A COMPARITIVE STUDY OF ETHANOLIC VERSUS TRITURATED DILUTIONS IN TERMS OF THE AMOUNT OF CAFFEINE EXTRACTED FROM *COFFEA TOSTA* BY MEANS OF HIGH PRESSURE LIQUID CHROMATOGRAPHY

BY

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I, Bronwyn Claire Harris do hereby declare that this dissertation represents my own work in both conception and execution

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DEDICATION

I would like to dedicate this work to my mother and late father. Thank you for your unconditional love and support.

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ABSTRACT

The purpose of this study was to compare the amount of caffeine extracted from triturated samples and ethanolic samples of *Coffea tosta* using high pressure liquid chromatography (HPLC) as a method of analysis.

The study wanted to expand on homoeopathic pharmaceutical knowledge, specifically looking at the two methods of remedy preparation of plant materials.

From the same batch of ground roasted coffee beans, using the decimal scale of dilution, the mother tincture (1xH) and the first triturated (1xH) samples were prepared.
The subsequent 2xH and 3xH triturated and ethanolic potencies were then made in accordance with homoeopathic methodology. Each group contained three different dilution levels (1xH, 2xH and 3xH), 18 samples per group giving a total of 36 samples that were analysed using HPLC. Three samples were analysed from the three dilution levels in each Group, in total there were 18 samples from the triturated group and 18 from the ethanolic group.

The samples were analysed quantitatively using the highly accurate and advanced method of high pressure liquid chromatography. This method gives accurate readings of the caffeine concentrations of a sample compared to a caffeine standard. This allowed for quantification of the caffeine concentration of each sample. The percentage caffeine was calculated from each sample.

The aim of the study was to evaluate the difference in each method of preparation by measuring the amount of caffeine extracted from the samples.

The results obtained from the inter-Group Mann-Whitney and ANOVA tests showed that there was a significant difference between the ethanolic dilutions and triturated dilutions with regards to the 1xH and 2xH dilutions. In the 1xH dilution the ethanolic method retained

relatively more caffeine. However there was no difference between the dilution methods at the 3xH level. For this reason, one cannot say that one method is better than the other.

The results obtained when comparing the three dilution levels of the triturated samples showed there was a significant difference between each level. The results obtained from analysis of the ethanolic dilutions showed no significant difference between each dilution level.

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DEFINITION OF TERMS

ALKALOID

A nitrogen containing substance which tends to have strong actions and can effect a wide range of functions in the body (Balkam, 1995: 32).

CENTESIMAL POTENCY

A homoeopathic potency scale whereby one part of the crude drug is combined with 99 parts of the determined vehicle. The mixture is either violently shaken or ground. (Gaier, 1991: 449.)

CHROMATOGRAPHY

A group of laboratory separation techniques based on selective absorption by which components of complex mixtures (vapours, liquid and solutions) can be identified. The compounds to be separated are distributed between a stationary phase and a mobile phase. (Meyer, 1997: 5.)

COLUMN

A tube which contains the stationary phase. The stationary phase differentially interacts with the sample's constituent compounds as they are carried along in the mobile phase. (Meyer, 1998: 97.)

COLUMN PERFORMANCE

As a band of solute moves through the chromatographic system it becomes dispersed, the longer the solute spends in the system the more dispersed it becomes, however, the more efficient the chromatographic column, the less band spreading will occur (Lindsay, 1992: 19).

DECIMAL POTENCY

A homoeopathic potency scale by which one part of the crude drug is combined with 9 parts of the determined vehicle. The mixture is either violently shaken or ground. (Gaier, 1991: 449.)

DYNAMIZATION

Imparting and passing along the pharmacological message of the original substance through serial dilution. It is the process of modification of medicines invented by the founder of homoeopathy, Samuel Hahnemann. (Gaier, 1991: 144.)

ELUTION

The process of passing a liquid or gas through a chromatographic column is called elution (Harris, 1995: 628).

GRADIENT ELUTION

A technique for decreasing separation time by increasing mobile phase strength over time during a chromatographic separation. It is also known as solvent programming. Gradients can be continuous or step-wise. (Harris, 1995: 679.)

PHARMACOPOEIA

An authoritative reference work containing monographs of medicines and other therapeutic agents, as well as specifications for the sources of and standards for the strength and purity of, base substances and mother tinctures, formulae and methods of preparation of these substances and their derivative potencies, and descriptive processes for the testing of starting material (Gaier, 1991: 398).

POTENCY

The stage of altered remedial activity to which a drug has been taken by means of a measured process of deconcentration, either by succussion or trituration which introduces kinetic energy into the medicine (Gaier, 1991: 432).

SUCCUSSION

The action of shaking up, or the condition of being shaken up vigorously, of a liquid dilution of a homoeopathic medicine in a bottle, where each stroke ends in a jolt usually by pounding the hand engaged in the shaking action against the other palm (Gaier, 1991: 532).

TRITURATION

This process is used for drug substances which are insoluble in liquid vehicles. An ideal process for the trituration of medicines is by using milk sugar as a vehicle, and made by constant rubbing through. (Banerjee, 1987: 54.)

ABBREVIATIONS

xH – Decimal Hahnemanian

HPLC – High Pressure Liquid Chromatography

GHP – German Homoeopathic Pharmacopoeia

CHAPTER ONE

INTRODUCTION

Humans have been plagued by disease ever since inhabiting the planet, cures for which are continuously being developed. The quality of medicines that a healer uses is of extreme importance if the patient is to be helped as medicines are an indispensable tool to any physician. Therefore attention must be placed on the initial production steps of preparing and processing the crude drug, as the healing process will be compromised if the medicines are of poor quality. (Vithoulkas, 1980: 157-158.)

Allopathic medicine is rigorously subjected to chemical standardisation and clinical trials where Federal Drug Administration approved methods of extraction of ingredients remain constant throughout. Homoeopathic medicines should therefore also enjoy scrutinization of its manufacturing quality.

Medicinal substances have to be processed before they can be potentized. This may be done by producing a solution, a mother tincture, or by triturating the substance with lactose. According to Samuel Hahnemann, the founder of homoeopathy, the 3 methods are not interchangeable, because they produce medicines with different levels of homoeopathic activity. (Delmour, 1994.)

Homoeopathic medicines are derived from various sources namely; animal, plant, biological and chemical. Plant medicines make up approximately 65% of all the medicines used in homoeopathy. Species of the plant, the parts taken and extraction methods are unique to each pharmacopoeia. (Kayne, 1997: 43-46.)

Today the standard method of extraction for coffee is to produce a mother tincture by soaking the ground toasted beans in ethanol (German Homoeopathic Pharmacopoeia 1985: 19). This homoeopathic product is then known as *Coffea tosta.*

However, investigations by Dellmour indicate that ethanolic extraction may not be the most effective means for extracting the full strength of active ingredients of plant material. His investigations indicated that trituration may be more effective. This study expands on this investigation. (Dellmour, 1994.)

The coffee bean has a long and well documented history. Today, being the world's most popular beverage, more than 400 billion cups are consumed annually. Coffee is a commodity second to oil in worldwide trade. (koffeekorner, 2000.)

There are approximately seventy recognised varieties of coffee species that range from small shrubs to tall trees. Commercially 2 main species are used *Coffee arabica* and *Coffee robusta.* (Stern, 2000: 456.)

Coffee contains a wide range of constituents, such as fats, carbohydrates, organic acids, phenolic acids, nitrogen compounds, and traces of the alkaloids trigonelline, theophylline and theobromine (Demarque, *et al.,* 1997: 123).

Caffeine is an extremely well researched and important alkaloid found in the bean. It is available in numerous foods and beverages as well as over the counter medicines. (Stern, 2000: 456.)

High pressure liquid chromatography (HPLC) is an important analytical technique that provides both qualitative and quantitative information. It is able to provide separation of compounds in short periods of time thereby improving pharmacological and medical research by being able to detect the purity levels of drugs. (Kromidas, 1999: 3.)

The impact of HPLC is considerable, and is an indispensable tool in most advanced laboratories, providing cost effective extensive and in depth structural analysis (Filmore and Lee, 2001).

1.1 AIM OF THE STUDY

The aim of this study was to compare two methods of homoeopathic medicine preparation, namely ethanolic and triturated potencies, of *Coffea tosta*, derived from *Coffea arabica*, using high pressure liquid chromatography to measure the amount of caffeine extracted from the samples.

1.2 STATEMENT OF THE OBJECTIVES

1.2.1 THE FIRST OBJECTIVE

The first objective was to determine the amount of caffeine extracted from 1xH, 2xH and 3xH samples of *Coffea tosta* prepared according to the ethanolic method using high pressure liquid chromatography.

1.2.2 THE SECOND OBJECTIVE

The second objective was to determine the amount of caffeine extracted from 1xH, 2xH and 3xH samples of *Coffea tosta* prepared according to the trituration method using high pressure liquid chromatography.

1.2.3 THE THIRD OBJECTIVE

The third objective was to compare the amount of caffeine extracted by the two methods of preparation.

1.3 THE HYPOTHESIS

1.3.1 HYPOTHESIS ONE

There is no difference in the amount of caffeine extracted by either method of preparation.

1.4 DELIMITATIONS

1.4.1 DELIMITATION ONE

The study was limited to measuring the amount of caffeine extracted from *Coffea tosta* and not any other of the plant's constituents.

1.5 THE ASSUMPTIONS

1.5.1 ASSUMPTION ONE

The manufacture of the *Coffea tosta* tincture was in accordance with the principles of the German Homoeopathic Pharmacopoeia (GHP) methods of preparation.

1.5.2 ASSUMPTION TWO

The high pressure liquid chromatography runs were reliable and constant.

CHAPTER TWO

REVIEW OF THE RELATED LITERATURE

2.1 INTRODUCTION

The elements of this research are coffee and caffeine, two methods of homoeopathic medicine preparation, namely trituration and ethanolic potencies, and high pressure liquid chromatography. This review will attempt to cover each aspect of the research and their relevance to the study.

2.2 COFFEE AND CAFFEINE

2.2.1 THE HISTORY OF COFFEE

Coffee is known to have originated in Ethiopia, where it was initially eaten whole or crushed. During the thirteenth century the custom of making a brew from this bean increased throughout Arabia. (Mackley, 1989: 28.) Distribution was tightly controlled and export material was boiled destroying its ability to germinate (Knox and Huffaker, 1997: 16).

At the beginning of the seventeenth century cultivation outside Arabia began, and soon grew to play an important socio-economic role in the world (Knox and Huffacker, 1997: 17). In France it was controlled by physicians who sold the bean more as a drug than as a beverage (Carper, 1988: 170-171). During the latter half of the seventeenth century and into the eighteenth century, coffee houses opened throughout Europe and the idea spread quickly. Today coffee is one of the worlds most consumed beverages. (Carper, 1988: 170-171.)

2.2.2 THE COFFEE PLANT AND BEAN

The coffee plant requires a moderate temperature along with an abundant rainfall in order for

optimal germination. A matured plant will reach a height of approximately seven meters when it will bear fruit which ripens from a green to red colour in approximately 7 months. (Mackley, 1989: 29.)

After an adequate rainfall, trees produce white flowers which bloom for 3 days before falling off. Each pollinated flower becomes a cherry called so due to its red colour. A cherry contains 2 seeds tightly packed against each other. The bean is 2 seeds of each fruit. (Stern, 2000: 456-457.)

The main growing species are *arabica* and *robusta. Coffea arabica* constitutes three quarters of the worlds coffee production. It originated in Arabia, and has a relatively low caffeine content. The plants are large, reaching heights of up to 20 feet. The fruit is oval shaped and contains two flat seeds. *Coffea robusta* thrives in higher altitudes and is more resistant to parasites. As it contains a larger content of caffeine it is used in speciality blends. The plants grow to a height of up to a height of 32 feet. This species has a round fruit which can take up to a year to mature.

The bean needs to be extracted from the surrounding pulp within a few days of harvesting and there are two processes which can be used, namely the wet and dry. The wet process involves a complicated and expensive process of berry cleaning, maceration, desiccation and peeling. In the dry process the beans are exposed to the sun for 15-20 days while being continuously stirred. The beans are then milled to remove the dry capsule. (Mashall, 1983: 33-34.)

When the coffee bean is roasted certain chemical changes occur. Firstly chlorogenic acid releases caffeine, vitamin PP is produced from trigonelline and lastly cafeone is given off which is responsible for the characteristic odour of coffee. (Demarque, *et al.*, 1997: 123.)

2.2.3 CAFFEINE

Caffeine is a bitter, odourless alkaloid found naturally in tea, coffee, kola nuts, mate and guarana (Lee and Lee, 1994: 21). Caffeine falls into a group of compounds, along with theophylline and theobromine, called the xanthines (Martindale, 1989: 1522). Chemically this alkaloid is known as 1,3,7-trimethylxanthine (Katzung, 1989: 245).

Caffeine is readily available in certain beverages and over the counter combination analgesics (e.g. Migril®, Antipyn®) and stimulants (e.g. Bioplus®, Liviton®) (Snyman, 2002: 57, 60, 373, 374).

Within an hour of ingesting a cup of coffee, caffeine reaches the bloodstream and remains in circulation for approximately 4 to 6 hours (Carper, 1988: 170). There are several physiological actions that caffeine stimulates. Caffeine is predominantly a central nervous system stimulant, where it acts mainly on the higher neural centres. It is known to inhibit phosphodiesterase enzyme activity and have an antagonistic effect on central adenosine receptors. (Martindale, 1989: 1523.)

Caffeine is commonly used to improve alertness and attention and to prevent sleep. Even in small doses of 32 milligrams, caffeine boosts mental functioning, reaction speed and accuracy. (Carper, 1988: 170.)

Cardiovascular effects are less profound. However, caffeine increases the force of cardiac contractions as well as affecting periodicity. It exhibits this effect by increasing calcium influx into cardiac muscle. (Katzung, 1989: 246.) Caffeine causes vasoldilation in most blood vessels, but in the cerebral blood vessels it can cause vasoconstriction (Rang and Dale, 1991: 409-411). In an evaluation of the electrocardiographic and haemodynamic effects of caffeine,

one study showed that blood pressure was slightly elevated after ingestion of the alkaloid (Ammar, *et al.*, 2001). This effect can be beneficial in the elderly as their blood pressure tends to fall after eating. Consuming a cup of coffee after a meal may prevent this (Carper, 1988: 173). However, caffeine toxicity can cause severe cardiac arrhythmias, atrial fibrillation and ventricular tachycardia (Donnerstein, *et al.*, 2001).

One of the major therapeutic effects of the methylxanthines is bronchodilation, and the ability to inhibit the release of histamine from lung tissue (Katzung, 1989: 247). Caffeine also reduces fatigue of the respiratory muscles. In one study, airway function was still improved up to 4 hours after caffeine consumption. (Bara and Barley, 1998.) In patients suffering with obstructive lung diseases, caffeine increases diaphragmatic contractions and reverses fatigue (Katzung, 1989: 247).

Caffeine is reported to increase gastric acid and digestive enzyme secretion (Katzung, 1989: 246).

2.2.4. COFFEE AND HOMOEOPATHY

2.2.4.1 THERAPEUTIC EFFECT

From a homoeopathic point of view, there is no difference between the materia medica of *Coffea cruda* (raw coffee) and *Coffea tosta* (Vermeulen, 1994: 343).

Clinical symptoms susceptible to treatment with *Coffea tosta* include: hyperethesia of the senses, i.e. sensitivity to pain, odours and noises; hyperactivity of the nervous system including neuralgia , excitability, unusual activity of mind and body and insomnia (Vermeulen, 1994: 343-344; Jouanny, 1984: 125).

2.2.4.2 ANTIDOTE EFFECT

Along with mint and camphor, coffee as a beverage is one of the most well known homoeopathic antidotes (Taylor, 1998).

Gaier defines an antidote as being a substance which is similar in bioenergetic effects, but which will neutralise the competing substance's field of influence, thereby cancelling it's effects (Gaier, 1991: 39-40).

In aphorism 259 of the Organon of Medicine, Samuel Hahnemann explains that any substance which could exert a medicinal influence on the patient should be removed while they are on homoeopathic treatment. The small homoeopathic dose must not be extinguished or disturbed by a foreign medicinal irritant. (Boericke, 1994: 281.)

For this reason, therefore, homoeopaths generally request their patients to stop drinking coffee while they are on homoeopathic medication (Cummings and Ullman, 1997: 35-36).

2.3 HOMOEOPATHIC PHARMACY

Official pharmacopoeia have been established, for example, Germany, Britain, the United States of America and India (Banerjee, 1987: 6-7). These reference works contain specific standards for base substance and mother tincture preparation, potency preparation and methods for testing starting materials (Gaier, 1991: 398).

Potentization of medicines is a method unique to homoeopathy. It involves standardised physical and mathematical attenuations which increase the bioavalibility and physical solubility of medicines. Each stage of attenuation is accompanied by vigorous shaking, known as succussion. (Gaier, 1991: 432.)

Three potentization scales have been developed, namely the decimal, centesimal and millesimal (Banerjee, 1987: 48).

In the decimal scale, introduced by Constantine Hering, one tenth of the crude drug is combined with the appropriate vehicle and the mixture is shaken vigorously. This then forms the first decimal potency or 1xH. This process is repeated in a stepwise progression such that the second decimal potency will then contain one tenth of the first decimal potency etc. etc.

The centesimal scale, developed by Samuel Hahnemann, is a one-in-a-hundred dilution (Banerjee, 1987: 48-50). The first potency contains one hundredth of the base substance, and is referred to as 1cH. Each potency following that contains one hundredth of the preceeding potency. (Gaier, 1991: 447-449.)

In the millesimal scale of dilution, the proportion of base substance to diluent is 1 to 50 000 (Banerjee, 1987: 55).

Dynamization of a substance is a reliable and accurate method for producing a stable medicine. Hahnemann states that succussion and trituration ensure that medicine and diluent are correctly mixed and that the relevant medicinal powers are released. (Boericke, 1994: 288.)

Ainsworth (1983: 164) recommends that the production of homoeopathic medicines should be manufactured through professional channels as they should be considered as medicine.

The xH scale of dilution was chosen in this research because the *Coffea tosta* tincture in the GHP is prepared using a 1:10 ratio of crude coffee to ethanol (1985: 19). In order to keep the

dilution levels the same throughout, the triturated dilutions were prepared on the xH scale as well.

2.3.1. ETHANOLIC DILUTIONS

This applies to all soluble base materials, which includes all plant, most animal and certain mineral substances. The base material is placed in a designated aqueous and alcoholic mixture in proportional quantities as stated in the pharmacopoeia, and left to macerate for a set time. The resultant solution is a mother tincture. It is homoeopathic medicine in its most concentrated form. The mother tinctures are the starting points for the production of the homoeopathic potencies. (Kayne, 1997: 46-48.) From the mother tincture either decimal or centesimal potencies can be manufactured.

Ethanol is used as a diluent because it has numerous advantages;

- in small doses it is safe to consume and is soluble in water in all concentrations;
- ethanol preserves both animal and plant material, and is able to extract medicinal properties from them;
- long storage of materials in an alcoholic solution is possible (Bannerjee, 1987: 158-159).

2.3.2. TRITURATED DILUTIONS

All insoluble materials (such as gold, and silver) are triturated in a base/vehicle of saccharum lactis. Saccharum lactis is a disaccharide and is obtained from skimmed goats milk which has undergone extensive refining and filtration procedures. Saccahrum lactis is a milky white crystalline powder that is odourless, sweet to taste, insoluble in alcohol and soluble in water. (Bannerjee, 1987: 54-55.)

Trituration involves grinding the base substance with saccharum lactis, in a 1:10 or 1:99 proportion. The saccharum lactis is divided into three equal portions by weight. One third of the saccharum lactis is incorporated into a mortar along with the base substance. The mixture is ground in a uniform circular manner for six minutes. The mixture is then scraped from the mortar for three minutes and then stirred for one. These steps are then repeated. Another third of the saccharum lactis is added and the above mentioned process repeated. The last third is added and the mixing repeated. This then forms the first dilution. The next dilution is made using one-tenth or one-hundredth of the first dilution with nine or ninety-nine parts of saccharum lactis. (Banerjee, 1987: 55-57.)

Each triturated dilution involves a 1 hour interaction between medicine and vehicle, where both are subjected to intense mechanical and energy factors. This enhances the dynamization so that all of the natural constituents are included in the final product, whereas ethanol only extracts the soluble components. (Dellmour, 1994.)

Hahnemann in the 1st edition of Chronic Disease compared medicines made from solutions and triturations, and stated his preference for trituration. In the same concentration and with the same number of potentizing stages, the trituration-based medicine proved more powerful. (Dellmour, 1994.) He stated that plant material should be triturated with saccharum lactis 3 distinct times before potentising the medicine in an ethanolic solution (Boericke, 1994: 288-290).

Dellmour investigated work by Madaus, who provided evidence that triturated medicines contained practically all the constituents of fresh plants, whereas tinctures suffered serious losses due to the selective nature of ethanolic extraction (see Figure 2.1). Madaus also asserted that triturated medicines had a better shelf life than ethanolic medicines. The reason

for this is that raw materials of animal and plant origin have different constituents which are housed in separate locations in different cell compartments which ensures their stability. During the production of an ethanolic mother tincture all these highly reactive biochemical substances are combined into a single fluid phase which results in countless chemical reactions. However, during the trituration process, the dryness of the vehicle and the mechanical process involved results in rapid separation of the constituents which therefore prevents interaction thereby preserving the stability of the individual constituents. (Dellmour, 1994.)

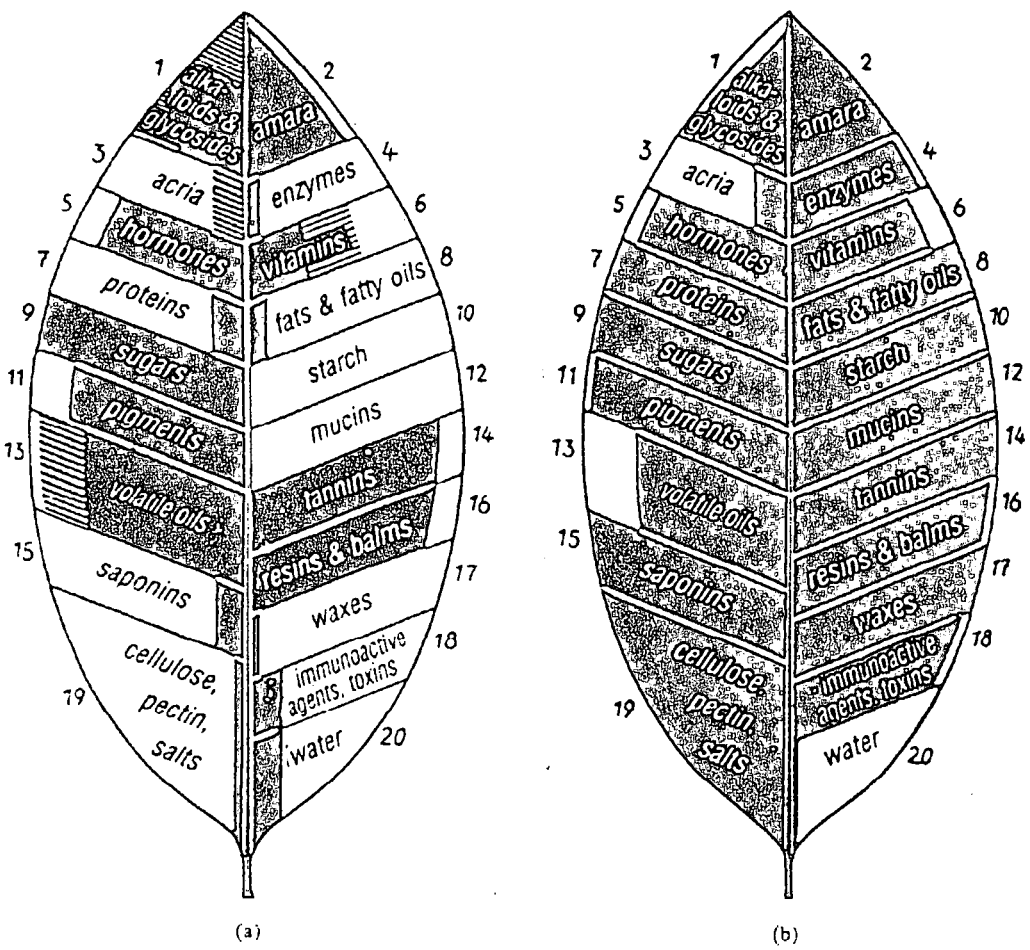

Figure 2.1 Constituents of triturated plant material and mother tinctures. Estimated percentage of concentration in fresh plant material. Black areas: natural constitutions; white areas: losses.
a) Ethanolic tincture b) Trituration of fresh plant material. (Dellmour, 1994.)

2.4 HIGH PRESSURE LIQUID CHROMATOGRAPHY

Chromatography is a method by which different components are separated between two phases. One phase is stationary while the other is mobile. It is a method that is applicable to large areas of research and industry. (Kromidas, 1999: 173.)

HPLC was developed in the 1960s as research into chromatography indicated what was necessary to achieve higher separation efficiencies and consequently shorter analysis times. Smaller and better packing of the particles was essential in order for the system to function optimally. This resulted in the development of high pressure pumps in order to force the mobile phase through the column at the required flow rate. (Robards, *et al.,* 1994: 7.)

HPLC provides both a quantitative and qualitative analysis of each compound within the sample. Each compound has its own unique retention time that is characteristic under certain conditions. (Lindsay, 1992: 4.)

The separation of components is distributed between a stationary and mobile phase. A continuous supply of mobile phase is injected into the system to act as a transport for the sample components as they come into contact with the stationary phase. As the sample is passed through the column, separation results from the different affinities of the sample components. A compound with a high affinity for the stationary phase will move through the system at a slower rate. The lower the affinity, the faster the time taken to move through. (Robards, *et al.,* 1994: 7.)

2.4.1 RETENTION MEASUREMENTS AND PEAK DISPERSION

The eluted compounds are recorded by the detector in bell shaped curves. The signals are known as peaks. Peaks give both quantitative and qualitative information regarding the

mixture.

- Quantitative: both the area and the height of the peak are proportional to the amount of compound injected. A calibration graph can be derived from peak areas obtained for various solutions of precisely known concentrations and a peak size comparison then can be used to determine the concentration of the known sample.
- Qualitative: retention time of the components of a mixture is always constant under identical chromatographic conditions. The column dimensions, the stationary and mobile phase composition and flow, sample size and temperature provide the chromatographic conditions. Therefore the peak can be identified by injecting the relevant substance and comparing it to retention times. (Meyer, 1998: 20-22.)

Retention time is the period between sample injection and recording of the maximum peak. Two compounds are able to be separated if they have different retention times. The mobile phase flow velocity needs to be at suitable levels, otherwise a compound cannot be characterised. (Meyer, 1998: 23.)

2.4.2 PUMPS AND SAMPLE INJECTION

The pump or solvent delivery system passes the mobile phase through the system at a constant flow rate. Pumps are constructed of inert materials that are resistant to chemical attack from the mobile phase.

A mobile phase reservoir stores the solvent before it is fed into the pump. The sample is transferred from a microlitre syringe into the sample loop or container while the valve is turned on to "Load". By shifting the switch to "Inject" the sample loop is connected to the high pressure mobile phase stream, thereby transferring the contents of the syringe into the column. A small amount of sample remains in the needle port valve and needs to be flushed

out either by the mobile phase or a few injections of the following sample. (Lindsay, 1992: 57-59.)

2.4.3 MOBILE PHASE

In order to interact with a suitable stationary phase and to separate a mixture effectively, the mobile phase must be correctly selected. The mobile phase must not react with the compounds within the sample, it should not be detected. (Meyer, 1998: 57.)

The polarity of the stationary and mobile phases should not be similar as this leads to poor separation of the compounds. Therefore silica, which is highly polar, requires a mobile phase of low polarity. The polarity of the mobile phase directly influences the retention of solutes. (Lindsay, 1992: 118-119.)

Water for HPLC must be specially purified through a multiple stage purification system (Meyer, 1998: 151-152).

2.4.4 COLUMNS

The majority of HPLC columns are constructed with high grade stainless steel that is resistant to pressure and is chemically inert. HPLC tends to favour short diffusion paths, therefore a short column with a small packing size gives optimal results. (Meyer, 1998: 100-102.)

Tubing within the system must be free of roughness and any micro porous structure on the surface (Parris, 1989: 38-40).

Porous silica micro particles are most commonly used for HPLC columns. Silica has OH or silanol groups on the surface that can be modified. The manufacturing process occurs at high pressures, thus ensuring a narrow distribution of particle and pore size, which results in stable

and highly efficient columns. The silica column can have limitations at extremes of pH, therefore other packing materials such as alumina and carbon have been developed. (Lindsay, 1992: 149-152.)

2.4.5 DETECTORS

Detectors monitor the mobile phase as it emerges from the column by converting change in mobile phase composition into an electrical signal which then is displayed as a deviation from a baseline. Various characteristics are required for a detector to be suitable, such as sensitivity to eluted peaks that are of interest, ability to monitor trace amounts of the compounds, stability in the face of changes in the composition and temperature of the mobile phase, quick reaction, convenience and reliability. (Meyer, 1998: 76.)

2.4.6. REVERSED-PHASE CHROMATOGRAPHY

This is a form of HPLC that is also known as bonded phase chromatography. It is the most widely used of the liquid separation methods, and has wide applications in the pharmaceutical industry.

The term reversed-phase arises from the fact that this separation mode utilizes a non-polar stationary phase with a polar mobile phase which is the reverse of the situation in normal phase (adsorption) chromatography. With this separation technique the more polar the solute the lower the retention. Thus, by increasing the polarity of the mobile phase, an increase of solute retention occurs.

Columns used in this method of chromatography are identified by the nature of their bonded R group. The most common functional group on the bonded reversed phase is C 18 octa decyl silane. Carbon load refers to the amount of material bonded onto the silica.

The greater the carbon load of the stationary phase the better the sample handling capacity. (Robards, *et al.*, 1997: 317-320.)

In reversed-phase systems the greater the amount of water present in the eluent the longer the retention time. The mobile phase consists of a water-miscible solvent in a mixture of water or an aqueous buffer. (Meyer, 1998: 149.)

In an experiment to determine the concentration of caffeine in soft drinks by the Department of Chemistry at the University of Adelaide, a reverse phase C18 column was used. The components were selectively detected using a UV absorption (University of Adelaide, 2001).

In homoeopathy, manufactured mother tinctures are subjected to a range of tests to determine their purity. HPLC is often used to carry out purity and strength tests in order to determine their quality. (Kayne, 1997: 56.)

The applications of HPLC are varied as most organic compounds can be analyzed. Samples can be separated and the quantity of each component estimated. (Fritz and Schenk, 1979: 402.)

CHAPTER THREE

METHODS AND MATERIALS

3.1 STUDY DESIGN

The aim of this project was to show which method of homoeopathic remedy preparation, namely triturated or ethanolic dilutions, extracted the most caffeine from *Coffea tosta* using high performance liquid chromatography.

The mother tincture was prepared in the W. Last Flora Force homoeopathic laboratory in Cape Town according to method 4a in the GHP (1985: 19). The dilutions and the triturations were prepared in the same laboratory according to method 4a and 7 of the GHP respectively (GHP, 1985: 19, 37). The high pressure liquid chromatography procedures were conducted at the Durban Institute of Technology Department of Chemistry laboratory.

The coffee sample used was 100% ground, medium roasted, Colombia House of Coffees® brand. The ground bean was derived from *Coffea arabica.* The bean was roasted at the House of Coffees® factory roasterie for approximately 13 to 20 minutes at a temperature between 220°C and 300°C[1](Bove, 2002.)

The study group consisted of

- ethanolic dilutions of *Coffea tosta*, which included the mother tincture or 1xH , 2xH and 3xH.
- triturated dilutions of *Coffea tosta*, which included the 1xH, 2xH and 3xH.

[1] The company would not divulge any exact specifications

Three samples were analysed from each method and dilution level. There were 18 samples of ethanolic dilutions and 18 samples of triturated dilutions. The decimal scale of dilution was chosen as the mother tincture *Coffea tosta* is prepared in the GHP as a one-in-ten tincture. In order to keep the dilutions uniform throughout, the decimal scale was used for both the ethanolic and triturated dilutions.

3.2 DILUTIONS

3.2.1 PREPARATION OF TRITURATED DILUTIONS

These were prepared according to method 7 in the GHP (1985: 37).

3.2.1.1 PREPARATION OF THE 1XH DILUTION

i. 20g of *Coffea tosta* was weighed out.

ii. 180g saccharum lactis (obtained from W. Last Flora Force, Cape Town) was weighed out into 3 portions of 60g each.

iii. 20g of *Coffea tosta* was combined with the first 60g portion of saccharum lactis in a sterilised mortar.

iv. The mixture was then triturated in a uniform circular manner for 6 minutes.

v. A clean spatula was used to scrape the mixture for 3 minutes.

vi. The mixture was then stirred for 1 minute.

vii. Steps iv to vi were repeated.

viii. The second 60g portion of saccharum lactis was added to the mortar.

ix. Steps iv to vii were repeated.

x. The third 60g portion of saccharum lactis was added to the mortar.

xi. Steps iv to vii were repeated.

xii. The triturated material was stored in a clean, sealed glass container.

Each dilution took one hour to complete.

3.2.1.2 PREPARATION OF THE 2XH DILUTION

The 2xH dilution was made by adding one part of the 1xH to nine parts by weight of saccharum lactis and repeating the process as outlined in 3.2.1.1.

3.2.1.3 PREPARATION OF THE 3XH DILUTION

The 3xH dilution was made by adding one part of the 2xH to nine parts by weight of saccharum lactis and repeating the process as outlined in 3.2.1.1.

3.2.2 PREPARATION OF THE ETHANOLIC DILUTIONS

3.2.2.1 PREPARATION OF THE MOTHER TINCTURE (1XH DILUTION)

The mother tincture was prepared at the W. Last Flora Force homoeopathic laboratory in Cape Town in accordance with method 4a in the GHP (1985: 19).

According to this method the mother tincture is in fact a 1xH dilution.

i. 50g of *Coffea tosta* was combined with 450ml of 92% ethanol.

ii. The mixture was shaken and left to stand for 7 days in a cool room where the temperature was not higher than 20°C.

iii. The solution was agitated every morning for 30 seconds.

iv. After 7 days the mixture was strained and sealed in an amber glass bottle.

3.2.2.2 PREPARATION OF THE 2XH ETHANOLIC DILUTION

The 2xH was prepared using 10ml of the 1xH dilution and combining it with 90ml of 62% ethanol solution and succussing 10 times (as per GHP).

3.2.2.3 PREPARATION OF THE 3XH ETHANOLIC DILUTION

The 3xH was prepared using 10ml of the 2xH dilution and combining it with 90ml of 62% ethanol solution and succussing 10 times (as per GHP).

3.3 HIGH PRESSURE LIQUID CHROMATOGRAPHY

3.3.1 CAFFEINE STANDARD

Pure, powdered caffeine, manufactured by Sigma, was obtained from the Durban Institute of Technology Department of Chemistry laboratory.

Three different dilutions of the caffeine standard were made up, namely, 0,1mg/ml, 0,2mg/ml and 0,3mg/ml. These dilutions were passed through the HPLC system and the results recorded.

The standards were run in order to calibrate the HPLC system.

3.3.2 8-CHLOROTHEOPHYLLINE

8-Chlorotheophylline is used as an internal standard. Like caffeine it is a purine with similar physical properties. It monitors any changes in volume that occur within the solution which could result in quantitative errors. It is often added to samples in analytical techniques where preparation of the samples might result in a loss of part of the sample. A certain concentration of the internal standard is added before preparation. If for example 10% of the internal standard is lost then the implication is that there should be a loss of 10% of the compound/substance of interest within the sample. It thereby compensates for any losses.

The 8-chlorotheophylline (0,16mg/ml solution) used in this study was obtained from Sigma. During this study, no 8-chlorotheophylline was lost. (Adamson, 2002.)

3.3.3 PREPARATION OF THE HPLC MACHINE

The mobile phase consisted of 30% methanol and 70% HPLC grade water containing 1% acetic acid.

The column used was a 150mm Novapak C18 reverse phase column.

The Varian UV 50 variable wavelength detector was set at 280 namometers (nm).

The flow rate was set to 1,0ml/min.

The run time set for each sample was 5 minutes. (Adamson, 2001a.)

3.3.4 PREPARATION OF THE SAMPLES

3.3.4.1 TRITURATED

3.3.4.1.1 1xH

i. Approximately 0.5g of the sample was weighed out and the exact mass recorded.

ii. This was added to 25ml of 8-chlorotheophylline solution.

iii. The mixture was stirred for 5 minutes using a glass stirring rod.

iv. The mixture was then filtered through Whatman No. 1 filter paper to remove any particulate materials.

v. The collected filtrate was collected and stored in a clean glass bottle which was clearly labelled, prior to HPLC analysis.

3.3.4.1.2 2xH

i. Approximately 10g of the 2xH sample was weighed out and the exact mass recorded.

ii. This was added to 25ml of 8-chlorotheophylline solution.

iii. The procedures stated in 3.3.3.1 iii-v were repeated.

3.3.4.1.3 3xH

i. Approximately 10g of the 3xH sample was weighed out and the exact mass recorded.

ii. This was added to 25ml of 8-chlorotheophylline solution.

iii. The procedures stated in 3.3.3.1 iii-v were repeated.

3.3.4.2 ETHANOLIC

These samples required no special preparation because they were injected directly into the HPLC system.

3.4 CHROMATOGRAPHIC ANALYSIS

10µl of each sample was manually injected into the system using a Rheodyne sample introduction valve.

Between each dilution level of sample injection, the sample injection valve was flushed out by injecting 3 loads of 100µl of the new sample to flush out any residual material from the previous sample.

Results were printed and stored on computer. See Appendix C.1 for an example of an HPLC run.

3.5 DERIVATION OF THE CAFFEINE STANDARD

The values for three peak areas of the standardised caffeine concentrations were as follows:

0,1mg/ml – 132341

0,2mg/ml – 252390

0,3mg/ml – 383252

The values were added together and divided by six (1 x 0,1 + 2 x 0,1 + 3 x 0,1) to obtain an average value for 0,1mg/ml.

$$\frac{132341 + 252390 + 383252}{6} = 127997 : 0{,}1\text{mg/ml}$$

This was then used to derive a reciprocal factor with which to mathematically establish the mg/ml value of the analysed samples.

$$\frac{127997}{1279970} = 0{,}1$$

The peak areas of caffeine from each HPLC result was divided by 1279970 to obtain mg/ml of caffeine for each sample (Adamson, 2001b).

3.6 STATISTICAL ANALYSIS

Both parametric and non-parametric statistical tests were used to analyse the data.

Sample sizes per group were small, namely $n_1 = 16$ and $n_2 = 14$.

Data entry and analysis were performed using the Statistical Package for Social Sciences (SPSS) version 9+.

The 2 groups analysed were:

Group 1: triturated dilution samples;

Group 2 : ethanolic dilution samples.

3.6.1 MANN-WHITNEY U-TEST

The Mann-Whitney U-test was used to compare Group 1 and Group 2 with one another. The two groups were treated as being independent of one another, hence unpaired. (Swinscow, 1996: 92-94.)

The purpose of this test was to determine whether there was any difference between Group 1 and 2.

Hypothesis testing:

H_o : there is no difference between the two groups;

H_1 : there is a difference between the two groups.

The level of significance, α, was set at 0,05 in each case.

Decision rule:

If $p \geq 0,05$ accept H_o;

If $p < 0,05$ reject H_o.

P is the observed significance level of the test or P-value.

3.6.3 ANOVA ANALYSIS

The second method used to analyse the data was the ANOVA test. In an ANOVA test the data must be independent from each other, the distribution of each Group in the original data is normal and the variances are the same for each Group.

n – number of observations

m – mean

s^2 – variance

k – Groups

$n_1, n_2 \ldots\ldots n$ – observations

$m_1, m_2 \ldots\ldots m_k$ – Group means. (Riffenburgh, 1999: 311.)

Hypothesis testing:

H_o: there is no difference between the two groups;

H_1 : there is a difference between the two groups.

The level of significance, α, was set at 0,05 in each case.

Decision rule:

If $p \geq 0,05$ accept H_o;

If $p \leq 0,05$ reject H_o.

P is the observed significant level of the test or P-value.

3.6.3 TABLES AND SUMMARIES

The calculated p-values of the Mann-Whitney unpaired test and the ANOVA tests were demonstrated in the form of tables.

The summary statistics (mean, mode, median, standard error and the coefficient of variation) were provided.

CHAPTER FOUR

RESULTS OF THE STUDY

4.1 THE CRITERIA GOVERNING THE ADMISSIBILITY OF RESULTS

The data utilised was percentage caffeine extracted from each sample. Only data that produced an HPLC peak reading was used.

4.2 CONVERSION OF PEAK AREA INTO PERCENTAGE CAFFEINE

4.2.1 TRITURATED DILUTIONS

20g *Coffea tosta* was the base amount of coffee used as the starting point for the 1xH dilution.

The caffeine concentration was determined by using the peak area values of the individual HPLC results for each potency and dividing them by the factor 1279970 (see 3.5.1). Because 25ml solution samples were made up, the caffeine concentration was multiplied by 25 to determine the caffeine concentration in 25ml.

The mass of trituration that was added to each 25ml solution was recorded.

1xH sample number 1 – 0,4999g of triturate
sample number 2 – 0,5004g of triturate
sample number 3 – 0,5005g of triturate

2xH sample number 1 – 10,004g of triturate
sample number 2 – 10,0015g of triturate
sample number 3 - 10,007g of triturate

3xH sample number 1 – 10,007g of triturate
sample number 2 – 10,0004g of triturate
sample number 3 – 9,9998g of triturate

The starting volume of *Coffea tosta* and saccharum lactis was 200g (180g of saccharum lactis and 20g of *Coffea tosta*). The caffeine concentration was calculated for each sample.

The 1xH caffeine concentration in 25ml was multiplied by the total mass of the initial *Coffea tosta* and saccharum lactis (20g + 180g) and divided by the sample amount that was added into the 25ml (as noted above).

The 2xH caffeine concentration in the 25ml was multiplied by the total mass of powder i.e. 200 x 10, divided by the sample mass that was added to the 25ml.

The 3xH the caffeine concentration per 25ml was multiplied by the total mass of powder 200 x 100, and then divided by the sample mass that was added to the 25ml.

To convert to a percentage the amount of caffeine in milligrams was divided by the total mass of *Coffea tosta* that was added to the first triturate, and then multiplied by 10. (Adamson , 2001b.)

See Appendix A.1 for data.

4.2.2 ETHANOLIC DILUTIONS

The recorded starting amount of *Coffea tosta* was 50 g which was incorporated into 450ml of 92% ethanol that resulted in the 1xH dilution.

For the 1xH dilutions (1 x 10-1) the caffeine concentration was multiplied by 450ml to obtain the total amount of caffeine in 450ml.

For the 2xH (2 x 10-2) dilutions the caffeine concentration was multiplied by 4500ml.

For the 3xH (3 x 10-3) dilutions the caffeine concentration was multiplied by 45000ml.

The answers obtained from the above calculations were multiplied by the total mass of the

original amount of coffee that was incorporated into the tincture (50g = 50 000mg) and multiplied by 100 to obtain a percentage caffeine. (Adamson , 2001b.)

See Appendix A.2 for data.

4.3 INTER-GROUP COMPARISONS

Figure 4.1 is a graphical representation of the mean quantity (percentage) caffeine extracted at each dilution.

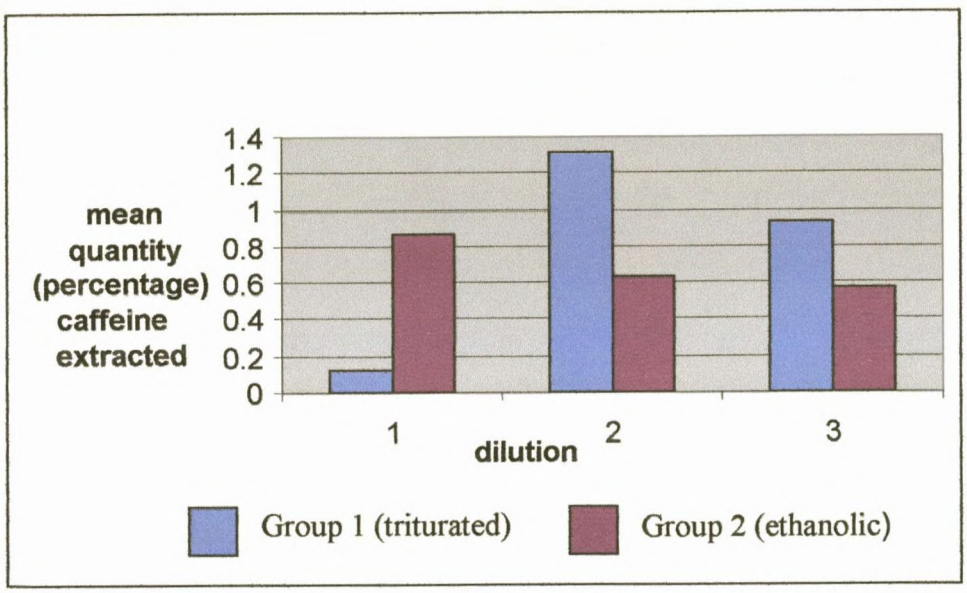

Figure 4.1 Mean quantity (percentage) of caffeine extracted per Group per dilution level.

4.3.1 INTER-GROUP COMPARISONS AT EACH DILUTION LEVEL

The Mann-Whitney test for inter-group comparison revealed that for the 1xH and 2xH dilutions, the p-value was smaller than 0,05 (see Table 4.1). The null hypothesis was thus rejected and it was concluded that there was a significant difference between the ethanolic and triturated potencies of the 1xH and 2xH. The results of the 3xH potency showed a p-value that was larger than 0,05 which indicated that there was no significant difference between the 2 groups. See Appendixes B.1 – B.3 for further analysed data.

Table 4.1 Mann Whitney inter-group comparison with regards to percentage caffeine at each potency level.

POTENCY	P-VALUE	CONCLUSION
1 xH	0,04	Difference
2 xH	0,04	Difference
3 xH	0,165	No difference

The ANOVA test for inter-group comparison revealed that for the 1xH and 2xH dilutions, the p-value was smaller than 0,05. Therefore the null hypothesis was rejected and it was concluded that there was a significant difference between the ethanolic and triturated dilutions of the 1xH and 2xH. The results of the 3xH dilution showed that a p-value that was larger 0,05 which indicates that there was no significant difference between the 2 Groups.

Table 4.2 ANOVA inter-group comparison with regards to percentage caffeine at each potency level.

POTENCY	P-VALUE	CONCLUSION
1xH	0,000(< 0,001)	Difference
2xH	0,000(< 0,001)	Difference
3xH	0,433	No difference

4.3.2 INTER-GROUP COMPARISON IRRESPECTIVE OF DILUTION.

Figure 4.2 is a graphical representation of the mean quantity (percentage) caffeine produced by each Group as a whole.

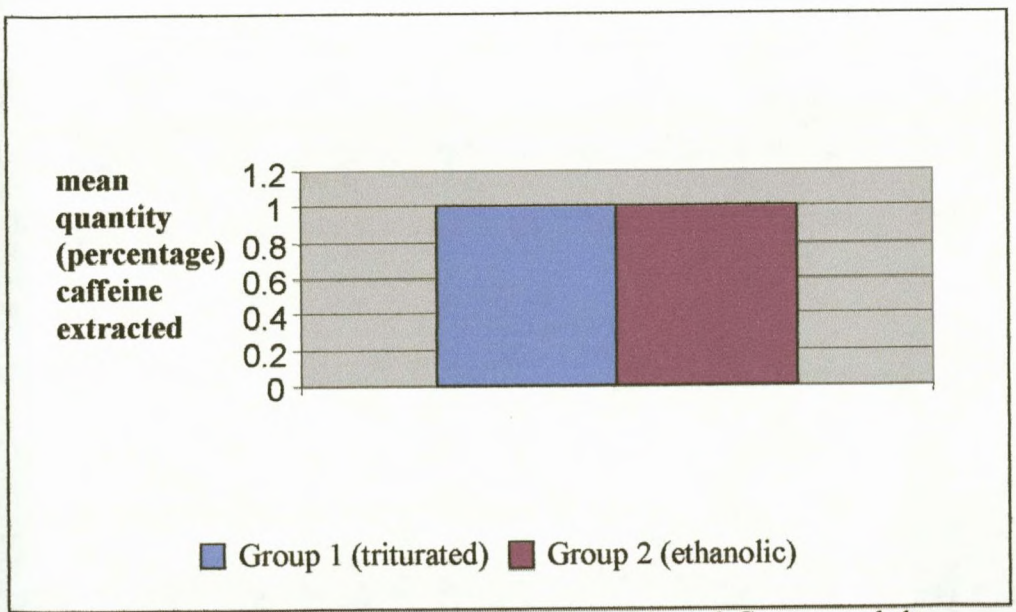

Figure 4.2 Mean quantity (percentage) of caffeine produced by each Group as a whole.

The Mann-Whitney tests showed that the calculated p-value (0,589) was greater than 0,05 (see Table 4.2). The null hypothesis was thus accepted and it was concluded that there was no overall difference in the amount of caffeine extracted from the 2 Groups. See Appendixes B.4 for further analysed data.

Table 4.3 Mann-Whitney test for inter-group comparison with regards to percentage caffeine extracted irrespective of dilution.

TEST	P – VALUE	CONCLUSION
Group 1 versus Group2	0,589	No difference

The ANOVA test revealed a p-value of 0,804. Therefore the null hypothesis was accepted and it was concluded that there was no difference in the percentage caffeine extracted between Group 1 and Group 2. See Appendix B.5 and B.6 for further analysed data.

Table 4.4 ANOVA test for inter-group comparison with regards to percentage caffeine extracted irrespective of dilution.

TEST	P-VALUE	CONCLUSION
Group 1 versus Group2	0,804	No difference

4.4 INTRA-GROUP COMPARISONS

4.4.1 INTRA-GROUP COMAPARISON WITHIN GROUP 1

Figure 4.3 is a graphical representation of the mean quantity (percentage) of caffeine extracted within Group 1.

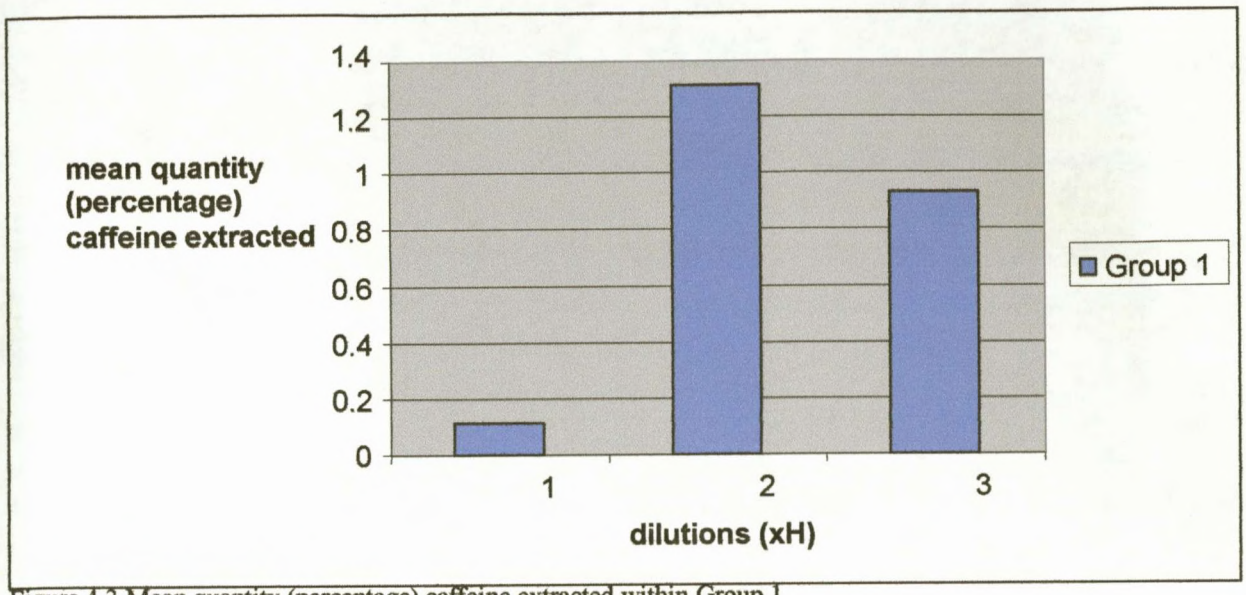

Figure 4.3 Mean quantity (percentage) caffeine extracted within Group 1.

The ANOVA test for the triturated samples revealed a p-value of less than 0,05 (see Table 4.5). The null hypothesis was therefore rejected and it was concluded that there was a difference between the 3 dilution levels. See Appendix B.7 for further analysed data.

Table 4.5 ANOVA test of the triturated samples (Group1) with regards to the different dilutions.

TEST	P-VALUE	CONCLUSION
1Xh, 2xH and 3xH	0,000 (<0,001)	Difference

4.4.2 INTRA-GROUP COMPARISON WITHIN GROUP 2

Figure 4.4 is a graphical representation of the mean quantity (percentage) of caffeine extracted within Group 2.

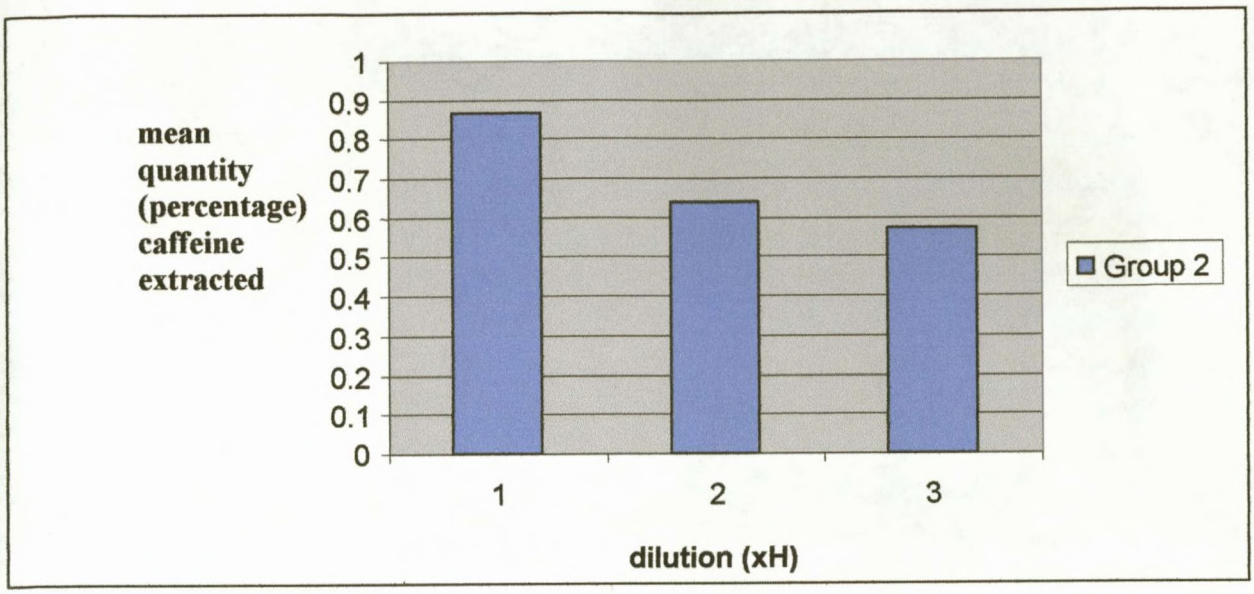

Figure 4.4 Mean quantity (percentage) of caffeine extracted within Group 2.

The ANOVA test for the ethanolic samples revealed a p-value of 0,05 which is equal to α.

Therefore it was concluded that there was no difference between the 3 dilution levels.

See Appendix B.8 for further analysed data.

Table 4.6 ANOVA test of the ethanolic samples (Group 2) with regards to the different dilutions.

TEST	P-VALUE	CONCLUSION
1xH, 2xH and 3xH	0,05	No difference

CHAPTER FIVE

DISCUSSION

The results obtained from the inter-Group Mann-Whitney and ANOVA tests showed that there was a significant difference between the ethanolic dilution and the triturated dilution with regards to the 1xH and 2xH dilutions. In the 1xH dilution the ethanolic method retained relatively more caffeine, whereas in the 2xH dilution the triturated method retained relatively more caffeine. However, there was no difference between the dilution methods at the 3xH level. For this reason, one cannot say that one method is better than the other. This is not what was expected when compared to Dellmour's investigations (1994).

The fact that there was no difference between the two dilution groups at the 3xH dilution could have been due to the small sample size that was statistically analysed. It could also have been due to the fact that HPLC could not distinguish the smaller peak readings from the base noise level. The small peak reading could have been due to the small quantity of caffeine at the 3xH dilution.

The results obtained when comparing the three dilution levels of the triturated samples showed there was a significant difference between each dilution level.

The results obtained from analysis of the ethanolic dilutions showed no significant difference between each dilution level.

Thus it can be said that the one ethanolic dilution is not distinguishable from the next. However, the triturated dilutions can be differentiated from each other in terms of caffeine concentration.

The triturated dilutions showed a trend of extracting a higher amount of caffeine than the ethanolic dilutions with each successive dilution level.

This was expected based on former investigations into the extraction of active ingredients from plants (Hahnemann as per Dellmour, 1994). This observation is important, because if more of the active ingredient is extracted and retained the higher will be the quality of the resulting medicine.

There was also a clear distinction between the triturated samples whereas the ethanolic samples showed little change as the dilution levels increased. Homoeopathically this is important as each potency level is unique, and different potency levels are utilised in different pathological conditions. For instance, low dilution levels (5cH) are normally used for acute conditions, whereas higher dilution levels (30cH) are usually used for chronic conditions (Jouanny, 1993: 94).

The 3xH sample in the HPLC process did not produce all the results as the HPLC system battled to pick up the level of caffeine. A larger amount of sample should have been taken i.e. instead of 20g, 50g should have been used. A larger amount of caffeine present could have produced a reading.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSIONS

Results from the study are inconclusive so it cannot be said that one method is superior to the other. However, there is a trend indicating that the triturated samples retain a higher level of caffeine than do the ethanolic samples as the dilution level increases. There was also no quantitative difference between the ethanolic samples implying that the dilutions were not unique from each other.

Further studies into the efficacy of the 2 methods of homoeopathic remedy preparation are warranted.

6.2 RECOMMENDATIONS FOR FURTHER STUDIES.

1.Raw material to be verified with a voucher lodged at a herbarium if possible (McGraw, *et.al.*, 2000).

2. A larger number of samples should be used for statistical stability.

3. A wider range of active ingredients should be analysed. This study looked at only one active ingredient, namely caffeine. Plants contain a large number of active ingredients that contribute to its pharmacological and homoeopathic action.

4. The research should be performed in the centesimal scale of dilution. This was originally advised by Samuel Hahnemann, who recommended trituration of plant material to the 3cH dilution.

5. Fresh plant material should be triturated. The time period between harvesting the plant and triturating it should be kept to a minimum to avoid degradation of the pharmacologically

active constituents.

6. Samples should be tested over a period of time in order to determine degradation and possibly shelf life of the dilutions produced (as per Dellmour quoting Hahnemann, 1994).

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LIST OF APPENDICES

APPENDIX A.1

THE TABULATED RESULTS OF THE PEAK AREA, CAFFEINE CONCENTRATION AND PERCENTAGE CAFFEINE EXTRACTED FROM EACH TRITURATED SAMPLE.

	PEAK AREA	CAFFEINE CONCENTRATION MG/ML	% CAFFEINE
1xH sample 1 run 1	29713	0,0232	0,166
1xH sample 1 run 2	25234	0,0197	0,0985
1xH sample 2 run 1	31383	0,0245	0,1225
1xH sample 2 run 2	30167	0,0236	0,117
1xH sample 3 run 1	31705	0,0248	0,124
1xH sample 3 run 2	31851	0,0249	0,124
2xH sample 1 run 1	68358	0,0534	1,334
2xH sample 1 run 2	68313	0,0534	1,334
2xH sample 2 run 1	68750	0,05372	1,343
2xH sample 2 run 2	70678	0,0552	1,379
2xH sample 3 run 1	65888	0,0514	1,285
2xH sample 3 run 2	61585	0,0481	1,202
3xH sample 1 run 1	1520	0,0012	0,29
3xH sample 1 run 2	7546	0,0059	1,474
3xH sample 2 run 1	-	-	-
3xH sample 2 run 2	4725	0,0037	0,925
3xH sample 3 run 1	-	-	-
3xH sample 3 run 2	5284	0,0041	1,025

APPENDIX A.2

THE TABULATED RESULTS OF THE PEAK AREA, CAFFEINE CONCENTRATION AND PERCENTAGE CAFFEINE EXTRACTED FROM EACH ETHANOLIC SAMPLE

	PEAK AREA	CAFFEINE CONCENTRATION MG/ML	PERCENTAGE CAFFEINE
1xH sample 1 run 1	1146580	0,895	0,81
1xH sample 1 run 2	1131942	0,884	0,796
1xH sample 2 run 1	1173303	0,9167	0,825
1xH sample 2 run 2	1139206	0,89	0,801
1xH sample 3 run 1	1633146	1,2759	1,148
1xH sample 3 run 2	1180416	0,9222	0,83
2xH sample 1 run 1	93868	0,073	0,657
2xH sample 1 run 2	103255	0,081	0,729
2xH sample 2 run 1	82000	0,064	0,576
2xH sample 2 run 2	83381	0,065	0,585
2xH sample 3 run 1	81315	0,064	0,576
2xH sample 3 run 2	99403	0,078	0,702
3xH sample 1 run 1	3739	0,003	0,27
3xH sample 1 run 2	-	-	-
3xH sample 2 run 1	-	-	-
3xH sample 2 run 2	-	-	-
3xH sample 3 run 1	12431	0,0097	0,873
3xH sample 3 run 2	-	-	-

APPENDIX B.1

SPSS STATISTICAL ANALYSIS OF THE TABULATED COMPARISON BETWEEN GROUP 1 AND GROUP 2 WITH REGARD TO THE 1XH DILUTION IN ACCORDANCE WITH MANN-WHITNEY U-TEST.

Npar Tests:

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
1x Group	12	.4927	.4032	.10	1.15
	12	1.5000	.5222	1.00	2.00

Mann-Whitney U-Test

Ranks

Group		N	Mean Rank	Sum of Ranks
1x	1.00	6	3.50	21.00
	2.00	6	9.50	57.00
	Total	12		

Test Statistics∇

	1x
Mann-Whitney U-Test	.000
Wilcoxon W	21.000
Z	-2.887
Asymp.Sig. (2 tailed)	.004
Exact Sig. [2*(1 tailed Sig.)]	.002∘

∘ .Not corrected for ties

∇. Grouping variables:GROUP

APPENDIX B.2

SPSS STATISTICAL ANALYSIS OF THE TABULATED COMPARISON BETWEEN GROUP 1 AND GROUP 2 WITH REGARD TO THE 2XH DILUTION IN ACCORDANCE WITH MANN-WHITNEY U-TEST.

Npar Tests

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
2x Group	12	.9752	.3581	.58	1.38
	12	1.5000	.5222	1.00	2.00

Mann-Whitney U-Test

Ranks

Group	N	Mean Rank	Sum of Ranks
2x 1.00	6	9.50	57.00
2.00	6	3.50	21.00
3.00	12		

Test Statistics∇

	2x
Mann-Whitney U-Test	.000
Wilcoxon W	21.000
Z	-2.892
Asymp.Sig. (2 tailed)	.004
Exact Sig. [2*(1-tailed Sig.)]	.002∘

∘. Not corrected for ties

∇. Grouping Variable : GROUP

APPENDIX B.3

SPSS STATISTICAL ANALYSIS OF THE TABULATED COMPARISON BETWENN GROUP1 AND GROUP 2 WITH REGARD TO THE 3XH DILUTION IN ACCORDANCE WITH MANN-WHITNEY U-TEST.

Npar Tests

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
3x Group	6	.8095	.4618	.27	1.47
	12	1.5000	.5222	1.00	2.00

Mann-Whitney Test

Ranks

Group		N	Mean Rank	Sum of Ranks
3x	1.00	4	4.25	17.00
	2.00	2	2.00	4.00
	Total	6		

Test Statistics∇

	3x
Mann-Whitney U	1.000
Wilcoxon W	4.000
Z	-1.389
Asymp.Sig. (2-tailed)	.165
Exact Sig. [2*(1-tailed Sig.)]	.267∘

∇. Grouping variable: GROUP

∘. Not corrected for ties

APPENDIX B.4

SPSS STATISTICAL ANALYSIS OF THE TABULATED INTER-GROUP COMPARISON BETWEEN GROUP 1 AND GROUP 2 WITH REGARD TO PERCENTAGE CAFFEINE (IRRESPECTIVE OF DILUTION) IN ACCORDANCE WITH MANN-WHITNEY U-TEST.

Npar Tests:

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Percaf Group	30	.7490	.4430	.10	1.47
	30	1.4667	.5074	1.00	2.00

Mann Whitney

Ranks

Group	N	Mean	Sum of Ranks
Percaf 1	16	16.31	261.00
2	14	14.57	204.00
Total	30		

Test Statistics∇

	Percaf
Mann-Whitney U	99.000
Wilcoxon W	204.000
Z	-.541
Asymp.Sig. (2-tailed)	.589
Exact Sig. [2*(1-tailed Sig.)]	.608∘

∘. Not corrected for ties

∇. Grouping Variable: GROUP

APPENDIX B.5

SPSS STATISTICAL ANALYSES OF THE TABULATED INTER-GROUP COMPARISON BETWEEN GROUP 1 AND GROUP 2 WITH REGARD TO PERCENTAGE CAFFEINE (IRRESPECTIVE OF DILUTION) IN ACCORDANCE WITH AN ANOVA INDEPENDENT SAMPLES TEST.

T-Test

Group Statistics

Group	N	Mean	Std. Deviation	Std. Error Mean
Percaf 1.	16	.7683	.5866	.1466
2.	14	.7270	.1992	5.325E-02

Independent Samples Test

		Levene's Test for equalityof Variances	
		F	Sig
Percaf	Equal Variances Assumped	58.992	.000
	Equal Variances Not Assumed		

	T	df	Sig. (2-tailed)	Mean Difference
Percaf Equal Variances Assumed	.251	28	.804	4.131E-02
Equal Variances Assumed	.265	18.838	.794	4.131E-02

APPENDIX B.6

SPSS STATISTICAL ANALYSIS OF THE TABULATED INTER-GROUP COMPARISON BETWEEN GROUP 1 AND GROUP 2 IN RESPECTIVE DILUTIONS IN ACCORDANCE WITH ANOVA ONEWAY ANALYSIS.

Oneway

Descriptives

		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound
1x	1.00	6	.1170	9.711E-03	3.964E-03	.1068	.1272
	2.00	6	.8683	.1376	5.619E-02	.7239	1.0128
	Total	12	.4927	.4032	.1164	.2365	.7489
2x	1.00	6	1.3128	6.204E-02	2.533E-02	1.2477	1.3779
	2.00	6	.6375	6.817E-02	2.783E-02	.5660	.7090
	Total	12	.9752	.3581	.1034	.7476	.7489
3x	1.00	4	.9285	.4880	.2440	.1519	1.7051
	2.00	2	.5715	.4264	.3015	-3.2594	4.4024
	Total	6	.8095	.4618	.1885	.3249	1.2941

Descriptives

		Minimum	Maximum
1x	1.00	.10	.12
	2.00	.80	1.15
	Total	.10	1.15
2x	1.00	1.20	1.38
	2.00	.58	.73
	Total	.58	1.38
3x	1.00	.29	1.47
	2.00	.27	.87
	Total	.27	1.47

ANOVA

	Sum of Squares	df	Mean Sqaure	F	Sig.
1x Between Grps	1.694	1	1.694	177.888	.000
Within Grps	9.520E-02	10	9.520E-03		
Total	1.789	11			
2x Between Grps	1.368	1	1.368	322.115	.000
Within Grps	4.248E	10	4.248E-03		
Total	1.411	11			

3x Between Grps	.170	1	.170	.758	.433
Within Grps	.896	4	.224		
Total	1.066	5			

APPENDIX B.7

SPSS STATISTICAL ANALYSIS OF THE TABULATED ONEWAY COMPARISON WITH REGARD TO THE DILUTION OF GROUP 1 IN ACCORDANCE WITH AN ANOVA ANALYSIS.

Oneway

ANOVA

TRIT

	Sum of Squares	Df	Mean Square	F	Sig
Between Grps	4.427	2	2.213	39.187	.000
Within Grps	.734	13	5.648E-02		
Total	5.161	15			

Post Hoc Tests

Multiple Comparisons

Dependant Variable: Trit

(I) Group	(J) Group	Mean difference (I-J)	Std. Error	Sig.	Lower Bound ◊	Upper Bound ◊
LSD 1.00	2.00	-1.1958*	.1372	.000	-1.4923	-.8994
	3.00	-.8115*	.1534	.000	-1.1429	-4801
2.00	1.00	1.1958*	.1372	.000	.8994	1.4923
	3.00	.3843*	.1534	.026	5.291E-02	.7158
3.00	1.00	.8115*	.1534	.000	.4801	1.1429
	2.00	-.3843*	.1534	.026	-.7158	-5.29E-02

*. The mean difference is significant at the .05 level

◊. 95% Confidence Interval

Homogenous Subsets

Trit

GROUP	N	1	2	3
Student-Newman-Keuls □°				
1.00	6	.1170		
2.00	4		.9285	
3.00	6			1.3128
Sig.		1.000	1.000	1.000

Means for groups in homogenous substets are displayed

Subset for $\alpha = .05$

□. Uses Harmonic Mean Sample Size = 5.143.

°. The group sizes are unequal. The harmonic mean of the group sizes is used. Type 1 error levels are not guaranteed.

APPENDIX B.8

SPSS STATISTICAL ANALYSIS OF THE TABULATED ONEWAY COMPARISON WITH REGARD TO THE DILLUTIONS OF GROUP 2 IN ACCORDANCE WITH AN ANOVA ANALYSIS.

ANOVA

ETH

	Sum of Squares	Df	Mean square	F	Sig.
Between Grps	.216	2	.108	3.968	.050
Within Grps	.300	11	2.725E-02		
Total	.516	13			

Post Hoc Tests

Multiple Comparisons

Dependant Variable: ETH

(I) Group	(J) Group	Mean difference	Std. Error	Sig.	Lower Bound ◊	Upper Bound ◊
LSD 1.00	2.00	.2308*	9.531E-02	.034	2.106E-02	.4406
	3.00	.2968*	.1348	.050	1.677E-04	.5935
2.00	1.00	-.2308*	9.531E-02	.034	-.4406	-2.11E-02
	3.00	6.600E-02*	.1348	.634	-2307	.3627
3.00	1.00	-.2968*	.1348	.050	.5935	-1.68E-04
	2.00	-6.6000E-02*	.1348	.634	.3627	.2307

* The mean difference is significant at the .05 level.

◊ 95% Confidence Interval

Homogenous Subsets

ETH

Group	N	1
Student – Newman-Keuls□°		
3.00	2	.5715
2.00	6	.6375
1.00	6	.8683
Sig.		.081

Mean for groups in homogenous subsets are displayed.

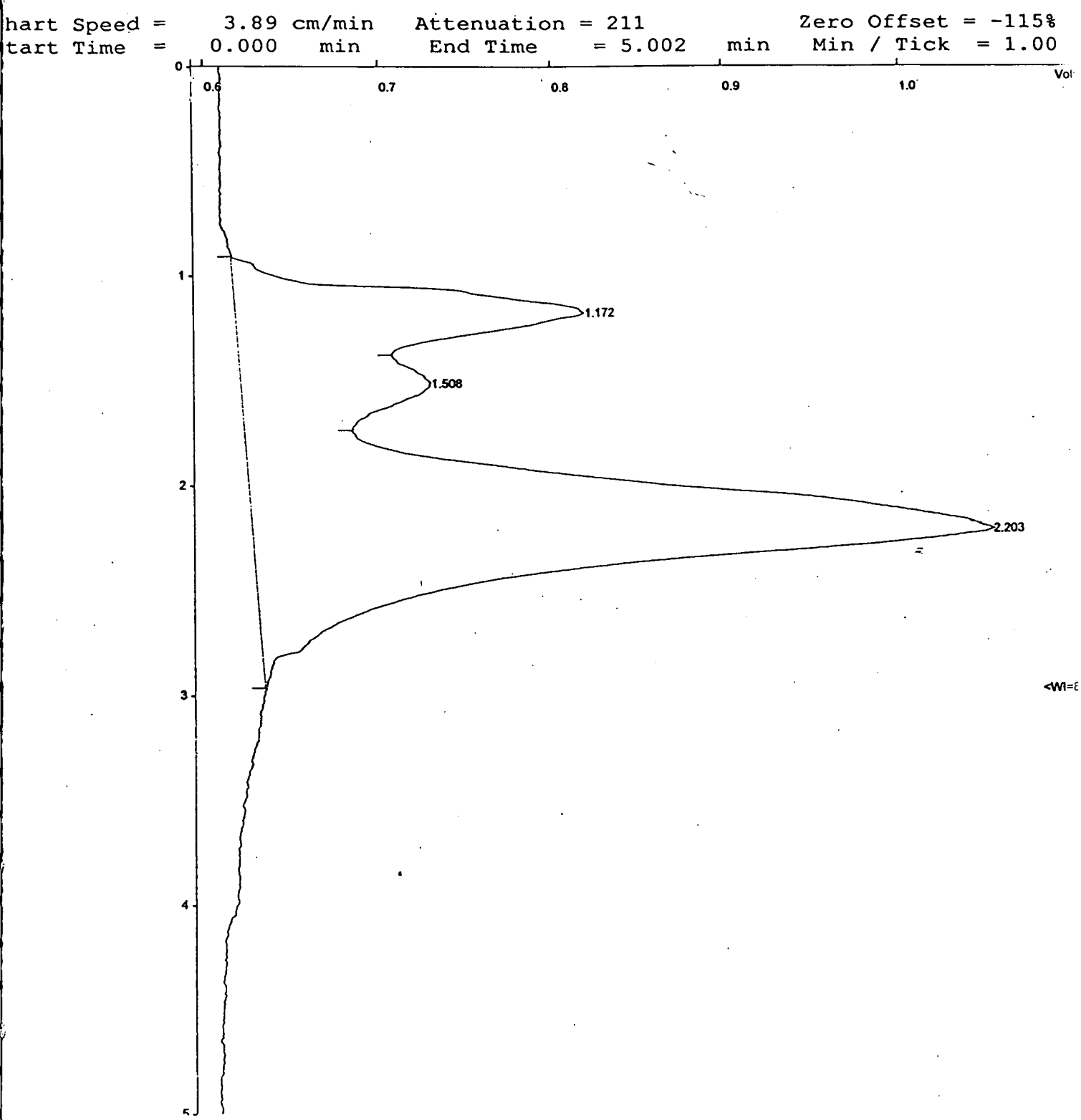
Subset for alpha = 3.600

□. Uses Harmonic Mean Sample Size =3.600.

°. The group sizes are unequal. The harmonic mean of the group sizes is used. Type 1 error levels are guaranteed.

APPENDIX C.1

EXAMPLE OF AN HPLC RUN.



Star Chromatography Workstation Version 5.50 ** 00398 3228 0cc 2304 **

n Mode : Analysis
ak Measurement: Peak Area
lculation Type: Percent

eak No.	Peak Name	Result ()	Rel. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1		18.1721	1.172	0.000	304682	BV	16.6	
2		11.4244	1.508	0.000	191547	VV	0.0	
3		70.4034	2.203	0.000	1180416	VB	24.8	
	Totals:	99.9999		0.000	1676645			

tal Unidentified Counts : 1676645 counts

tected Peaks: 3 Rejected Peaks: 0 Identified Peaks: 0

ltiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

aseline Offset: 609680 microVolts

ise (used): 2230 microVolts - monitored before this run

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