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UNIVERSITY OF ALBERTA

INVESTIGATION OF MENTHOL RESIDUES IN ALBERTA HONEY AND BEESWAX

BY

MAOJUAN LI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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IN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled INVESTIGATION OF MENTHOL RESIDUES IN ALBERTA HONEY AND BEESWAX submitted by MAOJUAN LI in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in FOOD CHEMISTRY.

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Date July 28 1992

TO MY FATHER AND MOTHER

ABSTRACT

L-menthol was applied to hives of bees to control the honey bee tracheal mite (*Acarapis woodi*). The L-menthol residues in honey and beeswax were extracted by simultaneous steam distillation and solvent extraction, and analysed by gas chromatography (GC). The analysis of residue levels in honey revealed that pastes and strips of L-menthol at 30 g and 60 g dosages were effective in controlling the honey bee tracheal mite (*Acarapis woodi*) in northern Alberta during late spring and early summer. The results indicated that all honey samples from brood nest, with the exception of untreated controls, contained L-menthol residue levels from ≤ 0.1 to 18.0 ppm, with an average of 2.4 ppm. All honey samples from supers added after the L-menthol treatments contained no detectable L-menthol. L-menthol was also found in beeswax samples at higher levels. The residue levels of L-menthol in honey and beeswax were related to the methods of application. The L-menthol dosage, position in the hive and container had a great effect on the L-menthol residue levels. The detection of a minty taste of L-menthol in honey by untrained panelists appeared to be at a level of about 35 ppm L-menthol. There was no loss and detectable change of L-menthol in honey after a couple of months of storage at room temperature.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisor, Dr. P. Sporns, for his kindness in giving me the chance to study at this University; for his guidance, continuous encouragement and support throughout the course of this work.

My acknowledgment is also extended to Leonard Steele for his assistance in technical difficulties, and to various individuals for their participation in the sensory evaluations.

Finally, I would like to thank my husband, Zhida, for his endless support, encouragement, patience and love, and also for his helpful scientific suggestions and technical assistance throughout my research.

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LIST OF ABBREVIATIONS

A. woodi	*Acarapis woodi*
Am/Astd	Peak area ratio of L-menthol and internal standard
AOAC	Official Methods of Analysis of the Association of Official Analytical Chemists
AR	Peak area ratio of L-menthol and internal standard
CD	Cyclodextrin
DMAB	Dimethylaminobenzaldehyde
EPA	Environmental Protection Agency
GC	Gas Chromatography
HBTM	Honey bee tracheal mite
Hm/Hstd	Peak height ratio of L-menthol and internal standard
HPLC	High Performance Liquid Chromatography
HR	Peak height ratio of L-menthol and internal standard
SD	Standard deviation
TLC	Thin Layer Chromatography
Trt	Treatment
U.H.P.	Ultra High Purity
2,6-DMP	2,6-dimethylphenol

1. INTRODUCTION

The honey bee tracheal mite (*Acarapis woodi*) was first discovered in North America in 1982. This mite infects only young adult bees and is parasitic, residing in the breathing tube of the bee. The infestation of tracheal mite is the cause of decreased honey production, reduced brood production, and increased mortality of honey bees in over-wintering colonies.

It has been found that L-menthol fumes are effective in controlling the honey bee tracheal mite (*Acarapis woodi*). L-menthol crystals, used directly or mixed with vegetable shortenings (paste), have been applied to the hives. The mixture of L-menthol and vegetable shortening is either spread on a piece of cardboard placed on the bottom board of the hive or L-menthol is soaked directly into a foam strip which is then hung between brood frames. Generally, the efficacy of L-menthol treatment is dependent on factors such as hive temperature, L-menthol dosage applied, treatment period and L-menthol position in the hives. Although one of the applications of L-menthol is its use as a food flavoring additive, the contamination of honey and beeswax by L-menthol after application is still of concern to beekeepers and scientists. Significantly high residue levels of L-menthol could change the flavor of honey.

One of the important objectives of this project was to examine the residue levels of L-menthol in honey samples from the hives treated by different methods of L-menthol application in northern Alberta. Beeswax samples from these hives were also analysed and the partition coefficients of L-menthol between beeswax and honey were determined. Factors affecting the residue levels were studied.

Analyses of L-menthol in both honey and beeswax were carried out on gas chromatography (GC). The internal standard 2,6-dimethylphenol was used to calibrate the analytical data. Calibration curves were constructed for ratios of the height and area of L-

menthol and the internal standard (Hm/Hstd and Am/Astd) and these ratios were used to determine the L-menthol residue levels in the honey and beeswax samples.

A sensory evaluation procedure using untrained panelists was carried out to determine a flavor threshold of L-menthol in honey.

L-menthol was registered in the United States for use to control the honey bee tracheal mite (*Acarapis woodi*) in November 1989. In Canada, L-menthol which must be food grade with purity greater than 98% has been available for use by beekeepers to control honey bee tracheal mite (*Acarapis woodi*) since the spring of 1992.

2. LITERATURE REVIEW

2.1 Sources and uses of menthol

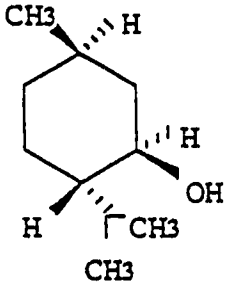
Menthol's chemical name is 5-methyl-2-(1-methylethyl) cyclohexanol. The structure is shown in Fig. 1. From this structure, it can be seen that there are three asymmetric centers. This compound can exist in four diastereomeric forms (Fig. 1) which are commonly known as menthol, isomenthol, neomenthol and neoisomenthol. Probably, due to the unfavorable steric effect of larger groups in an axial position, only menthol occurs abundantly in nature (Emberger and Hopp, 1985; Leung, 1980; Klouwen and Heide, 1962). Menthol has two enantiomeric forms, but only the L-form is found naturally (Fig. 1) (Emberger and Hopp, 1985).

L-menthol occurs as a colorless, crystalline material, with a very penetrating odor. The crystal structure is hexagonal, usually needle-like. It is slightly soluble in water, but very soluble in alcohol, chloroform, ether, petroleum ether, glacial acetic acid, fixed and volatile oils (Walker, 1967; Merck Index, 1983).

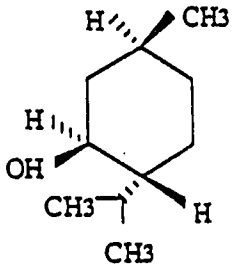
L-menthol is naturally obtained from oils of peppermint (*Mentha piperita*) and commint (*Mentha arvensis*) which have high L-menthol contents of 50%-60% and 46%-80%, respectively (Clark, 1988; Leung, 1980; Walker, 1967). L-menthol is produced by steam distilling the mint plants, cooling the oils to crystallize the L-menthol and separating the supernatant liquid from the L-menthol crystals (Walker, 1967; Biyani, 1982; Leffingwell and Shackelford, 1974). Due to the ever increasing demand for L-menthol throughout the world, synthetic methods of L-menthol production have been developed. Leffingwell and Shackelford (1974) and Walker (1967) reviewed the various synthetic routes and potential raw material for L-menthol production. Popular manufacturing methods include using thymol and citronella as starting materials. The synthetic routes are shown in Fig. 2.

L-menthol is the second most important flavoring substance of the aroma chemicals

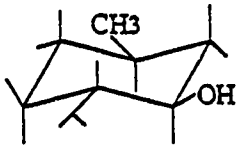
Fig. 1. Stereochemical structure of menthol



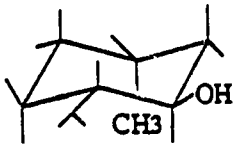
L-menthol
(1R, 2S, 5R)



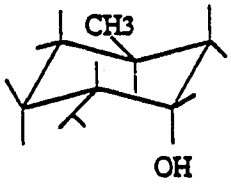
D-menthol
(1S, 2R, 5S)



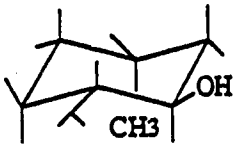
Menthol



Isomenthol

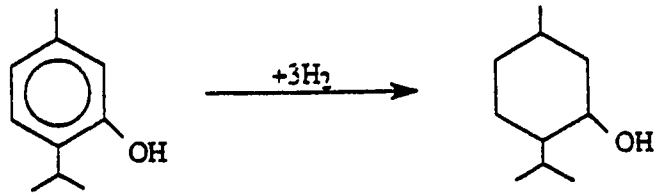


Neomenthol

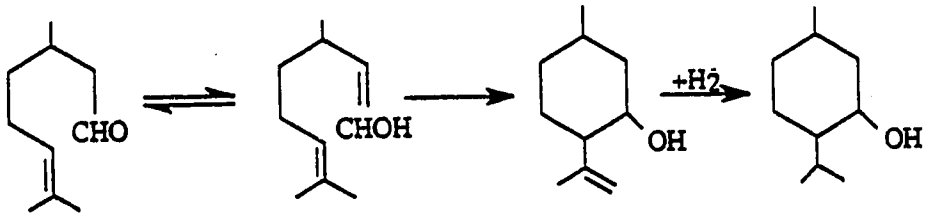


Neoisomenthol

Fig. 2. Synthesis of menthol



Thymol Menthol



Citronella Menthol

after vanilla (Emberger and Hopp, 1985) and 5600 M tons were consumed worldwide in 1988 (Clark, 1988). It is extensively used for flavoring chewing gums, candies and chocolates, alcoholic and nonalcoholic beverages, frozen dairy desserts, baked goods and puddings. In pharmacy and medicine, L-menthol is used as an aromatic, stomachic, stimulant, antiseptic, local anesthetic, and antispasmodic in treating indigestion, nausea, sore throat, diarrhea, colds, cramps, headaches and toothaches. It also finds applications in cosmetics as a fragrance component in perfumes, creams, lotions, detergents, soaps, toothpastes and mouthwashes (Leung, 1980; Walker, 1967). Another important use of L-menthol is for flavoring cigarettes. L-menthol used in the tobacco industry represented more than 60% of total usage in U. S. in 1988 (Clark, 1988).

In recent decades, particularly recent years, the fumes from L-menthol have been reported to be an effective agent against the honey bee tracheal mite (*Acarapis woodi*) infestations (Giavarini and Giordani, 1966; Giordani, 1977; Cox et al., 1989; Herbert et al., 1988; Wilson et al., 1988, 1990). It was estimated that 50-100 tons of L-menthol were used by U. S. beekeepers in 1989 (Wilson et al., 1990).

2.2 Toxicity of L-menthol

L-menthol vapors are an irritant to eyes, nose and throat, and ingestion of this chemical has been reported to cause abdominal pain, vomiting, staggering gait and sopor (Dangwal, 1980). It may cause allergic reactions (e.g., contact dermatitis, flushing and headache) in certain individuals and applying a menthol-containing ointment to the nostrils of infants for the treatment of cold symptoms may cause instant collapse (Leung, 1980).

The acute oral LD_{50} values were reported as 3300 mg/kg in rats (Herken, 1961) and as 800-1000 mg/kg in cats (Flury, 1920). For humans, the World Health Organization has established an Average Daily Intake of 0.2 mg/kg body weight per day (Murrell, 1988).

Psychotropic activity of L-menthol, particularly when administered orally, is weak.

The psychotropic action of this substance is always transient and it is unlikely that it would have any effect of this kind in man at the concentrations that occur in food or drinks (Le Bourhis and Soenen, 1973). The toxicity of L-menthol to honey bee has been reported. Giavarini and Giordani (1966) said that L-menthol was harmless to bees. Wilson et al. (1988) observed that toxicity of L-menthol to adult bees and brood was unnoticeable when L-menthol was applied in the fall; but, brood damage could occur under some conditions. It was also reported that L-menthol could become repellent to bees and result in unusual behavior such as uncapping and removal of honey or live immature capped brood (Clark, 1989). Cox et al. (1989) reported that early summer L-menthol exposure in Nebraska repelled bees from some of the honey supers, and consequently reduced honey production. They also observed significantly smaller colony weight gain and adult populations from the L-menthol treated colonies.

2.3 Honey

The Canadian Food and Drug Act of 1975 (Duhamel, 1975) regulated that: "honey should be the food produced by honey bees and derived from the nectar of blossom or from secretion of or on living plants. Honey derived mainly from blossom shall contain not more than 20% moisture, not more than 0.6% ash and not more than 5% sucrose. Honey derived from secretion of or on living plants shall contain not more than 20% moisture, not more than 1.0% ash and not more than 10% sucrose." Honey is classified according to origin as blossom or nectar honey and honeydew honey, and by processing mode as comb, extracted or pressed honey (Codex Alimentarius Commission, 1969).

Honey is produced by honey bees mainly from nectar and from honeydew. The average composition of honey is shown in Table 1. Nectar is an aqueous, sugar-containing secretion of plant glands called nectaries. Besides water and sugars, other substances such as nitrogenous compounds, organic acids, minerals, and aromatic substances are presented in small amounts. Honeydew is a sugar-containing substance excreted by certain plant-

Table 1. Average composition of honey produced in four countries(1) and in Alberta(2)

Component	USSR	USA	Romania	Australia	Average	Range	Alberta	Range
Water(%)	18.6	17.2	16.5	15.6	17.0	13.4-26.6	16.5	13.4-21.0
Fructose(%)	37.4	38.2	38.4	43.3	39.3	21.7-53.9	37.2	35.6-40.2
Glucose(%)	35.9	31.3	34.0	30.2	32.9	20.4-44.4	34.6	30.4-39.3
Sucrose(%)	2.1	1.3	3.1	2.5	2.3	0.0-7.6	0.6	0-6.5

(1) Crane, E. 1990. Bees and Beekeeping: Science, Practice and World Sources. p.389. Cornell University Press. Ithaca, New York.

(2) Sporns et al., 1992. Alberta Honey Composition. This paper will be published in Food Research International in 1992.

sucking insects. It always contains enzymes derived from the secretion of the salivary glands and the gut of the insects. Honeydew also contains amino acids, organic acids, etc., and some of the sugars in it are replaced or accompanied by sugar alcohols (Crane, 1975).

The honey bee ingests raw sugar-containing material (nectar, honeydew or other liquid) through its mouthparts into the honey sac and gut. In the honey sac, the raw material is mixed with saliva, which comes from the hypopharyngeal and salivary, and diluted. As collected, the raw material contains too much water to allow storage and it must be ripened by honey bees by evaporation of water. At the same time, invertase (α-glucosidase) is added by bees to the raw materials which eventually leads to hydrolysis of most of the sucrose to glucose and fructose. Finally, the honeybees fill the cells completely with ripened honey which must contain 20% water or less, and seal the cells with an airtight wax capping (Crane, 1975).

Honey has natural antibiotic properties. It is known that honey can be kept for long periods of time without spoilage. Glucose oxidase which is from hypopharyngeal glands of honeybees converts some glucose to gluconic acid and hydrogen peroxide. The hydrogen peroxide and acids in honey are the major contributors to the antibiotic effect. High osmotic pressure in honey also increases the resistance to spoilage by microorganisms (Tan et al. 1988).

Honey is a natural product with unique flavor. It was man's first sweetener before the discovery of cane and beet sugar. Although it is in competition with sucrose, glucose and high fructose syrup, there is still a large market for honey because of its flavor, texture, keeping qualities, and other factors. About 90% of honey is used on its own as table honey which is either spread on bread, biscuits or crackers, or used directly to sweeten drinks (tea, coffee, fruit juice) or fruit or cereals. It is also used in baking, confectionery, preserves, spread and syrups, meat packing, tobacco manufacture, and cosmetics. The antibiotic properties of honey make it useful as an antiseptic dressing for wounds and burns because the high osmotic pressure causes the death of microorganisms and hydrogen

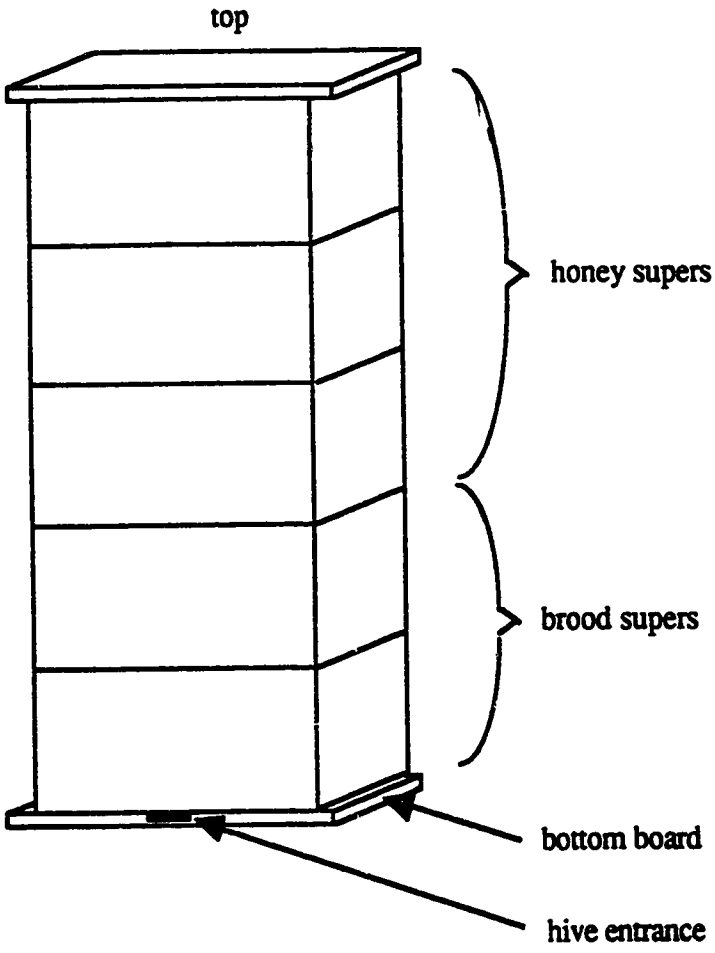
peroxide produced enzymatically in diluted honey is toxic to pathogens. When taken with lemon juice, honey has a smoothing effect to ease coughs and sore throats. In the tobacco industry, honey has the particular advantage that its hygroscopic nature helps to keep the tobacco moist (Crane, 1975; 1990).

The total recorded world honey production is nearly a million tons a year. China, Mexico and Argentina are the largest honey exporters and Germany, the USA and Japan are the largest importers at present. In Canada, honey production was 43,298 tons in 1984 and 44% of this production was exported (Crane, 1990).

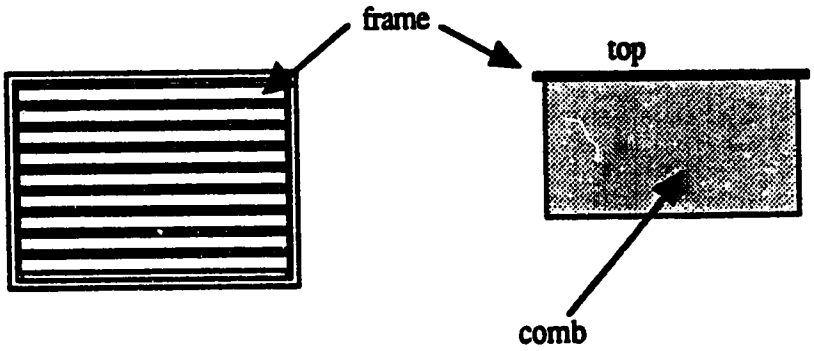
Fig. 3 shows the structure of a standard beehive. The standard beehive consists of two brood supers and three honey supers. Each super has nine frames. Honeybees build up combs with beeswax on each frame and store honey in the combs. The commercial honey is from honey supers. Any chemical treatments to a beehive during honey flow season are "abusive" treatments.

2.4 Honey Bee Tracheal Mite (HBTM)

Honey bees have evolved elaborate behavioral and chemical mechanisms to protect themselves and their food. A strict system of sanitation is foremost. Bees are constantly preening and cleaning themselves and their hivemates. Their bodies are peculiarly equipped for grooming; and antennas of worker bees periodically wipe off any minute particles that have accumulated. The hive or nest itself is also patrolled and cleaned. Foreign objects, debris, and dead bees are routinely carried off and deposited many meters from the hive, actions that help suppress the spread of pathogens and pests (Morse and Nowogrodzki, 1990). In addition, methods of storing nectar and pollen in the hive help to maximize the safety of the colony. Pollen from a single source is stored in a single cell. This procedure prevents contamination of all the pollen crop if one batch is found to be toxic. The modification of the nectar collected results in a raw honey product with low pH (3.2-4.5), low moisture content (<20%) and high osmotic pressure which is also a means of defense



Top of a super



against the growth of bacteria, fungi, and molds (Assil, 1991).

Although honey bees possess instinctive ability to protect themselves, their hives and hive products, they also suffer from diseases caused by honey bee pathogens which include viruses, bacteria, fungi, microspora and protozoa, parasitic mites and insect pests (Bailey and Ball, 1991). Two bacterial diseases that can be very damaging are caused by bacteria affecting young larvae and are known as American Foul Brood (AFB) and European Foul Brood (EFB). AFB is caused by *Bacillus larvae* and EFB is caused by *Melissococcus pluton*, and both kill the larvae of honeybees before they can develop into adult bees. A viral disease of honeybee brood is sac brood which infects larvae. Infected larvae do not pupate in the same way as healthy larvae. Nosema disease is the most widespread of adult bee diseases. It is caused by the pathogen *Nosema apis* and related disorders to the digestive system of adult honeybees. Nosema debilitates the bees and shortens their life span (Bailey and Ball, 1991; Crane, 1990; Morse and Nowogrodzki, 1990).

'Acarine disease' in adult bees has been a big concern of apiarist and scientists in North America in recent years. It is caused by infestation of the prothoracic tracheae by the mite *Acarapis woodi* which is normally called the honey bee tracheal mite (HBTM) (Crane, 1990). HBTM infests only young adult bees and feeds on blood by piercing the bee's wall with its stylet-like chelicerae, placing its mouth opening near the puncture, and sucking haeomolymph (Morse and Nowogrodzki, 1990; Crane, 1990). It has been confirmed that tracheal mite infestation is the cause of decreased honey production (Eischen et al., 1989); reduced brood production (Eischen, 1987); and increased mortality of honey bees in over-wintering colonies (Furgala et al., 1989). The number of honey bee colonies that died in the U. S. in 1988-1989 as a result of HBTM was at least 10,000 and probably 50,000 or more (Wilson et al., 1990).

Adult female and male mites are found in the honey bee respiratory system. The respiratory system is composed of seven spiracles and the internal ramifications of the

tracheae. Female mites enter the first thoracic spiracle of young adult bees and oviposit in the tracheae. The mites undergo biological stages of egg, larva, adult and are mated in the same honey bee trachea in which they developed. The mated female mite migrates out of the spiracle and attaches to a hair on the thorax of her host, and waits at the tip of the hair until stimulated by the hair of another bee brushing up against the initial host. Then the mite turns her front legs toward the stimulus, soon attaching firmly to the hairs of the passing bee (Morse and Nowogrodzki, 1990).

HBTM (*Acarapis woodi*) was first discovered in Scotland by Rennie et al. in 1921 (Crane, 1990; Bailey and Ball, 1991). Since then, European bees have suffered from HBTM with a lower infestation level during recent decades (Bailey and Ball, 1991). *Acarapis woodi* was first reported in North America by Wilson and Nunamaker in 1982 and samples of honey bees collected in 1984 in Texas were confirmed to be infested with *A. woodi* (Morse and Nowogrodzki, 1990). In Canada, the HBTM was first found in Manitoba and Ontario in 1986, in Saskatchewan in 1987, in British Columbia and Quebec in 1988 (Murrell, 1988). Up to 1990, the HBTM had been found in all Canadian provinces except the Maritimes. Besides the common impact of HBTM, the Canadian infested bees show high mortality for over-wintering due to the cold weather in Canada.

2.5 L-menthol treatment to control HBTM

Control of honey bee tracheal mite (HBTM) is a major concern to beekeepers. Several chemicals such as Folbex and Folbex VA, L-menthol, Apitol and Amitraz have been studied (Eischen and Dietz, 1986; Giavarini and Giordani, 1966; Vecchi and Giordani, 1968; Guzman-Novoa and Zozayap-Rubio, 1984; Cox et al., 1986; Rivera et al., 1987; Dietz et al., 1987). Natural and synthetic L-menthol are an excellent acaricide for the treatment of tracheal mite (*Acarapis woodi)* (Murrell, 1988; Wilson et al., 1988). Another isomer, D-menthol, has been tested and found to be less effective for killing HBTM (Wilson et al., 1989). Only food-grade, purity greater than 98%, L-menthol can be used

(Anonymous, 1992). On November 28, 1988, the EPA issued a general use registration for L-menthol in the United States (Federal Register, 1988) and in Canada L-menthol is now available for use by any beekeeper who has tracheal mite infestation (Anonymous, 1992).

The efficacy of L-menthol treatment to control HBTM is dependent on temperature. At low temperatures L-menthol will not vaporize and will not be effective. At high temperature L-menthol can melt, run on the comb, kill brood and adult bees, and irritate bees to the point that hive activities and beekeeping manipulation are interfered with (Wilson, 1990). The other factors, such as L-menthol placement in the hives, L-menthol containers, L-menthol dosage applied, and treatment period, have a great effect on the efficacy (Herbert, et al., 1988; Cox et al., 1989; Moffett et al., 1987; Wilson et al., 1988).

2.6 L-menthol residue level in honey

The major concern of the beekeepers after applying L-menthol to the infested colonies is the L-menthol residue level in hive products, mainly honey and beeswax. The determination of L-menthol residues in honey was done by Gas Chromatography (GC) or High Performance Liquid Chromatography (HPLC) (Herbert et al., 1988). Menthol has been naturally found in some honey and beeswax since honey bees (*Apis mellifera* L.) gather nectar from mint plants (Rivera and Wilson, 1989; Rivera et al., 1987).

Rivera and Wilson (1989) analysed 100 menthol-untreated honey and 80 wax samples from various parts of the U. S., Mexico, Central and South America and found that the range of L-menthol in these honey samples was from 0 to 1.03 ppm (average 0.11 ppm) and was from 0 to 8.4 ppm (average 1.07 ppm) in wax samples.

Wilson et al. (1988) tested the L-menthol residue levels in honey by GC after applying different amounts of L-menthol to the hive. They found that the resulting residues were extremely low, only 0—0.68 ppm for largest dosage of 90 g.

Herbert et al. (1987) examined the samples of newly capped honey from the treated

colonies by GC and found 0—12.3 ppm L-menthol in those samples.

It has been reported that the L-menthol residue increases in honey as the dosage applied increases (Rivera et al., 1987). It was also found that location of the L-menthol package in the infested colonies and the container of L-menthol have a great effect on the residue level in honey and the effectiveness of mite control (Herbert et al., 1988).

2.7 Analytical methods for quantitation of L-menthol

2.7.1 Extraction of L-menthol

Isolation of L-menthol from different materials has been achieved by steam distillation and/or solvent extraction. Both conventional and simultaneous steam distillation and solvent extraction have been employed. Sur et al. (1991) isolated major monoterpenoids such as menthol, menthone and menthylacetate in peppermint leaves by simultaneous steam distillation and solvent extraction with hexane. The full retention of above components by hexane was achieved during distillation because of the high partition coefficient between hexane and water.

Bicchi and Pisciotta (1990) used conventional steam distillation to separate peppermint oil from plant material and Atzl et al. (1972) carried out steam distillation to separate menthol from urine after enzymatic hydrolysis. Steam distillation is applied to liquids which are completely immiscible or liquids which are miscible to a slight extent. Also, simultaneous steam distillation and solvent extraction has been employed for isolation of pesticides and industrial chemicals from water and sediments (Veith and Kiwus, 1977; Peters, 1980). The problems of emulsions and interferences commonly observed in solvent extraction of the distillate after conventional steam distillation are eliminated with simultaneous steam distillation and solvent extraction. This is one of the advantages of this technique.

In steam distillation the boiling point at which the mixture distills is attained when the sum of the vapor pressures of the components present is equal to that of the

atmosphere. Steam distillation at atmospheric pressure results in separation of the immiscible higher boiling components at temperatures below 100°C. The use of steam distillation is of considerable value because water has a low molecular weight and comparatively moderate vapor pressure, therefore allowing isolation of large amounts (weight) of substances of high molecular weight and low vapor pressure.

Extraction of L-menthol in honey and beeswax by acetone has been reported (Anonymous, 1991). In addition, removal of L-menthol from honey and beeswax was achieved by heating at 65°C in a water bath for two days for honey and three days for wax (Rivera and Wilson, 1989). But this method is not feasible in commercial honey production because it is time consuming and may change the flavor of honey.

2.7.2 Quantitation of menthol

Quantitation of menthol has been accomplished by thin layer chromatography (TLC), colorimetric methods and gas chromatography (GC). The determination of menthol in tobacco by a colorimetric method and GC has been standardized in AOAC methods 968.02 and 968.03 (1990). Menthol in drugs can be determined by the methods described in AOAC method 929.14 (1990).

TLC provides a rapid method of identification. Metwally (1975) used benzol as the developing solvent to quantitate L-menthol in peppermint oil by TLC on silica gel. Although this method can be used to determine L-menthol, its precision and accuracy are not as good as GC.

Two colorimetric methods for the determination of menthol in air were reported by Dangwal (1980) based on the development of color on dilution of the reaction product of menthol with dimethylaminobenzaldehyde (DMAB) or vanillin in concentrated sulfuric acid medium. These are simple and reliable methods for the determination of menthol in air but less sensitive than GC.

Extensive survey of the literature has indicated that GC methods are the most

popular for the determination of menthol. Determination of menthol in different samples such as: human urine, aerosol spray lotions, peppermint oils and medicinal plants has been performed on both packed and capillary GC columns (Kaffenberger and Doyle, 1990; Bell et al., 1981; Atzl et al., 1972; De Fabrizio, 1981; Sang, 1982; Sur et al., 1991).

Kaffenberger and Doyle (1990) used a 30 m x 0.25 mm DB-5 (J&W Scientific, Folsom, CA) capillary column to quantitate menthol in human urine after enzymatic hydrolysis. Menthol was recovered by ethyl acetate extraction with recovery averaging 100±10%. Observation of the retention times showed menthol was free of interference from the urine matrix in DB-5 column.

Lee and Huang (1991) used a 15 m x 25 um SP-2100 fused silica column (Supelco Co.) to quantitate menthol in Shi-Di-Shui formulations which are widely used in Asia as aromatic-analgesics to relieve symptoms of cold sores, fever blisters, sunburn and insect bites. Quantitation was performed by calculation of peak area ratios, using n-octanol as the internal standard. They found that GC measurement yielded excellent results and provided a simple, rapid and accurate method for quantitative purposes.

In the majority of GC analyses, quantitation of menthol is associated with an internal standard calibration. The internal standard must be well resolved from all of the components of the mixture and its retention time must be reasonably close to those of the compounds that are being quantified. This will ensure that error made on the quantitative determination of the peak of the internal standard is comparable to the error made on the measurement of the peak of the measured components (Guiochon and Guillemin, 1988). Furthermore, the internal standard should be stable, behave in a predictable manner during extraction and analysis, and be susceptible to the same procedure variation as the compounds being analysed. The main advantage of internal standard calibration is simplicity and the ability to compensate for variations in extraction efficiency, injection volume and changes in detector response (Burgard and Kuznicki, 1990).

As stated in section 2.1 menthol has two optical isomers: L-menthol and D-

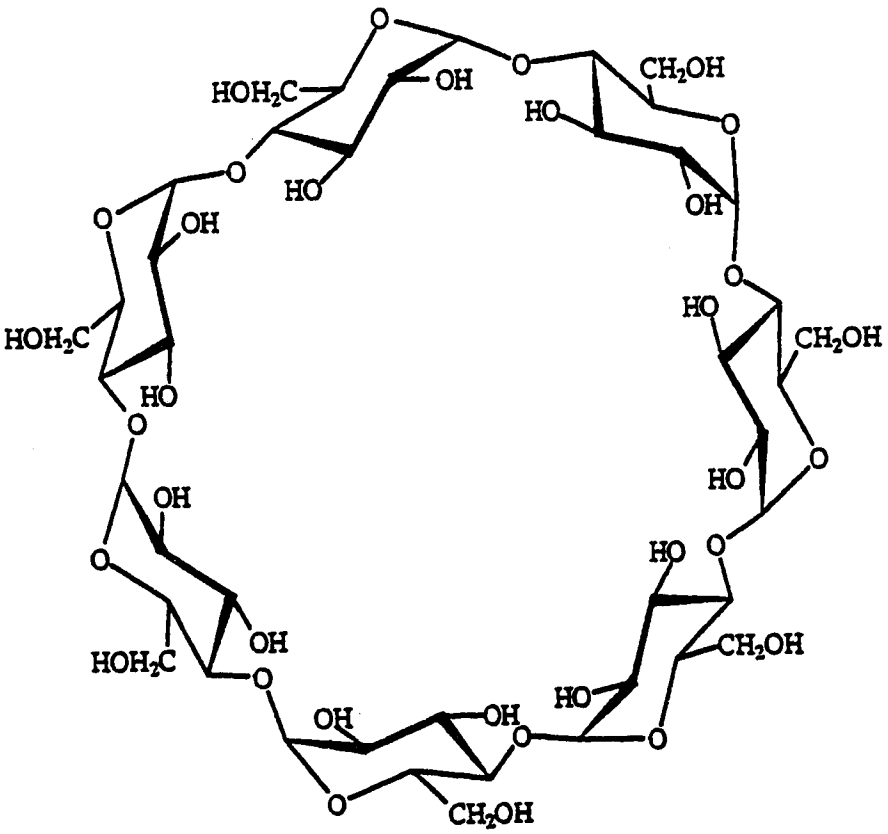
menthol. The separation of these enantiomers is important in many areas such as the food and beverage industry, medicine, pharmacy and cosmetics industry. GC analyses using new chiral stationary phase have been developed which will resolve racemic mixtures of chemicals. These techniques have been demonstrated as a convenient, rapid and reliable technique to separate enantiomers. In particular, there are two different types of successful chiral stationary phases: Ni(II), Co(II), and Mn(II) derivatives of bis-3-heptafluorobutanoyl camphorate and variously modified α-, β-, and γ-cyclodextrins (Bicchi and Pisciotta, 1990).

Bicchi and Pisciotta (1990) described the chiral resolution of menthol enantiomers in essential oils by applying two dimensional gas chromatography with a second column coated with a chiral stationary phase of Ni(II) bis[3-heptafluorobutanoyl-(IR)-camphorate]. Menthol enantiomers could be well resolved and their methods were shown to be rapid, easy and effective in enantiomer analysis of components in an essential oil.

However, in recent years the use of derivatized cyclodextrin as a chiral stationary phase in capillary GC has been the subject of intense research. Various α-, β-, and γ-cyclodextrin derivatives have been synthesized and modified cyclodextrins are used directly, mixed with silicone phase or incorporated in silicone backbones (Sandra, 1990). The differently derivatized cyclodextrins show different enantioselectivities for various racemic mixtures (Bicchi et al., 1991; Keim et al., 1991; Schurig et al., 1990).

Cyclodextrins (CD) are cyclic, nonreducing oligosaccharides composed of six to twelve D-(+) glucopyranose units which are bonded by α-(1→4)-linkages. They are chiral, toroidal-shaped molecules with all of the glucose units in a C-1(D) chair conformation. According to the number of glucose units per cyclodextrin, the molecule which has six, seven, or eight glucose units per cyclodextrin is named as α-CD, β-CD, γ-CD, respectively. The structural diagram of β-cyclodextrin is shown in Fig. 4. The hydroxyl groups on C-2, C-3, C-6 of glucose unit can be alkylated, acylated, or silylated to form various derivatives of cyclodextrin. The interior of CD is hydrophobic and forms inclusion

Fig. 4. Structural diagram of β-cyclodextrin



complexes which can retain one enantiomer longer than the other to achieve enantioselective separation.

Schurig et al. (1990) investigated enantiomer separation of compounds belonging to different classes on three different CD stationary phases. The menthol enantiomers could be well resolved on a 25 m x 0.25 mm capillary column coated with permethylated-β-CD in OV-1701 under isothermal condition (T=85°C). Bicchi et al. (1991) studied the resolution of a racemate mixture including menthol on three kinds of CD stationary phases which were: 30% trimethyl-α-CD in OV-1701-OH (phase A), 30% trimethyl-β-CD in OV-1701-OH (phase B), and 30% trimethyl-γ-CD in OV-1701-OH (phase C). Menthol racemate had a good resolution on phase A and phase B with temperature programming from 95°C to 210°C at 2°C/min, but had a poor resolution on phase C with temperature programming from 110°C to 210°C at 2°C/min. They also found that temperature programming had no influence on the enantiomeric resolution of menthol.

The quantitation of racemic menthol using cyclodextrin as a chiral stationary phase of capillary GC column has not been reported. Racemic menthol was only used to test the performance of various modified cyclodextrins as stationary phases. However, the commercial product, cyclodex-B, is available from J&W Scientific. The stationary phase of this column is 10% permethylated β-cyclodextrin in the moderately polar DB-1701 and this column is stable and capable of chiral separations from 30°C to 230°C. Menthol and other terpenes can be resolved using this column (Dinnauer et al., 1990).

2.8 Sensory evaluation

Sensory evaluation has been defined as "a scientific discipline used to evoke, measure, analyze, and interpret reactions to those characteristics of foods and materials as they are perceived by the senses of sight, smell, taste, touch, and hearing" (Sensory Evaluation Division of IFT, 1981). In sensory evaluation, factors such as testing environment, sample preparation and method of presentation are controlled in order to

minimize external influences on judgment. Another important consideration is the selection and training of panelists.

There are three fundamental types of sensory tests: preference/acceptance tests, discriminatory tests and descriptive tests. Preference/acceptance tests are based on a measure of preference or a measure from which relative preference can be determined. Discriminatory tests are either difference tests, which measure the difference between samples, or sensitivity tests, which measure the ability of individuals to detect sensory characteristics. Difference tests include the triangle test and sensitivity tests include the threshold test. Descriptive tests are used to identify sensory characteristics and quantify them. These tests have been classified and documented (Larmond, 1987).

In the triangle test, the panelist receives three coded samples. The panelist is told that two of the samples are the same and one is different and asked to identify the odd sample. This method is very useful in quality control work to ensure that samples from different production lots are the same. It is also used to determine if ingredient substitution or some other change in manufacturing results in a detectable difference in the product. The triangle test is often used for selecting and training panelists (Larmond, 1987), but it has limited use with products that involve sensory fatigue, carryover, or adaptation and with a panelist who finds testing three samples too confusing (Meilgard et al., 1987). The triangle test is appropriate for determining thresholds, provided fatigue, carry-over, and/or other constraints do not eliminate the use of the method. Analysis of the data of triangle tests is based on the probability that if there is no detectable difference, the odd sample will be selected by chance one-third of the time. The results indicate whether or not there is a detectable difference between two samples. Tables for rapid analysis of triangle test data have been prepared (Roessler et al., 1978).

The paired comparison test (simple difference test) is another method used to determine thresholds. In this method, the panelists are asked to determine whether a sensory difference exists between two samples. This method has applications similar to the

triangle test and is useful for comparisons between samples with a strong or lingering flavor (Meilgard et al., 1987). Fewer samples are required and there is less tasting, but the statistical efficiency is not as great. The probability of a panelist selecting a samples by chance is 50% (Larmond, 1987).

Thresholds are the limit of sensory capacities. It is convenient to distinguish between the detection threshold, the recognition threshold, the difference threshold and the terminal threshold. The detection threshold is the lowest stimulus capable of producing a sensation. The recognition threshold is the level of a stimulus at which the specific stimulus can be recognized and identified. The recognition threshold is usually higher than the detection threshold. The difference threshold is the extent of change in the stimulus necessary to produce a noticeable difference. The difference threshold is usually determined by presenting a standard stimulus which is then compared with a variable stimulus. The terminal threshold is that magnitude of a stimulus above which there is no increase in the perceived intensity of the appropriate quality for that stimulus (Meilgard et al., 1987).

L-menthol has a minty, light, refreshing odor that at most practical concentrations is complicated by an intense cooling sensation. At very low concentrations, L-menthol displays a slight warming sensation along with the refreshing mint odor. At moderate concentrations, the cooling effect develops, which, as concentration increases, becomes overwhelming and produces an anesthetic reaction (Clark, 1988).

Emberger and Hopp (1985) did sensory evaluation of menthol enantiomers. Menthol in L- and D- forms, as well as racemic mixtures, were tested by experienced flavorists in a 5% sucrose solution which was found to be the best test medium to recognize all of the flavor aspects. Their results were shown in Table 2. Also, the sensation profiles for L-menthol, D-menthol, and D,L-menthol were shown in their paper. L-menthol has a clean taste with a cooling property and freshness, while D- and D,L-menthol have remarkably, disagreeable notes such as phenolic, medicated, camphor and musty.

Table 2. Sensory evaluation of L-, D- and D,L-menthol

	L-menthol (ppm)	D-menthol (ppm)	D,L-menthol (ppm)
taste threshold	0.4	0.3	0.4
cooling threshold	0.8	3.0	1.33
best applicable dosage	1.66-5.5	1.33-5.0	1.66-5.0
bitter threshold	15.0	25.0	15.0

Test medium: 5% sucrose solution

Table was converted from a graph. Error might be introduced to the above data.

Emberger, R.; Hopp, R. 1985. Topics in Flavor Research Proceedings of the International Conference. 201-218

3. EXPERIMENTAL

3.1 GC equipment and conditions

Analyses of honey and beeswax were performed on a Varian Model 3700 Gas Chromatography (Georgetown, Ont.) equipped with a flame ionization detector (FID). A piece of deactivated fused silica capillary column (0.4 m x 0.25 mm, J&W Scientific, Folsom, CA), used as a guard column, was connected to an analytical DB-5 capillary column (30 m x 0.25 mm, J&W Scientific, Folsom, CA). The carrier gas was U. H. P. helium (Linde, Edmonton, AB) passed through oxygen traps (Chromatographic Specialties Inc., Brockville, Ont.). The velocity of carrier gas was 26.6 cm/sec. Samples were injected using a 1.0 ul syringe (7000 series, Hamilton Company, Reno, Nevada) with a splitter ratio 42:1. The analyses were carried out using temperature programming: 80°C, hold for 1 min, followed by a temperature ramp of 5°C/min until a final temperature of 120°C, hold for 10 min, was attained. Injector and detector were at 140°C and 280°C, respectively. Chromatographic data were recorded using a 3388A integrator terminal (Hewlett-Packard, Avondale, PA)

3.2 Preparation of standard solutions

(1) solution I—10.0 ppm 2,6-dimethylphenol (2,6-DMP) standard solution:

0.0020 g of 2,6-dimethylphenol (Aldrich Chemical Company, Milwaukee, WI) was weighed and dissolved in distilled hexane in a 200 ml volumetric flask. The solution was protected from light and kept at room temperature for two days.

(2) solution II—20.0 ppm L-menthol, 10.0 ppm 2,6-DMP standard solution:

0.0010 g of L-menthol (Aldrich Chemical Company, Milwaukee, WI) was weighed and dissolved by solution I in a 50 ml volumetric flask.

(3) standard solutions for calibration curve:

solution II was diluted to various concentrations of L-menthol and 10.0 ppm 2,6-DMP by solution I. Table 3 shows the dilution ratio.

Table 3. Preparation of standard solutions for calibration curve

L-menthol (ppm)	0.1	0.5	1.0	2.5	5.0	10.0
solution II (ml)	0.125	0.625	1.25	3.125	6.25	12.5
total volume (ml)	25.0	25.0	25.0	25.0	25.0	25.0

3.5 g of Na_2SO_4 (Anachemia, Montreal, PQ) was added to 10 ml of each of the above solutions which were in 20 ml scintillation vials (Fisher Scientific, Edmonton, AB). The suspensions of Na_2SO_4 were stirred vigorously using a Vortex JR. Mixer (Scientific Industries Inc., Springfield, MA) for 1 min and the supernatant was removed by filtration. The precipitated Na_2SO_4 was washed with 5 ml of distilled hexane and washing solvent was filtered and combined with the original filtrate. A gentle stream of N_2 (Linde, Edmonton, AB) was used to concentrate the filtrate to 1 ml. The remaining filtrate was then transferred to a 7 ml scintillation vial (Fisher Scientific, Edmonton, AB) and could be injected into the GC. Then the calibration curves were constructed according to the GC data.

3.3 Effect of adding sodium sulfate (Na_2SO_4)

5.0 ppm L-menthol, 10.0 ppm 2,6-DMP standard solution was used to check the effect of adding sodium sulfate.

3.5 g sodium sulfate was added to 10 ml of the above solution. Using the procedures described in section 3.2(3), the final samples for GC injection were prepared. At the same time, 10 ml of the above solution without sodium sulfate drying was concentrated to 1 ml and prepared for injection into the GC. The height and area ratios of L-menthol and internal standard for each sample were calculated and compared.

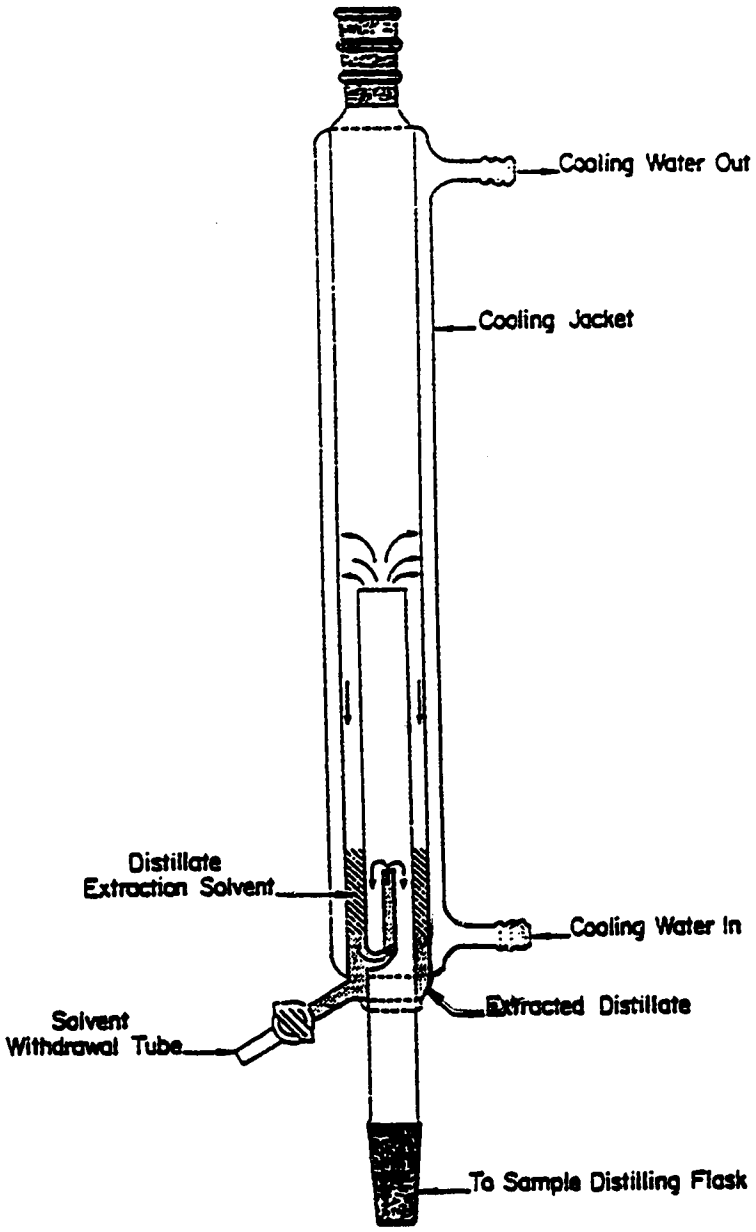
3.4 Sample Preparation

Simultaneous steam distillation and solvent extraction was applied to prepare honey and beeswax samples. The apparatus used is shown in Fig. 5. The honey solution or beeswax blended with water was placed in a 500 ml round bottom flask fitted with a 24/40 glass joint. 10.0 ml of 10.0 ppm 2,6-DMP standard solution in hexane was added between the inner tube and inside walls of the cooling jacket. The sample solution was boiled and the steam distillate passed through the inner tube and condensed on the inside walls of the cooling jacket. The condensate passed through 2,6-DMP standard solution into which the trace amounts of L-menthol and other volatile compounds were partitioned. The extract was removed through the stopcock and the inner walls of the unit were rinsed with distilled hexane to assure a quantitative transfer of the extract.

This steam distillation and solvent extraction unit had overall dimensions of 55 mm x 560 mm with a 24/40 joint. It was made by the machine shop in the Department of Chemistry, University of Alberta.

The sample in the round bottom flask was heated using a heating mantle. For honey samples, a certain amount (from 4 to 10 g) was mixed with 100 ml of distilled water and boiled vigorously for 20 min. Because L-menthol is fat soluble and beeswax contains more L-menthol than honey, 200 ml of distilled water was blended with a certain amount (from 0.4 to 2 g) of beeswax and boiled vigorously for 30 min.

Fig. 5. Steam distillation and solvent extraction apparatus



Veith, G. D.; Kiwus, L. M. 1977. An Exhaustive Steam-Distillation and Solvent-Extraction unit for Pesticides and Industrial Chemicals. *Bulletin of Environmental Contamination 17*(6): 631-636

The L-menthol extracts of honey and beeswax were dried over 3.5 g of Na_2SO_4 and concentrated to 1 ml by a gentle stream of N_2 using the procedure described in section 3.2(3).

Honey could not be completely separated from beeswax. To obtain pure wax samples for analysis, beeswax was washed using 50 ml of distilled water, and the washed beeswax sample was dried on a paper towel.

3.5 Investigation of possible interference of components in honey with 2,6-DMP and L-menthol

Two 100 ml portions of a 10 g honey sample were boiled for 20 min. One distillate was extracted using 10 ml of pure hexane and the other was extracted using 10 ml of 10.0 ppm 2,6-DMP in hexane standard solution. Both extracts were dried over sodium sulfate, concentrated using a gentle stream of N_2 to 1 ml, and injected into the GC.

3.6 Recovery of L-menthol in honey by steam distillation and solvent extraction

100 ml of a 10 g honey sample was spiked with 0.0010 g of L-menthol, resulting in 100 ppm L-menthol concentration. Each solution was boiled for 10, 15, and 20 min and the distillate extracted using 10 ml of 10.0 ppm 2,6-DMP in hexane standard solution. The concentration of L-menthol in the extract was out of the linear range of detector response. Thus, the extract was diluted to 3.3 ppm L-menthol (w/w) by adding 1.25 ml of the extract to a 25 ml volumetric flask and diluted with 10.0 ppm 2,6-DMP hexane solution. 10.0 ml of diluted extract was dried over 3.5 g of sodium sulfate, concentrated to 1 ml using gentle stream of N_2 and injected into the GC.

3.7 Repeatability of the methods of sample preparation

100 ml of 10 g honey solutions was spiked using 0.0010 g of L-menthol and boiled for 20 min. The extract was diluted to 3.3 ppm L-menthol honey (w/w) using the procedure described above, dried over 3.5 g of sodium sulfate, concentrated to 1 ml, and injected into the GC. This was repeated twice and the analytical data were compared.

3.8 Repeatability of GC

A honey sample containing 3.3 ppm L-menthol (w/w) sample was prepared the same as described in section 3.6 and 1.0 ul of this sample was injected to GC five times. 5.0 ppm L-menthol standard solution prepared using the procedures described in section 3.2(3) was also used to check the repeatability of GC injections.

3.9 Stability of L-menthol in honey

Four bottles of honey, each containing 200 g of honey, were spiked using 0.0200 g of food grade L-menthol (provided courtesy of H&R, Springfield, NJ). The mixtures were kept in a water bath at 50°C for 1 hour and then shaken vigorously. Two bottles of honey were capped and the other two were uncapped after shaking. All of them were kept at room temperature. There was no L-menthol source near the samples. Each bottle was marked and the honey was repeatedly analysed over a period of two months. Each honey sample in the bottles was stirred before analysis.

3.10 Sensory evaluation

Taste threshold determination of L-menthol in honey was performed using the triangle test. In the triangle test (Appendix 1), panelists were asked to choose the odd sample following the given procedures (Appendix 2) and to give their comments.

Testing was done periodically from February 7, 1992 to March 6, 1992 on Thursday or Friday with sessions held from noon to 12:30 p.m or from 1:30 p.m to 3:30

p.m in the Sensory Testing Room equipped with individual booths in the Department of Food Science, University of Alberta.

Untrained panelists consisted of 9—15 members of staff and students of the University of Alberta. They were randomly selected including both men and women and participated throughout the period of sensory evaluation. The panelists varied slightly during the course of the tests because some members were unavailable.

Honey used throughout the evaluation was purchased from G. Bruns, who collected honey in August 1988 from hives without the use of L-menthol or any other chemicals. Analysis by GC confirmed that this honey was L-menthol free. It was contained in a white pail with cover and kept in -30°C freezer until December 1991. From then on to the end of sensory evaluation, it was stored in a 4°C walk-in cooler.

All samples for sensory evaluation were prepared two days before the testing day. About 300 g honey was weighed into a 1000 ml beaker used only for honey and the beaker was covered with parafilm. The beaker containing honey was kept in a water bath at 50°C for one hour and the liquified honey was weighed into two glass jars in 100 g and 200 g portions. A known amount of food grade L-menthol crystals (H&R, Springfield, NJ) was added to one of the two jars and both jars were sealed immediately. The honeys with and without L-menthol were placed back into the 50°C water bath for one hour and were shaken vigorously for 10 min. The honeys were then kept at room temperature overnight and analysed by GC the next day.

Honey samples, about 3 g, were presented to panelists in 7 ml scintillation vials and served with coffee stirrers. The instructions given to the panelists were to use 1% lemon water as a mouth rinse between samples. Honey was not swallowed and retasting was allowed.

The evaluations were conducted under yellow light to mask color difference between honey samples with and without L-menthol.

All apparatus such as vials, coffee stirrers, cups were disposable and were replaced for each session of sensory evaluation.

Four evaluations were carried out in total. Three of them were performed in the patterns of two control samples (no L-menthol) vs. one L-menthol containing sample (2:1), while the other test was carried out using one control sample vs. two samples containing L-menthol (1:2).

To avoid the interference of the aftertaste of peppermint, the panelists were told not to chew gum one hour prior to flavor testing.

3.11 L-menthol treatments

3.11.1 Treatments

The field trials, using L-menthol, were conducted at a yard located at Fairview College, Alberta.

The L-menthol treatments were started on May 17, 1991. Table 4 lists the different methods used in treatments. Honey frames and extracted honey were sampled for residue tests. For background analyses, honey frames from brood chambers were taken from hives before treatment started on May 17, 1991. On June 7, 1991, honey frames from brood chambers were pulled from treated hives for residue analyses. Frames were taken from the brood chamber as well as from the honey super on July 11, 1991 for residue tests. On August 14, 1991, frames from the honey super were taken from treated hives but frames from brood chambers were taken only from treatments A and F for L-menthol residue analyses.

3.11.2 Extraction of honey and beeswax

The brood and super frames from hives treated with L-menthol were sent to Edmonton from Fairview by bus and delivered to our laboratory in cardboard or wooden boxes. Each frame was marked by codes (the codes and treatment methods were originally

Table 4. L-menthol treatments

Treatment	Period	Position in hive
Trt. A—Control	5/17/91—6/7/91	No L-menthol
Trt. B—L-menthol paste, 60 g L-menthol applied on v-notched cardboard.	5/17/91—6/7/91	Bottom board
Trt. C—6 L-menthol strips, each containing 10 g L-menthol applied on plastic foam.	5/17/91—6/7/91	Between the frame near the front and back of the hive, and between frames No. 2&3, 5&6 8&9.
Trt. D—3 L-menthol strips, each containing 10 g L-menthol applied on plastic foam.	5/17/91—6/7/91	Between the frame near the back of the hive, between frames No.2&3, 5&6 and 8&9.
Trt. E—L-menthol paste, 30 g L-menthol, applied on v-notched cardboard.	5/17/91—6/7/91	Bottom board
Trt. F—L-menthol cardboard, 30 g L-menthol applied on cardboard.	5/17/91—6/7/91 6/17/91—7/11/91 —8/14/91	Bottom board

known only by the apiculturist at Fairview College) representing the treatment date and method, as well as the position of the frame in the hive. These frames were processed as soon as they were obtained. Honey and beeswax, from capped and/or uncapped combs, were cut from different places in the frames and strained through four layers of cheesecloth. Collected honey and remaining beeswax were kept in glass jars at -20°C separately until they were analysed. The positions and sampling size on the frame were recorded for data analysis.

3.12 GC conditions for enantiomer separation of D,L-menthol

At the beginning of the study, some effort was put into the separation of racemic menthol using a chiral capillary column—cyclodex B (30 m x 0.25 mm, J&W Scientific, Folsom, CA). The equipment used was the same as that described in section 3.1, but the operating conditions were different. The velocity of carrier gas was 29 cm/sec which was in the recommended value range by manual. Sample size was 1.0 ul with a splitter ratio 110:1. The analyses were carried out using temperature programming: 100°C, 1 min, 1°C/min, 120°C, 10 min. Injector and detector were at 140°C and 300°C, respectively. This analysis procedure was used to confirm the authenticity of L-menthol used for treating the bee colonies, and for the analysis of a few of the initial honey samples.

4. RESULTS and DISCUSSIONS

4.1 Repeatability of GC

Repeatability of the GC analysis was checked with both standard solutions and honey extracts. Tables 5 and 6 show the results. It can be seen that the repeatability of GC was good because the standard deviations based on height and area ratios were small. In addition, the consistent retention time of L-menthol and internal standard over the period of study confirmed the repeatability of the GC performance. The retention times of L-menthol and internal standard were 9.00±0.10 min and 7.35±0.10 min, respectively.

4.2 Effect of adding sodium sulfate

The hexane extract of a honey distillate may contain some water. 3.5 g of sodium sulfate was used to dry the extract, but sodium sulfate may also bind some L-menthol and internal standard. To examine this possibility 5.0 ppm L-menthol in a 10.0 ppm 2,6-DMP hexane standard solution was either dried over 3.5 g of sodium sulfate or had no sodium sulfate added. The analytical data from these experiments are compared in Table 7.

It can be seen that 3.5 g of sodium sulfate did not have a noticeable effect on the amount of L-menthol and internal standard. This is in accordance with the observation made by Dix and Fritz (1987) who found that L-menthol recovery was 98.4% after adding 7.1 g of sodium sulfate to 5 ml distillate. Thus, drying the honey extracts over 3.5 g of sodium sulfate became part of a standardized procedure in the preparation of samples.

4.3 Calibration curve

The internal standard method on which quantitation of L-menthol is based provides an accurate determination, because any loss of L-menthol is reflected in a corresponding loss in internal standard. The internal standard 2,6-DMP is suitable because it is not found in honey and its retention time is similar to that for L-menthol, yet there is allowed an

Table 5. Repeatability of GC determination of L-menthol in standard solution

Injection No.	HR	mean	SD	AR	mean	SD
I	0.607			0.522		
II	0.615			0.530		
III	0.600	0.600	0.012	0.527	0.530	0.008
IV	0.594			0.529		
V	0.584			0.543		

Tested solution: 5.0 ppm L-menthol in 10.0 ppm 2,6-DMP hexane standard solution.

HR: peak height ratio of L-menthol and internal standard

AR: peak area ratio of L-menthol and internal standard

SD: standard deviation

Each data was the average of duplicate sampling

Table 6. Repeatability of GC determination of L-menthol in honey extract

Injection No.	HR	mean	SD	AR	mean	SD
I	0.358			0.279		
II	0.362			0.307		
III	0.347	0.356	0.006	0.288	0.290	0.011
IV	0.353			0.283		
V	0.359			0.291		

Tested sample: hexane extract of honey containing 3.3 ppm L-menthol (w/w)

HR: peak height ratio of L-menthol and internal standard

AR: peak area ratio of L-menthol and internal standard

SD: standard deviation

Each data was the average of duplicate sampling.

Table 7. Repeatability of GC determination of L-menthol in standard solutions dried and not dried over sodium sulfate

	Sample dried			Sample not dried		
Sample No.	HR	mean	SD	HR	mean	SD
I	0.597			0.588		
II	0.598	0.599	0.003	0.586	0.594	0.012
III	0.603			0.608		

Tested sample: 5.0 ppm L-menthol in 10.0 ppm 2,6-DMP hexane standard solution

HR: peak height ratio of L-menthol and internal standard

AR: peak area ratio of L-menthol and internal standard

SD: standard deviation

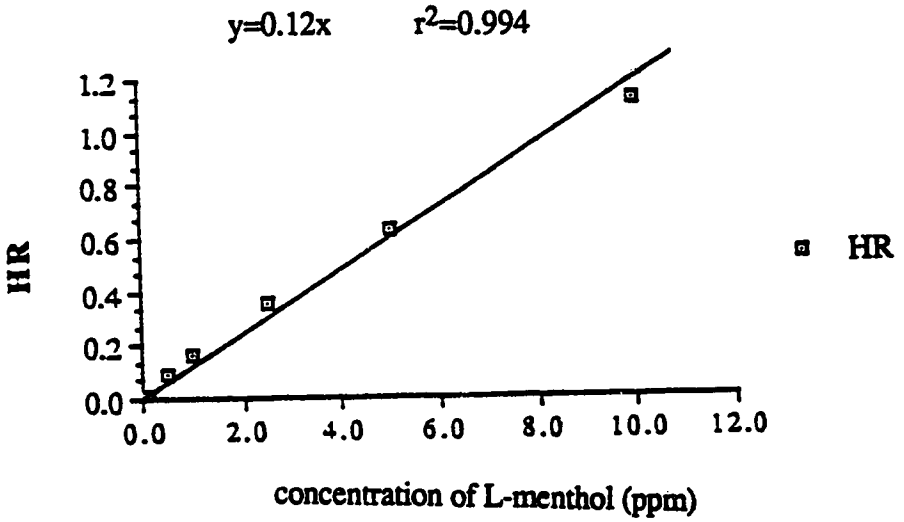
Each data was the average of duplicate sampling.

excellent separation. 2,6-DMP had previously been used as an internal standard for honey volatiles (Daharu, 1983).

The flame ionization detector (FID) is a sensitive detector with a wide dynamic linear range. However, other factors such as hydrogen gas, air and flame temperature may affect the sensitivity and linearity of the FID, and different compounds may have a different linear range.

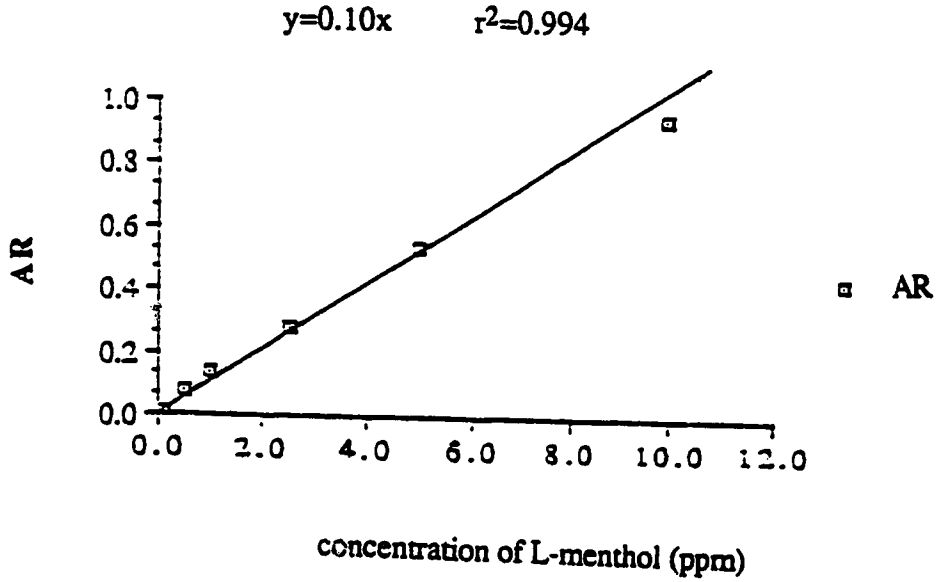
To quantitate L-menthol, concentrated standard solutions with consistent concentration of internal standard and various concentrations of L-menthol were analysed. The calibration curves (Fig. 6 & 7) were constructed for ratios of the height and area of L-menthol and the internal standard (Hm/Hstd & Am/Astd) against the concentration of L-menthol. The Hm/Hstd verses the L-menthol concentration in ppm (x) was fitted to the equation $y=mx$ and the slope calculated using least-square estimates. The slope (m) was 0.12 with a coefficient of determination (r^2) of 0.994. Relative areas of the two peaks were also compared and found to have a slope of 0.1[illegible] a coefficient of determination of 0.994. While both sets of data fit a straight line relationship, it was obvious, especially for samples containing more than 10 ppm L-menthol, that there was a straight curvature to the peak ratios relationship. However, for simplicity all quantations were determined using the above relationships. Since numerous corrections for varying peak heights and areas would also require an accurate accounting for volumes throughout the procedure, this would defeat many of the advantages of using an internal standard. Systematic errors introduced by using a linear relationship were small. In all following L-menthol determinations, any sample containing more than 10 ppm L-menthol was volumetrically diluted with the appropriate amount of 10.0 ppm 2,6-DMP hexane solution to insure that the peak ratio was within the 0.1 to 10 ppm L-menthol range.

Fig. 6. Calibration curve of peak height ratio of L-menthol and internal standard vs. concentration of L-menthol



HR=height of L-menthol/height of internal standard (Hm/Hstd).

Fig. 7. Calibration curve of peak area ratio of L-menthol and internal standard vs. concentration of L-menthol



AR=area of L-menthol/area of internal standard (Am/Astd).

4.4 Investigation of possible interference in the detection of L-menthol

Investigation of possible interfering compounds arising from steam distillation of honey and, perhaps, co-eluting with L-menthol and 2,6-DMP was examined. A typical chromatogram of honey extract with L-menthol and internal standard is illustrated in Fig. 8. From the chromatograms obtained, there were no other peaks at the retention times of L-menthol and of the internal standard. This means that there was no interference of honey components with L-menthol and internal standard.

4.5 Repeatability of the methods of sample preparation

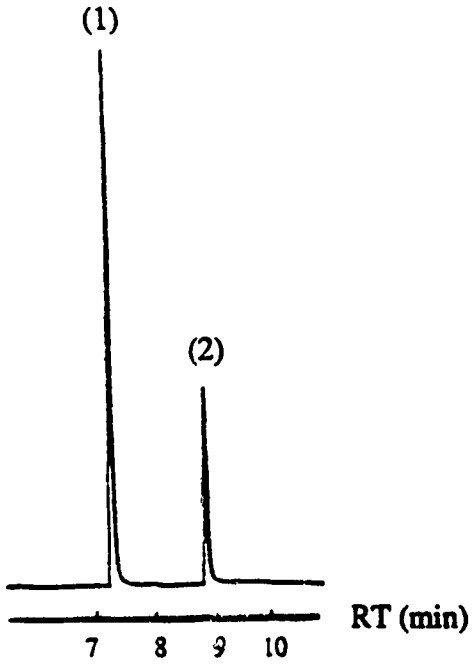
It is essential to check the repeatability of the methods used in sample preparation. The honey samples with the same concentration of L-menthol but prepared independently were examined. The results are shown in Table 8. From the analytical data, it can be seen that the repeatability of distillation, drying and concentration procedures used to prepare the samples was good due to the small standard deviation of the analytical data.

4.6 Recovery of L-menthol in honey by steam distillation and solvent extraction

The melting point and boiling point of L-menthol are 41-43^{o}C and 210-213^{o}C, respectively. When the honey-water solution boils, the L-menthol in it evaporates with the distillate. The rate of distillation and extraction varies for different compounds depending on their volatility, their solubility in the sample matrix, and the extraction solvent (Burgard and Kuznicki,1990). It was our purpose to find out how long it took to obtain complete recovery of L-menthol by simultaneous steam distillation and solvent extraction.

Water solutions of 10 g honey spiked with 0.0010 g of L-menthol were tested. The recovery for different distillation times is presented in Table 9. All L-menthol spiked into honey could be distilled out of the honey-water solution within 10 min. The concentration of L-menthol in these spiked honey samples was 100 ppm (i.e. 0.0010 g L-menthol/10 g

Fig. 8. Gas chromatogram for honey extract with L-menthol and internal standard on a DB-5 column



(1)=internal standard—2,6-DMP, RT=7.32 min

(2)=L-menthol, RT=8.96 min

Table 8. Repeatability of methods used in sample preparation

Sample No.	HR	mean	SD	AR	mean	SD
I	0.342			0.314		
II	0.356	0.351	0.008	0.287	0.309	0.020
III	0.356			0.326		

Tested sample: hexane extracts of honey containing 3.3 ppm L-menthol (w/w)

HR: peak height ratio of L-menthol and internal standard

AR: peak area ratio of L-menthol and internal standard

SD: standard deviation

Each data was the average of duplicate sampling.

Table 9. Recovery of 100 ppm L-menthol honey (w/w) solution by steam distillation and solvent extraction

Distillation time (min)	Recovery(%)
10	93.9
15	102.2
20	91.5

Each data was the average of duplicate sampling.

honey). Most of our honey samples from L-menthol treated hives did not have such a high L-menthol residue level, but during the extraction of honey from frames, we observed that frames from L-menthol strip treatments contained solid L-menthol particles in the combs. Thus, the honey samples from these frames would have higher L-menthol residue levels than the others. To distill out the L-menthol in these samples and standardized the analytical procedure, 20 min of distillation time was used.

4.7 L-menthol residue in honey

The L-menthol residue levels of honey samples are presented in Table 10. The first capital letter represents the different treatment methods (Table 4). The first, second, and third Arabic numerals indicate the replication of the treatments, the date of pulling the frames from hives and the frame number, respectively. The detailed meaning of each code is stated in Table 10.

In the samples selected for background check, one honey sample contained an unexpected 0.5 ppm L-menthol. This sample (A-3-1) was collected prior to any L-menthol treatment in a control hive. The reason for this may be due to the contamination of this sample by L-menthol during handling or transportation.

The L-menthol treatments for each group of hives were started on May 17/91. After 21 days of exposure, L-menthol was removed from the hives on June 7/91, and the frames from the brood chambers were pulled out on the same day. No L-menthol was applied to the hives during the honey season, except group F . One month after the treatments were over, frames from brood chambers and honey supers were pulled out of the hives on July 11/91. Frames from honey supers were pulled on August 14/91, two months after the treatments were over. For the hives in group F, a 30 g L-menthol cardboard was placed on the bottom board of the hives on June 17/91, and replaced by another 30 g L-menthol cardboard on July 11/91 and removed from the hives on August 14/91. Frames from both brood chambers and honey supers in groups F and A were pulled on July 11/91 and

Table 10. L-menthol residue levels in honey

Sample[1]	L-menthol (ppm)
(1) For background level taken on May 17/91:	
A-3-1	<0.5
B-2-1	ND
C-5-1	ND
D-1-1	ND
E-4-1	ND
(2) For brood nest samples taken on June 7/91:	
A-2-2-9	ND
A-4-2-9 TS	0.3
A-4-2-9 BS	<0.1
B-1-2-9	1.8
B-4-2-9	1.7
C-2-2-9 TS (X)	9.0
C-2-2-9 BS	3.0
C-3-2-9 TS (X)	8.8
C-3-2-9 BS	4.0
D-2-2-9 TS (X)	18.0
D-2-2-9 TS	1.8
D-2-2-9 BS (X)	4.5
D-2-2-9 BS	1.7
D-5-2-9 TS (X)	3.8
D-5-2-9 TS	2.0

D-5-2-9 BS (X)	2.8
D-5-2-9 BS	1.4
E-3-2-9	0.8
E-5-2-9	1.6
F-1-2-9 UP	0.7
F-1-2-9 DN	1.4
F-3-2-9 UP	0.4
F-3-2-9 DN	0.8
F-3-2-9 (3)	0.8

(3) For brood nest and honey supers taken on July 11/91:

A-2-3-10	ND
A-4-3-1	ND
A-2-3-H-7	ND
A-4-3-H-8	ND
B-1-3-1	0.3
B-4-3-10	0.3
B-1-3-H-4	ND
B-4-3-H-4	ND
C-1-3-10	0.7
C-4-3-2	0.5
C-1-3-H-7	ND
C-4-3-H-6	ND
D-2-3-2	0.9
D-5-3-10	<0.1
D-5-3-H-3	ND
D-2-3-H-3	ND

E-3-3-10	<0.1
E-5-3-1	<0.1
E-4-3-H-3	ND
E-5-3-H-3	ND
F-1-3-10	0.5
F-3-3-10	0.7
F-1-3-H-4	ND
F-3-3-H-4	ND

(4) For honey supers taken on August 14/91:

A-2-4-H-5 29/6 2.25	ND
A-4-4-H-5 9/7 2.6	ND
B-1-4-H-5 9/7 2.5	ND
B-4-4-H-5 29/6 2.3	ND
C-3-4-H-5 29/6 1.5	ND
C-5-4-H-5 9/7 2.2	ND
D-2-4-H-5 29/6 2.5	ND
D-5-4-H-5 9/7 2.4	ND
E-3-4-H-5 29/6 2.4	ND
E-5-4-H-5 9/7 2.5	ND
F-1-4-H-5 29/6 2.3	≤0.1
F-3-4-H-5 29/6 2.3	0.4

(5) For brood nest taken on August 14/91:

A-2-4-2	ND
A-4-4-2	ND

F-1-4-9	0.4
F-3-4-2	0.8

(6) For three honey samples[2] from the first honey extracted from the FFF91 experimental hives:

FFF91-2/8/91 I	ND
FFF91-2/8/91 II	ND
FFF91-2/8/91 III	ND

[1]Note of coding:

A—control

B—60 g L-menthol paste

C—60 g L-menthol strips

D—30 g L-menthol strips

E—30 g L-menthol paste

F—30 g L-menthol cardboard

1st Arabic numeral=replication of treatment

2nd Arabic numeral=date code

'1'=for background level taken on May 17/91.

'2'=for brood nest taken on June 7/91.

'3'=for brood nest and honey supers taken on July 11/91.

'4'=for honey supers and two brood nest taken on August 14/91.

3rd Arabic numeral=frame number

for example: '9'=brood nest frame number 9 counted from left to right when looking at the hive from the front.

'H-9'=honey super frame number 9.

ND=non detectable

TS=top side of frame

BS=bottom side of frame

UP=upper part of frame

DN=lower part of frame

(X) indicates position of L-menthol strips.

(X) indicates the L-menthol strips was on the other side of the frame.

For honey supers taken on August 14/91, the date (29/6, 9/7) in the code were the dates that the honey supers were placed in the colonies. The weight was in kilogram and was the weight of the frame.

Each data was the average of duplicate sampling.

(2)These three honey samples were from the mixture of all honey which were produced in the experimental beehives.

August 14/91. All of the frames were shipped to the University of Alberta for L-menthol residue study.

All of the samples which were taken immediately after the treatments were completed on June 7/91 contained L-menthol residues. The different treatment methods resulted in different residue levels. Furthermore, the same treatments but in different hives led to different residue levels. The L-menthol in these samples ranged from 0.4 ppm to 18.0 ppm (average 3.3 ppm). For two samples from untreated hives taken at this time, one (A-2-2-9) contained no L-menthol and the other (A-4-2-9) contained a trace amount (0.2 ppm) again possibly due to contamination.

For the brood nest sampled on July 11/91, all honey samples except the control had L-menthol ranging from ≤0.1 ppm to 1.2 ppm (average 0.4 ppm), while the honey supers taken on the same day did not contain any L-menthol.

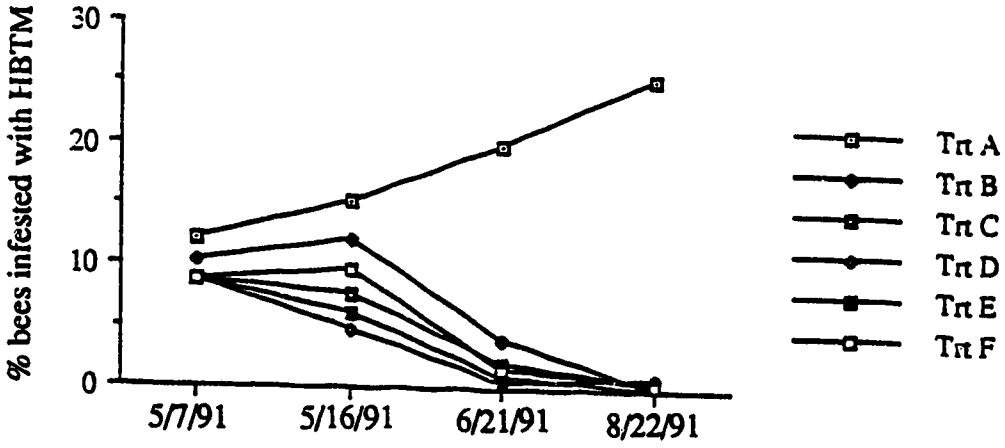
For honey supers sampled on August 14/91, all honey samples except F-1-4-H-5 29/6 2.3 and F-3-4-H-5 29/6 2.3 contained no L-menthol residues. The trace amount of L-menthol found in F-1-4-H-5 29/6 2.3 and F-3-4-H-5 29/6 2.3 resulted from successive "abusive" use of L-menthol cardboard from June 17/91 to August 14/91 in these hives. Low levels of L-menthol were also found in brood nest samples taken on the same day from these hives (treatment F).

Finally, honey extracted from all these L-menthol treated hives had no detectable L-menthol

4.8 L-menthol treatments and residue levels in honey

Fig. 9 illustrates the percentage of bees infested with HBTM during the period of field work. The data for this figure was supplied by Dr. Don Nelson who was in charge of the application of L-menthol at Fairview College, Alberta. This figure shows the efficacy of different L-menthol treatments to control the HBTM. All hives treated with L-menthol had decreased infestation levels which were much lower than the control (Treatment A) (see

Fig. 9. Percentage of bees infested with HBTM with different treatments of L-menthol



See Table 4 for detailed treatments

The data for this graph was supplied by Dr. Don Nelson who co-operated in this project.

Table 11). On May 17/91 the hives had 60 g or 30 g L-menthol paste applied and L-menthol strips 60 g or 30 g for 21 days. There was no detectable HBTM or ≤1% infestation level after about three months. While the L-menthol hives subjected to abusive L-menthol treatment had about 1% infestation after the same period of time. Without L-menthol, the control hives had an increased HBTM infestation level of up to 25% in the end. 30 g of L-menthol cardboard was the most effective treatment with a highest HBTM decrease rate. L-menthol paste resulted in a more rapid decrease of infestation level than L-menthol strips with the same dosage. Thus, L-menthol is effective to control the HBTM in northern Alberta during late spring and early summer.

It has been reported that L-menthol residue increases in honey as the dosage applied to the hives is increased (Rivera et al., 1987). This was generally true for brood nest samples in our experiments. Table 11 shows the average L-menthol residue level in brood nests and honey supers. The brood nest samples from Trt B and C had a higher average residue level than those from Trt E and D because double the amount of L-menthol was applied in Trt B and C. For samples from honey supers added later, no L-menthol was detected from these treatments.

The L-menthol residue levels in honey is related to the position of L-menthol application in the hive. More residue was found in the samples close to L-menthol. For example, C-2-2-9 TS (X) had higher residue levels than C-2-2-9 BS because the latter was not right beside the applied L-menthol strip. The same result of higher level of L-menthol was apparent in C-3-2-9 TS (X), D-5-2-9 TS (X), D-2-2-9 TS (X) compared with C-3-2-9 BS, D-5-2-9 BS (X), D-2-2-9 BS (X), respectively. The higher L-menthol level in C-2-2-9 TS (X) and C-3-2-9 TS (X) was due to the L-menthol strips being right beside the honey cells where these samples came from. White crystals which smelled and looked like L-menthol were found in these cells. These cells were uncapped and contained liquid honey. D-2-2-9 TS (X) possessed the highest L-menthol residue level of all honey samples.

Table 11. Average residue level (ppm) of L-menthol in honey after L-menthol treatment of hives

Treatment	Brood nest June 7/91	Honey supers July 11/91
A—Control	<0.1	ND
B—60 g L-menthol paste	1.7	ND
E—30 g L-menthol paste	1.3	ND
C—60 g L-menthol strips	6.2	ND
D—30 g L-menthol strips	4.5	ND
F—abusive (3x30 g) L-menthol cardboard	0.8	ND

Although the L-menthol strip was right beside the cells containing this honey, no L-menthol crystals were found in these cells.

Comparing all treatments, 30 g of L-menthol on cardboard was the most effective way resulting in the lowest L-menthol residue level in honey and the fastest decrease in HBTM infestation level. L-menthol strip applications were least effective because of higher L-menthol residues with slower decrease in HBTM infestation level.

4.9 L-menthol residue in beeswax

Table 12 shows the L-menthol residue level in beeswax and the partition coefficient of L-menthol between wax and honey. The data shows that the beeswax has a higher L-menthol concentration than corresponding honey samples. As L-menthol in honey increased, its concentration in beeswax increased also. For each group of samples, the partition coefficient was fairly consistent. For example, the mean value of partition coefficients in the beeswax samples of group (II) and of group (III) are 63.4 and 176.8, respectively. It is apparent from the data that the partition coefficient differs markedly between these two groups of beeswax samples. The partition coefficient of one compound between two phases of the same amount should be a constant. The big difference between the partition coefficients of group (II) and group (III) may be due to the following reason. When the L-menthol paste and cardboard were placed on the bottom board of the hive, the L-menthol fumes equilibrated between beeswax and honey. When the L-menthol strips were hung between brood frames, besides the L-menthol fume equilibrium, honey bees tracked L-menthol crystals from the strips to the beeswax and honey. This bee behavior was confirmed by the observation of L-menthol crystals in some cells and by noting that bees attended to L-menthol strips trying to cover them with propolis. However, bees would not go near the cardboard pastes at the bottom of the hives. Thus, the group (III) values which were from L-menthol strip treatments had what seemed as a higher

Table 12. L-menthol residues in beeswax and partition coefficients between honey and wax samples

	Sample	L-menthol (ppm)	Partition coefficient	mean	SD
group (I)	A-2-2-9	0.5			
	E-3-3-10	6.9			
	E-5-3-H-3 DN	ND			
	B-4-4-H-5 29/6 2.3	ND			
group (II)	B-4-2-9 (2)	106.0	53.0	63.4	12.2
	F-3-4-H-5 29/6 2.3	30.7	76.8		
	F-3-3-10 UP	60.4	60.4		
group (III)	C-2-2-9 TS	1787.6	198.6	176.8	21.8
	C-3-2-9 TS	1554.0	176.6		
	D-2-2-9 TS	2792.0	155.1		

group (I)=no L-menthol in the corresponding honey samples.
group (II)=low L-menthol concentration (0.4—2 ppm) in the corresponding honey samples. All from paste treatments.
group (III)=high L-menthol concentration (8—18 ppm) in the corresponding honey samples. All from strip treatments.
See Table 10 for the notes of sample coding. SD: standard deviation
Each data was the average of duplicate sampling.

partition coefficient. We think that the partition coefficient of group (II) is more likely to be the true value.

The purpose of determining the L-menthol partition coefficient between beeswax and honey was to confirm the higher affinity of beeswax for L-menthol. It is a common practice for beekeepers to reuse frames after collection of only the honey. The L-menthol in the old frames may migrate from beeswax to newly formed honey. This kind of migration had been found for phenol which was used as a bee repellent (Daharu, 1983).

4.10 Stability of L-menthol in honey

Table 13 shows the concentration of L-menthol, initially added to honeys, over a period of storage. Generally, there was no change or loss of L-menthol in the acidic honey at room temperature.

Daharu (1983) observed that there was no change of phenol in sealed honey after seven months. In this experiment, there was no change of L-menthol levels in honey in sealed or open containers. This indicates that honey retains the organic residue, at least at room temperature. This also indicates that there was no notable chemical change in L-menthol over this time period.

4.11 Enantiomer separation of menthol

Fig. 10 shows a typical GC chromatogram of D,L-menthol separation using a chiral capillary column—cyclodex B. It is obvious that a racemic menthol can be separated on this column. However, after the injections of honey samples, the performance of this column deteriorated resulting in poor resolution and tailing peaks which could not be used in quantitative analysis.

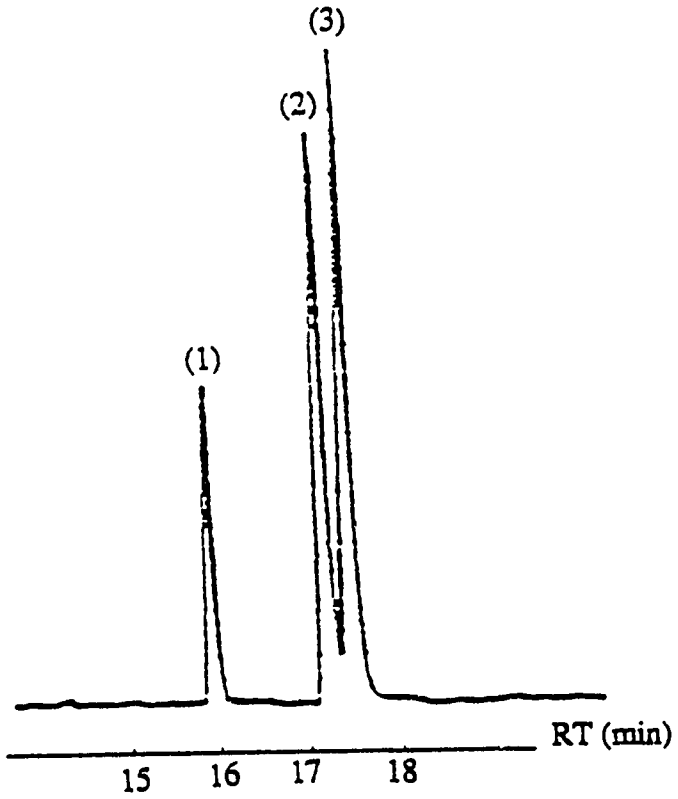
The honey samples injected onto this column were extracted directly using acetone rather than hexane. Both L-menthol and water in the honey were in the acetone extract because water is miscible with acetone. Before injection into the GC, the extract was dried

Table 13. Stability of L-menthol in honey during storage at room temperature

Time (day)	Sample (open) (ppm)	Sample (closed) (ppm)
0	100.0	97.9
7	97.3	97.9
16	93.1	95.6
36	100.5	99.7
55	96.6	89.9

Each data was the average of duplicate sampling.

Fig. 10. Gas chromatogram for acetone solution of D and L-menthol and internal standard on a Cyclodex-B column



(1)=internal standard—2,6-DMP, RT=15.90 min

(2)=D-menthol, RT=17.23 min

(3)=L-menthol, RT=17.47 min

over 3.5 g sodium sulfate. However, it was not certain that the extract was free of water because the dried extract might absorb more moisture from the air especially when concentrated. The water in the extracts might be hydrolytic to the stationary phase of the column. This might be the main reason for the rapid damage of the column after injection of honey samples.

The honey samples taken on May 17/91 to provide background levels were analysed on this column. The detection limit under the experimental conditions described in section 3.12 was 0.5 ppm.

4.12 Sensory evaluation

The experimental results of sensory evaluation of L-menthol in honey by triangle test are shown in Table 14.

As the concentration of L-menthol increased, the number of correct responses increased as well. The minty taste could be identified by all of panelists who chose the odd samples at 54.5 ppm level. Therefore, we can say that there was significant difference between these L-menthol containing samples and pure honey.

There were two patterns in triangle test. One pattern was to present samples as two controls and one L-menthol containing sample (2:1), the other was one control and two L-menthol containing samples (1:2). Theoretically, the results from these two patterns should be the same. But in our experiments, the testing results were much different if the pattern was changed from 2:1 to 1:2. With the pattern of two control honey samples and one 36.2 ppm L-menthol honey mixture, there was a significant difference between these two samples. While with pattern of one control and two 38.3 ppm L-menthol honey mixture samples, the panelists could not tell the difference between samples. The reason for this was probably that L-menthol has considerable aftertaste which becomes more of a problem when two out of three samples contain it, than only one sample out of three. We tried to overcome the L-menthol aftertaste by encouraging panelists to rinse their mouths with 1%

Table 14. Taste results of L-menthol in honey using triangle test

pattern of sample	[] of L-menthol (ppm) compared	I(1)	II(2)	III(3)
2:1	0 vs. 20.7	13	3	0.861
2:1	0 vs. 36.2	15	9	0.031
2:1	0 vs. 54.5	14	10	0.004
1:2	0 vs. 38.3	9	3	0.624

(1) total No. of panelists taking part in evaluation

(2) total No. of panelists choosing the odd sample

(3) probability that samples were correctly chosen at random

2:1=two pure honey samples and one L-menthol containing honey sample

1:2=one pure honey sample and two L-menthol containing honey samples

lemon juice in between samples. Obviously, this precaution was not sufficient to overcome the aftertaste with two L-menthol containing samples. Overall, however, it seemed as though even the highest L-menthol containing honey sample that we found in our experimental hives (18 ppm) would not be detected by the average consumer. It is known that when panelists are trained to detect L-menthol in a 5% sucrose solution, they can detect it at levels of as low as 0.5 ppm (Emberger and Hopp, 1985). The sweetness of honey (80+% sugar content) does seem to mask the flavor of L-menthol (noted before for other volatiles, Daharu, 1983), but it is likely that if panelists were trained to detect L-menthol in honey they could decrease the detection limit somewhat from the 36 ppm level that we have observed.

Determination of the exact taste threshold of L-menthol in honey requires more experimentation. Conclusive results can be established only if problems with lingering aftertaste can be reduced. With the diversity of honey flavors there are probably a wide variety of threshold levels for L-menthol in honey.

Canadian government regulations now allow the use of L-menthol to control HBTM. The threshold level of L-menthol in honey may be very important. L-menthol can be added to honey to produce a minty tasting honey which some people like. Also, L-menthol residue levels in honey should be controlled at lower than threshold levels to retain the traditional flavor of honey.

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APPENDIX 1

Questionnaire for triangle test

Test product: honey

Name:

Date:

You will receive a set with three samples. Two of these samples are identical and the other is different. Taste each sample following the procedures on the other sheet (Appendix 2). Circle the number of the odd sample and give your comments.

Sample No.

Comments:

APPENDIX 2

Sensory evaluation procedures for honey

Please taste the samples from left to right. Retasting is allowed.

(1) Rinse your mouth with water. Do not swallow water. Wait 30 sec before proceeding.

(2) Take 1/3 amount of honey in each vial into your mouth using coffee stirrer.
Wait before rinsing your mouth to evaluate notes.

(3) Wipe your mouth with napkin.

(4) Rinse your mouth with lemon water. Do not swallow water.

(5) Repeat from (1) to evaluate the other sample.