Quality Evaluation of Essential Oil from Nova Scotia Grown Mint

by

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Glossary

antipruritic
: agent to relieve itching of the skin

carbuncle
: a painful infection of the skin, deeper and more painful than a boil

carminative
: relieving flatulence

decoction
: a liquid extract prepared by boiling a substance

fusion
: a blending together so that the component parts are not distinguishable

gustative
: aspect of the sense of taste

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ABSTRACT

The purpose of this project was to evaluate the potential of mint stands to be established in Nova Scotia, Canada and to produce commercially acceptable mint oil. Three species of plants were used in the establishment of the mint stands (*Mentha spicata*, *Mentha piperita* cv Black Mitchum and *Mentha piperita* cv Murray Mitchum). The plants were grown in two locations in Nova Scotia (Bible Hill and Maitland) over two seasons. The plants were harvested in each season at 3 different bloom stages (50%, 75% and 100%). The samples were steam distilled to recover the essential oil and complete quality analysis (specific gravity, refractive index and percent composition) of the oil performed to determine its viability as a commercial product. The oil recovered was unexpectedly low for all 3 plant varieties, ranging from 0.21-0.55% rather than the expected 2-4%. The specific gravity of the *Mentha spicata* at the 3 bloom stages ranged from 0.87-0.90 g/mL which corresponds to the literature value of 0.9 g/mL. The two peppermint cultivars were marginally acceptable with specific gravity values of 0.80-0.82 g/mL. All plants had acceptable refractive indices of 1.46-1.49. With respect to the two major components of mint oil (menthol and menthone) the 2 cultivars of peppermint had acceptable levels with menthol ranging from 31.84-41.49% and menthone ranging from 3.12-14.18%. The menthol:menthone ratios of the two cultivars of peppermint ranged from 2.25:1 to 12.12:1. Also completed was an antioxidant test trial to measure the effect, if any, of a commercial mint oil and its individual components as antioxidants. It was found that there were no significant differences between the mint oil components and the control with respect to antioxidant capacity.

1. Introduction

Plants are natural chemical factories producing a wide variety of substances. For example, plants produce many different metabolites such as vitamins, sugars and amino acids which have very direct, known roles in the plant's survival as well as serving as macronutrients for organisms further up the food chain. These chemicals are known as primary metabolites. Plants also produce other chemicals, such as alkaloids and terpenoids, which in many cases have as yet unknown functions. These are known as secondary metabolites.

The substances known as secondary metabolites are produced in most higher plants and are commonly used as flavours, odours, pigments and biologically active substances (e.g. drugs and nutraceuticals). As time goes on, more and more knowledge is being accumulated on these secondary metabolites; their chemical structures, synthesis, functions and purposes are now being elucidated.

One group of secondary metabolites, terpenes, have a wide variety of industrial applications. They are used mostly as flavourants and scents in deodorizers, cleaners and foods. Peppermint and spearmint are plants which produce many terpenes with industrial applications. Mint flavour may be found in such products as toothpaste, mouthwash and confectionaries. Consistency within the mint flavour of the product (i.e. spearmint, peppermint, etc.) over time is a large factor in its marketability.

The flavour of mint oil is determined by the amount and type of components found in the oil. Menthone and menthol are the two major components in mint flavouring. The

oil quality is measured as the relative ratio of menthone to menthol. Currently the best and most accurate method of determining the percent composition of the oil is by gas chromatography. There are other factors in determining the oil quality. Density and refractive index are two factors which are to be completed within this study.

Two of the main factors which will affect changes in the flavour (which is determined by the component composition of the product) are:

1. The genetic history of the plant (species, cultivars, and cross breeding within the cultivars and species) will play a large part in determining the flavour produced by the plant. An example is the difference between the menthol concentrations found in peppermint and spearmint plants.
2. The environment in which the mint plants are grown will affect the composition of the components in the oil. The geographic location affects the local climate (weather, temperature, water availability, soil conditions, etc.) as well as farming practices (irrigation and fertilization).

Changes in environment and the type of plant, affect the biochemistry of the plant and how the components are formed. Due to agronomic factors, the oil composition does not remain completely consistent from year to year, so the mint oil may be combined with another mint oil (a process referred to as blending) to balance the flavour and achieve consistentcy.

The main objectives of this project are to establish and harvest stands of the mint plants in Nova Scotia, and to extract and qualify the essential oil. These criteria will be used to determine if mint may be grown in this area as a commercially viable crop.

2.0.0. Natural Products

Natural products are formed by plants and animals. There are many different types of natural products, which consist of primary and secondary metabolites. Some higher plants produce numerous compounds in one or several of the classes of secondary metabolites, for example conifer trees have a very wide range of terpenes and alkaloids. Erdtman (1973) explains the basis and theories behind chemotaxonomy. Various species of plants produce chemicals which others do not. The presence or absence of the metabolites may be used to characterize, classify and identify various plant species. This may also be used to trace the evolution of a plant species by providing links with other plant classifications with the same chemical characteristics. Mint plants have been subclassified into species based on the relative terpene concentrations. For example spearmint is a *Mentha* species characterized by low menthol levels when compared to peppermint (Murray et al. 1972).

There are many different types of secondary metabolites (terpenes, alkaloids, flavonoids, phenolic acids and many others) which are differentiated by their structures. These are discussed in more detail below. Terpenes are water insoluble and may be volatile or non-volatile, alkaloids are characterized by the presence of nitrogen in their structure, flavonoids are most readily recognized as plant pigments and phenolic acids are precursors of other secondary metabolites such as coumarins and tannins.

2.0.1. Terpenes

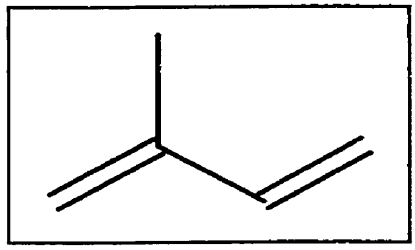

Fig. 2-1: Isoprene Unit

Terpenes are a major group of secondary metabolites. All terpenes are comprised of 5-carbon isoprene units (Fig. 2-1). The number of isoprene units in the structure determines the classification of a terpene. Terpenes are found in a limited range of plants and in many of these plants they have an unknown function. Where the function is known, or suspected, it is generally associated with the regulation of plant growth. Such regulatory functions allow the plant to adapt to biological stresses or changes in the environment, such as disease or drought, by adjusting what type and amount of metabolites are produced in the plant (Srivastava and Luthra 1991). Many types of terpenes are volatile and are major components of essential oils. Essential oils are one type of natural product produced by plants which are characterized by significant volatility at ambient temperatures and upon separation from the plant material, immiscibility in aqueous solutions. Terpenes, with specific references to the genus *Mentha*, is discussed in detail later.

2.0.2. Alkaloids

Alkaloids are one of the largest and most diverse of all groups of natural compounds. Alkaloids are carbon chains and rings which are classified based upon the presence of nitrogen in their structure. They are found in many higher plants ranging from flowering plants to trees. Complex molecular structures arise from simple reactions of starting materials and are derived from amino acids and small biological molecules.

Alkaloids are generally classified based upon the amino acids from which they are derived. For instance, one group of alkaloids is derived from amino acids such as ornithine and lysine, another alkaloid group is derived from tyrosine and phenylalanine and yet another from tryptophan (Herbert 1989).

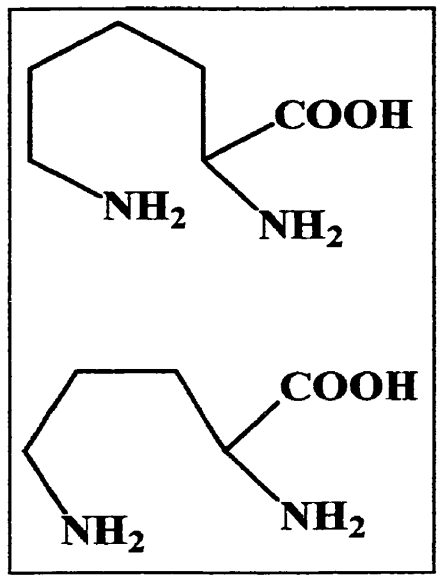

Fig. 2-2: Base Structure of Alicyclic Alkaloids

Alkaloids which are derived from ornithine and lysine are known as alicyclic alkaloids (Fig 2-2). They have simple molecular skeletons of 5-6 carbon atoms and 2 nitrogen atoms. This group may be further divided into 4 smaller groups: piperidine alkaloids, pyrrolizidine alkaloids, quinolizidine alkaloids and pyridine alkaloids on the basis of structure, shape and functional groups (Robinson 1974).

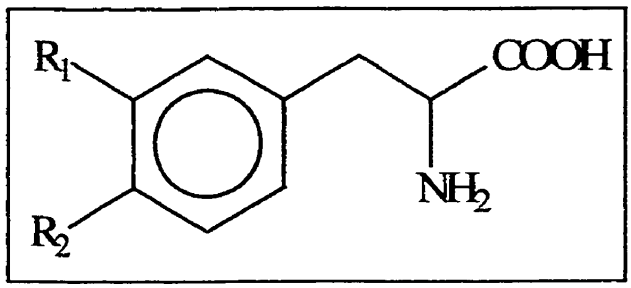

Fig 2-3: Base Structure of Phenylalanine Alkaloids

Alkaloids which are derived from phenylalanine and tyrosine contain aryl sub-units are known as phenylalanine alkaloids (Fig 2-3). This group may be further divided into monocyclic, isoquinoline, benzylisoquinoline and amaryllidaceae alkaloids on the basis of structure, shape and functional groups (Robinson 1974).

Alkaloids which originate from tryptophan contain indole sub-units and are called indole alkaloids (Fig 2-4). Simple alkaloids of this group have undergone minor chemical modification of tryptophan or incorporation of a C_2 unit. The rest of the alkaloids formed from tryptophan are produced from mixed metabolism involving tryptophan (Robinson 1974).

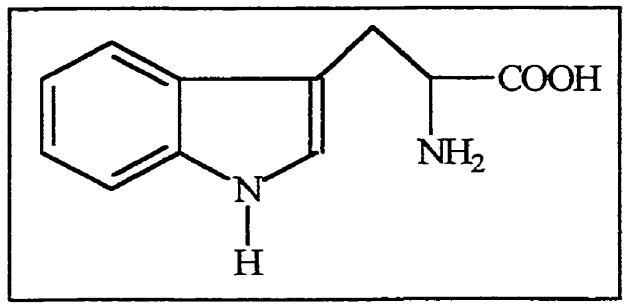

Fig 2-4: Base Structure of Indole Alkaloids

2.0.3. Flavonoids

Flavonoids are found in many higher plants. Flavonoids are most commonly found as flower pigments, however they are found in all parts of the plant (Hahlbrock 1981). They are very water soluble compounds which are generally coloured red, crimson, purple or yellow. Flavonoids protect the plant against damage from UV light, infection by phytopathegenic organisms and attract animals to further fertilization (Hahlbrock 1981). The basic structure of flavonoids are two aromatic rings connected by a three carbon chain (Fig 2-5). Most flavonoids exist as glycosides with different combinations of sugars attached to the hydroxyl group (Hahlbrock 1981). The number of hydroxyl and methoxy groups present, the types, numbers and sites of sugars attached to the molecule as well as the types and numbers of aliphatic or aromatic acid groups attached to those sugars are all used in defining and

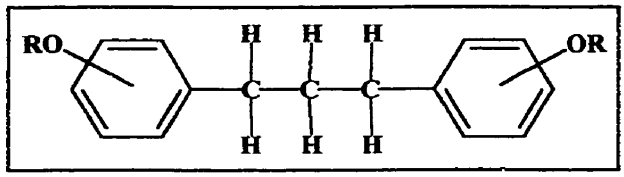

Fig. 2-5: Flavonoid Skeleton

classifying flavonoids. All classes of flavonoids are formed from compounds of intermediate cell metabolism through the action of two pathways, the general phenylpropanoid pathway and the flavonoid glycoside pathway. Phenylpropanoid units are derived from the shikimate pathway (Herbert 1989).

2.0.4. Other Groups of Secondary Metabolites

Phenolic acids are natural products normally formed from such precursors as benzoic acid (Fig. 2-6) and cinnamic acid (Fig. 2-7). Phenolic acids are themselves important, but mostly because they are the precursors of a variety of more complex natural products. For example tannins are natural products consisting of high molecular weight polyphenolic compounds.

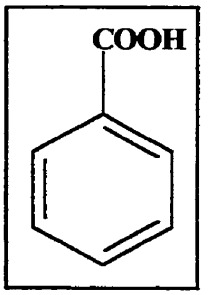

Fig. 2-6: Benzoic Acid

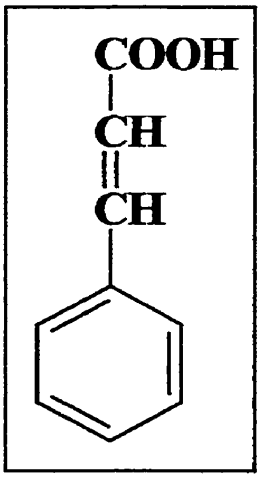

Fig. 2-6: Cinnamic Acid

Tannins have the ability to form cross-linkages with proteins and other biopolymers which play a role in the aging of organisms and also in commerce (tanning industry). Though there is no rigorous definition for tannins, as many of the structures are so varied (Pavia et al. 1982), a functional definition of tannins is that they are water soluble polyphenolic compounds with molecular weights ranging from 500-3000 with the ability to precipitate alkaloids, gelatin and other proteins from aqueous solutions (Pavia et al. 1982). There are two major groups of tannins, condensed tannins (form condensation polymers of catechin) and hydrolyzed tannins (form esters of gulcose and gallic acid when hydrolyzed) (Pavia et al. 1982).

Coumarins are a smaller group of secondary metabolites with a structure based upon cinnamic acid. Not a lot is known about the functions of coumarins in plants, but they are of great interest due to their medicinal and industrial value. Coumarins have been used as anticoagulant drugs and as laser dyes (Robinson 1967).

2.1. Extraction of Natural Products

There are various methods for extracting natural products, the more common techniques employ steam distillation, organic solvent extraction and mechanical expression. The distillation of natural products requires that the natural product be volatile, as is the case with essential oils. For distillation, the plant material is cut into small segments and placed in a container to which hot steam or water is added. As heat is released into the container, the essential oils volatilize and are diverted, along with steam, into an opening at the top of the vessel. The volatile oil and steam mixture is then channelled through cooling units which condense the vapour into a liquid. The liquid mixture is collected in a vat and allowed to separate with water, and the essential oil is separated based on differences in density. Since mint oil is less dense than water it rises to the top and can be skimmed off.

To extract natural products which are not volatile, such as alkaloids, it is common to use an organic solvent to remove the natural product from plant tissues. The solvent may then be removed from the natural product by evaporation. This method is also popular for obtaining some of the more expensive volatile natural products such as rose oil for perfumes. The process is slower, however heat degradation of the natural product does not occur as is the case with distillations (Schay 1975).

Mechanical pression, also known as expression, is popular in essential oil production procedures with such materials as citrus fruits. This involves the juice and oil being mechanically pressed from the plant material by a pressurized device. The liquid containing both the aqueous and essential oil fractions is then collected and centrifuged allowing the essential oil to be separated from the aqueous portion according to Phillips (1993).

2.2.0. Importance of Natural Products in Industry

Natural products are very important in today's way of life. Specifically, essential oils are found in many areas of the world, and are used in everyday North American households. Essential oils are used in cleaners, pharmaceutical products (e.g. cough syrups), personal care products (e.g. mouthwash, toothpaste) and foods (e.g. baked goods, candy, gum, etc.). Several recognized essential oil flavours such as peppermint and spearmint are obtained from the genus *Mentha*.

2.2.1. Natural Products as Flavours

Flavourants, including naturally derived essential oils are very popular and widely used in many food products. Exclusive use of these natural products as flavourant additives enables companies to use packaging and product advertising that can claim the product "contains no artificial flavour" (Minister of Supply and Services Canada 1986). Spices have long been prized for their sensory characteristics which is attributed to the content of essential oils and oleoresins. In 1492 the economic potential of the spice trade led to Christopher Columbus' attempt to sail around the world in search of a trade route to the Far East where a variety of spices were abundant.

2.2.2. Medicinal Uses of Natural Products

For centuries natural products have been used as medicines. For example, vitamin C, naturally occurring in citrus fruits, may be useful in the treatment of influenza and colds (Pauling 1970) as well as act in the prevention and treatment of scurvy. Folk medicines include remedies which are based on the pharmacological properties of natural products; steeped willow bark for headaches and burdock infusions and red clover fusions for treating skin disorders caused by impurities in the blood (Lacey 1989) are just a few of numerous examples. The active ingredient in steeped willow bark is naturally occurring salicylic acid, a precursor to aspirin. This decoction has also been prepared from poplar trees and wintergreen and may be used to treat and relieve pain, reduce fevers and inflammation and overcome mild insomnia (Dominick 1992).

Essential oils, as a category of natural products, also have medical benefits. For example, the essential oil from coriander has been used as a carminative, stomach and digestive stimulant (Taylor 1990). Eucalyptus essential oils have been used as a bactericide against problems of the respiratory tract like bronchitis, influenza, coughs and asthma as well as afflictions of the urinary tract and colibaccilosis (Taylor 1990). Mint, especially preparations of the fresh plant, have been used to treat and prevent intestinal problems and headaches (Reynolds et al. 1989, Hutchens 1973). Both peppermint and spearmint oil have been used as carminatives (Phillips et al. 1992, Reynolds et al. 1989). The major constituent of mint oil, menthol, has been credited with relief of many ailments. Menthol may be used as an antipruritic and as a local anesthetic as well as to relieve symptoms of bronchitis,

sinusitis and nasal congestion (Phillips et al. 1992, Osol and Pratt 1973). An external application of menthol has been shown to relieve headaches (Osol and Pratt 1973). Menthol has been used to counter symptoms of nausea and gastrodynia (Osol and Pratt 1973). Preparations of menthol have been used to control inflammations such as boils and carbuncles (Osol and Pratt 1973). Menthol is used as a decongestant (British Pharmacopoeia Commission 1990); a 2% solution of menthol is used in inhalers to treat respiratory infections (Osol and Pratt 1973). One of the less predominant constituents of mint, cineole, has been used to treat bronchitis (Phillips et al. 1992).

2.2.3. Natural Products as Antimicrobial Agents in Food

Essential oils from some plants possess biological activity against prokaryotic and eucaryotic organisms (Sivropoulou et al. 1995). In folk medicines these essential oils have been used as antiseptics to clean wounds and help keep them infection free (Müller-Riebau et al. 1995). In addition the aqueous extracts and essential oils of several aromatic plants have exhibited antifungal properties (Müller-Riebau et al. 1995).

Many food borne illnesses are the result of consumption of foods which have been contaminated with pathogenic bacteria and/or their toxins (Sivropoulou et al. 1995). The goal of many food processors is to produce a microbially safe, shelf-stable, high moisture food product without the loss of the fresh-like quality (Cerrutti et al. 1997). The use of chemically manufactured preservatives is increasingly being questioned by consumers in light of the safety of synthetic food additives and preservatives (Moleyar and Narasimham 1992). Current trends in food preservation include a reduction in the use of synthetic

preservatives and/or an increase in the use of naturally derived antimicrobial agents of animal, vegetable or microbial origin (Cerrutti et al. 1997).

When used in food preparation many spices such as basil, oregano, rosemary and thyme serve a dual purpose, in offering both flavour as well as playing a role in food preservation. Four members of the Lamiaceae family (which includes the genus *Mentha*), winter savory (*Satureja montana L.)*, rosemary (*Rosmarinus officinalis L.*), thyme (*Thymus vulgaris L.*), and calamint (*Calamintha nepeta L. Savi*) all have demonstrated biotoxic activity against various strains of bacteria such as *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae* and *Candida albicans* (Panizzi et al. 1993). Vanillin, a natural product occurring in vanilla beans, is used as an antimicrobial agent against mold and yeast in fruit purées and fruit based agar systems. This has several advantages, as a dilute vanilla flavour (concentrations not exceeding 3000 ppm) is accepted by the consumer and is compatible with many fruit flavours (Cerrutti et al. 1997). *In vitro* testing indicated that vanillin is an effective antimicrobial (inhibiting growth of microorganisms for longer than 60 days) at levels higher than that normally used in foodstuffs. Hence a potential problem in using essential oils as antimicrobials is the need to use the essential oil at a level higher than that normally associated with optimal flavour, thus the true flavour of the foodstuff may be distorted (Cerrutti et al. 1997).

Menthol is an active germicide which is more powerful than phenol of equal concentration (Osol and Pratt 1973). In dilutions of 3.1% it was found that the oil of *Mentha spicata* and *Mentha pulegium* completely inhibited the growth of *Staphylococcus aureus* and *Escherichia coli* (Sivropoulou et al. 1995). However in this high concentration, the mint

flavour becomes overpowering in the foodstuff. As the concentration of the mint oil is decreased, its effectiveness as an antimicrobial agent falls dramatically. With *S. aureus* (a gram positive bacteria), mint oil dilutions of 0.2% showed a small decrease initially in the growth of the bacteria, but lower than in the control. With *E. coli* (a gram negative bacteria) mint oil dilutions of 0.2% showed a small decrease initially, followed by an increase in growth when compared to the control. With both *S. aureus* and *E. coli*, their growth was completely inhibited with treatments of 1.0% dilutions (Sivropoulou et al. 1995).

2.2.4. Natural Products as Antioxidants

Many plants such as basil (*Ocimum basilicum*), oregano (*Originum vulgare*) (Madsen and Bertelsen 1995; Schwarz and Ernst 1996; Tsimidou et al. 1995), rosemary (*Rosmarinus officinalis*) (Frankel et al. 1996; Madsen and Bertelsen 1995; Schwarz and Ernest 1996) and thyme (*Thymus vulgaris*) (Scharz and Ernst 1996) contain natural products which inhibit the oxidation of foodstuffs such as oils, cereals, etc. These herbs and their components serve a dual role in a food system, acting both as a flavour component and as a preservative by inhibiting the formation and decomposition of hydroperoxides during lipid oxidation (Madsen and Bertelsen 1995).

Antioxidant compounds are effective when they are more susceptible to oxidation than the oxidizable food components (e.g. lipids and vitamins), thus resulting in the preferential oxidation of the antioxidants. Antioxidant compounds will thus protect the foodstuff as long as they are present. The oxidation of food components leads to several reactions including a loss of the nutritional value, as in the oxidation of vitamin C in fruit

juices. It can also result in lipid rancidity which adversely affects the gustative properties of the food. One important finding of antioxidant research is that free radical production associated with oxidation has been strongly linked to a number of diseases such as cancer and atherosclerosis (Wang et al. 1996), thus antioxidant agents may be one mechanism to prevent these diseases.

During lipid oxidation, the production of radicals keeps the reaction self-perpetuating until two radicals bond together and the oxidation reaction is terminated (Cuvelier et al. 1994). There are three main steps in lipid oxidation reactions: initiation, propagation and termination. The initiation step is the formation of radicals (Fig. 2-8). These radicals join with other molecules to create more radicals, the propagation step. The reaction ends when two radicals come together to join, thus no radical is formed and the reaction is terminated. The initially formed products also further react to form secondary oxidation products such as aldehydes and ketones.

There are many tests to detect oxidation, including headspace analysis of volatile components formed during oil oxidation (Frankel et al. 1996), UV analysis for specific compounds in the test material (Frankel et al. 1996) and oxygen radical absorbance capacity (ORAC) (Wang et al. 1996). Headspace analysis by gas chromatography can be used to measure changes in the composition of volatile aldehydes and ketones in the food product over time. Increases and decreases of these components can be correlated to the level and stage of rancidity and hence can be used to monitor the development of rancidity (Frankel et al. 1996). Ultra-violet analysis of specific by-products of oxidation (e.g. aldehydes and ketones) within the foodstuff can be used to monitor the level of rancidity in the product by

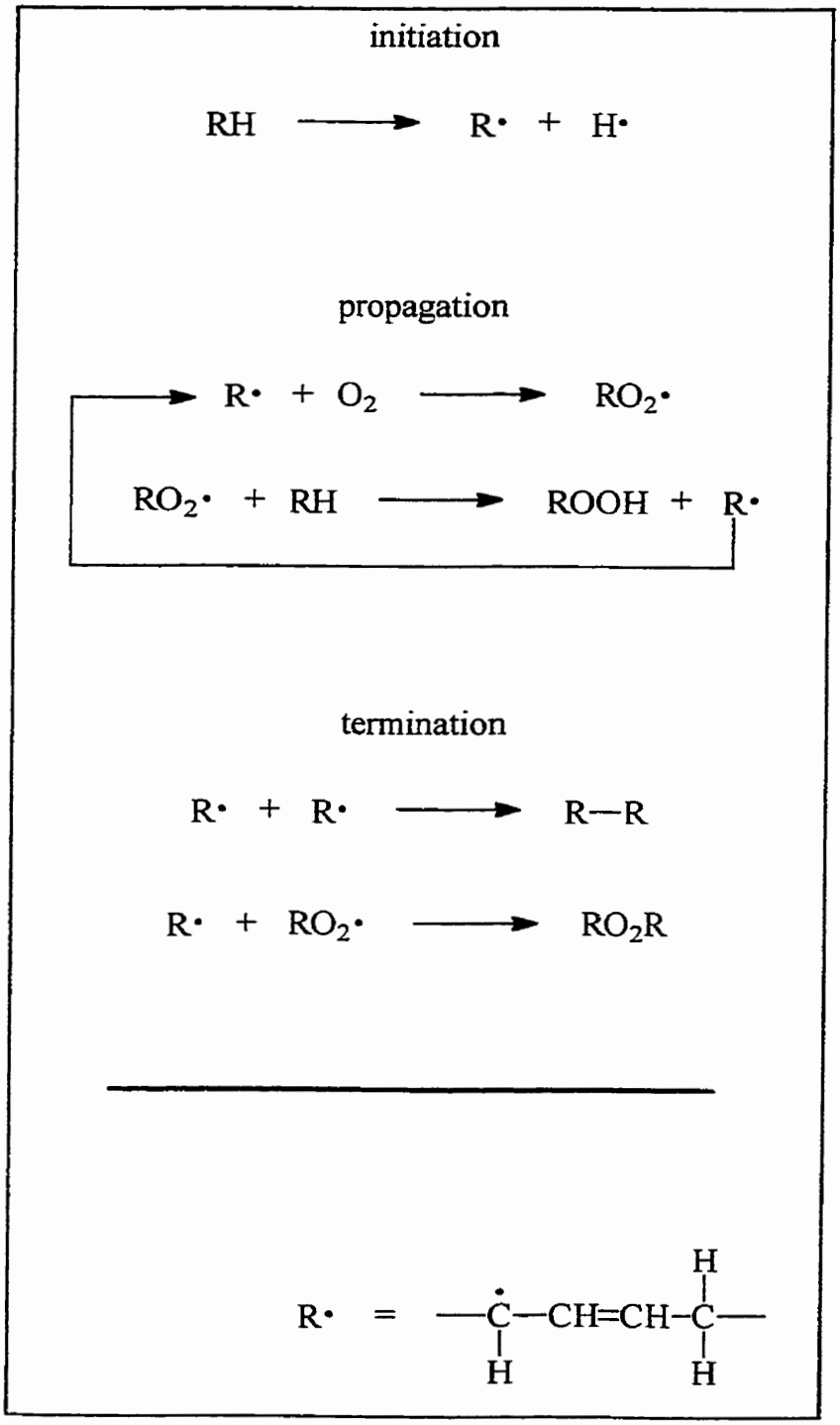

Fig. 2-8: Lipid Oxidation Reactions (deMan 1980)

measuring the relative increases and decreases of the by-products (Frankel et al. 1996). ORAC uses fluorescence detection to measure the radical capacity of the product which is correlated to the stage of rancidity (Wang et al. 1996).

Some tests measure products of the propagation stage (Fig. 2-8) of the oxidation. One such test, the peroxide value test, determines the amount of primary oxidation products (hydroperoxides and peroxides) which has been produced by the oxidation reaction. The peroxide value results are expressed in units of milliequivalents of peroxides per kg of sample. The thiobarbituric acid (TBA) test is used to determine the final termination stage (Fig. 2-8) of lipid oxidation as determined by the amount of aldehydes and ketones present in the sample mixture.

Based upon their structure and functional groups, some components of mint oil may exhibit antioxidant activity. Of the major components of mint oil (referring specifically to the functional group attached to the carbon atom in the number one position of pulegone (Fig. 2-9), menthone, menthol and menthyl acetate), pulegone and menthone are the most oxidized (loss of electrons) and menthyl acetate is the least oxidized (or most reduced). Based upon the reactions carried out within the plant (Fig. 2-10 and 2-11), a change in the oxidation state of the molecules may be observed. Pulegone would be more oxidized than menthone due to the addition of an external double bond at the two position.

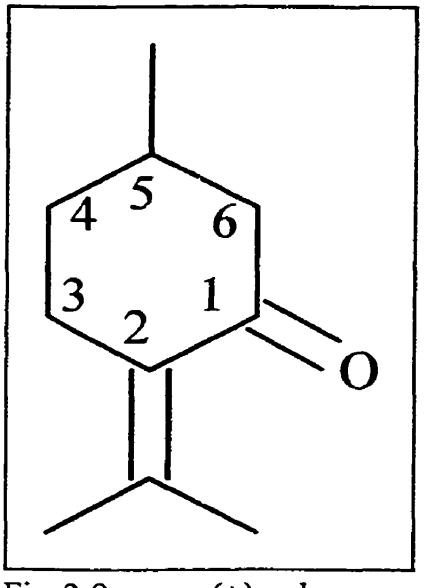

Fig. 2-9: (+)-pulegone

2.3.0. Mint: A Source of Natural Products

Terpenes, a major group of secondary metabolites, consist of covalently linked C_5 isoprene units (Fig. 2-1). Isoprene units are structures containing five carbon atoms and two double bonds; they form the skeleton of all of the terpenes. Terpenes may have one or more isoprene units. It is through a series of reactions that terpene molecules of varying lengths are produced. Terpenes are biosynthetically produced by the addition of consecutive isoprene units containing pyrophosphate groups. The pyrophosphate portion connected to an isoprene unit is replaced by another isoprene unit as terpenes are anabolically built, thus there are 2 linked isoprene units in a monoterpene, with one pyrophosphate tail. This reaction may terminate, depending on the plant and the conditions present, or the molecule may react further to form one of the other classes of terpenes.

The major classes of terpenes are distinguished by the number of isoprene units. These are monoterpenes which have two isoprene units (10 carbon atoms), sesquiterpenes consist of 3 isoprene units (C_{15}), diterpenes consist of 4 isoprene units (C_{20}), sesterpenes consist of 5 isoprene units (C_{25}), triterpenes consist of 6 isoprene units (C_{30}), tetraterpenes consist of 8 isoprene units (C_{40}) and polyterpenes consist of over 40 carbon atoms.

With respect to monoterpenes there are many different molecular structures, each sharing a common base structure, but differing chemically due to various functional groups which are covalently bonded to the molecule. The structural differences are primarily due

to cellular oxidation and reduction reactions of monoterpenes which are enzyme catalyzed. While some of the enzymes in these biochemical transformations are known, not all have yet been determined.

The main constituents of the essential oil of mint are monoterpenes consisting primarily of menthol and menthone. There are hundreds of terpenes present, but only 18 are present in concentrations over 1% (Table 2-1) (Lawrence 1993).

Table 2-1: Peppermint Oil Compounds Generally Found in Concentrations over 1%

Compound	Concentration
(-)-menthol	28.0 - 35.6
(-)-menthyl acetate	10.6 - 20.1
(-)-menthone	4.2 - 11.6
menthofuran	4.4 - 8.7
1,8-cineole	2.0 - 6.7
(+)-pulegone	1.6 - 6.7
(-)-limonene	1.8 - 6.3
β-caryophyllene	2.0 - 5.0
germacrene D	2.1 - 3.7
myrcene	1.0 - 3.1
trans-piperitone oxide	0.5 - 3.1
(+)-neomenthol	2.1 - 2.9
α & β-pinene	0.5 - 2.0
(+)-isomenthone	0.9 - 1.9
α-terineol	0.1 - 1.9
piperitone	0.5 - 1.3
viridiflorol	0.5 - 1.3

The pathways for the formation of these compounds are complex and involved (Fig. 2-10 and 2-11). The figure is a compilation of a number of different authors

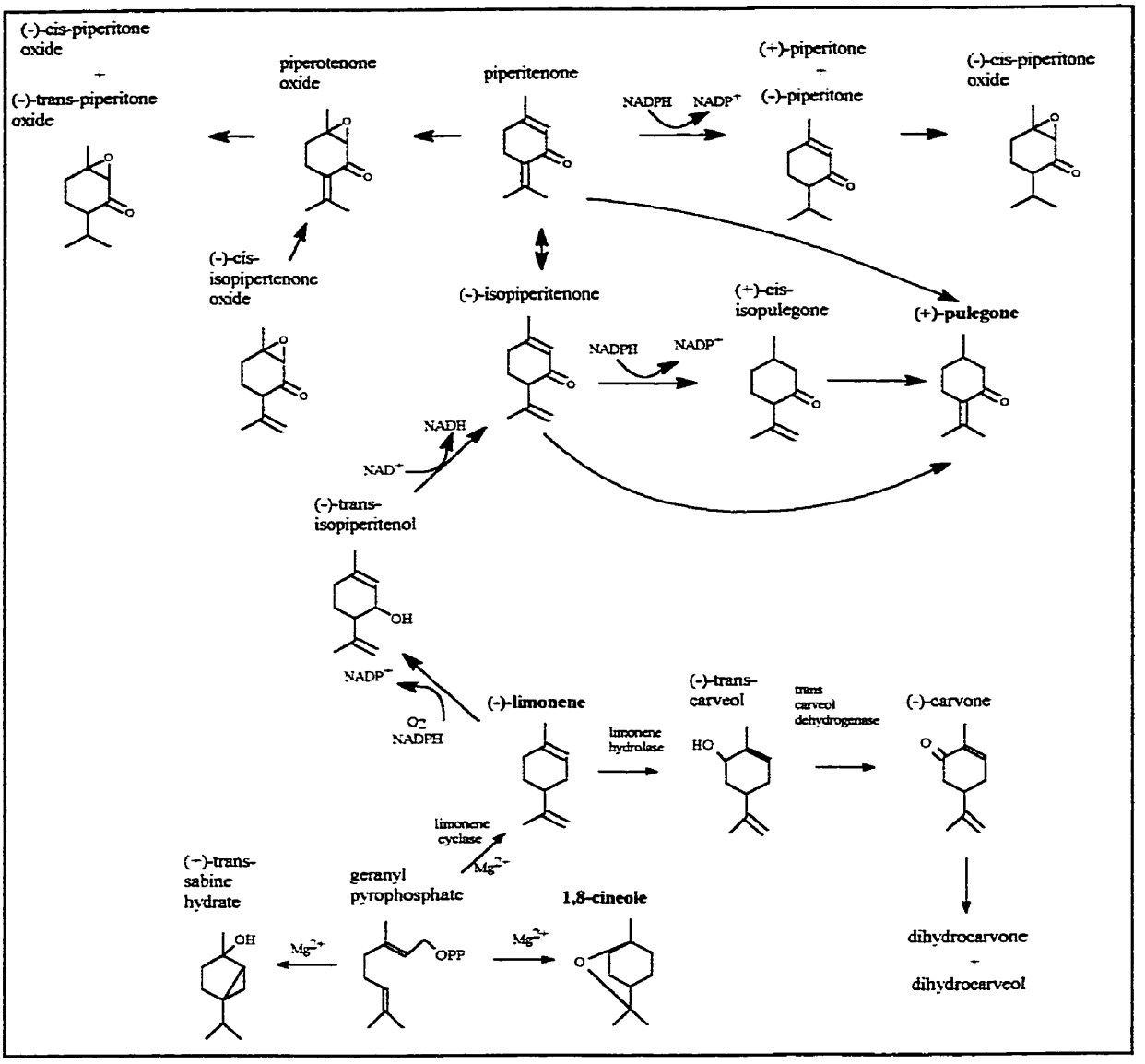

Fig. 2-10: Pathways Leading to the Formation of the Major Constituents in the Essential Oil of Mint - Part I

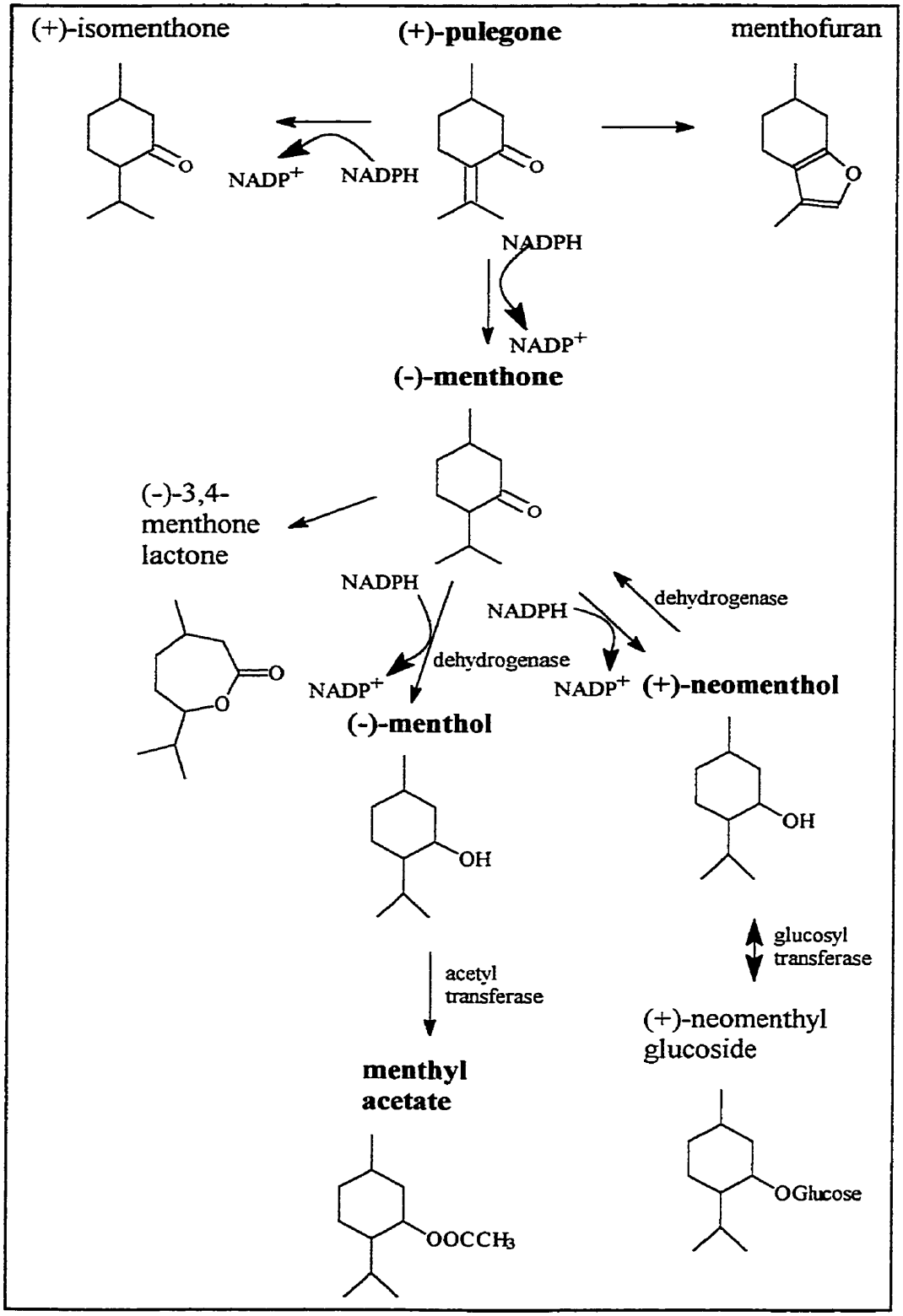

Fig. 2-11: Pathways Leading to the Formation of the Major Constituents in the Essential Oil of Mint - Part II

(Croteau 1988; Croteau et al. 1991; Kjonaas and Croteau 1983; M°Caskill et al. 1992; Murray et al. 1988; Kjonaas et al. 1982; Gershenzon et al. 1989) and this is the first time known to the present author that this extensive of a collection of pathways has been compiled in one figure. There is still much to be learned about the effect of stress and growing conditions on the pathways and which pathway, and hence, secondary metabolite is favoured by specific conditions.

A simplified summary of Figs. 2-10 and 2-11 is as follows: geranyl pyrophosphate is the starting molecule of the monoterpene production in the *Mentha* genus. From geranyl pyrophosphate, 1,8-cineole and limonene are formed. Dependent upon the conditions, limonene may react to form dihydrocarvone and dihydrocarveol or piperitenone. Piperitenone reacts to form piperitenone oxides or pulegone. Pulegone is the precursor of the desirable, and undesirable, monoterpenes found in mint oil. Pulegone may react to form menthofuran or menthone. Menthone then reacts to form menthone lactone or menthol. Menthol may react to form menthyl acetate. The extent of each of these reactions are dependent upon the environmental conditions which will be discussed in detail below.

2.4.0. Mint Production

The majority of the herbs grown in Nova Scotia are by hobbyists who grow herbs in their home gardens which provides them with access to fresh herbs for culinary and medicinal preparations. Popular dishes of mid-Eastern origin such as Tabboulah use fresh mint. Mint tea is a popular herbal tea and its flavouring is largely due to the essential oil content of the mint. Some preserves, such as mint jelly, use the essential oil as a flavouring

and a preservative. Herbs and fragrant flowers are also used in gardens as insect repellents (Feeny 1992). The plants may be positioned throughout a garden of fruits and vegetables to act as an insect repellent. A rare member of the mint family, *Diceranda frutescens*, has been observed to repel ants (Segelken 1991). Applications of the essential oil from this plant to insects resulted in severe irritation to the insects. Upon further analysis a component of mint oil, trans-pulegol, was found to act as a natural insect repellant (Segelken 1991).

Mint oil produced in the USA has been valued at over US$125 million/year for 1987-1996 (United States Department of Agriculture 1997). The essential oil industry also generates considerable financial benefits throughout the entire economy by the incorporation of the oils into numerous products (Craker 1990). The gross production of the mint oil industry in the USA in 1996 was over $150 million (Table 2-2) (United States Department of Agriculture 1997). As a result of the small mint production market in Canada, there is not any information available (Waddell 1995).

2.4.1. Harvesting and Extraction of Mint Oil

During harvesting, the top of the plant is taken and cut into segments with forage harvesters at a specific bloom stage (75-90% of full bloom). The mint oil from the crop is either extracted immediately or the crop is allowed to wilt for a period of 12-36 hours. The most common and economical method of extracting the mint oil is by steam distillation (Phillips 1993). The plant material is gathered in a container such as a large forage wagon and taken to the steam generator location. Hoses are used to convey the hot steam to the

Table 2-2: Mint Oil: Production and Value in the USA, 1987-1996.

species	year	production / 10^6 lbs	price/pound / $	production value / US10^6
peppermint	1987	4.4	11.6	51.7
	1988	5.4	15.9	85.3
	1989	6.7	13.1	87
	1990	7	13.9	96.6
	1991	6.6	13.3	87.4
	1992	7.4	12.8	94.7
	1993	6	13.3	80.1
	1994	7.5	14.6	109.3
	1995	9.4	13.8	130
	1996	9.4	13.6	128.5
spearmint	1987	2.1	12.1	24.1
	1988	1.7	12.8	22.4
	1989	1.8	13.9	25.7
	1990	2.6	14.9	38.2
	1991	3.1	13.9	43.1
	1992	3.6	12.8	46.6
	1993	2.7	12.3	33.5
	1994	2.2	12.5	27.6
	1995	2.3	12.3	27.9
	1996	2.2	12.2	26.2

bottom of the wagon. As the steam is released into the wagon, the essential oils volatilize and are diverted, along with the steam into an opening at the top of the wagon. The volatile oil and steam mixture is then channelled through a series of condensers which condense the vapour into a liquid. The liquid is collected and allowed to separate into the heavier water and the lighter oil fractions. The mint oil is removed by draining or skimming the oil off the top as it is immiscible in water and is less dense.

2.5. Commercially Acceptable Mint Oil

In the essential oil industry, the quality of the mint oil is evaluated based upon various characteristics, such as composition of the individual terpene constituents, specific gravity, refractive index, etc. (Fenaroli 1975). The ideal characteristics of mint oil will vary depending upon the specific type of mint flavour desired, for example spearmint versus peppermint. There are certain industry guidelines as to what the percentage compositions of the monoterpenes in the peppermint oil should be (Table 2-3).

Commercially acceptable mint oil has a specific gravity of 0.9 (20°C), a refractive index of 1.46 (20°C), an optical rotation between -10° and -30° and having a solubility of 1:3.5 to 1:5 in a 70% solution of ethanol (Fenaroli 1975).

The flavour of spearmint or peppermint in a food product or formulation should be consistent to ensure consumer repeat purchase. However mint oil composition varies significantly from season to season due to changes in the weather, the age of the plants, infections, etc. To achieve consistency, flavour house companies require an oil with very specific chemical and flavour characteristics to produce the desired flavour. In order to

Table 2-3: Percentage Composition of Peppermint Oil / % ($^{w}/_{v}$)

Chemical Constituent	IUPAC name	1	2	3	4
β-caryophyllene	4,11,11-trimethyl-8-methylenebicyclo [7.2.0]undec-4-ene	—	1.21	—	—
1, 8-cineole	1,3,3-trimethyl-2-oxabicycle[2.2.2]-octane	—	—	5.9	4.18
limonene	1-methyl-4-(1-methylethenyl) cyclohexene	—	2.41	2.4	1.39
menthyl acetate	5-methyl-2-(1-methylethyl) cyclohexanol acetate	4.5-10	4.72	5.9	3.88
menthol	(1α,2β,5α)-5-methyl-2-(1-methylethyl) cyclohexanol	>44	34.54	44.6	46.50
menthone	(-)-5-methyl-2-(1-methylethyl) cyclohexanone	15-32	24.02	17.2	24.83
neomenthol	(1β,2β,5α)-5-methyl-2-(1-methylethyl) cyclohexanol	—	2.97	3.6	—
α-pinene	2,6,6-trimethylbicyclo [3.1.1]hept-2-ene	—	1.37	0.9	—
pulegone	5-methyl-2-(1-methylethylidene) cyclohexanone	—	2.26	1.2	—

[1] Reynolds et al. 1989
[2] Lawrence 1984
[3] Murray et al. 1988
[4] Clark and Menary 1980

overcome seasonal variation, companies blend oils of different specific characteristics to produce a mixture which will result in the desired characteristics of the mint flavour which they wish to use.

2.6.0. Conditions Affecting Mint Production

The amount and quality of oil produced by a mint crop is affected by several factors. Two of the major factors affecting the quality and yield are the genetic ancestry of the species and the agronomic environment. It has been demonstrated that the biosynthesis and metabolism of monoterpenes in plants can be influenced by environmental conditions such as day length, precipitation, soil type and over-wintering conditions (Maffei 1988). The yield and physicochemical characteristics of mint oil are both influenced by climatic conditions, soil conditions, and cultural practices such as cultivation, irrigation, harvesting and processing (Watson and St. John 1955). The quality of mint oil exhibits both regional and seasonal variation, as well as changes in response to shading, fertilization and water availability and maturity at harvest (Burbott and Loomis 1967).

A marked geographic variation has been reported with regards to some secondary metabolites of plants (Khanna and Shukla 1990). Buyers have noted differences in the oil of *Mentha piperita* from Washington and Oregon, the two major mint production areas in the USA (Watson and St. John 1955). The areas of major mint production, or test sites as

noted from the literature have been characterized by their latitudinal positions represented in Table 2-4. Washington, Oregon, Idaho, Wisconson, Indiana, Michigan and Alberta represent the current areas in which mint production has been established in North America. Trials of mint production in Florida exhibited good plant growth, but poor oil quality as the menthol concentration was extremely low (Clark and Menary 1981). Greece, Turkey, Italy (Maffei 1988), Egypt (Clark and Menary 1981), Lincoln, New Zealand, and Tasmania are areas capable of producing a commercially acceptable mint oil. Bolivia and Israel both produced mint oil which was unacceptable for commercial sale in North America due to low menthol concentrations (Munoz-Collazos et al. 1993).

Table 2-4: Latitudinal Coordinates of Mint Producing Areas

Geographic Area	Latitudinal Range	Geographic Area	Latitudinal Range
Washington	46-48°N	Greece - northern and western	40°N
Oregon	42-46°N	Turkey - northern and western	40°N
Idaho	42-48°N	Italy - northwest	45°N
Wisconson	42-47°N	Israel	10-35°N
Indiana	38-42°N	Egypt	22-32°N
Michigan	42-46°N	Lincoln, New Zealand	43.4°S
Florida	25-30°N	Tasmania	41-44°S
Alberta	50°N	Bolivia	17.0°S

In looking at the geographic areas where mint production has been successful, it can be deduced that there is an area of "superior" mint production (Burbott and Loomis 1967), which is, in general, above the 40th parallel North (Tucker 1992) and below the 40th parallel

South. However, when considering Egypt's location and its successful production of mint oil, the strict use of geographical boundaries cannot be the sole criteria on which to judge the feasibility of mint oil production (Clark and Menary 1981).

Temperature will influence the plant's growth, flowering and the resultant oil yield (Burbott and Loomis 1967). As evidenced by peppermint plants (*Mentha piperita*), cooler growing conditions facilitate the conversion of menthone to menthol (Murray et al. 1986). Further, it has been found that warmer nights deplete respiratory substrates which result in oxidizing conditions, while cooler nights preserve high levels of respiratory substrates and maintain reducing conditions (Burbott and Loomis 1967). In *Mentha arvensis* (Japanese Mint) the oil yield and plant growth was found to be highest at 30°C (Duriyaprapan et al. 1986). According to these authors, menthol concentrations exhibited little change with temperature while menthone increased significantly in trials having higher day temperatures.

Typically, secondary metabolites are produced in several organs of a plant during various stages of growth. The terpene producing cells (specifically secretory cells located above the epidermis layer in the leaf) are affected by the growth stage of the plant; this in turn will affect of the terpene composition of the essential oil (Burbott & Loomis 1967). If the oil is produced primarily in one specific part of the plant, then it is important to identify this fact and harvest at the right stage of growth or maturity in order to optimize oil

production. If this optimum growth stage is missed, then the desired product may be metabolized to another less desirable secondary metabolite (Khanna & Shukla 1990), Fig. 2-12.

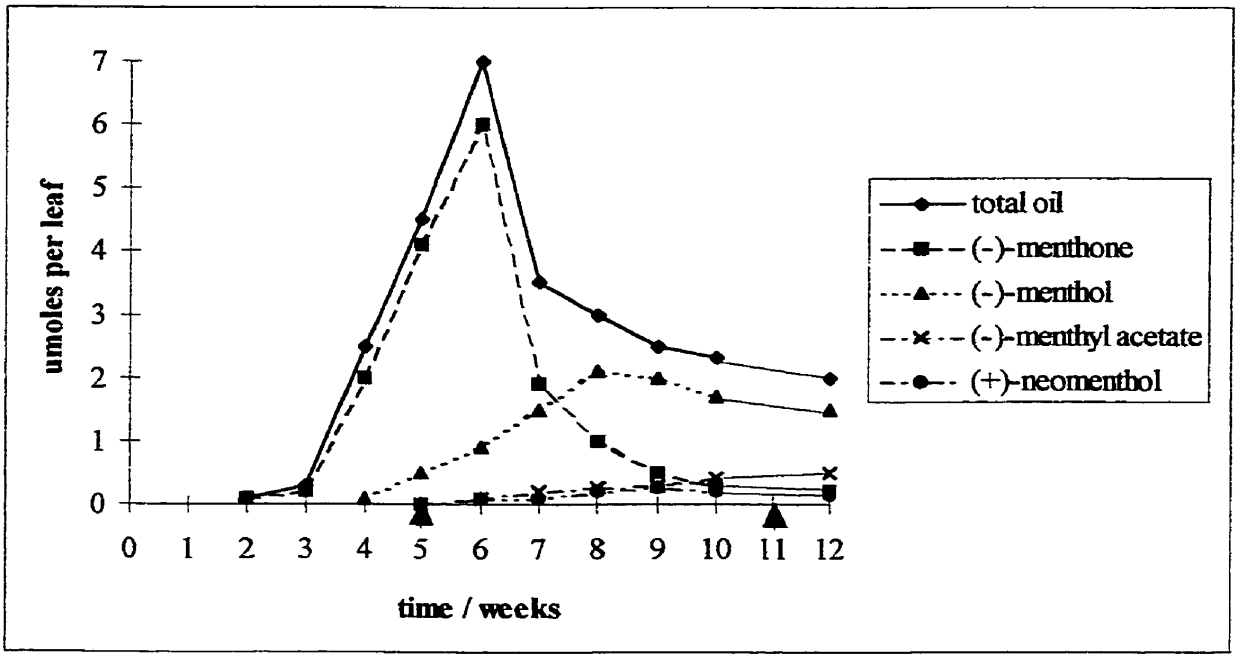

Fig. 2-12: Monoterpene Composition of Midstem Peppermint Leaves as a Function of Development. The first arrow indicates the approximate time of floral initiation and the second arrow indicates the approximate time of full bloom.

The total amount and relative concentrations of terpenes in peppermint plants changes over time (Croteau and Martinkus 1979). These changes may be correlated to the onset of flowering and the development of blooms on the plant (Fig. 2-12) (Croteau 1988). During production of mint oil, the oil initially consists of menthone, which peaks at the time of floral initiation (Croteau and Martinkus 1979). As the flowering process advances, the terpenes are catabolized to neomenthyl glucosides and transferred to other parts of the plant for conversion to primary metabolites (Croteau 1988). This would explain the decrease in the total oil concentration as the plant reaches the full bloom stage. As the concentration

of menthone falls, the concentrations of other major terpenes such as menthol and menthyl acetate increase (Fig. 2-12). For commercial purposes a high oil yield with a ratio of menthol to menthone of 2:1 is desired (Lawrence 1984).

2.7.0. Problems Facing Mint Production - Day Length

Day length affects the photosynthetic potential of the plant by limiting the time during which solar absorption may occur. The longer the day length, the longer the period of time in which photosynthesis may occur, leading to a greater accumulation of primary (and secondary) metabolites which are required for sustained and continual growth of the plant. Day length is affected by the geographic area of the region (i.e. regions at the equator (0° latitude) receive more light on a yearly basis then regions at the the 45th parrallel north and south).

Limited photosynthesis, such as that resulting from excessive shading, stresses the plant in such a way that the ability of the plant to produce primary metabolites (e.g. sugars) is severely restricted. In this scenario, a greater portion of the primary metabolites that are formed are required to maintain growth. As a result there is relatively less raw photosynthetic material available for the secondary pathways and this suppresses the production of carbon-based secondary metabolites (Chapin 1991). While the concentration of the carbon-based secondary metabolites is lowered, the concentration of nitrogen-based secondary metabolites exhibit a relative increase (Jones and Coleman 1991) due to the increase of nitrogen to carbon.

A major factor in commercial mint production is the day length required to produce commercially acceptable mint oil as defined by the total yield of the volatile secondary metabolite production and its constituent composition. It is preferential to both the total essential oil yield (Burbott and Loomis 1967; Clark and Menary 1979) and the biomass of the plant (Burbott and Loomis 1967) that day length for mint plants used in oil production should be in the range of 10-16 hours. A day with more than 10 hours of light favours the production of menthone, menthol and neomenthyl acetate at the expense of menthofuran, pulegone and menthyl acetate (Clark and Menary 1979). These attributes are desirable for commercially acceptable mint oil.

In an experiment similar to Clark and Menary (1979), mint experiencing short, cool nights with full light during the day exhibited increases in menthone content while at the same time the menthofuran and pulegone concentrations decreased (Burbott and Loomis 1967). However, this seems at variance with other findings where a decrease in menthone and menthol concentrations and a rise in menthofuran concentration was observed (Clark and Menary 1979) under similar light conditions. These findings can best be explained by Burbott and Loomis (1967) in terms of changing oxidation-reduction levels within the plant. The oxidation-reduction level of the monoterpenes reflect the redox state of the respiratory coenzymes of the terpene-producing cells. This affects the concentration of the respiratory substrates in the cells. Warm nights tend to cause a depletion of respiratory substrates, which result in oxidizing conditions. Cool nights preserve high levels of respiratory substrates and maintain reducing conditions. Hence, warm nights would be expected to

yield higher levels of menthofuran and pulegone, and relatively lower levels of menthone and menthol, which are oxidized and reduced, respectively.

2.7.1. Problems Facing Mint Production - Water

Water stress, as both an excess and a deficiency in plants, causes changes in essential oil production because water affects the plant in many ways. The changing availability of water will alter the concentration of the secondary metabolites in a plant, either decreasing or increasing them depending on the duration, type and severity of the water stress (Jones and Coleman 1991). Drought stress will decrease ion uptake of a plant, decrease the turgor pressure and disrupt the metabolism of a plant (Levitt 1980). Decreased ion uptake will lead to nutrient deficiencies (Levitt 1980). Decreased turgor pressure will result in inhibited growth and stomatal closures, both of which will lead to starvation of the plant. A disruption of the metabolism of the plant changes the enzyme activity within the plant, increasing activity in some cases resulting in increased respiration and protein breakdown and decreasing activity in others, leading to hormone imbalances within the plant (Levitt 1980).

Specific to mint, under conditions of drought, the concentration of the menthol and menthone in mint were not affected, however the concentration of pulegone fell while the concentration of menthofuran increased (Denys et al. 1990). The total sesquiterpene fraction of the oil also increased (Denys et al. 1990). Sesquiterpenes, being a higher order of terpenes, are formed through the addition of isoprene units to a potential monoterpene

unit, thus if the sesquiterpene concentration increases, then the monoterpene concentration should decrease, having a detrimental effect on the total oil yield.

2.7.2. Problems Facing Mint Production - Nutrients

Nutrients play a major role in the growth and development of a plant. Excesses or deficiencies of nutrients can be detrimental to the plant, or at the very least will affect the biochemical regulators governing production of both primary and secondary metabolites in the plant.

Nitrogen is a primary nutrient required for plants. Deficiencies in nitrogen will cause the nitrogen-based secondary metabolite concentrations to fall, while resulting in a relative increase in the carbon-based secondary metabolite production (Jones and Coleman 1991). Nitrogen is not directly involved in the molecular structure of terpenes, but it is essential to the growth and health of the plant. Nevertheless, researchers have shown that the use of high levels of nitrogen fertilizer on mint caused an increase in the total oil yield (Praszna and Bernáth 1993; Piccaglia et al. 1993; Baird 1957; Singh et al. 1989), the biomass (Piccaglia et al. 1993; Baird 1957) and the percentage of oil recovered (Praszna and Bernáth 1993). Also, under high levels of nitrogen, the concentration of menthol was decreased and the menthofuran concentration increased (Baird 1957). This was due to conditions which caused pulegone to be converted into menthofuran rather than directed towards the synthesis of menthone and menthol (Fig. 2-13). Clark and Menary (1980) also found that the nitrogen applied affects mint oil production; as the level of nitrogen applied was increased (50, 100, 200 and 300 kg/ha), the oil yield increased. This was offset by the fact that the

concentration of menthone increased and the concentration of menthol decreased which lowered the commercial value of the mint oil (Clark and Menary 1980). The concentration of the menthofuran did not change significantly.

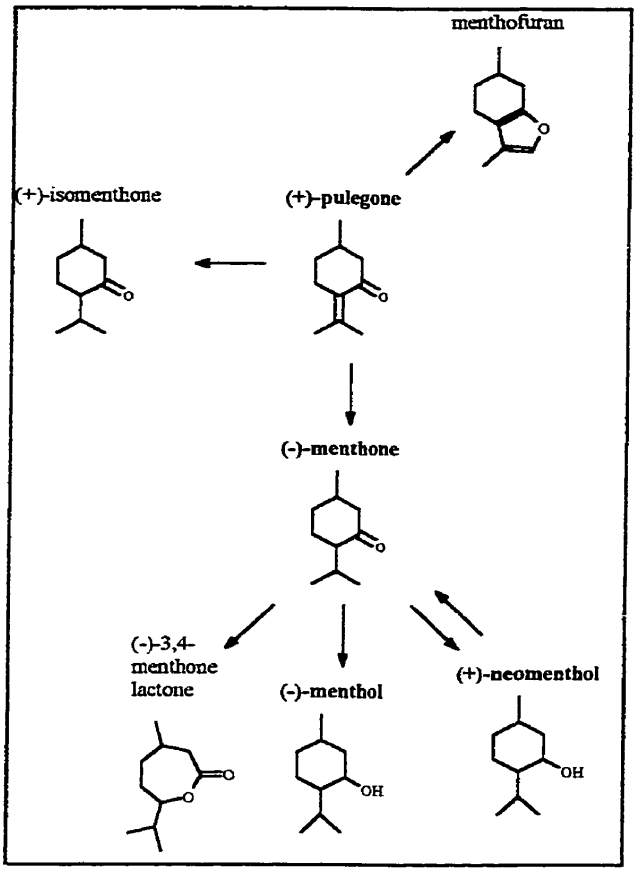

Fig. 2-13: Biochemical Pathways of (+)-Pulegone

As can be expected, as nitrogen is increased (thereby increasing the amount of nitrogen to carbon within the plant), the compounds containing nitrogen such as amino acids, imino acids, polyamines, diamines, and low molecular weight polypeptides also increase (Jones and Coleman 1991). In one study, Emongor and Chweya (1992) found that the oil yield of chamomile flowers increased with increased nitrogen. The increased oil yield was directly proportional to the nitrogen application (0, 75, 150 and 225 mg/pot), however it appeared that there was a point where the oil yield peaked (150 mg/pot), and after additional nitrogen (225 mg/pot), the oil yield decreased dramatically. This may have possibly been due to a problem with ammonia toxicity (Emongor and Chweya 1992). It was also found that some of the essential oil constituents increased or decreased significantly due to the increased levels of nitrogen.

Specific to mint, when there was a nitrogen deficiency, the total oil yield, the plant

biomass and the percentage of mint oil recovered decreased (Praszna and Bernáth 1993). The relative concentration of menthyl acetate increased greatly and was accompanied by a decrease in the concentrations of menthone and menthol (Praszna and Bernáth 1993). Lammerink and Manning (1971) determined that as nitrogen levels were increased, the oil yield also increased. In a similar experiment, increased levels of nitrogen, increased the biomass and thus the total oil yield, but decreased the ratio of leaves over biomass (Piccaglia et al. 1993). These authors also reported that the only essential oil constituent to change significantly was pulegone. Pulegone increased from 1% with an amendment of 0 kg of nitrogen per hectare to 1.9% with an amendment of 200 kg nitrogen per hectare.

Potassium is a macronutrient which will affect the plant if it is not available in suitable amounts. Under conditions of excess potassium, the total oil yield of mint was decreased and the percent of oil quantity recovered from the plant also decreased. The quality of the oil, as judged by commercial flavourist standards, was decreased in an undescribed fashion (Praszna and Bernáth 1993). This was due to menthone and menthol being replaced by less desirable secondary metabolites. Under conditions of potassium deficiency the total oil yield and the plant biomass fell and the concentrations of menthol and menthone decreased (Praszna and Bernáth 1993).

Phosphorous is a macronutrient which will affect the plant in deficient or excess amounts. This element plays a very important role in plant metabolism as pyrophosphates are involved in the anabolic production of terpenes and energy from the sun is transferred via molecules of NADP (Fig. 2-10 and 2-11), ATP and ADP, which contain high energy phosphorous bonds. Hence, changes in the available phosphorous to the plant may result

in changes to the metabolism. When phosphorous was in excess, the plant biomass increased, however, the percentage of the mint oil recovered decreased. When phosphorous was found to be present at low levels, the total essential oil yield decreased as did the plant biomass. While under a phosphorous deficiency, the concentrations of menthol tended to decrease and the concentration of menthone increased (Praszna and Bernáth 1993).

A deficiency of manganese adversely affects plants. The biomass of the plant was decreased as was the total oil yield (Srivastava and Luthra 1994). To trace the interactions between the primary and secondary metabolic pools two radio-labelling experiments were completed on manganese deficient plants. The incorporation of $^{14}CO_2$ in both the primary and secondary metabolite pools was found to have decreased while the ^{14}C-sucrose incorporation in each metabolite pool rose (Srivastava and Luthra 1994).

When mint plants experience an iron deficiency, a trend similar to that of manganese deficiency was noticed within both the primary and secondary metabolite pools (Srivastava and Luthra 1993). These trends indicate a limited availability of photosynthates for oil biosynthesis which will affect oil accumulation (Srivastava and Luthra 1993). If the substrate (photosynthates) is lacking, then the amount of products (oil) will also be less due to shortage of the starting material. The lack of photosynthates does not influence the oil biosynthesis via the mevalonate-isoprenoid pathway (Srivastava and Luthra 1994) and therefore does not affect the relative concentration of the oil constituents.

Nutrients play a vital role in the growth and development of plants. Nutrients, when in the correct proportions, provide the plant with the sustenance it requires for optimal growth. However, problems may result due to the toxicty caused by excess nutrients. In

cases of deficiencies of the nutrients, the result may be the plant not being able to function and grow properly.

2.7.3. Problems Facing Mint Production - Diseases and Insects

Diseases and insects also harm and stress plants. These are non-photosynthetic growth stressors which have a direct impact on the photosynthetic capabilities of the plant. Disease and insect damage decrease the leaf area which will in turn decreases the ability of the plant to photosynthesize. This results in a reduction of metabolites and hence oil accumulation.

A form of rust, *Puccinia menthae*, is a common infection of the mint plant. Rust infections tend to lower the total oil yield of the plant (Clark and Menary 1984; Nadaska et al. 1995). In infected plants, the menthol concentration fell (Clark and Menary 1984), while the menthone and menthofuran content increased slightly (Margina and Zheljazkov 1994). In a study by Nadaska et al. (1995), rust-free plants yielded 4.9 and 3.5 times the oil than rust infected plants over a two year period, but the oil from the rust infected plants contained 1.7 times as much menthol and menthyl acetate. Thus the rust affected the quality of the oil by increasing the concentration of the undesirable menthyl acetate.

In examining spider mite injury to mint it was noted that the concentration of pulegone decreased while the concentrations of menthol and neomenthol increased (DeAngelis et al. 1983). The injury to the plant caused the enzyme activity to increase which then specifically changed pulegone to a more oxidized form (DeAngelis et al. 1983).

2.7.4. Problems Facing Mint Production - Pollution

Air pollution decreases the plant's ability to absorb carbon and decreases the carbon to nitrogen ratio in plants. Thus the carbon-based secondary metabolites such as terpenoids tend to decrease while nitrogen based secondary metabolites increase. In addition, in areas of high pollution, plants exhibited evidence of tissue injury. The plant may respond by catabolizing material from the secondary metabolite pool to the primary metabolite pool in order to repair the damage (Jones and Coleman 1991).

3.0. Researching Mint in Nova Scotia

Spearmint and peppermint plants are grown in many parts of the world for their specific culinary attributes. Currently within Nova Scotia mint is grown on a small scale for fresh use in restaurants and by hobbyists for personal uses. On a world-wide scale mint is grown on a commercial basis for use in food additives, pharmaceutical formulations, aromatherapy and for other homeopathic applications.

The major areas for mint production in the USA occur in a concentration of American states situated along the 45th Parallel. The 45th Parallel also passes through Nova Scotia, within 50 km of Bible Hill and Maitland. Nova Scotia is therefore within this apparently "superior growth region" for mint. This lends support to the hypothesis that Nova Scotia may have the capability to produce a commercially acceptable mint oil.

3.1. Objectives of the Project: Qualitative Evaluation of Essential Oil from Nova Scotia Grown Mint

There were 3 main objectives of this project:

1. To establish a stand of mint in Nova Scotia and extract the oil.

2. To characterize the extracted mint oil and to determine its viability for commercial production based on physicochemical characteristics (composition of the individual terpene constituents, specific gravity, refractive index, etc.), oil yield, biomass production and observe agronomic characteristics.

3. To determine if the mint oil produced or any of its major constituents exhibited antioxidative properties.

4.0. Establishment and Processing of the Mint Stands for Essential Oil Production

Mint plants currently grow in many areas of Nova Scotia as competitive weeds and plants in greenhouses and hobbyist's gardens. Thus, if mint is grown on a small scale, it may be speculated that Nova Scotia may be able to sustain an economically viable mint crop for commercial oil production. In order to determine the suitability of mint for regional agronomic and essential oil production, the project required the establishment and evaluation of various species and cultivars of the genus *Mentha* (*Mentha spicata* cv Native Spearmint, *Mentha piperita* cv Black Mitchum, *Mentha piperita* cv Murray Mitchum).

4.1. Objective

To establish a stand of mint in Nova Scotia and extract the oil.

4.2. Plant Material

The plants, propagated by commercial tissue culture, were purchased by Maitland Greenhouses, Maitland, Nova Scotia. The plants were supplied with USDA phytosanitary certificates and certified to be *Verticillium* free. The plants were subsequently grown out in greenhouses to an approximate height of 15 cm at which time they were transplanted to the field.

4.3. Experimental Design

The experimental design was a Repeated Measures Analysis (bloom stages - 50%, 75% and 100% bloom) based on a split-split plot with blocking factors for location, where the plants were grown (Maitland and Bible Hill), species and cultivar (*Mentha spicata* cv Native Spearmint, *Mentha piperita* cv Black Mitchum and *Mentha piperita* cv Murray Mitchum) and year (1994 and 1995).

There were a total of 36 plots arranged in 4 rows of 9 plots at each of the two locations. Each row represented a replication and was a complete randomized block of bloom stages and species. The 9 plots on each row contained a plot for each cultivar at each bloom stage (3 cultivars, 3 bloom stages). Individual plots were 1.5 x 5 m and separated by 1 m pathways. There were 45 plants transplanted to each plot. The plants were planted in rows 0.5 m apart and 0.25 m from the edge of the plot. Within their row spacings the plants were 0.3 m apart and 0.4 m from each end. The plots (Fig. 4-1) were arranged so the rows represented complete replications of the mint cultivars. The mint cultivars were used as blocking factors and were individually randomized within each row with the repeated measure statistic, bloom stage. The location and the year were fixed blocking factors and could not be randomized.

4.4. Mint Stand Establishment

In addition to the main experimental plan, 4 demonstration plots, with the same plot spacing as described above, were planted. These consisted of 2 plots each of *Mentha spicata* cv Scotch Spearmint and *Mentha piperita* cv Todd's Mitchum. The demonstration plots

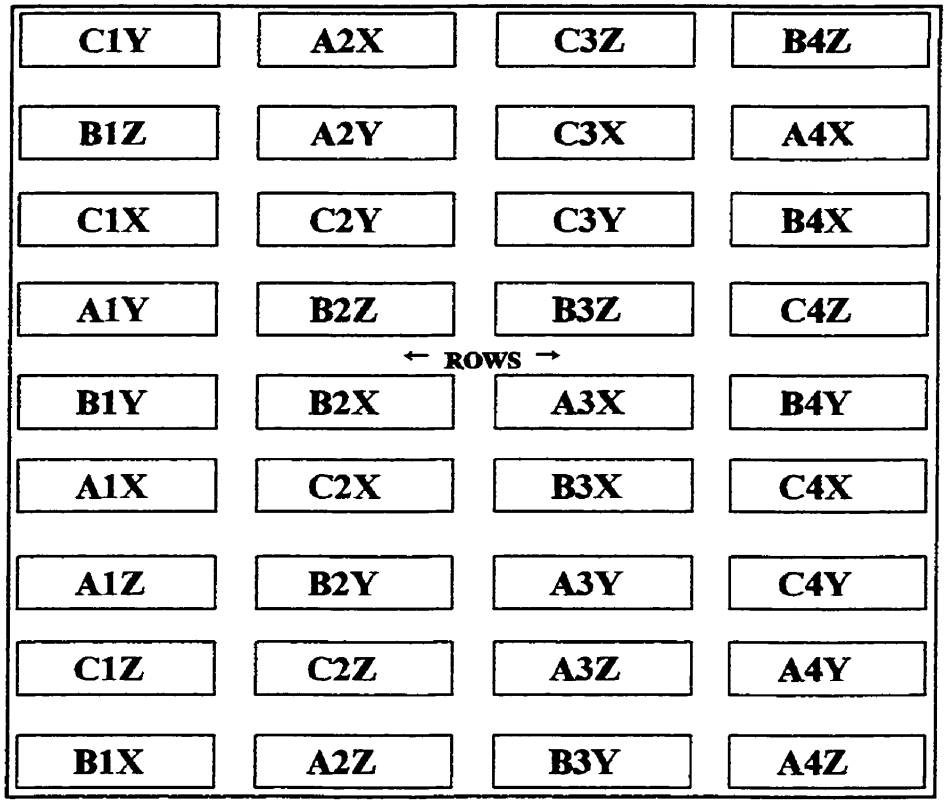

Figure 4-1: Plot Design for both Maitland and Bible Hill Sites

A - Native Spearmint
B - Black Mitchum Peppermint
C - Murray Mitchum Peppermint

X - 50% bloom stage
Y - 75% bloom stage
Z - 100% bloom stage

were planted, harvested and analyzed to gather preliminary information on commercialization of other potential mint species. The data obtained were not statistically analyzed within the model of the project due to a limited number of samples, however useful information was gained from their evaluation (Appendix 8-1).

Soil testing was completed at both sites to determine site comparability (Appendix 8-2). The plants were recieved by Riverview Herbs of Maitland in May of 1994. Maitland Herbs allowed the plants to be grown out in the greenhouse. When the plants had reached a height of 15 cm, all of the mint plants were transplanted to the Bible Hill field on June 18,

1994 and to the Maitland site June 19, 1994. Due to excessively dry conditions the plants at the Bible Hill site were hand watered from a portable tank on June 23. The plants at both sites were hand weeded weekly.

During the summer of 1994 the mint plants blossomed prematurely in response to heat stress. The plant material present was not sufficient to harvest, so the blossoms were removed from the plant to promote more vegetative growth (Goodyear 1994).

On August 9, 1994, a rust infection (*Puccini mentha*) was found on the plants. The infection was identified by Dr Bruce Gray, Department of Biology, NSAC and confirmed by Rick Durbridge, Nova Scotia Department of Agriculture and Marketing, Plant Industry Branch, Kentville, N. S. The infection was determined to be very severe and an immediate threat to the summer harvest and possible infection to other indigenous plants. For this reason harvesting was initiated on August 16, 1994. Hand harvesting of the centre row was carried out, leaving the outer guard rows untouched. No further intervention was made at that time. On September 28, 1994 a count of the plants which survived the summer was recorded.

On May 23, 1995 both sites were flame weeded using a flame weeder obtained from Dr N. Rafai, Department of Agricultural Engineering, NSAC to combat the rust infection as well as to aid in the prevention of weed growth in the upcoming 1995 season. Fish bone meal was applied to both sites as a nitrogen supplement (Goodyear 1995).

On July 14, 1995 the first plants of the second season began to emerge. The emergence of the plants was delayed due to a delay in the thawing of the ground frost. On July 28, 1995 a count of the plants which survived the winter was recorded. A survival

rating was calculated by comparing the ratio of the number of plants alive in the fall of 1994 to the number of plants alive in the spring 1995.

At the end of July a rust infection returned despite of the flaming process used. At this time, it was decided that a fungicide was to be used to prevent the spread of the rust. On August 13-14, 1995 an organic sulfur fungicide was applied at a rate of 375 g/plot (as directed on the packaging). After the second growing season the entire plot was harvested, including the centre and guard rows, due to a lack of plant material.

4.5.0. Survival Rating

The survival rating of the mint plants was determined strictly on the number of plants which survived the winter. In the fall of 1994 a count of the plants was taken. Subsequently in July of 1995 another count was taken (Appendix 8-3). A comparison of the plants alive in 1995 with those in 1994 was completed to provide the survival rating.

The statistical design for the survival rating of the mint plants was a split-plot design and analyzed using the following model:

$$\text{survival} = \mu + \alpha + \beta + \alpha * \beta + \epsilon$$

μ = mean
α = location
β = cultivar
ϵ = error

In using the above model it was found that there were no significant differences in the survival rating of the plants among the cultivars using a Tukey's test ($P \leq 0.05$). The Native Spearmint, Black Mitchum Peppermint and Murray Mitchum Peppermint had survival ratings of 23%, 23% and 17%, respectively over the winter of 1994-5. The

survivability of the mint was unexpectedly low. This may have been caused by the combination of a dry summer and a severe infection by *Puccinia menthae* in the summer of 1994 which may have weakened the plants. In addition, during the winter of 1994-5, unseasonably warm temperature conditions caused premature growth of the mint plants. This growth ended when the temperature dropped below freezing and returned to normal. These freeze-thaw cycles may have contributed to the low survival rating of the plants.

4.5.1. Leaf Area Index

Leaf Area Indices (LAI) of the mint plants were measured at regular intervals throughout the summer of 1994. Using the LAI 2000 (a photocell attached to a data recorder), the amount of sunlight which is filtered down through the foliage is measured. The LAI measurements are based upon the amount of light which is measured by the photocell. The area of the foliage that was measured is inversely related to the amount of light reaching the sensor. The mint plants were expected to reach a height of 60-90 cm, however, due to environmental stresses the tallest plants were only approximately 25 cm high. It is suspected that the height of the plants, the poor vegetative growth and the poor winter survival of the mint plants greatly affected the leaf canopy which is crucial in obtaining accurate ratios of the light filtering through to the LAI detector. For this reason the 1994 results were difficult to interpret (Appendix 8-4). The canopy present in 1995 was similarly not conducive to LAI measurements and hence the LAI readings were dropped from the data collection.

4.6. Distillation Procedures

At the appropriate time, as defined by the bloom stage, the plants were hand harvested with a knife by cutting the plant at ground level, leaving the roots to over-winter. After harvesting, the plant material was bagged and transported to the laboratory where the plant material was massed and sub-sampled twice by quartering the sample until it was in the required weight range. The sub-samples ranged from 100-500 g depending on the amount of plant material available at harvest. The sub-samples were subjected to immediate distillation or stored double-bagged in sealed freezer bags in an ultra-cold freezer (-80°C) for steam distillation at a later time.

The plant material was chopped on a cutting board into pieces approximately 1.5-3 cm long prior to distillation. The distillation apparatus consisted of a steam generator which supplied steam directly to the plant material which had been placed in a 5 L 3- neck round bottomed flask. A Cleavenger (light oil) trap (Sigma) was used to collect and separate the condensed oil as the distillation proceeded (Fig. 4-2).

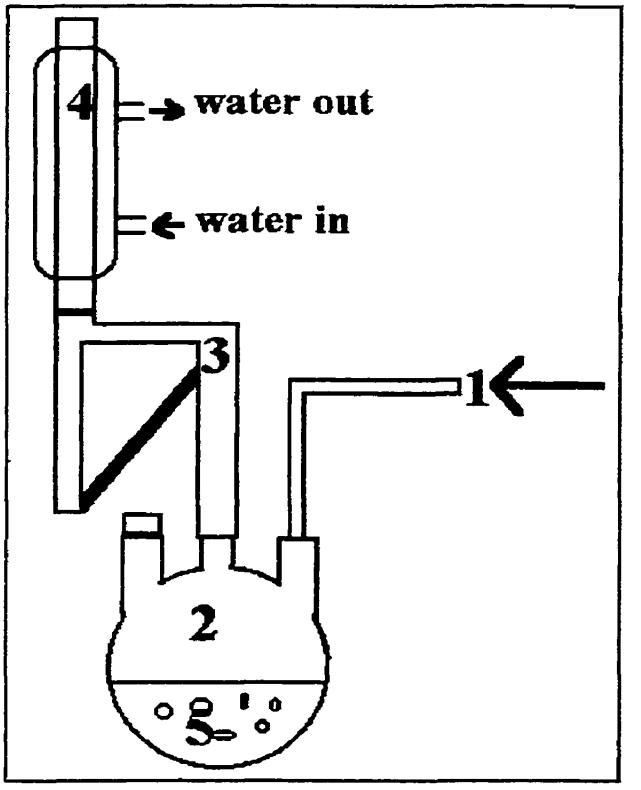

Fig.. 4-2: Steam Distillation Apparatus
1 - steam source 2 - sample flask
3 - light oil trap 4 - condenser
5 - sample

The distillation was continued 30 minutes beyond the last visible increase in

the oil volume (total distillation time was normally 90-150 minutes). After distillation was completed, the trap was brought to room temperature and then the volume of the oil collected was recorded. The oil fraction was removed with a disposable pipet and stored in Teflon sealed screw capped glass bottles under nitrogen gas. The recovered oil was stored at -80°C for future analysis.

4.7. Oil Recovery Yields

The amount of oil which may be recovered from the plant material is of great importance in commercial production of a viable essential oil crop. The oil recovered is a ratio of the volume of oil (mL) recovered to the mass of the plant material (g). Oil recovery yields were analyzed by SAS utilizing a Tukey's test ($P \leq 0.05$). It was found that the 3 cultivars harvested at 50% bloom (Table 4-1) were significantly different from each other with respect to oil yield. Murray Mitchum Peppermint had the highest oil yield (0.55%) followed by Black Mitchum Peppermint (0.47%) with the lowest yield being from Native Spearmint (0.34%). At 75% and 100% bloom (Table 4-1) the 3 cultivars were not significantly different in oil yield (0.21-0.39%) from each other.

The recovered oil yields in all cases were less than 0.6%. This differs greatly from the reported literature values of both species which were typically in the range of 2-4% (Lawrence 1984). This may be due to the lack of plant foliar growth because of the environmental conditions at the time. The highest oil containing portions of the plant, the leaves, were significantly damaged and desiccated due to the onset of the rust. These two factors played a major role in the below average yield of the essential oil.

Table 4-1: Oil Recovery of Three Different Mint Types at Three Bloom Stages / mL/100g

Cultivar\Bloom Stage	50%	75%	100%
Native Spearmint	0.34 C [1]	0.25 A	0.21 A
Black Mitchum Peppermint	0.47 B	0.31 A	0.39 A
Murray Mitchum Peppermint	0.55 A	0.36 A	0.39 A

[1] different letters following the means denote significant differences ($P \leq 0.05$) within a bloom stage

5.0. Quality Analysis of the Mint Oil

5.1. Objectives

The primary objective of this portion of the research was to characterize the mint oil recovered with respect to its physicochemical properties (percentage of the individual essential oil constituents, density and refractive index).

5.2. Essential Oil Component Analysis

Essential oils are made of many different constituents. The most important aspect of mint oil is its content and the relative ratio of essential oil components (i.e. menthone:menthol) (Clark and Menary 1980). A raw commercial mint oil (Table 5-1) was obtained from Givauden-Roure, a commercial distributor of flavours, to compare with the mint oil produced under Nova Scotia growing conditions. There were differences between the reported values by Givauden-Roure and my analysis of the commercial mint oil. These may be attributed to a number of factors; two major factors which could attribute to the differences are the different detectors, my analysis was completed by GC-MS and the Givauden-Roure analysis was by GC-FID. The second factor could be the stability of the mint oil. The oil will change over time due to storage conditions (heat, light, exposure to oxygen, etc.).

Table 5-1: Percentage Composition of Commercial Peppermint Oil

Chemical Component	GC-MS Analysis of Commercial Mint Oil / g/100 g	Certificate of Analysis from Givauden-Roure / %
β-caryophyllene	2.12	1.60
1,8-cineole	5.68	4.77
(-)-limonene	2.98	1.53
(-)-menthyl acetate	1.87	5.15
(-)-menthol	46.63	42.74
(-)-menthone	14.75	not provided
(+)-neomenthol	2.81	3.06
α-pinene	2.52	0.96
(+)-pulegone	1.91	0.37

The same model was used to analyze all data sets (percent composition, density and refractive index).

$$\text{quality factor} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha_i * \beta_j + \alpha_i * \gamma_k + \gamma_k * \beta_j + \alpha_i * \beta_j * \gamma_k + \epsilon_{ijk}$$

α_i = location
β_j = cultivar
γ_k = year
ϵ_{ijk} = error

The model was originally intended to employ a Repeated Measure Analysis with the bloom stage to be used as the repeated measure. This analysis had to be altered due to the agronomic problems that were experienced, as discussed above. In 1994 and 1995 the rust infection led to irregular harvesting periods. As the infection spread, the leaf area was

depleted and this forced the early harvest of some plants which were scheduled to be harvested at the 75% and 100% bloom stages. The larger number of samples at 50% bloom as compared to the 75% and 100% bloom stages, led to an unbalanced model. In an unbalanced model, SAS estimates values. The assumed values could potentially lead to a skewing of the results. Therefore it was determined that analyzing the data separately (50%, 75% and 100% bloom stage) would be best (Astatkie 1996).

5.3.0. Individual Essential Oil Constituent Analysis by Gas Chromatography

A mixed standard was prepared from chemicals purchased from Sigma-Aldrich, Fluka, and Fisher Scientific (Table 5-2). Relative ratios of components in the standard were selected so as to simulate the ratios of components naturally occurring in mint oil. Approximately 0.5 g (-)-menthol, 0.5 mL each of (-)-limonene, β-caryophyllene, (+)-neomenthol, 1,8-cineole, α-pinene and (+)-pulegone and 0.75 mL (-)-menthone were combined in the mixed standard; exact masses of each were mixed together and combined to a total volume of 10.00 mL with hexane.

The standards were diluted with hexane (Fisher Scientific) as per Table 5-3. The 100% standard was mixed so that it simulated the highest possible amount of each constituent in naturally occurring mint oil. The 66% and 33% mixes were dilutions of the 100% standard used to prepare the standard curve for the quantification of the samples.

Table 5-2: Mint Oil Constituent Standards

mint oil constituent	company purchased from	purity / %	lot number
α-pinene	Aldrich	99	10120EF
β-caryophyllene	Sigma	99	74H2512
1,8-cineole	Aldrich	97	06015CG
(-)-limonene	Aldrich	97	10107HG
(+)-neomenthol	Aldrich	99	11715DF
(-)-menthol	Aldrich	99	0906BN
(-)-menthone	Aldrich	90	06301LG
(-)-menthyl acetate	Fluka	99	2572031189
(+)-pulegone	Aldrich	98	28618EF

Table 5-3: Preparation of the Standards

	standard mix		hexane		internal standard		total	
dilution ratio	volume / mL	mass / g	volume / mL	mass / g	volume / mL	mass / g	volume / mL	mass / g
100%	3.000	0.5485	0.000	0.000	0.030	0.0077	3.030	0.5562
66%	2.000	0.2755	1.000	0.1581	0.030	0.0077	3.030	0.4413
33%	1.000	0.1743	2.000	0.2810	0.030	0.0078	3.030	0.4631
0% (blank)	0.000	0.0000	3.000	0.4479	0.030	0.0076	3.030	0.4555

An internal standard served as a reference point for all peak area measurements. For this particular experiment 1-octanol (Fisher-Scientific) was used. The internal standard minimizes any error from small variations in injection size or GC detector sensitivity. The peak area of each compound was determined by the software controlling the GC and the inputted integration parameters. The area response for a fixed amount of internal standard

(30 μL in 3 mL) was determined. The peak area of a sample compound can be directly correlated to the mass of the component. When using a constant mass, the same detector response should be determined each time, with small changes in the response attributed to slight differences in the injection volume and detector sensitivity. These small changes can be corrected by utilizing an internal standard. By determining the true (average) peak area response to mass ratio, a mathematical adjustment may be made to compensate for the peak area changes in the internal standard. The correction factor may then be applied to all of the peaks of interest. A correction factor for each sample injection was calculated and applied to each of the peaks of interest. This correction factor adjusted the raw peak area values to a corrected value (Shugar and Ballinger 1996) (Appendix 8-6) (Microsoft Excel 5.0).

The distilled mint oil was removed from storage (the ultra-cold freezer) the night before the analysis and brought to room temperature. The oil was prepared for injection into the gas chromatograph (GC) as follows. A sample of 50 μL (≈ 40 μg) of sample was massed on an analytical balance into a 4 mL test tube. To this, 10 μL (≈ 6 μg) of 1-octanol (used as an internal standard) was added directly to the sample and massed accurately. The mixture was diluted with a known quantity of hexane, 940 μL (≈ 660 μg) for a total volume of 1 mL (≈ 710 μg). This mixture was vortexed prior to analysis. All volumes were weighed for accuracy and mass:mass ratios were used in the calculations.

Gas chromatography is a method used for the separation of volatile materials which takes advantage of differences in partition coefficients through the use of a variety of factors (stationary phase, flow rates, temperature programs, etc). Two different GC systems were used, and due to the different column sizes and detectors, operating parameters were

optimized for each system (Table 5-4). Using the standard mixture and several trial runs, a temperature program was determined which provided the greatest separation and best resolution in the shortest period of time for both GC methods.

Table 5-4: Gas Chromatograph Conditions

model	Hewlett Packard	Varian 6500
detector	mass spectrometer (MS)	flame ionization detector (FID)
carrier gas	helium	nitrogen
flow rate	1 mL/minute	10 mL/minute
column	J & W Scientific DB-5 length - 30 m i.d. - 0.32 μm film thickness - 1 μm	J & W Scientific DB-5 length - 30 m i.d. - 0.53 μm film thickness - 0.5 μm
sample size	1 μL	2 μL
split ratio	1:50	---
temperature program	10 min @ 60°C 15 min @ 100°C @ 5°C/min 5 min @ 200°C @ 10°C/min	15 min @ 50°C 10 min @ 60°C @ 2°C/min 2 min @ 180°C @ 10°C/min

A qualitative identification of the compounds of interest was made by comparing the retention times of the compounds to the retention time (t_R) of the standards. The use of two different GC systems (GC-FID; GC-MS) allowed for enhanced confirmation of qualitative information. As a function of the software of the mass spectrometer detector, the identity of the peaks were confirmed by cross referencing the MS data to a data library (Hewlett-Packard Chemstation).

The corrected peak area responses of the FID detector were compared to the corrected peak area responses of the MS detector. Correlations of the standards showed that the peak area responses provided by the FID and MS detector for all compounds of interest, except β-caryophyllene, could be directly correlated with each other (Table 5-5).

Table 5-5: Area Correlation Results Between FID and MS Detectors

component of interest	correlation between detectors (r^2) (α=0.05)
α-pinene	0.912
(+)-neomenthol	0.951
(-)-menthol	0.967
(-)-menthone	0.989
(+)-pulegone	0.975
(-)-menthyl acetate	0.882
β-caryophyllene	0.687

Unexpectedly, two of the peaks, (-)-limonene and 1,8-cineole, could not be adequately resolved on the 0.53 μm column used with the GC-FID system. The lack of resolution can be attributed to the temperature program required to adequately resolve the more important peaks of (-)-menthone, (-)-menthol and (+)-neomenthol. For this reason all FID data were dropped from the analysis and only the MS data were used for the calculations of the percent composition of the mint oil.

Standard curves for each of α-pinene, (-)-limonene, 1,8-cineole, (-)-menthone, (-)-neomenthol, (-)-menthol, (+)-pulegone, (-)-menthyl acetate and β-caryophyllene were prepared using the 100, 66 and 33% standard mixtures. In the analysis of the samples, peaks

and their areas were compared with the appropriate standard curve. The amount of each component in the oil was calculated and expressed as a percentage of the total oil. A check standard was run daily using the 66% standard mix. When the analysis was completed, the check standards were compared. A correction factor based upon the check standard was applied to the data set associated with the day upon which the sample was run (Appendix 8-6).

For the GC-MS, limits of detection were calculated based upon the standard curve. The lowest reported peak area response was 5000 area units. Using this value as the initial peak area, the calculations from the standard curve were used to determine the limit of detection as per Table 5-6.

Table 5-6: Limits of Detection for the GC-MS

constituent	mass detected / μg	percent detected / g/100 g
α-pinene	0.033	0.075
(-)-limonene	0.033	0.075
1,8-cineole	0.037	0.084
(-)-menthone	0.035	0.082
(+)-neomenthol	0.029	0.066
(-)-menthol	0.031	0.072
(+)-pulegone	0.038	0.089
(-)-menthyl acetate	0.015	0.034
β-caryophyllene	0.037	0.085

5.3.1. Analysis of Essential Oil Components

When the mint plants were at 50% bloom some of the plants were harvested and analyzed. Using a Tukey's test ($P \leq 0.05$) it was found that the concentrations of α-pinene and β-caryophyllene were not significantly different in each of the 3 cultivars tested (Fig. 5-1). The concentrations of 1,8-cineole, (-)-menthyl acetate, (+)-neomenthol and (+)-pulegone in Native Spearmint were found to be significantly lower than those of the two peppermint cultivars. The concentration of (-)-limonene in the Native Spearmint was found to be significantly higher than in the two cultivars of peppermint. The (-)-menthol content (3.18%) of the Native Spearmint was significantly lower than the (-)-menthol content of the Black Mitchum (38.01%) and Murray Mitchum (52.16%) Peppermints; the Black Mitchum and Murray Mitchum peppermint cultivars did not differ significantly in (-)-menthol content from each other. With respect to the (-)-menthone concentrations, all cultivars differed significantly from each other. Within the peppermint oils the concentrations of β-caryophyllene, 1,8-cineole, (-)-limonene, α-pinene and (+)-pulegone all compared favourably to the commercial mint oil analyzed (Table 5-1). The concentrations of (-)-menthyl acetate, (-)-menthone and (+)-neomenthol were found to be lower than that analyzed in the commercial mint oil. The (-)-menthol concentrations were found to be higher than that analyzed in the commercial mint oil. The major constituents of the spearmint oil ((-)-menthone and (-)-menthol) were very low in comparison to the commercial mint oil.

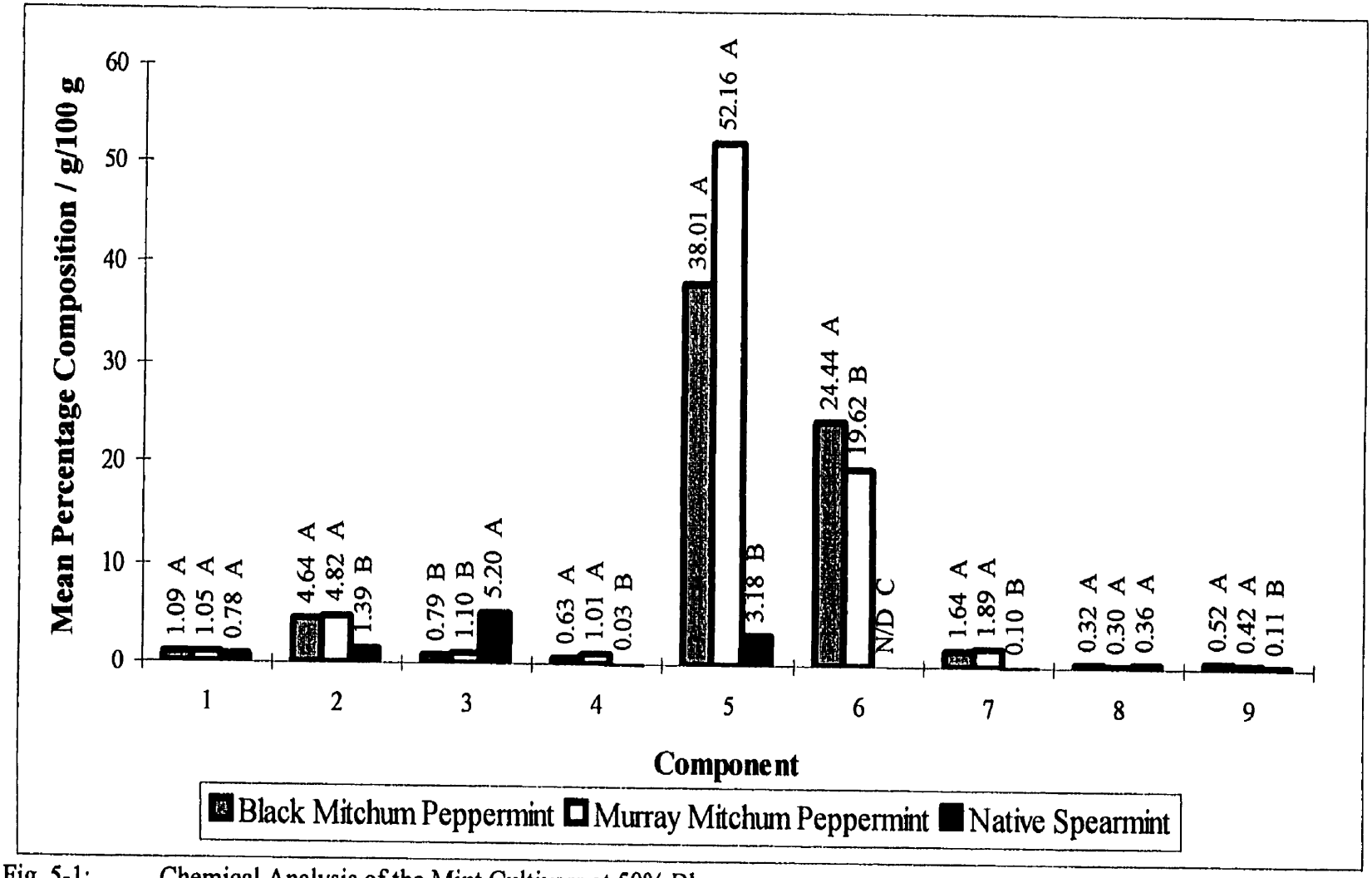

Fig. 5-1: Chemical Analysis of the Mint Cultivars at 50% Bloom

β-caryophyllene (1), 1,8-cineole (2), limonene (3), menthyl acetate (4), menthol (5), menthone (6), neomenthol (7), α-pinene (8), pulegone (9)

N/D - not detectable: means followed by the same letter indicate there are no significant differences in the level of constituents between cultivars for the same constituent and bloom stage

Chemical analysis of the essential oil from the mint plants at 75% bloom showed that the (-)-limonene concentration was significantly higher in the Native Spearmint than the other 2 species of peppermint (Fig. 5-2 & Table 8-6). As at the 50% bloom stage, the (-)-menthol content of Native Spearmint remained significantly lower than that of Black Mitchum and Murray Mitchum peppermint. By the 75% bloom stage the accumulated (-)-menthyl acetate was different for all 3 cultivars, Murray Mitchum peppermint had 3.92% (-)-menthyl acetate followed by Black Mitchum peppermint (2.46%).

Within the peppermint oils the concentrations of β-caryophyllene, 1,8-cineole, (-)-limonene, (+)-neomenthol and α-pinene all compared favourably to the commercial mint oil analyzed (Table 5-1). The (-)-menthyl acetate, (-)-menthone and (+)-pulegone concentrations were found to be lower than that analyzed in the commercial mint oil. The (-)-menthol concentration was found to be higher than that analyzed in the commercial mint oil. The major constituents of the spearmint oil ((-)-menthone and (-)-menthol) were very low in comparison to the commercial mint oil.

Within the plants harvested at 100% bloom it was found that the (-)-limonene concentration was still significantly higher using the Tukey's test ($P \leq 0.05$) in Native Spearmint (3.87%) than in the Murray Mitchum peppermint (0.05%). The Murray Mitchum Peppermint had a significantly higher (-)-menthyl acetate (7.45%) content than the Native Spearmint while the Black Mitchum Peppermint was not significantly different from either the Native Spearmint or the Murray Mitchum Peppermint. The (-)-menthol content of the

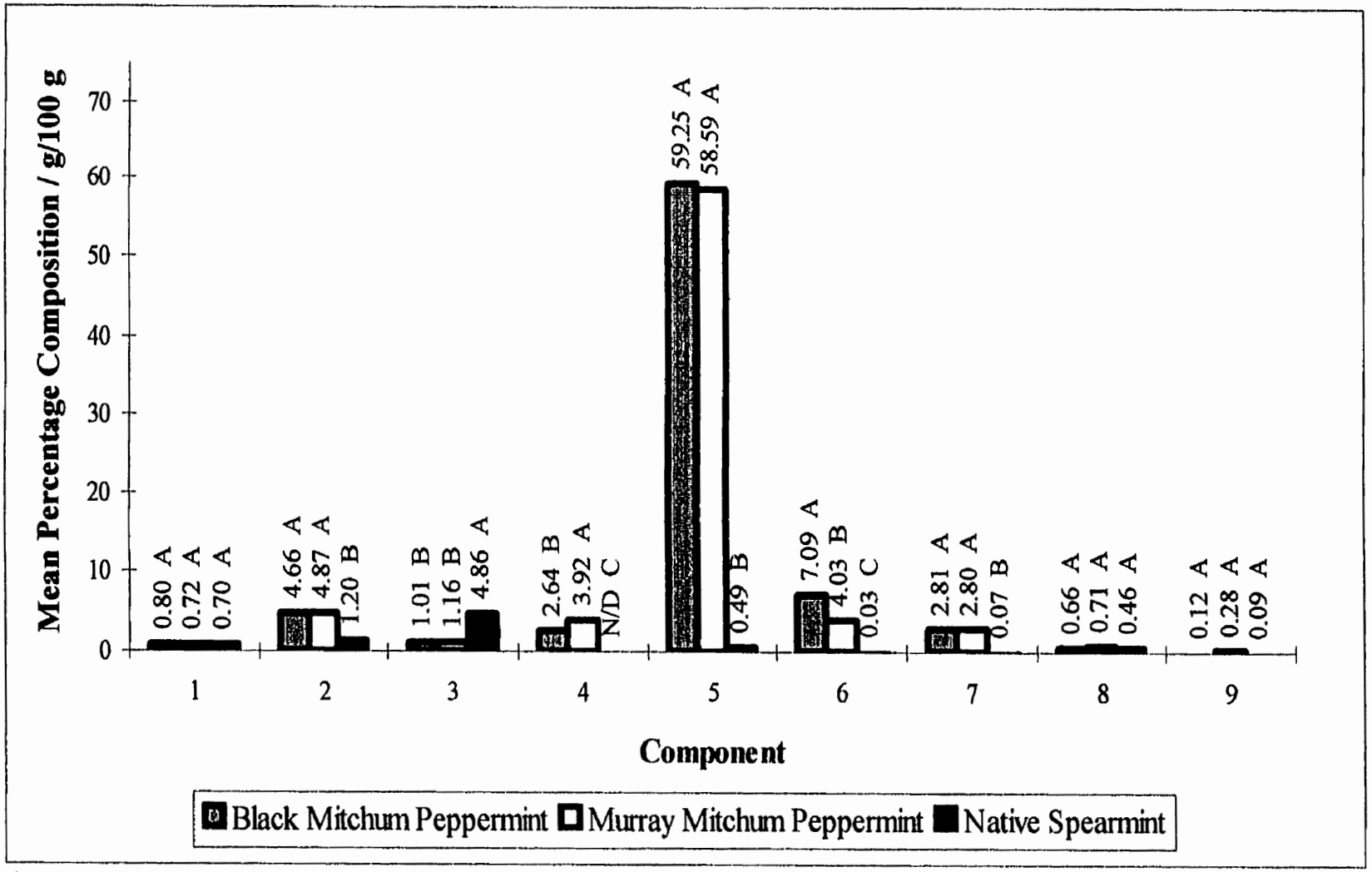

Fig. 5-2: Chemical Analysis of the Mint Cultivars at 75% Bloom

β-caryophyllene (1), 1,8-cineole (2), limonene (3), menthyl acetate (4), menthol (5), menthone (6), neomenthol (7), α-pinene (8), pulegone (9)

N/D - not detectable: means followed by the same letter indicate there are no significant differences in the level of constituents between cultivars for the same constituent and bloom stage

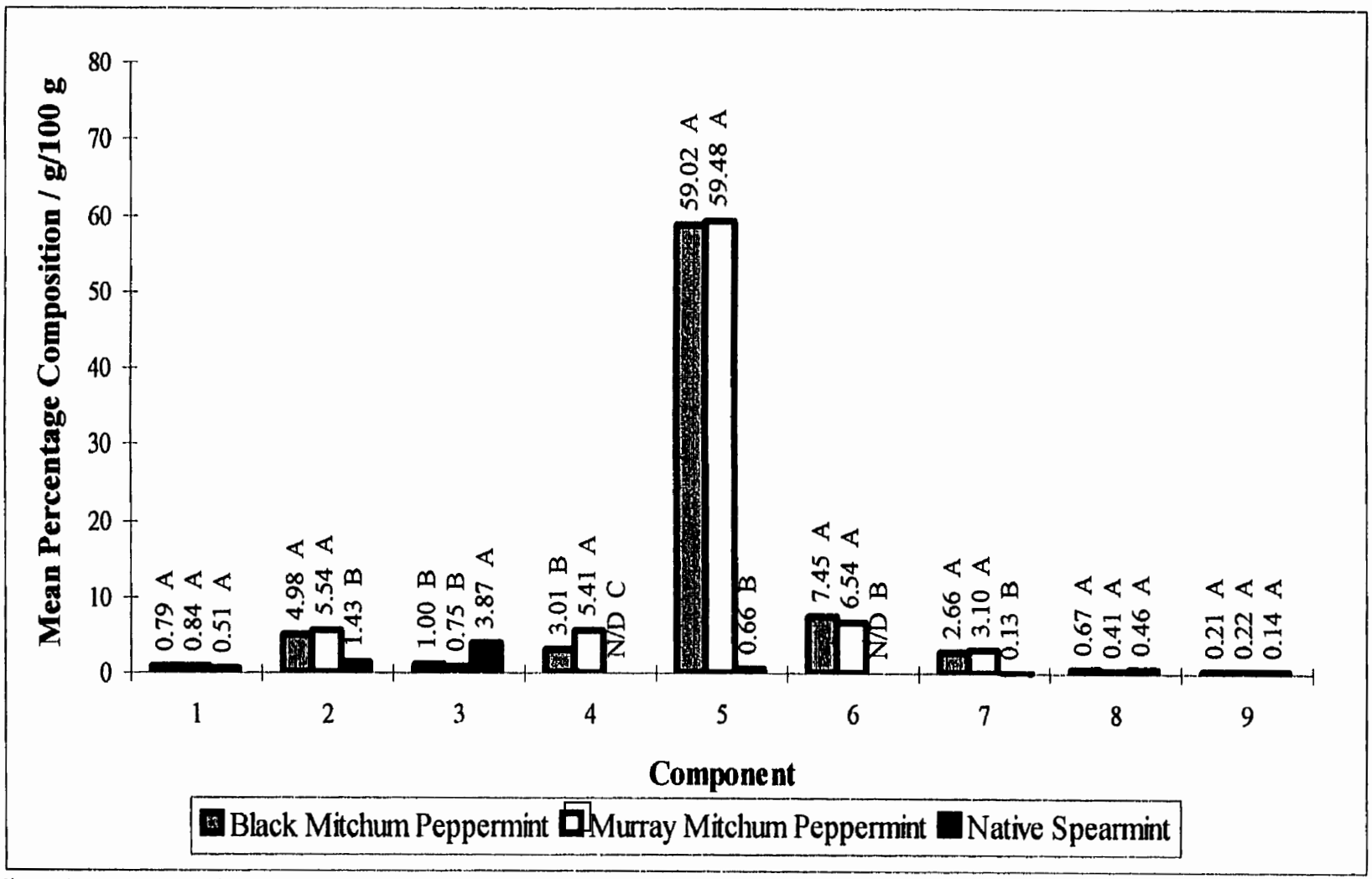

Fig. 5-3: Chemical Analysis of the Mint Cultivars at 100% Bloom

β-caryophyllene (1), 1,8-cineole (2), limonene (3), menthyl acetate (4), menthol (5), menthone (6), neomenthol (7), α-pinene (8), pulegone (9)

N/D - not detectable; means followed by the same letter indicate there are no significant differences in the level of constituents between cultivars for the same constituent and bloom stage

the Native Spearmint was significantly lower than the (-)-menthol content of the Black Mitchum and Murray Mitchum Peppermints which were not significantly different from each other. As with the 75% bloom stage, the concentrations of (-)-limonene, (+)-neomenthol and α-pinene in the peppermint oils compared favourably to the commercial mint oil analyzed (Table 5-1). The β-caryophyllene, (-)-menthol and (+)-pulegone concentrations were found to be lower than that analyzed in the commercial mint oil. The (-)-menthyl acetate concentration was found to be lower

than that analyzed in the commercial mint oil. The major constituents of the spearmint oil ((-)-menthone and (-)-menthol) were very low in comparison to the commercial mint oil.

The (-)-menthol to (-)-menthone ratios (Table 5-7) of the peppermint cultivars at the 50% bloom stage were best for commercial flavourant requirements of 2:1 (Lawrence 1984), however the ratios at the 75% and 100% bloom stages were higher. Within the spearmint data, the samples had either undetectable levels of (-)-menthol or (-)-menthone. The ratio was determined by dividing the percentage composition of (-)-menthol with (-)-menthone. Due to the undetectable levels, the ratios were taken to be zero. This may have been caused by the presence of the rust. An infection by *Puccinia Mentha* may result in depleted levels of (-)-menthol (Nadaska et al. 1990). This is evidenced in the undetectable levels of menthol found in Native Spearmint (Figs. 5-1, 5-2 and 5-3).

Table 5-7: Analysis of the (-)-Menthol:(-)-Menthone Ratios of the Three Mint Varieties at Three Different Bloom Stages

Bloom Stage	50%	75%	100%
Native Spearmint	0 A[1]	0 A	0 A
Black Mitchum Peppermint	2.25:1 A	12.12:1 A	8.31:1 A
Murray Mitchum Peppermint	2.44:1 A	11.88:1 A	6.51:1 A

[1] different letters following the means denote significant differences ($P \leq 0.05$) within a bloom stage

5.3.2. Environmental Effects on the Oil

There were many problems faced during the growing season which were beyond the control of the experimenter. These conditions affected the biochemical pathways of the plant and altered the percent composition of the constituents within the mint oil. One of the initial problems was an early drought. A lack of water will affect the ability of the plant to absorb required nutrients from the soil. Due to a deficiency of water, a lower oil yield may result due to conversion of monoterpenes to higher orders of terpenes (i.e. sesquiterpenes) (Denys et al. 1990).

Another major problem was the infection by rust (*Puccinia menthae*). The desiccation of the leaves as the infection spread seriously reduced the viable leaf area available for production of the mint oil; thus the total oil yield fell considerably. Nadaska et al. (1990) explains that the infection of the rust may be responsible for the lower concentration of (-)-menthol according to expected literature values.

It is speculated that due to stress on the plants, as a result of the rust, drought and heat, the plants bloomed early. At that time there was not enough plant material to justify harvesting, so the blooms were removed to try to delay the onset of floral initiation. After

an attempt to allow an increase in the foliar growth by deblossoming, the total oil yield and the (-)-menthone concentration was low, and in the later blossoming stages the (-)-menthyl acetate was high. This may be explained as a natural process where the total oil yields have been shown to peak at the beginning of floral initiation and continue to drop off until full bloom is reached (Murray et al. 1988). As the total oil yield drops, the total yield of (-)-menthol falls and the total yield of (-)-menthone drops considerably. Also the total yield of (+)-pulegone and (-)-menthyl acetate rises as blossoming of the plants progress (Fig. 2-12).

5.4. Density Analysis

Density is one of the several determinations used to qualify the commercial value of the oil. The optimum density of the oil is 0.9 g/mL (Fenaroli 1975). The density was determined using a gravimetric-volumetric technique. Density is defined as the amount of mass contained in a known volume and is temperature dependent. The density was calculated using the mass of 50 μL of oil which was used to make the sample mixture which was injected into the GC. This small volume was used due to the limited sample size. The density was calculated as:

$$\frac{\text{mass (g)}}{50\ \mu\text{L}} \times \frac{10^3\ \mu\text{L}}{1\ \text{mL}} = \frac{\text{mass (g)}}{0.050\ \text{mL}}$$

The density of the oil from different species and cultivars were analyzed using a Tukey's test ($P \leq 0.05$) for significant differences. At 50% and 75% bloom it was found that there were significant differences between the 3 varieties of mint ($P=0.0001$). The density of Native Spearmint was significantly higher than the 2 cultivars of peppermint. It was found that there was no significant difference between the two cultivars of peppermint (Table 5-8). At 100% bloom the 3 cultivars were not significantly different ($P=0.5553$) from

Table 5-8: Analysis of the Density of the Three Mint Plants at Three Different Bloom Stages

Bloom Stage	50%	75%	100%
Native Spearmint	0.897 ±0.035[1] A[2]	0.895 ±0.039 A	0.898 ±0.026 A
Black Mitchum Peppermint	0.818 ±0.046 B	0.830 ±0.058 B	0.869 ±0.056 A
Murray Mitchum Peppermint	0.825 ±0.039 B	0.807 ±0.021 B	0.904 ±0.000 A

[1] standard deviation of the mean

[2] different letters following the means denote significant differences ($P \leq 0.05$) within a bloom stage

each other (Table 5-8). The density of Native Spearmint meets the commercial quality specifications (0.9 g/mL (Fenaroli 1975)) at 50% (0.90 g/mL), 75% (0.89 g/mL) and 100% (0.90 g/mL) bloom. The density of the Black Mitchum Peppermint oil: 0.82 g/mL, 0.83 g/mL and 0.87 g/mL at 50%, 75% and 100% bloom respectively, and the density of the Murray Mitchum Peppermint: 0.82 g/mL, 0.81 g/mL and 0.90 g/mL at 50%, 75% and 100% bloom are only marginally acceptable as a commercial mint oil product.

5.5. Refractive Index Analysis

The refractive index of mint oil is commonly used to evaluate the quality of the oil for commercial value. The optimum refractive index of mint oil is 1.46-1.49 at 20°C (Fenaroli 1975). Refractive index is defined as the ratio of the velocity of light in air to the velocity of light in the medium being measured. Refractive index is a measure of the total components. On a per mole basis it is the sum of the refraction by all bonds and atoms present in the sample. Hence it is an approximate measure of the actual total volume of the molecules in one gram mole (Weast 1977). With respect to the mint oil, the more constituents present in the sample the greater the refractive index and therefore the higher the oil quality. The essential oil fractions were subjected to refractive index measurements. A drop of oil was placed on the sample area of a refractometer (Fisher-Scientific Refractometer) and the measurement recorded at 20.2-21.9°C.

The results from different cultivars were analyzed using a Tukey's test ($P \leq 0.05$) for significant differences. At 50% and 75% bloom it was found that the refractive index of

Native Spearmint was significantly higher than the 2 cultivars of peppermint which were not significantly different from each other (Table 5-9). At full bloom, the refractive index of the two peppermint cultivars were approximately equal to the value of the refractive index of Native Spearmint. It is important to note that the refractive indices of all three cultivars, at all three bloom stages, meet the commercial quality specifications of 1.46-1.49 (Fenaroli 1975).

Table 5-9: Analysis of the Refractive Indices of the Three Mint Plants at Three Different Bloom Stages

Bloom Stage	50%		75%		100%	
Native Spearmint	1.4925 ±0.0058[1]	A[2]	1.4952 ±0.0048	A	1.4941 ±0.0111	A
Black Mitchum Peppermint	1.4630 ±0.0029	B	1.4628 ±0.0030	B	1.4606 ±0.0052	B
Murray Mitchum Peppermint	1.4634 ±0.0035	B	1.4628 ±0.0034	B	1.4601 ±0.0001	B

[1] standard deviation of the mean

[2] different letters following the means denote significant differences ($P \leq 0.05$) within a bloom stage

6.0 Antioxidant Properties of Mint Oil and Its Constituents

6.1. Objective

The purpose of this part of the project was to determine if mint oil and selected constituents of mint oil ((-)-menthol, (-)-menthone, (-)-menthyl acetate and (+)-neomenthol) exhibit any antioxidant effect on a model system of unsaturated vegetable oil. The progression and extent of any oxidation or antioxidation effect was followed using tests to determine the peroxide value (PV) and thiobarbituric acid (TBA) value of the oil system over time.

6.2. Mint Oil as an Antioxidant

Mint oil from the genus *Mentha* is comprised of many components, some which because of their interconversion via oxidation-reduction reactions may, as speculated by the present author, exhibit antioxidant activity. (+)-Pulegone is the most oxidized of the major mint oil constituents and (-)-menthyl acetate is the least oxidized (i.e. most reduced). It is speculated that due to the oxidation level (-)-menthyl acetate may serve well as an antioxidant followed in antioxidant capability by (-)-menthol, (-)-menthone and (+)-pulegone. (+)-Pulegone can theoretically be expected to have the poorest antioxidant capability because of its highly oxidized chemical structure as can be seen in Fig. 2-10 and 2-11.

The peroxide test determines the extent of primary oxidation reaction which has occurred by measuring the resultant concentrations of hydroxides and peroxides. A peroxide value (PV) is obtained via the reaction of thiosulphate and peroxide using starch and iodine to indicate the end point. Peroxide values are expressed in units of milliequivalents of peroxides per kg of sample.

The thiobarbituric acid test (TBA) detects the amount of tertiary oxidation products in the form of aldehydes and ketones. These are final stage by-products of lipid oxidation. There is a colourimetric reaction between the thiobarbituric acid and the carbonyl groups to result in a production of a red complex. Absorbance (540 nm) is used to quantify the extent of the reaction. TBA values are expressed as milliequivalents per kg of sample (Dirinck et al. 1996).

6.3. Experimental Design

The sampling period to test the oxidation was 28 days with 8 sampling times (0, 1, 2, 4, 8, 15, 22, and 28 days). The primary treatments consisted of commercial mint oil (GR) and selected constituents (menthol, menthone, menthyl acetate and neomenthol). The PV and TBA values were measured in order to determine the type and extent of oxidation over time. The experiment was conducted as a Repeated Measures Analysis. The model used was:

$$\text{response (PV or TBA)} = \mu_i + \alpha_j + \epsilon_{ij}$$

μ_i - mean
α_j - treatment
ϵ_{ij} - error

6.4. Sample Preparation and Storage

The vegetable oil was Northern Lite Canola Oil, containing canola oil and dimethylpolysiloxane purchased from a local grocery store. Approximately 800 g of oil were weighed and 1% w/w of the treatment constituent was added (Table 6-1).

Table 6-1: Concentration of Antioxidant in Oil

treatment	oil mass / g	constituent mass / g	Total mass / g	Percentage of Treatment / %
Givauden-Roure Mint Oil	801	8.7275	810	1.078
(-)-menthol	801	8.2050	809	1.014
(-)-menthone	821	8.9196	830	1.075
(-)-menthyl Acetate	801	5.5041	807	0.682
(+)-neomenthol	800	7.8112	808	0.967
control	804	0.0000	804	0.000

A mass of 30-35 g of the vegetable oil mixture was placed in an Erlenmeyer flask with 25-30 mL of water according to a method previously used for this type of study (Frankel et al. 1996). The top was covered with cheesecloth to allow the entry of air and to prevent contamination from the environment. The samples were stored at 20°C in the dark except during mechanical aeration.

6.5. Oxidation Method

From day 0 through day 14 each sample was shaken daily for 30 minutes on a rotary shaker (300 rpm; Fermentation Design) (Frankel et al. 1996). From day 15 until the end of the experiment, the aeration period was increased from 30 to 60 minutes due to a lack of

oxidation. The experiment was lengthened by 29 days with 2 added sampling times to a total of 57 days to allow more time for oxidation to occur. In lengthening the experiment it was expected that over the 57 day experiment there would be a significant level of oxidation within the control with which the test treatments could be compared.

6.6. Peroxide Value Test

The peroxide values of the samples were measured at the times specified in the design. The method for determining the peroxide value of the sample was a modification of an AOAC method (Coniff 1995). Approximately 50 mL of a 3:2 mixture of acetic acid:chloroform (Fisher Scientific) was added to 10 g of the sample. To this a saturated solution of potassium iodide (Fisher Scientific) (1 mL) was added and the mixture was shaken manually for 1 minute. About 100 mL of distilled water was added to the mixture followed by 1 mL of fresh 1% starch indicator solution. The aqueous layer became a deep blue colour as the iodine formed from the reaction of the peroxides, complexed with the starch. The mixture was titrated with 0.02 N sodium thiosulphate to the equivalence point. The titrant volume was converted to milliequivalents of peroxide per kilogram of vegetable oil using the following formula:

$$M = \frac{T \times N}{W} \times 1000$$

M - milliequivalents of peroxide per kilogram of oil
T - millilitres of thiosulphate
N - normality of thiosulphate
W - mass of sample

Statistical analysis of the Peroxide Values (Table 6-2, Fig. 6-1) showed that there was significant difference ($P \leq 0.05$) using the model for Repeated Measures Analysis of Variance. Further analysis of the data also showed that for menthyl acetate, the PV was significantly higher than the other five test components which were not significantly different from each other ($P \leq 0.05$) (Figure 6-1). The commercial mint oil sample was found to be not significantly different ($P \leq 0.05$) from the control or from the following test components of (-)-menthol, (-)-menthone and (+)-neomenthol. This indicated that there was not any significant antioxidant effect exhibited by mint oil and selected components against the oxidation of vegetable oil.

(-)-Menthyl acetate, showing a significantly higher oxidation value than the control, may have actually increased the oxidation of the oil and thus acted as a pro-oxidant. A reaction between (-)-menthyl acetate and the vegetable oil may have catalysed the oxidation process.

Table 6-2: Peroxide Values Determined Over Time

treatment\day	0	1	2
commercial mint oil	3.61 ±1.22	2.86 ±0.97	4.54 ±1.06
(-)-menthyl acetate	5.76 ±0.92	6.40 ±0.99	7.59 ±0.79
(-)-menthol	4.27 ±0.56	5.44 ±1.08	4.92 ±1.17
(-)-menthone	5.29 ±0.92	4.59 ±0.56	5.14 ±1.29
(+)-neomenthol	3.69 ±0.38	3.49 ±0.34	6.29 ±1.11
control	3.54 ±0.24	4.65 ±0.79	4.39 ±1.24
treatment\day	4	8	15
commercial mint oil	5.85 ±0.97	8.29 ±1.92	7.26 ±0.87
(-)-menthyl acetate	7.35 ±2.81	10.07 ±1.34	12.00 ±1.62
(-)-menthol	7.21 ±2.35	7.05 ±1.37	11.19 ±1.43
(-)-menthone	6.75 ±0.62	7.92 ±2.38	9.29 ±1.79
(+)-neomenthol	6.36 ±1.66	6.59 ±1.23	8.78 ±2.31
control	5.20 ±0.18	7.56 ±1.62	10.74 ±4.30
treatment\day	28	43	57
commercial mint oil	11.32 ±3.90	19.51 ±2.03	35.37 ±12.78
(-)-menthyl acetate	12.68 ±2.99	20.23 ±1.55	81.56 ±28.26
(-)-menthol	21.66 ±0.30	28.83 ±13.20	35.02 ±6.92
(-)-menthone	14.97 ±4.95	21.41 ±7.60	24.07 ±3.64
(+)-neomenthol	17.56 ±4.50	24.43 ±5.68	26.83 ±3.42
control	26.16 ±8.94	13.79 ±3.20	27.05 ±6.55

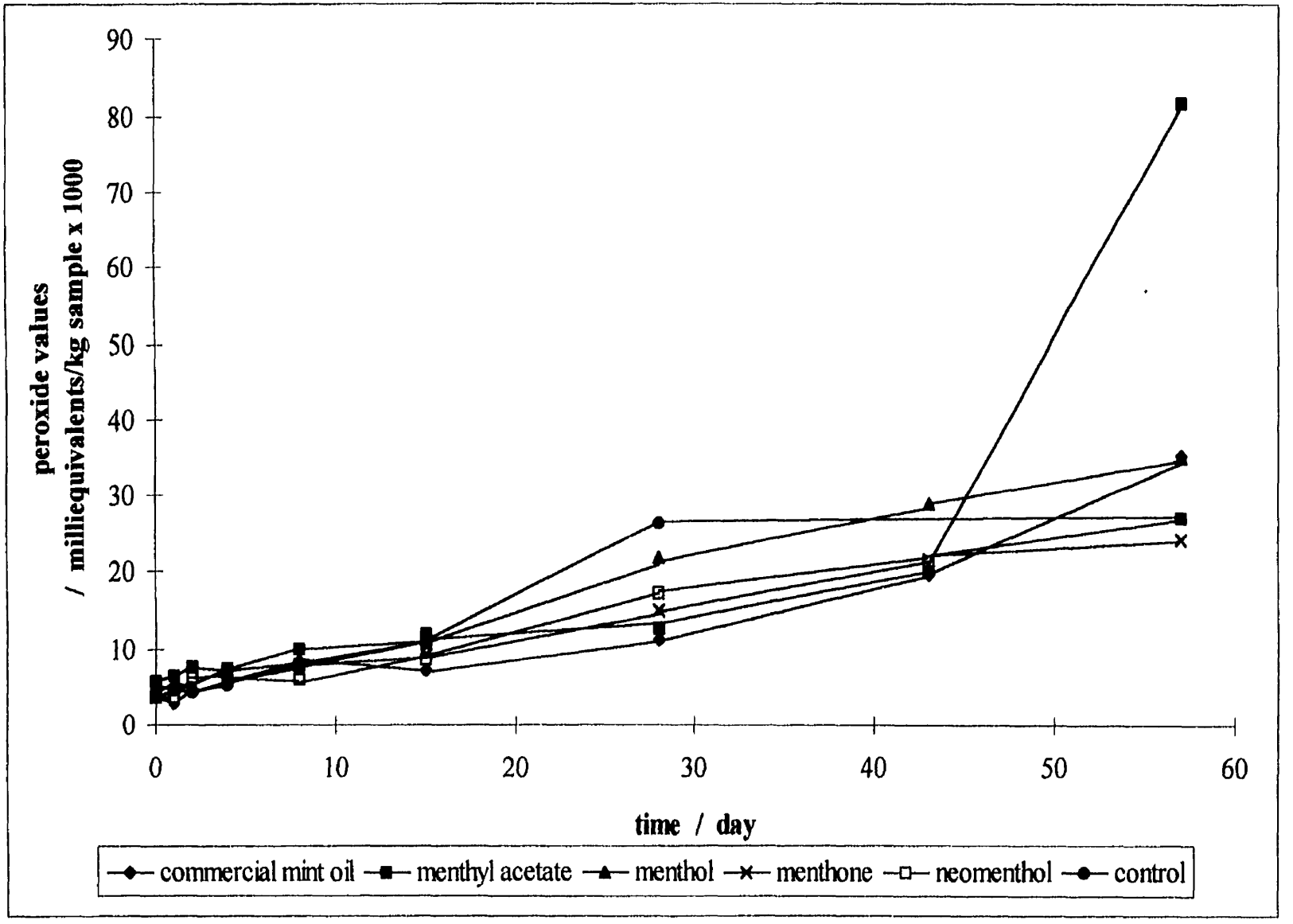

Fig. 6-1: Oxidation (as Measured by Peroxide Values) Over Time
error bars not included for clarity

6.7. Thiobarbituric Acid Test

The vegetable oil samples were measured at intervals specified in the design for the thiobarbituric acid (TBA) value. In determining the TBA value a modification of a method used in Tsimidou et al. (1995) was used. A 1.5 g vegetable oil sample was massed into a teflon-lined screw-capped test tube. Each set of samples was run with a blank which consisted of a test tube containing water, but no vegetable oil. Each sample was dissolved in 5 mL of chloroform (Fisher Scientific). To this 5 mL of TBA reagent (1:1 solution of acetic acid:0.67 g/L thiobarbituric acid (Fisher Scientific) in water) was added. The samples were capped and shaken by hand for four minutes and the upper, aqueous layer, was removed and transferred to a second test tube. The aqueous layer in the test tube was placed in a boiling water bath for 20 minutes. After cooling, the solution was measured at a wavelength of 540 nm on a UV-Visible spectrophotometer (Beckman DU-70). The TBA value was calculated in the following manner:

$$\text{TBA value} = \frac{(A - B) \times 100}{W}$$

A - absorbance of sample
B - absorbance of blank
W - mass of oil sample

Statistical analysis of the TBA Values (Table 6-3, Fig. 6-2) found that there was no significant difference ($P \leq 0.05$) using the model Repeated Measures Analysis of Variance.

Table 6-3: Thiobarbituric Acid Values Determined Over Time

treatment\day	0	1	2	4
commercial mint oil	0.98 ±0.10	3.77 ±2.75	N/D	22.51 ±10.85
(-)-menthyl acetate	3.93 ±1.96	0.91 ±0.98	3.79 ±2.31	16.86 ±10.46
(-)-menthol	2.04 ±2.00	3.62 ±1.27	3.96 ±3.82	25.02 ±7.62
(-)-menthone	3.24 ±1.90	4.02 ±2.38	8.78 ±6.07	13.77 ±4.66
(+)-neomenthol	5.94 ±1.92	6.75 ±7.61	14.98 ±11.87	7.37 ±2.65
control	1.42 ±0.82	12.05 ±2.47	13.82 ±7.03	21.67 ±5.82
treatment\day	**8**	**28**	**43**	**57**
commercial mint oil	16.92 ±21.38	32.79 ±20.92	106.12 ±11.79	132.62 ±80.68
(-)-menthyl acetate	14.57 ±11.12	75.18 ±54.12	65.12 ±50.49	149.97 ±52.81
(-)-menthol	25.41 ±17.68	80.66 ±17.89	78.08 ±55.37	118.42 ±46.60
(-)-menthone	29.37 ±20.27	45.85 ±32.40	67.56 ±47.89	105.17 ±39.16
(+)-neomenthol	4.22 ±3.50	46.49 ±26.82	86.48 ±24.72	163.34 ±18.74
control	4.27 ±5.88	99.08 ±38.79	104.69 ±78.54	97.54 ±68.63

N/D - not detectable

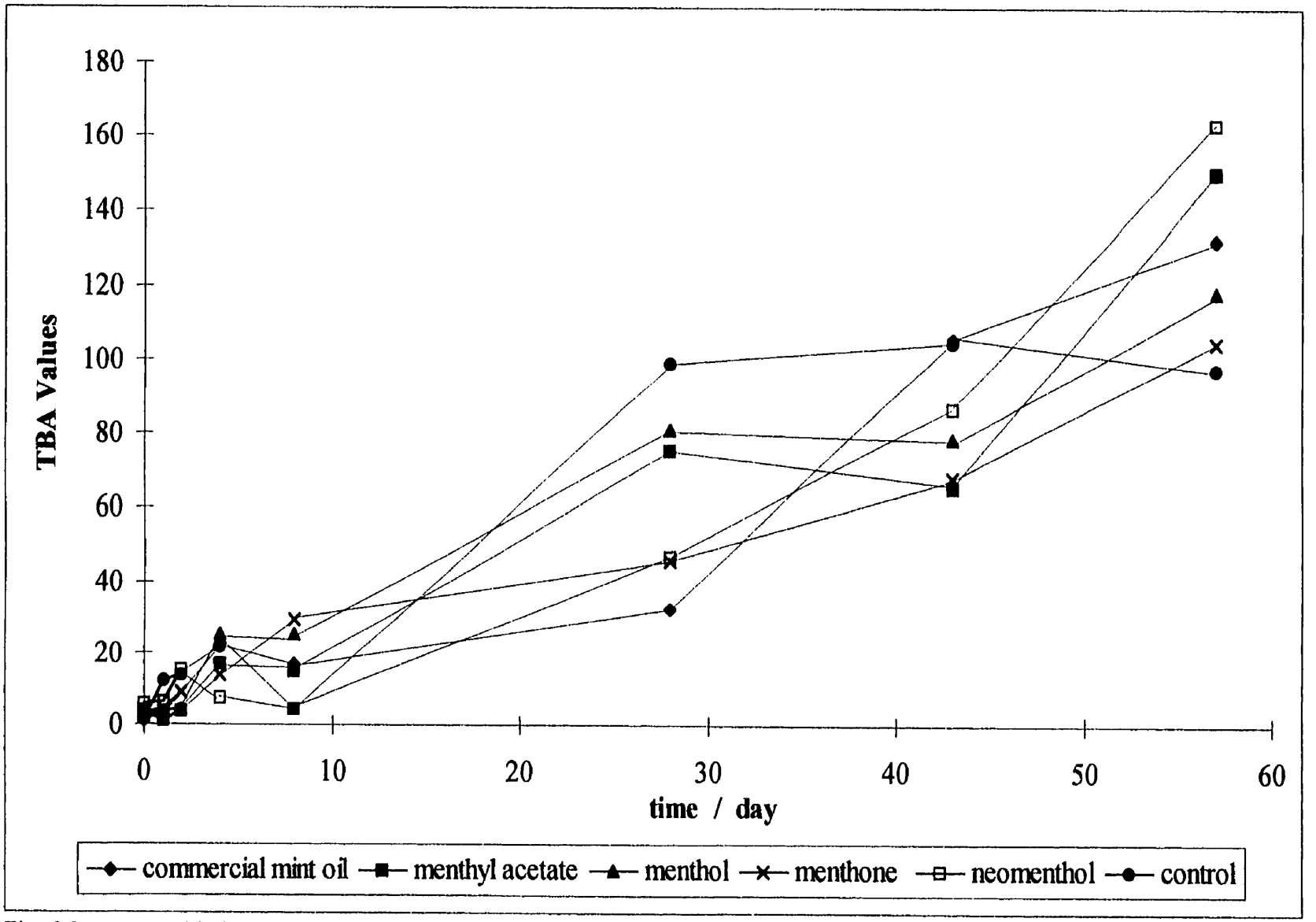

Fig. 6-2: Oxidation (as Measured by Peroxide Values) Over Time
error bars not included for clarity

6.8. Antioxidant Effect of the Mint Oil Constituents

Comparison of the TBA and Peroxide Values indicated that there was no apparent antioxidant effect exerted by commercial mint oil or its selected components in the vegetable oil system. Based on peroxide value the Givauden-Roure mint oil and the individual constituents may have suppressed the first stage of oxidation (day 15-27), indicating the treatments were preventing oxidation of the vegetable oil as intended, but by the end of two months there was no antioxidant effect on primary oxidation as evidenced by a rise in perioxide value at day 28 and TBA values at days 43 and 57.

The increase in the Peroxide Value of the menthyl acetate trial is not supported by the corresponding TBA values. The oxidation may not have progressed enough to be able to detect the formation of the secondary oxidation products by the TBA tests. Changes would be required for future experiments of this type. The foremost would be an extended analysis period, 120 days, the second would be an oxidation system, which would have an increased environmental temperature, 30-35°C and an oxygenating system which would bubble into the oil (Frankel et al. 1996).

7.0. Conclusions

7.1. Establishment of Mint in Nova Scotia for Commercial Production

In establishment of the mint stands during this study, many difficulties were experienced. There was a drought during the first six weeks of the field trial. Stress from the summer heat and lack of water resulted in the premature floral initiation of the plants. Due to a lack of foliar growth at the time of floral initiation, the blossoms were removed to promote further vegetative growth. At floral initiation the total oil yield is expected to peak, and then rapidly decrease as time progresses (Fig. 2-12). The oil yields recovered from this study followed the expected trend; there was relatively higher oil yields recovered for peppermint and spearmint at the 50% bloom stage, than for samples taken at 100% bloom stage (Table 4-1). Another agronomic factor adversely affecting the oil yields was the presence of a rust infection (*Puccinia menthae*), noted soon after deblossoming. The rust infection resulted in the desiccation of the leaves. This lowered the oil yield (0.21-0.55%) from the expected 2-4%.

7.2. Quality of the Nova Scotian Produced Mint Oil

There were three aspects of the mint oil which were considered when the quality of the oil was evaluated: percentage composition, density and refractive index. The percentage composition of the *Mentha spicata* cv. Native Spearmint, when compared to the literature, exhibited lower than expected concentrations of (-)-menthol and (-)-

menthone. The premature floral initiation of the Native Spearmint and the subsequent deblossoming may account for this occurrence. After floral initiation, the concentration of (-)-menthone will decrease (Fig. 2-12). In addition, an infection by rust may lead to a decrease in the concentration of (-)-menthol (Nadaska et al. 1990).

The (-)-menthol concentrations of the two cultivars of peppermint (*Mentha piperita* cv. Black Mitchum and *Mentha piperita* cv. Murray Mitchum) ranged from 58.6-59.5 g/100 g at the 75% and 100 % bloom stages. These are high when compared with the commercial peppermint oil analysis provided by Givauden-Roure (Table 5-1) and the acceptable standards for peppermint oil (Table 2-3). At the 75% and 100 % bloom stages the (-)-menthone content of the two peppermint cultivars was low (4.0 - 7.5 g/100 g) when compared with the standards. At the 50% bloom stage the concentration of (-)-menthone (19.6 - 24.4 g/100 g) of both cultivars of peppermint was comparable to the standards found for the oil (Table 2-3), but was higher than that analyzed in the oil provided by Givauden-Roure. The concentration of (-)-menthol in the Black Mitchum Peppermint (38.0 g/100 g) was comparable to the values associated with the standard peppermint oils, but slightly lower than that found in the oil from Givauden-Roure. The concentration of (-)-menthol in the Murray Mitchum Peppermint (52.2 g/100 g) was higher than those associated with the standard peppermint values and the commercial peppermint oil. Depending upon the end use of the peppermint oil it is likely that this would be acceptable since commercially, oils are blended to produce various peppermint flavours.

Although slight differences were observed, the other constituents (β-caryophyllene, 1,8-cineole, (-)-limonene, (-)-menthyl acetate, (+)-neomenthol, α-pinene and (+)-pulegone) all compared favourably with the standard peppermint oils and the commercial peppermint oil provided by Givauden-Roure.

The density (0.90 g/mL) of the Native Spearmint compared favourably with the literature value (0.90 g/mL), as did those of the two peppermints (0.81-0.90 g/mL). The refractive indices of the Native Spearmint, Black Mitchum Peppermint and Murray Mitchum Peppermint ranged from 1.46-1.49 which matches the desired value of 1.46-1.49. This would indicate that Nova Scotia is suitable for the growth of peppermint as a commercial crop, but further investigation is required for the potential of Native Spearmint. This may be that an infection by *Puccinia menthae* is more damaging to Native Spearmint than peppermint. A relatively unstressed, rust-free crop of Native Spearmint may produce significantly different results.

7.3. Antioxidant Effect of the Mint Oil Constituents

An examination of the TBA and Peroxide Values indicated that there was no antioxidant effect exerted by commercial mint oil or its selected components in the oil system. Based on the peroxide values of the Givauden-Roure mint oil and the individual constituents, intial oxidation of the vegetable oil may have been suppressed (day 15-27), but by the end of two months there was no evidence of antioxidant effect on primary oxidation as evidenced by a rise in perioxide value of the treatments and the control at day 28 and TBA values at days 43 and 57.

The short duration of this oxidation study may not have allowed sufficient time for oxidation to proceed to the formation and detection of the secondary oxidation product by the TBA test. For future study, modifications of the experiment would be required. These should include an extended analysis period (e.g. 120 days), an environment with an increased temperature (30-35°C) and an aeration system which would continuously bubble oxygen or air into the oil (Frankel et al. 1996). This modification would be expected to increase the oxidation within the control sample. Increases in the oxidation of untreated sample when compared to the treated samples should provide stronger evidence towards the inhibition, if any, of oxidation within the sample.

7.4. Potential of Nova Scotia Produced Mint Oil

Due to the adverse conditions of the first season as well as the continual rust infection, the results of the first season were unreliable. The Native Spearmint offered little hope for potential as a new crop within Nova Scotia. The oil yield was low and the menthone and menthol concentrations were unacceptable to the commercial flavour industry. The menthol and menthone concentrations were less then 1 g/100 g in all cases except for menthol at 50% bloom (3.18 g/100 g). It is speculated that this was due to the rust infection, to which spearmint appears to be more susceptible than peppermint. Within both cultivars of peppermint, the potential for its use as a new crop is hopeful. The mint oil had a low oil yield. The rust infection which affected the peppermint plants, although not as severe as the spearmint plants, did decrease the resultant oil yield.

Further, it is speculated that the low oil yield was a result of the climatic conditions of the 1994 and 1995 growing season. The concentration of the mint oil constituents indicated that Nova Scotia may be able to produce a peppermint oil of acceptable commercial quality.

It is the recommendation of this author that a new project to evaluate the potential of peppermint growth in Nova Scotia should be attempted. This should involve a scale up of the project to simulate a small commercial operation. A larger scale distillation unit should be employed to determine the amount that would be recovered on a commercial scale. Infection problems with diseases, fungi and insects could be diminished or inhibited through the use of plant materials obtained by tissue culture. Advances in tissue culture may be used to remove a potential infection (as is with the case with the systemic infection of *Puccinia menthae*), or to endow the plant with genes to enable it to repel and better survive an infection. Soil amendments and irrigation of the plot areas should be carefully planned and implemented. Other species and cultivars, including Native Spearmint, Scotch Spearmint, Todd's Mitchum Peppermint, etc. should be grown on a small scale and tested for their potential as a commercial crop.

8.0. Appendices

8.1. Percentage Composition of *Mentha* Test Species

Table 8-1: Percentage Composition of *Mentha* Test Species

	Todd's Mitchum Peppermint	Scotch Spearmint
Oil Recovery / mL/100 g	0.32 ±0.10[1]	0.47 ±0.09
	Percentage Composition / g/100 g	
Components		
β-caryophyllene	0.60 ±0.08	0.46 ±0.52
1,8-cineole	5.63 ±0.95	1.84 ±1.18
(-)-limonene	1.00 ±0.78	10.84 ±1.18
(-)-menthyl acetate	2.69 ±0.77	N/D
(-)-menthol	42.07 ±22.94	0.38 ±0.47
(-)-menthone	18.08 ±15.64	0.61 ±0.48
(+)-neomenthol	2.68 ±0.60	0.27 ±0.46
α-pinene	0.74 ±0.58	1.48 ±1.02
(+)-pulegone	0.21 ±0.20	0.43 ±0.48
Density / g/mL	0.905 ±0.029	0.884 ±0.021
Refractive Index	1.4624 ±0.0059	1.4958 ±0.0035

[1] standard deviation of the mean
N/D - not detectable

8.2. Soil Characteristics

Table 8-2: Soil Characteristics of the Two Nova Scotia Sites as Tested in 1994 (Harnish 1994)

Characteristic	Bible Hill Site	Maitland Site
organic matter	5.6%	5.4%
pH	7.2	5.6
phosphate	479 kg/ha	215 kg/ha
potash	237 kg/ha	171 kg/ha
calcium	3106 kg/ha	1183 kg/ha
magnesium	768 kg/ha	105 kg/ha

8.3. Survival Rating Data for the Mint Plants Over the Winter of 1994-1995

Table 8-3: Survival Rating Data for the 1994-1995 Season

	Bloom Stage / %	Location	Surviving Plants in 1995	Plants in 1994	Survival Rating / %
Native Spearmint	50	Maitland	55	180	30.6
Native Spearmint	50	Bible Hill	8	180	4.4
Native Spearmint	75	Maitland	70	180	38.9
Native Spearmint	75	Bible Hill	8	180	4.4
Native Spearmint	100	Maitland	90	180	50.0
Native Spearmint	100	Bible Hill	14	180	7.8
Black Mitchum Peppermint	50	Maitland	46	180	25.6
Black Mitchum Peppermint	50	Bible Hill	43	180	23.9
Black Mitchum Peppermint	75	Maitland	67	180	37.2
Black Mitchum Peppermint	75	Bible Hill	9	180	5.0
Black Mitchum Peppermint	100	Maitland	59	180	32.8
Black Mitchum Peppermint	100	Bible Hill	29	180	16.1
Murray Mitchum Peppermint	50	Maitland	36	180	20.0
Murray Mitchum Peppermint	50	Bible Hill	9	180	5.0
Murray Mitchum Peppermint	75	Maitland	71	180	39.4
Murray Mitchum Peppermint	75	Bible Hill	5	180	2.8
Murray Mitchum Peppermint	100	Maitland	45	180	25.0
Murray Mitchum Peppermint	100	Bible Hill	19	180	10.6

8.4. LAI Data

Table 8-4: Leaf Area Data

Location	Cultivar	Replication	Bloom Stage / %	Aug 15-16	Aug 17-23	Aug 30-Sept 2	Sept 6-9	Sept 14-16	20-Sep	21-Sep
BH	A	1	50	6.18	6.06	.	.	.	.	.
BH	A	1	75	4.47	4.79	3.51	.	2.43	.	2.05
BH	A	1	100	5.51	5.08	4.77	.	3.17	.	2.56
BH	A	2	50	4.87	5.11	.	.	.	.	.
BH	A	2	75	4.76	4.19	3.79	.	3.39	.	2.69
BH	A	2	100	5.08	5.39	4.44	.	3.31	.	2.7
BH	A	3	50	4.73	5.85	.	.	.	.	.
BH	A	3	75	4.37	4.86	.	.	.	.	.
BH	A	3	100	4.31	4.74	3.75	.	2.88	2.43	.
BH	A	4	50	4.81	4.57	.	.	.	.	.
BH	A	4	75	5.76	4.73	3.75	.	2.43	1.95	.
BH	A	4	100	5.8	4.86	3.85	.	3.13	2.38	.
BH	B	1	50	5.71	5.6	.	.	.	.	.
BH	B	1	75	5.19	5.55	3.79	.	2.73	.	1.92
BH	B	1	100	7.94	7.28	4.79	.	3.98	.	2.58
BH	B	2	50	6.04	6.26	.	.	.	.	.
BH	B	2	75	7.57	6.64	4.79	.	3.13	.	2.36
BH	B	2	100	6.2	5.85	3.93	.	3.11	.	2.45
BH	B	3	50	5.81	5.94	5.295	.	.	.	.

LAI data continued on following page

LAI data continued

BH	B	3	75	4.78	5.03	4.64	.	.	2	.
BH	B	3	100	3.94	4.82	3.3	.	2.4	.	.
BH	B	4	50	.	4.5	.	.	.	.	.
BH	B	4	75	6.45	5.97	3.91	.	2.59	1.91	.
BH	B	4	100	4.69	4.75	3.54	.	2.77	2.21	.
BH	C	1	50	3.53	3.23	.	.	.	.	.
BH	C	1	75	3.03	3.21	4.12	.	2.54	.	2.21
BH	C	1	100	4.46	4.45	5	.	4.03	.	3.16
BH	C	2	50	3.55	3.86	.	.	.	.	.
BH	C	2	75	1.77	2.44	2.97	.	2.27	.	2.07
BH	C	2	100	2.71	3.55	4.58	.	3.52	.	2.23
BH	C	3	50		1.05	0.78	.	0.68	0.62	.
BH	C	3	75	1.54	1.36	1.24	.	1.76	1.12	.
BH	C	3	100	1.46	1.56	1.17	.	1.25	1.22	.
BH	C	4	50	3.6	3.43	.	.	.	.	.
BH	C	4	75	1.37	1.11	1.55	.	1.24	1.04	.
BH	C	4	100	1.22	1.44	1.45	.	0.94	1.03	.
MA	A	1	50	2.22	4.23	.	.	.	.	.
MA	A	1	75	2.14	5.71	.	.	.	.	.
MA	A	1	100	2.32	4.54	3.69	3.77	2.8	2.77	.
MA	A	2	50	2.65	3.54	.	.	.	.	.
MA	A	2	75	2.36	3.75	.	.	.	.	.

LAI data continued on following page

LAI data continued

MA	A	2	100	2.9	3.93	3.13	2.93	2.45	2.16	.
MA	A	3	50	3.59	3.9	.	.	.	.	.
MA	A	3	75	3.39	4.36	3.75	3.71	3.73	3.74	.
MA	A	3	100	3.65	4.26	3.45	2.65	2.75	2.64	.
MA	A	4	100	4.04	4.06	3.46	3.5	2.77	2.49	.
MA	B	1	50	4	3.46	.	.	.	.	.
MA	B	1	75	2.61	3.93	2.84	2.47	1.86	1.75	.
MA	B	1	100	2.66	2.96	2.72	2.3	1.57	1.7	.
MA	B	2	50	3.35	4.08	.	.	.	.	.
MA	B	2	75	3.66	4.61	4.05	3.62	2.17	2.48	.
MA	B	2	100	3.36	3.25	3.08	2.16	1.6	1.86	.
MA	B	3	50	3.58	3.93	.	.	.	.	.
MA	B	3	75	2.73	2.94	2.19	2.01	1.63	1.53	.
MA	B	3	100	3.1	3.94	3.3	2.24	2.07	1.94	.
MA	B	4	50	3.85	4.09	.	.	.	.	.
MA	B	4	75	3.68	3.98	2.9	2.58	2.53	1.86	.
MA	B	4	100	3.24	3.39	2.99	2.42	2.18	1.68	.
MA	C	1	50	1.22	2.45	.	.	.	.	.
MA	C	1	75	1.08	1.75	2.12	2.08	1.85	1.65	.
MA	C	1	100	1.23	2.63	2.79	2.6	1.66	2.43	.
MA	C	2	50	1.57	2.63	3.23	2.645	.	.	.
MA	C	2	75	2.78	4.68	5.1	.	3.89	3.58	.

LAI data continued on following page

LAI data continued

MA	C	2	100	1.74	2.33	2.95	3.21	3.39	3.22	.
MA	C	3	50	1.68	2.41	2.12	.	.	.	.
MA	C	3	75	2.1	2.98	3.74	3.12	3.73	2.7	.
MA	C	3	100	2.17	2.44	2.71	2.54	2.22	1.4	.
MA	C	4	50	2.59	2.845	3.18	3.72	3.16	.	.
MA	C	4	75	2.64	.	.	.	.	3.52	.
MA	C	4	100	2.51	2.93	2.81	2.57	2.88	2.08	.

Symbols:

BH - Bible Hill Site
MA - Maitland Site

A - Native Spearmint
B - Black Mitchum Peppermint
C - Murray Mitchum Peppermint

8.5. Statistics Programs Used within SAS

8.5.0. To Analyze All Mint Plot Data

```
DATA mmratx;
OPTIONS ls=70;
   INPUT cultivar $ rep bloomstg $ blmstg $ location $ year subsam $ mmratx;
PROC GLM;
   CLASS year location cultivar;
      MODEL mmratx = year location year*location cultivar year*cultivar
location*cultivar year*location*cultivar;
      MEANS year / tukey;
      MEANS cultivar / tukey;
      MEANS location / tukey;
      OUTPUT out=new R=resids P=predicts;
   TEST h=location e=year*location;
   TEST h=cultivar e=year*cultivar;
   TEST h=location*cultivar e=year*location*cultivar;
PROC PLOT;
   PLOT resids*predicts;
PROC UNIVARIATE normal plot;
   VAR resids;
PROC PRINT;
   VAR resids predicts;
RUN;
```

8.5.1. To Analyse the Antioxidant Data

```
DATA ANTIOX;
OPTIONS ls=70;
    INPUT treat $ rep $ PV1 PV2 PV3 PV4 PV5 PV6 PV7 PV8 PV9;
PROC GLM;
    CLASS treat;
        MODEL PV1-PV9=treat;
    REPEATED DAY 8 (0 1 2 4 8 15 28 43 56) polynomial;
    REPEATED DAY 8 (0 1 2 4 8 15 28 43 56) contrast (1);
    REPEATED DAY 8 (0 1 2 4 8 15 28 43 56) profile;
        OUTPUT out=new R=resids P=predicts;
PROC PLOT;
    PLOT resids*predicts;
PROC PRINT;
    VAR resids predicts;
PROC UNIVARIATE;
    var resids;
RUN;
```

8.6. Sample Calculation of Percentage Composition

raw area data entered
Internal Standard (IS) mass entered

ratio of IS area:IS mass
IS area / IS mass

IS correction factor
average IS area:IS mass correction factor **x** (IS mass / IS area)

IS corrected area
IS correction factor **x** raw area

Check Standard (CS) correction factor
average of individual CS component / individual CS component

CS corrected area
CS correction factor **x** IS corrected area

concentration of standards were calculated as g of standard in 10 mL
mass in 1 uL was calculated
standard curves were calculated as ug vs IS corrected area

mass in each injected sample was calculated
$x = (y - b) / m$

mass in diluted sample was calculated
mass in injected sample **x** (1 / 1 uL) **x** 1000 uL

mass of oil sample was entered

% composition in oil was calculated
(mass in diluted sample / mass of oil sample) **x** (1 g / 1000000 ug)

8.7. Percentage Composition of Mint Oil

Table 8-5.: Mint Oil Composition at 50% Bloom (Fig. 5-1)

	Black Mitchum Peppermint		Murray Mitchum Peppermint		Native Spearmint	
β-caryophyllene	1.09 ±0.40[1]	A[2]	1.05 ±0.40	A	0.78 ±0.40	A
1,8-cineole	4.64 ±0.74	A	4.82 ±0.69	A	1.39 ±0.68	B
limonene	0.79 ±0.39	B	1.10 ±0.54	B	5.20 ±1.21	A
menthyl acetate	0.63 ±0.43	A	1.01 ±0.66	A	0.03 ±0.14	B
menthol	38.01 ±18.94	A	52.16 ±25.75	A	3.18 ±12.38	B
menthone	24.44 ±4.64	A	19.62 ±7.96	B	N/D	C
neomenthol	1.64 ±0.62	A	1.89 ±0.59	A	0.10 ±0.41	B
α-pinene	0.32 ±0.23	A	0.30 ±0.34	A	0.36 ±0.35	A
pulegone	0.52 ±0.25	A	0.42 ±0.31	A	0.11 ±0.20	B

[1] standard deviation of the mean

[2] different letters following the means denote significant differences ($P \leq 0.05$) within a bloom stage

N/D - not detectable

Table 8-6: Mint Oil Composition at 75% Bloom (Fig. 5-2)

	Black Mitchum Peppermint		Murray Mitchum Peppermint		Native Spearmint	
β-caryophyllene	0.80 ±0.40[1]	A[2]	0.72 ±0.48	A	0.70 ±0.52	A
1,8-cineole	4.66 ±0.95	A	4.87 ±1.51	A	1.20 ±0.88	B
limonene	1.01 ±0.57	B	1.16 ±0.61	B	4.86 ±1.30	A
menthyl acetate	2.64 ±0.45	B	3.92 ±1.24	A	N/D	C
menthol	59.25 ±4.67	A	58.59 ±4.75	A	0.49 ±0.93	B
menthone	7.09 ±2.84	A	4.03 ±2.73	B	0.03 ±0.13	C
neomenthol	2.81 ±0.63	A	2.80 ±0.40	A	0.07 ±0.23	B
α-pinene	0.66 ±0.55	A	0.71 ±0.53	A	0.46 ±0.54	A
pulegone	0.12 ±0.19	A	0.28 ±0.29	A	0.09 ±0.20	A

[1] standard deviation of the mean

[2] different letters following the means denote significant differences ($P \leq 0.05$) within a bloom stage

N/D - not detectable

Table 8-7: Mint Oil Composition at 100% Bloom (Fig. 5-3)

	Black Mitchum Peppermint		Murray Mitchum Peppermint		Native Spearmint	
β-caryophyllene	0.79 ±0.49[1]	A[2]	0.84 ±0.52	A	0.51 ±0.41	A
1,8-cineole	4.98 ±1.19	A	5.54 ±0.99	A	1.43 ±0.88	B
limonene	1.00 ±0.61	B	0.75 ±0.82	B	3.87 ±1.50	A
menthyl acetate	3.01 ±0.38	B	5.41 ±4.28	A	N/D	C
menthol	59.02 ±14.15	A	59.48 ±8.30	A	0.66 ±1.89	B
menthone	7.45 ±3.42	A	6.54 ±2.76	A	N/D	B
neomenthol	2.66 ±0.84	A	3.10 ±1.38	A	0.13 ±0.59	B
α-pinene	0.67 ±0.72	A	0.41 ±0.58	A	0.46 ±0.46	A
pulegone	0.21 ±0.41	A	0.22 ±0.39	A	0.14 ±0.29	A

[1] standard deviation of the mean

[2] different letters following the means denote significant differences ($P \leq 0.05$) within a bloom stage

N/D - not detectable

9.0. Literature Cited

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