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IDENTIFICATION OF RISK CONCENTRATIONS OF HAZARDOUS COMPOUNDS ON TEXTILES

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SUMMARY OF THE THESIS

Title of the thesis:	IDENTIFICATION OF RISK CONCENTRATIONS OF HAZARDOUS COMPOUNDS ON TEXTILES
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Abstract

Public concern over pesticide residues in food and crops has been increased for the past several decades. There is a perception among many consumers that organically grown cotton is superior in some aspects to cotton grown with conventional agriculture. 'Organic apparel' and 'organically produced' are now useful marketing concepts. A simplistic approach, such as an association of 'natural' with 'good' and 'synthetic' with 'bad' is useful in advertising but is difficult to justify due to the dependency of a lot of factors. A pesticide chemical may be very toxic which can be considered as being dependent on its intrinsic properties but the level of risk to the consumer associated with the chemical will be dependent on the level of exposure. The dissertation is a study of risk assessment based on processes in order to decide if the risk is low and acceptable in scientific terms. The thesis is a combination of study of qualitative and quantitative analytical measurements. For qualitative analysis, Biosensor approach and Interaction with algae have been implemented. The method is utilized for real cotton samples extracted with different solvents. We are not only able to estimate the inhibition % of each individual sample but also we can compare this inhibition with the standard control points. The pesticide residues were determined by Gas Chromatography coupled to triple Quadrupole 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). Trueness, Repeatability, Specificity, Limit of detection (LOD), Limit of determination (LOQ) and Applicability have been experimentally determined for each individual representative analyte. 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.

Keywords: Risk Assessment, Cotton, Biosensor, Gas Chromatography, GC, Tandem mass spectrometry, MS/MS

Anotace

Za poslední desítky let rezidua pesticidů v potravinách a plodinách vyvolávají veřejné znepokojení. Mezi mnoha spotřebiteli panuje představa, že ekologicky pěstovaná bavlna je v některých ohledech lepší než bavlna pěstovaná v konvenčním zemědělství. „Organické oblečení" a „Vyrobeno ekologicky" jsou často užívanými marketingovými koncepty v dnešní době. Zjednodušené přístupy jako organický – dobrý a syntetický – špatný jsou použitelné v reklamě, ale obtížně se zdůvodňují vzhledem k závislosti na mnoha faktorech. Vzhledem ke svým vlastnostem jsou pesticidy velmi toxické, ale úroveň rizika pro spotřebitele závisí na úrovni vystavení se pesticidům. Tato disertační práce posuzuje míru rizika založeného na procesech s účelem rozhodnout, zda je riziko nízké a přijatelné ze zdravotního hlediska. Tato práce je kombinací studia výsledků kvalitativních a kvantitativních analytických měření. Pro kvalitativní analýzu byla použita metoda přístupu biosenzorů a také interakce se zelenými řasami. Metoda se používá na vzorky pravé bavlny extrahované různými rozpouštědly. Nejenže jsme schopni odhadnout % inhibice každého jednotlivého vzorku, ale také můžeme porovnávat tuto inhibici se standardními kontrolními body. Rezidua pesticidů byla stanovena pomocí plynové chromatografie ve spojení s až trojnásobkem kvadrupólové tandemové hmotnostní spektrometrie (GC-MS/MS). Vyvinutou metodou bylo úspěšně detekováno 57 pesticidů z celkových 76. Kvantifikace a potvrzení pesticidů bylo provedeno v režimu sledování vybrané reakce (SRM). Správnost, opakovatelnost, specifičnost, mez detekce (LOD), limit kvantifikace (LOQ) a aplikovatelnost byly experimentálně stanoveny pro každý reprezentativní analyt. Metoda přinesla dostatečné analytické parametry provedení pro většinu cílových pesticidů a analýza reálných vzorků prokázala její využitelnost pro daný účel.

Klíčová slova: Zhodnocení rizik, bavlna, biosenzor, plynová chromatografie, GC, tandemová hmotnostní spektrometrie, MS/MS

Summary

1 Introduction

Cotton has been part of the fabric of human existence for thousands of years. Cotton is the most important natural textile fibre, as well as cellulosic textile fiber, in the world, used to produce apparel, home furnishings, and industrial products. Cotton has always been a major part of the textile industry and today provides almost 38% of the world textile consumption, second only to polyester, which recently took the lead. There has been a wide range of cotton made wearing apparel like shirts, dresses, children's wear, active wear, blouses, suits, jackets, skirts, pants, sweaters, hosiery, neckwear due to its unique characteristics of comfortable Soft hand, good absorbency, color retention, machine-washable, dry-cleanable, good strength, easy to handle and sew.
Public concern over pesticide residues in food and crops has been increased for the past several decades. There is a perception among many consumers that organically grown cotton is superior in some aspects to cotton grown with conventional agriculture. 'Organic apparel' and 'organically produced' are now useful marketing concepts. The market will supply the wants of those consumers especially concerned about the safety of pesticide residues and who are willing to pay a premium for reassurance of their health. However, there is still no convincing proof to believe that which production method is better regarding residual pesticides due to the involvement of a lot of factors. A simplistic approach, such as an association of 'natural' with 'good' and 'synthetic' with 'bad' is useful in advertising but is difficult to justify due to the dependency of a lot of factors.
A pesticide chemical may be very toxic which can be considered as being dependent on its intrinsic properties but the level of risk to the consumer associated with the chemical will be dependent on the level of exposure. If the pesticide leaves no residues on the cotton, then there would be no risk to the consumer. If on the other hand, the use of the pesticides leads to high residues, then this would result in a risk. The dissertation is a study of risk assessment based on processes in order to decide if the risk is low and acceptable in scientific terms. It is not possible to identify and quantify all residues of these pesticides on all the types of cotton within available resources. So a comparison of selected cotton samples of both modes of agriculture from the field has been analyzed in terms of their toxic effects. All the important factors for analytical process like proper sampling, handling, pre-treatment (cryogenic homogenization), extraction and analysis have been taken into account.

2 Purpose and the aim of the thesis

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

2.1 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.

2.2 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.

2.3 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.

3 Overview of the current state of the problem

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].

4 Experimental methods

4.1 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.

4.2 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.

4.2.1 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.

4.2.2 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.

4.3 Techniques Utilized

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

4.3.1 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.

4.3.2 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.

4.3.3 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.

5 Evaluation of results and new findings

5.1 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.

Table 1: 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	

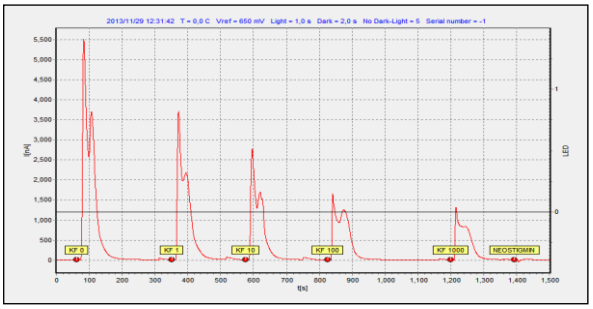

Figure 2: Amperometric response of calibration samples with optimized concentrations

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.

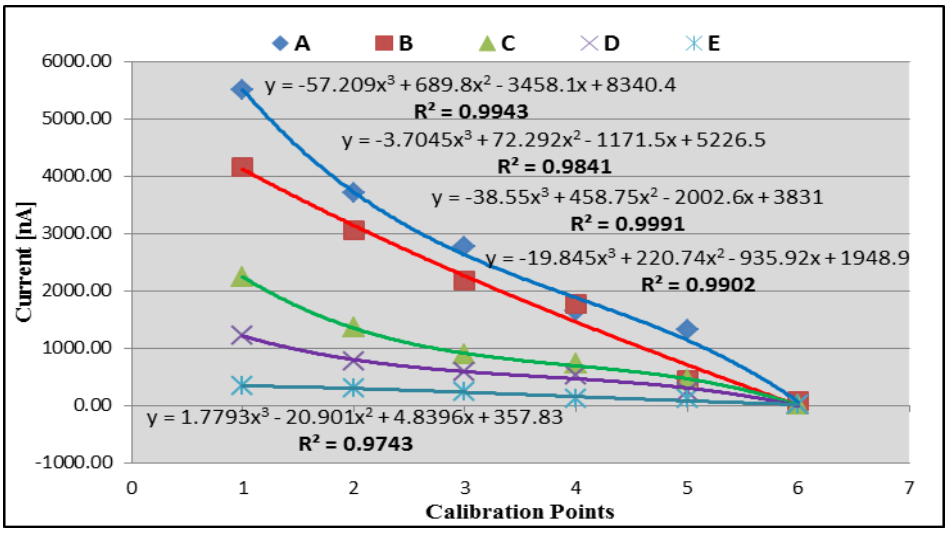

Figure 3: Amperometric response of different calibration samples; *n*=5

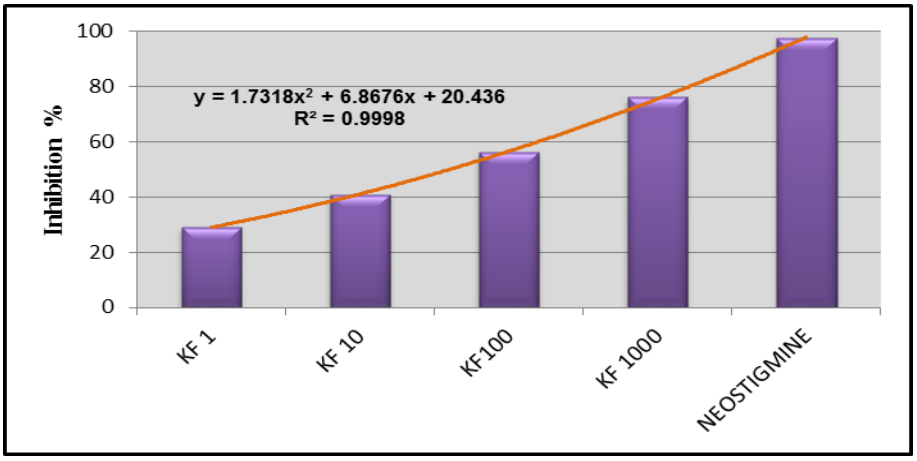

Figure 4: Average AChE-inhibition caused by different concentrations; *n*=5

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.

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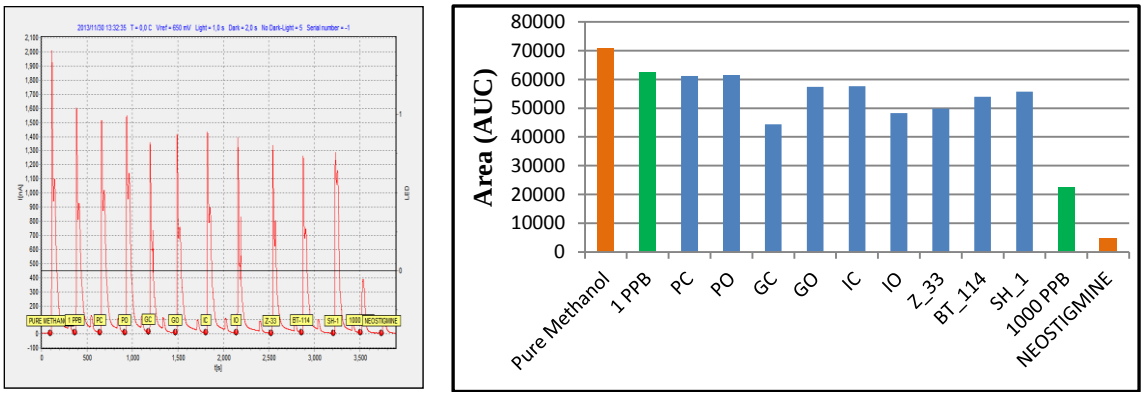

Figure 5: Amperometric response & AUC of all cotton samples extracted with methanol

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.

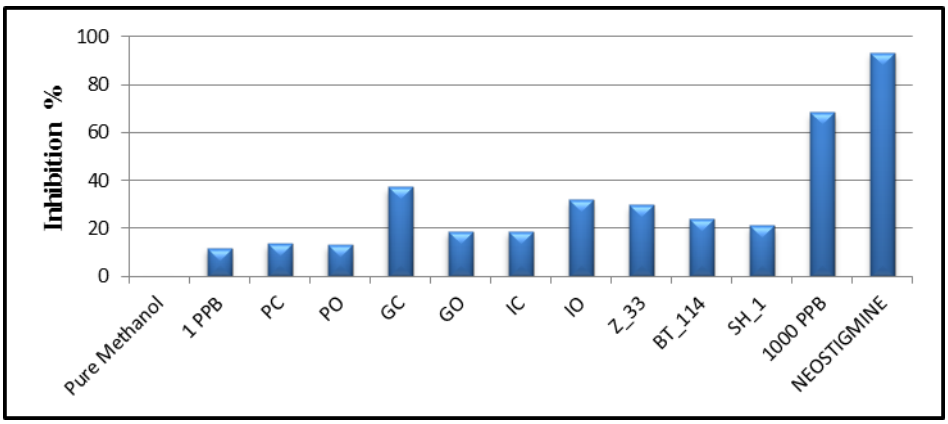

Fig 6: AChE-Inhibition caused by all cotton samples extracted with methanol

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.

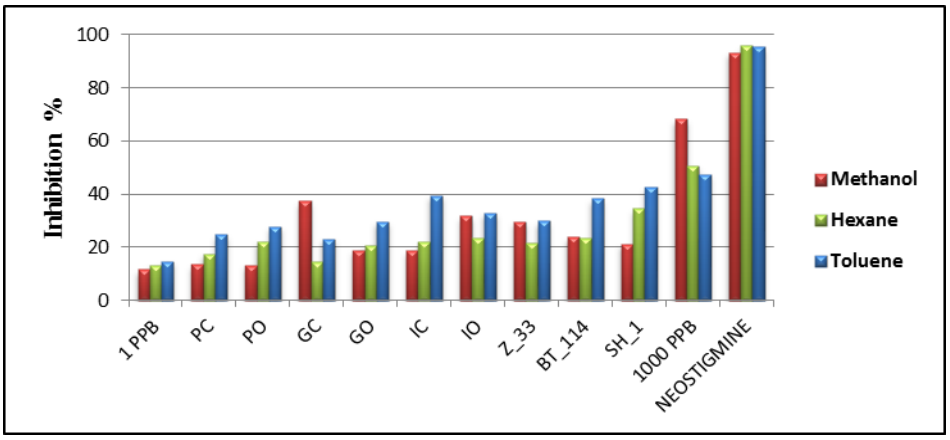

Figure 7: Summary of AChE-Inhibition caused by cotton samples with all solvents

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.

5.2 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.

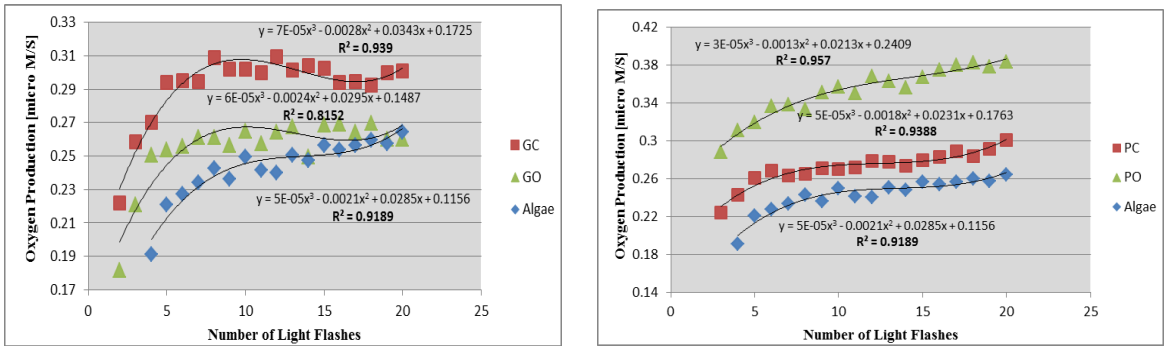

Figure 8: Comparison of GC & GO samples (L.H.S) and PC & PO (R.H.S)

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.

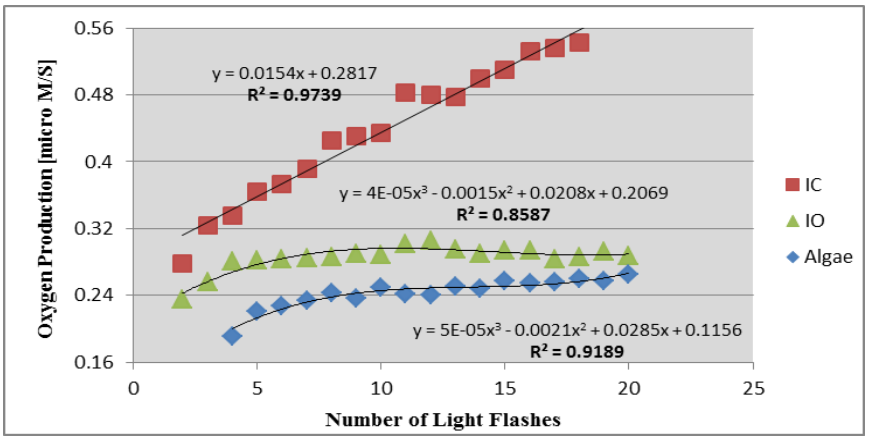

Figure 9: Comparison of IC and IO samples

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.

5.3 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.

5.3.1 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.

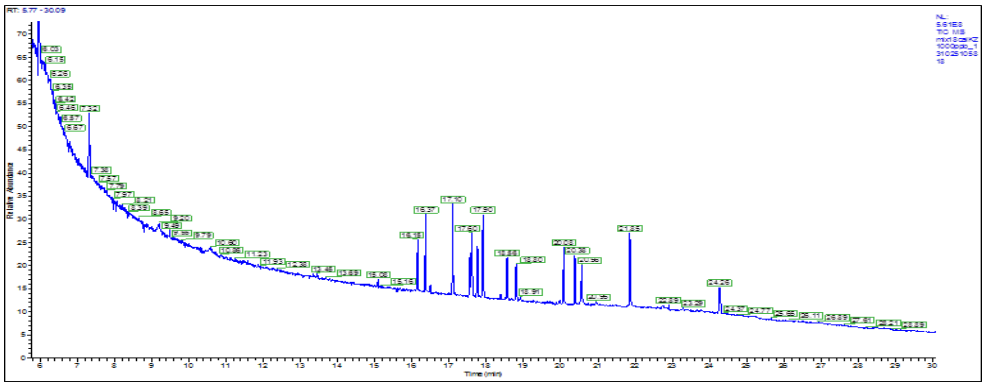

Figure 10: Gas Chromatogram for Pesticide Mix KZ

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.

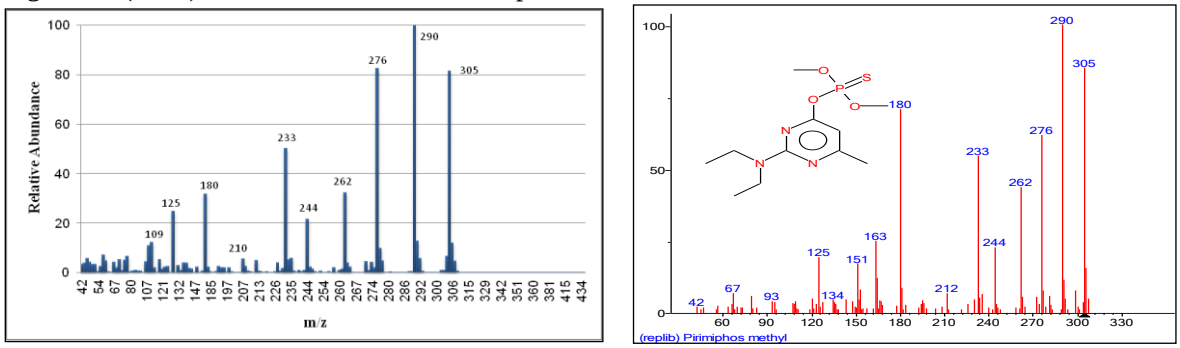

Figure 11: Mass to charge ratio for Primiphos-methyl (LHS) & EI spectra for Primiphos-methyl from NIST database (RHS)

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).

5.3.2 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].

5.3.3 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

5.3.4 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.

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7 List of published papers by the author

7.1 Publications in 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

7.2 Contribution in conference proceedings

[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

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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

8 Conclusion

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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Stanovisko školitele k doktorské disertační práci:

Identification of risk concentrations of hazardous compounds on textiles

Autora: **Ing. Syeda Zameer Ul Hassana**

Disertační práce je zaměřena na hodnocení míry zdravotního rizika spojeného s možnou přítomností zbytků pesticidů několika různých typů bavln klasických a pěstovaných bez použití pesticidů („organické" bavlny). Byl navržen systém výběru vzorků, manipulace V úvahu byly vzaty všechny důležité faktory, jako je výběr řádných vzorků, manipulace (kryogenní homogenizace), extrakce a vlastní hodnocení efektů využívající jak kvalitativní tak i kvantitativní analýzy. Pro kvalitativní analýzu míry zdravotního rizika spojeného s možnou přítomností zbytků pesticidů byla použit speciální biosenzor a interakce se zelenými řasami. Byly hodnoceny bioelektrické signály způsobené inhibicí enzymatické acetyl-cholinesterázy (AChE) s použitím analyzátoru biosensorové toxicity (BTA). Změny těchto signálů jsou způsobeny interakcí biologických látek a zbytků pesticidů.
Byla také hodnocena interakce zbytků pesticidů a zelených řas měřením inhibice kyslíku vznikajícím fotosyntézou.
Pro kvantitativní analýzu zbytků pesticidů byla použita plynová chromatografie spojená s trojitým kvadrupólovým tandemovým hmotnostním spektrometrem (GC/MS/MS). Bylo analyzováno 76 pesticidních systémů běžně používaných pro bavlnu, nejen pro identifikaci, ale také pro kvantifikaci. Kalibrace byla v rozsahu od 1 ppb do 1000 ppb.
Je potřeba zvláště ocenit, že prakticky všechny zajímavé resp. původní výsledky byly autorem již publikovány v průběhu jeho doktorandského studia to nejen na mezinárodních konferencích (celkem 8 příspěvků) ale také v časopisech (4 příspěvky), takže autorovy výsledky byly již vlastně kladně posouzeny mezinárodní odbornou komunitou. To ukazuje na systematickou kvalitní práci uchazeče spojenou s řadovou publikovatelných výsledků.
Práce je psána stručně, s vynecháním známých detailů a popisu běžných postupů, ale s dostatečným objasněním zejména vlastních výsledků.
Část současného stavu je psána jen přehledově a v některých případech i s detaily. Obsahuje dostatečný popis zejména různých typů pesticidů a jejich praktické použitelnosti včetně vlivu na životní prostředí. Jsou uvedeny také relevantní informace o stavu řešení v oblasti měření zbytků pesticidů základními metodami a metodami využitými ve vlastní práci.
V experimentální části jsou popsány základní charakteristiky tří typů bavln (Egyptská bavlna Giza 86, Pakistánská bavlna MNH 93 a Indická bavlna) vždy pěstovaných konvenčně a bez použití pesticidů. Je popsán postup kryogenní homogenizace a extrakce možných zbytků pesticidů z bavln a vlastní postupy jak kvalitativní tak i kvantitativní analýzy.
Tady lze ocenit především množství provedených experimentů. Na druhé straně jsou kalibrační závislosti statisticky zpracovány pouze na základní úrovni.
Je uvedena řada formálních empirických modelů popisujících získané výsledky. Některé však mají pouze omezenou platnost a chybí také hlubší statistická analýza.
V práci jsou další, nepřesnosti a nedostatky zejména formálního charakteru, které však výrazně nesnižují její celkově dobrou odbornou úroveň. Výsledky práce jsou zajímavé a budou využitelné pro reálné posouzení vlivu reziduí pesticidů na míru zdravotního rizika.
Práce je celkově na postačující úrovni a **doporučuji ji** k obhajobě.

V Liberci 22/05/2014

Školitel: prof. Ing. Jiří Militký, CSc., EUR ING

Opponents' reviews

Review of the PhD Thesis

Identification of risk concentrations of the hazardous compound on textiles

The dissertation work has 140 pages, 105 figures and 33 tables. The literature contains 165 citations. It is organized in the standard style of thesis.

The annotation emphasizes the key point of this thesis. It is association of the word natural with good and synthetic with bad. It clearly demonstrates the difficult task of the thesis – prove if there is some probability that the pesticides (and may be other compounds) can survive all technological process from collection of cotton fibres to final tissue which is used by customer as wearable clothing. The difficulty of this task consists of three major influences:

- Precise description of the sample origin and its history
- Evaluation of the influence of original hazardous compounds introduced in the cotton during cultivation with hazardous compounds introduced during next cotton treatment.
- The methods of hazardous compounds extraction and analysis.

These last points are leading idea which can be identified in whole thesis and which is necessary to have in the mind in discussion of all results.

The topic of the thesis is highly actual and of very high scientific importance. In fact, two fully independent methods are compared. The method based on biosensors measures the toxic activity on the target point. In case of organophosporous and carbamate pesticides it is inhibition of Acetylcholine Esterase. These method measures the real toxic activity. The HPLC and GC methods measure the actual physical concentration of the toxic analyte. In different conditions due to different bioavailability the methods can differ in significant manner. The biosensor methods can be applied in low cost format as qualitative information but they can be also used in quantitative format. The inhibition rate can be a result in this case, for example. The fig. 70 confirms that the method works "quite well". The quotation marks are very important here. There is a problem of extraction solvents which are not soluble in water. The optimum function of enzyme is in water conditions. The transfer of sample from solvent to water brings major source of errors. It is also not emphasized that in case of biosensor measurement it is necessary to optimize significantly more parameters as in case of GC and HPLC. It is pH of sample, ionic strength, composition of buffers and many others. The biosensor can offer reliable and cheap measurement but the procedure of its development can be more expensive than use of GC or HPLC. The optimization of the biosensor measurement is very laborious. Therefore this comment should not depreciate the thesis. It is highly worth full that the author had courage to input in this field.

There is another difference between biosensor measurement and GC/ HPLC measurement. Biosensor can detect not only artificial pesticides which are detected by GC/ HPLC but also natural compound which can be present in the cotton. It means that the result in table 11 (cotton from Giza) where the higher inhibition was detected for organic cotton need not to be experimental error.

The results in chapter 4.3 should be more stressed from my point of view. The human cells were cultivated on the cotton fibres and the growth rate using algae on cotton was measured too. These tests are wide spectrum bioassays which detect all possible toxic activities of sample (not only chemical but also mechanical for example). No significant

effects of the cotton extracts were observed. It has significant consequences, namely that the risk of hazardous compounds at samples is very low. This part of thesis should be repeated (if possible) in exact methodological procedure:

- collection of samples including their documentation
- evaluation of Cytotoxicity
- evaluation by Algae
- evaluation by biosensor
- GC evaluation

All samples should be evaluated in approximately same time. Pesticides are unstable and their time decomposition should be eliminated.

I do not have any comment to the GC/HPLC part of the thesis because I am not expert in this field.

The thesis is work out in very good formal manner. There are only a few of formal mistakes (p.2 immunoassay, p. 48 varieties, p.75 repetition,..)

The methodology of the thesis is clear from the formal point of view. The thesis is divided to the standard form of such work. I did not find the chapter between chapter 2 and 3 where the state of art in this scientific field is described (as result of the literature review) and where the aim of thesis is specified. The sample collection in chapter 3 is not described sufficiently from the point of precision and methodology. There are many standards of sample collections. They are not mentioned. It is necessary at least to specify the place where the sample was taken, time of sample collection, storage of sample including temperature, wetness and light. (Light and wetness can stimulate the pesticides decomposition). The lack of the information about sample and its storage depreciates all effort spent in next analyses. The figures are work out in excellent quality. The content of figures is sometimes misleading. It is necessary to estimate the physical processes which are behind phenomena which should be expressed on the picture. In principle it is an error to approximate the data by polynomial of order 3 if no reason is for it, for example. It can be demonstrated on the fig. 45 for example. I suppose that the data 1 – 7 is an experimental error and the real response starts at calibration point 7. In many graphs there are the numbers of calibration points not their values. (It is not obvious. I suppose for example fig. 44 and 45.). However then the correlation does not have any physical meaning. The correlation correlates the values of the independent and depended variable. There is no physically reasonable correlation between number of measurement and dependent variable. The similar is valid for the fig. 100 – 102 for example. The linear correlation of the data which are ranged in extend of 3 orders brings unacceptable error. All values near zero contribute as 1 value and second value is 1 point at high concentration.

I have a question to the author: Can you demonstrate the fig. 99 in format where the both axes will be in logarithm scale?

The thesis is very good introduction in the problem of detection of hazardous compounds in cotton. The work is done carefully and with big effort. I recommend to accept this work without any changes.

Additional comment:

From my point of view there is no significant difference between the organic and standard cotton. The division to organic and standard cotton is marketing trick. I suppose the results which show the possibility of simple and cheap detection of pesticides can be used by people

who really cultivate the cotton. The information about toxicity of plants in the field, the measurement of activity of Acetylcholin esterase in their blood (similar as glucose strips at diabetic peoples) has significantly higher importance than to measure the traces of pesticides in the final products. It can improve their health and life. If they can use optimum amount of pesticides it also decreases the amount of pesticide traces in the final product. However it is my personal opinion.

Jan Krejci

BVT Technologies a.s.

Strazek 206,

28.4.2014

Review of PhD. dissertation of Ing.Syed Zameer Ul Hassan entitled:
Identification of Risk Concentrations of Hazardous Compounds on Textiles.

The work reported is dealing with very important subject – the contamination of textiles by pesticides and related compounds. Today, the approach to such problem is critically dependent on the instrumentation and advanced techniques of analytical chemistry. That is why, that many techniques are available to the analytical chemist and many strategies are ready for use. It has been pointed out, that having many advanced instrumentations at hand, the simplest methods are usually the best one.

The thesis submitted includes a study of three different techniques for the investigation of residual pesticides on cotton. I would like to deal with two of them.

1. A rapid , sensitive and low-cost method based on specific enzyme inhibition utilizing biosensor technology have been developed. It is claimed that all variables have been studied and optimized. There are five variables in this thesis (concentration of both enzyme and substrate, buffer used, pH, and incubation time. So it means five dimensional optimization. Consequently, my question is: How such multidimensional task has been accomplished?

2. GC-MS/MS, is extremely sensitive and at the same time it is able to furnish the broad range of data. Surprisingly, I did not find any experiment using tandem MS/MS technique. Moreover, the software supplied with any instrument of this kind is well developed and usually as user friendly as possible. The vast range of analytical data can be obtained from routine measurement. It is not very clear to me which data shown in Thesis have been obtained from routine GC-MS experiments and which data have been obtained using original experiments or treatment. Too many data are gathered in thesis – some of them are not very important - one example: Figure 90 on page 93 has hardly any information content.

Consequently, I would like to ask following questions:

a) Separation conditions.
In Table 17, page 93, GC parameters are listed – they have been originally developed for this work or it is routine setting supplied with instrument? Could you describe in more detail the GC column that has been used and why?

b) Calibration curves (pages 100-103)
They are sometimes ridiculous – what about those on page 108? Have they any statistical significance?

c) GC-MS Databases.
Are there any need to compare two commercial GC-MS database. Such databases are in routine use for decades. They should be very similar. Did you find any important difference?

In summary, thesis goals have been fully attained and results obtained can be used directly. In addition to it, solutions belonging to very important topics have been developed. I am in the position to recommend the thesis to be accepted as good basis for PhD. degree award to Ing. Syed Zameer Ul Hassan.

In Liberec, May 16th, 2014

Prof.Ing.Ivan Stibor,CSc.