ABBREVIATIONS

% A/A_o	% Maximal absorbance
Ab	Antibody
Ag	Antigen
A_{max}	Maximum absorbance
AMPA	Amino methyl phosphonic acid
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
APC	Agricultural Pesticides Committee
BDL	Below detectable level
BSA	Bovine serum albumin
CCME	Canadian Council of Ministers of the Environment
CFA	Complete Freund's adjuvant
CLIA	Chemiluminescent immunoassay
CS_2	Carbon disulfide
Cyp450	Cytochrome P450
Da	Dalton
DAD	Diode-array detection
DCM	Dichloromethane
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
d-SPE	dispersive-Solid phase extraction
EBDCs	Ethylene-bisdithiocarbamates
EC	Emulsifiable concentrate
ECD	Electron capture detection
EDC	1-ethyl-3-(3-diaminopropyl) carbodiimide hydrochloride
EFSA	European Food Safety Authority
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EPA	Environmental Protection Agency

EPSPS	Enolpyruvylshikimate-3-phosphate synthase
ETU	Ethylene thiourea
EU	Ethyleneurea
Fab	Antibody fragment containing the antigen binding site
FAO	Food and Agriculture Organization
Fc	Fixation of complement
FD	Fluorescence detection
FIA	Fluorescence immunoassay
FMOC-Cl	9-Fluorenylmethyl chloroformate
FPD	Flame photometric detection
G	Granules
GABA	γ -aminobutyric acid
GC	Gas chromatography
GCB	Graphitized carbon black
GLC	Gas liquid chromatography
h	Hour
ha	Hectare
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IA	Immunoassay
IARC	International Agency for Research on Cancer
ID	Internal diameter
IFA	Incomplete Freund's adjuvant
Ig	Immunoglobulin
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
kDa	Kilodaltons
KLH	Keyhole limpet hemocyanin
L'ELISA	Linker-assisted enzyme-linked immunosorbent assay
LC	Liquid chromatography
LLE	Liquid liquid extraction
LOD	Limit of detection

LOQ	Limit of quantitation
mAb	Monoclonal antibody
MRL	Maximum residue limit
MS	Mass spectrometry
MW	Molecular weight
NHS	N-hydroxysuccinimide
NPD	Nitrogen phosphorous detection
O.D	Optical density
OM	Organic matter
OPA	*o*-phthalaldehyde
OVA	Ovalbumin
pAb	Polyclonal antibody
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline with Tween 20
pH	Potential of hydrogen
PHI	Pre-harvest interval
ppm	Part per million
PSA	Primary secondary amine
QuEChERS	Quick, easy, cheap, effective, rugged and safe
R^2	Correlation coefficient
RIA	Radioimmunoassay
RSD	Relative standard deviation
RT	Room temperature
SAS	Saturated ammonium sulphate
SC	Suspension concentrate
SDS	Sodium dodecyl sulfate
SE	Standard error
SG	Soluble granules
SL	Soluble concentrate
SLE	Solid liquid extraction
SPE	Solid phase extraction
Sulfo-NHS	N-hydroxysulfosuccinimide

$t_{½}$	Half-life
TBS	Tris buffer saline
TG	Thyroglobulin
TMB	3,3′,5,5′-tetramethylbenzidine
USA	United State of America
UN	United Nation
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet
Vis	Visible
WG	Water dispersible granules
WHO	World Health Organization
WP	Wettable powder

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SUMMARY AND CONCLUSION

Biotechnology is unique in its global range of applications, combining engineering with molecular biology and chemistry to detect and quantitate chemicals, whether environmental and food contaminants, products of industrial processes, metabolites of drugs of abuse in urine as well as in medical diagnostics. Pesticides are unusual among environmental pollutants in that they are used deliberately for the purpose of killing some form of life. The ideal situation, of course, is that pesticides be highly selective, destroying target organisms while leaving non target organisms unharmed. In reality, most pesticides are not so selective. In considering the use of pesticides, the benefits must be weight against the risk to human health and environmental quality. Pest control is among the benefits of pesticides. A major risk is environmental contamination, especially translocation within the environment where pesticides may enter both food chains and natural water systems. Factors to be considered in this regard are persistence in the environment and potential for bioaccumulation judged by the most precise and accurate analytical procedures.

The need to evaluate the risk to the environment from the use of chemicals has been a significant part of the regulation to control pesticides for many years in the world. There has been an increased awareness and concern from the public and regulatory authorities regarding the potential for pesticides to contaminate air, soil and water sources. This pressure has resulted in the evaluation of different analytical methods and detection techniques in an effort to lower detection limits and improve confirmation procedures for pesticides in environment.

Immunoanalysis is recognized as a major analytical method applicable to numerous analytical needs including detection and quantitation of drugs, pesticides and other chemicals, in body fluids and chemicals in environmental samples (e.g. rivers, underground water or soil extracts). Enzyme linked immunosorbant assay (ELISA) is the dominant format used at present time. Briefly, the antigen (Ag) is adsorbed on the surface of microtiter plate wells and the primary antibodies (Abs) in the immune serum are allowed to bind to the coating Ag. A secondary Ab, linked to an enzyme (e.g. horseradish peroxidase), is added followed by a substrate solution. A colored product appears and its density is measured with a plate reader (a special spectrophotometer). The presence of the

analyte during the first incubation competes coating antigen for binding with the primary Ab, thereby reducing the signal quantitatively.

Fipronil, mancozeb and glyphosate are applied to control various species of pests in agriculture. Due to their widespread uses, these pesticides should be continuously monitored especially in environmental samples and therefore a rapid, reliable, convenient and inexpensive method for their analysis is required. Many established analytical techniques have been employed for the determination of fipronil, ETU and glyphosate in soil, water, vegetables, fruits, etc. These include gas chromatography (GC) employing electron capture detection or nitrogen phosphorous detection, high performance liquid chromatography (HPLC) with UV detection, fluorescence detection or diode array detection and GC or LC coupled with mass spectrometry (MS) or tandem mass spectrometry (MS/MS). These methods are accurate, but also time-consuming and expensive. Antibody-based assays represent an effective alternative to instrumental methods. Recently, ELISAs have proven to be rapid, cost effective and highly sensitive analytical methods.

The aim of this study was to develop technique for fipronil, ETU and glyphosate residues analysis. Since pesticides (haptens) are small molecules, development of immunoassay for pesticide residues requires coupling them to a large immunogenic carrier, such as protein. The conjugation was a step for producing specific antibodies for further detection using ELISA to determine fipronil, ETU and glyphosate in the environment samples. The study included the following:

First : Fipronil as a phenyl pyrazole insecticide, ETU; the main metabolite of mancozeb as a EBDCs fungicide and glyphosate as an N-(phosphonomethyl) glycine herbicide have been selected. These pesticides are widely used to control various species of pests in agriculture.

Second : The approach was followed to conjugate the amine group of hapten (fipronil, ETU or glyphosate) with the carboxylic group of carrier protein (KLH, TG or BSA) to produce hapten-protein conjugate. The conjugates were confirmed by UV spectrophotometry.

Third : The laboratory rabbits were immunized with the respect immunogen to elicit an appropriate antibody response.

Fourth : The immunochemical technique (ELISA) has been used to develop a signal to be measured of the conjugated analyte. A competitive indirect ELISA assay has been used to determine optimum concentrations of the antigen and antibody by checkerboard in the absence and presence of analyte (fipronil, ETU or glyphosate). Also, optimization of assay conditions such as solvent concentration, ionic strength concentration and pH value of the corresponding buffer were studied for fipronil and ethylene thiourea, whereas derivatization with succinic anhydride using 50 mM TBS, pH 9 was studied for glyphosate.

Fifth : Comparison between the conventional method (HPLC) and the immunochemical technique (ELISA) was undertaken for spiked−recovery studies.

Sixth : Determination of tested compounds residues in environmental samples using ELISA.

A field experiment was conducted in the Agriculture Research station, Alexandria University. Fipronil (Couch 20 % SC) and mancozeb (Dithane 80 % WP) were applied on tomato and potato crops, whereas glyphosate (Round up 48 % WSC) was applied in uncultivated field. Samples of soil, tomato and potato were collected before application and 0 (1h), 1, 3, 5, 7 and 10 days after applications. The residues were quantified using indirect competitive ELISA.

The results of the present study can be summarized as follows:

I. Preparation of the hapten-protein conjugates:

1) Hapten-protein conjugates were carried out by form a covalent linkage between a function group (amino group) of the tested compounds with a carboxylic group of the corresponding protein using EDC and Sulfo-NHS.
2) Two conjugates for each hapten were prepared. One for immunizing laboratory animals as an immunogenic conjugate (fipronil-KLH, ETU-KLH and glyphosate-TG) and the other for ELISA format as coating conjugate (fipronil-BSA, ETU-BSA and glyphosate-BSA).
3) Each hapten-protein conjugate was confirmed using UV spectroscopy, by following the shift of the conjugate peak to a different position compared with corresponding hapten and protein which approves the successful

conjugation.

II. Antibody production:

1) Immunization of conjugated haptens elicited appropriate antibody response to fipronil, ETU and glyphosate in rabbits, and the antibodies were obtained from the serum.
2) The antibody concentration (titer) in the sera was determined by ELISA. The antibody of the tested conjugates showed high levels of polyclonal antibodies, with titer reached to 1:128,000 for fipronil and ETU, whereas 1:250,000 for glyphosate.

III. Assay optimization:

1) A competitive indirect ELISA assay has been used to determine optimum concentrations of the coating antigen and antibody using checkerboard titration in the absence and presence of analyte.
2) Optimum reagent concentrations were 3.125, 1.56 and 1.56 µg/ml of hapten-BSA conjugates and 1:2000, 1:4000 and 1:10000 dilutions of polyclonal antibodies for fipronil, ETU and glyphosate, respectively.
3) The optimal conditions for assay were 50 mM PBS, pH 7.4 containing 1 % methanol for fipronil and ETU or 50 mM TBS, pH 9 with derivatization using succinic anhydride for glyphosate.
4) From the standard curve (dose-response curve) of each analyte it was found that IC_{50} (concentration of the analyte that causes 50 % inhibition) values were 0.325, 3.71 and 0.018 µg/ml for fipronil, ETU and glyphosate, respectively. LOD (least concentration of the analyte that produces response = 90 % A/A_o) were 0.026, 0.2 and 0.8 ng/ml for fipronil, ETU and glyphosate, respectively. From these values it is clear that the developed ELISA techniques for all the tested compounds are very sensitive and accurate.

IV. Also, the developed ELISA technique for each hapten was successfully demonstrated its accuracy and reliability when applied in spiked−recovery studies and compared to established conventional analytical techniques (HPLC) in different matrixes. The recovery values of fipronil, ETU and glyphosate from soil, tomato and potato matrixes fortified at 0.01, 0.1, 1.0 and 10 µg/g levels

were ranged from 85.5 to 102.7 % indicating that the ELISA technique can be considered good. Also, the data illustrated that the analysis of tested analytes in soil, tomato and potato samples by ELISA and HPLC yielded a good correlation between two methods.

V. The determination of fipronil, ETU and glyphosate residues in the field trials after different time intervals was conducted using the development ELISA techniques. The fipronil residues in soil and tomato samples were degraded by time, with a dissipation rate of 73.81 and 76.84 %, respectively at 10 days. The decay of fipronil followed the first order kinetics with half-life period of 5.2 and 4.7 days for soil and tomato samples, respectively. No detectable residues were recorded in potato tubers until 3rd day of fipronil spraying, whereas the concentration of 1.3 µg/kg was observed at the 5th day of application and the concentration was increased until the 10th day (6.5 µg/kg). The concentration of ETU was gradually increased in soil with time after application from 0.0125 to 1.416 mg/kg. Whereas the residues of ETU in both tomato and potato samples were gradually increased until 3rd and 5th day, respectively, then dissipated with half-life of 1.33 and 4.33 days for tomato and potato samples, respectively. The glyphosate was slowly degraded in uncultivated soil reaching to dissipation rate of 18.94 % at the end of experiment (10 days).

From this study, we can conclude that, conjugation of the small molecule (hapten) with carrier proteins (KLH or TG) in such a way that they elicit the immune response of rabbits and subsequent generation of specific antibodies with high titer. ELISA can be affected by many factors and optimization process is necessary to improve the sensitivity, accuracy and reproducibility. ELISA provided lower detection limits and higher recoveries and was considerably faster than a classical HPLC procedure.

Also, we could be used the locally produced antibodies to determine the analyte concentrations in environmental samples collected from Agriculture Research Station, Alexandria University. The developed ELISA described in this study is promising for monitoring of fipronil, ETU and glyphosate residues in a variety of environmental samples (soil, tomato and potato) and generate a large number of samples for which ELISAs are ideally suited. For screening samples, ELISA could also be applied directly to the sample

without clean up of the extract. So, ELISA is especially effective when many samples have to be quickly screened for pesticide residues or in the detection of illegal pesticide applications.

The maximum residue levels (MRLs) of fipronil in vegetables are 10 µg/kg for potato and 5 µg/kg for tomato **(EFSA, 2012)**. Although, ETU is polar, water-soluble compound and its control in fruits and vegetables is essential due to greater toxicological concern, ETU is not regulated by the same system of maximum residue limits (MRLs) as the parent pesticide (mancozeb) in Europe **(López-Fernández *et al.*, 2014)**. **Startin *et al.*, (2005)** adopted 0.01 mg/kg as the target reporting limit for the alkylenethioureas. Also, **Özhan and Alpertunga (2008)** mentioned that MRL of ETU admitted on agricultural crops and food products is 0.05 mg/kg. Therefore, the development of simple and sensitive method for fast monitoring of EBDC metabolite in food products is of great importance.

Our findings show that, the ELISA method satisfies the requirement which provide excellent performance and make it suitable for fast fipronil, ETU and glyphosate monitoring. Where, the rapidity and simplicity of the method, combined with the low detection limit and satisfactory recoveries, make it valuable for the routine analysis of pesticide residues particularly in low-budgeted laboratories.

Finally, it was concluded that the routine monitoring of pesticide residues is required in order to protect the environment and ensure the food safety of consumer's health. Analytical methods using gas and liquid chromatography are sensitive and reliable for the detection of pesticide residues; however, they require well-trained personnel, sophisticated instrumentation, a well-equipped laboratory and time-consuming sample preparation. Consequently, there is an increasing demand for more rapid and economic methods for analyzing pesticide residues. Immunoassays, especially ELISA, have been emerging as an attractive alternative or complementary method to the traditional chromatographic methods for the determination of pesticides residues. Due to their simplicity, easy performance, selectivity, good sensitivity and cost-effectiveness, immunoassays can be used for the high sample throughput and on-site screening of pesticide residues.