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A solid phase extraction system for the isolation of the germination cue in smoky water when subjected to the light sensitive lettuce Lactuca Sativa v. Grand Rapids

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A SOLID PHASE EXTRACTION SYSTEM FOR THE ISOLATION OF THE GERMINATION CUE IN SMOKY WATER WHEN SUBJECTED TO THE LIGHT SENSITIVE LETTUCE *LACTUCA SATIVA* v. GRAND RAPIDS.

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USE OF THESIS

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ABSTRACT

The search for the chemical or set of chemicals that act as a germination stimulant in *Lactuca sativa* v. Grand rapids (a variety of lettuce seed) was the underlying basis of this project. Through the development of a solid phase extraction (SPE) system different fractions of smoky water were tested for their effects on germination enhancement. The project targeted streamlining the amount of solvents as well as time used in past research on smoky water. Previously, several steps involving high performance liquid chromatography (HPLC), thin layer chromatography (TLC) and Gas Chromatography (GC) were employed.

This project used SPE cartridges to separate fractions and isolate components. Cartridges of the non-polar varieties were C_{18}, C_8, Phenyl and Cyanopropyl. After cartridge loading with smoky water and flushing, three fractions of increasing concentration of methanol from a buffered ethanoic acid/ methanol system were created. These fractions were extracted into hexane before application to bioassays.

Polar cartridges were also loaded with smoky water and fractionated with mixtures of hexane, and increasing concentrations of dichloromethane and methanol. These four fractions were not extracted but applied directly to bioassays.

Fraction activity was prevalent in early eluting fractions from non-polar cartridges but the pattern was more varied from the polar cartridges. Solutions not retained

by cartridges enhanced germination except for solutions from 2OH and Amino cartridges.

GC fractions from the three C_{18} fractions highlighted differences in peak number and comparative peak ratios within chromatograms. This emphasised that component separation was resulting from the SPE system. An adsorption test on seed coats of *L. sativa* was unable to reject the hypothesis that germination enhancing components do adsorb irreversibly to the seed coat.

An application of 1, 8 – cineole as a result of a poor Mass Spectrometry (MS) library match proved to be a source of germination enhancement at low concentrations.

DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

(i) incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education;

(ii) contain any material previously published or written by another person except where due reference is made in the text; or

(iii) contain any defamatory material.

Troy David Adriansz

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CHAPTER 1. INTRODUCTION

1.1 Introduction

De Lange and Boucher (1990) recently discovered that plant-derived smoke could stimulate germination. Subsequent research has been redirected from an emphasis on aspects of germination such as the role of phytochrome, to the area of smoke enhanced germination. This work has focused on germination studies performed in a total absence of light, hence removing the effects of phytochrome, with the application of smoky water (the product of bubbling smoke through water).

Fire is known to increase the germination of many plant species. This has for many years been attributed to the effect of heat, in particular, breaking the seed coats of hard seed coated species. Recently, it has been noted that heat is not always the mechanism for increasing germination and for some species the chemical components of smoke can cause this stimulation (Brown & van Staden, 1997). Early studies conducted on Californian chaparral species showed a remarkable number were found to have smoke as their germination cue, rather than heat shock as previously thought (Keeley & Fotheringham, 1997).

Subsequent work in South Africa on the germination of *Erica hebecalyx* did not focus solely on heat treatment (Van de Venter & Esterhuizen, 1988). Other tests were also performed, with the seeds being treated with ethylene and ammonia. The results indicated that fire products might be influential in germination, however, smoke was not tested alone as a cold application. This is required to isolate the effect of chemicals in smoke from the influence of the heat of fire.

In South Africa, the stimulatory effect of smoke was overlooked until 1990 when it was discovered that the fynbos species, *Audouinia capitata*, displays smoke stimulated germination (De Lange & Boucher, 1990). This species was found to be influenced by the cold application of smoke. This removed the assumption that the heat characteristic of fire was the stimulatory agent. It has since been discovered that other fynbos species, formerly thought to respond to heat from fire, in fact show similar responses to *A. capitata* with regard to smoke (Brown & van Staden, 1997). These smoke influenced fynbos species include five species of Asteraceae and two species each of *Ericaceae*, *Restionaceae* and *Proteaceae*.

The discovery of smoke enhanced germination has important applications for a number of different areas, these include conservation biology, restoration ecology and rangeland management. In terms of conservation biology, the benefit of creating carbon sinks from rapid germination in barren areas after mining is an important factor for large mining corporations. If the native plants removed whilst mining the earth beneath could be replanted or germinated in a short time span, a mining corporation's argument for licensing to mine certain vegetated areas would be improved. This is termed 'landscape revegetation' and the impact of mining on fire-influenced plants would be reduced (Brown & van Staden, 1997). Another possibility is that smoky water could be applied to the soil to improve seed germination. This could be either by application of the smoky water to the soil on site or to the application of previously treated soil (Drewes et al., 1995).

Other commercial impacts could flow from the enhanced germination in the wide variety of seeds that are influenced by smoke. Examples could be in the propagation of hard to germinate, yet attractive, species. Another commercial

product, (smoke extracted food flavouring solutions), initially intended to give indoor cooking an outdoor feel, have been found to promote germination when tested on *Themeda triandra* (Jager et al., 1996b).

It is probable that the 'infochemicals' (Drewes et al., 1995) in smoky water are fire-derived volatiles that act as cues in germination. Further knowledge of the function of these infochemicals will benefit productivity and maximise the germination of a set of seed. In addition, it has the potential to uncover information about the physiological process of germination (Drewes et al., 1995).

The species chosen for this study was *Lactuca sativa* v. Grand Rapids. Grand Rapids has a high requirement for light and water in order to germinate and an optimum temperature of 25 °C (Drewes, et al., 1994). However, smoke is capable of replacing the light requirement and so this seed can be used to test smoke and smoke fractions for activity. This will help discern the exact chemical or set of chemicals within the smoke that assist germination. The seed has a very short germination period and is therefore ideal for such an assay (van Staden et al., 1995b).

1.2 Species affected by smoke

Initial work on the effect of fire and germination was studied in California but the majority of research is now done in South Africa (Brown & van Staden, 1997) and Western Australia (Dixon et al., 1995).

1.2.1 Studies in South Africa

The original work performed in South Africa was on the monotypic fynbos species of *Audouinia capitata*. It was on this species that the promotive effects of

smoke were discovered (distinct from previous work perf ed where heat was assumed to influence germination). In the wild, *A. capitata* is found in a region subjected to periodic fires hence the initial assumption that the key element was heat (Brown & van Staden, 1997).

Subsequent work by Brown et al. (1993) went on to show that 25 out of 40 *Erica* species and 25 out of 32 *Restionaceae* species were positively influenced by smoke treatment. Positive results of smoke treatment were seen in *Themeda triandra*, which is significant because this species is not from a fire-influenced habitat.

Due to lengthy germination time for other species, the lettuce seed bioassay (*Lactuca sativa*) was chosen to check for all promotive and inhibitory effects with regard to smoke by some South African researchers (Drewes et al., 1995).

1.2.2 Application of smoke to Australian species

In Western Australia germination tests were performed on 94 species using the application of cold smoke, and of these 45 responded positively (Dixon et al., 1995). Similar results also occurred when the tests were performed using smoke fumigated filter papers instead of direct smoke application. Amongst these species are several that are important components of the Western Australian flora and were previously very difficult to propagate. These include species in genera such as *Stirlingia, Hibbertia* and *Verticordia* (Dixon et al., 1995). Testing on horticulturally important species such as *Anigozanthos manglesii* and *Lysinema ciliatum* has also been described in detail (Dixon et al, 1995) and commercial production of smoky water is being undertaken by staff at Kings Park Botanical Gardens in Western Australia.

The importance of the promotive effect of smoke on seeds to conservation is exhibited best by the germination of the endangered *Epacris stuartii*. Seeds of this hard-to-germinate Australian shrub are currently being germinated after application of smoke (Keith, 1997). The magnitude of this example is that the species is restricted to a single population in south-eastern Tasmania.

In order to maximise the number of species being regenerated in areas mined for bauxite, *Eucalyptus marginata* and other native species were treated with smoky water or aerosol smoke. This has resulted in greater percentage germination and a higher species diversity being established in mined pits (Roche et al., 1997). As a result the revegetation practices of Alcoa of Australia Ltd. have been modified to incorporate smoke treatments for all revegetation programs.

Thus the studies in Australia and South Africa proved that treating seeds with aqueous extracts of smoke, whether from fire- prone areas or not, can have a stimulatory effect on germination

1.2.3 Rapid bioassay using *Lactuca sativa*

Many species that respond to smoke are still slow to germinate (e.g. *Anigozanthos manglesii* (Langkamp, 1987), *Epacris stuartii* (Keith, 1997)) even after smoke treatments. Therefore, they are not particularly useful in trying to determine the chemical stimulant for germination.

The first work on smoke and *Lactuca sativa* germination was reported by Drewes et al. (1995). They concluded that the seed was fast in its germination time and showed a positive response over many concentrations of smoky water. Their experiments were conducted in a dark room and red light {R}(660 nm) and far red

light (FR) (730 nm) were found to influence germination. When applied as the last light before incubation in the dark, red light was found to stimulate germination while far red light was found to inhibit it. This inhibition can be overcome by application of smoky water. The conclusion was to conduct the experiments without either sources of light present, in order to minimise variables.

In subsequent experiments the only illumination in the room was a green 'safelight' which was expected to have no influence upon germination. The radiation of the light was only 0.5 μmol $m^{-2}s^{-1}$. This proved that light had a positive influence on germination and they were able to minimise the effect of this variable in their experiments. Drewes et al. (1995) eliminated the rapid germination of *Lactuca sativa* in the presence of light by applying distilled water and the relevant test solutions whilst in a dark room.

The effects of seasonal variation in temperature and humidity preclude the use of a native species as the bioassay. In studies by Roche et al. (1998) the seasonal aspect of smoke application was shown to be profound on Australian natives. Only 3 out of 37 species showed no change in seasons. No reports of seasonal effects on *Lactuca sativa* with respect to germination have been published.

1.3. Smoky water

1.3.1 Application of smoke treatments

Smoke treatment on seeds is performed in one of two ways. The first is to pump air from the smoke drum gently into a pipe found at the top of the drum. By burning a mixture of green and dry leaf along with twigs, and with regular restocking, a constant smoke source can be established. Through use of a long

outlet pipe, the smoke is cooled considerably and then delivered into a smoke tent. In the smoke tent the seed trays are multi-layered so many trays can be treated at once (Roche et al., 1997). In Australia the work has been performed with burning a mix of *Banksia* and *Eucalyptus* for anywhere between 60 and 90 minutes (Dixon et al., 1995). However, other studies have found *Eucalyptus* smoke to be inhibitory (Jager et al., 1996a).

An alternative method of smoke enhanced germination is reported in South African studies by Jager et al. (1996a). This involved burning *Themeda triandra* leaf in a muffle furnace for 30 minutes and then pouring on 50 mL of distilled water immediately after removal from the furnace. After standing for ten minutes the solution was filtered with Whatman No.1 filter papers. A variation of this indirect method is to obtain the active ingredients of aqueous smoke by forcing the smoke to bubble through distilled water under high compression. In these cases heavy acrid smoke can be generated by slow combustion of raw materials (Brown & van Staden, 1997).

Variables in smoke creation such as temperature of the furnace and sources to be burned have been investigated in analysing smoky water generation. Jager et al. (1996a) investigated different furnace temperatures on *Themeda triandra* from 140 to 240 °C. A pattern was noted where the highest germination stimulation occurred when furnace temperature had been between 160 and 200°C. Higher temperatures exhibited a loss in activity.

Keeley and Pizzorno (1986) suggested that the active components of smoke are derived from hemicellulose and cellulose, which were common to all plant materials. To verify this, Jager et al. (1996a) performed experiments to determine

the activity of combustion products of agar, tissue paper, cellulose, starch, glucose, galactose and glucuronic acid. These compounds were combusted at 200 °C, and activity was tested using the rapid *Lactuca sativa* bioassay. The results indicated that starch, glucose, galactose and glucuronic acid did not act as germination stimulants when their smoke was bubbled through distilled water. However, agar and cellulose did act as germination stimulants (Jager et al, 1996a).

The concentration of smoky water has been shown to be important in stimulating germination. Undiluted Kings Park smoky water and dilutions of 1:10 are inhibitory, whilst enhancement of germination occurs in the dilution range of 1:100 to 1:1000. This concentration dependence is a widespread property and has been observed with smoky water produced from a range of sources from tissue paper to *Eucalyptus* wood (Jager et al., 1996a). This dependence on concentration is a limiting factor for research in the search for the germination cue as an increase in the concentration of the germination cue or other smoke components actually leads to inhibition.

1.3.2 Commercial products

Work performed on commercially available smoke products has also enhanced germination. For example, commercially available food flavouring products produced from smoke were tested for activity on the lettuce seed bioassay and increased germination. From further testing on fractions of the smoke after separation using HPLC, fractions from two different commercial products produced very similar results with respect to which fractions were active (Jager et al., 1996b). This is indicative of the similarity in the products through similarity in elution times of active fractions from HPLC. Of 50 fractions collected from each

product, fractions 12 - 18 of one and fractions 14 – 19 of the other exhibited activity when subjected to the same test procedure.

The commercially available products were of a much stronger concentration then normally applied to germination tests. Dilutions of 1: 1,000 proved to be too concentrated and showed inhibition of germination, whereas concentrations of 1: 10,000 and 1: 1,000,000 proved to act as germination stimulants (Jager et al., 1996b).

1.4 Chromatography

Chromatography is a valuable technique for separating different chemicals by virtue of their different polarities, as well as other factors such as charge and size. Components of a mixture are carried through a stationary phase by a flow of a liquid or gaseous mobile phase. Interaction between the sample components in the mixture and the stationary phase separates them on the basis of their retention rates (Skoog et al., 1992).

Chromatography can be separated into two forms. The first is where a narrow tubing holds the stationary phase and the mobile phase is pushed through by gravity or applied pressure, this is called column chromatography. The other type is planar chromatography and it has the stationary phase in the pores of a paper or on a flat plate, as in thin layer chromatography (TLC). Instead of applied pressure, the mobile phase moves by gravity or capillary action (Harris, 1991).

Two chromatographic approaches have been used in past research on smoky water; HPLC and GC. In this project only GC was used.

1.4.1 HPLC

HPLC is a powerful analytical tool because of its accuracy and consistency in elution times. The column is packed with stationary phase particles with diameters typically 8μm, which create a large surface area for sample components to be retained. The components emerging from the column (the eluates) (Harris, 1991) can be separated by time, which corresponds to how much they are retained by the stationary phase. The more a sample component is similar to the stationary phase, the more it is retained. With the mobile phase within the column only moving between 0.1 and 10 $mLmin^{-1}$ and pressures up to 6,000 psi, the ability to partition components into different portions is very high (Skoog et al., 1992).

1.4.2 GC

Preliminary separations with HPLC have in other research projects on smoky water produced samples suitable for subsequent GC analysis (van Staden et al., 1995c). In this way the two techniques can be used in the identification of components from an HPLC fraction; working in a complementary manner.

GC analysis was first introduced in 1952 (James & Martin, 1952). Separation of components in a mixture for individual detection is the key feature of the technique. The separation is on the basis of time and involves injecting a sample into a heated injector to ensure that the sample is volatilised. The sample is then propelled along a column, by a carrier gas such as nitrogen or helium, toward a detector.

A stationary phase in the column attracts certain components within the sample and retards their movement along the column toward a detector. Thus separation

of components is distributed between the balance of adhesion to the stationary phase and the carrier gas. GC uses an inert carrier gas like nitrogen or helium as the mobile phase. (Skoog et al., 1992). Components eluting early in the column run spend little time in the stationary phase and need limited conditional change to force their removal. Others require a temperature increase and this is a feature of GC. Components of similar poarity but differing volatility can also be separated with the components requiring a lower temperature to boil, eluting first.

GC operates within an oven where overall temperature increases and rates of increase can be finely controlled. Compounds of low volatility require this temperature increase for separation (Schomburg, 1990). Eventually the components cannot be retained but are analysed by a detector. Flame ionisation, thermal conductivity and electron capture detection are some of the available methods but mass spectrometry (MS) was used in this project.

The feature of volatility is a requirement in GC not found in HPLC. Advantages of GC over HPLC are that it provides better resolution and GC can be used for different types of samples e.g. headspace samples from volatile solutions (Boyd-Boland, 1998).

The use of GC is restricted to volatile samples. Analysis of non-volatile samples is possible through derivitisation to produce more volatile species. However this form of chromatography is not suitable for thermally labile substances such as sugars due to the likely conversions (Skoog et al., 1992).

The use of GC is limited due to sample destruction (does not occur in HPLC) and it cannot directly analyse aqueous samples. This is because such samples are too

dilute and hydrated components can become too complex (Hennion & Pichon, 1994). The sample needs to be volatile below about 300°C

A refinement of GC has linked GC to MS. MS bombards sample components with electrons and a fragmentation pattern is produced. This can be recorded for all components as they are sequentially eluted from the GC column. The fragment ion pattern (mass spectrum) can then be compared to a database library spectra and matched for similarity. A good match infers the likely identity of the component being eluted (Skoog et al., 1992).

1.4.3 Stationary phases

HPLC, GC and solid phase extraction (SPE) cartridges (described below) all require a stationary phase to attract like compounds from a sample run through them. The choice of stationary phase depends largely on the sample components that require separation. Considered selection of the stationary phase is an important step in HPLC, GC and SPE. In the case of non-polar components within an aqueous material, the choice is a non-polar stationary phase that will retain the components and allow their subsequent, sequential elution. There are several non-polar phases, which have C_{18} or C_8 chains bonded to a silica support. The terms C_{18} and C_8 indicate the length of the carbon chain, these chains cap a certain percentage of a Si-OH base. As can be noted in Figure 1.1, there are two types of capping for a given cartridge. In this example of C_{18} the predominant capping is presented on the capped example on the right. The small percentage of uncapped Si bases would retain the structure presented on the left.

Si – O – H Uncapped	Si – O – C_{18} Capped with a carbon chain

Figure 1.1. The two possible cappings of silica base in a C_{18} SPE cartridge.

The property of like attracting like molecules makes the C_{18} ideal for extracting organics from aqueous solution. The carbon chain is reciprocated in most organic compounds. Shorter length organic compounds could benefit from a smaller chain to separate them due to their intermediate polarity (Skoog et al, 1992).

The term for a stationary phase that is less polar than the mobile phase is reverse phase whilst a normal phase set up has the stationary phase more polar than the mobile phase (Harris, 1991). Thus the C_{18} cartridge is termed reverse phase (Skoog et al., 1992).

1.5 Fractionation of the aqueous samples of smoky water

In analysing smoky water the solutions are separated into fractions according to their retention by the stationary phases. Once a set of preliminary fractions are created they undergo bioassay to determine which contain germination stimulating components. The active fractions then undergo GC-MS library matching to ascertain the probable components (Hennion & Pichon, 1994).

Previous procedures have partitioned aqueous smoke extracts against ethyl acetate, which were then dried and suspended in methanol of HPLC grade (van Staden et al., 1995b). After passing through a HPLC analytical column, fractions were collected which were then dried and applied to thin layer chromatography,

before testing on the *L. sativa* bioassay. The methods of smoke analysis outlined used HPLC separation systems with an aqueous buffer/methanol mobile phase. The pH was buffered to a level of 3.5 to ensure protonation of acids. The methanol and water mixture used to elute the sample components, through increasing concentrations of methanol over time, led to fractions of decreasing polarity being eluted with respect to time.

Biological activity was found to be more prevalent in specific fractions when applied to the *L. sativa* bioassay (van Staden et al., 1995c). The discovery of active fractions around the 70 percent mark of elution held firm with previous work on elution of smoke fractions when the 0.7 and 0.8 R_f values had activity when applied to the lettuce seed bioassay (van Staden et al., 1995c). The activity gave the same pattern as dilutions of smoky water, where concentrated samples actually inhibited germination of seeds and other dilutions of these products increased germination. Suggestions have led to individual chemical applications to the bioassay. The following compounds have been suggested to be active compounds; 4- hydroxy-acetophenone, as well as its isomers 2- and 3-, and 2-hydroxybenzonitrile and two of its isomers 3- and 4-, 4-hydroxy-3-methylbenzaldehyde, methyl ester and octadenoic acid. The assumption was based on their elution times and analysis of those elution fractions. However, none of these proved to be the germination stimulant when applied to the lettuce seed bioassay.

Recent approaches in purification of the smoke product have examined variations on work involving steps of liquid-liquid partitioning, (semi-preparative) HPLC, twice repeated TLC and then HPLC (for analysis) (van Staden et al., 1995c).

Current approaches attempt to reduce the number of solvents, materials and procedures in creating and analysing fractions. This trend has pioneered a comprehensible path to work with the comparatively efficient SPE.

1.6 Solid Phase Extraction Cartridges

A common method of initial separation for aqueous samples is SPE. The choice of the correct cartridge sorbent has borrowed concepts from work in HPLC, where components in solutions that have similar structures to the stationary phase are retained more by the structure than the others.

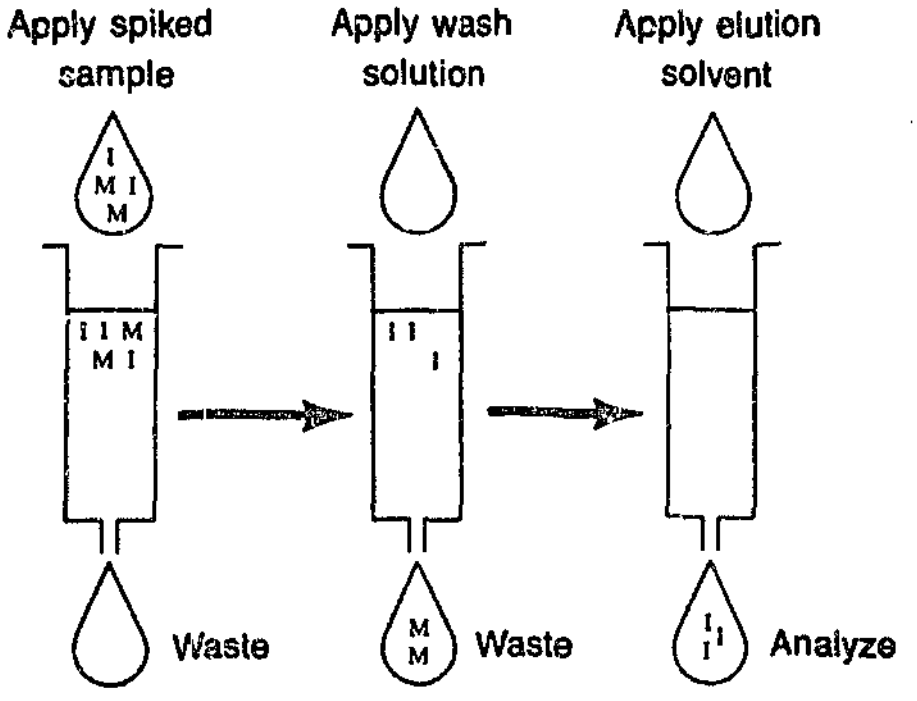


Figure.1.2. A diagram of a simplified experiment using SPE cartridges to concentrate an isolate into a final eluti

(Anon., 1998. *Instruction Manual* Varian Sample Preparation Products.)

The SPE cartridges are utilised by loading of a solution of interest onto the cartridge. In a simplified experiment a wash to remove unwanted components would be run through the solid phase and discarded before a solution more aligned structurally to the components of interest was flushed through. The solvent structurally aligned to the components of interest would be termed an eluting solvent and the component(s) of interest termed isolate(s). The waste solution can be retained for analysis or discarded but the main emphasis would be on analysis of this refined solution with a higher level of purity. As noted in Figure 1.2 a simplified experiment might look something along these lines. The

diagram shows the selective ability of SPE when well-chosen solvents are used. However, more complicated experiments might involve several washes of the column with marginally different mixtures in each solution to maximise isolation of components within elution. This process is an efficient analogue of the process of fractionation in HPLC.

Selection of cartridges is an important emphasis for SPE work and is dependent on the isolate required. In broad terms there are two divisions of SPE cartridges; polar and non-polar.

The C_{18} silica cartridge is probably the best choice for analysis of non-polar to moderately polar neutral compounds. The optimum elution solvent would be either methanol or acetonitrile when using a reversed phase system. In this study methanol was decided upon due to its availability and initial success. Sorbent choice is dependent on the nature of the aqueous sample. Bare silica and silica with polar groups does not act as well because the water does not easily elute the organic compounds of interest that are required (Hennion & Pichon, 1994).

The most polar cartridge is generally regarded to be the Si cartridge (Simpson & Van Horne, 1993). The polar cartridges are limited by their tendency to absorb water from the air but are the solid phase of choice for separation of vegetable oils and other fatty structures.

SPE cartridges have been used in the analysis of water pollutants (Hennion & Pichon, 1994) and this research bears relevance to the work in separation of chemicals from smoky water. Hennion and Pichon (1994) point toward the C_{18} silicas having higher retention of organic components than C_8 silicas as a reason they should be used for extracting organics from water. However, retention may

vary from one C_{18} cartridge to another according to the varying number of C_{18} chains bonded to the silica. This acts as a limitation of these columns. The value of C_{18} chains may also be limited with moderately polar organics, where the amount of sorbent cannot be increased.

SPE may use either vacuum or positive pressure. The bed volumes of the packed columns have varied from 100 to 1000 mg in offline studies that were said to save a substantial amount of solvent (Hennion & Pichon, 1994). Organic and aqueous materials are used to prime the support material before the solution to be analysed is applied. After application of the test solution a solvent wash is applied to remove interfering material. Then the solvent to elute the sample components of interest is applied. Constituents of solvents may include methanol, organic acids and organic bases as well as acetonitrile (Plumb et al., 1997). The pH is lowered in some instances to ensure acid protonation.

Other research has involved the activation of the sorbent bed by passing 4 mL of acetone followed by 4 mL of methanol through the $0.166 g.mL^{-1}$ C_{18} sorbent extraction cartridge. After double rinsing with distilled water and maintenance of a wet sorbent, the extraction was performed at 5 $mLmin^{-1}$ (Edwards & Peterson, 1994). These two instances are examples of work on biological fluids where trace elements were removed and analysed using gas-liquid chromatography.

Other studies, performed on different cartridges indicate that, depending on the task, careful choice of cartridge can enhance the separation. In testing for separation of phenolics, previous workers (Puig & Barcelo, 1996) compared C_{18}, C_{18}/OH, C_8, C_2, CH, CN, PH, and PLRP-S (styrene-divinylbenzene copolymer), and led to the discovery that PLRP-S was the most appropriate for that task. Their

example represents the number of cartridges requiring initial testing before an optimum one can be decided upon.

In their work, 80% recovery was the best possible once the correct cartridge had been determined for a particular separation task whilst other cartridge sorbents had lesser recovery percentages. (Puig & Barcelo, 1996). In this work, previous chromatography indicates a good starting point for cartridge choice and eluting solvent.

1.7 Offline and online procedures

Use of cartridges in an analysis procedure can be performed in two separate categories. The first is the off-line procedure where there is total separation between use of the SPE and the ensuing chromatographic analysis. In this method a sorbent packed with disposable cartridges has the samples percolated through it. In comparison to liquid-liquid extraction SPE is faster and does not use as much solvent. This was the method used in this project. Sample contamination is a limiting factor for offline procedures. This problem can be minimised by online separation. Online separation eliminates sample manipulation between preconcentration and the analytical steps. The process eliminates a switch between the SPE and the analytical column (Hennion & Pichon, 1994).

1.8 Project Aims

The project had a specific aim:

to determine the capacity of SPE cartridges to reduce the complexity of smoky water extracts prior to subsequent analysis. This was achieved by examination of a number of different types of SPE cartridges including polar and non-polar

cartridges and the fractions created from the cartridges were assayed by using the *L. sativa* bioassay.

2.0 MATERIALS AND METHODS

2.1 Smoky water

The smoky water used in this project was purchased from Kings Park Botanical Gardens, Western Australia. Three 500 mL bottles were used and germination tests were conducted to ensure consistency from one bottle to the next. 1:10, 1:100, 1:500 and 1:1000 dilutions were tested for bioactivity by seed germination tests described below. Germination percentages were consistent from each bottle when compared for bioactivity at each dilution.

2.2 Seeds

The *Lactuca sativa* seeds chosen for the project were obtained from R.B. Dessert Seeds, Kununurra, Western Australia

2.3 Seed germination testing

Twenty-five seeds were sown onto two pieces of Whatman filter papers in Petri dishes. The seeds were centred in each dish and test solutions were applied to the outer perimeter of the filter papers, the papers were then allowed to dry in the fume-cupboard. Ten replications of each treatment were used to assist in statistical differentiation of active fractions.

Petri dishes containing seeds were transferred to a dark room equipped with a green safelight of low intensity. The safelight was tested to determine if its wavelength influenced germination of *L. sativa*. Germination of seeds exposed to this safelight was similar to that reported by Drewes et al. (1995). Germination of

seeds in the light (16 hour daylight 8 hour darkness) was greater, as would be expected for this species.

In the dark room each Petri dish was watered with 2 mL of Milli-Q water. When watering, Petri dish lids were lifted, but not taken off completely, in order to minimise fungal contamination. Water was dispensed directly onto the seed, leading to a dispersion effect that scattered the seeds randomly over the surface of the filter paper. The dishes were then sealed with plastic and placed in a light proof cardboard box.

For each experiment involving hexane-derived fractions, a treatment of blank hexane was compared to the water control for mean percentage germination. If the results were found to show any statistically significant variation from the water control, then the experiment was rejected and repeated. Hexane not allowed to dry properly on the filter papers would often result in significant or total inhibition.

In experiments where the compounds being tested were dissolved in water, the seeds were sown and 2 mL of aqueous solution was applied in the dark room.

All samples were placed into a growth cabinet and maintained in complete darkness at 25°C ± 1 °C

Germination was scored after 48 hours ±1 hour. Germination at 48 hours was the same as germination after 7 days.

Seeds were considered to have germinated once the radicle was visible.

2.4 Solutions

All chemicals used were of analytical grade, and included: 1, 8 - cineole (Aldrich Chemical Company, Michigan USA), dichloromethane (BDH, Poole, England), methanol (Burdick and Jackson, Muskegon, USA), hexane (Burdick and Jackson, Muskegon, USA), ethyl acetate (Ajax Chemicals, Auburn, NSW), triethylamine (Ajax Chemicals, Auburn, NSW) and ethanoic acid (BDH, Poole, England).

All acids were protonated with 0.2M ethanoic acid buffered to pH 3.5 ± 0.1 with triethylamine.

2.5 Cartridge preparation

SPE cartridges (Bond-Elut, Harbor City, CA, USA) were categorised into either polar or non-polar cartridges. Within the two different categories the methods of loading of smoky water onto the cartridges were the same, but the eluting solvent mixtures used to flush through the column were different.

Cartridges were loaded by pipetting 1 mL of solution into the cartridge and a positive pressure applied via a 2 mL syringe inserted in the top of the cartridge. Two mL of smoky water was applied to each cartridge at a time, and subsequent solvent applications were also 2 mL.

To produce more concentrated samples for GC, using 100 mL of smoky water varied the procedure.

2.5.1 Non polar cartridges

The non-polar cartridges chosen for this experiment were capped as illustrated in Figure 2.1.

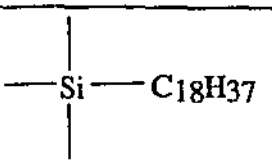
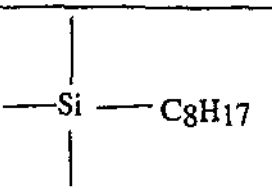
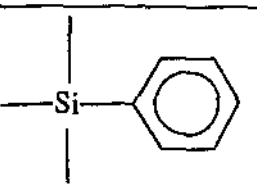
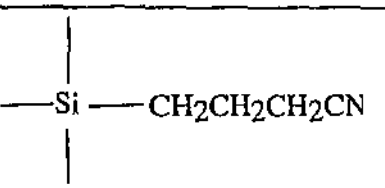
Octadecyl C_{18}	
Octyl C_8	
Phenyl **PH**	
Cyanopropyl **CN**	

Figure 2.1. The set of non-polar cartridge cappings examined in this project.

The non-polar cartridges were loaded with smoky water and the unretained run-off collected. The cartridge was then flushed with Milli-Q water then a series of solutions altering the ratio of buffered ethanoic acid: methanol. This series included: 80% buffered ethanoic acid 20% methanol, 70 % buffered ethanoic acid/ 30 % methanol, 60% buffered ethanoic acid / 40% methanol solution and finally 20% buffer / 80% methanol solution. The eluent from the 80% buffered ethanoic acid/ 20% methanol was discarded, all other eluents were collected and extracted into hexane (Table 2.1).

Solution flushed through column	Resulting fraction
100% Smoky water	Retain solution, extract into hexane
100% Milli-Q water	Discard
80% Buffered ethanoic acid/ 20% Methanol	Discard
70% Buffered ethanoic acid/ 30% Methanol	Retain solution, extract into hexane
60% Buffered ethanoic acid/ 40% Methanol	Retain solution, extract into hexane
20% Buffered ethanoic acid/ 80% Methanol	Retain solution, extract into hexane

Table 2.1 Solutions flushed through non-polar SPE cartridges and fractions created.

The fractions were extracted into 2 mL of hexane in a fume-cupboard. The hexane extracts were tested using the seed bioassay described. To remain in accordance with past research of 10 plates per replication it was necessary to use 2 cartridges each time and their fractions were combined giving 4 mL of each fraction. Ten replications of each test sample also assisted in verifying whether a solution was statistically significant in its germination enhancement. The resultant 4 mL was sufficient to apply 0.25 mL to each of the 10 plates

2.5.2 Polar cartridges

The polar cartridges chosen for this experiment were capped as shown in Figure 2.2.

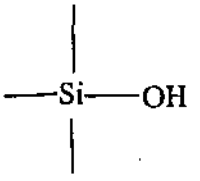
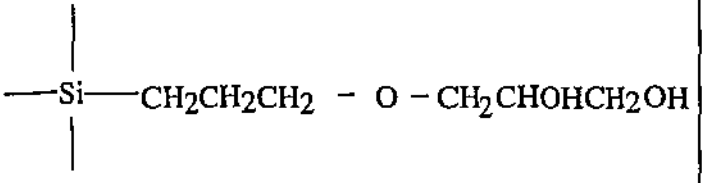
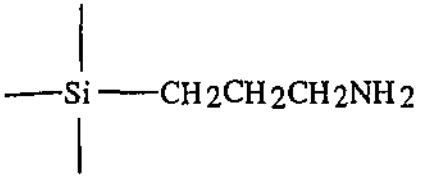
Silica **Si**	
Diol **2OH**	
Aminopropyl NH_2	

Figure 2.2. The set of polar cartridge cappings examined in this project.

The polar cartridges were also loaded with smoky water and the unretained run-off was collected. The cartridge was then flushed with Milli-Q water, from which the run-off was not collected. The cartridges were then flushed with a series of solutions altering the ratio of hexane: dichloromethane: methanol. This series included: 100% hexane, 90% hexane/ 10% dichloromethane, 85% hexane - 10% dichloromethane 5% methanol and finally 80% hexane 10% dichloromethane 10 % methanol. This series of eluents were retained (Table 2.2).

Solution flushed through column	Result
100% Smoky water	Retain solution, extract into hexane
100% Milli-Q water	Discard
100% Hexane	Retain
90% Hexane/ 10% Dichloromethane	Retain
85% Hexane/ 10% Dichloromethane/ 5% Methanol	Retain
80% Hexane/ 10% Dichloromethane/ 10% Methanol	Retain

Table 2.2 Solutions flushed through polar SPE cartridges and fractions created.

Unlike the non-polar cartridges these fractions were not extracted, as they were already in volatile solvents. As such the fractions were bioassayed directly. Again the 10 plate protocol meant that the eluents flushed out of 2 cartridges were combined for an adequate 4 mL of test solution.

2.6 Statistical analysis

Percentage germination per plate was scored after 24 or 48 hours, this was used to determine mean germination per plate from 10 replicates. Means were compared using one-way analysis of variance (ANOVA). Where a significant effect ($p \leq 0.05$) was obtained, Dunnett's test (Dunnett, 1955) was then applied to determine if treatments were different from the control (water).

2.7 GC-MS Analysis

2.7.1 GC Method development

GC-MS was performed on a Varian star 3400Cx. . An Alltech Econocap column was chosen that had a 30 m length, an internal diameter of 0.25 mm and a column coating 0.25µm thick. Helium was used as the carrier gas. The method used was: initial column temperature of 40°C maintained for 1 minute after an injection temperature of 260°C. The temperature was then increased at $10°Cmin^{-1}$ up to a temperature of 150°C. This temperature was maintained for 5 minutes. A further increase at $10°Cmin^{-1}$ up to a temperature of 260°C ensured that nothing was left on the column between injections. A 2µl injection from a SGE 5 µL syringe was used for sample introduction under a splitless injection protocol.

2.7.2 Seed adsorption tests

Replicate tests were conducted where a non-polar C_{18} cartridge was loaded with 100 mL of smoky water before flushing with 2 mL of Milli-Q water and 2 mL of 80% buffered ethanoic acid/ 20% methanol. The next flush of 2 mL of 70% buffered ethanoic acid/ 30% methanol was used in this test.

750 seeds weighing 0.67g were immersed in 2 mL of 70% buffered ethanoic acid/ 30% methanol fraction for 1 hour. The solution was then extracted with 6 mL of hexane and run on the GC. This was compared to a blank hexane extract that did not have seeds soaked in it.

2.7.3 MS

Library matching of peaks in the mass spectrum of each peak eluting from the column used the Terpene library supplied with Varian Star 3400Cx GC (Anon., 1992).

3.0 SPE CARTRIDGES

3.1 Introduction

Disposable SPE cartridges are used in sample preparation to concentrate and purify components from solution. Sorption of the components in question onto the sorbent bed, within the cartridge, depends on the similarity of the component's structure to that of the bed. Elution of the components of interest is performed by selection of a solvent, appropriate for both extraction and eventual analysis (Thurman & Mills, 1998). Similar sorbents are used in the relatively new method of SPE as found in traditional liquid chromatography.

Testing of drugs from urine (Katagi et al., 1995) and plasma (Lin et al., 1995) incorporates both non-polar and polar sorbents. A polar sorbent not utilised in this project, Alumina, has the capacity to test sugars and caffeine in cola type beverages (Thurman & Mills, 1998). Other sorbents like benzenesulfonic acid or carboxylic acid are used for cation exchange whilst an effective anion exchange sorbent is found in a quaternary amine structure (Anon., 1998).

The C_{18} and C_8 solid phases are distinctly similar in structure and would be expected to have similar results with respect to which fractions are active. The other non-polar solid phases of PH and CN-U depart from the hydrocarbon structure. The PH structure is described as having the same polarity as the C_8 but with an aromatic ring its component selectivity may vary (Simpson & Van Horne, 1993). CN-U varies again, being the most polar of this non-polar group and championed in its ability to elute very non-polar components that would be almost

impossible to remove from C_{18} and C_8 solid phases (Simpson & Van Horne, 1993).

Of the cartridges tested as polar, Si remains the most polar structure by virtue of its lack of capping. A less polar structure is found in the 2OH cartridge for which its interaction with isolates by hydrogen bonding closely mimics the Si cartridge. The NH_2 cartridge has been used for non-polar extractions and this highlights its dependency on the solvent solution run through the solid phase to determine what it will retain (Simpson & Van Horne, 1993).

SPE Cartridges were used in this project to examine the creation of active fractions in an efficient manner without the level of expertise of HPLC or TLC. Along with the reduction in skill requirements of users, a cost reduction was a priority of the research into SPE as a clean up method for GC.

This chapter details the bioactivity of fractions created for selected non-polar and polar cartridges. The structure of the cappings of the solid phase in each of these cartridges has already been presented in Figure 2.1 for the non-polar C_{18}, C_8, PH and CN-U sorbents and Figure 2.2 for the polar Si, 2OH and NH_2 sorbents.

3.2 Methods

Non-polar cartridges C_{18}, C_8, PH and CN-U were used to produce fractions using eluent compositions as detailed in section 2.5.1. These fractions were assayed for germination stimulating activity as described in section 2.3. In addition the C_{18} cartridge derived fractions were diluted to 1:10 and the germination compared with the neat fraction and water.

Polar cartridges, Si, 2OH and NH_2, were tested under a different solvent protocol. Variations in proportions of hexane/ dichloromethane/ methanol used as eluents as detailed in section 2.5.2. These fractions were assayed for germination stimulating activity as described in section 2.3. The series of eluting solvents were subjected to seed germination tests without being first passed through cartridges to ensure that no germination enhancing components were in any of the mixtures of hexane/ dichloromethane and methanol.

A further test was conducted with the polar Si cartridge. The unretained fraction was diluted 1:1 with Milli-Q water and this solution was further diluted to give 1:10, 1:20 and 1: 50 in aqueous solutions. These dilutions were compared as before (section 2.3. for solutions dissolved in water) for germination enhancement to the neat fraction.

Each experiment was performed at least twice with the exception of the C_{18} dilution tests, performed only once to note differences in germination. Mean percentage germination was compared using ANOVA, where a significant response ($p \leq 0.05$) was obtained, Dunnett's test was also performed.

3.3 Results.

3.3.1 Non-polar fractions

C_{18} Cartridge

The different fractions collected from the C_{18} cartridges produced germination percentages ranging from 46 – 78%. Three of the fractions consistently produced significantly higher germination than the control ($p = 0.000$ for each trial). These germination enhancing fractions were the unretained, the 70% buffered ethanoic

acid/ 30% methanol and 60% buffered ethanoic acid/ 40% methanol. This resulted in an approximate 27-37% increase in germination. The 20% buffered ethanoic acid/ 80% methanol fraction had the same germination as the water control in each trial ($p = 0.211$).

When the neat fraction was diluted 1:10 there was no significant difference ($p = 0.202$) in the germination obtained.

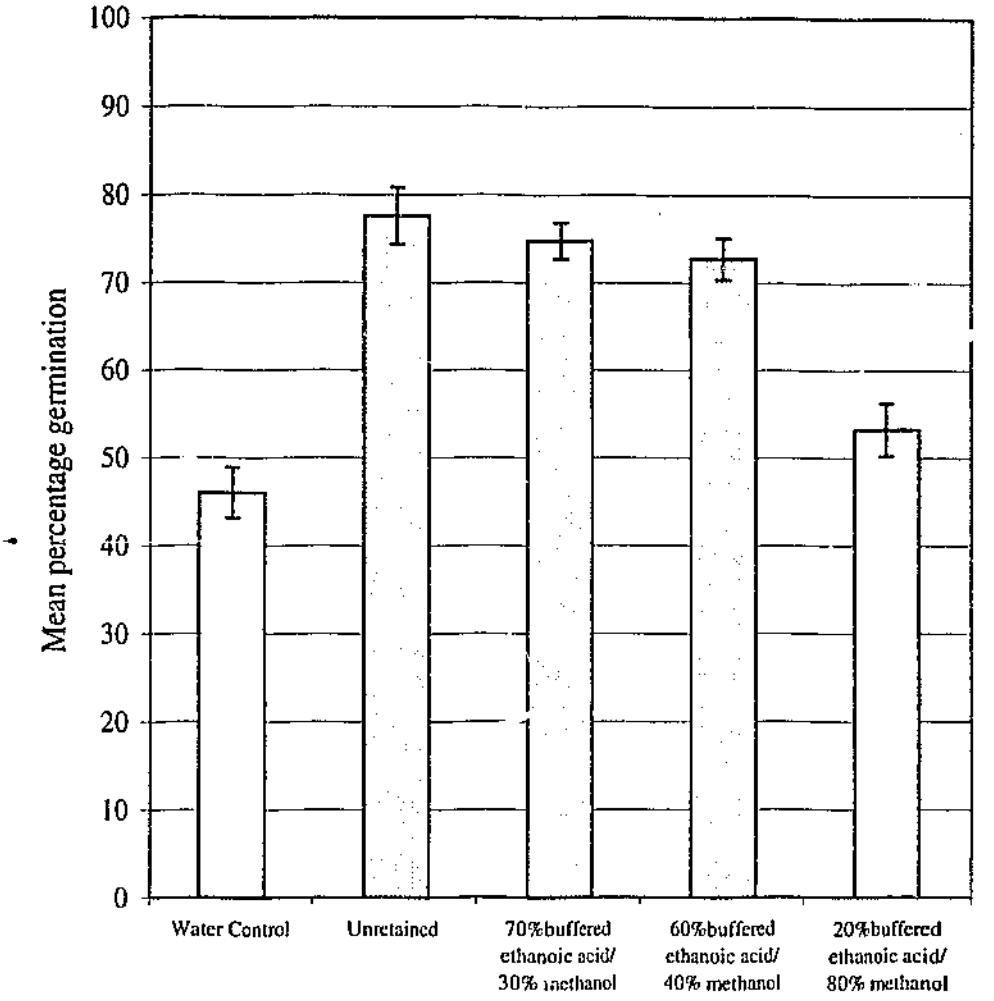


Figure 3.1. Germination of *L. sativa* 48 hours following the application of non-polar C_{18} cartridge derived fractions. Stippled columns indicate significant differences ($p \leq 0.05$) from the control, vertical bars represent standard errors of the mean. The experiment was repeated thrice, the data presented is from one trial and typical of the results obtained in each trial.

C_8 Cartridge

The same cartridge derived fractions were active in germination enhancement from the C_8 (Octyl) non-polar cartridge as found for the C_{18} cartridge. These statistically significant fractions were the unretained ($p = 0.000$) as well as fractions of 70% buffered ethanoic acid/ 30% methanol ($p = 0.003$) and 60% buffered ethanoic acid/ 30% methanol ($p = 0.001$). Percentage germinations ranged from 52% to 72% with approximately 16 – 20% increases in the significant fractions (Figure 3.2).

PH Cartridge

When the non-polar PH (Phenyl) cartridge was utilised a distinct change from the C_{18} and C_8 results was found. Only the unretained fraction and the 70% buffered ethanoic acid/ 30% methanol fraction were found to be statistically significant ($p = 0.000$ for both trials) in their germination enhancement. Up to a 26% increase in germination was found by the unretained fraction. These results are represented in Figure 3.3.

CN-U Cartridge

Germination percentages ranged from 50% to 72%. In the CN-U cartridge the mean germination percentages for all of the retained fractions were not statistically different from the water control (Figure 3.4). Only the unretained components showed any activity when applied to the assay. The unretained fraction exhibited approximately 72% germination, which was a statistically significant increase ($p = 0.000$) on the water control percentage of 50%. These results are represented in Figure 3.4.

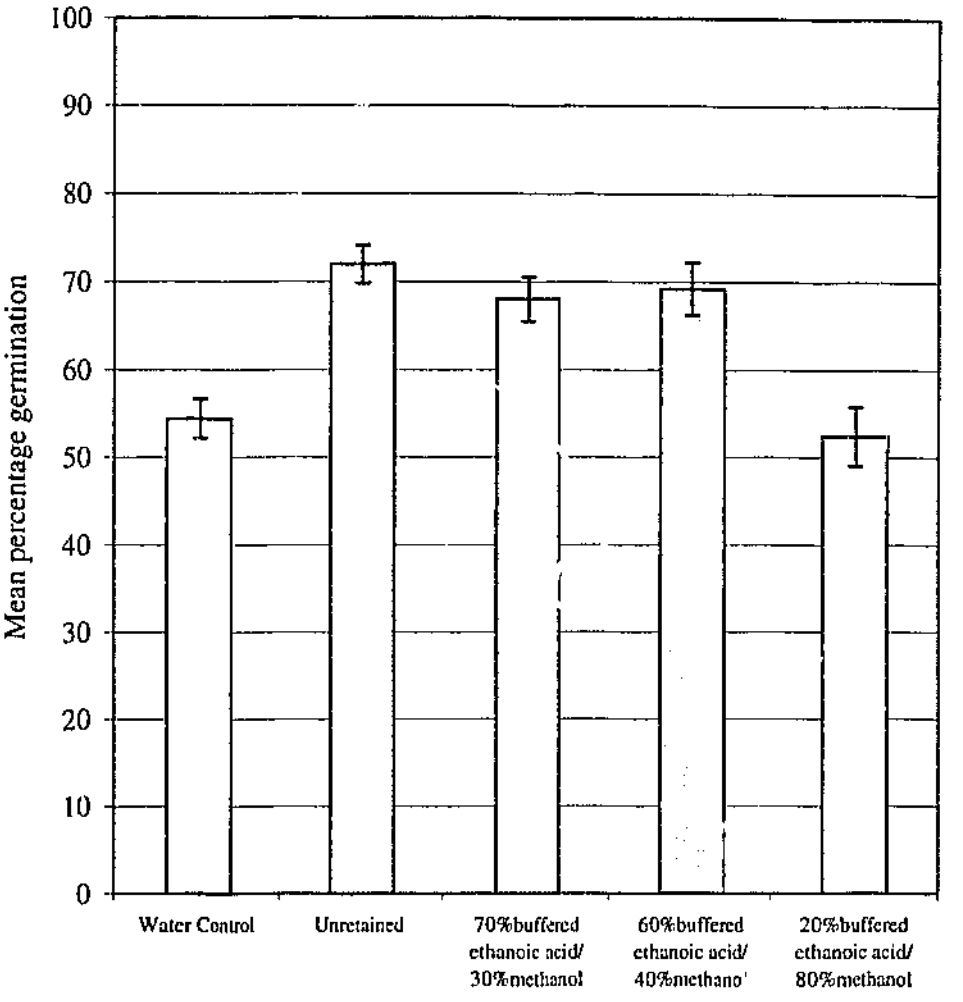


Figure 3.2. Germination of *L. sativa* 48 hours following the application of non-polar C_8 cartridge derived fractions. Stippled columns indicate significant differences ($p \leq 0.05$) from the control, vertical bars represent standard errors of the mean. The experiment was repeated twice, the data presented is from one trial and typical of the results obtained in each trial.

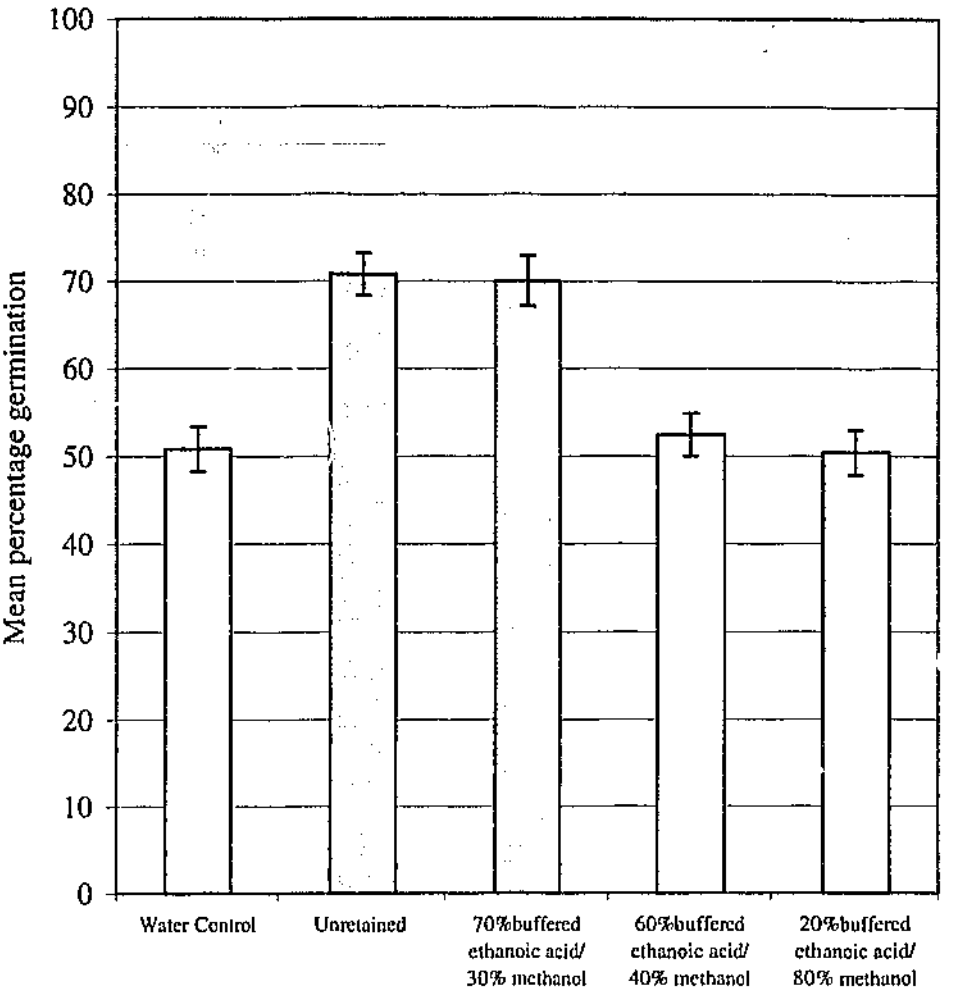


Figure 3.3. Germination of *L. sativa* 48 hours following the application of non-polar PH cartridge derived fractions. Stippled columns indicate significant differences ($p \leq 0.05$) from the control, vertical bars represent standard errors of the mean. The experiment was repeated twice, the data presented is from one trial and typical of the results obtained in each trial.

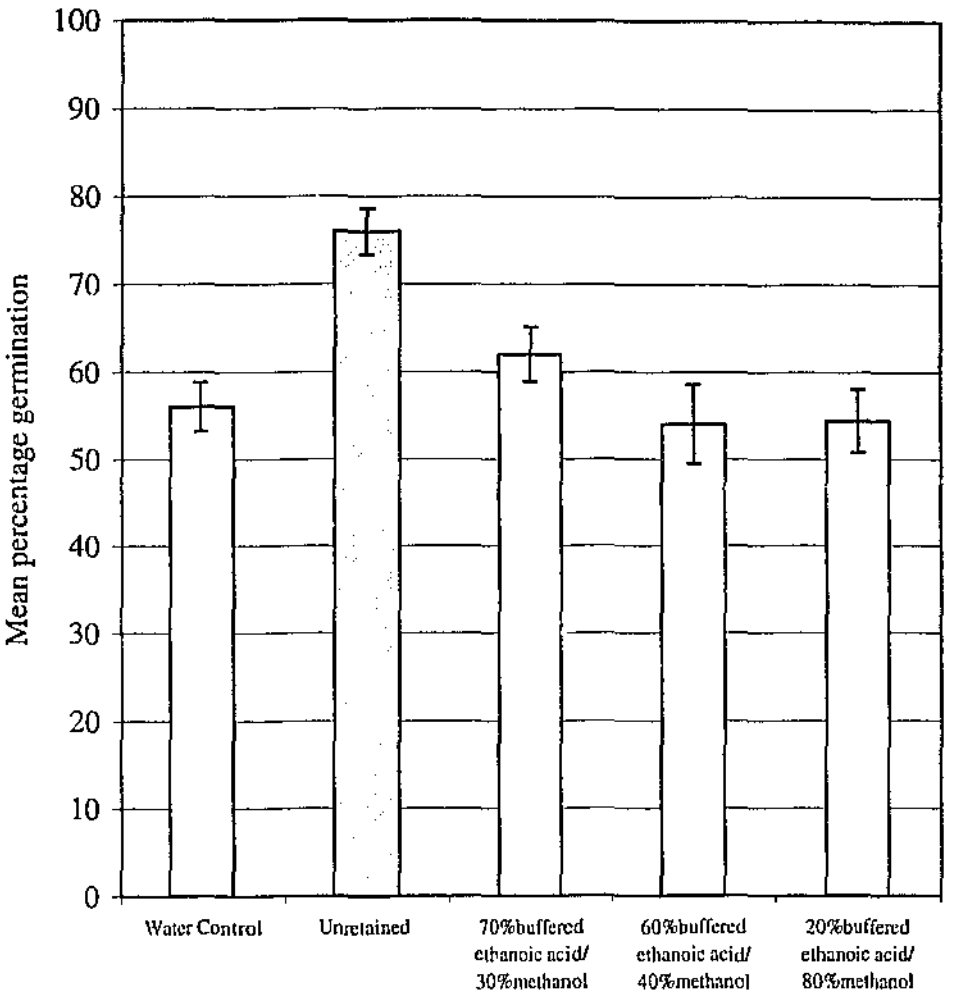


Figure 3.4. Germination of *L. sativa* 48 hours following the application of non-polar CN-U cartridge derived fractions. Stippled columns indicate significant differences ($p \leq 0.05$) from the control, vertical bars represent standard errors of the mean. The experiment was repeated twice, the data presented is from one trial and typical of the results obtained in each trial

In summary of the results found for the non-polar fractions the following Table 3.1 highlights the diminishing number of active fractions as more polar cartridges were tested in this non-polar section.

Solutions run through cartridges	C_{18}	C_8	PH	CN-U
Unretained	+	+	+	+
70% buffered ethanoic acid/ 30% methanol	+	+	+	
60% buffered ethanoic acid/ 40% methanol	+	+		
20% buffered ethanoic acid/ 80% methanol				

Table 3.1 Germination enhancing fractions of non-polar cartridges.

3.3.2 Polar Fractions

Si Cartridge

The uncapped polar Si (Silica) cartridge exhibited activity for some of its fractions. The unretained fraction ($p = 0.000$), 100% hexane ($p = 0.000$) and 90% hexane/ 10% dichloromethane ($p = 0.001$) fractions from the column showed significant germination enhancement. Germination was increased up to 77% for the unretained fraction, which was an increase of approximately 29% percent from the water control (Figure 3.5).

Further testing of the non-retained components from a Si cartridge without extraction into hexane presented results of both inhibition and enhancement. A straight application of 1:1 dilution of non-retained components with Milli-Q water showed significant inhibition ($p = 0.000$) resulting in a germination percentage of 31%. However when these fractions were recreated and diluted further each fraction was significant in its germination enhancement ($p = 0.001$ for 1:10; $p = 0.000$ for 1:20 & 1:50), with results ranging from 64-70% germination; an enhancement upwards of 15%. A graphical representation is presented for each dilution in Figure 3.6.

2OH Cartridge

Fractions created from a 2OH cartridge produced germination enhancing solutions in some instances. Statistically significant enhancement was exhibited for the 100% hexane, 90% hexane/ 10% dichloromethane ($p = 0.000$ for both trials) and 85% hexane/ 10% dichloromethane/ 5% methanol ($p = 0.011$) fractions. Germination percentages were as high as 79% for the 100% hexane fraction, an increase of 29% on the water control (Figure 3.7)

NH_2 Cartridge

The NH_2 (Aminopropyl) cartridge was also found to have germination enhancing fractions. The NH_2 cartridge was unusual in that it was the only cartridge that presented active fractions after non-active ones were already eluted. The unretained and 85% hexane/ 10% dichloromethane/ 5% methanol fractions were the only ones to not show any activity. Germination percentages peaked in the final fraction at approximately 82% a significant increase ($p = 0.000$) from the water control of 57%. The other two active fractions of 100% hexane ($p = 0.007$)

and 90% hexane/ 10% dichloromethane fell within the ranges of these two extremes (Figure 3.8).

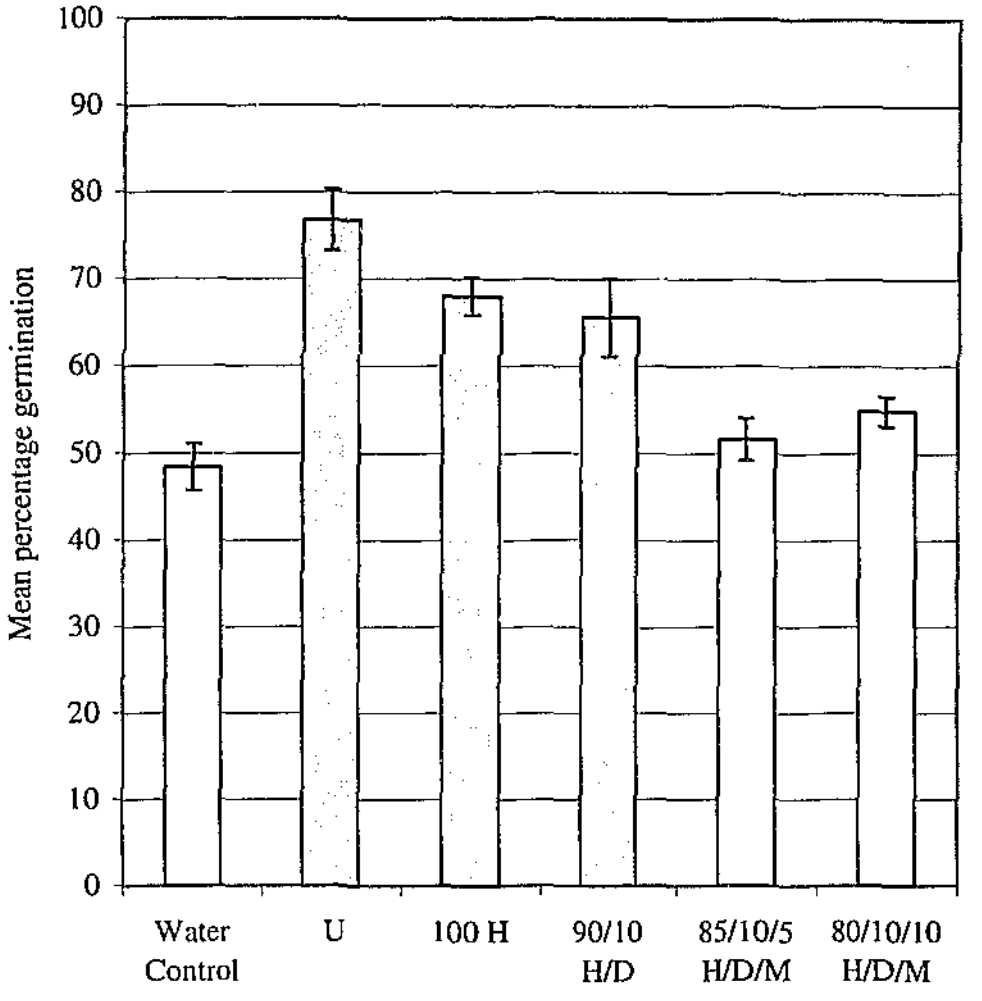


Figure 3.5. Germination of *L. sativa* 48 hours following the application of polar Si cartridge derived fractions. Stippled columns indicate significant differences ($p \leq 0.05$) from the control, vertical bars represent standard errors of the mean. The experiment was repeated twice, the data presented is from one trial and typical of the results obtained in each trial. U : Unretained; H : Hexane;

D : Dichloromethane; M : Methanol.

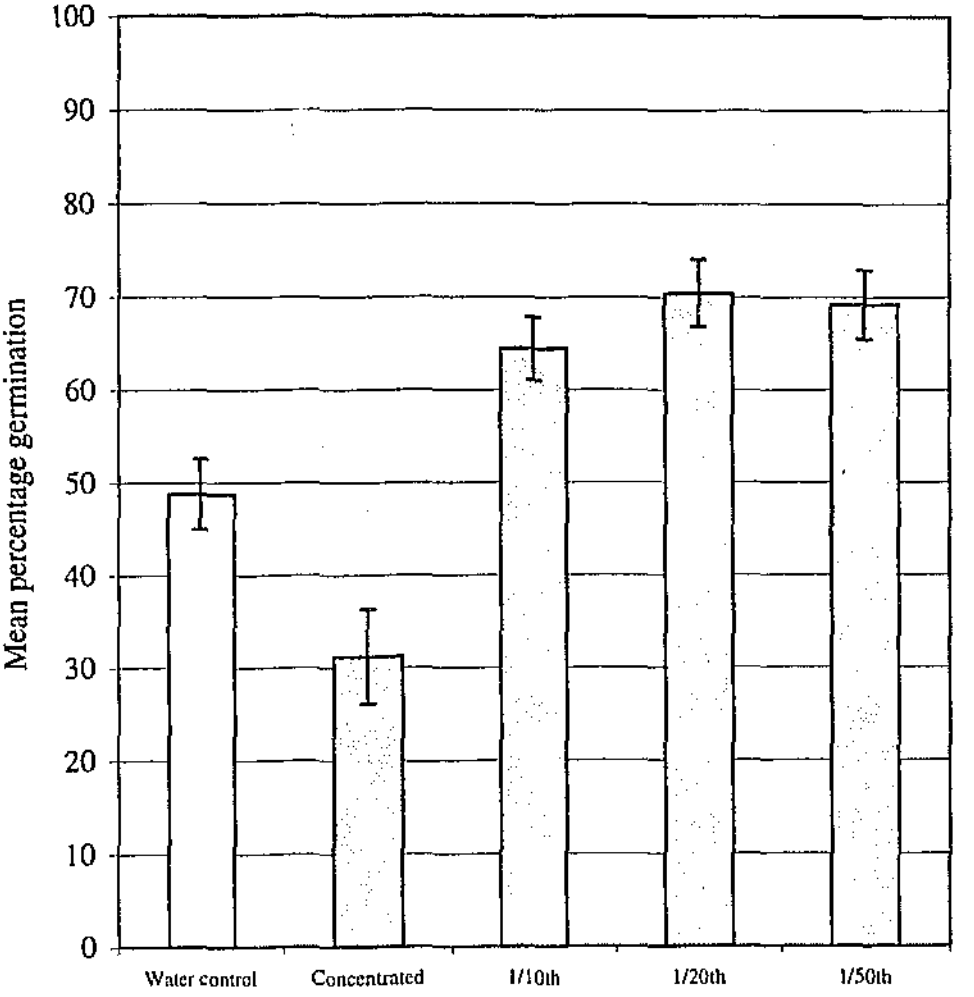


Figure 3.6. Germination of *L. sativa* 48 hours following the application of dilutions of the unretained components of a polar Si cartridge. Stippled columns indicate significant differences ($p \leq 0.05$) from the control, vertical bars represent standard errors of the mean.

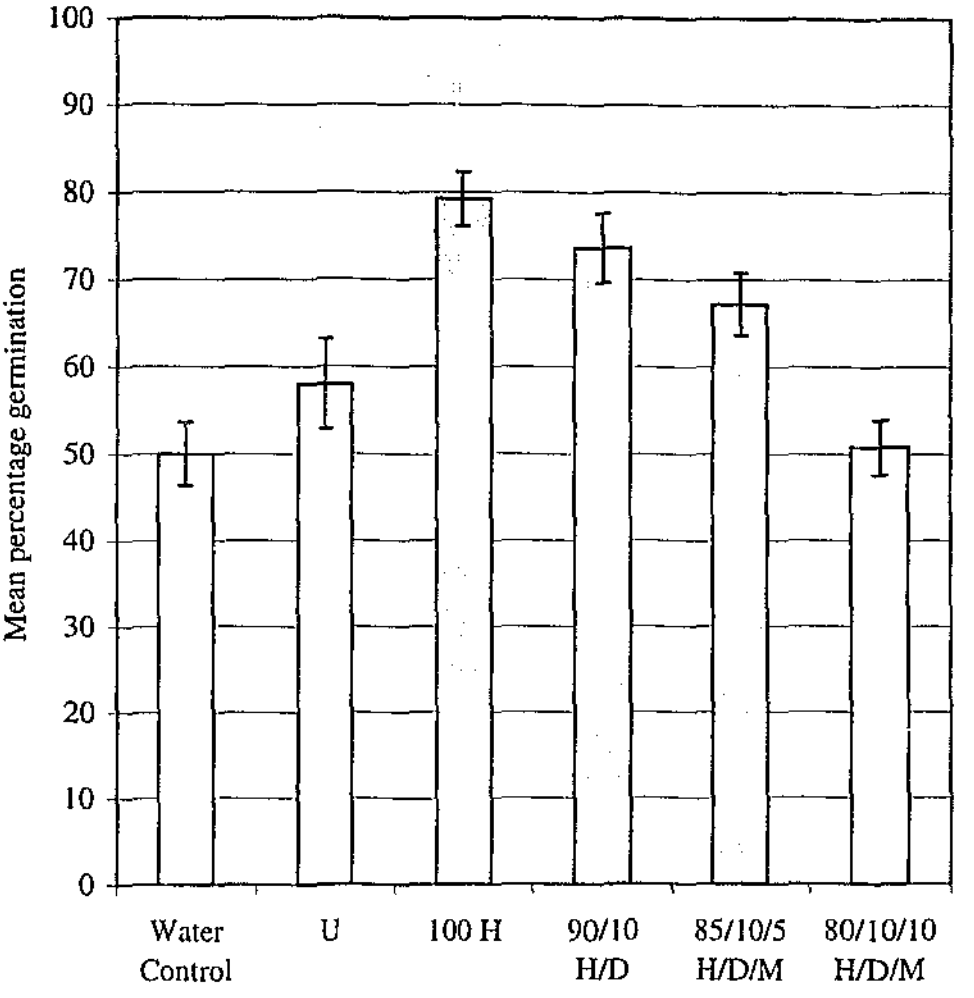


Figure 3.7. Germination of *L. sativa* 48 hours following the application of polar 2OH cartridge derived fractions. Stippled columns indicate significant differences ($p \leq 0.05$) from the control, vertical bars represent standard errors of the mean. The experiment was repeated twice, the data presented is from one trial and typical of the results obtained in each trial. U : Unretained; H : Hexane; D : Dichloromethane; M : Methanol.

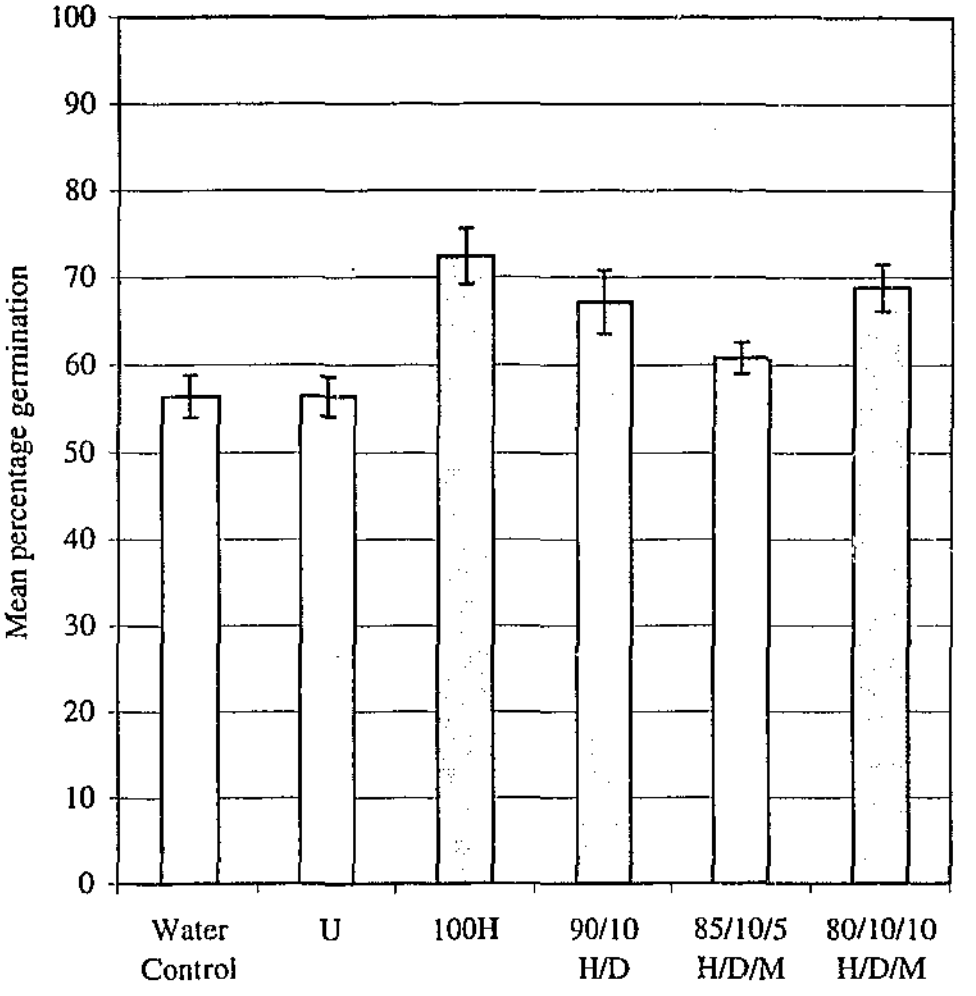


Figure 3.8. Germination of *L. sativa* 48 hours following the application of polar NH_2 cartridge derived fractions. Stippled columns indicate significant differences ($p \leq 0.05$) from the control, vertical bars represent standard errors of the mean. The experiment was repeated twice, the data presented is from one trial and typical of the results obtained in each trial. U : Unretained; H : Hexane; D: Dichloromethane; M : Methanol.

In summary of the results found for the polar fractions the following Table 3.2 highlights the gap in active fractions in the NH_2 cartridge compared to all others that were eluted consecutively from their respective cartridges.

Solutions run through cartridges	Si	2OH	NH_2
Unretained	+		
100% hexane	+	+	+
90% hexane/ 10% dichloromethane	+	+	+
85% hexane/ 10% dichloromethane/ 5% methanol		+	
80% hexane/ 10% dichloromethane/ 10% methanol			+

Table 3.2 Germination enhancing fractions of polar cartridges.

3.4 Discussion

3.4.1 Non-polar cartridges

Non-polar cartridges provided interesting and varied results from the fractions created with increasing percentages of methanol as well as the portions that were initially not a research priority, the unretained components. Non-polar cartridges were consistent in their inability to withhold at least one active component. In each of the unretained fractions from non-polar cartridges at least one component was exhibiting a positive effect on germination.

The consistent germination enhancement of the 70% buffered ethanoic acid/ 30% methanol fraction and 60% buffered ethanoic acid/ 40% methanol fraction, in the C_{18} and C_8 cartridges might indicate that the same component is being gathered by the slightly greater percentage of methanol in the latter solution. The cartridge does serve its purpose in concentrating out non-germinating components that can be visibly seen discolouring the fraction with 20% buffered ethanoic acid/ 80% methanol.

The likelihood that the very non-polar components are being lodged irreversibly in the hydrocarbon sorbent bed of these cappings is a likely explanation. The result of having the same fractions active between the two trials is not unusual when considering this characteristic.

The trend with the four non-polar cartridges is of increasing polarity in the series of C_{18}, C_8, PH and CN-U. The germination results from these cartridges show an increasing inability to retain active components, as the cartridges become more polar. This may result from two factors: the inability of the solvents chosen for elution to collect components of interest or from the inability of hexane to extract the components.

The next two non-polar cartridges that were examined, PH and CN-U exhibited no activity for the 60% buffered ethanoic acid/ 40% methanol fraction and the CN-U cartridge showed no activity for any of the retained fractions. A possible paradigm might be then that the unretained components from these fractions would have a comparably greater germination enhancement than the results presented for the C_8 and C_{18} cartridges. However this result did not eventuate.

A question exists whether fraction results can be compared between experiments. With respect to the consistency of water control results (46-57%) between experiments a comparison of unretained components between experiments may be justified. It is clear in examining Figures 3.1 through to 3.4 that the mean germination percentage remains between 70 and 80 percent for all trials. In fact it was the C_{18} cartridge that was closest to achieving 80% germination. Thus compounding of germination components into the unretained fraction, as might be expected from the lack of activity from 5 of the 6 solvent derived fractions from PH and CN-U (see Table 3.1), has not been converted into greater germination percentages.

The possibility that the fraction of 20% buffered ethanoic acid/ 80% methanol, which was inactive in all four non-polar cartridges, actually contains germination enhancing compounds has still not been eliminated by the course of experiments undertaken in this project. With each fraction requiring extraction into hexane, the possibility that active components were not extracted by the pure hexane solution remains a disputable point limiting the discarding of any of the final fractions.

Another point that limits the ability to discard all final fractions is the possibility that a narrow dilution window, e.g. between one-eleventh and one-twelfth dilutions might actually prove to be germination enhancing or inhibitory. Without an exhausting investigation into all the possible dilutions of the 20% buffered ethanoic acid/ 80% methanol fraction, a vehement defence of labelling them as redundant would be futile.

3.4.2 Polar fractions

Three polar cartridges were examined in this experiment. Results from these polar cartridge fractions still were significantly more variable than the results found for the non-polar cartridges. The results from the 2OH and NH_2 cartridges, indicating no significant change in germination percentage from a water control for the unretained fraction serves as a point of interest. The implication is that these two cartridges have retained all active components within smoky water. The supposition, could be considered in light of the trend of decreasing polarity in the series of polar cartridges of Si, 2OH and NH_2. The suggestion is that under the protocol used for the polar cartridges a slightly less polar cartridge could retain all of the components of interest. Such an assumption though would be premature without further research to eliminate the concentration of the fraction being the cause of the result.

The most significant result was obtained from the NH_2 cartridge. Increasing solvent polarity from the first solvent of 100% hexane down to the last of 80% hexane/ 10% dichloromethane/ 10% methanol did not show a smooth increase or decrease in activity of the fraction. The NH_2 cartridge has proved capable of separating two different active fraction and further work should be based on this result.

3.4.4 Limitations

The 2 major limitations were:

- concentration dependence of the germination cue

- extracting from polar fractions into hexane.

The limitation of concentration is a major factor that applies to not only the non-polar cartridges but the polar ones as well. For all fractions that showed no significant enhancement it is possible they actually are active at different concentrations. One point that does strengthen the argument against analysing all dilutions of fractions is that previous work on smoky water (Drewes et al., 1995;Jager et al., 1996a; Jager et al., 1996b; van Staden et al., 1995a) has noted germination inhibition at too concentrated a level and enhancement at weaker dilutions. Thus when a fraction's mean percentage germination shows neither inhibition nor enhancement it can almost be safely branded as a low priority for further separation.

The basis of these results was supported here when the unretained components of Si were tested in a neat application. The significant inhibition that resulted from an initial experiment led to the entire experiment design to test dilutions up to 1:50. Based on the results of the researchers finding inhibition or germination from smoky water but rarely any change at all, the results where no change occurred can tentatively be sidelined.

4.0 GAS CHROMATOGRAPHY

4.1 Introduction

The linkage of GC to MS, for characterisation of the components of complex organic mixtures, is the most effective of the chromatography separation methods (Evershed, 1993). The mass spectrometer is beneficial to trace analysis because, like GC, it performs well with components in the nanogram range. Analysis of volatile or semi-volatile mixtures, like those found in smoky water are ideally suited to GC-MS.

When a highly pure, base compound (e.g. hexane) can be eluted early from a GC with a steady column temperature increase, a well-controlled experimental environment is created. The capacity to compare peaks between different chromatogram samples is increased when runs of the column are performed on the same day with the pure solvent as a blank in-between. Hexane's ability to extract components from an aqueous sample and exhibit little or no interfering peaks in a chromatogram at higher temperatures can be exploited to confirm that peaks eluting at the latter stages of a column run are indeed from extracted components. Hexane, with a boiling point of 69°C is a capable solvent (Budavari, 1989).

4.1.1 GC Temperature Programming

The stationary phase of the GC column is of primary importance for separation of mixtures, but the temperature of the programme is also an important consideration. The absolute and relative chromatographic volatilities are dependent on the column temperature and thus total analysis time and resolution are all reliant on such parameters as initial column temperature, injection temperature, rate of change of temperature and final column temperature (Schomburg, 1990; Fowlis, 1995).

A low initial column temperature after a high injection temperature (250°C - 270°C) allows the less volatile hexane to elute first before the more adsorbed components elute from the column. The importance of having a high injection temperature is to ensure that all components are in a gaseous form when injected onto the column (Skoog et al., 1992). The large drop in initial column temperature aids further separation, via a method termed cold trapping. Within the column run, the rate of temperature increase is important because an optimum temperature increase can maximise the ratio of peak height to background interference which is termed a signal to noise ratio (Chapman, 1994). The optimum temperature increase must be slow enough to achieve good peak separation yet the demands of efficiency require the shortest possible run times, thus the greatest rate of temperature increase that can still effect separation is selected.

4.1.2 GC-MS library matching

The mass spectrometer involves a bombardment of organic matter with electrons to create fragments that are recorded to give a fragmentation pattern. The closeness of the fragmentation pattern in comparison to database library spectra is of value to predict the possible compounds in the smoky water.

Match perfection is a preference for increasing the confidence of stating which organic compound is being bombarded with electrons. Values that become critical are the purity, fit and reverse fit. A match on the basis of purity matches the similarity between the sample mass spectrum and the library mass spectrum. A match of over 900 is acceptable (Anon., 1992).

The fit search looks only for library spectrum peaks in the sample mass spectrum. A reverse fit search is aptly named because it performs the opposite task, which is looking for sample spectrum peaks in the library spectrum. The reverse fit is useful because peaks that are present in the sample that are not present in the library match reduce the value of the fit (Anon., 1992). The reverse fit values are lowered by low purity samples, or multiple components coeluting.

Even when a known analytical grade compound is introduced into the GC-MS, the purity match through the library is sometimes only around 80% in similarity. Thus values that are below 800 (80%) when being introduced from a mix of components may be given serious consideration but will require further investigation.

4.1.3 Seed absorption

The probability that seed coat adsorption or absorption, as a dormancy breaking agent, does occur and is irreversible has been examined by Brown et al. (1998) in a study on *Syncarpha vestita* and *Rhodocoma gigantea*. Their procedure soaked seeds in smoky water for 24 hours and then dried at 18°C for up to one year. Seeds were taken out monthly and tested for germination enhancement and compared to control seeds that were soaked in distilled water for 24 hours instead. The enhanced germination of seeds from both species in every experimental period implies an irreversible adsorption by seed coats in those species. An assumption that absorption of certain components of smoky water could occur with *L. sativa* based on the similarity in the effect of smoky water with other species is a point worthy of inspection.

4.1.4 1, 8 – Cineole

One of the essential oils found commonly in the leaves of Australian eucalypts of the southern and south - western regions of Australia is 1,8-Cineole (Bignell et al., 1995; Doran et al., 1995). Its full role in animal-plant interactions is only beginning to be understood. The different amounts of this component and other fragrant oils within the leaves of varying species of *Eucalyptus* are used as a taxonomic tool to identify one species from another.

1, 8 - Cineole is known by other names such as cajeputol and eucalyptol and in IUPAC terminology is 1, 8 – Epoxy- p menthane or 1, 3, 3 –Trimethyl – 2

oxabicyclo [2.2.2.] - octane. In the oil of eucalypt it is the chief constituent (Budavari, 1989).

Structurally it is written as:

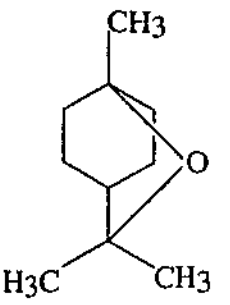


Figure 4.1. 1, 8 – Cineole.

1, 8 - Cineole is considered to be practically insoluble in water (Budavari, 1989) but with heating, a solution of very low concentration can be formed. It is miscible with chloroform, alcohol and ether and would be thus considered likely to dissolve in hexane. With a boiling point 76°C above that of water (Budavari, 1989), it would be relatively stable once an aqueous solution has been formed.

Overall the key elements in this part of the project were the identification of peaks from library matches and acknowledgement of any differences between experiments from either fractionation or soaking of seeds in fractions

4.2 Methods

4.2.1 C_{18} Fractions [Experiment 1]

Neat smoky water (2 mL) was extracted into 2 mL of hexane. The hexane component was injected onto the GC and as described in section 2.7.1.

To provide adequate peak size in chromatograms for analysis for each of the last three fractions from a C_{18} cartridge, 100 mL of smoky water was loaded onto a C_{18} cartridge for each experiment created in this section. The cartridge was then flushed with 2 mL of Milli-Q water and then 2 mL of a series of solutions altering the ratio of buffered ethanoic acid: methanol. This series included: 80% buffered ethanoic acid/ 20% methanol, 70 % buffered ethanoic acid/ 30 % methanol, 60% buffered ethanoic acid/ 40% methanol solution and finally 20% buffered ethanoic acid/ 80% methanol solution. The eluates of the last three of the series were retained and extracted into hexane (2 mL). These extractions were run on the GC (section 2.7.1) to note differences in eluate peaks between fractions.

Each fraction was analysed on the GC-MS and the spectra were compared to the Terpene library in the Saturn Software which is supplied with the Varian star 3400Cx (Anon., 1992) to obtain a tentative identification of the peaks.

4.2.2 Seed Absorption [Experiment 2]

To test the absorption properties of *L. sativa* seed/seedcoats, 0.67 g of seed was soaked in 2 mL of a 70% buffered ethanoic acid/ 30% methanol fraction derived from a C_{18} cartridge for 1 hour. The seeds were then removed, the remaining solution extracted in 2 mL of hexane and analysed by GC-MS as described in

section 2.7.1. The same fraction, derived from another C_{18} cartridge, without seed soaked in it was used for comparison

4.2.3. 1, 8 – Cineole [Experiment 3]

Experiment 3.1

GC-MS library matching from (section 4.2.1) indicated that a peak eluting between 417 and 419 seconds could possibly be 1, 8 - cineole. 1, 8 – Cineole was dissolved into 100 mL of Milli-Q water by heating for 1 hour at 90°C. Standard solutions up to 0.01% v/v and 0.1% v/v were produced. Onto each filter paper 0.25 mL of these solutions with 1.75 mL of Milli-Q water was dispensed for bioassay. Conditions for germination were as outlined in section 2.3.

Experiment 3.2

1, 8 – Cineole was dissolved in hexane to give standard solutions of 0.02% v/v, 0.1% v/v, 1.0% v/v, 2.0% v/v and 10% v/v. Onto each filter paper 0.25 mL of each solution was dispensed and allowed to dry. The filter papers were later watered with 2 mL of Milli-Q water. Two controls were set up; one with blank hexane on filter paper and the other with water. The germination conditions outlined in section 2.3 were followed.

4.3 Results

4.3.1 Experiment 1 Results

The smoky water chromatogram proved to consistently give the same peaks over 4 runs. An abundant number of peaks were found, and are represented in Figure 4.2. Several peaks eluted throughout the run of the GC from the neat smoky water

extraction. Clearly separate peaks eluted from the first few seconds of the run and continued up to the 1500 seconds mark.

Ten peaks were selected for comparisons between neat smoky water extract and the fractions obtained from C_{18} cartridges.

The comparisons show that :

- the C_{18} cartridge has significantly reduced the number of components in each fraction compared to neat smoky water.
- the fractions are of different composition.
- as the non-polar nature of the eluting solvent increased, an increase in peaks in the latter part of the chromatogram was observed, as expected.

The peaks are labelled in Figures 4.3, 4.4 and 4.5. Table 3 indicates selected comparative peak heights as percentages compared to peak 7 and highlights the different concentrations of components in each fraction. The scan of 951 seconds is thus scaled to read as 100% height in the ensuing table. These chromatograms were indicative of repeatable results from separate cartridge derived fractions when run on the GC.

Peak elution times (s)	70%B/ 30%M	60%B/ 40%M	20%B/ 80%M
Peak 2 417	30.5	28.9	45.6
Peak 3 530	20.0	7.2	3.5
Peak 4 572	41.9	21.6	7.8
Peak 5 651	33.4	4.5	4.4
Peak 7 951	(100.0)	(100)	(100)
Peak 8 1116	1.0	1.8	12.3
Peak 10 1212	4.8	1.0	0.0

Table 4.1. Comparative percentages of selected peak heights from retained fractions of a C_{18} cartridge normalised against a scan 951 seconds into each run of the GC. B: buffered ethanoic acid; M: methanol.

The first peak of interest, Peak 2, was considerably greater in its percentage comparison to peak 7 in the final fraction than in the first two fractions. With a greatly increased value to that found in the first two retained fractions (45.6 compared to 30.5), there is an indication that the component was more soluble in an increased concentration of methanol, or that peak 7 was decreasing relative to this peak under the higher concentration of methanol.

Peak 3 again exhibited a noticeably larger percentage in one fraction compared to the other two. A 20% value in the 70% buffered ethanoic acid/ 30% methanol

fraction is almost double the combined percentages of the next two fractions. This is indicative of a component or group of components that are more miscible with a higher concentration of buffer relative to Peak 7.

Peak 4 and Peak 5 are further examples of a gradual decrease in peak height in comparison to the normalised peak at 951 seconds. With each sequential fraction of the cartridge, lesser relative amounts of these components are being eluted. Peak 10 is another example of a component that decreased to the point in the 20% buffered ethanoic acid/ 80% methanol fraction that it was no longer distinguishable from the baseline.

Peak 8 is an example of components beginning to emerge later on in the elutions. The peak is noticeable in the final fraction but barely noticeable in the others. It follows a similar pattern to that exhibited in Peak 2 where the greatest peak height was seen in the final fraction.

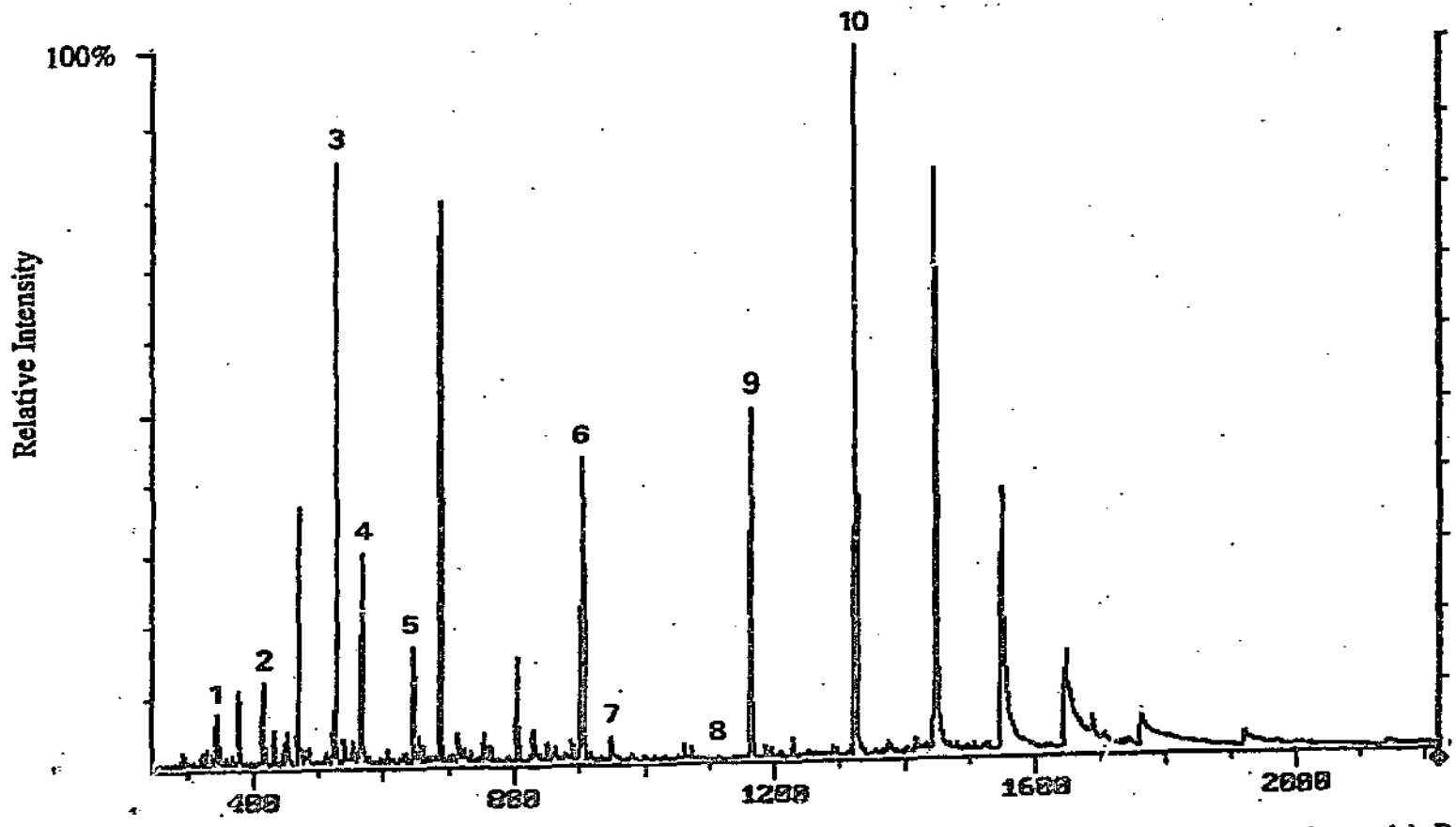


Figure 4.2. Smoky water chromatogram run on a Varian Star 3400Cx GC. Peaks of interest are in order of elution (seconds). Peak heights are normalised in comparison to peak 10..

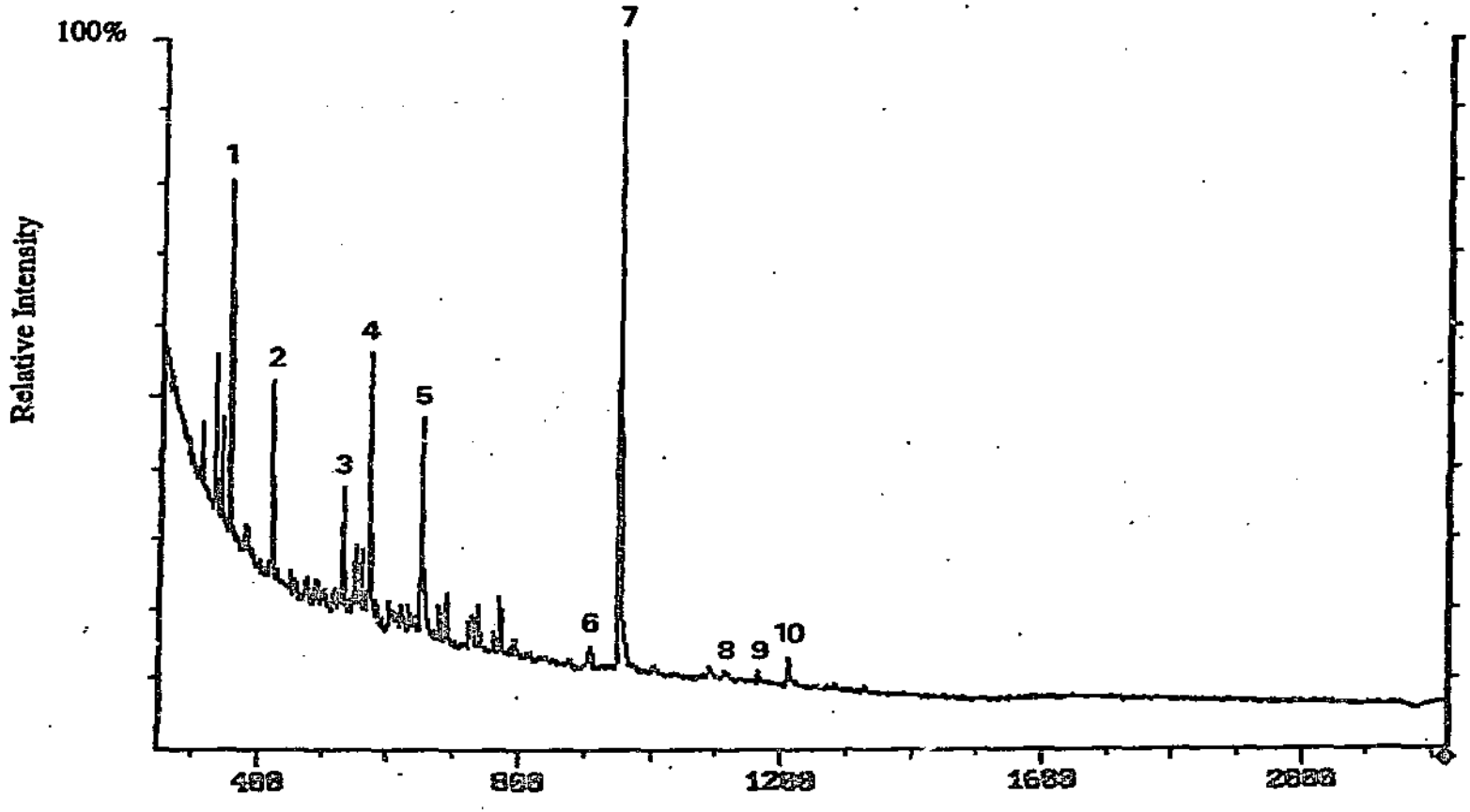


Figure 4.3 Smoky water chromatogram of concentrated 70% buffered ethanoic acid/ 30% methanol elution from a C_{18} cartridge run on a Varian Star 3400Cx GC. Peaks of interest are in order of elution (seconds). Peak heights are normalised in comparison to peak 7.

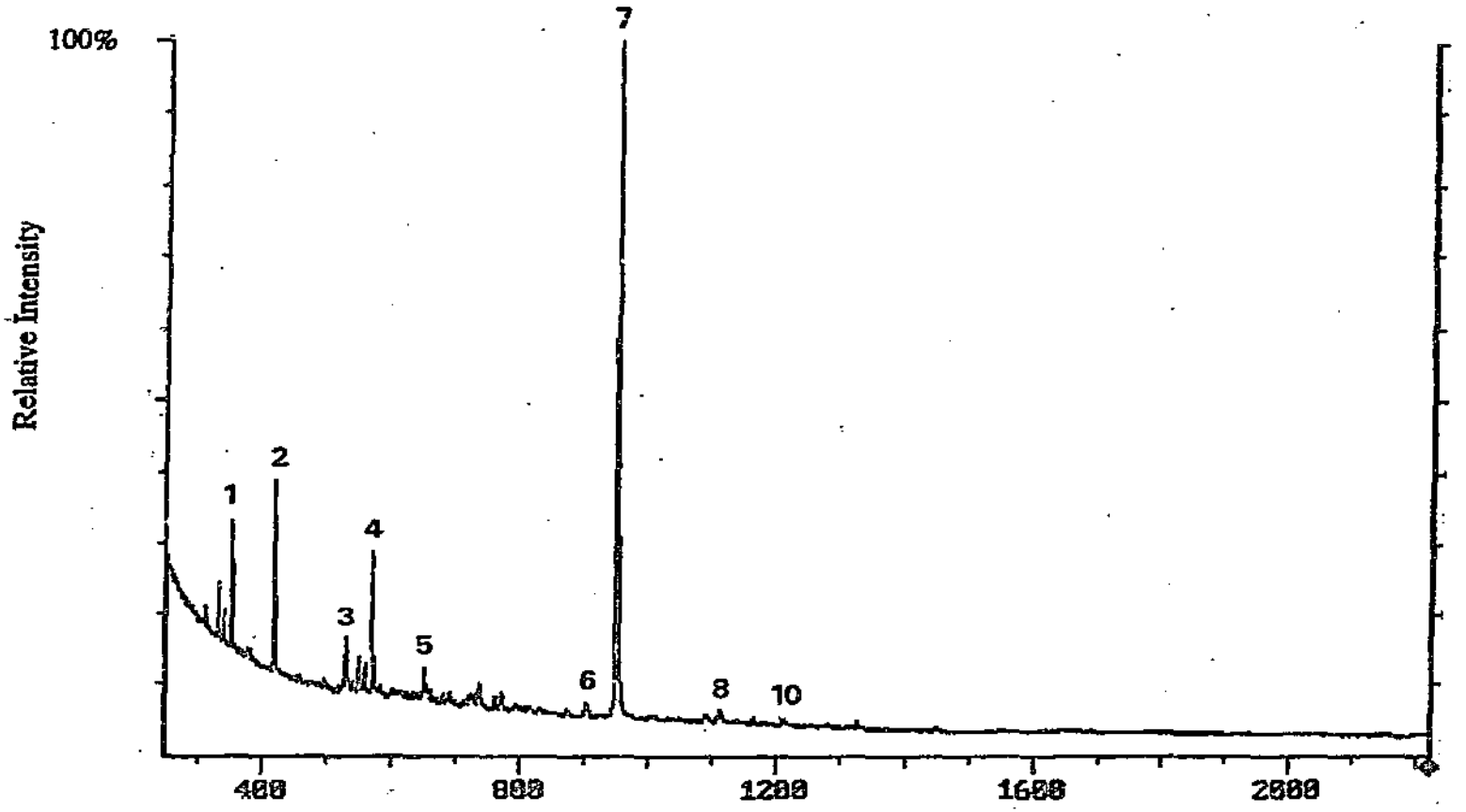


Figure 4.4 Smoky water chromatogram of concentrated 60% buffered ethanoic acid/ 40% methanol elution from a C_{18} cartridge run on a Varian Star 3400Cx. Peaks of interest are in order of elution (seconds). Peak heights are normalised in comparison to peak 7.

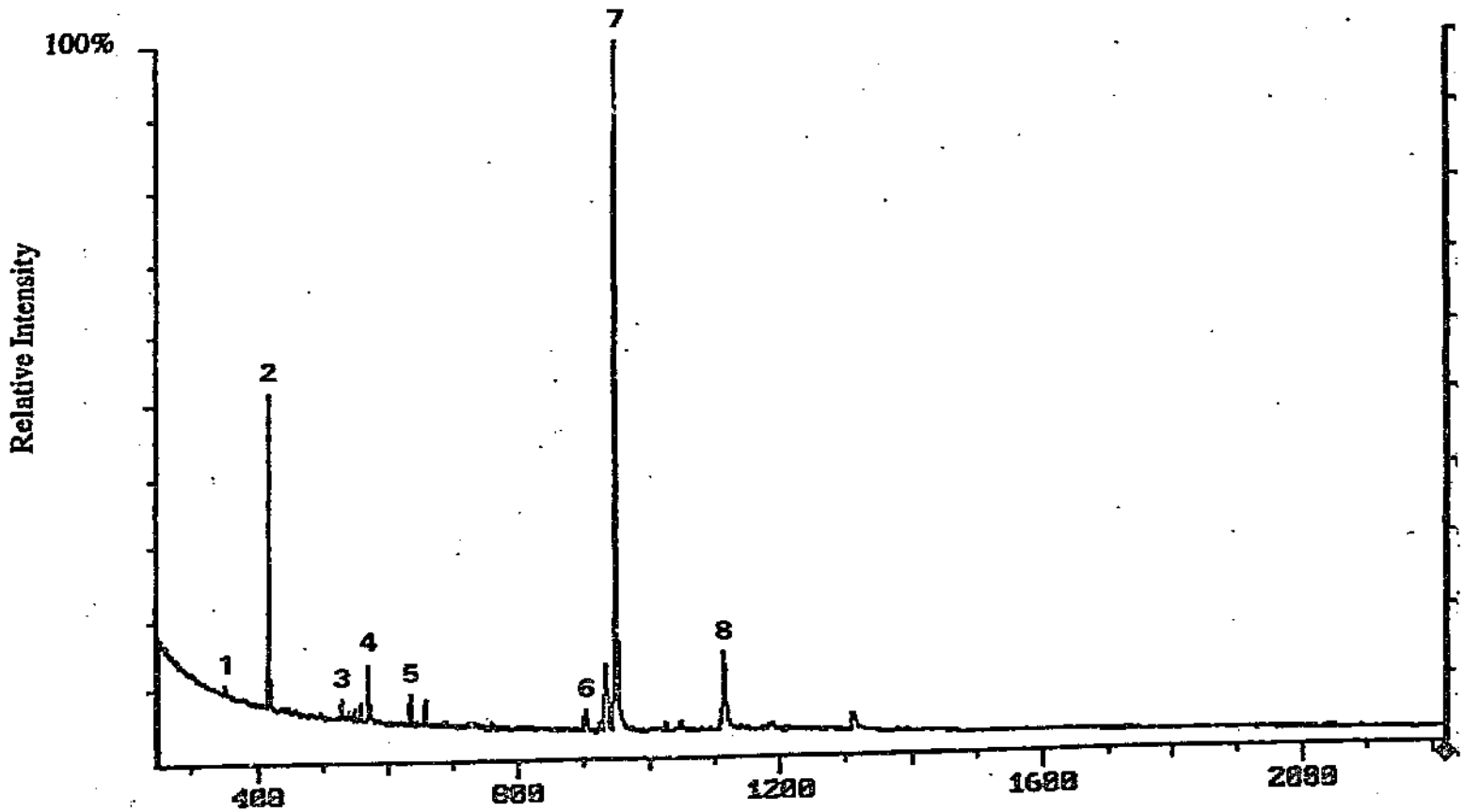


Figure 4.5 Smoky water chromatogram of concentrated 20% buffered ethanoic acid/ 80% methanol elution from a C_{18} cartridge run on a Varian Star 3400Cx. Peaks of interest are in order of elution (seconds). Peak heights are normalised in comparison to peak 7.

Library matches on each of the peaks that have been labelled in this experiment have been attempted. Only 1, 8 – cineole gave a match that could be interpreted with any confidence as shown in Table 4.2

Run time (s)	Best matched peak	Purity	Fit	Reverse Fit
353	Octadecene	679	720	759
417	1, 8 – cineole	888	957	900
531	Dehydroabietal	22	383	46
572	Ethyl hydroquinone	802	844	832
649	Pulegone	547	640	582
910	Farmesol (trans)	38	205	56
950	Dihydroxy p- cymene (2,4)	436	672	471
1116	Coniferyl alcohol	388	704	473
1167	Cinammic acid	19	368	40
1211	Pentacosane	483	872	536

Table 4.2 Table of the best matches available from the Terpene library on the bases of purity, fit and reverse fit for peaks highlighted in smoky water chromatograms.

The chromatograms of the retained fractions were variable in their peak heights. A select group of peaks are labelled to make comparisons between chromatograms. A limitation exists in making direct comparisons between the

smoky water chromatogram and other chromatograms. This is because the smoky water chromatogram was created from only 2 mL of solution. With the ability of the SPE cartridge to retain components, a peak barely noticeable in the smoky water fraction becomes clearly distinguishable as it may be increased up to 50 times in one fraction through the use of 50 times more aqueous solution.

4.3.2 Experiment 2 Results.

The resultant chromatogram after soaking *L. sativa* seeds in a 2 mL solution of a concentrated 70% buffered ethanoic acid/ 30% methanol fraction (Figure 4.7) highlights a considerable change in the peak heights between a typical unaffected first fraction (Figure 4.6.). Peak 7 is no longer the normalised peak because a substantial percentage of its components may have adsorbed onto the seed coats or absorbed into the seed of the *L. sativa*. The key peak of this graph is from a scan 353 seconds into the column run, which is labelled Peak 1. The unaffected fraction extracted with 6 mL of hexane exhibited the same ratio of peaks to that shown in Figure 4.3, thus a second 70% buffered ethanoic acid/ 30% methanol fraction has not been included.

In contrasting comparative percentage heights of peaks between the test solution and a blank fraction A, percentage heights compared to the peak at 353 seconds were created in Table 4.3.

Peak elution time in seconds		Unaffected 70%B/ 30%M	Treated 70%B/ 30%M
Peak 1	353 seconds	100	100
Peak 2	417 seconds	58.9	7
Peak 3	530 seconds	35.7	5
Peak 4	572 seconds	7.6	5
Peak 5	651 seconds	62.5	11
Peak 6	910 seconds	7.1	32
Peak 7	951 seconds	187.5	8
Peak 9	1167 seconds	3.6	42

Table 4.3. Comparative percentages of selected peak heights from a 70% buffered ethanoic acid/ 30% methanol fraction of concentrated smoky water from a C_{18} cartridge and a treated fraction subjected to *L. sativa* seeds soaking for 1 hour. Results are compared to a peak at 353 seconds. B: buffered ethanoic acid; M: methanol.

The most noticeable change between comparative peak heights is that for the peak from a scan 951 seconds into the column run. The drop indicates that probable extraction is taking place for the components that normally elute at this point of the column run. With respect to the consideration that components that elute at the 353 second mark have actually been added to the solution, the scans at 417 seconds, 530 seconds and 651 seconds have all lost around 85% of their original

percentage. A loss of 96% is thus considerably larger and implies that possibly the seeds extracted the components eluting after 951 seconds. By virtue of all the other peaks not having the same percentage in comparison to the scan at 353 seconds it can cautiously be proposed that extraction took place.

The gain in peak height cited after 910 seconds might be attributed to chemicals from the seed coat dissolving in solution over the 1 hour the experiment was set up. Alternatively the peak of peak 6 might be the components that were least effected by the seed coats and thus all peaks relative to that would have undergone even more extraction than proposed by using Peak 1 as the reference peak.

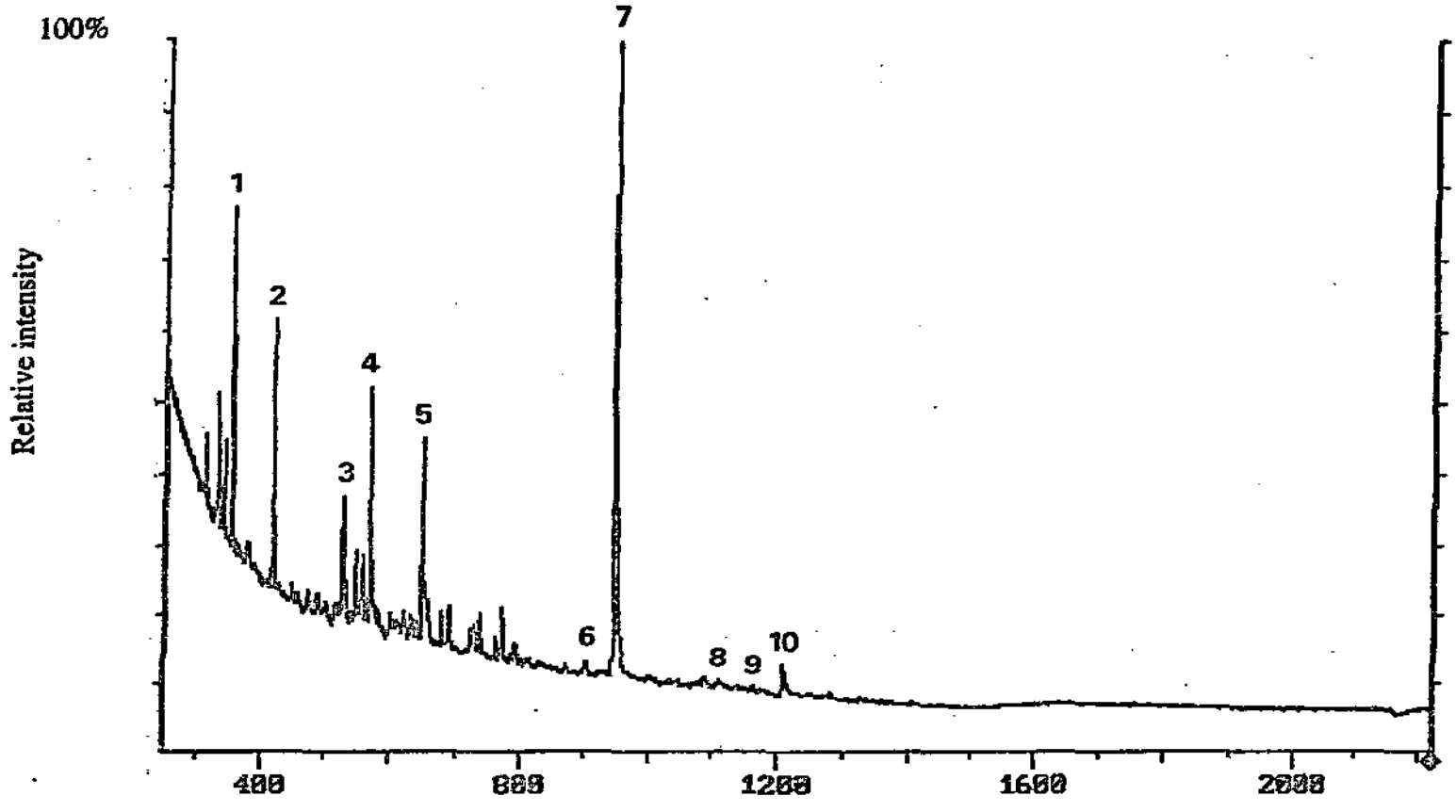


Figure 4.6 Chromatogram of concentrated 70% buffered ethanoic acid/ 30% methanol elution from a C_{18} cartridge, the unaffected fraction, run on a Varian Star 3400Cx. Peaks of interest are in order of elution (seconds). Peak heights are normalised in comparison to peak 7.

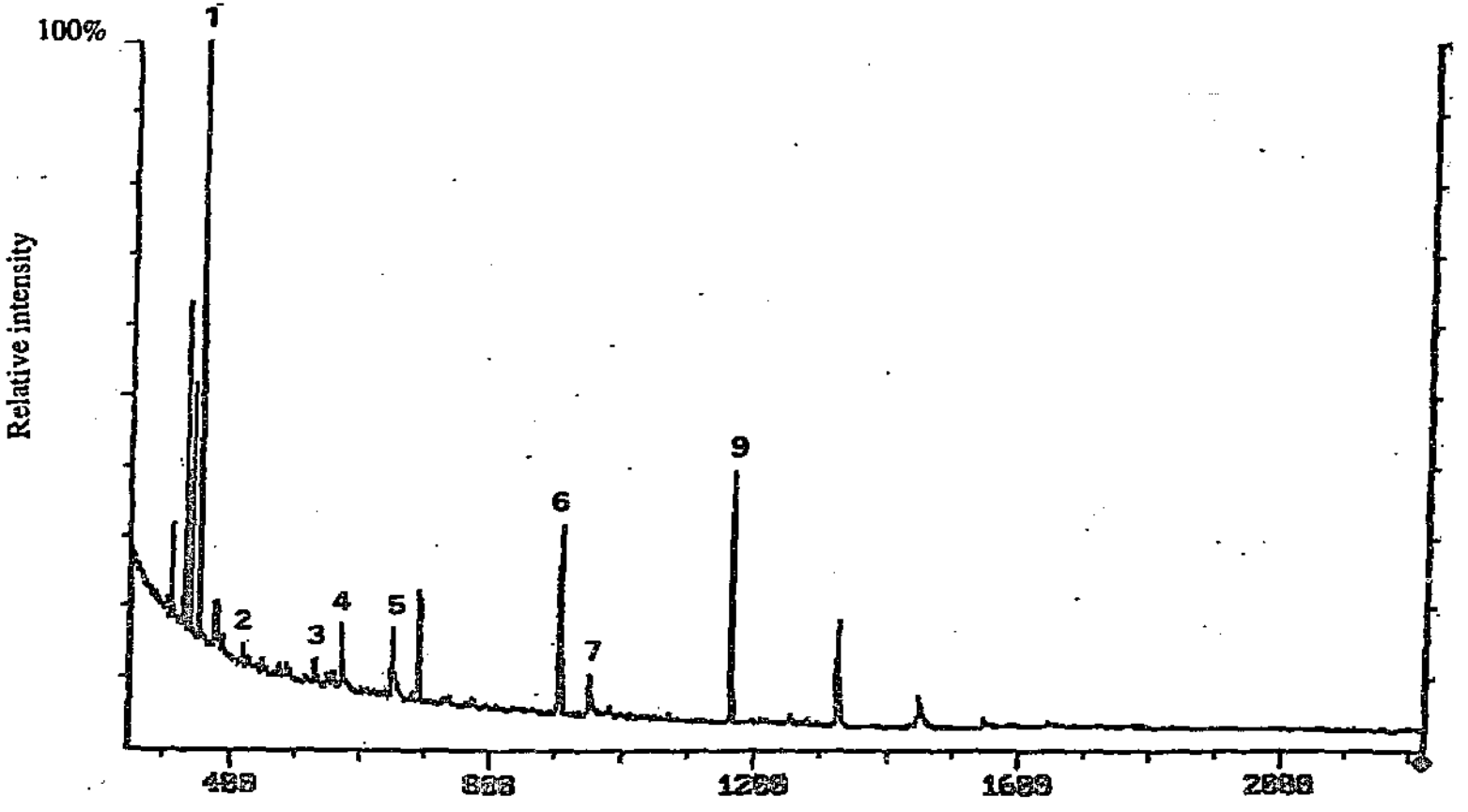


Figure 4.7 Chromatogram of concentrated 70% buffered ethanoic acid/ 30% methanol elution from a C_{18} cartridge, after having *L. sativa* soaked in it, run on a Varian Star 3400Cx. Peaks of interest are in order of elution (seconds). Peak heights are normalised in comparison to peak 1.

4.3.3 Experiment 3 Results

Experiment 3.1 Results

Aqueous solutions of 1, 8 – cineole provided interesting results. Germination percentage ranged from 58 – 63% for the aqueous cineole solutions this is a 10% increase on controls treated with Milli-Q water. 1, 8 – Cineole was found to be statistically significant in its enhancement of germination of *L. sativa* ($p = 0.017$; $p = 0.000$; $p = 0.011$ over three trials) when an aqueous solution, of concentration not over 0.1% v/v, was bioassayed. A weaker concentration, 0.01% v/v of 1,8-cineole in hexane, could not be differentiated from a blank control of Milli-Q water. These results are represented graphically in Figure 4.8.

Experiment 3.2 Results.

Solutions of 1, 8 – cineole in hexane were successfully dissolved into hexane. For these solutions of 1, 8 – cineole created by dissolution in hexane, only the weaker dilutions proved to be germination enhancing when applied to the *L. sativa* bioassay. These results were statistically significant for 0.02% v/v ($p = 0.039$), 0.1% v/v ($p = 0.003$) and 1.0% v/v ($p = 0.000$) cineole in hexane. Concentrations of 2.0% v/v and greater could not be differentiated from the Milli-Q water control even through repetition of the experiment. A graphical representation of these results can be seen in Figure 4.9.

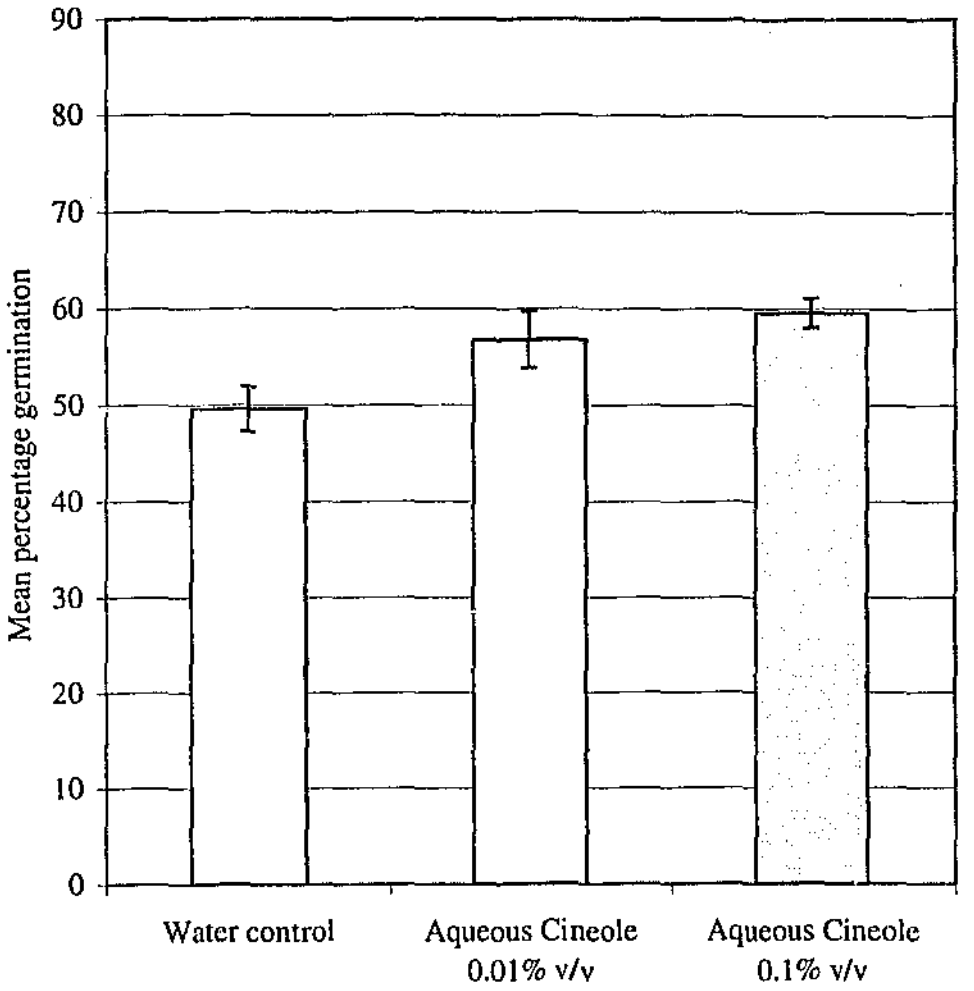


Figure 4.8. Germination of *L. sativa* 48 hours following the application of an aqueous dilution of 1, 8 – cineole. Stippled columns indicate significant differences from the control, vertical bars represent standard errors of the mean. The experiment was repeated thrice, the data presented is from one trial and typical of the results obtained in each trial.

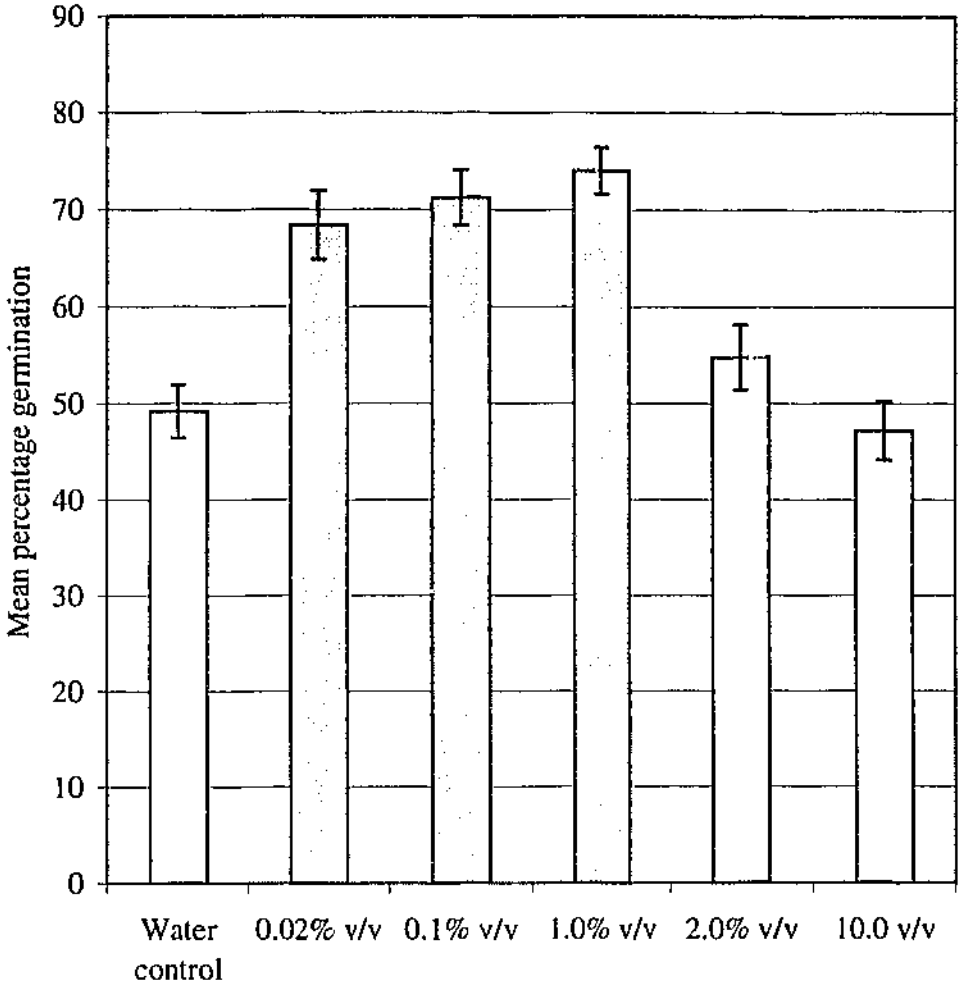


Figure 4.9. Germination of *L. sativa* 48 hours following the application of dilutions of 1, 8 – cineole in hexane. Stippled columns indicate significant differences from the control, vertical bars represent standard errors of the mean. The experiment was repeated twice, the data presented is from one trial and typical of the results obtained in each trial.

4.4 Discussion

4.4.1 C_{18} Fractions

Smoky water chromatograms and C_{18} fraction derived chromatograms were very consistent over 4 runs of the column, in their peak heights and elution times. Creation of consistent chromatograms for fractions of a C_{18} cartridge proved to be a difficult task. Nuances in peak heights were overcome by injection of hexane blanks between GC column runs of interest, as well as running samples on the same day. Results agreed with the expectation that as more non-polar solvents were used, peaks eluted later in chromatogram and early eluting peaks were diminished.

In the fractionating experiment, caution must still be adhered to when interpreting the library matches of peaks emerging in the 20% buffered ethanoic acid/ 80% methanol fraction. The second peak, (Peak 2 at 417 seconds) would have been dismissed as an improbable germination stimulant because it is more prevalent in the final fraction than the others and this fraction never exhibited any germination enhancement for *L. sativa*. The unusual result was that a statistically significant result was found for germination enhancement from that compound.

Ready availability of 1, 8 – cineole influenced its choice as a test compound. As it is characterised as being practically insoluble in water, it is a reasonable suggestion that 1, 8 - cineole may have been previously undetected from GC results from other researchers. The ability of the SPE to concentrate these trace components with the assistance of a solvent other than water is championed in this example.

SPE cartridges, with respect to the example of the eighth labelled peak (Peak 8 at 1116 seconds), highlight components in an aqueous solvent that would remain otherwise undetectable. Only through concentration of 100 mL of smoky water on a C_{18} cartridge and then flushing through a very high percentage solution of methanol did the small yet noticeable peak emerge.

With regards to referencing peak heights to the seventh peak (Peak 7 at 951 seconds), the variation in height should be considered. Peak 7 may very well have fluctuated in its own right but still remains the highest normalised peak from the computer software printout. Regardless of whether that peak varied or not, or whether component extraction varied, the relative percentages compared to that peak, vary. This detail alone highlights the value of using SPE to isolate components.

In the same manner, the referencing of the first peak (Peak 1 at 353 seconds), for the second experiment is useful because an assumption is made that regardless of whether the fraction peaks of reference change or not, a comparative percentage peak height change indicates that something has been altered. In the case of the second experiment this assumption is extraction by the seed.

4.4.2 Absorption by seeds

From analysis of the chromatograms it can be clearly seen that the solution has been altered. Clearly in experiment 2 the comparative percentage height of peak 7 to peak 1 has declined. A question arises as to whether that can be confirmed as seed coat adsorption, a seed absorption or just more of the component that elutes at 353 seconds leaching into the solution from the seeds. Without a perfectly known history of the seeds and an elemental analysis of the seed coat, any

increase in organic compounds on its surface cannot be solely concluded as adsorption.

A statement can be made that seed coat adsorption is ? likely outcome from soaking *L. sativa* seed in a fraction of smoky water and that these results have done little to reject the hypothesis. The reductions in comparative percentage height of the second, third, fifth and seventh peaks (at 417, 530, 651, and 951 seconds respectively) are distinct and supportive to a theory involving seed coat adsorption or seed absorption.

A question arising from the augmented comparative percentage height of the sixth and ninth peaks (at 910 and 1167 seconds respectively) as to why values were increased in the test sample might be attributed to seed dust. The other possibility implies that all these other components have undergone considerably more extraction then initially hypothesised by referencing peak 1. The enormity of answering that question was not tackled in this project.

4.4.3 1, 8 – Cineole

The heating of 1, 8 – cineole into a solution of Milli-Q water was based on a whim that one of the possibly several germination stimulants must have been undetected in previous research and was thus very low in concentration. The description of 1, 8 – cineole as 'practically insoluble' (Budavari, 1989) heightened the attraction toward testing for it along with a reasonable library match. As seen from the reasonable separation achieved in the pure smoke fraction of 1, 8 – cineole (as well as all the other C_{18} derived fractions) it is a puzzle as to why it has not been tested or eliminated as a germination stimulant. A speculative suggestion

is that 1, 8 – cineole remains undetected when extracted into something other than hexane.

The evidence that 1, 8 – cineole did not enhance germination at concentrations of 2.0% v/v and 10% v/v in hexane correlates to a concentration relationship found in the fractions made in experiment 1. The percentage peak height of peak 2 in the 20% buffered ethanoic acid/ 80% methanol fraction exhibits a greater value than that found in the first two fractions, on a comparative basis, as can be noted from Table 4.1. Thus an inference can be formed that 1, 8 – cineole does enhance germination and ceases to do so beyond a certain concentration. This inference is dependent upon an assumption that components eluting as the reference peak are consistent in concentration; certainly a vincible argument. Thus the inference is of the most premature and unsubstantiated manner.

5.0 GENERAL DISCUSSION

The work performed in this project is an innovative adaptation to research attempting to identify chemical components of smoke that stimulate germination of *L. sativa*. The more recent works on smoky water have involved longer-term studies on slow to germinate species (Roche et al, 1997; Brown et al., 1998) rather than rapid adsorption studies. Neither have studies been published that present a single component as one of the germination stimulants.

The SPE work conducted in this experiment incorporated the experience of past research (van Staden et al, 1995c) where the buffered ethanoic acid/ methanol mix varied the percentages of methanol from 20% to 80%. Without the use of HPLC, fast, cheap, disposable cartridges were able to eliminate the peaks of disinterest through rapid preparation of fractions. These fractions were useful in that biological assays performed on the fractions created were to prioritise active fractions as solutions for further research. The reduced number of components in such an active fraction, by elution with a buffered ethanoic acid/ methanol mix, and then by the extraction into a solvent, is a creditable tool for elimination.

The research performed comparing the non-polar and polar cartridges of smoky water yielded useful results. Essentially, the project met its stated aims and even found a possible germination cue through research on 1, 8 – cineole. The most important result was the difference in germination percentages found in fractions derived from the polar NH_2 cartridge. A consistent lack of statistically significant germination enhancement in the second last fraction eluted from the cartridge, yet

significant enhancement in the last fraction gave weight to a theory of multiple germination cues (van Staden et al., 1995c).

As a development of the capabilities of SPE, this research adds another dimension to the fields which SPE is already used. The C_{18} cartridge is already widely used in biological research (Katagari, 1995; Lin, 1995), water pollutant research (Hennion & Pichon, 1994), many environmental analysis projects as well as its role in commercial drug research for anion and cation exchange (Thurman & Mills, 1998). With the comparatively minimal collection time of SPE fractions as opposed to those derived from HPLC (Skoog et al., 1992) the SPE disposable cartridge stands as a reasonable tool for further research on smoky water. With amounts of $400,000 being spent annually on seed alone for bauxite mine rehabilitation by one company in Western Australia (Roche et al., 1997), SPE could prove to be a vital to improve the efficiency of this area of research with smoke-dependent species.

Recent works by Brown et al. (1998) involved the increased germination of *Syncarpha vestita* and *Rhodocoma gigantea* after soaking of these seed in smoky water for 24 hours. The seeds were dried and then rehydrated at regulated intervals throughout a 1 year period. The conclusion drawn from their work was that some germination stimulating components will adhere to the seeds, be it adsorbtion or absorption, and retain the increased ability to germinate for a ' year period. Research in this experiment on soaking of the *L. sativa* seed in smoky water, for only 1 hour, does little to discount the adherence theories presented by Brown et al. (1998) in this similarly behaving species. The reduction in peak height, on a repeatable basis, supports the theory that seeds, known to be

influenced by smoky water, actively uptake components from smoke or smoky water.

1, 8 – cineole was found to be present in smoky water and when concentrated by loading 100 mL of smoky water onto a C_{18} cartridge the Terpene library matches did little to dissuade pinpointing its presence. Further research into a direct application of this chemical proved to be successful in germination enhancement and exhibition of a characteristic of concentration dependency.

The concentration dependence of 1, 8 – cineole in hexane found agreement in past research on different dilutions of smoky water (Drewes et al., 1995;van Staden et al., 1995a; Jager et al., 1996b; Keeley and Fotheringham, 1998). Work in this area supports a finding that a dilution too weak in concentration would not exhibit enhanced germination and one too strong would exhibit inhibition. This research found no such inhibition as a very concentrated solution of 1, 8 – cineole was unable to be formed. A theory must then be promoted that another component of smoky water is responsible for inhibition, when concentrations are higher.

SPE would thus stand as a tool worthy of consideration to all researchers in biological science due to its low cost as a preparatory tool for GC compared to a HPLC. The encouraging preliminary results of the NH_2 cartridge in not creating adjunct active fractions is worthy of support to the suggestion that there is more than one active component in smoky water and that they can be separated. The role of 1, 8 – cineole in the germination of other hard to germinate species that are fire dependent would be another insight that might naturally progress from this research.

6.0 REFERENCES

Anon., (1992). *Saturn II GCMS Getting Started Manual.* Walnut Creek Ca:Varian Associates Inc.

Anon., (1998). *Instruction Manual* Varian Sample Preparation Products

Bignell, C.M., Dunlop, P.J., Brophy, J.J., & Jackson, J.F. (1995). Volatile leaf oils of some south-western and southern Australian specie of the genus *Eucalyptus.* Part V. subgenus *Symphyomyrtus*, section Bisectaria, series Oleosae. *Flavour and Fragrance Journal.* **10 (5)**, 313-317.

Boyd-Boland, A.(1998). Solid-phase Microextraction : Solvent-free sample preparation for GC and HPLC. *Chemistry in Australia,* **65(4)**, 10-12.

Brown, N.A.C., Kotze, G., & Botha, P.A.(1993) The promotion of seed germination of Cape *Erica* species by plant derived smoke. *Seed Science and Technology*, **21**, 179-185.

Brown, N.A.C., & van Staden, J. (1997) Smoke as a germination cue: a review. *Plant Growth Regulation,* **22**, 115-124.

Brown, N.A.C., Prosch, D.S., & Botha P.A. (1998). Plant derived smoke: An effective pre-treatment for seeds of *Syncarpha* and *Rhodocoma* and potentially for many other Fynbos species. *South African Journal of Botany*, **64(1)**, 90-92.

Budavari, S. (1989). *The Merck Index : an encyclopedia of chemicals, drugs and biologicals*, 11th edition. New Jersey: Merck and Co. Inc.

Chapman, J.R. (1993). *Practical Organic Mass Spectrometry: A guide for Chemical and Biochemical Analysis*, 2nd edition. New York: John Wiley and Sons.

De Lange, J.H., & Boucher, C. (1990). Autoecological studies on *Audouinia capitata* (Bruniaceae). I. Plant-derived smoke as a seed germination cue. *South African Journal of Botany*, **56**: 700-703.

Dixon, K.W., Roche S., & Pate J.S.(1995). The promotive effect of smoke derived from burnt vegetation on seed germination of Western Australian plants. *Oecolgia*, **101**, 185-192.

Doran. J.C., Williams, E.R., & Brophy, J.J. (1995). Patterns and variation in the seedling leaf oils of *Eucalyptus Urophylla, E. pellita* and *E. scias. Australian Journal of Botany*, **43(3)**, 327-336.

Drewes, F.E., Smith.M.T., & van Staden, J. (1995). The effect of a plant-derived smoke extract on the germination of light-sensitive lettuce seed. *Plant Growth Regulation*, **16**. 205-209.

Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. *Journal of American Statistics Association*, **50**, 10961-11121.

Evershed, R.P. (1993). Combined gas chromatography – mass spectrometry. In P.J. Baugh (Ed.), *Gas Chromatography: A Practical Approach*. New York: Oxford University Press.

Fowlis, I.A. (1995).*Gas Chromatography*, 2nd edition. Chichester: John Wiley and Sons.

Harris, D.C. (1991). *Quantitative chemical analysis*, 3rd edition. New York : W.H.Freeman and Company.

Hennion, M-C, & Pichon, V. (1994). Solid Phase Extraction of Polar Organic Pollutants from Water. *Environmental Science and Technology*, **28**, 576-583.

Jager, A.K., Light, M.E., & van Staden, J. (1996a) Effects of source of plant material and temperature on the production of smoke extracts that promote germination of light-sensitive lettuce seeds. *Environmental and Experimental Biology*, **36 (4)**, 421-429.

Jager, A.K., Rabe, T., & van Staden, J. (1996b). Food-flavouring smoke extracts promote seed germination. *South African Journal of Botany*, **62(5)**, 282-284.

James, A.T., & Martin, A.J.P.(1952). Gas liquid partition chromatography. *Biochemical Journal*, **50** 679.

Katagi, M., Nishioka, H., Nakajima, K., Nishikawa, M., Tsuchihashi, H., Takino, M., Yamaguchi, K. (1995). Automated extraction, derivatization & GCMS of methanphetamine & amphetamine in human urine. *Japanese Journal of toxicology and Environmental Health*, **41(2)**, 148-155.

Keeley, J.E., & Fotheringham, C.J. (1997). Trace Gas Emissions and Smoke-Induced Seed Germination. *Science*, **276**, 1248-1250.

Keeley, J.E. & Fotheringham, C.J. (1998). Mechanism of smoke-induced seed germination in a post-fire chaparral annual. *Journal of Ecology*, **86**, 27-36.

Keeley, S.C., & Pizzorno, M. (1986). Charred wood stimulated germination of two fire-following herbs of the California chaparral and the role of hemicellulose. *American Journal of Botany*, **73**, 1289-1297.

Keith, D.A.(1997). Combined effects of heat shock, smoke and darkness on germination of *Epacris stuartii* Stapf., an endangered fire-prone Australian shrub. *Oecolgia*, **112**, 340-344.

Lin, Y., Stewart, J.J., & Zhang, H. (1995). A comparison of disc & cartridge solid phase extraction for the LC determination of rifampin & 25- desacetylrifampin in

human serum. *Journal of Pharmaceutical and Biomedical Analysis*, **13(9)**, 1185-1188.

Mangas, J.J., Gonzalez, M.P. Rodriguez, R., & Blanco D.(1996). Solid-Phase Extraction and Determination of Trace Aroma and Flavour Components in Cider by GC-MS. *Chromatographia*, **42 (1/2)**, 101-105.

McMahon, G.P., & Kelly, M.T. (1998). Determination of Aspirin and Salicylic Acid in Human Plasma by Column-Switching Liquid Chromatography Using On-Line Solid-Phase Extraction. *Analytical Chemistry*, **70**, 409 - 414.

Plumb, R.S., Gray, R.D.M., & Jones, C.M. (1997).Use of reduced sorbent bed and disk membrane solid-phase extraction for the analysis of pharmaceutical compounds in biological fluids, with applications in the 96-well format. *Journal of Chromatography B*, **694 (1)**, 123-133.

Puig, D., & Barcelo, D. (1996). Comparison of different sorbent materials for on-line liquid-solid extraction followed by liquid chromatographic determination of priority phenolic compounds in environmental waters. *Journal of Chromatography A*, **733**, 371-381.

Roche, S., Dixon, K.W., & Pate, J.S.(1998). For everything a season: Smoke induced seed germination & seedling recruitment in a Western Australian Banksia woodland. *Australian Journal of Ecology*, **23**(2), 111.

Roche, S., Koch, J.M., & Dixon, K.W. (1997). Smoke Enhanced germination for Mine rehabilitation in the Southwest of Western Australia. *Restoration Ecology* **5(3)**, 191-203.

Schomburg, G. (1990). *Gas chromatography: A Practical Course.* Weinheim: VCH Publishers Inc.

Simpson, N., & Van Horne, K.C. (1993*). Sorbent Extraction Technology Handbook.* Harbor City, CA: Varian Sample Preparation Products.

Skoog, D.A., West, D.M. & Holler, F.J. (1992). *Fundamentals of Analytical Chemistry*, 6th edition. Fort Worth : Saunders College Publishing.

Thurman, E.M., & Mills, M.S. (1998). Solid-Phase Extraction: Principles and Practice. New York: John Wiley and Sons.

Van de Venter, H.A., & Esterhuizen, A.D. (1988). The effects of factore associated with fire on seed germination of *Erica sessiflora* and *E.hebecalyx* (*Ericaceae*). *South African Journal of Botany,* **54**, 301-304.

Van Staden, J., Jager, A.K., & Strydom, A. (1995a). Interaction between a plant-derived smoke extract, light and phytohormones on the germination of light-sensitive lettuce seeds. *Plant Growth Regulation*, **17**, 213-218.

Van Staden, J., Drewes, F.E., & Brown, N.A.C. (1995b). Some chromatographic characteristics of germination stimulants in plant-derived smoke extracts. *Plant Growth Regulation,* **17**, 241-249.

Van Staden, J., Drewes, F.E., & Jager, A.K. (1995c). The search for germination stimulants in plant-derived smoke extracts. *South African Journal of Botany,* **61(5)**, 260-263.

Wahlen, E, Tamasawa, N, Ichimiya, H, Egestad, B, & Sjovall, J.(1994). Conditions for solid-phase extraction of urinary bile acids octadecylsilane-bonded silica. *Journal of Lipid Research*, **35**, 1902- 1907.