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Boronia Extracts

Increasing Yield and Quality

A report for the Rural Industries Research and Development Corporation

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Foreword

Research by MacTavish (1995) illustrated key areas where there was the potential for significant increases in the concentration of floral extract, including volatiles such as β–ionone, to be achieved via management practices. These include harvest timing, post-harvest incubation of flowers and the extraction protocol used; the potential of the first two practices are modified by clonal differences. All of this work was preliminary, and on a small-scale. It was imperative for the essential oils industry in Tasmania that the potential for using this information on a commercial-scale be investigated.

The possibility that biosynthesis of floral volatiles or hydrolysis of precursors in boronia flowers continued after harvest was novel and required extensive verification. The opportunity to extract an increased yield of floral volatiles from a standard quantity of flower material has economic significance, and implications for *in vitro* production.

Research presented in this report has illustrated that not only is post-harvest production of floral volatiles reproducible, it has considerable potential in some clones under specific environmental conditions. Our research has also shed some light on the biological processes behind this and the limitations to post-harvest changes. In addition, specific information regarding accumulation of extract throughout flowering has been obtained for each of the commercial clones, allowing optimisation of harvest timing.

The opportunity for production of floral extracts with new, and perhaps improved, organoleptic properties is demonstrated via several means: harvest timing, post-harvest incubation, solvent extraction protocol and pre-extraction flower treatment. Thus the objectives of the project: 'To produce boronia concrete at higher yields and with improved quality by developing pre-harvest, harvesting, and post-harvest management practices and by value-adding the existing product' have in the main been achieved.

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Peter Core
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Executive Summary

The demonstration of how rapidly the boronia plant responds to changes in the external environment and how these changes manifest as changes in pools of secondary metabolites, particularly floral volatiles is significant.

In general, clones #3 and #5 had a greater concentration of extract compared with clones #17 and #250. This suggests that there is genetic control of the concentration of extract accumulated by particular plants. It is also possible that the clones differ in the 'extractability' of extract. It appears from this research that all four clones are best harvested at one time. Clones #3 and #5 progress through flowering earlier than clones #17 and #250 and yet the former two clones maintain extract yield/plant in late stages of flowering despite a decline in the concentration of extract at this time. The organoleptic quality of floral extracts from all four clones vary, however extract from clones #3 and #5 is most desirable in late stages of flowering, and from clones #17 and #250, at earlier stages.

The pool of volatiles bound in forms other than glycosides may represent ‘real’ precursors, in that the flowers actually metabolise these compounds into volatiles throughout flowering. The increase in glycosidically bound volatiles throughout flowering may result from a decline in glycosidase activity, however such forms may represent a step in the catabolism of non-emitted volatiles, enabling transportation from the flower prior to floral abscission. The role of glycosidically bound volatiles in producing post-harvest increases in volatiles may not be so clear cut, since excision from the plant may alter normal metabolic functions. ATP is required for *de novo* biosynthesis of volatiles (Rogers *et al.*, 1967), however it does not appear to be required for hydrolysis of precursors into free volatiles (Günata *et al.*, 1990).

There appears to be a maximum level of floral extract and volatiles that can be accumulated by a whole flower, evidenced by the decline in extract after 80% open flowers. The *in vivo* equilibrium position may be set by the number and volume of storage sites available for free volatiles in plastids, by enzyme kinetics and by supply of substrates.

Increases in free volatiles in boronia flowers after harvest occur only in fresh, undamaged open flowers, which suggests that enzymic activity brings about the changes. The greatest increases occur in flowers which have the potential for continued aerobic respiration: unhampered by extremes of temperature or high concentrations of nitrogen gas, and without physical disruption caused by freezing and thawing. The limit to post-harvest production of volatiles may be the supply of precursors or cofactors within the flower at harvest, the activity of enzymes, the toxicity of products, or changed conditions such as cell pH. A limit to available photosynthate or starch for respiratory processes may limit production of volatiles after harvest (Loomis and Croteau, 1973).

Increases in the concentration of extract including total volatiles and β-ionone as a result of post-harvest incubation are reproducible phenomena, occurring throughout flowering, at different incubation temperatures and at different rates depending on the clonal type. It is possible to make suggestions as to the best incubation procedures for each of the four currently available commercial clones.

There is significant potential for the production of floral boronia products with different oganoleptic properties to the standard extract through the manipulation of extraction conditions, and also through pre-treatment of boronia flowers. Rolling the flowers almost doubled recovery of floral extract, albeit with inferior organoleptic qualities.

1. Introduction

Boronia megastigma Nees. (Brown boronia, family *Rutaceae*) is an endemic shrub grown commercially in Tasmania for production of a highly valued floral extract. Boronia flowers are harvested using a combing apparatus, frozen for storage and extracted with petroleum ether to yield between 0.3 and 0.7% (fresh flower weight) of product, or 'concrete', from which an alcohol soluble fraction (absolute) may be prepared. Many components of boronia extract have been identified, β–ionone is the major component of the volatile fraction, present as 1.2 - 4.5% of product (Guenther, 1974; Davies & Menary, 1983; Weyerstahl *et al*., 1994). Several clones have been produced by the University of Tasmania and released to growers to comprise the main crop that is harvested annually. Work by MacTavish (1995) showed that the major areas where significant improvements in yield and quality of concrete were possible include the correct timing of harvest, the post-harvest treatment of flowers, and the extraction protocol used.

Brown boronia flowers normally mature over a period of two months; uniform maturation is a rare occurrence. The yield of floral extract per hectare is proportional to the number of plants per hectare, flower size, number of flowers per node, number of flowers per plant and the concentration of extract (Roberts and Menary, 1994a,b). In steam distilled crops, essential oil yield generally peaks at anthesis, and subsequently declines (Cernaj *et al*., 1983; Holm *et al*., 1988). In crops grown for production of floral oils, for example chamomile, a shorter flowering period produces a reduced oil yield as there is less time for essential oil accumulation. In rose flowers, monoterpenes increase rapidly after calyx splitting until the flowers are fully opened, after which the concentration plateaus and subsequently declines (Francis and Allcock, 1969). The effects of flower maturity and harvest timing on extract from brown boronia flowers of one clone have been described (MacTavish and Menary, 1997a). Significant quantities of product with the typical floral bouquet may be extracted from medium and large buds. The presence of such buds at harvest does not cause depletion of the potential yield of extractable product ('extract') per plant.

Genetically different boronia plants have variable chemical composition (Davies and Menary, 1983); flowers on such plants may reach anthesis at different times within the flowering season. Environmental differences between North and South Tasmania manifest in disparate times for commencement of flowering, North tending to flower earlier than the South. Variations in this pattern are brought about through variations in climate and management. The harvest date for each clone which optimises the yield of flower and extract per plant may also differ (MacTavish, 1995). In boronia, shade increases the length of the flowering period and may delay flowering (Bussell, unpublished), however flower initiation and development is not photoperiodically sensitive (Roberts and Menary, 1994a,b). Extract concentration is less variable than extract composition in non-clonal plants, suggesting that concentration is more heritable than composition (MacTavish and Menary, 1997b). Despite this, environmental conditions may have a major effect on both extract concentration and composition, suggesting that both have relatively low heritability.

A distinction between genotypic and environmental control of flowering is important commercially, as selection of clones with disparate flowering times may extend the flowering 'window'. The identification of the optimum harvest date for each clone, and the constancy of this value between seasons and sites is also important commercially.

Currently flowers are cooled as soon as possible after harvest and frozen rapidly for storage until extraction. Work by Bugorskii and Zaprometov (1983, 1988) showed increases in volatiles in rose flowers during post-harvest incubation. MacTavish (1995) exposed boronia flowers to various conditions post-harvest and found increases of up to 50% in β–ionone and other volatiles. This illustrates the large potential that exists for increasing the quality of boronia concrete produced from existing plantations, simply by adding an additional stage to harvesting/storing procedures. Large-scale experiments were required to assess the most suitable post-harvest incubation conditions, and the feasibility of adding such a process to variable on-site situations. Preliminary work showed that increases in the concentration of volatiles including β–ionone were greater when fresh flowers were

incubated after harvest at 20°C compared with 4°C, in whole flowers compared with simulated harvester-damaged (hand-squeezed) flowers, and in open flowers compared with less mature developmental stages.

Many floral species emit ionones and related compounds *in vivo*, including Osmanthus *fragrans* (Mookherjee *et al*., 1986, 1989) and *Boronia megastigma* (MacTavish, 1995). Documented changes in the concentration of ionones in flowers after harvest include an increase in the % of dihydro-β–ionol in yellow tea rose flowers, and depletion of β–damascenone, dihydro-β–ionol, β–ionone and 4-keto-β–ionone and an increase in dihydro-β–ionone in *Osmanthus* flowers (Mookherjee *et al*., 1989). Purging harvested flowers with nitrogen gas during headspace analysis altered the composition of emitted volatiles compared with purging with air (Mookherjee *et al*. 1986, 1989). Essential oil production in rose flowers has been shown to be proportional to respiratory activity, with non-respiratory oxidative reactions depleting oil yield (cited in Tyutyunnik and Ponomaryova, 1977). Roses produced 6-8% more essential oil after being fermented for 10 hours at 45°C in solutions of plumbagene, which limits oxidative reactions. In the same study, fermentation for 4-6 hours at 25-30°C in air produced increased yields of rose essential oil, however fermentation in atmospheres of helium or hydrogen increased the essential oil yield to a greater extent. After the initial increase during aerobic fermentation, essential oil yield in roses declined, and it is suggested that depletion of carbohydrate and amino acid reserves during fermentation caused this (Tyutyunnik and Ponomaryova, 1977). High proportions of alcohols including phenyl ethyl alcohol, an aromatic compound with high floral impact, were produced in fermented rose petals (Bayrak and Akgül, 1994).

There is extensive literature on the existence, structure, analysis, occurrence and identification of volatile-glucoside moieties, particularly in grapes and grape products (Gunata et al, 1985; Sefton et al, 1993; Williams et al, 1992; Winterhalter et al, 1990a,b). Glucosides of monoterpene alcohols were initially discovered in rose flowers (Francis and Allcock, 1969) and since then much work has focussed on identifying precursor-product relationships (Ackermann et al, 1989). Large changes in levels of free and bound volatiles have been noted for several floral species during flower development, however no diurnal changes have been discovered as yet (Francis and Allcock, 1969; Loughrin et al, 1992). Enzymes from tea (Yano et al, 1990) and several flower species (including *Osmanthus* which accumulates β–ionone) have been prepared and examined for their activity in hydrolysing precursors made from the same flowers at various maturity stages (Watanabe et al, 1993). It is postulated that fragrance precursors are enzymatically transformed into volatile compounds during flower opening by the synthesis and/or activation of hydrolytic enzymes. Several ionones and derivatives have been found as glycosides (Pabst et al, 1992), however Watanabe (Watanabe et al, 1993) was unable to release such compounds from aqueous extracts made from *Osmanthus* flowers after incubation with enzyme from the same flowers. Such bound forms also play an important role in the biogenesis and accumulation of essential oils (Stahl-Biskup 1987).

It is possible that increases in boronia volatiles after harvest may result from the release of bound volatiles from glycosidic bonds. This valuable pool of bound volatiles, present in flowers at harvest, could be converted into free volatiles with very little input of extra labour and money. Similarly, the potential of different clones to produce additional volatiles after harvest may depend on the concentration of bound volatiles or of hydrolytic enzymes at harvest. MacTavish (1995) studied the levels of free and bound volatiles during post-harvest incubations however, and found an increase in the concentration of glycosidically bound volatiles as a result of prolonged incubations. No other work has been published on the occurrence of glucosides in native Australian flowers to our knowledge. The significance of glucosides in harvested flowers, representing a source of potential volatiles that could be extracted after hydrolysis is apparent.

Boronia flowers are extracted by solvent using a protocol of several solvent replenishments, bulking of solvent and concentration of concrete by solvent removal. MacTavish and Menary (1998) studied the effect of various regimens on the yield and quality of boronia concrete released. Further refinements can still be made to reduce solvent usage and make the extraction process more efficient. There are published studies on the extraction of boronia flowers, mostly pertaining to the type of solvent used (Leggett, 1979; Naves and Parry, 1947). High purity petroleum ether (pet. ether) with a boiling range

of 40-70°C is most commonly used for extraction of flowers (Guenther, 1972). The products of solvent extraction are 'concretes': semi-solid products comprising volatiles, waxes and pigments. Solvents penetrate the floral organs, dissolving the flower perfume, waxes, albuminous material and pigments which are released by diffusion (Guenther, 1972). The amount of time required for complete extraction may depend on the volume of solvent used, especially if particular compounds saturate the solvent during extraction. Pet. ether does not penetrate well into plant material with a high moisture content (Georgiev and Balinova-Tsvetkova, 1977), therefore extraction of floral oils is usually incomplete. It was not known how the duration of each wash affects the yield of extract obtained, and if the effect is constant throughout the extraction process. An 'absolute' is obtained by alcohol extraction of the concrete to remove waxes and some pigments.

The yield and composition of an extract can be influenced by the extraction conditions (Verzár-Petri *et al*., 1984). Processes such as chopping vegetative material prior to extraction have been shown to increase oil yield (Smallfield *et al*., 1994) and this may be due to better plant/solvent, or plant/steam contact as a result of breaking up tissue and glands in which oils accumulate. A rapid, precise and reasonably complete extraction method was required for routine analysis of boronia flowers. Commercially, the solvent used for the extraction of boronia flowers incurs one of the major costs for production. The effect on extract yield of extraction constraints such as the time, the number of changes of solvent (washes) and the volume of solvent used, required consideration. The potential for producing different products using different extraction strategies was investigated.

2. Objectives

To produce boronia concrete at higher yields and with improved quality by developing pre-harvest, harvesting, and post-harvest management practices and by value-adding the existing product.

Pre-Harvest: To examine patterns in the emission of volatiles from boronia flowers in order to quantify rates of production of volatiles or conversion of precursors into free volatiles under different lighting conditions.

Precursors to Volatiles: To establish methods for the analysis of precursors to floral volatiles and the activity of enzymes that convert them into free volatiles; including glycosidically bound compounds and other bound forms.

Phenology: To produce a standard method for sampling and analysing flower material to identify the flower maturity index. To identify the optimum time to harvest each clone in order to maximise the concentration and composition of floral oil and volatiles including β–ionone at harvest. To assess the change in concentrations of free and bound volatiles and volatile-producing enzymes throughout flowering.

Post-Harvest: To identify the optimum conditions in terms of clonal type, harvest time, temperature and atmospheric conditions of incubation and the duration of incubation under which boronia flowers produce increased floral extract and volatiles after harvest. To assess the viability of commercial-scale incubations. To assess the change in concentrations of free and bound volatiles and volatile-producing enzymes during post-harvest incubation.

Extraction: To optimise the efficiency of extraction by considering the effect of tissue disruption on a commercial scale and by examining different flower weight : solvent volume ratios in the commercial extractor.

3. Methodology & Results

3.1 General

Plant material: Clonal plants of *Boronia megastigma* developed by the University of Tasmania and grown on several sites in Tasmania were used for all studies.
Temperature loggers: Hobo^{R}-Temp temperature data loggers (Onset Computer Corp. Pocasset, MA 02559, 508-563-9000) were used to monitor the temperatures inside and outside boxes, inside bags of flowers during incubation, and in some cases on the outside of pilot scale extractors during extraction.

Solvent: The solvent used for extraction of boronia flowers was a mixture of n-pentane and n-hexane. Industrial grade n-pentane is imported from BP Belgium, and re-distilled prior to use. Industrial grade n-hexane is obtained from BP Australia, acid washed and re-distilled prior to use. GC and organoleptic assessment before use test the solvent mixture for impurities and residues. Hexane used for solubilisation of extract prior to GC analysis was analytical grade, obtained from Mallinckrodt.

Extraction methods:

Small scale (20 -100 g): Fresh or thawed flowers were extracted in triplicate using 2 exchanges of solvent, each of 2 hours, at 20°C. The solvent volume used was enough to cover the flowers. The combined solvent/extract from the two washes was dried down under reduced pressure at 60°C for 5 minutes following removal of the last visible traces of solvent, yielding the extract. The concentration of extract was calculated (% fr. and dr. wt.).

Pilot scale (10 - 15 kg): Fresh or thawed flowers were extracted in 200L stainless steel pilot scale extractors during 6 hours incorporating 3 washes of solvent at ambient temperature. The extractors rotated throughout extraction, temperatures were generally 12-15°C. Solvent from the three washes was combined, the recovered volume calculated, and three sub-samples were taken for analysis of extract yield by drying down as described above. Solvent losses occurred due to vapourisation and solvent remaining in the flowers at the completion of the extraction. The yield of extract was calculated using final (recovered) solvent volumes, as a % of fresh flower weight (% fr. wt.) and dry flower weight (% dr. wt.). Standard errors were calculated using Statview^{TM}.

Gas chromatography (GC): A Hewlett Packard 5890 Series II GC equipped with auto injection, a flame ionisation detector (FID), a split-injection system and a HP1 fused silica capillary column (30 m x 0.2 mm i.d., 0.33 µm film thickness) was used. Carrier gas: N_2 @ 2 ml/min, head pressure 12 psi and split ratio of 1:50. Oven temp program: 50°C for one minute, then 10°C/min to 250°C. Injector temp: 250°C, detector temp: 280°C. Injection volume: 1 ml of a 20-25 mg/ml solution of extract or absolute in hexane. Quantitative peak estimation was achieved by addition of octadecane as an internal standard, a FID response factor of one unit was used. Peaks were initially identified by GC/MS of splitless injections of 1 µL samples on a HP 5890 GC coupled via an open split interface to a HP 5970B mass selective detector (MSD). The GC was equipped with an HP1 fused silica capillary column (25 m x 0.32 mm i.d., 0.17 µm film thickness). Oven temp program: 50°C held for one min., then 30°C/min to 220°C, then 10°C/min to 290°C held for 5 min. Injector temp: 250°C. Electron ionisation was undertaken with a source temperature of 200°C and electron energy of 70 eV. Total volatiles were calculated as the fraction of the GC-analysable material eluting before n-heneicosane (Davies and Menary, 1983).

Conversion of extract into absolute: To prepare absolute, boronia extract was dissolved in ethanol and heated to 40°C. The solution was cooled to -18°C and filtered, the filtrate was dried down at 40°C, reduced pressure; the yield of absolute was calculated as a % of the extract. Absolute and extract (30 mg) were redissolved in analytical grade hexane prior to GC analysis.

Preparation of acetone powders: Fresh flower material was blended in a stainless steel blender with re-distilled acetone (-18°C). The slurry was filtered under vacuum through Whatman #1 filter paper and the particulates washed many times with chilled acetone until the filtrate was clear. The particulates were then spread over filter paper and left to air dry over night at room temperature followed by storage at 4°c in sealed containers.

Organoleptic assessment: Organoleptic tests were performed in a 'double blind' test, in an aqueous medium at a dilution of $2 \times 10^{-3}\%$. Solutions were ranked according to their floral, citrus and green bouquet, immediately and again 15 min later; solutions were tasted at the completion of the organoleptic test and ranked according to taste.

3.2 Pre-Harvest

Emission of Volatiles:

Methodology: Clonal plants of brown boronia (*Boronia megastigma* Nees) developed by the University of Tasmania and grown in pots containing a 1:1 sand : peat bark mixture were used. Plants were previously housed outside with av. day temperatures of 12-14°C, and 12 hour days from 6 am. Plants were placed in experimental conditions (19°C) at 8am; conditions were A) continuous light, B) continuous dark or C) 12:12:12 light : dark : light cycles. Plants under light were not enclosed between samples; for sampling plants were placed in a 27 L glass vessel with lid bearing a small hole into which the fiber assembly was inserted. Lighting in addition to laboratory lighting comprised 2, 60 cm, 18W Crompton white fluorescent lights held 45 cm away from plants. Plants in the dark were held in 27.5 L stainless steel vessels with several layers of shadecloth over the open top, the plant and vessel were kept in a darkened room during and between sampling. For sampling, a glass lid was placed on top, bearing a small hole into which the fiber assembly was inserted. At the completion of the experiment, samples of the headspace above plants from which all flowers had been removed and from a pot with potting mixture but no plant taken to quantify volatiles emanating from non-floral tissues and apparatus.

A solid phase microextraction fiber (SPME: Supelco Co., Supelco Park Bellefonte, PA) coated with polydimethylsiloxane (PDMS, 1 cm long, 100 mm thickness) was used to collect and concentrate volatiles by virtue of its sorption characteristics. The SPME device consisted of a retractable fiber enclosed in a sheath. The exposed fiber was preconditioned for 1 hour at 260°C in the GC injector. During sampling, after entering the sample container, the fiber was extended, exposing the sorption surface for the appropriate time. The fiber was then retracted prior to removal from the sample container. Volatiles were desorbed from the fiber for 30 s at 200°C into the glass-lined, splitless injector port of the GC (HP-5890, Hewlett Packard Co.). All SPME samples were taken at 19-20°C, plants were sampled once every 90 minutes throughout 36 hours. Adsorption times of 30 minutes followed by 30 s desorption were selected as optimum after examining the peak area for β–ionone resulting from adsorption times of 5, 10, 20, 30, 60 and 120 minutes and desorption times of 30 and 60 s. Adsorption times longer than 30 minutes increased the adsorption of β–ionone, however 30 min was selected to maximise the number of samples within the 36 hour period; 30 minutes was the GC run time. A desorption time of 30 s was sufficient to desorb all volatiles onto the GC column for analysis as described previously: the temperature program was isothermal for 5 minutes at 30°C, then raised at the rate of 10°C/min to 280°C. The peak areas for all peaks were used for calculations, after subtraction of peak areas resulting from artefacts of adsorption from plants without flowers, pots without plants and blank injections. At the completion of the experiment all flowers were removed from each plant, were weighed, frozen and subsequently extracted and the extract analysed by GC.

Results: SPME and GC were used to analyse changes in the emission of 16 volatiles from brown boronia flowers on clonal plants held in either continuous light (A), continuous dark (B) or 12 light : 12 dark : 12 light cycles (C). Three of the volatiles had insufficient signal to noise ratio to be quantified with confidence. Only a few of the volatiles have been identified at this stage, most of them being present in the headspace above 'absolute' prepared from boronia flowers by solvent extraction and captured by SPME, but not previously observed in GC/MS analysis of solubilised absolute (Davies and Menary, 1983). One compound of quantitative significance has almost identically the same mass spectra as β–ionone, however it elutes 2.33 minutes before β–ionone, this is referred to as 'β–ionone-like'. The compounds tentatively identified by GC/MS include an acyclic monoterpene (peak '26' in Davies and Menary, 1983) with mass spectra with m/z 71 (100%), 43 (86), 82 (77) and 67 (59), very similar to hotrienol, the 'β–ionone-like' compound, dihydro-β–ionone, β–ionone, dodecyl acetate and (Z)-heptadec-8-ene.

There appear to be two distinct groups of volatiles with different emission patterns over 36 hours. The first group includes: the acyclic monoterpene peak '26' (Davies and Menary, 1983), the 'β–ionone-like'

compound (Figure 1), compounds eluting at 17.916 min, 18.684 min, dodecyl acetate (Figure 2), 20.99 min, (Z)-heptadec-8-ene, and 21.403 min.

Figure 1. Emission patterns of the 'β–ionone-like' compound from plants under continuous light ( ), continuous dark ( ), or 12 light: 12 dark : 12 light cycles ( ).

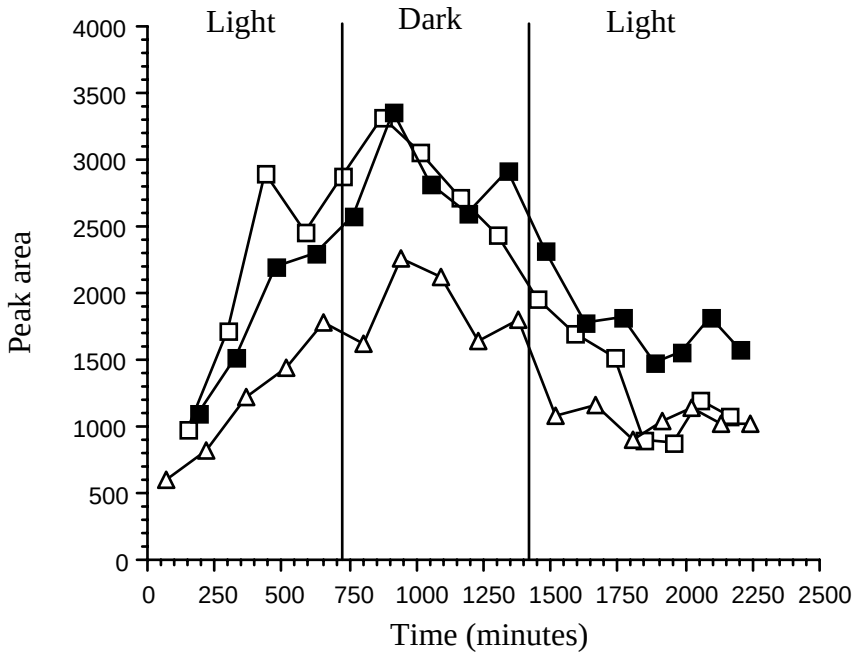
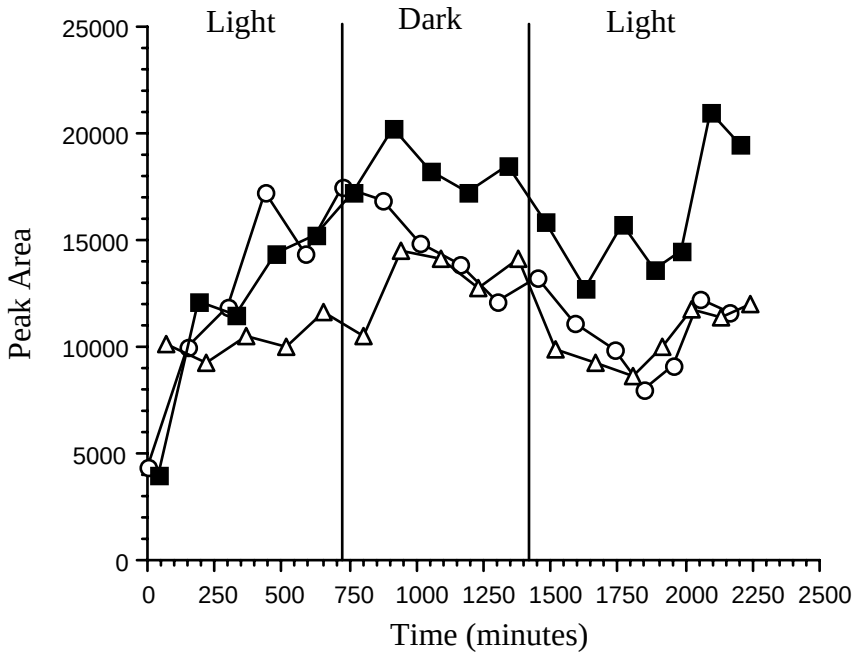


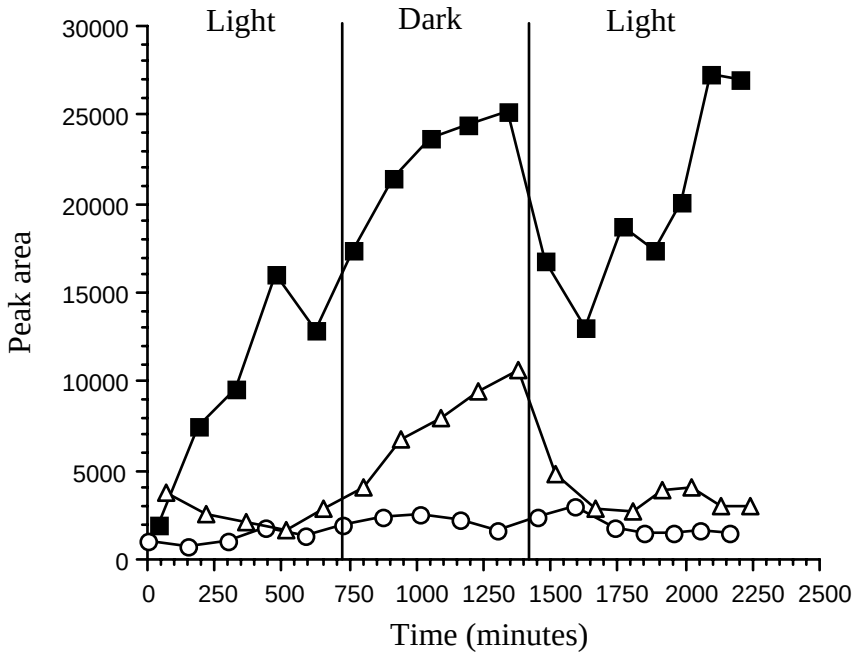
Figure 2. Emission patterns of dodecyl acetate from plants under continuous light ( ), continuous dark ( ), or 12 light: 12 dark : 12 light cycles ( ).



The general trend for these 8 compounds is that emission increases throughout the first 12-14 hours of the experiment and declines during the subsequent 12-14 hours. In most cases, emission begins to increase again in the third 12 hour period, however this does not occur with peak '26' or the 'β–ionone-like' compound. Similar patterns occurred whether plants were held in continuous light or dark, but the maxima were generally reduced in plants held in 12:12:12 light : dark : light cycles.

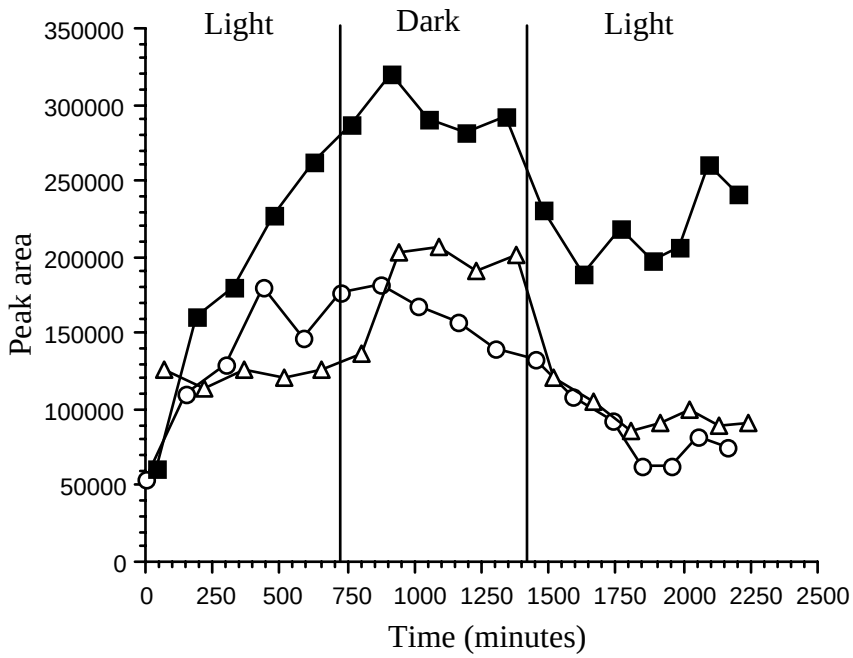
The second group of compounds include compounds eluting at 16.828 min, 17.718 min, 17.916 min, dihydro-β–ionone (Figure 3), β–ionone (Figure 4) and 18.306 min. Emission of these compounds was significantly greater in the dark compared with in the light. There was virtually no emission of dihydro-β–ionone in continuous light or in the plants under 12 light : 12 dark : 12 light cycles when under the light phases (Figure 3). In the second 12 hour cycle (dark), emission rapidly increased in the plant under light/dark cycles and declined again once the plant was placed in the light. Under continuous dark, emission increased dramatically for the first 24 hours of the experiment, declined over the subsequent 5 hours and then began to increase again. Several compounds were emitted only when plants were in the dark.

Figure 3. Emission patterns of dihydro-β-ionone from plants under continuous light ( ), continuous dark ( ), or 12 light: 12 dark : 12 light cycles ( ).



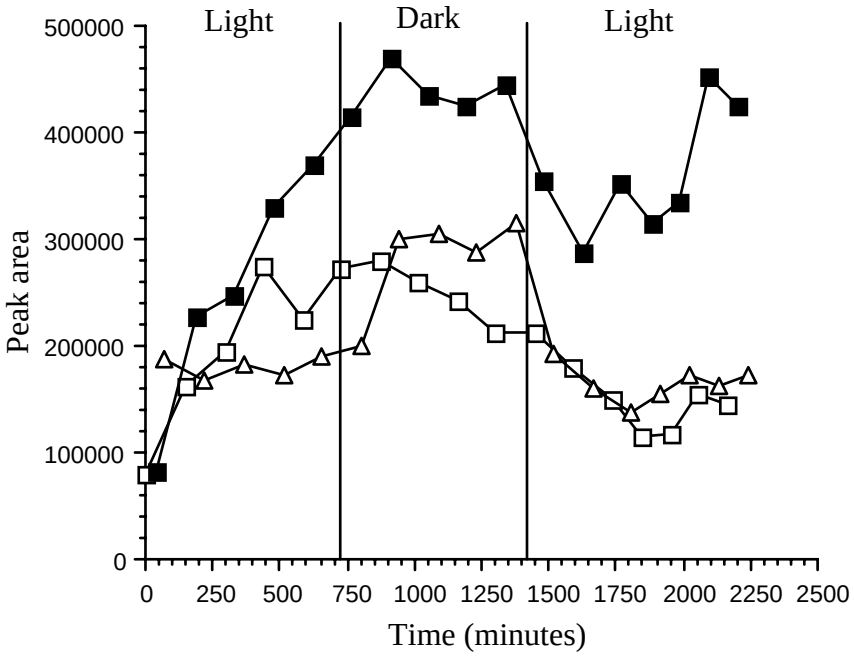
Emission of β–ionone increased during the first 12-14 hours and declined over the subsequent 12-14 hours in both continuous light and dark, however maxima were almost two-fold greater in the dark (Figure 4). Compared with the plant under continuous light, emission from plant C did not increase to the same extent within the first 12 hours (light), was enhanced in the second 12 hours (dark) and in the final 12 hours (light), emission from plants A and C were similar (Figure 4).

Figure 4. Emission patterns of β–ionone from plants under continuous light ( ), continuous dark ( ), or 12 light: 12 dark : 12 light cycles ( ).



Similar patterns were observed in the emission of 'total' volatiles, ie. all volatiles pooled together (Figure 5).

Figure 5. Emission patterns of total volatiles from plants under continuous light ( ), continuous dark ( ), or 12 light: 12 dark : 12 light cycles ( ).



At the completion of the experiment, flowers were removed from the plants, extracted with solvent and analysed by GC (Table 1).

Table 1. Concentration of extract and volatiles in flowers from plants held under different light regimens for 36 hours.

Values	Continuous light (A)	Continuous dark (B)	12 light : 12 dark : 12 light (C)
Extract conc. (% fr. wt.)	0.467	0.449	0.422
Volatiles in flowers (% fr. wt.)	0.061	0.061	0.045
β–ionone in flowers (% fr. wt.)	0.018	0.018	0.011

The concentration of total volatiles and β–ionone were similar in plants held under continuous light or dark, however they were reduced in plants held under 12:12:12 cycles by 25 and 40% respectively.

A different clone was used to examine the volatiles emanating into the headspace above separated flowers and leaf/stem material, collected by SPME and analysed by GC/MS. Compounds emanating from the different plant parts are identified where possible (Table 2).

Table 2. Compounds identified in the headspace emanating from different parts of the boronia plant: flowers, and leaf/stem material.

Compound	Open flowers	Leaf/stem
α–pinene	*	*
Camphene	*	*
Myrcene	*	*
β–pinene	*	*
limonene/β–phellandrene	*	*
δ-3-carene	*	*
monoterpene alcohol (peak '26')	*	
Caryophyllene	*	*
β–ionone	*	
Bicyclogermacrene	*	*
Sesquieucalyptol	*	*
Sabinene	* probable	*
m/z 79/94		*
bicyclo elemene		*
α–copaene/α– cubebene		*
β–elemene		*
cyperene (tentative)		*
β–sesquiphellandrene		*
Humulene		*
Sesquicineole		*
δ-cadinene		*

In this particular clone, the 'β–ionone-like' compound, dodecyl acetate, (Z)-heptadec-8-ene and methyl jasmonates were not observed, although most of these are believed to be exclusively floral. Compounds exclusive to flowers include β–ionone and the monoterpene alcohol (peak '26' from Davies and Menary, 1983).

There are several volatiles, emission of which is clearly enhanced in dark conditions; some are not emitted at all in the light. For most of these volatiles, particularly dihydro β–ionone and the compound eluting at 18.306 minutes, it is the absence of light that stimulates emission, there appears to be little regulation by the plant. The emission of two volatiles, eluting at 17.718 and 17.916 minutes, appear to have a cycle of 12 hours with maxima at midday and between 11 pm and 4 am. However, emission in the third 12 hour period is completely inhibited in plants under continuous light or in the last light phase of the plant under the 12:12:12 regimen. The loss of emission variation after 24 hours may be due to reduced stimulation of diurnal patterns as a result of the constant temperature. Some typically floral volatiles such as dodecyl acetate have approximately 24 hour cycles in emission

irrespective of light changes, yet other floral volatiles such as b-ionone have similar patterns but the maxima are enhanced in the absence of light to a greater or lesser extent.

Despite the changes in emission, there was little difference in the tissue levels of total volatiles and b-ionone except in the plant under 12:12:12 cycles in which tissue concentrations were reduced by 25-30%. Lack of an appropriate standard means one cannot calculate the proportion of the compound present in the tissue that is emitted. However, it is likely that only a fraction of the tissue concentration is emitted due to, perhaps, saturation of the headspace near the flowers with compounds such as b-ionone. Oxidative destruction of compounds such as b-ionone by light may explain the enhancement of detection during the dark periods also. However, the plant may also diurnally regulate emission of b-ionone in that the absence of light triggers synthesis or emission.

It appears that regulation of emission of dodecyl acetate amongst other volatiles may be circadian, ie. emission is to coincide with pollinator activity since emission patterns continue irrespective of light/dark. Dodecyl acetate is a sex pheromone for moths, and moths are perceived to be the main pollinator of *B. megastigma* in its native Western Australia.

3.3 Precursors to Volatiles

Methodology:
Commercial Enzymes and Chemicals: β–Glucosidase from almonds (4-5.6 units/mg) and all other reagents were obtained from Sigma Chemicals except where specified.

Acetone Powders From Boronia Flowers were prepared and stored at 4°C until required (approx. 11% protein g/g). To resolubilise, approximately 1:1.5 (g/g) acetone powder : PVPP was resuspended in 0.05M citrate buffer with 0.1% Tween 80 overnight at 4°C. This mixture was either used directly, or filtered and used (protein concentration approximately 0.2 mg/ml).

Ammonium Sulphate Fractionation of Boronia Enzymes was achieved by solubilising acetone powder in 0.1M citrate buffer as above. The filtrate was fractionated with increasing concentrations of ammonium sulphate from 25% to 80%. After centrifugation at 18,000 rpm for 30 minutes at 4°C, the pellet from each fraction was resuspended in buffer and dialysed against four changes of buffer with 1% $MgCl_2$. The protein content, hydrolytic activity against para nitrophenyl-β–D-galactopyranoside and volatile-producing activity from standard precursor solutions of each fraction were assayed.

Protein estimation was achieved using the Bio-Rad Protein Assay (BioRad Laboratories, 32 nd & Griffin Ave, Richmond, CA, 94804), which utilises the method of Bradford (1976); involving the shift in A_{595nm} caused by the binding of Coomassie Brilliant Blue G-250 to protein. The kit was used as per the manufacturer's instructions. A standard curve was prepared with BSA.

Crude Aqueous Extracts From Flowers were made by blending frozen boronia flowers with 0.05 - 0.2 M citrate buffer, pH 4-5 with PVPP and Tween 80. Homogenates were stored for 1 hour at 4°C and subsequently filtered through 8 layers of cheesecloth. For some experiments, crude extracts were evaporated to dryness under vacuum at 70°C, made up to constant volume in buffer and re-filtered under vacuum to remove particulates.

Enzymic Hydrolysis of Glycosides in Aqueous Extracts was achieved by incubation of 10 ml aliquots of the aqueous extracts with either β–glucosidase, 100-300 mg acetone powder or 1-2 ml of solubilised protein from same, with Tween 80 and PVPP for up to 24 hours at 37 °C. The reactions were stopped by addition of DCM with an octadecane internal standard. After a minimum of 4 hours at 4°C, the DCM layer was removed and analysed by GC or GC/MS. In all assays, production of free volatiles, if any, after incubation of either aqueous extracts or the enzyme fraction was accounted for and subtracted from combined assays. In addition, the yield of free volatiles present in combined assays

prior to incubation were subtracted from the yield as a result of concentration of volatiles after incubation.

Hydrolysis of Synthetic pnp-Glycosides including para nitrophenyl-α– and β–D-galactopyranosides, para nitrophenyl acetyl-β–D-N-galactosaminide and para nitrophenyl acetyl-β–D-N-glucosaminide, para nitrophenyl-β–D-glucopyranoside and para nitrophenyl-α–D-mannopyranoside (Sigma Chemicals, all 25 mM unless specified otherwise). The galactosaminide and glucosaminide were not very soluble in 0.1 M citrate buffer, pH 4.6, the final concentration was probably closer to 10 mM; the mannopyranoside took time, heat and stirring to dissolve. Glycosides were incubated with enzymes at 30°C for 30 minutes followed by addition of 0.2M Na_2CO_3; the adsorption at 420 nm was measured spectrophotometrically.

Fractionation of Aqueous Extract Using C18 and Sephadex LH-20. A crude aqueous extract was prepared by blending flowers with 0.05M citrate buffer and Tween 80. The slurry was filtered through cheesecloth, boiled and cooled, excess PVPP was added for 1 hour and then filtered off. The filtrate was concentrated by dialysis against solid sucrose for 12 hours at 4°C and filtered. The filtrate was passed through 11, 900 mg C18 Sep-Pak cartridges in series which were then washed with methanol to remove volatiles and water to remove sugars; glycosides were eluted with 100% ethanol, filtered and stored at 4°C until use*. A second flower sample was blended whilst frozen, extracted with pet. ether to remove some volatiles and pigments, then extracted in methanol with pH adjustment to 7. The methanol was concentrated under reduced pressure at 30°C, treated with molecular sieves and PVPP for 12 hours at 4°C to adsorb water and phenolics, then filtered and dried down under reduced pressure at 60°C. The solid was extracted three times with hexane and subsequently diethyl ether; re-dissolved in methanol, and filtered prior to loading (5 mL) onto the LH-20 column (30 mm x 900 mm), the mobile phase was 100% methanol. The first 226 ml of the eluate after the void volume were collected in 16 x 9 ml fractions and 2 fractions of 40 ml. The fractions were pooled into 9 fractions by similarity of spectrophotometric adsorption. The pooled samples were dried down under vacuum, resuspended in citrate buffer and A) incubated separately with β–glucosidase and boronia enzymes for 24 hours at 37°C after which time DCM was added. After a minimum of 4 hours at 4°C, the DCM layer was removed and analysed by GC or GC/MS. Frozen, aqueous sub-samples of fractions 1, 2 and 3 from the LH-20 column were passed back through C18 Sep-Paks, washed with methanol and water and the glycosides sequentially eluted with ethanol at 30%, 60% and 100%. The eluates were dried down, resuspended in 0.2 M acetate buffer pH 5 (to aid GCMS analysis), and hydrolysed as from A) above.

Thin Layer Chromatography was done on the sample from A) above using Silica 60 F_{254} plates and 95:5 (v:v) ethyl acetate : methanol as the mobile phase. Plates were sprayed with a 10% phosphomolybdic solution and heated at 110°C for 10 minutes. Six fractions were collected from preparatory scale plates, compounds were dissolved in 0.1M citrate buffer pH 4.6 for 1 hour at 20°C and subsequently filtered. Each sample was divided into 3, one part was extracted with DCM, the second part was incubated with solubilised boronia enzyme and the third part with 1 mg β–glucosidase for 1 hour at 37°C and subsequently the free volatiles were extracted with DCM; DCM extracts were analysed by GC.

GC Analysis of DCM extracts from hydrolysed samples A Hewlett Packard 5890 Series II GC equipped with auto injection, a flame ionisation detector (FID), a split-injection system and a BPX-70 fused silica capillary column (50 m x 0.32 mm i.d., 0.25 μm film thickness) was used. Carrier gas: N_2 @ 50 ml/min, head pressure 25 psi and split ratio of 50:1. Oven temp program: 50°C for one minute, then 10°C/min to 280°C. Injector temp: 250°C, detector temp: 280°C. Injection volume: 1-5 μL of the DCM sample.

Results:

Ammonium Sulphate Fractionation Hydrolysis of pnp-β–D-galactopyranoside/mg protein was greatest in solubilised boronia enzyme saturated to between 45 and 65% with ammonium sulphate (Table 3). The fractions that produced the greatest amount of total volatiles from aqueous extracts/mg of fractionated protein included the crude, unfractionated enzyme followed by the 0-25 and 75-80

fractions (Table 3). Production of β–ionone/mg protein from aqueous extracts was greatest in the 0-25% and 75-80% fractions, however there was some activity in the crude enzyme, and the 25-45% fractions. In this experiment, commercial β–glucosidase from almonds produced more volatiles than the boronia enzymes from the same aqueous extract, hydrolysing twice the amount of total volatiles/mg protein compared with the crude enzyme, and almost three times as much β–ionone/mg as the 0-25% and 75-80% fractions (data not shown). In summary, the fractions most active in hydrolysis of pnp-glycosides were least active in production of typical floral volatiles. It appears that some volatiles are bound as glycosides, including β–ionone or a compound that is converted into β–ionone upon hydrolysis, but that other forms of bound volatiles exist, the hydrolytic enzymes for which are easily separated from glycosidases.

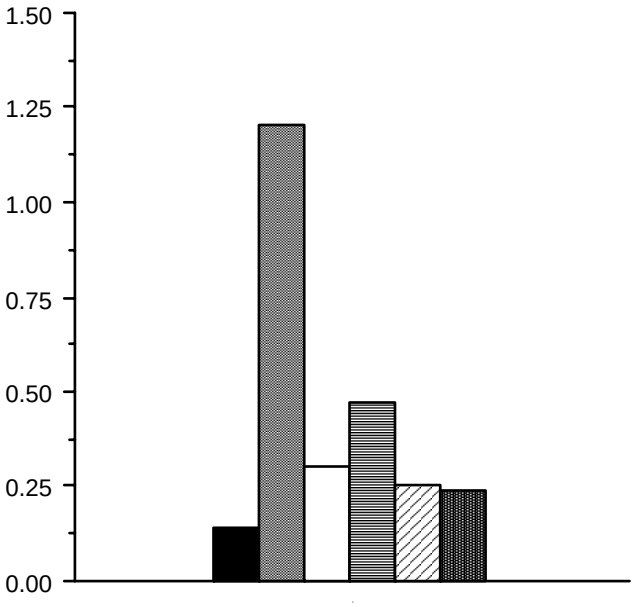
Table 3. Fractions from ammonium sulphate fractionation of solubilised boronia enzymes: concentration of protein, β–galactosidase activity and release of β–ionone and total volatiles from incubation with a standard aqueous extract.

% $(NH_4)_2SO_4$	Protein (mg/ml)	β–Galactosidase @(AU)/mg protei	β–Ionone hydrolysed/mg protein	Total volatiles hydrolysed/mg protein
unfractionated	3.03	0.59	15.00	5150
0-25	2.77	0.47	36.17	885.1
25-35	2.15	0.19	11.20	(81.3)
35-45	3.11	0.43	12.50	(86.5)
45-55	3.34	1.62	(6.86)	(386.6)
55-65	3.20	1.09	(33.25)	(26.5)
65-75	2.24	0.49	(0.78)	(326.8)
75-80	2.10	0.26	37.87	1269.5

NB figures in brackets denote a decrease in volatiles as a result of incubation.

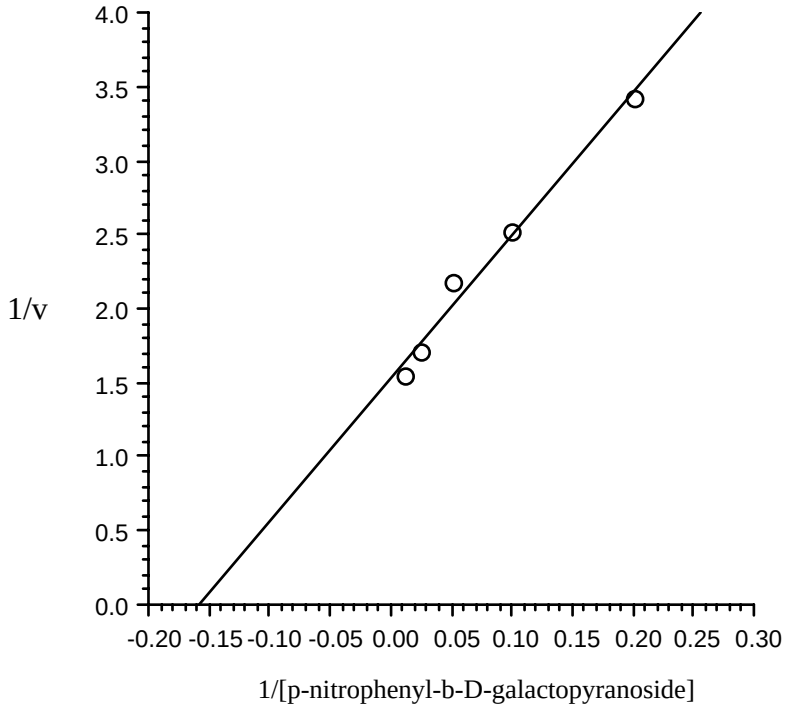
Substrate Specificity and Concentration The hydrolytic activity of manno-, gluco- and galactosidase enzymes from boronia flowers was studied using commercial glycosides including para nitrophenyl-α– and β–D-galactopyranosides, para nitrophenyl acetyl-β–D-N-galactosaminide and para nitrophenyl acetyl-β–D-N-glucosaminide, para nitrophenyl-β–D-glucopyranoside and para nitrophenyl-α–D-mannopyranoside. The greatest activity was observed upon incubation with p-nitrophenyl-β–D-galactopyranoside (Figure 6), which may indicate that glycosides of this type, ie. β–D-galactosides, are most prevalent in boronia flowers.

Figure 6. Comparison of hydrolysis of synthetic glycosides with solubilised boronia enzyme after incubation at 30°C for 30 min., measured as change in A_{420nm}: p-nitrophenyl-α–D-galactopyranoside ( ), pnp-β–D-galactopyranoside ( ), pnp-β–D-N-acetylgalactosaminide ( ), pnp-β–D-N-acetylglucosaminide ( ), pnp-β–D-glucopyranoside ( ) and pnp-α–D-mannopyranoside ( ).



In a study of glycosides in many different plant species, 80% of the aglycones were bound to glucose as β–D-glucopyranosides with galactose being the second most common sugar moiety (Stahl-Biskup, 1987). The effect of substrate concentration on the velocity of the hydrolysis reaction was determined at 30°C. Figure 7 shows the data plotted by the method of Lineweaver and Burk (1934).

Figure 7. Effect of concentration of pnp-β–D-galactopyranoside on the velocity of hydrolysis by solubilised boronia enzymes, incubation at 30°C for 30 min. The data are plotted by the method of Lineweaver and Burk (1934).



The K_m value was 6.25 x 10^{-3} M for hydrolysis of pnp-β–D-galactopyranoside with boronia enzyme. There was relatively little hydrolysis of pnp-α–D-galactopyranoside and pnp-β–D-glucopyranoside, even up to 80 mM. The K_m for β–galactosidase of *Phaseolus vulgaris* was 9.18 x 10^{-4} M, but for β–glucosidase from the same source was 8.3 x 10^{-3} M (Agrawal and Bahl, 1972), closer to the K_m observed here in a crude, unfractionated mixture. Crudely solubilised boronia enzymes may contain inhibitors to glycosidases as described previously, and the plethora of enzymes present may lack substrate specificity. There was a linear rate of hydrolysis of 25 mM pnp-β–D-glucopyranoside with increasing amounts of boronia enzyme (data not shown). The slope of 0.45 and the fact that saturating amounts of enzyme had not been reached indicates that boronia enzymes contain at least 0.4 units/ml of β–glucosidase activity (1 unit = amount that will hydrolyse 1 mmole pnp-β–D-glucopyranoside/min at 30°C). This indicates that glucosidases are present amongst boronia flowers, but they may be non specific or present at a lower activity level than β–galactosidases.

Fractionation of Bound Volatiles: Bound volatiles were crudely fractionated using C18 cartridges, hydrolysed using heat and low pH, and the free volatiles were examined by GC/MS (methods not discussed in detail). Acid hydrolysis in this way produced a miscellany of volatile compounds including many naphthalene derivatives, and it was concluded that this method was unsuited to a study of glycosidically bound volatiles in boronia flowers as has also been concluded by Ackermann *et al*., (1989) in other species. TLC of the un-hydrolysed product after C18 fractionation identified six different regions which were collected, solubilised and hydrolysed with both β–glucosidase and boronia enzymes. The baseline sample point released β–ionone upon hydrolysis with β–glucosidase, as did the fraction with Rf values between 0.065 and 0.2. The fraction between the baseline sample point and up to Rf value 0.065 released 8-hydroxy linalool upon hydrolysis with β–glucosidase. No other volatiles were identified in hydrolysed fractions, possibly due to insufficient concentration or incubation time.

The product from C18 fractionation was fractionated further by passing it through a Sephadex LH-20 column followed by enzymatic hydrolysis of the aqueously resuspended fractions. Fractions 1 (0-84 ml after V_t), 2 (85-150 ml after V_t) and 3 (151-245 ml after V_t) contained extra volatiles or higher concentrations of volatiles after incubation with β–glucosidase. Some volatiles were observed after incubation with both β–glucosidase and boronia enzymes, 1 compound in fraction 2 was released only after hydrolysis with boronia enzyme. Compounds that increased with β–glucosidase treatment included linalool, 8-hydroxy linalool, β–ionone, 3-hydroxy 5,6-dihydro β–ionone (2 epimers), a hydroxy β–ionone with m/z 43, 175, 109 and 208, methyl jasmonate and several compounds related to β–ionone.

Fraction 2 from the LH20 column was fractionated once again via a C18 column using 30% ethanol to elute. This produced a further 11 compounds after hydrolysis with b-glucosidase, 2 further compounds appeared only after hydrolysis with boronia enzymes. When 60% ethanol was used to elute fraction 2 from the second C18 step, 5 compounds were present after GC analysis, 2 of that were different to those observed in the 30% ethanol eluate. The 30% ethanol eluate of fraction 3 produced 2 compounds after hydrolysis with β–glucosidase, both were different compared with compounds from the other fractions. Most of the additional compounds were ionone related, and identification of them has so far proved inconclusive. The presence of so many ionone related compounds is not unexpected, since β–ionone itself occurs in such high concentrations in boronia flowers (Penfold and Phillips, 1927). Norisoprenoids are commonly found after enzymatic or acid hydrolysis, especially of wine products and fruit extracts (Humpf and Schreier, 1992; Krammer *et al*., 1991; Pabst *et al*., 1992; Williams *et al*., 1989; Winterhalter *et al*., 1990a, b). Rarely, however, is β–ionone identified amongst the products of hydrolysis, probably due to lack of an alcohol group. One notable exception is the identification of β–ionone after enzymatic hydrolysis of extract from stem, leaf and floral tissue of *Hysoppus officinalis* L. (Schulz and Stahl-Biskup, 1991). In Schulz and Stahl-Biskup's study, β–ionone was released only after treatment with Pectinol C, a less specific enzyme than β–glucosidase. The present study is the first to our knowledge to show production of β–ionone after treatment with β–glucosidase.

3.4 Phenology

Floral Extract:

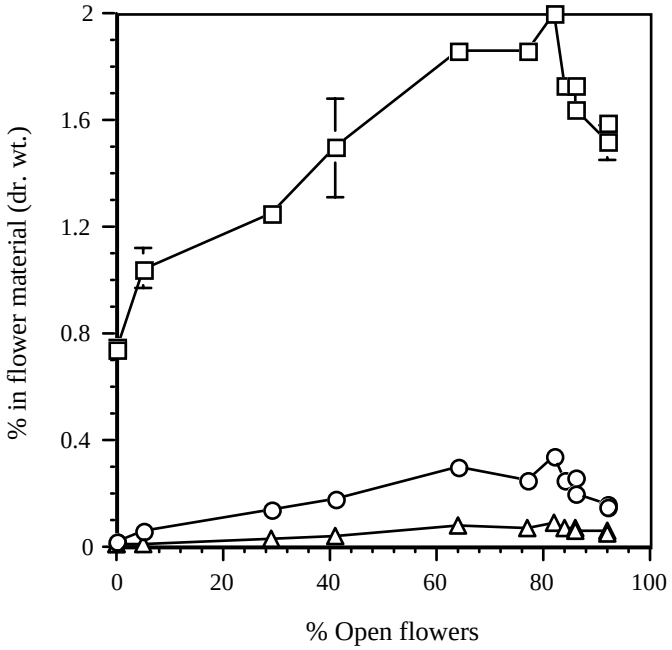
Methodology: Flowers and flower buds (200 g, clone #250) were harvested from randomly selected plants in one site throughout one season, from 0% open flowers to well past 100% open flowers. Flowers were passed through a series of mesh grids:

5.6 mm holes separate open flowers from the rest of the sample;
4.0 mm holes separate large buds from the rest of the sample;
3.36 mm holes separate medium buds from the rest of the sample;
2.83 mm holes separate small buds from the rest of the sample;
1.41 mm holes separate very small buds from the rest of the sample

From each harvest, after separation, half of each sample was made into an acetone powder and the remainder was frozen. Each sample was subsequently extracted on a small scale, in triplicate, and analysed by GC.

Results: The concentration of extract and floral volatiles (% dr. wt.) throughout flowering reached a maximum at 80% open flowers and declined rapidly (Figure 8). This illustrates the potential for clonal, seasonal or environmental differences to alter the optimum time for harvest. Previously clone #5 was shown to reach a maximum at 70% open flowers (MacTavish, 1995).

Figure 8. Concentration of floral extract, total volatiles and β–ionone (% dr. wt.) in the whole sample, throughout flowering. Floral extract ( ), total volatiles ( ), and β–ionone ( ).



Clonal Harvest Timing

Methodology: There are two studies presented in this work: 1) a small scale study that used flower material (100-200 g/sample) from four clones developed by the University of Tasmania grown on commercial plantations in the north-west, north-east, south-east and southern parts of Tasmania; and 2) a pilot scale study that used clonal flower material (15 kg/sample) from a site near Dover in southern Tasmania (average min-max temperatures in September = 2-13°C; October = 6-17°C). The % of open flowers was determined in all samples by shaking the fresh sample over a sieve with holes, 5.6 x 5.6 mm square. The two fractions: open flowers retained by the grid, and small, medium and large flower buds which passed through the grid were weighed and the % of open flowers calculated. Once the % of open flowers reached 80%, visual assessment of the bud sample was made, because stalks (flowers from which the petals had abscised) and abscised petals passed through the grid with the buds, giving an over-estimation of the weight of buds present. Flower and bud fractions were subsequently re-mixed and frozen at -18°C for storage. A sub-sample was dried at 70°C for 24 hours to determine the % dry weight.

Results: Throughout the flowering season, the concentration and composition of extract from all four clones grown in Tasmania was assessed in two ways: by extraction of small samples from the different sites, and on a pilot scale by extraction of one large sample (15 kg) of each clone from one site at each of three harvest dates. For each clone, there appear to be similarities in the concentration of extract resulting from either small or pilot scale sampling and extraction (Figs. 9A-D). The concentration of extract in all clones at most times was between 1.5 and 2% (dr. wt.). Extract concentration (% dr. wt.) in clone #3 increased up to approximately 70-75% open flowers, after which it declined (Figure 9A). This trend was more apparent in the pilot scale study where the differences between each sampling time were significant at the 5% level of significance; in the small-scale study, only one sample at >85% open flowers was identified. A third order polynomial function was used to describe the relationship between the small-scale data points.

There was a plateau in extract concentration in clone #5 in late flowering, at approximately 80-85% open flowers (Figure 9B). The pilot scale data shows a non-significant decline in extract concentration between 82 and 90% open flowers. In clone #17, there was a linear increase in the concentration of extract as flowering progresses, observed in both studies: $r^2 = 0.75$ for the small scale study (Figure 9C). At least within the experimental sampling period there was no plateau or decline observed for clone #17. The plateau in extract concentration observed in clone #250 in the small scale study at approximately 80-90% open flowers was not observed in the pilot scale study (Figure 9D).

Figures 9A-D. The concentration of extract (% dr. wt.) in small- and pilot-scale extracts from four clones throughout flowering. A = clone #3 small-scale ( ), pilot-scale ( ); B = clone #5 small-scale ( ), pilot-scale ( ); C = clone #17 small-scale ( ), pilot-scale ( ); D = clone #250 small-scale ( ), pilot-scale ( ).

Figure. 9A

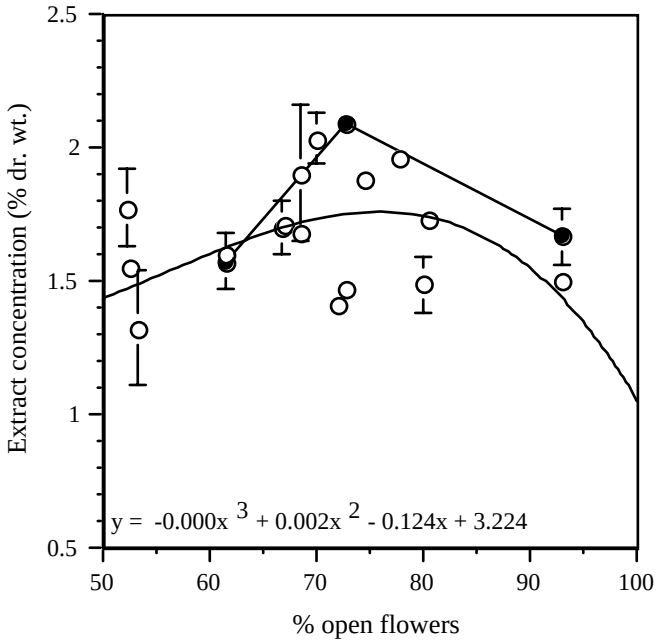


Figure 9B.

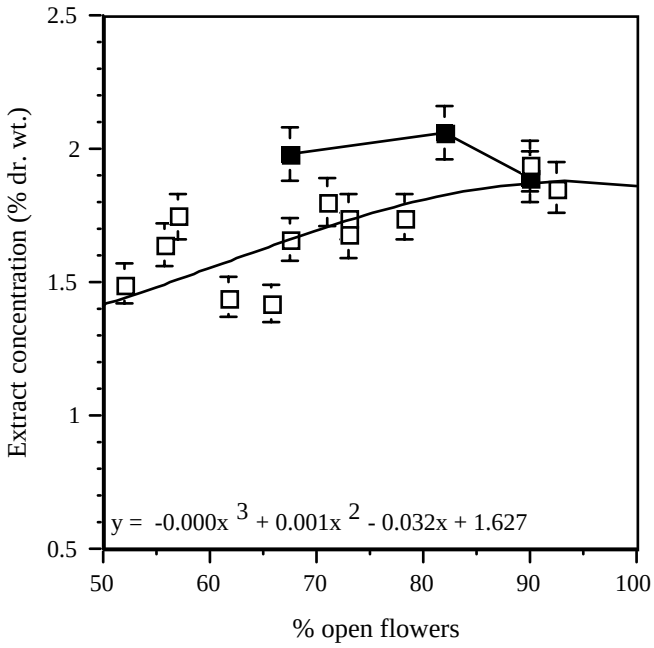


Figure 9C

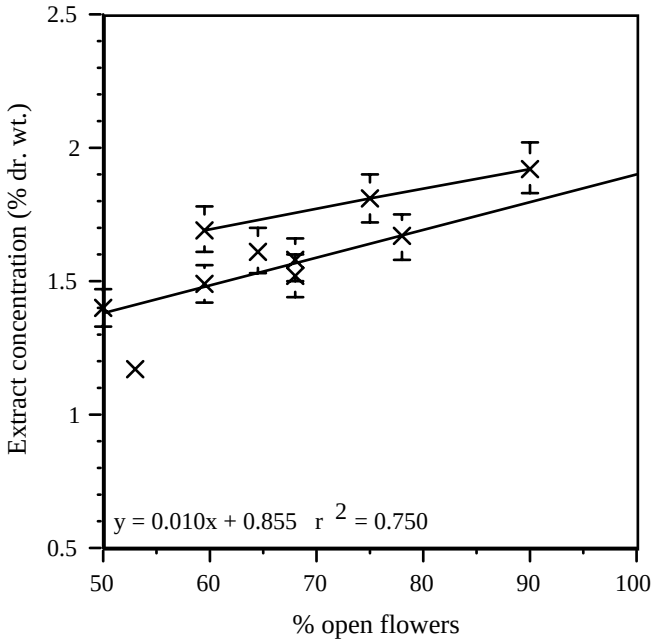
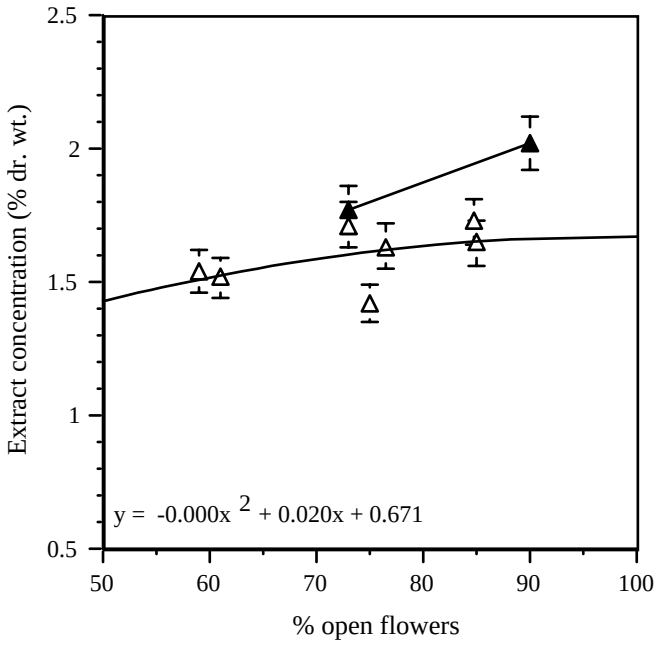


Figure 9D



In general, there were significantly less total volatiles (Figure 10A-D) and β–ionone (Figure 11A-D) in pilot scale extractions compared with the small scale extractions in all clones. This is unlikely to be due to differences between sites because samples from the site harvested on a pilot scale were also included in the small scale study; it is more likely to be caused by incomplete extraction of volatiles, or rather, preferential extraction of non-volatile compounds due to insufficient time and excess solvent used during the pilot scale extraction (MacTavish and Menary, 1998a). Due to inter- and intra-sample variation, significant differences in the concentration of total volatiles throughout flowering occurred only in clone #3 between 60 and 80% open flowers when the concentration of total volatiles increases from 0.3 to approximately 0.5 % (dr. wt.) (Figure 10A-D). All clones produced about 0.1% volatiles (% dr. wt.) when extracted on a pilot scale, differences between harvest dates were not significant. When extracted on a small scale, clone #5 produced approximately 0.5% total volatiles (Figure 10B), clone #17 approximately 0.4% (Figure 10C) and clone #250 approximately 0.2% (Figure 10D). There was no evidence of a significant decrease in total volatiles in late flowering in any clone.

Figures 10A-D. The concentration of total volatiles (% dr. wt.) in small- and pilot-scale extracts from four clones throughout flowering. A = clone #3 small-scale ( ), pilot-scale ( ); B = clone #5 small-scale ( ), pilot-scale ( ); C = clone #17 small-scale ( ), pilot-scale ( ); D = clone #250 small-scale ( ), pilot-scale ( ).

Figure 10A

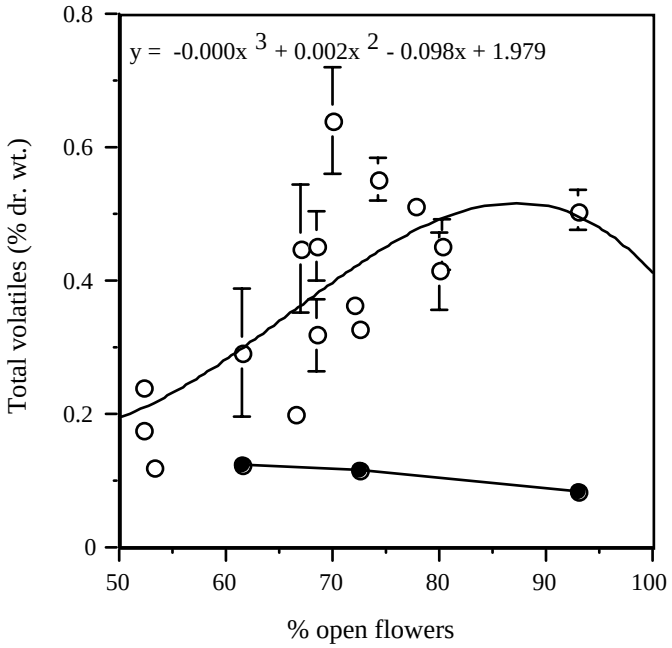


Figure 10B

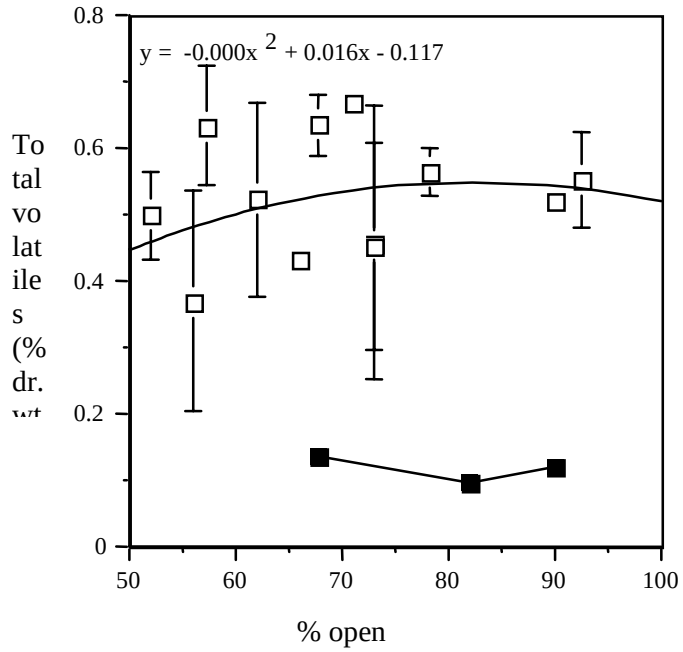


Figure 10C

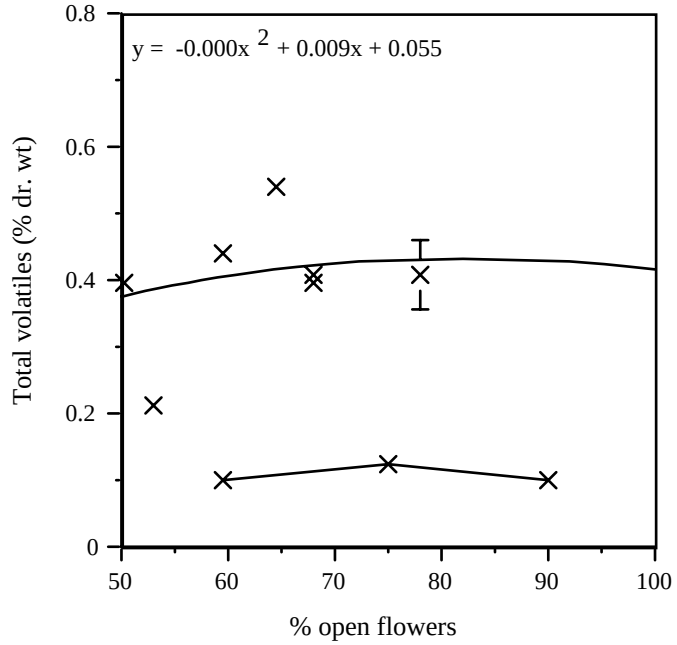
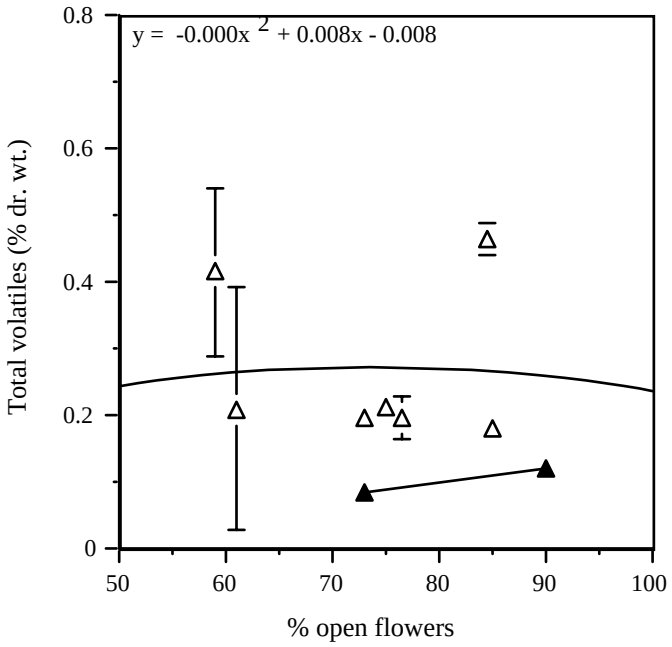


Figure 10D



Figures 11A-D. The concentration of β–ionone (% dr. wt.) in small- and pilot-scale extracts from four clones throughout flowering. A = clone #3 small-scale ( ), pilot-scale ( ); B = clone #5 small-scale ( ), pilot-scale ( ); C = clone #17 small-scale ( ), pilot-scale ( ); D = clone #250 small-scale ( ), pilot-scale ( ).

Figure 11A

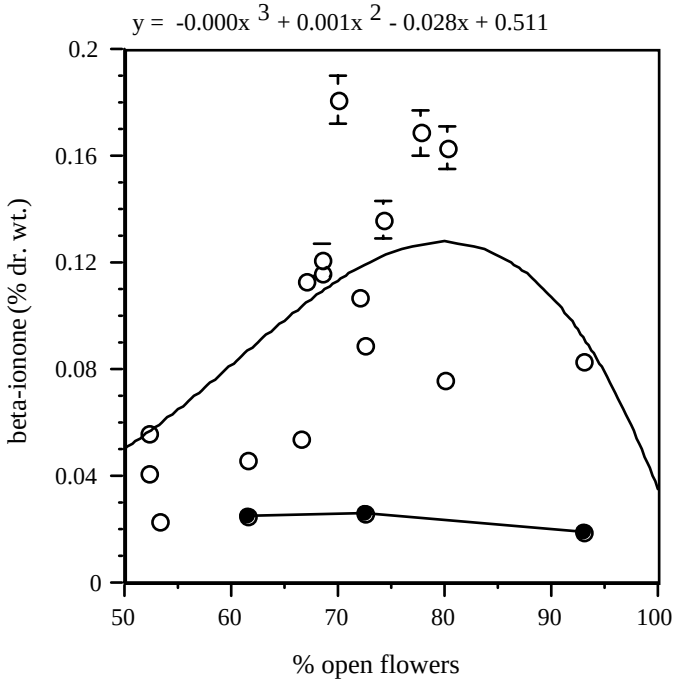


Figure 11B

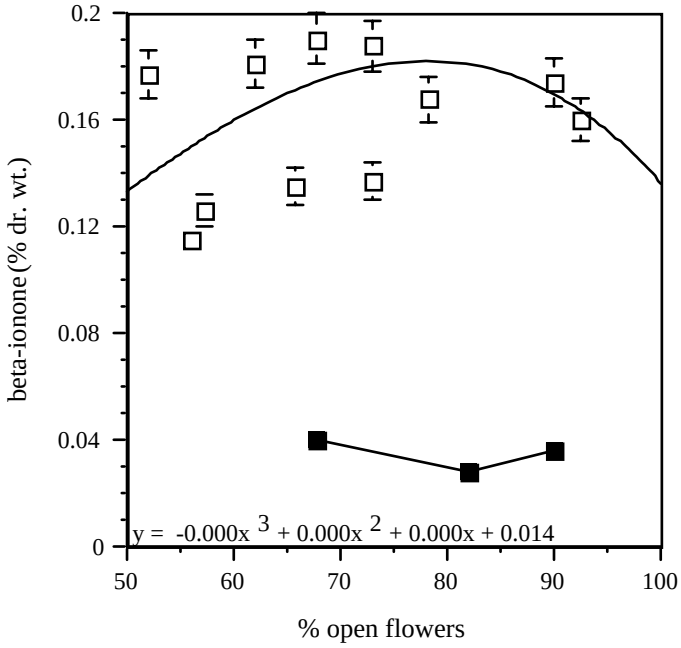


Figure 11C

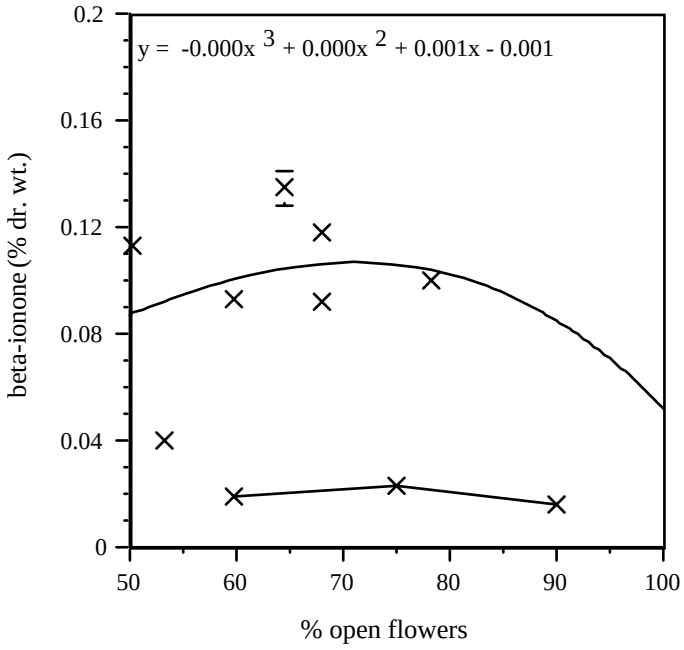
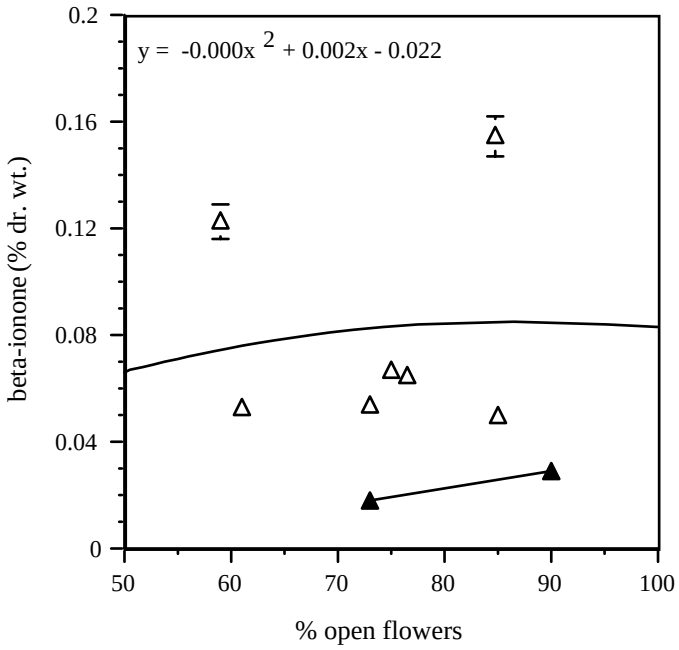


Figure 11D



In clone #3, the concentration of β–ionone throughout flowering increased between 50 and 80% open flowers, with some evidence for depletion later (Figure 11A). There were no significant trends in the content of β–ionone in extracts from any of the clones extracted on a pilot scale. This was due to the low concentration of β–ionone and the small number of samples (Figure 11A-D). When clones #5, #17 and #250 were extracted on a laboratory scale, there were no significant trends in the concentration of β–ionone throughout flowering, due in part to variation between the samples, and insufficient sample number, although the third order polynomial functions show a potential for decline after 75% open flowers in clones #5 and #17 (Figs. 11B, C).

In clones #5 and #17, when extracted on a laboratory scale, there were significant positive relationships between the concentration of extract and the concentration of total volatiles (% dr. wt.), with r^2 values of 0.848 and 0.636 respectively (Figs. not shown). The slopes of the lines of best fit were 0.59 and 0.46 respectively, indicating that floral extract accumulates more rapidly than volatiles.

At the Dover site, where flowers of all four clones were extracted on a pilot scale, the rates at which all clones progressed through flowering were similar; clone #3 began flowering before clone #5 which was closely followed by clone #250, and subsequently clone #17 (Figure 12A). The dry flower yield per plant in clone #3 declined between 62 and 72% open flowers and subsequently stabilised (Figure 12B). Dry flower yield from clone #17 declined between 60 and 90%, and in clone #250 between 73 and 90% respectively (Figure 12B). Dry flower yield increased in clone #5 throughout flowering (Figure 12B). At 75% open flowers, clone #5 yielded the greatest amount of dry flower material per plant (34 g) followed by clone #17 (31 g), clone #3 (20 g) and clone #250 (19 g). On a commercial scale, dry flower yield throughout flowering will have as much effect on the overall yield of extract/ha as the concentration of extract within the flower material, if not more. In the latter case there is an economic advantage through increased yield without an increase in harvesting and extraction costs. There is distinct clonal variation in dry flower weight/plant throughout flowering despite similarities in all clones in the rates at which flowers open (Figure 12A). This variation, namely a decline in clones #17 and #250, and a steady yield, especially between 85-95% open flowers in clones #3 and #5, could be caused by abscission of petals from clones #17 and #250 at earlier stages of senescence than the other 2 clones, however this is not observed in the field. Another factor may be differences in the sequence or rates by which compounds within the flower are catabolised by the plants, or are transported from the flower, and differences in the rates of moisture-loss from different floral organs.

Figure 12A. The progression through flowering of 4 clones from one site. Clone #3 ( ); clone #5 ( ); clone #17 ( ); clone #250 ( ).

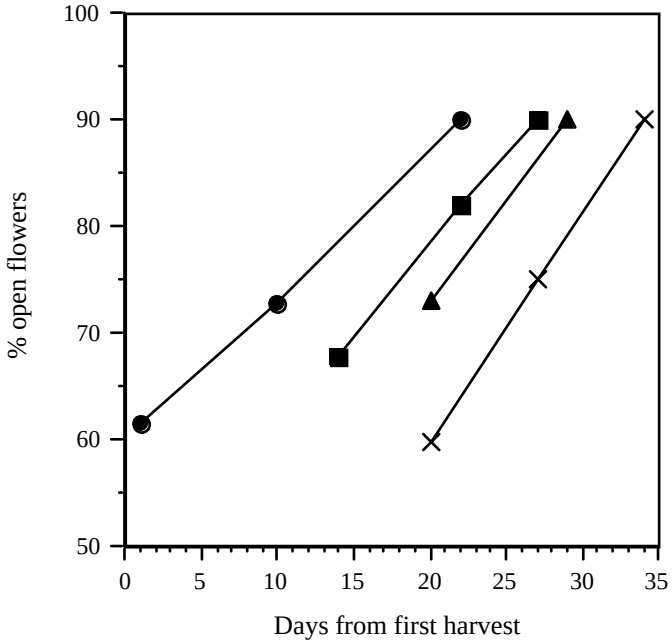
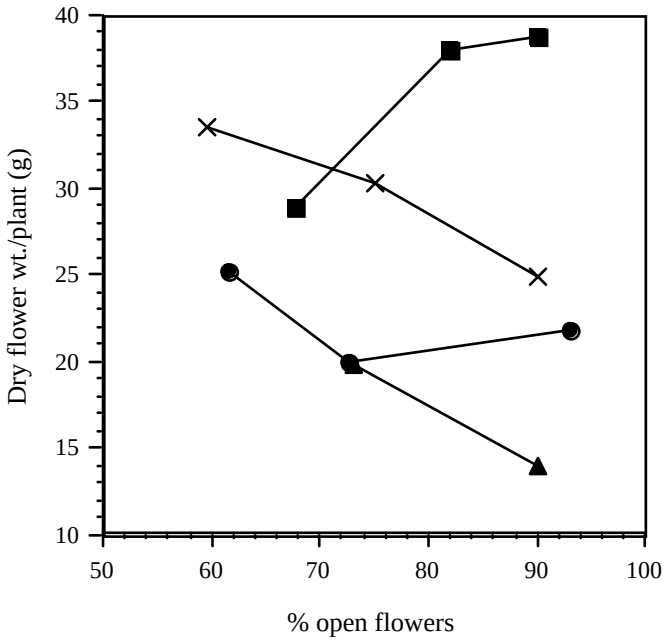


Figure 12B. Yield of dry flower weight per plant from pilot scale (10-15 kg) harvests of 4 clones from one site throughout flowering. Clone #3 ( ); clone #5 ( ); clone #17 ( ); clone #250 ( ).

Figure 12B

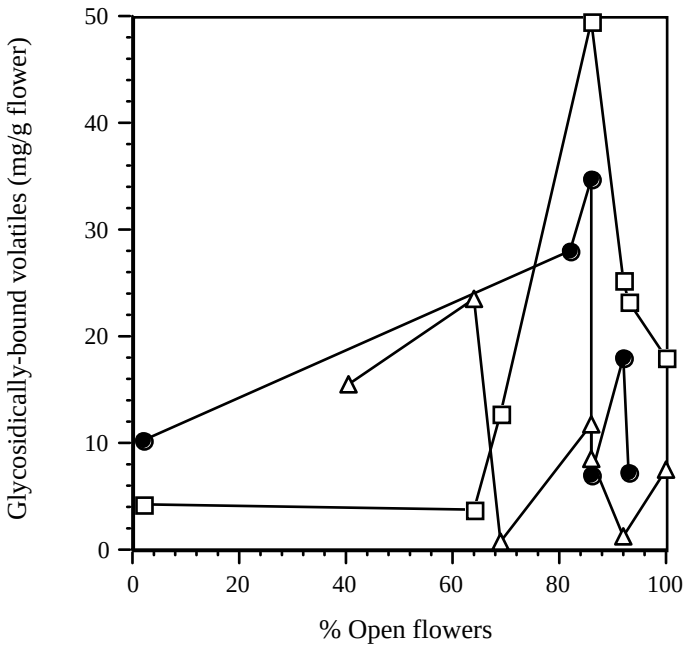


Absolutes from extracts made on a pilot scale were assessed by qualitative organoleptic procedures. Absolute from clone #3 flowers had a green/citrus odour with some astringency at early harvests, and an intense floral odour with notes of jasmine at 90% open flowers; at all times the absolute had distinct woody notes. Absolute from early harvests of clone #5 was fruity and floral but also had a strong citrus odour, at later stages of flowering the floral character was reduced in intensity but the deep background 'body' of boronia absolute was evident at 90% open flowers. Absolute from early harvests of clone #17 had a floral background with a strong fruit odour and a tropical, persimmon-type note, later harvests produced an absolute which was less fruity but more intense with the typical 'body' of boronia. At 90% open flowers, clone #17 absolute was the least intense, with a citrus and fruity top note masking a floral background. Absolute made from clone #250 flowers harvested at 75% open flowers had intense violet and rose odours, this absolute was considered more acceptable and rated higher than the absolute made from 90% open flowers. Clear distinctions were apparent in the organoleptic properties of absolutes made from each clone, and in some cases, from each harvest date. The choice of harvest date to produce a desired absolute would depend on the end use, however in general, a fruity/floral body with jasmine and rose notes, and a woody dry out are desirable.

Changes in Free and Bound Volatiles

Methodology: As per previous section on Precursors to Volatiles.
Results: The concentration of glycosidically bound volatiles in aqueous extracts made from sequential stages of flower development was analysed by hydrolysis with β–glucosidase (Figure 13).

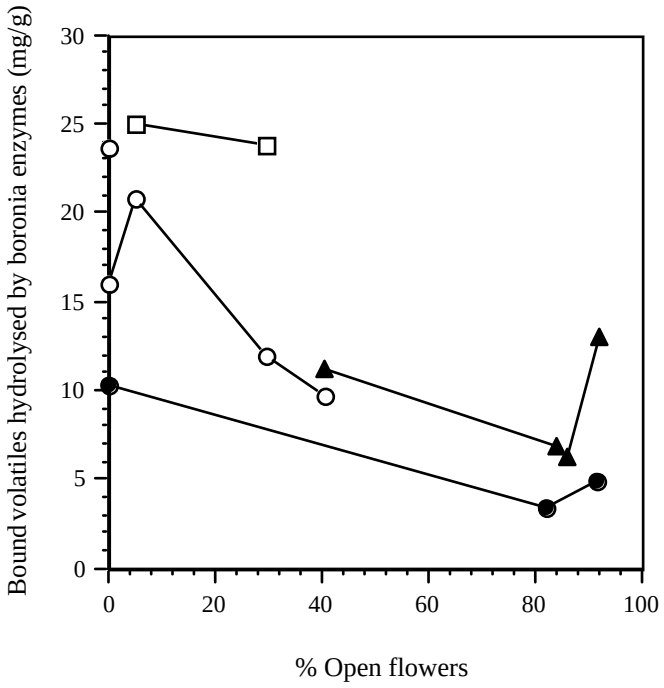
Figure 13. Concentration of glycosidically bound volatiles (mg/g flower) in several developmental stages throughout flowering, hydrolysed by β–glucosidase. Medium-sized buds ( ), large buds ( ), and open flowers ( ).



The concentration of glycosidically-bound volatiles in large buds was variable, but in medium-sized buds and open flowers, glycosidically bound volatiles increased after 60% open flowers and declined rapidly after 84% open flowers. Incubation of the same extract with boronia enzymes resulted in a reduced amount of volatiles being released compared with hydrolysis with β–glucosidase (Figure 14

compared with Figure 13). Generally, the concentration of volatiles bound in forms suitable for hydrolysis with boronia enzyme declined throughout flowering, with a slight increase in large buds/open flowers after 84% open flowers (Figure 14).

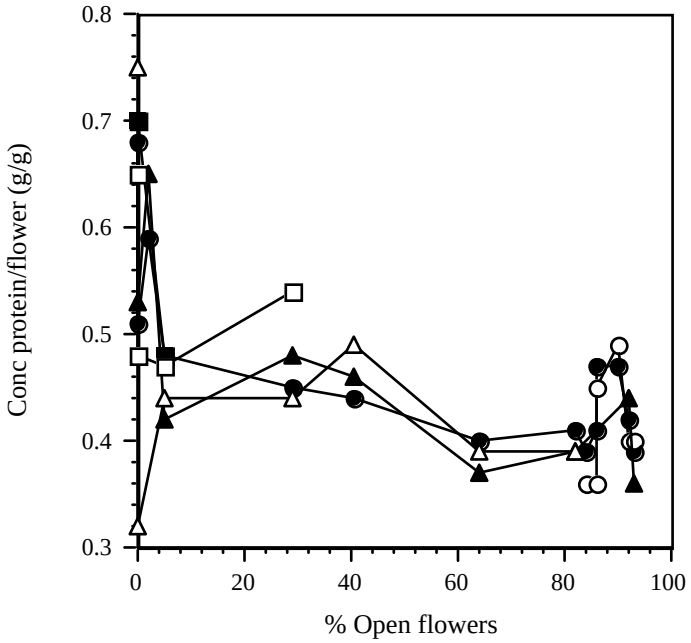
Figure 14. Volatiles released after hydrolysis with boronia enzymes (mg/g) in several developmental stages, and the whole sample, throughout flowering. Small and medium-sized buds ( ), medium-sized and large buds ( ), large buds and open flowers ( ), and the whole sample ( ).



Protein Levels and Enzyme Activity

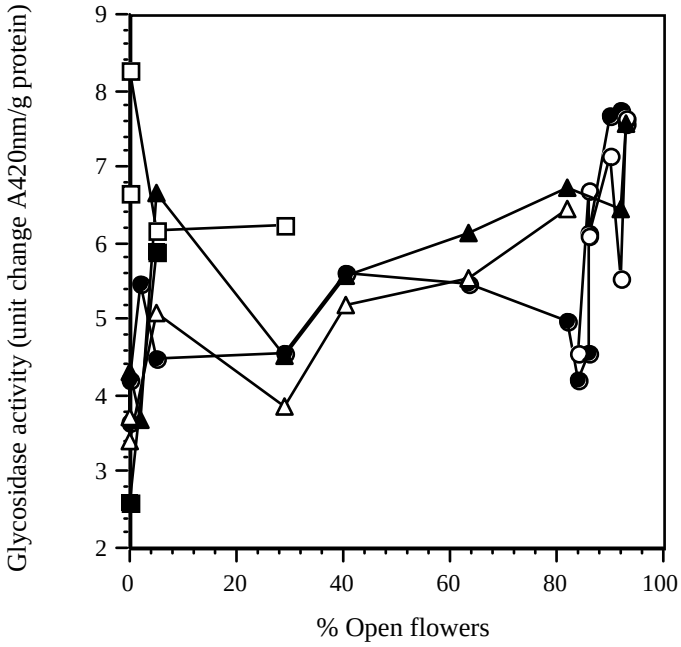
Methodology: As per previous section on Precursors to Volatiles.
Results: The protein content of flower material was relatively high in early stages of flowering when small buds predominated (MacTavish and Menary, 1997a), and generally declined throughout flowering until 85% open flowers when there was a rapid increase and subsequent decline (Figure 15).

Figure 15. Concentration of protein (g/g) in several developmental stages, and the whole sample, throughout flowering. Very small buds ( ), small buds ( ), medium-sized buds ( ), large buds ( ), open flowers ( ), and the whole sample ( ).



The proportion of the protein in flower material comprised of glycosidase activity increased in medium-sized and large buds throughout flowering, with amounts in open flowers controlling the levels observed in the whole sample which declined after 40% open flowers (Figure 16). There was a rapid increase and subsequent decline in glycosidase activity/g protein in open flowers and the whole sample between 84 and 92% open flowers.

Figure 16. Glycosidase activity (unit change in A_{420nm}/g protein) with pnp-β–D-galactopyranoside in several developmental stages, and the whole sample, throughout flowering. Very small buds ( ), small buds ( ), medium-sized buds ( ), large buds ( ), open flowers ( ), and the whole sample ( ).



3.5 Post-Harvest

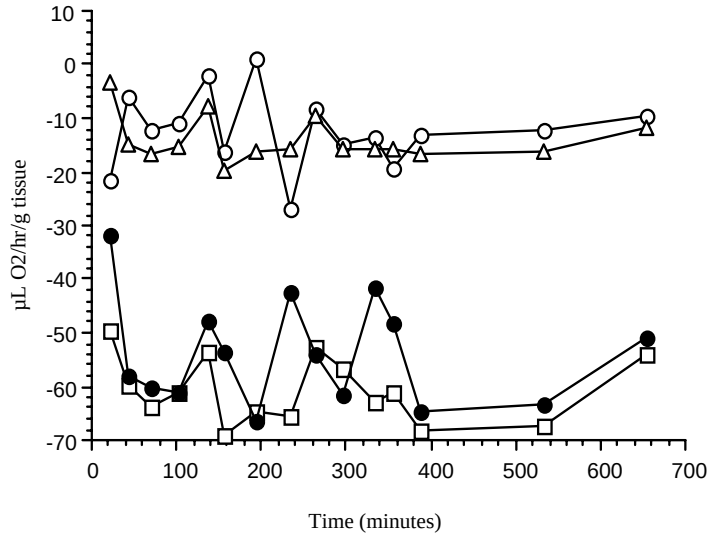
Oxygen Consumption

Methodology: Oxygen consumption in flowers and flower buds (clone #17) of several developmental stages was measured using a Warburg Respirometer. Duplicate (weighed) samples of small buds (12/vial), medium-sized buds (10/vial), large buds (8/vial) and open flowers (8/vial) were analysed. The Warburg Respirometer was used as per published methods, with the addition of KOH in half of the vials to adsorb CO_2.

Flowers for the second oxygen consumption experiment (30-40 per sample) were infiltrated under vacuum with water or with various solutions: sodium azide at 100 μM (low concentration) and 2 mM (high concentration), potassium cyanide at 100 μM (low concentration) and 3 mM (high concentration), and 2,4-dinitrophenol at 10 μM (low concentration) and 600 μM (high concentration); Tween 80, polyoxyethylenesorbitan monooleate: a mixture of oleic, linoleic, palmitic and stearic acids, was added to all solutions as a surfactant. Eight flowers were placed in each vial of the Warburg Respirometer and oxygen consumption was measured over 24 hours; there were duplicate vials for each treatment. The Warburg Respirometer was used as per published methods, with the addition of KOH in half of the vials to adsorb CO_2.

Results: The rates of oxygen consumption in different developmental stages were compared (Fig. 17).

Fig. 17. Oxygen consumption in sequential developmental stages (clone #17) at 20°C for 11 hours after harvest measured by Warburg Respirometer. Small buds ( ); medium-sized buds ( ); large buds ( ); and open flowers ( ).



Small and medium-sized buds (described fully in MacTavish and Menary, 1997a) consumed 17 µL oxygen/hr/g after the first hour of incubation at 20°C and continued to consume this amount per hour for up to 11 hours after harvest. Large buds and open flowers consumed 60 µL oxygen/hr/g for the first 10 hours, after which the rate of consumption reduced to 53 µL oxygen/hr/g during the subsequent 2 hours. There were fluctuations in the rates of oxygen consumption throughout incubation with cycles of approximately 100 minutes, most pronounced in the open flowers (Fig. 17). The respiratory quotient (RQ) in open flowers was on average 1.04, which is indicative of glucose being the energy source for respiration after harvest (Wills *et al*., 1998).

Rates of oxygen consumption were compared in open flowers before and after infiltration with water under vacuum. Infiltration of flowers with water did not adversely affect oxygen consumption. Open flowers that were infiltrated with water had variable oxygen consumption within the first 5 hours of incubation, stabilising at 6.5 µL/min/mg dr. wt. and steadily decreasing to 1 µL/min/mg dr. after a total of 25 hours at 20°C (Figs. 18A-D).

Fig. 18. Oxygen consumption in flowers incubated at 20°C for 26 hours after harvest on the Warburg apparatus. A = flowers infiltrated with water (control) ( ); flowers infiltrated with 100 µM sodium azide ( ); flowers infiltrated with 2 mM sodium azide ( ). B = flowers infiltrated with water (control) ( ); flowers infiltrated with 10 µM 2,4-dinitrophenol ( ); flowers infiltrated with 600 µM 2,4-dinitrophenol ( ); C = flowers infiltrated with water (control) ( ); flowers infiltrated with 100 µM potassium cyanide ( );

flowers infiltrated with 3 mM potassium cyanide ( ); D = flowers infiltrated with water (control) ( ); flowers infiltrated with water and purged with nitrogen gas ( ).

Fig. 18A.

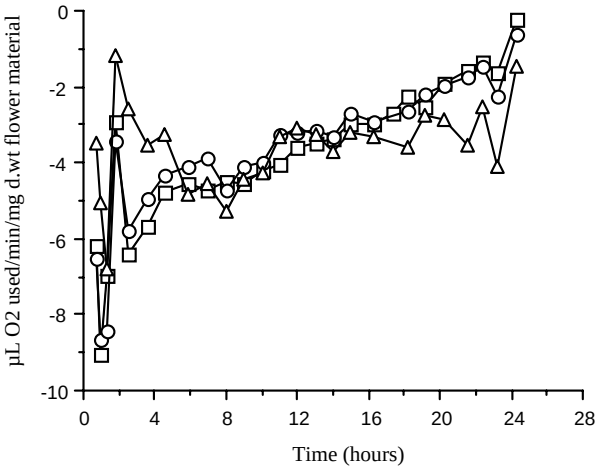


Fig. 18B.

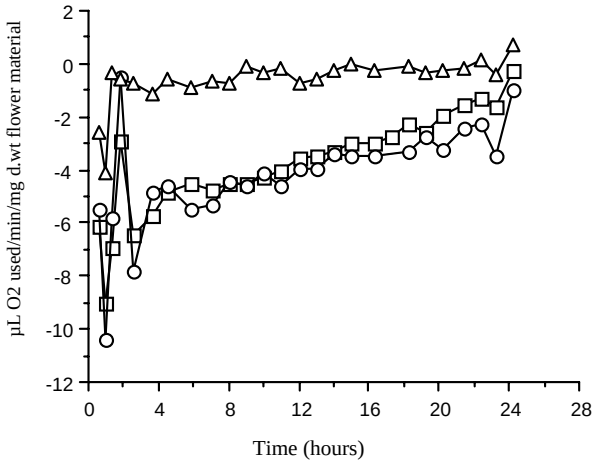


Fig. 18C.

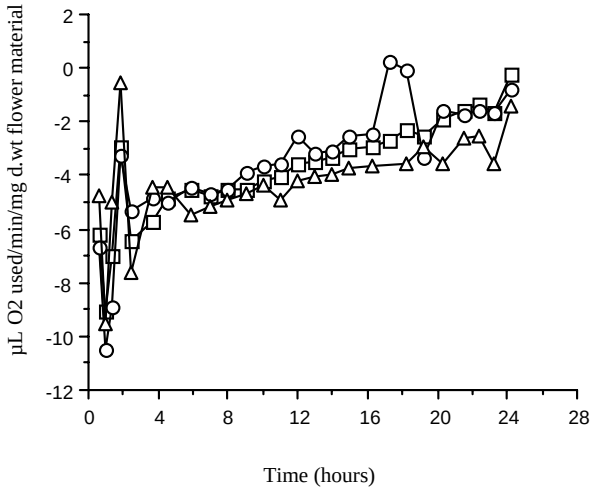
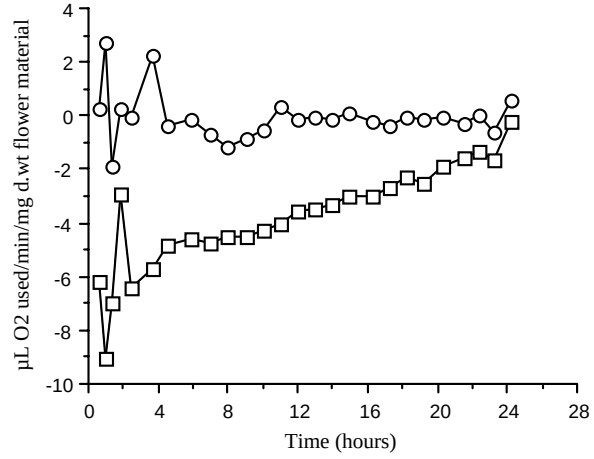


Fig. 18D.



Open flowers that were infiltrated with a low concentration of sodium azide (NaN_3, 100 µM) had similar rates of oxygen consumption (Fig. 18A) and concentrations of total volatiles and β–ionone (Table 4) compared with the control flowers.

Table 4. Concentration of volatiles in flowers after treatment with various exogenous compounds and incubation at 20°C for 24 hours, respiration rates as per Figs. 18A-D.

Solution	Respiration rates compared with control	Total volatiles μg/8 flowers	β–ionone μg/8 flowers
Control (water only)		151.1	39.4
Low sodium azide (100 μM)	Similar	148.3	34.7
High sodium azide (2 mM)	Slightly higher	97.6	21.6
Low 2,4-DNP (10 μM)	Slightly higher	139.5	39.0
High 2,4-DNP (600 μM)	Virtually nil	88.6	23.2
Low KCN (100 μM)	Similar	97.6	18.6
High KCN (3 mM)	Slightly higher	105.7	22.0
Nitrogen gas (+ water)	Virtually nil	73.9	21.6

Flowers infiltrated with high concentrations of NaN_3 (2 mM) had reduced oxygen consumption in the first 5 hours of incubation and enhanced consumption relative to the control in the final 10 hours (Fig. 18A). In these flowers, the concentration of total volatiles was reduced by 35% and β–ionone by 45% (Table 4).

Flowers treated with a relatively low concentration of DNP (10 mM) had slightly higher rates of oxygen consumption throughout incubation compared with control flowers, especially after 16 hours of incubation (Fig. 18B). Flowers treated with relatively high concentrations of DNP (600 μM) consumed almost negligible oxygen throughout incubation (Fig. 18B). At low concentrations of DNP, the concentrations of total volatiles and β–ionone after incubation were largely unaffected compared with the control, and were reduced in flowers treated with high concentrations of DNP (Table 4).

Flowers treated with a low concentration of KCN (100 μM) had largely similar rates of oxygen consumption compared with the control (Fig. 18C), however the concentrations of total volatiles and β–ionone were reduced in these flowers (Table 4). After 5 hours, oxygen consumption was slightly higher in flowers treated with high concentrations of KCN (3 mM) (Fig. 18C), however the concentrations of total volatiles and β–ionone were also reduced in these flowers (Table 4).

Flowers that were infiltrated with water and purged with nitrogen gas had reduced concentrations of total volatiles and β–ionone compared with the control (Table 4). Rates of oxygen consumption in such flowers were mostly between zero and 1 μL/min/mg dr. wt throughout incubation (Fig. 18D). In summary: nitrogen gas and high concentrations of DNP inhibited or significantly reduced oxygen consumption in open flowers. High concentrations of KCN, NaN_3 and low concentrations of DNP enhanced oxygen consumption, particularly during 12-16 hours of incubation. The concentrations of total volatiles and β–ionone were reduced by 25-40% relative to the control in flowers incubated with nitrogen gas, low and high concentrations of KCN and high concentrations of NaN_3 and DNP.

Respiration has been demonstrated to be critical for the production of volatiles after harvest. There appears to be a large increase in the respiratory activity of boronia flower buds between medium-sized and large buds (described in MacTavish and Menary, 1997a). In flowers treated with exogenously supplied chemicals, only flowers in which oxygen consumption was similar to or greater than the control produced the 25-40% increase in volatiles after harvest. In some treatments, including so-called high concentrations of NaN_3 and low and high concentrations of KCN, despite rates of oxygen consumption similar to or better than the control, the concentration of total volatiles and β–ionone did not increase.

Atmosphere During Incubation

Methodology: Flowers (3-5 kg) were incubated in atmospheres of air or nitrogen in 980 x 835 mm long, 100 µ poly plastic non-gusseted bags, #87022, obtained from EC. Blackwood Packaging. Bags were purged for 10 minutes with either compressed air or gaseous nitrogen, allowing outflow of gas for the first 5 minutes during which time the flowers were shaken; a headspace of gas above the flowers was maintained while the bags were closed by twisting the top five or six times, folding and securing it. The headspace was reduced slightly during the incubation period, indicating the seal was not air tight. Bags were incubated at 25°C for up to 20 hours in random positions in a closet incubator, then frozen rapidly in a blast freezer (-35°C) and subsequently stored at -18°C until they were thawed at 20°C for 60 minutes prior to extraction on a pilot scale, one extraction per treatment.

Results: Some gas was lost from each bag during incubation indicating that bags were not airtight, therefore oxygen levels may have increased in the bags purged with nitrogen, enabling respiration to occur. In both cases, the concentration of extract (% fr. wt.) increased during incubation and subsequently declined, the maximum increase being from 0.48 to 0.54% in air-purged flowers stored for 10 hours at 25°C (Table 5). The increases were not as great (max. 0.515%), and the decline greater in nitrogen-purged flowers. Statistical analysis of this data is not possible, however the differences are greater than normally expected from biological and extraction variability. Extract concentration was reduced upon incubation of frozen and thawed flowers, purged with air (data not shown).

The concentration of total volatiles increased steadily from 0.75 to 0.88% (x 10^{-1}, fr. wt.) during incubation of air-purged flowers, and did not change to a great extent in nitrogen-purged flowers (Table 5).

Table 5. Concentration and composition of floral extract from flowers purged with air or nitrogen prior to incubation at 25°C for 4, 10 and 20 hours.

	Control	Air 4 h[3]	Air 10 h[3]	Air 20 h[3]	N_2 4 h[4]	N_2 10 h[4]	N_2 20 h[4]
Extract conc.[1]	0.481	0.517	0.540	0.505	0.484	0.515	0.488
α-pinene[2]	0.36	0.49	0.53	0.75	0.51	0.64	0.55
β-pinene[2]	1.10	1.29	1.46	1.92	1.35	1.53	1.53
β-ionone[2]	1.43	1.21	1.34	1.48	1.21	1.05	0.83
dodecyl acetate[2]	0.33	0.30	0.24	0.17	0.24	0.21	0.18
methyl jasmonate[2]	0.06	0.07	0.06	0.08	0.06	0.07	0.06
methyl epijasmonate[2]	0.39	0.45	0.33	0.36	0.35	0.32	0.30
(Z)-heptadec-8-ene[2]	2.02	2.05	1.88	1.73	1.76	1.75	1.64
Total volatiles[2]	7.48	8.02	7.82	8.82	7.39	7.63	7.21

[1] % fr. wt.

[2] % fr. wt. x 10^{-2}

[3] Purged with air and incubated at 25°C.

[4] Purged with nitrogen gas and incubated at 25°C.

The concentration of α– and β–pinene increased in both treatments, although larger increases occurred in air-purged flowers compared with nitrogen-purged flowers (Table 5). The concentration of β–ionone declined in both treatments after 4 hours incubation, subsequently increasing in air-purged flowers and continuing to decline in nitrogen-purged flowers (Table 5). Dodecyl acetate, (Z)-heptadec-8-ene and methyl epijasmonate declined in all treatments compared with the control, except for a slight increase in methyl epijasmonate in air-purged flowers after 4 hours of incubation (Table 5). There are distinct differences in the extract made from air-purged flowers compared with nitrogen-purged flowers, especially with regard to the concentration of β–ionone.

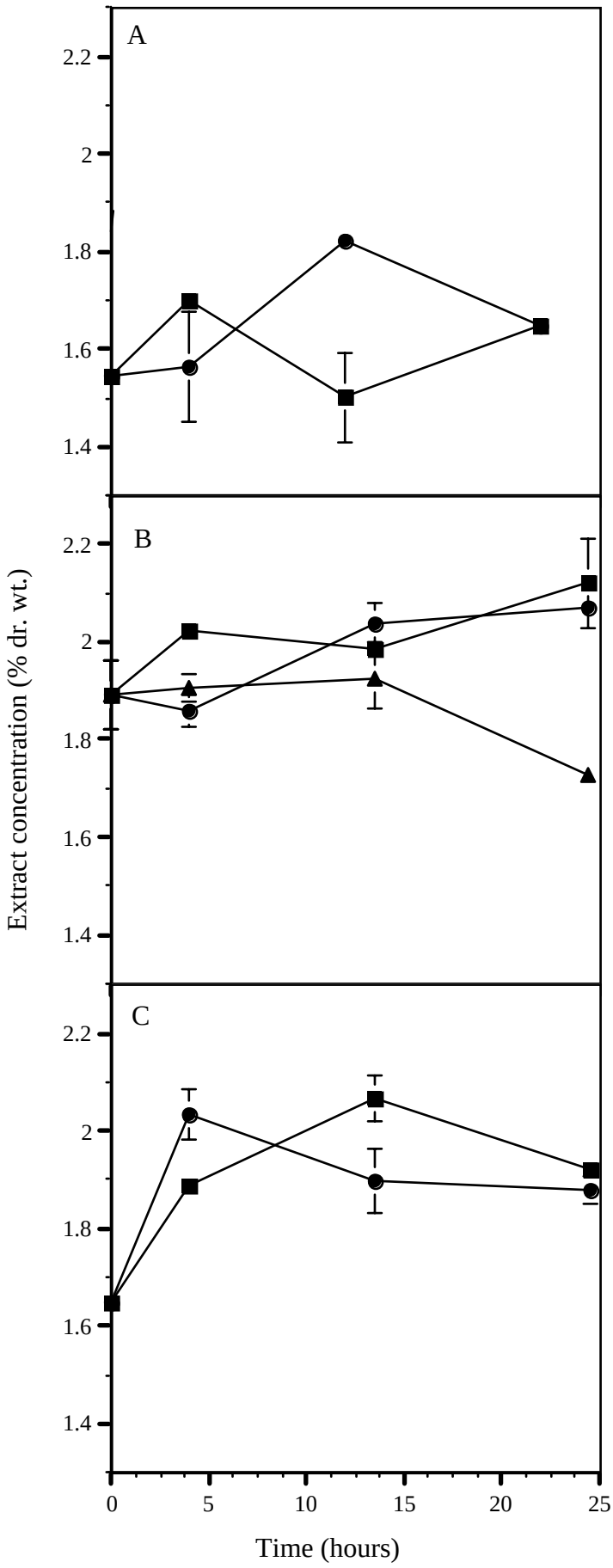
The extract produced from boronia flowers that were incubated after harvest after purging with either air or nitrogen contained different compositions of volatiles. Air-purged flowers had larger increases in extract and an increase in β–ionone, whereas β–ionone was depleted in nitrogen-purged flowers (Table 5). Mookherjee *et al* (1986, 1989) observed that the concentration of emitted β–ionone declined in several floral species upon harvesting and nitrogen purging, and the content of modified ionones including dihydro-β–ionol, dihydro-β–ionone increased.

Temperature and Harvest Timing

Methodology: Flowers of clone #5 (3 - 5 kg) harvested throughout flowering (68, 82 and 90% open flowers) were placed in the bags described above (#87022), however bags were open to the atmosphere during incubation in walk-in incubators for up to 24 hours. Samples harvested at 68% and 90% open flowers were incubated at 12 and 23°C (+/- 2°C); samples harvested at 82% open flowers were incubated at 12°C, 23°C and 32°C (+/- 2°C). After incubation, bags were placed on the floor of an -18°C freezer for rapid freezing, and stored at this temperature until extraction. Random samples (100 g) were taken from each bag, thawed at 20°C for 30 min. and extracted in triplicate, on a small scale.

Results: At harvest, the concentration of extract increased from 1.55% (dr. wt.) at 68% open flowers to 1.9% at 82% open, and subsequently declined to 1.65% at 90% open flowers (Figs. 19A, B, C).

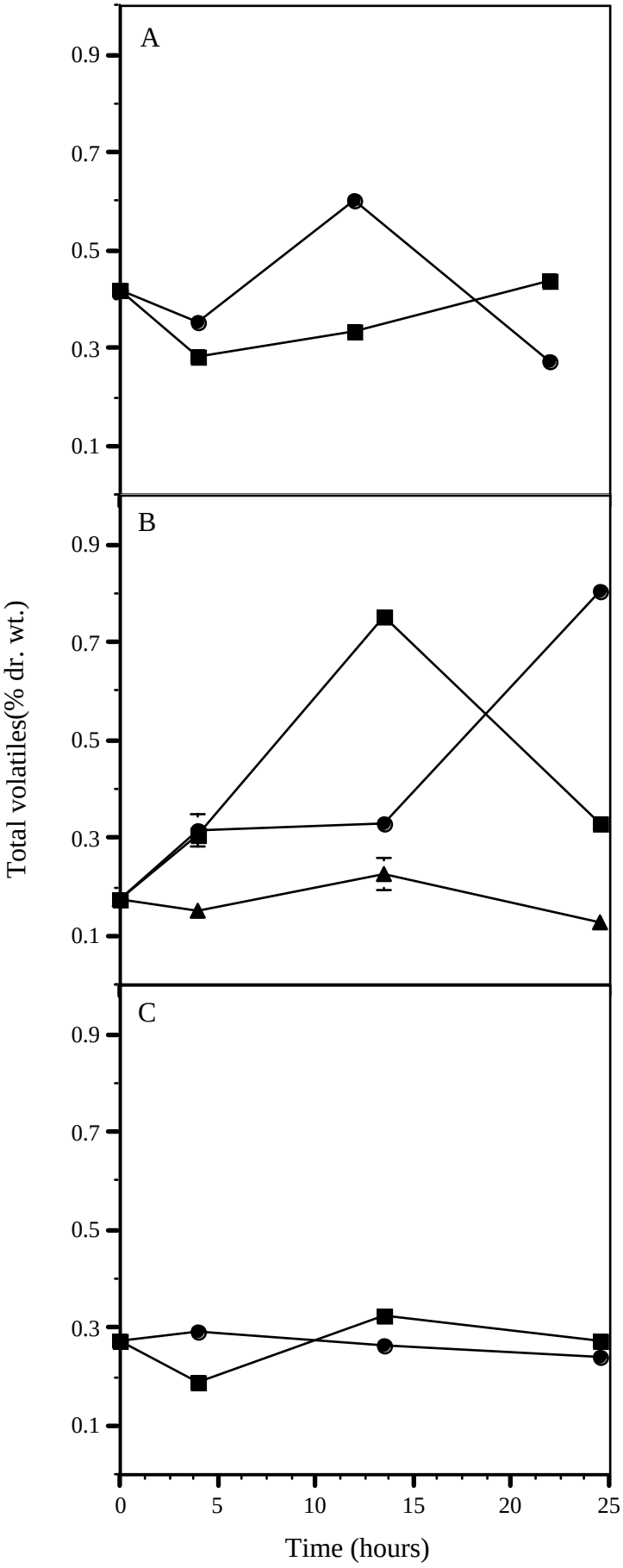
Fig. 19: Concentration of extract (% dr. wt.) in flowers harvested at 68% open (A), 82% open (B), and 90% open (C) and stored at several temperatures for up to 24.5 hours. A: 68% open, 12°C ( ), 23°C ( );
B: 82% open, 12°C ( ), 23°C ( ), 32°C ( );
C: 90% open, 12°C ( ), 23°C ( ).
Bars represent 2 s.e., n = 3. In some cases, error bars are obscured by symbols.


A
B
C
2.2
2
1.8
1.6
1.4
0
5
10
15
20
25
Extract concentration (% dr. wt.)
Time (hours)

As a result of incubation, flowers at 68% open had significantly more extract (1.72%) after 4 hours at 23°C and even more (1.83% dr. wt.) after 12 hours at 12°C (Fig. 19A). The concentration of extract declined after prolonged incubation. Flowers harvested at 82% open had significantly more extract (2.13%) after 25 hours at 23°C. In this case, no other changes were significant, however there was a trend toward increased extract after 24 hours at 12°C similar to that observed at 23°C, and a declining trend in extract concentration after 14 hours at 32°C (Fig. 19B). The most significant increase in extract concentration, from 1.65 to approximately 2.05% (dr. wt.), an increase of approximately 25% overall, occurred in flowers harvested at 90% open and incubated at either 12 or 23°C for between 4 and 25 hours (Fig. 19C). This enhanced concentration of extract was maintained for the duration of the incubation period sampled, no significant decline was apparent.

The concentration of total volatiles and β–ionone followed similar patterns in all samples, β–ionone comprised approximately 35-40% of total volatiles at all times (data not shown). The concentration of total volatiles in flowers harvested at 68% open, initially 0.4% (dr. wt.), declined during the first 4 hours of incubation at 12 and 23°C, increased significantly to 0.60% (dr. wt.) after 12 hours at 12°C and subsequently declined again (Fig. 20A).

Fig. 20. Concentration of total volatiles (% dr. wt.) in flowers harvested at 68% open (A), 82% open (B), and 90% open (C) and stored at several temperatures for up to 24.5 hours. A: 68% open, 12°C ( ), 23°C ( );
B: 82% open, 12°C ( ), 23°C ( ), 32°C ( );
C: 90% open, 12°C ( ), 23°C ( ).
Bars represent 2 s.e., n = 3. In some cases, error bars are obscured by symbols.


A
B
C
0.9
0.7
0.5
0.3
0.1
0
5
10
15
20
25
Total volatiles(% dr. wt.)
Time (hours)

This increase did not occur at 23°C, however there was a trend toward increased volatiles between 4 and 23 hours of incubation at 23°C. In flowers harvested when 82% open, there were significant increases in the concentration of total volatiles as a result of incubation (Fig. 20B). Total volatiles increased from 0.2 to 0.8% (dr. wt.), a 300% increase, during 14 hours at 23°C, and 25 hours at 12°C. No change occurred at 32°C. At 23°C, the maximum concentration was followed by a rapid decline to 0.33%. Flowers harvest at 90% open did not undergo any change in the concentration of total volatiles during incubation at 12 or 23°C for up to 25 hours (Fig. 20C).

Floral extract made at harvest from three successive harvests (68, 82 and 90% open flowers) showed the same patterns as those observed previously, notably the increase in extract to 80% open flowers and subsequent decline as the majority of flowers at harvest become increasingly senescent (Fig. 19). The maximum concentration of total volatiles at harvest occurred when flowers were 68% open, and subsequently declined (Fig. 20), which is not typical (MacTavish, 1995). The potential for post-harvest increases in extract and volatiles also changes with harvest time. The early harvested sample, which comprised 32% medium and large buds, had an increased extract concentration (% dr. wt.) as a result of incubation, bringing the concentration of extract to a level equal to that observed in flowers harvested later (82% open), before incubation (Fig. 19A,B). Bud material did not have significant post-harvest activity with regard to the biosynthesis of volatiles; therefore the increased volatiles at 68% open are most likely to be produced in the open flowers in the sample. Other researchers have found aroma-producing enzymes to be most active in open flowers (Watanabe *et al.*, 1993, 1995).

Flowers harvested when 82% open, the optimum harvest time, produced increases in extract concentration (% dr. wt.) at 12 or 23°C to the highest concentration observed overall, an increase of approximately 10% (Fig. 19B). The concentration of extract after incubation of late-harvested flowers (90% open), when many flowers were visibly senescent (i.e. pigments had begun to fade and some petals had abscised), was similar to the concentration achieved by optimally harvested flowers after incubation. However, the increased extract in late-harvested flowers occurred in less time, and the overall increase compared with zero time levels was relatively greater at almost 25% than that observed after incubation of optimally-harvested flowers (Fig. 19B,C). In contrast, the concentration of volatiles (% dr. wt.) did not change during incubation of late-harvested flowers, but increased dramatically in flowers harvested at 82% open, by up to 300% (Figs. 20B,C). Increases in the concentration of β–ionone occurred to a similar extent (data not shown). Increases in total volatiles and β–ionone were slight at 68% open (Figs. 20A).

Commercial-Scale Incubations

Methodology: Clone #5 flowers grown at Scottsdale in Tasmania's NorthEast were used for this trial. 600 kg of flowers at 88 to 90% open flowers were harvested mechanically over two days, the flowers were more senescent than desirable. Flowers were divided into 4 batches, each incorporating 16, 9 kg boxes (144 kg/batch). One batch was taken to the 'Alfoods' freezers in Launceston for rapid freezing in boxes (= normal frozen control). The second batch was spread to a maximum depth of 20 cm over palettes and shadecloth on the floor of a 3 x 3 m room (12°C +/- 1°C) at Scottsdale for 20 hours; the average temperature amongst the flowers was 16°C (= incubated sample #1). After 20 hours, flowers were bagged in shadecloth bags, transported to Launceston (2 hours) and cooled by a vacuum cooling apparatus to 0°C over 2 hours. The vacuum cooler had a capacity of 93 cubic feet, and operated at 1.3 atm pressure, it was designed and built by John Goodricke, 'Tas Freeze Dried', 181 Westbury Rd., Launceston Tasmania 7250. Subsequently flowers were boxed as before (9 kg/box) and frozen in the Alfoods freezer. A third batch was boxed, transported to the vacuum cooler and cooled to 0°C as described above (= vacuum cooled control) and subsequently frozen in the 'Alfoods' freezer. The fourth batch was incubated and treated as per incubated sample #1 (= incubated sample #2); in this case the maximum temperature within flowers during incubation was 18°C.

Flowers were thawed slightly and extracted on a commercial scale at Essential Oils of Tasmania Ltd. using 4 washes: Wash 1 = 1 : 3.3 kg flowers : L solvent, 2 hours; W2 = 1 : 2.7 kg : L, 3 hours; W 3 = 1 : 2 kg : L, 1 hour; W 4 = 1 : 1.3 kg : L, 30 minutes. The extracts were evaporated to dryness under vacuum and weighed; extracts were analysed by GC.

Results: There was an average increase in floral extract of 13% from an average of 0.5437% (fr. wt.) within the two controls to an average of 0.6152% (fr. wt.) in the two incubated samples (Table 6). Total volatiles and β–ionone (% fr. wt.) increased by 28.6% and 39% respectively (Table 6). Between the two controls and the two incubated samples, extract concentration was more variable than extract composition. There were considerable limitations to the scaling up of this procedure, notably the re-boxing of flowers after spreading them over the floor of the room, and the distances traveled between harvest site, incubation site, vacuum cooling apparatus and storage freezers. Also, the large size of the samples precluded statistically useful replication - to triplicate everything, almost 2 tons of flowers of one clone from one site would have been required in a short period of time. A modified experiment was proposed for future work involving incubation of flowers in mesh bags allowing for airflow under ambient, on-farm conditions and rigorous temperature measurement.

Table 6. Concentration and composition of extract from commercial scale incubations of clone #5 flowers.

Sample	Extract conc. (% fr. wt.)	Total volatiles (% in extract)	β–Ionone (% of total volatiles)	Total volatiles (% fr. wt.)	β–Ionone (% fr. wt.)
Normal frozen	0.5721	17.34	27.52	0.099	0.0273
Vacuum cooled	0.5153	17.36	29.62	0.090	0.0265
Incubated #1	0.6337	19.61	29.05	0.124	0.0361
Incubated #2	0.5966	19.96	32.46	0.119	0.0387

Clonal Type

Methodology: Plants from one site in Tasmania were used. Clone #3 flowers were harvested at 64% open, clone #5 at 79% open, clone #17 at 74% open and clone #250 at 77% open. Flowers were harvested by hand using combs, sieved through a mesh grid with holes 10 mm square to remove branches and laterals, and placed immediately under incubation conditions. Flowers (4 - 5 kg) were incubated in unsealed 980 x 835 mm long, 100 µ poly plastic non-gusseted bags, #87022, obtained from EC. Blackwood Packaging, 9 Sunmont St. Lutana Tasmania 7009. Bags were incubated at 12°C (+/- 2°C) and 30°C (+/- 3°C) for up to 48 hours in random positions in incubating rooms. Random samples (100-200g) were removed at zero time and regularly throughout incubation. Half the sample was made into an acetone powder for other work; the other half was frozen rapidly to -18°C and stored at this temperature until being thawed at 20°C for 30 minutes prior to extraction and GC analysis, in triplicate. All incubations were done in the dark. It was intended that flowers for this study would be harvested when close to 80% open, all except clone #3 were within 6% of this target. It was also intended that flowers would be incubated at 12-14°C and 20-25°C for up to 48 hours, however temperatures in the warmer incubating room were higher than anticipated (30 +/- 3°C).

Results: In clone #3 flowers (64% open) that were incubated at 12°C, the concentration of extract (% dr. wt.) declined during the first 12 hours of incubation, subsequently increasing to a maximum of 1.75% after 36 hours of incubation (Fig. 21A), a maximum change of some 6%. At 30°C, the concentration of extract in flowers from the same clone did not alter significantly throughout 48 hours (Fig. 21A). The concentration of total volatiles increased by 40% after 36 hours at 12°C, however there was little change at 30°C (Fig. 21B). At 12°C there was an 88% increase in β–ionone after 36 hours, and a 35% at 32°C (Fig. 21C).

Fig. 21. Changes in floral extract in clone #3 flowers incubated after harvest for 48 hours at 12°C (_) and 30°C (´ O '). A = concentration of extract (% dr. wt.); B = concentration of total volatiles (% dr. wt.); C = concentration of β–ionone (% dr. wt.). Bars represent 2 s.e., n =3. In some cases, error bars are obscured by symbols.

Fig. 21A

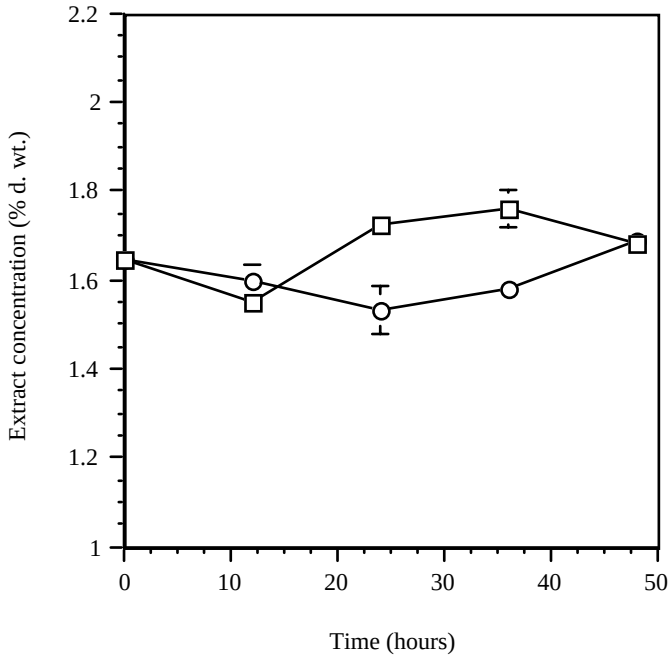


Fig. 21B.

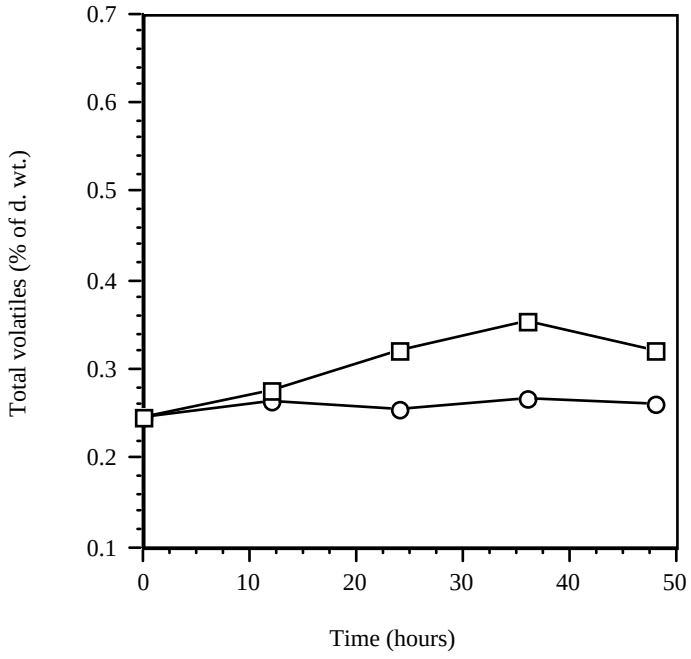
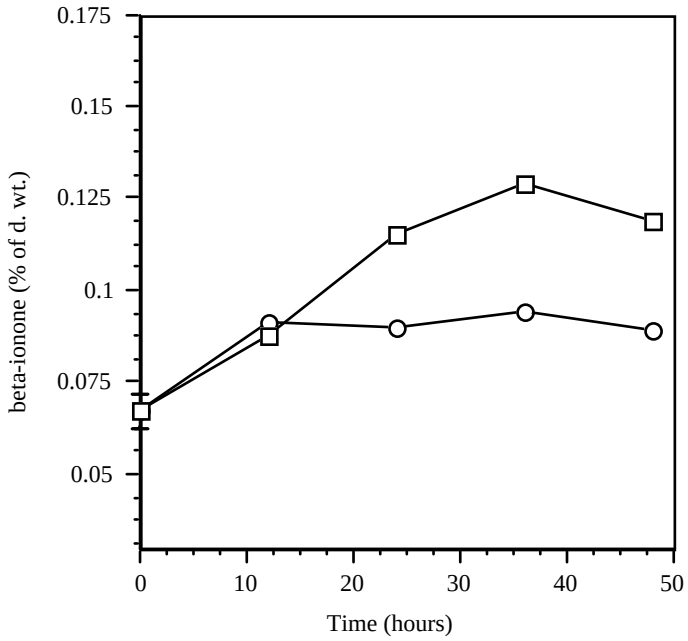


Fig. 21C.



In clone #5 flowers (79% open) incubated at 12°C, the concentration of extract increased during the first 24 hours and remained at this level, an increase of 16% (Fig. 22A). At 30°C, the concentration increased slightly after 12 hours but subsequently decreased steadily over the remaining 36 hours (Fig. 22A). Similar patterns were observed in the concentration of total volatiles with maximum increases of 25% at 12°C and decreases of 28% at 30°C over 24 hours (Fig. 22B). The concentration of β–ionone increased by 45% at 12°C and decreased by 43% at 30°C (Fig. 22C).

Fig. 22. Changes in floral extract in clone #5 flowers incubated after harvest for 48 hours at 12°C (_) and 30°C (´ O '). A = concentration of extract (% dr. wt.); B = concentration of total volatiles (% dr. wt.); C = concentration of β–ionone (% dr. wt.). Bars represent 2 s.e., n =3. In some cases, error bars are obscured by symbols.

Fig. 22A

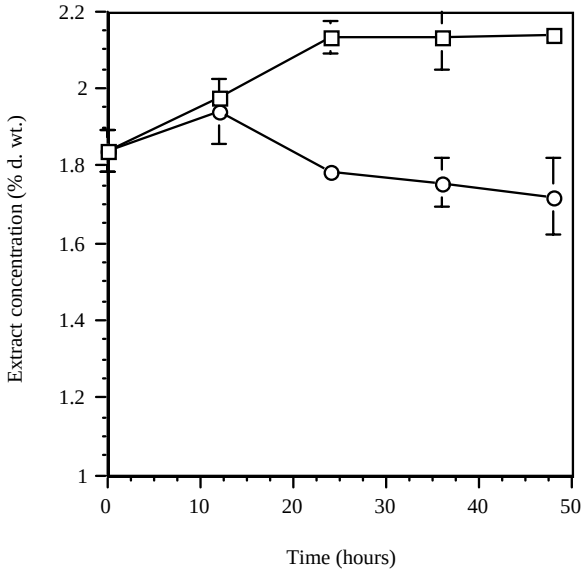


Fig. 22B.

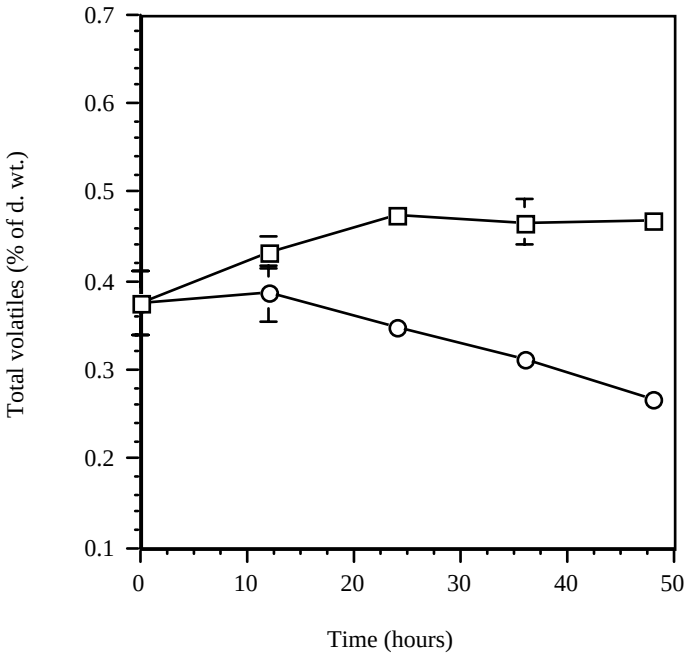
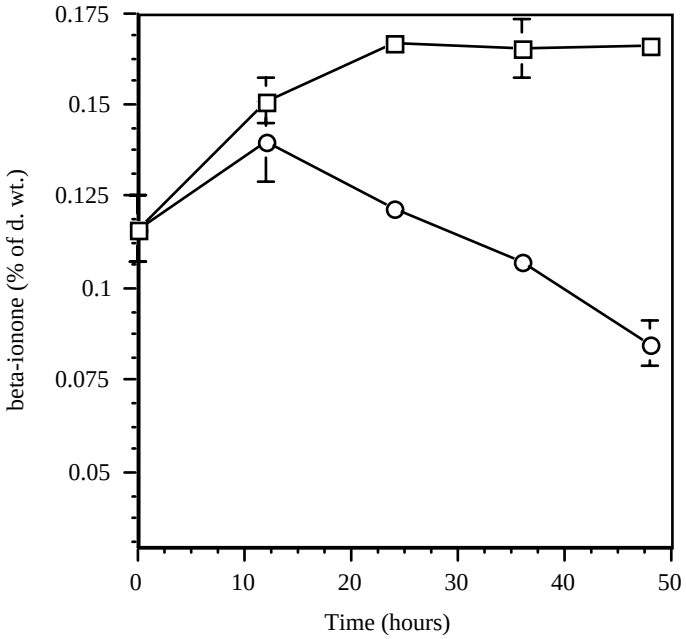


Fig. 22C.



The concentration of extract in clone #17 flowers (74% open) underwent little change during incubations at 12°C, however at 30°C, extract concentration declined by 38% after 12 hours of incubation (Fig. 23A). At 12°C, the concentration of total volatiles increased steadily for the first 24 hours and was subsequently maintained at this higher level, an increase of 62% (Fig. 23B). At 30°C the concentration of volatiles increased during the first 12 hours and subsequently declined (Fig. 23B). The concentration of β–ionone increased during the first 12 hours at both temperatures, subsequently declining at 30°C and continuing to increase at 12°C, producing a 100% increase at 12°C compared with zero time concentrations.

Fig. 23. Changes in floral extract in clone #17 flowers incubated after harvest for 48 hours at 12°C ( ) and 30°C ( ). A = concentration of extract (% dr. wt.); B = concentration of total volatiles (% dr. wt.); C = concentration of β–ionone (% dr. wt.). Bars represent 2 s.e., n =3. In some cases, error bars are obscured by symbols.

Fig. 23A

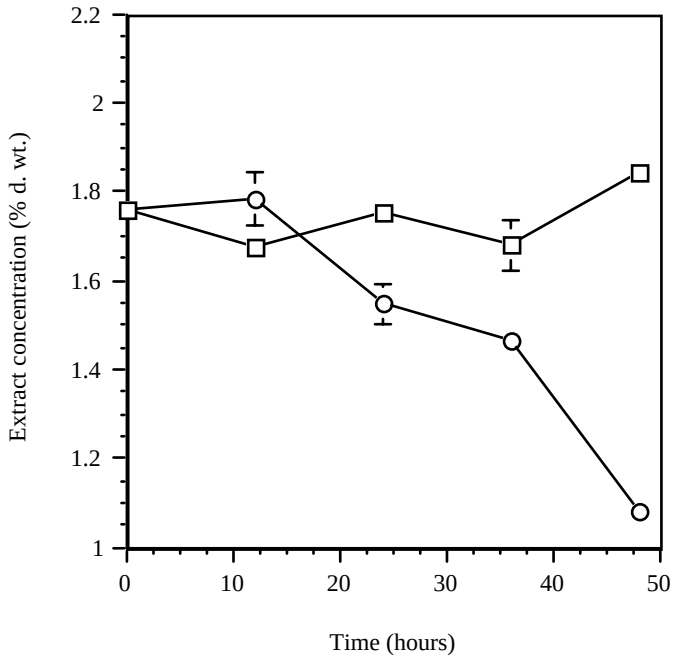


Fig. 23B.

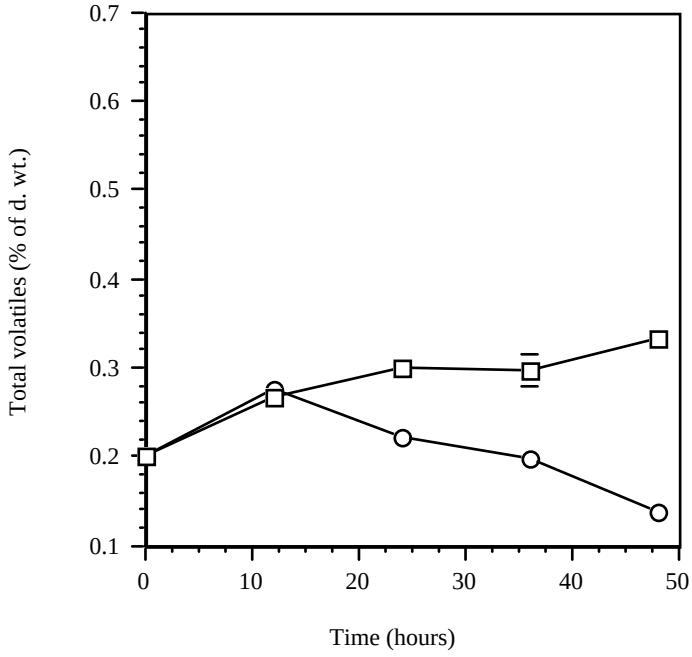
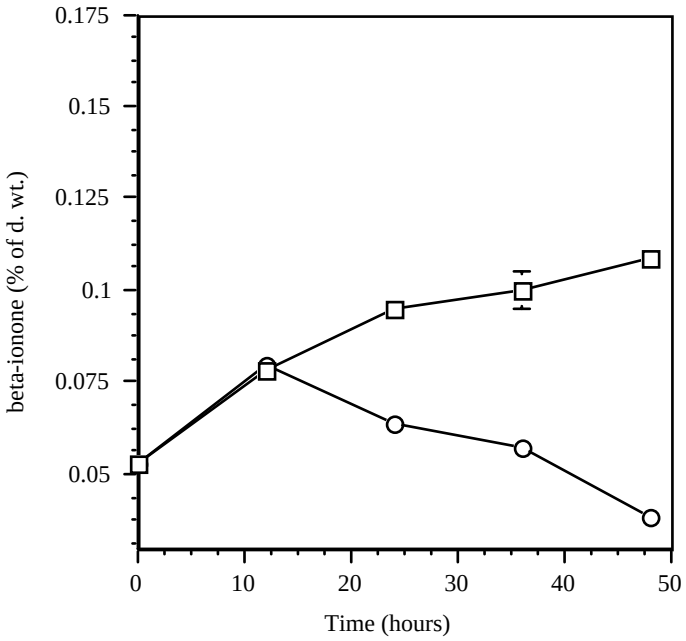


Fig. 23C.



In clone #250 (77% open flowers), the concentration of extract increased by 15% during 12 hours at 30°C and subsequently declined (Fig. 24A). At 12°C the 19% increase extended over 48 hours (Fig. 24A). Similar patterns were observed in the concentrations of both total volatiles and β–ionone: an increase at both temperatures followed by a decline after 12 hours at 30°C and a gradual plateau at 12°C after 24-36 hours (Figs. 24B, C). After 48 hours at 12°C, the concentration of total volatiles increased by 117% and β–ionone by 191%.

Fig. 24. Changes in floral extract in clone #250 flowers incubated after harvest for 48 hours at 12°C ( ) and 30°C ( ). A = concentration of extract (% dr. wt.); B = concentration of total volatiles (% dr. wt.); C = concentration of β–ionone (% dr. wt.). Bars represent 2 s.e., n =3. In some cases, error bars are obscured by symbols.

Fig. 24A

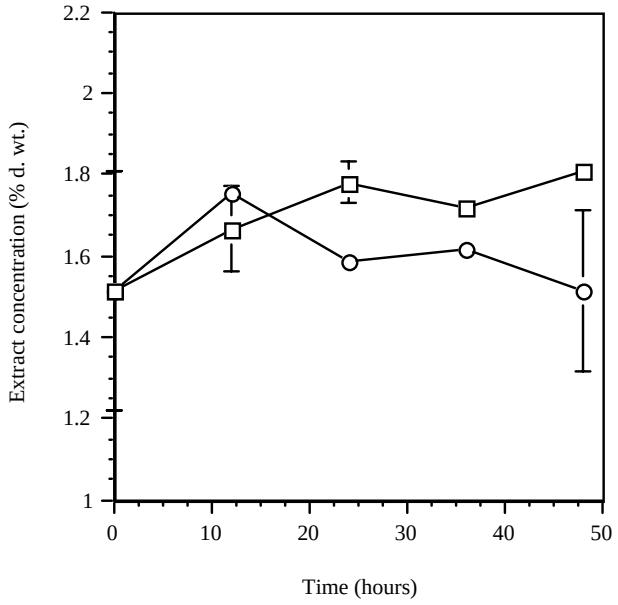


Fig. 24B.

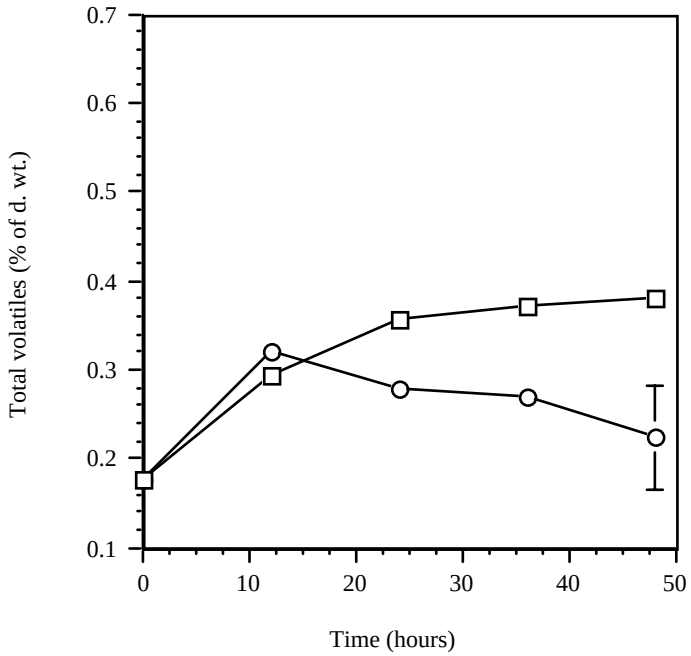
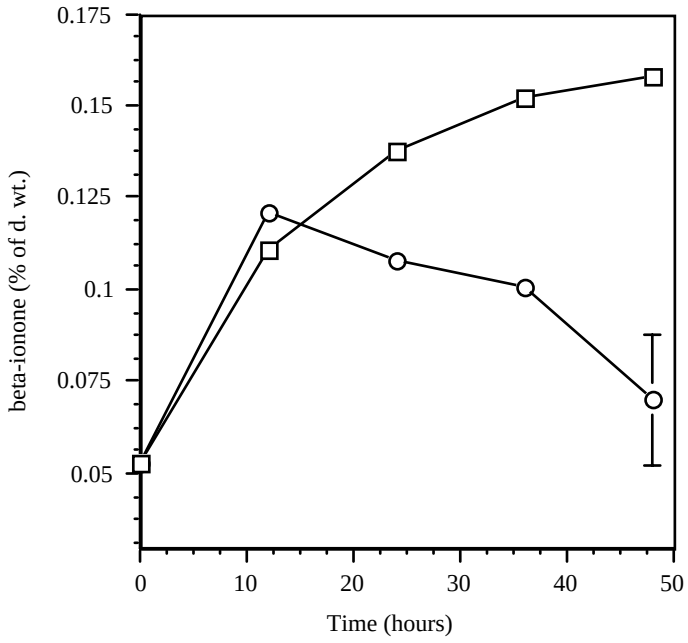


Fig. 24C.



The results of the experiments at 12°C are summarised in Table 7.

Table 7. Summary of changes in extract, total volatiles and β–ionone in four clones during post-harvest incubation at 12°C and 30°C.

	Clone #3, 64% open			Clone #5, 79% open			Clone #17, 74% open			Clone #250, 77% open		
Value	at harvest	12°C	30°C	at harvest	12°C	30°C	at harvest	12°C	30°C	at harvest	12°C	30°C
Conc. of extract (% dr. wt.)	1.65	1.75	1.67	**1.84**	**2.13**	**1.94**	1.76	1.83	1.80	1.52	1.80	1.75
Hours of inc. to max		36	36		24	12		48	12		**48**	12
Max % change/clone		6%			16%			3%			**19%**	
Concentration of ttl volatiles (% dr. wt.)	0.245	0.35	0.27	**0.375**	**0.47**	0.39	0.201	0.325	0.26	0.175	0.38	0.33
Hours of inc. to max		36	36		24	12		48	12		**48**	12
Max % change/clone		43%			25%			62%			**117 %**	
Concentration of β–ionone (% dr. wt.) (hr)	0.067	0.128	0.092	**0.114**	**0.165**	0.138	0.053	0.108	0.08	0.055	0.155	0.12
Hours of inc. to max		36	36		24	12		48	12		**48**	12
Max % change/clone		91%			45%			103 %			**181 %**	

The greatest % increases in extract, total volatiles and β–ionone as a result of post-harvest incubation occurred in clone #250 flowers after 24 hours at 12°C. However, under the same conditions, the final concentrations of extract, total volatiles and β–ionone were at a maximum in clone #5 flowers at 2.13%, 0.48% and 0.17% respectively.

There are large differences between the 4 clones in the increases in floral extract and total volatiles after harvest when calculated as a % of dry flower weight. For example in clones #3 and #17, the increase in floral extract is relatively small and is probably brought about entirely by the relatively large increase in total volatiles. In clone #17, the increase in volatiles more than explains the increase in extract, indicating that non-volatile and other components of the extract have declined concomitantly. In clones #5 and #250, the increase in floral extract as a % of dry weight is greater than the increase in volatiles, indicating either that other components of the extract have also increased, or that they were extracted more easily. The former appears to be more likely, since the dry weights changed very little during incubation, although other processes occurring during incubation, both enzymatic and oxidative, may enable better penetration of solvent into the floral tissues during extraction.

In the light of the present work it is possible to make suggestions as to the best incubation procedures for each of the four currently available commercial clones. If the entire harvest is harvested optimally at 75-80% open flowers, with appropriate incubation, it is possible to bring all flower material up to an optimum 'standard' of 1.8-2.0% floral extract (dr. wt. basis), 0.35-0.5% total volatiles including 0.1-0.15% β–ionone.

Changes in Free and Bound Volatiles, Protein Levels and Enzyme Activity

Methodology: Flowers were harvested using hand-held combs and were either a) made in to acetone powders immediately, or were frozen at -18°C for storage until aqueous extracts were made; or b) were incubated at 12-30°C for up to 48 hours (MacTavish and Menary, 1998a) followed by treatment as per a). Clone #3 was harvested at 64% open flowers, clone #5 at 79%, clone #17 at 74% and clone #250 at 77% open flowers.

1. Analysis of Boronia Enzymes From Incubated Flowers: Acetone powders (2 g) were resolubilised in 25 ml of 0.1 M citrate buffer pH 4.3, with 0.1% Tween 80 and 0.2-0.3 g PVPP, overnight at 4°C. The mixtures were filtered and the filtrate made up to 45 ml with buffer; the protein contents were measured.
A Standard Aqueous Extract was prepared by blending frozen flowers (740 g) with 0.1 M citrate buffer pH 4.3, with excess PVPP and 0.1% Tween 80. The slurry was then heated to boiling point, cooled and filtered under vacuum using Whatman #1 filter papers. The filtrate was stirred for 2 hours with excess PVPP at 20°C, filtered, and the filtrate dialysed against solid sucrose at 4°C. The concentrated extract was filtered, frozen for storage, and subsequently made up to 250 ml with buffer.
Incubation of Boronia Enzymes with Standard Aqueous Extracts: 10 ml aliquots of the solubilised boronia enzymes were incubated with 1 ml standard aqueous extract for 6 hours at 37 °C. The reactions were stopped by addition of DCM with an octadecane internal standard; 10 ml aliquots of each enzyme and 1 ml of the standard aqueous extract were extracted with DCM to enable quantitation of free volatiles prior to incubation. After 24 hours at 4°C, the DCM layer was removed and analysed by GC.

2. Analysis of Bound Volatiles in Incubated Flowers: Aqueous extracts from incubated flowers (20-30 g) were prepared by blending frozen flowers (20-30 g) with 0.1 M citrate buffer pH 4.3, with excess PVPP and 0.1% Tween 80. The slurry was then heated to boiling point, cooled and stirred for 2 hours with excess PVPP at 20°C. The filtrate plus excess PVPP was dialysed against solid sucrose at 4°C, filtered, frozen for storage, and subsequently made up to 50 ml with buffer; 15 ml was used/sample.
A Standard Boronia Enzyme was prepared by mixing 7 acetone powders together which had previously been shown to be highly active. 10 g of acetone powder was resolubilised in 200 ml 0.1 M citrate buffer pH 4.3, with excess PVPP and 0.1% Tween 80 overnight at 4°C. The filtrate was made up to 140 ml; 5 ml was used/sample. *Incubation of Aqueous Extracts with Standard Boronia Enzyme:* 5 ml aliquots of the solubilised standard boronia enzyme and 20 mg of β–glucosidase were incubated separately with 15 ml of each aqueous extract for 24 hours at 37 °C. The reactions were stopped by addition of DCM with an octadecane internal standard. After 24 hours at 4°C, the DCM layer was removed and analysed by GC. Aliquots (15 ml) of each aqueous extract and 5 ml of the standard boronia enzyme were extracted with DCM to enable quantitation of free volatiles prior to incubation.

Results: During post-harvest incubation of clone 3 flowers, the concentration of protein increased during 24 hours at 12°C and 36 hours at 30°C and subsequently declined (Fig. 25A); maximum change was 18%. At 12°C the proportion of the protein comprising glycosidase hydrolytic activity (change in A_{420nm}/g protein) increased, but did not alter significantly at 30°C (Fig. 25B); maximum change was 17%. The concentration of glycosidically bound volatiles (free volatiles released by treatment with β–glucosidase) declined throughout 36 hours, and increased between 36 and 48 hours at 30°C, with final concentrations (mg/g) similar to at harvest (Fig. 25C); most significant decrease was 93%. Production of volatiles after enzymatic hydrolysis of aqueous extract, so-called 'volatile-producing activity' (mg total volatiles hydrolysed/g protein) increased in the first 12-24 hours at 30 and 12°C respectively and subsequently declined to negligible levels (Fig. 25D); maximum change was 20%.

Fig. 25. Clone #3 flowers (64% open flowers) incubated at 12°C ( ) and 30°C ( ). for up to 48 hours after harvest. A = Concentration of protein (g/g). B = Proportion of protein comprising glycosidase hydrolytic activity (change in A420nm/g protein). C = Concentration of

glycosidically bound volatiles (mg/g). D = Volatile-producing activity (mg total volatiles hydrolysed/g protein).

Fig. 25A.

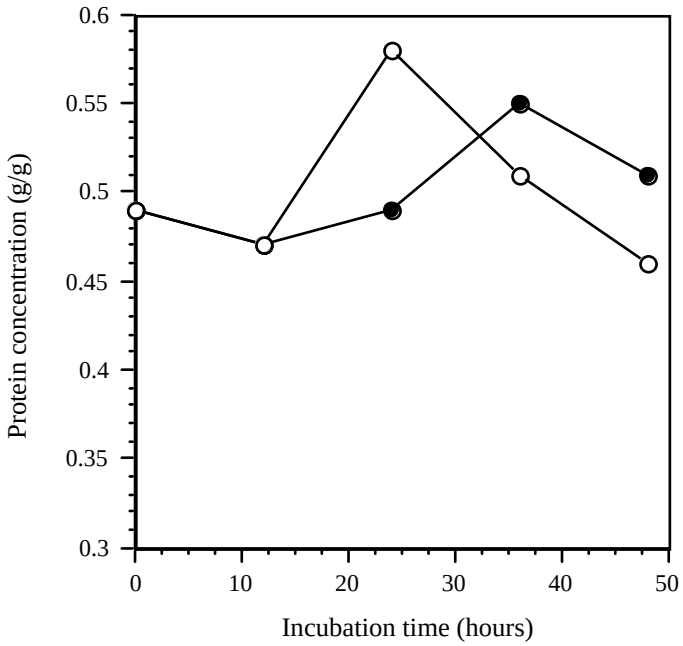


Fig. 25B.

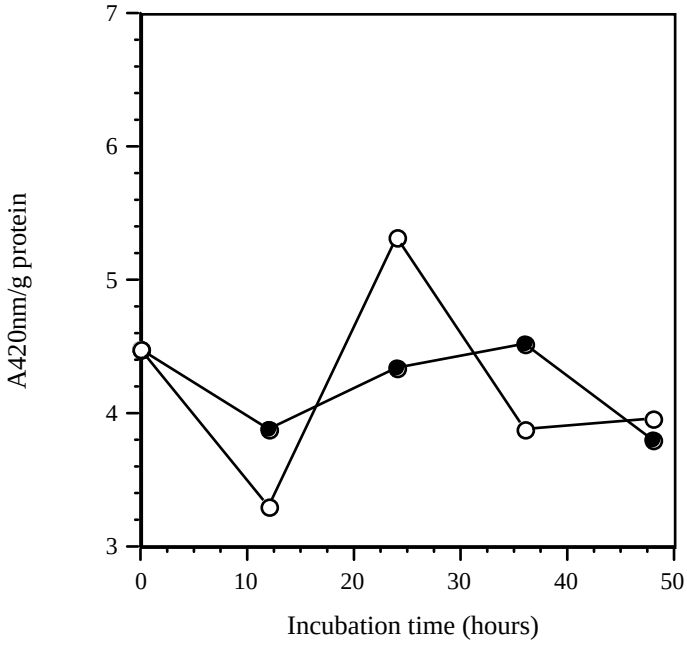


Fig. 25C.

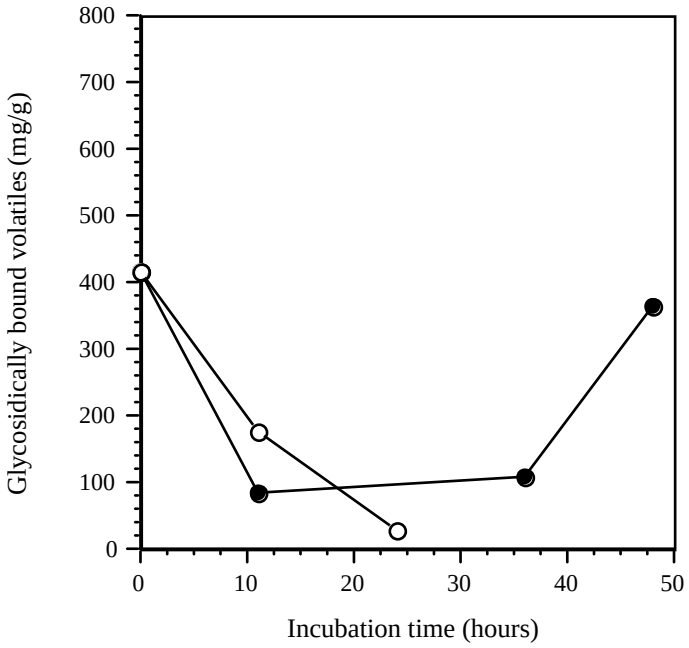
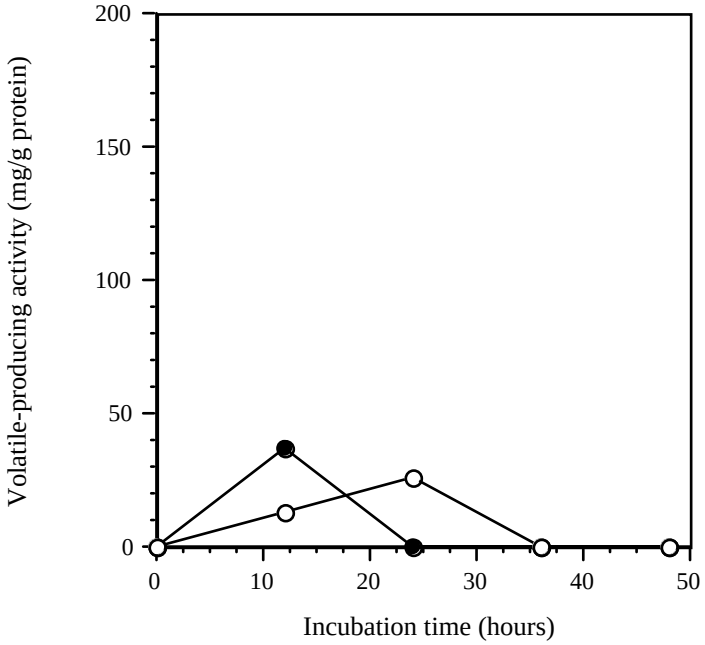


Fig. 25D.



In clone #5 flowers, the concentration of protein increased between 24 and 36 hours at 12°C and after 24 hours at 30°C; protein concentration decreased subsequently at 30°C (Fig. 26A); maximum change was 11%. Glycosidase activity/g protein increased rapidly during the first 12 hours at 30°C, subsequently declined over the next 24 hours and increased again to levels similar to those at harvest; maximum change was 27%. The concentration of glycosidically bound volatiles declined during the first 12 hours at 12°C; at 30°C there was a subsequent increase between 12 and 36 hours followed by an equally rapid decline at stabilisation at levels similar to those at harvest (Fig. 26C), maximum change was 140%. The activity of volatile-producing enzymes increased steadily throughout 24 hours at 30°C, subsequently declining to zero after 36 hours; at 12°C the increase was delayed until between 24 and 36 hours, also subsequently declining (Fig. 26D); maximum change was 225%.

Fig. 26. Clone #5 flowers (79% open flowers) incubated at 12 ( ) and 30°C ( ) for up to 48 hours after harvest. A = Concentration of protein (g/g). B = Proportion of protein comprising glycosidase hydrolytic activity (change in A420nm/g protein). C = Concentration of glycosidically bound volatiles (mg/g). D = Volatile-producing activity (mg total volatiles hydrolysed/g protein).

Fig. 26A

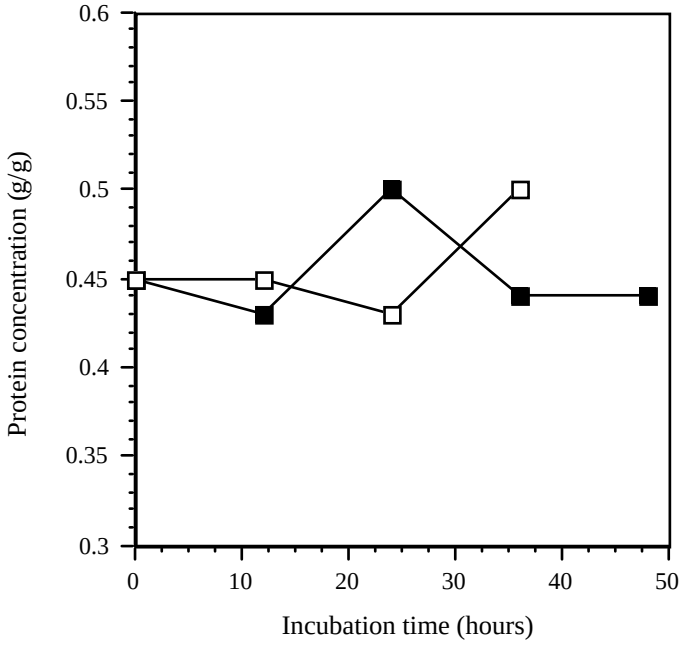


Fig. 26B.

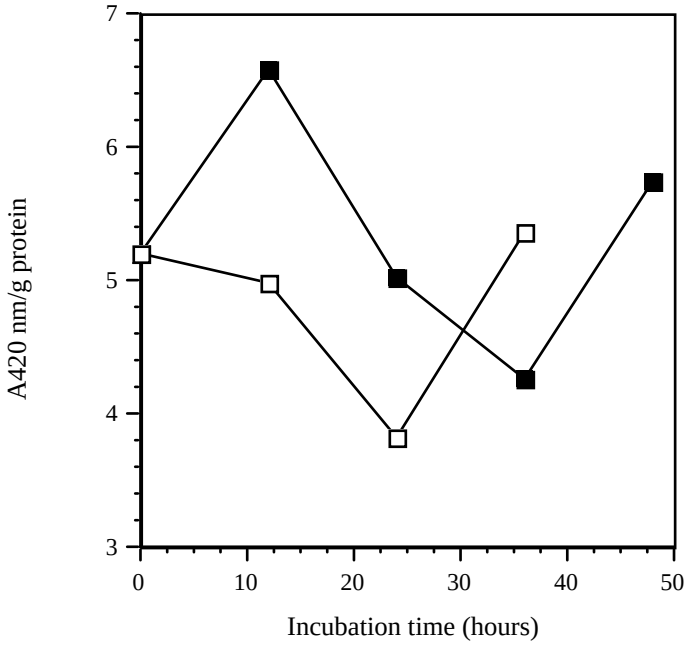


Fig. 26C.

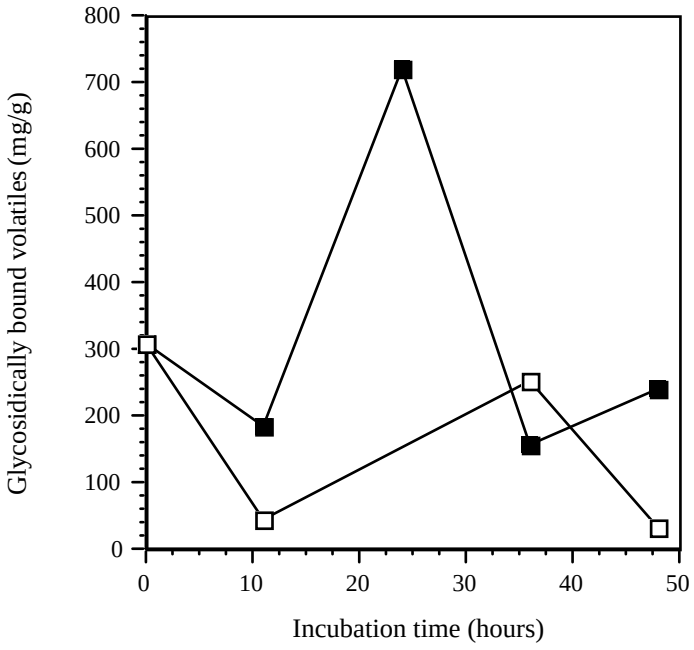
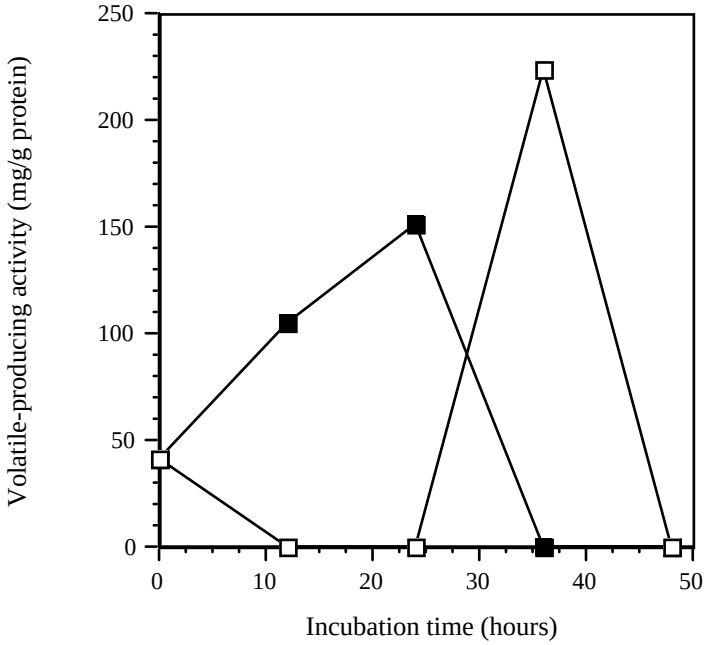


Fig. 26D.



In clone #17 flowers, protein concentrations at 12 and 30°C were similar, with a slight increase after 12 hours and subsequent maintenance at this level throughout incubation (Fig. 27A); an increase of 16%. The changes in glycosidase activity/g protein were relatively minor, with a tendency to decline at 30°C and a slight incline at 12°C, however limited data points are available (Fig. 27B); maximum change was 3.6%. The concentration of glycosidically volatiles declined between 12 and 48 hours at 12°C, and increased slightly between 24 and 48 hours at 30°C (Fig. 27C). There was no change in the activity of volatile-producing enzymes at 12°C and an increase after 12 hours at 30°C (Fig. 27D); maximum increase was 114%.

Fig. 26. Clone #17 flowers (74% open flowers) incubated at 12 (△) and 30°C (▲) for up to 48 hours after harvest. A = Concentration of protein (g/g). B = Proportion of protein

comprising glycosidase hydrolytic activity (change in A420nm/g protein). C = Concentration of glycosidically bound volatiles (mg/g). D = Volatile-producing activity (mg total volatiles hydrolysed/g protein).

Fig. 26A.

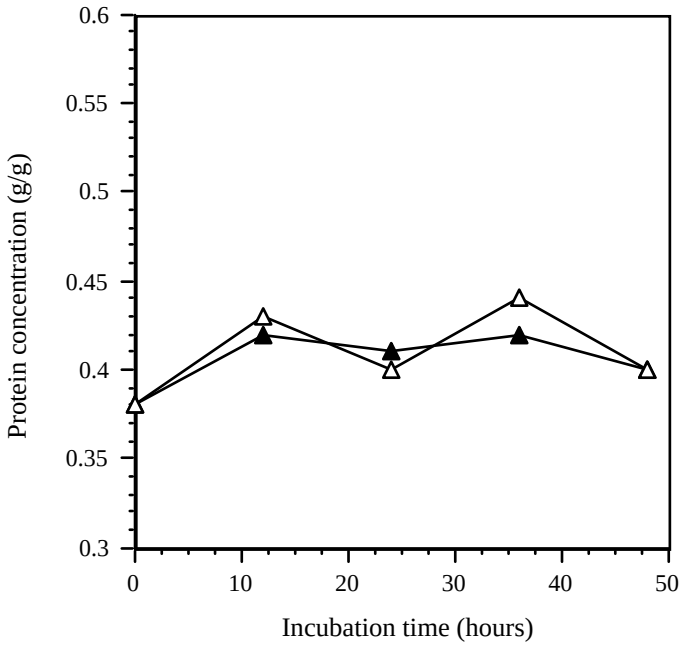


Fig. 26B.

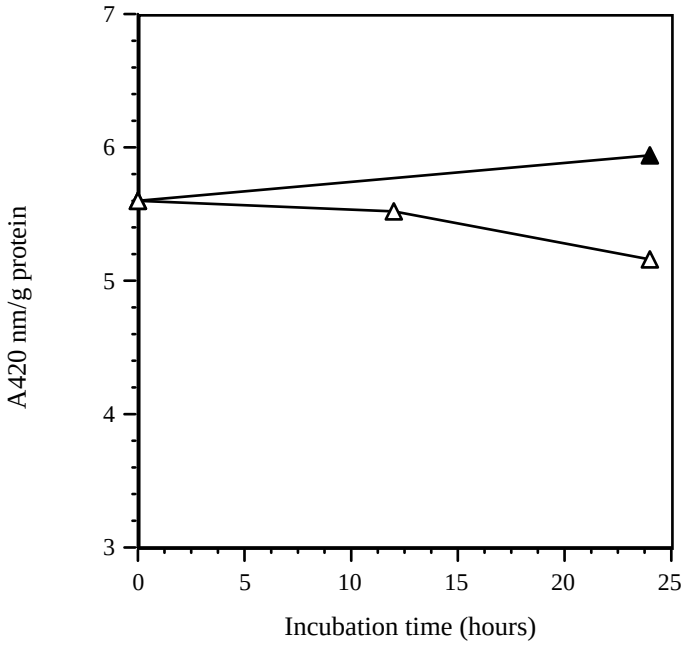


Fig. 26C.

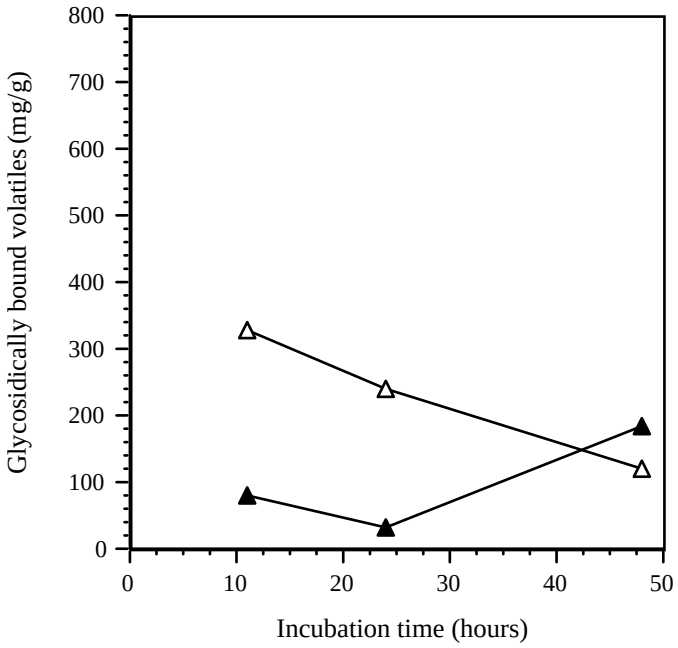
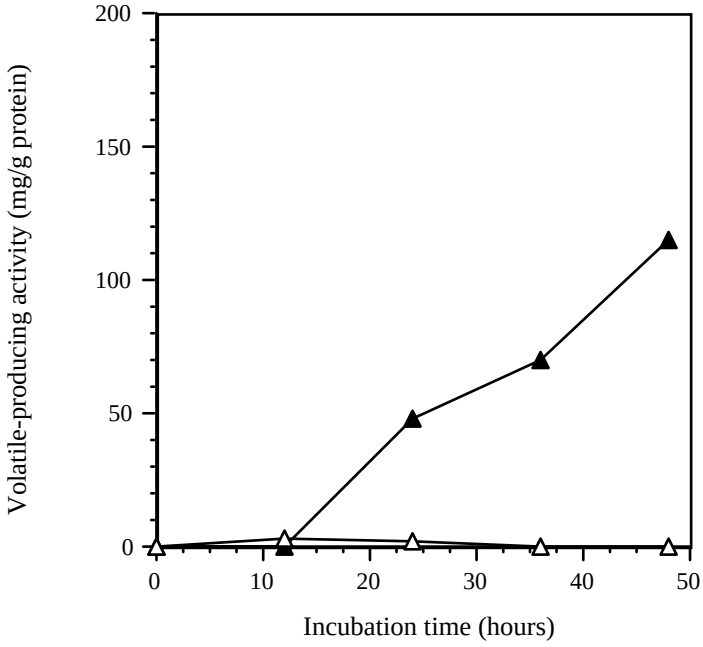


Fig. 26D.



In clone #250 flowers the concentration of protein varied only slightly during incubation at either temperature (Fig. 27A); maximum change was 8.3%. The activity of glycosidase enzymes was variable, but only exceeded harvest levels after 48 hours at 12°C (Fig. 27B); maximum increase was 9.4%. Limited data points are available for the concentration of glycosidically-bound volatiles, however a rapid decline was observed between 24 and 36 hours at 30°C (Fig. 27C). Volatile-producing activity increased at 12°C, especially after 36 hours, and subsequently declined rapidly (Fig. 27D); maximum change was 150%.

Fig. 27. Clone #250 flowers (77% open flowers) incubated at 12 ( ) and 30°C ( ) for up to 48 hours after harvest. A = Concentration of protein (g/g). B = Proportion of protein comprising glycosidase hydrolytic activity (change in A420nm/g protein). C = Concentration of

glycosidically bound volatiles (mg/g). D = Volatile-producing activity (mg total volatiles hydrolysed/g protein).

Fig. 27A.

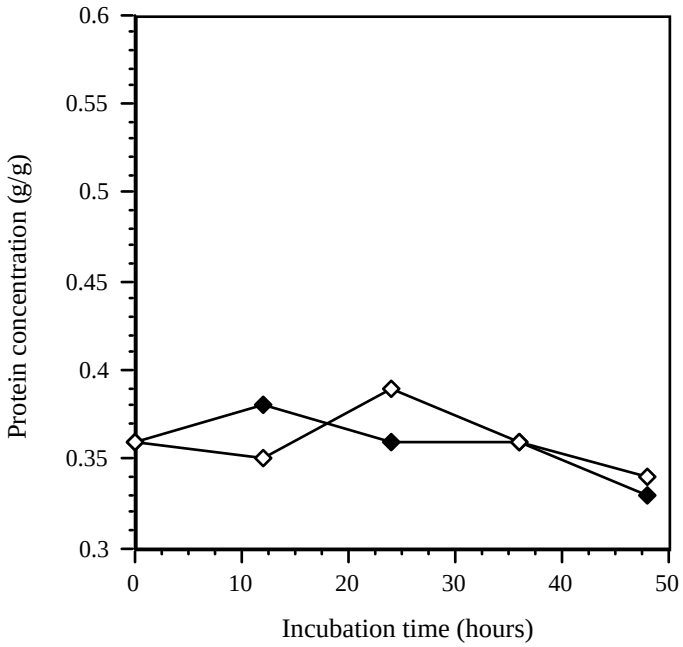


Fig. 27B.

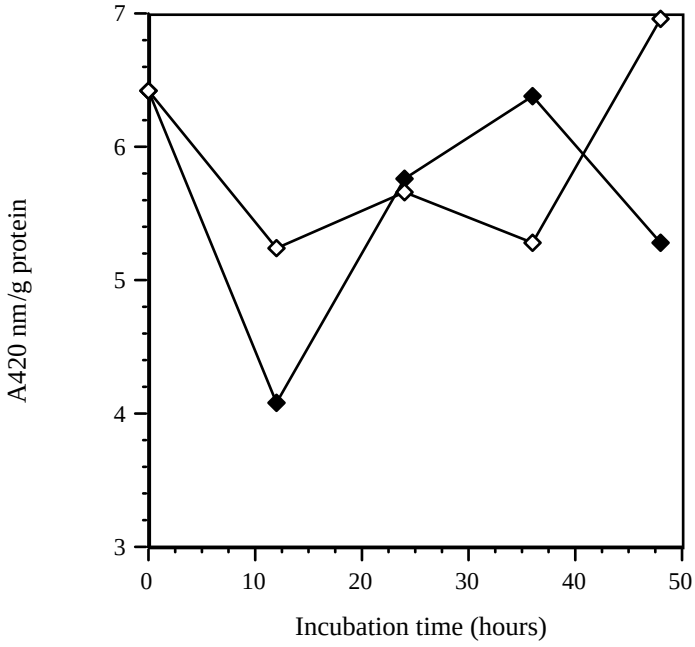


Fig. 27C.

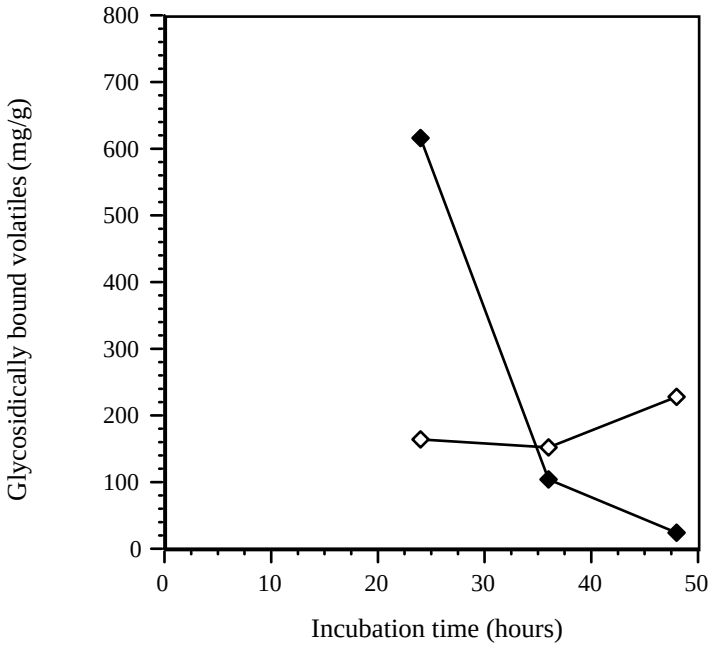
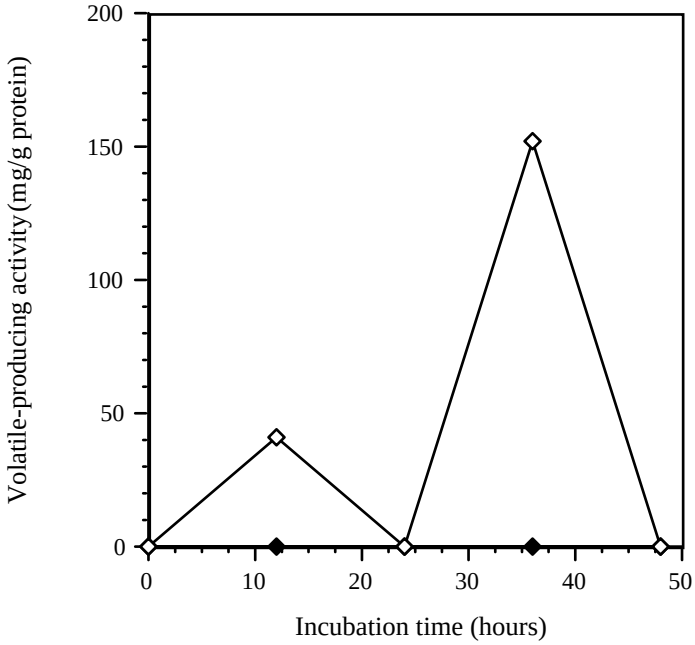


Fig. 27D.



As a result of changes taking place during post-harvest incubations, the maximum increase and final concentrations of protein (g/g) were observed in clone #3 flowers; the maximum glycosidase activity/g protein was observed in clone #250 flowers, the maximum change in clone #5 flowers; the maximum concentration of free volatiles in floral extract, and the maximum change and level of activity of volatile-producing enzymes and concentration of glycosidically-bound volatiles were observed in clone #5.

If we consider, as an example, the observed changes in clone #5 flowers harvested at 79% open flowers and incubated at 12°C for 24 hours, there is an additional 90 mg of total volatiles comprising 50 mg β–ionone per 100 g flowers retrieved via the pet. ether extraction process as a result of post-harvest incubation. There is a 265 mg per 100 g increase in free volatiles as a result of incubation of

aqueous extracts made from the same flowers upon incubation with β–glucosidase. The quantity of volatiles bound in non-glycosidic linkages was not assessed successfully, yet we must assume from other work that such bound forms exist given the greater increase in volatiles from incubation of aqueous extracts with boronia enzymes compared with β–glucosidase. This indicates that hydrolysis of glycosidically bound volatiles alone is sufficient to explain the production of additional volatiles in floral tissues: the excess of volatiles hydrolysed from glycosides and other moieties presumably being either metabolised in the flower, lost through emission or are unavailable to, or insoluble in, pet. ether.

3.6 Extraction

Flower weight: Solvent Volume Ratio & Extraction Time - Commercial Extractions:

Methodology: Three commercial-scale extractions of clone #5 flowers done at Essential Oils of Tasmania Ltd. allow comparison of different extraction times and washing regimens; four washes were used for each extraction. The flower weight: solvent volume ratios, extraction time per wash and resultant extract concentration and composition are presented in Table 8.

Table 8. Extraction protocol and resultant floral extract from three different commercial-scale extractions.

Protocol for extraction	Conc. of extract (% fr. wt.)	Total volatiles (% fr. wt.)	β–Ionone (% fr. wt.)	Organoleptic assessment
A) 1:7 (kg:L) 9 hours	0.470	0.049	0.013	Very floral, strong, ranked first. Taste: floral, passionfruit, tannin after-taste.
B) 1:6 (kg:L) 8 hours	0.485	0.052	0.014	Floral, not as strong as 9 hr. Taste: tannin stronger than 9 hr extract.
C) 1:4.5 (kg:L) 7 hours	0.410	0.048	0.012	Floral, violet odour. Taste: less strong than other two, tannin very strong.

Results: Extraction B (8 hours, 1:6 kg:L) produces the most desirable yield of extract including higher levels of total volatiles and β–ionone. Organoleptic assessment ranked the extract from A, the 9 hour, 1:7 (kg:L) extraction as the most desirable in terms of strength of bouquet, floral notes and pleasant taste. Although extract from C, the 7 hour, 1:4.5 (kg:L) extraction, had more strength to it than B which was ranked second, C also had a bitter after-taste with strong tannin over tones making it the least desirable organoleptically.

Rolling Flowers Prior to Extraction:

Methodology: Clone #5 flowers were passed through the blackcurrant bud roller at Essential Oils of Tasmania Ltd. whilst frozen; flowers were rolled to a thickness of 2.5-3 mm, sufficient to squash the stigmas. Pilot scale extractions of rolled and un-rolled flowers (18 kg/batch) were done in triplicate, the extracts were analysed by GC and organoleptically.

Results: There was a 90% in extract (% fr. wt.) from flowers which were rolled prior to extraction compared with whole flowers (Table 9). This incorporated a 48% increase in total volatiles and a 23% increase in β–ionone (% fr. wt.). In addition, the conversion of extract into absolute was improved by rolling the flowers: 127% more absolute (% fr. wt.) compared with whole flowers, indicating increased extraction of polar compounds.

Table 9. Concentration of floral extract, volatiles and absolute from flowers that were rolled prior to extraction compared with un-rolled (whole) flowers.

Component	Flower treatment	
	Whole flowers	Rolled flowers
Concentration of extract (% fr. wt.)	0.5076 +/- 0.0205	***** 0.9641 +/- 0.0309
Concentration of total volatiles (% fr. wt.)	0.0875	0.129
Concentration of β–ionone (% fr. wt.)	0.0185	0.227
Concentration of absolute (% fr. wt.)	0.373	0.846

The proportion of the extract or absolute made up of volatile compounds is reduced in products from flowers which were rolled compared with whole flowers, however the actual yield of these compounds (% fr. wt.) increases because of the large increases in extract yield. Thus the product from flowers which were rolled is actually a different product to that produced from whole flowers. Extracts of flowers which were rolled prior to extraction were dark coloured and disagreeable organoleptic properties. By using less rigorous rolling procedures and with further work on the purification and fractionation of the products from rolled flowers, we hope to improve these high yielding products and demonstrate the potential for product diversity from boronia flowers.

4. Discussion

Objectives Pre-Harvest:

To examine patterns in the emission of volatiles from boronia flowers in order to quantify rates of production of volatiles or conversion of precursors into free volatiles under different lighting conditions.

Rapid changes in emission of volatiles are observed as a result of changes in light conditions. For many volatiles, cycles of approximately 24 hours are apparent in emission levels, continuation of which under constant temperature conditions varies. Emission of several volatiles was completely inhibited by the presence of light. Emission of some distinctly floral compounds appears to be under circadian control in that emission patterns do not differ under different light patterns, eg. dodecyl acetate. Emission of other volatiles, β–ionone for example, varies depending on the light environment. The demonstration of how rapidly the boronia plant detects changes in the external environment and how these changes manifest as changes in pools of secondary metabolites, particulalry floral volatiles is significant. Changes in emission generally follow or manifest in changes in the concentrations of volatiles in tissues, although in this particular study it is difficult to explain the reduced concentration of volatiles in, and emitted from the plant that underwent alternating light and dark periods relative to the plants in constant environments. Perhaps reduction in emissions results in short-term (ie. seconds, minutes) accumulation of extract, and the plant regulates itself by reducing production of volatiles in the longer (ie. hourly) term.

Objectives Precursors to Volatiles:

To establish methods for the analysis of precursors to floral volatiles and the activity of enzymes that convert them into free volatiles; including glycosidically bound compounds and other bound forms.

A clear separation between enzyme fractions active in hydrolysis of glycosidic conjugates and those active in production of free volatiles from miscellaneous precursors was achieved using ammonium sulphate as a preliminary step. Ammonium sulphate fractionation of boronia enzymes demonstrates that hydrolytic enzymes other than glycosidases are present, as would be expected. It is apparent also that a large proportion of volatiles are bound in forms that are hydrolysed by unspecified enzymes in boronia flowers, perhaps even a larger pool than that comprising glycosidically bound volatiles.

β-ionone is often released from aqueous extracts after treatment with β–glucosidase, and sometimes not after treatment with boronia enzymes, suggesting that β–ionone is bound as a glycoside, and yet how does this occur since β–ionone lacks an alcohol group? One possibility is that β–ionol is bound as a glycoside, converting to β–ionone after hydrolysis of the sugar moiety, however we have no information to confirm this as yet. The fact that β–ionone production, or at least emission, is not regulated by circadian rhythms suggests that biosynthesis may be via carotenoid degradation, producing a plethora of ionone related compounds, glycosylated for further metabolism or transportation from the plastid and eventually the flower prior to abscission.

Objectives Phenology:

To produce a standard method for sampling and analysing flower material to identify the flower maturity index. To identify the optimum time to harvest each clone in order to maximise the concentration and composition of floral oil and volatiles including β–ionone at harvest. To assess the change in concentrations of free and bound volatiles and volatile-producing enzymes throughout flowering.

Newly opened flowers tend to have higher concentrations of volatiles than flowers that have been open for 5-6 days (MacTavish, 1995). The concentration of volatile compounds have been shown to decline after full bloom in rose, ie. between 9 and 10.5 days after calyx splitting (Francis and Allcock,

1969). A net decrease in monoterpene content in late stages of development as a result of catabolism is well documented (Croteau, 1987). In the present study, depletion of volatiles in boronia flowers in late flowering was not apparent, perhaps due to an insufficiency of samples in very late flowering, ie. >95% open flowers. The samples of flower material extracted in this study contained flowers that had been open for different periods of time, designated as 'open flowers' only because of their separation from buds due to size differences. This may be another reason why the concentration of volatiles was maintained in late flowering in this study.

In general, clones #3 and #5 had a greater concentration of extract compared with clones #17 and #250. This suggests that there is genetic control of the concentration of extract accumulated by particular plants. This may also be related to the observation that clones #3 and #5 maintained dry flower weight after 80% of flower opening: perhaps catabolic enzymes are on the whole more active in clones #17 and #250. It is also possible that the clones differ in the 'extractability' of extract. A large proportion of floral extract is contained within the stigma and extraction of the stigmatic contents is best achieved by some form of tissue disruption (MacTavish, 1995). Clones with particularly large stigmas may not fully release the extract present without tissue disruption (MacTavish and Menary, 1998a), a process not used in this particular study.
It appears from this research that all four clones are best harvested at one time. Clones #3 and #5 progress through flowering earlier and the % dry weight is maintained longer than in clones #17 and #250. This latter factor maintains extract yield/plant in late stages of flowering despite a decline in the concentration of extract at this time in clones #3 and #5. The organoleptic quality of floral extracts from all four clones vary, however extract from clones #3 and #5 is most desirable in late stages of flowering, and from clones #17 and #250, at earlier stages.

It has been observed that a large proportion of so-called ‘precursors’ to volatiles are not bound as glycosides, and yet major compounds such as β–ionone are converted from precursor into free volatile, specifically by β–glucosidase. The concentration of glycosidically-bound volatiles increases between 60 and 84% open flowers, at a time when the concentration of free volatiles in floral extract is also increasing. However, the concentration of non-glycosidically bound volatiles generally decreases throughout flowering, with a slight increase in very late flowering (>85% open flowers). Protein content of flowers also decreases during flowering with a small increase and subsequent decrease again between 84 and 90% open flowers; glycosidase activity as a proportion of protein content in flowers increases in early flowering then follows the same trend as bulk protein after 40% open flowers. From these results it appears that the pool of volatiles bound in forms other than glycosides may represent ‘real’ precursors, in that the flowers actually metabolise these compounds into volatiles throughout flowering. The increase in glycosidically bound volatiles throughout flowering may result from a decline in glycosidase activity, but it also indicates that such forms may represent a step in the catabolism of non-emitted volatiles and/or enabling transportation from the flower prior to floral abscission. As such, glycosidically bound volatiles may be converted into free volatiles *in vitro* but may not represent ‘real’ precursors *in vivo*, in fact, they may be function in quite the opposite way.

Objectives Post-Harvest:

To identify the optimum conditions in terms of clonal type, harvest time, temperature and atmospheric conditions of incubation and the duration of incubation under which boronia flowers produce increased floral extract and volatiles after harvest. To assess the viability of commercial-scale incubations. To assess the change in concentrations of free and bound volatiles and volatile-producing enzymes during post-harvest incubation.

The role of glycosidically bound volatiles in producing post-harvest increases in volatiles may not be so clear cut, since excision from the plant may alter normal metabolic functions. If not, one would expect free volatiles in excised flowers to decline as glycosylation or metabolism reduces the pool of volatiles available for extraction by an organic solvent. It is likely then, that post-harvest changes should not be seen as enhanced senescence, but as unregulated and perhaps aberrant activity.

Exogenously supplied chemicals were applied to boronia flowers after excision to block different parts of the normal respiratory pathway in plants: a) complex IV or earlier; b) the alternative oxidase system which is activated upon inhibition of complex IV (Wagner and Krab, 1995), as well as c) the enzymes that hydrolyse precursors into free volatiles. The technique used for infiltrating the floral tissues was variable, producing pigmentation changes where tissues were saturated with solution. Solutions entered the free space between cells and then were directly adsorbed through the plasma membrane into cells. The actual concentration reaching the mitochondria was probably lower than that stated which may explain the lack of response at 100 μM NaN_3 (Lynnes and Weger, 1996). In analysing this experiment, we are making an assumption that activation of the alternative oxidase system changes the profile of oxygen consumption.

Thus, in cases where oxygen consumption was slightly greater than in the control, especially in later stages of incubation, this may result from the activity of the alternative oxidase pathway (Lance *et al*., 1985). Alternatively, the chemicals may not have reached the mitochondria and the variation in oxygen consumption may be artefactual. If the former is the case, low concentrations of DNP and high concentrations of NaN_3 and KCN inhibit cytochrome oxidase and activate the alternative oxidase system. High concentrations of DNP inhibit cytochrome oxidase and the alternative pathway, as previously demonstrated by Larhrissi *et al*., (1994), as does the presence of nitrogen gas, which presumably limits respiration due to lack of oxygen. In both these treatments, there is no increase in volatiles, indicating a requirement for energy in the production of volatiles after harvest. Production of volatiles is also inhibited with high KCN and NaN_3, but not with low concentrations of DNP. It seems likely that KCN and NaN_3 also interfere with enzymes required for the production of volatiles after harvest. The alternative pathway does not produce ATP (Lance *et al*., 1985), however this does not seem to inhibit production of volatiles: low concentrations of DNP probably activated the alternative system and yet production of volatiles also occurred. ATP is required for *de novo* biosynthesis of volatiles (Rogers *et al.*, 1967), however it does not appear to be required for hydrolysis of precursors into free volatiles (Günata *et al.*, 1990).

During post-harvest incubation of boronia flowers, an increase in the concentration of floral extract may occur prior to an increase in free volatiles (Fig. 18), may occur to a reduced extent compared with the increase in free volatiles (Figs. 19B, C), or may occur to a greater extent than the change in free volatiles (Figs. 19C, 20C). There appears to be a maximum level of floral extract and volatiles that can be accumulated by a whole flower, evidenced by the decline in extract after 80% open flowers. The *in vivo* equilibrium position may be set by the number and volume of storage sites available for free volatiles in plastids, by enzyme kinetics and by supply of substrates. The decline in the concentration of extract and volatiles in late flowering and after prolonged post-harvest incubation may occur by the action of senescence-related processes which become more significant after anthesis, such as oxidation, loss of volatiles through emission, and formation of non volatile moieties such as glycosides, compounds which may be transported from the flower or may be the preliminary step in the catabolism of volatiles (Croteau and Martinkus, 1979; Ackermann *et al*., 1989). In early stages of post-harvest incubation, saturation of the headspace in enclosed bags of flowers by floral volatiles may prevent further emission and loss of volatiles. Reduced emission of volatiles may explain part of the observed increases in volatiles, the subsequent depletion being caused by oxidative and other processes. Unregulated processes, and the reduced capacity for transportation of glycosylated compounds in harvested flowers may allow accumulation of free volatiles to levels greater than that possible in flowers *in vivo*.

Volatiles may be produced after harvest at the cost of, or via metabolism of other components of the extract, or by *de novo* synthesis. There are three fractions present in boronia extract: volatiles, arbitrarily defined as those compounds eluting from the column before n-heneicosane (Davies and Menary, 1983), non volatiles including hydrocarbons identified by Davies and Menary (1983) and cinnamate terpene esters, esters of hydroxy linalool, a tiglamide and α– and β–amyrin identified by Weyerstahl *et al*. (1994), and the fraction which is not amenable to GCMS. Recent unpublished work has identified compounds in this latter portion of the extract including long chain fatty acid esters (eg palmitate, stearate) of α– and β–amyrin and related triterpenes, as well as triglycerides (Davies pers.

comm.). Also present in boronia extract are carotenoid pigments such as α– and β–carotene, lutein, violaxanthin and neoxanthin (MacTavish, 1995). Many of these compounds represent potential precursors for compounds found in the volatile fraction, for example β–carotene or other carotenoids with cyclic end groups are potential precursors for β–ionone and other norisoprenoids (Enzell, 1985; Croteau and Karp, 1991).

Increases in free volatiles in boronia flowers after harvest occur only in fresh, undamaged open flowers, which suggests that enzymic activity brings about the changes. The greatest increases occur in flowers which have the potential for continued respiration: unhampered by extremes of temperature or high concentrations of nitrogen gas, and without physical disruption caused by freezing and thawing. The limit to post-harvest production of volatiles may be the supply of precursors or cofactors within the flower at harvest, the activity of enzymes, the toxicity of products, or changed conditions such as cell pH. A limit to available photosynthate or starch for respiratory processes may limit production of volatiles after harvest (Loomis and Croteau, 1973).

The quantity of glycosidically-bound volatiles hydrolysed during post-harvest incubations is more than sufficient to explain the production of additional free volatiles in floral extract. Thus simultaneous hydrolysis or conversion of volatiles that are bound in other forms together provide an excess of free volatiles in terms of the amount actually retrieved via solvent extraction. Excess free volatiles may be emitted or catabolised by the flower. Other work (MacTavish, 1995) has shown an increase in the concentration of glycosidically-bound volatiles after extended incubations after harvest (> 60 hours for example). This is in line with previous suggestions that glycosides represent the first step in catabolism of volatiles, and thus production of these compounds is a normal part of flower senescence, and perhaps an unregulated part of flower death after excision.

Increases in the concentration of extract including total volatiles and β-ionone as a result of post-harvest incubation are reproducible phenomena, occurring throughout flowering, at different incubation temperatures and at different rates depending on the clonal type. The experiment examining the effect of temperature of incubation on clone #5 (82% open flowers), in particular at 12°C, resulted in a 10% increase in floral extract and a 350% increase in floral volatiles. However, in the study examining the four clones at different temperatures, clone #5 (79% open flowers) incubated at 12°C resulted in 16% more extract including 25% more floral volatiles. The major differences between the two experiments, both using clone #5 flowers from one site but over two successive seasons, are the higher initial concentration of total volatiles in the second study (0.375% compared with 0.175%) and the ten-fold greater increase in volatiles during incubation in the first study. The initial difference may be brought about by different climatic conditions prior to harvest, or to the effect of one year's further growth on the habit or biosynthetic ability of the plants, events which may have caused increased emission of volatiles prior to harvest in the first study. The vast difference in post-harvest production of volatiles may be brought about by widely differing concentrations of precursors to free volatiles in flowers at harvest, or variations in incubation conditions between the two experiments in ways that were not measured, such as availability of oxygen.

From the differences between the two experiments using clone #5 flowers we can conclude that either the flower material or the incubation conditions in the second experiment may not have been optimal. Subsequently, increases actually observed as a result of on-farm incubation or upon repetition of the experiment with all four clones may be more significant. Also evident is that a low initial concentration of volatiles resulted in a higher final concentration of volatiles, ie. after incubation. Free volatiles may be released from bound forms by enzymatic hydrolysis both pre- and post-harvest. If so, a low concentration of free volatiles may indicate either that the concentration of bound volatiles is high or that overall, stores of both free and bound volatiles are reduced. Given the rapid increase in free volatiles within 12-24 hours, particularly in clone #5, the former is more likely. Other research has shown that the concentration of glycosidically bound volatiles declines during the first 12-16 hours of incubation (MacTavish, 1995).

There are large differences between the 4 clones in the increases in floral extract and total volatiles after harvest when calculated as a % of dry flower weight. For example in clones #3 and #17, the increase in floral extract is relatively small and is probably brought about entirely by the relatively large increase in total volatiles. In clone #17, the increase in volatiles more than explains the increase

in extract, indicating that non-volatile and other components of the extract have declined concomitantly. In clones #5 and #250, the increase in floral extract as a % of dry weight is greater than the increase in volatiles, indicating either that other components of the extract have also increased, or that they were extracted more easily. The former appears to be more likely, since the dry weights changed very little during incubation, although other processes occurring during incubation, both enzymic and oxidative, may enable better penetration of solvent into the floral tissues during extraction (see MacTavish and Menary, 1998b). The increases in floral extract and total volatiles as a result of the larger scale incubations were of similar magnitude to those observed in the pilot-scale, clonal study.

Objectives Extraction:

To optimise the efficiency of extraction by considering the effect of tissue disruption on a commercial scale and by examining different flower weight : solvent volume ratios in the commercial extractor.

The commercial scale extractions using different solvent volumes and extraction times illustrate the increased extraction of floral volatiles through the use of excess solvent. Although the median extraction in terms of time and solvent volume was the most efficient at recovering floral extract, the comparative reduction in organoleptic properties of this extract compared with the extraction with longer time and greater solvent volumes must be taken into account. There is significant potential for the production of floral boronia products with different oganoleptic properties to the standard extract through the manipulation of extraction conditions, and also through pre-treatment of boronia flowers. Rolling the flowers almost doubled recovery of floral extract, albeit with inferior organoleptic qualities. Fractionation and purification of this product, or of a product of less rigorous rolling procedures could obtain fractions high in β–ionone or jasmonates, or even bereft of pigments: ie a product more suited to the fragrance market.

Overall Objectives:

To produce boronia concrete at higher yields and with improved quality by developing pre-harvest, harvesting, and post-harvest management practices and by value-adding the existing product.

Optimisation of harvest time and post-harvest incubation conditions is now possible for each clone due to information about the changes in floral extract throughout flowering and after harvest.

5. Recommendations and Implications

Harvest timing:

The most appropriate point for harvest in terms of % open flowers has been established for each clone, to optimise the concentration of extract and volatiles and organoleptic quality of the extract. This will enable harvest planning for each site and statewide, and optimal returns to growers if paid on oil yield or quality.

Post-Harvest:

Post-harvest increases in floral extract and volatiles are reproducible and have the potential to produce 20-25% more extract and 30-300% more volatiles from present flower material. Clones vary in their ability to produce volatiles after harvest, and the effect of harvest timing is pronounced. Flowers must be respiring actively and thermal control is critical. Uncontrolled incubations, for example in open boxes of flowers, often lead to enhanced temperatures and oxygen depletion which not only reduce post-harvest activity but can reduce volatiles and delay freezing such that flower material begins to decay before frozen. For on-farm incubations, specific incubation procedures are required; incubation in either open or closed boxes is largely ineffective.

Extraction:

There is significant potential for the production of floral boronia products with different oganoleptic properties to the standard extract through the manipulation of extraction conditions such as time and solvent volume. Rolling flowers prior to extraction enhances floral extract by 75-100%; fractionation and purification of the product from rolled flowers could result in fractions high in β–ionone or jasmonates, or even bereft of pigments: ie a product more suited to the fragrance market. This has implications for market diversity from existing plantations.

Pre-Harvest:

Regulation of the pool of volatiles and precursors in boronia flowers responds rapidly to changes in the external environment. There are significant differences in the emission of volatiles in the light and dark conditions, however in the preliminary study these did not manifest as differences in tissue concentrations and therefore a change in the harvesting schedule (ie. from day to night time) is not suggested. This information is important for future research on pre-harvest application of biosynthetic effectors of floral volatiles.

Clonal Selection:

The importance of genetic control of phenology, extract concentration and quality and potential for post-harvest production of volatiles has been demonstrated. Inclusion of these factors in clonal selection programmes should continue.

6. References

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