

AN ABSTRACT OF THE THESIS OF

Cheng C. Yao for the degree of Master of Science in
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Title: Environmental Influences on Monoterpenes and Phenolics
of *Mentha piperita*.

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Dr. W. D. Loomis

Vegetatively propagated peppermint (*Mentha piperita* L., cv. Black Mitcham) was used to determine the effects of night temperatures (8°C vs 25°C) on the plant growth in terms of fresh weight and leaf area, and on metabolite contents. The plants were sampled after 14 days, 21 days and 28 days in the controlled environments.

Warm-night plants grew faster than cool-night plants, seen in larger leaf area and greater fresh weight on whole plant or leaf basis. Cool night (8°C) favored anthocyanin synthesis and accumulation. Warm night (25°C) resulted in very low levels of anthocyanin pigment.

Monoterpene content and composition were also influenced by the night temperatures. Warm-night plants produced more essential oil than cool-night plants at all three sampling dates, approximately in proportion to the additional growth. However, the cool-night plants had higher percentages of reduced forms of monoterpenes such as

menthone and menthol, and lower percentages of the oxidized monoterpenes pulegone and menthofuran.

Although warm-night plants showed higher total measured amounts of starch and sugars , the specific contents per unit area tended to be lower than in cool-night plants.

Leaf expansion growth apparently utilized substrates that otherwise might have been used in secondary product synthesis or retained as reserves.

**Environmental Influences on Monoterpenes and
Phenolics of Mentha piperita**

by

Cheng C. Yao

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ENVIRONMENTAL INFLUENCES ON MONOTERPENES AND PHENOLICS OF MENTHA PIPERITA

I. INTRODUCTION

It is generally accepted that environmental factors influence the synthesis and metabolism of plant secondary metabolites, though the mechanisms of environmental effects are not well understood. Burbott and Loomis (6) reported that day length and night temperature influence the composition of oil of peppermint (Mentha piperita L.). Cool nights, and/or short days, produced an oil containing a higher content of reduced monoterpenes than did warm nights with short days. Photoperiod influenced plant growth and flowering, but it appeared that there was no direct photoperiod effect on monoterpene metabolism.

Clark and Menary (10) made similar observations but claimed a direct photoperiod effect on monoterpene metabolism, in spite of the fact that there were gross morphological differences in their plants under the different growing conditions.

Burbott and Loomis (6) suggested that synthesis and reductive metabolism (Figure 1) of monoterpenes compete with growth and respiration for the available carbohydrates from photosynthesis. High temperature speeds up growth and respiration and thus presumably

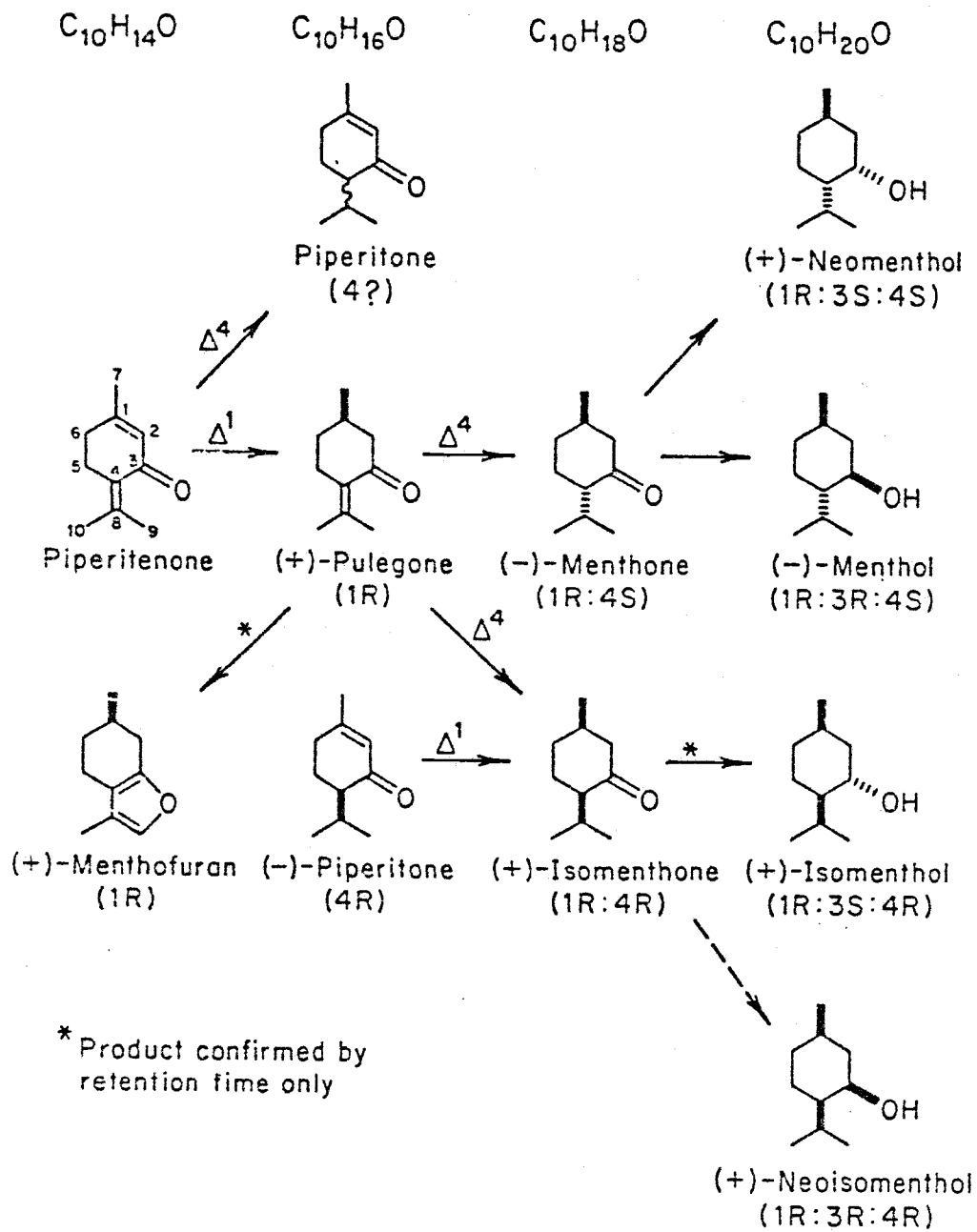


Figure 1. Peppermint monoterpenes and their metabolic interconversions (Burbott and Loomis, 1984 in preparation).

makes less carbohydrate available for "differentiation" processes (39, 40) such as secondary product synthesis and reductive metabolism of monoterpenes (38). Although the concept of "partitioning" or "growth/differentiation balance" (39, 40) is widely accepted, quantitative data in support of it are meager.

Carbohydrates are the primary source of reserve energy stored in the vegetative organs of plants. Analyzing the levels of carbohydrates such as starch and soluble sugars can provide information on plant energy status and vigor. Webb (58) reported that the starch content generally is a good indicator of Douglas-fir and white fir vigor, and is correlated both with crown growth at the time of bud burst and with survival of trees after tussock moth attack.

Starch is an important product of carbon fixation during photosynthesis. It acts as both a long-term and a short-term storage polysaccharide (31). Starch formed during photosynthesis is converted to sugars for transport to physiological sinks or for metabolism in the leaf. The night-time temperature has its influence on plant metabolism in terms of respiration rate. High temperature facilitates the reactions involved in respiration and growth, which directly influence starch degradation and indirectly influence secondary metabolic pathways such as the monoterpene synthesis pathway.

Peppermint produces large quantities of two classes of secondary metabolites: essential oil, primarily monoterpenes (Figure 1), and phenolic compounds (6, 35). Secondary metabolites are not uniformly distributed throughout all the tissues of a plant. The ability for synthesis and the capacity for storage of secondary metabolites depend on the tissue types, physiological age, environmental conditions and the genetic make-up (43).

The phenolic compounds are generally considered as important plant secondary products, many of which are synthesized by the shikimic acid pathway and the polyacetate pathway (26). It has been estimated that about 2% of all carbon assimilated by plants is converted into flavonoids or closely related compounds (52). The phenolic compounds range from relatively simple compounds to complex tannin type substances as reviewed by Robinson (48) and Harborne (30). Anthocyanins, the conspicuous flavonoid pigments which are red to purple at physiological pH, are of great importance in plant biochemistry, plant taxonomy and food science research.

According to Burbott and Loomis (6), peppermint plants grown under cool night conditions (25°C day and 8°C night, 14-hour photoperiod) showed visually higher red pigmentation on the lower leaf surface than those grown

under warm night conditions (25°C day and night, 14-hour photoperiod). It appeared that the cool night temperature favored the activity of anthocyanin synthesizing enzyme systems. Or, conversely, warm nights had a negative effect on anthocyanin synthesis and accumulation.

Croteau et al. (14) reported that glucose and CO₂ were the most efficient substrates, and acetate and mevalonate were the least effective compounds as monoterpene precursors. However when unlabelled sucrose was fed along with labelled mevalonate, the incorporation of mevalonate into mono- and sesqui-terpenes in peppermint (Mentha piperita L.) was markedly increased (13). DeAngelis (17) found that sucrose is the predominant soluble carbohydrate of peppermint leaf tissue, and that stachyose and raffinose are the predominant soluble carbohydrates of stems and rhizomes.

These results suggested that the sugars may serve as a transportable source of both carbon and energy (ATP and reducing power) for monoterpene synthesis.

The present study was undertaken to gain further insight into the effects of night-temperature on monoterpenes, anthocyanins and total phenolics of peppermint and to attempt to correlate these with effects on the content of starch and soluble sugars.

II. LITERATURE REVIEW

2.1 Starch analysis

The methods currently favored depend on specific enzymatic hydrolysis of starch (e.g. by glucoamylase), followed by measurement of the resulting glucose with glucose oxidase (9, 18, 19, 20, 25, 27, 41, 57), or on extraction of the starch with perchloric acid (28, 31, 42, 46), followed by colorimetric assay (usually with anthrone). Perchloric acid extracts contain other polysaccharides (pectic materials ?) in addition to starch, and to make this assay specific an iodine-precipitation step is added (45). Disadvantages of the enzyme method are:

(a). Most commercial amylases are contaminated or impure and must be dialyzed and purified (41);

(b). The enzymes may interact with the phenolic compounds in the extract. If polyphenol oxidase (PPO) is present (55), PPO decreases the starch-cleaving enzyme activity and may create errors in the final starch determination;

(c). It is more expensive than the perchloric acid method.

The acid extraction method does not have these disadvantages for starch extraction and determination.

Nielsen (45) first described perchloric acid (HClO_4)

as an efficient solvent for starch extraction. Perchloric acid of concentrations higher than 20%, will completely dissolve starch at room temperature but has a minimal effect on the solubility or hydrolysis of cellulose only at the concentration of 35% (28). Hansen and Møller (28) pointed out that perchloric acid influenced the carbohydrate-anthrone reaction, but the iodine precipitation step eliminates this problem.

2.2 Sugar analysis

The established methods for soluble carbohydrate analysis include the extraction of soluble carbohydrates at different concentrations of ethanol such as 80% (28, 31), 70% (62), or 30% (17). Results of DeAngelis (17) showed that 30% ethanol is more efficient than 80% ethanol (or intermediate concentrations (DeAngelis, personal communication)) in extracting soluble carbohydrates. DeAngelis (17) tested and found that 30% ethanol will solubilize only 0.1 % of starch, which is insignificant in comparison to the starch content of subsequent extracts.

2.3 Phenolic and anthocyanin analysis

Singleton (52) reported that the total phenolic content of grape berries increased rapidly over a considerable portion of the development and ripening

period. Considerable metabolism of phenolic compounds in grapes during ripening was observed up to the last stages wherein some components increase while others decrease in relation to total phenolics (52). It has been shown that keeping grapes for several days at 35°C, leads to a slight diminution in the amount of anthocyanins suggesting metabolic turnover of the compounds (47). The anthocyanins and flavonoids are localized in the vacuoles of epidermal cells (32). Burbott and Loomis (6) have observed that peppermint plants grown under cool night conditions (either 14 or 16 hour photoperiod) show visually higher red pigmentation on the lower leaf surface than those grown under warm night conditions. The activity of anthocyanin-synthesizing enzyme system is induced or increased by low night temperature. These red pigments can be used as a visual indicator of the physiological state of M. piperita. They may reflect the different energy status of secondary metabolism under warm and cool night temperatures.

According to Amerine and Ough (1), the two preferred methods for determination of total phenolics in solution are: the Neubauer-Lowenthal method and the Folin-Denis method. The former was the AOAC (Association of Official Analytical Chemists) official method before it was replaced by the latter. More

recently, the Folin-Denis method has been improved by replacing the Folin-Denis reagent with the Folin-Ciocalteu reagent, which provides greater sensitivity in the colorimetric determination of total phenolics. The Folin-Ciocalteu absorption is considerably greater than that observed with Folin-Denis reagent, except at the shorter wavelengths (51).

Total anthocyanin content can be determined in solution, independently of other naturally occurring phenolics, by measuring the absorption maximum at a single wavelength. The spectral maximum for phenolics in general is in the range of 350-380 nm, which is far away from the absorption maximum of most anthocyanins, 510-550 nm (29). Reported values of anthocyanin extinction coefficient range from 11,000 to 48,400 (23, 60). The absorption maximum of anthocyanins in peppermint is at 528 nm (Figure 2).

The anthocyanin absorption maximum and molar absorptivity used in the spectroscopic method is markedly affected by pH (52), temperature, solvents (29), the presence of metals (33), and the time of standing. In addition, a straight line relationship between concentration and absorptivity can be expected only at low concentration (23).

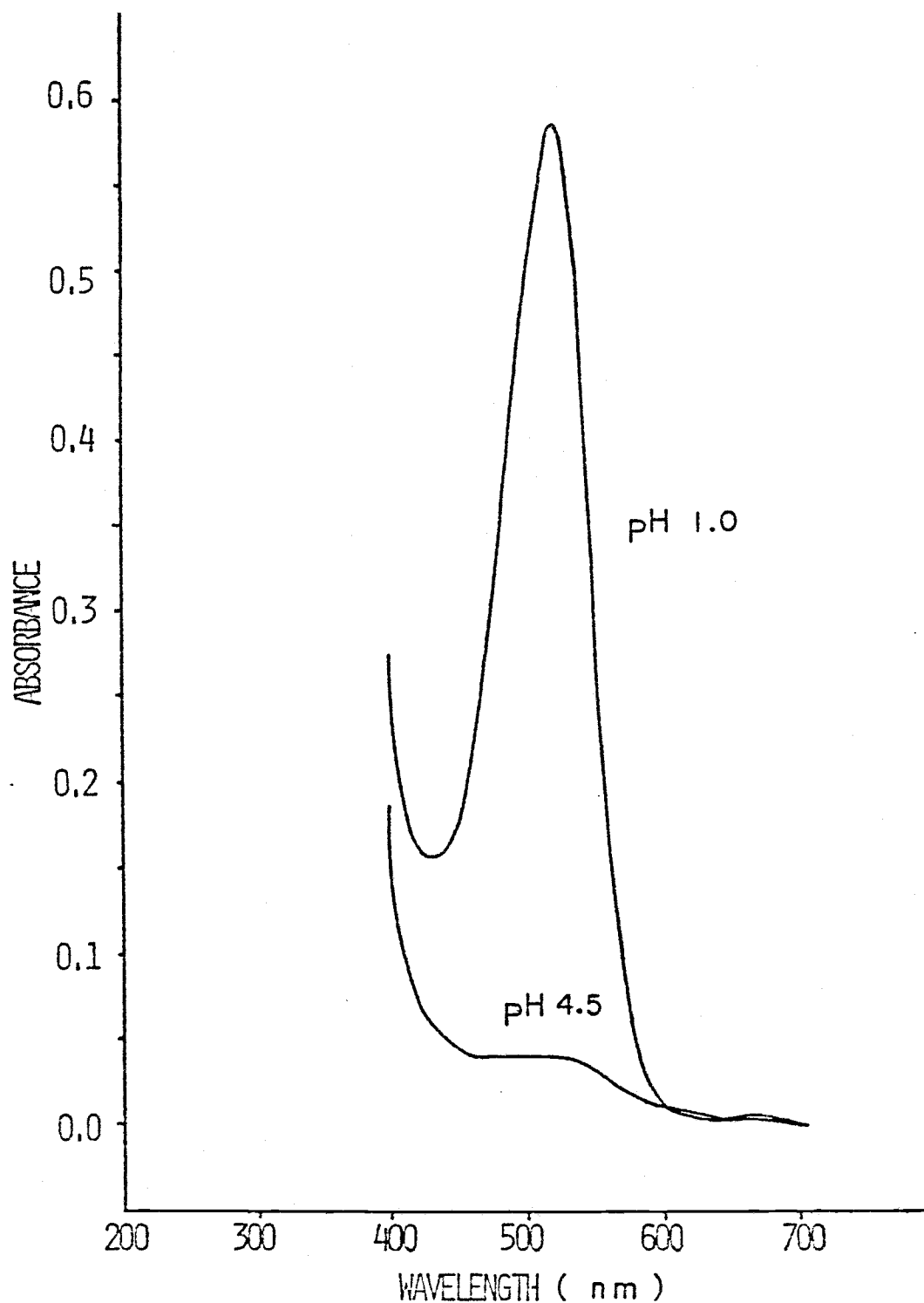


Figure 2. Absorption spectra of anthocyanin (at pH 1.0 and pH 4.5) of leaf #4 of cool-night plant at 28 days, illustrating the change in absorbance at 528 nm which is used for specific quantitative determination of anthocyanin.

2.4 Monoterpenes

The peppermint plants grown at cool night showed shorter internode and smaller leaf size than the warm night plants. Since peppermint is clonal, the differences in leaf color, oil composition and other characteristics from different controlled environments must be environmental in origin. Burbott and Loomis (7) concluded that photoperiod does not directly influence the terpene composition, but long days induce flowering and short days bring on a characteristic winter growth habit with many stolons. Guenther (24) reported that the production of peppermint oil requires a day length of 15-16 hours, but Burbott and Loomis (6) showed the situation is more complex than that. Biggs and Leopold (4) also pointed out that temperature greatly influences growth, flowering and essential oil yield in peppermint.

III. MATERIALS AND METHODS

3.1 Plant materials

Peppermint plants were the Black Mitcham cultivar of Mentha piperita L. vegetatively propagated from the same clone as described by Burbott and Loomis (6). Cuttings consisted of the tuft of the youngest leaves at the growing tip and the next two leaf pairs were used for all of the experiments described later. These were planted in perlite in 250 ml plastic beakers with drain holes cut in the bottom. Cuttings were visually matched as closely as possible for each experiment before being put into the controlled-environment chambers.

3.2 Growth chamber and growth conditions

The chambers were identical in size (57x59x113 cm) and were put in the controlled environment room. Temperature was controlled by heaters and thermostats at 25 °C day and night for warm-night condition and 25 °C day, 8 °C night for cool-night condition and the air circulation from the room into the chamber was controlled by blower to provide continuous air circulation. The humidity in the chambers was not controlled. The relative humidity ranged from 35% (warm night) to 60% (cool night). The photoperiod was 14 hours with a photosynthetically available radiation

(PAR) range between 110 $\mu\text{Einstein}/\text{m}^2 \cdot \text{sec}$. (at floor of chamber) and 195 $\mu\text{Einstein}/\text{m}^2 \cdot \text{sec}$. (at top of chamber), which was supplied by 20 Sylvania F96T12 8-foot VHO GRO-LUX and 20 Sylvania F96T12 GRO/VHO/WS Fluorescent lamps 80 watts each, approximately 150 cm above the floor of chambers. The PAR was measured with a LI-COR Model LI-185 Quantum/Radiometer/photometer.

3.3 Sampling methods

Peppermint leaves are paired, two per node, and the two leaves of a pair are highly correlated in terms of monoterpene composition and the content of total phenolics (35). This allows the two leaves of a pair to be regarded as matched samples for the analysis of various components. The leaf pairs were numbered from base of plant upward.

Rooted cuttings subjected to cool- and warm-night treatments were sampled at three different dates, 14 days, sampling date (i); 21 days, sampling date (ii) and 28 days, sampling date (iii) after transferring to the controlled environments:

(a). At sampling date (i), one leaf was removed from the specified leaf pair of each of six plants to be used for replicate monoterpene analyses. The remaining leaf of 3 plants was used for total phenolic (including total anthocyanin) analyses and the leaves from the

other 3 replicate plants used for replicate starch and soluble sugar analyses;

(b). At sampling dates (ii) and (iii), the same technique as described for sampling date (i) was applied, and twelve plants were used. This was done for the purpose of providing more plant material for analysis;

(c). At each sampling date the plants from different controlled environments were removed from the growth chambers approximately three hours after the start of the photoperiod.

Leaf pairs were removed from the main stem. Fresh weight and leaf area were measured. Leaf area was measured with a Lambda Instruments LI-3100 area meter to the nearest 0.01 cm². Fresh weight was determined immediately after the removal from the plant, to the nearest mg.

3.4 Methods for phenolic and anthocyanin determination

3.4.1 Total phenolic determination

The extraction procedure is summarized in Figure 3. The solvent used for the extraction of phenolics and anthocyanins was 0.1% aqueous HCl (44). The Singleton-Rossi method (51) with modification of final reaction mixture volume to 10 ml was used in this study to determine absorbance of total phenolics. The total

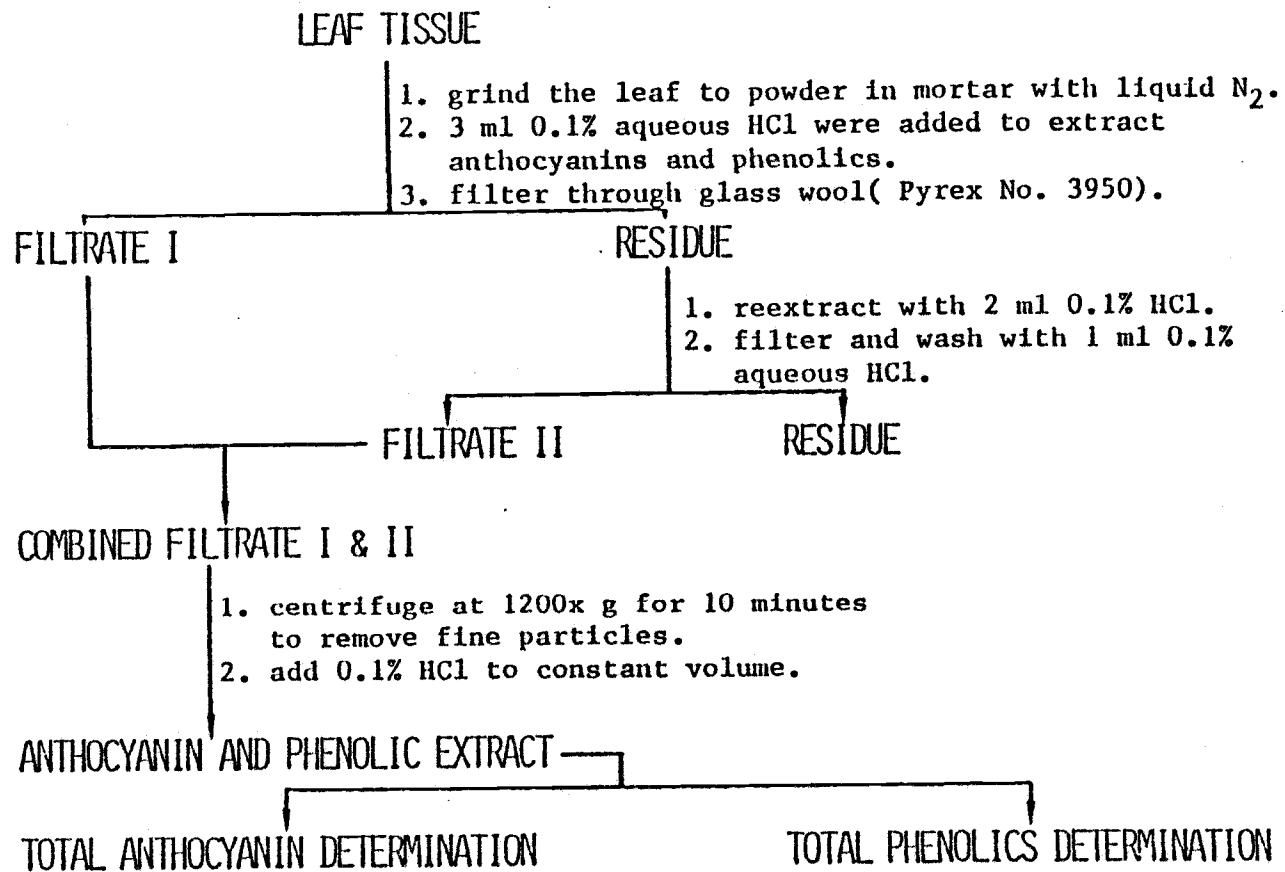


Figure 3. Flow chart of anthocyanin and phenolic isolation.

phenolics content of extracts was expressed as gallic acid equivalent (GAE) by Folin-Ciocalteu type colorimetry at 765 nm. Refer to Figure 4 for gallic acid standard curve. The method for total phenolic determination is as follows:

(a). 0.1 ml sample or gallic acid standard is pipetted into 25 ml test tube (1.5 cm in diameter and 15 cm in length) and 0.9 ml distilled water are added to it;

(b). 5 ml 0.2 N Folin-Ciocalteu solution are added and mixed completely;

(c). After an interval of 30 seconds to 8 minutes, add 4 ml of 1.3M Na_2CO_3 solution and mix thoroughly;

(d). The color is allowed to develop for two hours at room temperature, and the absorbance is measured against a reference blank at 765 nm in a Varian 219 spectrophotometer;

(e). The final phenolic content is calculated by taking the dilution factor into consideration.

The solution preparation for the above method is:

(a). 0.2 N Folin-Ciocalteu solution: 50 ml Sigma 2N Folin-Ciocalteu solution is diluted to 500 ml total with distilled water;

(b). 1.3M Na_2CO_3 solution: 37.5 g J.T. Baker reagent grade Na_2CO_3 is dissolved in distilled water to make a total of 500 ml;

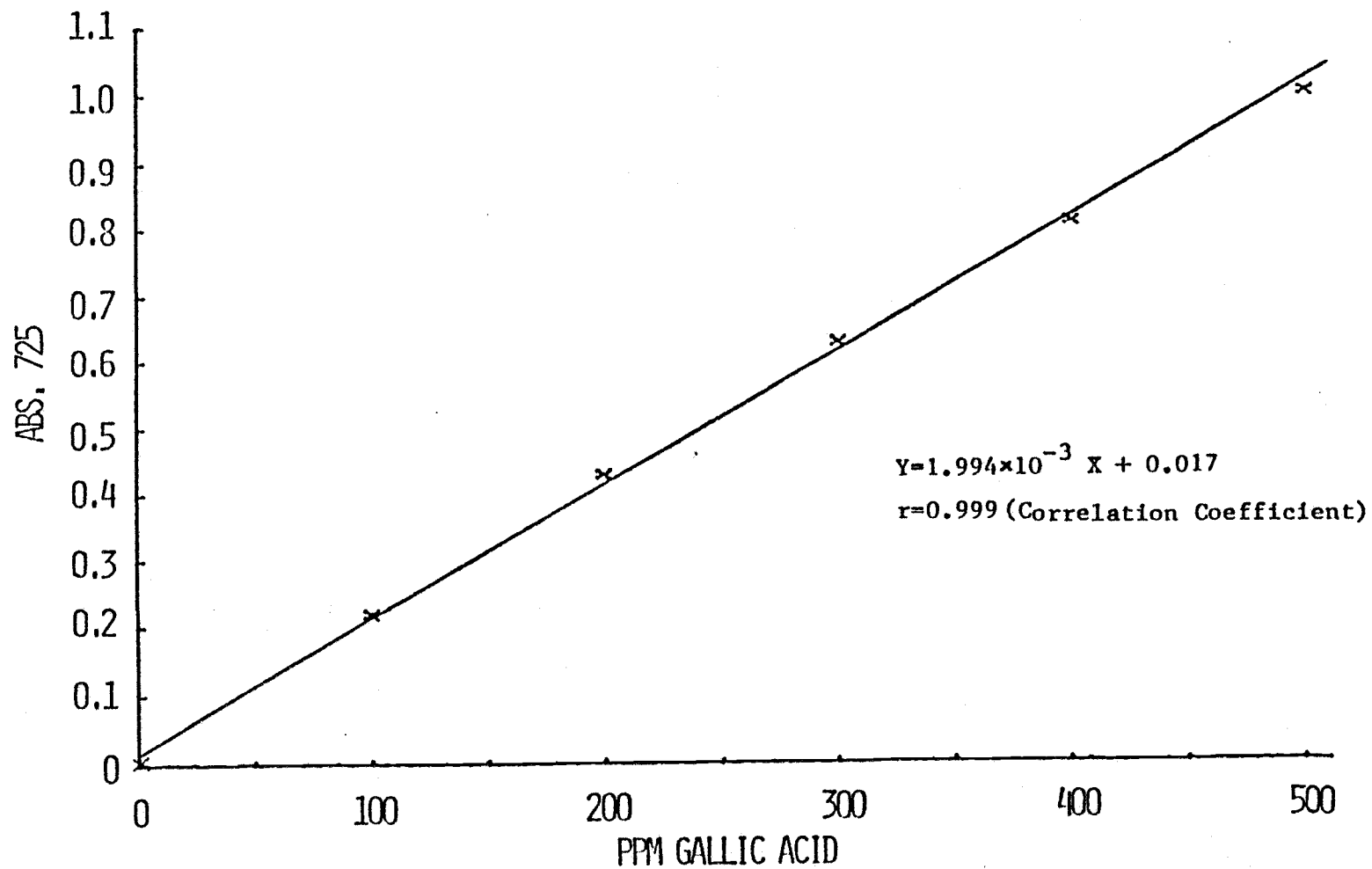


Figure 4. Gallic acid standard curve for total phenolic determination.

(c). Gallic acid standard: 500 ppm stock solution was made. Stock diluted to 100, 200, 300, 400 ppm prior to each analysis. The gallic acid (J.T. Baker, reagent grade) used was recrystallized twice from water and dried over sodium sulfate for 48 hours.

3.4.2 Total anthocyanin determination

The method used in this research is adapted from Wrolstad et al. (61), which is based on the structural transformation of anthocyanin between pH 1.0 and 4.5. Refer to Figure 2 for absorption spectra. The anthocyanin content was determined as follows:

(a). Dilute samples with pH 1.0 buffer so that the maximum absorbance is less than 1.0 O.D.;

(b). Dilute a second identical sample to same concentration with pH 4.5 buffer;

(c). Determine absorbance at wavelength of maximum absorption 528 nm and at 700 nm;

(d). Subtract absorbance at 700 nm (haze) from that at 528 nm;

(e). Determine difference in absorbance (A) at 528 nm (corrected for haze) between pH 1.0 and 4.5 samples;

(f). Calculate anthocyanin pigment concentrations (as cyanidin-3-glucoside) by the following formula:

$$\text{Anthocyanin pigment (mg/l)} = (A \times \text{MW} \times \text{DF} \times 10^3) / (\epsilon \times L)$$

$E = 29,600 \text{ l/Mole cm,}$

$MW = 445 \text{ g/mole,}$

$L = \text{pathlength} = 1.0 \text{ cm,}$

$DF = \text{dilution factor.}$

The solution preparation for the above method is:

(a). pH 1.0 buffer: combine 125 ml 0.2 N KCl and 385 ml 0.2 N HCl, dilute to 1 liter with water and adjust to pH 1.0;

(b). pH 4.5 buffer: combine 400 ml 1 M sodium acetate and 240 ml 1 N HCl. Dilute to 1 liter with water and adjust pH to 4.5 with same solutions.

3.5 Method for monoterpene determination

3.5.1 Extraction of the essential oil

Matched leaves from matched cuttings were ground with liquid nitrogen in a mortar, then extracted with technical grade hexane (13) in the presence of anhydrous Na_2SO_4 (Mallinkrodt, reagent grade), and the extracts were decolorized with Norit A activated charcoal (Sigma) as described previously (6). Before GLC analysis, the extract was concentrated down to slightly less than 200 μl . One μl of n-Tridecane (which was measured by Hamilton microliter gas-tight syringe) was added as internal standard and volume brought to 200 μl with hexane. For extraction procedure, refer to Figure 5.

3.5.2 GC analysis of monoterpenes

Essential oil extracts were analyzed by gas chromatography on a Perkin-Elmer gas chromatograph model 990 with FID (flame ionization detector) as described by Croteau et al. (13). The column used was 6.1 m x 3.175 mm stainless steel with 1% PDEAS (phenyl diethanolamine succinate) and 1.5% SAIB (sucrose acetate isobutyrate) coated on 100-120 mesh Chromosorb G (8, 13). Temperature was programmed from 100-160°C at 4°C per minute, with 25 minutes at the final temperature. Carrier gas was nitrogen at a flow rate of 25 ml/minute. One μ l injections were used. The peak area and retention time were integrated and recorded individually by a programmable Hewlett Packard 3130A integrator. The retention time of each individual peak was confirmed by running individual standards under the same conditions. Refer to Figure 6 for GC trace of leaf #4 of warm-night plant at 28 days. Reproducible quantitative injection of the 1 μ l samples was found to be impossible, and for this reason an n-Tridecane was used as an internal standard. The sample size injected was corrected for internal standard. GC response was quantitatively calibrated using menthol and limonene.

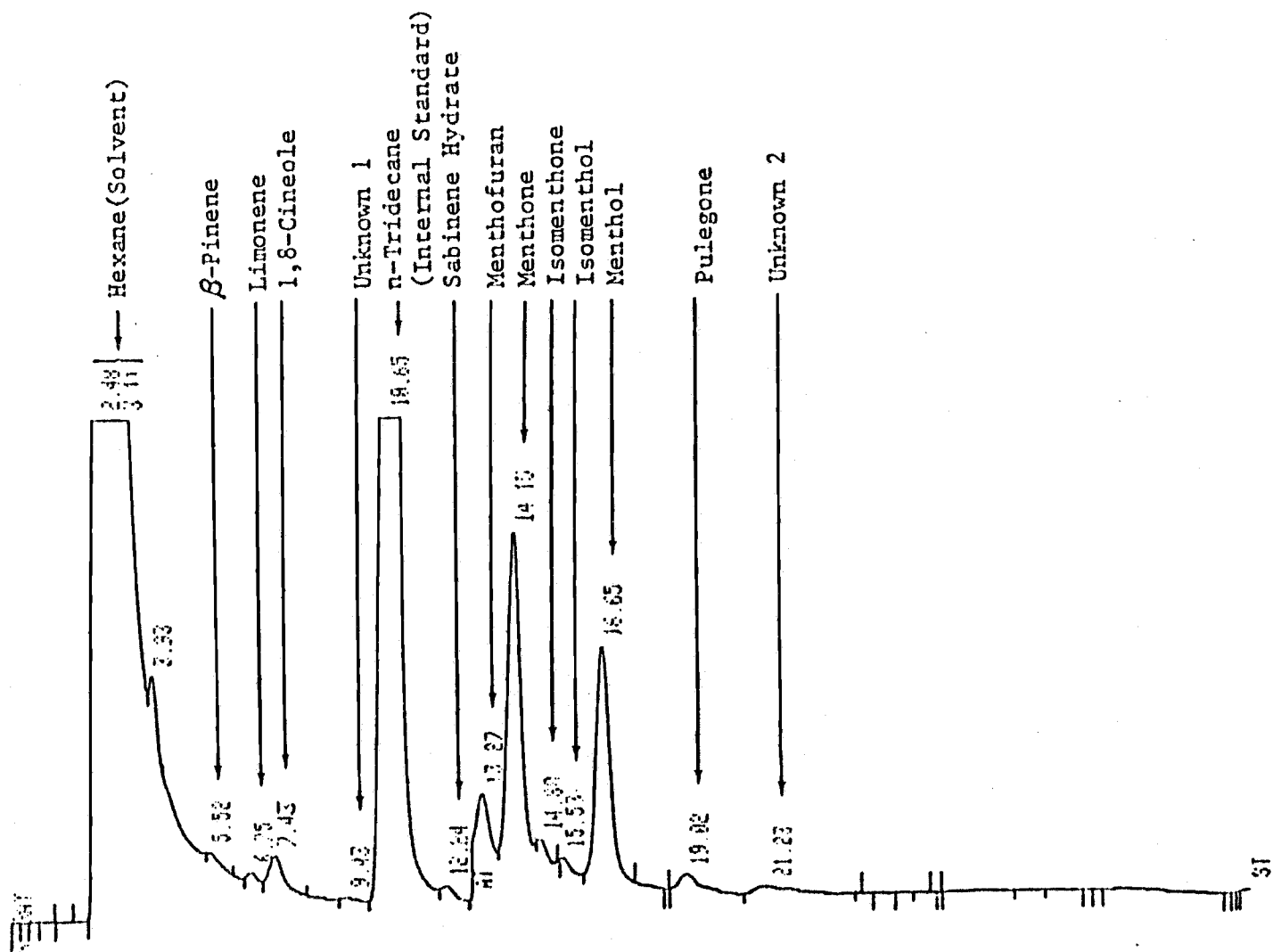


Figure 6. GC trace of leaf #4 of warm-night plant at 28 days.

3.6 Methods for soluble sugar and starch determination

3.6.1 Soluble sugars

The peppermint leaf samples were extracted twice with 5 ml 30% ethanol. The solution was heated to 80°C for 30 minutes for the first extraction and 80°C for 20 minutes for a second extraction (17, 18). During the heating, the tube was sealed with a clean rubber stopper to minimize evaporation. Refer to Figure 7 for extraction procedures.

Insoluble PVP (polyvinylpyrrolidone), acid washed (37), was then added to the combined extract in 1:1 ratio (by leaf fresh weight) (49). The mixture was stirred occasionally for at least 30 minutes to allow PVP to adsorb interfering components (mostly phenolics) in the extract. The PVP step was repeated (49), and 200 μ l saturated neutral lead acetate ($\text{Pb}(\text{OAc})_2$) solution was added to the filtered extract to precipitate additional interfering materials. After 25 minutes, 1 ml saturated sodium oxalate solution was added to precipitate the excess amount of Pb^{+2} (50). The final solution was filtered through Whatman #42 filter paper. One ml aliquots were used for anthrone determination of sugars. Refer to Figure 8 for glucose standard curve used for sugar determination.

LIQUID NITROGEN PREPARATION OF LEAF TISSUE

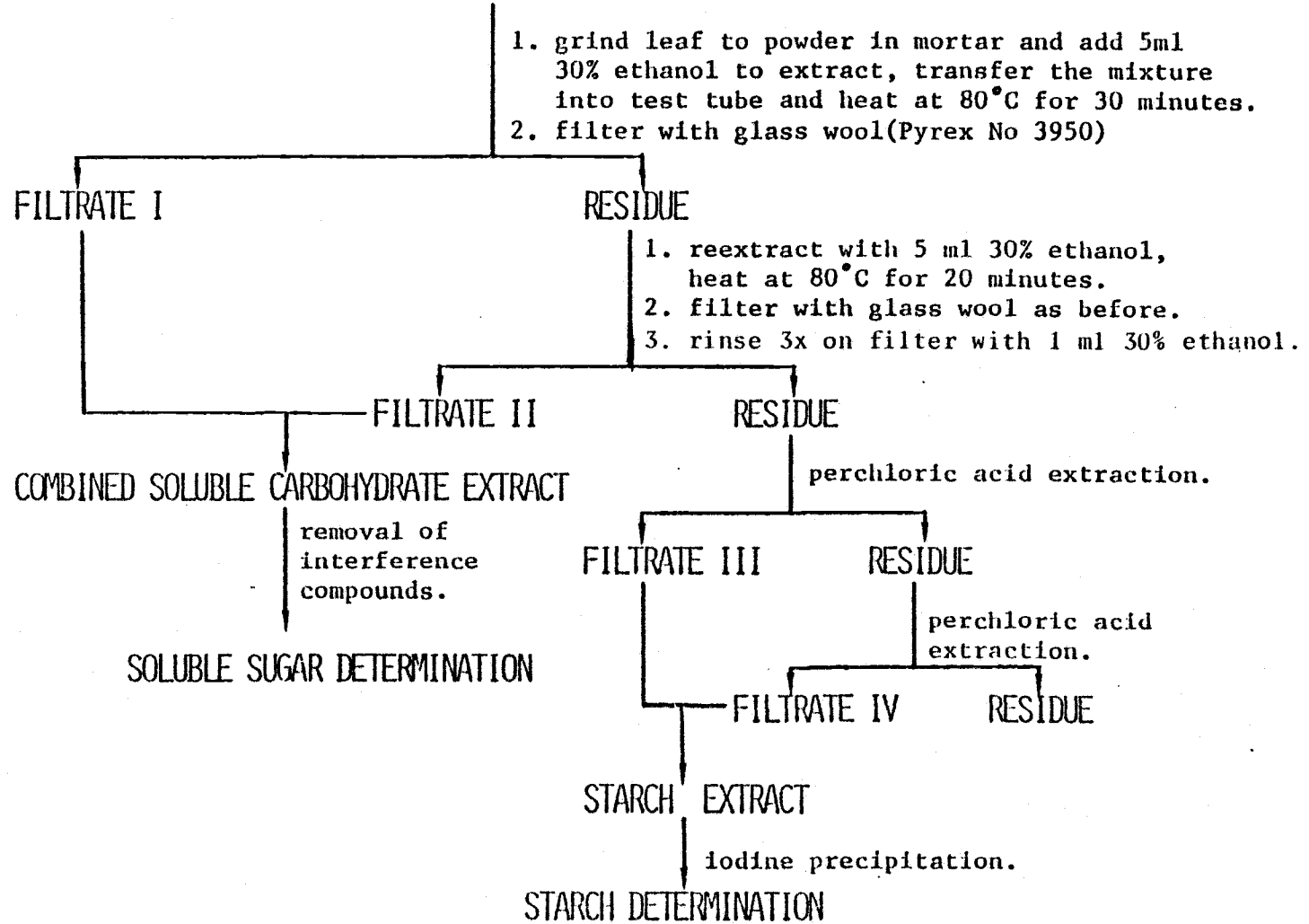


Figure 7. Flow chart of starch and soluble carbohydrate isolation.

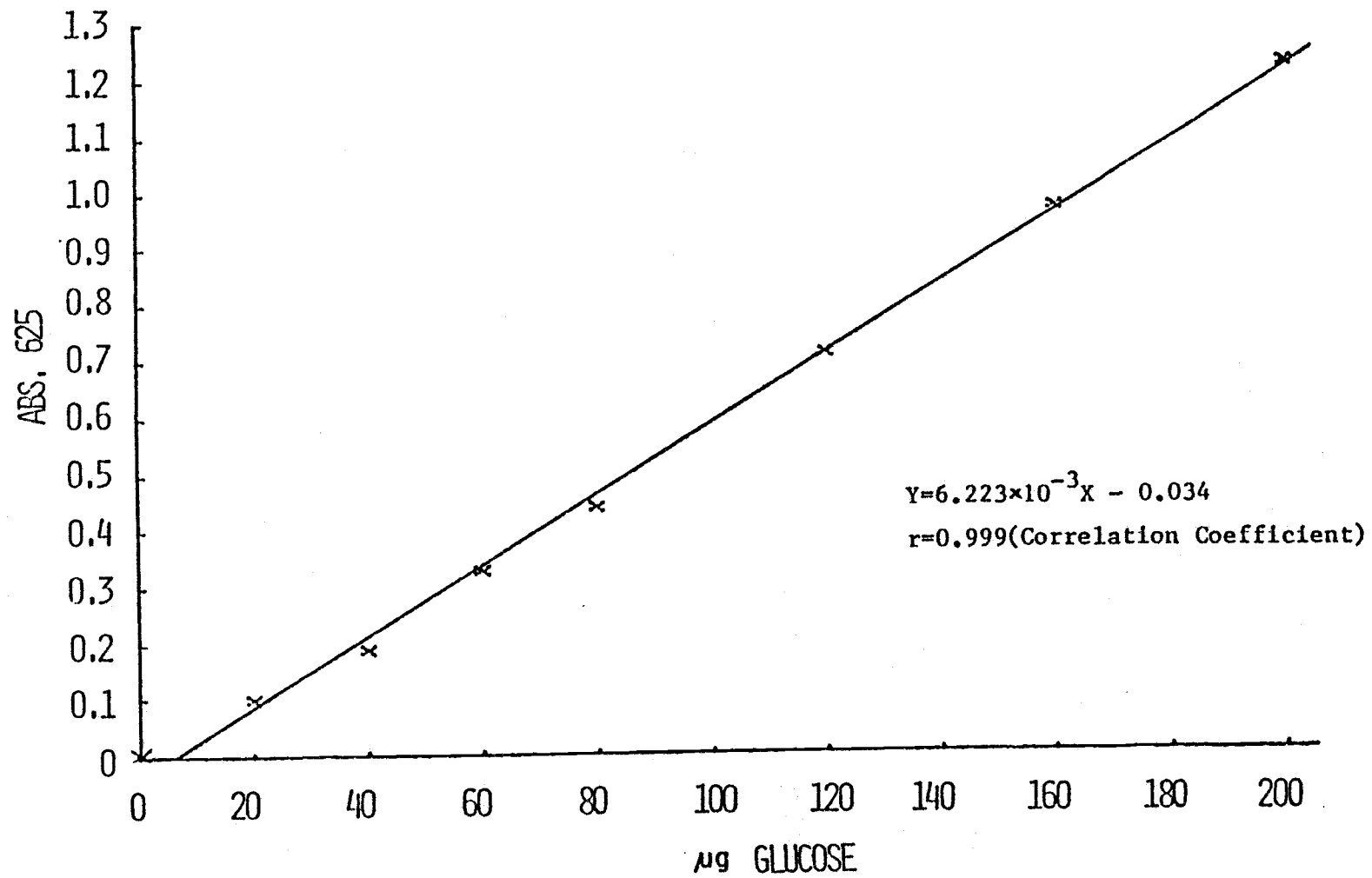


Figure 8. Glucose standard curve for colorimetric carbohydrate determination.

3.6.2 Starch

The plant material after removal of the soluble carbohydrates was placed in a 1.5 cm diameter x 15 cm long test tube. The sample was heated exactly 15 minutes in 5 ml distilled water in a boiling water bath and cooled to room temperature. Five ml of 70% perchloric acid (HClO_4) were added with stirring. The solution was allowed to stand for at least 30 minutes, filtered through glass wool and the starch extract collected in a 10 ml calibrated tube. The residue (and glasswool) were extracted without heating by another 5 ml 52% perchloric acid (31) for at least 30 minutes, as before. The second extract was combined with the first extract and distilled water was added to 10 ml. For extraction procedure, refer to Figure 7.

The perchloric acid extract was transferred to a 30 ml conical centrifuge tube, and 5 ml 20% sodium chloride and 2 ml I_2 -KI reagent (see solution make-up) were added and mixed (31). After standing for 20 minutes, the sample was centrifuged (5000xg, 15 minutes) and the supernatant carefully removed with a fine tip pipette. The precipitate was suspended in 5 ml 2% ethanolic sodium chloride solution (NaCl /ethanol, w/v), centrifuged and supernatant removed carefully as before. This step was repeated once more. To the packed precipitate, 2 ml 0.25 N ethanolic sodium hydroxide was

added, the tube covered and gently shaken until the blue color disappeared. The liberated starch was then centrifuged and washed twice with 5 ml portions of 2% ethanolic sodium chloride. The purified starch precipitate was redissolved in 2 ml hot water (31). The final solution was brought to constant volume (3 ml) with distilled water. One ml aliquots were used for anthrone determination of starch. Refer to Figure 8 for glucose standard curve used for determination of starch. For starch, the same curve as for sugar was used, except that a 10% correction was applied for the difference in molecular weight of glucose (M.W.=180) and of anhydroglucose residues (M.W.=162).

3.6.3 Colorimetric determination of carbohydrate using anthrone

A 1.0 ml aliquot of sample solution was placed into a test tube (3 cm in diameter x 20 cm in length). 0.5 ml distilled water and 0.5 ml of anthrone reagent (2% anthrone in ethyl acetate), then 6 ml of conc. H_2SO_4 were added and mixed thoroughly. The mixed solution was allowed to stand at room temperature until cool, then the tubes were placed in a 25°C water bath to obtain uniform temperature. Absorbance was read at 625 nm against a reagent blank. A series of glucose standards ranging from 0 to 200 μg glucose/per assay was run at

the same time with each set of samples and treated in exactly the same way as the samples. Samples too concentrated for spectrophotometer measurement were diluted with 75% H_2SO_4 (34).

3.6.4 Solution preparation

(a). 35% perchloric acid: 500 ml 70% perchloric acid was added to 500 ml distilled water.

(b). I_2 -KI reagent: 7.5 g I_2 and 7.5 g KI were ground together with 150 ml distilled water, and the resulting solution was diluted to 250 ml and filtered if necessary.

(c). 2% ethanolic sodium chloride: 350 ml 95% ethanol, 80 ml distilled water and 50 ml 20% sodium chloride were added together and diluted to 500 ml with distilled water.

(d). 0.25 N ethanolic sodium hydroxide: 350 ml ethanol, 100 ml distilled water and 25 ml 5 N sodium hydroxide were added and diluted to 500 ml with distilled water.

(e). 2% anthrone reagent: 2 g anthrone was added to reagent grade ethyl acetate to make a total volume 100 ml, filtered if necessary, and store in brown glass-stoppered bottle.

(f). 75% sulfuric acid: 75 ml conc. sulfuric acid was added to 25 ml distilled water and additional water

added to make a total 100 ml.

(g). Glucose standard: 10, 20, 40, 60, 80, 100, and 200 μg glucose/ml water standard were made and stored in volumetric flask.

(h). 30% ethanol: 158 ml 95% ethanol was added to 300 ml distilled water and the volume brought up to 500 ml with distilled water.

IV. RESULTS

Preliminary data for a 16-hour photoperiod with 8°C night and 25°C night showed a lack of marked compositional differences in the oil between the two night-temperature treatments (Appendices 1 & 2). When peppermint was grown under 14-hour day, the two night-temperature regimes (8°C vs. 25°C) produced marked difference in growth and in metabolite contents of the plants. Leaf pairs #3, were initiated before the cuttings were subjected to controlled environments and were also stressed by the rooting process. Therefore, leaf pairs #3 do not fully reflect the effect of different night temperatures. Analytical values for leaf pair #3 are shown here, but in general, the discussion will be based on leaf pair #4 and above.

Results (whole-plant values) are summarized in Table 1. It can be seen that warm nights resulted in greater growth, seen both as fresh weight and as leaf area.

Total soluble sugars were increased under the warm night regime, but not in proportion to the increased leaf area and fresh weight. This presumably reflects the balance between increased photosynthesis due to increased growth (greater biomass) and the energy and carbon required to produce that growth.

Total phenolics behaved similarly to leaf area and

Table 1. Summed whole plant measurements of fresh weight, leaf area and metabolite content.

Component	Summed value per plant*					
	14 Days		21 Days		28 Days	
	8°C night	25°C night	8°C night	25°C night	8°C night	25°C night
Fresh weight(mg)	304	568	732	1283	1290	2109
Leaf area(cm ²)	19	35	43	75	67	110
Fresh wt./leaf area(mg/cm ²)	16	16	17	17	19	19
Total phenolics(µg)	1389	1340	3657	4883	6001	7512
Total anthocyanins(µg)	45	14	227	76	241	41
Starch content(µg)	155	350	470	700	724	976
Soluble sugars(µg)	1016	2339	5452	7154	6021	6055
Total terpenes(nmoles)	826	1553	943	1798	1690	3478

* Sum of measurements of leaf pair #3 and above.

fresh weight, while conversely, anthocyanin production was greatly stimulated by cool nights.

Total terpenes increased approximately in proportion to total leaf area. However, it will be seen later that the composition of the essential oil differed dramatically between the two night-temperature regimes.

Table 2 summarizes the metabolite content results (whole-plant values) per unit leaf area. This presentation takes the leaf expansion into consideration as contrasted with the total measured values in Table 1.

4.1 Plant growth

4.1.1 Whole plant growth

Warm-night plants had one more leaf pair than cool night plants at each sampling date. The internodes of warm-night plants were longer than those of cool-night plants, making the warm-night plants taller.

4.1.1.1 Fresh weight

Table 1 shows that the warm-night plants had a greater fresh weight than cool-night plants at all three sampling dates. The fresh weight ratio of warm-night plants to cool-night plants (Table 1) at 14 days was 1.9; at 21 days was 1.8; and at 28 days was 1.6. The ratio decreased as the growth time increased. This

Table 2. Calculated value of metabolite contents on per unit leaf area.

	14 Days		21 Days		28 Days	
	8°C night	25°C night	8°C night	25°C night	8°C night	25°C night
Fresh weight (mg/cm ²)	16	16	17	17	19	19
Total phenolics (µg/cm ²)	75	38	85	65	90	68
Anthocyanins (µg/cm ²)	2.4	0.4	5.3	1.0	3.6	0.4
Starch (µg/cm ²)	8.4	10.0	10.9	9.4	10.9	8.9
Sugars (µg/cm ²)	54.9	66.8	126.5	95.8	90.3	55.2
Terpenes (nmoles/cm ²)	44.6	44.4	21.9	24.1	25.3	31.7

indicated that the fresh weight of cool-night plants increased more slowly in the early stage but became faster in later stages.

4.1.1.2 Leaf area

Table 1 also shows that warm-night plants had greater leaf area than cool-night plants because of greater growth. The leaf area ratio of warm-night plants to cool night plants was 1.9 at 14 days, 1.7 at 21 days, and 1.6 at 28 days. This ratio slowly declined with the increased growth time. Table 2 shows that the fresh weight was approximately proportional to the leaf area by showing similar fresh weight to leaf area ratio of warm-night and cool-night plants at three different stages. The ratio of fresh weight to leaf area of both warm-night and cool-night plants increased slightly with age.

4.1.2 Leaf pair growth

Figure 9 shows the average fresh weights of individual leaves at 14, 21 and 28 days. Figure 10 shows the areas of the same leaves, plus leaf areas at 40 days. It can be seen that warm-night plants had more leaves and larger leaves than cool-night plants.

Table 3 shows the detailed index of fresh weight to unit leaf area for individual leaves. It appears that

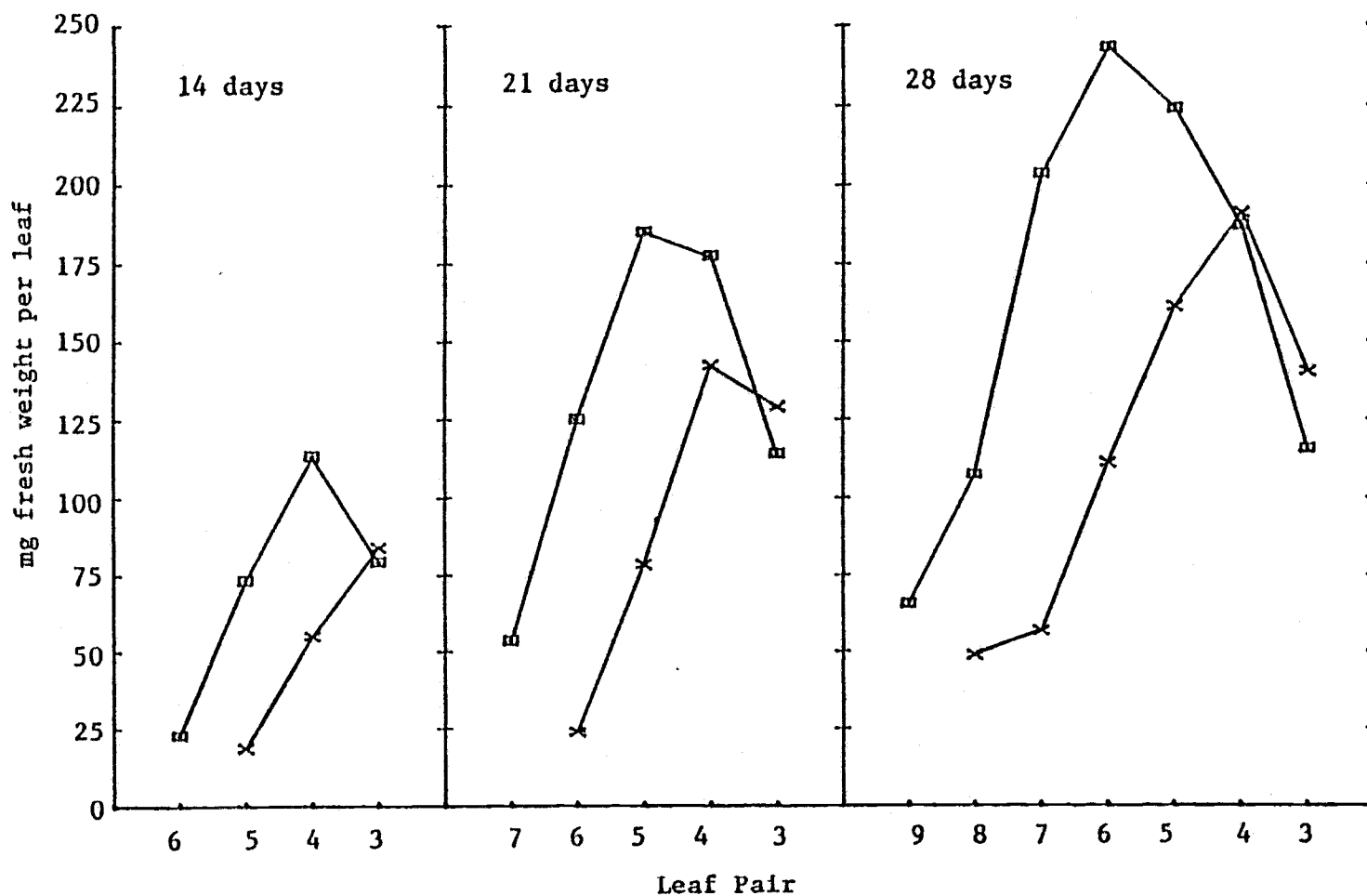


Figure 9. Fresh weight of peppermint leaves grown on 14-hour day with 8°C and 25°C night(X = 8°C night, □ = 25°C night).

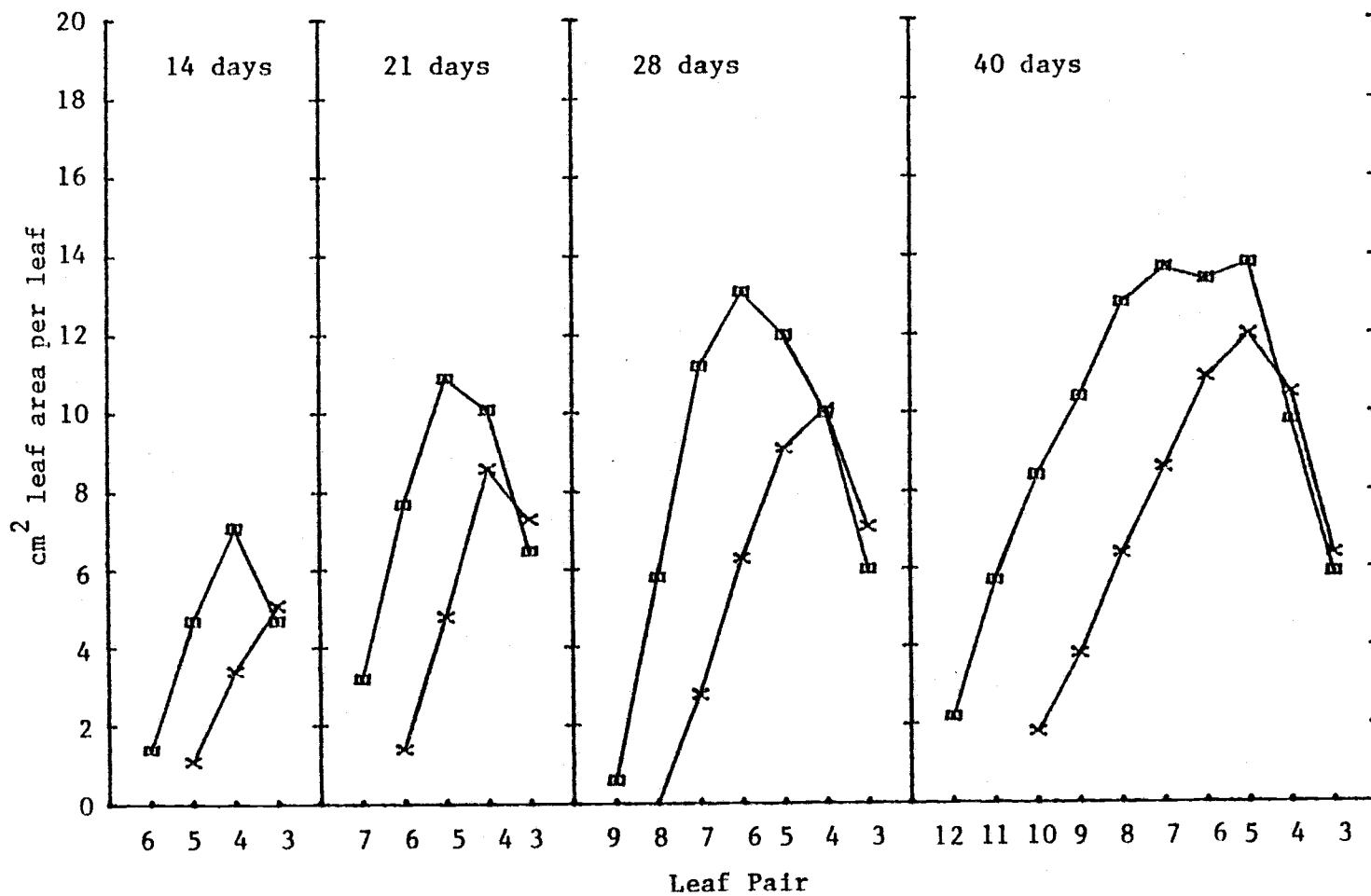


Figure 10. Leaf area of peppermint leaves grown on 14-hour day with 8°C and 25°C night (x = 8°C night, □ = 25°C night).

Table 3. Index of fresh weight/unit leaf area.

Leaf pair	Fresh weight/leaf area index(mg/cm ²)					
	14 Days		21 Days		28 Days	
	8°C night	25°C night	8°C night	25°C night	8°C night	25°C night
9						11.6
8					9.8	18.6
7				16.8	20.4	18.2
6		16.9	17.2	16.3	17.7	18.5
5	17.4	15.6	16.3	17.1	17.7	18.7
4	16.2	16.0	16.6	17.7	19.0	18.8
3	16.4	17.0	17.8	17.7	19.9	19.2

this index gradually increases with growth time and is little affected by night temperature.

4.2 Carbohydrate balance

4.2.1 Total starch content

The starch analyses as described in Chapter III were performed on individual leaves sampled about 3 hours after the start of the light period. Figure 11 shows that the total starch content was higher in warm-night plants than in cool-night plants, except in some old leaves. Both warm- and cool-night plants showed a tendency toward lower starch content per leaf, at higher positions.

At three sampling dates, when the whole plant is considered, warm-night plants had a higher starch content than cool-night plants. Table 1 shows that the difference in starch content between warm- and cool-night plants increased with increased growth time. However, when starch is expressed on a unit area basis (Figure 12) the young leaves of cool-night plants had higher starch content per cm^2 than those of warm-night plants. This suggests that warm night plants had more night-time depletion of starch and at the time of sampling the depletion had not been compensated (22).

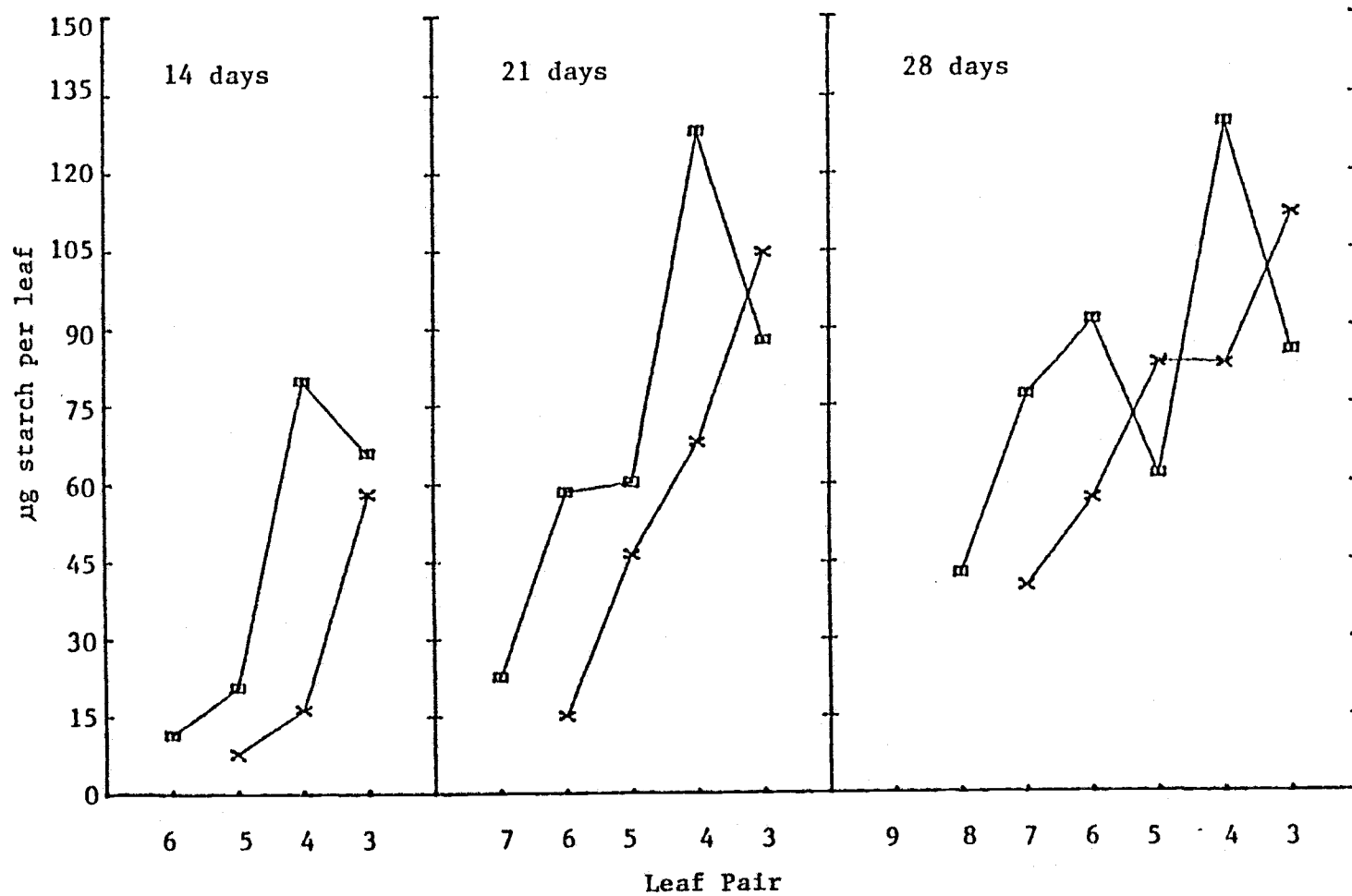


Figure 11. Starch content(per leaf) of peppermint leaves grown on 14-hour day with 8°C and 25°C night(x = 8°C night, □ = 25°C night).

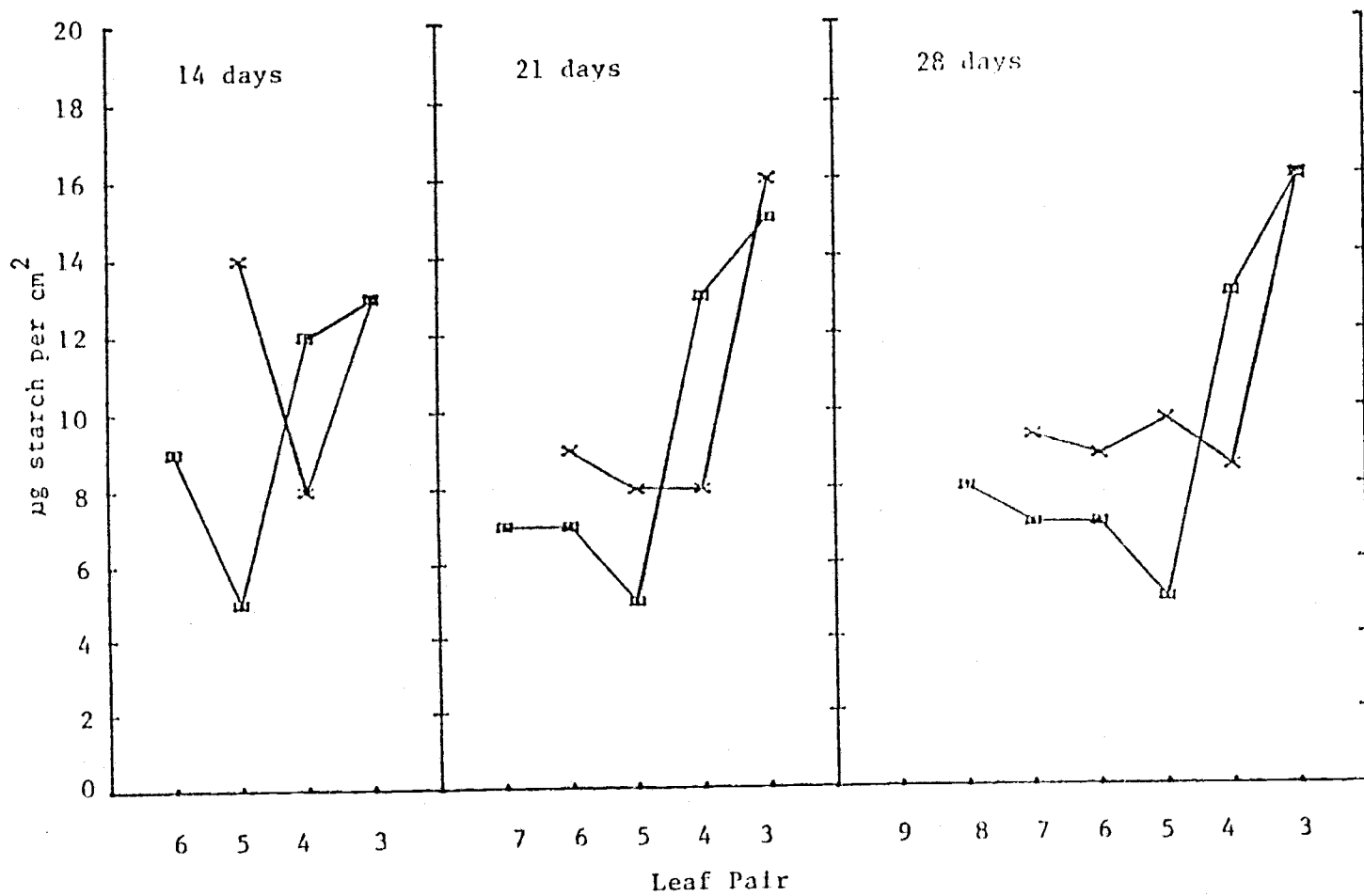


Figure 12. Starch content(per cm²) of peppermint leaves grown on 14-hour day with 8°C and 25°C night(× = 8°C night, u = 25°C night).

4.2.2 Soluble sugar content

The soluble sugar ratio of warm-night plants to cool-night plants is 2.3 at 14 days, 1.3 at 21 days, 1.0 at 28 days (Table 1). When the whole plant is considered, cool-night plants had less soluble sugar than the warm night plants (Table 1), and the difference increased with growth time. Figure 13 shows that soluble sugar content was higher at all leaf positions in warm-night plant except leaf pair #3 of the 21st day and leaf pairs #3, 4, and 5 of the 28th day. Figure 14 shows that generally cool-night plants had higher soluble sugar content per cm^2 leaf area.

4.3 Secondary products

4.3.1 Anthocyanins

The cool-night plants had consistently higher anthocyanin content than warm-night plants at three sampling dates (Table 1) and the difference increased as the growth time increased. The ratio of anthocyanin content of cool-night plants to warm-night plants was 3.2 at 14 days, 3.0 at 21 days and 5.9 at 28 days.

The cool-night plants had higher anthocyanin content at all leaf positions except newly initiated leaves, whether expressed per leaf (Figure 15) or per cm^2 (Figure 16). For example, comparing leaf pair # 5 at 28

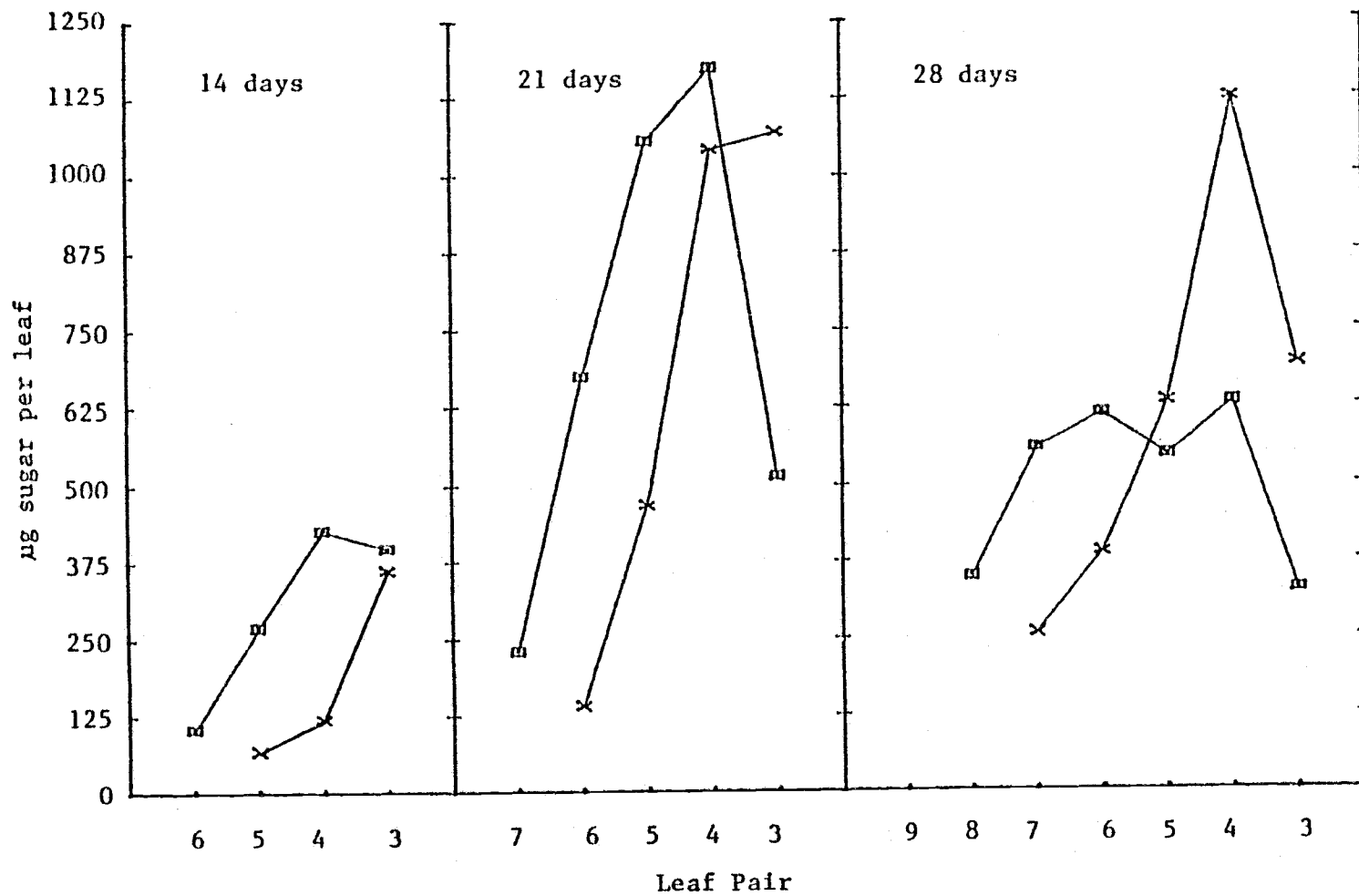


Figure 13. Sugar content(per leaf) of peppermint leaves grown on 14-hour day with 8°C and 25°C night(x = 8°C night, □ = 25°C night).

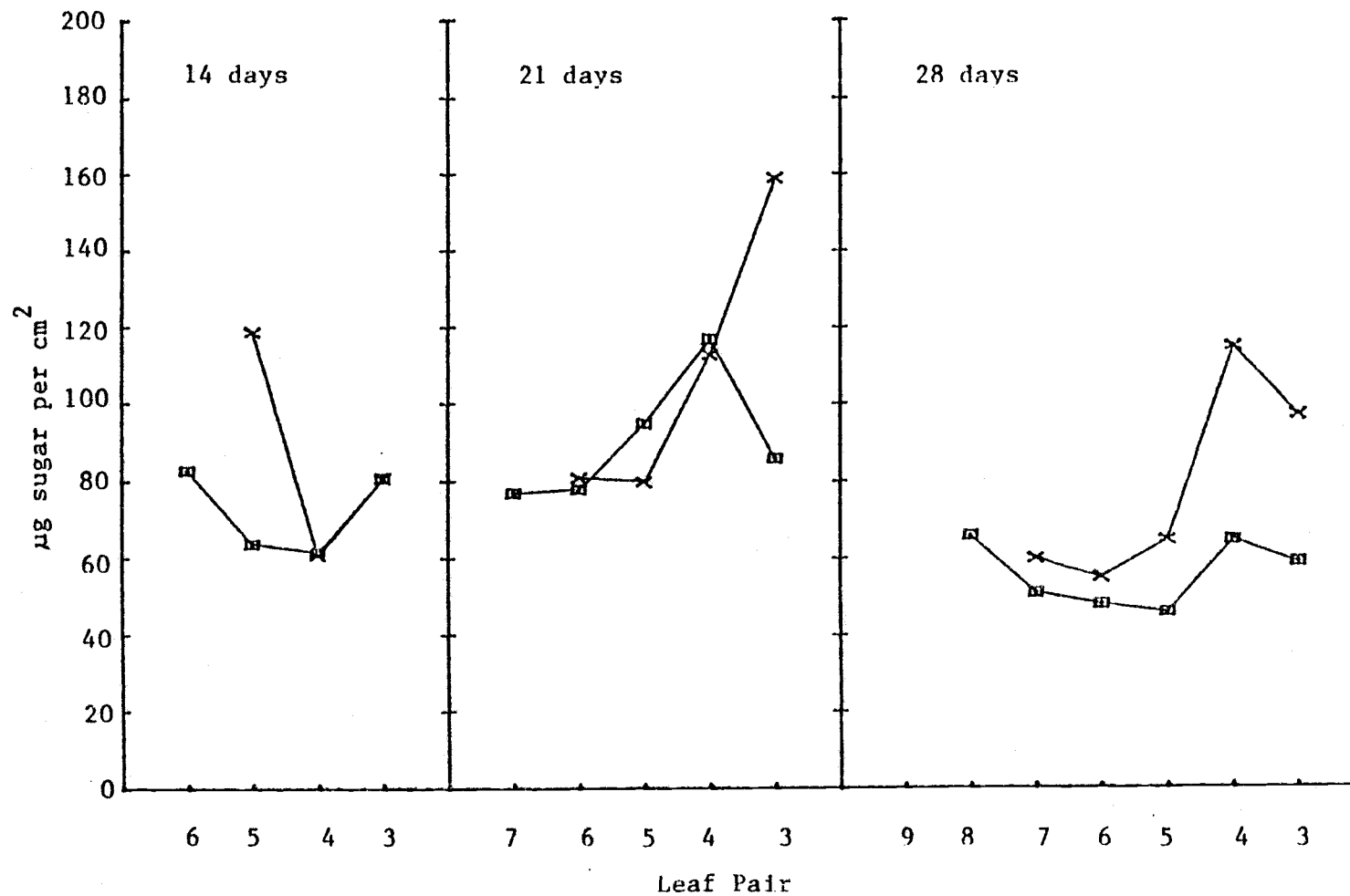


Figure 14. Sugar content(per cm²) of peppermint leaves grown on 14-hour day with 8°C and 25°C night(X = 8°C night, □ = 25°C night).

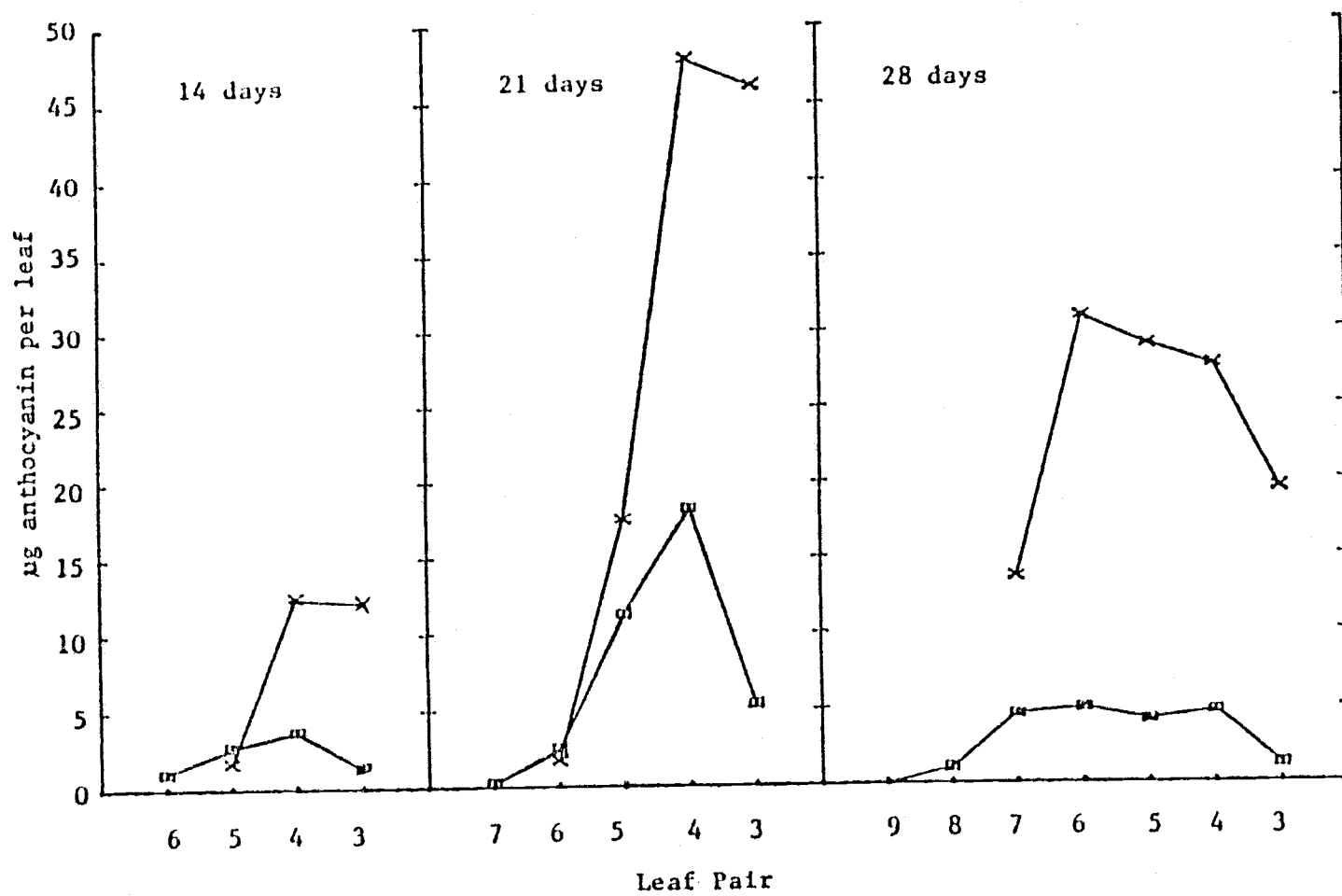


Figure 15. Anthocyanin content(per leaf) of peppermint leaves grown on 14-hour day with 8°C and 25°C night(X = 8°C night, □ = 25°C night).

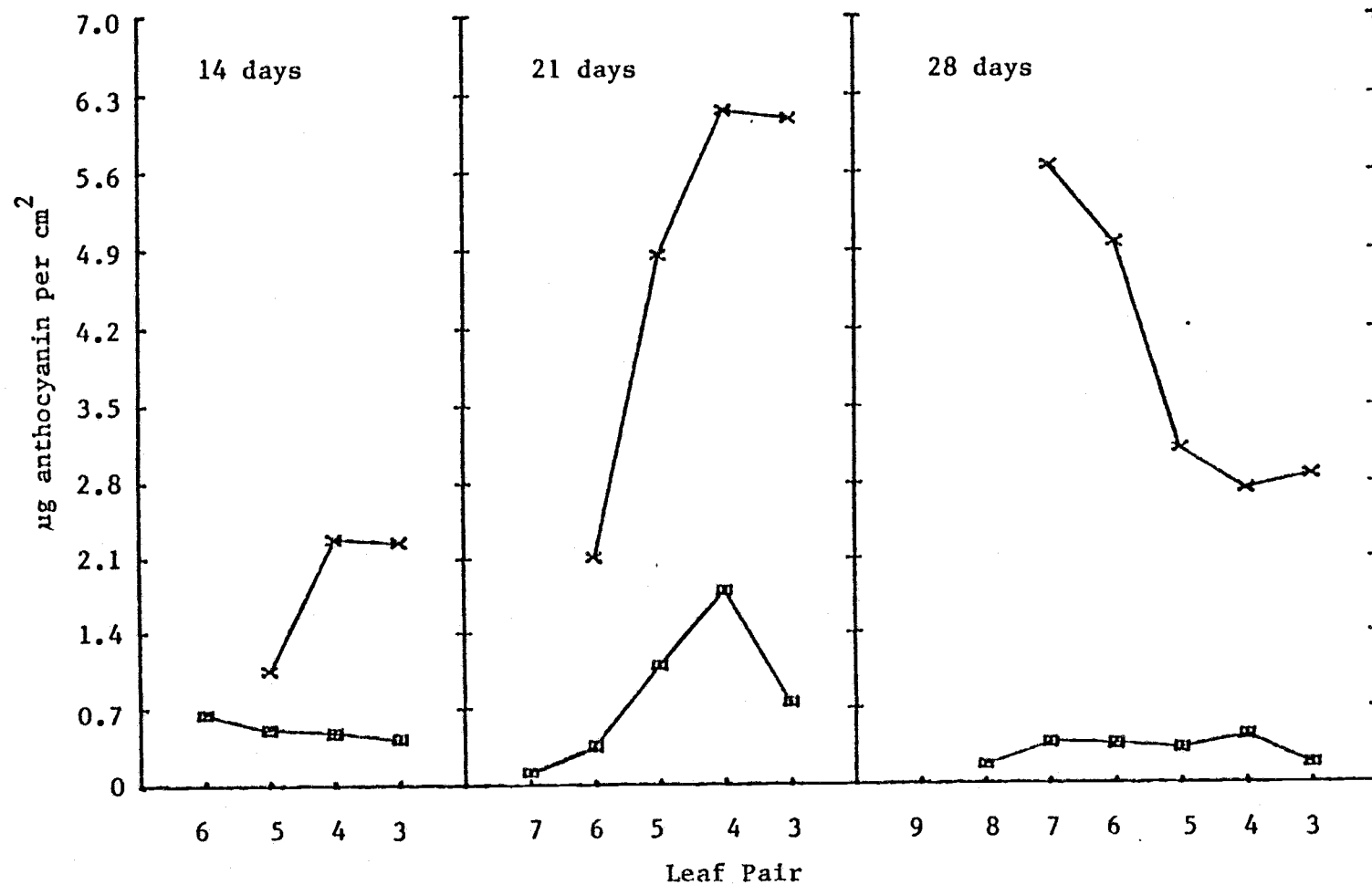


Figure 16. Anthocyanin content(per cm²) of peppermint leaves grown on 14-hour day with 8°C and 25°C night(x = 8°C night, □ = 25°C night).

days, cool nights produced 7 times as much anthocyanin per leaf and 9 times as much per cm^2 as warm nights. This indicates that cool nights greatly favored the synthesis and accumulation of anthocyanin. Ribereau-Gayon (47) reported lower amounts of anthocyanin of grape berries at 35°C . This agrees with our result that cool night favored the production of anthocyanin. Since anthocyanin is localized in the vacuoles of epidermal cells (32), one might anticipate that further analyses would show a relationship between surface area and limiting anthocyanin values.

4.3.2 Phenolics

Under both warm-night and cool-night conditions the total phenolic content increased with increased growth time (Table 1). This is consistent with the results of Singleton in grape berry studies (52). The ratio of total phenolics of warm-night plants to cool-night plants was 0.9 at 14 days, 1.3 at 21 days, and 1.3 at 28 days.

Figure 17 shows that warm-night plants had higher total phenolic content in young upper leaves. However, on a leaf-area basis (Figure 18) the cool-night plants generally had a higher content of total phenolics, especially in the youngest leaves.

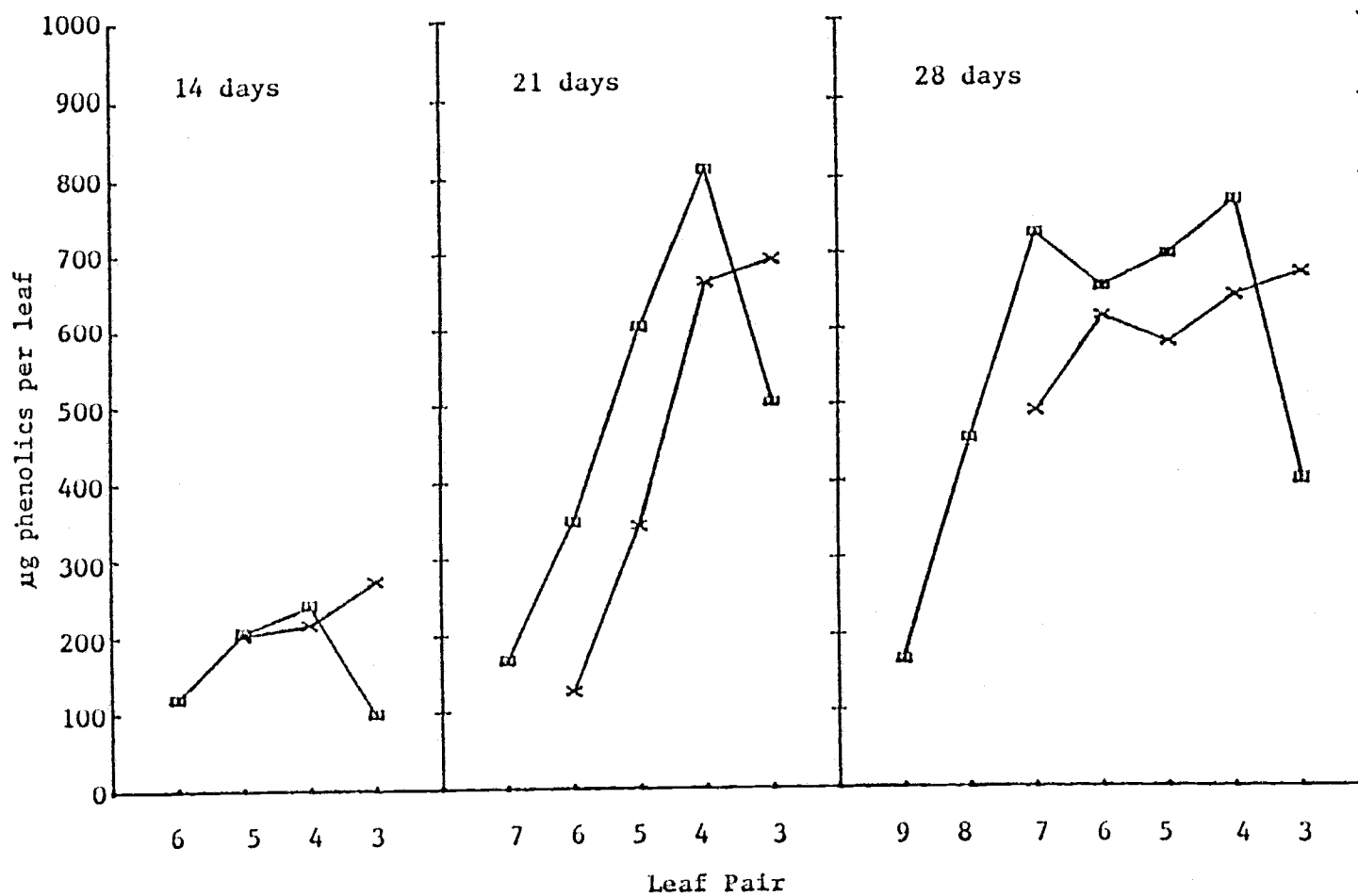


Figure 17. Total phenolic content(per leaf) of peppermint leaves grown on 14-hour day with 8°C and 25°C night(x = 8°C night, □ = 25°C night).



Figure 18. Total phenolic content(per cm²) of peppermint leaves grown on 14-hour day with 8°C and 25°C night(x = 8°C night, □ = 25°C night).

4.3.3 Terpenes

In the whole plant, the warm-night plants consistently had higher oil content at three sampling dates and the difference between warm and cool night plants increased with increased growth time (Table 1). The total terpene ratio of warm-night plant to cool-night plant was 1.8 at 14 days, 1.9 at 21 days and 2.0 at 28 days.

Figure 19 indicates that warm-night plants had higher total terpene per leaf in young leaves, while in old leaves there was little if any difference. Figure 20 shows that generally the cool-night plants had higher terpene content per cm^2 leaf area at upper leaf positions because of the smaller leaf size of the cool-night plants. As the leaves expanded and became older the terpene content did not increase as much as the leaf area increased; thus, the young leaves showed a higher terpene content per cm^2 than the old leaves. Table 4 compares the terpene content per leaf on different sampling dates at each of two temperatures instead of different temperatures at each sampling date.

Table 5 shows the percentage composition of the essential oil calculated for the whole plant at the three sampling dates.

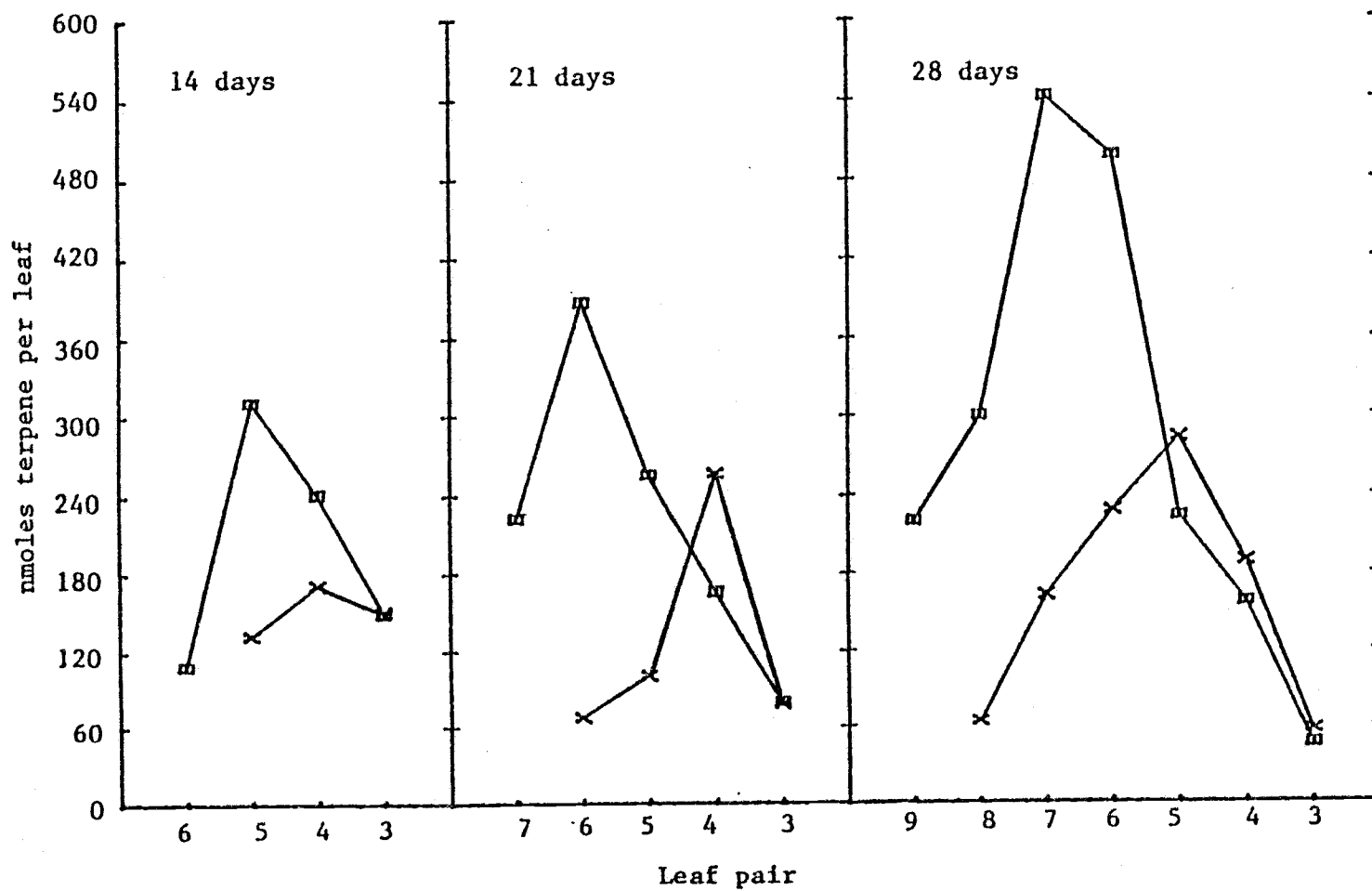


Figure 19. Total terpenes(per leaf) of peppermint leaves grown on 14-hour day with 8°C and 25°C night(X = 8°C night, □ = 25°C night).

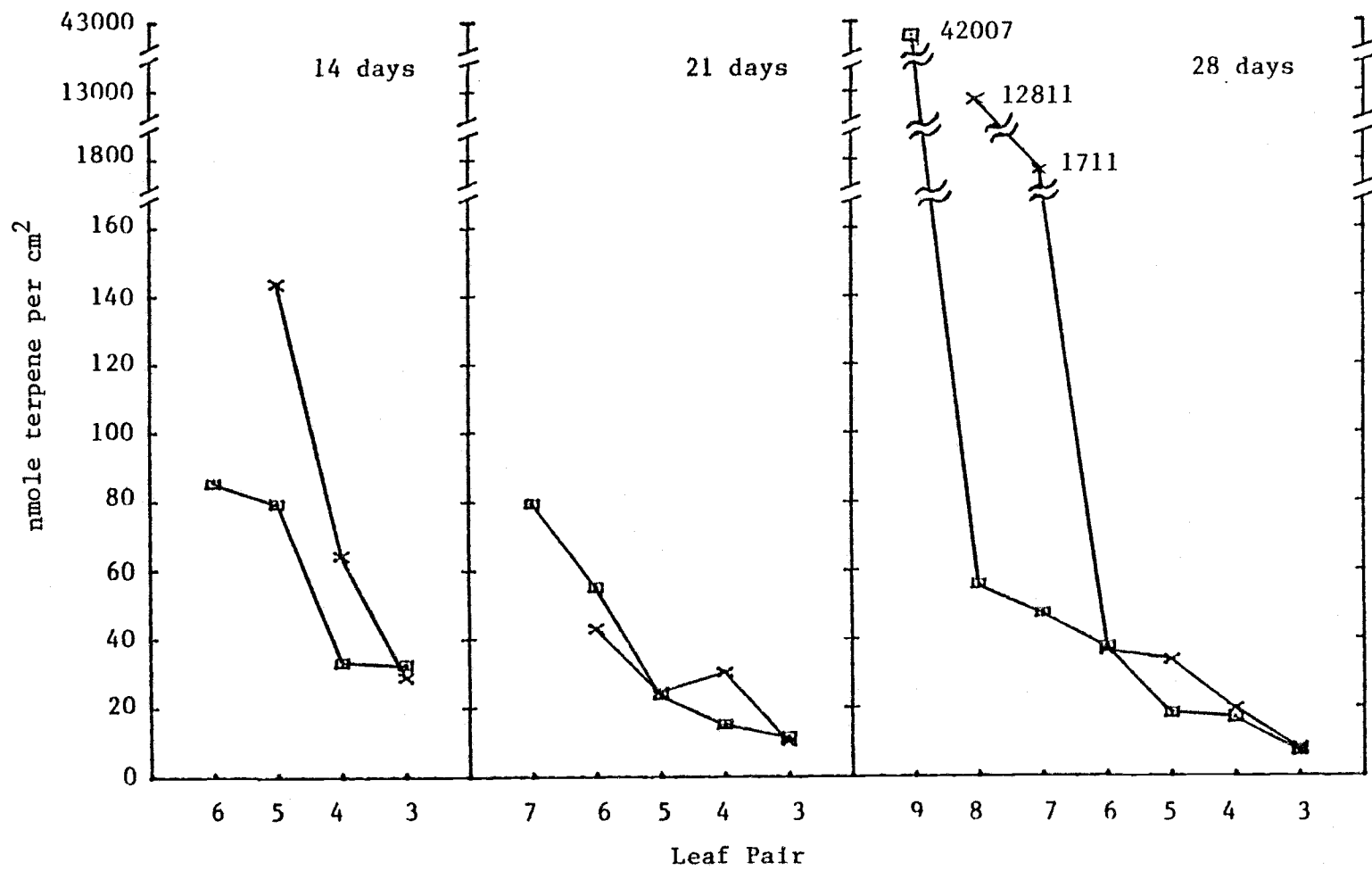


Figure 20. Total terpenes(per cm²) of peppermint leaves grown on 14-hour day with 8°C and 25°C night(x = 8°C night, □ = 25°C night).

Table 4. Total terpenes of individual peppermint leaves.

Leaf pair	Total terpenes (nmoles/leaf)					
	8°C night			25°C night		
	14 days	21 days	28 days	14 days	21 days	28 days
9						220
8			64			300
7			163		223	542
6		69	228	109	388	498
5	133	101	283	312	256	224
4	172	257	189	242	167	158
3	152	79	57	150	80	46

Table 5. Percent composition of essential oil of whole plant**.

Component	Mole % of total oil					
	14 Days		21 Days		28 Days	
	8°C night	25°C night	8°C night	25°C night	8°C night	25°C night
Limonene	3.56	1.82	2.15	0.96	2.62	1.36
1,8-Cineole	4.19	5.12	1.78	8.26	1.60	4.28
Unknown 1	0.20	1.09	1.61	0.67	0.90	0.46
Sabinene hydrate	3.32	3.05	3.61	5.74	3.48	8.12
Menthofuran	10.27	13.24	7.77	11.83	1.52	5.41
Menthones	58.04	50.19	62.63	55.18	75.15	63.68
Menthol*	4.92	9.15	12.56	5.20	10.40	8.37
Pulegone	12.36	13.95	6.54	9.92	2.39	7.09
Unknown 2	3.15	2.44	1.37	2.24	1.95	1.24
Total terpenes per plant(nmoles)	826	1554	943	1797	1692	3478

* "Menthol" here included up to 2% isomenthol in some cases.

** Based on summation of leaf oil analyses.

Menthone is the main component of the essential oil at these stages. Warm-night plants contained higher percentages of 1,8-cineole, sabinene hydrate, menthofuran and pulegone than cool-night plants, while, cool-night plants showed a higher percentage of menthones than warm-night plants. This was not because cool-night plants contained more total menthones than warm-night plants but because cool-night plants contained less total oil than warm-night plants.

The individual percentages of monoterpene are:

(a). Limonene: The percentage of limonene in cool-night plants approximately doubled those in warm-night plants throughout the three sampling dates. At both warm and cool night, the percentage of limonene decreased at the second sampling time;

(b). 1,8-Cineole: Plants grown at warm night had higher percentage of 1,8-Cineole at the three sampling dates. In cool night plants, the percentage of 1.8-Cineole decreased with increased growth time;

(c). Sabinene hydrate: The percentage of sabinene hydrate increased with longer growth time in warm-night plants, but was almost constant in cool-night plants. The percentage of sabinene hydrate was almost the same at the 14 days for cool-night and warm-night plants. However, the difference became greater at later sampling dates;

(d). Menthofuran: The percentage of menthofuran decreased with increased growth time, and was higher in warm-night plants;

(e). Menthone: The percentage of menthone increased with increased growth time, and was consistently higher in the cool-night plants;

(f). Menthol: Warm-night plants had a higher percentage of menthol on the 14th day. Cool-night plants had a higher percentage composition of menthol on the 21st and the 28th day;

(g). Pulegone: The percentage of pulegone decreased with increased growth time, and was higher in warm-night plants. The difference was greater with increased growth time.

Menthofuran, menthone (plus isomenthone), menthol and pulegone which make up more than 80 percent of the total terpenes were considered as "major" components. Figures 21 to 24 present these four major components respectively. The data are plotted with the youngest leaf at the left. In general, the warm-night leaves contained more monoterpene per leaf, reflecting their larger size. However, individual components behaved in differing ways. Warm nights generally resulted in greatly increased amounts of menthofuran and pulegone, especially in the younger leaves. The amounts of menthone and menthol increased with time from 14 to 28 days

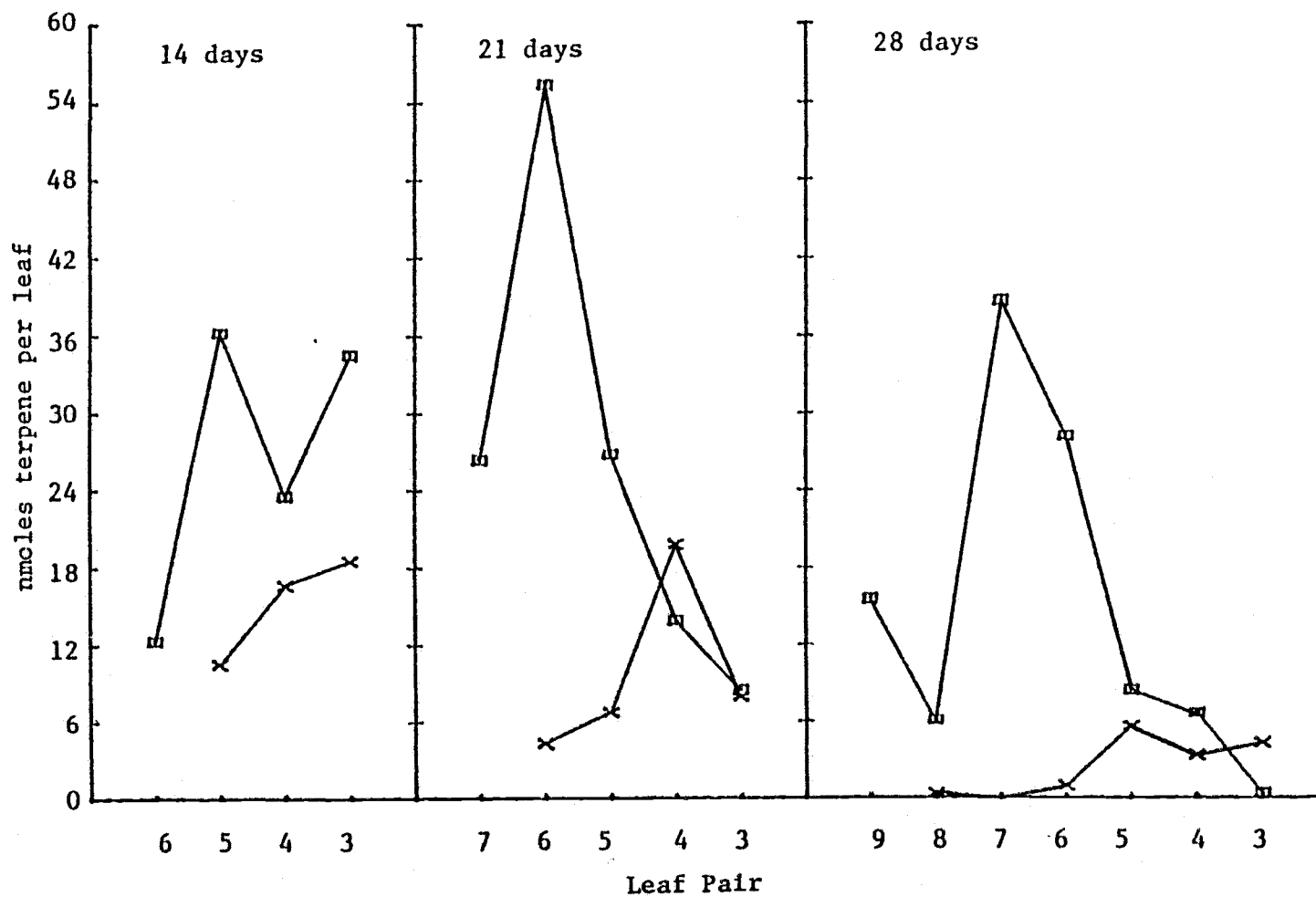


Figure 21. Menthofuran content of peppermint leaves grown on 14-hour day with 8°C and 25°C night (x = 8°C night, □ = 25°C night).

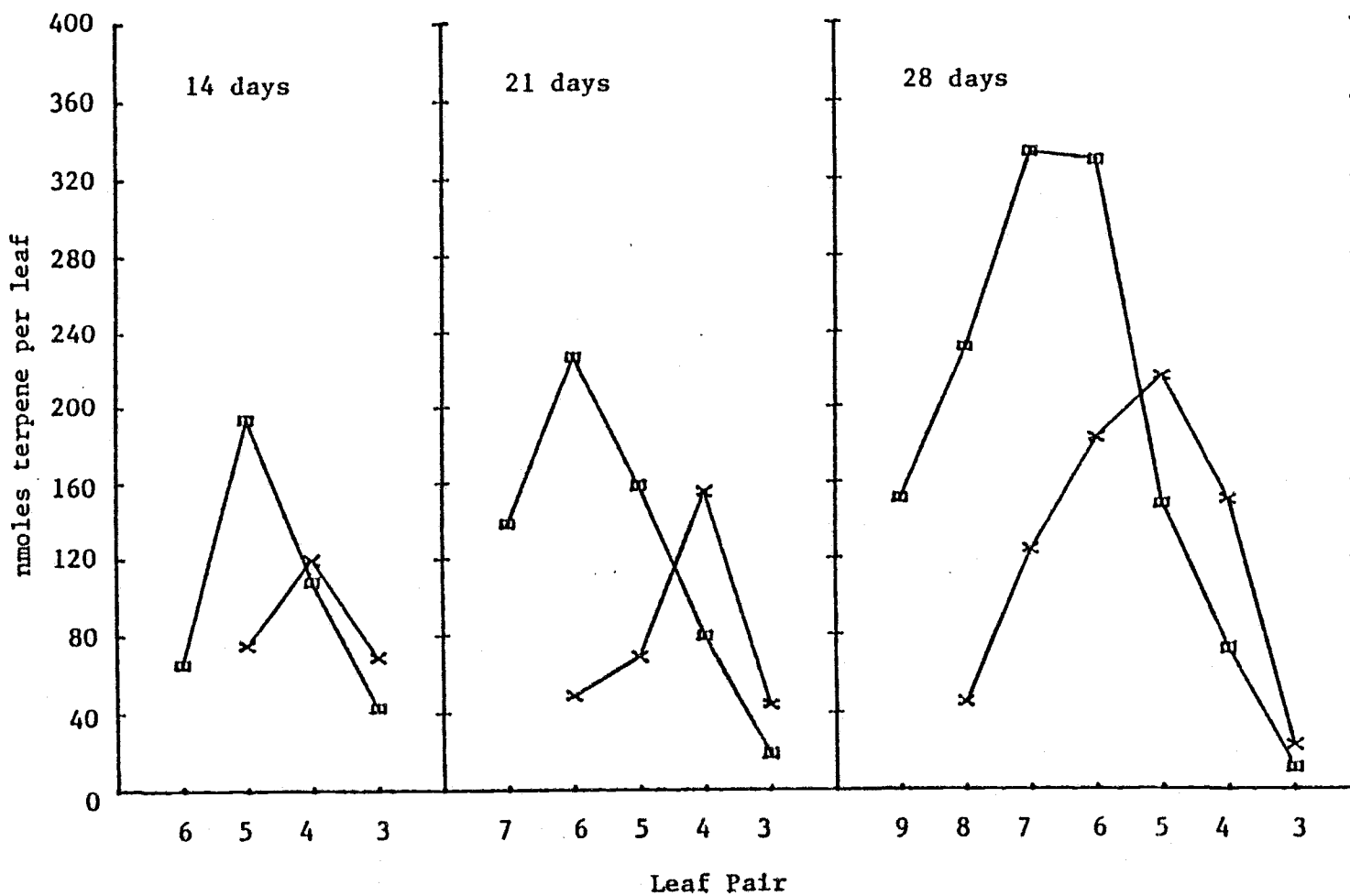


Figure 22. Menthone content of peppermint leaves grown on 14-hour day with 8°C and 25°C night(x = 8°C night, B = 25°C night).

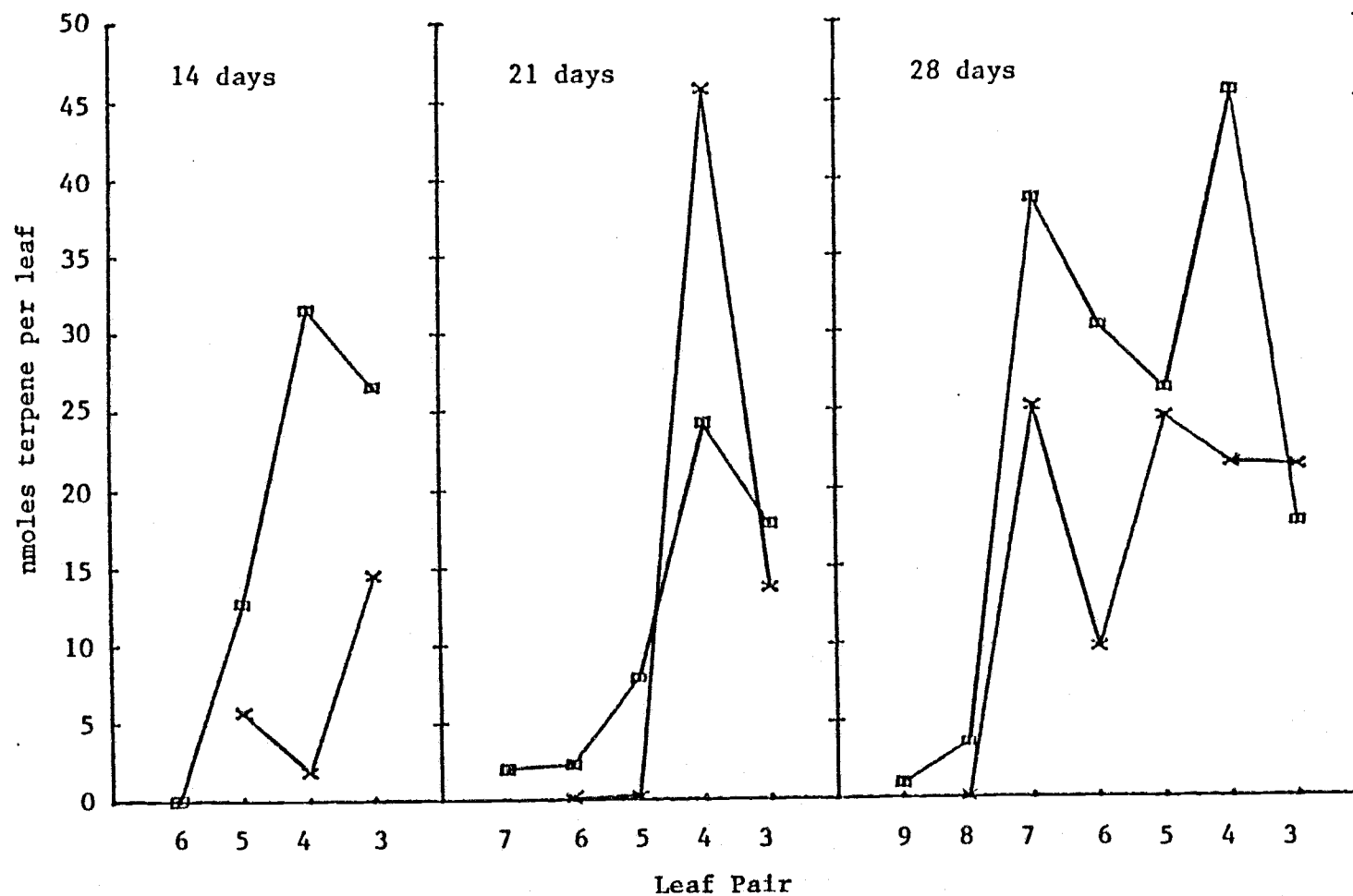


Figure 23. Menthol content of peppermint leaves grown on 14-hour day with 8°C and 25°C night (X = 8°C night, □ = 25°C night).

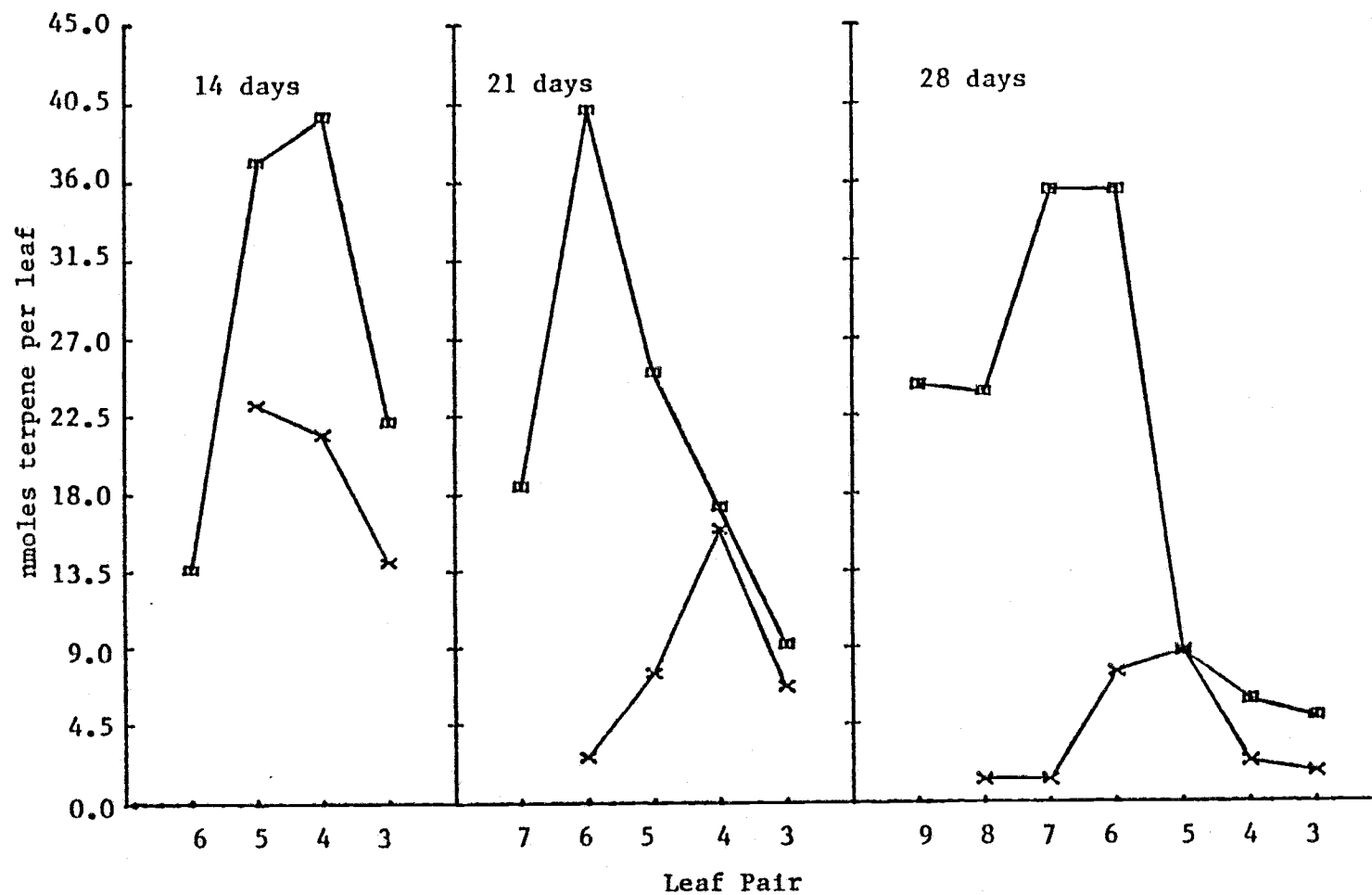


Figure 24. Pulegone content of peppermint leaves grown on 14-hour day with 8°C and 25°C night(X = 8°C night, □ = 25°C night).

(Figure 22, 23). Cool nights stimulated menthone production as the leaves aged (cf. leaf pairs 4 and 5 of Figure 22)

The warm-night plants had higher terpene content per leaf than cool-night plants at upper young leaves. Table 6 shows that when the whole plant is considered, the warm-night plants had higher contents of menthofuran, menthone and pulegone. But menthofuran and pulegone are relatively much higher than is menthone at all three sampling dates. It also indicates that warm nights favored the relatively oxidized compounds, pulegone and menthofuran, while the cool nights favored the more reduced compound, menthone. This is consistent with the findings of Burbott and Loomis (6). In addition, it shows that for cool-night plants, the total monoterpene content change with respect to growth time is that menthofuran and pulegone contents decrease while menthone content increases.

Figures 25a, b, c present the above data in a different way. This presentation shows the individual-leaf values for the individual major components, and the night temperature effect on differences in oil composition.

Table 6. Amount of individual essential oil components of whole plant.

Component	Individual oil component(nmoles) per plant*					
	14 Days		21 Days		28 Days	
	8°C night	25°C night	8°C night	25°C night	8°C night	25°C night
Limonene	29	28	20	17	44	47
1,8-Cineole	35	80	17	148	3	149
Unknown 1	2	17	15	12	1	16
Sabinene hydrate	27	47	34	103	59	282
Menthofuran	85	206	73	213	26	188
Menthones	479	779	591	992	1270	2215
Menthol**	41	142	118	94	116	291
Pulegone	102	217	62	178	40	247
Unknown 2	26	38	13	40	33	43
Total terpenes	826	1554	943	1797	1692	3478

* Sum of essential oil components of leaf pairs #3 and above.

** "Menthol" here included up to 2% isomenthol in some cases.

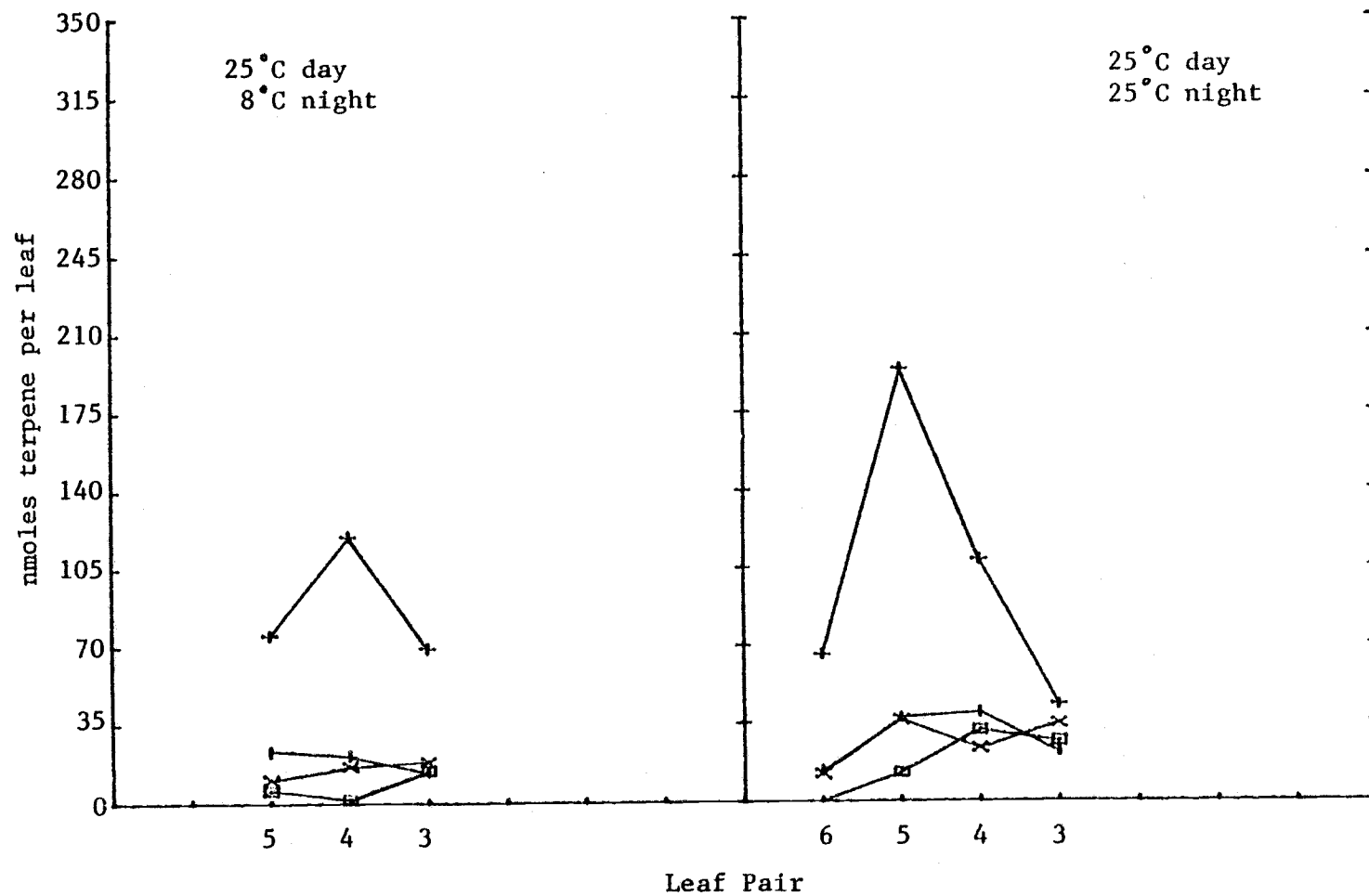


Figure 25a. Major monoterpenes of peppermint leaves grown on 14-hour day with 8°C and 25°C night for 14 days(+ = menthones, X = menthofuran, □ = menthol, | = pulegone).

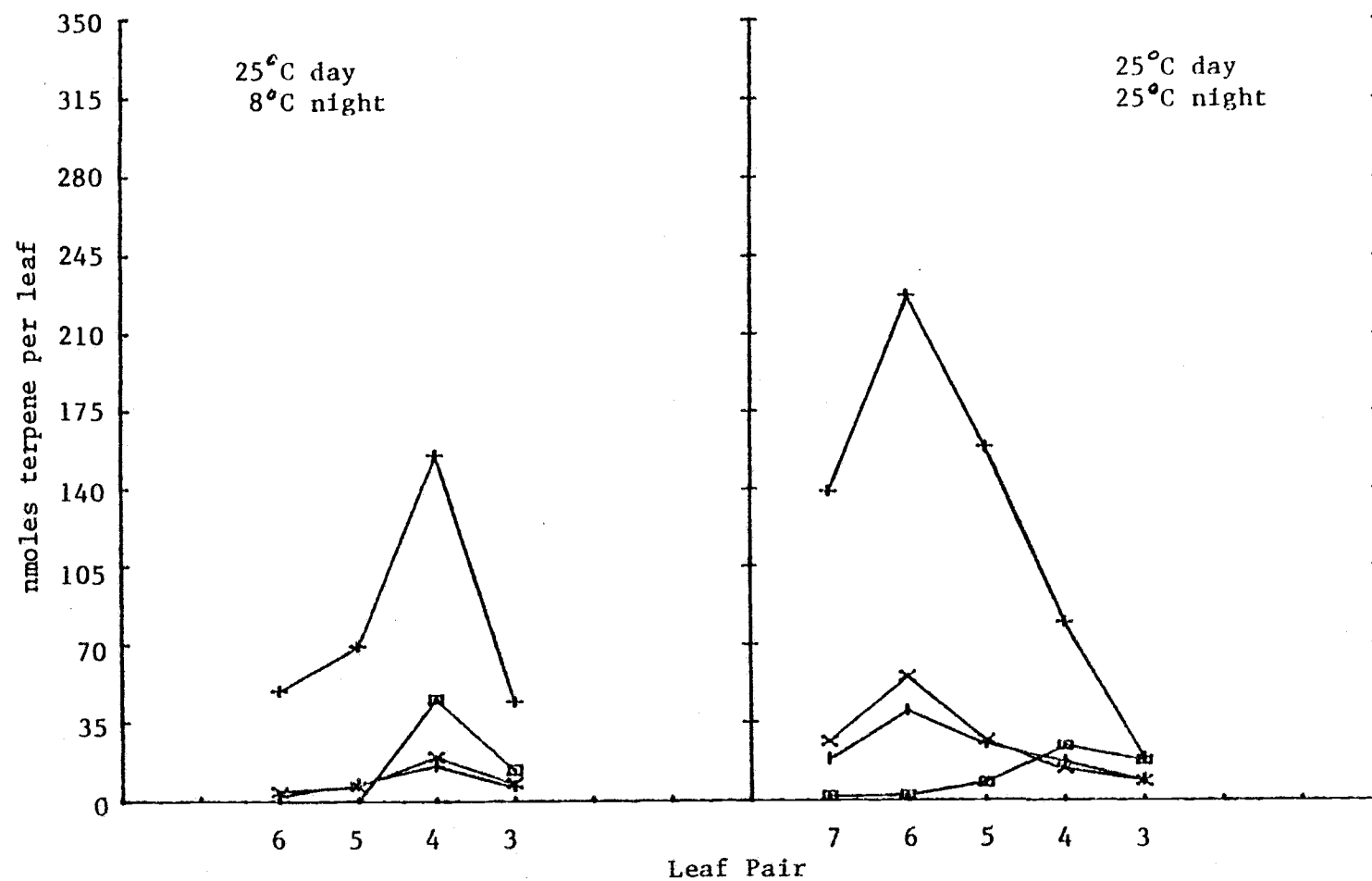


Figure 25b. Major monoterpenes of peppermint leaves grown on 14-hour day with 8 C and 25°C night for 21 days(+ = menthones, x = menthofuran, □ = menthol, | = pulegone).

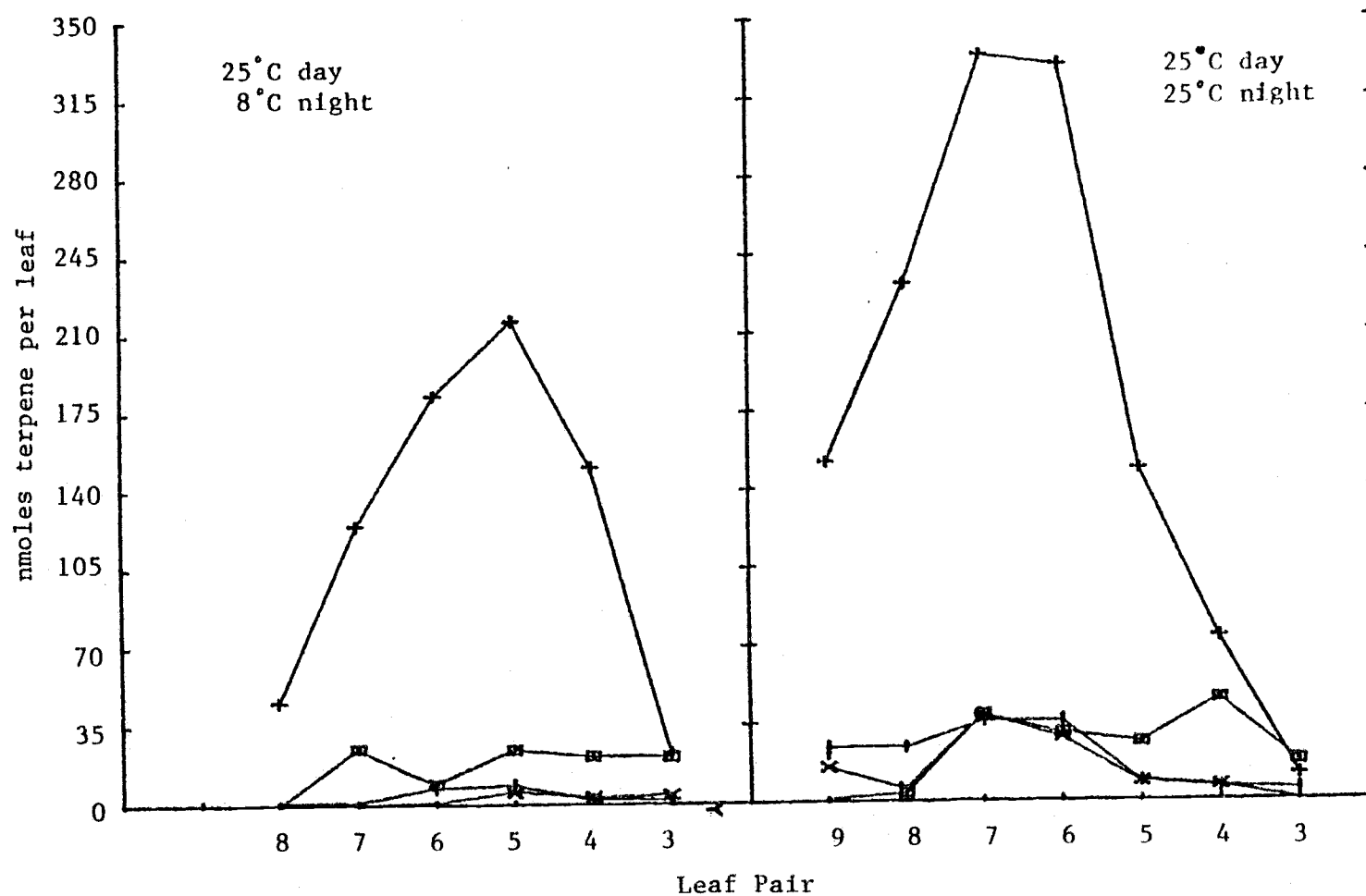


Figure 25c. Major monoterpenes of peppermint leaves grown on 14-hour day with 8°C and 25°C night for 28 days(+ = menthone, x = menthofuran. □ = menthol, | = pulegone).

V. DISCUSSION

Environmental effects on plant growth and development are complex. Changes in one factor will consequently change other factors. Thus, the final growth and development difference is an integrated effect of the various environmental factors.

The plant material in this research was propagated vegetatively, and was therefore genetically uniform. Though the cuttings were matched as closely as possible at the start of the experiment, it was impossible to match them exactly.

The research described here was designed to test the hypothesis that synthesis and metabolism of secondary products in plants is controlled by the competition for photosynthate. Burbott and Loomis (6) had reported earlier that effects of light and night temperature on the oxidation/reduction state of peppermint monoterpenes were consistent with this hypothesis. In their work, long-day conditions, or interrupted nights, greatly stimulated growth, with a resulting increase in monoterpene production. Their results suggested that effects on the essential oil were secondary to effects on growth, i.e. more growth means more oil glands and more photosynthetic tissue.

Clark and Menary (10) using 13 hours continuous illumination, compared with 12 hours + 1 hour (the

latter to simulate long-day condition) obtained similar results. In spite of the fact that night interruption doubled the dry-matter content of the plants and produced a typical upright summer growth habit in contrast to a recumbent winter growth habit with small leaves and many stolons, they concluded that effects on monoterpenes were direct photoperiod effects. In subsequent publications (11, 12), they concluded that some of the temperature effects could be explained by competition for carbohydrate. We feel that the experiments of Burbott and Loomis (6) ruled out a direct photoperiod effect on monoterpene synthesis or metabolism, but carbohydrate metabolism was not examined.

In the current study, we initially grew plants on 16 hour days, a long-day condition for peppermint, not tested by Burbott and Loomis. These plants did not show pronounced effects of night temperature on monoterpenes, so the 14-hour day, marginally short-day for peppermint, was used. Warm-night plants produced more and larger leaves than cool-night plants. These large warm-night leaves contained more essential oil per leaf than the cool-night leaves. We cannot say whether this was due to the initiation of more oil glands, or to a better filling of the glands. The warm-night leaves had greater amounts of oxidized monoterpenes, menthofuran

and pulegone, and smaller amounts of phenolic compounds (μg phenolics/ cm^2 leaf area) than the cool-night plants. They also contained less starch and sugar per cm^2 , when sampled at 3 hours after the start of the light period. Peppermint (Burbott, unpublished) apparently behaves like sugar beet (22) in its diurnal pattern of carbohydrate levels. If so, the 3-hour sampling time would represent an early stage in the recovery from night-time carbohydrate depletion. We regard the analyses presented here as tentative evidence for greater night-time depletion of carbohydrates in warm nights, and as a preliminary to experiments in which the total diurnal pattern of carbohydrate levels in peppermint leaves will be correlated with secondary product synthesis and metabolism.

The results presented here indicated that the sugar content was positively correlated with total terpene content and was negatively correlated with total anthocyanin content. It appears that sucrose serves as an important transportable energy source for the synthesis of terpenes and anthocyanins.

DeAngelis (17) found that sucrose is the predominant soluble carbohydrate in peppermint. Thimann and Edmondson (55) found the addition of sucrose increased the synthesis of anthocyanins in the study of Spirodela oligorrhiza. Ribereau-Gayon (47) also found a

similar relation existed in grapes. Croteau et al.(13) reported that when unlabelled sucrose is fed along with ¹⁴C-mevalonate, the incorporation of mevalonate into mono- and sequi-terpenes is markedly increased in peppermint.

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APPENDICES

APPENDIX 1

The percent composition of essential oil of peppermint
grown under 16-hour photoperiod for 35 days.

Leaf pair #	Mole % of total oil (25 ° C day & night)									
	3	4	5	6	7	8	9	10	11	12
Component										
limonene	0	3.4	-	0.1	0.1	0.1	0.1	0.1	1.9	0
1,8-Cineole	2.4	10.4	-	9.0	10.2	7.7	5.6	5.6	2.3	0
Sabinene hydrate	7.3	4.2	-	5.7	5.8	5.5	5.9	5.2	4.0	2.7
Menthofuran	20.5	14.4	-	6.3	5.7	6.1	5.7	5.0	2.5	6.4
Menthones	29.2	18.6	-	42.6	53.2	66.8	73.8	78.3	87.7	76.7
Menthol	38.3	49.0	-	34.9	24.1	11.8	7.3	3.2	1.3	9.2
Pulegone	2.3	0	-	0.1	0.1	1.5	1.4	2.0	0.1	5.1