Carbohydrate Composition and Structure Changes as Phloem Sap is Converted to Nectar in *Borago officinalis* L. and Select *Brassica* spp. L.

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ABSTRACT

Nectar is a carbohydrate-rich solution produced by nectary organs as a reward to pollinators and animal mutualists. Nectar production involves the upload of carbohydrate (i.e., sucrose) from the phloem sap, intracellular (symplastic) and/or intercellular (apoplastic) transport of carbohydrates into the nectary, and secretion of carbohydrates to the nectary exterior as nectar.

To investigate carbohydrate composition and structure changes during nectar production, the carbohydrate composition of phloem sap, nectary fluid, and nectar of *Borago officinalis* L. and two *Brassica* spp. L. (*Brassica napus* L. var. *AC Excel*, *B. napus* L. transgenic var. *AV 225 R. R.*, and *B. rapa* L. var. *AC Parkland*) were determined employing high performance anion exchange-pulsed amperometric detection (HPAE-PAD) and capillary gas chromatography-flame ionization detection (CGC-FID) chromatographic methods. To elucidate the mechanism(s) of carbohydrate transformation during nectar production, substrate hydrolysis experiments were conducted on both nectaries and nectar, and nectary proteomics analysis was also employed.

Carbohydrate composition results showed that: a) sucrose (S; >95% w:v) was present in the phloem sap of both genera; b) fructose (F; >50%), glucose (G; ~45%), and non-sucrose oligosaccharides were present in the nectary fluids of *B. officinalis* and *Brassica* spp., indicating that sucrose hydrolysis and carbohydrate synthesis occurred; c) F, G, S, and non-sucrose oligosaccharides were detected in the nectars of both genera with significant concentration differences; d) *B. officinalis* nectar was sucrose-dominant (S; 61%), whereas *Brassica* spp. nectars were hexose-dominant (average, F + G; 99%) indicating that sucrose was resynthesized in *B. officinalis*; and e) common non-sucrose oligosaccharides were detected in *B. officinalis* and *Brassica* spp. nectars and unique non-sucrose oligosaccharides were detected in both genera.

The observed hydrolysis of sucrose and the synthesis of non-sucrose oligosaccharides in the nectaries and nectars of *B. officinalis* and *Brassica* spp. can be explained by the presence of carbohydrases (α-glucosidase, β-fructosidase β-glucosidase) and synthases (sucrose synthase, sucrose phosphate synthase) as confirmed by select substrate and proteomics experiments. The significant difference in the sucrose concentration of the floral nectar of *B. officinalis* is attributed to sucrose phosphate synthase activity in *B. officinalis* when compared to *Brassica* spp., and by the type of carbohydrate transport pathway (symplast vs apoplast) followed.

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LIST OF ABBREVIATIONS

1D: One-dimensional

2D: Two-dimensional

ANOVA: Analysis of variance

BLAST: Basic local alignment search tool

BSA: Bovine serum albumin

CGC-FID: Capillary gas chromatography-flame ionization detection

ELSD: Evaporative light scattering detector

ESI: Electrospray ionization

FT: Fourier transform

HPAE-PAD: High performance anion exchange chromatography-pulsed amperometric detection

HPLC: High performance liquid chromatography

IPI: International Protein Index

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LED: Light emitting diode

MALDI: Matrix-assisted laser desorption/ionization

MS: Mass spectrometry

NCBI: National Centre for Biotechnology Information

PC: Paper chromatography

PDB: Protein Data Bank

PIR: Protein Information Resource

PNPG: p-nitrophenyl-α-D-glucopyranoside

PRF: Protein Research Foundation

QIT: Quadrupole ion trap

QTOF: Quadrupole time-of-flight

RI: Refractive index

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TCA: Trichloroacetic acid

TFA: Trifluoroacetic acid

TLC: Thin layer chromatography

TMSI: N-trimethylsilylimidazole

TOF: Time-of-flight

UniProtKB: UniProt Knowledgebase

1. INTRODUCTION

Nectars are sweet aqueous solutions that are secreted by plant organs called nectaries in order to attract and reward animal mutualists (Terrab et al., 2007). These mutualists include pollinators like insects, birds, and bats that are attracted to floral nectar and defending arthropods like ants and parasitoids that are attracted to extrafloral nectar (González-Teuber & Heil, 2009). Active regulation of floral nectar production has been suggested to be an adaptive trait to improve reproduction success (Nicolson, 1995; Biernaskie et al., 2002) as animal pollination ensures directional, accurate, and efficient transfer of pollen when compared to wind pollination. Many angiosperms produce floral nectar to attract insect or vertebrate pollinators to achieve adequate fertilization and outcrossing (De la Barrera & Nobel, 2004; Bradenburg et al., 2009). As the chemical composition of nectar is adapted to pollinator type, studies on this composition are important to understand plant-pollinator interactions.

The chemical composition of nectar is dominated by three carbohydrates, the disaccharide sucrose and its component monosaccharides, glucose and fructose (Baker & Baker, 1983). In addition, nectar has been shown to contain trace amounts of a number of other compounds including, but not limited to: alkaloids (Baker & Baker, 1975; Adler et al., 2006); amino acids (Baker & Baker, 1975, 1983); antioxidants (Vitamin C; Bukatsch & Wildner, 1956; Baker & Baker, 1975); lipids/fatty acids (Vogel, 1971; Baker & Baker, 1975; Bernardello et al., 1999; Vesprini et al., 1999; Varassin et al., 2001); minerals (Hiebert & Calder, 1983; Varassin et al., 2001); odour compounds (Raguso, 2004a, b); phenolics (Baker, 1977; Forcone et al., 1997); and proteins (Carter & Thornburg 2004a, b, c; Carter et al., 2007; Kram et al., 2008; González-Teuber et al., 2009, 2010; Hillwig et al., 2010, 2011).

It is generally accepted that nectar carbohydrates are derived from photosynthesis in the nectary, and/or in other floral, and/or vegetative parts of the plant (e.g., pedicel, calyx, ovary, and adjacent leaves) that are generally close to the flower (Pacini et al., 2003; Pacini & Nepi, 2007; Heil, 2011; Lüttge, 2013). For carbohydrates from non-nectary sources, they can be translocated as sucrose in the phloem sap with subsequent biochemical/enzymatic conversion in the nectary

(Nicolson & Thornburg, 2007). A portion of the carbohydrates from the photosynthesis of nectary and non-nectary parts of the plant may also be stored as starch and may also be used for nectar carbohydrate production (Paiva & Martins, 2014).

Information on how carbohydrates are uploaded from the phloem to the nectary, how they are structurally altered or synthesized in this organ, and if further changes in carbohydrate composition and structure occur in the nectar, are still under investigation. Studies on the carbohydrate composition of phloem have shown: the presence of sucrose only (Riens et al., 1991; Winter et al., 1992; Giavalisco et al., 2006; Lohaus & Schwerdtfeger, 2014); the presence of monosaccharides such as glucose and fructose (Richardson et al., 1982; van Bel & Hess, 2008); and others have identified the presence of non-sucrose oligosaccharides such as maltose and raffinose (Haritatos et al., 1996, 2000; Lu et al., 2006). Literature reports on nectar carbohydrate composition have identified plant nectars as hexose dominant, hexose-rich, sucrose-rich, and sucrose dominant (Baker & Baker, 1983). When combined, these interesting literature reports generated the following scientific questions that this research program sets out to investigate: What is the carbohydrate composition of the phloem sap of *Borago officinalis* L. (*B. officinalis*) and two *Brassica* spp. L. (*B. napus* and *B. rapa*)?; Do changes in carbohydrate (sucrose) composition and structure occur as the carbohydrate moves into the nectaries of these plants, and if so, what mechanisms are responsible for these changes?; and do further changes in carbohydrate composition and structure occur in the nectar?

The central hypothesis of this research study was that changes in carbohydrate composition and structure occur as phloem sap is converted to nectar and that these changes are due to the presence of carbohydrases and synthases in the nectary.

To address this central hypothesis, two plant species differing in the major carbohydrate profiles of their nectar were selected for this research study. The nectar of *B. officinalis* is known to be sucrose-dominant with fructose and glucose also being present (Percival, 1961), whereas *Brassica* spp. nectars are hexose-dominant with very low sucrose levels (<1.0%) (Davis et al., 1994, 1998). It was hypothesized that these differences in the major carbohydrate profiles of the nectar for each plant species should provide mechanistic clues on how carbohydrate hydrolysis and formation occur as phloem sap moves into the nectary and becomes converted to nectar.

The selection of these two plant genera for this study was not only based on their differences in major nectar carbohydrate profiles but also on their economic importance to the

province of Saskatchewan. *Borago officinalis* L. is a herbaceous annual plant that is native to Europe, North America, and Asia Minor (Janick et al., 1989). It has been cultivated for centuries for culinary and medicinal purposes and has been grown for pollination, honey production, and as an ornamental plant (Fairbairn, 1994). *Borago officinalis* L. is well-adapted to the cooler and moist areas of Saskatchewan and approximately 13000 acres was planted in 2014 (Saskatchewan Ministry of Agriculture, 2014). *Borago officinalis* L. is a self-incompatible plant that requires pollinators (e.g., honey bees) for seed production. A minimum of two hives of honey bees per hectare (one per acre) is generally required to meet seed production requirements (Fairbairn, 1994; Foundations of Saskatchewan Agriculture, 2010).

Canola is officially defined as the "seeds of the genus *Brassica* (*B. napus*, *B. rapa*, or *B. juncea*) from which the oil shall contain less than 2% erucic acid in its fatty acid profile and the solid component shall contain less than 30 micromoles of any one or any mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate per gram of air-dry, oil-free solid" (Canola Council of Canada, 2005). *Brassica napus* L. is the important canola genus grown in Saskatchewan with 10.5 million acres planted in 2014 (Saskatchewan Ministry of Agriculture, 2014). *Brassica napus* L. is a self-pollinating crop but insect visitations confer benefits to the plant by increasing seed production (Morandin et al., 2006) or by producing heavier seeds with higher oil and lower chlorophyll contents (Bommarco et al., 2012) whereas *B. rapa* L. is a diploid, self-incompatible plant and requires cross-pollination for reproduction (Stewart, 2002). Being a well-known insect pollinator attractant, canola is also a good food source for honey bees and therefore, for honey production (Canola Council of Canada, 2005).

Based on the aforementioned research questions, the overarching goal of this research project was to elucidate the mechanisms responsible for carbohydrate composition and structure changes in the phloem, nectary fluid, and nectar of *B. officinalis* and select *Brassica* spp. The following three objectives were designed to meet this goal: (a) determine changes in carbohydrate composition and structure from phloem to nectar in *B. officinalis* and two *Brassica* spp. employing high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) and capillary gas chromatography with flame ionization detection (CGC-FID); (b) elucidate the mechanism(s) of carbohydrate composition and structure changes by determining nectary carbohydrase activities employing select carbohydrate substrates; and (c) identify nectary

carbohydrases and synthases that are most likely responsible for carbohydrate composition and structure changes via proteomics.

2. LITERATURE REVIEW

Nectar is a carbohydrate-rich solution secreted by plant organs called nectaries. Nectaries have been divided into floral and extrafloral, with the first occurring in flowers and the second on aerial, vegetative organs (Caspary, 1848, cited in Fahn, 1979). Floral nectaries reward animals that carry pollen for reproduction purposes whereas extrafloral nectaries provide energy reward for animals that defend plants against herbivores.

The reward of nectar enhances the probability of pollination by insects and/or other vertebrates so that visited plants can achieve adequate fertilization and outcrossing (De la Barrera & Nobel, 2004; Bradenburg et al., 2009). As an example, in canola, cross-pollination by the honey bee increases crop yield, and quality and oil content of the seed (Abrol & Shankar, 2012). In more than a hundred plant families, the presence of extrafloral nectar has served as an attractant to ants, parasitoids, and generalist predators that serve as an indirect defense against herbivores (Heil, 2008; Marazzi et al., 2013). Nectar can be ingested directly by flower visitors like birds and bats or it may be carried back to a hive/nest and used in the nourishment of larval stages of insects like the Hymenoptera (Baker & Baker, 1983). In the case of honey bees (*Apis* spp.), nectar is usually concentrated and biochemically altered to produce a carbohydrate-rich food called honey.

Early scientific literature on nectar reported that phloem was directly transported to nectar without chemical change (Agthe, 1951; Frey-Wyssling et al., 1954; Zimmermann, 1954; Lüttge, 1961; Fahn, 1988; De la Barrera & Nobel, 2004). However, it has been found that chemical composition differences between phloem and nectar exist, particularly in terms of hexose carbohydrates and nectar proteins (Escalante-Pèrez & Heil, 2012; Orona-Tamayo et al., 2013). These results clearly show that nectar/nectar production is not the simple transport of phloem sap but is a dynamic process involving the phloem and the nectary as a whole (Vesprini et al., 2012). This dynamic process involves a number of events, including carbohydrate unloading from the vascular bundle (phloem), the transport of molecules into the nectary organ, and chemical and enzymatic molecule transformations, which lead to nectar release (or exudation) from the nectary (Nepi, 2007).

The solution that is transported into the nectary organ for molecular transformation(s) is referred to as pre-nectar (Nepi, 2007). This material is processed in the nectary parenchyma cells.

2.1 Phloem Sap

Phloem is the conducting tissue associated with the transport of substances for nectar production (Vassilyev, 2005; Heil, 2011). Phloem is an extremely important vascular tissue in plants as it is responsible for the transportation of photosynthesis products (e.g., glucose) from source (e.g., leaves) to sink organs, with the nectaries being an important sink organ.

Carbohydrates are the main non-aqueous components of the phloem sap, with sucrose generally predominating. Reported sucrose concentrations in phloem sap range from 10 to 25% (w/v; w/w) depending on the plant species and the time of day the phloem sap was collected (Swanson, 1959; Kallarackalet al., 2012; Jensen et al., 2013). Select phloem sap sucrose concentrations include 340 mM in *Arabidopsis* (Deeken et al., 2002) and 1.8 M in *Solanum tuberosum* (potato) (Pescod et al., 2007). Enzymatic (Galtier et al., 1993) and high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) analyses of *Brassica napus* phloem sap showed the presence of sucrose at concentrations of 600 mM and 947 mM, respectively, and that fructose and glucose were not detected (Giavalisco et al., 2006; Lohaus & Schwerdtfeger, 2014).

Research has also shown that non-sucrose carbohydrates have been identified in phloem sap at appreciable concentrations. These include the non-reducing galactosyl-oligosaccharides (i.e., raffinose, stachyose, and verbascose) and carbohydrate alcohols/polyols (e.g., mannitol and sorbitol), which do not contain aldehyde or ketone functional groups (van Bel & Hess, 2008). The raffinose family oligosaccharides are abundant in the Cucurbitaceae family (e.g., squash) at concentrations reaching 600 mM, with reported raffinose and stachyose concentrations of 70 and 330 mM, respectively (Haritatos et al., 1996). Although trace amounts of reducing carbohydrates such as fructose and glucose have been identified in phloem sap, it is generally believed that these arise from the detachment/cutting action of stems and leaves and/or as contaminants from other cells (Ziegler, 1975; Dinant & Lemoine, 2010). This paradigm has been questioned based on the finding that phloem exudates from members of the Ranunculaceae and Papaveraceae families contained >80% of their carbohydrate as hexoses (van Bel & Hess, 2008). These findings have since been questioned by Liu et al. (2012) based on the experimental method employed, where

phloem sap was obtained by detaching leaves and placing the cut ends of the petioles in EDTA. Liu et al. (2012) found almost identical results when the leaf blades were removed and only the petiole stubs were EDTA treated. The authors concluded that the EDTA solution represented compounds extracted from the petioles rather than carbohydrates in transit in the phloem.

The presence and high concentration of carbohydrate in plant phloem sap is believed to provide a metabolically available source of carbon for energy, and/or for storage as polysaccharides (e.g., starch), and may also be a driving force for sap movement according to the mass flow model (Dinant & Lemoine, 2010). Munch (1930) proposed the widely accepted mass flow model for phloem transport. In the mass flow model, phloem transport is driven by an osmotically generated pressure gradient between the source and sink organs. At the source, carbohydrates are loaded in the phloem causing a decrease in water potential at that site. As a result, water from the xylem enters the phloem by osmosis, creating a high turgor pressure. The resulting turgor pressure moves carbohydrates and other phloem constituents to the sink organs as water is moving in and out of the phloem. Unloading of carbohydrates at the sink increases the water potential causing water to move out of the phloem and back to the xylem. It is this mass flow mechanism that operates in the nectaries during the transport of sucrose from the phloem into this organ. Invertases present in the nectaries have been postulated to play a role in the establishment of a sucrose gradient between the source to the sink (Ruhlmann et al., 2010). These enzymes can hydrolyze sucrose into its constituent hexoses so as to decrease sucrose concentration in the nectary. Enzymatic hydrolysis of sucrose and the subsequent metabolism of the cleavage products have been postulated to control sink strength so as to attract sucrose to the nectary and to maintain a concentration gradient favouring its diffusion (Eschrich, 1980; Nicolson, 1998). In addition, because sucrose is a non-reducing carbohydrate, it is the ideal major transported molecule in phloem as it has low chemical reactivity with non-enzymatic molecules that it encounters in the transport route (Arnold, 1968).

2.2 Nectar

Literature supports the hypothesis that carbohydrates, which are the major non-aqueous component of nectar, are derived from photosynthesis in the nectary, and/or in other floral, and/or vegetative parts of the plant (e.g., pedicel, calyx, ovary, and adjacent leaves) that are generally close to the flower (Pacini et al., 2003; Pacini & Nepi, 2007). The carbohydrate source for nectar

production is not mutually exclusive but one source usually dominates. Also, this source can vary cyclically according to the plant's circadian physiological changes, as has been observed in *Ipomoea cairica* (Paiva & Martins, 2014). In this case, when sunlight was readily available, photosynthesis within the nectary supplied the carbohydrates for nectar production with a portion stored as starch in the plastids. Photoassimilates from other non-nectary photosynthetic parts can also act as a supply route for starch synthesis during the day. During the absence of sunlight (i.e., night), hydrolysis of stored starch sustained nectar secretion. Therefore, photosynthesis in the nectary, stored starch hydrolysis, and the phloem supply of photosynthates from non-nectary organs can contribute synergistically to nectar production by operating at different times of the day (Paiva & Martins, 2014).

2.2.1 Photosynthesis in Non-Nectary Organs

A number of floral nectaries are not green, and as such are not able to photosynthesize. In these cases, photosynthesis occurs in distant source tissues with the photoassimilate transported by the phloem so as to supply substrates for energy metabolism and carbohydrates for nectar (Lüttge, 2013). Photosynthesis at non-nectary plant parts and the subsequent photosynthate transport have been reported to be the predominant source of nectar carbohydrates through girdling of floral shoots, darkening, and defoliation experiments. The girdling of flowering shoots (von Czarnowski, 1952; Wykes, 1952) darkening, and defoliation studies (von Czarnowski, 1952; Pleasants & Chaplin, 1983; Nepi et al., 2005) showed either the absence or the reduction of transported photosynthate in the phloem and decreased nectar production. Experiments with ^{13}C-labelled CO_2 also revealed that the extrafloral nectar of *Phaseolus lunatus* (lima bean) contained carbohydrates that were assimilated during the last hours before nectar secretion (Radhika et al., 2008). In tobacco, photosynthesis in non-nectary plant parts and the transport of the photosynthate to the nectary provided carbohydrates during periods of high nectar flow (Ren et al., 2007b).

In Brassicaceae, phloem alone supplies the nectaries and a direct relationship has been demonstrated between the abundance of phloem supply and nectar carbohydrate production (Davis et al., 1998). For Boraginaceae, phloem branches were observed to enter the glandular tissue of the nectaries (Frei, 1955; Weryszko-Chmielewska, 2003). However, it has been shown that about 40% of floral nectaries lack any direct vascularization (Fahn, 1979) and may be compensated by

the vasculature of organs (e.g., flower receptacle) close to them (Fahn, 1988, 2000; Ma et al., 2011).

Photosynthate transport from the phloem to the nectary has been reported to occur in two ways: a) Apoplastic route: where the photosynthate is unloaded from the sieve elements and companion cells via intercellular spaces and cell walls (Davis et al., 1988; Peng et al., 2004); and b) Symplastic route: where the photosynthate is passed through the plasmodesmata from the phloem parenchyma cells to the nectary parenchyma cells (Fahn, 1979). There is also scientific evidence for the existence of both routes within the same nectary (Wergin et al., 1975; Davis et al., 1986, 1988; Wist & Davis, 2006).

2.2.2 Photosynthesis in Nectary

There are many examples where both the floral and extrafloral nectaries are green. These include but are not limited to the nectaries of *Brassica napus* (Davis et al., 1986), *Cerinthe major* (Boraginaceae) (Nocentini et al., 2012), *Cyclanthera pedata* (Pacini et al., 2003), and *Helleborus* (Vesprini et al., 1999) where chlorophyll has been observed, which is a strong indicator of the presence of chloroplasts. Photosynthesis in these nectaries would provide energy as well as carbohydrates and reduction equivalents (e.g., NADPH) to the nectar, making them partially or completely autonomous organs (i.e., self-sufficient) (Lüttge, 2013). In *Helleborus bocconei*, nectary photosynthesis was believed to contribute significantly to nectar production based on the following observations: a) The flower was more open and as such the nectaries were exposed to light; b) Nectar was offered as soon as the flower opened, that is when the nectaries were exposed; c) Intense autofluorescence of chlorophyll; d) Carbohydrate production was constant over time; and e) Small quantities of starch were observed in the cells of more superficial parts of the nectary closer to the vascular bundles (Vesprini et al., 1999).

A study by Lüttge (2013) on a range of plants with green nectaries showed that nectary photosynthesis was sufficient to make a significant contribution to the amount of carbohydrate secreted in the nectar. Also, Pacini and Nepi (2007) reported that photosynthetic nectaries are beneficial to the plant because of the following: long-term production of nectar (days or months); production of carbohydrates close to where nectar is secreted; and nectar is produced following pollinator removal. However, the disadvantages of photosynthetic nectaries include: nectar

production only during the day; low rate of nectar production; low carbohydrate concentration; and increased microbial growth (e.g., pathogens) because of the long nectar production period.

It has been shown that photosynthesis in some green nectaries appears to be unlikely because they are often concealed and they receive only diffuse light (Nepi, 2007; Pacini & Nepi, 2007). For example, nectaries of the Ericaceae (heath family) and Scrophulariaceae (figwort family) lie below the ovary, whereas nectaries of the Fabaceae (legume family) form a protuberance at the base of the ovary, which is often covered by the calyx, corolla, or stamen filaments (Davis et al., 1988; Pacini & Nepi, 2007).

2.2.3 Starch Storage in Nectary

Nectaries have been reported to store starch in different quantities or not at all (Figueiredo & Pais, 1992; Galetto & Bernardello, 1992; Thomas & Dave, 1992). Carbohydrate storage is essential for plants that produce nectar at night (Pacini & Nepi, 2007; Gaffal, 2012) or when a large amount of nectar is needed in a short time period (Nepi et al., 1996). Carbohydrates from both phloem and chloroplasts are often stored as starch in amyloplasts, which are non-pigmented organelles. Amyloplasts in the nectary serve as a constant source of carbohydrate and they also provide energy through starch hydrolysis (Durkee et al., 1981). Also, storage of carbohydrates as starch provides the most efficient means of accumulating nectar precursors for fast nectar producers.

Cruden et al. (1983) suggested that there are three classes of nectar producers: slow producers, which secrete 5-10% of their maximum accumulation per hour; fast producers, which secrete 22-68% of their maximum per hour; and super producers that secrete two or three times as much nectar as fast producers. Starch storage in parenchyma cells ensures the availability of nectar carbohydrates especially for fast producers (Belmonte et al., 1994).

Starch accumulation and subsequent breakdown into monosaccharides and disaccharides were observed in the nectaries of ornamental *Nicotiana* and *Arabidopsis* at the start of nectar secretion (Horner et al., 2007; Ren et al., 2007b). The accumulation and subsequent breakdown of starch was also observed in cucumber and other cucurbits, glory flower, orchid, and *Passiflora* spp. (Durkee et al., 1981; Belmonte et al., 1994; Nepi et al., 1996; Peng et al., 2004; Stpiczyńska et al., 2005). The nectaries of many species have been observed to contain amyloplasts (Pacini et al., 2003) with the contained starch degraded by vacuolar hydrolytic enzymes during active nectar

secretion (Gaffal et al., 2007). However, it has been shown that starch storage/hydrolysis accounts for only a portion of the carbohydrate that is secreted during the peak activity of floral nectaries, with transported photosynthate from the phloem also contributing to the flow of carbohydrates into nectar (Gaffal et al., 2007; Ren et al., 2007b). Starch accumulation has also been rarely reported in extrafloral nectaries; therefore, the presence of this polysaccharide is not an absolute requirement for nectar secretion (Escalante-Pèrez & Heil, 2012).

Starch storage in the nectaries confers benefits and disadvantages to the plant (Pacini & Nepi, 2007). The benefits of storing starch prior to nectar secretion include: the exposure of nectar to consumers at any time of the day; production of large amounts of nectar at any time of the day; and the production of nectar containing a high carbohydrate concentration. The disadvantages of starch storage include: the cessation of nectar production following pollinator consumption; nectaries that may disintegrate after nectar secretion; and photosynthate for nectar production that comes from non-nectary plant organs can be affected by the environment.

2.3 Nectary

Schmid (1988) defined the nectary as "a more or less localized, multicellular glandular structure that occurs on vegetative or reproductive organs and regularly secretes nectar, a sweet solution containing mainly carbohydrates and generally serving as a reward for pollinators or for protectors (e.g., ants) against herbivores or in carnivorous plants as a lure for animal prey". Structurally, nectaries vary widely in ontogeny, morphology, and anatomy (Fahn, 1979, 1988; Durkee, 1983; Smets et al., 2000). They can take on diverse forms in different species and can occur in different areas of the flower. From an ecological point of view, the diversity of nectary location is attributed to the type of pollinator being attracted and their corresponding foraging behaviour (Nepi, 2007).

Floral nectaries of both *Brassica* spp. and *B. officinalis* (Figures 2.1 and 2.2) were investigated in this study. Nectaries of Brassicaceae family are variable in shape, located at the base of the flower, and concealed by the sepals, with four general nectary types: 1) Annular: a continuous ring of tissue fused to the ovary at the base of the flower; 2) Two-nectary type: with two lateral nectaries at the floral base; 3) Four-nectary type: made up of two pairs of nectaries classified as lateral and median; and 4) Eight-nectary type: with two pairs each of both the median and lateral nectaries (Davis et al., 1986, 1998; Bernardello, 2007). Nectaries of the two *Brassica*

spp. used in the study were of the four-nectary type. Only the nectars from the lateral nectaries were collected since they are innervated by phloem and 95% of the nectar and total nectar carbohydrates are produced by this organ (Davis et al., 1998). In Boraginaceae family, nectaries form a disc-like ring below the ovary and each ring is referred to as an annular nectary (Kugler, 1970).

Anatomically, nectaries are comprised generally of three tissue layers (Nepi, 2007): the epidermis, parenchyma, and subnectary parenchyma (Figure 2.3). Epidermal cells are generally small (e.g., 6 μm mean diameter [Stpiczyńska et al., 2005]), have large vacuoles, are polyhedric in shape, and may have an anticlinal orientation (Nepi, 2007). The epidermis mediates nectar release via its secreting cells called trichomes or by pores of stomata. Nectar exudation through the stomata appears to be the most common manner of nectar release (Bernardello, 2007), but if secretion does not occur through the stomata, the epidermis itself is involved in the secretion process via epidermal secreting cells or secreting trichomes, pores in the cuticle, microchannels, or cuticle breakage leading to permeation (Nepi, 2007).

Below the epidermis is the parenchymatous tissue, which is generally composed of a few to several layers of small (e.g., 12.5 μm mean diameter [Stpiczyńska et al., 2005]), thin walled isodiametric cells, containing a dense granular cytoplasm, small vacuoles, and relatively large nuclei (Caspary, 1848, cited in Fahn, 1979; Behrens, 1879; Bonnier, 1879; Fahn, 1952, 1974). It is in this tissue where pre-nectar is transformed into nectar.

The last layer of nectary tissue is the subnectary parenchyma, which is located below the nectary parenchyma. It consists of larger diameter cells (e.g., 26 μm diameter [Stpiczyńska et al., 2005]) with large vacuoles, a less dense cytoplasm, and large intercellular spaces (Nepi, 2007). It is generally richer in chloroplasts than the nectary parenchyma and always contains vascular bundles. As an active metabolic organ, nectaries are able to transform substances that pass through them for nectar production (Orona-Tamayo et al., 2013).

Figure 2.1 The dark green floral nectaries evident within an open flower of *Brassica napus* L.; LN = lateral nectary; MN = median nectary.

Figure 2.2 The pale green, ring-like floral nectary of *Borago officinalis* L. is an annular nectary (AN) that surrounds the base of the ovary.

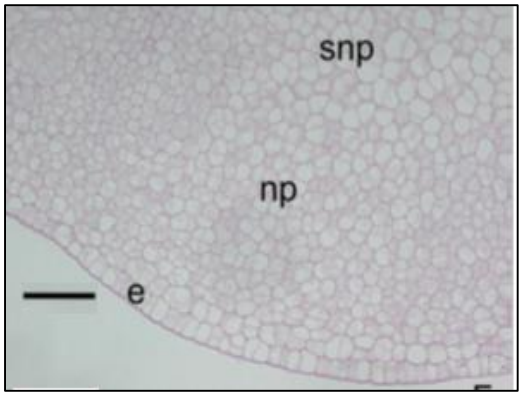

Figure 2.3 Floral nectary of *Cerinthe major*; e = epidermis; np = nectary parenchyma; snp = subnectary parenchyma; Bar = 20 µm (adapted from Nocentini et al., 2012).

2.4 Nectar Secretion

Nectar secretion or the transport of nectar outside the protoplast of the parenchyma cells, occurs by two mechanisms: 1) Eccrine secretion: the molecular transport of individual carbohydrate molecules across the cell membrane by a carrier molecule; or 2) Granulocrine secretion: the transport of a carbohydrate solution into vesicles derived from dilated cisternae of the endoplasmic reticulum or dictyosomes that fuse with the plasmalemma, releasing nectar into the wall area (Fahn, 2000). Following secretion, the nectary parenchyma cells together with the epidermal cells can remain intact throughout the process (merocrine secretion) or in a few cases, cell death may follow secretion (holocrine). If cell death does not occur as is the case for most plants, the nectary parenchyma can either be involved in nectar reabsorption (Nepi et al., 1996) or differentiate into another tissue (Nepi, 2007).

In summary, phloem photosynthate (produced from non-nectary vegetative plant organs) seems to be the predominant source of nectar carbohydrates for nectar production. The process involves the uploading of carbohydrates as sucrose from the phloem to the nectary where they are stored and/or processed (Wenzler et al., 2008; Kram & Carter, 2009). The enzymes in the nectary enable the transformation of the substances that pass through this organ for nectar production. For instance, an invertase cleaves sucrose to produce a hexose-rich nectar and creates the source-sink relationship to prevent reloading of sucrose into the phloem (Agthe, 1951; Zimmermann, 1953; Frey-Wyssling et al., 1954; Peng et al., 2004).

2.5 Nectar Chemical Composition

According to literature, nectar is primarily made up of carbohydrates comprising >90% of its dry weight (Lüttge, 1977). The remainder is made up of other chemical compounds, including amino acids, antioxidants, lipids, minerals, volatiles, and secondary plant metabolites, including alkaloids, and phenolics (Lüttge & Schnepf, 1976; Baker, 1977). An extensive review of nectar composition literature by Heil (2011) revealed that its chemistry is tailored to fulfill two roles: to attract mutualists and to provide protection from non-mutualists, including nectar robbers and nectar-infecting microorganisms. Each of the aforementioned major chemical groups will be discussed separately in the following sections with specific examples of both their chemical composition and plant species.

2.5.1 Carbohydrates

Nectar chemistry is dominated by carbohydrates. Early research by Percival (1961) and Baker and Baker (1983) showed that nectar was primarily comprised of the carbohydrates, fructose, glucose, and sucrose. The predominance of carbohydrates in nectar makes it an excellent reward for pollinators by providing a good source of energy and as an aqueous solution, nectar is readily utilized (Nicolson, 2007).

Based on their fructose (F), glucose (G), and sucrose (S) concentration ratios, nectars have been divided into four classes as shown in Table 2-1. Alternative approaches (Freeman et al., 1984) for the representation of nectar carbohydrates in analyses include the expression of the proportion of fructose, glucose, and sucrose as percentages of total carbohydrate and the percent carbohydrate composition of nectar, which is the most common reporting method in the current literature (Perret et al., 2001; Torres & Galetto, 2002; Galetto & Bernardello, 2003; Petanidou, 2005; Wolff, 2006).

Table 2-1 Nectar classes based on carbohydrate concentration ratios; S = sucrose; G = glucose; F = fructose (adapted from Baker and Baker, 1983).

Nectar Class	**S/(G+ F)**
Sucrose-dominant	>1.0
Sucrose-rich	0.5-1.0
Hexose-rich	0.1-0.5
Hexose-dominant	<0.1

In addition to fructose, glucose, and sucrose, trace (<1% of the total carbohydrate concentration) amounts of other carbohydrates have been detected in nectar (Baker & Baker, 1983). These include monosaccharides such as arabinose, galactose, mannose, and xylose, as well as oligosaccharides such as lactose, maltose, melezitose, melibiose, raffinose, stachyose, and trehalose (Maurizio, 1959; Percival, 1961; Baskin & Bliss, 1969; Bowden, 1970; Jeffrey et al., 1970; Watt et al., 1974; Baker & Baker, 1982b; Gottsberger et al., 1984; Nicolson & Van Wyk, 1998; Petanidou, 2005). In general, the presence and identification of these carbohydrates were made by paper or thin-layer chromatography, followed by elution and detection employing colourimetric and titrimetric methods.

2.5.2 Alkaloids

Alkaloids are a class of nitrogen containing organic bases produced by a number of plant species as secondary metabolites and are present in the nectar of some species (Baker & Baker, 1975). Probable functions of these compounds include the attraction of a specialized pollinator, repelling nectar thieves, inhibition of microbial growth, and the alteration of pollinator behaviour (Adler, 2000).

The presence of alkaloids in nectar as an attractant for pollinators was observed for *Coffea* and *Citrus* spp. nectars, which contain caffeine. It was found that the presence of this compound in nectar was most likely responsible for increased pollinator visitation behaviour by honey bees. Caffeine can pharmacologically alter a pollinator's behaviour by enhancing their memory of reward. Pollinators are more likely to remember a learned floral scent, resulting in increased pollinator fidelity (Wright et al., 2013).

Alkaloids may also deter pollinators and regulate the duration of pollinator visits (e.g., the probing of fewer flowers) to reduce the possibility of self-pollen transfer (Irwin & Adler, 2008). As examples, bee pollinators probed fewer flowers and spent less time per flower in *Gelsemium sempervirens* (Carolina jessamine) with nectars containing high gelsemine concentrations (Adler & Irwin, 2005). The presence of the alkaloid nicotine in *Nicotiana attenuata* was found to significantly decrease the length of hawkmoth and hummingbird visitation (Kessler & Baldwin, 2006) and reduce nectar visitations by florivores and nectar robbers such as ants and carpenter bees (Kessler et al., 2008). Laboratory behaviour studies using artificial nectar enriched with alkaloids have also demonstrated the deterrence of bumble bees (Adler & Irwin, 2005; Gegear et al., 2007; Manson et al., 2013), honey bees (Detzel & Wink, 1993; Köhler et al., 2012), and hummingbirds (Kessler et al., 2012). However, it was found that this deterrence only occurred at nectar alkaloid concentrations that were approximately 50 times higher than those present naturally. Tiedeken et al. (2014) employed naturally occurring nectar concentrations of caffeine, nicotine, and quinine to determine their deterrence effects on bumble bees and found a response for quinine only.

Antimicrobial or antiparasitic properties of nectar alkaloids may play a role in controlling and reducing pathogen and parasitic infections in pollinators. A feeding experiment employing artificial nectar containing gelsemine showed that bumble bees inoculated with an intestinal parasite developed a less intense infection (Manson et al., 2010). It has been found that the presence of pathogens and parasites in pollinators can negatively impact foraging efficiency (Gegear et al., 2005, 2006; Otterstatter et al., 2005). As such, the presence of natural control compounds that are consumed during normal nectar foraging is of benefit both to the pollinator and in the case of honey bees, to the beekeeper.

2.5.3 Amino Acids

Floral and extrafloral nectars contain amino acids (Baker & Baker, 1975, 1983; Gardener & Gillman, 2001). All 20 of the common amino acids found in protein have been identified in plant nectars and the presence of these compounds is most likely an important nitrogen source for pollinators (Nicolson, 2007). High performance liquid chromatography (HPLC) was one technique employed for a more precise and accurate determination of amino acids in nectars (Gardener & Gillman, 2001).

The first report of the presence of amino acids in nectar was from Ziegler (1956) based upon the observation of ninhydrin-reactive compounds. Subsequently, Lüttge (1961, 1962) identified Ala, Asn, Cys, Gln, Met, Pro, Ser, and Tyr in the nectar of plantain bananas and five other plant species through descending paper chromatography. A large scale study of nectar amino acid composition was performed by Baker and Baker (1973). In this work, the amino acid content of 266 species of flowering plants was estimated by ninhydrin staining of nectar spots using a histidine scale. Of the species studied, only six nectars failed to show the presence of amino acids by this assay. Differences in the concentrations of nectar amino acids among plant species have been observed and are most likely related to the principal pollinator (Baker & Baker, 1973, 1986). Generally, lower concentrations of nectar amino acids are observed if the principal pollinator has access to other amino acid containing food sources (Terrab et al., 2007).

Specialized flowers that attract carrion and dung flies as well as butterfly-pollinated flowers have nectars that are especially rich in amino acids (Nicolson & Thornburg, 2007). It has been proposed that amino acids may promote butterfly fitness; however, the evidence is equivocal (Jervis & Boggs, 2005). A study by Mevi-Schütz and Erhardt (2003) showed that nectar amino acids enhanced fecundity in the butterfly, *Araschnia Levana*, only when the larval diet was poor. Experimental studies showed that female butterflies fed with a poor (e.g., low nitrogen) larval diet were smaller, whereas females fed with an enriched (e.g., high nitrogen) diet were larger. A study by Cahenzli and Erhardt (2012) on the small heath butterfly (*Coenonympha pamphilus* L.) showed that nectar amino acids enhanced its fitness. Female adults fed with nectar mimics containing 16 µmoles mL^{-1} amino acid with Ala, Arg, Asn, Glu, Gln, Gly, Pro, Ser, Thr, Tyr, and Val produced heavier larvae and had higher egg hatching success over the oviposition period.

2.5.4 Antioxidants

Ascorbic acid (Vitamin C) is an essential nutrient for many insects and also serves as an antioxidant by acting as a free radical scavenger (Barbehenn et al., 2001). Vitamin C was initially identified by Griebel and Hess (1940) in the nectars of three Labiatae species and since this discovery, has been identified in the floral nectars of many plants (Bukatsch & Wildner, 1956; Baker & Baker, 1975; Carter & Thornburg, 2004a, b; Naef et al., 2004).

2.5.5 Lipids

Lipids have been detected in numerous plant nectars (Vogel, 1971; Baker & Baker, 1975; Bernardello et al., 1999; Vesprini et al., 1999; Varassin et al., 2001). These nutritional compounds act as an excellent storage source of energy for pollinators and also yield the most energy per gram when compared to carbohydrates and proteins (Nicolson & Thornburg, 2007). Baker and Baker (1975) tested 220 flowering species for the presence of lipids in their nectars employing the OsO_4 qualitative test and found that 75 (34%) of the samples gave a positive result. Forcone et al. (1997) analyzed the nectar of 29 flowering species native to the Patagonia region of Argentina and found the presence of lipids in 50% of the nectars. This study was extended by Bernardello et al. (1999) with 32% (9/28) of the species tested being lipid-containing.

A variety of lipids have been identified in nectars. For example, squalene was identified as the major lipid component in the parasitic plant *Dactylanthus taylorii* (wood rose), with minor amounts of the ethyl and benzyl esters of hexadecanoic acid, along with C_{18} to C_{23} polyunsaturated fatty acids, and C_{21} to C_{31} hydrocarbons (Ecroyd et al., 1995). Also, cholesterol and phospholipids have been identified in the nectars of select *Passiflora* spp. (Varassin et al., 2001).

2.5.6 Minerals

Literature reports on the presence and concentration of minerals in nectar are few. Aluminum, calcium, iron, magnesium, manganese, potassium, and sodium were detected in the nectars of ~20 different plant species using the laser microprobe mass analyzer (LAMMA) (Heinrich, 1989). Chloride, potassium, and sodium concentrations were also determined in the nectar of 19 species of flowering plants visited by hummingbirds (e.g., *Aloe*, *Delphinium*, *Penstemon*). Results from this study showed that the average concentrations of chloride, potassium, and sodium were 9.9, 24.7, and 3.4 mM, respectively (Hiebert & Calder, 1983). A study on *Passiflora* spp., another hummingbird-pollinated flower, also showed the presence of both potassium and sodium with average concentrations of 4.25 and 2.85 meq L^{-1}, respectively (Varassin et al., 2001). Calcium was also observed in the nectar of *Passiflora* spp. at a mean concentration of 1.45 mg L^{-1}. The focus of these studies on flowers visited by hummingbirds illustrates the importance of nectar compounds such as minerals to pollinators, as these electrolytes must be replaced daily through the diet (Hiebert & Calder, 1983; Varassin et al., 2001).

Nectars of bat-pollinated flowers have significantly higher calcium concentrations when compared to those of other flowers, and this is important because calcium is a limiting electrolyte in a bat's diet (Barclay, 2002). Therefore, plants depending on bats for pollination may have evolved to increase mutualist visitation by producing higher levels of this mineral. High calcium and magnesium concentrations have been reported in *Mucuna sempervirens*, a subtropical climbing vine of the bean family that may serve to attract mutualist pollinators such as honey bees (Liu et al., 2013).

Minerals can also have a deterring effect on pollinators. Avocado nectar was observed to contain a wide range of minerals including boron, calcium, copper, iron, lead, magnesium, phosphorus, potassium, silicon, sodium, sulphur, and zinc. The low attractiveness to pollinators of avocado nectar is believed to be partly due to its high minerals' concentration, with potassium dominating at a concentration of ~4000 mg kg^{-1} (Afik et al., 2006). It has also been postulated that high mineral concentrations in nectar may be harmful to honey bees through interference with their sensory mechanisms (Afik et al., 2008).

2.5.7 Odour Constituents

Floral fragrances are a mixture of different chemical compounds including amino acid-derived compounds, aromatics, fatty acids, and terpenoids (Raguso & Pichersky, 1999). These volatile organic compounds (VOC) have a variety of functions that include, but are not limited to: pollinator attraction; as a deterrent to nectar robbers and florivores; as antimicrobial agents; and as pleiotropic agents involved in plant defense and communication (Raguso, 2004b).

It has been proposed that nectar scent can come from the volatiles emitted by the floral tissue which are absorbed and solubilized in nectar (Raguso, 2004b) or may be from the nectar itself (Kessler & Baldwin, 2006). Ecroyd et al. (1995) found that the ethyl esters of benzoic, cinnamic, and salicylic acids imparted a sweet odour that allyl methyl sulphide gave an onion or garlic odour, and that terpene derivatives such as geraniol, nerol, nerol oxide, and pentanyl alcohols were responsible for the floral and citrus notes of select floral nectars. The relationship between nectar VOC and pollinators was observed with *Osmia* spp. bees being attracted to *Penstemon caesius* (San Bernardino Beardtongue) flowers based on nectar volatiles and ultimately being nectar-rewarded (Howell & Alarcon, 2007). Butterflies and moths were also shown to prefer artificial flowers with scented nectars rather than nectars with pure carbohydrates alone (Weiss,

2001). Benzylacetone is the abundant attractant compound in *N. attenuata* nectar (Kessler & Baldwin, 2006) and may be associated with increased pollinator visits (Kessler et al., 2008). Alternately, nicotine is a volatile alkaloid found in *N. attenuata* nectar but functions as a deterrent (Kessler & Baldwin, 2006; Kessler et al., 2008) as discussed earlier in Section 2.5.2 on nectar alkaloids.

Volatile organic compounds may also be involved in the plant's defense strategy against herbivores. Plants may be able to recruit the enemies of herbivores via odour emissions and/or to help these enemies locate food sources. For example, parasitoid wasps known to attack noctuid larvae were shown to be able to locate the extrafloral nectar of cotton by its odour (Röse et al., 2006).

2.5.8 Phenolics

Phenolics are a class of organic compounds that have a hydroxyl group/groups covalently bound to an aromatic ring. They are secondary plant metabolites derived from phenylalanine. The presence of phenolics in nectar was established by Baker (1977) in 528 plant species from California and Colorado, U.S.A., and Costa Rica employing p-nitraniline (a colourimetric test). Results showed that >30% of the nectars analyzed were positive, indicating the presence of phenolics. Phenolics were also observed in 60% of the 48 samples of plant species native to the Argentinian Patagonia region (Forcone et al., 1997).

It has been postulated that the major functions of these compounds in nectar are as attractants or deterrents to pollinators/nectar robbers. Phenolic compounds may also: be a visual cue for insects because of their fluorescent properties (Thorp et al., 1975); render carbohydrates and amino acids less or non–metabolizable to non-pollinators; impart an unfavourable nectar taste for non-pollinators and a favourable taste for pollinators; and protect nectar carbohydrates from degradation due to their antimicrobial properties (Hagler & Buchmann, 1993).

Bitter phenolics in the dark nectar of the South African succulent shrub, *Aloe vryheidensis*, deterred morphologically incompatible pollinators like honey bees and sunbirds (Johnson et al., 2006). However, the more effective pollinators of *A. vryheidensis* (e.g., passerine birds) were unaffected by the bitter taste. Honey bees were observed to prefer low concentrations of caffeic and genistic acids in a 30% sucrose solution whereas higher concentrations (2.1 and 6.3 mg mL^{-1}) were deterrent (Hagler & Buchmann, 1993). Inhibition of nectar collection by honey bees was

also observed when nectar solutions were mixed with a >12.5% concentration of the phenolic-rich nectar of *Aloe* (Hagler & Buchmann, 1993).

2.5.9 Proteins/Enzymes

The presence of protein in the nectar of flowering plants was detected more than 80 years ago (Buxbaum, 1927). Literature reports on this topic following this discovery were few due to the low concentration of proteins in nectar coupled with the low sensitivity of detection methods. Improvements in protein detection methods (e.g., SDS-PAGE, LC-MS/MS) have enabled a better characterization of nectar protein profiles, which has resulted in a number of literature citations on this topic (Carter & Thornburg, 2004a, b, c; Carter et al., 2007; Kram et al., 2008; González-Teuber et al., 2009, 2010; Hillwig et al., 2010, 2011). The two general classes of proteins that have been identified in floral nectars include those involved in carbohydrate metabolism and those that inhibit/delay microbial invasion (Nepi et al., 2012).

Invertase is one of the carbohydrate metabolizing enzymes known to be active in nectar. The term invertase has been widely used to explain the enzymatic hydrolysis of the disaccharide sucrose into its monosaccharide components, glucose and fructose. As such, there is some ambiguity in the literature as this interpretation of invertase activity covers both α-glucosidase and β-fructosidase. True invertases (EC 3.2.1.26) are β-fructosidases and in plants they catalyze the hydrolysis of sucrose to glucose and fructose so as to create a source-sink relationship (Agthe, 1951; Zimmermann, 1953; Frey-Wyssling et al., 1954; Pate et al., 1985; Kram & Carter, 2009; Ruhlmann et al., 2010; Nepi et al., 2012; Lohaus & Schwerdtfeger, 2014).

The first literature report on the detection of invertase activity in nectar was made by Beutler (1935), when conducting research on the floral nectar of *Tilia* spp. Since that initial report, this carbohydrase activity has been identified in *Acacia*, *Cucurbita*, *Nicotiana*, and other genera (Baker & Baker, 1983; Heil et al., 2005; Nepi et al., 2012; Seo et al., 2013) however, the basic chemical and kinetic properties of this enzyme in nectar remains poorly studied. According to Heil (2011), invertase may be involved in the following steps of nectar production: 1) the uploading of sucrose from the phloem into the nectary; 2) the formation of the sink required for carbohydrate secretion into the extracellular space (Agthe, 1951; Zimmermann, 1953; Frey-Wyssling et al., 1954; Kram & Carter, 2009; Ruhlmann et al., 2010); and 3) the formation of hexose-rich nectars. Physical sites in the plant of invertase activity include the nectary and the

nectar, however the presence of invertase activity in nectar is still debated (Pacini & Nepi, 2007). Pate et al. (1985) reported that invertase was present in the stipel nectary of *Vigna* but not in the nectar, and the authors suggested that invertase was present at the point of nectar secretion so as to enable the regulation of sink strength. Nichol and Hall (1988) demonstrated that sucrose hydrolysis occurred at the final stage of nectar secretion and reported that no invertase activity was found in nectar. In contrast, Heil et al. (2005) reported invertase activity in the extrafloral nectar of four myrmecophyte *Acacia* spp. and three related non-myrmecophyte species. The authors reported that the average invertase activity values ranged from 0.73 to 1.52 μg of glucose μL^{-1} min^{-1} for myrmecophytes and 0.01 to 0.09 μg of glucose μL^{-1} min^{-1} for non-myrmecophytes. Invertase activity was also found in the nectar of *Cucurbita* with a maximum activity of 1.24 μM mL^{-1} h^{-1} at pH 6.0 (Nepi et al., 2012). The authors reported that it was possible that the presence of invertase activity in *Cucurbita* may be due to a cell-wall bound invertase that leached from the nectary.

Other enzyme activities involved in carbohydrate metabolism have also been reported in nectar. For example, the presence of transglucosidase and transfructosidase activities in *Robinia pseudoacacia* (black locust tree) and in the extrafloral nectar of *Impatiens holstii* (Zimmermann, 1953, 1954); xylosidase activities in *Cucurbita pepo* (winter squash and pumpkin) and *Nicotiana* nectars (Nepi et al., 2011; Seo et al., 2013); and galactosidase activity in *Nicotiana* (Zha et al., 2012; Seo et al., 2013) have been reported. The functions of these enzymes in these nectars include, but are not limited to: oligosaccharide formation for pollinator attraction/nutrition; degradation of oligosaccharides in the nectary cell walls so as to limit pathogen activity; and induction of cell-wall restructuring in the early stages of fruit development (Nicolson & Thornburg, 2007; Nepi et al., 2011; Zha et al., 2012). LC-MS/MS analysis of the nectar of *N. attenuata* also showed the presence of β-glucosidase, however the exact role of this enzyme was not reported (Seo et al., 2013).

Proteins that can aid in plant defense have also been reported in nectar. As an example, the nectar from the cross of *Nicotiana langsdorffii* x *N. sanderae* was found to contain five novel proteins, identified as nectarins 1-5 (*NecI-V*), which are postulated to be involved in the nectar redox cycle. These proteins include superoxide dismutase (*NecI*), carbonic anhydrase/monodehydroascorbate reductase (*NecIII*), endoglucanase inhibitor (*NecIV*), and

glucose oxidase (*NecV*) (Carter et al., 1999; Carter & Thornburg 2000, 2004a, b, c; Naqvi et al., 2005).

The postulated role of the nectar redox cycle is the production of hydrogen peroxide, which acts as a floral defense compound against microbial growth. Nectarins were also detected in nectar of *N. attenuata*, and similar defense-related proteins (e.g., chitinase) were found in the extrafloral nectar of *Acacia* spp. myrmecophytes and related non-myrmecophytes (González-Teuber et al., 2009, 2010).

Other enzyme activities reported in nectar include: esterase, lipase, and malate dehydrogenase (Scogin, 1979; Kram et al., 2008); oxidase (Zauralov, 1969); phosphatase (Cotti, 1962; Nicolson & Thornburg, 2007); RNAse (Hillwig et al., 2010, 2011; Seo et al., 2013); tyrosinase (Lüttge, 1961; Nicolson & Thornburg, 2007); and unidentified lipid transfer proteins (Seo et al., 2013).

2.6 Nectar and Microorganisms

The presence of microorganisms (e.g., yeasts, bacteria) in plant nectars is ubiquitous and appears to be irrespective of environmental habitat (Gilliam et al., 1983; Sandhu & Waraich, 1985; Ehlers & Olesen, 1997; Brysch-Herzberg, 2004; de Vega et al., 2009; Herrera et al., 2009; Alvarez-Pérez et al., 2012; Belisle et al., 2012; Fridman et al., 2012; Alvarez-Pérez & Herrera, 2013). The major sources of microorganisms in plant nectars are pollinators (e.g., bees, birds, and ants) (Brysch-Herzberg, 2004; Belisle et al., 2012; de Vega & Herrera, 2012, 2013).

The presence of yeasts in nectar has been shown to decrease its nutritional quality for pollinators by carbohydrate concentration reduction (de Vega & Herrera, 2012, 2013). It has been found that sucrose hydrolysis by yeast carbohydrases results in the alteration of the 'natural' carbohydrate profile of nectars, which can lead to the non-stoichiometric 1:1 monosaccharide ratio expected from sucrose hydrolysis due to preferential selection and metabolism of fructose (D' Amore et al., 1989; von der Ohe, 1994; Barnett, 1997; Berthels et al., 2004). In addition to carbohydrate conversion and metabolism, the presence of yeasts in plant nectar may reduce the amino acid content (Peay et al., 2012) and release ethanol as a byproduct of fermentation (Wiens et al., 2008). Either of these situations can have a negative impact on the foraging activity of the pollinator and pollination success (Eisikowitch et al., 1990; Herrera et al., 2013).

2.7 Nectary Enzymes and their Activities

There is a strong metabolic contribution of the nectary to nectar production, which is based on the presence and activity of a variety of enzymes in the nectary that are active directly before and during the peak hours of nectar production (Orona-Tamayo et al., 2013). The active metabolic capacity of the nectary has been studied by *in vitro* experiments showing that excised nectaries continue to secrete nectar for a period of time when carbohydrates are supplied (Bieleski & Redgwell, 1980; Findlay et al., 1982; Nichol & Hall, 1988). For example, nectar secretion in excised *Ricinus communis* nectaries was found to be sustained when fructose, glucose, and sucrose were provided; however, in the presence of 3-*O*-methyl-D-glucopyranose, raffinose, sorbitol, or xylose, nectar secretion did not take place (Nichol & Hall, 1988). Enzyme activities associated with carbohydrate metabolism that have been reported in nectaries include acid and alkaline phosphatase, ATPase, fructokinase, hexokinase, invertase, phosphoglucomutase, phosphohexoisomerase, pyrophosphatase, starch hydrolases and phosphorylases, sucrase, sucrose synthetase, and UTP-glucose-1-phosphate-uridyltransferase (de Fekete et al., 1967; Nichol & Hall, 1988; Orona-Tamayo et al., 2013). For nectaries storing starch for nectar production, the presence of starch metabolic enzymes such as ADP-glucose pyrophosphorylase has also been reported and confirmed by Western blot analysis (Ren et al., 2007a).

A number of literature reports on the presence of invertase in nectaries, as indicated by its activity, have been made (Pate et al., 1985; Nichol & Hall, 1988; Nicolson, 2002; Kram & Carter, 2009; Ruhlmann et al., 2010; Orona-Tamayo et al., 2013; Lohaus & Schwerdtfeger, 2014). For example, it has been shown that invertase activity was present in: the stipel and inflorescence nectaries of cowpea (Pate et al., 1985); the extrafloral nectaries of *Acacia* and *Ricinus* (Nichol & Hall, 1988; Orona-Tamayo et al., 2013); and the nectaries of plum and pear (Bieleski & Redgwell, 1980).

Enzymes involved in sucrose synthesis may also be present in the nectary (de Fekete et al., 1967). The presence of a sucrose synthesizing enzyme in some plant species is supported by their nectars having a greater sucrose than hexose concentration due to sucrose synthesis in the nectary prior to, or during, nectar secretion (Zauralov & Pavlinova, 1975; Nichol & Hall, 1988). Previously, the carbohydrate synthesis and catabolism enzyme, sucrose synthase (EC 2.4.1.13) has been implicated as the enzyme responsible for sucrose synthesis as this enzyme catalyzes the reversible chemical reaction between UDP (uridine diphosphate)-glucose and fructose, to produce

UDP and sucrose (Koch, 2004). However, sucrose synthase has been proven to have a catabolic function *in vivo* (Sturm & Tang, 1999).

In addition to carbohydrases, enzymes that use amino acids and proteins as substrates have been observed in nectaries. Select examples and their possible functions include: glutamine synthetase and methionine synthase for amino acid metabolism (Orona-Tamayo et al., 2013); the nectarins and terpene synthase for wound protection and plant pathogen control (Song et al., 2000; Carter & Thornburg, 2003, 2004a, b, c; Tholl et al., 2005); serine carboxypeptidase for protein turn-over or processing (Lehfeldt et al., 2000; Kram et al., 2009; Escalante-Peréz et al., 2012); and calreticulin for the chaperoning and regulation of Ca^{+2} homoeostasis (Nelson et al., 1997; Michalak et al., 1999).

2.8 Carbohydrate Analysis

A number of chromatographic methods have been applied to nectar carbohydrate analysis including: capillary gas chromatography (CGC); high performance liquid chromatography (HPLC); paper chromatography (PC); and thin layer chromatography (TLC). The two techniques that were used in this research were HPLC and CGC and as such, these two analytical techniques will be discussed in the following sections.

2.8.1 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is an analytical technique wherein compounds in a mixture are separated based on differences in their partitioning between a liquid mobile and a solid stationary phase. The application of HPLC for the analysis of carbohydrates in biological materials has received a great deal of research attention based on a number of important factors, which include but are not limited to: both accurate and precise analytical results are obtained; minimal sample preparation is required; sample analysis times are short; and the separated carbohydrates can be isolated for further analysis (Low, 1994). A number of HPLC separation modes have been applied to the analysis of carbohydrates in biological materials and the three major techniques will be discussed in the following sections.

2.8.2 HPLC Separation Methods

2.8.2.1 Normal Phase Chromatography

Normal phase chromatography employs a polar stationary phase coupled with either a non-polar or an intermediate polarity mobile phase. Stationary phases that are commonly used in normal phase chromatographic analysis of carbohydrates include polar solid adsorbents such as microparticulate silica or pellicular resins alone and this same material covalently modified with aminopropyl, cyanopropyl, and diol functional groups (Riley, 1996). For carbohydrate analysis, these stationary phases are most often used in combination with an intermediate polarity mobile phase such as acetonitrile:water (70-80%:30-20%). Solute affinity for the stationary phase (i.e., retention) increases with increasing polarity of both the analyte and the stationary phase and with decreasing polarity of the mobile phase (Jandera, 2011). Select examples of normal phase chromatographic separation of carbohydrates and polyols in biological samples include the analysis of: arabinose, fructose, glucose, lactose, maltose, mannose, rhamnose, sucrose, and xylose in chicory root, grapefruit juice, honey, milk powder, and molasses samples (Wight & van Niekerk, 1983); arabinose, fructose, galactose, glucose, kestose, maltose, mannitol, mannose, nystose, raffinose, sucrose, and xylose in corn stover (Agblevor et al., 2007) and onion (Davis et al., 2007; Downes & Terry, 2010); and fructose, glucose, sorbitol, and sucrose in apple and pear juice (Thavarajah & Low, 2006a).

Carbohydrate analysis employing normal phase chromatography suffers from a number of limitations including: Schiff's base formation between reducing carbohydrates and the amino functions of the stationary phase which can negatively impact analyte separation and quantitation and also reduce column lifetime; mobile phase solvents (e.g., acetontitrile) are expensive to purchase and dispose; and limited pH range of the mobile phase due to stationary phase solvation (silica gel at pH >8) and functional group hydrolysis (covalently modified silica gel or pellicular resins at pH <2) (Porsch, 1982; Brons & Olieman, 1983; Yamauchi et al., 1993; Hicks, 1995).

2.8.2.2 Reverse Phase Chromatography

Reverse/Reversed phase chromatography employs a non-polar stationary phase coupled with either a polar or intermediate polarity mobile phase. Non-polar stationary phases traditionally consist of microparticulate silica or pellicular resins with covalently bound alkyl (e.g., octadecyl [C_{18}], octyl [C_8], butyl [C_4]) or aryl functional groups with mobile phases consisting of unbuffered

or buffered aqueous-organic solvent mixtures containing acetonitrile, isopropanol, methanol, n-propanol, or tetrahydrofuran (El Rassi, 1996). Reverse phase chromatography has been employed for the separation of both small (monosaccharides) and large (oligosaccharides) carbohydrate molecules in biological samples. Solute affinity for the stationary phase increases with increasing hydrophobicity of both the analyte and the stationary phase and with increasing polarity of the mobile phase (Jandera, 2011). Select examples of reverse phase chromatographic separation of monosaccharides and oligosaccharides in biological samples include the analysis of: raffinose, stachyose, sucrose, and verbascose from soybeans, lupin seeds, and fermented soybean products (Wight & Datel, 1986); glucose, mannitol, sucrose, and xylose from the rhizomes of *Picrorhiza* species (Bhandari et al., 2008); and fructose, glucose, kestose, nystose, and sucrose in onion (Downes & Terry, 2010).

With the exception of Schiff's base formation, the limitations of reverse phase chromatography for carbohydrate analysis are similar to those observed for normal phase. Additional disadvantages include: poor resolution of monosaccharides employing an octadecyl stationary phase because of short analyte retention times and the presence of anomers (Verzele et al., 1987; El Rassi, 1995); and poor resolution of oligosaccharides within the same class (i.e., for the separation of structurally similar di- and trisaccharides) (Hicks, 1995; Brokl et al., 2011)

2.8.2.3 Ion Exchange Chromatography

Ion exchange chromatography is based on the electrostatic attraction (or repulsion) of the analyte to the stationary phase. Ion exchange chromatography is subdivided into cation and anion exchange depending upon the functional groups on the stationary phase. In cation exchange chromatography, the stationary phase is negatively charged and has positively charged counterions (cations) available for exchange; whereas in anion exchange chromatography, the stationary phase is positively charged with negatively charged counterions (anions) available for exchange. Also, anion or cation stationary phases are further classified as being either weak or strong according to the pH range where the functional group is charged (Riley, 1996). Strong anion exchangers are immobilized quaternary ammonium ions and strong cation exchangers are immobilized sulfonic acids. Weak anion exchangers are immobilized amines and weak cation exchangers are immobilized carboxylic acids. Analyte interaction with the stationary phase is determined by the pKa/pK_b of its functional group(s) (e.g., hydroxyl, amine), the pH and ionic strength of the mobile

phase, and the stationary phase temperature (Riley, 1996). A major advantage of the use of ion exchange chromatography for carbohydrate analysis is the use of water or aqueous based mobile phases (e.g., 100 mM sodium hydroxide).

Cation exchange employing sulfonated polystyrene with Ca^{+2}, Ag^{+}, or Pb^{+2} as counterions has been widely used for carbohydrate analysis (Scobell & Brobst, 1981). Select examples include: the analysis of a range of floral nectars for their fructose, glucose, and sucrose content (Van Wyk et al., 1993; Barnes et al., 1995; Nepi et al., 2010; Nocentini et al., 2012); and the separation of fructose, glucose, and sucrose in juices, molasses, and wines (Duarte-Coelho et al., 1985). Major limitations of cation exchange chromatography for carbohydrates include the lack of resolution for oligosaccharides and the problem that the early elution of oligosaccharides (immediately after the void volume) can have on monosaccharide resolution (Huber & Bonn, 1995; Stefansson & Westerlund, 1996).

A major advancement in carbohydrate analysis was the development by Dionex Corporation of a microparticulate anion exchange-polymeric stationary phase comprised of negatively charged pellicular latex beads (5 to 10 µm in diameter) to which positively charged pellicular beads (0.1 µm in diameter) are either electrostatically or covalently bound. This stationary phase has been shown to have a number of benefits for carbohydrate analysis including: a large surface area for ion exchange; excellent resolution of both monosaccharides and structurally similar oligosaccharides; high mechanical and pH stability; and rapid mass transport and fast diffusion separation properties, which result in rapid sample analysis times (Lee, 1996; Cataldi et al., 2000; Fritz, 2004). This stationary phase is used in conjunction with a high pH aqueous mobile phase (e.g., 80-150 mM sodium hydroxide) as the weak acidic nature of carbohydrates (pKa values of 12 to 14) results in their partial ionization. Under these analysis conditions, carbohydrate separation is based on electrostatic interactions with the stationary phase, however size exclusion (due to the crosslinked nature of the latex beads) and adsorption (due to the small aqueous layer that surrounds the stationary phase) also play a role (Low, 1994).

Carbohydrate elution is a function of pKa with the analyte with the highest value eluting first and the general elution order is polyols, monosaccharides, disaccharides, other oligosaccharides, and polysaccharides. The addition of sodium acetate to the basic mobile phase increases the ionic strength without affecting the overall pH and effectively competes with carbohydrate anions for the charged sites on the resin, resulting in sharper peak shape and more

rapid elution times of strongly retained oligo- and polysaccharides (Wong & Jane, 1995; Lee, 1996; Weiss & Jensen, 2003; Corradini et al., 2012). This type of ion chromatography is called high performance anion exchange (HPAE) chromatography and is used in conjunction with pulsed amperometric detection (PAD), which is discussed in detail in the following section. Select examples on the use of HPAE for the analysis of carbohydrates in biological materials include the analysis of: 20 structurally-similar carbohydrates (e.g., glucose, fructose, sucrose, maltose, erlose, panose) in alfalfa, alsike, canola, and trefoil unifloral honeys (Swallow & Low, 1990); oligosaccharides in a selection of New Zealand (Weston & Brocklebank, 1999) and Algerian (Ouchemoukh et al., 2010) honeys; fructose, glucose, and sugar alcohols in needles, twigs, and wood (Raessler et al., 2010); and inulin fragments, kestose, raffinose, starch, and sucrose in artichokes (Ronkart et al., 2007). In addition, HPAE-PAD has been utilized in a wide range of applications in agriculture, biotechnology, environmental and food sciences, and medicine (Cataldi et al., 2000; Corradini et al., 2012).

The major limitations of this separation method relate to the strong alkaline conditions to which the carbohydrates are exposed. These conditions can result in carbohydrate enolization and interconversion via a 1, 2-enediol intermediate (Lobry de Bruyn-van Ekenstein [1895] reaction) and the possible base catalyzed hydrolysis of glycosides (Speck, 1958; Wong, 1989).

2.8.3 HPLC Detection Systems

Although a number of detection systems have been employed in conjunction with analyte separation by HPLC, those that are commonly used for carbohydrate detection are limited to evaporative light scattering, pulsed amperometric, and refractive index. Each of these detection systems will be discussed in the following sections.

Refractive index (RI) is a physical property of a medium and as such this detection system is referred to as non-selective or bulk property. The principle of operation of this detection system is based on Snell's Law, which states that the index of refraction of a solution is equal to the ratio of the sine of the angle of incidence to the sine of the angle of refraction (Munk, 1983). Analyte detection is afforded by exposure of the RI cell, which contains a reference cell (pure mobile phase) and the sample cell (mobile phase plus analyte) to a beam of electromagnetic radiation from a light source (generally 660-880 nm). The change in refractive index between the two cells is measured

by comparing the relative intensity of the electromagnetic signal produced at a pair of photodiodes (one reference and one sample).

Refractive index is the most common detection system employed for carbohydrate analysis. Advantages of RI detection for carbohydrates include: its universality (all classes of carbohydrates can be detected); wide linear response range; and reasonable sensitivity (Low, 1994). Limitations of this detection system include: low sensitivity for carbohydrates (µg range for monosaccharides); that only isocratic mobile phase systems can be employed unless computer assisted gradient adjustment is employed; and the fact that RI measurements are sensitive to changes in mobile phase flow rate, pressure, and temperature (Low, 1994).

The evaporative light scattering detector (ELSD) is a non-selective detector of non-volatile analytes, which was first described by Charlesworth (1978). The principle of operation of this detection system is based upon the nebulization of the eluent from the HPLC column in a stream of hot (40-90ºC) inert gas (e.g., N_2). Under these conditions, the mobile phase is evaporated and small, uniform-sized analyte particles are produced, which are then exposed to a beam of electromagnetic radiation that is normally generated by a light emitting diode (LED; 660 nm for the red LED) or a laser. The amount of scattered light is measured at an angle of 30, 45, or 60º (depending upon the manufacturer of the instrument) employing a photomultiplier. The amount of scattered light is proportional to the concentration of analyte in the sample. Evaporative light scattering detection has been employed in the analysis of mono-, oligo-, and polysaccharides in foods (Churms, 2002; Lafosse & Herbreteau, 2002; Davis et al., 2007; Estevinho et al., 2009; Muir et al., 2009; Morlock & Sabir, 2011). Advantages in the use of ELSD for carbohydrate analysis include its universality (all classes of carbohydrates can be detected), sensitivity (ng detection limits for monosaccharides), and that it can be employed with gradient elution (Clement et al., 1992; Meyer, 1998; Lafosse & Herbreteau, 2002; Estevinho et al., 2009).

Carbohydrate detection employing electrochemistry is based on the presence of a selection of electroactive functional groups on these analytes including amine, aldehyde, hydroxyl, and ketone. In electrochemical detection, as an analyte elutes from the HPLC column, it passes through the electrochemical cell where it undergoes either oxidation or reduction at the working electrode. The original electrochemical detection system which employed Pt as the electrode suffered from the accumulation of oxidized products on the working electrode, which resulted in decreased detector response over time (Breiter, 1963). The development of commercial triple pulsed

amperometric/pulsed electrochemical detection (PAD/PED) systems employing noble metal (e.g., Au) electrodes in the late 1970's overcame this fouling problem. The combination of HPAE chromatography with this detection system (HPAE-PAD) resulted in an excellent analytical system for carbohydrate analysis (Olechno et al., 1987; La Course, 1997; La Course, 2002).

The triple pulsed waveform employed for carbohydrate detection at a solid gold electrode employing a flow-through electrolysis cell is shown in Figure 2.4. Initially the gold electrode is maintained at a potential <+200 mV and carbohydrate oxidation (aldehyde, hydroxyl, and ketone functional groups) occurs at the electrode (E_{det}) for a time period of about 430 milliseconds (ms) (t_1). Following the detection process, a two-step cleaning process of the electrode surface is conducted employing a positive oxidation potential step (E_{oxd}) of +600-800 mV for a period ranging from 50 to 200 ms (t_2) and a cathodic reduction potential step (E_{red}) of -800 to -200 mV for a duration of 100 to 600 ms (t_3) (Cataldi et al., 2000; Corradini et al., 2012).

The mechanism of aldehyde, hydroxyl, and ketone oxidation at the gold electrode in an alkaline mobile phase has been proposed to follow a free radical reaction on the basis of an oxide-catalyzed oxygen transfer mechanism (Johnson & Pola, 1986; Larew & Johnson, 1989); [Figure 2.5]). The first step involves the formation of AuOH at the gold electrode surface (Step 1). In alkaline media, the hydroxyl group in carbohydrates forms RO^- (Step 2). The resulting RO^- is adsorbed on the gold electrode surface (Step 3) followed by hydrogen abstraction from the ROAu complex to form a stabilized free radical at the gold electrode surface (Step 4). The free radical complex is then replaced by a hydroxyl ion on the gold surface resulting in the oxidation of the hydroxyl group into an aldehyde or ketone (Step 5).

Carbohydrate analysis employing pulsed amperometry with a gold electrode has a number of advantages including: no pre- or post-column derivatization; selectivity (as only carbohydrates are oxidized under the specific potentials employed); and excellent sensitivity (low picomole range) (Raessler, 2011). HPAE-PAD has been extensively utilized for carbohydrate analysis as supported by the separation of a series of neutral oligosaccharide standards (triose to undecaose) based on their molecular size, composition, and anomeric linkage (Hardy & Townsend, 1988); and the separation of structurally similar disaccharides and trisaccharides in honey (Swallow & Low, 1990).

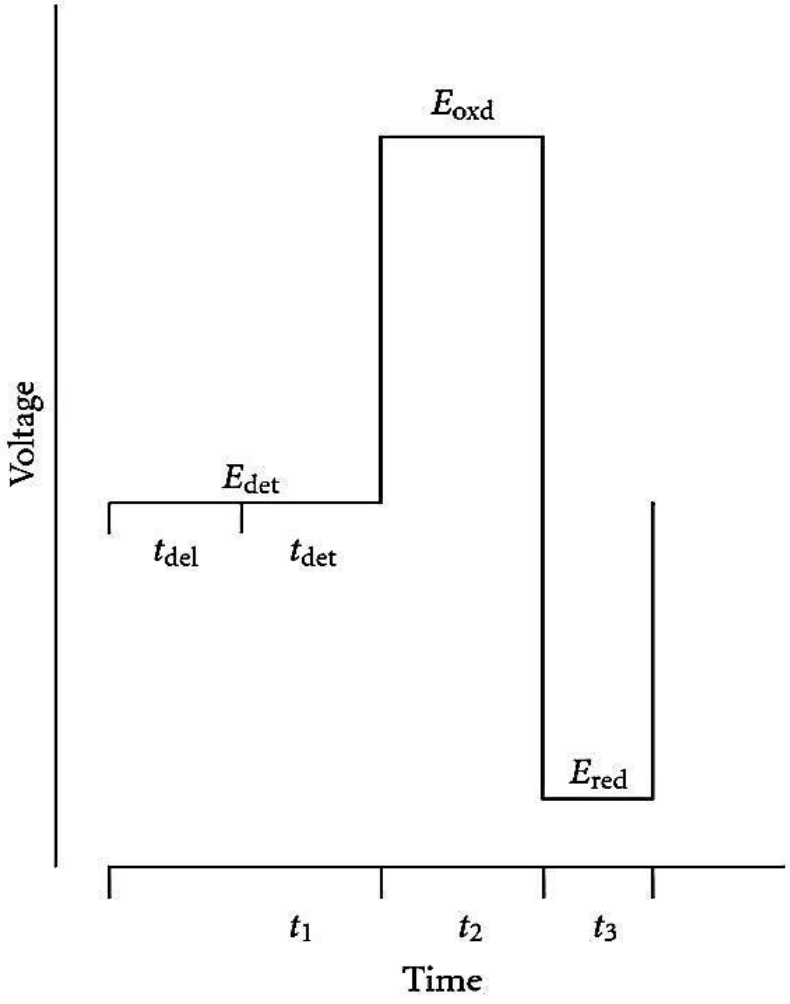

Figure 2.4 Triple-pulsed potential waveform designed for carbohydrate detection. Qualitative waveform, where E_{det} is the constant potential E_1 applied for time t_1; E_{oxd} is the potential E_2 (full oxidation on the working gold electrode), applied for time t_2; E_{red} is the negative potential applied during time t_3 to convert gold oxide back to native metal (adapted from Corradini et al., 2012).

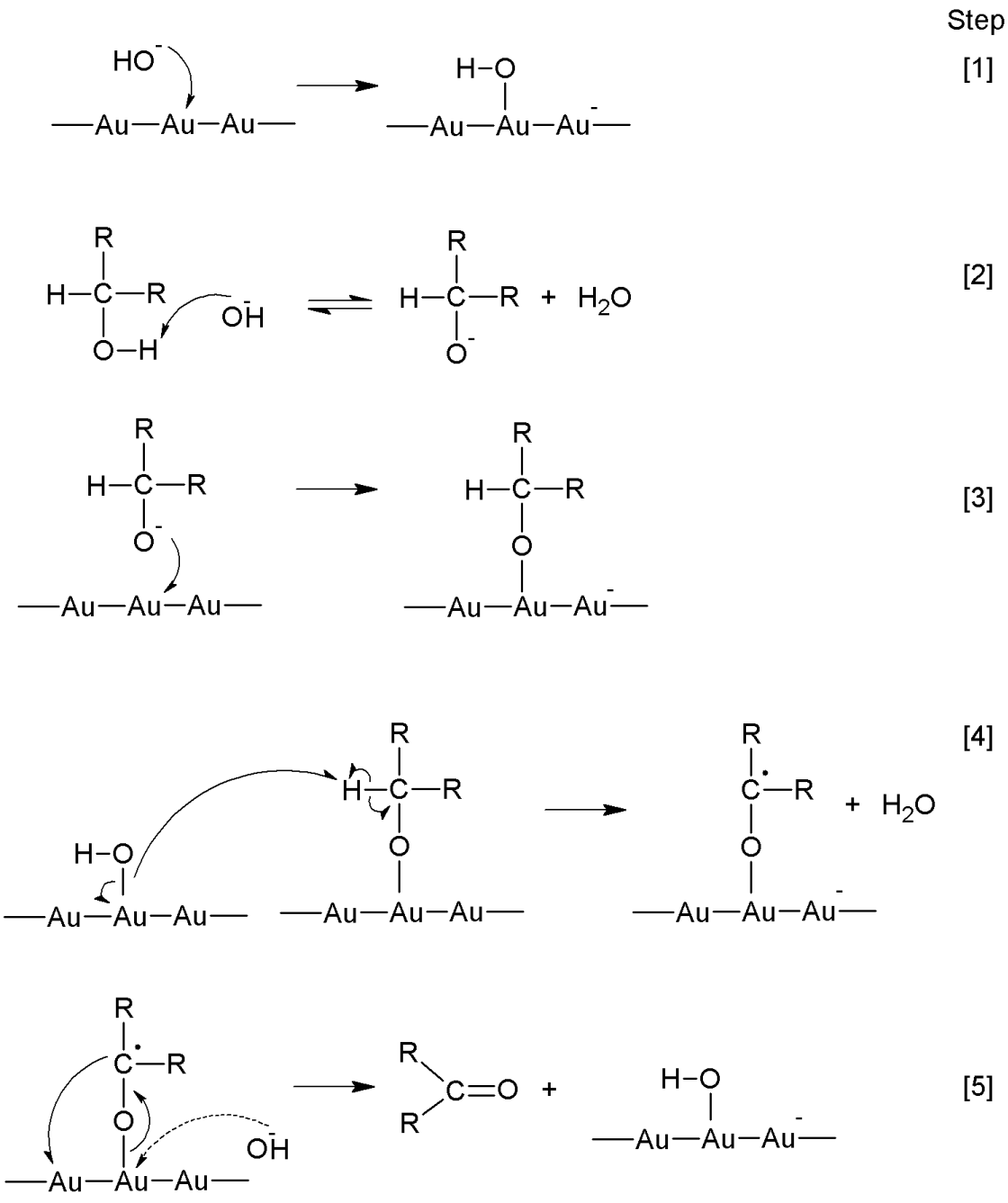

Figure 2.5 The mechanism of hydroxyl group oxidation at a gold electrode in alkaline media (adapted from Larew and Johnson, 1989).

2.8.4 Nectar Carbohydrate Analysis by HPLC

Literature contains a number of examples of the HPLC analysis of nectar carbohydrates and select examples are: (a) HPLC-RI: detection of fructose, glucose, and sucrose in the nectars of *Erica* (Barnes et al., 1995), eucalyptus (Morrant et al., 2009), honeywort (Nocentini et al., 2012), and species of Alooideae, Gentianales, and Proteaceae (Van Wyk et al., 1993; Nicolson & Van Wyk, 1998; Wolff, 2006); and (b) HPAE-PAD: where fructose, glucose, sucrose, and other carbohydrates (arabinose, lactose, maltose, melibiose, trehalose) were successfully resolved, detected, and quantitated in the nectars of *Brassica napus*, *Lophospermum erubescens*, *Maurandya barclayana*, and the perennial herbs, *Aquilegia* spp. and *Helleborus foetidus* (Davis et al., 1994, 1998; Petanidou, 2005; Herrera et al., 2006; Canto et al., 2007; Lohaus & Schwerdtfeger, 2014).

2.8.5 Capillary Gas Chromatography (CGC) with Flame Ionization Detection (FID)

An alternate chromatographic method for the analysis of carbohydrates is CGC. In this chromatographic method, a non-polar stationary phase, such as 5% diphenyl-95% dimethyl-polysiloxane (e.g., DB-5) with a film thickness of 0.25 µm, chemically bound to a 30 m x 0.25 mm (i.d.) fused silica column, is used in conjunction with a gaseous mobile phase (H_2, He, or N_2). Analytes are separated based on their affinity for the stationary phase and their vapourizaton temperature, which is generally achieved employing a temperature gradient. Analytes are generally quantified employing a flame ionization detector (FID), which is a universal detector for organic compounds with detection limits of ng to pg for carbohydrates. The principle of operation of the FID involves analyte pyrolysis/combustion that results in the production of ions and electrons, which are collected so as to produce an ion current (Skoog et al., 1996).

Analyte volatility is an essential criterion for CGC analysis, which is one of the main limitations of CGC for carbohydrate analysis. As such, derivatization of all hydroxyl groups is required for CGC analysis of carbohydrates and two common methods for derivatization are acetylation and silylation (Sanz & Martínez-Castro, 2007; Ruiz-Matute et al., 2011). Trimethylsilylation is often the method of choice for carbohydrates because: the derivatives formed are more volatile leading to shorter analysis times; capillary columns can be operated at lower temperatures, prolonging column lifetime and decreasing baseline noise; and silyl derivatives generally result in sharper peaks than their corresponding acetylated derivatives (Laker, 1979; Low, 1994). However, this derivatizaton requirement for CGC analysis has a

number of drawbacks including, but not limited to: hydroxyl group derivatization must be performed at high temperatures (>60°C) and extremely low sample moisture content so as to prevent reagent deactivation; and the addition of a trimethylsilyl (or an acetyl) moiety to each hydroxyl group of a carbohydrate significantly increases its molecular weight, which generally limits the usefulness of CGC to polyols, monosaccharides, disaccharides, and trisaccharides only (Low, 1994). A second limitation of carbohydrate analysis by CGC is the fact that reducing carbohydrates may exist in the following four tautomeric forms, α, β-pyranoside and α, β-furanoside. As each of these compounds can be detected by CGC, the resulting chromatograms can become quite difficult to interpret. This issue can be alleviated by reduction of the carbonyl group using sodium borohydride (alditol formation) or hydroxylamine-HCl (oxime formation) prior to derivatization and CGC analysis (Low, 1994).

Select examples on the use of CGC for the analysis of carbohydrates in nectars include the analysis of: fructose, glucose, raffinose, sucrose, and other oligosaccharides in the extrafloral nectars of different orchid species (Baskin & Bliss, 1969); arabinose, fructose, glucose, maltose, raffinose, sucrose, and xylose in the extrafloral nectar of the savannah grass, *Andropogon* (Bowden, 1970); and fructose, glucose, and sucrose in the nectars of *Mucuna* (Agostini et al., 2011) and from other species in the temperate forests of Patagonia (Forcone et al., 1997; Chalcoff et al., 2006).

2.9 Nectary Proteomics

The metabolic contribution of the nectary to both nectar chemical composition and production is significant (Orona-Tamayo et al., 2013). Therefore, the identification of the proteins/enzymes (e.g., carbohydrases) involved in carbohydrate metabolism in the nectary are integral to elucidating the mechanisms of nectar carbohydrate formation and to better understand the function of the nectary in nectar production and chemical composition. Proteomics is an analytical technique that can be applied to study the protein composition of nectaries. This technique will be discussed in the next section with specific examples of its application to nectaries presented.

2.9.1 Proteomics Introduction and Definition

The term "proteome" was first coined by Marc Wilkins (Swinbanks, 1995) and refers to the total set of proteins or gene products found in the organelle, cell, tissue, organ, organism, population, and ecosystem at a specific developmental stage and environmental conditions (Jorrin-Novo, 2014). Proteomics is the large-scale study of proteins and covers much of the functional analysis of gene products or 'functional genomics' and includes the large-scale identification, localization, and interaction studies of proteins (Pandey & Mann, 2000). However, it is more than the simple cataloguing of proteins, as the goals of proteomics include the determination of the function, site and time of production, and mode of formation of proteins within a specified system (e.g., cell, tissue). Proteomics can also be used to help explain protein-protein interactions and protein interactions with other molecules. Finally, proteomics can be used to identify, quantify, and elucidate the structure and function of a complete protein complement (Phizicky et al., 2003).

Proteomics emerged from protein chemistry/biochemistry methods including extraction, purification (e.g., gel electrophoresis and liquid chromatography-based techniques), labeling, identification (e.g., Western blot), and sequencing (e.g., Edman degradation) (Jorrin-Novo, 2014). However, the most significant breakthroughs in proteomics were made possible by the development of mass spectrometry (MS) and systematic sequencing technologies. Mass spectrometry is a highly sensitive, high-throughput technology that extended protein analysis beyond mere visualization (Pandey & Mann, 2000) and enabled the determination of protein/peptide molecular weight and sequence information (Jonscher & Yates, 1997). An improved system for protein identification was also made possible by the development of systematic sequencing technologies and the inclusion of this information into large sequence databases. Proteins are identified by the correlation of experimental information from the analysis of peptides with sequence information in databases (Aebersold & Goodlett, 2001).

In addition to MS, other analytical advances that have contributed to proteomics development include, but are not limited to: cell imaging by light and electron microscopy, array and chip technologies, and genome-wide two-hybrid protocols (e.g., yeast two-hybrid assay). Based on its important role in proteomics development and because it was the technology utilized in the analysis of the protein complement of nectaries in this study, only MS-based proteomics will be discussed in this literature review.

2.9.2 Mass Spectrometry (MS)-Based Proteomics

A generic MS-based proteomics experiment typically consists of five stages (Aebersold & Mann, 2003; Steen & Mann, 2004): i) protein isolation from cell lysate or tissues by biochemical fractionation or affinity selection often including the final step of one-dimensional gel electrophoresis (SDS-PAGE); ii) in-gel protein digestion into peptides (trypsin digestion); iii) peptide separation/purification on- or off-line using single or multiple dimensions of separation (e.g., HPLC); iv) introduction of peptides into a MS ion source (usually via electrospray) and MS analysis of peptides; and v) matching of peptide-sequencing data (mass spectrum) against protein databases using appropriate searching programs.

The next sections of this literature review will be used to discuss the details of each of the aforementioned steps and will cover the experimental methods specifically used in completing this research objective.

2.9.3 Protein Extraction Methods

Sample proteins require extraction and isolation before proteomic analysis because they are often compartmentalized in a specific cell or tissue and the presence of other compounds in the sample can interact with these biomolecules making their analysis difficult (Zhang et al., 2013). A number of chemical compounds present in plants such as proteases, polyphenols, starch, lipids, and secondary metabolites have been shown to interfere with protein analysis (Granier, 1988; Gegenheimer, 1990; Tsugita & Kamo, 1999). For example, the presence of cell wall and storage polysaccharides, lipids, phenolic compounds, and a variety of secondary metabolites were found to cause horizontal and vertical streaking, smearing, and/or decreased resolution of protein bands/spots during total plant protein electrophoresis (Saravanan & Rose, 2004). The abundance of proteins such as Rubisco or seed storage proteins may also dominate protein profiles and interfere with protein analysis (Chen & Harmon, 2006). Ideally, a method that will reproducibly extract all proteins from a sample with minimal interference from non-protein substances is desired. However, finding a universal protocol for sample protein extraction is difficult because of the dynamic range in protein abundance and molecular weight, charge, post-translational modifications, distribution, and interactions with non-protein compounds (Rose et al., 2004). Due to these issues, a number of protocols using a variety of physical treatments, solvents, and buffers have been developed for protein extraction from various cells and tissues (Rabilloud, 1996).

The most common method for protein extraction is based on its precipitation employing trichloroacetic acid (TCA) and acetone (Damerval et al., 1986; Santoni et al., 1994). The TCA/acetone method is based on protein denaturing under acidic and/or hydrophobic conditions, which results in protein concentration with minimal precipitation of interfering compounds such as salts or polyphenols (Görg et al., 2004; Chen & Harmon, 2006). The method is also very effective at inhibiting unwanted enzyme activities including, but not limited to protease, phenoloxidase, and peroxidase (Damerval et al., 1986; Granier, 1988; Saravanan & Rose, 2004). However, the resulting protein pellet can be difficult to dissolve (Chen & Harmon, 2006) and protein losses can occur because of incomplete solubilization and/or precipitation of sample proteins (Görg et al., 2004). In addition, TCA can cause the precipitation of nucleic acids longer than 20 nucleotides and protein hydrolysis (Chen & Harmon, 2006).

Alternate protein precipitation methods have been developed and include protein solubilization in phenol, with or without sodium dodecyl sulfate (SDS), followed by precipitation via the addition of methanol and ammonium acetate (Hurkman & Tanaka, 1986; Meyer et al., 1988). A widely employed protein precipitation method involves the extraction of sample tissue powder in extraction buffer/buffered phenol (pH 8.0) followed by methanol or acetone precipitation of the phenol phase (Wang et al., 2008). It has been reported that protein extracts produced with this method were of better quality (i.e., higher purity) because polysaccharides, nucleic acids, and salts are separated into a discrete aqueous phase or are centrifuged into a separate pellet from the protein-rich phenol layer (Isaacson et al., 2006). However, the method is laborious, time consuming, and solubilization of the protein pellet can be difficult (Wang et al., 2003; Chen & Harmon, 2006).

A study by Saravanan and Rose (2004) showed that the combination of the TCA/acetone and phenol methods improved the results obtained from the proteomic analysis of plant tissues. Based on this work, Wang et al. (2006) combined TCA/acetone and phenol extraction and included methanol washes so as to develop a universal and rapid protein extraction protocol for recalcitrant tissues. This protocol combined the benefits of TCA/acetone precipitation (reduced extraction of lipids, phenolics, and pigments) as well as phenol extraction (reduced extraction of nucleic acids, polysaccharides, and salts and further elimination of lipids, phenolics, and pigments) (Wang et al., 2008). The developed protocol was relatively simple and rapid as minimal sample handling was required. The protein pellet could then be solubilized in the buffer of choice, such as the SDS

sample buffer or the isoelectric focusing rehydration buffer. The use of this combined protocol virtually eliminates protein pellet solubility difficulties observed when only one of these methods is employed for sample protein extraction. However, if these difficulties are observed, they can be alleviated by prolonged incubation of the protein pellet in the buffer accompanied by shaking (Wang et al., 2008), or by increasing the buffer volume (Isaacson et al., 2006). The aforementioned protocol proved to be effective for high-quality protein extraction from a wide range of tissues including: high-polyphenol containing leaves (olive and pine); and fruits that have low protein (apple and pear), high carbohydrate (banana), acid (grape and orange), and pigment (olive and tomato) concentrations (Wang et al., 2006). Sample proteins extracted with this protocol were found to be suitable for electrophoretic analysis with less vertical and horizontal streaking and smearing observed. Based on this literature information, the protocol published by Wang et al. (2006) was used in this research with slight modifications based on nectary protein concentration.

2.9.4 Protein Separation by One-Dimensional Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein mixtures extracted from plant tissues can be separated by SDS-PAGE so as to elucidate sample protein profiles and determine their molecular weights (Laemmli, 1970). For proteomics research, individual separated protein bands on the gel can be excised for enzymatic hydrolysis followed by MS analysis. Additional advantages of SDS-PAGE analysis include a large molecular mass range for protein separation, high sample protein solubility, and further protein purification (Pandey & Mann, 2000).

In SDS-PAGE, the anionic detergent SDS forms a micellular complex with proteins making the surface negatively charged (Arndt et al., 2012). When the resulting charged and denatured proteins are subjected to an external electric field, they move towards the anode. In principle, migration distance depends upon size, with smaller proteins moving faster and is negatively correlated to the log of the molecular weight of the protein. The relative molecular weight and abundance of sample proteins can be determined when using SDS-PAGE prior to MS. It has been shown that the confidence in protein identification employing dynamic range measurements with comparison to database results increases when SDS-PAGE is utilized prior to MS (Steen & Mann, 2004).

2.9.5 Protein Digestion

Prior to proteomic analysis, sample proteins are digested to peptides using proteolytic enzymes (proteases) or chemical reagents (Lundell & Schreitmüller, 1999). This step which is most often used as the direct MS analysis of proteins suffers from a number of limitations including, but not limited to: handling issues (protein instability), protein solubility, and decreased sensitivity (ionization issues) (Steen & Mann, 2004; Zhang et al., 2013). Proteases are commonly used for protein digestion rather than chemical reagents (e.g., cyanogen bromide), as the latter generates smaller numbers of peptides, which can limit protein identification. Protease (e.g., trypsin) digestion of proteins normally generates a significant peptide map resulting in thousands of mass spectral data points, which affords more reliable protein identification (Hustoft et al., 2012).

Prior to protease digestion, proteins are denatured, reduced, and alkylated so as to more effectively produce a significant peptide map. Urea, guanidine hydrochloride, or organic solvents (e.g., acetonitrile) are commonly used to solubilize and denature proteins (Medzihradszky, 2005). However, the disulphide bonds that stabilize protein structure (Wedemeyer et al., 2000) are not broken using simple denaturing methods and must be cleaved employing reducing agents (e.g., dithiothreitol) followed by alkylation (e.g., iodoacetamide) so as to prevent disulphide bond reformation (Cleland, 1964; Lundell & Schreitmüller, 1999).

Proteolytic enzymes cleave peptide bonds on either the N- or C-terminus of a specific amino acid residue, or combination of residues (Zhang et al., 2013). Examples of these enzymes include trypsin, chymotrypsin, subtilisin, and elastase. One of the most important requirements for protein digestion for proteomics analysis is protease specificity, as the cleavage activity of less sequence-specific proteases results in overlapping peptide signals that can complicate peptide analysis and protein identification (Steen & Mann, 2004).

The serine protease, trypsin, is most often employed for peptide production for proteomic analysis. Trypsin hydrolyses peptide bonds at the carboxyl side (C-terminus) of arginine and lysine residues but with some exceptions (Hustoft et al., 2012). Trypsin hydrolysis is prevented when lysine is N- or C-linked to aspartic acid, or if lysine is N-linked to cysteine and C-linked either to aspartic acid, cysteine, histidine, or tyrosine. Hydrolysis is also prevented if: arginine is N-linked to aspartic acid; arginine is N-linked to arginine and C-linked to histidine; arginine is N-linked to cysteine and C-linked to lysine; or arginine is N- and C-linked to arginine. Trypsin

hydrolysis is also prevented when proline is present on the carboxyl side of lysine or arginine however, it may be circumvented when lysine is: N-linked to tryptophan; arginine is N-linked to methionine; or glutamic acid is present at the N-terminal of arginine or lysine (Gasteiger et al., 2003).

The advantages of the use of trypsin for protein digestion for proteomics analysis include: production of peptides in the preferred mass range (800-2000 Da) for MS, based on arginine and/or lysine residue frequencies in proteins at the approximate rate of one every 10-12 amino acids; and homogeneous fragmentation under collision-induced dissociation methods yielding easily interpretable, information-rich fragmentation spectra, where if included in an appropriate database, these peptide fragments will provide sufficient sequence information for protein identification (López-Ferrer et al., 2006).

2.9.6 Sample Purification

Following proteolytic enzyme digestion and prior to MS analysis, digests are often purified using a ZipTip® for buffer and salt removal and sample concentration (Capelo et al., 2009). A ZipTip® is a pipette tip that contains a bed of C_{18} silica that can be employed for single-step desalting, enrichment, and purification of protein and proteolytic enzyme treated samples (Hustoft et al., 2012). A second purification step involves peptide/protein separation prior to MS analysis employing reverse phase chromatography employing a capillary (50-150 µm i.d.; 150 mm long) LC column, which is directly coupled to, or is on-line with the MS instrument (LC-MS; Steen & Mann, 2004). Separated peptides are then directly introduced into the ionization source of the MS instrument.

2.9.7 Mass Spectrometry (MS)

A mass spectrometer consists of an ion source, mass analyzer, and ion detector. Mass spectrometry is based on the determination of the molecular mass of individual molecules by transforming them into ions *in vacuo* followed by the measurement of their trajectory response to electric and/or magnetic fields (Fenn et al., 1989).

The first step in mass spectrometry involves molecule conversion into gas-phase ions by their transfer from a solution or solid phase into the gas phase. Electrospray ionization (ESI) (Fenn et al., 1989; Fenn, 2003) and matrix-assisted laser desorption/ionization (MALDI) (Karas et al.,

1985; Karas & Hillenkamp, 1988) are the commonly used ionization methods for proteomic studies. These are often called 'soft' ionization methods because they are able to generate ions from large, non-volatile analytes such as proteins and peptides without significant analyte fragmentation (Aebersold & Goodlett, 2001). Electrospray ionization is the most popular choice as it is readily interfaced with modern analyte separation techniques including HPLC, CGC, and capillary electrophoresis (Lee et al., 1988). Following ionization, sample analytes are separated in the mass analyzer based on their mass-to-charge-ratio (m/z) (Aebersold & Mann, 2003). There are four basic types of mass analyzers used in modern mass spectrometer instruments, which differ in design, performance, and how they separate charged (e.g., m/z ratio) analytes such as peptides. They include: time-of-flight (TOF), ion trap (Quadrupole ion trap, QIT; linear ion trap, LIT or LTQ), quadrupole time-of-flight (QTOF), and Fourier transform ion cyclotron (FT-MS) analyzers.

As the separated ions strike the MS detector, a mass spectrum is recorded, which is comprised of a record of the signal intensity of the ion at each value of the m/z scale (Yates, 2004). For protein identification, the obtained peptide mass spectra can be interpreted manually to determine their amino acid sequences (*de novo* sequencing) or they can be compared to appropriate databases (Chen & Harmon, 2006).

In this study, reverse phase LC separation using a high-capacity HPLC chip was employed prior to peptide fragment analysis. Positive-ion electrospray mass spectra were acquired and analyzed using a QTOF mass analyzer/spectrometer.

2.9.8 Database Searching for Peptide and Protein Identification

Database searching involves the comparison of the theoretical mass spectrum of a protein digest to that obtained by experimentation, wherein peptide identification depends on the best match between theoretical and observed spectra. The method involves the *in silico* digest of a proteome database using the known specificity of the enzyme used (e.g., protease) and a theoretical mass spectrum is predicted for each peptide, which is compared with the experimental mass spectrum (Mallick & Kuster, 2010).

The four main comprehensive protein/peptide databases that are commonly used in proteomics are the International Protein Index (IPI), the UniProt Knowledgebase (UniProtKB), Ensembl, and National Centre for Biotechnology Information's (NCBI) non-redundant (nr) database (Griss et al., 2011). These protein databases have different emphases and hence, they

differ in terms of completeness, degree of redundancy, and quality of annotations. For example, non-redundant protein databases contain a consensus sequence for each protein and known variants are collapsed into a single entry, whereas a comprehensive, non-identical database explicitly represents every known protein sequence (Cottrell, 2011).

The International Protein Index is a non-redundant protein database created from different source databases (e.g., Swiss-Prot, TrEMBL, Ensembl, RefSeq) where entries that represent the same protein from the different source databases are clustered (Kersey et al., 2004). Although IPI is balanced in terms of redundancy and completeness, it was discontinued in 2011. UniProtKB/TrEMBL is a combination of the high quality manually annotated protein knowledgebase (UniProtKB/Swiss-Prot) and the automatically annotated records of UniProtKB/TrEMBL (UniProt Consortium, 2011). UniProtKB has high quality records with a minimal degree of redundancy. Ensembl is a database of automatic annotations from the human genome sequence and produces protein sequence sets based on gene predictions (Flicek et al., 2011). This enables Ensembl to establish the connection among proteins, genes, and transcripts. Lastly, the NCBInr database compiles protein sequences from: GenBank translations, Protein Data Bank (PDB), UniProtKB/Swiss-Prot, Protein Information Resource (PIR), and Protein Research Foundation (PRF) (Sayers et al., 2011). Information in the NCBI nr database provides a high degree of redundancy, however it is still a widely used resource for non-model organisms as they are well represented.

The type of protein sequence database used affects the sensitivity, specificity, and speed of the protein identification process. Longer search and protein identification times and more difficulty in protein identification are concomitant with database size (Cottrell, 2011). Also, larger and more inclusive databases may result in more false positive and reduced statistical significance (Edwards, 2011). Searching by employing a smaller database (e.g., Swiss-Prot) is more rapid and concise, but is less accurate if proteins are present in low amounts and produce only one or two peptide MS spectra, as these sequences may be missing in the database and protein identification is compromised (Cottrell, 2011).

The likelihood of the match of the experimentally obtained spectrum with the theoretical mass spectrum in the database is assessed using computer algorithms. Different methods of scoring peptide matches have been developed and have been classified as descriptive (e.g.,

Sequest, Sonar), interpretative (e.g., PeptideSearch, MS-Seq), stochastic (e.g., Scope, Olav), and probability-based (e.g., Mascot, OMSSA) (Sadygov et al., 2004).

These database algorithms are devised to calculate the score of an experimental mass spectral match to that of the expected theoretical mass spectrum. The candidate peptides may be narrowed down based on criteria such as: mass tolerance, proteolytic enzyme specificity, and types of post-translational modifications as specified by the user (Nesvizhskii et al., 2007). The database algorithms produce an output consisting of a list of fragment ion spectra matched to peptide sequences, ranked according to the search score or the degree of similarity between the experimental and theoretical mass spectra. Scoring schemes described in the literature include: spectral correlation functions, shared fragment counts, dot product empirically observed rules, and statistically derived fragmentation frequencies (Nesvizhskii et al., 2007). The score reported may be based on an arbitrary scale or may be converted to a statistical measure called the expectation (E) value or the number of times you would expect to get a score at least as high by chance. Therefore, small E values indicate a good match and matches with E values ≥ 1 indicate randomness.

Complete mass spectral matching of all peptides experimentally obtained with the predicted theoretical results rarely occurs. This is due to a number of factors that can occur before or during sample peptide MS analysis including, but not limited to: poor analyte solubility, select analyte chromatographic adsorption, ion suppression, select analyte ionization, and short peptide length; each of which can lead to analyte loss and poor detection (Aebersold & Goodlett, 2001). However, protein identification based on peptide mapping is still possible even though all of the predicted peptides are absent, because in most cases only a few peptide mass matches are required for protein identification. Alternatively, the presence of unmatched sample peptides is often a source of protein misidentification and may be caused by: changes in expected mass by post-translational modification, modifications caused by sample handling, low-fidelity proteolysis by contaminating proteases, the presence of more than one protein in the sample, and false positive identification, where the identified protein matches a sequence homologue or splice variant in the database (Aebersold & Goodlett, 2001).

Protein identification employing computer algorithms and comprehensive protein/peptide databases, is limited to organisms with available peptide sequence information or to homologous proteins from closely related species. Protein identification is compromised for any biological

family with a poorly conserved domain and/or strong sequence polymorphisms, or from species that are phylogenetically distant from model organisms (Seo et al., 2013).

2.10 Proteomic Studies of Nectar and Nectary Proteins

A limited number of proteins have been identified and characterized in nectars and nectaries by conventional analytical methods. This scarcity in data is mainly due to their low protein contents, the small physical size of nectaries, and the small volume of nectar produced by plants. The development of MS protocols with high m/z resolution and low detection limits for peptides, coupled with computer database peptide algorithms, have paved the way for the application of proteomics to nectary and nectar analysis.

Limited literature reports on the identification of proteins (e.g., carbohydrases) in nectar and/or nectaries exist. Nectar proteins of *Nicotiana attenuata* were separated by 2D gel electrophoresis, and individual gel bands were excised and digested with trypsin and the resulting peptides were analyzed by nano-ultra-performance liquid chromatography-tandem MS (LC-MS/MS). Peptide mass spectral results were generated and searched using MASCOT software against the NCBInr database combined with the *N. attenuata* protein subdatabase with α-galactosidase, α-glucosidase, nectarins, RNases, xylosidase, and unspecified lipid transfer proteins identified (Seo et al., 2013).

Nectar protein analyses of trypsin proteinase inhibitor silenced and wild-type *N. attenuata* were conducted using MALDI-TOF and nanoflow liquid chromatography-tandem MS (LC-MS/MS) (Bezzi et al., 2010). The authors reported the identification of aspartic proteinase, calcium-dependent protein kinase, germin-like proteins, glyceraldehyde-3-phosphate dehydrogenase, glycolate oxidase, methionine synthase, nectarins, and zeaxanthin epoxidase. Proteomic analysis of the nectars of *Acacia cornigera* showed the presence of pathogenesis-related enzymes including chitinase, β-1,3-glucanase, and peroxidase, as well as glycoside hydrolase and cell wall invertase (González-Teuber et al., 2009). Orona-Tamayo et al. (2013) analyzed the SDS-PAGE separated and trypsin digested proteomes of the nectary and nectar of *A. cornigera* by nano-electrospray liquid chromatography MS/MS. The nectary proteome showed the presence of two enzymes involved in carbohydrate metabolism, invertase and sucrose synthase. The nectar proteome was also observed to be a subset of the nectary proteome.

3. MATERIALS AND METHODS

3.1 Chemicals

Iodoacetamide was purchased from Acros Organics (Thermo Fisher Scientific, Ottawa, ON, CA). Bio-Rad 4–15% mini-protean TGX precast gel, Bio-Rad protein assay, Bio-safe coomassie stain, bovine serum albumin (BSA), glycine, 2x Laemmli sample buffer, Precision plus protein dual xtra molecular weight marker standard (2-250 kDa), sodium dodecyl sulfate (SDS), and Tris were purchased from Bio-Rad Laboratories, Inc. (Bio-Rad Laboratories Ltd, Mississauga, ON, CA). Bacto nutrient agar medium was purchased from Difco Laboratories (Detroit, MI, USA). Acetone, acetonitrile, ammonium bicarbonate (NH_4HCO_3), cysteine hydrochloric acid, formic acid, mass spectrometry grade water, sodium hydroxide (NaOH) solution (50% w/w), trichloroacetic acid (TCA), and trifluoroacetic acid were purchased from Fisher Scientific (Ottawa, ON, CA). Dithiothreitol was purchased from MP Biomedicals (Solon, OH, USA). Trypsin was purchased from Promega Corporation (Madison, WI, USA). Hydrochloric acid (HCl) was purchased from Ricca Chemical Company (Arlington, TX, USA). Cellobiose, D-fructose, β-fructosidase (I4504; from *Saccharomyces cerevisiae*), gentiobiose, D-glucose, α-glucosidase (G5003; from *Saccharomyces cerevisiae*), β-glucosidase (49290; from almond), isomaltose, kojibiose, maltose, maltulose, melezitose, melibiose, nigerose, p-nitrophenyl-α-D-glucopyranoside (α-PNPG), p-nitrophenyl-β-D-glucopyranoside (β-PNPG), palatinose, panose, raffinose, sucrose, sylon TP (TMSI + pyridine, 1:4), and trehalose were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, CA). Sodium acetate (NaOAc) was obtained from VWR Canada (Mississauga, ON, CA). Erlose (O-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-β-D-fructofuranoside) and 1-kestose (O-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside) were gifts from Dr. S. Chiba, Dept. of Agriculture, Hokkaido University (Japan). Laminaribiose (O-β-D-glucopyranosyl-(1→3)-D-glucopyranose) was a gift from Dr. E. Reese of the U.S. Army Natick Research and Development Laboratories. The water used throughout this

study was obtained from a Millipore Milli-Q™ water system (Millipore Corporation, Milford, MA, USA) with a pH of 6.26.

3.2 Plant Cultivation

Borago officinalis L. and two *Brassica* spp. L., *Brassica napus* L. (var. *AC Excel*), *B. napus* L. transgenic (var. *AV 225 R. R.*), and *B. rapa* L. (var. *AC Parkland*) were cultivated in 20 x 15 cm plastic pots containing approximately 650 g of Sunshine Mix 1 (Sun Gro Horticulture Inc., Bellevue, WA, USA) soil. Plants were grown in the University of Saskatchewan phytotron under the following growth chamber conditions: 18 h fluorescent and incandescent light per day (1 h ramping at the beginning and end); light intensity of 250 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR); day temperature of 22ºC; night temperature of 18ºC (2 h ramping at the beginning and end); relative humidity of 60%. Plants were watered daily and fertilized with 20:20:20 (N:P:K) every three days (Plant Products Co. Ltd., Brampton, ON, CA).

Plants were also grown in a field plot for nectar oligosaccharide studies only. For field plots, eight plants per species were cultivated in the growth chamber as described above for one month. The seedlings were then transplanted in a plot (field) near the W. P. Thompson building at the University of Saskatchewan in June 2010. Emerged flowers were removed and the inflorescences were bagged with nylon mesh (approx. 2 mm^{-1}) for each plant species.

3.3 Phloem Analyses

3.3.1 Phloem Sap Collection

Phloem sap was collected from the cut surface of the flower stem (pedicel) using filter paper wicks (Mc Kenna & Thomson, 1988) 30 min to 2 h post cutting. Wicks containing the phloem sap were placed in 2.0 mL microcentrifuge tubes (Thermo Fisher Scientific, Ottawa, ON, CA) on ice and then stored at -40ºC. *Borago officinalis* L. phloem sap was not readily available by simply cutting the pedicel, as such, phloem sap collection was afforded just before the 18 h fluorescent and incandescent light treatment in the phytotron, whereas *Brassica* spp. phloem saps were collected throughout the light treatment period.

3.3.2 Phloem Carbohydrate Extraction and Sample Preparation for HPAE-PAD and CGC-FID Analysis

To individual single wicks containing the phloem sap was added 200.0 µL of water followed by vortexing (Fisherbrand Vortex Genie 2, Allied Fisher Scientific, Morristown, NJ, USA; speed 4) for 15 min. Phloem soluble solids concentration (ºBrix) was determined using refractometry (Leica Refractometer, Buffalo, NY, USA). For phloem carbohydrate analysis by high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD), individual samples were syringe filtered (nylon syringe filter, 13 mm diameter, 0.2 µm pore size; Chromatographic Specialties, Brockville, ON, CA) directly into a 2.0 mL microcentrifuge tube and analyzed immediately. For oligosaccharide analysis by capillary gas chromatography with flame ionization detection (CGC-FID), samples were transferred to individual 300.0 µL glass microinsert vials (National Scientific, Rockwood, TN, USA) within 1.5 mL glass vials (12 x 32 mm; Chromatographic Specialties Inc., Brockville, ON, CA) followed by lyophilization for 60 min (Heto Lab Equipment, Allerod, Denmark). Following lyophilization, the resulting syrup/foam was silylated via treatment with N-trimethylsilylimidazole (TMSI) + pyridine (1:4, v:v; Sylon TP). A 5:1 (v:v) ratio of derivatizing agent to sample solution (@ 5.5 ºBrix) was employed (e.g., 100.0 µL of lyophilized 5.5 ºBrix phloem sap was derivatized with 500.0 µL of Sylon TP). A minimum of 10.0 µL of Sylon TP was added to samples with total solids concentrations equal to or less than 0.05 ºBrix. Vials were capped and heated at 70ºC for 1 h in a block heater (Denville Scientific Inc. Metuchen, NJ, USA).

3.3.3 Phloem Carbohydrate Analysis by HPAE-PAD

Analysis of phloem sap for its fructose, glucose, and sucrose content was carried out (LC Method 1) employing a Dionex ICS 5000 HPLC system (Thermo Fisher Scientific, Ottawa, ON, CA) equipped with an autosampler and pulsed amperometric detector (PAD; electrochemical cell), which was equipped with a disposable gold electrode. The potentials and durations (i.e., the E_1 potential was held from time 0.00 to 0.41 s) of the PAD were as follows: E_1= 0.10 V, t_1= 0.00 s; E_2= -2.00 V, t_2= 0.41 s; E_3= 0.60 V, t_3= 0.43 s; E_4= -0.10 V, t_4= 0.44 s; E_5= -0.10 V, t_5= 0.50 s. Data acquisition was afforded with Dionex Chromeleon 7.0 software. Carbohydrates were separated using a Dionex CarboPac PA1 column (4 x 250 mm; Dionex, Sunnyvale, CA, USA) in series with a CarboPac PA1 guard column (4 x 50 mm; Dionex, Sunnyvale, CA, USA). Elution

of the carbohydrates was carried out using an isocratic mobile phase of 80.0 mM NaOH (50% w/w NaOH) at a flow rate of 1.0 mL min^{-1}. The sample injection volume was 25.0 µL. Standard curves for fructose, glucose, and sucrose were prepared in water at concentrations ranging from 10.0 to 200.0 ppm for carbohydrate quantitation. Standard curves had R^2 values of 0.998 or greater. All samples and standards were analyzed in duplicate.

Phloem sap oligosaccharide analysis (LC Method 2) was performed on a Dionex Bio LC 4000 gradient HPLC system (Dionex, Sunnyvale, CA, USA). The presence of oligosaccharides was determined using a pulsed amperometric detector equipped with a gold electrode employing triple pulsed amperometry at a sensitivity of 10K. The electrode was maintained at the following potentials and durations: E_1= 0.05 V, t_1= 120 ms; E_2= 0.80 V, t_2= 120 ms; E_3= -0.60 V, t_3= 420 ms. Oligosaccharide separation was accomplished on a Dionex CarboPac PA1 column in series with a CarboPac PA1 guard column. A gradient elution program (Table 3-1) was employed for oligosaccharide separation with the following mobile phases: 160.0 mM NaOH (A); 160.0 mM NaOH/1.0 M NaOAc (B); 1.0 M NaOH (C). The mobile phase flow rate was 1.0 mL min^{-1}. Sample injection volume was 50.0 µL. All samples were analyzed in duplicate.

Table 3-1 HPAE-PAD gradient program for oligosaccharide separation and analysis.

Time (min)	%A	%B	%C
0.0	100.0	0.0	0.0
1.5	100.0	0.0	0.0
100.0	0.0	100.0	0.0
101.0	0.0	100.0	0.0
101.1	0.0	0.0	100.0
105.0	0.0	0.0	100.0
105.1	100.0	0.0	0.0
115.0	100.0	0.0	0.0

A=160.0 mM NaOH; B=160.0 mM NaOH/1.0 M NaOAc; C=1.0 M NaOH

3.3.4 Phloem Oligosaccharide Analysis by CGC-FID

Phloem sap oligosaccharide profiles were determined employing an Agilent 6890 Series gas chromatographic system (Agilent Technologies Canada Ltd., Mississauga, ON, CA) equipped with an Agilent 6890 autosampler. Oligosaccharide analysis was performed using a J&W DB-5 (95% dimethyl-(5%)-diphenyl-polysiloxane) open tubular capillary column (30 m x 0.25 µm i.d., 0.25 µm film thickness; Chromatographic Specialties, Inc., Brockville, ON, CA). Samples were analyzed in the splitless mode with ultra-pure hydrogen as the carrier gas that was delivered at a constant flow rate of 1.4 mL min^{-1}, with ultra-pure nitrogen delivered at a flow rate of 30.0 mL min^{-1} as the makeup gas. The injection port temperature was 250ºC and the FID was maintained at 300ºC. The sample injection volume was 4.0 µL. Oligosaccharide profiles were obtained using both disaccharide and trisaccharide temperature programs. The disaccharide temperature program was: 210ºC for 10 min, 1ºC min^{-1} from 210 to 248ºC, 248ºC for 1 min, 20ºC min^{-1} from 248 to 295ºC, and 295ºC for 15 min (total run time of 66.35 min) (Low, 1994). The trisaccharide temperature program was 250ºC for 10 min, 1ºC min^{-1} from 250 to 290ºC, 290ºC for 3 min, 30ºC min^{-1} from 290 to 295ºC, and was held at this temperature for 12 min (total run time of 65.17 min). All samples were analyzed in duplicate.

3.4 Nectar and Nectary Analyses

3.4.1 Nectar and Nectary Collection

The nectar of ten flowers from six phytotron grown plants of *B. officinalis* and all *Brassica* spp. (from both lateral nectaries) (i.e., 60 samples for *B. officinalis* and 120 samples for each *Brassica* sp.) was sampled as follows: two days after flowering, fully-opened flowers were excised and their nectars immediately collected using individual filter paper wicks (Mc Kenna & Thomson, 1988) under a dissecting microscope (10 x 40; SZ-ST; Olympus, Japan). Nectar containing wicks were dried at room temperature (21-22ºC) for 1 h, transferred to individual sterile 2.0 mL microcentrifuge tubes, and were stored at -40ºC until they were used for carbohydrate analyses.

Nectar from field grown flowers from *B. officinalis* and each *Brassica* sp. were also collected. Fully-opened flowers inside the bags of each plant from each species were excised and their nectars immediately collected as described for phytotron-grown plants.

Following nectar collection, the perianth of *B. officinalis* and each *Brassica* sp. was removed for nectary excision. *Borago officinalis* L. (annular) and *Brassica* spp. (lateral) nectaries

were excised using a scalpel blade, placed in individual 2.0 mL microcentrifuge tubes on ice, and following collection, were stored at -40ºC until they were used for select experiments.

3.4.2 Nectar Volume, Soluble Solids, and pH

The nectar of six flowers from each of three plants of *B. officinalis* and from each *Brassica* sp. (from the two lateral nectaries) (i.e., 18 samples for *B. officinalis* and 36 samples for each *Brassica* sp.) was collected using individual Drummond microcaps (1.0 µL) (#1, Drummond Scientific Co., Broomall, PA, USA). Sample volume, total soluble solids, and pH were determined for nectar samples immediately as follows: by direct measurement from the calibrated microcap; use of a portable refractometer (Bellingham and Stanley Ltd., UK); and by pH indicator strips (Colorphast pH 2.5-4.5 and 4.7-7.0; EM Science, Gibbstown, NJ, USA), respectively. Nectar volume, soluble solids concentration, and pH were reported as the mean ± standard deviation. A one-way analysis of variance (ANOVA) with a Scheffe post-hoc test was utilized for the measurement of the statistical differences in these nectar parameters. All statistical analyses were performed employing the SPSS program version 17.0 software (SPSS Inc., 2008, Chicago IL, USA).

3.4.3 Nectary Carbohydrate Extraction and Sample Preparation for HPAE-PAD and CGC-FID Analyses

In order to obtain sufficient carbohydrate concentrations for HPAE-PAD and CGC-FID analysis for each plant sample: (a) 40 *B. officinalis* nectaries were transferred to a 2.0 mL microcentrifuge tube followed by the addition of 900.0 µL of water; and (b) 50 lateral nectaries for each *Brassic*a sp. were transferred to individual 2.0 mL microcentrifuge tube followed by the addition of 500.0 µL of water. Samples were vortexed for 15 min. The nectary fluid soluble solids concentration (ºBrix) was determined by refractometry. For each sample, a 300.0 µL aliquot was removed and syringe filtered for fructose, glucose, and sucrose analysis and for non-sucrose oligosaccharide analysis by HPAE-PAD as outlined in Section 3.3.3 (LC Methods 1 and 2). For CGC-FID analysis, a 200.0 µL sample aliquot for each sample was individually transferred into a 300.0 µL glass microinsert vial within a 1.5 mL glass vial and the sample was freeze-dried and derivatized as outlined in Section 3.3.4. The nectary ºBrix of individual nectaries of *B. officinalis* and each *Brassica* sp. was also determined by transferring single nectaries to a 2.0 mL

microcentrifuge tube followed by the addition of 200.0 µL of water, vortexing, and measuring the ºBrix by refractometry.

3.4.4 Nectar Carbohydrate Extraction and Sample Preparation for HPAE-PAD and CGC-FID Analyses

Nectars from ten flowers of six plants of *B. officinalis* and from ten flowers (from the two lateral nectaries) of six plants of each *Brassica* sp. were randomly selected. Nectars from each of the individual wicks were placed in 2.0 mL microcentrifuge tubes followed by the addition of 1.0 mL and 200.0 µL of water for *B. officinalis* and for each *Brassica* sp., respectively. Samples were vortexed for 15 min followed by syringe filtration. Each nectar sample was diluted to fit within standard curve values as follows: for *B. officinalis*, 10.0 or 25.0 µL of extracted *B. officinalis* nectar was diluted with either 1990.0 or 1975.0 µL of syringe filtered water for fructose, glucose, and sucrose analysis, whereas for each *Brassica* sp., 10.0 µL of extracted nectar was diluted with 490.0 µL of water for fructose and glucose analysis, and the original extraction solution was used for sucrose analysis. In some cases, because of the negligible amount of sucrose in the *Brassica* spp. nectar samples, the original extraction solution was lyophilized for 3 h to ensure complete dryness, and the dried material was solubilized in 100.0 µL of syringe filtered water, and this sample was employed for sucrose analysis.

For oligosaccharide analysis, nectars of *B. officinalis* and *Brassica* spp. cultivated in the growth chamber and field were randomly selected. For the growth chamber plants, three nectar containing wicks for *B. officinalis* and four nectar containing wicks for each *Brassica* sp. from six plants (i.e., 18 samples for *B. officinalis* and 72 samples for each *Brassica* sp.) were analyzed. For the field-grown plants, four nectar containing wicks for *B. officinalis* and three nectar containing wicks for each *Brassica* sp. from different plants were randomly selected. Each nectar containing wick was placed in an individual 2.0 mL microcentrifuge tube followed by the addition of either 1.0 mL or 200.0 µL of water for *B. officinalis* and *Brassica* spp. samples, respectively. Samples were vortexed for 15 min followed by syringe filtration. A 200.0 µL aliquot for each *B. officinalis* and *Brassica* sp. sample was transferred to individual 300.0 µL glass microinsert vials within 1.5 mL glass vials followed by lyophilization for 60 min. Samples were analyzed for their oligosaccharide content by CGC-FID as outlined in Section 3.3.4. For nectar oligosaccharide

analysis by HPAE-PAD the syringe filtered samples were analyzed directly as outlined in Section 3.3.3.

3.4.5 Nectar Major Carbohydrate Analyses by HPAE-PAD

Nectar samples were individually analyzed for their fructose, glucose, and sucrose content (LC Method 3) employing a Waters 625 metal-free gradient high performance liquid chromatographic system (Waters Chromatography, Milford, MA, USA) equipped with Waters 712 Wisp autosampler. The sample injection volume was 25.0 μL. Major nectar carbohydrates (fructose, glucose, and sucrose) were separated using a Dionex CarboPac PA1 column in series with a Dionex CarboPac PA1 guard column. Carbohydrate elution was afforded with an isocratic mobile phase of 80.0 mM NaOH maintained at a flow rate of 1.0 mL min^{-1} (Swallow, 1992; Davis et al., 1998). Carbohydrates were detected using a Waters model 464 PAD with a dual gold electrode and triple pulsed amperometry at a sensitivity of 50 μA. The electrode was maintained at the following potentials and durations: E_1= 0.05 V, t_1= 0.299 s; E_2= 0.60 V, t_2= 0.299 s; E_3= -0.80 V t_3= 0.499 s. Chromatograms were obtained employing Millennium 2010 Chromatography Manager Software (Waters Chromatography, Milford, MA, USA). Carbohydrate quantification was afforded using external standard curves with R^2 values of 0.998 or greater. Standards for fructose, glucose, and sucrose ranged from 10.0-200.0 ppm. Samples were analyzed in duplicate and reported as the mean ± standard deviation. A one-way analysis of variance (ANOVA) with a Scheffe post-hoc test was utilized for the measurement of the statistical differences in nectar carbohydrate content (w/v). All statistical analyses were performed employing the SPSS program version 17.0 software.

3.5 Nectar Microbial Assay

Nectars were examined for the presence of microorganisms employing the method of Gilliam et al. (1983). Briefly, the freshly collected nectar from three flowers from three plants for each species was collected using a Drummond micro-pipette and was streaked via a platinum loop onto individual plates (9 x 1.3 cm) containing Bacto nutrient agar medium (Difco Laboratories, Detroit, MI, USA) under sterile conditions. These inoculated plates were incubated at 30ºC upside-down in a biological safety cabinet (Nuaire, Plymouth, MN, USA) and their microbial content was determined at day 1, 15, and 29 post-streaking. Positive controls were run in

conjunction with the aforementioned samples for organisms that may be present in nectar (Gilliam, 1975; Baker & Baker, 1983; Eisikowitch et al., 1990) and included *Saccharomyces cerevisiae* (ATCC 24859), *Staphylococcus aureus* (ATCC 25923), and Gram-negative/positive rod-shaped bacteria (*Escherichia coli*; ATCC 11303)/*Bacillus subtilis*; ATCC 6051) conducted under the same experimental conditions.

3.6 Enzyme Substrate Assay of Nectary and Nectar α- and β-Glucosidase, and β-Fructosidase Activities

The α-, β-glucosidase, and β-fructosidase activities present in *B. officinalis* and *Brassica* spp. nectars and nectaries were determined using individual 100.0 ppm solutions of the following carbohydrate substrates: maltose, cellobiose, and raffinose, respectively. Positive controls were prepared by adding 1.0 µL of commercial α-glucosidase (G5003; from *Saccharomyces cerevisiae*; ≥10 units mg^{-1} protein) to 2.0 mL of a 100.0 ppm aqueous maltose solution; 1.0 µL of commercial β-glucosidase (49290; from almonds; ≥6 units mg^{-1}) to 2.0 mL of a 100.0 ppm aqueous cellobiose solution; and 1.0 µL of commercial β-fructosidase (I4504; from *Saccharomyces cerevisiae*; >300 units mg^{-1} solid) to 2.0 mL of a 100.0 ppm aqueous raffinose solution. One unit of α-glucosidase is defined as the amount of enzyme required to liberate 1.0 µmol of D-glucose from p-nitrophenyl-α-D-glucopyranoside (α-PNPG) per minute at pH 6.8 at 37ºC. For β-glucosidase, one unit is defined as the amount of enzyme needed to liberate 1.0 µmol of glucose from salicin per minute at pH 5.0 at 35ºC. For β-fructosidase, one unit of activity is defined as the amount of enzyme required to hydrolyze 1.0 µmol of sucrose to invert sugar per minute at pH 4.5 at 55ºC. Negative controls consisting of 2.0 mL solutions of 100.0 ppm of maltose, cellobiose, and raffinose without enzyme addition were also run. All reactions were conducted at 30ºC for three h in a water bath with intermittent stirring and quenched by placing samples in boiling water for five min. Samples were filtered (0.2 µM pore size) and analyzed by HPAE-PAD (LC Method 3).

For each of the three carbohydrase enzyme activity determinations, the following protocol was employed: 20 nectaries for each plant species were individually placed in an ultrafiltration cell (Model 52; Amicon Corp., Lexington, MA, USA) equipped with a YM 10 ultrafiltration membrane (10,000 daltons cut-off; Millipore Co., Bedford, MA, USA) and washed with 10.0 mL of water. Sample filtration employing nitrogen was maintained at a flow rate of approximately 1.0 mL min^{-1} in a cold room maintained at 4ºC. The filtrate was collected and analyzed by HPAE-PAD (LC Method 3) to determine the presence/absence of major carbohydrates (e.g., fructose,

glucose, and sucrose) in the nectaries. When the nectaries were almost dry, an additional 10.0 mL of water was added until virtually all (<0.1 ppm) of the major carbohydrates were removed as determined by HPAE-PAD. Detection limits (6x the signal to noise [S/N] ratio for quantitation and approximately 3x S/N for qualitative analysis) for carbohydrates (i.e., fructose and glucose) were established by preparing serial dilutions of 50 ppm standards of fructose and glucose. Following carbohydrate removal, a final washing of the nectaries with an additional 10.0 mL of water was employed. Following this washing, 1.0 mL of the 100.0 ppm substrate solution was added to the carbohydrate-free nectaries in the ultrafiltration cell. A 200.0 µL aliquot was immediately removed, syringe filtered, and analyzed by HPAE-PAD (LC Method 3) and was reported as the 0 h sample. Carbohydrate-free nectaries plus enzyme solutions were left static for 24 to 72 h at 4ºC in the cold room. Following the appropriate reaction period, a 200.0 µL aliquot was removed, syringe filtered, and analyzed by HPAE-PAD (LC Method 3).

Nectar enzyme activity was also determined employing the aforementioned protocols using approximately 400.0 µL of *B. officinalis* and *Brassica* spp. carbohydrate-free nectars in the ultrafiltration process. The nectars of different flowers of *B. officinalis* and each *Brassica* sp. (from the two lateral nectaries) were collected using individual Drummond microcaps (1.0 µL) (#1, Drummond Scientific Co., Broomall, PA, USA) and were collected in sterile 2.0 mL microtubes on ice and immediately stored at -40ºC until further analysis.

In addition to the HPAE-PAD determination of the three specified carbohydrase nectary and nectar activities, colourimetric assays employing α- and β-PNPG (p-nitrophenylglucoside) were also employed (Siegenthaler, 1977; Low et al., 1986) for the nectaries only. For this assay, two nectaries from each species were placed in individual test tubes (13 x 100 mm) to which was added 1.0 mL of 20 mM α-PNPG in 0.10 M phosphate buffer (pH 6.0) or 20 mM β-PNPG in 0.10 M acetate buffer (pH 5.0). Test tubes were covered with a parafilm, vortexed for 15 s, and incubated at 30ºC in a water bath for 20 min. Following incubation, a 200.0 µL aliquot of 3.0 M Tris buffer (pH 10.0) was added to terminate enzyme activity.

Positive controls consisted of 1.0 µL of commercial α-glucosidase or β-glucosidase in 1.0 mL of α- or β-PNPG employing the aforementioned assay conditions. Negative controls consisted of the same nectary samples with the immediate addition of a 200.0 µL aliquot of 3.0 M Tris buffer (pH 10.0) to the sample solution. Sample blanks consisted of 1.0 µL of water, 1.0 mL of 20 mM

α- or β-PNPG in their appropriate buffer, and 200.0 µL of 3.0 M Tris buffer (pH 10). All controls and blanks were incubated for 20 min at the appropriate temperature.

All samples, controls, and blanks were cooled to room temperature, individually transferred to spectrophotometer cuvettes (12.5 x 12.5 x 45 mm; Cole-Parmer Instrument Co., Vernon Hill, IL, USA) with their absorbance read at 400 nm (Spectronic 1201; Milton Roy Inc., Rochester, NY, USA). All analyses were conducted in triplicate.

3.7 Nectary Protein

3.7.1 Nectary Protein Extraction and Concentration Determination

For protein concentration determination, 15 nectaries for each individual plant species were ground with 1.0 mL of homogenization buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M sucrose, 0.1% w/v ascorbic acid, 0.1% w/v cysteine HCl). The resulting mixture was quantitatively transferred into a sterile 2.0 mL microcentrifuge tube and vortexed for 5 min. For *B. officinalis*, 50.0 µL of the extract was diluted with 750.0 µL homogenization buffer, whereas for each of the *Brassica* spp., 300.0 µL of the extract was diluted with 500.0 µL homogenization buffer. Protein concentration was determined using the Bio-Rad Protein Assay employing bovine serum albumin for standard curve preparation (125 to 1500 µg mL^{-1} for *B. officinalis* and 1.25 to 15 µg mL^{-1} for *Brassica* spp.). For *B. officinalis*, the standard assay procedure was followed using 20.0 µL of the diluted extract. The microassay procedure was followed for all *Brassica* spp. using 800.0 µL of the diluted extract. Sample and standard absorbances were read at 595 nm in duplicate (Genesys 10S; Thermo Fisher Scientific, Ottawa, ON, CA). All sample analyses were performed in triplicate.

Nectary proteins were extracted following the procedure of Wang et al. (2006) with modifications. Briefly, 100 nectaries from each plant species were ground with 2.0 mL of 10% TCA/acetone (w:v). Approximately, 2.0 mL of the extract was transferred into a sterile 2.0 mL microcentrifuge tube. The tubes were mixed by inversion and centrifuged at 15000 rpm for 5 min (Eppendorf Microcentrifuge 5424; Eppendorf, Mississauga, ON, CA). The supernatant was removed by decanting and approximately 2.0 mL of 80% aqueous methanol with 0.1 M ammonium acetate solution was added. The tubes were vortexed for 30 s and centrifuged at 15000 rpm for 5 min. The supernatant was discarded and approximately 2.0 mL of 80% aqueous acetone (v:v) was added, followed by vortexing (30 s) and centrifugation at 15000 rpm for 5 min. The supernatant was discarded and the tubes were left open for sample air-drying at room temperature

(20-22ºC) for 15 min. To each microcentrifuge tube was added 800.0 µL of 1:1 phenol (pH 8.0)/SDS buffer (v:v) and the contents were mixed by inversion. The samples were then left static at room temperature for 5 min and were then centrifuged at 15000 rpm for 5 min. The upper phenol phase was transferred into a new 2.0 mL microcentrifuge tube and approximately 2.0 mL of methanol containing 0.1 M ammonium acetate was added, and the resulting sample mixture was stored at -20ºC overnight. The tubes were centrifuged at 15000 rpm for 5 min and the supernatant was discarded. Approximately 2.0 mL of methanol was added to the sample pellet followed by vortexing (30 s), centrifugation (15000 rpm/5 min), and decanting. This procedure was repeated employing 80% aqueous acetone (v:v). The resulting pellet was air dried at room temperature overnight and was dissolved in 30.0 µL of 2x Laemmli sample buffer.

3.7.2 Separation of Proteins by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The extracted proteins (30 µL; protein concentration not determined) together with a protein standard (3 µL; Precision Plus Protein Dual Xtra Molecular Weight Marker Standard; Bio-Rad Laboratories Ltd, Mississauga, ON, CA) were loaded onto a Bio-Rad 4–15% Mini-Protean TGX precast gel (Bio-Rad Laboratories Ltd, Mississauga, ON, CA) and electrophoresis was carried out using a Bio-Rad Mini-Protean Tetra Cell (Bio-Rad Laboratories Ltd, Mississauga, ON, CA) at 100 V for 90 min with Tris/glycine/SDS buffer (pH 8.3). Proteins were visualized on the gel by overnight treatment with Bio-safe coomassie stain (Bio-Rad Laboratories Ltd, Mississauga, ON, CA). Gels were then destained using Milli-Q water until the background appeared clear. Protein bands were visualized using a ChemiDoc XRS Imager with Image Lab Software (Bio-Rad Laboratories Ltd, Mississauga, ON, CA). Individual protein bands (1 mm wide sections) were excised from the gels using a razor blade, were transferred to 2.0 mL microcentrifuge tubes, and were stored overnight at -4ºC.

3.7.3 In-Gel Protein Digestion

Individual protein bands (contained in 2.0 mL microcentrifuge tubes) were treated with 100.0 µL of destaining solution (200 mM ammonium bicarbonate containing 50% acetonitrile) followed by incubation at 30ºC for 20 min with intermittent sample vortexing (30 s) after 10 min of the incubation period. The supernatant was discarded and the procedure was repeated using 50.0 µL of the destaining solution with incubation at 30ºC for 10 min. Following incubation, 10.0

µL of 200 mM ammonium bicarbonate was added to the gel samples followed by vortexing for 30 s, and incubation at 30ºC for 10 min. The supernatant was discarded and a 100.0 µL aliquot of acetonitrile was added followed by incubation at room temperature for 10 min. The supernatant was discarded and the gel samples were dried in a speed vac (Labconco, Kansas City, MO, USA) for 15 min. To the individually dried samples was added 100.0 µL of reducing buffer (10 mM dithiothreitol in 100 mM ammonium bicarbonate) followed by incubation at 56ºC for 1 h. Excess reducing buffer was removed by pipette and 100.0 µL of alkylating solution (100 mM iodoacetamide in water) was added with sample incubation in the dark at room temperature for 30 min. The supernatant was discarded and individual samples were treated twice with 100.0 µL of 200 mM ammonium bicarbonate followed by incubation at room temperature for 5 min with supernatant removal between treatments. Sample dehydration was afforded by duplicate treatment with 100.0 µL of acetonitrile followed by incubation at room temperature for 5 min with supernatant removal between treatments. The resulting white protein-gel samples were rehydrated with 50.0 µL of 200 mM ammonium bicarbonate followed by incubation at room temperature for 5 min and the removal of the supernatant. This step was repeated. Following rehydration, protein-gel samples were dried by speed vac for 15 min.

Trypsin digestion of the protein-gel samples was afforded by the addition of 10.0 µL of digestion buffer (50 ng/µL trypsin in 1 mM HCl/100 mM ammonium bicarbonate) followed by incubation at room temperature for 5 min. A 20.0 µL of 200 mM ammonium bicarbonate was then added to each sample followed by incubation at room temperature for 5 min. To this solution was added an additional 10.0 µL of 200 mM ammonium bicarbonate and samples were incubated at 30ºC overnight (10 h) with shaking (Eppendorf Thermomixer, Eppendorf, Mississauga, ON, CA) at 300 rpm. Trypsin action was quenched by the addition of 4.0 µL of 10% trifluoroacetic acid (TFA) and individual supernatants were transferred into new 2.0 mL microcentrifuge tubes (sample fraction 1). A 100.0 µL aliquot of extraction buffer (0.1% TFA in 60% acetonitrile) was added to the original protein-gel sample followed by incubation at 30ºC for 40 min with sample vortexing after 20 min of incubation. The supernatant was removed and was combined with sample fraction 1 (sample fraction 2). This procedure was repeated and the combined supernatants were evaporated to dryness by speed vac, and were stored at -80ºC for LC-MS/MS analysis.

3.7.4 Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)

The dried tryptic peptide samples were reconstituted in 20.0 µL of mass spectrometry grade water: acetonitrile: formic acid (97:3:0.1, v:v:v) followed by vortexing for 1 to 2 min to achieve peptide solubility. The resulting sample solutions were centrifuged at 14000 rpm for 10 min at 4ºC. A 15.0 µL aliquot of each sample was transferred to a mass spectrometry vial (Agilent Technologies Canada Ltd., Mississauga, ON, CA) for LC-MS/MS analysis.

All mass spectral analyses were performed on an Agilent 6550 iFunnel quadrupole time-of-flight (QTOF) mass spectrometer equipped with an Agilent 1260 series liquid chromatography instrument and an Agilent Chip Cube LC-MS interface (Agilent Technologies Canada Ltd., Mississauga, ON, CA). Chromatographic peptide separation was accomplished using a high-capacity HPLC chip consisting of a 160-nL enrichment column (Agilent Technologies Canada Ltd., Mississauga, ON, CA) and an analytical column (75 µm x 150 mm; Agilent Technologies Canada Ltd., Mississauga, ON, CA), both packed with Zorbax 300 SB-C18, 5 µm, 300 Å silica. Samples were loaded onto the enrichment column with 97% solvent A (0.1% formic acid) and 3% solvent B (90% acetonitrile: 10% water containing 0.1% formic acid) at a flow rate of 2.0 µL min^{-1}. The following linear gradient program was employed for peptide separation and eluted with a linear gradient of 8–30% solvent B for 55 min and then 30–90% solvent B for 10 min at a flow rate of 0.3 µL min^{-1}. Positive-ion electrospray mass spectral data was acquired using a capillary voltage set at 1900V, the ion fragmentor set at 360V, and the drying gas (nitrogen) set at 225ºC with a flow rate of 12.0 L min^{-1}. Spectral results were collected over a mass range of 250–1700 (mass/charge; m/z) at a scan rate of 8 spectra sec^{-1}. MS/MS data was collected over a range of 100–1700 m/z and a set isolation width of 4 atomic mass units. The top 20 most intense precursor ions for each MS scan were selected for tandem MS with active exclusion for 0.25 min.

3.7.5 Peptide and Protein Identification

Select spectral data was converted to a mass/charge data format using the Agilent MassHunter Qualitative Analysis Software (Agilent Technologies Canada Ltd., Mississauga, ON, CA) and was processed against the NCBI non-redundant green plant database using Spectrum Mill (Agilent Technologies Canada Ltd., Mississauga, ON, CA) as the database search engine. Search parameters included a fragment mass error of 50 ppm, a parent mass error of 20 ppm, trypsin cleavage specificity, and carbamidomethyl as a fixed modification of cysteine. Oxidation of

methionine to methionine sulfoxide and sulfone was specified as a variable modification. Results of the database search were analyzed by Scaffold software (Scaffold_4.4.1.1, Proteome Software Inc., Portland, OR, USA). Peptide identifications were accepted if they could be established at >90% probability. Protein identifications were accepted if they could be established at >90% probability and if they contained ≥2 identified peptides. Protein identification was confirmed if the following parameters were achieved: a False Discovery Rate (FDR) less than 1%; presence of at least two unique peptides; a protein score greater than 9; and a scored peak intensity (SPI) greater than 60%. In some cases, a FDR greater than 1% or a score lower than 9 was accepted if the SPI was still greater than 60%. The obtained peptide sequences were also compared for sequence similarities in the non-redundant protein sequences plant database using the BLAST (Basic Local Alignment Search Tool) program to provide additional scientific evidence for protein identification (Madden, 2002). In BLAST, the calculation of sequence similarity takes into account the alignment of similar or identical residues together with the gaps introduced to align the sequences. A key element in the calculation of sequence similarity is the substitution matrix (e.g., BLOSUM 62) which assigns a score in the alignment of possible pair of residues. To evaluate the quality of the pairwise sequence alignment, the substitution matrix, blosum 62, and the universal compositional score matrix adjustment were utilized to compensate for the amino acid composition of sequences. An expect threshold (i.e., the statistical significance threshold for reporting matches against database sequences) of 10 and a maximum E (i.e., number of hits one can expect to see by chance when searching a database of a particular size) value of <5 was utilized in the database search in this study.

4. RESULTS AND DISCUSSION

4.1 Phloem Sap

Carbohydrate Composition and Concentration

Due to the lack of consistent production and low volume available for collection, crude phloem sap samples from the plants grown in this study were insufficient for direct analysis of their carbohydrate concentration (ºBrix). As such, phloem sap samples were collected via the filter paper wick method (Mc Kenna & Thomson, 1988), diluted in 200.0 µL water, and were analyzed for their ºBrix content by refractometry. By definition, ºBrix is the grams of sucrose/100 grams of sample, which is equivalent numerically to percent sucrose on a wt/wt basis. Although refractive index measurements as ºBrix are accurate only for pure sucrose samples, they are widely used to approximate the percent total soluble solids or carbohydrate in a sample (Low, 1994). Therefore, ºBrix will be used throughout the remainder of this thesis rather than soluble solids. Although the original volume of the phloem sap was unknown, the mean ºBrix values of the diluted samples were 0.03 (±0.01) for *B. officinalis*, 0.05 (±0.04) for *Brassica napus*, 0.03 (±0.01) for *B. napus* transgenic, and 0.04 (±0.02) for *B. rapa*. The mean diluted phloem sap ºBrix content for *B. officinalis* and all *Brassica* spp. ranged from 0.03 to 0.05 ºBrix. No published ºBrix values for *B. officinalis* and *Brassica* spp. phloem saps were found for comparison.

In the case of phloem sap, the presence and abundance of carbohydrates contributed significantly to the ºBrix content as measured by refractometry. Lohaus and Schwerdtfeger (2014) reported that the total soluble solids (no ºBrix value reported) in *B. napus* phloem sap were mainly due to sucrose, with a small contribution from amino acids. In citrus, the phloem sap ºBrix values obtained using centrifugation and EDTA-enhanced exudation techniques were 9.46 and 10.74, respectively (Hijaz & Killiny, 2014). Citrus phloem sap was found to contain a variety of amino acids, carbohydrates other than sucrose (e.g., fructose, glucose), and organic acids (e.g., fumaric, malic, succinic).

In this study, the predominance of sucrose and the presence/absence of other carbohydrates in the phloem sap were determined employing HPAE-PAD and CGC-FID. Both methods were used as CGC-FID yielded improved oligosaccharide (disaccharide and trisaccharide) separation and detection and HPAE-PAD afforded improved major carbohydrate (fructose-glucose-sucrose) separation and detection. Oligosaccharide analysis by CGC-FID has the ability to separate structurally similar disaccharides (e.g., isomaltose and maltose), separate the α- and β-anomers of reducing disaccharides, and the retention time (RT) for sucrose (~30 min) is significantly different from other disaccharides such as isomaltose (~45 min). A significant limitation of this method is the ability to detect fructose and glucose as these compounds elute very close to that of the derivatizing agent used in this study under the temperature program conditions employed. Sample analysis by HPAE-PAD affords the separation and detection of fructose, glucose, and sucrose and can also readily separate structurally similar disaccharides. In this study, one of the main limitations of this method was the high concentration of sucrose in the phloem sap samples, which masked the detection of structurally similar oligosaccharides.

For CGC-FID analysis, phloem sap was eluted from filter paper wicks with water followed by freeze drying (to produce a concentrated phloem sap sample), which was then derivatized with 10.0 µL of derivatizing agent. For HPAE-PAD analysis, phloem sap collected using filter paper wicks were eluted with 200.0 µL of water and this dilution was used for analysis (i.e., 20x more dilute). Phloem sap carbohydrate analysis for *B. officinalis* and all *Brassica* spp. samples by HPAE-PAD (LC Method 2; Section 3.3.3) showed only the presence of sucrose with a retention time (RT) of ~9.4 min (Figure 4.1). Phloem sap samples did not show the presence of fructose or glucose at quantitation detection limits (6x signal/noise [S/N]) of 5.0 ppm. When compared to sucrose standards prepared at the same concentrations observed for this compound in sample phloem saps, this carbohydrate accounted for >95% of the ºBrix value for all samples. Phloem sap sample analysis by CGC-FID confirmed the presence and concentration of sucrose with a RT of ~31.5 min and also did not show the presence (detection limit of 5.0 ppm for maltose) of non-sucrose oligosaccharides (Figure 4.2).

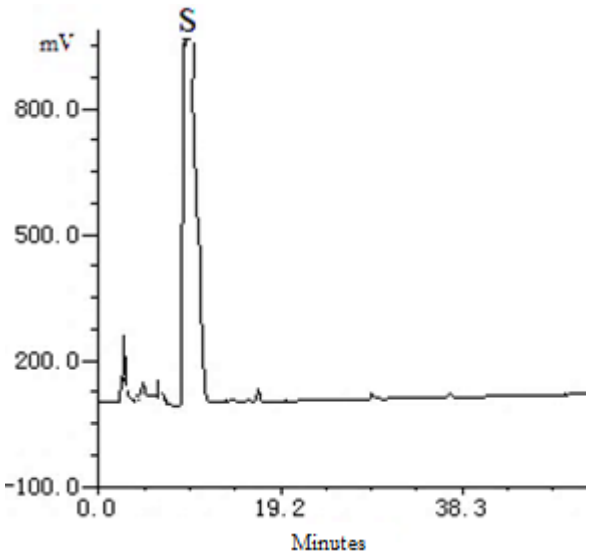

Figure 4.1 HPAE-PAD chromatogram (LC Method 2) of *Brassica napus* L. transgenic (var. *AV 225 R. R.*) phloem sap carbohydrates showing the presence of sucrose (S; RT of ~9.4 min.) as identified by retention time comparison to a standard under gradient mobile phase conditions.

The presence and relative abundance of sucrose in the phloem saps analyzed in this study is supported by literature. As examples, *Arabidopsis*, *B. napus*, and *Solanum* phloem sap samples were all found to contain very high concentrations of sucrose with no free hexoses detected (Galtier et al., 1993; Deeken et al., 2002; Dinant & Lemoine, 2010). Sucrose is an ideal transport molecule in phloem because it is a non-reducing carbohydrate, has low chemical reactivity, has high water solubility, and generates a modest osmotic pressure when compared to other carbohydrates (e.g., fructose and glucose) and carbohydrate alcohols (Arnold, 1968; Lang, 1978).

Literature also exists on the presence of reducing carbohydrates (e.g., fructose and glucose) and oligosaccharides (e.g., maltose and raffinose) in phloem sap. It is possible that the presence of these carbohydrates in samples identified as phloem sap may be due to the detachment/cutting action of stems and leaves and/or as contaminants from other cells/tissues (Ziegler, 1975; Dinant & Lemoine, 2010). It is also possible that acid catalyzed hydrolysis of sucrose could account for the presence of hexoses in the phloem sap. According to literature, sucrose hydrolysis does not occur at pH values ≥5.0 and temperatures of <50ºC (Bemiller, 1967). The pH of *B. napus* phloem sap was determined by pH strips and was found to be ~6.5. The observed slightly acidic pH of *Brassica* sp. phloem sap may be due to the presence of organic acids in this fluid (Hijaz & Killiny, 2014). Based on the aforementioned results, the low pH conditions required for both the

production of reducing carbohydrates from sucrose and acid-catalyzed oligosaccharide (e.g., maltose) synthesis (Thavarajah & Low, 2006b) would not be possible in the phloem saps of the plants used in this study.

4.2 Nectary Fluid

Carbohydrate Composition and Concentration

Nectary fluid ºBrix results determined on a single analysis of 40 *B. officinalis* nectaries/900.0 µL of water and 50 lateral nectaries of each *Brassica* sp./500.0 µL of water gave values of 0.37 for *B. officinalis*, 0.36 for *B. napus*, 0.49 for *B. napus* transgenic, and 0.11 for *B. rapa*. Individual nectaries (analyzed in triplicate) diluted in 200.0 µL of water gave average ºBrix values of 0.06 (±0.01), 0.03 (±0.02), 0.05 (±0.01), and 0.02 (±0.01) for *B. officinalis*, *B. napus*, *B. napus* transgenic, and *B. rapa*, respectively. In general, nectary ºBrix values for *B. officinalis* and *B. napus* were similar, whereas those observed for *B. rapa* were consistently lower. As an active metabolic organ, a number of water soluble compounds including carbohydrates, organic acids, and proteins are present in nectaries (Orona-Tamayo et al., 2013) and would contribute to the ºBrix value of the fluid contained within this organ.

The major carbohydrate and oligosaccharide composition of nectary fluid was determined by HPAE-PAD using LC Method 2 (Section 3.3.3). Chromatographic results for both *B. officinalis* and *Brassica* spp. nectary fluids showed the presence of both glucose (RT of ~5.5 min) and fructose (RT of ~6.2 min) with non-detectable (<5.0 ppm) sucrose and non-sucrose oligosaccharide levels (Figure 4.3). The carbohydrate composition of nectary fluids as determined by HPAE-PAD was: 52.86% for fructose, 47.14% for glucose, and <0.05% for sucrose for *B. officinalis* whereas for *Brassica* sp. (*B. rapa* var. *AC Parkland*), the carbohydrate composition of the nectary fluids was 55.02% for fructose, 44.98% for glucose, and sucrose was not detected.

However, CGC-FID analyses of *B. officinalis* and *Brassica* spp. nectary fluids showed the presence of sucrose (RT of ~30.4 min). Based on peak height comparisons, the sucrose concentration in *Brassica* spp. nectary fluids was approximately 50% greater than that found in *B. officinalis* nectary fluid (Figure 4.4). The greater sensitivity of CGC-FID compared to HPAE-PAD enabled the detection of sucrose in these nectary fluid samples.

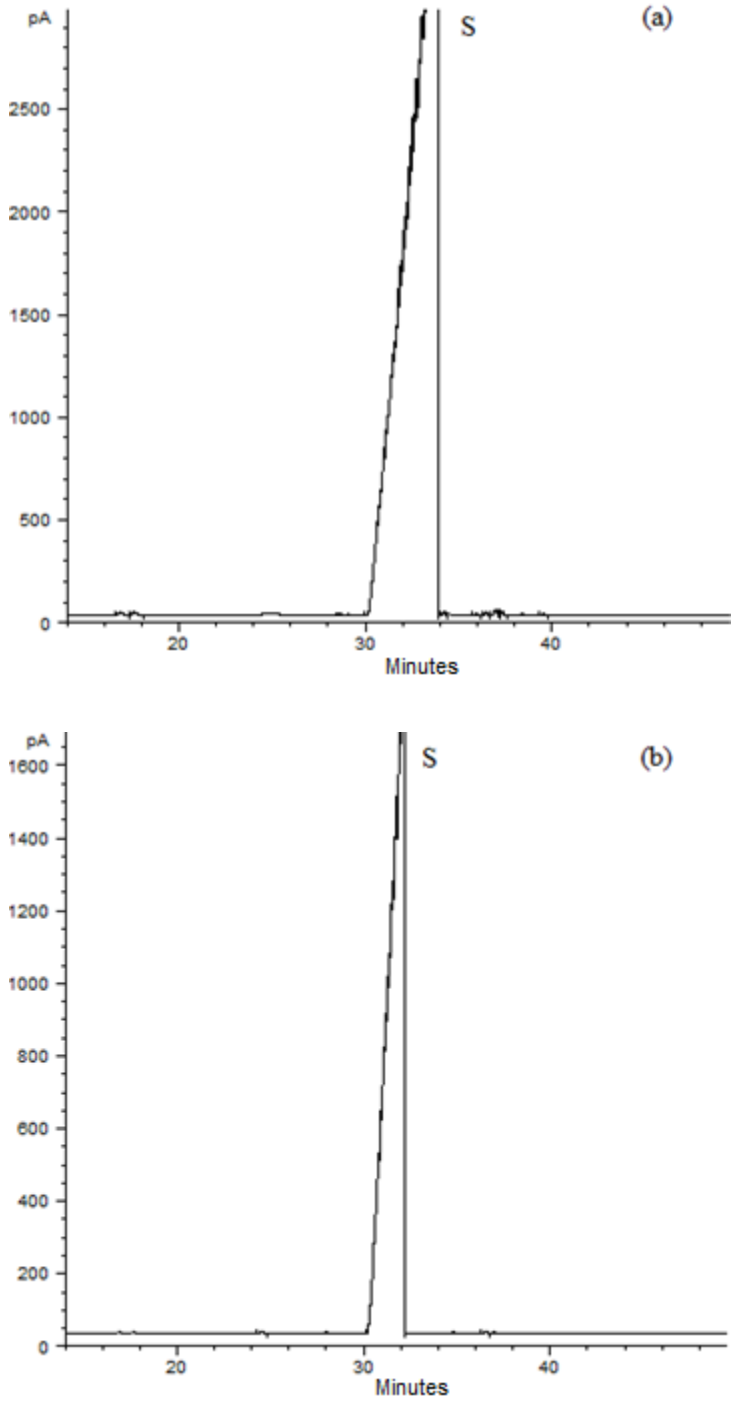

Figure 4.2 CGC-FID chromatograms of Sylon TP derivatized carbohydrates in (a) *Borago officinalis* L. and (b) *Brassica napus* L. (var. *AC Excel*) phloem saps employing temperature programming showing the presence of sucrose (S; RT of ~31.5 min) as identified by retention time comparison to a standard.

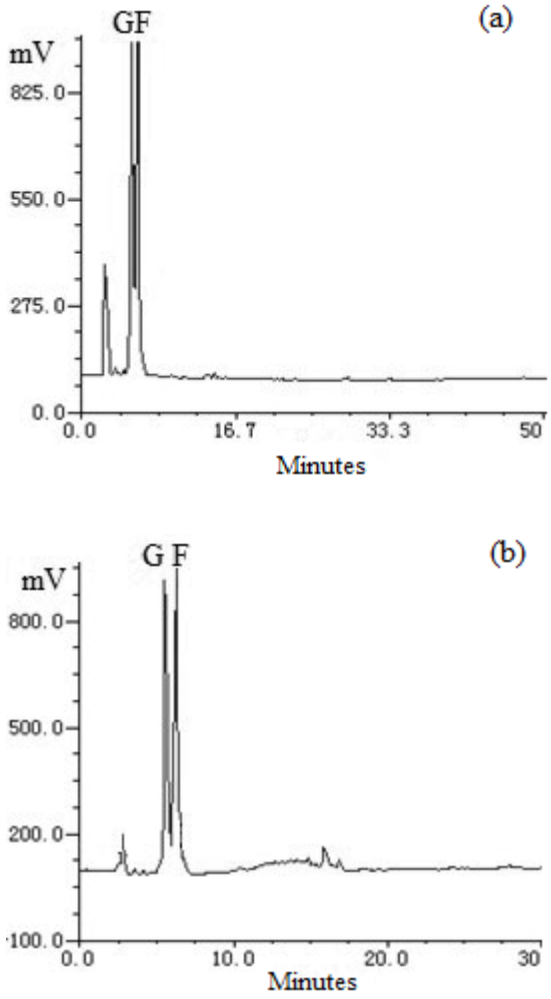

Figure 4.3 HPAE-PAD (LC Method 2) chromatograms of the major carbohydrates present in (a) *Borago officinalis* L. and (b) *Brassica rapa* L. (var. *AC Parkand*) nectary fluids showing the presence of glucose (G; RT of ~5.5 min) and fructose (F; RT of ~6.2 min) under gradient mobile phase conditions.

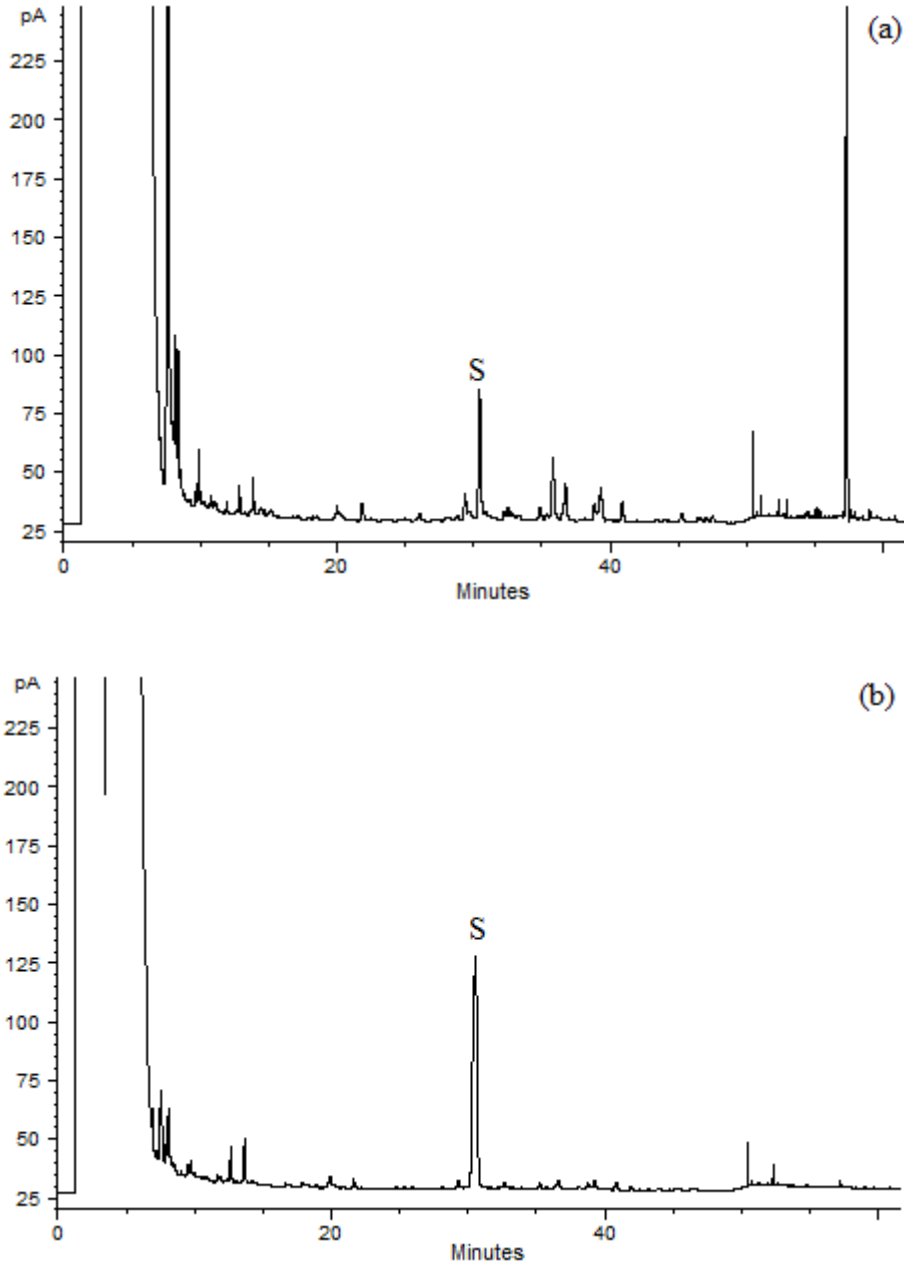

Figure 4.4 CGC-FID chromatograms showing the Sylon TP derivatized oligosaccharide profile of (a) *Borago officinalis* L. and (b) *Brassica napus* L. (var. *AC Excel*) nectary fluids employing temperature program conditions (S: sucrose).

These results clearly showed that there was a significant carbohydrate composition change between phloem sap and nectary fluids. The observed almost complete hydrolysis of sucrose to produce glucose and fructose occurs either within the nectary or during phloem sap transport into this organ. Although few literature reports on nectary carbohydrate composition exist, fructose, glucose, and sucrose were found to be present in tobacco nectaries at anthesis at a molar ratio of 1:1:1 (Ren et al., 2007b; Liu & Thornburg, 2012) whereas, the analysis of the phloem sap of tree tobacco (*Nicotiana glauca* Grah.) showed the dominance of sucrose accounting for over 95% of the total carbohydrate content (Hocking, 1980). As tobacco phloem sap has been shown to be rich in sucrose and with no detected hexoses, this result supports those obtained in this study for *B. officinalis* and *Brassica* spp. that carbohydrate transformation most likely takes place (i.e., sucrose hydrolysis with concomitant glucose and fructose production) within the nectary.

Sink activity is governed by the model of Munch (1930), where photoassimilate unloading and loading in conducting tissues is driven by concentration and/or osmotic gradients. The establishment of concentration/osmotic gradients can be achieved in several ways: sucrose may be removed by chemical alteration (e.g., hydrolysis) to create a concentration gradient; synthesis of high molecular weight compounds like starch or lipids to create both a concentration and osmotic gradient; and the removal of sucrose symplastically and apoplastically by compartmentation (Herbers & Sonnewald, 1998). The control of sink strength appears to involve a futile cycle of sucrose hydrolysis and resynthesis involving the enzymes invertase, sucrose synthase, and sucrose phosphate synthase (Stitt et al., 1995). The observed carbohydrate transformation (i.e., sucrose hydrolysis) in *B. officinalis* and *Brassica* spp. nectary fluid that occurred when sucrose was unloaded from the phloem is the mechanism postulated for nectary control of sink strength.

Both sucrose and a number of non-sucrose oligosaccharides were observed by CGC-FID in both *B. officinalis* and *Brassica* spp. nectary fluids with retention times ranging from approximately 20-60 min as illustrated in Figure 4.4. At approximately equivalent ºBrix values, the relative abundance of these compounds was greater in *B. officinalis* than in *Brassica* spp. nectary fluids. Oligosaccharide formation in nectary fluids and possible mechanism(s) for their formation will be discussed in Section 4.3.

4.3 Nectar

4.3.1 Nectar Volume, ºBrix, and pH

Nectars of *B. officinalis* and each *Brassica* sp. were characterized by measuring their mean volume (µL), pH, and soluble solids content (ºBrix) and these results are presented in Table 4-1. The mean volume and range values for *B. officinalis*, *B. napus*, *B. napus* transgenic, and *B. rapa* were 1.94, 0.43, 0.66, and 0.34, respectively. The pH mean and range values for *B. officinalis*, *B. napus*, *B. napus* transgenic, and *B. rapa* were 4.52, 4.70, 4.77, and 4.87, respectively. The ºBrix mean and range values for *B. officinalis*, *B. napus*, *B. napus* transgenic, and *B. rapa* were 64.94, 61.08, 58.83, and 54.89, respectively.

Table 4-1 Mean volume, pH, ºBrix, standard deviation, and range results for the nectars of *Borago officinalis* L. and *Brassica* spp. L. plants[1].

Species	Volume (µL)	pH	ºBrix
Borago officinalis L.	$1.94 \pm 0.22^{a*}$ (1.59-2.31)	4.52 ± 0.15^{a} (4.40-4.70)	64.94 ± 1.73^{a} (62.40-67.80)
Brassica napus L. (var. *AC Excel*)	0.43 ± 0.19^{b} (0.16-0.88)	4.70 ± 0.00^{b} (4.70-4.70)	61.08 ± 4.47^{b} (52.00-69.00)
B. napus L. transgenic (var. *AV 225 R. R.*)	0.66 ± 0.23^{c} (0.36-1.00)	4.77 ± 0.13^{bc} (4.70-5.00)	58.83 ± 4.89^{b} (50.00-67.20)
B. rapa L. (var. *AC Parkland*)	0.34 ± 0.10^{b} (0.10-0.48)	4.87 ± 0.15^{c} (4.70-5.00)	54.89 ± 2.49^{c} (50.00-60.00)

[1]Results based on nectar analysis from three different plants per species with six flowers per plant analyzed, for a total of 18 samples for *Borago officinalis* L. and 36 samples from the lateral nectaries of *Brassica* spp. L.; for *Brassica* spp. L., nectar data pertain to the pair of lateral nectaries per flower only; *p <0.05; significant differences between plant species and varieties are indicated by different letters.

The significantly higher nectar volume observed for *B. officinalis* when compared to all *Brassica* spp. may be explained by the larger flower (~2.5 cm^2) and nectary size of this species compared to *Brassica* spp. Although the nectar volume of *B. officinalis* has not been reported in literature, a value of 2.05 µL for *Cerinthe major*, which is a member of the same family, has been determined (Nocentini et al., 2012) and is identical to that observed for *B. officinalis* in this study.

It has been reported that small flowers contain less water and produce less nectar when compared to larger flowers (Plowright, 1981; Cresswell & Galen, 1991). In addition, flowers with

a large nectary were shown to secrete more nectar than those with smaller ones (Weryszko-Chmielewska et al., 2004). As an example, floral nectar collected from *Ipomoea* species showed that total secreted volume correlated with nectary size and flower length (Galetto & Bernardello, 2004). The authors observed that as flower size and length increased, there was a concomitant increase in both nectar volume and nectar secretion.

The relationship between flower size and nectar volume was also observed in *Brassica* spp. in this study. *B. napus* flowers were larger and produced more nectar than those for *B. rapa*. Also, *B. napus* transgenic had the statistically highest *Brassica* nectar volume ($p < 0.05$), followed by *B. napus*, and lastly *B. rapa*. The visual observation that *B. napus* flowers were larger than those of *B. rapa* confirmed previous reports on *Brassica* spp. flower size, where mean flower dry weights for *B. napus* was the highest (6.87 mg) compared to the diploid (3.23 mg) and tetraploid (4.63 mg) *B. rapa* flowers (Davis et al., 1996). Mohr and Jay (1987) observed that *B. napus* cultivars had larger flowers than *B. campestris* (syn. *B. rapa*) and produced more nectar (0.90 µL vs. 0.68 µL). A study on the nectar secretion of 28 varieties and breeder's lines of two *Brassica* species found that nectar yield per flower was higher in the larger *B. napus* (0.348 µL/24 h) than the smaller *B. campestris* (0.165 µL/24 h) flowers (Szabo, 1982).

The ºBrix value for *B. officinalis* nectar was significantly higher than those observed for all *Brassica* spp. nectars. For *B. officinalis* nectar, the value of approximately 65 was higher than literature reported values of 61 for *Anchusa officinalis* and 62 for *Echium plantagineum*, both of which are members of the Boraginaceae (Corbet & Delfosse, 1984; Weryszko-Chmielewska & Chwil, 2007). In *Brassica* spp., the nectar ºBrix values of both varieties of *B. napus* were significantly ($p < 0.05$) higher than that observed for *B. rapa*. The nectar ºBrix results of approximately 55 to 61 determined for the *Brassica* spp. samples analyzed in this study were in agreement with those reported by Mohr and Jay (1990) of 57 and 62 for *B. campestris* and *B. napus*, respectively.

Variations in nectar carbohydrate concentration can be a result of nectary activities including secretion and reabsorption, by forager removal, and by nectar carbohydrate composition (Nicolson & Thornburg, 2007). Hexose rich nectars would be expected to evaporate more slowly than those that are sucrose rich (at the same concentration on a w/w basis), because of the water binding/interacting properties of these lower molecular mass carbohydrates (Corbet, 1978). For example, it has been shown that at the same carbohydrate concentration, hexose-rich nectars have

much higher osmolalities than sucrose-rich nectars (Corbet, 1978; Corbet et al., 1979; Nicolson, 1994), leading to slower water evaporation and lower final carbohydrate concentrations at equivalent ambient relative humidities. Higher nectar osmolalities may also draw water into the nectar resulting in a significant increase in volume (Nicolson, 2002; Nicolson & Thornburg, 2007).

The observed mean pH values for the *Brassica* spp. of 4.70 (*B. napus*), 4.77 (*B. napus* transgenic), and 4.87 (*B. rapa*) were similar and all were significantly different ($p < 0.05$) from that of *B. officinalis* at 4.52. As each of these plant nectars had pH values that were significantly lower than pH 7.0, they can all be described as acidic. Although nectar pH is rarely reported in literature, the observed range is wide from 3 for *Silene alba* (Caryophyllaceae) to 10 for *Viburnum costaricanum* (Caprifoliaceae) (Baker & Baker, 1983).

4.3.2 Major Carbohydrate Composition and Concentration

Analysis of *B. officinalis* and *Brassica* spp. nectars for their carbohydrate content by HPAE-PAD (LC Method 3) showed the presence of fructose, glucose, and sucrose (Figures 4.5a-c). Carbohydrate elution order and their RTs (min) under the experimental conditions employed were: glucose (~4.3 min), fructose (~4.8 min), and sucrose (~8.4 min). The observed HPAE-PAD RTs for fructose, glucose, and sucrose in the nectar samples were different than those observed in the nectary fluids because different HPAE-PAD systems and programs were used for sample carbohydrate analysis.

Percent mean (w:v) for glucose, fructose, and sucrose for sample nectars was: (a) *B. officinalis*: 21.56, 17.38, and 61.05; (b) *B. napus*: 54.39, 44.87, and 0.73; (c) *B. napus* transgenic: 54.81, 44.45, and 0.84; and (d) *B. rapa*: 53.47, 45.15, and 0.77, respectively (Table 4-2). Significant differences were not observed ($p > 0.05$) for the glucose, fructose, and sucrose concentration values among the *Brassica* spp. used in this study. However, the glucose, fructose, and sucrose concentrations of *B. officinalis* were found to be significantly different ($p < 0.05$) when compared to those of the *Brassica* spp., with glucose and fructose concentrations being lower and sucrose higher. These results clearly show that fructose, glucose, and sucrose were the major carbohydrates present in these plant nectars. The presence of glucose, fructose, and sucrose as an aqueous solution in the nectar of flowers enables easy ingestion, digestion, and absorption of these nutrients by pollinators, making nectar a major floral reward (Nicolson, 2007).

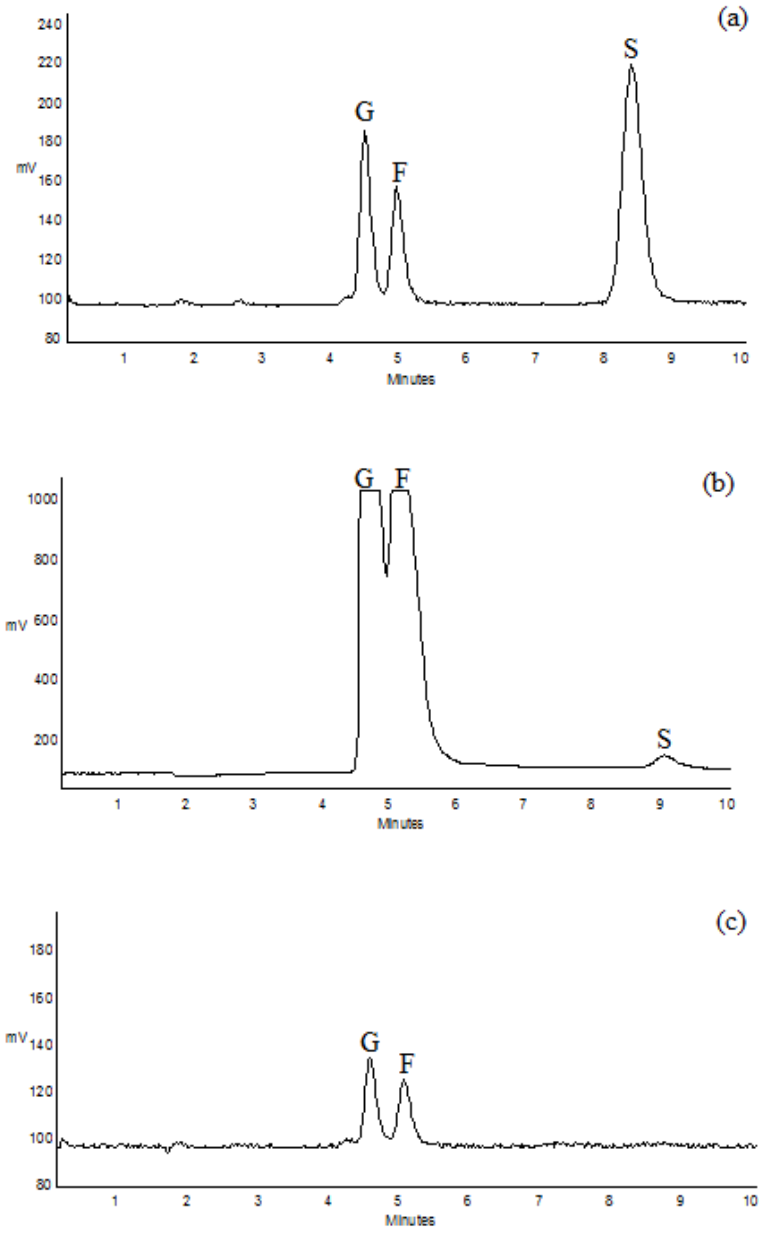

Figure 4.5 HPAE-PAD chromatograms showing the major carbohydrates present in (a) *Borago officinalis* L., (b) and (c) *Brassica napus* L. (var. *AC Excel*) nectar conducted under isocratic (80.0 mM NaOH) mobile phase conditions. A more concentrated sample (b) was employed for *Brassica* spp. L. nectars (e.g., *B. napus* [var. *AC Excel*]) so as to illustrate quantifiable sucrose levels. Diluted nectar samples (c) were employed for *Brassica* spp. L. (e.g., *B. napus* [var. *AC Excel*]) glucose and fructose quantification. Major carbohydrates were identified as: glucose (G; Retention Time [RT] of ~4.3 min), fructose (F; RT of ~4.8 min), and sucrose (S; RT of ~8.4 min) based on retention time comparisons with standards.

Table 4-2 Mean, standard deviation, and range values for percent glucose, fructose, and sucrose in *Borago officinalis* L. and *Brassica* spp. L. nectars[1].

Species	Glucose (%)	Fructose (%)	Sucrose (%)
Borago officinalis L.	21.56 ± 2.12[a*] (17.34-25.86)	17.38 ± 2.03[a] (13.28-22.90)	61.05 ± 3.66[a] (52.88-67.99)
Brassica napus L. (var. *AC Excel*)	54.39 ± 1.59[b] (50.87-58.20)	44.87 ± 1.65[b] (40.93-48.66)	0.73 ± 0.47[b] (0.16-2.56)
B. napus L. transgenic (var. *AV 225 R. R.*)	54.81 ± 2.11[b] (50.18-59.62)	44.45 ± 2.20[b] (40.02-49.46)	0.84 ± 0.47[b] (0.14-2.06)
B. rapa L. (var. *AC Parkland*)	53.47 ± 1.92[b] (49.38-58.15)	45.15 ± 2.29[b] (39.79-49.28)	0.77 ± 0.41[b] (0.07-1.95)

[1]Results based on nectar analysis from six different plants per species with ten flowers per plant analyzed in duplicate; for a total of 60 samples for *Borago officinalis* L. and 120 samples from the lateral nectaries of *Brassica* spp. L.; for *Brassica* spp. L., nectar data pertain to the pair of lateral nectaries per flower only; *p <0.05; significant differences between plant species and varieties are indicated by different letters.

Literature supports these experimental results as the aforementioned carbohydrates have been shown to account for >70% (w:w) of nectar (Baker & Baker, 1983; Nicolson & Thornburg, 2007; Nepi et al., 2012). Davis et al. (1994, 1998) showed that the nectar carbohydrates of *B. napus* and *B. rapa* consisted almost exclusively of glucose (~52%) and fructose (~48%), with a low concentration of sucrose (0.4%) reported. Similarly, Pierre et al. (1999) showed that glucose and fructose were the major carbohydrates present in 71 genotypes of *B. napus* with mean values of 52.0% and 47.4%, respectively. Sucrose values were very low or undetectable with the highest value reported as 2%. A recent report by Lohaus and Schwerdtfeger (2014) showed that *B. napus* had glucose and fructose values of 52.3% and 47.4% respectively, and a low concentration of sucrose (0.4%). The quantitative nectar carbohydrate results for *Brassica* spp. obtained in this study were in agreement with those reported in literature and are also in agreement with those that describe *Brassica* nectars as hexose-dominant (Baker & Baker, 1979, 1982a, 1983). The qualitative and quantitative carbohydrate results on *B. officinalis* nectar from this research have not been previously reported. Based on the analytically determined mean sucrose/hexose ratio of 1.57 (61.1%/39.0%), *B. officinalis* nectar is sucrose dominant, which agrees with that reported in literature (Percival, 1961; Baker & Baker, 1982a).

Mean monosaccharide (G/F) ratio results in this study were 1.25, 1.21, 1.24, and 1.19 for *B. officinalis*, *B. napus*, *B. napus* transgenic, and *B. rapa*, respectively. Results showed that the *B. officinalis* G/F ratio significantly differed ($p < 0.05$) from those of *B. napus* and *B. rapa*. However, the *B. officinalis* G/F ratio was not significantly different from *B. napus* transgenic. Comparison of the mean G/F ratios of *Brassica* spp. showed significant differences ($p < 0.05$). Nectar monosaccharide results for all plants consistently showed higher glucose than fructose levels in all 420 samples analyzed. The G/F results obtained in this study were higher than those reported in literature for *Brassica* spp. of 1.02-1.13 (Davis et al., 1994, 1998) and 1.10 (Pierre et al., 1999).

No literature report on the G/F ratio of *B. officinalis* nectar was available for comparison to the results obtained in this study. As sucrose hydrolysis results in a 1:1 concentration ratio of glucose to fructose, the observed deviation from this ratio is not readily explained by enzymatic (e.g., invertase) hydrolytic activity and may possibly involve other mechanisms and/or cellular events. Possible reasons for the deviation from the 1:1 concentration ratio of glucose to fructose include, the cycling of sucrose, glucose, and fructose in different biochemical pathways before nectar secretion (Wenzler et al., 2008), microbial degradation (Lüttge, 1961; Herrera et al., 2008), and the formation of oligosaccharides (Lüttge, 1962).

The analysis of nectar carbohydrates in these plant samples further established that carbohydrate transformation occurred during nectar production in the nectary. In *B. officinalis*, as phloem sap carbohydrate (i.e., sucrose) was transported through the nectary it was hydrolyzed to produce a monosaccharide-rich (fructose and glucose) solution (>99%) and was then resynthesized back to a sucrose-rich solution (~60%) before being secreted as nectar. In *Brassica* spp., nectary fluid carbohydrates appear to have been either directly secreted as nectar based on similar sucrose concentrations or sucrose may have been resynthesized during passage from the nectary followed by hydrolysis in nectar.

These results bring into question several literature reports that phloem sap components are directly transported to the nectar without chemical change (Agthe, 1951; Frey-Wyssling et al., 1954; Zimmermann, 1954; Lüttge, 1961; Fahn, 1988; De la Barrera & Nobel, 2004). The results from this study support more recent literature results of chemical composition differences between phloem fluid and nectar, particularly in terms of hexose carbohydrates and proteins (Escalante-Pérez & Heil, 2012; Orona-Tamayo et al., 2013; Lohaus & Schwerdtfeger, 2014). For the *B.*

officinalis and *Brassica* spp. samples analyzed in this study, nectar production was not the mere secretion of phloem sap and involved metabolic processes occurring in the nectaries and/or nectar.

4.3.3 Non-Sucrose Oligosaccharide Composition

In addition to determining their major carbohydrate (fructose, glucose, and sucrose) composition, nectars from *B. officinalis* and *Brassica* spp. were analyzed for their oligosaccharide content by CGC-FID. Oligosaccharide profiles for *B. officinalis* and each of the *Brassica* spp. nectars grown under phytotron conditions were determined by CGC-FID and representative chromatograms are shown in Figure 4.6. Chromatographic results from these analyses clearly showed the presence of non-sucrose oligosaccharides in all nectars with RTs ranging from ~25 to 57 min for *B. officinalis* and ~30 to 40 min for all *Brassica* spp., with sucrose having a RT of ~35 min under the experimental conditions employed.

Based on CGC-FID results, a set of common and unique or marker oligosaccharides were identified in sample nectars based on peak retention time data and these results are reported in Table 4-3. The CGC-FID protocol used in this work is quite specific for carbohydrate derivatization (e.g., phenolics and organic acids are not derivatized) and the temperature program employed was designed for oligosaccharide (i.e., disaccharide and trisaccharide) separation.

Table 4-3 CGC-FID retention times of major non-sucrose oligosaccharides in the nectars of *Borago officinalis* L. and *Brassica* spp. L. employing disaccharide/trisaccharide temperature program.

Sample	**Non-Sucrose Oligosaccharide Retention Times (min)**					
Borago officinalis L.	25.38	29.18		37.87	40.40	57.80
Brassica napus L. (var. *AC Excel*)			29.42	37.84	40.42	
B. napus L. transgenic (var. *AV 225 R. R.*)			29.43	37.82	40.44	
B. rapa L. (var. *AC Parkland*)			29.42	37.84	40.44	

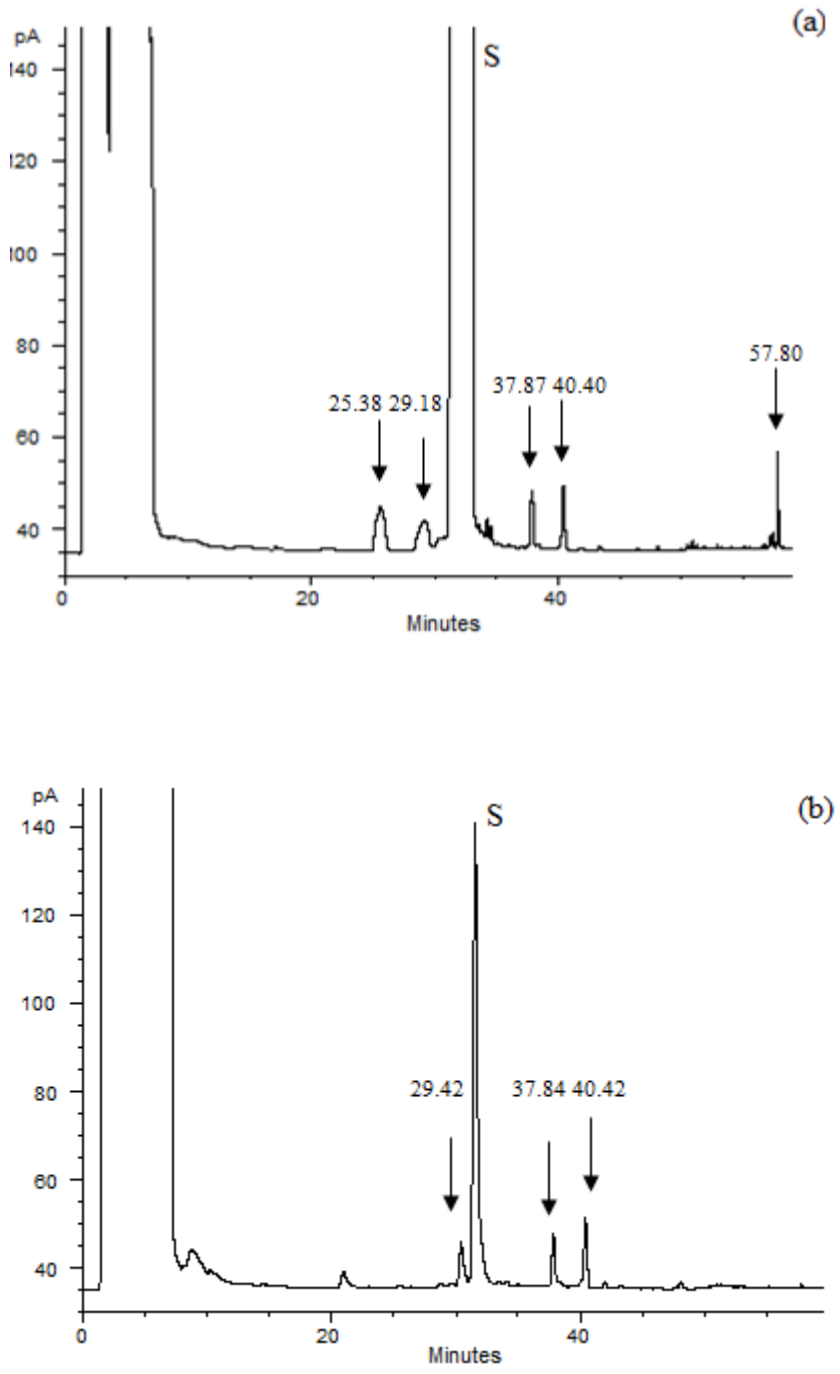

Figure 4.6 CGC-FID chromatograms showing the Sylon TP derivatized oligosaccharide profile of (a) *Borago officinalis* L. and (b) *Brassica napus* L. (var. *AC Excel*) nectars employing temperature program conditions (S: sucrose).

Non-sucrose oligosaccharide peaks that were common to both genera had approximate RTs of 37.9 and 40.4 min and all *Brassica* spp. showed identical oligosaccharide patterns but at differing concentrations (i.e., peak height differences). Unique (i.e. fingerprint) oligosaccharide peaks present in *B. officinalis* had RTs of 25.38, 29.18, and 57.80 min, and at ~29.42 min for all *Brassica* spp. The presence of these oligosaccharides may offer an alternative or supporting evidence to melissopalynology (i.e., identifying the botanical origin of honey through pollen analysis) for determining the botanical origin of honey from these plant species. As reducing carbohydrates can exist in a number of tautomeric forms, it is possible that a single oligosaccharide could yield multiple peaks under the reaction conditions employed in this study.

Based on their chromatographic retention times and carbohydrate composition (fructose, glucose, and sucrose), attempts to identify the non-sucrose oligosaccharides present in *B. officinalis* and *Brassica* spp. nectars by RT comparison to a series of commercially available disaccharides and trisaccharides were conducted. These glucose-glucose and glucose-fructose disaccharide and trisaccharide standards included: cellobiose, gentiobiose, isomaltose, kojibiose, laminaribiose, maltose, maltulose, melibiose, nigerose, palatinose, and trehalose for the disaccharides, and erlose, 1-kestose, melezitose, panose, and raffinose for the trisaccharides.

The common and systematic (IUPAC) names and their CGC-FID RTs under the chromatographic conditions employed are shown in Table 4-4. Chromatographic results for reducing oligosaccharides (e.g., maltose) showed the presence of both α- and β-anomers, whereas non-reducing oligosaccharides (e.g., raffinose and trehalose) gave a single detector response. The RTs of series of these standards ranged from 37 to 41 min (e.g., trehalose and maltulose), which included the two common oligosaccharides (37.8 and 40.4 min) identified in both *B. officinalis* and *Brassica* spp., however spiking experiments did not support their identification. Unfortunately, the RTs of the marker oligosaccharides identified in *B. officinalis* and *Brassica* spp. were not a match for any of the standards analyzed in this study. The most likely explanation for the lack of identification of the marker oligosaccharides in these nectars was due to the small number of standards available and the minimal coverage of fructose-fructose and sucrose-fructose linked disaccharides and trisaccharides, respectively.

Trace oligosaccharide levels have been reported in a selection of nectars as determined by paper, thin-layer, high performance, and gas chromatography. These oligosaccharides include cellobiose, gentiobiose, lactose, maltose, melibiose, melezitose, raffinose, stachyose, turanose, and

trehalose (Percival, 1961; Baskin & Bliss, 1969; Bowden, 1970; Jeffrey et al., 1970; Watt et al., 1974; Baker & Baker, 1982a; Nicolson & Van Wyk, 1998; Petanidou, 2005).

As discussed previously, many of the aforementioned oligosaccharides were not identified in the samples analyzed in this study by CGC-FID based on retention time comparison to standards. In summer rape nectar, several oligosaccharides were observed by CGC-FID and reported by our research group, however these oligosaccharides were not identified (Pernal & Currie, 1997).

The presence of the same marker oligosaccharide(s) in the nectars of all *Brassica* spp. indicates a certain level of chemical and/or biochemical synthetic control, which is further supported by the differences in marker peaks observed in *B. officinalis* nectar. These results lead to the postulate that the presence of oligosaccharides in nectar is plant specific and could be employed as either an alternative or corroborative method to melissopalynology. These results also show that carbohydrate synthesis in the form of non-sucrose oligosaccharides occur as phloem fluid moves to the nectary and to nectar.

Borago officinalis L. and *Brassica* spp. were also grown under field conditions in order to investigate the role of a less controlled environment on nectar oligosaccharide formation and composition. The oligosaccharide profiles for these nectars matched (chromatograms not shown) those observed for plants grown under phytotron conditions showing that these compounds would be present in commercially grown *B. officinalis* and *Brassica* spp. plants.

Table 4-4 CGC-FID retention times for oligosaccharide standards employing the disaccharide/trisaccharide temperature program.

Common Name	Systematic Name	Retention Times (min)	
Maltose	O-α-D-glucopyranosyl-(1→4)-D-glucopyranose	33.93	36.63
Cellobiose	O-β-D-glucopyranosyl-(1→4)-D-glucopyranose	34.49	41.08
Nigerose	O-α-D-glucopyranosyl-(1→3)-D-glucopyranose	36.11	37.57
Trehalose	α-D-glucopyranosyl-β-D-glucopyranoside	37.14	
Kojibiose	O-α-D-glucopyranosyl-(1→2)-D-glucopyranose	37.40	41.52
Maltulose	O-α-D-glucopyranosyl-(1→4)-D-fructose	38.18	41.37
Palatinose	O-α-D-glucopyranosyl-(1→6)-D-fructofuranose	38.45	
Laminaribiose	O-β-D-glucopyranosyl-(1→3)-D-glucopyranose	39.97	42.01
Isomaltose	O-α-D-glucopyranosyl-(1→6)-D-glucopyranose	45.35	48.95
Melibiose	O-α-D-galactopyranosyl-(1→6)-D-glucopyranose	44.55	46.14
Gentiobiose	O-β-D-glucopyranosyl-(1→6)-D-glucopyranose	48.03	
Raffinose	O-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-β-D-fructofuranoside	36.36	
1-Kestose	O-β-D-glucopyranosyl-(1→2)-β-D-fructofuranosyl-(1→2)-β-D-fructofuranoside	36.91	
Panose	O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→4)-D-glucopyranose	38.50	
Erlose	O-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-β-D-fructofuranoside	38.70	
Melezitose	O-α-D-glucopyranosyl-(1→3)-β-D-fructofuranosyl-α-D-glucopyranoside	40.00	

4.4 Nectar Microbial Assay

Microorganisms are also known to alter the carbohydrate composition of nectars (D'Amore et al., 1989; von der Ohe, 1994; Barnett, 1997; Berthels et al., 2004). In order to investigate the possibility of this mechanism for carbohydrate transformation and synthesis, the presence of microorganisms (specifically yeasts and bacteria) in the nectars of *B. officinalis* and each of the *Brassica* spp. used in this study was investigated employing a nutrient medium that would support the growth of these organisms (i.e., Bacto nutrient agar; Gilliam et al., 1983). Positive controls included the yeast, *Saccharomyces cerevisiae* (ATCC 24859), the bacteria, *Staphylococcus aureus* (ATCC 25923), and Gram-negative/positive rod-shaped bacteria (*Escherichia coli* [ATCC 11303]/*Bacillus subtilis* [ATCC 6051]). No microbial growth was observed for any of the plant nectars, however all of the positive controls showed growth under the same experimental conditions. The lack of microbial growth in the *B. officinalis* and *Brassica* spp. nectars studied may be due to their high carbohydrate concentrations (≥55 ºBrix), acidic pH (4.5-4.9), and the nectar redox cycle, which results in the production of hydrogen peroxide (Carter & Thornburg, 2004a). The absence of microorganisms in these nectar samples would eliminate their role in the observed carbohydrate hydrolysis and synthesis as phloem sap is transformed to nectar.

The non-sucrose oligosaccharides observed in the nectars and nectary fluids of *B. officinalis* and *Brassica* spp. are most likely synthesized via an enzymatic mechanism such as transglycosylation and/or glycosyl transferase. An alternative method for oligosaccharide formation is based on acid-catalyzed reversion reactions; however, based on the pH of the nectars in this study and the low pH conditions required for this reaction (<3.0), it is postulated that this reaction does not occur in either the nectary or nectar (Thavarajah & Low, 2006b). Based on the aforementioned phloem sap, nectary fluid, and nectar carbohydrate results, the next objective in this research was to investigate and identify the carbohydrases present in the nectaries of *B. officinalis* and *Brassica* spp. employing both specific substrate assays and proteomics.

4.5 Nectary and Nectar Carbohydrase Activities

Select Substrate Hydrolysis Reactions Employing α- and β-Glucosidase, and β-Fructosidase

Based on the observed changes in carbohydrate composition and structure as sucrose in the phloem was converted to nectar in *B. officinalis* and *Brassica* spp., possible mechanisms for these transformations were investigated. As outlined previously, sucrose hydrolysis in the nectary and

nectar have been reported in literature. Because carbohydrases are the catalysts involved in the hydrolysis of oligosaccharides and polysaccharides, the presence and activity of these enzymes as the reaction mechanism(s) for nectary and nectar sucrose hydrolysis, as observed in this study, was investigated. In these experiments, the nectaries and nectars of *B. officinalis* and *Brassica* spp. were separately subjected to ultrafiltration (10,000 daltons cut-off) treatment with water so as to remove virtually all of their carbohydrate content (<0.1 ppm as determined by HPAE-PAD; Section 3.6). The resulting carbohydrate-free nectaries and nectars of *B. officinalis* and *Brassica* spp. samples were individually incubated in water (pH 6.26) with a selection of oligosaccharide substrates including cellobiose, maltose, and raffinose and two commercial substrates, p-nitrophenyl-α-D-glucopyranoside (α-PNPG) and p-nitrophenyl-β-D-glucopyranoside (β-PNPG), to determine specific carbohydrase activities (Section 3.6).

As positive controls, commercial α-glucosidase, β-glucosidase, and β-fructosidase were incubated with substrate specific (e.g., cellobiose for β-glucosidase) solutions (100 ppm) of maltose, cellobiose, and raffinose, respectively. These solutions together with negative controls (100 ppm solutions of the same substrates without enzyme addition) were incubated at 30ºC for 3 h and were analyzed by HPAE-PAD (Section 3.4.5 LC Method 3) for their carbohydrate composition. Carbohydrate elution order from these hydrolysis reactions was glucose (~4.0 min), fructose (~5.2 min), melibiose (~6.0 min), cellobiose (~9.5 min), raffinose (~13.1 min), and maltose (~14.4 min). In the positive controls, maltose was hydrolyzed by α-glucosidase (~99%; based on peak area reduction) with concomitant formation of glucose; cellobiose was hydrolyzed by β-glucosidase (~80%; based on peak area reduction) with concomitant formation of glucose; and fructose and melibiose were produced from raffinose hydrolysis (~100%; based on peak area reduction) by β-fructosidase (Figure 4.7). Substrate hydrolysis was not observed in the negative control samples. In addition, non-substrate specific incubation (e.g., cellobiose with α-glucosidase) with each of the aforementioned carbohydrases showed no hydrolysis.

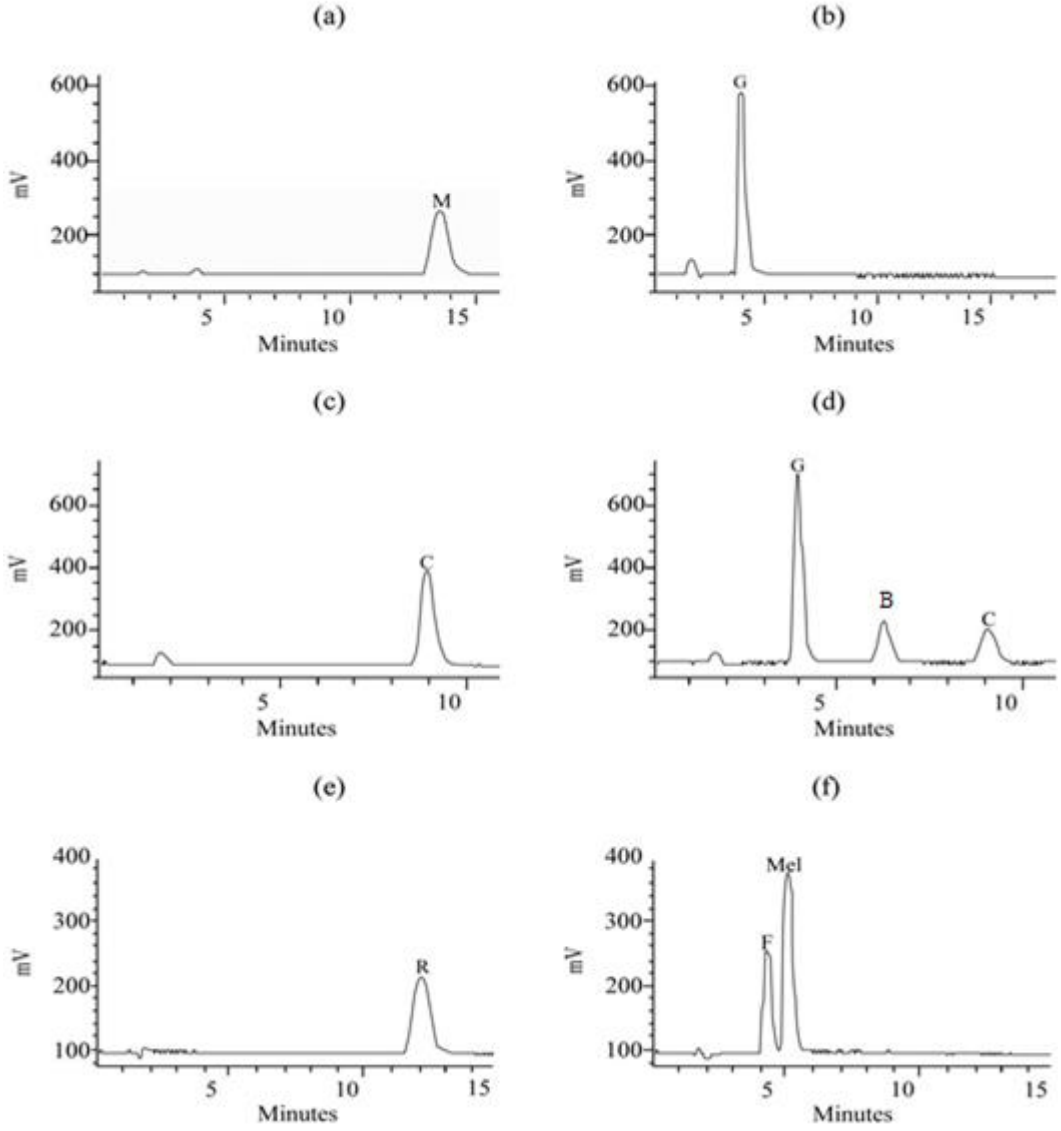

Figure 4.7 Substrate hydrolysis experiments for select commercial carbohydrases. (a) 2.0 mL of 100 ppm maltose; (b) 2.0 mL of 100 ppm maltose + 1.0 µL (≥10 units mg^{-1} protein) of commercial α-glucosidase; (c) 2.0 mL of 100 ppm cellobiose; (d) 2.0 mL of 100 ppm cellobiose + 1.0 µL (≥6 units mg^{-1}) of commercial β-glucosidase; (e) 2.0 mL of 100 ppm raffinose; and (f) 2.0 mL of 100 ppm raffinose + 1.0 µL (>300 units mg^{-1}) of commercial β-fructosidase. Symbols, G: glucose; F: fructose; C: cellobiose; Mel: melibiose; M: maltose; R: raffinose and B: commercial β-glucosidase constituent. All reactions were maintained at 30ºC for 3 h.

The incubation of 20 carbohydrate-free nectaries of *B. officinalis* with individual solutions of cellobiose, maltose, and raffinose solutions (100 ppm) for 72 h at 4ºC resulted in the hydrolysis of all three substrates. These results demonstrated the presence of α-glucosidase, β-glucosidase, and β-fructosidase activities in *B. officinalis* nectaries (Figure 4.8). To estimate the relative activity of these carbohydrases in the nectaries, their activities were compared after 24 h of incubation at 4ºC. Experimental results are reported as percent reduction in the peak area of the substrate (Table 4-5). Based on these results, the ranking of carbohydrase activities after 24 h observed in *B. officinalis* nectaries were: β-glucosidase/β-fructosidase (45% peak area reduction) > α-glucosidase (13% peak area reduction). However, this ranking is debatable due to: the limited number of nectaries and samples used in these experiments; hydrolysis reactions being conducted at non-optimum environmental conditions; and unknown carbohydrase concentrations and substrate specificities.

With the exception of substrate/carbohydrate-free nectary incubation times (ranging from 24 to 72 h), the same experimental conditions were followed for the carbohydrate-free nectaries of each *Brassica* sp. Experimental results showed substrate hydrolysis for each species (Figures 4.9-4.11), demonstrating the presence of α-glucosidase, β-glucosidase, and β-fructosidase activities in *Brassica* spp. nectaries.

Comparison of the carbohydrate-free nectary carbohydrase activities in *Brassica* spp. after 24 h incubation showed more rapid hydrolysis of raffinose (~24% average peak area reduction), followed by maltose (~18% average peak area reduction), and cellobiose (~7% average peak area reduction) (Table 4-5). These results demonstrated that the carbohydrase activity ranking observed for each *Brassica* sp. was similar, which may be due to similar nectary enzyme concentrations and/or substrate activities for all three varieties. Based on these results, the ranking of carbohydrase activities observed in *Brassica* spp. nectaries were: β-fructosidase > α-glucosidase > β-glucosidase. However, the aforementioned experimental limitations for *B. officinalis* also hold for the ranking of *Brassica* spp. nectary carbohydrases.

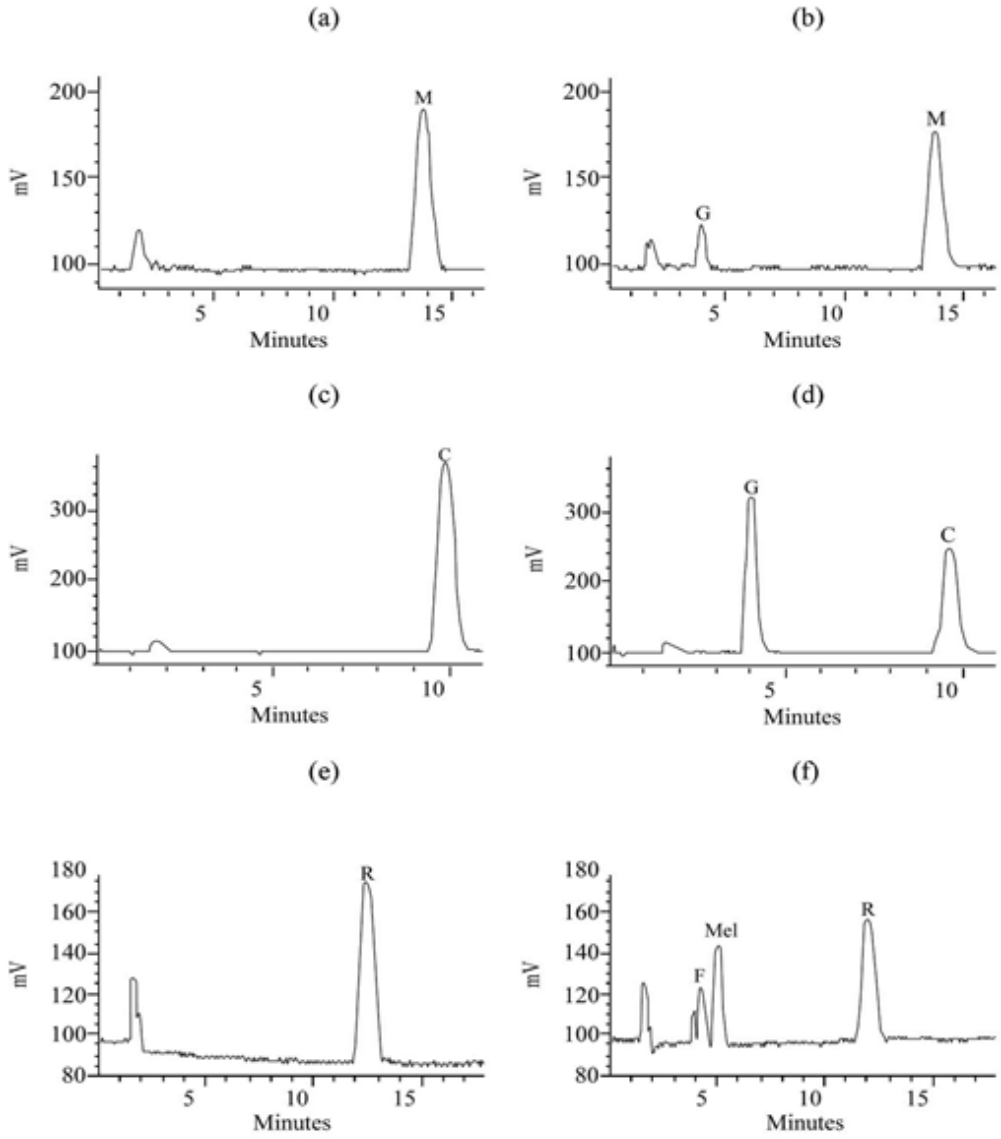

Figure 4.8 Substrate hydrolysis experiments for carbohydrate-free *Borago officinalis* L. nectaries. (a) 2.0 mL of 100 ppm maltose; (b) 1.0 mL of 100 ppm maltose + 20 carbohydrate-free nectaries incubated for 24 h; (c) 2.0 mL of 100 ppm cellobiose; (d) 1.0 mL of 100 ppm cellobiose + 20 carbohydrate-free nectaries incubated for 24 h; (e) 2.0 mL of 100 ppm raffinose; and (f) 1.0 mL of 100 ppm raffinose + 20 carbohydrate-free nectaries incubated for 24 h. Symbols, G: glucose; F: fructose; C: cellobiose; Mel: melibiose; M: maltose; and R: raffinose. All reactions were maintained at 4ºC.

Table 4-5 Hydrolysis activities reported as % peak area reduction of the substrate due to carbohydrase activities in 20 carbohydrate-free nectaries of *Borago officinalis* L. and *Brassica* spp. L. All reactions were maintained at 4ºC for 24 h.

Substrate/ Carbohydrase	***Borago officinalis* L.**	***Brassica napus* L. (var. *AC Excel*)**	***B. napus* L. transgenic (var. *AV 225 R. R.*)**	***B. rapa* L. (var. *AC Parkland*)**
maltose/α-glucosidase	13%	16%	22%	16%
cellobiose/β-glucosidase	45%	3%	7%	11%
raffinose/β-fructosidase	45%	22%	26%	24%

A comparison of the carbohydrase activities for carbohydrate-free nectaries of *B. officinalis* and *Brassica* spp. under the experimental conditions employed showed that β-fructosidase and β-glucosidase predominated for *B. officinalis* and β-fructosidase predominated for all *Brassica* spp. samples. In addition to the use of oligosaccharides as substrates, the presence of α- and β-glucosidase activities in the carbohydrate-free nectaries of *B. officinalis* and *Brassica* spp. were examined employing α- and β-PNPG as substrates. Results from these experiments showed both enzyme activities in all nectary samples analyzed as indicated by a positive colourimetric response measured spectrophotometrically at 400 nm (indicating the formation of p-nitrophenol). These experiments were conducted to confirm the oligosaccharide substrate results as a yes/no indicator for the presence of α- and β-glucosidase activities.

Carbohydrases, specifically O-glycoside hydrolases (E.C. 3.2.1.*x*) are highly efficient and specific catalysts for the hydrolysis of the glycosidic linkages of oligosaccharides and polysaccharides (Henrissat, 1998). The hydrolysis of glycosidic bonds by carbohydrases can follow at least two different mechanisms based on the different spatial arrangement of catalytic groups (e.g., carboxylic acids) leading to two possible stereochemical outcomes, inversion or retention of the anomeric configuration (Figure 4.12; Zechel & Withers, 2000).

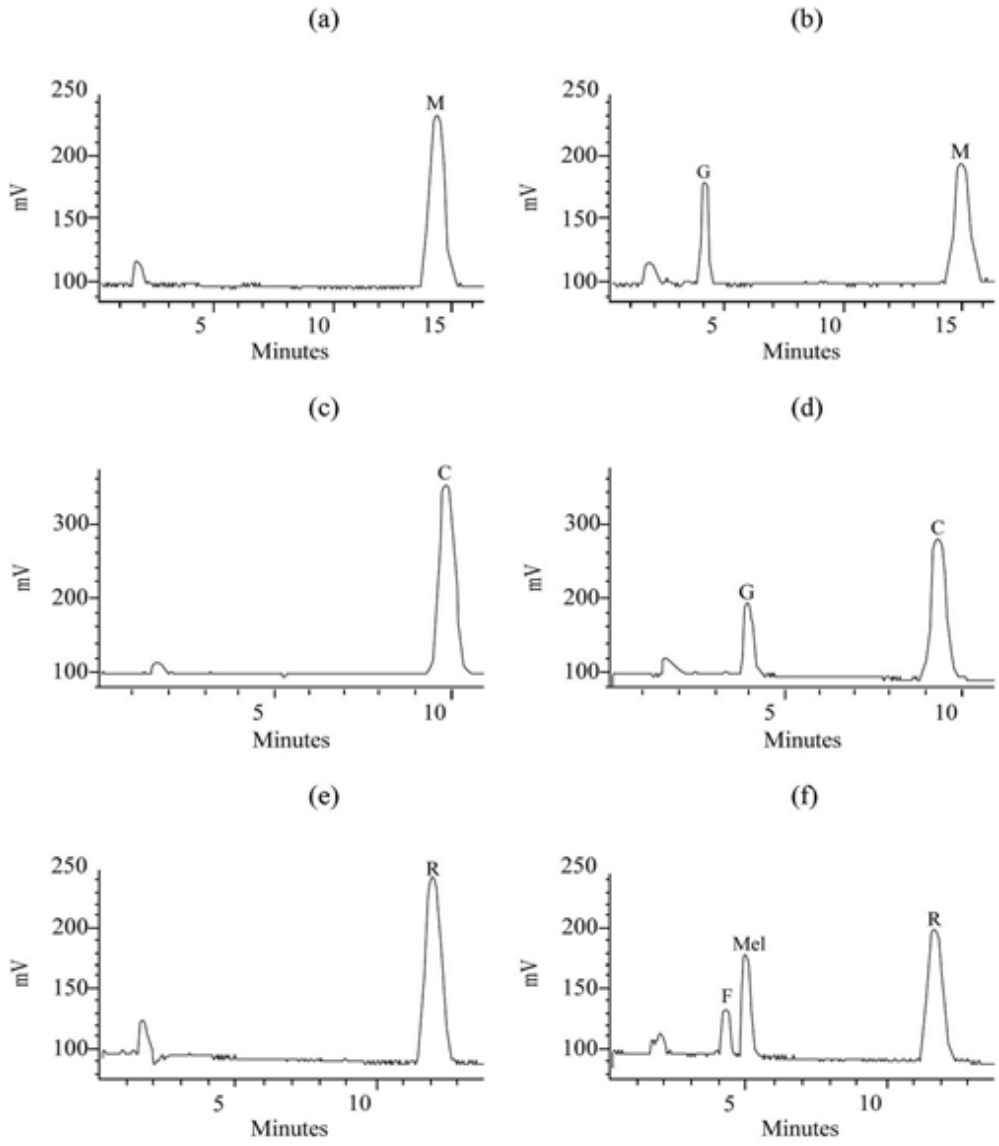

Figure 4.9 Substrate hydrolysis experiments for 20 carbohydrate-free *Brassica napus* L. (var. *AC Excel*) nectaries. (a) 2.0 mL of 100 ppm maltose; (b) 1.0 mL of 100 ppm maltose + 20 carbohydrate-free nectaries incubated for 48 h; (c) 2.0 mL of 100 ppm cellobiose; (d) 1.0 mL of 100 ppm cellobiose + 20 carbohydrate-free nectaries incubated for 72 h; (e) 2.0 mL of 100 ppm raffinose; and (f) 1.0 mL of 100 ppm raffinose + 20 carbohydrate-free nectaries incubated for 24 h. Symbols, G: glucose; F: fructose; C: cellobiose; Mel: melibiose; M: maltose; and R: raffinose. All reactions were maintained at 4ºC.

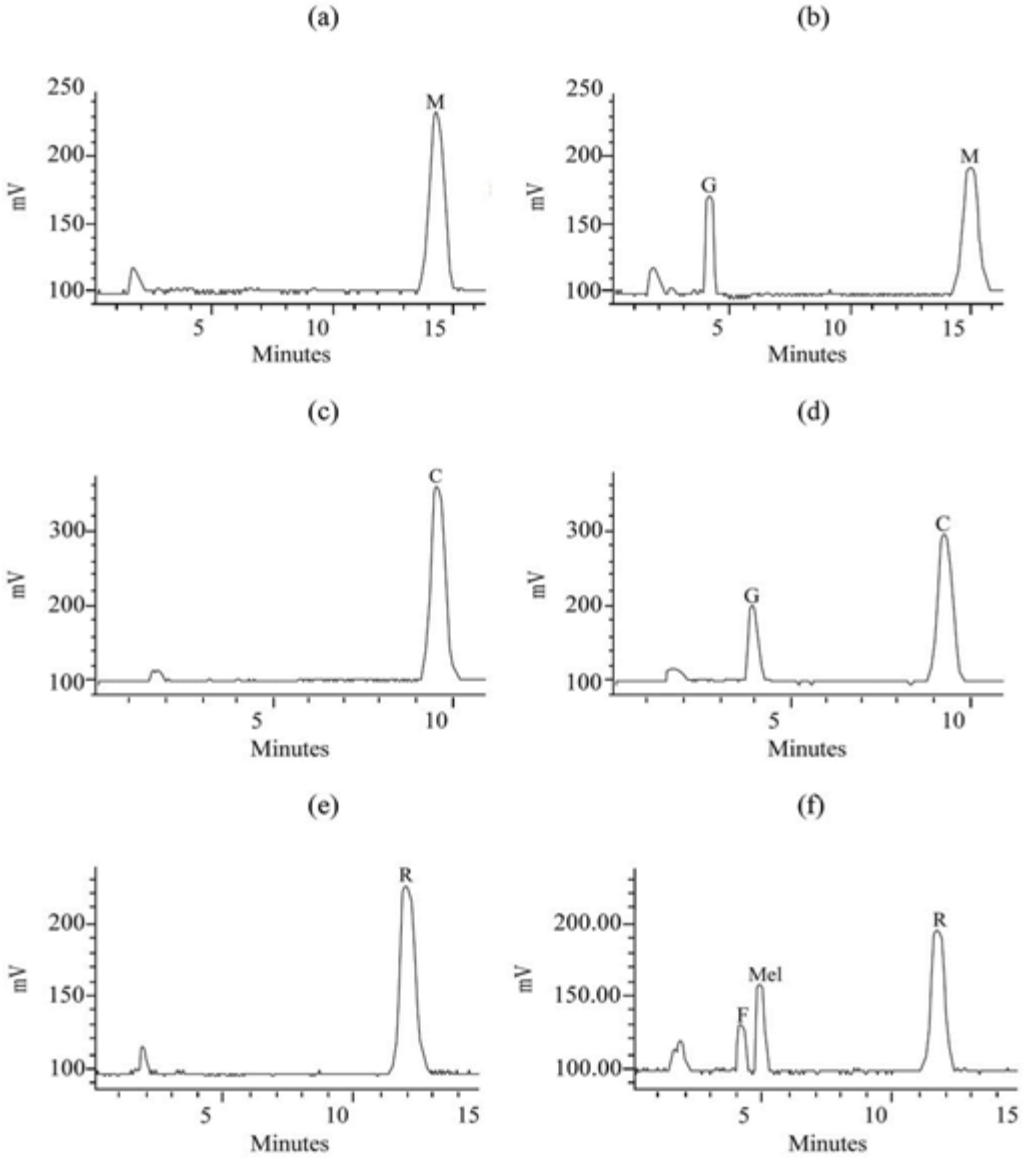

Figure 4.10 Substrate hydrolysis experiments for 20 carbohydrate-free *Brassica napus* L. transgenic (var. *AV 225 R. R.*) nectaries. (a) 2.0 mL of 100 ppm maltose; (b) 1.0 mL of 100 ppm maltose + 20 carbohydrate-free nectaries incubated for 48 h; (c) 2.0 mL of 100 ppm cellobiose; (d) 1.0 mL of 100 ppm cellobiose + 20 carbohydrate-free nectaries incubated for 72 h; (e) 2.0 mL of 100 ppm raffinose; and (f) 1.0 mL of 100 ppm raffinose + 20 carbohydrate-free nectaries incubated for 24 h. Symbols, G: glucose; F: fructose; C: cellobiose; Mel: melibiose; M: maltose; and R: raffinose. All reactions were maintained at 4ºC.

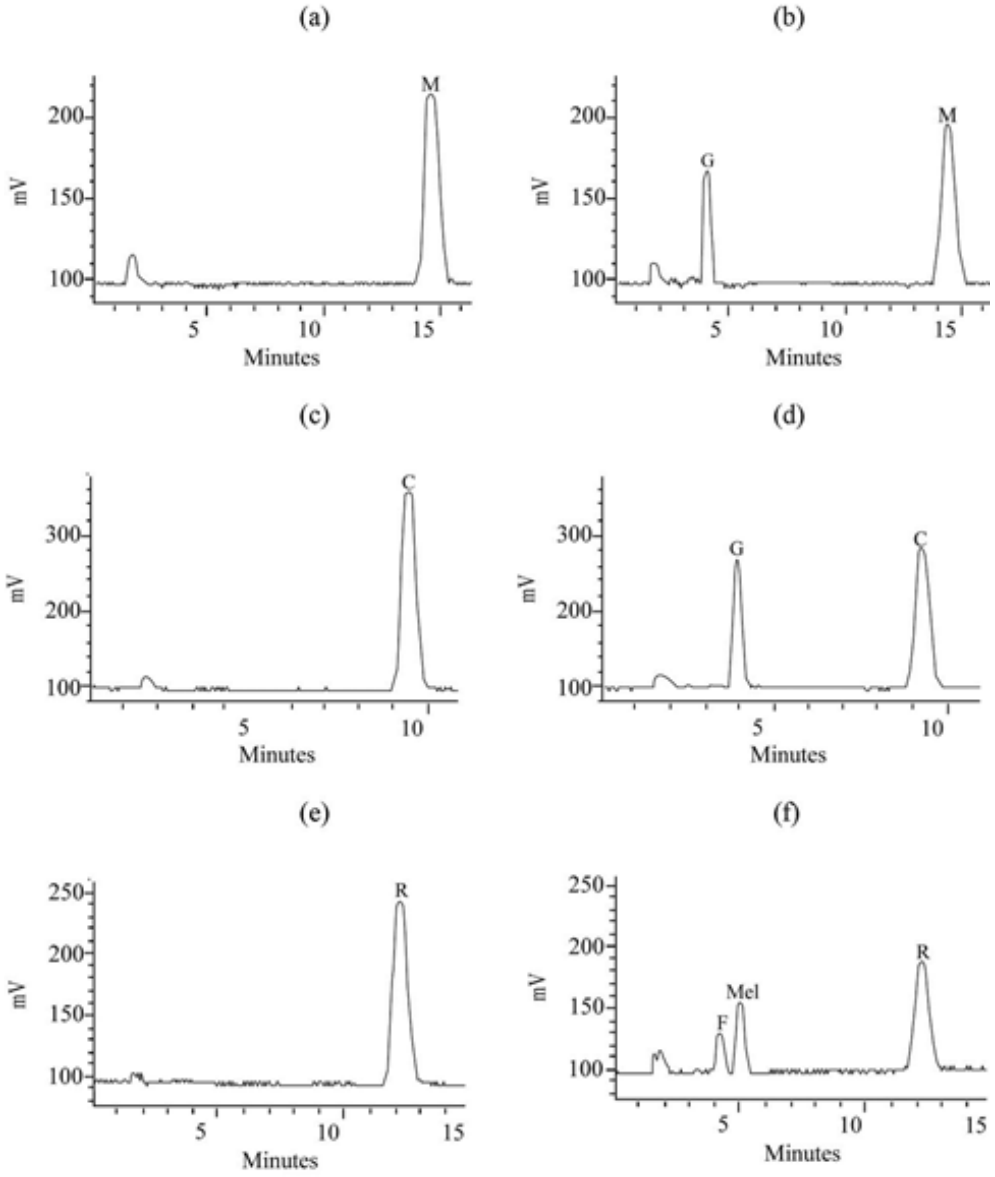

Figure 4.11 Substrate hydrolysis experiments for 20 carbohydrate-free *Brassica rapa* L. (var. *AC Parkland*) nectaries. (a) 2.0 mL of 100 ppm maltose; (b) 1.0 mL of 100 ppm maltose + 20 carbohydrate-free nectaries incubated for 48 h; (c) 2.0 mL of 100 ppm cellobiose; (d) 1.0 mL of 100 ppm cellobiose + 20 carbohydrate-free nectaries incubated for 72 h; (e) 2.0 mL of 100 ppm raffinose; and (f) 1.0 mL of 100 ppm raffinose + 20 carbohydrate-free nectaries incubated for 24 h. Symbols, G: glucose; F: fructose; C: cellobiose; Mel: melibiose; M: maltose; and R: raffinose. All reactions were maintained at 4ºC.

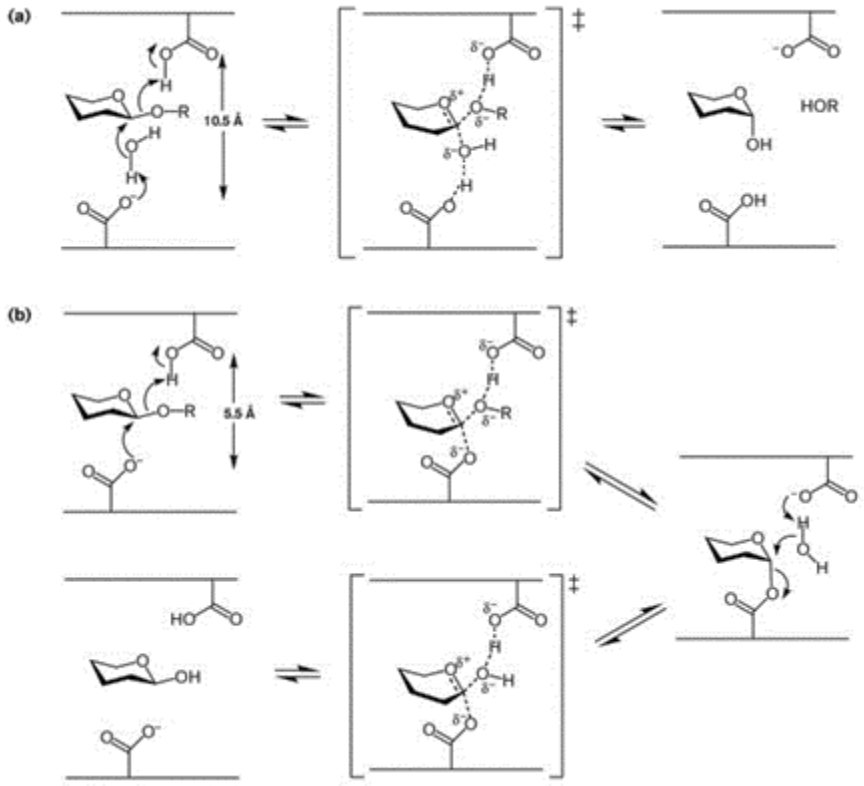

Figure 4.12 General mechanisms for inverting (a) and retaining (b) carbohydrases (adapted from Zechel and Withers, 2000).

The carbohydrase mechanism that results in the inversion of the anomeric configuration of the starting material involves the formation of an oxocarbonium ion-like transition state. The two carboxylic acid groups of inverting carbohydrases serve as general acid and general base catalysts and are located approximately 10.5 Å apart (McCarter & Withers, 1994). This distance between the carboxylic acid groups allows the binding of the substrate and a water molecule in the active site. The reaction proceeds via a single-displacement mechanism wherein one of the carboxylic acid residue protonates the scissile glycosidic oxygen while the other interacts with a nucleophile (i.e., water) resulting in inversion at the anomeric carbon and completion of the hydrolysis reaction (Figure 4.12a; Bras et al., 2012).

In the retention mechanism, the initial conformation of the anomeric carbon is maintained, the two carboxyl groups are approximately 5.5 Å apart and the reaction proceeds as a double-displacement mechanism involving both a glycosylation and deglycosylation step (Koshland, 1953; Zechel & Withers, 2000). In the glycosylation step, one of the carboxylic acid groups functions as a general acid catalyst leading to the protonation of the glycosidic oxygen and the

hydrolysis of the glycosidic bond. Concurrently, the other carboxylic acid residue acts as a nucleophile attacking the anomeric carbon at the oxocarbonium ion-like transition state to form a covalent glycosyl-enzyme intermediate (McCarter & Withers, 1994). This glycosylation step results in an anomeric configuration opposite that of the starting material. The deglycosylation step involves the hydrolysis of the glycosyl-enzyme intermediate, where the carboxylic acid group that initially functioned as an acid catalyst now acts as a base by deprotonating a water molecule. Simultaneously, the water molecule attacks the carbohydrate-enzyme linkage resulting in the formation of a hemiacetal with the same anomeric configuration as the starting material (Figure 4.12b; Bras et al., 2012). In the retention mechanistic pathway, the protonation of the glycosidic oxygen can also be afforded by $-B^{+}H$, where B is a basic amino acid such as histidine (Walsh, 1979).

The nectary carbohydrase activity experiments conducted in this study, where both β-fructosidase and α-glucosidase activities were observed in carbohydrate-free nectaries, readily explain the observed sucrose hydrolysis of phloem sap in *B. officinalis* and *Brassica* spp. and the mechanism of formation of glucose and fructose in nectary fluids (Section 4.2.1). In plants, sucrose hydrolysis enzymes that have been postulated to be present in sink organs (e.g., nectary) include invertase, α-glucosidase, and the glycosyl transferase enzyme, sucrose synthase (Quick & Schaffer, 1996).

Invertase is an O-glycoside hydrolase that is commonly associated with the hydrolysis of sucrose to glucose and fructose, and this activity can involve either a β-fructosidase or an α-glucosidase. However, true invertases (EC 3.2.1.26) are β-fructosidases and their presence in plants are further classified as apoplasmic (cell wall; CWIN), vacuolar (VIN), or cytoplasmic (CIN) isoforms depending on their optimum pH and subcellular locations. CWINs and VINs are acidic invertases with pH optima in the 4.5-5.0 range and are found in the cell walls and lumen of vacuoles, respectively, whereas CINs hydrolyze sucrose in the neutral or slightly alkaline range and are found in multiple subcellular compartments (Sturm, 1999).

The presence of invertase activity in plant nectaries has been reported in numerous studies (Pate et al., 1985; Nichol & Hall, 1988; Nicolson, 2002; Kram et al., 2009; Ruhlmann et al., 2010; Orona-Tamayo et al., 2013; Lohaus & Schwerdtfeger, 2014). Sucrose hydrolysis by invertase results in the production of hexose-rich nectars so as to create the required source-sink relationships for sucrose unloading from phloem (Heil, 2011). Ruan (2014) postulated that

invertase activity in the cell wall lowers sucrose concentration so as to create a steep concentration difference between phloem and the surrounding cell wall matrix. The importance of nectary invertase activity for nectar production was shown in *Arabidopsis*, where flowers of the reduced invertase activity mutant *Atcwin4* failed to secrete nectar (Ruhlmann et al., 2010).

Alpha-glucosidases (E.C. 3.2.1.20) are a highly diverse group of O-glycoside hydrolases that can hydrolyze α-1-1, -1-2, -1-3, -1-4, and -1-6 glycosidically-linked glucose oligosaccharides, polysaccharides, and glycoproteins and are present in different subcellular locations including the apoplast and cytoplasm (Monroe et al., 1999). Although widely found in nature, the presence of α-glucosidase in nectaries has not previously been reported. The presence of this enzyme activity in the nectaries of *B. officinalis* and *Brassica* spp. provides an additional mechanistic pathway for sucrose hydrolysis, which further supports the source-sink carbohydrate relationship and nectar secretion. Also, the observed α-glucosidase activity in the nectaries of both *B. officinalis* and *Brassica* spp. provides a mechanistic pathway for the observed changes in carbohydrate composition between phloem saps and nectary fluids for the plant samples analyzed in this study (Section 4.2.1).

Beta-glucosidases (E.C. 3.2.1.21) are glycosyl hydrolases catalyzing the hydrolysis of the β-O-glycosidic bond at the anomeric carbon at the non-reducing end of oligosaccharides and polysaccharides containing β-D-glucopyranose. Beta-glucosidase belongs to the glycosyl hydrolase 1 family, which are abundant in plants. The presence of this enzyme in plants has been reported to be related to biotic and abiotic stresses, herbivore defense, phytohormone activation, lignification, and cell wall remodeling (Opassiri et al., 2006). The presence of this enzyme in the nectary has not been previously reported in literature and as such its role in nectar production has not been established. As no simple (e.g., cellobiose) substrate for this enzyme was detected in the phloem sap of *B. officinalis* and *Brassica* spp., it can be hypothesized that its presence is not involved in source-sink carbohydrate relationships and nectar secretion. However, it has been shown that hemicellulose/cellulose hydrolysis via β-glucosidase is part of a phytochemical response employed by some plants (e.g., cabbage) to herbivores and pathogens (Mattiacci et al., 1995) and this may partially explain its presence in *B. officinalis* and *Brassica* spp. nectaries.

In addition to hydrolysis by β-fructosidase and α-glucosidase, the observed conversion of phloem sap sucrose to glucose and fructose in the nectaries of *B. officinalis* and *Brassica* spp. could also have been catalyzed by the cytosolic enzyme, sucrose synthase (E.C. 2.4.1.13). This

glycosyl transferase enzyme catalyzes the hydrolysis of sucrose into hexoses via the reversible transglycosylation reaction: UDP-glucose + fructose ↔ sucrose + UDP (Quick & Schaffer, 1996). Sucrose synthases have been identified in many plant tissues and are especially high in sink tissues (Avigad, 1982). As an example, Orona-Tamayo et al. (2013) observed the presence of this enzyme in the extrafloral nectary of *Acacia cornigera*. Sucrose synthase is also known to catalyze the synthesis of sucrose, however reports on the participation of this enzyme in sucrose formation are equivocal. Physiological and kinetic studies have shown that sucrose synthase is primarily involved in sucrose hydrolysis *in vivo* (Sturm & Tang, 1999). However, evidence for its participation in sucrose synthesis has also been reported in castor bean cotyledon, Jerusalem artichoke, and potato tubers (Geigenberger & Stitt, 1993; Nöel & Pontis, 2000).

Carbohydrase activities in the nectars of *B. officinalis* and a single *Brassica* sp. (*B. napus* var. *AC Excel*) were also determined employing the aforementioned oligosaccharide substrates. The choice of a single *Brassica* sp. was based on: the similar nectary enzyme substrate hydrolysis activities that were observed in all *Brassica* spp.; and that enzymes solubilized in the nectary fluid are expected to be present in the nectar. Because nectar was not collected from the same plants used for nectary carbohydrase studies, enzyme activity results between the nectary and nectar of this *Brassica* sp. can only be correlated.

Based on *B. officinalis* nectar substrate hydrolysis results as determined by HPAE-PAD: (1) α-glucosidase and β-fructosidase activities were detected (Figure 4.13); (2) no β-glucosidase activity was observed following 72 h of incubation at 4ºC; and (3) carbohydrase activity ranking after 24 h incubation at 4ºC was α-glucosidase (12% peak area reduction)/β-fructosidase (10% peak area reduction) > β-glucosidase (-; no peak area reduction observed) (Table 4-6). These results differed from those observed for *B. officinalis* nectaries where all three enzyme activities were higher, and all three (i.e., α-glucosidase, β-glucosidase, and β-fructosidase) activities were observed (Table 4-5). These differences in enzyme activity results are most likely explained by the lower concentration of these enzymes in the nectar when compared to the nectary.

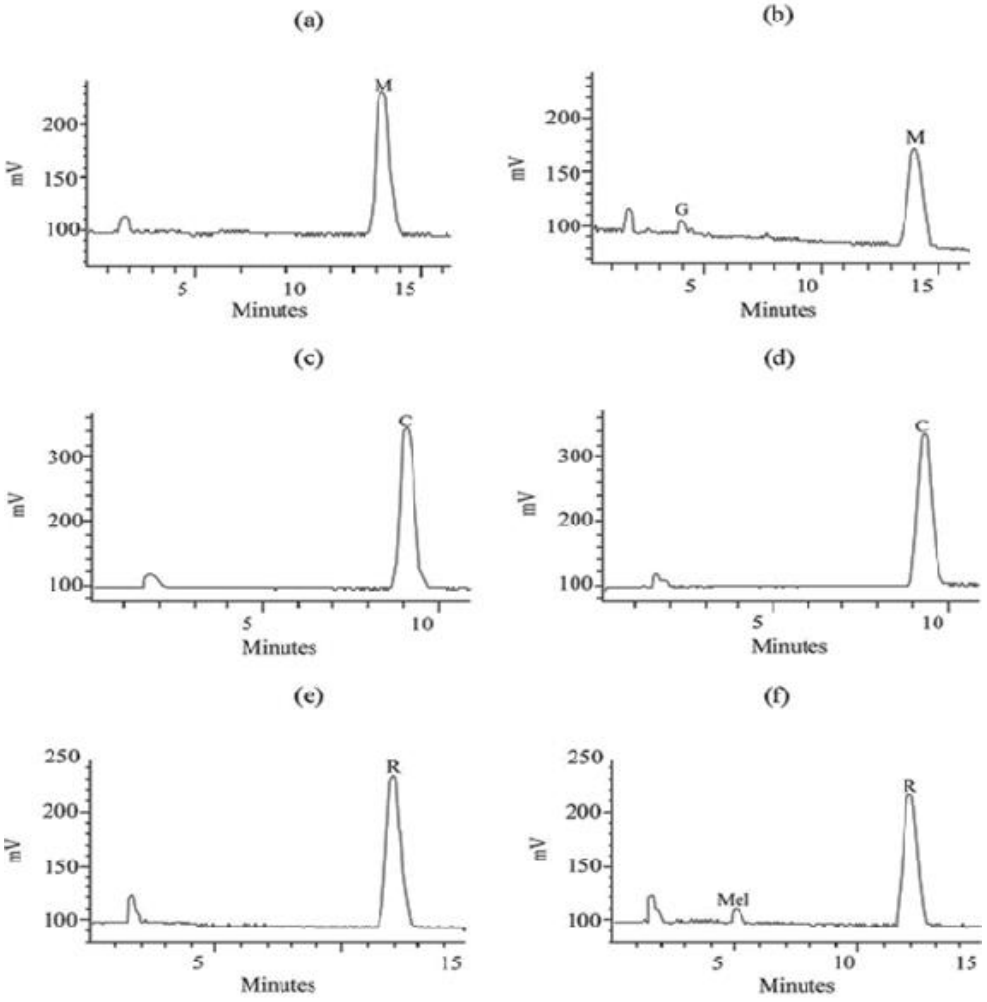

Figure 4.13 Substrate hydrolysis experiments for 400 µL *Borago officinalis* L. nectar. (a) 2.0 mL of 100 ppm maltose; (b) 1.0 mL of 100 ppm maltose + carbohydrate-free nectar incubated for 48 h; (c) 2.0 mL of 100 ppm cellobiose; (d) 1.0 mL of 100 ppm cellobiose + carbohydrate-free nectar incubated for 72 h; (e) 2.0 mL of 100 ppm raffinose; and (f) 1.0 mL of 100 ppm raffinose + carbohydrate-free nectar incubated for 48 h. Symbols, G: glucose; C: cellobiose; Mel: melibiose; M: maltose; and R: raffinose. All reactions were maintained at 4ºC.

Table 4-6 Hydrolysis activities reported as % peak area reduction of substrate due to carbohydrase activities in carbohydrate-free nectars of *Borago officinalis* L. and *Brassica napus* L. (var. *AC Excel*). All reactions were maintained at 4ºC for 24 h.

Substrate/Carbohydrase	***Borago officinalis* L.**	***Brassica napus* L. (var. *AC Excel*)**
maltose/α-glucosidase	12%	24%
cellobiose/β-glucosidase	0%	11%
raffinose/β-fructosidase	10%	29%

In *B. napus* (var. *AC Excel*) nectar substrate hydrolysis experiments, HPAE-PAD results showed that: (1) α- and β-glucosidase, and β-fructosidase activities were detected (Figure 4.14); (2) carbohydrase activity ranking after 24 h incubation at 4ºC was β-fructosidase (29% peak are reduction) > α-glucosidase (24% peak area reduction) > β-glucosidase (11% peak area reduction) (Table 4-6). These results were similar in ranking to those observed for the corresponding *B. napus* (var. *AC Excel*) nectary, however the enzyme substrate activities observed were found to be higher in the nectar than in the nectary (Table 4-5). Possible explanations for these results would be the high volume of nectar used in these studies and/or the fact that analytical samples were obtained from different plant nectaries and nectar.

Published reports on plant nectars have identified both invertase (Baker & Baker, 1983; Heil et al., 2005; Nepi et al., 2012; Seo et al., 2013) and β-glucosidase activities (Seo et al., 2013). These carbohydrase activities were used to explain the source-sink relationship for nectar secretion and the production of hexose-dominant nectars in the plants studied. Results from this research is the first to report the presence of α-glucosidase activity in both the nectaries and nectars of *B. officinalis* and *Brassica* spp. studied and support their source-sink carbohydrate relationships, and the production of hexose-dominant nectars in *Brassica* spp.

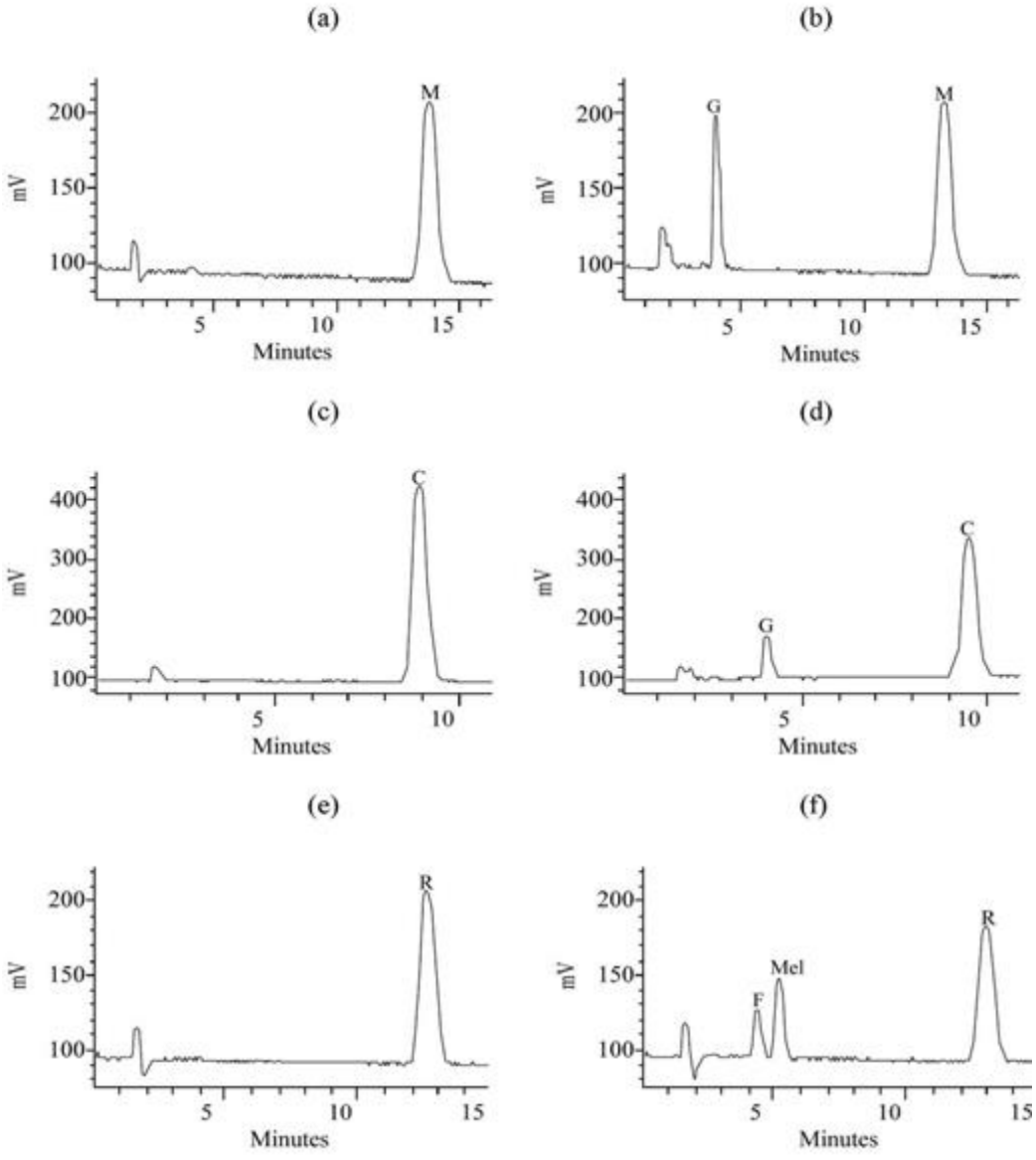

Figure 4.14 Substrate hydrolysis experiments for 400 µL *Brassica napus* L. (var. *AC Excel*) nectar. (a) 2.0 mL of 100 ppm maltose; (b) 1.0 mL of 100 ppm maltose + carbohydrate-free nectar incubated for 48 h; (c) 2.0 mL of 100 ppm cellobiose; (d) 1.0 mL of 100 ppm cellobiose + carbohydrate-free nectar incubated for 72 h; (e) 2.0 mL of 100 ppm raffinose; and (f) 1.0 mL of 100 ppm raffinose + carbohydrate-free nectar incubated for 24 h. Symbols, G: glucose; F: fructose; C: cellobiose; Mel: melibiose; M: maltose; and R: raffinose. All reactions were maintained at 4ºC.

A significant result from this work was the observed sucrose concentration changes in *B. officinalis* from phloem sap (~100%), to the nectary fluid (~ <5%) and to nectar (~61%). A possible explanation for these results was that sucrose hydrolysis via the aforementioned carbohydrases occurred in the nectary, followed by sucrose resynthesis prior to secretion as nectar. The enzymatic system most likely responsible for this resynthesis is sucrose phosphate synthase, and the presence of this enzyme in the nectary is hypothesized as the mechanism responsible for the high sucrose concentration observed in *B. officinalis* nectar.

It is also possible that sucrose was transported intact as pre-nectar, and was only partially hydrolyzed during nectar secretion because either, the concentrations of α-glucosidase and β-fructosidase were too low to cause sufficient sucrose hydrolysis, and/or environmental conditions (e.g., pH) were such that complete sucrose hydrolysis did not occur.

For *Brassica* spp., the major observed change in sucrose concentration occurred from phloem sap (~100%) to the nectary fluid (~ <5%), with minimal further changes observed in nectar (0.7-0.8%). These results indicate that either sucrose resynthesis prior to nectar secretion did not occur, or that resynthesized sucrose (via sucrose phosphate synthase) was hydrolyzed by the identified carbohydrases in the nectary and/or nectar. It is not clear from this research which of these two mechanistic pathways was followed, however the observed carbohydrase activities in *B. napus* (var. *AC Excel*) nectar (Table 4-6) could explain the almost complete hydrolysis of sucrose observed.

The observed carbohydrate structure and concentration changes as sucrose moves from phloem to the nectary and to nectar in *B. officinalis* and *Brassica* spp. is consistent with previous literature on the fate of carbohydrates in sink organs. Carbohydrates are metabolized as they are transported from phloem into the sink organs to ensure the continuous movement of the photosynthate from the source and also to provide direction to the movement of the photosynthate into sinks (Beevers, 1969). From phloem, sucrose is transported into sink organs following three different pathways: apoplastic, symplastic, and symplastic interrupted by an apoplastic step (Patrick & Offler, 1996). The symplastic is the most common pathway, whereas the others exist to serve specialized functions. In nectaries, both the apoplastic (Davis et al., 1988; Wist & Davis, 2006) and symplastic pathways (Fahn, 1988; Nepi, 2007) are known to exist. In the apoplastic pathway, the pre-nectar flows intercellularly by passing along intercellular spaces and in-between cell walls whereas in the symplastic route the pre-nectar flows intracellularly via the

plasmodesmata (Nichol & Hall, 1988; Pacini & Nepi, 2007; Vassilyev, 2010). The symplastic pathway has been suggested as the more general pathway for nectar transport because: (a) apoplastic barriers can exist for nectaries that have trichomes as the secretory structures for nectar secretion; (b) vesicles are commonly found in nectary tissues; (c) secreted nectars are characterized by a wide range of concentrations and sucrose:hexose ratios; (d) non-carbohydrate components such as lipids and proteins are likely to be synthesized in the nectary tissues and are added into the pre-nectar before secretion; and (e) nectar secretion is dependent on very rapid control mechanisms (Heil, 2011).

For pre-nectar transport following the apoplastic pathway (Figure 4.15), sucrose is unloaded from the phloem (i.e., sieve tubes and companion cells) endings into the apoplast of the nectary as pre-nectar (Vassilyev, 2010). The pre-nectar is transported via intercellular spaces in the nectary with the possibility of being hydrolyzed into hexoses by β-fructosidase and/or α-glucosidase since these enzymes are known to be present in the apoplast (Monroe et al., 1999; Sturm, 1999). Alternately, in the symplastic pathway (Figure 4.15), sucrose is unloaded from the phloem into the cytoplasm of the nectary parenchyma cells. The carbohydrate then moves in the cytoplasm of the nectary cells as pre-nectar via the plasmodesmata (Vassilyev, 2010). Since carbohydrases (e.g., invertase, α-glucosidase, sucrose synthase) are present in the cytoplasm of nectary cells, sucrose may be hydrolyzed by the carbohydrases with the possibility of being resynthesized by sucrose phosphate synthase before secretion as nectar. The enzyme activities in the apoplast and symplast lead to carbohydrate structure changes in phloem sap sucrose (pre-nectar) resulting in the production of nectar. The resulting carbohydrate profile (i.e., hexoses, sucrose, and other oligosaccharides) from apoplastic or symplastic transport is released into the apoplasm for secretion as nectar via the modified stomata or epidermal openings.

Based on the carbohydrate results for *B. officinalis* in this study, it is hypothesized that the pre-nectar is transported symplastically and that the hexoses produced from sucrose hydrolysis are resynthesized into sucrose before secretion as nectar. The aforementioned would explain the sucrose-dominant classification and high sucrose content of *B. officinalis* nectar and is further supported by the low nectar carbohydrase activity observed for this species.

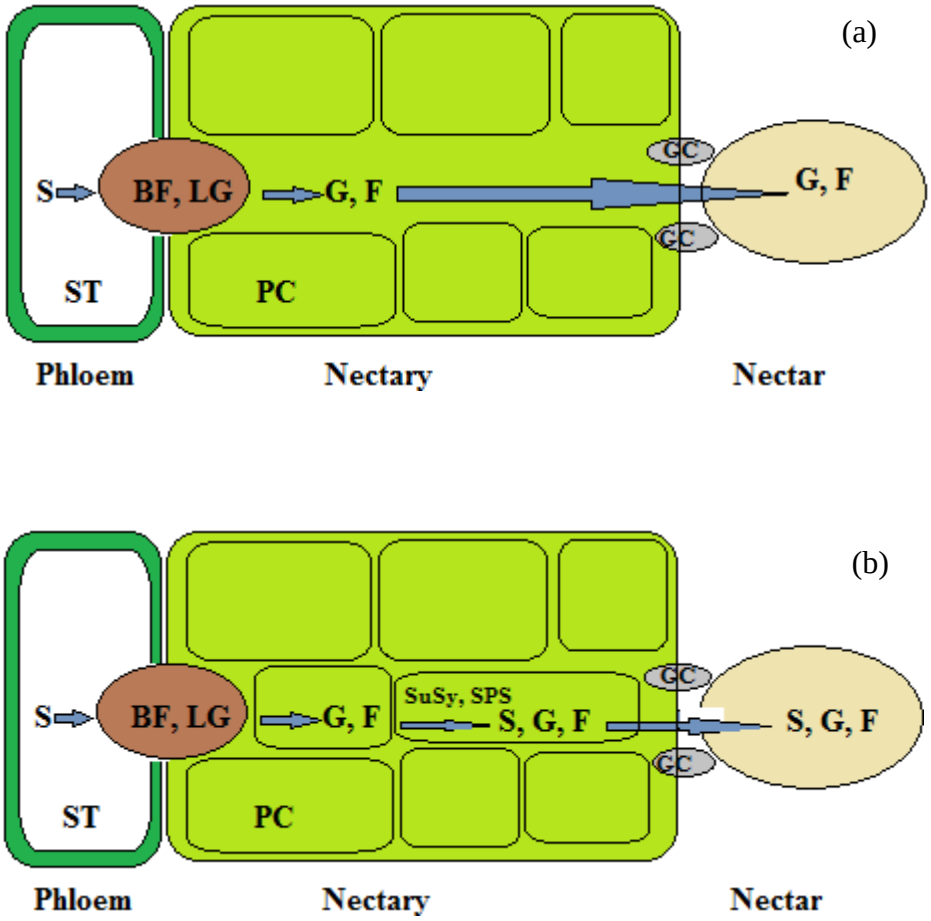

Figure 4.15 Pathways (a: apoplastic; b: symplastic) of pre-nectar transport from the phloem to the nectary; BF: β-fructosidase; F: fructose; G: glucose; GC: guard cells; LG: α-glucosidase; PC: parenchyma cell; S: sucrose; SPS: sucrose phosphate synthase; SuSy: sucrose synthase; ST: sieve tube.

For *Brassica* spp., the observed similarity in carbohydrate composition of the nectary fluid and nectar implies the existence of the apoplastic pathway leading to the direct secretion of the hexoses produced from sucrose hydrolysis by extracellular invertase (i.e., β-fructosidase and/or α-glucosidase) in the pre-nectar into the nectar. The direct secretion of nectary chemical compounds via an apoplastic transport is supported by substrate hydrolysis experiments as the carbohydrase activities in *Brassica* spp. nectary fluid and nectar (Tables 4-5 and 4-6) were also found to be similar.

However, it is also possible that the carbohydrate in pre-nectar was transported symplastically and sucrose entered the nectary parenchyma cells and was hydrolyzed by cytoplasmic β-fructosidase, α-glucosidase, and/or sucrose synthase resulting in hexose-rich nectar

with a minimal sucrose concentration. The lack of change in carbohydrate composition between pre-nectar and nectar also poses the question of whether a sucrose synthesizing enzyme such as sucrose phosphate synthase is present in the nectary of *Brassica* spp. Nectar carbohydrase activity results for *Brassica* spp. also showed the presence of both α-glucosidase and β-fructosidase, which were approximately 2x and 3x greater than those observed for *B. officinalis*, respectively. This higher hydrolytic activity may also contribute to the low sucrose concentration observed in *Brassica* spp. nectar and its classification as hexose-dominant.

In addition to carbohydrate hydrolysis, the aforementioned carbohydrases have also been shown to function in the synthesis of oligosaccharides via a reaction known as transglycosylation (Bras et al., 2009). The proposed catalytic mechanism for the transglycosylation reaction, is shown in Figure 4.16 and can occur when the carbohydrate concentration in the reaction medium is high.

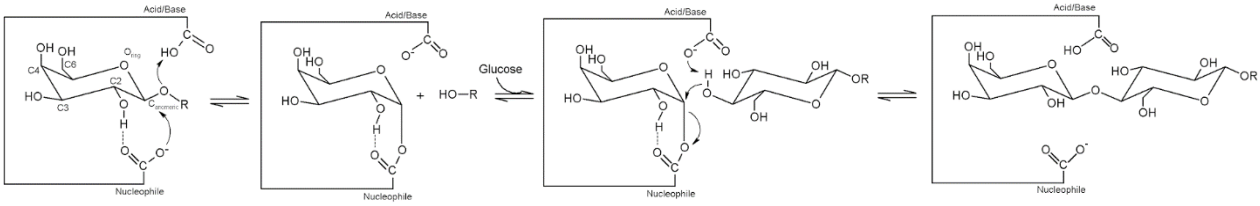

Figure 4.16 Reaction mechanism for the formation of oligosaccharides via transglycosylation by retaining glycosidases (adapted from Bras et al., 2012).

In the transglycosylation mechanism, the first step involves the departure of the aglycon group coupled with the formation of the covalent glycosyl-enzyme intermediate. The second step involves the attack of the intermediate by non-water nucleophiles (i.e., substrate and/or hydrolysis products) with proton transfer from the nucleophile to the active site of the acid/base carboxylate (Bras et al., 2012). This leads to glycosidic bond formation and oligosaccharide synthesis. The transglycosylation product yields for this enzyme mediated reaction are generally low due to the abundance of water in the reaction medium and the fact that the reaction product itself is a possible substrate for hydrolysis (Nakatani, 2001; Bras et al., 2009).

The presence of non-sucrose oligosaccharides in both the nectary fluids and nectars of all *B. officinalis* and *Brassica* spp. samples analyzed in this study (Figures 4.4 and 4.6) are hypothesized to be formed via the transglycosylation activity of carbohydrases in the nectary and nectar. The relative high concentrations of non-sucrose oligosaccharides in *B. officinalis* and *Brassica* spp. nectars (Figure 4.6) when compared to their nectary fluid (Figure 4.4) can be

explained by the higher carbohydrate concentration (i.e., ºBrix value; Table 4-1) observed in the former. In addition, the relative RTs (~20-40 minutes) of non-sucrose oligosaccharides correspond to those for disaccharides (Table 4-4). These disaccharides can be formed by the addition of fructose (β-fructosidase activity) or glucose (α- and β-glucosidase activities), to fructose and glucose (i.e., the major monosaccharides present in pre-nectar and nectar). The formation of trisaccharides, which appear in the high temperature [250-290ºC] region of the chromatogram (e.g., RT of 57.80 min; Table 4-3/Figure 4.6a), can be explained by the addition of sucrose to either fructose or glucose. Due to the lack of volatility of higher molecular weight trimethylsilyl derivatized carbohydrates (degree of polymerization ≥4), they do not appear in these chromatograms (Low, 1994). The absence of trisaccharide peaks in the high temperature region of the chromatograms of *Brassica* spp. nectars (Figure 4.6b) can be explained by the low sucrose concentration in this nectar (Table 4-2). It has been shown that the transglycosylation activities of α-glucosidase and β-glucosidase from bees are involved in the synthesis of α-linked (e.g., maltose, 1-kestose) and β-linked (e.g., gentiobiose) oligosaccharides in honey (Low et al., 1986; Pontoh & Low, 2002).

The fact that microorganisms were absent in the nectars of *B. officinalis* and *Brassica* spp. (Section 4.5) and that identical oligosaccharide patterns were found in the nectars of plants grown under phytotron and field conditions, where the flowers were bagged, clearly shows that carbohydrase activities due to microbes were not responsible for the oligosaccharide profiles observed in the nectars analyzed in this study.

Based on results from this research it is hypothesized that the observed non-sucrose oligosaccharide patterns in the nectary fluids and nectars of *B. officinalis* and *Brassica* spp. studied are due to the transglycosylation activity of carbohydrases such as β-fructosidase and α-, and β-glucosidase. Therefore, non-sucrose oligosaccharide formation is based on plant biochemistry. Based on the presence of unique non-sucrose oligosaccharide profiles in each of these two plant genera, it is possible that these compounds could be valuable indicators of the botanical origin of honey derived from these plants.

The transglycosylation reactions of β-fructosidase, and α-, and β-glucosidase cannot readily explain the observed significant resynthesis of sucrose in *B. officinalis* as pre-nectar is converted and transported as nectar. It is hypothesized that *B. officinalis* pre-nectar is transported symplastically and that sucrose resynthesis involves the sucrose anabolic enzyme, sucrose

phosphate synthase. In *Brassica* spp., the similarities in carbohydrate and carbohydrase profiles observed for the nectary fluid and nectar demonstrate that the carbohydrates in pre-nectar are either secreted directly apoplastically, or that sucrose is resynthesized enzymatically in the nectary and then hydrolyzed post-secretion. To determine which mechanistic pathway is correct, proteomic analysis of nectary fluid was utilized in the next section of this study. Proteomics can provide a comprehensive survey of nectary fluid proteins and would confirm the presence of the carbohydrases previously identified in the nectary substrate assays. Also, information on the presence/absence of enzymes that are involved in sucrose hydrolysis (e.g., sucrose synthase) or formation (e.g., sucrose phosphate synthase) can be used to identify the mechanism(s) responsible for the carbohydrate composition of *B. officinalis* and *Brassica* spp. nectary fluids and nectars.

4.6 Nectary Proteomics

Nectary Protein Concentration and SDS-PAGE Analysis

Sample nectary protein concentrations were determined using the Bradford assay employing 15 nectaries for *B. officinalis* and 15 lateral nectaries from each *Brassica* sp. Average total protein concentration results from three replicates per plant were 0.523 ± 0.128 mg mL^{-1} for *B. officinalis*, 0.028 ± 0.003 mg mL^{-1} for *B. napus*, 0.033 ± 0.007 mg mL^{-1} for *B. napus* transgenic, and 0.028 ± 0.002 mg mL^{-1} for *B. rapa* nectaries, respectively. Average protein concentration was significantly ($p < 0.05$) higher for *B. officinalis* when compared to *Brassica* spp., however the analytical values obtained for each *Brassica* sp. were not significantly different ($p > 0.05$). The observed ~19-fold higher protein amount in *B. officinalis* nectaries when compared to those of *Brassica* spp. may be explained by: a) the larger nectary size (~three-fold bigger than canola); b) higher α-glucosidase, β-glucosidase, and β-fructosidase concentrations as supported by higher substrate hydrolysis rates (Table 4-5); and c) the presence of additional enzymes as supported by the resynthesis of sucrose in the nectaries of *B. officinalis* but not in *Brassica* spp.

A review of literature did not uncover any published reports on the protein content of nectaries, however the total protein content of tobacco nectars of wild-type and transformed (trypsin protease inhibitor silenced) *N. attenuata* plants as determined by the Bradford assay were 0.17 ± 0.01 µg $µL^{-1}$ and 0.047 ± 0.002 µg $µL^{-1}$, respectively (Bezzi et al., 2010). Also, nectar protein concentrations ranging from 0.035 to 3.5 µg $µL^{-1}$ have been reported for a selection of garden plants including *Campsis*, *Impatiens*, *Nicotiana*, *Passiflora*, and *Ricinus* as determined by

SDS-PAGE (Chen & Kearney, 2015). Although the protein content of nectar from the plants used in this study were not determined, the much larger concentrations observed in the nectaries of *B. officinalis* and *Brassica* spp. samples analyzed when compared to literature nectar values and the fact that a high nectar volume was utilized for the substrate hydrolysis experiments (Section 4.6) could indicate that protein transport to nectar appears to be limited.

A review by Nicolson and Thornburg (2007) reported that a limited number of proteins (mostly enzymes) were present in nectar, however their concentration range can be large. The presence of a limited number of proteins in nectar when compared to the nectary is most likely due to the physical interactions and bonding between nectary proteins and nectary tissue, which minimizes their transport to nectar. As an example, experimental evidence based on both feeding experiments and enzyme assays showed that invertase was present in the stipel nectary tissue of cowpea (*Vigna unguiculata*) but not in the secreted nectar (Pate et al., 1985).

Reports comparing the nectary and nectar composition (e.g., proteins, carbohydrates) remain limited, however reports showing significant differences in the chemical composition of phloem sap and nectar exist (Ziegler, 1956; Baker et al., 1978; Pate et al., 1985; Lohaus & Schwerdtfeger, 2014). The existence of compositional differences indicates that the pre-nectar composition is altered as it passes through the nectaries and is transformed as nectar. The occurrence of compositional differences between the pre-nectar and nectar may be due to the metabolic processes occurring in the nectaries, selective secretion of compounds into the nectar, and/or selective resorption of compounds from the nectar into the nectary (Lüttge, 1961). For example, nectars of *Ricinus* and *Vigna* showed lower amino acid and ion (e.g., N, P, K, and Mg) concentrations when compared to the phloem sap indicating the selective retention of these compounds in the nectaries. The amino acids were most likely utilized for protein synthesis as indicated by the presence of high amounts of polyribosomes in the nectaries (Baker et al., 1978).

SDS-PAGE was utilized in the next section of this study as a preparatory step to separate the proteins present in the nectaries of selected samples prior to MS analysis. Only one *Brassica* sp. (*B. napus* var. *AC Excel*) was utilized in the proteomic analysis procedure due to equipment, time/cost constraints, and the consistency of substrate analysis results for all *Brassica* spp. as outlined in Section 4.6.1. SDS has the universal ability to bind proteins at high stoichiometry, hence, SDS-PAGE is able to analyze multiple proteins present in biological materials (Bischoff et al., 1998). In SDS-PAGE, the anionic SDS detergent denatures proteins and forms a micellar

SDS-protein complex with a negatively-charged surface. As such, protein migration in SDS-PAGE is dependent on protein size (i.e., molecular mass), with lower sized proteins moving faster in an applied electric field. However, different proteins may have close or very similar molecular masses resulting in the migration of the protein in the same region of the gel (Rabilloud, 2009). For example, a SDS-PAGE band may correspond to a denaturation product of a heavier protein or may be an aggregate of two or more lighter ones. For example, in petunia nectar, the proteomics analysis of the 35 kDa region of the SDS-PAGE gel corresponded to presence of four proteins, which were identified as an aldolase, ATP synthase, malate dehydrogenase, and properoxidase (Hillwig et al., 2011). In *B. napus* phloem sap, only 40 protein bands were detected after one-dimensional SDS-PAGE (1DE) but were further separated into more than 600 spots after two-dimensional electrophoresis (2DE). These results clearly show that size alone may not be a good protein separation parameter and the utilization of other structural features such as amino acid protein composition may be required (Rabilloud, 2009).

Sample nectary protein separations by SDS-PAGE (1DE) are shown in Figure 4.17. Protein bands in the 15 to >250 kDa range with varying concentrations as indicated by the depth of colour (visual determination) of the band were observed for *B. officinalis* and *B. napus*. Because these bands corresponded to the denatured nectary proteins, and SDS-PAGE separation is a function of molecular mass, a specific protein may be multiplied represented by gel bands at different regions, (i.e., if the protein was cleaved into its subunits) or different proteins may be represented by the same gel bands (i.e., if the proteins have similar molecular weights). For *B. officinalis*, bands were present (i.e., molecular mass approximations) at 12, 18, 20, 25, 30, 37, 40, 50, 65, 75, 80, and >250 kDa. For *B. napus*, bands were present at 15, 18, 30, 37, 40, 50, 65, 70, 85, and 100 kDa. Species-specific proteins/peptides for *B. officinalis* were observed at 12, 20, 25, 75, 80, and >250 kDa whereas for *B. napus*, species-specific proteins/peptides were observed at 15, 70, 85, and 100 kDa. Similar protein/peptide bands for *B. officinalis* and *B. napus* were also observed at 18, 30, 37, 40, 50, and 65 kDa.

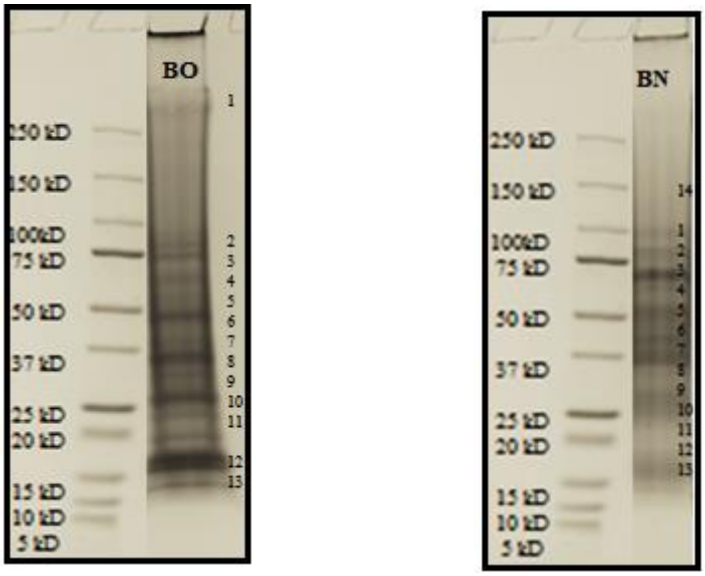

Figure 4.17 One-dimensional SDS-PAGE separation of *Borago officinalis* L.; (BO) and *Brassica napus* L. (var. *AC Excel*; BN) nectaries with a high molecular weight protein standard (Precision Plus Protein Dual Xtra MW Marker Standard [Bio-Rad Laboratories Ltd, Mississauga, ON, CA]) at the far left; numbers on the right indicate the region of the gel (i.e., band number) that was excised for trypsin digestion and LC-MS analysis.

Literature reported studies on SDS-PAGE electrophoretic separation of nectar/nectary proteins were found for nectar only. In tobacco, nectar protein bands were observed to be distributed between the 20 and 60 kDa region after 1DE (Seo et al., 2013). In a second 1DE study of tobacco nectar proteins, six distinct bands with molecular weights ranging from 10 to 25 kDa (Zha et al., 2012) were identified. Other studies where SDS-PGE has been applied to protein separation include, the nectar proteins of *Acacia cornigera*, *Brugmansia suaveolens*, *Cestrum* x '*Newellii*', *Lycium barbarum*, and various *Nicotiana* spp., (Orona-Tamayo et al., 2013; Kerchner et al., 2015). The higher number (i.e., >6) of gel bands observed in the nectaries of *B. officinalis* and *B. napus* in this study could indicate that nectaries contain a wider range of proteins when compared to nectar and/or that nectary proteins are comprised of multiple subunits that were denatured during 1DE analysis.

Following 1DE protein separation, the gel was segmented into either 13 (*B. officinalis*) or 14 bands (*B. napus*), and each band was excised and subjected to reduction, alkylation, and trypsin digestion (Figure 4.17; Section 3.7.3). The role of tryptic protein digestion was to generate a peptide map for *B. officinalis* and *B. napus* nectaries that could be analyzed by LC-MS for their amino acid composition and this data was subsequently used for nectary protein identification.

Each of the gel bands was treated as a single sample and was introduced into the LC-MS for analysis. To interpret the MS results, the data was converted to a mass/charge format using Agilent MassHunter Qualitative Analysis Software and was processed against the NCBI non-redundant green plant database for peptide sequence assignment.

In order to reduce the vast information contained in the NCBI database, a customized NCBI non-redundant green plant database was created that included information restricted to the enzymes of interest in the study (i.e., carbohydrases and select synthases). The NCBI non-redundant green plant database was filtered to include information of only the carbohydrases observed in the nectary substrate hydrolysis experiments that is α-glucosidase, β-glucosidase, and β-fructosidase/invertase (Section 4.6). In addition, NCBI data on sucrose synthase, a common carbohydrase present in the sink organs of plants, and sucrose phosphate synthase, a sucrose anabolic enzyme, in order to determine the mechanism of sucrose synthesis especially in *B. officinalis* nectar, were also included for peptide sequencing. Following data processing employing the customized NCBI non-redundant green plant database for the aforementioned carbohydrases/synthases, protein identification was confirmed if the following literature based parameters were achieved: a False Discovery Rate (FDR) less than 1%; presence of at least two unique peptides; a protein score greater than 9; and a scored peak intensity (SPI) greater than 60% (Vannini et al., 2013; Lucini & Bernardo, 2015). Following the recommended thresholds for each parameter ensures the accurate identification of proteins through database matching. False discovery rate measures the accuracy of the database search and is defined as the percentage of the false identifications in all the identifications above the score threshold (Zhang et al., 2011) whereas unique peptides refer to peptide sequences that are unique to a protein or sequences that correspond to a specific protein (Nesvizhskii & Aebersold, 2005). The protein score is calculated as the sum of the ion scores of all the peptides identified for the protein and refers to the score of the over-all protein, whereas %SPI refers to the percentage of the extracted MS/MS ion current explained by the theoretical fragmentation of the database hit (Agilent Technologies, 2012).

For some of the proteins of interest, a FDR greater than 1%, and a score lower than 9 was accepted if the SPI was still greater than 60%. These conditions were followed in the identification of α-glucosidase in *B. officinalis* and *B. napus* and β-glucosidase and sucrose synthase in *B. napus*. Due to the high resolution mass spectrometry system employed (i.e., quadrupole time of flight [QTOF]), femtomole peptide detection and mass resolutions of 0.035 Da were possible. With this

accurate mass measurement capability, lower peptide scoring results are still considered valid (Chernushevich et al., 2001; Agilent Technologies, 2012), as they could indicate that the peptides are short or are present in low abundance. However, for this work, a minimum SPI of 60% for nectary protein identification was maintained.

LC-MS results showed that the following carbohydrases/synthases, α-glucosidase, β-glucosidase, β-fructosidase/invertase, sucrose synthase, and sucrose phosphate synthase were present in both *B. officinalis* and *B. napus* nectaries. Detailed information of the enzymes identified including their accession numbers and sequences are presented in Tables 4-7 to 4-16.

Different forms (i.e., isoforms) of some of the enzymes were observed in this study. Isoforms are different forms of the same protein produced from different genes or from the same gene by alternative splicing (Giavalisco et al., 2006). Previous studies have shown that post-translational modification together with differential splicing can produce 5-10 protein variants from a gene (Collins et al., 2004; Fröhlich & Arnold, 2006). In this study, different isoforms of β-glucosidase (i.e., lysosomal β-glucosidase, β-glucosidase 24, and β-glucosidase 32), β-fructosidase, sucrose synthase, and sucrose phosphate synthase for *B. officinalis,* and sucrose synthase and sucrose phosphate synthase for *B. napus,* were observed supporting the post-translational modification-differential splicing mechanism.

Previous literature results have reported the existence of different forms of the aforementioned five carbohydrases/synthases identified in this study. As examples, in *Arabidopsis*, at least three α-glucosidase genes (*Aglu-1, -2, and -3*) were identified and two proteins with molecular masses of 96 and 81 kD were expressed from *Aglu-2* in vegetative tissue (Monroe et al., 1999). Similar results were obtained for different forms of β-glucosidase and sucrose phosphate synthase (Singhania et al., 2013; Taneja & Das, 2014), β-fructosidase (Sturm, 1996; Tymowska-Lalanne & Kreis, 1998), and sucrose synthase (Sturm et al., 1999) based on molecular masses, biochemical properties, and cellular and tissue locations. The existence of multiple forms of proteins makes protein structures more heterogeneous compared to the genome (Black, 2000; Bruce et al., 2013). However, since mass spectrometry has the capacity to identify protein isoforms (Sànchez-Pèrez et al., 2012), the presence of multiple forms of the aforementioned proteins in the nectaries of *B. officinalis* and *B. napus* did not reduce the confidence/accuracy of the identification process.

Table 4-7 Alpha-glucosidase identified in *Borago officinalis* L. nectaries using the customized NCBI green plant database.

Band number	MW (Da)	Species	Accession number	Entry name	Previous amino acid	Sequence	Next amino acid
4	103114.2	*Oryza brachyantha*	573952448	PREDICTED: neutral alpha glucosidase AB-like	(K)	IDSSIQAK	(Q)

Table 4-8 Alpha-glucosidase identified in *Brassica napus* L. (var. *AC Excel*) nectaries using the customized NCBI green plant database.

Band number	MW (Da)	Species	Accession number	Entry name	Previous amino acid	Sequence	Next amino acid	Modification
5	103631.8	*Medicago truncatula*	355516040	alpha-glucosidase	(K)	IYGPDIPLLRFyAkHETEnR	(L)	y:Phosphorylated Y k:Acetyl n:Deamidated

Table 4-9 Beta-glucosidase isoforms identified in *Borago officinalis* L. nectaries using the customized NCBI green plant database.

Band number	MW (Da)	Species	Accession number	Entry name	Previous amino acid	Sequence	Next amino acid	Modification
3	69193.6	*Glycine max*	356534700	PREDICTED: lysosomal beta glucosidase	(K)	IGEATALEVR	(A)	
3	70536.2	*Oryza brachyantha*	573938770	PREDICTED: lysosomal beta glucosidase-like	(K)	IGEATALEVR	(A)	
3	69193.6	*Glycine max*	356534700	PREDICTED: lysosomal beta glucosidase	(K)	SLVLLK	(N)	
3	70536.2	*Oryza brachyantha*	573938770	PREDICTED: lysosomal beta glucosidase-like	(K)	SLVLLK	(N)	
7	60249.3	*Prunus mume*	645268255	PREDICTED: beta-glucosidase 24-like	(R)	IPkVSAK	(W)	k:Carbamylated lysine

Table 4-10 Beta-glucosidase identified in *Brassica napus* L. (var. *AC Excel*) nectaries using the customized NCBI green plant database.

Band number	MW (Da)	Species	Accession number	Entry name	Previous amino acid	Sequence	Next amino acid	Modification
6	73926.7	*Morus notabilis*	587906290	Beta-glucosidase-like protein	(E)	WADGYGPKFGLVAVDR	(A)	
7	73926.7	*Morus notabilis*	587906290	Beta-glucosidase-like protein	(K)	LAGILVTVTVATnAFSFSR	(Y)	n:Deamidated

Table 4-11 Beta-fructosidase isoforms identified in *Borago officinalis* L. nectaries using the customized NCBI green plant database.

Band number	MW (Da)	Species	Accession number	Entry name	Previous amino acid	Sequence	Next amino acid
3	66486.8	*Setaria italica*	514787667	PREDICTED: beta-fructofuranosidase, insoluble isoenzyme 4-like	(R)	IDYGNYYASK	(S)
3	66390.6	*Solanum tuberosum*	359431057	apoplastic invertase	(R)	LDYGNYYASK	(T)
3	67155.2	*Daucus carota*	18324	beta-fructofuranosidase	(K)	LEEYTPVFFR	(V)
4	64608.5	*Medicago truncatula*	657403142	beta-fructofuranosidase	(R)	LREKEVGINN	(K)
6	66251	*Vicia faba* var. *minor*	861157	cell wall invertase II:beta-furanofructosidase	(R)	TLIDHSVVESFGGEGK	(A)
7	67155.2	*Daucus carota*	18324	beta-fructofuranosidase	(K)	GVYHLFYQYNPK	(G)

Table 4-12 Beta-fructosidase identified in *Brassica napus* L. (var. *AC Excel*) nectaries using the customized NCBI green plant database.

Band number	MW (Da)	Species	Accession number	Entry name	Previous amino acid	Sequence	Next amino acid	Modification
1	41948.6	*Brassica rapa*	239911778	cell wall invertase 4, partial	(K)	YVPnGDTPDGWDGLR	(F)	n:Deamidated
3	41948.6	*Brassica rapa*	239911778	cell wall invertase 4, partial	(L)	ATPDLEEYTPVFFR	(V)	

Table 4-13 Sucrose synthase isoforms identified in *Borago officinalis* L. nectaries using the customized NCBI green plant database.

Band number	MW (Da)	Species	Accession number	Entry name	Previous amino acid	Sequence	Next amino acid	Modification
1	93255.1	*Jatropha curcas*	468181080	sucrose synthase	(R)	ELANLVVVGGDR	(R)	
1	89331.6	*Ricinus communis*	223544696	sucrose synthase	(R)	ELANLVVVGGDR	(R)	
1	92476.7	*Orobanche ramosa*	345286417	sucrose synthase 1	(R)	KLAEAVPLAVE	(-)	
1	92476.7	*Orobanche ramosa*	345286417	sucrose synthase 1	(K)	SIGnGVEFLNR	(H)	n:Deamidated
2	92955.1	*Vitis vinifera*	225444613	PREDICTED: sucrose synthase	(K)	DLEEQSEMK	(K)	
2	93064.8	*Cicer arietinum*	332367442	nodule-enhanced sucrose synthase	(T)	IYFPYTETSRR	(L)	
2	92825.5	*Medicago falcata*	145687787	sucrose synthase	(T)	IYFPYTETSRR	(L)	

Table 4-13 Sucrose synthase isoforms identified in *Borago officinalis* L. nectaries using the customized NCBI green plant database (Cont'd).

Band number	MW (Da)	Species	Accession number	Entry name	Previous amino acid	Sequence	Next amino acid	Modification
2	93271.3	*Gossypium arboreum*	392050914	sucrose synthase 3	(K)	LDGQFR	(W)	
2	92476.7	*Orobanche ramosa*	345286417	sucrose synthase 1	(K)	LAEAVPL AVE	(-)	
2	175585	*Medicago truncatula*	657383073	sucrose synthase	(K)	LGVtQATI AHALEK	(T)	t:Phosphorylated T
2	93271.3	*Gossypium arboreum*	392050914	sucrose synthase 3	(K)	LRELANL VVVGGDR	(R)	
2	92476.7	*Orobanche ramosa*	345286417	sucrose synthase 1	(R)	LRPGVWE YVR	(V)	
2	92757.7	*DAUCA*	1351139	RecName: Full=Sucrose synthase isoform 1	(K)	STQEAIVS PPWVALA IR	(L)	
2	92476.7	*Orobanche ramosa*	345286417	sucrose synthase 1	(R)	VVHGIDV FDPK	(F)	

Table 4-14 Sucrose synthase isoforms identified in *Brassica napus* (var. AC Excel) nectaries using the customized NCBI green plant database.

Band number	MW (Da)	Species	Accession number	Entry name	Previous amino acid	Sequence	Next amino acid	Modification
2	175584.7	*Medicago truncatula*	657383073	sucrose synthase	(K)	LGVTQAtI AHALEK	(T)	t:Phosphorylated T
2	92216.2	*Mangifera indica*	425875161	sucrose synthase	(K)	YTWKIYS QR	(L)	
3	93571.5	*Arabidopsis thaliana*	22331535	sucrose synthase 4	(R)	IKQQGLNI TPR	(I)	

Table 4-15 Sucrose phosphate synthase isoforms identified in *Borago officinalis* L. nectaries using the customized NCBI green plant database.

Band number	MW (Da)	Species	Accession number	Entry name	Previous amino acid	Sequence	Next amino acid	Modification
4	86541.9	*Actinidia deliciosa*	2754746	sucrose-phosphate synthase, partial	(R)	kIFDAVAk	(E)	k:Acetyl
6	120894.5	*Citrus clementina*	557522836	PREDICTED: probable sucrose-phosphate synthase 3-like	(K)	TLIMkGV VEK	(G)	k:Acetyl

Table 4-16 Sucrose phosphate synthase isoforms identified in *Brassica napus* L. (var. *AC Excel*) nectaries using the customized NCBI green plant database.

Band number	MW (Da)	Species	Accession number	Entry name	Previous amino acid	Sequence	Next amino acid	Modification
5	118898	*Vitis vinifera*	526117782	sucrose-phosphate synthase 1	(R)	WGIDLSkMVVFVGEK	(G)	k:Carbamylated lysine
6	120502	*Selaginella moellendorffii*	300167770	sucrose phosphate synthase	(R)	NKLEQLLVQGR	(Q)	
8	65989.1	*Gossypium hirsutum*	110339461	putative sucrose phosphate synthase, partial	(Q)	LLKQGRQSR	(E)	
9	74135	*Actinidia chinensis*	19223856	sucrose phosphate synthase, partial	(K)	AVLTWSVQkSTQK	(S)	k:Carbamylated lysine
9	116335	*Populus trichocarpa*	550325324	sucrose-phosphate synthase family protein	(K)	kLIEAITQmAPSnGk	(A)	k:Carbamylated lysine m:Oxidized methionine n:Deamidated

Results showed that each enzyme appeared in the same and/or different regions of the gel. Specifically, (a) α-glucosidase was identified in bands 4 and 5 (~60 kDa) in *B. officinalis* and *B. napus*, respectively; (b) β-glucosidase was identified in bands 3 (~70 kDa) and 7 (~37 kDa) in *B. officinalis*, and in bands 6 (~45 kDa) and 7 (~40 kDa) in *B. napus*; (c) β-fructosidase/invertase was identified in bands 3 (~70 kDa), 4 (~60 kDa), 6 (~40 kDa), and 7 (~37 kDa) in *B. officinalis*, and in bands 1 (~110 kDa) and 3 (~75 kDa) in *B. napus*; (d) sucrose synthase was identified in bands 1 (>250 kDa), 2 (~90 kDa), and 3 (~94 kDa) in *B. officinalis*, and in bands 2 (~90 kDa) and 3 (~75 kDa) in *B. napus*; and (e) sucrose phosphate synthase was identified in bands 4 (~86 kDa) and 6 (~40 kDa) in *B. officinalis*, and in bands 5 (~60 kDa), 6 (~45 kDa), 8 (~37 kDa), and 9 (~30 kDa) in *B. napus*. Similarities in the region of migration were observed for the same enzyme in both *B. officinalis* and *B. napus*. For example, α-glucosidase was observed in the ~60 kDa region whereas β-glucosidase was observed at the ~40 kDa region in both species. However, similar enzymes were also observed to migrate in different regions of the gel. For example, β-fructosidase was identified in ~40 and ~70 kDa regions in *B. officinalis* and in ~75 and ~110 kDa regions in *B. napus*. The identification of similar proteins (e.g., β-glucosidase) within different gel regions may indicate the presence of subunits that were denatured during 1DE analysis, post-translational modifications, or the existence of genetic isoforms. It is also important to note that under the environmental condition used in this study, the proteomics results provided information on protein expression (i.e., within the nectary) levels only, irrespective of the genes that encode them and their rates of transcription.

The proteomics data established the presence of α-glucosidase, β-fructosidase, and β-glucosidase in these plant nectaries and confirmed the substrate hydrolysis experimental results (Section 4.6). In addition to the carbohydrases observed during the substrate hydrolysis experiments, proteomics was able to identify the sucrose catabolism enzyme sucrose synthase, as well as a sucrose synthesizing enzyme, sucrose phosphate synthase in both *B. officinalis* and *B. napus*. The use of the BLAST program further confirmed the identity of the proteins by searching sequence similarities in a protein database (Altschul, et al., 1997). Results showed that the peptide sequences obtained from proteomics experiments matched those of other plant species for the specific enzyzmes identified employing the non-redundant protein sequences plant database. The LC-MS-identification of these enzymes in the nectaries of *B. officinalis* and *B. napus* supports the postulated enzymatic mechanism for the observed hydrolysis of sucrose as it was transported from

the phloem into the nectaries. It is hypothesized that the carbohydrase (i.e., α-glucosidase and β-fructosidase) activities observed in the substrate hydrolysis experiments and the presence of a glycosyl transferase, sucrose synthase, are responsible for the sucrose hydrolytic activity occurring in the nectaries. In addition, the transglycosylation mechanism(s) of these carbohydrases are responsible for the formation of non-sucrose oligosaccharides in the nectaries and nectars of *B. officinalis* and *Brassica* spp.

The presence of sucrose phosphate synthase supports the hypothesis that a sucrose synthesizing enzyme is present in the nectaries that catalyzes significant resynthesis of sucrose in pre-nectar prior to secretion as nectar, especially in *B. officinalis*. Sucrose phosphate synthase (E.C. 2.3.1.14) is a cytoplasmic plant enzyme that has an important role in sucrose biosynthesis through the catalysis of the reaction: Fru-6-P + UDP-glu → sucrose phosphate + UDP (Leloir & Cardini, 1955). A study by Lin et al. (2014) showed that the genes responsible for sucrose phosphate synthase transcription are highly expressed in the nectaries of *A. thaliana*, *B. rapa*, and *N. attenuata* and play a major role in nectar secretion. It was observed that the genes *SPS1F* and *SPS2F* were upregulated in maturing nectaries and inhibition of the genes led to the loss of nectar secretion. Gene expression studies of *A. thaliana* also showed that sucrose biosynthesis genes including the sucrose phosphate synthase gene were also upregulated in nectaries (Kram et al., 2009). In this study, the observed presence of sucrose phosphate synthase in both *B. officinalis* and *B. napus* nectaries is the first to report the presence of this enzyme in this plant organ, and supports the role of this enzyme in nectar secretion in these plant species.

Sucrose phosphate synthase is reported to be localized in the cytoplasm (Leloir & Cardini, 1955), and as such supports the hypothesis of a symplastic mechanism for pre-nectar transport to nectar in *B. officinalis*. In this mechanistic pathway, sucrose is unloaded from the phloem intracellularly into the nectaries and sucrose is hydrolyzed by the carbohydrases, α-glucosidase, β-fructosidase/invertase, and sucrose synthase. A portion of the hexoses produced from sucrose hydrolysis is resynthesized to form sucrose by sucrose phosphate synthase before secretion as nectar. Because nectar carbohydrase activity in *B. officinalis* nectar was observed to be low (Figure 4.13; Section 4.6), sucrose hydrolysis in this carbohydrate-rich fluid was also observed to be low. This mechanistic pathway results in the sucrose-dominant classification and high sucrose content of *B. officinalis* floral nectars.

For *B. napus*, the presence of apoplastic invertase explains the hexose-dominant classification of the nectar for this species. Cell wall invertase was the only β-fructosidase isoform observed in *B. napus* and its presence confirmed a previous study implicating the enzyme as an absolute requirement for nectar production in the Brassicaceae (Ruhlmann et al., 2010). The presence of a cell wall β-fructosidase(s) in *B. napus* supports the postulated apoplastic mechanism for pre-nectar transport for this species. In this mechanistic pathway, sucrose from the phloem is transported intercellularly to the nectaries and is hydrolyzed by extracellular β-fructosidase with the resulting hexoses directly secreted as nectar. However, the existence of a symplastic mechanism for pre-nectar transport in *Brassica* spp. cannot be excluded, as sucrose may also enter the nectary intracellularly and be cleaved into hexoses by the carbohydrases (e.g., sucrose synthase, α-glucosidase) present in the cytoplasm. Because sucrose phosphate synthase was identified in the *B. napus* sample used in this study by proteomics, sucrose may have been resynthesized in the nectary followed by hydrolysis (as β-fructosidase is present in the cell wall) at the point of secretion as nectar. It is not clear to this author why the process of sucrose hydrolysis and synthesis occur in both *Brassica* spp. and *B. officinalis* nectaries as it seems to be a futile cycle of sucrose/hexose interconversion. However, the futile cycle involving invertase, sucrose synthase, and sucrose phosphate synthase was studied in tomato and was implicated in the regulation of carbohydrate metabolism and determination of sink strength in tomato fruit (Nguyen-Quoc & Foyer, 2001).

The presence of β-fructosidase in the cell walls of *Brassica* spp. nectary tissue may also lead to the leakage of this enzyme into the nectar and may explain the high levels of nectar β-fructosidase activity observed for this species (Table 4-6; Section 4.6.1). From literature, it was proposed that the observed hexoses in the nectar of cowpea were due to phloem sucrose hydrolysis by an invertase on the cell walls or plasma membranes of secretory cells, or by an invertase released during the lysis of such cells (Pate et al., 1985).

In the present study, the postulate of a cell wall β-fructosidase in the nectaries of *Brassica* spp. explains the almost complete hydrolysis (0.73%; Table 4-2; Section 4.3.2) of phloem sucrose prior to secretion as nectar and explains the hexose-dominant classification of *Brassica* spp. nectars reported in this study (Section 4.6).

The results of this study clearly demonstrate that there is a strong metabolic contribution of the nectary in the carbohydrate composition and profile in nectar. Results from this study

support those in literature that contend that nectar is not the mere secretion of phloem sap but involves metabolic processes involving enzymes to produce nectars with significantly different carbohydrate compositions and structures than those present in phloem. The results of this study are unique as they follow carbohydrate composition and structure changes as phloem sap from *B. officinalis* and *Brassica* spp. is transported to the nectary and finally to nectar, and in elucidating the possible enzymatic mechanisms responsible for these changes.

5. CONCLUSIONS

Nectar is a carbohydrate-rich solution produced from processes occurring in the nectar-producing organ, the nectaries. Nectar formation typically involves the upload of carbohydrate from phloem sap, processing of this carbohydrate in the nectary, and secretion of the resulting carbohydrates through the nectary openings as nectar (i.e., carbohydrate-rich fluid).

To determine carbohydrate composition and structure changes during nectar formation, the phloem sap, nectaries/nectary fluid, and nectar of two plant genera, *Borago officinalis* L., *Brassica napus* L. (var. *AC Excel*), *B. napus* L. transgenic (var. *AV 225 R. R.*), and *B. rapa* L. (var. *AC Parkland*) were analysed by HPAE-PAD and CGC-FID. In order to elucidate the mechanism(s) of carbohydrate composition and structure changes as phloem sap is transformed into nectar, substrate hydrolysis experiments (i.e., carbohydrase activities) on the nectary and nectars of all samples were performed, and proteomic studies (LC-MS) of the nectaries of *B. officinalis* and a single *Brassica* sp. (*B. napus* var. *AC Excel*) were conducted.

To the best of the author's knowledge, there have been no published reports that have determined carbohydrate composition and structure changes in plants as phloem sap is transported to the nectary, and ultimately to nectar. Therefore, this study was the first to show that carbohydrate composition and structure changes occurred during nectar production in *B. officinalis* and *Brassica* spp. These changes included: sucrose hydrolysis to fructose and glucose in the nectary in both plant genera, with sucrose resynthesis in borage as nectary fluid was transported as nectar; and non-sucrose oligosaccharide formation in both the nectaries and nectar of both plant genera. In addition, this study was the first to elucidate the mechanisms responsible for these changes in carbohydrate composition and structure due to the presence of carbohydrases and synthases in the nectaries of these plants as shown by specific substrate hydrolysis and proteomics experiments.

The presence of α-glucosidase and β-glucosidase in the nectaries of these two genera was unique to this study. The presence of α-glucosidase is postulated to be involved in both sucrose hydrolysis and non-sucrose oligosaccharide formation (i.e., transglycosylation) activities. The

presence of β-glucosidase was postulated to be involved in herbivore and microbial protection for the plant. In addition to carbohydrases, this study also identified other carbohydrate metabolic enzymes in the nectaries that have hydrolytic (e.g., sucrose synthase) or anabolic (e.g., sucrose phosphate synthase) activities and were postulated to also play a role in nectar carbohydrate composition.

Although literature reports on the presence of non-sucrose oligosaccharides in nectar exist, this study was the first to postulate a transglycosylation mechanism of carbohydrases for the formation of these carbohydrates in the nectary fluid and nectars of *B. officinalis* and *Brassica* spp. In addition, the detection of unique non-sucrose oligosaccharides in the nectars of *B. officinalis* and *Brassica* spp. may serve as botanical markers, or as supporting and/or, alternative method to melissopalynology for honey botanical origin determination. The unique structural differences in non-sucrose oligosaccharides, as indicated by their retention time (RT) differences, in the nectars of these plant genera is postulated to be due to carbohydrate composition differences in their nectars (i.e., sucrose dominant in *B. officinalis* and hexose dominant in *Brassica* spp.) and enzyme catalytic sites.

The study was able to elucidate how carbohydrates are formed in nectars. The study showed that nectar production is not a mere secretion of the phloem sap but involves metabolic processes that occur in the nectaries.

Analysis of the phloem sap of both plants showed that sucrose was the only carbohydrate identified in *B. officinalis* and all *Brassica* spp. and as such was the major starting material for nectar production. The concentration of sucrose in phloem sap was >95% (w:v) as determined by HPAE-PAD and CGC-FID, and no other carbohydrates (i.e., fructose, glucose, and non-sucrose oligosaccharides) were detected (quantitation detection limit of 5.0 ppm). However, analysis of the nectary fluids of both plants showed that glucose and fructose were the major carbohydrates in the nectaries. The carbohydrate composition (w:v) of nectary fluids as determined by HPAE-PAD were: fructose: 52.86%, glucose: 47.14%, and sucrose: <0.05% for *B. officinalis* and fructose: 55.02%, glucose: 44.98%, and sucrose: not detected for *Brassica* sp. (*B. rapa* var. *AC Parkland*). These results show that almost complete hydrolysis of phloem sap sucrose to fructose and glucose occurred in the nectaries of these two genera. Also, non-sucrose oligosaccharides were detected by CGC-FID in the nectary fluids of both genera, indicating that carbohydrate synthesis occurred.

These results clearly show that carbohydrate composition changes occurred within the nectary organs of *B. officinalis* and *Brassica* spp.

Analysis of the nectars of *B. officinalis* showed that the mean carbohydrate composition (w:v) as determined by HPAE-PAD were: fructose: 17.38%, glucose 21.56%, and sucrose: 61.05%. The preponderance of sucrose in this nectar supports its classification as sucrose-dominant. These results show that sucrose was resynthesized in the nectary of *B. officinalis* prior to secretion as nectar. On the other hand, the mean carbohydrate composition (w:v) data for the *Brassica* spp. nectars as determined by HPAE-PAD were: fructose: 44.87%, glucose: 54.39%, and sucrose: 0.73% for *B. napus*; fructose: 44.45%, glucose: 54.81%, and sucrose: 0.84% for *B. napus* transgenic; and fructose: 45.15%, glucose: 53.47%, and sucrose: 0.77% for *B. rapa*. The high levels of fructose and glucose coupled with low sucrose concentration support the classification of *Brassica* spp. nectars as hexose-dominant, confirming previous literature findings.

Non-sucrose oligosaccharides were also detected in nectar samples of both plants as determined by CGC-FID. Based on RT comparisons to standards, these non-sucrose oligosaccharides were identified as disaccharides and trisaccharides. Non-sucrose oligosaccharides that were common to both *B. officinalis* and *Brassica* spp., albeit with different concentrations, had approximate RTs of 37.9 and 40.4 min. Unique non-sucrose oligosaccharides in *B. officinalis* had RTs of 25.38, 29.18, and 57.80 min whereas *Brassica* spp. had a unique non-sucrose oligosaccharide with RT of 29.42 min. These non-sucrose oligosaccharides may have been secreted from the nectaries or may have been synthesized in the nectars.

A comparison of CGC-FID oligosaccharide profiles of field-grown versus phytotron-grown plants showed the same non-sucrose oligosaccharide profiles in the nectars, indicating that insects were not involved in the synthesis of these compounds. In addition, no microorganisms were also identified in the nectars of both genera and therefore, microorganisms do not play a role in the synthesis of non-sucrose oligosaccharides in nectars of both genera. This information further strengthens the postulate that the carbohydrase activities were responsible for non-sucrose oligosaccharide formation in the nectaries and nectars of these plants.

Based on specific substrate (i.e., cellobiose, maltose, and raffinose) reactions, β-glucosidase, α-glucosidase, and β-fructosidase activities were detected in the nectaries of *B. officinalis* and all *Brassica* spp. nectaries and nectars. However, only α-glucosidase and β-fructosidase activities were detected in the nectars of *B. officinalis*. The carbohydrase (i.e., α-

glucosidase and β-fructosidase) activities were postulated to be responsible for the hydrolysis of sucrose from the phloem sap to the nectaries. In addition to carbohydrate hydrolysis, carbohydrases, (i.e., α-glucosidase, β-glucosidase, and β-fructosidase) have also been shown to catalyse transglycosylation reactions. It is hypothesized that the observed non-sucrose oligosaccharides detected by CGC-FID in *B. officinalis* and *Brassica* spp. nectary fluids and nectars were synthesized via transglycosylation reaction mechanisms. Fructose (via β-fructosidase transglycosylation activity), and/or glucose (via α- and β-glucosidase transglycosylation activities) could be covalently linked to hydroxyl groups of fructose and glucose (i.e., the major monosaccharides present in pre-nectar) to form disaccharides, and/or fructose or glucose could be covalently linked to sucrose leading to the formation of trisaccharides.

LC-MS analyses of the nectaries confirmed the presence of the carbohydrases, α-glucosidase, β-glucosidase, and β-fructosidase/invertase in both *B. officinalis* and *B. napus*. In addition, LC-MS identified sucrose synthase and sucrose phosphate synthase in the nectaries of both *B. officinalis* and *B. napus*. Sucrose synthase is a catabolic enzyme present in the cytoplasm and is also hypothesized to be responsible for the hydrolysis of sucrose in the nectaries of both genera whereas sucrose phosphate synthase is a cytoplasmic sucrose anabolic enzyme, and may be responsible for the resynthesis of sucrose. The identification of sucrose phosphate synthase in the nectaries explains the resynthesis of sucrose and the predominance of this carbohydrate in *B. officinalis* nectar. Proteomics results for carbohydrase identification was further supported by BLAST results for each of the experimentally obtained peptide sequences.

The significant nectar carbohydrate composition difference of *B. officinalis* and *Brassica* spp. cannot be explained by the presence of the carbohydrases alone because all enzyme activities were observed in both genera. Therefore, the pathway that the carbohydrate (i.e., sucrose) follows from the phloem sap to the nectary is postulated to play an important role in determining the carbohydrate composition of nectars. Carbohydrate transport from phloem sap through/within the nectaries is known to follow either an apoplastic (intercellular) or symplastic (intracellular) pathway. For the apoplastic pathway, sucrose is unloaded from phloem sap into the apoplast of the nectary. Alternately, for the symplastic pathway, sucrose is unloaded from phloem sap into the cytoplasm of the nectary parenchyma cells via the plasmodesmata. The resulting carbohydrates produced during apoplastic or symplastic transport are secreted into the apoplasm of the nectary for nectar secretion.

The low sucrose concentration in *Brassica* spp. nectars may indicate that carbohydrates are transported apoplastically (i.e., intercellularly). In this pathway, phloem sucrose is hydrolyzed into monosaccharides by the carbohydrases (i.e., α-glucosidase and β-fructosidase) present in the cell walls and the resulting carbohydrates (i.e., hexose dominant) are directly secreted as nectar. However, for *B. officinalis*, the presence of sucrose phosphate synthase in the cytoplasm of nectary cells may indicate that carbohydrates are transported via the symplastic pathway (i.e., intracellularly) from the phloem into the nectaries of *B. officinalis* resulting in sucrose resynthesis in the cytoplasm by sucrose phosphate synthase before secretion as nectar (i.e., sucrose dominant).

Other nectar parameters were also measured in this study including ºBrix, pH, and volume, to further characterize these plant nectars. The soluble solids (ºBrix) mean values for *B. officinalis*, *B. napus, B. napus* transgenic, and *B. rapa* nectars were: 64.94, 61.08, 58.83, and 54.89, respectively confirming that nectars are carbohydrate-rich solutions. Nectar pH was found to be acidic for *B. officinalis* and all *Brassica* spp. The pH mean values for *B. officinalis, B. napus, B. napus* transgenic, and *B. rapa* were: 4.52, 4.70, 4.77, and 4.87, respectively. Significant differences ($p < 0.05$) in nectar volume was observed between *B. officinalis* and the *Brassica* spp. but not within *Brassica* spp. Average nectar volume (μL) values were: 1.94, 0.43, 0.66, and 0.34 for *B. officinalis, B. napus*, *B. napus* transgenic, and *B. rapa*, respectively.

Results from this study clearly show carbohydrate composition differences as phloem sap is transported to the nectary and ultimately as nectar. The observed carbohydrate changes can be explained by the hydrolytic and transglycoslation activities of carbohydrases (i.e., β-glucosidase, α-glucosidase, β-fructosidase, and sucrose synthase) and by the activity of sucrose phosphate synthase in the nectary. The observed differences in the sucrose concentrations in the nectary fluids and nectar of *B. officinalis* and *Brassica* spp. may also be explained by carbohydrate transport pathway followed (i.e., symplastic for *B. officinalis* and apoplastic for *Brassica* spp.).

6. FUTURE DIRECTIONS

The analyses of the carbohydrates of the phloem sap, nectary, and nectars using HPAE-PAD and CGC-FID and the investigation of the proteins of the nectaries and nectars using enzyme substrate assay experiments and proteomics enabled the elucidation of the mechanism of nectar carbohydrate formation in *B. officinalis* and *Brassica* spp. However, further studies (i.e., temporal analyses at pre-secretory, secretory, post-secretory) need to be conducted to enable the complete understanding of nectar production. A study on the confirmation of the source of nectar carbohydrates in *B. officinalis* and *Brassica* spp. is suggested. Girdling and/or defoliation studies may be conducted to confirm if the phloem sap is the source of the nectar carbohydrates. In addition, starch production in the nectaries may also be investigated by anatomical staining and observation. The movement of the carbohydrate (symplast vs apoplast) from the phloem to the nectar may also be confirmed by labelling and/or structural characterization of the cells/tissues (e.g., phloem, nectary) involved in nectar production.

The composition changes as the carbohydrate enters the nectary may be further elucidated by the isolation of non-sucrose oligosaccharides observed in the nectaries and nectars and the NMR identification of the carbohydrates. When these non-sucrose oligosaccharides are identified, these carbohydrates may also be used as marker compounds as an alternative method for honey botanical origin determination.

The enzymatic mechanism of nectar carbohydrate transformation may be further studied by the characterization of the chemical and kinetic properties of the carbohydrases and synthases (i.e., pH/temperature optimum, substrate specificities, molecular mass, subunits, Michaelis-Menten kinetics) in the nectaries, nectars, and phloem sap. Understanding the production and regulation of the enzymes may also be possible by genomic and transcriptomic studies of the nectaries.

7. REFERENCES

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