

**EFFECTS OF AMITRIPTYLINE AND
NORTRIPTYLINE ON TIME OF DEATH
ESTIMATIONS IN THE LATER POSTMORTEM
INTERVAL USING INSECT DEVELOPMENT**

by

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Effects of Amitriptyline and Nortriptyline on Time of Death Estimations in the Later Postmortem Interval Using Insect Development

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Abstract

Forensic entomology can be used to estimate the postmortem interval in human death investigations. However, the presence of drugs can impact the development of the insects used to estimate the time since death. An *in vitro* model, consisting of beef liver, powdered whole egg and agar was used to simulate the natural rearing media of *Sarcophaga bullata* (Parker) (Diptera: Sarcophagidae). Since amitriptyline is a commonly abused antidepressant in British Columbia, it was added to the *in vitro* model to determine its impact on the development of *S. bullata*. Further, nortriptyline, a major metabolite of amitriptyline, was added along with amitriptyline to the *in vitro* model. The levels of amitriptyline and nortriptyline added to the *in vitro* model were obtained from an earlier entomotoxicological investigation (Goff *et al.*, 1993), and represented concentrations commonly found in amitriptyline related human deaths. Gas chromatography with a nitrogen-phosphorus detector was used to confirm the homogeneity of the antidepressant spiked rearing media. First instar *S. bullata* larvae were raised to adulthood on the antidepressant spiked rearing media, and on antidepressant free rearing media. No significant difference in overall larval development was noted, but the duration of the pupal stage for the insects reared on the drug-spiked artificial foodstuff was affected.

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Glossary and Abbreviations

Acceptance Criteria: Acceptance criteria specify the acceptance/rejection thresholds for parameters, such as accuracy and precision, which are described for a given analytical method during the process of method validation.

Accuracy: How closely the measurement result agrees with the true value (EURACHEM Working Group, 1998).

Amitriptyline: Tricyclic antidepressant. The chemical formula for amitriptyline is $C_{20}H_{23}N$. The molecular weight of amitriptyline is 277.41 amu (Budavari *et al.*, 1996).

For an illustration of the chemical structure of amitriptyline see Appendix C.

AMT: Amitriptyline.

Analytical Toxicology: An area of scientific inquiry that is concerned with the detection, identification and measurement of drugs and other poisons in biological materials, such as blood, and other relevant substances (e.g. pill residues). The purpose of such investigation is to assist in the diagnosis and treatment of poisoning. In some situations, information derived from toxicological inquiries may be used to prevent future poisoning (Flanagan, 1993).

Analysis of Variance: In analysis of variance, the mean and variance of two variables are used to determine if the measured difference between the variables is statistically significant (Rutherford, 2001). ANOVA makes several assumptions concerning the data, including that the data compared should be continuous and normally distributed with equal variances.

Analyte: The component or components present in the sample, for which the analysis was conducted (Skoog *et al.*, 1996).

ANOVA: Analysis of Variance.

Apolysis: The separation of the old skin from the new skin. For example, at the beginning of the pupal stage, the outer skin of the larva detaches from the inner wall of the puparium (Greenberg and Kunich, 2002).

Autoinjector: An autoinjector is an instrument that injects programmed sample volumes with a high degree of accuracy and precision (Sadek, 2000).

Band Broadening: In reference to a **chromatographic band**. Band broadening is the increase in the baseline width of a chromatographic peak, as the solute attributed to that peak moves from the point of injection to the detector (Harvey, 2000).

Bartlett's Test: A statistical test that is used to test for unequal variances. Bartlett's test assumes that the data is normally distributed; therefore, Bartlett's test is sensitive to departures from the normal distribution (Sall *et al.*, 2001).

Baseline: The baseline is the chromatographic trace formed when the response of the detector is due only to the injection of the mobile phase into a fully equilibrated chromatographic system (Sadek, 2000). All chromatographic parameters, such as peak height and peak area are dependent on the accuracy of the baseline (Sadek, 2000).

Blank Correction: A blank correction is done when the calibration curve prepared does not accurately take into account the baseline noise (i.e. the curve was not prepared in a matrix of either the same, or similar, composition to the sample matrix. Blank correction involves subtraction of the signal present in the blank (analyte free), at a given retention time, from the signal present at the same retention time in the sample.

Box and Whisker Plot: A box and whisker plot presents the median of a data set as a thick bar, and the interquartile range as a box. The range for the data set is illustrated using two lines extending from the top and bottom of the interquartile box. Outliers, data points that are well outside the range of other data points observed in the data set are illustrated as individual points (Dytham, 2003).

Brown-Forsythe Test: A robust statistical test that is used to detect the existence of unequal variances. The Brown-Forsythe test measures the differences from the median, and then tests those differences using an *F*-test (Sall *et al.*, 2001).

Calibration Curve: A calibration curve is a plot of detector response *versus* analyte

concentration. The ideal calibration curve passes through the origin. Furthermore, in an ideal calibration curve a directly proportional relationship exists between concentration (independent variable) and detector response (dependent variable). In addition, the ideal calibration curve is constructed from evenly spaced data points that bracket the entire expected analyte range (Sadek, 2000).

Calliphoridae: Family of carrion feeding flies that are commonly referred to as blow flies (Greenberg and Kunich, 2002).

Chromatography: is an analytical method where the components of a mixture are separated based upon the rates at which they are carried through or over a stationary phase by a gaseous or liquid mobile phase (Skoog *et al.*, 1996).

Chromatogram: A chromatogram is a plot of detector response (*y*-axis) *versus* time (*x*-axis). In general, time increases from left to right, and the intensity of the response increases from bottom to top (Sadek, 2000). Sample chromatograms from the present thesis are presented in Appendix A.

Chromatographic Peak: Also referred to as a chromatographic band. A chromatographic peak can be defined mathematically as the distribution of a chemical species about a central value (Skoog *et al.*, 1996). Ideally, chromatographic peaks are symmetrical and exhibit a Gaussian distribution (Skoog *et al.*, 1996). In the present thesis, chromatographic peaks are referred to simply as **peaks**.

CI: Confidence Interval.

Coefficient of Variation: See **relative standard deviation**.

Coeluting: Multiple peaks that elute at the same, or nearly the same, retention time. Coeluting peaks can also be referred to as interferences.

Column: The component of the chromatographic system that performs the separation.

Confirmation of Identity: The process of confirming that a peak present in a chromatogram, that has been attributed to be due to the presence of a specific analyte, is in fact, due to the presence of that analyte, and that analyte alone (EURACHEM Working Group, 1998).

Constant Systematic Error: With constant systematic errors, the error is independent of the concentration of the analytes analyzed. The result of the presence of a constant systematic error is the parallel displacement of the calibration curve with

respect to the ideal calibration curve (i.e. y -intercept = zero). Constant systematic errors may result from the co-detection of a matrix component, and indicates that the specificity of the method must be improved (Funk *et al.*, 1995).

Crop: A region of the foregut in insects that serves as a food storage organ in fly larvae (Greenberg and Kunich, 2002).

Developmental Maximum: The highest temperature at which insect development will still occur (Higley and Haskell, 2001).

Developmental Minimum: The lowest temperature at which insect development will still occur (Higley and Haskell, 2001).

Discontinuous Batch Extraction: Liquid-liquid extraction where the extracting solvent is added and then removed in discrete steps.

Efficiency: A measure of column suitability or goodness (Sadek, 2000).

Elution: Elution is the process in which solutes are pushed through the stationary phase by movement of the mobile phase (Skoog *et al.*, 1996).

Eluent: An eluent is the solvent that is used to transport the components of a mixture through the stationary phase. Synonymous with mobile phase (Skoog *et al.*, 1996).

Entomotoxicology: Entomotoxicology is a relatively new area of research that can be included under the auspices of forensic entomology. Investigations within the area of entomotoxicology include (Introna *et al.*, 2001):

1. studying the effects of drugs and other toxins on the developmental rate of forensically important insects
2. using insects as alternative toxicological specimens when traditional specimens, such as blood and liver, are no longer available or suitable for analysis.

Exoskeleton: Skeleton outside the body.

Extraction: The process of isolating one or more components from the bulk of the matrix.

Forensic entomology: The application of entomology to law.

Fundamental Analytical Procedure: Procedure in which only analyte standards in pure solvents are analyzed (Funk *et al.*, 1995).

Fundamental Calibration Curve: Calibration curve generated from the **fundamental analytical procedure** (Funk *et al.*, 1995).

Gas chromatography: Chromatographic technique that employs a gaseous mobile phase and a solid or liquid stationary phase (Skoog *et al.*, 1996).

GC-NPD: Gas Chromatography using a nitrogen-phosphorous detector.

Holometabolous: Complete metamorphosis. Immature individuals (e.g. larvae) do not resemble the adults (Elzinga, 2000).

Homogeneous: A material of uniform consistency and composition.

Imaginal discs: Imaginal discs clusters of embryonic cells in larvae that remain viable after the majority of the larval structures are destroyed during the pupal stage. The imaginal discs give rise to adult structures such as legs and wings (Greenberg and Kunich, 2002).

In-House Quality Control: A quality control that is prepared according to strict guidelines within a laboratory. For a specimen to be considered a proper in-house quality control, the control must be prepared by someone other than the person conducting the analyses requiring the quality control.

Injection: The process of introducing a sample of known volume in the chromatographic system (Sadek, 2000).

Instar: An instar is the period of time between larval moults. Fly larvae generally have three larval instars, and therefore two moults (Greenberg and Kunich, 2002).

Integument: Synonymous with **exoskeleton**.

Interference: Components present in the sample that interfere with the signal attributed to the analyte. For example, substances that coelute with the analyte of interest are interferences.

Intermediate Precision: According to Snyder *et al.*, (1997), intermediate precision refers to the agreement between complete measurements, of both standards and samples, when the same method is applied several times in the same laboratory. The evaluation of intermediate precision may require multiple analyses of samples and

standards within the same day or on different days, depending on the frequency in which the method will be used.

Internal Standard: A known quantity of a chemical species that is added to the sample under investigation, at the beginning of the sample preparation procedure. The concentration of analyte present in the sample is calculated using the ratio of the analyte response to the internal standard response (Skoog *et al.*, 1996). The chemical species chosen as the internal standard should have chemical and physical properties similar to that of the analyte, and should respond to the chromatographic detection system in a manner similar to that of the analyte(s) (Robards *et al.*, 1994). Further, the internal standard must be well separated from other sample components, but still be relatively close to the peak(s) attributed to the analyte(s) of interest. In addition, the internal standard must not react with any components of the sample, and must not be found in the sample as a common constituent (Robards *et al.*, 1994). Furthermore, according to Robards *et al.* (1994), the internal standard should be incorporated into the sample in exactly the same way as the analyte(s); however, such an ideal is not normally achieved in analytical practice. Internal standards are commonly used in chromatographic analyses because their use minimizes the uncertainty introduced by variations in the chromatographic system (e.g. variations in sample injection, flow rate, and column condition) (Skoog *et al.*, 1996).

Interquartile Range: The interquartile range is a measure of spread in a data set. The interquartile range is determined by placing the data in rank order, and then selecting the range that encompasses the data from the 25% value to the 75% value (Dytham, 2003).

Isolation Phase: The phase in an analytical procedure that involves the separation of the target analyte from the bulk of the matrix in which it was held. For example, extraction techniques for part of the isolation phase. Chromatographic techniques, which separate the components of a mixture according to physical and chemical properties of the analytes themselves can also be viewed as part of the isolation phase.

Larva: The actively moving and feeding stages of immature flies. The series of stages before the pupa, but after the egg (Greenberg and Kunich, 2002). Synonymous with **maggot**. Plural form is larvae.

Larviparous: Female flies that deposit first instar larvae rather than eggs. These insects can also be referred to as **ovoviviparous**.

Larviposition Media: The food substrate introduced to an insect species to stimulate the deposition of larvae. Liver is a commonly used larviposition media in carrion

insects, such as *Sarcophaga bullata*. Often, the substrate used as the larviposition media is the same substance that is used as the rearing media.

Least Squares Method: According to Rutherford (2001), least squares estimation is the most frequently applied method of parameter estimation. The method of least squares minimizes the sum of the squared differences, called **residuals**, between the measured values and predicted values. Proper application of least squares estimation requires that the data be normally distributed (Rutherford, 2001).

Levene's Test: A statistical test that tests for unequal variances. Levene's test estimates the mean of the absolute differences from each group, and then tests the estimates of the means using an *F*-test (Sall *et al.*, 2001).

Limit of Detection: The limit of detection is the lowest concentration of analyte that can be statistically distinguished from a sample that does not contain the analyte (Wu *et al.*, 1999).

Limit of Quantitation: The limit of quantitation is the lowest concentration of analyte that can be quantitated with a predetermined level of statistical confidence (e.g. 95% confidence) (Wu *et al.*, 1999).

Linearity: The linearity of a method is a measure of how well a plot of signal response *versus* concentration approximates a straight line (Snyder *et al.*, 1997). The magnitude of the coefficient of determination (R^2) is the parameter used to describe the linearity of a method. For most analytical work, an R^2 value of 0.95 or better is required.

Linear Regression: A regression technique that assumes the relationship between two variables is best described by a straight line (Dytham, 2003).

Liquid-liquid Extraction: Liquid-liquid extraction (LLE) is a method of sample preparation in which an excess of inert, water immiscible organic solvent is used to isolate the analytes from an aqueous solution. The extraction of the analytes from the aqueous solvent must be done at an appropriate pH. The organic solvent and the aqueous solution containing the analytes are then mixed together to facilitate the distribution of the analytes to the organic solvent. The two layers are usually separated after mixing by centrifugation. The excess solvent is usually removed under a stream of nitrogen gas, and then the dried extract is reconstituted using a small volume of an appropriate solvent (Flanagan, 1993).

LOD: Limit of Detection.

LOQ: Limit of Quantitation.

Maggot: See **larva**.

Maprotyline: Tricyclic antidepressant. The chemical formula for maprotyline is $C_{20}H_{23}N$. The molecular weight of maprotyline is 277.41 amu (Budavari *et al.*, 1996).

For an illustration of the chemical structure of maprotyline see Appendix C.

Matrix: The matrix is the material that encases the analytes.

Measurement Phase: The phase in an analytical procedure where the analyte(s) isolated from the bulk of the matrix are measured either qualitatively (i.e. identified) or quantitatively (i.e. identified and quantitated).

Metamorphosis: The process by which an organism changes from one shape to another during the life cycle (Elzinga, 2000).

Method Validation: The process by which it is established, by laboratory studies, that the performance characteristics of a particular analytical method are described and are confirmed to be appropriate for the intended purpose of the method (EU-RACHEM Working Group, 1998).

Mobile phase: The chromatographic phase that moves over or through the stationary phase. The mobile phase carries the analyte through column or across the surface of the stationary phase (Skoog *et al.*, 1996).

Moulting: The process by which insects and other arthropods shed their exoskeleton (Elzinga, 2000).

Necrophagous: An organism, such as an insect, that feeds on that on carrion or other decomposing animal material.

Nitrogen-phosphorous Detector: The nitrogen-phosphorous detector consists of a heated ceramic bead that is coated with an alkali metal such as rubidium or cesium. The alkali metal, when heated, promotes the selective ionization of compounds containing nitrogen or phosphorous (Stafford, 1992). The use of a specific detector, such as the nitrogen-phosphorous detector can significantly increase the sensitivity of the chromatographic system to compounds containing nitrogen or phosphorus. However, the use of a selective detector cannot replace selective sample preparation techniques; components that are present in the sample, but are not detected because of their chemical composition may still interfere with the chromatographic separation

(Stafford, 1992).

Noise: Any disturbance in the system that results in a detector response that is not generated by either components of the matrix or by the analytes themselves (e.g. electronic noise and random noise) (Sadek, 2000).

Nortriptyline: Tricyclic antidepressant. The chemical formula for nortriptyline is $C_{19}H_{21}N$. The molecular weight of amitriptyline is 263.38 amu (Budavari *et al.*, 1996).

For an illustration of the chemical structure of nortriptyline see Appendix C.

NPD: Nitrogen-phosphorous detector.

O'Brien's Test: A statistical test that is used to test for unequal variances. In O'Brien's test, the variances are treated as means, and then evaluates the variances using an *F*-test (Sall *et al.*, 2001).

Outlier: An anomalous observation that lies well away from the rest of the observations in a given data set (Dytham, 2003).

Ovoviviparous: Insects in which the eggs hatch within the body of the female fly (Elzinga, 2000).

Peak: See **Chromatographic Peak**.

Peak Area: The area under a chromatographic peak, determined by integration.

Peak Height: The height of a chromatographic peak, calculated from the baseline to the apex of the peak.

pA: A unit of detector response (picoamps).

pH: A unit of hydrogen activity.

Poikilotherm: An organism that lacks a temperature regulating system, and as a result has a body temperature that varies with the temperature of its surroundings (Elzinga, 2000).

Postmortem Interval: The length of time between the death of an individual or animal and the discovery of the corpse by humans (Catts and Goff, 1992). Synonymous with time since death, and elapsed time since death.

Postfeeding Larva: Behaviourally distinct stage in the third instar of a fly. During the postfeeding stage the fly larvae ceases to feed, and migrates away from the food source. During the postfeeding stage, the gut contents are digested, and a single layer of fat cells is deposited underneath the cuticle (Greenberg and Kunich, 2002).

Precision: Precision refers to the level of agreement among individual test results, where each result is generated by repeatedly applying the same procedure, from sampling to analysis, to a homogeneous sample (Snyder *et al.*, 1997).

Prepupa: A relatively short, behaviourally distinct stage within the third instar during which the puparium begins to harden and darken. Plural form is prepupae.

Proportional Systematic Error: With proportional systematic errors, the error is dependent on the concentration of the analytes analyzed. The result of the presence of a proportional systematic error is the deviation of the slope from the ideal (i.e. slope = 1). Proportional systematic errors may result from problems caused during individual sample preparation steps, such as matrix digestion (Funk *et al.*, 1995).

Pupa: The intermediate stage between larva and adult in holometabolous fly species (Elzinga, 2000). During this stage the majority of the larval tissues are destroyed in order to form adult structures, such as legs (Greenberg and Kunich, 2002). Plural form is pupae.

Pupal Stage: The intermediate stage between larva and adult in holometabolous fly species (Elzinga, 2000).

Pupariation: The process that shrinks the postfeeding maggot, and makes it skin dark shiny and brittle (Greenberg and Kunich, 2002).

Puparium: The puparium is the last larval exoskeleton. The pupa detaches from the interior of the puparium during pupariation. In addition, during pupariation, the puparium becomes hard and brittle, and changes from white to dark brown in colour (Elzinga, 2000).

Pupation: The formation of the pupa within the fly puparium (Greenberg and Kunich, 2002).

Quadratic Fit: Also referred to as second order polynomial regression. Regression technique where the relationship between the two variables is assumed to be best described by a quadratic equation (Dytham, 2003).

Range: The range, in terms of analyte concentration, is defined as the lower and upper analyte concentrations for which the analytical method has satisfactory precision, linearity, and accuracy (Snyder *et al.*, 1997).

Recovery Function: Plot of extracted (recovered) concentration *versus* spiked (actual) concentration. Used to investigate constant systematic errors and proportional systematic errors in sample preparation procedures (Funk *et al.*, 1995).

Reference Standard: Most reference standards are obtained from the *National Institute of Standards and Technology* (NIST) (Skoog *et al.*, 1996). Reference standards are materials of known composition that have been analyzed extensively by a variety of different analytical methods, and are certified to contain certain levels of analyte (Skoog *et al.*, 1996).

Regression Analysis: According to Rutherford (2001), regression analysis is a statistical technique that attempts to explain the dependent variable(s) in terms of the independent variable. In regression analysis, the calculated relationship between two or more independent variables and a dependent variable is referred to as the model, and the portion of the data that the model does not explain is referred to as the residual component, or error (Rutherford, 2001). In analytical work, regression analysis is commonly used to compute the line of best fit for a set calibration data (Skoog *et al.*, 1996).

Relative Retention Time: The retention time of the analyte(s) with respect to the retention time of the internal standard. The relative retention times should remain constant even if the actual retention times for each analyte drift slightly with variations in the chromatographic system.

Relative Standard Deviation: Synonymous with **coefficient of variation** when the relative standard deviation is expressed as a percentage. The percent relative standard deviation (%RSD) is computed by dividing the standard deviation by the mean, and then expressing that value as a percentage (Skoog *et al.*, 1996). The %RSD is useful because it incorporates both the standard deviation and the mean of a data set in a single measure. However, the %RSD can only be used to compare normally distributed data (Lang and Secic, 1997).

Repeatability: Repeatability describes the precision of an analytical method over a short period of time (Snyder *et al.*, 1997)

Reproducibility: Reproducibility measures the precision of an analytical method between different laboratories (Snyder *et al.*, 1997).

Residual: The variation in the data left over after a statistical model has been applied to the data set. The model exhibiting the best fit for a given data set is the model that produces the smallest amount of residual variation (Dytham, 2003).

Resolution: The degree of separation between two adjacent chromatographic peaks (Harvey, 2000).

Retention Time: The time required for the mobile phase to move the analyte from the time of injection onto the stationary phase, through the stationary phase, and to the detector. The retention time is determined from the apex (signal maximum) of the analyte peak (Dean, 1995).

Sample: (1) The material containing the analytes of interest (Sadek, 2000), (2) The solution, ready to be introduced into the chromatographic system that contains the analytes of interest (Sadek, 2000), (3) The portion of the population taken for statistical analysis (Dytham, 2003).

Sample Preparation: The steps required to ready a sample for analysis.

Sarcophagidae: Family of carrion feeding flies that are commonly referred to as flesh flies (Greenberg and Kunich, 2002).

Selectivity: 100% specificity.

Silylation: Derivatization process that uses a silane as the derivatizing agent. Silylation is commonly used to deactivate the silanol groups present on chromatographic system components such as the glass liner in the injector.

Silanol: The terminal functional group on a silica stationary phase or other silica containing sorbent. Si-OH (Sadek, 2000). Free silanol groups are responsible for silanophilic interactions.

Silanophilic Interaction: Interactions that result from hydrogen bonding between the solute and surface silanol groups. Silanophilic interactions are a major cause of tailing peaks in the chromatographic separation of basic compounds, such as amines (Sadek, 2000).

Slope: A value that describes how a trend line deviates from zero (Dytham, 2003).

Specificity: A term that describes the ability of a method to accurately measure the concentration of analyte in a sample in the presence of other sample components (Snyder *et al.*, 1997).

Spiking: Addition of a known amount of a chemical species.

Spiracle: An opening in the rear-end of a larva's body through which air passes into the tracheae or breathing tubes of the insect (Greenberg and Kunich, 2002).

SD: Standard Deviation

Standard Operating Procedure: A written authorized procedure which gives instructions for performing an analytical procedure.

Stationary Phase: the chromatographic phase that remains fixed either in a column or on a planar surface (Skoog *et al.*, 1996).

Tailing Peaks: A peak with the tail at the end of it is referred to as a tailing peak. Tailing peaks usually result from the presence of active sites in the stationary phase (Harvey, 2000).

Thermal History: Term used by Greenberg and Kunich (2002) to describe the temperatures experienced by larvae feeding on animal carrion prior to discovery of the carrion by humans. Accurate knowledge of an insect's thermal history is essential to the accurate and precise estimation of the postmortem interval.

Volume of Distribution: The volume of distribution is the apparent volume in which a substance (e.g. a drug) is distributed, following absorption and allocation to different tissues within the body (Klaassen and Watkins, 1999). The volume of distribution is an apparent volume that is calculated based on the concentration of the substance in the blood. The volume of distribution does not correspond to a real value; therefore it is not directly meaningful from a physiological perspective (Medinsky and Klaassen, 1996). A large volume of distribution, sometimes even greater than the total volume of the body, is a well-established characteristic of a substance with a high tissue affinity (Medinsky and Klaassen 1996). Chemicals with high tissue affinity are likely to accumulate within tissues with high blood-flow to mass ratios, such as the liver, heart and lungs (Stine and Brown 1996). Examples of substances with high volumes of distribution are amitriptyline and nortriptyline.

Preface

This thesis was intended to be a multidisciplinary project, and therefore it was written in a form designed to be accessible to a variety of disciplines. As a result, sections of this thesis that would have likely been excluded or shortened for a more focused audience, were retained for the benefit of other disciplines.

Chapter 1

Introduction

1.1 Forensic Entomology

The accurate estimation of the **postmortem interval** is extremely critical to the successful completion of death investigations, both criminal and noncriminal. For example, knowledge of the postmortem interval may reduce the number of suspects in a homicide investigation, or in cases where the identity of the deceased is not known, knowledge of the time of death may aid in the identification of the deceased. At the present time, there are several methods available for estimating the postmortem interval. Most of these methods are based on changes that occur to the corpse after death. These changes can be collectively termed postmortem changes (Buchan and Anderson, 2001). In the early postmortem period¹ these processes include *livor mortis*, *algor mortis*, *rigor mortis*, autolysis and putrefaction (Kashyap and Pillay, 1989). In the later postmortem interval, which begins approximately 72 hours after death, animal carrion passes through a series of decompositional stages, including: fresh, bloat, active decay, advanced decay, dry decay and remains (Payne 1965). These stages are easily recognizable, but the boundaries between each stage are diffuse and overlap considerably (Campobasso *et al.*, 2001). Furthermore, their rate of progress can be affected by a number of factors, including humidity, temperature, the presence

¹The early postmortem period lasts up to 72 hours after death.

or absence of clothing, burial and depth of burial (Buchan and Anderson, 2001). As a result, the accurate determination of the onset and duration of these stages may be considerably difficult, thus hindering the accurate and precise estimation of the postmortem interval.

Forensic entomology is the most well-researched method of determining the time since death in the later postmortem interval (Buchan and Anderson, 2001), and can be defined as the application of the study of insects and other arthropods to investigations of violent crimes such as rape, physical abuse, and murder (Keh, 1985; Catts and Goff, 1992). In forensic entomology, the arthropod evidence associated with the corpse is most often used to estimate the elapsed time since death, or postmortem interval (Catts and Goff, 1992).

Numerous living organisms contribute to the decomposition of animal carrion, including insects, fungi, bacteria, and other scavengers such as rodents. Human or other animal carrion, when viewed from a biological perspective is a rich source of proteins, lipids, carbohydrates and other nutrients (Erzinclioglu, 1992). However, insects remove soft tissue from animal carrion with remarkable speed and completeness (Haskell *et al.*, 1997). As a result, in terms of carrion decomposition, insects are considered to be the most influential group of living organisms (Haskell *et al.*, 1997). For instance, animal carcasses left exposed and uncovered can lose up to 90% of their body weight within seven days during the summer depending on the location and treatment of the corpse² (Lord and Rodriguez, 1989).

At present, there are two approaches available for estimating the postmortem interval using insect evidence, and the application of either one largely depends on the state of decomposition of the corpse at the time of discovery by humans. The first method involves the analysis of the pattern of colonization of the carrion by successive waves of insects and other arthropods (Haskell *et al.*, 1997). The second method relies on the development of immature flies that are deposited on the carrion shortly after death. Insect succession can generally be used from about one month after death to one year after death, or until the carrion is completely skeletonized

²e.g. buried or exposed

(Anderson, 1999). In contrast, insect development can be used up to one month after death, but rarely any longer than one month. After this time period, the immature stages that were deposited shortly after death would have already completed their development, and therefore, would no longer be useful for estimating the postmortem interval (Anderson, 1999). However, the times given for each method are general; and are based on the assumption that the insects arrived at the corpse within a few hours after death. The use of either approach will also depend on factors such as season, climate, location of the corpse³ and treatment of the corpse⁴.

Postmortem interval estimates, calculated using insect development data, are generally more precise than the estimates generated using insect succession data (Haskell *et al.*, 1997). The differences in the precision of the two methods is largely due to the difference in time frames over which the two methods are applied. According to Haskell *et al.* (1997), estimates of the postmortem interval can be within 12 hours or less of the actual time of death when the remains have been exposed for at least 15-20 days. Even so, the calculation of accurate and precise estimates of the postmortem interval requires extensive knowledge of the life histories of the insects present, the ability of the insect species to arrive at the corpse shortly after death, and the ability of the insect species to then deposit their offspring on the corpse (Haskell *et al.*, 1997).

According to Smith (1986), the insects associated with decomposing animal carrion can be divided into four distinct groups:

1. Necrophagous species.
2. Parasites and predators of the necrophagous species.
3. Omnivorous species.
4. Adventive species.

From a forensic perspective, adventive species, which are transient arthropods⁵ are not useful for estimating the postmortem interval. The time of arrival of adventive

³e.g. indoors or outdoors

⁴e.g. buried, wrapped, submerged, etc.

⁵e.g. spiders

species in the pattern of insect succession cannot be predicted with certainty, because it is not associated with a particular stage of decomposition. Adventive species do not rely on the carrion for sustenance, but rather use the carrion as an extension of their habitat (Smith, 1986).

Omnivorous insects⁶ are also, in general, not useful for estimating the postmortem interval because they feed on both the carrion and its inhabitants. However, data on omnivorous insects such as ants may prove useful in conjunction with data from other insects species present on the carrion. For example, the time required for the establishment of an ant colony, specifically a colony of *Anoplolepis longipes* (Jerdon) (Hymenoptera: Formicidae), was used in conjunction with development data for *Hermetia illucens* (Linnaeus) (Diptera: Stratiomyidae) to estimate the postmortem interval for human remains that were discovered inside a toolbox (Goff and Win, 1997). However, omnivorous insect species usually pose the same problem as adventive insect species in that the time of their arrival in the pattern of insect succession cannot be reliably associated with a particular stage of decomposition (Smith, 1986).

In terms of estimating the postmortem interval, the **necrophagous** insects, and their predators are the most valuable. These species are the carrion feeders, and include members of the Orders Diptera (true flies) and Coleoptera (beetles). Of the many species of Diptera, the blow flies (Family Calliphoridae) and the flesh flies (Family Sarcophagidae) are usually the first colonizers of carrion (Smith, 1986). These insects generally arrive within hours of death depending on the season, weather conditions and location of the corpse (Erzinclioglu, 1983). In situations where blood, body fluids, or both, are present, the insects may arrive within minutes of death (Nuorteva, 1977). In general, species of the Family Calliphoridae arrive first, within hours after death, followed by insects of the Family Sarcophagidae, which generally arrive a few days after death (Turner, 1991). However, there have been some instances where in inclement weather, the Sarcophagidae, which are stronger fliers than the Calliphoridae, have arrived before the Calliphoridae (Erzinclioglu, 1983).

⁶e.g. wasps, ants

1.2 Insect Development

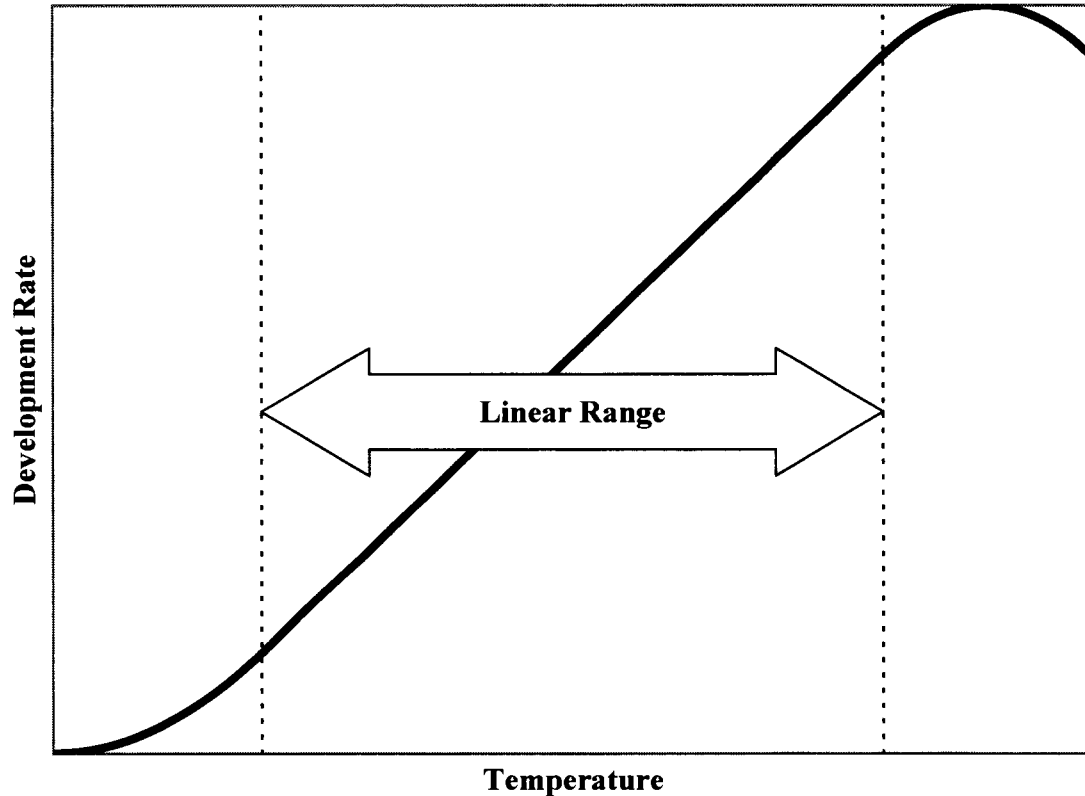


Figure 1.1: Generalized relationship between the rate of insect development and temperature.

Insects are **poikilotherms**, and therefore they lack a temperature regulating system. As a result, their development is largely dependent on the ambient temperature (Higley and Haskell, 2001). The general relationship between insect development rate and temperature is illustrated in Figure 1.1.

As illustrated in Figure 1.1, the relationship between the rate of insect development and temperature is linear in the middle and nonlinear at the ends (Higley and Haskell, 2001). The curved areas at either end of the generalized development curve represent developmental thresholds, which are the minimum and maximum temperatures at which development will still occur. (Higley and Haskell, 2001). The peak on the

right side of the curve represents the highest temperature at which development will occur, and is referred to as the **developmental maximum** (Higley and Haskell, 2001). The trough on the left side of the curve represents the lowest temperature at which development will occur, and is referred to as the **developmental minimum** (Higley and Haskell, 2001). Estimating these developmental thresholds can be very difficult because development is either extremely slow at these thresholds, or occurs at the lethal limits for the species. As a result, maintaining colonies at or near the developmental thresholds is considerably difficult because of high mortality rates at these extreme temperatures (Higley and Haskell, 2001). Although the details of the curve (e.g. the developmental thresholds and the slope of the linear portion of the curve) are species dependent, the development curve presented in Figure 1.1 is similar for all species of Diptera (Higley and Haskell, 2001). Variation within a species further complicates the estimation of the developmental thresholds; therefore, describing the developmental curve for a given species is highly time consuming and labour intensive. Description of the developmental curve for a given species requires developmental data from multiple temperatures, multiple generations and multiple individuals (replicates) (Higley and Haskell, 2001). The accurate and precise estimation of the postmortem interval, by insect development, requires accurate and detailed elucidation of the specific relationship between the external temperature and rate of development for the insect species of forensic interest.

1.3 Insect Development and its Use in Forensic Entomology

Insect evidence discovered at a death scene is primarily used to provide an estimate of the postmortem interval, and the insect evidence most commonly collected, particularly during the early stages of carrion decomposition, are fly larvae (Nuorteva, 1977). According to Catts and Goff (1992), the deposition of offspring on decomposing animal carrion by female flies can be viewed as the activation of a biological clock,

which can be used to estimate the postmortem interval. Four major requirements govern the use of this biological clock as a means to estimate the postmortem interval, specifically:

1. The species present must be correctly identified.
2. Accurate and precise developmental data must be available for that species. In addition, the development data available must encompass a variety of the temperatures expected for the region where the corpse was discovered.
3. The climatic conditions (e.g. temperature, amount of rainfall) of the location where the corpse was found must be obtained for the time period between colonization and human discovery of the remains.
4. The age of the larvae found at the time of human discovery of the remains must be accurately determined.

Furthermore, accurately establishing the age of the larvae collected from the corpse at the time of discovery is dependent on the accurate determination of the first three requirements listed above.

The generalized life cycle of a flesh fly (Sarcophagidae) is presented in Figure 1.2. Unlike blow flies (Diptera: Calliphoridae), most flesh fly species are **larviparous**, and deposit live first instar larvae rather than eggs. Other than differences in the stage of offspring initially deposited, the development of all species of Diptera is the same and consists of two moults, three feeding stages called **instars**, a **pupal stage** and an adult stage (Greenberg and Kunich, 2002).

Adult females generally deposit their offspring in wounds or orifices, such as the mouth and nose, first, because the external tissues in those areas are softer, and, as in the case of wounds, the presence of blood and other body fluids provides a readily accessible source of nourishment to young first instar larvae. First instar larvae are extremely small, and their mouthparts are not strong enough to puncture hard external tissues such as unbroken skin. The first instar is the first of three feeding stages, and during the first instar the larvae usually increase in size from about 2 mm to 4 mm (Greenberg and Kunich, 2002).

The cuticle, or outer skin of the larva, is quite flexible, and expands considerably within a given stage to accommodate the rapid increase in size that accompanies the voracious feeding of the larva. However, further increases in size require that the larva shed its outer skin in a process called **moulting**. Once a first instar larva moults, it becomes a second instar larva.

The second instar is the second of three feeding stages, and during this stage, the larva grows to about 8 mm in length (Greenberg and Kunich, 2002). More feeding is accomplished in this stage compared to the first instar, largely due to the increased size of the larvae (Haskel *et al.*, 1997). In addition, the pH of the carrion tissue has usually changed by this time, becoming more alkaline. The increased alkalinity of the tissues facilitates the breakdown of connective tissue and muscle, which in turn facilitates the penetration of the tougher tissues by the insects' mouthparts (Haskell *et al.*, 1997). The second instar is typically the shortest in duration of the three feeding stages, lasting approximately 8 to 12 hours in most species at moderate temperatures (Haskell *et al.*, 1997). During the second instar, the **crop**, a food storage organ, becomes visible and starts to increase in size as the rate of food ingested starts to exceed the rate at which food is digested (Greenberg and Kunich, 2002). The cuticle continues to expand to accommodate the rapid growth of the second instar larva, until further increases in size can no longer be accommodated. At this point, the second instar larva moults, and becomes a third instar larva. During the third instar,

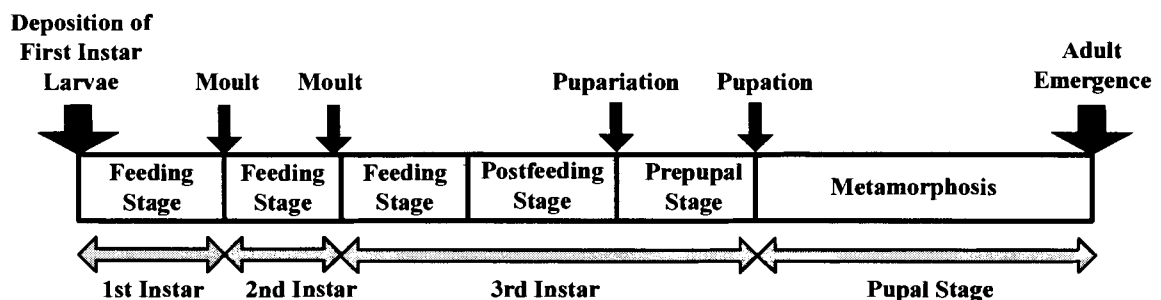


Figure 1.2: Generalized flesh fly (Diptera: Sarcophagidae) life cycle. The size of the boxes representing each stage are not drawn to scale (i.e. the size of the box does not indicate the relative length of the developmental stage).

the larva grows from about 8 mm to between 15 mm and 22 mm, depending on the species (Greenberg and Kunich, 2002).

The third instar can be divided into two, behaviourally distinct stages: (1) the feeding stage, and (2) the postfeeding stage (Anderson, 2000). The feeding stage of the third instar is the last of the three feeding stages, and in this stage, the larvae feed rapaciously. Both the larvae themselves, and their crops increase in size until a maximum size⁷ is attained (Anderson, 2000; Greenberg and Kunich, 2002). Once this maximum size has been attained, the third instar larvae stop feeding and wander away from the food source in search of a safe place to pupate (Anderson, 2000). This wandering stage is called the postfeeding stage, and it can last for several days. During this postfeeding stage, the larvae begin to use the food stored within their crops (Greenberg and Kunich, 2002). The size of the crop gradually decreases as the contents are consumed until it is no longer visible. At the same time, an opaque layer of fat bodies are produced under the surface of the **integument** (Anderson, 2000). The formation of opaque fat bodies serves to block the internal structures, including cephalic structures, such as the mouthparts, from view (Catts, 1990). As a result, the white colour of the larval cuticle is transformed to an opaque, creamy whitish yellow.

After several days of wandering, the larvae begin to pupariate (Greenberg and Kunich, 2002). **Pupariation** is a developmental process that transforms the pliable whitish-yellow larval cuticle into a hard, brownish-black protective shell (Greenberg and Kunich, 2002). The process of pupariation includes several morphological and physiological changes, including, but not limited to (Greenberg and Kunich, 2002):

1. reduction of the speed of locomotion
2. contraction of longitudinal muscles
3. longitudinal shrinkage of the cuticle
4. hardening and darkening (tanning) of the cuticle

⁷i.e. the maximum larval size attained is both species dependent and nutrition dependent

The stage at the start of pupariation is called the **prepupal stage**, and it is relatively short in duration (Greenberg and Kunich, 2002). The prepupal stage should not be confused with the **postfeeding stage**. Pupariation and the formation of the pupa are actually discrete events. In addition, a prepupal larva is still considered to be a third instar larva (Greenberg and Kunich, 2002). The skin of the prepupal third instar larva is called the **puparium**, and after the puparium hardens and darkens, **pupation** begins (Greenberg and Kunich, 2002). During pupation, the insect detaches from the interior surface of the puparium, in a process that is called larval-pupal **apolysis**. At the end of apolysis, the insect, or pupa, is encased in a shell created from its own hardened skin (Greenberg and Kunich, 2002). Approximately 40% of the total development time for a fly is taken up by pupariation and pupation (Catts, 1990).

Since flies are **holometabolous** insects, they undergo complete metamorphosis while in the pupal stage. During the pupal stage, the majority of the larval structures are broken down by **histolysis** (Elzinga, 2000). Adult structures are formed within the hardened puparium at the same time in a process called **histogenesis**. The adult structures are formed from **imaginal discs**, which are regions of embryonic tissue that remain after histolysis (Elzinga, 2000). When metamorphosis is complete, the fully formed adults emerge from the puparia. Newly emerged adults have soft cuticles, very small abdomens, and are unable to fly because their wings have not yet expanded. Within a few hours of emergence, the cuticle will have hardened and the wings and abdomen will have expanded (Greenberg and Kunich, 2002). At this point, the adult is referred to as the teneral adult. Teneral adults are not reproductively mature (Greenberg and Kunich, 2002). In most fly species, protein consumption by both males and females is usually required to initiate the maturation of their respective reproductive organs (Rasso and Fraenkel, 1954; Avancini, 1988; Stoffolano *et al.*, 1995).

1.4 Accuracy, Precision and Reliability of the Entomological Method with Emphasis on Postmortem Interval Estimations Using Insect Development

As noted previously, the deposition of offspring by female flies can be regarded as the start of a biological clock, which can be used to estimate the postmortem interval (Catts and Goff, 1992). However, in order to estimate the postmortem interval using insect development, the age of the developing larvae present on the corpse at the time of discovery must first be established (Catts and Goff, 1992). The age of the oldest larvae present at the time of discovery by humans will give a minimum time since death; for example, if the oldest larvae discovered on the corpse are five days old, then the decedent has been dead for at least five days (Anderson, 1995). The age of the oldest insects collected at the time of discovery give a minimum estimate of the postmortem interval because if the insects are of a given age, the duration of the postmortem interval cannot be less than the number of days required for the insects discovered to have reached that age (Erzinclioglu, 1992). Therefore, accurate estimation of the age of the larvae at the time of discovery of the corpse is absolutely essential for the calculation of a realistic and justifiable estimate of the postmortem interval (Catts, 1992). Furthermore, the calculated estimate of the postmortem interval must be defensible on both scientific and legal⁸ grounds (Catts, 1992).

The age of a fly larva is determined primarily by its thermal history⁹, species, length or weight and developmental stage (instar) (Greenberg and Kunich, 2002). Insect development studies typically report only one variable of insect size¹⁰ versus time, and the developmental curves created from these studies then serve as the reference material for determining the age of the larvae collected from a corpse.

⁸i.e. in court

⁹i.e. the temperatures that it was exposed to during development

¹⁰e.g. length or weight

Larval length, on its own, is not a reliable indicator of larval age. Considerable overlap in larval length exists between the developmental stages, particularly between the second and third instars (Anderson, 2000). The overall size of the larvae discovered, especially for larvae in the second and third instars, largely depends on the nutritional quality of the food source (Greenberg and Kunich, 2002). In addition, during pupariation, the larvae shrink in length as the longitudinal muscles and cuticle contracts. However, the degree of shrinkage experienced by a given larva during pupariation varies both between and within species (Greenberg and Kunich, 2002). Once the larva has entered the wandering stage of the third instar the length of the larva can no longer be used to determine the age of the larva (Greenberg, 1991). Therefore, the length of a larva should never be used as the sole determinant of the age of the larva.

Likewise, weight should never be used as the sole determinant of larval age. Considerable overlap exists in the weight distributions for larvae of different ages (Wells and LaMotte, 1995; Williams, 1984). As with larval length, the nutritional quality of the food source will impact the weight of the larvae that were feeding on the substrate, especially during the feeding stages of the second and third instars. In addition, the larval weight decreases sharply during the postfeeding stage of the third instar because the contents of the crop are being consumed in preparation for pupation.

The estimation of larval age usually involves the comparison of preserved larvae that were collected from the corpse with development data generated from larvae of known ages (Wells and LaMotte, 1995). Since a comparative approach is used, the larvae of unknown age must be measured in the same way as the larvae used to generate the reference data (Wells and LaMotte, 1995). Two types of larval samples are usually collected from the corpse, either at the death scene or at autopsy. In some cases, larvae are collected at both the death scene and at autopsy¹¹. One of the two samples of larvae is kept alive and reared to adulthood in order to facilitate species identification, and the other sample is preserved for later age estimation, and for possible presentation in court.

¹¹Preferably, the insects are collected at the death scene, or at both the death scene and at the autopsy, rather than just at autopsy.

Considerable attention must be given to the preservation method used to collect and store the larval evidence, as the method chosen may lead to further error if the sample insects were preserved using a method different from that used to preserve the reference insects.

For example, Tantawi and Greenberg (1993) determined that live peak-feeding third instar larvae of *Protophormia terranova* (Robineau-Desvoidy) (Diptera: Calliphoridae) shrink when they are placed in San Veino, a surface disinfectant commonly used at autopsy to kill any arthropods still present on the corpse. The degree of larval shrinkage due to storage in San Veino was sufficient to reduce the apparent age of the larvae by 11 hours (Tantawi and Greenberg, 1993). A similar effect was also observed when *P. terranova* larvae of the same age were killed using formalin. However, in the case of the formalin preserved insects, the under-age error was considerably greater than for the *P. terranova* larvae preserved in San Veino; *P. terranova* larvae appear approximately 17 hours younger when they are killed in formalin (Tantawi and Greenberg, 1993). A solution of 70% alcohol was also determined to shrink live peak-feeding third instar *P. terranova* larvae, and even though the degree of shrinkage was less, it was still significant enough to reduce the apparent age of the larvae by ten hours (Tantawi and Greenberg, 1993). In addition, the degree of shrinkage is not constant over the larval lifespan; larvae of different ages shrink by different amounts. For instance, Tantawi and Greenberg (1993) determined that the shrinkage exhibited by young third instar larvae was greater than the shrinkage exhibited by old third instar larvae. Greater shrinkage was observed in the young third instar larvae because the cuticle in younger larvae is thinner and has greater plasticity (Tantawi and Greenberg, 1993).

These errors can significantly undermine the accuracy and precision of postmortem interval estimates generated by analysis of preserved insect specimens. The potential problems associated with larval shrinkage can be circumvented by ensuring that the preservation method used to collect and store insects from the crime scene is the same as the method used to generate the reference curves for the development of the insect species of forensic interest.

Another way to circumvent the shrinkage problem is to kill the live larvae in hot

water. According to Greenberg and Kunich (2002) larvae killed in this manner will remain extended regardless of the preservative used to store them. Even though this does circumvent the problem of shrinkage due to the preservative used, it does not eliminate the requirement that the insect development curves used to determine the age of the larvae collected should be generated using the same preservation method, particularly when length is used as a determinant of larval age.

In forensic situations, the length or weight of the largest larvae collected are usually used to determine the postmortem interval (Greenberg and Kunich, 2002). Therefore, in addition to preserving the insects obtained from the scene in the same manner as those used to generate the reference curves, the reference curves should be generated using the largest larvae present at each sampling period (Greenberg and Kunich, 2002). Some entomologists, such as Erzinclioglu (1990) do not advocate the use of only the largest insects collected from a corpse. Instead, Erzinclioglu (1990) advocates the use of the largest insects from the developmental stage present on the corpse in the greatest abundance.

1.5 Factors confounding use of insect development for estimating postmortem interval

To date, forensic entomology is the most well-researched method of estimating the elapsed time since death in the later postmortem interval (Buchan and Anderson, 2001). As a result, it is considered to be the most accurate, reliable and precise method in use world-wide (Buchan and Anderson, 2001), and extensive research has been conducted to support this claim. For instance, in a study conducted by Kashyap and Pillay (1989), the entomological evidence provided an estimate of the postmortem interval that was both more accurate and more precise compared to the estimate generated on the basis of changes in decomposition.

However, there are several factors that, if left unaccounted for, significantly decrease the accuracy and precision of the postmortem estimates calculated using insect

development data. Many of these confounding factors relate to temperature fluctuations, which can alter the rate of insect development. In many cases, the exact temperatures to which the insects were exposed to prior to discovery are impossible to determine, and instead they have to be estimated from regional weather reports, or from consideration of the degree of corpse exposure to sun and or shade. The formation of maggot masses is another factor that can impact the thermal history of the larvae feeding on a corpse. A considerable amount of metabolic heat is generated by a mass of feeding larvae, and may cause an increase in the rate of insect development (Higley and Haskell, 2001).

Forensic entomology is usually used to estimate the postmortem interval in human death investigations, and humans, with the exception of household pets¹², may be viewed as somewhat unique when compared to other animal carrion. While alive, many humans ingest a variety of drugs, both illegal and legal, on a continual basis, and in many cases the drug consumed may accumulate in a variety of human tissues, such as the liver, skeletal muscle and blood. Therefore, when a human dies, the insects deposited on the corpse may be feeding on drug contaminated tissues, and the drugs present may alter the rate of insect development. If the impact of the drugs present in human corpses on the development of the insects is not accounted for, the estimate of the postmortem interval calculated from insect development data may be inaccurate. As a result, the reliability of the post-mortem interval estimate obtained using developmental data from insects reared on drug-contaminated tissue may be highly questionable, and therefore a matter of primary concern.

1.6 Entomotoxicology

Entomotoxicology is a relatively new area of research that can be included under the auspices of forensic entomology. Investigations within the area of entomotoxicology include (Introna *et al.*, 2001):

1. studying the effects of drugs and other toxins on the developmental rate of forensically important insects

¹²e.g. cats and dogs

2. using insects as alternative toxicological specimens when traditional specimens, such as blood and liver, are no longer available or suitable for analysis.

As discussed earlier in the previous section, the use of entomological evidence to determine the postmortem interval has been shown to be extremely accurate, reliable and precise (Kashyap and Pillay, 1989; Buchan and Anderson, 2001). However, previous research indicates that the developmental patterns of forensically important insects may be altered if the insects are feeding on drug contaminated tissues. The impact of several commonly abused drugs, both legal and illegal, on the developmental patterns of forensically important insects have been researched in a number of countries, including Brazil, France, South Africa and the United States.

Ideally, the effects of drugs on the development of forensically important fly species would be conducted using human tissue contaminated with a known concentration of a particular drug or combination of drugs, as this would eliminate problems associated with the use of non-human animal models. However, the use of human tissues in entomotoxicological research would be problematic for a number of reasons, such as ethical restrictions, health restrictions, problems with producing replicable results¹³ and lack of proper experimental control¹⁴.

For this reason, most of the research done to date, regarding the impact of drugs on insect development, has been conducted using rabbits as the experimental model. Typically, live rabbits are infused via ear artery perfusion (e.g. Bourel *et al.*, 1999) or injected via cardiac puncture (e.g. Goff *et al.*, 1989) with a known quantity of drug. The experimental rabbits are then allowed to metabolize the drug for a specified period of time, and after that time are then euthanised. Their carcasses, or a portion of their carcasses¹⁵ then serve as the drug-contaminated food source used to rear the insects. Any alterations in the development of the insects reared on the drug-contaminated

¹³The likelihood of obtaining several samples of human tissues with the same concentration of a specific drug, or combination of specific drugs is highly unlikely.

¹⁴For the same reason as before, the likelihood of receiving human tissue samples that are free of drugs or contaminants is highly unlikely, simply due to the fact that the majority of the human population consumes a wide variety of different drugs or medicaments on a regular basis. The disease state of the tissues obtained would have to be matched as well.

¹⁵e.g. the liver

tissues are recorded by measuring the length, weight or developmental stage of insects sampled at specified sampling periods.

To date, the impact of several commonly abused drugs, such as cocaine, morphine, phencyclidine, diazepam and amitriptyline, on the development of a forensically important fly species (Diptera) has been investigated. For example, a substantial amount of research has been conducted on *Parasarcophaga ruficornis* (Fabricius) (Diptera: Sarcophagidae) and *Boettcherisca peregrina* (Robineau-Desvoidy) (Diptera: Sarcophagidae), both of which are species of considerable importance in Hawaii (Goff *et al.*, 1986). Other researchers have focused on fly species from the Family Calliphoridae, including *Lucilia sericata* (Meigen), which is a prominent necrophagous species in Europe, and flies of the species *Chrysomya*, such as *Chrysomya albiceps* (Wiedemann) and *Chrysomya putoria* (Wiedemann), which are species of forensic importance in southeastern Brazil.

The impact of cocaine and its major metabolite benzoylecgonine on the development of *Boettcherisca peregrina* (Diptera: Sarcophagidae) was investigated by Goff *et al.* (1989). The larvae were reared on tissue derived from three different rabbits that were injected with 35 mg, 69 mg or 137 mg of cocaine. These doses were calculated to represent median sublethal, sublethal, median lethal and twice median lethal doses, respectively. The second and third instar larvae reared on rabbit tissues containing the metabolic products of the median lethal and twice median lethal doses of cocaine developed 12 to 18 hours more rapidly than either the larvae reared on the cocaine-free rabbit tissue, or the rabbit tissues containing the metabolic products of the sublethal dose (Goff *et al.*, 1989). In addition, the onset of pupariation occurred earlier in the larvae reared on the median lethal and twice median lethal dosed rabbit tissues. However, the duration of pupation, when compared with the control colony, was not altered in any of the colonies reared on tissues containing cocaine and its major metabolite (Goff *et al.*, 1989).

In another study conducted by Goff *et al.* (1991), the effects of heroin, as morphine¹⁶, on the development of *Boettcherisca peregrina* (Diptera: Sarcophagidae) were

¹⁶Morphine is a major metabolite of heroin, and intravenously administered heroin is rapidly

investigated by injecting rabbits with 6 mg, 12 mg, 18 mg and 24 mg of heroin. The levels of heroin injected were calculated to represent the morphine levels found in heroin related human fatalities. The rate of larval growth was accelerated in the colonies reared on the rabbit tissues containing morphine up to 29 hours. In contrast, the duration of the pupal period was extended by an average of 18-36 hours for the four experimental colonies; however, in the case of the 6 mg dose of heroin, the extended duration of the pupal period was not significant. Furthermore, the difference in mean pupal weight between the control colony and the four experimental colonies was also not significant (Goff *et al.*, 1991).

Bourel *et al.* (1999) also investigated the effects of morphine on the development of a forensically important insect species, specifically *Lucilia sericata* (Diptera: Calliphoridae). However, in contrast to the research conducted by Goff *et al.* (1991), Bourel *et al.* (1999) reared the *L. sericata* larvae on the entire rabbit carcass, and not just on rabbit liver. With this in mind, the research conducted by Bourel *et al.* (1999) is likely more applicable to human cases. In their investigation, Bourel *et al.* (1999), dosed experimental rabbits with 12.5 mg, 25 mg, and 50 mg of morphine by ear artery infusion. The use of ear artery infusion allowed for greater control over the resulting blood and tissues levels of morphine in the rabbits. In addition, the use of ear artery perfusion enabled the researchers to achieve end morphine concentrations in the experimental rabbits that more closely matched the concentrations found in morphine related human fatalities (Goff and Lord, 2001). The presence of morphine in the tissues used to rear *L. sericata* decreased the rate of larval growth, in a dose dependent manner; larvae reared on the rabbit tissues that received the largest dose of morphine developed more slowly than those reared on the rabbit tissues containing less morphine. These results are opposite of those found by Goff *et al.* (1991) in their investigation on the effects of heroin, as morphine, on the development of *B. peregrina*. The contrasting results obtained by Goff *et al.* (1991) and Bourel *et al.* (1999) indicate the possible existence of Family specific differences in physiological response to the presence of morphine in the rearing media. Clearly, additional studies

metabolized to morphine. For instance, the half-life of heroin is 2-6 minutes (Baselt, 2000).

with different forensically important species of Calliphoridae and Sarcophagidae are required.

The preceding paragraphs have discussed only a small portion of the research that has been conducted regarding the effects of commonly used or abused drugs on insect development. Although the use of experimental animals, such as rabbits, incorporates aspects of drug metabolism, its major drawback is, in fact, the extensive use of experimental animals. Given the number of drugs, drug combinations, and insect species to be studied, it is not feasible based on financial and ethical grounds, to rely solely on the use of animals as the experimental model.

1.7 Thesis Rationale and Objectives

Entomotoxicological data for Canada is limited, and if forensic entomology is to be used to its fullest extent in Canada, this situation must change. The presence of drugs, including alcohol, in British Columbian deaths is not unusual; nor is it unusual for such death investigations to require the techniques of forensic entomology to establish time of death. For instance, in an analysis of 42 death investigations that used insect evidence in British Columbia between 1988 and 1994, 48% (20) of the cases required toxicological analyses. Of the cases that required toxicological analyses, 80% (16) of the cases tested positive for drugs and/or alcohol (Anderson 1995). Therefore, investigating the effects of drugs on the development of forensically significant insects in British Columbia is extremely important. Such knowledge is required to ensure that the estimate of elapsed time since death is as accurate as the quality of evidence obtained will allow.

Therefore, the present thesis had two objectives:

1. To investigate the impact of a commonly used or abused drug in British Columbia on the development of a forensically important insect species in British Columbia.
2. To investigate the use of a non-live animal model for use in entomotoxicological investigations.

The tricyclic antidepressant amitriptyline was the drug chosen for study in the

present thesis for several reasons, including its relatively low cost, and the existence of a large amount of peer-reviewed literature available on amitriptyline pharmacokinetics, postmortem tissue concentrations and chromatographic analysis. Furthermore, given the fact that the experimental research necessary for this thesis was conducted at three different locations¹⁷, it was important to pick a drug that did not require special facilities or licenses to have in the laboratory. Since amitriptyline is not an illegal drug, regular laboratory facilities were appropriate for its storage, and therefore amitriptyline was more appropriate for the present thesis than an illegal substance such as cocaine. In addition, tricyclic antidepressants such as amitriptyline are commonly prescribed antidepressants in British Columbia. During the six year period of 1997-2002, 1,388,166 prescriptions for tricyclic antidepressants were received by BC pharmacies, and of this number 57.6% (793,429) of the prescriptions were for amitriptyline (BC Ministry of Health, 2003)¹⁸. Furthermore, a review of the Judgment of Inquiry reports produced by the BC Coroners' Service indicated that 213 deaths during the six year period of 1997-2002 were the result of poisoning by tricyclic antidepressants, and of those 213 deaths, 156 (73.2%) were due to poisoning by amitriptyline. Given these results, amitriptyline was a reasonable choice for an entomotoxicological investigation¹⁹.

Since the second objective of the present thesis was to evaluate the use of a non-live animal model for investigating the effects of commonly used or abused drugs on the development of forensically important insects, it was also necessary, for comparative purposes, to choose a drug on which entomotoxicological research had already been conducted. In an earlier study, Goff *et al.* (1993) investigated the impact of

¹⁷The method validation experiments were conducted at the Provincial Toxicology Centre, the artificial foodstuff was prepared at the British Columbia Institute of Technology, and the insect development studies were conducted in the Forensic Entomology laboratory at Simon Fraser University.

¹⁸Neglecting the fact that certain age groups do not typically receive prescriptions for tricyclic antidepressants, and based on the 1996 Census population of 3,724,500 people for British Columbia, this amounts to 0.37 amitriptyline prescriptions per person over the six year period (BC Stats: Ministry of Management Services, no date).

¹⁹The data obtained from both the BC Ministry of Health and the BC Coroners' Service was obtained under SFU ethics approval for a previous project entitled "The effects of commonly abused drugs in Canada on time of death determinations in the later postmortem interval, using insect development." See Appendix D.

amitriptyline on the development of *Parasarcophaga ruficornis* (Diptera: Sarcophagidae). Therefore, it was important that either the same species, or a closely related species, was used in the present experiment. Unfortunately, the species used by Goff *et al.* (1991) is not native to Canada, and as a result a different, albeit closely related, species had to be chosen²⁰.

Insects of the Family Sarcophagidae are insects of considerable forensic importance in tropical areas, such as Hawaii or the southern mainland of the United States (Byrd and Castner, 2001). Of the insect species recovered from corpses located indoors, during the summer months in the southeastern United States, the majority of the species belong to the Family Sarcophagidae (Byrd and Castner, 2001). In regions where they are commonly associated with animal carrion, flies from the Family Sarcophagidae normally arrive at an animal carcass at the same time, or slightly after blow flies (Family Calliphoridae) (Byrd and Castner, 2001). In comparison, insects from the Family Sarcophagidae are rarely recovered from decomposing carrion in British Columbia (Anderson, 1995). In British Columbia, the majority of the insect species collected from decomposing remains are species from the Family Calliphoridae. For example, immature insects from the Family Calliphoridae, during the period of 1988 to 1994, were associated with 60 cases involving human remains, compared to only four cases with immature insects of the Family Sarcophagidae (Anderson, personal communication, 2003). This is somewhat unfortunate, since the results from the present project may not be immediately applicable to forensic case work in British Columbia. Even so, this does not negate the validity of the present project since the second objective of the present thesis was to investigate the suitability of a non-live animal model for use in entomotoxicological investigations.

With this in mind, another fly species from the Family Sarcophagidae was chosen, namely the species *Sarcophaga bullata* (Parker). The Family Sarcophagidae is a large family, consisting of over 2000 species world-wide, with the majority of the species

²⁰For the purposes of the present thesis, obtaining a closely related fly species to the one used by Goff *et al.* (1993), for comparative purposes, was viewed to be more important than obtaining a fly species that was of great forensic importance in British Columbia.

found in tropical or warm temperate regions (Byrd and Castner, 2001). Approximately 327 of these species can be found in the United States and Canada (Byrd and Castner 2001). *S. bullata* is most commonly found in the southern United States, but the species can also be found in Canada. *S. bullata* is closely related to another species commonly found in Canada, *Sarcophaga haemorrhoidalis* (Fallén), and the two species exhibit similar behaviour and habitat preferences (Byrd and Castner 2001). In addition, *Sarcophaga bullata* was a readily available species from the Family Sarcophagidae. Furthermore, the behaviour and biology of *S. bullata*, in the absence of drugs, has been researched by a number of researchers, from both a forensic and a biological perspective (e.g. Mitchell and Soucie, 1992; Christopherson and Gibo, 1997).

Previous research has already indicated that the developmental responses of blow flies (Diptera: Calliphoridae) and flesh flies (Diptera: Sarcophagidae) to some commonly abused drugs are not the same (Goff *et al.*, 1991; Bourel *et al.*, 1999). Therefore, the results obtained from the present study can be used to determine if a given drug response are consistent among insects of the Family Sarcophagidae.

The non-live animal model, or **rearing media**, used in the present thesis was previously used in an entomotoxicological investigation on the accumulation and elimination of amitriptyline *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae) larvae (Sadler *et al.*, 1997). The artificial food medium was prepared using homogenized beef liver, powdered whole egg and agar. Amitriptyline and its major metabolite, nortriptyline, were added to the artificial rearing media in order to evaluate the impact of the drugs on *S. bullata* development. For comparative purposes, the amitriptyline and nortriptyline were added to the artificial foodstuff according to the levels of amitriptyline and nortriptyline quantitated in the rabbit livers used by Goff *et al.* (1993) to evaluate the impact of amitriptyline and nortriptyline on the development by *Parasarcophaga ruficornis* (Diptera: Sarcophagidae).

Spiking artificial food media with chemicals to test the effects of those chemicals on insect development is not a new technique in itself; the technique is commonly employed in insecticide investigations (Brown, 1960). However, in insecticide studies,

only the parent compound is normally under investigation, and not the parent compound in combination with the metabolite(s) specifically produced by the metabolism of the parent compound by humans.

Therefore, the present thesis expanded the traditional food-spiking technique by adding both the parent compound and the primary human metabolite to the artificial food media. The major metabolite of amitriptyline, nortriptyline, was added to the artificial rearing media in order to simulate the metabolism of the drug by humans. The benefits of a validated non-live animal model for use in entomotoxicological investigations are considerable. For instance, research costs would be lower; beef liver, agar and powdered whole egg are relatively inexpensive and readily obtainable, especially when compared to the costs associated with experimental animals. In addition, the artificial foodstuff could be custom spiked according to the toxicology results obtained for a case requiring forensic entomology to estimate the postmortem interval. For example, if the toxicology results indicated that a specific amount of amitriptyline and codeine were present in the tissues sampled from the deceased, the same concentrations of each drug could be easily added to the artificial foodstuff. The custom prepared artificial foodstuff could then be used to rear insects of the same species, and any changes in development observed could be directly incorporated into the estimate of the postmortem interval, rather than extrapolating from research that at this time is still fairly limited in scope.

1.8 Thesis Overview

This chapter has introduced insect development and its relationship to forensic entomology. A look at a few of the limitations of forensic entomology explained the motivation for this thesis. Chapter 2 will present the procedures used to quantitate the amitriptyline and nortriptyline, while Chapter 3 will present the procedures used to extract and analyze the presence of amitriptyline and nortriptyline from the rearing media. Chapter 4 will present the methods and results of the rearing experiments conducted with insects of the species *Sarcophaga bullata* (Diptera: Sarcophagidae). Finally, the thesis conclusion is in Chapter 5.

Chapter 2

Method Validation I: Amitriptyline and Nortriptyline in Ethyl Acetate

2.1 Introduction to Method Validation

Method validation can be defined as the process through which the performance of the method under investigation is described and confirmed to be appropriate for the intended purpose of the method (EURACHEM Working Group, 1998). Method validation is essential to all analytical work because it is important to not only obtain the correct result, it is also important to demonstrate that the result obtained from a particular analytical method is correct within a described level of confidence (EURACHEM Working Group, 1998).

Knowing that a method meets a described level of confidence is critical in chemical analysis because in most cases, the cost of the analysis in terms of equipment, reagents, labour and time is considerable. Furthermore, there are usually additional consequences, both economic and social, associated with the analytical results. For instance, critical legal decisions may depend on the results of analyses on blood, urine or other tissues for the presence of illegal substances. Confirmation of the presence of such illegal substances could result in fines, job loss, or imprisonment (EURACHEM Working Group, 1998).

Method validation or revalidation is conducted under a variety of circumstances,

including, but not limited to: the development of a new method for a given problem or the extension of an established method to a new problem (EURACHEM Working Group, 1998).

In terms of the research project described in this thesis, an already established gas chromatographic method for the analysis of tricyclic antidepressants from blood and liver, which is used by the Provincial Toxicology Centre in British Columbia, was extended to a new problem. Specifically, the method was extended to the isolation and analysis of tricyclic antidepressants from a unique matrix¹. With this in mind, the method had to be re-evaluated in terms of its suitability for the analytical task because the tricyclic antidepressants had to be extracted from a matrix² different from the matrices originally defined for the method³. In the present project, all analytical work was conducted using a Hewlett-Packard (HP) 6890 gas chromatograph, with a **nitrogen-phosphorous detector**. The actual gas chromatographic method used in the present thesis is described in Appendix A.

In summary, the overall purpose of method validation is to ensure the generation of high-quality data. The method validation process must specify the method's suitability for its intended purpose, report the method's performance characteristics, and provide an estimate of the uncertainty of the results obtained with the method to a given level of confidence.

In the context of this research project, it is important to confirm that amitriptyline and nortriptyline have both been added to the artificial foodstuff, and to quantify the amount of each drug present. Another requirement for this project is homogeneity; amitriptyline and nortriptyline must be evenly dispersed throughout the artificial foodstuff, and as a result, the level of drug homogeneity in the prepared foodstuff must be evaluated for each batch of artificial foodstuff prepared.

Therefore, the purpose of this chapter is to introduce some of the major components of the method validation process:

¹The artificial foodstuff is discussed further in Chapter 3.

²i.e. artificial foodstuff

³i.e. blood and liver tissues

- Selectivity
- Linearity
- Range
- Precision
- Limit of detection
- Limit of quantitation

Furthermore, this chapter will describe the procedures used to evaluate each of the above validation criteria for the analytical method used in the present project. In addition, the results of each of these validation tests will be presented and discussed.

Several other components of the method validation process, such as analyte recovery must also be investigated. However, these criteria will be investigated in Chapter 3.

2.2 Confirmation of Identity and Specificity/Selectivity

In most cases, an analytical method consists of an **isolation phase** followed by a **measurement phase** (EURACHEM Working Group, 1998). During the method validation process, it is vital to verify that the signal produced in the measurement stage, which has been ascribed to the presence of a specific analyte, is in fact, due to the presence of that that analyte, and not to the presence of another substance or combination of substances. In other words, the signal should not result from the presence of another substance with similar chemical and/or physical properties to the analyte of interest (EURACHEM Working Group, 1998). The process of establishing the chemical origination of a signal is known as **confirmation of identity** (EURACHEM Working Group, 1998).

The goal of the isolation phase is to isolate the analyte from its matrix while at the same time minimizing the amount of interfering substances (technically known as **interferences**) that are retained. The effectiveness of the isolation phase and the selectivity of the measurement phase determines whether or not other substances interfere with the analyte of interest (EURACHEM Working Group, 1998).

The ability of a method to accurately and reliably measure the concentration of an analyte in the presence of interfering substances is known as **selectivity** (EURACHEM Working Group, 1998). Selectivity must be verified, otherwise all other method performance characteristics, such as linearity, precision and method accuracy are suspect (Snyder *et al.*, 1997).

Selectivity is a term that is often used interchangeably with **specificity**. This is not good practice, as in general, specificity is defined as 100% selectivity (EURACHEM Working Group, 1998). In cases where a complex matrix is involved, it is often difficult to effectively isolate the analytes of interest, while still maintaining an acceptable rate of analyte recovery. As a result, the measurement phase is often non-specific when the analytes of interest must be isolated from a complex matrix (EURACHEM Working Group, 1998). Even so, the analyst can state, after investigation, that certain substances, such as material from the sample matrix, do not interfere with the measurement of the analytes of interest (EURACHEM Working Group, 1998). Such a method can be described as a selective method. It is unlikely that the analyst will ever be able to state that a method is specific (100% selective) because new, previously undocumented substances may appear in later samples that interfere with the previously validated method (Snyder *et al.*, 1997; EURACHEM Working Group, 1998). With this in mind, selectivity must be reassessed frequently during method development and validation, and even after the method is in routine use (Snyder *et al.*, 1997).

One method of evaluating the selectivity of a chromatographic method is to compare the retention times of the peaks present in the chromatogram of the sample, with the retention times, generated by the same method, of a reference material containing the same analytes of interest. However, this method is only suitable for determining selectivity in certain cases because the method can be very unreliable if **coeluting**

species exist. Usually this problem is solved by confirmation of peak identity using another, comparable analytical method, or another detector (EURACHEM Working Group, 1998).

In terms of the initial method validation for the present project, ethyl acetate is the matrix, and it is known that only three compounds, namely amitriptyline, nortriptyline and maprotyline, will be added to the matrix. As a result, the method described below was suitable for determining selectivity of the method during the initial method validation. However, once the artificial foodstuff matrix is introduced, the evaluation of selectivity will have to be much more rigorous because interfering peaks are very common in complex matrices, such as biological materials. With this in mind, the selectivity will have to be checked each day, before sample analysis is conducted.

Amitriptyline, nortriptyline, and maprotyline were obtained from Sigma-AldrichTM (Oakville, Canada) in the form of their hydrochloride salts. Their chemical structures are illustrated in Appendix C. The selectivity of the method for amitriptyline, nortriptyline and maprotyline in a matrix of ethyl acetate was determined by injecting and analyzing a series of series of standard analyte solutions on the HP 6890 gas chromatograph. This was done to ensure that all analyte peaks were appropriately resolved and that there were no coeluting peaks present. The first standard run was an ethyl acetate blank. Running this blank demonstrated that the matrix did not contain any interfering peaks. Next, separate standards of amitriptyline, nortriptyline and maprotyline were analyzed to determine their respective retention times. Once the retention time for each of the three compounds of interest was determined, a mixed standard containing equal concentrations of amitriptyline, nortriptyline and maprotyline was injected in order to determine whether the three drugs could be separated to an appropriate level of **resolution**. A chromatogram illustrating the baseline separation of the three components, amitriptyline, nortriptyline and maprotyline is presented in Figure A.1 (Appendix A).

2.3 Linearity, Range and Precision

The **linearity** of a method is simply a measure of how closely a calibration curve,

generated by plotting the instrument response⁴ versus concentration, approximates a straight line (Snyder *et al.*, 1997). The **range** of an analytical method is defined as the lower and upper analyte concentrations for which the method has adequate linearity, accuracy and precision (Snyder *et al.*, 1997). In analytical work, the range of a particular method refers to the concentrations of analyte actually measured with the method, not the range of analyte concentrations present in the original, unprocessed material. If the linearity of a method can be established over only a small portion of the range of concentrations present in the original, unprocessed material, the analyte concentrations present in the processed samples can usually be diluted or concentrated so that they can be analyzed by the method.

The range of concentrations that can be reliably analyzed with the method will depend on a number of factors. For example, at the lower end of the range, limiting factors are the **limit of detection (LOD)** and the **limit of quantitation (LOQ)** (EURACHEM Working Group, 1998). At the upper end of the range, the constraints are dependent on the response of the instrument (EURACHEM Working Group, 1998).

The linearity of a method is evaluated by analyzing samples with known analyte concentrations that cover the entire range of expected concentrations. Evaluation of linearity usually involves mathematical treatment of the instrument response versus analyte concentration data. Usually, the mathematical treatment involves calculating a **regression** line, using a regression technique such as the **least squares method**. In **linear regression**, the linearity can be evaluated by examining the variance associated with the slope of the regression line. The sensitivity of the method depends on the magnitude of the slope (Meyer, 1998). Ideally, in a linear calibration function, the slope should be equal to, or near 1 (or -1). This is important because if the slope is not equal to, or near 1 (or -1), small errors present in one parameter⁵ could result in comparatively large errors in the other parameter⁶. Therefore, the accuracy of the method may also be affected if the magnitude of the slope is too high or too low

⁴i.e. more specifically, the detector response

⁵e.g. detector response

⁶e.g. concentration

(Meyer, 1998). The y -intercept in an ideal situation is equal to zero⁷. The magnitude of the y -intercept is a measure of the potential assay bias (Meyer, 1998).

The actual range of concentrations examined for a method will depend on the type of method and its intended purpose. For the present project, the required concentration ranges for each analyte are known. The range of expected concentrations for amitriptyline in the artificial foodstuff is 20 mg/kg to 200 mg/kg, and for nortriptyline, the expected range is 0.9 mg/kg to 20 mg/kg. Therefore, the method used in the present project must provide linear results for each of these analytes within their expected concentration ranges.

Precision refers to the level of agreement among individual test results, where each result is generated by repeatedly applying the same procedure, from sampling to analysis, to a homogeneous sample (Snyder *et al.*, 1997). Precision can be divided into three types: (1) reproducibility, (2) repeatability and (3) intermediate precision (Snyder *et al.*, 1997).

Reproducibility measures the precision between different laboratories (Snyder *et al.*, 1997). Since the method used in the present project was intended for use only at the Provincial Toxicology Centre, this aspect of precision will not be examined further.

Repeatability describes the precision of a method under the same operating conditions⁸ over a short period of time (Snyder *et al.*, 1997). Several different procedures for determining the repeatability of an analytical method can be found in the validation literature. One method involves five or six repetitive injections of the same homogeneous sample at two or three concentrations. (Huber, 2001). The EU-RACHEM Working Group (1998) suggests that for each concentration, ten repetitive injections of the same homogeneous sample should be done. Another publication indicates that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure. Further, the minimum of nine determinations should be made at three different concentrations, with three replicate

⁷i.e. the calibration curve runs through the origin

⁸e.g. same instrument, same analyst

injections each (Snyder *et al.*, 1997).

Since the above methods involve the repeat injection of the same homogeneous samples, the precision being measured is instrument precision. Furthermore, the procedures listed above rely on the same basic theme to evaluate instrument precision; namely, the repeat injection of one or more samples of a homogeneous nature. However, considerable discrepancy concerning the number of concentrations to test and the number of repeat injections required, exists in the validation literature. Even so, the statistical treatment of the repeatability data remains the same. From each set of replicate determinations the mean, standard deviation, and **relative standard deviation (RSD)**, expressed as a percentage %, should be calculated. The number of repeat determinations must be stated along with the calculated level of precision.

The acceptance criteria for precision depends on the type and purpose of the analysis. In terms of quality control for pharmaceutical compounds, precision of better than 1% relative standard deviation (RSD) is often required (Huber, 2001). For biological samples, the precision, or relative standard deviation required is often 15% at the concentration limits, and 10% for other concentration levels (Huber, 2001). Considerable discrepancy regarding the acceptance criteria also exists in the literature. Some researchers indicate that 5-10% RSD is only acceptable for low-level impurities (Snyder *et al.*, 1997). In the end, the acceptance criteria for precision must be determined by the goals of the analysis. Other factors that will also affect the precision acceptance criteria include the time, equipment and finances available for method validation.

For the present project, the acceptance criteria for precision for amitriptyline and nortriptyline in ethyl acetate was set at a maximum of 10% RSD at the concentration limits and 5% for all other concentrations. However, given the complexity of the artificial foodstuff, the precision acceptance criteria for the analytes extracted from the foodstuff matrix was set at a maximum of 15% RSD at the concentration limits and a maximum of 10% RSD for all other concentrations. In addition, due to time constraints and the number of samples to be analyzed, only three repeat injections of each sample were conducted. Therefore, the analytical method developed for the present project must meet the acceptability criteria for precision within three replicate

injections.

With the above in mind, all precision evaluations for the present project were done with three repeat injections of each sample. Furthermore, the RSD for three repeat injections for 11 different calibration standards, covering the entire expected range of analyte concentrations was conducted. The instrument response versus concentration data generated for the precision evaluation was also used to evaluate the linearity of the method.

Additionally, since the method will be used on more than one occasion, over a period of several months, the **intermediate precision** of the method must be determined. Intermediate precision is the agreement of the assay results when the same method is applied over a period of time within the same laboratory, by the same analyst, using the same equipment. Therefore, the intermediate precision expresses the within laboratory variation (Snyder *et al.*, 1997). Intermediate precision is evaluated by conducting the same repeatability tests over a series of days⁹ or weeks¹⁰ and then calculating the overall relative standard deviation. The RSD should be within the previously chosen precision **acceptance criteria**. If the method is to be used over an extended period of time, the intermediate precision should be evaluated at appropriate intervals. For example, in the present project, column contamination was a continual problem, and regeneration of the column using chlorobutane was necessary on several occasions. As a result, the intermediate precision was re-evaluated after each column regeneration.

2.4 Procedure for the Evaluation of Linearity, Range and Precision

Ten calibration standards using maprotyline as the internal standard, were prepared from a mixed 1 mg/mL amitriptyline and 0.1 mg/mL nortriptyline stock solution (in methanol). In order to determine the background noise, a blank containing

⁹i.e. over at least three different days

¹⁰i.e. over at least three different weeks

only maprotyline was also prepared. Each of the 11 calibration standards were prepared in a 10 mL volumetric flask. The concentrations prepared were as follows:

1. 0 mg/L nortriptyline + 0 mg/L amitriptyline (blank)
2. 0.5 mg/L nortriptyline + 5 mg/L amitriptyline
3. 1 mg/L nortriptyline + 10 mg/L amitriptyline
4. 2.5 mg/L nortriptyline + 25 mg/L amitriptyline
5. 5 mg/L nortriptyline + 50 mg/L amitriptyline
6. 7.5 mg/L nortriptyline + 75 mg/L amitriptyline
7. 10 mg/L nortriptyline + 100 mg/L amitriptyline
8. 12.5 mg/L nortriptyline + 125 mg/L amitriptyline
9. 15 mg/L nortriptyline + 150 mg/L amitriptyline
10. 18 mg/L nortriptyline + 180 mg/L amitriptyline
11. 20 mg/L nortriptyline + 200 mg/L amitriptyline

The standards were prepared by adding 100 μ L of 1 mg/ml maprotyline (the internal standard), followed by the appropriate volume of the mixed amitriptyline and nortriptyline stock solution. The aliquoted analyte and internal standard solutions were then diluted to the mark on the volumetric flask with ethyl acetate. The final internal standard concentration in each calibration standard was 10 mg/L. After thorough mixing, an aliquot of each calibration standard was pipetted into a clean autoinjector vial and capped. Fresh aliquots were prepared for each day of analysis. The remainder of the prepared calibration standards were stored at -10°C for later use. Solutions of less than 1 mg/L amitriptyline or nortriptyline are stable¹¹ if stored in the freezer for not more than one month (Huckin, personal communication, 2003).

¹¹i.e. do not degrade

The glass liner present in the injector of the gas chromatograph is a common site of analyte adsorption. The glass of the injector liner contains free **silanol** groups that can cause significant problems when analyzing active compounds (Rood, 1999). Active compounds are those compounds that contain groups such as $-\text{NH}_2$ and $-\text{OH}$. These active compounds can interact with free silanol groups, and cause a significant amount of analyte adsorption. Adsorption of the analytes decreases the sensitivity of the chromatographic system to the analytes. Furthermore, the interaction of active compounds with the silanol groups can cause the peaks to broaden and to exhibit tailing. Broad and/or **tailing peaks** are unsuitable for quantitative analysis.

Therefore, in order to prevent or reduce the amount of analyte lost to adsorption, the glass injector liner was silylated. Silylation converts the active silanol groups to groups that do not interact with groups containing active functional groups (Rood, 1999).

Analyte loss due to adsorption by active silanol groups is common for amitriptyline, nortriptyline and maprotyline since they all contain functional groups that interact well with silanols. With this in mind, a repeatability of injection test was conducted to determine if the glass insert was adequately silylated. For this test the mid-range calibration standard (10 mg/L nortriptyline + 100 mg/L amitriptyline) was injected into the HP 6890 gas chromatograph with a nitrogen-phosphorus detector, 45 times in succession. The standard was run on the gas chromatograph according to the previously developed method (Appendix A). The relative standard deviation of the response, in terms of **peak height** ratio (analyte peak height/internal standard peak height) for the 45 injections of the mid-range standard was then calculated to determine the precision of the results.

Evaluation of method repeatability was conducted by injecting the above set of eleven calibration standards, three times each. The entire series of calibration standards, from lowest (0 mg/L nortriptyline + 0 mg/L amitriptyline) to highest concentration (20 mg/L nortriptyline + 200 mg/L amitriptyline), was injected three times in series. Linear least squares regression analysis of the signal response *versus* concentration data was then performed using JMP IN[®] (SAS Institute, Inc., Cary, NC, USA) in order to determine linearity and range. Intraday precision was evaluated by

calculating the relative standard deviation and repeatability limit for the response data from the three successive injections for each of the 11 calibration standards analyzed. The standards were run on an HP 6890 gas chromatograph, according to the previously developed method (Appendix A).

Intermediate precision was evaluated by injecting the above set of eleven calibration standards over a period of six days. Every two days during the six days, the entire series of calibration standards, from lowest (0 mg/L nortriptyline + 0 mg/L amitriptyline) to highest concentration (20 mg/L nortriptyline + 200 mg/L amitriptyline), was injected and run on a HP 6890 gas chromatograph, according to the previously developed method, three times each, in succession. The relative standard deviation and repeatability limit for the response from the nine repeat determinations of each concentration was then calculated to determine the intermediate precision. The calibration curves generated over the six days were tested for equivalency by calculating the relative standard deviation of the slopes of the calibration curves created. If the RSDs were less than 10%, based on nine determinations for each calibration standard¹², the calibration curves were considered to be equivalent for the purposes of the present research project.

2.5 Results of the Linearity, Range and Precision Evaluations

The results of the repeatability of injection assay, for the mid-range calibration standard, are shown in Table 2.1. As stated earlier, the precision acceptance criteria for all analyte concentrations except those at the concentration limits was a relative standard deviation (RSD) of 5%. The RSD for the amitriptyline/maprotyline peak height ratio for this set of injections was 15.8%, and the RSD for the nortriptyline/maprotyline peak height ratio was 12.0%. Neither of these calculated RSDs met the precision acceptance criteria. In fact, both of the RSDs for analyte peak height/internal standard peak height ratios significantly exceeded the RSD cut-off of

¹²Three determinations on each of three days of validation.

5% (Table 2.1).

Measure of Precision	Peak Height Ratio (AMT Peak Height/MAP Peak Height)	Peak Height Ratio (NOR Peak Height/MAP Peak Height)
Average Peak Height Ratio	30.7	1.6
Standard Deviation	4.8	0.2
Relative Standard Deviation (%)	15.8	12.0

Table 2.1: Precision measurements for 45 repeat injections of a mid-range mixed calibration standard containing 10 mg/mL nortriptyline, 100 mg/L amitriptyline and 10 mg/L maprotyline (internal standard). AMT = amitriptyline, NOR = nortriptyline; MAP = maprotyline.

Figure 2.1 is a plot of peak height ratio (amitriptyline peak height/maprotyline peak height) versus injection number. As can be seen from the plot, the magnitude of the peak height ratio decreased as the injection number decreased. Stabilization of the peak height ratio did not happen quickly. In fact, the stabilization did not occur until after the 40th repeat injection of the homogenous, mid-range calibration standard.

Based on these results, it is evident that some of the analytes were adsorbed somewhere in the chromatographic system, most likely either adsorption on to active sites present on the glass insert of the injector, or adsorption on to active sites present in the column.

If the strength¹³ of the amitriptyline and maprotyline signals over the 45 repeat injections are examined more closely, it appears that maprotyline was preferentially adsorbed to the active sites present on the glass insert compared with amitriptyline. Except for approximately the first three injections, the amitriptyline peak height over the 45 injections remained fairly constant (Figure 2.2). In comparison, maprotyline exhibited a trend of increasing peak height over the 45 repeat injections (Figure 2.3). Smaller peak heights at the start of the analysis would be expected if a smaller proportion of the maprotyline injected into the system was able to reach the detector.

¹³i.e. peak height

A situation such as this could occur if some of the maprotyline was adsorbed by the glass insert. As the number of injections increased, and the active sites present along the glass insert were filled, a greater proportion of the maprotyline injected would reach the detector. As a result, the maprotyline peak heights would get larger as the number of injections progressed. This trend toward increasing maprotyline peak height is illustrated in Figure 2.3.

Nortriptyline also exhibited a trend of increasing peak height over the 45 repeat injections, indicating that it was also adsorbed by the glass insert to some degree (Figure 2.4). However, the trend was not as pronounced for nortriptyline as it was for maprotyline (Figure 2.3). Therefore, of the three tricyclic antidepressants studied, maprotyline was more readily adsorbed to the active sites on the glass insert than either amitriptyline or nortriptyline.

However, it is interesting to note that the RSD for nortriptyline was several percentage points better than the RSD for amitriptyline. It appears that the steep increase in amitriptyline peak height, observed in the first five injections, increased the RSD for amitriptyline. The peak height for nortriptyline did not increase as steeply as did the peak height for amitriptyline during the same initial set of five injections. Perhaps, amitriptyline was picked up by the active sites faster than the other two tricyclic antidepressants due to its greater concentration in the standard.

The signal data generated from injection number 7 appeared to be an outlier. In all three plots of peak height or peak height ratio versus injection number, injection number 7 was observed to deviate considerably from the perceived trend (Figures 2.1, 2.2, 2.3, and 2.4). The signal data generated from injection number 25 was an outlier as well, as it was observed to significantly deviate from the perceived trend in Figures 2.1, 2.3, and 2.4.

The variation evident in the peak height ratios were unacceptable for quantitative analysis because results calculated from data generated at the beginning of the run would indicate a greater concentration of amitriptyline compared with results calculated from data generated at the end of the run. In an effort to reduce the amount of drug adsorption to the glass insert, the old insert was replaced with a clean insert that had been silylated twice. The double silylated glass insert was prepared by placing

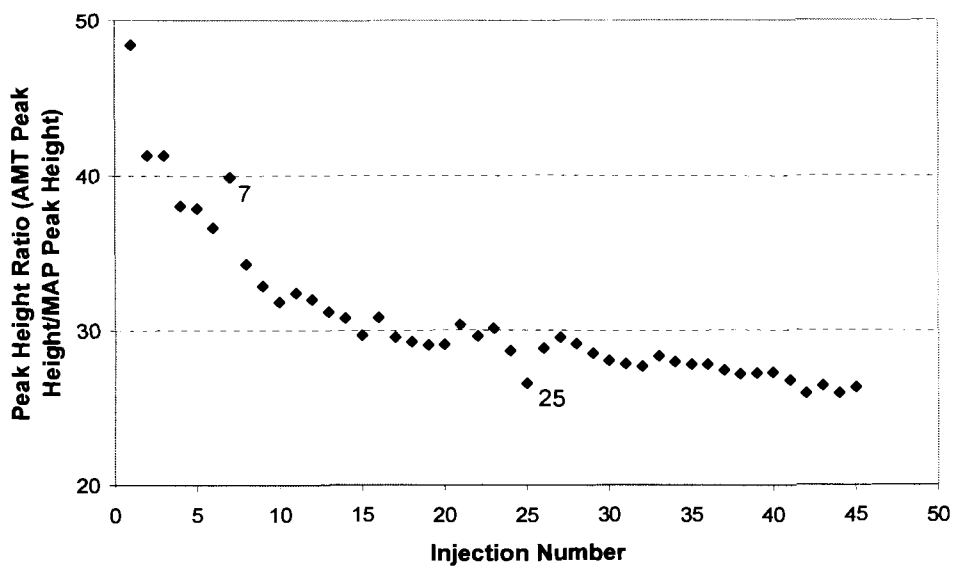


Figure 2.1: Plot of peak height ratio (amitriptyline peak height/maprotyline peak height) versus injection number for a mid-range calibration standard containing 10 mg/L nortriptyline, 100 mg/L amitriptyline and 10 mg/L maprotyline. AMT = amitriptyline; NOR = nortriptyline and MAP = maprotyline.

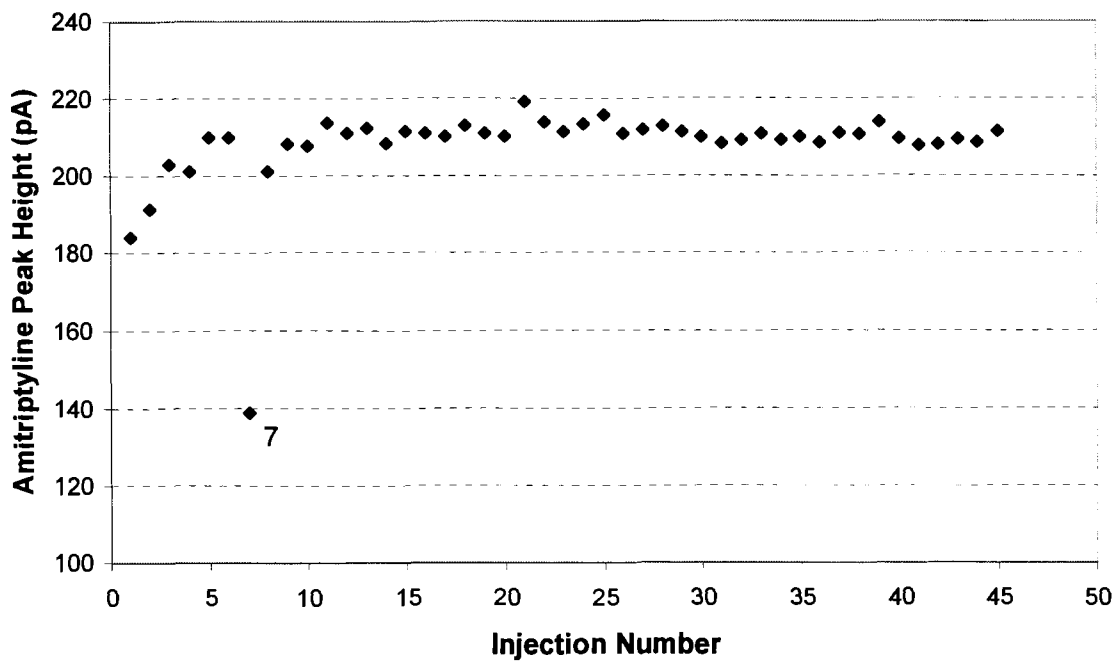


Figure 2.2: Plot of amitriptyline peak height (pA) versus injection number.

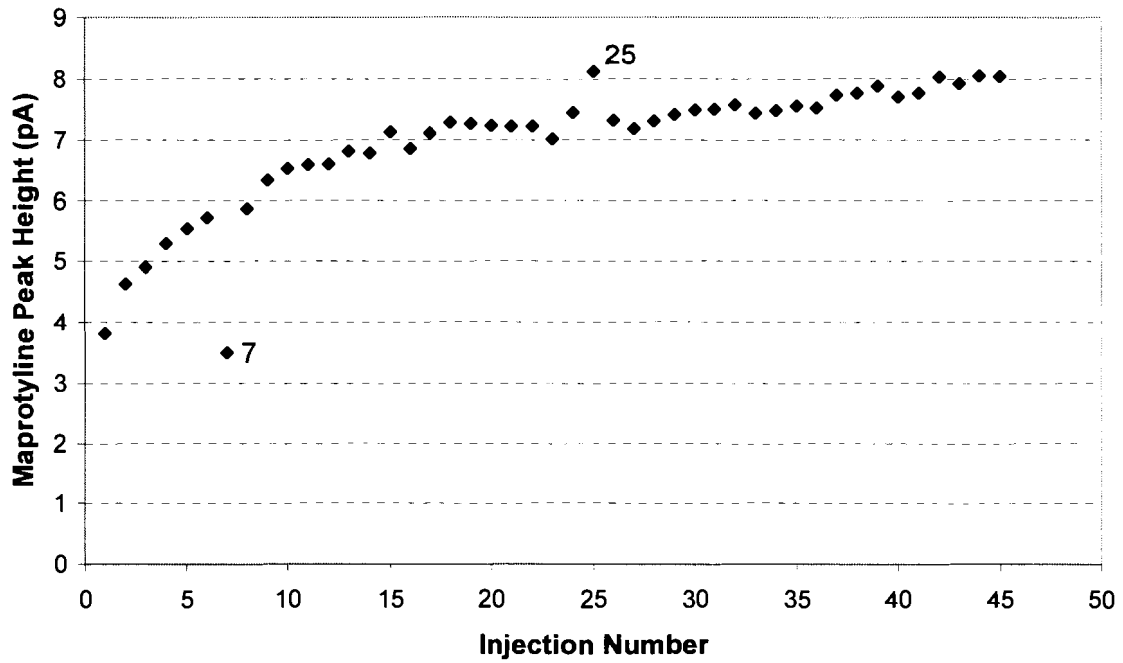


Figure 2.3: Plot of maprotyline peak height (pA) versus injection number.



Figure 2.4: Plot of nortriptyline peak height (pA) versus injection number for a mid-range calibration standard containing 10 mg/L nortriptyline.

an already silylated, clean glass insert in a 1:10 dilution of a silylating agent with acetonitrile for 24 hours. After 24 hours, the insert was removed from the silylating agent and placed in the oven of a gas chromatograph at 260°C for one hour in order to remove any excess silylating agent and acetonitrile.

Measure of Precision	Peak Height Ratio (AMT Peak Height/MAP Peak Height)	Peak Height Ratio (NOR Peak Height/MAP Peak Height)
Average Peak Height Ratio	15.6	1.24
Standard Deviation	0.42	0.02
Relative Standard Deviation (%)	2.7	1.3

Table 2.2: Precision measures for the double-silanized glass insert. For this test, 45 repeat injections of a mid-range mixed calibration standard containing 10 mg/mL nortriptyline, 100 mg/L amitriptyline and 10 mg/L maprotyline (internal standard) were conducted. AMT = amitriptyline, NOR = nortriptyline; MAP = maprotyline.

Once the new double silanized glass insert was in placed in the injector, the repeatability of injection test was duplicated using a fresh homogeneous aliquot of the mid-range calibration standard. Forty-five repeat injections of the calibration standard were completed and the relative standard deviation of the signal data was calculated (Table 2.2).

As can be seen from Table 2.2, the RSD for both the amitriptyline/maprotyline peak height ratio and the nortriptyline/maprotyline peak height ratio were significantly improved with the introduction of a double-silanized glass insert. The RSD of 2.7% for the amitriptyline/maprotyline peak height ratio, the RSD of 1.3% for the nortriptyline/maprotyline peak height ratio are well within the precision acceptance criteria of 5%. Once again, the RSD for the nortriptyline/maprotyline peak ratio is better than the peak height ratio for amitriptyline/maprotyline.

Peak height as a function of injection number for amitriptyline, nortriptyline and maprotyline for the double-silanized glass insert are illustrated in Figure 2.5, Figure 2.6 and Figure 2.7, respectively. Unlike the first repeatability of injection test, the peak heights for amitriptyline, nortriptyline and maprotyline were all observed to decrease during the first ten injections of the same homogeneous mid-range calibration

standard. This trend indicated that adsorption to the glass insert did not occur right away, most likely due to the decreased number of active sites present along the glass insert.

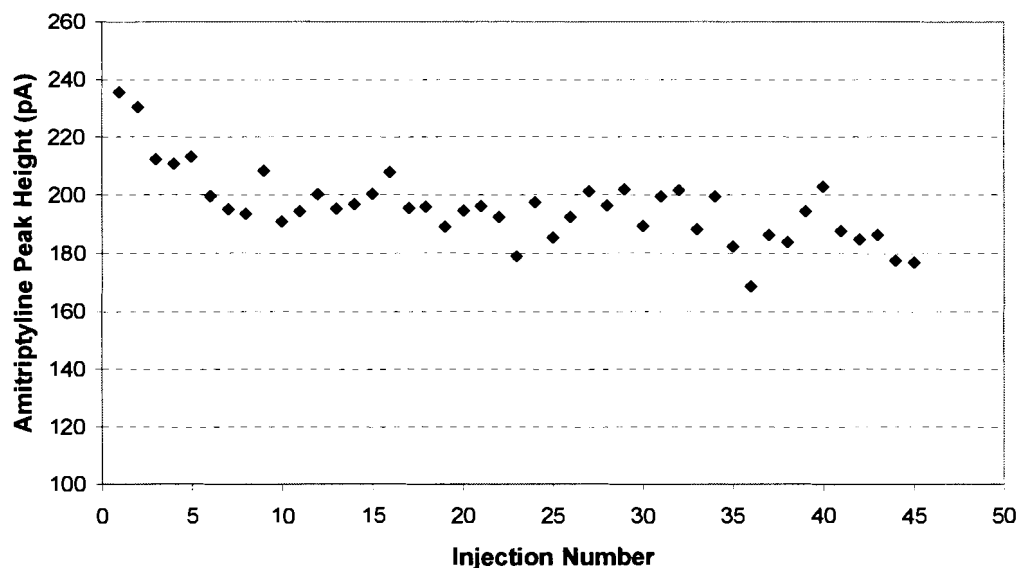


Figure 2.5: Plot of amitriptyline peak height (pA) versus injection number for the second repeatability of injection test using a double-silylated glass insert. The calibration standard injected 45 times contained 100 mg/L of amitriptyline.

Double silylation of the glass liner considerably improved the RSD for peak height ratio for both the amitriptyline/maprotyline peak height ratio and for the nortriptyline/maprotyline peak height ratio (Table 2.2). However, the most significant improvement that resulted from double silylation of the glass liner was the rapid stabilization of the peak height ratios. As illustrated in Figures 2.8 and 2.9, stabilization of the peak height ratio occurred much faster with the double silylated glass liner than with the single silylated glass liner. Therefore, results obtained with the double silylated liner were more appropriate for quantitative analysis.

Note that the figures still indicate that some adsorption is occurring, although it has been significantly reduced. The effect of adsorption is near the effect of run-to-run variation. Future work to reduce adsorption could improve the RSD.

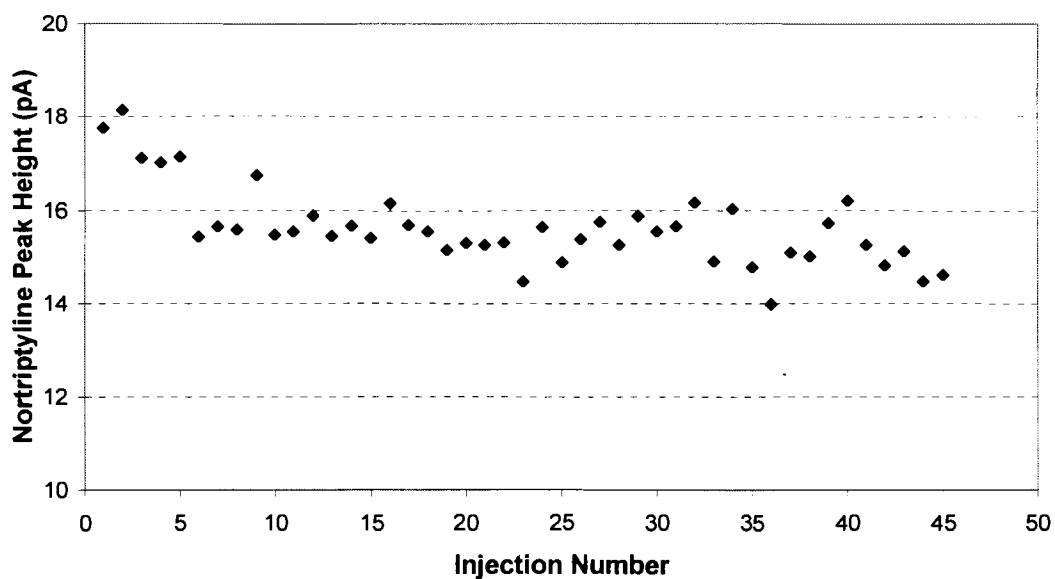


Figure 2.6: Plot of nortriptyline peak height (pA) versus injection number for the second repeatability of injection test using a double-silylated glass. The calibration standard injected 45 times contained 10 mg/L of nortriptyline.

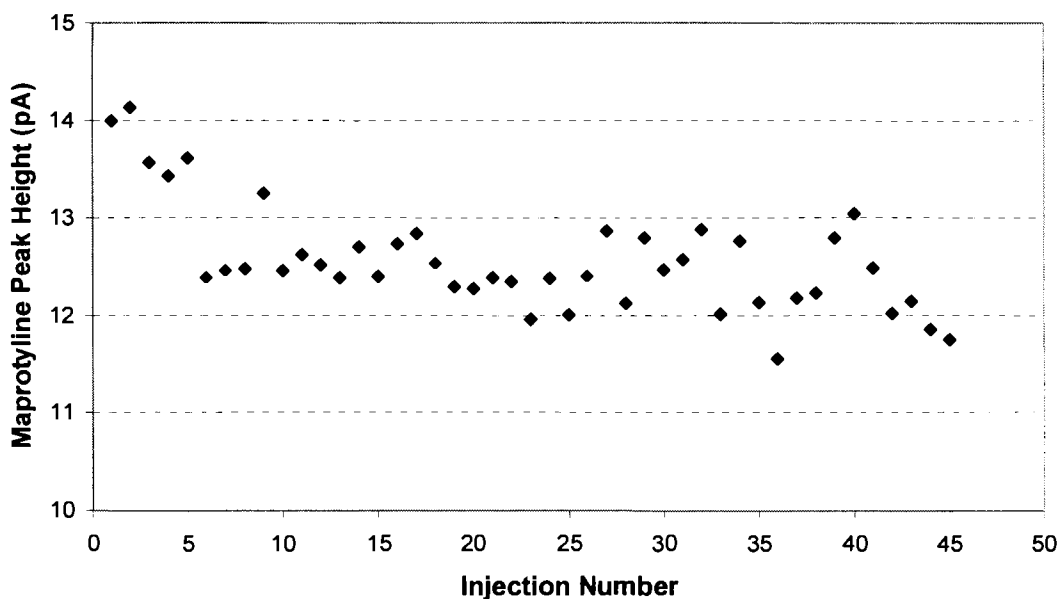


Figure 2.7: Plot of maprotyline peak height (pA) versus injection number for the second repeatability of injection test using a double-silanized glass liner. The calibration standard injected 45 times contained 10 mg/L of maprotyline.

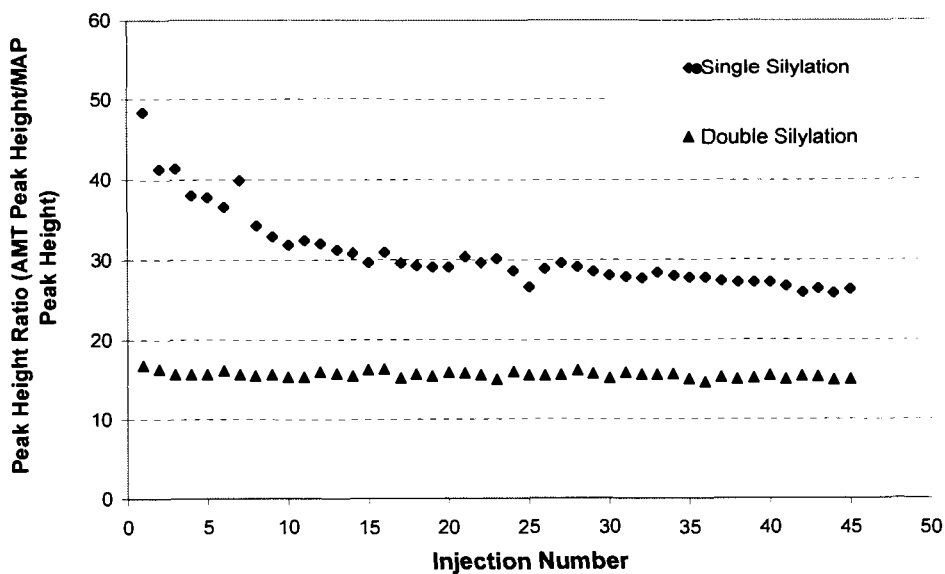


Figure 2.8: Comparison of the amitriptyline/maprotyline peak height ratio as a function of injection number for both the single silylated glass liners and the double silylated glass liners. AMT = amitriptyline; MAP = maprotyline.

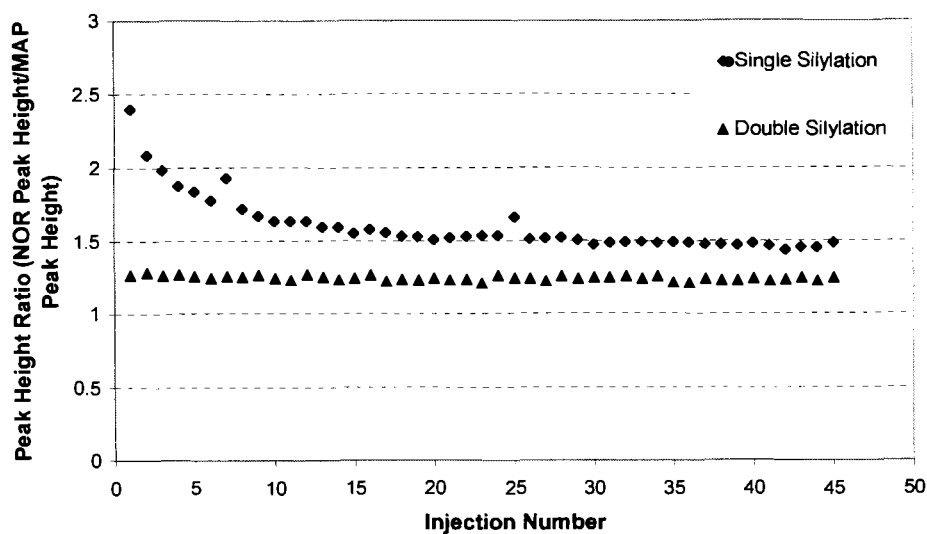


Figure 2.9: Comparison of the nortriptyline/maprotyline peak height ratio as a function of injection number for both the single silylated glass liners and the double silylated glass liners. NOR = amitriptyline; MAP = maprotyline.

2.5.1 Amitriptyline Intraday Precision

The intraday precision for the three replicate injections of each amitriptyline calibration standard, for each of the three days of validation are shown in Table 2.3. The RSD(%) for the sample blank on each of the three validation days, was greater than the 15% precision acceptance criteria set for the concentration limits. The precision acceptance criteria set for all other concentrations within the working linear range of the analyte was 10%. Based on the data in Table 2.3, all of the other calibration concentrations examined had peak height ratio RSDs less than the 10% threshold value.

The RSD(%) is expected to be high in the sample blank due to random fluctuations in the baseline. Based on the rapid decrease in the RSD between the sample blank and the 5 mg/L standard, it appears that the limit of detection and limit of quantitation for amitriptyline was equivalent to some concentration between 0 and 5 mg/L. This assumption will be confirmed in the next section. However, all of the other calibration standard concentrations have RSDs that were less than the 10% threshold set for all other concentrations within the working concentration range for the analyte.

The amitriptyline calibration equations obtained from three replicate injections of each calibration standard, for each of the three days of validation are as follows:

1. Day One (April 07, 2003): $y = 0.167x + 0.476$ (95% CI(slope) = 0.159 to 0.174, $t_{df=1} = 51.24$, $P < 0.0001$, $R^2 = 0.997$).
2. Day Two (April 09, 2003): $y = 0.177x + 0.446$ (95% CI(slope) = 0.170 to 0.184, $t_{df=1} = 56.88$, $P < 0.0001$, $R^2 = 0.997$).
3. Day Three (April 11, 2003): $y = 0.151x + 1.07$ (95% CI(slope) = 0.145 to 0.157, $t_{df=1} = 54.32$, $P < 0.0001$, $R^2 = 0.997$).

The calibration curves, and the standard deviation associated with the data, for each individual validation day, are presented in Figures 2.10, 2.11, and 2.12. The R^2 values for each of the calibration curves generated during the validation process were all above 0.990, indicating that the method was linear within the expected concentration range for amitriptyline.

Amitriptyline Calibration Standard Concentration (mg/L)	April 07, 2003 Validation Day 1 RSD(%)	April 09, 2003 Validation Day 2 RSD(%)	April 11, 2003 Validation Day 3 RSD(%)
0 ($n = 3$)	28.5	35.8	14.9
5 ($n = 3$)	4.6	1.4	3.3
10 ($n = 3$)	5.2	5.6	4.9
25 ($n = 3$)	1.7	2.8	4.3
50 ($n = 3$)	3.2	2.8	1.3
75 ($n = 3$)	1.4	0.5	0.9
100 ($n = 3$)	1.1	1.6	1.3
125 ($n = 3$)	2.6	0.4	4.4
150 ($n = 3$)	1.8	0.6	0.5
180 ($n = 3$)	1.5	1.4	1.9
200 ($n = 3$)	1.5	0.5	0.5

Table 2.3: Intraday precision, expressed in terms of the RSD(%) for each of the amitriptyline calibration standards, for each of the three validation days (April 7, 9, and 11, 2003). Triplicate injections of each calibration standard were conducted on each of the three validation days. Therefore, a total of nine determinations were conducted for each of the eleven calibration standards.

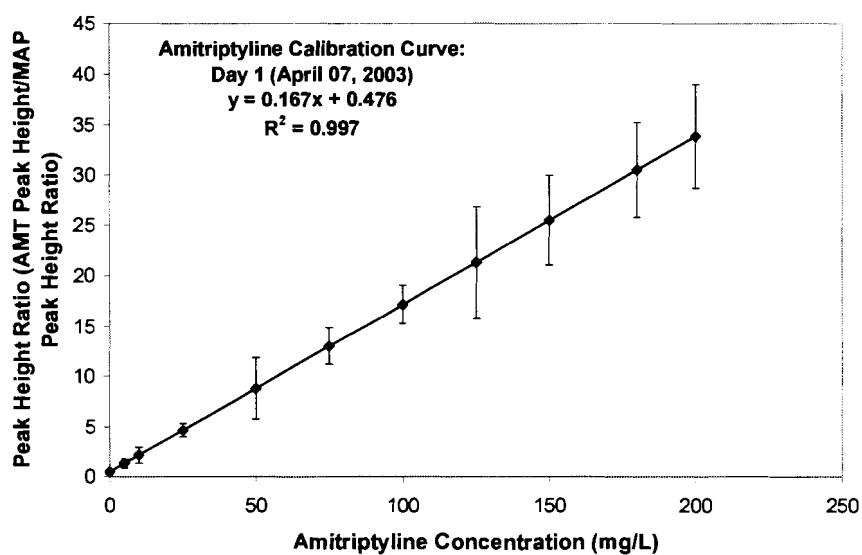


Figure 2.10: Amitriptyline Calibration curve for validation Day 1 (April 07, 2003). Least squares linear regression was performed on the calibration data, weighted for errors in y . Error bars represent the standard deviation, scaled by a factor of +10, so that most of the error bars were clearly visible.

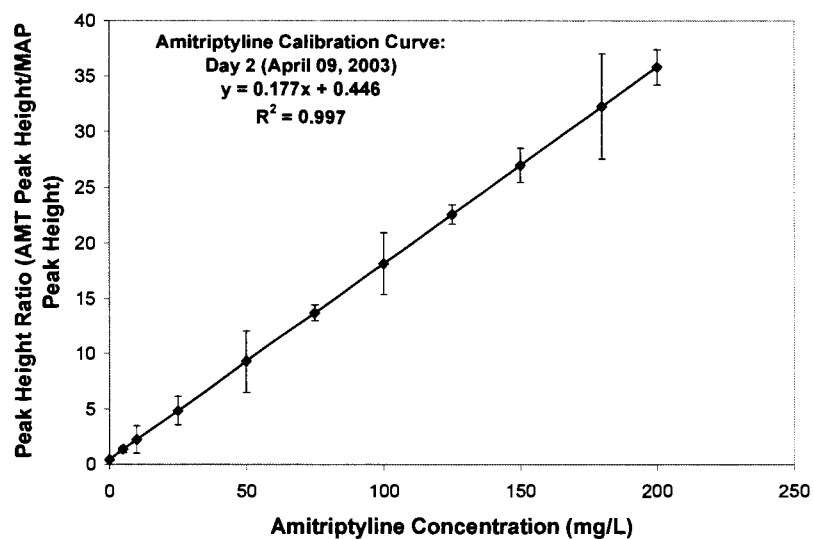


Figure 2.11: Amitriptyline Calibration curve for validation Day 2 (April 09, 2003). Least squares linear regression was performed on the calibration data, weighted for errors in y . Error bars represent the standard deviation, scaled by a factor of +10, so that most of the error bars were clearly visible.

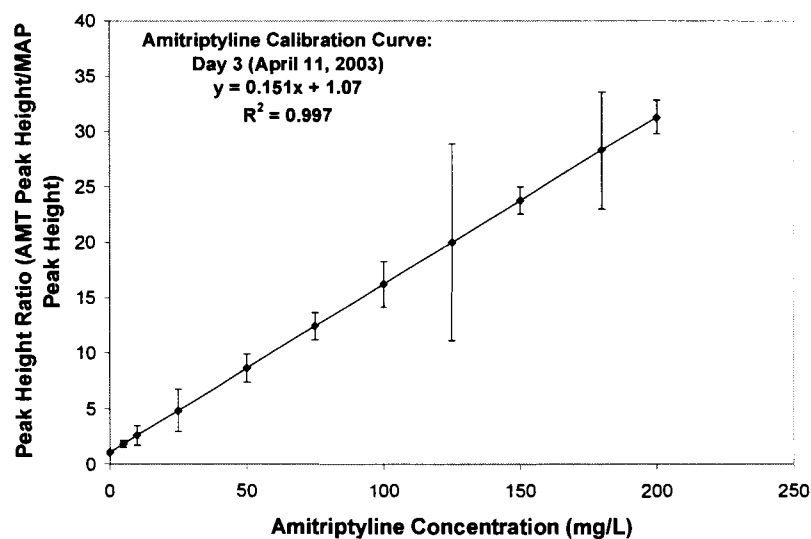


Figure 2.12: Amitriptyline Calibration curve for validation Day 3 (April 11, 2003). Least squares linear regression was performed on the calibration data, weighted for errors in y . Error bars represent the standard deviation, scaled by a factor of +10, so that most of the error bars were clearly visible.

2.5.2 Amitriptyline Interday Precision

The peak height ratios for all nine injections of each calibration standard, over the entire validation period were compiled, and their respective relative standard deviations calculated. These results are presented in Table 2.4. As can be seen in Table 2.4, the RSD for the 0 mg/L, 5 mg/L and 10 mg/L amitriptyline calibration standards, was extremely high, and exceeded the precision acceptance criteria of 15% at the concentration limits, and 10% for all other concentrations. Aside from these three lower concentration standards, the RSDs for all of the other amitriptyline calibration standards met the precision acceptance criteria, because their respective RSDs were all less than 10%.

Amitriptyline Calibration Standard Concentration (mg/L)	Interday Precision (April 07, 09 and 11, 2003) RSD(%)
0 ($n = 9$)	34.3
5 ($n = 9$)	31.4
10 ($n = 9$)	16.5
25 ($n = 9$)	9.1
50 ($n = 9$)	3.4
75 ($n = 9$)	3.5
100 ($n = 9$)	3.4
125 ($n = 9$)	4.2
150 ($n = 9$)	5.0
180 ($n = 9$)	6.4
200 ($n = 9$)	6.7

Table 2.4: Interday precision, expressed in terms of the RSD(%) for each of the amitriptyline calibration standards, for each of the three validation days (April 7, 9, and 11, 2003).

The slope is equivalent to the mean of a distribution, since like a mean, it represents the best estimate from the available data (Meier and Zünd, 2000). With this in mind, the mean, standard deviation and relative standard deviations for the distribution of slopes obtained from the three days of validation can be calculated. The same calculations were conducted on the y -intercepts of the calibration curves as well. These values are presented in Table 2.5.

The relative standard deviation for the y -intercepts for the amitriptyline calibration curves, over the three validation days was extremely large (53.1 %). This bias reduces the ability of the method to quantify low concentrations of amitriptyline. Figure 2.13 graphically compares the calibration curves from the three days of validation.

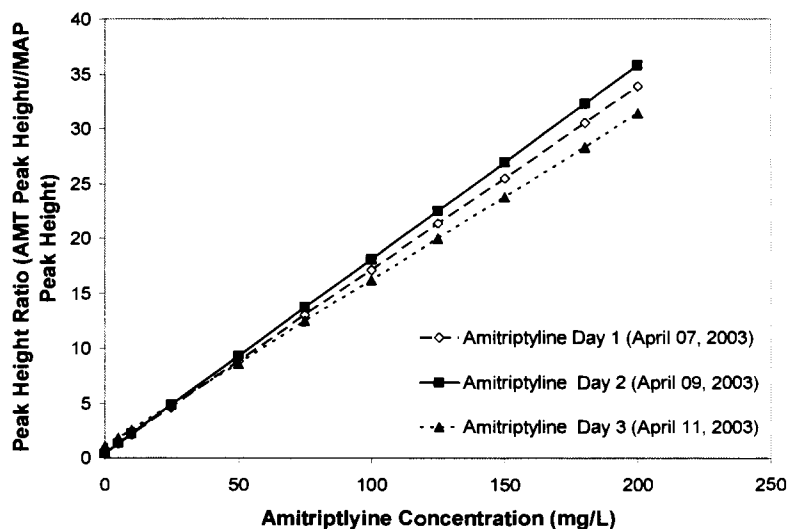


Figure 2.13: Amitriptyline calibration curves generated by least squares regression, weighted for errors in y , for each of the three validation days (April 7, 9, and 11, 2003).

The relative standard deviation for the slopes for the amitriptyline calibration curves, over the three days of validation days was less than 10%. This was a good indication that the slopes were not significantly different from each other.

Calibration Curve Element	Mean (\pm SD)	Relative Standard Deviation (%)
Slope	0.165 ± 0.013	7.7
Intercept	0.661 ± 353	53.1

Table 2.5: Mean, standard deviation and relative standard deviation for the slopes and intercepts of the amitriptyline calibration curves obtained from triplicate injections of the calibration standards on three different days.

2.5.3 Nortriptyline Intraday Precision

The intraday precision for the three replicate injections of each nortriptyline calibration standard, for each of the three validation days are shown in Table 2.6. The RSD for the sample blank was extremely high on both the first and the third day of validation. On both of these days, the RSD was much greater than the 15% precision acceptance criteria set for the concentration limits. In comparison, on the second day of validation, the RSD for the sample blank was only a few percentage points greater than the 15% threshold value. Based on the data presented in Table 2.3, all of the other calibration standards examined had peak height ratio RSDs of less than 10%, and therefore met the acceptance criteria for precision.

Based on the rapid decrease in the RSD between the sample blank and the 0.5 mg/L standard, it appeared that the limit of detection and limit of quantitation for amitriptyline in ethyl acetate was between 0 mg/L and 0.5 mg/L. This assumption will be confirmed in the next section.

Nortriptyline Calibration Standard Concentration (mg/L)	April 07, 2003 Validation Day 1 RSD (%)	April 09, 2003 Validation Day 2 RSD (%)	April 11, 2003 Validation Day 3 RSD (%)
0 (n=3)	73.3	17.9	60.2
0.5 (n=3)	1.1	5.6	4.8
1 (n=3)	1.2	3.5	3.6
2.5 (n=3)	0.6	1.0	1.2
5.0 (n=3)	1.0	1.2	1.9
7.5 (n=3)	0.9	1.4	0.7
10 (n=3)	1.1	0.4	0.8
12.5 (n=3)	0.9	1.2	2.9
15 (n=3)	1.2	0.4	0.3
18 (n=3)	2.8	1.0	1.6
20 (n=3)	0.7	0.2	0.6

Table 2.6: Intraday precision, expressed in terms of the RSD(%) for each of the nortriptyline calibration standards, for each of the three validation days (April 7, 9, and 11, 2003).

The nortriptyline calibration equations obtained from three replicate injections of each calibration standard, for each of the three days of validation are as follows:

1. Day One (April 07, 2003): $y = 0.177x + 0.009$ (95% CI(slope) = 0.168 to 0.186, $t_{df=1} = 45.65$, $P < 0.0001$, $R^2 = 0.996$).
2. Day Two (April 09, 2003): $y = 0.186x + 0.026$ (95% CI(slope) = 0.178 to 0.193, $t_{df=1} = 56.12$, $P < 0.0001$, $R^2 = 0.997$).
3. Day Three (April 11, 2003): $y = 0.170x + 0.047$ (95% CI(slope) = 0.165 to 0.175, $t_{df=1} = 71.28$, $P < 0.0001$, $R^2 = 0.998$).

The calibration curves, and the standard deviation associated with the data, for each of the three days of validation, are presented in Figures 2.14, 2.15, and 2.16. The R^2 values for each of the calibration curves generated during the validation process were all above 0.99, indicating that the method was linear within the expected concentration range for nortriptyline.

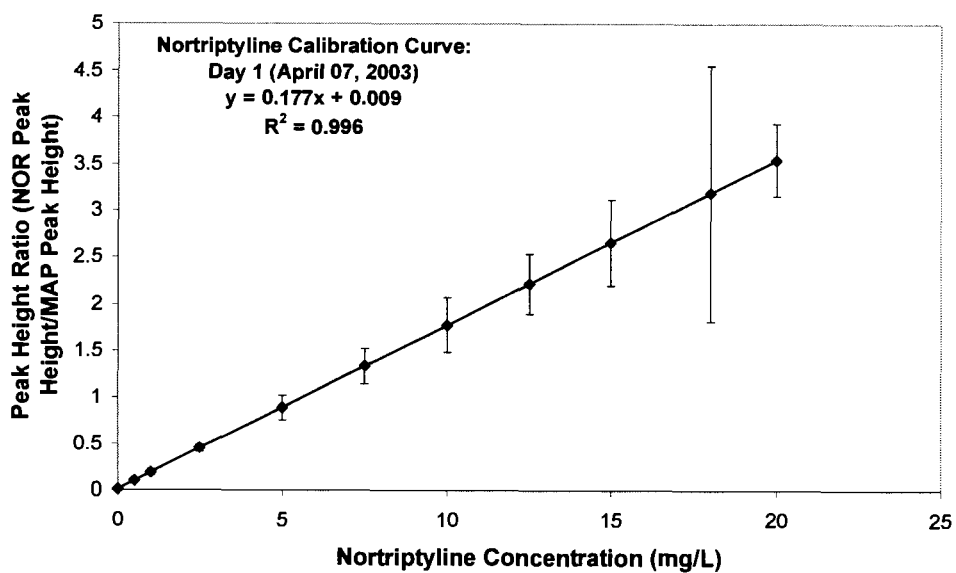


Figure 2.14: Nortriptyline Calibration curve for validation Day 1 (April 07, 2003). Least squares linear regression was performed on the calibration data, weighted for errors in y . Error bars represent the standard deviation, scaled by a factor of +15, so that most of the error bars were clearly visible.

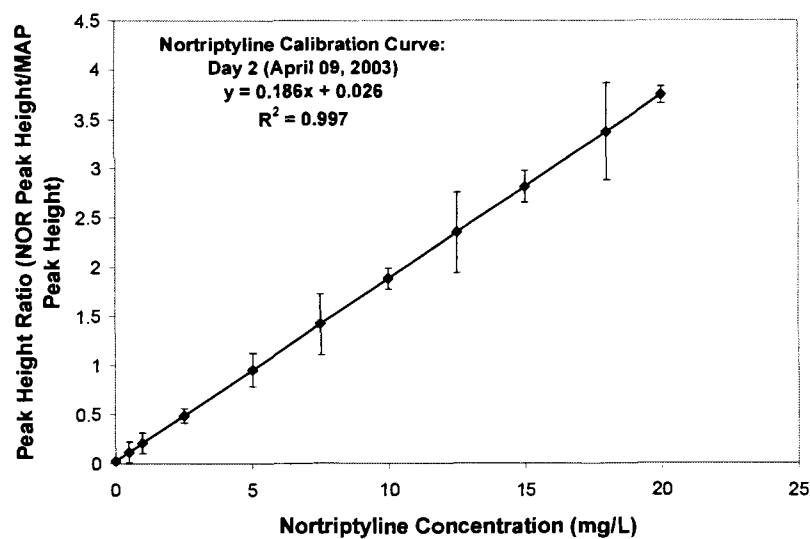


Figure 2.15: Nortriptyline calibration curve for validation Day 2 (April 09, 2003). Least squares linear regression was performed on the calibration data, weighted for errors in y . Error bars represent the standard deviation, scaled by a factor of +15, so that most of the error bars were clearly visible.

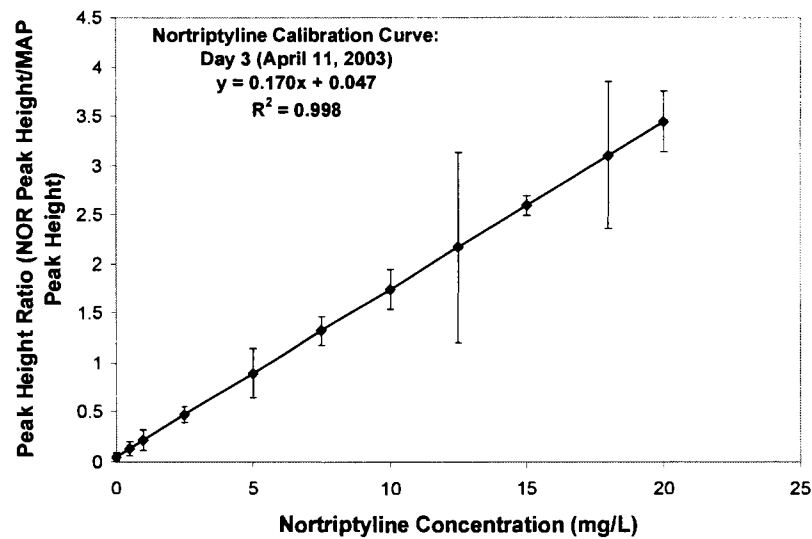


Figure 2.16: Nortriptyline calibration curve for validation Day 3 (April 11, 2003). Least squares linear regression was performed on the calibration data, weighted for errors in y . Error bars represent the standard deviation, scaled by a factor of +15, so that most of the error bars were clearly visible.

2.5.4 Nortriptyline Interday Precision

Nortriptyline calibration Standard Concentration (mg/L)	Interday Precision (April 07, 09 and 11, 2003) RSD (%)
0 ($n = 9$)	94.4
0.5 ($n = 9$)	19.8
1 ($n = 9$)	42.7
2.5 ($n = 9$)	37.4
5 ($n = 9$)	22.9
7.5 ($n = 9$)	12.5
10 ($n = 9$)	13.8
12.5 ($n = 9$)	8.5
15 ($n = 9$)	10.9
18 ($n = 9$)	4.8
20 ($n = 9$)	3.4

Table 2.7: Interday precision, expressed in terms of the RSD(%) for each of the nortriptyline calibration standards, for each of the three validation days (April 7, 9, and 11, 2003).

The peak height ratios for all nine injections for each calibration standard over the validation period (three replicate injections per day of validation) were compiled, and their respective relative standard deviations calculated. These results are presented in Table 2.7. As can be seen in Table 2.7, the RSD for all of the nortriptyline calibration standards except the 12.5 mg/L, 18 mg/L and 20 mg/L standards, exceeded the precision acceptance criteria of 15% at the concentration limits, and 10% for all other concentrations.

Calibration Curve Element	Mean (\pm SD)	Relative Standard Deviation (%)
Slope	0.178 \pm 0.008	4.5
y -Intercept	0.027 \pm 0.019	71.4

Table 2.8: Mean, standard deviation and relative standard deviation for the slopes and intercepts of the nortriptyline calibration curves obtained from nine determinations of a series of calibration standards (three replicate injections on each of three validation days).

Overall, it may be more valuable to compare the RSDs for the slopes and the y -intercepts of the calibration curves generated from the calibration data, rather than simply the RSDs for each of the calibration standards, because the calibration curve as a whole is used to quantitate the analytes present in the sample. As stated earlier, the slope is equivalent to the mean of a distribution, and therefore, it represents the best estimate from the available data (Meier and Zünd, 2000). As a result, the mean, standard deviation and relative standard deviation for the slopes and intercepts of the calibration curves obtained during the validation process were calculated. These values are presented in Table 2.8.

The relative standard deviation (%) of the y -intercepts for the nortriptyline calibration curves, over the three days of validation days was extremely large (71.4%). This bias reduces the ability of the present method to quantitate extremely low concentrations of nortriptyline (Table 2.8). However, the high RSD associated with the y -intercept is not as important as the value of the RSD associated with the slopes of the calibration curves (Huckin, personal communication, 2003). Figure 2.17 graphically compares the calibration curves from the three validation days.

The relative standard deviation for the slopes for the nortriptyline calibration curves, over the three validation days was less than 10% (Table 2.8). This was a good indication that the slopes were not significantly different from each other.

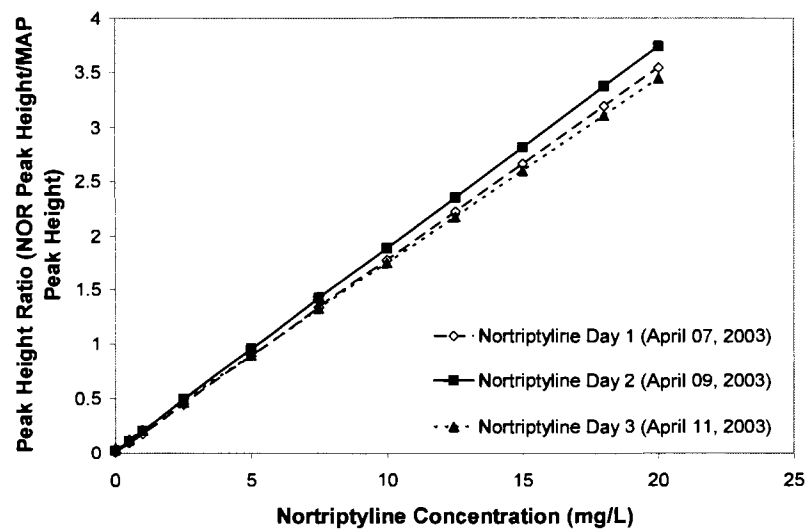


Figure 2.17: Nortriptyline calibration curves generated by least squares regression, weighted for errors in y , for each of the three days of validation (April 7, 9, and 11, 2003).

2.6 Discussion for Precision, Linearity and Range

2.6.1 Injection Repeatability

Since there is always going to be some degree of fluctuation in the system conditions, peak height (or **peak area**) on its own, without reference to some other component, either present or added to the sample, is usually unsuitable for quantitative analysis. For this reason, most quantitative analyses require the use of a well-characterized, pure material, with chemical and physical properties similar to that of the analyte to serve as a reference material. When the same amount of reference material is added to each standard and sample prior to sample preparation and analysis, the reference material is called an **internal standard**. When an internal standard is used, the ratio of analyte peak height to the internal standard peak height becomes the analytical parameter that is used to quantitate the analytes of interest (Skoog *et al.*, 1996). In summary, the purpose of an internal standard, such as maprotyline, is to minimize the uncertainties introduced during sample preparation, or in the gas chromatographic system. Such fluctuations could occur, for example, in the target analyte isolation during sample preparation phase, during the injection of the sample into the gas chromatograph, carrier and support gas flow rates, and variations in column conditions such as temperature (Skoog *et al.*, 1996).

Stabilization of the analyte/internal standard peak height ratio occurred much faster in the second repeatability of injection test. In the first test, with the insufficiently silylated liner, it took approximately 20 injections before the analyte/internal standard peak height ratio appeared to stabilize. Considering that the run time for the method used in this analysis was five minutes, approximately two hours worth of time (Appendix A), carrier and support gases would be wasted waiting for the signal data generated to stabilize at the beginning of each run. This is obviously unacceptable, and therefore the condition of the glass liner must be properly maintained in order to generate signal data that is appropriate for quantitative analysis. In comparison, with the double silylated glass liner, the analyte/internal standard peak height ratio appeared to stabilize within the first five injections.

For this reason, before the start of each analysis, five injections of a mid-range calibration standard containing amitriptyline, nortriptyline and maprotyline were conducted, and the resulting chromatograms examined for broad peaks or tailing peaks. Broad or tailing peaks reduce the resolution of adjacent peaks, and therefore, adversely affects the accuracy of the quantitated results. With this in mind, once the separation between adjacent analyte peaks was decreased to the point where the peaks were no longer suitable for quantitative analysis¹⁴, the liner was removed and replaced with a fresh, double silylated glass liner. The preliminary runs required approximately 25 minutes to complete, which in terms of the total run time for a set of calibration standards and samples, was relatively insignificant, and well worth the time spent.

In conclusion, frequent liner maintenance is required, particularly with the analysis of dirty samples and/or samples containing compounds with active functional groups (Rood, 1999). In addition, frequent liner maintenance is especially important for the analysis of samples with low analyte concentrations. The silanol groups present on the glass liner become more active with continued liner use. Essentially, the silanol groups become less and less deactivated as the silylating agent wears off. A highly active liner will reduce the ability of the detector to detect these analytes, and as a result, increase both the limit of detection and limit of quantitation. When this happens, the liner must either be replaced with a new one or cleaned and resilylated (Rood, 1999).

2.6.2 Linearity

The linearity requirements for analytical work are quite stringent. According to Sadek (2000), correlation coefficients of 0.995 or greater are required for critical analytical work. For less critical work, correlation coefficients of 0.990 or less may be permissible (Sadek, 2000). The linearity requirement, in terms of the correlation coefficient, will depend on the purpose of the method being tested, and as a result will be constrained by financial and time considerations. For the present experiment, correlation coefficients of 0.98 or greater were acceptable for stating that the method was linear over the expected concentration range. In an ideal situation, the slope

¹⁴i.e. Baseline separation of adjacent peaks is required for quantitative analysis.

of a calibration curve should be constant¹⁵, and the value of the y -intercept should be equal to zero. If the calibration curve does not run through the origin, constant systematic errors are present, and proportional systematic errors exist when the slope is not constant (Meyer, 1998). The presence of either of these two types of bias, or both, indicate that improvements could be made to the chromatographic separation or sample preparation (Meyer, 1998). These biases will have to be investigated and either removed or improved when the analyte recovery from the artificial matrix is determined (Chapter 3). Additionally, the slope of the calibration curve should be near one. If the slope is extremely small or large, then the method will be sensitive to errors.

2.6.3 Intraday and Interday Precision

Snyder *et al.* (1997) indicated that the interday precision, expressed in terms of the relative standard deviation (RSD), for major components should not be greater than 1 or 2 percent. However, for many compounds, including amitriptyline and nortriptyline, precision acceptance criteria of less than 15% is unrealistic given their extensive interaction with system components, such as the injector liner. These interactions result in broader peaks compared with the peaks generated by compounds that do not interact with system components. In general the peaks for amitriptyline exhibit less tailing than nortriptyline peaks, because amitriptyline is a tertiary amine. In contrast, nortriptyline is a secondary amine, and as a result the amine group in nortriptyline can interact more freely with system components, such as injector liner. Therefore, nortriptyline peaks are more likely to exhibit tailing, and therefore the precision with which they can be measured decreases. Maprotyline is also a secondary amine, and as a result, can also exhibit significant tailing.

For the present project, the intraday precision acceptance criteria of 15% for the concentration limits and 10% for all other concentrations were met by all of the amitriptyline and nortriptyline calibration standards, except for the sample blanks. The large RSDs obtained for the sample blanks are not unexpected since they are simply a measure of random fluctuations in the baseline at the appropriate retention

¹⁵i.e. Curve should be a straight line.

times for each analyte. Most interesting was the rapid decreasing in RSD from sample blank to the 0.5 mg/L nortriptyline calibration standard, and from the sample blank to the 5 mg/L amitriptyline calibration standard. The sharp decrease in RSD between these standards indicates that the limit of detection and limit of quantitation probably exists at some concentration between them.

However, the interday precision for both amitriptyline and nortriptyline requires improvement. In the case of amitriptyline, only the 5 mg/L and 10 mg/L standards exceeded the precision acceptance criteria of 10%. In the case of the 5 mg/L amitriptyline standard, the RSD was 31.4%. In comparison, the RSD for the 0 mg/L amitriptyline standard was 34.3%. Therefore, although the intraday precision for the 5 mg/L standard was more than adequate, the interday precision was not acceptable.

The interday precision for nortriptyline needs substantial improvement as well, because the precision acceptance criteria for all but three of the 11 calibration standards exceeded the precision acceptance criteria. The extremely low precision observed with nortriptyline is likely the result of peak broadening and tailing due to its interaction with free silanol groups either on the injector liner or in the column itself.

However, the interday RSDs for the slopes, for both amitriptyline and nortriptyline, were considerably below 10%. In fact, better interday precision was obtained for the nortriptyline calibration curves (4.5%) compared with the amitriptyline calibration curves (7.9%). Therefore, despite the poor precision obtained for each of the nortriptyline calibration standards, the RSD calculated for the slopes of the nortriptyline calibration curves met the precision acceptance criteria of 10%.

In the present project, nine separate determinations were made for each calibration standard (three per standard on each of the three days of validation). The poor precision obtained for most of the nortriptyline standards, and for two of the amitriptyline standards, indicates that more than three determinations of each calibration standard and sample should be made in order to improve the accuracy of the quantitated results.

2.7 Determination of the Limit of Detection and Limit of Quantitation In Ethyl Acetate

2.7.1 Introduction

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) are two important performance characteristics of a method (Snyder *et al.*, 1997). The LOD is the smallest concentration of analyte that can be reliably detected by the method (Snyder *et al.*, 1997; EURACHEM Working Group, 1998). By definition then, the LOD is the lowest concentration where the measured value of that concentration is greater than the uncertainty associated with it (Huber, 2001). With this in mind, the method must be able to detect the lowest concentration of analyte to an acceptable degree of uncertainty (EURACHEM Working Group, 1998).

In contrast, the limit of quantitation is the smallest concentration of analyte that can be reliably quantitated within an acceptable degree of uncertainty (Snyder *et al.*, 1997; EURACHEM Working Group, 1998). By definition, the LOQ is then the smallest concentration of analyte for which precise measurements can be obtained (Huber, 2001).

According to the EURACHEM Working Group (1998), the LOD and LOQ do not represent concentrations where quantitation is impossible; however, near the LOD, the magnitude of the uncertainty associated with the results are approximately equal to the results obtained using the analytical method. Considerable discrepancy exists in the scientific literature regarding which method is the most appropriate for determining the LOD and LOQ. The various methods can be separated into two major types: (1) statistical methods and (2) empirical methods.

In the statistical method, a series of blank samples are analyzed and mean blank value and standard deviation calculated. In this method, the LOD is the mean blank value plus two or three standard deviations, and the LOQ is the mean blank value plus five, six or ten standard deviations. A sample blank is a sample that contains no analyte but with a matrix, including the internal standard, identical to that of the samples to be analyzed (Armbruster *et al.*, 1994).

The statistical method assumes that a signal more than three standard deviations above the signal of the sample blank could only have resulted from the blank itself less than 1% of the time (EURACHEM Working Group, 1998). Therefore, any signals greater than three standard deviations above the blank are most likely due to the presence of other substances, such as the analytes of interest (EURACHEM Working Group, 1998).

Compared with the LOD, the number of standard deviations for the LOQ is set to a much higher value. Setting the number of standard deviations to a higher value for the LOQ increases the probability that the measurement signal represents the real presence of the analyte (Armbruster *et al.*, 1994). In addition, setting the standard deviations to a greater value also reduces the measurement error (Armbruster *et al.*, 1994).

In comparison, the empirical method involves the analysis of standards with increasingly lower concentrations of analyte. In this method, the LOD is the lowest concentration of analyte that still meets some predetermined acceptance criteria (Armbruster *et al.*, 1994). The acceptance criteria is typically a series of constraints that if met 90% of the time, then the data can be retained for the analysis. For example, common acceptance criteria in chromatography are (Armbruster *et al.*, 1994):

1. Peaks that are sharp and symmetrical¹⁶.
2. Peaks that are separated to within 10% of the baseline.

These criteria usually have to be relaxed slightly in the determination of the LOD and LOQ, because suboptimal peaks regularly occur at the concentration limits for the analytical method. Even so, accurate quantitation requires that such criteria be met to the fullest extent possible, and as a result the LOD and LOQ obtained using the statistical method are usually of a considerably lower concentration than the LOD and LOQ obtained using the empirical method (Armbruster *et al.*, 1994). Furthermore, both the LOD and LOQ are matrix dependent, and therefore must be re-evaluated if the analyte matrix is altered.

¹⁶i.e. peaks that do not do not exhibit significant levels of tailing or fronting

2.7.2 Procedure for LOD and LOQ in Ethyl Acetate

Due to time constraints, the statistical method was used in the present thesis. Additionally, three standard deviations and ten standard deviations of the blank mean were chosen as the threshold values for the limit of detection and for the limit of quantitation, respectively.

Calibration curves are still required for the determination of the LOD and LOQ, and therefore, the samples tested included not only the blanks, but also the calibration standards. Nine calibration standards were prepared from a mixed 0.1 mg/mL amitriptyline and 0.1 mg/mL nortriptyline stock solution (in methanol). Each of the nine calibration standards were prepared in a 10 mL volumetric flask. The concentrations prepared were as follows:

1. 0 mg/L nortriptyline + 0 mg/L amitriptyline
2. 0.1 mg/L nortriptyline + 0.1 mg/L amitriptyline
3. 0.2 mg/L nortriptyline + 0.2 mg/L amitriptyline
4. 0.35 mg/L nortriptyline + 0.35 mg/L amitriptyline
5. 0.5 mg/L nortriptyline + 0.5 mg/L amitriptyline
6. 0.75 mg/L nortriptyline + 0.75 mg/L amitriptyline
7. 0.9 mg/L nortriptyline + 0.9 mg/L amitriptyline
8. 1.0 mg/L nortriptyline + 1.0 mg/L amitriptyline
9. 1.25 mg/L nortriptyline + 1.25 mg/L amitriptyline

Each of the nine calibration standards were prepared by adding 50 μ L of 0.1 mg/ml maprotyline (internal standard), followed by the appropriate volume of the mixed amitriptyline and nortriptyline stock solution, and then diluting to the mark on the volumetric flask with ethyl acetate. The final concentration of the internal standard in each of the nine calibration standards was 0.5 mg/L. In order to determine the extent of the baseline noise, a 0.5 mg/L maprotyline standard, in the absence of

amitriptyline and nortriptyline was also prepared. After thorough mixing, an aliquot of each calibration standard was pipetted into clean autoinjector vials and capped.

The calibration standards were run on a HP 6890 gas chromatograph with a nitrogen-phosphorus detector, according to the previously developed method (Appendix A). The calibration standards were injected from lowest concentration (0.1 mg/L) to highest concentration (1.5 mg/L), and the entire series of calibration standards, from lowest to highest concentration, was injected seven times in succession. The target number of analyses for each standard was ten. However, the autoinjector needle became plugged during the run, and as a result, only seven complete sets of calibration data were obtained.

Twenty replicate injections of the 0.5 mg/L maprotyline standard were conducted in order to estimate the baseline noise at the retention times of amitriptyline and nortriptyline. In addition, 10 replicate injections of the calibration standard solvent, ethyl acetate, was conducted in order to estimate the baseline noise under the maprotyline peak. The average (\pm SD) noise, in units of peak height (pA), at the retention times of interest, calculated from these 20 replicate injections of the 0.5 mg/L maprotyline standard. This 0.5 mg/L, maprotyline standard is referred to as the sample blank as it contains neither amitriptyline nor nortriptyline.

As mentioned previously, the LOD, calculated using the statistical method, is the mean peak height (pA) at each of the retention times of interest from the blank standard, plus three standard deviations (SD). In order to determine the concentration equivalent to the LOD for each analyte, the appropriate mean peak height, plus three SDs, must be converted to a peak height ratio by dividing it by the mean maprotyline (internal standard) peak height (Table 2.11) for the 20 replicate injections of the sample blank. The concentration of each analyte equivalent to the LOD can then be calculated by inserting the calculated peak height ratio (y) into the equation for the appropriate calibration curve, and solving for the concentration (x).

The LOQ is calculated in much the same way as the LOD. However, the peak height used to calculate the LOQ for each analyte is the appropriate mean peak height, plus ten standard deviations. This value must also be converted to a peak height ratio so that the concentration equivalent to the LOQ, for each analyte, can

be determined using their respective calibration curves.

2.7.3 Results of LOD and LOQ in Ethyl Acetate

The calibration curves for both nortriptyline and amitriptyline were constructed by plotting the peak height ratio¹⁷ versus the concentrations of the calibration standards. Weighted least squares linear regression, using JMP IN[®] (SAS Institute Incorporated, Cary, NC, USA) was used to obtain the best fit for the calibration data, with y as the peak height ratio, and x as the concentration (mg/L). Since the calibration standards were prepared to a certain concentration, it is assumed that any indeterminate errors in x are negligible¹⁸. However, since each calibration standard was injected seven times each, errors in y are possible. Therefore, for the determination of the LOD and LOQ, the linear calibration curve was generated by least squares regression, weighted for errors in y (peak height ratio).

The calibration curves from the weighted linear regression for amitriptyline and nortriptyline are illustrated in Figure 2.18 and Figure 2.19. The calibration curve for amitriptyline is $y = 0.567x + 0.068$ (95% CI(slope) = 0.510 to 0.624, $t_{df=1} = 23.81$, $P < 0.0001$, $R^2 = 0.988$). Likewise, the calibration equation for nortriptyline is $y = 0.234x + 0.026$ (95% CI(slope) = 0.218 to 0.251, $t_{df=1} = 33.57$, $P < 0.0001$, $R^2 = 0.994$).

The mean, standard deviation and relative standard deviation (RSD) for peak height ratios calculated from the seven repeat injections of each calibration standard are presented in Table 2.9. The data presented in Table 2.9 were used to generate the calibration curves for amitriptyline and nortriptyline. As can be seen from Table 2.9, the relative standard deviations are quite high for these low concentration calibration standards. It is interesting to note that the 0.35 mg/L nortriptyline standard has incredibly high RSD at 56.2%, considering that it is well above the LOQ and LOD for nortriptyline. The same situation is observed with amitriptyline. The LOQ for amitriptyline is 0.36 mg/L, and yet the 0.5 mg/L calibration standard has a greater

¹⁷i.e. analyte peak height/internal standard peak height

¹⁸i.e. the errors in x are significantly less than the errors in y .

RSD (27.1%) than the RSD for the 0.35 mg/L calibration standard (17.9%). It is expected that at the LOQ, the relative standard deviations would be smaller in comparison to the RSD for concentrations less than the LOQ. With this in mind, it is also expected that concentrations greater than the LOQ would have lower relative standard deviations. Based on the results presented in Table 2.9, this did not occur in the present experiment.

Concentration of the Calibration Standard (mg/L)	Amitriptyline		Nortriptyline	
	Mean Peak Height Ratio (AMT/MAP) \pm SD	RSD (%)	Mean Peak Height Ratio (NOR/MAP) \pm SD	RSD (%)
0 ($n = 7$)	0.052 \pm 0.013	26.5	0.028 \pm 0.007	23.5
0.1 ($n = 7$)	0.123 \pm 0.026	21.5	0.030 \pm 0.004	11.7
0.2 ($n = 7$)	0.169 \pm 0.007	4.0	0.063 \pm 0.006	10.3
0.35 ($n = 7$)	0.268 \pm 0.048	17.9	0.108 \pm 0.061	56.2
0.5 ($n = 7$)	0.382 \pm 0.103	27.1	0.137 \pm 0.021	15.4
0.75 ($n = 7$)	0.480 \pm 0.089	18.5	0.201 \pm 0.038	19.0
0.9 ($n = 7$)	0.583 \pm 0.123	21.1	0.237 \pm 0.046	19.4
1.0 ($n = 7$)	0.608 \pm 0.201	33.1	0.273 \pm 0.054	19.8
1.25 ($n = 7$)	0.797 \pm 0.195	24.5	0.313 \pm 0.095	30.0

Table 2.9: Mean peak height ratio (\pm SD) and relative standard deviation (RSD) for the seven repeat injections of each calibration standard used to generate the LOD and LOQ calibration curves for amitriptyline and nortriptyline in a matrix of ethyl acetate. AMT/MAP = amitriptyline peak height/maprotyline peak height; NOR/MAP = nortriptyline peak height/maprotyline peak height.

The mean noise, (\pm SD), in units of peak height (pA) for the two analytes of interest, amitriptyline and nortriptyline, are presented in Table 2.10. The mean peak height, (\pm SD), for maprotyline, calculated from the 20 replicate injections of the 0.5 mg/L maprotyline standard is presented in Table 2.11.

The calculated limits of detection and limits of quantitation for amitriptyline and nortriptyline in ethyl acetate are presented in Table 2.12. The LOD for amitriptyline in a **matrix** of ethyl acetate was 0.05 mg/L, and the LOQ for amitriptyline was 0.36 mg/L. The LOD for nortriptyline in a matrix of ethyl acetate was 0.09 mg/L,

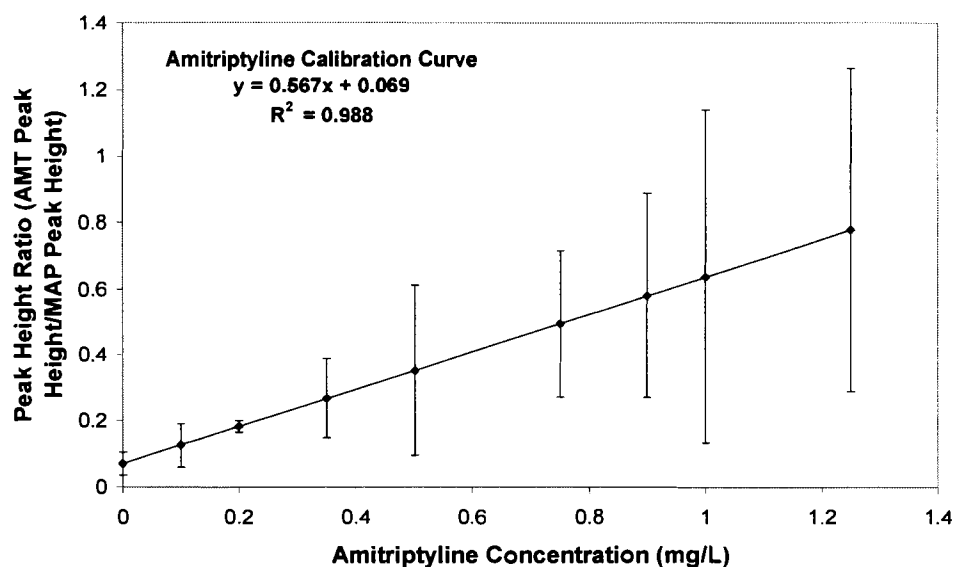


Figure 2.18: Limit of detection and limit of quantitation calibration curve for amitriptyline. Least squares linear regression performed calibration data weighted for errors in y . Error bars represent the standard deviation, scaled by a factor of +2.5, so that all errors bars were clearly visible.

Analyte Identity	Mean Noise \pm Standard Deviation (pA)	Relative Standard Deviation (%)
Amitriptyline	0.068 ± 0.020	29.6
Nortriptyline	0.037 ± 0.009	24.0

Table 2.10: Average baseline noise (\pm SD) and relative standard deviation (%) for amitriptyline and nortriptyline, in units of peak height (pA). Data was obtained from 20 replicate injections of a sample blank containing 0.5 mg/L maprotyline as the internal standard.

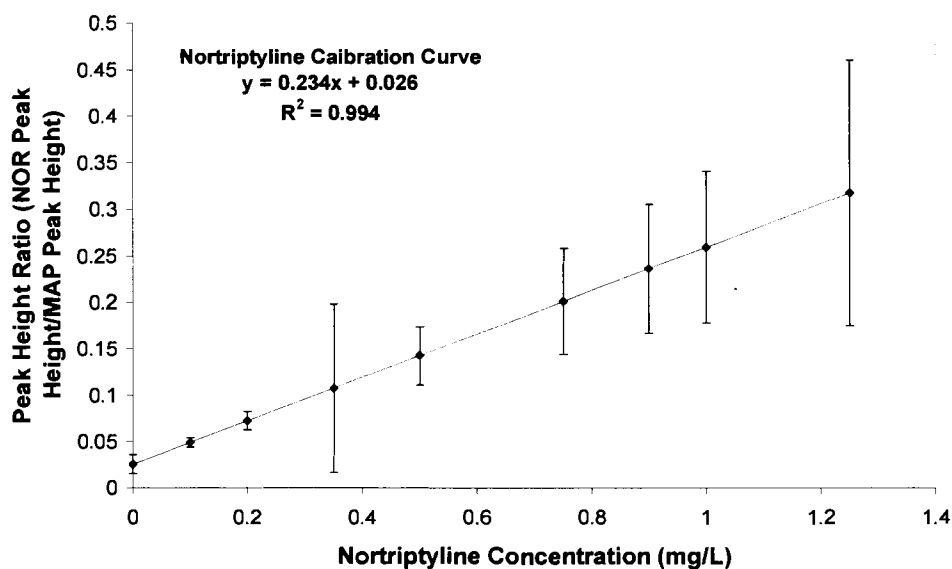


Figure 2.19: Limit of detection and limit of quantitation calibration curve for nortriptyline. Least squares linear regression performed calibration data weighted for errors in y . Error bars represent the standard deviation, scaled by a factor of +1.5, so that most of the error bars were clearly visible.

Analyte Identity	Mean Peak Height \pm Standard Deviation (pA)	Relative Standard Deviation (%)
Maprotyline	1.32 ± 0.248	18.7

Table 2.11: The mean, standard deviation, and relative standard deviation (RSD) of peak height (pA) for the internal standard maprotyline used in the calculation of the limit of detection and limit of quantitation of amitriptyline and nortriptyline in ethyl acetate. Data was obtained from 20 replicate injections of a sample blank containing 0.5 mg/L maprotyline.

and the LOQ for nortriptyline was 0.29 mg/L (Table 2.12). Based on these results, nortriptyline can be quantitated to a lower concentration than amitriptyline using the analytical method developed for the HP 6890 gas chromatograph (Appendix A), which is surprising given the propensity of nortriptyline to produce broad and tailing peaks.

The noise under the maprotyline peak, in units of pA, calculated from the injection of 10 samples of ethyl acetate was 0.041 ± 0.010 (RSD = 24.7%). This mean value represents only 3.1% of the mean maprotyline peak height of 1.32 pA. Therefore, the noise under the maprotyline peak was negligible in the matrix of ethyl acetate.

Analyte	Limit of Detection (LOD)		Limit of Quantitation (LOQ)	
	Peak Height Ratio	Concentration (mg/L)	Peak Height Ratio	Concentration (mg/L)
Amitriptyline	0.098	0.05	0.205	0.36
Nortriptyline	0.048	0.09	0.094	0.29

Table 2.12: The statistically determined limit of detection and limit of quantitation for amitriptyline and nortriptyline in a matrix of ethyl acetate.

2.7.4 Discussion of LOD and LOQ in Ethyl Acetate

The concentrations equivalent to the LOD and LOQ are calculated values because they were determined using the statistical method rather than the empirical method. Since the statistical method produces a calculated result, the reliability of such a result is indicated by the magnitude of the relative standard deviations associated with the raw data used to calculate the LOD and LOQ.

The large relative standard deviations observed with each of calibration standards (Table 2.9) and the peak heights used to solve for the concentrations equivalent to the LOD and LOQ (Table 2.10; Table 2.11) indicate that the statistical method may have underestimated the LOD and LOQ for both amitriptyline and nortriptyline. For instance, a decrease in the relative standard deviation, as a function of increasing analyte concentration, was not observed for the analyte calibration data. Given the fact that the LOQ is supposed to represent a concentration that can be reliably detected and quantitated using the applied chromatographic method, it was expected that the RSD would decrease at concentrations greater than the LOQ. Unfortunately, this was not observed, and therefore, it appears that both the LOD and LOQ have been underestimated. Therefore, if further research is to be conducted, the LOD and LOQ, for both amitriptyline and nortriptyline, should be re-evaluated using the more accurate, but more time consuming, empirical method.

However, the LOD and LOQ results for both amitriptyline and nortriptyline are encouraging, since it appears that the method may be sensitive enough to detect fairly low levels of amitriptyline and nortriptyline, even if the reliability at the lower concentration range was less than desirable. In addition, given the extremely low interday precision observed, it would be interesting to determine the interday precision for the LOD and LOQ calculated in the present section. Given more time, this researcher would have conducted interday precision experiments for the LOD and LOQ determinations as well.

2.8 Conclusion

No significant problems with the analytical method were encountered for the analysis of amitriptyline and nortriptyline in a matrix of ethyl acetate. All three of the tricyclic antidepressants analyzed were resolvable to within 10% of the baseline. This degree of separation was particularly important for amitriptyline and nortriptyline due to their high degree of chemical and structural similarity. The intraday precision acceptance criteria of 15% at the concentration limits and 10% for all other concentrations was met by all of the tested calibration standards. Even though the interday precision acceptance criteria, in terms of the relative standard deviations for the nine total determinations conducted for each calibration standard were not met, the RSD associated with the slopes of the three different calibration curves created from that data was less than 10%. This indicates that the curves were sufficiently equivalent for the purposes of the present thesis. Furthermore, the linearity, expressed as the R^2 value, for both the amitriptyline and nortriptyline calibration curves, was greater than 0.98. Therefore, the method used in the present project produced linear results within the expected concentration range of both nortriptyline and amitriptyline, when the analytes were present in a non-complicated matrix of ethyl acetate.

This chapter described the method validation process for analytes within an uncomplicated matrix of ethyl acetate. Validation of the previously developed gas chromatographic method for the quantitation of amitriptyline and nortriptyline in the artificial foodstuff is presented in Chapter 3.

Chapter 3

Method Validation II:

Amitriptyline and Nortriptyline in the Artificial Foodstuff Matrix

3.1 Introduction

In the present project, amitriptyline and nortriptyline were added to an artificial foodstuff consisting of beef liver, powdered whole egg and agar, which was then used to rear larvae of the species *Sarcophaga bullata* (Diptera: Sarcophagidae). The goal of this thesis was to quantify any observed changes in the rate of *S. bullata* development, resulting from the presence of either amitriptyline or nortriptyline in their food source. With this in mind, the amount of amitriptyline and nortriptyline added to each batch of foodstuff had to be quantified in order to confirm both the homogeneity of the drugs in the foodstuff, and that each drug was present in the required concentration. However, due to the complexity of the analyte matrix¹, the concentration of amitriptyline and nortriptyline could not be determined without first appropriately processing the samples.

Sample preparation is an integral part of chromatographic analysis. For the most

¹i.e. the artificial foodstuff

part, the majority of samples are too dilute, too complex or simply not compatible with the chromatographic system, and must be processed in some fashion before introduction to the chromatographic system (Robards *et al.*, 1994).

For most chromatography techniques, including gas chromatography, the sample must be in solution in order for it to be analyzed. Furthermore, the sample must be dissolved in a solvent that is compatible with the detector. For example, if a nitrogen-phosphorus detector is the detector of choice, then the solvent used to introduce the analyte(s) to the chromatographic system must not contain either nitrogen or phosphorus, otherwise the signal from the solvent will mask the signal of the analyte in the sample.

In some cases, sample preparation can be as simple as dissolving the sample in a suitable solvent, as was done to prepare the amitriptyline and nortriptyline calibration standards used in the initial method validation process (Chapter 2). However, this approach is only suitable for gas chromatography if there are no non-volatile lipids or proteins present in the sample (Robards *et al.*, 1994). In most cases, the samples under investigation are more complex, and as a result, multiple steps are required to overcome the problems associated with sample complexity and system incompatibility (Robards *et al.*, 1994).

The principal goal of sample preparation is to provide a homogeneous sample extract that is both suitable for chromatographic analysis and free from interfering matrix materials (Robards *et al.*, 1994). The steps involved in the sample preparation process may be collectively referred to as **analyte recovery**. According to Robards *et al.* (1994), analyte recovery usually involves four basic steps:

1. Fractionation/homogenization of the sample
2. Isolation of the analyte(s) from the rest of the matrix
3. Sample clean-up to remove any coextracted matrix components
4. Preconcentration of the isolated analyte(s)

Analyte isolation from the bulk of the matrix is usually achieved by some type of

extraction technique². Analyte isolation, in most cases, is not 100% efficient, and as a result, some interferences originating from the matrix are commonly extracted along with the analyte(s). Therefore, post-extraction clean-up is normally required to remove the unwanted, co-extracted matrix components and to improve the overall rate of analyte recovery (Robards *et al.*, 1994).

In general, clean-up procedures are time consuming and labour intensive. Therefore, clean-up procedures significantly increase the complexity of the sample preparation process. Following clean-up and extraction, preconcentration of the extracted analyte(s) is normally required before the extract can be introduced into the chromatographic system. Preconcentration is usually necessary for two reasons (Robson *et al.*, 1994):

- The concentration of the analyte(s) in the original sample is usually too low
- The isolation and clean-up steps of the recovery process dilutes the concentration of the analyte(s) even further

Overall, the most suitable sample preparation process is the one that minimizes analyte losses while at the same time eliminating the most interferences. In general, the amount of analyte loss is directly proportional to the number of steps required for the preparation of the sample extract (Robards *et al.*, 1994). Therefore, the most suitable procedure is also the procedure that produces the best rate of analyte recovery, with the least amount of interfering substances, in the fewest number of steps.

Biological tissue samples require stringent sample preparation procedures if the extracts are to be analyzed using gas chromatography. Non-volatile components of the sample matrix, such as lipids and proteins, must be removed in order to prevent the production of charred residues during the sample vapourization process. The production of burnt lipid and protein residues can lead to column degradation if they are allowed to build up within the chromatographic system (Robards *et al.*, 1994).

²e.g. liquid-liquid extraction

In this project, the recovery of amitriptyline and nortriptyline from the foodstuff was a time-consuming process, taking three days from the start of sample preparation to the introduction of the sample extract into the chromatographic system.

The artificial foodstuff was very complex, and as a result the efficient extraction of amitriptyline and nortriptyline from the foodstuff was problematic. Tricyclic antidepressants, including amitriptyline and nortriptyline, are highly lipid soluble, and as a result they bind tightly to tissues (Frommer *et al.*, 1987). Tricyclic antidepressants preferentially accumulate in a variety of tissues, such as the brain, liver and lungs. For example, the concentration of tricyclic antidepressants in liver cells have been found to be 30 times greater than their concentration in blood plasma (Frommer *et al.*, 1987).

Tissue binding makes the isolation of analytes from the matrix time consuming and less efficient. The extraction of tissue bound, lipid soluble components from a fatty matrix is particularly problematic because significant co-extraction of unwanted non-volatile lipids and proteins usually occurs (Robards *et al.*, 1994).

The artificial foodstuff was prepared from beef liver and powdered whole egg, and as a result, the protein and lipid content of the artificial foodstuff was quite high. Therefore, given the propensity of amitriptyline and nortriptyline to bind to tissues, it is expected that the majority of the amitriptyline and nortriptyline will be bound to the proteins and lipids present in the artificial foodstuff.

The purpose of this chapter is to describe and present the results of the procedures used to evaluate the following method validation criteria concerning the isolation and measurement of amitriptyline and nortriptyline from the artificial foodstuff:

- Analyte recovery
- Accuracy
- Extracted linearity
- Limit of detection
- Limit of quantitation

- Homogeneity of the drug-spiked artificial foodstuff

The results of each of these validation tests will be presented and discussed in this chapter.

3.2 Preparation of the Foodstuff for Extraction: Homogenization and Acid Digestion

3.2.1 Introduction

Before the level of amitriptyline and nortriptyline added to the artificial foodstuff can be quantitated, the analytes must first be extracted from the matrix. The analytes of interest may be present in a variety of forms in the sample. For instance, in the artificial foodstuff, amitriptyline and nortriptyline likely exists in two basic forms:

1. “Free” analyte; analyte that is not bound to endogenous components, such as lipids and liver proteins, present in the artificial foodstuff matrix
2. “Bound” analyte; analyte that is bound to endogenous components, such as proteins and lipids, present in the artificial foodstuff matrix

Since the total concentration of each analyte is of interest in the present project, the sample preparation method employed must be able to break down the foodstuff matrix in order to release the bound amitriptyline and nortriptyline so that it can be recovered along with the free amitriptyline and nortriptyline during sample preparation.

The breakdown of the foodstuff was facilitated in two ways: (1) homogenization of the foodstuff and (2) digestion of the foodstuff matrix. Homogenization at the correct pH should facilitate the release of the unbound amitriptyline and nortriptyline into solution, whereas digestion of the actual matrix components should release the matrix bound drugs into solution. These two steps should result in the total recovery of free and bound amitriptyline and nortriptyline from the matrix, and therefore make the drugs available for quantitation by gas chromatography.

Digestion of the foodstuff matrix could be accomplished using either chemical digestion or enzymatic digestion. Chemical digestion was the method chosen for this project. It is important that the method of digestion be selective; in other words, the method of digestion employed should digest the foodstuff matrix yet leave the analytes intact. Acid digestion was the method of digestion employed in this project since a wide variety of acids were readily available in the lab. In addition, acid digestion is a commonly used method during preparation of organs such as the liver and brain for toxicological analysis (Flanagan, 1993). Hydrochloric acid was the choice of acid for digestion of the foodstuff matrix because it was a readily available, non-oxidizing acid. Amitriptyline and nortriptyline are readily oxidized, and therefore oxidizing acids such as nitric acid or phosphoric acid were avoided because their use would have destroyed the analytes. Before using hydrochloric acid to digest the matrix of real samples, the stability of amitriptyline and nortriptyline in hydrochloric acid must be determined.

3.2.2 Stability Test Procedure

Ten percent by volume hydrochloric acid was chosen as the starting concentration of acid for digestion of the foodstuff matrix. Concentrations less than this would likely have had little effect on the agar in the matrix, and concentrations greater than this would likely have resulted in substantial analyte degradation.

Two test solutions of amitriptyline and two test solutions of nortriptyline were prepared from 1 mg/mL aqueous amitriptyline and 1 mg/mL aqueous nortriptyline stock solutions, respectively. A test solution for maprotyline, the internal standard, was also prepared from a 1 mg/mL aqueous maprotyline stock solution. Each of the five test solutions were prepared in separate 25 mL volumetric flasks by adding the appropriate volume of analyte stock solution and then diluting to the mark with distilled water. Distilled water was used as the solvent for the stability test because the foodstuff was originally prepared from an aqueous homogenate of liver, egg and agar. The concentrations prepared were as follows:

1. 0.9 mg/L nortriptyline

2. 7 mg/L nortriptyline
3. 25 mg/L amitriptyline
4. 165 mg/L amitriptyline
5. 10 mg/L maprotyline

These concentrations were chosen because they represented the minimum and maximum expected concentrations of each analyte in the foodstuff. The internal standard used for the present thesis was maprotyline, and the maprotyline concentration chosen for the stability assay represented the concentration used to quantitate the amitriptyline and nortriptyline in the artificial foodstuff. The volumes of amitriptyline, nortriptyline and maprotyline stock solutions required for the preparation of the 10% (v/v) hydrochloric acid stability test solutions are presented in Table 3.1.

Target Concentration (mg/L) in Distilled Water	Volume (μ L) of 1 mg/mL AMT Stock Solution	Volume (μ L) of 1 mg/mL NOR Stock Solution	Volume (μ L) of 1 mg/mL MAP Stock Solution	Final Volume (mL)
0.9	-	22.5	-	25
7	-	175	-	25
25	625	-	-	25
160	4000	-	-	25
10	-	-	250	25

Table 3.1: Volume of amitriptyline, nortriptyline and maprotyline stock solutions used to prepare the test solutions required for the 10% (v/v) hydrochloric acid stability test. The test solutions were prepared in 25 mL volumetric flasks from the aqueous stock solutions and then diluted to the mark with distilled water (AMT = amitriptyline; NOR = nortriptyline; MAP = maprotyline).

The procedure outlined below was completed for all five of the test solutions listed in Table 3.1. However, for ease of explanation, the procedure used will be described for only one of the five test solutions, specifically the 25 mg/L amitriptyline test solution.

One millilitre of the 25 mg/L amitriptyline test solution was added to each of three borosilicate glass test tubes, followed by 1 mL of distilled water to three of the six

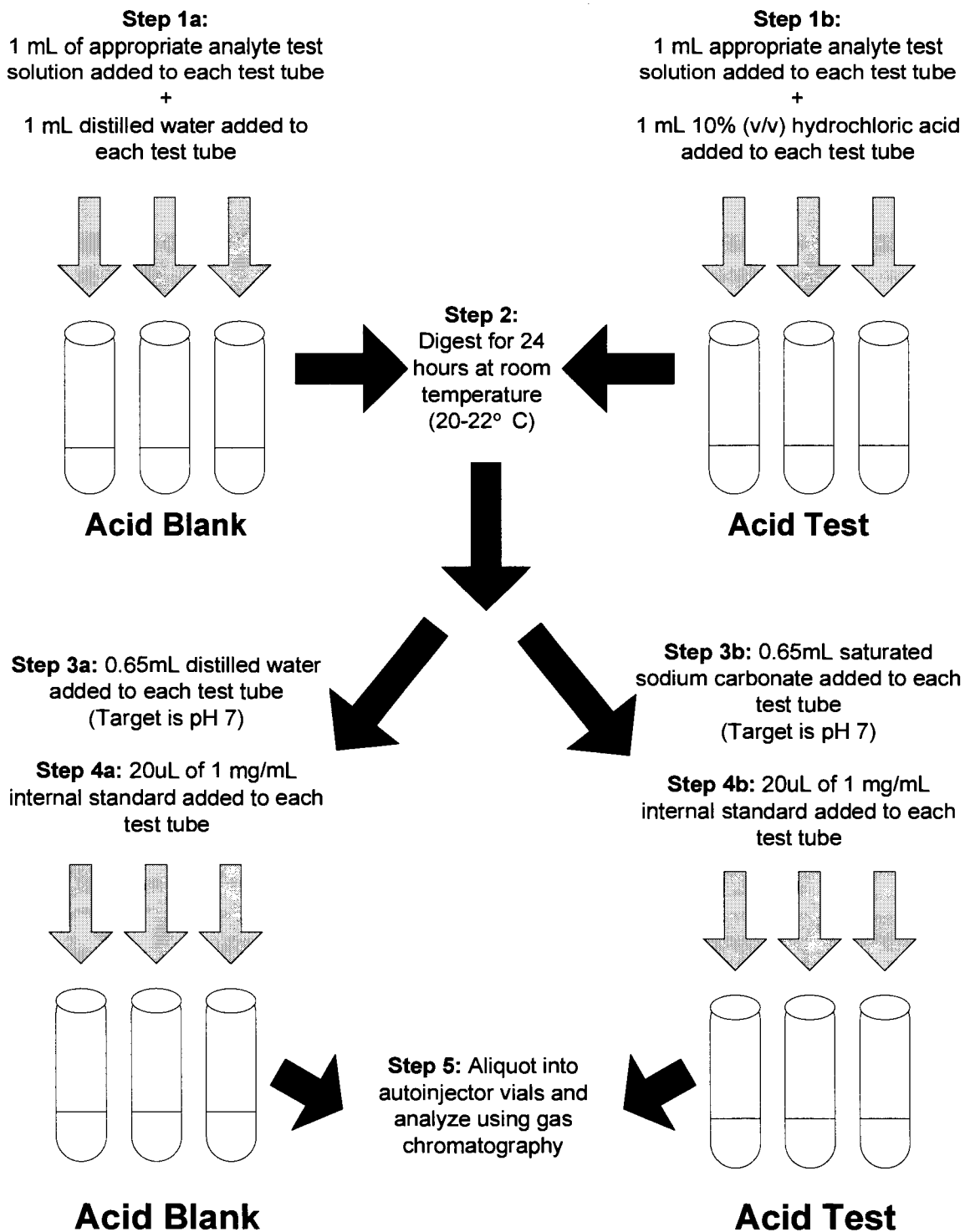


Figure 3.1: Procedural schematic for the acid stability assay.

test tubes (Figure 3.2, Step 1a). These three tubes served as the control replicates for the assay since the analyte solution added to these three test tubes was not exposed to hydrochloric acid. These three tubes are labelled “acid blanks” in Figure 3.2.

One millilitre of the 25 mg/L amitriptyline test solution was added to the remaining three test tubes, followed by 1 mL of 10% (v/v) hydrochloric acid (Figure 3.2, Step 1b). These three test tubes are labelled “acid test” in Figure 3.2, and they served as the experimental replicates for the stability assay because the analyte solution added to each of these three tubes was digested with hydrochloric acid. The six test tubes were then capped and allowed to sit for 24 hours at room temperature (20-22°C) (Figure 3.2, Step 2).

A neutralization test was then performed by adding 1 mL of 10% (v/v) hydrochloric acid, followed by 1 mL of distilled water to a clean borosilicate glass test tube. Saturated sodium carbonate was then added in 50 μL increments until the acid present in the tube was neutralized (pH 7). In this situation, 650 μL was required to neutralize the hydrochloric acid present.

At the end of the 24 hour digestion period, 650 μL of saturated sodium carbonate was added to each of the three “acid test” tubes and then mixed using a vortex mixer for ten seconds (Figure 3.2; Step 3b). This step was required to neutralize the hydrochloric acid added in Step 1b because extremely low pH values can damage the stationary phase inside the column.

The pH of the neutralized “acid test” solutions were checked to ensure that the neutralization step was effective³. In addition, 650 μL of distilled water was added to each of the three “acid blank” tubes, and then mixed using a vortex mixer for 10 seconds (Figure 3.2; Step 3a) to ensure that the total volume for the “acid blank” solutions remained the same as the total volume for the “acid test” solutions.

The pH of distilled water is between 5.6 and 7, which is a fairly wide range; therefore, the pH of the acid blanks were checked. The pH of the acid blanks were determined to be approximately pH 6, which was adequate for the purposes of the present experiment.

³i.e. the target pH was approximately 7

After neutralization of the “acid test samples”, 20 μL of 1 mg/mL maprotyline stock solution (in MEOH) was added to each of the three tubes, and then mixed using a vortex mixer (Figure 3.2; Step 4b). The same amount of 1 mg/mL maprotyline (in MEOH) was also added to each of the three “acid blank” test tubes, after the addition of the distilled water from Step 3a (Figure 3.2; Step 4a).

Maprotyline was used as the internal standard for both the amitriptyline and nortriptyline stability test solutions; however, for the stability test of the 10 mg/L maprotyline test solution, 20 μL of 1 mg/L amitriptyline (in MEOH) was used as the internal standard.

Each of the “acid test” samples and “acid blank” samples were then aliquoted into autoinjector vials and analyzed by GC-NPD. The samples were analyzed using the previously validated method (Appendix A). Triplicate injections were made for each sample. The analyte/internal standard peak height ratio results for all three “acid test” samples were pooled together to obtain a mean, standard deviation and relative standard deviation for peak height ratio data. The analyte/internal standard peak height ratio results for all three “acid blank” samples were pooled together as well, and a mean, standard deviation and relative standard deviation of the peak height ratio results were calculated. The mean peak height ratio for the “acid test” samples and “acid blank” samples were then compared statistically using a two-tailed Students *t*-test in JMP IN[®] (SAS Institute, Inc., Cary, NC, USA). Since triplicate injections were conducted for each “acid blank” sample and each “acid test” sample, the Student’s *t*-test was weighted for errors in peak height ratio. If the peak height ratio results for the “acid test” samples were significantly less ($\alpha = 0.05$) than the “acid blank” samples, then the mean percent drug loss was also calculated.

3.2.3 Stability Test Results

Based on the stability test conducted, a 10 mg/L aqueous maprotyline solution is remarkably stable when exposed to 10% (v/v) hydrochloric acid for 24 hours at room temperature (20-22°C). The mean peak height ratios for the “acid blank” samples were shown not to be significantly different from the mean peak height ratios for the

“acid test” samples (95% CI = 0.36 to 0.39; $t = 0.14$; $df = 4$; $P = 0.73$; Table 3.2).

In addition, aqueous solutions of nortriptyline appear to be stable upon exposure to 10% (v/v) hydrochloric acid as well. The mean peak height ratios for the “acid test” samples were shown not be significantly different from the mean peak height ratios for the “acid blank” samples at both 0.9 mg/L (95% CI = 0.05 to 0.06; $t = 3.52$; $df = 4$; $P = 0.13$; Table 3.2) and 7 mg/L (95% CI = 0.37 to 0.50; $t = 1.36$; $df = 4$; $P = 0.31$; Table 3.2).

Also, aqueous solutions of 25 mg/L amitriptyline were shown to be stable upon exposure to 10% (v/v) hydrochloric acid for 24 hours (95% CI = 1.72 to 1.89; $t = 6.91$; $df = 4$; $P = 0.06$; Table 3.2). However, the same results were not observed for the 165 mg/L aqueous amitriptyline solutions. Aqueous solutions of 165 mg/L amitriptyline were not stable upon exposure to 10% (v/v) hydrochloric acid for 24 hours (95% CI = 9.76 to 13.39; $t = 8.99$; $df = 4$; $P = 0.04$; Table 3.2). In this case, the percent drug loss was found to be $16.2 \pm 0.9\%$ (Table 3.2).

3.2.4 Stability Test Discussion

The stability test conducted indicated that a solution of 10 mg/L maprotyline will be stable under acid digestion with 10% (v/v) hydrochloric acid. Likewise, nortriptyline was also shown to be stable under acid digestion. Both the 0.9 mg/L and 7 mg/L aqueous solutions of nortriptyline were shown to be stable under acid digestion, therefore it is likely nortriptyline will be stable within the concentration range expected for the present project. The aqueous solutions of amitriptyline, on the other hand, were stable at 25 mg/L but not at 165 mg/L. For the 165 mg/L aqueous solution, the mean drug loss was $16.2 \pm 0.9\%$. This was an unacceptable level of drug loss given the complexity of the foodstuff matrix. Given these results, it appears that amitriptyline may not be stable upon digestion with 10% (v/v) hydrochloric acid within the concentration range expected for the present project. However, based on the stability assay, it appears that lower concentration solutions of amitriptyline may be more stable than higher concentration solutions. The significant drug loss observed for the aqueous 165 mg/L amitriptyline solutions will likely adversely affect the recovery of the analyte from the foodstuff matrix. However, given the stability of

Aqueous Test Solution	Acid Blank		Acid Test		Mean Drug Loss Due to Acid Digestion (%)	t-Test ($\alpha = 0.05$)
	Mean (\pm SD) Peak Height Ratio	RSD (%)	Mean (\pm SD) Peak Height Ratio	RSD (%)		
0.9 mg/L Nortriptyline [n = 3]	0.06 \pm 0.0002	0.4	0.06 \pm 0.0001	0.2	-	t = 3.52; df = 4; P = 0.13
7 mg/L Nortriptyline [n = 3]	0.47 \pm 0.003	0.7	0.44 \pm 0.001	0.4	-	t = 1.36; df = 4; P = 0.31
25 mg/L Amitriptyline [n = 3]	1.99 \pm 0.02	1.2	1.81 \pm 0.01	0.4	-	t = 6.91; df = 4; P = 0.06
165 mg/L Amitriptyline [n = 3]	13.82 \pm 0.60	4.3	11.58 \pm 0.21	1.8	16.2 \pm 0.9	t = 8.99; df = 4; P = 0.04
10 mg/L Maprotyline [n = 3]	0.38 \pm 0.002	0.5	0.38 \pm 0.001	2.2	-	t = 0.14; df = 4; P = 0.73

Table 3.2: Mean, standard deviation (SD), and relative standard deviation (RSD) for the pooled peak height ratios from triplicate injections of each “acid blank” and each “acid test” for the test solutions. The mean percent drug loss and the associated error were calculated for those test solutions where the mean peak height ratios for the “acid test” samples were significantly different from the mean peak height ratios for the “acid blank” samples. Significance was tested using a two-tailed Students t-Test in JMP IN[®] ($\alpha = 0.05$).

nortriptyline and maprotyline to acid digestion, digestion with hydrochloric acid will be investigated further during the recovery experiment (Section 3.4).

3.3 Extraction of Amitriptyline and Nortriptyline from the Foodstuff Matrix

3.3.1 Introduction

The purpose of an extraction is to increase the concentration of the analyte(s) of interest while at the same time removing them from the bulk of the sample matrix. **Liquid-liquid extraction** is one type of extraction, and can be defined as the selective transfer of one or more compounds from one liquid (usually aqueous) to another immiscible liquid (usually organic) (Meloan, 1999). The degree of partition between the two liquids depends on the physical and chemical properties of each compound (Meloan, 1999). In general, the more hydrophobic compounds will prefer the organic layer while the more hydrophilic compounds will prefer the polar aqueous phase (Snyder *et al.*, 1997). In order for liquid-liquid extraction to be useful at isolating the analyte(s) of interest from the matrix, the analyte(s) must exhibit a different solubility in the extracting solvent compared to the majority of the matrix components (Robards *et al.*, 1994).

Liquid-liquid extraction offers several advantages, including relatively low equipment costs. In addition, a wide variety of selectivities can be exploited by simply changing the identity of the extracting solvent, or by altering various characteristics of the sample solution, such as the pH (Robards *et al.*, 1994). Furthermore, the steps involved in liquid-liquid extraction are relatively easy to execute. Even so, liquid-liquid extraction also has several disadvantages such as the use of relatively large amounts of possibly toxic and flammable solvents. In addition, even relatively simple extraction procedures can become time consuming and labour intensive, especially if multiple extractions must be performed, or if emulsions occur, and cannot be eliminated using simple techniques⁴. Emulsions adversely affect analyte recovery and

⁴e.g. such as the addition of a small amount of a different organic solvent, centrifuging

frequently occur with samples from fatty matrices, because they prevent the analytes from partitioning properly between the two liquid phases (Snyder *et al.*, 1997).

In liquid-liquid extraction, the analytes are separated from interferences by partitioning the analytes between an aqueous phase and an organic phase. For example, if the analytes of interest are present in the aqueous phase, but are more soluble in the organic phase, the analytes will preferentially move into the organic phase until it becomes saturated. Any further transfer of the hydrophobic analytes into the organic layer would require replacement of the saturated organic layer with fresh organic solvent. The transfer from one liquid to the other is seldom complete in a single step because of phase saturation (Meloan, 1999). In general, there is always a small amount of the compound left behind, even after several replacements of the extracting solvent (Meloan, 1999). The amount left behind in the aqueous phase contributes to analyte loss and as result, reduces the rate of analyte recovery.

In the present thesis, **discontinuous batch** liquid-liquid extraction was employed to isolate amitriptyline and nortriptyline from the majority of the foodstuff matrix. Discontinuous batch extraction is usually quite simple, albeit time consuming, to execute, and it involves adding the two phases to a mixing vessel, such as a test tube, mixing the two phases together, allowing the two liquids to separate and then recovering the desired layer (Robards *et al.*, 1994). In general, analyte recovery can be improved by repeating the extraction several times. Several small extractions are potentially better than one large extraction because the addition of fresh extraction solvent reduces the problem of solvent saturation by the extract (Zubrick, 1984). However, very little in the way of analyte recovery is gained after repeating the extraction process more than three times (Robards *et al.*, 1994).

The organic solvent used to extract amitriptyline and nortriptyline from the foodstuff matrix was chlorobutane. Chlorobutane has a calculated (MDL[®] QSAR⁵) $\log(P_{ow})$ ⁶ of 2.64 (Blaha *et al.*, 1998), and as a result, is immiscible in water, and

⁵MDL[®] QSAR is a quantitative structure activity modeling system used to identify quantitative structure-activity and structure-property relationships for chemical substances.

⁶The value of $\log(P_{ow})$ is the octanol/water partition coefficient. A large $\log(P_{ow})$ indicates that the compound preferentially distributes into the organic layer. The partition coefficient can be

therefore forms two distinct layers upon mixing with an aqueous solution. Furthermore, chlorobutane has been shown to provide some selectivity in the isolation of pharmaceuticals from biological fluids and tissues (Robards *et al.*, 1994). As a result, chlorobutane is an appropriate choice for drug extraction from the aqueous foodstuff homogenate.

Amitriptyline has a calculated (MDL[®] QSAR) $\log(P_{ow})$ of 4.88, and nortriptyline has a calculated (MDL[®] QSAR) $\log(P_{ow})$ of 4.35 (NIAID, no date). Due to their large partition coefficients, amitriptyline and nortriptyline will preferentially distribute into an organic solvent such as chlorobutane. It is their large partition coefficients that also makes amitriptyline and nortriptyline highly lipophilic, and also leads to their accumulation in various tissues, such as the liver.

3.3.2 Extraction Procedure

The following extraction protocol was designed for the extraction of amitriptyline and nortriptyline from artificial foodstuff, and was adapted from the extraction protocol used for amitriptyline and nortriptyline from liver and blood at the Provincial Toxicology Centre, Riverview Hospital, Port Coquitlam, British Columbia. A schematic of the extraction protocol used in the present thesis is presented in Figure 3.2.

The first step of the extraction procedure was to prepare the 1:1 foodstuff: distilled water homogenate⁷. Each cube of foodstuff was accurately weighed into a plastic urine collection container using an analytical balance, followed by the addition of an equal weight of distilled water, and then the mixture was homogenized until smooth.

One millilitre of the foodstuff homogenate was then transferred to an accurately weighed borosilicate glass test tube. The test tube and its contents were then weighed and the weight of the aliquoted 1:1 foodstuff homogenate was determined by subtracting the weight of the test tube. Twenty-five microlitres of 1 mg/mL maprotyline (in MEOH) were added to each test tube, and the contents were vortexed for 10 seconds.

calculated using the following equation (Walker *et al.*, 2001):

$$\log(P) = \log(K_{ow}) = \log\left(\frac{\text{concentration in octanol}}{\text{concentration in water}}\right)$$

⁷Hereby referred to as the 1:1 foodstuff homogenate.

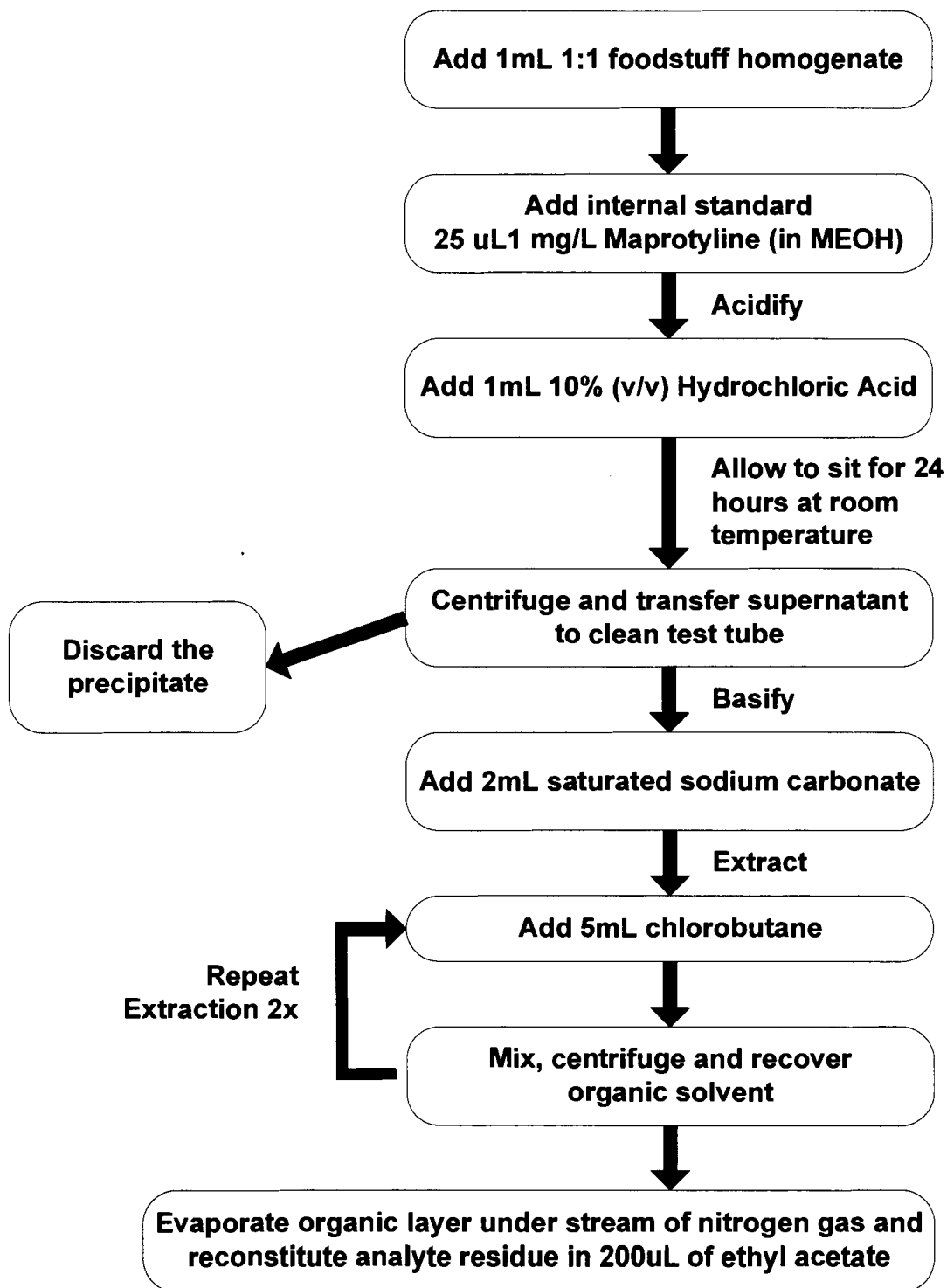


Figure 3.2: Schematic outline of the extraction protocol used to isolate amitriptyline and nortriptyline from the artificial foodstuff matrix using liquid-liquid extraction.

One millilitre of 10% (v/v) hydrochloric acid was then added to each test tube containing the foodstuff homogenate. The mixture was then vortex mixed for 10 seconds, capped and allowed to digest for 24 hours at room temperature (20-22°C).

Homogenization of the foodstuff with an equal weight of distilled water resulted in a thick, free-flowing, slightly frothy homogenate that was light pink in colour. Addition of hydrochloric acid resulted in a thin, runny homogenate with free floating particulate matter. No colour change was observed in the foodstuff homogenate upon addition of the 10% hydrochloric acid.

After 24 hours, the tubes were vortexed briefly to resuspend the remaining particulate matter, and then centrifuged for 15 minutes at 3,000 rpm. Centrifuging forced the solid material present in the foodstuff homogenate into a solid plug at the bottom of the test tube. The aqueous portion of the foodstuff homogenate, the supernatant, was light yellow in colour, clear, and free of particulate matter. The supernatant from each tube was gently decanted into clean, borosilicate glass test tubes. The decanting step had to be performed with care in order to reduce the amount of particulate matter that was decanted along with the aqueous supernatant. Digestion with 10% hydrochloric acid reduced the solid portion of the foodstuff homogenate to an extremely friable material, and disturbance of the surface resuspended the extremely fine-textured pieces of particulate matter that rested on the surface of the foodstuff plug.

Saturated sodium carbonate (2 mL) was added to each of the test tubes containing the supernatant to raise the pH of the solution above pH 9. Amitriptyline has a pK_a of 9.4 and nortriptyline has a pK_a of 9.7, and as a result, both are basic compounds (Budavari *et al.*, 1996). Therefore, in order to extract them from an aqueous solution using an organic liquid, the aqueous solution must be altered so that it has a pH greater than that of the analytes themselves. With this in mind, the target pH for the aqueous foodstuff homogenate was pH 10. The pH of the aqueous phase was checked to ensure that it was sufficiently alkaline.

Chlorobutane (5 mL) was then added to the alkaline supernatant and mixed using a rocking agitator for 20 minutes. After 20 minutes, the mixture was centrifuged for 15 minutes at approximately 3,000 rpm to separate the organic and aqueous phases.

The density of the organic layer was less than that of water; therefore, the organic layer formed on top of the aqueous layer, and was easily recovered using a Pasteur pipette. The recovered organic layer was transferred into a clean borosilicate glass test tube.

Another 5 mL of chlorobutane was added to the alkaline aqueous supernatant, and then mixing, centrifuging and organic phase recovery procedure described above was repeated, and the second organic layer was added to the previously collected layer.

The combined organic layer was then evaporated to dryness at 50°C, under a stream of nitrogen gas. Once the organic layer was completely evaporated, the residue was reconstituted in 200 μ L of ethyl acetate. The reconstituted extract was vortex mixed and allowed to sit for 30 minutes at room temperature (20-22°C). After 30 minutes, the reconstituted extracts were aliquoted into autoinjector vials and analyzed using the previously developed GC-NPD method (Appendix A).

3.3.3 Extraction Discussion

Emulsions formed quite regularly during the extraction of amitriptyline and nortriptyline due to the high fat content of the foodstuff matrix. For the most part, the emulsions were easily broken by centrifuging until a sharp boundary between the aqueous phase and the organic phase was restored. If centrifuging alone was not able to break up the emulsion, a small amount of another organic solvent was added, and then the contents were centrifuged again. The high lipid content of the artificial foodstuff samples was probably what contributed to the extensive emulsion formation. Therefore, further experimentation, concerning the production of lipid-free extracts from the artificial foodstuff, is required.

In biological samples, it is quite common for the analytes to bind to high molecular weight compounds such as proteins, and this binding adversely affects the rate of analyte recovery. In the present project, hydrochloric acid was used to disrupt the association between the analytes and proteins present in the matrix. Acetonitrile precipitation can also be used to precipitate the proteins present in the sample once the association between the proteins and analytes has been disrupted, such as with acid digestion. However, when the protein precipitation step was included in the extraction

protocol used in the present thesis, no improvement in the recovery of amitriptyline or nortriptyline was observed. These results will be presented and discussed in the following section.

3.4 Recovery of Amitriptyline and Nortriptyline from the Foodstuff Matrix

3.4.1 Introduction

Other components present in the artificial foodstuff matrix may interfere with the separation, detection and accurate quantitation of amitriptyline and nortriptyline. Therefore, the impact of the matrix components must be investigated. One method of investigating the effectiveness of the sample preparation procedure is via a recovery experiment. In a recovery experiment, blank foodstuff matrix is spiked with several different analyte concentrations, and the recovery of the analyte at each concentration is determined by comparison to the known amount of analyte added (Snyder *et al.*, 1997). Comparison of the amount of analyte recovered during the sample preparation procedure, to the known amount added, is achieved through the generation of a **recovery function**. A recovery function is simply the plot of the amount of analyte recovered *versus* the known amount added. The recovery function generated for a specific set of data can be used to identify the type and magnitude of systematic errors introduced by the sample preparation process (Meyer, 1998). In conclusion, the purpose of a recovery experiment, and the creation of a recovery function, is to determine the influence of sample modification, such as extraction, on the analytical process (Funk *et al.*, 1995). Knowledge of the type and magnitude of any systematic errors present can be then be used to improve the sample preparation procedure.

In many recovery experiments, test portions of blank sample matrices are used to determine the efficiency of the sample preparation procedure. However, the test portions of sample matrices used are often homogenized, aqueous solutions of the matrix, and are therefore not true representations of the actual environment of the analyte(s) when present in natural samples. It is not likely that analytes added just

prior to extraction would be as strongly held by the matrix as the analytes present in a natural sample. As a result, it is not uncommon for recovery assays using fortified sample matrices to give unrealistically high impressions of the efficiency of the sample preparation process (EURACHEM Working Group, 1998).

As mentioned earlier, amitriptyline and nortriptyline are highly lipophilic molecules, and as a result form strong associations with high molecular weight molecules such as proteins and lipids. Given the high protein and lipid content of the artificial foodstuff, it was expected that both analytes would be strongly held by these high molecular weight components present in the foodstuff matrix.

In order to accurately determine the efficiency of the proposed sample preparation procedure, the analytes had to be introduced in such a way that would sufficiently mimic the protein and lipid binding of the analytes in the actual foodstuff. With this goal in mind, real foodstuff samples, containing a variety of analyte concentrations, were prepared to test the efficiency of the sample preparation procedure.

However, the protocol used to prepare the spiked foodstuff required for the recovery experiment was slightly different from the actual procedure used to prepare the spiked foodstuff required for rearing the larvae of *S. bullata* (Diptera: Sarcophagidae). The difference in the preparation protocol was simply one of scale; significantly less foodstuff was required for the recovery experiment compared to the insect development experiments. However, great care was taken to ensure that the ratio of ingredients⁸ in the foodstuff used in the recovery experiment matched the ratio of ingredients in the foodstuff used to rear the insects.

3.4.2 Amitriptyline and Nortriptyline Recovery Procedure

Establishment of the Calibration Function for the Fundamental Analytical Procedure

The first step in the recovery experiment is to establish the calibration curve of the **fundamental analytical procedure** (Funk *et al.*, 1995). To do this, 11 calibration standards were prepared from two mixed amitriptyline and nortriptyline stock

⁸e.g. ratio of agar to liver homogenate

solutions (in methanol):

1. 0.1 mg/mL amitriptyline + 0.1 mg/mL nortriptyline
2. 1 mg/mL amitriptyline + 1 mg/mL nortriptyline

The 0.1 mg/mL stock solutions were prepared by a 1:10 dilution (in methanol) of the 1 mg/mL mixed amitriptyline and nortriptyline stock solution. Each of the eleven calibration standards were prepared by adding 25 μ L of 1 mg/ml maprotyline (internal standard), the appropriate volume of amitriptyline and nortriptyline stock solution, and then diluting to the mark in a 10 mL volumetric flask with ethyl acetate. The final internal standard concentration in each calibration standard was 2.5 mg/L. The concentrations prepared were as follows:

1. 0 mg/L amitriptyline + 0 mg/L nortriptyline
2. 0.1 mg/L amitriptyline + 0.1 mg/L nortriptyline
3. 0.25 mg/L amitriptyline + 0.25 mg/L nortriptyline
4. 0.5 mg/L amitriptyline + 0.5 mg/L nortriptyline
5. 1 mg/L amitriptyline + 1 mg/L nortriptyline
6. 5 mg/L amitriptyline + 5 mg/L nortriptyline
7. 10 mg/L amitriptyline + 10 mg/L nortriptyline
8. 15 mg/L amitriptyline + 15 mg/L nortriptyline
9. 20 mg/L amitriptyline + 20 mg/L nortriptyline
10. 25 mg/L amitriptyline + 25 mg/L nortriptyline
11. 35 mg/L amitriptyline + 35 mg/L nortriptyline

The volumes of amitriptyline, nortriptyline and maprotyline stock solutions used to prepare the calibration standards required for the recovery experiment are presented in Table 3.3.

Each of the 11 calibration standards were injected in triplicate and analyzed by GC-NPD according to the previously developed method (Appendix A). The data generated was used to create the calibration curve by plotting peak height ratio (analyte peak height/maprotyline peak height) versus the concentration of the calibration standard. Weighted linear regression analysis (weighted for errors in y) was used to obtain the equation of the fundamental calibration curve for the analytical procedure. The calibration curve was then used to quantitate the levels of amitriptyline and nortriptyline subsequently extracted from the foodstuff.

Preparation of the Spiked Artificial Foodstuff Required for the Recovery Experiment

Eight different samples of artificial foodstuff were prepared for the recovery experiment, each with a different concentration of amitriptyline and nortriptyline. The target amitriptyline and nortriptyline concentrations for the foodstuff samples were as follows:

1. 0 mg/kg amitriptyline + 0 mg/kg nortriptyline (blank)
2. 0.25 mg/kg amitriptyline + 0.25 mg/kg nortriptyline
3. 0.75 mg/kg amitriptyline + 0.75 mg/kg nortriptyline
4. 1.5 mg/kg amitriptyline + 1.5 mg/kg nortriptyline
5. 3.0 mg/kg amitriptyline + 3.0 mg/kg nortriptyline
6. 6.0 mg/kg amitriptyline + 6.0 mg/kg nortriptyline
7. 12.5 mg/kg amitriptyline + 12.5 mg/kg nortriptyline
8. 25 mg/kg amitriptyline + 25 mg/kg nortriptyline

Target Concentration (mg/L) in Ethyl Acetate	Volume (μ L) of Mixed 0.1 mg/mL AMT and 0.1 mg/mL NOR Stock Solution	Volume (μ L) of Mixed 1 mg/mL AMT and 1 mg/mL NOR Stock Solution	Volume (μ L) of 1 mg/mL MAP Stock Solution	Final Volume (mL)
0	0	-	25	10
0.1	10	-	25	10
0.25	25	-	25	10
0.5	50	-	25	10
1	100	-	25	10
5	-	50	25	10
10	-	100	25	10
15	-	150	25	10
20	-	200	25	10
25	-	250	25	10
35	-	350	25	10

Table 3.3: Volume of amitriptyline, nortriptyline and maprotyline stock solutions used to prepare the calibration standards required for the analyte recovery experiment. The calibration standards were prepared in 10 mL volumetric flasks with ethyl acetate as the diluting solvent (AMT = amitriptyline; NOR = nortriptyline; MAP = maprotyline).

The foodstuff samples were prepared in several steps over a period of two days. On the first day, approximately 20 g of liver homogenate was added to each of eight, seven ounce glass jars. Next, the appropriate volumes of amitriptyline and nortriptyline were added to each jar. For this purpose, separate 1 mg/mL stock solutions (in distilled water) of both amitriptyline and nortriptyline were prepared. The total volume of liquid added must be equal across all samples. With this in mind, the appropriate volume of distilled water was added to each sample of foodstuff homogenate in order to adjust the total volume of liquid added to 2.5 mL (Table 3.4). The contents of each jar were swirled by hand for 30 seconds after the addition of each liquid in order to evenly disperse the drug within the liver homogenate. The liver homogenate-drug mixture was then allowed to equilibrate overnight for 24 hours at room temperature (20-22°C).

Approximate Target Concentration (mg/kg)	Volume of 1 mg/mL Amitriptyline Added (mL)	Volume of 1 mg/mL Nortriptyline Added (mL)	Volume of Distilled Water Added (mL)
0	0	0	2.5
0.30	0.015	0.015	2.47
0.70	0.035	0.035	2.43
1.4	0.070	0.07	2.36
2.8	0.140	0.140	2.22
6.0	0.300	0.300	1.90
12.0	0.600	0.600	1.30
24.0	1.2	1.2	0.10

Table 3.4: Volumes of 1 mg/mL amitriptyline and 1 mg/L nortriptyline stock solutions (in distilled water) added to eight 20 g samples of liver homogenate.

On the second day, liquid agar was prepared by adding 11.26 g agar into 250 mL distilled water, and heating the mixture on a hot plate until the agar was completely dissolved. The liquid agar was then allowed to cool to approximately 50°C, and just before it gelled, approximately 25 g of the agar was added to each of the seven jars containing the foodstuff and drug mixture. This mixture was then swirled until the constituents were evenly mixed. The liver homogenate mixture was a dull red in

colour, while the liquid agar was a slightly opaque, dull yellow. Even mixing was assumed when no red or yellow streaks were visible and when the overall colour of the liver homogenate-agar mixture changed to a pale pink. This mixture was allowed to set overnight for 24 hours at room temperature (20-22°C). The weight of liquid (drug solution and distilled water), agar and the overall weight of each foodstuff sample is presented in (Table 3.5).

The final concentration of amitriptyline and nortriptyline in the eight spiked foodstuff samples are shown in Table 3.6. The final concentration of amitriptyline and nortriptyline present in each sample of spiked foodstuff was calculated by dividing the amount of drug added (in mg) by the total weight of foodstuff prepared (in kg).

Extraction of Amitriptyline and Nortriptyline from the Spiked Artificial Foodstuff

Amitriptyline and nortriptyline were isolated from the spiked foodstuff samples using the previously described extraction protocol. However, 25 μL of 0.1 mg/mL maprotyline was used as the internal standard for the recovery experiments, due to the low concentrations involved in the recovery experiments, rather than 25 μL of 1 mg/mL maprotyline.

A sample of blank foodstuff matrix was also extracted and analyzed using GC-NPD to determine whether or not any of the matrix components remaining in the sample after extraction interfered with any of the analyte peaks.

Given the propensity of amitriptyline and nortriptyline to bind to proteins, the effectiveness of adding an additional step to the extraction protocol, specifically a protein precipitation step, was also investigated. Once the association between the analytes and the proteins present in the matrix have been disrupted, protein precipitation should prevent analyte-protein re-association once conditions favouring the re-association have been restored. For example, in the extraction protocol used for the present thesis, an acid pH was used to disrupt the analyte-protein association. However, there is the possibility that any proteins or other high molecular weight

Approximate Target Concentration (mg/kg)	Weight of Liver Homogenate Added (g)	Weight of Agar Added (g)	Weight of Liquid Added (Drug Solution + Water) (g)	Total Weight of Foodstuff (g)
0	20.77	26.03	2.5	49.30
0.30	20.78	25.47	2.5	48.75
0.70	20.84	25.31	2.5	48.65
1.4	20.88	25.27	2.5	48.65
2.8	21.01	25.53	2.5	49.04
6.0	20.79	25.21	2.5	48.50
12.0	20.80	25.53	2.5	48.83
24.0	20.82	25.23	2.5	48.55

Table 3.5: The weight of the liver homogenate (g), agar (g), liquid(g), and the overall weight(g) of each spiked foodstuff sample prepared for the recovery experiment.

molecules remaining in the supernatant once it has been decanted, could rebind analytes when the pH of the supernatant is increased to pH 10.

Therefore, in the interest of increasing the rate of analyte recovery, a second recovery experiment was conducted, using acetonitrile as the protein precipitating reagent. The acetonitrile step was added after acid digestion, but before acid neutralization, and the modified extraction procedure is outlined below:

1. One millilitre of the 1:1 foodstuff homogenate was added to each test tube, followed by the addition of 25 μL 0.1 mg/mL maprotyline (internal standard) and 1 mL of 10% (v/v) hydrochloric acid. The mixture was then allowed to digest for 24 hours at room temperature (20-22°C).
2. The samples were vortex mixed, and then centrifuged for 15 minutes at 3,000 rpm. Each aqueous supernatant was then decanted into clean test tubes.
3. Two millilitres of acetonitrile was then added to each aqueous supernatant, and the mixture centrifuged for 10 minutes at 3,000 rpm. The precipitated proteins were forced into a plug at the base of the test tube. Each supernatant was decanted into clean test tubes.
4. The acetonitrile was then evaporated at 50°C under a stream of nitrogen gas. Aqueous solutions are miscible with acetonitrile, and therefore, the solution was blown down until the solution volume remaining approximated the volume of aqueous solution originally decanted into the test tube.
5. Two millilitres of saturated sodium carbonate was then added to each supernatant to increase the pH of the solution to pH 10.
6. After the addition of the saturated sodium carbonate, the extraction protocol proceeded as already outlined in Section 3.3.

Amitriptyline		Nortriptyline	
Approximate Target Amitriptyline Concentration (mg/kg)	Prepared Amitriptyline Concentration (mg/kg)	Approximate Target Nortriptyline Concentration (mg/kg)	Prepared Nortriptyline Concentration (mg/kg)
0	0	0	0
0.30	0.31	0.30	0.31
0.70	0.72	0.70	0.72
1.4	1.44	1.4	1.44
2.8	2.85	2.8	2.85
6.0	6.18	6.0	6.18
12.0	12.29	12.0	12.29
24.0	24.72	24.0	24.72

Table 3.6: Comparison of the approximate target concentration (mg/kg) and the prepared concentration (mg/kg) for the amitriptyline and nortriptyline spiked foodstuff required for the recovery experiment.

3.4.3 Amitriptyline Recovery from the Artificial Foodstuff

Table 3.7 presents the calibration data used to generate the fundamental calibration function for amitriptyline. The relative standard deviations for the triplicate injections of each amitriptyline calibration standard were within the precision acceptance criteria outlined in Chapter 2. The limit of quantitation for amitriptyline was 0.36 mg/L, and it is interesting to note that the RSDs for the three calibration standards below the limit of quantitation (0 mg/L, 0.1 mg/L and 0.25 mg/L) were all below 15%, but above 10%. Of the amitriptyline calibration standards analyzed, the 0.5 mg/L standard was the first standard immediately above the experimentally determined limit of quantitation, and it was interesting to observe that the RSD for this calibration standard, at 5.2%, was considerably less than the RSDs calculated for the 0 mg/L, 0.1 mg/L, and 0.25 mg/L amitriptyline calibration standards (Table 3.7).

Concentration of Amitriptyline Calibration Standard (mg/L)	Mean Peak Height Ratio (AMT Peak Height/MAP Peak Height) \pm SD	Relative Standard Deviation (%)
0 ($n = 3$)	0.014 \pm 0.002	12.1
0.1 ($n = 3$)	0.108 \pm 0.009	8.2
0.25 ($n = 3$)	0.195 \pm 0.023	11.9
0.5 ($n = 3$)	0.353 \pm 0.018	5.2
1 ($n = 3$)	0.638 \pm 0.008	1.3
5 ($n = 3$)	3.09 \pm 0.024	0.8
10 ($n = 3$)	6.62 \pm 0.170	2.6
15 ($n = 3$)	9.70 \pm 0.438	4.5
20 ($n = 3$)	11.6 \pm 0.141	1.2
25 ($n = 3$)	15.3 \pm 0.353	2.3
35 ($n = 3$)	22.1 \pm 0.340	1.5

Table 3.7: The mean peak height ratio, standard deviation and relative standard deviations for the triplicate injections of the eleven amitriptyline calibration standards prepared in ethyl acetate (AMT = amitriptyline; MAP = maprotyline).

The calibration curve of the fundamental analytical procedure for amitriptyline is given in Figure 3.3. The calibration equation for amitriptyline was $y = 0.619x + 0.154$

(95% CI (slope) = 0.588 to 0.650; $t_{df=1} = 45.59$; $P = < 0.0001$; $R^2 = 0.996$; Figure 3.3).

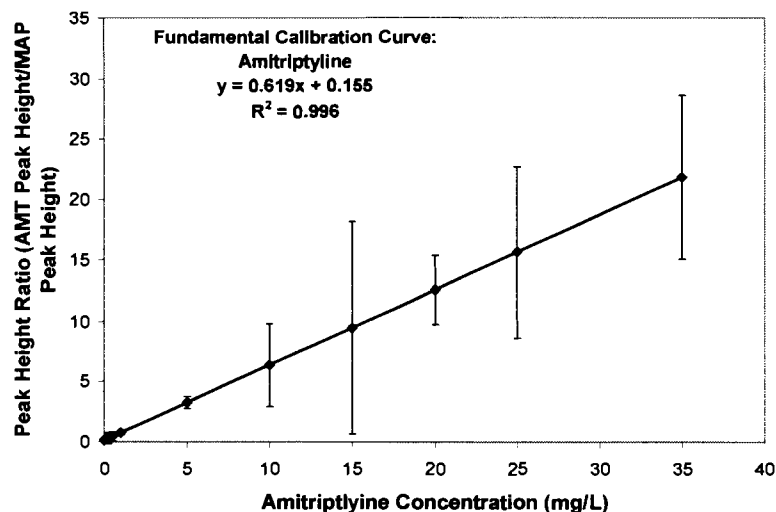


Figure 3.3: Calibration curve of the fundamental analytical procedure for amitriptyline quantitation. Data generated from triplicate injections of eleven amitriptyline calibration standards (in ethyl acetate). Least squares linear regression, weighted for errors in y , was performed on the calibration data. Error bars represent the standard deviation, scaled by a factor of +20, so that majority of the error bars were visible. AMT = amitriptyline; MAP = maprotyline.

Unfortunately, the magnitude of the y -intercept obtained for the fundamental amitriptyline calibration curve (Figure 3.3) was too large to allow the quantitation of the noise in the drug-free foodstuff sample, for either of the two extraction procedures⁹.

The calibration standards were prepared in a matrix different from that of the samples, and therefore the calibration curve will not correct for baseline noise generated by the drug-free foodstuff sample. In order to correct for this, the peak height ratio obtained for the extracts of the amitriptyline-spiked foodstuff samples must be **blank corrected**, and in order to do this, the magnitude of the baseline noise must be quantitated. In addition, the magnitude of the y -intercept for the fundamental

⁹Without acetonitrile protein precipitation, and with acetonitrile protein precipitation.

calibration function for amitriptyline was also too large to quantitate the amount of amitriptyline extracted from the foodstuff sample spiked at a level of 0.31 mg/kg. To illustrate this problem, the mean peak height ratios obtained for both of the extractions procedures that were tested are presented in Table 3.8.

The fundamental calibration curve for amitriptyline exhibited excellent linearity, based on its R^2 value of 0.996. Therefore, rather than just subjectively removing the data from one or more of the eleven calibration standards until a lower y -intercept was obtained, it was decided to force the fundamental calibration curve through zero. This effectively eliminated the y -intercept, thereby allowing the quantitation of all the extracts for level of amitriptyline present.

Forcing the y -intercept through zero can be justified based on *a priori* knowledge that, if no analyte is present, the detector response should be zero. Further, a y -intercept of zero is within the 95% confidence level of the fundamental amitriptyline curve presented in Figure 3.3. Therefore, from a statistical perspective it was reasonable to force the regression line of the amitriptyline calibration data through zero. The equation for the fundamental calibration curve, obtained by forcing the curve through zero, was $y = 0.625x$ (95% CI (slope) = 0.613 to 0.638; $t_{df=1} = 114.92$; $P < 0.0001$). It was interesting to note that the 95% confidence interval for slope of the first curve included the slope of the second curve, and *vice versa*. This indicated that from a statistical perspective, the two slopes were equivalent.

Furthermore, the relative standard deviation (RSD) of the two slopes were calculated in order to estimate the equivalence of the two calibration curves (Table 3.9). The calculated RSD for the two slopes was extremely small (0.7%), and therefore, it appears that forcing the curve through zero did not appreciably affect the slope of the fundamental calibration curve for amitriptyline.

Therefore, the equation for the fundamental amitriptyline calibration curve forced through zero was used to quantitate the level of amitriptyline recovered with the two different extraction procedures.

Amitriptyline Extraction Without Prior Protein Precipitation			Amitriptyline Extraction With Prior Protein Precipitation		
Concentration of Amitriptyline Calibration Standard (mg/L)	Mean Peak Height Ratio (AMT Peak Height/MAP Peak Height) \pm SD	Relative Standard Deviation (%)	Concentration of Amitriptyline Calibration Standard (mg/L)	Mean Peak Height Ratio (AMT Peak Height/MAP Peak Height) \pm SD	Relative Standard Deviation (%)
0	0.013 \pm 0.002	11.5	0	0.014 \pm 0.001	3.0
0.31	0.147 \pm 0.002	1.4	0.31	0.108 \pm 0.001	0.4
0.72	0.323 \pm 0.003	0.9	0.72	0.247 \pm 0.012	4.9
1.44	0.563 \pm 0.019	3.4	1.44	0.513 \pm 0.006	1.1
2.85	0.999 \pm 0.020	2.0	2.85	0.879 \pm 0.006	06
6.18	2.171 \pm 0.024	1.1	6.18	2.038 \pm 0.002	0.1
12.29	4.138 \pm 0.089	2.1	12.29	3.831 \pm 0.073	1.9
24.72	8.069 \pm 0.015	0.2	24.72	7.681 \pm 0.004	0.1

Table 3.8: The mean peak height ratio, standard deviation and relative standard deviations for the duplicate injections of each set of sample extracts (AMT = amitriptyline; MAP = maprotyline).

Extraction of Amitriptyline Without Prior Protein Precipitation

The mean and standard deviation for the concentration of amitriptyline (mg/kg) extracted, without protein precipitation, from each of the artificial foodstuff samples are presented in Table 3.10. The mean and standard deviation of the extracted amitriptyline concentration were calculated using the equation for the calibration curve that was forced through zero.

The recovery function for the extraction of amitriptyline without prior protein precipitation was established by least squares regression analysis, weighted for errors in y , of the amitriptyline recovery plot data, which was generated by plotting the extracted concentration of amitriptyline versus the spiked concentration of amitriptyline (Table 2.9). The recovery function for the extraction of amitriptyline without prior protein precipitation is presented in Figure 3.4.

The equation for the recovery function for amitriptyline extracted without prior protein precipitation is $y = 0.305x - 0.038$ (95% CI (slope) = 0.286 to 0.323; $t_{df=1} = 43.08$; $P < 0.0001$; $R^2 = 0.997$). Since the slope of the recovery function is not equal to 1, a proportional systematic error, resulting from the sample preparation and analysis exists. In addition, since the intercept of the recovery function is not equal to zero, a constant systematic error resulting from the sample preparation and analysis exists as well (Meyer, 1998).

The rate of amitriptyline recovery from the extraction without prior protein precipitation is presented in Table 3.11.

	Slope
Mean \pm SD	0.622 \pm 0.004
RSD (%)	0.7

Table 3.9: The mean and relative standard deviation (RSD) for the slopes of the two fundamental calibration curves for amitriptyline.

Spiked Amitriptyline Concentration (mg/kg)	Mean \pm SD Extracted Amitriptyline Concentration (mg/kg)
0.31	0.11 \pm 0.002
0.72	0.23 \pm 0.002
1.44	0.47 \pm 0.016
2.85	0.93 \pm 0.018
6.18	1.91 \pm 0.021
12.29	3.61 \pm 0.078
24.72	7.70 \pm 0.015

Table 3.10: Concentration of amitriptyline (mg/kg) extracted without protein precipitation from the spiked artificial foodstuff, and the corresponding rate of amitriptyline recovery (%). The recovery rate was calculated using the recovery function, $y = 0.625x$, where y = extracted amitriptyline concentration (mg/kg) and x = spiked amitriptyline concentration (mg/kg).

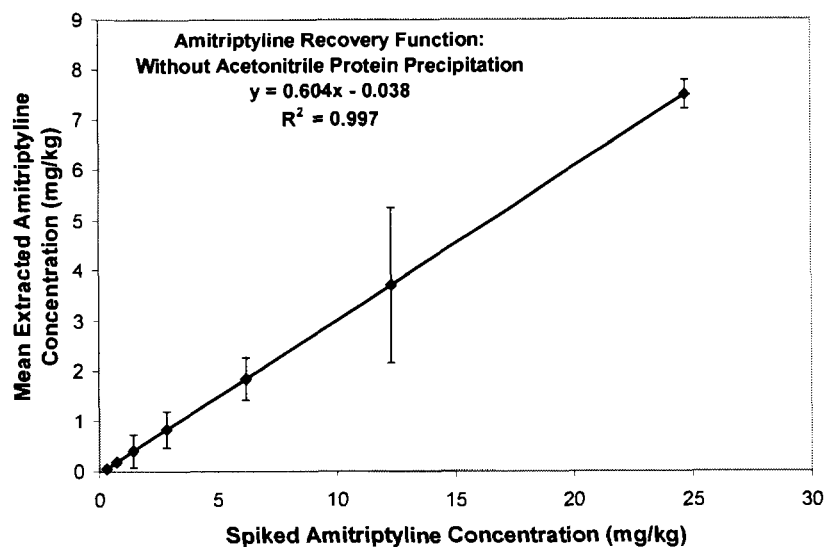


Figure 3.4: Amitriptyline recovery function for the extraction of amitriptyline from the artificial foodstuff without prior protein precipitation with acetonitrile. Error bars represent the standard deviation, scaled by a factor of +20, so that majority of the error bars were visible.

Spiked Amitriptyline Concentration (mg/kg)	Mean Extracted Amitriptyline Concentration (mg/kg)	Recovery Rate (%) without Acetonitrile Protein Precipitation
0.31	0.11	18.3
0.72	0.23	25.3
1.44	0.47	27.9
2.85	0.93	29.2
6.18	1.91	29.9
12.29	3.61	30.2
24.72	7.70	30.3

Table 3.11: Concentration of amitriptyline (mg/kg) extracted with prior protein precipitation from the spiked artificial foodstuff, and the corresponding rate of amitriptyline recovery (%). The recovery rate was calculated using the mean extracted amitriptyline concentration (mg/kg) and the recovery function, $y = 0.305x + 0.038$, where y = spiked amitriptyline concentration (mg/kg), and x = extracted amitriptyline concentration (mg/kg), and as a result, the biases introduced during sample preparation and analysis were incorporated into the calculation of the recovery rate.

Extraction of Amitriptyline With Prior Protein Precipitation

The mean and standard deviation for the concentration of amitriptyline (mg/kg) extracted, with prior protein precipitation, from each of the artificial foodstuff samples are presented in Table 3.12. The mean and standard deviation of the extracted amitriptyline concentration were calculated using the equation for the calibration curve that was forced through zero.

Spiked Amitriptyline Concentration (mg/kg)	Mean \pm SD Extracted Amitriptyline Concentration (mg/kg)
0.31	0.11 \pm 0.002
0.72	0.23 \pm 0.002
1.44	0.47 \pm 0.016
2.85	0.93 \pm 0.018
6.18	1.91 \pm 0.021
12.29	3.61 \pm 0.078
24.72	7.70 \pm 0.015

Table 3.12: Mean and standard deviation for the concentration of amitriptyline (mg/kg) extracted with protein precipitation from the spiked artificial foodstuff.

The recovery function for the extraction of amitriptyline with prior protein precipitation is presented in Figure 3.5. The equation of the recovery function for amitriptyline extracted with prior protein precipitation is $y = 0.277x - 0.038$ (95% CI (slope) = 0.260 to 0.295; $t_{df=1} = 40.01$; $P < 0.0001$; $R^2 = 0.997$; Figure 3.5). The slope of the recovery function does not equal one, and therefore a **proportional systematic error** from the sample preparation and analysis exists. In addition, the intercept of the amitriptyline recovery function is not equal to zero, and therefore a **constant systematic error** exists as well.

Extraction with prior protein precipitation by acetonitrile did not improve the rate of amitriptyline recovery (Figure 3.5; Table 3.13). In fact, prior treatment with acetonitrile decreased the recovery rate for all foodstuff samples, except the sample with the lowest spiked concentration of amitriptyline, 0.31 mg/kg. With acetonitrile protein precipitation, the calculated amitriptyline recovery rate for the 0.31 mg/kg

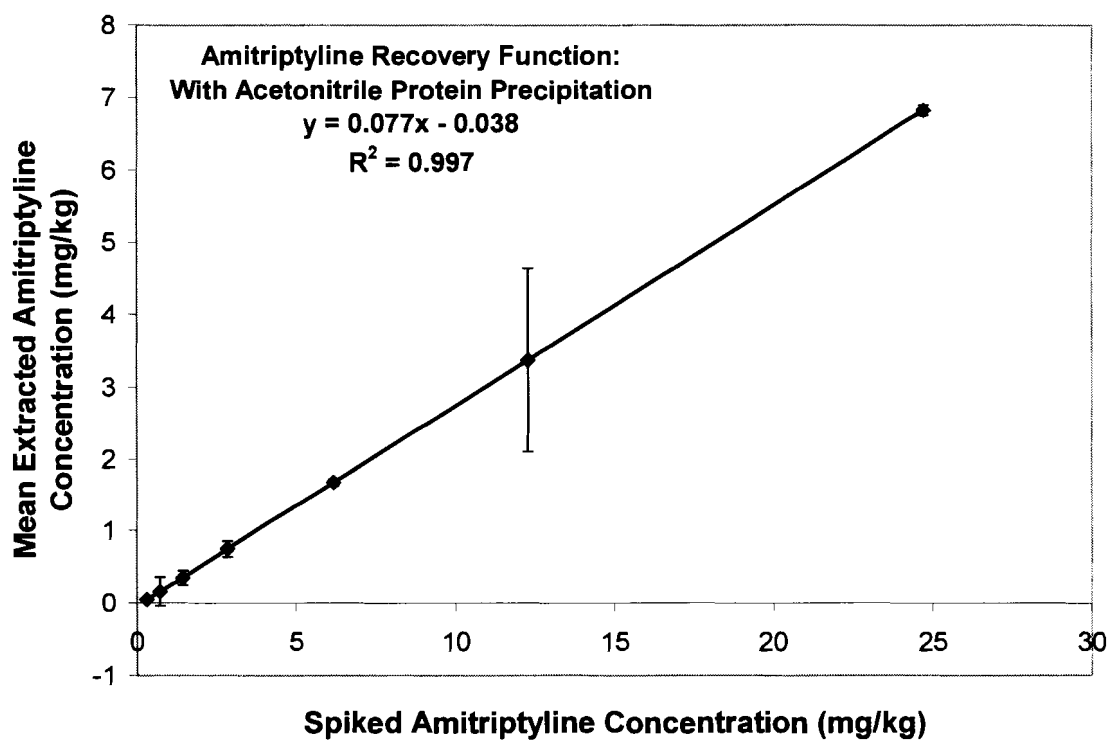


Figure 3.5: Amitriptyline recovery function for the extraction of amitriptyline from the artificial foodstuff with prior protein precipitation with acetonitrile. Error bars represent the standard deviation, scaled by a factor of +20.

sample was 18.3% (Table 3.11), compared to 15.3% recovery rate achieved without prior protein precipitation (Table 3.13).

Spiked Amitriptyline Concentration (mg/kg)	Mean Extracted Amitriptyline Concentration (mg/kg)	Recovery Rate (%) with Acetonitrile Protein Precipitation
0.31	0.11	15.3
0.72	0.23	22.4
1.44	0.47	25.07
2.85	0.93	26.4
6.18	1.91	27.1
12.29	3.61	27.4
24.72	7.70	27.6

Table 3.13: Mean concentration of amitriptyline (mg/kg) extracted with prior protein precipitation from the spiked artificial foodstuff, and the corresponding rate of amitriptyline recovery (%). The recovery rate was calculated using the recovery function $y = 0.277x + 0.038$, where y = extracted amitriptyline concentration (mg/kg), and x = spiked amitriptyline concentration, and as a result, the biases introduced during sample preparation and analysis are incorporated into the calculation of the recovery rate.

Figure 3.6 presents the amitriptyline recovery rate for both recovery experiments, as a function of the spiked amitriptyline concentration (mg/kg). As illustrated in the plot, prior protein precipitation does not improve the isolation of amitriptyline from the matrix.

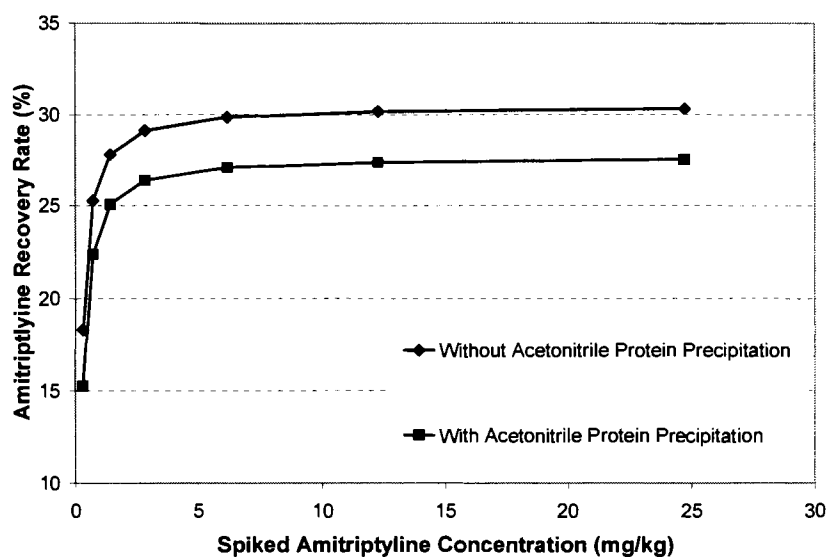


Figure 3.6: Comparison of the calculated amtriptyline recovery rates for the extraction of amtriptyline from artificial foodstuff, both without prior protein precipitation and with prior protein precipitation.

3.4.4 Nortriptyline Recovery from the Artificial Foodstuff

The calibration curve of the fundamental analytical procedure for nortriptyline is given in Figure 3.7. The calibration equation for nortriptyline in a matrix of ethyl acetate was $y = 0.617x - 0.756$ (95% CI (slope) = 0.566 to 0.668; $t_{df=1} = 27.32$; $P < 0.0001$; $R^2 = 0.988$; Figure 3.7). The R^2 value for the fundamental nortriptyline calibration curve was lower than expected given the high linearity obtained during the intraday and interday precision experiments. However, an R^2 value of 0.988 was still acceptable for the present thesis. Even so, if more time had been available, this experiment would have been repeated with new calibration standards to improve the linearity of the fundamental analytical procedure for nortriptyline.

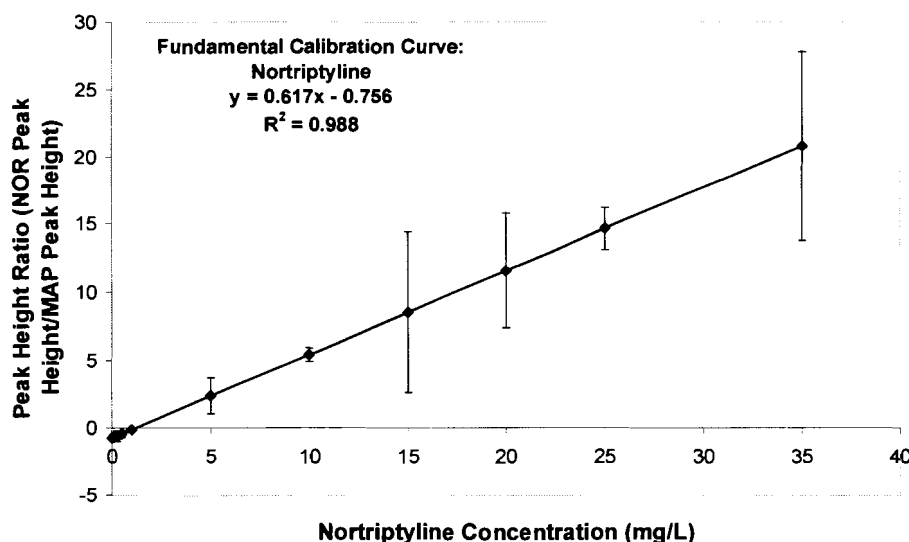


Figure 3.7: Calibration curve of the fundamental analytical procedure for nortriptyline quantitation. Data generated from triplicate injections of eight nortriptyline calibration standards (in ethyl acetate). Least squares linear regression, weighted for errors in y , was performed on the calibration data. Error bars represent the standard deviation, scaled by a factor of +25, so that majority of the error bars were visible. NOR = nortriptyline; MAP = maprotyline.

Table 3.14 presents the calibration data used to generate the fundamental calibration function for nortriptyline, which is presented in Figure 3.7. The relative

standard deviations for the triplicate injections of each calibration standard containing nortriptyline was within the precision acceptance criteria outlined in Chapter 2. As expected, the RSD for the 0 mg/L standard was fairly large given the fact that it is a measure of transient baseline noise. The limit of quantitation (LOQ) for nortriptyline was 0.29 mg/L, and it is interesting to note that the RSDs for the 0.1 mg/L and 0.25 mg/L nortriptyline calibration standards were all below 10%, despite the fact that they represented concentrations lower than the calculated LOQ for nortriptyline. Of the nortriptyline calibration standards analyzed, the 0.5 mg/L standard was the first standard immediately above the experimentally determined LOQ, and it was interesting to observe that the RSD for this calibration standard, at 3.3%, was considerably smaller than the RSDs calculated for the 0.1 mg/L and 0.25 mg/L calibration standards (Table 3.14).

Concentration of Nortriptyline Calibration Standard (mg/L)	Mean Peak Height Ratio (NOR Peak Height/MAP Peak Height) \pm SD	Relative Standard Deviation (%)
0 ($n = 3$)	0.026 \pm 0.004	16.8
0.1 ($n = 3$)	0.108 \pm 0.011	9.9
0.25 ($n = 3$)	0.159 \pm 0.015	9.7
0.5 ($n = 3$)	0.296 \pm 0.010	3.3
1 ($n = 3$)	0.579 \pm 0.003	0.5
5 ($n = 3$)	2.92 \pm 0.053	1.8
10 ($n = 3$)	5.54 \pm 0.021	0.4
15 ($n = 3$)	21.4 \pm 0.236	2.7
20 ($n = 3$)	10.3 \pm 0.168	1.6
25 ($n = 3$)	13.6 \pm 0.061	0.5
35 ($n = 3$)	21.4 \pm 0.280	1.3

Table 3.14: The mean peak height ratio, standard deviation and relative standard deviations for the triplicate injections of the eleven nortriptyline calibration standards prepared in ethyl acetate (NOR = nortriptyline; MAP = maprotyline).

Nortriptyline Extraction Without Prior Protein Precipitation

The concentrations of nortriptyline (mg/kg) extracted, without acetonitrile protein precipitation, from each of the seven samples of artificial foodstuff samples are presented in Table 3.15.

Spiked Nortriptyline Concentration (mg/kg)	Mean Extracted \pm SD (Without Protein Precipitation) Nortriptyline (mg/kg)
0.31	0.07 \pm 0.003
0.72	0.15 \pm 0.012
1.44	0.35 \pm 0.002
2.85	0.58 \pm 0.001
6.18	1.52 \pm 0.002
12.29	3.81 \pm 0.015
24.72	5.81 \pm 0.025

Table 3.15: Mean extracted \pm SD concentration of nortriptyline (mg/kg). Extracted without prior acetonitrile protein precipitation from the spiked artificial foodstuff.

The recovery function for the extraction of nortriptyline without prior protein precipitation was established by linear least squares regression, weighted for errors in y , of the nortriptyline recovery data presented in Table 3.15. The recovery function was generated by plotting the extracted concentration of nortriptyline *versus* the spiked concentration of nortriptyline, and is presented in Figure 3.8. The equation for the nortriptyline recovery function was $y = 0.232x + 0.265$ (95% CI (slope) = 0.187 to 0.277; $t_{df=1} = 13.36$; $P < 0.0001$; $R^2 = 0.973$; Figure 3.8). Unfortunately, the linearity of the nortriptyline recovery function was much lower than expected ($R^2 = 0.973$). Figure 3.8 presents both the least squares regression line calculated for the data and the mean concentration of nortriptyline extracted from each of the seven foodstuff samples. Based on the regression line calculated from the recovery data, a greater proportion of nortriptyline was extracted from the 12.3 mg/kg foodstuff sample than expected, and as a result, the linearity of the recovery function was compromised.

With this in mind, the 95% and 99% confidence intervals for the regression line of the nortriptyline recovery function were investigated to determine whether the amount

of nortriptyline extracted from the 12.3 mg/kg foodstuff sample was an **outlier**. The amount of nortriptyline extracted from the 12.3 mg/kg nortriptyline spiked foodstuff was outside the 95% confidence region for the regression line, and right on top of the upper 99% confidence limit for the regression line (Figure 3.9). Therefore, the data point at $(x,y) = (12.3 \text{ mg/kg}, 3.8 \text{ mg/kg})$ was considered an outlier and removed from the analysis. The data point at $(12.3 \text{ mg/kg}, 3.8 \text{ mg/kg})$ will hencefore be referred to as the “outlier”.

When the outlier was excluded from the analysis, the linearity of the nortriptyline recovery function increased significantly (Figure 3.10). The equation for the nortriptyline recovery function with the outlier removed was $y = 0.235x - 0.005$ (95% CI (slope) = 0.233 to 0.238; $t_{df=1} = 293.43$; $P < 0.0001$; $R^2 = 0.999$; Figure 3.10).

However, before continuing with the rest of the analysis, the resulting two recovery functions were investigated further to determine their equivalence. Therefore the relative standard deviation (RSD) for the two curves was calculated and the results are presented in Table 3.16.

	Slope	Intercept
Mean \pm SD	0.233 \pm 0.002	0.131 \pm 0.192
RSD (%)	1.0	146.7

Table 3.16: The mean and relative standard deviation (RSD) for the slopes and intercepts of the two nortriptyline recovery functions, calculated with and without the data point classified as an outlier.

Based on the extremely large RSD for the y -intercepts (146.7%), it appears that the outlier, when included, artificially increased the magnitude of the y -intercept of the nortriptyline recovery function (Figure 3.11). In contrast, based on the small RSD associated with the slopes (1.0%), removal of the outlier did not significantly alter the slope of the nortriptyline recovery function (Figure 3.11). Therefore, eliminating the outlier from the analysis reduced the amount of the proportional systematic error associated with the nortriptyline recovery function.

Furthermore, despite the large differences in the y -intercepts, the curves were not statistically different based on a Student’s two-tailed t -test at an alpha level of 0.05

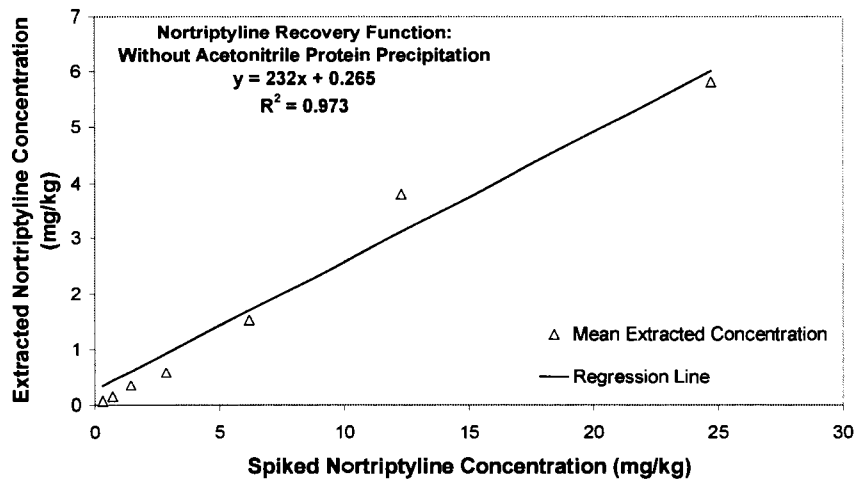


Figure 3.8: Nortriptyline recovery function for the extraction of nortriptyline from the artificial foodstuff without prior protein precipitation with acetonitrile. Both the regression line and the mean extracted concentration of nortriptyline are presented in the plot.

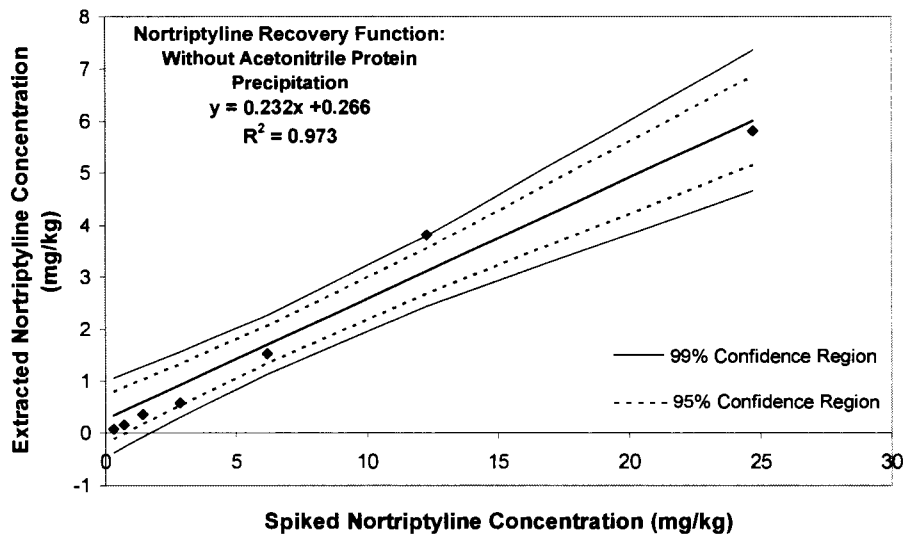


Figure 3.9: The 95% and 99% confidence regions of the nortriptyline recovery function for the extraction of nortriptyline from the artificial foodstuff without prior protein precipitation.

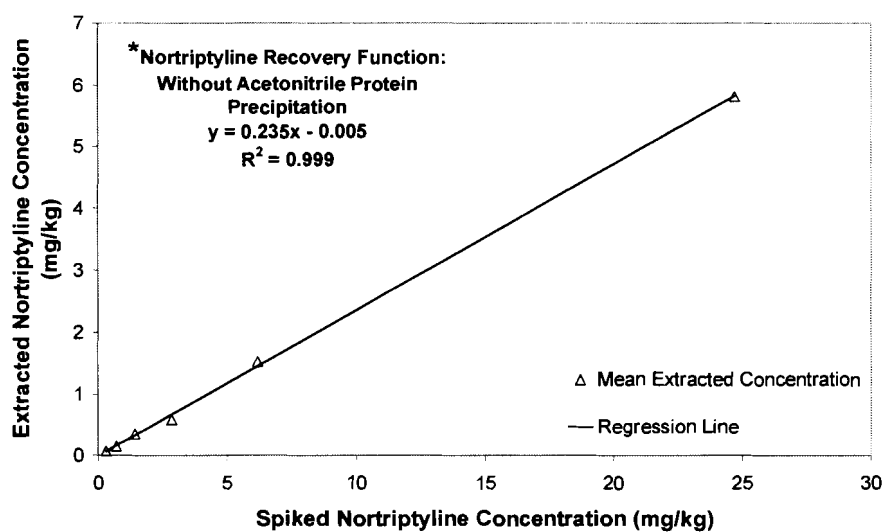


Figure 3.10: Nortriptyline recovery function for the extraction of nortriptyline from the artificial foodstuff without prior protein precipitation with acetonitrile. Both the regression line and the mean extracted concentration of nortriptyline, calculated from triplicate injections of each extract are presented in the plot. *With the outlier excluded.

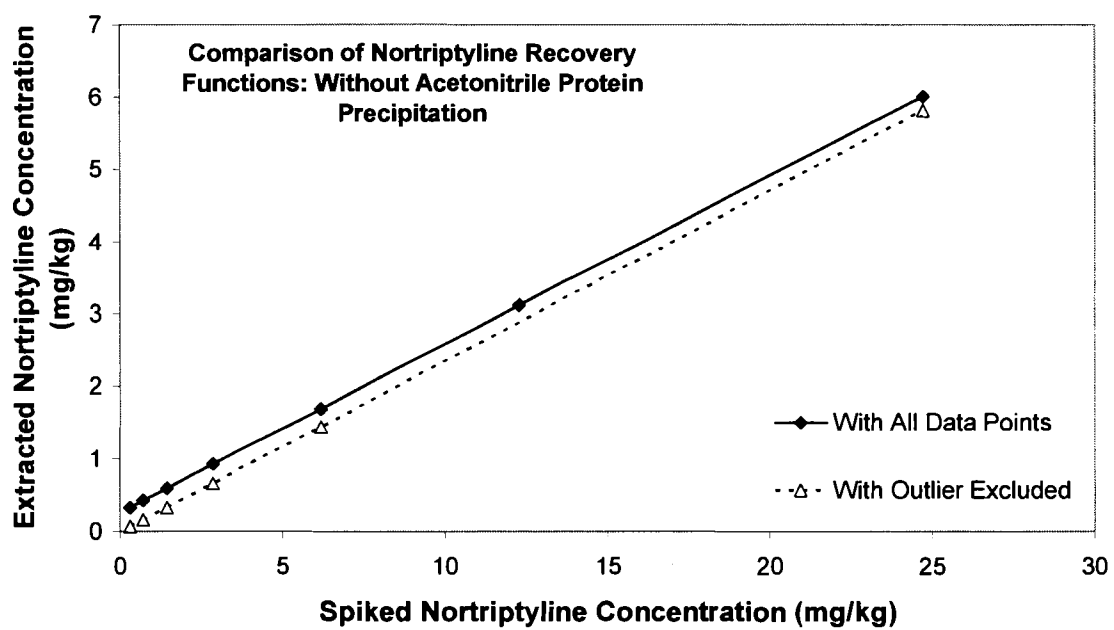


Figure 3.11: Comparison of the nortriptyline recovery function with all data points included to the nortriptyline recovery function with the outlier excluded.

($t_{df=1} = 0.032$; $P = 0.8619$)¹⁰. Given the exceptionally small RSD calculated for the slopes, these results were not unexpected.

There was strong statistical evidence to indicate that the amount of nortriptyline recovered from the foodstuff sample spiked with 12.3 mg/kg nortriptyline was abnormally high. However, according to Meier and Zünd (2000), data should never be removed on the basis of statistical interpretation alone. Meier and Zünd (2000) advocate a more conservative approach, where the results from both the calculations with the outlier and the calculations without the outlier are presented. Interpretation of the data is then accomplished by examining both sets of calculated results. Therefore, Table 3.17 presents the calculated rate of nortriptyline recovery using both recovery functions.

Both recovery functions obtained for the extraction of nortriptyline without prior protein precipitation indicate the presence of both constant and proportional systematic errors. However, the proportional systematic error associated with each of the two curves are considerably different. The proportional systematic error associated with the recovery function calculated for all seven data points, including the outlier was significantly larger than the proportional systematic error associated with the recovery function calculated when the outlier is excluded from the analysis.

The larger proportional systematic error associated with the recovery function calculated from all seven data points artificially inflated the rate of nortriptyline recovery at the smaller spiked concentrations of nortriptyline in the artificial foodstuff (Table 3.17).

In comparison, a constant rate of nortriptyline recovery, albeit much lower, was obtained using the recovery function calculated with the outlier removed (Table 3.17; Figure 3.12). Exclusion of the outlier from the analysis revealed a much more conservative, yet constant, estimate for the rate of nortriptyline recovery. The concentrations of nortriptyline (mg/kg) extracted, with protein precipitation, from each of the artificial foodstuff samples are presented in Table 3.18. Given the complexity of the matrix, the conservative estimate is more appropriate, and indicates that further

¹⁰Comparison of two linear curves using a t -test requires that the two curves be parallel. It was assumed based on Figure 3.11, that the two curves were parallel.

optimization of the sample preparation procedure is required.

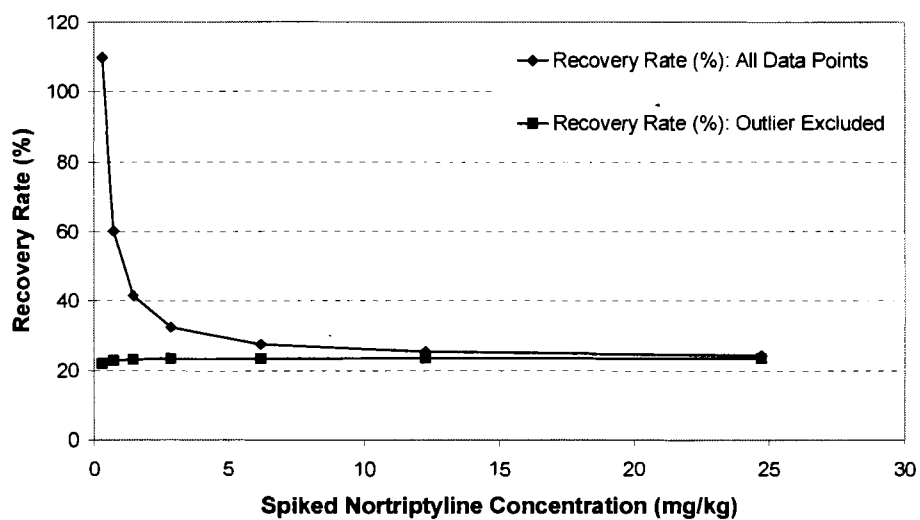


Figure 3.12: Comparison of the nortriptyline recovery rate calculated using both the nortriptyline recovery function generated using all seven data points, and the nortriptyline recovery function generated with the outlier excluded from the analysis.

Spiked Nortriptyline Concentration (mg/kg)	Extracted Nortriptyline Concentration (mg/kg)	Recovery Rate (%) without Acetonitrile Protein Precipitation (Outlier Included)	Recovery Rate (%) without Acetonitrile Protein Precipitation (Outlier Excluded)
0.31	0.07	109.7	22.0
0.72	0.15	60.2	22.9
1.44	0.35	41.7	23.2
2.85	0.58	32.5	23.4
6.18	1.52	27.5	23.5
12.29	3.81	25.4	-
24.72	5.81	24.3	23.5

Table 3.17: Concentration of nortriptyline (mg/kg) extracted without prior protein precipitation from the spiked artificial foodstuff, and the corresponding rate of nortriptyline recovery (%). The recovery rate was calculated using the recovery function, and as a result, the biases introduced during sample preparation and analysis are incorporated into the calculation of the recovery rate. Furthermore, the recovery rate was calculated using both the recovery function with the outlier included, and the recovery function without the outlier included, in order to evaluate how the large difference in the magnitude of the y -intercept affected the calculated recovery rate. The recovery rate was calculated using the recovery function, and as a result, the biases introduced during sample preparation and analysis are incorporated into the calculation of the recovery rate.

Spiked Nortriptyline Concentration (mg/kg)	Mean Extracted \pm SD (with Protein Precipitation) Nortriptyline (mg/kg)
0.31	0.05 \pm 0.002
0.72	0.13 \pm 0.009
1.44	0.34 \pm 0.003
2.85	0.41 \pm 0.004
6.18	1.53 \pm 0.012
12.29	3.66 \pm 0.059
24.72	5.74 \pm 0.071

Table 3.18: Mean extracted \pm SD concentration of nortriptyline (mg/kg). Extracted with protein precipitation from the spiked artificial foodstuff.

Nortriptyline Extraction With Prior Protein Precipitation

The recovery function for the extraction of nortriptyline with prior protein precipitation was established by linear least squares regression, weighted for errors in y , of the nortriptyline recovery data presented in Table 3.17. The nortriptyline recovery function, with prior protein precipitation is illustrated in Figure 3.13). The equation for the nortriptyline recovery function was $y = 0.216x + 0.550$ (95% CI (slope) = 0.166 to 0.267; $t_{df=1} = 11.02$; $P < 0.0001$; $R^2 = 0.960$; Figure 3.13).

Unfortunately, the linearity of the recovery function for the extraction of nortriptyline with acetonitrile protein precipitation was also much lower than expected ($R^2 = 0.960$). In fact, the linearity of the recovery function for the extraction of nortriptyline with prior protein precipitation was even lower than the linearity of the recovery function for the extraction of nortriptyline without prior protein precipitation ($R^2 = 0.973$; Figure 3.8)

Figure 3.13 presents both the least squares regression line calculated for the data presented in Table 3.18, and the mean concentration (\pm SD) of nortriptyline extracted from each of the seven foodstuff samples. As in the nortriptyline extraction without prior precipitation, there appears to be an outlier in the nortriptyline recovery data, specifically at $(x, y) = (12.3 \text{ mg/kg}, 3.66 \text{ mg/kg})$, which appears to have artificially increased the y -intercept of the regression line. Therefore, the proportional systematic error associated with the extraction of nortriptyline with prior protein precipitation was extremely large.

If the apparent outlier is removed from the analysis, the linearity of the nortriptyline recovery function for the extraction with protein precipitation improves remarkably (Figure 3.14). For example, the R^2 value increases to 0.999.

Given the remarkable improvement in the linearity of the nortriptyline recovery function when the apparent outlier was removed, the 95% and 99% confidence intervals for the regression line were investigated.

The amount of nortriptyline extracted from the 12.3 mg/kg nortriptyline spiked foodstuff was outside the 95% confidence region for the regression line, and just inside the upper 99% confidence limit for the regression line (Figure 3.15). Therefore, the

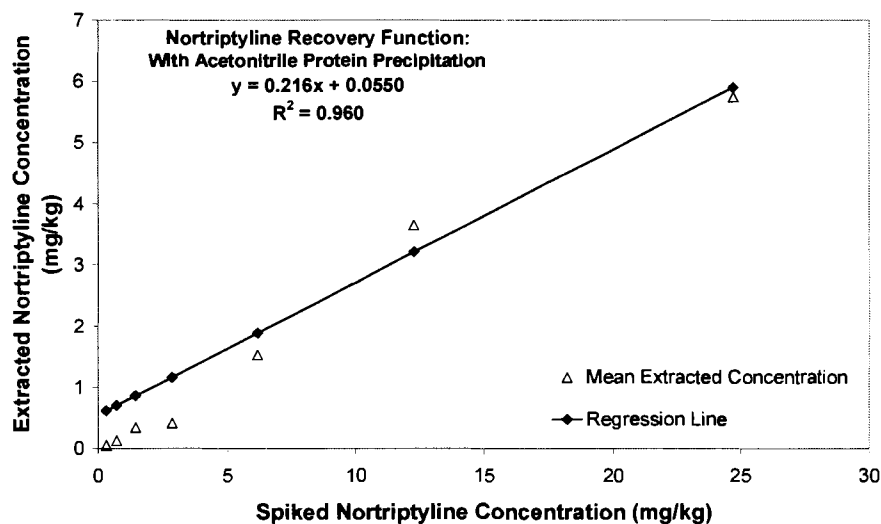


Figure 3.13: Nortriptyline recovery function for the extraction of nortriptyline from the artificial foodstuff with prior protein precipitation with acetonitrile. Both the regression line and the mean extracted concentration of nortriptyline are presented.

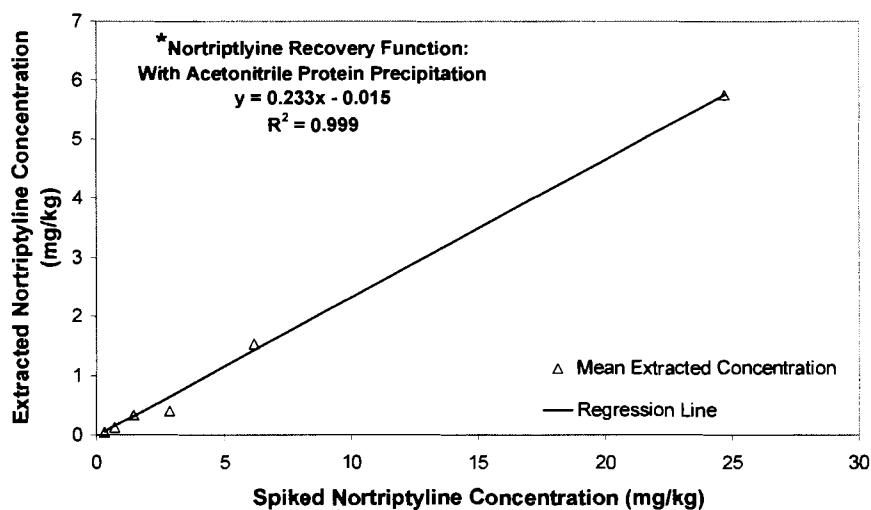


Figure 3.14: Nortriptyline recovery function for the extraction of nortriptyline from the artificial foodstuff with prior acetonitrile protein precipitation. Both the regression line, with the apparent outlier excluded, and the mean extracted concentration ($n = 3$) of nortriptyline are presented.

data point at $(x,y) = (12.29 \text{ mg/kg}, 3.66 \text{ mg/kg})$ is likely an outlier, and will hereby be referred to as the “outlier”.

A second recovery function was then calculated using weighted least squares regression (weighted for errors in y) on the remaining six data points. The relative standard deviation (RSD) for the two curves was calculated in order to estimate the equivalency of the two recovery functions, and the results are presented in Table 3.19.

	Slope	Intercept
Mean \pm SD	0.225 ± 0.012	0.268 ± 0.400
RSD (%)	5.2	149.3

Table 3.19: The mean and relative standard deviation (RSD) for the slopes and intercepts of the two recovery functions calculated with and without the data point classified as an outlier.

The calculated RSD for the slopes, at 5.2%, was higher than expected. Therefore, it appears that when the outlier was removed the slope of the recovery function was altered slightly. The shift in slope is illustrated in Figure 3.16. However, a statistical analysis of the two recovery functions revealed that the curves were not statistically different at an alpha level of 0.05 (Student’s t -test; $t_{df=1} = 0.5854$; $P = 0.4638$)¹¹.

In comparison, the calculated RSD for the y -intercepts of the two recovery functions was exceptionally large. Given the fact that the outlier was located significantly outside the 95% confidence regions, the large RSD was not unexpected.

As mentioned earlier, data should not be excluded based on statistical reasoning alone (Meier and Zünd, 2000) For this reason, the rate of nortriptyline recovery, with prior protein precipitation, was calculated using both recovery functions, and then the results generated using the two curves were compared.

The recovery rate for nortriptyline with prior protein precipitation is presented in Table 3.20. The recovery rate was calculated using both the recovery function for all seven extracted foodstuff samples, and the recovery function with the outlier excluded. The extreme proportional systematic error observed for the recovery function that was generated from all seven data points severely overestimated rate of nortriptyline

¹¹Once again, it was assumed that the two recovery functions were parallel.

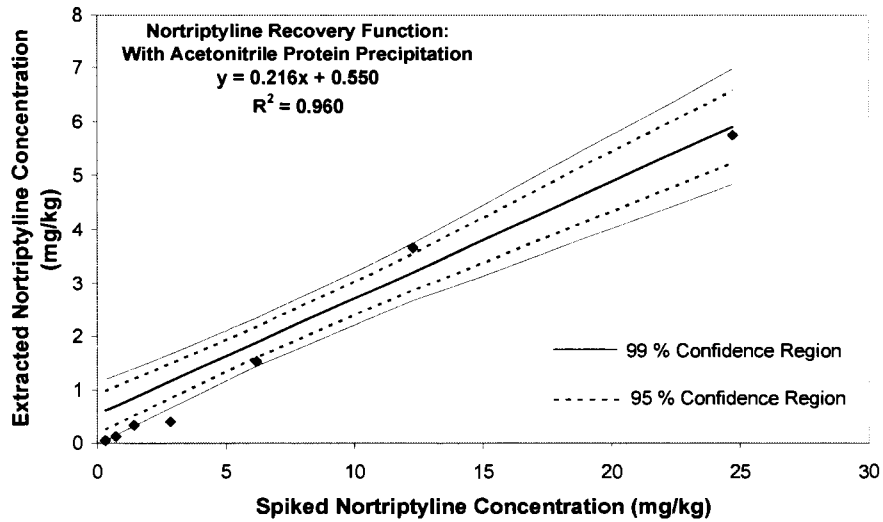


Figure 3.15: The 95% and 99% confidence regions of the nortriptyline recovery function for the extraction of nortriptyline from the artificial foodstuff with prior protein precipitation.

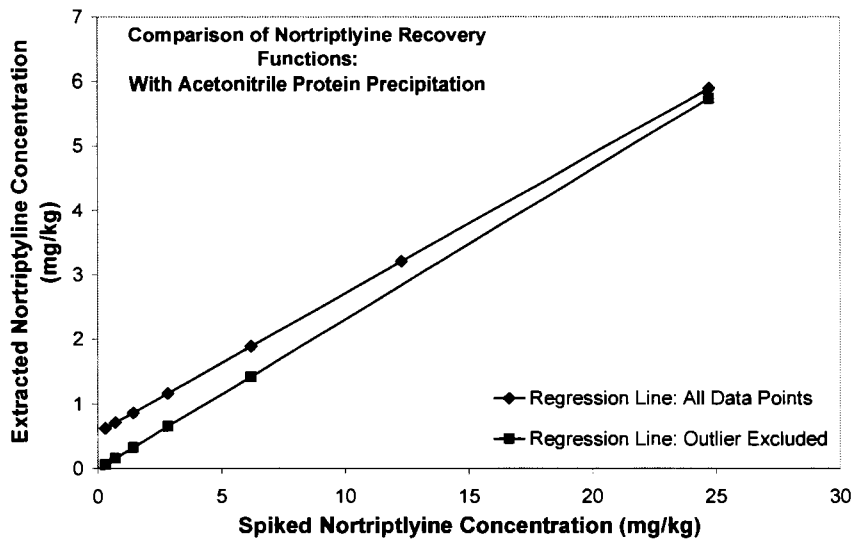


Figure 3.16: Comparison of nortriptyline recovery function with all data points included to the nortriptyline recovery function with the outlier excluded.

recovery, especially for the artificial foodstuff samples containing lower concentrations of nortriptyline. Therefore, there is good evidence, both statistical and empirical, to support the removal of the outlier at $(x,y) = (12.29 \text{ mg/kg}, 3.66 \text{ mg/kg})$. With this in mind, the accepted rate of nortriptyline recovery from an extraction with prior protein precipitation was calculated with the recovery function presented in Figure 3.14. The rate of recovery rate for nortriptyline, calculated using recovery function in Figure 3.14, was much more realistic, albeit extremely low (Table 3.20).

The rate of nortriptyline recovery from artificial foodstuff by extraction, both with prior protein precipitation and without prior protein precipitation are compared in Figure 3.17. As can be seen in Figure 3.17, the rate of nortriptyline recovery is not improved with prior protein precipitation. Based on these results, it appears that the acid digestion is not complete, and some of the nortriptyline remains bound to the proteins, and therefore is removed upon protein precipitation.

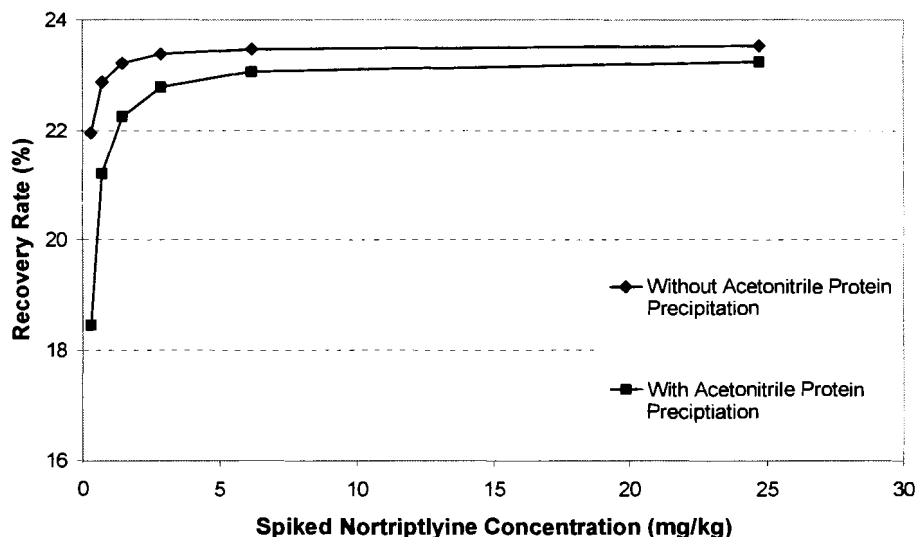


Figure 3.17: Comparison of the calculated nortriptyline recovery rates for the extraction of nortriptyline from artificial foodstuff, both with prior protein precipitation and without prior protein precipitation.

Spiked Nortriptyline Concentration (mg/kg)	Mean Extracted Nortriptyline Concentration (mg/kg)	Recovery Rate (%) without Acetonitrile Protein Precipitation (Outlier Included)	Recovery Rate (%) without Acetonitrile Protein Precipitation (Outlier Excluded)
0.31	0.05	200.4	18.4
0.72	0.13	98.1	21.2
1.44	0.34	59.8	22.3
2.85	0.41	40.9	22.8
6.18	1.53	30.5	23.1
12.29	3.66	26.1	-
24.72	5.74	23.9	23.2

Table 3.20: Mean extracted \pm SD concentration of nortriptyline (mg/kg), and the corresponding rate of nortriptyline recovery (%). Extracted with prior protein precipitation from the spiked artificial foodstuff. The recovery rate was calculated using the recovery function, and as a result, the biases introduced during sample preparation and analysis are incorporated into the calculation of the recovery rate. Furthermore, the recovery rate was calculated using both the recovery function with the outlier included, and without, in order to evaluate how the difference in magnitude of the y -intercept affected the calculated recovery rate.

3.4.5 Discussion

The plot of recovery rate *versus* spiked amitriptyline concentration was not constant¹², and therefore, the calibration curves prepared in a matrix of ethyl acetate were not suitable for the quantitation of amitriptyline in the artificial foodstuff matrix. As a result, the calibration standards must be prepared by spiking drug-free foodstuff homogenate with the appropriate volumes of amitriptyline stock solution, in order to incorporate the matrix effects into the accurate quantitation of amitriptyline in the artificial foodstuff.

Sample preparation and analysis can introduce systematic errors, and these systematic errors may bias the quantitative results. Therefore, if these biases are not accounted for in the calculation of the recovery rate, the recovery rate may be underestimated or overestimated. If no biases are present, the recovery rate could simply be calculated by dividing the extracted concentration by the known (spiked) concentration and multiplying the quotient by 100. Since the slope of the recovery function is not equal to one, a proportional systematic error, resulting from the sample preparation and analysis exists. In addition, since the intercept of the recovery function is not equal to zero, a constant systematic error resulting from the sample preparation and analysis exists as well (Meyer, 1998).

The sample preparation procedure requires considerably more optimization, as revealed by the extremely poor rate of analyte recovery. Unexpectedly, the recovery rate did not increase with prior protein precipitation. This may indicate that the method used to release the bound fraction of the analytes, namely acid digestion, was not appropriate for the artificial foodstuff matrix. Therefore, other digestion methods, such as enzymatic digestion should be investigated for use with this particular matrix. According to Flanagan (1993), proteolytic enzymes often improve rates of analyte recovery. In addition, after enzymatic digestion, the samples can be analyzed using the methods and calibration standards generally reserved for simple matrices such as plasma.

The formation of emulsions during the extraction process was another issue that

¹²i.e. linear

adversely affected the recovery of amitriptyline and nortriptyline in the present thesis. Emulsions formed readily and often when extracting the analytes from the artificial foodstuff matrix. Therefore, future extraction trials should strive to eliminate the lipid material in the sample prior to the actual extraction.

3.5 Accuracy

3.5.1 Introduction

Accuracy can be defined as the closeness of the measured value to the true value (Snyder *et al.*, 1997). Ideally, a method with high accuracy should produce a measured result that is identical, or nearly identical, to the true value. However, a caveat to this definition is that in order to determine the closeness of the measured value to the true value, the true value must first be known.

The accuracy of a method is usually evaluated using some variation of the analyte recovery experiment. There are three variations of the recovery experiment that are commonly used to evaluate method accuracy (Snyder *et al.*, 1997):

1. Comparison of method results to those obtained for a **reference standard**
2. Method of standard addition
3. Spiking blank matrix with the analyte and measuring percent recovery

It is important to be aware of the fact that not every method for evaluating accuracy is suitable for each analytical problem; however, all three methods require analytes that are well-characterized and of established purity (Snyder *et al.*, 1997).

If suitable reference standards are available, comparison to a reference standard would be the preferred method for evaluating accuracy. However, in many cases, the analyte matrix may be too complex, and as a result a suitable reference standard may not be available (Snyder *et al.*, 1997). The present research project is an example of a situation where a certified reference standard was unavailable. Instead, one of the other two methods, or a combination of the three methods, must be used to evaluate the method accuracy.

In cases where it is difficult to either obtain or prepare the matrix without the analyte(s) of interest, the method of standard addition is usually the best choice for evaluating accuracy. This method involves spiking known amounts of the analyte(s), at a number of different levels, into the sample matrix. Unspiked and spiked samples are then put through the sample preparation procedure and analyzed with the chosen

method. After analysis, the concentration of the analyte in the original sample can be determined mathematically by plotting the measured amounts *versus* the amount added (Snyder *et al.*, 1997). The major advantage of this method is that an unmodified sample matrix can be used, even if it already contains some unknown quantity of the analyte of interest (Snyder *et al.*, 1997). This method would have been suitable for the present project, but was not necessary because blank matrix was easily prepared.

If blank sample matrices are readily available, the percent recovery method may be the most practical method for evaluating method accuracy. In this method, a blank matrix is spiked with known amounts of analyte(s) and the amount of analyte(s) recovered, after sample preparation and analysis, is compared to the amount added. Except for one major difference, this method is essentially the same as the method that was used in the previous section to determine the efficiency of the sample preparation procedure (Section 3.4). The major difference lies in the preparation of the calibration standards. In the previous section, the purpose of the recovery experiment was to determine how effective the sample preparation procedure was at isolating the analyte(s) from the matrix. As a result, the calibration standards were prepared in ethyl acetate so that when analyzed, the data would represent the calibration curve of the fundamental analytical procedure¹³. However, as discussed previously, the rate of recovery, for both amitriptyline and nortriptyline, was extremely low, and varied with the concentration of analyte added. Therefore, in order to account for the observed concentration dependence in the extraction efficiency, the calibration standards used to quantitate real samples were prepared in blank artificial foodstuff matrix, and then exposed to the same preparation procedure as the samples themselves.

In the present project, the accuracy of the chosen method was evaluated using a combination of two of the three above methods: (1) percent recovery, and (2) comparison to amitriptyline and nortriptyline “reference” standards.

Since analyte-free artificial foodstuff was relatively easy to prepare, known concentrations of amitriptyline and nortriptyline were added to samples of the blank foodstuff. However, since the amitriptyline and nortriptyline solutions used in the

¹³The instrument response to the analytes in the absence of interferences

present thesis were prepared by the author, and therefore, were not certified reference standards, the accuracy of both their preparation, and their addition to the foodstuff, must be evaluated.

Therefore, two sets of artificial foodstuff were spiked with known amounts of amitriptyline and nortriptyline. The first set was spiked by this researcher, using amitriptyline and nortriptyline solutions that were also prepared by this researcher. The second set of foodstuff was spiked by a research technician at the Provincial Toxicology Centre, using the amitriptyline and nortriptyline **In-House Quality Control** solutions. The samples spiked by the research technician became the reference samples for the accuracy evaluation.

3.5.2 Procedure

Preparation of the Reference Set of Artificial Foodstuff Used in the Accuracy Experiment

The Reference Set of artificial foodstuff samples were prepared in the same manner as the artificial foodstuff samples used in the evaluation of analyte recovery (Section 3.4).

The appropriate amount of each In-House quality control, or methanol, was added to the appropriate liver homogenate sample in the Reference Set by the research technician. The concentration of both the amitriptyline In-House quality control, and the nortriptyline In-House Quality control, was 1 mg/mL. Since the total volume of liquid added to each liver homogenate sample must remain constant, a calculated volume of methanol was added to each sample to ensure that the total volume of liquid added to the liver homogenate was equal to 3.0 mL. The total volumes of 1 mg/mL amitriptyline, 1 mg/mL nortriptyline and methanol added to the samples in the Reference Set is presented in Table 3.21.

The weight of liver homogenate, liquid (drug solution and methanol) and agar used in the preparation of each foodstuff sample in the Reference Set is presented in Table 3.22. In addition, the total weight of each foodstuff sample prepared for the Reference Set is also presented in Table 3.22. Furthermore, the density of methanol is

approximately 0.79 g/mL; therefore, 3 mL of methanol weighs approximately 2.37 g. The presence of amitriptyline and nortriptyline, in this cases, does not appreciably alter the density of methanol.

The concentration of amitriptyline and nortriptyline in each of the foodstuff samples in the Reference Set is presented in Table 3.23. The concentrations of each analyte present in each sample was calculated by dividing the weight of each analyte added (mg) by total weight of each sample (kg).

Preparation of the Experimental Set of Artificial Foodstuff Used in the Accuracy Experiment

The foodstuff samples in the Experimental Set were prepared in the same way, and at the same time, as the foodstuff samples in the Reference Set, with one exception. The amitriptyline and nortriptyline solutions used to spike the artificial foodstuff were prepared by the author. In addition, the foodstuff samples in the Experimental Set were also spiked by the author.

The stock solutions (in methanol) prepared for addition to the foodstuff samples in the Reference Set were as follows:

1. 1 mg/mL amitriptyline
2. 1 mg/mL nortriptyline

Set	Sample	Volume of 1 mg/mL AMT Added (mL)	Volume of 1 mg/mL NOR Added (mL)	Volume of MEOH Added (mL)
Reference	REF-Blank	0	0	3
	REF-A	1.5	0.2	1.3
	REF-B	2.5	0.05	0.45

Table 3.21: Volumes of 1 mg/mL amitriptyline and 1 mg/L nortriptyline In-House Quality Control (in MEOH) added to each liver homogenate sample in the Reference Set of the accuracy experiment (AMT = amitriptyline; NOR = nortriptyline; MEOH = methanol; REF = Reference Set).

The above stock solutions were prepared in methanol, rather than in distilled water, because the In-House quality control stock solutions from the Provincial Toxicology Centre were in methanol.

The volumes of each amitriptyline and nortriptyline stock solution added to each of the foodstuff samples in the Experimental Set are presented in Table 3.24. Since the intent of the accuracy experiment is to compare the quantitation results from the Reference Set with the quantitation results from the Experimental Set, effort was taken to ensure that approximately the same concentration of amitriptyline and nortriptyline were added to the foodstuff samples in each set.

The weight of liver homogenate, liquid (drug solution and methanol) and agar used in the preparation of each foodstuff sample in the Experimental Set is presented in Table 3.25. In addition, the total weight of each foodstuff sample prepared for the Experimental Set is also presented in Table 3.25. As mentioned earlier, the density of methanol is approximately 0.79 g/mL; therefore, 3 mL of methanol weighs approximately 2.37 g. The presence of amitriptyline and nortriptyline, in this cases, does not appreciably alter the density of methanol.

Sample	Weight of Liver Homogenate Added (g)	Weight of Agar Added (g)	*Weight of Liquid Added (MEOH) (g)	Total Weight of Foodstuff (g)
REF-Blank	20.01	25.18	2.37	47.56
REF-A	20.00	25.20	2.37	47.57
REF-B	20.00	26.03	2.37	48.40

Table 3.22: The weight of the liver homogenate (g), agar (g), liquid (g), and the overall weight (g) of each spiked foodstuff sample in the Reference Set that was prepared for the accuracy experiment.

* Note: The density of methanol is approximately 0.79 g/mL; therefore, 3 mL of methanol weighs approximately 2.37 g. It is assumed that the presence of amitriptyline and nortriptyline in methanol does not appreciably alter the overall density of the drug solution.

Sample	Amitriptyline			Nortriptyline		
	Weight of AMT Added (mg)	Weight of Foodstuff (g)	AMT Con- centration (mg/kg)	Weight of NOR Added (mg)	Weight of Foodstuff (kg)	NOR Con- centration (mg/kg)
REF-Blank	0	47.56	0	0	47.56	0
REF-A	1.5	47.57	31.53	0.2	47.57	4.20
REF-B	2.5	48.40	51.65	0.05	48.40	1.03

Table 3.23: The concentration (mg/kg) of amitriptyline and nortriptyline added to each sample of artificial foodstuff in the Reference Set (AMT = amitriptyline; NOR = nortriptyline; REF = Reference Set).

Set	Sample	Volume of 1 mg/mL AMT Added (mL)	Volume of 1 mg/mL NOR Added (mL)	Volume of MEOH Added (mL)
Experimental	EXP-Blank	0	0	3.0
	EXP-A	1.5	0.2	1.3
	EXP-B	2.5	0.05	0.45

Table 3.24: Volumes of 1 mg/mL amitriptyline and 1 mg/L nortriptyline stock solutions (in MEOH) added to each liver homogenate sample in the Experimental Set of the accuracy experiment (AMT = amitriptyline; EXP = Experimental Set; NOR = nortriptyline; MEOH = methanol).

Sample	Weight of Liver Homogenate Added (g)	Weight of Agar Added (g)	*Weight of Liquid added (MEOH) (g)	Total Weight of Foodstuff (g)
EXP-Blank	20.01	25.67	2.37	48.05
EXP-A	20.04	25.25	2.37	47.56
EXP-B	20.01	26.53	2.37	48.91

Table 3.25: The weight of the liver homogenate (g), agar (g), liquid (g), and the overall weight (g) of each spiked foodstuff sample in the Experimental Set prepared for the accuracy experiment (MEOH = methanol).

* Note: The density of methanol is approximately 0.79 g/mL; therefore, 3 mL of methanol weighs approximately 2.37 g. It is assumed that the presence of amitriptyline and nortriptyline in methanol does not appreciably alter the overall density of the drug solution.

Sample	Amitriptyline			Nortriptyline		
	Weight of AMT Added (mg)	Weight of Foodstuff (g)	AMT Con- centration (mg/kg)	Weight of NOR Added (mg)	Weight of Foodstuff (g)	NOR Con- centration (mg/kg)
EXP-Blank	0	48.05	0	0	48.05	0
EXP-A	1.5	47.56	31.54	0.2	47.56	4.20
EXP-B	2.5	48.91	51.11	0.05	48.91	1.02

Table 3.26: The concentration (mg/kg) of amitriptyline and nortriptyline added to each sample of artificial foodstuff in the Experimental Set (AMT = amitriptyline; EXP = Experimental Set; NOR = nortriptyline).

Extraction of Amitriptyline and Nortriptyline from the Reference and Experimental Sets of Artificial Foodstuff

The artificial foodstuff samples of both the Reference Set and the Experimental Set were extracted according to the procedure outlined previously (Section 3.3).

Preparation of the Calibration Standards for the Accuracy Experiment

The calibration standards required for the accuracy experiment were prepared from the following stock amitriptyline and nortriptyline solutions (in methanol):

1. 0.1 mg/mL amitriptyline + 0.1 mg/mL nortriptyline
2. 1 mg/mL amitriptyline + 1 mg/mL nortriptyline

The 0.1 mg/mL stock solution was prepared by a 1:10 dilution (in methanol) of the mixed 1 mg/mL amitriptyline and nortriptyline stock solution.

Eight calibration standards were prepared by adding 1 mL of a 1:1 blank foodstuff homogenate to each test tube, followed by the appropriate amount of each mixed amitriptyline and nortriptyline stock solution, 25 μ L of 1 mg/ml maprotyline (internal standard), and then the contents of each test tube was vortex mixed. The target amitriptyline and nortriptyline concentrations for each of the ten calibration standards samples were as follows:

1. 0 mg/L amitriptyline + 0 mg/L nortriptyline
2. 1 mg/L amitriptyline + 1 mg/L nortriptyline
3. 2.5 mg/L amitriptyline + 2.5 mg/L nortriptyline
4. 5 mg/L amitriptyline + 5 mg/L nortriptyline
5. 10 mg/L amitriptyline + 10 mg/L nortriptyline
6. 25 mg/L amitriptyline + 25 mg/L nortriptyline
7. 50 mg/L amitriptyline + 50 mg/L nortriptyline

8. 100 mg/L amitriptyline + 100 mg/L nortriptyline

The volumes of amitriptyline, nortriptyline and maprotyline stock solutions used to prepare the calibration standards required for the accuracy experiment are presented in Table 3.27. The prepared calibration standards were then extracted alongside the foodstuff samples using the sample preparation procedure previously described.

Target Concentration (mg/L)	Volume (μ L) of MIXED 0.1 mg/mL AMT and 0.1 mg/mL NOR Stock Solution	Volume (μ L) of MIXED 1 mg/mL AMT and 1 mg/mL NOR Stock Solution	Volume (μ L) of 1 mg/mL MAP Stock Solution
0	0	-	25
1	10	-	25
2.5	25	-	25
5	50	-	25
10	100	-	25
25	-	25	25
50	-	50	25
100	-	150	25

Table 3.27: Volume of amitriptyline, nortriptyline and maprotyline stock solutions used to prepare the calibration standards required for the accuracy experiment. The calibration standards were prepared in 1 mL of 1:1 foodstuff homogenate (AMT = amitriptyline; NOR = nortriptyline; MAP = maprotyline).

Amitriptyline and Nortriptyline Quantitation

The levels of amitriptyline and nortriptyline present in the foodstuff samples from both the Experimental and Reference Sets were quantitated using the chromatographic method described in Appendix A.

The percent difference between the spiked concentrations and the measured concentrations of each analyte, for both the Experimental Set and the Reference Set was calculated using the following formula:

$$\% \text{ difference} = \frac{\text{mean measured concentration} - \text{spiked concentration}}{\text{spiked concentration}} \times 100\%$$

A negative value for the percent difference indicates that the mean measured value was less than that of the actual (spiked) value. In contrast, a positive value for the percent difference indicates that the mean measured value was greater than that of the actual (spiked) value.

3.5.3 Accuracy Results

Evaluation of Method Accuracy for Amitriptyline

The calibration data for amitriptyline, generated by triplicate injections of each of the eight calibration standards are presented in Table 3.28.

The calibration curve for amitriptyline, generated from the data presented in Table 3.28, is illustrated in Figure 3.18. Least squares linear regression, weighted for errors in y , was performed on the calibration data. The calibration data was generated from triplicate injections of each calibration standard. The equation for the calibration curve was $y = 0.072x + 0.400$ (95% CI (slope) = 0.069 to 0.076; $t_{df=1} = 50.91$; $P < 0.0001$; $R^2 = 0.998$; Figure 3.28).

However, this calibration equation was problematic due to the large y -intercept. The 95% confidence interval y -intercept of the calibration curve was 0.142 to 0.659. Therefore, a linear fit may not provide the best mathematical representation of the amitriptyline calibration data because the 95% confidence interval for the y -intercept did not contain zero. Clearly, if no analyte or interfering material is present, and if

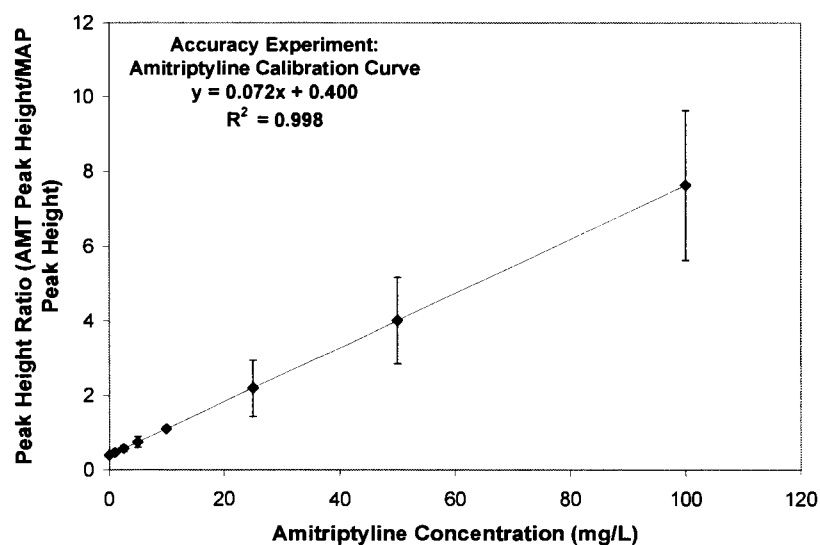


Figure 3.18: Amitriptyline calibration curve for the accuracy experiment. Linear least squares regression was conducted on the calibration data. The errors bars represent the standard deviation associated with each signal, multiplied by a factor of 20 so that most of the error bars were visible. Each calibration standard was prepared in a matrix of blank artificial foodstuff homogenate and the extracted with chlorobutane by liquid-liquid extraction (AMT = amitriptyline; MAP = maprotyline).

baseline noise is neglected, the detector response should be zero, and as a result, the y -intercept should equal zero.

Further examination of the amitriptyline calibration data revealed that if the data for the calibration standard with the largest amitriptyline concentration was excluded from the fit, the confidence interval of the y -intercept became -0.076 to 0.416, and therefore contained zero. In addition, the exclusion of the largest amitriptyline calibration standard from the analysis increased the R^2 value from 0.997 to 0.999.

However, signal data from calibration standards should not simply be dropped from the analysis, especially when the expected range for the analyte would no longer be bracketed by the calibration data. Therefore, rather than eliminating any data points, several different mathematical fits of the data, such as a **quadratic fit**, were investigated.

The calibration curve for amitriptyline, generated using a centered, second order polynomial (quadratic) fit, weighted for errors in y , of the calibration data for amitriptyline is presented in Figure 3.19. The equation for the quadratic calibration curve was $y = -0.0001x^2 + 0.0887x + 0.0706$. The results of the second order polynomial regression are presented in Table 3.29.

The results for the quantitation of amitriptyline from the spiked foodstuff samples in both the Reference Set and Experimental Set of the accuracy experiment are presented in Table 3.31. The mean percent difference between the mean measured concentration and spiked concentrations are presented in Table 3.31 as well.

The accuracy of the method was adequate for amitriptyline. The percent difference between the spiked concentrations and the measured concentrations for the Reference Set were less than 5%. For both REF-A and REF-B the measured concentrations were less than the spiked concentrations. The percent difference between the spiked concentrations and the measured concentrations for the Experimental Set were less than 4.0%. For the sample EXP-A the measured concentration was greater than the spiked concentration and for EXP-B, the measured concentration was less than the spiked concentration.

Concentration of Amitriptyline Calibration Standard (mg/L)	Mean Peak Height Ratio (AMT Peak Height/MAP Peak Height)	Relative Standard Deviation (%)
0 ($n = 3$)	0.001 ± 0.0003	22.8
1 ($n = 3$)	0.081 ± 0.0023	2.8
2.5 ($n = 3$)	0.245 ± 0.0038	1.6
5 ($n = 3$)	0.447 ± 0.0075	1.7
10 ($n = 3$)	0.745 ± 0.0029	0.4
25 ($n = 3$)	2.287 ± 0.0377	1.6
50 ($n = 3$)	4.133 ± 0.0577	1.4
100 ($n = 3$)	7.598 ± 0.1000	1.3

Table 3.28: The mean (\pm SD) peak height ratio (amitriptyline peak height/maprotyline peak height ratio), and the relative standard deviation (%) for the data generated from triplicate injections of each amitriptyline calibration standard. Each calibration standard was prepared in a matrix of blank artificial foodstuff homogenate and the extracted with chlorobutane by liquid-liquid extraction.

Variable	Confidence Interval	t -Ratio	Prob $> t $	R^2
y -intercept	0.4736 to 0.8198	9.60	0.0002	1.0
x	0.0693 to 0.0728	104.95	< 0.0001	1.0
x^2	-0.0002 to -0.0001	-5.07	0.0039	1.0

Table 3.29: Results of the centered second-order polynomial regression (quadratic) analysis, weighted for errors in y , of the calibration data for amitriptyline. The equation of the regression line was $y = -0.00001x^2 + 0.0889x + 0.0706$, where $x =$ amitriptyline concentration (mg/L).

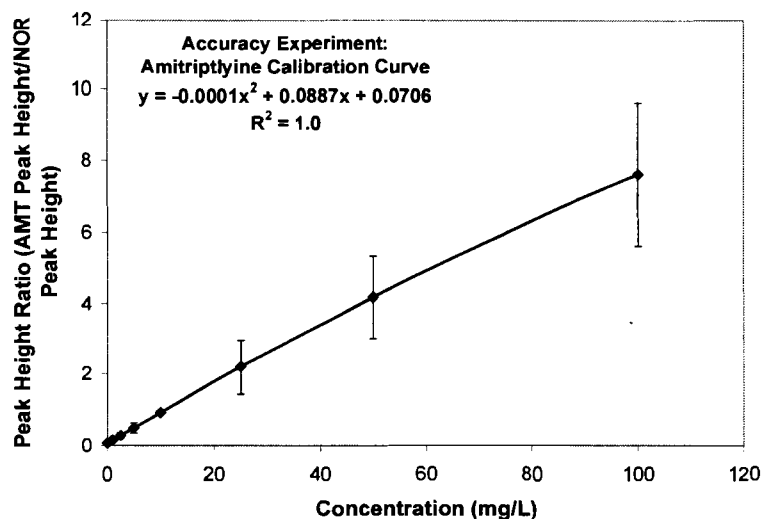


Figure 3.19: Amitriptyline calibration curve for the accuracy experiment. Second-order polynomial least squares regression, weighted for errors in y , was conducted on the calibration data. Each calibration standard was prepared in a matrix of blank artificial foodstuff homogenate and the extracted with chlorobutane by liquid-liquid extraction. The errors bars represent the standard deviation associated with each signal, multiplied by a factor of +20 so that most of the error bars were visible (AMT = amitriptyline; MAP = maprotyline).

Set	Sample	Mean (\pm SD) Peak Height Ratio (AMT Peak Height/MAP Peak Height)	Relative Standard Deviation (%)
Reference	REF-A	1.224 ± 0.026	2.1
	REF-B	2.046 ± 0.082	4.0
Experimental	EXP-A	1.220 ± 0.032	2.6
	EXP-B	1.980 ± 0.103	5.2

Table 3.30: The mean (\pm SD) peak height ratio (amitriptyline peak height/maprotyline peak height) for triplicate injections of each artificial foodstuff extract, from both the Reference Set of spiked foodstuff samples and the Experimental Set of spiked foodstuff samples. Each sample of artificial foodstuff was extracted using chlorobutane by liquid-liquid extraction (AMT = amitriptyline; MAP = maprotyline).

Set	Sample	Spiked Amitriptyline Concentration (mg/kg)	Measured Amitriptyline Concentration (mg/kg)		Mean Percent Difference (%)
			Mean \pm SD	RSD (%)	
Reference	REF-A	31.53	30.18 \pm 0.68	2.3	-4.3
	REF-B	51.65	50.12 \pm 2.16	4.3	-3.0
Experimental	EXP-A	31.54	32.56 \pm 0.92	2.8	3.2
	EXP-B	51.11	50.99 \pm 2.85	5.6	0.2

Table 3.31: Comparison of the measured (\pm SD) amitriptyline concentration with the spiked concentration for the foodstuff samples from both the Reference Set and the Experimental Set of the accuracy experiment. The percent difference was calculated using the mean measured value for each sample. A negative percent difference indicates that the measured concentration was less than the spiked concentration.

Evaluation of the Method Accuracy for Nortriptyline

The calibration data for nortriptyline, generated by triplicate injections of each calibration standard are presented in Table 3.32. Least squares linear regression, weighted for errors in y , was performed on the calibration data presented in Table 3.32. The equation for the calibration curve was $y = 0.057x + 0.304$ (95% CI (slope) = 0.054 to 0.061; $t_{df=1} = 42.40$; $P < 0.0001$; $R^2 = 0.997$). The nortriptyline calibration curve generated by the data presented in Table 3.32 is presented in Figure 3.20.

Concentration of Nortriptyline Calibration Standard (mg/L)	Mean Peak Height Ratio (NOR Peak Height/MAP Peak Height)	Relative Standard Deviation (%)
0 ($n = 3$)	0.001 ± 0.0004	36.9
1 ($n = 3$)	0.059 ± 0.0012	2.0
2.5 ($n = 3$)	0.181 ± 0.0021	1.2
5 ($n = 3$)	0.339 ± 0.0038	1.1
10 ($n = 3$)	0.586 ± 0.0024	0.4
25 ($n = 3$)	1.727 ± 0.0162	0.9
50 ($n = 3$)	3.360 ± 0.0278	0.8
100 ($n = 3$)	6.004 ± 0.0995	1.7

Table 3.32: The mean (\pm SD) peak height ratio (nortriptyline peak height/maprotyline peak height ratio), and the relative standard deviation (%) for the data generated from triplicate injections of each nortriptyline calibration standard. Each calibration standard was prepared in a matrix of blank artificial foodstuff homogenate and the extracted with chlorobutane by liquid-liquid extraction (NOR = nortriptyline; MAP = maprotyline).

However, this calibration equation was problematic due to the large y -intercept, and as a result, the peak height ratios for the REF-A, REF-B, EXP-A and EXP-B samples were too small to be quantitated using the calibration curve presented in Figure 3.20 (Table 3.33).

The 95% confidence interval y -intercept of the calibration curve was 0.028 to 0.581. Since the level of nortriptyline in the foodstuff samples from both the Reference Set

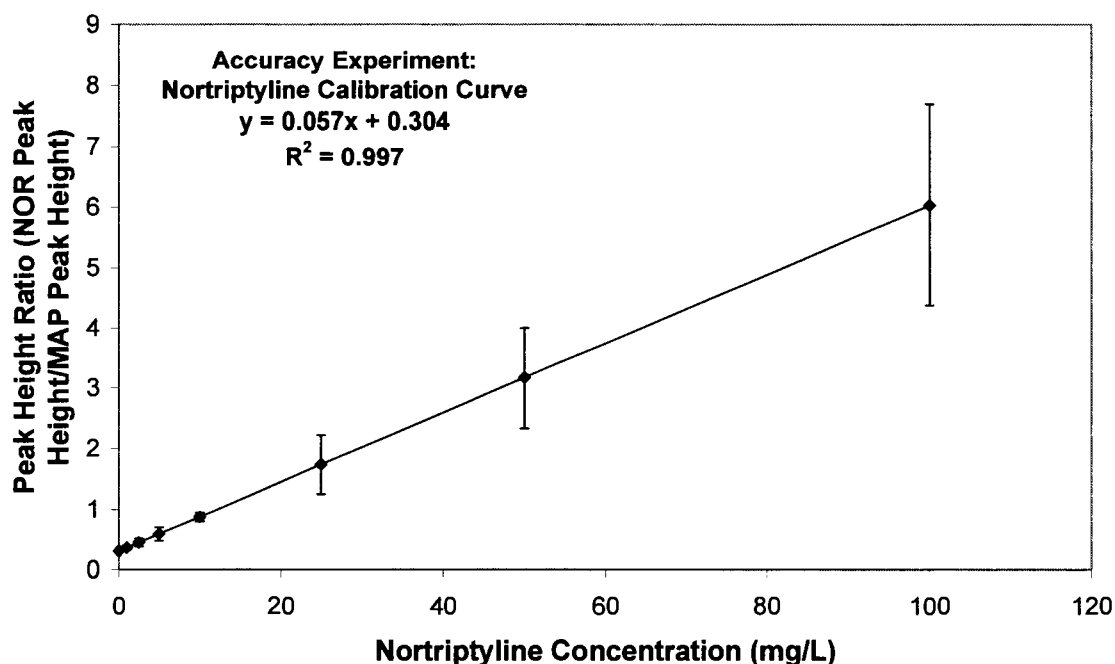


Figure 3.20: Nortriptyline calibration curve for the accuracy experiment. Linear least squares regression was conducted on the calibration data. The errors bars represent the standard deviation associated with triplicate determinations of each calibration standard, multiplied by a factor of +30 to make the majority of the error bars visible. Each calibration standard was prepared in a matrix of blank artificial foodstuff homogenate and the extracted with chlorobutane by liquid-liquid extraction. NOR = Nortriptyline; MAP = maprotyline.

Set	Sample	Mean (\pm SD) Peak Height Ratio	Relative Standard Deviation (%)
Reference	REF-A	0.1276 ± 0.0011	0.9
	REF-B	0.0450 ± 0.0014	3.1
Experimental	EXP-A	0.1166 ± 0.0085	7.2
	EXP-B	0.0402 ± 0.0044	10.9

Table 3.33: The mean (\pm SD) nortriptyline peak height (pA), the mean (\pm SD) maprotyline peak height (pA), and the mean peak height ratio (nortriptyline peak height/maprotyline peak height) for each foodstuff sample in both the Reference Set and the Experimental Set of the accuracy experiment.

and the Experimental Set is expected to be considerably lower than 50 mg/L¹⁴, a second nortriptyline calibration curve was constructed, this time using only seven of the eight calibration standards. The second nortriptyline calibration curve was constructed using linear least squares regression, weighted for errors in y , on the signal data generated for the 0 mg/L, 1 mg/L, 2.5 mg/L, 5 mg/L, 10 mg/L, 25 mg/L and 50 mg/L nortriptyline calibration standards. The calibration curve generated from the seven calibration standards listed previously, using weighted least squares regression (weighted for errors in y), is presented in Figure 3.21. The equation for the second nortriptyline calibration curve is $y = 0.067x + 0.017$ (95% CI (slope) = 0.065 to 0.069; $t_{df=1} = 90.27$; $P < 0.0001$; $R^2 = 0.999$; Figure 3.21).

Furthermore, when the data for the calibration standard with the greatest concentration of nortriptyline (100 mg/L) was excluded from the analysis, the confidence interval for the y -intercept became -0.056 to 0.090, and therefore contained zero. In addition, the magnitude of the R^2 value increased from 0.997 to 0.999 when the data point for the 100 mg/L nortriptyline standard was removed from the analysis.

However, signal data from calibration standards should not simply be dropped from the analysis, especially in the absence of statistical and practical evidence to support the claim that the data point(s) are outliers. Therefore, second-order centered polynomial regression analysis of the nortriptyline calibration data was also investigated. The calibration equation, generated from all eight nortriptyline calibration standards using second-order polynomial regression analysis is presented in Figure 3.22.

Given the higher quality of the second-order (quadratic) polynomial fit of all eight nortriptyline calibration standards ($R^2 = 0.999$), compared to the linear fit of all eight calibration standards ($R^2 = 0.996$), it appears that that detector was exhibiting a sub-linear response. Based on the data presented above, the linear range of the detector

¹⁴The density of healthy human liver is approximately 1.05 g/mL (ICRP, 2002), and the specific gravity of water is 1.0 mg/L. It was assumed that the density of healthy beef liver is approximately equal to the density of healthy human liver. The specific gravity of the artificial foodstuff, determined by the volume of water displaced using a piece of artificial foodstuff of known weight, was approximately 0.98 mg/L ($n = 1$). Therefore, for the purposes of this research project, the specific density of the artificial foodstuff was approximated to be 1.0 g/L.

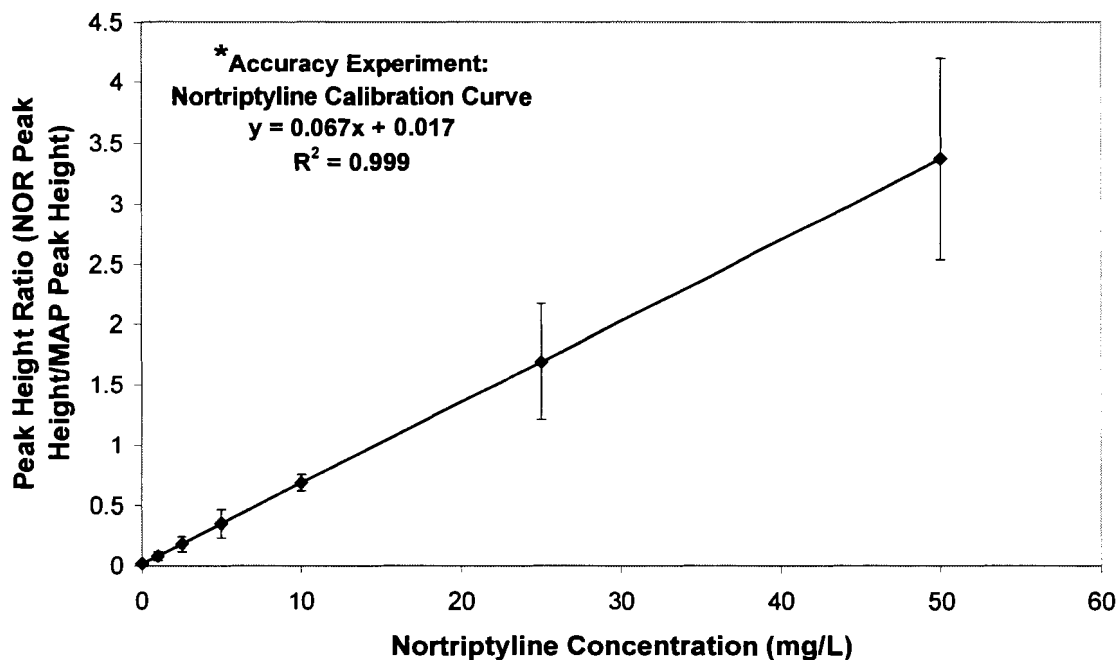


Figure 3.21: Nortriptyline calibration curve for the accuracy experiment, constructed from the signal data generated from the following seven nortriptyline calibration standards: 0 mg/L, 1 mg/L, 2.5 mg/L, 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L. The error bars represent the standard deviation associated with triplicate determinations of each calibration standard, multiplied by a factor of +30 to make the majority of the error bars visible. Each calibration standard was prepared in a matrix of blank artificial foodstuff homogenate and extracted with chlorobutane by liquid-liquid extraction (NOR = nortriptyline; MAP = maprotyline).

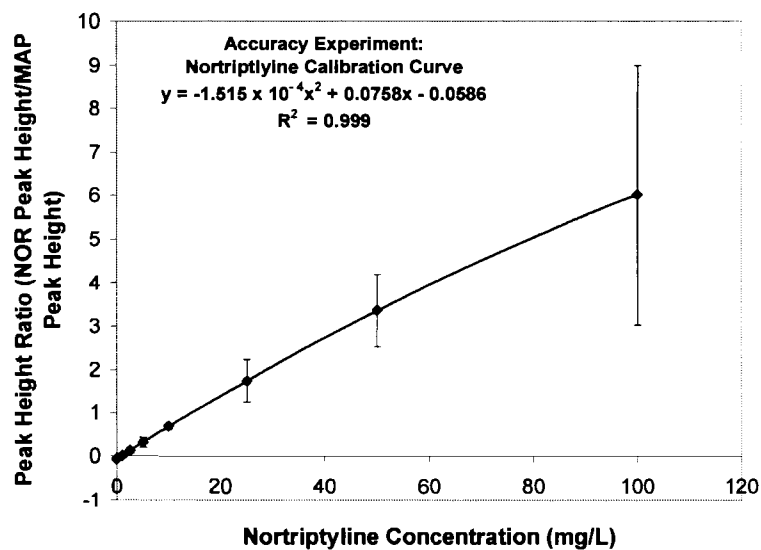


Figure 3.22: Nortriptyline calibration curve for the accuracy experiment, generated by second-order centered polynomial regression analysis of the signal data generated from all eight nortriptyline calibration standards: 0 mg/L, 1 mg/L, 2.5 mg/L, 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L, 100 mg/L. The errors bars represent the standard deviation associated with triplicate determinations of each calibration standard, multiplied by a factor of +30 to make the majority of the error bars visible. Each calibration standard was prepared in a matrix of blank artificial foodstuff homogenate and the extracted with chlorobutane by liquid-liquid extraction (NOR = nortriptyline; MAP = maprotyline).

for nortriptyline, for this present method, extends from approximately 0 mg/L to somewhere between 50 and 100 mg/L. Therefore, the signal data generated from the seven nortriptyline calibration standards, 0 mg/L, 1 mg/L, 2.5 mg/L, 5 mg/L, 10 mg/L, 25 mg/L, and 50 mg/L, were used to construct the calibration equation used to quantitate the amount of nortriptyline present in each of the foodstuff samples. Furthermore, the 0 mg/L to 50 mg/L calibration range more appropriately bracketed the expected nortriptyline concentration range than did the 0 mg/L to 100 mg/L calibration range.

Since linear fits are desired for analytical work because of their simplicity and amenability to statistical analysis, the 0 mg/L to 50 mg/L linear fit and the 0 mg/L to 100 mg/L quadratic fit were graphically compared in Figure 3.23. As can be seen in Figure 3.23, the linear fit for the 0 mg/L to 50 mg/L calibration data was a good approximation of the 0 mg/L to 50 mg/L region of the quadratic fit for the same nortriptyline calibration data. With this in mind, the amount of nortriptyline present in each of the artificial foodstuff samples in both the Reference Set and the Experimental Set, were quantitated using the calibration equation presented in Figure 3.21.

The results for the quantitation of nortriptyline from the spiked foodstuff samples in both the Reference Set and Experimental Set of the accuracy experiment are presented in Table 3.34. The percent difference, between the mean measured concentrations and the spiked concentrations for nortriptyline are also presented in Table 3.34.

The accuracy of the method was very poor for nortriptyline. The percent difference between the spiked concentrations and the measured concentrations for the Reference Set for REF-A was 11.0% and for REF-B 12.7%. In addition, measured concentrations for both REF-A and REF-B were lower than the spiked concentrations. The percent difference between the spiked concentrations and the measured concentrations for the EXP-A was 12.9% and for EXP-B it was 23.5%. In addition, the measured concentrations for both EXP-A and EXP-B were less than the spiked concentrations.

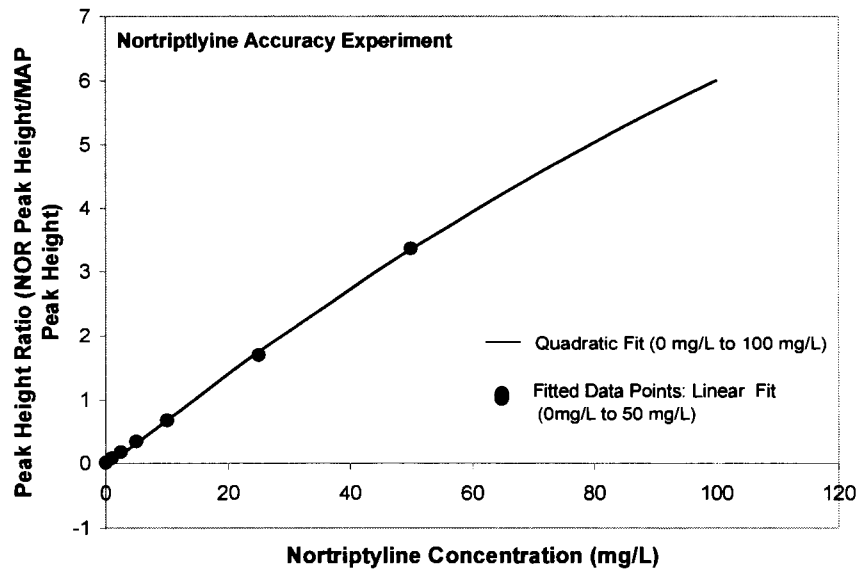


Figure 3.23: Second-order (quadratic) polynomial regression analysis of the signal data generated by triplicate injections of all eight of the nortriptyline calibration standards: 0 mg/L, 1 mg/L, 2.5 mg/L, 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L, 100 mg/L. The triangles represent the fitted data points for the least squares linear regression analysis of seven of the nortriptyline calibration standards: 0 mg/L, 1 mg/L, 2.5 mg/L, 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L. Based on the data presented above, the linear fit for the 0 mg/L to 50 mg/L is a good approximation of the 0 mg/L to 50 mg/L region of the quadratic fit of the same nortriptyline calibration data (NOR = nortriptyline; MAP = maprotyline).

Set	Sample	Spiked Nortriptyline Concentration (mg/kg)	Measured Nortriptyline Concentration (mg/kg)		Mean Percent Difference (%)
			Mean \pm SD	RSD (%)	
Reference	REF-A	4.20	3.74 \pm 0.04	1.0	-11.0
	REF-B	1.02	0.89 \pm 0.05	5.1	-12.7
Experimental	EXP-A	4.19	3.65 \pm 0.31	8.5	-12.9
	EXP-B	1.02	0.78 \pm 0.15	19.1	-23.5

Table 3.34: Comparison of the measured (\pm SD) nortriptyline concentration with the spiked concentration for the foodstuff samples from both the Reference Set and the Experimental Set of the accuracy experiment. The percent difference was calculated using the mean measured value for each sample. A negative percent difference indicates that the measured concentration was less than the spiked concentration.

3.5.4 Accuracy Discussion

A non-linear detector response was observed at high concentrations of both amitriptyline and nortriptyline when extracted from an artificial foodstuff matrix. The concentration of amitriptyline expected for the artificial foodstuff ranged from 0 mg/L to 200 mg/L. Unfortunately, the linear range of the detector appears to have ranged from 0 mg/L to somewhere between 50 mg/L and 100 mg/L. Therefore, using a linear fit for the amitriptyline and nortriptyline calibration curves would underestimate, at relatively high concentrations¹⁵, the levels of amitriptyline and nortriptyline present. As a result, the sample preparation procedure employed in the present research project requires further optimization.

In an ideal situation, when a non-linear response is observed, the sample extracts can be diluted so that the signal response would then fit within the linear range of the detector. However, this technique is often not feasible because it would decrease the sensitivity of the system to small analyte concentrations. An alternate solution in this case would be to perform a transformation of the data for the control variable that would allow a linear fit to be performed over the entire concentration range. Unfortunately, the appropriate transformation could not be found for the present project.

Therefore, an alternative fit of the amitriptyline calibration data, namely a quadratic fit, was used to quantitate the level of amitriptyline present in the artificial foodstuff samples prepared for the accuracy experiment. Unfortunately, polynomial fits make curve comparisons difficult. As a result, curve comparisons were conducted using a linear fit over the restricted range¹⁶.

The short linear range of the detector did not cause a problem for the quantitation of nortriptyline, because the highest concentration of nortriptyline expected in the artificial foodstuff was less than 20 mg/L¹⁷. Since a linear fit is so important to analytical work because of its amenability to statistical analysis, the nortriptyline

¹⁵Approximately greater than 50 mg/L.

¹⁶0 mg/L to 50 mg/L

¹⁷Recall that for the purposes of this experiment that the specific density of the artificial foodstuff is approximately 1.0 g/L.

calibration curve was limited to 50 mg/L, the approximate linear range of the detector. Given the expected concentrations of nortriptyline in the artificial foodstuff, a linear fit of the 0 mg/L to 50 mg/L calibration data was appropriate for the quantitation of nortriptyline in the present thesis.

The accuracy of the method was quite good for amitriptyline, as the percent difference between the measured and spiked concentrations, for both the Reference Set and the Experimental Set were less than 5%. However, the accuracy for nortriptyline was surprisingly poor, and as a result, the method requires further optimization. Overall, based on the negative percent differences obtained for all four nortriptyline samples (two from the Reference Set and two from the Experimental Set), it appears that the method seriously underestimates the level of nortriptyline present. The percent difference associated with EXP-B samples was considerably greater than the percent difference associated with the REF-B sample. This indicates the presence of an error in the spiking of the EXP-B foodstuff sample.

In addition, relatively large relative standard deviations were observed with the measured concentrations of nortriptyline, and therefore, the precision of the method was low as well. The largest relative standard deviations were associated with the two samples from the Experimental Set. This indicates an error in the sample preparation procedure, most likely in the extraction of the analytes from the matrix. One option, albeit time consuming, for increasing the accuracy is to average the results obtained from multiple analyses¹⁸ (Tipler, 1993).

In conclusion, the results of the accuracy experiment were acceptable for amitriptyline, but less than satisfactory for nortriptyline. Given more time, this researcher would have worked to improve the accuracy of the method by further optimizing the sample preparation procedure. Overall, the level of amitriptyline added to the artificial foodstuff can be quantitated with a great deal of confidence. On the other hand the level of nortriptyline added to the artificial foodstuff can be quantitated using this method; however, the results should be accepted cautiously.

¹⁸i.e. replicate injections

3.6 LOD and LOQ of Amitriptyline and Nortriptyline in the Artificial Foodstuff Matrix

3.6.1 Introduction

A procedure similar to the one used to determine limit of detection (LOD) and the limit of quantitation (LOQ) in the matrix of ethyl acetate (Chapter 2) was used to determine the LOD and LOQ for amitriptyline and nortriptyline in the artificial foodstuff matrix. However, the calibration standards used in the artificial foodstuff LOD and LOQ determination, were prepared in artificial foodstuff rather than in ethyl acetate.

3.6.2 LOD and LOQ Procedure

Six different samples of artificial foodstuff were prepared for the LOD and LOQ experiment, each with a different concentration of amitriptyline and nortriptyline. The target amitriptyline and nortriptyline concentrations for the foodstuff samples were as follows:

1. 0 mg/kg amitriptyline + 0 mg/kg nortriptyline (blank)
2. 0.1 mg/kg amitriptyline + 0.1 mg/kg nortriptyline
3. 0.2 mg/kg amitriptyline + 0.2 mg/kg nortriptyline
4. 0.4 mg/kg amitriptyline + 0.4 mg/kg nortriptyline
5. 0.6 mg/kg amitriptyline + 0.8 mg/kg nortriptyline
6. 0.8 mg/kg amitriptyline + 0.8 mg/kg nortriptyline

The foodstuff samples listed above were prepared according to the procedure previously outlined, and will be used as the calibration standards for the amitriptyline and nortriptyline LOD and LOQ determination in the artificial foodstuff matrix. The

volume of mixed amitriptyline and nortriptyline stock solution added to each of the six samples of liver-egg homogenate are presented in Table 3.35.

Approximate Target Concentration (mg/kg)	Volume of Mixed (1 mg/L AMT + 1 mg/L NOR) Stock Solution added (μL)	Volume of Distilled Water Added (μL)
0	0	100
0.1	5	95
0.2	10	90
0.4	20	80
0.6	30	70
0.8	40	60

Table 3.35: The volume of mixed stock solution (1 mg/mL amitriptyline + 1 mg/L Nortriptyline), prepared in distilled water, added to each of six 20 g samples of blank liver homogenate. The specified volume of distilled water was added to each sample in order to ensure that an equal volume of liquid was added to each sample.

The weight of liver-egg homogenate, agar and the overall weight of each foodstuff sample is presented in (Table 3.36). The total volume of liquid¹⁹ was only 100 μL , and therefore the impact of this small volume (approximately 0.2% of the total sample weight) on the total sample weight is negligible, because the volume represents approximately 0.2% of the total sample weight.

The final concentration of amitriptyline and nortriptyline in the six spiked foodstuff samples are shown in Table 3.37. The final concentration of amitriptyline and nortriptyline present in each sample of spiked foodstuff was calculated by dividing the amount of drug added (in mg) by the total weight of foodstuff prepared (in kg).

In addition, a second drug-free sample of artificial foodstuff was prepared, in the same manner as the 0 mg/kg amitriptyline + 0 mg/kg nortriptyline foodstuff calibration standard. Once the six foodstuff calibration standards, and the drug-free foodstuff sample were prepared they were extracted according to the sample preparation procedure outlined in Section 3.4.2²⁰.

¹⁹ Aqueous drug solution and distilled water.

²⁰ Twenty-five microlitres of 0.1 mg/mL maprotyline (internal standard) was added to the 1 mL

Approximate Target Concentration (mg/kg)	Weight of Liver Homogenate Added (g)	Weight of Agar Added (g)	Total Weight of Foodstuff (g)
0	20.08	24.91	44.99
0.1	20.11	24.63	44.74
0.2	20.19	25.22	45.41
0.4	20.05	25.67	45.72
0.6	20.70	27.48	48.18
0.8	20.14	24.96	45.10

Table 3.36: The weight of the liver homogenate (g), agar (g), and the overall weight(g) of each spiked foodstuff sample prepared for the recovery experiment.

Amitriptyline		Nortriptyline	
Approximate Target Amitriptyline Concentration (mg/kg)	Prepared Concentration (mg/kg)	Approximate Target Nortriptyline Concentration (mg/kg)	Prepared Nortriptyline Concentration (mg/kg)
0	0	0	0
0.1	0.11	0.1	0.11
0.2	0.22	0.2	0.22
0.4	0.44	0.4	0.44
0.6	0.62	0.6	0.62
0.8	0.89	0.8	0.89

Table 3.37: Comparison of the approximate target concentration (mg/kg) and the prepared concentration (mg/kg) for the amitriptyline and nortriptyline spiked foodstuff samples required for the determination of the LOD and the LOQ of amitriptyline and nortriptyline in the artificial foodstuff.

Two separate 1 mL aliquots of the 1:1 drug-free foodstuff homogenate were prepared using the second drug-free sample of artificial foodstuff. One of these two aliquots received 0.1 mg/mL maprotyline, and the other aliquot was left completely drug-free.

The extract resulting from the maprotyline spiked 1:1 blank foodstuff homogenate was used to estimate the magnitude of the noise underneath amitriptyline and nortriptyline, and the extract resulting from the drug free 1:1 blank foodstuff homogenate was used to estimate the noise underneath maprotyline²¹. Each extract was analyzed using GC-NPD according to method outlined in Appendix A.

Seven replicate injections of each of the six extracted calibration standards were conducted. Ten replicate injections of the maprotyline only extract, and ten replicate injections of the drug-free extract were also conducted.

3.6.3 Results for the LOD and LOQ of the Analytes in the Artificial Foodstuff Matrix

The calibration curves required for the determination of the LOD and LOQ for amitriptyline and nortriptyline in the artificial foodstuff were generated by plotting mean peak height ratio obtained from the seven repeat injections of each extract, for each analyte, *versus* the spiked concentration. Least squares linear regression, weighted for errors in y , was performed on the data using JMP INTM (SAS Institute, Inc., Cary, NC, USA), and the resulting calibration curves are presented in Figure 3.24 for amitriptyline and in Figure 3.25 for nortriptyline.

The calibration equation for amitriptyline was $y = 2.562x + 0.212$ (95% CI(slope) = 2.261 to 2.862, $t_{df=1} = 23.64$, $P < 0.0001$, $R^2 = 0.994$). Likewise, the calibration equation for nortriptyline was $y = 2.359x + 0.087$ (95% CI(slope) = 2.125 to 2.593, $t_{df=1} = 27.98$, $P < 0.0001$, $R^2 = 0.995$).

The mean, standard deviation and relative standard deviation (RSD) for the seven aliquots of 1:1 foodstuff homogenate, prepared from each of the foodstuff calibration standards, prior to acid digestion.

²¹The magnitude of the baseline noise is estimated at the expected retention time for each analyte.

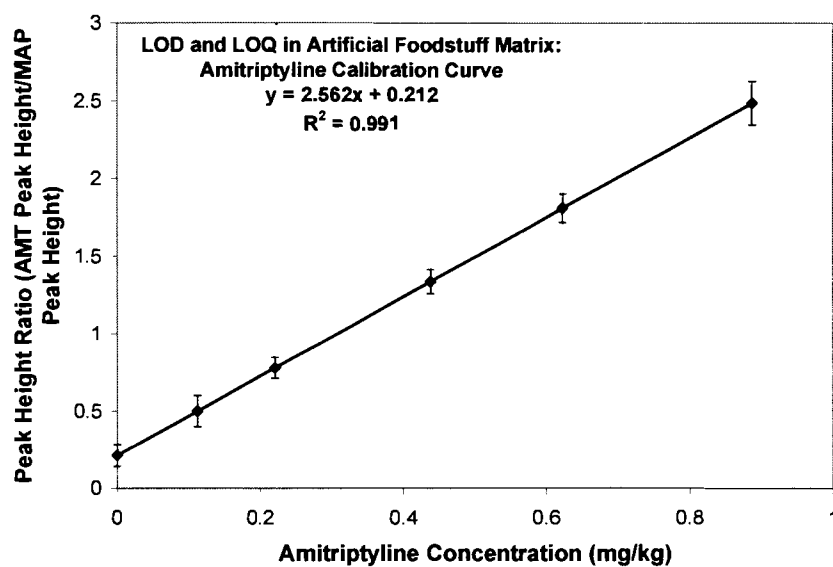


Figure 3.24: Limit of detection and limit of quantitation calibration curve for amitriptyline in artificial foodstuff. Least squares linear regression, weighted for errors in y , was performed on the calibration data. The error bars represent the standard deviation associated with seven repeat injections of each extract used to generate the calibration curve.

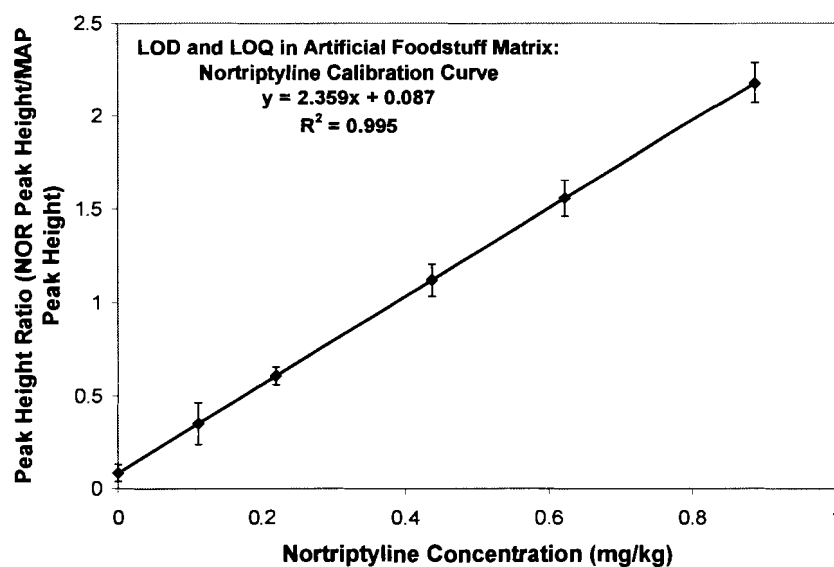


Figure 3.25: Limit of detection and limit of quantitation calibration curve for nortriptyline in artificial foodstuff. Least squares linear regression, weighted for errors in y , was performed on the calibration data. The error bars represent the standard deviation associated with seven repeat injections of each extract used to generate the calibration curve.

repeat injections for each of the six extracted calibration standards used to generate the calibration curves for amitriptyline and nortriptyline are presented in Table 3.38.

Concentration of the Calibration Standard (mg/kg)	Amitriptyline		Nortriptyline	
	Mean Peak Height Ratio (AMT/MAP) \pm SD	RSD (%)	Mean Peak Height Ratio (NOR/MAP) \pm SD	RSD (%)
0 ($n = 7$)	0.255 \pm 0.070	27.2	0.163 \pm 0.046	28.4
0.11 ($n = 7$)	0.553 \pm 0.100	18.0	0.341 \pm 0.112	33.0
0.22 ($n = 7$)	0.760 \pm 0.068	8.9	0.634 \pm 0.047	7.3
0.44 ($n = 7$)	1.182 \pm 0.078	6.6	1.014 \pm 0.084	8.3
0.62 ($n = 7$)	1.771 \pm 0.091	5.1	1.585 \pm 0.097	6.1
0.89 ($n = 7$)	2.539 \pm 0.140	5.5	2.199 \pm 0.107	4.8

Table 3.38: Mean peak height ratio (\pm SD) and relative standard deviation (RSD), calculated from seven repeat injections of each calibration standard. The extracted calibration standards were prepared in order to generate the amitriptyline and nortriptyline calibration curves required for the determination of the LOD and LOQ for the analytes in the artificial foodstuff matrix (AMT/MAP = amitriptyline peak height/maprotyline peak height; NOR/MAP = nortriptyline peak height/maprotyline peak height).

The average peak height (pA), standard deviation, and relative standard deviation of the baseline noise at both the retention time for amitriptyline and the retention time for nortriptyline, that were calculated from the 10 repeat injections of the extract prepared from the maprotyline spiked 1:1 blank foodstuff homogenate, are presented in Table 3.39.

Analyte Identity	Mean Noise \pm Standard Deviation (pA)	Relative Standard Deviation (%)
Amitriptyline	0.707 \pm 0.221	31.2
Nortriptyline	0.359 \pm 0.175	48.8

Table 3.39: Average baseline noise (\pm SD) and relative standard deviation (%) for amitriptyline and nortriptyline, in units of peak height (pA). Data was obtained from 10 replicate injections of a sample extract prepared by the extraction of a maprotyline (internal standard) spiked, 1:1 blank foodstuff homogenate.

The average peak height (pA), standard deviation, and relative standard deviation for the maprotyline peak height, calculated from the 10 replicate injections of the extract prepared from the maprotyline spiked 1:1 blank foodstuff homogenate, are presented in Table 3.40.

Mean Peak Height \pm Standard Deviation (pA)	Relative Standard Deviation (%)
5.892 \pm 0.668	11.3

Table 3.40: The mean, standard deviation, and relative standard deviation (RSD) of the peak height (pA) for the internal standard maprotyline, calculated from 10 replicate injections of the extract prepared from the maprotyline spiked 1:1 blank foodstuff homogenate.

The calculated limits of detection and limits of quantitation for amitriptyline and nortriptyline in the artificial foodstuff matrix are presented in Table 3.41. The LOD for amitriptyline in the artificial foodstuff matrix was 0.01 mg/kg, and the LOQ for amitriptyline was 0.11 mg/kg.

The LOD for nortriptyline in the artificial foodstuff matrix was 0.15 mg/kg, and the LOQ for nortriptyline was 0.24 mg/kg (Table 3.41). Based on these results, amitriptyline can be quantitated to a lower concentration than nortriptyline using the analytical method developed for the HP 6890 gas chromatograph, for the present project (Appendix A).

The noise under the maprotyline peak, in units of pA, calculated from 10 replicate injections of the extract prepared from the blank (drug-free) 1:1 foodstuff homogenate was 0.087 ± 0.033 (RSD = 39.2%). This mean value represents only 1.5% of the mean maprotyline peak height of 5.892 pA that was used to calculate the peak height ratios necessary for the determination the LOD and LOQ. Therefore, the noise under the maprotyline peak was negligible.

Analyte	Limit of Detection (LOD)		Limit of Quantitation (LOQ)	
	Peak Height Ratio	Concentration (mg/kg)	Peak Height Ratio	Concentration (mg/kg)
Amitriptyline	0.232	0.01	0.494	0.11
Nortriptyline	0.449	0.15	0.657	0.24

Table 3.41: The limit of detection and the limit of quantitation for amitriptyline and nortriptyline in the artificial foodstuff matrix, using a method developed for use on the HP 6890 gas chromatograph at the Provincial Toxicology Centre, Riverview Hospital, British Columbia, Canada.

3.6.4 Discussion

In contrast to the determination of the LOD and LOQ in ethyl acetate, the LOD and LOQ in the artificial foodstuff was greater for nortriptyline, then amitriptyline. This may be due to the fact that the nortriptyline peaks tended to broaden out more when analyzing extracts derived from the artificial foodstuff. Unfortunately, non-volatile components from the artificial foodstuff, which could not be eliminated during the sample preparation procedure, contributed to significant column degradation, which reduced the sensitivity of the chromatographic system to the analytes of interest. As a result, the column was regularly washed with at least 10 mL of chlorobutane to eliminate the build-up of non-volatile residues from the artificial foodstuff extracts. Furthermore, although washing the column with chlorobutane was able to restore baseline separation of the peaks, the peak produced with the chlorobutane-rinsed column were generally broader overall (Appendix A).

In addition, the concentration range between the LOD and LOQ for amitriptyline in the artificial foodstuff was greater than the range between the LOD and LOQ for nortriptyline in the artificial foodstuff. The lowest expected concentration for amitriptyline in the artificial foodstuff was 24 mg/kg, and the lowest expected concentration for nortriptyline in the artificial foodstuff was 0.9 mg/kg. Therefore, even though the sensitivity of the system was reduced due to column degradation, the chosen sample preparation procedure and GC-NPD method was still sensitive enough to detect amitriptyline and nortriptyline, at least as low as the lowest concentrations expected for each analyte in the artificial foodstuff.

3.7 Preparation and Analysis of the Artificial Foodstuff Used in the Insect Development Experiment

3.7.1 Introduction

The purpose of this section is to describe both the preparation and analysis of the

artificial foodstuff used in the insect development experiment. Even though a known amount of amitriptyline and nortriptyline was added to each batch of foodstuff, the homogeneity of the foodstuff must be evaluated. In addition, the end concentrations in the artificial foodstuff must be quantitated because the amount of each drug added was based on the ideal weight of the prepared artificial foodstuff. The ideal weight of the foodstuff was calculated by summing the weights of all the materials used to make the foodstuff. However, the actual weight of the foodstuff will likely be less due to component loss during preparation. All reasonable precautions, such as scraping the remaining foodstuff material from the sides of the mixing containers, were undertaken to minimize component loss during the foodstuff preparation.

Homogenization of the animal tissue is an effective and practical method of ensuring that the selected drugs, once added, will be distributed evenly throughout the food source. However, this presents a problem for the larvae because they cannot feed on liquid media, and homogenization effectively serves to liquefy animal tissue. This problem will be circumvented through the addition of agar, which will provide the larvae with a solid surface for feeding. Bacteriological agar is used as a solidifying agent in microbiological culture media. Agar possesses a unique property known as hysteresis; agar melts at a temperature different from the temperature at which it solidifies. Bacteriological agar melts at approximately 90°C and solidifies at approximately 35°C (Selby and Selby, 1959). Bacteriological agar is sold as a fine, granular, free flowing and creamy white powder that dissolves in boiling water. As the agar solution cools it begins to thicken until it solidifies at approximately 35°C. Another valuable characteristic of agar is that it remains firm at temperature as high as 65°C. The degree of firmness obtained in the final solidified product can be altered by changing the final concentration of agar in the solution (Selby and Selby, 1959).

3.7.2 Preparation of the Amitriptyline and Nortriptyline Spiked Artificial Foodstuff

The recipe and protocol for the preparation of the artificial foodstuff used in the present thesis was obtained from Sadler *et al.* (1997). However, due to the potential thermal instability of amitriptyline and nortriptyline, the liver and egg homogenate

was not heated prior to the addition of the aqueous solution of bacteriological agar, as was done by Sadler *et al.* (1997).

Artificial Foodstuff Ingredients

- 500 g Beef liver
- 30 g Powdered whole egg
- 300 mL Distilled Water (for dissolving the powdered whole egg)
- 100 mL Distilled Water (for dissolving the drug(s) under investigation)
- 1000 mL Distilled boiling Water (for dissolving the agar)

The total weight of the prepared foodstuff is approximately 1.97 kg.

Artificial Foodstuff Preparation Protocol

Thirty grams of powdered whole egg (CANASOY, Vancouver, Canada) was dissolved in a 500 mL glass beaker with 250 mL of distilled water, and then stirred until no clumps were visible. Five hundred grams of fresh beef liver was cut into small pieces and divided into five portions of approximately 100 g each. Two hundred grams of the beef liver was then homogenized at high speed for 30 seconds with 100 mL of the aqueous egg mixture using an Osterizer[®] blender with a glass container. Another 200 g of chopped beef liver and 100 mL of the aqueous egg mixture was added to the blender and the blended at high speed for another 30 seconds. The remaining 100 g of beef liver was added to the blender, and the liver-egg mixture was then blended at high speed until its consistency resembled that of a smooth, thick milkshake.

Amitriptyline and nortriptyline were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Canada) in the form of amitriptyline hydrochloride and nortriptyline hydrochloride, respectively. Amitriptyline and nortriptyline commonly adsorb to glass surfaces. Therefore, in order to avoid cross-contamination of different batches of artificial foodstuff prepared at the same time, the drug solution was not added to the liver-egg homogenate while the homogenate was in the glass container of the blender. Instead, the liver-egg homogenate was poured into a 3.8 L glass bowl (PYREX[®]

Prepware, Corning Inc., Corning, USA). The glass container of the blender was held upside down until the majority of the liver-egg homogenate remaining in the glass container had dripped into the mixing bowl. To prevent cross-contamination, separate glass bowls were used for each target concentration of amitriptyline and nortriptyline. The remaining 100 mL of distilled water was then used to rinse out the glass mixing container, and the rinsate was added to the liver-egg homogenate in the glass bowl.

The amount of amitriptyline and nortriptyline, in the form of their respective hydrochloride salts, required to produce the target concentrations of amitriptyline and nortriptyline in the artificial foodstuff were accurately weighed using an analytical balance, and then transferred accurately into a 100 mL glass volumetric flask. Distilled water was added to the dilution mark, and the flask was capped and gently inverted ten times to dissolve the drugs. The drug solution was allowed to sit for 20 minutes at room temperature to ensure the homogeneity of the aqueous drug solution. The aqueous drug solution was then added to the liver-egg homogenate, and the mixture was blended at medium speed for 2 minutes with a 10 speed hand mixer to evenly distribute the drugs throughout the liver-egg homogenate. The liver-egg homogenate was then covered with plastic wrap and allowed to equilibrate in the fridge for 48 hours.

After 48 hours, 1 L of distilled water was heated in a 2 L glass beaker, on a hot plate, until the distilled water reached a rolling boil. The boiling water was then poured into a 4 L metal mixing bowl. Forty-five grams of agar was added to the boiling water and blended at high speed until smooth using the 10 speed hand mixer. To reduce clumping, the agar was spread evenly over the surface of the hot water rather than adding it in one large mass. Blending with a hand mixer at high speed proved efficient enough to breakdown the majority of the undissolved clumps of agar.

Agar starts to gel relatively quickly once it starts to cool, therefore as soon as the majority of the agar clumps were removed, the agar solution was added to the liver-egg homogenate. The mixture was then blended at high speed using the 10 speed hand mixer for two minutes. Before the agar was added, the liver-egg homogenate was a dark red in colour. Once the agar was added, and thoroughly mixed with the liver-egg homogenate, the red colour was muted to a light pink.

Once the agar and liver homogenate were thoroughly mixed, the mixture was poured into a 2.8 L, 23 x 33 cm glass baking dish (PYREX[®] Bakeware, Corning Inc., Corning, USA). The baking dish was shaken slightly during pouring to create a smooth, level surface. The foodstuff mixture was then allowed to set for 30 minutes. Once the foodstuff had solidified, the foodstuff was cut into approximately 120, 3 x 2 x 2 cm pieces. Five pieces were randomly selected from each batch, placed into appropriately labelled plastic Ziploc[™] bags and frozen for later analysis by GC-NPD using the previously developed method (Appendix A).

From the foodstuff remaining, pieces were chosen at random, and placed, into wax-paper lined, square plastic Ziploc[™] containers. Each row of foodstuff placed in the containers was separated by a piece of wax paper. The artificial foodstuff was stored at -10°C until required. The frozen foodstuff pieces were thawed for 24 hours at 4°C before use.

Separate metal mixing rods were maintained for the dissolution of the agar and the mixing of the agar with the liver-egg homogenate. In addition, between the preparation of each batch of foodstuff, the glass portion of the blender, the metal mixing rods for the hand mixer, and all other glass or metal equipment used during the preparation of the foodstuff were washed with a dilute aqueous solution of Extran 300[®], a phosphate free surface cleanser, and then rinsed with methanol.

Preparation of Control (Drug-Free) Foodstuff

The control (drug-free) batches of the artificial foodstuff were also prepared using the above procedure. However, the liver-egg homogenate prepared for the control batches received 100 mL of distilled water in lieu of the 100 mL aqueous solution of amitriptyline and nortriptyline.

3.7.3 Analysis of the Prepared Artificial Foodstuff

Target Amitriptyline and Nortriptyline Concentrations in the Artificial Foodstuff

A preliminary study conducted by Goff *et al.* (1993) investigated the effect of amitriptyline on the development of *Parasarcophaga ruficornis* (Fabricius) (Diptera: Sarcophagidae). In their study, three different doses of amitriptyline were administered to three different experimental rabbits by ear vein infusion. A control rabbit was given an equivalent volume of a saline solution by ear vein infusion. According to Goff *et al.* (1991), the concentrations of amitriptyline administered to the experimental rabbits were calculated to represent sub-lethal, median lethal, and twice the median lethal levels of amitriptyline by body weight. After the administration of amitriptyline, livers from each of the four rabbits were removed and each liver was used to rear approximately 210 *P. ruficornis* larvae. Before introduction of the larvae to the liver, a small sample of each liver was retained for quantitation of the levels of amitriptyline and nortriptyline present. The levels of amitriptyline and nortriptyline that were found to be present in the three rabbit livers used to rear the larvae of *P. ruficornis* in the study conducted by Goff *et al.* (1991) are presented in Table 3.42.

Level of Analyte in Rabbit Liver	Dosages of Amitriptyline Administered by Ear Vein Infusion		
	300 mg	600 mg	1000 mg
Amitriptyline Concentration (mg/kg)	24.0	154.0	49.0
Nortriptyline Concentration (mg/kg)	3.3	7.1	0.9

Table 3.42: Concentrations of amitriptyline and nortriptyline in the livers obtained from three experimental rabbits that were administered three different doses of amitriptyline by ear vein infusion (Goff *et al.*, 1993: 318).

Therefore, the target concentrations of amitriptyline and nortriptyline in the artificial foodstuff used in the present thesis were the concentrations presented in Table 3.42.

3.7.4 Preparation of Artificial Foodstuff Used in the Insect Development Section

Each batch of artificial foodstuff was prepared according to the procedure outlined earlier in this chapter. The target weight and actual weight of amitriptyline hydrochloride and nortriptyline hydrochloride (Sigma-Aldrich Canada, Ltd., Oakville, Ontario) used to prepare the aqueous drug solutions that were added to each drug-spiked batch of artificial foodstuff are presented in Table 3.43.

Foodstuff Batch	Target Concentration in Foodstuff (mg/kg)	Amitriptyline		Nortriptyline	
		Target (g)	Actual (g)	Target (g)	Actual (g)
Batch A	154.0 AMT + 7.1 NOR	0.3042	0.3041	0.0140	0.0142
Batch B	24.0 AMT + 3.3 NOR	0.0474	0.0477	0.0062	0.0064
Batch C	49.0 AMT + 0.9 NOR	0.0968	0.0967	0.0012	0.0015
Batch D	49.0 AMT	0.0968	0.0967	0	0
Blank	0	0	0	0	0

Table 3.43: The calculated (target) weight of amitriptyline hydrochloride and nortriptyline hydrochloride required for addition to each batch of artificial foodstuff, and the actual weight of amitriptyline hydrochloride and nortriptyline hydrochloride dissolved in 100 mL of distilled water for addition to the artificial foodstuff. (AMT = amitriptyline; NOR = nortriptyline).

3.7.5 Quantitation of Amitriptyline and Nortriptyline in the Artificial Foodstuff

Preparation of the Calibration Standards for the Quantitation of Amitriptyline and Nortriptyline

The stock solutions used for the preparation of the calibration standards were mixed amitriptyline and nortriptyline stock solutions (in methanol) of the following concentrations:

1. 0.1 mg/mL amitriptyline + 0.1 mg/mL nortriptyline

2. 1 mg/mL amitriptyline + 1 mg/mL nortriptyline

The 0.1 mg/mL stock solution was prepared by a 1:10 dilution (in methanol) of the mixed 1 mg/mL amitriptyline and nortriptyline stock solution.

Ten calibration standards were prepared by adding 1 mL of blank (drug-free) 1:1 foodstuff homogenate to each test tube, followed by the appropriate amount of each mixed amitriptyline and nortriptyline stock solution, 25 μ L of 1 mg/ml maprotyline (internal standard), and then the contents of each test tube were vortexed.

The target amitriptyline and nortriptyline concentrations for each of the ten calibration standards samples were as follows:

1. 0 mg/L amitriptyline + 0 mg/L nortriptyline (blank)
2. 1 mg/L amitriptyline + 1 mg/L nortriptyline
3. 2.5 mg/L amitriptyline + 2.5 mg/L nortriptyline
4. 5 mg/L amitriptyline + 5 mg/L nortriptyline
5. 10 mg/L amitriptyline + 10 mg/L nortriptyline
6. 25 mg/L amitriptyline + 25 mg/L nortriptyline
7. 50 mg/L amitriptyline + 50 mg/L nortriptyline
8. 100 mg/L amitriptyline + 100 mg/L nortriptyline
9. 150 mg/L amitriptyline + 150 mg/L nortriptyline
10. 200 mg/L amitriptyline + 200 mg/L nortriptyline

Since the maximum concentration of nortriptyline expected in the artificial foodstuff was less than 7.1 mg/kg (Table 3.42), only the signal data obtained from the first seven calibration standards were used to quantitate nortriptyline. Furthermore, since it was shown earlier that the linear response range of the detector extended from approximately 0 mg/L to 50 mg/L of nortriptyline; therefore, the use of only

the first seven calibration curves allowed a linear calibration curve to be constructed for nortriptyline (Section 3.7).

The volumes of amitriptyline, nortriptyline and maprotyline stock solutions used to prepare the calibration standards required for the experiment are presented in Table 3.43. The prepared calibration standards were then extracted alongside the foodstuff samples using the sample preparation procedure previously described.

Target Concentration (mg/L)	Volume (μ L) of Mixed 0.1 mg/mL AMT and 0.1 mg/mL NOR Stock Solution	Volume (μ L) of Mixed 1 mg/mL AMT and 1 mg/mL NOR Stock Solution	Volume (μ L) of 1 mg/mL MAP Stock Solution
0	0	-	25
1	10	-	25
2.5	25	-	25
5	50	-	25
10	100	-	25
25	-	25	25
50	-	50	25
100	-	100	25
150	-	150	25
200	-	200	25

Table 3.44: Volume of amitriptyline, nortriptyline and maprotyline stock solutions used to prepare the calibration standards required for the accuracy experiment. The calibration standards were prepared in 1 mL of drug-free (blank) 1:1 foodstuff homogenate (AMT = amitriptyline; NOR = nortriptyline; MAP = maprotyline).

Sampling and Extraction of the Artificial Foodstuff

Five pieces of foodstuff were randomly chosen from each batch of foodstuff. Each sample was then homogenized and extracted without prior acetonitrile precipitation according to the procedure described in Section 3.

3.7.6 Quantitation of Amitriptyline in the Artificial Foodstuff

Amitriptyline Calibration Curve

The calibration data for amitriptyline, generated by triplicate injections of each of the ten calibration standards are presented in Table 3.45.

Concentration of Amitriptyline Calibration Standard (mg/L)	Mean Peak Height Ratio (AMT Peak Height/MAP Peak Height)	Relative Standard Deviation (%)
0 ($n = 3$)	0.019 ± 0.011	57.5
1 ($n = 3$)	0.114 ± 0.005	4.2
2.5 ($n = 3$)	0.351 ± 0.009	2.6
5 ($n = 3$)	0.638 ± 0.015	2.4
10 ($n = 3$)	1.271 ± 0.010	0.8
25 ($n = 3$)	3.474 ± 0.061	1.7
50 ($n = 3$)	6.419 ± 0.101	1.6
100 ($n = 3$)	9.061 ± 0.045	0.5
150 ($n = 3$)	13.263 ± 0.171	1.3
200 ($n = 3$)	16.346 ± 0.134	0.8

Table 3.45: The mean (\pm SD) peak height ratio (amitriptyline peak height/maprotyline peak height ratio), and the relative standard deviation (%) for the data generated from triplicate injections of each amitriptyline calibration standard prepared to quantitate the level of amitriptyline present in each batch of artificial foodstuff. Each calibration standard was prepared in a matrix of blank artificial foodstuff homogenate, and then extracted with chlorobutane by liquid-liquid extraction (AMT = amitriptyline; NOR = nortriptyline).

The calibration curve for amitriptyline, generated from the data presented in Table 3.45, is illustrated in Figure 3.26. Second-order, least squares polynomial regression

(centered), weighted for errors in y , was performed on the calibration data using JMP IN[®] (SAS Institute, Inc., Cary, NC, USA). The equation for the calibration curve was $y = -0.001x^2 + 0.1043x + 0.8341$.

The results of the second order polynomial regression of the calibration data provided in Table 3.44 are presented in Table 3.45.

Variable	Confidence Interval	t Ratio	Prob > $ t $	R^2
y -intercept	1.5128 to 3.6857	5.66	0.0008	0.994
x	0.0671 to 0.0792	28.57	< 0.0001	
x^2	-0.0003 to -0.00002	-2.75	0.0284	

Table 3.46: Results of the centered second-order polynomial regression (quadratic) analysis, weighted for errors in y , of the calibration data for amitriptyline. The equation of the regression line was $y = -0.0001x^2 + 0.1043x + 0.8341$, where y = the mean peak height ratio ($n = 3$) and x = amitriptyline concentration (mg/L).

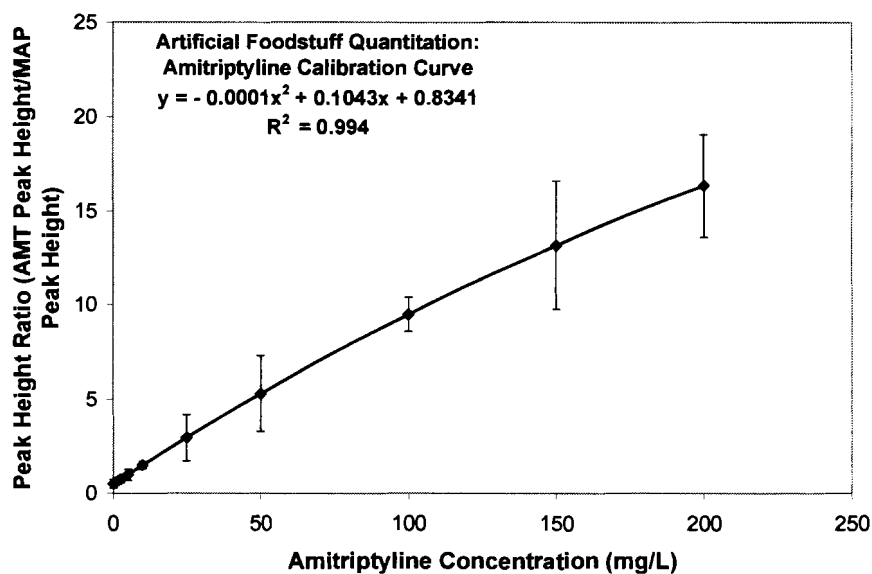


Figure 3.26: Amitriptyline calibration curve for the quantitation of the level of amitriptyline present in each of the four batches of artificial foodstuff prepared for the insect development experiment. Second-order least-squares polynomial regression, weighted for errors in y , was conducted on the calibration data. The errors bars represent the standard deviation associated with each signal, multiplied by a factor of +20 so that most of the error bars were visible. Each calibration standard was prepared in a matrix of blank artificial foodstuff homogenate and the extracted with chlorobutane by liquid-liquid extraction. Triplicate analyses of the extract, for each calibration standard, were conducted by GC-NPD. (AMT = amitriptyline; MAP = maprotyline).

Concentration and Homogeneity of Amitriptyline in the Artificial Foodstuff

Five pieces of foodstuff were randomly selected from each batch, extracted and analyzed using GC-NPD (Appendix A). Triplicate analyses of each of the five extracts, from each batch of foodstuff were conducted. The mean concentration of amitriptyline in each batch of foodstuff was calculated by averaging the average results obtained from the triplicate injections from each of the five pieces of foodstuff²². The target concentration²³, mean measured concentration \pm standard deviation, and relative standard deviation (RSD) of the amitriptyline concentration added to each batch of artificial foodstuff is presented in Table 3.47. The RSD was calculated in order to estimate the homogeneity of the foodstuff in terms of amitriptyline concentration; the greater the RSD, the less homogeneous the foodstuff. In order for a batch of foodstuff to be considered homogeneous, the calculated RSD had to be less than 15%. The discrepancy between the target amitriptyline concentration for each batch of foodstuff and the mean quantitated nortriptyline concentration for each batch is also presented in Table 3.47.

²²The mean, standard deviation and relative standard deviation of the measured amitriptyline concentration calculated for each of the five pieces of artificial foodstuff, for each batch of foodstuff, are presented in Appendix B.

²³From Goff *et al.* (1993).

Foodstuff Batch	Target Amitriptyline Concentration (mg/kg) Goff <i>et al.</i> (1993)	Mean (\pm SD) Actual Amitriptyline Concentration	Relative Standard Deviation (%)	Percent Difference (%)
A ($n = 5$)	154.0	168.41 \pm 13.30	7.9	+9.4
B ($n = 5$)	24.0	9.55 \pm 4.47	46.7	-60.2
C ($n = 5$)	49.0	41.38 \pm 6.71	16.2	-15.6
D ($n = 5$)	49.0	42.59 \pm 5.66	13.3	-13.1

Table 3.47: The target concentration, mean concentration \pm standard deviation and relative standard deviation (RSD) for the concentration of amitriptyline present in each batch of artificial foodstuff. The RSD indicates the level of amitriptyline homogeneity in each batch of artificial foodstuff; the greater the RSD, the less homogeneous the foodstuff. The discrepancy between the target amitriptyline concentration and the actual mean concentration is represented by the percent difference. The concentration of amitriptyline present in each batch of foodstuff was calculated by analyzing five randomly selected pieces of foodstuff from each batch by GC-NPD.

3.7.7 Quantitation of Nortriptyline in the Artificial Foodstuff

Nortriptyline Calibration Curve

The calibration curve for nortriptyline was constructed using the signal data generated from triplicate injections of the first seven calibration standards. The calibration signal data for nortriptyline is presented in 3.48.

Concentration of Nortriptyline Calibration Standard (mg/L)	Mean Peak Height Ratio (NOR Peak Height/MAP Peak Height)	Relative Standard Deviation (%)
0 ($n = 3$)	0.019 ± 0.011	57.5
1 ($n = 3$)	0.063 ± 0.004	4.2
2.5 ($n = 3$)	0.204 ± 0.003	2.6
5 ($n = 3$)	0.396 ± 0.005	2.4
10 ($n = 3$)	0.785 ± 0.044	0.8
25 ($n = 3$)	2.103 ± 0.072	1.7
50 ($n = 3$)	4.170 ± 0.123	1.6

Table 3.48: The mean (\pm SD) peak height ratio (nortriptyline peak height/maprotyline peak height ratio), and the relative standard deviation (%) for the data generated from triplicate injections of each nortriptyline calibration standard used to quantitate the level of nortriptyline present in each batch of artificial foodstuff. Each calibration standard was prepared in a matrix of blank artificial foodstuff homogenate and the extracted with chlorobutane by liquid-liquid extraction. (NOR = nortriptyline; MAP = maprotyline).

The calibration curve for nortriptyline, is illustrated in Figure 3.27. Least squares linear regression, weighted for errors in y , was performed on the calibration data presented in Table 3.49. The calibration data was generated from triplicate injections of each calibration standard. The equation for the nortriptyline calibration curve was $y = 0.084x - 0.017$ (95% CI (slope) = 0.084 to 0.085; $t_{df=1} = 157.82$; $P < 0.0001$; $R^2 = 0.999$; Figure 3.27).

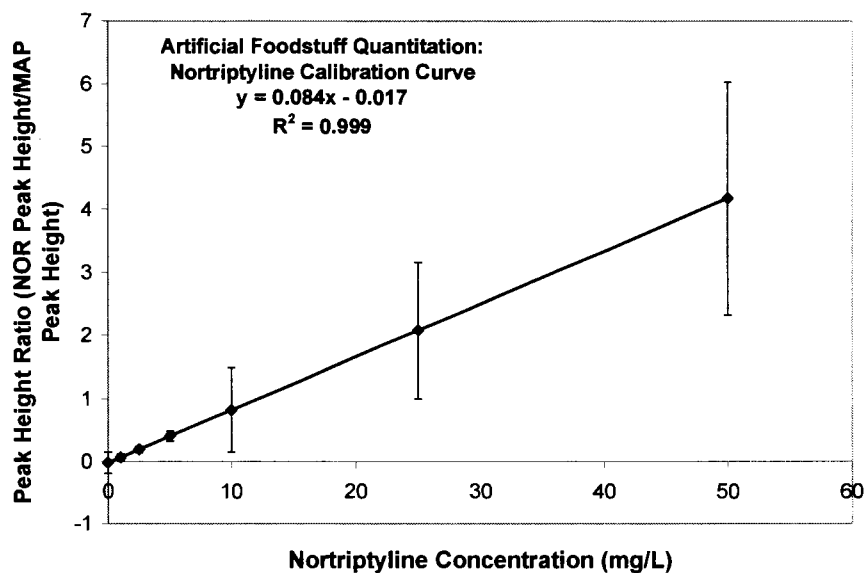


Figure 3.27: Nortriptyline calibration curve for the quantitation of the level of amitriptyline present in each of the four batches of artificial foodstuff prepared for the insect development experiment. Least-squares linear regression, weighted for errors in y , was conducted on the calibration data. The errors bars represent the standard deviation associated with each signal, multiplied by a factor of +20 so that most of the error bars were visible. Each calibration standard was prepared in a matrix of blank artificial foodstuff homogenate and the extracted with chlorobutane by liquid-liquid extraction. Triplicate analyses of the extract, for each calibration standard, were conducted by GC-NPD. (NOR = nortriptyline; MAP = maprotyline).

Concentration and Homogeneity of Nortriptyline in the Artificial Foodstuff

The mean concentration of nortriptyline in each batch of foodstuff was calculated by averaging the average results obtained from the triplicate injections from each of the five pieces of foodstuff²⁴.

In addition, the target concentration²⁵, mean quantitated concentration \pm standard deviation, and relative standard deviation (RSD) of the nortriptyline concentration present in each batch of artificial foodstuff is presented in Table 3.49. The RSD was calculated in order to estimate the homogeneity of the foodstuff in terms of nortriptyline concentration; the greater the RSD, the less homogeneous the foodstuff. As with amitriptyline, the calculated RSD had to be less than 15% for a batch of foodstuff to be considered homogeneous for nortriptyline. In addition, the discrepancy between the target nortriptyline concentration for each batch of foodstuff and the mean quantitated nortriptyline concentration for each batch is also presented in Table 3.49.

²⁴The mean, standard deviation and relative standard deviation of the quantitated nortriptyline concentration calculated for each of the five pieces of artificial foodstuff, for each batch of foodstuff, are presented in Appendix B.

²⁵From Goff *et al.* (1993).

Foodstuff Batch	Target Nortriptyline Concentration (mg/kg) Goff <i>et al.</i> (1993)	Mean (\pm SD) Nortriptyline Concentration	Relative Standard Deviation (%)	Percent Difference (%)
A ($n = 5$)	7.1	7.65 ± 0.28	3.7	+7.7
B ($n = 5$)	3.3	3.21 ± 0.11	3.3	-2.7
C ($n = 5$)	0.9	1.92 ± 0.07	3.7	+113
D ($n = 5$)	0	1.22 ± 0.06	5.1	-

Table 3.49: The target concentration, mean quantitated concentration \pm standard deviation and relative standard deviation (RSD) for the concentration of nortriptyline present in each batch of artificial foodstuff. The RSD indicates the level of amitriptyline homogeneity in each batch of artificial foodstuff; the greater the RSD, the less homogeneous the foodstuff. The discrepancy between the target nortriptyline concentration and the actual mean concentration is represented by the percent difference. The concentration of nortriptyline present in each batch of foodstuff was calculated by analyzing five randomly selected pieces of foodstuff from each batch by GC-NPD.

3.7.8 Discussion of the Quantitation Results

For ease of discussion, the four batches of drug spiked artificial foodstuff are referred to in the following chapters according to the labels presented in Table 3.50.

Label Used in Thesis	Measured Concentration
Batch A	168.41 mg/kg AMT + 7.65 mg/kg NOR
Batch B	9.55 mg/kg AMT + 3.21 mg/kg NOR
Batch C	41.38 mg/kg AMT + 1.92 mg/kg NOR
Batch D	49.0 mg/kg AMT + 1.22 mg/kg NOR

Table 3.50: Labels assigned to each set of artificial foodstuff prepared for use in the insect development study.

The intent of the present thesis was to expose insects of the species *S. bullata* (Diptera: Sarcophagidae) to the same amitriptyline and nortriptyline concentrations found in the rabbit livers used in the study conducted by Goff *et al.* (1993). Unfortunately, based on the magnitude of the percent difference calculated for both amitriptyline and nortriptyline, for each of the four drug-spiked batches of artificial foodstuff, the target concentrations were not adequately achieved. Overall, the quantitated nortriptyline concentrations for each batch of artificial foodstuff more closely matched the target concentrations than did the quantitated amitriptyline concentrations.

The percent differences calculated for each batch containing nortriptyline were positive, indicating that the concentrations present in the artificial foodstuff were higher than the target concentrations. Given the fact that the artificial foodstuff lost a substantial amount of water on thawing, the higher concentration of nortriptyline present in each batch of foodstuff was not unexpected. Nortriptyline is a lipid soluble drug with a correspondingly large **volume of distribution**. As a result, nortriptyline tends to bind tightly to tissues, and therefore, the loss of water from the foodstuff results in an overall increase in the concentration of nortriptyline in the foodstuff.

In comparison, only Batch A was observed to have a quantitated amitriptyline concentration greater than that of the target concentration. Batches B, C and D were all observed to have quantitated amitriptyline concentrations less than that of

their respective target concentrations. Given the fact that amitriptyline also binds tightly to tissues, the lower concentrations of amitriptyline, compared with the target concentrations, in Batches B, C and D, were unexpected.

However, extremely poor within batch homogeneity was observed for amitriptyline. For example, the target concentration of amitriptyline in Batch B was 24 mg/kg, but the mean quantitated concentration was only 9.55 mg/kg. The RSD associated with the five pieces of artificial foodstuff quantitated for Batch B was 46.7%. Therefore, due to the extreme inhomogeneity of Batch B, the percent difference between the target concentration and the mean quantitated concentrations will be large, and will likely not reflect the overall increase in amitriptyline concentration in the foodstuff, resulting from the water lost from the foodstuff during thawing.

Unfortunately, the relatively large discrepancy between the target concentrations and mean quantitated concentrations for both amitriptyline and nortriptyline makes comparisons between the results obtained from the present study and the study conducted by Goff *et al.* (1993) difficult.

Furthermore, the inhomogeneity of some of the foodstuff batches will significantly complicate the analysis of the insect development results presented in Chapter 4, because the non-uniformity of the food source will make the existence of dose-dependent changes in the rate of *S. bullata* development difficult, if not impossible, to detect. Overall, Batches A and D can be considered homogeneous in terms of amitriptyline concentration because their calculated RSDs were both less than 15%. The RSD for the quantitated amitriptyline concentration in Batch A was 7.9%, and the RSD was 13.3% for Batch D. As indicated earlier, the homogeneity of Batch B for amitriptyline was extremely poor, as indicated by an RSD of 46.7%. The RSD for Batch C was 16.2%, which was just outside the 15% homogeneity acceptance criteria, and as a result, the amitriptyline levels for Batch C cannot be considered homogeneous.

It is interesting to note that the homogeneity results for nortriptyline in all of the foodstuff batches were considerably better than the homogeneity results obtained for amitriptyline. Given the fact that for each batch, the concentration of nortriptyline present was significantly lower than the concentration of amitriptyline present, it was expected that their quantitation would be less precise. In addition, given the poor

accuracy results obtained for nortriptyline in the accuracy experiment (Section 3.7) it was expected that the nortriptyline RSDs for each batch would be greater than the RSDs actually observed. However, this was not the case because the all four batches of the artificial foodstuff were shown to be homogeneous for nortriptyline.

Furthermore, the presence of nortriptyline in Batch D, at a level of 1.22 mg/kg, was unexpected. Batch D was intended to only contain amitriptyline, at a concentration equal to the concentration of amitriptyline present in Batch C. The purpose of this was to determine whether the presence or absence of nortriptyline altered the development of *S. bullata* when the amitriptyline concentration was held constant. With this in mind, nortriptyline was not added to Batch D during its preparation. Contamination from other sources is unlikely since precautions to avoid this situation were undertaken. For example, completely separate preparation equipment was used for each foodstuff batch and all equipment was thoroughly washed and then rinsed with methanol 3.

Therefore, the appearance of nortriptyline indicates that during the preparation of the foodstuff, some breakdown of the amitriptyline occurred, most likely from catabolism of the parent drug by liver enzymes. During foodstuff preparation, the drugs were added to the liver homogenate and then the drug-liver homogenate mixture was stored at 4°C for 24-48 hours before the addition of the agar. This was done to allow the drug to equilibrate throughout the homogenate. Despite the cold storage temperatures, the activity of the liver enzymes may have been sufficient enough to produce the levels of nortriptyline seen in Batch D (Huckin, personal communication, 2003). Similar activity of the liver enzymes was also observed in one of the amitriptyline and nortriptyline quality controls prepared for this experiment. The concentration of the quality control was reduced by approximately half within two weeks of fridge storage, indicating ongoing catabolism of amitriptyline and nortriptyline by liver enzymes.

Many of problems encountered in the present chapter, including the poor precision and accuracy associated with nortriptyline and the non-linear detector response for amitriptyline are likely the result of column contamination by non-volatile residues in the extracts derived from the artificial foodstuff. According to Rood (1999), the

most common symptoms of column contamination are peak shape problems, such as tailing or broadening, and adsorption. Nortriptyline exhibited the greatest sensitivity to peak shape and adsorption problems. Nortriptyline is a secondary amine, and compared to amitriptyline, which is a tertiary amine, can more readily associate with active sites present in the chromatographic system.

3.8 Conclusion

Solvent rinsing, which was used in the present thesis to recover the column after contamination, is only a temporary solution. Solvent rinsing, in some cases can restore a column to its original performance parameters, even after several episodes of contamination. However, such was not the case in the present thesis. Solvent rinsing was able to restore the column to reasonable condition twice before the rinsing process ceased to recover the column. Since the majority of the validation problems discussed in this chapter were likely due to column contamination by non-volatile residues, the sample preparation procedure must be substantially improved in order to produce a less contaminating sample. Given the complexity of the artificial foodstuff matrix, it is unlikely that a sample preparation procedure will be able to eliminate non-volatile contaminants 100%. However, with further investigation, they can be significantly reduced.

The results of the *S. bullata* insect development experiment, conducted using the foodstuff prepared and analyzed according to the procedures discussed in the present chapter will be discussed in Chapter 4. Unfortunately, only Batches A and D can be considered homogeneous for both amitriptyline and nortriptyline. Further modification of the foodstuff preparation procedure is required to increase the homogeneity of the foodstuff. Non-homogeneity of the test substances in the foodstuff will unfortunately decrease the probability of attributing changes in the development of *S. bullata* to a specific concentration, or ratio of concentrations, of amitriptyline and nortriptyline.

Chapter 4

Insect Development Experiment

4.1 Introduction

As discussed in the introduction, the present thesis had two objectives; the first was to determine the effects of amitriptyline and nortriptyline on the development of an insect species of forensic importance in British Columbia, and the second was to evaluate the use of a non-live animal model. However, in order to meet the requirements of the second objective, an insect species closely related to the one used by Goff *et al.* (1993) had to be chosen. With this in mind, *Sarcophaga bullata* (Parker) (Diptera: Sarcophagidae) was chosen as the test species.

In order to evaluate the effects of amitriptyline and nortriptyline on the development of *S. bullata*, five different batches of artificial foodstuff, prepared from homogenized beef liver, powdered whole egg, and agar were each spiked with different concentrations of amitriptyline and nortriptyline. The proportions of amitriptyline and nortriptyline added were obtained from a previous entomotoxicological experiment conducted by Goff *et al.* (1993), and were selected to represent levels of the drugs commonly found in human deaths involving amitriptyline.

The artificial foodstuff used to determine the impact of a specific drug, or drugs, on insect development must be homogeneous with respect to the drugs of interest. As a result, each batch of amitriptyline and nortriptyline spiked artificial foodstuff prepared was analyzed to confirm both its amitriptyline and nortriptyline content, and

homogeneity using the previously developed GC-NPD method (Appendix A). The method used to analyze the artificial foodstuff was modified from the method used at the Provincial Toxicology Centre, Riverview Hospital, Port Coquitlam, British Columbia; therefore, before this method could be used to analyze the actual artificial foodstuff used in the development experiment for *S. bullata*, the modified analytical method had to be validated. With this in mind, the purpose of Chapters 2 and 3 was to describe both the procedures and the results of the process used to evaluate the performance, from sample preparation to final quantitation, of the analytical method used in the present thesis.

Now that the performance characteristics of the analytical method have been described the results of the insect development experiment can be presented and discussed. Therefore, the following chapter presents and discusses the results of the insect development experiment completed for the present thesis.

4.2 Experimental Methods

4.2.1 Maintenance of the Adult *Sarcophaga bullata* Colony

One hundred and fifty *Sarcophaga bullata* (Parker) (Diptera: Sarcophagidae) pupae were obtained from WARD's Biology (Ontario, Canada), and separated into three groups of approximately 50 pupae. Each group of 50 pupae was placed in the lid portion of a Petri dish on top of a slightly water dampened piece of filter paper. The Petri dishes were then placed into a 75 x 75 x 75 cm rearing cage at room temperature and allowed to emerge. The emerging adult *S. bullata* flies were given water, sugar and skim milk powder *ad libitum*. Three days after emergence, beef liver was added to the rearing cage to allow for ovarian and testes development, and to serve as the larviposition media. Fresh liver was added each day until sufficient larvae had been laid. These larvae were transferred to a rearing jar containing fresh beef liver and were reared until the insects had pupated. The pupae were then collected and placed into another 75 x 75 x 75 cm rearing cage. This F1 generation colony served as the stock colony for the experimental colonies.

Once the pupae of the F1 generation had emerged, they were provided with water, sugar and skim milk *ad libitum*, followed by fresh beef liver as the larviposition media. When sufficient larvae were deposited, they were transferred to rearing jars and reared until the insects had pupated. The pupae of the F2 generation was then divided into three groups of approximately 50 pupae. Each set of 50 pupae was then placed into separate 75 x 75 x 75 cm rearing cages. Each of the three cages was provided with water, sugar and skim milk powder *ad libitum*. These three F2 colonies provided all the insects used in the experiments and are referred to as the experimental or working colonies.

4.2.2 Experimental Design

Three pieces of fresh beef liver¹ were introduced into each of the three experimental colonies, and checked every 20 minutes for larvae. The experimental colonies did not deposit a sufficient quantity of larvae for the experiment until three hours after the liver was introduced into the rearing cages. After three hours, the liver pieces were removed from the rearing cages. The time of larviposition was recorded as 1.5 hours after the initial introduction of the larviposition media.

Eighteen rearing jars, three per type of rearing media, were set up with sawdust and labelled. One hundred grams of the appropriate rearing media was placed in each of the 18 rearing jars.

Approximately 400 larvae were placed in each of the 18 rearing jars. The larvae were transferred from the larviposition media to the rearing media using a blunt-tipped metal spatula. Only clumps of larvae were selected for transfer from the larviposition media to the rearing jars. Although the larvae on the perimeter of the larval mass may be damaged during transfer, larvae in the middle of the mass should be protected from damage caused during transfer. Therefore, only a subsection of the larvae deposited on each of the nine² pieces of liver used as larviposition media were used in the present experiment. In addition, preferentially selecting clumps of larvae

¹larviposition media

²Recall that three pieces of liver were placed in each rearing cage to serve as the larviposition media.

resulted in the selection of more larvae from one of the three experimental colonies compared with the other two. This was simply due to the fact that one experimental colony deposited substantially more larvae compared with the other two. Therefore larval selection was non-random. To alleviate the non-random selection of the insects, each clump of insects was transferred to a randomly selected rearing jar. As a result, the order in which the insects were transferred to each of the eighteen rearing jars was randomized through the use of a random number table.

Overall, three replicates were set up for each of the six types of rearing media. Figure 4.1 outlines the experimental design for the insect development experiment. Batch A refers to the artificial foodstuff containing 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline. Batch B refers to the artificial foodstuff containing 9.55 mg/kg amitriptyline and 3.21 mg/kg nortriptyline. Batch C refers to the artificial foodstuff containing 41.38 mg/kg amitriptyline and 1.92 mg/kg nortriptyline. Batch D refers to the artificial foodstuff containing 42.59 mg/kg amitriptyline and 1.22 mg/kg nortriptyline.

4.2.3 Sampling

As indicated in Figure 4.1, ten individual larvae, from each of the 18 jars, were to be sampled every eight hours. The larvae from each sample were then killed by placing them in hot water (approximately 90°C) for 30 seconds. After removal from the hot water, the larvae were placed in 75% ethanol for storage until the length of each larvae could be measured and its developmental stage determined.

During the first instar and second instar, the first ten larvae observed at each sampling period were collected. In the case of the liver control, selecting the first larvae observed was not a problem, for most of the larvae were still present on the outer surface of the liver. However, the artificial foodstuff was soft enough that the young first instar larvae could burrow into it, and as a result, the majority of the larvae were concealed from view.

When the larvae reached the third instar, the sampling of larvae from the rearing jars containing artificial foodstuff became difficult because the larvae were concealed

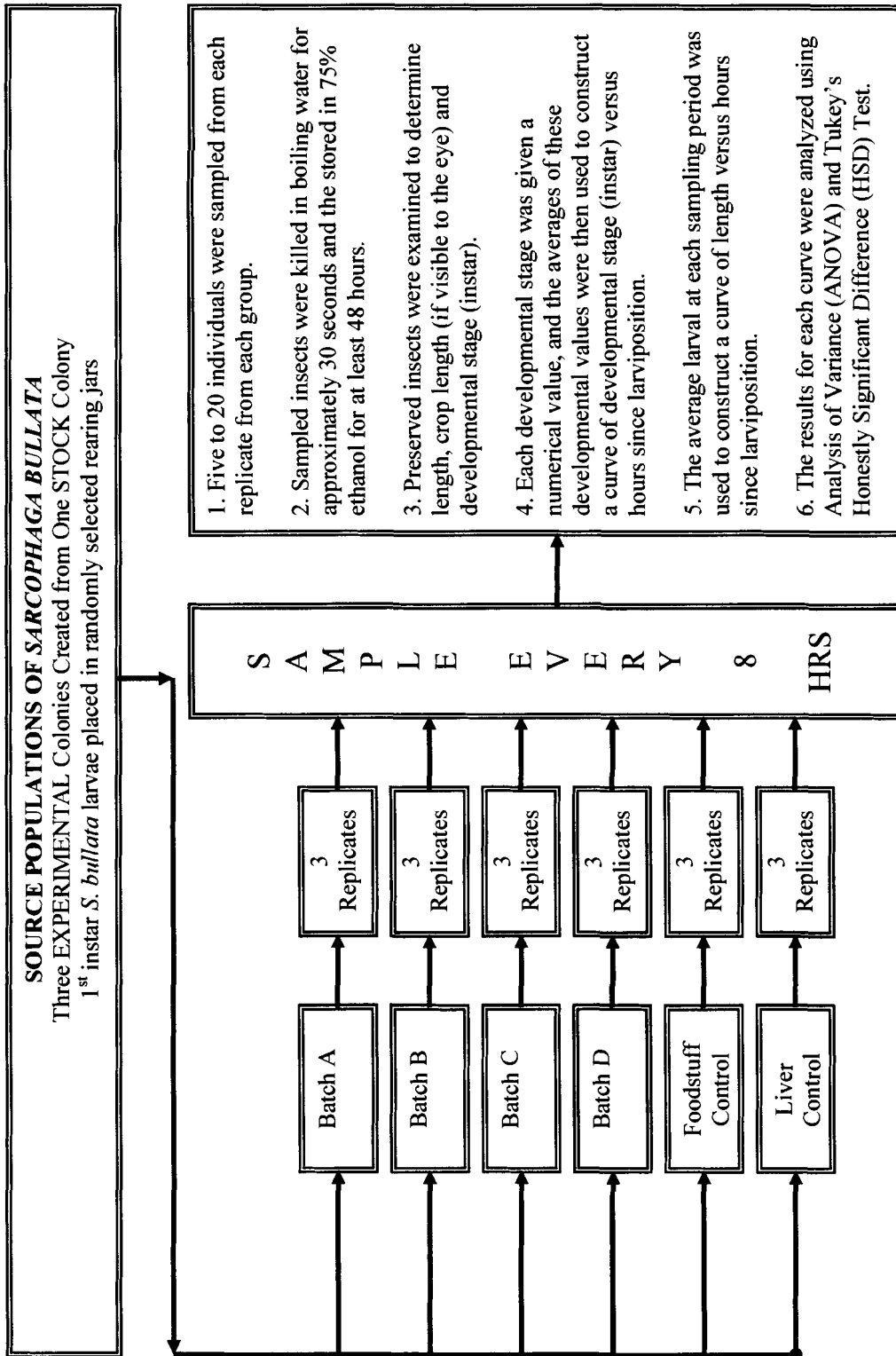


Figure 4.1: Outline of the experimental design used to investigate the effects of amitriptyline and nortriptyline on the development of *Sarcophaga bullata*. Batch A = 168.41 mg/kg AMT + 7.65 mg/kg nortriptyline; Batch B = 9.55 mg/kg AMT + 3.21 mg/kg NOR; Batch C = 41.38 mg/kg AMT + 1.92 mg/kg NOR; Batch D = 42.59 mg/kg AMT + 1.22 mg/kg NOR. (AMT = amitriptyline; NOR = nortriptyline).

by undigestable³ portions of the foodstuff. Rather than pick through the undigested portions of the foodstuff, a small handful of the undigested portions were removed at each sampling period and placed into the hot water. The hot water killed larvae present in the handful of indigestible foodstuff were collected and placed in 75% ethanol. Sampling of the third instar larvae from the liver proceeded in much the same way, except that the larvae reared on the liver could be removed directly from the surface of the liver, killed with hot water and then placed in the preservative.

Although the behaviour of the larvae and the condition of the rearing media made random sampling of the larvae difficult, no attempt was made to sample only the largest larvae observed in each rearing jar because the statistical tests used in the present thesis require normally distributed data in order to appropriately apply them to a data set. Sampling larvae of only a certain size has the potential to skew the distribution.

However, it should be noted that it is common practice in forensic entomology to use the largest larva present in the sample collected from the corpse to estimate the postmortem interval. Even so, some researchers, as discussed in the introduction, consider this method to be problematic, and advocate that the age of the largest larva from the most abundant larval stage collected from the corpse should be used to estimate the postmortem interval (Erzinclioglu, 1990). With this in mind, a substantial amount of larvae, of various sizes, should be collected from the corpse. The sampling procedure used in the present thesis was intended to more closely resemble the collection of a large number of larvae of various sizes, and possibly, ages from the corpse.

4.2.4 Rearing Jars

The rearing jars used throughout the experiment consisted of 4.5 L wide-mouthed glass jars that were lined with 10 cm of slightly water dampened sawdust, followed by a piece of folded paper towel. Beef liver or foodstuff, depending on the experimental treatment, was then placed on top of the paper towel. First instar larvae were then transferred from the larviposition media to the liver or foodstuff in the rearing jar

³post-consumption by larvae

using a blunt-tipped spatula. The jar was closed using a piece of 2-ply cotton netting approximately 30 cm², followed by two layers of paper towel. Both the cloth and the paper towel were secured with several elastic bands.

The cotton netting was necessary to prevent the *S. bullata* larvae from escaping. Previous rearings by the author demonstrated that the third instar *S. bullata* larvae could chew through two layers of paper towel but not the combination of paper towel and cotton netting.

4.2.5 Rearing Conditions

The larvae were reared in a Conviron E7A incubator (Controlled Environments Limited, Winnipeg, Manitoba, Canada) at 26°C, 75% relative humidity with a photoperiod of 16 hours light and 8 hours dark.

4.2.6 Data Analysis

The rate of *S. bullata* development was evaluated in the present thesis by two different techniques: (1) larval length, (2) developmental stage. All statistical analyses were performed in JMP IN[®] (SAS Institute, Inc., Cary, NC, USA).

One-way analysis of variance (ANOVA) was used to compare both the pooled mean maximum lengths, and the mean time required for the larvae reared on each of the six types of foodstuff to reach the mean maximum length. ANOVA assumes that the data is normally distributed, and that the variances associated with each of the groups are equal. JMP IN[®] (SAS Institute, Inc., Cary, NC, USA) provides four different tests for determining if the variance between the groups is unequal: Levene's test, the Brown-Forsythe test, O'Brien's test and Bartlett's test. If, at an alpha level of 0.05, any of these four tests revealed that the variances between the groups were unequal, the results of the Welch ANOVA for unequal variances was reported instead. Furthermore, if ANOVA indicated that there was a difference present between the larvae reared on the six types of rearing media, Tukey-Kramer's Honestly Significant Difference (HSD) test was used to determine which of the six groups were different from each other.

The development of *S. bullata* larvae was divided into two major stages, the larval

stage and the pupal stage, and one-way analysis of variance was also used to compare the mean duration of both the larval stage and pupal stage between the insects reared on the six different types of rearing media. The development of *S. bullata* larvae was further divided into three feeding stages (first, second and feeding third instars), and a prepupal stage. One way analysis of variance was then used to compare the mean duration of the each of these stages between the six groups of insects.

As with the mean maximum length comparisons, Levene's test, O'Brien's test, Bartlett's test, and the Brown-Forsythe test were used to test for the presence of unequal variances, and if, at an alpha level of 0.05, any of the four tests revealed that the variances between the groups were unequal, the results of the Welch ANOVA for unequal variances was reported. Further, if the results of the one-way ANOVA indicated that there was a difference present between the larvae reared on the six types of rearing media, Tukey-Kramer's Honestly Significant Difference (HSD) test was used to determine which groups were significantly different.

The mean pupal weight, and adult weight for the insects reared on the six different types of rearing media were also compared using one-way analysis of variance. Unequal variances were determined using the procedures described earlier in the present section.

4.2.7 Larval Length as an Indicator of Larval Age

The total length and crop length (if visible) of each larva preserved at each sampling period was measured using a 10 x 10 mm sheet of laminated graph paper under a binocular microscope. The larvae were measured crop side up, with their posterior end lined up with the edge of one of the thickened gradations. The length was measured to the nearest 0.1 mm. All of the insects sampled were measured over a period of two months (July and August 2003), after storage in 75% ethanol for at least 24 hours. Preservation of the killed larvae was required because of the large number of larvae sampled at each sampling period, and because of the length of time required to sample the insects from each of the 18 rearing jars. Length measurements were not taken from any of the preserved larvae until after they had been in the preservative for at least 24 hours because research conducted by Adams and Hall (2003) indicated

that the majority of the postmortem changes in the length of hot water killed larvae occurred within the first 12 hours of placing the larvae in the preservative. However, it should be noted that Adams and Hall (2003), investigated the effects of 80% ethanol on hot water killed postfeeding third instar larvae of the species *Calliphora vomitoria* (L.). Therefore, these results may not be directly transferable to the larvae of the species *Sarcophaga bullata*. At the present time data on the postmortem changes in the length of hot water killed larvae are not available for species of the Family Sarcophagidae.

The average maggot length from each sampling period, for each experimental set and control was plotted against the hours since larviposition using Microsoft®Excel 2002 (Microsoft Corporation, 1981-2001). The larvae at each sampling period were not separated according to developmental stage (i.e. instar). Instead, the length of each maggot was measured, and then an average larval length was computed for each sampling period. This is a commonly used technique for investigating the rate of insect development, and has been use by a number of researchers, including Goff *et al.* (1993) and Bourel *et al.* (1999).

Larvae increase in length over time until a maximum length is reached. The maximum length is generally attained at peak feeding during the third instar. Length can be used as a measure of insect growth until the onset of pupariation. When pupariation begins, the length of the larvae decrease in length until pupation.

With this in mind, the time required for the larvae to reach the mean maximum length can be used as a comparative measure for evaluating differences in the rate of development between sets of larvae reared under different experimental conditions.

In this research project both the time required to reach the mean maximum length, and the mean maximum length, were compared for each type of rearing media. The statistical significance of any observed differences in mean maximum length, and the time required to reach the mean maximum length, were evaluated using one-way analysis of variance (ANOVA). The Tukey-Kramer Honestly Significant Difference (HSD) test for multiple comparisons was used to identify which groups were significantly different from each other. All statistical tests were performed in the statistical program JMP IN®(SAS Institute, Inc., Cary, NC, USA).

In addition, because the measurement process extended over a fairly long period of time, ten third instar larvae were selected for a measurement test. These ten insects were numbered and stored in separate vials of 75% ethanol. Both the body length and the crop length for each of the ten larvae were recorded at five different times during the two month measurement period. The same ten insects were measured once at the beginning of the measurement period, once at the end, and three times in the middle. Overall, the length measurements for the measurement test larvae were conducted at roughly two week intervals. The purpose of this test was to estimate the amount of intra-observer error present in the insect measurement process.

4.2.8 Developmental Stage as an Indicator of Maggot Age

The development of *Sarcophaga bullata* was divided into six stages. Stages 1-3 represented the feeding instars, including the feeding phase of the third instar, stage 4 represented the prepupal stage, stage 5 represented the pupal stage, and stage 6 represented the adult stage. Each developmental stage was assigned a numerical value, with the value increasing according to increasing insect age (Table 4.1).

Developmental Stage	Developmental Marker	Numerical Value
First Instar	Two sets of poorly defined spiracular slits.	1
Second Instar	Two sets of two well-defined, chitinized spiracular slits	2
Feeding and Postfeeding Stage (Third Instar)	Two sets of three well-defined spiracular slits	3
Prepupal Stage (Third Instar)	No visible crop	4
Pupal Stage	Formation of pupa	5
Adult	Emergence of adult fly	6

Table 4.1: The developmental marker for each developmental stage, and the numerical value assigned to each development stage of *Sarcophaga bullata* larvae in the insect development study.

First, second and third instar larvae were distinguished primarily on the basis of the number of posterior spiracular slits. Third instar larvae have two sets of three

well-differentiated posterior spiracular slits, second instar larvae have two sets of well-differentiated spiracular slits, and first instar larvae have two sets of two poorly differentiated spiracular slits that are attached at the bottom. The posterior **spiracles** of the flesh flies (Family Sarcophagidae), including *S. bullata*, are located at the bottom of a depression at the tip of the abdomen, the edges of which are rimmed with fleshy tubercles (Byrd and Castner 2001).

When the crop was no longer visible under the dissecting microscope, the larva was identified as a prepupal larva. Even though the crop was not visible to the eye, it may not be completely empty at this point. Therefore, it is important to note that the definition used in the present thesis likely overestimated the duration of the prepupal stage. As mentioned in the introduction, it is important that the prepupal stage not be confused with the postfeeding stage, because during the prepupal stage, which is relatively short in duration, the larvae start to irreversibly shrink in size (Greenberg and Kunich, 2002)

One other drawback to the developmental stage classification scheme used in the present thesis, was the fact that the scheme was unable to distinguish between the feeding stage of the third instar and the postfeeding stage of the third instar. The differentiation between the third instar and the prepupal stage was defined as the point in which the crop was no longer visible to the eye. Previous to this point in time, the crop would have reached a maximum length, and then would have started to shrink in size. However, given the changes that were observed during the measurement test, changes in crop length would not have been a consistent age marker for *S. bullata* larvae. Therefore, decreases in visible crop length were not used to determine the stage of the developing larvae during this investigation. This is unfortunate, because for many species (e.g. *Calliphora vicina*), decreases in crop length can indicate the age of a postfeeding larva at forensically useful intervals, such as three hours, nine hours, etc. (Greenberg and Kunich, 2002).

Insects that were in the middle of a moult were given a value half way between the insect's previous stage and the insect's next stage. Moulting, for the purposes of this experiment was considered to be imminent when the spiracular slits of the upcoming developmental stage were visible behind the spiracular slits of the preceding stage.

For example, if three spiracular slits were visible behind two spiracular slits, the larvae was given a numerical value of 2.5. If the larvae were shaped like a pupa, and displayed a corresponding reduction in length, but had yet to tan⁴, the insect was given a numerical value of 4.5. There were two drawbacks to this method of assigning developmental values. First, a value of 3.5 could not be accurately assigned because the assignment would have been too subjective. For this experiment, the goal was to assign developmental values based on simple objective criteria, such as the number of spiracular slits. Similarly, a value of 5.5 could not be assigned because pupae would have to be sacrificed in order to do so.

In the present research project, the onset of the next developmental stage is defined as the time at which 50% of the sample had entered the next developmental stage. Therefore, the duration of the larval period was defined as the time from larviposition to the time at which 50% of the sampled population had pupated. Likewise, the duration of the pupal period was defined as time at which 50% of the sampled population had pupated to the time at which 50% of the sampled population had emerged as adults. With this in mind, when the larval development was divided into instars, the duration of an instar was defined as the time at which 50% of the sample had entered the next instar. For example, given a sample of ten maggots, if five of the maggots were second instar and the other five third instars, then onset of the third instar would be taken as the time that this sample was collected.

4.3 Insect Development Experiment

4.3.1 Measurement Test Results: Estimation of Intra-observer Error

The mean body length (\pm SD) and the mean crop length (\pm SD) for the ten third instar *Sarcophaga bullata* larvae set aside for the measurement test are presented in Table 4.2. The same 10 larvae were measured five times over a period of two

⁴During pupation, the puparium hardens and darkens in colour. Tanning occurs over a period of approximately 10 hours, and the puparium changes from white to dark brown in colour.

months (July and August, 2003). The relative standard deviation (RSD), expressed as a percentage, associated with the five measurements of both total length and crop length, on each of the ten larvae are also presented in Table 4.2.

Insect #	Total Length		Crop Length	
	Mean \pm SD (mm)	RSD (%)	Mean \pm SD (mm)	RSD (%)
1	16.6 \pm 0.15	0.92	6.8 \pm 0.27	4.0
2	16.4 \pm 0.08	0.51	7.5 \pm 0.15	2.0
3	16.3 \pm 0.13	0.80	7.6 \pm 0.22	2.8
4	16.0 \pm 0.05	0.34	6.5 \pm 0.46	7.0
5	16.4 \pm 0.19	1.1	7.7 \pm 0.31	4.1
6	18.0 \pm 0.04	0.25	9.2 \pm 0.18	1.9
7	17.1 \pm 0.09	0.52	6.7 \pm 0.27	4.1
8	15 \pm 0	0	5 \pm 0	0
9	17.2 \pm 0.1	0.58	7.2 \pm 0.43	6.0
10	16.6 \pm 0.05	0.33	7.1 \pm 0.08	1.2

Table 4.2: The mean total length \pm SD and the mean crop length \pm SD for the ten third instar *Sarcophaga bullata* larvae, measured five times over a period of two months (July and August, 2003). The relative standard deviation, RSD, (%) of the five measurements made for both total length and crop length, on each of the ten larvae are presented as well.

Table 4.2 indicates that the measurement of total larval length as a function of time is quite precise. In fact, the highest coefficient of variation is only 1.1%. Therefore, the precision is known to be better than 1.1%. In comparison, a lower degree of precision was obtained for the crop length measurements. The precision of the crop length measurements were only known to be better than 7.0%. In addition, a trend of decreasing crop length for each insect over time was observed. This may be an indication that the crop becomes less visible after storage in 75% ethanol, and as a result, the decrease in the visibility of the crop over time may be a storage artefact. This has important implications for age estimations based on the ratio of crop length to total body length, particularly when the larvae have been stored in a preservative such as 75% ethanol for a considerable period of time. Therefore, because the measurement of visible crop length was shown to be unreliable due to the

preservation technique, visible crop length was not used in the present thesis as an indicator of larval age. Furthermore, since crops are also visible in the second instar, the effect of preservation and storage time on the visible crop length of second instar larvae should also be investigated.

In addition, it should be noted that this measurement test does not provide a measure of intra-observer measurement accuracy. A determination of intra-observer measurement accuracy would require the comparison of larval lengths obtained with several different methods. In addition, the above method only measured the precision of the measurement process for fully elongated third instar larvae. Given the fact that the same technique for determining both total length and crop length was used for all three stages of larval development, it was assumed that measurement precision would not change as a function of developmental stage. This assumption should be tested in future work.

4.3.2 Larval Length as an Indicator of Larval Age: Results

Mean Time Required to Reach the Mean Maximum Length

The mean, the standard deviation (SD), and the relative standard deviation (RSD), for the time required for the larvae in each experimental group to reach the mean maximum length is presented in Table 4.4. The larvae reared on foodstuff containing 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline (Batch A), at a mean of 83.3 hours, required the greatest amount of time to reach the mean maximum length (Table 4.4). In contrast, the larvae reared on the foodstuff control⁵, at a mean of 53.5 hours, required the least amount of time to reach their mean maximum length. Overall, the order, in terms of decreasing mean time required reach mean maximum length, was as follows (Table 4.4):

Batch A > Liver Control > Batch C > Batch B > Batch D > Foodstuff Control

One-way analysis of variance (ANOVA) of the data presented in Table 4.3 indicated that the differences between each group of foodstuff, in terms of the time required for the larvae to reach the mean maximum length, was not statistically significant at an alpha level of 0.05 ($F_{5,12} = 1.90$; $P = 0.17$; Table 4.3).

Given the high variation, measured in terms of relative standard deviation, observed between the three replicates within each type of rearing media, the differences in the mean time required to reach the mean maximum length for each type of rearing media was not unexpected. For instance, the variation between the three replicates in Batch A, at 28.8%, was substantial (Table 4.4). Considerable variation was also observed between the replicates within each of the remaining groups of drug-spiked artificial foodstuff. For instance, the variation in the time required to reach the mean maximum length between the replicates was 23.4% for Batch C, 17.7% for Batch B, and 17.5 % for Batch D (Table 4.4). In contrast, the variation in the times required to reach the mean maximum length, between the three foodstuff control replicates, at 3.0%, was considerably lower compared to the variation observed within the other

⁵i.e. drug-free foodstuff

groups of artificial foodstuff. Overall, the smallest variation between replicates, in terms of the time required for larvae reared on a particular type of foodstuff to reach their mean maximum length, was observed for the Liver Control replicates, as the variation between the three Liver Control replicates was only 1.3% (Table 4.4).

Source of Variation	DF	Sums of Squares	Mean Square	<i>F</i>	<i>P</i>
Rearing Media	5	1721.30	344.26	1.90	0.17
Error	12	2177.10	181.43	-	-

Table 4.3: Results of one-way ANOVA for the mean time required for the larvae reared on six different types of rearing media to reach the mean maximum length, at 26°C.

Foodstuff ($n = 3$)	Mean Time \pm SD Required to Reach Mean Maximum Length (hr.)	Relative Standard Deviation (%)
Liver Control	71.8 \pm 0.9	1.3
Foodstuff Control	53.5 \pm 1.6	3.0
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	83.3 \pm 24.0	28.8
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	63.5 \pm 11.2	17.7
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	71.4 \pm 16.7	23.4
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	58.5 \pm 10.2	17.5

Table 4.4: Mean, standard deviation (SD), and relative standard deviation for the time (hrs) required for *Sarophaga bullata* larvae, for each type of rearing media, at 26°C to reach their respective mean maximum length (AMT = amitriptyline; NOR = nortriptyline).

Mean Maximum Larval Length

The mean maximum length, standard deviation and relative standard deviation (RSD) for each replicate, within each type of rearing media, are summarized in Table 4.5. The number of larvae sampled in each replicate varied from a minimum of ten to a maximum of 34. The difference in sample size was due to the problems associated with extracting the larvae from the undigestible portion of the artificial foodstuff.

Considerable variation in larval length was observed for insects of relatively the same age (± 1.5 hours). This variation is illustrated by the rather large relative standard deviations associated with each replicate, within each type of rearing media (Table 4.5). The maggots used to generate the mean maximum lengths were mostly third instar, except for a few postfeeding larvae. Normally, postfeeding larvae, especially those nearing the onset of pupariation, would not be used to generate the mean maximum lengths since the overall maggot length starts to shrink at this stage. However, the lengths of the postfeeding maggots were within the range of maggot lengths observed for the feeding third instar maggots, and as a result, there was no reason to exclude the lengths of the prepupal maggots.

Since each of the three replicates for each type of rearing media were set up in an identical manner, at the start of the experiment, with approximately the same number of larvae, it is reasonable to pool together the larval lengths from each of the three replicates for each type of rearing media. Further, given the fact that only three replicates were set up for each type of rearing media, the removal of outliers revealed through statistical tests is not warranted. The mean pooled length, standard deviation and relative standard deviation for each type of rearing media is also presented in Table 4.5.

The greatest mean maximum length, at 20.4 ± 1.4 mm, was observed in the Liver Control replicates. In comparison, the smallest mean maximum length was observed in the larvae collected from the rearing media containing 55.7 mg/kg amitriptyline and 1.7 mg/kg nortriptyline (Batch C). In fact, the mean maximum lengths observed for the individual replicates of Batch C were considerably different. The mean maximum length observed for Replicate 3 of Batch C was quite different from the mean maximum

Rearing Media	Mean Maximum Maggot Length per Replicate			Pooled Mean Maximum Lengths	
	Replicate	Mean \pm SD (mm)	RSD (%)	Mean \pm SD (mm)	RSD (%)
Liver Control	R1 ($n = 10$)	20.1 \pm 2.2	11.0	20.4 \pm 1.4	6.9
	R2 ($n = 21$)	19.9 \pm 1.1	5.4	20.4 \pm 1.4	
	R3 ($n = 20$)	21.0 \pm 0.9	4.2		
Foodstuff Control	R1 ($n = 21$)	16.3 \pm 1.5	8.9	16.9 \pm 1.6	9.2
	R2 ($n = 21$)	16.6 \pm 0.8	5.0		
	R3 ($n = 23$)	17.6 \pm 1.9	10.5		
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	R1 ($n = 20$)	16.9 \pm 0.8	4.9	17.2 \pm 1.1	6.1
	R2 ($n = 20$)	17.9 \pm 1.0	5.6		
	R3 ($n = 10$)	16.3 \pm 0.6	3.6		
Batch B (22.0 mg/kg AMT; 3.21 mg/kg NOR)	R1 ($n = 33$)	17.2 \pm 2.0	11.4	16.6 \pm 1.8	11.1
	R2 ($n = 34$)	16.7 \pm 0.7	4.2		
	R3 ($n = 21$)	15.7 \pm 2.4	15.4		
Batch C (55.7 mg/kg AMT; 1.7 mg/kg NOR)	R1 ($n = 13$)	17.3 \pm 0.9	4.9	15.5 \pm 2.0	12.9
	R2 ($n = 17$)	16.0 \pm 1.1	6.6		
	R3 ($n = 20$)	14.0 \pm 2.0	14.3		
Batch D 1.0 (59.3 mg/kg AMT; mg/NOR)	R1 ($n = 20$)	16.8 \pm 0.9	5.1	16.6 \pm 0.8	5.1
	R2 ($n = 10$)	16.6 \pm 1.0	5.8		
	R3 ($n = 10$)	16.4 \pm 0.7	4.3		

Table 4.5: The mean larval length, standard deviation (SD), and relative standard deviation (RSD) for each replicate for each type of rearing media, and the mean pooled larval length, standard deviation, relative standard deviation for each type of rearing media used to rear larvae of the species *Sarcophaga bullata* (Diptera: Sarcophagidae) at 26°C.

lengths observed for Replicates 1 and 2 of Batch C. The differences in the mean maximum lengths observed between the Batch C replicates may be the result of several factors, including, but not limited to, genetic differences between the larval groups placed in each of the rearing jars, and differences in the number of insects added to each rearing jar. The effect of these two factors on the experiment were considered in the experimental design, but perhaps more rigorous procedures are necessary in future experiments. For instance, approximately 400 larvae were placed in each rearing jar, and the number of larvae was estimated based on the size of the larval groups being transferred. A more rigorous procedure would be to accurately count the number of larvae placed in each rearing jar. In any event, no statistical tests for outliers were conducted given the fact that there exists only three replicates for each batch of foodstuff.

One-way ANOVA analysis of the pooled length data indicated that the differences in mean maximum length between the six different forms of rearing media were statistically significant at an alpha level of 0.05 ($F_{5,338} = 58.97$; $P < 0.0001$; Table 4.6). Further analysis with Tukey's Honestly Significant Difference (HSD) pairwise comparison procedure to control for multiple testing, indicated that the mean maximum lengths (\pm SD) for Batches A, B, C, D, and the Foodstuff Control were significantly less than the mean lengths (\pm SD) for the Liver Control (Table 4.4; Table 4.5). Furthermore, only the mean maximum lengths from the larvae reared on artificial foodstuff containing 41.38 mg/kg amitriptyline and 1.92 mg/kg nortriptyline (Batch C) were shown to be significantly different from the mean maximum lengths obtained for the larvae reared on the other four types of artificial foodstuff (Table 4.4; Table 4.5). However, given the much lower mean maximum length observed for Replicate 3, Batch C, this result was not unexpected.

As discussed in Chapter 3, only Batches A and D were determined to be homogeneous in terms of the spiked amitriptyline concentration. With this in mind, it is possible that the inhomogeneity of Batch C resulted in one replicate with a considerably lower mean maximum length compared to the other two replicates. Overall, the mean maximum lengths of the larvae reared on artificial foodstuff were significantly different from the lengths of larvae reared on beef liver (Control). This trend is clearly

illustrated in the box and whisker plot of the pooled maximum length data for each type of rearing media (Figure 4.2).

The order, in terms of decreasing mean maximum pooled length was as follows (Table 4.5):

Liver Control > Batch A > Foodstuff Control > Batch B = Batch D > Batch C

Source of Variation	DF	Sums of Squares	Mean Square	<i>F</i>	<i>P</i>
Rearing Media	5	719.12	143.83	58.97	< 0.0001
Error	338	824.39	2.44	-	-

Table 4.6: The results for the one-way ANOVA analysis of the pooled mean maximum length data for *Sarcophaga bullata* larvae reared on six different types of rearing media.

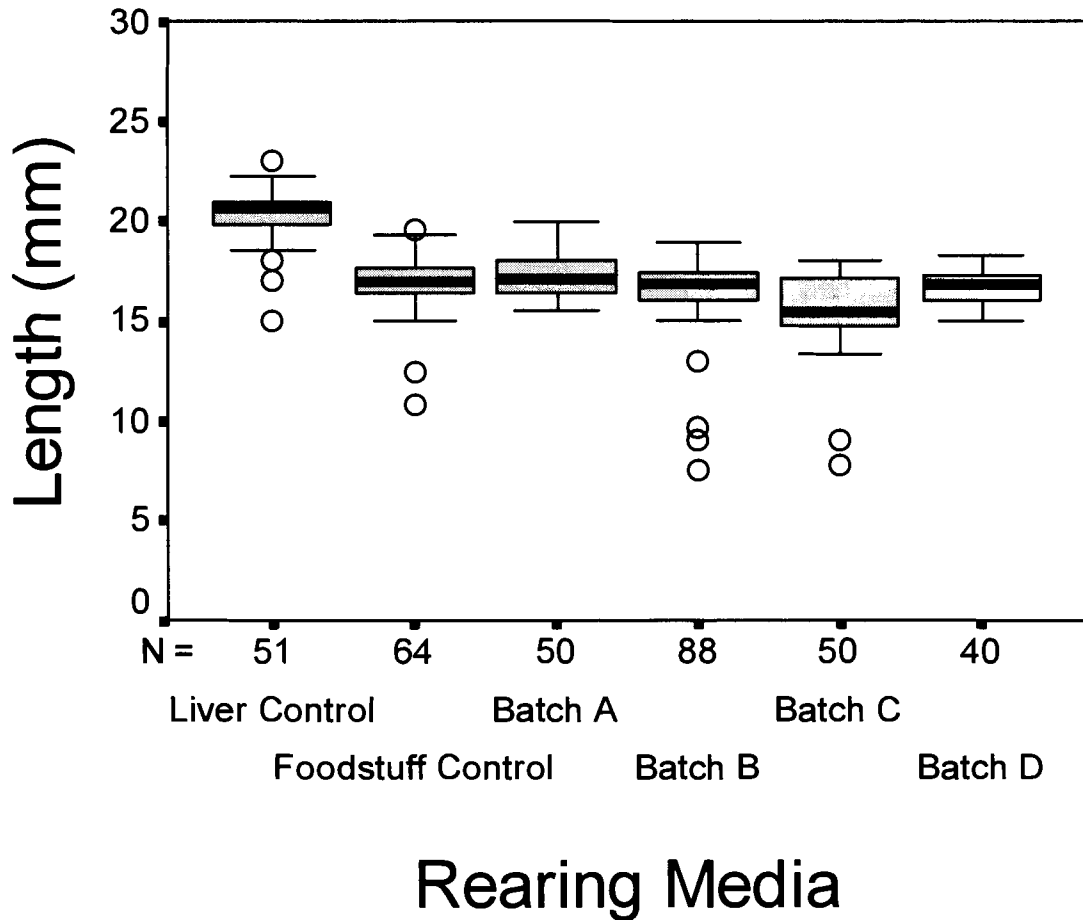


Figure 4.2: Box and whisker plot of the length data used to calculate the mean maximum larval length for larvae reared on each of the six types of rearing media at 26°C. The box and whisker plot was constructed using the length data from all three replicates from each type of rearing media. The plot shows the median length for each type of rearing media as a thick black bar. The interquartile range of the length data for each type of rearing media is shown as a box. The range of the length data for each type of rearing media is illustrated by the thin black bars (whiskers). Data points that are outside the range of the other points observed for the data set are illustrated by circles.

Larval Development Curves: Mean Length *versus* Time

The development curves created from the length data for the first experimental set are illustrated in Figures 4.3 through 4.8. Replicates 1 and 2 from Batch B required a slightly different procedure to obtain the mean maximum lengths. As illustrated in Figure 4.4, the mean maggot lengths for these two replicates of Batch B plateaued for two sampling periods. The mean lengths for the larvae of Replicate 1 plateaued between approximately 70 and 81 hours, and for Replicate 2, the mean lengths plateaued between approximately 53 and 71 hours. Therefore, the time at which the mean maximum length was achieved for Replicates 1 and 2 of Batch B was estimated by calculating the average of their respective plateau times.

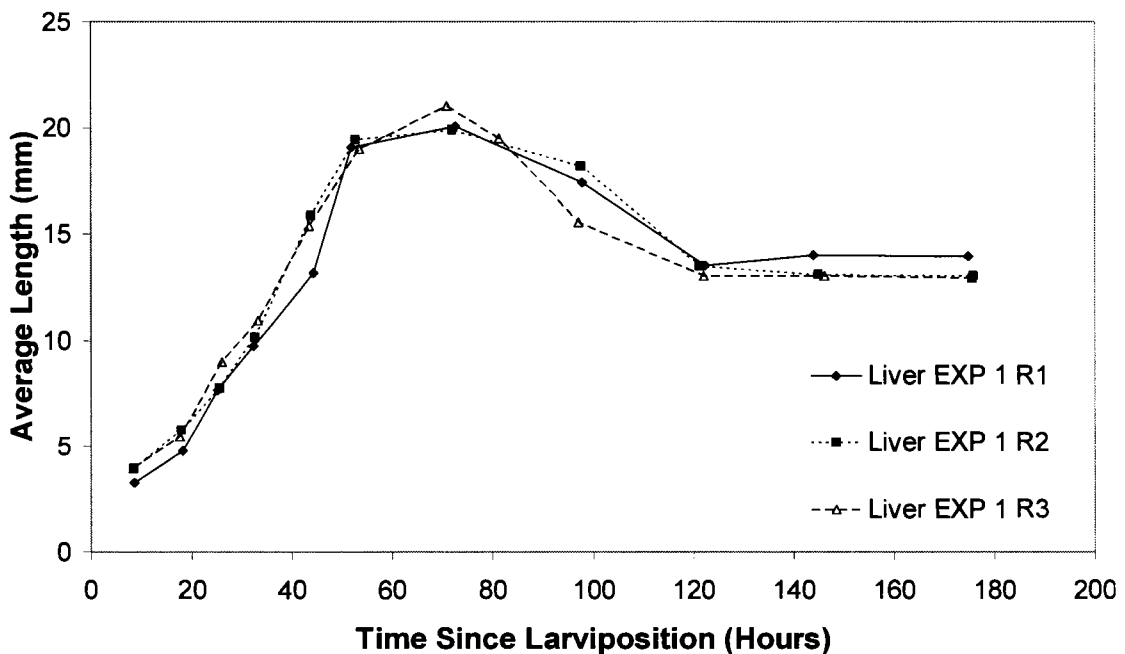


Figure 4.3: Mean larval length vs. time since larviposition (hrs) for *Sarcophaga bullata* larvae reared on the Liver Control at 26°C.

All three replicates of larvae reared on the liver (Liver Control) experienced a relatively smooth and constant increase in mean body length until the mean maximum length was achieved (Figure 4.3). The same relatively smooth and constant increase in mean body length was observed in all three replicates of larvae reared on the blank

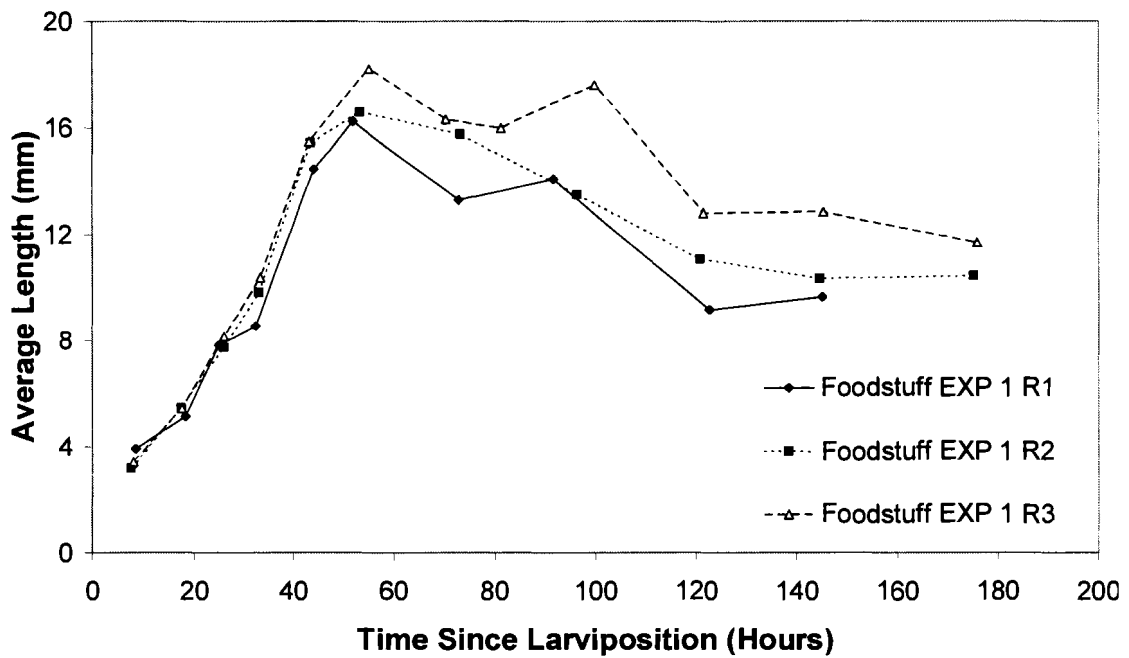


Figure 4.4: Mean larval length vs. time since larviposition (hrs) for *Sarcophaga bullata* larvae reared on the Foodstuff Control at 26°C.

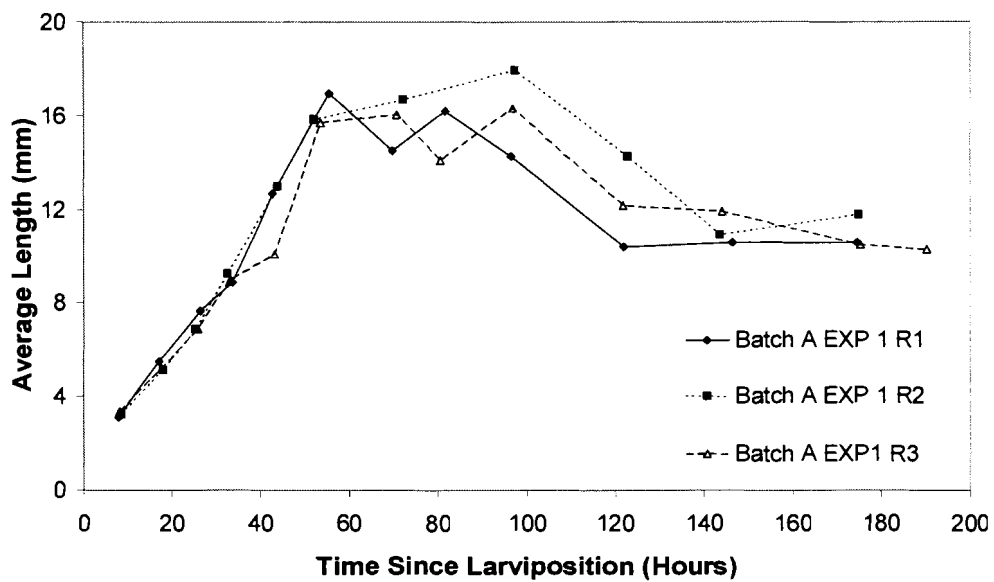


Figure 4.5: Mean maggot lengths vs. time since larviposition (hrs) for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline (Batch A) at 26°C.

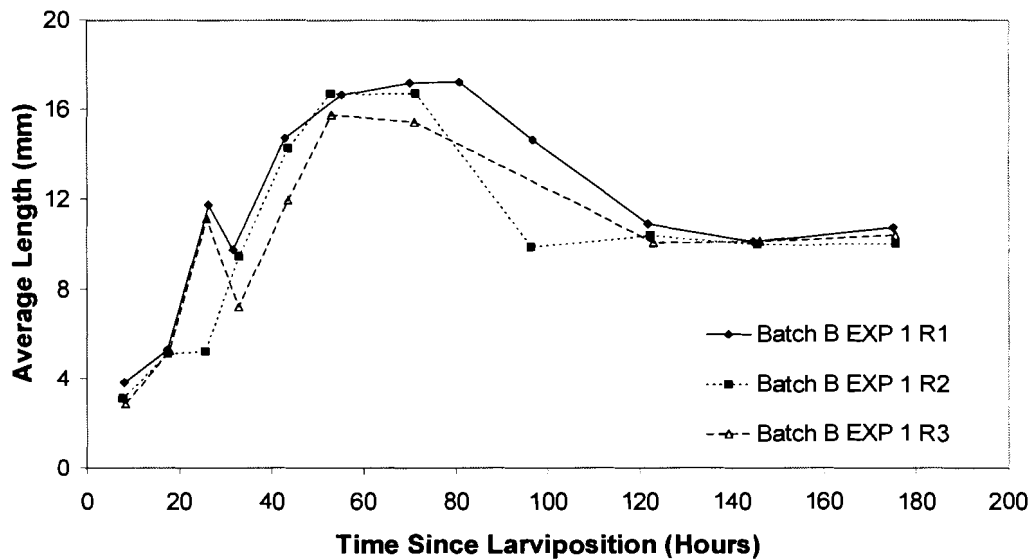


Figure 4.6: Mean maggot lengths vs. time since larviposition (hrs) for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 22.0 mg/kg amitriptyline and 3.21 mg/kg nortriptyline (Batch B) at 26°C.

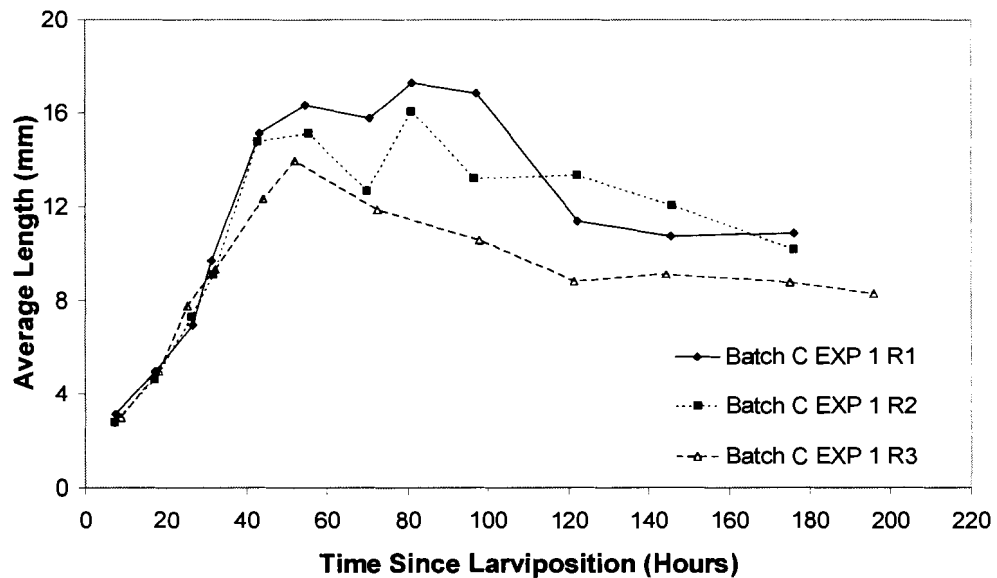


Figure 4.7: Mean maggot lengths vs. time since larviposition (hrs) for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 41.38 mg/kg amitriptyline and 1.92 mg/kg nortriptyline (Batch C) at 26°C.

artificial foodstuff (Foodstuff Control) (Figure 4.4).

In comparison, only one of the three larval replicates reared on the artificial foodstuff containing 22.0 mg/kg amitriptyline and 3.21 mg/kg nortriptyline (Batch B) experienced a relatively smooth increase in length over time. Given the inhomogeneity of Batch B, this result was not surprising. Both Replicates 1 and 3 of Batch B experienced a sharp decrease in mean larval length at approximately 33 hours since larviposition (Figure 4.6). The larvae sampled from Batch B Replicate 1 at approximately 33 hours since larviposition were a mix of moulting second instars and feeding third instars. Larval lengths at that time ranged from a minimum of 8.7 mm to a maximum of 10.6 mm. In Batch B Replicate 2, larval lengths ranged from 8.6 mm to 10.1 mm, and all of the insects sampled were second instars. In contrast, the larvae sampled from Batch B Replicate 3 at approximately 33 hours since larviposition were a mix of feeding second instars and moulting second instars. Larval lengths at that time ranged from 2.6 mm to 10.6 mm.

In addition, the variation in larval lengths at 33 hours since larviposition for Batch B Replicate 3 were quite large, as indicated by the magnitude of the error bars in Figure 4.12C. The errors bars in the figure represented the standard deviation about the mean. The variation in larval lengths at 33 hours since larviposition for Batch B Replicate 1 (Figure 4.12A) and Replicate 2 (Figure 4.12B) was considerably lower than the variation in length observed for Batch B Replicate 3 (Figure 4.12C).

The larvae sampled from Replicates 1 and 2 of Batch C also exhibited a sharp decrease in mean body length before the mean maximum length was achieved (Figure 4.13). The sharp decrease in the mean larval length occurred approximately 70 hours after larviposition in both Replicates 1 and 2. In contrast, although Replicate 3 of Batch C exhibited a similar sharp decrease in mean larval length at approximately 70 hours, the following sharp increase in mean larval length, which was observed in both Replicates 1 and 2 of Batch C at approximately 96 hours. In fact, the overall mean maximum length for larvae from Batch C Replicate 3 was achieved at approximately 52 hours after larviposition. Therefore, it appears that the larvae in Replicate 3 of Batch C were developing at faster rate compared to the larvae in Replicates 1 and 2.

Larval lengths for the larvae sampled from Batch C Replicate 1 ranged from

13.2 mm to 18 mm. Furthermore, all of the insects sampled were feeding third instar larvae. In comparison, the larvae sampled from Batch Replicate 2, 70 hours after larviposition, were a mixture of feeding and postfeeding third instar larvae. Larval lengths for Replicate 2 ranged from 5 mm to 15.3 mm. The shortest larva (5 mm) in sample obtained from Replicate 2 was a postfeeding larva. In comparison, the larvae sampled from Batch C Replicate 3, ranged from 9.5mm in length to 13.8 mm in length, and were also a mixture of feeding and postfeeding third instar larvae.

Graphs illustrating the mean length (\pm SD) *versus* time since larviposition, for each replicate, for each type of rearing media are illustrated in Figures 4.9 through 4.12.

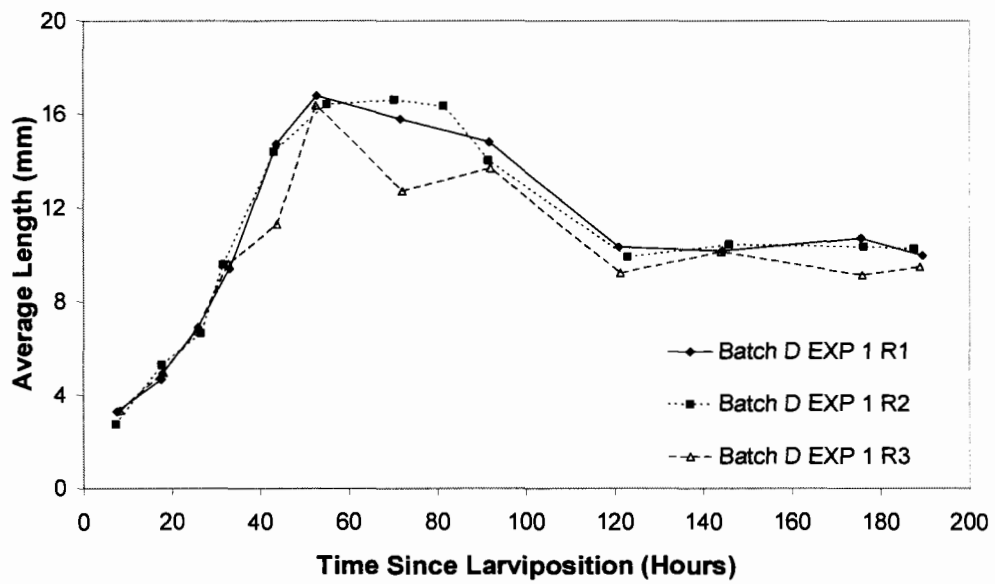
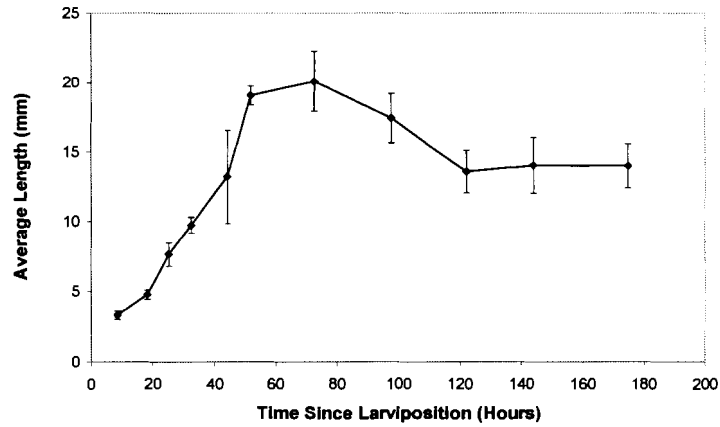
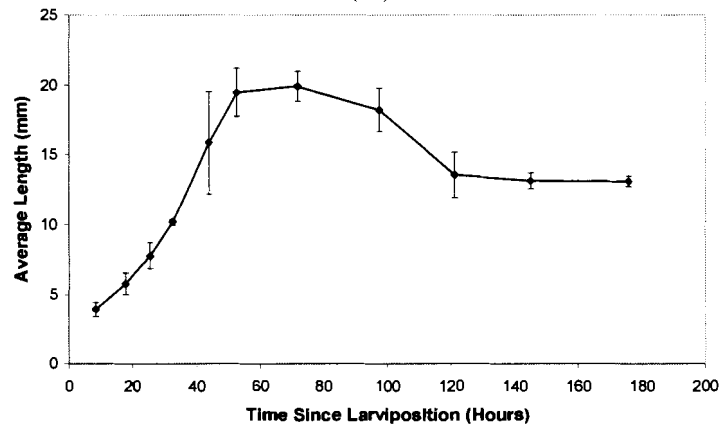


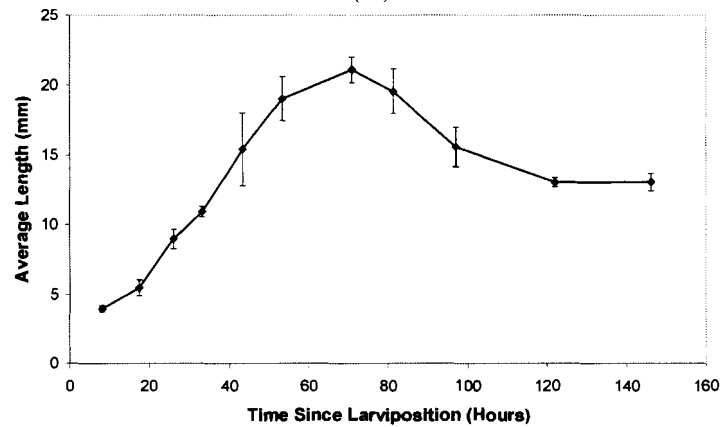
Figure 4.8: Mean maggot lengths vs. time since larviposition (hrs) for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 42.59 mg/kg amitriptyline and 1.22 mg/kg nortriptyline (Batch D) at 26°C.



(A)



(B)



(C)

Figure 4.9: Mean larval length (\pm SD) versus time since larviposition for the three replicates of *Sarcophaga bullata* larvae reared on liver at 26°C. (A) Liver Control Replicate 1, (B) Liver Control Replicate 2 and (C) Liver Control Replicate 3.

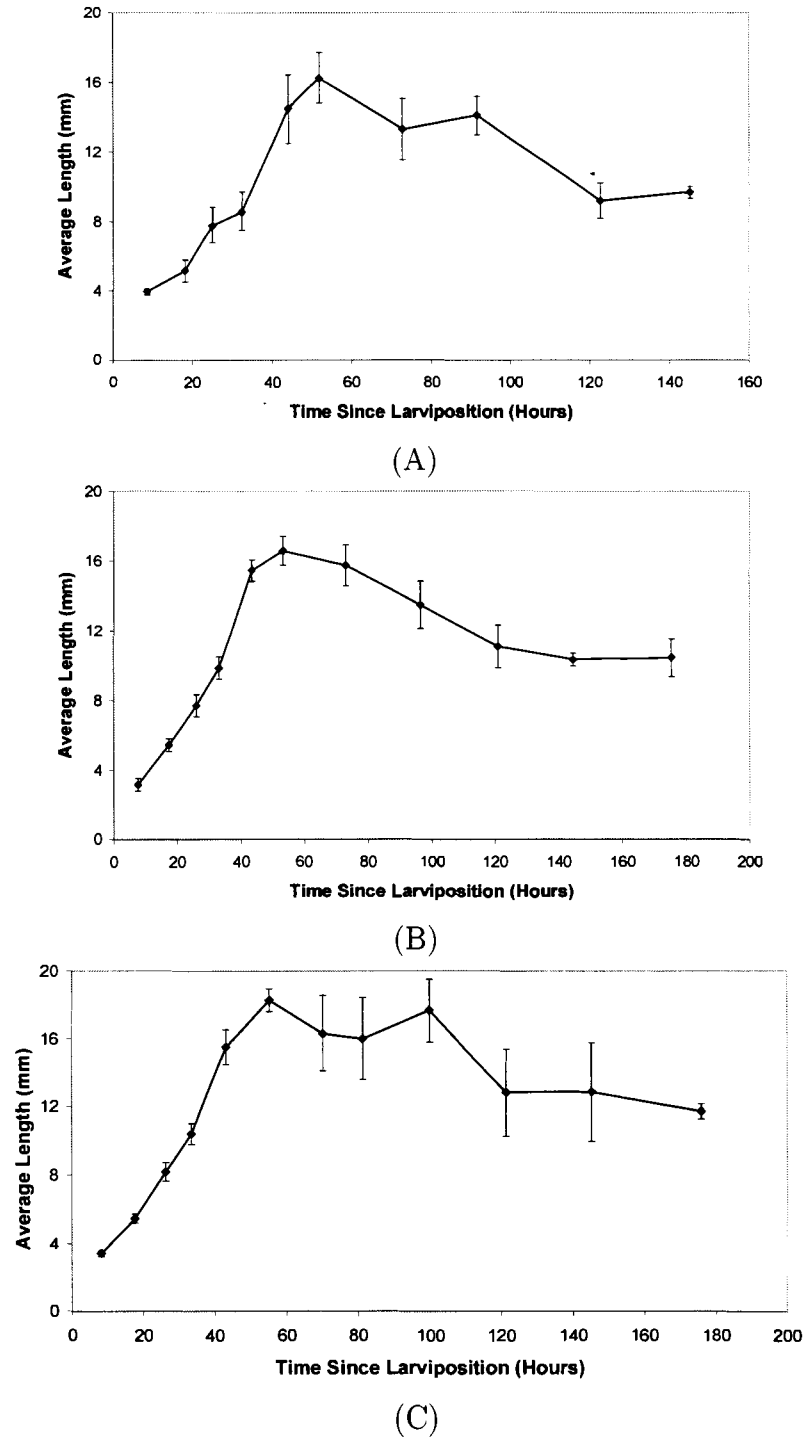
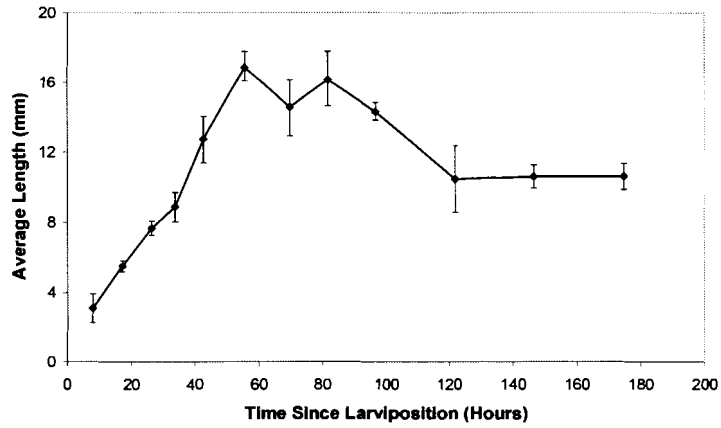
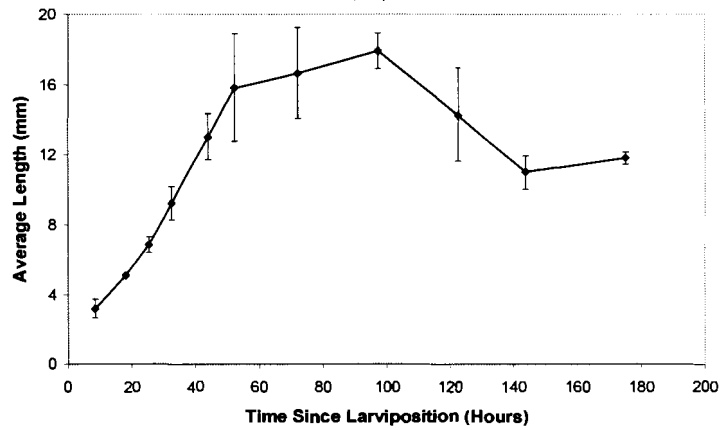


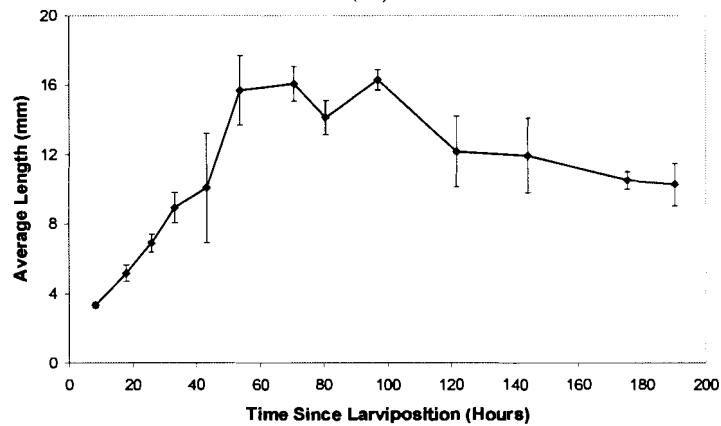
Figure 4.10: Mean larval length (\pm SD) *versus* time since larviposition for the three replicates of *Sarcophaga bullata* larvae reared on blank artificial foodstuff (Foodstuff Control) at 26°C. (A) Foodstuff Replicate 1, (B) Foodstuff Replicate 2, and (C) Foodstuff Replicate 3.



(A)

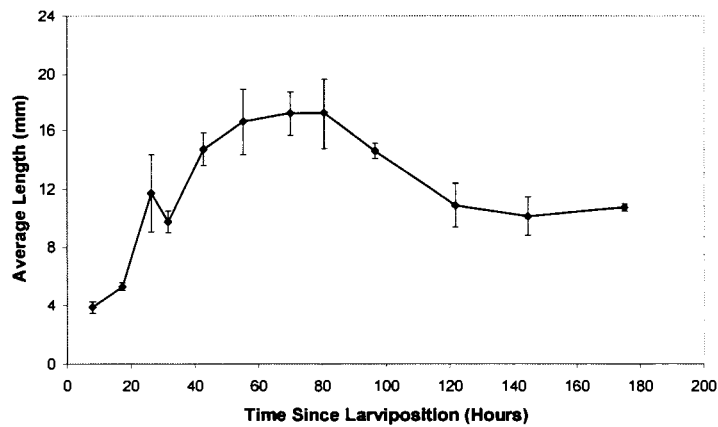


(B)

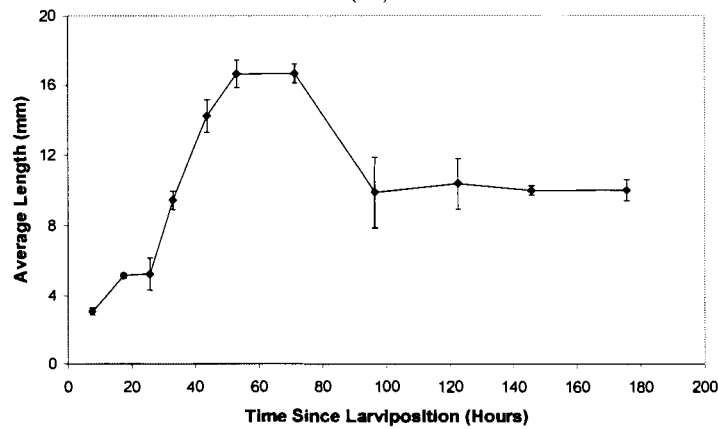


(C)

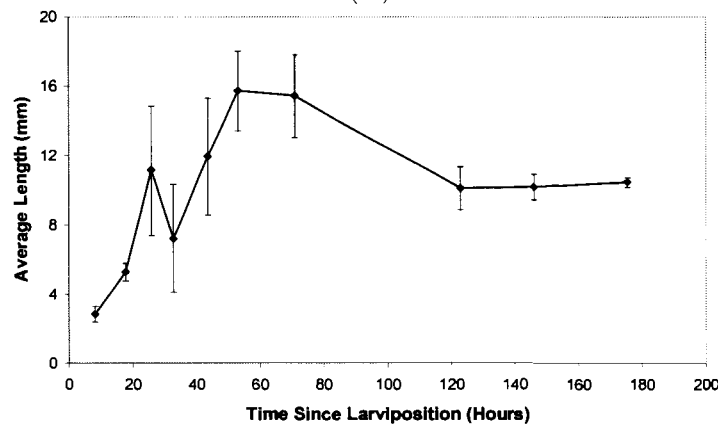
Figure 4.11: Mean larval length (\pm SD) versus time since larviposition for the three replicates of *Sarcophaga bullata* larvae reared on artificial foodstuff containing 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline (Batch A) at 26°C. (A) Batch A Replicate 1, (B) Batch A Replicate 2, and (C) Batch A Replicate 3.



(A)

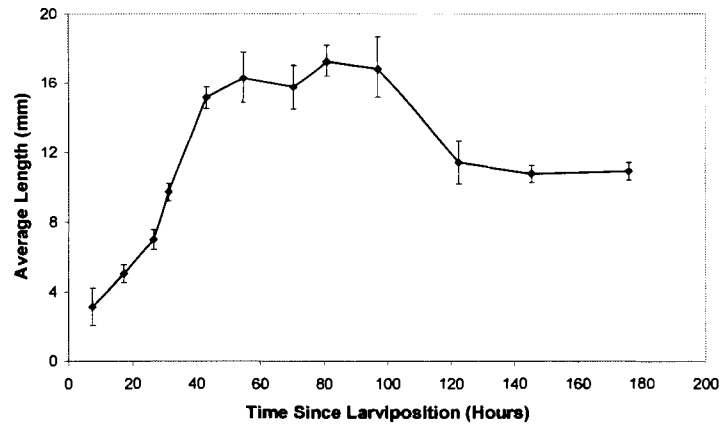


(B)

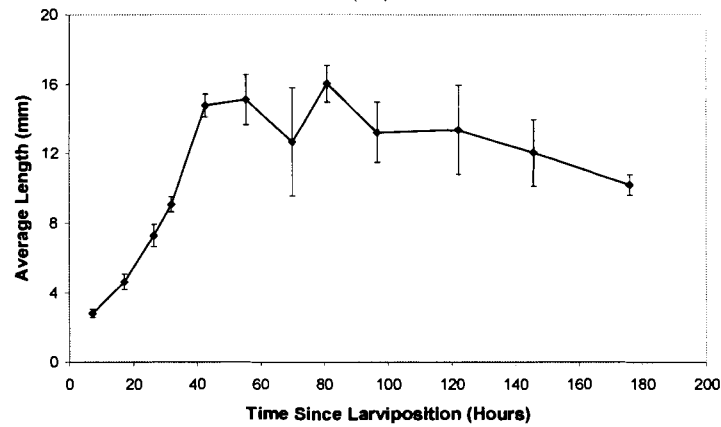


(C)

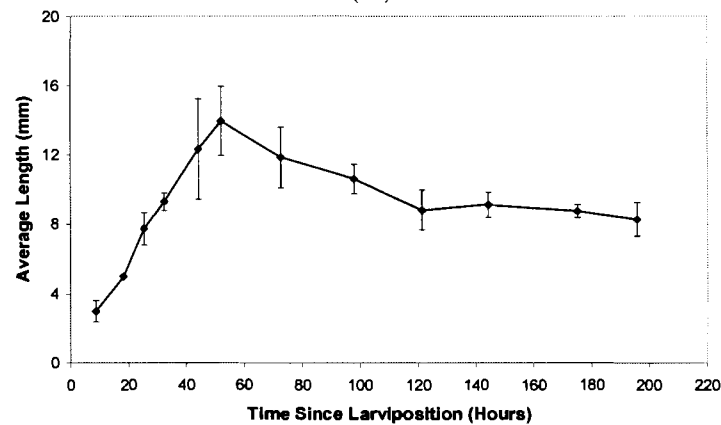
Figure 4.12: Mean larval length (\pm SD) versus time since larviposition for the three replicates of *Sarcophaga bullata* larvae reared on artificial foodstuff containing 9.55 mg/kg amitriptyline and 3.21 mg/kg nortriptyline (Batch B) at 26°C. (A) Batch B Replicate 1, (B) Batch B Replicate 2, and (C) Batch B Replicate 3.



(A)

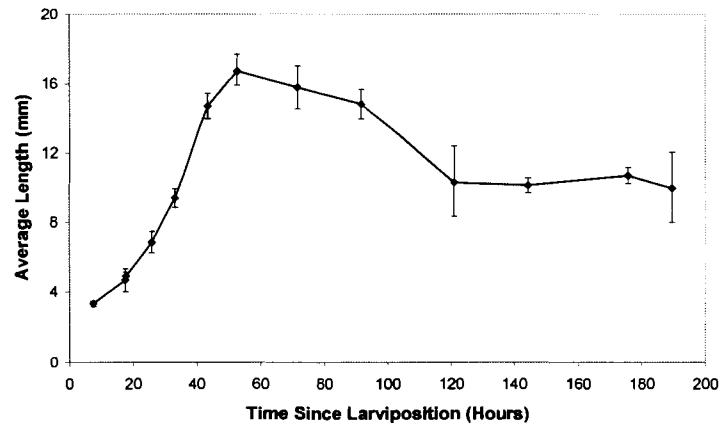


(B)

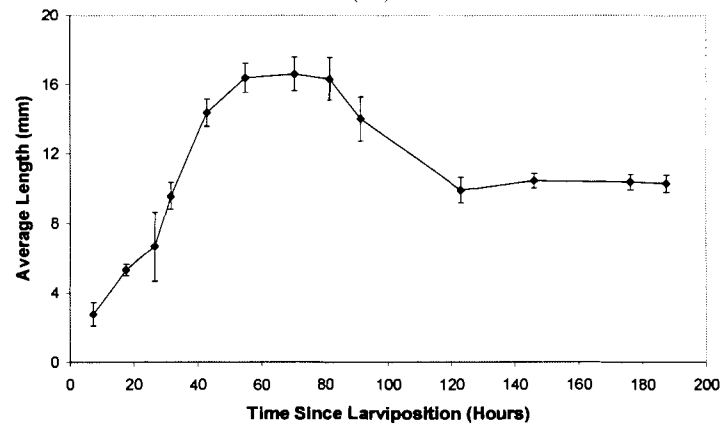


(C)

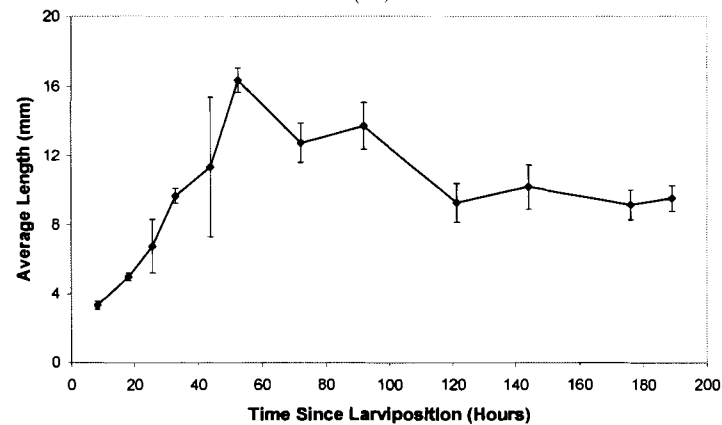
Figure 4.13: Mean larval length (\pm SD) versus time since larviposition for the three replicates of *Sarcophaga bullata* larvae reared on artificial foodstuff containing 55.7 mg/kg amitriptyline and 1.7 mg/kg nortriptyline (Batch C) at 26°C. (A) Batch C Replicate 1, (B) Batch C Replicate 2, and (C) Batch C Replicate 3 for Experiment 1.



(A)



(B)



(C)

Figure 4.14: Mean larval length (\pm SD) versus time since larviposition for the three replicates of *Sarcophaga bullata* larvae reared on artificial foodstuff containing 42.59 mg/kg amitriptyline and 1.22 mg/kg nortriptyline (Batch D) at 26°C. (A) Batch D Replicate 1, (B) Batch D Replicate 2, and (C) Batch D Replicate 3.

4.3.3 Discussion of the Effects of Amitriptyline and Nortriptyline on Larval Length

Based on these results, it appears that while the addition of drugs to the rearing media increased the variability in the time required for the maggots to reach their respective mean maximum lengths, the presence of a specific amount of either amitriptyline or nortriptyline did not appear to significantly alter the time required for the larvae reared on the six different sets of rearing media to reach their respective mean maximum length. In the study conducted by Goff *et al.* (1993), no difference in the time required to reach the mean maximum length was observed among any of the colonies, control or drug-spiked.

Further, no obvious pattern, in terms of amitriptyline batch inhomogeneity was observed for the variability in the time required for the larvae reared on each batch of foodstuff to reach their respective mean maximum length. For example, the RSD associated with the amitriptyline concentration in Batch B was 46.7%, while RSD associated with the time required to reach the mean maximum length for the larvae reared on Batch B foodstuff was only 17.7%. In comparison, the RSD associated with the amitriptyline concentration in Batch A was only 7.9%, while the RSD associated with the time required to reach the mean maximum length was 28.8%.

In the study conducted by Goff *et al.* (1993), the greatest mean lengths were observed for the colony reared on the liver from the rabbit that had received 1000 mg of amitriptyline by ear vein infusion. The concentration of amitriptyline and nortriptyline present in the liver of this rabbit was 49.0 mg/kg and 0.9 mg/kg, respectively. These concentrations were comparable to those found in Batch C of the present experiment. Even so, no statistically significant difference in the mean maximum length was observed across the four different insect colonies studied by Goff *et al.* (1993).

However, in the present experiment, the lowest mean maximum lengths were observed for the larvae reared on the foodstuff containing 41.38 mg/kg amitriptyline and 1.92 mg/kg nortriptyline (Batch C), which was the exact opposite of what was observed by Goff *et al.* (1993). The percent difference between the mean concentration of amitriptyline present in Batch C and the target amitriptyline concentration

was -15.5%. Although this difference is substantial, it is unlikely that the difference between the target concentration and the mean measured concentration of amitriptyline was influential enough to have caused a complete reversal of the results for mean maximum length observed by Goff *et al.* (1993).

Three possible explanations for this, and any other differences observed between the results observed by Goff *et al.* (1993), and the results observed by the author, are:

1. Species difference; Goff studied the effects of amitriptyline and nortriptyline on the development of *P. ruficornis*, and the present thesis studied the effects of amitriptyline and nortriptyline on the development of *S. Bullata*
2. Goff *et al.* (1993), used rabbit tissue for their rearing media. The present thesis used beef liver.
3. Goff *et al.* (1993), used a single drug-contaminated rabbit tissue for their experiment. Given the relatively high density of larvae added to the liver, a food shortage may have occurred. In contrast, in the present experiment, fresh food was added on a continual basis by the author.

Therefore, although both the present experiment, and the experiment conducted by Goff *et al.* (1993) used fly species from the Family Sarcophagidae, it is possible that differences, in terms of the physiological response to the presence of amitriptyline and nortriptyline in the rearing media contributed to the different results. Or, the species difference, with respect to the rearing media, may have contributed to the reversal in results observed for *S. bullata* compared with *P. ruficornis*.

In the present study, the mean maximum lengths obtained from the larvae reared on both the drug-spiked artificial foodstuff (Batches A, B, C and D), and the drug-free artificial foodstuff (Foodstuff Control) were significantly different from the mean maximum lengths of the larvae reared on liver (Liver Control). Since a relationship between drug concentration and mean maximum length was not observed in the data, the differences in length are more likely due to the use of the artificial foodstuff, and not the presence of amitriptyline and nortriptyline in the artificial foodstuff.

Overall, the pooled length data for the three replicates of Batch B and the replicates of Batch C exhibited the greatest variability in mean maximum larval length,

with RSDs of 11.1% and 12.9%. Given the fact that these two batches were heterogeneous in terms of amitriptyline concentration, the greater variability is not unexpected if amitriptyline and nortriptyline actually have an effect on *S. bullata* development. However, the effects of amitriptyline and nortriptyline on *S. bullata* development, if there are any, are indistinguishable from the effect of the artificial foodstuff.

Overall, the inhomogeneity of the artificial foodstuff with respect to amitriptyline appeared to contribute to the variability observed in mean maximum length, but not to the variability observed in the time required to reach the mean maximum length.

4.3.4 Developmental Stage as an Indicator of Maggot Age: Results

Total Development Time, from Larva to Adult for *Sarcophaga bullata*

The mean total development time for the larvae reared on each of the six types of rearing media is presented in Table 4.7.

Rearing Media	Mean (\pm SD) Mean Total Development Time (hrs)	Relative Standard Deviation (%)
Liver Control	438.1 \pm 1.48	0.3
Foodstuff Control	409.7 \pm 19.29	4.7
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	422.5 \pm 21.51	5.1
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	404.5 \pm 23.90	5.9
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	397.5 \pm 17.9	4.5
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	384.4 \pm 12.23	3.2

Table 4.7: The mean, standard deviation (\pm SD), and relative standard deviation for the total development time for *Sarcophaga bullata* reared on six different types of rearing media.

Based on the results presented in Table 4.7, the order, in terms of decreasing mean total development period of *S. bullata* was as follows:

Liver > Batch A > Foodstuff Control > Batch B > Batch C > Batch D

One-way ANOVA analysis of the mean total development time indicated that the differences in the mean total development time for *S. bullata*, from larviposition to adult emergence, were significant ($F_{5,12} = 3.432$; $P = 0.037$; Table 4.8).

Further analysis by Tukey's Honestly significant difference test indicated that the total development time for the insects reared on the liver control differed significantly from the total development time observed for larvae reared on Batch B, Batch C and Batch D. In addition, the total development time for the insects reared on Batch D

significantly differed from the total development time for the insects reared on Batch A.

Source of Variation	DF	Sums of Squares	Mean Square	F	P
Rearing Media	5	5373.11	1074.62	3.43	< 0.0372
Error	12	3757.31	313.11	-	-

Table 4.8: One-way ANOVA results for the mean total development time for *S. bullata* reared on six different types of rearing media at 26°C.

The development curves for each set of *Sarcophaga bullata* larvae reared on the six different types of rearing media are illustrated in Figures 4.15 through 4.20.

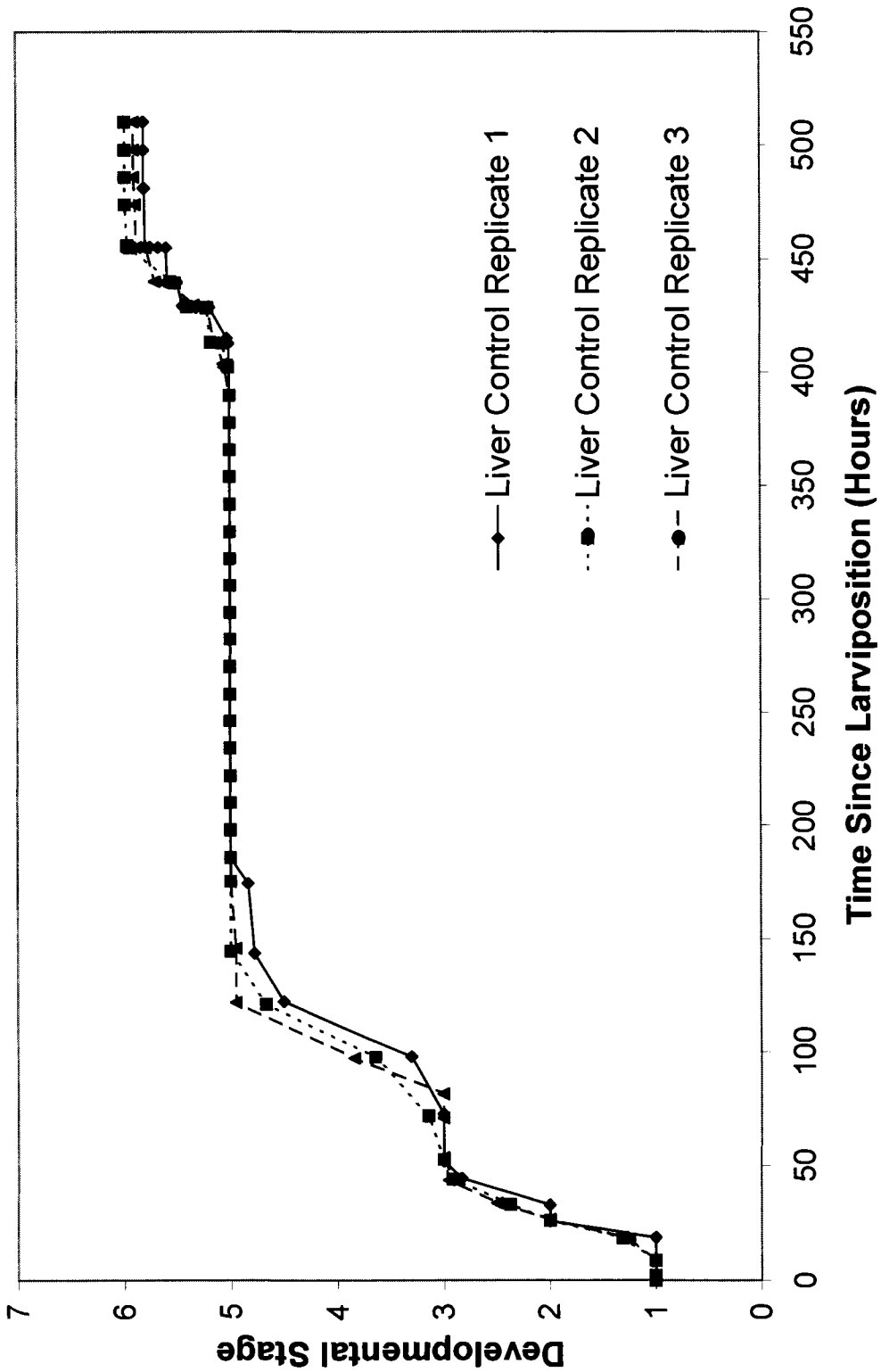


Figure 4.15: Development curve for *Sarcophaga bullata* larvae reared on liver at 26 °C. Stage 1 = first instar, Stage 2 = second instar, Stage 3 = third instar (feeding and postfeeding), Stage 4 = prepupal larvae, Stage 5 = pupal stage and Stage 6 = adult emergence.

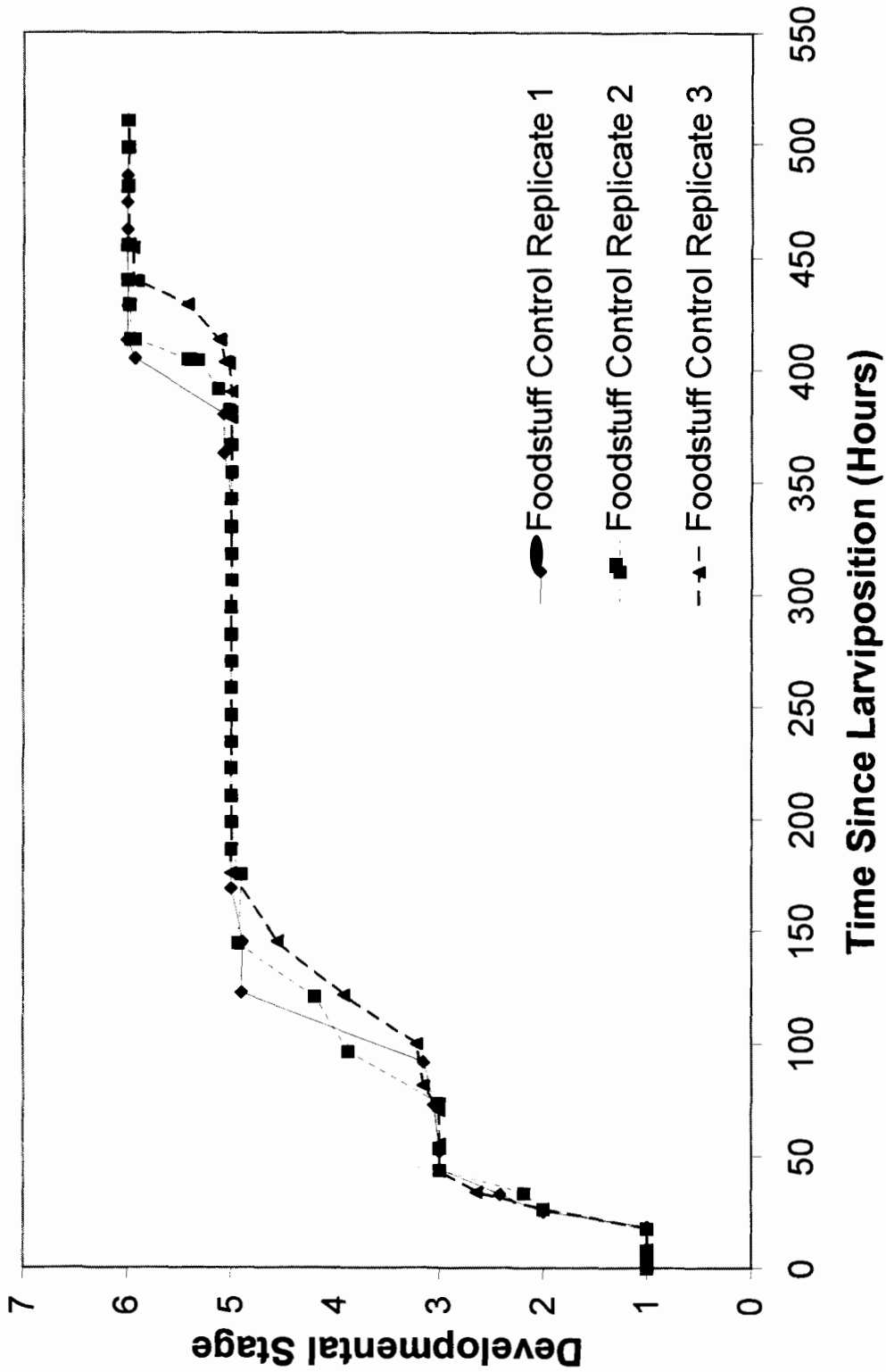


Figure 4.16: Development curve for *Sarcophaga bullata* larvae reared on blank artificial foodstuff (Foodstuff Control) at 26°C. Stage 1 = first instar, Stage 2 = second instar, Stage 3 = third instar (feeding and postfeeding), Stage 4 = prepupal larvae, Stage 5 = pupal stage and Stage 6 = adult emergence.

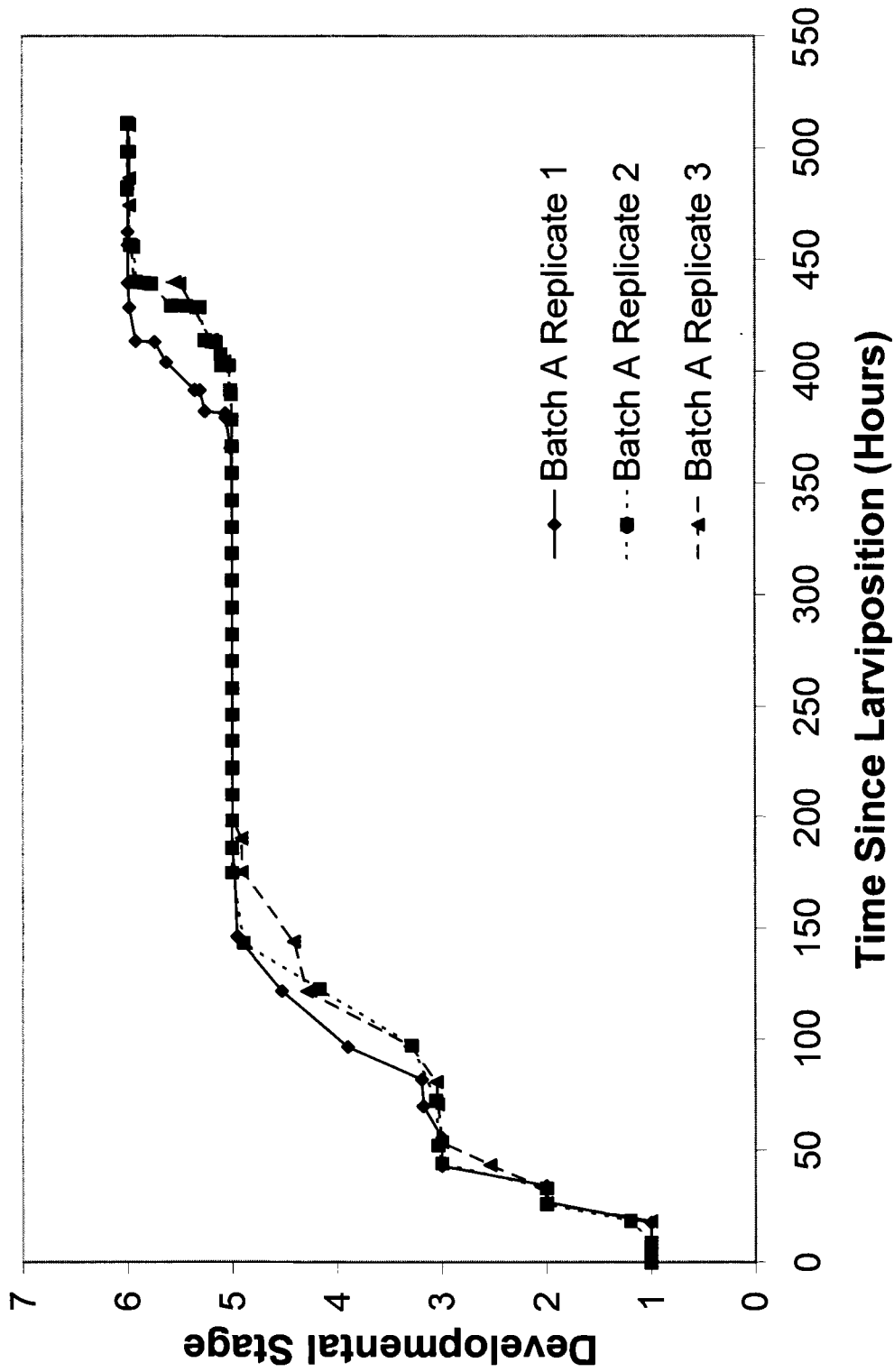


Figure 4.17: Development curve for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline (Batch A) at 26°C. Stage 1 = first instar, Stage 2 = second instar, Stage 3 = third instar (feeding and postfeeding), Stage 4 = prepupal larvae, Stage 5 = pupal stage and Stage 6 = adult emergence.

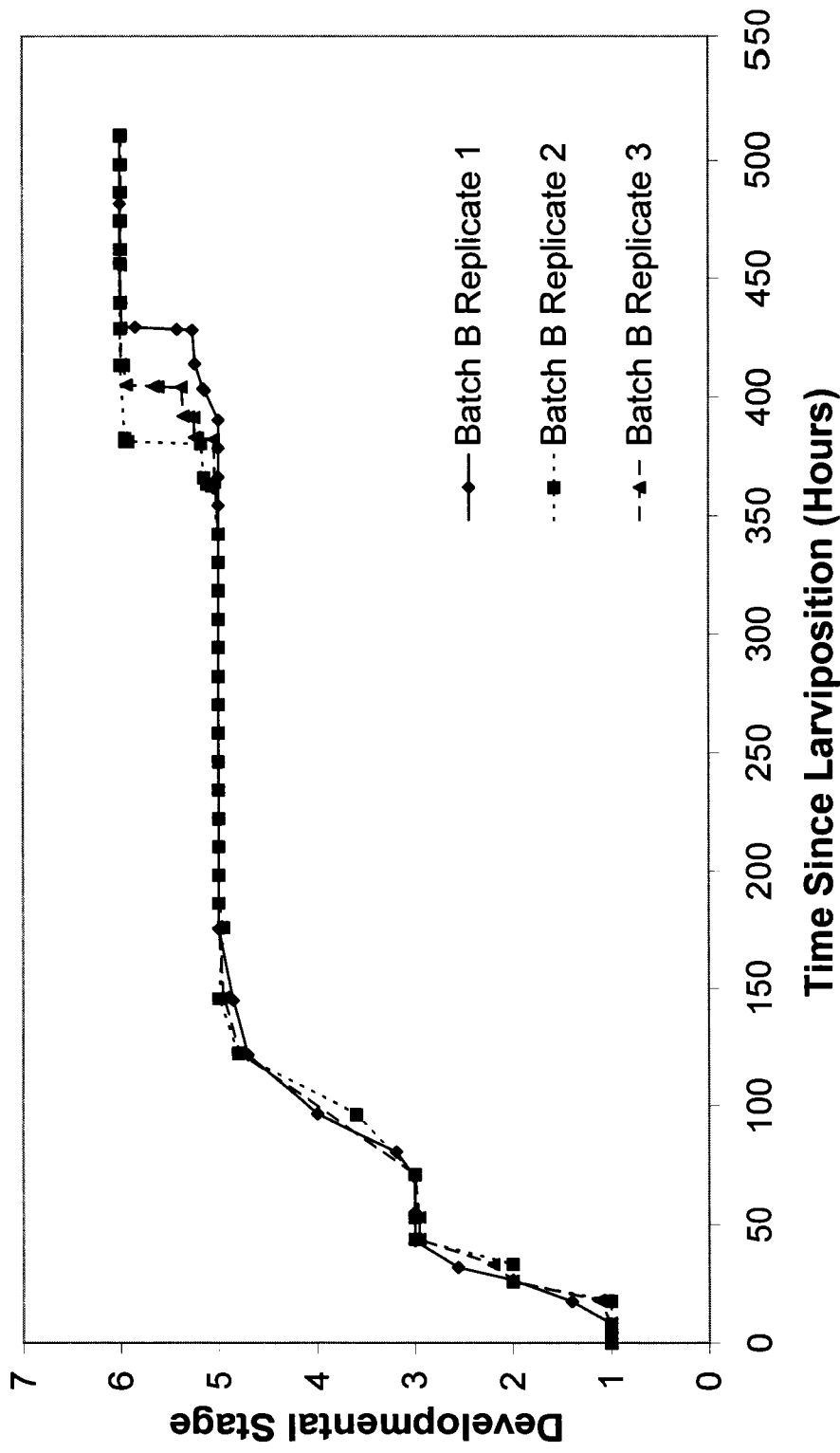


Figure 4.18: Development curve for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 9.55 mg/kg amitriptyline and 3.21 mg/kg nortriptyline (Batch B) at 26°C. Stage 1 = first instar, Stage 2 = second instar, Stage 3 = third instar (feeding and postfeeding), Stage 4 = prepupal larvae, Stage 5 = pupal stage and Stage 6 = adult emergence.

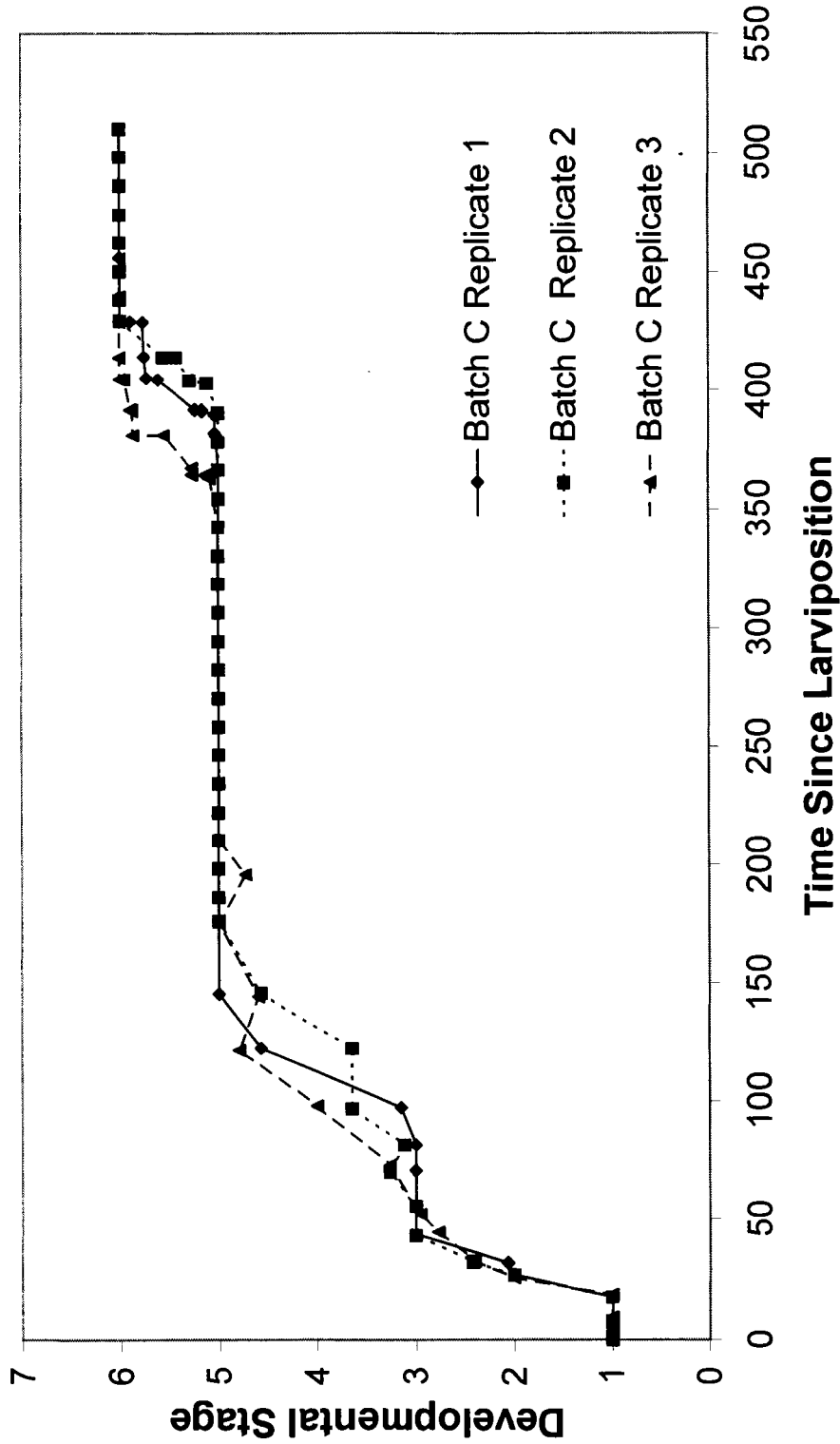


Figure 4.19: Development curve for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 41.38 mg/kg amitriptyline and 1.92 mg/kg nortriptyline (Batch C) at 26°C. Stage 1 = first instar, Stage 2 = second instar, Stage 3 = third instar (feeding and postfeeding), Stage 4 = prepupal larvae, Stage 5 = pupal stage and Stage 6 = adult emergence.

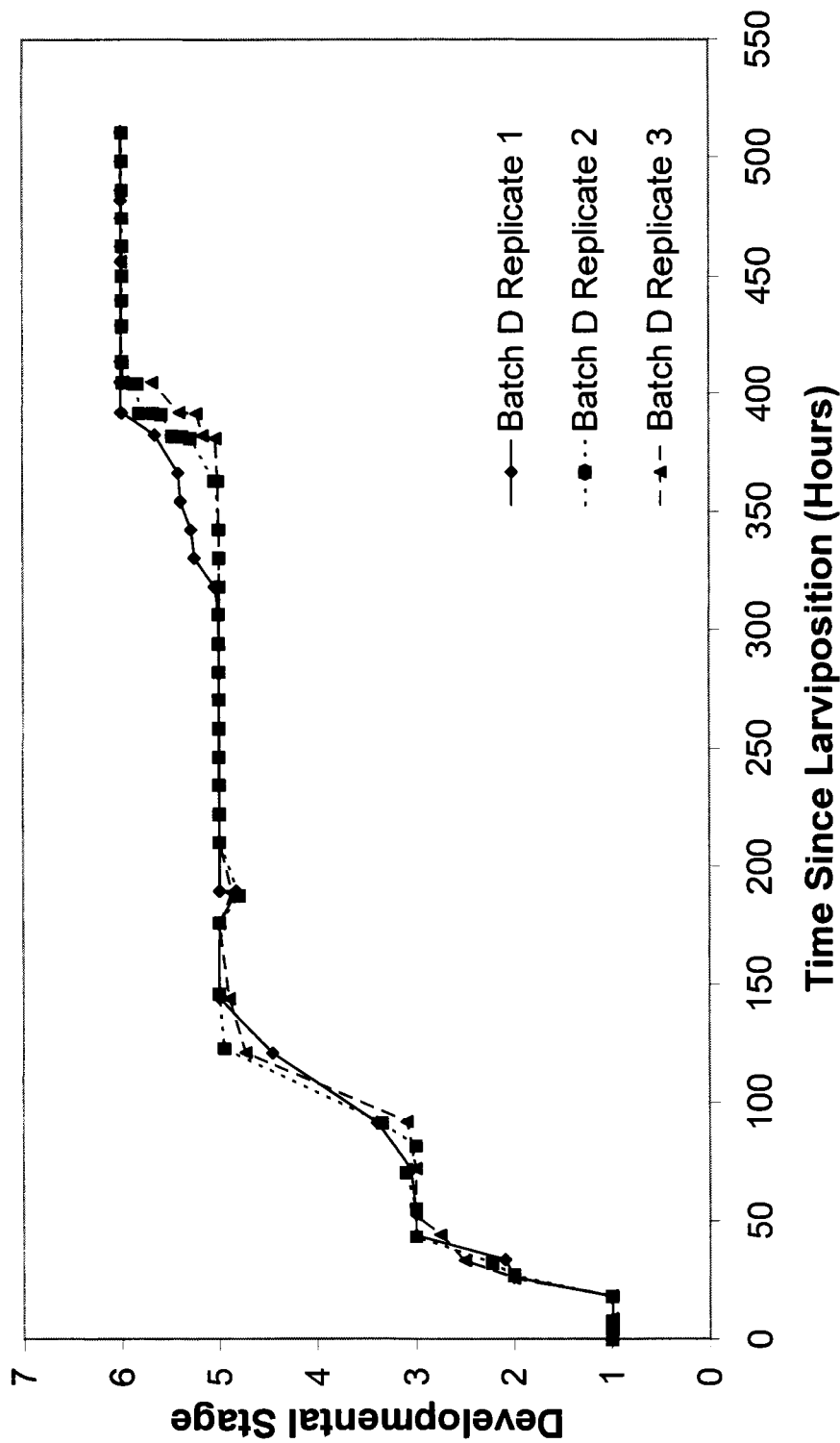


Figure 4.20: Development curve for *Sarcophaga bullata* larvae reared on artificial foodstuff containing 42.59 mg/kg amitriptyline and 1.22 mg/kg nortriptyline (Batch D) at 26°C. Stage 1 = first instar, Stage 2 = second instar, Stage 3 = third instar (feeding and postfeeding), Stage 4 = prepupal larvae, Stage 5 = pupal stage and Stage 6 = adult emergence.

Duration of the Larval Period

Preliminary examination of the results for the total development time indicate that the presence of amitriptyline and nortriptyline in the rearing substrate decreases the time required, from larviposition to adult emergence, for *S. bullata* development at 26°C. However, the effect of amitriptyline and nortriptyline may not be continuous over the entire development period. Flies are holometabolous insects, and as a result drastic physiological changes occur during the transition from larva to adult. With this in mind, the effect of amitriptyline and nortriptyline on the duration of the larval period on its own was also examined.

Rearing Media	Mean (\pm SD) Duration of Larval Period (hrs)	Relative Standard Deviation (%)
Liver Control	117.2 \pm 5.12	4.4
Foodstuff Control	129.8 \pm 13.97	10.8
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	136.2 \pm 16.92	12.4
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	115.2 \pm 0.65	0.57
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	125.8 \pm 16.21	12.9
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	118.1 \pm 4.49	3.8

Table 4.9: The mean, standard deviation (\pm SD), and relative standard deviation for the mean duration of the larval period for *Sarcophaga bullata* reared on six different types of rearing media.

Based on the results presented in Table 4.9, the order, in terms of decreasing mean duration of the larval period was as follows:

Batch A > Foodstuff Control > Batch C > Batch D \approx Liver Control > Batch B

One-way ANOVA analysis of the data presented in Table 4.9 indicated that the differences in the duration of the larval period between rearing medias were not significant ($F_{5,12} = 1.56$; $P = 0.24$; Table 4.10).

Duration of the Individual Larval Stages

However, the larval period can also be divided into its individual stages or instars, and then the duration of the each of these stages can be compared across the types of rearing medias. For the purposes of the present experiment, the larval stage was divided into the following four stages:

1. First instar
2. Second instar
3. Third instar
4. Prepupal stage

The mean duration of the first instar, for the larvae reared on each of the six types of rearing media is presented in Table 4.11.

Based on the results presented in Table 4.11, the order, in terms of decreasing mean duration of the first larval instar was as follows:

Batch C \approx Foodstuff Control = Batch A \approx Batch D > Batch B = Liver Control

However, a one-way ANOVA analysis of the differences in the mean duration of the first instar across the six different types of rearing media indicated that the differences between the types of rearing medias were not statistically significant ($F_{5,12} = 1.23$; $P = 0.35$; Table 4.12).

The mean duration of the second instar, for the larvae reared on each of the six types of rearing media is presented in Table 4.13).

Source of Variation	DF	Sums of Squares	Mean Square	F	P
Rearing Media	5	719.12	143.83	58.97	< 0.0001
Error	338	824.39	2.44	-	-

Table 4.10: One-way ANOVA results for mean duration of the larval period for *S. bullata* larvae reared on the six different types of rearing media at 26°C

Rearing Media	Mean (\pm SD) Duration of the First Instar (hr.)	Relative Standard Deviation (%)
Liver Control	20.7 \pm 0.89	4.3
Foodstuff Control	21.8 \pm 0.10	0.4
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	21.8 \pm 0.06	0.3
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	20.7 \pm 1.68	8.1
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	21.9 \pm 0.11	0.5
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	21.5 \pm 0.85	4.0

Table 4.11: The mean, standard deviation and relative standard deviation for the duration of the first instar for *Sarcophaga bullata* larvae reared on six different types of rearing media at 26°C.

Source of Variation	DF	Sums of Squares	Mean Square	F	P
Rearing Media	5	4.475	0.895	1.232	0.353
Error	12	8.716	0.726	-	-

Table 4.12: One-way analysis of variation (ANOVA) for the duration of the first instar of *Sarcophaga bullata* larvae reared in six different types of rearing media at 26°C.

Based on the results presented in Table 4.13, the order, in terms of decreasing mean duration of the second instar was as follows:

Batch A > Liver Control \approx Batch B > Batch D \approx Batch C > Foodstuff Control

However, a one-way ANOVA analysis of the difference in the mean duration of the second instar across the different rearing medias indicates that the mean difference in the duration of the second instar was not statistically significant for the six groups of *Sarcophaga bullata* larvae reared on the different types of rearing media ($F_{5,12} = 2.29$; $P = 0.11$; Table 4.14).

The mean duration of the third instar (feeding stage plus postfeeding stage), for the larvae reared on each of the six types of rearing media is presented in Table 4.15. Based on the results presented in Table 4.15, the order, in terms of decreasing mean duration of the third instar (feeding stage plus postfeeding stage) was as follows:

Foodstuff Control > Batch D > Liver Control \approx Batch A > Batch C > Batch B

However, one-way ANOVA analysis of the differences in the mean duration of the third instar (feeding stage plus postfeeding stage) across the six different types of rearing media were not statistically significant ($F_{5,12} = 0.618$; $P = 0.690$; Table 4.16).

The mean duration of the prepupal stage of the third instar, for the larvae reared on each of the six types of rearing media is presented in Table 4.17. Based on the results presented in Table 4.17, the order, in terms of decreasing mean duration of the prepupal stage of the third instar was as follows:

Batch A > Batch C > Foodstuff Control > Batch B > Liver Control \approx Batch D

However, the differences in the mean duration of the prepupal stage of the third instar for *Sarcophaga bullata* larvae were not significant based on a one-way ANOVA analysis ($F_{5,12} = 1.023$; $P = 0.447$; Table 4.18).

Since the third instar can be divided up into three behaviourally and/or physically distinct stages, it is also prudent to compare the mean duration of the third instar,

Rearing Media	Mean (\pm SD) Duration of Second Instar (hr.)	Relative Standard Deviation (%)
Liver Control	15.2 \pm 2.47	16.2
Foodstuff Control	12.6 \pm 2.69	21.4
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	18.2 \pm 2.11	11.6
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	15.0 \pm 2.44	16.3
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	13.4 \pm 1.66	12.4
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	13.9 \pm 1.87	13.5

Table 4.13: The mean, standard deviation and relative standard deviation for the duration of the second instar for *Sarcophaga bullata* larvae reared on six different types of rearing media at 26°C.

Source of Variation	DF	Sums of Squares	Mean Square	<i>F</i>	<i>P</i>
Rearing Media	5	54.471	11.494	2.294	0.111
Error	12	60.125	5.011	-	-

Table 4.14: One-way analysis of variation (ANOVA) for the duration of the second instar of *Sarcophaga bullata* larvae reared in six different types of rearing media at 26°C.

Rearing Media	Mean (\pm SD) Duration of the Third Instar: Feeding Stage + Postfeeding Stage (hr.)	Relative Standard Deviation (%)
Liver Control	58.4 \pm 3.59	6.1
Foodstuff Control	63.2 \pm 13.66	21.1
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	58.2 \pm 7.74	13.3
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	52.7 \pm 3.85	7.3
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	55.1 \pm 10.73	19.5
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	60.6 \pm 5.10	8.4

Table 4.15: The mean, standard deviation and relative standard deviation for the duration of the third instar (feeding stage plus postfeeding stage) for *Sarcophaga bullata* larvae reared on six different types of rearing media.

Source of Variation	DF	Sums of Squares	Mean Square	F	P
Rearing Media	5	213.519	42.704	0.618	0.690
Error	12	830.380	69.239	-	-

Table 4.16: One-way analysis of variation (ANOVA) for the duration of the third instar (feeding stage plus postfeeding stage) of *Sarcophaga bullata* larvae reared in six different types of rearing media.

Rearing Media	Mean (\pm SD) Duration of the Prepupal Stage of the Third Instar (hr.)	Relative Standard Deviation (%)
Liver Control	22.8 \pm 3.56	15.6
Foodstuff Control	32.2 \pm 13.43	41.2
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	38.0 \pm 12.63	33.2
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	26.8 \pm 2.78	10.4
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	35.5 \pm 19.76	55.7
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	22.0 \pm 5.62	25.6

Table 4.17: The mean, standard deviation and relative standard deviation for the prepupal stage of the third instar for *Sarcophaga bullata* larvae reared on six different types of rearing media at 26°C

Source of Variation	DF	Sums of Squares	Mean Square	F	P
Rearing Media	5	667.012	133.402	1.023	0.447
Error	12	1564.541	130.378	-	-

Table 4.18: One-way analysis of variation (ANOVA) for the duration of the prepupal stage of the third instar of *Sarcophaga bullata* larvae reared in six different types of rearing media at 26°C

as a whole⁶, across the six different types of rearing media (Table 4.19). Based on the results presented in Table 4.19, the order, in terms of decreasing mean duration of the prepupal stage of the third instar was as follows:

Batch A > Foodstuff Control > Batch C > Batch D > Liver Control \approx Batch B

Rearing Media	Total Mean (\pm SD) Duration of the Third Instar (hr.)	Relative Standard Deviation (%)
Liver Control	81.2 \pm 2.15	2.6
Foodstuff Control	95.5 \pm 15.20	15.9
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	96.2 \pm 14.79	15.4
Batch B (22.0 mg/kg AMT; 3.21 mg/kg NOR)	79.5 \pm 3.48	4.4
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	90.5 \pm 17.61	19.4
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	82.7 \pm 3.68	4.5

Table 4.19: The mean, standard deviation and relative standard deviation for total duration of the third instar (feeding stage + postfeeding stage + prepupal stage) of *Sarcophaga bullata* larvae reared on six different types of rearing media.

However, the differences in the mean duration of the third instar, as a whole, for *Sarcophaga bullata* larvae were also not significantly different based on one-way ANOVA analysis ($F_{5,12}1.023$; $P = 0.447$; Table 4.20).

⁶Feeding stage plus postfeeding stage plus prepupal stage

Source of Variation	DF	Sums of Squares	Mean Square	<i>F</i>	<i>P</i>
Rearing Media	5	824.441	164.822	1.252	0.345
Error	12	1579.670	131.639	-	-

Table 4.20: One-way analysis of variation (ANOVA) for the duration of third instar (feeding stage + postfeeding stage + prepupal stage) of *Sarcophaga bullata* larvae reared in six different types of rearing media at 26°C.

Duration of the Pupal Stage

The duration of the pupal stage of the insects reared on each of the six types of rearing media is presented in Table 4.21. Based on the results presented in Table 4.21, the order, in terms of decreasing mean duration of the pupal stage was as follows:

Liver Control > Batch B > Batch A > Foodstuff Control > Batch C > Batch D

Rearing Media	Duration of the Pupal Stage (hr.)	Relative Standard Deviation (%)
Liver Control	321.0 ± 3.66	1.1
Foodstuff Control	279.9 ± 6.39	2.3
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	286.4 ± 9.82	3.4
Batch B (9.55 mg/kg AMT; 3.21 mg/kg NOR)	289.3 ± 24.60	8.5
Batch C (41.38 mg/kg AMT; 1.92 mg/kg NOR)	271.7 ± 6.94	2.6
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	266.3 ± 15.49	5.8

Table 4.21: The mean, standard deviation and relative standard deviation for the duration of the pupal stage of *Sarcophaga bullata* larvae reared on six different types of rearing media at 26°C

Based on a one-way ANOVA analysis, the differences in the mean duration of the pupal stage, for *Sarcophaga bullata* across the six different types of rearing media larvae, was significantly different ($F_{5,12} = 6.426$; $P = 0.004$; Table 4.23).

Source of Variation	DF	Sums of Squares	Mean Square	F	P
Rearing Media	5	5591.369	1118.27	6.426	0.004
Error	12	2088.425	174.04	-	-

Table 4.22: One-way analysis of variation (ANOVA) for the duration of the pupal stage of *Sarcophaga bullata* larvae reared in six different types of rearing media at 26°C.

Further analysis by Tukey's HSD test revealed that the duration of the pupal

period for the insects reared on the liver control was significantly different from the duration of the pupal period for the insects reared on Batches C, D, and the foodstuff control. However, the duration of the pupal stage for the insects reared on the liver control did not significantly differ from the duration of the pupal stage for the insects reared on Batches A and B. Furthermore, the duration of the pupal period for the insects reared on Batches A and B significantly differed from the duration of the pupal period for the insects reared on Batches C, D and the foodstuff control. In comparison, the duration of the pupal stage for the insects reared on Batches C, D and the foodstuff control did not significantly differ from each other.

4.3.5 Discussion of the Effects of Amitriptyline and Nortriptyline on the Duration of the Developmental Stages

Based on the results presented above, amitriptyline and nortriptyline do not appear to have an effect on the duration of the larval period, or any effect on the duration of any of the individual larval stages. These results were the same as those observed by Goff *et al.* (1993). In the study conducted by Goff *et al.* (1993), no significant differences in the duration of the larval period were observed for larvae of the species *P. ruficornis*.

In the study conducted by Goff *et al.* (1993), the duration of the pupal period was significantly longer for insects of the species *P. ruficornis* that were reared on tissues representing the administration of 1000 mg⁷ and 600 mg⁸ of amitriptyline to the experimental rabbits by ear vein infusion.

Similarly, the duration of the pupal stage was shown to be significantly different across the six types of rearing media. Unfortunately, unlike in the study conducted

⁷The administration of 1000 mg of amitriptyline resulted in tissue concentrations of 49.0 mg/kg amitriptyline and 0.9 mg/kg nortriptyline, roughly equivalent to the concentrations present in Batch C

⁸The administration of 600 mg of amitriptyline resulted in tissue concentrations of 154.0 mg/kg amitriptyline and 7.1 mg/kg nortriptyline, roughly equivalent to the concentrations present in Batch A

by Goff *et al.* (1993), a clear pattern between the concentrations of amitriptyline and nortriptyline in the rearing media, and alterations in the duration of the pupal stage across the six types of rearing media, was not observed.

Of the four drug-spiked foodstuff batches tested, only Batches A and D were classified as being homogeneous. With this in mind, a tentative conclusion regarding the effects of amitriptyline and nortriptyline on the duration of the pupal stage can be made. It appears that 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline increased the duration of the pupal stage, and therefore increased the total duration of the development period, compared to the insects reared on the foodstuff control, but not compared to the insects reared on the liver control. A similar result was observed in the study conducted by Goff *et al.* (1993). However, in their study, the duration of the pupal period of *P. ruficornis* reared on the rabbit tissue containing 154.0 mg/kg amitriptyline and 7.1 mg/kg nortriptyline significantly differed from the rabbit liver control as well, and not just the other drug-spiked food substrates, as was observed in the present investigation.

The inhomogeneity of Batches B and C, with respect to amitriptyline, did not appear to increase the variability in the duration of the developmental stages between replicates of the same batch. However, three replicates are not enough to make a definitive conclusion in this regard.

Unfortunately, these results do not present any reliable conclusions regarding the effects of amitriptyline and nortriptyline on the duration of the pupal stage of *S. bullata*. The poor drug homogeneity associated with two batches of the artificial foodstuff, and the lower nutritional quality of the foodstuff as a whole, confounds such attempts.

4.4 Pupal Weight Comparison

4.4.1 Introduction

Pupal weight is a common measure used to evaluate the appropriateness of a given type of food source for rearing a given species of insect. An increase or decrease in the mean pupal size of the insects reared on the test substrate, compared to the mean pupal size of insects reared on the controls, provides insight into the relative nutritional quality of the test substance to the control substance. Pupal size, measured in terms of either weight or length, has been used by a number of researchers to evaluate the nutritional quality of a wide variety of food substrates for rearing a wide variety of insect species (e.g. Sherman and Tran, 1995; Okorie and Okeke, 1990; Daniels *et al.*, 1991 and Perotti and Lysyk, 2003).

In the present thesis, pupal weight was used as the comparative measure because pupal weight was the measure used by Goff *et al.* (1993) in their investigation of the effects of amitriptyline and nortriptyline on the development of *P. ruficornis*.

4.4.2 Results for the Pupal Weight Comparison

Pupation was first observed in the liver control, the foodstuff control and in all the drug-spiked artificial foodstuffs between 121 and 122 hours since larviposition. In the present research project, the first observation of pupae is not likely to be a good comparative measure as the sampling period prior to the time of observed onset of pupation was approximately 24 hours previous. This means that the error in the estimate of the time of first pupation is at least ± 12 hours. This is a significant error, and as such, any difference in first onset of pupation would be masked.

The mean pupal weights for each batch of artificial foodstuff and liver control are shown in Table 4.23. The mean pupal weights for the liver control and the blank foodstuff control were considerably heavier than the weights of the pupae that were reared on drug-spiked foodstuff. Furthermore, the mean weight for the pupae that developed from insects that were reared on liver were considerably heavier than the mean weight for the pupae that developed on artificial foodstuff, regardless of the presence of amitriptyline and nortriptyline. The box and whisker plot presented in

Rearing Media	Mean Pupal Weight per Replicate			Pooled Mean Pupal Weight	
	Replicate (<i>n</i> = 20)	Mean Pupal Weight \pm SD (g)	Relative Standard Deviation (%)	Mean Pupal Weight \pm SD (g)	Relative Standard Deviation (%)
Liver Control	R1	0.171 \pm 0.015	8.7	0.163 \pm 0.015 (9.3 %)	9.3
	R2	0.164 \pm 0.018	10.2		
	R3	0.155 \pm 0.010	6.3		
Foodstuff Control	R1	0.151 \pm 0.013	8.9	0.122 \pm 0.028 (23.2 %)	23.2
	R2	0.089 \pm 0.008	9.3		
	R3	0.127 \pm 0.014	10.8		
Batch A (168.41 mg/kg AMT; 7.65 mg/kg NOR)	R1	0.091 \pm 0.014	15.1	0.098 \pm 0.019 (19.4 %)	19.4
	R2	0.119 \pm 0.013	10.9		
	R3	0.085 \pm 0.009	10.5		
Batch B (22.0 mg/kg AMT; 3.21 mg/kg NOR)	R1	0.101 \pm 0.010	9.7	0.087 \pm 0.013 (15.3 %)	15.3
	R2	0.074 \pm 0.006	8.8		
	R3	0.086 \pm 0.005	5.8		
Batch C (55.7 mg/kg AMT; 1.7 mg/kg NOR)	R1	0.101 \pm 0.010	9.9	0.079 \pm 0.026 (33.0 %)	33.0
	R2	0.087 \pm 0.020	22.8		
	R3	0.048 \pm 0.004	7.6		
Batch D (42.59 mg/kg AMT; 1.22 mg/kg NOR)	R1	0.099 \pm 0.009	9.2	0.082 \pm 0.018 (21.3 %)	21.3
	R2	0.085 \pm 0.009	10.0		
	R3	0.062 \pm 0.009	14.9		

Table 4.23: Mean (\pm SD) *Sarcophaga bullata* pupal weights (g) for the insect reared on six different types of rearing media at 26°C.

Figure 4.21 clearly illustrates this trend.

All four of the tests for unequal variances, indicated that the variances in pupal weights were unequal at an alpha level of 0.05 ($P < 0.0001$), and therefore Welch's ANOVA was used rather than the results of the one-way ANOVA that assumed equal variances. Welch's ANOVA indicated that there was a significant difference between the pupal weights resulting from larvae reared on the six different types of rearing media ($F_{5,163.61} = 232.5112$; $P < 0.0001$).

Further analysis with Tukey's HSD pairwise comparison procedure revealed that the weight of the pupae from the liver control was significantly greater than the weight of pupae from both the foodstuff control and from the drug-spiked artificial foodstuff. In addition, Tukey's HSD test revealed that the mean pupal weight of the insects that were reared on the foodstuff control were significantly different from the mean pupal weight of the insects that were reared on Batches A, B, C and D. Furthermore, the mean pupal weight from the insects that were reared on the foodstuff containing 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline (Batch A) were significantly different from the mean pupal weights of the insects that were reared on Batches B, C, and D. Interestingly, Tukey's HSD test revealed that the mean pupal weights of the insects that were reared on Batches B, C and D were not significantly different from each other.

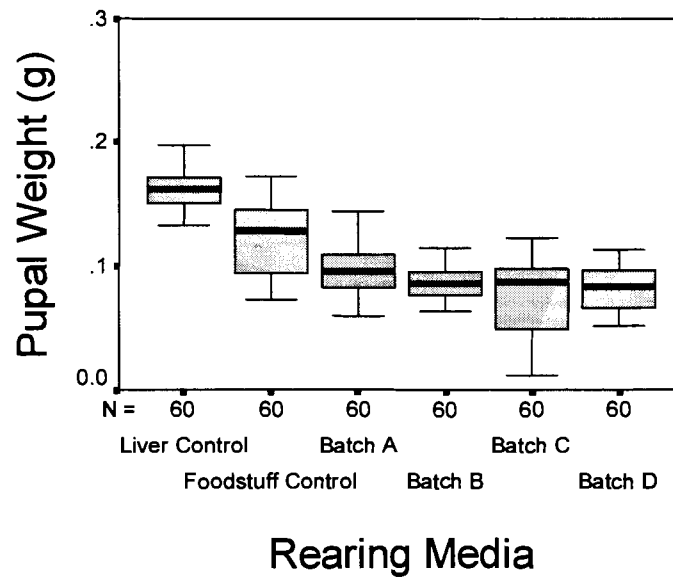


Figure 4.21: Box and whisker plot of the pupal weight data for the insects reared on each of the six types of rearing media at 26°C. The box and whisker plot was constructed using the pupal weight data from all three replicates from each type of rearing media. The plot shows the median pupal weight for each type of rearing media as a thick black bar. The interquartile range of the pupal weight data for each type of rearing media is shown as a box. The range of the pupal weight data for each type of rearing media is illustrated by the thin black bars (whiskers). Data points that are outside the range of other points observed for the data set are illustrated by circles.

4.4.3 Discussion of the Pupal Weight Comparison

The weight of the pupae obtained from the foodstuff control were significantly greater than the weight of the pupae from the drug spiked artificial foodstuff. Based on these results it appears that both the foodstuff and the drugs have an additive affect on pupal weight. In other words, larvae that are reared on liver produce heavier pupae than do insects that are reared on blank artificial foodstuff, and insects that are reared on blank foodstuff produce heavier pupae than do the pupae that are reared on the foodstuff containing amitriptyline and nortriptyline. However, despite the masking effect of the artificial foodstuff, it appears that larvae reared on 168.41 mg/kg amitriptyline and 7.65 mg/kg nortriptyline (Batch A) produce heavier pupae than the larvae that were reared on Batches B, C and D. Even more importantly, the result for Batch A can be viewed as a true reflection of the effects of amitriptyline and nortriptyline on the development of *S. bullata* larvae because Batch A was homogeneous for both amitriptyline and nortriptyline.

A similar result was observed by Goff *et al.* (1993) for *P. ruficornis*. In their study, heavier pupae were produced by the insect colonies that were reared on rabbit tissues containing 154.0 mg/kg amitriptyline and 7.1 mg/kg nortriptyline, concentrations that are roughly equivalent to those found in Batch A. However, in contrast to the present study, the greater pupal weights observed for *P. ruficornis* reared on the rabbit tissues containing 154.0 mg/kg amitriptyline and 7.1 mg/kg nortriptyline were also greater than the pupal weights observed for *P. ruficornis* reared on the drug-free rabbit tissue (Goff *et al.*, 1993).

Another clearly visible trend is the increased variation in pupal weight for the pupae that were reared on artificial foodstuff compared to the variation pupal weight observed for the pupae that were reared on liver (Table 4.23). The relative standard deviation for pupal weight, for Batches A, B, C, D, and the Foodstuff Control were considerably greater than the relative standard deviation observed for the pupae that were reared on liver. However, the inhomogeneity of Batches B and C, with respect to amitriptyline, did not appear to increase the variability in mean pupal weight between replicates of the same batch.

This common trend speaks to the suitability of the artificial foodstuff as a rearing media. The higher the coefficient of variation, the less useful the estimate. Any differences in development caused by the presence of amitriptyline and nortriptyline may be masked due to the high variation in the results. The high variation in the pupal weights for the insects reared on the artificial foodstuff indicates that the artificial foodstuff is of lower nutritional quality than the liver. Therefore, the lower nutritional quality of the artificial foodstuff may be contributing, or masking, the effects of amitriptyline and nortriptyline on the development of *S. bullata*.

4.5 Conclusion

Some similarities between the results from the present study and the study conducted by Goff *et al.* (1993) were observed. However, based on the results for the present study, it appears that nutritional improvements to the artificial foodstuff are required before further entomotoxicological experiments, using it as the experimental model, can be conducted. Furthermore, the poor nutritional quality of the food source and the inhomogeneity of some of the drug-spiked batches of artificial foodstuff made the reliable detection of dose-dependent developmental changes extremely difficult. Therefore, the homogeneity of the artificial foodstuff in terms of the added drugs, must also be improved before further experiments can be conducted.

Chapter 5

Conclusion

5.1 Evaluation of the Artificial Foodstuff for Use in Entomotoxicological Research

An ideal high quality larval diet should be easy to prepare from readily available, low cost materials (Sherman and Tran, 1995). In addition, when rearing necrophagous flies, the food source should be relatively free from offensive odours. Arguably the most important characteristic of a food source, however, is the production of healthy insects (Sherman and Tran, 1995).

The foodstuff used in the present experiment was relatively easy to prepare, and was prepared from low-cost, readily obtained materials. In addition, it proved to be less malodorous than liver. However, certain aspects of the preparation and storage of the foodstuff were problematic and may limit the successful use of the artificial foodstuff as a rearing media for entomotoxicological studies. For instance, in the present experiment, the foodstuff was stored in the freezer and thawed before use. However, the foodstuff exhibited significant water loss during thawing. In the case of amitriptyline and nortriptyline, analysis of the water lost from the foodstuff by GC-NPD indicated that a negligible amount of amitriptyline and nortriptyline were present. Given the fact that amitriptyline and nortriptyline are both drugs with a large volume of distribution, this result was not unexpected. However, if drugs with

small volumes of distribution (i.e. water soluble drugs) are investigated using this artificial foodstuff, there is the potential to lose a considerable amount of the drug during thawing if the foodstuff is frozen prior to use.

Furthermore, water loss from the foodstuff after quantitation but before introduction to the larvae indicates that the larvae likely received a higher concentration of both amitriptyline and nortriptyline than originally intended. This will be problematic when trying to attribute a given developmental response to a specific concentration of the drug. A simple solution to this problem would be to vacuum pack the foodstuff and store it at 4°C, rather than at -10.

However, the most serious problem encountered with the artificial foodstuff used in the present thesis was the fact that it altered the development of *S. bullata* compared to the beef liver control, and as a result ascribing specific changes in the duration of some of the developmental stages to the presence of amitriptyline and nortriptyline was not possible.

The foodstuff used in the present thesis is very similar the liver-agar rearing media evaluated by Sherman and Tran (1995) for rearing *Lucilia sericata* (Meigen) (Diptera: Calliphoridae). In addition, when the liver-agar foodstuff was prepared in a 1:1 ratio of liver weight (kg) to dissolved agar weight (kg), the development of *L. sericata* reared on the liver-agar foodstuff was observed not to differ from the development of the larvae reared on the liver control (Sherman and Tran, 1995). Furthermore, Sherman and Tran (1995) used a 3% agar solution by weight.

In comparison the artificial foodstuff used in the present thesis contained significantly less liver, and more agar. The artificial foodstuff prepared for the present thesis contained only a 0.5:1 ratio of liver weight (kg) to dissolved agar weight (kg) was used. Furthermore, a 4.5% agar solution was used compared to the 3% solution used by Sherman and Tran (1995).

With this in mind, it is apparent that the artificial foodstuff used in the present thesis to rear *S. bullata* larvae was not of an equivalent nutritional value to the liver control. The artificial foodstuff also contained powdered whole egg but it is unlikely that the protein and nutrient content present in the powdered whole egg was sufficient to make up for the nutritional losses resulting from a reduced liver content. Therefore,

for future research, the artificial foodstuff should be modified to more accurately match the nutritional level of the specific type of animal tissue being used as a the control.

Based on the results presented in this thesis, the use of a non-live animal model for use in insect development experiments is feasible, but requires significantly more preliminary research to use effectively and reliably, compared to the use of a live animal model. For instance, density controlled experiments should be conducted to determine if the blank artificial foodstuff alters the development of the insect under investigation compared to the animal tissues used as the control. If a difference is noted, the composition of the rearing media should be altered so that the development of the insects reared on the artificial foodstuff more closely matches the development of the insects reared on the control tissues. However, changing the composition of the foodstuff to any great extent may require revalidation of the analytical method used to quantitate the drug levels present in the foodstuff. The extent of revalidation required would depend on the extent of the alterations made to the artificial foodstuff. In general, significant alteration of the proportion of each component present in the matrix would require revalidation of the following parameters: recovery, accuracy, limit of quantitation, limit of detection and homogeneity.

5.2 Recommendations

1. The sample preparation procedure used to isolate amitriptyline and nortriptyline for the artificial foodstuff matrix should be further optimized. Further optimization of the sample preparation procedure will improve both the precision and the accuracy of the results obtained using GC-NPD.
2. The degree of shrinkage, caused by storage in 75% ethanol, in the length of *S. bullata* larvae should be investigated. In addition, the investigation of larval shrinkage should not be limited to one larval stage; the degree of shrinkage should be assessed for all larval stages of development.
3. Visible crop length was observed to decrease as a function of time since preservation of the larvae in 75% ethanol. Since in many insect species, the ratio of

crop length to body length is an excellent indicator of larval age, future research should perhaps include the dissection of the crops in order to determine crop length.

4. However, given the number of insects usually required for developmental research, the time required to dissect the crops would likely be prohibitive. With this in mind, experiments could be conducted to mathematically describe the degree of visible crop shrinkage as a function of time since preservation. This would then enable the reliable use of the preserved, visible crop length, as an indicator of larval age.
5. Insect mortality was not sufficiently investigated in the present thesis, and therefore, mortality experiments need to be conducted in future research to fully characterize the growth and development of an insect species reared on the artificial foodstuff.
6. In the experiment conducted by Goff *et al.* (1993), no additional food was supplied to the insect colonies, other than the first initial liver sample. In contrast, in the present experiment, fresh food was added on a continual basis. Therefore, the effects of food shortage and food desiccation should be investigated in future experiments.
7. Preliminary experiments indicated that the procedure used to prepare the artificial foodstuff for the insect development experiment produced a rearing substrate that was homogeneous for amitriptyline and nortriptyline (within a %RSD of 15%). However, based on the fact that two of the four drug-spiked batches used in the insect development experiment were determined to be inhomogeneous for amitriptyline, the present preparation method is not sufficiently reliable. Therefore, alternative procedures for the preparation of the foodstuff should be investigated.
8. Before use of the artificial foodstuff with a new insect species, density controlled experiments should be conducted using drug-free artificial foodstuff to

determine the impact of the foodstuff itself on the development of the insects. If the artificial foodstuff was shown to impact the development of the insects, its composition should be modified to more closely match that of the tissue used as the control.

9. If the composition of the artificial foodstuff (e.g. ratio of liver to agar) has to be altered to suit the specific nutritional requirements of a given fly species, the extent of analytical method revalidation required will have to be assessed, and subsequently conducted.
10. Given the propensity of the foodstuff to lose water, the foodstuff should not be frozen prior to use. Instead, it should be vacuum-sealed and stored at 4°C.

Appendix A

Chromatography

A.1 Introduction to Chromatography

Chromatography is an analytical method that is used to separate and identify chemical components in complex mixtures (Skoog *et al.*, 1996). In chromatography, the substances present in a complex mixture are differentially distributed between a **mobile phase** and a **stationary phase** (Ravindranath, 1989).

In chromatography, the chemical and physical properties of the molecules within a mixture are exploited to effect the separation of that mixture into its individual components. For example, the polarity, molecular size and chemical reactivity of a molecule may all be used to separate it from the rest of the compounds in a mixture (Ravindranath, 1989).

Chromatography is an extremely powerful separation technique, and therefore, it is a technique that can facilitate the identification of an unknown component or components present in a mixture. However, chromatography is not an identification technique; it cannot, on its own, determine the identity of the separated components (Stafford, 1992).

Essentially, there are two types of chromatography (Skoog *et al.*, 1996):

1. Planar chromatography
2. Column chromatography

The stationary phase in planar chromatography is supported on a flat plate or in the pores of a piece of chromatographic paper. The mobile phase moves over the surface of the planar stationary phase by capillary action. In comparison, the stationary phase in column chromatography is held in a cylindrical tube, and the mobile phase moves through the stationary phase under pressure or by gravity (Skoog *et al.*, 1996). Column chromatography, in the form of capillary gas-solid chromatography (GSC), was used in the present thesis.

A.2 The Chromatographic System

The gas chromatographic system can be divided into five functional areas, specifically:

1. *The gas supply system:* The purpose of the gas supply system is to provide clean carrier gas and support gases to the injector under controlled, reproducible conditions (Stafford, 1992).
2. *The injector:* The purpose of the injector is to introduce a known volume of the sample onto the column. The goal is to introduce the sample, originally at atmospheric pressure onto the column at operating pressure, with minimal **band broadening** (Stafford, 1992).
3. *The column and oven:* The part of the chromatographic system that effects the separation of the sample components. Column temperature is an important factor that can be used to increase the efficiency of the separation. The samples introduced into the chromatographic system in gas chromatography must be volatile in order for separation to occur. The oven maintains the gaseous state of the sample components (Skoog *et al.*, 1996).
4. *The detector:* The detector is a component of the chromatographic system that responds to characteristic present in the sample, and converts that response to a measurable signal (e.g. peaks on a chromatogram) (Skoog *et al.*, 1996).

5. *The data system:* The purpose of the data system is to capture and store the large amount of chromatographic data generated during an analysis.

A.3 General Method Description

The following method was developed and validated for the quantitation of amitriptyline and nortriptyline in an artificial foodstuff, prepared from beef liver, powdered whole egg, and agar, that was used to rear insects of the species *S. bullata* (Diptera: Sarcophagidae). The method was validated for use on a HP (Hewlett-Packard) 6890 gas chromatograph fitted with a nitrogen-phosphorus detector (GC-NPD).

A.3.1 Instrumental Parameters

Front Inlet

Split mode (split ratio of 20:1) with a split flow of 23.1 mL/min and a total flow of 27.2 mL/min. Initial temperature of 300°C with a pressure of 12 psi.

Carrier gas

Helium.

Oven Temperature

An isocratic oven temperature was used, ranging from 240°C and 260°C. Analyses for the analytes in ethyl acetate were conducted at an isocratic oven temperature of 260°C. However, interferences present in the artificial foodstuff matrix varied somewhat with each set of samples analyzed. Therefore, the specific temperature used for each set of samples varied between 240°C and 260°C, depending on the matrix interferences present. Isocratic oven temperatures were still used with samples extracted from the artificial foodstuff. Essentially, the oven temperature was varied in order to maintain baseline separation of the analytes, both from each other, and other components derived from the artificial foodstuff matrix.

Column

A 30.0 m, HP-5 (5% Phenyl methyl siloxane) capillary column with diameter of 320 μm film thickness of 0.25 μm and a maximum temperature of 325°C. Constant pressure of 12 psi and initial flow rate of 1.2 mL/min.

Detector

Nitrogen-phosphorous detector, with a temperature of 240°C hydrogen gas flow of 4.0 mL/min and a medical air flow of 60.0 mL/min. Helium flow rate of 15 mL/min.

Run Time

Five minutes.

Relative Analyte Retention Time

The relative retention times for amitriptyline and nortriptyline were calculated in reference to the retention time of the internal standard, maprotyline¹

The relative retention time for amitriptyline was 0.80 min, and the relative retention time for nortriptyline was 0.82 min.

¹i.e. Relative retention time is calculated by dividing the analyte retention time by the retention time for the internal standard

A.3.2 Sample Chromatograms

Amitriptyline, Nortriptyline and Maprotyline in Ethyl Acetate

Figure A.1 illustrates the baseline separation achieved for amitriptyline, nortriptyline and maprotyline in a matrix of ethyl acetate. The amitriptyline peak represents a concentration of 100 mg/L, the nortriptyline peak a concentration of 10 mg/L and the maprotyline peak represents a concentration of 10 mg/L.

Figure A.2 illustrates the baseline separation achieved for amitriptyline, nortriptyline and maprotyline in a matrix of ethyl acetate after rinsing the column with chlorobutane. A non-ideal consequence of the solvent wash was peak broadening; therefore solvent rinsing reduced the **efficiency** of the column. The amitriptyline peak represents a concentration of 100 mg/L, the nortriptyline peak a concentration of 10 mg/L and the maprotyline peak represents a concentration of 10 mg/L.

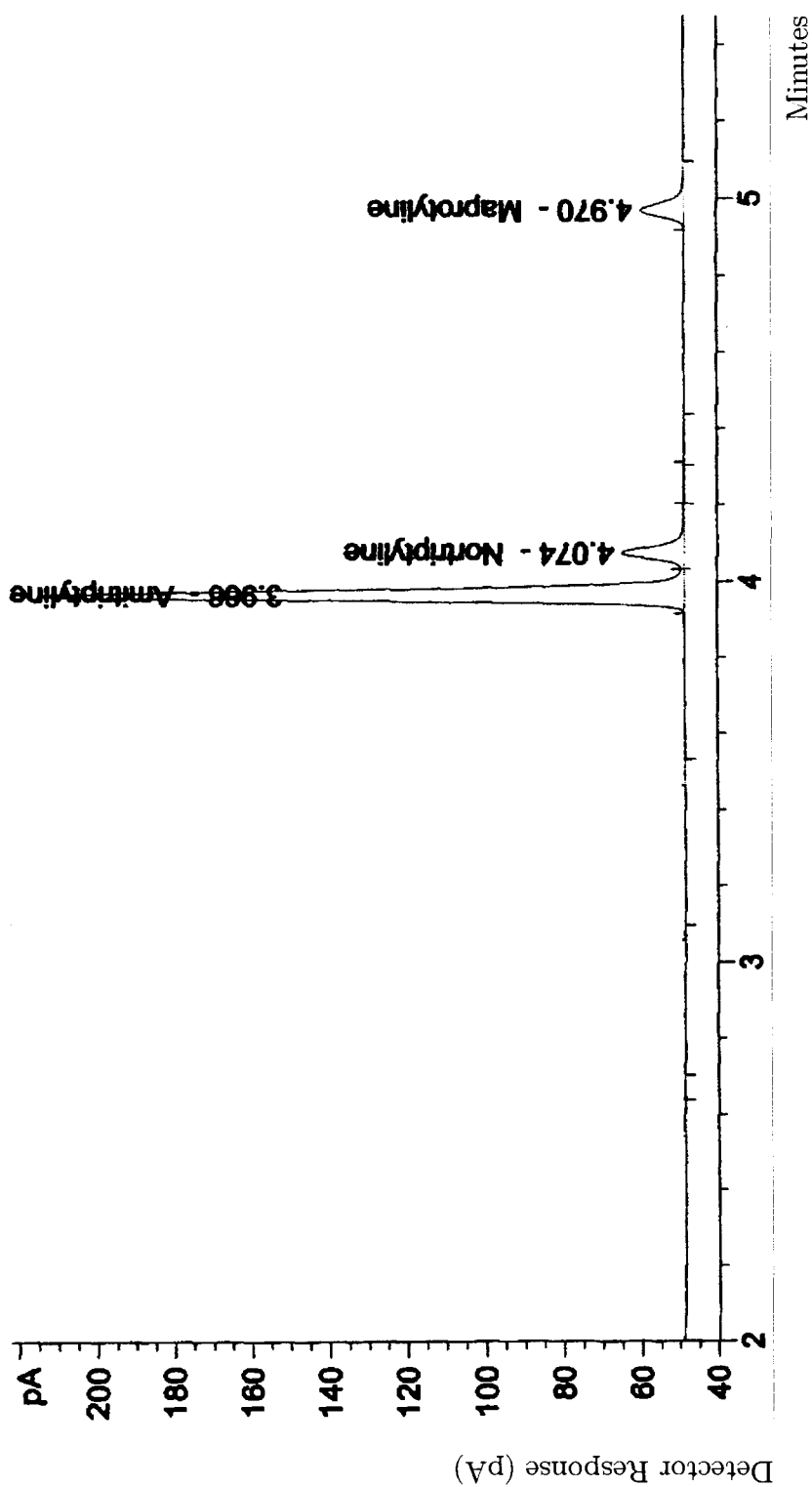


Figure A.1: Sample chromatogram of amitriptyline, nortriptyline, and the internal standard, maprotyline, in ethyl acetate. The chromatogram illustrates the baseline separation achieved for all three analytes. Amitriptyline 100 mg/L. Nortriptyline 10 mg/L. Maprotyline 10 mg/L.

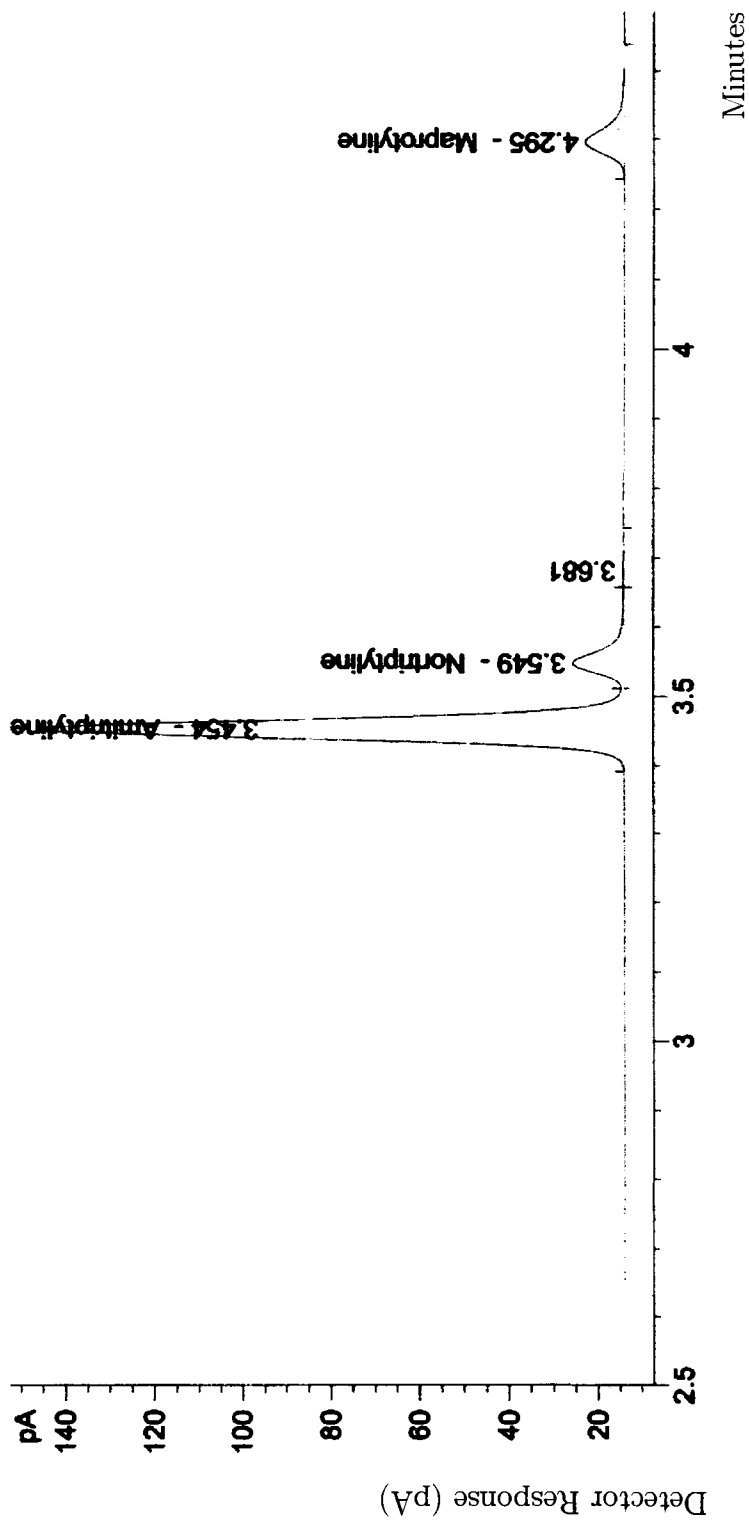


Figure A.2: Sample chromatogram which illustrates the peak broadening that occurred after rinsing the column with chlorobutane (Amitriptyline = 100 mg/L; Nortriptyline = 10 mg/L; Maprotyline = 10 mg/L).

Amitriptyline, Nortriptyline and Maprotyline Extracted from the Artificial Foodstuff

Figure A.3 illustrates the baseline separation achieved for amitriptyline, nortriptyline and maprotyline extracted from the artificial foodstuff matrix. The amitriptyline peak represents a concentration of 25 mg/L, the nortriptyline peak a concentration of 25 mg/L and the maprotyline peak represents a concentration of 25 mg/L.

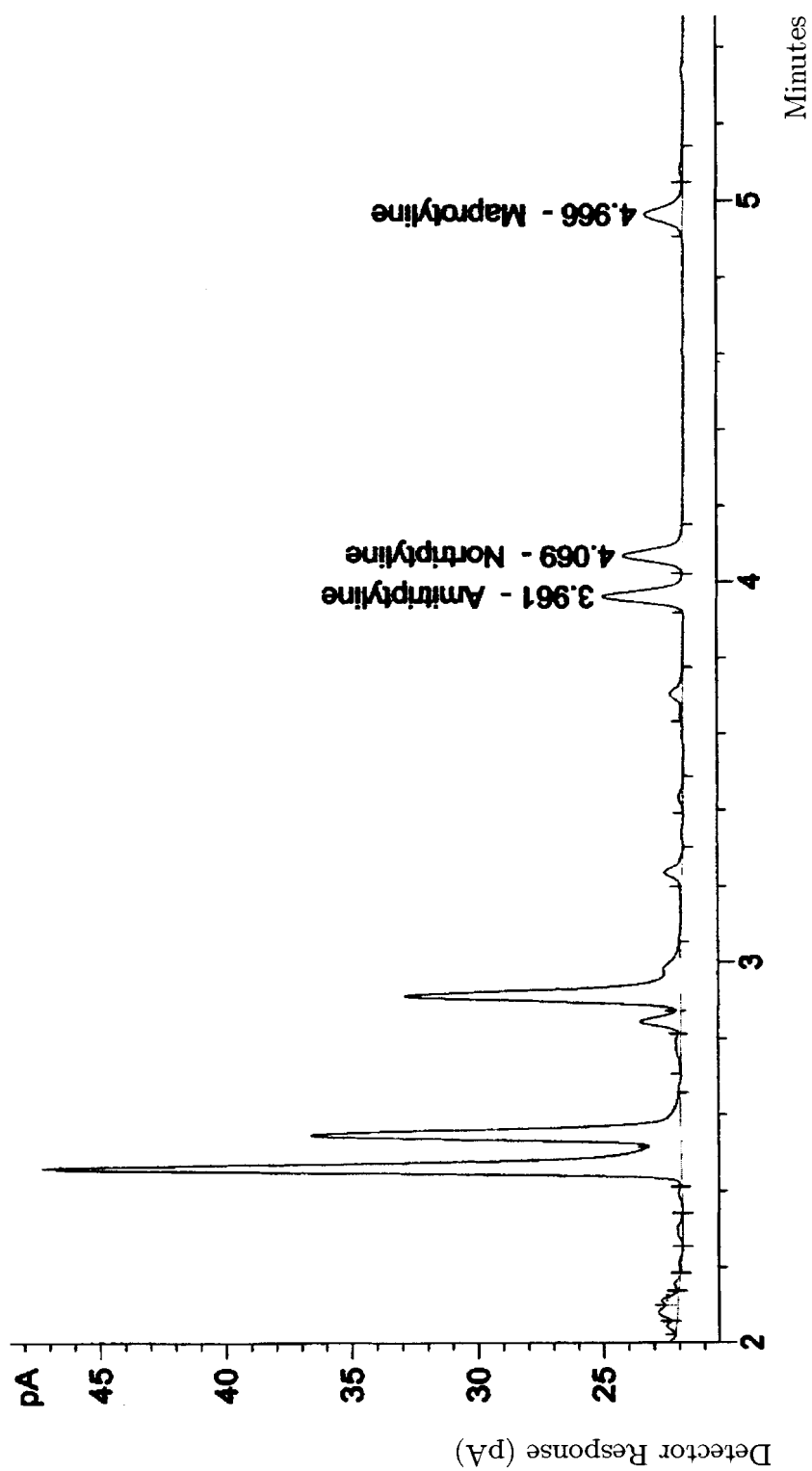


Figure A.3: Sample chromatogram for a calibration standard prepared in, and extracted from, the artificial food-stuff which illustrates the baseline separation achieved for amitriptyline, nortriptyline and maprotyline (Amitriptyline = 25 mg/L Amitriptyline; 25 mg/L Nortriptyline; 25 mg/L maprotyline).

Appendix B

Additional Data

Foodstuff Batch	Sample	Mean (\pm SD) Amitriptyline Concentration (mg/kg)	Relative Standard Deviation (%)
Batch A Target: 154.0 mg/kg	A-1 ($n = 3$)	177.15 \pm 3.61	2.0
	A-2 ($n = 3$)	182.78 \pm 3.62	2.0
	A-3 ($n = 3$)	167.33 \pm 3.16	1.9
	A-4 ($n = 3$)	147.87 \pm 6.88	4.7
	A-5 ($n = 3$)	166.94 \pm 2.92	1.8
Batch B Target: 24.0 mg/kg	B-1 ($n = 3$)	5.05 \pm 0.37	7.4
	B-2 ($n = 3$)	7.62 \pm 0.98	12.8
	B-3 ($n = 3$)	7.24 \pm 0.44	6.1
	B-4 ($n = 3$)	16.42 \pm 0.89	5.3
	B-5 ($n = 3$)	11.43 \pm 0.37	3.2
Batch C Target: 49.0 mg/kg	C-1 ($n = 3$)	47.81 \pm 1.94	4.1
	C-2 ($n = 3$)	37.87 \pm 1.46	3.8
	C-3 ($n = 3$)	31.84 \pm 1.12	3.5
	C-4 ($n = 3$)	42.09 \pm 1.58	3.7
	C-5 ($n = 3$)	47.29 \pm 2.53	5.4
Batch D Target: 49.0 mg/kg	D-1 ($n = 3$)	32.76 \pm 0.80	2.4
	D-2 ($n = 3$)	45.37 \pm 1.09	2.4
	D-3 ($n = 3$)	42.82 \pm 1.18	2.7
	D-4 ($n = 3$)	45.66 \pm 0.20	0.4
	D-5 ($n = 3$)	46.36 \pm 0.92	1.9

Table B.1: The mean, standard deviation and relative standard deviation for the measured amitriptyline concentrations, for each of the five randomly selected pieces, from each batch of artificial foodstuff. Triplicate analyses on each extract were conducted by GC-NPD.

Foodstuff Batch	Sample	Mean (\pm SD) Nortriptyline Concentration (mg/kg)	Relative Standard Deviation (%)
Batch A Target: 7.1 mg/kg	A-1 ($n = 3$)	7.42 \pm 0.08	1.1
	A-2 ($n = 3$)	7.78 \pm 0.12	1.5
	A-3 ($n = 3$)	7.62 \pm 0.10	1.3
	A-4 ($n = 3$)	7.89 \pm 0.51	6.5
	A-5 ($n = 3$)	7.52 \pm 0.20	2.7
Batch B Target: 3.3 mg/kg	B-1 ($n = 3$)	3.11 \pm 0.09	2.9
	B-2 ($n = 3$)	3.11 \pm 0.08	2.7
	B-3 ($n = 3$)	3.29 \pm 0.05	1.6
	B-4 ($n = 3$)	3.31 \pm 0.08	2.4
	B-5 ($n = 3$)	3.22 \pm 0.03	0.8
Batch C Target: 0.9 mg/kg	C-1 ($n = 3$)	1.90 \pm 0.07	3.4
	C-2 ($n = 3$)	1.95 \pm 0.06	3.3
	C-3 ($n = 3$)	1.90 \pm 0.11	5.6
	C-4 ($n = 3$)	1.90 \pm 0.08	4.3
	C-5 ($n = 3$)	1.96 \pm 0.05	2.7
Batch D Target: 0.9 mg/kg	D-1 ($n = 3$)	1.26 \pm 0.08	6.7
	D-2 ($n = 3$)	1.20 \pm 0.05	4.3
	D-3 ($n = 3$)	1.28 \pm 0.05	3.8
	D-4 ($n = 3$)	1.23 \pm 0.003	0.3
	D-5 ($n = 3$)	1.15 \pm 0.03	2.4

Table B.2: The mean, standard deviation and relative standard deviation for the measured amitriptyline concentrations, for each of the five randomly selected pieces, from each batch of artificial foodstuff. Triplicate analyses on each extract were conducted by GC-NPD.

Appendix C

Chemical Structures of Analytes

The following diagrams were created using ISISTM Draw 2.4 (MDL Information Systems Inc., San Leandro, California).

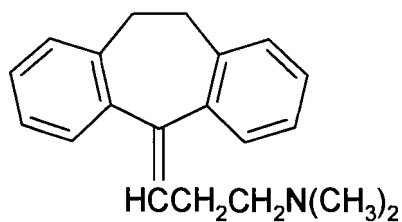


Figure C.1: Chemical structure of Amitriptyline.

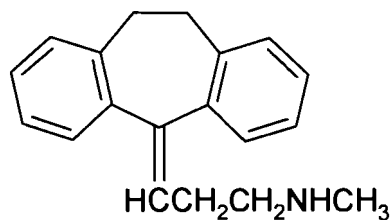


Figure C.2: Chemical structure of Nortriptyline.

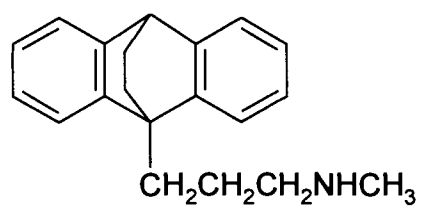


Figure C.3: Chemical structure of Maprotyline.

Appendix D

Ethics Approval

This section presents the ethics approval obtained for the previous research study “The effects of commonly abused drugs in Canada on time of death determinations in the later postmortem interval, using insect development.”

The data obtained from this previous research study provided the background information on tricyclic antidepressant use in British Columbia, which was necessary for the present thesis.



Simon Fraser University

Office of Research Ethics

<http://www.sfu.ca/vp-research/ethics>

Duke

Record Number

4 - 195

Code

33337

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Title The effects of commonly abused drugs in Canada on time of death determinations in the later postmortem interval, using insect development.

Co Investigator none

Supervisor Gail Anderson

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Application Date 4/11/2002

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Current Review

Start Date 5/1/2002

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Previous Review

Date Modified 4/13/2002

End Date 12/30/2003

Medical

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Approval Approved

Minimal Risk

Non Minimal Risk

Approval Date 4/16/2002

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Entered into Grant Track

Grant Track Pending

Grant Track No



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