according to the following formula: Y = [(A - B)/1 - (x/C)D] + B, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance, and D is the slope at the inflection point of the sigmoid curve.

For cross-reactivity determinations, standard curves were prepared for deltamethrin, and other structurally related compounds, i.e., permethrin, α-cypermethrin, paraquat, vomitoxin, and simazine in PBST (0.64 nM - 100 μM) and measured with the ELISA. For some analytes it was possible to build a standard curve that fitted to the four-parameter equation mentioned above. The cross-reactivity values were calculated according to the equation: (IC_{50} deltamethrin/IC_{50} tested compounds) x 100.

EIS biosensor

Electrical impedance spectra were obtained using an Ivium compactStat mobile measurement station (Ivium technologies, De Zaale, NT). IviumSoft v2.2 software (Ivium technologies, De Zaale, NT) was used for impedance data treatment and an equivalent circuit fitting. Data were fitted to a conventional Randless equivalent circuit (Additional file 1: Figure S1). Commercial interdigitated gold electrodes ED-IDE3-Au (Micrux technologies, Oviedo, Asturias, Spain) were used as transducers, and measurements were performed in ALL-IN-ONE PLATFORM (ref. ED-AIO-CELL) electrochemical cell (Micrux technologies, Oviedo, Asturias, Spain). Characterization of sensors was performed by impedance measurements in a 1 kHz – 1 MHz frequency range with a 25 mV (amplitude) voltage exci-

tation. The impedance response of an interdigitated electrode (IDE) in the absence of faradaic processes was emulated by an electrical equivalent circuit presented in Additional file 1: Figure S1 [33]. It was formed by the following components: R_C is the contact resistance introduced by wires and collector bars of the electrodes; C_G is the geometrical capacitance between two interdigitated electrodes in a water solution; R_S is the resistance of the solution between two electrodes of the array; and CPE_{DL}, which is a constant phase element associated with the capacitance of the electrical double layer at the electrode - water solution interface.

3-(glycidoxypropyl)trimethoxysilane (GPTS) was used to derivatize the transducer surface were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Methoxy-poly (ethylene-glycol)-amine (PEG-NH_2) was purchased from Laysan Bio, inc. (Arab, Alabama, USA).

Before chemical treatment the IDE samples were first cleaned with absolute ethanol and Milli-Q water and dried under N_2 flow. Immediately afterwards the samples were plunged in a 10 M NaOH aqueous solution at RT for 30 min and rinsed with Milli-Q water in order to neutralize the action of the base. After drying them with N_2, samples were silanized in gas phase with GPTS under vacuum conditions at 60°C. The terminal epoxy group of silane attached to the surface of the SiO_2 readily reacts with amino groups of biomolecules [34, 35], fixing them covalently to the surface. In this way immobilization of the deltamethrin antigen on the sensor surface modified with GPTS was performed. The sensors were immersed in a solution of antigen (1µg mL^{-1} in carbonate buffer) 0.6 mL for 4 hours at RT with orbital stirring. Afterwards, capping of the non-reacted epoxy groups was performed adding 0.1 mL of a solution of PEG-NH_2 (35 mg mL^{-1}) in ethanol anhydrous and reaction was left overnight (12 h) at RT with orbital stirring. Finally, the reaction liquid was washed away and sensors were rinsed with PBST buffer, Milli-Q water and dried with a N_2 stream.

First, biofunctionalized electrodes were characterized by impedance spectrometry in 5 µM PBS using the conditions explained before. Ten consecutive spectra were recorded until stabilization of the signal and only the data of the 3 last spectra were used. The measures were performed in triplicates to calculate the mean and the SD. Deltamethrin standards 0.1 nM - 10 µM, were prepared in PBST and added to the electrodes (50 µL/electrode) followed by theantisera (1/500, in PBST, 50 µL/electrode). After 30 min of incubation time at RT and with orbital stirring, the electrodes were washed with PBST buffer, Milli-Q water and dried with a N_2 stream. Afterwards, the electrodes were analysed again using impedance spectrometry with the same protocol an the Rs parameter was used for monitoring the difference in the impedance spectra due to antibody binding. The final results

corresponded to the difference with the Rs parameter of the intact biofunctionalized electrode. Calibration curves were analysed as for ELISA using a four-parameter equation.

Lateral Flow Immunoassay (LFIA)

Blank test strips were obtained from Unisensor Co. (Liège, Belgium). Finally, intensities of the gold spots were quantified using digital imaging public domain software (Image J 1.42, National Institute of Health, USA).

Goat anti-rabbit IgG labelled with gold nanoparticles (40 nm size, AuNP-anti-rabbit-IgG) were obtained from BBI solutions (Cardiff, UK). Rabbit anti-goat IgG whole molecule was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). One drop of coating antigen 2 mg mL^{-1} (1 µL) and anti-goat IgG 1/100 (1 µL) was added to the test strips. After that, test strips were dried in an oven at 40°C for 30 min.

For the Lateral flow immunoassay (LFIA), deltamethrin antiserum (1/50 in PBST, 30 µL) was added to a mixture of deltamethrin standards (0.1 nM - 10 µMin PBST, 105 µL) and AuNP-anti-rabbit IgG (1/10 in PBST, 15 µL). After 5 min incubation with orbital stirring the test strips were introduced in the solution for further 5 min. Finally, test strips were dried and photographed. Intensity levels for every strip were analysed with ImageJ (National Institutes of Health, USA) in two steps: (i) defining a circular region of interest (200 pixels diameter) on the test spot and quantifying the mean intensity of the selected region with the histogram function; (ii) repeating the same procedure to quantify the mean intensity of the white region right before the test spot. The results of the LFIA measurements were obtained calculating the difference between the two mean intensity values. The LFIA calibration curves with deltamethrin standards were analysed in the same way as for ELISA.

Accuracy Assessment

Four samples from LifeNet and Interceptor nets, obtained from the sonication assisted method, were measured using the immunochemical analytical methods established for deltamethrin. The analyses were made in triplicate and on 3 different days. Data were compared with the GC-ECD results.

Results

Extraction

Sonication is a known reference extraction method that has been used for evaluation of the extraction efficiency of the nets with the tested solvents. The use of this method with n-hexane, methanol and ethanol showed recoveries in the order of 50%, which led us to discard them as feasible choices (results not shown). Conversely, dichloromethane and dioxane provided recoveries of 101±2% (mean ± standard deviation) and 120±25%, respectively, for the extraction of α-cypermethrin on coated Interceptor nets (Figure 1 and Table 1). In the case of the deltamethrin incorporated into the LifeNet ITN, those two solvents provided extraction recoveries of 75±10% and 98±6%, respectively (Figure 1).

Keeping in mind the field tests, we also evaluated a simple approach for insecticide recovery such as direct infusion of the nets with the selected extraction solvents (Figure 1 and Table 1). Infusion of the Interceptor and LifeNet samples with dichloromethane, provided recoveries of 106±3.5% and 79±6.2%, respectively. The recoveries of infusion with dioxane were 109±4.5% and 73±1.2% in the two types of nets, respectively. Thus, both solvents provided complete extraction of the pyrethroid in the coated nets and recoveries of about 70-80% in the nets with incorporated pyrethroid.

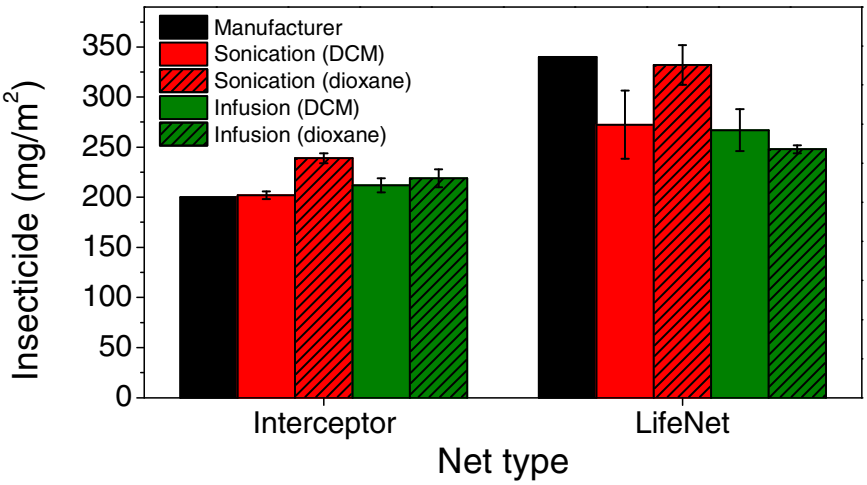

Figure 1. Insecticide extraction recovery from ITN. Comparison between the nominal concentration given by the manufacturer (black) and the concentrations measured by GC-ECD, after sonication assisted extraction (red) and direct infusion extraction (green). DCM: dichloromethane.

Table 1. Insecticide extraction efficiency (in %) from ITN comparing the extraction method (ultrasonication and direct infusion) and the extraction solvent (dichloromethane and dioxane). Percentages were obtained from dividing the concentrations measured by GC-ECD and the nominal values given by the ITN manufacturers.

	Sonication		Infusion	
Net type	**DCM**	**Dioxane**	**DCM**	**Dioxane**
Interceptor (BASF)	101 ± 2	120 ± 3	106 ± 4	110 ± 5
LifeNet (BAYER)	80 ± 10	98 ± 6	79 ± 6	73 ± 1

ELISA performance

Because of the higher high-throughput capabilities, microplate-based ELISA was used for the characterization of the immunoreagents and to assess performance of the assay on different conditions, as well as to know its response to the presence of several potential cross-reactants. The influence of the extraction solvent, i.e. methanol, ethanol, dioxane and dimethylformamide, on the ELISA performance was also studied. In all cases, the assay preserved good response when the presence of the solvent in the assay buffer was equal to or below 10%. Regarding the specificity of the immunoassay different insecticide analytical standards were tested in the assay to check their cross-reactivity (Figure 2 and Table 2). The immunoassay was responsive to the pyrethroids family (especially to Type II pyrethroids, such as deltamethrin and α-cypermethrin) and had low response to non-related compounds, where the maximum signal variation was close to the limit of detection for deltamethrin.

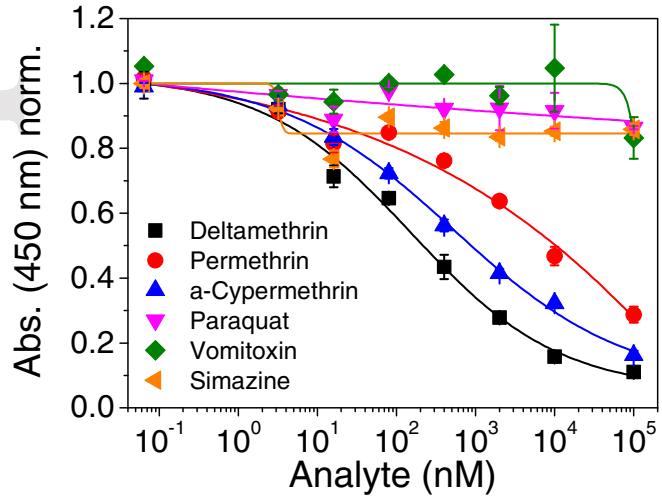

Figure 2. ELISA cross-reactivity studies with different types of insecticides and chemical compounds (deltamethrin, black square, permethrin, red dot; α-cypermethrin, blue regular triangle; paraquat, magenta inverted triangle; vomitoxin, green rhombus; simazine, orange tilted triangle).

Table 2. Results of the ELISA cross-reactivity studies[a].

Insecticide	C.R. (%)
Deltamethrin	100
Permethrin	48
α-Cypermethrin	73
Paraquat	<1
Vomitoxin	<1
Simazine	<1

[a] the results expressed in % correspond to the ratio between the IC50 of each tested compound and the IC50 of deltamethrin used as reference

Comparative analysis of the performance of the three immunochemical analytical methods used in this study: microplate-ELISA, EIS biosensor and LFIA

Full calibration tests with analytical standards, to determine the dynamic range (80% to 20% of the total signal variation) and the limit of detection (LOD: 90% of the total signal variation) for each technique were performed (Figure 3D and Table 3). Microplate-ELISA showed a wide dynamic range within three orders of magnitude of analyte concentration (12 nM – 1.5 µM; IC_{50} at 138 nM), and a LOD of 3.2 nM. The dispersion at every calibration point was very low, which gives good precision to this technique (mean coefficient of variation, CV, within the dynamic range below 5%). The EIS biosensor showed the lowest limit of detection at 0.6 nM, with a dynamic range within two orders of magnitude (1.7 nM – 61 nM; IC_{50} at 10.4 nM). However, this technique exhibited large dispersion in most of the calibration points, leading to poor precision (mean CV in the dynamic range was around 14%). Finally, the LFIA was the less sensitive technique, with a LOD of 67 nM, and showed a very narrow dynamic range within one decade (150 nM – 2 µM; IC_{50} at 505 nM).

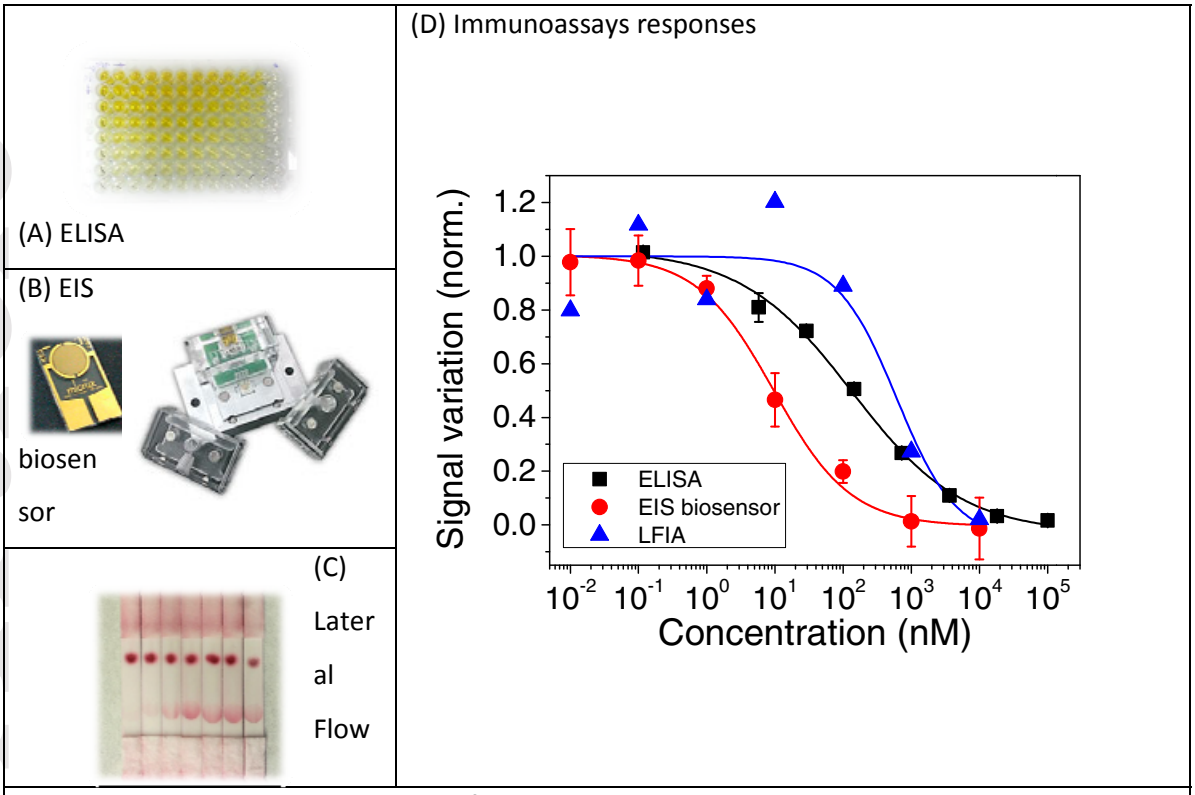

Figure 3. Analytical characterization of the immunochemical techniques used in this study. Photographs of the three techniques used: (A) ELISA, (B) EIS biosensor, and (C) Lateral flow. (D) Immunochemical responses to different deltamethrin concentrations recorded on each of the immunochemical analytical approaches. These curves provide the information to calculate the dynamic range of the assay (from 20% to 80% of the total signal variation), the IC_{50} (concentration that gives 50% of the total signal variation), and the limit of detection, LOD (90% of the total signal variation).

Validation with ITN samples analysis

The three immunochemical techniques established and the GC methods were used for comparative analysis of the insecticide content of the net samples (Figure 4). ELISA results showed good correlation with the quantification by GC-ECD (R^2 = 0.99257 for LifeNet and R^2 = 0.99919 for Interceptor nets). Moreover, a good accuracy was observed and also a very good precision: (i) for Interceptor α-cypermethrin coated nets, the relative difference percentages were around 1% with CV values below 6% (slope of the linear regression fitting: 0.99±0.01); (ii) in the case of LifeNet deltamethrin incorporated nets, both relative differences to GC-ECD values and CV values were below 5% (slope of the linear regression fitting: 0.98±0.04). In contrast, for the EIS biosensor the CV values were close to 30% (showing again a large dispersion around the reference values obtained by GC-ECD). Given the large dispersion, we focused on the use of the EIS biosensor on the ITN containing deltamethrin (compound to which the antibodies used in the assay are more specific).

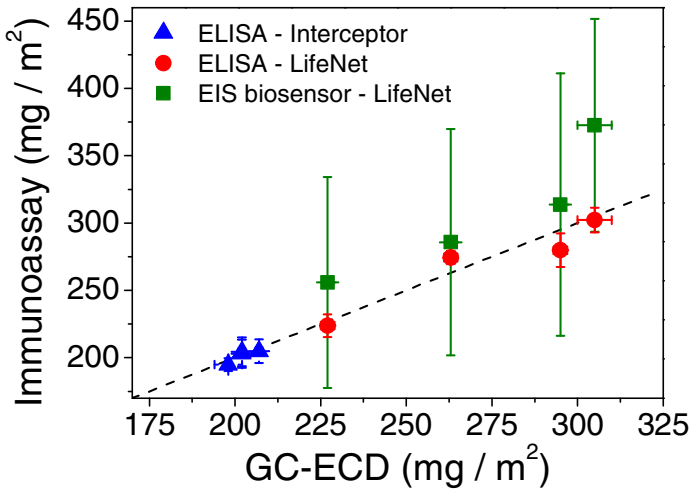

Figure 4. Evaluation of insecticide levels on commercial ITNs. Correlation between the insecticide levels measured with the immunochemical techniques and those obtained with the reference method (GC-ECD).

Discussion

ITN sampling

With the aim of defining a universal extraction method that could be easily used in most types of pyrethroid treated nets, for diverse compounds, i.e. deltamethrin, α-cypermethrin, permethrin, and type of net treatment, i.e. coated or incorporated, a selection of extraction solvents (dichloromethane and dioxane) and extraction methods (sonication and direct infusion) were evaluated. We found that extraction/recovery efficiencies in pyrethroid-coated nets were, in general, higher than in pyrethroid-incorporated nets. In surface-coated nets, the insecticide was more accessible for extraction than in those incorporating the compound into every fiber. The use of dioxane showed insecticide recoveries around 100% with sonication-assisted extraction.

With a simple direct infusion of a net patch in any of the selected extraction solvents (dichloromethane or dioxane), comparable results to those obtained with sonication (in both, insecticide coated and incorporated nets) can be achieved. However, dichloromethane is strongly irritating through skin contact, it is highly inhalable, and it is not soluble in water, which can compromise its handling for non-experts and its use/compatibility in the immunoassay. Conversely, dioxane is strongly water soluble and does not produce strong reactions with the skin, which leads us to propose this extraction solvent as the best option for this application. Furthermore, dichloromethane is classified 2A by IARC (probable carcinogen for humans [36]), whereas dioxane is classified 2B (possible carcinogen for humans [36]) which obviously involves much lower risk. These results can be used as first step to define a non-destructive sampling method for bednet analysis.

Evaluation of distinct immunochemical analytical techniques: advantages and limitations

In this study, we propose the use of immunochemical analytical techniques as an alternative to the laborious and expensive chromatographic analysis, in the specific case of determining the insecticide content in treated bednets. We also show how those studied immunochemical methods respond specifically to pyrethroids, and especially to Type II pyrethroids. The main features of the implemented immunochemical methods are summarized in Table 3. ELISA performed very well, both with analytical standards and real samples from commercial bednets. It allowed accurate quantification of the insecticide concentrations in the samples and afforded a reasonable high-throughput format (e.g., 96 well plates), and in an affordable price range (i.e., about 3€/test). Thus, ELISA is a good alternative to GC in the laboratory. However, the degree of automation required for good accuracy and precision in the quantitative measurements means that ELISA could not be conceived yet as a portable system for field applications.

For portability and integration, we propose the use of biosensors, and in this study we evaluated an immunosensor based on Electrical Impedance Spectroscopy (EIS biosensor). The immunosensor showed good sensitivity and dynamic range, also reducing the analysis time from 100 minutes, required in ELISA, to 30 minutes. The shorter time is due to the use of a label-free detection (with just one incubation during the competitive assay, avoiding any enzymatic amplification). This approach has similar unit price, around 5€/test (at a prototype cost with commercially available components; not considering reduced cost due to mass production). Nevertheless, the dispersion found, both in the calibration curves and in the analysis of real samples, decreases the accuracy and precision of the quantification of insecticide levels. This dispersion could be partially reduced by standardizing some critical steps, as for instance automation of electrodes surface modification and cleaning steps. However, there are also intrinsic contributions to the impedimetric measurement, e.g., temperature variations or buffer ionic strength, that affect the double layer contribution, and to the label-free approach, i.e. avoiding amplification steps, and thus extra incubation times, limits the maximum signal variation, which are not always easy to limit. Thus, this approach must be considered as a semi-quantitative technique.

Finally, LFIA combines most of the features desired for a field test analytical tool: (i) it is integrated and portable; (ii) it is very simple to use and to manipulate; (iii) it is fast (about 5 minutes per test); and (iv) it is cheap (1€ or less per test). However, due to its current narrow dynamic range, lateral flow strips provide binary information (i.e., below or beyond a threshold value), being more

qualitative than quantitative. This limitation can be improved by standardizing the fabrication and using a reader, as for the commercial lateral flow tests implemented for other applications (e.g., clinical, veterinary, food and beverage, pharma, environment [37]), which can turn it into a semi-quantitative assay.

Therefore, the requirements of each analysis of the ITN will determine the selection of the right immunochemical approach : (i) LFIA will be useful when it is need to quickly determine if a ITN has enough insecticide to be effective against mosquitos (setting the threshold value to the value where mosquito mortality is around 80% [38]). (ii) The EIS biosensor can provide additional information in the cases in which the insecticide content is half-way between the highest concentration and the threshold value requiring net replacement. (iii) ELISA cannot be performed *in-situ* since it requires many steps of manipulation. However, this laboratory technique can analyse multiple samples at the same time, with good accuracy and precision for the quantification of active bednets ingredient.

The implemented immunoassay relies on anti-deltamthrin polyclonal antibodies that showed some cross-reactivity to pyrethroid analogues [19]. However, this issue is not a problem, since nets are usually treated with a defined insecticide or a mixture of them. In case other pyretrhoids have also been used, the cross-reactivity of the antibody used in this study can be an advantage, since their levels in the net could also be quantified. Nevertheless, the specificity of the antibodies can be modulated by an appropriate hapten design or/and by an exhaustive monoclonal antibody screening. This would allow developing more specific test, including tests for new molecules currently under evaluation [39]. This is an important advantage of this method as: (1) it could be more specific if required, and (2) it could be adapted/used for other molecules, including new tests being under evaluation. This can be interesting for IVCC and other industrial partners.

Table 3. Comparison of the competitive immunoassay in the three formats studied: ELISA, EIS biosensor and LFIA.

Method	Antigen	Antisera	IC_{50}	LOD	Dynamic range	Analysis time	Cost	Pros	Cons
ELISA	0.5 µg/mL (100 µL/well)	1/2000 (50 µL/well)	138 nM	3.2 nM	12 nM – 1.5 µM	100 min	3 €	Quantitative Specific	Lab system Analysis time
EIS biosensor	1 µg/mL (600 µL/sensor)	1/500 (50 µL/sensor)	10.4 nM	0.6 nM	1.7 nM – 61 nM	30 min	5€	Portable Specific Label-free	Standardization Semi-quantitative
LFIA	2 mg/mL (1 µL/strip)	1/50 (30 µL/strip)	505 nM	67 nM	150 nM – 1.5 µM	5 min	1€	Portable Simple Cheap Specific	Qualitative

Conclusions

Direct infusion of the net fabric samples into the extraction solvent is a simple and non-destructive alternative to sonication. This extraction method could be easily implemented in the field.

ELISA can be a good surrogate for chromatography as laboratory technique. The EIS biosensor in its current label-free approach cannot provide the accurate quantification of insecticide levels required. However, if the signal to noise ratio is increased (e.g. use of secondary antibody), the EIS biosensors could provide an integrated and portable version of the electrochemical ELISA-like. Finally, the LFIA test prototype developed in this study is the most promising approach to a field-friendly and affordable semi-quantitative test for insecticide concentration in ITN.

The immunochemical approaches presented here could also be useful for testing other new molecules as active compounds of IRS and ITN, by just developing the appropriate immunoreagents.

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Authors' contribution

AP and MN conceived and supervised the research, and MC coordinated the different experimental studies. Competitive immunoassay validation and ELISA studies were performed by JRA. MC and JRA implemented and characterised the EIS biosensor and the lateral flow competitive immunoassays. Sample extraction and chromatography studies were performed and analysed by YGQ, JL and JOG. Competitive immunoassays data analysis was performed by MC and JRA. MC, JRA, JOG, MPM, MN and AP contributed to the preparation of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.