THE DETERMINATION OF β-ENDOSULFAN AND ENDOSULFAN SULFATE IN HUMAN SERUM WITH DIALKYLPHOSPHATE METABOLITES AS URINARY MARKERS USING LC-MS/MS ELECTROSPRAY IONIZATION

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Dissertation submitted in fulfillment of the requirements for the degree

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DECLARATION

It is herewith declared that this dissertation for the degree Master of Medical Science (Clinical Pharmacology) at the University of Cape Town is the independent work of the undersigned and has not previously been submitted by him at any other University or Faculty for a degree. In addition, copyright of this dissertation is hereby ceded in favour of the University of Cape Town.

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Declaration certifying the candidate's personal contribution towards the research, which is the subject of this M.(Med). Sc (Clinical Pharmacology).

Candidate: Mr Werner Bergh

Title: The determination of β-endosulfan and endosulfan sulfate in human serum with dialkylphosphate metabolites as urinary markers using LC-MS/MS electrospray ionization.

Supervisor: Dr Lubbe Wiesner

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We, the undersigned, declare that under our supervision, Mr. Bergh performed the bioanalytical work contained in this dissertation. Under our supervision, Mr. Bergh personally compiled and typed the dissertation in its present form.

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28 Nov 2016

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ABSTRACT

Two separate bioanalytical methods were developed, validated and applied to determine agricultural exposure to organochlorine and organophosphorus pesticides using different biological matrices as reference sources.

The method that was validated for the quantification of the organochlorine compounds was used to simultaneously determine β-endosulfan [6, 7, 8, 9, 10, 10- hexacloro-1, 5, 5a, 6, 9, 9a- hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepin-3- oxide] and one of its main metabolites, endosulfan sulfate, in human serum. In a second bioanalytical method, urinary dialkylphosphate metabolites have been assessed as markers to estimate the exposure to organophosphorus pesticides, focusing on three of the six organophosphorus urinary metabolites, namely dimethyl phosphate, dimethyl thiophosphate and diethyl phosphate.

For both the bioanalytical methods, liquid-liquid extraction was used for sample preparation and high performance liquid chromatography with tandem mass spectrometry as detection method due to its high sensitivity and selectivity.

Chromatographic separation for both bioanalytical methods was achieved by performing reverse phase chromatography on C18 analytical columns. Isocratic elution with a mobile phase composed of acetonitrile, methanol and water was employed for the analysis of the organochlorine compounds while the organophosphorus compounds were eluted using gradient elution with a mobile phase consisting of acetonitrile and 20 mM ammonium acetate.

A triple quadrupole mass spectrometer equipped with an electrospray ionization source operating in the negative ionization mode was used for mass detection of all the analytes, employing multiple reaction monitoring as scan mode.

Calibration standards and quality control samples for both analyses were prepared in the biological matrix in which the samples for each determination were collected, i.e. serum for the determination of the organochlorine compounds and stripped urine for the organophosphorus compounds. Deuterated internal standards were used in the bioanalytical method for the determination of the organophosphorus compounds whereas the organochlorine compounds were determined without the use of an internal standard due to unavailability of suitable internal standards.

The calibration ranges for the determination of β-endosulfan and endosulfan sulfate were 0.8 ng/ml to 200 ng/ml and 0.117 ng/ml to 30 ng/ml, respectively, and 1.0 ng/ml to 30 ng/ml for the dialkylphosphate metabolites of the organophosphorus compounds.

These sensitive and robust quantitation methods were successfully applied to quantify 219 serum and 187 urine samples that were collected from agricultural workers with the purpose to determine whether they were exposed to any of the investigated organochlorine or organophosphorus compounds.

No traces of β-endosulfan and endosulfan sulfate were found in any of the serum samples that were analyzed, however, significant amounts of the three organophosphorus compounds dimethyl phosphate, dimethyl thiophosphate and diethyl phosphate were present in the urine samples.

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GLOSSARY OF DEFINITIONS AND ABBREVIATIONS

AAPS	American Association of Pharmaceutical Scientists
AB	Applied Biosystems
ACN	Acetonitrile
Analyte	The substance or chemical constituent that is determined in an analytical procedure.
APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric Pressure Ionization
APPI	Atmospheric Pressure Photo Ionization
AUX	Auxillary gas
A	Alpha
BASD	Bioanalytical Services Division
B	Beta
°C	Degrees Celsius
C	Carbon
C_8	Octa-carbon silica
C_{18}	Octadeca-carbon silica
CAD	Collision Activated Dissociation (Collision gas)
Calc	Calculated
CE	Collision energy
CID	Collision Induced Dissociation
Cl	Chlorine
Conc	Concentration
CUR	Curtain gas
CV	Coefficient of Variation
CXP	Collision cell exit potential
Da	Dalton
DAP	Dialkylphosphate
DC	Direct current
DDT	Dichlorodiphenyltrichloroethane
DEDTP	Diethyl dithiophosphate
DEP	Diethyl phosphate
DETP	Diethyl thiophosphate
DMDTP	Dimethyl-dithiophosphate
DMP	Dimethyl phosphate
DMTP	Dimethyl thiophosphate
DNOC	Dinitro-ortho-cresol
DP	Declustering potential
EMEA	European Medicines Agency
EP	Entrance potential
ESI	Electrospray Ionization
eV	elctronVolt
FA	Formic acid
FDA	Food and Drug Administration
FIA	Flow Injection Analysis
G	Gravitation force (Relative Centrifugal force)
G	Gram

GC	Gas Chromatography
H	Hydrogen
HPLC	High Performance Liquid Chromatography
H2O	Water
ISTD	Internal standard
LC	Liquid chromatography
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
LC-MS	Liquid chromatography mass spectrometry
LIMS	Laboratory Information Management System
LLE	Liquid-liquid extraction
LOD	Lower limit of detection
LLOQ	Lower Limit of Quantification
LTS	Long-term Stability
$[M]^+$	Molecular ion
$[M+H]^+$	Protonated molecular ion
$[M-H]^-$	Deprotonated molecular ion
MeOH	Methanol
MF	Matrix Factor
Mg	Milligram
Min	Minute(s)
Ml	Millilitre
mM	Millimolar
MRM	Multiple Reaction Monitoring
Ms	Millisecond(s)
m/z	Mass-to-charge ratio
N	Number
N	Nitrogen
NEB	Nebuliser
Ng	Nanogram
NH_4OH	Ammonia solution
NO^+	Nitrosonium ion
O	Oxygen
OCP	Organochlorine pesticides
OP	Organophosphate pesticides
OTC	Over-the-Counter
P	Phosphorus
pH	Power of hydrogen
Q1	Quadrupole 1
Q2	Collision cell
Q3	Quadrupole 3
QC	Quality Control
QC_{low}	Quality control 2 - 3 times that of the LLOQ
QC_{high}	Quality control about 80% of the ULOQ
®	Registered trademark
Resp	Response
RF	Radio frequency
RP	Reverse phase

r^2	Coefficient of Determination
S	Sulfur
S/N	Signal-to-noise ratio
SPE	Solid phase extraction
STD	Calibration standard
STDEV	Standard deviation
SYS	System Suitability Sample
TBME	Tertiary-butyl-methyl ether
TCA	Trichloroacetic
™	Trademark
Mg	Microgram
Ml	Microliters
ULOQ	Upper Limit of Quantification
UV	Ultraviolet
V	Volt
v/v	volume to volume ratio
Watson LIMS™	Watson is a Laboratory Information Management System (LIMS) for professionals who are involved in drug metabolism and pharmacokinetic studies. Watson supports the process of study design, sample receipt, scheduling analytical runs, interfacing with instruments, regressing analytical results, and analysing and summarizing the resulting concentrations and pharmacokinetic parameters.
%	Percentage
% Bias	The difference between the true nominal value and the value obtained, expressed as a percentage: % Bias = ((Measured value – Nominal value) ÷ Nominal value) x 100 (Equal to % Dev)
% CV	Percentage Coefficient of Variation: % CV = (Standard Deviation ÷ Mean) x 100
% Dev	Percentage Deviation
% Diff	Percentage Difference
% Nom	Percentage Nominal
% RSD	Percentage Relative Standard Deviation
~	Approximately

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CHAPTER 1

INTRODUCTION

1.1 STUDY RATIONALE

Pesticides are used worldwide in the agriculture industry against pests that can damage crops, in order to avoid or reduce losses and improve product quality. However, with this extensive use of pesticides, human health has become a major concern as these chemicals are designed to have adverse biological effect on a variety of organisms. Pesticides are organic pollutants and are the only toxic substance released on an international scale into the environment to kill living organisms [1].

Based on their functional groups and toxicity, pesticides are generally divided into five categories namely organophosphorus pesticides, organochlorine pesticides, carbamate pesticides, pyrethroids and herbicides [2] [3].

Many contemporary agricultural pesticides are hormonally active [4] with the potential to cause male reproductive health effects in exposed persons. The Clinical Study Protocol titled “Male reproductive health effects due to pesticides among farm residents in the Western Cape” was approved by the Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town in 2005 (REC REF: 279/2005) and proposed the investigation of the effects of exposure to pesticides on male subjects in the target area. A clinical trial was conducted in compliance with this Clinical Study Protocol and ethical principles that have their origins in the Declaration of Helsinki of the 25th World Medical Assembly [5].

South Africa is the principal user of pesticides in Southern Africa and previous investigations indicated pesticide use in the Western Cape agricultural areas to be substantial [6] [7]. Some of the most commonly used pesticides in the Western Cape, identified in previous surveys [6] [7] [8], were reported to have the potential to adversely affect the male reproductive systems of laboratory animals, and/or wildlife, or have been related to adverse male reproductive outcomes in humans. Candidate pesticides included chlorpyrifos, cypermethrin, deltamethrin, dichlorvos, dinitro-ortho-cresol (DNOC), endosulfan, fenarimol, fenvelerate, glyphosate, iprodione, parathion, procloraz, vinclozolin, linuron and procymidon.

Previous studies reported the detection of endosulfan, chlorpyrifos, fenarimol, deltamethrin and iprodione in Western Cape rural surface and groundwater, including drinking water [8] [9] [10]. Endosulfan and chlorpyrifos concentrations exceeding the World Health Organization (WHO) drinking water standard of 0.1 µg/l were reported in about a third of the samples tested.

The proposed study based on the above-mentioned Clinical Study Protocol was aimed at monitoring exposure to organochlorine or organophosphate pesticides in biological samples collected from rural residents in the Western Cape. Therefore, serum and urine samples from such subjects were collected and analytical methods were developed to determine the levels of the pesticides and/or the metabolites thereof in the biological matrix. The samples were obtained from male farm workers and farm residents, including children, adolescents and adults from four rural areas in the Western Cape. Three of these areas are agriculturally intensive where pesticides have previously been detected in water supplies [8] [9] [10], including the Hex River Valley where grape farming is practiced, Grabouw where pome fruit farming is predominant and Piketberg where wheat and fruit farming is practiced. Controls were collected from similar male subjects residing in neighboring non-agricultural areas.

Venous blood samples, 10 ml, for the determination of the organochlorine pesticides and first morning void urine samples for the determination of organophosphates were collected into labeled tubes. The blood samples were allowed to clot and serum was prepared from the clotted blood. Urine samples were collected in plastic containers topped with a plastic cap. The urine and serum samples were stored frozen at ~ -20°C in solvent pre-cleaned containers.

Occupational effects were investigated amongst adults from all four areas using both years of exposure as calculated using a questionnaire to describe the application status. A physical examination of the reproductive system was performed by a professional health practitioner, recording height, weight, secondary sexual characteristics and sexual maturity rating according to the Tanner score [11].

1.1.1 Endosulfan

Endosulfan, the common name for 6, 7, 8, 9, 10, 10- hexacloro-1, 5, 5a, 6, 9, 9a- hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepin-3- oxide (see Table 1.1), is an organochlorine insecticide [12] developed and introduced by Farbwerke-Hoechst A.G under the registered trademark “Thiodan” [13], and was first registered for use in the United States of America in 1954 [14]. Chemically similar to dichlorodiphenyltrichloroethane (DDT), the agricultural industry uses this insecticide to protect a variety of crops against several species of pests [15]. Although most organochlorine pesticides have been forbidden in most parts of the world, the compounds and their metabolites are still in existence in biological matrices due to their high lipophilic properties [16]. Manual and aerial spray used for agricultural purposes are the major contributors of endosulfan residue in soil and water.

Contact with this insecticide is common, especially for agricultural workers, as well as for

consumers buying the food products from crops on which the pesticides are used [17]. Degradation of endosulfan in soil is affected by environmental conditions, and studies have shown that degradation is reduced at lower temperatures and lower water content [18].

Endosulfan is a solid substance that ranges from brown to cream colored. It has been reported to be light sensitive and therefore it is recommended that samples containing endosulfan be prepared, stored and analyzed under protected lighting condition [19]. Technical endosulfan consists of two stereoisomers that differ in the configuration of the 7-member dioxothiepin-oxide ring. The two isomers are known as the alpha-isomer (α-isomer) and beta-isomer (β-isomer) and are present in the ratio of 2:1 to 7:3, depending on the mixture applied [14] [20] [21]. Schmidt *et al*. indicated that the α-isomer is more toxic than the β-isomer and that conversion of the β-isomer to the α-isomer can occur, which may be irreversible [22]. Significant conversion of the β-isomer to the α-isomer has been reported, but only one instance of the conversion from the α-isomer to the β-isomer has been described. The conversion quantities of the α-isomer was insignificant (less than 1% conversion), and additional work would be required to confirm this reported conversion process [22] [23] [24].

Similar to other chlorinated pesticides, the isomers of endosulfans are semi-volatile and have similar vapour pressures causing volatilization into the atmosphere and allowing subsequent atmospheric transport and deposition. The vapour pressures of the α- and β-isomers are similar, but the aqueous solubility of the β-isomer is much greater than that of the α-isomer allowing the β-isomer to have a lower Henry's Law constant (H) and will therefore more readily partition to aqueous phases [18].

The chemical properties of endosulfan, α-endosulfan and β-endosulfan, are listed in Tables 1-1, 1-2 and 1-3, respectively.

Table 1.1 Chemical properties of endosulfan

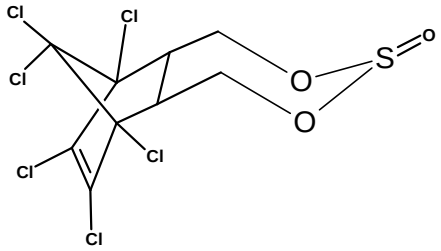
Chemical Structure	
Chemical name	Endosulfan
Chemical name Synonyms	6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide;Endosulfan technical; 5-Norbornene-2,3-dimethanol1,4,5,6,7,7-hexachlorocyclic sulfit
Registered trade names	Thiodan; Thionex; Thionate Malix; HOE 2671; FMC 5462; Cyclodan; Thifor; Beosit; Chlorthiepin; Endosulphanb
Chemical formula	$C_9H_6Cl_6O_3S$
Molecular weight:	406.95Da
Monoisotopic mass:	403.816Da

Table 1-2 Chemical properties of α- endosulfan

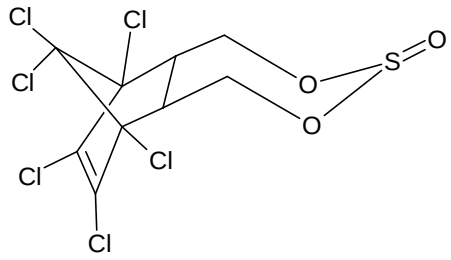
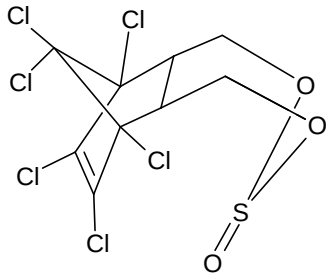
Chemical Structure	
Chemical name	α- Endosulfan
Chemical name Synonyms	Endosulfan I; Endosulfan A, 6,9-Methano2,4,3- benzodioxathiepin, 6,7,8,9,10,10-hexachloro1,5,5a,6,9,9a- hexahydro-, 3-oxide (3α, 5a β, 6α, 9a α, 9β)-; 5- Norbornene-2,3-dimethanol, 1,4,5,6,7,7-hexachloro-, cyclic sulfite, endo-[b]
Registered trade name(s)	α-Benzoepin; α-Thiodan; α -Thionex[c]
Chemical formula	$C_9H_6Cl_6O_3S$
Molecular weight:	406.95Da
Monoisotopic mass:	403.816Da

Table 1-3 Chemical properties of β-endosulfan

Chemical Structure	
Chemical name	β-Endosulfan
Chemical name Synonyms	EndosulfanII; Endosulfan B,6,7,9,10,10-- Hexachloro1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3- benzodiozathiepin3-oxide, (3α, 5aα, 6β, 9β, 9aα)-; 5 Norbornene-2,3-dimethanol, 1,4,5,6,7,7-hexachloro-, cyclic sulfite, endo-[b]
Registered trade name(s)	β-Benzoepin; β-Thiodan; β -Thionex[c]
Chemical formula	$C_9H_6Cl_6O_3S$
Molecular weight:	406.95Da
Monoisotopic mass:	403.816Da

Exposure to endosulfan is not only determined by the presence of the two intact isomers in biological matrices, but also by the presence of its metabolites. In humans, endosulfan is known to be metabolized to endosulfan sulfate, endosulfan diol, endosulfan ether, endosulfan hydroxy-ether and endosulfan lactone [16]. Endosulfan sulfate, formed metabolically from both the α-isomer and β-isomer, is equally toxic and is more persistent in the environment than both of its parent compounds [18] [25]. The chemical properties of endosulfan metabolites are listed in Tables 1-4, 1-5, 1-6, 1-7 and 1-8.

Table 1-4 Chemical properties of endosulfan sulfate

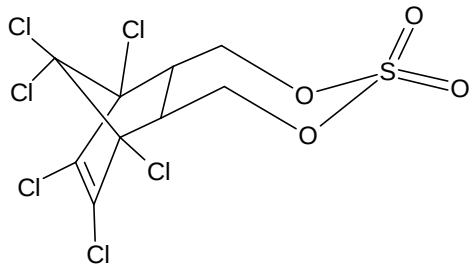
Chemical Structure	
Chemical name	Endosulfan sulfate
Chemical formula	$C_9H_6Cl_6O_4S$
Molecular weight:	422.924Da
Monoisotopic mass:	419.811Da

Table 1-5 Chemical properties of endosulfan ether

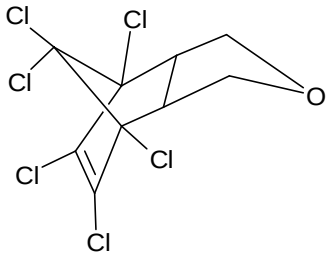
Chemical Structure	
Chemical name	Endosulfan ether
Chemical formula	$C_9H_6Cl_6O$
Molecular weight:	342.861Da
Monoisotopic mass:	339.854Da

Table 1-6 Chemical properties of endosulfan hydroxy ether

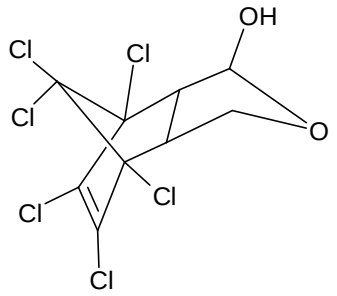
Chemical Structure	
Chemical name	Endosulfan hydroxyl ether
Chemical formula	$C_9H_6Cl_6O_2$
Molecular weight:	358.860Da
Monoisotopic mass:	355.849Da

Table 1-7 Chemical properties of endosulfan diol

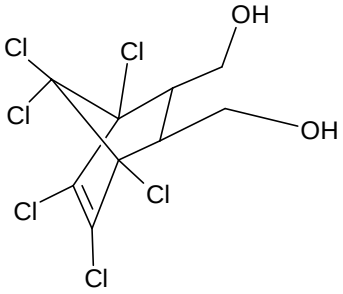
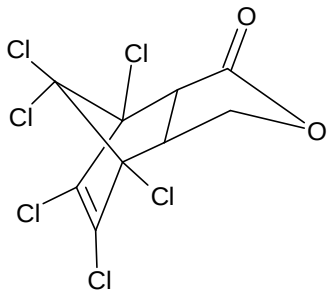
Chemical Structure	
Chemical name	Endosulfan diol
Chemical formula	$C_9H_8Cl_6O_2$
Molecular weight:	360.876Da
Monoisotopic mass:	357.865Da

Table 1-8 Chemical properties of endosulfan lactone

Chemical Structure	
Chemical name	Endosulfan lactone
Chemical formula	$C_9H_4Cl_6O_2$
Molecular weight:	356.844Da
Monoisotopic mass:	353.834Da

Endosulfan can enter the body through the skin, by inhalation and by drinking and/or eating contaminated products [26]. Once absorbed, endosulfan accumulates in fatty tissues and affects the nervous system to cause headaches, nausea, seizures and in extreme cases can lead to death [27]. Additionally, various studies have shown that pesticides such as endosulfan causes male reproductive organ abnormalities [14] due to its anti-androgenic and estrogenic capabilities demonstrated *in vitro* [28]. Endosulfan is also extremely toxic to fish and is implicated in genotoxicity and neurotoxicity in mammal species [29].

The most widely published analytical method for the determination of organochlorine pesticides is gas chromatography (GC) with electron capture detection [16] [26] [30] [20]. In this study the emphasis was however on the employment of the fast developing technology of high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to detect and quantify these molecules in biological matrices. This very well developed technology, focused on sensitive and highly specific detection, is now commonly available in most bioanalytical laboratories. The development of an LC-MS/MS assay to analyze organochlorine pesticides is therefore considered as a worthy pursuit.

1.1.2 Organophosphates

Organophosphate pesticides are also widely used in developed and developing countries, both in agricultural and residential applications and these compounds have the same adverse effects on human health than that indicated for the organochlorine pesticides [31] [32]. Because of the widespread use of organophosphate pesticides, well documented studies have shown that exposure to these pesticides are almost impossible to avoid [33].

Most organophosphates are metabolised in humans to six common dialkylphosphate (DAP) metabolites, namely dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), dimethyl-dithiophosphate (DMDTP), diethyl phosphate (DEP), diethyl thiophosphate (DETP) and diethyl dithiophosphate (DEDTP) [34] [35] [36] [37], which are detectable in urine. The presence of these metabolites in urine is used as markers for exposure to organophosphates [32] [34].

The chemical structures of the six dialkylphosphate metabolites are listed in Tables 1-9, 1-10, 1-11, 1-12, 1-13 and 1-14.

Table 1-9 Chemical properties of dimethylphosphate

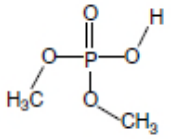
Chemical Structure	
Chemical name	Dimethylphosphate
Chemical formula	$C_2H_7O_4P$
Molecular weight:	126.048Da
Monoisotopic mass:	126.008Da

Table 1-10 Chemical Identity of Dimethyl thiophosphate

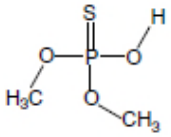
Chemical Structure	
Chemical name	Dimethyl thiophosphate
Chemical formula	$C_2H_7O_3PS$
Molecular weight:	142.113Da
Monoisotopic mass:	141.985Da

Table 1-11 Chemical properties of dimethyl dithiophosphate

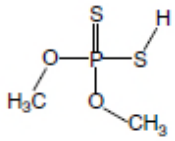
Chemical Structure	
Chemical name	Dimethyl-dithiophosphate
Chemical formula	$C_2H_7O_2PS_2$
Molecular weight:	158.179Da
Monoisotopic mass:	157.962Da

Table 1-12 Chemical properties of diethyl phosphate

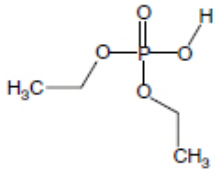
Chemical Structure	
Chemical name	Diethyl phosphate
Chemical formula	$C_4H_{11}O_4P$
Molecular weight:	154.101Da
Monoisotopic mass:	154.039Da

Table 1-13 Chemical Identity of diethyl thiophosphate

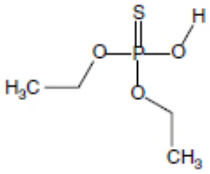
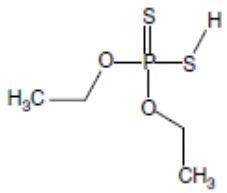
Chemical Structure	
Chemical name	Diethyl thiophosphate
Chemical formula	$C_4H_{11}O_3PS$
Molecular weight:	170.167Da
Monoisotopic mass:	170.016Da

Table 1-14 Chemical properties of diethyl dithiophosphate

Chemical Structure	
Chemical name	Diethyl dithiophosphate
Chemical formula	$C_4H_{11}O_2PS_2$
Molecular weight:	186.232Da
Monoisotopic mass:	185.993Da

Analytical methods for the determination of DAP metabolites described in the literature mainly involve gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) [33] [38] [32]. This study describes a sensitive bioanalytical method based on LC-MS/MS, which was used to screen urine samples of agricultural workers exposed to organophosphate pesticides.

1.2 INTRODUCTION TO BIOANALYTICAL METHODS

In the modern era, bioanalytical methods are mainly used to generate quantitative concentration data to establish bioavailability, bioequivalence, pharmacokinetic and toxicokinetic parameters of bio-active exogenous molecules administered to humans and other mammals [39].

To obtain knowledge regarding these parameters, measurable concentrations in different biological matrices such as plasma, serum, blood, urine and saliva are determined. For acceptance, such data needs to meet strict criteria set by the regulatory authorities and therefore a bioanalytical method which is the basis for obtaining the data, also needs to conform to strict criteria [40] [41]. A well-defined work-flow is used to establish an acceptable analytical method, starting with literature research to obtain as much as possible available information, followed by method development to establish an acceptable, well performing analytical method, which is then validated before it can be used for the measurement of study samples.

1.3 BIOANALYTICAL METHOD DEVELOPMENT

1.3.1 Method development

During method development, different experiments are conducted to ascertain the optimal techniques with which an analyte can be determined and measured in a biological matrix.

Development of an analytical method is complex and expensive and involves many considerations such as concentration levels, biological matrix of a sample, chemical properties of the analyte and speed of the analyses [40]. Method development consists of three essential parts: sample preparation and extraction of the analyte from the biological matrix, chromatographic separation of the analyte from extracted matrix components and detection of the analyte by a sensitive and specific detection method [41].

A literature survey of published analytical methods supplies useful information to set up a strategic action plan for method development. Such a survey should be as specific as possible, focused on the specific analyte detected in the specific biological matrix [42] [43]. The strategic plan for method development should include steps to optimize the method concerning aspects such as selectivity, accuracy, precision, recovery, sensitivity, reproducibility and stability of the analyte in spiked biological matrices [42] [44]. An example of the work-flow involved in such a plan is depicted in Figure 1.1.

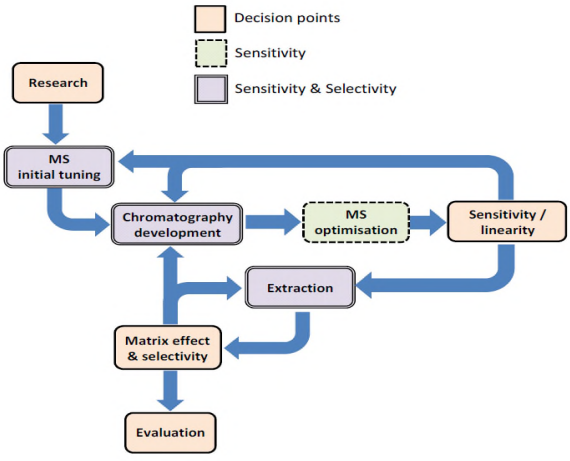


Figure 1.1 Strategic plan for bioanalytical method development [45]

Since a bioanalytical method will be used for the creation of important data which may eventually be employed by the Toxicology industry in the development of vital medicinal preparations, strict rules are applied to this scientific field and the foundation of adherence to these rules is a well-

developed robust and reliable analytical method. Much attention should therefore be given to the development thereof and the major points of interest in method development are briefly discussed in the following sections [42] [43].

1.3.2 Analyte

The analyte is the specific chemical moiety being measured, which can be an intact drug, a biomolecule or its derivative, metabolite and/or degradation product present in the biologic matrix [42] [43]. This compound has to be acquired as a pure reference standard which is usually commercially available.

1.3.3 Internal Standard

The use of an internal standard is common practice in bioanalysis and involves the addition of a compound which is similar to the analyte, to each sample being analyzed, in order to compensate for variations in the analytical procedure. Each sample being analyzed should therefore contain the same amount of the internal standard, added as soon as possible during sample preparation. The similarity to the analyte depends on the nature of the detection method. If mass spectrometry is used to detect the analyte, the preferred internal standard would be a stable isotope-labeled molecule of the analyte, which means that the internal standard has the same chemical characteristics as the analyte, but can be separately detected based on the different mass properties of the two molecules. Alternatively, a molecule which is structurally and chemically similar, but not identical to the analyte may be used as internal standard. It is important to assure that the difference between the analyte and the internal standard is maintained throughout the analytical process and also that the internal standard is not intrinsically present in the biological matrix, such as for instance a metabolite or break-down product of the analyte. When acquiring an internal standard it is also important to assure that the preparation used does not contain the analyte as an impurity, such as the non-labeled analyte being present in a preparation of the stable isotope-labeled compound used as an internal standard [43] [46].

1.3.4 Biological matrix

In bioanalysis, the matrix is the medium in which the analyte has to be measured and which can be defined as a discrete material of biological origin that can be sampled and processed in a reproducible manner. These discrete materials can be blood or its separable components such as serum and plasma, or other body fluids such as urine, sputum, saliva, cerebrospinal fluid etc. [43].

The analytical method will be developed to specifically analyze the analyte in the biological matrix and therefore an appropriate volume of the biological matrix, devoid of the analyte (blank matrix),

has to be available for the development of the method. This blank matrix will have to be available later on as well, during method validation and also for the routine analysis of study samples, in order to prepare calibration standards (STDs) and quality control samples (QCs). The blank matrix is normally collected from voluntary donors who give consent to the use of the matrix [46].

1.3.5 Sample preparation and extraction procedures

General considerations such as the environment and management of a sample must be considered during sample preparation and extraction. The suitability of sample containers needs to be explored as some analytes may adsorb onto plastic and/or glass surfaces and certain solvent mixtures may dissolve molecular components from the container material, increasing the complexity of the sample. The thermal stability and photo stability of the analyte must also be taken into account, necessitating the preparation being performed at lower or higher temperatures and/or the use of light protected containers [45]. Once the appropriate containers and handling conditions have been established, such measures can be applied to the sample collection process.

The study samples *per se* are usually not suitable for analysis since the matrix may be too complex, too dilute for direct sample injection, or incompatible with the mobile phase. In such instances, some sample cleanup procedures such as extraction must be investigated and evaluated.

Sample extraction techniques are the processes whereby biological samples are subjected to procedures that would transfer the analyte from the aqueous biological matrix into a suitable solvent. During this process, background components in the biological matrix, which may interfere with the detection and measurements of the analyte, can be removed. The sensitivity of the method may also be increased due to the concentrating effect that can be applied during extraction. The end product of extraction should thus be focused on the reconstitution of the analyte in a solvent in which it is optimally detectable. [47].

Frequently used sample preparation techniques include protein precipitation, liquid-liquid extraction and solid phase extraction.

1.3.5.1 Protein precipitation

Protein precipitation is used to remove proteins from the biological matrix resulting in a relatively clean preparation containing the analyte. This technique is thus relevant to samples in which the matrix contains proteins, such as plasma and/or serum.

Proteins can be precipitated by the addition of solvents that render the proteins insoluble, upon which it can be removed by centrifugation, resulting in the analyte being present in the supernatant.

For this purpose, water soluble organic solvents such as acetonitrile, methanol or ethanol can be used. Proteins will also precipitate due to the addition of certain salts, such as ammonium sulfate or by drastically adjusting the pH by the addition of strong organic acids such as trichloroacetic acid (TCA) to the biological sample [45].

The protein-free supernatant can either be analyzed directly or evaporated to dryness and thereafter reconstituted in a more suitable injection solvent prior to analysis. Protein precipitation is a simple and less time consuming technique when compared to liquid-liquid extraction (LLE) and solid-phase extraction (SPE). The disadvantage of protein precipitation is however that it is relatively non-selective and many endogenous compounds that can interfere during analysis may still be present in the supernatant [48].

1.3.5.2 Liquid-liquid extraction

This sample preparation technique is based on the partitioning of an analyte between two immiscible solvents and entails the addition of an organic solvent to the sample which is in an aqueous solution. The purpose is to optimally displace the analyte into the organic phase. To achieve this, the polarity of the analyte can be manipulated by adjustment of the pH of the sample (aqueous phase) to render the analyte as non-polar as possible. The required pH will depend on the pKa of the analyte and in general, basic analyte molecules will optimally transfer to the organic phase at high pH (where they are less protonated), whereas acidic molecules will optimally transfer to the organic phase at low pH (where they will be protonated). A series of experiments assessing the recovery of the analyte in the organic phase at different pH values is therefore always necessary as part of the method development process [49].

Although the adjustment of the sample pH can manipulate the polarity of the analyte by controlling ionization of basic and acidic groups, each molecule has an intrinsic polarity even when not ionized, based on the functional groups within the molecule. An intrinsically polar molecule will therefore not optimally transfer to an organic phase that is too non-polar and a highly non-polar molecule will not transfer to a high degree to an intrinsically polar organic solvent. Because there are differences in the polarity of different water-immiscible solvents, the extraction can be optimized by choosing an organic solvent with the correct polarity. The different polarity indexes of some commonly used organic solvents are indicated in Table 1.15 where the most non-polar solvent, pentane, has a polarity index of 0.0 as compared to water with a polarity index of 10.2. To obtain the optimal polarity of the organic solvent, mixtures of solvents can also be used and the adjustment of this should also be an important part of the method development process.

Table 1-15 Polarity indexes of commonly used organic solvents [50]

Solvent	Polarity Index (P')
Pentane	0.0
Hexane	0.1
Cyclohexane	0.2
Toluene	2.4
Methyl *t*-Butyl Ether	2.5
Ethyl Ether	2.8
Dichloromethane	3.1
Chloroform	4.1
Ethyl Acetate	4.4
Water	10.2

Liquid-liquid extraction is practically executed by adding an appropriate volume of a suitable organic solvent to the sample, usually three times that of the volume of the sample, followed by thorough mixing for an appropriate period to allow optimal transfer of the analyte. After the mixing process, the separation of the phases is allowed to take place (can be expedited by low-g centrifugation). The organic phase, containing the analyte, is then separated from the aqueous phase by decanting the organic phase to a clean container. The volatile organic solvent is then removed by evaporation and the residue reconstituted in a solvent in which the analyte is soluble and ready for analysis.

Liquid-liquid extraction is often preferred over sample preparation by simple protein precipitation as it results in a "cleaner" preparation of the analyte in which most of the unwanted, mostly more polar compounds, have been removed. Liquid-liquid extraction also allows for the enrichment of the analyte, because the final reconstitution of the sample can be done in a lower volume than that of the initial sample volume.

1.3.5.3 Solid-phase extraction (SPE)

This preparation technique can be used to establish three important prerequisites during sample preparation, namely removal of interfering matrix components, changing the solvent from matrix to an appropriate reconstitution solution for subsequent analysis, and enrichment of the analyte [51].

Similar to liquid-liquid extraction, the analyte is partitioned between two immiscible phases during SPE. Instead of using a liquid organic phase, the second phase is a stationary phase bonded to insoluble, solid particles (the solid phase). Depending on the chemical character of the analyte, a bonded phase will be selected that has an affinity for the analyte. The aqueous sample containing

the analyte is then passed through a column packed with the bonded phase resulting in the analyte associating with the bonded phase and therefore being retained in the column. In this way unwanted compounds in the sample pass through the column and are washed out of the column. The analyte can then be eluted from the column by passing a liquid phase through the column for which the analyte has a higher affinity than the bonded phase. Alternatively, the bonded phase and the condition of the sample can be selected so that unwanted compounds in the sample are retained on the bonded phase and the analyte pass through, devoid of the unwanted material [51] [52].

The principle by which association between the analyte and the bonded phase takes place is normally based on either hydrophobic or ionic interactions. Similar to liquid-liquid extraction, the pH of the sample can be adjusted so as to render the analyte in the most favorable polarity condition to induce the desired interaction. The work-flow by which SPE is accomplished is explained graphically in Figure 1.2.

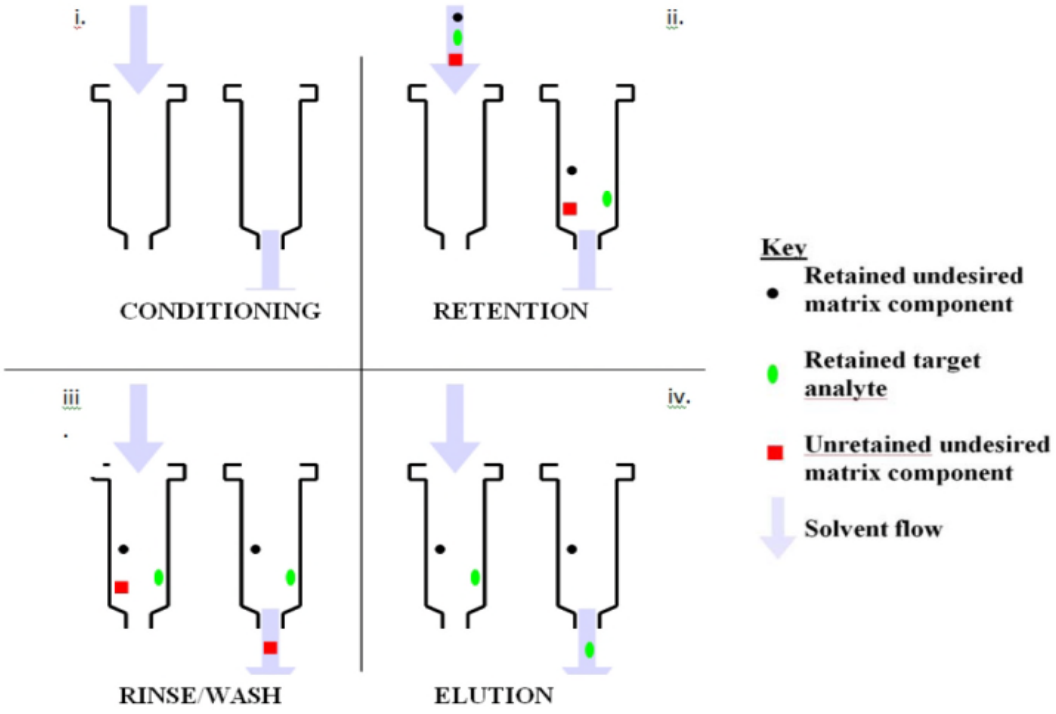


Figure 1.2 Schematic representation of a solid phase extraction process [45]

1.3.6 Separation by Liquid Chromatography

Following sample preparation as described in the previous sections, the sample is usually not ready for analysis on the detection system, since there are still biological components present that may

interfere with the detection of the analyte which could lead to incorrect results during quantitative or qualitative analysis. The final preparative step for the introduction of the analyte into the detection system is therefore usually a chromatographic procedure that would separate the analyte from most of the matrix components. The most commonly used chromatographic technique for the determination of small molecules in extracts of biological matrices is high performance liquid chromatography (HPLC) [53].

A typical HPLC chromatograph consists of a solvent delivery system comprising a solvent degasser and a high pressure pump that delivers the mobile phase to a packed column. Between the pump and the column, an automated injector introduces the sample into the solvent flow. Separation takes place in the column which is packed with a stationary phase and the separation process depends on the chemical nature of the analyte which will allow it to associate to a specific degree with the packing material within the column. This association will then retard the flow of the analyte through the column and under the ideal conditions of packing material and mobile phase, the analyte will separate from other compounds still in the sample extract, so that only the analyte will flow from the column into the detection system at a given time (the retention time) [54].

The principles controlling the association of the analyte with the material in the column is the same as that employed during SPE, therefore relying on the polarity of the analyte and the material in the column. The most widely used association is that of hydrophobic interaction resulting in reverse phase chromatography during which hydrophobic groups on the packing material in the column will associate with hydrophobic groups in the analyte molecule, therefore retaining it in the column and allowing more polar compounds to elute from the column before the analyte. By adjusting the organic content in the column, the retention time of the analyte in the column can be controlled [54] [55] [56].

Because HPLC can employ volatile mobile phases, this separation technique can readily be connected directly to mass spectrometry in order to create a very versatile and specific detection technique widely used in the bioanalytical field, termed LC-MS or LC-MS/MS [57]. Both quantitative and qualitative analyses are accomplished using this methodology.

1.3.7 Mass spectrometry

Mass spectrometry is an analytical technique that can be used selectively to detect and determine the presence and amount of many biological relevant compounds in biological matrices. With this powerful technique, one can quantify known compounds, identify unknown compounds and reveal the chemical properties of different compounds [58]. By mass spectrometry the *mass to charge*

ratio (m/z) of ions is determined providing information regarding the molecular mass of a molecule as well as information as to its structural composition through fragmentation of the ion and analysis of the mass of its fragments [54] [59].

A mass spectrometer can be divided into three basic components, namely an ionization source, a mass analyzer, and an ion detector. The analyzer and detector of the mass spectrometer, and often the ionization source too, are maintained under high vacuum inside the instrument (vacuum chamber), enabling the ions to travel from one end of the instrument to the other without any interruption from air molecules [54].

Mass spectrometers used as detectors for HPLC during bioanalysis should be selective and sensitive, and characterized by a linear response for concentrations over a wide dynamic range. The detector should be reliable with good reproducibility and stability, and have a fast response time [60]. Quadrupole mass spectrometers, mostly employed as tandem instruments, provide the combination of an effective and sensitive mass analyzer with versatile analytical possibilities based on fragmentation analysis of solute molecules [51]. These instruments were used for the study described here and will therefore be discussed in more detail.

A schematic representation of a triple quadrupole mass analyzer is shown in figure 1.3.

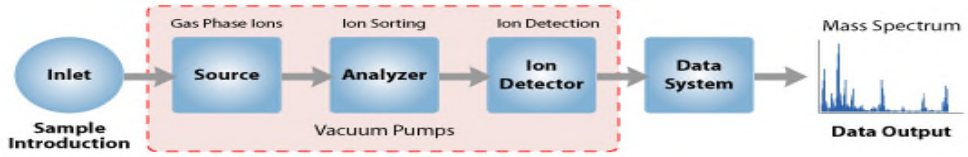


Figure 1.3 Components and schematic view of a triple quadrupole mass spectrometer [58]

1.3.8 The ionization source and ionization techniques relevant to bioanalysis

Triple quadrupole mass spectrometers used during bioanalysis mostly employ atmospheric pressure ionization (API) to accomplish three basic tasks, namely conversion of solutes to gaseous phase while performing ionization, separation of ions according to their *m/z* ratio and then detecting the separated ions [52] [54]. The API techniques most commonly employed in current LC-MC interphases are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI). These techniques allow the ionization of various polar and non-polar compounds at trace level concentrations.

1.3.8.1 Electrospray ionizaion (ESI)

In an electrospray ionization (ESI) interface, the inflow of solvent containing the sample is coupled to a metal capillary on which a high direct current (DC) charge (about 4000V) is applied, either positive or negative. The charge is transferred to the surface of the solvent which cause the solvent stream to break up in a spray of small droplets, all of which are covered with the same charge so that they repel each other, giving rise to the typical electrospray [59].

The charged capillary is usually situated within another capillary through which a flow of nitrogen gas is applied. The source is also heated and the combination of these two factors causes evaporation of the solvent from the charged droplets, resulting in ever decreasing sizes of the droplets. Surface charges will therefore be compressed until the repulsion of like charges overcome the surface tension of the droplets, at which stage the droplets will break apart in a process termed Coulombic explosion. Except for the solvent, the droplets will also contain ions of the same charge than that on the surface of the droplets. These ions will also be subjected to the increasing Coulombic forces until they escape the droplets and thus enter the gas phase. Finally, the total dissolution of the droplets will result in total desolvation with the ions left in the gas phase. Ions will then be drawn into the analyzer section of the instrument while uncharged particles can be excluded by applying an orthogonal flow of gas [51] [59]. The electrospray process is graphically demonstrated in figure 1.4.

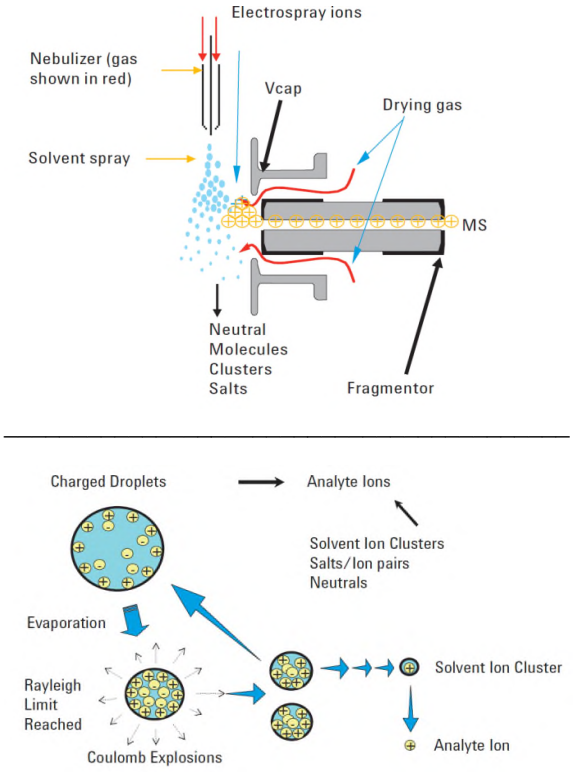


Figure 1.4 Electrospray interface and schematic ESI process [61]

Electrospray ionization is considered a mild ionization process, since the ionization of the analyte molecules already takes place to a large extent in the solvent before the electrospray process. Cations are mostly formed by protonation resulting in pseudo-molecular cations with *m/z* of one mass unit more than the monoisotopic mass of the molecules. An anion is formed from a molecule that more readily deprotonates resulting in a pseudo-molecular anion which will have a *m/z* value of one mass unit lower than the monoisotopic mass of the molecule. Due to the mildness of the ionization process, adducts of the molecular ions with other cations or anions present in the source can also be created, these then having *m/z* values equal to the sum of the monoisotopic masses of the molecule and the specific cation or anion [59]. These ionization mechanisms are summarized in Scheme 1.

Positive Mode

1. Volatile acid (formic acid or acetic acid) added to solvent to aid **protonation:**

(Pseudo molecular ion)

$[A+H]^+_{m/z} = A_{mass} + 1$

2. Metal ions (Na^+, Ag^+ etc.) or other cations ($NH4^+$) added to solvent to aid **cationation:**

$[A+H]^+_{m/z} = A_{mass} + 23$

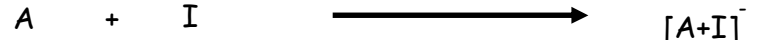
Negative mode ESI:

1. Volatile base (ammoniumhydroxide) added to solvent to aid **deprotonation:**

(Pseudo molecular ion)

$[A-H]^-_{m/z} = A_{mass} - 1$

2. Various anions (Cl^-, I^-) added to solvent to aid **anionation:**



$[A+I]^-_{m/z} = A_{mass} + 127$

Scheme 1: Mechanisms of ionization during electrospray ionization

1.3.8.2 Atmospheric pressure chemical ionization (APCI)

In an APCI source the capillary through which the solvent is delivered ends in a short ceramic tube which can be heated to high temperatures. Heated nitrogen acting as a nebulizing gas also flows through the tube unidirectional to the flow of the solvent. The combination of heat and gas rapidly vaporizes the solvent so that dry vapor exits in the tube. In front of the opening a metal pin is situated to which as high voltage is applied causing a corona discharge at the tip of the pin. The

vaporized solvent molecules entering this area of corona discharge are then ionized. As for electrospray ionization, APCI can be either in the positive or the negative mode. Due to the presence of water in the solvent, the primary ion species formed during positive ionization is oxonium (H_3O^+). Organic solvents in the mobile phase can result in the formation of for instance protonated methyloxonium ions ($CH_3OH_2^+$) if methanol is present or methylidyne ammonium ($CHNH^+$) if acetonitrile is present. In the negative mode, mainly hydroxide (OH^-) ions result from the deprotonation of water while the presence of methanol or acetonitrile can give rise to methanolate ions (CH_3O^-) or cyanide ions (CN^-), respectively. If an analyte is present in the solvent, it will thus also be transformed to the gas phase and enter the corona discharge. Direct ionization of the analyte may occur, but mostly, chemical ionization will occur by transfer of the charge from the solvent ions to the analyte molecules, forming protonated pseudo-molecular ions in the positive mode or deprotonated pseudo-molecular ions in the negative mode. Analyte ions formed during this process will then be extracted and transferred to the rest of the instrument as explained above for ESI [51] [54] [59].

The elevated temperature and more robust vaporization functionality during APCI allow the use of higher solvent flow rates from the HPLC than is possible with electrospray ionization. The process is graphically demonstrated in figure 1.5.

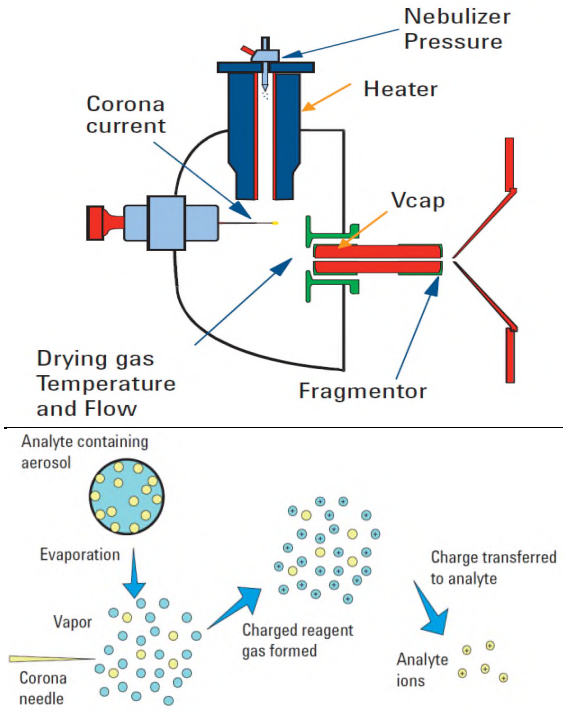
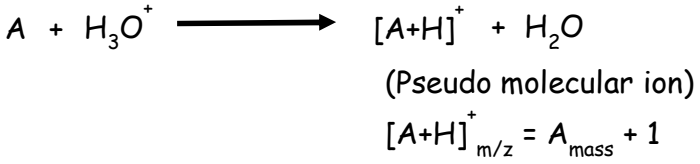


Figure 1.5 Atmospheric pressure chemical ionization interface and schematic APCI process [61]

Atmospheric pressure chemical ionization is typically used for less polar compounds which may not readily be ionized by the milder electrospray ionization process. The ionization mechanisms relevant to APCI are summarized in Scheme 2.

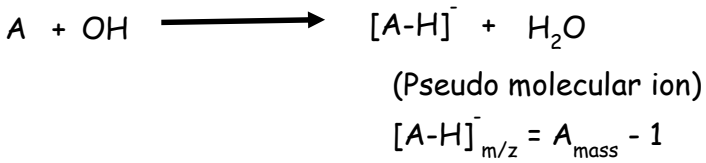
Positive mode APCI:

Protonation



Negative mode APCI:

Deprotonation



Scheme 2: Mechanisms of ionization during atmospheric pressure chemical ionization

1.3.8.3 Atmospheric pressure photo ionization (APPI)

Atmospheric pressure photo ionization (APPI) technology is an evolution of APCI. Vaporization in an APPI source is identical to that in the APCI source. However, instead of creating ions with a corona discharge needle, an ultraviolet (UV) light source is situated in front of the outflow from the vaporization tube.

Ionization depends on the action of high energy photons emitted by the UV source. Although direct ionization of the vaporized solvent molecules is possible, a dopant solvent is normally added to promote ionization by UV radiation. Due to their intrinsic photo-reactivity, the most common dopants are toluene and acetone, which would absorb the high energy photons causing ionization of the dopant which further leads to a cascade of ion-molecule reactions involving solvent molecules introduced by the mobile phase. If vaporized analyte molecules are present, ionization may occur directly if the molecule is photo-reactive. Most commonly however, ionization will occur by either proton transfer or by charge exchange between the solvent or dopant ions and the

analyte molecules. In the positive ion mode, both $[M+H]^+$ as well as $[M]^+$ ions can be formed depending on the molecule and whether the ions were created by proton transfer or charge transfer. In the negative ion mode, $[M-H]^-$ ions are predominantly formed by deprotonation [54] [62].

A schematic representation of an APPI source is shown in figure 1.6, while ionization mechanisms during APPI are summarized in Scheme 3.

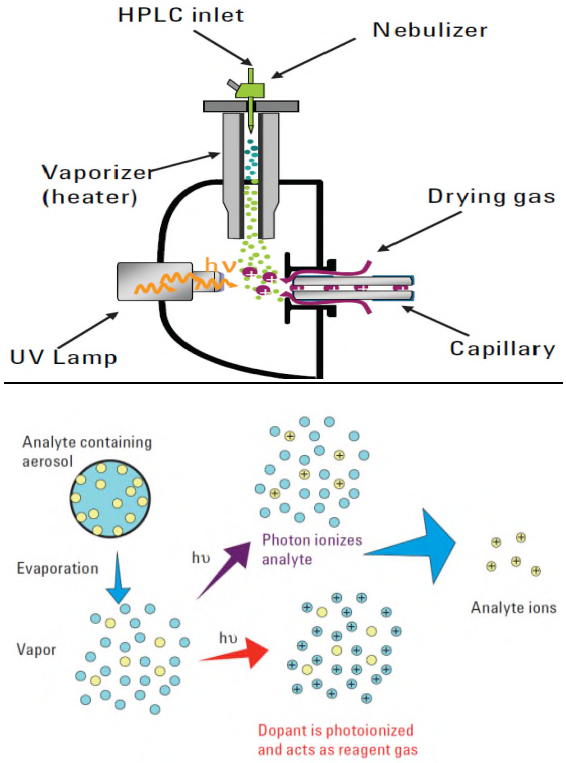
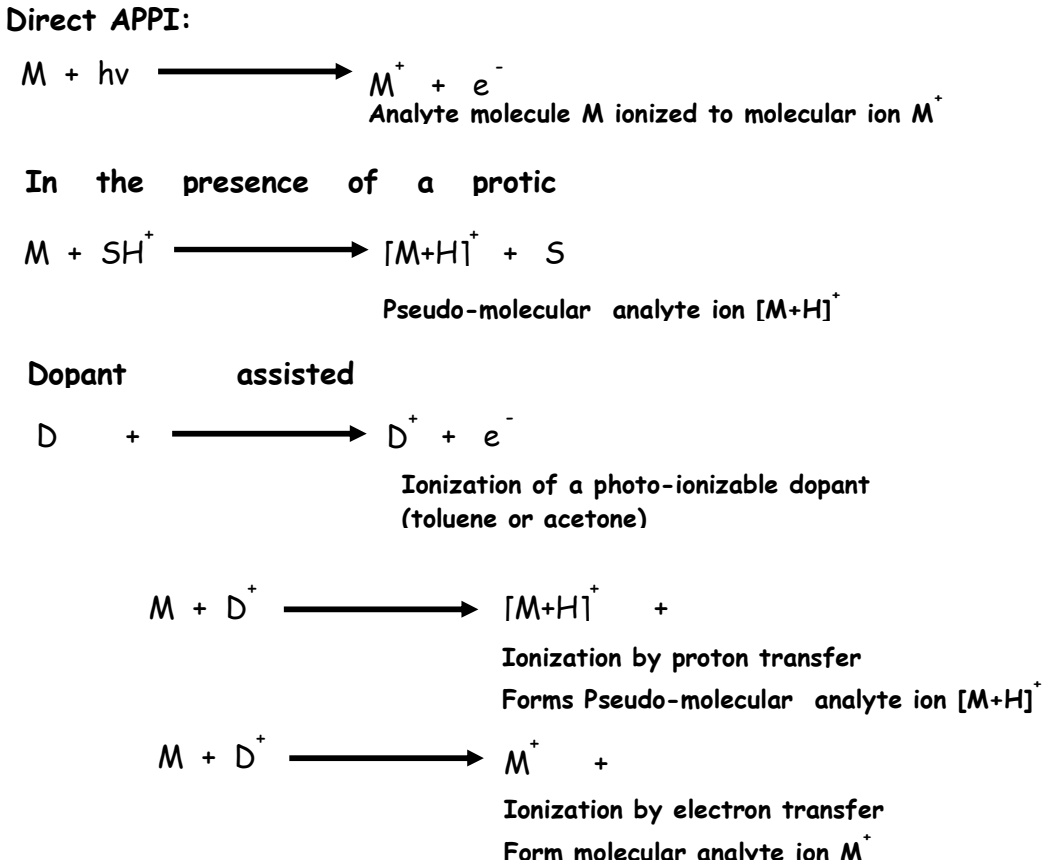


Figure 1.6 Atmospheric pressure photo ionization interface and schematic APPI process [61]



Scheme 3: Mechanisms for ionization in the positive mode during atmospheric pressure photo ionization

1.3.9 Mass analyzers: the quadrupole analyzer

As mentioned before, the instruments relevant to this study employ quadrupoles as mass analyzers. These are structures within the vacuum chamber, consisting of four separate metal rods arranged so that a channel is formed between them. Ions formed in the ionization source are focused by electrostatic lenses and drawn into the vacuum compartment by the opposing electrostatic force of the ion lenses. The quadrupoles are aligned in such a way that the ions will move through the channel formed by the four rods of the quadrupole. Opposing direct current (DC) and alternating radio frequencies (RF) voltages are applied to the rods which create electrostatic forces on the surfaces of the rods. By keeping the frequency of the applied RF voltages constant and increasing the amplitude thereof, only ions of a specified mass-to-charge ratio (*m/z*) will follow a stable oscillation path and thus pass through the quadrupole analyzer and impinge on the detector at a given instance. Quadrupole analyzers are therefore also termed mass filters. Collision of ions on

the surface of the detector will cause an increasing cascade of electrons, multiplying the signal strength to a level where the event can be detected as an electronic signal which can then be recorded and integrated so that the signal strength is proportional to the abundance of ions. This then forms the basis by which the quantity of an analyte extracted from a matrix and subjected to LC/MS analysis, can be measured [54] [59].

To increase the specificity and sensitivity by which analytes can be measured, the more advanced instruments used for quantitative analysis employ two quadrupole mass analyzers in tandem, separated in space by a non-analyzing quadrupole in which ions can be fragmented. This fragmentation process is termed collision induced dissociation (CID) and will be further discussed in section 1.3.9.2 below.

The tandem array of quadrupoles resulted in these instruments being termed triple quadrupole mass spectrometers and the arrangement of major components therein is graphically demonstrated in figure 1.7.

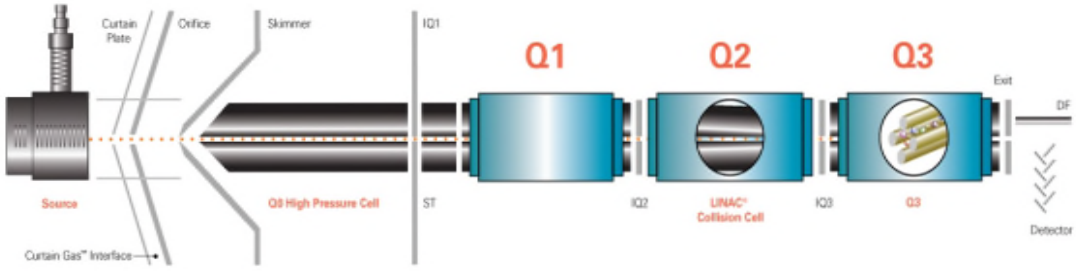


Figure 1.7. Main components of a triple quadrupole mass spectrometer [63]

The tandem array of quadrupole mass analyzers in the triple quadrupole mass spectrometer allows for a range of scan types possible with this instrument. The three scan types applicable to this study, precursor ion scans, product ion scans and multiple reaction monitoring will be briefly discussed in the following sections [59] [63].

1.3.9.1 Precursor ion scans (Q1 scan)

The *m/z* values of all ions produced in the ionization source can be determined by continuous scanning of a pre-set *m/z* range using Q1. The data will then be in the form of a mass spectrum, indicating the *m/z* value of all ions detectable and their relative abundance. This data is valuable to ascertain the presence of a particular ion, and also to adjust the settings of the ionization source to

obtain the optimal intensity of the expected ion. Such settings will include the flow rate by which the solvent is delivered into the ionization source, the flow of nebulizing gas and the control of the temperature of the ionization source. For ESI, the voltage of the capillary can also be adjusted, while for APCI the charge on the corona discharge pin can be optimized. Furthermore, the energy by which the ions are extracted into the instrument can be adjusted, so that unwanted clusters of the analyte and other ions such as solvent ions can be prevented. If this energy setting (termed the declustering voltage in some instruments) is however set too high, the increase in kinetic energy can cause collisions between the analyte ions and residual atmospheric gas ions, resulting in unwanted fragmentation of the analyte ions and thus reduction in the intensity thereof.

1.3.9.2 Product ion scans

The first quadrupole (Q1) can be set to pass ions of only one specific *m/z* value. Such ions will then be directed into the fragmentation cell (Q2). Introduction of an inert gas into Q2 will result in collisions between the gas molecules and the introduced ions. The energy of this collisions can be controlled by adjustment of the kinetic energy of the ions (collision energy) producing fragment ions of the specific ion. Such fragment ions (also termed product ions) are characteristic to the structure of the introduced ion (termed the precursor ion) and the *m/z* values of the product ions can be determined by introducing it into the second analyzing quadrupole (Q3) and scanning the *m/z* range through set values. The product of such a scan is a mass spectrum in which the *m/z* values of the product ions are indicated. The mass spectrum is thus valuable for the interpretation of the molecular structure of the precursor ion.

1.3.9.3 Multiple reaction monitoring (MRM)

Product ions produced during fragmentation are characteristic and unique to the precursor ion and can therefore be used to represent the precursor ion. If Q1 is therefore set to select and introduce only the specific precursor ion into Q2 where fragmentation occurs, Q3 can be set to allow only one of the product ions to pass through and impinge on the detector. The transition of the precursor ion to a characteristic product ion therefore produces a very selective mechanism for detection of the specific precursor ion. Because the abundance of the product ion will depend on the concentration of the introduced precursor ion, this scanning mode, termed multiple reaction monitoring (MRM), can also be used in a quantitative detection mechanism. The result of MRM scan mode is a chromatogram, indicating the exit of the precursor ion from a chromatographic system such as HPLC, with the area of the representative chromatographic peak as indication of the concentration of the selected precursor ion. Since the transition from precursor to product ion is relatively unique, the specificity of MRM detection is superior to many other detection methods

and this uniqueness also renders it very sensitive because it excludes most non-specific background detection, therefore resulting in a very low baseline for the chromatogram.

MRM detection was the scanning mode used for the determination of specific compounds in biological matrices in this study.

1.3.10 Optimization of chromatographic and detection conditions

The optimization of chromatagraphic conditions for sufficient separation of the analyte and settings for optimal detection thereof on the mass spectrometer are of the most important criteria to be considered when developing an analytical method based on LC-MS/MS. Mass spectrometric optimization is performed by optimizing the specific source and compound dependent parameters to achieve maximum sensitivity, whilst the chromatographic optimization involves optimization of the physical separation technique. The following three steps summarize the normal procedures involved to achieve an optimized analytical method.

Step 1: A primary stock solution of the analyte is prepared at a high concentration, typically 1 mg/ml, usually in a volatile solvent such as methanol. A working solution containing a relatively low concentration of the analyte, typically about 200 ng/ml, is prepared from the primary stock solution and is infused into the mass spectrometer at a continuous low flow rate (typically 10 µl/min) using a syringe pump. The pseudomolecular ion of the analyte can then be observed in the mass spectrum if a Q1 scan is performed. Fragmentation of the pseudomolecular ion (the precursor ion) can be monitored by performing a product ion scan and the most abundant ion is then selected to set up an MRM method, representing the presence of the analyte as a peak in a chromatographic run [62]. Once the combination of precursor ion and product ion *m/z* values are selected (termed the transition), optimization of the sensitivity of detection can be done by tuning of the compound dependent parameters, including ionization settings such as the capillary voltage and the declustering potential (DP), as well as settings within the analyzers such as the collision energy (CE) and collision cell entrance and exit potentials.

Step 2: Chromatography optimization during method development is a critical factor to establish a robust analytical method since it affects both the sensitivity and the selectivity of the method. Careful consideration should therefore be given to all aspects of the chromatography process.

The most commonly used chromatographic separation technique is reverse phase chromatography. Optimal chromatography, resulting in symmetric peak shapes at short run times, is achieved by selecting the correct combination of mobile phase pH, composition and flow-rate, and the correct analytical column at the optimal column temperature. The flow-rate should be acceptable for optimal ionization in the ionization source of the mass spectrometer and volatile pH modifiers

should be used. Acetic acid, formic acid and buffer salts such as ammonium acetate and ammonium formate are the most common additives used. The flow of the mobile phase can be either isocratic (the same ratio of aqueous and organic phases throughout the run) or by applying a gradient, therefore linearly increasing the ratio of organic to aqueous phases throughout the run.

A final aspect to consider during the optimization of the chromatographic method is the injection volume and composition of the injection solution used to apply the sample to the column. The injection volume should be as small as possible and as a rule the injection solution should have the same or slightly higher polarity than the mobile phase, to assure that the sample is applied as a narrow band on the column [45].

Step 3: Once satisfactory chromatographic conditions have been determined, the ionization source parameters are adjusted and optimized to accommodate the flow-rate and composition of the mobile phase which will be used for sample analysis. Repeated injections are performed during which source settings such as temperatures, gasses and voltages are optimized. This is usually performed automatically during the process termed flow injection analysis [45].

1.4 METHOD VALIDATION

The measurements of drug concentration in biological matrices such as blood, serum, plasma, urine and saliva are a vital aspect in product development [42]. The validation of the bioanalytical method used to perform such measurements includes processes and procedures that generate quantitative concentration data of an analyte in a given biological matrix to prove that the method is accurate, precise, repeatable and robust and that the integrity of the analyte is maintained throughout the quantification process to yield reliable and reproducible results [42] [64].

According to international standards as outlined in the Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation, different types and levels of method validation are required depending on the application of the bioanalytical method.

- A full validation must be performed when a newly developed bioanalytical method is used for the first time.
- A full validation must be performed when an existing validated method is altered by adding metabolites for quantification or for the addition of a new analyte entity to the method.
- A partial validation is performed when minor changes or modifications are made to a bioanalytical method that has previously been validated. Changes that require a partial

validation include transfer of a bioanalytical method to another analyst or laboratory, change in detection equipment, another biological fluid, concentration range, sample processing and storage conditions, etc.

- Cross validations are performed when different bioanalytical methods are used to generate data across different studies or within the same study. Data obtained from cross validations are used to compare results between newly validated methods and results obtained from previously validated methods.

To fulfill validation requirements, fundamental parameters such as selectivity and specificity, accuracy and precision, recovery, reproducibility, sensitivity, stability and suitability has to be demonstrated.

1.4.1 Selectivity/specificity

Selectivity is the ability of the analytical method to secure the quantification of an analyte in the presence of other endogenous components in the sample whilst specificity is the ability to observe solely the analyte rather than the unknown or interfering components. For selectivity/specificity, analysis of blank samples of the appropriate biological matrix such as blood, urine, serum and plasma should first be tested for interfering/endogenous components which may consist of a variety of impurities, metabolites, matrix components or even concomitantly administered medication [42] [43].

1.4.1.1 Blanks samples

At least 6 blank samples from different individual sources are assessed to determine if endogenous matrix components will interfere with the detection of the analyte. If the intended use of the method is for more than one analyte, each analyte should be tested to ensure that there is no interference. Interference by components in the blank matrix should be less than 20% of the lower limit of quantification (LLOQ; see section 1.4.3) of the analyte and less than 5% of the LLOQ when such interference originate from the internal standard [42].

1.4.1.2 Matrix effects

Matrix components can have a negative effect on the analytical process since they can cause suppression or enhancement of the ionization of the analyte. Therefore, when performing quantitative analysis using LC-MS/MS, matrix effects should be tested using blank matrix from six individual donors. Pooled matrix should not be used.

The assessment of matrix effects can typically be determined by calculating the internal standard

normalised matrix factor or alternatively by using Matuszewski's approach as described below [65] [66].

The matrix factor (MF) for the analyte and the internal standard (IS) should be determined for each individual source of blank matrix by calculating the ratio of the peak area in the presence of matrix to the peak area in the absence of matrix. The data is acquired by analysing a blank sample to which the analyte is added after extraction and comparing it to the analysis of a pure solution of the analyte. The internal standard normalised MF takes the internal standard (IS) into account and is calculated by dividing the MF of the analyte by the MF of the internal standard. The percentage coefficient of variation (% CV) of the IS-normalised matrix factors is calculated and should be less than 15%. This determination should be done at low and high levels of concentrations of the analyte and at one concentration of the internal standard.

When assessing the matrix effects according to the Matuszewski approach, six individual blank sources, spiked with the analyte at a low and at a high concentration level and the internal standard at one concentration, are analyzed separately to determine the variability of the response from source to source. The peak responses of the analyte and of the IS are documented for each individual sample and the % CV calculated for the peak responses should be smaller than 15% [42] [43].

When performing matrix effects, it is recommended to investigate other possible sources of potential interference such as haemolysed and lipidaemic samples and the presence of relevant concomitantly administered drugs, to ensure that the selectivity and precision are not compromised by such components within the matrix [65] [66] [67].

1.4.2 Accuracy, precision and recovery

The reliability of an analytical method is determined by its precision and accuracy, which is demonstrated by the analysis of quality control samples.

Accuracy of an analytical method, expressed as percentage nominal (% Nom), can be described as the closeness of the measured concentration obtained by the analytical method to the nominal concentration of the analyte. The accuracy of a method is calculated using the following formula:

$$\%\ Nominal = \frac{Mean\ measured\ concentration}{Nominal\ concentration} \times 100$$

Accuracy determination is done by performing replicate analysis of quality control samples containing known amounts of the analyte. A minimum of five determinations should be done of QC's at each of three concentration levels, namely at low, medium and high concentration levels. It is recommended that the low QC should be within three times the LLOQ, the medium QC at approximately the 50% mark of the calibration curve series and the high QC at 75% of the concentration of the upper limit of quantification (ULOQ). Deviation from the mean value (CV %) should be within 15% of the actual value except at LLOQ, where it should not be more than 20%.

Precision of an analytical method can be described as the closeness of repeated individual measurements of the analyte when the procedure was repeated with multiple aliquots of a single homogeneous volume of biological matrix containing a certain concentration of the analyte.

As for accuracy assessment, precision is assessed by performing at least five determinations of QC's at each of the three concentration levels used for accuracy determination. Using the formula indicated below, the precision should not exceed 15% of the coefficient of variation (CV), except for the LLOQ, for which it should not exceed 20%.

$$\%\ CV = \frac{Standard\ deviation}{Mean}\ X\ 100$$

Accuracy and precision assessments are performed during two sub categories of the validation process, enabling evaluation based on within-batch and between-batch calculations, respectively.

Within-batch accuracy and precision calculations are performed within in a single validation batch, analyzing a minimum of five samples per level at a minimum of four concentration levels that covers the calibration ranges. Between-batch accuracy and precision calculations must include data from QC samples from three separately analyzed validation batches over a period of two days. This includes the LLOQ, low, medium and high QC samples from the individual batches.

Assessment of the **recovery** of an analyte measures the efficiency by which the analyte is extracted from the matrix. Therefore recovery assessment is performed by comparing the instrument response of a blank matrix sample spiked with analyte and undergoing the extraction process (test sample), with the response of a blank matrix sample first undergoing extraction and then being spiked with analyte to contain the same concentration as the test sample (theoretical sample). This comparative assessment is done at three different concentrations for the analyte (low, medium and high) and for the internal standard at the concentration at which it will be used.

The recovery of the analyte does not have to be 100%, but the degree of recovery of an analyte and of the internal standard should be precise, consistent and reproducible. It is calculated using the

following formula:

$$\% \text{ Recovery} = \frac{\text{Mean response of the test samples}}{\text{Mean response of the theoretical samples}} \text{ X } 100$$

1.4.3 Sensitivity and the Lower Limit of Quantification (LLOQ)

The sensitivity of an analytical method is determined by the lowest concentration of analyte that can measurably be detected in the biological matrix. For quantitative analysis, this concentration is termed the lower limit of quantification (LLOQ) and defined as the lowest concentration that can be distinguished from the background noise. If an adequately signal to noise ratio is calculated, the LLOQ is suitable for quantification of the samples. Therefore the LLOQ should meet the following criteria:

- The analyte peak should be at least 5 times more compared to the response of a blank sample similarly analyzed.
- The analyte peak should be detectable, distinct, and reproducible with accuracy between 80-120% and a precision of 20%.

1.4.4 Calibration curve

A calibration curve is used to define the relationship between the instrument response and the known concentration of the analyte over the specified calibration range [42] [68]. A calibration curve should be generated for each analyte in the same blank matrix as the intended study samples. Blank matrix is spiked with a known concentration of the analyte to define an analyte concentration to instrument response relationship.

Concentrations of calibration standards should be selected on the basis of the range expected for a particular study, covering the calibration range from the lowest calibration standard (LLOQ) up to the highest calibration standard (ULOQ), for each analyte.

After defining the expected concentration range of the analyte involved, a calibration curve must be evaluated. A calibration curve must consist of a minimum of six calibration standards including a blank sample and a zero sample. The blank and the zero samples are not taken into consideration when calculating the calibration parameters. Each calibration standard is analyzed in duplicate [42] [43].

Calibration standards should be evaluated against different regression models. The simplest model that adequately describes the concentration-response relationship should be chosen. The

relationship between the response and the concentration should be continuous and reproducible.

A standard curve should meet the following criteria:

- At least 75% of the calibration standards must have an accuracy within 15% of the nominal value, except for the LLOQ which should be within 20%
- At least one of the duplicate calibration standards at each concentration level must have an accuracy of 15% of the nominal value (20% at the LLOQ)

1.4.4.1 Carry-over

Carry-over is defined as residue of an analyte that is carried over from a previous injected sample to a subsequent injected sample. It can affect the accuracy and precision of study samples during analysis and should be addressed and minimised during method development. The primary causes of carry-over can be ascribed to column carry-over, when residues of the analyte are retained on the analytical column and auto sampler carry-over where the analyte is trapped within the injection port. During validation, carry-over should be assessed by including a blank sample after a high concentration sample, usually at the upper limit of quantification (ULOQ). Carry-over measured in the blank sample following the high concentration sample should be less than 20% of the LLOQ, and 5% or for the internal standard [69] and can be calculated using the following formula:

$$\%\ Carry\text{-}over = \frac{Peak\ area\ of\ analyte\ in\ blank\ sample\ after\ ULOQ}{Peak\ area\ of\ analyte\ in\ LLOQ} \times 100$$

1.4.4.2 Dilution integrity

Accuracy and precision of a sample is set within specified criteria and dilution of a sample should not affect this, however the dilution integrity should cover the dilution applied to the entire range of study samples.

Dilution integrity should be performed by spiking the matrix with an analyte at a concentration above the highest calibration standard concentration (ULOQ), and diluting this sample with blank matrix, performing at least five determinations per dilution factor.

1.4.5 Reproducibility

Reproducibility of a method represents the precision of a method under the same operating condition over a short period of time. It can also represent the precision between two different laboratories [43].

When reinjection reproducibility has been demonstrated an analytical batch can be reanalyzed (reinjected) in the case of instrument interruptions or failures.

1.4.6 Stability

The stability of a chemical compound in a given biological matrix must be evaluated under specific conditions for given time periods at low and high concentration levels during method validation. The parameters of stability that should be evaluated are the storage condition, the matrix and the container system. The condition used in stability experiments should reflect situations likely to be encountered during routine analysis [43].

Assessment of analyte stability under the following conditions is required by the FDA and EMA:

- Freeze and thaw stability
- Short-term matrix temperature stability (on-bench stability)
- Long term matrix stability
- Stock solution stability
- Post preparative stability (on-instrument stability)

1.4.6.1 Freeze and thaw stability

Analyte stability should be determined after three freeze and thaw cycles, using at least three aliquots at both low and high concentrations. These aliquots must be stored frozen at the intended temperature for a period of at least 24 hours and then thawed unassisted at room temperature. When completely thawed, samples must be frozen again at the same temperatures for a period of 12-24 hours and the thawing process repeated under the same conditions. After the third thawing cycle the samples are analyzed using freshly prepared calibration standards. Stability sample results should be within 15% of the nominal concentrations [43].

1.4.6.2 Stock solution stability

Stability of the analyte and internal standard in a stock solution should be evaluated to determine their stability under different storage conditions in the solvent used to prepare the stock solutions. The storage conditions used in the assessment should reflect those used for normal storage of such solutions, thus at room temperature, refrigerated in a fridge (approximately 5ºC) and in a freezer (approximately -20ºC). The time of storage should not be less than 6 hours. The stored stocks solutions should be tested by comparing the instrument response thereof to that of freshly prepared stock solutions at the same concentration [43].

1.4.6.3 Short term stability

Short-term matrix stability (on-bench stability) assesses the stability of the analyte in the biological matrix if left standing under laboratory conditions for an extended period. Therefore at least three samples at both low and high concentrations in a biological matrix is thawed at room temperature and kept at these conditions for a period of 4 to 24 hours before analysis using freshly prepared calibration standards. Stability sample results should be within 15% of the nominal concentrations [43].

1.4.6.4 Long term stability

Long term stabilities of an analyte should be determined by storing at least three aliquots at low and high concentrations of the analyte in the biological matrix under the same conditions as the intended study samples. The time of storage for the assessment must exceed the period for which the study samples will be stored, therefore from the date of the first sample collection until the last day of analysis [43]. Stability assessment samples can be retrieved and analyzed at different times during this period of long term storage as long as it is analyzed using freshly prepared calibration standards. Stability sample results should be within 15% of the nominal concentrations.

1.4.6.5 Post-preparative stability

Post-preparative stability (on-instrument stability) of the analyte indicates the stability of the analyte in processed format, in other words following extraction and awaiting injection for analysis on the analytical instrument. The storage condition will thus be that of the conditions within the analytical instrument, which is normally the auto sampler of the HPLC. The period of storage should cover the time resident on the instrument from submitting the samples of an analytical batch for injection until completion of injection. As for the other stability assessments, this assessment should also be performed using samples at low and high concentration. The concentration of the samples should be calculated against freshly prepared or freshly extracted calibration standards. Stability sample results should be within 15% of the nominal concentrations [42].

1.4.7 Suitability

Suitability can be subdivided in to two categories, the first being the suitability of an analytical method and the second the suitability of the system (instrumentation) used for analysis.

Suitability of an analytical method indicates that validation requirements have been met and that the method is suitable for the quantification of a compound in the biological matrix of choice.

System suitability is determined by scientifically qualified and properly maintained instruments for conducting of the analytical methods ensuring that the system operates properly during time of use. Suitability checks are conducted during each batch by ten injections (five at the beginning of the batch and five at the end of the batch) of a sample at a suitable concentration of the analyte and containing the internal standard, if one is used, to evaluate instrument performance during an analytical batch. The responses obtained from the injections at the beginning of the batch are compared with the responses obtained at the end of the batch [42] [43]. Any erratic changes or upward or downward trends in peak responses are an indication of possible problems with the instrument assembly and are investigated and resolved.

1.5 ACCEPTANCE CRITERIA OF AN ANALYTICAL AND VALIDATION BATCH

Acceptance criteria of an analytical batch are based on guidelines set by regulatory authorities such as the FDA and EMA. Evaluation of these criteria confirms acceptance of a validation batch, analytical batch and subsequently the analytical method.

A validation batch consists of a minimum of six STDs. All the calibration standards from ULOQ to LLOQ must be analyzed in duplicate. A minimum of three concentration levels of QCs at low, medium and high concentration and at the LLOQ are included. Additional QC's can be added at the medium level if the calibration range is judged too wide. QCs in six fold at each concentration level are spread throughout the batch but placed so as to control the analysis appropriately and detect possible carry-over. If an internal standard is used, a zero sample, containing neither analyte nor internal standard, should be included as well as blank samples, which contain only the internal standard to confirm that there is no interference from the internal standard on analyte analysis. Blank samples should be placed following high concentration calibration standards or QCs to assess carry-over. In an analytical batch, the sequence is constructed similar to a validation batch accept that the study samples are included.

1.5.1 Acceptance criteria of a validation and analytical batch

A validation batch and an analytical batch are accepted based on the performance of the calibration curve and the quality control samples.

Acceptance criteria for the calibration curve (applicable to a validation and analytical batch):

- The calibration curve, including the ULOQ and LLOQ, must consist of a minimum of six calibration standard levels and the calculated regression line should have a coefficient of determination (r^2) not less than 0.990.

- At least 75% of the calibration standard concentrations must be within 15% of the nominal concentrations, except at LLOQ where it should be within 20% of the nominal concentration. One of each (50%) of the duplicates per concentration level should meet this criterion.
- If one of the calibration standards does not meet the above criterion, it should be rejected, and the calibration curve without this calibration standard must be re-evaluated. This includes re-calculating the regression line of the regression model used.
- The batch should be rejected in the event that both the duplicates of the ULOQ or the LLOQ are not within the acceptance criteria.

Acceptance criteria for the quality control samples (applicable to a validation batch):

- At least four of the six QC samples of each level included in a validation batch must be within 15% of their individual nominal concentrations and within 20% of the nominal concentration at LLOQ. The mean within-batch accuracy of each QC level included in a validation batch must be within 15% for each QC and 20% at the LLOQ. The within-batch precision of each QC level included in a validation batch must be equal to, or less than 15% and equal to, or less than 20% at LLOQ. This last criterion is not applicable to batches with only two QC samples per concentration level.

Acceptance criteria for the quality control samples (applicable to an analytical batch, as well a validation batch that contains only two QCs per concentration level):

- At least 67% of the total number of QC samples must be within 15% and within 20% at LLOQ of their respective nominal concentrations. At least one (50%) QC sample per concentration level must be within its individual nominal concentration.

1.5.2 Acceptance criteria for an analytical method

In addition, the following acceptance criteria must be met for an analytical method:

- Between-batch accuracy, expressed as % Bias, must be within 15% over the range and within 20% at the LLOQ, calculated over all three successive validation batches.
- Between-batch precision, expressed as % CV, must be equal to or less than 15% or equal to or less than 20% at the LLOQ, calculated over all three successive validation batches.
- Selectivity, stability and recovery criteria as detailed under the relevant headings in section 1.4 must be met.

1.6 ROBUSTNESS OF AN ANALYTICAL METHOD

Robustness of an analytical method is the ability to reproduce the method in different laboratories, under different circumstances without the occurrence of unexpected variances in the obtained result(s). A robustness test is an experimental set-up to evaluate the robustness of a method by performing validation batches on separate days and not on the same day. This robustness test can also be verified by using different instrumentation of the same type. If the results remain unaffected by these small, but deliberate procedures, the method reliability is proven for routine analysis.

1.7 STUDY OBJECTIVES

The aim of the development and validation for the quantitative determination of endosulfan in serum was to obtain data that would correlate exposure and possible levels in the biological matrix and to compare this to published results.

Similarly, the use of the validated method obtained from UCT to study levels of organophosphate metabolites in urine of exposed subjects provided data that can be used to monitor such exposure and correlate it with similar data in the literature.

CHAPTER 2

DEVELOPMENT AND VALIDATION OF A BIOANALYTICAL METHOD FOR THE DETERMINATION OF β-ENDOSULFAN AND ENDOSULFAN SULFATE IN HUMAN SERUM PREPARED FROM BLOOD COLLECTED FROM AGRICULTURAL WORKERS

2.1 INTRODUCTION

Analytical methods for the determination of the organochlorine pesticide endosulfan and its metabolites in biological matrices described in the literature mainly employed gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) [16] [20] [26]. This was also the prescribe method of choice in the Clinical Study Protocol on which this study is based (REC REF: 279/2005). The superior versatility of LC-MS/MS was however demonstrated in this study by the application of this technology to develop and validated a sensitive bioanalytical method to quantitatively determine β-endosulfan and its major metabolite, endosulfan sulfate, in human serum.

Although protein precipitation and solid phase extraction were also investigated, the best sample preparation procedure proofed to be liquid-liquid extraction. Chromatographic separation was optimized using a Supelco® Discovery C18 (2.1mm x 150 mm, 5 μm) analytical column fitted with a Phenomenex® Security Guard™ system with a C18, 4 x 2 mm precolumn and applying an isocratic mobile phase consisting of acetonitrile, methanol and water (80:80:40 v/v/v).

Mass spectrometer detection was done by MRM scanning using negative mode electrospray for both β-endosulfan and endosulfan sulfate. Although a second metabolite, endosulfan lactone could also be detected, this metabolite could only be observed in the positive mode and since polarity switching could not be achieved on the available instrument, the quantification of this metabolite was not performed in this study. The literature also indicates that the most abundant metabolite is the sulfate [12], and since the aim of the project was the optimal detection of endosulfan and its metabolites to proof exposure of human subjects to the pesticide, it was decided to concentrate on the most abundant species. For this reason, α-endosulfan was also excluded since the lower level of quantification achieved was much higher than that of β-endosulfan.

The lower level of quantifications were 1 ng/ml and 0.1 ng/ml for β-endosulfan and endosulfan sulfate, respectively, and was applied to analyze individual serum samples collected from healthy workers reported to have been exposed to agricultural pesticides in the Western Cape. Although the sensitivity of the method was deemed adequate to be able to detect these compounds [20] [16],

no indication of the presence of any of the two tested compounds could be found in any of the samples. However, previous studies reported the detection of endosulfan, chlorpyrifos, fenarimol, deltamethrin and iprodione in Western Cape rural surface and groundwater, including drinking water [8] [9] [10]. Endosulfan and chlorpyrifos concentrations exceeding the WHO drinking water standard of 0.1 µg/l as reported in about a third of the samples tested.

2.2 METHOD DEVELOPMENT PROCEDURE

2.2.1 Chemicals and reagents

Analytical reference standards of α-endosulfan, β-endosulfan, endosulfan sulfate and endosulfan lactone, with purities higher than 99% were obtained from Sigma Aldrich. Internal standards tested during method development were sourced from a suitable supplier. All chemicals and reagents used in this study were of analytical grade, as indicated in the Table 2.1.

Table 2.1 Chemicals and reagents

Reagents	Grade	Supplier
Acetonitrile	High Purity	Honeywell
Ammonium formate	Reagent	Sigma-Aldrich
Ammonium acetate	High Purity	Honeywell
Dichloromethane	High Purity	Honeywell
Dimethyl sulfoxide	High Purity	Radchem
Ethanol	High Purity	Honeywell
Formic acid	99%	Radchem
Hexane	High Purity	Honeywell
Isoamyl alcohol	High Purity	Sigma-Aldrich
Methanol	High Purity	Honeywell

Ultrapure water used to prepare solutions was prepared by a Millipore Elix 10 reverse osmosis and Milli-Q® (Millipore, USA) Advantage A 10® polishing system.

2.2.2 Preparation of stock solutions

Separate primary stock solutions for α-endosulfan, β-endosulfan, endosulfan sulfate and endosulfan lactone were prepared in methanol, each at a concentration of 100 µg/ml as indicated in table 2.2. The stock solutions were stored at approximately -20ºC and were used to prepare secondary reference solutions (10 ng/ml) of each analyte.

Table 2.2 Preparation of primary stock solutions

Analyte	Solvent used	Solvent density	Mass analyte (mg)	Mass solvent (g)	Volume solvent (ml)	Concentration analyte (µg/ml)
α-endosulfan	Methanol	0.791	1.00	7.91	10	100
β-endosulfan	Methanol	0.791	1.00	7.91	10	100
Endosulfan lactone	Methanol	0.791	1.00	7.91	10	100
Endosulfan sulfate	Methanol	0.791	1.00	7.91	10	100

2.2.3 LC-MS/MS optimization for endosulfan and its metabolites

2.2.3.1 Infusion (Spectral Analysis)

Spectra of the different compounds were obtained from direct infusion of the reference solutions (10 ng/ml), prepared from the primary stock solutions, by using a Harvard syringe pump at a constant flow rate of 10 µl/min. During this process compound parameters are optimized including the declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP).

Detection was performed on an AB Sciex API 4000 mass spectrometer and the ionization intensities of three different atmospheric pressure ionization techniques namely electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) were evaluated for both positive and negative polarity scan types, using the principles as described in Chapter 1, section 1.3.8.

ESI was found to be the best ionization technique. α-endosulfan, β-endosulfan and endosulfan sulfate were successfully ionized in the negative ion mode forming the respective deprotonated pseudo molecular ions, while endosulfan lactone could only be observed as the protonated pseudo molecular ion in the positive mode.

The presence of six chlorine atoms in each of these molecules resulted in complex mass spectra indicating the patterns expected due to the stable chlorine isotopes (see figure 2.1). Ions at the following *m/z* values were selected to act as precursor ions for the creation of MRM detection methods for the four compounds:

α-endosulfan: *m/z* = 405
β-endosulfan: *m/z* = 405
Endosulfan sulfate: *m/z* = 421
Endosulfan lactone: *m/z* = 357

Figure 2.2 indicates the mass spectra of the fragments ions obtained when the precursor ions of the

four molecules are subjected to collision induced dissociation. The proposed structures of the prominent fragment ions selected as transition for the detection of each compound are also indicated in figure 2.2, and therefore the transition settings on the instrument were as follows:

α-Endosulfan: 405→287

β-Endosulfan: 405→305

Endosulfan sulfate: 421→97

Endosulfan lactone: 357→85

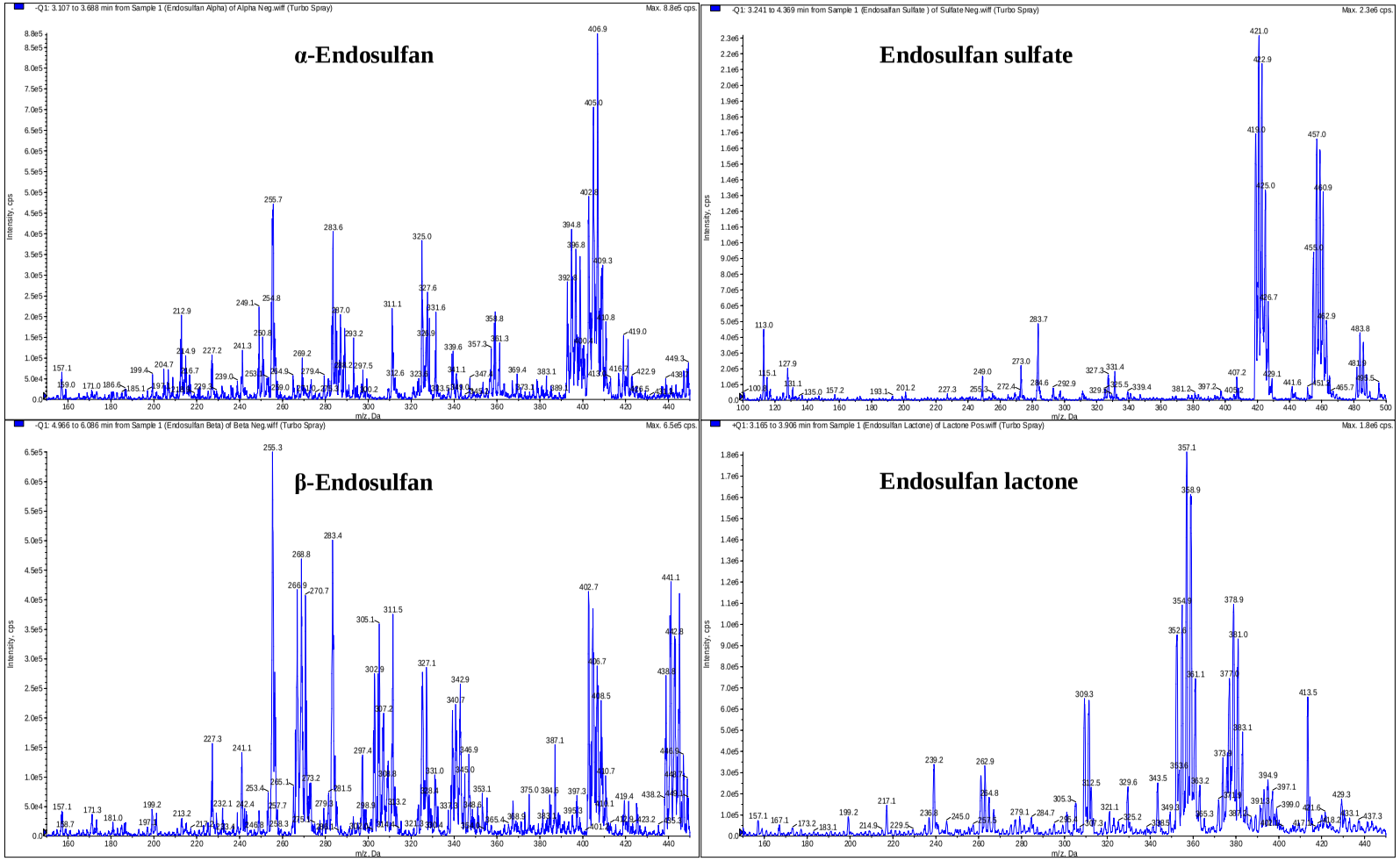


Figure 2.1 Q1 mass spectra of α-endosulfan, β-endosulfan, endosulfan sulfate and endosulfan lactone

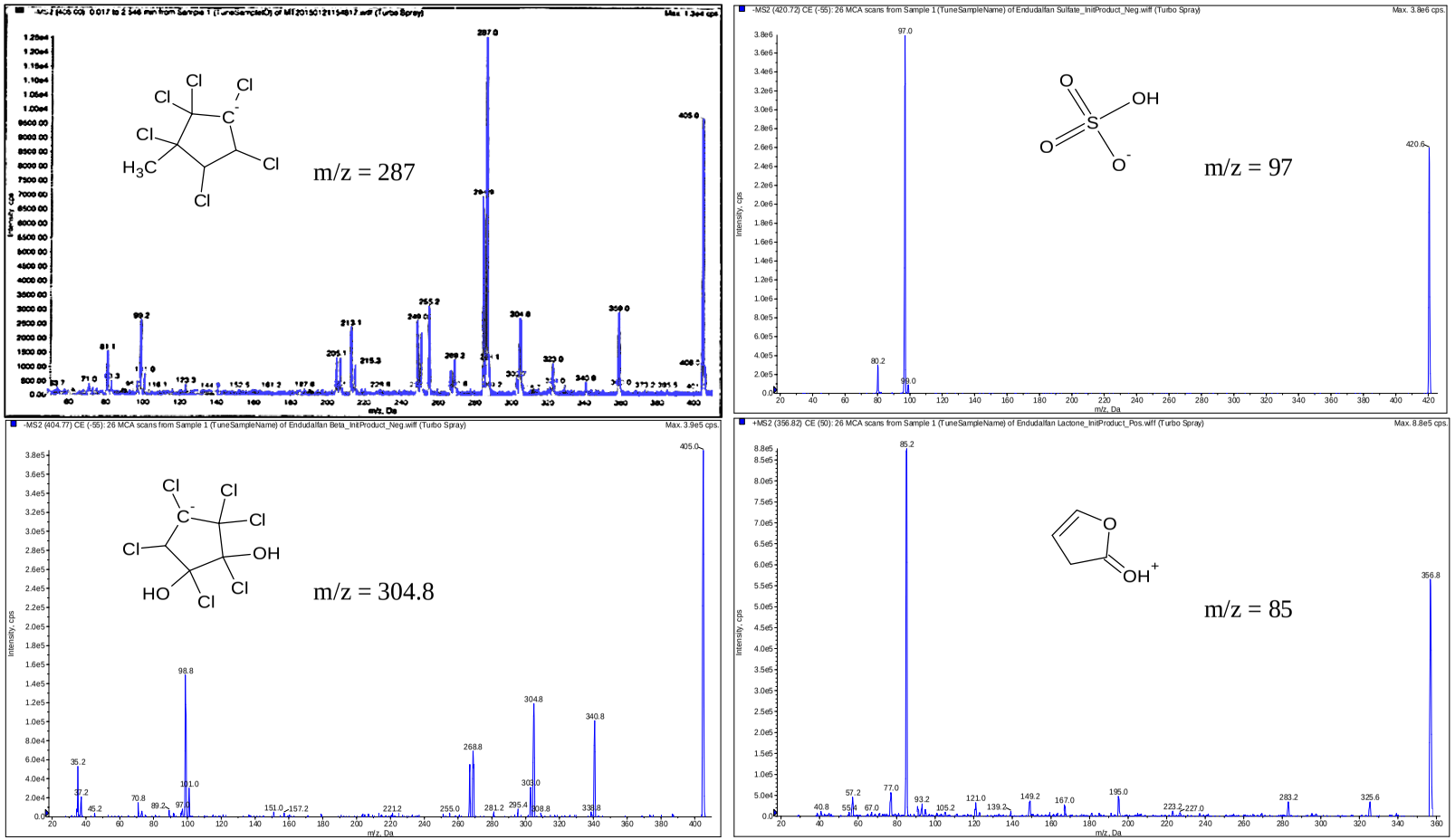


Figure 2.2 Mass spectra of the fragments of α-endosulfan, β-endosulfan, endosulfan sulfate and endosulfan lactone, following CID of the respective precursor ions. Proposed structures of the prominent ions used for MRM detection are indicated.

2.2.3.2 Flow Injection Analysis

Following infusion and the establishment of transitions for the detection of the analytes, flow injection analyses (FIA) was performed automatically to optimize individual parameters. The FIA optimization process was conducted by injecting multiple samples of endosulfan and its metabolites onto a Discovery C18 (2.1 mm x 150 mm, 5 µm) analytical column with a constant flow rate of 200 µl/min. Based on the evaluated chromatographic conditions; acetonitrile, methanol and water (80:80:40 v/v/v) were used as the mobile phase and acetonitrile and methanol (50:50 v/v) as the injection solution.

During the FIA process the optimum source temperature, nebulizing and auxiliary gas flows, as well as ionization voltage settings were obtained, by ramping the various parameters. Each parameter was evaluated in triplicate and based on average results the parameter with the highest average intensity was logged for each compound. Examples of source parameters settings are present in figure 2.3.

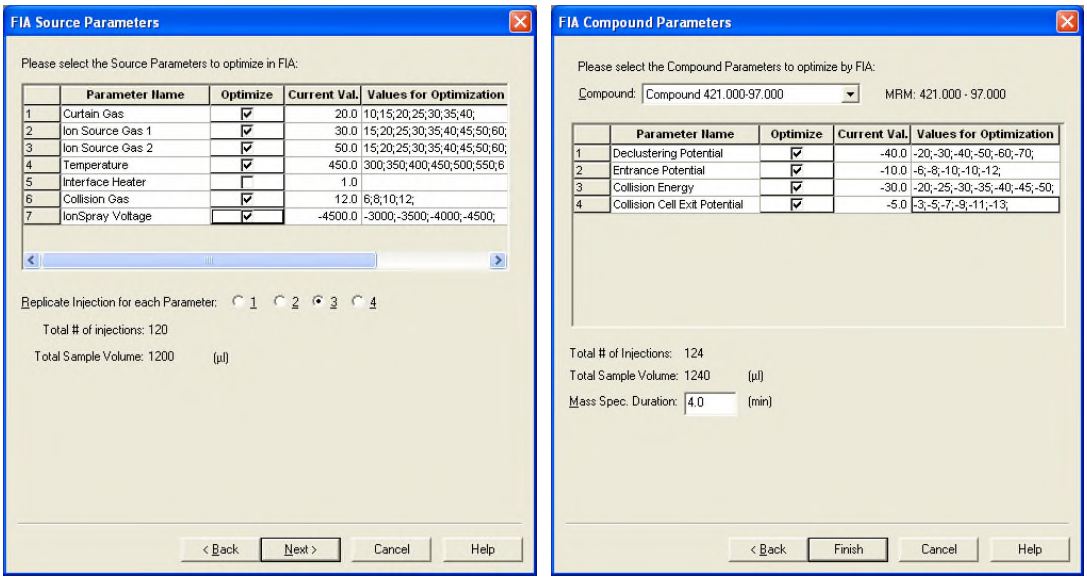


Figure 2.3 Source parameter settings during FIA

2.2.4 Chromatographic development

Chromatographic conditions were optimized using combinations of mobile phases, various types of reverse phase analytical columns, different flow rates, injection volumes and injection solutions.

For the selection of mobile phases, various mixtures of solvents such as acetonitrile, methanol,

acetonitrile and methanol (50:50 v/v), acetonitrile, methanol and water (80:80:40 v/v/v) were tested to achieve efficient separation, good peak shapes and optimal ionization. Small percentages of volatile organic acids such as formic acid and acetic acid, ranging from 0.1 to 0.5%, and volatile buffers such as ammonium acetate and ammonium formate, ranging from 5 mM to 20 mM, were added to the mobile phase solutions to improve ionization and gain maximum sensitivity. All solvents were degassed by sparging with helium gas for 3-5 minutes depending on the volume of the mobile phase.

The injection solvent was optimized by testing the various combinations of mobile phases as injection solutions. The optimum injection volume was assessed by injecting different volumes of the various injection solutions by increments of 3 µl, starting from 2 µl up to 20 µl.

Various types of reverse phase analytical columns, namely Kinetex® EVO C18 (2.1 mm x 150 mm, 5 µm), Discovery® C18 (2.1 mm x 150 mm, 5 µm), Discovery® C8 (2.1 mm x 150 mm, 5 µm), Discovery® Cyano (2.1 mm x 150 mm, 5 µm), Agilent® Poroshell C18 (2.1 mm × 150 mm, 5 µm), Luna C18 (2.0 mm x 150 mm, 5 µm), Luna C8 (2.0 mm x 150 mm, 5 µm), and Luna Phenyl Hexyl (2.0 mm x 150 mm, 5 µm) were tested. Different flow rates were applied to separate columns ranging from 150 µl/min up to 1 ml/min, to achieve a short but stable retention time. Chromatographic parameters such as stable retention time, highest peak intensities good symmetrical peak shapes were optimised.

2.2.5 Procedures for the selection of a sample extraction method

Serum samples were submitted to a series of sample preparation techniques such as protein precipitation, liquid-liquid extraction (LLE) and solid phase extraction (SPE). The recoveries and sensitivities of detection of each compound was determined and compared to select the ideal extraction procedure. A stock solution of each compound was prepared at a concentration of 100 µg/ml in methanol. Pooled blank serum donated by healthy volunteers was spiked with 180 µl of each stock solution to obtain a serum sample at a concentration of 30 ng/ml of each analyte. This was used to assess the different extraction procedures as summarised below.

2.2.5.1 Protein precipitation

Protein precipitation was evaluated by using solvents or solvent mixtures as precipitation solution. The following precipitation solutions were evaluated: acetonitrile; methanol; acetonitrile : methanol (50 : 50 v/v); acetonitrile : 0.1% formic acid in water (50 : 50, v/v); methanol : 0.1% formic acid in water (50 : 50, v/v); acetonitrile : methanol : 0.1% formic acid: (50:50 v/v 0.1% formic acid).

This following procedure was performed for each of the precipitation solutions mentioned above.

- Aliquot 200 µl of the serum sample into a 2 ml microfuge tube.
- Add 500 µl precipitation solution.
- Vortex the samples for 30 seconds.
- Place the sample on ice for approximately 15 minutes.
- Centrifuge at 3000 x g for 5 minutes.
- Transfer the supernatants to 96-well collection plates for injection onto the chromatographic system.

2.2.5.2 Liquid-liquid extraction (LLE)

The organic solvents evaluated for liquid-liquid extraction were hexane : ethyl acetate (50 : 50, v/v), hexane : dichloromethane (60 : 40, v/v), hexane : iso-amyl alcohol (98 : 2, v/v) and tert-butyl methyl ether (TBME). Each of these solvents was tested separately with and without the universal Britton Robinson buffers at various pH values, ranging from 2.5 up to 12. The following procedure was applied:

- Aliquot 200 µl of the serum sample into a 10 ml amber glass ampoule.
- Add 200 µl of each of the buffer, if applicable.
- Add 5 ml of the applicable organic solvents.
- Vortex the samples for 90 seconds.
- Centrifuge at 1300 x g for 5 minutes.
- Freeze the aqueous phase in an alcohol freezing bath at approximately -30°C.
- Decant the organic phase into a 5 ml amber glass ampoule.
- Evaporate the solvent under a gentle stream of nitrogen at 40°C until completely dry.
- Add 200 µl reconstitution solution (acetonitrile : methanol [50 : 50 v/v]).
- Vortex for 30 seconds.
- Transfer the extracts to 96-well collection plates for injection onto the chromatographic system.

This procedure was performed for the entire buffer range at various pH levels (bullet 2), for each of the above mentioned organic solvents (bullet 3).

2.2.5.3 Solid phase extraction (SPE)

Solid phase extraction was performed using Waters Sep-Pak®Vac 100 mg tC18® SPE columns with a SPEEDISK® 48 place positive pressure system. Different organic solvents such as acetonitrile, hexane and methanol were tested as elution solvents. Each of the organic solvents was tested separately with and without buffers at various pH values, ranging from 2.5 up to 12.

- Activate the solid phase cartridges by adding 1 ml of organic solvent (SPE Conditioning).
- Wash the column with 1 ml buffer.
- Aliquot 200 µl of the serum sample (with and without water dilution) onto the activated solid phase cartridge.
- Perform a wash step with 1 ml of buffer.
- Elute with 200 µl organic buffer into polypropylene tubes.
- Evaporate the samples under a gentle stream of nitrogen at 40°C until completely dry.
- Add 200 µl of reconstitution solution (acetonitrile : methanol [50 : 50 v/v]).
- Vortex briefly for 30 seconds.
- Transfer the reconstitute to 96-well collection plates for injection onto the chromatographic system.

2.3 DESCRIPTION OF THE OPTIMIZED ANALYTICAL PROCEDURE

2.3.1 Optimization of the sample preparation

Amongst all the extraction procedures that were evaluated, liquid-liquid extraction proved to be the best and therefore it was applied for the extraction of α-endosulfan, β-endosulfan, endosulfan sulfate, and endosulfan lactone from serum.

The optimal pH of extraction and the choice of solvent were made based on the data depicted in figures 2.4 and 2.5, respectively. At a pH of 9, the recovery of α-endosulfan, β-endosulfan and endosulfan sulfate was consistently approximately 82%, while that of endosulfan lactone was approximately 50%. As all the compounds were extracted simultaneously from the same serum sample, the lower recovery of endosulfan was regarded as acceptable.

The organic solvent of choice for the extraction of α-endosulfan, β-endosulfan, endosulfan sulfate, and endosulfan lactone from serum was hexane : dichloromethane (60 : 40, v/v) as it resulted in the highest extraction efficiency of approximately 80% for all the compounds.

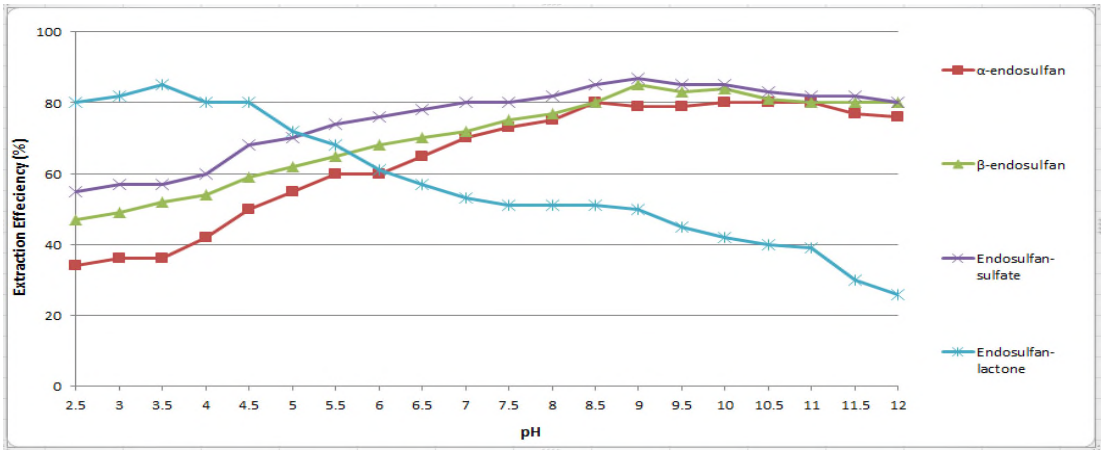


Figure 2.4 The influence of pH on sample on extraction efficiency of α-endosulfan, β-endosulfan, endosulfan sulfate, and endosulfan lactone from serum

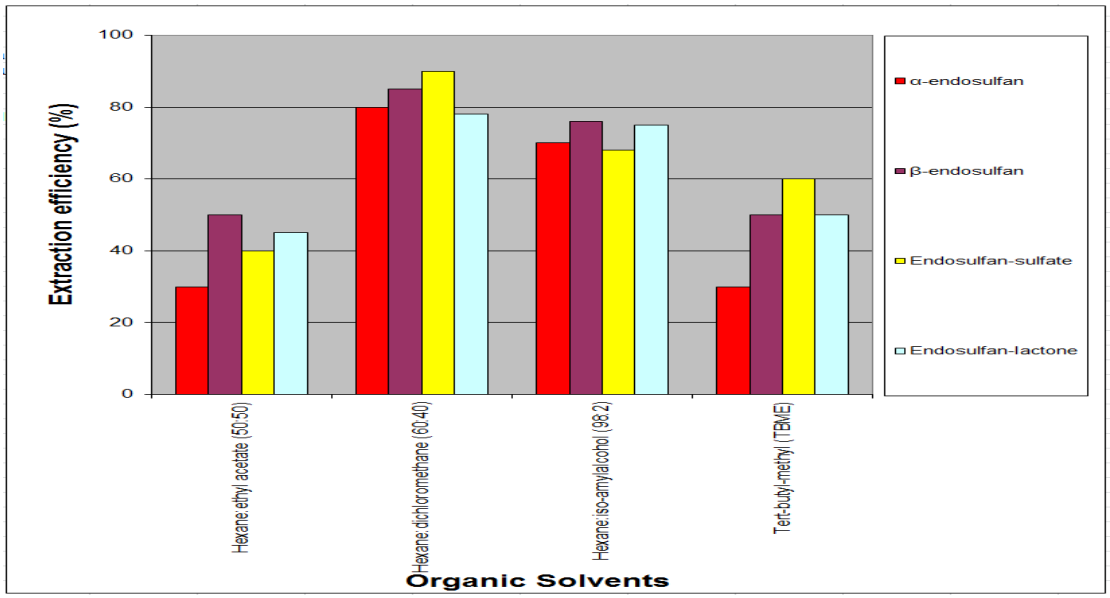


Figure 2.5 Organic solvent extract efficiency of, α-endosulfan, β-endosulfan, endosulfan sulfate, and endosulfan lactone from serum

The final extraction procedure can be summarized as follows:

- Thaw the serum samples unassisted at room temperature (~ 23 ^{o}C).
- Vortex briefly for 10 seconds.
- Aliquot serum (200 µl) into a 10 ml amber glass ampoule.
- Add 200 µl of a Britton Robinson buffer at pH 9.

- Add 5 ml hexane : dichloromethane (60 : 40, v/v).
- Vortex the samples for 90 seconds.
- Centrifuge for 5 minutes at 1300 x *g* (~ 5 ^{o}C).
- Freeze the aqueous phase in an alcohol freezing bath (~ -30 ^{o}C).
- Decant the organic phase into a clean 5 ml amber glass ampoule and discard the aqueous phase left in the 10 ml amber glass ampoule.
- Evaporate the organic solvent in the 5 ml amber glass ampoule under a gentle stream of nitrogen at ~ 40°C until completely dry (approximately 25 minutes).
- Add 200 µl reconstitution solution into the 5 ml amber glass ampules (acetonitrile : methanol (50 : 50 v/v)).
- Vortex for 30 seconds.
- Transfer the extracts to 96 well plates.
- Inject 10 µl onto the HPLC column.

2.3.2 Chromatographic separation and detection of the analytes

HPLC analysis and detection were performed using the following instruments and conditions:

2.3.2.1 Chromatography

For the analysis of α-endosulfan, β-endosulfan and endosulfan sulfate, which was detected by negative mode electrospray ionization, the best analytical column was a Supelco® Discovery C18 (2.1 mm x 150 mm, 5 µm) fitted with a Phenomenex® Security Guard™ system with a C18, 4 x 2 mm precolumn. Endosulfan lactone was detected in the positive mode and the column of choice for its analysis was a Phenomenex® Kinetex C18 (2.1 mm x 100 mm, 2.6 µm). The columns were kept at 40°C in an Agilent 1200 series column compartment.

Acetonitrile : methanol : water (80 : 80 : 40, v/v/v) was used as the isocratic mobile phase to perform analysis in the negative ionization mode for the detection of α-endosulfan, β-endosulfan and endosulfan sulfate, and acetonitrile : 0.1 % formic acid (80 : 20, v/v) was used (also isocratically) for the detection of endosulfan lactone in the positive ionization mode. The respective mobile phases were used as reconstitution and injection solutions.

An Agilent 1200 series binary pump, combined with a degasser, was used to deliver the mobile phase at a constant flow rate of 200 µl/min.

An Agilent 1200 series auto sampler, equipped with an Agilent cooling device (sample cooler) set

at 5^{o}C, was used to inject 10 µl sample onto the HPLC column. The auto sampler was equipped with a flush port with needle wash for 20 seconds.

2.3.2.2 Detection

Detection was performed on an AB SCIEX API 4000 triple quadrupole mass spectrometer (AB SCIEX Toronto Canada) equipped with an electrospray ionization (ESI) source operating in the positive or negative ion mode as applicable. Settings used for the detection of α-endosulfan, β-endosulfan, endosulfan sulfate are indicated in Tables 2.3 to 2.5 and for endosulfan lactone as listed in Tables 2.6 to 2.8.

Table 2.3 Source parameter settings for α-endosulfan, β-endosulfan and endosulfan sulfate

Electrospray Ionization Settings	Value
Nebulizer gas (Gas 1)	30
Turbo gas (Gas 2)	50
CUR (curtain gas)	20
CAD (collision gas)	12
TEM (source temperature) (°C)	450
IS (Ion Spray Voltage) (V)	-4500

Table 2.4 MS/MS parameter settings for α-endosulfan, β-endosulfan and endosulfan sulfate

MS/MS Settings	α-Endosulfan	B-Endosulfan	Endosulfan sulfate
Monoisotopic mass*	403.816	403.813	419.811
Deprotonated isotope used (*m/z*)	405	405	421
Product ion (*m/z*)	287	305	97
Dwell time (ms)	150	150	150
DP (declustering potential) (V)	-30	-30	-40
EP (entrance potential) (V)	-10	-10	-10
CE (collision energy) (eV)	-15	-15	-5
CXP (collision cell exit potential) (V)	-12	-15	-10

**As calculated using Analyst® software*

Table 2.5 Scan description for α-endosulfan, β-endosulfan and endosulfan sulfate

Scan Description	
Scan type	MRM
Polarity	Negative
Pause time	5 ms

Table 2.6 Source parameter settings for endosulfan lactone

Electrospray Ionization Settings	Value
Nebulizer gas (Gas 1)	45
Turbo gas (Gas 2)	55
CUR (curtain gas)	15
CAD (collision gas)	12
TEM (source temperature) (°C)	450
IS (Ion Spray Voltage) (V)	5000

Table 2.7 MS/MS parameter settings for endosulfan lactone

MS/MS Settings	Endosulfan lactone
Monoisotopic mass*	353.834
Protonated isotope used (*m/z*)	357
Product ion (*m/z*)	85
Dwell time (ms)	150
DP (declustering potential) (V)	40
EP (entrance potential) (V)	12
CE (collision energy) (eV)	30
CXP (collision cell exit potential) (V)	5

**As calculated using the Analyst® software*

Table 2.8 Scan description for endosulfan lactone

Scan Description	
Scan type	MRM
Polarity	Positive
Pause time	5 ms

2.3.3 Chromatographic results

The total runtime for each sample was 4.5 minutes. A representative chromatogram of an extracted serum sample containing α-endosulfan (300 ng/ml), β-endosulfan (200 ng/ml) and endosulfan sulfate (30 ng/ml), in the negative ionization mode, is present in figure 2.6 and that for endosulfan lactone (100 ng/ml) analyzed in positive ionization mode is present in figure 2.7.

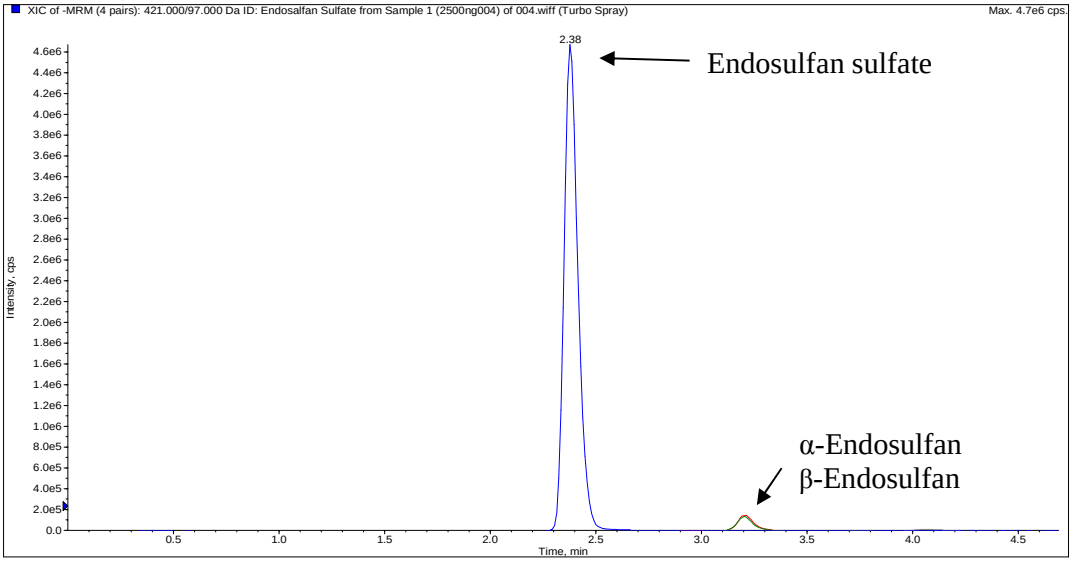


Figure 2.6 A Representative Chromatogram of α-endosulfan, β-endosulfan and endosulfan sulfate

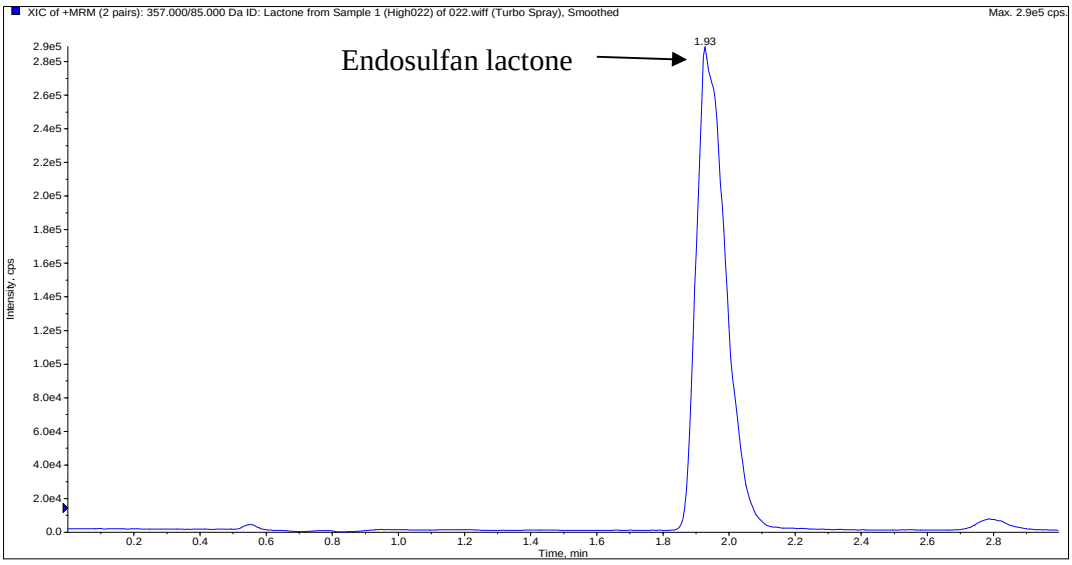


Figure 2.7 A Representative Chromatogram of endosulfan lactone

2.3.3.1 Limit of Detection

According to literature, gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) was mostly employed to determine endosulfan and its metabolites in biological matrices such as serum and urine [20] [26].

Arrebola *et al*. [20] reported limits of detection of 0.014 ng/ml, 0.015 ng/ml, 0.01 ng/ml and 0.019 ng/ml for α-endosulfan, β-endosulfan, endosulfan sulfate and endosulfan lactone, respectively,

using human serum as matrix. Limits of quantification were however 0.051 ng/ml for α-endosulfan, 0.055 ng/ml for β-endosulfan, 0.034 ng/ml for endosulfan sulfate and 0.068 ng/ml for endosulfan lactone. The method was applied to nine serum samples from agricultural workers, eight males and one female that were occupationally and non-occupationally exposed. The α and β-isomers were found in all the samples with concentration levels between 3.88 ng/ml and 12.84 ng/ml for α-endosulfan and 1.68 ng/ml and 6.86 ng/ml for β-endosulfan with no traces of endosulfan sulfate or endosulfan lactone in any of the samples. The levels of β-endosulfan that can be observed with the method reported in this study, therefore falls within the expected range following exposure as indicated by Arrebola *et al*. [20].

Vidal *et al*. [26] performed a similar GC-MS/MS-based study in human urine and reported limits of detection at 0.009 ng/ml for α-endosulfan, 0.018 ng/ml for β-endosulfan, 0.013 ng/ml for endosulfan sulfate and 0.009 ng/ml for endosulfan lactone. However, the limits of quantification were 0.031 ng/ml for α-endosulfan, 0.060 ng/ml for β-endosulfan, 0.044 ng/ml for endosulfan sulfate and 0.031 ng/ml for endosulfan lactone.

The electrospray ionization technique employed in this study successfully ionized β-endosulfan in the negative mode. Under the same conditions, the ionization intensity of α-endosulfan was much lower so that the quantification limit thereof was not comparable to that of the β-isomer. For this reason, α-endosulfan was omitted from the study. The sulfate metabolite ionized stronger than the parent compounds and the anion was formed under the same conditions as applied for the ionization of the β-isomer. These two analytes could also be successfully extracted and chromatographically separated from the matrix components using the same column.

The lactone metabolite did not ionize in the negative mode but formed a strong cation during electrospray ionization. The extraction and chromatographic characteristics of the lactone also significantly differed from that of β-endosulfan and the sulfate metabolite, to such a degree that the lactone had to be analyzed in a separate run, including a separate extraction procedure. The available sample volume was not enough to allow two separate analytical procedures per sample and therefore β-endosulfan and the sulfate metabolite were used as markers for the exposure to the pesticide.

2.3.3.2 Repeatability

Repeatability of an analytical method should be proven during the method development process. This is evaluated by performing 100 injections in pure solution (sample prepared in reconstitution

solution) followed by 100 injection of an extracted sample. As indicated in figure 2.8 and 2.9, the instrument response was stable and the % CV of β-endosulfan and endosulfan sulfate were 5.39 and 2.28 in pure solution, and 6.25 and 3.14 in the extracted sample.

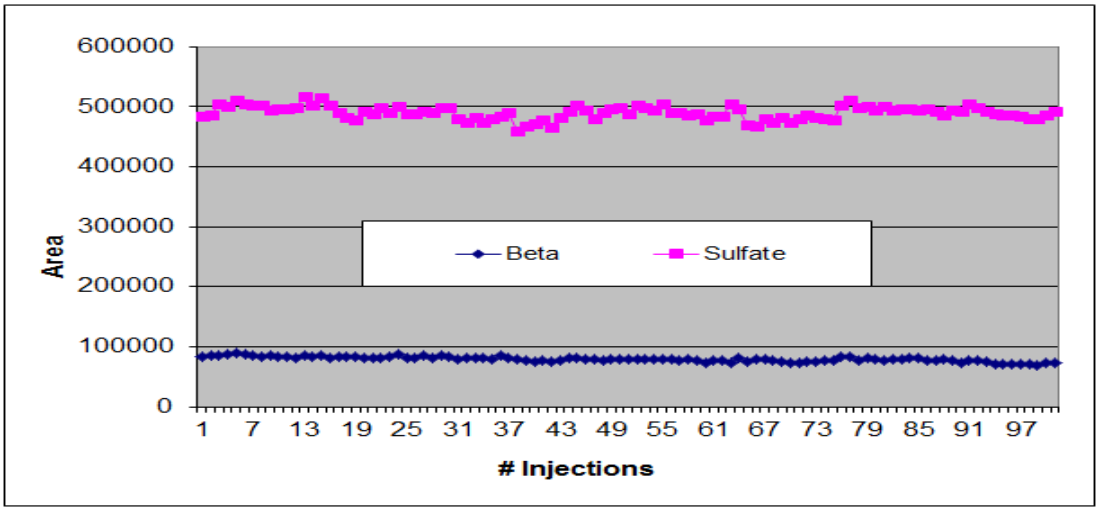


Figure 2.8 Repeatability of β-endosulfan and endosulfan sulfate in pure solution

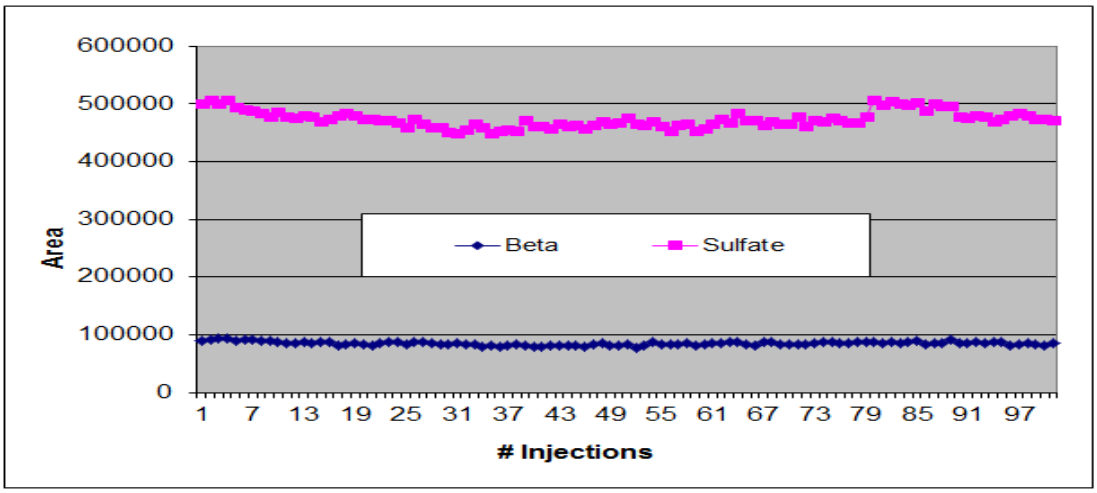


Figure 2.9 Repeatability of β-endosulfan and endosulfan sulfate after extraction

The results prove that the instrument response and retention times are consistent during sample injection.

2.3.4. Choice of internal standard

Both β-endosulfan and endosulfan sulfate contain six chlorine atoms which results in multiple isotope peaks representing the molecular ions as presented in figures 2.10 and 2.11 [70]. The complexity of the mass spectrometric characteristics prevents the use of deuterated molecules of

these compounds as internal standards. Such material is also not commercially available.

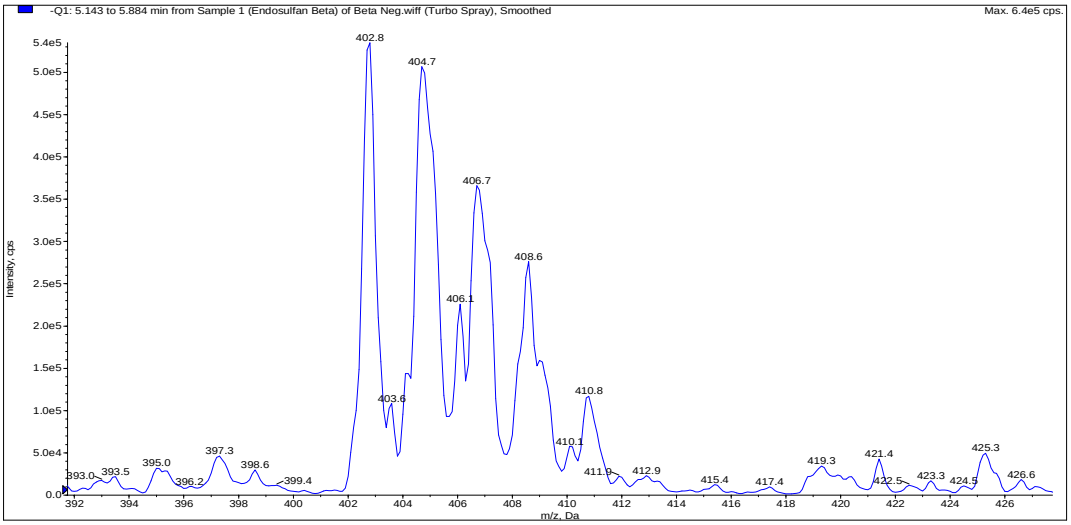


Figure 2.10 Q1 mass spectrum of β-endosulfan indicating the chlorine isotope peaks

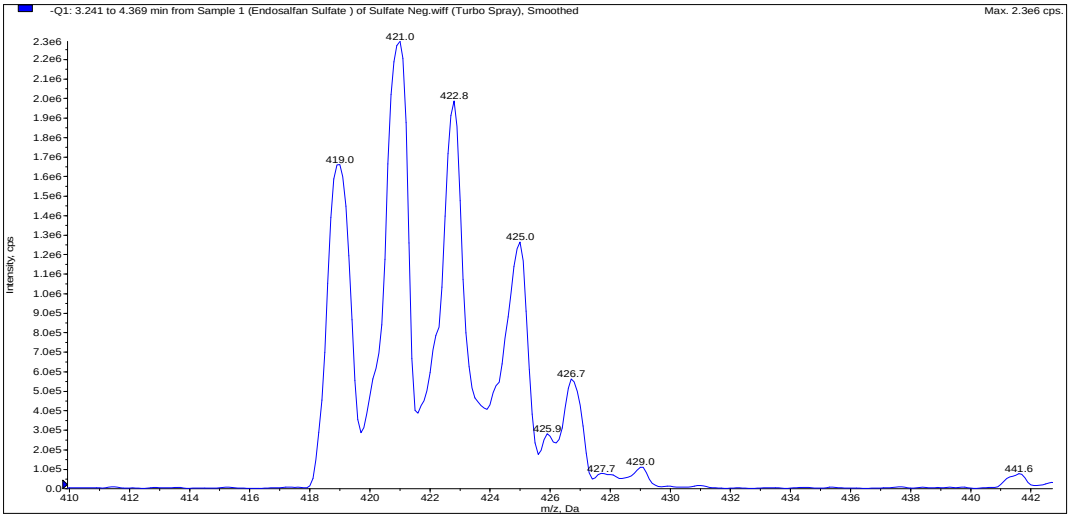


Figure 2.11 Q1 mass spectrum of endosulfan sulfate indicating the chlorine isotope peaks

Other chlorinated pesticides such as dieldrin and dichlorodiphenyltrichloroethane (DDT) were considered as internal standards, but due to their chemical characteristics and lack of ionisable functional groups, could not be ionized under the same conditions as for the endosulfans. Chlorinated compounds containing ionisable functional groups such as diclofenac and dichorophenol were evaluated but their chemical characteristics were found to be too diverse to the endosulfans so that they could not compensate during ionization and extraction. It was therefore decided to perform the detection of β-endosulfan and endosulfan sulfate without an internal

standard.

2.3.5 Method conclusion and application

Both β-endosulfan and endosulfan sulfate are to be determined simultaneously from the same sample if the acceptance criteria are met for both calibration ranges. Having two different calibration ranges also implies that calibration standards and quality control samples would each contain the two compounds at different concentrations. All analysis performed on the instrument was interfaced to a DELL® work station running Analyst® software version 1.5.2, and all data generated was captured and stored on this workstation. Watson™ LIMS software version 7.1 was used to process the raw data.

2.4 INTRODUCTION TO THE VALIDATION PROCESS

Before subject samples can be analyzed with the developed analytical method, the reliability and reproducibility of the method have to be confirmed by performing a validation. This is done in a complete and well defined understandable format, proving accurate determination of β-endosulfan and endosulfan sulfate in human serum over a suitable concentration range. A complete validation includes three validation batches consisting of repeated analysis of calibration standards and quality control samples in three successive batches, each analyzed on different occurrences.

2.4.1 Calibration range

The calibration range of β-endosulfan was within 1 % from 0.800 ng/ml to 200 ng/ml and 0.117 ng/ml to 30.0 ng/ml for endosulfan sulfate.

2.4.1.1 Preparation of calibration standards (STDs)

Primary stock solutions of each analyte were prepared in HPLC grade methanol to a final concentration as indicated in Table 2.2. The primary stock solutions were used to spike an accurate volume of pooled normal blank serum so that the ULOQ of each analyte is reached. This serves as the highest concentration calibration standard (identified in this case as STD J). Serial dilutions of STD J were then performed gravimetrically (1:1 dilutions), using the same blank pool of serum, until the full range from ULOQ to LLOQ is achieved, i.e. in this case STD J - STD B (see Table 2.9). The individual calibration standards were divided into aliquots of 1 ml into three individual sets of polypropylene tubes. A set of aliquots were used after preparation for the first validation batch and the rest of the aliquots were stored at approximately -20°C to be used in validation batches 2 and 3 and for the analysis of the study samples. The storage conditions of the prepared calibration standards were the same as applied to the storage of the subject samples.

Table 2.9 Preparation of calibration standards

Sample Identification	Source Solution	A (g)	B (g)	C (ng/ml)	D (ng/ml)
STD J	Stock solution	30.029		204	30.0
STD I	STD J	7.000	14.000	102	15.0
STD H	STD I	7.000	14.001	51.1	7.49
STD G	STD H	7.001	14.007	25.6	3.75
STD F	STD G	6.999	14.002	12.8	1.87
STD E	STD F	7.001	14.001	6.39	0.937
STD D	STD E	7.000	14.001	3.20	0.468
STD C	STD D	7.002	14.004	1.60	0.234
STD B	STD C	4.999	10.003	0.799	0.117

A = Mass of container + normal blank serum
B = Total mass of container + normal blank serum + spiked serum
C = Concentration of β-endosulfan
D = Concentration of endosulfan sulfate
Note: Density of human serum is 1.0269kg/l

2.4.2 Preparation of quality controls (QCs)

Quality control samples were similarly prepared gravimetrically in pooled blank serum by using the primary stock solutions of β-endosulfan and endosulfan sulfate prepared for the calibration standards. An accurate volume of normal blank serum was spiked to the highest concentration of each individual analyte (QC H). QC H was then serially diluted with normal blank serum to achieve the desired concentrations (QC H - QC A; see Table 2.10). The quality control samples were aliquoted into three individual sets of polypropylene tubes. A set of aliquots were used after preparation for the first validation batch and the rest of the aliquots were stored at approximately -20°C to be used in validation batches 2 and 3 and for the analysis of the study samples. The prepared quality control samples were stored under the same conditions as the subject samples.

Table 2.10 Preparation of quality control samples

Sample Code & No.	Source Solution	A (g)	B (g)	C (ng/ml)	D (ng/ml)
QC H	**Stock solution**	30.021		323	48.0
QC G	**QC H**	8.002	15.998	162	24.0
QC F	**QC G**	8.001	15.999	80.8	12.0
QC E	**QC F**	8.001	16.000	40.4	6.00
QC D	**QC E**	8.002	16.001	20.2	3.00
QC C	**QC D**	7.998	16.002	10.10	1.15
QC B	**QC C**	10.000	12.399	1.95	0.230
QC A	**QC B**	5.000	8.500	0.805	0.117

A = Mass of container + normal blank serum
B = Total mass of container + normal blank serum + spiked serum
C = Concentration of β-endosulfan
D = Concentration of endosulfan sulfate

Note: Density of human serum is 1.0269kg/l

2.4.3 Preparation of stability samples

Quality Control samples prepared for the validation batches were used as stability samples. The concentration of the sample must be determined immediately after preparation by using freshly prepared calibration standards. The stability assessments were performed during the analysis of the first validation batch.

2.4.4 Calibration curve

To test the acceptability of the calibration standards, duplicates of a set of the calibration standards were analyzed using the validated analytical method. The responses were plotted against the known nominal concentrations of these calibration standards. The calibration curves for β-endosulfan and endosulfan sulfate, obtained using Watson™ LIMS version 7.1 software, covers the concentration ranges of 0.800 ng/ml to 200 ng/ml and 0.117 ng/ml to 30 ng/ml. See figures 2.12 and 2.13 and Tables 2.11 and 2.12.

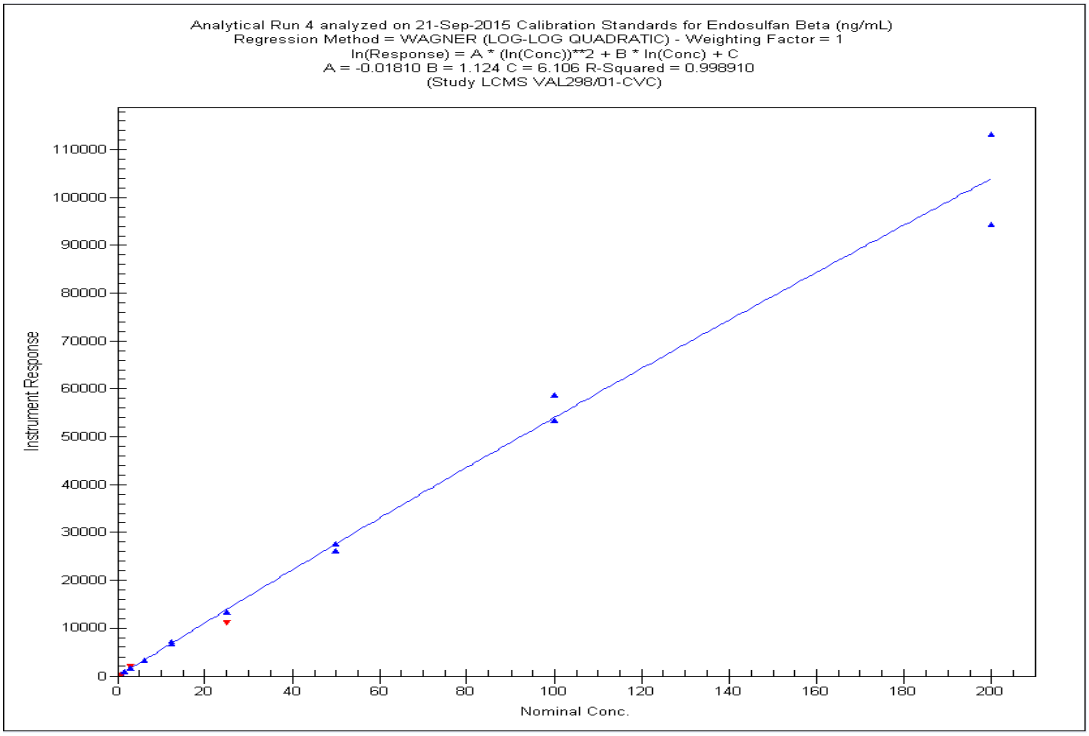


Figure 2.12 Calibration curve of β-endosulfan

Table 2.11 Results of the analysis of the calibration standards for β-endosulfan

Sample ID	Replicates	Calibration Concentration(ng/ml)	Concentration Found (ng/ml)
STD B	1	0.781	0.719
STD B	2	0.781	*0.368
STD C	1	1.56	1.69
STD C	2	1.56	1.66
STD D	1	3.13	*3.83
STD D	2	3.13	3.08
STD E	1	6.25	6.00
STD E	2	6.25	6.22
STD F	1	12.5	12.4
STD F	2	12.5	13.1
STD G	1	25.0	*20.1
STD G	2	25.0	24.0
STD H	1	50.0	49.9
STD H	2	50.0	47.3
STD I	1	100.0	109
STD I	2	100.0	98.6
STD J	1	200.0	180
STD J	2	200.0	219

Code: * = Deactivated calibration standard (% Dev > 15%)

Results	%Dev	Response per ng/ml
Mean	4.9	521.744
S.D	3.5	46.174

Calibration curve parameters	
Regression method	WAGNER
R-Squared	0.998
ULOQ	200 ng/ml
LLOQ	0.781 ng/ml

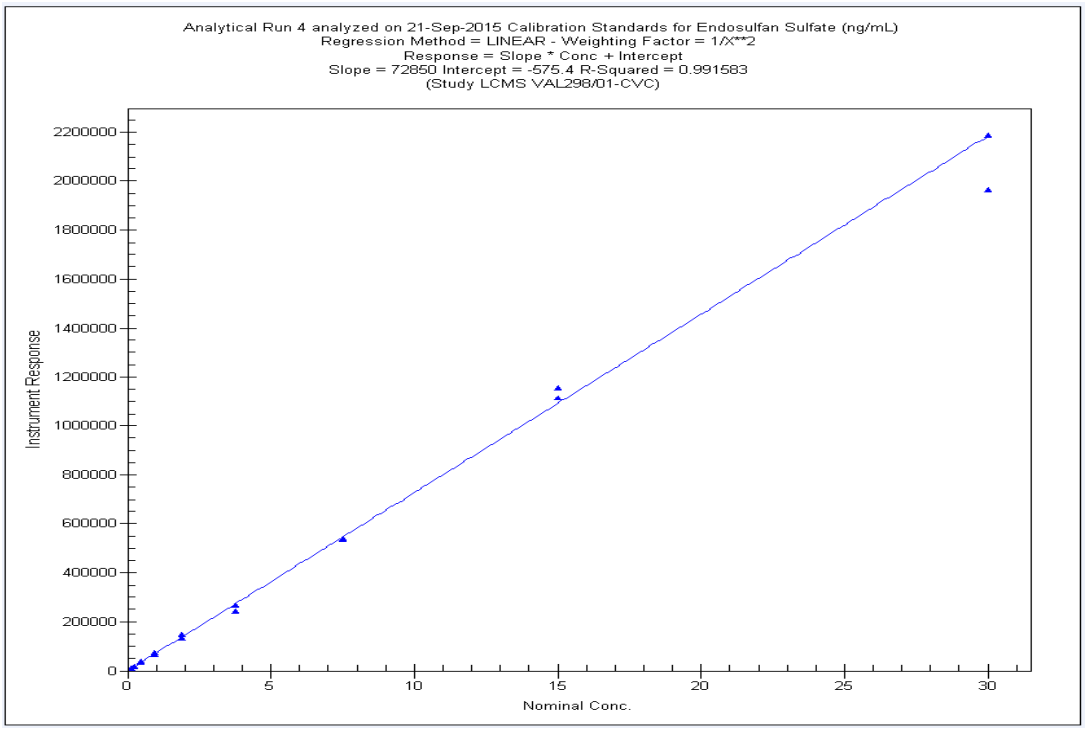


Figure 2.13 Calibration curve of endosulfan sulfate

Table 2.12 Results of the analysis of the calibration standards for endosulfan sulfate

Sample ID	Replicates	Calibration Concentration (ng/ml)	Concentration Found (ng/ml)
STD B	1	0.117	0.130
STD B	2	0.117	0.103
STD C	1	0.234	0.247
STD C	2	0.234	0.207
STD D	1	0.469	0.536
STD D	2	0.469	0.448
STD E	1	0.938	0.944
STD E	2	0.938	0.996
STD F	1	1.88	1.86
STD F	2	1.88	2.03
STD G	1	3.75	3.35
STD G	2	3.75	3.69
STD H	1	7.50	7.43
STD H	2	7.50	7.35
STD I	1	15.0	15.9
STD I	2	15.0	15.3
STD J	1	30.0	27.0
STD J	2	30.0	30.1

Results	%Dev	Response per ng/ml
Mean	6.0	71757.200
S.D	4.7	5865.730
Calibration curve parameters		
Regression method		LINEAR (1/X**2)
R-Squared		0.991
ULOQ		30 ng/ml
LLOQ		0.117 ng/ml

2.5 RECOVERY

The absolute recovery of the extraction procedure was assessed by comparing the responses measured for samples spiked with β-endosulfan and endosulfan sulfate post-extraction (reference samples) to responses measured for extracted quality controls. Six samples of each were injected at high, medium and low concentrations. Recovery was determined (expressed as a percentage) by calculating the ratio of the β-endosulfan and endosulfan sulfate peak areas after extraction to the reference samples peak areas. The precision of the measured recovery expressed as percentage coefficient of variation should not exceed 15%. See tables 2.13 and 2.14 for the recovery assessment results for β-endosulfan and endosulfan sulfate, respectively.

Table 2.13 Recovery of β-endosulfan

Injection No.	β-Endosulfan extract (Peak areas)			B-Endosulfan reference (Peak areas)		
	High Conc.	Medium Conc.	Low Conc.	High Conc.	Medium Conc.	Low Conc.
1	145300	26590	1447	128800	28200	1519
2	108700	33910	1326	125200	32800	1413
3	125500	33410	1255	129800	31200	1509
4	127100	30350	1746	115100	32060	1901
5	122800	29830	1775	112600	32190	1692
6	119100	26600	1487	109400	33200	1371
Average	124750	30115	1506	12150	31608	1567.5
% CV	9.6	10.5	14.2	7.4	5.7	12.6

Recovery	High Concentration (159.6 ng/ml)	Medium Concentration (40.7 ng/ml)	Low Concentration (2.3 ng/ml)
	103.8	**95.3**	**96.1**

Table 2.14 Recovery of endosulfan sulfate

Injection No.	Endosulfan-sulfate extract (Peak areas)			Endosulfan-sulfate reference (Peak areas)		
	High Conc.	Medium Conc.	Low Conc.	High Conc.	Medium Conc.	Low Conc.
1	2117000	508500	29130	2278000	553600	32020
2	1757000	641600	27850	2240000	606600	29800
3	1950000	620100	27870	2171000	590300	31660
4	2150000	562000	30070	2111000	588300	32450
5	2140000	524200	34110	2048000	617900	32620
6	2061000	486600	31010	2095000	621200	32770
Average	2029166.67	557166.67	30006.67	2157166.67	596313.67	31886.67
% CV	7.5	11.2	7.9	4.1	4.2	3.5

Recovery	High Concentration (24.1 ng/ml)	Medium Concentration (6.2 ng/ml)	Low Concentration (0.3 ng/ml)
	94.1	**93.4**	**94.1**

β-endosulfan and endosulfan sulfate at high (159.6 ng/ml and 24.1 ng/ml, respectively), medium (40.7 ng/ml and 6.2 ng/ml, respectively) and low (2.8 ng/ml and 0.3 ng/ml, respectively) concentrations were used to determine the recovery of the analytes. Results obtained from the samples peak areas after extraction compared to the reference samples peak areas indicated an extraction efficiency of 103.8%, 95.3% and 96.1% for β-endosulfan and an extraction efficiency of 94.1%, 93.4% and 94.1% for endosulfan sulfate.

2.6 SELECTIVITY ASSESMENT

Using the quality control samples, several assessments were performed to prove the selectivity of the analytical method for the specific analytes. It is important to establish selectivity since the biological matrix is a complex mixture of components with the potential for interfering with the analytical process which may lead to aberrant conclusions regarding the quantification of the analytes in the matrix.

2.6.1 Blank selectivity

Blank serum samples from six different sources were analyzed by the developed method for the determination of β-endosulfan and endosulfan sulfate to prove analytical specificity. Figure 2.14 depicts a chromatogram resulting from this analysis showing that no interfering chromatographic peaks were found at the expected retention times of 3.25 minutes for β-endosulfan and 2.38 minutes for endosulfan sulfate. The detection of either of the analytes in serum samples can therefore not be ascribed to interfering components extracted from the serum.

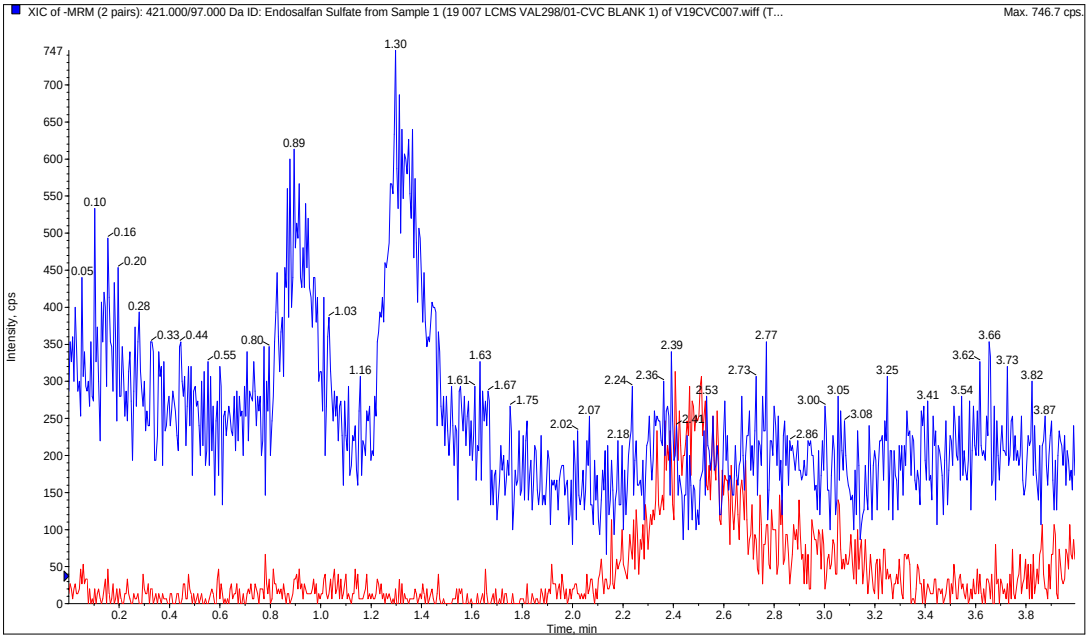


Figure 2.14 A Representative chromatogram of a blank serum extract

2.6.2 Matrix Effects

The effect of matrix components on the ionization of β-endosulfan and endosulfan sulfate was assessed at high and low concentration levels, by comparing the peak areas of two sets of samples.

The first set of samples contained no matrix ions, but consisted of β-endosulfan and endosulfan sulfate in the reconstitution solution (reference samples). For the second set of samples containing matrix ions, six different lots of blank serum samples were extracted. The extracted samples (test samples) were reconstituted with the reconstitution solution containing β-endosulfan and endosulfan sulfate at the same concentrations as that of the reference samples. The reference and test samples were analyzed together.

This assessment was performed at high (160 ng/ml and 24.0 ng/ml) and low (3 ng/ml and 0.3 ng/ml) concentrations of β-endosulfan and endosulfan sulfate, respectively.

As described before, the analyte matrix factor (MF) is determined by dividing the analyte peak areas in presence of matrix (test samples) by the corresponding analyte peak area in the absence of matrix (reference samples). An absolute MF value of 1.00 indicates no matrix effect, while a matrix factor of greater than one indicates ionization enhancement and a matrix factor less than one indicates ionization suppression. The relative matrix effect is expressed as the coefficient of variation (% CV) and is acceptable when it has a value equal or less than 15%.

Tables 2.15 and 2.16 indicate that matrix components had a slight effect, especially at low concentration of both the analytes. The % CV in both cases is however low, indicating the constancy of this effect and making it acceptable.

Table 2.15 Assessment of the influence of matrix components on the analysis of β-endosulfan at high and low concentrations

Matrix Identification	High Concentration Relative to Calibration Curve (160 ng/ml)			Low Concentration Relative to Calibration Curve (3 ng/ml)		
	Analyte Peak Area in Presence of Matrix Ions	Analyte Peak Area in Absence of Matrix Ions	Analyte Matrix Factor	Analyte Peak Area in Presence of Matrix Ions	Analyte Peak Area in Absence of Matrix Ions	Analyte Matrix Factor
Matrix 1	131100	104900	1.08	1200	819.2	1.263
Matrix 2	138700	111400	1.15	1237	761.4	1.30
Matrix 3	130600	119800	1.08	945.2	1029	0.99
Matrix 4	114200	128400	0.94	1200	971	1.26
Matrix 5	127600	131700	1.06	1151	1051	1.21
Matrix 6	135700	129300	1.12	1081	1068	1.14
	Average	120917	1.07	**Average**	950	1.20
	% CV of Matrix Factors		6.6	**% CV of Matrix Factors**		9.5

Table 2.16 Assessment of the influence of matrix components on the analysis of endosulfan sulfate at high and low concentrations

Matrix Identification	High Concentration Relative to Calibration Curve (24.0 ng/ml)			Low Concentration Relative to Calibration Curve (0.3 ng/ml)		
	Analyte Peak Area in Presence of Matrix Ions	Analyte Peak Area in Absence of Matrix Ions	Analyte Matrix Factor	Analyte Peak Area in Presence of Matrix Ions	Analyte Peak Area in Absence of Matrix Ions	Analyte Matrix Factor
Matrix 1	1775000	1426000	1.231	18960	12240	1.419
Matrix 2	1715000	1418000	1.19	17750	12660	1.33
Matrix 3	1583000	1449000	1.10	14740	13690	1.10
Matrix 4	1374000	1445000	0.953	18720	13570	1.401
Matrix 5	1621000	1442000	1.12	16880	13900	1.26
Matrix 6	1781000	1468000	1.24	16440	14100	1.23
	Average	1441333	1.14	**Average**	13360	1.29
	% CV of Matrix Factors		9.4	**% CV of Matrix Factors**		9.1

2.6.3 Effect of haemolysis

Serum prepared from haemolysis blood contains higher concentrations of the ingredients of red blood cells, such as heme, the presence of which may have an effect on the ionization and the

analysis of analytes in such a matrix. To assess such a possibility, haemolyzed serum was artificially prepared by the addition of haemolysed blood to normal serum and comparing the responses of samples prepared in this matrix to the responses obtained from samples prepared in normal serum.

A set of samples containing the analyte at concentrations equal to that of the high and low QCs was prepared in severely haemolysed drug-free serum (about 5% haemolysis). The estimated QC_{low} was 2 - 3 times that of the LLOQ and estimated QC_{high} were about 80% of the ULOQ.

A second set of samples were prepared similarly in normal (not haemolysed) drug-free serum to the same concentration levels. Both sets of samples were analyzed in six-fold at each concentration level. The analyte peaks areas obtained by the analysis of the haemolysed matrix samples were compared with that of the normal matrix samples.

For both β-endosulfan and endosulfan sulfate, the % difference (which should not be exceeding 15%) between the mean peak areas observed for the haemolysed samples and normal samples indicates a negligible effect due to the presence of haemolysis components. The precision, indicated as % CV of the peak areas of both the haemolysed and normal samples, was within 15%, which demonstrates acceptable reproducibility of analysis (see Tables 2.17 and 2.18).

Table 2.17 Assessment of the influence of a matrix containing components from haemolysed blood on the analysis of β-endosulfan at high and low concentrations

Replicate	β-Endosulfan Peak Area			
	High Analyte Concentration		Low Analyte Concentration	
	Haemolysis Matrix (160 ng/ml)	Normal Matrix (160 ng/ml)	Haemolysis Matix (0.3 ng/ml)	Normal Matrix (0.3 ng/ml)
1	197400	177600	3711	3482
2	193300	195600	3045	3272
3	200200	179600	3075	3248
4	199700	195300	3269	3824
5	184200	207100	3408	3329
6	211900	193300	3053	3345
Average	197783	191417	3260	3417
% Diff	3.3	N/A	-4.6	N/A
% CV	4.6	5.8	8.1	6.3

Table 2.18 Assessment of the influence of a matrix containing components from haemolysed blood on the analysis of endosulfan sulfate at high and low concentrations

Replicate	Endosulfan sulfate Peak Area			
	High Analyte Concentration		Low Analyte Concentration	
	Haemolysis Matrix (24.0 ng/ml)	Normal Matrix (24.0 ng/ml)	Haemolysis Matix (0.3 ng/ml)	Normal Matrix (0.3 ng/ml)
1	3264000	2762000	55130	48830
2	3221000	2782000	54890	47590
3	3234000	2766000	51650	47830
4	3186000	2895000	51480	50070
5	3103000	2985000	50570	49780
6	3406000	2859000	49350	47930
Average	3235667	2841500	52178	48672
% Diff	13.9	N/A	7.2	N/A
% CV	3.1	3.1	4.5	2.2

The results meet the required acceptance criteria, indicating that haemolysis had no significant effect on the analysis of β-endosulfan and endosulfan sulfate at high and low concentrations, and hence β-endosulfan and endosulfan sulfate can be accurately quantified in the presence of severe hemolysis.

2.6.4 Effect of Lipemia

Due to physiological conditions, serum prepared from the blood of individual donors/study subjects may contain high levels of lipids. Such samples are judged to be lipemic and because some of these endogenous lipids can also interfere with the analytical method, an assessment of such a possibility is usually performed similar to that performed to assess the effect of haemolysis.

A set of samples at both high and low concentrations of the analytes are therefore prepared in lipemic matrix and analytically compared to a similar set of samples prepared in normal (non lipemic) drug-free matrix at the same concentration levels. These samples were analyzed in six-fold at each concentration level according to the prescribed analysis procedure.

The acceptance criteria, similar to the haemolysis assessment, state that no effect is observed if both the mean % difference and % of coefficient of variation do not exceed 15%. Table 2.19 and 2.20 however, clearly indicates that both parameters are noticeably affected by lipemic serum. This was also noted at both high and low concentration of both analytes.

The results of this assessment therefore indicate that both β-endosulfan and endosulfan sulfate cannot accurately be quantified in lipemic samples, and should be excluded from the analysis and reported as such.

Table 2.19 Assessment of the influence of a matrix containing components from lipemic serum on the analysis of β-endosulfan at high and low concentrations

Replicate	β-endosulfan Peak Area			
	High Analyte Concentration		Low Analyte Concentration	
	Lipemic Matrix (160 ng/ml)	Normal Matrix (160 ng/ml)	Lipemic Matix (3 ng/ml)	Normal Matrix (3 ng/ml)
1	197500	77060	3622	1326
2	139000	86720	1978	1804
3	80710	193500	1563	3547
4	76670	194000	1077	3801
5	74310	208200	1526	3280
6	83630	200000	1384	3143
Average	108637	159913	1858	2817
% Diff	-32.1	N/A	-34.0	N/A
% CV	45.9	38.0	49.1	35.8

Table 2.20 Assessment of the influence of a matrix containing components from lipemic serum on the analysis of endosulfan sulfate at high and low concentrations

Replicate	Endosulfan sulfate Peak Area			
	High Analyte Concentration		Low Analyte Concentration	
	Lipemic Matrix (24.0 ng/ml)	Normal Matrix (24.0 ng/ml)	Lipemic Matix (0.3 ng/ml)	Normal Matrix (0.3 ng/ml)
1	3053000	1927000	50820	33790
2	2551000	2091000	38730	36710
3	2161000	2549000	31480	42590
4	2060000	2800000	30940	47070
5	2011000	2932000	32850	46160
6	2066000	2846000	31790	47670
Average	2317000	2524167	36102	42332
% Diff	-8.2	N/A	-14.7	N/A
% CV	17.7	16.7	21.5	13.8

2.7 VALIDATION PERFORMANCE

The validation of an analytical method is defined as a process by which the accuracy, precision and repeatability of an analytical method is proven. It is performed by analyzing the calibration standards in three batches of which two are done on one day and the third on a consecutive day. For this study, calibration standards (STD's) were analyzed in duplicate ranging from 0.780 ng/ml to 200 ng/ml and 0.117 ng/ml to 30 ng/ml for β-endosulfan and endosulfan sulfate, respectively. Since no internal standards were employed, the instrument response expressed as the integral of the chromatographic peaks, was proportionally equated to the concentration of the analytes in the sample, according to the regression function used.

Quality control samples (QC's) were analyzed for each validation batch by extracting 6 replicates to cover the range of each concentration level (QC H – QC A), to determine the accuracy and precision of the method.

Within-batch accuracy and precision were assessed for each of the three consecutive validation batches that were successfully completed. A Wagner calibration curve weighted by 1 and a Linear $1/concentration^2$ were selected for β-endosulfan and endosulfan sulfate, respectively.

The accuracy of the method was determined by comparing the analytically acquired concentrations of the quality control samples to the nominal (theoretical) concentrations and expressed as a percentage (% nom). The precision of the method was determined by calculating the percentage relative standard deviation (% RSD) determined between the concentrations obtained from quality control samples at the same concentration level.

Each validation batch consisted of:

- Ten System Suitability Samples (SYS)
- Calibration standards (STDs) in duplicate
- Blank samples and zero samples
- Quality control samples (QCs) in six fold

The results of each of the three individual validation batches are displayed in section 2.7.1 to 2.7.4.

2.7.1 Intra-batch accuracy and precision for validation batch 1

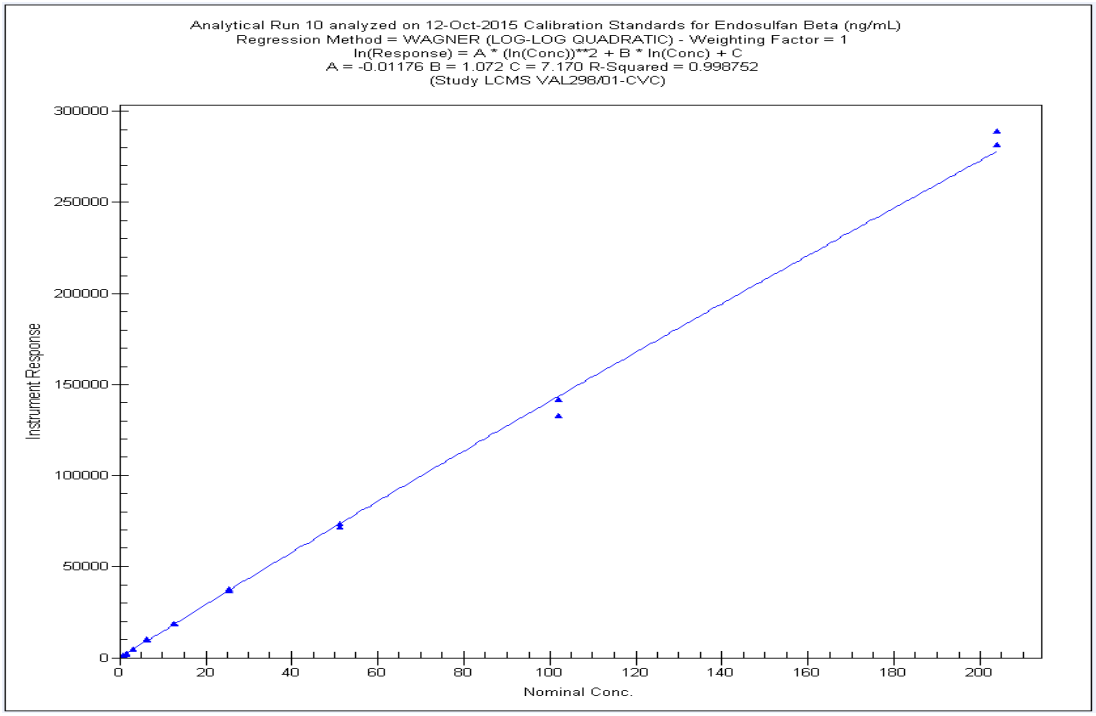


Figure 2.15 Calibration curve constructed by analysis of the STD's in validation batch 1, indicating the β-endosulfan concentration in each

Table 2.21 Back- calculated STD concentrations in ng/ml (Validation 1)

Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J
	0.800	**1.60**	**3.19**	**6.39**	**12.8**	**25.6**	**51.1**	**102**	**204**
1	0.884	1.36	3.26	7.07	13.0	25.3	51.3	101	213
% Bias	10.5	-15	2.2	10.6	1.6	-1.2	0.4	-1.0	4.4
2	0.823	1.46	3.27	6.67	12.8	26.0	50.2	-94.3	207
% Bias	2.9	-8.8	-0.6	4.4	0.0	1.6	-1.8	-7.5	1.5

Table 2.22 Quality control samples summary in ng/ml (Validation 1)

Replicates	QC A	QC B	QC C	QC D	QC E	QC F	QC G	QC H (Dil)
	0.805	**1.95**	**10.1**	**20.2**	**40.4**	**80.8**	**162**	**323**
1	0.716	2.02	9.90	20.5	42.2	85.1	165	304
2	0.800	2.11	10.3	21.6	44.1	84.6	167	346
3	0.785	1.95	10.9	19.7	36.8	77.5	160	324
4	0.671	1.90	10.5	19.5	39.6	87.5	179	334
5	0.824	1.92	9.86	19.6	37.8	84.7	159	287
6	0.818	#1.53	9.18	18.6	38.1	73.5	152	307
Average	0.769	1.98	10.1	19.9	39.8	82.2	164	317
% CV	8.0	4.3	5.9	5.1	7.1	6.6	5.6	6.8
% Bias	-4.5	1.5	0.0	-1.5	-1.5	1.7	1.2	-1.9
N	6	5	6	6	6	6	6	6

Code: # = % Dev > 15%

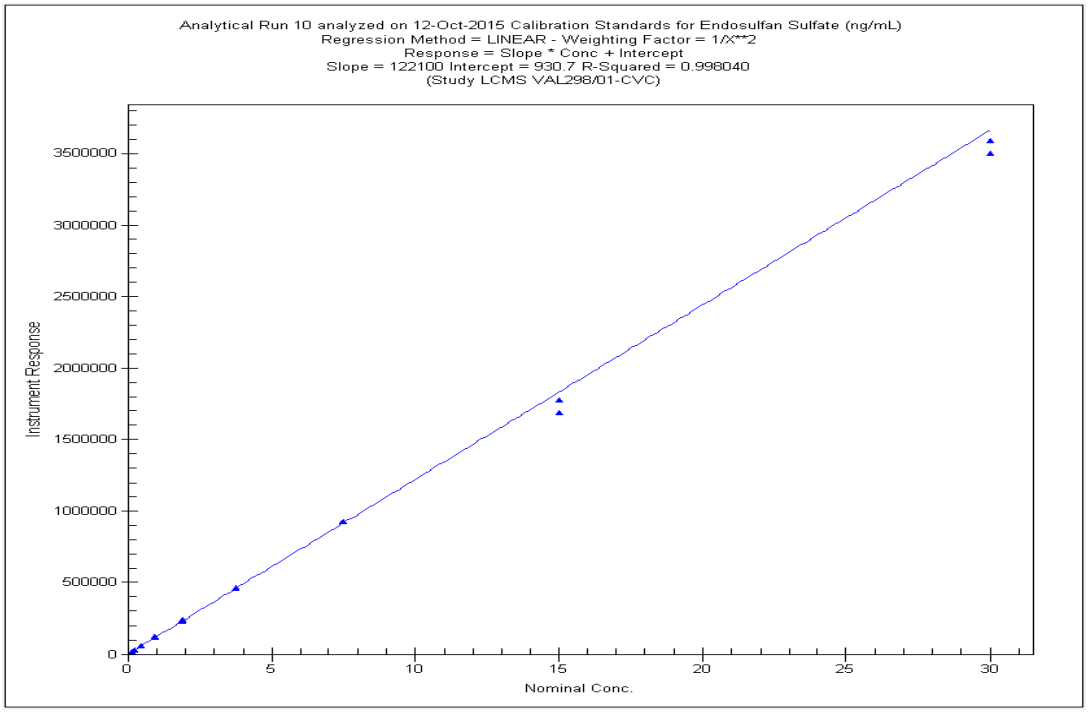


Figure 2.16 Calibration curve constructed by analysis of the STD's in validation batch 1, indicating the endosulfan sulfate concentration in each

Table 2.23 Back- calculated STD concentrations in ng/ml (Validation 1)

Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J
	0.117	**0.234**	**0.468**	**0.936**	**1.87**	**3.74**	**7.49**	**15.0**	**30.0**
1	0.119	0.221	0.457	0.955	1.93	3.71	7.63	14.9	30.0
% Bias	1.7	-5.6	-2.4	2.0	3.2	-0.8	1.9	-0.7	0.0
2	0.119	0.230	0.490	0.942	1.80	3.76	7.60	17.1	30.8
% Bias	1.7	-1.7	4.7	0.6	-3.7	0.5	1.5	-6.0	2.7

Table 2.24 Quality control samples summary in ng/ml (Validation 1)

Replicates	QC A	QC B	QC C	QC D	QC E	QC F	QC G	QC H (Dil)
	0.121	**0.294**	**1.52**	**3.04**	**6.07**	**12.1**	**24.3**	**48.6**
1	0.130	0.311	1.53	3.14	6.50	12.9	24.7	46.8
2	0.130	0.302	1.53	3.12	6.50	12.6	25.4	51.0
3	0.129	0.293	1.63	3.09	5.94	12.6	25.0	50.1
4	0.114	0.306	1.61	3.12	6.26	13.4	26.4	51.8
5	0.119	0.284	1.48	3.00	5.69	12.6	24.7	47.1
6	0.130	0.291	1.47	3.02	6.19	12.1	23.0	47.3
Average	0.125	0.298	1.54	3.08	6.18	12.7	24.9	49.0
% CV	5.6	3.4	4.3	1.9	5.2	3.4	4.5	4.5
% Bias	3.3	1.4	1.3	1.3	1.8	5.0	2.5	0.8
N	6	6	6	6	6	6	6	6

The results obtained from validation batch one met all validation criteria for β-endosulfan and endosulfan sulfate analyzed in serum.

2.7.2 Intra-batch accuracy and precision for validation batch 2

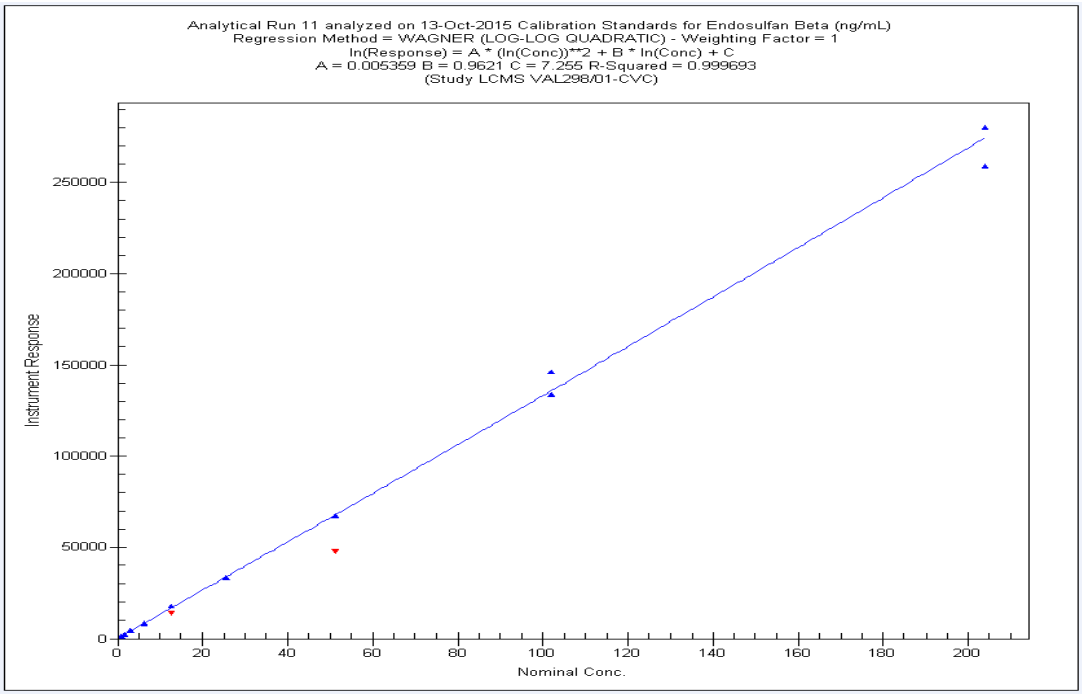


Figure 2.17 Calibration curve constructed by analysis of the STD's in validation batch 2, indicating the β-endosulfan concentration in each

Table 2.25 Back- calculated STD concentrations in ng/ml (Validation 2)

Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J
	0.800	**1.60**	**3.19**	**6.39**	**12.8**	**25.6**	**51.1**	**102**	**204**
1	0.822	1.57	3.30	6.38	*10.3	25.5	*36.2	100	208
% Bias	2.8	-1.9	3.4	-0.2	-19.5	-0.4	-29.2	-2.0	2.0
2	0.803	1.57	3.16	6.07	13.4	25.3	50.9	110	192
% Bias	0.4	-1.9	-0.9	-5.0	4.7	-1.2	-0.4	7.8	-5.9

Code: * = Deactivated calibration standard (% Dev > 15%)

Table 2.26 Quality control samples summary in ng/ml (Validation 2)

Replicates	QC A	QC B	QC C	QC D	QC E	QC F	QC G
	0.805	**1.95**	**10.1**	**20.2**	**40.4**	**80.8**	**162.0**
1	0.663	1.96	10.8	19.8	#32.9	74.0	150
2	0.719	#1.57	10.6	20.5	42.0	78.9	148
3	0.825	1.99	9.94	#15.7	36.9	86.4	159
4	0.770	2.01	11.1	23.2	43.1	83.2	160
5	##0.992	2.09	#11.7	#7.31	39.8	82.2	161
6	##0.609	2.00	10.5	21.3	37.8	76.0	156
Average	0.763	1.93	10.8	20.10	38.8	80.1	156
% CV	17.8	9.5	5.5	13.8	9.6	5.8	3.5
% Bias	-5.2	-1.0	6.9	-0.5	-4.0	-0.9	-2.7
N	6	6	6	5	6	6	6

Code: # = % Dev > 15%
= % Dev 20%

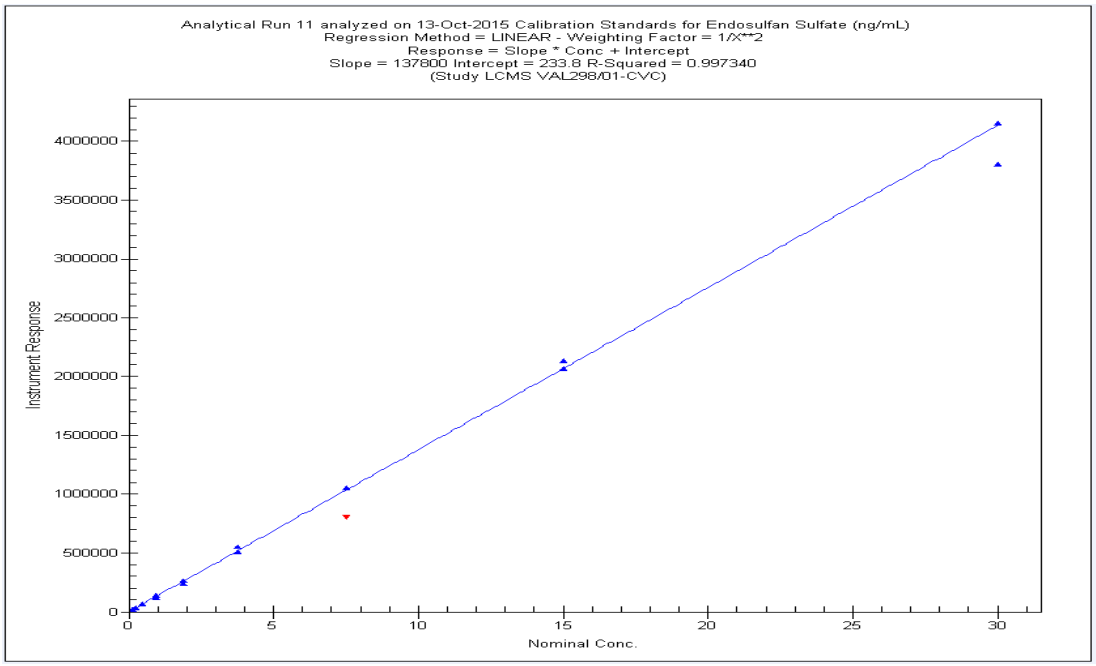


Figure 2.18 Calibration curve constructed by analysis of the STD's in validation batch 2, indicating the endosulfan sulfate concentration in each

Table 2.27 Back- calculated STD concentrations in ng/ml (Validation 2)

Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J
	0.117	**0.234**	**0.468**	**0.936**	**1.87**	**3.74**	**7.49**	**15.0**	**30.0**
1	0.118	0.238	0.490	0.988	1.73	4.00	*5.84	15.0	30.2
% Bias	0.9	1.7	4.7	5.6	-7.5	7.0	-22.0	0.0	0.7
2	0.116	0.231	0.456	0.874	1.92	3.70	7.66	15.5	27.6
% Bias	-0.9	-1.3	-2.6	-6.6	2.7	-1.1	2.3	3.3	-8.0

Code: * = Deactivated calibration standard (% Dev > 15%)

Table 2.28 Quality control samples summary in ng/ml (Validation 2)

Replicates	QC A	QC B	QC C	QC D	QC E	QC F	QC G
	0.121	**0.294**	**1.52**	**3.04**	**6.07**	**12.1**	**24.3**
1	0.126	0.314	1.58	3.07	5.27	11.9	24.5
2	0.121	0.303	1.57	3.18	6.35	12.2	22.9
3	0.129	0.313	1.47	#2.48	5.55	12.6	23.8
4	0.121	0.289	1.60	3.23	6.12	11.8	23.0
5	0.125	0.299	1.53	#1.15	5.83	12.0	23.2
6	0.119	0.287	1.56	3.10	5.76	11.3	22.6
Average	0.124	0.301	1.55	3.01	5.81	12.0	23.3
% CV	3.0	3.8	3.0	10.1	6.7	3.6	3.0
% Bias	2.5	2.4	2.0	-0.9	-4.3	-0.8	-4.1
N	6	5	6	5	6	6	6

Code: # = % Dev > 15%

The results obtained from validation batch two met all the criteria for β-endosulfan and endosulfan sulfate analyzed in serum.

2.7.3 Intra-batch accuracy and precision for validation batch 3

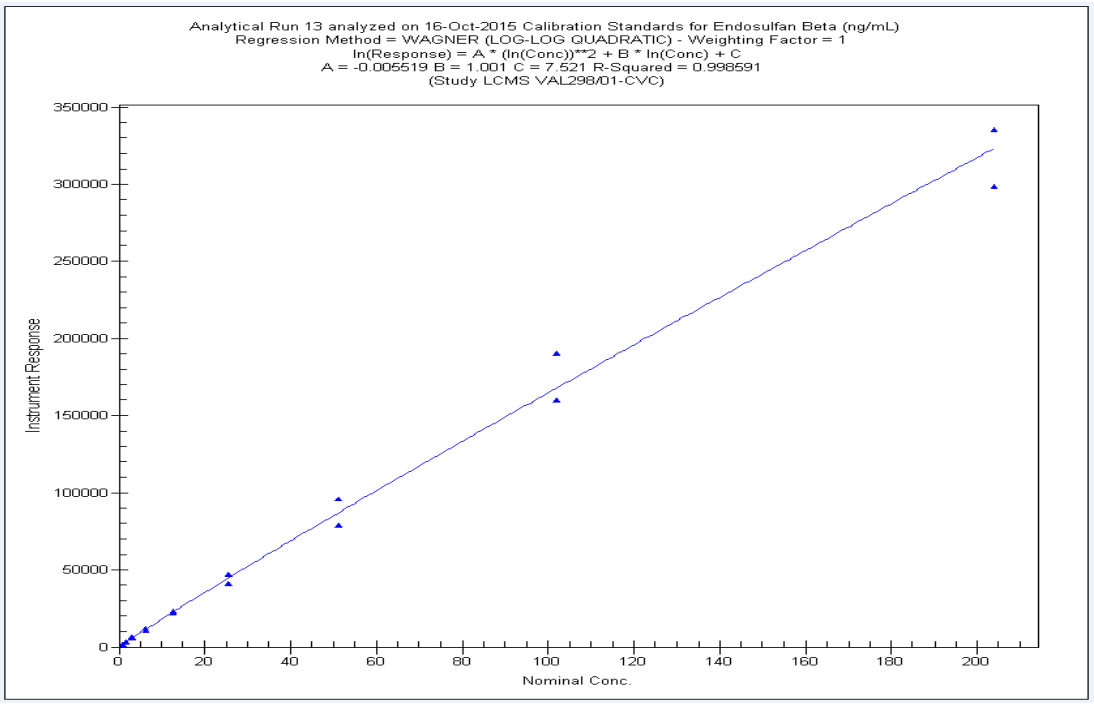


Figure 2.19 Calibration curve constructed by analysis of the STD's in validation batch 3, indicating the β-endosulfan concentration in each

Table 2.29 Back- calculated STD concentrations in ng/ml (Validation 3)

Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J
	0.800	**1.60**	**3.19**	**6.39**	**12.8**	**25.6**	**51.1**	**102**	**204**
1	0.755	1.73	3.19	5.92	12.5	23.4	46.2	97.2	212
% Bias	-5.6	8.1	0.0	-7.4	-2.3	-8.6	-9.6	-4.7	3.9
2	0.825	1.53	3.37	6.57	12.9	27.0	56.7	117	188
% Bias	3.19	-4.4	5.6	2.8	0.8	5.5	11.0	14.7	-7.8

Table 2.30 Quality control samples summary in ng/ml (Validation 3)

Replicates	QC A	QC B	QC C	QC D	QC E	QC F	QC G
	0.805	**1.95**	**10.1**	**20.2**	**40.4**	**80.8**	**162**
1	0.921	2.06	9.89	#16.7	37.0	79.2	164
2	0.737	1.89	9.90	18.9	40.1	86.2	168
3	0.757	2.08	10.9	19.6	40.5	85.0	176
4	0.780	2.21	11.1	19.9	43.4	90.2	185
5	0.886	2.06	11.1	21.9	42.5	#95.6	#187
6	0.951	#2.32	10.8	22.5	40.7	84.4	#188
Average	0.839	2.10	10.6	19.9	40.7	86.8	178
% CV	10.9	7.0	5.4	10.6	5.5	6.4	5.8
% Bias	4.2	7.7	5.0	-1.5	0.7	7.4	9.9
N	6	6	6	6	6	6	6

Code: # = % Dev > 15%

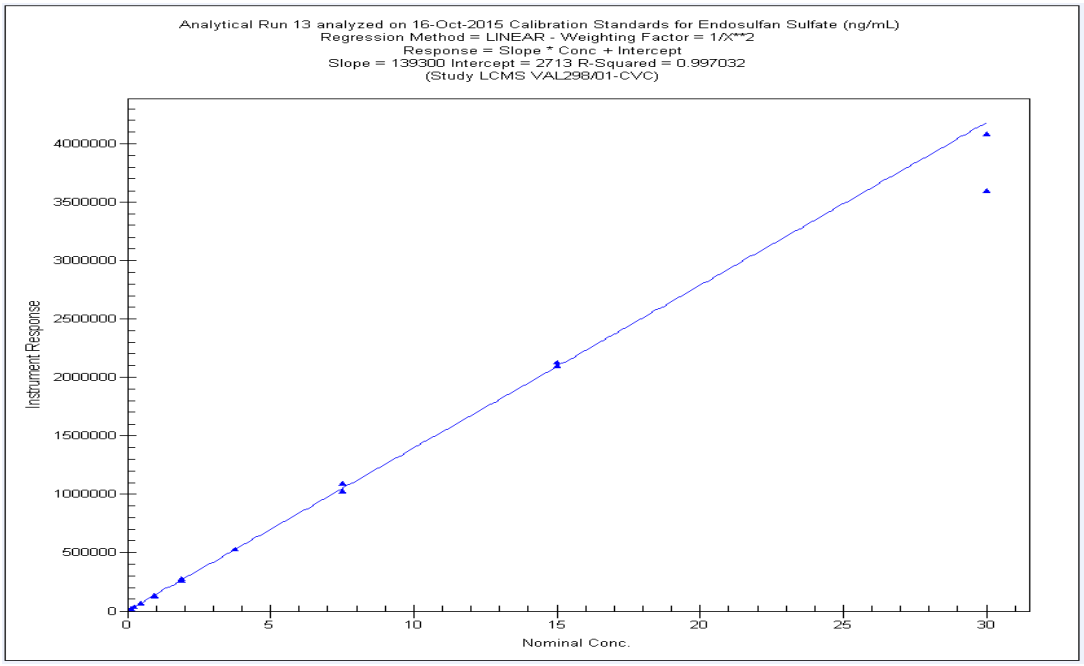


Figure 2.20 Calibration curve constructed by analysis of the STD's in validation batch 3, indicating the endosulfan sulfate concentration in each

Table 2.31 Back- calculated STD concentrations in ng/ml (Validation 3)

Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J
	0.117	**0.234**	**0.468**	**0.936**	**1.87**	**3.74**	**7.49**	**15.0**	**30.0**
1	0.11	0.244	0.490	0.906	1.95	3.76	7.36	15.0	29.3
% Bias	-5.1	4.3	4.7	-3.2	4.3	0.5	-1.7	0.00	-2.3
2	0.119	0.231	0.488	0.959	1.83	3.78	7.82	15.3	25.8
% Bias	1.7	-1.3	4.3	2.5	-2.1	1.1	4.4	2.0	-14.0

Table 2.32 Quality control samples summary in ng/ml (Validation 3)

Replicates	QC A	QC B	QC C	QC D	QC E	QC F	QC G
	0.121	**0.294**	**1.52**	**3.04**	**6.07**	**12.1**	**24.3**
1	0.108	0.313	1.55	2.72	6.10	12.4	23.3
2	0.115	0.307	1.57	3.09	6.23	12.1	23.0
3	0.106	0.317	1.66	3.05	6.39	12.5	25.0
4	0.122	0.306	1.67	3.04	6.40	12.9	25.2
5	##0.096	0.292	1.60	3.23	6.16	13.2	24.5
6	0.132	0.318	1.68	3.17	5.91	12.3	24.2
Average	0.113	0.309	1.62	3.05	6.20	12.6	24.2
% CV	11.2	3.1	3.4	5.8	3.0	3.2	3.7
% Bias	-5.6	5.1	6.6	0.3	2.1	4.1	-0.4
N	6	6	6	6	6	6	6

Code: ## = % Dev > 20%

The results obtained from validation batch three met all the validation criteria for β-endosulfan and endosulfan sulfate analyzed in serum.

2.7.4 Intra-batch accuracy and precision for reinjection batch

To prove the robustness of the method, one of the validation batches was reinjected and the results of the reinjected batch compared to those of the original batch. Validation batch 2 was chosen as the reinjection validation batch and the results are indicated in figure 2.21 and 2.22 and in tables 2.33 to 2.36.

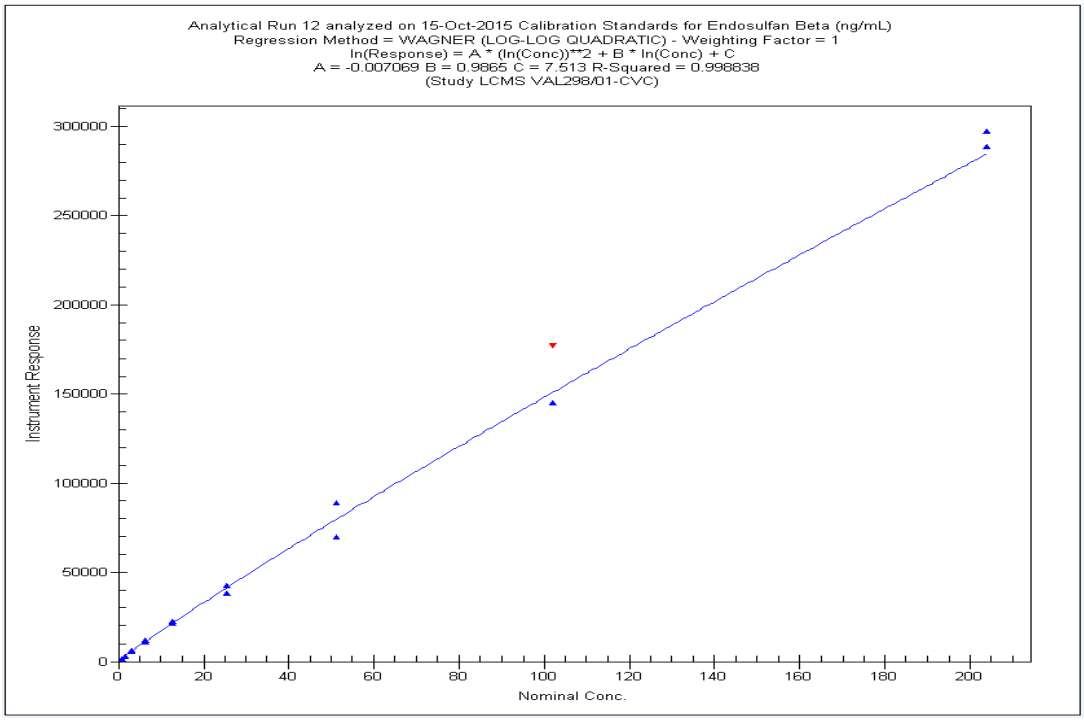


Figure 2.21 Calibration curve constructed by analysis of the STD's during the reinjection of validation batch 2, indicating the β-endosulfan concentration in each

Table 2.33 Back- calculated STD concentrations in ng/ml (Re-injection validation batch 2)

Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J
	0.800	**1.60**	**3.19**	**6.39**	**12.8**	**25.6**	**51.1**	**102**	**204**
1	0.811	1.62	3.22	6.00	13.0	22.7	*43.0	93.7	204
% Bias	1.4	1.3	0.9	-6.1	1.6	-11.3	-15.9	-8.1	0.0
2	0.763	1.62	3.38	6.69	12.4	25.6	55.5	117	197
% Bias	-4.6	1.3	6.0	4.7	-3.1	0.0	8.6	14.7	-3.4

Code: * = Deactivated calibration standard (% Dev > 15%)

Table 2.34 Quality control samples summary in ng/ml (Re-injection validation batch 2)

Replicates	QC A	QC B	QC C	QC D	QC E	QC F	QC G
	0.805	**1.95**	**10.1**	**20.2**	**40.4**	**80.8**	**162.0**
1	0.702	1.92	9.24	#16.8	36.8	89.6	163
2	##0.630	2.02	10.4	20.4	39.3	80.5	184
3	0.721	2.17	10.6	19.8	40.5	86.2	185
4	0.840	2.03	10.5	18.9	42.9	91.4	#193
5	0.680	2.04	10.3	21.5	42.2	92.0	186
6	0.891	2.19	10.7	21.5	40.3	86.4	#194
Average	0.744	2.06	10.3	19.8	40.3	87.7	184
% CV	13.4	4.9	5.2	9.0	5.4	4.9	6.1
% Bias	7.6	-5.6	-2.0	2.0	0.2	-8.5	-13.6
N	6	6	6	6	6	6	6

Code: # = % Dev > 15%
= % Dev 20%

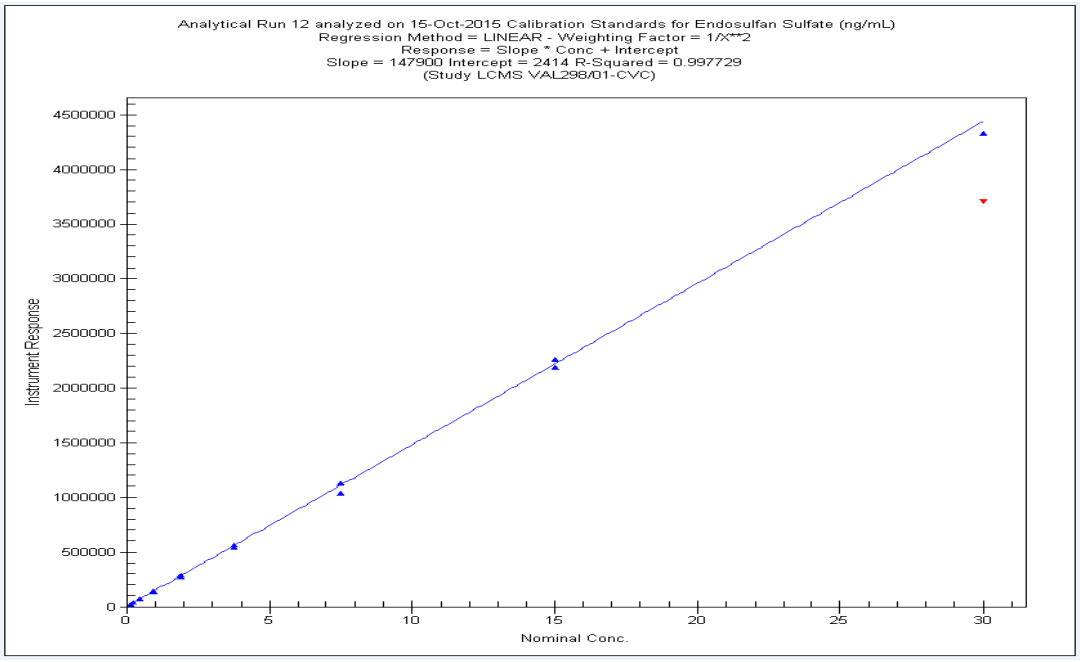


Figure 2.22 Calibration curve constructed by analysis of the STD's during the reinjection of validation batch 2, indicating the endosulfan sulfate concentration in each

Table 2.35 Back- calculated STD concentrations in ng/ml (Re-injection validation batch 2)

Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J
	0.117	**0.234**	**0.468**	**0.936**	**1.87**	**3.74**	**7.49**	**15.0**	**30.0**
1	0.0.106	0.239	0.468	0.921	1.94	3.68	6.99	14.8	29.3
% Bias	-9.4	2.1	0.0	-1.6	3.7	-1.6	-6.7	-1.3	-2.3
2	0.124	0.236	0.493	0.962	1.82	3.76	7.64	15.3	25.1
% Bias	6.0	0.9	5.3	2.8	-2.7	0.5	2.0	2.0	-16.3

Table 2.36 Quality control samples summary in ng/ml (Re-injection validation batch 2)

Replicates	QC A	QC B	QC C	QC D	QC E	QC F	QC G
	0.121	**0.294**	**1.52**	**3.04**	**6.07**	**12.1**	**24.3**
1	0.117	0.326	1.59	2.93	6.06	13.3	23.3
2	0.116	0.301	1.61	3.06	5.63	12.0	23.5
3	0.116	0.304	1.59	3.07	6.02	12.4	24.3
4	0.119	0.303	1.63	2.91	6.17	12.3	24.6
5	0.120	0.298	1.57	3.16	6.15	12.9	24.6
6	0.125	0.307	1.61	3.11	5.79	11.9	23.4
Average	0.119	0.307	1.60	3.04	5.97	12.5	24.0
% CV	2.9	3.3	1.3	3.3	3.6	4.3	2.6
% Bias	-1.7	4.4	5.3	0.0	-1.6	3.3	-1.2
N	6	6	6	6	6	6	6

The results obtained from the reinjection of validation batch 2 compared well with the values obtained for the original batch and therefore met all validation criteria set for this assessment

2.7.4.1 Summary of the intra-batch accuracy and precision

All results were calculated using the WATSON LIMS™ (Laboratory Information Management System) data capturing software interfaces automatically with the Analyst 1.5.2 software used on the instruments. Acquired data files are automatically analyzed and summarised and the data points fitted to the pre-set regression model, calculating the concentrations of the injected samples by comparison to the theoretical concentrations of the calibration standards.

The intra-batch accuracy and precision of each validation batch were evaluated separately by calculation of the regression equation and constructing the calibration curve based on the best results.

The results of the intra-batch validation are summarized in tables with the performance statistics per batch as summarised in table 2.21 to table 2.36. All acceptance criteria for the intra-batch validations were met.

2.7.5 Inter-batch accuracy and precision for all the validation batches

2.7.5.1 Inter-batch accuracy and precision results

The between-batch accuracy and precision were evaluated by calculating the accuracy and precision statistics over all three validation batches. Assessment of the β-endosulfan content was as follows:.

Table 2.37 Parameters of the calibration curves used for the analysis of β-endosulfan over the three validation batches (ln(Resp.) = A * (ln(Conc.))2 + B * ln(Conc.) + C)**

Validation batch	Calibration Curve Parameters (β-endosulfan)			
	A	**B**	**C**	**R-Squared (r^2)**
Validation 1	-0.01176	1.072	7.170	0.998752
Validation 2	0.005359	0.9621	7.255	0.999693
Validation 3	-0.005519	1.001	7.521	0.998591
Average	-0.003973	1.012	7.315	0.999012
% CV	-218.1	5.5	2.3	0.1

Table 2.38 Back-calculated calibration standard concentrations of β-endosulfan in ng/ml for the three validation batches

Validation batch	Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J
		0.800	**1.60**	**3.19**	**6.39**	**12.8**	**25.6**	**51.1**	**102**	**204**
Validation 1	**1**	0.884	1.36	3.26	7.07	13.0	25.3	51.3	101	213
	2	0.823	1.46	3.17	6.67	12.8	26.0	50.2	94.3	207
Validation 2	**1**	0.822	1.57	3.30	6.38	*10.3	25.5	*36.2	100	208
	2	0.803	1.57	3.16	6.07	13.4	25.3	50.9	110	192
Validation 3	**1**	0.755	1.73	3.19	5.92	12.5	23.4	46.2	97.2	212
	2	0.825	1.53	3.37	6.57	12.9	27.0	56.7	117	188
Average		0.819	1.54	3.24	6.45	12.9	25.4	51.1	103	203
% CV		5.1	8.1	2.6	6.5	2.5	4.6	7.3	8.3	5.3
% Bias		2.4	-3.8	1.6	0.9	0.8	-0.8	0.0	1.0	-0.5
N		6	6	6	6	5	6	5	6	6

Code: * = Deactivated calibration standard (% Dev > 15%)

Table 2.39 Statistics for the β-endosulfan contents of quality control samples for the three validation batches (concentration is in ng/ml)

Validation batch	Replicates	QC A	QC B	QC C	QC D	QC E	QC F	QC G
		0.805	**1.95**	**10.1**	**20.2**	**40.4**	**80.8**	**162**
Validation 1	**1**	0.716	2.02	9.90	20.5	42.2	85.1	165
	2	0.800	2.11	10.3	21.6	44.1	84.6	167
	3	0.785	1.95	10.9	19.7	36.8	77.5	160
	4	0.671	1.90	10.5	19.5	39.6	87.5	179
	5	0.824	1.92	9.86	19.6	37.8	84.7	159
	6	0.818	#1.53	9.18	18.6	38.1	73.5	152
Validation 2	**1**	0.663	1.96	10.8	19.8	#32.9	74.0	150
	2	0.719	#1.57	10.6	20.5	42.0	78.9	148
	3	0.825	1.96	9.94	#15.7	36.9	86.4	159
	4	0.770	2.01	11.1	23.2	43.1	83.2	160
	5	##0.992	2.09	#11.7	[7.31]	39.8	82.2	161
	6	##0.609	2.00	10.5	21.3	37.8	76.0	159
Validation 3	**1**	0.921	2.06	9.89	#16.7	37.0	79.2	164
	2	0.737	1.89	9.90	18.9	40.1	86.2	168
	3	0.757	2.08	10.9	19.6	40.5	85.0	176
	4	0.780	2.21	11.1	19.9	43.4	90.2	185
	5	0.886	2.06	11.1	21.9	42.5	#95.6	#187
	6	0.951	#2.32	10.8	22.5	40.7	84.4	#188
Average		0.790	1.98	10.5	19.97	39.7	83.0	166
% CV		12.8	9.6	5.9	9.5	7.4	6.9	7.5
% Bias		-1.9	1.5	4.0	-1.1	-1.7	2.7	2.5
N		18	18	18	17	18	18	18

Code: # = % Dev > 15%
= % Dev 20%
[ ] = Outlier not included in statistics (MNR outlier test)

The inter-batch accuracy and precision for endosulfan sulfate over all three validation batches were as follows:

Table 2.40 Parameters of the calibration curves used for the analysis of endosulfan sulfate over the three validation batches (Resp. = Slope * Conc. + Intercept)

Validation batch	Calibration Curve Parameters (Sulfate)		
	Slope	**Intercept**	**R-Squared (r^2)**
Validation 1	122100	930.7	0.998040
Validation 2	137800	233.8	0.997340
Validation 3	139300	2713	0.997032
Average	133100	1293	0.997471
% CV	7.2	98.9	0.1

Table 2.41 Back-calculated calibration standard concentrations of endosulfan sulfate in ng/ml for the three validation batches

Validation batch	Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J
		0.117	**0.234**	**0.468**	**0.936**	**1.87**	**3.74**	**7.49**	**15.0**	**30.0**
Validation 1	**1**	0.117	0.223	0.468	0.980	1.97	3.75	7.59	14.6	28.7
	2	0.117	0.233	0.502	0.966	1.84	3.80	7.56	13.8	29.4
Validation 2	**1**	0.118	0.238	0.490	0.988	1.73	4.00	*5.84	15.0	30.2
	2	0.116	0.231	0.456	0.874	1.92	3.70	7.66	15.5	27.6
Validation 3	**1**	0.111	0.244	0.490	0.906	1.95	3.76	7.36	15.0	29.3
	2	0.19	0.231	0.488	0.959	1.83	3.78	7.82	15.3	25.8
Average		0.116	0.233	0.482	0.946	1.87	3.80	7.60	14.9	28.4
% CV		2.4	3.1	3.5	4.8	4.8	2.7	2.2	4.1	5.5
% Bias		-0.9	-0.4	3.0	1.1	0.0	1.6	1.5	-0.7	-5.0
N		6	6	6	6	6	6	5	6	6

Code: * = Deactivated calibration standard (% Dev > 15%)

Table 2.42 Statistics for the endosulfan sulfate contents of quality control samples for the three validation batches (concentration is in ng/ml)

Validation batch	Replicates	QC A	QC B	QC C	QC D	QC E	QC F	QC G
		0.121	**0.294**	**1.52**	**3.04**	**6.07**	**12.1**	**24.3**
Validation 1	**1**	0.128	0.316	1.57	3.18	6.49	12.6	23.7
	2	0.129	0.307	1.57	3.16	6.48	12.4	24.3
	3	0.128	0.299	1.66	3.13	5.94	12.3	24.0
	4	0.111	0.311	1.65	3.16	6.25	13.1	25.3
	5	0.117	0.289	1.51	3.04	5.70	12.4	23.8
	6	0.129	0.296	1.50	3.06	6.19	11.9	22.2
Validation 2	**1**	0.126	0.314	1.58	3.07	5.27	11.9	24.5
	2	0.121	0.303	1.57	3.18	6.35	12.2	22.9
	3	0.129	0.313	1.47	#2.48	5.55	12.6	23.8
	4	0.121	0.289	1.60	3.23	6.12	11.8	23.0
	5	0.125	0.299	1.53	#[1.15]	5.83	12.0	23.2
	6	0.199	0.287	1.56	3.10	5.76	11.3	22.6
Validation 3	**1**	0.108	0.313	1.55	2.72	6.10	12.4	23.3
	2	0.115	0.307	1.57	3.09	6.23	12.1	23.0
	3	0.106	0.317	1.66	3.05	6.39	12.5	25.0
	4	0.122	0.306	1.67	3.04	6.40	12.9	25.2
	5	##0.0960	0.292	1.60	3.23	6.16	13.2	24.5
	6	0.132	0.318	1.68	3.17	5.91	12.3	24.2
Average		0.12	0.30	1.58	3.01	6.06	12.30	23.80
% CV		8.1	3.4	3.9	6.2	5.6	3.8	5.2
% Bias		-0.8	3.4	3.9	0.8	-0.2	1.7	-2.1
N		18	18	18	17	18	18	18

Code: # = % Dev > 15%
= % Dev > 20%
[] = Outlier not included in statistics (MNR-ESD outlier test)

The overall % CV of the quality control samples of endosulfan sulfate was 6.2 as indicated in the appendix C3 with one QC D as an outlier in the second validation batch.

2.7.5.2 Summary of the inter-batch accuracy and precision

The evaluation of the inter-batch accuracy and precision was assessed by the overall performance of the quality control in all three validation batches. The statistical outlier for endosulfan sulfate in validation batch 2 (injection 5; see Table 2.42) influenced the analytical performance of quality control standard levels. After the exclusion of this statistical outlier, the overall statistics for the combined validation batches were again evaluated and found to be within the acceptance criteria.

The decision that this one value can be interpreted as a statistical outlier is based on the MNR-ESD outlier test being applied to QC level D and the results of this are included in the appendix section of this dissertation.

The % CV (precision) of the quality control samples of β-endosulfan ranged from 5.9 % to 12.8 % and endosulfan sulfate ranged from 3.4 % to 8.1 %, respectively with one QC D as an outlier in the second validation batch.

The % Bias (accuracy) of the quality control samples of β-endosulfan ranged from -1.9 % to 4.0 % and endosulfan sulfate ranged from -2.1 % to 3.9 %, respectively with one QC D as an outlier in the second validation batch

The results confirmed that the % CV values for the intra- and inter-batch accuracy and precision were less than 15% and less than 20% at the LLOQ making this a valid method.

2.7.6 Dilution integrity

The dilution integrity was assessed during the first validation batch by using the prepared quality control sample (QC H) that was above the upper limit of quantification (ULOQ).

QC H was diluted (1:1) with normal blank serum and analyzed in order to validate the dilution of unknown sample concentrations that does not fall within the validated range of the analysis. Evaluated results confirmed that this dilution may be applied to β-endosulfan and endosulfan sulfate samples with concentration levels up to 323.0 ng/ml and 48.0 ng/ml, respectively.

2.7.7 Lower limit of quantification (LLOQ)

The lower limit of quantification was assessed with accuracy and precision of below 20%, and the produced signal to noise ratio was indicated to be 8.9 and 22.6 for β-endosulfan and endosulfan sulfate, respectively. See figures 2.23 and 2.24.

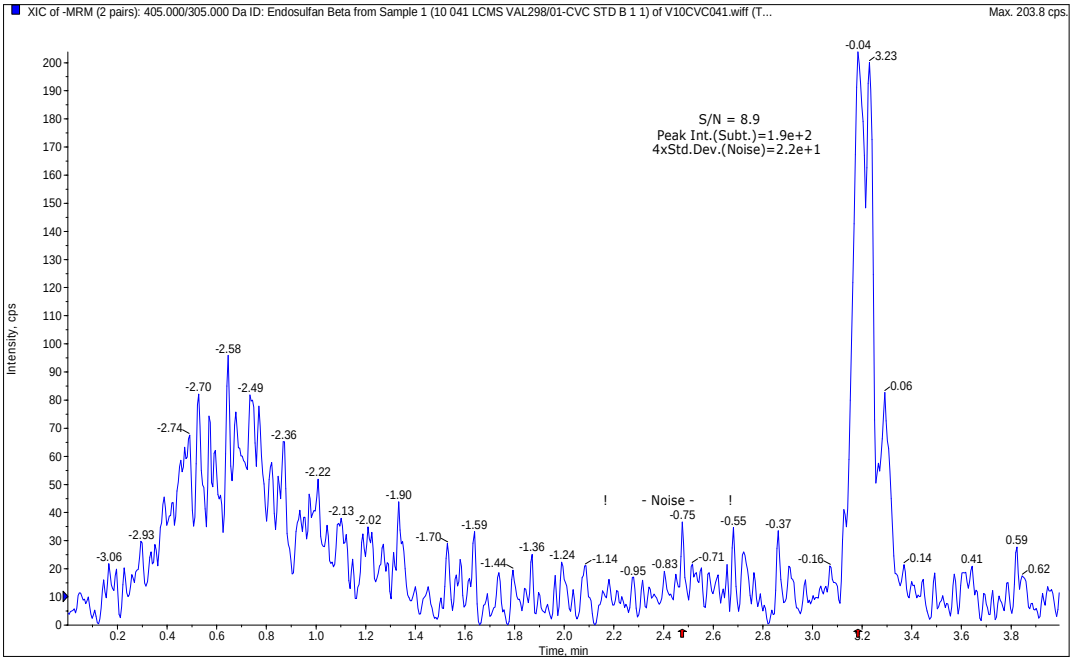


Figure 2.23 A representative chromatogram of β-endosulfan extracted from STD B (LLOQ). S/N ratio is 8.9

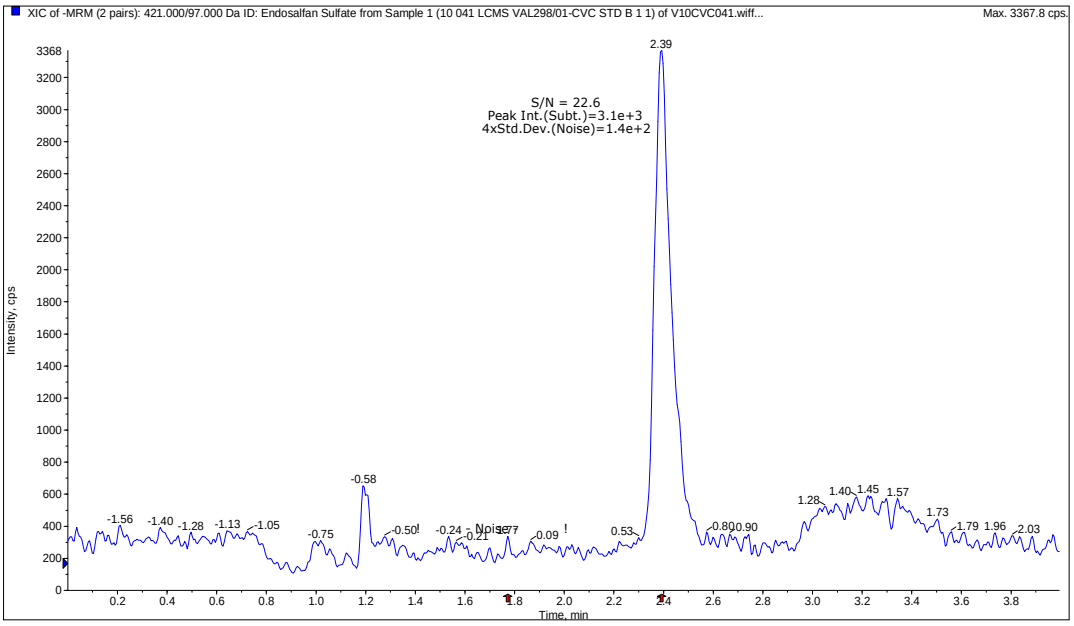


Figure 2.24 A representative chromatogram of endosulfan sulfate extracted from STD B (LLOQ). S/N ratio is 22.5

2.8 STABILITY ASSESMENTS

2.8.1 Short term stability (On bench)

Short-term matrix stability of β-endosulfan and endosulfan sulfate was determined in serum at high and low concentration levels. Serum samples were spiked with β-endosulfan and endosulfan sulfate at high concentration (162 ng/ml and 22.9 ng/ml, respectively), and low concentration (1.95 ng/ml and 0.308 ng/ml, respectively) and stored frozen at approximately -20°C until used.

Six aliquots of the stability samples at each concentration level were thawed and allowed to stand at room temperature for approximately 8 hours. The stability samples were analyzed and the concentrations were determined using a calibration curve derived from freshly prepared calibration standards.

The means of the measured concentrations were compared with the nominal concentrations and indicated that the % Bias and the % CV are within 15%, resulting in a reliable stability assessment (see Table 2.43).

Table 2.43 Results of the short term stability assessment of β-endosulfan and endosulfan sulfate in human serum (concentration is in ng/ml)

Replicate	Short term stability			
	β-Endosulfan		Endosulfan sulfate	
	High Concentration	Low Concentration	High Concentration	Low Concentration
Nominal	**162**	**1.95**	**22.9**	**0.308**
1	164	2.02	23.1	0.296
2	164	2.09	23	0.341
3	158	2.35	22	0.311
4	155	2.04	23.4	0.327
5	156	2.43	23.3	0.316
6	160	2.33	21.8	0.312
Average	159.5	2.21	22.77	0.32
% Diff	-1.5	13.3	-0.6	3.0
% CV	2.4	8.1	3.0	4.8

Results obtained from short-term stability met the required acceptance criteria for the compounds left in the biological matrix for 8 hours at room temperature.

2.8.2 Freeze-thaw stability

Freeze-thaw stability of β-endosulfan and endosulfan sulfate was determined in serum at high and low concentration levels, over three freeze-thaw cycles. The concentration levels were the same as that used for short term stability assessment.

Six aliquots of the stability samples that have been frozen for 24 hours were thawed unassisted at room temperature. When completely thawed, they were refrozen for at least 12 hours.

After a third freeze-thaw cycle, the samples were analyzed and the concentrations were determined using a calibration curve derived from freshly prepared calibration standards.

As indicated in Table 2.44, the means of the measured concentrations were compared with the nominal concentration that indicated that the % Bias and the % CV are within 15 %, resulting in a reliable stability assessment.

Table 2.44 Results of the freeze-thaw stability assessment of β-endosulfan and endosulfan sulfate in human serum (concentration is in ng/ml)

Replicate	Freeze thaw stability			
	β-Endosulfan		Endosulfan sulfate	
	High Concentration	Low Concentration	High Concentration	Low Concentration
Nominal	**162**	**1.95**	**22.9**	**0.308**
1	131	1.75	19.2	0.296
2	161	2.05	22.7	0.317
3	154	2.35	21.9	0.319
4	154	1.87	23.4	0.326
5	166	2.43	23.5	0.334
6	153	2.41	21.8	0.301
Average	153.17	2.14	22.08	0.32
% Diff	-5.5	9.9	-3.6	2.4
% CV	7.8	13.8	7.2	4.6

Results obtained from freeze thaw stability met the required acceptance criteria for the compounds over three freeze thaw cycles.

2.8.3 On-Instrument stability

On-instrument stability of β-endosulfan and endosulfan sulfate was assessed to demonstrate that the compounds are stable in the extract (after sample preparation) when stored on the instrument at approximately +5°C.

For the assessment of on-instrument stability, six individual aliquots of the stability samples at high and low concentration levels of the two compounds, as used for the stability assessments above, were extracted and the extracts were pooled. The pooled extracts were placed on the auto sampler at 5°C and analysed after a period of 24 hours has elapsed, against a freshly prepared set of calibration standards.

As indicated in Table 2.45, the % Bias and the % CV were within 15%, indicating reliable stability of β-endosulfan and endosulfan sulfate left as extracts on instrument.

Table 2.45 Results of the on instrument stability assessment of β-endosulfan and endosulfan sulfate in human serum (concentration is in ng/ml)

Replicate	On-Instrument stability			
	β-Endosulfan		Endosulfan sulfate	
	High Concentration	Low Concentration	High Concentration	Low Concentration
Nominal	**162**	**1.95**	**24.3**	**0.294**
1	206	2.42	29.5	0.362
2	185	2.29	28	0.342
3	179	2.09	27.6	0.339
4	168	2.31	26.2	0.304
5	161	2.15	26.4	0.31
6	176	2.13	25.3	0.325
Average	179.17	2.23	27.17	0.33
% Diff	10.6	14.4	11.8	12.4
% CV	8.7	5.7	5.5	6.7

2.8.4 Stability of stock solutions

Separate stock solutions of β-endosulfan and endosulfan sulfate were prepared in methanol at concentrations of 100 µg/ml of β-endosulfan and 22 µg/ml of endosulfan sulfate. Aliquots of each solution were kept at room temperature for at least 8 hours and at approximately 5ºC and at ~ -20ºC, for up to 12 days. Just prior to analysis, the solutions were spiked into the reconstitution solutions to achieve concentrations of 200 ng/ml for β-endosulfan and 30.0 ng/ml endosulfan sulfate. These solutions were then chromatographically analyzed together with freshly prepared reference stock solutions of the compounds at the same concentrations and following the same dilutions.

The peak areas of the stored stock solutions were compared to that of the freshly prepared stock solutions and the % difference between the two preparations was found to be within 15%. The

precision, expressed as % CV, was also within 15% (see Tables 2.46 and 2.47 for β-endosulfan and endosulfan sulfate respectively).

Table 2.46 Stability assessment of β-endosulfan as a working solution in methanol, at 200 ng/ml, kept at the temperature conditions indicated

Replicate	Working Solution Peak Area (β-Endosulfan)			
	Room Temperature	~ 5°C	~ -20°C	Fresh (Reference)
1	139000	139400	143800	148900
2	141000	139700	147200	148000
3	140500	140800	146700	151000
4	140000	140900	144900	150200
5	138100	138600	146800	147900
6	140000	144000	147600	149000
Average	139767	140567	146167	149167
% of Reference	93.7	94.2	98.0	N/A
% CV	0.8	1.3	1.0	0.8

Table 2.47 Stability assessment of endosulfan sulfate as a working solution in methanol, at 30 ng/ml, kept at the temperature conditions indicated

	Working Solution Peak Area (Endosulfan sulfate)			
Replicate	Room Temperature	~ 5 °C	~ -20 °C	Fresh (Reference)
1	4718000	4623000	5041000	4771000
2	4700000	4659000	5004000	4831000
3	4785000	4654000	4995000	4791000
4	4815000	4547000	5015000	4758000
5	4731000	4664000	4964000	4749000
6	4688000	4657000	4996000	4777000
Average	4739500	4634000	5002500	4779500
% of Reference	99.2	97.0	104.7	N/A
% CV	1.1	1.0	0.5	0.6

The results indicate that β-endosulfan and endosulfan sulfate are stable in methanol when stored at room temperature, at approximately 5ºC and at approximately - 20ºC for 12 days.

2.8.5 Long term stability

The storage period for long-term stability evaluation should exceed the time between the date of first sample collection during the clinical trial and the date of last sample analysis. Long-term stability was not determined during this study for the period that the study samples was stored due to the fact that sample collection during the clinical trial was performed at least a year before the start of the study and there was not enough time available to store QCs for the required time to assess stability covering the full storage period.

A long-term stability assessment was however performed for the period of 1 month, covering the period from the first day of study sample analysis to the last day of study sample analysis, using quality control samples (QC) that were stored from the date of preparation during method development until completion of the analysis of the last study sample.

Quality control samples containing β-endosulfan and endosulfan sulfate at high concentration (162 ng/ml and 22.9 ng/ml, respectively) and low concentration (1.95 ng/ml and 0.308 ng/ml, respectively) were stored frozen at ~ -20°C for a period of 30 days. The samples were analyzed against a calibration curve consisting of freshly prepared STDs and the measured concentrations were compared to the nominal concentrations of the samples. The results obtained met the required acceptance criteria for both compounds, proving stability in the matrix for at least 30 days (see Table 2.48).

Table 2.48 Long term stability assessment of β-endosulfan and endosulfan sulfate in human serum stored at approximately -20°C for one month (concentration is in ng/ml)

Replicate	Long term stability			
	β-Endosulfan		Endosulfan sulfate	
	High Concentration	Low Concentration	High Concentration	Low Concentration
Nominal	**162**	**1.95**	**22.9**	**0.308**
1	144	2.22	22.6	0.311
2	147	2.13	23.3	0.29
3	151	2.15	23.7	0.297
4	156	1.53	24.2	0.3
5	142	1.99	24	0.296
6	144	1.62	21.6	0.307
Average	147.33	1.92	23.23	0.300
% Diff	-9.1	-1.7	1.5	-2.5
% CV	3.6	14.6	4.2	2.6

2.9 SAMPLE ANALYSIS RESULTS

2.9.1 β-Endosulfan and endosulfan sulfate

A total of 219 serum samples which were collected during the clinical trial, were received for analysis. The samples were divided into three equal quantities and analyzed in three separate batches, referred to as production batches, to confirm the presence of β-endosulfan or endosulfan sulfate. The samples were collected from agricultural farm workers and other individuals reported to have been exposed to endosulfan during treatment of fruit trees with the pesticide.

The production batches each consisted of STDs that were extracted in duplicate, ranging from 0.800 ng/ml to 200 ng/ml and 0.117 ng/ml to 30 ng/ml for β-endosulfan and endosulfan sulfate, respectively. The sequence of the production batches was designed so that the STDs were placed in decreasing order of concentration, starting from the highest STD (ULOQ) to the lowest standard (LLOQ). The first replicate of the STDs was positioned in the first part of the batch and the second replicate at the end of the batch.

Two replicates of the QCs at each concentration level (QC G – QC A), covering the entire calibration range, were included in each production batch in order to determine the accuracy and precision of the method. The QCs were distributed throughout each production batch in groups of decreasing concentration order after a STD.

A Wagner calibration curve and a linear curve, weighted by 1/concentration^2 were selected for β-endosulfan and endosulfan sulfate, respectively.

The production batch acceptance criteria are described in section 1.5 and were determined by using the calculated concentration of the STDs and QC samples.

Each production batch contained ten system suitability samples (SYS) to evaluate the system during the run. No upwards or downward trends in the responses of the SYS samples were noticed in any of the production runs.

The performance of the STDs and QCs over the three production runs are displayed in tables 2.49 to 2.54.

Table 2.49 Back-calculated calibration standard concentrations of β-endosulfan in ng/ml for the three production batches

Production batch	Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J
		0.799	**1.60**	**3.20**	**6.39**	**12.8**	**25.6**	**51.1**	**102**	**204**
Production 1	**1**	0.748	*3.64	3.51	6.76	13.3	26.3	*38.1	104	194
	2	0.901	1.4	3.05	*3.78	*16.3	25.2	44	108	215
Production 2	**1**	0.858	1.42	*2.24	6.81	13.4	*31.7	56.9	112	223
	2	0.824	1.6	3.15	5.74	12.1	28.7	44.4	99.6	175
Production 3	**1**	0.794	1.48	3.09	6.72	13.1	26.8	45.1	103	197
	2	0.804	1.41	3.17	6.15	12.4	25.4	47.6	105	204
Average		0.821	1.46	3.19	6.44	12.9	26.5	47.6	105	201
% CV		6.5	5.7	5.7	7.3	4.5	5.3	11.3	4.1	8.4
% Bias		2.8	-8.6	-0.1	0.7	0.4	3.4	-6.8	3.2	-1.3
N		6	5	5	5	5	5	5	6	6

Code: * = Deactivated calibration standard (% Dev > 15%)

Table 2.50 Statistics for the β-endosulfan contents of quality control samples for the three production batches (concentration is in ng/ml)

Production batch	Replicates	QC A	QC B	QC C	QC D	QC E	QC F
		1.95	**10.1**	**20.2**	**40.4**	**80.8**	**162**
Production 1	**1**	##2.67	10.9	#23.9	43.3	83	159
	2	2.1	#8.30	18.8	34.7	76.9	#212
Production 2	**1**	2.15	11	#23.9	#51.1	90.8	184
	2	2.06	#8.29	23.2	41.2	82.2	165
Production 3	**1**	2.08	10.4	19.6	38.7	79.8	164
	2	2.04	10.9	20.3	41.2	81.6	166
Average		2.08	10.8	20.47	39.82	82.38	167.6
% CV		2.0	2.5	9.4	8.3	5.7	5.7
% Bias		7.0	6.9	1.4	-1.4	2.0	3.5
N		6	6	6	6	6	6

Code: # = % Dev > 15%
= % Dev > 20%

Table 2.51 Back-calculated calibration standard concentrations of endosulfan sulfate in ng/ml for the three production batches

Production batch	Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I	STD J
		0.117	**0.234**	**0.468**	**0.937**	**1.87**	**3.75**	**7.49**	**15.0**	**30.0**
Production 1	**1**	0.111	0.239	0.507	1.03	2.01	3.98	7.14	14.6	26.5
	2	0.115	0.249	0.446	0.889	1.98	4.02	7.62	13.6	26.8
Production 2	**1**	0.125	0.26	0.46	0.978	2.12	4.11	7.68	14.9	28.7
	2	0.108	0.215	0.461	0.854	1.93	3.78	6.93	13.4	*22.0
Production 3	**1**	0.11	0.258	0.485	0.933	1.99	3.94	8.05	15.1	28.1
	2	*0.0650	*0.190	*0.338	0.817	2.09	3.68	7.06	*12.1	26.2
Average		0.114	0.244	0.472	0.917	2.02	3.92	7.41	14.3	27.3
% CV		5.9	7.5	5.1	8.7	3.5	4.1	5.9	5.4	4
% Bias		-2.6	4.3	0.9	-2.1	8	4.5	-1.1	-4.7	-9
N		5	5	5	6	6	6	6	5	5

Code: * = Deactivated calibration standard (% Dev > 15%)

Table 2.52 Statistics for the endosulfan sulfate contents of quality control samples for the three production batches (concentration is in ng/ml)

Production batch	Replicates	QC A 0.294	QC B 1.52	QC C 3.03	QC D 6.07	QC E 12.1	QC F 24.3
Production 1	**1**	0.33	#1.76	3.45	6.43	12.1	23
	2	0.326	1.64	3.12	6.43	13.2	26.3
Production 2	**1**	0.318	1.49	3.23	6.72	12.1	26.4
	2	0.25	#1.07	3.12	5.63	10.8	21.6
Production 3	**1**	0.306	1.56	2.71	6.37	13.3	23.8
	2	##0.219	#1.21	3.18	6.5	12.4	21.6
Average		0.292	1.45	3.14	6.35	12.3	23.8
% CV		15.8	17.9	7.7	5.9	7.4	9.1
% Bias		-0.7	-4.6	3.6	4.6	1.7	-2.1
N		6	6	6	6	6	6

Code: # = % Dev > 15%
= % Dev > 20%

Table 2.53 Summary of calibration curve parameters over the three production batches for β-endosulfan (ln(Resp.) = A * (ln(Conc.))2 + B * ln(Conc.) + C))**

Production batch	Calibration Curve Parameters (β-endosulfan)			
	A	B	C	R-Squared (r^2)
Production 1	-0.003410	1.237	6.634	0.998752
Production 2	0.001017	1.016	6.634	0.999693
Production 3	-0.003550	1.110	6.730	0.998591
Average	-0.00198	1.121	6.666	0.999012
% CV	-131.1	9.9	0.8	0.1

Table 2.54 Summary of calibration curve parameters over the three production batches for endosulfan sulfate (Resp. = Slope * Conc. + Intercept)

Production batch	Calibration Curve Parameters (Sulfate)		
	Slope	Intercept	R-Squared (r^2)
Production 1	169100	1604	0.992944
Production 2	88790	31.25	0.992206
Production 3	111200	-628.9	0.992104
Mean	123030	335.45	0.992418
% CV	33.68	341.96	0.04

Evaluation of the % CV of the QC B's of endosulfan sulfate across the three production batches resulted in a value of 17.9%, which is outside of the accepted criteria. This was however accepted due to the fact that these QCs passed within each batch and the similar values for the validation batches were well within the acceptable range.

Notwithstanding this good performance as judged by the acceptance criteria, the presence of neither β-endosulfan nor endosulfan sulfate could be indicated in any of the subject samples. Due to the fact that each sample only contained about 200 µl of serum, no re-analysis of any of the samples could be performed. Results obtained from each of the three individual production batches are displayed in table 2.55 to 2.57.

Table 2.55 Results summary of production batch 1 for β–endosulfan and endosulfan sulfate

Production batch 1			Production batch 1		
Sample Name	β-Endosulfan	Endosulfan sulfate	Sample Name	β-Endosulfan	Endosulfan sulfate
	Calc. Conc. (ng/ml)	Calc. Conc. (ng/ml)		Calc. Conc. (ng/ml)	Calc. Conc. (ng/ml)
Subject 1	BLQ	BLQ	Subject 54	BLQ	BLQ
Subject 2	BLQ	BLQ	Subject 55	BLQ	BLQ
Subject 3	BLQ	BLQ	Subject 56	BLQ	BLQ
Subject 4	BLQ	BLQ	Subject 57	BLQ	BLQ
Subject 5	BLQ	BLQ	Subject 59	BLQ	BLQ
Subject 8	BLQ	BLQ	Subject 60	BLQ	BLQ
Subject 9	BLQ	BLQ	Subject 61	BLQ	BLQ
Subject 11	BLQ	BLQ	Subject 62	BLQ	BLQ
Subject 13	BLQ	BLQ	Subject 64	BLQ	BLQ
Subject 14	BLQ	BLQ	Subject 65	BLQ	BLQ
Subject 15	BLQ	BLQ	Subject 67	BLQ	BLQ
Subject 16	BLQ	BLQ	Subject 68	BLQ	BLQ
Subject 17	BLQ	BLQ	Subject 69	BLQ	BLQ
Subject 20	BLQ	BLQ	Subject 70	BLQ	BLQ
Subject 22	BLQ	BLQ	Subject 71	BLQ	BLQ
Subject 23	BLQ	BLQ	Subject 72	BLQ	BLQ
Subject 25	BLQ	BLQ	Subject 73	BLQ	BLQ
Subject 27	BLQ	BLQ	Subject 75	BLQ	BLQ
Subject 28	BLQ	BLQ	Subject 76	BLQ	BLQ
Subject 29	BLQ	BLQ	Subject 77	BLQ	BLQ
Subject 31	BLQ	BLQ	Subject 78	BLQ	BLQ
Subject 32	BLQ	BLQ	Subject 79	BLQ	BLQ
Subject 33	BLQ	BLQ	Subject 80	BLQ	BLQ
Subject 34	BLQ	BLQ	Subject 81	BLQ	BLQ
Subject 35	BLQ	BLQ	Subject 82	BLQ	BLQ
Subject 39	BLQ	BLQ	Subject 83	BLQ	BLQ
Subject 40	BLQ	BLQ	Subject 84	BLQ	BLQ
Subject 41	BLQ	BLQ	Subject 85	BLQ	BLQ
Subject 42	BLQ	BLQ	Subject 86	BLQ	BLQ
Subject 43	BLQ	BLQ	Subject 87	BLQ	BLQ
Subject 44	BLQ	BLQ	Subject 88	BLQ	BLQ
Subject 45	BLQ	BLQ	Subject 89	BLQ	BLQ
Subject 46	BLQ	BLQ	Subject 91	BLQ	BLQ
Subject 50	BLQ	BLQ	Subject 92	BLQ	BLQ
Subject 52	BLQ	BLQ	Subject 93	BLQ	BLQ
Subject 53	BLQ	BLQ	Subject 94	BLQ	BLQ

Table 2.56 Results summary of production batch 2 for β–endosulfan and endosulfan sulfate

Production batch 2			Production batch 2		
Sample Name	β-Endosulfan	Endosulfan sulfate	Sample Name	β-Endosulfan	Endosulfan sulfate
	Calc. Conc. (ng/ml)	Calc. Conc. (ng/ml)		Calc. Conc. (ng/ml)	Calc. Conc. (ng/ml)
Subject 95	BLQ	BLQ	Subject 135	BLQ	BLQ
Subject 96	BLQ	BLQ	Subject 136	BLQ	BLQ
Subject 97	BLQ	BLQ	Subject 137	BLQ	BLQ
Subject 98	BLQ	BLQ	Subject 138	BLQ	BLQ
Subject 99	BLQ	BLQ	Subject 139	BLQ	BLQ
Subject 100	BLQ	BLQ	Subject 140	BLQ	BLQ
Subject 101	BLQ	BLQ	Subject 141	BLQ	BLQ
Subject 102	BLQ	BLQ	Subject 142	BLQ	BLQ
Subject 103	BLQ	BLQ	Subject 143	BLQ	BLQ
Subject 104	BLQ	BLQ	Subject 144	BLQ	BLQ
Subject 105	BLQ	BLQ	Subject 145	BLQ	BLQ
Subject 106	BLQ	BLQ	Subject 146	BLQ	BLQ
Subject 107	BLQ	BLQ	Subject 147	BLQ	BLQ
Subject 109	BLQ	BLQ	Subject 148	BLQ	BLQ
Subject 110	BLQ	BLQ	Subject 149	BLQ	BLQ
Subject 111	BLQ	BLQ	Subject 151	BLQ	BLQ
Subject 112	BLQ	BLQ	Subject 154	BLQ	BLQ
Subject 113	BLQ	BLQ	Subject 155	BLQ	BLQ
Subject 114	BLQ	BLQ	Subject 156	BLQ	BLQ
Subject 115	BLQ	BLQ	Subject 157	BLQ	BLQ
Subject 116	BLQ	BLQ	Subject 158	BLQ	BLQ
Subject 117	BLQ	BLQ	Subject 159	BLQ	BLQ
Subject 118	BLQ	BLQ	Subject 160	BLQ	BLQ
Subject 119	BLQ	BLQ	Subject 161	BLQ	BLQ
Subject 121	BLQ	BLQ	Subject 162	BLQ	BLQ
Subject 122	BLQ	BLQ	Subject 163	BLQ	BLQ
Subject 123	BLQ	BLQ	Subject 164	BLQ	BLQ
Subject 124	BLQ	BLQ	Subject 165	BLQ	BLQ
Subject 125	BLQ	BLQ	Subject 166	BLQ	BLQ
Subject 126	BLQ	BLQ	Subject 167	BLQ	BLQ
Subject 127	BLQ	BLQ	Subject 168	BLQ	BLQ
Subject 128	BLQ	BLQ	Subject 169	BLQ	BLQ
Subject 129	BLQ	BLQ	Subject 170	BLQ	BLQ
Subject 130	BLQ	BLQ	Subject 171	BLQ	BLQ
Subject 131	BLQ	BLQ	Subject 172	BLQ	BLQ
Subject 134	BLQ	BLQ	Subject 173	BLQ	BLQ

Table 2.57 Results summary of production batch 3 for β–endosulfan and endosulfan sulfate

Production batch 3			Production batch 3		
Sample Name	β-endosulfan	Endosulfan sulfate	Sample Name	β-endosulfan	Endosulfan sulfate
	Calc. Conc. (ng/ml)	Calc. Conc. (ng/ml)		Calc. Conc. (ng/ml)	Calc. Conc. (ng/ml)
Subject 174	BLQ	BLQ	Subject 215	BLQ	BLQ
Subject 176	BLQ	BLQ	Subject 216	BLQ	BLQ
Subject 177	BLQ	BLQ	Subject 217	BLQ	BLQ
Subject 178	BLQ	BLQ	Subject 218	BLQ	BLQ
Subject 179	BLQ	BLQ	Subject 219	BLQ	BLQ
Subject 180	BLQ	BLQ	Subject 220	BLQ	BLQ
Subject 181	BLQ	BLQ	Subject 221	BLQ	BLQ
Subject 182	BLQ	BLQ	Subject 222	BLQ	BLQ
Subject 183	BLQ	BLQ	Subject 223	BLQ	BLQ
Subject 184	BLQ	BLQ	Subject 224	BLQ	BLQ
Subject 185	BLQ	BLQ	Subject 225	BLQ	BLQ
Subject 186	BLQ	BLQ	Subject 226	BLQ	BLQ
Subject 187	BLQ	BLQ	Subject 227	BLQ	BLQ
Subject 188	BLQ	BLQ	Subject 228	BLQ	BLQ
Subject 189	BLQ	BLQ	Subject 229	BLQ	BLQ
Subject 190	BLQ	BLQ	Subject 230	BLQ	BLQ
Subject 191	BLQ	BLQ	Subject 231	BLQ	BLQ
Subject 192	BLQ	BLQ	Subject 232	BLQ	BLQ
Subject 193	BLQ	BLQ	Subject 233	BLQ	BLQ
Subject 194	BLQ	BLQ	Subject 235	BLQ	BLQ
Subject 195	BLQ	BLQ	Subject 236	BLQ	BLQ
Subject 196	BLQ	BLQ	Subject 237	BLQ	BLQ
Subject 198	BLQ	BLQ	Subject 238	BLQ	BLQ
Subject 199	BLQ	BLQ	Subject 239	BLQ	BLQ
Subject 200	BLQ	BLQ	Subject 240	BLQ	BLQ
Subject 201	BLQ	BLQ	Subject 241	BLQ	BLQ
Subject 202	BLQ	BLQ	Subject 247	BLQ	BLQ
Subject 203	BLQ	BLQ	Subject 248	BLQ	BLQ
Subject 204	BLQ	BLQ	Subject 250	BLQ	BLQ
Subject 205	BLQ	BLQ	Subject 257	BLQ	BLQ
Subject 206	BLQ	BLQ	Subject 259	BLQ	BLQ
Subject 207	BLQ	BLQ	Subject 260	BLQ	BLQ
Subject 208	BLQ	BLQ	Subject 262	BLQ	BLQ
Subject 209	BLQ	BLQ	Subject 263	BLQ	BLQ
Subject 210	BLQ	BLQ	Subject 264	BLQ	BLQ
Subject 211	BLQ	BLQ	Subject 265	BLQ	BLQ
Subject 213	BLQ	BLQ	Subject 266	BLQ	BLQ
Subject 214	BLQ	BLQ			

2.10 CONCLUSION

A robust and accurate analytical method for the quantitative determination of β-endosulfan and endosulfan sulfate in human serum was developed and validated using LC-MS/MS with electrospray ionization and MRM scanning in the negative mode.

During the validation, intra- and inter-batch accuracy and precision were demonstrated by analyzing calibration standards (STDs) and quality control samples (QCs) in three consecutive validation batches each containing the calibration standards (STD J – STD B) in duplicate to yield one calibration curve and six replicates of quality control samples (QC H – QC A). The method was shown to be specific and selective.

The selected regression models adequately described the concentration-response relationships for each compound. Based on a range that consisted of nine calibration levels, a Wagner regression equation provided the best fit for β-endosulfan, while a linear equation, weighted by1/concentration^2 provided the best fit for endosulfan sulfate. The regression models selected during the validation were used for the quantification of the study samples.

No suitable internal standards could be found for either of the compounds. Stable isotope-labeled congeners of the compounds were not commercially available and would not have been sufficiently specific to separate their *m/z* values from that of the complex isotope patterns of the compounds that each contains six chlorine atoms. Furthermore, related chlorinated pesticide molecules such as dichlorodiphenyltrichloroethane (DDT) and dieldrin are not ionizable with electrospray and could therefore not been used as internal standards. These compounds were used as internal standards in published studies based on GC-MS/MS in which electron ionization was applied [20] [26]. The fact that good retention times of both compounds were achieved during chromatography and that no significant effect from normal matrix components could be evidenced, contributed to the satisfactory performance of the method without using internal standards.

Although the matrix effect assessment indicated no significant influence on the analysis by the normal matrix components, it should be noted that lipemia in the serum had noticeable effects on the analysis of both compounds. This can be ascribed to the fact that high concentrations of phospholipids in lipemic serum can possibly influence the successful formation of cations of both analytes during the negative ionization process [70].

During the first validation batch the QC at the highest concentration level (QC H) were diluted (1:1) with blank normal serum to evaluate dilution integrity and the results demonstrated that samples with concentration levels above the upper limit of quantification (ULOQ) (up to 323 ng/ml

and 48 ng/ml for β-endosulfan and endosulfan sulfate, respectively) can be accurately quantified with this method.

System suitability tests were performed during each validation batch to ensure that the instrument did not lose response (sensitivity) during the validation batches. This was also performed for the three production batches.

The lower limit of quantification (LLOQ) was confirmed and successfully quantified with a signal to noise ratio of 8.9 and 22.6 for β-endosulfan and endosulfan sulfate, respectively. No carry-over was observed as assessed during the analysis of the three validation batches. Reinjection reproducibility was demonstrated by reinjecting the second validation batch. Stability assessments were performed during the validation and as summarized in section 2.8, no indication of instability of either of the two compounds could be found during any of the assessments.

The validation results indicated that the performance of the analytical method met the acceptance criteria as stipulated in the European Medical Agency (EMA) and Food and Drug Administration (FDA) guidelines [42] [43]. The method is therefore regarded to be suitable for the quantification of β-endosulfan and endosulfan sulfate in human serum samples over a concentration range of 0.800 ng/ml to 200 ng/ml and 0.117 ng/ml to 30 ng/ml, respectively.

The applicable clinical protocol (REC REF: 279/2005) mentions the detection of endosulfan and other pesticides in surface and groundwater of rural Western Cape areas [8] [9] [10]. The samples analyzed in this study originated from farm workers active in the same area and therefore thought to have been exposed to the pesticides. However, no evidence for the exposure to endosulfan pesticides could be found in any of the serum samples. This was notwithstanding the fact that the STDs and QCs in the production batches conformed to the same standards found during the validation performance. The fact that none of the compounds could be observed could therefore not be ascribed to poor performance of the analytical method. Furthermore, the signal to noise ratio of the LLOQs for both compounds, especially for endosulfan sulfate, was high enough to still observe sub-LLOQ concentrations of the compounds. No such observations were made. The reasons for the negative results may reside in seasonal variation in the application of pesticides used for pest control. This possibility can be explored by correlating the pesticide products used during or before the period of sampling. However, it may indicate that the time of sampling was too long after exposure or exposure was not high enough to result in levels of the compounds within the calibration range.

CHAPTER 3

DEVELOPMENT AND VALIDATION OF A BIOANALYTICAL METHOD FOR THE DETERMINATION OF DIALKYLPHOSPHATE METABOLITES IN HUMAN URINE BY LC-MS/MS

3.1 INTRODUCTION

As indicated in the rationale for the clinical trial (REC REF: 279/2005), South Africa is the highest pesticide user in Southern Africa and exposure to these harmful pesticides needs to be determined to incorporate protective practices. Pesticide exposure can occur through a number of sources as indicated in Chapter 1 and the approach to biological monitoring for organophosphates is based on the analysis of diakylphosphate metabolites in urine [71] [72] [73]. Organophosphates are rapidly hydrolyzed to the diakylphosphate (DAP) metabolites detectable in urine, and can be measured several days after exposure [74]. Identification of these metabolites can be used to monitor the occasional exposure to organophosphate pesticides [75] and the non-invasive sampling procedure is preferable [76].

Previous investigations have shown that the use of first morning void urine samples accurately represent total daily exposure to organophosphates [77] although variable urine production may influence the concentration levels [71]. The ubiquitous use of house-hold pesticides containing organophosphates poses a further disadvantage when measuring the DAP metabolites as markers for specific agricultural exposure [71]. Environmental routes of exposure to pesticides through contaminated food, soil, water and spray drift in addition to occupational exposure might be important for rural residents in the Western Cape. The use of a control group of subjects to act as a "population background level" is therefore essential.

Due to the superior specificity and sensitivity that can be achieved with high performance liquid chromatography with tandem mass spectrometer detection (LC-MS/MS), this technique was applied in this study to quantitatively measure three of the DAP metabolites, dimethyl phosphate (DMP), diethyl phosphate (DEP), and dimethyl thiophosphate (DMTP), using the corresponding deuterated molecules as internal standards. One method was developed to extract the three metabolites from urine and to determine the concentrations by LC-MS/MS. Although other known metabolites could also be assessed, the lack of appropriate internal standards to accurately quantify all of the molecules restricted the method to the three mentioned above.

The developed and partially validated method used for the quantitative determination of dialkylphosphate (DAP) metabolites in urine was performed at the University of Cape Town. This

method was used to investigate the possible correlation between exposure to the pesticides and urinary levels of the metabolites, data which can also be compared to literature reports.

3.2 CHEMICALS AND MATERIALS USED

3.2.1 Chemicals and reagents

Analytical reference standards of the three DAP metabolites, with purities higher than 99%, as well as the corresponding deuterated internal standards were obtained from Sigma Aldrich. All chemicals and reagents used in this study were of analytical grade.

3.2.2 Preparation of stock solutions

Separate primary stock solutions for the three dialkylphosphate metabolites were prepared at a concentration of 100 µg/ml in acetonitrile as indicated in Table 3.1.

Similarly, stock solutions of the internal standards were prepared in water as indicated in Table 3.2.

All stock solutions were stored at approximately -20ºC and were used to prepare secondary reference solutions.

Table 3.1 Preparation of primary stock solutions

Analyte	Solvent used	Solvent density	Mass analyte (mg)	Mass solvent (g)	Volume Solvent (ml)	Concentration analyte (µg/ml)
Dimethyl phosphate (DMP)	Acetonitrile	0.791	1.00	7.91	10	100
Dimethyl thiophosphate (DMTP)	Acetonitrile	0.791	1.00	7.91	10	100
Diethyl phosphate (DEP)	Acetonitrile	0.791	1.00	7.91	10	100

Table 3.2 Preparation of internal standard working solutions

Deuterated Internal standard	Solvent used	Solvent density	Mass analyte (mg)	Mass solvent (g)	Volume Solvent (ml)	Concentration analyte (µg/ml)
Dimethyl phosphate (DMP)-D6	Water	1	1.00	10	10	100
Dimethyl thiophosphate (DMTP)-D6	Water	1	1.00	10	10	100
Diethyl phosphate (DEP))-D5	Water	1	1.00	10	10	100

3.2.3 Biological matrix

The biological matrix for this study was human urine and therefore it was used to prepare the calibration standards (STD) and quality control (QC) samples. The acquisition of completely blank (drug-free) urine was however problematic as exposure to even low levels of organophosphates leads to the presence of the DAP metabolites in urine. Thus the urine which was used for the preparation of STDs and QCs had to be treated to remove potential interfering molecules. Stripping with activated charcoal is an acceptable method for removing unwanted molecules from biological matrices and the following procedure was used in this case:

1. To a round bottom flask (volumetric), add 5 g of activated charcoal for every 50 ml of urine.
2. Stopper the flask and incubate overnight at room temperature on an orbital shaker.
3. Transfer to 50 ml tubes and centrifuge at 13000 x g for 20 minutes.
4. Repeat the stripping procedure with the supernatant (steps 1 and 3), while discarding the pellet.
5. After the final stripping, repeat the centrifugation at 13000 x g for 20 minutes.
6. Transfer the supernatant to a clean 50 ml tube.
7. Pre-filter the stripped urine using qualitative filter paper under vacuum to remove the charcoal.
8. Finally, filter the stripped urine through a 0.45 µM filter under vacuum.
9. Store the stripped urine at ~ -20^{o}C until required.

3.2.4 LC-MS/MS optimization by spectral analysis

Appropriate dilutions of the stock solutions were prepared for infusion to determine their mass spectra of the analytes and the internal standards. The acidic nature of the phosphate groups ensured successful ionization with electrospray in the negative mode. The molecular ions were identified according to the expected molecular weights and fragment mass spectra were produced by collision induced dissociation of the precursor ions. The product ion mass spectra and proposed structures of selected fragment ions are depicted in figures 3.1 to 3.3 for DMP, DMTP and DEP, and the internal standards d6-DMP, d6-DMTP and d10-DEP respectively.

Optimized compound parameters were obtained during infusion. The declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP) were all adjusted to achieve optimal signal intensity. The fragment ions indicated in figures 3.1 to 3.3 were selected and the ion transitions indicated in Table 3.4 were selected for the detection of each individual compound.

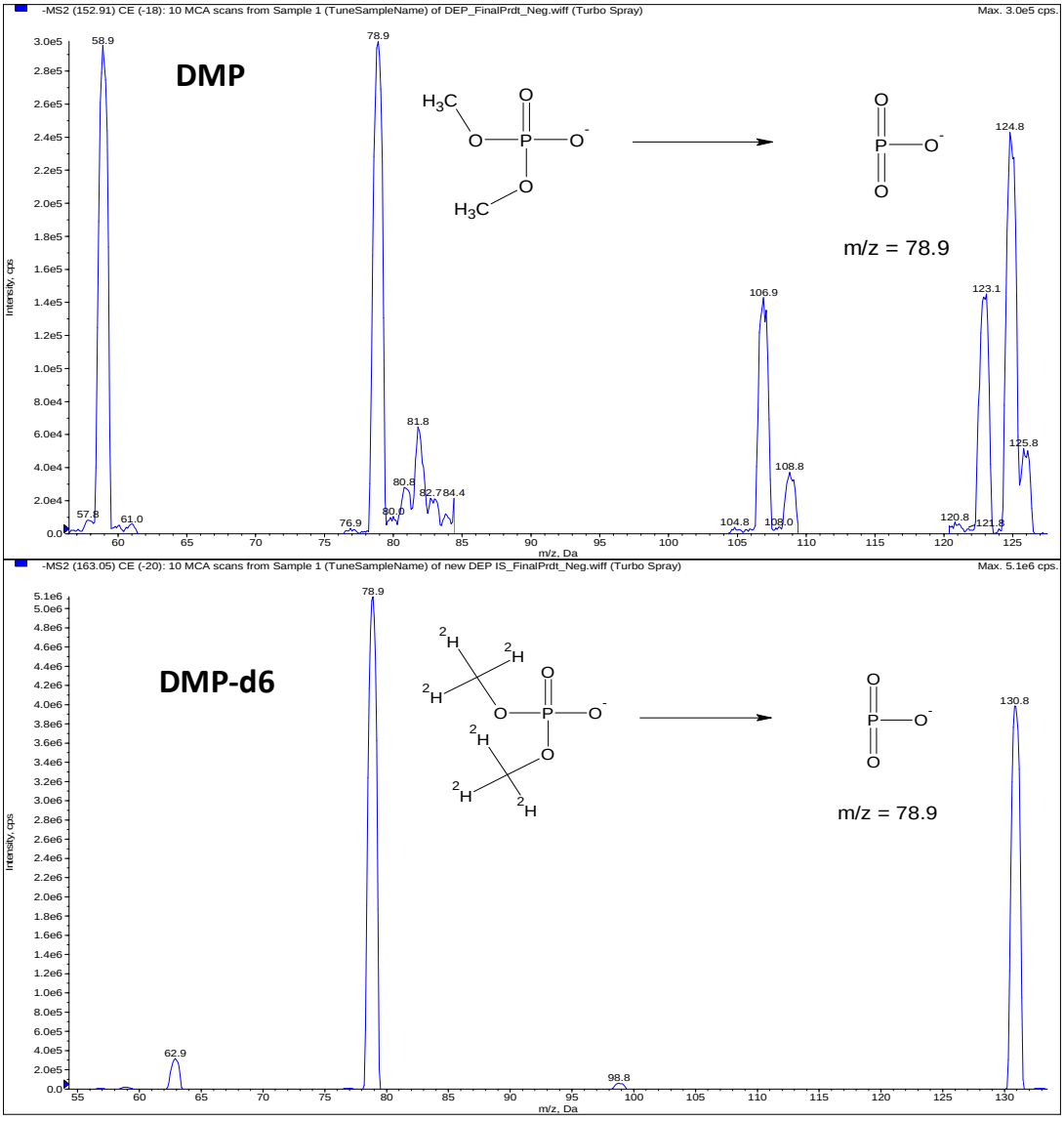


Figure 3.1 The mass spectra of the fragment ions of deprotonated dimethyl phosphate (DMP) and its deuterated internal standard, DMP-d6. The structures of the fragment ions based on their *m/z* values are proposed.

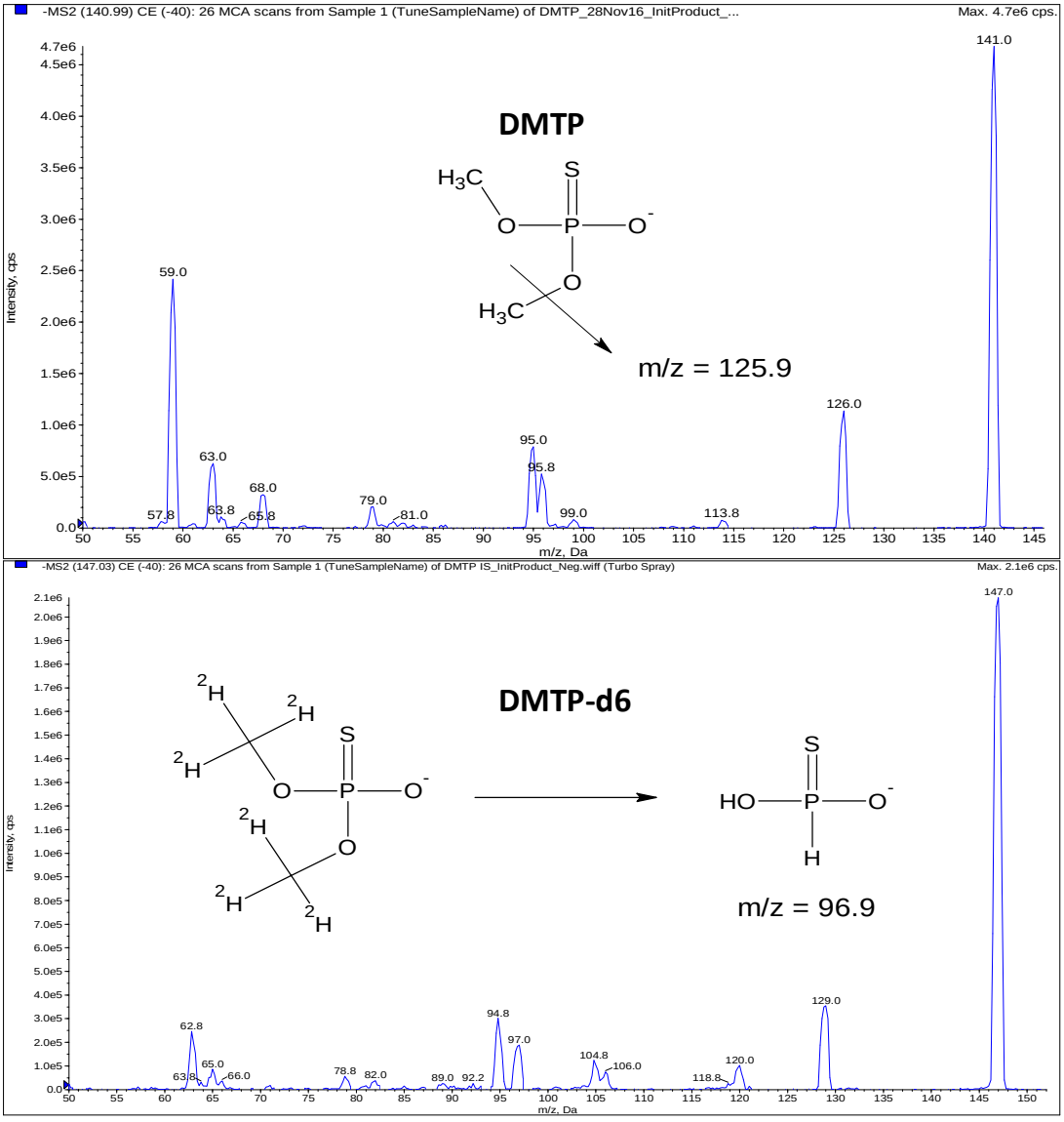


Figure 3.2 The mass spectra of the fragment ions of deprotonated dimethyl thiophosphate (DMTP) and its deuterated internal standard, DMTP-d6. The structures of the fragment ions based on their *m/z* values are proposed

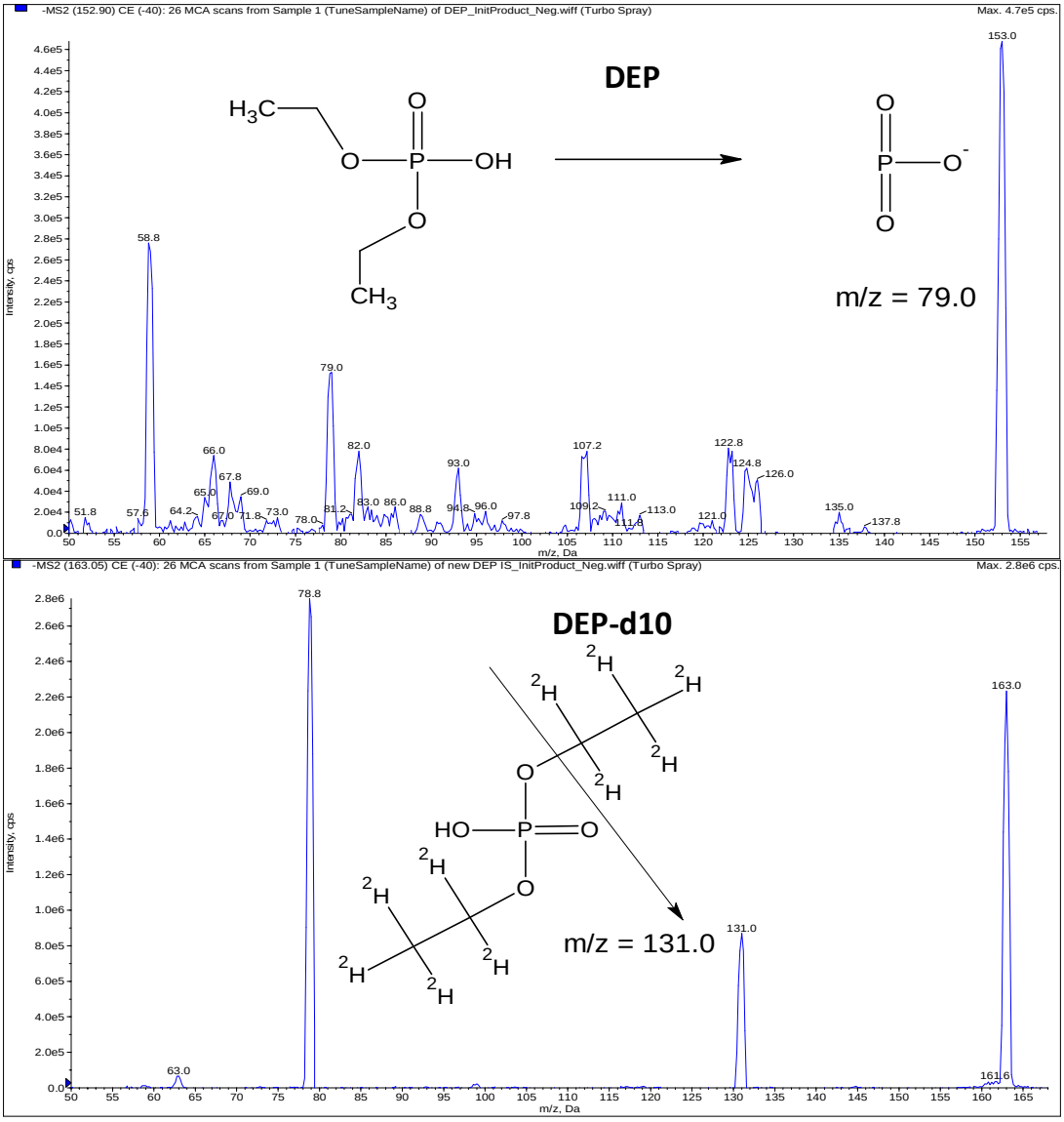


Figure 3.3 The mass spectra of the fragment ions of deprotonated diethyl phosphate (DEP) and its deuterated internal standard, DEP-d10. The structures of the fragment ions based on their *m/z* values are proposed.

3.3 ANALYTICAL METHODOLOGY

3.3.1 Sample preparation

Urine samples, used for the determination of the DAP metabolites were processed as follows:

- Aliquot 1 ml urine into a 5 ml polypropylene tube.
- Add 20 µl of internal standard (5 µg/ml) to the tube.
- Vortex for 30 seconds.
- Freeze dry the sample overnight.
- Add 2 ml acetonitrile to the tube.
- Vortex for 30 seconds.
- Ultrasonicate for 10 minutes.
- Vortex briefly.
- Centrifuge for 10 minutes at 3800 x *g* (~ 5^{o}C).
- Transfer the supernatant to a glass tube.
- Evaporate using a MiVac evaporator until completely dry (approximately 45 minutes).
- Add 200 µl injection solvent (20 mM ammonium acetate : acetonitrile [95:5, v/v]) to the glass tube
- Vortex briefly.
- Transfer 150 µl of the extract to a 96-well plate.
- Inject 10 µl onto the HPLC column.

3.3.2 Chromatographic separation

Chromatography was performed on a Phenomenex® Luna C18 (4.6 mm x 150 mm, 5 µm) analytical column. The column was kept at 30°C in an Agilent 1200 series column compartment.

The mobile phase was pumped through the column by an Agilent 1200 binary pump at a constant flow-rate of 600 µl/minute. A gradient was created by mixing solvent A (acetonitrile) and solvent B (20 mM ammonium acetate prepared in water) according to table 3.3

Table 3.3 Time table for gradient elution

Step	Time (min)	% A	% B
0	0.00	98	2
1	3.00	2	98
2	3.10	98	2
3	7.50	98	2

A volume of 10 µl of each sample was injected onto the column by an Agilent 1200 auto sampler, equipped with an Agilent cooling device (sample cooler) set at 5^{o}C. The auto sampler was equipped with a flush port to enable a needle wash cycle of 20 seconds before each injection.

3.3.3 Detection

Tandem mass spectrometric analysis was performed on an AB SCIEX API 5500QTRAP triple quadrupole mass spectrometer (AB SCIEX Toronto Canada) equipped with an electrospray ionization (ESI) source operating in the negative ion mode. Mass spectrometer settings used for the detection of DMP, DMTP and DEP, together with their respective internal standards, are indicated in Table 3.4 to 3.7. These settings were selected from data acquired by the infusion of each compound (see figures 3.1 to 3.3).

Table 3.4 Source parameter settings for the optimal detection of the dialkylphosphate (DAP) metabolites and their internal standards

Electrospray Ionization Settings	Value
Nebulizer gas (Gas 1) (arbitrary unit)	65
Turbo gas (Gas 2) (arbitrary unit)	35
CUR (curtain gas) (arbitrary unit)	30
CAD (collision gas) (arbitrary unit)	Medium
TEM (source temperature) (°C)	500
IS (Ion Spray Voltage) (V)	-3500

Table 3.5 MS/MS settings for the optimal detection of the dialkylphosphate (DAP) metabolites

MS/MS Settings	Dimethyl phosphate (DMP)	Dimethyl thiophosphate (DMTP)	Diethyl phosphate (DEP)
Monoisotopic mass	126.008	141.985	154.039
Deprotonated monoisotopic mass (*m/z*)	125	141	153
Product ion monoisotopic mass (*m/z*)	79	126	79
Dwell time (ms)	150	150	150
DP (declustering potential) (V)	-85	-195	-55
EP (entrance potential) (V)	-10	-10	-10
CE (collision energy) (eV)	-32	-16	-26
CXP (collision cell exit potential) (V)	-7	-11	-9

Table 3.6 MS/MS settings for the optimal detection of the dialkylphosphate (DAP) metabolites internal standards

MS/MS Settings	DMP-d6	DMTP-d6	DEP-d10
Monoisotopic mass	132.000	148.000	164.000
Deprotonated monoisotopic mass (*m/z*)	131	147	163
Product ion monoisotopic mass (*m/z*)	79	97	131
Dwell time (ms)	150	150	150
DP (declustering potential) (V)	-85	-120	-120
EP (entrance potential) (V)	-10	-10	-10
CE (collision energy) (eV)	-32	-30	-16
CXP (collision cell exit potential) (V)	-7	-5	-15

Table 3.7 Scan description for the optimal detection of the dialkylphosphate (DAP) metabolites and their internal standards

Scan Description	
Scan type	MRM
Polarity	Negative
Pause time	5 ms

3.3.4 Chromatographic results

The total runtime for each sample was 7.5 minutes. A representative chromatogram of an extracted urine sample containing 25 ng/ml of each of DMP, DMTP and DEP together with the internal standards (at 20 ng/ml) is presented in figure 4.9.

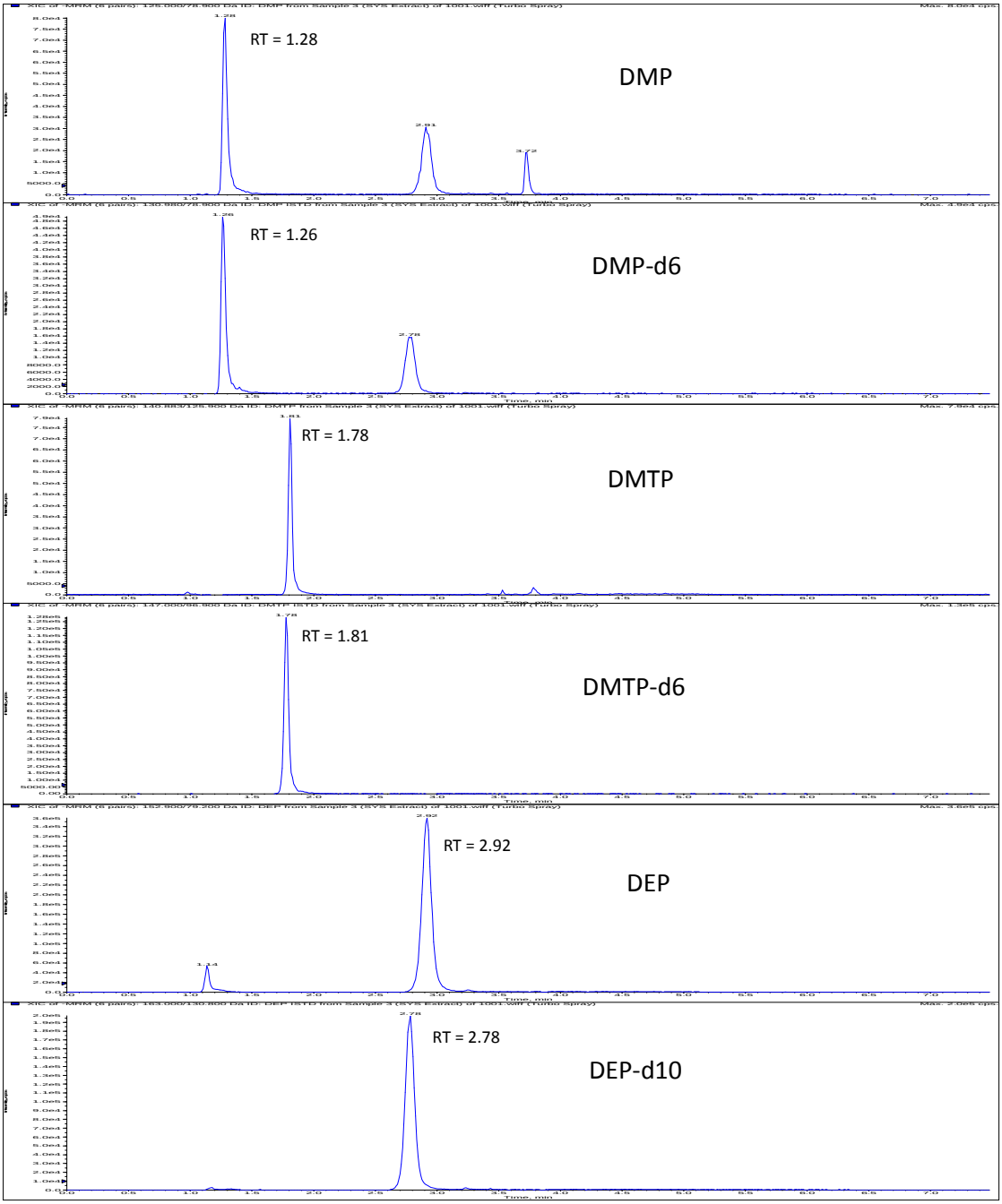


Figure 3.4 A representative chromatogram of dialkylphosphate (DAP) metabolites and their deuterated internal standards extracted from stripped human urine.

3.3.5 Blank selectivity

Blank stripped urine samples from six different sources were analyzed and no interfering components were evident at the retention times of the compounds as indicated in the representative chromatogram of one of the sources shown in figure 3.5.

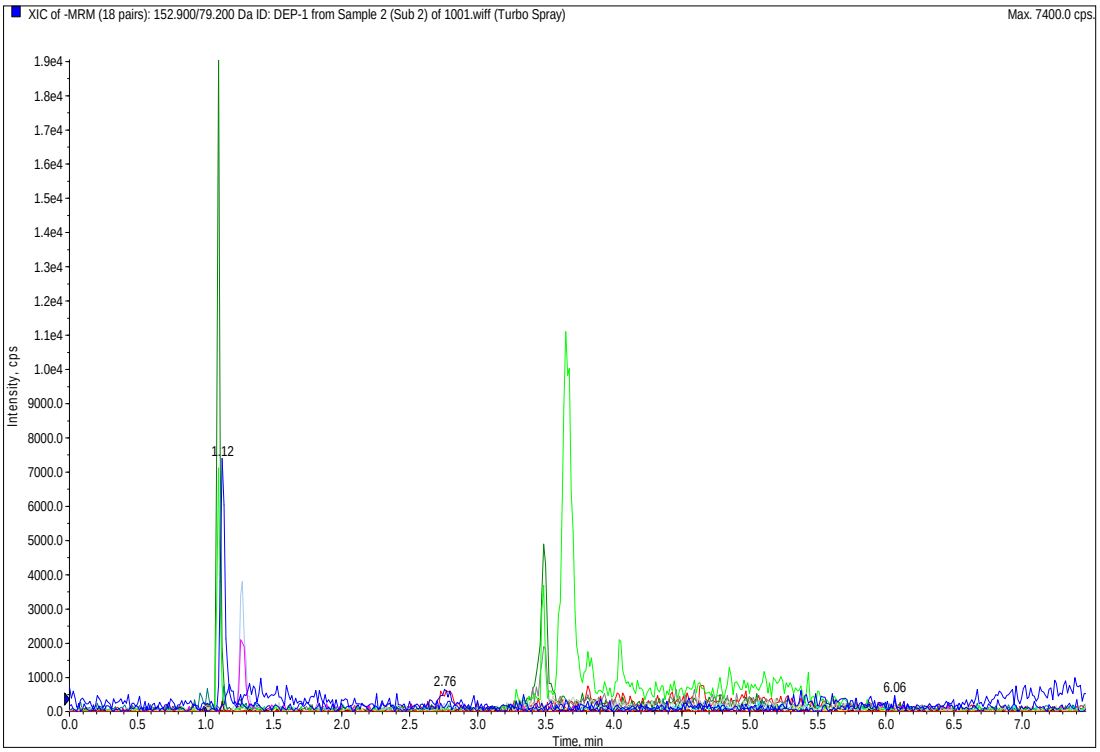


Figure 3.5 A representative chromatogram of a blank urine extract

3.3.6 Marix effects and selectivity

The matrix effects were assessed by comparing six different urine sources, spiked with each of the compounds at three concentration levels (25 ng/ml, 12.5 ng/ml and 2 ng/ml) and at one concentration with each of the internal standards. Employing the analytical method, the peak areas of each of the compounds and each of the internal standards were determined for each sample. The peak area ratios were then calculated for each compound by dividing the analyte peak areas by that of the internal standards peak areas. By graphically plotting the concentration versus the peak area ratio, a regression line for each urine pool was obtained. The slopes of these regression lines should be similar between the different urine pools (% CV ≤15%) if matrix components do not influence the analysis.

Selectivity is indicated when the % CV of the area ratios of the six pools at a certain concentration

is less than or equal to 15%. .

The results of these assessments for each analyte are depicted in Tables 3.8, 3.9 and 3.10 for DMP, DMTP and DEP respectively.

Table 3.8 Peak area ratios of DMP at low, medium and high concentrations in six different stripped urine pools

Stripped Urine	Peak Area Ratio High Conc. 25.0 ng/ml	Peak Area Ratio Medium Conc. 12.5 ng/ml	Peak Area Ratio Low Conc. 2.00 ng/ml	Slope of Regression Line Area Ratio vs Conc.
Pool 1	60.8	36.6	5.84	2.38
Pool 2	66.9	40.0	7.07	2.59
Pool 3	72.0	36.3	7.65	2.80
Pool 4	75.6	41.2	6.98	2.98
Pool 5	68.7	41.2	7.95	2.63
Pool 6	69.3	40.5	10.0	2.57
Average	68.9	39.3	7.58	2.66
STDEV	4.99	2.27	1.39	0.207
% CV	7.2[b]	5.8[b]	18.3[b]	7.8[a]

[a] Matrix effects are measured by the % CV of the slopes of the regression lines obtained by plotting the peak area ratios against the concentration.

[b] Selectivity is measured by the % CV of the peak area ratios at each concentration level.

Table 3.9 Peak area ratios of DMTP at low, medium and high concentrations in six different stripped urine pools

Stripped Urine	High Conc. 25.0 ng/ml Peak Area Ratio	Medium Conc. 12.5 ng/ml Peak Area Ratio	Low Conc. 2.00 ng/ml Peak Area Ratio	Slope of Regression Line Area Ratio vs Conc.
Pool 1	26.4	16.4	2.62	1.03
Pool 2	28.1	14.3	1.86	1.14
Pool 3	24.8	13.2	2.57	0.96
Pool 4	24.4	14.5	2.19	0.96
Pool 5	27.6	12.8	2.48	1.10
Pool 6	30.5	13.3	2.10	1.24
Average	27.0	14.10	2.30	1.07
STDEV	2.29	1.31	0.304	0.110
% CV	8.5[b]	9.3[b]	13.0[b]	10.2[a]

[a] Matrix effects are measured by the % CV of the slopes of the regression lines obtained by plotting the peak area ratios against the concentration.

[b] Selectivity is measured by the % CV of the peak area ratios at each concentration level.

Table 3.10 Peak area ratios of DEP at low, medium and high concentrations in six different stripped urine pools

Stripped Urine	High Conc. 25.0 ng/ml Peak Area Ratio	Medium Conc. 12.5 ng/ml Peak Area Ratio	Low Conc. 2.00 ng/ml Peak Area Ratio	Slope of Regression Line Area Ratio vs Conc.
Pool 1	225	110	15.1	9.15
Pool 2	221	99.3	14.9	8.99
Pool 3	237	122	17.5	9.55
Pool 4	266	124	17.2	10.8
Pool 5	211	116	15.9	8.46
Pool 6	294	109	16.6	12.1
Average	243	113	16.2	9.85
STDEV	31.4	9.31	1.08	1.37
% CV	13.0[b]	8.2[b]	6.6[b]	13.9[a]

[a] **Matrix effects are measured by the % CV of the slopes of the regression lines obtained by plotting the peak area ratios against the concentration.**

[b] **Selectivity is measured by the % CV of the peak area ratios at each concentration level.**

The assessment of matrix effects indicates that matrix components in stripped urine do not significantly influence the analysis of the DAP metabolites.

The selectivity of the method is also acceptable, although it was observed that the % CV of the peak area ratios at low concentration of DMP did not meet the acceptance criteria (% CV > 15). This was most probably due to a too high value incurred from the last injection and this point can probably be regarded as a statistical outlier.

3.4 VALIDATION

The method was partially validated at the University of Cape Town as indicated in the following paragraphs.

3.4.1 Accuracy and precision

Accuracy and precision were assessed by analyzing three consecutive, independent validation batches. The calibration curves fit quadratic regressions, weighted by 1/concentration, for all three DAP metabolites across the concentration range of 1 – 32 ng/ml. No indications of carry over or interfering peaks were observed during the analysis of the validation batches and no analyte peaks were observed in chromatograms of the blank samples.

The results are expressed as those for the three independent validation (intra-batch accuracy and precision) and then a statistical comparison is made between the three batches (inter-batch accuracy and precision). Lastly, validation batch two was re-injected to prove acceptability of the

re-injection of a complete validation batch.

3.4.1.1 Intra-batch accuracy and precision

3.4.1.1.1 Validation batch 1

The calculation of the concentrations of DMP, using the analytical method and applying the acquired peak area ratios (using the relevant internal standard) to the calibration curve (figure 3.6), resulted in the values for the calibration standards and quality control standards as indicated in tables 3.11 and 3.12 respectively.

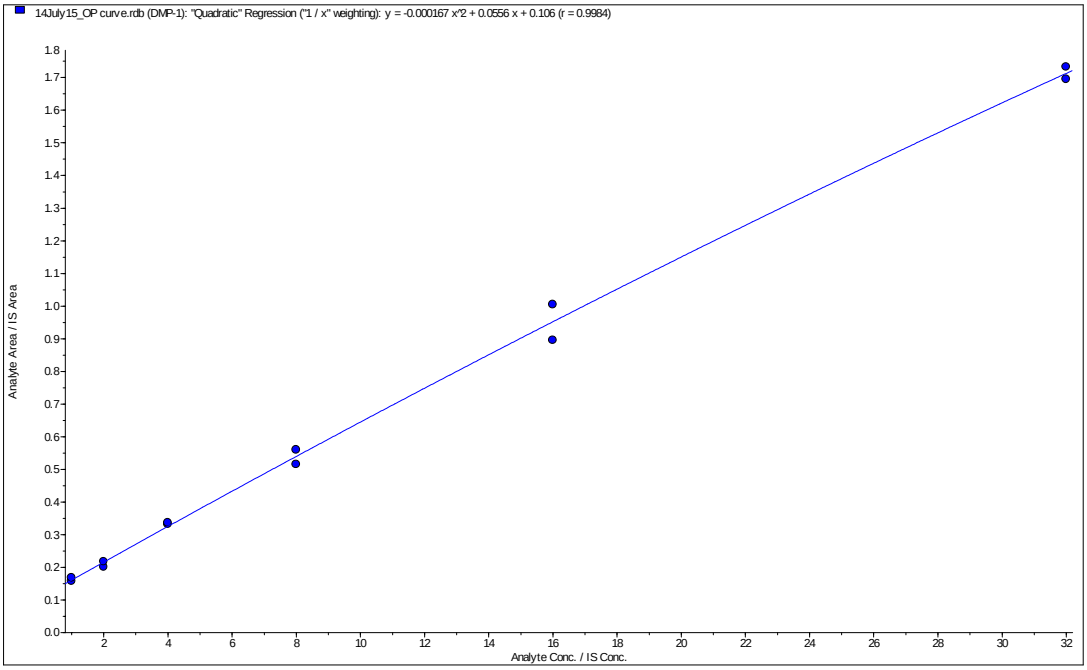


Figure 3.6 Calibration curve constructed by analysis of the STDs in validation batch 1, indicating the DMP concentration in each.

Table 3.11 Summary of the performance of the calibration standards of DMP of validation batch 1

Nominal Concentrations (ng/ml)	STD 6 1.00	STD 5 2.00	STD 4 4.00	STD 3 8.00	STD 2 16.0	STD 1 32.0
Mean Observed Concentration (ng/ml)	0.967	2.10	4.14	7.73	16.1	32.0
Standard Deviation	0.122	N/A	0.369	1.204	0.770	0.774
% CV	12.6	N/A	8.9	15.6	4.8	2.4
% Accuracy	96.7	104.7	103.6	96.6	100.8	99.9
N	2 of 2	1 of 2	2 of 2	2 of 2	2 of 2	2 of 2

Table 3.12 Summary of the accuracy and precision of the quality control standards of DMP of validation batch 1

Nominal Concentrations(ng/ml)	QC 6 (LLOQ) 1.00	QC 5 (L) 2.00	QC 4 3.50	QC 2 (M) 12.5	QC 1 (H) 25.0
Mean Observed Concentration (ng/ml)	0.992	1.89	3.41	12.7	23.9
Standard Deviation	0.139	0.152	0.238	0.079	1.16
% CV	14.1	8.1	7.0	8.5	4.9
% Accuracy	99.3	94.4	97.5	101.2	95.6
N	6 of 6	4 of 6	6 of 6	6 of 6	6 of 6

The calculation of the concentrations of DMTP, using the analytical method and applying the acquired peak area ratios (using the relevant internal standard) to the calibration curve (figure 3.7), resulted in the values for the calibration standards and quality control standards as indicated in tables 3.13 and 3.14 respectively.

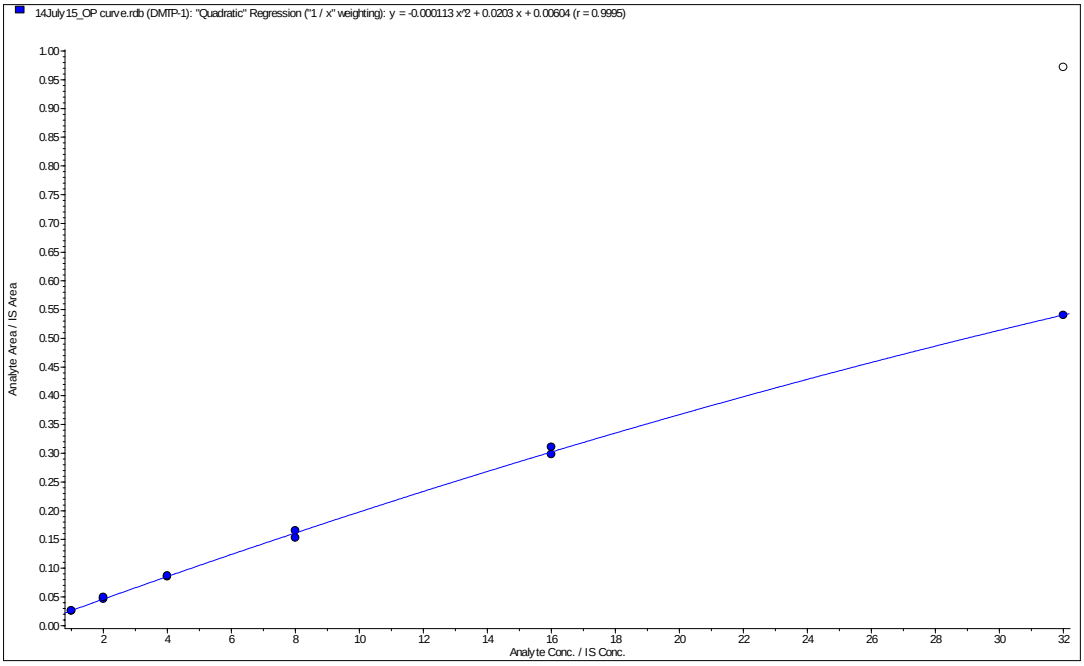


Figure 3.7 Calibration curve constructed by analysis of the STDs in validation batch 1, indicating the DMTP concentration in each.

Table 3.13 Summary of the performance of the calibration standards of DMTP of validation batch 1

Nominal Concentrations (ng/ml)	STD 6 1.00	STD 5 2.00	STD 4 4.00	STD 3 8.00	STD 2 16.0	STD 1 32.0
Mean Observed Concentration (ng/ml)	1.02	1.86	4.16	8.52	15.15	32.3
Standard Deviation	N/A	0.024	0.270	0.474	0.938	3.13
% CV	N/A	1.3	6.5	5.6	6.2	9.7
% Accuracy	101.6	93.1	104.0	106.5	94.6	101.0
N	1 of 2	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2

Table 3.14 Summary of the accuracy and precision of the quality control standards of DMTP of validation batch 1

Nominal Concentrations (ng/ml)	QC 6 (LLOQ) 1.00	QC 5 (L) 2.00	QC 4 3.50	QC 2 (M) 12.5	QC 1 (H) 25.0
Mean Observed Concentration (ng/ml)	1.01	1.93	3.46	12.3	24.0
Standard Deviation	0.140	0.179	0.346	0.856	1.09
% CV	13.9	9.3	10.0	7.0	4.6
% Accuracy	101.0	96.6	98.2	98.1	96.0
N	5 of 6	4 of 6	6 of 6	6 of 6	6 of 6

The calculation of the concentrations of DEP, using the analytical method and applying the acquired peak area ratios (using the relevant internal standard) to the calibration curve (figure 3.8), resulted in the values for the calibration standards and quality control standards as indicated in tables 3.15 and 3.16 respectively.

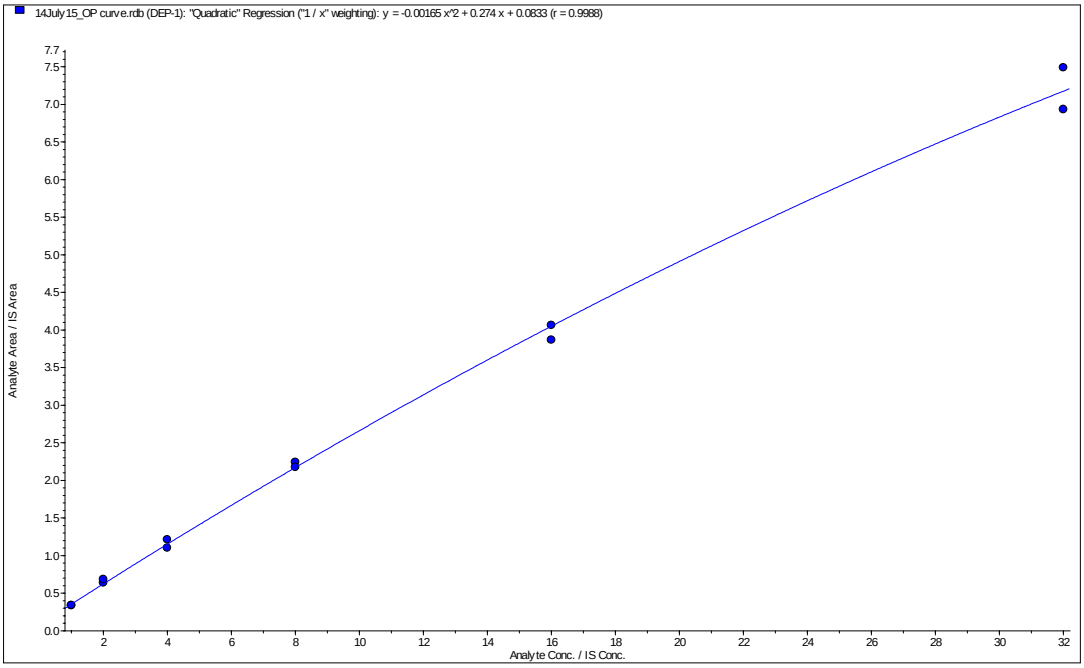


Figure 3.8 Calibration curve constructed by analysis of the STDs in validation batch 1, indicating the DEP concentration in each

Table 3.15 Summary of the performance of the calibration standards of DEP of validation batch 1

Nominal Concentrations (ng/ml)	STD 6 1.00	STD 5 2.00	STD 4 4.00	STD 3 8.00	STD 2 16.0	STD 1 32.0
Mean Observed Concentration (ng/ml)	1.04	1.93	4.02	7.74	16.4	31.8
Standard Deviation	0.917	0.050	0.359	0.371	0.816	1.23
% CV	8.8	2.6	8.9	4.8	5.0	3.9
% Accuracy	104.4	96.2	100.4	96.8	102.7	99.5
N	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2

Table 3.16 Summary of the accuracy and precision of the quality control standards of DEP of validation batch 1

Nominal Concentrations (ng/ml)	QC 6 (LLOQ) 1.00	QC 5 (L) 2.00	QC 4 3.50	QC 2 (M) 12.5	QC 1 (H) 25.0
Mean Observed Concentration (ng/ml)	0.965	2.07	3.73	12.6	23.8
Standard Deviation	0.087	0.091	0.216	0.880	1.79
% CV	9.1	4.4	5.8	7.0	7.5
% Accuracy	96.5	103.5	106.5	100.4	95.3
N	6 of 6	6 of 6	4 of 6	6 of 6	6 of 6

3.4.1.1.2 Validation batch 2

The calculation of the concentrations of DMP, using the analytical method and applying the acquired peak area ratios (using the relevant internal standard) to the calibration curve (figure 3.9), resulted in the values for the calibration standards and quality control standards as indicated in tables 3.17 and 3.18 respectively.

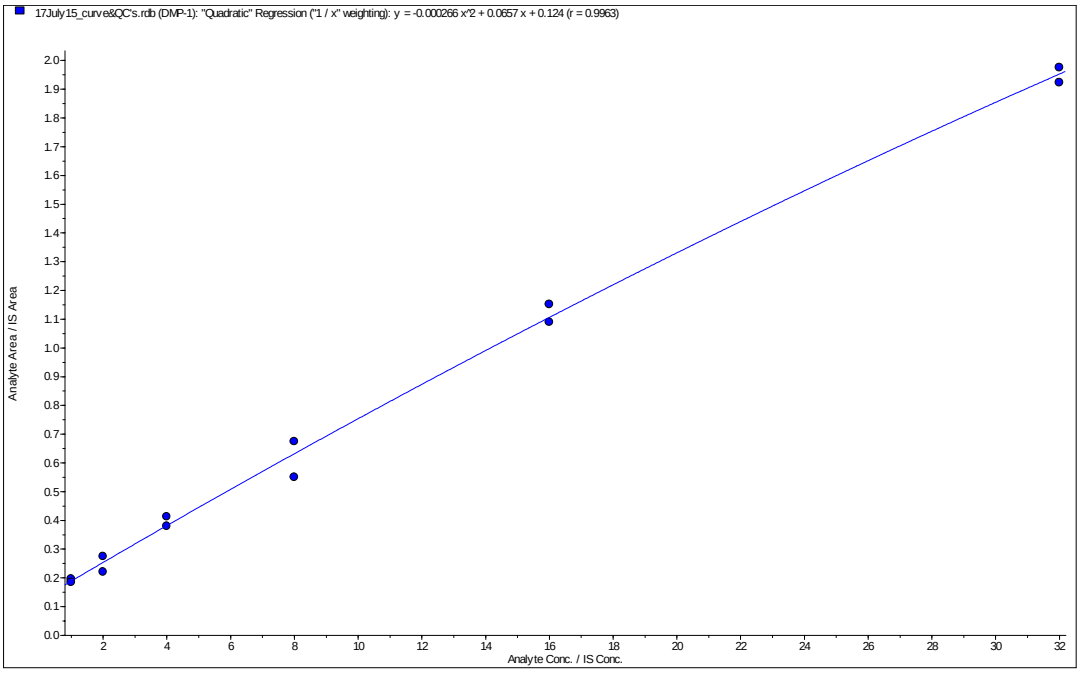


Figure 3.9 Calibration curve constructed by analysis of the STDs in validation batch 2, indicating the DMP concentration in each.

Table 3.17 Summary of the performance of the calibration standards of DMP of validation batch 2

Nominal Concentrations (ng/ml)	STD 6 1.00	STD 5 2.00	STD 4 4.00	STD 3 8.00	STD 2 16.0	STD 1 32.0
Mean Observed Concentration (ng/ml)	0.952	1.95	4.28	8.47	14.9	32.5
Standard Deviation	0.010	0.116	0.119	0.361	0.033	2.25
% CV	1.1	6.0	2.8	4.3	0.2	6.9
% Accuracy	95.2	97.3	107.1	105.9	93.2	101.5
N	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2

Table 3.18 Summary of the accuracy and precision of the quality control standards of DMP of validation batch 2

Nominal Concentrations (ng/ml)	QC 6 (LLOQ) 1.00	QC 5 (L) 2.00	QC 4 3.50	QC 2 (M) 12.5	QC 1 (H) 25.0
Mean Observed Concentration (ng/ml)	1.06	1.99	3.60	12.7	25.9
Standard Deviation	0.099	0.164	0.182	0.731	0.742
% CV	9.4	8.3	5.1	5.8	2.9
% Accuracy	106.2	99.4	102.9	101.7	103.8
N	5 of 6	6 of 6	6 of 6	6 of 6	6 of 6

The calculation of the concentrations of DMTP, using the analytical method and applying the acquired peak area ratios (using the relevant internal standard) to the calibration curve (figure 3.10), resulted in the values for the calibration standards and quality control standards as indicated in tables 3.19 and 3.20 respectively.

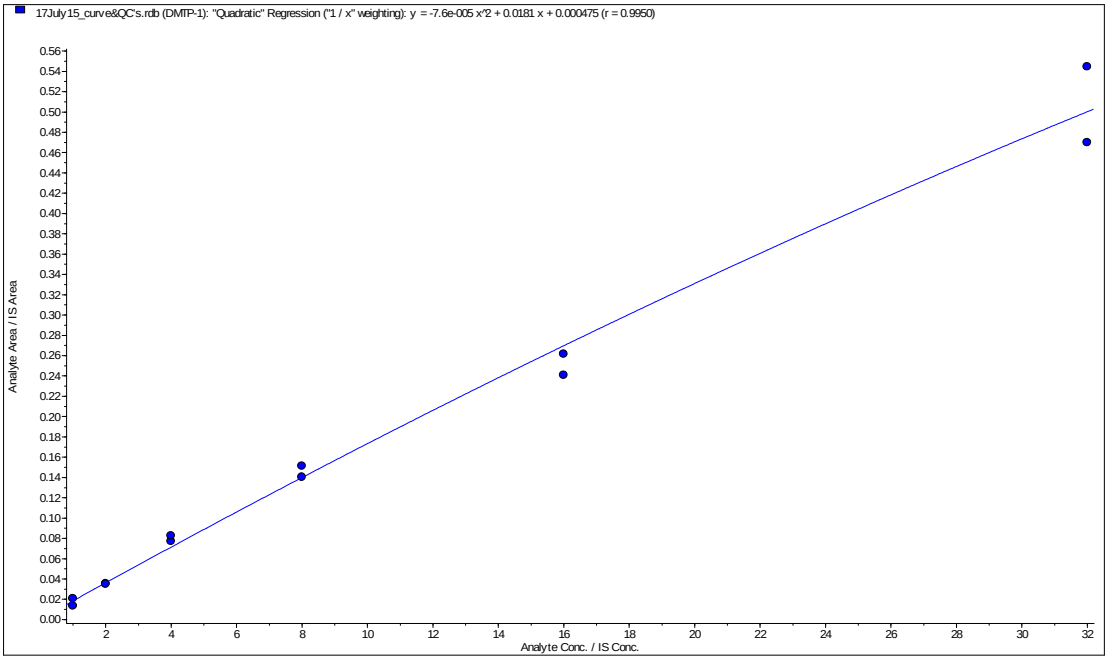


Figure 3.10 Calibration curve constructed by analysis of the STD's in validation batch 2, indicating the DMTP concentration in each.

Table 3.19 Summary of the performance of the calibration standards of DMTP of validation batch 2

Nominal Concentrations (ng/ml)	STD 6 1.00	STD 5 2.00	STD 4 4.00	STD 3 8.00	STD 2 16.0	STD 1 32.0
Mean Observed Concentration (ng/ml)	1.01	2.07	3.84	7.78	16.5	31.8
Standard Deviation	0.052	0.143	0.136	0.45	1.32	1.50
% CV	5.2	6.9	3.6	5.8	8.0	4.7
% Accuracy	100.8	103.4	96.0	97.2	103.3	99.3
N	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2

Table 3.20 Summary of the accuracy and precision of the quality control standards of DMTP of validation batch 2

Nominal Concentrations (ng/ml)	QC 6 (LLOQ) 1.00	QC 5 (L) 2.00	QC 4 3.50	QC 2 (M) 12.5	QC 1 (H) 25.0
Mean Observed Concentration (ng/ml)	1.00	2.06	3.59	12.4	24.3
Standard Deviation	0.115	0.191	0.258	0.718	0.560
% CV	11.5	9.3	7.2	5.8	2.3
% Accuracy	100.5	102.8	102.5	99.3	97.0
N	6 of 6	5 of 6	6 of 6	6 of 6	6 of 6

The calculation of the concentrations of DEP, using the analytical method and applying the acquired peak area ratios (using the relevant internal standard) to the calibration curve (figure 3.11), resulted in the values for the calibration standards and quality control standards as indicated in tables 3.21 and 3.22 respectively.

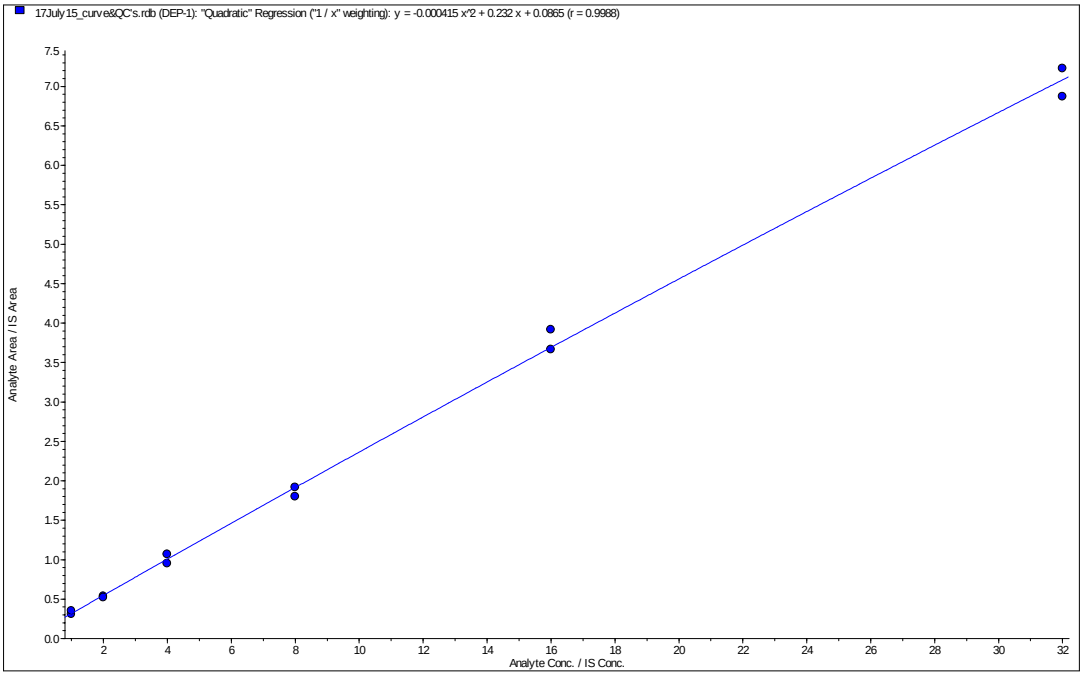


Figure 3.11 Calibration curve constructed by analysis of the STDs in validation batch 2, indicating the DEP concentration in each.

Table 3.21 Summary of the performance of the calibration standards of DEP of validation batch 2

Nominal Concentrations (ng/ml)	STD 6 1.00	STD 5 2.00	STD 4 4.00	STD 3 8.00	STD 2 16.0	STD 1 32.0
Mean Observed Concentration (ng/ml)	0.983	1.99	3.94	8.54	15.3	32.2
Standard Deviation	0.053	0.089	0.245	0.048	0.0235	1.22
% CV	5.4	4.5	6.2	0.6	0.2	3.8
% Accuracy	98.3	99.9	98.6	106.8	95.7	100.7
N	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2

Table 3.22 Summary of the accuracy and precision of the quality control standards of DEP of validation batch 2

Nominal Concentrations (ng/ml)	QC 6 (LLOQ) 1.00	QC 5 (L) 2.00	QC 4 3.50	QC 2 (M) 12.5	QC 1 (H) 25.0
Mean Observed Concentration (ng/ml)	1.01	2.13	3.77	12.9	24.9
Standard Deviation	0.091	0.076	0.183	0.313	1.08
% CV	9.1	3.6	4.9	2.4	4.3
% Accuracy	100.6	106.7	107.7	103.5	99.7
N	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6

3.4.1.1.3 Validation batch 3

The calculation of the concentrations of DMP, using the analytical method and applying the acquired peak area ratios (using the relevant internal standard) to the calibration curve (figure 3.12), resulted in the values for the calibration standards and quality control standards as indicated in tables 3.23 and 3.24 respectively.

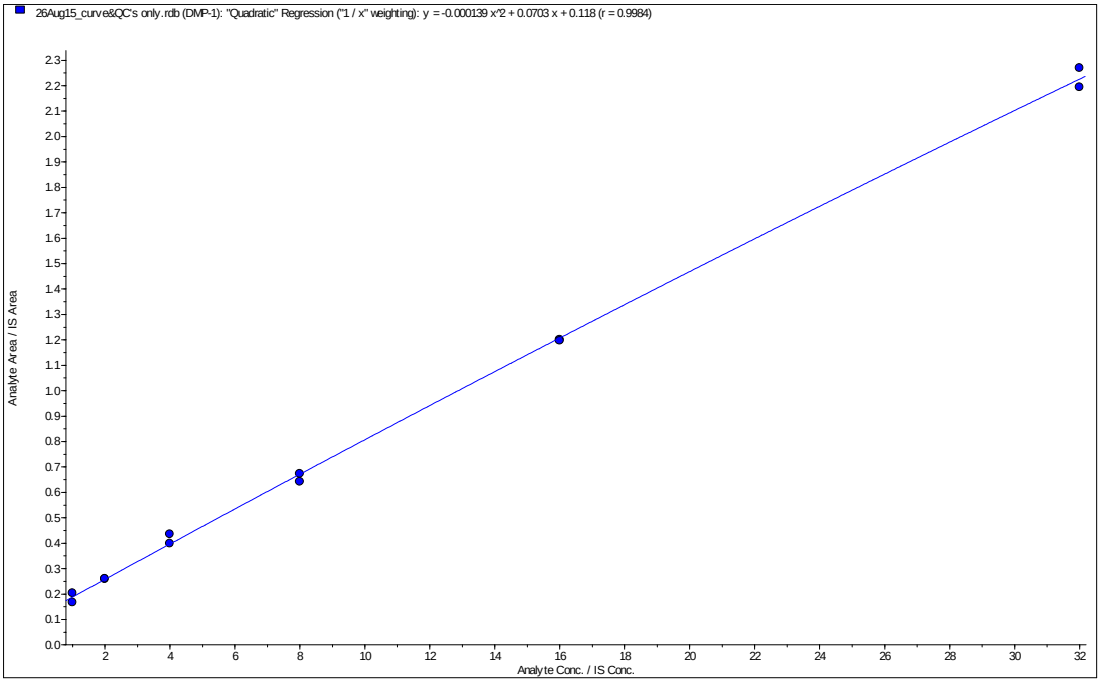


Figure 3.12 Calibration curve constructed by analysis of the STDs in validation batch 3, indicating the DMP concentration in each.

Table 3.23 Summary of the performance of the calibration standards of DMP of validation batch 3

Nominal Concentrations (ng/ml)	STD 6 1.00	STD 5 2.00	STD 4 4.00	STD 3 8.00	STD 2 16.0	STD 1 32.0
Mean Observed Concentration (ng/ml)	0.996	1.91	4.21	8.14	15.6	32.2
Standard Deviation	0.061	0.054	0.039	0.116	0.773	0.374
% CV	6.1	2.9	0.9	1.4	5.0	1.2
% Accuracy	99.6	95.6	105.2	101.7	97.5	100.5
N	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2

Table 3.24 Summary of the accuracy and precision of the quality control standards of DMP of validation batch 3

Nominal Concentrations (ng/ml)	QC 6 (LLOQ) 1.00	QC 5 (L) 2.00	QC 4 3.50	QC 2 (M) 12.5	QC 1 (H) 25.0
Mean Observed Concentration (ng/ml)	1.02	2.01	3.61	12.8	26.9
Standard Deviation	0.100	0.190	0.234	0.789	0.759
% CV	9.8	9.5	6.5	6.2	2.8
% Accuracy	102.2	100.6	103.3	102.5	107.5
N	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6

The calculation of the concentrations of DMTP, using the analytical method and applying the acquired peak area ratios (using the relevant internal standard) to the calibration curve (figure 3.13), resulted in the values for the calibration standards and quality control standards as indicated in tables 3.25 and 3.26 respectively.

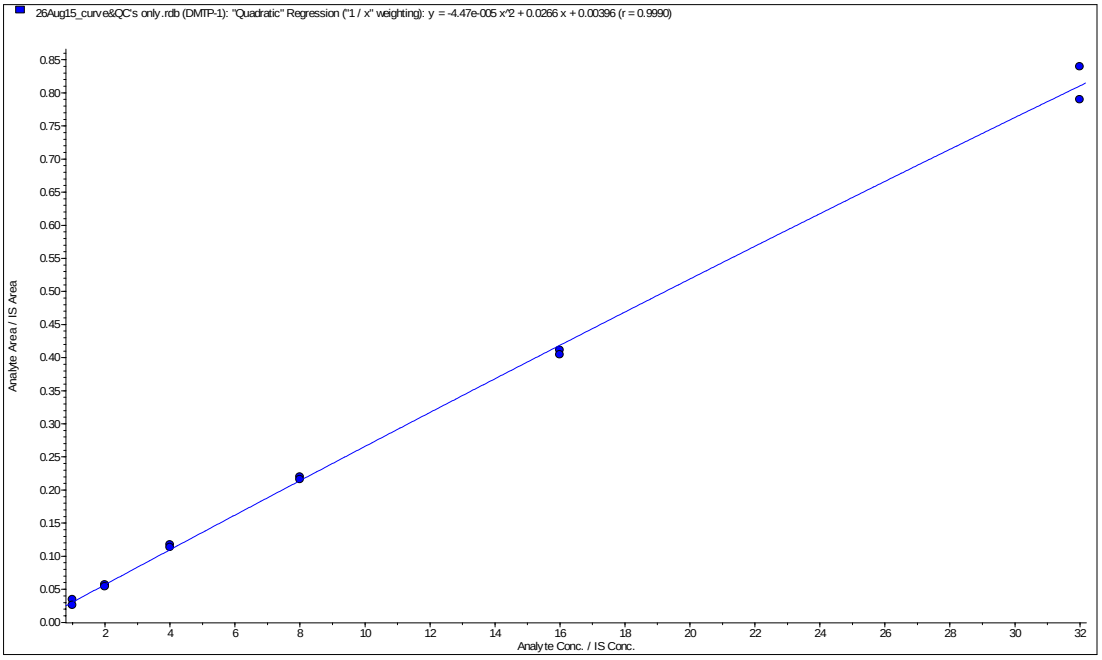


Figure 3.13 Calibration curve constructed by analysis of the STDs in validation batch 3, indicating the DMTP concentration in each.

Table 3.25 Summary of the performance of the calibration standards of DMTP of validation batch 3

Nominal Concentrations (ng/ml)	STD 6 1.00	STD 5 2.00	STD 4 4.00	STD 3 8.00	STD 2 16.0	STD 1 32.0
Mean Observed Concentration (ng/ml)	1.08	1.86	3.96	7.87	16.4	31.8
Standard Deviation	0.085	0.078	0.116	0.460	0.420	1.37
% CV	7.9	4.2	2.9	5.8	2.6	4.3
% Accuracy	107.6	93.1	98.9	98.4	102.6	99.5
N	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2

Table 3.26 Summary of the accuracy and precision of the quality control standards of DMTP of validation batch 3

Nominal Concentrations (ng/ml)	QC 6 (LLOQ) 1.00	QC 5 (L) 2.00	QC 4 3.50	QC 2 (M) 12.5	QC 1 (H) 25.0
Mean Observed Concentration (ng/ml)	1.00	2.06	3.63	12.8	26.1
Standard Deviation	0.095	0.090	0.198	0.505	1.66
% CV	9.9	4.3	5.5	3.9	6.3
% Accuracy	96.8	105.2	103.6	102.6	104.4
N	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6

The calculation of the concentrations of DEP, using the analytical method and applying the acquired peak area ratios (using the relevant internal standard) to the calibration curve (figure 3.14), resulted in the values for the calibration standards and quality control standards as indicated in tables 3.27 and 3.28 respectively.

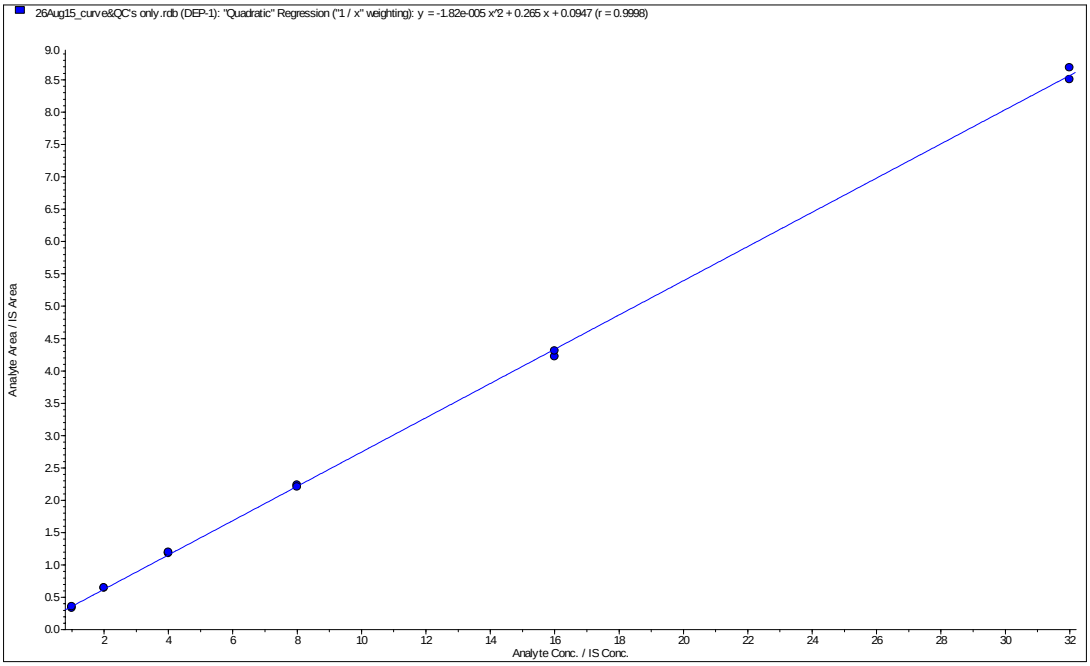


Figure 3.14 Calibration curve constructed by analysis of the STDs in validation batch 3, indicating the DEP concentration in each

Table 3.27 Summary of the performance of the calibration standards of DEP of validation batch 3

Nominal Concentrations (ng/ml)	STD 6 1.00	STD 5 2.00	STD 4 4.00	STD 3 8.00	STD 2 16.0	STD 1 32.0
Mean Observed Concentration (ng/ml)	1.04	1.96	3.87	7.97	16.3	31.9
Standard Deviation	0.033	0.087	0.168	0.019	0.396	1.18
% CV	3.2	4.5	4.4	0.2	2.4	3.7
% Accuracy	104.4	98.0	96.7	99.7	101.7	99.6
N	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2

Table 3.28 Summary of the accuracy and precision of the quality control standards of DEP of validation batch 3

Nominal Concentrations (ng/ml)	QC 6 (LLOQ) 1.00	QC 5 (L) 2.00	QC 4 3.50	QC 2 (M) 12.5	QC 1 (H) 25.0
Mean Observed Concentration (ng/ml)	0.976	2.13	3.64	12.9	26.5
Standard Deviation	0.074	0.083	0.119	0.373	0.988
% CV	7.6	3.9	3.3	2.9	3.7
% Accuracy	97.6	106.4	103.9	103.2	105.9
N	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6

3.4.1.1.4 Reinjection of validation batch 2

The calculation of the concentrations of DMP, using the analytical method and applying the acquired peak area ratios (using the relevant internal standard) to the calibration curve (figure 3.15), resulted in the values for the calibration standards and quality control standards as indicated in tables 3.29 and 3.30 respectively.

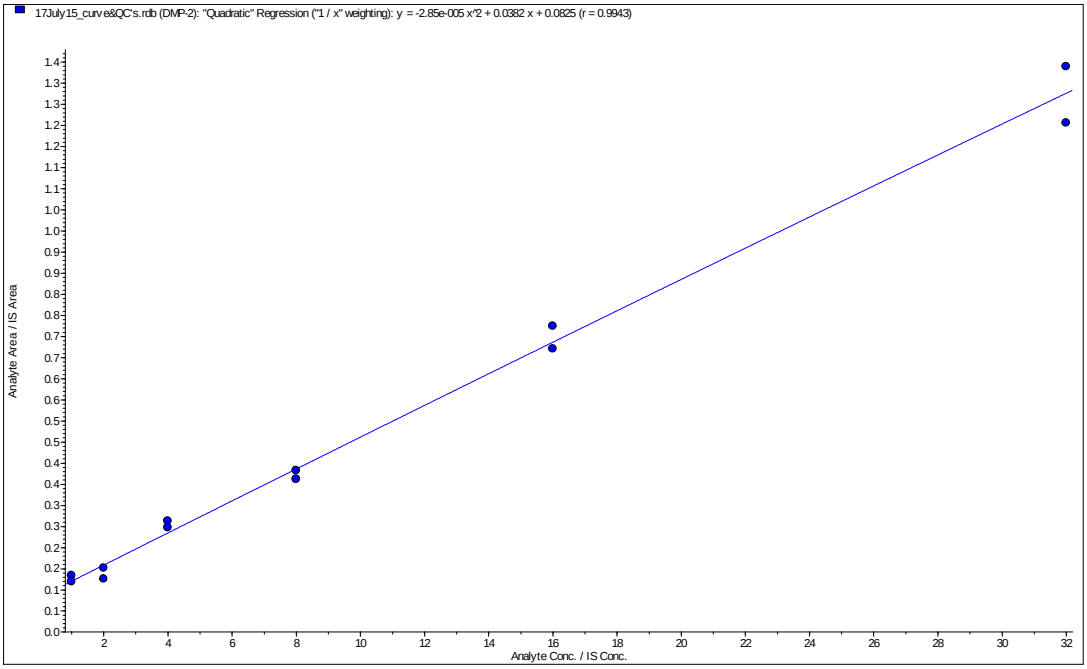


Figure 3.15 Calibration curve constructed by analysis of the STDs in the reinjection of validation batch 2, indicating the DMP concentration in each

Table 3.29 Summary of the performance of the calibration standards of DMP of reinjection of validation batch 2

Nominal Concentrations (ng/ml)	STD 6 1.00	STD 5 2.00	STD 4 4.00	STD 3 8.00	STD 2 16.0	STD 1 32.0
Mean Observed Concentration (ng/ml)	1.04	1.95	3.84	8.30	15.9	32.0
Standard Deviation	0.005	0.161	0.223	0.326	1.091	0.076
% CV	0.5	8.3	5.8	3.9	6.9	0.2
% Accuracy	103.6	97.6	96.0	103.7	99.1	100.1
N	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2

Table 3.30 Summary of the accuracy and precision of the quality control standards of DMP of reinjection of validation batch 2

Nominal Concentrations (ng/ml)	QC 6 (LLOQ) 1.00	QC 5 (L) 2.00	QC 4 3.50	QC 2 (M) 12.5	QC 1 (H) 25.0
Mean Observed Concentration (ng/ml)	1.03	2.26	3.69	12.8	27.0
Standard Deviation	0.103	0.110	0.239	0.621	0.929
% CV	10.1	4.9	6.5	4.9	3.4
% Accuracy	102.8	112.8	105.3	102.5	108.2
N	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6

The calculation of the concentrations of DMTP, using the analytical method and applying the acquired peak area ratios (using the relevant internal standard) to the calibration curve (figure 3.16) resulted in the values for the calibration standards and quality control standards as indicated in tables 3.31 and 3.32 respectively.

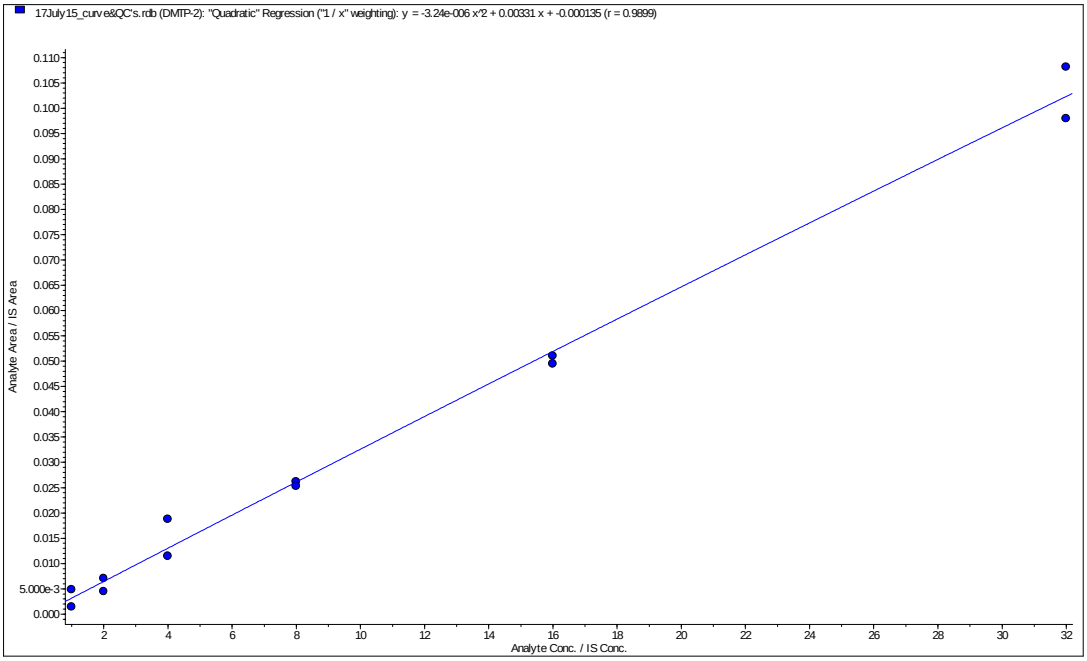


Figure 3.16 Calibration curve constructed by analysis of the STDs in the reinjection of validation batch 2, indicating the DMTP concentration in each.

Table 3.31 Summary of the performance of the calibration standards of DMTP of reinjection of validation batch 2)

Nominal Concentrations (ng/ml)	STD 6 1.00	STD 5 2.00	STD 4 4.00	STD 3 8.00	STD 2 16.0	STD 1 32.0
Mean Observed Concentration (ng/ml)	1.02	1.91	3.99	8.15	15.9	32.1
Standard Deviation	0.112	N/A	0.215	0.202	1.372	0.173
% CV	11.1	N/A	5.4	2.5	8.7	0.5
% Accuracy	101.6	95.3	99.6	101.9	99.1	100.1
N	2 of 2	1 of 2	2 of 2	2 of 2	2 of 2	2 of 2

Table 3.32 Summary of the accuracy and precision of the quality control standards of DMTP of reinjection of validation batch 2

Nominal Concentrations (ng/ml)	QC 6 (LLOQ) 1.00	QC 5 (L) 2.00	QC 4 3.50	QC 2 (M) 12.5	QC 1 (H) 25.0
Mean Observed Concentration (ng/ml)	1.08	2.20	3.81	12.4	24.2
Standard Deviation	0.062	0.142	0.143	0.580	0.920
% CV	5.8	6.5	3.8	4.7	3.8
% Accuracy	107.9	109.8	108.9	99.5	96.8
N	6 of 6	6 of 6	6 of 6	6 of 6	6 of 6

The calculation of the concentrations of DEP, using the analytical method and applying the acquired peak area ratios (using the relevant internal standard) to the calibration curve (figure 3.17), resulted in the values for the calibration standards and quality control standards as indicated in tables 3.33 and 3.34 respectively.

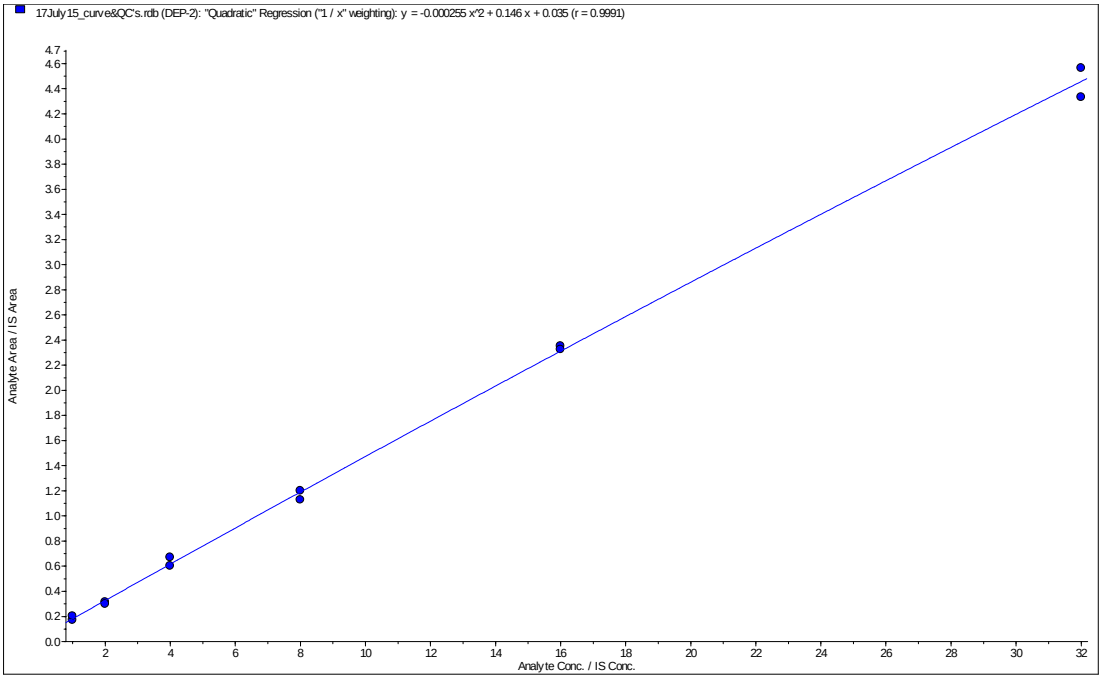


Figure 3.17 Calibration curve constructed by analysis of the STDs in the reinjection of validation batch 2, indicating the DEP concentration in each

Table 3.33 Summary of the performance of the calibration standards of DEP of reinjection of validation batch 2

Nominal Concentrations (ng/ml)	STD 6 1.00	STD 5 2.00	STD 4 4.00	STD 3 8.00	STD 2 16.0	STD 1 32.0
Mean Observed Concentration (ng/ml)	1.00	1.99	3.92	8.28	15.7	32.1
Standard Deviation	0.092	0.047	0.260	0.123	1.40	1.56
% CV	9.3	2.4	6.6	1.5	8.9	4.8
% Accuracy	100.1	99.9	98.0	103.5	98.2	100.3
N	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2	2 of 2

Table 3.34 Summary of the accuracy and precision of the quality control standards of DEP of reinjection of validation batch 2

Nominal Concentrations (ng/ml)	QC 6 (LLOQ) 1.00	QC 5 (L) 2.00	QC 4 3.50	QC 2 (M) 12.5	QC 1 (H) 25.0
Mean Observed Concentration (ng/ml)	1.03	2.14	3.82	12.9	25.3
Standard Deviation	0.095	0.041	0.137	0.355	0.753
% CV	9.3	2.0	3.6	2.8	3.0
% Accuracy	102.8	106.9	109.1	102.9	101.4
N	6 of 6	4 of 6	6 of 6	6 of 6	6 of 6

The acceptance criteria for intra-batch accuracy and precision were met for all the compounds.

3.4.1.2 Inter-batch perfomance of the method

The inter-batch validation is a comparison of the values of the calibration standards, the quality control standards and the parameters of the calibration curves of the three independent validation batches.

For the analysis of DMP, the inter-batch comparison of the calibration curve parameters is depicted in table 3.35.

Table 3.35 The inter-batch comparison of the calibration curve parameters of DMP measured during the three validation batches

Validation batch	Calibration Curve Parameters (DMP)			
	A	**B**	**C**	**R-Squared (r^2)**
Validation 1	-0.000292	0.0663	0.126	0.9980
Validation 2	0.000283	0.0636	0.104	0.9978
Validation 3	-0.000927	0.0566	0.133	0.9994
Mean	-0.000212	0.06216	0.121	0.9984
% CV	-61.094	8.053	12.506	0.1

Note: Quadratic regression model, weighted by 1/concentration (Resp. = $AX^2 + BX + C$)

Comparison of the values of the DMP calibration standards between the validation batches are shown in table 3.36.

Table 3.36 Back-calculated calibration standard concentrations of DMP in ng/ml for the three validation batches

Validation batch	Replicates	STD 6 1.00	STD 5 2.00	STD 4 4.00	STD 3 8.00	STD 2 16.0	STD 1 32.0
Validation 1	**1**	1.05	2.09	4.41	8.58	16.7	31.4
	2	0.88	*1.59	3.88	6.88	15.6	32.5
Validation 2	**1**	0.96	1.86	4.37	8.73	14.9	34.1
	2	0.94	2.03	4.20	8.22	14.9	30.9
Validation 3	**1**	0.95	1.87	4.18	8.22	16.1	32.4
	2	1.04	1.95	4.24	8.05	15.1	31.9
Mean		0.971	1.960	4.213	8.11	15.55	32.20
% CV		6.6	5.1	4.5	8.1	4.7	3.4
STDEV		0.064	0.100	0.187	0.655	0.731	1.110
%Accuracy		97.1	98.0	105.3	101.4	97.2	100.6
N		6	5	6	6	6	6

Code: * = Deactivated calibration standard (% Dev > 15%)

Comparison of the values of the DMP quality control samples between the validation batches are shown in table 3.37.

Table 3.37 Statistics of quality control samples of DMP for the three validation batches concentration in ng/ml

Validation batch	Replicates	QC 6 LLOQ 1.00	QC 5 Low 2.00	QC 4 3.50	QC 2 Med 12.5	QC 1 High 25.0
Validation 1	1	0.902	2.01	3.41	13.5	25.2
	2	0.99	#2.71	3.39	14.1	23.0
	3	1.25	#1.55	3.81	11.8	25.1
	4	0.997	1.68	3.30	12.1	23.7
	5	0.848	1.86	3.48	11.3	24.0
	6	0.965	2.00	3.08	13.1	22.3
Validation 2	1	0.909	2.08	3.56	12.2	25.1
	2	1.10	2.02	3.93	13.1	26.2
	3	1.13	1.78	3.56	13.1	26.8
	4	1.15	1.79	3.44	13.2	26.7
	5	1.02	2.07	3.67	11.5	25.6
	6	#1.22	2.18	3.44	13.3	25.2
Validation 3	1	1.09	2.12	3.63	12.8	26.3
	2	0.939	1.79	3.93	12.7	28.2
	3	1.06	1.93	3.56	13.7	27.0
	4	0.878	2.27	3.44	13.2	26.3
	5	1.15	2.13	3.67	13.1	26.2
	6	1.01	1.83	3.44	11.4	27.2
Mean		1.022	1.971	3.542	12.73	25.56
% CV		10.9	8.6	6.4	6.5	6.0
STDEV		0.111	0.168	0.228	0.826	1.539
Accuracy		102.3	98.6	101.2	101.9	102.2
N		17	16	18	18	18

Code: # = Deactivated quality control sample

For the analysis of DMTP, the inter-batch comparison of the calibration curve parameters are depicted in table 3.38.

Table 3.38 The inter-batch comparison of the calibration curve parameters of DMTP measured during the three validation batches.

Validation batch	Calibration Curve Parameters (DMTP)			
	A	B	C	R-Squared (r^2)
Validation 1	-0.0000549	0.0172	0.00328	0.9968
Validation 2	-0.000163	0.0289	0.00235	0.9985
Validation 3	-0.0000796	0.0253	0.00745	0.9991
Mean	-0.000099	0.0283	0.00436	0.99813
% CV	-57.120	25.179	62.296	0.1

Note: Quadratic regression model, weighted by 1/concentration (Resp. = $AX^2 + BX + C$)

Comparison of the values of the DMTP calibration standards between the validation batches are shown in table 3.39

Table 3.39 Back-calculated calibration standard concentrations of DMTP in ng/ml for the three validation batches

Validation batch	Replicates	STD 6	STD 5	STD 4	STD 3	STD 2	STD 1
		1.00	**2.00**	**4.00**	**8.00**	**16.0**	**32.0**
Validation 1	**1**	*0.584	1.88	4.35	8.18	14.5	30.1
	2	1.02	1.85	3.97	8.85	15.8	34.5
Validation 2	**1**	1.04	1.97	3.74	8.09	15.6	30.7
	2	0.97	2.17	3.94	7.46	17.5	32.8
Validation 3	**1**	1.14	1.92	3.87	7.55	16.7	30.9
	2	1.02	1.81	4.04	8.20	16.1	32.8
Mean		1.04	1.93	3.99	8.06	16.0	32.0
% CV		6.0	6.6	5.2	6.3	6.4	5.2
STDEV		0.062	0.128	0.205	0.505	1.02	1.68
%Accuracy		103.8	96.7	99.6	100.7	100.2	99.9
N		5	6	6	6	6	6

Code: * = Deactivated calibration standard (% Dev > 15%)

Comparison of the values of the DMTP quality control standards between the validation batches are shown in table 3.40

Table 3.40 Statistics of quality control samples of DMTP for the three validation batches concentration in ng/ml

Validation batch	Replicates	QC 6 LLOQ 1.00	QC 5 Low 2.00	QC 4 3.50	QC 2 Med 12.5	QC 1 High 25.0
Validation 1	1	1.10	1.75	3.09	10.9	23.3
	2	#0.766	1.82	3.45	13.5	23.0
	3	0.807	#1.54	3.06	12.2	23.8
	4	1.07	2.01	3.52	12.6	23.4
	5	0.925	2.14	3.65	12.0	24.6
	6	1.15	#2.64	3.98	12.4	25.9
Validation 2	1	0.854	#1.65	3.35	12.2	23.9
	2	0.925	1.86	3.42	11.8	24.0
	3	0.929	1.86	3.31	12.5	24.0
	4	1.09	2.08	3.77	11.8	24.7
	5	1.13	2.20	3.93	12.5	25.2
	6	1.09	2.28	3.74	13.7	23.7
Validation 3	1	0.955	2.14	3.64	12.8	25.5
	2	0.854	2.21	3.42	12.5	26.7
	3	0.935	1.98	3.49	12.4	23.5
	4	0.925	2.02	3.47	12.8	27.0
	5	1.00	2.09	3.86	13.8	25.5
	6	1.14	2.19	3.87	12.7	28.4
Mean		0.992	2.04	3.56	12.5	24.8
% CV		11.2	7.9	7.6	5.6	6.0
STDEV		0.111	0.160	0.269	0.700	1.48
Accuracy		99.3	102.1	101.6	100.0	99.1
N		17	15	18	18	18

Code: # = Deactivated quality control sample

For the analysis of DEP, the inter-batch comparison of the calibration curve parameters are depicted in table 3.41

Table 3.41 The inter-batch comparison of the calibration curve parameters of DEP measured during the three validation batches

Validation batch	Calibration Curve Parameters (DEP)			
	A	B	C	R-Squared (r^2)
Validation 1	-0.000447	0.233	0.0807	0.9989
Validation 2	0.000118	0.237	0.111	0.9989
Validation 3	-0.000341	0.239	0.0875	0.9995
Mean	-0.000302	0.236	0.0930	0.9991
% CV	-55.602	1.293	17.083	0.1

Note: Quadratic regression model, weighted by 1/concentration (Resp. = $AX^2 + BX + C$)

Comparison of the values of the DEP calibration standards between the validation batches are shown in table 3.42

Table 3.42 Back-calculated calibration standard concentrations of DEP in ng/ml for the three validation batches

Validation batch	Replicates	STD 6	STD 5	STD 4	STD 3	STD 2	STD 1
		1.00	**2.00**	**4.00**	**8.00**	**16.0**	**32.0**
Validation 1	**1**	0.979	1.96	3.76	7.48	15.9	31.0
	2	1.11	1.89	4.27	8.00	17.0	32.7
Validation 2	**1**	1.02	2.06	4.12	8.58	15.3	31.4
	2	0.945	1.94	3.77	8.51	15.3	33.1
Validation 3	**1**	1.07	1.90	3.99	7.99	16.6	32.7
	2	1.02	2.02	3.75	7.96	16.0	31.1
Mean		1.02	1.96	3.943	8.09	16.0	32.0
% CV		5.8	3.4	5.6	5.0	4.3	2.9
STDEV		0.059	0.067	0.219	0.405	0.685	0.933
%Accuracy		102.4	98.1	98.6	101.1	100.1	100.0
N		6	6	6	6	6	6

Comparison of the values of the DEP quality control standards between the validation batches are shown in table 3.43

Table 3.43 Statistics of quality control samples of DEP for the three validation batches concentration in ng/ml

Validation batch	Replicates	QC 6 LLOQ	QC 5 Low	QC 4	QC 2 Med	QC 1 High
		1.00	**2.00**	**3.50**	**12.50**	**25.0**
Validation 1	**1**	0.835	1.91	3.44	11.5	23.4
	2	0.902	2.04	3.70	12.2	21.6
	3	0.944	2.13	#4.12	12.2	26.6
	4	1.00	2.14	3.95	12.7	23.0
	5	1.05	2.15	3.81	12.7	25.3
	6	1.06	2.04	#4.15	14.1	23.1
Validation 2	**1**	0.874	2.13	3.43	12.4	25.0
	2	0.940	2.26	3.95	12.8	24.7
	3	1.11	2.07	3.87	13.3	25.5
	4	1.01	2.04	3.81	12.9	25.6
	5	0.999	2.14	3.86	13.1	25.9
	6	1.1	2.15	3.70	13.1	22.9
Validation 3	**1**	0.865	2.11	3.70	13.2	26.8
	2	0.933	2.02	3.50	12.5	27.7
	3	0.996	2.16	3.59	12.5	26.5
	4	0.991	2.06	3.52	13.4	24.9
	5	1.04	2.20	3.80	13.1	27.1
	6	1.07	2.24	3.71	12.7	25.9
Mean		0.982	2.11	3.71	12.8	25.1
% CV		8.3	4.0	4.6	4.5	6.7
STDEV		0.081	0.084	0.170	0.569	1.68
Accuracy		98.2	105.5	106.0	102.4	100.3
N		18	18	16	18	18

Code: # = Deactivated quality control sample

The acceptance criteria for accuracy and precision were met for the inter-batch validation.

3.4.2 Stability assessments

3.4.2.1 On-Instrument stability

Six individual aliquots of the stability samples at high and low concentration levels (estimated QC_{low} at 2-3 times that of the LLOQ and estimated QC_{high} at about 80% of the ULOQ) were extracted according to the sample preparation method. These were immediately injected and the peak area ratios calculated. The extracted samples were then left for a period of 24 hours in the auto sampler at 5°C and then re-analyzed and the peak area ratios then compared to those of the first injection series.

The results, expressed as the area ratios, are depicted for DMP, DMTP and DEP in tables 3.44 to

3.46

Table 3.44 Results for the assessment of on-instrument stability of DMP following extraction from stripped urine

Sample	High Concentration (25 ng/ml)		Low Concentration (2 ng/ml)	
	First Injection Peak Area Ratio	Second injection Peak Area Ratio	First injection Peak Area Ratio	Second injection Peak Area Ratio
1	1.52	1.59	0.2352	0.241
2	1.56	1.63	0.2320	0.259
3	1.61	1.62	0.2163	0.242
4	1.60	1.64	0.2175	0.237
5	1.55	1.61	0.2343	0.253
6	1.53	1.51	0.2414	0.260
Average	1.56	1.60	0.2295	0.249
STDEV	0	0.047	0.01021	0.009
% CV	2.4	2.9	4.4	4.0
% Difference	-	2.6	-	8.6

Table 3.45 Results for the assessment of on-instrument stability of DMTP following extraction from stripped urine

Sample	High Concentration (25 ng/ml)		Low Concentration (2 ng/ml)	
	First Injection Peak Area Ratio	Second injection Peak Area Ratio	First injection Peak Area Ratio	Second injection Peak Area Ratio
1	0.60	0.62	0.0495	0.066
2	0.60	0.63	0.0554	0.058
3	0.60	0.66	0.0553	0.066
4	0.61	0.66	0.0617	0.061
5	0.62	0.66	0.0649	0.067
6	0.60	0.61	0.0670	0.068
Average	0.61	0.64	0.0590	0.064
STDEV	0	0.022	0.00668	0.004
% CV	1.7	3.4	11.3	6.2
% Difference	-	5.8	-	6.8

Table 3.46 Results for the assessment of on-instrument stability of DEP following extraction from stripped urine

Sample	High Concentration (25 ng/ml)		Low Concentration (2 ng/ml)	
	First Injection Peak Area Ratio	Second injection Peak Area Ratio	First injection Peak Area Ratio	Second injection Peak Area Ratio
1	5.96	5.84	0.6149	0.636
2	5.90	6.07	0.6460	0.629
3	6.07	5.98	0.6012	0.643
4	6.08	5.98	0.5944	0.620
5	6.16	6.05	0.6171	0.680
6	5.47	5.65	0.6205	0.686
Average	5.94	5.93	0.6157	0.649
STDEV	0	0.159	0.01793	0.027
% CV	4.2	2.7	2.9	4.2
% Difference	-	-0.2	-	5.5

The results obtained from the on-instrument stability assessment met the required acceptance criteria of % Dev < 15% as well as a % CV < 15% for the analytes and indicated stability for at least 24 hours.

This stability assessment also proves on-instrument stability of the internal standards since these were included in the sample preparation method.

3.4.2.2 Freeze-thaw stability

Six samples prepared in stripped urine at a concentration of 25 ng/ml of all three analytes, were stored at -80°C for 12 hours. The samples were then left at room temperature to thaw unassisted. After complete thawing, the samples were refrozen for 12 hours and the process repeated twice. Following the third thawing cycle, the samples were analyzed together with a similar set of six quality control samples at the same concentration of the analytes. Comparison of the two sets of data will indicate the stability of the analytes during three freeze-thaw cycles.

Table 3.47 – 3.49 indicates the values obtained during this comparative analysis for each of the analytes.

Table 3.47 Results for the freeze-thaw stability of DMP

Peak area	Freeze-thaw stability (DMP)	
	Fresh (Reference)	Control (~ -80 °C Freeze Thaw)
1	295000	295000
2	283000	290000
3	274000	299000
4	284000	299000
5	266000	299000
6	273000	293000
Average	279167	295833
STDEV	10265	3817
% CV	3.7	0.3
% Difference	N/A	6.0

Table 3.48 Results for the freeze-thaw stability of DMTP

Peak area	Freeze-thaw stability (DMTP)	
	Fresh (Reference)	Control (~ -80 °C Freeze Thaw)
1	128000	103000
2	134000	101000
3	134000	100000
4	137000	101000
5	130000	111000
6	131000	100000
Average	132333	102667
STDEV	3266	4227
% CV	2.5	4.1
% Difference	N/A	-22.4

Table 3.49 Results for the freeze-thaw stability of DEP

Peak area	Freeze-thaw stability (DEP)	
	Fresh (Reference)	Control (~ -80 °C Freeze Thaw)
1	625000	593000
2	644000	583000
3	640000	556000
4	631000	586000
5	628000	622000
6	670000	592000
Average	639667	588667
STDEV	16525	21201
% CV	2.6	3.6
% Difference	N/A	6.0

The data indicate that DMP and DEP are stable during three freeze thaw cycles. DMTP is however unstable under these conditions and consequently samples were not subjected to three freeze thaw cycles.

3.4.3 Stability of stock solutions

Separate stock solutions of DMP, DMTP and DEP were prepared in methanol. Aliquots of each solution were kept at room temperature and at approximately - 20ºC and - 80ºC for at least 8 hours (test solutions). The test solutions were then prepared for analysis. Similar reference solutions were prepared from fresh stock solutions. Analytical comparison was then made between the reference solutions and the test solutions. The results for the three analytes are shown in tables 3.50 to 3.52.

Table 3.50 Results for the stock solution stability of DMP. Results in peak areas are shown.

Replicate	Stock Solution Peak Area (DMP)		
	Fresh (Reference)	Control~ -20 °C	Control~ -80 °C
1	295000	285000	312000
2	283000	294000	290000
3	274000	276000	289000
4	284000	292000	299000
5	266000	291000	309000
6	273000	292000	313000
Average	279167	288333	302000
STDEV	10265	6772	10881
% CV	3.7	2.3	3.6
% Difference	N/A	3.3	8.2

Table 3.51 Results for the stock solution stability of DMTP. Results in peak areas are shown.

Replicate	Stock Solution Peak Area (DMTP)		
	Fresh (Reference)	Control~ -20 °C	Control~ -80 °C
1	128000	103000	116000
2	134000	111000	112000
3	134000	109000	104000
4	137000	100000	110000
5	130000	111000	104000
6	131000	112000	107000
Average	132333	107667	108833
STDEV	3266	4967	4750
% CV	2.5	4.6	4.4
% Difference	N/A	-18.6	-17.8

Table 3.52 Results for the stock solution stability of DEP. Results in peak areas are shown.

Replicate	Stock Solution Peak Area (DEP)		
	Fresh (Reference)	Control~ -20 °C	Control~ -80 °C
1	625000	554000	558000
2	644000	578000	601000
3	640000	555000	576000
4	631000	543000	564000
5	628000	594000	585000
6	670000	581000	608000
Average	639667	567500	582000
STDEV	16525	19665	19910
% CV	2.6	3.5	3.4
% Difference	N/A	3.3	8.2

The results indicate that DMP and DEP are stable in methanol for 8 days, when stored at room temperature and at ~ -20ºC and -80ºC. DMTP however proofed to be unstable when stored at -20°C and -80°C and consequently stock solutions were freshly prepared when used. No assessments were performed on the stability of stock solutions of the internal standards.

3.5 RECOVERY

The extraction recovery pertains to the extraction efficiency of the analytical process within the limits of variability. It is determined by comparing the analytical response of blank matrix spiked with the analyte and extracted with the response of the blank matrix first extracted and then spiked with analyte (theoretical, represents 100% recovery).

No recovery of the ISTD was calculated.

Extracted (test) samples: A minimum of six QCs at each concentration level (low, medium and high) are extracted as per the analytical method.

Theoretical samples: Samples are spiked at each concentration level (relative to the final concentration of the corresponding extracted QC's level) in three fold using extracted blank matrix.

The analyte peak areas found after extraction are compared to the theoretical peak area expressed as a percentage recovery.

The mean recovery of a quantitative drug assay should be consistent and the precision of the measured recovery expressed as percentage coefficient of variation should not exceed 15 % for any particular concentration of the analyte at which it is determined.

Table 3.53 Recovery of DMP

Sample No.	High Concentration (25 ng/ml)		Medium Concentration (12.5 ng/ml)		Low Concentration (2 ng/ml)	
	Corrected Reference Peak Area	Test Peak Area	Corrected Reference Peak Area	Test Peak Area	Corrected Reference Peak Area	Test Peak Area
Sample 1	473000	158000	249000	84100	44200	24700
Sample 2	476000	175000	245000	89400	45200	23900
Sample 3	450000	164000	251000	88700	49000	22500
Sample 4	-	173000	-	90400	-	22400
Sample 5	-	158000	-	79100	-	25300
Sample 6	-	156000	-	84600	-	22400
Average	466333	164000	248333	86050	46133	23533
STDEV	14224	8622	3055	2879	2532	1114
% CV	3.1	5.3	1.2	3.3	5.5	4.7
% Recovery	35.2		34.7		51.0	
					%Average Recovery	40.3
					STDEV	9.3
					% CV	23.1

Table 3.54 Recovery of DMTP

Sample No.	High Concentration (25 ng/ml)		Medium Concentration (12.5 ng/ml)		Low Concentration (2 ng/ml)	
	Corrected Reference Peak Area	Test Peak Area	Corrected Reference Peak Area	Test Peak Area	Corrected Reference Peak Area	Test Peak Area
Sample 1	188000	175000	96300	88300	13400	13800
Sample 2	189000	187000	97800	96000	15000	16300
Sample 3	190000	177000	103000	94800	15000	15600
Sample 4	-	183000	-	101000	-	17100
Sample 5	-	179000	-	101000	-	16100
Sample 6	-	175000	-	96600	-	15200
Average	189000	179333	99033	96283	14467	15683
STDEV	1000	6429	3516	4143	924	1290
% CV	0.5	3.6	3.6	4.3	6.4	8.2
% Recovery	94.9		97.2		108.4	
					%Average Recovery	100.2
					STDEV	7.2
					% CV	7.2

Table 3.55 Recovery of DEP

Sample No.	High Concentration (25 ng/ml)		Medium Concentration (12.5 ng/ml)		Low Concentration (2 ng/ml)	
	Corrected Reference Peak Area	Test Peak Area	Corrected Reference Peak Area	Test Peak Area	Corrected Reference Peak Area	Test Peak Area
Sample 1	4140000	2040000	2230000	999000	274000	214000
Sample 2	4170000	2230000	2180000	1150000	329000	219000
Sample 3	4250000	2190000	2170000	1080000	349000	208000
Sample 4	-	2250000	-	1130000	-	214000
Sample 5	-	2150000	-	1060000	-	224000
Sample 6	-	1990000	-	1100000	-	206000
Average	4186667	2141667	2193333	1086500	317333	214167
STDEV	56862	100167	32146	75567	38837	5508
% CV	1.4	4.7	1.5	7.0	12.2	2.6
% Recovery	51.2		49.5		67.5	
					%Average Recovery	56.1
					STDEV	9.9
					% CV	17.7

The results indicate reproducible extraction efficiency for DMP, DMTP and DEP at high, medium and low concentrations.

3.6 APPLICATION OF THE ANALYTICAL METHOD

Urine samples originating from agricultural workers thought to be exposed to organophosphate pesticides were collected and stored at ~ -20°C until analysis. The validated method described in this study was used to assess the presence of the DAP metabolites, DMP, DMTP and DEP in the urine samples as markers to such exposure.

3.6.1 The performance of the analytical method

A total of 187 subject urine samples were prepared according to the validated method and were analyzed in three separate production batches, each including a full set of calibration standards and six quality control standards at each concentration level as used during the validations. Representative calibration curves for DMP, DMTP and DEP are presented in figures 3.18 to 3.20, respectively, as well as the results obtained from the STDs and QCs of each of the three individual production batches are displayed in tables 3.56 to 3.61

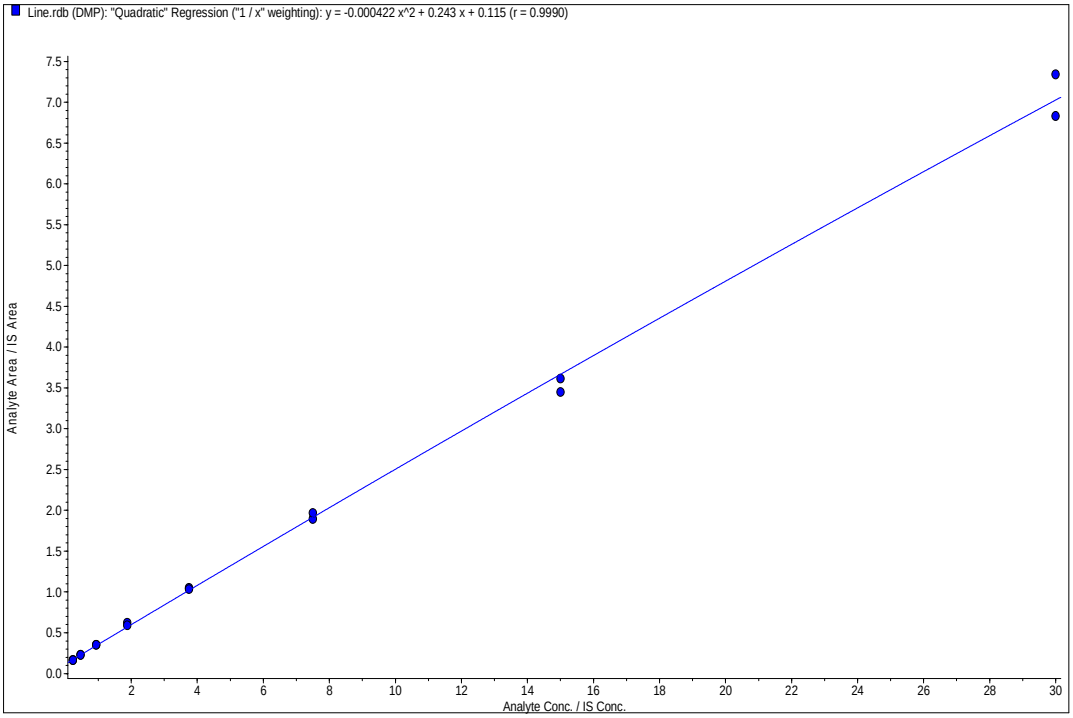


Figure 3.18 Calibration curve for the determination of DMP in subject samples. An unweighted quadratic calibration curve was selected with the coefficient of determination (r^2) = 0.9990.

Table 3.56 Back-calculated calibration standard concentrations of DMP in ng/ml for the three production batches

Production batch	Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I
		0.780	**1.56**	**3.13**	**6.25**	**12.5**	**25.0**	**50.0**	**100**
Production 1	**1**	0.726	1.63	3.07	6.29	12.1	25.7	50.2	99.9
	2	0.833	1.49	3.28	6.16	12.4	24.7	49.9	100
Production 2	**1**	0.722	1.48	3.38	6.27	12.1	25.3	49.4	103
	2	0.865	1.45	2.98	6.45	13.2	25.6	48.6	98.0
Production 3	**1**	0.837	1.48	3.50	6.69	12.7	25.6	49.2	*
	2	0.658	1.59	3.00	5.9	12.7	24.5	49.3	101
Mean		0.773	1.52	3.20	6.29	12.5	25.2	49.4	100
% CV		10.69	4.74	6.75	4.23	3.37	2.03	1.13	1.81
% Bias		-0.833	-2.56	2.29	0.693	0.266	0.933	-1.133	0.380
N		6	6	6	6	6	6	6	5

Code: * = Deactivated calibration standard (% Dev > 15%)

Table 3.57 Statistics for the DMP contents of quality control samples for the three production batches (concentration is in ng/ml)

Production batch	Replicates	QC A	QC B	QC C	QC D	QC E
		2.00	**4.00**	**12.0**	**30.0**	**80.0**
Production 1	**1**	2.03	4.28	11.8	29.0	79.5
	2	2.22	3.91	11.4	29.4	79.0
Production 2	**1**	2.27	3.88	11.6	29.3	78.5
	2	2.06	4.31	11.7	29.1	76.7
Production 3	**1**	2.18	4.2	12.4	29.2	77.6
	2	2.01	3.71	12.6	29.2	78.3
Mean		2.12	4.04	11.9	29.2	78.3
% CV		5.12	6.12	3.98	0.484	1.27
% Bias		6.4	1.2	-0.694	-2.66	-2.16
N		6	6	6	6	6

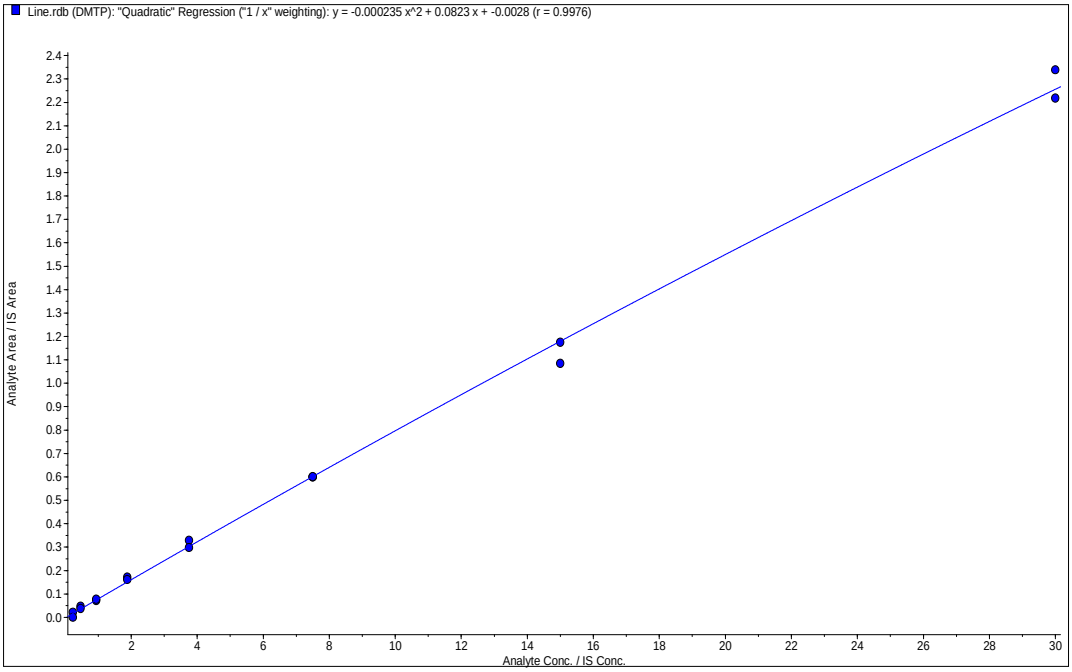


Figure 3.19 Calibration curve for the determination of DMTP in subject samples. An unweighted Quadratic calibration curve was selected with the coefficient of determination (r^2) = 0.9976.

Table 3.58 Back-calculated calibration standard concentrations of DMTP in ng/ml for the three production batches

Production batch	Replicates	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I
		0.780	**1.56**	**3.13**	**6.25**	**12.5**	**25.0**	**50.0**	**100**
Production 1	**1**	0.792	1.56	3.14	6.11	12.7	25.4	49.9	102
	2	0.700	1.59	3.38	6.32	12.4	24.0	50.5	97.9
Production 2	**1**	0.666	1.45	3.2	6.3	12.4	24.7	49.6	103
	2	0.719	1.65	3.33	7.31	12.9	24.2	49	97.6
Production 3	**1**	0.652	1.88	3.44	6.28	12.3	25.5	49.7	*
	2	0.726	1.34	3.39	6.24	12.5	24.9	48.8	101
Mean		0.709	1.57	3.31	6.42	12.5	24.8	49.6	100
% CV		7.03	11.67	3.55	6.83	1.79	2.46	1.24	2.42
% Bias		-9.08	1.17	5.85	2.82	0.266	-0.866	-0.833	0.3
N		6	6	6	6	6	6	6	5

Code: * = Deactivated calibration standard (% Dev > 15%)

Table 3.59 Statistics for the DMTP contents of quality control samples for the three production batches (concentration is in ng/ml)

Production batch	Replicates	QC A	QC B	QC C	QC D	QC E
		2.00	**4.00**	**12.0**	**30.0**	**80.0**
Production 1	**1**	2.05	3.89	11.4	30.2	77.9
	2	1.92	3.93	11.5	28.1	76.1
Production 2	**1**	1.79	3.47	12.3	28.9	75.0
	2	1.97	3.79	13.1	30.1	78.1
Production 3	**1**	1.87	3.65	12.0	29.1	82.7
	2	1.74	3.55	12.7	28.1	78.8
Mean		1.89	3.71	12.16	29.08	78.1
% CV		6.06	5.02	5.49	3.16	3.40
% Bias		-5.5	-7.16	1.38	-3.05	-2.37
N		6	6	6	6	6

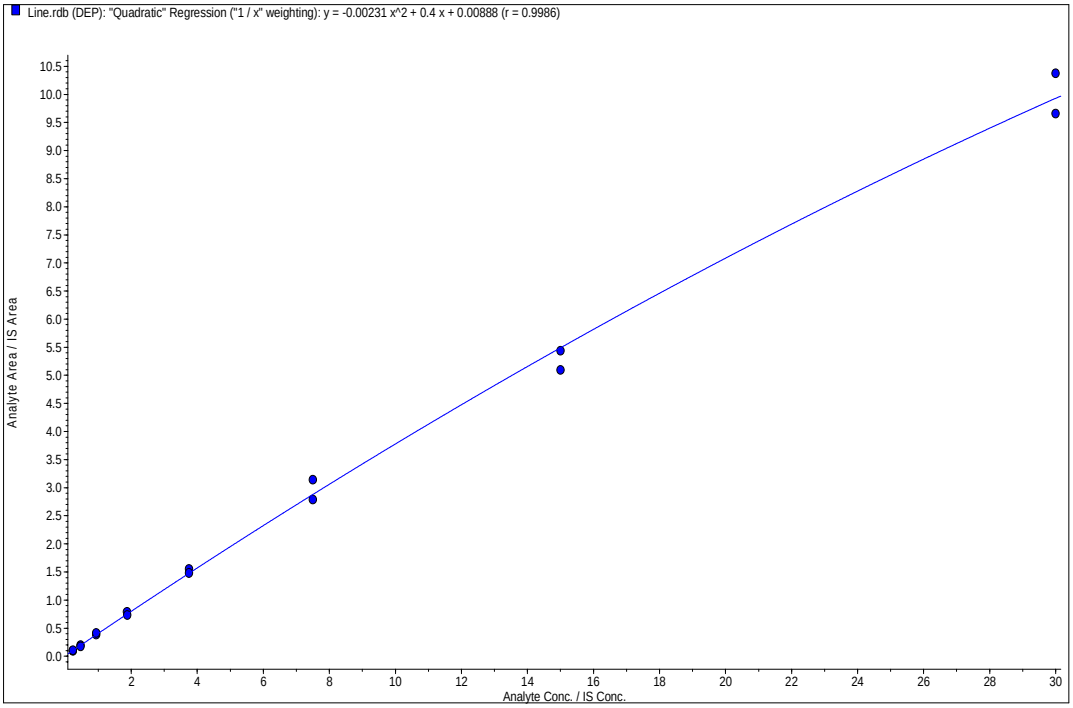


Figure 3.20 Calibration curve for the determination of DMP in subject samples. An unweighted quadratic calibration curve was selected with the coefficient of determination (r^2) = 0.9986.

Table 3.60 Back-calculated calibration standard concentrations of DEP in ng/ml for the three production batches

Production batch	Replicates	STD B 0.780	STD C 1.56	STD D 3.13	STD E 6.25	STD F 12.5	STD G 25.0	STD H 50.0	STD I 100
Production 1	**1**	0.711	1.48	3.16	6.38	23.3	24.7	49.9	100
	2	0.762	1.69	3.39	6.09	13.0	24.1	51.0	99.7
Production 2	**1**	0.550	1.57	3.39	6..19	12.9	25.1	48.6	105
	2	0.793	1.69	3.19	6.70	13.1	24.2	49.5	96.0
Production 3	**1**	0.626	1.69	3.63	6.59	12.8	24.6	48.7	*
	2	0.633	1.68	3.21	6.38	12.4	24.7	49.9	101
Mean		0.679	1.63	3.32	6.48	14.58	24.56	49.6	100
% CV		13.6	5.43	5.37	3.64	29.3	1.49	1.79	3.21
% Bias		-12.92	4.70	6.33	2.84	16.66	-1.73	-0.8	0.34
N		6	6	6	6	6	6	6	5

Code: * = Deactivated calibration standard (% Dev > 15%)

Table 3.61 Statistics for the DEP contents of quality control samples for the three production batches (concentration is in ng/ml)

Production batch	Replicates	QC A 2.00	QC B 4.00	QC C 12.0	QC D 30.0	QC E 80.0
Production 1	**1**	2.06	4.10	11.6	28.8	76.7
	2	2.06	3.94	12.7	28.1	74.7
Production 2	**1**	1.99	3.91	13.1	30.6	76.6
	2	2.16	4.03	12.2	28.4	75.7
Production 3	**1**	2.0	4.14	12.1	28.1	80.3
	2	2.15	3.88	13.9	28.9	76.1
Mean		2.07	4.00	12.6	28.8	76.7
% CV		3.48	2.65	6.50	3.25	2.49
% Bias		3.5	0	5	-3.94	-4.14
N		6	6	6	6	6

The results obtained from the analysis of the quality control samples met the acceptance criteria for accuracy and precision proving the validity of the results and therefore the acceptance of the production batch.

Blank samples included following high concentration calibration controls and quality control samples indicated the specificity regarding the internal standards and that no carry over was observed.

Each production batch contained ten System Suitability Samples (SYS) to evaluate the instrument response during the production batch. No upwards or downward trends were noticed in each of the

production batches.

3.6.2 Test samples results

The analytical results obtained from the analysis of the subject samples are indicated in tables 3.62, 3.63 and 3.64.

Table 3.62 Results summary of production batch 1 for DMP, DMTP and DEP

DAP-II batch 1-290116				DAP-II batch 1-290116			
Sample Name	Calc. Conc. (ng/mL)			Sample Name	Calc. Conc. (ng/mL)		
	DMP	DMTP	DEP		DMP	DMTP	DEP
RED 002	3.99	47.1	87.4	RED 056	9.39	43.8	34.5
RED 003	8.37	71.4	47.2	RED 057	3.40	55.9	56.2
RED 004	4.02	37.3	30.4	RED 059	1.05	23.8	22.5
RED 005	4.79	104*	147	RED 060	17.2	355*	167
RED 008	8.45	17.7	10.7	RED 061	23.2	195*	113
RED 009	32.8	321*	126*	RED 062	14.9	308*	34.8
RED 011	16.0	354*	202*	RED 064	5.60	23.1	8.01
RED 013	2.16	179*	233*	RED 065	1.34	8.83	3.83
RED 015	21.0	136*	181*	RED 067	2.11	17.4	3.38
RED 016	42.4	327*	365*	RED 068	13.9	13.7	14.3
RED 020	5.49	24.2	13.2	RED 069	9.77	44.7	15.5
RED 023	1.48	15.9	9.93	RED 070	2.52	13.0	12.4
BLUE 025	4.20	9.97	10.6	RED 071	5.83	144*	16.2
BLUE 027	BLQ	5.71	5.59	RED 072	11.8	47.1	30.8
BLUE 028	2.84	63.5	60.6	RED 073	3.94	8.77	4.09
BLUE 029	8.63	30.0	17.9	RED 075	5.54	40.9	15.4
RED 031	16.7	31.5	12.3	RED 076	22.2	25.4	30.8
BLUE 033	4.28	13.2	15.3	RED 078	1.82	45.5	21.0
RED 034	BLQ	BLQ	3.62	RED 079	6.70	8.58	3.93
BLUE 035	BLQ	BLQ	6.42	RED 080	18.8	4.13	5.11
RED 039	5.74	35.8	31.0	RED 081	23.3	29.1	6.78
RED 041	2.59	13.4	14.2	RED 083	BLQ	6.38	6.20
RED 042	10.7	90.5	48.3	RED 084	4.56	41.8	18.8
RED 043	4.98	62.8	92.5	RED 085	1.42	16.0	5.30
RED 044	13.5	157	288	RED 086	7.53	31.6	11.0
RED 045	4.81	11.0	30.3	RED 088	22.1	49.6	58.6
RED 046	BLQ	3.14	1.93	RED 089	244*	62.8	16.0
RED 046	BLQ	3.14	1.93	RED 091	7.51	40.4	13.1
RED 052	2.09	11.6	10.5	RED 092	8.51	29.5	15.2
RED 053	18.5	54.8	129	RED 093	3.17	27.6	18.0
RED 054	8.03	87.3	104	RED 094	6.55	57.0	39.3
RED 055	55.7	27.6	61.4				

Table 3.63 Results summary of production batch 2 for DMP, DMTP and DEP

DAP-II batch 2-290116				DAP-II batch 2-290116			
Sample Name	Calc. Conc. (ng/mL)			Sample Name	Calc. Conc. (ng/mL		
	DMP	DMTP	DEP		DMP)	DMTP	DEP
RED 095	13.3	9.47	7.58	RED 134	2.24	17.9	15.3
RED 097	5.26	73.9	40.2	RED 136	6.13	12.6	13.4
RED 098	5.34	75.5	25.0	RED 137	22.6	26.8	16.7
RED099(A)	4.50	21.3	30.7	RED 138	9.14	44.7	74.3
RED099(B)	0.642	11.2	14.6	RED 139	2.74	7.05	7.13
RED 100	15.1	104*	43.9	RED 140	6.34	67.5	20.3
RED 101	2.45	38.3	6.56	RED 141	3.07	32.4	42.3
RED 102	2.78	131	30.3	RED 142	8.60	29.3	14.7
RED 103	0.664	7.91	8.05	RED 143	6.74	63.6	32.7
RED 104	40.4	30.6	8.23	RED 144	199*	37.7	13.1
RED 106	5.83	323*	45.4	RED 145	16.0	9.38	14.5
RED 107	5.03	85.5	24.5	BLUE 147	BLQ	1.65	BLQ
RED 109	7.14	125*	43.4	BLUE 148	30.4	28.4	44.2
RED 110	6.15	170*	31.2	BLUE 149	1.01	4.72	6.89
RED 111	7.31	120*	40.2	BLUE 151	11.2	35.7	36.4
RED 113	BLQ	17.1	4.93	BLUE 154	1.05	11.2	4.15
RED 114	7.92	76.3	45.3	BLUE 155	2.10	14.9	9.88
RED 115	7.81	124*	58.9	BLUE 156	3.93	5.62	3.45
RED 116	7.29	406*	48.9	BLUE 157	4.97	35.7	16.7
RED 117	6.10	21.8	9.46	BLUE 158	2.84	1.41	3.25
RED 118	13.6	55.6	7.88	BLUE 159	7.86	24.5	8.63
RED 119	1.23	60.3	5.50	BLUE 160	4.18	52.1	60.9
RED 121	BLQ	4.90	4.86	BLUE 161	BLQ	6.00	10.9
RED 122	2.13	58.4	16.4	BLUE 163	14.9	17.2	10.3
RED 124	1.87	19.7	19.7	BLUE 165	2.22	4.42	5.38
RED 125	1.8	7.29	2.20	BLUE 166	3.27	52.3	56.3
RED 127	1.62	69.5	7.15	BLUE 167	1.96	29.3	41.1
RED 128	2.44	61.4	12.5	BLUE 168	10.9	73.7	3.99
RED 129	2.19	6.09	1.98	BLUE 169	BLQ	1.34	1.88
RED 130	2.29	6.46	5.90	BLUE 170	4.67	29.5	23.4
RED 131	BLQ	40.7	9.72	BLUE 171	BLQ	5.98	8.82

Table 3.64 Results summary of production batch 3 for DMP, DMTP and DEP

DAP-II batch 3-290116				DAP-II batch 3-290116			
Sample Name	Calc. Conc. (ng/mL)			Sample Name	Calc. Conc. (ng/mL)		
	DMP	DMTP	DEP		DMP	DMTP	DEP
BLUE 172	28.8	51.2	33.6	BLUE 206	5.90	44.2	60.3
BLUE 173	1.73	7.19	1.55	BLUE 207	14.7	31.9	40.3
BLUE 174	BLQ	6.46	6.61	BLUE 208	48.7	236*	56.8
BLUE 176	BLQ	10.8	5.32	BLUE 209	67.8	55.2	28.4
BLUE 177	BLQ	BLQ	BLQ	BLUE 210	47.5	44.6	43.4
BLUE 178	BLQ	8.95	12.1	BLUE 211	20.2	8.45	14.9
BLUE 179	BLQ	18.2	16.1	RED 216	20.7	23.8	29.4
BLUE 180	BLQ	BLQ	1.38	RED 217	11.1	18.7	9.29
BLUE 181	3.53	9.18	7.18	BLUE 220	15.3	42.0	82.8
BLUE 182	12.4	245*	221*	RED221	7.99	15.7	27.7
BLUE 183	19.8	58.3	48.4	RED 222	4.82	7.42	6.44
BLUE 184	4.53	34.1	47.8	RED 223	13.8	68.7	29.3
BLUE 185	BLQ	32.6	65.3	RED 224	5.69	66.3	24.9
BLUE 186	1.24	10.2	4.96	RED 225	BLQ	5.89	3.07
BLUE 187	17.8	13.8	12.2	RED 226	17.5	93.4	38.3
BLUE 188	8.34	37.8	43.9	RED 228	8.33	20.6	14.4
BLUE 189	25.5	60.0	35.6	RED 229	9.33	26.1	26.2
BLUE 191	3.14	17.6	7.27	RED 230	2.60	3.57	8.31
BLUE 192	2.61	97.7	2.07	RED 231	10.8	45.5	38.4
BLUE 193	3.93	27.5	23.0	RED 232	BLQ	76.2	2.79
BLUE 194	2.49	1.23	1.86	RED 233	3.86	59.0	24.5
BLUE 195	19.2	70.0	120	RED 235	15.1	48.9	13.9
BLUE 196	2.04	7.50	4.15	RED 236	1.82	39.5	20.2
BLUE 198	4.76	101*	104*	RED 237	29.0	47.6	29.8
BLUE 199	11.6	116*	94.8	RED 238	21.4	125*	113
BLUE 200	36.6	80.0	64.4	RED 239	21.4	280*	36.8
BLUE 201	54.0	58.1	55.9	RED 240	BLQ	5.78	6.00
BLUE 202	1.67	26.7	26.0	RED 247	10.7	48.2	35.8
BLUE 203	1.12	62.6	66.8	RED 248	3.36	16.6	9.34
BLUE 204	14.1	117*	70.0	RED 257	2.56	12.3	7.19
BLUE 205	114*	61.7	BLQ				

Code: * = Values > 100 ng/ml as per dilution procedure

Although some samples were diluted four times according to the validated dilution procedure, these still had values above 100 ng/ml for some of the analytes, especially for DMTP and DEP. Such samples were labeled with * following the estimated concentrations.

There are also some samples with concentration values for the analytes below the lower limit of quantifications. Such samples were labeled BLQ.

According to the results obtained from the subject samples, exposure to organophosphate pesticides can be inferred for all subjects tested.

3.7 CONCLUSION

An LC-MS/MS method was developed and partially validated in the laboratory of Clinical Pharmacology at the University of Cape Town and this method was used in this study to evaluate the levels of three dialkylphosphate (DAP) metabolites of organophosphates in urine samples of agricultural workers reported to have been exposed to organophosphate pesticides during normal working conditions. The detection and quantification of DMP, DMTP and DEP can be used as markers to indicate organophosphate exposure and the fact that all samples but one, contained at least one of the three metabolites, indicates that the sensitivity of the method is sufficient for this purpose.

DuLaurent *et al* [36] reported a LC-MS/MS method for quantification of DAP metabolites in urine with a lower limit of quantification (LLOQ) of 2 ng/ml, while Sinha *et al* [37] developed a LC-MS/MS method with a LLOQ of 0.028 ng/ml. Although quantification of such low levels of the DAP metabolites can thus be achieved using LC-MS/MS, the LLOQ obtained in this study was regarded as suitable for the quantification of the DAP metabolites in the urine samples collected during the clinical trial as very few of these samples had concentrations below the lower limit of quantification (BLQ).

The reported presence of the three metabolites in most of the samples is evidence of definite exposure to the pesticides and such values were also reported in the literature from similarly exposed persons (Ueyama *et al* [78] Dulaurent *et al* [36]). These studies could however not make distinctions between persons being exposed to normal house-hold levels and those exposed in the work-place. Comparison between the statistical evaluation reported in literature and similar evaluation of the results reported in this present study (see Table 3.65) can also not unequivocally proof the nature of the exposure.

Table 3.65 Statistical evaluation of the values of DMP, DMTP and DEP in urine samples from 187 subjects supposedly exposed to organophosphates

Diakylphospahtes	Detected (%)	Geometric mean (ng/ml)	Median (ng/ml)	95th percentile (ng/ml)
DMP	88 (96*/84**))	6.45(17*)	6.14(17.7*/20.1**)	32.99(117.4*/50**)
DMTP	97(78*/79**))	23.01(1.3*)	28.75(1.1*/4.6**)	76.86(83.3*/139.2**)
DEP	98(80*/100**))	16.99(0.7*)	76.86(0.4*/4.5**)	93.08(14.7*/85.4**)

Comparisons are made between values reported in this study and similar values reported by Ueyama *et al* [78] * and DuLaurent *et al* [36]**.

Ueyama *et al* [78] reported urinary concentration levels of DAP metabolites using 23 healthy volunteers (controls) and 25 exposed operators (sprayers). Urinary concentration levels from the control and exposed group did not show a significant difference.

The values reported by DuLaurent *et al* [36] were compiled during a study using 19 non-occasional exposed volunteers and some of the values, especially those representing maximum levels of DMTP and DEP (95^{th} percentile) are even higher than values obtained from definite reported exposure.

The obvious uncertainty about baseline levels of organophosphate metabolites and the relevance thereof to pesticide exposure, compounds the difficulty to use data such as this reported by the present study to make conclusions regarding exposure. Well controlled studies are needed which imply difficult ethical decisions regarding the use of study subjects.

This study however focused mainly on the technical aspects regarding the development of analytical methods and has proven that LC-MS/MS can successfully be used to detect and quantify the relevant compounds in a biological matrix.

CHAPTER 4

SUMMARY

The aim of the development and validation for the quantitative determination of endosulfan in serum was to obtain data that would correlate exposure and possible levels in the biological matrix and to compare this to published results.

Similarly, the use of the validated method obtained from UCT to study levels of organophosphate metabolites in urine of exposed subjects provided data that can be used to monitor such exposure and correlate it with similar data in the literature.

The OCPs assay was used for the simultaneous quantification of β-endosulfan and one of its main metabolites, endosulfan sulfate, in human serum as markers for endosulfan exposure to farm workers. An AB Sciex API 4000 mass spectrometer at unit resolution in the multiple reaction monitoring (MRM) mode, using electrospray ionization (ESI) in negative ionization mode was used to monitor the transitions of the deprotonated precursor ions *m/z* 405.0 and 421.0 to the product ions *m/z* 305.0 and *m/z* 97.0 for β-endosulfan and the metabolite endosulfan sulfate, respectively.

Sample preparation was performed using liquid-liquid extraction (LLE). The analytes were extracted from serum, adjusted to pH 9, using a solvent mixture of hexane and dichloromethane (60:40, v/v). The absolute recovery at high, medium and low concentrations of β-endosulfan and endosulfan sulfate varied between 93% and 104% with the coefficient of variation within a batch and between batches, varying from 3% to 14%.

The dried extracts were reconstituted in a mixture of methanol and water (80:20, v/v) and injected onto the analytical column. Chromatographic separation was achieved on a Supelco® Discovery® C18 (2.1 mm x 150 mm, 5 µm) analytical column using a mobile phase of acetonitrile, methanol and water in the ratio 80:80:40, v/v/v at a flow-rate of 200 µl/ml.

The calibration ranges for β-endosulfan and endosulfan sulfate were 0.8 ng/ml to 200 ng/ml and 0.117 ng/ml to 30 ng/ml, respectively. The accuracy and precision of the method over these calibration ranges were demonstrated by performing three independent validation batches. A linear regression model, weighted by1/concentration^2 was used to describe the concentration-response relationship for the analyte and metabolite. A coefficient of determination (r^2) of not less than 0.990 was achieved in each validation batch using this regression model throughout the validations for each batch. Precision and accuracy conformed to the acceptance criteria through the complete concentration ranges.

The analytes were proven to be stable in serum when left on-bench at room temperature for 8 hours and when left overnight as an extract in the auto sampler at 5°C. Freeze-thaw stability was proven for three cycles. Long term stability of the analytes, stored at the same temperature as subject samples, could not be proven for the complete storage period but was proven to be stable under these conditions for at least one month.

The quantification of the analytes was not significantly influenced by co-extracted matrix components. Haemolysis which can result in high concentrations of red blood cell components in the serum was shown not to influence the analytical process. However, highly lipemic serum negatively influenced analysis, most probably due to the fact that ionization was in the negative mode and the presence of high concentrations of phospholipids in lipemic serum would then have an effect.

The quantification of β-endosulfan and endosulfan sulfate in this study was performed without the inclusion of internal standards. The endosulfans contain six chlorine atoms resulting in extensive and complicated isotope patterns. Stable isotope labelled congeners of these molecules, containing either deuterium or carbon-13, would not be specific enough to act as internal standards, since the resultant isotope patterns of labelled molecules will still coincide with isotope mass-peaks of unlabelled molecules. Such stable isotope labelled isomers of β-endosulfan and endosulfan sulfate could also not be sourced commercially. Other molecules known to ionize well in the negative mode, and to also contain chlorine atoms such as diclofenac were tested, but found not to compensate well as internal standards for the endosulfans. Similar chlorinated pesticide molecules tested were dieldrin and dichlorodiphenyltrichloroethane (DDT), but they could not be used due to their lack of functional groups ionisable by electrospray. It was therefore decided to perform the quantifications without internal standard. No adverse effect due to this omission was evident and since the retention times of both analytes were well separated from the ion front, compensation due to ionic effects was not deemed necessary.

The validated method was used to analyze 219 serum samples of agricultural workers thought to have been exposed in their working environment to endosulfan pesticides. No indication of the analyte and metabolite could be found in any of the serum samples. The reasons for the negative results may reside in seasonal variation in the application of pesticides used for pest control. This possibility can be explored by correlating the pesticide products used during or before the period of sampling.

The second analytical method was used to quantitatively measure urinary dialkylphosphate (DAP) metabolites as markers to indicated exposure of farm workers to organophosphorus pesticides. The

developed method was used to determine three DAP metabolites, namely dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP) and diethyl phosphate (DEP).

The method was used to quantitatively analyze 187 urine samples from agricultural workers suspected of being exposed to organophosphate pesticides in their working environment.

Different concentrations of the three DAP metabolites could be found in all the samples tested from agricultural workers from the Hex River Valley where grape farming is practiced, Grabouw where pome fruit farming is predominant and Piketberg where wheat and fruit farming is practiced.

These OP's are considered by the World Health Organization [79] as highly hazardous (Class I-b) and moderate hazardous (Class II), respectively. In the present study, DAP metabolites were indicated in 100% of the samples with (DMP) in 88%, (DMTP) in 97% and (DEP) in 98% of the samples tested. Comparatively, in other studies, the frequencies of detection of OPs found in urine of farm workers were as follows: 96% and 94% for DMP and DEP [80] and 51% and 68% for DMP and DEP [81], respectively. The results should however be stratified to indicate results from the control group to determine the level of environmental exposure, as this can also be a source of DAB metabolites in urine [82].

Some samples contained concentrations much higher than the upper limit of quantification and even though dilution integrity was proven for concentrations as high as 100 ng/ml, higher levels will have to be validated to determine the full range of concentrations. On average, the metabolite found at the highest concentrations was DMTP, followed by DEP and DMT.

Keywords: analytical method, validated, liquid chromatography, multiple reaction monitoring, electrospray ionization, β-endosulfan, endosulfan sulfate, chromatographic separation, calibration range, sample preparation, recovery, mass spectrometry, serum, urine, dialkylphosphate.

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APPENDIX A: CERTIFICATES OF ANALYSIS

A1. α-Endosulfan

SIGMA-ALDRICH

CERTIFICATE OF ANALYSIS

Sigma-Aldrich Laborchemikalien GmbH D-30918 Seelze
Telefon: +49 5137 8238-150

Seelze, 03.12.2010/150956/08/25049

Order-No.:
Customer-No.:

Order-Code:

Quantity:

Production Date: 15.Oct.2008
Expiry Date: 15.Oct.2015

Article/Product: 45468
Batch : SZB8289XV

Alpha-Endosulfan PESTANAL®

Reference Material (RM)

1. General Information

Formula: C9H6Cl6O3S
CAS-No.: [959-98-8]
Usage : Insecticide

Molar mass: 406.93 g/Mole
Recomm. storage temp.: roomtemp.

The estimated uncertainty of a single measurement of the assay can be expected to be 0.5 % relative (confidence level = 95%, n= 6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

Identity (NMR)	complying
Assay (GC)	99.6 area %
Water (Karl Fischer)	0.04 %
Melting range	107.4-108.8 °C
Date of Analysis	07.Nov.2008

3. Advice and Remarks

- The minimum shelf life is based on the current knowledge and holds only for proper storage conditions in the originally closed flasks/ packages.
- Whenever the container is opened for removal of aliqout portions of the substance, the person handling the substance must assure, that the integrity of the substance is maintained and proper records of all its handlings are kept. Special care has to be taken to avoid any contamination or adulteration of the substance.
- We herewith confirm that the delivery is effected according to the technical delivery conditions agreed.
- Particular properties of the products or the suitability for a particular area of application are not assured.
- We guarantee a proper quality within our General Conditions of Sales.

Sigma-Aldrich Laborchemikalien GmbH
Quality Management SA-LC

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A2. β-Endosulfan

SIGMA-ALDRICH®

CERTIFICATE OF ANALYSIS

Sigma-Aldrich Laborchemikalien GmbH D-30918 Seelze
Telefon: +49 5137 8238-150

Seelze, 26.09.2013/439510/13/19483

Order-No.:
Customer-No.:

Order-Code:

Quantity:

Production Date: 04.Sep.2013
Expiry Date: 04.Sep.2018

Article/Product: 33385 Batch : SZBD247XV

beta-Endosulfan PESTANAL®

Reference Material (RM)

1. General Information

Formula: C9H6Cl6O3S
CAS-No.: [33213-65-9]
Usage : Insecticide

Molar mass: 406.93 g/Mole
Recomm. storage temp.: roomtemp.

The estimated uncertainty of a single measurement of the assay can be expected to be 0.5 % relative (confidence level = 95%, n= 6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

Identity (NMR)	complying	
Assay (GC)	99.6	area %
Melting range	212.0-213.1	°C
Water (Karl Fischer)	<0.1	%
Date of Analysis	25.Sep.2013	

3. Advice and Remarks

- The expiry date is based on the current knowledge and holds only for proper storage conditions in the originally closed flasks/ packages.
- Whenever the container is opened for removal of aliqout portions of the substance, the person handling the substance must assure, that the integrity of the substance is maintained and proper records of all its handlings are kept. Special care has to be taken to avoid any contamination or adulteration of the substance.
- We herewith confirm that the delivery is effected according to the technical delivery conditions agreed.
- Particular properties of the products or the suitability for a particular area of application are not assured.
- We guarantee a proper quality within our General Conditions of Sales.

Sigma-Aldrich Laborchemikalien GmbH
Quality Management SA-LC

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A3. Endosulfan sulfate

SIGMA-ALDRICH®

CERTIFICATE OF ANALYSIS

Sigma-Aldrich Laborchemikalien GmbH D-30918 Seelze
Telefon: +49 5137 8238-150

Seelze, 06.08.2012/416811/12/16599

Order-No.:
Customer-No.:

Order-Code:

Quantity:

Production Date: 31.Jul.2012
Expiry Date: 31.Jul.2017

Article/Product: 36676 Batch : SZBC213XV

Endosulfan sulfate PESTANAL®

Reference Material (RM)

1. General Information

Formula: C9H6Cl6O4S
CAS-No.: [1031-07-8]
Usage : Metabolite

Molar mass: 422.92 g/Mole
Recomm. storage temp.: roomtemp.

The estimated uncertainty of a single measurement of the assay can be expected to be 1 % relative (confidence level = 95%, n= 6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

Identity (NMR)	complying	
Assay (GC)	96.6	area %
Melting range	180.5-181.6	°C
Water (Karl Fischer)	0.02	%
Date of Analysis	03.Aug.2012	

3. Advice and Remarks

- The minimum shelf life is based on the current knowledge and holds only for proper storage conditions in the originally closed flasks/ packages.
- Whenever the container is opened for removal of aliqout portions of the substance, the person handling the substance must assure, that the integrity of the substance is maintained and proper records of all its handlings are kept. Special care has to be taken to avoid any contamination or adulteration of the substance.
- We herewith confirm that the delivery is effected according to the technical delivery conditions agreed.
- Particular properties of the products or the suitability for a particular area of application are not assured.
- We guarantee a proper quality within our General Conditions of Sales.

Sigma-Aldrich Laborchemikalien GmbH
Quality Management SA-LC

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A4 Endosulfan ether

SIGMA-ALDRICH®

CERTIFICATE OF ANALYSIS

Sigma-Aldrich Laborchemikalien GmbH D-30918 Seelze
Telefon: +49 5137 8238-150

Seelze, 25.08.2011/230221/08/25033

Order-No.:
Customer-No.:

Order-Code:

Quantity:

Production Date: 15.Oct.2008
Expiry Date: 15.Oct.2015

Article/Product: 36673 Batch : SZB8289XV

Endosulfan ether PESTANAL®

Reference Material (RM)

1. General Information

Formula: C9H6Cl6O
CAS-No.: [3369-52-6]
Usage : Metabolite

Molar mass: 342.86 g/Mole
Recomm. storage temp.: roomtemp.

The estimated uncertainty of a single measurement of the assay can be expected to be 0.5 % relative (confidence level = 95%, n= 6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

Identity (NMR)	complying	
Assay (GC)	99.9	area %
Melting range	205.0-205.2	°C
Water (Karl Fischer)	0.02	%
Date of Analysis	07.Nov.2008	

3. Advice and Remarks

- The minimum shelf life is based on the current knowledge and holds only for proper storage conditions in the originally closed flasks/ packages.
- Whenever the container is opened for removal of aliqout portions of the substance, the person handling the substance must assure, that the integrity of the substance is maintained and proper records of all its handlings are kept. Special care has to be taken to avoid any contamination or adulteration of the substance.
- We herewith confirm that the delivery is effected according to the technical delivery conditions agreed.
- Particular properties of the products or the suitability for a particular area of application are not assured.
- We guarantee a proper quality within our General Conditions of Sales.

Sigma-Aldrich Laborchemikalien GmbH
Quality Management SA-LC

This document was produced electronically and is valid without a signature

A5. Endosulfan lactone

SIGMA-ALDRICH®

CERTIFICATE OF ANALYSIS

Sigma-Aldrich Laborchemikalien GmbH D-30918 Seelze
Telefon: +49 5137 8238-150

Seelze, 15.12.2011/285493/10/09195

Order-No.:
Customer-No.:

Order-Code:

Quantity:

Production Date: 29.Apr.2010
Expiry Date: 29.Apr.2015

Article/Product: 36675 Batch : SZBA119XV

Endosulfan lactone PESTANAL®

Reference Material (RM)

1. General Information

Formula: C9H4Cl6O2
CAS-No.: [3868-61-9]
Usage : Metabolite

Molar mass: 356.84 g/Mole
Recomm. storage temp.: roomtemp.

The estimated uncertainty of a single measurement of the assay can be expected to be 0.5 % relative (confidence level = 95%, n= 6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

Identity (NMR)	complying
Assay (GC)	99.9 area %
Melting range	265.5-267.3 °C
Water (Karl Fischer)	0.02 %
Date of Analysis	14.May.2010

3. Advice and Remarks

- The minimum shelf life is based on the current knowledge and holds only for proper storage conditions in the originally closed flasks/ packages.
- Whenever the container is opened for removal of aliqout portions of the substance, the person handling the substance must assure, that the integrity of the substance is maintained and proper records of all its handlings are kept. Special care has to be taken to avoid any contamination or adulteration of the substance.
- We herewith confirm that the delivery is effected according to the technical delivery conditions agreed.
- Particular properties of the products or the suitability for a particular area of application are not assured.
- We guarantee a proper quality within our General Conditions of Sales.

Sigma-Aldrich Laborchemikalien GmbH
Quality Management SA-LC

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A6. O,O-Dimethylphosphorothioate (DMTP), potassium salt

Cambridge Isotope Laboratories, Inc.

Certificate of Analysis

RECEIVED
05-06-2015

Quality Standards:
ISO Guide 34 • ISO/IEC 17025 • ISO 13485 • cGMP

DMTP

Product Name: O,O-DIMETHYLPHOSPHOROTHIOATE, POTASSIUM SALT
(Isotopic Label & Enrichment Specification) UNLABELED 1000 UG/ML IN METHANOL 97%+PURE

Lot Number: SDEG-008

Catalog Number: ULM-8905-S

Product Information

Chemical Purity Specification:	≥ 97%
Labeled CAS Number:	NA
Unlabeled CAS Number:	28523-79-7
MW*:	180.20
Chemical Formula:	C2H6KO3PS
Storage:	Store refrigerated (-5°C to 5°C). Protect from light.*
Stability:	See storage and expiration date.

Exact mass = 140.978

Certification

Cambridge Isotope Laboratories, Inc. guarantees that this material meets or exceeds the specifications stated. Absolute identity as well as chemical and isotopic purities are assured by the use of unambiguous synthetic routes and multiple chemical analyses whenever possible. Results are representative of QC testing at time of release from Quality Control unless otherwise stated.

Volumetric measurements were made with Class A glassware. Gravimetry is traceable to the NIST through calibrated balances and certified, calibrated, standard weights. The calibrations are traceable to the NIST under Test No. 822/270236-04. The calibrations also meet specifications outlined in ISO 9001, ISO/IEC 17025, ANSI/NSCL Z540-1-1994, NCR Document 10CFR50 Appendix B, and applicable subdocuments.

This COA references the bulk catalog number before packaging. The COA also applies to the CIL finished good catalog number. Some possible packaging sizes and their corresponding suffix are -1.2, -1, -0.5, -10, or -0.1.

** For isotopically labeled compounds, MW listed is for the fully enriched product.*

Approved by: signature removed
Marina Kliorsky, Quality Assurance

Quality Control Tests and Results

QC Release Date	4/23/2015
Expiration Date	4/23/2016*
Concentration Based on Gravimetry	1000 ± 10 µg/mL (k=2)
Chemical Purity of Neat Material(s)	100.0%

50 Frontage Road, Andover, MA 01810-5413 USA • 1.800.322.1174 (North America) • 1.978.749.8000 (International) • isotope.com

A7. Diethyl hydrogen phosphate (DEP)

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Analytical Reference Standards
a SIGMA-ALDRICH company

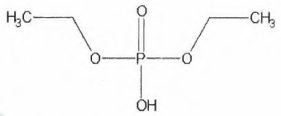
ERD-118
ER013012-02
Revision 0
Page 1 of 6
Product of USA

Cerilliant Quality
ISO GUIDE 34
ISO/IEC 17025
ISO 13485
ISO 9001
GMP/GLP

DEP

Certificate of Analysis

Diethyl hydrogen phosphate



Catalog Number:	ERD-118
Solution Lot:	ER013012-02
Expiration Date:	April 2017
Solvent:	Methanol
Volume per Ampule:	Not less than 1.2 mL
Storage:	Store unopened in freezer.
Shipping:	Ambient. See Stability Section.
Intended Use:	For R&D/ analytical purposes only. Not suitable for human or animal consumption.
Safety:	Flammable, Poison

- Expiration Date has been established through real time stability studies.
- Ampules are overfilled to ensure a minimum 1.2 mL volume fill. We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.

Component	Solution Purity	Certified Concentration
Diethyl hydrogen phosphate	97.7%	1000 ± 6 µg/mL

- Uncertainty of the concentration is expressed as an expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k = 2 and has been calculated by statistical analysis of our production system and incorporates uncertainty of the purity factor, material density, and balance and weighing technique.
- This standard is prepared gravimetrically and mass results are reported on the conventional basis for weighing in air. Concentration is calculated based on: the actual measured mass; Purity Factor of the analyte(s), measured mass of the solution; and the density of the pure diluent at 20°C.
- Concentration is corrected for chromatographic purity, residual water, residual solvents and residual inorganics.

Solution Standard Verification and Homogeneity

Standard Solution	Lot Number	Verified Concentration (µg/mL) Actual Results	Acceptance Crtieria	%RSD - Homogeneity Actual Results	Acceptance Crtieria
New Lot	ER013012-02	1024	± 3%	1.0	≤ 3%
Previous Lot	ER031708-03	1019	± 3%	2.0	≤ 3%

- Concentration is verified through multiple analyses and is calculated as the average of multiple analyses compared to an independently prepared calibration solution.
- Homogeneity of the New Lot is ensured through rigorous production process controls statistically analyzed to evaluate risk and verified by analysis. The % RSD of samples pulled from across the lot demonstrate homogeneity of the New Lot.
- The % RSD of the Previous Lot represents variability of the analysis performed at the time of release.

Traceability

- Gravimetrically prepared using qualified balances calibrated semi-annually by Mettler Toledo using NIST traceable weights. Calibration verification performed weekly and prior to each use utilizing NIST traceable weights. Each balance has been assigned a minimum weighing by Mettler Toledo taking into consideration the balance and installed environmental conditions to ensure weighing complies with USP tolerances of no more than 0.1% relative error.
- Concentration is verified against an independently prepared calibration solution; gravimetrically prepared using balances calibrated to NIST.
- In addition, each neat material utilized has been identified and thoroughly characterized through the use of multiple analytical techniques. Spectral data is provided on subsequent pages of the COA.

Cerilliant certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration/retest date when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules stored after opening.

ACLASS ACCREDITED REFERENCE MATERIAL PRODUCER

signature removed

Lara Sparks, Quality Assurance Director

May 7, 2012

Date

Cerilliant Corporation 811 Paloma Drive, Suite A, Round Rock, TX 78665 800-848-7837 / 512-238-9974

A8. O,O-Diethyl hydrogen thiophosphate (DETP), potassium salt

Cerilliant
Analytical Reference Standards
a SIGMA-ALDRICH company

RECEIVED
05-06-2015

CIL

Revision 1
Page 1 of 7

Cerilliant Quality
ISO GUIDE 34
ISO/IEC 17025
ISO 13485
ISO 9001
GMP/GLP

DETP

Certificate of Analysis

O,O-Diethyl hydrogen thiophosphate, potassium salt

Catalog Number:	ERD-119
Solution Lot:	FN05301302
Expiration Date:	August 2018
Solvent:	Methanol (LC-MS Chromasolv®)
Volume per Ampule:	Not less than 1.2 mL
Storage:	Store in Freezer.
Intended Use:	For R&D/analytical purposes only. Not suitable for human or animal consumption.
Safety:	Flammable, Poison

- Expiration Date has been established through real time stability studies.
- Ampules are overfilled to ensure a minimum 1.2 mL volume can be transferred when using Class A volumetric pipettes. We advise laboratories to quantitatively transfer desired volumes of this standard using established good laboratory practices to dilute to the desired concentration.

Component	Certified Concentration
O,O-Diethyl hydrogen thiophosphate, potassium salt	1000 ± 5 µg/mL (as free acid)

- Uncertainty of the concentration is expressed as an expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k = 2 and has been calculated by statistical analysis of our production system and incorporates uncertainty of the purity factor, material density, and balance and weighing technique.
- This standard is prepared gravimetrically and mass results are reported on the conventional basis for weighing in air. Concentration is calculated based on: the actual measured mass; Purity Factor of the analyte(s); measured mass of the solution, and the density of the pure diluent at 20°C.
- Concentration is corrected for chromatographic purity, residual water, residual solvents and residual inorganics.

Solution Standard Verification and Homogeneity

Standard Solution	Lot Number	Verified Concentration (µg/mL) Actual Results	Verified Concentration (µg/mL) Acceptance Crtieria	%RSD - Homogeneity Actual Results	%RSD - Homogeneity Acceptance Crtieria
New Lot	FN05301302	995.8	± 3%	0.3	≤ 3%
Previous Lot	FN092811-05	1006	± 3%	0.3	≤ 3%

- Concentration is verified through multiple analyses and is calculated as the average of multiple analyses compared to an independently prepared calibration solution.
- Homogeneity of the New Lot is ensured through rigorous production process controls statistically analyzed to evaluate risk and verified by analysis. The % RSD of samples pulled from across the lot demonstrate homogeneity of the New Lot.
- The % RSD of the Previous Lot represents variability of the analysis performed at the time of release.

Traceability

- Gravimetrically prepared using qualified balances calibrated semi-annually by Mettler Toledo using NIST traceable weights. Calibration verification performed weekly and prior to each use utilizing NIST traceable weights. Each balance has been assigned a minimum weighing by Mettler Toledo taking into consideration the balance and installed environmental conditions to ensure weighing complies with USP tolerances of no more than 0.1% relative error.
- Concentration is verified against an independently prepared 4-point calibration curve gravimetrically prepared using balances calibrated to NIST.
- In addition, each neat material utilized has been identified and thoroughly characterized through the use of multiple analytical techniques. Spectral data is provided on subsequent pages of the COA.

Cerilliant certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration/retest date when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules stored after opening.

ACLASS ACCREDITED REFERENCE MATERIAL PRODUCER

signature removed

Darron Ellsworth, Quality Assurance Manager

October 29, 2013

Date

Cerilliant Corporation 811 Paloma Drive, Suite A, Round Rock, TX 78665 800-848-7837 / 512-238-9974

A9. Dimethyl phosphate-d6 (d6-DMP), sodium salt (Internal Standard)

Toronto Research Chemicals Inc.

CERTIFICATE OF ANALYSIS

2 Brisbane Road, North York, ON. M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

1. Identification

CAS Number:

Catalogue Number:
D477602

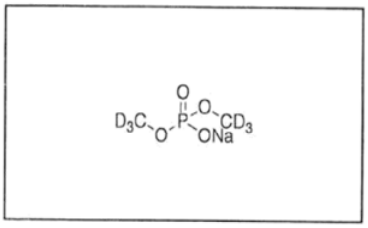
Product:
Dimethyl Phosphate-d6 (Major) Sodium Salt

(DMP IS) TRC 16/2/15

signature removed

Synonyms:
Phosphoric Acid Dimethyl Ester-d6 Sodium Salt; Dimethyl-d6 Monosodium Phosphate; Dimethyl-d6 Sodium Phosphate; Monosodium Dimethyl-d6 Phosphate; Sodium Dimethyl-d6 Phosphate;

Structure:



Molecular Formula:
$C_2D_6NaO_4P$

Molecular Weight:
154.07

Source of Product:

2. Analytical Information

Lot Number:
7-UPA-58-1

Melting Point:
232 - 234°C

Boiling Point:
N/A

Atmosphere:
Inert Atmosphere

Appearance of Product:
Off-White Solid

Solubility
DMSO, Water

Method for Determining Identity:
^{1}H NMR (DMSO-d_6), ^{31}P NMR (DMSO-d_6), and MS

Stability
Not Determined

Purity:
95%

Long Term Storage Condition:
Hygroscopic, -20°C Freezer, Under Inert Atmosphere

Additional Information:
^{1}H NMR, ^{31}P NMR, and MS conform to structure.
Elemental Analysis: (Found) %C: 16.15, %H: 3.80; (Calculated) %C: 15.59, %H: 3.92
Sodium Contant: 5.50%
Normalized Intensity: d_0 = 0.37%, d_1 = 0.00%, d_2 = 0.00%, d_3 = 8.22%, d_4 = 0.00%, d_5 = 0.41%, d_6 = 91.00%

signature removed

Philip Chan, Head of Quality Assurance

QC Test Date
January 31, 2014

Retest Date
January 29, 2019

A10. O,O-Dimethyl phosphorothionate-d6 (d6-DMTP), ammonium salt (Internal standard)

Toronto Research Chemicals Inc.

CERTIFICATE OF ANALYSIS

2 Brisbane Road, North York, ON. M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

1. Identification

CAS Number:

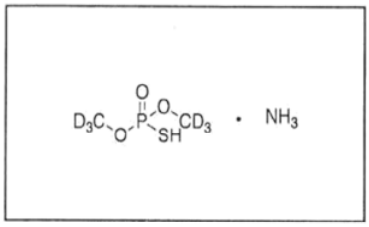
Catalogue Number:
D477622

Product:
O,O-Dimethyl Phosphorothionate-d6 Ammonium Salt (DMTP IS)

MC 16/2/15
signature removed

Synonyms:
Phosphorothioic Acid O,O-Dimethyl Ester-d6 Ammonium Salt; Ammonium Methyl Phosphorothioate-d6; Phosphorothioic Acid O,O-Dimethyl Ester-d6 Ammonium Salt; Ammonium O,O-Dimethyl Phosphorothioate-d6; Ammonium O,O-Dimethyl Thiophosphate-d6; Ammonium Dimethyl Phosphorothioate-d6;

Structure:



Molecular Formula:
$C_2H_4D_6NO_3PS$

Molecular Weight:
165.18

Source of Product:
Synthetic

2. Analytical Information

Lot Number:
1-VBN-51-1

Melting Point:
91 - 93°C

Boiling Point:
N/A

Atmosphere:
Inert Gas

Appearance of Product:
White Solid

Solubility
DMSO, Methanol

Method for Determining Identity:
^{1}H NMR (DMSO-d_6), ^{31}P NMR (DMSO-d_6), and MS

Stability
Hygroscopic

Purity:
Chemical Purity: 98%
Isotopic Purity: 99.9%

Long Term Storage Condition:
Hygroscopic, -20°C Freezer, Under Inert Atmosphere

Additional Information:
TLC Conditions: C_{18}; Acetonitrile : Water = 9 : 1; Visualized with Ninhydrin; Rf = 0.55.
^{1}H NMR, ^{31}P NMR, and MS conform to structure.
Normalized Intensity: d_0 = 0.01%, d_1 = 0.03%, d_2 = 0.03%, d_3 = 0.01%, d_4 = 0.02%, d_5 = 0.16%, d_6 = 99.74%

signature removed
Philip Chan, Head of Quality Assurance

QC Test Date
January 28, 2014

Retest Date
January 26, 2017

A11. O,O-Di(ethyl-d5) phospate (d5-DEP), sodium salt (Internal Standard)

TRC Toronto Research Chemicals Inc.

CERTIFICATE OF ANALYSIS

2 Brisbane Road, Toronto, ON. M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

1. Identification

CAS Number: N/A

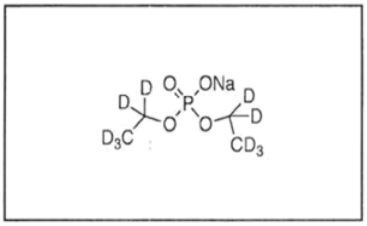
Catalogue Number: D444723

Received 17/03/15
Jenna signature removed

Product: O,O-Di(Ethyl-d5)phosphate Sodium Salt

Synonyms: (Internal std for DEP)

Structure:



Molecular Formula: $C_4D_{10}NaO_4P$

Molecular Weight: 186.14

Source of Product: Synthetic

2. Analytical Information

Lot Number: 14-JWA-86-3

Melting Point: 215.0 - 218.0°C

Boiling Point: N/A

Atmosphere: Inert Gas

Appearance of Product: White Solid

Solubility Methanol, Water

Method for Determining Identity: ^1H NMR (D_2O), ^{31}P NMR (D_2O), and MS

Stability Hygroscopic

Purity:
Chemical Purity: 95%
Isotopic Purity: 99.7%

Long Term Storage Condition: Hygroscopic, -20°C Freezer, Under inert atmosphere

Additional Information:
TLC Conditions: C_{18}; Methanol : Water = 9 : 1; Visualized with $KMnO_4$; Single Spot, Rf = 0.65.
^1H NMR, ^{31}P NMR, and MS conform to structure.
Elemental Analysis: (Found) %C: 25.01, %H: 5.45; (Calculated) %C: 25.81, %H: 5.42
Normalized Intensity: d_0 = 0.06%, d_1 = 0.01%, d_2 = 0.00%, d_3 = 0.00%, d_4 = 0.00%, d_5 = 0.00%, d_6 = 0.00%, d_7 = 0.00%, d_8 = 0.00%, d_9 = 2.37%, d_{10} = 97.56%
Sodium Content: 12.66%

signature removed
Philip Chan, Head of Quality Assurance

QC Test Date March 6, 2015

Retest Date March 4, 2018

APPENDIX B: CALIBRATION CERTIFICATES OF EQUIPMENT

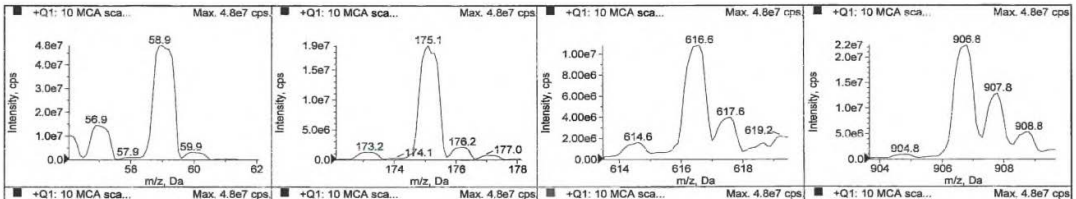
B1. Mass Spectrometer Calibration

Q1 Positive

Printing Date: 23 July 2015
Printing Time: 08:48:23

Acq. File: MT20150723084646.wiff
Polarity/Scan Type: Positive Q1 MS

*ASD621 (S/N: V1142107O5)
Workstation: BFN000998



Peak List for " +Q1: 10 MCA scans from Sample 1 (TuneSampleID) of MT20150723084646.wiff (Turbo Spray)"

	Target Mass (Da)	Found At (Da)	Intensity (cps)	Width (Da)	Mass Shift (Da)
1	59.0500	59.0458	4.8168e7	0.7314	4.1880e-3
2	175.1330	175.1338	1.9114e7	0.7192	-7.5043e-4
3	616.4640	616.4664	1.0829e7	0.7777	-2.3721e-3
4	906.6730	906.6975	2.2408e7	0.7060	-0.0245
5	1254.9250	1254.9643	3.5893e6	0.6461	-0.0393
6	1545.1340	1545.1346	1.3775e6	0.7363	-5.7428e-4
7	2010.4690	2010.4809	3.5179e6	0.7241	-0.0119
8	2242.6370	2242.6210	2.6674e6	0.7497	0.0160

Acq. Time: 08:46
Acq. Date: Thursday, July 23, 2015

*PPG LOT NR: A4315
*PPG Expiry Date: 06/Nov/2015

*PAGE 2 OF 2
Operator: Bergh, Werner

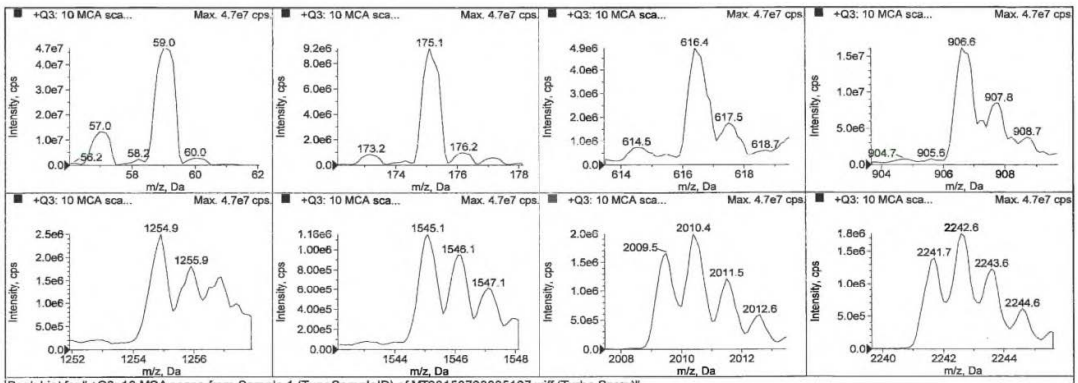
signature removed
23/Jul/2015

Q3 Positive

Printing Date: 23 July 2015
Printing Time: 08:53:15

Acq. File: MT20150723085127.wiff
Polarity/Scan Type: Positive Q3 MS

*ASD621 (S/N: V114210705)
Workstation: BPN000998



Peak List for " +Q3: 10 MCA scans from Sample 1 (TuneSampleID) of MT20150723085127.wiff (Turbo Spray)"

	Target Mass (Da)	Found At (Da)	Intensity (cps)	Width (Da)	Mass Shift (Da)
1	59.0500	59.0547	4.6546e7	0.7245	-4.6926e-3
2	175.1330	175.1509	9.2038e6	0.6578	-0.0179
3	616.4640	616.4900	4.9200e6	0.7897	-0.0260
4	906.6730	906.6790	1.6164e7	0.6628	-6.0249e-3
5	1254.9250	1254.8530	2.5025e6	0.6896	0.0720
6	1545.1340	1545.0985	1.1571e6	0.7215	0.0355
7	2010.4690	2010.4546	1.9877e6	0.7181	0.0144
8	2242.6370	2242.6141	1.7695e6	0.7133	0.0229

Acq. Time: 08:51
Acq. Date: Thursday, July 23, 2015

*PPG LOT NR: A4315
*PPG Expiry Date: 06/Nov/2015

*PAGE 2 OF 2
Operator: Bergh, Werner

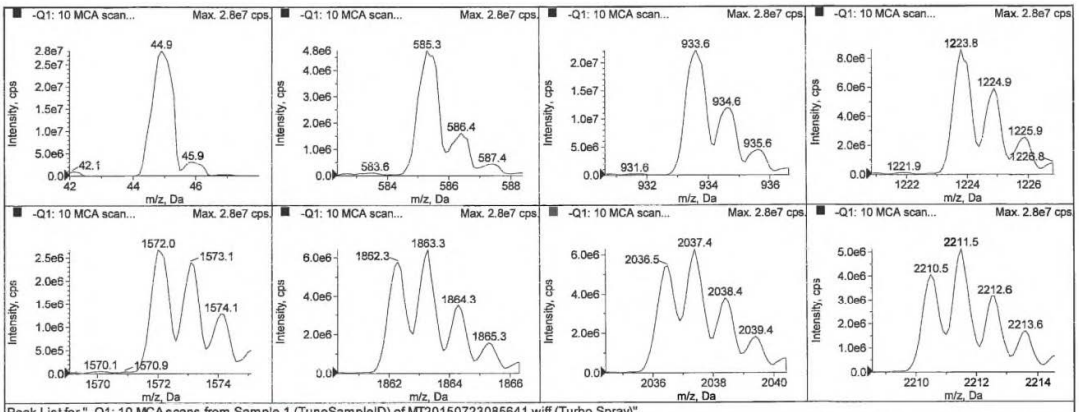
signature removed
23/Jul/2015

Q1 Negative

Printing Date: 23 July 2015
Printing Time: 08:58:55

Acq. File: MT20150723085641.wiff
Polarity/Scan Type: Negative Q1 MS

*ASD621 (S/N: V114210705)
Workstation: BFN000998



Peak List for "-Q1: 10 MCA scans from Sample 1 (TuneSampleID) of MT20150723085641.wiff (Turbo Spray)"

	Target Mass (Da)	Found At (Da)	Intensity (cps)	Width (Da)	Mass Shift (Da)
1	44.9980	44.9616	2.8118e7	0.7684	0.0364
2	585.3850	585.3542	4.7576e6	0.7640	0.0308
3	933.6360	933.6022	2.2246e7	0.7426	0.0338
4	1223.8450	1223.8476	8.6139e6	0.6604	-2.6124e-3
5	1572.0970	1572.0744	2.6678e6	0.6922	0.0226
6	1863.3060	1863.2506	6.4143e6	0.6372	0.0554
7	2037.4310	2037.3851	6.2768e6	0.7016	0.0459
8	2211.5570	2211.4834	5.1425e6	0.6120	0.0736

Acq. Time: 08:56
Acq. Date: Thursday, July 23, 2015

*PPG LOT NR: A4370
*PPG Expiry Date: 08/Oct/2015

*PAGE 2 OF 2
Operator: Bergh, Werner

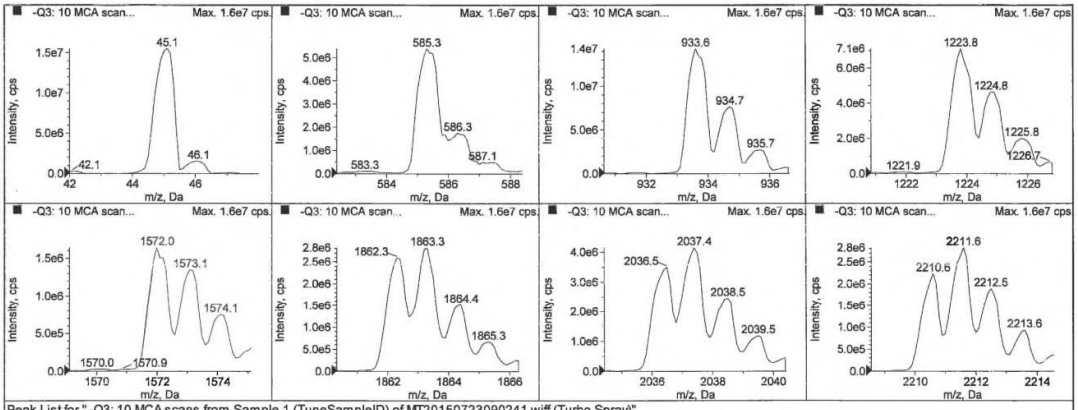
signature removed
23/July/2015

Q3 Negative

Printing Date: 23 July 2015
Printing Time: 09:04:02

Acq. File: MT20150723090241.wiff
Polarity/Scan Type: Negative Q3 MS

*ASD621 (S/N: V114210705)
Workstation: BFK000998



Peak List for "-Q3: 10 MCA scans from Sample 1 (TuneSampleID) of MT20150723090241.wiff (Turbo Spray)"

	Target Mass (Da)	Found At (Da)	Intensity (cps)	Width (Da)	Mass Shift (Da)
1	44.9980	45.0322	1.5542e7	0.7032	-0.0342
2	585.3850	585.3386	5.3908e6	0.7736	0.0464
3	933.6360	933.6508	1.4295e7	0.6931	-0.0148
4	1223.8450	1223.8274	7.1172e6	0.7641	0.0176
5	1572.0970	1572.0586	1.6418e6	0.7205	0.0384
6	1863.3060	1863.2605	2.8209e6	0.7456	0.0455
7	2037.4310	2037.3469	4.1513e6	0.7809	0.0841
8	2211.5570	2211.5187	2.8244e6	0.7003	0.0383

Acq. Time: 09:02
Acq. Date: Thursday, July 23, 2015

*PPG LOT NR: AG30
*PPG Expiry Date: 08/Oct/2015

*PAGE 2 OF 2
Operator: Bergh, Werner

signature removed
23/Jul/2015

B2. Micro balance

4 Jan Van Riebeeck Street
Dan Pienaar, Bloemfontein
Tel No: 051 436 5379; Fax No: 086 219 9676
ramtech@internext.co.za

R.A.M. INSTRUMED SERVICES cc
REGISTERED ENGINEERING TECHNICIANS

sanas Calibration Laboratory 1437

Certificate No: RAM 7822

CALIBRATION CERTIFICATE

This certificate is issued under the authority and conditions granted by SANAS and may not be reproduced, except in full,without prior written approval.The results relate only to the items calibrated.

Calibrated for:	Farmovs Parexel (PTY) Ltd Attention : Mr R Henson University of the Free State Bloemfontein
Location:	Analytical Lab Room F225
Calibration of:	METTLER XP6 Capacity: 6,1 g Division size: 0,000001 g Serial number: B140304875 ASD736
Date of calibration:	2015-04-07 Date of issue : 2015-04-08

Validity: The values in this certificate are correct at the time of calibration. Subseqeuntly the accuracy will depend on such factors as the care exercised in the handeling and use of the instrument as well as the frequency of use. Re - calibration should be performed after the period, which has been chosen to ensure that the instruments remain within the desired limits.

Laboratory Standards used	Serial No	Traceability Certificate No
RAM set	RAM 01	M15-230

Procedures

Compared with standard masspieces in accordance with Quality Procedure(s) number QP/RAM/01, QP/RAM/02,

Results see page 2 of 2.

Remarks

Uncertainty:"The reported expanded uncertainty is based on a standard uncertainty multiplied by a coverage factor k=2, providing a coverage probability of approximately 95%.
The uncertainty evaluation has been carried out in accordance with SANAS requirements".

General Comment:

A settling time of approximately 15 sec was allowed during all test performed under this calibration.
Adjustment was made to the Balance.
The highest cold start drift and eccentric errors were taken into account.

Calibrated by : Mr F R Clementz
Metrologist

Checked by : Mr F R Clementz
Technical Signatory

signature removed

Page 1 of 2

Certificate No: RAM 7822

Results:

Pre-calibration results:

Linearity:

Range 1	Run - up/down	
Cert Value of std in g	Reading in g	Error in g
0,001013	0,001014	1E-06
2,00004	2,000040	0
6,00004	6,000019	-2,1E-05

Post-calibration results:

Linearity:

	Run - up/down	
Cert Value of std in g	Reading in g	Error in g
0,001013	0,001012	-1E-06
0,500010	0,500008	-2E-06
2,00004	2,000033	-7E-06
3,00007	3,000062	-8E-06
6,00004	6,000019	-2,1E-05
-	-	-

Repeatability test:

The repeatability test was performed at approximately full capacity of the instrument and the calculated standard deviation was: ± 3,1623E-07 g

Eccentric test.:

The eccentric test was performed at approximately 1/4 of the capacity of the instrument and the biggest corner departure was found to be: 3E-06 g

Uncertainty of Measurement.:

The calculated expanded uncertainty of measurement for the calibration is: ± 7,01E-05 g

Enviromental Conditions:

21,3 °C
45 %
861 mb

Calibrated by : Mr F R Clementz
Metrologist

signature removed

Checked by : Mr F R Clementz
Technical Signatory

THIS CERTIFICATE IS VALID FOR ONE YEAR FROM DATE OF CALIBRATION
2016-04-10

End of Certificate

signature removed

Page 2 of 2

B3. Top loader balance

4 Jan Van Riebeeck Street
Dan Pienaar, Bloemfontein
Tel No: 051 436 5379; Fax No: 086 219 9676
ramtech@internext.co.za

R.A.M. INSTRUMED SERVICES cc
REGISTERED ENGINEERING TECHNICIANS

sanas 1437

Certificate No: RAM 7677

CALIBRATION CERTIFICATE

This certificate is issued under the authority and conditions granted by SANAS and may not be reproduced, except in full,without prior written approval.The results relate only to the items calibrated.

Calibrated for: Farmovs Parexel (Pty)Ltd
Attention : Mr Rob Henson
Anaiytical Lab Room F230A
University of the Free State
Bloemfontein

Calibration of: Mettler XP 1203S ASD 578
Capacity: 1210 g
Division size: 0.001 g
Serial number: 1127220003

Date of calibration: 2014-12-19 **Issue Date:** 2014-12-22

Validity The values in this certificate are correct at the time of calibration. Subseqeuntly the accuracy will depend on such factors as the care exercised in the handeling and use of the instrument as well as the frequency of use. Re - calibration should be performed after the period, which has been chosen to ensure that the instruments remain within the desired limits.

Laboratory Standards used	Serial No	Traceability Certificate No
RAM Set	RAM 01	M14-171

Procedures

Compared with standard masspieces in accordance with Quality Procedure(s) number QP/RAM/01, QP/RAM/02,

Results see page 2 of 2.

Remarks

Uncertainty:"The reported expanded uncertainty is based on a standard uncertainty multiplied by a coverage factor k=2, providing a coverage probability of approximately 95%.
The uncertainty evaluation has been carried out in accordance with SANAS requirments".

General Comment:

A settling time of approximately 15 sec was allowed during all test performed under this calibration.
Adjustment was made to the Balance.
The highest linearity and eccentric errors were taken into account.

Calibrated by : Mr F R Clementz
Metrologist

Checked by : Mr F R Clementz
Technical Signatory

signature removed

Page 1 of 2

Certificate No: RAM 7677

Results:

Pre-calibration results:

Linearity:

Range 1	Run - up/down	
Cert Value of std in g	Reading in g	Error in g
1.000	0.997	-0.001
499.996	499.994	-0.002
1000.001	1000.000	-0.001

Post-calibration results:

Linearity:

Cert Value of std in g	Run - up/down Reading in g	Error in g
1.000	1.000	0
99.999	99.998	-0.001
499.996	499.998	0.002
699.995	700.000	0.005
1000.001	1000.000	-0.001
-	-	-

Repeatability test:

The repeatability test was performed at approximately full capacity of the instrument and the calculated standard deviation was: ± **0.0003162 g**

Eccentric test.:

The eccentric test was performed at approximately 1/4 of the capacity of the instrument and the biggest corner departure was found to be: **0.001 g**

Uncertainty of Measurement.:

The calculated expanded uncertainty of measurement for the calibration is: ± **0.04235 g**

Enviromental Conditions:

22 °C

Calibrated by : Mr F R Clementz
Metrologist

Checked by : Mr F R Clementz
Technical Signatory

signature removed

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End of Certificate

signature removed

Page 2 of 2

B4. Pipette Calibration

R.A.M Instrumed Services
4 Jan van Riebeeck Street
Dan Pienaar
Bloemfontein
9301

Certificate Number : QC_0184.2

Pipette Calibration Report

Pipette Make	: **Finnpipette**
Pipette Model	: **F1**
Volume Range	: **20 - 200 µl**
Serial Number	: **LH18037**

Pipette Calibrated In Accordance With: Manufacturer's Specification
Finnpipette F1 20 - 200 µl

Test Series : 1 Test Volume : **20.00 µl** Number of Readings : **10**

Corrected Measured Values in Microlitres

19.7571	20.0580	19.9577	19.9577	20.0580	20.1583	19.9577	19.6568
20.1583	20.0580						

Mean Value		:	**19.9780 µl**		Test Specification	Pass/Fail
Inaccuracy	(d)	:	**-0.1110 %**	±	**2 %**	**Pass**
Imprecision	(CV)	:	**0.8130 %**	<=	**2 %**	**Pass**

Test Series : 2 Test Volume : **200.00 µl** Number of Readings : **10**

Corrected Measured Values in Microlitres

199.1759	201.6832	201.6832	201.1817	201.4826	200.6803	200.7806	201.0815
200.3794	200.5800						

Mean Value		:	**200.8710 µl**		Test Specification	Pass/Fail
Inaccuracy	(d)	:	**0.4350 %**	±	**0.8 %**	**Pass**
Imprecision	(CV)	:	**0.3740 %**	<=	**1 %**	**Pass**

Temperature	: **21.00°C**	Z Factor Correction: **1.0029 µl/mg**	
Calibrated With	: **Scaltec SBC 32 0.0001g**	Medium: **Distilled Water**	
Calibrated By	: **Mr FR Clementz**	Signature : signature removed	
Date Calibrated	: **2015-01-15**		

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www.bio-medical.co.uk

www.pipettedoctor.co.uk

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signature removed
19-01-2015

BIOHIT
www.biohit.com

R.A.M Instrumed Services
4 Jan van Riebeeck Street
Dan Pienaar
Bloemfontein
9301

Certificate Number : QC_0185.2

Pipette Calibration Report

Pipette Make	: Finnpipette
Pipette Model	: F1
Volume Range	: 100 - 1000 µl
Serial Number	: LH19204

Pipette Calibrated In Accordance With: Manufacturer's Specification
Finnpipette F1 100 - 1000 µl

Test Series : 1 Test Volume : 100.00 µl Number of Readings : 10

Corrected Measured Values in Microlitres
100.5909 100.4906 100.2900 100.1897 99.4877 99.6883 99.6883 99.9891
99.7886 100.0894

					Test Specification	Pass/Fail
Mean Value		:	100.0290 µl			
Inaccuracy	(d)	:	0.0290 %	±	1 %	Pass
Imprecision	(CV)	:	0.3667 %	<=	1 %	Pass

Test Series : 2 Test Volume : 1000.00 µl Number of Readings : 10

Corrected Measured Values in Microlitres
997.6849 999.2896 999.3899 998.8884 997.0832 997.3841 999.2896 998.1864
997.8855 999.9916

					Test Specification	Pass/Fail
Mean Value		:	998.5070 µl			
Inaccuracy	(d)	:	-0.1490 %	±	0.6 %	Pass
Imprecision	(CV)	:	0.0991 %	<=	0.6 %	Pass

Temperature	: 21.00°C	Z Factor Correction: 1.0029 µl/mg	
Calibrated With	: Scaltec SBC 32 0.0001g	Medium: Distilled Water	
Calibrated By	: Mr FR Clementz	Signature :	signature removed
Date Calibrated	: 2015-01-15		

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19/01/2015

APPENDIX C: MNR-ESD OUTLIER TEST

C1. Analytical Performance of QC level D after Exclusion of Statistical Outliers (β-Endosulfan)

Key:
% Nom = Accuracy (the measured concentration expressed as percentage of the nominal concentration)
% Bias = Accuracy (the percentage difference between the measured and the nominal concentration)
% CV = Precision (Coefficient of Variation)
N = Number of Determinations

Run Date	Curve Number	QC D (ng/ml)
		20.20
		20.500
		21.600
		19.700
		19.500
		19.600
		18.600
		19.800
		20.500
		15.700
		23.200
		[7.31]
		21.300
		16.700
		18.900
		19.600
		19.900
		21.900
		22.500
	MEAN	19.97
	%CV	9.5
	%Nom	98.9
	%Bias	-1.1
	N	17

[ ] = Statistical Outlier not included in statistics
Note: QC concentrations in bold type indicates % deviation from nominal > 15%

C2. MNR-ESD Outlier Test of QC level D (β-Endosulfan)

	Flag	Data	Absolute Difference From Mean	
1		20.5	1.233	
2		21.6	2.333	
3		19.7	0.433	
4		19.5	0.233	
5		19.6	0.333	
6		18.6	0.667	
7		19.8	0.533	
8		20.5	1.233	
9		15.7	3.567	
10		23.2	3.933	
11	MDV	7.31	11.957	Outlier
12		21.3	2.033	
13		16.7	2.567	
14		18.9	0.367	
15		19.6	0.333	
16		19.9	0.633	
17		21.9	2.633	
18		22.5	3.233	

C3. Analytical Performance of QC level D after Exclusion of Statistical Outliers (Endosulfan-sulfate)

Key:
% Nom = Accuracy (the measured concentration expressed as percentage of the nominal concentration)
% Bias = Accuracy (the percentage difference between the measured and the nominal concentration)
% CV = Precision (Coefficient of Variation)
N = Number of Determinations

Run Date	Curve Number	QC D (ng/ml)
		3.04
		3.18
		3.16
		3.13
		3.16
		3.04
		3.06
		3.07
		3.18
		2.48
		3.23
		[1.15]
		3.10
		2.72
		3.09
		3.05
		3.04
		3.23
		3.17
	MEAN	3.06
	%CV	6.2
	%Nom	100.8
	%Bias	0.8
	N	17

[ ] = Statistical Outlier not included in statistics
Note: QC concentrations in bold type indicates % deviation from nominal > 15%

C4. MNR-ESD Outlier Test of QC level D (Endosulfan- sulfate)

1		3.18	0.222	
2		3.16	0.202	
3		3.13	0.172	
4		3.16	0.202	
5		3.04	0.082	
6		3.06	0.102	
7		3.07	0.112	
8		3.18	0.222	
9		2.48	0.478	
10		3.23	0.272	
11	MDV	1.15	1.808	Outlier
12		3.1	0.142	
13		2.72	0.238	
14		3.09	0.132	
15		3.05	0.092	
16		3.04	0.082	
17		3.23	0.272	
18		3.17	0.212	

APPENDIX D: ADDITIONAL INFORMATION

D1. Examples of a sequence file setup

Validation batch sequence

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Analytical Run 10 (Validation Core1) for Study LCMS VAL298/01-CVC
Assay: Endosulfan - Human - Serum
Analyst: W Bergh
Assay Date: 12-Oct

	ID	Rep.	Dilution	Volume	Saved Position	Plate/Cell Position	Rack Number	Prep Date	Cycles	Temperature	Time Hours	Long Term Time	Long Term Time Units	Stability Type	Batch Id	Analyte Endosulfan Beta	Analyte Endosulfan Sulfate
1	SYS		1					07-Oct-2015									
2	SYS		1					07-Oct-2015									
3	SYS		1					07-Oct-2015									
4	SYS		1					07-Oct-2015									
5	SYS		1					07-Oct-2015									
6	STD J	1	1														
7	BLANK		1					07-Oct-2015									
8	STD I	1	1														
9	STD H	1	1														
10	STD G	1	1														
11	QC H DIL	1	2														
12	QC G	1	1														
13	QC F	1	1														
14	QC E	1	1														
15	QC D	1	1														
16	QC C	1	1														
17	QC B	1	1														
18	QC A	1	1														
19	STD F	1	1														
20	STD E	1	1														
21	BLANK		1					07-Oct-2015									
22	QC H DIL	2	2														
23	QC G	2	1														
24	QC F	2	1														
25	QC E	2	1														
26	QC D	2	1														
27	QC C	2	1														
28	QC B	2	1														
29	QC A	2	1														
30	STD D	1	1														
31	STD C	1	1														
32	BLANK		1					07-Oct-2015									
33	QC H DIL	3	2														
34	QC G	3	1														
35	QC F	3	1														
36	QC E	3	1														
37	QC D	3	1														
38	QC C	3	1														
39	QC B	3	1														
40	QC A	3	1														
41	STD B	1	1														
42	STD J	2	1														
43	BLANKS		1					07-Oct-2015									
44	QC H DIL	4	2														
45	QC G	4	1														

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Analytical Run 10 (Validation Core1) for Study LCMS VAL298/01-CVC
Assay: Endosulfan - Human - Serum
Analyst: W Bergh
Assay Date: 12-Oct

	ID	Rep.	Dilution	Volume	Saved Position	Plate/Cell Position	Rack Number	Prep Date	Cycles	Temperature	Time Hours	Long Term Time	Long Term Time Units	Stability Type	Batch Id	Analyte Endosulfan Beta	Analyte Endosulfan Sulfate
1	SYS		1					07-Oct-2015									
2	SYS		1					07-Oct-2015									
3	SYS		1					07-Oct-2015									
4	SYS		1					07-Oct-2015									
5	SYS		1					07-Oct-2015									
6	STD J	1	1														
7	BLANK		1					07-Oct-2015									
8	STD I	1	1														
9	STD H	1	1														
10	STD G	1	1														
11	QC H DIL	1	2														
12	QC G	1	1														
13	QC F	1	1														
14	QC E	1	1														
15	QC D	1	1														
16	QC C	1	1														
17	QC B	1	1														
18	QC A	1	1														
19	STD F	1	1														
20	STD E	1	1														
21	BLANK		1					07-Oct-2015									
22	QC H DIL	2	2														
23	QC G	2	1														
24	QC F	2	1														
25	QC E	2	1														
26	QC D	2	1														
27	QC C	2	1														
28	QC B	2	1														
29	QC A	2	1														
30	STD D	1	1														
31	STD C	1	1														
32	BLANK		1					07-Oct-2015									
33	QC H DIL	3	2														
34	QC G	3	1														
35	QC F	3	1														
36	QC E	3	1														
37	QC D	3	1														
38	QC C	3	1														
39	QC B	3	1														
40	QC A	3	1														
41	STD B	1	1														
42	STD J	2	1														
43	BLANKS		1					07-Oct-2015									
44	QC H DIL	4	2														
45	QC G	4	1														

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On-Bench Stability, Freeze-thaw Stability and Long-term Stability in Matrix

Watson Version 7.1 PAREXEL Bloemfontein | Report generated by Bergh, Werner | 04-May-2016 12:44:00

Analytical Run 14 (Stability Run (OBS, LTS, FTS)) for Study LCMS VAL298/01-CVC
Assay: Endosulfan - Human - Serum
Analyst: W Bergh
Assay

	ID	Rep.	Dilution	Volume	Saved Position	Plate/Cell Position	Rack Number	Prep Date	Cycles	Temperature	Time Hours	Long Term Time	Long Term Time Units	Stability Type	Batch Id	Analyte Endosulfan Beta	Analyte Endosulfan Sulfate
1	SYS		1					27-Oct-2015									
2	SYS		1					27-Oct-2015									
3	SYS		1					27-Oct-2015									
4	SYS		1					27-Oct-2015									
5	SYS		1					27-Oct-2015									
6	STD J	1	1														
7	BLANK		1					27-Oct-2015									
8	STD I	1	1														
9	STD H	1	1														
10	QC G	1	1														
11	OBS High		1					27-Oct-2015									
12	OBS Low		1					27-Oct-2015									
13	FTS High		1					27-Oct-2015									
14	FTS Low		1					27-Oct-2015									
15	LTS High		1					27-Oct-2015									
16	LTS Low		1					27-Oct-2015									
17	STD G	1	1														
18	STD F	1	1														
19	QC F	1	1														
20	OBS High		1					27-Oct-2015									
21	OBS Low		1					27-Oct-2015									
22	FTS High		1					27-Oct-2015									
23	FTS Low		1					27-Oct-2015									
24	LTS High		1					27-Oct-2015									
25	LTS Low		1					27-Oct-2015									
26	STD E	1	1														
27	QC E	1	1														
28	STD D	1	1														
29	QC D	1	1														
30	STD C	1	1														
31	QC C	1	1														
32	OBS High		1					27-Oct-2015									
33	OBS Low		1					27-Oct-2015									
34	FTS High		1					27-Oct-2015									
35	FTS Low		1					27-Oct-2015									
36	LTS High		1					27-Oct-2015									
37	LTS Low		1					27-Oct-2015									
38	STD B	1	1														
39	QC B	1	1														
40	STD J	2	1														
41	STD I	2	1														
42	STD H	2	1														
43	OBS High		1					27-Oct-2015									
44	OBS Low		1					27-Oct-2015									
45	FTS High		1					27-Oct-2015									

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Analytical Run 14 (Stability Run (OBS, LTS, FTS)) for Study LCMS VAL298/01-CVC
Assay: Endosulfan - Human - Serum
Analyst: W Bergh
Assay

	ID	Rep.	Dilution	Volume	Saved Position	Plate/Cell Position	Rack Number	Prep Date	Cycles	Temperature	Time Hours	Long Term Time	Long Term Time Units	Stability Type	Batch Id	Analyte Endosulfan Beta	Analyte Endosulfan Sulfate
46	FTS Low		1					27-Oct-2015									
47	LTS High		1					27-Oct-2015									
48	LTS Low		1					27-Oct-2015									
49	STD G	2	1														
50	QC G	2	1														
51	STD F	2	1														
52	QC F	2	1														
53	OBS High		1					27-Oct-2015									
54	OBS Low		1					27-Oct-2015									
55	FTS High		1					27-Oct-2015									
56	FTS Low		1					27-Oct-2015									
57	LTS High		1					27-Oct-2015									
58	LTS Low		1					27-Oct-2015									
59	STD E	2	1														
60	QC E	2	1														
61	STD D	2	1														
62	QC D	2	1														
63	STD C	2	1														
64	QC C	2	1														
65	OBS High		1					27-Oct-2015									
66	OBS Low		1					27-Oct-2015									
67	FTS High		1					27-Oct-2015									
68	FTS Low		1					27-Oct-2015									
69	LTS High		1					27-Oct-2015									
70	LTS Low		1					27-Oct-2015									
71	QC B	2	1														
72	STD B	2	1														
73	SYS		1					27-Oct-2015									
74	SYS		1					27-Oct-2015									
75	SYS		1					27-Oct-2015									
76	SYS		1					27-Oct-2015									
77	SYS		1					27-Oct-2015									

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On- instrument stability

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Analytical Run 17 (Stability Run (On-Instrument)) for Study LCMS VAL298/01-CVC
Assay: Endosulfan - Human - Serum
Analyst: W Bergh
Assay

	ID	Rep.	Dilution	Volume	Saved Position	Plate/Cell Position	Rack Number	Prep Date	Cycles	Temperature	Time Hours	Long Term Time	Long Term Time Units	Stability Type	Batch Id	Analyte Endosulfan Beta	Analyte Endosulfan Sulfate
1	SYS		1					27-Oct-2015									
2	SYS		1					27-Oct-2015									
3	SYS		1					27-Oct-2015									
4	SYS		1					27-Oct-2015									
5	SYS		1					27-Oct-2015									
6	STD J	1	1														
7	BLANK		1					27-Oct-2015									
8	STD I	1	1														
9	STD H	1	1														
10	QC G	1	1														
11	OIS High		1					16-Nov-2015									
12	OIS Low		1					16-Nov-2015									
13	STD G	1	1														
14	STD F	1	1														
15	QC F	1	1														
16	OIS High		1					16-Nov-2015									
17	OIS Low		1					16-Nov-2015									
18	STD E	1	1														
19	QC E	1	1														
20	STD D	1	1														
21	QC D	1	1														
22	STD C	1	1														
23	QC C	1	1														
24	OIS High		1					16-Nov-2015									
25	OIS Low		1					16-Nov-2015									
26	STD B	1	1														
27	QC B	1	1														
28	STD J	2	1														
29	STD I	2	1														
30	STD H	2	1														
31	OIS High		1					16-Nov-2015									
32	OIS Low		1					16-Nov-2015									
33	STD G	2	1														
34	QC G	2	1														
35	STD F	2	1														
36	QC F	2	1														
37	OIS High		1					16-Nov-2015									
38	OIS Low		1					16-Nov-2015									
39	STD E	2	1														
40	QC E	2	1														
41	STD D	2	1														
42	QC D	2	1														
43	STD C	2	1														
44	QC C	2	1														
45	OIS High		1					16-Nov-2015									

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Watson Version 7.1 PAREXEL Bloemfontein | Report generated by Bergh, Werner | 04-May-2016 12:45:10

Analytical Run 17 (Stability Run (On-Instrument)) for Study LCMS VAL298/01-CVC
Assay: Endosulfan - Human - Serum
Analyst: W Bergh
Assay

	ID	Rep.	Dilution	Volume	Saved Position	Plate/Cell Position	Rack Number	Prep Date	Cycles	Temperature	Time Hours	Long Term Time	Long Term Time Units	Stability Type	Batch Id	Analyte Endosulfan Beta	Analyte Endosulfan Sulfate
46	OIS Low		1					16-Nov-2015									
47	QC B	2	1														
48	STD B	2	1														
49	SYS		1					27-Oct-2015									
50	SYS		1					27-Oct-2015									
51	SYS		1					27-Oct-2015									
52	SYS		1					27-Oct-2015									
53	SYS		1					27-Oct-2015									

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Production batch

Watson Version 7.1 PAREXEL Bloemfontein | Report generated by Bergh, Werner | 04-May-2016 12:45:35

Analytical Run 18 (Production Samples first 50) for Study LCMS VAL298/01-CVC
Assay: Endosulfan - Human - Serum
Analyst: W Bergh
Assay D

	ID	Rep.	Custom ID	Subject	Alias	Period	Treatment	Study Day	Time	Matrix-Split	Dilution	Volumns	Saved Position	Plate/Cell Position	Rack Number	Prep Date	Cycles	Temperature	Time Hours	Long Term Time	Long Term Time Units	Stability Type	Batch Id	Analyte Endosulfan Beta	Analyte Endosulfan Sulfate
1	SYS										1					27-Oct-2015									
2	SYS										1					27-Oct-2015									
3	SYS										1					27-Oct-2015									
4	SYS										1					27-Oct-2015									
5	SYS										1					27-Oct-2015									
6	STD J	1								Serum	1														
7	BLANK										1					27-Oct-2015									
8	STD I	1								Serum	1														
9	STD H	1								Serum	1														
10	QC G	1								Serum	1														
11	STD G	1								Serum	1														
12	Subject 1										1					18-Nov-2015									
13	Subject 2										1					18-Nov-2015									
14	Subject 3										1					18-Nov-2015									
15	Subject 4										1					18-Nov-2015									
16	Subject 5										1					18-Nov-2015									
17	STD F	1								Serum	1														
18	QC F	1								Serum	1														
19	Subject 6										1					18-Nov-2015									
20	Subject 9										1					18-Nov-2015									
21	Subject 11										1					18-Nov-2015									
22	Subject 13										1					18-Nov-2015									
23	Subject 14										1					18-Nov-2015									
24	STD E	1								Serum	1														
25	QC E	1								Serum	1														
26	Subject 15										1					18-Nov-2015									
27	Subject 16										1					18-Nov-2015									
28	Subject 17										1					18-Nov-2015									
29	Subject 20										1					18-Nov-2015									
30	Subject 22										1					18-Nov-2015									
31	STD D	1								Serum	1														
32	QC D	1								Serum	1														
33	Subject 23										1					18-Nov-2015									
34	Subject 25										1					18-Nov-2015									
35	Subject 27										1					18-Nov-2015									
36	Subject 28										1					18-Nov-2015									
37	Subject 29										1					18-Nov-2015									
38	STD C	1								Serum	1														
39	QC C	1								Serum	1														
40	Subject 31										1					18-Nov-2015									
41	Subject 32										1					18-Nov-2015									
42	Subject 33										1					18-Nov-2015									
43	Subject 34										1					18-Nov-2015									
44	Subject 35										1					18-Nov-2015									
45	STD B	1								Serum	1														
46	QC B	1								Serum	1														
47	STD J	2								Serum	1														
48	STD I	2								Serum	1														
49	Subject 39										1					18-Nov-2015									
50	Subject 40										1					18-Nov-2015									
51	Subject 41										1					18-Nov-2015									
52	Subject 42										1					18-Nov-2015									
53	Subject 43										1					18-Nov-2015									
54	STD H	2								Serum	1														
55	STD G	2								Serum	1														
56	QC G	2								Serum	1														
57	Subject 44										1					18-Nov-2015									
58	Subject 45										1					18-Nov-2015									
59	Subject 48										1					18-Nov-2015									
60	Subject 50										1					18-Nov-2015									
61	Subject 52										1					18-Nov-2015									
62	STD F	2								Serum	1														
63	QC F	2								Serum	1														
64	Subject 53										1					18-Nov-2015									
65	Subject 54										1					18-Nov-2015									
66	Subject 55										1					18-Nov-2015									
67	Subject 56										1					18-Nov-2015									
68	Subject 57										1					18-Nov-2015									
69	STD E	2								Serum	1														
70	QC E	2								Serum	1														
71	Subject 99										1					18-Nov-2015									

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Analytical Run 18 (Production Samples first 50) for Study LCMS VAL298/01-CVC
Assay: Endosulfan - Human - Serum
Analyst: W Bergh
Assay D

	ID	Rep.	Custom ID	Subject	Alias	Period	Treatment	Study Day	Time	Matrix-Split	Dilution	Volume	Saved Position	Plate/Cell Position	Rack Number	Prep Date	Cycles	Temperature	Time Hours	Long Term Time	Long Term Time Units	Stability Type	Batch Id	Analyte Endosulfan Beta	Analyte Endosulfan Oxfate
72	Subject 60										1					18-Nov-2015									
73	Subject 61										1					18-Nov-2015									
74	Subject 62										1					18-Nov-2015									
75	Subject 64										1					18-Nov-2015									
76	STD D	2								Serum	1														
77	QC D	2								Serum	1														
78	Subject 66										1					18-Nov-2015									
79	Subject 67										1					18-Nov-2015									
80	Subject 68										1					18-Nov-2015									
81	Subject 69										1					18-Nov-2015									
82	Subject 70										1					18-Nov-2015									
83	STD C	2								Serum	1														
84	QC C	2								Serum	1														
85	QC B	2								Serum	1														
86	STD B	2								Serum	1														
87	SYS										1					27-Oct-2015									
88	SYS										1					27-Oct-2015									
89	SYS										1					27-Oct-2015									
90	SYS										1					27-Oct-2015									
91	SYS										1					27-Oct-2015									