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A dehydrochlorinase-based pH change assay for determination of DDT in sprayed surfaces

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ABSTRACT

A glutathione S-transferase (GST) from the mosquito *Aedes aegypti* (*aagste2*), selected in the field as a major metabolic resistance enzyme for this parasite vector, was employed to produce a highly specific assay for the determination of DDT [1,1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene]. Detection is based on the pH change occurring in an appropriate buffer system by the concomitant release of H^+ during the *aagste2*-catalyzed dehydrochlorination reaction and is monitored potentiometrically or colorimetrically in the presence of a pH marker. The theoretical limit of detection (LOD) of the assay is 3.8 μg/ml, and the linear range of quantification is 12 to 250 μg/ml. The method does not recognize biologically inactive DDT analogues or major DDT photodegradants and breakdown molecules, and it is highly specific for the insecticidal *p.p'*DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane]. The biosensor was validated with a number of insecticide swabs from DDT-sprayed surfaces and found to be reproducible and reliable as compared with high-performance liquid chromatography (HPLC) (correlation coefficient $R^2 = 0.98$). Given the current expansion of DDT residual sprayings in many regions of Africa as a key strategic intervention for malaria vector control, this simple assay to monitor DDT levels for vector control spraying programs could have an important impact on malaria control.

The controversial organochlorinated insecticide DDT [1,1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene][2] has been used extensively for many decades for the control of malaria-carrying mosquitoes worldwide. Although internationally banned for general use by the Persistent Organic Pollutants (POPs) treaty due to its hazardous environmental impact [1], DDT is still registered by the World Health Organization Pesticides Evaluation Scheme (WHOPES) and actively promoted by the WHO for indoor residual spraying (IRS) in stable endemic areas [2]. It has both insecticidal and repellence action, and it remains the cheapest and most effective long-term weapon against malaria.

Although IRS with DDT is a key measure for malaria transmission control, there are a number of unresolved operational and technical issues that prevent it from being used to maximum benefit and restrict its epidemiological impact. Among them is the lack of quality control (QC) tools at both the procurement and intervention levels. The WHO provides guidance to national health authorities for the quality assurance of IRS applications [3]. These measures include consignment sampling of insecticides and shipping to independent analytical laboratories or conducting bioassays to ensure that the formulations conform to the specifications (e.g., test amount of biologically inactive impurities) and to verify the efficacy of the intervention (e.g., confirm spray, test quality of application). The practicality of these QC tools is limited in that they either are reliant on sophisticated and expensive systems (e.g., outsourcing high-performance liquid chromatography [HPLC]) or are nonquantitative bioassays of limited accuracy (being dependent on live insects).

Alternative field-applicable technologies to measure insecticide residues include chemical, antibody, and biosensor enzyme assays [4–6]. Enzyme-based assays are environmentally benign and have

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[2] *Abbreviations used:* DDT, 1,1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; WHO, World Health Organization; IRS, indoor residual spraying; QC, quality control; HPLC, high-performance liquid chromatography; GST, glutathione S-transferase; *aagste2, Aedes aegypti* GST epsilon 2; *p.p'*-DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane; *o.p'*-DDT, 1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl) ethane; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene; dicofol, 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloro ethanol; α-cypermethrin, (RS)-α-cyano-3-phenoxybenzyl (1S,3S)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate; λ-cyhalothrin, (RS)-α-cyano-3-phenoxybenzyl(1RS)-*cis*-3-[(Z)-2-chloro-3,3,3-trifluoropropenyl]-2,2-dimethylcyclopropanecarboxylate; permethrin, 3-phenoxybenzyl (1RS)-*cis-trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, reduced glutathione; SPB, sodium potassium phosphate buffer; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation; ELISA, enzyme-linked immunosorbent assay; SPME, solid-phase microextraction.

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advantages in specificity and accuracy of quantitation. Most often these assays are based on the inhibition of the biosensor enzymes by xenobiotics that block substrate turnover and indirectly allow the detection of the xenobiotics [7,8]. Although these systems are sensitive, their specificity is often compromised by broad affinity for xenobiotics, meaning that irrelevant nontarget molecules can also act as inhibitors. A lot of effort is required to screen or tailor enzymes to recognize target xenobiotic molecules as specific substrates and adapt their catalytic reactions into simple detection assays [9,10].

We used the *Aedes aegypti* glutathione S-transferase (GST) epsilon 2 (*aagste2*, acc. no. AY819710), selected in the field by the persistent DDT insecticide as a major metabolic resistance enzyme for this mosquito species [11]. The *aagste2*–DDT interaction is highly specific, enabling the development of a specific assay for determination of DDT concentrations on sprayed surfaces. Detection is based on the pH change occurring in the appropriate buffer system as a result of the concomitant release of H^+ during the *aagste2*-catalyzed DDT dehydrochlorination reaction, and it is monitored potentiometrically and colorimetrically.

Materials and methods

Chemicals and materials

p.p′-DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane, 99.5% purity], *o.p′*-DDT [1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl) ethane, 99.1% purity], DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene, 99.5% purity], dicofol [1,1-bis(*p*-chlorophenyl)-2,2,2-trichloro ethanol, 95% (*p.p′* 93% and *o.p′* 2%) purity], α-cypermethrin [(RS)-α-cyano-3-phenoxybenzyl (1S,3S)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate, 99.5% purity], λ-cyhalothrin [(RS)-α-cyano-3-phenoxybenzyl(1RS)-*cis*-3-[(Z)-2-chloro-3,3,3-trifluoropropenyl]-2,2-dimethylcyclo-propanecarboxylate, 99% purity], and permethrin [3-phenoxybenzyl (1RS)-*cis-trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate, 46% *cis* and 52% *trans*] were purchased from Chem Service (West Chester, UK). Vivaspin 15R concentrator with a 100,000 molecular weight cutoff was purchased from Sartorius Stedim Epsom (Surrey, UK). Standard biochemical and chemical reagents were purchased from Sigma–Aldrich (Dorset, UK). Glass filter surface swab filters were purchased from SKC (Houston, TX, USA).

Cloning, expression, and purification of GST

The cloning of *A. aegypti aagste2* (acc. no. AY819710) into a pET3a vector, the expression in *Escherichia coli* BL21(DE3) plysS, and the purification of the recombinant enzyme were conducted as described previously [11,12]. The eluted enzyme was concentrated using Vivaspin 15R concentrator and exchanged using a PD-10 column into 50 mM sodium potassium phosphate (pH 7.4), 10 mM dithiothreitol, and 40% glycerol according to the manufacturer's instructions. Samples were stored at −80 °C.

Assay of enzyme activity and protein

Standard GST activity spectrophotometric assays were performed by monitoring the formation of the conjugate of 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) according to a published method [13]. One unit of enzyme is defined as the amount of enzyme that gives 1.0 μmol of conjugate product per minute at pH 6.5 at 30 °C. Protein concentration was determined as in Ref. [14] using bovine serum albumin as standard.

DDT dehydrochlorinase activity assays were carried out at 25 °C in a total volume of 1 ml. The buffer system was 2 mM sodium phosphate buffer (SPB) (pH 7.6), 10 mM NaCl, 2.5 mM GSH, and 1 U enzyme in the presence of 0 to 300 μg/ml insecticide dissolved in acetonitrile (final 10%, v/v). Control wells contained the same reagent mixture without the insecticide and/or recombinant enzyme. At defined time intervals, an aliquot of the reaction mixture was transferred to a microtiter plate or glass tube. Subsequent pH measurements were performed either spectrophotometrically by adding 10 μg/ml bromothymol blue and recording at 616/433 nm on a SpectraMax M2 microplate reader (Molecular Devices, Berkshire, UK) or potentiometrically using a single pore glass electrode (Hamilton, Reno, NV, USA). The sensor signal was calculated as a difference between the reference signal and the reaction signal. The remaining aliquot was extracted with chloroform twice, evaporated to dryness, redissolved in acetonitrile, and analyzed by HPLC on a reverse-phase Dionex Acclaim C18 column (120 Å, 250 × 4.6 mm, 5 μ, Dionex, Camberley, UK). A mobile phase of acetonitrile/water 90:10 was used at a flow rate of 1 ml/min. The quantities of DDT and DDE were calculated from standard curves established by known concentrations of authenticated standards. Peaks were detected at 232 nm with the Ultimate 3000 UV detector and were analyzed with Dionex Chromeleon software.

Insecticide swabs from treated surfaces

DDT-sprayed tile surfaces (7.5 × 15 cm^2) in the range of WHO-recommended concentrations (100–200 μg/cm^2) were swabbed with hexane-saturated Whatman number 1 filter paper or glass filter surface swabs for bioaerosols and xenobiotic contaminations (SKC, Eighty Four, PA, USA). Each filter paper was transferred to a glass tube, and DDT residue was extracted by 2 ml (3×) of mixture solvent ethyl acetate/hexane (1:9, v/v). The extracts were combined, evaporated to dryness, and redissolved in 4 ml of acetonitrile. Then 1 ml of each sample was filtered through a 0.45-μm membrane syringe filter into an HPLC vial. After that, 10 μl of each sample was injected in HPLC for DDT quantification and 35 μl was used for the biosensor enzyme assay.

Determination of limits of detection and quantification

Theoretical values for the limit of detection (LOD) and the limit of quantification (LOQ) were determined as described previously [15].

Results

Establishing the basis of a potentiometric assay for the determination of DDTase activity

The GST-catalyzed DDT dehydrochlorination reaction results in concomitant release of H^+ protons (Fig. 1). The concentration of released protons reflects the progress of the DDTase reaction and can be measured by the pH change occurring in the appropriate buffer system either potentiometrically using a pH electrode or colorimetrically in the presence of a pH marker such as bromothymol blue. Fig. 2A shows typical enzymatic reaction rate curves obtained for substrate concentrations of 0 to 250 μg/ml DDT. The kinetics of the reaction was biphasic for all concentrations of DDT used. Rate constants for the reaction exhibiting biphasic kinetics were calculated from pH versus time (in min) using the double exponential decay equation [16,17]:

$$\mathrm{pH} = \mathrm{A}_{0_1} e^{-k_{fast} t} + \mathrm{A}_{0_2} e^{-k_{slow} t} + \mathit{Offset}, \quad (1)$$

where A_{01} and A_{02} are the initial activity for the fast and slow phases of reaction, respectively, and k_{fast} and k_{slow} are the rate constants for the fast and slow phases of the reaction, respectively.

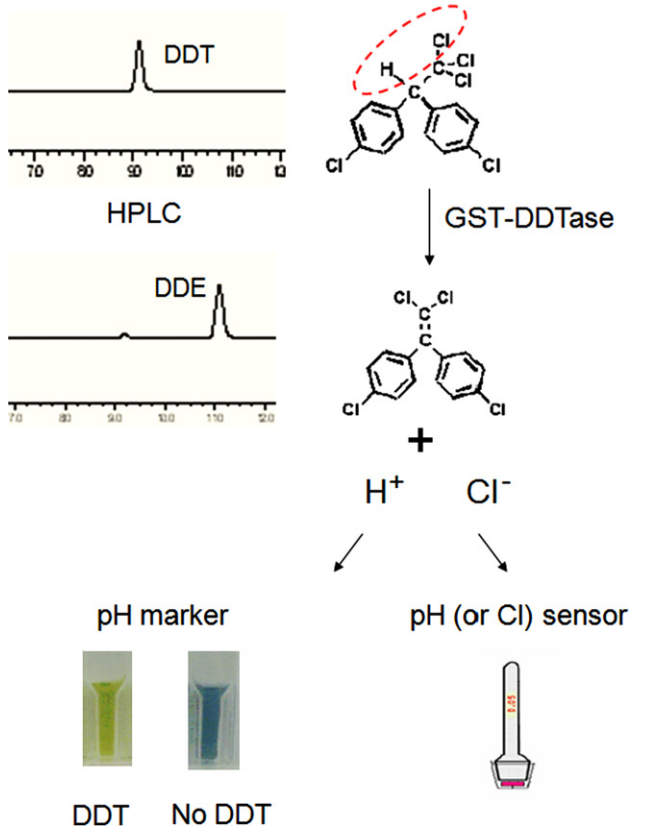

Fig. 1. Schematic graph of the recombinant dehydrochlorinase-based pH change assay for determination of DDT. The concomitant release of H^+ during the GST-catalyzed DDT dehydrochlorination reaction results in pH change in the appropriate buffering system that is measured potentiometrically or colorimetrically in the presence of pH marker. The release of Cl^- can also be recorded with the appropriate halogen sensor.

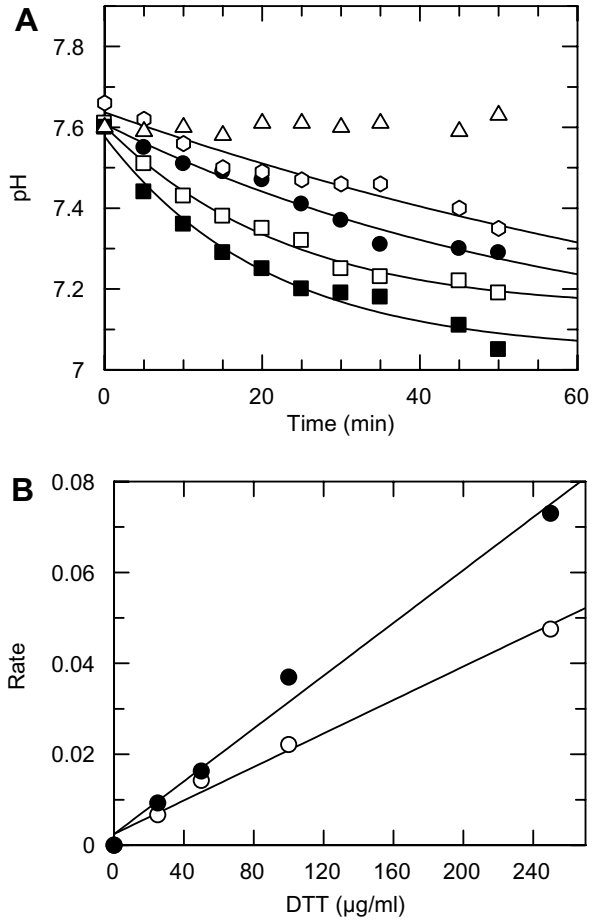

Fig. 2. Potentiometric determination of DDT concentrations. (A) Potentiometric assay of GST-catalyzed DDT dehydrochlorination reaction. Enzymatic reaction rate curves shown are for the following DDT concentrations:Δ, blank experiment (0.0 - μg/ml); ○, 25 μg/ml; ●, 50 μg/ml; □, 100 μg/ml; ■, 250 μg/ml. (B) Enzyme–substrate response curve using the data from panel A. The absolute values of the fast (●) and slow (○) phases of reaction were used as signals to plot the curves. Experimental conditions were as follows: 2 mM sodium phosphate buffer, 10 mM NaCl, 2 mM GSM, initial pH 7.6, temperature 25 °C.

The observed reaction rates for the fast and slow phases were dependent on DDT concentration, as illustrated in Fig. 2B. For both phases, a linear dependence of the observed rate of inactivation on the reagent concentration was observed.

The optimal concentration of phosphate in the buffer solution was 2 mM. Higher signals were obtained for lower buffering capacity reaction mixtures (e.g., 1 mM phosphate) but compromised sensor response and reproducibility due to the inherent instability of these dilute buffer solutions (data not shown). The incorporation of 10 mM NaCl into the reaction mixture did not inhibit the enzymatic reaction for a range of DDT concentrations. Finally, variable initial pH values in the range of 7.0 to 8.0 were tested and did not significantly affect the rate of the reaction. The assay pH was set to 7.6 based on the maximization of the colorimetric difference occurring in the presence of bromothymol blue.

Determination of DDT concentration from pH changes in reaction mixture

The progress of the *aagste2*-catalyzed dehydrochlorination reaction, as determined by the monitoring of pH change, was correlated with the initial DDT concentration under assay conditions. Fig. 2B shows the enzyme–substrate response curve using the absolute value of the fast rate of reaction (Fig. 2A) as a signal. The response curve was linear for concentrations of 25 to 250 μg/ml. The reproducibility of pH response to DDT, expressed by relative standard deviation (RSD), was on the order of ±5% ($n = 4$). The change in pH resulted in a clear spectral and visual change (elimination of blue color and formation of yellow color) in the presence of bromothymol blue (Fig. 3A). The kinetics of the spectrophotometric assay was similar to that of the potentiometric one, with a fast and slow rate of reaction. The optimal correlation of 616/433 nm end point values with initial DDT concentration was obtained after 60 min incubation time at 25 °C. The linear range of the spectrophotometric determinations under assay conditions was 20 to 100 μg/ml (Fig. 3B). The slightly lower linear response range obtained for the spectrophotometric determination is possibly explained by the ionization properties of bromothymol blue or the cloudy effects observed at higher DDT concentrations, which may have interfered with the spectrophotometric determination.

Sensitivity and specificity of potentiometric assay

The specificity of the method was tested against a number of nontarget DDT analogues and biologically inactive DDT breakdown products often present in DDT formulations as impurities or as result of UV degradation on sprayed surfaces. No reaction signal in either the potentiometric or dye indicator assay was observed when *o.p′*DDT, DDE, and dicofol were used as substrates at concentrations equivalent to the assay range for DDT (20–250 μg/ml). In addition, the method does not detect any of the pyrethroid insecticides λ-cyhalothrin, β-cypermethrin, α-cypermethrin, permethrin, and deltamethrin also used in IRS applications. Incubation of

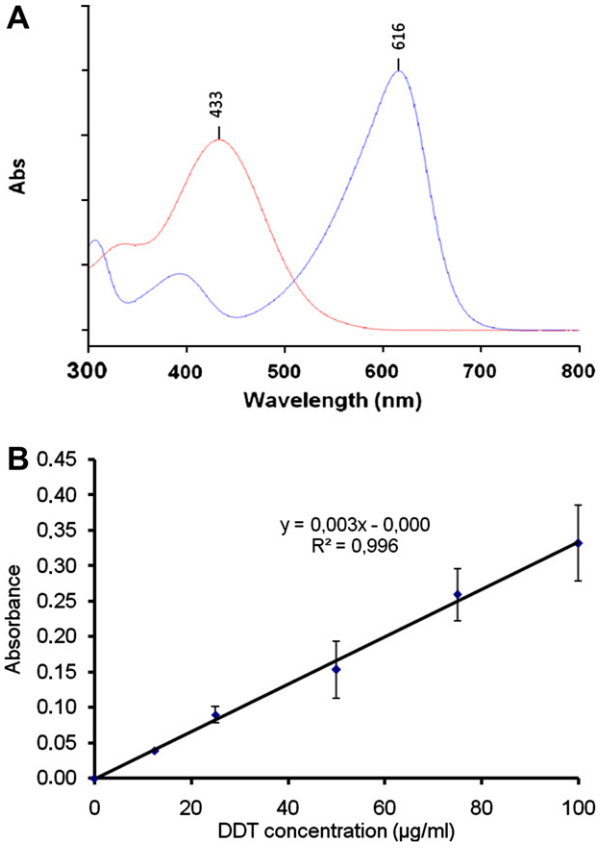

Fig. 3. Spectrophotometric determination of DDT concentrations. (A) The change in pH during the dehydrochlorination reaction resulted in a clear spectral peak shift from 613 to 433 nm in the presence of bromothymol blue. The change (elimination of blue color measured at 616 and formation of yellow color measured at 433) is clearly visible. Experimental conditions were as follows: 2 mM sodium phosphate buffer, 10 mM NaCl, 2 mM GSH, 100 μg/ml DDT, initial pH 7.6, temperature 25 °C, incubation 60 min. (B) Calibration curve for the spectrophotometric determination of DDT concentrations. Experimental conditions were as in Fig. 2A except that bromothymol blue (10 mg/ml) was included in the reaction mixture. Absorbance was recorded as an end point at 616 and 433 nm after 1 h incubation. Each point represents the abstraction of 433 nm value from 616 nm value and is the average of three independent determinations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

aagste2 with the aforementioned DDT analogues or breakdown products and/or pyrethroid insecticides under assay conditions also did not have any effect on the metabolic profile determined by HPLC, confirming that these molecules are not being recognized as substrates by the enzyme *aagste2*.

The theoretical LOD and LOQ values of the assay were 3.8 and 11.5 μg/ml, respectively. The LOQ is equivalent to the amount of DDT present in approximately 0.1 cm^2 sprayed surface, which is well within the WHO-recommended dose of 100 to 200 μg/cm^2 for vector control.

Determination of DDT from sprayed surfaces using aagste2

DDT swabs from sprayed tiles were quantified by the recombinant dehydrochlorinase assay developed in this study, and results were compared with those obtained from standard HPLC methodology in a blind trial approach. DDT quantities were calculated by converting results obtained spectrophotometrically into concentrations using the standard curve derived by analytical standards. A standard curve was similarly prepared from known concentrations of authenticated standards for the HPLC analysis. Fig. 4 depicts the correlation between the analysis of 12 swabs by the recombinant biosensor assay and that by HPLC. Quantification showed the same pattern whether measured by HPLC or by the biosensor assay with a correlation coefficient of $R^2 = 0.98$.

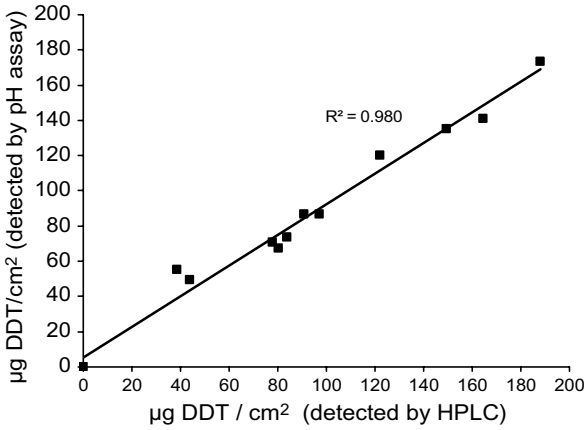

Fig. 4. Comparison of determination of DDT swaps from sprayed surfaces with recombinant dehydrochlorinase and HPLC method. DDT swabs from sprayed tiles in the range of WHO-recommended application dosage were quantified by standard analytical HPLC and the recombinant dehydrochlorinase assay. Experimental conditions of the pH assay were as follows: 2 mM sodium phosphate buffer, 10 mM NaCl, 2 mM GSH, 10 mg/ml bromothymol blue, initial pH 7.6, temperature 25 °C, incubation time 1 h. Quantification showed the same pattern with a correlation coefficient of $R^2 = 0.98$.

Discussion

We have developed a highly specific GST dehydrochlorinase (*aagste2*)-based potentiometric assay for the determination of DDT. Quantification is achieved by measuring the pH change occurring in the mixture by the concomitant release of H^+ during the *aagste2*-catalyzed DDT dehydrochlorination reaction. The difference in the pH can be linearly correlated with the initial DDT concentration in the range of 12 to 250 μg/ml. A colorimetric version was also developed by incorporating the pH marker bromothymol blue in the reaction mixture to obtain visual or spectrophotometric determination. The linear range of the latter was narrower (20–100 μg/ml) compared with the potentiometric methodology; however, this version might be more easily adaptable to a simple test format.

The assay is highly specific for the biologically active *p.p′*DDT molecule, which is recognized as enzymatic substrate by the recombinant *aagste2*, a protein selected in the field for its ability to metabolize this particular highly toxic insecticide enantiomer [11]. None of the DDT breakdown products or low-toxicity analogues and other structurally similar compounds tested interfered with the assay. This high specificity provides an advantage over current immunoassay-based detection systems [18].

The assay is also more practical compared with standard analytical methodology (e.g., HPLC) in a field situation and provides a much-needed tool for measuring DDT in the frame of malaria control interventions in Africa [3]. Toward this end, the assay was tested on a number of insecticide swabs from DDT-sprayed tile surfaces, and the DDT measurements were found to be highly reproducible and reliable against the standard HPLC methodology (correlation coefficient $R^2 = 0.98$). The sensitivity of the assay (12 μg) is lower than enzyme-linked immunosorbent assay (ELISA) tests but is still sufficient for the determination of insecticide residues on a very small area of sprayed surfaces (< 1 cm^2). One can also alleviate interference by dirt or chemicals through appropriate dilution of swab extracts from large sampling areas that may be necessary to obtain representative sampling from sprayed surfaces. Alternative improved extraction protocols applicable to the variety

of wood and house wall surfaces in the field can be developed. The elimination of ethyl acetate from the extraction solvent that might affect the assay if reacted with strong alkali compounds present in the field, the addition of an immiscible water phase for removal of variable alkali or acids, and/or the use of a solid-phase microextraction (SPME) cleanup step using commercially available cartridges [19] should be explored during the next stage of development. The latter (SPME cartridge) would substantially reduce sample preparation time, but the increased cost would need to be taken into consideration for the design of a low-cost field kit for DDT detection.

The adaptation of the DDT assay into a cost-effective practical format is greatly facilitated by the simple pH detection assay. However, it is also possible to measure the Cl− ion release that occurs during the dehydrochlorination reaction using a Cl^- electrode (e.g., halide electrode ISM-146Cl, Lazar Research Laboratories, Los Angeles, CA, USA). This was explored at the early phase of assay development and was successful in DDT detection (data not shown). Nevertheless, the cost of halide electrodes (> $700) and the difficulty in adapting this for field sampling favored the simpler pH change-based detection system.

In conclusion, we have developed a highly specific recombinant GST- based assay for the determination of DDT using a simple pH detection technique. The potentiometric and colorimetric versions described here combine the most desirable features of a specific analytical tool with the low cost and ease of use required for a field test kit. The enzyme system employed (GST) is generally stable and easily reproducible [11,20]. The adaptation of the assay into a simple test format, possibly with the employment of immobilized markers and appropriate enzyme forms [21], and its evaluation in the field situation is the next stage of development. We anticipate that this will have direct application in routine testing and quality control of IRS spray programs in Africa and could have an important impact on malaria control.

Acknowledgments

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