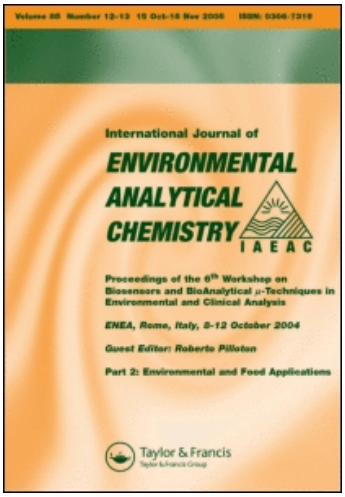

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Development of a colourimetric pH assay for the quantification of pyrethroids based on glutathione-S-transferase

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Development of a colourimetric pH assay for the quantification of pyrethroids based on glutathione-S-transferase

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Recombinant glutathione-S-transferases (GSTs) can be used as analytical tools for the development of simple insecticide quantification assays. This assay explores the ability of pyrethroids to promote inhibition of the GST-catalysing 1-chloro-2,4-dinitrobenzene (CDNB)/glutathione (GSH) conjugation reaction. The sensing scheme is based on the pH change occurring in a weak buffer system by the GST reaction, which is measured using a spectrophotometer and the dye indicator bromothymol blue (616 nm). Practical use depends on the recognition affinity of the GST for insecticides, inhibition kinetics, enzyme stability and compatibility with the detection assay. In this study we compared the recombinant GSTs AgGSTD1-6 and AdGSTD1-1 from the mosquito vectors *Anopheles gambiae* and *Anopheles dirus*, respectively, with high affinity for pyrethroids, for their suitability for detecting pyrethroids in vector disease control programmes. The results showed that AgGSTD1-6 was the most suitable enzyme with the best structural stability at higher temperatures ($T_m = 57^\circ C$) and pH optima in the alkaline range (pH 7.7). Using the AgGSTD1-6, we subsequently developed a pH-change colourimetric assay for detecting pyrethroids. Linear calibration curves were obtained for deltamethrin ($R^2 = 0.99$) with useful concentration ranges of 0–50 $\mu g\,mL^{-1}$. The effect of temperature in the range 25–40°C on the pyrethroid quantification assay was negligible. The assay was validated with extracts from insecticide sprayed surfaces and found to be reproducible and reliable compared with the standard reverse-phase high performance liquid chromatography (Rp-HPLC) method. The potential of the assay for monitoring insecticide residues in the frame of insecticide based malaria control interventions is discussed.

Keywords: pesticides/endocrine disruptors; bioanalytical methods; enzymes

1. Introduction

Pyrethroids are among the most successful insecticides ever used [1]. They have several distinct advantages, such as great selectivity and potency for insect sodium channels, low mammalian-toxicity and rapid killing effect [2]. Thus, they have become a widely favoured

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class of insecticide commanding approximately 20% of the world insecticide market. They are extensively used in disease vector control interventions, particularly for insecticide residual sprays (IRS) and insecticide treated bednets (ITNs), currently the most effective means of blocking the transmission of malaria to man.

While ITNs and IRS are the major preventive tools in the global fight against malaria and other diseases carried by insects, a number of unresolved operational issues prevent their potential benefits being maximised. One limitation is the lack of cheap and simple routine testing systems for quality control to determine adequate insecticide treatment. This is particularly important in order to monitor distribution, or to advise on the re-treatment of insecticide treated material. The only way to check that protection is being provided by a spray or treatment intervention is to measure the actual amount of insecticide residue remaining. Such information is particularly important for local agencies/users in disease endemic countries.

Enzyme systems based on glutathione-S-transferases (GSTs) have been recently reported as a viable option for detecting xenobiotics. They have been considered as advantageous, in light of the cost and complexity vs. specificity and quantification accuracy, compared with bioassays, analytical methods (gas chromatography-mass spectrometry [GC/MS]) and immunological techniques [3–5].

Insect GSTs are detoxification enzymes that play an important role in insecticide resistance. They can have high affinity for specific insecticide molecules and have therefore been employed for biosensor applications that target insecticides used for mosquito control [6–8]. The delta class of GSTs have been extensively studied in mosquito species. These include AgGSTD1-6 and the AdGSTD1-1 from the mosquito vectors *Anopheles gambiae* and *An. dirus,* respectively. They show remarkably high affinity for pyrethroid insecticides, which dramatically inhibit their activity against model enzymatic substrates [9,10].

With the exception of the GSTE2 DDT dehydrochorinase assay [7], the majority of the GST-analytical systems are based on the inhibition of GST activity by xenobiotics [6,7,11,12]. Detection and quantification of the xenobiotics can thus be based on inhibition measurements of reaction rates using spectrophotometric or ion-selective potentiometric methods [6,8]. A colourimetric GST assay has also previously been developed for the quantification of pyrethroid insecticides, using iodometric titration [6,11,13]. However, the assay is technically demanding and provides moderate accuracy, as it relies on the detection of the non conjugated substrate GSH, only a small fraction of which is utilised in the enzymatic reaction.

In this study, we have compared mosquito recombinant GSTs, AgGSTD1-6 and AdGSTD1-1 with characterised affinity for pyrethroids, for their suitability as analytical tools for the quantification of pyrethroids. This information has been used to develop a simple and robust pH-change colourimetric assay using the dye indicator bromothymol blue, which can be adapted for use in the field.

2. Experimental

2.1 *Materials*

Insecticides were obtained from Greyhound (Birkenhead, Merseyside, UK) and Sigma-Aldrich (Poole, Dorset, UK). Unless previously indicated, all other reagents and analytical grade chemicals were acquired from Sigma-Aldrich.

2.2 *Methods*

2.2.1 *Expression and purification of biosensor GSTs*

Escherichia coli strains of BL21(DE3) containing the *Anopheles gambiae* Zands recombinant AgGSTD1-6 and the *Anopheles dirus* AdGSTD1-1 genes were prepared as described in [10] and [9], respectively.

Recombinant enzymes were purified from 200 mL cultures grown in Luria-Bertani (LB) Broth. Following induction with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and overnight growth at 25°C, bacterial cells were harvested by centrifugation at 10,000 *g* for 10 min and the cell pellet resuspended in 20 mL of buffer A (50 mM Tris-HCl pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl). After two rounds of freeze-thawing, the cells were resuspended in buffer B (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 10 mM dithiothreitol, and 250 U Benzonase^{TM}) and sonicated 4 times for 30 s in a Sonics Materials Vibracell. The cell debris was removed by centrifugation (10,000 *g* for 20 min). AdGSTD1-1 and AgGSTD1-6 were purified from the cell supernatant using a 1 mL GSTrap 4B column (GE Healthcare, Little Chalfont, Bucks, UK) equilibrated in buffer A. The bound proteins were eluted with 20 mM glutathione (GSH) in buffer A. The fractions containing active enzyme were passed through a PD-10 column (GE Healthcare) to remove the GSH, and the enzymes were stored in 50 mM sodium phosphate, pH 7.4, 10 mM dithiothreitol and 40% (v/v) glycerol.

2.2.2 *Assay of enzyme activity and protein*

Assays for GST activity were performed by monitoring the formation of the conjugate of 1-chloro-2,4-dinitrobenzene (CDNB) and GSH at 340 nm ($\varepsilon = 9.6\,mM^{-1}\,cm^{-1}$) and 25°C, according to the method of Habig [14]. AdGSTD1-1 and AgGSTD1-6 were incubated in 0.1 M sodium phosphate, pH 6.5 buffer supplemented with 2.5 mM GSH, 1 mM CDNB and 20% (v/v) methanol in either a reaction volume of 1 mL (spectrophotometric assay), or a reaction volume of 250 µL (microtitre plate assay). Protein concentrations were measured using the Bio-Rad protein assay reagent with bovine serum albumin (BSA) as the protein standard [15].

2.2.3 *Kinetic studies of GST enzymes*

Initial velocities for the GST-catalysed reaction with GSH as the variable substrate were determined at 25°C in a total volume of 250 µL, containing 0.1 M sodium phosphate, pH 6.5, 1.0 mM CDNB and different concentrations of GSH. With CDNB as the variable substrate, the 250 µL reaction mixture contained 0.1 M sodium phosphate, pH 6.5, 2.5 mM GSH (AdGSTD1-1, AgGSTD1-6) and different concentrations of CDNB. The apparent kinetic parameters (k_{cat} and K_m) were determined using the program GraFit (Version 5, Erithacus Software, Horley, Surrey, UK).

2.2.4 *Interaction of recombinant GSTs with various pyrethroid insecticides*

The inhibition caused by six pyrethroid insecticides (cyfluthrin, etofenprox, deltamethrin, alpha-cypermethrin, permethrin and lambda-cyhalothrin) on the catalytic activity of AdGSTD1-1 and AgGSTD1-6 was evaluated. Insecticide stocks of 12.5 µM, 25 µM and 50 µM in methanol, which were equivalent to the ranges 5.6–22.5 $\mu g\,mL^{-1}$ for lambda-cyhalothrin, 4.9–19.6 $\mu g\,mL^{-1}$ for permethrin, 5.2–20.8 $\mu g\,mL^{-1}$ for alpha-cypermethrin,

6.3–25.3 μg mL^{-1} for deltamethrin, 4.7–18.8 μg mL^{-1} for etofenprox and 5.4–21.7 μg mL^{-1} for cyfluthrin, were added to the reaction mixture containing GSH (2.5 mM), 1 mM CDNB, 40–100 ng enzyme, in 0.1 M sodium phosphate, pH 6.5. The final concentration of methanol in the reaction mixtures was 10% (v/v).

2.2.5 *pH optima of GST enzymes*

The pH dependence of AdGSTD1-1 and AgGSTD1-6 were determined using the microtitre plate assay method described above, with the following buffers: 0.1 M citrate (from pH 2.5 to 3.75), 0.1 M citrate-phosphate (from pH 4.6 to 7.3), 0.1 M sodium phosphate (from pH 7.4 to 7.8), 0.1 M Tris-HCl (from pH 8.2 to 9.2) and 0.1 M glycine-NaOH (from pH 9 to 9.7). The salt concentration of each buffer was adjusted to 0.1 M using a 1 M NaCl stock solution. In addition, as GSH spontaneously conjugates to CDNB at higher pH values, controls with no added enzyme were assayed, and these data were subtracted from the enzyme activity data to obtain a reliable estimate for the activities at higher pH values.

2.2.6 *Enzyme stability and stability upon storage at room temperature*

The GST enzymes were incubated at temperatures between 30°C and 72°C for 10 min. The protein concentrations of AdGSTD1-1 and AgGSTD1-6 in 0.1 M sodium phosphate buffer were both 5 μg mL^{-1}. Following incubation, the enzymes were subsequently assayed for residual activities using the microtitre plate assay as described above. Melting temperature (T_m) values were determined from the plot of residual activity (%) vs. temperature (°C). The stabilities on storage of AdGSTD1-1 (0.081 μg) and AgGSTD1-6 (0.083 μg) at room temperature (20–25°C) were measured in 0.1 M sodium phosphate pH 6.5 in the absence, or presence, of GSH (0.1 mM). The time-course of enzyme inactivation was determined at regular intervals by removing aliquots of sample for assaying CDNB conjugation activity using the spectrophotometric assay as described above.

2.2.7 *pH-change colour assay of the GST-catalysed reaction*

Thirty μL of deltamethrin standard or sample in methanol, prepared as described above, were applied to the wells of a microtitre plate. Sodium phosphate buffer, pH 7.6 at a concentration of 2 mM, 10 mM NaCl, 2.5 mM GSH, 0.06 U enzyme and 1 mM CDNB were subsequently added to a volume of 250 μL. The final methanol concentration of the reaction was 20% (v/v). The reaction was incubated for 30 min at 25°C to allow for optimum H^+ ion production. A 12.5 μL aliquot of the bromothymol blue indicator dye solution diluted 1/1.5 in methanol (12.5 μg mL^{-1} final concentration) was subsequently added to each well of the microtitre plate. The absorbance value of each sample or standard was measured at 616 nm, and these results were subtracted from the equivalent negative control absorbance to give a value for OD_{616}. The standard curve for each enzyme was obtained by plotting deltamethrin concentration (0, 5, 10, 20, 30, 40, and 50 μg mL^{-1}) versus OD_{616}.

To measure the effect of temperature on the colourimetric assay, AdGSTD1-1 or AgGSTD1-6 (0.06 U of each enzyme) was added to a microtitre plate in the presence of 0, 13, 25 or 50 μg mL^{-1} deltamethrin and 2 mM sodium phosphate pH 7.6, 10 mM NaCl, 2.5 mM GSH and 1 mM CDNB in a final volume of 250 μL. The reaction was incubated

for 30 min at 25°C, 30°C, 35°C or 40°C before adding the bromothymol solution (12.7 μg mL^{-1} final concentration) and determining a value of OD_{616} as described above.

2.2.8 *Determination of insecticide residues on treated surfaces*

Insecticides sprayed on tile surfaces in the range of World Health Organization (WHO) recommended concentrations were extracted from tiles (54 cm^2 total sprayed area) by swabbing. For the swabbing method, glass filter surface swabs of 5 cm diameter (SKC, Eighty Four, PA, USA) for bioaerosols and xenobiotic contaminations were used. Filters were subsequently transferred to a glass tube, and insecticide residue was extracted by acetone (3×). Extracts were combined, evaporated to dryness, and re-dissolved in 2 mL of methanol. Aliquots were used either for HPLC analysis or the GST enzyme assay. HPLC analyses were performed on a reverse-phase Dionex Acclaim C18 column (120 Å, 250 × 4.6 mm, 5 μ, Dionex, Camberley, UK) with an Ultimate 3000 UV detector, as previously described [7]. The quantities of insecticides were calculated from standard curves established by known concentrations of authenticated standards.

3. Results

3.1 *Inhibition analysis and kinetic characterisation of AdGSTD1-1 and AgGSTD1-6 enzymes*

The most suitable enzymes for use in analytic applications are those with the highest sensitivity for an analysis, thus reducing the threshold limit of detection as low as possible. Therefore a comparison of the catalytic efficiency of the two mosquito recombinant GSTs AdGSTD1-1 and AgGSTD1-6 against the substrate CDNB, and the inhibition potency by pyrethroids, a property tightly associated with their detection efficiency, were initially performed. In line with previous studies, the enzymes showed similar catalytic efficiencies ($k_{cat}/K_m = 787.4\ mM^{-1}\ s^{-1}$ and $406.6\ mM^{-1}\ s^{-1}$ for AdGSTD1-1 and AgGSTD1-6, respectively) with the substrate CDNB [9]. The recognition affinity (inhibition potency) of the AgGSTD1-6 and AdGSTD1-1 was evaluated against six pyrethroid insecticides registered for use on insecticide treated material. From the data it is evident that AdGSTD1-1 and AgGSTD1-6 have similar inhibition profiles, both being inhibited >90% by all pyrethroids at concentrations above 50 μM (Figure 1).

The pH optimum profiles for AdGSTD1-1 and AgGSTD1-6 were also evaluated and the results are summarised in Table 1. All were within or close to the detection range of the indicator bromothymol blue, which changes colour from blue at pH 7.6 to yellow at pH 6.0. AgGSTD1-6 showed a slight alkaline preference (7.7) relative to AdGSTD1-1 (7.0).

3.2 *Structural stability*

Heat inactivation studies were carried out to compare the structural stabilities of the two enzymes. As shown in Figure 2a, the inactivation profiles for the enzymes exhibited a clear transition with inflection points corresponding to T_m values of 57°C for AgGSTD1-6, and 49°C for AdGSTD1-1. These compare with T_m values of 56–58°C measured for a maize GST I [16], *Ochrobactrum anthropi* GST [17] and human GSTA1-1 [18].

As a further measure of stability at room temperature (25°C), the catalytic activities of the three GSTs were monitored over a period of 120 hours (Figure 2b).

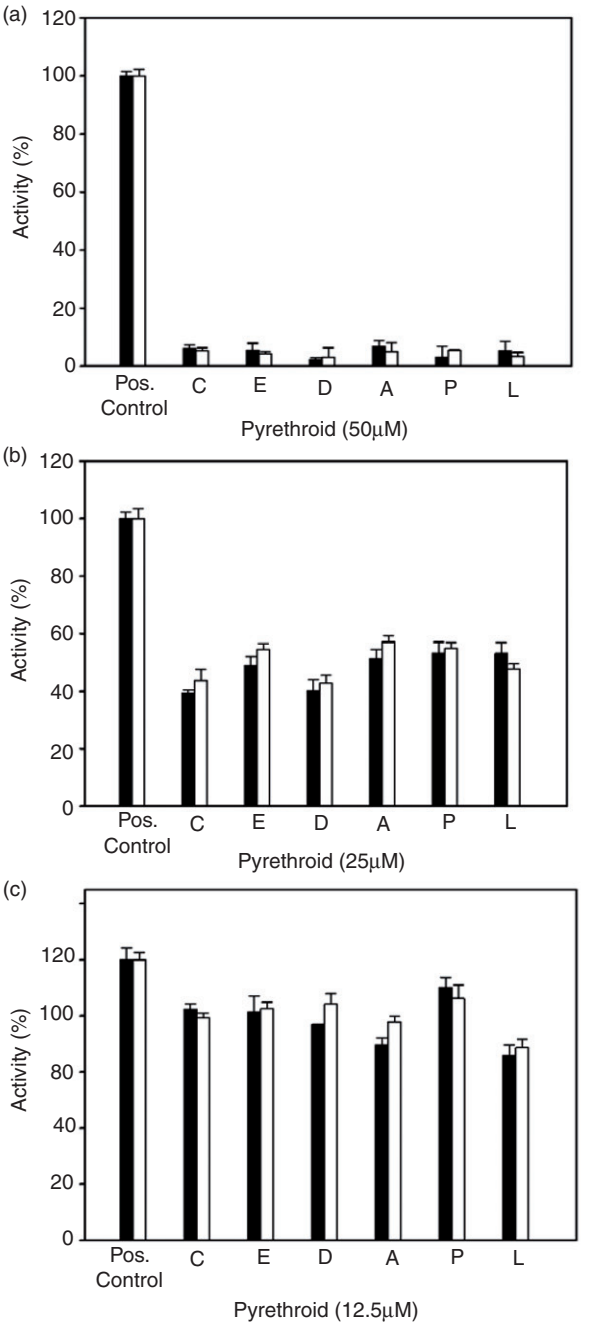

Figure 1. Pyrethroid inhibition of *Anopheles* GST enzymes. AdGSTD1-1 (9 mU, black bars) and AgGSTD1-6 (11 mU, white bars) were incubated with 50 μM, 25 μM and 12.5 μM pyrethroid as indicated. Pyrethroids shown are cyfluthrin (C), etofenprox (E), deltamethrin (D), alpha-cypermethrin (A), permethrin (P) and lambda-cyhalothrin (L). CDNB conjugating activity was measured using a microtitre plate assay as described in 'Materials' and 'Methods'. Activity was expressed as a percentage of the CDNB conjugating activity of the positive control (no added pyrethroid).

Table 1. pH optima of GST enzymes.

Enzyme	pH maximum	pH range 50% activity	pH range 25% activity
AdGSTD1-1	7	5.8–8.5	5.4–8.9
AgGSTD1-6	7.7	6.4–9.1	5.6–9.2

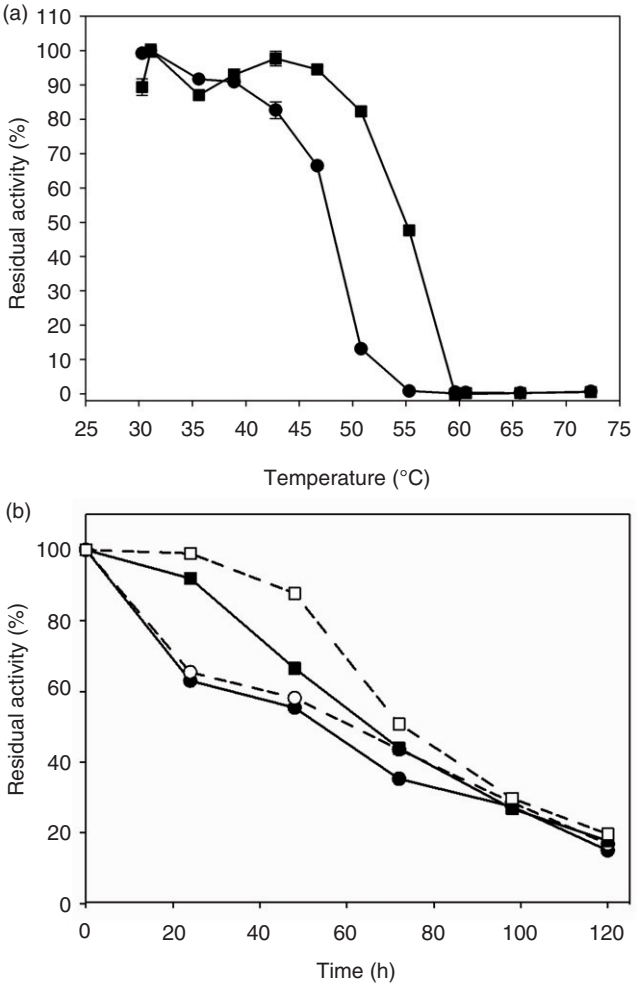

Figure 2. Structural stability of *Anopheles* GST enzymes. (a) Thermal inactivation of AdGSTD1-1 (circles) and AgGSTD1-6 (squares) in 100 mM sodium phosphate pH 6.5. (b) Stability comparison of AdGSTD1-1 (circles) and AgGSTD1-6 (squares) at 25°C in 100 mM sodium phosphate in the absence (solid lines) or presence of 0.1 mM glutathione (dashed lines).

In general, AgGSTD1-6 and AdGSTD1-1 followed a linear decay with AgGSTD1-6 maintaining higher levels of activity than AdGSTD1-1. Glutathione at a concentration of 0.1 mM was also added to each of the GST enzymes as it was shown to enhance the stability of a mutant maize GST I [8]. Our data show that addition of

GSH slightly enhances the stability of AgGSTD1-6 but had minimal effect on the AdGSTD1-1.

Based on catalytic activity and pyrethroid inhibition, AdGSTD1-1 and AgGSTD1-6 appeared to be similarly good candidates as analytical tools for pyrethroid detection. However, the AgGSTD1-6 showed slightly better structural stability at higher temperatures compared to the AdGSTD1-1 and higher pH optimum towards the alkaline area. Thus, AgGSTD1-6 was used for further development of a simple colourimetric assay.

3.3 *Development of a AgGSTD1-6-based pH change colourimetric assay for detecting pyrethroids*

The sensing scheme is based on the ability of GST to catalyse the conjugation of GSH with CDNB with concomitant proton release (H^+Cl^-). The concentration of released protons is proportional to the amount of conjugated substrate and can be measured spectroscopically using a pH dye-indicator [7]. The concentration of pyrethroids can be determined indirectly by measuring the different degree of pH change due to enzyme inhibition by pyrethroids. This pH change can be related to the amounts of pyrethroids using a calibration curve.

A visual-colourimetric assay for detecting pyrethroids was produced by incorporation of pH indicator dye (bromothymol blue) into the optimised AgGSTD1-6/pyrethroid inhibition reaction mixture. Standard curves for the quantification of deltamethrin by the AgGSTD1-6 are shown in Figure 3. Linear calibration curves were obtained for deltamethrin ($r^2 = 0.99$), with a useful concentration range of 0–50 $\mu g\,mL^{-1}$.

The effect of temperature on the assay was also determined (Figure 4). An incubation temperature of 25°C–30°C exhibited the best dynamic range, thus this is the optimum proposed temperature for performing this test. However, no appreciable difference was observed at the higher temperatures (up to 40°C).

3.4 *Determination of pyrethroid insecticides from sprayed surfaces using the AgGST1-6 pH change colourimetric assay*

We tested the practical use of the assay by comparing its efficiency against the gold standard high performance liquid chromatography (HPLC) analytical methodology with a number of insecticide swabs from deltamethrin-sprayed tile surfaces (at WHO recommended dosages, thus insecticide levels expected in the field, for example, for a post spray QC application). Insecticide quantities were calculated by converting results obtained spectrophotometrically into concentrations using the standard curve derived from analytical standards. A standard curve was similarly prepared from known concentrations of authenticated standards for the HPLC analysis. Figure 5 depicts the correlation between the analysis of deltamethrin swabs by the recombinant biosensor assay and by HPLC. Quantification showed the same pattern whether they were measured by HPLC or by the colourimetric assay with a correlation coefficient of $R^2 = 0.86$. The insecticide measurements were found to be highly reproducible and reliable against the standard HPLC methodology.

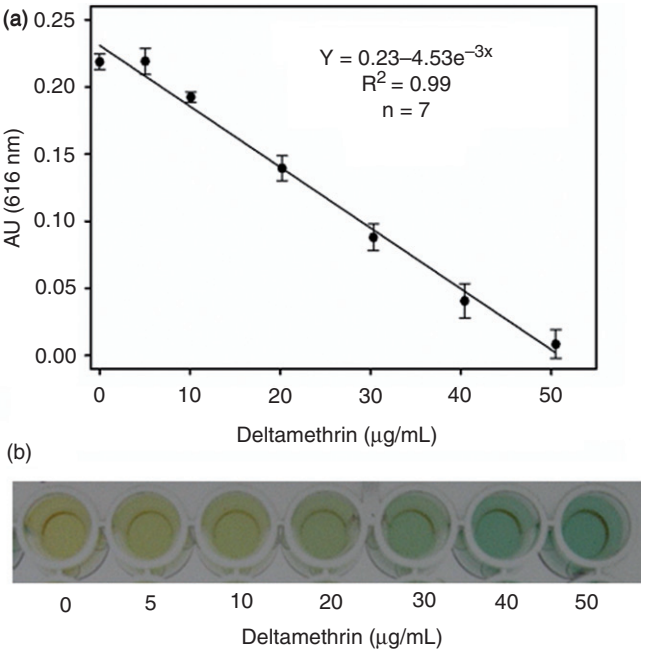

Figure 3. Potentiometric-pH colour assay standard curves. (a) Standard curve obtained for deltamethrin using AgGSTD1-6. (b) Microtitre plate demonstrating the colour changes associated with the incubation of GST (0.06 U) in the presence of different concentrations of deltamethrin (0–50 µg mL^{-1}). Colour development was achieved and measured as described in 'Materials' and 'Methods'.

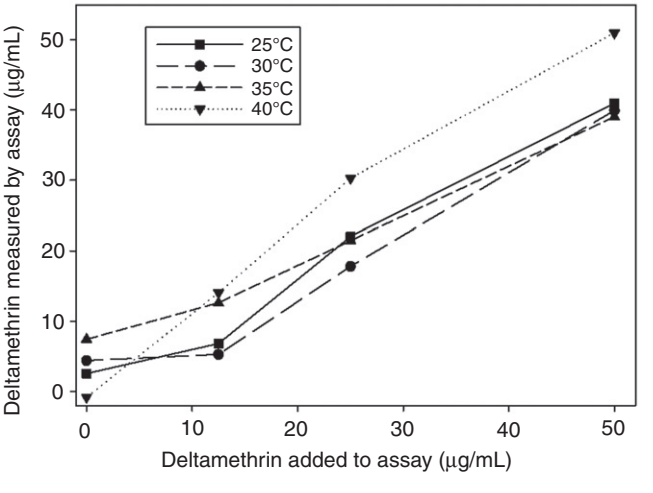

Figure 4. Effect of temperature on the colorimetric-pH colour assay. AgGSTD1-6 (0.06 U) was incubated for 30 min at 25°C (■), 30°C (●), 35°C (▲) and 40°C (▼) with either 50 µg mL^{-1}, 25 µg mL^{-1}, 13 µg mL^{-1} or 0 µg mL^{-1} deltamethrin in 2 mM phosphate buffer, pH 7.6 supplemented with 10 mM NaCl, 2.5 mM glutathione, 1 mM CDNB in a total volume of 250 µL. Colour development was achieved and measured as described in 'Materials' and 'Methods'.

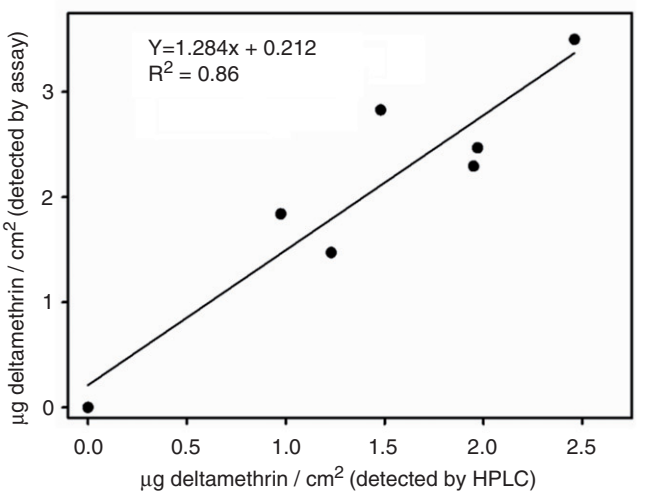

Figure 5. Comparison of the AgGSTD1-6 pH colour assay with HPLC for the determination of insecticide concentration of extracts from surfaces sprayed with deltamethrin. Deltamethrin swabs from sprayed tiles at the range of WHO recommended application dosage were quantified by standard analytical HPLC and the recombinant AgGSTD1-6 assay. Quantification showed a similar pattern with a correlation coefficient of $R^2 = 0.86$.

4. Discussion

Recombinant GSTs have been previously employed in simple insecticide quantification assays [8,11]. Here, we have optimised a GST system for measuring pyrethroids recommended by the WHO for disease vector control interventions, using a pH-change colourimetric format.

We have compared two mosquito delta class GSTs, AgGSTD1-6 and AdGSTD1-1, for their suitability for quantification against six different pyrethroids. AgGSTD1-6 was previously used for the quantification of permethrin and deltamethrin in an iodometric assay [6] while AdGSTD1-1 had shown a significantly higher degree of inhibition by both type I and II pyrethroids compared to the AgGSTD1-6 [9].

As with previous studies [9], the catalytic efficiency of the AdGSTD1-1 and the AgGSTD1-6 enzymes were found to be similar. The inhibition profile was evaluated against different concentrations within the solubility limits of the six pyrethroids. This was done for side-by-side comparison of the dynamic range of the two GSTs. The analysis showed that the AdGSTD1-1 and AgGSTD1-6 were inhibited at an equally low concentration (50 µM) for all pyrethroids. Amongst the GSTs tested AgGSTD1-6 was considered the best enzyme for further development of a pH-change colourimetric assay for detecting pyrethroids. This was based on the strength of inhibition by all six pyrethroids, stability at highest temperatures, and pH optima at 7.7.

Kapoli *et al.* [8] recently reported a potentiometric assay for the detection of malathion via the monitoring of associated GST inhibition. The sensing scheme utilised the ability of GST to catalyse the conjugation of GSH with CDNB. The reaction releases protons proportional to the amount of conjugated substrate and thus is measurable using a pH electrode. In order to simplify the assay for high throughput analysis, we have converted the inhibition assay into a colourimetric format by incorporating the indicator dye

bromothymol blue. This allows end-point measurements of reactions to be done spectrophotometrically using a microtitre plate. Compared with the potentiometric analysis, the assay requires minimal sample and enzyme volumes and can be visually determined (i.e. semi-quantitative scoring by eye), which facilitates use for field applications. The recombinant enzyme can be produced consistently in large quantities, it is stable in its lyophilized form (80–100% stability at 4°C, for more than 6 months, Dowd and Morou, unpublished results), and thus, it can be easily adapted for use in basic labs.

In order to test the practical application of the assay, a preliminary test on a number of insecticide swabs from pyrethroid-sprayed tile surfaces was performed, and the results were compared with those obtained from standard HPLC methodology. The sensitivity of the assay was sufficient for the determination of insecticide residues on a 100 cm^2 area of surfaces sprayed according to WHO recommended dosing regimens (i.e. 1–5 $\mu g\,cm^{-2}$ for type II pyrethroids). The correlation between the analysis of deltamethrin swabs by the recombinant biosensor assay and HPLC was high ($R^2 = 0.86$), and the insecticide measurements were reproducible and reliable.

The possible interferences of coextracts from a variety of actual treated surfaces (e.g. wood, house wall, etc.), such as dust, soil, oil, moisture will be evaluated during the adaptation of the assay into a Kit format and its application in field trials, which is the next stage of development. Existence of alkaline or acidic contaminants in high concentration in the real field extracts that might influence colourimetric results and the assay performance cannot be discounted at this stage.

In conclusion, we have shown that the inhibition of AgGSTD1-6 by pyrethroids can be translated into a simple colourimetric assay for the determination of deltamethrin. The proposed assay displays good precision, accuracy and short response time, and the operational use of the system for monitoring pyrethroid insecticides is being evaluated.

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