

**ANALYSIS OF THE POTATO SPROUT INHIBITOR
1,4-DIMETHYLNAPHTHALENE: HPLC METHOD
DEVELOPMENT AND APPLICATIONS**

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In the name of Allah, Most Gracious, Most Merciful

“Verily, all things have We created in proportion and measure”

The Holy Qur'an, 54:49

“And We send down water from the sky according to (due) measure, and We cause it to soak in the soil; and We certainly are able to drain it off (with ease)”

The Holy Qur'an, 23:18

“It is He Who has let free the two bodies of flowing water: one palatable and sweet, and the other salt and bitter; yet has He made a barrier between them, a partition that is forbidden to be passed”

The Holy Qur'an, 25:53

Abstract

1,4-Dimethylnaphthalene (1,4-DMN) is a pesticide used for inhibiting the sprouting of stored potatoes, and therefore prolonging the storage time. It is registered for commercial use in different parts of the world (e.g. USA and New Zealand) and its registration process in the EU is at an advanced stage. Limited information is available regarding the behaviour and fate of this pesticide in the environment, and therefore, various studies are required in this field. In such studies, analytical methods for the determination of 1,4-DMN in the different environmental samples are a core element.

This work aims to contribute to the knowledge about this pesticide, particularly in the analytical and environmental aspects. Several methods were developed in this work for the determination of 1,4-DMN in environmental samples. HPLC was selected for the final separation and quantification. The development of HPLC separation methods for 1,4-DMN and other related compounds were achieved by the practical step-by-step approach, the use of chromatographic-simulation software, or by a combination of the two approaches. A mixture of seven dimethylnaphthalene isomers and other related naphthalene compounds was used to study the behaviour of these compounds toward the different chromatographic conditions in reversed-phase HPLC with UV detection. This study provides a good understanding of the effect of different chromatographic conditions on the HPLC separation of the compounds studied, and forms a background for the subsequent work in method development. Optimised methods for the separation of this mixture were finally achieved which provide good separation of most of the mixture's components. In the light of the above study, an HPLC-UV separation method for routine analysis of 1,4-DMN was developed and validated. This method provides good linearity ($r^2 > 0.999$) in the range 0.2–300 µg/ml, and good precision with %RSD values of 3.45 % and 0.37 % for 0.02 (method LOD) and 50 µg/ml levels. The method was found to be accurate by comparing it statistically to a gas chromatographic method. The two methods were found to produce results which were not significantly different (at the 5% level) by using a regression test.

Several extraction procedures were then compared for their efficiency in extracting 1,4-DMN residues in potato samples, and also for their suitability for routine HPLC analysis. A final HPLC method (TMP/Heat method) for the analysis of 1,4-DMN residues in potato samples was then achieved and validated. This method is based on extracting 1,4-DMN from potato peel with a mixture of ethanol and 2,2,4-trimethylpentane (7:3) by heating at 50 °C. A liquid-liquid extraction is achieved (in the same extraction flask) with the water

derived from the fresh peel, to end up with 1,4-DMN concentrated in the 2,2,4-trimethylpentane layer. The evaluation of the volume of this layer, in addition to the correction of any loss of 1,4-DMN during the analysis, was achieved by using 2-methylnaphthalene as an internal standard. 2-methylnaphthalene was selected as a suitable internal standard for 1,4-DMN analysis after comparing it with several other compounds (2-ethylnaphthalene, 1-ethylnaphthalene and n-butylbenzene) for the similarity of their behaviour to 1,4-DMN in the extraction and chromatographic separation processes. An aliquot of the 2,2,4-trimethylpentane layer was then analysed directly by HPLC. This method was validated in the range 0.015 to 3 µg/g of potato fresh weight. It was found to have adequate speed, detection sensitivity (LOQ of 0.015 µg/g of potato fresh weight), accuracy (recovery between 90.3 to 106 %) and precision (%RSD between 1.7 to 10.5 %) for routine analysis of 1,4-DMN residues in treated potatoes.

For the determination of natural 1,4-DMN in potatoes, a new analytical method was developed for the extraction and quantification of 1,4-DMN at trace levels. The method (ACN/PROP method) uses the advantage of injecting large volumes (100 µl) of the extracts containing 1,4-DMN directly to the HPLC as a means of enhancing the detection sensitivity. This advantage was achieved by using a mixture of acetonitrile : 2-propanol (7:3) as the extraction solution, which is compatible with the mobile phase and miscible with the water derived from potato peel. The resulting extracts were ready for direct analysis with HPLC with no further clean up. In addition, a high ratio of sample : solvent (1:1) was used for further enhancement of the detection sensitivity. This method was validated at 7.5 and 15 µg/kg of potato fresh weight. It was found to be adequate for trace analysis of 1,4-DMN in potatoes with a limit of quantification of 4.5 µg/kg of potato fresh weight, recovery values between 86.4 to 87.1 % and a precision expressed by %RSD between 4.0 to 7.9 %.

The ACN/PROP method was used for the determination of the natural levels of 1,4-DMN in potato peel and flesh, in addition to some other plant materials. A small peak was detected in the chromatogram of potato peel extracts, at the right retention time for 1,4-DMN, with an area equivalent to a level of about 4 µg/kg of potato fresh weight. However, it was not possible to confirm the identity of this peak due to its low level and the high background noise. There was no sign of the presence of 1,4-DMN in any of the rest of the plant materials analysed which were potato flesh, apples, orange, celery, spring onion, carrots, rhubarb and poppy seeds. A headspace method and preliminary work using Soxhlet extraction were also examined for the determination of natural 1,4-DMN in

potatoes. However, some shortcomings in the development of the methods obstructed the achievement of adequate results.

The ACN/PROP method was also optimised for rapid routine analysis of the residues of 1,4-DMN in treated potatoes. The optimised method was validated in the range of 0.03 to 3 µg/g of potato fresh weight, and found to provide good accuracy (recovery between 89.6 to 93.2 %) and precision (%RSD between 1.6 to 7.3 %). In addition, it is a rapid, easy and straightforward procedure. Because of its advantages, this method was used for different applications regarding the distribution and removal of 1,4-DMN residues in treated potatoes.

To investigate the distribution and removal of 1,4-DMN residues, potato tubers treated and stored for about 18 weeks under commercial storage conditions were analysed for 1,4-DMN residues. 1,4-DMN residues in individual tubers were in the range of 0.63 to 1.16 µg/g fresh weight after 18 weeks of storage with a low variability factor of about 1.5. The residues were found to be concentrated in the peel layer of the tuber and have relatively even distribution across the different parts of the tuber surface. Washing 1,4-DMN-treated potatoes with water and some other solutions was found to remove insignificant amounts of 1,4-DMN residues from potato tubers. In contrast, heating the peel of 1,4-DMN-treated potatoes in an oven at 75 ± 5 °C removed up to 96 % of 1,4-DMN residues..

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Author's declaration

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions. This thesis has not been submitted, in part or in whole, for any other degree.

A summary of the parts of the work in the chapters dealing with the development and validation of the TMP/Heat method was presented at the 12th Symposium on Sample Handling for Environmental and Biological Analysis, Zaragoza, Spain, October 18-20, 2006.

Mohammed Dhafer Yahya Oteef

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List of abbreviations

DMN	=	Dimethylnaphthalene
EtOH	=	Ethanol
TMP	=	2,2,4-trimethylpentane
ACN	=	Acetonitrile
PROP	=	2-propanol
LOD	=	Limit of detection
LOQ	=	Limit of quantification
SD	=	Standard Deviation
%RSD	=	Percentage relative standard deviation
min	=	Minute
psi	=	Pound per square inch
MPa	=	Mega Pascal
r.p.m.	=	Revolutions per minute (in centrifuge)

Chapter 1: General introduction

1.1 The potato crop

The potato is the fourth most important food crop in the world, after maize, wheat and rice. The homeland of potatoes is the western region of South America. Potatoes were brought to Europe by Spanish explorers, and from Europe they were then spread to the rest of the globe. The total world production of the potato crop was 314.37 million tonnes in 2006. With about 80 % of the world's production in 2006, Asia and Europe are the world's major potato producers. In Europe, a total of 7.35 million hectares of harvested area produced 126.33 million tonnes of potatoes in the year 2006 (FAO, 2007). The home supply of potato crop in the UK in 2006 was, in total, about 5.95 million tonnes with 0.42 million tonnes from the previous season and the rest from the current crop (BPC, 2007).

Potato tubers contain an average of about 24 % of dry matter, with the remaining 76 % being water. Starch is the main constituent of the tuber's dry matter, with a mean percentage (of the tuber's fresh weight) of about 18 %, followed by proteins (2-2.5 %), fibres (1-1.8 %) and fatty acids (0.15 %) (FAO, 2007; Lisinska and Leszczynski, 1989). Because of this high nutritional value, potatoes are used for various purposes.

The main use of potatoes is for human food. Just over 66 % of the world's production of potatoes in 2005 was for human consumption in different forms (fresh and processed). The rest were used for animal feed, seed tubers or industrial purposes (FAO, 2007). In the UK, the total human consumption of potatoes in 2006 was about 73 % of the total supply of 7.57 million tonnes. However, only about 52 % of the UK potatoes for human consumption was consumed as fresh produce. The rest was consumed as processed products, with about 31 % as frozen or chilled products, 13 % as crisps and 4 % dehydrated and other products (BPC, 2007).

Another major use of potatoes is for animal feed. In some parts of the world, up to half of the potato harvest is used for animal feed (FAO, 2007). In addition, potato-processing wastes (peel and other potato materials) are traditionally used for animal feeding.

Potatoes are also used as a source of starch, which is an important material for a variety of industries. Starch (or its modified materials) is used in the food industry, the textile industry, the wood and paper industry, and in the manufacturing of glues. It is also widely used in the medical and pharmaceutical fields (Lisinska and Leszczynski, 1989; FAO, 2007).

Potato is a seasonal crop, and therefore some of the harvested crop has to be stored or imported from other countries, to maintain continuous supply for all the above types of potato demand.

1.2 Potato storage

Being a seasonal crop, potato production is limited by climatic conditions. The main factor affecting the cultivation of potatoes is climate temperature. Mean daily temperatures of 18 to 20 °C are required for optimum yields, while night temperatures below 15 °C are necessary for tuber initiation (FAO, 2007). In the UK, potatoes are planted in the late winter (early potatoes) or spring (main crop) and harvested in late summer or autumn. After harvesting, potato tubers start a period of dormancy where no visible growth of any plant structure occurs. The length of the dormancy period is fairly variable. It is affected by both genetic and environmental factors. Different potato cultivars have different dormancy durations. In addition, environmental conditions (such as temperature, photoperiod and water supply) pre- and post-harvest have an important effect on dormancy duration. However, most cultivars grown for the potato processing industry have short dormancy periods, and therefore it is necessary to use storage techniques to ensure continuous supply (Sonnewald, 2001; Suttle, 2007).

At the end of the dormancy period, potato tubers start to develop sprouts. Sprouting is one of the main problems that can affect the quality of stored potatoes and cause major loss. To preserve the quality of stored potatoes, sprouting has to be effectively controlled. Sprouting control can be achieved by storing tubers at low temperatures or with the use of chemical sprout suppressants. However, low storage temperature causes the degradation of potato starch into reducing sugars. The accumulation of these sugars is correlated with the dark fry colour which is not acceptable in the potato processing industry (Sonnewald, 2001; Kalt et al., 1999). Therefore this storage technique is not commonly used for potatoes prepared for the processing industry.

The use of chemical sprout inhibitors is the commercially preferred option for successful long-term storage of potato tubers, particularly the ones used for the processing industry. It is estimated that 52 % of ware potatoes stored in the UK from the 2004 harvest received chemical pesticide treatments (Anderson et al., 2006). The primary method for controlling sprouting of stored potatoes is by the application of the sprout-inhibiting chemical chlorpropham (Anderson et al., 2006; Kleinkopf et al., 2003).

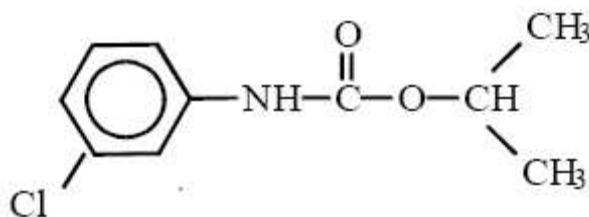


Figure 1.1 Chemical structural formula for chlorpropham

Chlorpropham (Figure 1.1) [Isopropyl 3-chlorocarbanilate, Isopropyl N-(3-chlorophenyl)carbamate, Chlor-IPC or CIPC] is a post-harvest sprout inhibitor used successfully for controlling sprouting in stored potatoes for more than 40 years. It is commonly applied to stored potatoes as an aerosol fog (diluted in an organic solvent) at rates of 17 to 22 ppm (mg/kg) to bulk potatoes. The rate may vary based on the potato cultivar, storage temperature, and the length of intended storage. Two applications may be required when the storage period required is more than 8 months (Kleinkopf et al., 2003). However, some other formulations are used such as dust powder as in Greece (Lentza-Rizos and Balokas, 2001). In the UK, 95 % of the total ware potatoes treated in stores (2.1 million tonnes) in 2004 was treated with chlorpropham using a total of 72.88 tonnes of chlorpropham (Anderson et al., 2006). This would be equivalent to an average treatment of 34.70 g of chlorpropham per tonne of ware potatoes. The maximum total dosage in the UK is 63.75 g of chlorpropham per tonne of potatoes in a storage season. Up to 4 treatments per season may be applied with a minimum interval of 45 days between applications and the latest time of application is 21 days before removal from store for sale or processing (Product label of “MSS SPROUT NIP[®]”, a 100 % chlorpropham formulation).

Despite the successful use of chlorpropham for potato storage, its availability may be restricted in the future (Lewis et al., 1997). Due to the increasing safety and environmental concerns regarding this synthetic chemical, many countries have started to reassess the use of chlorpropham, and limits of the allowable levels of chlorpropham residues (MRL) in potatoes entering the market place have been set in many countries. A maximum residue

limit of 10 mg/kg was approved in the EU countries and is in place since 2007 (PRC, 2007a). In the USA, the tolerance value is 30 mg/kg (Kleinkopf et al., 2003). The concerns regarding levels of chlorpropham residues and its toxicity have contributed to an interest in finding safer and more natural sprout inhibitors.

A wide range of compounds and materials has been studied as potential sprout inhibitors. Several natural compounds were found to be effective sprout inhibitors, including several monoterpenes (e.g. carvone), spearmint and peppermint oils, purified extracts from clove and substituted naphthalenes (Kleinkopf et al., 2003; Vaughn and Spencer, 1991; Meigh et al., 1973; Beveridge et al., 1981a; Beveridge et al., 1981b). Many of these natural sprout inhibitors are commercially marketed in different countries.

Naphthalene and some of its alkyl-substituted compounds have been identified as natural volatiles produced by potatoes (Meigh et al., 1973; Buttery et al., 1970; Nursten and Sheen, 1974; Coleman et al., 1981; Oruna-Concha et al., 2001). Dimethylnaphthalene (DMN) isomers are a group of naphthalene-substituted compounds in which 2 methyl groups substituted two hydrogen atoms in the naphthalene ring. Ten isomers of dimethylnaphthalene have been identified (Shinbo et al., 2000; Shinbo et al., 1998; Alexander et al., 1983). Some of them, such as the 1,4- and 1,6- isomers, have shown potato sprout inhibiting effects (Beveridge et al., 1981a; Meigh et al., 1973; Filmer and Rhodes, 1985).

1.3 The sprout inhibitor 1,4-dimethylnaphthalene

1,4-dimethylnaphthalene (1,4-DMN) was extracted from potato peel as a natural volatile and was shown to be an effective sprout inhibitor with inhibition activity comparable with that provided by chlorpropham (Beveridge et al., 1981b; Meigh et al., 1973). 1,4-DMN is used as a sprout inhibitor on potatoes in order to maintain their quality during storage and transport. It has been recently introduced to the market in the United States and some other countries such as New Zealand. It is marketed with the commercial names 1,4SIGHT[®], 1,4SHIP[®] and 1,4SEED[®]. The introduction of 1,4-DMN to the European markets is at an advanced stage.

1,4-DMN is a pale-yellow liquid chemical at room temperature with a petroleum distillate odour. It has low water solubility with a value of 11.4 mg/l at 25 °C. It has a melting point of 7.6 °C and a boiling point of 268 °C. Its vapour pressure at 25 °C is 0.0214 mm Hg (SRC, 2005). The chemical structure of 1,4-DMN is depicted in Figure 1.2 below.

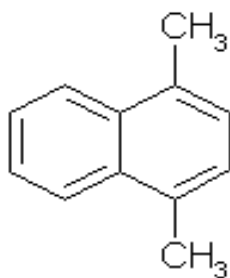


Figure 1.2. Chemical structural formula for 1,4-dimethylnaphthalene

Because it is in a liquid phase, 1,4-DMN can be applied to potatoes without dilution with an organic solvent, which removes concern regarding the safety of using such solvents. In addition, the high volatility of 1,4-DMN helps to provide a more even distribution of this sprout inhibitor through the potato store. This should help in ensuring effective control with lower levels and less variability of the residues, and therefore less risk to consumers. Another advantage of 1,4-DMN as a sprout inhibitor is that 1,4-DMN is a natural potato volatile and therefore has been considered in the USA to be a biopesticide (USEPA, 1997) and has been exempted from the requirement of tolerance when applied post-harvest to potatoes (USEPA, 1995). Furthermore, the effect of 1,4-DMN as a sprout inhibitor is reversible which makes it suitable for seed potatoes (Lewis et al., 1997; Beveridge et al., 1981b).

Although 1,4-DMN is used successfully for sprout inhibition, its mode of action is not well known, but it is thought to inhibit sprouting through hormonal action (Kleinkopf et al., 2003; Knowles et al., 2005).

1,4-DMN can be applied as a thermal fog or as a mist with a recommended label rate of 20 ppm (ml of 1,4-DMN per tonne of potatoes). Reapplication may be required in the case of extended storage periods; however, a maximum total dose of 80 ppm must not be exceeded in a storage season (1,4SIGHT[®] product label). After application, some 1,4-DMN residues can remain in the stored potatoes despite the volatile characteristics of the compound. With the proper use of 1,4-DMN for controlling sprouting in stored potatoes, residues in the range of 0.5 to 1.5 mg/kg (potato fresh weight basis) can typically remain in commercially treated potato tubers (John Forsythe, personal communication).

The use of 1,4-DMN as a commercial sprout inhibitor requires some investigation regarding the safety of the compound and its environmental fate. The level of 1,4-DMN in stored potatoes needs to be monitored to assure effective sprout suppression and to avoid applying unnecessary quantities of the compound. In addition, 1,4-DMN residues need to

be monitored prior to introducing the potatoes to the outlets to ensure the consumer's safety and avoid the risk of intake of hazardously high levels of 1,4-DMN. In addition to the possible risk to humans, the use of the chemical on a commercial scale should be accompanied by the assessment of the possible risks to the environment. The wastes produced after processing potato tubers treated with 1,4-DMN (particularly at a large scale in potato processing plants) such as peel wastes, sediments and waste wash water can pose an environmental risk. All the above monitoring objectives require reliable and rapid analytical methods for the analysis of 1,4-DMN in potatoes and the other different environmental samples.

1.4 Analytical methods for the analysis of pesticides in environmental samples

The analysis of pesticides in environmental samples can best be achieved by chromatographic-based methods. The analysis of environmental samples is often a complicated procedure and normally has many operations. This usually includes selection of sampling strategy, the storage of the sample, sample pre-treatment, sample extraction and, if required, extract clean up and/or pre-concentration, and at the end, the determination of the pesticides in the extracts. A brief summary of the different processes in pesticide analysis in environmental samples is reported in the next sections. For the purpose of this discussion, the different processes were categorised into three sections: sampling and sample storage; extraction and clean up; and the chromatographic techniques used for pesticide determination.

1.4.1 Sampling and sample storage

For any analysis, it is important to set the objectives clearly and draw up a plan for achieving them. Sampling is an important step that should be carefully planned to ensure the achievement of the analysis' objectives. Sampling contributes largely to the validity of the analysis. The main objective of sampling strategies is to get a representative portion of the total population which is analysed, and the analyte level in this portion is assumed to be the same in the whole population. Sampling is required because it is not possible in most cases to analyse the whole population, and therefore well-planned sampling should provide an accurate and precise estimate of the total population.

In most cases, the analyte level differs from unit to unit in a population and, therefore, the level of representation required to achieve the analysis' objective has to be well defined. The analysis' objective therefore controls the sampling strategy required. For example, in

the analysis of pesticides in produce, samples can be collected from different locations in a field, different plants and different positions in a plant. In addition, samples can be mixed to give representation of all levels, or samples of each level can be analysed separately.

Sampling time (i.e. when to take a sample) is another important factor in planning a sampling strategy. For example, in sampling potatoes for the analysis of post-harvest pesticide residues, samples can be collected at different times after the application of the pesticide to study the various aspects of residue levels.

Another important aspect that should be considered when planning for sampling is how to take a representative sample out of a population. Usually, a number of individual units is taken from the population with random or systematic approaches and mixed well to provide the representative sample. The required number for good representation is dependant upon different factors such as the lot size, individual unit size and the nature of the unit (e.g. produce, meat, liquid material, etc.). Composite sampling method is used for evaluating the pesticide residue levels in foodstuffs and assures its consistency with the set regulatory limits. However, the analysis of individual units is sometimes required to evaluate the risk of acute dietary intake. In this case, representative samples should be prepared from the different parts in the unit analysed.

After deciding the optimum method for sampling, samples have to be taken in a proper container and might be stored before the analysis. The correct sampling container should be carefully selected. For example, in samples where the analyte is a volatile compound, a sealed container should be used to minimise the volatilisation of the analyte during the transportation of the sample, or during sample storage. In addition, it is important to select the right material for the sampling containers in order to minimise the contamination of the sample by compounds from the container material (such as some plastics) and also to minimise the adsorption of the analytes in the container walls, particularly at low levels of the pesticide. In the EU guidelines for pesticide residue analysis, it was stated that sample transportation must be in clean containers or robust packaging. Polyethylene bags were considered acceptable but low-permeability bags (such as nylon film) need to be used for residues of fumigants (European-Commission, 2006).

When samples arrive at the laboratory, they should be analysed as soon as possible to minimise any changes in the sample components or the analyte level. However, this is not practical in many cases. Therefore, samples need to be stored correctly. Storing environmental samples is commonly conducted by refrigerating them at 4 °C or freezing

them. Using this technique, the breakdown of the analytes due to microbial activities is normally minimised. However, some other processes may still pose a risk of losing the analyte, such as the adsorption process, particularly at low levels of the analyte. In addition, some samples are photodegradable, so they require to be stored in dark containers or places. Therefore, it is important to conduct the analysis as soon as applicable to avoid the adverse effects of these processes on the sample.

1.4.2 Extraction and clean up methods

Sample extraction aims mainly to remove the compound of interest (analyte) from a complex matrix to a simpler matrix with an optimum yield and selectivity. In many determination techniques, such as most chromatographic techniques, samples must be in a liquid state in order to be analysed. Liquid matrices may be directly determined, or after some treatment such as filtration, solvent exchange, clean up or concentration. However, for solid samples, solvents are normally used to extract the analytes and bring them to liquid solutions. The solutions containing analytes are then separated from solid residues by decanting, filtration or centrifugation. Then these solutions may need some form of clean up or concentration prior to the chromatographic determination.

Pesticide analysis in plant materials normally follows the above general scheme for the solid sample analysis. Many different methods are reported in literature for the analysis of pesticide residues in plant materials. They usually include an extraction step for removing the pesticides from the sample matrix, a clean up step to remove interfering co-extractives, and possibly a step for concentrating the extract to get it ready for instrumental analysis.

In the area of method development for pesticide analysis in plant materials, much effort has been put into the separation of pesticide residues from the plant tissues (Tekel and Hatrik, 1996). Different extraction techniques are used for extracting pesticide residues from plant materials. Some of these methods have been used for more than a hundred years while others are recently applied to pesticide analysis. Most of these techniques are based on the release of the pesticide due to interaction with a selected solvent (or mixture of solvents). Solvent extraction techniques are crucially affected by sample matrix characteristics, chemical characteristics of the analyte and solvent, temperature, pressure and extraction time (Colmsjo, 1998). A brief description of the most common extraction techniques used in pesticide analysis is summarised below.

The simplest and least instrumentally demanding form of extraction methods is the called pure solvent extraction method. In this method, analytes are extracted by adding the

solvent to the sample in one particular vessel. The extraction can be achieved by simply soaking the sample for several hours or sometimes days. Shaking (manually or automated) and/or heating are commonly used to speed up this type of extraction. In this method, the sample should be finely divided to help the leaching process. In addition, the analytes need to be highly soluble in the solvent used, in order to maintain the highest equilibrium concentration of the analytes and minimise the adsorption of the analytes by the solid surfaces. An example of this method was reported by Bushway and co-workers for the analysis of a fungicide residues in fruit and vegetables (Bushway et al., 1998). In this method, a food sample of 5 or 10 g was extracted into a 30-ml polypropylene bottle by hand shaking with 20 ml of methanol for 10 min. An aliquot was then centrifuged and analysed by HPLC. A similar technique has been also used by Navickiene and colleagues (Navickiene et al., 1999).

Sonication or ultrasonic extraction is another simple method of extraction. It applies acoustic vibrations, with frequencies above the range detected by the human ear, to the sample immersed in the extraction solvent. This is a safe and more rapid method compared to some other methods. Ultrasonic extraction is accepted by many regulatory agencies (e.g. USEPA method 3550B) for extracting non-volatile and semi-volatile organic compounds from solids. An example of this method for pesticide analysis was reported by Escuderos-Morenas and co-workers for the extraction of three herbicide residues in potato samples (Escuderos-Morenas et al., 2003). They extracted 2.5 g of potato samples in a 40-ml tube by shaking in an ultrasonic bath with 8 ml of methanol for 30 s. Some clean up processes followed the extraction prior to the analysis by GC.

Homogenisation is another form of solvent extraction. It is based on extracting pesticides from the plant materials by blending the plant sample with the extraction solvent in a blender. By this method, the plant sample is converted into a finely homogenised state, which speeds up and enhances the efficiency of extracting the pesticides by increasing the surface area of the sample that will be in contact with the extraction solvent. Homogenisation is one of the most frequently reported methods of extraction of pesticide residues from plant material. Many publications have reported the use of this extraction method (Fernandez-Cruz et al., 2004; Boulaid et al., 2005; Moreno et al., 2006; Lagana et al., 1997). This method has frequently been used for the extraction of pesticides used for controlling potato sprouts. An example is a method reported by Dias and Duncan (1999a) for the extraction of maleic hydrazide residues in potato products. In this method, maleic hydrazide was extracted from potato chips by homogenising 40 g of potato sample with 80 ml of hexane for 90 s. Another example was reported by Lentza-Rizos and Balokas (2001)

for the extraction of chlorpropham (CIPC) residues from potato tubers. Chopped potatoes (25 g) were homogenised with 25 ml of propan-2-ol and 50 ml of toluene for 3 min followed by filtration and clean up prior to the analysis with GC.

As a development of the pure solvent extraction, refluxing of the solvent at its boiling temperature was introduced by Franz von Soxhlet to enhance the extraction efficiency. Samples are extracted dynamically by fresh hot solvent in an all-glass device. This method has been used for many years and has proved to provide high yield of analytes. The Soxhlet method is accepted by many regulatory agencies (e.g. USEPA method 3540C) and is used as a standard method to which other solid-extraction methods are compared (Snyder et al., 1997). Many examples of methods applying Soxhlet extraction for pesticide analysis in plant materials can be found in published literature, for example (El-Nagar et al., 2005; Chu et al., 2006; Quan et al., 2004).

Soxhlet extraction has also been used for extraction of the residues of sprout control chemicals from potato tubers. Baloch (1999) reported a method for the extraction of chlorpropham (CIPC) residues from potato tubers by Soxhlet extraction. This method is based on extracting a 30-g representative subsample of chopped potato tubers with 150 ml of hexane in the Soxhlet device. Anhydrous sodium sulphate (10 g) is added to adsorb the water derived from the potato tissues. Samples are refluxed for 2 h and cooled down prior to concentration by rotary evaporation. The determination of CIPC is carried out by GC-FID. This method, with slight modification, is still used for the analysis of CIPC residues in our laboratory in Glasgow. It has also been used for the analysis of other sprout inhibitors including 1,4-dimethylnaphthalene.

More modern techniques have also been used for pesticide analysis in plant materials such as microwave-assisted extraction (Barriada-Pereira et al., 2005; Singh et al., 2004; Barriada-Pereira et al., 2007), supercritical fluid extraction (Nunes et al., 2002; Rissato et al., 2005; Stuart et al., 1999; Ono et al., 2006) and pressurized liquid extraction (sometimes called accelerated solvent extraction) (Blasco et al., 2005; Adou et al., 2001; Cho et al., 2007). The use of these methods aims to avoid some of the disadvantages of traditional methods such as the large use of solvents and the long extraction time. However, modern methods need more sophisticated instruments and procedures which raise the cost of extraction compared to traditional methods.

Microwave-assisted extraction (MAE) uses the electromagnetic radiation with a frequency at 2.45 GHz to heat solvents in contact with a sample in order to desorb the analytes from

their sample matrices (Dean, 1998). The extraction is carried out in open-vessel or closed-vessel systems. In the open-vessel system, individual samples are extracted sequentially. The extraction vessel in this type is in principle similar to the Soxhlet device. The sample is placed in a glass vessel connected to a condenser to prevent solvent and analyte loss through evaporation. In close-vessel systems, up to 12 sample vessels can be extracted simultaneously at elevated temperatures. In a typical extraction, sample sizes in the range 0.5 to 10 g are normally extracted with 10 to 30 ml of the solvent in 15 to 30 minutes (Sparr Eskilsson and Bjorklund, 2000; Buldini et al., 2002). The main advantage of this technique is the rapid heating of the sample-solvent mixture which increases the extraction throughput with, in most cases, improved recoveries and reproducibility compared to the conventional extraction methods (Sparr Eskilsson and Bjorklund, 2000).

Supercritical fluid extraction (SFE) is another modern method which is reported frequently for the extraction of pesticides from different matrices. In this technique, samples are extracted with a fluid above and relatively close to its critical temperature and pressure. The critical temperature is defined as “the temperature above which a gas cannot be liquefied by an increase of pressure” (Isaacs et al., 1999). Consequently, critical pressure is the pressure of a fluid when it is at its critical temperature and critical volume (Isaacs et al., 1999). Supercritical fluids combine some properties of both gas and liquid. They have good diffusivity through solids, good solvating power and minimal surface tension (Dean, 1998). These properties make them ideal for solvent extraction. Supercritical carbon dioxide (CO₂) is the most widely used solvent in this technique because it is non-toxic, non-flammable and has low critical values and low chemical reactivity. However, an organic solvent, such as methanol, acetone or acetonitrile is sometimes added to supercritical CO₂ to improve the recoveries of some polar analytes (Motohashi et al., 2000; Buldini et al., 2002). In this technique, samples (about 1-3 g) are placed in a small cell and extracted in static, dynamic or recirculation mode. In the static mode, extraction is achieved by filling the cell with the supercritical fluid, applying pressure and allowing the mixture to equilibrate. In the dynamic mode, a continuous flow of fresh supercritical fluid is passed through the sample cell. In the circulating mode, the same supercritical fluid is passed through the sample for a number of cycles before pumping to the collection vial (Buldini et al., 2002). Supercritical fluid extraction has the advantage of generating cleaner extracts with fewer co-extractives compared to conventional organic solvent extraction, in addition to concentrating the analyte in the final extract (Lehotay, 1997; Buldini et al., 2002).

Pressurized liquid extraction (sometimes called pressurized fluid extraction or accelerated solvent extraction) uses organic solvents at high temperature (between 80-200 °C) and high pressure (between 10 to 20 MPa) to extract the analytes from sample matrices. The high temperature disrupts the interaction between the analyte and sample matrix. It also decreases the viscosity and surface tension of the organic solvents, and increases their solvation and diffusion. At these conditions, the extraction rate increases. The high pressure keeps the solvent in a fluid state (Buldini et al., 2002; Dean, 1998). The extraction is conducted in a special cell (normally constructed of stainless steel) designed to withstand the high pressure and temperature. The sample is placed in the cell and the organic solvent is then pumped through. The cell is then heated under pressure in an oven and maintained static when reaching the required conditions, to allow for the analyte diffusion from the matrix to the solution. The extract is then flushed with another solvent portion and finally the solvent is flushed into a vial (normally 40 or 60 ml) with nitrogen gas (Ezzell, 1999). The extraction time required in pressurized liquid extraction is normally between 15 and 20 minutes (Buldini et al., 2002).

In many cases, extraction methods of pesticides from plant materials are followed by a separate clean up step. The clean up step is commonly carried out to remove co-extractive compounds that may interfere with the analytes during the determination by the instrumental equipment. The most frequently reported techniques for the clean up of extracts of plant materials in pesticide analysis are: liquid-liquid partitioning or extraction (LLE) (Lentza-Rizos and Balokas, 2001; Ishii et al., 2006; Lekkas and Nikolaou, 2006), column chromatography (Hirahara et al., 1997; Wilson et al., 1981) and, more recently, solid-phase extraction (SPE) (Barriada-Pereira et al., 2005; Lee et al., 2001).

In clean up by liquid-liquid partitioning, the sample is partitioned between two immiscible phases of solvents in which the analyte and matrix have different solubilities. One of the two phases is usually aqueous and the other is an organic solvent. Hydrophobic compounds will be concentrated in the organic layer while the hydrophilic ones prefer the aqueous layer. By selecting the right solvents for the analyte-matrix system, good isolation of the analyte from the co-extractives can be achieved. Neutral salts are normally added to aid the separation of the two phases and reduce the solubility of the hydrophobic analytes in the aqueous phase. In addition, shaking is also used to help in speeding up the partitioning process. At the end of the partitioning process, the layer containing the analytes is separated from the other layer for the determination, or it may be concentrated or evaporated to dryness and the solvent changed to a more suitable one for the determination technique. Although in many cases the extracts obtained from the liquid-liquid partitioning

can be analysed directly by the chromatographic technique, further clean up steps might be required in some cases. For this purpose, adsorption column chromatography and solid phase extraction are possible choices.

Adsorption column chromatography is one of the older sample clean up techniques. A tubular column is packed with a gel material in the laboratory or bought pre-packed from a supplier. The most common materials used in this type are inorganic packings such as silica, alumina and Florisil (Snyder et al., 1997). The sample is placed on the top of the column and pushed down with an organic solvent using external pressure, or by gravity. The fraction including the analytes is then collected at the end of the column for the determination.

Solid phase extraction (SPE) is a newer version of column chromatography for sample clean up. However, it is prepared in small cartridges, disks or as coated fibres. The cartridges are the most common form of SPE devices (Snyder et al., 1997). The inorganic packing materials used in adsorption column chromatography are also available in SPE. However, silica particles coated with bonded organic materials are used more often. In SPE, the liquid extract is passed through the cartridge that retains the analyte and some other co-extractives that interact with the packing material. Many of these co-extractives can be either washed off the cartridge before eluting the analyte or left behind after eluting the analyte depending on the combination of the solid phases and eluting solvents used.

Although the clean up steps are separated from the extraction step in many cases, some recent methods have combined these steps. An examples of this situation was reported by Steinwandter (1990) for the analysis of phosphorous pesticides in produce samples. The method developed by Steinwandter (1990) omits the filtration and separate partitioning in separation funnel, and minimises the use of solvents. With a minimum use of solvents and glassware, the method aims to overcome the disadvantages of the common extraction and clean up methods that require large amounts of solvents and laborious work. The extraction and clean up steps are combined into one step. Extraction is based on homogenising produce samples with a mixture of organic solvents and clean up is driven by the salting-out effect of added salt. Five grams of sample are extracted by homogenising with 20 ml of a mixture of acetone/dichloromethane (1:1) and 2 g of sodium chloride (NaCl) in a small glass vessel using a probe homogenizer for 15-30 s. The homogenate is then left to stand for 5 min, after which the organic extract is poured into a 50-ml beaker and dried with anhydrous sodium sulphate (Na_2SO_4) for 5-10 min with stirring. An aliquot of 10 ml of the

extract is reduced to 1 ml by rotary evaporation and diluted with hexane and evaporated again twice. The final extract is made up to 1 ml in a volumetric flask for GC analysis.

The main advantage of this method is the combination of the extraction and the partitioning clean up in one vessel, in addition to the minimum use of solvents and glassware. This combination saves a great deal of the time and effort needed for sample preparation.

Another recent method which combines the extraction and clean up in one vessel was introduced by Anastassiades and co-workers (2003) for the analysis of pesticide residues in food samples. The method is called “QuEChERS” which stands for quick, easy, cheap, effective, rugged and safe. This method is based on extracting pesticide residues in acetonitrile (10 ml) by vortex shaking of 10-g sample (fresh fruits or vegetables) for 1 min in a 40-ml Teflon centrifuge tube. The extraction is followed by liquid-liquid partitioning formed by the addition of 4 g of anhydrous magnesium sulphate (MgSO_4) and 1 g of sodium chloride salt and mixing for 1 min. These salts are added to induce phase separation of the organic and aqueous layers. An aliquot of 1 ml of the acetonitrile extract is then transferred to a 1.5-ml centrifuge tube containing 150 mg anhydrous MgSO_4 and 25 mg of primary-secondary amine sorbent and vortex shaken for 30 s to remove residual water and clean up of the co-extractives. Aliquots of the clean extracts are then analysed by GC-MS. This method has been found to produce good recovery and precision with quick, easy, cheap, effective, rugged and safe analysis. In addition, the use of vortex shaking for the extraction of incurred pesticides was compared to the standard blending method and found to produce similar results (Anastassiades et al., 2003). The 1:1 sample to solvent ratio provides concentrate extracts and eliminates the need for solvent evaporation, which is a common practice in many pesticide analytical methods.

The QuEChERS method has been evaluated, modified and used for different matrices by many researchers since it was published in 2003 (Cunha et al., 2007; Wang et al., 2007; Diez et al., 2006; Hercegova et al., 2006; Pan et al., 2006; Plossl et al., 2006).

1.4.3 Chromatographic techniques for pesticide determination

The use of chromatographic techniques has revolutionised the field of pesticide analysis. This revolution started in the late 1960s with the introduction of gas chromatography (GC). Liquid chromatography (LC) has also contributed to this revolution with the rapid growth of its use for pesticide analysis since its introduction in late 1970s (Tribaldo, 2006). In addition to GC and LC, some other chromatographic techniques are also used in the field

of pesticide analysis such as thin layer chromatography and supercritical fluid chromatography (Toribio et al., 2004; Dost et al., 2000).

In gas chromatography, the vaporised sample is carried into a chromatographic column by an inert gas (the mobile phase). The sample components that are carried in the mobile phase are separated through their partitioning between the gaseous mobile phase and the stationary phase in the column at elevated temperatures. Because of the gaseous nature of the mobile phase in this technique, the analytes are required to have a high enough volatility. These are normally compounds with low molecular weight and low polarity. In addition, they need to be thermally stable in order to be volatilised at the high temperatures used in this technique without adverse changes to their nature.

In liquid chromatography, the mobile phase is a liquid solvent which carries the sample components to a column packed with the stationary phase. The sample components are separated by partitioning (among some other mechanisms such as adsorption, size exclusion, etc.) between the liquid mobile phase and the stationary phase. In liquid chromatography, a wider range of compounds can be separated because samples are carried in a liquid mobile phase at low temperatures. In addition, the wide variety of separation modes (Reversed Phase, Normal Phase, Ion Pairing, etc.) contributes to widening the applications of this technique. Therefore, many of the compounds that are not suitable for GC separation, such as the ones with low volatility or thermal instability, are normally separated using the LC technique. Nowadays, the LC technique is being extensively used in the analysis of many pesticides from different classes including carbamates, phenylurea herbicides, triazines, organophosphorus, quaternary ammonium salts and benzoimidazolic fungicides (Torres et al., 1996; Pico et al., 2000).

The diversity of the LC and GC detectors contributed largely to the spread of the applications of these two techniques. In LC, the most commonly used detectors are the UV detectors, diode array detectors (DAD), fluorescence and mass spectroscopic detectors; whereas in GC, the flame ionization detector (FID), the nitrogen phosphorus detector (NPD), the electron capture detector (ECD) and the mass spectroscopic detectors are the most commonly used ones.

Supercritical fluid chromatography combines some of the best features of GC and LC. The mobile phase in this technique is a supercritical fluid, normally supercritical carbon dioxide. The columns containing the stationary phase can be similar to the capillary GC columns or to the ones used in partitioning HPLC. The detection of the separated

compounds in this technique can be achieved by FID, MS, UV, Fluorescence, DAD and other detectors (Skoog et al., 1998).

In addition to the chromatographic techniques, capillary electrophoresis is used for pesticide determination. Capillary electrophoresis is a relatively new separation technique used for charged species. In this technique, analyte ions are separated according to the difference in rates at which they migrate, across a surface or through a column, under the influence of an electric field. Capillary electrophoresis has the advantage of high separation efficiency and resolution power, low analysis time, and low consumption of sample and reagents. However, it has some limitations in the reproducibility and sensitivity, but these can be solved by several approaches (Ravelo-Perez et al., 2006).

1.5 Distribution and removal of pesticide residues in produce

Pesticides are extensively used in modern agriculture for various applications. Agriculture and food storage and shipping use about 90 % of all pesticides worldwide (Cunningham et al., 2003). Despite the precautions taken to minimise the level of pesticides in produce, about 50.5 % of fruit and vegetable samples (1791 samples) collected from retail sources in the UK in 2006 was found to contain pesticide residues, including about 3.3 % with residues higher than the allowable limits (PRC, 2007a). The presence of a pesticide with a certain risk level is not the only factor in evaluating the actual risk for consumers. It is also important to know the quantity, location of the pesticide in the produce, accessibility and who is exposed to that pesticide.

Many countries and organisations have set up certain limits (such as Maximum Residue Limit values) to control the risk levels of pesticides. In the evaluation of pesticide residue levels in produce samples, composite samples comprised of 5 to 10 individual units are routinely used (Rawn et al., 2006). However, the residue level in a single unit of a fruit or vegetable can be higher than the average residue in the lot estimated by the analysis of composite samples (Hamilton et al., 2004; Hill and Reynolds, 2002; Rawn et al., 2007; Lentza-Rizos and Balokas, 2001). The pesticide residue level can be acceptable in a composite sample because of averaging the levels in the individual units comprising the composite sample. However, because of the variability found between individual units, all of the residues may concentrate in a few units or perhaps, theoretically, in only a single unit of the composite sample (Hamilton et al., 2004). The level of pesticide residues present in single or few units can exceed the acceptable level of residues and may cause a

risk for the consumer. As an example, during the pesticide residue monitoring programme conducted by the UK Pesticide Residues Committee, a potato sample was found to have a residue level of the sprout inhibitor chlorpropham of 47 mg/kg, which is more than four times the Maximum Residue Limit (MRL) recently set for this compound (PRC, 2007a). Therefore, the concept of the variability factor was introduced as an indicator for the variability of pesticide residues.

The variability factor was first defined as “the highest residue level found in any one crop item divided by the level found in a composite sample from the same batch” (Harris, 2000). This definition was then refined for a crop sample taken from a controlled trial as “an upper percentile (97.5th percentile) of the residue levels found in individual units (or single serving portion for large or small unit crops) divided by sample mean” (Harris et al., 2000). Variability factor describes the relationship between the unit with high level and the average residue of the lot, which is best estimated by the measured residue concentration in a composite sample (Hamilton et al., 2004).

A typical value of the variability factor is around 2 to 3 (Hamilton et al., 2004; Lentza-Rizos and Balokas, 2001; Caldas et al., 2006). A default value of 3 was concluded as a good estimate for the variability factor in the 2005 joint meeting of the FAO and WHO experts on pesticide residues (FAO and WHO, 2005). However, wider range from 1.4 to up to 19 has also been reported (Hill and Reynolds, 2002; Rawn et al., 2006).

The causes of variability in pesticide residues between individual crop units were discussed only in the last few years. Harris (2000) reviewed several possible causes of pesticide variability in carrots, including carrot size or variety, position of the top of the carrot in relation to the soil, and the mode of action of the pesticide, but no correlation was found between these factors and the variability in the level of pesticides. Hill and Reynolds (2002) investigated the residue levels of 36 pesticides in 11 different fruit and vegetables and calculated the variability factors for the pesticides in the different commodities. They found no correlation between the variability factor and the commodity, country of origin, residue concentration or the physicochemical characteristics of the pesticide.

Variability in pesticide residues among individual crop units was primarily attributed to the pesticide application method and conditions (Harris et al., 2000; Hamilton et al., 2004; Hill and Reynolds, 2002; Lentza-Rizos and Tsioumplekou, 2001). Application method and conditions affect the quantity of pesticide initially received by the crop unit at or about the time of treatment. Therefore, improving the application method and conditions to provide

more even distribution of pesticides among the individual crop units should reduce the variability levels. However, a major improvement in this direction is not likely to be achievable in near future (Hill and Reynolds, 2002). Therefore, the risk of consuming a crop unit with high pesticide residue will continue to exist and needs to be minimised.

Washing fruit and vegetables with water and/or other washing solutions was reported to reduce the levels of residues of many different pesticides in produce as summarised below.

Jamieson (1988) studied the residue levels of the post-harvest fungicide thiabendazole in potatoes. Potato tubers (Record variety) were sprayed with thiabendazole and mixed well before storing in 10-kg boxes for four months at 8 °C. Washing tubers was conducted by hand with cold water prior to preparing samples for the analysis. A significant amount of 84 % of thiabendazole residues was found to be removed due to washing, compared to the unwashed tubers. This was thought to be an indication of the little movement of thiabendazole into the tuber. The same experiment was repeated after 6 months of storage. There was no significant difference between the unwashed tubers (control) after 6 months and the ones after 4 months. However, washing reduced only 76 % of the residues after 6 months of storage. This may indicate that storage time has an effect on reducing the available pesticide for washing removal.

Tsumurahasegawa and co-workers (1992) studied the residue levels of dichlorvos, chlorpropham and pyrethrins in post-harvest-treated potatoes during storage or processing into starch. The effect of washing treated tubers on the residues of the pesticides was investigated, as washing tubers is a step in the process of producing starch. Potato tubers were sprayed with emulsified solutions of the pesticides and stored at 5 °C for six weeks prior to processing. Washing was conducted by placing treated tubers in a beaker with 5 times their weight of distilled water and shaken for 1 min in a shaker at a speed of 120 shake/min. Water was replaced and samples were shaken again for 2 minutes. This last step was again repeated for a third time. Using this rigorous washing with water, 96 % of dichlorvos, 88 % of chlorpropham and 20-71% of the pyrethrins were removed from treated potatoes.

Cabras and co-workers (1998) investigated the residue levels of several pesticides during prune processing. Prunes were grown and sprayed with diazinon, bitertanol, iprodione, phosalone, and procymidone in an experimental field trial. Samples were taken for processing and analysis after harvesting following 21 days of pre-harvest time after the last application. One subsample was washed with water for 5 min and another subsample for

20 min to evaluate the effect of solubilisation in the removal of pesticide residues. Washing prune samples for 5 min caused a reduction in the residues by a factor of 2 for iprodione and a factor of 3 for phosalone, while not affecting the residue levels of the rest of the pesticides detected. The prolonged washing for 20 min did not result in any further reduction in the residue levels of the pesticides studied. The reduction of the residues of iprodione and phosalone in the first 5 min of washing was thought to be attributed to the removal of the dust adsorbing the pesticides during the washing process. To explain the ineffectiveness of the prolonged washing after the first 5 min wash, the pesticides were assumed to penetrate the epicuticular layer and the cuticle of prunes after the treatment, which prevented the contact between the wash water and the pesticide, therefore avoiding the solubilization.

Baloch (1999) studied the distribution and environmental fate of chlorpropham in potatoes stored in commercial stores. Potato samples were collected from box and bulk commercial stores. Samples from the box store were collected from different sites and heights of the store to check the variability of chlorpropham residue levels. The effect of washing on the residue levels was studied by rinsing treated tubers under running cold tap water sometimes with slight brushing to remove attached soil residues. The effectiveness of washing on removing chlorpropham residues was found to vary according to the store type (bulk or box), storage conditions (cold 3-4 °C or conventional 8-10 °C) and position of the tubers in the store. In general, washing according to the above method was considered to be ineffective in removing considerable amounts of chlorpropham residues because chlorpropham residues were thought to be tightly attached to tuber surfaces, with an exception where surface deposition occurred. The highest reduction of chlorpropham residues was found to be in the cold box store with percentages in the range 48-90 % for the top box and 29-31 % for the bottom box in the store. The lowest reduction due to washing was reported to be in the tubers stored at 8-10 °C in the conventional box store with percentages between 0-18 % and 0-14 % for the top and bottom boxes respectively.

Krol et al. (2000) examined the effect of the simple household technique of rinsing with tap water in reducing pesticide residues in produce. Produce samples (14 fruits and vegetables) were grown in a field for the experiment and treated with a mixture of pesticides, or collected at local farms and grocery stores. The samples were rinsed in a plastic colander under running cold tap water for 15-30 seconds with gentle rotation by hand, to mimic actual household food preparation. Residue levels were found to be reduced for nine of the twelve pesticides studied as a result of the short rinse with cold tap water. The reduction of pesticide residues was not correlated to the water solubility of the

pesticides. It was thought that the majority of the pesticides resided on the surface of produce and the removal was achieved by the mechanical action of the rinsing.

Lentza-Rizos and Balokas (2001) studied the effect of water washing on the residues of chlorpropham in potato tubers. Potato tubers (Lizetta variety) were treated with chlorpropham formulated as dust powder. Tubers were subsampled for washing and analysis after 10 and 28 days after application. Tubers were hand washed individually under running cold tap water for 20 s then analysed for residues. Using the above application and washing technique, chlorpropham residues in potato tubers were reduced by 33 % and 24 % for the 10 days and 28 days post-application sampling respectively. This seems to suggest a decline in the washable residues as the storage period after the application increased.

Soliman (2001) studied the effect of washing and home preparation on the residue levels of several organochlorine and organophosphorous pesticides in potatoes. Potato samples were collected from local retail outlets and the residue levels of the detected pesticides were evaluated. The samples were washed with tap water or aqueous solutions of acetic acid or sodium chloride at concentrations of 2,4,6,8 and 10 %. The washing procedure was not clearly described. Tap water was reported to remove between 12-23 % of the residues of the different pesticides. Reduction percentages in the residues of the different pesticides were reported to be 18-98 % and 17-90 % for acetic acid and sodium chloride washes respectively. The reduction percentage was found to increase with the increase in the concentration of both acetic acid and sodium chloride in the washing solutions.

Pugliese and co-workers (2004) evaluated the effect of washing nectarines with 10 different aqueous solutions on the reduction of pesticide residues in the fruit samples compared to simple tap water washing. Nectarine samples were brought from an organic farming source and dipped in a mixed solution of pesticides for one minute. The samples were left for 24 hours at room temperature in a fume hood prior to the washing process. For each aqueous washing solution, treated samples were washed by immersing in the washing solution for 3 min after which they were sprayed with cold (16-20 °C) tap water for 15 s with gentle rotation by hand. The aqueous solutions studied were citric acid (5 % w/v), ethanol (70 % v/v), glycerol (15 % v/v), hydrogen peroxide (3 % v/v), potassium permanganate (25 mg/l), sodium metabisulfite (5% w/v), sodium laurylsulfate (5 % w/v), sodium hypochlorite (70 mg/l), and urea (15 % w/v) in addition to a mixture of ethanol, glycerol and sodium laurylsulfate. Using the above treatment and washing procedure, the authors reported a reduction of 30-60 % of pesticide residues in nectarines when washed

with ethanol (most effective), glycerol and sodium laurylsulfate. The rest of the solutions used gave washing effect comparable to washing with tap water which removed between 7-34 % of the residues. The effectiveness of a washing solution in removing the residues of a pesticide was thought to be related to the pesticide solubility in the washing solution. In addition, the reduction of pesticide residues due to washing was also found to be related to the water solubility of the pesticide and its octanol-water partitioning coefficient (as all the washing solutions used were aqueous solutions). Pugliese and co-workers pointed out what seems to be a discrepancy in the literature regarding the effectiveness of water rinsing in the removal of pesticide residues. They related this irregularity in results to the many different factors affecting this process, such as differences in pesticide application (post-harvest, in-vitro or in-field conditions) and washing mode (immersing, spraying, water temperature, etc.).

Boulaid and co-workers (2005) investigated the effect of household processing on the residues of the pesticides pyrifenoxy, pyridaben, and tralomethrin in treated tomatoes. Tomatoes were grown in an experimental greenhouse and treated twice with the pesticides during a period of 5 weeks. Several samples were collected after each treatment at different times up to 21 days and subsampled for the different processing steps. A tomato subsample was intensively washed with tap water (no more details reported for the washing procedure) and further dried with absorbent paper to evaluate the effect of washing on the pesticide residue levels. The intensive washing used was found to have a negligible effect on the residues of the three pesticides studied. The mean washing factors (calculated as the ratio between the pesticide level in the washed sample and the mean value of the pesticide levels in the unprocessed samples) were found to be 1.1, 0.9 and 1.2 for pyrifenoxy, pyridaben, and tralomethrin respectively. The ineffectiveness of washing was thought to be justified by the quick adsorption and strong retention of the three pesticides by the waxes of the tomato skin, due to the high liposolubility of the three pesticides as indicated by the octanol-water coefficients.

Radwan and co-workers (2005) compared the effect of various washing solutions and household processing on the residues of the pesticide profenofos in field-grown pepper and eggplant. The plants were grown in an experimental field and sprayed once with profenofos. Samples were examined for the residue removals (by washing) on the 10th and 7th day after treatment for the pepper and eggplant respectively. To examine the effect of washing, samples were soaked in a jar filled with any of the solutions examined for 1 min and left to dry on a paper towels. The washings examined were tap water, potassium permanganate (0.01 %), acetic acid (2 %) soap (1 %), sodium chloride (1 %) and sodium

hydroxide (0.1 %). Reduction values were reported of between 52 % to up to about 100 % removal of pesticide residues due to washing with the different solutions for the different samples. Tap water reduced the residues of profenofos with a percentage of 81 %, 85 % and 99 % for hot pepper, sweet pepper and eggplant respectively. Acetic acid reduced the residues of profenofos with a percentage of 61 %, 85 % and about 100 % for hot pepper, sweet pepper and eggplant respectively. The effect of washing was found to vary depending on the type of crop. The removal of profenofos residues from eggplant was in general higher than from the pepper samples for the different washing solutions.

Randhawa and co-workers (2007) studied the effect of household processing on the residue levels of the pesticides chlorpyrifos and 3,5,6-trichloro-2-pyridinol (TCP) in six vegetables including potatoes. The vegetables were either grown in a supervised field trial or collected from a local farmer's field. The vegetables grown in the supervised field were sprayed twice or 3 times with the pesticides and harvested after 7 days from the last application. Sample washing was conducted in a plastic colander under running tap water for 30 s with gentle rotation by hand, and dried with paper towels. This washing technique was found to reduce the residues of chlorpyrifos significantly with a percentage reduction of between 10-33 % in all the tested vegetables (24-30 % for potatoes). In contrast, the residues of TCP were not affected by the washing process. The researches reported that water solubility was not found to be the most important factor in reducing pesticide residues in vegetables during the washing process. However, such a generalisation needs to be supported by studying more than two pesticides.

A summary of the brief review of the above literature regarding the effect of washing fruit and vegetables with water and/or other washing solutions can be made. The effectiveness of washing produce in removing pesticide residues varies from one experiment to another. The effectiveness of washing is affected by many different factors including the nature of the pesticide and its physicochemical properties, nature of the produce surface, nature of the washing solution, washing process nature and duration, and the final factor is the storage time after application.

The levels and effect of washing on 1,4-DMN residues in potatoes treated with the chemical was studied in a private project sponsored by DormFresh Ltd., Glasgow, Scotland, and performed by Inveresk Research Laboratories, Tranent, Scotland (Gubert et al., 2003). Potato tubers (Russet Burbank and Bintje varieties) were treated with 1,4-DMN using mist application technique. Six applications were conducted at 30-day intervals. A set of tubers was washed and air dried (which is not the common way performed at home)

at ambient temperature before sampling at different time intervals starting from 42 days to 72 days after the last application to study the effect of these treatments on the residue levels of 1,4-DMN. Samples were collected for the analysis at 0-30 days after washing (42-72 days after last application). The residue levels of 1,4-DMN were found to be comparable for the two varieties with no noticeable difference. The residue levels of 1,4-DMN were found to increase steadily from each of the six applications. 1,4-DMN residues were found to have a very slight decline over the 25 days after the last application. Washing and airing tubers as explained above was found to reduce the residues of 1,4-DMN to levels between 19-55 % and 33-50 % for Bintje and Russet Burbank varieties respectively, with no clear trend for time period after washing.

Despite the fact that water washing was widely found to be effective in removing pesticide residues from produce, several commercial washing products for fruit and vegetables are appearing on market shelves with claims that they are more effective than washing with water alone. Some of these products claim to be more than 92 % more effective than water (Juiceland-Limited, 2007).

Michaels and co-workers (2003) compared one of the commercial produce cleaners (brand not specified) with tap water washing and some other produce cleaning methods for the removal of gross dirt, wax and environmental contaminants (including pesticide residues)(Michaels et al., 2003). Various combinations of produce cleaner (used as directed by manufacturer), water rinse, produce cleaning brushes or paper towels were evaluated. Among the various combinations examined, water rinsing with paper towel drying was found superior to all the other methods tested. In addition, wiping produce with paper towels was found to increase the effectiveness of any treatment compared to the same treatment without the use of paper towels.

Krieger and co-workers (2003) compared the use of the commercial product Fit[®] Fruit & Vegetable Wash to tap water washing for the removal of pesticide residues in fruit. The study aimed to evaluate the manufacturers claim that the product is 98 % more effective than water in removing the residues of pesticides most commonly found in produce. Captan and the relatively more water-soluble pesticide methomyl were the two pesticides studied. The crop used was not reported as it was considered “not material to relative measurements of residue reduction”. However, in the discussion of the possible causes of the variation in the reduction of pesticide residues, the authors mentioned the characteristics of the produce surface as a possible cause. Therefore, crop type is an important element which should be mentioned in such studies. Produce samples placed in a

colander were soaked for 30 s in a bowl containing 2 litres of water or water with added 58 ml of Fit[®] Fruit & Vegetable Wash. Samples were then gently rinsed with water four times at successive 30-second intervals, before a final rinse for 5 s in 2 litres of fresh tap water. The samples were then drained on paper towels. The percentages of the reduction in captan residue levels using water wash and the solution of Fit[®] wash were 39 % and 45 % for a residue level of 6.7 mg/kg and 81 % and 90 % for a 0.52 mg/kg residue level. Despite the significant reduction of the residues by the two washing methods compared to the unwashed samples, captan residues in the samples washed by Fit[®] Fruit & Vegetable Wash were not significantly different to the samples washed with water only. For methomyl, both water-rinsed (18 % reduction) and detergent-rinsed (39 % reduction) produce had residues with no significant difference from the residues in the unwashed samples. The difference between the two pesticides in their removal behaviour was attributed to the surface nature of captan residues and the higher penetration ability of methomyl into plant cuticular waxes.

Angioni and co-workers (2004) studied the effect of domestic washing with tap water and a commercial vegetable detergent (Fresh & Clean Natura) on the residues of the pesticides azoxystrobin, fenhexamid and pyrimethanil in strawberries following field treatments. Strawberries were grown in a field-experiment and treated with a mixture of the three pesticides. Mature fruits were collected and washed with either tap water or the detergent before the analysis. For the detergent washing, fruit samples were hand-shaken in a plastic tray containing a water solution of the detergent (1:5, w/v) for 3 min after which they were rinsed twice with about 2 litres under running tap water for 10 s. The samples were then left to dry at room temperature before freezing until the analysis. The water washing method was not clearly described. The authors reported that washing with tap water significantly reduced the residues of fenhexamid (43 %) and azoxystrobin (no percentage reported) while no effect was noticed for the more water-soluble pesticide pyrimethanil. Based on this, the authors concluded that the decrease in the residues of pesticides after water washing is not correlated to their water solubility, but it was thought to be caused by the removal of dust and soil particles adsorbing the pesticides. The use of the vegetable detergent was reported to reduce the residues of all the three pesticides with an average value of about 50 %. The authors concluded that washing with the detergent removed greater amounts of the residues. This was attributed to the ability of the detergent to dissolve epicuticular waxes trapping the pesticides, therefore removing the trapped pesticides which are not removed by water washing.

In most of the above studies, washing produce with water and/or other washing solutions contributed to removing some of the residues of many pesticides. This highlights the importance of washing produce before consumption in reducing the levels of pesticide residues in the consumers' diet. Because of the surface nature of many pesticides and the accumulation of residues in the outer surface of the produce, removing these layers should contribute significantly in reducing the risk of the intake of pesticide residues on the consumers' health. In the case of fruit and vegetables that are normally consumed without the skin, peeling was found to reduce the residue levels of many pesticides by about 80 % to undetectable levels (Lentza-Rizos and Balokas, 2001; Mondy et al., 1992; Boulaïd et al., 2005; Orejuela and Silva, 2004; Fernandez-Cruz et al., 2004; Randhawa et al., 2007; Fernandez-Cruz et al., 2006; Dalziel and Duncan, 1980; Kraish, 1990; Jamieson, 1988).

Many researchers have studied the penetration and distribution of different pesticides in the outer layers of different fruit or vegetables by comparing the residue levels in the peel to the rest of the fruit or vegetable. However, some researchers evaluated the penetration and distribution of the pesticide into the different depths of the fruit or vegetable.

Jamieson (1988) evaluated the penetration of thiabendazole into the layers of a large potato tuber (about 80 mm diameter) by comparing the residues detected in four layers with 10-mm depth. Thiabendazole residues were detected only in the first 10 mm layer at level of 5.2 mg/kg while no residues were detected in all of the other three layers. The inhibition of the movement of the moderately-polar compound thiabendazole into the inner layers of potato tuber was thought to be attributed to the hydrophobic nature of the suberin surrounding the tuber.

Kraish (1990) studied the levels of trifluralin in potato peel, the first centimetre of the flesh and the rest of the flesh. The researcher found that all residues of trifluralin were accumulated in the peel (at levels of 12.3-19.2 mg/kg) and no residues were detected (LOD= 2 ng/kg fresh potato weight) in the rest of the other tuber layers. The absence of trifluralin in the sub-peel layers was thought to be related to its low water solubility which prevented the penetration of trifluralin into the water-rich flesh.

Dias and Duncan (1999) studied the distribution of free and bound residues of maleic hydrazide in potato tubers. Unlike other sprout suppressants, maleic hydrazide is applied to the potato crop in the field before harvesting, and is then translocated from the foliage to tubers. To study the distribution of the pesticide within-tuber, washed tubers were fractionated into three layers: peel, first 10-mm layer of the flesh, and the rest of the flesh.

The distribution of maleic hydrazide was found to be uniform throughout the different layers of the tuber - in contrast to the other post-harvest sprout suppressants - which was thought to be related to the application of maleic hydrazide on the crop and not directly on the tubers. Free maleic hydrazide was found to be gradually converted to bound form with time after treatment, as indicated by the substantial amount of maleic hydrazide released after acid hydrolysis from older tubers compared to the newly-treated ones.

Using radiolabelled pesticides is a valuable technique in studying the distribution of pesticides in crops. Coxon and Filmer (1985) studied the fate and distribution of chlorpropham by applying radiolabelled chemical to potato tubers. To study the distribution of the pesticide within-tuber, potato samples were divided into four layers: attached soil to the tuber surface, 1-mm peel layer, first 10-mm layer below the peel and the rest of the tuber. Chlorpropham was found to concentrate on the peel layer and little penetration was found beyond this layer even after 6 months of storage. The researchers reported that there was evidence of bound non-extractable chlorpropham residues.

Spackman and Cobb (2000) investigated the uptake and distribution of radiolabelled imazethapyr in potato tubers (Spackman and Cobb, 2000). Imazethapyr was found to penetrate the tuber periderm, as some residues were detected beneath this layer. However, minimal residues were detected further into the tuber in the cortical layers. Penetration was found to be slow, but over a prolonged time course it could be significant. Imazethapyr penetration was found to be affected by the pH, as imazethapyr is a weak acid.

The distribution of 1,4-DMN residues in potato tubers is evaluated in Chapter 8. In addition, an investigation was conducted to study the effect of washing and some other treatments on the removal of 1,4-DMN residues.

1.6 Development of HPLC separation methods

The development of separation methods is a major step in any analytical method which uses chromatographic techniques for the determination of the analytes. One of the main goals for HPLC method development is to find the optimum experimental conditions that produce the required separation of the sample components in a reasonable run time.

In reversed-phase HPLC (RP-HPLC), the separation of a mixture of compounds can be improved by optimising the different effective factors such as mobile phase composition, column characteristics and column temperature.

Mobile phase composition (or solvent strength) plays an important role in RP-HPLC separation. In general, compounds are less retained in RP-HPLC columns when using a stronger (more non-polar) mobile phase. The type of organic solvent and its percentage in the mobile phase define the strength of the mobile phase. Acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF) are commonly-used solvents for RP-HPLC. All the three solvents are miscible with water and with each other. In addition, they have low UV cutoff (lowest usable wavelength) of 190, 205 and 212 for acetonitrile, methanol and tetrahydrofuran respectively. Miscibility of the mobile phase components ensure the homogeneity of the mobile phase, which is required to avoid deterioration of the separation, and UV cutoff has a clear effect on the ease and sensitivity of UV detection in HPLC. Any of the aforementioned solvents is normally mixed with water at different percentages to provide the required mobile phase strength for a separation. Mixtures of acetonitrile/water provide good mobile phases for UV detection at low wavelengths. This mixture is the best initial choice for the mobile phase during method development (Snyder et al., 1997). A higher concentration (%B) of ACN in the mobile phase is a good start for the first run of a new separation as it provides quick results. The separation can then be optimised for the mobile phase composition by reducing the percentage of the organic solvent in the mobile phase.

In many cases, an HPLC column packed with a stationary phase of C18-bonded silica is a common start for a new RP-HPLC separation of a wide range of organic compounds. This type of column and mode of HPLC separation are the most widely used for the analysis of different types of pesticides (Tadeo et al., 2000). The C18 stationary phase provides strong retention in RP-HPLC, particularly for non-polar compounds such as naphthalene and its alkylated substituents such as 1,4-DMN. Columns with a C18 stationary phase are nowadays produced by many manufacturers in different parts of the world. C18 columns from different manufacturers, but with the same bonded phase, may provide different separation selectivity for a certain separation components (Snyder et al., 1997). Even differences between column production batches from the same manufacturer have been reported to affect the separation (Dolan et al., 2002).

Several column parameters control the separation of a mixture of compounds in C18 columns. Column dimensions, silica substrate properties and bonded stationary phase characteristics are the main ones. The use of silica-based packing is favoured in most of the present HPLC columns due to several physical characteristics. Silica substrates are available in spherical or irregular shapes and can be prepared with different surface areas, pore sizes and particle sizes, which make them suitable for most HPLC applications.

Totally porous silica particles with 5 μm diameter provide the desired characteristics for most HPLC separations (Snyder et al., 1997).

The surface of silica particles can be modified by a variety of bonded ligands. These ligands are bonded to the silica surface by a reaction between the surface silanol and a silane reagent. C18 columns are usually prepared by using mono-, di- or trichlorooctadecylsilane reagents (Snyder et al., 1997; Poster, 1998). The use of different reagents and reaction conditions affects the final characteristics of the bonded phase. The use of monofunctional silanes in the reaction with silica surface, under certain conditions, forms single bond linkage with the silica as shown in Figure 1.3. Phases prepared with this technique are called monomeric phases.

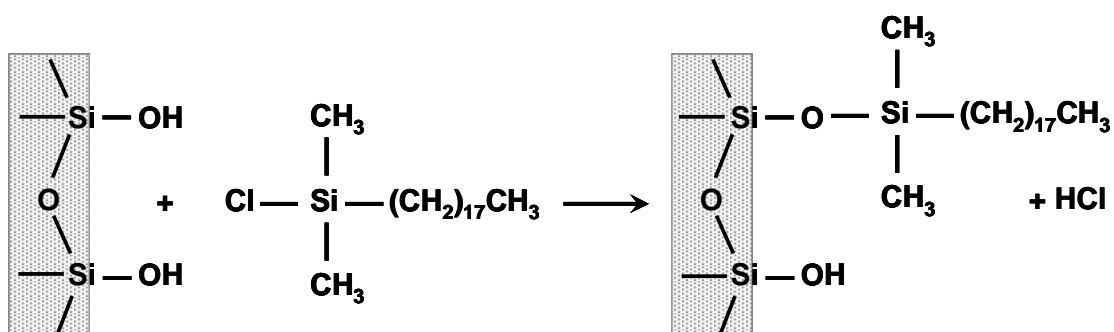


Figure 1.3 Monomeric stationary phase ligand binding in C18 columns

In contrast, the reaction of silica surface with di- or trifunctional silanes under certain conditions forms a polymerised surface layer on the silica surface as shown in Figure 1.4. The phases in this case are called polymeric phases (Poster, 1998; Snyder et al., 1997).

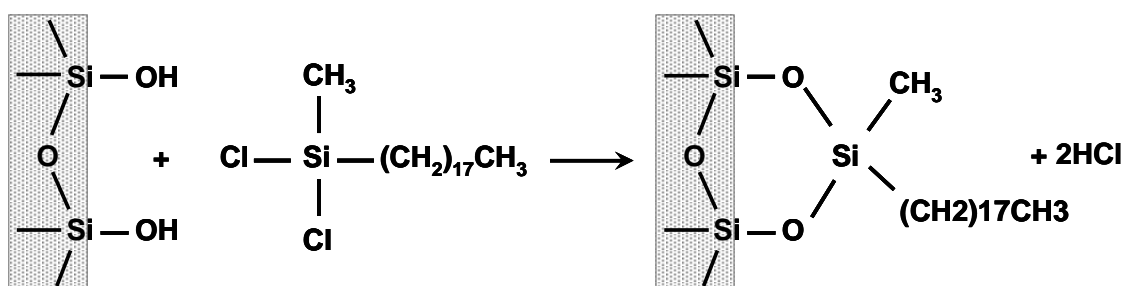


Figure 1.4 Polymeric stationary phase ligand binding in C18 columns

Separation characteristics of these two types of bonded phases of C18 (monomeric and polymeric) may differ remarkably in many applications. In general, polymeric C18 columns can provide better overall separations of isomers and other mixtures of structurally similar compounds compared with monomeric C18 columns (Poster, 1998).

At the end of the reaction with the silane reagents, almost 50 % of the silanol on the silica surface remains unreacted (Snyder et al., 1997). These unreacted groups may interact with appropriate solutes causing problems in their separation such as peak tailing. To reduce the number of unreacted silanol groups, column manufacturers use a process called endcapping. In this process, a subsequent silanization reaction of the bonded packing is conducted with a small-molecule reagent such as trimethylchlorosilane (Jandera and Novotna, 2006).

Column temperature is another condition that plays an important role in controlling the level of separation in RP-HPLC. By selecting the right column temperature, the separation of many samples can be enhanced. Using a higher column temperature helps in reducing system backpressure by decreasing mobile phase viscosity, which in turn should allow the use of longer columns with higher separation efficiency. However, with many samples, an overall loss of resolution between mixture components is expected by increasing column temperature (Dolan, 2002b). The optimum temperature is largely dependant upon the nature of the mixture components in a specific separation, and therefore different temperature values should be tested during the method development to obtain the optimum temperature. In many cases, changes in column temperature affect the separation less than changes in mobile phase composition (Greibrokk and Andersen, 2003; Dolan, 2002b). However, combining both factors in the optimisation can provide a very powerful effect in enhancing the separation.

Resolution in chromatographic separations is a quantitative measurement to define the degree of separation of two adjacent peaks (Dolan, 2002a; Snyder et al., 1997). In liquid chromatography, resolution is defined by three main factors: retention, selectivity and efficiency. As a rough approximation, the three factors may be considered as independent from each other and changes in one of them may not affect the other factors. Conditions that provide higher retention of compounds in the column (i.e. longer retention times, e.g. decrease in mobile phase strength) usually improve resolution. An increase in selectivity (e.g. by changing column stationary phase or solvents in the mobile phase) moves bands apart, and therefore increases resolution significantly. Efficiency affects the peaks' width without changing their relative positions in the chromatogram. Higher efficiency (e.g. by using columns with smaller particle sizes) produces narrower peaks and therefore higher resolutions. Retention and selectivity are generally more affected by changes in the composition of the mobile phase or stationary phase where these changes have less effect on efficiency. Changes in conditions that affect both retention and selectivity are a good start in method optimisation. Efficiency is affected more by column conditions such as

column length and particle size, and also by the flow rate. These conditions are changed at a later stage in method optimisation. Temperature can affect the three factors but has a more direct effect on retention and selectivity (Snyder et al., 1997).

Resolution values can be measured practically for any pair of peaks in a chromatogram using the following equation:

$$R_s = 2(t_2 - t_1) / 1.7(w_1 + w_2)$$

Where t_1 and t_2 are the retention times of the first and second peak in the pair of interest and w_1 and w_2 are their bandwidths at half of the peak height as shown in Figure 1.5. Baseline bandwidths can also be used in measuring resolution. However, resolution measurements based on half-height bandwidth are more commonly used by data systems because it is easier to measure half-height bandwidth than baseline width (Dolan, 2002a).

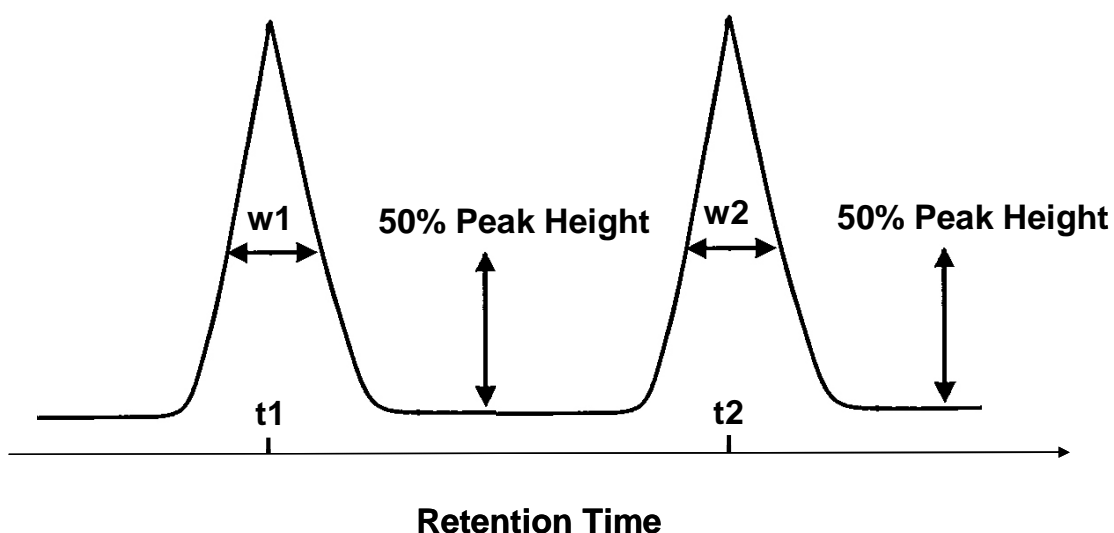


Figure 1.5 Peak characteristics required to measure the resolution

A resolution value of $R_s > 1.5$ (Figure 1.6) describes baseline separation of peaks with a similar size. However, for a robust method that allows for small changes in conditions or when peak sizes are not similar, a resolution value of 2.0 or greater is a desirable target (Snyder et al., 1997).

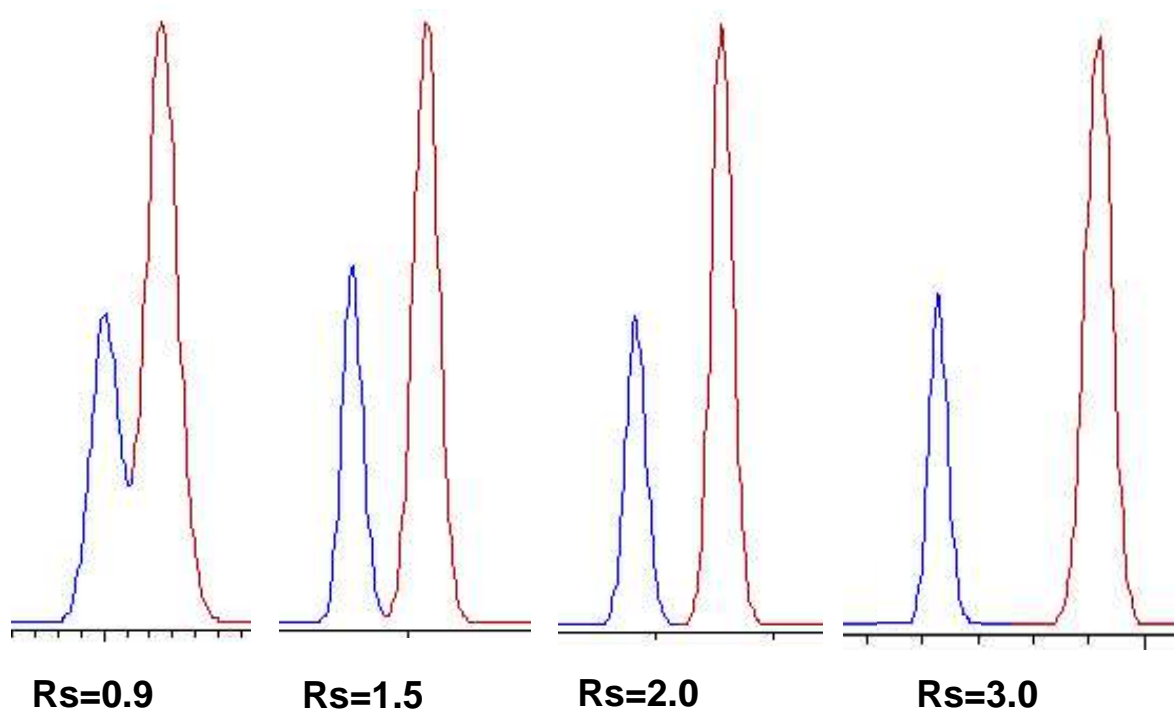


Figure 1.6 Two adjacent peaks with different resolution values

Optimising a separation for adequate resolution in a reasonable run time can be achieved through different procedures. One of these procedures is the practical step-by-step approach. In such an approach, systematic changes in chromatographic conditions are made, and the effect of each change is studied before moving to the next step. This approach is useful in minimising the total number of experiments required for samples that are easy to separate. Although acceptable HPLC separation, in most cases, can be achieved easily with a few number of experiments, some separation problems require a considerable amount of experimentation (Snyder et al., 1997). For the more complex samples, computer-assisted method development can provide a faster and less laborious approach for getting the targeted method.

Computer-assisted method development can be helpful, particularly in situations in which two or more variables are required to be optimised (Snyder et al., 1997). Chromatographic simulation software (such as DryLab[®]) can be used to predict separations for different conditions, by calculation, after loading data from a small number of well-chosen experiments to the software. The software is based on the fact that many of the rules and relationships that relate separation to a change in conditions are quantitative, and therefore it will be possible to carry out accurate calculations to make a better prediction of conditions based on the loaded experimental data (Snyder et al., 1997).

The use of chromatographic simulation software in method development helps to reduce the time, effort and materials needed to generate more reliable methods and therefore more reliable scientific results. This in turn will reduce the cost of developing a new method, and also contributes to reducing environmental pollution. Chromatographic simulation software can also help to give a better understanding of the highly complex relationships that control chromatographic separations by visually showing the effect of the different variables on the resulting chromatogram (Molnar, 2002; Snyder et al., 1997).

1.7 Validation of chromatographic methods

Any developed analytical method needs to be examined to prove that it fits the intended purpose of use. Many definitions and criteria for method validation are set by different regulatory organisations and in different sectors of analytical chemistry. Validation is defined by the the International Organization for Standardization (ISO) as “verification, where the specified requirements are adequate for an intended use”, where the term verification is defined as “provision of objective evidence that a given item fulfils specified requirements” (ISO/IEC, 2007). In the field of pesticide analysis, method validation is defined according to the EC guidelines for pesticide residues analysis as “the process of characterising the performance to be expected of a method in terms of its scope, specificity, accuracy (bias), sensitivity, repeatability and reproducibility” (European-Commission, 2006). This definition includes characteristics that are typically evaluated during the process of method validation for pesticide analysis. Most of the different characteristics required in method validation are generally well defined. However, few criteria are available to define the acceptability of a method. This, in part, may be due to the wide range of purposes served by analytical methods and a broad overview of validation cannot address the differing requirements of each area of analysis (Hill and Reynolds, 1999). In the next paragraphs, a brief summary is provided of the different characteristics that are frequently reported in the validation of quantitative methods, with particular concentration on the field of pesticide analysis.

1.7.1 Applicability and scope of a method

When validating any analytical method, its applicability and scope should be defined before starting the validation process. This can include defining the analyte (or analytes), the sample matrices, the analyte concentration range, description of the equipment and procedures, and the validation level and criteria required.

A description of the analytes' identity and their expected range of concentration is essential. The validated range is defined by IUPAC as "the interval of analyte concentration within which the method can be regarded as validated" (Thompson et al., 2002). This range does not have to be the highest and lowest possible levels of the analyte that can be determined by the method. Instead, the validated range should be defined on the basis of the intended purpose of the method (Hill and Reynolds, 1999). Most methods, in practice, are validated at only one or two concentration levels, and the validated range may be defined as a reasonable extrapolation of these two levels on the concentration scale (Thompson et al., 2002).

In addition to the identification of the analytes, the sample matrix (e.g. potato tubers, soil, water, etc.) in which the analytes are going to be determined should be defined sufficiently. Ideally, method validation would be carried out before use for every analyte-matrix combination, but this is unlikely to be practical (Hill and Reynolds, 1999). Some changes in sample matrices require re-validation of the analytical method, whereas other changes can have minor effects on the method performance and therefore can be accepted without re-validation. For example, in the analysis of pesticides, different varieties of fruit or vegetables, different parts or different manufactured variants may be generally (though not invariably) represented by a single variant. However, some variants may be known or suspected to have a different effect on the method performance than the general effect of the other variants. In this case, the method needs to be revalidated for such variants (Hill and Reynolds, 1999). In contrast, major changes in the sample matrix will of course require re-validation, or, in many cases, further development of the method. For example, a method validated for the analysis of a pesticide in potatoes may not be suitable for the analysis of the same pesticide in animal tissue.

The analytical procedures and equipment should also be defined clearly in any method to be validated. Procedures may include sampling, sample processing, extraction and the determination process. Major changes in these procedures may affect the method performance and therefore require method validation. Some of the examples of these major changes in the analytical procedure are: a change in extraction solvent with significant difference in polarity or chemistry, a change in extraction temperature that may affect solubility and/or partitioning, major changes in the clean up process such as using a different principle, adsorbent or omitting the clean up step entirely. In addition, major changes in the equipment such as the detection technique or changing to a very different chromatographic separation system will require method re-validation (Hill and Reynolds, 1999).

Taking all of the above issues into consideration, the level of validation required has to be set carefully. The method can be validated for use as a screening (qualitative), semi-quantitative (e.g. above 5, below 10 ppm) or quantitative method. In addition, it can be validated for use on single equipment, different equipment in the laboratory, different laboratories or even for international use at different climatic and environmental conditions. The criteria of each type of validation will of course be different with the validation level required (Thompson et al., 2002).

1.7.2 Selectivity and specificity

Selectivity and specificity are two terms that are defined variously in literature in the context of method validation. They are sometimes used interchangeably to describe the same concept in method validation. There has been a debate for decades on the proper definitions and uses of both terms, and many publications have attempted to define and distinguish between the two terms (Vessman et al., 2001; Danzer, 2001; Aboul-Enein, 2000; Stone, 1994; Ward, 1991; Denboef and Hulanicki, 1983).

Selectivity of a measuring system (analytical method in this context) is defined by the ISO as “property of a measuring system, used with a specified measurement procedure, whereby it provides measured quantity values for one or more measurands such that the values of each measurand are independent of other measurands or other quantities in the phenomenon, body, or substance being investigated”. A measuring system was defined as a “set of one or more measuring instruments and often other devices, including any reagent and supply, assembled and adapted to give information used to generate measured quantity values within specified intervals for quantities of specified kinds” and a measurement procedure as a “detailed description of a measurement according to one or more measurement principles and to a given measurement method, based on a measurement model and including any calculation to obtain a measurement result” (ISO/IEC, 2007). No definition of specificity has been provided in this ISO report.

In the EC guidelines for analytical methods for pesticide analysis, the two terms have been defined. Selectivity was defined as “The ability of the extraction, the clean-up, the derivatisation, the separation system and (especially) the detector to discriminate between the analyte and other compounds”. Specificity was related more to the detection technique used instead of the whole analytical method, and was defined as “The ability of the detector (supported by the selectivity of the extraction, clean-up, derivatisation or separation, if necessary) to provide signals which effectively identify the analyte”.

However, only the term specificity has been used in these guidelines in the definition of method validation and reported to be the required characteristic that has to be evaluated during method development, with no mention of the term selectivity (European-Commission, 2006; European-Commission, 2004).

IUPAC defined selectivity as “the extent to which the method can be used to determine particular analytes in mixtures or matrices without interference from other components of similar behaviour”. The use of the term ‘selectivity’ for the above purpose was recommended to be promoted. In contrast, the use of the term specificity to describe the same concept of selectivity was considered incorrect, as specificity is an absolute term and cannot be graded, i.e. a method is either specific or it is not. This use of specificity instead of selectivity was recommended to be discouraged. Specificity was considered the ultimate of selectivity. Therefore, only a few, if any, methods are specific (Vessman et al., 2001).

The Codex Alimentarius Commission followed the IUPAC definition and recommendations and defined method selectivity as “the extent to which a method can determine particular analyte(s) in mixtures or matrices without interferences from other components of similar behaviour”. Codex stressed the IUPAC recommendation to use selectivity as the term for expressing the extent to which a particular method can determine analyte(s) in the presence of interference from other components. It was also emphasised that the use of specificity to describe the same concept of selectivity is to be discouraged as it often leads to confusion. However, the same report used specificity instead of selectivity as one of the general validation criteria required for the selection of a method of analysis (Codex, 2006).

The ambiguity and the tendency to mix up these two terms can cause confusion. Therefore, it is important to emphasise the discussion of the concept required for method validation apart from the terminology debate.

During method validation, it is necessary to demonstrate that the method is capable of providing a unique detected response attributable to the analyte and free from interferences from other compounds present in the sample matrix. Ideally, the method should be evaluated for every important interference that is likely to be present in the sample. However, this may not be practicable and therefore, as a general principle, the method should be sufficiently capable of distinguishing the analyte to the extent that the effect of any potential interferent can be ignored.

Potential interference can be caused by the equipment, reagents or natural constituents of samples. The analysis of reagent blanks should identify the source of equipment and reagent interference, and then they should be removed or at least minimised. To estimate the effect of sample interference, blanks containing sample extracts with the absence of the analyte should be used. In this case, changes in the clean up or determination technique might be required if the interferent signal is overlapping with that of the analyte (European-Commission, 2006; Hill and Reynolds, 1999).

The various steps in the analytical method from sampling to detection can be evaluated and adjusted to provide a unique detected response attributable to the analyte and free from interference from other compounds present in the sample matrix. The effect of the various major steps will be briefly discussed in the context of analytical methods for pesticide analysis. Sampling can help to reduce the interferents present in the final extract. For example, it is sometimes appropriate to select the parts of the plant (e.g. potato peel instead of whole tuber) where the analyte is concentrated and avoid other parts that may add more interferents. Sample extraction may have an important role in reducing interferences. The characteristics of the extraction solvent used are a clear example. Some solvents can extract more compounds from the sample matrix than others. The selection of the appropriate solvent that has good extraction of the analyte with minimum extraction of interfering compounds is desirable. Acetonitrile, for example, has the merit of extracting less of the lipophilic plant materials such as fats and waxes, which helps in obtaining extracts with only a minor load of co-extractives (Tekel and Hatik, 1996). After extraction, the sample may require a clean up step to remove co-extractives. The clean up technique used can be adjusted to remove many of the potential interfering compounds, for example, by selecting the appropriate solvent in liquid-liquid partitioning or the right adsorbent in solid-phase extraction. A further reduction of the potential interference can be achieved by chromatographic separation prior to the detection of the analyte. By careful adjustment of the chromatographic conditions, the remaining co-extractives that may interfere with the analyte determination can usually be separated from the analyte. The detector type and operation will largely control the level of adjustment required in the other preceding steps in the analysis because these steps affect the detectable response of the sample components including the analytes and potential interferences. Some detectors such as refractive index detectors can respond, at least theoretically, to any compound present in the sample. Despite the need for such detectors in certain cases (such as impurity detection in a new material), this type is inappropriate when specific analytes need to be determined. The flame ionisation detector (FID), which is the most widely used detector in GC, responds to most organic compounds and therefore it can be considered less generic than

refractive index detectors. Fewer compounds can be detected by the UV detectors used in LC as they respond only to compounds that absorb UV light, and therefore provide a less generic response compared to the preceding detectors. Diode array detectors (DAD) are based on the same principle of absorbing UV light, but they can produce UV spectra for the sample components. Therefore, they can be operated at different wavelengths in the same run and certain interferences can be eliminated or at least minimised. In addition, UV spectra can be used as a tool for the identification of unknown peaks by comparison with spectra of analyte standards. Coupling GC or LC with techniques such as mass spectroscopy (MS) or nuclear magnetic resonance spectroscopy (NMR) provides very powerful tools for identifying the analytes in complex sample matrices, to the extent that sometimes only minor clean up and chromatographic separations are required. The use of LC-MS-MS, for example, allows the analyst to quantify accurately two or more compounds coeluted in a single peak in the chromatogram without the need for chromatographically separating them.

1.7.3 Accuracy and precision

Errors in experimental measurements are divided into three types: gross error, systematic error and random error. Gross error is a serious error that ruins the experiment and requires the repetition of the experiment from the start. A breakdown of the instrument used, serious contamination of a standard or using a different chemical to prepare a standard, are some examples of this type of error. This type of error is easily identified (Miller and Miller, 2005).

The second type of error in experimental measurements is the systematic error, which is defined as a “component of measurement error that in replicate measurements remains constant or varies in a predictable manner” (ISO/IEC, 2007). Systematic errors and their causes may be known or unknown. When known, a correction can be applied to compensate for their effect (ISO/IEC, 2007). Some examples of systematic errors include: using volumetric glassware at temperatures that differ significantly from the calibration temperature (equipment systematic error), slowness or incomplete reactions when a method is based on complete reactions (method systematic error) or the systematic error caused by the analyst’s judgment of the liquid level with respect to the graduation in a burette (analyst systematic error) (Skoog et al., 1996).

Random error is the third type of error in experimental measurements. It is defined by the ISO as a “component of measurement error that in replicate measurements varies in an

unpredictable manner” (ISO/IEC, 2007). Errors of this type cause the replicate results to spread around the average on both sides. They mainly affect the precision of an experiment (Miller and Miller, 2005). Some examples of this type of error include small variations in temperature, slight uncertainty in weight and volume measurements, or fluctuations in the electrical supplies for analytical instruments.

Accuracy of measurement is defined by the International Organization for Standardization (ISO) as “closeness of agreement between a measured quantity value and a true quantity value of a measurand”. Accuracy is a qualitative characteristic that cannot be expressed as a numerical value. However, it is stated quantitatively in terms of bias which is an “estimate of a systematic measurement error”. Sometimes accuracy is understood as closeness of agreement between measured quantity values that are being attributed to the measurand (ISO/IEC, 2007). According to this definition, the accuracy of a single result may be influenced by both random and systematic errors. When applied to the average of a set of test results, accuracy is more a measure of the systematic error. Accuracy has an inverse relation to both random and systematic errors, where higher accuracy means lower errors (Miller and Miller, 2005; European-Commission, 2006).

Precision is defined by the ISO as “closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions” (ISO/IEC, 2007). Based on the conditions selected under which the precision is evaluated, three levels of precision can be characterised. The first level of precision is called repeatability. This is evaluated when the same analyst conducts repeated analysis of the same sample over a short period of time, using the same procedure and instrument in the same laboratory and under the same operating conditions. The second level of precision is intermediate precision. This level is achieved when the same sample is repeatedly analysed over an extended period of time using the same analytical procedure in the same laboratory, but may include changes in the other conditions such as the analyst, instrument, calibrators and reagents, and operation conditions. The third level of precision is reproducibility. In this level, different analysts analyse the same sample in different laboratories using possibly different analytical procedures (Jenke, 1998; ISO/IEC, 2007). In addition, precision can be divided based on its source within the procedure. For example, system precision will include the evaluation of only the instrument performance, whereas method precision will include the evaluation of the analytical method as a whole, including sampling, sample preparation and instrumental determination (Jenke, 1998).

Repeatability is the precision level that is usually evaluated during method validation, particularly in the field of pesticide analysis. It is normally expressed as the relative standard deviation (RSD) of repeated measurements of a reference material, or during recovery experiments as discussed below. EC guidelines for analytical methods for pesticide residues recommend that repeatability level (expressed as %RSD) should not, in general, exceed 20 % for each commodity and level of analyte concentration (European-Commission, 2004).

Accuracy of an analytical method can be evaluated by the analysis of reference materials, comparing the method to a reference method or other methods, or by spiking and recovery studies.

A reference material is defined as a “material, sufficiently homogeneous and stable with reference to specified properties, which has been established to be fit for its intended use in measurement or in examination of nominal properties” (ISO/IEC, 2007). Reference materials can be analysed for the levels of certain elements or compounds, and the level of these substances accompanied by the measurement uncertainties is then reported in a certificate issued from an authoritative body. These materials are called “Certified Reference Materials”. Reference materials are certified by analysing the homogenous sample using a “definitive or primary” method (a method of high precision for which all sources of bias have been rigorously investigated), analysis using two or more independent and reliable methods, or by analysing the samples of the lot in several laboratories participating in a multi-laboratory comparison exercise (Wise et al., 2006). Certified reference materials are primarily used for validation of the accuracy of analytical methods. However, because of the homogeneity and stability of these materials, they are ideal for assessing the precision of analytical methods. They are also useful for analytical method development for compounds which are present but for which no certified values have been assigned (Wise et al., 2006; ISO/IEC, 2007).

Many certified reference materials are currently available for different applications that require reliable measurements. In environmental analysis, there are a number of certified reference materials available for many organic and inorganic compounds and elements, and in different matrices including air, water, soil, sediments, plant and animal tissues. However, because of the huge number of analyte-matrix combinations required in environmental analysis, certified reference materials are offered in “typical” matrices for analytes of global interest. For organic trace analysis, the range of environmental certified reference materials is more limited and, usually, restricted to compounds regarded as

persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), polychlorinated dibenzo-p-dioxins and dibenzofurans PCDD/Fs, and organochlorine pesticides (OCPs) (Ulberth, 2006).

Analysis of a reference material and comparison of the results obtained by the method under validation to the value stated for the reference material is the ideal means for validation (Hill and Reynolds, 1999). Statistical comparison methods such as significance tests are recommended to evaluate the difference between the results of the method validated and the values assigned for the reference material (Thompson et al., 2002). However, due to the limitation in the available reference materials that can be used for pesticide analysis, alternative methods of evaluating the accuracy of analytical methods are more commonly used.

The second method used to evaluate the accuracy of an analytical method under validation is to compare it to a reference method or other previously validated methods. A reference method or more generally a “reference measurement procedure” is defined as a “measurement procedure accepted as providing measurement results fit for their intended use in assessing measurement trueness of measured quantity values obtained from other measurement procedures for quantities of the same kind, in calibration, or in characterizing reference materials” (ISO/IEC, 2007). To evaluate the accuracy of a method by comparing it to another method, both methods are used to analyse a number of typical samples, preferably with analyte levels covering the required range, and then statistical tests (e.g. paired t-test or regression graphs) are used to compare the results of the two methods. By making this comparison, analysts aim to identify any systematic error that might be produced by the new method (Miller and Miller, 2005; Thompson et al., 2002). In the field of pesticide analysis, some examples of using this method of evaluating the accuracy of new analytical methods are found in literature (Garcia-Reyes et al., 2006; Rodil and Popp, 2006; Walorczyk, 2007).

The third and probably the most common method of evaluating the accuracy (and also precision) of an analytical method under validation is the use of spiking and recovery studies. Recovery studies are a crucial component of the validation and use of all analytical methods. Different practices are found in the area of the estimation and use of recovery, with the differences most obvious in the field of the determination of veterinary drug residues and pesticide residues in complex matrices, such as foodstuffs, and in environmental samples. The spiking-recovery method is a less costly expedient and one that is very commonly applied (Thompson et al., 1999). The EC guidelines for the analysis

of pesticides require the use of spiking and recovery studies for evaluating accuracy in validating analytical methods. Recovery is defined as “the proportion of analyte remaining at the point of the final determination, following its addition (usually to a blank sample) immediately prior to extraction” (European-Commission, 2006). Using this method, specific amounts of the analyte are added to a number of blank samples (a sample matrix known to contain no detectable levels of the analyte) and then analysed according to the analytical method under validation. The recovery is then calculated as the ratio (usually as a percentage) of the concentration of analyte found to that stated to be added. When the recovery is significantly different from the unity (or 100 %) it indicates that a bias is affecting the method.

In the EC guidelines for analytical methods for the analysis of pesticides residues in plants and plant products, five spiked samples are required to be analysed at concentration levels equivalent to the method limit of quantification (LOQ), and at ten times this limit or the maximum residue level (MRL) of the pesticide when it is higher than the 10x LOQ. Mean recovery within the range 70-110 should be demonstrated by the analytical method at validation (European-Commission, 2006; European-Commission, 2004).

Some considerations need to be borne in mind when using spiking and recovery to evaluate the accuracy and precision of an analytical method. The first is that the recovery and precision can be affected by the concentration level of the analytes and may differ substantially between high and low levels of the analyte. This is particularly clear at low levels where some of the analyte can be adsorbed irreversibly to the adsorption sites in surfaces. The effect of adsorption should be less apparent at high levels of the analyte, as all the adsorption sites will be occupied by a small fraction of the analyte. This effect of analyte concentration on recovery should be investigated and taken into consideration during the validation of analytical methods (Thompson et al., 1999; Thompson et al., 2002). Secondly, the spiking-recovery procedure, as defined by the EC guidelines and described above, evaluates any bias that may affect the analyte from the point of adding the spike to the point of measuring its level by the instrument. The native analyte may not necessarily be affected to the same extent as the spike added. Although it is not a guarantee of accuracy, high recoveries are a good indication of the accuracy of the analytical method.

1.7.4 Calibration and linearity

The output of chromatographic instruments is normally produced as peak areas or height related to the analyte amount received by the instrument detectors. The instrumental

response is related to the analyte mass or concentration through some type of calibration within the same chromatographic run or in different runs. Three main standard calibration methods are commonly used in chromatographic analysis. These are external standard calibration, internal standard calibration and the method of standard addition.

In external standard calibration, standard solutions of the pesticide in an organic solvent (or extract of blank samples) are prepared at different concentrations covering the required range for the analysis. These standards are analysed by the chromatographic method and the peak areas (or height) are plotted against the nominal concentrations of the pesticide in the standards. The samples are then analysed by the chromatographic instrument in exactly the same manner as the standards, and the concentration of the pesticide is then determined from the calibration plot graphically or by calculations.

When using external standard calibration, the analyst assumes that all the procedural steps (e.g. extraction, partitioning, clean up, preconcentration by evaporating solvents, etc.) prior to the detection by the chromatographic technique have a negligible effect on the analyte level. However, this is not true in many cases. Losses of the pesticide can occur at various stages during sample preparation due to incomplete extraction of the pesticide from the sample, adsorption of the pesticide on surfaces, losses during the partitioning of the pesticide between solvent phases, incomplete recovery during different clean up methods or losses during preconcentrating the extracts. In addition, some variation (caused by matrix mismatch for example) may occur during the chromatographic separation that may affect the level of the pesticide in the external standard and in the sample differently. Losses of up to 90 % have been found to occur when using traditionally recommended procedures for the solvent extraction of pesticides from samples combined with quantitative analysis by the external standard calibration (Ostroukhova and Zenkevich, 2006).

The above effects of sample preparation on the pesticide levels are normally evaluated during method validation, particularly recovery and precision studies. When the effect is consistent (high precision), the recovery data can be used to apply a correction factor for the results to compensate for the effects of sample preparation. Another approach to overcome the effect of the sample preparation on the analyte level when using external calibration is to pass the calibration solutions through all the sample preparation procedure exactly as the real samples. With this treatment, the different sample preparation steps affect the analyte in both the calibrators and the real samples in the same way and should, therefore, minimise the effect of the sample preparation. However, this approach adds cost

and labour, and is more time consuming. The third approach that can be used to compensate for losses during sample preparation is to use internal standard calibration or the method of standard addition.

In the method of internal standard calibration, a compound different from the analyte but with very similar behaviour (the internal standard) is added to both the samples and the calibration standards. Based on the point in the procedure where the internal standard is added, the effect of various processes in the analytical method can be compensated for. When the internal standard is added to the samples at the start of sample preparation, it should compensate for losses during the different processes in sample preparation in addition to the chromatographic determination by adding the internal standard to the calibration solutions. However, it should be noted that although the internal standard can be selected to mimic the analyte during the different processes in the sample preparation, it may express some differences in the adsorption/desorption behaviours compared to an analyte that has been in contact with the sample material for a significantly longer period of time. The internal standard is usually added to the samples and calibrators at fixed concentration, preferably close to the expected analyte levels.

The selection of a suitable internal standard for quantitative chromatographic analysis is often a difficult prediction, particularly when the method includes sample preparation or clean up before the chromatographic determination. In this case, the internal standard should behave similarly to the analyte in any sample preparation procedure (Wieling et al., 1992). In addition to this criterion in selecting an internal standard candidate, some other criteria should be available in the internal standard used in chromatographic methods including:

- Commercially available in pure form
- Not detected in the original sample
- Has similar chemical behaviour to the analyte
- Stable and not subjected to any reaction except for sample preparation procedure
- Well-resolved in the separation method from the analyte and any other peaks
- Detectable under the same conditions as the analyte (Snyder et al., 1997; Wieling et al., 1992).

- Deuterated analogues of analytes are commonly used internal standards when using mass detection - e.g. in LC-MS-MS, as they can be differentiated from the analytes based on their molecular weight.

Perhaps the most challenging requirement is the necessity for the internal standard to mimic the analyte in any sample preparation step, and at the same time be well separated from all compounds in the chromatographic separation. Similarity between the analyte and the internal standard in their physicochemical properties is favourable for tracking the analyte during sample preparation. However, this similarity is not normally favourable for chromatographic separation, particularly in HPLC, as the retention is known to correlate with the physicochemical properties of the substances, particularly with partitioning coefficients (Li, 2004; Snyder et al., 1997). Therefore, it is important to find a balance between the two challenges.

The third calibration method used in chromatographic analysis is the standard addition method. This method is required, in particular, when the analyte is originally present at detectable levels in the sample to be analysed. In this method, known amounts of the same analyte are added to the same sample at different concentrations sufficient to construct a calibration line. The original concentration of the analyte present in the sample can then be obtained by extrapolating the calibration line. This method can also be used in samples where the analyte is not originally present in order to overcome the effects of sample preparation on the analyte levels. However, it requires more time, resources and effort compared to the other calibration methods, as a calibration plot has to be generated for each sample.

The linearity of the calibration curve is essential to obtain instrumental responses proportional to the analyte concentrations. Linearity should be evaluated firstly by visual examination. Then, statistical approaches can be used for numerical evaluation of the linearity. Peak areas (or heights) of the calibration standards are usually plotted in the y-axis against the nominal standard concentration, and the linearity of the plotted curve is evaluated through the value of the correlation coefficients (r^2) which can be obtained from the software (e.g. Excel[®] or Minitab[®]) used to generate the calibration plot. Generally, a value of $r^2 > 0.998$ is considered as evidence of an acceptable fit of the data to the regression line (Shabir et al., 2007). Linearity of the calibration curve is required to determine the useful range at which the instrumental response is proportional to the analyte concentration. This is normally conducted as part of the validation of the analytical method. However, in routine analysis, repeated two-point calibration can be used when it has been demonstrated (during the validation) that the calibration curve is linear and passes

through the origin (i.e. has zero intercept) and the calibration values are not affected by the sample matrix (Thompson et al., 2002). The significance of the deviation of the intercept of the calibration line from the origin value of zero can be evaluated statistically by determining confidence limits for the intercept, generally at the 95 % level (Miller and Miller, 2005). These limits can easily be obtained from software such as Excel[®] or Minitab[®].

1.7.5 Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) are two parameters which are frequently reported as part of analytical method validation. Various definitions and approaches are reported in literature and set by different regulatory organisations for these two terms. Limit of detection is defined as the lowest amount of an analyte in a sample which can be detected with acceptable certainty, but not necessarily quantified as an exact value with acceptable precision. The limit of quantification is defined as the lowest amount of analyte (concentration or mass) in a sample which can be quantitatively determined with suitable precision and accuracy (European-Commission, 2006; ICH, 1994).

There are different approaches used for the determination of these two limits for analytical methods. Signal-to-noise approach is common for analytical methods that use instruments which exhibit baseline noise such as chromatographic techniques. Using this approach, LOD is defined as the analyte amount that gives a signal-to-noise ratio of 1:3 and LOQ is the analyte amount that gives a signal-to-noise ratio of 1:10 (ICH, 1994). This approach is useful for chromatographic methods in which the sample is clean enough to have a clear chromatogram baseline for evaluating the signal-to-noise ratio. However, in most environmental methods this approach is not often practical due to background peaks around the analyte peak.

Another approach for determining the LOD and LOQ is based on the use of the standard deviation of detector response, which is then converted to mass or analyte concentration by using the slope of the calibration line. In this method, the two terms are calculated as follows: $LOD = 3.3 \text{ SD}/S$ and $LOQ = 10 \text{ SD}/S$ where SD is the standard deviation of the detector response and S is the slope of the calibration line. Using 3.3 times the standard deviation for defining the LOD should give an error of only 5 % in the probability that the sample does not differ from the blank, when in fact it does. However, many analysts use 3 times SD which gives a probability of error of 7 % as a reasonable definition of LOD (Miller and Miller, 2005). The detector SD can be estimated practically in several ways. It

can be based on analysing replicate blank samples and calculating the standard deviation of the responses (ICH, 1994). However, this method of estimating the response is not applicable for most quantitative chromatographic methods as the response is usually expressed by a peak area which is not measurable in most blank samples. Instead of using a blank sample, a spiked blank (or a real sample) at a low concentration level can be analysed in replicates, and the standard deviation of the peak areas is obtained and converted to concentration of the analyte by using the calibration line (Yang et al., 1995; Hartmann et al., 1998). Another way of estimating the detector response SD is by using the residuals standard deviation of the calibration regression line (ICH, 1994). It is important to use a low calibration range close enough to the LOD for this estimation (Peters and Maurer, 2002).

Miller and Miller (2005) describe a statistical method of estimating the limit of detection of an analytical method using the calibration approach. Based on this method, the limit of detection is defined as the concentration of the analyte that gives a peak area significantly different from the blank or the background peak area. The estimation of the analyte peak area which is significantly different from the blank peak area is based on the equation:

$$\text{LOD peak area} = y_B + 3 s_B$$

where y_B is the blank peak area and s_B is the standard deviation of the blank peak area. In practice, the value of y_B and s_B can be estimated from the calibration graph at the lower range by using values from the regression analysis of variance. The value of y_B can be replaced by the intercept value of the regression equation of the calibration graph. Similarly, the term s_B can be replaced by the residuals standard deviation, which is the square root of the error mean square. Both terms can easily be estimated using the regression function in Excel[®] software.

All the above approaches for the determination of LOD and LOQ are based on using standard solutions or spiked blanks and extracts. These methods will therefore measure the LOD and LOQ of the chromatographic separation method used (with or without the sample matrix effect) but not the whole analytical procedure including extraction. The LOD and LOQ values obtained for the chromatographic separation method can, in many cases, be adjusted for the dilution effect and related back as a concentration value of the original sample. However, the values obtained by this adjustment may not necessarily be identical to the ones used to characterise the complete analytical method

A more practical approach for determining the LOQ of the whole analytical procedure is to use a recovery and precision experiment to determine the lowest concentration of a sample that can still be quantified with acceptable recovery within the range 70-120 %, and with precision not exceeding 20 % (%RSD) (Peters and Maurer, 2002; Bansal and DeStefano, 2007; Pizzutti et al., 2007). The LOD can be then estimated by dividing the resulting LOQ by 3.

1.8 Analytical methods for 1,4-DMN analysis in potatoes

1,4-DMN is a relatively new sprout inhibitor for use in the potato storage industry. There is very limited information about analytical methods for the analysis of 1,4-DMN residues in potatoes. Some of these methods date back to the time of evaluating 1,4-DMN for its sprout control properties, such as the method reported by Beveridge (1979). Beveridge method was a modification of a method used by Dalziel (1978) for the analysis of the residues of the sprout inhibitor tecnazene in potatoes. The extraction of 1,4-DMN residues from potato tubers was achieved by homogenising a representative sample with ethanol in a blender followed by the addition of hexane. 1,4-DMN was partitioned into the hexane layer by liquid-liquid extraction. The hexane layer was then cleaned up by alumina column. The clean extract was concentrated by rotary evaporating the solvent to a known small volume. 1,4-DMN concentration in the final extract was determined by GC-FID.

Beveridge (1979) also proposed a spectrophotometric method for the detection and quantification of 1,4-DMN. It was reported that 1,4-DMN has a strong absorbance of UV light at 228 nm which enabled the detection of the compound at levels of 0.1 µg/ml in the solution. However, the presence of interfering plant residues was a major drawback of using the proposed spectrophotometric method for 1,4-DMN quantification in potato extracts.

The GC method reported by Beveridge (1979) for the extraction and determination of 1,4-DMN residues in potato samples was used by O'Hagan (1991) with further modification. The alumina clean up was replaced by a solid-phase extraction and the rotary evaporation conditions were optimised for better recovery of 1,4-DMN. The final extract was finally analysed by GC-FID.

O'Hagan (1991) also reported an HPLC method for the analysis of 1,4-DMN residues in potato samples. The method was a modification of a method developed for the analysis of the herbicide trifluralin [2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)benzenamine]. The

extraction of 1,4-DMN residues from potato tubers was achieved by homogenising a representative sample with methanol in a blender. The extract was partitioned with methylene dichloride and the methylene dichloride layer was reduced to about 4 ml. The extract was then cleaned up with a silica SPE cartridge and an aliquot was analysed by HPLC with UV detection at 230 nm. The separation was conducted on a C18 column with the mobile phase composed of a mixture of methanol/water (7:3) with the addition of acetic acid.

All the above methods reported for the analysis of 1,4-DMN residues in potato samples include lengthy procedures with the use of large volumes of solvents. These methods will not be the right choice for routine analysis of 1,4-DMN residues in potatoes and environmental samples.

A slightly less laborious method for the analysis of 1,4-DMN residues in potato samples is currently used in our laboratory at Glasgow University for routine analysis. This method is a modification of the method reported by Baloch (1999) for chlorpropham (see Section 1.4.2) which uses Soxhlet extraction with hexane and GC-FID for the determination of 1,4-DMN in the extracts. The main modification is the use of potato peel for the analysis instead of whole chopped potatoes in the original method, as most of 1,4-DMN is concentrated in the peel as is the case with many other pesticides (Dalziel and Duncan, 1980; Lentza-Rizos and Balokas, 2001; Orejuela and Silva, 2004). Potato peel samples are extracted by refluxing with hexane for two hours. After cooling, extracts are concentrated by rotary evaporation and made up to a known small volume prior to the analysis by GC-FID.

Another method for the routine analysis of 1,4-DMN residues in potato sample was developed in 1,4Group laboratories in Idaho, USA. This method (will have the abbreviation of 'TMP/Heat method' throughout this thesis) involves the extraction of 1,4-DMN with a mixture of ethanol : 2,2,4-trimethylpentane (TMP) 7:3 v/v containing 2-ethylnaphthalene as an internal standard. The extraction is carried out in a water bath at 50 °C for 15 min. The extract is then cleaned up by liquid-liquid partitioning induced by the addition of NaCl solution and centrifugation. An aliquot of the resulting TMP extract is then analysed by GC-FID (John Forsythe, personal communication).

As shown in the above brief review of the accessible literature for 1,4-DMN analytical methods, most of the methods use gas chromatography with flame ionisation detection as the determination technique. The use of GC (particularly with FID) in analysing plant

tissue extracts requires, in most cases, a clean up step and a concentration step prior to the injection of the extract to the GC. These steps are normally laborious and time consuming. Sample clean up might be more important for the GC-FID analysis due to the sensitivity of the FID detector towards a broad range of organic compounds. However, in UV detection fewer matrix compounds respond to the UV light. This in turn will provide much cleaner chromatograms. Therefore, it might be possible to eliminate the clean up step in order to save considerable time, effort and therefore cost of the analysis. In addition, the ability to inject larger volumes of extracts in HPLC (up to 500 μl or more) than what can be injected in GC [10^{-3} μl for capillary columns and from few tenths of μl to 20 μl for ordinary analytical columns (Skoog et al., 1998)] provides a valuable and easy means of increasing detection sensitivity. Therefore, it would be possible to save more time and effort by eliminating the pre-injection concentration step such as the rotary evaporation used in the Soxhlet method. For the above reasons, HPLC should provide a powerful and more convenient technique for the analysis of 1,4-DMN in environmental samples.

The separation behaviour of 1,4-DMN (and other related compounds) was investigated using HPLC in the reversed-phase mode during this work. This investigation is detailed in Chapter 2. Based on the information obtained from this investigation, a quantitative separation method was developed and validated for the analysis of 1,4-DMN in potato extracts.

The extraction methods that have been originally developed for GC analytical methods were then evaluated for its suitability for use with HPLC as the determination technique. This evaluation was the main objective of Chapter 3, 4 and 5. Two of the most recent methods which have been used for the routine analysis of 1,4-DMN residues in potato samples were evaluated. The first was the method used in our laboratory at Glasgow University which uses the Soxhlet extraction with hexane and the second method is the method developed and used in 1,4Group laboratories as described above. In addition to these two methods, ultrasonic extraction was also examined as a replacement of the heating at 50 °C in the second method.

The extracts obtained by the Soxhlet extraction method can be directly analysed by HPLC-UV (using the routine separation method reported in Section 2.3) without any modification in the extraction procedure. The amount of 1,4-DMN present in the final extract can be easily calculated as the final extract is made up to a known volume. In contrast, some modifications are necessary for the second method (and also the sonication method). This method is based on using 2-ethylnaphthalene as an internal standard for the calculation of

the final extract volume in addition to the correction of any losses of the analyte during the extraction procedure.

2-ethylnaphthalene has a good separation from 1,4-DMN in GC. However, due to the close properties of the two compounds, 2-ethylnaphthalene is not easily separated from 1,4-DMN by HPLC. Therefore, it was necessary either to develop an HPLC chromatographic method which provides a baseline separation of these two compounds in order to use 2-ethylnaphthalene as an internal standard, or to search for another suitable internal standard which has a baseline separation from 1,4-DMN at suitable HPLC conditions for a rapid routine method.

In Chapter 3, the first option of developing an HPLC separation method capable of providing baseline separation for 2-ethylnaphthalene from 1,4-DMN was evaluated. The main conclusion from that evaluation was that 1,4-DMN is not easily separated from 2-ethylnaphthalene in a reasonable time suitable for routine analysis. Therefore, it was decided to move to the other option, which was to search for another internal standard suitable for routine HPLC analysis.

For the purpose of selecting a replacement internal standard for 2-ethylnaphthalene, three internal standard candidates were initially selected to compare their properties and, in particular, their extraction behaviour to that of 1,4-DMN by comparing their relative recoveries (yield) to that of 1,4-DMN. In order to compare the relative recoveries of 1,4-DMN and the three internal standard candidates, in addition to 2-ethylnaphthalene (the original internal standard in the GC method which was added for comparison), an HPLC separation method capable of separating the five compounds had to be developed. The development of this method was aided by DryLab[®] chromatographic-simulation computer software, as detailed in Chapter 3. The method developed provides good separation for quantitative analysis of 1,4-DMN and the four internal standards. Basic validation of this separation method was also described in Chapter 3. In addition to the above method for 1,4-DMN and the four internal standards, the development of a method for separating the internal standard 1-ethylnaphthalene from 1,4-DMN under suitable conditions for routine analysis was also described in Chapter 3. This method was successfully developed with the aid of DryLab[®] software.

In Chapter 4, several recovery experiments were conducted to evaluate the similarity in the extraction behaviour of 1,4-DMN and the internal standard candidates in addition to 2-ethylnaphthalene in the TMP/Heat method. These recovery experiments used the HPLC

separation methods developed in Chapter 3 and the routine separation method reported in Chapter 2. The most suitable internal standard among the ones examined was then selected for the final modified TMP/Heat method for the analysis of 1,4-DMN residues in potatoes by HPLC.

In Chapter 5, the modified TMP/Heat method was compared to the Soxhlet extraction method, which uses hexane as the extraction solvent, and to the extraction by shaking in an ultrasonic bath with the same EtOH/TMP mixture and the same internal standard, as a third extraction method. The three methods were compared for their extraction efficiency of 1,4-DMN from potato samples which were treated with 1,4-DMN and stored under commercial storage conditions for weeks. Based on the evaluation of the methods compared, the final TMP/Heat method for the routine determination of 1,4-DMN residues in potato samples was selected.

For the purpose of investigating the natural levels of 1,4-DMN produced by potatoes, a new analytical method (ACN/PROP) was developed in Chapter 6. This method uses a mixture of acetonitrile/2-propanol as the extractant which is compatible with the mobile phase used. This compatibility allowed the injection of large volumes into the HPLC as a means of enhancing the detection sensitivity for trace analysis. The ACN/PROP method was also optimised and validated for routine analysis of 1,4-DMN residues in potatoes because it is an efficient, rapid, simple, straightforward and less laborious procedure.

In Chapter 7, the ACN/PROP method for trace analysis was used to investigate the natural levels of 1,4-DMN in potatoes and other plant materials. In addition to the ACN/PROP method, a dynamic headspace and a Soxhlet extraction method were also tried for the purpose of investigating the natural levels of 1,4-DMN in potatoes.

In Chapter 8, the ACN/PROP method for routine analysis was used for investigating the distribution and removal of 1,4-DMN residues in stored potatoes. The variability of 1,4-DMN residues between-tubers and within-tuber was evaluated. The effect of washing potatoes on the reduction of 1,4-DMN residues was investigated by analysing potato samples for 1,4-DMN residues before and after washing with tap water and several other washing solutions. The removal of 1,4-DMN residues from potato peel by oven-drying was also investigated in this chapter.

1.9 Thesis objectives

Published data about 1,4-DMN as a post-harvest pesticide is very limited, as many of the studies related to this relatively new pesticide were carried out under confidential conditions and very few are accessible. Because of this limitation, this work aims to provide publishable information regarding this pesticide, which is on its way to becoming a replacement candidate for other commercial sprout inhibitors in many countries.

The main objectives of the work in this thesis were as follows:

1. To study the separation behaviour of 1,4-dimethylnaphthalene and related compounds in HPLC with reversed-phase mode. This study intended to provide a good understanding of the effect of the different chromatographic conditions on the separation of the studied compounds, to form a basic background for the development of the required HPLC separation methods in the following parts of the work.
2. To develop and validate an HPLC separation method for routine quantitative analysis of 1,4-DMN in environmental samples, primarily potato extracts.
3. To review and compare the current extraction methods used for the analysis of 1,4-DMN residues in potatoes in order to select the most appropriate one for routine analysis, or develop a modified one if required.
4. As 1,4-DMN is present naturally in potatoes at trace levels, it was aimed to compare extraction methods for trace levels of natural 1,4-DMN and modify or develop a new one if required.
5. To investigate the natural levels of 1,4-DMN in potatoes and other plant materials
6. To investigate the levels and distribution of 1,4-DMN residues in treated potato tubers.
7. To investigate the effect of different domestic washing methods on the residue levels of 1,4-DMN in treated potato tubers.
8. To investigate the effect of different methods for removing 1,4-DMN residues from peel wastes and evaluate their applicability for industrial use.

Chapter 2: HPLC separation methods for dimethylnaphthalene isomers and related naphthalene compounds

2.1 Introduction

Naphthalene and some of its alkyl substituted compounds have been identified as natural volatiles produced by potatoes (Meigh et al., 1973; Buttery et al., 1970; Nursten and Sheen, 1974; Coleman et al., 1981; Oruna-Concha et al., 2001).

Dimethylnaphthalene (DMN) isomers are a group of substituted-naphthalene compounds in which two methyl groups substituted two hydrogen atoms in the naphthalene ring. Ten isomers of dimethylnaphthalene have been reported in literature (Shinbo et al., 2000; Shinbo et al., 1998; Alexander et al., 1983). Some of them have shown potato sprout suppressing effects such as 1,4- and 1,6- isomers (Beveridge et al., 1981a; Meigh et al., 1973; Filmer and Rhodes, 1985).

1,4-DMN is used as a sprout inhibitor on potatoes in order to maintain their quality during storage and transport. As with other pesticides, the fact that 1,4-DMN residues can remain in treated potato tubers poses a potential concern for consumers. In addition, the use of 1,4-DMN on a commercial scale in the potato storage industry poses the risk of introducing certain amounts of the chemical into the environment. In order to monitor the levels of 1,4-DMN residues in potatoes and other environmental samples routinely, a valid and fast analytical method is required.

The current method used in our laboratory for the routine analysis of 1,4-DMN residues in potato samples applies GC for the final separation and quantification of 1,4-DMN in potato extracts after a 2-hour Soxhlet extraction with hexane, followed by concentrating the extract by rotary evaporation of most of the solvent (see Section 1.8 for more details). Although GC has been widely used for pesticide determination in vegetables, many of the most recent publications have reported the use of HPLC to be a valuable choice in overcoming the problems associated with GC procedures (Mandic et al., 2005; Pous et al., 2001; Orejuela and Silva, 2004; Granby et al., 2004; Lee et al., 2001; Nunes et al., 2002; Obana et al., 2003; Obana et al., 2002; Singh et al., 2004).

Two objectives were established for the work in this chapter. The first was to study the HPLC separation behaviour of 1,4-DMN and some of the other related compounds under different chromatographic conditions. This study intended to provide a good understanding of the effect of the different chromatographic conditions on the separation of the studied compounds. This understanding of the various conditions should help in the future developments of methods for 1,4-DMN analysis in environmental samples.

The second objective was to develop and validate an HPLC separation method for routine analysis of 1,4-DMN in potatoes. The HPLC separation method developed in this chapter is the first step in developing a simple, fast, accurate and precise method for the routine analysis of 1,4-DMN residues in potato samples. The other steps which are concerned with sampling, extraction and clean up procedures will be discussed in the following chapters.

2.2 HPLC separation of dimethylnaphthalene isomers and other related naphthalene compounds

2.2.1 Introduction

Most of the early methods for the separation of naphthalene and its substituted compounds used gas chromatography (Meigh et al., 1973; Coleman et al., 1981; Alexander et al., 1983). However, difficulties were reported in separating some isomers, such as 2,6- and 2,7-dimethylnaphthalene whose separation is difficult in many analytical methods due to their very similar physicochemical properties (Putrawan and Soerawidjaja, 2004; Meigh et al., 1973; Kim et al., 2001; Shinbo et al., 1998).

The separation of isomeric compounds is generally more difficult than that of non-isomeric compounds due to their extreme structural similarity (Snyder and Dolan, 2000). In reversed-phase HPLC, the separation of isomeric compounds - like other compounds - can be improved by optimising the different effective factors such as mobile phase composition, column characteristics and column temperature, as discussed in the introduction in Section 1.6.

In general, compounds are less retained in RP-HPLC columns when using a stronger (more non-polar) mobile phase. An HPLC column packed with silica-based C18 stationary phase is a common start for a new RP-HPLC separation. The use of this type of column was found to enhance the separation of some structural isomers of PAH compounds (naphthalene and its substituents are considered among this family of compounds) (Kayillo et al., 2006). The excellent separation efficiency of PAH isomers by RP-HPLC on C18 columns contributed to the popularity of this technique for PAH separations (Poster, 1998).

Two types of C18 bonding chemistries (monomeric and polymeric) are used in preparing HPLC columns (see Section 1.6 for details). Separation characteristics of these two types of bonded phases of C18 may differ remarkably in many applications. In general, polymeric C18 columns can provide better overall separation of isomers and other mixtures of structurally similar compounds compared to monomeric C18 columns (Poster, 1998). Column temperature is another condition that plays an important role in controlling the separation in RP-HPLC separations. By selecting the right column temperature, the separation of many samples can be enhanced.

Shinbo and co-workers (1998 and 2000) used two new developed HPLC columns (packed with cyclophane bonded-silica stationary phase) for the separation of DMN isomers (10 isomers) and some other aromatic compounds (Shinbo et al., 2000; Shinbo et al., 1998). They also compared the new columns to a commercially produced C18 column and to a phenyl column. The new cyclophane columns were reported to be able to separate most of the examined substituted-naphthalene compounds better than the other two columns. They also examined the effect of temperature on the separation of alkylated naphthalenes using the new columns and compared that to the C18 column. An example was provided for the pair 2,6- and 2,7-DMN which were not separated by the ODS column at any temperature, but were well separated by the new cyclophane columns at low temperatures (<20 °C). However, these columns are not yet commercially available (T. Shinbo, personal communication).

The work in this section aimed to study the RP-HPLC separation behaviour of 1,4-DMN and some of the other related compounds under different chromatographic conditions in order to provide a good understanding of the effect of different chromatographic conditions on the separation of the studied compounds. This study was conducted using the available columns in the laboratory, and adjusting the different variables that can affect the chromatographic separation.

1,4-DMN absorbance was measured on a UV spectrophotometer to obtain the wavelength that gives the highest absorbance (λ_{max}) which was then set up as the detector wavelength throughout the method development. UV absorbance was selected in the detection of the present mixture components as it provides extremely sensitive and selective detection for such compounds (Poster, 1998).

An initial separation was conducted to obtain an overview of the retention of the mixture components. In the light of the initial separation, several experiments were conducted to optimise the separation by using three columns, and optimising the mobile phase composition and column temperature.

2.2.2 Materials and methods


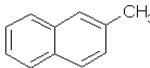
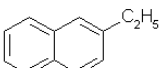
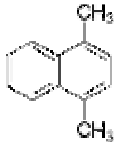

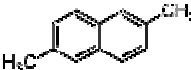
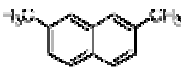
2.2.2.1 Materials and standards

The mobile phase was prepared by mixing certain volumes of acetonitrile (ACN) (HPLC grade, Fisher Scientific, UK) with deionised water to get the required concentration of

ACN (% v/v). The mixture was then degassed by sonication in an ultrasonic bath (Sonicor SC120T) for at least half an hour.

The development of HPLC separation methods for DMN isomers and the other related compounds was achieved by using a mixture of compounds listed in Table 2.1.

Table 2.1 Some characteristics of the mixture components used for the development of an HPLC method for the separation of DMN isomers and the other related naphthalene compounds

Compound Name	Abbreviation in the Thesis	Structure	Molecular Weight	Melting Point (°C)	Boiling Point (°C)
Naphthalene	Naph		128.2	80-82	218
2-methylnaphthalene	2-MeNaph		142.2	34.4	241
2-ethylnaphthalene	2-EtNaph		156.2	-7.4	258
1,4-dimethylnaphthalene	1,4-DMN		156.2	7.6	268
2,3-dimethylnaphthalene	2,3-DMN		156.2	103-104	269
2,6-dimethylnaphthalene	2,6-DMN		156.2	106-110	262
2,7-dimethylnaphthalene	2,7-DMN		156.2	94-97	263

A stock solution of each component was prepared at a concentration of 1000 µg/ml in acetonitrile. The mixture was prepared by dilution with acetonitrile to a concentration of 2 µg/ml.

2.2.2.2 Equipment

The water used to prepare the mobile phase was obtained from an Elga Purelab Option Deioniser.

The HPLC system used consisted of a Gilson 234 Auto-Injector, a Severn Analytical SA6410B solvent delivery system and a Severn Analytical SA6500 UV/Vis absorbance detector connected to a ChromJet integrator.

The characteristics of the HPLC columns used are detailed in Table 2.2.

Table 2.2 General characteristics of the HPLC columns used for the separation of DMN isomers and the other related naphthalene compounds

Column Manufacturer	Packing Material	Dimensions (mm)	Particle Size (µm)	Carbon Loading (%)	Endcapping
Jones	Spherisorb ODS-2	250 x 4.6	5	11.5	Fully
Supelco	Spherisorb ODS-2	250 x 4.6	5	11.5	Fully
Supelco	Supelcosil LC-PAH	150 x 4.6	5	High Load*	Fully

* The value was not specified in the manufacturer's website or column datasheet.

The effect of the column temperature on the separation of DMN isomers and the other naphthalene compounds has been studied by placing the column in a glass jacket full of running water from a water bath at 4 °C and 12 °C and compared with the ambient temperature.

2.2.3 Results and discussion

2.2.3.1 Initial separation of 1,4-DMN isomers and other related compounds

The starting conditions were chosen to give a quick overview of the retention behaviour of the mixture components. The following conditions were initially chosen for this purpose.

- Column: Jones Spherisorb ODS-2
- Mobile phase: 70 % acetonitrile : 30 % water
- Flow rate: 1.5 ml/min
- Injection volume: 10 µl
- Run time: 15 min

- Temperature: ambient ($\sim 20^{\circ}\text{C}$)
- Detector wavelength: 228 nm

The separation of the mixture of DMN isomers and the other related compounds under the starting chromatographic conditions is shown in Figure 2.1. This initial separation gives an idea of the retention and UV absorbance behaviour of the mixture components. 1,4-DMN has been reported to have a strong UV absorbance at a wavelength of 228 nm (Beveridge, 1979). This wavelength was also confirmed here by the spectrophotometric scanning of 1,4-DMN standard. Therefore, this wavelength was used here.

As shown in Figure 2.1, all the compounds examined had good retention in the column selected under the initial conditions. In addition, the mixture components showed good UV absorbance at the λ_{max} of 1,4-DMN, which supports the use of this wavelength in the rest of the separation method development for this mixture.

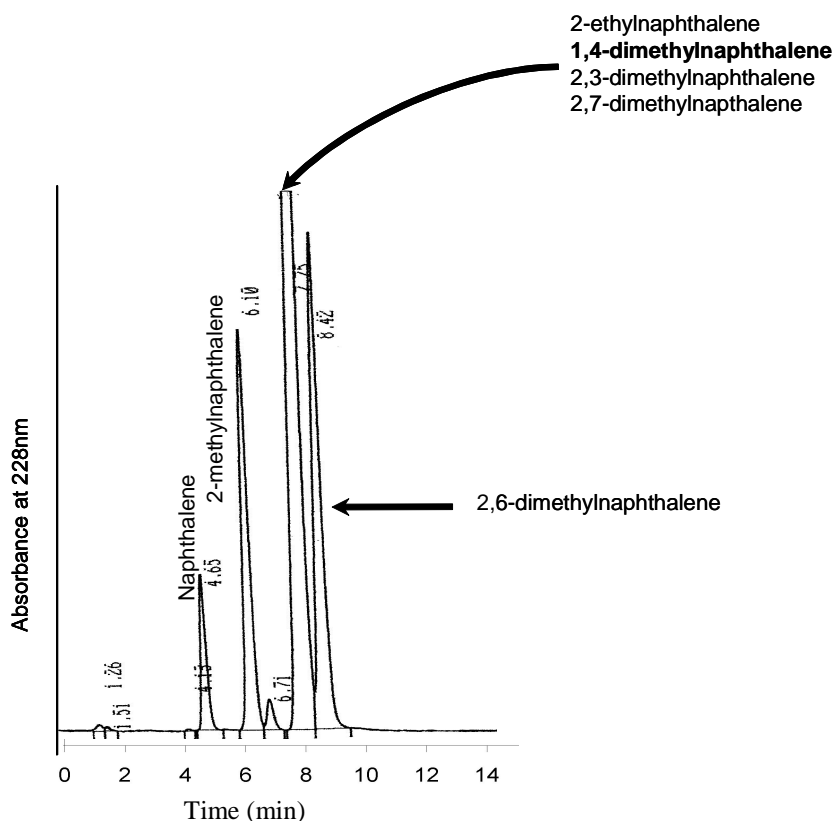


Figure 2.1. The separation of the mixture of DMN isomers and the other related compounds under the starting chromatographic conditions

Peak identification was achieved by comparing the retention times of the peaks in the mixture to those of pure standards containing a single compound and run under the same

chromatographic conditions. In addition, further confirmation was obtained by running four other mixtures in which a compound from the coeluted compounds was excluded.

The first two peaks were naphthalene and 2-methylnaphthalene respectively, and the last peak was for 2,6-DMN. The other four compounds were coeluted and appeared as a single peak at 7.75 min in the chromatogram. The four compounds were identified as 1,4-DMN; 2,3-DMN; 2,7-DMN and 2-ethylnaphthalene.

This initial separation revealed the need for optimising the chromatographic conditions to achieve better separation for the mixture components.

It is normally easier and more effective to start optimising a separation by changing the solvent strength (mobile phase composition). Therefore, several experiments were conducted to study the role of this factor in optimising the current separation.

2.2.3.2 Optimising the separation on the Jones ODS-2 column by using different solvent strengths

Using the Jones ODS-2 column, several concentrations of ACN in the mobile phase were examined. The concentrations tested were at 10 % intervals, i.e. 60 %, 50 % and 40 % ACN mixtures in water. Flow rate was 1.5 ml/min in all of these runs, except at 40 % where 2.0 ml/min was used to speed up the separation in addition to providing less broadened peaks at the higher flow rate.

At 60 % and 50 %, resolution increased only between the peaks that were already separated at 70 %, but no improvement was achieved in separating the coeluted peaks of the four compounds 1,4-DMN; 2,3-DMN; 2,7-DMN and 2-ethylnaphthalene.

The coeluted peaks started to separate at 40 % ACN/water, as shown in the chromatogram in Figure 2.2.

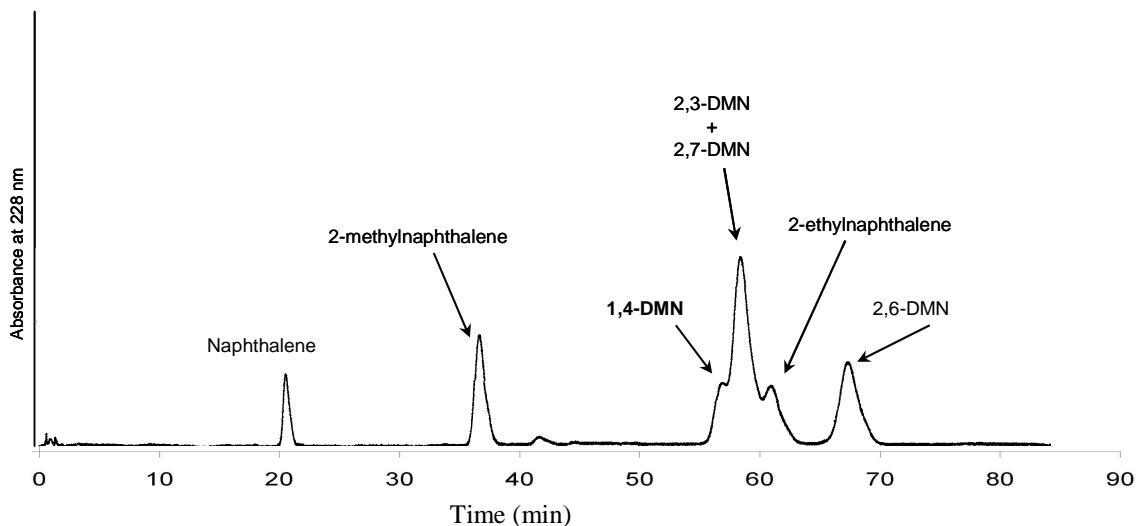


Figure 2.2. The separation of the mixture of DMN isomers and the other related compounds on Jones ODS-2 column using 40 % ACN/water at 2.0 ml/min and ambient temperature (~20 °C)

However, this degree of separation of the four compounds was not sufficiently encouraging to continue using this column. The low separation efficiency of this column was thought to be due to the age of the column, which had been used for a long time prior to this separation. Therefore, the Jones column was replaced with a brand new and more modern Supelco Spherisorb ODS-2 column.

2.2.3.3 Optimising the separation on Supelco ODS-2 column by using different solvent strengths

This column was packed with a Spherisorb ODS-2 stationary phase and had a length of 250 mm. The separation optimisation on this column started with optimising the mobile phase strength. The same conditions used with Jones ODS-2 were repeated here to compare the efficiency of this column in separating the mixture compounds. Mobile phase compositions of 70 %, 60 %, 50 % and 40 % ACN/water were examined.

The separation profiles of the mixture components on this column at 70 % and 60 % ACN/water were similar to the ones obtained by using the Jones column. However, slightly narrower and more symmetrical peaks were obtained on the Supelco column. It was considered that this improvement in peak shape provided the basis for enhancing the separation of the coeluted peaks, particularly at lower concentrations of ACN in the mobile phase at which the peaks are normally broader.

At 50 % ACN/water, the 2-ethylnaphthalene peak started to be resolved from the other three compounds that appeared as a single peak. This was a clear improvement in the

separation at this concentration of ACN compared to the Jones ODS-2 column. The separation of the mixture component at 50 % ACN is shown in Figure 2.3.

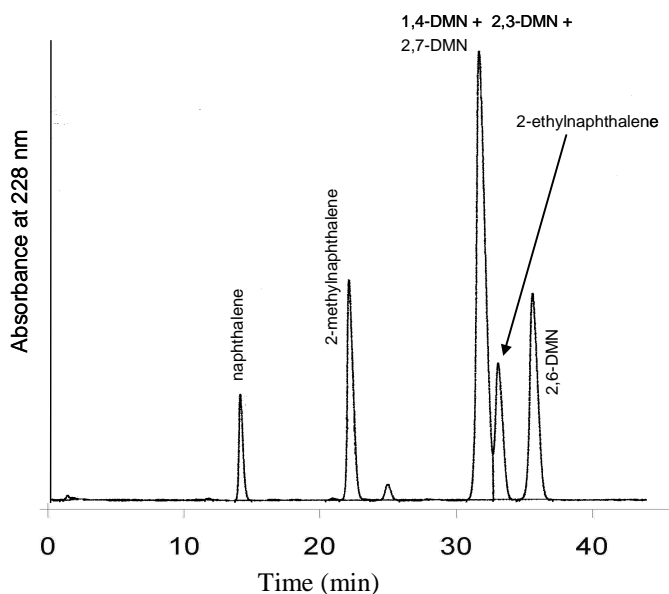


Figure 2.3. The separation of the mixture components on Supelco ODS-2 at 50 % ACN/water with a flow rate of 1.5 ml/min and at ambient temperature

The best separation of the mixture components on this column among the conditions examined was achieved at 40 % ACN/water with a flow rate of 2.0 ml/min and ambient temperature. This separation is shown in the chromatogram in Figure 2.4.

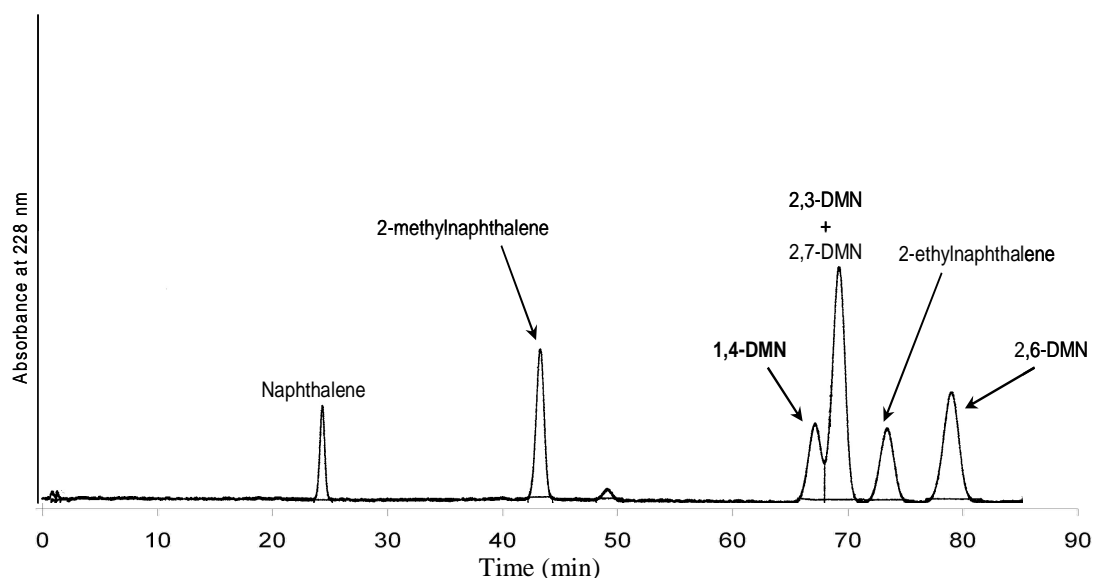


Figure 2.4. The best separation of the mixture components on Supelco ODS-2 column. Conditions: 40 % ACN/water, 2.0 ml/min and ambient temperature

Four compounds out of the seven comprising the mixture were completely resolved. In addition, 1,4-DMN was partially separated. The only coelution occurred for 2,3-DMN and

2,7-DMN, which might be related to the very close physicochemical properties of the two isomers.

By comparing the chromatograms in Figure 2.2 and Figure 2.4, it can be clearly noticed that the peaks produced by Supelco ODS-2 column are narrower and less tailed. This better peak shape contributed to enhancing the separation of 1,4-DMN and 2-ethylnaphthalene from the other coeluted DMN isomers. In addition, the higher retention of the mixture components on the Supelco ODS-2 column gave more time for the mixture components to be separated. Tailed peaks and a decrease in retention are commonly noticed with heavily used columns and are signs of column degradation (Snyder et al., 1997). This seems to be the case with the Jones column.

The main drawback of the separation shown in Figure 2.4 is the long run time required for the separation. This was expected from a column with this length at the low solvent strength used. However, this should not be a problem for a method which is not designed for routine analysis.

In a search for shorter run times with good separation in addition to comparison of more columns, the LC-PAH column was selected and similar experiments conducted.

2.2.3.4 Optimising the separation on Supelco Supelcosil LC-PAH column by using different solvent strengths

The third column chosen to be used to optimise the separation of DMN isomers and the other related naphthalene compounds was a Supelco Supelcosil LC-PAH (5 μm particle size, 150 mm x 4.6 mm). This column was designed specifically for the separation of polycyclic aromatic hydrocarbons, which include the compounds in the current mixture. The high carbon load of this column should provide more efficiency in the separation of such mixtures.

The separation optimisation on this column started with optimising the mobile phase strength. The same conditions used with the Jones ODS-2 and the Supelco ODS-2 were repeated here to compare the efficiency of this column in separating the mixture compounds. Mobile phase compositions of 70 %, 60 %, 50 % and 40 % ACN/water were examined.

The mixture components eluted faster in the LC-PAH column than in the previous two columns. This was to be expected, as the length of the LC-PAH column was only 150 mm.

At 70 % all the mixture components were eluted in less than 5 minutes on the LC-PAH column, but with four compounds coeluted.

A total of only four peaks appeared in the chromatogram at 60 % ACN/water, which indicated a coelution of four compounds in a single peak. However, despite the shorter length of this column - which was expected to cause lower resolution of the mixture components - the four coeluted compounds started to show a sign of separation at ACN concentration as high as 60 %. The shape of the peak representing the four coeluted compounds appeared to have a shoulder to the left. By lowering the ACN concentration in the mobile phase to 50 %, this shoulder was further separated as another peak which had a shoulder as well. The new peak was identified as 1,4-DMN and 2-ethylnaphthalene, which was separated from the peak representing 2,7-DMN and 2,3-DMN. The chromatogram in Figure 2.5 shows the separation of the mixture at 50 % ACN/water.

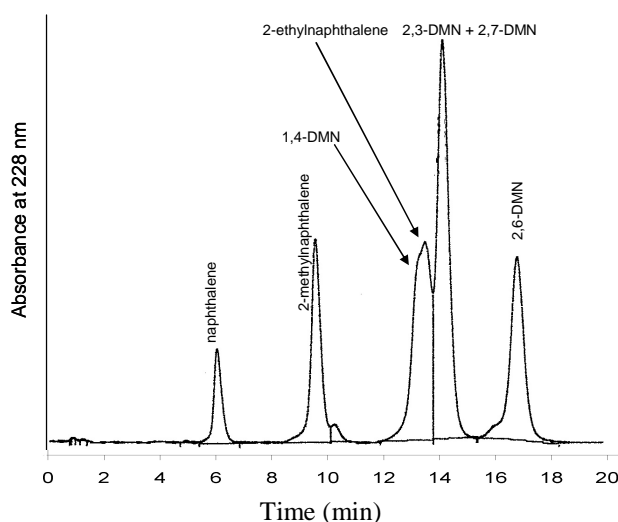


Figure 2.5. The separation of the mixture components on Supelco Supelcosil LC-PAH at 50 % ACN/water with a flow rate of 1.5 ml/min and at ambient temperature (~20 °C)

At a mobile phase concentration of 40 % ACN/water, 1,4-DMN had better separation as a single peak from the rest of the three other compounds which formed a peak with a shoulder corresponding to 2-ethylnaphthalene. This separation is shown in Figure 2.6.

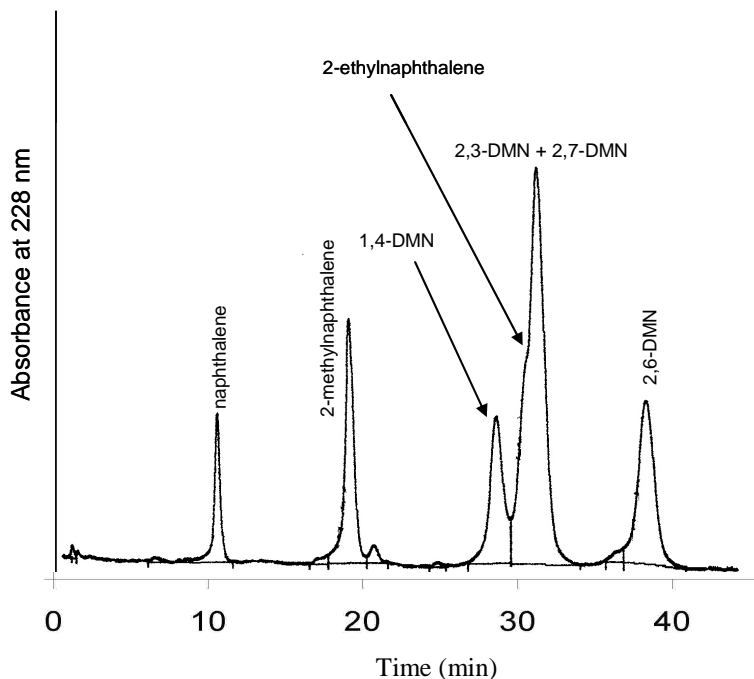


Figure 2.6. The separation of the mixture components on Supelco Supelcosil LC-PAH at 40 % ACN/water with a flow rate of 2.0 ml/min and at ambient temperature ($\sim 20^{\circ}\text{C}$)

Despite the shorter length of the LC-PAH column, it showed high separation efficiency in the separation of the current mixture of naphthalenes. This high efficiency could be attributed to the special design of its C18 bonded phase in addition to its high carbon load. The Supelcosil LC-PAH packing material was characterised as having a polymeric phase, which has better efficiency for the separation of PAH isomers and other mixtures of structurally similar compounds (Poster, 1998).

Because of the promising results obtained with the LC-PAH column, it was decided to use it to study the effect of column temperature on the separation of the current mixture. In addition, the short length of this column makes it suitable for examining the separation at lower temperatures, which are accompanied by a decrease in the mobile phase viscosity and therefore an increased system backpressure.

2.2.3.5 Temperature effect on optimising the separation on Supelco LC-PAH column

The effect of the column temperature on the separation of DMN isomers and the other naphthalene compounds was studied by placing the column in a glass jacket full of running water from a water bath at 4°C and 12°C and compared with the ambient temperature.

As noted from studying the effect of solvent strength on the LC-PAH, mobile compositions of 50 % and 40 % ACN/water provided promising results for the separation of the mixture

components. Therefore, these two concentrations were selected to study the effect of column temperature on the separation of the mixture components.

There was a significant effect of column temperature in separating the mixture of DMN isomers and the other related compounds. This effect can be clearly noticed by comparing the chromatogram in Figure 2.7 to that in Figure 2.5.

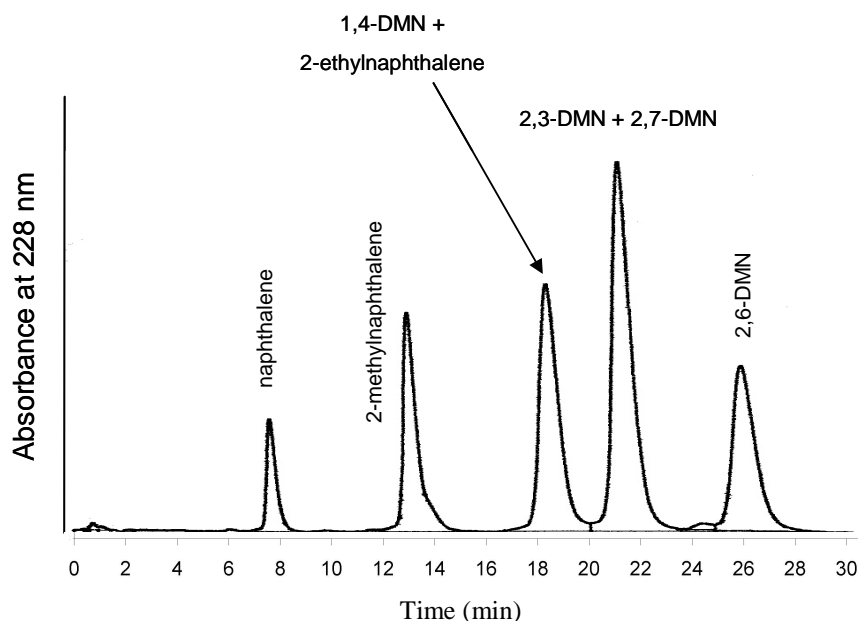


Figure 2.7. The separation of the mixture component on Supelco Supelcosil LC-PAH at 50 % ACN/water with a flow rate of 1.5 ml/min and at 4 °C

Both chromatograms were obtained at 50 % ACN/water and 1.5 ml/min. The only difference between the conditions used was the column temperature. Column temperature was about 20 °C in the separation in Figure 2.5 where it was lowered to about 4 °C for the separation in Figure 2.7. By lowering the column temperature to 4 °C, the separation of 1,4-DMN from the other DMN isomers was enhanced. However, 2-ethylnaphthalene formed a single peak with 1,4-DMN as a result of lowering the temperature. The single peak of the two compounds was completely resolved from the single peak representing 2,7-DMN and 2,3-DMN as a result of the low column temperature.

Sub-ambient temperatures have been found to enhance the separation of complex isomer mixtures of PAHs using various C18 columns (Sander and Wise, 1989). Shinbo and co-workers have also achieved better separation of DMN isomers at sub-ambient temperatures on their laboratory-prepared columns with cyclophane-bonded stationary phases (Shinbo et al., 2000; Shinbo et al., 1998).

Because 2-ethylnaphthalene had some separation from the 1,4-DMN peak in Figure 2.5 at 20 °C whereas the two are completely coeluted at 4 °C, a temperature between the two was examined to try to optimise the separation of 2-ethylnaphthalene. It was thought that such a temperature may cause 2-ethylnaphthalene to elute in the middle between 1,4-DMN peak and the peak representing 2,7-DMN and 2,3-DMN. Column temperature was adjusted to 12 °C with all of the other conditions fixed. However, there was no significant improvement in the separation of the mixture components. 2-ethylnaphthalene still completely coeluted with 1,4-DMN, and 2,7-DMN and 2,3-DMN still forming a single peak in the chromatogram.

It was then decided to investigate the effect of column temperature on the separation of the isomers using a mobile phase consisting of 40 % ACN/water. The chromatogram in Figure 2.8 shows the separation of the mixture components at 40 % ACN/water with the column temperature set at 4 °C.

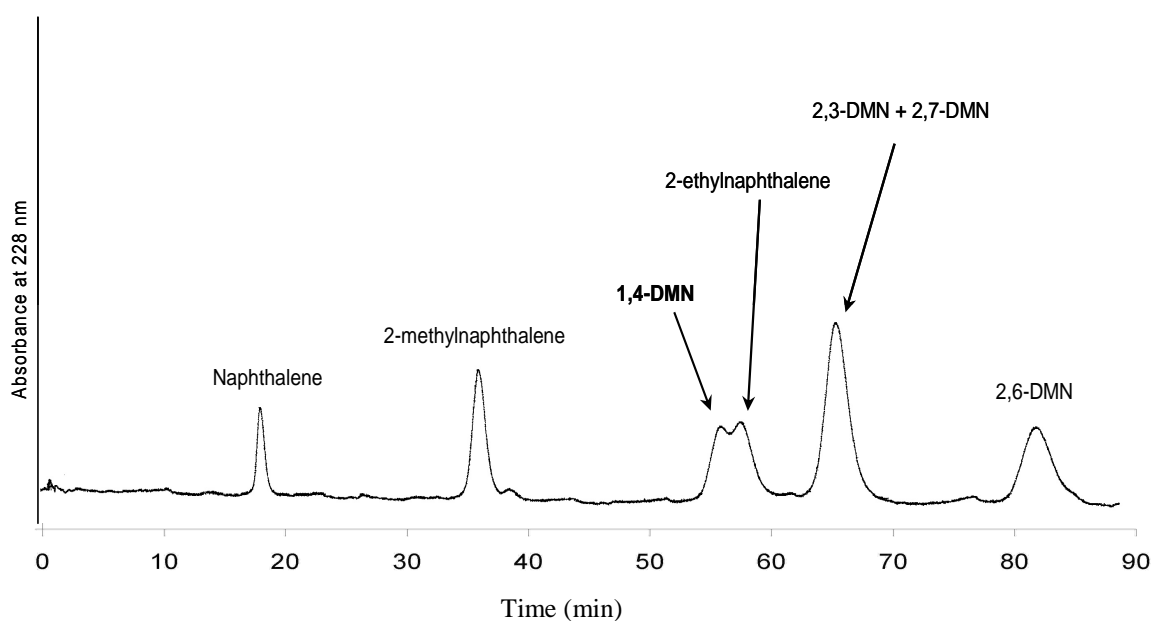


Figure 2.8. The separation of the mixture component on Supelco Supelcosil LC-PAH at 40 % ACN/water with a flow rate of 1.5 ml/min and at 4 °C

Using these conditions, 2-EtNaph started to separate from 1,4-DMN showing a slightly longer retention time. In comparison to Figure 2.7, this slight separation between the two peaks was attributed to lowering the solvent strength, as it was the only condition which was changed.

The separation was then examined at 12 °C in a trial to enhance the resolution between 2-EtNaph and 1,4-DMN. This separation is shown in the chromatogram in Figure 2.9.

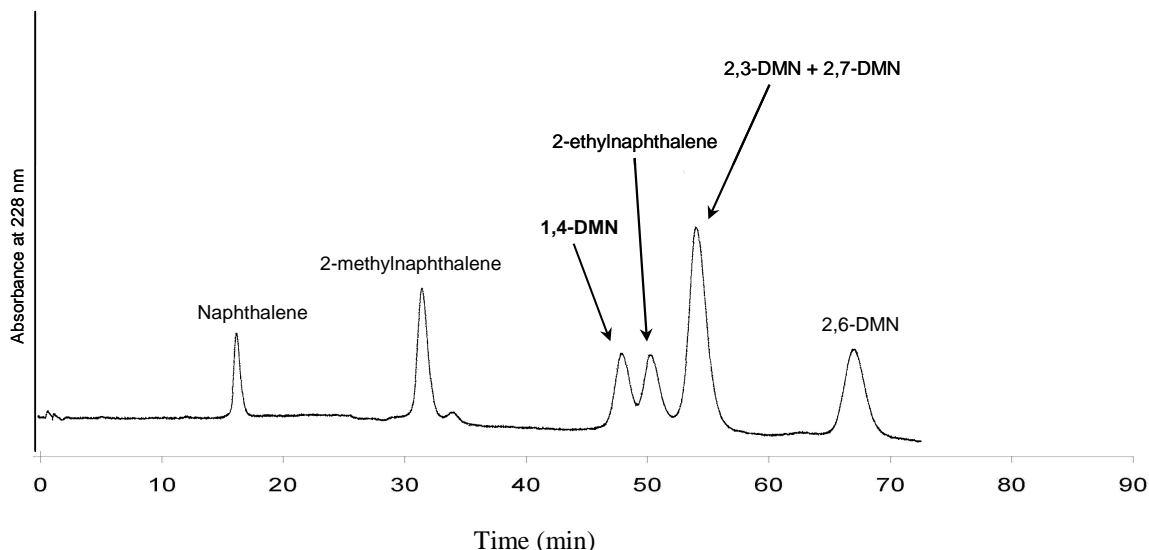


Figure 2.9. The separation of the mixture component on Supelco Supelcosil LC-PAH at 40 % ACN/water with a flow rate of 1.5 ml/min and at 12 °C

Using these conditions, both 2-EtNaph and 1,4-DMN were better resolved from each other and from the peak of 2,7-DMN and 2,3-DMN. These conditions of mobile phase composition, temperature and flow rate were the optimum practical conditions achieved for separating the mixture components on this column.

2.2.3.6 Summary and application of the best separation method

The Jones ODS-2 column used gave insufficient separation of the DMN isomers and the other related naphthalene compounds. It produced fairly broad and tailed peaks, particularly at low solvent strengths. The age and possible degradation of the column seemed to be responsible for this low separation efficiency of the mixture.

In contrast, the Supelco ODS-2 column provided narrower and more symmetrical peaks, even at low concentrations of ACN in the mobile phase. These advantages contributed to producing a good separation of the mixture components. The best separation on this column was achieved at the conditions used to generate the chromatogram in Figure 2.4, as follows:

- Column: Supelco Spherisorb ODS-2 (250 x 4.6 mm)
- Mobile phase: 40 % acetonitrile : 60 % water
- Flow rate: 2.0 ml/min
- Injection volume: 10 μ l

- Run time: 85 min
- Temperature: ambient (~20 °C)
- Detector wavelength: 228 nm

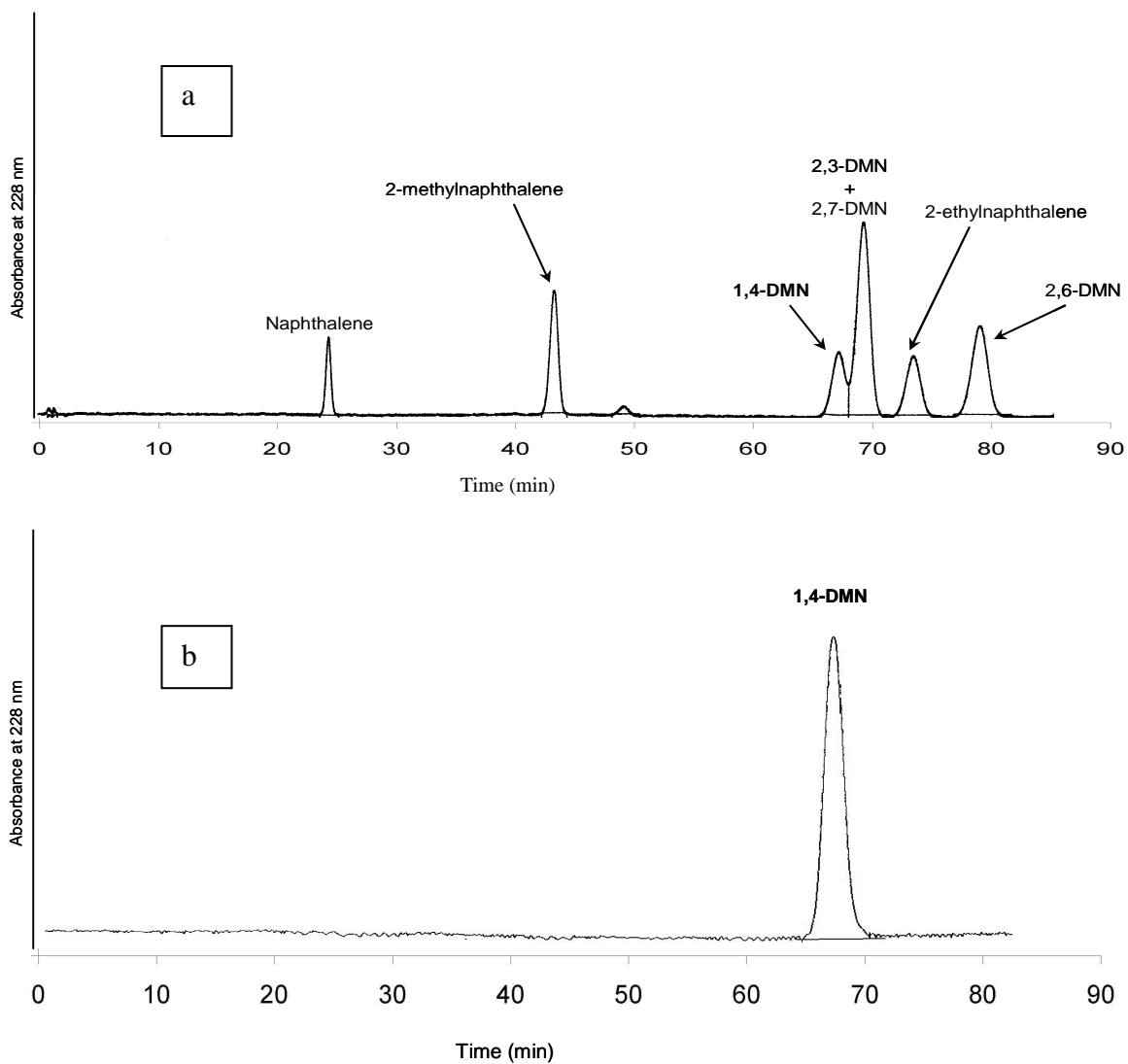
The Supelco Supelcosil LC-PAH column is specifically designed for PAH compounds, which includes the present mixture components. This column has the advantage of being short, which should reduce the separation time. However, longer columns with the same specifications should provide better separation as they will allow more time for the separation to be developed. By optimising the mobile phase compositions and column temperature, the LC-PAH column provided very good separation of the mixture components, as shown in Figure 2.9.

For the best separation of the mixture components, two combinations might be used. The first is the use of Supelco ODS-2 column with 40 % ACN/water at a flow rate of 2.0 ml/min and at ambient column temperature. The separation was achieved in about 85 minutes. The other alternative is the use of Supelco Supelcosil LC-PAH with 40 % ACN/water at 1.5 ml/min and at column temperature of 12 °C. The separation time using these conditions was about 75 minutes. Both combinations provided a good separation of the mixture components, with only two isomers coeluted. However, the optimum separation achieved on the Supelco ODS-2 column was at ambient temperature without any need for column temperature control, which reduces the effort and time needed for setting up the right temperature. These savings in time and effort may overcome the 10 min difference in the separation time between this column and the LC-PAH column, particularly when bearing in mind that this separation was not designed for a routine method.

The separation method using Supelco ODS-2 column was applied to examine the presence of any of DMN isomer or any of the other naphthalenes examined in a commercial product of 1,4-DMN sprout inhibitor called 1,4-Ship[®], produced and used in the United States. In addition, a potato extract (extracted based on the Soxhlet method used in our laboratory which is described in Section 1.8) of tubers treated with the commercial 1,4-DMN sprout inhibitor was also examined.

Figure 2.10.a-d show chromatograms of the mixture of 1,4-DMN isomers and the other naphthalenes (a), a 1,4-DMN standard in ACN (b), a solution of a commercial product of

1,4-DMN sprout inhibitor (1,4-Ship[®]) diluted appropriately in ACN (c), and a representative chromatogram of an extract of potato peel (in hexane) for tubers treated with the commercial sprout inhibitor 1,4-DMN (d). The chromatograms show no presence of any of the mixture components used in developing the separation method other than 1,4-DMN. This should help in the further development of a routine method for the analysis of 1,4-DMN residues in potato extracts by considering only the isomer 1,4-DMN.



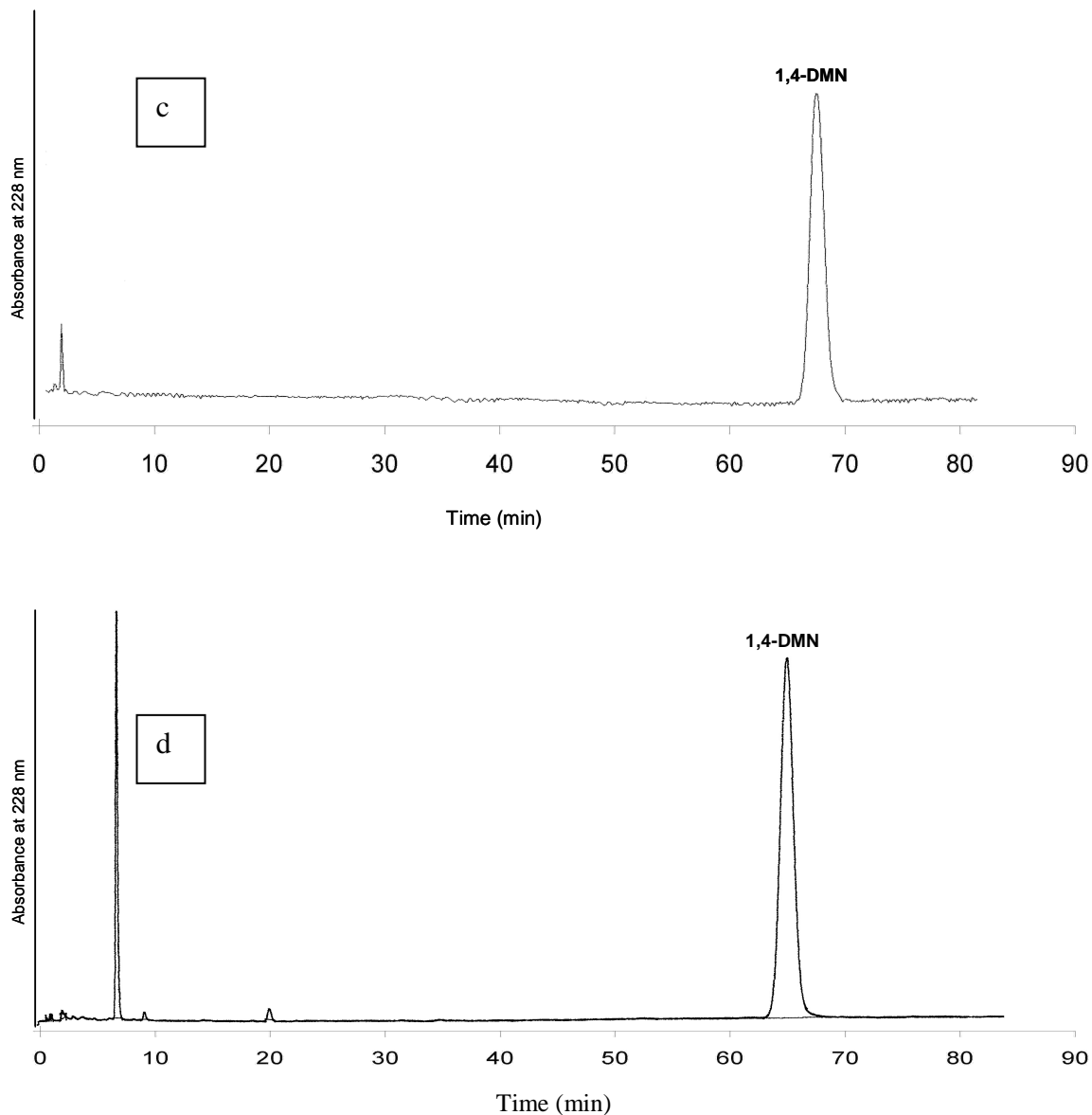


Figure 2.10. Chromatograms obtained by the best separation method for DMN isomers and the other related compounds a) A mixed standard of the DMN isomers and the other naphthalene compounds. b) A 1,4-DMN standard in ACN. c) a diluted solution of a commercial product of 1,4-DMN sprout inhibitor (1,4-Ship) in ACN. d) A representative chromatogram of an extract of potato peel (in hexane) for tubers treated with 1,4-DMN. Chromatographic conditions: Supelco Spherisorb ODS-2 (250 x 4.6 mm), 40 % ACN/water, 2.0 ml/min, 10 μ l injection volume, 85 min run time, ambient temperature and detector wavelength of 228 nm

2.3 HPLC separation method for routine analysis of 1,4-dimethylnaphthalene residues in potato samples

2.3.1 Introduction

The analysis of 1,4-DMN in potato samples is carried out largely by gas chromatographic methods (Beveridge, 1979; Boylston et al., 2001; O'Hagan, 1991; Knowles et al., 2005). In many of these methods, a lengthy sample preparation is normally required. The extracts containing 1,4-DMN need a multi-step clean up procedure in addition to concentrating the final extract by evaporating most of the organic solvent. During this step, some loss of 1,4-DMN has been reported (Beveridge, 1979).

Very limited information is available for methods which apply HPLC for the determination of 1,4-DMN residues in potato samples. An HPLC method for this purpose was reported by O'Hagan (1991). The method was a modification of a previous method developed for the analysis of the herbicide trifluralin [2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl) benzenamine]. The method modified by O'Hagan (1991) includes a lengthy extraction and clean up procedure prior to the analysis by HPLC. A RP-HPLC separation method was used for the determination of 1,4-DMN in the final extract. The separation was achieved on a C18 column with the mobile phase composition of methanol/water (70:30) mixed with acetic acid. The flow rate was 2.0 ml/min and the detection wavelength was 230 nm. The use of acetic acid was thought to enhance the separation of 1,4-DMN. However, there was no reason reported for this. Acetic acid may affect the separation of a chemical such as trifluralin which has some functional groups that might be affected by the pH of the mobile phase. However, no such functional groups are present in 1,4-DMN and therefore, the use of acetic acid might not be appropriate.

In Section 2.2, valuable information was obtained for the RP-HPLC separation of 1,4-DMN and the other naphthalene compounds examined. 1,4-DMN was found to be retained well on C18 columns. The Supelco ODS-2 column was found to provide good peak shapes. This column in conjunction with the initial conditions (see Section 2.2.3.1) was selected for the development of the routine separation method for 1,4-DMN residues in potatoes.

The HPLC separation method reported in this work aimed to provide a simple, fast, accurate and precise separation for the routine analysis of 1,4-DMN residues in potato extracts.

2.3.2 Materials and methods

2.3.2.1 HPLC equipment and chromatographic conditions

The same HPLC equipment described in Section 2.2.2.2 was used here. Chromatographic conditions were set as follows:

- Column: Supelco Spherisorb ODS-2 (5 μ m particle size, 250 mm x 4.6 mm)
- Mobile phase: 70 % acetonitrile : 30 % water
- Flow rate: 1.5 ml/min
- Injection volume: 10 μ l
- Run time: 10 min
- 1,4-DMN retention time : ~8 min
- Temperature: ambient
- Detector wavelength: 228 nm

2.3.2.2 Stock solution and standards

A stock solution of 1,4-DMN was prepared at a concentration of 1000 μ g/ml in acetonitrile. The rest of the standards were prepared by diluting the stock solution with acetonitrile to the required concentration. All solutions were stored at 4 °C.

2.3.2.3 System precision

The system precision was examined by analysing replicate injections (n=7) of 1,4-DMN standards at the lowest concentration examined (0.02 μ g/ml) and at 50 μ g/ml 1,4-DMN.

The relative standard deviation (%RSD) was calculated for each level as follows:

$$\%RSD = 100 \text{ Standard deviation of peak areas} / \text{Mean peak area}$$

2.3.2.4 Linearity assessment

Linearity was tested in two ranges, with five concentration levels for the low range and 15 levels for the high range. The lower range was 0.2 - 1.0 µg/ml and the higher range was 20 - 300 µg/ml. Two series of calibration standard solutions were prepared for this test. Aliquots of these solutions (10 µl) were injected into the HPLC system in triplicate and the detector response (peak area) plotted against the actual concentration to generate the calibration graphs. The linearity of the graph was statistically assessed using Excel[®] software.

2.3.2.5 Limit of detection

The limit of detection (LOD) of the separation method was calculated statistically based on the data from the lower range calibration, following a method described by Miller and Miller (2005) and summarised in the introduction in Section 1.7.5. The limit of detection is defined, based on this method, as the concentration of the analyte that gives a peak area significantly different from the blank or the background peak area. The calibration curve at low levels of 1,4-DMN was used to statistically estimate the 1,4-DMN peak area that was significantly different from the blank peak area. The equation used to estimate this peak area was:

$$\text{LOD peak area} = y_B + 3 s_B$$

Where y_B is the blank peak area and s_B is the standard deviation of the blank peak area. In practice, the value of y_B and s_B can be estimated from the calibration graph at the lower range by using values from the regression analysis of variance. Therefore, the value of y_B was replaced by the intercept value of the regression equation of the calibration graph of 1,4-DMN at the lower range of 0.2 - 1.0 µg/ml. Similarly, the term s_B was replaced by the residuals standard deviation, which is the square root of the error mean square. Both terms were estimated using the regression function in Excel[®] software. The concentration of the analyte corresponding to this peak area was the estimated LOD of the separation method.

The estimated value of LOD was examined experimentally by replicate injections of a standard containing the 1,4-DMN at that value.

2.3.2.6 Assay accuracy

The accuracy of this HPLC separation method was examined by comparing the values produced by the HPLC method of 1,4-DMN in potato extracts to those obtained by the

standard method used in our laboratory which apply gas chromatography. The extracts used here were in hexane and prepared by Soxhlet extraction of potato tubers treated with 1,4-DMN. A brief description of the Soxhlet method used for preparing these samples is found in Section 1.8.

Twenty two samples were analysed by both methods and the results were plotted and statistically evaluated following the method described by Miller and Miller (2005) as detailed in the discussion below.

2.3.3 Results and discussion

2.3.3.1 Chromatographic conditions

This method was designed for the routine quantitative determination of 1,4-DMN in environmental samples, in which the speed, accuracy and precision are the most important elements.

The Supelco Spherisorb ODS-2 column was chosen for the routine method due to its good performance shown in Section 2.2. It has been shown to provide narrow and symmetrical peaks which help to provide better resolution for mixture components. The importance of high column efficiency is apparent for separating 1,4-DMN in potato extracts which, may contain a variety of other naturally interfering compounds.

The mobile phase composition was chosen to be 70 % ACN/water with flow rate of 1.5 ml/min, as it has been shown (in combination with the other conditions) to provide a good enough peak shape with a fast elution of 1,4-DMN, which is required in a routine method. In addition, the use of partial loop filling with only 10 μ l of injection volume should contribute to speeding up the separation method by reducing the injection time cycle.

The detection wavelength was set at 228 nm which is the wavelength that gives the highest absorbance of the UV light by 1,4-DMN molecules (λ_{max}). This should enhance the sensitivity of the method, particularly at low concentrations of 1,4-DMN. This wavelength was used by Beveridge (1979) in a proposed spectrophotometric method for the detection and quantification of 1,4-DMN. It was reported that 1,4-DMN has a strong absorbance of UV light at this wavelength which enabled the detection of the compound at low levels of 0.1 μ g/ml in the solution.

2.3.3.2 System precision

The first step in validating the separation method was to examine the suitability of the HPLC instrument to generate results with sufficient precision. System precision was examined by analysing replicate injections ($n=7$) of the 1,4-DMN standards at the limit of detection (LOD) (see Section 2.3.3.4) of $0.02 \mu\text{g/ml}$ and at $50 \mu\text{g/ml}$ 1,4-DMN. The precision was expressed by the relative standard deviation (%RSD) of peak areas in the replicate injections. %RSD value was 3.45 % at $0.02 \mu\text{g/ml}$, which is good at this very low concentration of 1,4-DMN. Better precision was achieved at higher concentrations as indicated by the %RSD at $50 \mu\text{g/ml}$ 1,4-DMN with a value of only 0.37 %. These values demonstrate that the method is able to provide precise and consistent results.

2.3.3.3 Linearity assessment

The linearity was tested to verify a proportional relationship of the detector response to 1,4-DMN concentrations. Figure 2.11 and 2.12 show this relationship for the low range ($0.2 - 1.0 \mu\text{g/ml}$) and the high range ($20 - 300 \mu\text{g/ml}$) of 1,4-DMN concentrations respectively.

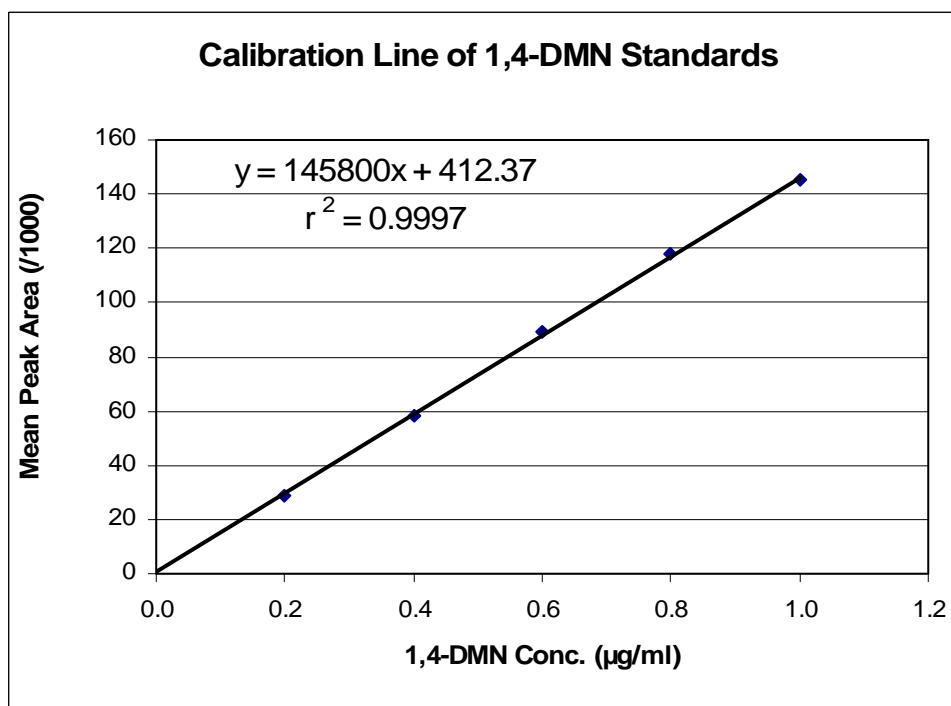


Figure 2.11. Calibration graph for the low range ($0.2 - 1.0 \mu\text{g/ml}$) of 1,4-DMN

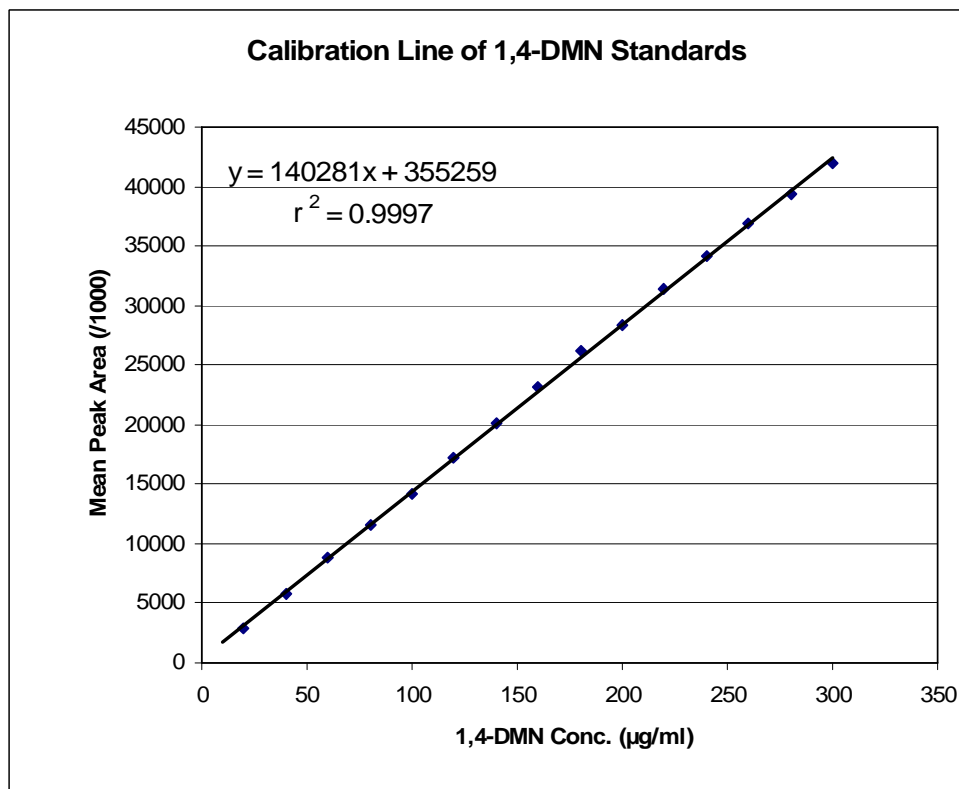


Figure 2.12. Calibration graph for the high range (20 – 300 µg/ml) of 1,4-DMN

The equations of the regression lines for the two ranges are shown in the calibration graphs. The correlation coefficients obtained for the two regression lines ($r^2 > 0.999$) demonstrate the excellent linear relationship between peak areas and 1,4-DMN concentrations. Generally, a value of $r^2 > 0.998$ is considered to be evidence of acceptable fit of the data to the regression line (Shabir et al., 2007). The y-intercepts of the regression lines in the two graphs are less than 2 % of the middle of the range in each graph. These values fit well in the acceptable range for the intercept of calibration lines, which should not exceed a few percent of the response obtained for the analyte at the target level (Shabir et al., 2007; Bruce et al., 1998). The wide range of linearity examined here provides high flexibility in developing methods for various applications of 1,4-DMN analyses.

2.3.3.4 Limit of detection

The statistical method described by Miller and Miller (2005) for estimating the limit of detection of analytical methods based on the calibration curves was used here. The calibration curve in the range of 0.2 - 1.0 µg/ml of 1,4-DMN was used here. An Excel[®] spreadsheet with the aid of the regression function was used to estimate the required statistical values shown in Table 2.3.

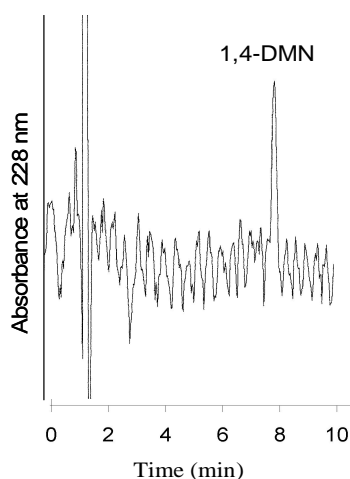
Table 2.3 Summary of the statistical values used in the estimation of the limit of detection of the routine HPLC separation method

Statistic	Value
Slope	145800
Intercept (estimation for y_B)	412
Residuals standard deviation (estimation for s_B)	1234
Peak area for LOD	4115
LOD ($\mu\text{g/ml}$)	0.02

The slope and the intercept of the calibration curve at the low range were used to replace the two terms in the equation:

$$\text{LOD peak area} = y_B + 3 s_B$$

The peak area corresponding to the LOD was then calculated using the above equation and finally the LOD as a concentration ($\mu\text{g/ml}$) was calculated from the calibration line equation. As shown in Table 2.3, a concentration of 1,4-DMN of 0.02 $\mu\text{g/ml}$ was estimated as the LOD. This value was examined experimentally by replicate injections of a standard containing 0.02 $\mu\text{g/ml}$ of 1,4-DMN in ACN, as described in the system precision sections. A representative chromatogram is shown in Figure 2.13.

**Figure 2.13. A representative chromatogram of 1,4-DMN standard at the LOD of 0.02 $\mu\text{g/ml}$ analysed according to the conditions of the routine method**

The chromatogram in Figure 2.13 shows a 1,4-DMN peak that can be clearly distinguished from the background noise, which agrees with the definition of the limit of detection.

2.3.3.5 Assay accuracy

Several procedures are reported in literature for the assessment of method accuracy as detailed in the introduction in Section 1.7.3. One of these methods is based on comparing the results of the proposed method to those of a second reputable method which is widely accepted (Jenke, 1998; Miller and Miller, 2005). By making this comparison, analysts aim to identify any systematic error that might be produced by the new method. The values obtained by the two methods are plotted in a regression graph with one axis used for the results obtained from the new method, and the other axis for the results from the other method. The use of this regression method in comparing analytical methods is widely used due to the valuable information it provides about the nature of any differences between the two analytical methods. By evaluating the slope and intercept of the regression line, the analyst can obtain valuable information about the nature of any systematic errors between the two methods. In addition, the value of the correlation coefficient (r^2) for the regression line generated to compare the two methods can provide valuable information of the precision of the two analytical methods. The ideal situation is when the two methods produce identical results. In this model situation the regression graph will have a line with a slope of 1, a correlation coefficient of 1 and an intercept of zero. However, this never happens in practice due to the various errors - random or possibly systematic - associated with analytical procedures. Therefore, statistics can be applied to evaluate the significance of the deviation of the regression line from the ideal situation (Miller and Miller, 2005). The precision of each of the two analytical methods can be evaluated as described in Section 2.3.3.2. The significance of the deviation of the slope and the intercept of the regression line from the ideal values can be evaluated by determining confidence limits for the slope and the intercept, generally at the 95 % level (Miller and Miller, 2005).

For the current comparison of the HPLC and GC methods, Excel[®] was applied to plot the regression line of the data obtained for the twenty two samples, and also to calculate the required statistical values through the regression function available in the software.

The comparison of the two methods is illustrated in Figure 2.14.

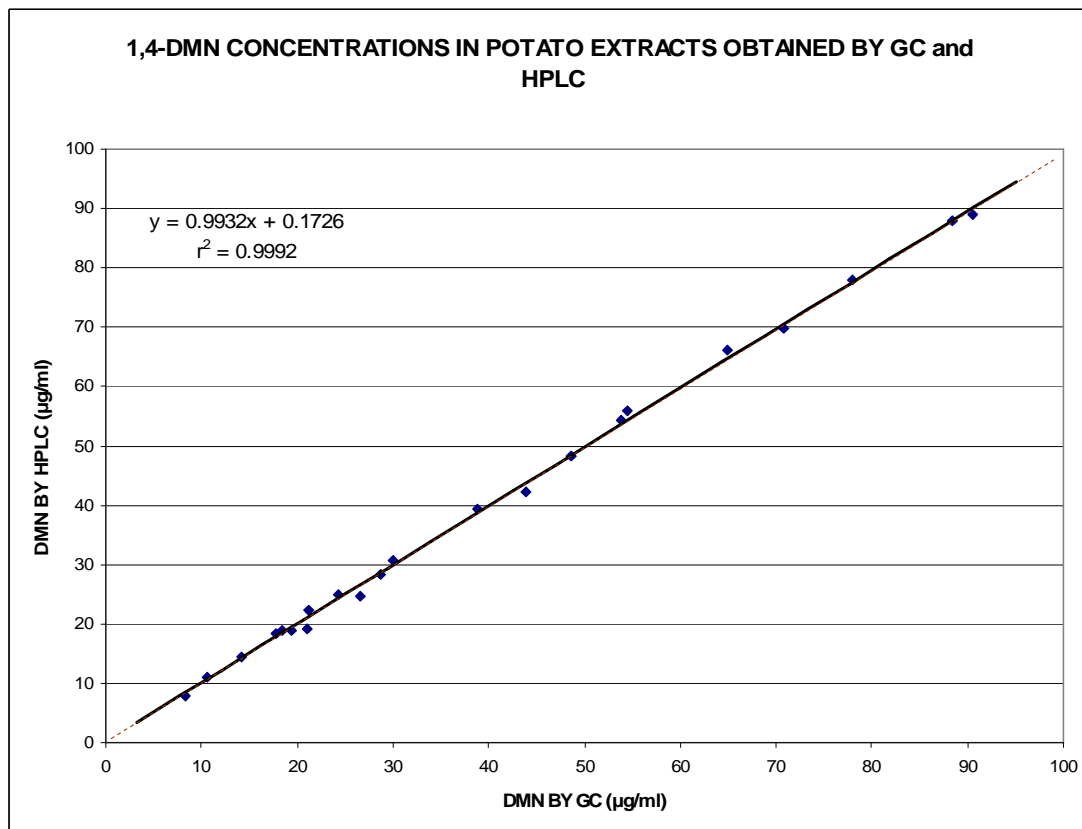


Figure 2.14. Comparison of 1,4-DMN values in potato extracts obtained by HPLC routine method and by GC method. The solid line shows the regression line (best fit) and the dashed one represents the equality line

The graph in Figure 2.14 shows the regression line of 1,4-DMN concentrations obtained by the two analytical techniques in 22 samples. The set of data covered a wide range of 1,4-DMN concentrations between less than 10 µg/ml to above 90 µg/ml of 1,4-DMN in potato extracts. The HPLC data were plotted on the y-axis and the GC results on the x-axis. The regression line shows a very good agreement between the two methods. This agreement was described well by evaluating the statistical values obtained from the Excel[®] output which are summarised in Table 2.4.

Table 2.4. Summary of the statistical values obtained from Excel[®] for the regression line of the comparison between HPLC and GC methods

Statistic	Value
Correlation Coefficient (r^2)	0.9992
Intercept	0.1726
Intercept 95 % Confidence Interval	-0.6727 to 1.0179
Slope	0.9932
Slope 95 % Confidence Interval	0.9752 to 1.0112

The high correlation ($r^2 > 0.999$) between the data points in the graph indicates the high precision of the two methods. The 95 % confidence interval of the intercept and the slope of the regression line includes the ideal values of 0 and 1 respectively. These statistical values confirm that there is no significant difference between the two analytical methods and therefore illustrates the accuracy of the routine HPLC separation method.

Despite the good agreement in quantification, differences were noticed in the quality of the GC chromatograms compared to the HPLC chromatograms, as shown in Figure 2.15 for the same potato extract sample analysed by both methods.

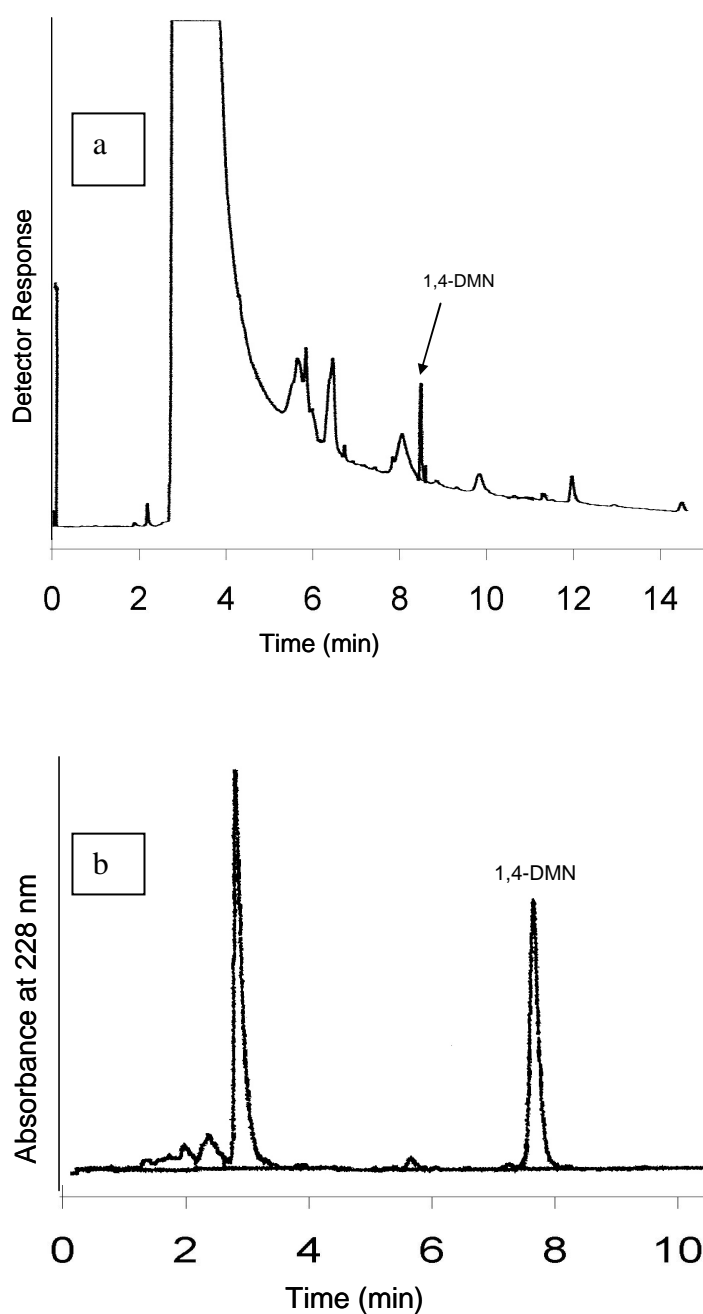


Figure 2.15. An extract (in hexane) of potato sample treated with 1,4-DMN analysed by two methods. a) GC chromatogram. b) HPLC chromatogram.

Figure 2.15.a shows a GC chromatogram for an extract of potato sample treated with 1,4-DMN. The analysis time was 15 minutes with 1,4-DMN eluted at 8.6 minute. The chromatogram has several other peaks before and after the 1,4-DMN peak including a large solvent peak (and possibly some other volatile compounds) at the start of the chromatogram.

In contrast, HPLC chromatogram contains fewer peaks around the 1,4-DMN peak, as shown in Figure 2.15.b. This lower number of peaks might be expected with UV detection, as fewer compounds respond by absorbing the UV light at a specific wavelength compared to the wide range of compounds responding to the flame ionisation detectors in GC.

Because of the cleanness of the chromatogram, particularly from late eluted compounds, a shorter run time of only 10 min is required for the HPLC separation compared to 15 min in GC. In addition, the less noisy baseline with the lack of interfering peaks around the 1,4-DMN peak enables the quantification of lower concentrations of 1,4-DMN. Therefore, it might be possible to analyse potato extracts directly after the solvent extraction without the need for concentrating the extracts by evaporating the solvent which was found to contribute to the loss of some 1,4-DMN from the extracts (Beveridge, 1979).

From all of the above results, the HPLC separation method proved to be fast, accurate and precise enough for routine analysis of 1,4-DMN in potato samples.

Chapter 3: Separation and extraction methods for 1,4-DMN and internal standards - HPLC separation methods

3.1 Introduction

The development of separation methods is a major step in any analytical method which uses chromatographic techniques for the determination of analytes. Two main approaches can be used for developing HPLC separation methods. The first is the practical step-by-step approach, where systematic changes in chromatographic conditions are made and the effect of each change is studied before moving to the next step. The other approach is computer-assisted method development, where chromatographic simulation software is employed.

Several chromatographic-simulation software packages are currently available. Some of them are stand-alone programmes while others are integrated with the system controller or data systems of HPLC instruments. The combination of the two modes are also available in some programmes (Snyder et al., 1997). The packages also differ in their capability and features. Some packages are limited to predicting the separation as a function of only one retention variable each time, while others allow simultaneous optimisation of more than one variable (Hoang et al., 2003; Jupille et al., 2002; Snyder et al., 1997). The ability to predict separation for changes in column parameters (e.g. dimensions, particle size, flow rate) and gradient conditions is also different from one package to another (Molnar, 2002; Snyder et al., 1997).

DryLab[®] is the most published software for method development (Dolan et al., 1989; Snyder and Dolan, 1998). Many published studies have reported the use of DryLab[®] during method development for optimising separation conditions. DryLab[®] was used to optimise the different factors that affect HPLC separation including solvent gradient (Dolan et al., 1998; Eeva et al., 2004; Krauze-Baranowska et al., 2004), percentage of the organic component in the mobile phase (%B) in isocratic separations (Hartman et al., 2003; Hoang et al., 2003; Krauze-Baranowska et al., 2004), column temperature (Dolan et al., 1998; Hoang et al., 2003), pH (Hartman et al., 2003; Hoang et al., 2003) and column parameters (Hoang et al., 2003).

DryLab[®] software is available in both stand-alone and system-integrated versions. It is capable of optimising separations based on changes in any two variables (e.g. percentage of organic component in the mobile phase, gradient time, temperature, and pH) simultaneously. Moreover, it can predict separation for any changes in column parameters (e.g. Column diameters, size of packing particles and flow rate) (Hoang et al., 2003; Jupille et al., 2002; Molnar, 2002; Snyder et al., 1997).

The main objective of this chapter is to develop HPLC separation methods for 1,4-DMN and some internal standard candidates for the TMP/Heat method that is described briefly in Section 1.8 (experimental details in Section 5.2.5). It was aimed to develop three methods. The first method was designed for the separation of 1,4-DMN and 2-ethylnaphthalene which is the internal standard used in the original GC method. This method was intended to be used for routine analysis of extracts containing 1,4-DMN and 2-ethylnaphthalene as the internal standard. The second method was developed for the separation of 1,4-DMN and four internal standard candidates. The targeted mixture contains 1,4-dimethylnaphthalene (1,4-DMN), 2-methylnaphthalene (2-MeNaph), 1-ethylnaphthalene (1-EtNaph), 2-ethylnaphthalene (2-EtNaph) and n-butylbenzene (BuBenz). This method was intended to be used for the simultaneous evaluation of the extraction behaviour of the four internal standards compared to 1,4-DMN extraction behaviour. The third HPLC separation method was developed for the routine analysis of extracts containing 1,4-DMN and 1-ethylnaphthalene as the internal standard.

The development of the second and third methods was aided by the chromatographic-simulation software DryLab[®], whereas in the first method practical experiments were used in the development. The simulation model, developed through DryLab[®], for the separation of the mixture in the second method was used to evaluate the usefulness and accuracy of DryLab[®] software in HPLC method development.



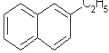
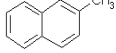
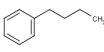
3.2 Materials and methods

3.2.1 Chemicals and solvents

The chemicals used during the development of the three methods are listed in Table 3.1 with some main characteristics. The ethanol and 2,2,4-trimethylpentane used in preparing standards were HPLC grade from Fisher Scientific, UK. The acetonitrile used in preparing the mobile phase for HPLC analysis was HPLC grade from Fisher Scientific, UK. The water used in the mobile phase was deionised water obtained from a PURELAB Option

deioniser from ELGA. The solvents used to prepare the mobile phase were degassed by sonication in an ultrasonic bath (Sonicor SC120T) for at least half an hour.

Table 3.1 Some main characteristics of the chemicals used

Compound	1,4-DMN	1-EtNaph	2-EtNaph	2-MeNaph	BuBenz
Structure					
Molecular Weight	156.2	156.2	156.2	142.2	134.1
Melting Point (°C)	7.6	-13.9	-7.4	34.4	-87.9
Boiling Point (°C)	268.0	258.6	258.0	241.1	183.3
Water Solubility (mg/l)	11.4	10.7	8.0	24.6	11.8
Octanol/Water Coefficient-Log Ko/w	4.37	4.40	4.38	3.86	4.38

[Values obtained from SRC PhysProp Database (SRC, 2005)]

3.2.2 Equipment and software

Equipment: The HPLC system used consisted of a Merck-Hitachi L-7100 pump, a L7200 autosampler and L4500 Diode Array Detector. The signal and UV spectra were processed by Merck-Hitachi Chromatography Data Station software.

Simulation Software: DryLab[®] 2000 Plus, Chromatography Optimization Software, Version 3.6.1, Rheodyne LLC, California, USA.

3.2.3 Separation method for 1,4-DMN and 2-ethylnaphthalene

The starting point in the development of this method was a method reported in Chapter 2 (see Section 2.2.3.3 and Section 2.2.3.6) for the separation of 1,4-DMN and some other related naphthalene compounds including 2-ethylnaphthalene. Practical step-by-step optimisation was followed by changing mobile phase composition (percentage of acetonitrile in the mobile phase) and column temperature, and studying the effect of each change. The separation was conducted on a Supelco Spherisorb ODS-2 (5 µm, 250 mm x 4.6 mm) column.

3.2.4 Separation method for 1,4-DMN and four internal standards

3.2.4.1 UV spectra for 1,4-DMN and four internal standards

To check the UV characteristics and to have a look at the retention behaviour of the five sample components, a 10 µg/ml standard of each compound was prepared from a 1000 µg/ml (in ethanol) stock by dilution with 2,2,4-trimethylpentane (isooctane). 10 µl of each standard were injected to the HPLC-DAD instrument. Chromatographic conditions were set as described in Section 2.3.2.1 for the routine method [Supelco ODS-2 (5 µm 250 x 4.6 mm), 70 % ACN/water, 1.5 ml/min and 25 °C]. The diode array detector (DAD) was set to get the spectrum of the eluted peak in the range 200-400 nm with a bandwidth of 4 nm.

3.2.4.2 Preliminary optimisation experiments

These practical experiments aimed to get an overview of the separation of the five components in a mixture. Different combinations of mobile phase composition (percentage of acetonitrile in the mobile phase, abbreviated as %B), flow rate and column temperature were studied for their effect on the separation as shown in Table 3.2.

Table 3.2 Chromatographic conditions for the preliminary optimisation experiments. Column used was Supelco Spherisorb C18 5 µm particles (250 x 4.6 mm). Solvent A = water, B= acetonitrile.

% B	Flow Rate (ml/min)	T °C
70	1.5	25
70	1.50	20
45	1.50	20
40	2.00	20
37	1.50	20
37	1.50	30
50	1.00	15

3.2.4.3 Instrument checks required for DryLab® software

The instrument checks conducted here aimed to check the suitability of the instrument to generate accurate data for DryLab® software. Several gradient performance checks were conducted following a method described by Gilroy and Dolan (2004) with slight modifications. Three performance checks were conducted since the pump used was a low-pressure mixing system. The checks started with gradient proportioning-valve (GPV) test followed by a linearity test and lastly, a step test.

Before starting the checks, the HPLC column was removed and replaced with about 50 cm of 0.005-inch internal diameter tubing to get sufficient backpressure for the pump to

operate properly. Solvent reservoir A was filled with 100 % water, whereas a 0.2 % or 1 % acetone/water solution was in solvent reservoir B. The flow rate was set at 2.00 ml/min and the detector at 265 nm.

Gradient proportioning-valve (GPV) test

The gradient pump used consisted of four solvent systems or channels (a, b, c and d). Therefore, all the combinations of the four proportioning valves were tested. The inlet lines for a and b were placed in a reservoir containing water, and c and d in a reservoir of acetone/water solution. A series of two-minute steps was programmed as described in Table 3.3.

Table 3.3 Gradient pump programming for the GPV Test

Time (min)	Water Reservoir		Acetone/Water Reservoir	
	a (%)	b (%)	c (%)	d (%)
0.0-2.0	50	50	0	0
2.1-4.0	90	0	10	0
4.1-6.0	50	50	0	0
6.1-8.0	90	0	0	10
8.1-10.0	50	50	0	0
10.1-12.0	0	90	10	0
12.1-14.0	50	50	0	0
14.1-16.0	0	90	0	10
16.1-18.0	50	50	0	0

Gradient linearity test

This test was performed by placing the inlet of the solvent line a in the water reservoir and b in the acetone/water reservoir. The pump was programmed to generate a linear gradient from 0 % b to 100 % b in 10 minutes with a hold at 100 % b for 5 minutes. All the other combinations of the four solvent systems in the pump were examined with the same procedure.

Gradient step test

The different combinations of the four solvent systems (a to d) were examined here. For each two systems, one inlet line was placed in the water reservoir and the other in the acetone/water reservoir. The pump was programmed to generate a series of 4-min steps in 10 % increments of the acetone/water concentration plus an extra step at 45 % and at 55 %. A 4-min hold was added to get a clear plateau at 100 % acetone/water. The pump programme is shown in Table 3.4 for the solvent systems a and b.

Table 3.4 Pump programme for the gradient step test for solvent systems a = water, b= acetone/water.

Time (min)	a (%)	b (%)
0.0-4.0	100	0
4.1-8.0	90	10
8.1-12.0	80	20
12.1-16.0	70	30
16.1-20.0	60	40
20.1-24.0	55	45
24.1-28.0	50	50
28.1-32.0	45	55
32.10-36.0	40	60
36.1-40.0	30	70
40.1-44.0	20	80
44.1-48.0	10	90
48.1-56.0	0	100

3.2.4.4 Input data collection for DryLab[®] software

Dwell volume measurements

The experimental method of determining system dwell volume followed here was described in the “help” topics of DryLab[®] software and also described by Gilroy and Dolan (2004). System dwell volume was determined from the plots generated for the gradient linearity test for solvent systems a and b, as described in the discussion section. The reported value is the mean for three replicate runs.

Gradient scouting run: Gradient or isocratic?

This scouting run was used to determine whether isocratic or gradient elution is recommended for this separation. The method used here was described by Snyder et al. (1997) and also in DryLab[®] notes on the website of the software’s company (Rheodyne, 2005).

A mixture of the five compounds (1,4-DMN and the four internal standards) was prepared in 2,2,4-trimethylpentane at 10 µg/ml. Mobile phase solvents were 100 % water (solvent A) and 100 % acetonitrile (solvent B). A gradient run from 5 – 100 % acetonitrile in 60 min gradient run time (t_G) at 35 °C was conducted using a Supelco Spherisorb ODS-2 5 µm (250 x 4.6 mm) column and 1.5 ml/min flow rate.

Input experiments for simultaneous optimisation of percentage organic solvent in the mobile phase and temperature using DryLab[®]

The DryLab[®] model selected for aiding the separation of the current mixture was a model for the simultaneous optimisation of the percentage of the organic component (acetonitrile here) in the mobile phase (%B) and column temperature (T). Four isocratic runs at two different %B and two different T values had to be carried out experimentally with all the other conditions remaining the same for all the four runs (Rheodyne, 2004). The four runs were conducted using an ACE C18 5 μ m (250 x 4.6 mm). The solvents used in the mobile phase were water (A) and acetonitrile (B) at a flow rate of 1.5 ml/min. The four different %B and T values were:

50 % B at 20 °C
60 % B at 20 °C
50 % B at 40 °C
60 % B at 40 °C

The retention data out of these experiments were used to generate the DryLab[®] simulation model. The preparation of the DryLab[®] model for this separation and the technical help was kindly provided by Stephen Ball, a DryLab[®] Support Specialist in Hichrom Limited, UK.

3.2.4.5 Evaluating the output results from the DryLab[®] software

Evaluating the output results on the ACE 5 μ m column

The output of the model generated based on the experimental data described above was evaluated by studying the resolution maps, resolution tables, result tables and the predicted chromatograms for the effect of changes in the percentage of the non-polar component in the mobile phase (%B) and for changes in temperature.

Optimum separation on the ACE 5 μ m column

The optimum conditions for the separation of the mixture of 1,4-DMN and the four internal standards on the ACE column were then concluded in the light of the DryLab[®] output. These optimum conditions, along with several other simulated conditions, were applied experimentally to evaluate the accuracy of DryLab[®] predictions.

Comparing different 5 μm columns at the optimum predicted conditions

In a search for better resolution for the mixture components at the optimum separation predicted by DryLab[®], four other 5 μm C18 columns were compared. The characteristics of the columns compared are listed in Table 3.5. The chromatographic conditions were 51 % ACN/water at flow rate of 1.5 ml/min and 18 °C.

Table 3.5 Main characteristics of the columns compared at the optimum chromatographic conditions predicted for the separation of the mixture of 1,4-DMN and the four internal standards

Column	Dimensions (mm)	Particle Size (μm)	Surface Area (m^2/g)	Carbon Load (%)
ACE C18	250 x 4.6	5 μm	300	15.5
Dionex Acclaim 120	250 x 4.6	5 μm	300	17.9
Hypersil HyPurity Elite	250 x 4.6	5 μm	200	13.0
Waters Spherisorb ODS-2	250 x 4.6	5 μm	220	11.5
Phenomenex Sphereclone ODS-2	250 x 4.6	5 μm	200	12.0

[Source: Columns datasheets and manufacturers' websites]

Optimising the separation on a column with 3 μm particles

The optimisation was carried out on a TechSphere ODS 3 μm (250 x 4.6 mm) column manufactured by HPLC Technology Ltd. Several chromatographic conditions were predicted by the DryLab[®] model to select the optimum simulated conditions for the separation on a 3 μm column. The optimum conditions predicted by the software, which reached a balance between the resolution and the practicality of the chromatographic conditions (such as affordable system backpressure), were concluded. These optimum predicted conditions were examined practically. However, more practical optimisations were conducted to get the final separation conditions on the TechSphere ODS 3 μm column. The conditions of the experiments conducted are shown in Table 3.6.

Table 3.6 Chromatographic conditions examined during the optimisation of the separation of 1,4-DMN and the four internal standards on the 3 μ m column

% B	T °C	Flow Rate (ml/min)
50	20	1.00
47	20	1.00
45	20	0.90
45	25	1.00
46	23	0.90

3.2.4.6 Final method and basic validations

The conditions for the final method for the separation of 1,4-DMN and the four internal standards were selected based on the understanding of the separation behaviour of the mixture components concluded from the combination of the DryLab[®] simulations and practical optimisation experiments. Basic validation checks were carried out on the final separation method. System precision and linearity of the detector response for each component were the main ones. For the system precision test, five replicate injections of 10 μ l of 10 μ g/ml mixed standard of the five compounds were carried out and the %RSD of the resulting peak areas were calculated for each component. For the linearity test, duplicate injections of 10 μ l were made of standards in the range 2-20 μ g/ml (2, 4, 6, 8, 10, 15 and 20 μ g/ml). Peak areas were plotted against the nominal concentration of each component.

3.2.5 Separation method for 1,4-DMN and 1-ethylnaphthalene

The aim of this method was use in routine analysis of mixtures containing 1,4-DMN and 1-ethylnaphthalene. The optimum conditions for the separation were predicted by the DryLab[®] model and applied practically. Several other conditions around the optimum conditions were examined to evaluate the ruggedness of the separation method. The conditions that were examined practically are shown in Table 3.7 with the optimum conditions at the first line.

Table 3.7 Chromatographic conditions for the practical experiments examined for the separation of 1,4-DMN and 1-ethylnaphthalene

% B	T (°C)	Flow Rate (ml/min)
65	18	1.50
65	25	1.50
67	25	1.50
62	25	1.50

3.3 Results and discussion

3.3.1 Separation method for 1,4-DMN and 2-ethylnaphthalene

In the TMP/Heat method which was briefly described in Section 1.8 (details in Chapter 5), 2-ethylnaphthalene was used as the internal standard. 2-ethylnaphthalene has a good separation from 1,4-DMN in GC. However, this might not be the case in HPLC, as can be predicted from the experiments in Section 2.2. This part of the work aimed to develop and optimise a separation method for mixtures of 1,4-DMN and 2-ethylnaphthalene, and evaluate the applicability of using the method for routine analysis of extracts containing the two compounds.

The starting point was a method reported in Chapter 2 (see Section 2.2.3.3 and Section 2.2.3.6) for the separation of 1,4-DMN and some other related naphthalene compounds including 2-ethylnaphthalene. The conditions of that method were as follows:

- Column: Supelco Spherisorb ODS-2 (5 μ m, 250 mm x 4.6 mm)
- Mobile phase: 40 % acetonitrile: 60 % water
- Flow rate: 2.0 ml/min
- Injected volume: 10 μ l
- Run time: 85 min
- 1,4-DMN retention time : ~ 67 min
- 2-Ethylnaphthalene retention time: ~74 min
- Temperature: ambient (~20 °C)
- Detector wavelength: 228 nm

This method provided good separation for 2-ethylnaphthalene from 1,4-DMN. However, it is not suitable for routine quantitative measurements due to the very long run time. Some optimisations of the mobile phase composition and column temperature were conducted practically in an attempt to reduce the run time.

It was noticed from these optimisation experiments that the resolution between the two peaks increased with the decrease in the percentage of acetonitrile in the mobile phase. It was also noticed that lower temperatures enhanced the separation, but to a lesser extent. However, lower solvent strength of the mobile phase (lower percentage of acetonitrile) and

lower temperatures lead to longer retention times. The best achievement was under the following conditions:

- Column: Supelco Spherisorb ODS-2 (5 μ m, 250 mm x 4.6 mm)
- Mobile phase: 37 % acetonitrile: 63 % water
- Flow rate: 1.5 ml/min
- Injection volume: 10 μ l
- Run time: 55 min
- 1,4-DMN retention time: ~ 47 min
- 2-Ethyl-naphthalene retention time: ~50 min
- Temperature: 30 °C
- Detector wavelength: 228 nm

A representative chromatogram of the separation of 1,4-DMN and 2-ethyl-naphthalene in a standard solution according to the above conditions is shown in Figure 3.1.

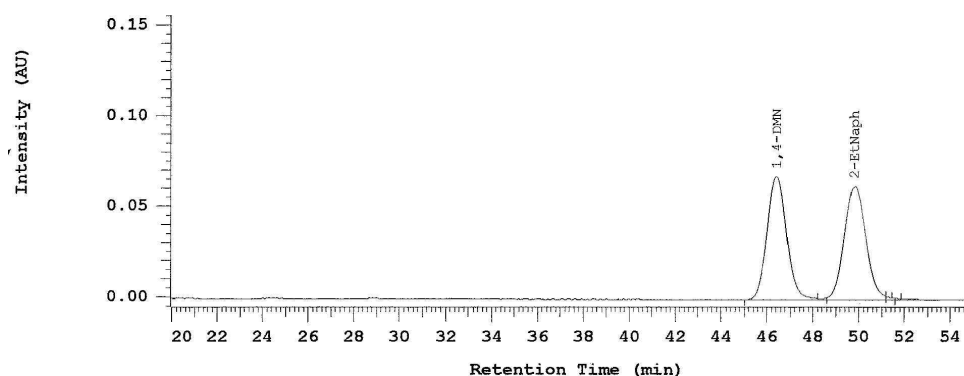


Figure 3.1 A representative chromatogram of the separation of 1,4-DMN and 2-ethyl-naphthalene in a standard of 30 μ g/ml. Conditions: Supelco Spherisorb ODS-2 5 μ m (250 x 4.6 mm), 37 % acetonitrile: 63 % water, 1.5 ml/min, 30 °C and 228 nm

Although the run time has been reduced to 55 minutes (compared to 86 minutes in the starting point) and the two compounds have good separation ($R_s > 2$), the method is not suitable for routine quantitative measurements. It was concluded that 2-ethyl-naphthalene cannot be easily separated from 1,4-DMN in suitable conditions for a routine method and therefore other compounds should be evaluated for their applicability to function as internal standards for the TMP/Heat method.

3.3.2 Separation method for 1,4-DMN and four internal standards

This method was intended to be used for the simultaneous evaluation of the extraction behaviour of four internal standards compared to the extraction behaviour of 1,4-DMN in the TMP/Heat method (see Chapter 4 for the use of the method). The targeted mixture

contains 1,4-dimethylnaphthalene (1,4-DMN), 2-methylnaphthalene (2-MeNaph), 1-ethylnaphthalene (1-EtNaph), 2-ethylnaphthalene (2-EtNaph) and n-butylbenzene (BuBenz). The method aimed to provide reproducible and rugged separation of the five compounds with a minimum resolution of at least $R_s=2$ for any pair of peaks. This resolution value or greater is a desirable target for method development (Snyder et al., 1997).

The first step in any method development is collecting data about the nature of the sample. The present sample consists of four substituted naphthalene compounds, three of which are structural isomers, in addition to a benzene substituted compound. Although acceptable HPLC separation, in most cases, can be achieved easily with a small number of experiments, some separation problems require a considerable amount of experimentation (Snyder et al., 1997). One of the main challenges during HPLC method development is finding the optimum experimental conditions that produce the required resolution in a reasonable time (Hoang et al., 2003).

Because UV absorbance detection was the detection method selected, the spectrum of each compound was generated by the Diode Array Detector (DAD) and stored in the chromatographic software's library for future identification of the compounds.

Preliminary experiments were conducted to get an idea about the nature of the separation. From these experiments, it was indicated that obtaining the optimum separation for this mixture may require a considerable amount of time and experimentation. Therefore, to assist in finding the optimum conditions, DryLab[®] chromatographic-simulation software was employed. The accuracy and usefulness of the software were evaluated as an additional objective.

Before starting the optimisation of the separation using DryLab[®], some instrument checks had to be performed. Gradient performance checks and dwell volume measurements were the main ones.

A scouting gradient run of 5-100 % acetonitrile was conducted for the prediction of the suitable elution mode (Gradient or Isocratic) for this separation.

Four practical experiments were then conducted to generate the input data for the DryLab[®] simulation model. DryLab[®] was used to study the simultaneous effect of the percentage of organic component in the mobile phase (%B) and column temperature (T) on the

resolution of the five peaks for the mixture components. The optimum chromatographic conditions for separating this mixture were then predicted.

The optimum predicted conditions along with some other predicted conditions were then applied experimentally and the resulting resolution and retention times were then compared to the predicted ones, to evaluate the accuracy of DryLab[®] predictions.

All the previous experiments were based on the use of an ACE C18 5 μm (250 x 4.6 mm) column, which was not able to provide the targeted resolution for this separation ($R_s \geq 2$). Therefore, five other columns with the same particle size were tested before moving to lower particle sizes, which should provide better resolution.

A C18 column with 3 μm particle size was then used in order to obtain higher efficiency (plate number) and therefore better resolution. This effect of lowering the column particle size was studied by DryLab[®] to discover the optimum conditions for such a column, which was then applied experimentally.

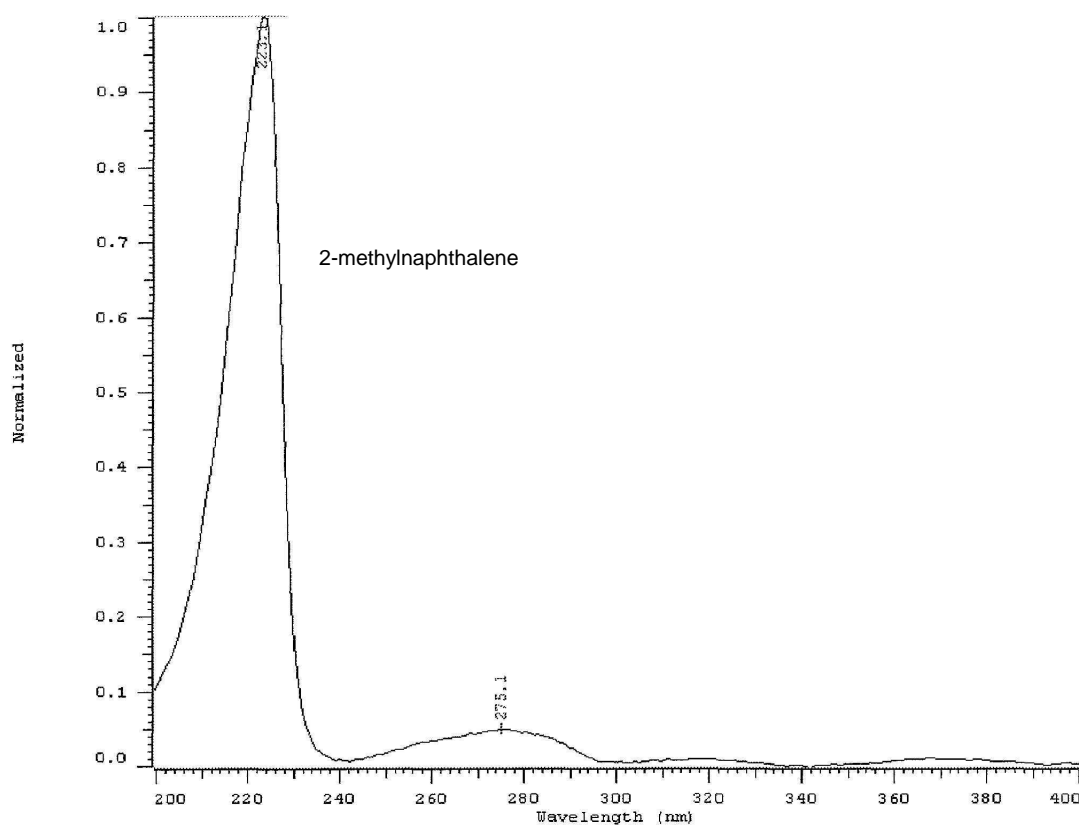
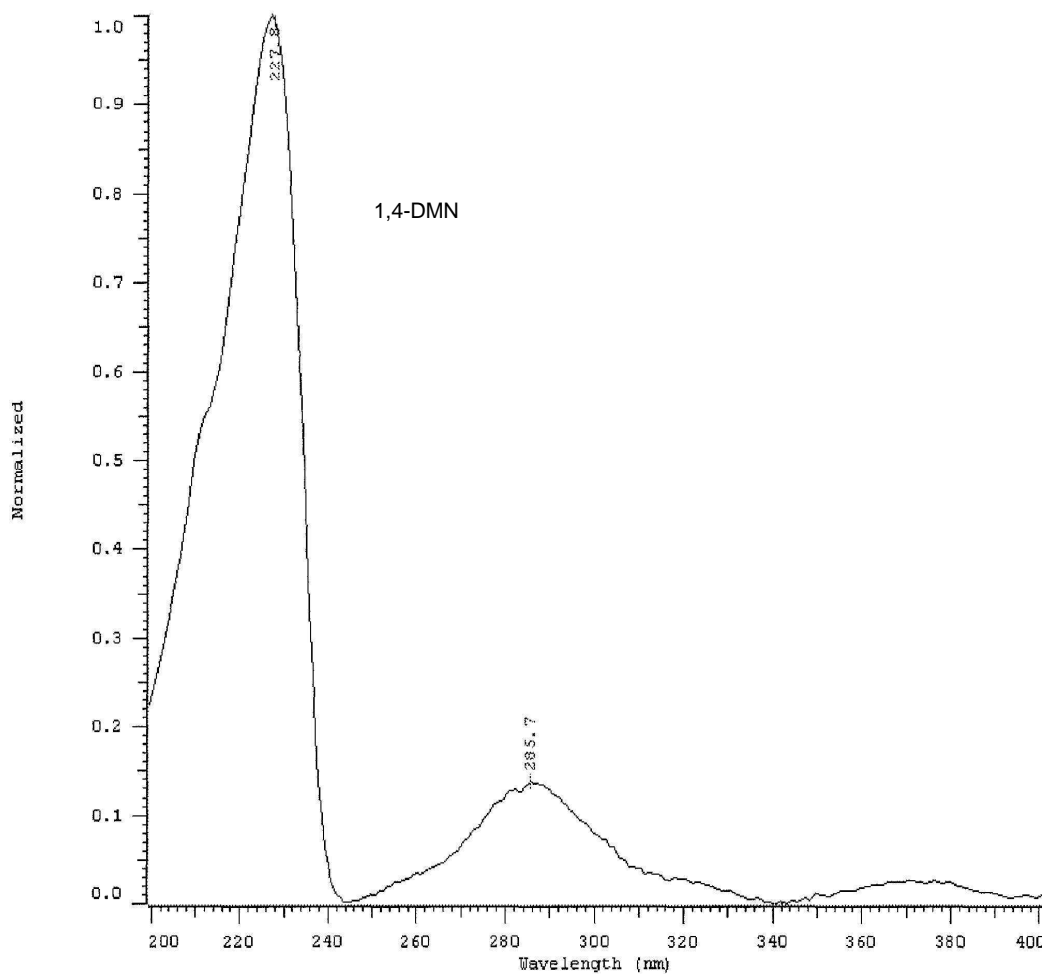
Some adjustments had to be carried out experimentally on the optimum conditions predicted by DryLab[®] for a 3 μm column to get the final separation method for the mixture of 1,4-DMN and the four internal standards. These adjustments were required mainly because of using a column with a C18 packing material different from the ACE C18 packing material used to generate the DryLab[®] model.

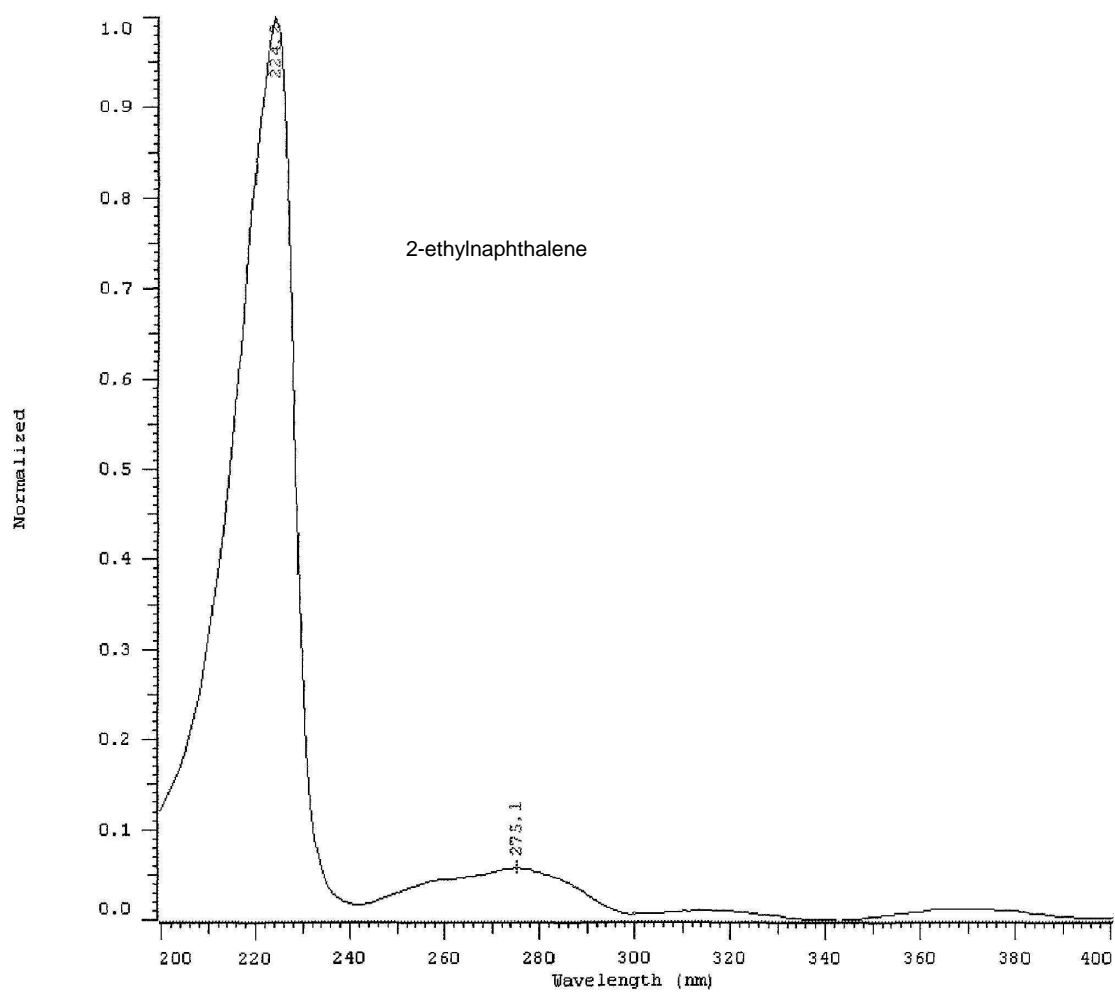
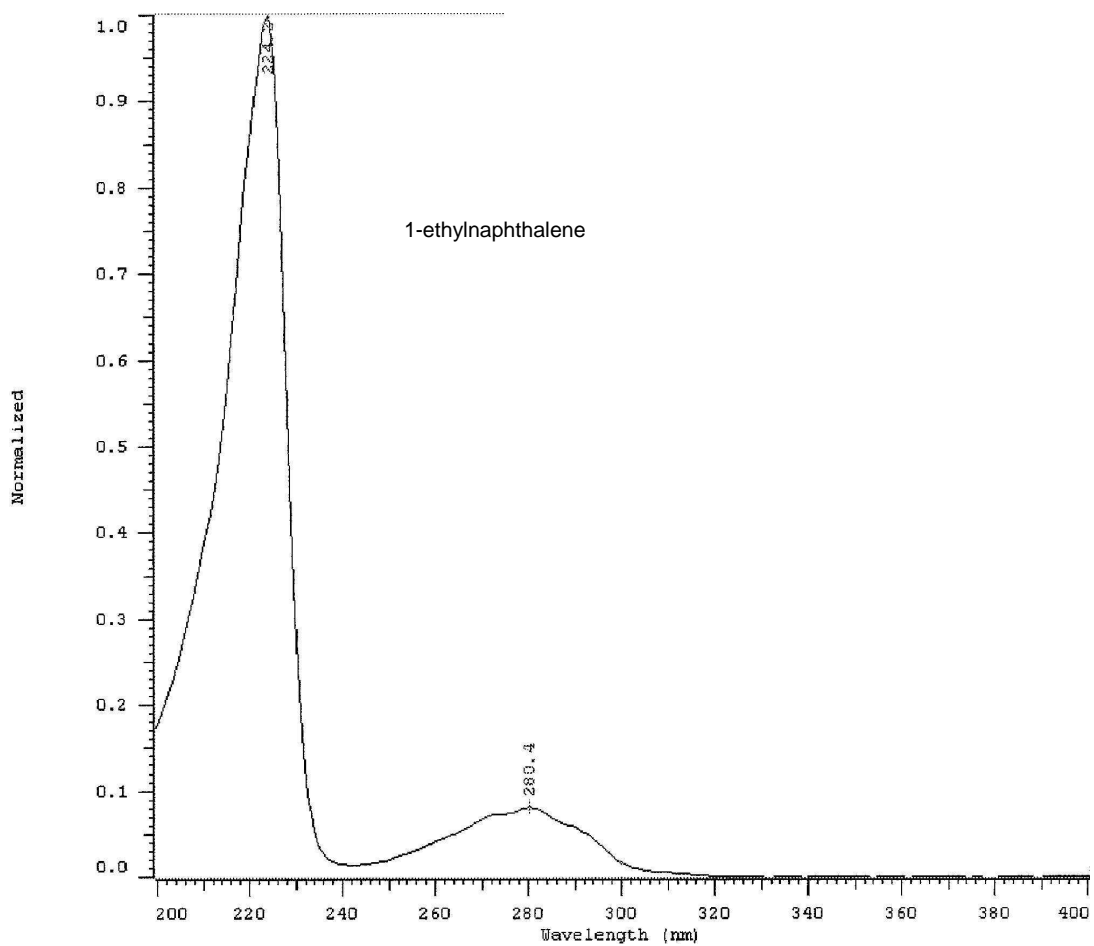
After getting the optimum conditions for the final method using the 3 μm column, basic validation tests were carried out on the final method before using it to study the suitability of the four candidates to work as internal standards for 1,4-DMN in the TMP/Heat method.

The details of each stage summarised above are given in the following sections.

3.3.2.1 UV spectra for 1,4-DMN and the four internal standards

This experiment aimed to check the UV characteristics and to take a look at the retention behaviour of the five compounds in the sample mixture. The spectra of 1,4-DMN and the four internal standards are shown in Figure 3.2.





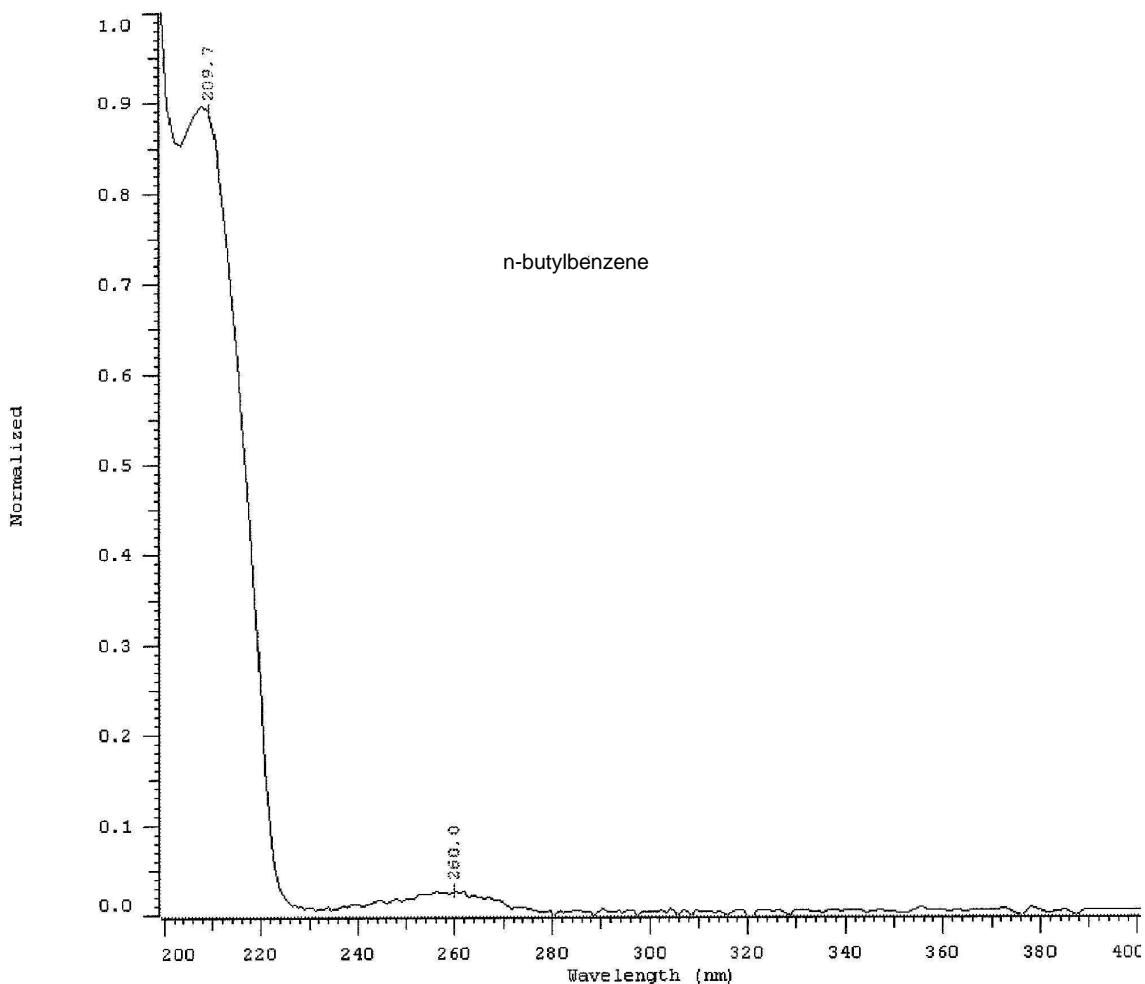


Figure 3.2 UV spectra for 1,4-DMN and the four internal standards in the range 200-400 nm with a bandwidth of 4 nm

As shown in Figure 3.2 and Table 3.8, all the sample components show strong UV absorbance at the optimum wavelength for 1,4-DMN at 228 nm except n-butylbenzene that has very weak absorbance at this wavelength. n-butylbenzene has a better absorbance at about 210 nm.

Table 3.8 Retention times and the wavelength for maximum absorbance (λ_{max}) for 1,4-DMN and the four internal standards in single-component standards. Chromatographic conditions: Supelco ODS-2 (5 μm 250 x 4.6 mm), 70 % ACN/water, 1.5 ml/min and 25 °C

Compound Name	Retention Time (min)	λ_{max} (nm)
2-methylnaphthalene	5.70	223
1-ethylnaphthalene	6.85	224
1,4-dimethylnaphthalene	7.21	228
2-ethylnaphthalene	7.22	224
n-butylbenzene	7.87	210

Fortunately, the DAD can easily be used to generate multi-wavelength chromatograms based on the suitable wavelength for each compound (or group of compounds), which will help to detect all the five components in this sample by setting the wavelength at 210 nm for n-butylbenzene and 228 nm for the rest of the compounds. However, to make a wavelength change for detecting n-butylbenzene, there has to be enough of a gap to measure a baseline before its peak. Therefore, the “Best Chromatogram” option with double wavelength was set for the rest of the chromatograms that included a BuBenz peak.

Retention times of 1,4-DMN and the four internal standards when analysing single-component standards are also listed in Table 3.8. 2-MeNaph ($T_R = 5.70$ min) and BuBenz peaks ($T_R = 7.87$ min) were expected to be well-separated from the other peaks if a mixture of the five compounds was run under the same conditions. In contrast, the retention times of 1-EtNaph ($T_R = 6.85$ min) and 1,4-DMN ($T_R = 7.21$ min) are too close which may cause some overlapping. The case might be worse with 2-EtNaph peak, which had almost the same retention time of that for 1,4-DMN peak at these conditions. This experiment indicate that the separation of the three isomers (1,4-DMN, 1-EtNaph and 2-EtNaph) might be the most challenging part of this separation.

3.3.2.2 Preliminary optimisation experiments

The experiments conducted here were based on a practical step-by-step method to give an overview of the nature of the separation and in a trial to optimise the separation. Some useful information about the nature of the separation was achieved by running a mixture of the five compounds at different chromatographic conditions. The experiments started with running the mixture of the five compounds at the conditions used for the routine separation method reported in Chapter 2 (see Section 2.3.2.1) with some adjustment of the temperature and wavelength. The resulting chromatogram is shown in Figure 3.3.

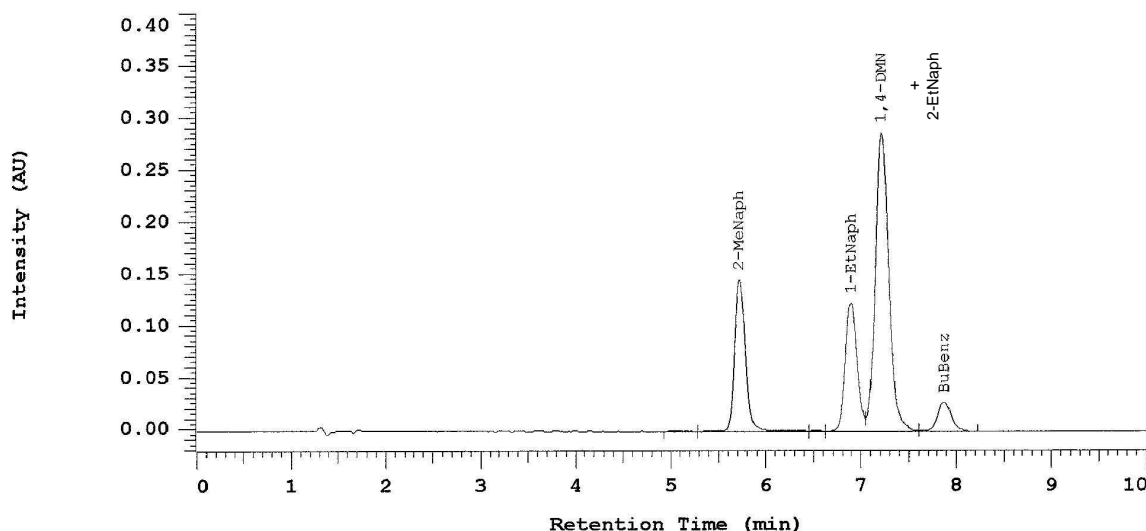


Figure 3.3 Chromatogram of the mixture of 1,4-DMN and the four internal standards at the initial conditions. Chromatographic conditions: Supelco ODS-2 (5 μ m 250 x 4.6 mm), 70 % ACN/water, 1.5 ml/min, 25 $^{\circ}$ C, and 228 nm before the 7.55 min and 210 nm thereafter

The chromatogram in Figure 3.3 confirms the expectations mentioned in the previous section regarding the separation of the mixture components at the conditions of the routine 1,4-DMN method. 2-MeNaph and BuBenz peaks were well separated from all the other peaks. In contrast, the retention times of 1-EtNaph and 1,4-DMN were too close, causing them to slightly overlap at the baseline. The case was worse with 2-EtNaph peak, which completely coeluted with 1,4-DMN peak at these conditions. The mixture was then separated at the different conditions shown in Table 3.9.

Table 3.9 Critical pair of peaks in the chromatogram of the mixture of 1,4-DMN and the four internal standards at different chromatographic conditions. Other conditions: Column= Supelco Spherisorb C18 5 μ m (250 x 4.6 mm), Solvent A = Water, B= acetonitrile

% B	Flow Rate (ml/min)	T ($^{\circ}$ C)	Critical Pair
70	1.50	25	1,4-DMN/2-EtNaph
70	1.50	20	1,4-DMN/2-EtNaph
45	1.50	20	1,4-DMN/2-EtNaph
40	2.00	20	1,4-DMN/2-EtNaph
37	1.50	20	1-EtNaph/1,4-DMN
37	1.50	30	1-EtNaph/1,4-DMN
50	1.00	15	1,4-DMN/2-EtNaph

Some general conclusions regarding the separation nature of the mixture components were obtained from running the mixture at the different chromatographic conditions described in Table 3.9. The order of the five peaks according to their retention times was as follows: 2-MeNaph, 1-EtNaph, 1,4-DMN, 2-EtNaph, and the last eluted peak was for BuBenz. As

might be predicted from their physicochemical properties (close K_o/w values), the three isomers (1,4-DMN, 1-EtNaph and 2-EtNaph) were the least resolved peaks (critical pairs) in this separation at all the different conditions examined, as shown in Table 3.9.

These preliminary experiments revealed that the major difficulty in this separation might be the opposite response of the two pairs, 1-EtNaph/1,4-DMN and 1,4-DMN/2-EtNaph, towards changes in chromatographic conditions, particularly the percentage of acetonitrile in the mobile phase. The 1,4-DMN peak was in the middle between the 1-EtNaph and 2-EtNaph peaks. The 1-EtNaph peak seemed to have better separation from the 1,4-DMN peak at a higher percentage of acetonitrile in the mobile phase, whereas lower percentages of acetonitrile in the mobile phase were favourable for the separation of the 1,4-DMN/2-EtNaph pair.

The response of the two critical pairs for changes in column temperature seemed to be more consistent. Lower temperatures (20 °C) seemed to enhance the separation of all the peaks.

Changes in the flow rate of the mobile phase seemed to have less effect on this separation, compared to the effect of changes in the percentage of acetonitrile in the mobile phase or column temperature.

The previous preliminary experiments suggested that the simultaneous effect of the percentage of acetonitrile in the mobile phase and column temperature needs to be studied with a more detailed and systematic approach, in order to obtain the optimum separation for this mixture. Such a study would need a large number of step-by-step experiments. Even so, the best separation achieved by these experiments may not necessarily be the 'real optimum' separation for the mixture. The real optimum separation that provides sufficient resolution and short analysis time, as well as a rugged method, may be obtained more easily by the aid of computer chromatographic-simulation software packages (Lammerhofer et al., 1997). Therefore, after these preliminary experiments, it was decided to use the chromatographic simulation software DryLab[®] for optimising this separation. Before starting the method development with the aid of DryLab[®], some instrument checks were conducted.

3.3.2.3 Instrument checks required for DryLab[®] software

It is strongly recommended to start the RP-HPLC method development by a scouting gradient elution run, which can be used for the prediction of the suitable elution mode for a

separation (Jupille et al., 2002; Snyder et al., 1997). For accurate predictions from such a scouting gradient, it was necessary to check the performance (linearity and accuracy) of the gradient system. Three performance checks were conducted here since the pump used has a low-pressure mixing system. The checks started with gradient proportioning-valve (GPV) test followed by a linearity test and lastly, a step test.

Gradient proportioning-valve (GPV) test

The results of the gradient proportioning-valve test are shown in the chromatogram in Figure 3.4 and in Table 3.10.

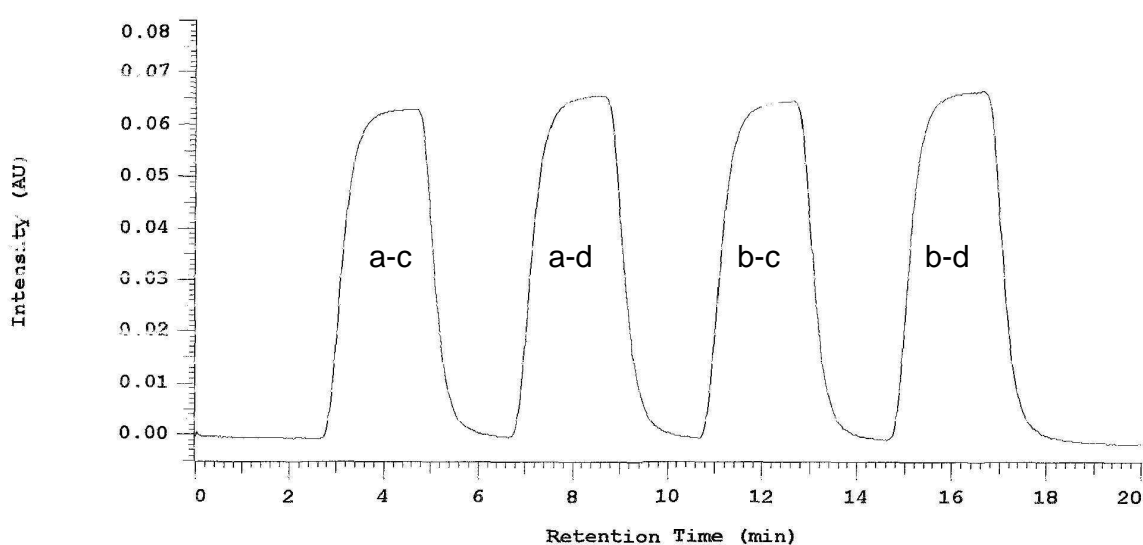


Figure 3.4 The resulting plot of the gradient proportioning-valve test

Table 3.10 Results of the proportioning-valve tests.

Valve Combinations	Plateau Heights (AU)		
	Test 1	Test 2	Test 3
a (water) and c (acetone/water)	0.06305	0.06337	0.06355
a (water) and d (acetone/water)	0.06540	0.06550	0.06625
b (water) and c (acetone/water)	0.06440	0.06465	0.06445
c (water) and d (acetone/water)	0.06620	0.06655	0.06605
Average Height	0.06476	0.06502	0.06508
Maximum Difference in Heights (%)	4.9	4.9	4.1

The baseline of the chromatogram in Figure 3.4 was generated when pumping a mixture of 50:50 a:b (water only), where the steps are formed by the 90:10 mixtures that contain the UV-absorbing solvent acetone. Three replicates of this test were conducted.

The difference between the highest and lowest plateaus was calculated as a percentage of the average height of the four plateaus in each chromatogram. The mean difference of the three replicates was 4.6 % which is approaching, but still less than, the maximum allowable value of 5 % for passing the test (Gilroy and Dolan, 2004). Therefore, the performance of the gradient system in this test was accepted.

Gradient linearity test

All the different combinations of the four solvent systems in the pump were examined. Representative results for the gradient linearity test for system a (water) vs b (acetone/water) are plotted in Figure 3.5.

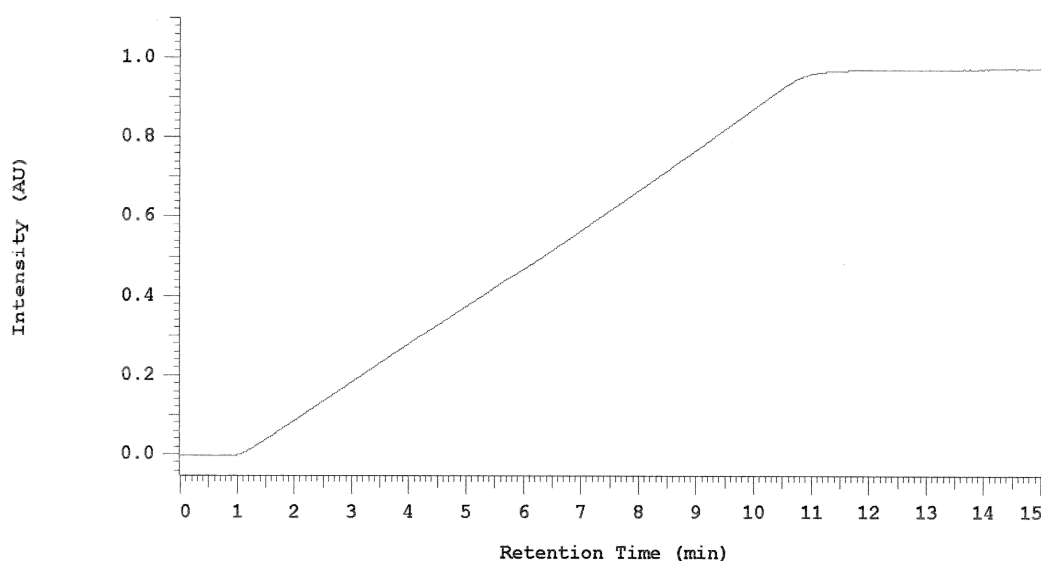


Figure 3.5. Plot of linear gradient for the valve combination a (water) vs b (acetone/water)

The gradient trace should have a linear ramp between the initial and final plateaus to pass the test (Gilroy and Dolan, 2004; Snyder et al., 1997). This requirement for the Gradient Linearity Test was fulfilled in all the different combinations of the four valves in the pump and therefore the instrument was considered to have successfully passed the second test.

Gradient step test

The plot for the gradient step test for valve a (water) vs b (acetone/water) is shown in Figure 3.6 as a representative of the other different combinations of the four solvent systems in the pump.

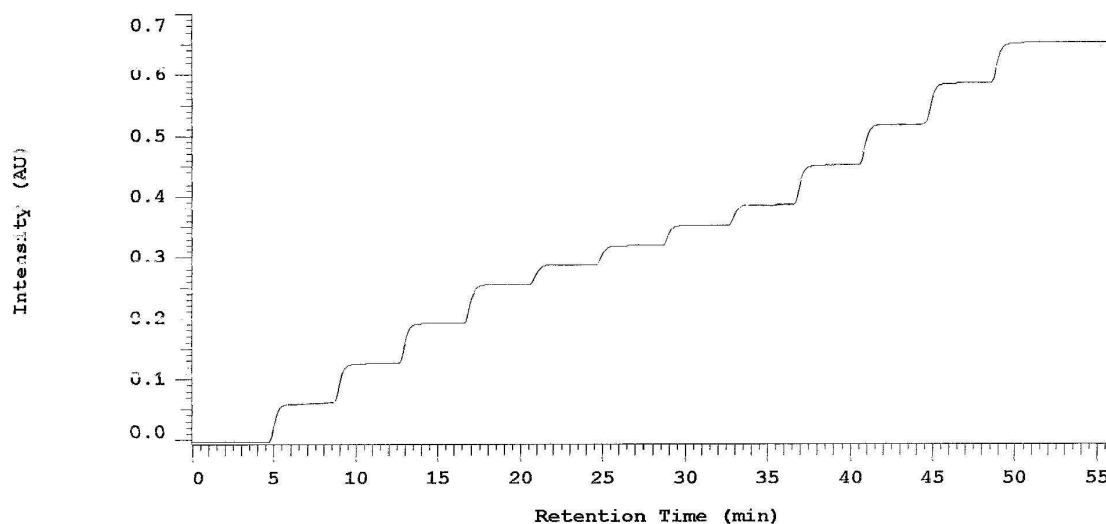


Figure 3.6 Gradient step test for valve a vs b. Baseline is generated by 100 % a (water) and steps were programmed for 10, 20, 30, 40, 45, 50, 55, 60, 70, 80, 90 and 100 % b (acetone/water solution)

All the different combinations of the four solvent systems show good shape of all the steps in the plot. The steps have flat plateaus with similar degree of rounding. In order to examine the results quantitatively, the maximum difference between the theoretical and practical step sizes was calculated and the results are shown in Table 3.11.

Table 3.11 Gradient Step Test results

Theoretical Step Size (%)	Step Height (AU)	Practical Step Size (%)	Deviation (%)
0	0.00000	0.0	0.0
10	0.06335	9.7	0.3
20	0.12840	19.7	0.3
30	0.19350	29.6	0.4
40	0.25710	39.4	0.6
45	0.28975	44.4	0.6
50	0.32095	49.1	0.9
55	0.35425	54.2	0.8
60	0.38755	59.3	0.7
70	0.45245	69.3	0.7
80	0.51875	79.4	0.6
90	0.58620	89.7	0.3
100	0.65320	100.0	0.0
Mean Deviation			0.5 %

The height of each step was determined in UV absorbance units (AU) and the difference in UV absorbance between zero and 100 % was used to calculate the practical step size for each step. The difference between the practical and theoretical steps was obtained. The average deviation was only 0.5 % with a maximum value of 0.9 %. These values are less than the maximum allowable deviation of 1 % specified by the pump manufacturer in the pump manual and also reported by Gilroy and Dolan (2004). Therefore, the instrument passed the third test for gradient mixing performance and can be used reliably for performing the scouting run and for generating data for the DryLab[®] software if gradient separation is required.

3.3.2.4 Input data collection for DryLab[®] software

Dwell volume measurements

Instrument dwell volume is the volume held up by the instrument between the point where the gradient is formed (mixer) and the inlet of the column. In a high pressure mixing system, this includes mixers, autosampler, injector, loop and connecting tubing between them. In low pressure mixing systems (such as the one used in this study), proportioning valves and pump head are also included (Snyder et al., 1997; Gilroy and Dolan, 2004). Dwell volume is the cause of the isocratic step at the start of a gradient elution profile (Molnar, 2002). This step is the flat part at the start of the linear gradient plot showed at 0 AU in Figure 3.5. The value of the dwell volume is different from a system to another. Typical values range between 2-8 ml. However, values can be as low as 0.5 ml or higher than 10 ml (Snyder et al., 1997).

Changes in dwell volume - either by using a different instrument or by changing some parts of the instrument (such as the loop or the autosampler) - will affect gradient separation primarily by shifting sample retention times to higher or lower values. This change in retention times is related to the dwell time. Dwell volume changes can also affect peak selectivity and resolution of a gradient separation, particularly for early eluting peaks. As a result of these changes in retention times, selectivity and resolution, gradient methods may not transfer well between different HPLC instruments. These effects of dwell volume on gradient separation make it very important to determine system dwell volume before starting any gradient method development (Snyder et al., 1997; Gilroy and Dolan, 2004).

Instrument dwell volume is one of the parameters required for DryLab[®] software models when using gradient elution mode. It was determined from the plots generated for the gradient linearity test as shown in Figure 3.7.

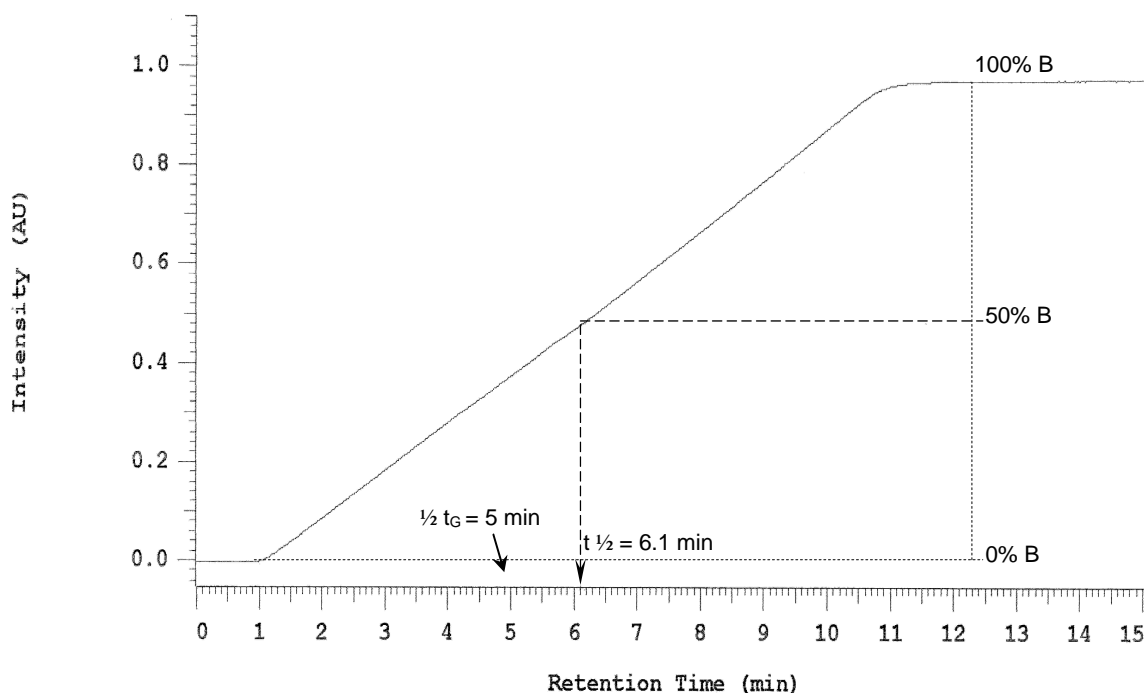


Figure 3.7 Determination of the dwell volume of the HPLC instrument from the plot generated for the linear gradient test

The midpoint of the gradient (50 % B) was located as half way between initial and final isocratic segments. The time at this midpoint ($t_{1/2}$) was determined by drawing a vertical line from the midpoint to on the time axis. Half of the gradient time ($1/2 t_G$) was then subtracted from $t_{1/2}$ to get the dwell time t_D which was then converted to dwell volume by multiplying with the flow rate 2.00 ml/min.

As shown in Figure 3.7, the gradient time was 10 min and therefore $1/2 t_G$ was 5 min. The mean value for the midpoint time $t_{1/2}$ from 3 replicates was 6.1 min. By subtracting $1/2 t_G$ from this value, the resulting dwell time for the HPLC instrument used is 1.1 min which is converted to a dwell volume of 2.2 ml at the 2.0 ml/min flow rate used. This value falls in the typical range of dwell volume of 2 - 8 ml (Snyder et al., 1997).

Gradient scouting runs: Gradient or isocratic?

It is strongly recommended to start HPLC method development with such a scouting gradient elution run to predict the most suitable elution mode (Jupille et al., 2002; Snyder et al., 1997). The scouting run was used to determine whether isocratic or gradient elution

is recommended for this separation in order to select the right model in DryLab[®] for the current separation. The mixture of the five compounds was separated using the gradient run from 5-100 % ACN in 60 min. The resulted chromatogram is shown in Figure 3.8.

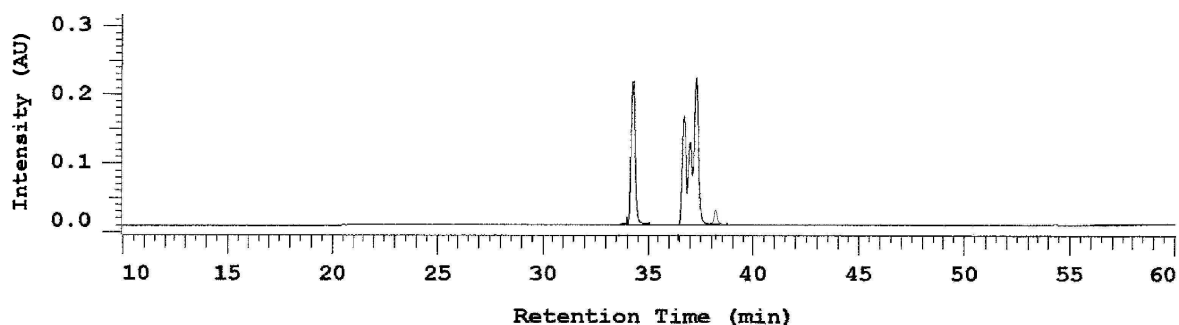


Figure 3.8 The resulting chromatogram from the gradient scouting run. Chromatographic conditions: gradient 5 – 100 % ACN in 60 min, 1.5 ml/min, 35 °C and Supelco Shperisorb ODS-2 C18 5 μ m (250 x 4.6 mm) column

Retention data obtained for the five component peaks in the scouting run are shown in Table 3.12

Table 3.12 Retention data obtained for the five peaks in the scouting run

Compound No.	Compound Name	Retention Time (min)	Peak Area	Tailing Factor	Half-Height Peak Width (min)
1	2-MeNaph	34.30	1060162	1.33	0.15
2	1-EtNaph	36.73	770150	Peaks not resolved at 5% height	0.16
3	1,4-DMN	37.02	594190		0.17
4	2-EtNaph	37.30	1155550		0.15
5	BuBenz	38.33	112673	1.89	0.17

By examining the data in Table 3.12, it can be noticed that all the five peaks eluted in a small fraction of time compared to the gradient time. The difference between the retention time of first eluted peak (2-MeNaph, t_R =34.30 min) and the last one (BuBenz, t_R =38.33) was only 4.03 min. The ratio $\Delta t_R / t_G = 4.03/60 = 0.07$ means that the t_R difference is only about 7 % of the gradient time. This ratio can be used to estimate the necessity for a gradient elution. When the difference Δt_R is less than 25 % of the gradient time then isocratic elution is feasible and gradient elution is not required (Snyder et al., 1997). In the current separation, the difference Δt_R was only 7 % of the gradient time and therefore isocratic separation is possible.

Chromatographic methods based on isocratic separation are fairly robust, often preferred and might be required. Many chromatographers prefer isocratic separations to gradient-based ones for different reasons. Some of these reasons are related to gradient equipment unavailability, more elution complexity, higher baseline noise, longer run times due to the equilibration step and more problems with method transferability (Snyder et al., 1997; Molnar, 2002). Therefore, based on the results of the scouting gradient run and due to the advantages of the isocratic separation mode, it was selected for the optimisation of the current method for 1,4-DMN and the four internal standards.

It can also be noticed from Table 3.12 that the peaks were quite asymmetrical (tailed). Poor symmetry of peaks can result in poor retention reproducibility, inaccurate plate number and resolution measurements, and imprecise quantification. The high values of tailing factor to above a value of about 1.3 may indicate that the column is dying and should be replaced (Snyder et al., 1997). The large tailing factors obtained here, particularly for the last peak of 1.9, may suggest replacing the Supelco column at least for the next few experiments that use DryLab[®] software, in order to get accurate predictions and good evaluation of the software. Therefore, to reduce the effects of peak tailing, a new ACE 5 μm (250 x 4.6 mm) column with ultrapure silica particles was used in the next experiments.

Input experiments for simultaneous optimisation of percentage organic solvent in the mobile phase and temperature using DryLab[®]

Based on the previous scouting run, isocratic elution was selected for the separation of 1,4-DMN and the four internal standard candidates. In addition, the preliminary experiments showed that temperature has a significant effect on the separation of this mixture. Hence, a DryLab[®] model for the simultaneous optimisation of the percentage of the organic component (acetonitrile here) in the mobile phase (%B) and column temperature (T) has been chosen for aiding this separation.

To use this model, four isocratic runs at two different percentages of acetonitrile in the mobile phase (%B) and two different temperature values (T) were carried out experimentally. The two %B points were 10 % apart and the temperatures values were 20 °C apart. All the other conditions kept the same for all the four runs.

Table 3.13 shows the retention data used as input data to generate the DryLab[®] model for the simultaneous optimisation of the percentage of acetonitrile in the mobile phase and column temperature. The chromatograms for the four runs are shown in Figure 3.9 a-d.

Table 3.13 Retention input data used to generate the DryLab[®] simulation model for the separation of 1,4-DMN and the internal standard candidates

Conditions	Compound No.	Compound Name	Retention Time (min)	Peak Area	Tailing Factor	Half-Height Peak Width (min)
50 % ACN/H ₂ O 20 °C	1	2-MeNaph	25.35	1228571	1.06	0.42
	2	1-EtNaph	35.65	950022	1.07	0.60
	3	1,4-DMN	37.27	702870	1.03	0.64
	4	2-EtNaph	39.12	1253703	1.03	0.67
	5	BuBenz	46.53	118438	1.20	0.85
60 % ACN/H ₂ O 20 °C	1	2-MeNaph	12.59	1228959	1.08	0.20
	2	1-EtNaph	16.55	962261	1.06	0.27
	3	1,4-DMN	17.49	511713	Peaks not resolved at 50% height	
	4	2-EtNaph	17.77	1463255		
	5	BuBenz	20.67	121280	1.05	0.37
50 % ACN/H ₂ O 40 °C	1	2-MeNaph	18.94	1219301	1.05	0.31
	2	1-EtNaph	26.35	946885	Peaks not resolved at 5% height	0.44
	3	1,4-DMN	27.21	712726		0.46
	4	2-EtNaph	28.74	1250316	1.05	0.48
	5	BuBenz	34.63	125108	1.14	0.73
60 % ACN/H ₂ O 40 °C	1	2-MeNaph	9.81	1217297	1.06	0.15
	2	1-EtNaph	12.71	953681	1.08	0.20
	3	1,4-DMN	13.22	687676	Peaks not resolved at 5% height	0.22
	4	2-EtNaph	13.56	1283497		0.22
	5	BuBenz	15.95	119539	1.10	0.27

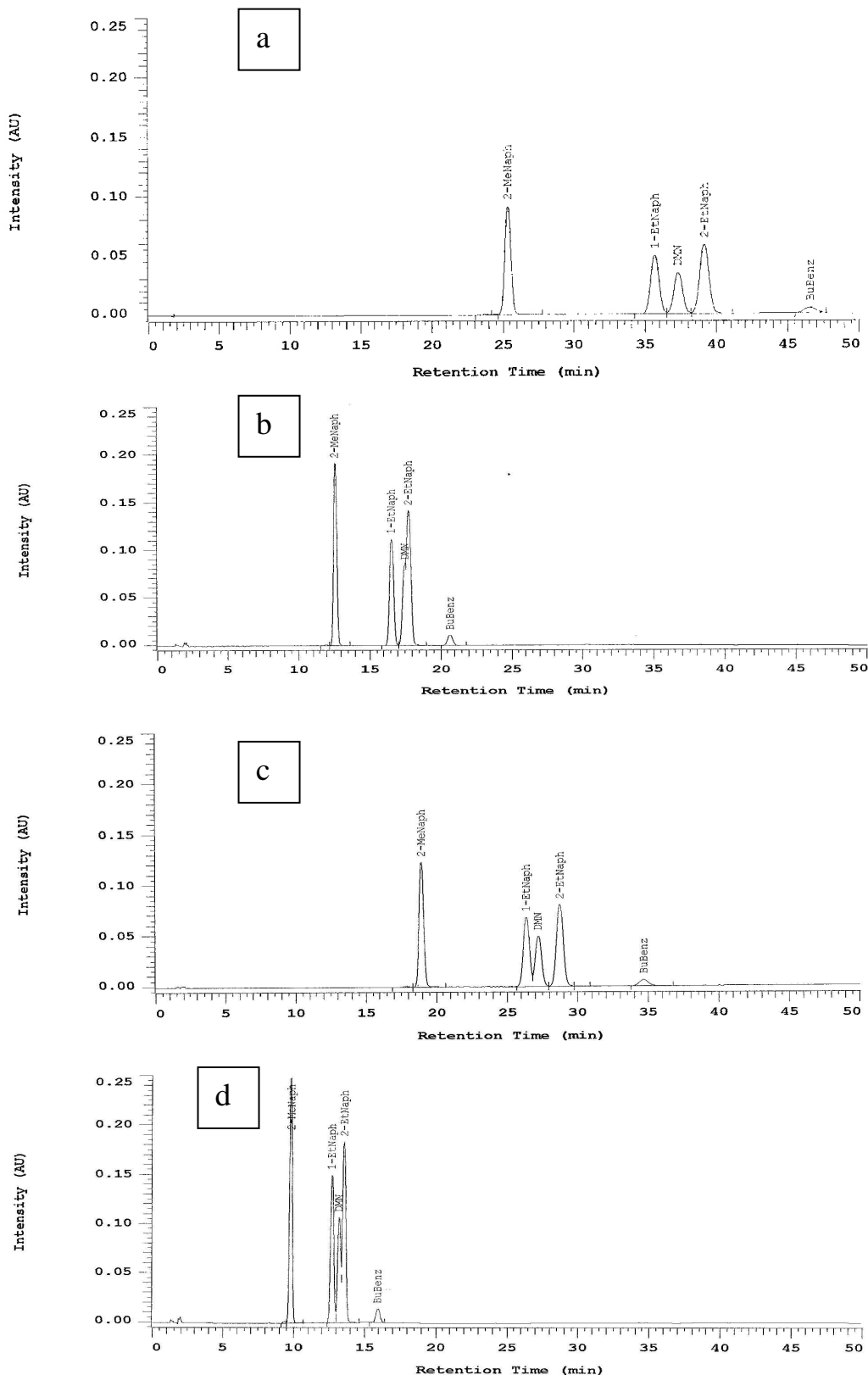


Figure 3.9 HPLC chromatograms for the four DryLab® input runs. Conditions: a. 50 % B at 20 °C, b. 60 % B at 20 °C, c. 50 % B at 40 °C, d. 60 % B at 40 °C. Other conditions: ACE C18 5 μ m (250 x 4.6 mm), mobile phase solvents: A=H₂O and B=ACN, flow rate 1.5 ml/min

By examining the data in Table 3.13 and the chromatograms in Figure 3.9, some observations may be noticed. Firstly, peak symmetry is better in this ACE column than in the Spherisorb one. Secondly, the three isomers 1-EtNaph, 1,4-DMN and 2-EtNaph formed

the critical pairs (the least resolved pairs) in all the runs. This would not be unexpected behaviour for isomers due to similarity in their properties. Thirdly, the separation of the 1-EtNaph/1,4-DMN pair seems to behave in an opposite way to the pair 2-EtNaph/1,4-DMN towards the changes in the chromatographic conditions. Lastly, the best separation among the four above was at 50 % acetonitrile and 20 °C (first conditions). All the previous observations support the ones obtained from the preliminary experiments. However, better details of these observations and more can be achieved through the DryLab[®] simulation output.

3.3.2.5 Evaluating the output results from the DryLab[®] software

Evaluating the output results on the ACE 5 µm column

The information in Table 3.13 along with the chromatographic conditions were input to the model called “LC-RP Isocratic %B / Temperature (4 runs)” in DryLab[®] software to start the simulation. The output of the simulation is presented as resolution tables, resolution maps and predicted chromatograms with result tables containing peak information at any selected chromatographic conditions in the simulated range.

The resolution table for the present separation shows resolution values (along with the identifying numbers of the critical peaks) at any given percentage of acetonitrile in the mobile phase (%B) and column temperature (T). By selecting any combination of the percentage of acetonitrile in the mobile phase and column temperature from the table, a predicted chromatogram and table of peak retention data can be presented. A part of the resolution table reported by DryLab[®] for the current separation is shown in Figure 3.10. The range of the percentage of acetonitrile in the mobile phase for the complete table was from 0 to 100 % with a column temperature range from 18 to 42 °C.

Page 2

DryLab Resolution Table

04/01/2006 18:42:01

DryLab Version 3.6.1 Serial Number: Not Specified

File name: UPDATED GLASGOW UNIVERSITY DATA, 3D MODEL, 14-9-05.DLB Revision number: 2

Separation mode: LC - RP Isocratic %B/Temperature (4 runs)

Message:

		43.0	46.0	49.0	52.0	55.0	58.0	61.0	%R 64.0	67.0	70.0	73.0	76.0	79.0	82.0
deg C	18.0	1.19 (2,3)	1.33 (2,3)	1.46 (2,3)	1.39 (3,4)	1.04 (3,4)	0.71 (3,4)	0.39 (3,4)	0.09 (3,4)	0.20 (4,3)	0.46 (4,3)	0.69 (4,3)	0.89 (4,3)	0.97 (2,4)	0.77 (2,4)
	19.2	1.17 (2,3)	1.31 (2,3)	1.44 (2,3)	1.40 (3,4)	1.06 (3,4)	0.73 (3,4)	0.41 (3,4)	0.11 (3,4)	0.17 (4,3)	0.43 (4,3)	0.67 (4,3)	0.86 (4,3)	0.96 (2,4)	0.76 (2,4)
	20.4	1.16 (2,3)	1.29 (2,3)	1.41 (2,3)	1.41 (3,4)	1.08 (3,4)	0.75 (3,4)	0.43 (3,4)	0.13 (3,4)	0.15 (4,3)	0.41 (4,3)	0.64 (4,3)	0.83 (4,3)	0.94 (2,4)	0.75 (2,4)
	21.6	1.14 (2,3)	1.27 (2,3)	1.39 (2,3)	1.43 (3,4)	1.09 (3,4)	0.77 (3,4)	0.45 (3,4)	0.15 (3,4)	0.12 (4,3)	0.38 (4,3)	0.61 (4,3)	0.80 (4,3)	0.93 (2,4)	0.74 (2,4)
	22.8	1.12 (2,3)	1.25 (2,3)	1.37 (2,3)	1.44 (3,4)	1.11 (3,4)	0.78 (3,4)	0.47 (3,4)	0.18 (3,4)	0.10 (4,3)	0.35 (4,3)	0.58 (4,3)	0.77 (4,3)	0.92 (2,4)	0.73 (2,4)
	24.0	1.10 (2,3)	1.23 (2,3)	1.34 (2,3)	1.45 (3,4)	1.12 (3,4)	0.80 (3,4)	0.49 (3,4)	0.20 (3,4)	0.08 (4,3)	0.33 (4,3)	0.55 (4,3)	0.74 (4,3)	0.90 (4,3)	0.72 (2,4)
	25.2	1.08 (2,3)	1.21 (2,3)	1.32 (2,3)	1.43 (2,3)	1.14 (3,4)	0.82 (3,4)	0.51 (3,4)	0.22 (3,4)	0.05 (4,3)	0.30 (4,3)	0.53 (4,3)	0.71 (4,3)	0.86 (4,3)	0.71 (2,4)
	26.4	1.06 (2,3)	1.18 (2,3)	1.30 (2,3)	1.41 (2,3)	1.16 (3,4)	0.84 (3,4)	0.53 (3,4)	0.24 (3,4)	0.03 (4,3)	0.28 (4,3)	0.50 (4,3)	0.69 (4,3)	0.83 (4,3)	0.70 (2,4)
	27.6	1.04 (2,3)	1.16 (2,3)	1.28 (2,3)	1.38 (2,3)	1.17 (3,4)	0.85 (3,4)	0.55 (3,4)	0.27 (3,4)	0.00 (4,3)	0.25 (4,3)	0.47 (4,3)	0.66 (4,3)	0.81 (4,3)	0.69 (2,4)
	28.8	1.03 (2,3)	1.14 (2,3)	1.25 (2,3)	1.36 (2,3)	1.19 (3,4)	0.87 (3,4)	0.57 (3,4)	0.29 (3,4)	0.02 (4,3)	0.23 (4,3)	0.44 (4,3)	0.63 (4,3)	0.78 (4,3)	0.68 (2,4)
	30.0	1.01 (2,3)	1.12 (2,3)	1.23 (2,3)	1.33 (2,3)	1.20 (3,4)	0.89 (3,4)	0.59 (3,4)	0.31 (3,4)	0.04 (4,3)	0.20 (4,3)	0.42 (4,3)	0.60 (4,3)	0.75 (4,3)	0.67 (2,4)
	31.2	0.99 (2,3)	1.10 (2,3)	1.21 (2,3)	1.31 (2,3)	1.22 (3,4)	0.91 (3,4)	0.61 (3,4)	0.33 (3,4)	0.06 (4,3)	0.18 (4,3)	0.39 (4,3)	0.57 (4,3)	0.72 (4,3)	0.66 (2,4)
	32.4	0.97 (2,3)	1.08 (2,3)	1.19 (2,3)	1.28 (2,3)	1.23 (3,4)	0.93 (3,4)	0.63 (3,4)	0.35 (3,4)	0.09 (4,3)	0.15 (4,3)	0.36 (4,3)	0.55 (4,3)	0.69 (4,3)	0.65 (2,4)
	33.6	0.95 (2,3)	1.06 (2,3)	1.16 (2,3)	1.26 (2,3)	1.25 (3,4)	0.94 (3,4)	0.65 (3,4)	0.37 (3,4)	0.11 (4,3)	0.13 (4,3)	0.34 (4,3)	0.52 (4,3)	0.66 (4,3)	0.64 (2,4)
	34.8	0.93 (2,3)	1.04 (2,3)	1.14 (2,3)	1.23 (2,3)	1.26 (3,4)	0.96 (3,4)	0.67 (3,4)	0.39 (3,4)	0.13 (4,3)	0.10 (4,3)	0.31 (4,3)	0.49 (4,3)	0.64 (4,3)	0.63 (2,4)
	36.0	0.92 (2,3)	1.02 (2,3)	1.12 (2,3)	1.21 (2,3)	1.27 (3,4)	0.97 (3,4)	0.69 (3,4)	0.41 (3,4)	0.15 (4,3)	0.08 (4,3)	0.29 (4,3)	0.47 (4,3)	0.61 (4,3)	0.62 (2,4)
	37.2	0.90 (2,3)	1.00 (2,3)	1.09 (2,3)	1.18 (2,3)	1.26 (2,3)	0.99 (3,4)	0.70 (3,4)	0.43 (3,4)	0.18 (4,3)	0.06 (4,3)	0.26 (4,3)	0.44 (4,3)	0.58 (4,3)	0.61 (2,4)
	38.4	0.88 (2,3)	0.98 (2,3)	1.07 (2,3)	1.16 (2,3)	1.23 (2,3)	1.01 (3,4)	0.72 (3,4)	0.45 (3,4)	0.20 (4,3)	0.03 (4,3)	0.24 (4,3)	0.41 (4,3)	0.55 (4,3)	0.60 (2,4)
	39.6	0.86 (2,3)	0.96 (2,3)	1.05 (2,3)	1.13 (2,3)	1.20 (2,3)	1.02 (3,4)	0.74 (3,4)	0.47 (3,4)	0.22 (4,3)	0.01 (4,3)	0.21 (4,3)	0.39 (4,3)	0.53 (4,3)	0.60 (2,4)
	40.8	0.84 (2,3)	0.94 (2,3)	1.03 (2,3)	1.11 (2,3)	1.17 (2,3)	1.04 (3,4)	0.76 (3,4)	0.49 (3,4)	0.24 (4,3)	0.01 (4,3)	0.19 (4,3)	0.36 (4,3)	0.50 (4,3)	0.59 (2,4)

Figure 3.10 Selected part of DryLab® Resolution table for the separation of 1,4-DMN and the four internal standards

Despite the large amount of information about the separation provided by resolution tables, visualising this information gives a faster and more convenient way of presenting the simulation results. Resolution maps are the visual method used by DryLab® to present the simulation results in two-dimension or three-dimension plots based on the simulation model selected. These plots describe the relationship between a separation variable (or variables) and the lowest resolution value for any critical pair in the chromatogram.

The simultaneous effect of the percentage of acetonitrile in the mobile phase and column temperature on the resolution of all the five peaks in the current mixture is visually presented through the 3D resolution map shown in Figure 3.11.

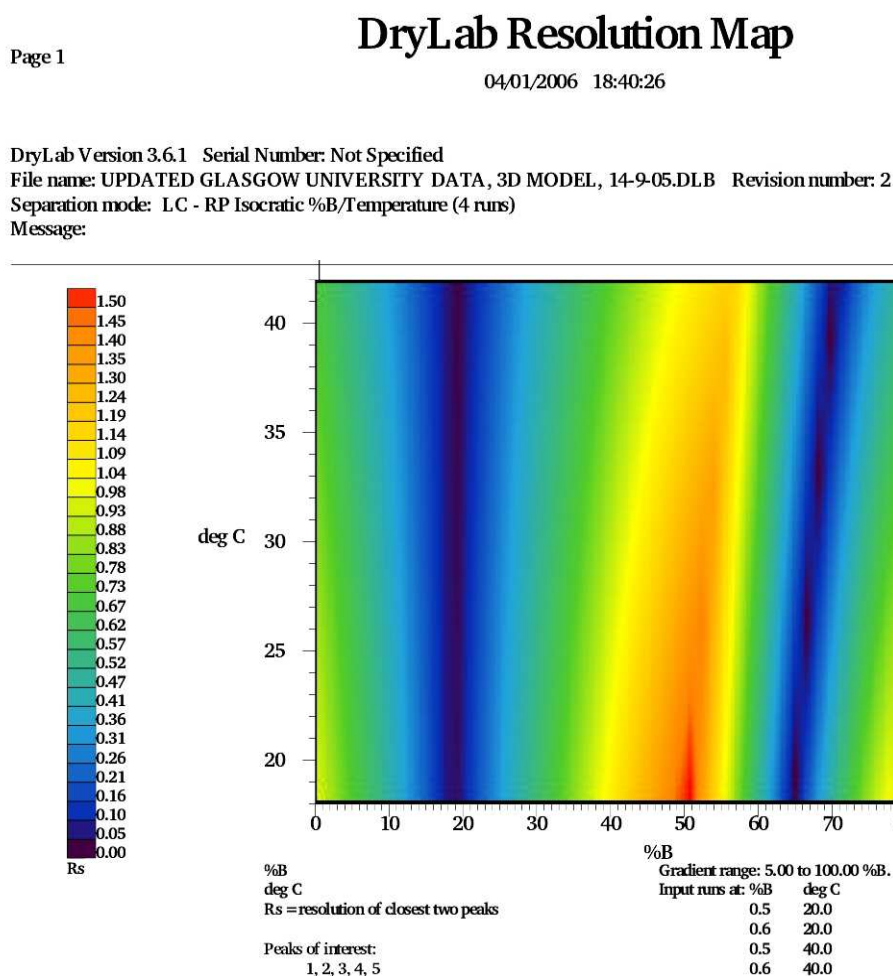


Figure 3.11 DryLab® 3D coloured resolution map for the simultaneous effect of the percentage of acetonitrile in the mobile phase and column temperature on the resolution of all the five peaks in the mixture of 1,4-DMN and the four internal standards

This coloured 3-dimensional plot shows the percentage of acetonitrile in the mobile phase on the X axis (from 0-100 % B) and column temperature on the Y axis (from 18-42 °C) where resolution values are represented by the colour shading (Z axis) of the map. The corresponding resolution range for each colour in the map is shown as a graded colour scale to the left of the resolution map. By choosing any point in the map, a simulated chromatogram is generated for the selected percentage of acetonitrile in the mobile phase and column temperature. In addition, simulated values of the minimum resolution of the critical pair, system backpressure and peak retention information are also provided for any selected point.

By examining the resolution map in Figure 3.11, it can be shown that sample resolution does not correlate to either the percentage of acetonitrile in the mobile phase or column temperature values in a simple way. However, an approximate generalisation can be made to compare the effect of these two variables on the separation based on the structure of the resolution map. The relatively vertical structure of the map indicates that the separation is mainly affected by the percentage of acetonitrile in the mobile phase and less effect of temperature is observed. However, at many values of %B (percentages of acetonitrile in the mobile phase), lower temperatures are preferred for higher peak resolution.

The map also shows clearly the conditions at which good separation is achieved and those where overlapping or coelution occurs. It shows that there are three coelution regions (identified by the black colour) that are more defined by the percentage of acetonitrile in the mobile phase. These coelution areas were at around 20 %, 65 % and above 90 % ACN/water (at almost all T values). In the first coelution area at about 20 %, 1-EtNaph completely coelutes with 1,4-DMN. For closer view of the effect of the different conditions on 1-EtNaph resolution from the other peaks, a new resolution map was rebuilt considering only the resolution of 1-EtNaph from any other peak in the mixture. This map is shown in Figure 3.12.a.

Page 1

a**DryLab Resolution Map**

04/01/2006 18:48:30

DryLab Version 3.6.1 Serial Number: Not Specified

File name: UPDATED GLASGOW UNIVERSITY DATA, 3D MODEL, 14-9-05.DLB Revision number: 2

Separation mode: LC - RP Isocratic %B/Temperature (4 runs)

Message:

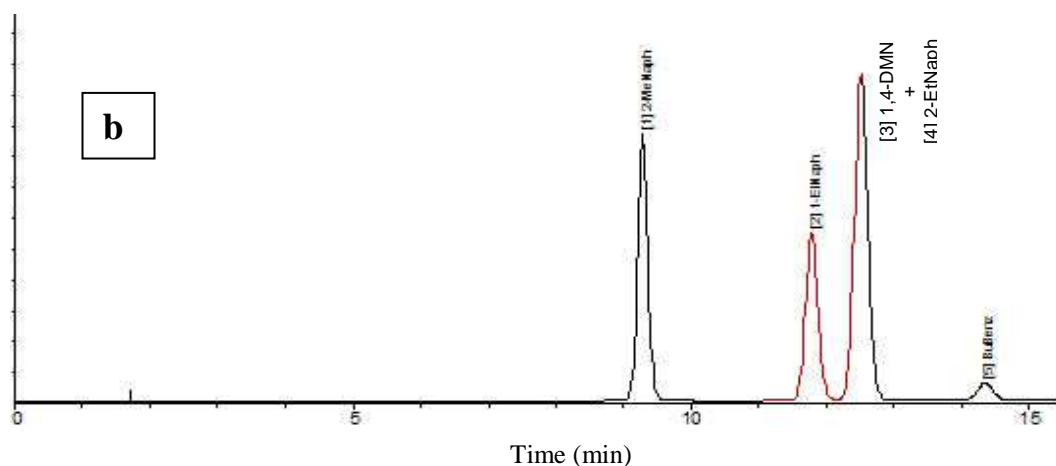
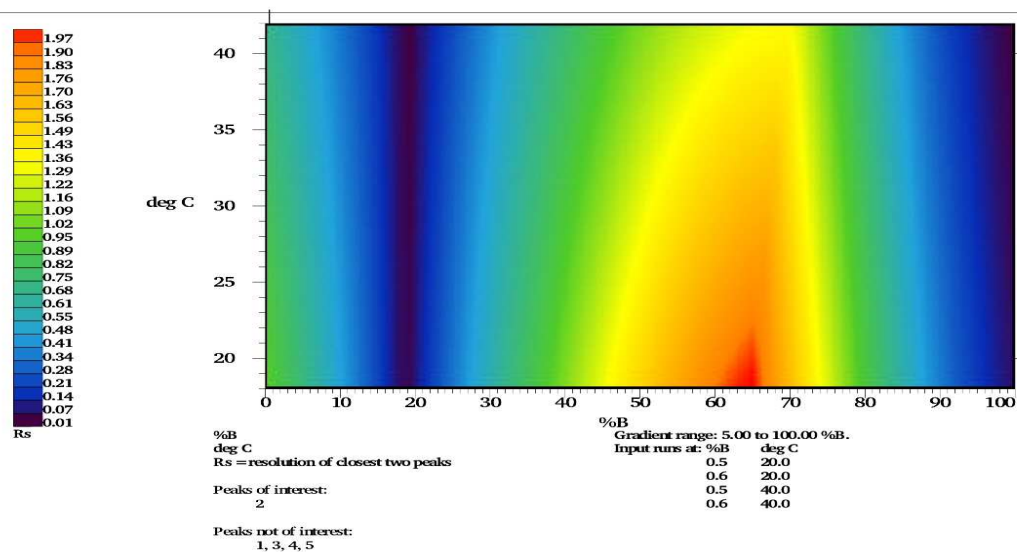


Figure 3.12 Simulation output for 1-EtNaph separation from any other component in the mixture. a) Resolution map (coloured). b) A chromatogram of the optimum conditions for separating the 1-EtNaph peak from all the other peaks in the mixture

As shown in the map above, the resolution of the 1-EtNaph peak from the other peaks remains below 1.00 till about 40 % acetonitrile, after which it starts to increase to get an optimum resolution of 2.01 at about 65 % acetonitrile and with a column temperature of 18 °C, with a run time of only 16 min. The predicted chromatogram at these conditions is shown in Figure 3.12.b.

However, the optimum conditions for the separation of 1-EtNaph from the other compounds in the mixture are the worst for the separation of 2-EtNaph. In these conditions, the 1,4-DMN and 2-EtNaph peaks are completely overlapping and form the second coelution region in the main resolution map (Figure 3.11). To study the separation

behaviour of 2-EtNaph in more detail, the simulation output was based on only the 2-EtNaph peak. The output of this simulation is shown in the resolution map in Figure 3.13.a which considers only the separation of 2-EtNaph peak from all the other peaks.

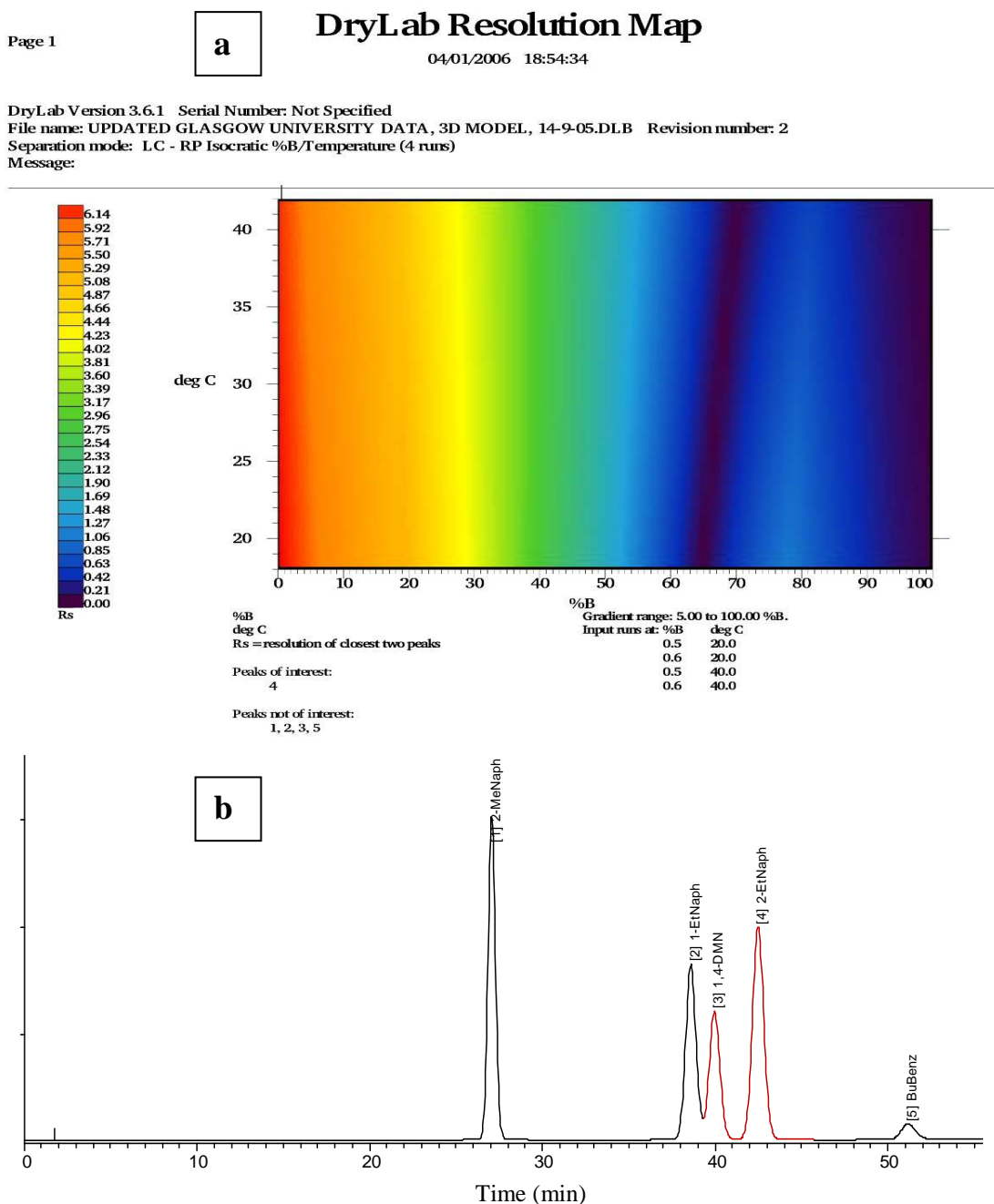


Figure 3.13 Simulation output for 2-EtNaph separation from any other component in the mixture. a) Resolution map (coloured). b) A chromatogram at the optimum conditions for separating the 2-EtNaph peak from all the other peaks in the mixture

The above resolution map shows that the peak of 2-EtNaph starts to have baseline separation ($R_s=1.5$) from the 1,4-DMN peak at about 50 % acetonitrile then increases by the decrease of the percentage of acetonitrile to get values of $R_s > 2$ at 46 % acetonitrile and 20 °C in 70 min run time. Lower temperatures generally enhance 2-EtNaph resolution. However the separation time can be reduced by raising the temperature to about 30 °C. The

optimum conditions that provide the required separation ($R_s \geq 2$) for 2-EtNaph from the other peaks in the shortest possible run time can be achieved at 47 % acetonitrile and 30 °C. The chromatogram under these conditions is shown in Figure 3.13.b, with the 2-EtNaph peak at 42.45 min with a minimum resolution of 2.06 from the adjacent 1,4-DMN peak.

These observations clearly confirm the opposite response of the two critical pairs 1-EtNaph/1,4-DMN and 2-EtNaph/1,4-DMN towards the changes in the percentage of acetonitrile in the mobile phase which was noticed previously in the preliminary experiments. While 2-EtNaph is well separated from 1,4-DMN with a low percentage of acetonitrile in the mobile phase, higher concentrations of ACN are required for better separation of the 1-EtNaph-DMN pair.

By reviewing the main resolution map for the five mixture components (Figure 3.11) and the predicted chromatograms generated for each point in the map in DryLab® software, it was possible to track peak order and check for any peak reversal. It was interesting to see that after the overlapping of the 2-EtNaph and 1,4-DMN peaks at about 65 % acetonitrile, the order of the two peaks started to change when moving to a higher percentage of acetonitrile in the mobile phase. 1,4-DMN started to elute later than 2-EtNaph, as shown in the simulated chromatogram in Figure 3.14 for 78 % acetonitrile at 18 °C.

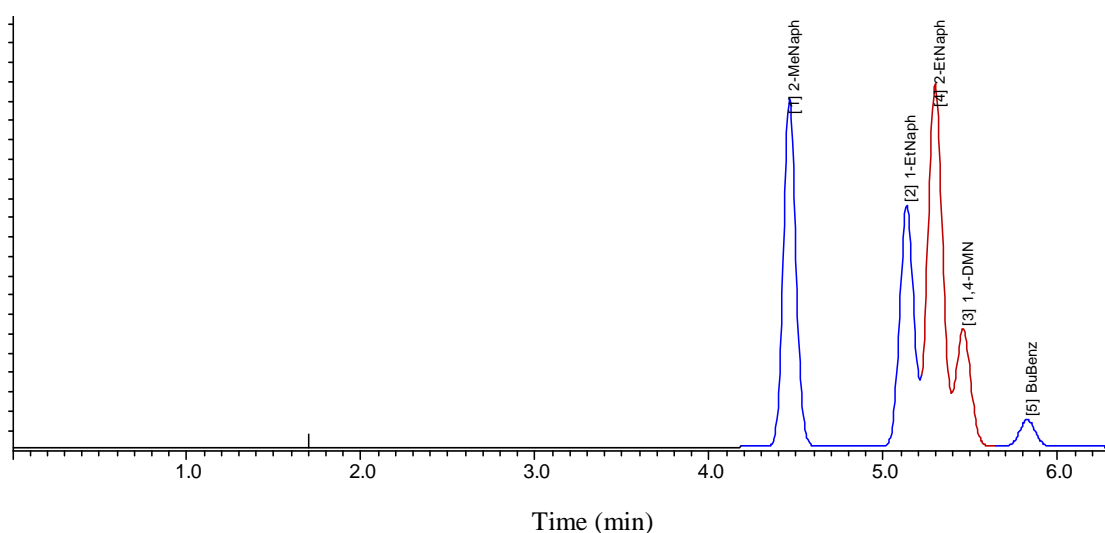


Figure 3.14 The reversal of the elution order of the 1,4-DMN and 2-EtNaph peaks at 78 % B and 18 °C

The third and last overlapping region shown in the main resolution map in Figure 3.11 was at 90 % acetonitrile and higher, at which all the five peaks started to overlap. This is a

normal effect of a very high concentration of ACN that causes a fast elution of all the mixture components out of the stationary phase in the column.

The previous discussion was an overview of some of the information that can be extracted from the DryLab[®] simulation results, which help in understanding the separation behaviour of the mixture of 1,4-DMN and the four internal standards. However, the main purpose of the simulation was to search for the optimum conditions that provide the best separation of the mixture, and this is discussed in the next paragraphs.

Optimum separation on the ACE 5 μ m column

The optimum separation, which provided the maximum resolution for the critical pair, predicted by DryLab[®] software for the mixture of 1,4-DMN and the four internal standards, is found to be in a narrow range around 51 % acetonitrile and at temperatures lower than 20 °C. This range of best separation is indicated by the red colour in the resolution map in Figure 3.11. The best predicted resolution for this separation (considering all the critical pairs) was 1.50 for the critical pair 1,4-DMN/2-EtNaph at 51 % acetonitrile and 18 °C. The simulated chromatogram at these conditions is shown in Figure 3.15.a. To verify the optimum simulated conditions, the predicted conditions were applied experimentally (3 replicates). A representative chromatogram is shown in Figure 3.15.b.

For further evaluation and validation of the accuracy of DryLab[®] predictions, several other chromatographic conditions (with predicted chromatograms and peak retention information) were simulated and then applied experimentally. The theoretical predictions of retention times and resolution were then compared to the experimental results. This comparison is shown in Table 3.14 and Table 3.15 for retention times and resolution respectively. The tables include the optimum conditions in addition to four other conditions.

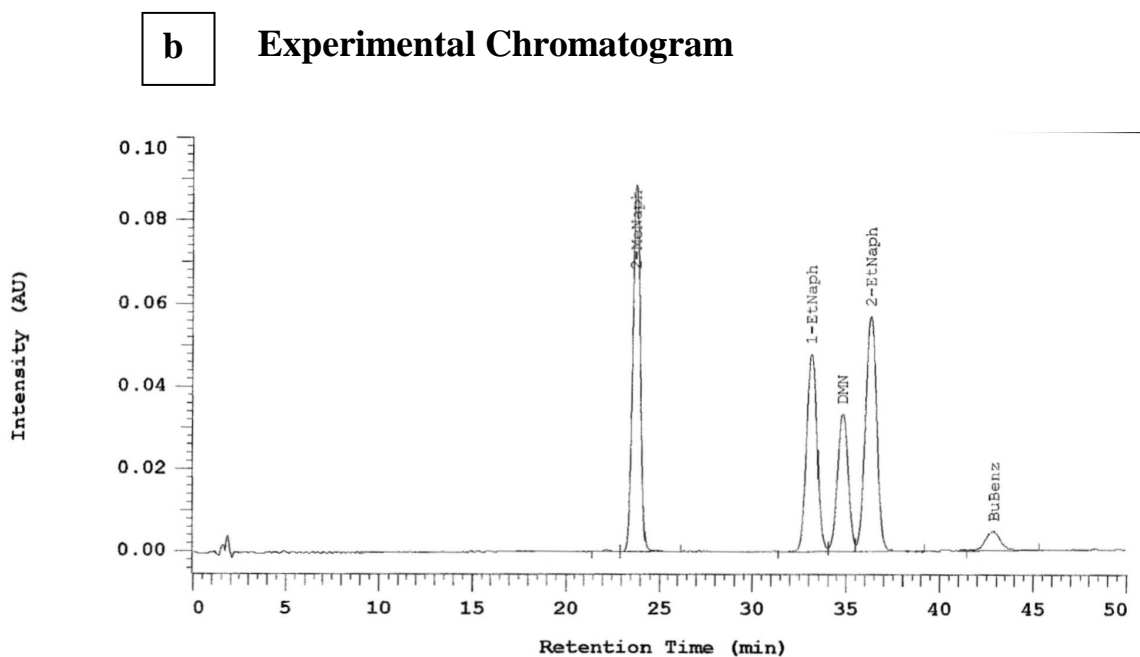
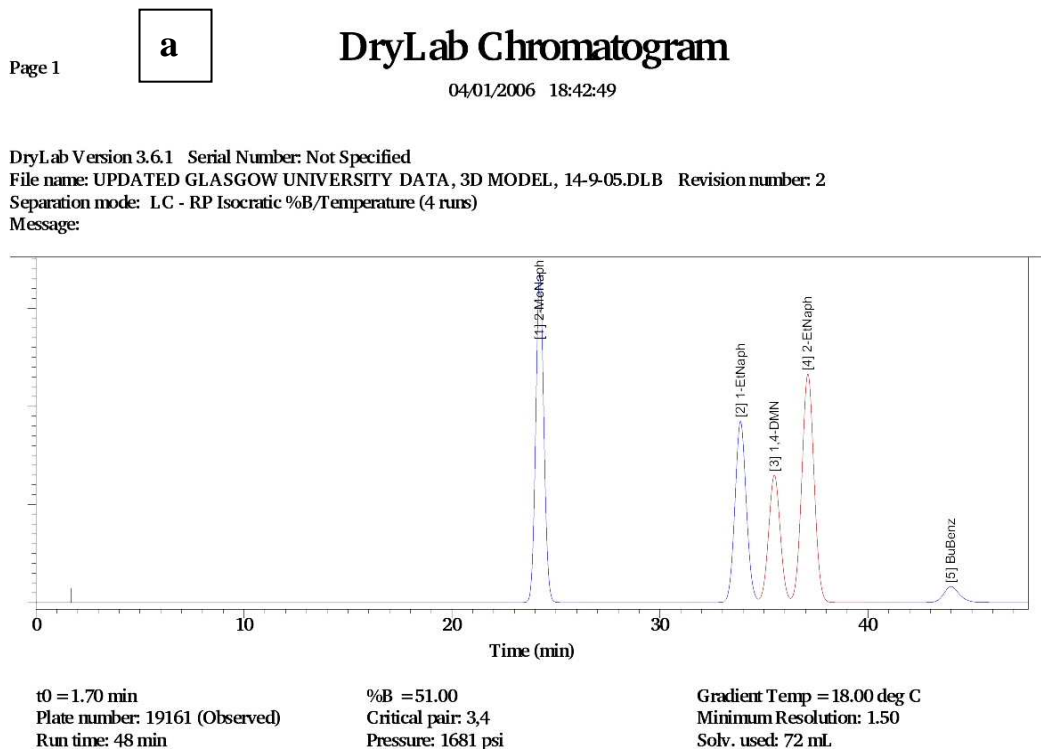


Figure 3.15 The best separation for 1,4-DMN and the four internal standards on the ACE 5 μ m column at 51 % B and 18 °C. a) Simulated chromatogram. b) Experimental chromatogram

Table 3.14 A comparison between DryLab® predictions of retention times and experimental results.

Conditions	Compound	Retention Time (min)				
		DryLab®	Experimental (Mean)	Exp. RSD (%)	Difference (min)	Difference (%of Exp)
51 % B 18 °C 1.5 ml/min	2-MeNaph	24.22	23.77	0.10	-0.45	1.89
	1-EtNaph	33.88	33.13	0.12	-0.75	2.26
	1,4-DMN	35.50	34.79	0.17	-0.71	2.04
	2-EtNaph	37.12	36.28	0.15	-0.84	2.32
	BuBenz	44.00	42.87	0.22	-1.13	2.64
	Average Error			0.15	-0.78	2.23
51 % B 18 °C 1.0 ml/min	2-MeNaph	35.62	35.95	0.03	0.33	0.92
	1-EtNaph	49.82	50.12	0.10	0.30	0.60
	1,4-DMN	52.21	52.61	0.13	0.40	0.76
	2-EtNaph	54.58	54.91	0.17	0.33	0.60
	BuBenz	64.70	65.01	0.18	0.31	0.48
	Average Error			0.12	0.33	0.67
57 % B 42 °C 1.5 ml/min	2-MeNaph	11.54	11.47	0.20	-0.07	0.64
	1-EtNaph	15.26	15.13	0.25	-0.13	0.84
	1,4-DMN	15.82	15.70	0.28	-0.12	0.76
	2-EtNaph	16.38	16.24	0.26	-0.14	0.88
	BuBenz	19.44	19.24	0.32	-0.20	1.02
	Average Error			0.26	-0.13	0.83
52 % B 35 °C 1.5 ml/min	2-MeNaph	17.68	17.82	0.07	0.14	0.79
	1-EtNaph	24.34	24.55	0.03	0.21	0.86
	1,4-DMN	25.26	25.49	0.09	0.23	0.90
	2-EtNaph	26.47	26.71	0.14	0.24	0.90
	BuBenz	31.67	32.01	0.14	0.34	1.06
	Average Error			0.09	0.23	0.90
57 % B 23 °C 1.5 ml/min	2-MeNaph	14.80	14.43	0.10	-0.37	2.56
	1-EtNaph	19.83	19.27	0.10	-0.56	2.91
	1,4-DMN	20.85	20.24	0.14	-0.61	3.01
	2-EtNaph	21.41	20.79	0.20	-0.62	2.98
	BuBenz	25.13	24.41	0.12	-0.72	2.95
	Average Error			0.13	-0.58	2.88

Table 3.15 Comparison between DryLab[®] predictions of peak resolution and experimental results.

Conditions	Compound	Resolution				
		DryLab [®]	Experimental (Mean)	Exp. RSD (%)	Difference (min)	Difference (% of Exp)
51 % B 18 °C 1.5 ml/min	2-MeNaph	11.07	11.66	0.35	0.59	5.06
	1-EtNaph	1.54	1.71	0.44	0.17	9.94
	1,4-DMN	1.50	1.47	0.90	-0.03	2.04
	2-EtNaph	5.75	5.53	1.03	-0.22	3.98
	BuBenz					
	Average Error			0.68	0.13	5.26
51 % B 18 °C 1.0 ml/min	2-MeNaph	10.88	11.43	0.55	0.55	4.81
	1-EtNaph	1.52	1.68	0.49	0.16	9.52
	1,4-DMN	1.48	1.46	0.87	-0.02	1.37
	2-EtNaph	5.65	5.53	0.98	-0.12	2.17
	BuBenz					
	Average Error			0.72	0.14	4.47
57 % B 42 °C 1.5 ml/min	2-MeNaph	9.55	10.02	0.62	0.47	4.69
	1-EtNaph	1.22	1.33	0.43	0.11	8.50
	1,4-DMN	1.19	1.19	1.28	0.00	0.28
	2-EtNaph	5.85	5.79	1.30	-0.06	1.04
	BuBenz					
	Average Error			0.91	0.13	3.63
52 % B 35 °C 1.5 ml/min	2-MeNaph	10.90	11.51	0.40	0.61	5.30
	1-EtNaph	1.26	1.37	0.92	0.11	8.03
	1,4-DMN	1.62	1.69	0.86	0.07	4.14
	2-EtNaph	6.16	5.85	1.26	-0.31	5.30
	BuBenz					
	Average Error			0.86	0.12	5.69
57 % B 23 °C 1.5 ml/min	2-MeNaph	9.96	10.62	0.29	0.66	6.21
	1-EtNaph	1.72	1.80	0.33	0.08	4.44
	1,4-DMN	0.93	0.95	0.68	0.02	2.11
	2-EtNaph	5.56	5.50	0.84	-0.06	1.09
	BuBenz					
	Average Error			0.54	0.18	3.46

As shown in Table 3.14 and Table 3.15, a good agreement was obtained between DryLab[®] predicted values for retention times (T_R) and resolution (R_s), and the experimentally observed ones. The average error of prediction was in the range ± 0.67 to ± 2.88 %, and ± 3.46 to 5.69 % for retention times and resolution respectively. The maximum experimental contribution in this error (evaluated by the %RSD of three replicates) was, in the worst case, ± 0.26 % and ± 0.91 % for T_R and R_s respectively. The average errors reported in the literature for diverse RP-HPLC applications of DryLab[®] were in the range ± 0.3 % to ± 9.6 %.

% and ± 6.5 % to ± 11.8 % for retention times and resolution respectively. Compared to the reported values, it should be emphasised that the errors of prediction reported here are well within the range of the reported values in literature (Lammerhofer et al., 1997; Hajnos et al., 2002; Didaoui et al., 2003; Lewis et al., 1992; Hoang et al., 2003; Chloupek et al., 1992; Wrisley, 1993). From this comparison, DryLab[®] software proved to be powerful and useful software for chromatographic method development by providing accurate predications.

As shown in Figure 3.15 and Table 3.15, the optimum separation of 1,4-DMN and the four internal standards on the ACE column achieved experimentally a maximum resolution of 1.47 for the critical pair 1,4-DMN/2-EtNaph. However, this resolution was still lower than the set goal for this method. The method aimed to provide reproducible and rugged separation of the five compounds with a minimum resolution of at least $R_s=2$ for any pair of peaks. This resolution value or greater is a desirable target for method development (Snyder et al., 1997).

The achieved resolution of 1.47 may not withstand small changes in experimental conditions, as indicated by the narrow range of the best separation in the resolution map in Figure 3.11. Simulated resolution values at conditions around the optimum conditions are shown in Table 3.16. An experimental error of 2 % in the percentage of the organic component in the mobile phase value is not unlikely in HPLC (Snyder et al., 1997). As can be seen from Table 3.16, such an error causes critical resolution to drop down to as low as 1.27 at 52 % acetonitrile, which result in an overlap between the 1,4-DMN peak and the 2-EtNaph peak. This value cannot of course be accepted for rugged quantitative analysis. Therefore, the optimum separation on the ACE 5 μm column at 51 % acetonitrile and 18 $^{\circ}\text{C}$ was not accepted for the purpose of internal standard quantification experiments, as it may not provide a sufficiently reproducible and rugged method.

Table 3.16 Critical resolution values predicted by DryLab® for the optimum separation (bold) and a range of other conditions around it

% B	T °C	Rs of Critical Pair	Critical Pair
48	18	1.42	1-EtNaph/1,4-DMN
49	18	1.46	1-EtNaph/1,4-DMN
50	18	1.50	1-EtNaph/1,4-DMN
51	18	1.50	1,4-DMN/2-EtNaph
52	18	1.39	1,4-DMN/2-EtNaph
53	18	1.27	1,4-DMN/2-EtNaph
50	19	1.48	1-EtNaph/1,4-DMN
50	20	1.46	1-EtNaph/1,4-DMN
50	21	1.44	1-EtNaph/1,4-DMN

Comparing different 5 µm columns at the optimum predicted conditions

Because of not achieving the targeted resolution on the ACE column, and in view of the fact that columns from different suppliers, but of the same bonded phase, can provide different selectivity (and therefore resolution) (Snyder et al., 1997), four other C18 columns with 5 µm particles were tested in a search for better resolution. The columns are listed in Table 3.17 along with some of their properties and the experimentally observed efficiency, tailing factors and critical resolution at 51% acetonitrile and 18 °C. The resulting chromatograms at these conditions are shown in Figure 3.16 a-d for the four columns.

Table 3.17 A comparison between the five C18, 5 µm columns examined for the best resolution of the mixture of 1,4-DMN and the 4 internal standards

Column	Surface Area (m ² /g)	Carbon Load %	Efficiency (Average Plate No.)*	Tailing Factor*	Critical Resolution	Run Time (min)
ACE C18	300	15.5	19161	1.02	1.47	47
Dionex Acclaim 120	300	17.9	17410	1.14	1.49	60
Hypersil HyPurity Elite	200	13.0	15991	1.30	1.28	30
Waters Spherisorb ODS-2	220	11.5	17741	1.22	1.11	32
Phenomenex Spherclone ODS-2	200	12.0	15082	1.26	0.79	40

* Average values obtained experimentally for the five peaks in the mixture at 51 % B and 18 °C

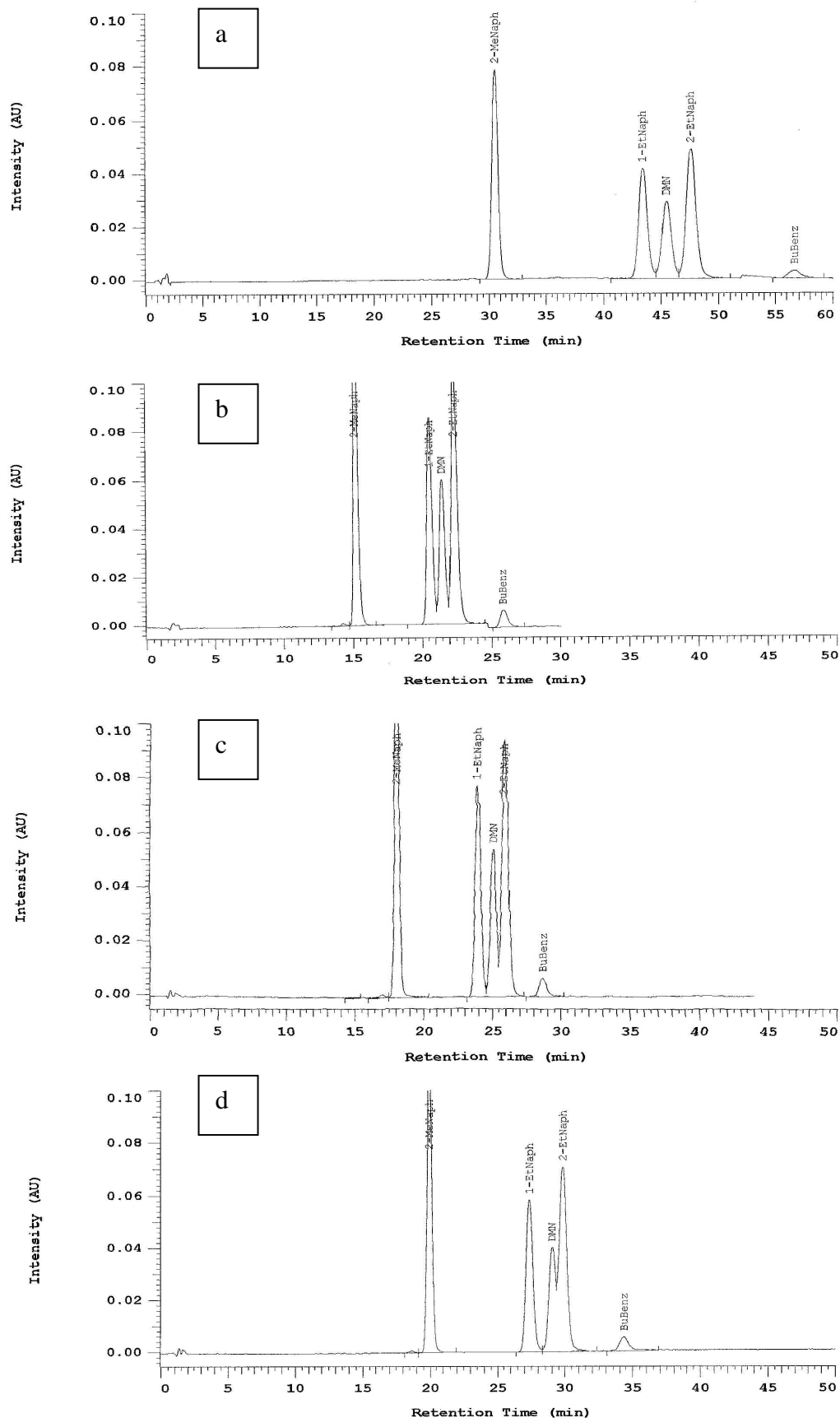


Figure 3.16 Separation of 1,4-DMN and the 4 internal standard mixture under the same chromatographic conditions on four different C18, 5 μ m columns. a) Dionex Acclaim 120. b) Hypersil HyPurity Elite. c) Waters Spherisorb ODS-2. and d) Phenomenex Sphereclone ODS-2

As can be seen from Table 3.17 and Figure 3.15.b and 3.16, the ACE and Dionex columns provided the best separation among the five columns examined, with a resolution value approaching 1.50. However, the Dionex column provided the resolution with the longest run time among the five columns. These two columns are characterised by having the highest carbon load and surface area for their solid phases. Carbon load, in particular, can give a rough guide for the retention ability of a column. The high resolution provided by the two columns might be attributed to their higher retention capability, as longer retention usually improves the separation (Snyder et al., 1997). The lower resolution provided by the other three columns (Hypersil, Waters and Phenomenex) seems to be a result of the higher asymmetry, lower efficiency, the need for more optimisation of the percentage of the acetonitrile in the mobile phase, or combinations of those three factors.

This comparison between the ACE C18 5 μm column and the other four columns shows that none of the other four columns tested provided considerably more enhancement in the resolution of the critical pairs than what had been provided by the ACE column. Therefore, it was decided to examine a column with a lower particle size.

Optimising the separation on a column with 3 μm particles

Due to not achieving sufficient and rugged resolution ($R_s \geq 2$) for the critical pairs on any of the five C18 5 μm columns, several simulated experiments were conducted using DryLab[®] to optimise column parameters, including mainly column particle size and flow rate, in a search for better resolution of the critical pairs. These experiments were based on using lower particle size for the C18 column, which should provide higher separation efficiency and therefore better resolution. However, by using smaller particle size, one should consider monitoring the higher system backpressure generated by these columns. As a result, flow rate has to be optimised here to maintain reasonable system backpressure. The simulated experiments here focused on obtaining the targeted resolution with a reasonable system backpressure (less than 3500 psi) and run time. Table 3.18 summarises some of the conditions that provide higher resolution value than the 1.50 that was approached by the 5 μm columns. It should be noted that all the predictions here were based on using a column with packing material having similar characteristics to the ACE C18 material that was used in generating the DryLab[®] model.

Table 3.18 Simulated chromatographic conditions that provide higher resolution than 1.50 based on using a 3 μ m column. Predicted critical resolution, system backpressure and run time are provided for each set of conditions

Flow Rate (ml/min)	%B	T ($^{\circ}$ C)	Critical Resolution	Pressure (psi)	Run Time (min)
1.50	51	18	1.80	4657	47
1.50	53	32	1.62	3427	33
1.50	54	35	1.61	3191	29
1.30	51	26	1.60	3430	49
1.30	51	18	1.77	4036	55
1.00	50	20	1.63	3016	76

As shown in Table 3.18, using an ACE column with 3 μ m packing particles would enhance the resolution of the critical pair to a maximum value of 1.80 at the optimum conditions predicted for 5 μ m columns, which are 51 % acetonitrile, 18 $^{\circ}$ C and 1.50 ml/min. However, at these conditions the system backpressure would be above the maximum affordable backpressure of 4000 psi for the pump used, which makes these conditions impractical. Therefore, the flow rate has to be reduced with some adjustment to the other conditions in order to maintain an affordable system backpressure. Unfortunately, this has to sacrifice some of the resolution.

Among the different simulated conditions tested, the last set of conditions (50 % acetonitrile, 20 $^{\circ}$ C and 1.00 ml/min) was found to provide a good balance between the resolution and reasonable backpressure. Although the run time at these conditions was predicted as 76 min, this can be an acceptable run time for the purpose of the method, as it is not going to be used for routine analysis. This set of conditions was predicted to provide a resolution value of 1.63 for the critical pair on an ACE column with 3 μ m packing particles.

It should be noted that all the previous simulations were based on using ACE packing materials. Unfortunately, the only ACE column available during this study was the ACE 5 μ m that was used for generating the DryLab[®] model. Instead, an HPLC Technology TechSphere ODS 3 μ m (250 x 4.6 mm) column was used to test the predicted conditions for the 3 μ m column. Due to the variations between the two packing materials, it would not be unexpected to have some differences between the predicted results for an ACE column and the practical data obtained by the TechSphere column. The main one would be the changes in the critical peak pairs (as noticed with the different 5 μ m column compared

above). Such changes in critical peak pairs from column to column are the major problem in method optimisation using different columns (Molnar, 2002)

The optimum predicted conditions of 50 % acetonitrile at 20 °C with a flow rate of 1.00 ml/min were examined experimentally using the TechSphere 3 µm column. The resulting chromatogram is shown in Figure 3.17.a.

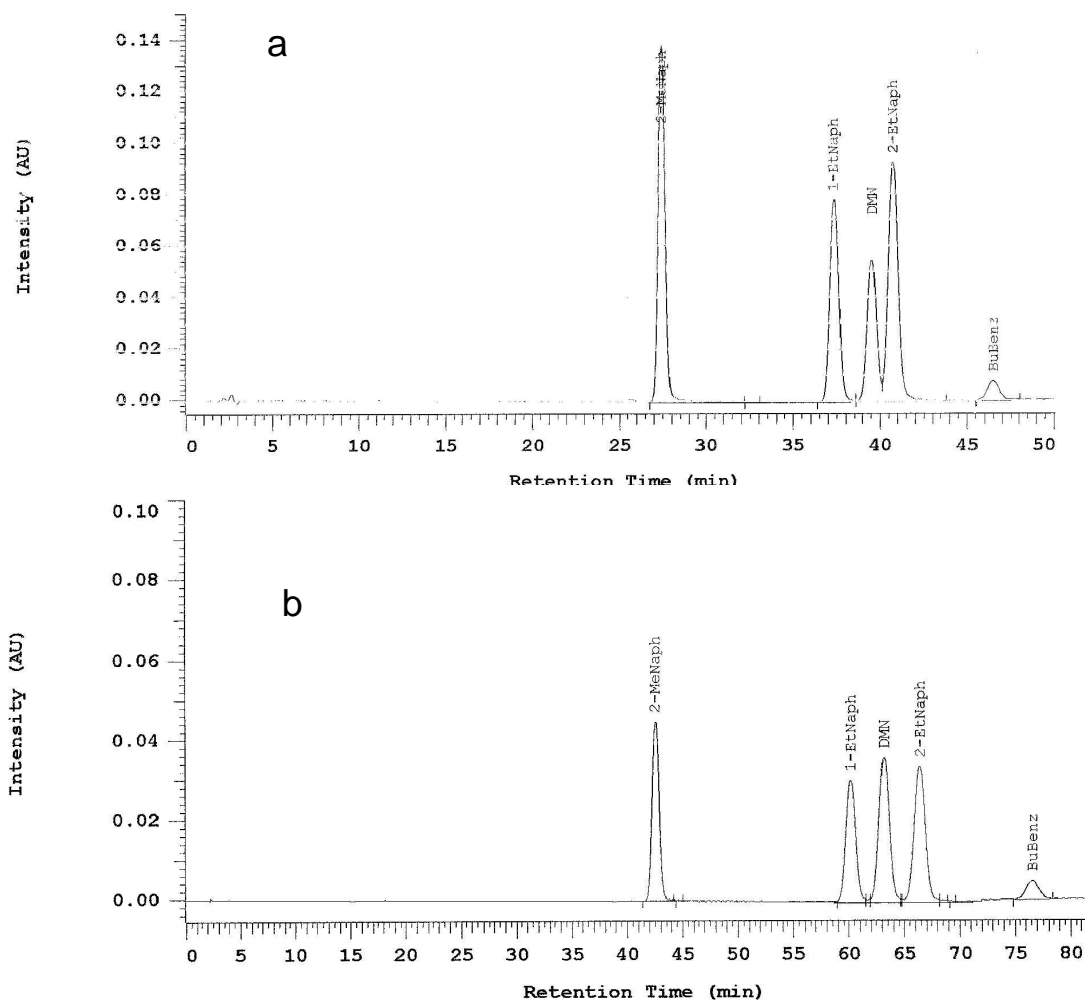


Figure 3.17 Typical chromatograms for the separation of the 1,4-DMN and the four internal standards on the TechSphere ODS 3 µm (250 x 4.6 mm) column. a) With 50 % B at 1.0 ml/min and 20 °C b) With 46 % B at 23 °C and a flow rate of 0.90 ml/min

As can be concluded from the chromatogram in Figure 3.17.a, the optimum conditions predicted for an ACE 3 µm column are not the same as those for the TechSphere column. An extra resolution of 2.45 between 1-EtNaph and 1,4-DMN peaks was achieved at the cost of only 1.30 between 1,4-DMN and 2-EtNaph peaks. As it was concluded previously, 1,4-DMN peak overlaps more with 2-EtNaph at a high percentage of acetonitrile in the mobile phase and with 1-EtNaph at a low percentage of acetonitrile in the mobile phase. By applying this conclusion to the present chromatogram in Figure 3.17.a, it can be concluded that a lower percentage of acetonitrile in the mobile phase is required to obtain

the optimum conditions for this column. However, lowering the percentage of acetonitrile in the mobile phase resulted in an increase in system backpressure, and therefore more practical step-by-step experiments were required to optimise the percentage of acetonitrile in the mobile phase, flow rate and column temperature for sufficient resolution with affordable system backpressure and in an acceptable run time. These experiments are summarised in Table 3.19.

Table 3.19 Practical experiments for optimising the separation of 1,4-DMN and the four internal standards using TechSphere 3 μ m (250 x 4.6 mm) column

% B	T (°C)	Flow Rate (ml/min)	Critical Pair	Critical Resolution	Run Time (min)
50	20	1.00	1,4-DMN/2-EtNaph	1.30	50
47	20	1.00	1,4-DMN/2-EtNaph	1.71	65
45	20	0.90	1,4-DMN/2-EtNaph	1.96	90
45	25	1.00	1-EtNaph/1,4-DMN	1.82	80
46	23	0.90	1-EtNaph/1,4-DMN	1.97	82

The optimum conditions that balance between sufficient resolution, reasonable system backpressure and time, using the TechSphere 3 μ m column, were found to be at 46 % acetonitrile and 23 °C with a flow rate of 0.90 ml/min. These conditions provided a resolution of 1.97 for the critical pair 1-EtNaph/1,4-DMN and 1.98 for the pair 1,4-DMN/2-EtNaph and with a run time of 82 min. A typical chromatogram for the separation of the mixture of 1,4-DMN and the four internal standards at these conditions is shown in Figure 3.17.b.

Despite the fairly long run time at these conditions, the achieved resolution overcomes it, particularly in the knowledge that the method was not designed for routine measurements but for a small number of measurements that require adequate separation of all the peaks in the mixture. These conditions have been chosen for the final method for separating and quantifying 1,4-DMN and the four internal standard candidates.

3.3.2.6 Final method and basic validations

The chromatographic conditions for the final method for separating and quantifying 1,4-DMN and the four internal standards were as follows:

- **Column:** HPLC Technology TechSphere ODS 3 μm (250 x 4.6 mm)
- **Mobile phase:** 46 % acetonitrile/water
- **Flow rate:** 0.90 ml/min
- **Column temperature:** 23 $^{\circ}\text{C}$
- **Run time:** 82 min
- **Detector wavelength:** 228 nm from 0 to 71 min then 210 nm

A typical chromatogram for the separation of 1,4-DMN and the four internal standards at these conditions is shown in Figure 3.17.b.

Because this method was designed for quantitative measurements, basic validation checks were carried out on the final separation method. System precision and linearity of the detector response for each component were the main ones. For the system precision test, five replicate injections of 10 $\mu\text{g/ml}$ mixed standard of the five compounds were carried out and the %RSD of the resulting peak areas were calculated for each component. %RSD values were 0.73, 0.35, 0.43, 0.53 and 2.61 for 2-methylnaphthalene, 1-ethylnaphthalene, 1,4-dimethylnaphthalene, 2-ethylnaphthalene and n-butylbenzene respectively. Good precision was achieved for the mixture components except, n-butylbenzene that expressed the highest variability between injections.

For the linearity test, duplicate injections were made of standards in the range 2-20 $\mu\text{g/ml}$ (2, 4, 6, 8, 10, 15 and 20 $\mu\text{g/ml}$). All five components showed good linear responses with $r^2 \geq 0.999$ for the range examined.

3.3.2.7 Summary

A RP-HPLC method for the separation and quantification of 1,4-DMN and four internal standards was successfully developed and validated. The method was based on using an HPLC Technology TechSphere ODS 3 μm (250 x 4.6 mm) column and 46 % acetonitrile/water as the mobile phase. The flow rate was 0.9 ml/min and the separation was carried out at a column temperature of 23 $^{\circ}\text{C}$. At these conditions, the mixture components were adequately separated in 82 min with a minimum resolution of 1.97 for the critical pair. The detector had to be programmed to generate two-wavelength chromatograms of 228 nm for all the mixture components, except BuBenz at 210 nm. Linearity of the detector response for the five compounds was found to be sufficient. System precision was satisfactory. However, the BuBenz peak showed lower reproducibility in peak areas than the other four peaks.

The poorer reproducibility for the BuBenz peak in addition to the need for a different detection wavelength may weigh against the choice of BuBenz to work as an internal standard for 1,4-DMN. BuBenz was initially selected as a candidate internal standard for 1,4-DMN because it has close $K_{o/w}$ coefficient and water solubility values to those for 1,4-DMN. However, the differences between the melting points and the boiling points between the two compounds suggest differences in the physicochemical properties between the two. These differences seem to cause BuBenz to behave differently in the HPLC separation (and possibly in the extraction process) compared to the rest of the compounds examined here.

The case is different for 2-EtNaph. Despite the good absorbance at 228 nm and the good peak area precision of 2-EtNaph, it has been shown that it needs about 50 min (see Figure 3.13.b) to have a sufficient and rugged separation from 1,4-DMN (using ACE 5 μ m column), which is too long for a routine method. This may count against 2-EtNaph as an internal standard for 1,4-DMN. 2-MeNaph and 1-EtNaph were shown to be chromatographically the most suitable internal standards for 1,4-DMN in an HPLC method.

Despite these chromatographic obstacles, it is important to study the extraction behaviour of the candidates in order to select the most suitable one.

The second objective of this part of the work was to evaluate the usefulness and accuracy of DryLab[®] chromatographic simulation software in assisting the separation development and optimisation. DryLab[®] software was found to be powerful and useful software for chromatographic method development. Based on only four practical input experiments, numerous simulated experiments can be evaluated in minutes. It has been found to generate accurate predictions with average errors as low as 0.7 % and 3.5 % in retention times and resolution respectively. It was very useful in determining the optimum separation for the present mixture through resolution maps. It also helped to gain more understanding of the separation behaviour of the mixture components and their responses towards changes in chromatographic conditions.

3.3.3 Separation method for 1,4-DMN and 1-ethylnaphthalene

This part of the work aimed to develop an HPLC separation method for the routine analysis of extracts containing the analyte 1,4-DMN and 1-ethylnaphthalene as the internal standard. The development of this method was aided by the DryLab[®] simulation model developed in the previous section. The separation behaviour of 1-EtNaph peak and 1,4-

DMN peak was studied using the resolution map shown above in Figure 3.12.a. As discussed above, the optimum resolution of the pair 1-EtNaph/1,4-DMN is predicted to be 2.01 at 65 % acetonitrile and with column temperature of 18 °C and a run time of only 15 min using the ACE 5 μ m column. The predicted chromatogram at these conditions is shown above in Figure 3.12.b.

The optimum conditions, along with some other conditions around the optimum, were tested experimentally to evaluate the ruggedness of the method. The conditions examined experimentally are shown in Table 3.20.

Table 3.20 Practical experiments for the separation of a mixture of 1,4-DMN and 1-ethylnaphthalene at different conditions using the ACE 5 μ m column

% B	T °C	Flow Rate (ml/min)	Critical Rs (Mean of 3 Replicates)
65	18	1.5	2.17
65	25	1.5	1.95
67	25	1.5	1.94
62	25	1.5	1.85

As shown in Table 3.20, better resolution of 1,4-DMN and 1-EtNaph was obtained experimentally. The separation of the two compounds at the optimum conditions is shown in the chromatogram in Figure 3.18.

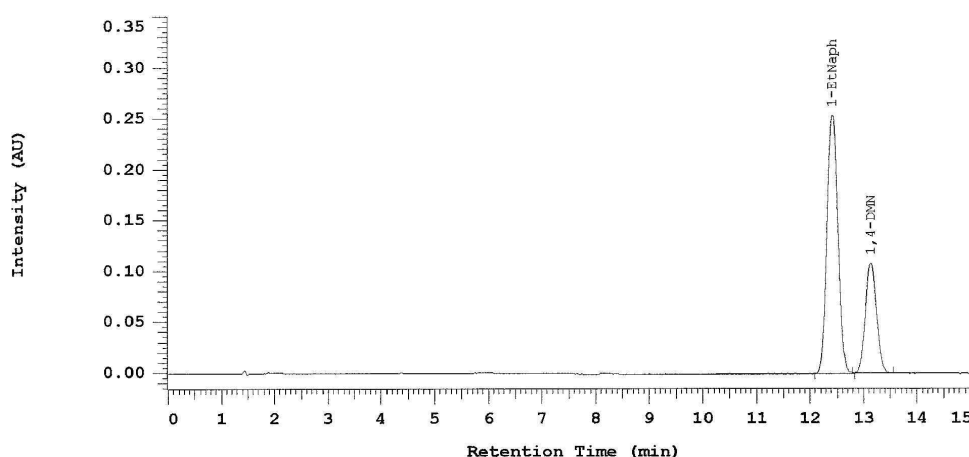


Figure 3.18 A chromatogram of a mixed standard of 1-ethylnaphthalene (50 μ g/ml) and 1,4-DMN (20 μ g/ml) showing the optimum separation of the two compounds. Chromatographic conditions: ACE C18 5 μ m (250 x 4.6 mm), 65 % acetonitrile: 35 % water, 1.5 ml/min, 18 °C and 228 nm

Table 3.20 also shows that the method was found to be sufficiently rugged and can tolerate variations of %ACN up to ± 2 % and temperatures up to 25 °C with only small reductions in the resolution value (lowest R_s was > 1.9).

Therefore, the following method was considered for routine analysis of samples containing 1,4-DMN and 1-ethylnaphthalene:

- **Column:** ACE C18 5 μm (250 x 4.6 mm)
- **Mobile phase:** 65 % acetonitrile: 35 % water
- **Flow rate:** 1.5 ml/min
- **Injection volume:** 10 μl
- **Temperature:** 18 °C
- **Detector wavelength:** 228 nm
- **Run time:** 15 min
- **1-ethylnaphthalene T_R :** ~12.4 min
- **1,4-DMN T_R :** ~13.1 min

Chapter 4: Separation and extraction methods for 1,4-DMN and internal standards - Comparison of extraction behaviours in the TMP/Heat method

4.1 Introduction

A main criterion in selecting a compound as an internal standard in an analytical method is to have similar behaviour in the analytical procedure to that of the analyte, particularly in the extraction and clean up steps. In addition, the internal standard has to be well separated (in most chromatographic techniques) from all other compounds in the chromatographic separation.

The original GC version of the TMP/Heat method (described briefly in Section 1.8) uses 2-ethylnaphthalene as an internal standard for the calculation of the final extract volume in addition to the correction of any losses of the analyte during the extraction procedure. However, because it was not possible to separate 2-ethylnaphthalene from 1,4-DMN in HPLC in a reasonable run time for routine analysis (see Section 3.3.1), this internal standard had to be replaced with a more suitable one for HPLC routine analysis.

For the purpose of selecting a replacement for 2-ethylnaphthalene in the TMP/Heat method, three internal standard candidates were initially selected based on their close physicochemical properties to 1,4-DMN. These candidates were: 2-methylnaphthalene, 1-ethylnaphthalene and n-butylbenzene. However, from the development and validation results in Chapter 3 (see Section 3.3.2), n-butylbenzene was excluded from any further investigations as an internal standard candidate because it has to be measured at a different wavelength than the one used for 1,4-DMN, in addition to its low precision in replicate analysis in the HPLC method used for the separation of 1,4-DMN and the four internal standards.

Since the TMP/Heat method includes a liquid-liquid extraction step with the use of the internal standard to correct for the final organic extract volume, it was essential to check the ability of the different candidates to mimic and track 1,4-DMN in this extraction, in addition to any losses of 1,4-DMN (e.g. by volatilisation or adsorption to surfaces) during the extraction procedure. In order to determine this ability, the relative recoveries (yield) of 1,4-DMN and the internal standards had to be determined. For this purpose, 1-

ethylnaphthalene and 2-methylnaphthalene (in addition to 2-ethylnaphthalene for comparison) were examined for their relative recoveries compared to 1,4-DMN .

The comparison between 1,4-DMN and the three internal standards (2-methylnaphthalene, 1-ethylnaphthalene and 2-ethylnaphthalene) was based on three recovery experiments. The first recovery experiment simultaneously compared the extraction behaviour of the three internal standards with that of 1,4-DMN by spiking potato peel with the four compounds and extracting them according to the TMP/Heat method. The extracts were analysed by the method for 1,4-DMN and the four internal standards described in Section 3.3.2.6. The second recovery experiment was conducted to examine the recovery of 1,4-DMN using the TMP/Heat method with 1-ethylnaphthalene as the internal standard, in the way it would be used for the routine analysis. In this experiment and the third experiment, potato peel was spiked with only 1,4-DMN and the internal standard was added to the extraction solution. The extracts obtained in the second experiment were analysed by the HPLC separation method for 1,4-DMN and 1-ethylnaphthalene described in Section 3.3.3. The third recovery experiment was designed to evaluate the recovery of 1,4-DMN using the TMP/Heat method with 2-methylnaphthalene as the internal standard, in the way it would be used in routine analysis. The analysis of the extracts containing 1,4-DMN and the internal standard 2-methylnaphthalene was achieved by using the routine HPLC method reported in Section 2.3. Based on the results of these recovery experiments, the most suitable internal standard among the ones examined was selected for the final routine analytical method which is based on the TMP/Heat extraction method.

4.2 Material and methods



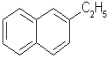
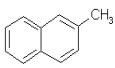
4.2.1 Solvents

The ethanol (EtOH) and 2,2,4-trimethylpentane (TMP) used in the extraction solution and in preparing standards were HPLC grade from Fisher Scientific, UK. The acetonitrile used in preparing the mobile phase for HPLC analysis was an HPLC grade from Fisher Scientific, UK.

4.2.2 Chemicals

Some main properties of 1,4-dimethylnaphthalene and the internal standard candidates used during this chapter are listed in Table 4.1.

Table 4.1 Some main characteristics of the chemicals used in this chapter

Compound	1,4-DMN	1-EtNaph	2-EtNaph	2-MeNaph
Structure				
Molecular Weight	156.2	156.2	156.2	142.2
Melting Point (°C)	7.6	-13.9	-7.4	34.4
Boiling Point (°C)	268.0	258.6	258.0	241.1
Water Solubility (mg/l)	11.4	10.7	8.0	24.6
Octanol/Water Coefficient-K _{ow}	4.37	4.40	4.38	3.86

[Values obtained from SRC PhysProp Database (SRC, 2005)]

Sodium chloride (NaCl) used in aiding the separation of the two phases in the liquid-liquid extraction was AnalaR grade from Hopkin and Williams, UK.

4.2.3 Standards and solutions

4.2.3.1 First recovery experiment

Stock solutions: 10000 µg/ml stock solutions of 1,4-DMN and the three internal standards candidates were prepared separately by accurately (4 decimal places) weighing approximately 0.5 g of each chemical, and dissolving it in EtOH in 50-ml volumetric flasks and making it up to volume.

1000 µg/ml Mixed standard: a 1000 µg/ml mixed standard of 1,4-DMN and the three internal standard candidates was prepared by transferring 1 ml of each stock solution into a 10-ml volumetric flask and making it up to volume with EtOH.

100 µg/ml Mixed standard: a 100 µg/ml mixed standard of 1,4-DMN and the three internal standard candidates was prepared by transferring 5 ml of the 1000 µg/ml mixed standard solution into a 50-ml volumetric flask and making it up to volume with TMP.

Calibration standards: Five calibration standards containing 4,10,20,30 and 50 µg/ml of 1,4-DMN and the three internal standards candidates were prepared by dilution from the 100 µg/ml mixed standard with TMP.

Spiking solutions: potato peel samples were spiked at two levels (4 and 20 µg/g peel) using the 1000 µg/ml mixed standard solution for the high level and a 200 µg/ml mixed

standard solution for the low level. The latter was prepared by dilution from the 1000 µg/ml mixed standard solution using EtOH.

Extraction solution: This solution was prepared by mixing 700 ml of EtOH with 300 ml of TMP using a measuring cylinder to get the 7:3 EtOH:TMP solution.

4.2.3.2 Second recovery experiment

Stock solutions: The 1,4-DMN and 1-ethylnaphthalene stock solutions prepared in Section 4.2.3.1 were used here.

Calibration standards: eight mixed calibration standards containing 0.02, 0.04, 0.08, 0.1, 1.0, 4.0, 10.0 and 20.0 µg/ml of 1,4-DMN, and 30 µg/ml of the internal standard 1-ethylnaphthalene were prepared by dilution from the stock solutions.

Spiking solutions: potato peel samples were spiked at two levels (0.02 and 4.0 µg/g peel) using a 1,4-DMN standard solution of 1 µg/ml for the low level and a 200 µg/ml for the high level. The spiking solutions were prepared by dilution from the stock solution.

Extraction solution: This solution was prepared containing 10 µg/ml of the internal standard 1-ethylnaphthalene by transferring 5 ml of a 1000 µg/ml 1-ethylnaphthalene standard solution into a 500-ml volumetric flask and diluting with a mixture of 7:3 EtOH:TMP solution to the volume.

4.2.3.3 Third recovery experiment

Stock solutions: The 1,4-DMN and 2-methylnaphthalene stock solutions prepared in Section 4.2.3.1 were used here.

Calibration standards: Four mixed calibration standards containing 0.05, 4.0, 10.0 and 50.0 µg/ml of 1,4-DMN, and 30 µg/ml of the internal standard 2-methylnaphthalene were prepared by dilution from the stock solutions.

Spiking solutions: potato peel samples were spiked at three levels (0.2, 4.0 and 20 µg/g peel) using 1,4-DMN standard solutions of 10 µg/ml, 200 µg/ml and 1000 µg/ml for the three levels respectively. The spiking solutions were prepared by dilution from the stock solution.

Extraction solution: This solution was prepared containing 10 µg/ml of the internal standard 2-methylnaphthalene by transferring 5 ml of a 1000 µg/ml 2-methylnaphthalene standard solution into a 500-ml volumetric flask and diluting with a mixture of 7:3 EtOH:TMP solution to the volume.

4.2.4 Equipment

The HPLC instrument used for the recovery experiments consisted of a Merck-Hitachi L-7100 pump, a L7200 autosampler and L4500 Diode Array Detector. The signal and UV spectra were processed by Merck-Hitachi Chromatography Data Station software. The centrifuge instrument used in aiding the separation of the two phases in the liquid-liquid extraction was a MSE Mistral 2000.

4.2.5 Procedure

The recovery experiments of 1,4-DMN and the internal standard candidates were conducted using organically grown potato tubers bought from a local supermarket. The tubers were washed under running tap water for about half a minute and left to dry. Tubers were peeled with a household peeler and the peel was chopped into small pieces (about 0.5 x 0.5 cm) using a knife and a wooden chopping board and mixed well. 10-g subsamples were transferred into 50-ml conical flasks. Five subsamples for each spiking level were prepared in addition to 3 control samples.

In the first experiment, peel subsamples were spiked with a mixture of 1,4-DMN and the three internal standards. Spiking of peel samples was conducted by adding 200 µl of the spiking solution containing the four compounds using a P200 Gilson micropipette to 10.0 g of potato peel in 50-ml conical flasks. The spiked peel samples were left to settle for about 1 h, after which the extraction process started.

In the second and third experiments, peel subsamples were spiked with only 1,4-DMN while the internal standard (1-ethylnaphthalene or 2-methylnaphthalene) was added to the extraction solution. In these two experiments, spiking of peel samples was achieved by adding 200 µl of the spiking solution containing only 1,4-DMN using a P200 Gilson micropipette to 10.0 g of potato peel in 50-ml conical flasks. The spiked peel samples were left to settle for about 1 h, after which the extraction process started.

Fifteen ml of the extraction solution were added to each of the conical flasks containing the spiked sample and the control samples and the flasks stoppered by lids. All samples were

heated with occasional swirling in a water bath at 50 °C for 15 min and left to cool for 10 min. The liquid phase was then transferred to centrifuge tubes and 2 ml of 0.2 M NaCl solution were added. The extracts were then centrifuged at 2000 r.p.m for 2 min. The upper TMP layer was then transferred into 4-ml vials and an aliquot of about 1 ml was transferred into HPLC vials through a 0.2 µm PTFE syringe filter. Samples were then analysed by HPLC using the appropriate method for each recovery experiment as described in the introduction of this chapter.

4.3 Results and discussion

The first recovery experiment aimed to evaluate the similarity of the three internal standards in their behaviour during the extraction procedure in the TMP/Heat method to that of 1,4-DMN. This experiment was conducted by spiking potato peel with the four compounds and extracting them according to the TMP/Heat method. The results of this experiment are shown in Table 4.2.

Table 4.2 Relative recoveries of 1,4-DMN and three internal standards extracted from spiked potato peel according to the TMP/Heat method

Internal Standard	Spiking Level (µg/g peel)	Recovered 1,4-DMN/Recovered Internal Standard (Mean %)	%RSD
2-methylnaphthalene	4 µg/g of peel	101.2	2.4
	20 µg/g of peel	104.5	1.3
	Mean of 2 Levels	102.9	-
1-ethylnaphthalene	4 µg/g of peel	97.9	0.6
	20 µg/g of peel	99.2	0.4
	Mean of 2 Levels	98.6	-
2-ethylnaphthalene	4 µg/g of peel	97.7	1.9
	20 µg/g of peel	98.8	0.6
	Mean of 2 Levels	98.3	-

In general, all three internal standards expressed high similarity in their behaviour during the extraction process to 1,4-DMN behaviour. This is clearly illustrated by the closeness of the relative recoveries to the 100 % value and the consistency (robustness) of the recoveries expressed by the low %RSD values. These two quantities (recoveries close to 100 % and robustness of the recovery values) are important to ensure accurate and precise assays by allowing for small variations in the extraction conditions in routine analysis (Wieling et al., 1993).

Statistical analysis of the recovery results using the general linear model with Tukey HSD shows that the relative recovery of 1,4-DMN is significantly affected by the internal standard used ($p < 0.001$) and also by the concentration level ($p < 0.001$). The use of 2-methylnaphthalene as the internal standard generated 1,4-DMN recoveries differed significantly ($p < 0.001$) from the results obtained by the other two internal standards. However, 1-ethylnaphthalene and 2-ethylnaphthalene produced values which were not significantly different from each other. This might be explained by the fact that the solubility of 2-methylnaphthalene in water (24.6 mg/l at 25 °C) is higher than that for 1,4-DMN (11.4 mg/l at 25 °C) whereas the solubility of the other two internal standards (10.7 mg/l for 1-EtNaph and 8.0 mg/l for 2-EtNaph at 25 °C) are lower than 1,4-DMN solubility. This solubility will of course affect the concentration of the internal standard in the final organic layer during the liquid-liquid partitioning, which in turn will affect the calculated recovery of 1,4-DMN based on the recovery of the internal standard.

The effect of the initial concentration level of 1,4-DMN and the internal standards on the final relative recovery is not unexpected. This is explained by the fact that the flux of the chemicals between the interface of two phases in a well mixed extraction system is proportional to the concentration of the analytes in the aqueous phase when the distribution constants (compound concentration in the organic phase divided by the concentration in aqueous phase) are high, such as those for the chemicals used here (Vanderwal and Snyder, 1981).

However, despite the statistical difference between the three internal standards in their recoveries, the difference should not be analytically (practically) important.

The slight differences in the %RSD values of the relative recoveries between the three internal standards could in part be due to the differences in their precision in the HPLC separation method used. The %RSD values of the three internal standards in the HPLC separation method as described in Section 3.3.2.6 follow similar trend as the values obtained in Table 4.2. The values of %RSD for replicate injections ($n=5$, 10 µg/ml standard) of 2-methylnaphthalene, 2-ethylnaphthalene and 1-ethylnaphthalene were 0.7 %, 0.5 % and 0.4 % respectively. In addition to the variations in the precision of the HPLC separation, the variation in the physicochemical properties of the three internal standards might have contributed to some extent in the value of the relative recoveries and their %RSD values. The main characteristic which is thought to have contributed to this difference is the water solubility of the three internal standards compared to 1,4-DMN, which may affect the amount of the compounds recovered in the organic TMP layer. As

shown in Table 4.1, 1-ethylnaphthalene has the closest water solubility to 1,4-DMN followed by 2-ethylnaphthalene. Both internal standards have lower water solubility than 1,4-DMN. This lower solubility compared to 1,4-DMN was thought to contribute in getting mean relative recoveries of less than 100 % and to contribute in the slight %RSD variations between the two concentration levels. 2-methylnaphthalene, on the other hand, has a slightly higher water solubility with a slightly larger difference to 1,4-DMN solubility. Therefore, the relative recoveries of this internal standard were slightly higher than 100 % and with a larger difference between the two concentration levels compared to what was achieved by the other two internal standards.

Despite the slight differences between the three internal standards discussed above, the general conclusion from the first recovery experiment is that all three internal standards have sufficiently similar behaviour to 1,4-DMN that they behave in a very similar way during the extraction method. Therefore, the other criteria should be used to select the most suitable internal standard for a routine HPLC method. The main criterion in this regard was the ease and speed of HPLC separation from 1,4-DMN and from any other interfering compound might be present in potato extracts.

As discussed in Section 3.3.1, 2-ethylnaphthalene requires a long run time in order to be separated from 1,4-DMN and it was used in the first recovery experiment just for comparison purposes, as it is the internal standard in the original GC method.

1-ethylnaphthalene on the other hand showed an attractive relative recovery and high precision. Because of this, a new HPLC method suitable for routine analysis was developed for the separation of 1-ethylnaphthalene and 1,4-DMN. The details of the development of this method was discussed in Section 3.3.3.

The above separation method for 1,4-DMN and 1-ethylnaphthalene was used in the second recovery experiment, which aimed to examine the recovery of 1,4-DMN using the TMP/Heat method with 1-ethylnaphthalene as the internal standard. 1-ethylnaphthalene was added to the extraction solution in the way it would be used for the routine method. Potato peel samples were spiked with 1,4-DMN at two concentration levels (0.02 and 4 $\mu\text{g/g}$ peel) and the internal standard 1-ethylnaphthalene was added to the extractant in a concentration of 10 $\mu\text{g/ml}$. Three control samples were prepared containing unspiked peel to test for any contamination. The extraction procedure was described in Section 4.2.5. The extracts were analysed using the routine method of separating 1,4-DMN and 1-ethylnaphthalene. The results of this recovery experiment are shown in Table 4.3.

Table 4.3 Recovery of 1,4-DMN from spiked peel extracted by the TMP/Heat method using 1-ethylnaphthalene as the internal standard

Sample	Fortification Level ($\mu\text{g/g}$ peel)	Peel Sample Weight (g)	Volume of Fortification Standard Added (ml)	Concentration of Fortification Standard Added ($\mu\text{g/ml}$)	Residues Detected ($\mu\text{g/g}$ peel)	Recovery (%)	Mean Recovery (%)	RSD (%)
Control A	NA	10.0	NA	NA	0.26	NA	NA	NA
Control B	NA	10.0	NA	NA	0.27	NA		
Control C	NA	10.0	NA	NA	0.24	NA		
Recovery A	0.02	10.0	0.2	10	0.30	1486.7	1423.4	4.1
Recovery B	0.02	10.0	0.2	10	0.29	1456.4		
Recovery C	0.02	10.0	0.2	10	0.27	1332.7		
Recovery D	0.02	10.0	0.2	10	0.29	1435.8		
Recovery E	0.02	10.0	0.2	10	0.28	1405.3		
Recovery A	4.00	10.0	0.2	200	3.36	84.3	86.7	2.3
Recovery B	4.00	10.0	0.2	200	3.38	84.6		
Recovery C	4.00	10.0	0.2	200	3.54	88.6		
Recovery D	4.00	10.0	0.2	200	3.52	87.9		
Recovery E	4.00	10.0	0.2	200	3.51	87.9		

The first note on the results in Table 4.3 was the detection of 1,4-DMN (or coeluted compound) in the control samples with a mean concentration of about $0.26 \mu\text{g/g}$ peel, whereas they should be free of 1,4-DMN as the potatoes used are organically grown and should not have been treated with any chemical. The presence of this peak affected the recovery values at the lower $0.02 \mu\text{g/g}$ peel level, which showed too high values. A chromatogram of one of the control samples is shown in Figure 4.1. The chromatogram shows the presence of the 1,4-DMN (or the coeluted compound) peak next to the peak of the internal standard 1-ethylnaphthalene.

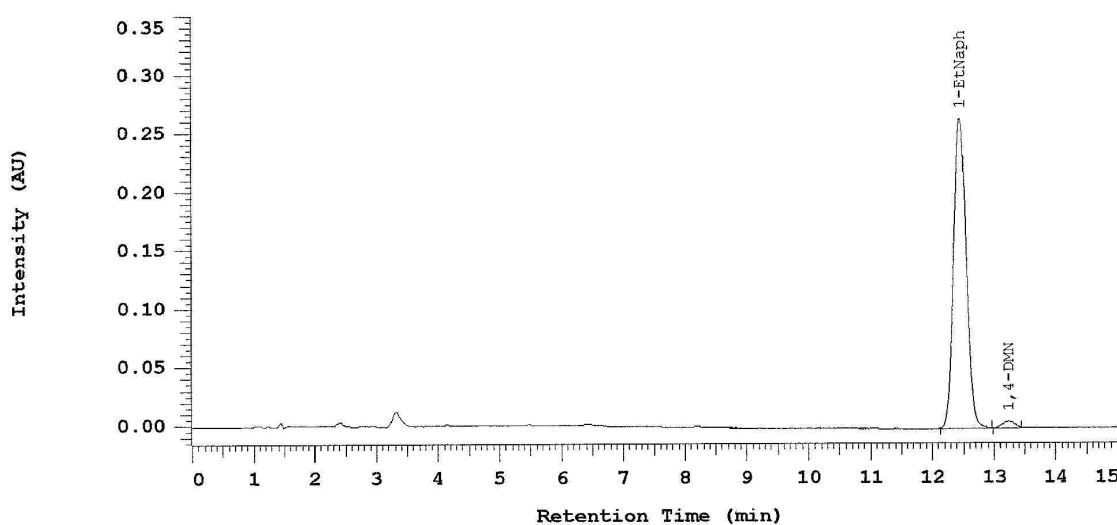


Figure 4.1 A chromatogram of an extract of a control sample of potato peel (not spiked with 1,4-DMN) showing a small peak at 1,4-DMN retention time next to the peak of the internal standard 1-ethylnaphthalene. Chromatographic Conditions: ACE C18 $5 \mu\text{m}$ (250 x 4.6 mm), 65 % acetonitrile: 35 % water, 1.5 ml/min, 18°C and 228 nm

In the first instance, it was thought that that the small peak that appeared at the retention time of the 1,4-DMN peak might be a contamination of 1,4-DMN. The possibility of getting the small peak due to a carryover from the standards used for the calibration, which were injected prior to the control samples, was eliminated by injecting several wash samples (with the mobile phase of 70 % ACN/water) between the calibrators and the control samples. Therefore, it was thought that the peak arose as a contamination during the extraction procedure. The extraction solution was then examined for any contamination and it was found to have the same small peak next to the peak of the internal standard. There were two possibilities here: either the solvents used in the extraction solution or the internal standard solution were contaminated. By examining samples of the ACN and TMP solvents used to prepare the extraction solution, the chromatograms were clear of any peak. In contrast, the examination of the internal standard solution (10 $\mu\text{g/ml}$) confirmed the presence of the contamination peak in the internal standard solution as shown in Figure 4.2.

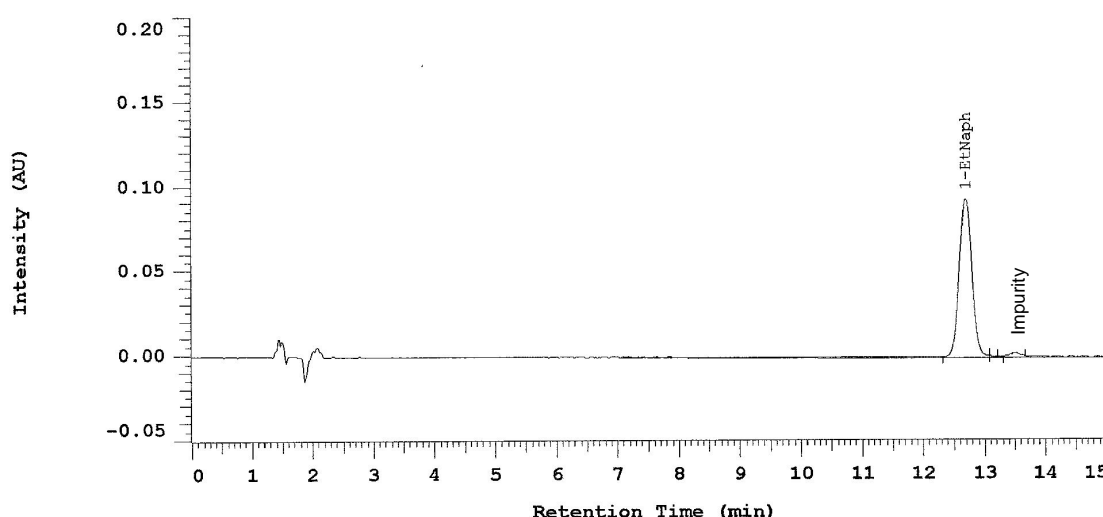


Figure 4.2 A chromatogram of 1-ethylnaphthalene standard (10 $\mu\text{g/ml}$) showing an impurity peak at 1,4-DMN retention time next to the 1-ethylnaphthalene peak. Chromatographic Conditions: ACE C18 5 μm (250 x 4.6 mm), 65 % acetonitrile: 35 % water, 1.5 ml/min, 18 $^{\circ}\text{C}$ and 228 nm

Unfortunately, the 1-ethylnaphthalene chemical used was not certified for the nature of impurities present (Sigma-Aldrich, personal communication). Therefore, to identify and quantify the impurity peak which was eluted at the retention time of 1,4-DMN a standard with higher concentration ($\sim 100 \mu\text{g/ml}$) of 1-ethylnaphthalene was analysed and the UV spectrum of the impurity peak was compared to the stored spectra in the library of the chromatographic software. The closest spectrum to the impurity spectrum was 2-ethylnaphthalene spectrum with a correlation factor of 0.9572. The comparison of the two spectra is shown in Figure 4.3.

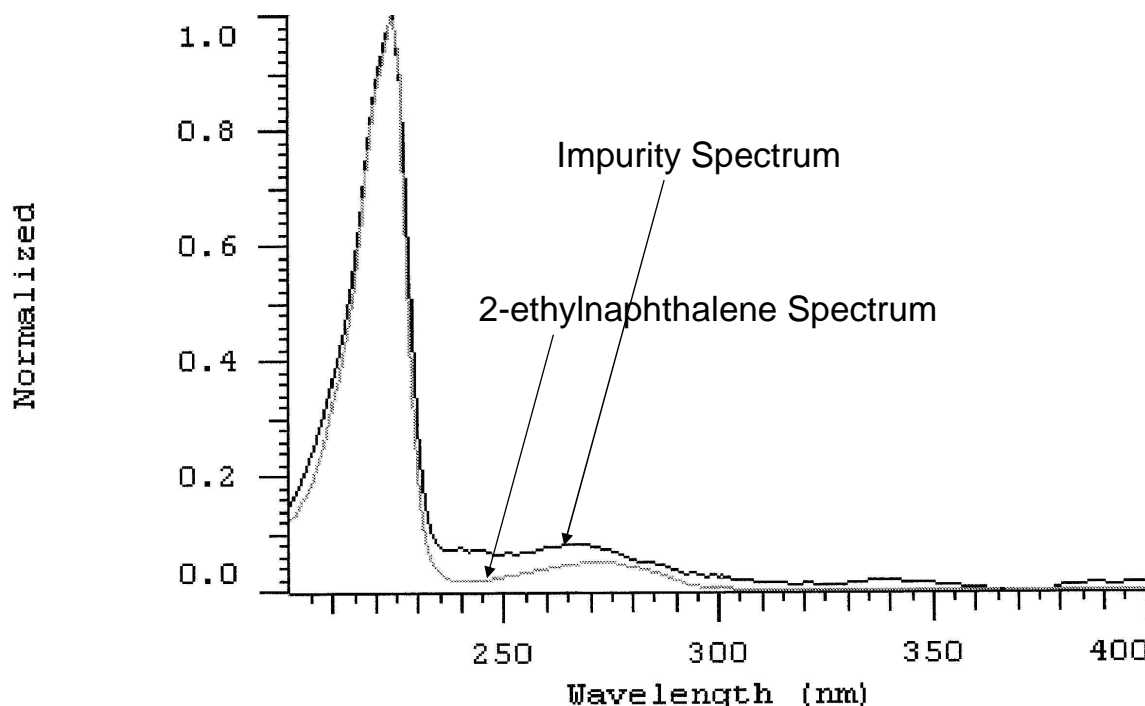


Figure 4.3 A comparison of the spectrum of the impurity peak eluted next to 1-ethylnaphthalene peak and the spectrum of 2-ethylnaphthalene

For clearer confirmation of the identity of the impurity peak, the 100 $\mu\text{g/ml}$ standard of 1-ethylnaphthalene was analysed at chromatographic conditions which provide good separation of 1-ethylnaphthalene, 1,4-DMN and 2-ethylnaphthalene. The conditions of this method are the ones detailed in Section 3.3.2.5 for the optimum separation of the mixture of 1,4-DMN and the four internal standards on the ACE column. In addition, the same 100 $\mu\text{g/ml}$ standard was spiked with 1,4-DMN at a level of about 20 $\mu\text{g/ml}$ to make sure that the impurity peak was not 1,4-DMN and to help in the confirmation of the identity of the impurity peak by the relative position of the impurity peak to 1,4-DMN and 1-ethylnaphthalene peaks in this separation method. 2-ethylnaphthalene was then added to the standard containing both 1-ethylnaphthalene and 1,4-DMN to confirm the identity of the impurity peak by checking the spectrum purity of the impurity peak after adding 2-ethylnaphthalene. The chromatograms of the unspiked and the spiked standards of 1-ethylnaphthalene are shown in Figure 4.4.

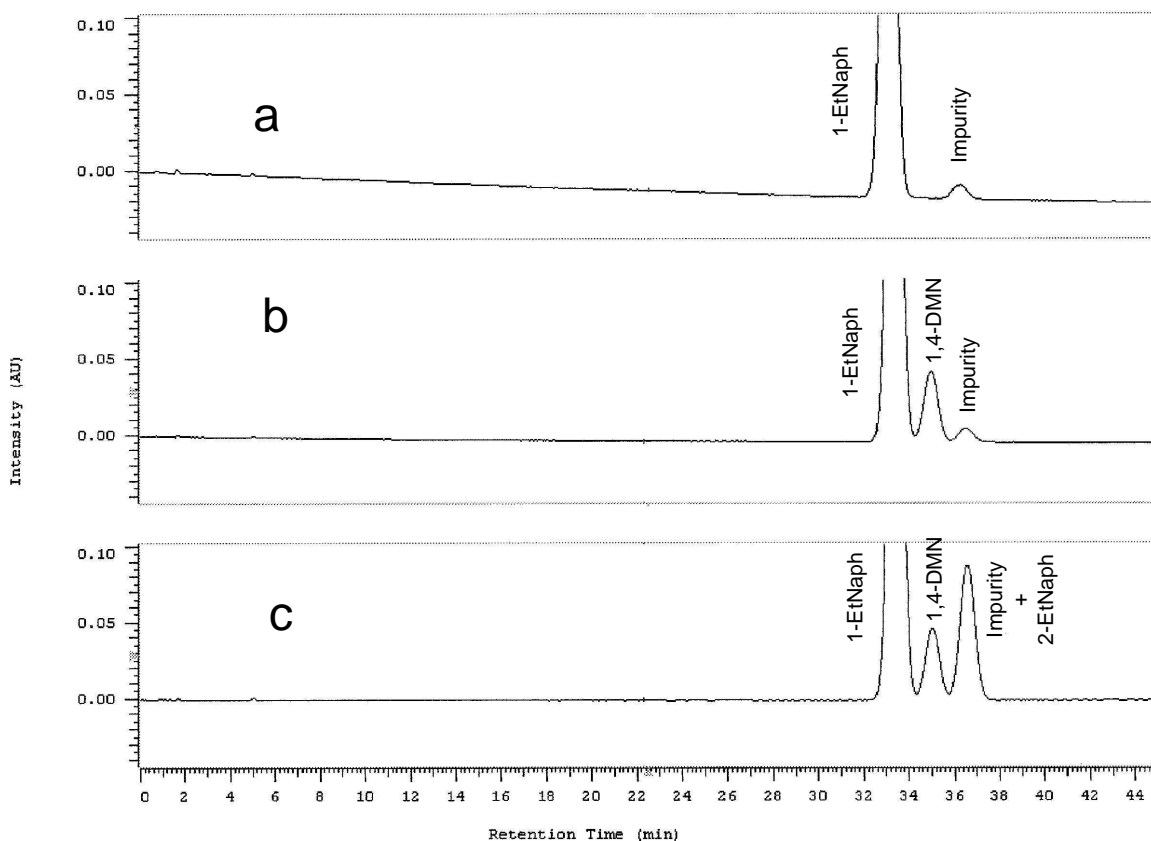


Figure 4.4 Chromatograms of 1-ethylnaphthalene standard (~100 µg/ml) showing an impurity peak at the retention time of 2-ethylnaphthalene. a) 1-ethylnaphthalene standard. b) the standard fortified with 1,4-DMN. c) The standard fortified with 1,4-DMN and 2-ethylnaphthalene. Chromatographic Conditions: ACE C18 5 µm (250 x 4.6 mm), 51 % acetonitrile: 49 % water, 1.5 ml/min, 18 °C and 228 nm

The chromatograms clearly confirm that the impurity peak was not for 1,4-DMN. In addition, the relative retention time of this peak to 1,4-DMN under the chromatographic conditions used can add more confirmation that the impurity present in the 1-ethylnaphthalene chemical is its isomer 2-ethylnaphthalene. The spectrum purity value of 0.983 when adding 2-ethylnaphthalene also adds more confirmation that the peak is 2-ethylnaphthalene.

2-ethylnaphthalene was found to be present at a level of 1.9 % (based on the relative peak areas of 1-ethylnaphthalene and the impurity of 2-ethylnaphthalene) in the 1-ethylnaphthalene chemical (98 + pure, Aldrich) which might be an impurity produced during the industrial production of the chemical. Other chemicals are available for chromatographic use with higher purity than the one used. However, they are very expensive for the purpose of working as an internal standard. One of these chemicals which is certified to be 99.5 % pure was bought from ChemService Inc., UK. A standard of this chemical was prepared and found to have only one peak corresponding to 1-ethylnaphthalene. However, the price for 100 mg of this chemical was £30 compared to less than £10 for one gram of the chemical bought from Aldrich.

The presence of 2-ethylnaphthalene in the 1-ethylnaphthalene chemical was a major disadvantage which led to favour the selection of 2-methylnaphthalene as the most suitable internal standard among the compounds examined for use with the TMP/Heat method.

2-methylnaphthalene is well separated from 1,4-DMN even at the conditions of the routine HPLC separation method reported in Section 2.3. A representative chromatogram of the separation of 2-methylnaphthalene and 1,4-DMN in a mixed standard (30 µg/ml) at the conditions of the routine HPLC separation method is shown in Figure 4.5.

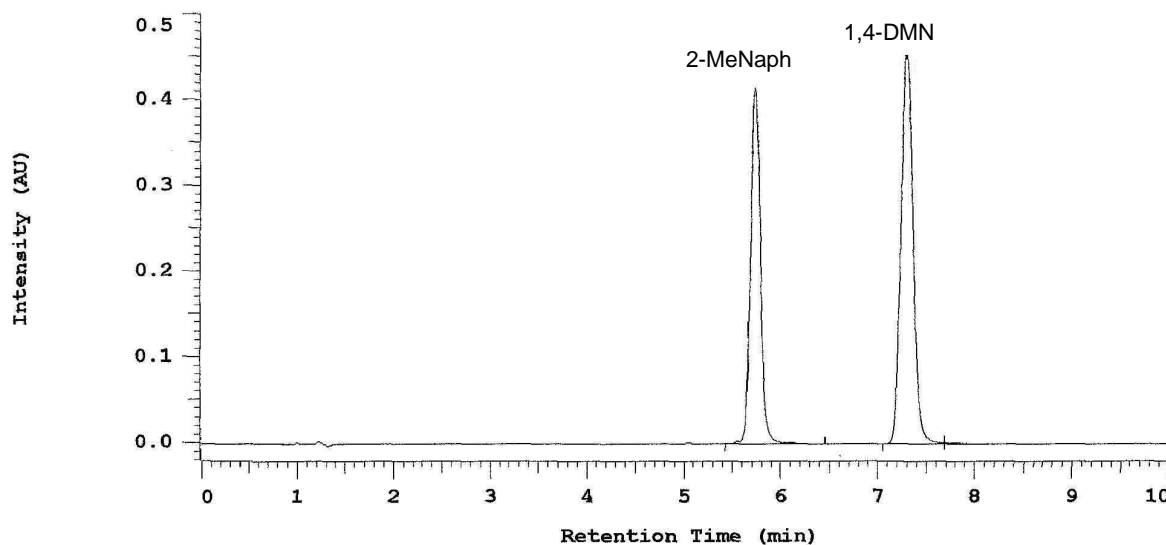


Figure 4.5 A representative chromatogram of the separation of 2-methylnaphthalene and 1,4-DMN at the conditions of the routine HPLC separation method. Chromatographic conditions: Supelco Spherisorb ODS-2 5 µm (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 228 nm and ambient temperature

The chromatogram in Figure 4.5 shows a good separation of the two compounds with large resolution between the two peaks. Due to the large resolution between the two peaks, a shorter column can be used to reduce the separation time which in turn reduces the consumption of the mobile phase (contains 70 % organic solvent). This advantage of using 2-methylnaphthalene as an internal standard for 1,4-DMN routine analysis is discussed in Chapter 6.

To examine the recovery of the TMP/Heat method using 2-methylnaphthalene as the internal standard, the third recovery experiment was conducted. Potato peel samples were spiked with 1,4-DMN at three concentration levels (0.2, 4 and 20 µg/g peel which are equivalent to about 0.03, 0.6 and 3.0 µg/g potato fresh weight) and the internal standard 2-methylnaphthalene was added to the extractant in a concentration of 10 µg/ml to examine the recovery of the final method, as it is going to be used in routine analysis. Three control samples were prepared containing unspiked peel to test for any contamination. The

extraction procedure was described in Section 4.2.5. The extracts were analysed using the routine HPLC separation method reported in Section 2.3. The results of this recovery experiment are shown in Figure 4.6 and Table 4.4.

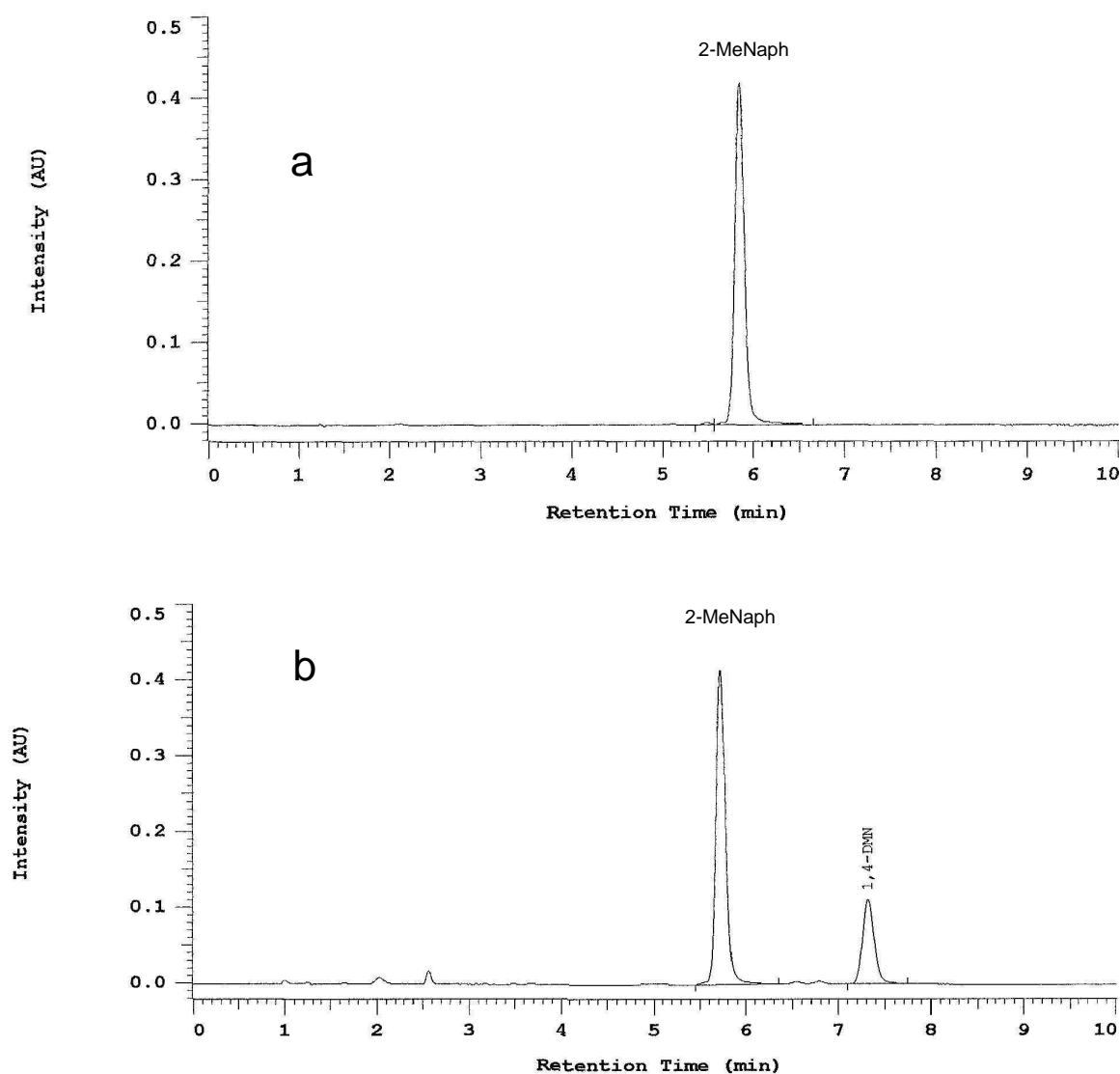


Figure 4.6 Representative chromatograms for extracts of potato peel samples extracted by TMP/Heat method with 2-methylnaphthalene as the internal standard. a) Control sample of unspiked potato peel. b) Spiked potato peel at 4 µg/g peel. Chromatographic conditions: Supelco Spherisorb ODS-2 5 µm (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 228 nm and ambient temperature

Table 4.4 Recovery of 1,4-DMN from spiked peel extracted by the TMP/Heat method using 2-methylnaphthalene as the internal standard. NA= Not Applicable and NQ=Not Quantifiable. Note: mean recovery values with the same subscript letter are not significantly different (Tukey HSD, $p<0.05$).

Sample	Fortification Level ($\mu\text{g/g}$ peel)	Peel Sample Weight (g)	Volume of Fortification Standard Added (ml)	Concentration of Fortification Standard Added ($\mu\text{g/ml}$)	Residues Detected ($\mu\text{g/g}$ peel)	Recovery (%)	Mean Recovery (%)	RSD (%)
Control A	NA	10.0	NA	NA	NQ	NA	NA	NA
Control B	NA	10.0	NA	NA	NQ	NA		
Control C	NA	10.0	NA	NA	NQ	NA		
Recovery A	0.20	10.0	0.2	10	0.21	106.2	106.6 _a	1.7
Recovery B	0.20	10.0	0.2	10	0.22	108.1		
Recovery C	0.20	10.0	0.2	10	0.21	105.1		
Recovery D	0.20	10.0	0.2	10	0.22	108.9		
Recovery E	0.20	10.0	0.2	10	0.21	104.7		
Recovery A	4.00	10.0	0.2	200	3.95	98.8	92.7 _b	6.5
Recovery B	4.00	10.0	0.2	200	3.93	98.2		
Recovery C	4.00	10.0	0.2	200	3.53	88.3		
Recovery D	4.00	10.0	0.2	200	3.40	85.1		
Recovery E	4.00	10.0	0.2	200	3.71	92.8		
Recovery A	20.00	10.0	0.2	1000	19.00	95.0	96.9 _b	5.2
Recovery B	20.00	10.0	0.2	1000	18.51	92.6		
Recovery C	20.00	10.0	0.2	1000	20.46	102.3		
Recovery D	20.00	10.0	0.2	1000	18.48	92.4		
Recovery E	20.00	10.0	0.2	1000	20.45	102.2		

The chromatograms in Figure 4.6 show the good separation of 1,4-DMN peak from the peak of the internal standard 2-methylnaphthalene. In addition, they show the absence of any contamination with 1,4-DMN or any possible interference at 1,4-DMN retention time.

The values in Table 4.4 show that the extraction with the TMP/Heat method using 2-methylnaphthalene provided enough accurate and precise results for the analysis of 1,4-DMN residues in potato samples. The recovery and precision values obtained here comply with the EU criteria for a quantitative method for the analysis of pesticide residues in food, which set up a minimum mean recovery between 70 to 110 % with a %RSD of 15 to 18 % for pesticides that have a maximum residue level (MRL) >0.1 -1 mg/kg (European-Commission, 2006).

Statistical analysis of the recovery data at the three levels, using ANOVA with Tukey's HSD test, shows that the recovery of 1,4-DMN at the lower level of 0.2 $\mu\text{g/g}$ peel differs significantly ($p=0.001$) from the other two levels, which are not significantly different.

However, despite the statistical difference, these differences should not be analytically important in practice.

By comparing the recovery values of 1,4-DMN at 4 and 20 $\mu\text{g/g}$ peel in Table 4.4 to those reported in Table 4.2, small differences can be noticed. These differences are thought to be due to differences in the adsorption level of the internal standard on the surfaces of the potato peel. The values in Table 4.2 were obtained by adding both 1,4-DMN and the internal standard directly to the peel. However, the values in Table 4.4 were obtained when only 1,4-DMN was added to the peel whereas the internal standard was added to the extraction solution. The addition of the internal standard to the extraction solution should reduce the contact between the internal standard and potato peel surfaces, which in turn reduces the loss of the internal standard by adsorption and increases the recovery of the internal standard in the final extract. Because the internal standard is used for correcting the final volume of the extract, the increase in the recovery of the internal standard will reduce the reported recovery of 1,4-DMN. This effect is discussed in more details in Section 6.3.3.3.

In summary, the three compounds compared here for their behaviour as internal standards in the extraction procedure of 1,4-DMN using the TMP/Heat method were found to have adequate similarity to 1,4-DMN. However, due to chromatographic considerations and the expense associated with the use of 1-ethylnaphthalene, 2-methylnaphthalene was selected as the most suitable internal standard for use with the TMP/Heat method when HPLC is used for the quantification.

The modified TMP/Heat method using 2-methylnaphthalene as the internal standard was then compared with two other extraction methods for their extraction efficiency of 1,4-DMN from potato samples which were treated with 1,4-DMN and stored in a commercial store for several weeks. This comparison is discussed in the next chapter.

Chapter 5: Separation and extraction methods for 1,4-DMN and internal standards - Comparison of extraction methods

5.1 Introduction

The main requirements for any extraction method are to produce valid data, rapidly, with less labour involvement and lower cost, and satisfying safety conditions (Dean, 1998). Many different methods are used for the analysis of pesticides in plant materials. The majority of them are based on solvent extraction by different techniques, ranging from such simple ones like soaking in a solvent to the use of sophisticated instruments such as pressurised liquid extraction or supercritical fluid extraction. These techniques vary with the method used to improve the action of the solvent for the extraction (see Section 1.4.2 for more details).

Several main factors affect the efficiency of solvent extraction of organic compound from solid samples such as plant materials. Among them are: solvent type, extraction time, extraction temperature, agitation or shaking and extraction surface area.

Many different solvents are used for the extraction of pesticides from plant materials. These solvents differ in their ability to solubilise the target analytes while leaving the sample matrix generally intact. Polarity is a main factor in controlling the solubility of analytes in the different solvents. The polarity of the extraction solvent should be close enough to that of the target analytes. Therefore, the choice of a solvent (or solvent mixtures) is a crucial factor in optimising an extraction method.

Shortening extraction times is one of the target goals of environmental analysis because of the large number of samples to be analysed every day. However, the speed of the analytical method should be balanced with the efficiency of the extraction. This balance can be achieved by extracting at high temperature and pressure in techniques such as pressurised liquid extraction (PLE) or by providing the mixture of sample and solvent with high energy such as microwave or ultrasound (Morales-Munoz et al., 2003). Efficient extraction time may vary from a few minutes to several hours or days according to the extraction technique used. In modern techniques such as pressurised liquid extraction 15 minutes has been found to provide comparable efficiency to that obtained with overnight extraction by the Soxhlet device (Berset et al., 1999).

Higher temperatures can generally increase the rate of extraction because the viscosity and the surface tension of the solvent reduce, while its solubility and diffusion rate into the sample increase (Buldini et al., 2002). In addition, the capability of solvents to disrupt matrix-analyte interaction increases at high temperatures (Morales-Munoz et al., 2003). However, care should be taken to avoid adverse effects on thermolabile compounds when conducting the extraction at elevated temperatures.

Agitation or shaking is used in assisting the extraction process. Shaking can be used as a separate extraction method such as in the shake-flask method. This method is effective when the analyte is highly soluble in the extraction solvent and the sample is quite porous (Snyder et al., 1997). Shaking can be performed by hand or with a mechanical shaker. An example of using shaking as the main extraction technique was reported by Bushway et al. (1998).

The efficiency of solvent extraction can be increased by increasing the surface area of the sample that is exposed to the solvent, because the solvent will be able to make more contact with the targeted analytes (Ezzell, 1999). Therefore, samples are usually ground, homogenised or chopped to fine pieces to increase the surface area of the extraction.

Numerous methods are reported in literature for the extraction and quantification of pesticides in plant materials. Most of these methods are evaluated for their extraction efficiency using recovery experiments either by fortified samples, which is more common (Arribas et al., 2007), or by comparing different methods using real samples known to have the pesticides of interest (Bushway et al., 1998).

Using real samples in recovery experiments should provide better evaluation of the recovery of extraction methods because the compound of interest is more incorporated into the sample matrix (Bushway et al., 1998).

The objective of this chapter was to evaluate three extraction methods for 1,4-DMN residues in treated potato samples. This evaluation aimed to compare their extraction efficiency of 1,4-DMN from potato samples which were treated with 1,4-DMN and stored under commercial storage conditions for several weeks, in addition to comparing the extraction procedures of the three methods. The three methods evaluated were the Soxhlet extraction with hexane, the extraction with ethanol: 2,2,4-trimethylpentane (TMP) 7:3 v/v by heating at 50 °C and by shaking in an ultrasonic bath. The last two methods used the internal standard 2-methylnaphthalene.

5.2 Material and methods

5.2.1 Solvents

The hexane used in the Soxhlet extraction and for preparing some standards was HPLC grade from BDH, UK. The ethanol and 2,2,4-trimethylpentane used in the heating and sonication extraction and in preparing some standards were HPLC grade from Fisher Scientific, UK. The acetonitrile used in preparing the mobile phase for HPLC analysis was an HPLC grade from Fisher Scientific, UK.

5.2.2 Chemicals

1,4-dimethylnaphthalene and the internal standard used during this chapter were described previously in Section 4.2.2. The sodium chloride (NaCl) used in aiding the separation of the two phases in the liquid-liquid extraction in the TMP/Heat and the sonication methods was AnalaR grade from Hopkin and Williams, UK.

5.2.3 Standards and solutions

Stock solutions: 10000 µg/ml stock solutions of 1,4-DMN and 2-methylnaphthalene were prepared by accurately (4 decimal places) weighing approximately 0.5 g of each chemical in 50-ml volumetric flasks and dissolving in TMP for 1,4-DMN standards and EtOH for 2-methylnaphthalene one. The solutions were then made up to volume.

1000 µg/ml standards: 1000 µg/ml standards of each of 1,4-DMN and 2-methylnaphthalene were prepared separately by transferring 10 ml of each stock solution into a 100-ml volumetric flask and made up to volume with TMP for 1,4-DMN and EtOH for 2-methylnaphthalene.

Extracting solution containing 20 µg/ml internal standard: This solution was used for the TMP/Heat method and the sonication method. 4 ml of the 1000 µg/ml 2-methylnaphthalene solution were transferred into a 200-ml volumetric flask using a pipette. It was then diluted to volume with a mixture of EtOH : TMP 7:3 (v/v).

50 µg/ml HPLC working (calibration) standard in TMP: This is a mixed standard of 1,4-DMN and 2-methylnaphthalene. It was prepared by pipetting 5 ml of 1000 µg/ml 1,4-DMN and 5 ml of the 1000 µg/ml 2-methylnaphthalene into a 100-ml volumetric flask and diluted to volume with TMP.

50 µg/ml HPLC working standard in hexane: It was prepared by pipetting 5 ml of 1000 µg/ml 1,4-DMN into a 100-ml volumetric flask and diluted to volume with Hexane.

0.2 M Sodium Chloride: 1.1688 g of NaCl was weighed out accurately into a 100-ml volumetric flask and dissolve in deionised water and made up to the volume.

70 % HPLC mobile phase: This solution was prepared by mixing 350 ml of ACN with 150 ml of deionised water in a 500-ml glass bottle. The solution was then degassed for 30 min in an ultrasonic bath.

5.2.4 Equipment

The HPLC instrument used for the comparison of the three methods was described in Section 2.3.2.1. The ultrasonic bath used was a Sonicator SC120T. The rotary evaporator used in the Soxhlet method was a Buchi Rotavapor-R with a Grant JB2 thermostated water bath. The centrifuge instrument used was a MSE Mistral 2000.

5.2.5 Procedure

5.2.5.1 Potato sample preparation

The comparison of the three extraction methods was conducted using potato tubers treated with 1,4-DMN and stored under commercial storage conditions for several weeks. The tubers were washed under running tap water for about half a minute and left to dry. Each tuber was weighed and peeled with a household peeler and the peel was weighed. The peel was chopped into small pieces (about 0.5 x 0.5 cm) using a knife and a chopping board and mixed well. The chopped and mixed peel of each tuber was divided into three subsamples, a subsample for each extraction method. Two 10-g subsamples were transferred into two 50-ml conical flasks for the extraction with EtOH/TMP extraction solution by heating and by sonication. The rest of the peel of each tuber (about 15-25 g) was transferred into a cellulose thimble for Soxhlet extraction. 1,4-DMN was extracted according to the following three methods.

5.2.5.2 TMP/Heat method

This method followed the method developed in 1,4Group laboratories with slight modifications. The extraction was conducted in a 50-ml conical flask. After transferring 10 g of potato peel into the flask, 15 ml of EtOH/TMP (7:3 v/v) containing the internal standard 2-methylnaphthalene were transferred accurately by a glass bulb pipette. The conical flask (with the stopper) was then heated with occasional swirling in a water bath at

50 °C for 15 min, after which it was cooled for about 10 min. The aqueous/organic phase was then transferred to a 50-ml centrifuge tube and 2 ml of 0.2 M NaCl solution were added. The extract was centrifuged at 2000 r.p.m for 2 min to help in the separation of the two phases. An aliquot of the upper TMP layer was transferred into a 4-ml vial. 1 ml of the TMP extract was then passed through a 0.2 µm PTFE syringe membrane filter into an HPLC vial for the analysis.

5.2.5.3 Soxhlet extraction method

The Soxhlet method used here follows the method reported by Baloch (1999) for the extraction of CIPC residues from potatoes with slight modification. After transferring potato peel samples into the thimble, 10 g of anhydrous sodium sulphate were added to the thimble and mixed with the peel. The thimble was plugged with cotton wool and inserted into a Soxhlet extraction unit, which was then connected to a flat bottom flask containing about 150 ml of hexane and to a condenser from the other end. The refluxing was conducted for two hours, after which the extract was cooled for about 20 min. The extract was then concentrated by evaporating the solvent to about 2 ml in a rotary evaporator with a water bath at about 35 °C. The concentrated extract was transferred quantitatively to a 5-ml volumetric flask and made up to the volume with hexane. An aliquot of 1 ml was passed through a 0.2 µm PTFE syringe membrane filter into an HPLC vial for the analysis.

5.2.5.4 Sonication method

The extraction steps in this method were exactly the same as in the TMP/Heat method (Section 5.2.5.2) except for the heating step. In this method, the conical flasks containing potato peel and the extractant with the internal standard were placed in an ultrasonic bath for 15 min instead of heating for 15 min. The rest of the steps were the same as described in the TMP/Heat method.

5.2.5.5 HPLC conditions

The analysis of the extracts obtained by the three extraction methods was carried out using the conditions of the routine HPLC method reported in Section 2.3.2.1.

5.3 Results and discussion

The three methods were compared for their extractability of 1,4-DMN from tubers treated with 1,4-DMN and stored for several weeks instead of using fortified samples. The comparison of the three extraction methods is shown in the graph in Figure 5.1.

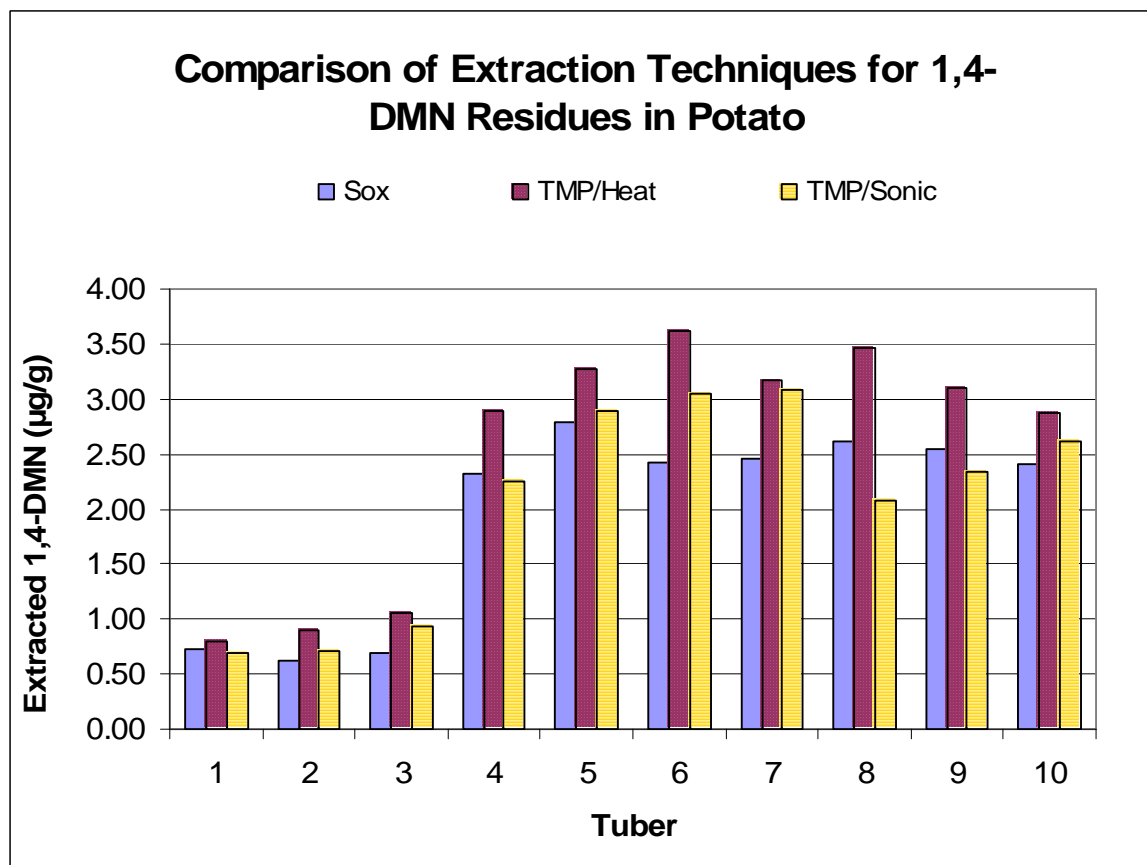


Figure 5.1 Comparison of extraction techniques for 1,4-DMN residues in potato peel samples. Extracted 1,4-DMN is expressed by µg/g of fresh potato weight

The graph shows that in all the ten trials (tubers), higher levels of 1,4-DMN were extracted using the TMP/Heat method, with Soxhlet extraction and TMP/sonication producing similar results.

The differences between the levels of extracted 1,4-DMN by the three methods were studied statistically using paired t-tests for each two methods. The regression method for comparing two analytical methods which was used in Chapter 2 (see Section 2.3.3.5) was not suitable for comparing the extraction methods here because the concentration levels do not have enough spread to generate an acceptable regression line. Therefore, paired t-tests were used instead. The results of the paired t-tests showed that TMP/Heat extracted significantly more 1,4-DMN than the Soxhlet method ($p < 0.001$) and the TMP/Sonication method ($p = 0.007$). The tests also showed that there was no significant difference between the Soxhlet and the TMP/Sonication methods ($p = 0.385$).

The lower efficiency of the sonication method might be attributed to the lower temperatures (only few degrees above room temperature) at which the extraction occurred. The lower efficiency of sonication has also been reported by Berset and his co-workers in comparing several techniques including Soxhlet and sonication for the extraction of PAHs

from contaminated soils (Berset et al., 1999). They found that sonication proved to be less efficient than the other techniques. Therefore, sonication was excluded from any further studies.

The comparison of the Soxhlet method and the TMP/Heat method is more complex. There are three main differences between the two methods. The first is the extraction solution. Hexane was used in the Soxhlet method while a mixture of EtOH/TMP (7:3 v/v) was used in the TMP/Heat method. The second main difference is the rotary evaporation of most of the solvent in the Soxhlet method to concentrate the extract whereas the extract in TMP/Heat method is concentrated through the use of high initial ratio of sample/solvent and also through the partitioning of 1,4-DMN into a small volume of TMP layer by using a only 30 % of TMP in the extracting solution. The third difference between the Soxhlet method and the TMP/Heat method is the use of an internal standard for the calculations of the extract volume in the TMP/Heat method, whereas the extract was made up to a known volume in the Soxhlet method.

To test the contribution of these three factors in raising the values obtained by the TMP/Heat method over the Soxhlet method, the two techniques were compared in a way that allowed the effect of the three differences to be minimised or removed. The extraction solution of EtOH/TMP (7:3 v/v) was used in both methods, the concentration by rotary evaporation was omitted and 2-methylnaphthalene was used as an internal standard for the correction of the extract volume in both methods. The results obtained for the comparison between the two methods after unifying the main factors affecting the extraction efficiency in both methods are shown in the graph in Figure 5.2.

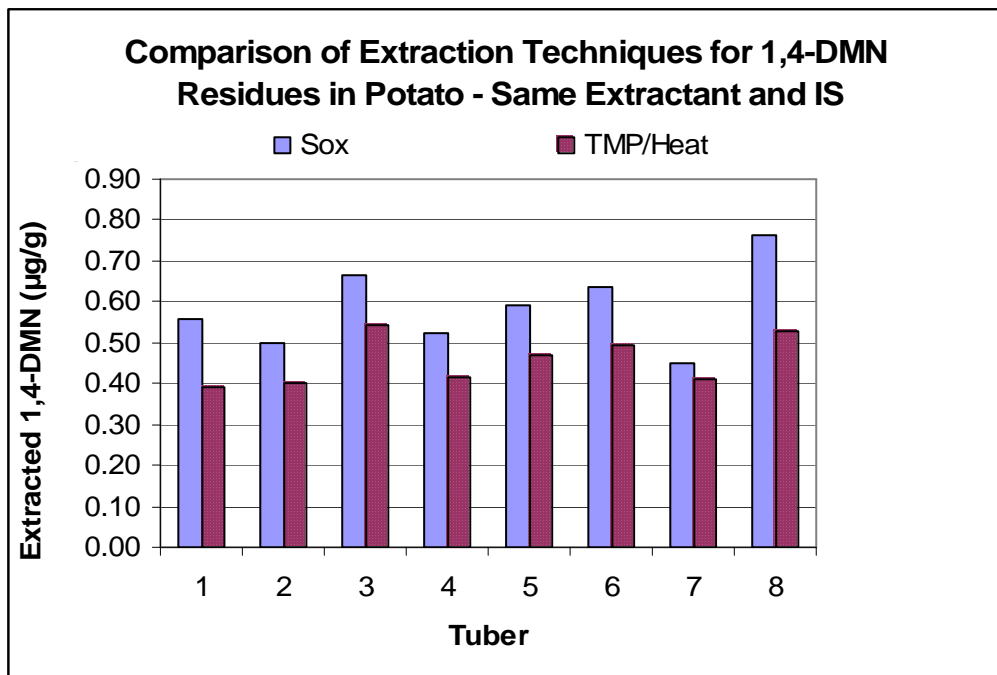


Figure 5.2. Comparison of extraction techniques for 1,4-DMN residues in potato peel samples using EtOH/TMP extraction solution and 2-methylnaphthalene as an internal standard in both techniques. Extracted 1,4-DMN is expressed by µg/g of fresh potato weight

The graph shows that in all the eight trials (tubers), the Soxhlet method gave higher values of the levels of extracted 1,4-DMN compared to the TMP/Heat method. A paired t-test also confirmed that there was a significant difference between the level of extracted 1,4-DMN obtained by Soxhlet and those obtained by TMP/Heat method ($p < 0.001$).

The higher results obtained by the Soxhlet method in the comparison shown in Figure 5.2 is not unexpected. The two methods agreed in using the same extraction solution and the same internal standard, but the extraction time in the Soxhlet method was 8 times longer than the extraction time in the TMP/Heat method. In addition, the extraction in the Soxhlet method is carried out at higher temperature (at about 70 °C for the mixture of EtOH : TMP 7:3 used here) and the sample is extracted by a fresh solvent by refluxing.

A recovery experiment was conducted to evaluate the contribution of rotary evaporation of the hexane solvent in the loss of 1,4-DMN in the original Soxhlet method. The experiment was based on reducing 100 ml of a standard of 2 µg/ml of 1,4-DMN in hexane to about 2-3 ml by rotary evaporating the hexane at 35 °C. The concentrate was then made up to 5 ml in a volumetric flask. The concentrate was analysed by the routine HPLC method described in Section 2.3.2.1. Five replicates were examined in this experiment. The mean recovery of 1,4-DMN for the five replicates was 91.1 % with a %RSD of 3.9 %. This indicates a mean loss of 8.9 % of 1,4-DMN during the rotary evaporation step in the original Soxhlet method. This loss of 1,4-DMN seems to be caused by the temperature of the water bath

used during the rotary evaporation. Beveridge (1979) studied the importance of controlling the temperature and pressure during the rotary evaporation of 1,4-DMN extracts in hexane. Optimum recoveries of 1,4-DMN was achieved by controlling the water bath temperature at 24 °C and using constant vacuum in the rotary evaporator. However, to balance between good recoveries and practical evaporation speed, temperatures of 31 ± 0.5 °C and vacuum of 470 mm Hg were used. O'Hagan (1991) evaporated 1,4-DMN extracts in dichloromethane solvent (which has lower boiling point of 40 °C compared to 69 °C for hexane) at 24 °C to avoid the loss of 1,4-DMN. In light of the recovery experiment conducted here, the evaporation step in the Soxhlet method should receive more optimisation studies to increase the recovery of 1,4-DMN.

The loss of 1,4-DMN in rotary evaporation contributed to about one third of the difference between the Soxhlet method and the TMP/Heat method which was noticed in the comparison illustrated in Figure 5.1. The rest of the difference between the two methods was thought to be mainly due to the lower extraction efficiency of hexane compared to EtOH/TMP mixture. This can be investigated by making a direct comparison between the extraction solvents used in both methods using a single extraction technique. However, due to time limitation this comparison was not made here.

To summarise the comparison between the three extraction methods as described in Section 5.2.5, TMP/Heating method with EtOH/TMP extraction solvent was found to be more efficient than the Soxhlet method with hexane and the sonication method with EtOH/TMP. In addition, the TMP/Heat method is more rapid, less laborious and uses much less solvent than the Soxhlet method. These are the main advantages for use as a routine extraction method for 1,4-DMN residues in treated potato samples. Therefore, the final method for the determination of 1,4-DMN residues in treated potato samples was based on the TMP/Heat extraction method.

The TMP/Heat method proved to have higher extractability of 1,4-DMN from real treated potato samples than the other two extraction methods examined in this chapter. The method was reported (see Section 4.3) to have good recovery (92.7 % to 106.6 %) and high precision ($\%RSD \leq 6.5$ %) using fortified potato peel samples at three different levels (0.2, 4 and 20 µg/g peel or 0.03, 0.6 and 3.0 µg/g potato fresh weight).

The limit of detection (LOD) and limit of quantification (LOQ) of this method can be estimated based on the LOD of the HPLC separation method reported in Section 2.3.3.4. The LOD of the HPLC method used in the TMP/Heat method was estimated (using the

calibration line at low levels of 1,4-DMN) to be 0.02 µg/ml of 1,4-DMN in solution which corresponds theoretically to approximately 0.02 µg/g of 1,4-DMN in peel. Therefore, the limit of quantification of the method can be estimated to be about 0.07 µg/g of 1,4-DMN in peel (i.e. about 0.01 µg/g of potato fresh weight). The lowest concentration measured experimentally in the recovery experiment reported in Chapter 4 using this method was 0.2 µg/g peel (or 0.03 µg/g of potato fresh weight) which is about three times higher than the estimated limit of quantification of this method. More discussion about the LOD and LOQ of this method is reported in Section 6.3.2.3.

This level of detection sensitivity of the TMP/Heat method is adequate for the purpose of the method, as the method was designed for the determination of 1,4-DMN residues in tubers that have been treated with 1,4-DMN. Typical values of residues in treated tubers are normally between 0.5 and 1.5 µg/g of potato fresh weight (John Forsythe, personal communication) which is well above the lower concentration level of 0.03 µg/g of potato fresh weight that can be quantified by this method. In addition, there is no Maximum Residue Limit (MRL) set for 1,4-DMN in Europe, as it is not yet used commercially. In the United States, the chemical is registered as a reduced-risk pesticide (a pesticide that poses less risk to human health and the environment) (Knowles et al., 2005) and is exempted from the requirement for a maximum residue level value (USEPA, 1995). This may lead to the expectation of relatively high values of 1,4-DMN residues in treated potato tubers. Therefore, the achieved level of detection sensitivity for the TMP/Heat method should be sufficient for the purpose of analysing 1,4-DMN residues in treated potato tubers.

5.4 Final HPLC method for the determination of 1,4-DMN residues in treated potato samples using EtOH/TMP solution

5.4.1 Solvents

The solvents required for this method should be HPLC grade. Ethanol (EtOH) and 2,2,4-trimethylpentane (TMP) are required for the extraction mixture. Acetonitrile is required for preparing the mobile phase for HPLC analysis.

5.4.2 Chemicals

1,4-dimethylnaphthalene and the internal standard 2-methylnaphthalene have to be in high purity for standard preparation. Sodium chloride (NaCl) is required to aid the phase separation.

5.4.3 Standards and solutions

Stock solutions: 10000 µg/ml stock solutions of 1,4-DMN and 2-methylnaphthalene are prepared by accurately (4 decimal places) weighing approximately 0.5 g of each chemical in 50-ml volumetric flasks and dissolving in TMP for 1,4-DMN standard and EtOH for 2-methylnaphthalene one. The solutions are then made up to volume.

1000 µg/ml standards: 1000 µg/ml standard of each of 1,4-DMN and 2-methylnaphthalene is prepared separately by transferring 10 ml of each stock solution into a 100-ml volumetric flask and made up to volume by TMP for 1,4-DMN and EtOH for 2-methylnaphthalene.

Extracting solution containing 10 µg/ml internal standard: 2 ml of the 1000 µg/ml 2-methylnaphthalene solution is transferred into a 200-ml volumetric flask using a pipette then diluted to volume with a mixture of EtOH : TMP 7:3 (v/v).

30 µg/ml HPLC working (calibration) standard in TMP: This is a mixed standard of 1,4-DMN and 2-methylnaphthalene. It is prepared by pipetting 3 ml of 1000 µg/ml 1,4-DMN and 3 ml of the 1000 µg/ml 2-methylnaphthalene into a 100-ml volumetric flask and diluted to volume with TMP. The 1,4-DMN concentration in this standard can be changed based on the expected level of 1,4-DMN in the samples analysed, or a series of calibrators might be used to generate a calibration line.

0.2 M sodium chloride: 1.1688 g of NaCl is weighed out accurately into a 100-ml volumetric flask and dissolved in water and then made up to the volume.

70 % HPLC mobile phase: This solution is prepared by mixing 350 ml of ACN with 150 ml of deionised water in a 500-ml glass bottle. The solution is then degassed for 30 min in an ultrasonic bath.

5.4.4 Procedure

1. Potato sample information is recorded.
2. 3 to 5 potato tubers are removed and washed gently under running cold tap water to remove soil. They are then left to dry or dried with paper towels.
3. The weight of fresh potatoes is taken using a top-pan balance.
4. Potatoes are peeled with a stainless steel household peeler and the total peel weight is recorded.
5. The peel is chopped to fine pieces (about 0.5 x 0.5 cm) using a knife and a chopping board.
6. The peel is mixed thoroughly and a 10-g subsample is taken randomly and transferred into a 50-ml conical flask.
7. 15 ml of the extracting solution (containing the internal standard) are added to the conical flask using a pipette.
8. The conical flask is capped with a stopper and placed in a heated water bath at 50 °C for 15 min with occasional swirling and then cooled for 10 min.
9. The aqueous/solvent liquid phase is transferred into a 50-ml glass centrifuge tube and the peel is discarded.
10. Two ml of the 0.2 M sodium chloride solution are added to the centrifuge tube to help the separation of the two layers
11. The tube is placed in the centrifuge instrument and is centrifuged for 2 min at 2000 r.p.m.
12. The TMP layer (top layer) is then pipetted using a glass Pasteur pipette into a 4-ml glass vial.
13. One millilitre of the extract is filtered through a 0.2 µm PTFE syringe filter into an HPLC vial and analysed by HPLC.

5.4.5 HPLC separation conditions

- **Column:** A C18 (5 μm particle size, 250 mm x 4.6 mm)
- **Mobile phase:** acetonitrile : water 70:30
- **Flow rate:** 1.5 ml/min
- **Injection volume:** 10 μl
- **Run time:** 10 min
- **2-methylnaphthalene retention time :** ~6 min
- **1,4-DMN retention time :** ~8 min
- **Temperature:** ambient
- **Detector wavelength:** 228 nm

5.4.6 Calculations

1. Measured 1,4-DMN in the Extract (Cm) ($\mu\text{g/ml}$) = $P_{\text{extr}} * C_{\text{wstd}}/P_{\text{wstd}}$
2. Measured Internal Standard in the Extract (Cmis) ($\mu\text{g/ml}$) = $P_{\text{exis}} * C_{\text{wis}}/P_{\text{wis}}$
3. Corrected Extract Volume (ml) = $C_{\text{adis}} * V_{\text{adis}}/C_{\text{mis}}$
4. 1,4-DMN weight in the extract (μg) = Cm * Corrected Extract Volume
5. 1,4-DMN weight in the peel ($\mu\text{g/g}$ peel) = 1,4-DMN weight in extract/ wt of Peel sample
6. 1,4-DMN in the Potato Sample ($\mu\text{g/g}$ Fresh Weight) = 1,4-DMN in peel* Tot. Peel wt/ Tot. Potato wt.

Where:

C_m = Measured 1,4-DMN in the extract

C_{mis} = Measured internal standard in the extract

P_{ex} = Peak area of 1,4-DMN in the extract

P_{exis} = Peak area of internal standard in the extract

P_{wstd} = Peak area of 1,4-DMN in the working HPLC standards

P_{wis} = Peak area of internal standard in the working HPLC standards

C_w = 1,4-DMN concentration in the working (calibration) HPLC standard (30 $\mu\text{g/ml}$)

C_{wis} = Internal standard concentration in the working (calibration) HPLC standard (30 $\mu\text{g/ml}$)

C_{adis} = Concentration of the added internal standard (10 $\mu\text{g/ml}$)

V_{adis} = Volume of the added internal standard (15 ml)

5.4.7 Example of calculations

An extracting solution containing 10 $\mu\text{g/ml}$ internal standard was used to extract 1,4-DMN from a 10.02 g of a potato peel sample selected randomly out of total peel sample of 54.82 g. The total weight of the fresh potato sample was 391.57 g. The extract was analysed by HPLC against a working standard containing 30 $\mu\text{g/ml}$ of both 1,4-DMN and the internal standard. 1,4-DMN peak areas were 4714902 and 4551857 for the working standard and the extract respectively. Internal standard peak areas were 5065394 and 4341495 for the working (calibration) standard and the extract respectively. The requirement is to calculate the amount of 1,4-DMN extracted from this potato sample and express the results in $\mu\text{g/g}$ (ppm) in potato fresh weight basis.

$$1. \text{ Measured 1,4-DMN in the Extract } (C_m) = 4551857 * 30 / 4714902 = 28.96 \mu\text{g/ml}$$

$$2. \text{ Measured Internal Standard in the Extract } (C_{mis}) = 4341495 * 30 / 5065394 = 25.71 \mu\text{g/ml}$$

$$3. \text{ Corrected Extract Volume} = 10 * 15 / 25.71 = 5.83 \text{ ml}$$

4. Corrected 1,4-DMN weight in the extract = **28.96 *5.83 =168.96** μg

5. 1,4-DMN weight in the peel = **168.96 /10.02=16.86** $\mu\text{g/g}$ peel

6. 1,4-DMN in the Potato Sample = **16.86 *54.82 /391.57=2.36** $\mu\text{g/g}$ Fresh wt (ppm)

Chapter 6: Analytical method for trace analysis of natural 1,4-DMN in potatoes

6.1 Introduction

The HPLC method reported in Chapter 5 for the analysis of 1,4-DMN residues in potato samples was successful as a routine method for the analysis of potato tubers treated with 1,4-DMN. The lowest concentration measured by this method was 0.2 µg/g peel. This level of sensitivity is thought to be inadequate for the determination of natural 1,4-DMN found in potatoes at trace levels (see Chapter 7). The levels of volatiles production by potato tubers was estimated to be equivalent to individual rates of the order 1 ng kg⁻¹ h⁻¹ (Meigh et al., 1973).

Several techniques are available to enhance the detection sensitivity of an analytical method. Extracts containing the analyte can be concentrated by reducing their volume through evaporating the solvent using, for example, a rotary evaporator. However, this was not a good choice for 1,4-DMN, as it is relatively volatile and was found to have some loss during rotary evaporation (see Section 5.3). In addition, 1,4-DMN is extracted in the TMP/Heat method in 2,2,4-trimethylpentane (TMP) which has a high boiling point of 99.2 °C. This high boiling point makes it difficult to concentrate the extract by evaporating the solvent without losing a great deal of 1,4-DMN.

Another option for obtaining a more concentrated extract and therefore enhancing detection sensitivity is to increase the sample : solvent ratio. The ratio used in the TMP/Heat method was 10 g : 15 ml sample : solvent. It might be possible to increase it to 10 g : 10 ml ratio, but this will not greatly increase the concentration of 1,4-DMN in the final extract.

A valuable way of enhancing the detection sensitivity is to inject a larger extract volume into the HPLC. Sample volumes ranging from several microlitres up to 500 µl or more can be injected to the HPLC depending on the column used, sample solvents, mobile phase composition and the retention characteristics of the analytes (Snyder et al., 1997).

With the right conditions of sample solvent and mobile phase composition, HPLC columns with 3.9 mm internal diameters and 150 mm length (5 µm particle size) can typically tolerate sample volumes between 40 and 160 µl without peak distortion (Neue and Serowik, 1996). Longer columns or columns with a larger inner diameter can tolerate more

volume loading capacity (Layne et al., 2001). A 150 x 4.6 mm column can tolerate sample volumes in the range 50-500 μ l without peak distortion (Snyder et al., 1997).

Sample solvent and mobile phase compositions play a crucial role in enabling the injection of large volumes of samples into HPLC columns without peak shape deteriorating. To avoid peak shape problems when injecting large volumes, the sample solvent should match the mobile phase in the elution strength and viscosity (Keunchkarian et al., 2006). In addition, a detailed study should be considered when injecting large volumes of solvents that are not miscible with the mobile phase (David et al., 2006).

The injection of large volumes of samples dissolved in solvents which mismatch the mobile phase can interfere with the adsorption of the sample onto the column top, and will result in a broadening and/or distortion in peak shapes such as fronting, tailing and other peak forms (Keunchkarian et al., 2006; Neue, 1997). This problem limits the injection volume to less than 25 μ l for columns with 0.46 cm internal diameter (Snyder et al., 1997).

The TMP/Heat method reported in Chapter 5 (see Section 5.4) was based on injecting 10 μ l of the extract in TMP into the HPLC instrument. A significant increase in the 1,4-DMN mass introduced to the HPLC (10 times) can be achieved by full loop (100 μ l) injection. However, when trying to take advantage of injecting larger volumes of 1,4-DMN in TMP into the HPLC as a mean of enhancing the detection sensitivity, the shape of 1,4-DMN and 2-methylnaphthalene started to deteriorate, as shown in Figure 6.1, for 100 μ l injection compared to 10 μ l injection.

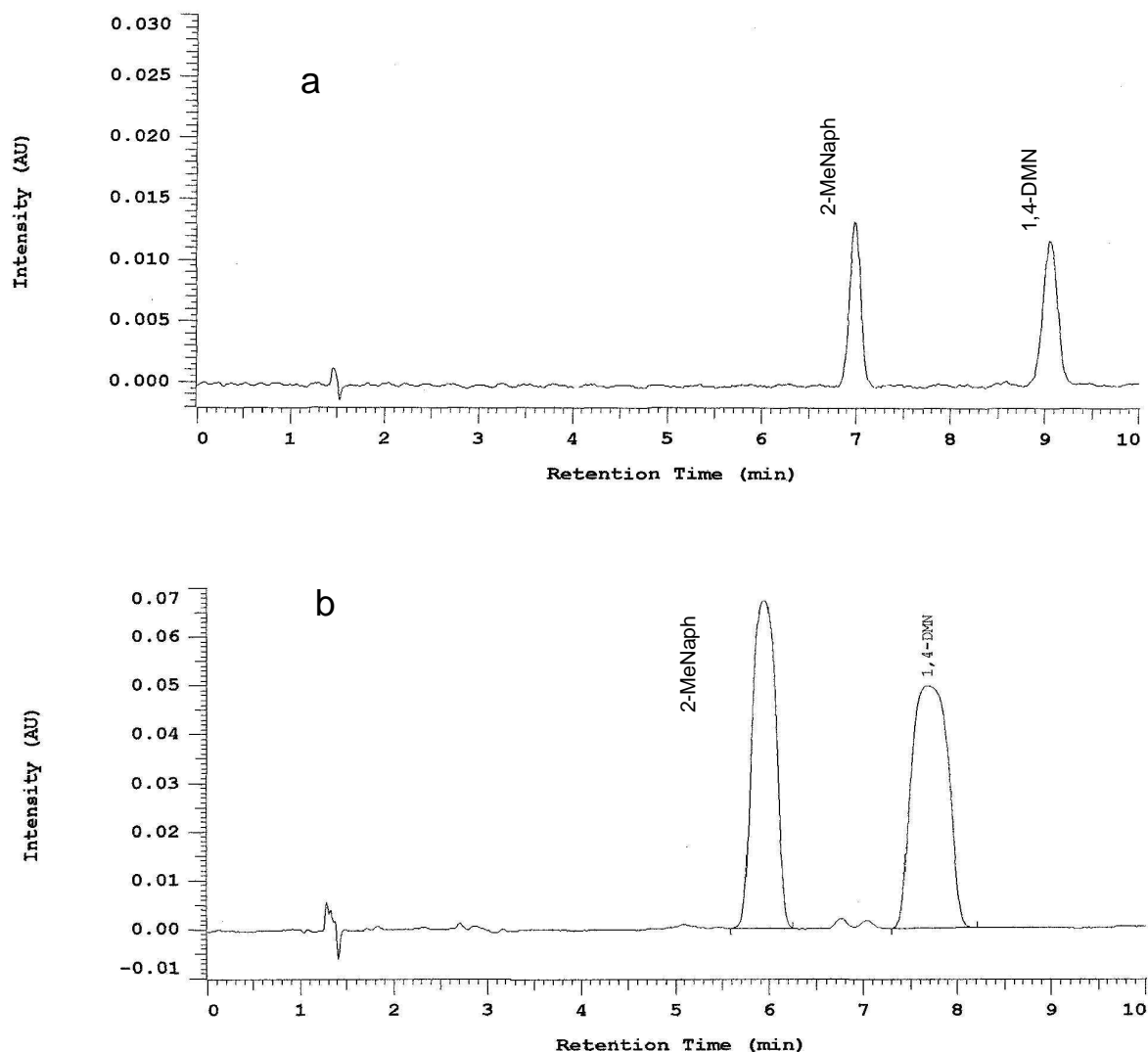


Figure 6.1 Effect of large volume injection on the peak shape of 1,4-DMN and 2-methylnaphthalene dissolved in 2,2,4-trimethylpentane solvent at 0.1 µg/ml. a) 10 µl injection. b) 100 µl injection. Chromatographic conditions: ACE C18 5 µm (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, ambient temperature and 228 nm

As shown in Figure 6.1, broad peaks were obtained when injecting large volumes of samples in the TMP solvent. In addition to the broadened peak shape, retention times of both 1,4-DMN and 2-methylnaphthalene peaks were about 13 % lower than the typical retention times obtained by injecting 10 µl.

This disadvantage is an obstacle to modifying the method for trace analysis of natural 1,4-DMN, as it will not be possible to use larger injection volumes to enhance the detection sensitivity. The cause of the deteriorated peak shape at the large injection volumes, as shown in Figure 6.1, can be related to the fact that 1,4-DMN and the internal standard are dissolved in TMP solvent which is stronger-eluting (more non-polar) and immiscible with the mobile phase used. This strength and immiscibility of the sample solvent might have interfered with the adsorption of the sample onto the column top, which resulted in the broadened peak shapes. This limitation in the injected volume restricts the use of the

method for samples with 1,4-DMN concentrations high enough for detection in low injected volumes. Because TMP is not miscible with water, it is not possible to dilute the extracts with water to reduce the mismatching with the mobile phase in order to use larger injection volumes.

In order to use the advantage of injecting large volumes of extracts to the HPLC to enhance the detection sensitivity, 1,4-DMN and the internal standard have to be loaded to the HPLC in a solvent more compatible with the mobile phase.

Different solvents have been used for the extraction of pesticide residues from fruits and vegetables. Acetonitrile is among the most commonly used extraction solvents for pesticides, particularly when liquid chromatographic methods are used for the final determination (Watanabe et al., 2004; Ishimitsu et al., 2003; Hetheron et al., 2004; Anastassiades et al., 2003). Because acetonitrile is a common constituent in mobile phases used for liquid chromatographic determination, using it as the sample solvent should help in enhancing the chromatographic separation. In addition, this solvent is miscible with the water derived from fresh fruits and vegetables, so it can form homogenous (single-phase) extracts for direct analysis when no clean up is required. In addition, acetonitrile has the merit of extracting less of the lipophilic plant materials such as fats and waxes, which helps in getting extracts with only a minor load of co-extractives (Tekel and Hatrik, 1996). This, in turn, should simplify the chromatographic separation and may eliminate the requirement for any sample clean up step. This advantage of acetonitrile was used by Caboni and co-workers (2005) who developed a fast method for the analysis of several pesticides using acetonitrile as the extraction solution, with no need for a clean up step prior to the chromatographic analysis. Omitting sample clean up helps in simplifying the analytical procedure which will reduce the analysis time, effort, solvent consumption and ultimately the cost of analysis.

For the advantages listed above, acetonitrile was selected as the main component in the new extraction mixture. In addition to acetonitrile, 2-propanol (isopropanol) was added in a small percentage to add more non-polarity to the extraction mixture, which was thought to be more favourable to the non-polar 1,4-DMN and 2-methylnaphthalene. Both solvents are miscible with the mobile phase and with the water derived from potato samples. This should help in forming a homogenous extract which can be injected directly into the HPLC possibly without any further clean up.

The objective of this chapter was to develop an extraction method for the analysis of trace levels of 1,4-DMN found naturally in potatoes. The method aimed to use the advantage of injecting large volumes of the extracts containing 1,4-DMN directly to the HPLC. For this purpose, a new extracting mixture of solvents, which is more compatible with the mobile phase and miscible with the water derived from potato peel samples, was compared to the EtOH/TMP mixture for its extraction efficiency of 1,4-DMN from potato samples. For the purpose of this comparison, potato tubers treated with 1,4-DMN and stored under commercial storage conditions were used. It was aimed to get a higher extraction efficiency of the new extractant or at least comparable efficiency to the EtOH/TMP mixture. The method was optimised and validated for trace analysis of the natural 1,4-DMN. In addition, it was also optimised and validated for rapid routine analysis of 1,4-DMN residues in tubers treated with 1,4-DMN.

6.2 Materials and methods

6.2.1 Solvents

All the solvents used were HPLC grade. The acetonitrile used in preparing the extraction solution and standards and also for preparing the mobile phase for HPLC analysis, was bought from Fisher Scientific, UK. The 2-propanol used in the extraction solution and standards was bought from Rathburn Chemicals Ltd., UK. The ethanol and 2,2,4-trimethylpentane used in the TMP/Heat extraction method and in preparing some standards were from Fisher Scientific, UK.

6.2.2 Chemicals

1,4-dimethylnaphthalene and the internal standard used during this chapter were described in Section 3.2.1. Sodium chloride (NaCl) used in the TMP/Heat method was described in Section 5.2.2.

6.2.3 Equipment

The HPLC instrument used was described in Section 4.2.4. The sonicator and centrifuge instruments were described in Section 5.2.4.

6.2.4 Efficiency comparisons of ACN/PROP and EtOH/TMP using treated potatoes

The new extraction solution acetonitrile (ACN) /2-propanol (PROP) (7:3 v/v) was compared for its extraction efficiency to the EtOH/TMP (7:3 v/v) mixture used in the

TMP/Heat method. The comparison was made by extracting 1,4-DMN residues in potato tubers treated with 1,4-DMN and stored under commercial storage conditions for several weeks.

The tubers were washed under running tap water for about half a minute and left to dry. They were then weighed and peeled with a household peeler and the peel was weighed. The peel was chopped into small pieces (about 0.5 x 0.5 cm) using a knife and a chopping board and mixed well before subsampling into 10-g portions in 50-ml conical flasks. The internal standard 2-methylnaphthalene was added to each extraction solution.

For the ACN/PROP extraction method, fifteen millilitres of ACN/PROP (7:3) extraction solution containing the internal standard were added to the flask using a pipette. The conical flask was capped with a stopper and placed in a preheated water bath at 50 °C for 15 min with occasional swirling and then cooled for 10 min. After cooling, the extract was decanted into 25-ml volumetric flasks along with several washings of the peel. The extract volume was then made up to 25 ml with 70 % ACN/PROP solution. One millilitre of the extract was filtered through a 0.2 µm PTFE syringe filter into an HPLC vial and analysed by HPLC.

For the samples extracted with the EtOH/TMP mixture, the extraction procedure reported for the final TMP/Heat method (Section 5.4.4) was followed exactly.

The HPLC analysis was carried out using the conditions detailed in Section 5.4.5 with a Supelco Spherisorb C18 5 µm (250 x 4.6 mm) column.

The evaluation of the final volume of the extracts was based on the use of the internal standard 2-methylnaphthalene in both solvent systems. In addition, the calculation method based on making up the extract to a known volume in the case of the ACN/PROP extraction was compared to the use of the internal standard.

Statistical analysis was used to evaluate the results based on the regression method described by Miller and Miller (2005) which was outlined and used in Chapter 2 (see Section 2.3.2.6 and Section 2.3.3.5). Excel[®] software was applied to plot the regression line and calculate the required statistical values for the data obtained for the comparison of the extraction efficiency of the extractants, and also to compare the use of internal standard compared to making up the extract to a known volume.

6.2.5 Optimising and validating the ACN/PROP method for trace analysis of natural 1,4-DMN in potatoes

6.2.5.1 Optimising the HPLC separation for large volume injection

The optimisation of the ACN/PROP method for the analysis of trace levels of natural 1,4-DMN was conducted using the ACE C18 5 μm (250 x 4.6 mm) column. Different volumes of standard solutions in ACN/PROP (7:3), ACN/PROP/water (49:21:30) and potato extracts containing 1,4-DMN and the internal standards were injected into the HPLC. The quality of the peak shape, retention time and peak areas were examined. The precision of the HPLC analysis of extracts containing 1,4-DMN at the lowest concentration examined was evaluated by replicate injections ($n=11$) of potato peel extract spiked with 1,4-DMN at 0.005 $\mu\text{g/ml}$.

6.2.5.2 Optimising the extraction procedure

The optimisation of the extraction procedure aimed to provide a greater concentration of 1,4-DMN in ACN/PROP extracts by increasing the ratio of sample to solvent. A ratio of sample : solvent of 1:1 was examined here by extracting 40 g of potato peel (finely chopped) by 40 ml of the extracting solution ACN/PROP (7:3) in a 100-ml conical flask. It was not possible to use the same ratio with a smaller scale of peel weight and solvent volume, as some of the peel was not covered by the solvent. The internal standard was added here by a micropipette. 50 μl of 1000 $\mu\text{g/ml}$ 2-methylnaphthalene in ACN were transferred into the conical flask containing the peel sample and the extraction solution. The conical flask was capped with a stopper and placed in a preheated water bath at 50 $^{\circ}\text{C}$ for 15 min with occasional swirling and then cooled for 10 min. About 1.5 ml of the extract was filtered through a 0.2 μm PTFE syringe filter into an HPLC vial.

100 μl of the final extract was injected in into the HPLC and separated on the ACE C18 5 μm (250 x 4.6 mm) column. The mobile phase was ACN/water (7:3) and the analysis was carried out at 25 $^{\circ}\text{C}$ and 228 nm wavelength.

6.2.5.3 Method validation

For the recovery experiment, spiking of potato peel was conducted at three levels: 0.01, 0.05 and 0.1 $\mu\text{g/g}$ peel. 200 μl of the spiking solution in acetonitrile containing 1,4-DMN (at levels of 2, 10 and 20 $\mu\text{g/ml}$ for 0.01, 0.05 and 0.1 $\mu\text{g/g}$ peel respectively) using a Gilson P200 micropipette to 40 g of potato peel in 100-ml conical flasks. The solutions were left to settle for 1 h, after which the extraction process conducted as described in

Section 6.2.5.2. Extracts were then analysed by HPLC using the chromatographic conditions described above in Section 6.2.5.2.

The calculations were based on using a calibration line generated by triplicate injections of five standards at levels of 0.008, 0.012, 0.04, 0.08 and 0.12 $\mu\text{g/ml}$ of 1,4-DMN and a constant concentration of the internal standard 2-methylnaphthalene at 1 $\mu\text{g/ml}$.

6.2.6 Optimising the ACN/PROP method for routine analysis of 1,4-DMN residues in potatoes

Although the ACN/PROP method was initially designed for the analysis of trace levels of 1,4-DMN, the method was also optimised and validated for routine analysis of 1,4-DMN residues in potatoes due to the different advantages found in this method compared to the TMP/Heat method. These advantages include the following:

- The extraction solution of ACN/PROP (7:3 v/v) is water miscible, and therefore more compatible with the HPLC mobile phase used and with the water derived from the fresh potato peel
- High extraction efficiency for 1,4-DMN from potato peel samples
- Cleaner extracts and therefore no clean up required
- Rapid, simple, straightforward and less laborious procedure

The optimisation for routine analysis included using a shorter (10 cm) column for the HPLC separation, and extracting only 10 g of peel sample with 15 ml of the extraction solution containing the internal standard. The method was then validated as detailed below.

6.2.6.1 Optimising the HPLC separation on a 10-cm column

Extracts and standard solutions were analysed on a 100 x 4.6 mm column (Phenomenex Sphereclone 5 μm) to shorten the run time of the HPLC analysis. Chromatograms were checked for the resolution of 1,4-DMN and 2-methylnaphthalene peaks from each other and from any interfering peak in addition to any late eluted peaks.

6.2.6.2 System precision

HPLC instrument precision was examined by analysing replicate injections ($n=7$) of the 1,4-DMN standards at levels of 0.5 $\mu\text{g/ml}$ and at 10 $\mu\text{g/ml}$ of both 1,4-DMN and the internal standard 2-methylnaphthalene. The precision was expressed by the relative standard deviation (%RSD) of peak areas in the replicate injections.

6.2.6.3 Method recovery and precision

This experiment was conducted to examine the accuracy and precision of the ACN/PROP method for the routine analysis of 1,4-DMN residues in potatoes. Organically grown potato tubers (Nicola variety) were bought from a local supermarket in Glasgow. The tubers were washed, peeled and subsampled as described in Section 5.4.4. Five spiked subsamples were prepared for each of the three spiking levels, in addition to three controls left without spiking. Three reagent blanks containing only the extraction solution and water, with no potato peel, were also included to check for any contamination in the reagents.

Recovery peel subsamples were spiked with 1,4-DMN at levels of 0.2, 4 and 20 µg/g peel. Spiking of peel samples was achieved by adding 200 µl of the spiking solution in acetonitrile containing 1,4-DMN (at levels of 10, 200 and 1000 µg/ml for 0.2, 4 and 20 µg/g peel respectively) using a Gilson P200 micropipette to 10 g of potato peel in 50-ml conical flasks. The solutions were left to settle for 1 h, after which the extraction process started. Fifteen millilitres of ACN/PROP (7:3) extraction solution containing the internal standard were added to the flask using a pipette. The conical flask was capped with a stopper and placed in a preheated water bath at 50 °C for 15 min with occasional swirling, and then cooled for 10 min. After cooling, one millilitre aliquot of the extract was filtered through a 0.2 µm PTFE syringe filter into an HPLC vial and analysed by HPLC.

HPLC analysis was conducted by injecting 10 µl of the final extract into the instrument. The separation was achieved on the Phenomenex Spherclone 100 x 4.6 mm column (5 µm). The mobile phase was ACN/water (7:3). The analysis was carried out at ambient temperature and at 228 nm wavelength.

The calculations were based on using a calibration line generated by duplicate injections of six standards at levels of 0.1, 0.5, 2, 4, 10 and 20 µg/ml of 1,4-DMN and a constant concentration of the internal standard 2-methylnaphthalene at 10 µg/ml.

6.2.6.4 Stability of 1,4-DMN in ACN/PROP (7:3) extracts at ambient temperature

This experiment was conducted to investigate the stability of 1,4-DMN in ACN/PROP extracts stored at ambient temperature for several days, a situation which could potentially occur in practice, e.g. when samples are prepared in a different location.

Potato samples treated with 1,4-DMN and stored under commercial storage conditions for weeks were used in this experiments. Two samples of three different varieties (Desiree,

Maris Piper and King Edward) were extracted and analysed according to the method described above in Section 6.2.6.3 for routine residue analysis. The conical flasks containing the samples (with the extraction solution) were then sealed with a plastic film and left on the bench for four days, after which another aliquot was taken for HPLC analysis to examine any changes in the extracted level of 1,4-DMN, or any new interfering compounds that might be produced due the deterioration of the extracts. These aliquots were also examined for any breakdown compounds that may have longer retention by increasing the run time to 10 min instead of 4 min.

6.3 Results and discussion

6.3.1 Efficiency comparisons of ACN/PROP and EtOH/TMP using treated potatoes

This experiment aimed to generate enough data for quantitative comparison of the extraction efficiency of the ACN/PROP extractant compared to the EtOH/TMP and also to evaluate the method of making up the extract to known volume compared to the use of internal standard. In addition, the necessity of a clean up step for potato extracts produced by the ACN/PROP method was evaluated by examining the chromatograms generated for such extracts for any interference.

The use of an internal standard minimises the uncertainty in the volumetric measurements in an analytical method. Providing that the internal standard and analyte behave identically in regard to their recoveries, and identical amounts of the internal standard are added to both the sample and reference standard, the rest of the volumetric operations become non-critical (Meyer and Majors, 2002). Therefore, during the comparison of the extraction efficiency of the two extractants, the calculations were based on using the internal standard.

The regression method described and used in Chapter 2 (see Section 2.3.2.6 and Section 2.3.3.5) was used here for the quantitative evaluation of the extraction efficiencies of the two solvent mixtures. The regression plot of the extraction efficiency (calculated using the internal standard) of ACN/PROP compared to EtOH/TMP is shown in Figure 6.2 and the statistical values in Table 6.1.

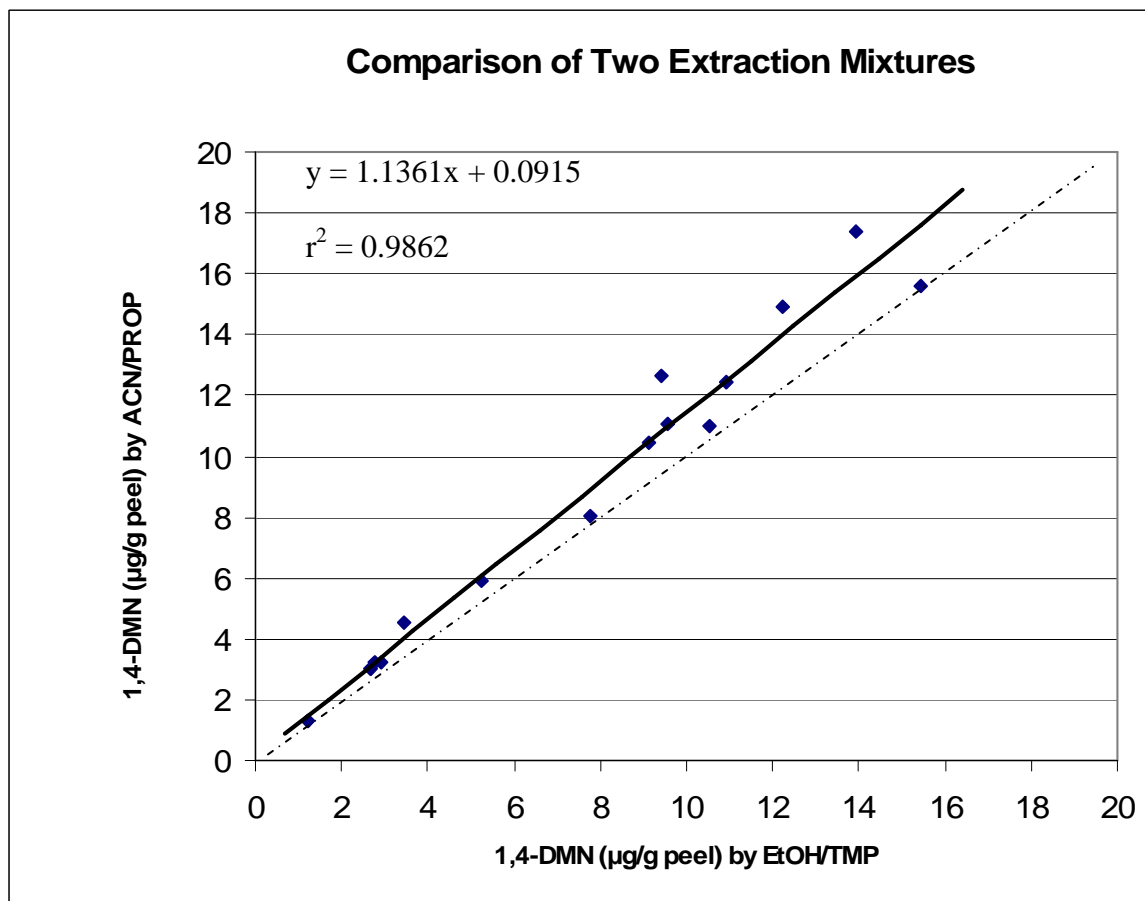


Figure 6.2 Comparison of the efficiency of extracting 1,4-DMN from treated potato samples by the two extraction mixtures ACN/PROP and EtOH/TMP. The solid line shows the regression line (best fit) and the dashed one represents the equality line

Table 6.1 Summary of the statistical values obtained from Excel® for the regression line of the comparison between the two extraction solvents

Statistic	Value
Correlation Coefficient (r^2)	0.9862
Intercept	0.0915
Intercept 95 % Confidence Interval	-0.7948 to 0.9777
Slope	1.1361
Slope 95 % Confidence Interval	1.0310 to 1.2412

The high correlation ($r^2 = 0.9862$) between the data points in the regression plot in Figure 6.2 indicates good precision of the extraction methods using the two extraction mixtures. The 95 % confidence interval of the intercept of the regression line includes the ideal value of zero and therefore, the intercept is not significantly different from the zero. This means

that there is no bias between the two methods in evaluating the 1,4-DMN level in the blank. In contrast, the 95 % confidence interval of the slope of the regression line does not include the ideal value of one and therefore, the slope differs significantly from the ideal value of one. The value of the slope of 1.1361 is higher than the slope of the equality line of one by about 14 %. This indicates that the ACN/PROP (7:3) mixture extracted about 14 % more 1,4-DMN than the extraction mixture EtOH/TMP (7:3). This might be related to the slightly higher polarity of the ACN/PROP mixture compared to EtOH/TMP mixture. This higher extraction efficiency of the method using ACN/PROP mixture is important in lowering the quantification limit of the method for trace levels of 1,4-DMN.

The comparison of the two methods of evaluating the final extract volume in the method using ACN/PROP mixture is shown in Figure 6.3 with the statistical values summarised in Table 6.2.

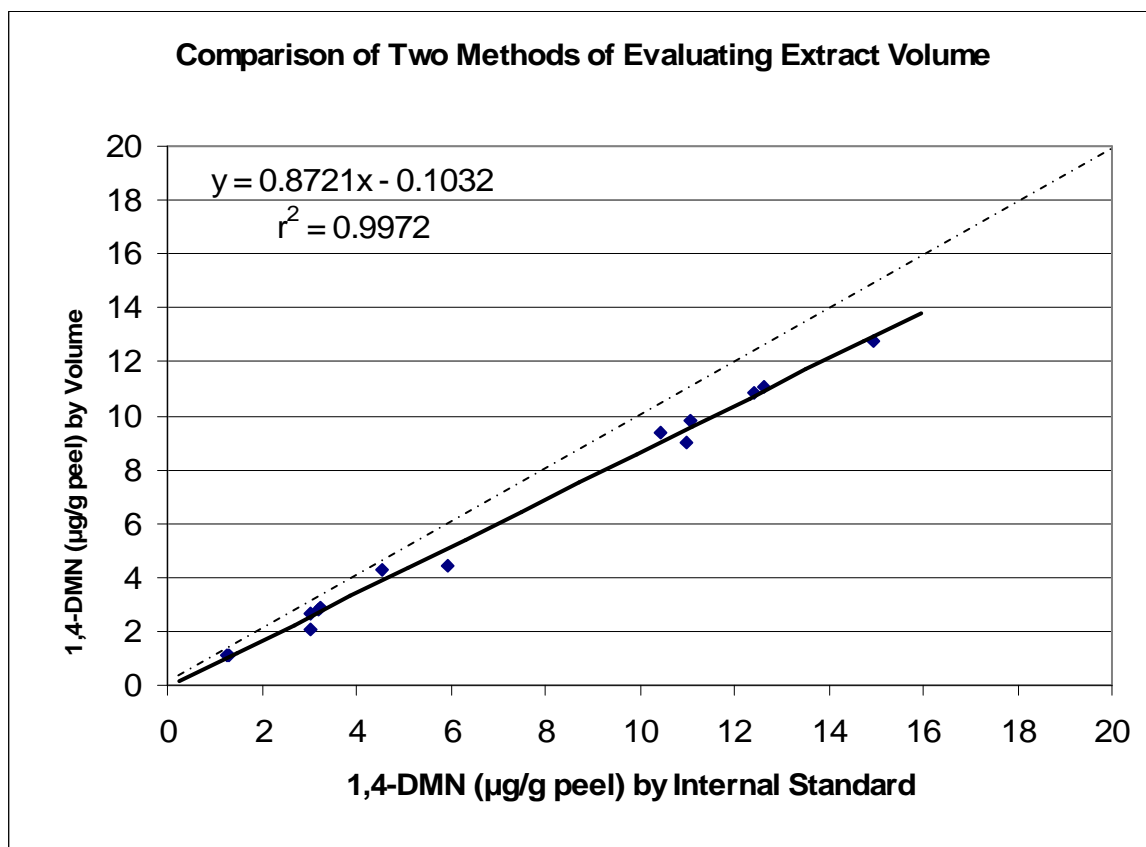


Figure 6.3 Comparison of the use of known extract volume and the internal standard for the evaluation of the final extract volume. The solid line shows the regression line (best fit) and the dashed one represents the equality line

Table 6.2 Summary of the statistical values obtained from Excel® for the regression line of the comparison between the two methods of evaluating the extract volume

Statistic	Value
Correlation Coefficient (r^2)	0.9972
Intercept	- 0.1032
Intercept 95 % Confidence Interval	-0.4463 to 0.2399
Slope	0.8721
Slope 95 % Confidence Interval	0.831231 to 0.912903

The two methods for evaluating the volume of the final extract showed good precision, as indicated by the high correlation factor of the regression line of $r^2=0.9972$. The intercept was not significantly different from zero because the ideal value of zero is included in the 95 % confidence interval which indicates that there is no offset in the measurement of blank samples. In contrast, the 95 % confidence limits showed that the slope of the regression line is significantly lower (with a value of about 13 %) from the slope of the equality line that has a value of one. This difference indicates that the use of the known volume (25 ml here) in the calculations of 1,4-DMN extracted with the ACN/PROP mixture leads to values significantly lower (with about 13 %) than what were obtained using the internal standard. The lower values of extracted 1,4-DMN when using the known volume in the calculation might not be unexpected, as some loss of the extracted 1,4-DMN can occur by volatilisation or adsorption on surfaces, in addition to the errors in adjusting the extract volume. This type of error can be fully correctable by the use of internal standardisation (Snyder and Vanderwal, 1981; Anastassiades et al., 2003). Based on these results, concentrations of 1,4-DMN in sample extracts will be calculated using an internal standard.

To evaluate the necessity for a clean up step in the ACN/PROP method, the chromatograms of the extracts obtained were examined to have an idea about the nature of the co-extractive compounds extracted. Representative chromatograms of samples extracted by using the ACN/PROP and the EtOH/TMP extractants are shown in Figure 6.4. The extract in ACN/PROP was run for a longer time to check for any late eluting peaks of more non-polar compounds.

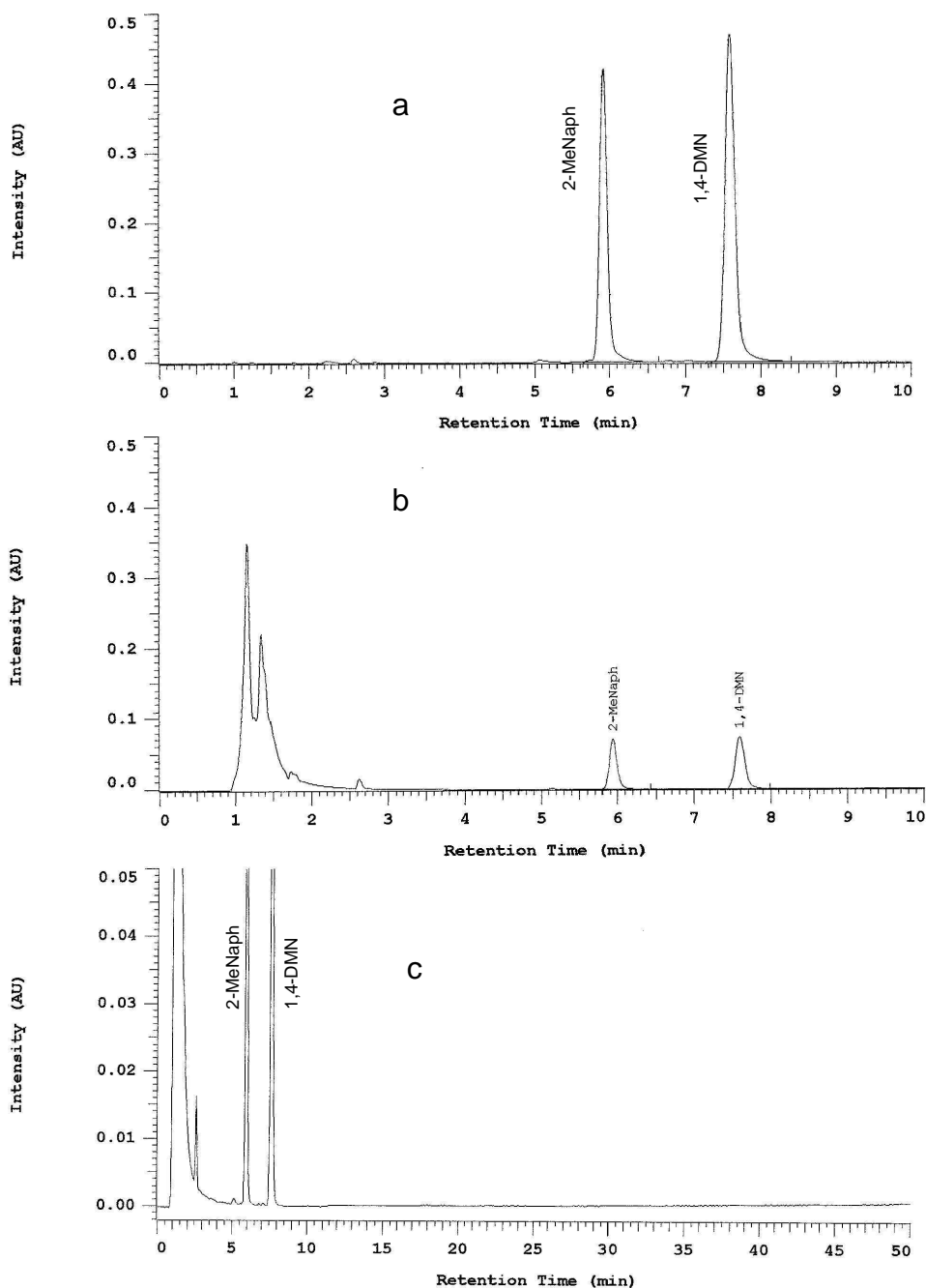


Figure 6.4 Representative chromatograms of extracts of potato peel containing 1,4-DMN at level of 15 $\mu\text{g/g}$ peel obtained by two different extraction mixtures. a) Ethanol/ 2,2,4-trimethylpentane (7:3). b) Acetonitrile/ 2-propanol (7:3). c) The same sample in b but with different absorbance scale and retention time. Chromatographic conditions: Supelco Spherisorb ODS-2 5 μm (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, ambient temperature and 228 nm

As shown in Figure 6.4, the extraction mixture EtOH/TMP provided a cleaner extract than the ACN/PROP mixture due to the efficient liquid-liquid extraction step used with the EtOH/TMP extracts. However, most of the co-extractive compounds extracted by the ACN/PROP mixture eluted earlier than 1,4-DMN and the internal standard 2-methylnaphthalene and no late eluting peaks were present, as shown in Figure 6.4.c. This nature of the co-extractives provides a chance to eliminate any further clean up step and

save more time, effort, solvent and cost of the analysis. Therefore, no further clean up step will be used in the final ACN/PROP method.

In summary, the ACN/PROP extraction solution was found to be a good replacement for the EtOH/TMP extraction solution, as it provides higher extraction efficiency compared to EtOH/TMP. This higher efficiency will help in enhancing the detection sensitivity of the method for trace levels of natural 1,4-DMN in potatoes. In addition, due to the polar nature of the co-extractives extracted by the ACN/PROP mixture, the extracts can be directly injected to the HPLC with no need for any further clean up. Furthermore, the compatibility of the extraction solvents with the mobile phase used in the chromatographic determination should allow the injection of large volumes of the extracts directly to the HPLC which will provide a valuable method of enhancing the detection efficiency of the method for trace levels of 1,4-DMN.

6.3.2 Optimising and validating the ACN/PROP method for trace analysis of 1,4-DMN in potatoes

6.3.2.1 Optimising the HPLC separation for large volume injection

The optimisation of the ACN/PROP method for the analysis of trace levels of 1,4-DMN was conducted on the ACE C18 5 μm (250 x 4.6 mm) column. This column provides high separation efficiency. High efficiency is required when analysing trace levels of the analytes as the background effect is normally high. In addition, the longer column used should tolerate higher volume loading (Layne et al., 2001).

The optimisation started with injecting gradually increased volumes of a standard solution containing 1,4-DMN and 2-methylnaphthalene at a level of about 1 $\mu\text{g}/\text{ml}$ dissolved in ACN/PROP (7:3). The injection volumes examined were 10, 20, 30, 40, 60, 80 and 100 μl . The shape of the two peaks was good up to 40 μl injection volume. At 60 μl injection volume, peaks showed some broadening with round heads instead of the usual sharp heads. Peak shapes started to deteriorate more at 80 μl injection volume where the head of each peak split into two peaks. This effect is shown in the chromatograms in Figure 6.5.

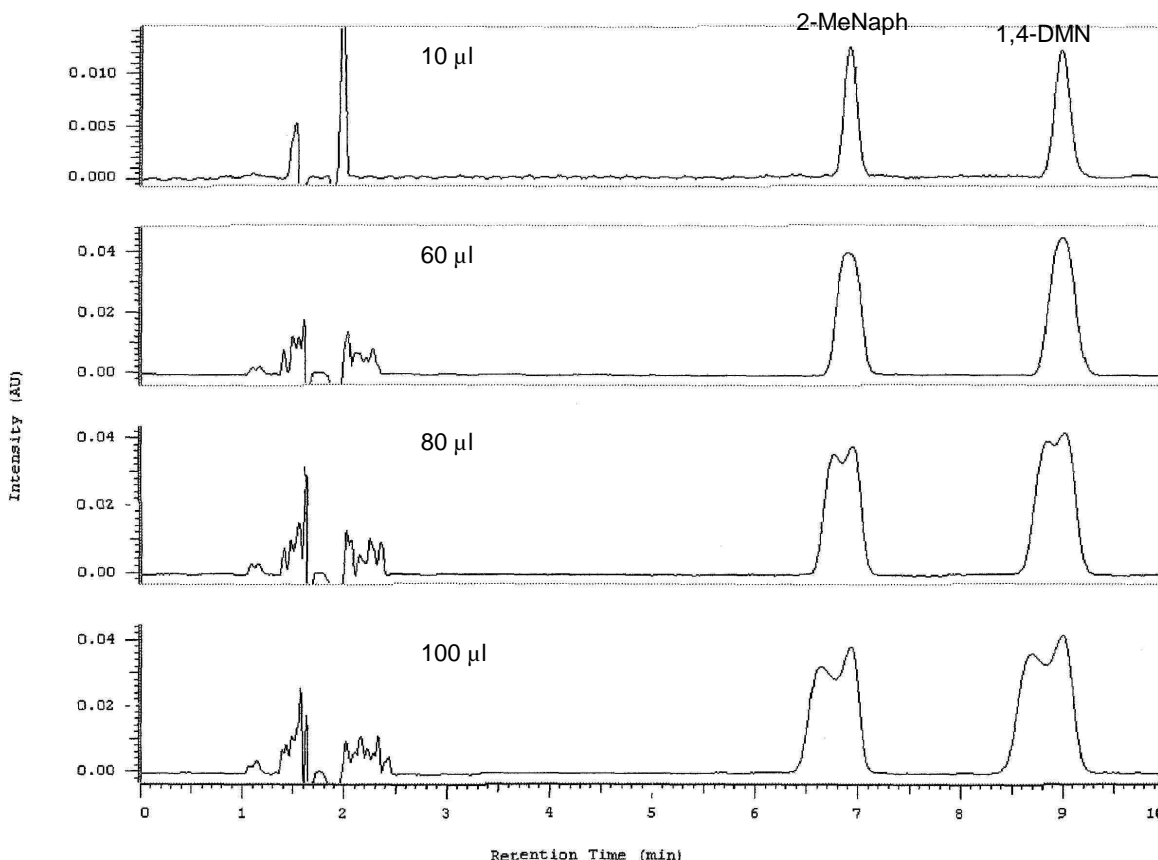


Figure 6.5 Effect of injecting large volumes of a standard solution (about 1 µg/ml) of 1,4-DMN and 2-methylnaphthalene in ACN/PROP (7:3). Chromatographic conditions: ACE C18 5 µm (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 25 °C and 228 nm.

Peak shape distortion is commonly caused by injecting large volumes of a sample dissolved in a solvent stronger (more non-polar, i.e. more organic in reversed-phase HPLC) than the mobile phase (Snyder et al., 1997; Dolan, 2004). In addition to the strength differences between the sample solvent and the mobile phase, differences in viscosities between the two was reported to result in severe distortion of peak shapes when injecting large sample volumes (Keunchkarian et al., 2006; Castells et al., 1997). Both differences seem to be present here for the sample dissolved in ACN/PROP (7:3). The sample solvent consists of two organic solvents with greater non-polarity than the mobile phase that contains 30 % of the polar component water. In addition, the presence of 30 % of the highly-viscous solvent 2-propanol (viscosity = 1.95 centipoise at 25 °C) increases the viscosity of the solvent mixture to about 0.61 centipoise compared to 0.59 centipoise for the mobile phase at 25 °C. Viscosity values used here were reported by Snyder et al. (1997) or calculated based on a method described in the same reference.

Two practices can be followed to avoid the peak distortion shown in Figure 6.5. The first is to minimise the injection volume, and the other is to minimise the differences between the sample solvent used and the mobile phase. The first practice can be used when 1,4-DMN concentration in samples is high enough for detection with small volume injections in

residue analysis. However, this practice will not be useful here as this method is intended to be used for trace levels of 1,4-DMN and hence a larger injection volume is required to enhance the detection sensitivity. Therefore, the second practice was used here.

A standard solution (about 1 $\mu\text{g/ml}$) of 1,4-DMN and 2-methylnaphthalene was prepared in ACN/PROP/water (49:21:30) in order to obtain better matching of the mobile phase and also to the real potato extract which contained water derived from potato peel. This standard was loaded to the HPLC with different injection volumes as described above for the standard in ACN/PROP (7:3). The resulting chromatograms are shown in Figure 6.6.

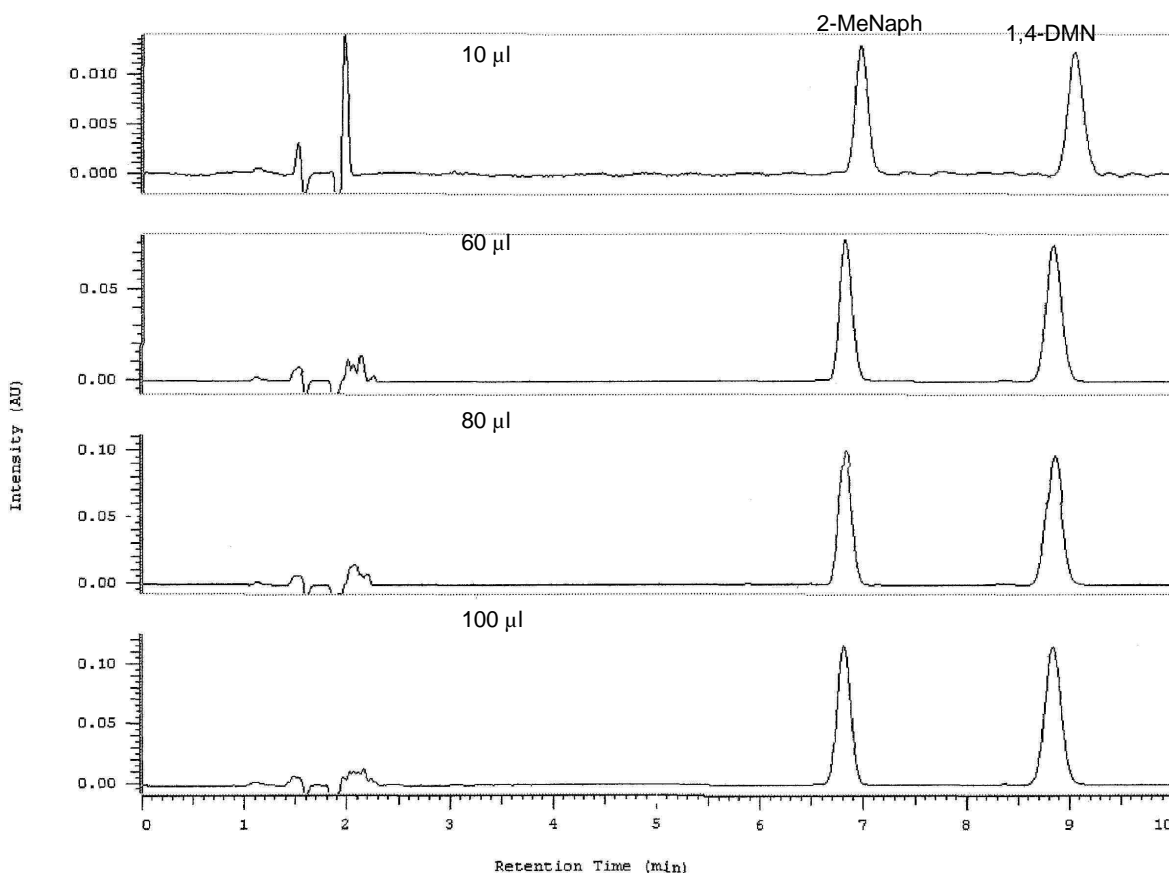


Figure 6.6 Effect of injecting large volumes of a standard solution (about 1 $\mu\text{g/ml}$) of 1,4-DMN and 2-methylnaphthalene in ACN/PROP/water (49:21:30). Chromatographic conditions: ACE C18 5 μm (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 25 $^{\circ}\text{C}$ and 228 nm.

Figure 6.6 shows clearly that the problem of peak distortion when injecting large volumes disappeared when injecting the standard in a mixture of ACN/PROP/water (49:21:30). The addition of 30 % of water to the mixture of ACN/PROP diluted the organic mixture and reduced the elution strength. In addition, reducing the proportion of the highly-viscous solvent 2-propanol reduces the viscosity of the sample solvent mixture. The sharp peak shape obtained indicates that the differences between the sample solvent and the mobile phase were minimised successfully.

Retention time precision was examined by making triplicate injections of the standard prepared in ACN/PROP/water (49:21:30) for each injection level. There was a slight reduction in the retention times of both the 1,4-DMN and 2-methylnaphthalene peaks between the 100 μ l and the 10 μ l injections of only 2.3 %. This slight variation is negligible and may occur in routine running conditions, due to slight variation in chromatographic conditions such as temperature.

It should be mentioned that the above problem of peak shape deterioration was not noticed when injecting large volumes (up to 100 μ l) of potato peel extracts because peel extracts contain a considerable amounts of water derived from the fresh peel tissues. This water was enough to minimise differences between the extract and the mobile phase.

To examine the effect of the background compounds extracted by the ACN/PROP solution from the peel on the chromatographic separation and quantification of 1,4-DMN and the internal standard at trace level, peel extracts of organically grown tubers were spiked with 1,4-DMN and 2-methylnaphthalene at a level of 0.005 μ g/ml and compared to the extracts without spiking. Representative chromatograms for spiked and non-spiked peel extracts are shown in Figure 6.7.

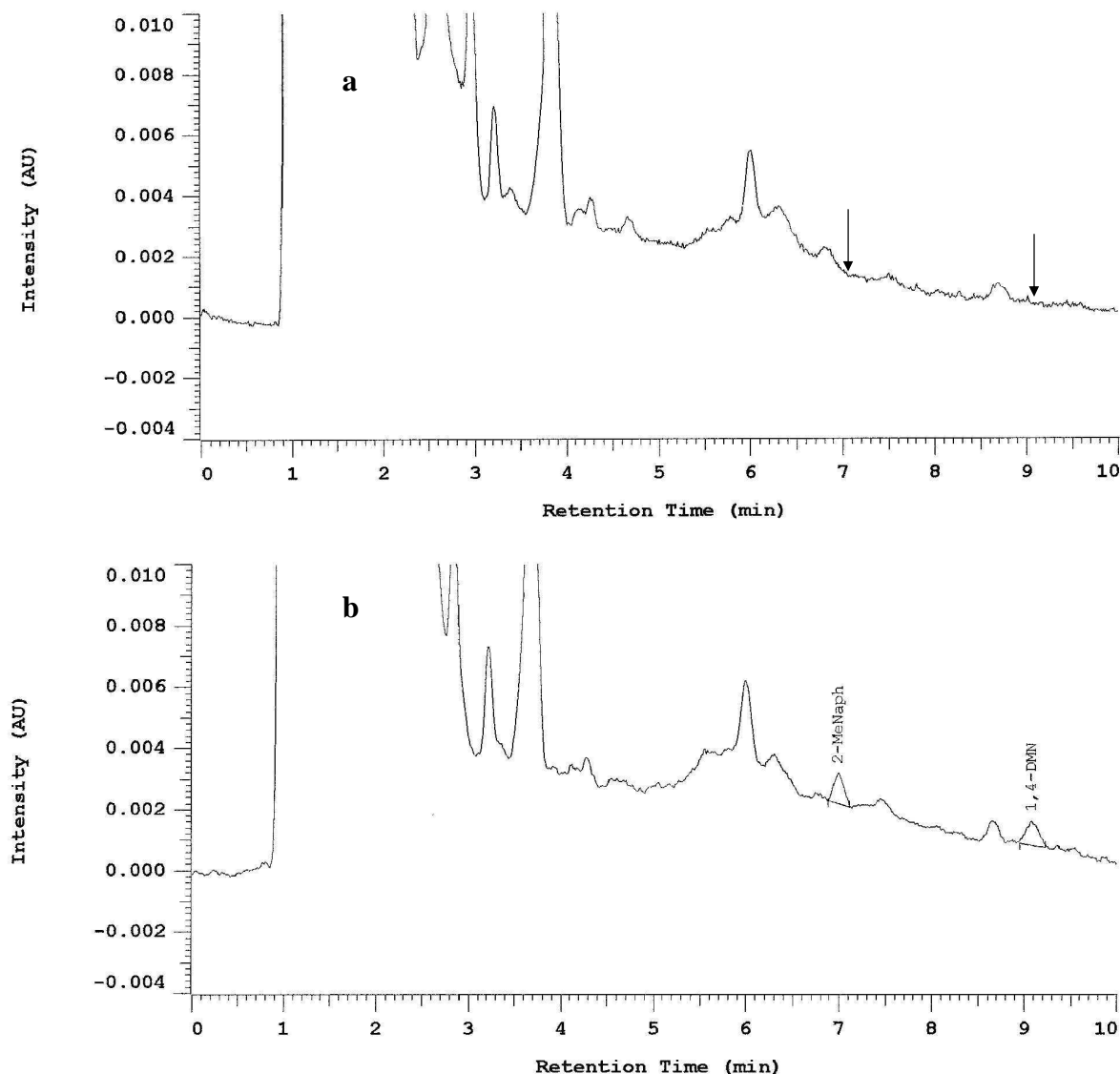


Figure 6.7 Representative chromatograms of potato peel samples extracted by ACN/PROP method and injected to the HPLC in large volume (100 μ l). a) Control sample without spiking. b) Extract spiked at a level of 0.005 μ g/ml. Arrows in the control chromatogram indicates 1,4-DMN and 2-methylnaphthalene retention times. Chromatographic conditions: ACE C18 5 μ m (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 25 $^{\circ}$ C and 228 nm

Fortunately, there were no interfering peaks at the retention times of either 1,4-DMN or 2-methylnaphthalene. However, small peaks were present just before the two peaks. This should not be a problem with the 2-methylnaphthalene peak, as high concentrations of this internal standard can be used, and therefore the percentage of the little interfering peak will be negligible. In contrast, more care should be taken to avoid any interference with the 1,4-DMN peak as the 1,4-DMN levels will be very low. Therefore, it will be important to have good control of the chromatographic conditions (such as column temperature) to avoid any deterioration in the resolution between the 1,4-DMN peak and the interfering background peaks.

The precision of the HPLC injection of 1,4-DMN levels as low as 0.005 μ g/ml in ACN/PROP extracts was evaluated by replicate injections (n=11) of a spiked extract under

the same chromatographic conditions described for Figure 6.7. Good precision was obtained at this low level with a %RSD value of 8.6 %.

To summarise the above discussion, the ACN/PROP method can be used successfully for the analysis of trace levels of 1,4-DMN in potato peel samples using large injection volumes to enhance the detection sensitivity. 1,4-DMN in extracts at levels as low as 0.005 µg/ml were successfully separated and quantified with adequate precision of %RSD of 8.6 %. Some consideration should be taken when using large volume injections. Extracts and standard solutions should have enough water content (30 % was used for standard solutions) to avoid peak shape distortion. Chromatographic conditions need to be controlled well to avoid any deterioration in peak resolution.

6.3.2.2 Optimising the extraction procedure

The main optimisation in the extraction procedure was the use of a higher ratio of sample : solvent to provide a more concentrated extract. Forty grams of peel sample were extracted with forty millilitres of ACN/PROP (7:3) solvent mixture added by a measuring cylinder. It was not possible to use the same ratio with a smaller scale of peel weight and solvent volume, as some of the peel was not covered with the solvent at the smaller scale. The internal standard was added here using a micropipette after adding the extraction solution to the peel sample. The volume of the added extraction solution should not be critical for quantification, as the internal standard was added separately. This method was then evaluated for its recovery and precision using samples spiked at low levels of 1,4-DMN. In addition, its detection sensitivity was evaluated through the determination of the method limit of detection and quantification.

6.3.2.3 Method validation

The precision of the HPLC injection of 1,4-DMN at levels of 0.005 µg/ml in peel extracts was described above in Section 6.3.2.1. Good precision was obtained at this low level with a %RSD value of 8.6 %.

The accuracy, precision and limit of detection and quantification of this method were evaluated through a recovery experiment at three low levels: 0.01, 0.05 and 0.1 µg/g peel (equivalent to approximately 1.5, 7.5 and 15 µg/kg fresh potato weight). A calibration line generated by triplicate injections of five standards was used for the calculations of the recovery and precision, and also for estimating the limit of detection and the limit of quantification statistically.

The calibration plot showed good linearity in the range examined (0.008 to 0.12 $\mu\text{g/ml}$) with a value of the regression coefficient $r^2 > 0.999$. The regression equation of the calibration line was $y = 636258x + 647$ where x represents the nominal concentration of calibrators and y value represents peak area. Statistical evaluation of the regression line (using Excel[®] software) showed that the intercept value of 647 is not significantly different from zero (95 % confidence interval was -1230 to 2524 which includes the zero value). Because of the good linearity of the calibration graph and the absence of any offset in the blank reading (intercept not significantly different from zero) a single calibration standard (defining two-point calibration with the origin) can be used in routine analysis.

The limit of detection (LOD) and limit of quantification (LOQ) are two parameters that are frequently reported as part of analytical method validation. The various definitions and approaches of estimating the two terms are detailed in the introduction in Section 1.7.5.

For the current ACN/PROP method for trace analysis of natural 1,4-DMN in potatoes, the limit of detection and limit of quantification was estimated by three different approaches. The first approach was by using the standard deviation (SD) of replicate injections ($n=11$) of a spiked potato extract at a concentration of 0.005 $\mu\text{g/ml}$. LOD and LOQ were then calculated as follows: $\text{LOD} = 3.3 \text{ SD}/S$ and $\text{LOQ} = 10 \text{ SD}/S$ where S is the slope of the calibration line. The values were then converted to the corresponding $\mu\text{g/g}$ peel using the typical final extract volume ($\sim 60 \text{ ml}$) and peel weight (40 g). The values in $\mu\text{g/g}$ peel can be converted to the basis of fresh potato weight by multiplying by 0.15 because the typical percentage of the peel weight was found to be 15 % of potato fresh weight.

The second approach for the determination of the LOD and LOQ of the ACN/PROP method for natural 1,4-DMN was based on the calibration line for 1,4-DMN standards. This method was described in Section 1.7.5 and used in Chapter 2 (see Section 2.3.2.5 and Section 2.3.3.4). In this method, the intercept of the calibration line and the residuals standard deviation (both values can easily be obtained using Excel[®] software) were used to estimate the LOD using the equation:

$$\text{LOD peak area} = \text{Intercept} + 3 \times \text{Residuals standard deviation}$$

The corresponding concentration (in $\mu\text{g/ml}$) of the LOD peak area was then calculated using the equation of the calibration line. The concentration was then converted to $\mu\text{g/g}$ peel by using the typical final extract volume (60 ml) and peel weight (40 g). The values in $\mu\text{g/g}$ peel can be converted to $\mu\text{g/g}$ of fresh potato weight by multiplying by 0.15.

A more practical approach for determining the LOQ of the whole analytical procedure is to use a recovery and precision experiment to determine the lowest concentration of a sample that can still be quantified with acceptable recovery within the range 70-120 %, and with precision not exceeding 20 % (%RSD) (Peters and Maurer, 2002; Bansal and DeStefano, 2007; Pizzutti et al., 2007). The LOD can then be estimated by dividing the resulting LOQ by 3.

The recovery approach was the third approach used for estimating the LOD and LOQ of the ACN/PROP method for trace 1,4-DMN levels. This experiment was conducted to examine the accuracy and precision of the method at three levels (0.01, 0.05 and 0.10 µg/g peel) close to the expected LOD and LOQ of the method. The standard deviation (SD) of the values of the recovered 1,4-DMN at the two levels 0.05 and 0.10 µg/g peel was used to calculate the LOQ and LOD values. This standard deviation was multiplied by 3.3 to get the LOD and by 10 to get the LOQ. The mean of the LOD and LOQ from the two levels was then used as the LOD and LOQ for this method, as this approach includes the whole analytical procedure in the determination of these two terms.

The values of the LOD and LOQ determined by the three methods are shown in Table 6.3.

Table 6.3 Limit of Detection (LOD) and quantification (LOQ) for the ACN/PROP method for natural 1,4-DMN determined by different approaches.

Term	Determination Approach		
	SD of Spiked Extract	Residuals SD of Calibration Line	SD of Real Recovery Experiment
LOD (µg/g peel)	0.002	0.006	0.01
LOQ (µg/g peel)	0.007	0.02	0.03

Although the differences in the resulting LOD and LOQ values given by the three approaches are small, these differences showed the need for a consensus on the proper way of determining these terms for environmental analytical methods, in order to allow for direct comparisons between methods.

The recovery and precision of the method for trace levels are shown in Table 6.4.

Table 6.4 Recovery of 1,4-DMN from spiked peel samples extracted by the ACN/PROP method for trace levels. Note: NA= Not Applicable and NP=No peak at 1,4-DMN retention time.

Sample	Fortification Level ($\mu\text{g/g}$ peel)	Peel Sample Weight (g)	Volume of Fortification Standard Added (ml)	Concentration of Fortification Standard Added ($\mu\text{g/ml}$)	Residues Detected ($\mu\text{g/g}$ peel)	Recovery (%)	Mean Recovery (%)	RSD (%)
Control A	NA	40.0	NA	NA	NP	NA	NA	NA
Control B	NA	40.0	NA	NA	NP	NA		
Control C	NA	40.0	NA	NA	NP	NA		
Recovery A	0.01	40.0	0.200	2	0.014	135.1	110.2	23.3
Recovery B	0.01	40.0	0.200	2	0.012	119.5		
Recovery C	0.01	40.0	0.200	2	0.010	95.8		
Recovery D	0.01	40.0	0.200	2	0.007	72.6		
Recovery E	0.01	40.0	0.200	2	0.013	128.1		
Recovery A	0.05	40.0	0.200	10	0.044	88.8	87.1	7.9
Recovery B	0.05	40.0	0.200	10	0.049	98.0		
Recovery C	0.05	40.0	0.200	10	0.042	84.4		
Recovery D	0.05	40.0	0.200	10	0.040	79.8		
Recovery E	0.05	40.0	0.200	10	0.042	84.5		
Recovery A	0.10	40.0	0.200	20	0.091	90.9	86.4	4.0
Recovery B	0.10	40.0	0.200	20	0.084	83.8		
Recovery C	0.10	40.0	0.200	20	0.089	89.3		
Recovery D	0.10	40.0	0.200	20	0.085	85.3		
Recovery E	0.10	40.0	0.200	20	0.083	83.0		

The values in Table 6.4 show that the method expressed higher variability of 23.3 % at the 0.01 $\mu\text{g/g}$ peel level exceeding the 20 % acceptable limit of the limit of quantification. This concentration level was estimated to be the LOD of the method, and therefore the method may not be capable of providing acceptable quantitative results at this level. However, semi-quantitative or qualitative results might be used at this level.

The good recovery and precision values obtained at the 0.05 $\mu\text{g/g}$ peel level indicates that the LOQ is below this value, which confirms the estimated value of 0.03 $\mu\text{g/g}$ peel as the limit of quantification of this method. Therefore, the values of LOD and LOQ obtained from the recovery experiment were accepted as a good estimation of the LOD and LOQ of the ACN/PROP method for natural 1,4-DMN. This means that the LOD of the ACN/PROP method for trace levels of natural 1,4-DMN has a LOD of 0.01 $\mu\text{g/g}$ peel and LOQ of 0.03 $\mu\text{g/g}$ peel. On the basis of potato fresh weight, LOD and LOQ values will correspond to 1.5 $\mu\text{g/kg}$ and 4.5 $\mu\text{g/kg}$ (ppb) respectively.

For comparison purposes, a similar recovery and precision experiment was conducted for the evaluation of the LOD and LOQ of the final TMP/Heat method reported in Section 5.4.

The experiment was conducted at the same spiking levels. The results of this recovery experiment are shown in Table 6.5.

Table 6.5 Recovery of 1,4-DMN from spiked peel samples extracted by the TMP/Heat method. Note: NA= Not Applicable and NP=No peak at 1,4-DMN retention time

Sample	Fortification Level ($\mu\text{g/g}$ peel)	Peel Sample Weight (g)	Volume of Fortification Standard Added (ml)	Concentration of Fortification Standard Added ($\mu\text{g/ml}$)	Residues Detected ($\mu\text{g/g}$ peel)	Recovery (%)	Mean Recovery (%)	RSD (%)
Control A	NA	10.0	NA	NA	NP	NA	NA	NA
Control B	NA	10.0	NA	NA	NP	NA		
Control C	NA	10.0	NA	NA	NP	NA		
Recovery A	0.01	10.0	0.100	1	NP	NA	NA	NA
Recovery B	0.01	10.0	0.100	1	NP	NA		
Recovery C	0.01	10.0	0.100	1	NP	NA		
Recovery D	0.01	10.0	0.100	1	NP	NA		
Recovery E	0.01	10.0	0.100	1	NP	NA		
Recovery A	0.05	10.0	0.100	5	0.062	123.7	105.9	22.1
Recovery B	0.05	10.0	0.100	5	0.040	80.9		
Recovery C	0.05	10.0	0.100	5	0.049	97.8		
Recovery D	0.05	10.0	0.100	5	0.068	136.6		
Recovery E	0.05	10.0	0.100	5	0.045	90.5		
Recovery A	0.10	10.0	0.100	10	0.078	77.7	90.3	10.5
Recovery B	0.10	10.0	0.100	10	0.102	102.1		
Recovery C	0.10	10.0	0.100	10	0.085	84.6		
Recovery D	0.10	10.0	0.100	10	0.096	95.6		
Recovery E	0.10	10.0	0.100	10	0.092	91.6		

The values in Table 6.5 show that the TMP/Heat method was not capable of recovering 1,4-DMN at the spiking level of 0.01 $\mu\text{g/g}$ peel. In addition, a higher variability of 22.1 % was shown at the level of 0.05 $\mu\text{g/g}$ peel which exceeds the 20 % acceptable limit for the limit of quantification. Therefore, the limit of quantification of the TMP/Heat method must be above this concentration level. The standard deviation of the values of the recovered 1,4-DMN at the two levels 0.05 and 0.10 $\mu\text{g/g}$ peel was used to calculate the LOQ and LOD values for this method. Based on these calculations, values of 0.03 and 0.10 $\mu\text{g/g}$ peel were estimated for the LOD and the LOQ respectively for the TMP/Heat method. These values correspond to 4.5 $\mu\text{g/kg}$ and 15 $\mu\text{g/kg}$ (ppb) for the LOD and LOQ respectively on the basis of potato fresh weight. The values reported here for the LOD and LOQ of the TMP/Heat method based on the practical recovery approach are very close to the values estimated based on the HPLC separation method which are reported at the end of Section 5.3. The LOD was estimated in Section 5.3 to be 0.02 $\mu\text{g/g}$ peel (3 $\mu\text{g/kg}$ fresh potato weight) and the LOQ with a value of 0.07 $\mu\text{g/g}$ peel (11 $\mu\text{g/kg}$ fresh potato weight).

In summary, the ACN/PROP method was found to be sensitive, accurate and precise for the extraction of 1,4-DMN at trace levels. The method was found to provide a quantification limit more than three times lower than the TMP/Heat method.

6.3.3 Optimising and validating the ACN/PROP method for routine analysis of 1,4-DMN residues in potatoes

The ACN/PROP method was found to be more efficient and less laborious than the TMP/Heat method, as discussed above. Therefore, the optimisation of this method for routine residue analysis was considered, as the method should provide rapid and simple analysis.

6.3.3.1 Optimising the HPLC separation on a 10-cm column

The chromatogram in Figure 6.8 is for a potato peel sample of tubers treated with 1,4-DMN and extracted by ACN/PROP (7:3 v/v). The separation of the extract was conducted on the 250 x 4.6 mm ACE 5 μ m column.

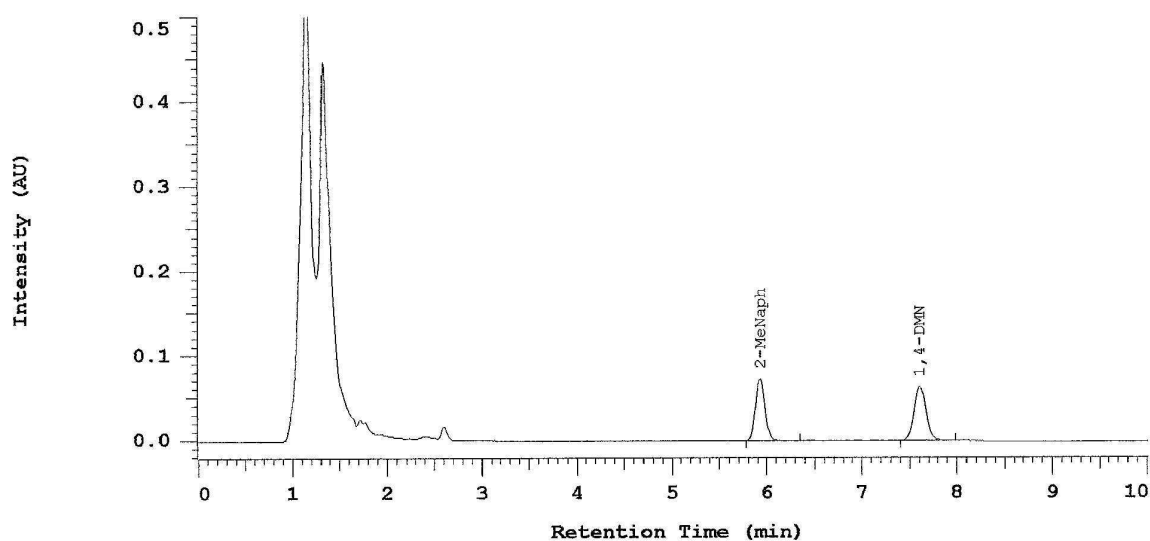


Figure 6.8 A chromatogram of a potato peel sample containing 12.6 μ g/g peel of 1,4-DMN extracted by ACN/PROP (7:3) and separated on the on the 250 x 4.6 mm ACE C18 5 μ m column. Other chromatographic conditions: 70 % acetonitrile: 30 % water, 1.5 ml/min, ambient temperature and 228 nm

This chromatogram shows the large resolution ($R_s > 8$) between the peak of 1,4-DMN and the internal standard 2-methylnaphthalene and also between 2-methylnaphthalene peak and the co-extractive compounds that are eluted earlier. This extra resolution can be reduced by shortening the length of the HPLC column used, and therefore saving considerable analysis time, mobile phase solvents and analysis cost.

A 100 x 4.6 mm column (Phenomenex Spherclone 5 μ m) was used to separate the ACN/PROP extract. Representative chromatograms of a control sample contains no 1,4-DMN and a sample with 1,4-DMN at 3.53 μ g/g peel are shown in Figure 6.9.

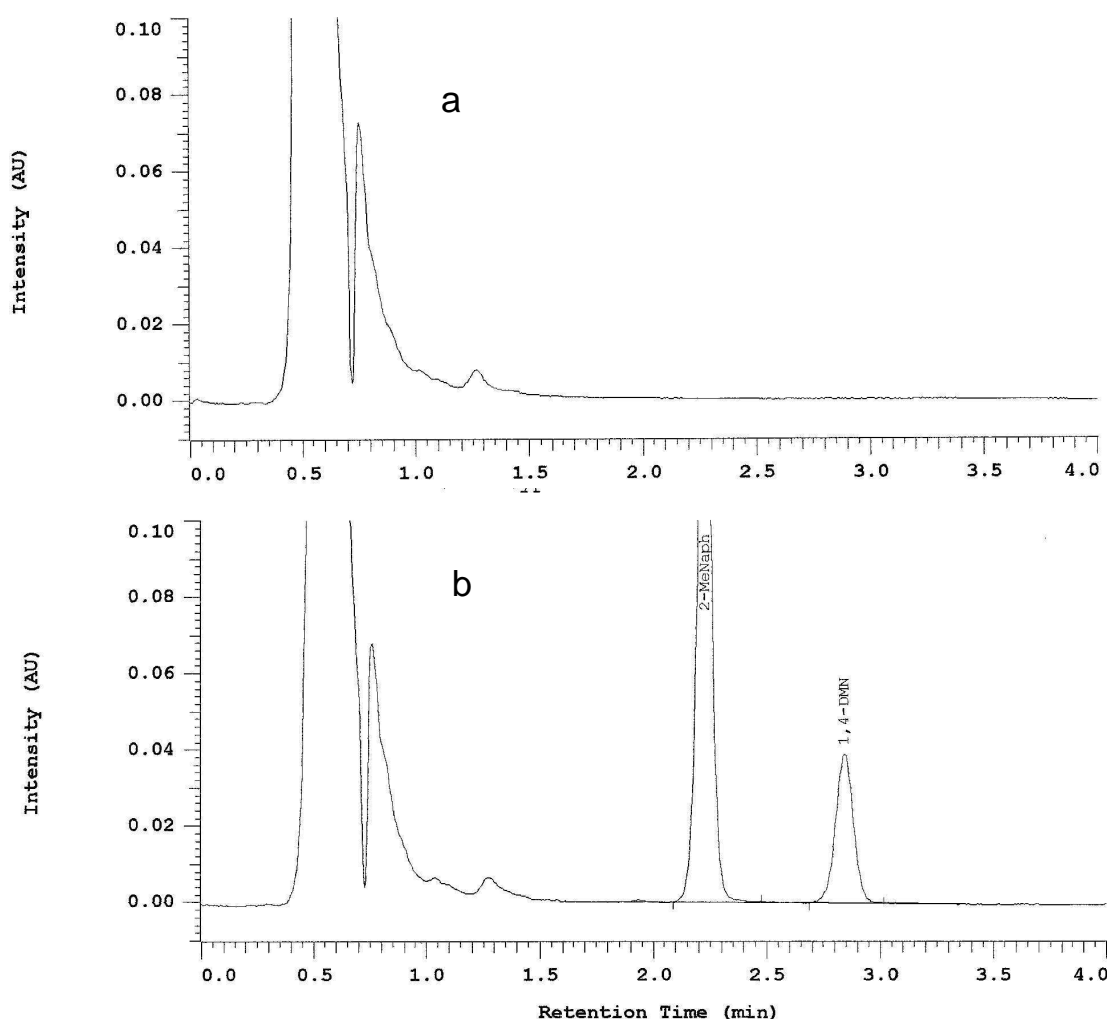


Figure 6.9 Representative chromatograms of potato peel samples extracted by ACN/PROP (7:3) and separated on a 100 x 4.6 mm column (Phenomenex Spherclone 5 μ m) a) Control sample containing no 1,4-DMN. b) Sample with 1,4-DMN at a level of 3.53 μ g/g peel. Other chromatographic conditions: 70 % acetonitrile: 30 % water, 1.5 ml/min, ambient temperature and 228 nm, 10 μ l injection volume

As shown in the chromatogram in Figure 6.9.a for the control sample (a peel extract of organic potato tubers), the extract has no interfering peaks at or around the retention times of 1,4-DMN and the internal standard. This allows the use of the short column safely without any risk of interference of the co-extractives with 1,4-DMN or the internal

standard. The two peaks of 1,4-DMN and 2-methylnaphthalene still have good separation from each other ($R_s \approx 5$) and from the other peaks of the co-extractive compounds.

By using the 100-mm column, a great saving is achieved in the HPLC run time from 10 min (in the 250-mm-length column) to 4 min here with no deterioration in the chromatographic separation. Therefore, this column was used in the final ACN/PROP method for routine residue analysis.

The absence of any interfering peaks around the retention time of 1,4-DMN and the internal standard eliminated the necessity for any further clean up step, which will also add greater savings in analysis time, effort and cost. In addition, the absence of any late eluted peaks (as discussed in Section 6.3.1) eliminates the necessity for any column cleaning step after running the sample in the HPLC. The omission of the clean up step to simplify the analytical procedure is frequently reported in literature (Nieva-Cano et al., 2001; Caboni et al., 2005; Fernandez-Alba et al., 2000). Therefore, no clean up step is conducted in the final ACN/PROP method for the routine analysis of 1,4-DMN residues in potato samples.

6.3.3.2 Precision of the HPLC injection

The HPLC instrument precision of the ACN/PROP method for residue analysis was examined by analysing replicate injections ($n=7$) of the 1,4-DMN standards at levels of 0.5 $\mu\text{g/ml}$ and at 10 $\mu\text{g/ml}$ of both 1,4-DMN and the internal standard 2-methylnaphthalene. The precision was expressed by the relative standard deviation (%RSD) of peak areas in the replicate injections. For the 1,4-DMN peak, %RSD values were 1.50 % and 0.36 % for 0.5 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ respectively. For 2-methylnaphthalene peak, %RSD values were 1.67 % and 0.34 % for 0.5 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ respectively.

These values demonstrate that the system is able to provide precise and consistent results using the chromatographic conditions for the routine method for 1,4-DMN residues in potatoes.

6.3.3.3 Method recovery and precision

The calibration curve used for the calculation in this recovery experiment showed excellent linearity $r^2 > 0.9999$ for 1,4-DMN peak area (y axis) against the nominal concentration of 1,4-DMN (x axis) in the standards. The regression equation was $y = 67050x - 3183$ with 95 % confidence interval of the intercept of (-11755 to 5389). This shows that the intercept is not significantly different from zero and therefore there is no bias in measuring blanks

using this method. Because of the excellent linearity and the absence of any bias in the blank reading, a single calibration standard can be used in routine analysis, which should define a two-point calibration line with the origin (Ake et al., 1998; Bruce et al., 1998; Thompson et al., 2002).

The recovery of 1,4-DMN from fortified peel samples are shown in Table 6.6.

Table 6.6 Recovery of 1,4-DMN from spiked peel samples extracted by the ACN/PROP final method for residue analysis of 1,4-DMN in potatoes. Note: NA= Not Applicable and NP=No peak at 1,4-DMN retention time

Sample	Fortification Level (µg/g peel)	Peel Sample Weight (g)	Volume of Fortification Standard Added (ml)	Concentration of Fortification Standard Added (µg/ml)	Residues Detected (µg/g peel)	Recovery (%)	Mean Recovery (%)	RSD (%)
Control A	NA	10.0	NA	NA	NP	NA	NA	NA
Control B	NA	10.0	NA	NA	NP	NA		
Control C	NA	10.0	NA	NA	NP	NA		
Recovery A	0.20	10.0	0.2	10	0.21	88.2	93.2	7.3
Recovery B	0.20	10.0	0.2	10	0.22	89.0		
Recovery C	0.20	10.0	0.2	10	0.21	102.6		
Recovery D	0.20	10.0	0.2	10	0.22	87.9		
Recovery E	0.20	10.0	0.2	10	0.21	98.1		
Recovery A	4.00	10.0	0.2	200	3.95	88.6	89.6	1.6
Recovery B	4.00	10.0	0.2	200	3.93	89.1		
Recovery C	4.00	10.0	0.2	200	3.53	88.7		
Recovery D	4.00	10.0	0.2	200	3.40	89.4		
Recovery E	4.00	10.0	0.2	200	3.71	92.1		
Recovery A	20.00	10.0	0.2	1000	19.00	91.1	89.6	2.9
Recovery B	20.00	10.0	0.2	1000	18.51	86.1		
Recovery C	20.00	10.0	0.2	1000	20.46	87.7		
Recovery D	20.00	10.0	0.2	1000	18.48	91.2		
Recovery E	20.00	10.0	0.2	1000	20.45	91.9		

The values in Table 6.6 show that the ACN/PROP final method provides accurate and precise results for the analysis of 1,4-DMN residues in potato samples. The recovery and precision values obtained here comply with the EU criteria for a quantitative method for the analysis of pesticide residues in food (document No. SANCO/10232/2006) which set a minimum mean recovery between 70 to 110 % with a %RSD of 15 to 18 % for pesticides that have a maximum residue level (MRL) >0.1-1 mg/kg (European-Commission, 2006).

The recovery values shown in Table 6.6 for the ACN/PROP method appear to express some loss of 1,4-DMN during the extraction procedure. A similar effect was also noticed for the TMP/Heat method, as shown in Table 4.4 in Chapter 4. The reason for the recovery

being lower than 100 % is related to the method used in conducting the recovery experiments, where 1,4-DMN was added to the peel sample while the internal standard was added to the extraction solution. In this case, 1,4-DMN has more contact with the peel surfaces than the internal standard and, hence, more 1,4-DMN is lost by adsorption in the peel than the internal standard. As a result of these adsorption differences, the reported recovery values of the methods are lower than the real recovery, with a factor equivalent to the difference in the recoveries of 1,4-DMN and the internal standard in the final extract.

This explanation is clearly confirmed with the recovery data reported in Table 6.7. The recovery experiments here were conducted by adding both 1,4-DMN and the internal standard to the peel sample in one spiking standard solution, and extracting the samples according to the ACN/PROP method or the TMP/Heat method. In the case of the ACN/PROP method, the extracts were made up to a known volume in order to evaluate the absolute recoveries of 1,4-DMN and the internal standard 2-methylnaphthalene. For the TMP/Heat method, the recovery values reported here are relative recoveries of 1,4-DMN calculated using the internal standard. It was not possible in this method to examine the absolute recoveries as the volume of the final extract containing 1,4-DMN and the internal standard, which is generated after the liquid-liquid partitioning, is not known and is evaluated based on the recovery of the internal standard.

Table 6.7 Recovery of 1,4-DMN from potato peel samples spiked at a level of 4 µg/g peel with a spiking solution containing both 1,4-DMN and the internal standard 2-methylnaphthalene and extracted according to two methods

Sample	ACN/PROP Method			TMP/Heat Method
	Absolute 1,4-DMN Recovery (%)	Absolute 2-MeNaph Recovery (%)	Relative Recovery (%)	Relative Recovery (%)
Control 1	ND	ND	ND	ND
Control 2	ND	ND	ND	ND
Control 3	ND	ND	ND	ND
Recovery 1	89.6	89.4	100.2	103.5
Recovery 2	86.3	89.2	96.7	101.8
Recovery 3	91.3	91.1	100.2	97.2
Recovery 4	84.6	88.7	95.4	100.7
Recovery 5	90.1	88.6	101.7	102.7
Mean	88.4	89.4	98.9	101.2
SD	2.8	1.0	2.7	2.5

In the ACN/PROP method, the mean absolute recovery values of 1,4-DMN and 2-MeNaph show losses of about 11.6 % and 10.6 % for the two compounds respectively. This loss is

thought to be caused mainly by the adsorption of the two compounds on the peel surface as suggested by comparing the results in this experiment and the results of the recovery experiment reported in Table 6.6. However, as the two compounds are affected in a very similar way, their relative recoveries (with a mean of 98.9) were not significantly different to 100 % (using t-test at 5 % significance level), which confirms the very similar behaviour of the internal standard used to 1,4-DMN behaviour during the analytical method.

As the relative recoveries obtained for the TMP/Heat method are similar to those for the ACN/PROP method, a similar adsorption effect on potato peel surface might be expected, and absolute recoveries might be expected to be close enough to the values obtained for the ACN/PROP method.

In real samples for residue analysis, the difference in the adsorption level between 1,4-DMN and the internal standard cannot be eliminated because 1,4-DMN has normally been in contact with the potato surface for much longer times which may reach several months. Therefore, the recovery of the method was evaluated by adding the internal standard to the extraction solution (the results in Table 6.6) which is more convenient in routine analysis.

The above discussion shows that the two extraction procedures have high recoveries and that no significant loss is occurring during the extraction procedure.

6.3.3.4 Stability of 1,4-DMN in ACN/PROP (7:3) extracts at ambient temperature

This experiment was conducted to investigate the stability of 1,4-DMN in ACN/PROP extracts stored at ambient temperature for several days, a situation which could potentially occur in practice, e.g. when samples are prepared in a different location.

Table 6.8 shows the residue levels of 1,4-DMN in the six samples analysed directly after the extraction and after four days.

Table 6.8 Residue levels of 1,4-DMN in six samples analysed directly after the extraction and after four days of extraction.

Sample	1,4-DMN ($\mu\text{g/g}$ fresh potato weight)		Difference (%)
	15 min Extraction	After 4 Days	
Maris Piper 1	1.15	1.19	2.86
Maris Piper 2	0.98	1.01	2.33
King Edward 1	1.38	1.39	0.97
King Edward 2	1.51	1.50	-0.63
Desiree 1	1.24	1.29	4.33
Desiree 2	1.25	1.27	1.83
Mean			1.95

The data in Table 6.8 shows significantly higher (t-test, 5 %) amounts of 1,4-DMN obtained when soaking peel samples in the extractant for four days. However, this slight difference of about 2 % should have no practical importance. The results suggest that there is no deterioration of the extract (or breakdown of 1,4-DMN and 2-methylnaphthalene) occurred during the four days.

Extracts were examined qualitatively to check for any sign of deterioration by increasing the HPLC run time to 10 min and carefully examining the chromatogram. A representative chromatogram is shown in Figure 6.10 for an extract analysed after four days of extraction.

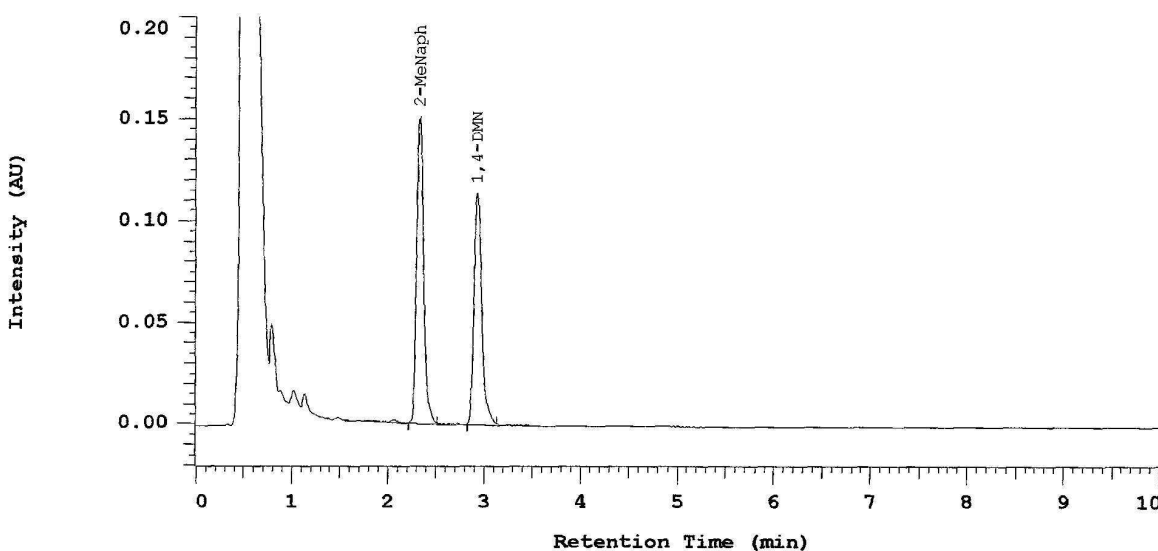


Figure 6.10 Representative chromatogram of an extract analysed after four days of extraction by the ACN/PROP final method. Chromatographic conditions: Phenomenex Spherclone 5 μm (100 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, ambient temperature and 228 nm

The chromatograms obtained for the extracts after aging were clear of any unusual peaks and any late eluted peaks.

This experiment shows that it would be safe to prepare the sample in a different location and send it to the laboratory for HPLC analysis, even if up to four days were required for the sample to get to the laboratory.

This was a quick test for extraction time effect. However, a more detailed study should provide better evaluation of the effect of extraction time on the quality of extracts and 1,4-DMN levels. More replicates for each sample should be analysed to provide enough data for statistical analysis. In addition, different time periods can be examined.

In summary, the final method for routine analysis of 1,4-DMN residues in potato peel samples using acetonitrile/2-propanol as the extractant was found to have adequate accuracy and precision. In addition, it is a rapid, easy and straightforward procedure.

6.4 Final ACN/PROP method for trace analysis of natural 1,4-DMN in potatoes

6.4.1 Solvents

The solvents required for this method need to be HPLC grade. Acetonitrile (ACN) is used in preparing the extraction solution and standards, and also in preparing the mobile phase for HPLC analysis. 2-propanol (PROP) is used in the extraction solution and for preparing standards.

6.4.2 Chemicals

1,4-dimethylnaphthalene and the internal standard 2-methylnaphthalene have to be of high purity for standard preparation.

6.4.3 Standards and solutions

Stock solutions: 10000 µg/ml stock solutions of 1,4-DMN and 2-methylnaphthalene are prepared by accurately (4 decimal places) weighing approximately 0.5 g of each chemical in 50-ml volumetric flasks and dissolving in acetonitrile. The solutions are then made up to volume.

1000 µg/ml standards: 1000 µg/ml standard of each of 1,4-DMN and 2-methylnaphthalene is prepared separately by transferring 10 ml of each stock solution into a 100-ml volumetric flask and making up to volume with acetonitrile.

Extracting solution: This solution is a mixture of acetonitrile: 2-propanol 7:3 (v/v). It is prepared by mixing 350 ml of acetonitrile and 150 ml of 2-propanol in a 500-ml bottle.

Calibration standard (s): A series of calibration standards can be used to generate the calibration graph used in the calculation. However, a single standard might be used (it should have close enough concentration to the expected level of 1,4-DMN in samples) with replication. The standards here are mixed standards of 1,4-DMN and 2-methylnaphthalene. They are prepared by dilution from the 1000 µg/ml standard of 1,4-DMN and 2-methylnaphthalene with a mixture of acetonitrile: 2-propanol: water 49:21:30 (v/v/v).

70 % HPLC mobile phase: This solution is prepared by mixing 350 ml of ACN with 150 ml of deionised water in a 500-ml glass bottle. The solution is then degassed for 30 min in an ultrasonic bath.

6.4.4 Procedure

1. Potato sample information is recorded.
2. A suitable number of potato tubers are removed and washed gently under running cold tap water to remove attached soil. They are then left to dry or dried with paper towels.
3. The weight of fresh potatoes is taken using a top-pan balance.
4. Potatoes are peeled with a stainless steel household peeler and the total peel weight is recorded.
5. The peel is chopped to fine pieces (the finest possible) using a knife and a chopping board.
6. The peel is mixed thoroughly and a 40-g subsample is taken randomly and transferred into a 100-ml conical flask.
7. 40 ml of the extracting solution are added to the conical flask using a measuring cylinder.
8. 0.050 ml (50 μ l) of the 1000 μ g/ml 2-methylnaphthalene internal standard is added to the flask and the flask is swirled gently.
9. The conical flask is capped with a stopper and placed in a preheated water bath at 50 °C for 15 min with occasional swirling and then cooled for 10 min.
10. About 1-1.5 ml of the extract is filtered through a 0.2 μ m PTFE syringe filter into an HPLC vial and analysed by HPLC.

6.4.5 Chromatographic conditions

Chromatographic conditions of this method are set as follows:

- **Column:** 250 mm x 4.6 mm C18 column with 5 μ m particle size
- **Mobile phase:** 70 % acetonitrile : 30 % water
- **Flow rate:** 1.5 ml/min
- **Injection volume:** 100 μ l
- **Run time:** 10 min
- **2-Methylnaphthalene retention time :** ~6.9 min

- **1,4-DMN retention time** : ~9.0 min
- **Temperature**: ambient (ca. 25 °C)
- **Detector wavelength**: 228 nm

6.5 Final ACN/PROP method for routine analysis of 1,4-DMN residues in potatoes

6.5.1 Solvents

The solvents required for this method need to be HPLC grade. Acetonitrile (ACN) is used in preparing the extraction solution and standards, and in preparing the mobile phase for HPLC analysis. 2-propanol (PROP) is used in the extraction solution and for preparing standards.

6.5.2 Chemicals

1,4-dimethylnaphthalene and the internal standard 2-methylnaphthalene have to be of high purity for standard preparation.

6.5.3 Standards and solutions

Stock solutions: 10000 µg/ml stock solutions of 1,4-DMN and 2-methylnaphthalene are prepared by accurately (4 decimal places) weighing approximately 0.5 g of each chemical in 50-ml volumetric flasks and dissolving in acetonitrile. The solutions are then made up to volume.

1000 µg/ml standards: 1000 µg/ml standard of each of 1,4-DMN and 2-methylnaphthalene is prepared separately by transferring 10 ml of each stock solution into a 100-ml volumetric flask and making up to volume with acetonitrile.

Extracting solution containing 10 µg/ml 2-methylnaphthalene: 2 ml of the 1000 µg/ml 2-methylnaphthalene solution is transferred into a 200-ml volumetric flask using a pipette then diluted to volume with a mixture of acetonitrile: 2-propanol 7:3 (v/v).

Calibration standard (s): A series of calibration standards can be used to generate the calibration graph used in the calculation. However, a single standard might be used (it should have close enough concentration to the expected level of 1,4-DMN in samples) with replication. The standards here are mixed standards of 1,4-DMN and 2-methylnaphthalene. They are prepared by dilution of the 1000 µg/ml standard of 1,4-DMN and 2-methylnaphthalene with acetonitrile: 2-propanol 7:3 (v/v).

70 % HPLC mobile phase: This solution is prepared by mixing 350 ml of ACN with 150 ml of deionised water in a 500-ml glass bottle. The solution is then degassed for 30 min in an ultrasonic bath.

6.5.4 Procedure

1. Potato sample information is recorded.
2. 3 to 5 potato tubers are removed and washed gently under running cold tap water to remove attached soil. They are then left to dry or dried with paper towels.
3. The weight of fresh potatoes is taken using a top-pan balance.
4. Potatoes are peeled with a stainless steel household peeler and the total peel weight is recorded.
5. The peel is chopped to fine pieces (about 0.5 x 0.5 cm) using a knife and a chopping board.
6. The peel is mixed thoroughly and a 10-g subsample is taken randomly and transferred into a 50-ml conical flask.
7. 15 ml of the extracting solution (containing the internal standard) are added to the conical flask using a pipette.
8. The conical flask is capped with a stopper and placed in a preheated water bath at 50 °C for 15 min with occasional swirling and then cooled for 10 min.
9. 1 ml of the extract is filtered through a 0.2 µm PTFE syringe filter into an HPLC vial and analysed by HPLC.

6.5.5 Chromatographic conditions

Chromatographic conditions for this method were set as follows:

- **Column:** 100 mm x 4.6 mm C18 column with 5 µm particle size
- **Mobile phase:** 70 % acetonitrile : 30 % water
- **Flow rate:** 1.5 ml/min
- **Injection volume:** 10 µl
- **Run time:** 4 min

- **1,4-DMN retention time** : ~2.9 min
- **2-Methylnaphthalene retention time** : ~2.3 min
- **Temperature**: ambient
- **Detector wavelength**: 228 nm

Chapter 7: Analysis of natural 1,4-DMN in potatoes and other plant materials

7.1 Introduction

Potato tubers have been found to produce volatile compounds that play different physiological roles. Some of these compounds are constituents of the potato aroma or flavour while others have been found to contribute to sprout suppression effects (Nursten and Sheen, 1974; Meigh et al., 1973; Coleman et al., 1981; Buttery et al., 1970; Oruna-Concha et al., 2001). Several dimethylnaphthalene isomers have been identified as natural potato volatiles produced by potatoes (Meigh et al., 1973; Coleman et al., 1981). Among them, 1,4-dimethylnaphthalene and 1,6-dimethylnaphthalene showed sprout suppression activity comparable to the commercial sprout suppression chemical chlorpropham (Meigh et al., 1973; Filmer and Rhodes, 1985; Beveridge et al., 1981b).

Different analytical techniques were used for the analysis of potato volatiles. They can be categorised into two main branches: direct solvent extraction and analysis of the volatiles collected from the headspace of potato tubers.

Direct solvent extraction was used successfully to extract volatiles from potatoes (Meigh et al., 1973; Petersen et al., 1998). Meigh (1973) and co-workers extracted potato volatiles (including DMNs) from 150 g freeze-dried potato peel using 250 ml of diethylether. The extract was then cleaned up and concentrated to about 0.5 ml under a gentle stream of nitrogen. The volatiles were separated and determined by GC-MS. Several dimethylnaphthalene isomers were identified by this method. 1,4- and 1,6- were positively confirmed to be present in addition to 2,6- or 2,7- dimethylnaphthalene. Peterson and co-workers (1998) extracted the volatiles from aqueous suspensions of homogenised potato material. The suspensions were homogenised and 200 g were extracted with 100 ml of diethylether / pentane (1:1). The extract was then concentrated under a gentle stream of nitrogen and analysed by GC-MS. A total of 29 compounds were identified but no DMNs or naphthalenes reported.

In a more recent study (2006) conducted in DiChlor laboratories (part of 1,4Group, USA), natural 1,4-DMN was solvent extracted from organically grown potatoes obtained from a commercial store. 1,4-DMN was reported to be present at levels of about 41 to 86 µg/kg (potato fresh weight basis) (John Forsythe, personal communication). However, some

uncertainty is present in these values as some 1,4-DMN background contamination was found in the reagent blanks, as will be discussed further in Section 7.3.2.

Another recent study (2006) for the natural 1,4-DMN levels in potatoes conducted in a commercial laboratory, as a part of the registration of 1,4-DMN with the USA authorities, reported 1,4-DMN levels of 20 µg of natural 1,4-DMN per kg of potato peel (John Forsythe, personal communication). This would be equivalent to a value of about 2-3 µg/kg on the basis of fresh potato weight. More details about this study are found in Section 7.3.2.

Headspace analysis is another important technique for the identification of potato volatiles. Potato volatiles can be collected by different trapping methods. Cold traps, steam distillation and the use of solid adsorbents are frequently reported collection methods for potato volatile analysis. After trapping, volatiles are desorbed by either thermal or solvent desorption prior to the chromatographic determination.

Cold trapping is one of the trapping methods used for the collection of potato volatiles. Meigh and co-workers (1973) tried a headspace method for the collection of volatiles evolved by respiring potatoes (5 kg) over 2-3 weeks. The oxygen required for respiration was supplied continuously while CO₂ was adsorbed by sodium hydroxide. Volatiles were accumulated in a cold trap (stainless steel trap immersed in liquid oxygen), cleaned up and then delivered to the GC for analysis. However, the yield of the volatiles using this method was reported to be inadequate for determination.

Beveridge (1979) conducted some headspace experiments to study potato volatiles that have sprout suppressing effects, such as 1,4-DMN. Two systems were designed for the collection of potato volatiles, both of which were based on using cold traps. In preliminary experiments, Beveridge aimed to collect the natural volatiles produced by healthy conventionally-stored potatoes. Potato tubers (25 kg) were placed in a sealed glass container (54 dm³) at 10-15 °C. Purified air was continuously flushed into the system with a flow rate of between 6 and 20 ml/min. The volatiles were collected in three cold traps (glass tubes immersed in liquid nitrogen, solid carbon dioxide-acetone or ice) connected in series. However, due to the insufficient air flow, moisture accumulated in the sample container causing the conditions to deviate from the normal storage conditions. In addition, some obstacles were also met in the GC analysis of the collected volatiles. Therefore, it was not possible to identify natural potato volatiles.

Coleman and co-workers (1981) collected the volatiles of baked potatoes using a headspace method with a much larger potato sample. The volatiles of a total potato sample of about 245 kg were accumulated in a cold trap and then extracted with diethylether. It was then cleaned up and concentrated prior to the analysis by GC-MS. The identification of some of the compounds was also aided by infrared spectroscopy. Using this method, a total number of 228 volatile compounds were identified, including three dimethylnaphthalene compounds and two trimethylnaphthalene compounds.

Filmer and Rhodes (1985) investigated the volatiles produced by raw potato tubers (one tonne) which were stored in a sealed galvanised tank with filtered air continuously drawn at 400 l/h. The volatiles were collected in a cold trap and extracted with ether. The ether volume was reduced and the residues were then diluted with 2 ml of ethanol. This final extract was fractionated and analysed by GC. Different fractions were investigated for their sprout inhibition activity using a potato shoot-tip bioassay. The identification of the compounds in the fractions of interest was achieved by mass spectroscopy. Several compounds were identified including diphenylamine which was shown to be an effective sprout suppressant. 1,4-DMN was also reported to be present among the collected volatiles in this study.

Steam distillation is another technique used for collecting potato volatiles. Buttery and co-workers (1970) used steam distillation for obtaining volatile potato oil from 4.5 kg of potato tubers under vacuum and at atmospheric pressure. The volatile compounds in the resulting oil were separated by GC and identified by mass spectroscopy and infrared spectroscopy. More than thirty compounds were identified in the potato oil collected through this method, including some naphthalenes, but not dimethylnaphthalenes. In another study, dimethylnaphthalene (no specific isomer) was reported to be present in the essence of unpeeled potatoes (60 kg of cooked chips) extracted by steam distillation followed by the separation and identification by GC-MS (Nursten and Sheen, 1974).

A more recent technique for the collection and concentration of potato volatiles is the use of polymeric adsorbents such as Tenax. Beveridge et al. (1983) used Tenax adsorbent successfully for a headspace analysis of potato tubers treated with 1,4-DMN and other sprout suppressing chemicals. In this experiment, potato tubers (5 kg) were stored in a cardboard box (10 kg capacity) at 9 °C. Tenax GC traps (glass tubes filled with 50 mg of the adsorbent) were inserted to a depth of about 40 mm into the top of the box and a headspace volume of 45 ml was drawn at a rate of 4.5 ml/min. The volatiles were then delivered to the GC by thermal desorption for analysis. The desorption procedure was

reported to provide accurate recoveries of 1,4-DMN with values greater than 99 % using Tenax precolumns spiked with known amounts of 1,4-DMN.

Tenax adsorbent has also been used by Boyd (1984) for adsorbing the natural volatiles produced by potatoes. Two systems for the collection of potato volatiles were designed by Boyd using Tenax for collecting the potato volatiles. In the first system, potato tubers were placed in a 250 dm³ aluminium tank with air (pressurised air cylinder, BOC Glasgow Ltd.) flow from the bottom of the tank at a rate of 50 ml/min and volatiles were trapped in a Tenax precolumn. However, background volatiles were found to be present in the air supply and also produced by the tank. The background volatiles from the air supply were removed by filtering the air before introducing it to the tank. However, the volatiles from the other parts of the system were a main obstacle in the continuation of using this system. To avoid the contamination of the aluminium tank, the second system developed by Boyd for the headspace analysis of potato volatiles was constructed entirely from glass and PTFE. In addition, instead of using an air cylinder for the air supply, oxygen and nitrogen were mixed from two separate cylinders. The mixture was then purified by passing through three cold traps prior to the introduction to the sample vessel at a flow of 200 ml/min. The vessel was connected at the other end to a Tenax precolumn for volatile collections. The contamination background was minimised and therefore this system was used to study potato natural volatiles. Eight kilograms of potatoes (sprouted) were loaded into the vessel and the system assembled and run as described above to collect 10 dm³. The collected volatiles were thermally desorbed into a GC column connected to a mass spectroscopic detector for the identification. Several potato volatiles were accumulated and separated. However, due to GC column bleeding, only tentative identification was obtained. A peak in the retention area of dimethynaphthalenes was tentatively identified as 1,3- or 2,3-dimethylnaphthalene.

Duckham et al. (2001) used Tenax TA to adsorb the volatiles given off by baked potato flesh. The volatiles were carried to the Tenax tube in purified nitrogen running at a flow rate of 120 ml/min over the potato sample (200 g) which was placed in a 1-l flask held in a water bath at 37 °C. The trapped volatiles were thermally desorbed into a GC-MS for the separation and identification. More than 75 compounds were collected and identified by this method including some aromatic hydrocarbons such as naphthalene.

In summary, many different volatiles were found to be produced by potatoes. 1,4-DMN was one of the volatiles which was identified by different techniques. However, most of the above studies were qualitative or semi-quantitative. The objective of this part of the

work was to investigate qualitatively and quantitatively the natural 1,4-DMN produced by potatoes. Three different analytical techniques were used for the analysis of natural 1,4-DMN in potatoes. In preliminary work, Soxhlet extraction was examined for the extraction of natural 1,4-DMN from potatoes. The ACN/PROP method for natural 1,4-DMN (see Section 6.4) was also used for the analysis of potatoes and some other plant materials for natural 1,4-DMN. The third technique examined was a headspace method for the analysis of the volatiles produced by potato tubers.

7.2 Materials and methods

7.2.1 Solvents

All the solvents used were of HPLC grade. The acetonitrile used in preparing the extraction solution and standards for the ACN/PROP method and the headspace analysis method, and also for preparing the mobile phase for HPLC analysis, was bought from Fisher Scientific, UK. The 2-propanol used in the extraction solution and standards for the ACN/PROP method was bought from Rathburn Chemicals Ltd., UK. The hexane used in the Soxhlet extraction and for preparing some standards was bought from BDH, UK.

7.2.2 Chemicals

1,4-dimethylnaphthalene and 2-methylnaphthalene used for preparing standards were described previously in Section 3.2.1.

7.2.3 Equipment

Some of the early work for the Soxhlet extraction was conducted on the HPLC instrument described in Section 2.2.2.2. The rest of the work was conducted on the HPLC-DAD system described in Section 3.2.2.

7.2.4 Potatoes and other plant materials

Potatoes (Princess and Nicola varieties) and other vegetables and fruits were organically grown and were bought from a local supermarket.

7.2.5 Using Soxhlet extraction method for the analysis of natural 1,4-DMN

The Soxhlet extraction method was described in Chapter 5 (see Section 5.2.5 for method details). Briefly, potato tubers were peeled and about 25 g of the peel transferred into the

extraction thimble with 10 g of anhydrous sodium sulphate and the thimble was plugged with cotton wool. Extraction was achieved by refluxing with about 150 ml of hexane for two hours. After cooling, the extract volume was reduced and made up to 5 ml. An aliquot of 1 ml was passed through a 0.2 μ m syringe membrane filter into an HPLC vial for HPLC analysis according to the separation method described in Chapter 2 (see Section 2.3.2.1).

Blank samples were prepared exactly as the potato samples but with the absence of peel in the thimble.

7.2.6 Using ACN/PROP method for the analysis of natural 1,4-DMN

The final ACN/PROP method for trace analysis of natural 1,4-DMN which was reported in Chapter 6 (see Section 6.4) was used here in an attempt to determine the levels of the natural 1,4-DMN in potatoes and other plant materials. The plant materials extracted according to this method were: potato peel and flesh (Princess and Nicola varieties), Gala apples, Navelate oranges, celery, spring onion, carrots, rhubarb and poppy seeds. Duplicate samples were extracted from each plant material.

In most of the analyses, the procedure reported in Section 6.4 was followed exactly. However, in some cases the HPLC separation method was modified by using a weaker mobile phase (50 % acetonitrile/water) to allow for more separation of overlapped peaks around 1,4-DMN retention time and therefore better identification of peaks. In addition, water was added to the poppy seeds to provide a final extract suitable for injecting large volume into the HPLC.

To confirm the presence (or absence) of the 1,4-DMN peak in the extracts of plant materials, the retention time and UV spectra of all the peaks around 1,4-DMN retention time were examined and compared to an authentic 1,4-DMN standard. In addition, the extracts were spiked with 1,4-DMN for further confirmation.

7.2.7 Headspace experiments

7.2.7.1 Collection system

The collection system is shown in the photo in Figure 7.1 with the main parts labelled.

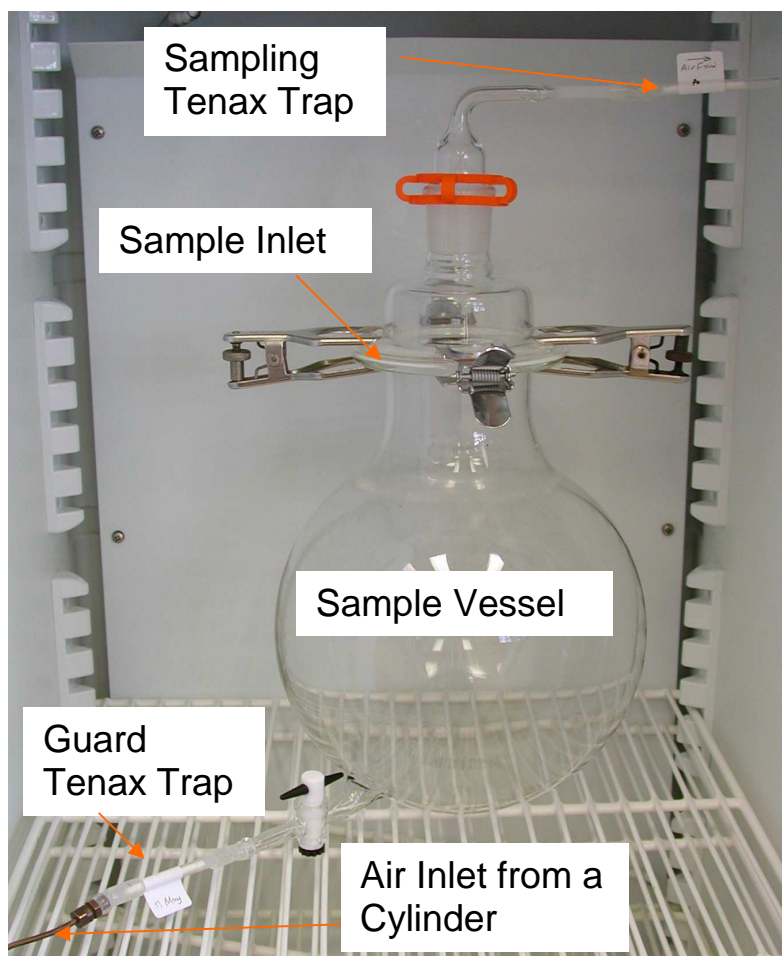


Figure 7.1 The headspace collection system of potato volatiles

It consisted of a modified 5-litre borosilicate glass flask with an inlet tube at the bottom and an outlet tube at the top. The inlet was connected to a ‘guard Tenax trap’ which was connected to a copper tube supplying air from a pressurised air cylinder. The vessel outlet was connected to the ‘sampling Tenax trap’. Tenax traps were connected to the other parts of the system by silicone rubber tubes. Potato samples were introduced to the vessel through the sample inlet. The two parts of the sample inlet were fixed with four spring clamps and a Teflon ring was placed in between to help in sealing the two parts.

Air was supplied from a pressurised air cylinder (BOC Glasgow Ltd.) fitted with a pressure regulator. Air was controlled to flow at a rate of 20 ml/min (measured by SKC Precision Dual Ball Rotameter 320, USA) using a flow controller (Porter Instrument Company, USA) and purified through the guard Tenax trap before entering the sample vessel.

Volatiles swept from the headspace of the vessel were accumulated in the sampling Tenax trap. For temperature control, the collection system was placed in an incubator set at 20 ± 0.5 °C.

7.2.7.2 Tenax traps preparation

Tenax traps were prepared using borosilicate glass tubes with a length of 80 mm and an internal diameter of 5 mm. The tubes were cleaned by soaking in Decon 90 detergent solution overnight followed by rinsing with tap water and deionised water. They were then dried in an oven at 110 °C. After cooling, tubes were rinsed with acetone followed by toluene. They were then immersed in a 5 % solution of hexamethyldisilazane (HMDS) in toluene for 15 min for the silation process to deactivate the glass surface. The tubes were then rinsed with toluene followed by acetone and dried in an oven.

Once cooled, columns were packed under slight vacuum with 100 mg of Tenax TA (80/100 mesh, Supelco) made as a slurry in acetonitrile and plugged at both ends with silanised glass wool.

Tenax traps were purified by washing with acetonitrile in a Soxhlet apparatus for 4 h and then dried in an oven at 110 °C overnight. After cooling, they were sealed with Teflon tape, wrapped in aluminium foil and stored in the fridge at 4 °C.

7.2.7.3 Recovery of 1,4-DMN from tenax traps

To examine the recovery of 1,4-DMN from the Tenax traps, four Tenax traps were spiked with 100 µl of 10 µg/ml 1,4-DMN standard in ACN using a P200 Gilson micropipette. The traps were left in a upright position for about 10 min to allow for the percolation of 1,4-DMN through the adsorbent column and also to allow for solvent evaporation. They were then sealed with Teflon tape and kept for 24 h after which they were eluted. Solvent elution was selected to recover the adsorbed volatiles in the Tenax trap. Each trap was eluted with two successive 3.5-ml volumes (the second portion was to examine for complete elution) of acetonitrile (ACN) into a 5-ml volumetric flask containing 1.5 ml of deionised water to get a solution of 70 % ACN/water which matches the mobile phase used in the chromatographic separation. The elution was in the reverse direction (back elution) of the inlet of 1,4-DMN spike. The resulting solutions were analysed by HPLC with the separation conditions as in the final ACN/PROP for natural 1,4-DMN (see Section 6.4.5).

7.2.7.4 Background test

Before using the collection system for collecting potato volatiles (and also for the recovery experiments) it was necessary to evaluate the background contamination level. In addition, these experiments aimed to investigate any leakage problems which may affect the amount of the collected volatiles.

Positive pressure was used in all the headspace experiments to avoid the introduction of any contaminants present in the laboratory air, particularly 1,4-DMN because the laboratory is used for 1,4-DMN analysis. Therefore, it was important to check for any leakage in the collection system which could contribute to losing some of the volatiles emitted by potatoes.

To check for leakage, the system was assembled as in Figure 7.1 with the two Tenax traps in position and air flow was adjusted to the desired flow rate (20 ml/min). Soap solution was used to check for any leak at the different connections in the system and particularly around the sample inlet.

For background evaluation, the sample vessel was carefully cleaned by soaking in a detergent solution overnight and rinsing with tap water followed by deionised water and finally rinsed several times with acetonitrile. After drying, the collection system was assembled as shown in Figure 7.1 but without the sampling Tenax trap. Air flow was run for about 4 h to flush the laboratory air out of the sample vessel. The sampling Tenax trap was then connected and the flow was adjusted at 20 ml/min. The collection was continued for 15 days after which the Tenax traps were disconnected, eluted and analysed by HPLC.

7.2.7.5 Recovery of 1,4-DMN from the collection system

These experiments were conducted to examine the amount of 1,4-DMN adsorbed and recovered from the sample Tenax trap when 1,4-DMN source is placed in the sample vessel. It aimed to simulate the real experiment of collecting the volatiles from potato sample and recovering them.

Three recovery experiments were conducted for different periods of times and different 1,4-DMN amounts loaded into the collection system. 100 μ l of 10 or 100 μ g/ml 1,4-DMN standards (1 or 10 μ g) in acetonitrile were loaded into the system in a 10-ml Pyrex conical flask. In the first experiment, 1 μ g was transferred into the conical flask which was placed in the sample vessel, and two sampling Tenax traps were connected in series for 24 h at a flow of 20 ml/min. In the second experiment, 1 μ g was transferred into the conical flask

which was then placed in the sample vessel, and the first sampling Tenax trap was connected for the first 24 h at a flow of 20 ml/min, then replaced with the second sampling Tenax trap which was disconnected after 6 days. The third experiment was conducted at higher level of 1,4-DMN. 10 µg were transferred into the conical flask which was then placed in the sample vessel, and two sampling Tenax traps were connected in series for 6 days at a flow of 20 ml/min. The traps were eluted and analysed as described above in Section 7.2.7.3. In the first and third experiments, the first sampling Tenax trap was eluted twice with 3.5 ml of acetonitrile to check for complete desorption.

7.2.7.6 Collection of potato volatiles

Two experiments were conducted for the collection of potato volatiles using the headspace collection system described above in a search for natural 1,4-DMN.

In the first experiment, potato volatiles were collected over 4 weeks at a flow rate of 20 ml/min. The system was placed on an open bench in the light with no temperature control. A digital thermotag was used for monitoring temperature variations over the sampling period. 3.5 kg of Valor organic potatoes (bought from a local supermarket) were placed into the sample vessel and the system was closed. The guard Tenax trap was connected and air was supplied for 4 h to flush out laboratory air before connecting the sampling Tenax trap. After 28 days, the Tenax traps were disconnected, eluted and analysed with HPLC as described in the above sections.

In the second experiment, the system was placed in an incubator in a different laboratory than the one used for conducting the first experiment. The sampling was conducted for 42 days in dark and the incubator temperature was set at 20 °C. 3.5 kg of Nicola organic potatoes were used here.

7.3 Results and discussion

7.3.1 Using Soxhlet extraction method for the analysis of natural 1,4-DMN

In these preliminary experiments, Soxhlet extraction was examined for the analysis of natural 1,4-DMN at trace levels. Organically grown potatoes which should have not received any pesticide applications including post-harvest chemicals, were analysed here. In the first attempt to use the Soxhlet extraction for trace levels, several tubers from two different varieties were analysed along with some reagent blanks to check for any

contamination at this low level. A representative chromatogram of a blank sample and a potato peel extract is shown in Figure 7.2.

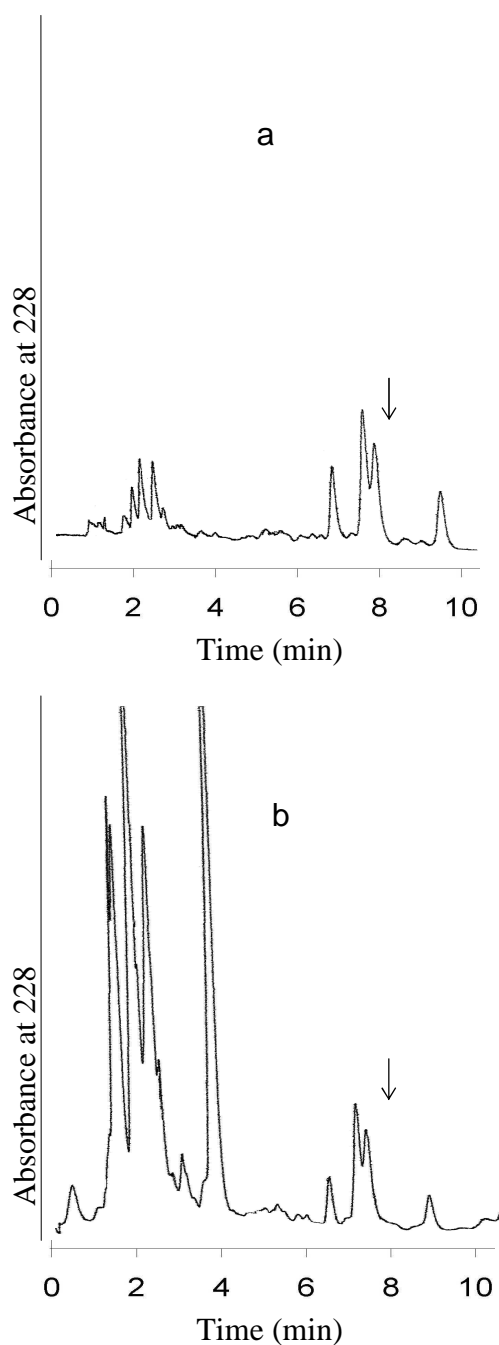


Figure 7.2 Representative chromatograms of a) Reagent blank sample for Soxhlet extraction with hexane at trace levels. b) Potato peel extract. The arrows indicate 1,4-DMN retention time

As shown in Figure 7.2, there were several peaks present in the blank sample. Two of them were too close to the retention time of 1,4-DMN at about 8 min. At the start, it was thought that the peak at about 7.8 min might be 1,4-DMN contamination, particularly with the small variation of 1,4-DMN retention times due to temperature changes. Therefore, the possible sources of contamination were investigated.

There were several possible sources for this contamination. Glassware, solvent, sodium sulphate, cellulose thimbles and the cotton wool plugs were the ones investigated. All the glassware was carefully cleaned, including the Soxhlet apparatus and condenser, and the rotary evaporator was rinsed with hexane several times. New batches of hexane, sodium sulphate, thimbles and cotton wool were used. The HPLC system was also cleaned with replicate injections of hexane.

Syringes and syringe filters were examined by passing hexane and acetonitrile through them and injecting the filtrate into the HPLC. Two types of syringe and three types of filter were examined. The main specifications of the used syringes and filters are shown in Table 7.1.

Table 7.1 Specifications of the filters and syringes examined for contamination.

Filter/Syrenge Name	Materials	Membrane	Porosity (µm)
PALL Acrodisc [®]	Modified acrylic	Supor [®] PES (polyethersulfone)	0.45
PALL Acrodisc [®] LC13	Polypropylene	PVDF (Polyvinylidene fluoride)	0.2
Phenomenex Phenex [®]	Polypropylene	Nylon	0.45
BD Plastipak [®] 1 ml syringe	Polyethylene (rubber tipped)	-	-
Norm-Ject [®] 1 ml syringe	Polypropylene and polyethylene (no rubber tip)	-	-

Both syringe types were found to be compatible with both hexane and acetonitrile. However, the Plastipak syringe had a rubber plunger which jammed when it came in contact with the two organic solvents. Therefore, it was decided to use the Norm-Ject syringe, as it has no rubber plunger.

The three filters were also found to be compatible with hexane. However, the Supor PES membrane was not compatible with acetonitrile as many compounds were present in the acetonitrile filtrate of this filter.

The above results show that neither the filters nor the syringes contributed to the contamination noticed in the control and potato samples showed in Figure 7.2. Therefore, it will be safe to use them in the following experiments.

The contribution of the glassware and the hexane to the contamination was evaluated by refluxing hexane in the Soxhlet apparatus without the presence of the thimble, and following the procedure described in Section 7.2.5. This test showed that all the glassware and the hexane used were free of contamination.

The cotton wool was examined by extracting it in the Soxhlet apparatus with hexane according to the procedure in Section 7.2.5 and the extract was found to be clear of any compound.

The thimbles were extracted in a similar way to the cotton wool and the extract was analysed by the HPLC-DAD instrument for precise control of column temperature (to obtain precise retention times) and also for some identification of any contamination peaks. The resulting chromatogram is shown in Figure 7.3.

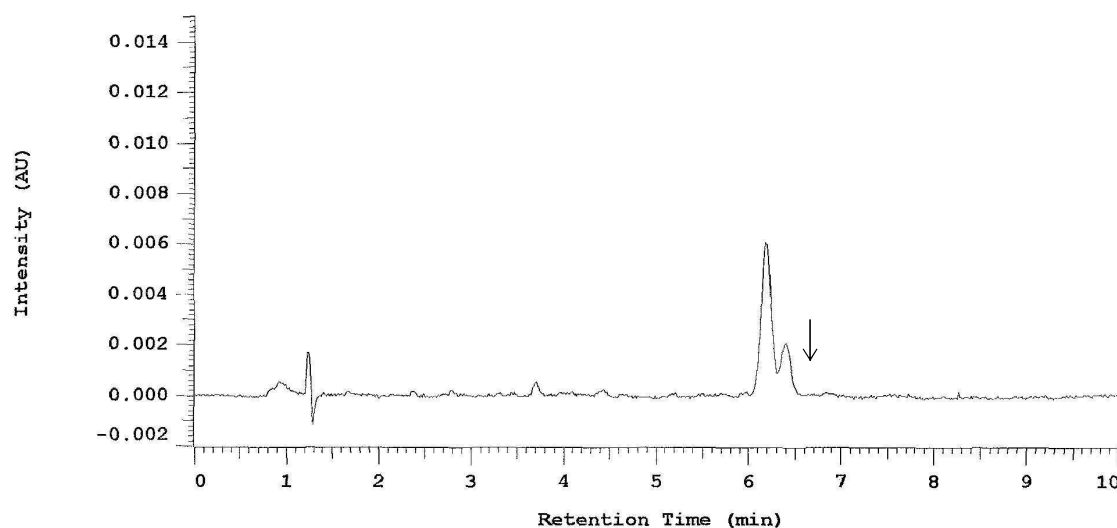


Figure 7.3 Hexane extract of a cellulose extraction thimble obtained by Soxhlet extraction for two hours. The arrow indicates 1,4-DMN retention time

As shown in Figure 7.3, the two contamination compounds that eluted around 1,4-DMN retention time (shown in Figure 7.2 in potato extracts) were extracted from the cellulose thimble. The UV spectra for both peaks and also the retention times (with the good control of chromatographic conditions in this instrument) indicated that neither of the two peaks was for 1,4-DMN. However, they were too close to 1,4-DMN retention time and had a large size, so they may interfere with the 1,4-DMN peaks at trace levels. Therefore, trials were conducted to clean the thimbles from these two contaminants.

By extracting several thimbles, the level of these two compounds was shown to vary from one thimble to another with a value of more than 50 %. However, by re-extracting the thimbles for another 2 h, the two contamination peaks were missing from the second extract as shown in Figure 7.4.a. This indicates that it is important to wash the thimbles (by Soxhlet extraction) prior to using them for the analysis of natural 1,4-DMN in potatoes.

Washing the thimbles for a shorter period of time (30 min) was found to remove most of the contaminants, as shown in the chromatogram in Figure 7.4.b for a blank sample of a thimble pre-washed for 30 min and then extracted for 2 h.

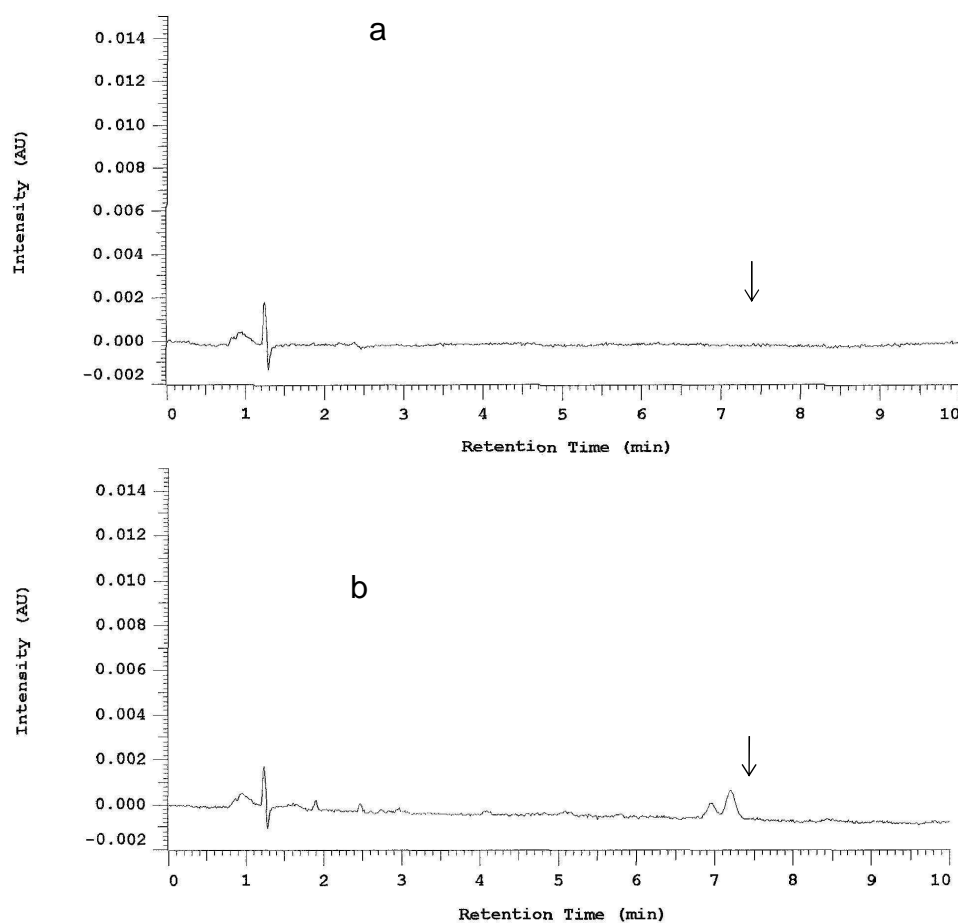


Figure 7.4 Hexane extracts (2 h Soxhlet extraction) of a blank sample using a thimble pre-washed by Soxhlet extraction for a) 2 hours b) 30 min. The arrows indicate 1,4-DMN retention time

The above investigation of the nature and source of the contamination peaks around 1,4-DMN retention time showed that none of the contamination peaks was for 1,4-DMN. In addition, their levels in the thimble were found to be minimised with Soxhlet washing with hexane for 30 min. Therefore, it was decided to repeat the extraction of potato peel samples with pre-washed thimbles. A representative chromatogram is shown in Figure 7.5.

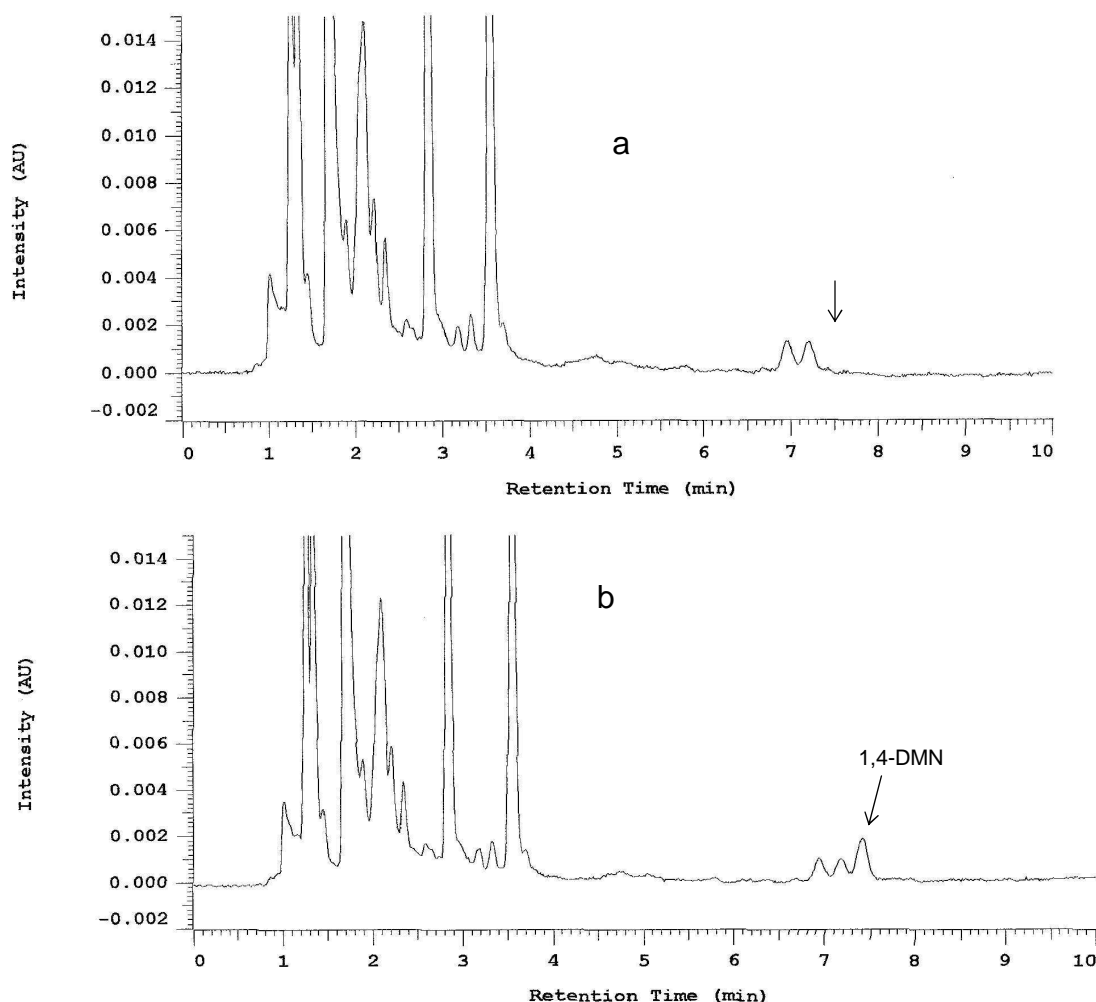


Figure 7.5 a) Hexane extract of a potato peel sample using a thimble pre-washed by Soxhlet extraction for 30 min and then extracted for 2 h. b) The same extract in (a) after fortifying with 1,4-DMN to a level of about 0.1 $\mu\text{g/ml}$.

Despite the success in identifying the nature and source of the contaminants found in hexane extracts generated by Soxhlet extraction and the success in minimising their interference with 1,4-DMN, the chromatogram in Figure 7.5.a shows no peak detected at the retention time of 1,4-DMN. This may indicate that the level of the natural 1,4-DMN is lower than the levels that can be detected by the method described in Section 7.2.5 or there might be no 1,4-DMN present in the samples in this instance. The LOD and LOQ of this method can be estimated based on the LOD of the HPLC separation method. The LOD of the HPLC method used for the determination of 1,4-DMN in the final extract of the Soxhlet method was estimated in Chapter 2 (see Section 2.3.2.5 and Section 2.3.3.4) using a calibration line generated with 1,4-DMN standard solutions at low levels. The LOD value was found to be 0.02 $\mu\text{g/ml}$. Based on the fact that 25 g of potato peel samples was extracted in the Soxhlet method in a final extract volume of 5 ml, the LOD value will correspond theoretically to approximately 0.004 $\mu\text{g/g}$ of 1,4-DMN in peel. Therefore, the limit of quantification of the Soxhlet method can be estimated to be about 0.013 $\mu\text{g/g}$ of 1,4-DMN in peel or about 0.002 $\mu\text{g/g}$ (i.e. 2 $\mu\text{g/kg}$) of potato fresh weight. Although this

level of quantification limit would be thought to be low enough for detecting natural 1,4-DMN, some factors may affect the values of LOD and LOQ that can be achieved practically. The values reported above were estimated based on using standard solutions of 1,4-DMN which should be clean enough to minimise any background effect. However, with the higher extractability of the Soxhlet method particularly with hexane as the extractant, many background compounds will be present in the final concentrated extract which would affect the level of 1,4-DMN that can be detected practically. In addition, some loss of 1,4-DMN was found to occur during the rotary evaporation of the extract, as reported in Section 5.3, which may contribute to raising the level of 1,4-DMN that can be detected practically. Therefore, another method with better detection capability is required. The ACN/PROP method for the analysis of natural 1,4-DMN in potatoes which has been practically shown to provide low LOD and LOQ levels (see Section 6.3.2.3) was used next.

7.3.2 Using the ACN/PROP method for the analysis of natural 1,4-DMN

The ACN/PROP method for trace analysis of 1,4-DMN which was reported in Section 6.4 was found to be capable of quantifying 1,4-DMN with good recovery and precision at levels as low as 4.5 µg/kg (ppb) of potato fresh weight (lower levels at 1.5 µg/kg of potato fresh weight were detected, but not quantified with acceptable accuracy and precision). This sensitive method was used for the determination of natural 1,4-DMN levels in potatoes and other plant materials. Figure 7.6 to 7.14 show chromatograms for the extracts obtained by the ACN/PROP method for the different plant materials examined. Arrows in the chromatograms indicate the position of the 1,4-DMN peak as identified by comparison to an authentic standard and also by spiking the same plant extract with 1,4-DMN.

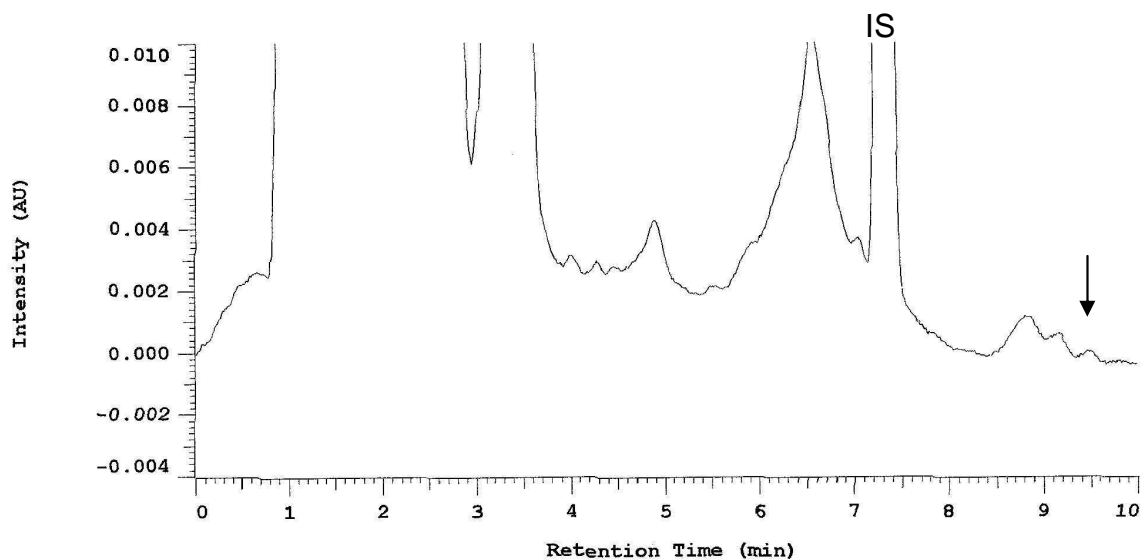


Figure 7.6 Representative chromatogram of potato peel sample extracted by ACN/PROP method. The arrow indicates 1,4-DMN retention time. Chromatographic conditions: ACE C18 5 μ m (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 25 °C and 228 nm

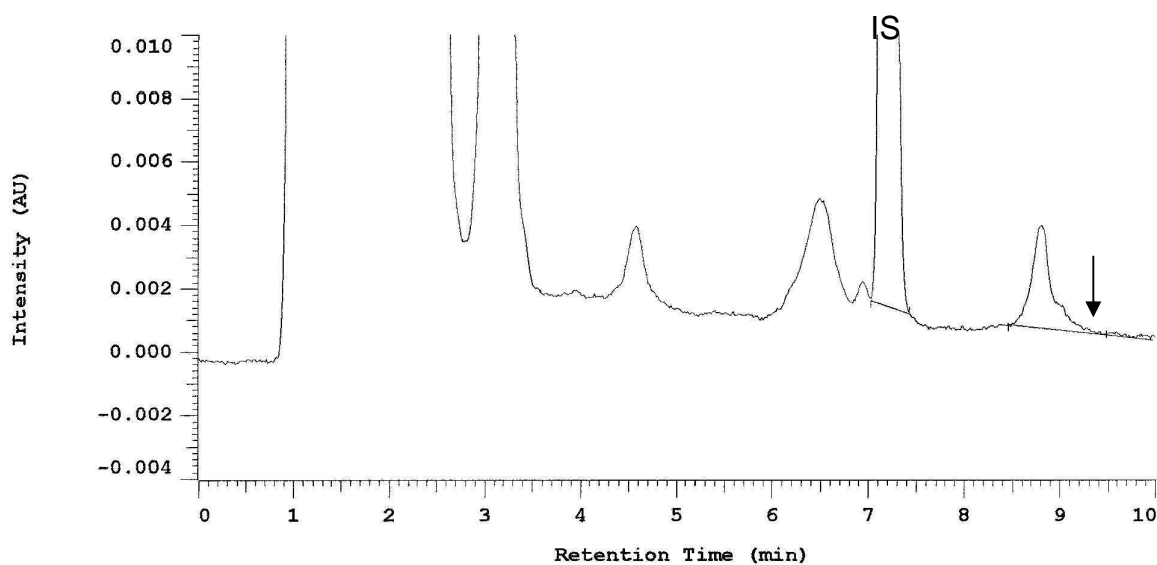


Figure 7.7 Representative chromatogram of potato flesh sample extracted by ACN/PROP method. The arrow indicates 1,4-DMN retention time. Chromatographic conditions: ACE C18 5 μ m (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 25 °C and 228 nm

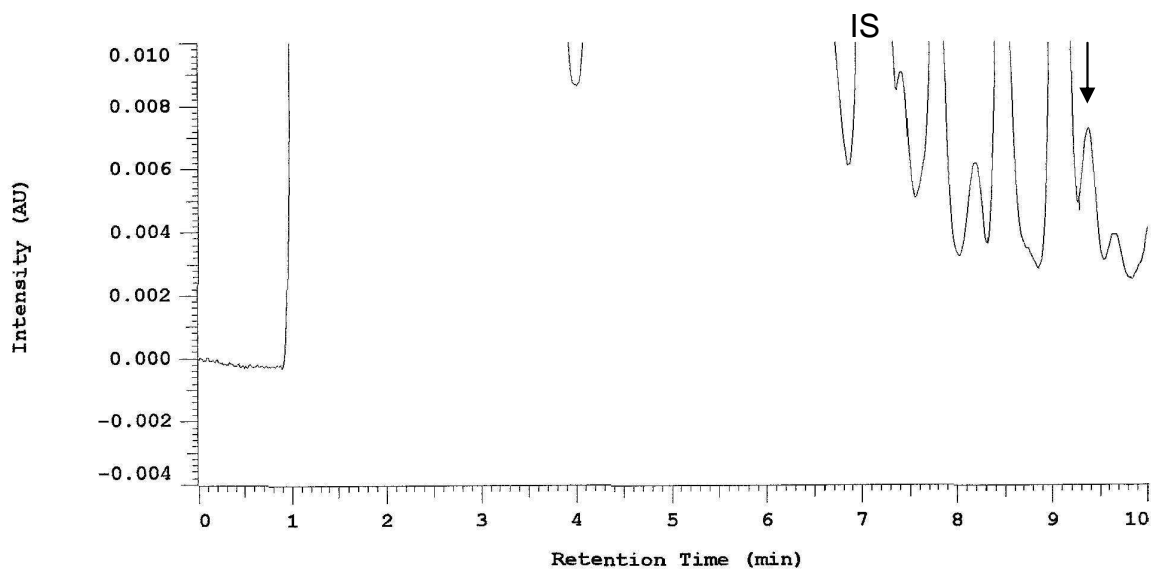


Figure 7.8 Representative chromatogram of spring onion sample extracted by ACN/PROP method. The arrow indicates 1,4-DMN retention time. Chromatographic conditions: ACE C18 5 μm (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 25 $^{\circ}\text{C}$ and 228 nm

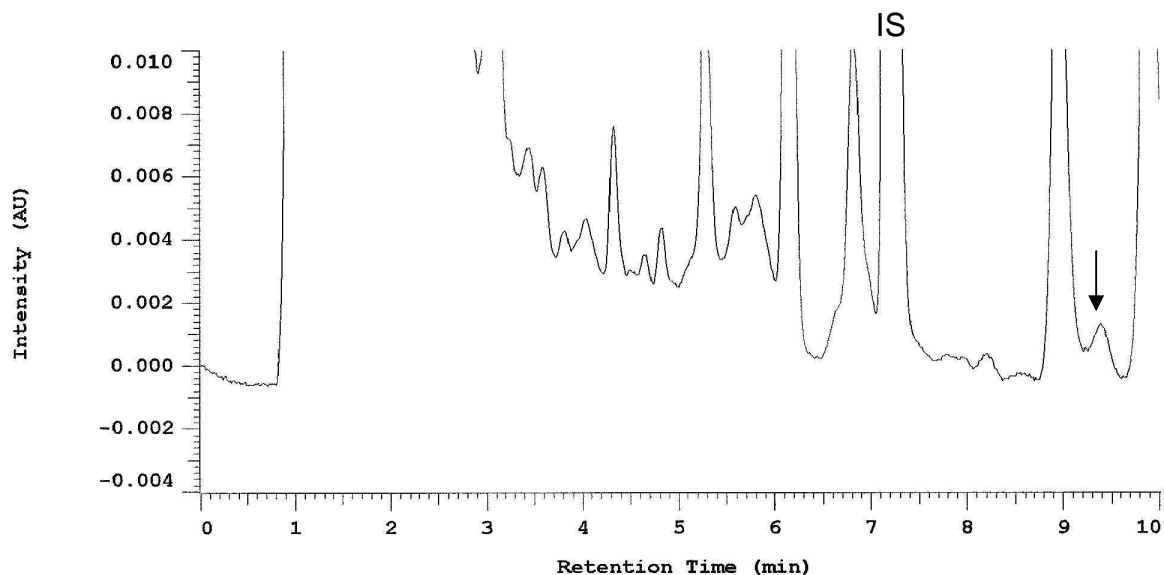


Figure 7.9 Representative chromatogram of carrot sample extracted by ACN/PROP method. The arrow indicates 1,4-DMN retention time. Chromatographic conditions: ACE C18 5 μm (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 25 $^{\circ}\text{C}$ and 228 nm

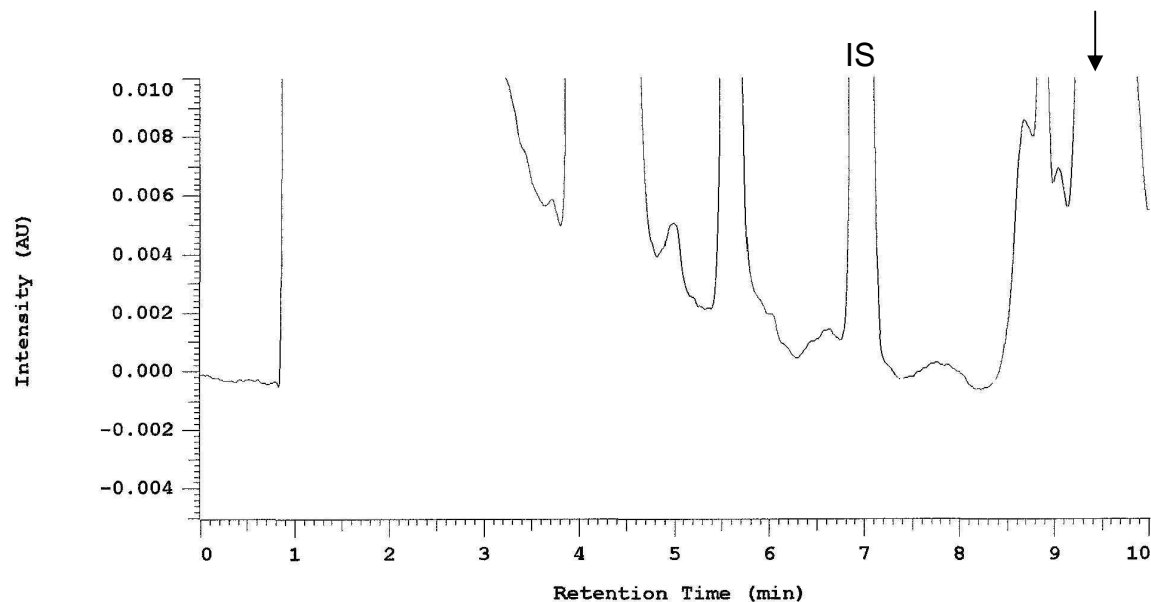


Figure 7.10 Representative chromatogram of rhubarb sample extracted by ACN/PROP method. The arrow indicates 1,4-DMN retention time. Chromatographic conditions: ACE C18 5 μ m (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 25 $^{\circ}$ C and 228 nm

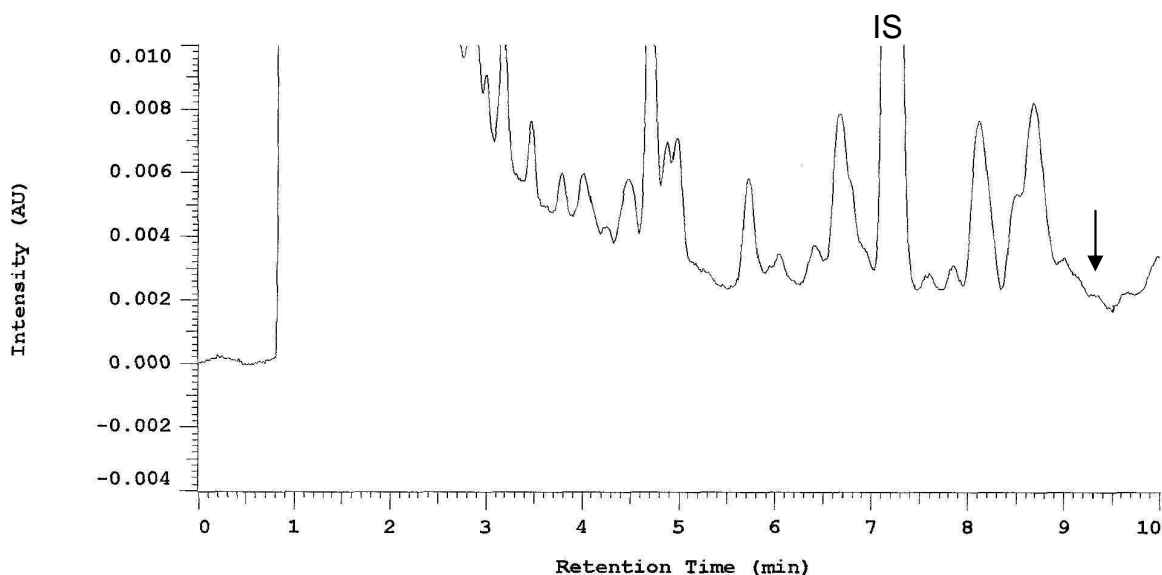


Figure 7.11 Representative chromatogram of apple sample extracted by ACN/PROP method. The arrow indicates 1,4-DMN retention time. Chromatographic conditions: ACE C18 5 μ m (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 25 $^{\circ}$ C and 228 nm

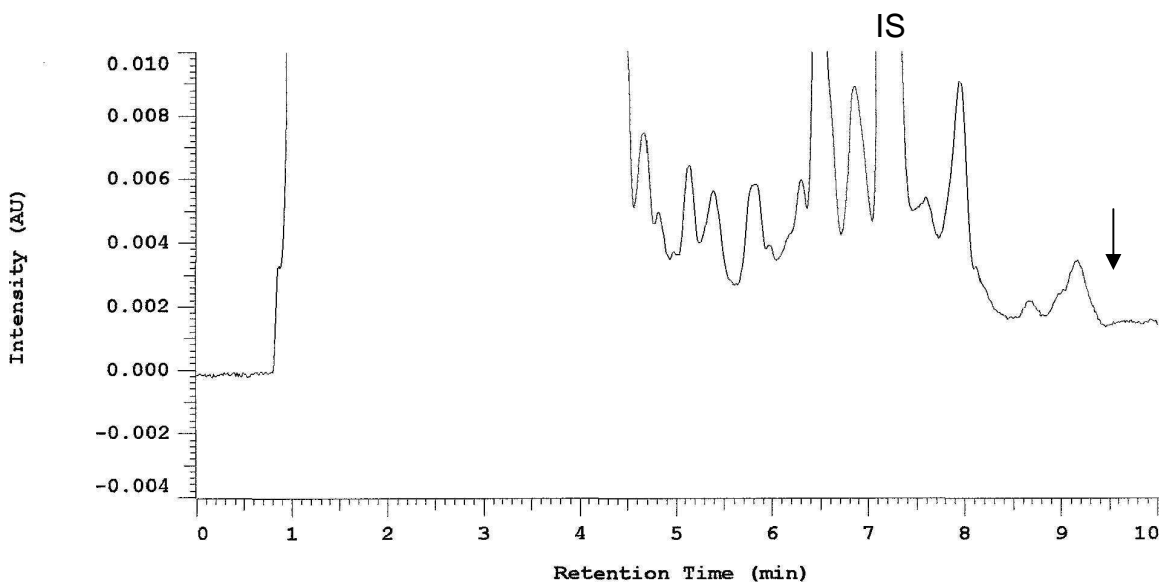


Figure 7.12 Representative chromatogram of orange sample extracted by ACN/PROP method. The arrow indicates 1,4-DMN retention time. Chromatographic conditions: ACE C18 5 μ m (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 25 °C and 228 nm

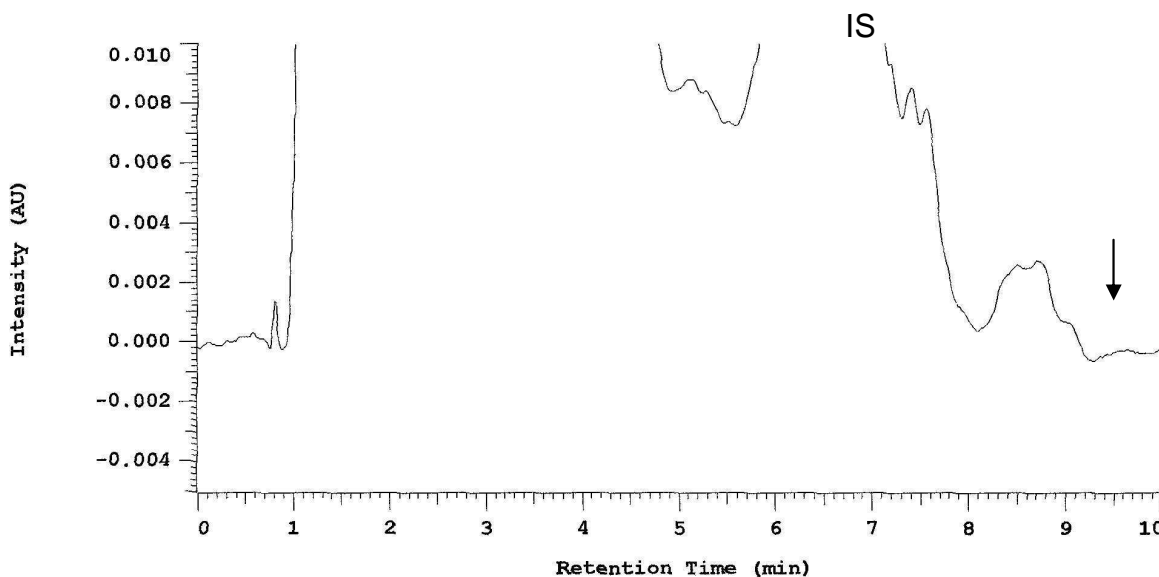


Figure 7.13 Representative chromatogram of poppy seeds sample extracted by ACN/PROP method. The arrow indicates 1,4-DMN retention time. Chromatographic conditions: ACE C18 5 μ m (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 25 °C and 228 nm

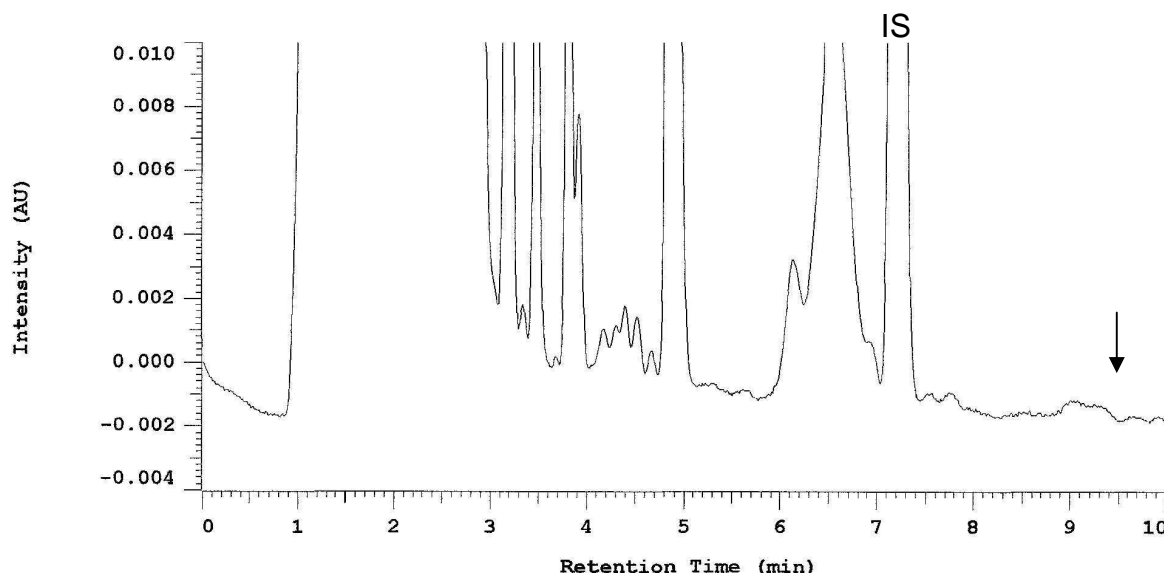


Figure 7.14 Representative chromatogram of celery sample extracted by ACN/PROP method. The arrow indicates 1,4-DMN retention time. Chromatographic conditions: ACE C18 5 μm (250 x 4.6 mm), 70 % acetonitrile: 30 % water, 1.5 ml/min, 25 $^{\circ}\text{C}$ and 228 nm

Potato volatiles are produced at a very low levels estimated to be in the order of $1 \text{ ng kg}^{-1} \text{ h}^{-1}$ (Meigh et al., 1973). Because of this low level, many workers have used different techniques for the accumulation and concentration of these volatiles to achieve the required detectable levels. The ACN/PROP method was reported to provide a sensitive approach for the detection of 1,4-DMN in potato peel. The method is capable of detecting 1,4-DMN in potatoes at levels as low as $1.5 \mu\text{g/kg}$ of potato fresh weight. The quantification limit of this method was found to be $4.5 \mu\text{g/kg}$ of potato fresh weight (see Section 6.3.2.3). This sensitivity level of the ACN/PROP method was thought to be enough to detect the natural 1,4-DMN found in potatoes and possibly other plant materials.

The chromatogram in Figure 7.6 for the potato peel sample shows a small peak detected at the right time for 1,4-DMN ($\sim 9.4 \text{ min}$) with an area equivalent to a level of about $4 \mu\text{g/kg}$ of potato fresh weight. This level is above the detection limit of the method but below the quantification limit of $4.5 \mu\text{g/kg}$ of potato fresh weight. It is possible that this peak was for 1,4-DMN. Unfortunately, because of the poor spectrum for this peak caused by the small size of the peak and the large background effect, it was not possible to confirm its identity by comparing its spectrum to the spectrum of 1,4-DMN in an authentic standard. By spiking the same peel extract to a level of about $6 \mu\text{g/kg}$ (potato fresh weight basis), the 1,4-DMN peak was completely coeluted with the small peak at about 9.4 min. However, even in this case it was not possible to confirm peak identity by spectrum comparison. In some cases, better resolving the peaks in extracts may help in the identification of closely eluting peaks. However, this is not applicable in this case as using weaker mobile phase,

for example, to separate the peaks would broaden the small peak too much and cause it to be non-detectable by the data software.

By examining the chromatogram in Figure 7.7 for potato flesh extract, it can be demonstrated that no 1,4-DMN was extracted from the potato flesh sample. This seems to indicate that 1,4-DMN is concentrated in the peel of potato tubers.

In a recent study conducted in DiChlor laboratories in 2006, 1,4-DMN was reported to be present at levels of about 41 to 86 $\mu\text{g/kg}$ (fresh weight basis) in organically grown potatoes obtained from a commercial store (John Forsythe, personal communication). In this study, potato peel was extracted by a solution of ethanol : 2,2,4-trimethylpentane (7:3 v/v) with shaking (hand + rotary shaker) followed by liquid-liquid extraction for clean up prior to the analysis by GC-MS. However, despite the fact that the experiment was conducted in a controlled environment to provide the most accuracy, traces (about 2 ppb, $\mu\text{g/l}$) of 1,4-DMN were reported to be present in the laboratory reagent blank. This level of contamination might have affected the level of the natural 1,4-DMN reported in this study. The contamination might be because the laboratory is used for routine analysis of samples containing 1,4-DMN at higher levels.

Another recent study (2006) for the natural 1,4-DMN levels in potatoes was conducted in a commercial laboratory as a part of the registration process of 1,4-DMN with the USA authorities. In this study, potatoes were grown in a greenhouse with a controlled environment for the purpose of the study. 1,4-DMN was extracted from the peel using a solvent extraction method. Natural 1,4-DMN was reported to be present at a level of 20 ppb ($\mu\text{g/kg}$) in potato peel, which is equivalent to about 2 to 3 $\mu\text{g/kg}$ of potato fresh weight (John Forsythe, personal communication). This recent study suggested a similar level of natural 1,4-DMN to the levels obtained by the ACN/PROP method of about 4 ppb ($\mu\text{g/kg}$) on the basis of potato fresh weight.

The chromatograms for the extracts of spring onion (Figure 7.8), carrot (Figure 7.9) and rhubarb (Figure 7.10) have several peaks with a good size around 1,4-DMN retention time. However, they were overlapped and could not be identified. Therefore, these three extracts were separated with weaker mobile phase (50 % acetonitrile/water). The chromatograms with this change in separation conditions are shown in Figure 7.15, 7.16 and 7.17 for spring onion, carrot and rhubarb respectively.

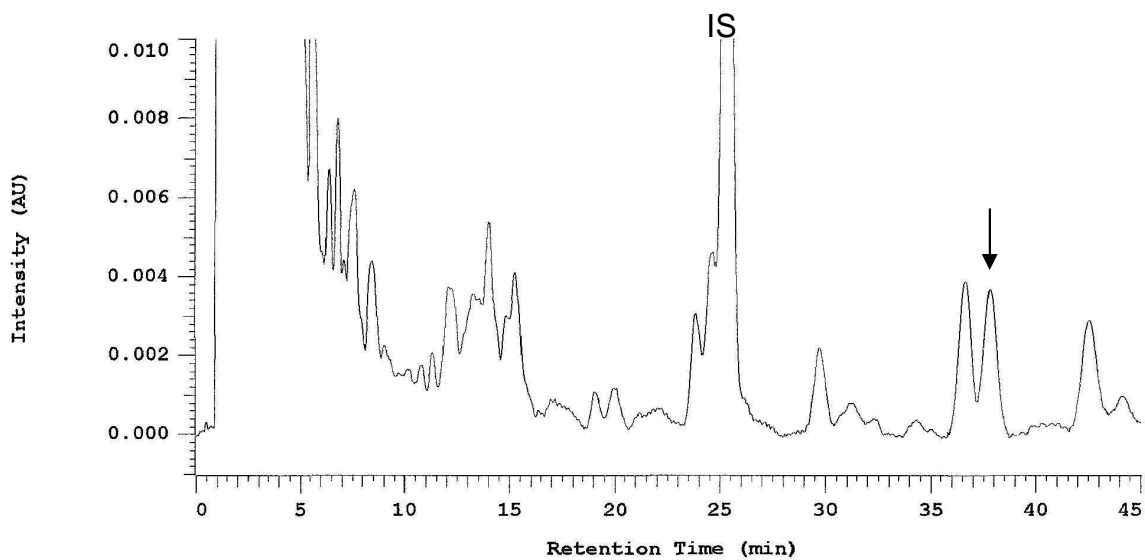


Figure 7.15 The spring onion extract separated with weaker mobile phase (50% ACN/water) with all the other conditions fixed. The arrow indicates 1,4-DMN retention time

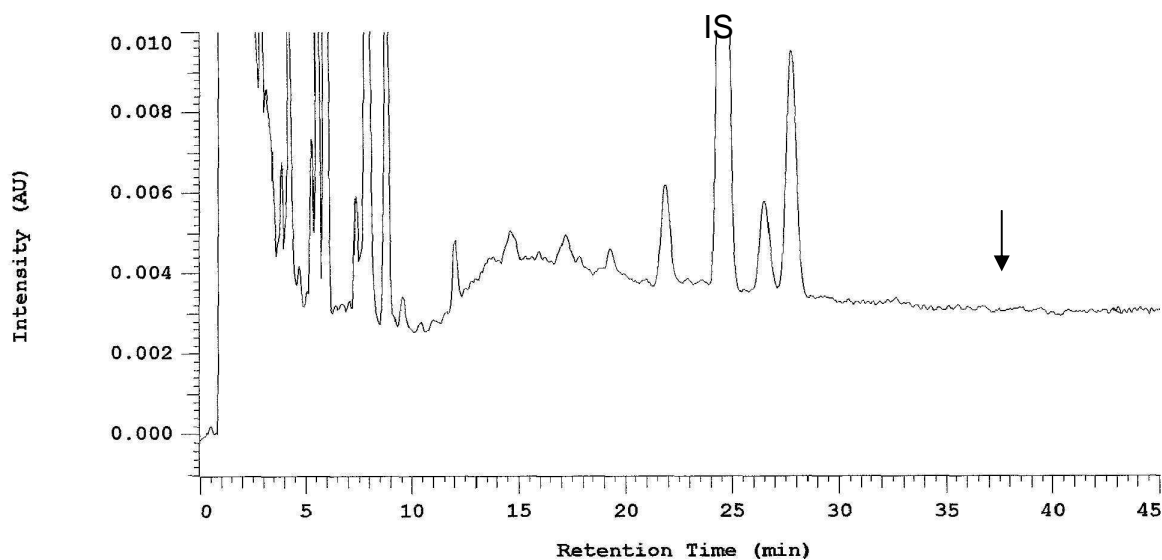


Figure 7.16 The carrot extract separated with weaker mobile phase (50% ACN/water) with all the other conditions fixed. The arrow indicates 1,4-DMN retention time

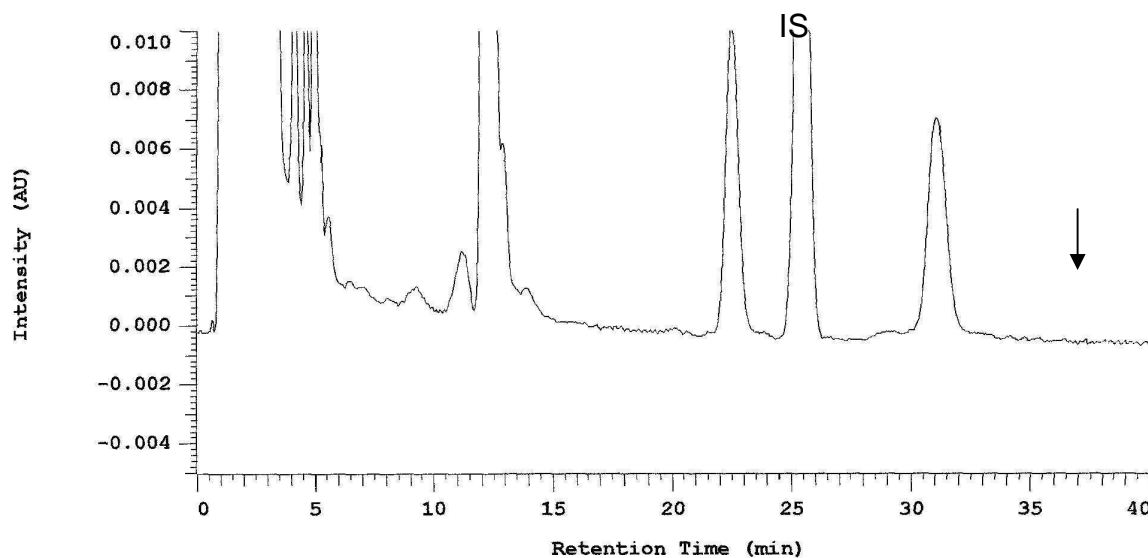


Figure 7.17 The rhubarb extract separated with weaker mobile phase (50% ACN/water) with all the other conditions fixed. The arrow indicates 1,4-DMN retention time

Spreading the chromatograms by using a weaker mobile phase helped to confirm that none of the good-sized peaks detected around 1,4-DMN retention time in both carrot and rhubarb extracts (as shown in Figure 7.9 and 7.10) were for 1,4-DMN because they were eluted at different retention times with the weaker mobile phase, as shown in Figure 7.16 and 7.17.

However, in the spring onion extract, a good-sized peak was still present at the retention time of 1,4-DMN at these conditions, as shown in Figure 7.15. The spectrum purity index (spectrum check across the peak) was 0.999, which confirms that the peak is for only one single compound. However, by comparing the spectrum generated for this peak with 1,4-DMN spectrum, a correlation factor of only 0.265 was reported. The spectra comparison is shown in Figure 7.18.

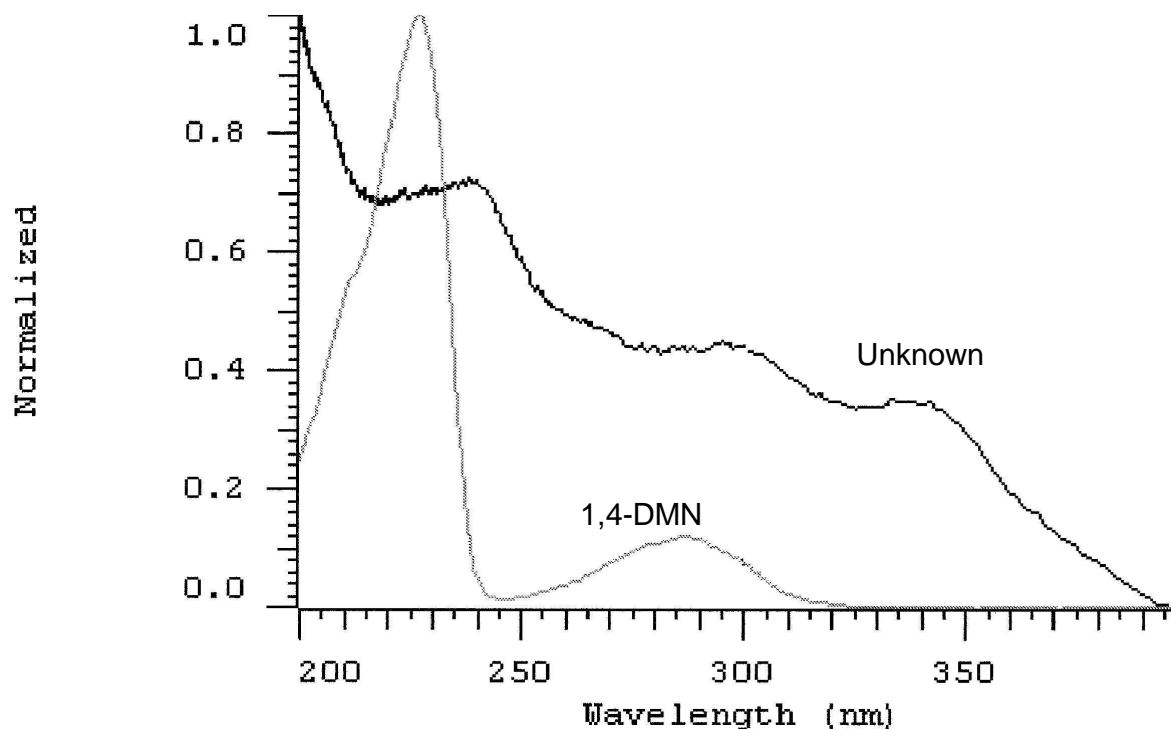


Figure 7.18 Comparison of the spectrum of the unknown peak in spring onion extract shown at about 37.5 in Figure 7.15 with the spectrum 1,4-DMN in a standard solution run at the same conditions

The above spectra comparison indicates that the peak detected in spring onion does not correspond to 1,4-DMN.

Better confirmation of the peaks' identity - particularly the ones in the potato peel extract and spring onion - could be achieved by using mass spectroscopy. However, there was no HPLC-MS facility available at the time of conducting these experiments.

The rest of the plant materials extracted showed no detectable peaks at 1,4-DMN retention time, as shown in Figure 7.11, 7.12, 7.13 and 7.14 for apple, orange, poppy seeds and celery respectively.

Because of uncertainty of the identity of the peak detected in potato peel extract due to its low level, it seemed to be necessary to try some other approaches to obtain sufficiently high concentrations of 1,4-DMN for quantitative study. One of the approaches might be the modification of the ACN/PROP method to add a concentration step for the extract, and possibly a clean up step to minimise the background effect. Both modifications might be achieved by using solid phase extraction (SPE) which can provide cleaner and more concentrated extract for quantitative analysis. In addition, it will be very useful to use an HPLC-MS for the final determination of natural 1,4-DMN in the extracts.

The other approach that might be examined for obtaining higher concentrations of the natural 1,4-DMN in potatoes is the collection and concentration of the volatiles produced in the headspace above potato tubers.

However, due to time and resource limitations, only the headspace approach was examined in this work, as discussed below.

7.3.3 Headspace experiments

This part of the work aimed to develop and use a method for the collection of the volatiles produced in the headspace of potato tubers in a search for natural 1,4-DMN. It was thought that more 1,4-DMN can be collected by this method compared to solvent extraction due to the dynamic nature of the biological systems producing plant volatiles (Augusto et al., 2003).

7.3.3.1 Recovery of 1,4-DMN from Tenax traps

This experiment aimed to examine the recovery of 1,4-DMN from the Tenax traps. Four traps were spiked each with 1 µg of 1,4-DMN and extracted by solvent elution. Solvent elution was selected because HPLC was the analytical technique used for the final separation, and this required the sample to be in a liquid form. Solvent elution has also been used for headspace methods using GC for the final determination, particularly when the instrument is not provided with a thermal desorption unit (Beltran et al., 2006). Due to the high affinity of the Tenax adsorbent for non-polar compounds such as the hydrocarbon 1,4-DMN, it was thought it would be accumulated close to the inlet of the Tenax trap. Therefore, back elution was used because it was thought this would help in achieving complete elution with a smaller volume of acetonitrile. In this recovery experiment, each spiked trap was eluted twice with 3.5-ml portions of acetonitrile into 5-ml volumetric flasks containing 1.5 ml of deionised water. The resulting solution was therefore in 70 % ACN/water, which is the same composition of the mobile phase used in the chromatographic separation. This matching was found to be important for the chromatographic separation when using large volume (100 µl) injections, as discussed in Chapter 6. The elution with the second 3.5-ml portion aimed to check for complete elution of 1,4-DMN in the first portion.

The amount of 1,4-DMN recovered in the first 3.5 elution ranged from 89.3 % to 92.4 % with a mean of 90.0 % and a %RSD of 1.9 %. There was no 1,4-DMN detected in the second elution in all the four replicates. These results show that solvent elution with 3.5 ml

of acetonitrile provides a sufficiently efficient method to recover the adsorbed 1,4-DMN from the Tenax traps. The deviation of the recovery from 100 % might be due to loss of some 1,4-DMN through evaporation before sealing the traps with the Teflon tape, or possibly due to irreversible adsorption in the trap, particularly at this low level of 1,4-DMN.

7.3.3.2 Background level evaluation

This experiment aimed to evaluate the level of the background contaminants that might be present, and also to check for any air leakage in the system, particularly with the use of positive pressure.

To test for any air leak, the system was assembled as in Figure 7.1 with the two Tenax traps in position, and air flow was adjusted to the desired flow rate. Soap solution was spread around the different connections in the system and particularly around the sample inlet.

Despite using 4 clamps to hold the two parts of the sample inlet together, some air leak was noticed around the sample inlet. To minimise this leak a Teflon ring was inserted between the two parts of the sample inlet. With the Teflon ring in place, the leak was successfully minimised but was not completely eliminated. It was thought that the remaining small level of leak should not have a great effect on the collection process, particularly when having good flow at the collection system outlet. Therefore, the system was accepted and used with this slight leak.

The background contamination level was evaluated by passing air through the collection system for 15 days and trapping any background contaminants in the Tenax traps. Both the guard Tenax trap and the sampling Tenax trap were eluted and analysed by HPLC. Figure 7.19 shows the chromatograms of the solutions resulted from eluting both traps along with a chromatogram for a 0.01 µg/ml 1,4-DMN standard.

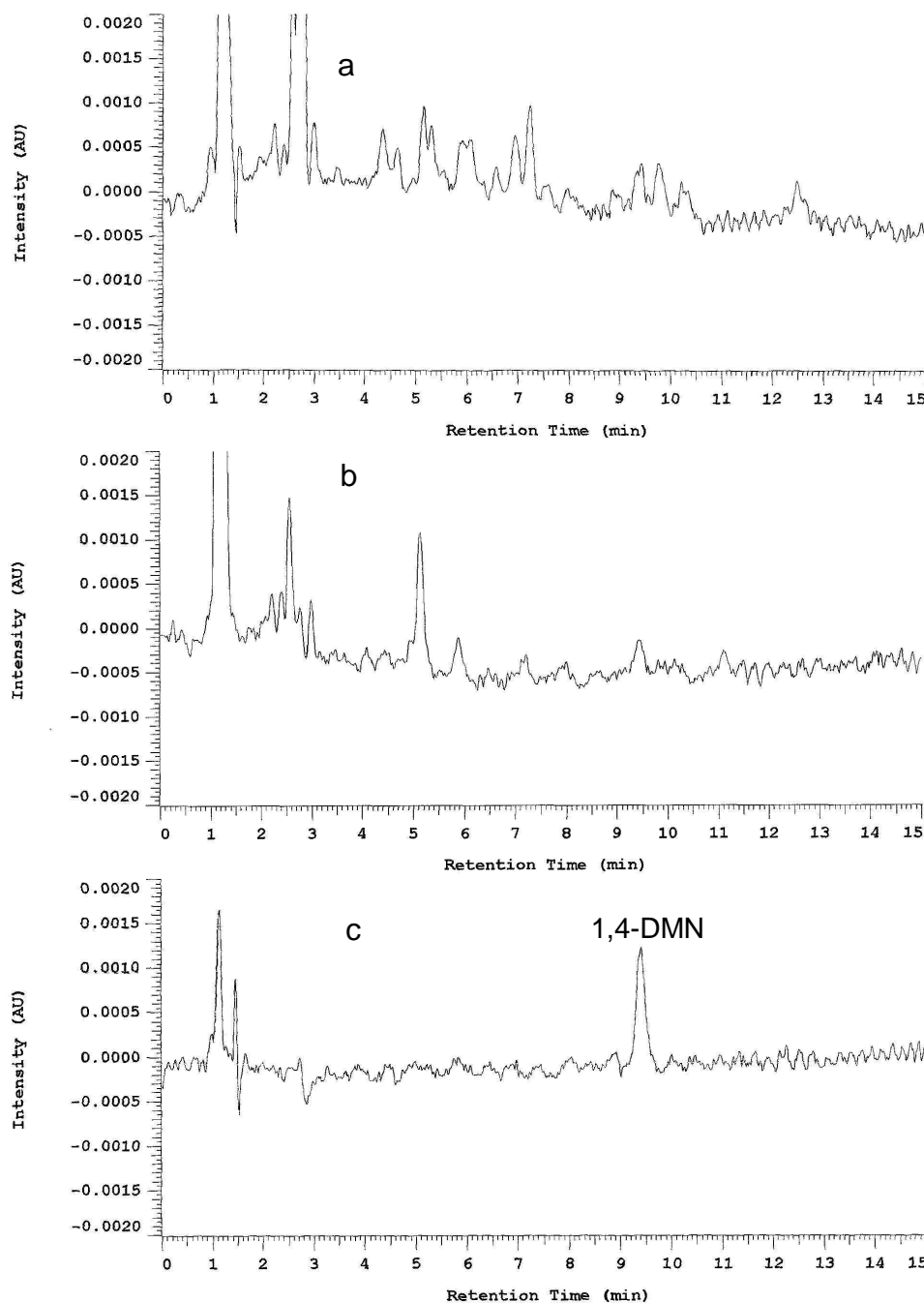


Figure 7.19 Chromatograms of a) eluate of guard Tenax trap of background sampling b) eluate of sampling Tenax trap of background sampling and c) 0.01 $\mu\text{g/ml}$ 1,4-DMN standard.

As shown in Figure 7.19a, some contamination compounds were trapped from the air cylinder supply with some major peaks at about 2-3 min. Fortunately, most of these compounds were adsorbed in the guard trap and only small minor peaks were noticed in the sampling Tenax trap. This shows the importance of purifying the air before being introduced to the collection system. Boyd (1984) used a mixture of oxygen and nitrogen as an air supply in a headspace experiment instead of using pressurised air cylinder, as it was found that the purity of the mixture was far higher than that of the pressurised air. In addition, the mixture of gases was purified by being passed through a cold trap. Despite the fact that both the pressurised air cylinders used by Boyd (1984) and those used in the

current work came from the same manufacturer (BOC Glasgow Ltd), the purity of the air might have increased. In addition, there should be a difference in the level and type of contaminants eluted from the Tenax traps when using solvent elution with acetonitrile compared to the thermal desorption used by Boyd. Therefore, pressurised air was used here for the air supply as the level of contaminants was considered acceptable, and most of the contaminants were trapped in the guard Tenax trap and prevented from entering the sample vessel.

A very small peak was detected at a retention time very close to that of 1,4-DMN in both the guard and sampling Tenax traps. This peak might be some 1,4-DMN contamination that occurred during the elution process. It was not possible to confirm the identity of the peak due to its small size. However, due to the limitation of time and resources, it was decided not to spend too long time investigating the source of this small peak in order to eliminate it. Rather, it was decided to accept its presence at this level and use the system for investigating the natural 1,4-DMN, as it was thought that a greater level of natural 1,4-DMN could be collected compared to this small peak.

7.3.3.3 Recovery of 1,4-DMN from the collection system

These experiments aimed to examine the efficiency of the collection system in trapping and desorbing 1,4-DMN at different periods of sampling times, and to check for any difficulties that may be encountered during the real experiment of collecting potato volatiles.

The first recovery experiment was conducted for 24 h collection time at a flow rate of 20 ml/min and 1,4-DMN level of 1 µg. 1,4-DMN was placed into a 10-ml conical flask (not directly added to the sampling vessel) to allow for rinsing the flask at the end of the experiment, and to examine for incomplete evaporation of 1,4-DMN. Two sampling Tenax traps were connected in series. The second trap was used to trap any 1,4-DMN that may escape the first trap due to incomplete adsorption. The first sampling Tenax trap was eluted twice with 3.5 ml of acetonitrile to check for complete desorption.

The recovered amounts of 1,4-DMN in each trap and in the conical flask relative to the initially loaded amount are shown in Table 7.2.

Table 7.2 Recovered amounts of 1,4-DMN in each trap and in the conical flask in the first experiment

Sample	1,4-DMN Recovery (%)
1 st Trap, 1 st Elution	26.9
1 st Trap, 2 nd Elution	Not Detectable
2 nd Trap	Not Detectable
Conical Flask Wash	6.9

This first experiment shows that the collected 1,4-DMN amount was relatively low. Most of the loaded 1,4-DMN was not recovered. Some of the loss was due to incomplete evaporation of 1,4-DMN, as suggested by the detection of 6.9 % of the loaded 1,4-DMN in the conical flask. Longer collection times will be required to allow for complete evaporation of 1,4-DMN from the conical flask. This was confirmed in the second experiment.

The absence of 1,4-DMN in the second elution of the first trap and also in the second trap (first elution) confirmed the high efficiency of the adsorption and desorption process of 1,4-DMN in the first trap.

The low recovery level obtained was assumed to be attributed to three causes. The first was the incomplete evaporation of 1,4-DMN from the source of 1,4-DMN (the conical flask). The second was the slight leak noticed around the sample inlet (see Section 7.3.3.2) which seems to have contributed to the loss of some 1,4-DMN out of the collection system. The third possible cause of recovering low 1,4-DMN in the sampling trap was thought to be due to the adsorption on the collection system surfaces, particularly at the low level of 1,4-DMN used in this first recovery experiment. To examine the contribution of each of the three causes in lowering the recovery of 1,4-DMN, the second and third recovery experiment were conducted.

In the second experiment, 1 µg was transferred into the conical flask which was placed in the sample vessel (without washing the vessel after the first recovery experiment to minimise the adsorption effect) and the first sampling Tenax traps was connected for the first 24 h at a flow of 20 ml/min, then replaced with the second sampling Tenax trap. The latter was disconnected after 6 days. The recovered amounts of 1,4-DMN in each Tenax trap and in the sample conical flask are shown in Table 7.3.

Table 7.3 Recovered amounts of 1,4-DMN in each trap and in the conical flask in the second experiment

Sample	1,4-DMN Recovery (%)
1 st Trap	37.1
2 nd Trap	4.5
Conical Flask Wash	Not Detectable

In this experiment, complete evaporation was achieved at the end of the experiment, as shown by detecting no 1,4-DMN in the sample conical flask. However, 24 h was confirmed to be not enough time to achieve complete evaporation of 1,4-DMN, as 4.5 % of the loaded 1,4-DMN was recovered in the second sampling trap which was connected after 24 h.

The total recovery of 1,4-DMN in this second experiment (41.6 %) was higher than the total in the first experiment (33.8 %). Two possible reasons were drawn for this increase in the total recovery. The first was the possibility of reducing the adsorption effect of the system surfaces by conducting the experiment without washing the sampling vessel. The second was the reduction of the air leak from the sample inlet by running this second experiment with lower backpressure, due to connecting only one sampling Tenax trap each time instead of two in series in the first experiment. To examine the effect of these two factors, the third recovery experiment was conducted.

The third experiment was conducted at a higher level of 1,4-DMN to minimise the adsorption effect, as it should have only a slight effect at higher levels of 1,4-DMN due to saturating the adsorption sites in the system surfaces. In addition, the experiment was conducted without washing the sample vessel for the same purpose. 10 µg were transferred into the conical flask which was placed in the sample vessel, and two sampling Tenax traps were connected in series for 6 days at a flow of 20 ml/min. The first sampling trap was eluted twice to check for complete desorption of 1,4-DMN. The recovered amounts of 1,4-DMN in each Tenax trap and in the sample conical flask are shown in Table 7.4.

Table 7.4 Recovered amounts of 1,4-DMN in each trap and in the conical flask in the third experiment

Sample	1,4-DMN Recovery (%)
1 st Trap, 1 st Elution	27.3
1 st Trap, 2 nd Elution	Not Detectable
2 nd Trap	Not Detectable
Conical Flask Wash	Not Detectable

In this third experiment, the level of 1,4-DMN collected and recovered in the first sampling trap was similar to the level in the first experiment and lower than the level in the sampling trap in the second experiment. In the first and third experiments, the backpressure in the collection system was higher than in the second experiment because of connecting two sampling Tenax traps. This backpressure should have increased the level of leakage through the sample inlet in the vessel. Therefore, despite the possible effect of adsorption into the system surfaces, the main factor in lowering the total recovery level was the loss of 1,4-DMN through the leak from the sample inlet.

From all the above three experiments, it was clear that some modifications in the collection system might be necessary to provide better sealing of the sample inlet and increase the recovery of 1,4-DMN, which should help in minimising the loss of natural 1,4-DMN which is produced by potatoes at very low levels. However, it was preferred to run the real headspace sampling of potato tubers using the current system without modification to have a look at the nature and levels of the volatiles produced, and defer any modification to a later stage.

7.3.3.4 Collection of potato volatiles

The aim of this part of the work was to collect the volatiles produced by potato tubers in a search for the natural 1,4-DMN. It was hoped to collect a sufficient amount of natural 1,4-DMN for qualitative and quantitative measurements. Two experiments were conducted for this purpose at different conditions and sampling time periods.

In the first experiment, the volatiles of 3.5 kg of Valor organic potatoes were collected over 4 weeks in light. Temperatures varied from 15-30 °C during the sampling period. Figure 7.20 shows the chromatograms of the solutions resulted from eluting both guard and sampling Tenax traps along with a chromatogram for a 0.01 µg/ml 1,4-DMN standard.

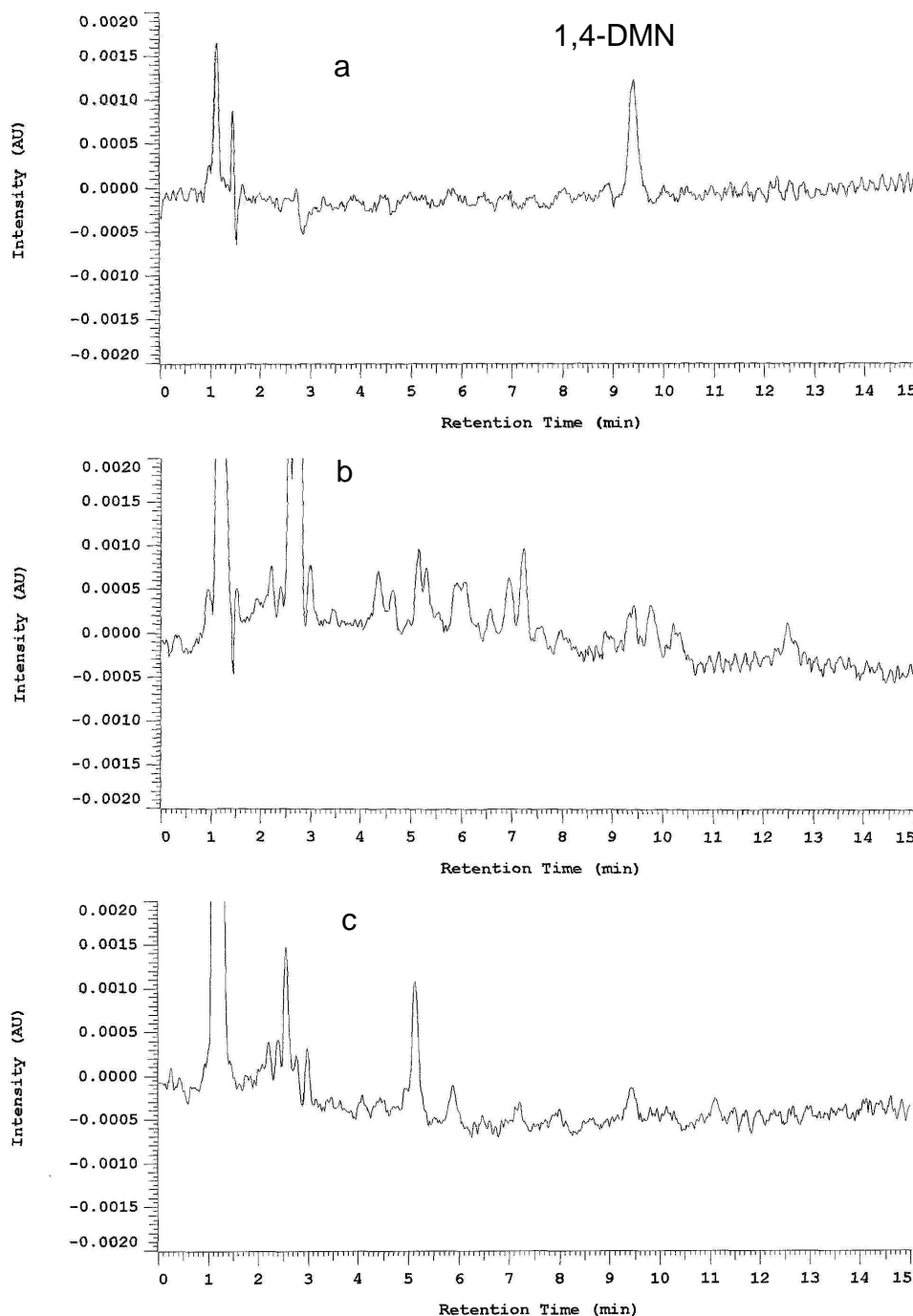


Figure 7.20 Chromatograms of the first experiment for potato volatile collection a) 0.01 $\mu\text{g/ml}$ 1,4-DMN standard b) eluate of guard Tenax trap and c) eluate of sampling Tenax trap.

The presence of some major peaks in the guard Tenax, but not the sampling Tenax, suggests that the guard Tenax trap was playing an important role in preventing the entry of several contaminant compounds from the air supply to the sample vessel.

A few small peaks were eluted around the retention time of 1,4-DMN in the guard Tenax traps and one small peak in the sampling Tenax trap. However, it was not possible to confirm the identity of these peaks by using the UV spectra, due to the small size of these peaks and the relatively high noise in the baseline. It is possible that the peaks in the guard trap were for compounds trapped from the air source and the ones in the sampling trap

were for different compounds produced by potatoes. However, the peaks in both traps might be for the same compounds, which may suggest a contamination during the elution process of the traps.

Because of the uncertainty regarding the peaks eluted around 1,4-DMN retention time and the low level of the collected volatiles, it was decided to repeat the experiment with more controlled conditions and for a longer time to collect more volatiles.

The collection system was placed in an incubator in a different laboratory than the one used for conducting the first experiment, for more temperature control and to help in avoiding any contamination with 1,4-DMN that may occur in the laboratory used for regular 1,4-DMN analysis. The concentration of a volatile chemical in the air surrounding the chemical source was found to increase with the increase in both temperature and time (O'Hagan, 1991). Therefore, the sampling was conducted for 6 weeks in the dark and the incubator temperature was set at 20 °C. 3.5 kg of Nicola organic potatoes were used here. Figure 7.21 shows the chromatograms of the solutions resulted from eluting both guard and sampling Tenax traps along with a chromatogram for a 0.01 µg/ml 1,4-DMN standard.

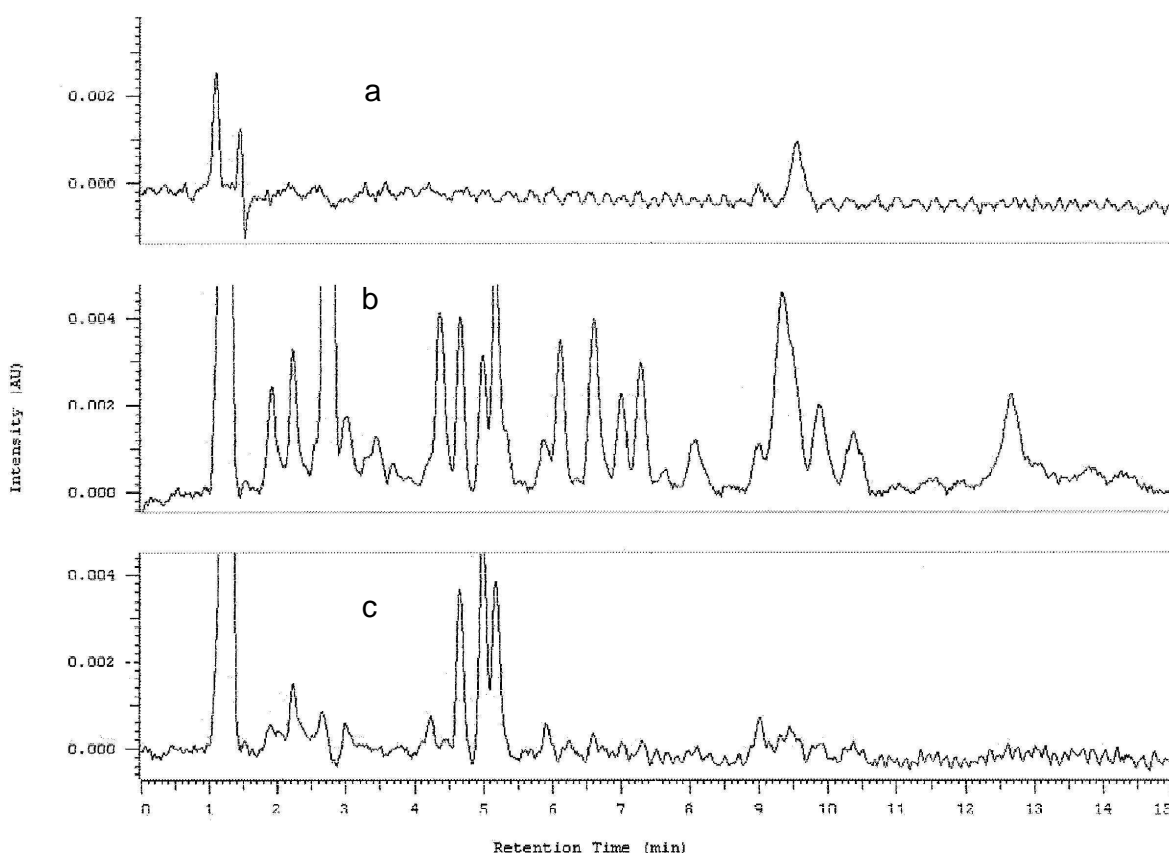


Figure 7.21 Chromatograms of the second experiment for potato volatile collection a) 0.01 µg/ml 1,4-DMN standard b) eluate of guard Tenax trap and c) eluate of sampling Tenax trap.

In this experiment, the guard Tenax provided good filtering of the air supply introduced to the collection vessel, as shown by the presence of many peaks in the chromatogram of the guard trap and only several small peaks in the sampling trap.

No peaks were quantified at or close to the retention time of 1,4-DMN. The level of volatiles produced by potatoes seems to be too low to be accumulated to a quantifiable level using only 3.5 kg of potatoes for the time period used. Filmer and Rhodes used one tonne of raw potato tubers to investigate potato volatiles, and they succeeded in collecting many of them including 1,4-DMN (Filmer and Rhodes, 1985). However, other authors reported the use of smaller amounts of potato sample for collecting volatiles than the one used by Filmer and Rhodes. This should be dependant upon the analytical method used for collecting the volatiles, and also upon the analytical technique used for the final determination. It was hoped that the collection method used, combined with the HPLC for the final determination, would provide enough sensitivity for quantifying the natural 1,4-DMN produced by potatoes. The use of a long collection period (4-6 weeks) aimed to add greater support for better sensitivity. However, some drawbacks in the analytical method used seem to affect the level of the collected volatiles. The air leak noticed around the sample inlet could have contributed to the loss of some of the volatiles out of the collection system, therefore reducing the collected amount of potato volatiles in the sampling trap. In addition, the potato sample used (3.5 kg) seems not to be large enough to produce quantifiable levels of natural 1,4-DMN. A larger collection vessel (or several ones connected in series) should be used to provide a larger potato sample for more 1,4-DMN production. However, as previously mentioned, there was not enough time or resources to conduct the above modifications in the analytical headspace method used for the collection of potato volatiles.

Chapter 8: Distribution and removal of the residues in stored potato tubers treated with 1,4-DMN

8.1 Introduction

The extensive use of pesticides in modern agriculture is normally associated with various risks to the consumers and to the environment. The presence of a pesticide with a certain risk level is not the only factor in evaluating the actual risk for consumers. It is also important to know the quantity, location of the pesticide in the produce, accessibility and who is exposed to that pesticide. The common practice in pesticide residue analysis is to evaluate the residue levels in composite samples. However, due to the possible risk of intake of high doses of pesticide residues by consuming individual units, the concept of 'variability factor' was introduced as an indicator for the variability of pesticide residues. The definition of this term, the typical values and the causes of variability are discussed in Section 1.5 in the introductory chapter.

To reduce the risk of consuming pesticide residues, fruit and vegetables are normally washed before consumption. Washing fruit and vegetables with water and/or other washing solutions was reported to reduce the levels of residues of many different pesticides in produce as summarised in the literature review in Section 1.5. In the literature reviewed, the effectiveness of washing produce in removing pesticide residues was found to vary from one experiment to another. The effectiveness of washing is affected by many different factors including the nature of the pesticide and its physicochemical properties, nature of the produce surface, nature of the washing solution, nature and duration of the washing process, and the final factor is the storage time after application.

In most of the reviewed studies, washing produce with water and/or other washing solutions contributed to removing some of the residues of many pesticides. More significant reduction of pesticide residues can be achieved by removing the outer layers of fruit and vegetables, because of the surface position of many pesticides and the accumulation of residues in the outer surfaces, as discussed in Section 1.5.

This part of the work aimed to investigate three issues. The first is the variability of 1,4-DMN residue levels in treated potato tubers between the different single tubers in a sample, between peel and flesh of a tuber and across the surface of a tuber. Knowledge

about the variability and distribution of 1,4-DMN residues has an important role for the safety of consumers in addition to its analytical experimental importance. From the consumer safety point of view, it is important to know if 1,4-DMN residues can penetrate to the inner layers of the tuber (flesh), which may expose the consumer to the risk of intake of the residues in the diet. In addition, the variability between tubers can provide a measure for the possibility of consuming high acute doses of the residues. The experimental importance of knowing the variability within-tuber and between-tubers appears for choosing the correct sampling procedure for analysis to provide a good representation of the original sample. The knowledge about the variability of the residues can shed light when taking a decision on the required number of tubers in a sample, the layer of the tuber that has to be analysed (peel vs. flesh) and the required levels of fragmenting and mixing the sample.

The second objective was to investigate the effect of washing potato tubers treated with 1,4-DMN on the reduction of 1,4-DMN residues. This investigation has importance for consumer safety and also for the environment. This study aimed to provide sound knowledge about the effectiveness of domestic household washing with water and a range of some other solutions on the removal of 1,4-DMN residues. In addition, knowledge about the level of 1,4-DMN residues that can be removed with water washing or the other range of solutions has importance for the environment, as the waste wash water will be disposed into the environment. This issue of waste wash water is more important for the potato processing industry, as large quantities of treated tubers are processed daily.

As waste wash water can be a significant issue for the potato processing industry, potato peel wastes have similar or perhaps even more significant importance. The peel wastes are normally fed to animals, so the presence of 1,4-DMN residues may pose a risk for these animals and for humans consuming their meat or products. Therefore, the third objective was to investigate the removal of 1,4-DMN residues from potato peel by oven-drying the peel. Two techniques were examined for this purpose. In the first, 10-g samples were oven-dried and in the second, larger (50-g or 120-g samples) were oven-dried with flushing air through the samples.

8.2 Materials and methods

8.2.1 Analytical method

Throughout this chapter, 1,4-DMN residues in all samples were analysed according to the ACN/PROP routine method detailed in Section 6.5.

8.2.2 Potato sample

A 75-kg sample of Desiree potato tubers were randomly selected out of a total crop weight of 5.983 tonnes in Sutton Bridge Experimental Unit (Lincolnshire, UK). The tubers had three applications of 1,4-DMN using a mist application technique on the dates detailed in Table 8.1 below. The tubers had no treatment with any other sprout inhibitor. They were held at 6 °C and 95 % relative humidity (Ajay Jina, personal communication).

Table 8.1 1,4-DMN application information for the potato sample used in the work in this chapter

Application	Date	Rate (g/tonne)
1	27/10/2005	10.14
2	16/12/2005	20.28
3	09/02/2006	10.14

The 75-kg sample was received on the 23rd of June 2006 in three paper bags, each containing about 25 kg. On arrival, the tubers were sorted to remove any that were too small or too large to end up with relatively uniformly-sized tubers for the experiment. They were then mixed well and stored in a cold room at 4 °C throughout the experiment period.

8.2.3 Distribution and variability of 1,4-DMN residues

This series of experiments aimed to evaluate the distribution and variability of 1,4-DMN residues in stored potatoes. Variability in 1,4-DMN residue levels was evaluated between tubers from the same 75-kg sample, and within a tuber by examining the distribution of 1,4-DMN between the skin and the flesh of the tuber, and also the surface distribution across the skin of the tuber.

8.2.3.1 Between-tubers variability of 1,4-DMN residues

This experiment aimed to evaluate the variability of 1,4-DMN residues between the individual tubers in the 75-kg sample. The variability level was evaluated in unwashed tubers and also for washed tubers.

For unwashed tubers, a representative sample of 15 tubers was randomly selected out of the 75-kg sample and left to warm up to room temperature (~ 1 h). Each individual tuber was then analysed as a separate sample by peeling it and analysing the peel according to the ACN/PROP routine method detailed in Section 6.5.

To evaluate the effect of the soil attached to the tubers on the variability of 1,4-DMN residues, 15 tubers were washed and analysed and compared to the unwashed tubers. The tubers were randomly selected out of the 75-kg sample and left to warm up to room temperature (~ 1 h). Each tuber was then washed gently under running cold tap water for 20 seconds and then wiped with paper towels to absorb residual water. They were then analysed separately by peeling each tuber and analysing the peel.

The flesh of each tuber was also chopped, mixed well and analysed for the purpose of comparing the distribution of 1,4-DMN between the peel and the flesh which will be discussed in later sections (see Section 8.2.3.2 and Section 8.3.1.2).

To calculate the variability factor of 1,4-DMN residues, another 15 tubers were divided into three composite samples each with 5 tubers. The tubers were randomly selected out of the 75-kg sample, left to warm up and then washed as described above. They were then peeled and the peel of each composite sample was mixed well before taking a 10-g sample for the analysis.

8.2.3.2 Within-tuber variability of 1,4-DMN residues

This experiment aimed to examine the vertical distribution of 1,4-DMN residues between peel and flesh and also the horizontal distribution of 1,4-DMN residues in the tubers' outer surface (peel).

The evaluation of the vertical distribution of 1,4-DMN residues between peel and flesh was conducted as part of the experiment described above in Section 8.2.3.1.

To examine the horizontal distribution of 1,4-DMN residues in the peel surface of treated tubers, the following experiment was conducted. Seven treated potato tubers were randomly selected out of the 75-kg sample and left to warm up to room temperature (~ 1 h). Each tuber was then washed gently under running cold tap water for 20 seconds and then wiped with paper towels to absorb residual water. Each tuber was then divided into four quarters and each quarter was analysed as a separate sample by peeling it and analysing the peel according to the ACN/PROP routine method detailed in Section 6.5.

8.2.4 The removal of 1,4-DMN residues from treated potato tubers by various washing solutions

This series of experiments aimed to compare the effect of several washing solutions in the removal of 1,4-DMN residues from the peel of treated tubers. The following washings were compared for this purpose:

- Cold water running for 20 seconds
- Cold water running for 1 minute
- Cold water running for 1 minute with brush scrubbing
- Hot (~50 °C) water running for 1 minute
- 5 % ethanol/water solution running for 1 minute
- 5 % acetic acid/water solution running for 1 minute
- Veggi-Wash vegetable and fruit detergent (soaked as directed in the label). Tubers were soaked for 2 min in a 0.5 % solution of the Veggi-Wash detergent (50 ml of the detergent concentrate in 10 l of water). Tubers were then rinsed with running cold tap water for 1 min.
- Water soaking treated as a blank for the Veggi-Wash treatment

For each of the above eight treatments, 15 tubers were washed using the washing solution and then wiped with paper towels. The tubers were then peeled and the peel was analysed for 1,4-DMN residues using the ACN/PROP routine method detailed in Section 6.5.

8.2.5 The removal of 1,4-DMN residues from potato peel by oven-drying

This series of experiments aimed to evaluate the effect of oven-drying potato peel samples on the removal of 1,4-DMN residues from the peel. For this series of experiments a large amount (about 1.3 kg) of potato peel containing 1,4-DMN residues was prepared by peeling several kilograms of potato tubers treated with 1,4-DMN. The peel was then chopped and mixed well before dividing into portions of about 170 g for each experiment. The portions were stored in aluminium foil in a freezer at -18 °C. Before using a portion, it was left to defrost and warm to room temperature for at least 3 hours.

8.2.5.1 The effect of oven-drying of 10-g samples of potato peel on 1,4-DMN residue level in potato peel

In this experiment, 10 g samples were transferred into 50-ml conical flasks (pre-weighed) and heated in an oven at 75 ± 5 °C for 1, 3, 6, 18 or 67 hours. For each period of time, 10

samples were dried in the oven and 5 were analysed directly as controls. After drying, samples were weighed again to calculate the moisture content and then analysed (after adding water to compensate for the water lost during the drying process) according to the ACN/PROP routine method detailed in Section 6.5.

8.2.5.2 The effect of oven-drying with air flushing on 1,4-DMN residue level in potato peel

In this experiment, 120-g samples of potato peel were transferred into a large (12 cm diameter x 6 cm height) Buchner funnel connected to an air cylinder to provide a continuous air flow up through the peel (flow rate = 2-3 l/min) during the drying period in an oven at 75 ± 5 °C. After drying for the different periods of times described in Section 8.2.5.1 and cooling, the peel was mixed well and replicate 10-g samples were analysed (after adding water to compensate for the water lost during the drying process). For each period of time, 5 samples were analysed directly without drying as controls.

To examine the effect of the amount of the peel in the Buchner funnel on the speed of removing 1,4-DMN residues, 50-g samples were compared to 120-g samples in the same experiment. Two funnels were placed in the oven and connected to the air supply. The air flow received by both funnels was adjusted to be at a similar rate. About 120 g of potato peel (out of the large pile of peel) were transferred into one funnel and about 50 g were transferred to the other funnel. The two samples were oven-dried at 75 ± 5 °C for 12 hours with air flushing. After drying and cooling, the peel was mixed well and replicate 10 g samples were analysed. Five samples were analysed out of each funnel in addition to 5 samples which were directly analysed without drying as controls for each experiment. This experiment was repeated four times.

8.3 Results and discussion

8.3.1 Distribution and variability of 1,4-DMN residues

The objective of these experiments was to evaluate the distribution and variability of 1,4-DMN residues in stored potatoes. Variability in 1,4-DMN residue levels was evaluated between tubers from the same 75-kg sample and within-tuber by examining the distribution of 1,4-DMN between the skin and the flesh of the tubers and also the surface distribution across the skin of the tubers.

8.3.1.1 Between-tubers variability of 1,4-DMN residues

This experiment aimed to evaluate the variability of 1,4-DMN residues between the individual tubers in the 75-kg sample. The variability level was evaluated in unwashed tubers and also in washed tubers. A variability factor was calculated from the data of the individual washed tubers and the washed composite samples. Table 8.2 shows 1,4-DMN residue levels in unwashed and washed individual tubers, and also in washed composite sample.

Table 8.2 Residues of 1,4-DMN in individual (washed and unwashed) tubers and composite samples

Tuber/Sample No.	1,4-DMN Residues (µg/g) Fresh Weight		
	Unwashed Individuals	Washed Individuals	Washed Composite
1	1.01	0.90	0.70
2	1.03	0.75	0.88
3	1.06	0.89	0.73
4	0.84	0.97	
5	0.63	1.05	
6	1.08	1.12	
7	0.82	0.55	
8	1.08	0.74	
9	0.69	0.90	
10	0.71	1.12	
11	1.01	0.79	
12	0.91	0.91	
13	1.16	0.83	
14	0.71	0.81	
15	0.87	1.07	
Mean	0.91	0.89	0.77
SD	0.17	0.16	0.10
RSD (%)	18.54	17.67	12.33

1,4-DMN residues in the unwashed individual tubers ranged from 0.63 to 1.16 µg/g fresh weight, with a mean of 0.91 µg/g and a %RSD of 18.54 %. To examine the effect of the attached soil and dust on the residue levels and variability, tubers were washed with tap water before the analysis. The results in Table 8.2 for the washed individuals showed 1,4-DMN residues ranged from 0.55 to 1.12 µg/g fresh weight, with a mean of 0.89 µg/g and a %RSD of 17.67 %. The similarity in the residue levels (difference is not statistically significant, see Section 8.3.2) and variability (indicated by the %RSD) between the washed and unwashed tubers indicate that the attached soil and dust on the surface of potato tubers have a minor effect on the levels and variability of the extracted residues of 1,4-DMN. This small effect of soil and dust removal suggests that little of the extractable 1,4-DMN

residues are in the soil and dust attached to the tuber surface. The long storage time after the last application of 1,4-DMN (more than 18 weeks) seems to contribute to lowering the effect of attached soil and dust on the residue levels and variability in two possible ways. The first is that the prolonged storage time may have caused 1,4-DMN residues to migrate from the soil and dust particles to the tuber tissues, therefore reducing the actual residues in these particles. The other possibility is that the prolonged storage period caused 1,4-DMN residues in the soil and dust particles to be converted to a bound form and therefore they cannot be extracted with the organic solvent extraction used. These possibilities can be better examined by using radiolabelled methods for tracking the residue movement processes or using rigorous extraction techniques to increase the free pesticide residues. Jamieson (1988) reported that the washable residues of thiabendazole after 6 months of storage were lower than the amount washed off after four months. The effect of storage time might be the cause for the reduction in the washable residues. Similar results have also been reported by Lentza-Rizos and Balokas (2001) for the effect of water washing on the residues of chlorpropham in potato tubers. Chlorpropham residues in potato tubers were reduced by 33 % and 24 % for the 10 days and 28 days post-application sampling respectively. This seems to suggest a decline in the washable residues as the storage period after the application increased.

1,4-DMN residue levels in the composite samples ranged from 0.70 to 0.88 $\mu\text{g/g}$ fresh weight, with a mean of 0.77 and a %RSD of 12.33 %. By dividing the highest residue level in individual potato tubers by the mean of composite samples, a variability factor of about 1.5 can be obtained for 1,4-DMN residues in this potato sample. This level is considerably lower (half) than the default value of 3 concluded by FAO and WHO for medium-sized crops (FAO and WHO, 2005). This lower variability of 1,4-DMN residues between tubers reduces the risk of acute dietary intake for consumers. Lentza-Rizos and Balokas (2001) reported a variability factor of up to 2 for chlorpropham applied to potato tubers as dust powder. From the analytical point of view, the lower variability between tubers helps in reducing the number of potato tubers required to get a representative sample during the routine analysis of 1,4-DMN residues in treated potatoes.

8.3.1.2 Within-tuber variability of 1,4-DMN residues

This part of the work aimed to examine the vertical distribution of 1,4-DMN residues between the peel and flesh of treated potato tubers in addition to the horizontal distribution of the residues across the tuber surface.

To evaluate the vertical distribution of 1,4-DMN residues between peel and flesh, treated potato tubers were washed and peeled as described in the experimental part. For each tuber, the peel and flesh were extracted and analysed as separate samples. 1,4-DMN residues in the peel ranged from 0.55 to 1.12 $\mu\text{g/g}$ potato fresh weight, with a mean of 0.89 $\mu\text{g/g}$ and a %RSD of 17.67 %. However, no 1,4-DMN residues were detected in any of the 15 flesh samples. The analytical method used is capable of quantifying levels of 1,4-DMN residues as low as 0.03 $\mu\text{g/g}$ (potato fresh weight basis) with acceptable accuracy and precision as described in Section 6.3.3.3. Therefore, 1,4-DMN residue levels in the flesh must be below this value. This indicates that 1,4-DMN residues are concentrated in the peel layer of the tuber which is good from the consumers' point of view. The removal of these layers should contribute significantly to reducing the risk of intake of 1,4-DMN residues on the consumers' health. Many authors reported similar results for the accumulation of the residues of different other pesticides on the outer surfaces of different produce (Lentza-Rizos and Balokas, 2001; Mondy et al., 1992; Boulaid et al., 2005; Orejuela and Silva, 2004; Fernandez-Cruz et al., 2004; Randhawa et al., 2007; Fernandez-Cruz et al., 2006; Dalziel and Duncan, 1980; Kraish, 1990; Jamieson, 1988). In some publications, some amounts of pesticide residues were reported to be present in the layers beneath the peel layer (Fernandez-Cruz et al., 2004; Fernandez-Cruz et al., 2006; Jamieson, 1988). The presence of these pesticides in the inner layers was attributed in some cases to the penetration and movement of the pesticide from the skin to the inner layers (Fernandez-Cruz et al., 2006). However, another likely reason for the detection of some pesticide residues in the layers beneath the peel layer (particularly when detected at low percentages) might be attributed to the non-uniform peeling of the produce sample or cross-contaminating the inner layers during the analysis (Fernandez-Cruz et al., 2004; Jamieson, 1988). From the analytical point of view, the accumulation and concentration of 1,4-DMN in the peel layer can help in using the peel safely for the analysis instead of the whole tuber which should help in concentrating the final extract by at least 10-16 % (peel percentage of the whole tuber). In addition, the use of the peel only for the analysis without the flesh may help in reducing the levels of some of the co-extractives which are accumulated in the flesh, and therefore, provide cleaner extracts.

To examine the horizontal distribution of 1,4-DMN residues in the peel surface of treated tubers, 7 treated potato tubers were divided into four quarters for each tuber and each quarter was analysed as a separate sample by peeling it and analysing the peel. Table 8.3 shows the residue levels of 1,4-DMN detected in the different quarters of the 7 tubers analysed.

Table 8.3 Residue levels of 1,4-DMN in the quarters of the 7 Desiree tubers

Tuber No.	Quarter No.	1,4-DMN Residues (µg/g fresh weight)	Mean 1,4-DMN per Tuber (µg/g fresh weight)	RSD (%)	Overall Mean (µg/g fresh weight)	Between-Tubers RSD (%)
1	1	0.87	0.72	21.55	0.81	12.97
	2	0.63				
	3	0.55				
	4	0.84				
2	1	0.80	0.86	5.30		
	2	0.85				
	3	0.88				
	4	0.90				
3	1	0.84	0.80	9.75		
	2	0.73				
	3	0.74				
	4	0.89				
4	1	0.70	0.80	10.91		
	2	0.87				
	3	0.87				
	4	0.75				
5	1	0.90	0.87	10.59		
	2	0.83				
	3	0.78				
	4	0.99				
6	1	0.86	0.96	16.27		
	2	1.16				
	3	1.00				
	4	0.82				
7	1	0.58	0.64	11.97		
	2	0.57				
	3	0.70				
	4	0.72				

As shown in Table 8.3, there is some variability in 1,4-DMN residue levels across the surface of a potato tuber. The variability ranged from 5.03 % to 21.55 % expressed by %RSD of the 1,4-DMN residue levels in the four quarters. This variability level shows that 1,4-DMN has relatively even distribution across the different parts of the tuber surface. Based on this, acceptable representation might be achieved for 1,4-DMN residues in the tuber by using parts (as discs by a corer) of the peel for the analysis instead of peeling the whole tuber and taking a subsample after chopping and mixing the peel. Such a method of sampling should speed up the analysis. However, to use this technique, several parts (discs) from different locations in the tuber should be analysed to minimise the variability. More investigations should be conducted to test the validity of such a sampling method compared to the use of a subsampling the whole chopped and mixed peel, or other

sampling techniques. A method based on the above sampling technique has been used for the analysis of 1,4-DMN residues in potato tubers (Knowles et al., 2005) but no validation data of the method was reported.

The level of within-tuber variability of 1,4-DMN residues is much lower than the level reported for the sprout-inhibiting chemical chlorpropham. The amount of chlorpropham was found to differ largely between the upper and lower halves (defined by the position of the tuber in the box after the application) of the tuber (Baloch, 1999). Upper portions were found to have more chlorpropham with a mean range of 21.60 to 236.56 mg/kg compared to a mean range of 8.58 to 32.07 mg/kg in the lower portions. The highest values of chlorpropham in the upper portions occurred in potatoes in the top of the top box in the store. This high level was a result of the fall-out of chlorpropham particulates on the top surface of the tubers, compared to the genuine distribution of the fog throughout the potato pile.

8.3.2 The removal of 1,4-DMN residues from treated potato tubers by various washing solutions

The objective of these experiments was to evaluate the effect of washing potato tubers treated with 1,4-DMN on the residue levels in the tubers. Eight washing treatments were evaluated. For each treatment, 15 treated tubers were used in order to minimise the effect of the between-tubers variability in 1,4-DMN residues. The washed tubers were compared to the unwashed set of tubers reported in Section 8.3.1.1 in addition to another set of unwashed tubers analysed at a later stage of the experiments to check for any changes with time in the level of 1,4-DMN residues. The levels of 1,4-DMN residues for the different washed and unwashed sets of samples are shown in Table 8.4.

Table 8.4 Effect of different washing treatments on 1,4-DMN residue levels

Treatment	Mean* 1,4-DMN Residues (µg/g fresh weight)	RSD (%)
Unwashed Tubers set # 1	0.91	18.54
Cold water running for 20 seconds	0.89	17.67
Cold water running for 1 minute	0.93	19.87
Cold water running for 1 minute with brush scrubbing	0.82	17.34
Hot (~50 °C) water running for 1 minute	0.85	19.36
5 % Ethanol/water solution running for 1 minute	0.81	16.56
5 % Acetic acid/water solution running for 1 minute	0.84	21.17
Unwashed tubers set # 2	0.91	16.01
Veggi-Wash vegetable and fruit detergent	0.83	18.83
Water soaking treated as a blank for the Veggi-Wash treatment	0.86	19.98

* Mean of 15 samples

The results in Table 8.4 show that the residue level of 1,4-DMN in the unwashed tubers remained unaffected throughout the experiment period, as indicated by the agreement between the mean value of the first unwashed set at the start of the experiment and the second set analysed at the later stage of the experiment.

The data in Table 8.4 were analysed statistically using ANOVA with Tukey's HSD test ($p=0.05$) in Minitab[®] software. The statistical analysis showed that 1,4-DMN residues in all the tuber sets (washed and unwashed) were not significantly ($p=0.438$) different. This shows that all the washing treatments did not remove significant amounts of 1,4-DMN residues from the potato peel. This could be because 1,4-DMN is strongly held in the peel particularly after the long period of storage of more than 18 weeks. Jamieson (1988) found that the washable residues of the fungicide thiabendazole in potatoes were reduced from 84 % (compared to unwashed samples) when washing after 4 months of storage to about 76 % when washing after 6 months after the last application. These findings seem to support the effect of storage time after application on the ease of removing pesticide residues from produce by washing. In the private project sponsored by DormFresh Ltd., Glasgow (detailed in Section 1.5), washing and air drying potato tubers was found to reduce the residues by a value up to 55 %. However, this reduction is more likely to be caused by air-

drying the tubers after washing rather than the washing process, particularly with the long air-drying times of up to 30 days. More investigations can be carried out to examine the effect of washing at different time periods after application with different washing solutions on the residues of 1,4-DMN in treated potatoes.

In the literature, the effect of washing produce (with water and/or other washing solutions and treatments) on the removal of pesticide residues varies considerably, as can be noticed in Section 1.5. Some researchers reported a negligible effect of washing on certain pesticide residues (Cabras et al., 1998; Boulaid et al., 2005; Baloch, 1999) while others reported reduction percentages of up to 100 % of the pesticide residues (Radwan et al., 2005).

The effectiveness of a washing solution in removing the residues of a pesticide from a produce sample is affected by a complex of different factors. Some of these factors that can be concluded from the literature review in Section 1.5 are: the nature of the pesticide and its physicochemical properties, the nature of the produce surface (amount of dust, soil or waxes), the nature of the washing solution (water only, acidic, basic or neutral, presence of organic solvents such as ethanol), the nature of washing process (simple soaking, soaking with shaking, running solution, scrubbing, airing, wiping with paper) and washing duration, and the finally the storage time after application.

Despite the weak effect of the above washing solutions in removing 1,4-DMN residues, the fact that 1,4-DMN residues are concentrated in the peel should provide a safer way, from the consumers' point of view, of removing 1,4-DMN residues simply by peeling the tubers before cooking. However, the residues of 1,4-DMN present in the peel waste will cause another problem, as discussed in the next section.

8.3.3 The removal of 1,4-DMN residues from potato peel by oven-drying

Many pesticides (including 1,4-DMN) have their residues concentrated in the outer layers of fruit and vegetables and removing these layers should contribute considerably to reducing the direct risk of intake of pesticide residues by consumers. However, the removed peel (particularly from produce processing factories) which contains pesticide residues is commonly used to feed cattle (Tsumurahasegawa et al., 1992; Dalziel, 1978). Tonnes of potato peel waste are generated by the potato processing industry every year. In 2006 alone, a total of 1,821,000 tonnes of potatoes were used for processing (1,105,000

tonnes for frozen or chilled, 702,000 tonnes for crisped and 15,000 tonnes for canned, dehydrated or other processing) in the UK (BPC, 2007). The peel waste out of this huge amount can be estimated at about 455,250 tonnes based on the fact that peeling losses can reach a value of 25 % to 30 % to produce a satisfactory product (Hung et al., 2006). The disposal of these wastes would be a major problem without cattle feeding operations. The presence of the 1,4-DMN residues in the peel can pose a risk to both the animals fed these wastes and to humans consuming the animals and their products. This in turn can restrict the use of the peel of treated tubers with 1,4-DMN for cattle feeding, unless the residues are reduced to acceptable levels. Therefore, the removal of 1,4-DMN residues from the peel of treated potatoes would be of great importance for safety, environmental and economic considerations.

Before processing for animal feeding, potato peel containing 1,4-DMN residues can be treated with different treatments to remove or at least reduce the residues of 1,4-DMN. Some of these treatments may include air drying, oven drying or composting. However, due to time and resource limitations, oven drying was the only one selected to be investigated in this work.

Several experiments were conducted with the objective of evaluating the effect of oven-drying on the removal of 1,4-DMN residues from potato peel. For this series of experiments, a large amount of potato peel containing 1,4-DMN residues was prepared by peeling several kilograms of potato tubers treated with 1,4-DMN. The peel was then chopped and divided into portions for the different experiments.

The experiments started on a small scale with heating 10-g samples in an oven at 75 ± 5 °C. Larger samples of 120 g were then used and the effect of flushing air through the heated peel samples was evaluated. A comparison between 120-g samples and 50-g samples for studying the effect of sample size on the removal of 1,4-DMN residues when heating with air flushing was also conducted.

8.3.3.1 The effect of oven-drying of 10-g samples of potato peel on 1,4-DMN residue level in potato peel

In this experiment, 10-g samples were heated in an oven at 75 ± 5 °C for 1, 3, 6, 18 or 67 hours. For each period of time, 10 samples were dried in the oven and 5 were analysed directly as controls. After drying, samples were weighed again to calculate the moisture content and then analysed for 1,4-DMN residues.

1,4-DMN residues in the peel samples after each heating period is shown in the graph in Figure 8.1. Each point in the graph is a mean of 10 replicate samples (except at zero time where 30 replicates used).

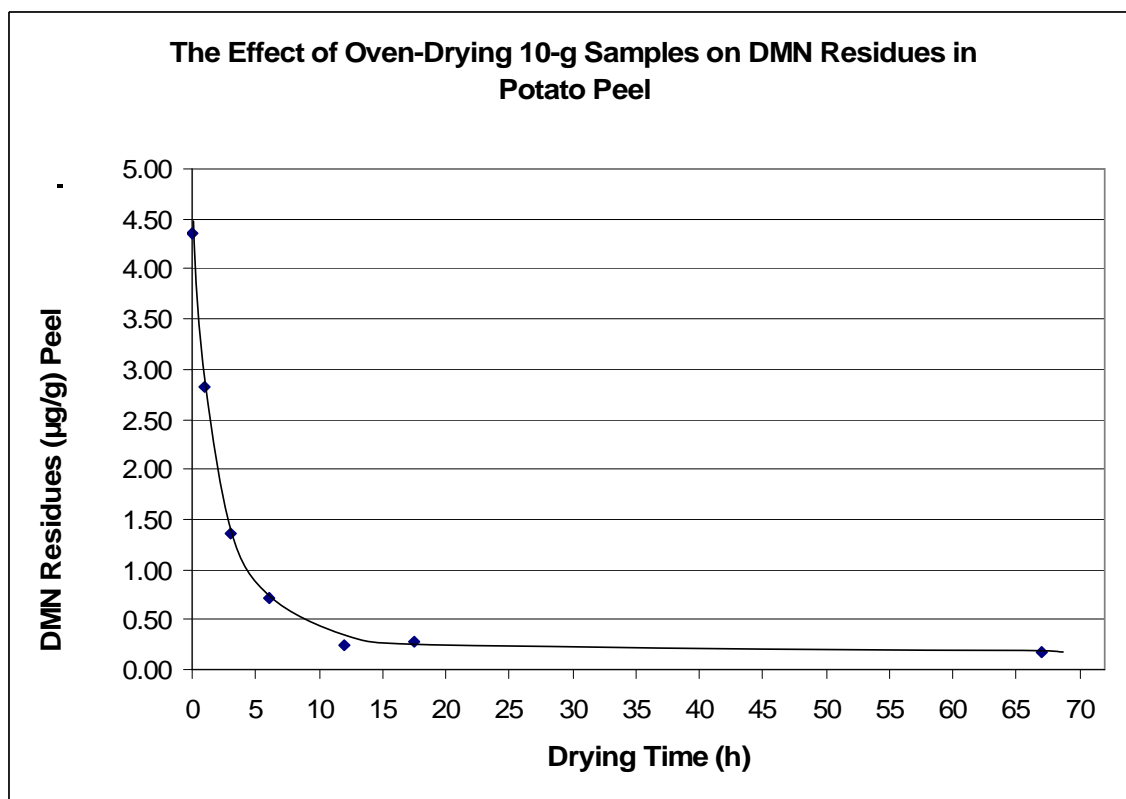


Figure 8.1 The effect of oven-drying 10-g samples on 1,4-DMN residues in potato peel

Control peel samples without drying have an average ($n=30$) 1,4-DMN residues of 4.35 $\mu\text{g/g}$ of peel weight basis. Heating the 10-g peel samples at $75 \pm 5^\circ\text{C}$ caused a quick reduction of 1,4-DMN residues with a percentage of 83 % of the total residues in the first 6 h. The rate of losing 1,4-DMN then slowed down and only 13 % more residues were lost during the rest of the drying period, to end up with a total loss of about 96 % of the extractable 1,4-DMN after 67 h of oven-drying and a residue level of 0.17 $\mu\text{g/g}$ of peel. The main mechanism of losing 1,4-DMN during the process of oven-drying is thought to be through volatilisation. It should be noted that about 4 % of the extractable residues of 1,4-DMN remained in the peel even after 67 h of oven-drying. This part of 1,4-DMN residues must be strongly held in the peel and not easily lost through volatilisation. In addition, there might be some bound residues of 1,4-DMN which are not extractable by the extraction method used in this experiment as has been found for chlorpropham and maleic hydrazide in stored potatoes (Coxon and Filmer, 1985; Dias and Duncan, 1999b).

The graph in Figure 8.2 shows the moisture content of the 10-g samples during oven-drying at $75 \pm 5^\circ\text{C}$ for different periods of time.

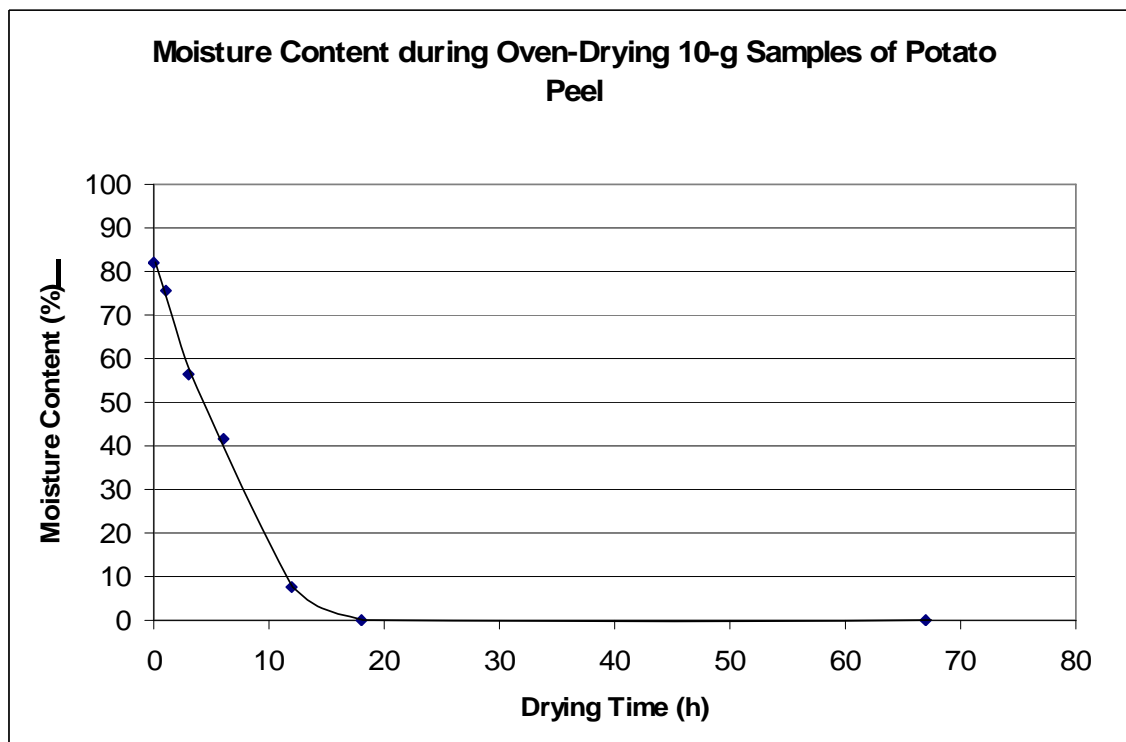


Figure 8.2 Moisture content during oven-drying 10-g samples of potato peel treated with 1,4-DMN

The moisture content in the potato peel after heating for time t (0, 1, 3, 6, 18 or 67 hours) was calculated as follows:

$$\text{Moisture Content (\%)} = 100 \times [(DW_t / FW_t) - (DW_{67} / FW_{67})]$$

Where:

DW_t = Weight of the dry peel after t h of drying

FW_t = Weight of the fresh peel initially used for the t h drying period

DW_{67} = Weight of the dry peel after 67 h of drying

FW_{67} = Weight of the fresh peel initially used for the 67 h drying period

Peel samples started to lose moisture slowly to reach a value near 0 % after 18 h, which was not changed even after 67 h of oven-drying. This value was considered as the total moisture content of the fresh peel.

The rate of losing water from potato peel was found to be slightly lower than the rate of losing 1,4-DMN. Only about 49 % of the total moisture in the fresh peel was lost after the first 6 h of oven-drying compared to 83 % of 1,4-DMN lost in the same drying period. This difference might be attributed to the higher volatility of 1,4-DMN compared to water.

8.3.3.2 The effect of oven-drying with air flushing on 1,4-DMN residue level in potato peel

This experiment aimed to test the effect of passing an air current through the peel which was oven-dried on the speed of losing 1,4-DMN residues. It was believed that flushing the peel with air during oven-drying should increase the rate of losing 1,4-DMN by carrying the volatilised 1,4-DMN away from the peel surface. In this experiment, 120-g samples of potato peel were oven-dried at 75 ± 5 °C in a Buchner funnel with continuous air flushing at a rate of 2-3 l/min. The experiment was repeated with different periods of time from 1-67 hours. The rate of losing 1,4-DMN residues when using this technique is shown in the graph in Figure 8.3.

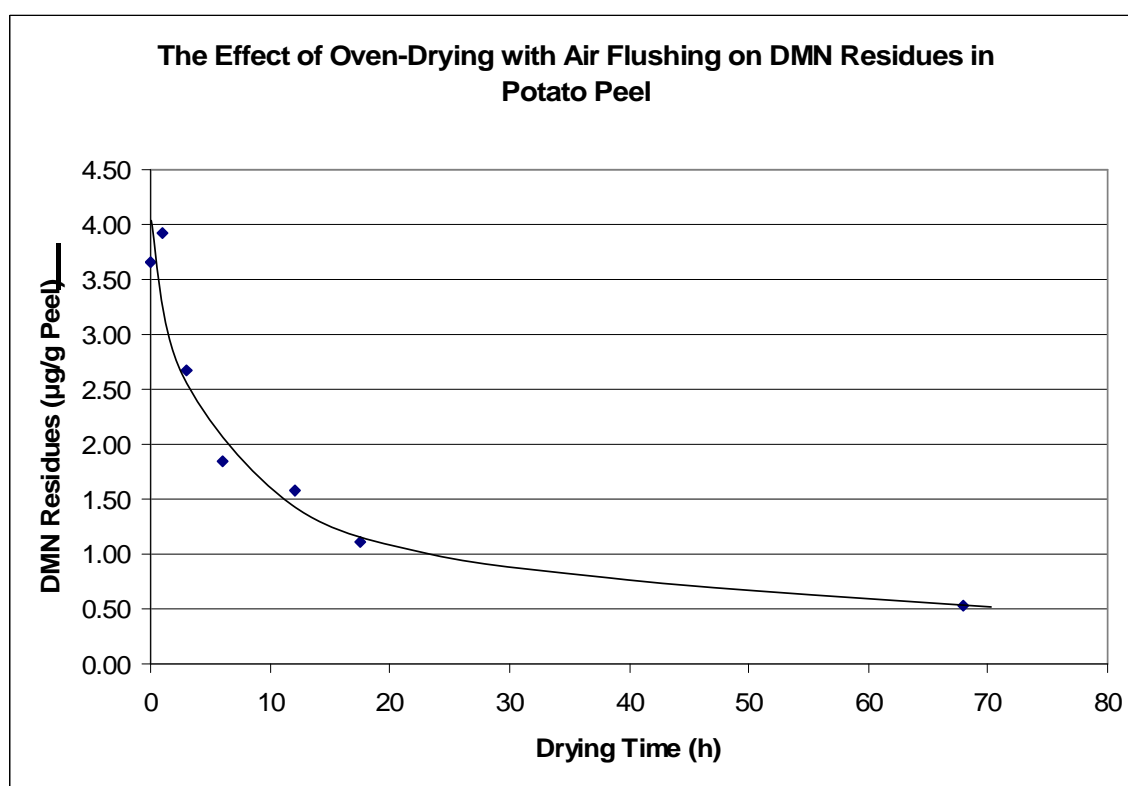


Figure 8.3 The effect of oven-drying 120-g samples of potato peel with air flushing on 1,4-DMN residues.

Heating the 120-g peel samples at 75 ± 5 °C with air flushing caused 1,4-DMN residues to drop from a level of 3.66 µg/g in the fresh peel to 0.54 µg/g at the end of the experiment. However, the reduction rate of 1,4-DMN residues was relatively slower compared to oven-drying the 10-g samples in the previous experiment. About 50 % of the extractable 1,4-DMN residues (out of 3.66 µg/g in the fresh peel) were lost in the first 6 h compared to 83 % lost when heating the 10-g samples. The rate of losing 1,4-DMN residues was then reduced after the first 6 h to reach a maximum total loss of 85 % of the original residues in the fresh peel at the end of the experiment after 67 h of oven-drying with air flushing.

The difference in the rate of losing 1,4-DMN between oven-drying 10-g samples without air flushing and oven-drying 120-g samples in the Buchner funnel with air flushing can be attributed to the difference in the methodologies used to carry out the two experiments. Although air flushing was thought to help in speeding up the rate of losing 1,4-DMN from potato peel when it is oven-dried, the large peel sample used in the second experiment (120 g) with the absence of peel mixing during the heating process is thought to be responsible for the slower rate of losing 1,4-DMN residues compared to the small 10-g samples. These two factors (the large sample size and the absence of mixing) are thought to have caused uneven distribution of the heat through the 120-g peel sample. The peel pieces directly in contact with the funnel walls received more heat (they were noticed to be much drier) than the rest of the sample (particularly the middle parts of the sample). Therefore, the middle parts of the peel sample should have lost only minor 1,4-DMN residues compared to the ones directly contacting the walls of the funnel, particularly in the first hours of heating. In addition, there is a possibility of readsorbing the volatilised 1,4-DMN from the lower layers of the peel sample by the upper layers. With good mixing of the peel sample before the residue analysis, the differences between the 10 replicate samples taken for the analysis were minimised to only 10 % (%RSD). However, for each period of time, the average reduction in the levels of 1,4-DMN residues of the ten samples was lower than the average of the 10-g samples heated directly in conical flasks due to the uneven distribution of the heat as discussed above.

These results show the importance of mixing peel samples during heating to assure even removal of 1,4-DMN residues from the peel sample, particularly when conducting such a process on a large scale in industry.

The moisture content of the 120-g samples during oven-drying at 75 ± 5 °C with air flushing is shown in the graph in Figure 8.4 for the different periods of time.

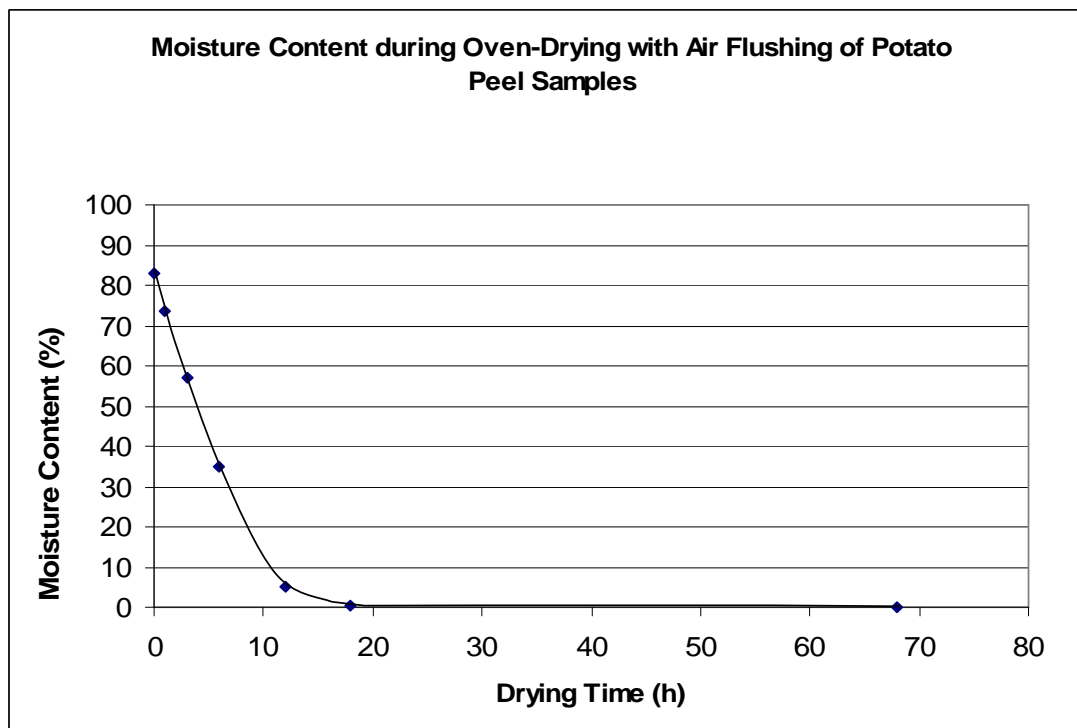


Figure 8.4 Moisture content during oven-drying 120-g samples of potato peel with air-flushing

In contrast to the levels of 1,4-DMN between the two experiments, the two moisture graphs show relatively similar rates of moisture loss throughout the different drying time periods. The uneven heating of the 120-g sample caused the parts of the peel sample contacting the funnel walls to be much drier (more moisture loss) than the rest of the sample. However, because the whole 120-g sample was used in evaluating the moisture content, the rate of the moisture loss was comparable to the rate when heating 10-g samples.

To examine the effect of the amount of peel in the Buchner funnel on the speed of removing 1,4-DMN residues, 50-g samples were compared to 120-g samples in the same experiment. Two funnels (one for the 50-g sample and the other for the 120-g sample) were placed in the oven and connected to the air supply at a rate of 2-3 l/min. The two samples were oven-dried at 75 ± 5 °C for 12 hours. After drying and cooling, the peel was mixed well and replicate 10-g samples were analysed. Five samples were analysed out of each funnel in addition to 5 samples which were directly analysed without drying as controls for each experiment. This experiment was repeated four times. The comparison between 1,4-DMN loss in the two sample sizes is shown in the graph in Figure 8.5.

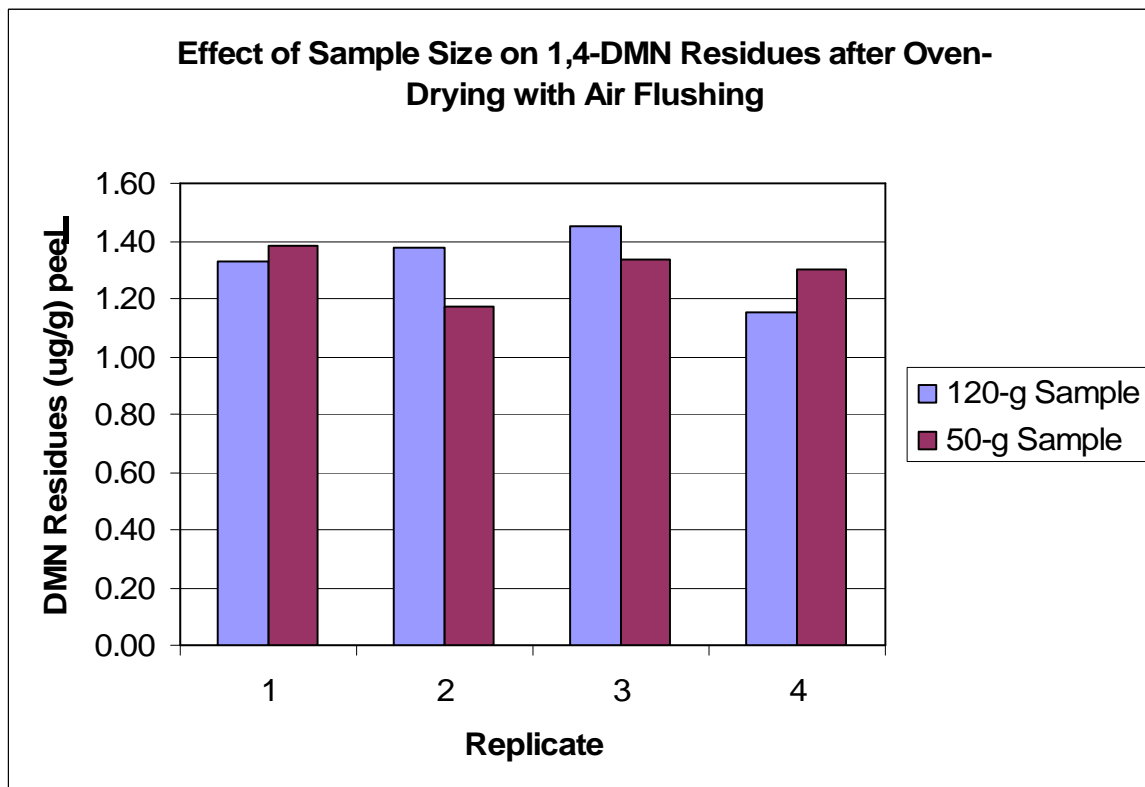


Figure 8.5 Effect of sample size on 1,4-DMN residues after oven-drying with air flushing

Statistical analysis (using pooled t-test at 95 % confidence level) of the levels of 1,4-DMN in Figure 8.5 shows that 1,4-DMN residue levels are not significantly different ($p=0.736$). The difference in the weight and in the distribution of the peel sample in the funnel between the 120-g samples and 50-g samples was not enough to cause a significant difference in 1,4-DMN residue levels to what has been shown when using 10-g samples. A better evaluation of the effect of the sample size can be achieved by heating replicate 10-g samples in small funnels in a comparison with 120-g replicates. However, due to time and resource limitations it was not possible to conduct this experiment.

To summarise the effect of oven-drying potato peel on the level of 1,4-DMN residues, the following points can be concluded. In general, 1,4-DMN residues can easily be reduced to low levels through oven-drying the peel. About 85 % to 96 % of the extractable residues were found to be removed through oven-drying at 75 ± 5 °C. The loss rate of 1,4-DMN was found to be relatively parallel to the loss of moisture. The majority of the extractable 1,4-DMN residues in the peel was removed by volatilisation. However, a small amount (only 4 % to 15 %) of the extractable 1,4-DMN was not volatilised even after 68 h of oven-drying at 75 ± 5 °C. This amount must be attached firmly to the peel; however, the use of higher temperatures might help in removing more residues. During oven-drying, it is important to have good mixing of the peel sample to ensure even distribution of the heat, and therefore even reduction of 1,4-DMN residues from the peel parts. Flushing air

through the heated peel should help in evenly distributing the heat and therefore increasing the loss rate. However, due to the experimental methodology used to evaluate this effect, it was not possible to support this idea.

The above experiments on the loss of 1,4-DMN during oven-drying potato peel samples give a good indication of the ease of losing 1,4-DMN from peel residues of treated potato tubers. This is an important advantage for reducing the risk of intake of 1,4-DMN residues when potatoes are eaten with the peel (e.g. baked potatoes) or when peel waste is fed to animals.

In the potato industry, steam peeling and abrasive peeling are the two main peeling procedures used in potato processing plants. Steam peeling is usually preferred by frozen and chilled potato processors, while abrasive peeling is commonly used by crisp manufacturers. Steam peeling uses steam with temperatures as high as 150 °C with pressure of up to 15 atmosphere followed by a rapid release of pressure which leads to loosening the attachment of the peel to the inner flesh layers (Crawshaw, 2004; Kotecha and Kadam, 1998). These severe conditions should cause a large reduction in 1,4-DMN residues. However, the effect of steam peeling on the residues of 1,4-DMN should be studied. Such a study may start with laboratory experiments using a pressure cooker, instead of the oven used in the current study, and evaluating the effect of different steaming times on the residues of 1,4-DMN in the peel or in whole tubers treated with 1,4-DMN. The experiment may then be extended to a larger industrial scale. In addition, the level of 1,4-DMN in the condensate generated usually in steam peelers should be studied as it may pose an environmental concern and may need to be treated before being disposed of in the environment. Some studies could be conducted for this purpose.

In contrast to steam peelers, thin layers of the peel are scraped physically from the inner flesh without using any heat in abrasive peelers (Crawshaw, 2004). The peel removed by these peelers may need more treatment to remove the residues of 1,4-DMN (either in the potato processing plants or in the feed industry) if intended to be used for animal feed. As shown in the oven-drying experiment, some form of drying process should be enough to remove the majority of 1,4-DMN residues. A dehydration unit, such as the drum dryers used normally in drying potatoes for the livestock industry, can be used to dry the peel in the potato processing plants and at the same time for removing 1,4-DMN residues. Such dryers are operated at much higher temperatures that can reach 500 °C (Lisinska and Leszczynski, 1989) which can increase the rate of losing 1,4-DMN. However, more studies are needed for evaluating the effect of such industrial processes on the residues of 1,4-DMN.

Reducing the water content of animal feed (such as potato peel wastes) would be of major benefit to the feed industry and may also be desirable for farmers in respect of storage cost. However, moist feed producers (such as potato processors) may not have the same enthusiasm for dewatering due to the cost required for the dewatering process and for treating the effluents produced by the dewatering before disposal to the environment. However, for distance potato processing sites such as ones in the North of Scotland, drying may represent the most economical choice as the cost of the drying process and effluent treatment can be overcome by the reduction in the storage and transport cost (Crawshaw, 2004).

Chapter 9: General discussion

The potato is one of the world's major crops. The total production of the potato crop in the world was 314.37 million tonnes in 2006. With about 80 % of the 2006 world's production, Asia and Europe are considered the world's major potato producers (FAO, 2007). The home supply of potato crop in the UK in 2006 was in total about 5.95 million tonnes with 0.42 million tonnes from the previous season and the rest from the current crop (BPC, 2007).

Because the potato is a seasonal crop, some of the produced crop has to be stored in order to maintain a continuous supply of potatoes. Sprouting is one of the major problems that can affect the quality of stored potatoes and cause a major loss. To preserve the quality of stored potatoes, sprouting has to be effectively controlled. The primary method for controlling sprouting of stored potatoes is by the application of the sprout-inhibiting chemical chlorpropham (Anderson et al., 2006; Kleinkopf et al., 2003). In the UK, 95 % of the total ware potatoes treated in stores (2.1 million tonnes) in 2004 were treated with chlorpropham using a total of 72.88 tonnes (Anderson et al., 2006).

Despite the intensive use of chlorpropham for potato storage, many countries have started to reassess its use due to increasing safety and environmental concerns regarding this synthetic chemical, and its availability may be restricted in the future (Lewis et al., 1997). Limits of the allowable levels of chlorpropham residues (MRL) in potatoes entering the market place have been set in many countries. A maximum residue limit of 10 mg/kg was approved in the EU countries and is in place since 2007 (PRC, 2007a). In the USA the tolerance value is 30 mg/kg (Kleinkopf et al., 2003). The maximum residue limits were set to minimise the risk of this chemical on the consumers' safety. However, some incidents were registered where chlorpropham residues in potatoes prepared for human consumption were considerably higher (47 mg/kg in 2006 and 20 mg/kg in 2003) than the EU MRL value set recently (PRC, 2007a; PRC, 2004). The sample containing 47 mg/kg was found to be treated in a potato store using fogging equipment (PRC, 2007b) which is the most popular method for chlorpropham application in commercial stores (Anderson et al., 2006; Baloch, 1999). In this method, chlorpropham is dissolved in a solvent such as methanol prior to the fogging process. The high variability of chlorpropham between individual tubers in potato storage facilities after application with thermal aerosol (fogging) is well documented and it was correlated to the application method of this solid chemical (Baloch, 1999; Kleinkopf et al., 1997). Due to all of the above issues regarding the residue levels

and variability of chlorpropham, in addition to the increasing public concern regarding chemical food additives, the use of safer alternatives for sprout controlling is essential.

1,4-DMN was extracted from potato peel as a natural product and was shown to be an effective sprout inhibitor with inhibition activity comparable with that provided by chlorpropham (Beveridge et al., 1981b; Meigh et al., 1973). It has been introduced into the market as a sprout inhibitor in the United States and some other countries in the 1990s and its introduction to the European countries is at an advanced stage.

Because it is in a liquid phase, 1,4-DMN can be applied without dilution with a solvent, which removes the concern regarding the safety of using the solvent. In addition, the volatility of 1,4-DMN helps to provide a more even distribution of this sprout inhibitor through the potato store. This should help in assuring effective control with less variability, and therefore, lower risk of acute intake. Furthermore, the effect 1,4-DMN as a sprout inhibitor is reversible, which makes it suitable for seed potatoes (Lewis et al., 1997; Beveridge et al., 1981b).

The use of 1,4-DMN on a commercial scale as a sprout inhibitor requires some investigation regarding the safety of the compound and its environmental fate. When applied to stored potatoes, the level of 1,4-DMN in potatoes needs to be monitored to assure effective sprout suppression and avoid applying unnecessarily excessive quantities of the compound. After application, some 1,4-DMN residues remain in the stored potatoes despite the volatile nature of the compound that causes these residues to be easily minimised. To assure the consumers' safety and avoid the risk of intake of high levels of 1,4-DMN, the residue levels in stored potatoes need to be monitored carefully prior to introducing the potatoes to the outlets. In addition to the possible risk to humans, the use of the chemical on a commercial scale should be accompanied with the assessment of possible risks to the environment. The wastes produced after processing potato tubers treated with 1,4-DMN (particularly on a large scale in potato processing plants) such as peel wastes, wastewater and sediments can pose an environmental risk. In order to achieve the monitoring objectives, reliable, fast, accurate and precise analytical methods for the analysis of 1,4-DMN in potatoes and the other different environmental samples are required.

The published data about the analytical methods for 1,4-DMN is very limited as most of the analyses are carried out under confidential conditions (for commercial and authority

use) and very little is accessible. Therefore, it was necessary to develop the required methods for the analysis of 1,4-DMN in different environmental samples in this work.

Gas chromatography (GC) has been widely used for pesticide determination in fruit and vegetables. However, the use of GC (particularly with FID) in analysing plant tissue extracts requires, in most cases, a clean up step and a concentration step prior to the injection of the extract to the GC. These steps are normally laborious and time consuming. In contrast, only minor clean up is, generally, required prior to the analysis by HPLC. In addition, the use of ultra violet (UV) absorption or fluorescence (in addition to coupling with mass spectroscopy) in HPLC detectors provides extremely sensitive and selective detection for a wide range of compounds. Furthermore, the ability to inject larger volumes of extracts in HPLC (up to 500 μ l or more) than can be injected in GC provides a valuable and easy means of enhancing detection sensitivity. Also, the presence of water in the samples required to be analysed (fresh potatoes, sediments, fresh soils and wastewater) is favourable for RP-HPLC analysis, in contrast to GC analysis that requires in most cases the removal of water residues from the extracts prior to the analysis. Hence, HPLC was selected here, as it should provide a powerful and more convenient technique for detecting, separating and quantifying 1,4-DMN in potatoes and the other environmental samples.

In this work, the separation behaviour of some dimethylnaphthalene isomers (including 1,4-DMN) and some other related naphthalene compounds towards the change in the different chromatographic conditions was investigated in a relatively detailed manner. Such an investigation can form a base for the development of the required HPLC methods. Two approaches were used when developing HPLC separation methods in this work. The first approach was the traditional practical step-by-step method. This approach includes many directed step-by-step experiments to understand the effect of the different chromatographic conditions on a particular separation problem. Method development using this approach is usually time-, effort- and money-consuming, in addition to consuming of more chemicals and solvents that pose an environmental issue. The second approach used in this work for developing HPLC separation methods is a more systematic way using computer chromatographic-simulation software. With only a few practical experiments required to generate the input data of the simulation software, numerous simulated experiments were conducted. The accuracy and precision of the output results of such simulated experiments were evaluated in this work and found to be impressive. Several HPLC separation methods for 1,4-DMN and some related compounds were developed in this work using the first approach, the second approach or a combination of the two.

Using the developed HPLC separation methods, several current extraction methods for 1,4-DMN were compared (with some modifications) for their extraction efficiency of 1,4-DMN from potato samples and also their applicability for routine HPLC analysis. A final HPLC method for the analysis of 1,4-DMN residues in potato samples was then achieved and validated. The method is based on extracting 1,4-DMN from potato peel with a mixture of ethanol and 2,2,4-trimethylpentane (7:3) by heating at 50 °C. A liquid-liquid extraction is then achieved with the aid of the water derived from the fresh peel to end up with 1,4-DMN concentrated in the 2,2,4-trimethylpentane layer. An aliquot of this layer is then analysed directly by HPLC. This method was found to have adequate speed, detection sensitivity, precision and accuracy for routine analysis of 1,4-DMN residues in treated potatoes.

The above method was evaluated for the analysis of trace levels of 1,4-DMN in a plan for the determination of the natural 1,4-DMN in potatoes. The detection sensitivity was thought to be inadequate for such a determination. Therefore, a new method (ACN/PROP method) was developed for this purpose. The method uses the advantage of injecting large volumes of the extracts containing 1,4-DMN directly to the HPLC as a means of increasing the detection sensitivity. This advantage was achieved by using a mixture of acetonitrile : 2-propanol (7:3) as the extraction solution which is more compatible with the mobile phase than 2,2,4-trimethylpentane used in the previous method and miscible with the water derived from potato peel. In addition a higher ratio of sample : solvent was used for additional enhancement in the detection sensitivity. This method was capable of quantifying 1,4-DMN with good recovery and precision at levels as low as 4.5 µg/kg (ppb) of potato fresh weight (lower levels at 1.5 µg/kg of potato fresh weight were detected but not quantified with acceptable accuracy and precision). It was hoped that this level of detection sensitivity would be adequate for the determination of natural 1,4-DMN in potatoes.

The method developed for trace levels of 1,4-DMN was used for the determination of the natural levels of 1,4-DMN in potato peel and flesh in addition to some other plant materials. A small peak was detected in the chromatogram of potato peel extracts at the right retention time for 1,4-DMN, with an area equivalent to a level of about 4 µg/kg of potato fresh weight basis. This level is above the detection limit of the method but below the quantification limit of 4.5 µg/kg of potato fresh weight. It is possible that this peak was for 1,4-DMN. Unfortunately, because of the poor UV spectrum for this peak caused by the small size of the peak and the large background effect, it was not possible to confirm its

identity. There was no sign of the presence of 1,4-DMN in the rest of the plant materials analysed including potato flesh.

Because of uncertainty of the identity of the peak detected in potato peel extract due to its low level, a headspace experiment was conducted as an approach to obtain sufficiently high concentrations of the natural 1,4-DMN for quantitative study. The method was based on a dynamic collection of the volatiles produced in the headspace above potato tubers contained in a sealed flask. The volatiles were adsorbed in Tenax traps and desorbed by elution with acetonitrile for HPLC analysis. However, the level of the volatiles collected was inadequate due to the low recovery efficiency of the collection system caused by some shortcomings in the design of the system.

In the literature, a main advantage reported to distinguish 1,4-DMN from most of the other commercial sprout inhibitors is that 1,4-DMN and some of its isomers have been identified as natural compounds in potatoes. Trace levels of the natural 1,4-DMN and some of its isomers were isolated from potato peel (Meigh et al., 1973), from the headspace above raw whole potatoes (Filmer and Rhodes, 1985) and also from unpeeled cooked potatoes (Coleman et al., 1981; Nursten and Sheen, 1974). Since then, the idea that 1,4-DMN is a natural volatile found in potato tubers has been widely reported in many publications such as the ones referenced here (Boylston et al., 2001; Beveridge et al., 1981b; Lewis et al., 1997; Knowles et al., 2005; Kalt et al., 1999).

Because 1,4-DMN is accepted as a natural potato volatile, it has been considered in the USA as a biopesticide (USEPA, 1997) and has been exempted from the requirement of tolerance when applied post-harvest to potatoes (USEPA, 1995). For the same reason, a petition has been made to allow the use of 1,4-DMN in organic potato storage. However, this petition has not been accepted, mainly because commercial 1,4-DMN is synthetically produced rather than extracted from potatoes (UC-SAREP, 2002).

In a project report for investigating the use of 1,4-DMN as a sprout inhibitor in Australia, the researchers claimed that 1,4-DMN and its isomers are found in potatoes as a result of general environmental contamination and not produced by potatoes (Walker et al., 2004). The researchers used two methods for the determination of 1,4-DMN in potatoes. The first was based on extracting 500 g of potato peel (tubers from commercial source) with 1000 ml of dichloromethane using the Soxhlet extraction technique followed by the concentration of the extract to 5 ml. A solvent concentrate of 500 ml dichloromethane was prepared to examine the level of contamination in the solvent. The levels of 1,4-DMN

(about 1 ppb) found in both potato peel extract and the solvent concentrate were similar. Therefore, the researchers concluded that the solvent extraction method was unsuitable for the determination of the natural 1,4-DMN in potatoes. They then developed a headspace-SPME (solid phase micro extraction) method with a detection limit of 0.01 ppb (exact unit was not specified in the report). Using this method, the 1,4-DMN level in the peel of untreated potatoes was reported in the range 0.143 to 0.244 ppb. Samples of Tasmanian soils were also analysed according to the headspace method. The researchers claim that the relative height and distribution of dimethylnaphthalene group of peaks in potato samples and in the soil samples were consistent with the pattern found in normal PAH profiles and environmental contaminants (but no further details or references reported). Based on the above data and statements, the authors concluded that 1,4-DMN does not appear to be an endogenous product in potatoes and the previously reported levels of 1,4-DMN in potatoes are likely to have resulted from the detection of contaminants.

The above report was not scientifically well-written, particularly as there was a lack of referencing for important statements. In addition, there was not enough data reported to support the above conclusions regarding the natural 1,4-DMN in potatoes. In the first method, the contamination of the blank solvent could not be avoided and therefore the method was discarded. However, there was no mention of analysing a procedural blank in the second method to check for any 1,4-DMN contamination. There was a similarity in the pattern of the dimethylnaphthalene group of peaks in potato peel samples and soil samples analysed. It might be possible that this similarity resulted from a contamination during the analysis and not an environmental contamination of the soil or the potato samples. The procedural contamination for analytical methods can be evaluated through the use of procedural blanks which were not mentioned in the above report. Despite the above notes regarding this report, having general environmental contamination of 1,4-DMN (and other PAHs) in potatoes is not impossible. However, the presence of 1,4-DMN as a contaminant in potatoes does not necessarily imply that 1,4-DMN is not being produced naturally by potatoes.

A detailed study is needed to determine the natural levels of 1,4-DMN in potatoes. In such a study, potatoes should be grown for the purpose of the experiment with care to avoid environmental contamination through soil, water or air. The levels of environmental contamination may be evaluated in soil, water and air for more assurance.

Another issue regarding the use of 1,4-DMN as a natural pesticide on a commercial scale is related to the levels of 1,4-DMN used commercially. Being natural should not justify the

use of 1,4-DMN with any quantity on potatoes. Some factors may help to avoid over-application of 1,4-DMN and limit the levels of 1,4-DMN used on potatoes, such as the economic factors or the delay in sprouting if 1,4-DMN is used for seed potatoes. However, some regulations should be made to control the use of 1,4-DMN as a pesticide.

The use of 1,4-DMN on a commercial scale will be accompanied by the need for many routine analyses of its residues in potatoes or other environmental samples. The ACN/PROP method was optimised for rapid routine analysis of the residues of 1,4-DMN in treated potatoes. The optimised method was found to provide good accuracy and precision. In addition, it is a rapid, easy and straightforward procedure. Because of these advantages, this method was used for different applications regarding the distribution and removal of 1,4-DMN residues in treated potatoes.

To investigate the distribution and removal of 1,4-DMN residues, potato tubers treated and stored under commercial storage conditions were analysed for 1,4-DMN residues. 1,4-DMN residues were in the range of 0.63 to 1.16 $\mu\text{g/g}$ of potato fresh weight (individual tubers) after 18 weeks of storage. The variability between single tubers in a sample was found to be low with a variability factor of about 1.5. The residues were found to be concentrated in the peel layer of the tuber and have relatively even distribution across the different parts of the tuber surface. The low residue levels of 1,4-DMN and its even distribution between tubers, in addition to the accumulation and concentration of the residues in the peel, minimise the risks on the consumers' health particularly in the knowledge that potatoes are normally consumed without the peel.

Washing 1,4-DMN-treated potatoes with water and some other solutions has been found to remove insignificant amounts of 1,4-DMN residues from potatoes stored for 18 weeks in the experiments conducted to mimic the normal household washing. However, in the potato processing industry or commercial storage facilities, large quantities of treated tubers are washed and processed daily, and more severe conditions during washing or processing (such as steam peeling) are used. As a result of these industrial processes, more 1,4-DMN residues may be removed from the 1,4-DMN-treated tubers into the industrial wastewater. In addition to wastewater, sediments containing the washed soils and some potato components normally result from the washing process of potatoes in storage facilities or processing plants. Also, when 1,4-DMN is used commercially, some effluents containing 1,4-DMN can be generated as a result of washing the application tools or the stores. The levels of 1,4-DMN in wastewater and washing effluents, and in the soil and sediments should be carefully monitored and minimised to avoid its impact on the

environment they are disposed into. 1,4-DMN is a highly toxic compound for fish and aquatic invertebrates with median lethal concentrations (LC_{50}) of 0.67 mg/l and 0.56 mg/l for Rainbow trout and *Daphnia magna* respectively (D-I-1-4, 2007). Therefore, some studies are required to evaluate the levels of 1,4-DMN in potato washing effluents and processing wastewater in addition to the solid wastes, and to suggest the appropriate methods for removing it.

An investigation of the levels and behaviour of 1,4-DMN in wastewater and sediments resulted from the washing process should be conducted at different storage times after applying 1,4-DMN to potatoes. If possible, real samples from industrial wastewater and sediments should be used in such studies. However, a laboratory experiment can provide valuable information about the expected levels and behaviour of 1,4-DMN in wastewater and sediments. For laboratory experiments, potato samples can be collected at different periods of times after application and washed with the collection of wash water and sediments. The levels of 1,4-DMN in potatoes, wash water and sediments can then be evaluated.

To remove the residues of 1,4-DMN from wastewater and effluents from storage and processing plants, some industrial techniques are available, but at rather high cost. Oxidation techniques are common methods for treating wastewater in order to reduce the level of contaminants and also reduce the organic content of these effluents. Potato processing wastewater will be rich in organic materials in addition to the presence of pesticide residues. Biological oxidation using bacterial cells is a valuable method for reducing the organic content of potato processing effluents with the production of a biomass with high protein content suitable for animal feed (Rubio and Molina, 1989). A bacterial strain (*Sphingomonas* sp. strain 14DN61) has been found to grow on 1,4-DMN as the sole source of energy and has the ability to degrade it to simpler compounds (Peng et al., 2005). It might be possible to utilise this bacterium for degrading 1,4-DMN residues in wastewater effluents during the biological oxidation processes.

In addition to the conventional biological oxidation, some other advanced oxidation processes are used to treat wastewater, such as photocatalytic oxidation (e.g. using ultraviolet radiation, near UV light or Sunlight in the presence of semi-conductor catalyst) and chemical oxidation (e.g. use of ozone or hydrogen peroxide). In an experimental work, the ozonolysis process has been found to be effective in degrading 1,4-DMN in water and converting it to simpler aldehydes and ketenes with no ring-methylated products (Gaul, 1984).

Although the above techniques may provide effective ways of treating wastewater from large potato processing plants, such methods may not be available for small storage facilities such as those in farms or small processing plants, and therefore, simple removal methods are required. Filter pads containing an adsorbent can be one of the cheap options for such small storages and plants. Different adsorbing materials can be examined for their effectiveness in adsorbing 1,4-DMN from wastewater. Some options are potato peel wastes, peat, bark, soils and other adsorbing materials. However, these filters will provide a means for removing 1,4-DMN from wastewater but further treatment may be required to safely dispose 1,4-DMN from these filters.

The behaviour of 1,4-DMN in soil and the ecological effects on soil organisms is another area that requires some investigation when 1,4-DMN is used as a pesticide. Such studies are required as potato tubers will be surrounded by soil during storage with the application of 1,4-DMN as a post-harvest pesticide. In addition, 1,4-DMN will come in contact with the soil through the wastewater and effluents from processing and storage facilities. Several areas can be studied in this context. For different soil types, soil conditions (such as pH, temperature and moisture content) the following can be investigated: 1) speed of degradation of 1,4-DMN in soil, 2) identity, quantity and toxicity of the different metabolites produced by degradation of 1,4-DMN by soil organisms.

To study 1,4-DMN degradation and metabolism, real soil systems can be used, or bacterial or fungal cultures may be used. The use of mass spectroscopy for the identification of compounds might be required in some stages of these studies. In addition, using radiolabelled compounds in these studies should provide more information regarding the different routes of losing 1,4-DMN such as adsorption, volatilisation or different mechanisms of metabolism.

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