# TECHNICKÁ UNIVERZITA V LIBERCI

## FAKULTA TEXTILNÍ





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# IDENTIFICATION OF RISK CONCENTRATIONS OF HAZARDOUS COMPOUNDS ON TEXTILES

## AUTOREFERÁT DISERTAČNÍ PRÁCE

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| Název disertační práce: | **IDENTIFICATION OF RISK CONCENTRATIONS OF HAZARDOUS COMPOUNDS ON TEXTILES** |
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# 1. Objectives

The research is focused on the identification of residual hazardous compounds on cotton fibers utilizing the following techniques.

### *1.1 Method Development utilizing Biosensors*

The major intention is the development of method based on the measurement of bio-electrical signals caused by enzymatic inhibition of Acetylcholinesterase to identify residual pesticides. The purpose is to measure the performance of biosensor responsible for evaluation of the signals by the interaction of biological substances and residues on cotton. Determination of the performance parameters and optimization of these parameters to evaluate such a biosensor is also the aim of the study.

### *1.2 The Impact of pesticides on the life cycle of Algae utilizing AGA*

This method is dependent on the measurement of life cycle responses following exposure in microorganisms with the help of Algae Growth Analyzer (AGA). These responses can be predictive for human health evaluation on the basis of the weight of evidence which include data from all of the hazard assessment and characterization studies.

### *1.3 Estimation of residual pesticides with GC-MS/MS*

Gas Chromatography coupled to quadrupole tandem mass spectrometry is used not only for identification but also for the quantification of the analytes present in the samples. The aim is to build up a procedure with the consideration of all the crucial parameters essential for the development of an analytical method recommended by the official authorities. The limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy have to be evaluated to have a trustworthy conclusion of the anlytes present in cotton samples.

# 2. Overview of current situation

Pesticides are widely used for the control of weeds, diseases, and pests all over the world, mainly since after Second World War, and at present, around 2.5 million tons of pesticides are used annually and the number of registered active substances is higher than 500 [1]. The cultivation of cotton has been estimated to consume 11% of the world's pesticides while it is grown on just 2.4% of the world's arable land [2]. Humans can be exposed to pesticides by direct or indirect means. Direct or primary exposure normally occurs during the application of these compounds and indirect or secondary exposure can take place through the environment or the ingestion of food [1]. Multiple reports exist on the unwanted side-effects of pesticides on wildlife. Over-spraying, accidents and aerial spraying are the most significant events affecting the environment. Pesticides applied in cotton production have also been documented as adversely affecting river ecosystems [3].

Of the many possible negative effects of pesticide use, the impact on human health remains a major concern [4]. The introduction of second generation pest control agents, largely synthetic organics such as DDT and ethyl parathion, from the 1940s on, had invited heightened consumer concern, regulatory attention and monitoring activity. The collection of residue monitoring data, begun in the 1950s, has played a major role in understanding how residues are deposited and dissipated [5]. As the pesticide residue is a potentially serious hazard to human health, the control and detection of pesticide residue plays a very important role in minimizing risk. Many methods have been developed in the last few years for the detection of pesticides [6]. The introduction of Biosensors was based on the Clark oxygen electrode and these are characterized by the direct spatial combination of a matrix-bound biologically active substance (receptor) with an electronic device [7]. Biosensors are increasingly becoming powerful tools in clinical diagnostics, drug detection, and food and environmental monitoring [8]. Electro analytical sensors and biosensors provide an exciting

and achievable opportunity to perform biomedical, environmental, food and industrial analysis due to their advantages such as high selectivity and specificity, rapid response, low cost of fabrication, possibility of miniaturization and easy to integrate in automatic devices [9]. Algae possess a number of distinct physical and ecological features and their ability to proliferate over a wide range of environmental conditions reflects their diversity [10]. The action of toxic substances on algae is therefore not only important for the organisms themselves, but also for the other links of the food chains [11]. Algal toxicity tests and Life-cycle toxicity tests are increasingly being used in bioassay test batteries and it has been observed in several studies that for a large variety of chemical substance algal tests are relatively sensitive bioassay tools [12].

The techniques of gas chromatography, liquid chromatography and thin film chromatography coupled with different detectors and the different types of spectroscopy are the most commonly used methods for the recognition of residual pesticides [13]. The Gas Chromatography has been the predominant tool in pesticides multiresidue methodology for over 30 years. It is the single most important tool for the identification and quantitation of volatile and semi volatile organic compounds in complex mixtures. It is the basis of official EPA methods [14]. Analyses must prove reliable, be capable of residue measurement at very low levels (sub ppb), and also provide unambiguous evidence of the identity and magnitude of any residues detected [15].

# 3. Experimental methods

## 3.1 Materials

The samples of three different varities of cotton namely, Egyptian cotton Giza 86, Pakistani cotton MNH 93 and Indian Cotton were collected from the cultivation season 2011/2012. Both varieties have classical conventional cotton and organic cotton. The samples were abbreviated as (GC&GO) for Giza, (PC&PO) for Pakistani and (IC&IO) for Indian cotton. Another three cotton samples (BT-114, SH-1 & Z-33) were taken after the first harvest from BahawalPur (Pakistan). These samples were collected from the cultivation season 2012/2013 and the analyses were made within three months of their collection from the field.

All the chemicals and reagents utilized were obtained commercially. Acetylcholinesterase (electric eel) (EC 3.1.1.7, 827 IU/mg), Acetylthiocholine chloride (A5626), Neostigmine methyl sulphate (N2126) and MOPSO Sodium Salt (M8767) were purchased from Sigma Aldrich. HPLC grade solvents (Hexane, Methanol, Toluene, Dichloromethane, Acetone, and Acetonitrile) have been purchased from Verkon.

A total of 76 different pesticides were purchased. Pesticide Mix 155 (KF) and Pesticide Mix 17 (KS) were purchased from Dr. Ehrenstorfer GmbH, Germany. Pesticide Mix 3 & 14 (KT) and Pesticide Mix 18 (KZ) were purchased from AccuStandard, USA.

## 3.2 Sample Preparation

The development of an appropriate sample preparation procedure involving extraction, enrichment, and cleanup steps becomes mandatory to obtain a final extract concentrated on target analytes. It is always necessary to carry out some pre treatments to get a homogeneous and representative subsample.

***3.2.1 Cryogenic Homogenization.*** CryoMill was used for the homogenization with 1 cm ball. All samples of cotton were arranged around the inside of a pre-chilled Teflon mill in the form of pallets which contained a concentric Teflon ring and Teflon puck in liquid nitrogen surrounding. Each sample was milled with two cycles. Each cycle consists of exactly two minutes for grinding with an interval of 15 seconds for cooling. After the milling the resulting powder was sampled.

***3.2.2 Ultrasound Assisted Extraction.*** Ultra sound extraction method was used for the extraction from all of the cotton samples. A total of 0.5 gm homogenized sample was transferred to the flask along with 10 ml of the solvent used. The flask was placed in the extraction apparatus Sonorex at a controlled temperature of 60 $^{O}$C. Samples were extracted for 30 minutes. The extracts were then filtered and stored for further analysis.

**3.3 Techniques Utilized**

Following three different techniques have been employed for the detection of residual pesticides on cotton samples.

***3.3.1 Biosensor based detection***

Biosensor toxicity analyzer (BTA) & Minithermostat have been used for monitoring the activity of the inhibition of AChE with the help of sensors equipped with an enzymatic membrane of AChE which is immobilized. AC1.W2.RS/AChE Sensors were used for the monitoring of AChE inhibition, provided by Bvt Technologies (Fig 1). The electrodes were connected to the Bioanalyzer. All measurements were performed at potential 350 mV.

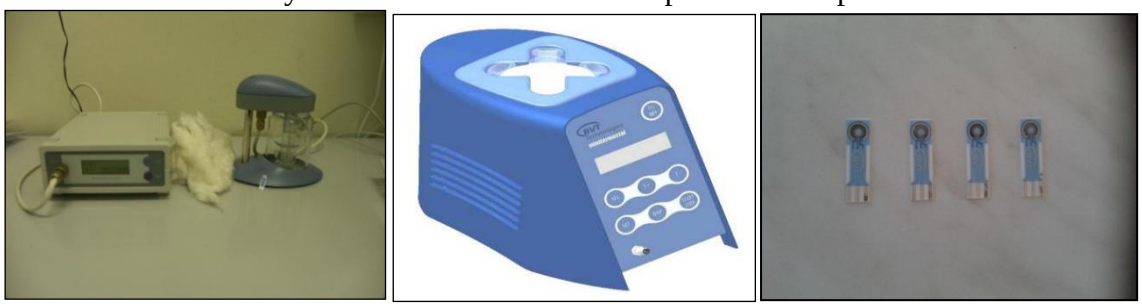

Figure 1: BTA (Left), Minithermostat (Middle) & AC1.W2.RS/AChE Sensors (Right)

The electrochemical measurements were performed at controlled room temperature (22±1$^{0}$C). Mopso & phosphate buffer solutions were used. Acetylthiocholine chloride (ATCh) and Neostigmine methyl sulfate were used as enzyme substrate and enzyme inhibitor, respectively.

***3.3.2 Life cycle assessment of single cell Algae***

Algae Growth Analyzer was used enabling to follow the lifecycle of algae producing oxygen. It is controlled by Bioanalyzer potentiostat that allows programming light and dark phases, measure and evaluating the oxygen electrode response. All the above mentioned extracts were analyzed by AGA for a duration of 30 minutes each. With the help of miniature Oxygen electrode, we have obtained the oxygen production activity of the algae in presence of the extracts by recording the oxygen produced in medium.

***3.3.3 Gas Chromatography coupled to Triple Quadrupole Mass Spectrometry***

The Thermo Scientific TRACE 1310 Gas Chromatograph coupled with triple quadrupole mass spectrometry is used. TSQ 8000 mass detector has the ability to analyze full scan data at the same time of targeted MRM analysis. Confirmation of pesticide and quantitation was performed in selected-reaction monitoring mode (SRM). The limit of detection (LOD), the limit of quantitation (LOQ) and precision have been worked out based on the guidelines for analytical measurements.

# 4. Results and discussions

**4.1 Method development utilizing Biosensors**

A rapid, sensitive and low cost method based on AChE-inhibition utilizing biosensor was developed. The working solutions of pesticide standard Mix 155 (KF) was prepared by taking 5 standard concentration levels (0, 1, 10, 100, 1000 ng/mL) along with the standard

inhibitor and analyzed in order of increasing concentration. The dilutions were prepared in methanol.

The enzyme activity has been analyzed following the method adopted by George L. Ellman, in which the determination of acetylcholinesterase activity was measured by following the increase of yellow colour produced from thiocholine by a photometric method [16].

Optimization of different variables involved in the process like enzyme and substrate concentrations, time of incubation, buffers and their pH has been executed. The degree of inhibition was calculated as a relative decay of the biosensor response.

$$I\,\% = 100 \times \frac{I_O - I_i}{I_O}$$

Where I is the degree of inhibition of AChE; $I_0$ and $I_i$ are the current values measured prior to and after the enzyme biosensor is treated with an inhibitor. There must be a certain positive correlation between *I* and the concentration of pesticides in principle [17].

The scheme of the final testing is described in Table 1.

Table1 : Scheme of final testing

| **Addition of Substances** | **Volume (µL)** |
|---|---|
| 0.1M Phosphate Buffer | 100 |
| ATCh (0.08 mM) | 100 |
| Calibration Std | 100 |
| **Stirring** | |
| AChE (0.5 IU/ µL) | 2 |
| **Stirring** | |
| **After 60 minutes** | |
| Neostigmine | 10 |
| **Final Stirring** | |

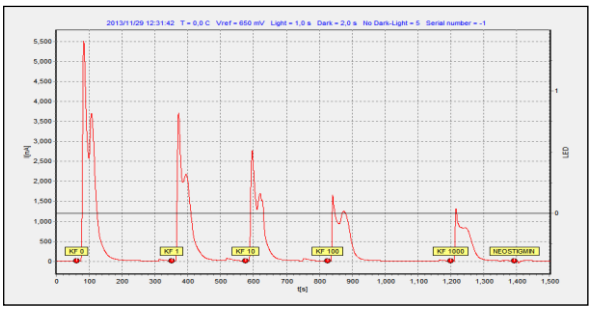


Figure 2: Amperometric response of calibration samples with optimized concentrations

The results of the above mentioned procedure are shown in Figure 2. A good correlation between AChE activity and the calibration points was observed. Five repititions (A, B, C, D, E) for the same test have been performed and the resultant graphs are shown in Figure 3, where as Figure 4 shows the overall average inhibition % with relevant concentration levels.

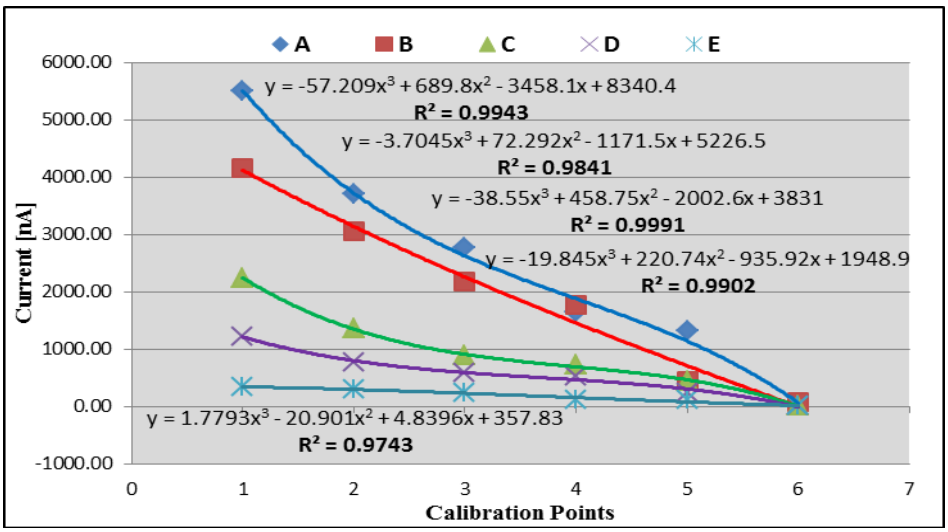


Figure 3: Amperometric response of different calibration samples; *n=5*

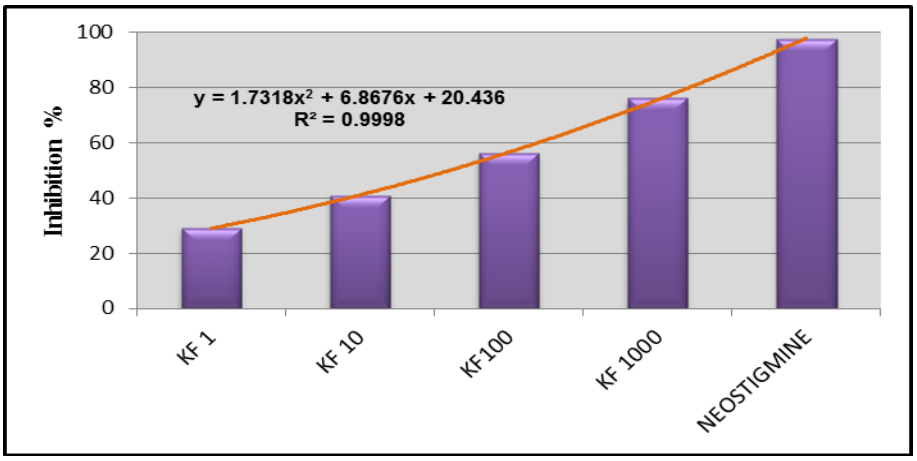

Figure 4: Average AChE-inhibition caused by different concentrations; *n*=5

The equation of the best fit line is as follows:

$$y = 1.7318x^2 + 6.8676x + 20.436$$

The value of predicted squared coefficient of correlation ($R^2$) is found to be 0.9998, which is excellent and shows a strong relationship between our variables i.e. Concentration and Inhibition %.

The method is utilized for real cotton samples extracted with different solvents (methanol, hexane, toluene, acetone & acetonitrile) after necessary sample pretreatments. The speciality of this method is that all the samples along with the control points can be tested in one run, The total time utilized for one complete test was approximately 50 ~ 55 minutes. The extracts of cotton samples were replaced by calibration points. After the complete procedure these final samples were introduced to the biosensor and the response is monitored. Figure 5 shows the activity of whole the experiment with solvent methanol and the graph which was plotted against the AUC and corresponding analytes. .

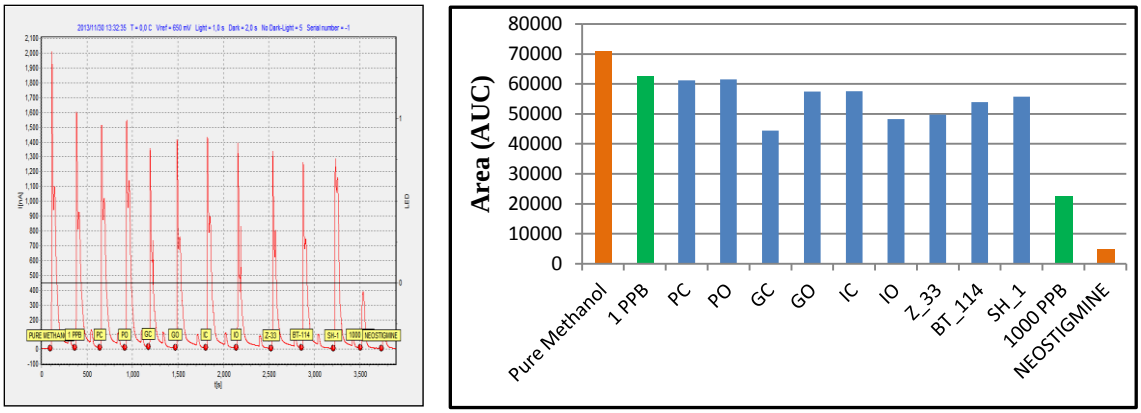

Figure 5: Amperometric response & AUC of all cotton samples extracted with methanol

We are able to compare our extracts with the help of minimum and maximum concentration‘s area. It is quite visible that almost all our samples have the area with in the range of 1 ppb to 1000 ppb but none of the samples exceed 1000 ppb limit.The inhibition % was calculated based on the area under the corresponding curves for each analyte and represented in Figure 6. It shows that all of our samples show the inhibition % (on average of < 40 ) but with some variations. PC and PO samples show almost same inhibition closer enough to 1ppb. There is a significant difference between GC and GO. GC show more inhibition than GO and the opposite trend is seen in the case of IC and IO. IO is responsible for more inhibition than IC.

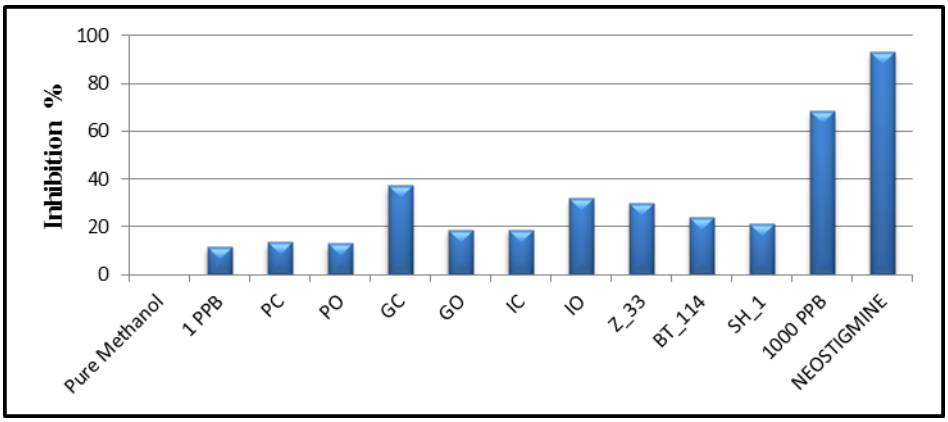


Fig 6: AChE-Inhibition caused by all cotton samples extracted with methanol

Same procedure was implemented to test the extracts with other solvents like hexane and toluene. With acetone and acetonitrile we experience a very poor response of detector which is not measueable. A summary of all the cotton samples with different solvents has been shown in Figure 7.

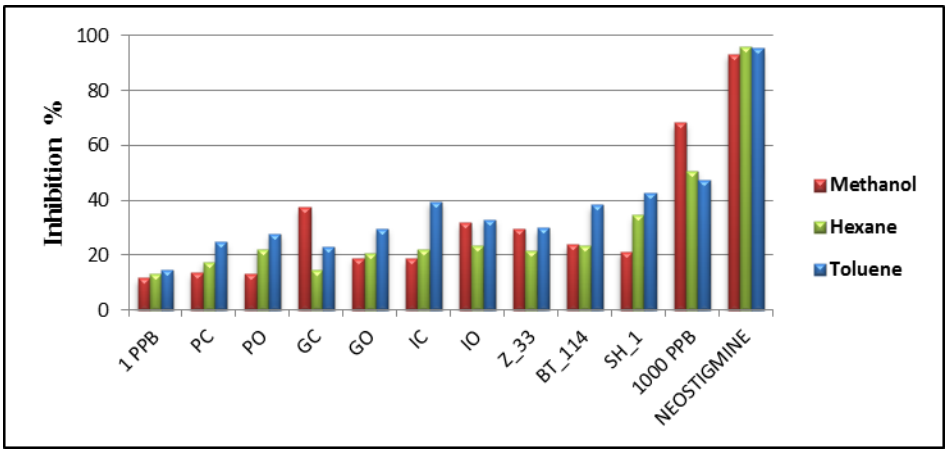


Figure 7: Summary of AChE-Inhibition caused by cotton samples with all solvents

Also the difference of inhibition between classical and organic cotton samples is also not substantial. We can conclude that there may be possibility of the presence of AChE inhibitors in almost all our samples without any discrimination.

**4.2 Life cycle assessment with Algae Growth Analyzer (AGA)**

Algae Growth Analyzer was used for the measurement of inhibition of photosynthetic activity of Algae. Green Algae of the family Scenedesmaceae and Genus SCENEDESMUS was arranged by Bvt technologies, Czech Republic. All the resulted extracts from cotton samples (GC, GO, PC, PO, IC, IO) were arranged. Calibration of the device was done with 1 gm $Na_2SO_4$ and 5 ml Distilled water to consume all the oxygen inside the glass cell repeatedly for three times. All the above mentioned extracts were analyzed by AGA for a duration of 30 minutes each. With the help of miniature Oxygen electrode, we have obtained the oxygen production activity of the algae in presence of the extracts by recording the oxygen produced in medium. The results of Giza & Pakistani Cotton are shown in Figure 8. There are the differences in the oxygen production but in each case the addition of extract increases the production of oxygen. However comparing the Pakistani classical and organic cotton, the stimulating agents in organic cotton are more and this is the cause of their high effect. Also it may be the possibility that the hazardous compounds in organic cotton are less than the classical one.

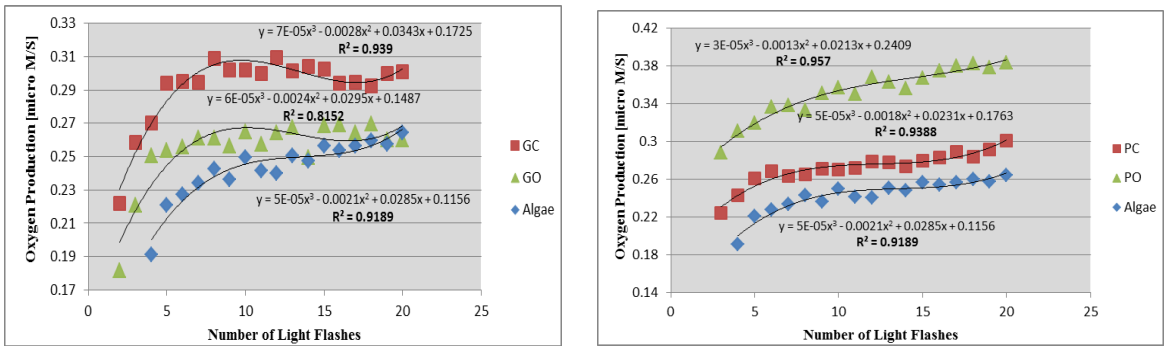


Figure 8: Comparison of GC & GO samples (L.H.S) and PC & PO (R.H.S)

The results of Indian cotton are shown in the Figure 9. It is quite visible that there is a significant difference in the oxygen production. Classical cotton shows higher production of oxygen in this case. Organic cotton extracts in this case may have some contaminants and pollutants which hinder in the streamline of oxygen production by the algae.

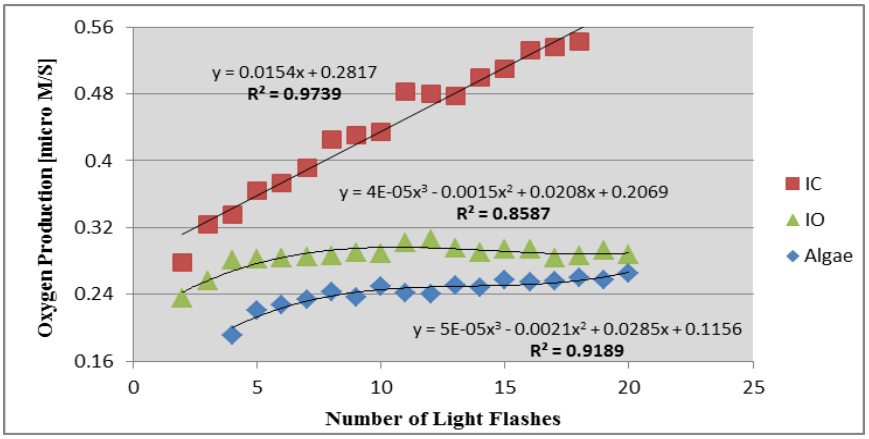


Figure 9: Comparison of IC and IO samples

we can see that there is measurable interaction between cotton samples and algae which can be observed according to the results of our experiments but we are not able to find out some convincing results. The variation in the behavior of different cotton samples has been observed but none of these samples show any harm to the algae rather the effect of extracts stimulated their behavior.

### 4.3 Method development utilizing GC-MS/MS

A multiresidue method for analysis of 76 pesticides with different physicochemical properties was developed. The method involves a rapid and small-scale extraction procedure of real cotton samples collected from different regions (Egypt, Pakistan & India) with five different solvents (Methanol, Acetonitrile, Acetone, Toluene, Hexane) from polar to non-polar region, using Ultra Sound assisted Extraction (USE). Cryogenic Homogenization was being implemented for sample Pre-treatment. After final extraction and filtration the extracts were concentrated. The pesticide residues were determined by gas chromatography with Tandem mass spectrometry (GC-MS/MS). 57 out of 76 pesticides were detected successfully by the method developed. Nineteen (19) pesticides could not be analyzed by GC-MS/MS using EI ionization, most often because of incompatibility with evaporation of the intact molecule in the GC injector.

All the essential parameters which are necessary for the method validation have been taken into account in the light of the document SANCO/12495/2011 for ‘Method Validation and

Quality Control Procedures for Pesticide Residues Analysis in Food and Feed'[18] which is the latest version of Commission Directive 96/46/EC. Moreover the document from Codex Alimentarius document 'Guidelines on Good Laboratory Practice in Pesticide Residue Analysis' has been also considered [19].

The stock solution of individual pesticide standards of 10µgmL$^{-1}$ were prepared by dissolving the appropriate amounts of the analytical standards in the relevant solvent. Working standard solutions were prepared by taking 10 standard concentration levels (1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL) for each standard pesticide mix (KF, KS ,KT & KZ), separately. The dilutions of the pesticide standards were made with the same solvent which they originally contain.

### *4.3.1 Evaluation of Retention time*

The working solution of 1µgmL$^{-1}$ of all the pesticide standard mixes (KF, KZ, KT, KS) was tested in EI-MS full scan mode for the typical mass range (35 to 500 amu). One of the resultant chromatograms has been shown in Figure 10 for KZ. Evaluation of retention time is accomplished by comparing the probability of the presence of related ions evaluated by the related chromatograms and electron impact mass spectra of the analyte from the two built in database of libraries i.e. NIST and Mainlib.

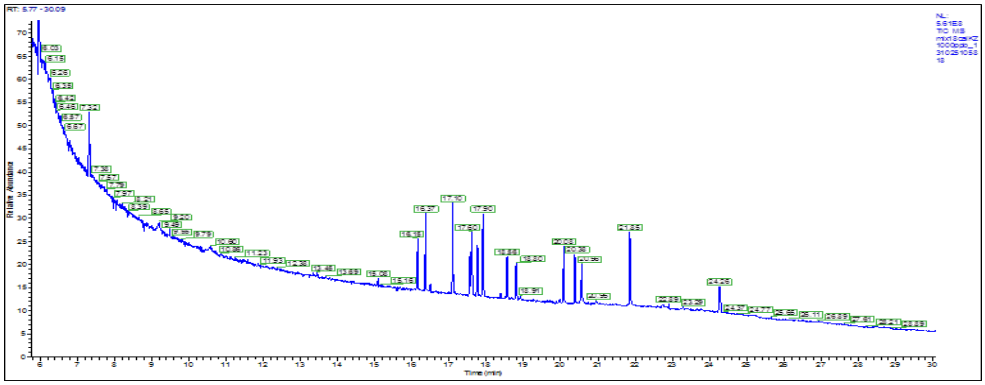

Figure 10: Gas Chromatogram for Pesticide Mix KZ

Each peak of the chromatogram is analysed for each compound of the standard mix by comparing the mass to charge ratios of precursor and product ions with that of the two built in libraries. The criteria of acceptance have been set for probability of the presence of the analyte > 85% in both the libraries. Figure 11 (LHS) shows the mass to charge ratio for Primiphos-methyl attained from this above mentioned chromatogram. This mass spectrum is compared with the above mentioned databases. Figure 11 (RHS) shows the resultant mass spectrum obtained from NIST database.

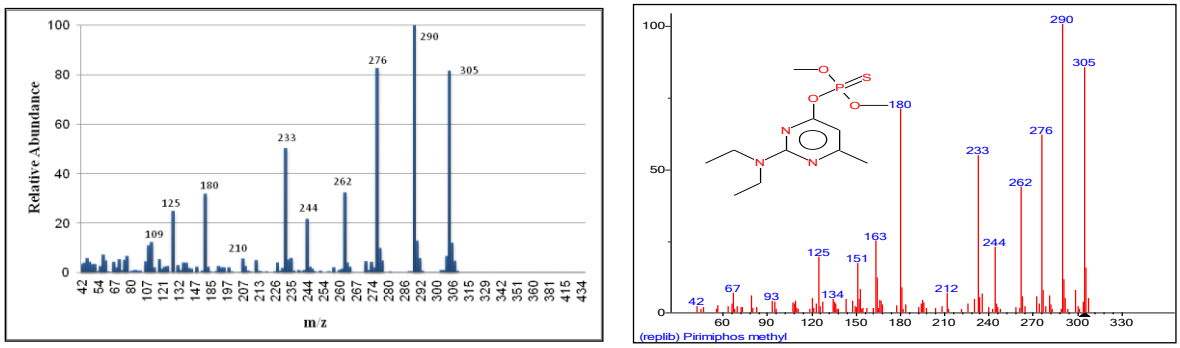


Figure 11: Mass to charge ratio for Primiphos-methyl (LHS) & EI spectra for Primiphos-methyl from NIST database (RHS)

The probability of presence of Primiphos-methyl in NIST is 97 % where as in Mainlib it was 97.03 %. These values are acceptable so the retention time evaluated for Primiphos methyl was 17.28 same as retention time of the corresponding peak in the main chromatogram.All the compounds of KZ and other all mixes were analyzed for the retention time in the same way. The summary of all the compounds of KF has been shown in Table 2.

Table 2: Retention time and precursor masses for KF

| KF_MIX 155 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Analyte** | **Retention Time (min)** | **Precursor Mass** | **Product Mass** | **Collision Energy** | **a** | **B** | **$r^2$** | **Concentration range (ng/mL)** |
| Thiometon | 15.23 | 247 | 89 | 40 | 0.46 | 64.99 | 0.9998 | 0 - 50 |
| Simazine | 15.48 | 201 | 173 | 5 | -15.01 | 3289.78 | 0.9966 | 0 - 100 |
| Terbumeton | 15.68 | 226 | 170 | 16 | 0.41 | 5.43 | 0.9962 | 0 - 50 |
| Terbuthylazine | 15.82 | 230 | 174 | 14 | -0.72 | 119.16 | 0.991 | 0 - 100 |
| Pirimicarb | 16.48 | 238 | 166 | 10 | -18.36 | 7523.22 | 0.9591 | 0 - 20 |
| Terbutryn | 17.21 | 242 | 186 | 25 | 0.76 | 63.47 | 0.9975 | 0 - 50 |
| Pirimiphos-methyl | 17.28 | 305 | 180 | 8 | -41.70 | 2166.69 | 0.8893 | 0 - 20 |
| Triadimefon | 17.64 | 208 | 111 | 20 | -28.54 | 2580.47 | 0.8992 | 0 - 20 |
| Procymidone | 18.38 | 283 | 96 | 10 | -51.52 | 5367.01 | 0.9298 | 0 - 20 |
| Vamidothion | 18.59 | 145 | 87 | 10 | 0.54 | -52.93 | 0.9997 | 0 - 500 |
| Tetrachlorvinphos | 18.65 | 329 | 109 | 38 | 0.30 | 109.22 | 0.9846 | 0 - 100 |
| Profenofos | 18.98 | 339 | 269 | 15 | 0.31 | 186.64 | 0.995 | 0 - 500 |
| Triazophos | 19.97 | 257 | 162 | 10 | 1.62 | 346.61 | 0.9978 | 0 - 500 |
| Pyrazophos | 22.06 | 374 | 222 | 35 | 0.0002 | 0.03 | 0.9955 | 0 - 1000 |

Calibration curves were constructed by plotting concentration of each pesticide versus GC response (peak area). For all analytes tested within a concentration range of 1–1000 ng/mL, the GC response was quadratic with excellent regression coefficients ($r^2 > 0.99$) as can be seen for KF in Table 2, with the exception of primicarb (0.9591), Primiphos-methyl (0.8893), triadimefon (0.8992), procymidone (0.9298), tetrachlorvinphos (0.9846).

### *4.3.2 Accuracy and precision of developed method*

The recovery, accuracy, and precision of the developed method were determined at the minimum concentration level i.e. 1 ng/mL for all mixes except KT for which it has been measured at 2 ng/mL. Each concentration contained ten replicates, although five replicates are recommended by [18]. Precision was calculated by using the relative standard deviation (R.S.D.). Accuracy was calculated by the following equation [20].

$$Accuracy = \frac{mean\ measured\ concentration}{nominal\ concentration} \times 100$$

According to the guidance document SANCO/12495/2011 of European Commission [18], the mean recovery should be in the range of 70–120% where as repeatability which is estimated by the relative standard deviation (RSD) of recoveries, should be ≤ 20% per commodity. According to Codex Guidelines, the acceptable range of recoveries should be in between 60-120 % with a RSD value of 30 % [19].

High accuracy, good precision, and good reproducibility for all analytes of the standard pesticide mixes were achieved at the tested concentrations. The range of recoveries for all analytes have been varied between 81- 120 % where RSD values lied between 0.93 - 14.16 %. The accuracy and precision results for all these analyses are within the acceptable range as prescribed by [18 & 19].

### *4.3.3 Determination of LOD and LOQ*

The limit of detection (LOD) is the minimum concentration of the analyte that can reliably be detected with a specified level of confidence. A linear calibration graph between GC responses versus initial 5 concentration levels was constructed for which the slope has been determined. The limit of detection (LOD) was then calculated with the following equation:

$$LOD = \frac{3.3 * s}{m}$$

where s is the standard deviation of the 10 replicate measurements of the lowest concentration level. The variable m represents the slope of the calibration graph including blanks [17].

The limit of quantitation (LOQ) is the lowest concentration of analyte that can be determined with an acceptable level of uncertainty. A value of 10s is frequently used (where s is the standard deviation of the results from replicate measurements of the lowest concentration level) [21]. For all analytes tested within a concentration range of 0-10 ng/mL, the GC response was linear with excellent regression coefficients ($r^2 > 0.99$) with a few exceptions. The Precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and regression coefficient for KF has been shown in Table 3. The LODs for KF are in the range of 0.17 - 9.84 ng/mL, whereas the LOQs for KF are in the range of 0.56 – 32.79 ng/mL.

Table 3: Precision, accuracy, LOD and LOQ description for KF

| Analyte | Nominal Concentration (ng/mL) | Concentration measured (ng/mL) | Precision (R.S.D.) | Accuracy (%) | Limit of Detection (LOD) ng/mL | Limit of Quantitation (LOQ) ng/mL | $r^2$ |
|---|---|---|---|---|---|---|---|
| Thiometon | 1 | 1.02 ± 0.15 | 14.16 | 102 | 9.26 | 30.87 | 0.9996 |
| Simazine | 1 | 0.87 ± 0.03 | 3.52 | 87 | 1.14 | 3.79 | 0.9987 |
| Terbumeton | 1 | 0.89 ± 0.12 | 13.57 | 89 | 5.72 | 19.06 | 0.9997 |
| Terbuthylazine | 1 | 0.99 ± 0.01 | 1.04 | 99 | 0.24 | 0.81 | 0.9998 |
| Pirimicarb | 1 | 0.98 ± 0.05 | 5.32 | 98 | 0.50 | 1.67 | 0.9610 |
| Terbutryn | 1 | 1.14 ± 0.10 | 8.88 | 114 | 9.84 | 32.79 | 0.9388 |
| Pirimiphos-methyl | 1 | 0.99 ± 0.02 | 2.27 | 99 | 0.54 | 1.80 | 0.9992 |
| Triadimefon | 1 | 0.97 ± 0.05 | 5.46 | 97 | 0.30 | 1.00 | 0.9918 |
| Procymidone | 1 | 1.01 ± 0.02 | 1.82 | 101 | 0.17 | 0.56 | 0.9543 |
| Vamidothion | 1 | 0.90 ± 0.05 | 5.12 | 90 | 1.54 | 5.13 | 0.9784 |
| Tetrachlorvinphos | 1 | 0.98 ± 0.02 | 2.4 | 98 | 0.24 | 0.80 | 0.9019 |
| Profenofos | 1 | 0.96 ± 0.02 | 1.98 | 96 | 0.31 | 1.04 | 0.9998 |
| Triazophos | 1 | 0.99 ± 0.02 | 1.87 | 99 | 0.45 | 1.50 | 0.9733 |
| Pyrazophos | 1 | 1.05 ± 0.02 | 1.77 | 105 | 0.55 | 1.83 | 0.9998 |

### *4.3.4 Method Application*

Identification of target analytes is accomplished by comparing the retention time and electron impact mass spectra of the analytes to that of a standard analyzed under the same conditions. The quantitative interpretation of a gas chromatogram is based on peak area. The procedure for quantitation by the peak area depends upon the measurement of the area of the peak of the compound from the extract solution to be analyzed and compared with the area of the peak measured for the compound from a standard (External or Internal), and from this comparison the amount of compound in the sample solution is calculated [22].

In order to evaluate the applicability of the developed method, real cotton samples extracted with different solvents were analyzed following the above mentioned methodology. With external standards, the area of mass chromatogram is calibrated with 10 standard concentration levels for each pesticide standard mixes (KF, KS, KT, KZ). Cotton samples extracted with different solvents (methanol, hexane, toluene, acetone & acetonitrile) were

injected for analysis. The maximum residue limit (MRL) for cottonseed were also mentioned which are recommended by EU Pesticide Database [23] and Codex Alimentarius Commission database [24], as MRL values for cotton fibers have still not been established. PCB 209 was used as an internal standard. An amount of 0.4 µg/mL was added homogeneously in all the cotton sample extracts along with method blanks and all calibration samples prior to the analysis. The overall residual pesticides obtained by this method from KF are summarized in Table 4.

Table 4: Description of residual pesticides detected with ESTD & ISTD from KF

| | | ESTD | | ISTD | |
|---|---|---|---|---|---|
| | **Analyte** | **Area** | **Amount in samples (mg/Kg)** | **Area Ratio** | **Amount in samples (mg/Kg)** |
| **Triazophos (0.2)** | BT114_A | 39724 | **0.331** | 0.1869 | 0.057 |
| | BT114_H | 38423 | **0.322** | 0.1810 | 0.055 |
| | PC_A | 36686 | **0.311** | 0.2466 | 0.074 |
| | PC_H | 43163 | **0.353** | 0.2513 | 0.076 |
| | PC_T | 38818 | **0.325** | 0.2863 | 0.086 |
| | PO_ACN | 2424 | 0.027 | 0.0099 | 0.003* |
| | PO_M | 1732 | 0.020 | 0.0071 | 0.002* |
| | SH1_M | 9299 | 0.096 | 0.0531 | 0.016 |
| | Z33_ACN | 73150 | **0.524** | 0.4253 | 0.126 |
| | Z33_M | 104084 | **0.673** | 0.7506 | **0.217** |
| **Terbuthylazine (0.1)** | GC_ACN | 173 | 0.006 | 0.0014 | 0.004 |
| | GC_H | 129 | 0.004 | 0.0004 | 0.001* |
| | GO_A | 201 | 0.007 | < LOD | |
| | GO_T | 125 | 0.004 | 0.0009 | 0.003* |
| | IC_H | 313 | 0.011 | < LOD | |
| | PC_A | 276 | 0.009 | 0.0015 | 0.004 |
| | PC_ACN | 266 | 0.009 | < LOD | |
| | PO_ACN | 501 | 0.017 | 0.0012 | 0.004 |
| | SH1_M | 1102 | 0.039 | < LOD | |
| | Z33_T | 1811 | 0.068 | | |
| | Z33_M | 1674 | 0.062 | | |

| | | ESTD | | ISTD | |
|---|---|---|---|---|---|
| | **Analyte** | **Area** | **Amount in samples (mg/Kg)** | **Area Ratio** | **Amount in samples (mg/Kg)** |
| **Profenofos (3)** | GC_ACN | 4263 | 0.088 | 0.0176 | 0.017 |
| | GC_H | 3331 | 0.069 | 0.0140 | 0.014 |
| | GO_ACN | 19768 | 0.368 | 0.0800 | 0.078 |
| | GO_H | 20850 | 0.386 | 0.0885 | 0.086 |
| | GO_M | 17930 | 0.338 | 0.0897 | 0.087 |
| | IC_A | 192 | 0.004* | < LOD | |
| | IC_ACN | 240 | 0.005 | 0.0028 | 0.003* |
| | IC_H | 285 | 0.006 | 0.0018 | 0.002* |
| | IC_M | 248 | 0.005 | 0.0018 | 0.002* |
| | IO_H | 361 | 0.008 | 0.0016 | 0.002* |
| | IO_M | 509 | 0.011 | < LOD | |
| | SH1_ACN | 1322 | 0.028 | 0.0071 | 0.007 |
| | SH1_H | 1231 | 0.026 | 0.0062 | 0.006 |
| | SH1_M | 1196 | 0.025 | 0.0052 | 0.005 |
| | Z33_T | 3084 | 0.064 | 0.0123 | 0.012 |

* Values >LOD but < LOQ.
Analytes that exceed MRL are in **bold** type.

Terbuthylazine, Profenofos, Terbutryn, Tetrachlorvinphos & Triazophos from KF were found present in the cotton samples. In case of Triazophos, 7 samples out of ten exceed MRL. The worth mentioning point is that PC (Pakistani classical) cotton samples contain more amount of residual pesticides than PO samples. In case of using ISTD, the residues of all insecticides in KF remained below MRL in all samples with the exception of Z33_M having residue more than MRL.

# 5. Author publications on the topic

### *5.1 International Journals*


[1]. Syed Zameer Ul Hassan, Jiri. Militký. "Acetylcholinesterase Based Detection of Residual Pesticides on Cotton." American Journal of Analytical Chemistry 3, no. 2 (2012): 93-98. ISSN:2156-8278

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[4]. Syed Zameer Ul Hassan, Jiri Militký, and Jan Krejci, "A Qualitative Study of Residual Pesticides on Cotton Fibers," Conference Papers in Materials Science, vol. 2013, Article ID 253913, 5 pages, 2013. doi:10.1155/2013/253913

### *5.2 International Conferences*

[1]. Syed Zameer Ul Hassan, Jiri. Militký. "Exploration of Residual Hazardous Compounds on Cotton Fibers." *Proceedings of World Cotton Research Conference-5.* New Delhi: Excel India Publishers, 2011. 561-567. ISBN: 978-93-81361-51-1

[2]. Syed Zameer Ul Hassan, Jiri. Militký. "Biosensor based Detection of Residual Pesticides on Cotton Fibers *Proceedings of 18th International Conference STRUTEX.* Liberec: Technical University of Liberec, Czech Republic, 2011. 203-208. ISBN: 978-80–7372–786–4

[3]. Syed Zameer Ul Hassan, Jiri. Militký. "Qualitative Detection of Residual Pesticides on Cotton." *Proceedings of The Beltwide Cotton Conference.* Orlando: National Cotton Council of America, 2012. ISSN: 1059-2644

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[8]. Syed Zameer Ul Hassan, Jiri. Militký, Jan Krejci. "A QUALITATIVE STUDY OF RESIDUAL PESTICIDES ON COTTON FIBERS." *Proceeding of 1st International Conference on Natural Fibers:Sustainable Materials For Future Applications.* Guimarães, 2013. ISBN: 978-989–20–3872–8

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# 7. Summary

A rapid, sensitive and low cost method based on AChE-inhibition utilizing biosensor has been developed for the identification of residual pesticides. It can be seen throughout the testing that the enzyme inhibition is a complicated mechanism. All the variables involved in AChE inhibition activity have been studied and optimized such as enzyme & substrate concentrations, buffer, pH and incubation time. Each of these variables has a significant role in this mechanism. Suitable calibration curves were obtained by preparing 5 standard concentration levels of Mix 155 along with Neostigmine as standard inhibitor and analyzed in order of increasing concentration. The values of RSD of inhibition % for 5 repetitions are found to be in a range of 1.51 – 34.45. The detection limit is found to be below 1 ppb. The method is utilized for real cotton samples extracted with different solvents (methanol, hexane, toluene). We are able not only to estimate the inhibition % of each individual sample but also we can compare this inhibition with the standard control points.
However in case of Algae testing, we can see that there is measurable interaction between cotton samples and algae which can be observed according to the results of our experiments but we are not able to find out some convincing results. The variation in the behavior of different cotton samples has been observed but none of these samples show any harm to the algae rather the effect of extracts stimulated their behavior. More concentrated samples must be employed in future to see some more interesting facts of this interaction. On the other hand algal species vary widely in their response to toxic chemicals and deferential sensitivity of green algae to the compounds has been observed in some reports.A multiresidue method for analysis of 76 pesticides with different physico-chemical properties has been developed for quantitative determination. The pesticide residues were determined by gas chromatography with Tandem mass spectrometry (GC-MS/MS). 57 out of 76 pesticides were detected successfully by the method developed. Confirmation of pesticide and quantitation was performed in selected-reaction monitoring mode (SRM). The range of recoveries for all analytes have been varied between 81- 120 % where RSD values lied between 0.93 - 14.16 %. The accuracy and precision results for all of these analyses have been found within the acceptable range as prescribed by [18 & 19].

The method was capable of detecting pesticides in real cotton samples. The GC-MS/MS method described in this work provides a reliable procedure for the determination of residual pesticides on cotton fibers. The procedure was proven to be effective, fast, sensitive and applicable to a wide range of pesticides. All validation criteria mentioned by European Commission document SANCO/12495/2011 for ‘Method Validation and Quality Control Procedures for Pesticide Residues Analysis in Food and Feed’ [18] were fulfilled. The method gave satisfactory analytical performance parameters for the most of the targeted pesticides and analysis of real samples proved its feasibility for the intended purpose.

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