Resistance and susceptibility to the invasive leaf miner *Cameraria ohridella* within the genus *Aesculus*

By

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Declaration of Authorship

I, Lilla D'Costa hereby declare that the research, experimental design and data analyses for this thesis was my own work with the exception of the following:

- NMR experiments and NMR structure elucidation were carried out by Dr Nigel C. Veitch at the Royal Botanic Gardens of Kew

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Abstract

The invasive horse chestnut leaf miner *Cameraria ohridella* Deschka & Dimic (Lepidoptera: Gracillariidae) was introduced into the UK in 2002 and since then it has spread across the country, causing significant aesthetic damage to the extensively planted horse chestnut (*Aesculus hippocastanum* L.) trees. Different species of *Aesculus* show varying susceptibility to the miner and the mechanisms responsible for these differences have not been investigated previously. The present study investigates the host plant range and oviposition choice of *C. ohridella* in the field and in experimental conditions. The study also investigates which leaf traits may convey resistance to the miner, by comparing leaf physical and chemical characteristics. Results indicate that the preference and performance link in *C. ohridella* is poor, as the miner lays eggs on host species where the larvae cannot develop. The study also found that both susceptibility to the leaf miner and foliar host chemistry is phylogenetically conserved within the genus *Aesculus*. Among the leaf physical traits that were investigated in this study, leaf toughness is likely to be an important resistance trait for some species, whereas different traits may convey resistance to others. Overall the study suggests that different resistance mechanisms are present in different species of *Aesculus*, and that traits which are responsible for female preference can be different from those traits which are important for the developing larvae.

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Abbreviations

%: Percentage

$[A+H]^+$: Molecular mass of the protonated aglycone

$[M+H]^+$: Molecular mass of the protonated molecule

$[M-H]^-$: Molecular mass of the deprotonated molecule

° C: Degrees Celsius

µ: Mean

µl/ml: Microlitre per millilitre

µl: Microlitre

µm: Micrometre

AICc: Akaike information criterion

ANOVA: Analysis of variance

B: Independent coefficient

CE: Capillary electrophoresis

cm: Centimetre

cm^2: Centimetre squared

DESI: Desorption electrospray ionisation

df : Degrees of freedom

DIMS: Direct infusion mass spectrometry

DNA: Deoxyribonucleic acid

EAG: Electroantennography

ESI: Electrospray ionisation

ESI-MS: Electrospray ionisation- mass spectrometry

F: F-ratio (test statistic used in ANOVA)

GC/EAD: Gas chromatography coupled to electroantennographic detection

GC/MS: Gas chromatography coupled to mass spectrometry

GLM: Generalised linear model

H: Kruskal-Wallis test statistic

HPLC: High performance liquid chromatography

ITS: Internal transcribed spacers

IUCN: International Union for Conservation of Nature

kV: Kilovolts

L/h: Litre per hour

LC-ESI-MS: Liquid chromatography-electrospray ionisation-mass spectrometry

LC-MS: Liquid chromatography coupled to mass spectrometry

lg: Logarithm

m/*z*: Mass to charge ratio

m: Metre

MALDI-MS: Matrix assisted laser desorption ionisation mass spectrometry

MEP: 2-C-methyl-D-erythritol 4-phosphate

mg/g: Milligram per gram

mg/l: Milligram per litre

mg: Milligram

min: Minute

ml/min: Millilitre per minute

ml: Millilitre

mm: Millimetre

mRNA: Messenger ribonucleic acid

N: Newton

n: Sample size

NetCDF: Network Common Data Form

nm: Nanometre

NMR: Nuclear magnetic resonance

ns: Non-significant

p, P: Probability

PC: Principal Component

ppm: Parts per million

RF lens: Radio frequency

rpm: Revolutions per minute

rps16: Radial spoke protein 16

Rt: Retention time

s: Second

SD, σ: Standard deviations

SE: Standard error

SLA: Specific Leaf Area

SPSS: IBM Software package used for statistical analysis

t: Time

UV: Ultra violet

v/v: Volume per volume

V: Volts

Z-factor: A measure of statistical analysis

λ_{max}: Wavelength at which a solution can absorb maximum light

τ: Kendall's tau (non-parametric correlation coefficient

Chapter 1

Introduction

1.1 Non-native herbivores

Invasive alien species pose a threat to ecosystems and biodiversity and can cause considerable economic damage. Many invasive herbivores are harmful to native plant populations to the extent of local extinctions (Kenis *et al*., 2009). Non-native herbivores can also affect local herbivore communities by competing for resources (Péré *et al*., 2010a; Péré *et al*., 2011), transferring new diseases (Kenis *et al*., 2009) and they can develop synergistic interactions with phytopathogenic fungi and bacteria, thus facilitating the emergence of more complex diseases. This was the case in the devastating Dutch elm disease which destroyed most of *Ulmus* spp. in Central Europe. This disease is caused by the mutualistic relationship between *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* fungi and the elm bark beetles *Scolytus* spp. and *Hylurgopinus rufipes* (Johne *et al*., 2008).

In Great Britain, 164 non-native, newly established invertebrate herbivores were recorded between 1970 and 2004. The majority of these species belonged to the orders Homoptera (33%) and Lepidoptera (25.5%) and their introduction was either human assisted or by natural colonisation. Almost half of these species originated from Europe and the major establishments were recorded on ornamental and woody plants. Interestingly, a higher proportion of these

herbivores established on non-native woody plants than on natives (Smith *et al*., 2007).

This thesis focuses on one of the non-native herbivore species which has recently arrived to the UK from Europe. The horse chestnut leaf miner (*Cameraria ohridella* Deschka & Dimic, 1986 (Lepidoptera: Gracillariidae)) originates from the Balkans (Valade *et al*., 2009; Lees *et al*., 2011), and during the last 25 years it has spread to most of the European countries. It causes significant aesthetic damage to horse chestnut trees (*Aesculus hippocastanum* L.), its native host, which is extensively planted as an ornamental tree throughout Europe (Girardoz *et al*., 2007a). In addition, *C. ohridella* has been reported to attack other species of the genus *Aesculus* as well as some species of maples (*Acer*) (Péré *et al*., 2010b; Straw & Williams, 2013), which are novel hosts to this herbivore. Some species of *Aesculus* are susceptible to the invader while others are not (Straw & Tilbury, 2006) and the mechanisms of resistance within the genus *Aesculus* are presently not known.

The herbivore's ability to expand its host range and utilise novel hosts is dependent on oviposition preference and larval performance. A good preference-performance linkage therefore is especially important for sessile insects such as leaf miners where the larvae is forced to feed on the host chosen by the ovipositing female (Forister *et al*., 2009). When ovipositing females encounter novel host plants the decision making of the females is affected by the altered oviposition environment (Gillespie & Wratten, 2011) and adults may accept potential new hosts if the traits which are responsible for

oviposition are similar to the host plant which the herbivore usually develops on. Often herbivores host plant range changes in their new distribution area, as is the case in the leaf mining moth *Phyllonorycter blancardella*, whose host range in Europe is broad on many genera within the Rosaceae. However, its host plant range is restricted to the genus *Malus* in North America (Schoonhoven *et al*., 2005). There are several factors which influence the colonisation of new hosts, such as voltinism or the number of generations produced per year, abiotic environment, natural enemies, heterophylly and host availability (Forister & Wilson, 2013). Ecological fitting which has been omitted from many preference-performance studies suggests that ecological interactions between species are not only the products of evolutionary processes but more often ecological match of traits and environment (Gillespie & Wratten, 2011; Forister & Wilson, 2013).

Although many studies investigated biological invasion of insect herbivores, very few studies concentrate on the interaction between a non-native herbivore and a non-native host. In this scenario, both the host plant and herbivore are introduced into a novel environment where they often lack specialist herbivores and natural enemies. The degree of preference-performance linkage between the herbivore and the novel host will depend on the synchronisation of their phenology, morphological, physical and chemical traits and their interaction, the effects of the environment and co-occurring species. In case of specialist insect herbivores, both the similarity of traits and the phylogenetic distance between the original and the non-native host will determine herbivory (Pearse & Hipp, 2009).

1.2 Plant resistance to herbivores

Plants produce a diversity of physical and chemical traits which mediate complex interactions between plants, herbivores and natural enemies (Kessler & Heil, 2011).

1.2.1 Physical and chemical characteristics of hosts and their impact on herbivores

Host physical and chemical traits are very important for both ovipositing females and herbivore development. Leaf size is an important plant trait for sessile insects and they usually prefer large leaves, as they need to complete their development within a single leaf (Bultman & Faeth, 1986). Leaf tissue toughness often provides a mechanical barrier for most herbivores (Kimmerer & Potter, 1987) and foliar water content and nitrogen are often found to be limiting factors for insects (Kimmerer & Potter, 1987; Ohgushi, 1992). Specialist herbivores feeding on their hosts with low nutritional quality can compensate for it by feeding on specific tissues which are richer in nutrients or water, low in allelochemicals and structural defences. As an example, most oak leaf miners feed in the palisade mesophyll, while birch leaf miner *Fenusa pusila* feeds in the spongy palisade mesophyll and *Messa nana* feeds selectively in the palisade (Kimmerer & Potter, 1987).

1.2.2 Secondary metabolites

Secondary plant metabolites are metabolic products which are not used by the plant for growth and development, but can influence the plant's fitness and survival. They have multiple roles and are known to play a role in the interactions with herbivores, pathogens, plant competitors, mycorrhizal fungi and N-fixing bacteria (Acamovic & Brooker, 2005; Kirk *et al*., 2005). Sterols are essential compartments of cell membranes, carotenoids can act as additional pigments in photosynthesis and protect the photosynthetic tissue from oxidation, dolichols can act as sugar carriers in cell wall and glycoprotein synthesis and lignin provides mechanical support (Taiz & Zeiger, 2006). Flavonoids play a role in auxin transport (Graham, 1998; Hartmann, 2007), male fertility and coloration of flowers (Ferreyra, *et al*., 2012). Plant secondary metabolites can also provide protection against abiotic stress like UV radiation and can also function as phytohormones that regulate physical changes (Weng & Noel, 2012).

The production of secondary metabolites is an adaptive strategy for sessile life style of plants. Genetic variations, for example, genome rearrangement, point mutations, deletions, insertions, gene translocations, chromosome loss and polyploidisation ensure metabolic diversity (Weng & Noel, 2012). Gene duplications are important sources of gene diversification and evolutionary novelty (Grotewold, 2005; Hartmann, 2007, Klibenstein & Osbourn, 2012). Following gene duplication, the genes can have either new function, secondary function or no function at all (Grotewold, 2005). In higher eukaryotes, tandem

duplicated genes at adjacent loci are common and support the expansion of specialised metabolic enzyme families (Weng & Noel, 2012).

Secondary metabolites are synthesised by metabolic enzymes in biosynthetic pathways. These enzymes can use either single or multiple substrates often with varying kinetic properties (Grotewold, 2005). In most cases, the enzymes in secondary metabolism are specific for a substrate and produce a single product, however, many enzymes can catalyse the synthesis of multiple products (Pichersky & Gang, 2000). The evolution of new biosynthetic pathways can be beneficial in adapting to new environmental niches, which is important in plant speciation (Klibenstein & Osbourn, 2012). Some pathways are present in all existing species (e.g. phenylpropanoid metabolism in terrestrial plants), while others are found in limited genera or in a single species (Weng & Noel, 2012). The biosynthetic pathways are regulated by transcription factors (Vom Endt *et al*., 2002; Grotewold, 2005). Transcription factors can regulate gene transcription in response to internal (e.g. plant hormones) or external (e.g. UV light, microbial elicitors) signals. Transcription factor activity can be regulated by posttranslational modifications, alteration of the production of the encoding mRNA and interactions with other proteins (Vom Endt *et al*., 2002).

Enzymes of secondary metabolites can be organised in complexes also known as “metabolic channels”, “metabolons” or “metabolic compartments”. The interaction of the enzymes can result in the channelling of pathway intermediates from one enzyme to the other (Dixon *et al*., 2001; Jørgensen *et*

al., 2005), which prevents the premature release of toxic intermediates (Weng & Noel, 2012). Metabolic channelling also relieves kinetic constraints and secures the conversion of toxic intermediates into more stable and less toxic constituents by sequestration, also it controls metabolic cross-talk. In the presence of biotic and abiotic stresses, additional enzymes can attach to metabolons to prevent access to their active sites to ensure the desired modifications (Jørgensen *et al*., 2005).

The main classes of secondary metabolites are phenolic compounds, terpenoids and isoterpenoids, nitrogen and sulphur containing compounds (Aharoni & Galili, 2011). The main biosynthetic pathways and their products are illustrated in Figure 1.1.

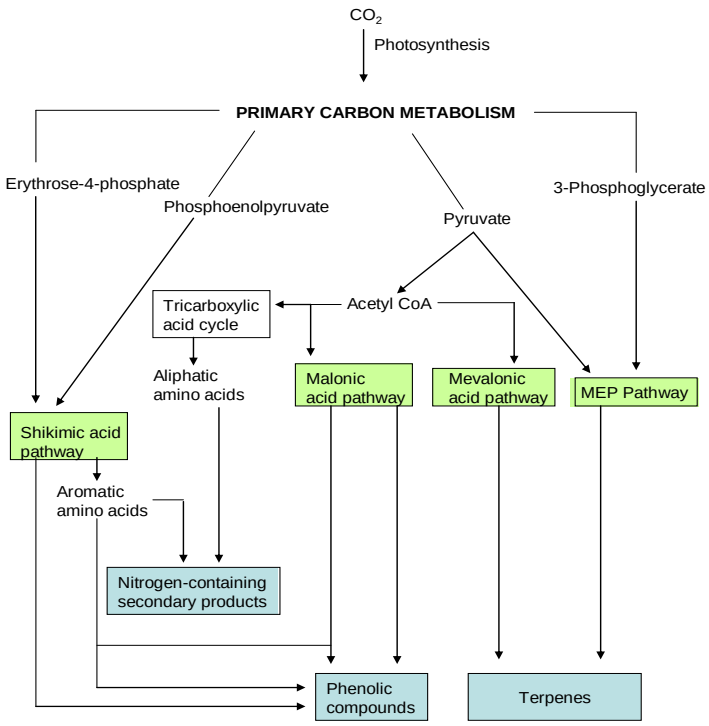


Figure 1.1 The pathways of secondary metabolite synthesis (Taiz & Zeiger, 2006)

These metabolites are usually accumulated in specific plant tissues and cell types (Acamovic & Brooker, 2005; Yazaki, 2005). Hydrophilic compounds are usually stored in vacuoles or idioblasts while lipophilic compounds are often stored in resin ducts, glandular hairs, trichomes, on the cuticle or in thylakoid membranes. However, some compounds may be stored in the epidermis itself (Acamovic & Brooker, 2005).

Many of these metabolites produced by plants are toxic, not only to pathogens and herbivores, but also to the producing plant itself (Bernays & Chapman, 1994; Sirikantaramas *et al*., 2008). Plants protect themselves from these toxic effects by vacuolar sesquestration, vesicle transport, extracellular biosynthesis or by excretion (Sirikantaramas *et al*., 2008). Many highly toxic secondary compounds are stored in less toxic forms. These precursors are then transformed to toxic compounds when needed by the plant, for example, when the plant is damaged (Schoonhoven *et al*., 2005).

Secondary metabolites in relation to insects can function as *allomones* (beneficial to the producing plant) or *kairomones* (beneficial to the insect). Allomones can be deterrents, oviposition and feeding repellents and harmful or toxic compounds, while kairomones can be attractants, oviposition and feeding stimulants (Smith, 1989). Successful chemical defence of plants is usually achieved either due to a wide range of moderately toxic compounds or due to a few highly toxic chemicals (Rattan, 2010).

Specialist insects are usually unaffected by high concentrations of secondary metabolites, which generally occur in their host plants. They use several

mechanisms to avoid poisoning: 1) prevention of noxious compounds passing through the gut wall (many molecules cannot pass biomembranes), therefore, fast excretion results in lower chances of toxicity, 2) detoxification by cytochrome P450s. These enzymes convert the noxious metabolites into more polar forms which then can be either excreted or further metabolised by secondary enzymes. Other groups of detoxifying enzymes are the glutathione-S-transferases and esterases. These enzymes are mainly located in the insect's midgut and in the fat bodies (Rattan, 2010) and upon exposure to the novel toxins the amount of detoxifying enzymes produced by the insect increases due to gene activation. This process is also called induction (Schoonhoven *et al*., 2005).

On the other hand, specialist insects can use secondary plant metabolites to protect themselves against parasitoids, predators and pathogens (Hartmann, 2004). Some species of *Chrysomela* and *Phratora* sesquester salicin and incorporate it into their eggs to protect the neonate larvae and secondary metabolites have also been known to be metabolised by insects to sex pheromones, as is the case of some male nymphalid butterfly species who utilise pyrrolizidine alkaloids (Opitz & Müller, 2009).

1.2.3 Plant hybridisation and its effects on leaf traits

The hybrid bridge hypothesis suggests that hybridisation affects host-herbivore associations by creating bridges in host phenotypes and thus facilitates host-switches (Pearse & Baty, 2012). Hybridisation can result in changes in

herbivory and it also affects the expression of leaf defensive traits. It is a very good study system to investigate the role of different leaf traits and resistance mechanisms. Genetic changes in hybrids can affect phenology, morphology and chemical and physical traits, which could affect herbivore load (Fritz, 1999). It has been previously reported that different hybrids of *Aesculus* experience different degrees of resistance towards *C. ohridella* (Straw & Tilbury, 2006). It would be therefore very useful to investigate the inheritance of different leaf traits of *Aesculus* hybrids and their effects on the resistant/susceptible status of the given hybrid towards the miner.

1.3 Leaf miners

The earliest leaf mining fossil records were found from the Triassic period, approximately 200 to 250 million years ago, which indicates that leaf mining evolved after boring, margin feeding, galling and skeletonising (Sinclair & Hughes, 2009). Leaf mining can be found in the orders of Coleoptera, Diptera, Hymenoptera and Lepidoptera (Rott & Godfray, 2000; Salvo & Valladares, 2007; Sinclair & Hughes, 2009). It occurs in 34 families in the order of Lepidoptera, with the highest number of species found in the Gracillariidae (Auerbach *et al*., 1995). Leaf miners usually have narrow host preferences and most of them are monophagous or oligophagous and only some species are polyphagous. Polyphagous and oligophagous species usually occur in the same plant genus (Hespenheide, 1991).

Most species of leaf miners are restricted to a single leaf as endophagous herbivores. However, in some cases, larvae can move to a different leaf, initiating a new mine (Connor & Taverner, 1997). The mining larvae can form a variety of mines within the leaf tissue e.g. linear tunnels or blotches (Sinclair & Hughes, 2009). The mining stage can be restricted in some taxa to a single instar or it can be the full duration of the larval and pupal period; hence adults need to break or chew through the leaf epidermis in order to emerge (Connor & Taverner, 1997). Tissue damage caused by the larvae can decrease the photosynthetic capacity of the leaves and hence causes premature leaf abscission, permitting pathogen entry into the plant tissue. This can cause severe aesthetic damage to the plant tissue and reduce the value of horticultural crops. Therefore, many leaf miners are considered as pests (Salvo & Valladares, 2007).

There are several advantages of endophagous feeding and the leaf miner habit. It is thought that mines offer a degree of protection to the herbivore feeding inside, such as protection from UV radiation, natural enemies, and environmental variations, such as wind and rain, which can dislodge the herbivore from its host plant. It was shown that over 95% of the UV radiation was absorbed by the leaf epidermis of *Quercus alba* and *Robinia pseudoacacia*, suggesting that larvae feeding inside the leaf tissue was indeed protected from radiation (Connor & Taverner, 1997). Leaf mining has also the advantage of fine scale selective-feeding as larvae can avoid feeding on certain plant tissues; therefore, avoiding some internal defences. This type of feeding

also provides protection from external plant defences such as spines and waxy cuticles (Connor & Traverner, 1997; Sinclair & Hughes, 2009).

There are, however, disadvantages of the leaf-mining habit as well, such as the higher mortality rates of parasitism compared to external feeders, as larvae are confined to the mine and cannot escape attack by natural enemies (Connor & Traverner, 1997).

1.4 Study species

The leaf miner *Cameraria ohridella* Deschka & Dimic, 1986 (Lepidoptera: Gracillariidae), (Figure 1.2) is the only representative of the genus *Cameraria* in Europe (Girardoz *et al*., 2006; Straw & Tilbury, 2006). Other species of the genus *Cameraria* are distributed in North America, Central Asia, Japan, Malaysia and India. Species in the genus can be associated with Fabaceae, Oleaceae, Betulaceae, Fagaceae, Aceraceae, Ulmaceae, Salicaceae, Caprifoliaceae, Ericaceae and Juglandaceae. The genus is very similar to the genus *Phyllonorycter* in adult features, with the exception of the outwardly black-margined white mark of the fore wing, the incomplete transtilla and the tegument with a pair of apical setae (Kumata, 1993).

Figure 1.2 *Cameraria ohridella* adult

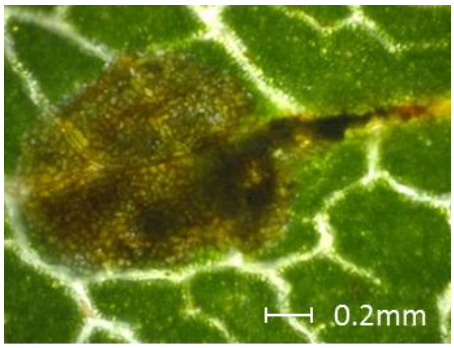
C. ohridella was first discovered near Lake Ohrid in Macedonia attacking *A. hippocastanum* trees (Deschka & Dimic, 1986). Adults are 3 to 5 mm long, metallic brown in colour with white stripes and dark grey hind wings (Tilbury & Evans, 2003). In the last 20-25 years this invasive miner has attracted much scientific, as well as public interest, due to its remarkable success story in colonising species of *Aesculus* in Europe in a very short space of time (Girardoz *et al*., 2006).

Females of *C. ohridella* usually lay between 30-80 eggs, but can lay up to 180 eggs (Girardoz *et al*., 2007a). Eggs (Figure 1.3) are laid from May to August along the lateral veins of the leaves, which hatch after 2-3 weeks, depending on weather conditions (Pschorn-Walcher, 1997; Tilbury & Evans, 2003). The larvae of the moth feed on the palisade parenchyma of the leaves. Larval development takes about four weeks to complete and includes four, sometimes five, feeding instars and two spinning instars (Šefrová, 2001). The first instar larvae are approximately 0.5 mm long, and the mine which they form is less

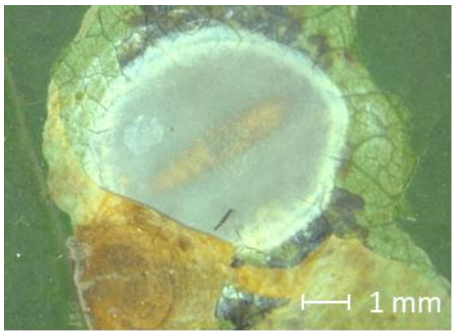
than 3mm in length (Šefrová & Skuhravy, 2000; Grabenweger, 2003). The second instar larvae are up to 1.5 mm long and form comma-shaped mines (Figure 1.3), while the third instar larvae, which are approximately 2 mm long, form circular mines. The fourth instar larvae (Figure 1.3) are about 3.5 mm in length and form irregular shaped mines (Šefrová & Skuhravy, 2000; Grabenweger, 2003). In this stage spinning glands are developed, which will be used for the next two non-feeding spinning instars to spin the cocoons (Figure 1.3) (Skuhravy, 1999). Pupal stage lasts from two to three weeks (Pschorn-Walcher, 1997), but in the case of overwintering pupae this stage can last several months, or even several years (Samek, 2003).

Egg of *C. ohridella*

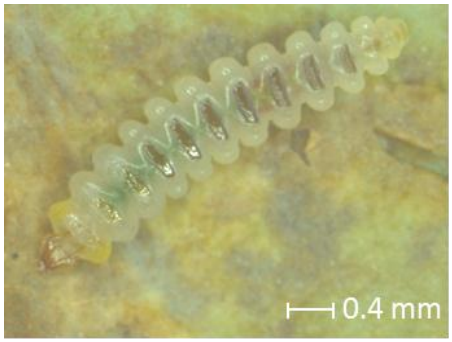
Early instar mine of *C. ohridella*



Larvae of *C. ohridella*



Cocoon of *C. ohridella*



Pupae of *C. ohridella*

Emerging pupae

Figure 1.3 The different developmental stages of *C. ohridella*

1.4.1 The spread of *C. ohridella* in Europe

The first recording of *C. ohridella* outside Macedonia was in Austria in 1989, and since then it has gradually spread throughout Europe (Figure 1.4).

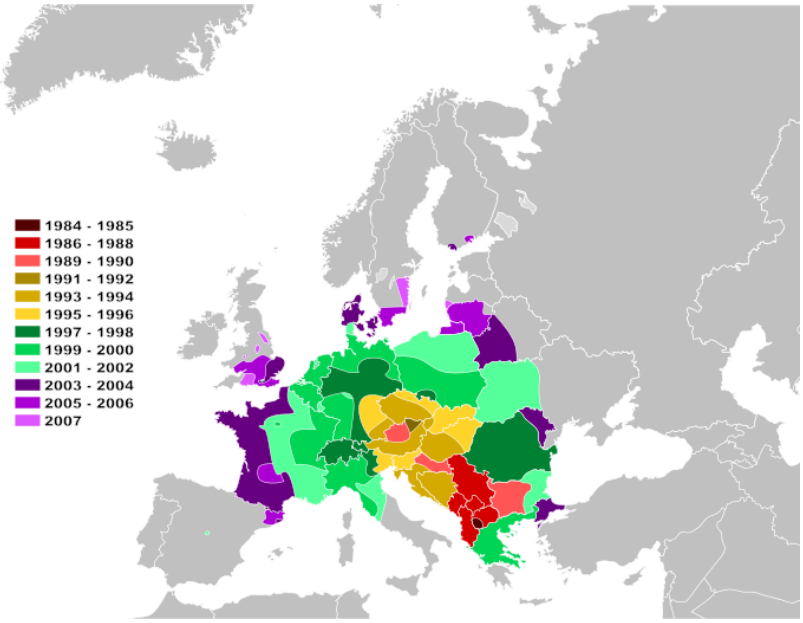


Figure 1.4 Spread of *C. ohridella* in Europe from 1984-2007 (http:// commons.wikimedia.org/wiki/file: cameraria-ohridella-distribution map.svg)

C. ohridella was first reported in the United Kingdom in Wimbledon; in 2002 (Straw & Tilbury, 2006). Since then the moth has effectively spread throughout England and Wales and reached as far as Scotland (Figure 1.5).

The rate of spread of *C. ohridella* in the UK is estimated to be between 40 and 70 km per year. Dispersal of the miner is achieved mainly by active flight and wind, whereas vehicular traffic of infected leaves can aid long distance distribution (Pocock *et al*., 2011).

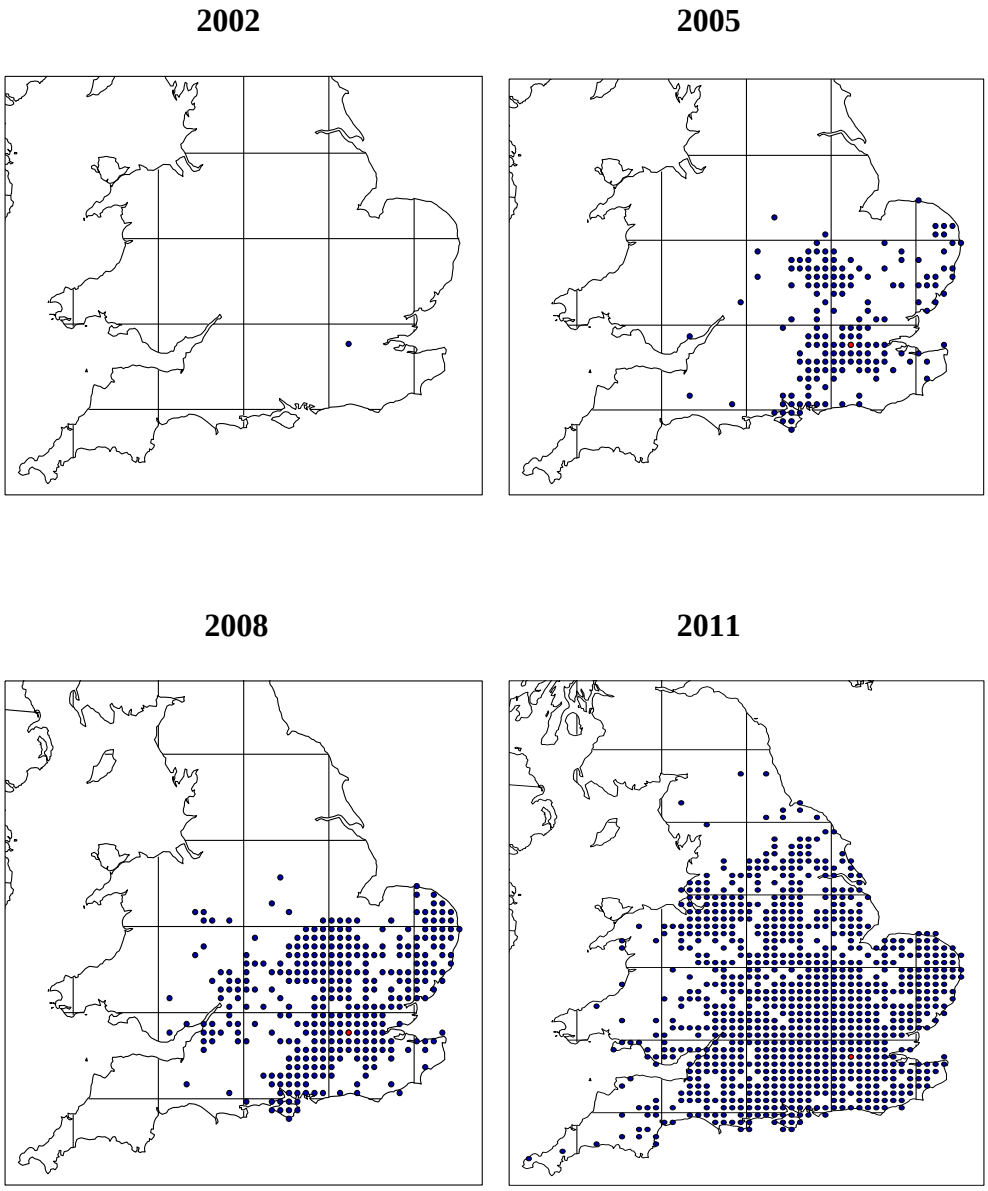


Figure 1.5 Distribution maps of *Cameraria ohridella* (www. forestry.gov.uk)

1.5 Host plants of *C. ohridella*

The main host plant of *C. ohridella* is the European horse chestnut tree *Aesculus hippocastanum* L. However, the miner can develop on other species of *Aesculus* and some species of *Acer* as well (Kenis *et al.*, 2003; Freise *et al.*, 2004; Gilbert *et al.*, 2005; Straw & Tilbury, 2006; Péré *et al.*, 2010b). Among

the species that are infested, *A. hippocastanum* and its sister species *A. turbinata* are the most suitable for development (Straw & Tilbury, 2006), (Figure 1.6).

Figure 1.6 First generation mines on *A. turbinata* growing in RBG Kew

Some of the North American species such as *A. glabra*, *A. flava* and *A. sylvatica* can also support successful development (Freise *et al.*, 2004; Kenis *et al*., 2005; Straw & Tilbury, 2006). However, *C. ohridella* does not develop on *A. assamica*, *A. californica*, *A. chinensis*, *A. indica*, *A. parviflora* and *A. pavia* (Kenis *et al*., 2005). Table 1.1 compares the susceptibility of some *Aesculus* species.

Differences in susceptibility are also reported for different cultivars of *A. hippocastanum*; some cultivars being more susceptible than others (Straw & Tilbury, 2006). Fully developed mines are sometimes found on *Acer pseudoplatanus* and *Acer platanoides*, but otherwise *Acer* trees are found to be resistant (Kenis *et al*., 2005; Péré *et al.*, 2010b).

Table 1.1 Suitability of different *Aesculus* species reported in the literature (Kenis *et al*., 2003; Straw & Tilbury, 2006)

Species	**Supporting larval development +/-**	
	Kenis et al 2003	Straw & Tilbury 2006
A. hippocastanum	+	+
A. turbinata	+	+
A. flava	+	+/-
A. pavia	+	+/-
A. slyvatica	+	+/-
A. glabra var glabra	+	+/-
A. chinensis	-	-
A. indica	-	-
A. assamica	-	-
A. wilsonii	no data	no data
A. californica	-	-
A. parviflora	-	-
A. parryi	no data	no data

1.5.1 Phylogeny of *Aesculus* L.

Resistance to *C. ohridella* within the genus *Aesculus* seems to be related to phylogeny (Straw & Tilbury 2006). The genus comprises of 13 species distributed in the Northern Hemisphere (Hardin 1957, 1960; Xiang *et al*., 1998; Forest *et al*., 2001). These species are divided into five taxonomical sections: *Aesculus*, *Pavia*, *Calothrysus*, *Macrothyrsus* and *Parryana* (Hardin 1957, 1960; Xiang *et al*., 1998, Figure 1.7).

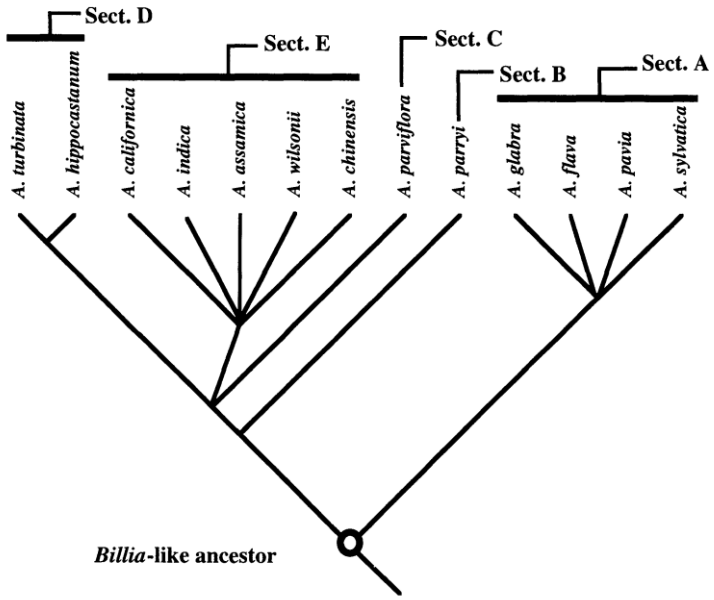


Figure 1.7 Phylogeny of *Aesculus* L. based on morphology. The letters represent the different sections (*Aesculus* (Sect. D), *Pavia* (Sect. A), *Calothyrsus* (Sect. E), *Macrothyrsus* (Sect. C) and *Parryana* (Sect. E)). Figure adapted from Xiang *et al*. (1998)

Species belonging to section *Aesculus* are distributed in Japan (*A. turbinata*) and in southeast Europe (*A. hippocastanum*). Species in the section *Pavia* are distributed in North America. Species in the section *Calothyrsus* can be found in eastern Asia (*A. indica*, *A. assamica*, *A. wilsonii* and *A. chinensis*) and in North America (*A. californica*). *A. parviflora*, the only member of the section *Macrothyrsus* can be found in North America and *A. parryi*, the only member of the section *Parryana* can be found in Mexico (Xiang *et al*., 1998). Six additional eastern Asian species of *Aesculus* have been described: *A. chuniana* Hu & Fang, *A. lansangensis* Hu & Fang, *A. megaphylla* Hu & Fang, *A. polyneura* Hu & Fang, *A. tsiangii* Hu & Fang and *A. wangii* Hu & Fang (Xiang *et al*., 1998; Harris *et al*., 2009). However, the species status of these taxa is uncertain (Harris *et al*., 2009). Molecular analyses of the genus based on DNA sequences of the chloroplast *mat*K gene supports Hardin's phylogeny.

However, molecular analyses of the internal transcribed spacers (ITS) from the nuclear 18S-5.8S-26S ribosomal gene family, place *A. californica* more closely to section *Macrothyrsus* (Xiang *et al*., 1998, Figure 1.8).

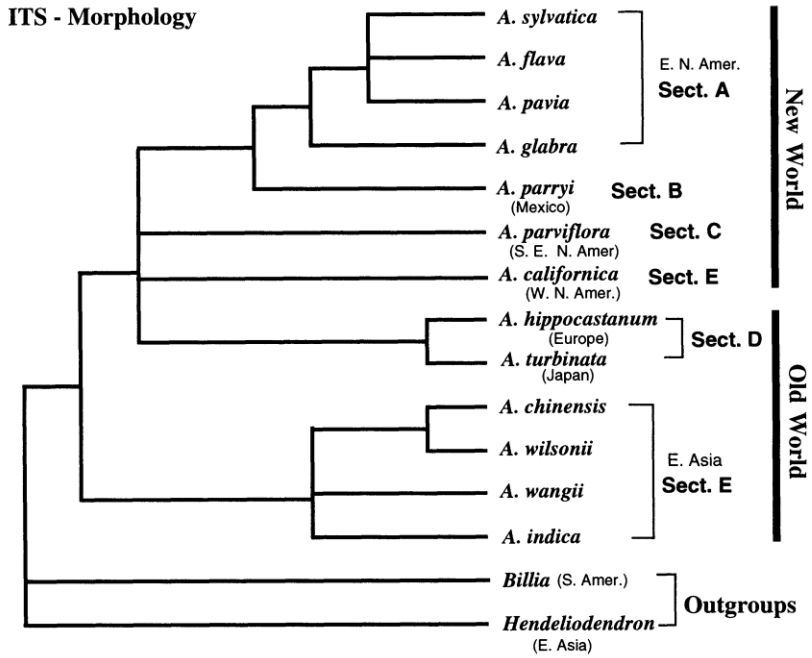


Figure 1.8 Phylogenetic tree from the combined ITS sequence and morphological dataset using branch-and-bound search adapted from Xiang *et al*. (1998). The letters indicate the sections of *Aesculus*; (*Aesculus* (Sect. D), *Pavia* (Sect. A), *Calothyrsus* (Sect. E), *Macrothyrsus* (Sect. C) and *Parryana* (Sect. E))

The placement of *A. californica* is therefore not fully resolved (Xiang *et al*., 1998; Forest *et al*., 2001; Harris *et al*., 2009). Phylogeny including DNA regions *rps16*, *trn*HK, *mat*K, ITS and part of LEAFY gene, combined with morphological characters, supported the major lineages in the genus described by other authors. This analysis also revealed a sister relationship between section *Aesculus*, section *Parryana* and section *Pavia* (Harris *et al*., 2009).

Anatomical and morphological description of the species of *Aesculus* is well documented in papers published by Hardin (1957, 1960). Table 1.2 describes the main morphological differences between the species and Figure 1.9 illustrates the leaves and flowers of the species.

Aesculus hippocastanum L.

Aesculus turbinata Blume

Aesculus pavia L.

Aesculus flava Aiton

Aesculus sylvatica Bartram

Aesculus glabra Willd.

Aesculus indica (Camb) Hook

Aesculus assamica Griffith

Aesculus wilsonii Rehder

Aesculus californica (Spach) Nuttal

Aesculus parviflora Walter

Figure 1.9 Different species of *Aesculus* growing in the Royal Botanic Gardens of Kew

Table 1.2 Description of the different species of *Aesculus* (Hardin (1957, 1960)

Species	Description
A. hippocastanum L.	Endemic to the Balkan Peninsula, can be found in Bulgaria, Greece and Albania. Widely cultivated in Europe and North America. 25-30 m in height, it has dense white flowers and 5 or 7 cuneate-obovate leaflets.
A. turbinata Blume	Native to Japan, also cultivated in North America, Europe and China. Can grow up to 30 m, has creamy white flowers, 5 or 7 leaflets. Lower surface of leaves is pale or glauscent.
A. pavia L.	North American species up to 10 m tall, has red flowers. Leaves have 5 or 7 leaflets, oblong obovate and narrowly elliptic.
A. flava Aiton	Distributed in North America, it is 20-30 m tall, has yellow flowers. Leaves have 5 or 7 leaflets.
A. sylvatica Bartram	Distributed in Southern Virginia, South and North Carolina, Georgia, Northeast Alabama and Tennessee. Can grow up to 15 m. Flowers are yellow but can be reddish. Leaves have 5 or 7 leaflets, oblong obovate, lanceolate, oblanceolate or elliptic.
A. glabra Willd.	10-30 m tall, can be found in Pennsylvania, Iowa, Arkansas, Tennessee and Alabama. It has yellow flowers. 5-7 leaflets, oblong-obovate or elliptic-obovate.
A. indica (Camb) Hook	Distributed in north-western Himalayas in Nepal, India, Pakistan and Afghanistan. Also cultivated in Europe. Grows to a height of 30 m. It has white flowers. Leaves have 5, 7 or 9 leaflets, lanceolate to elliptic-oblanceolate.
A. assamica Griffith	Distributed in Siam, Indo-China, Burma, Assam, east Pakistan, Bhutan and Sikkim. Grows up to 25 m tall. It has white flowers. Leaves have 5-9 leaflets, lanceolate or elliptic to oblong-oblanceolate.
A. wilsonii Rehder	Distributed in China and has white flowers. Leaves have 5-7 leaflets, oblong-lanceolate or oblong-oblanceolate.
A. chinensis Bunge	Distributed in China, up to 25 m tall and has white flowers. Leaves have 5-7 leaflets, oblong-lanceolate or oblong-oblanceolate.
A. californica (Spach) Nutall	Distributed in California. Grows up to 12 m, has white flowers. Its leaves have 4-7 leaflets.
A. parviflora Walter	North American species, up to 5 m tall. Leaves have 5 or 7 leaflets; elliptic to oblong-obovate. It has white flowers.
A. parryi Gray	A shrub or small tree, 1-6 m tall, distributed in Baja California, Mexico. It has white flowers and 5-7 leaflets.

1.5.2 Secondary chemistry of species of *Aesculus*

A vast amount of research has been carried out investigating the chemical composition of the different species of *Aesculus* (Zhang *et al*., 2010).

Different compounds with biological activity were previously detected in different organs of *Aesculus*; coumarins in the bark, flavonoids in the leaves and flowers, while saponins in the seeds (Stanic *et al*., 1999). However, most researches have concentrated on the seeds of these species because of their medicinal properties. Seed extracts of *Aesculus hippocastanum* are used for the treatment of varicose veins, inflammation of the veins, haemorrhoids, rheumatism, diarrhoea, fever, enlargement of the prostate gland and rectal complaints. The active ingredient extracted from the seeds of the plant is called aescin, which has been shown to reduce the symptoms of chronic venous insufficiency and it also has anti-inflammatory activity (Wilkinson & Brown, 1999). Seeds of *A. pavia*, *A. flava*, *A. glabra* and *A. californica* were often used by the native Americans to catch fish by tranquilizing them (Zhang *et al*., 2010).

Saponins are the main active constituents of *Aesculus* seed extracts. Saponins from *Aesculus* are classified as polyhydroxylated triterpenoid glycosides, based on four different aglycones such as 24-hydroxy-R_1-barringenol, R_1-barringenol, protoaescigenin and barringtogenol-C (Zhang *et al*., 2010). Saponins present in horse chestnut seeds have a wide range of therapeutic uses such as prevention and treatment of cellulites, vascular disorders, anti-inflammatory and anti-serotonic effects (Matsuda *et al*., 1997). Seed shells of *A. hippocastanum* are also known to contain A- and B-type proanthocyanidins

(Santos-Buelga *et al*., 1995) and flavonoids (Kapusta *et al*., 2007). Many flavonols, flavonones and flavonone-derivates were also isolated from seeds of *Aesculus*. Other compounds isolated from *A. hippocastanum* seeds are aesculin, fraxin, scopolin (coumarin derivates), tannins such as leucocyanidine, proanthocyanidin A_2, essentials oils such as oleic acid, linoleic acid) (Kapusta *et al*., 2007).

HPLC studies carried out on *A. hippocastanum* and *A. pavia* revealed that the main carotenoids present in the buds and pollen were aesculaxanthin, lutein, neoxanthin and *β*-carotene. Buds also contained 9-*cis*-neoxanthin and pollen also contained *β*-citraurin, *β*-cryptoxanthin and lutein 5,6-epoxide. The main compounds present in the petals were found to be lutein and lutein 5,6-epoxide (Deli *et al*., 2000).

The following type of compounds have been detected in *Aesculus* leaves: coumarins (Curir *et al*., 2007), *cis*-*trans*-polyprenols (Wellburn *et al*., 1967), saponins (Ferracini *et al*., 2010) and flavonoid-glycosides (Zhang *et al*., 2010). Volatiles emitted by leaves of *A. hippocastanum* have been investigated by using the combination of GC/MS, GC/EAD and EAG. Some of the volatiles from *A. hippocastanum* showed strong electroantennogram activity for *C. ohridella* (Svatoš *et al*., 2009). These compounds were tested in the field in mixtures and also individually. The overall attractiveness of the compounds was moderate.

Additional compounds were also detected from the host leaves which were attacked by the miner. Benzyl alcohol, methyl salicylate, 2-phenylethan-1-ol,

1,8-cineole, (*E*)-β-caryophyllene and (*E*,*E*)-α-farnesene were detected during the larval stages 1 and 3 in addition to leaf volatiles, while linalool oxides, nonanal, decanal, (*E*,*E*)-2,4-hexadienal were detected in the later stages and these compounds were found to be repellent towards ovipositing females (Svatoš *et al*., 2009).

1.6 Impact of *C. ohridella* on tree health

Infestation by *C. ohridella* causes considerable leaf damage to *A. hippocastanum* leaves as it is shown in Figure 1.10.

Figure 1.10 Leaf tissue damage of *A. hippocastanum* caused by *C. ohridella* in May, prior to *C. ohridella* infestation (top left), July, (top right) late August, (bottom left) and late September (bottom right)

Although the damage caused by the miner is very unsightly, it is mainly aesthetic. Infestation reduces the photosynthetically active tissue. This results in lower carbon allocation to the roots and reduced leaf-gas exchange which limits the water and nutrient uptake (Thalmann *et al*., 2003; Takos *et al*., 2008). *A. hippocastanum* trees compensate for this effect by more wood production through more false rings and wider xylem conduits. This increases the hydraulic efficiency of the wood and compensates for the reduced life span of the leaves (Raimondo *et al*., 2003; Salleo *et al*., 2003). Therefore, horse chestnut trees do not face serious risk of dieback as a result of *C. ohridella* damage. However, their reproduction is negatively affected (Salleo *et al*., 2003; Nardini *et al*., 2004). Seed weights of heavily infested trees are reduced considerably, which could affect the growth and survival of young horse chestnut seedlings (Thalmann *et al*., 2003; Nardini *et al*., 2004; Takos *et al*., 2008; Percival *et al*., 2011).

Infestation of *A. hippocastanum* by *C. ohridella* could also render the species more susceptible to other pests and pathogens. *A. hippocastanum* is known to be affected by a range of pests and diseases. *Guignardia aesculi* (*Botryosphaeriaceae*) is a leaf pathogen which causes damage to many *Aesculus* species. It is present in North America, Europe and Asia. The reddish brown leaf blotches caused by the fungi are very similar to that caused by *Cameraria ohridella* (Pastirčáková *et al*., 2009). *Erysiphe flexuosa*, also known as powdery mildew, covers the leaf surface with a greyish white coating and does not cause necrosis on the leaves as *Guignardia aesculi* does. Both *E. flexuosa* and *G. aesculi* can co-occur with *C. ohridella* (Johne *et al*., 2008).

Ganoderma and Armillaria are wood rotting fungi which cause decay on *A. hippocastanum* (www.forestry.gov.uk).

Bleeding canker of *A. hippocastanum* is a new disease and it is characterised by bleeding from the trunk and branches and often results in the death of the tree (Steele *et al*., 2010). The causal agent is the bacterium *Pseudomonas syringae* pv. *aesculi*, which was previously recorded in India as a pathogen of *Aesculus indica* (Camb) Hook. According to the Forestry Commission 40 to 70% of *A. hippocastanum* trees are affected by the disease, which in many cases causes the death of these trees. There is no indication however, that *C. ohridella* facilitates the spread of this disease (Straw & Williams, 2013).

1.7 The importance of pest management of *C. ohridella*

Aesculus hippocastanum is a very important ornamental tree all over Europe. It can be found in most of the cities, parks and avenues. In some countries horse chestnut is also an important source of food for animals in hunting districts (Samek, 2003) and horse chestnut extracts are also used in herbal remedies (Wilkinson & Brown, 1999). Unfortunately, seed size has been shown to be reduced due to the effect of high population densities of the moths, which in return has effects on the yield of the active ingredient of this medicinal plant (Thalmann *et al.*, 2003).

Although infestation by the moth has not to date caused serious impact on tree health, the aesthetic damage of the trees encourages councils to replace this

beautiful tree by other species (Kenis *et al*., 2005). It was estimated that replacing horse chestnut trees in Berlin would cost around €200 million (Kehrli & Bacher, 2004). In Great Britain, according to the National Woodland Inventory of Woodland Trees, there are 470,000 horse chestnut trees (www.forestry.gov.uk). Therefore it is increasingly important to establish good pest management practice for *C. ohridella*. Also, besides *A. hippocastanum*, other trees could also be in danger, such as other species of *Aesculus* and some species of *Acer* like *Acer pseudoplatanus* and *Acer platanoides* (Straw & Tilbury, 2006). Furthermore, *Aesculus hippocastanum* is also present on the IUCN plant red list of Albania (Valade *et al*., 2009), and the few native stands present in the Balkans could be in danger too, if the extensive outbreak of the moth continues for a long period of time (Valade *et al*., 2009).

1.8 Control options

1.8.1 Mating disruption

The discovery and the production of the synthetic sex pheromone of *C. ohridella* made monitoring the population densities an easier process. Synthesis was first achieved by using an acetylene-based approach which was later modified to obtain higher yields. However, this pheromone molecule is found to be unstable when exposed to environmental factors (e.g. humidity, light) and due to this reason pro-pheromones were synthesised. Although the sex pheromone is very successful in monitoring the emergence of *C. ohridella* adults it was found to be not a very effective control option for trapping and confusing males (Svatoš *et al*., 2009).

1.8.2 Leaf removal

The easiest and most effective way to decrease the build-up of infestation of *Cameraria* is the removal and burning of dead leaves during the autumn and winter. This decreases the number of over-wintering pupae. The process, however, can be expensive. An alternative way is to compost the horse chestnut leaf litter by covering the compost heap with compost foil to avoid emergence of *C. ohridella*. This method can reduce *C. ohridella*'s emergence in the spring by 96% (Kehrli & Bacher, 2004).

1.8.3 Chemical control

Non-ecological control options of *C. ohridella* are also available which are highly effective. Aerial spraying of the leaves and tree trunk injections with Dimilin are sometimes used but these can be hazardous to the environment (De Prins & De Prins, 2001; Ivinskis & Rimsaite, 2006). Dimilin, which has high efficacy, contains the active ingredient diflubenzuron, which reduces chitin deposition in the endocuticle (Salama *et al*., 2009). Although Dimilin has a long-term effectiveness, irregular dispersion of the compound was observed within the tree, where some branches within the tree were not protected from *C. ohridella* infestation. Besides Dimilin, Confidor with the active ingredient Imidacloprid, a neonicotinoid which is a potent insect neurotoxin acting on the central nervous system of insects is also used to control *C. ohridella*. Calypso with active ingredient Thiacloprid, which is also an insect neurotoxin, is also considered for pest management (Kuldová *et al*., 2007; Svatoš *et al*., 2009).

1.8.4 Natural enemies

Biological control of the miner is the most important ecological control option, however, so far native parasitoids and predators in the UK and Europe have failed to fulfil this role.

1.8.4.1 Parasitoids

Leaf miners are usually characterized by high parasitism rates (Tóth & Lukáš, 2005; Girardoz *et al*., 2006), which normally exceed 50% (Grabenweger *et al*., 2005a). In case of introduced leaf miners, native parasitoids usually quickly adapt to them and can provide substantial biological control, as it was the case of *Phyllocnistis citrell* (Staiton) which after its invasion of southern Europe and USA became substantially parasitized by native parasitoids (Girardoz *et al*., 2006). Similarly, the exotic leaf miners *Phyllonorycter robiniella* (Clemens) and *Phyllonorycter platani* (Staudinger), which were introduced in Central Europe about the same time as *C. ohridella*, suffer 45-50% parasitism rate (Girardoz *et al*., 2007b).

However, in case of *Cameraria ohridella* parasitism, rates are unusually low, even in regions where the moth has been established for a very long time (Grabenweger, 2003; Grabenweger, 2004; Grabenweger *et al*., 2005a; Tóth & Lukáš, 2005; Girardoz *et al*., 2006; Girardoz *et al*., 2007b; Béguinot, 2009). This could be due to the poor synchronisation between the parasitoids and *C. ohridella* (Grabenweger *et al*., 2005a; Girardoz *et al*., 2007b).

There are about 30 parasitoid species which are known to attack *C. ohridella* (Girardoz *et al*., 2007b; Pocock *et al*., 2011), which are all generalists, and with the exception of *Minotetrastichus frontalis*, they are all solitary (Pocock *et al*., 2011). None of the species parasitize the eggs of *C. ohridella*, and only the last feeding instars, the spinning stages and the pupae of the miner are parasitized by native parasitoids in Europe (Grabenweger, 2003; Pocock *et al*., 2011). The parasitoid complexes of *C. ohridella* in natural and artificial stands of *A. hippocastanum* are very similar (Grabenweger *et al*., 2005a).

1.8.4.2 Predators

Birds such as blue tit (*Parus caeruleu*), great tit (*Parus major*) and marsh tit (*Parus palustris*) are the most effective among the predators of *C. ohridella* (Grabenweger *et al*., 2005b). In addition, the bushcricket *Meconema meridionale* and the ant *Crematogaster scutellaris* and some spiders are also important predators (Grabenweger *et al*., 2005b).

1.9 Aims and objectives of the study

None of the current control options are adequate enough for controlling population densities of *C. ohridella*. Natural enemies, as discussed above, have no significant effect in controlling the outbreak densities of *C. ohridella*, and the present chemical controls available are not viable either, because of their high costs (tree injections), or due to the effect they exert on the ecosystem (aerial spraying which effects bee populations). Therefore, the understanding

of resistance mechanisms within the genus *Aesculus* is of utmost importance in order to develop new control strategies.

As it was pointed out earlier (section 1.5), different species of *Aesculus* show differences in their susceptibility to *C. ohridella*. The mechanisms responsible for these differences have not yet been investigated. None of the previous studies investigated oviposition patterns by *C. ohridella* on different species of *Aesculus* in a natural environment, nor have there been any studies investigating different leaf traits of species of *Aesculus*, and how these traits might explain the preference and performance of *C. ohridella*. Also, the comparison of leaf chemistry of the different species of *Aesculus* and their effect on *C. ohridella* development has not been conducted yet. Therefore, this study aims to address the following questions:

1) Which species and hybrids of *Aesculus* can support larval development and does successful development correlate with high egg densities?

2) How strong is the preference-performance relationship of *C. ohridella* on *A. hippocastanum* and other species of *Aesculus*, which are novel to the miner?

3) Is preference and performance of *C. ohridella* phylogenetically conserved on the different species of *Aesculus*?

4) Which leaf traits influence oviposition preference and larval performance of *C. ohridella*?

5) How does hybridisation of the main host *A. hippocastanum* affect leaf traits and the preference and performance of *C. ohridella*?

6) How do the phenolic and saponin profiles differ between species in the genus *Aesculus* and do these compounds affect the oviposition preference and larval performance of *C. ohridella*? Do the phenolic and saponin profiles of the species show phylogenetic conservatism?

7) Can metabolomic profiling differentiate resistant and susceptible species and hybrids of *Aesculus*?

1.10 Outline of the thesis

Chapter 2 investigates the host plant range of *C. ohridella* in 3 botanical gardens, compares oviposition and infestation patterns and levels at the different study sites and compares the results found in this study with that of other European studies.

Chapter 3 investigates the preference-performance relationship of *C. ohridella* on different species of *Aesculus* in an experimental setup and explores which leaf traits may influence the oviposition choice and larval performance of the miner.

Chapter 4 investigates how hybridisation of the susceptible *A. hippocastanum* and the resistant *A. pavia* affects preference and performance of *C. ohridella* in an experimental setup. It also examines how different leaf traits are inherited through hybridisation and how these traits influence oviposition preference and larval performance of *C. ohridella*.

Chapter 5 compares the profiles of secondary metabolites (phenolics and saponins) of the different species of *Aesculus* and investigates whether the distribution of phenolics and saponins are linked to the phylogeny of the genus. The effects of phenolic and saponin compounds obtained from *A. hippocastanum, A. indica* and *A. pavia* on the development of *C. ohridella* larvae are tested by conducting bioassays.

Chapter 6 compares the metabolite profiles of the different species and hybrids of *Aesculus* and investigates which compounds differ between resistant and susceptible species.

Chapter 7 summarises the main findings of the study, evaluates the results and discusses the implications of the findings.

Chapter 2

Oviposition patterns and host suitability: Monitoring species of *Aesculus* in botanical gardens for infestation by *Cameraria ohridella*

(Part of this chapter has been published in Ecological Entomology: D'Costa, L., Koricheva, J., Straw, N. & Simmonds, M.S.J. (2013) Oviposition patterns and larval damage by the invasive horse-chestnut leaf miner *Cameraria ohridella* on different species of *Aesculus*. Ecological Entomology, **38**, 456-462).

2.1 Introduction

The main host plant of *C. ohridella* is the white flowering horse-chestnut (*Aesculus hippocastanum* L.) However, females of *C. ohridella* have also been reported to lay eggs on other species of *Aesculus* (D'Costa *et al*., 2013), some of which are commonly used as ornamental trees in the UK and continental Europe. Not all of these species support larval development, and the knowledge of relative resistance of the different species of *Aesculus* to *C. ohridella* is useful for assessing the impact of the moth and selecting the tree species to be planted in the future (Straw & Tilbury, 2006). The mechanisms of resistance and susceptibility towards the leaf miner in the genus of *Aesculus* are presently not clear and individuals of certain species in this genus show great variability (Straw & Tilbury, 2006). Many observations are conflicting and the host plant range of *C. ohridella* is still not fully resolved. Also some of the previous studies were conducted on potted saplings rather than on mature trees

(Freise *et al*., 2004) and susceptibility of plants to herbivores may change through ontogeny (Barton & Koricheva, 2010). Furthermore, none of the previous studies examined oviposition by *C. ohridella* on different species of *Aesculus* under field conditions.

Straw and Tilbury (2006) suggested that the degree of resistance of different *Aesculus* species to *C. ohridella* depends on the taxonomic relationships between the tree species, rather than their geographic origin, and is not related to tree form, height or flower colour. Insect performance on non-native host plants has often been found to be related to host plant phylogeny, with host shifts often occurring among taxonomically closely related plants (Lopez-Vaamonde *et al*., 2003). For example, Rasmann and Agrawal (2011) showed that the performance of the root-feeding larvae of the red milkweed beetle *Teraopes tetraopthalms* on 18 species of *Asclepias* was related to host phylogeny. Host suitability decreased with increasing phylogenetic distance from the most susceptible host *Asclepias syriaca*. The same phenomena was also reported by Pearse and Hipp (2009), who found that leaf damage to non-native *Quercus* species caused by various insect herbivores, including leaf miners, was inversely related to the phylogenetic distance from the native oak species *Quercus lobata*. This could be partly explained by the fact that chemical, physical and ecological characteristics, which influence insect performance, are linked to phylogeny (Lopez-Vaamonde *et al*., 2003). In contrast, Desurmont *et al*. (2011) suggests that the lack of evolutionary history between a herbivore and a novel host can render the new host susceptible, due

to lack of specific defences against the herbivore, thus facilitating pest invasion.

Differences in susceptibility to *C. ohridella* have also been reported for different hybrids of *Aesculus* and for cultivars of *A. hippocastanum* (Straw & Tilbury, 2006). Understanding the prevalent patterns of inheritance of resistant traits in hybrids of *Aesculus* is potentially important for the future use of these hybrids for ornamental purposes. If resistance to *C. ohridella* is inherited mainly as dominant trait, then hybrids between resistant and susceptible species of *Aesculus* can be used. On the other hand, if susceptibility is dominant, then only hybrids between resistant species of *Aesculus* are viable.

This Chapter describes oviposition and infestation patterns of *C. ohridella* on introduced species and hybrids of *Aesculus* in three botanical gardens (Royal Botanic Gardens of Kew (RBG, Kew), Royal Horticulture Society Wisley (RHS Wisley) and Sir Harold Hillier Arboretum (Hillier)). Botanical gardens provide an ideal opportunity to compare herbivore infestation on a wide variety of plant species under similar environmental conditions. Also, *C. ohridella* has been present in these gardens over several years (it was first observed in RBG, Kew in 2003), allowing multiple generations to come in contact with the tree species studied. Therefore, the infestation patterns of the leaf miner population present in these gardens may be different from those observed in other studies, where the leaf miner was either only recently introduced to the country (De Prins & De Prins, 2001), or where oviposition patterns and larval survival were investigated in no-choice tests (Kenis *et al*., 2005). In addition, unlike previous

studies, leaf damage assessment in this study was carried out over multiple years at RBG, Kew and leaf tissue damage caused by the miner was assessed for each generation during the vegetative season, allowing annual comparison of infestation levels.

The following specific questions were addressed in this study:

1) Do females of *C. ohridella* oviposit on *Aesculus* species other than the main host *A. hippocastanum*?
2) On which species, hybrids and varieties of *Aesculus* can *C. ohridella* larvae develop and what are the relative infestation levels of these species?
3) Are the patterns of infestation related to host phylogeny as suggested by Straw & Tilbury (2006)?
4) Do observed infestation patterns differ between the three botanical gardens and study years?

2.2 Materials and methods

2.2.1 Infestation monitoring

Leaf miner damage on different species of *Aesculus* was recorded in RBG Kew between 2009-2011, three times during the growing season (early July, mid-August and late September), which corresponded to the three generations of *C. ohridella*. In Wisley and Hillier only the first two generations in 2011 were

monitored, to compare infestation patterns between the gardens. The species monitored in each garden are listed in Table 2.1.

During each sampling period, twenty leaves were sampled randomly from each tree, the number of trees per species sampled varied between 1 to 10, depending on availability and assessed for levels of damage using the score sheet developed by Gilbert and Gregoire (2003) (Figure 2.1). This damage scoring system gives a good estimation of infestation and is significantly correlated with measurements made by image processing (Gilbert & Gregoire, 2003). Leaf damage was categorized as 0%, 2%, 5%, 10%, 25%, 50%, 75% or 100%.

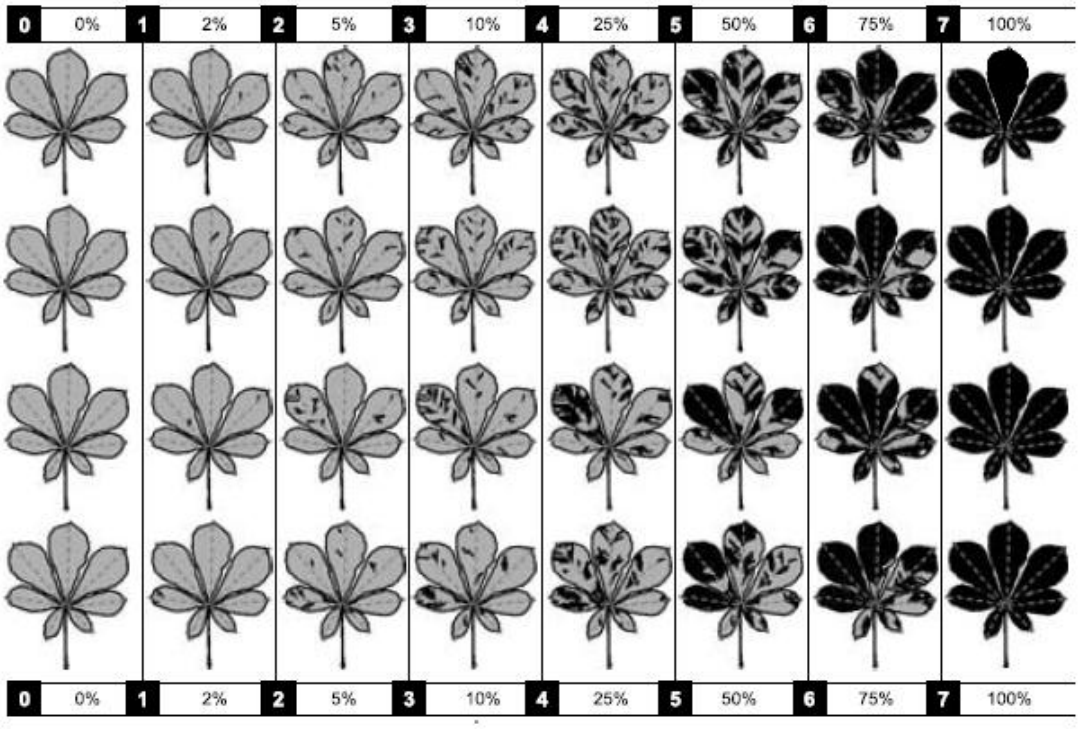


Figure 2.1 Score sheet used for leaf damage estimation (adapted from Gilbert & Gregoire, 2003)

Table 2.1 Species and hybrids of *Aesculus* monitored in the three botanical gardens. Small letters after the hybrids indicate the parent species: *f* (*A. flava*), *g* (*A. glabra*), *h* (*A. hippocastanum*), *p* (*A. pavia*), *s* (*A. sylvatica*)

Species and hybrids	**Collection reference**		
	RBG Kew	**RHS Wisley**	**Hillier Arboretum**
Aesculus hippocastanum	1969-10416, 1969-11006, 1969-10423, 1969-17282, 1969-10417, 2000-10347, 2000-10360, 1969-10368	832653A	19775936
Aesculus hippocastanum 'Baumannii'	1876-7601	-	-
Aesculus hippocastanum 'Incisa'	1973-18615	-	-
Aesculus hippocastanum 'Memmingeri'	1884-17501	-	-
Aesculus hippocastanum 'Pyramidalis'	1896-57102	833291A	19774588
Aesculus hippocastanum 'Digitata'	-	934621A	-
Aesculus hippocastanum 'Hampton Court Gold'	-	19983831A	19781921
Aesculus hippocastanum 'Pumila'	-	-	19772469
Aesculus turbinata	1887-58002, 1969-10450, 1999-153, 1998-461	832654A, 832654B	20010708, 19774585
Aesculus pavia	1969-10445, 1969-10446, 1969-10448	-	19782991, 19782992, 19774807
Aesculus flava	1969-10436, 1969-10438, 1969-10440, 1973-18564, 1912-23901, 1912-23909, 1910-65004, 1973-19596, 1973-19133	894601A	19774808
Aesculus flava 'Vestita'	-	915775A	-
Aesculus sylvatica	1969-10433, 1992-804, 1992-1264	20014693A	-
Aesculus glabra	1943-7101, 1922-45401, 1922-45402, 1922-45403	882672A	-
Aesculus glabra 'Klein's weeping'	-	-	20030061
Aesculus indica	1973-14435, 1995-1446, 1995-1447, 1995-1702, 1995-1703, 1994-1277, 1994-1278, 1889-59501, 1969-10430, 1969-10429	840303A, 19981944A, 20081797A	19820191, 775647
Aesculus assamica	1924-1601, 1924-1607	-	19823300, 19822937
Aesculus wilsonii	1986-2811, 1916-7601	-	-
Aesculus californica	1887-58003, 1979-2366, 1982-145	-	-
Aesculus californica 'Blue haze'	-	-	19776107
Aesculus parviflora	1969-10442	-	-
Aesculus × *arnoldiana* *g* × (*f* × *p*)	1986-2816	934620B	-
Aesculus × *bushii* *g* × *p*	1921-32801	833819A	19774433
Aesculus × *carnea* *h* × *p*	1969-10400, 1969-10402, 1969-10403	-	-
Aesculus × *carnea* Hayne 'Briotii'	1903-4801, 1903-4802	-	19822419
Aesculus × *carnea* Hayne 'Plantierensis' *h* × (*h* × *p*)	1900-1211	962958A	820205
Aesculus × *dallimorei* *h* × *f*	1963-52601, 1973-14653	-	783258
Aesculus × *glaucescens* *f* × *s*	1924-72103	-	-
Aesculus × *hybrida* *f* × *p*	1923-61008, 1923-61001, 1887-58001, 1973-18566	-	19774568
Aesculus × *marylandica* *g* × *f*	1969-10428	-	-
Aesculus × *mutabilis* *p* × *s*	1969-10431, 1924-72102	-	-
Aesculus × *dupontii* 'Hessei' *s* × *p*	-	-	19775933, 19976516
Aesculus × *neglecta* *f* × *s*	-	882620A	-
Aesculus × *neglecta* 'Erythroblastos' *f* × *s*	1934-2501	-	-

2.2.2 Egg density assessment

Egg density assessments were carried out only at RBG, Kew in May 2011. The following species were assessed: *A. hippocastanum, A. turbinata, A. flava, A. glabra, A. pavia, A. sylvatica, A. indica, A. californica, A. assamica, A. wilsonii* and *A. parviflora*. Twenty leaves per tree were collected and eggs, which were present on the upper surface of the leaves, were counted using a digital microscope. The number of trees monitored per species varied from one to five. To account for variation in leaf size among the different species, average leaf area was estimated by measuring the leaf surface area of ten leaves per species using the software package ImageJ (Abramoff *et al.,* 2004). Egg counts were then transformed to egg densities per square centimetre of leaf area.

2.2.3 Statistical Analysis

All analyses were conducted using SPSS version 18.0. Egg density data were analysed by One-Way ANOVA using species as the factor. The leaf damage data did not have a normal distribution (Kolmogorov-Smirnov test was significant) and transformations did not improve normality. Therefore, the Kruskal-Wallis non-parametric test was used when comparing several groups and the Mann-Whitney test was used when comparing two species. The Bonferroni correction was applied to adjust the level of significance for multiple tests. To aid data visualization on graphs, leaf tissue damage levels were transformed to $\log_{10}$ tissue damage +1. Kendall's correlation coefficient

was used to establish whether there was a correlation between egg density and leaf damage.

2.3 Results

2.3.1 Results of egg density assessment and infestation monitoring of *Aesculus* species in RBG Kew

C. ohridella eggs were found on all of the species of *Aesculus* studied, but egg density differed significantly between species ($F_{9,23}$ = 38.29, p< 0.001, Figure 2.2). The highest egg density was observed on *A. hippocastanum*, followed by *A. turbinata*. Egg density did not differ significantly between these two species (p = 0.252), however egg densities on *A. turbinata* and *A. hippocastanum* were significantly higher (p< 0.05) than on *A. flava*, *A. glabra*, *A. pavia*, *A. sylvatica*, *A. indica*, *A. californica*, *A. assamica*, *A. wilsonii* and *A. parviflora*.

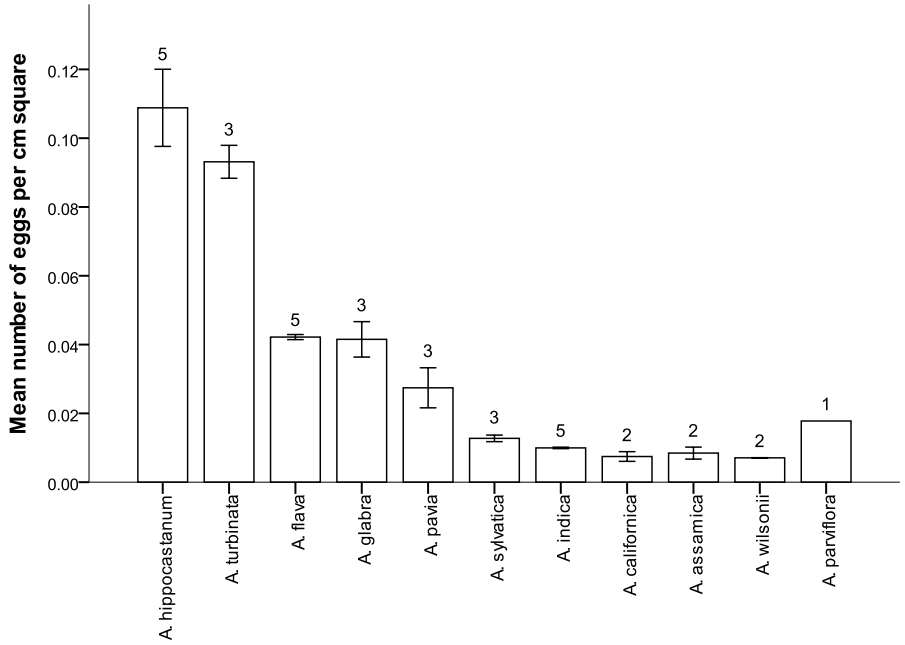


Figure 2.2 Egg density observed on different *Aesculus* species. The numbers above the bars indicate the number of individual trees sampled. Error bars represent +/- 1 SE

Mines of *C. ohridella* were recorded on six out of the eleven studied species of *Aesculus* (Figure 2.3). All individuals of *A. parviflora*, *A. wilsonii*, *A. assamica*, *A. californica* and *A. indica* remained free from *C. ohridella* mines during the three-year period of monitoring.

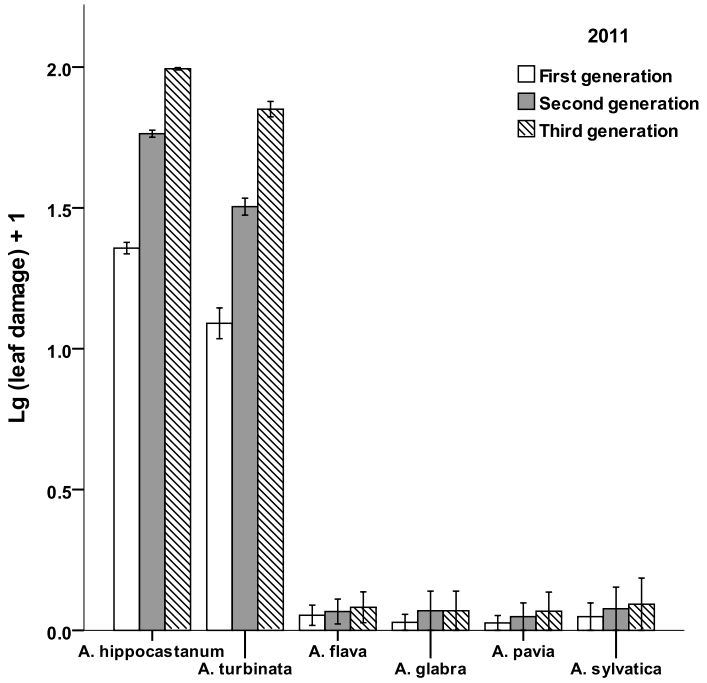


Figure 2.3 Leaf damage caused by *C. ohridella* larvae in RBG, Kew over the three generations in 2011. The data were lg transformed to aid visualisation. The error bars represent +/- 1 SE

The amount of leaf damage varied significantly among the species in all three years (2009: $H = 26.412$, $df = 5$, $p < 0.001$, 2010: $H = 25.836$, $df = 5$, $p < 0.001$, 2011: $H = 24.865$, $df = 5$, $p < 0.001$).

The most heavily damaged species were *A. hippocastanum* and *A. turbinata*. *A. hippocastanum* suffered significantly higher leaf damage than *A. turbinata* each year ($p < 0.05$). *A. pavia* and *A. sylvatica* were undamaged in 2009, but one individual of *A. sylvatica* became slightly infested in 2010 (1.1% leaf damage in the third generation) and in 2011 (0.9% leaf damage in the third generation). *A. pavia*, which was not infested in 2009 and 2010 (three individuals monitored), showed low infestation in 2011 (one tree with an infestation level of 0.6% in the third generation). Out of the four *A. glabra*

trees monitored, one was slightly infested in all three years (~1%) and three trees remained uninfested. Out of nine *A. flava* trees monitored, seven trees remained completely uninfested, whereas two trees were slightly infested each year (~ 1.2% damaged leaf area).

There was significant positive relationship between the number of eggs laid on a species and the percentage leaf area damaged by mines ($\tau = 0.786$, $n = 11$, $p < 0.01$).

Leaf damage was similar each year on *A. turbinata* ($H = 1.869$, $df = 2$, $p = 0.393$), *A. flava* ($H = 0.012$, $df = 2$, $p = 0.994$) and *A. glabra* ($H = 0.066$, $df = 2$, $p = 0.967$), but differed significantly between years on *A. hippocastanum* ($H = 15.321$, $df = 2$, $p < 0.001$) with the lowest leaf damage observed in 2009.

2.3.2 Infestation of different varieties of *A. hippocastanum*

The damage caused by the miner did not differ significantly among the varieties *A. hippocastanum* 'Baumannii', *A. hippocastanum* 'Incisa', *A. hippocastanum* 'Memmingeri' and *A. hippocastanum* 'Pyramidalis' in any of the studied years (2009: $H = 0.117$, $df = 3$, $p = 0.990$; 2010: $H = 0.450$, $df = 3$, $p = 0.930$; 2011: $H = 0.620$, $df = 3$, $p = 0.892$).

2.3.3 Infestation of hybrids of *Aesculus*

Out of the 10 different hybrids monitored, only *A.* × *dallimorei* and *A.* × *carnea* 'Plantierensis' were found to be infested. Infestation levels on these two hybrids increased gradually from spring to autumn. The remaining hybrids remained mine free during the three year monitoring period.

2.3.4 Comparison of infestation patterns in Kew, Wisley and Hillier

A. hippocastanum, *A. turbinata*, *A. flava* and the hybrid *A.* × *carnea* 'Plantierensis' were found to be infested by *C. ohridella* in all three gardens (Figure 2.4). Infestation levels of *A. hippocastanum* were highest in Hillier (48% and 86% leaf damage in the first and second generations, respectively) followed by Kew (30% and 57% leaf damage in the first and second generations, respectively) and Wisley (7% and 17% leaf damage in the first and second generations, respectively). Infestation levels were higher in Kew than at Hillier and Wisley for *A. turbinata*. Leaf damage of *A.* × *carnea* 'Plantierensis' was higher in Kew than in the other two gardens. In contrast, leaf tissue damage of *A. flava* was highest at Wisley.

A. glabra, *A. sylvatica* and *A. pavia* were only infested at Kew, whereas *A.* × *bushii* was only infested at Wisley in the second generation (~ 0.5% leaf damage). *A.* × *dallimorei*, which was only monitored in Kew and Hillier was found to be infested in both of these locations (Hillier 36% and 81%, Kew 14% and 35% leaf damage in the first and second generation, respectively).

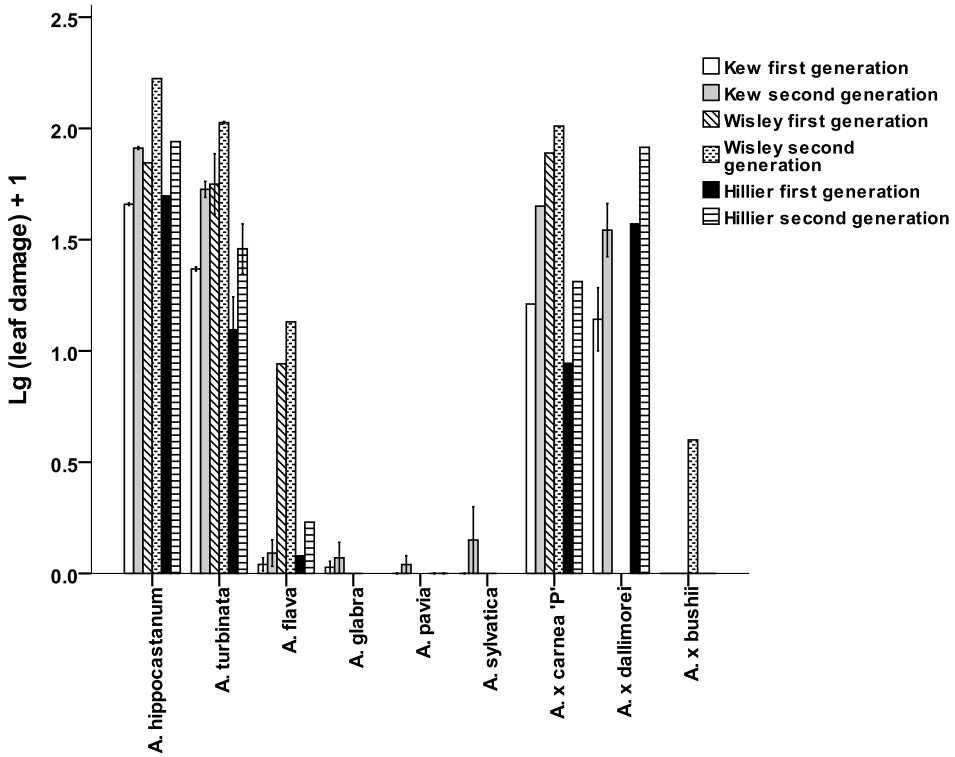


Figure 2.4 Comparison of infestation patterns of species and hybrids of *Aesculus* by *C. ohridella* at Kew, Wisley and Hillier botanical gardens in 2011. The data were lg transformed to aid visualisation.
The error bars represent +/- 1 SE

2.4 Discussion

2.4.1 Oviposition patterns of *C. ohridella* in RBG Kew

C. ohridella laid eggs on all 11 studied species of *Aesculus*, with a greater density of eggs on *A. hippocastanum* and *A. turbinata* than on any other species. Although there was a positive correlation between egg densities and infestation levels per species, *C. ohridella* females laid eggs on 5 *Aesculus* species that did not support larval development. This suggests poor link between oviposition preference and larval performance.

It is possible that females make oviposition decisions to maximise their realised fecundity instead of the performance of their progeny (Forister *et al*., 2009). Another possible explanation for the poor host discrimination by ovipositing adults is that females of *C. ohridella* may not be able to differentiate among some of the species of *Aesculus* and hence they oviposit on less suitable hosts. Insects often oviposit on plants which are chemically similar to suitable ones (Jaenike, 1990), which could be the case for *C. ohridella*. These 'oviposition mistakes' can offer an opportunity for host shifts or can select against less specific females (Thompson and Pellmyr, 1991).

Other factors which could influence adult oviposition decision are the quality and abundance of the host, inter- and intraspecific competition, natural enemies (Sugiura *et al*., 2007), physical traits, phenology and combinations of the above factors (Larson, 2002). Although adult oviposition choice can be influenced by parasitoids and predators (Videla *et al*., 2006), it is unlikely to be the case for *C. ohridella*, because natural enemies cause very little mortality of this species and the miner lacks specialist parasitoids and predators (Grabenweger, 2004; Grabenweger *et al*., 2005b). Temporal variation in host quality can also influence adult choice (Gripenberg *et al*., 2010), although Béguinot (2009) has shown that *C. ohridella* females show very low leaf selectivity on *A. hippocastanum*. Females of *C. ohridella* however, could be affected by intraspecific competition during the later generations.

The oviposition preference and performance of *C. ohridella* was not studied in detail, as larval performance was not measured in the present study. Therefore, a more detailed study is required to further investigate the preference and performance link of *C. ohridella* which will be described in Chapter 3.

2.4.2 Infestation patterns of species of *Aesculus*

The results indicated that the highest rates of damage caused by *C. ohridella* occurred on *A. hippocastanum* and *A. turbinata*. These species are of different geographic origin (Europe and Japan, respectively), but belong to the same section *Aesculus* of the genus *Aesculus* (Forest *et al*., 2001). No mines were found on any of the species belonging to sections *Calothyrsus* (*A. assamica*, *A. indica*, *A. wilsonii* and *A. californica*) and *Macrothyrsus* (*A. parviflora*), which are of Asian and/or North-American origin and which appear to be completely resistant to the miner (Straw & Tilbury, 2006). Species in the section *Pavia* (*A. pavia, A. sylvatica, A. glabra* and *A. flava*) of North-American origin, showed lower susceptibility to the miner, as compared to the section *Aesculus*. Some individuals in the section *Pavia* were slightly infested, while others were not. Therefore, the results indicate that host suitability for *C. ohridella* is not related to the continental origin of the tree species, but is linked instead to the phylogeny of the genus *Aesculus* as suggested by Straw & Tilbury (2006). This suggests that traits that determine susceptibility and resistance of the species are also likely to be linked to the phylogeny of the genus.

The infestation pattern of *C. ohridella* on different species of mature *Aesculus* trees growing at the botanical gardens are in accordance with results reported by Kenis *et al*. (2005), in that *A. hippocastanum* and *A. turbinata* are the most suitable hosts for *C. ohridella* and the leaf miner can develop on *A. flava*, *A. glabra* and *A. sylvatica*, whereas *A. californica*, *A. parviflora*, *A. assamica*, *A. wilsonii* and *A. indica* are unsuitable hosts. Kenis *et al*. (2005) also found that *A. pavia* is unsuitable for larval development, but during our monitoring we observed successful larval development on one tree in 2011, although the observed infestation was very low (0.6%). Also infestation slightly built up in 2010 and 2011 on *A. sylvatica*, a species which was uninfested in 2009 at RBG, Kew. This could indicate that *C. ohridella* slowly adapts to its new hosts. In contrast, the results did not entirely support the findings of De Prins & De Prins (2001), who reported that *A. californica* and *A. indica* were slightly infested, as these species were found to be completely resistant. Differences in susceptibility between the individual trees within the same species could be due to genotypic differences as well.

It might be expected that infestation would build up gradually on less suitable hosts, as the generations progress during the year, due to a lack of available space on the leaves of more suitable host plants nearby (by the end of the second generation, up to 80% of the foliage of *A. hippocastanum* at Kew was damaged by mines). However, infestation on *A. flava*, *A. glabra*, *A. sylvatica* and *A. pavia* remained low throughout the season in each year of the study, even though mine densities increased rapidly on *A. turbinata* and *A. hippocastanum*.

There were annual differences in infestation levels during our three year study period. The infestation was highest in 2010 and lowest in 2011. Additional monitoring in 2012 at RBG Kew revealed changes in infestation pattern and intensity. This could be due to the adverse weather experienced in the spring at this location.

2.4.3 Infestation patterns of hybrids

Out of the eleven monitored hybrids only two (*A.* × *dallimorei* and *A.* × *carnea* 'Plantierensis') were found to be heavily infested by *C. ohridella*. In addition, one *A.* × *bushii* tree, was found to be slightly (~ 0.5% leaf damage) infested in the second generation at RHS Wisley, but neither at Hillier Arboretum or at RBG Kew during the three-year monitoring period.

A. × *dallimorei* is a cross between the susceptible *A. hippocastanum* and the intermediately resistant *A. flava*. Susceptibility seems to be a dominant trait inherited from *A. hippocastanum*. No infestation was recorded on *A.* × *carnea*, (hybrid between the susceptible *A. hippocastanum* and the resistant *A. pavia*), suggesting that resistance is dominant in this case. Resistance of backcrosses is often found to be intermediate to the parents and the breakdown of resistance could also take place (Fritz *et al*., 1999), as it was the case with *A.* × *carnea* 'Plantierensis' a backcross between *A.* × *carnea* and *A. hippocastanum.*

Overall, the results of this study in regards to hybrid susceptibility are in accordance with those of reported by Straw & Tilbury (2006), with the exception of *Aesculus* × *glaucescens*, which was found to be resistant in this study but reported to be susceptible by Straw & Tilbury (2006).

In light of the present study, it is advisable to plant hybrids that are a cross between two resistant species as ornamental trees, as the study revealed that susceptibility of one of the parent species can be inherited as a dominant trait, thus resulting in hybrids which are prone to *C. ohridella* infestation.

2.4.4 Infestation patterns at RHS Wisley and Hillier Arboretum

Infestation patterns in regards to which species are susceptible and resistant were almost identical in the three botanical gardens (Kew, Wisely and Hillier), which suggests that resistance ranking of different *Aesculus* species is robust and independent of locality. There was, however, variation in mine densities among the three different locations, but none of the botanical gardens had consistently higher or lower densities for all examined species, which could be due to genotypic differences of host plants. Location and the distance of nearby *A. hippocastanum* trees could also result in varying infestation levels.

2.5 Conclusion

Overall, the study found that oviposition choice of *C. ohridella* is not perfect, as the miner lays eggs on species of *Aesculus* where larvae cannot develop. An explanation for this result could be that *C. ohridella* did not coevolve with most of the tested species. However, there are also cases where the lack of a co-evolutionary history makes it possible for non-native pests to colonize the novel hosts, as it could lack defences against the novel herbivore (Desurmont *et al*., 2011). Therefore, it is difficult to tease apart these factors and usually there is no single factor that determines the degree of host specificity (Thompson & Pellmyr, 1991), but rather the combination of multiple factors, such as herbivore and host genetics, insect behaviour, host plant quality, micro environment, interspecies interactions and macroevolution.

Further work is needed to: 1), identify factors that influence the egg laying behaviour of *C. ohridella* and to understand why the horse-chestnut leaf miner oviposits on host of poor quality and 2), determine what traits influence successful larval development and how these relate to the phylogeny of *Aesculus*.

In the light of the present findings, it is advisable to use species from the section *Calothyrsus* for future planting in parks and avenues. *A. indica* has very similar aesthetic properties to *A. hippocastanum*; therefore it would be a suitable candidate to replace *A. hippocastanum* trees in the future.

Chapter 3

The role of leaf traits of different species of *Aesculus* in determining oviposition preference and larval performance of *Cameraria ohridella*

3.1 Introduction

According to the preference-performance hypothesis, females of phytophagous insects oviposit on high quality plants where their offspring perform best (Jaenike, 1978; Videla *et al*., 2006; Gripenberg *et al*., 2010). This is especially important for leaf mining larvae as they have to feed on the leaf selected by the ovipositing female (Valladares & Lawton, 1991). However, a weak preference-performance linkage is often observed when the herbivore lacks coevolutionary history with the host plant (Jaenike, 1990; Thompson & Pellmyr, 1991; Kagata & Ohgushi, 2001; Gripenberg *et al*., 2010; Pearse, 2011).

Based on recordings of leaf tissue damage and egg density assessment on different species of *Aesculus* in botanical gardens, it was shown that the preference-performance linkage of *C. ohridella* is far from perfect, as females lay eggs on species of *Aesculus* where larvae do not develop (Chapter 2, D'Costa *et al*., 2013). However, larval performance in this case was only indirectly measured by the degree of leaf damage. Moreover, oviposition should ideally be studied in conditions when the plants are presented to females

in equal abundance and availability (Thompson, 1988), which is not the case at Kew Gardens (Chapter 2), where relative abundance and specific location of various species of *Aesculus* differs. Therefore, the present Chapter examines the preference-performance relationship of *C. ohridella* more closely, using an experimental set up. In addition, it investigates how different leaf traits relate to resistance and susceptibility to *C. ohridella*, as no studies have yet investigated this.

Different plant traits are known to influence herbivore preference and performance (Kessler & Baldwin, 2002, Pearse & Hipp, 2009). Chemical and physical traits not only influence the performance of the developing larvae, but also may act as recognition cues for ovipositing females (Ishino *et al*., 2011). Upon landing, females come in contact with physical and chemical leaf traits and some of these traits could influence oviposition choice.

Plant recognition in insect herbivores is often governed by secondary metabolites. However, water content and nutritional quality and structural defences also affects female decision making (Honda, 1995; Scheirs *et al*., 2003).

Leaf toughness, secondary metabolites or suboptimal nutrients can have negative effects on herbivores. The variations in physiological traits such as water and nitrogen content or phenological and morphological traits can influence plant susceptibility and resistance (Kimmerer & Potter, 1987; Ishino *et al*., 2011). Low plant quality can negatively affect growth rate and fecundity

and it could result in prolonged larval development, which increases the risk of mortality by parasitoids and predators, also known as the 'slow growth high mortality hypothesis' (Cornelissen & Stiling, 2006). Also, these traits can be important mechanistic determinants of herbivory across species (Pearse & Hipp, 2009).

Specialist herbivores who coevolved with their host can adapt to resistance traits present in their hosts, such as high levels of secondary metabolites, tough leaves or low nitrogen levels (Larson, 2002). Although specialist insects evolved mechanisms to detoxify plant secondary metabolites from their hosts, they usually have decreased capacity to detoxify novel plant secondary metabolites if they come in contact with novel hosts (Sorensen *et al*., 2005). Therefore, in most cases the herbivore will experience reduced fitness when interacting with a novel host. For *C. ohridella*, the European *A. hippocastanum* can be considered a native host, as *A. hippocastanum* is the main host of *C. ohridella* in the Balkans, from where the leaf miner originates (Lees *et al*., 2011). Also, *A. hippocastanum* has been present in Europe since the 16th century. On the other hand, other species of *Aesculus*, which have a different continental origin, are novel to *C. ohridella* as they share no evolutionary history.

Sometimes similarity of traits of non-native hosts to the original host can result in higher herbivory (Pearse & Hipp, 2009). In order for a herbivore to establish itself on a novel host, traits that determine oviposition and traits that determine larval performance are equally important. Trait similarity of novel hosts which

determine female preference to native ones can result in higher egg densities. However, if traits that determine larval performance are not suitable for the herbivore, colonisation of the novel host will not take place. On the other hand, if the novel host are similar in traits that determine larval performance to natives, but differ in traits that determine oviposition, will result in a scenario where the herbivore will fail to recognise the novel plant as a potential host and thereby will not establish high population densities, even though the novel host would be otherwise suitable for larval survival and optimal development.

To investigate the role of leaf traits of different species of *Aesculus* in determining oviposition preference and larval performance of *C. ohridella*, a choice experiment was carried out using five different species of *Aesculus*. Oviposition preference was assessed by egg density per unit area on the different species and performance was assessed by larval survival and pupal weight.

Physical characteristics such as leaf tissue thickness and toughness, and chemical characteristics such as total phenolics, free amino acids and total carbohydrate content of the different species were compared.

The study addressed the following questions:

1) To what extent *C. ohridella* oviposition preference among species of *Aesculus* corresponds to larval performance?

2) Which physical and chemical plant traits determine oviposition preference and larval performance?

3) Do more closely related *Aesculus* species show higher similarity in physical and chemical leaf traits than more distantly related species?

3.2 Materials and methods

3.2.1 Study plants and experimental design

Saplings of *A. hippocastanum, A. indica, A. chinensis, A. flava* and *A. turbinata* were used in this experiment and were obtained from Mallet Court Nursery (Taunton). The saplings were grown in 30 cm diameter pots and they were of approximately 1 meter in height. These species were chosen as they belong to different phylogenetic sections within the genus *Aesculus* and are from different geographic origins. *A. hippocastanum* and *A. turbinata* (both belonging to the section *Aesculus*) were found to be susceptible as described in Chapter 2, whereas, Asian species *A. indica* and *A. chinensis* (both belonging to the section *Calothyrsus*) were found to be resistant to the miner (Chapter 2).

The North American species *A. flava* (section *Pavia*) has both susceptible and resistant genotypes.

The experiment was carried out at Royal Holloway University of London (RHUL) from May-July 2011. Ten saplings per species were used for the experiment. Ten small greenhouses (approximately 1×2×2 meter) were erected in two rows (Figure 3.1). One sapling of each species was placed into each greenhouse in such a way that the position of each sapling varied relative to the other saplings from one greenhouse to the other.

Figure 3.1 Greenhouses used for the experiment

3.2.2 Oviposition and larval development

Newly emerged adult moths were collected from mature *A. hippocastanum* trees at RHUL and 40 individuals were introduced into each greenhouse. The sex ratio of the adult moths was not known, but it was assumed that roughly equal numbers of males and females were released at the same time. The adult moths were left to mate and oviposit for six days prior to leaf sampling.

Six days after the start of the experiment, 5 leaflets were randomly removed from each sapling (50 leaflets per species) and the number of eggs present on the upper surface of the leaflets was counted using a digital microscope (× 400 magnification). To account for the differences in the leaf size of different species of *Aesculus*, average leaf area of each species was measured (10 leaves per species) using ImageJ software (Abramoff *et al*., 2004) and egg counts were then transformed to egg densities per cm^2 of leaf area.

Mine development started approximately two weeks after oviposition. Mine numbers (>1mm) were counted on each leaf as the larval development progressed and expressed per unit of leaf area. Once the first emerging adults were noticed, leaves were collected and the mines were dissected.

Pupae from each sapling were collected and pupal weight of 100 pupae from *A. hippocastanum* (10 pupae per sapling) and 100 pupae from *A. turbinata* (10 pupae per sapling) were compared. These were the only two species on which successful pupation occurred.

3.2.3 Physical characteristics

Leaf thickness and leaf toughness were measured on ten leaves of each sapling during May 2011. Thickness was measured using a digital micrometer and toughness was measured using a Dial Tension gauge (Mitutoyo, Japan). The

measurements were standardised by restricting them to the middle section of the middle leaflet of each compound leaf.

3.2.4 Chemical characteristics

Five leaflets from each sapling were removed in May 2011 prior to oviposition by *C. ohridella* adults, and were immediately frozen at -20° C. The leaf samples were then lyophilized and homogenized to powder which was later used for the determination of chemical characteristics as described below.

3.2.4.1 Carbohydrate analysis

Homogenised plant material (20 mg per sample) was extracted in 80% ethanol (1 ml) three times and the pooled extract was centrifuged at 10000 rpm for 10 minutes and the clear solution was then carefully removed with a pipette and taken to dryness and redissolved in 80% ethanol (1 ml). 0.5 ml of this extract was again taken to dryness and redissolved in double distilled water (0.5 ml). This extract was filtered through a 45 µ nylon filter and the obtained extract was used for the total carbohydrate analysis.

Total carbohydrate concentration was determined by the phenol-sulphuric acid method described by Masuko *et al*. (2005) and analysed by Tecan (infinite M200) spectrophotometer using Tecan i-control 1.5 software. A calibration curve was prepared using glucose (Sigma Chemical Co.) as standard

(Appendix 1). All samples, standards and blanks were prepared and run in triplicates and their absorbance was measured at 490 nm.

3.2.4.2 Free amino acid analysis and total phenolics

Homogenous plant material (10 mg per sample) was extracted in 80% methanol (1 ml) three times and the pooled extract was centrifuged at 10000 rpm for 10 minutes and the clear solution was then carefully removed with a pipette and taken to dryness and redissolved in 80% methanol (1 ml). The resulting extract was filtered through a 45 µ nylon filter and this extract was used for the determination of the free amino acid and phenolic content.

Free amino acid concentration was determined by the ninhydrin method and analysed by a Tecan (infinite M200) spectrophotometer using Tecan i-control 1.5 software. A calibration curve was prepared using L-glutamine (Sigma Chemical Co.) as standard (Appendix 1). All samples, standards and blanks were prepared and run in triplicates. To 30 µl sample (in 96-well plate) 60 µl double distilled water and 60 µl ninhydrin solution was added and the mixture was heated at 90° C for 10 min. The absorbance of the samples was then measured at 570 nm.

The total phenolic content was determined by the Folin-Denis method and analysed by a Tecan (infinite M200) spectrophotometer using Tecan i-control 1.5 software. A calibration curve was prepared using chlorogenic acid (Sigma Chemical Co.) as standard (Appendix 1). All samples, standards and blanks

were prepared and run in triplicate. To 10 µl sample 90 µl double distilled water and 50 µl Folin-Denis solution was added and mixed thoroughly. After 3 min, saturated sodium carbonate (50 µl) was added and after 15 min incubation time the absorbance of the samples was measured at 690 nm.

3.2.5 Statistical analyses

Statistical analyses were conducted using SPSS version 18.0 and R software version 2.15.2 (R Development Core Team, 2012), with packages *AICcmodavg* and *pgirmess*. Differences in oviposition preference and larval performance between *Aesculus* species were analysed by the non-parametric Kruskal-Wallis' test. Multiple pairwise comparisons were carried out using the Mann-Whitney test with Bonferroni's adjustment. To compare the differences between weights of pupae collected from *A. hippocastanum* and *A. turbinata* independent samples t-test was used.

Differences in leaf traits between species were analysed by ANOVA. When assumptions of normality were not met, log transformation was applied to improve normality of distribution. To analyse which leaf traits contribute most to differences between species, all traits were then used in Principal Component Analysis (PCA). To test the contribution of each trait to oviposition preference; linear models with egg density as a response variable and each individual trait as a predictor were used.

Multiple regression was used to test for the influence of each trait on log transformed egg density. The corrected Akaike information criterion (AICc) weights (Hurvich and Tsai, 1989), were used to identify and remove non-influential traits. A set of 32 models was compiled, representing all trait combinations, from one to five traits per model, and AICc weights were calculated for each model. The model with the lowest AICc weight was retained. Model estimates and corresponding SE were reported for those traits retained in this model (Pearse, 2011). The influence of each trait was assessed in terms of the sum of the AICc weights of all models that included this particular trait.

3.3 Results

3.3.1 Oviposition and larval development

C. ohridella females oviposited on all the species used in this experiment, but the number of eggs differed significantly among species (H_4 = 136.9, $p < 0.001$, Figure 3.2).

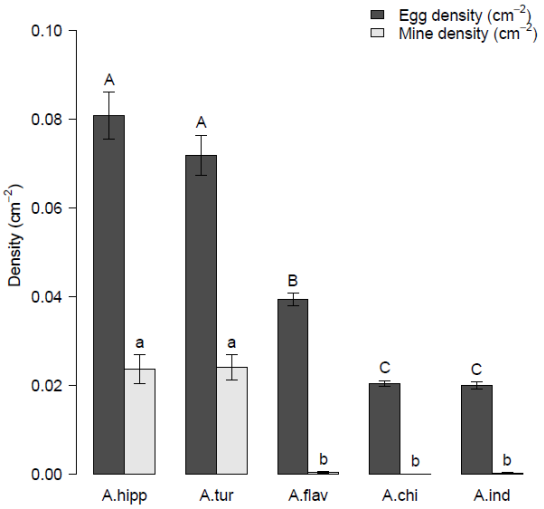


Figure 3.2 Egg and mine density on the different species per cm^2. Letters above bars indicate significant differences. Error bars represent +/- 1 SE. *A. hippocastanum* (A. hipp*), A. turbinata* (A. tur), *A. flava* (A. flav), *A. chinensis* (A. chi) and *A. indica* (A. ind)

A. hippocastanum and *A. turbinata* were the most preferred species. *A. flava* was an intermediate in terms of preference, while *A. chinensis* and *A. indica* were the least preferred by ovipositing females.

There were also significant differences in mine densities among the different species of *Aesculus* (H_4= 39.30, p < 0.001, Fig. 3.2). Larvae could only develop on *A. hippocastanum* and *A. turbinata*. A few mines were observed on *A. flava*, *A. indica* and *A. chinensis*. However, larvae died in an early stage on these species and no pupation occurred. The mean weight of pupae reared from *A. hippocastanum* (1.52 ± 0.020) and *A. turbinata* (1.53 ± 0.022) did not differ significantly (two-tailed value of p> 0.05), indicating that performance of *C. ohridella* was similar on these two species.

3.3.2 Leaf traits

There were significant differences in all the leaf traits measured among the different species (phenolics: $F_{4,45} = 11.43$, $p < 0.001$, amino acids: $F_{4,45} = 17.14$, $p < 0.001$, carbohydrates: $F_{4,45} = 6.48$, $p < 0.001$, toughness: $F_{4,45} = 21.12$, $p < 0.001$ and thickness: $F_{4,45} = 18.36$, $p < 0.001$, (Figure 3.3). The leaves of *A. indica* were the thickest and had the highest amino acid content, whereas the rest of the species did not differ significantly in these two traits. *A. indica* and *A. turbinata* also had higher carbohydrate content than the other species. The phenolic content was the highest in *A. hippocastanum* and *A. turbinata*, whereas *A. indica* and *A. chinensis* had the lowest phenolic content. Leaf toughness of *A. hippocastanum* and *A. turbinata* was significantly lower than in the rest of the species.

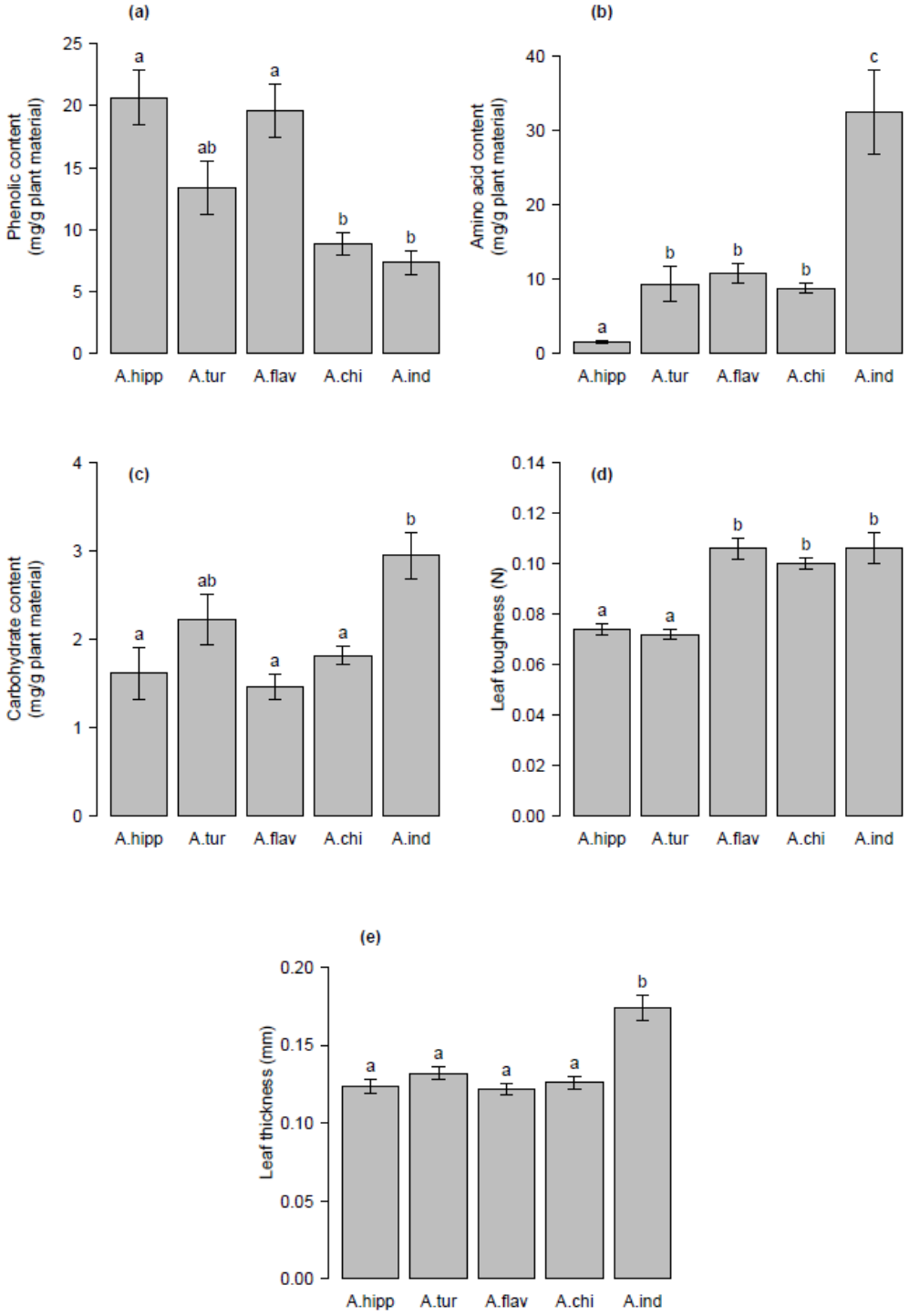


Figure 3.3 Comparison of leaf traits among the different species of *Aesculus*. Error bars represent +/- 1 SE. Letters above bars indicate significant differences. *A. hippocastanum* (A. hipp*), A. turbinata* (A. tur), *A. flava* (A. flav), *A. chinensis* (A. chi) and *A. indica* (A. ind). The mg/g plant material refers to freeze-dried plant material

Principal component analysis of the traits revealed that the first principal component axis (PC1) accounted for 49.5% of the variance in the dataset and was dominated by leaf thickness, amino acid content and phenolics (Table 3.1). This axis clearly separated *A. indica* which had significantly thicker leaves and the highest amino acid content from the rest of the species (Figure 3.4). The second principal component axis (PC2) accounted for 16.7% of the variance and was dominated by carbohydrate content and leaf toughness. This axis separated the two susceptible species *A. hippocastanum* and *A. turbinata* from *A. flava* and *A. chinensis*. There was an overlap in leaf traits between the susceptible *A. hippocastanum* and *A. flava* for which females of *C. ohridella* showed intermediate preference. The third axis (PC3) accounted for 12.3% of the variance and was dominated by leaf thickness.

Table 3.1 Contribution of traits to PC axes 1, 2 and 3

Traits	Principal component loadings		
	PC1	PC2	PC3
Thickness	0.777	0.023	0.658
Amino acid content	0.768	0.146	0.039
Phenolics	-0.716	0.105	0.264
Carbohydrates	0.651	0.552	0.316
Toughness	0.588	-0.704	0.106

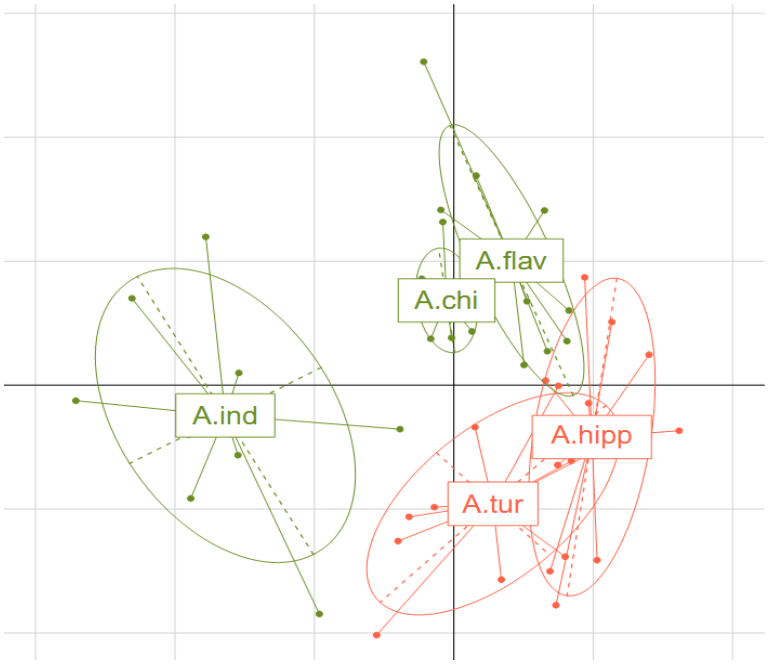


Figure 3.4 Separation of the five species of *Aesculus* according to the principal component analysis of variation in the leaf traits. *A. hippocastanum* (A. hipp), *A. turbinata* (A. tur), *A. indica* (A. ind), *A. chinensis* (A. chi) and *A. flava* (A. flav)

The best model obtained by model selection based on AICc weights retained leaf toughness, amino acid and phenolic content as the most influential traits for oviposition. The summed AICc weights of the different traits in all models are shown in Table 3.2.

Table 3.2 The best GLM model of leaf traits as predictors of egg density, based on AICc weights of all models generated by multiple regression. B = independent coefficient, SE = standard error, and *P* = the significance of each trait within the model

Traits	Egg density cm^2			
	B	SE	P	Summed AICc
Toughness	- 1.251	0.313	< 0.001	0.998
Amino acid content	- 0.166	0.071	< 0.05	0.851
Phenolics	0.246	0.123	ns	0.705
Thickness	-	-	-	0.268
Carbohydrate content	-	-	-	0.230

3.4 Discussion

3.4.1 Preference-performance linkage

The study revealed that although *C. ohridella* oviposited on all species of *Aesculus* used in this experiment, successful mine development and pupation occurred only on *A. hippocastanum* and *A. turbinata*. This experimental result is consistent with previous observational field studies (Chapter 2, D'Costa *et al*., 2013) and indicates that the preference-performance linkage of *C. ohridella* is indeed not perfect. This is often the case when a herbivore interacts with a novel host. These oviposition 'mistakes' are often observed in insects and are thought to be an important factor for expansion of the host plant range.

In this study the different species were confined to a small enclosed environment. The volatile bouquet of the different species probably influenced the oviposition behaviour of *C. ohridella*. Females could become confused by the volatile mix of the different species and therefore oviposit on species which otherwise would be rejected. On the other hand, some of the volatiles emitted from resistant species could be repellent to *C. ohridella*. However, the previous oviposition study on mature trees (Chapter 2) showed that even in botanical gardens, in the first generation, when plenty of oviposition sites are available on host plants, females still lay eggs on these species even though some of the non-host plants are not closely located to *A. hippocastanum* (e.g. the distance is more than 150 meters). This indicates that although the host plant may be more attractive to the females, other species of *Aesculus* probably do not repel them.

This study showed that the preference-performance linkage of *C. ohridella* was not perfect, as females laid eggs on species where larvae could not develop. This could be due to the fact that the traits that determine oviposition preference are different from traits that determine larval performance. It could also be that females are unable to assess some traits, which may be important for larval performance.

3.4.2 Physical and chemical traits

In most cases multiple plant traits play a role in resistance to herbivores (Agrawal, 2011), which was the case in this study. Oviposition preference by *C. ohridella* was influenced by low leaf toughness and amino acid content and high content of phenolics. Leaf toughness was not only important for female choice but was the only one trait which clearly separated the two susceptible hosts *A. hippocastanum* and *A. turbinata* from the rest of the species, suggesting that leaf toughness is a good predictor of resistance of the saplings. This was also supported by its large AICc weight (Table 3.2). Leaf toughness can cause significant larval mortality in leaf miners (Kimmerer & Potter, 1987). Newly hatched larvae of *C. ohridella* needs to burrow itself into the leaf tissue through the upper epidermis, therefore, leaf toughness possibly plays some role in resistance, reducing the likelihood of larval establishment in the leaf tissue.

The concentration of free amino acids in some of the resistant species was significantly higher than that of *A. hippocastanum* and *A. turbinata*. Within the

genus *Aesculus* there are great differences between the amino acid composition of the seeds of the different species and these differences are phylogenetically conserved. *A. turbinata* and *A. hippocastanum* have an almost identical profile, both lacking the C_6 and C_7 amino acids, which distinguish them from the remainder of the species (Fowden *et al*., 1970). Although, most studies of amino acids within the genus were carried out on seeds, it is possible that there are some differences between the leaf amino acid profiles of the species.

Phenolics are thought to play an important role in the resistance of deciduous trees against herbivores (Salminen *et al*., 2004a, Ossipov *et al*., 2001). In contrast, in this case high phenolic concentration had a positive effect on egg density. Secondary metabolites such as phenolics can be used during the host selection process and in case of specialist herbivores they can act as the so-called 'token stimuli' and hence specialist insects are not affected negatively by these plant metabolites originating from their hosts (Dicke, 2000).

Interestingly, *A. flava*, a species which was unsuitable for larval development, but for which ovipositing females showed intermediate preference, had high phenolic content, but also high leaf toughness. This again suggests that leaf toughness is an important factor which determines the ability of larvae to develop in leaves of *Aesculus* species, while leaf phenolics are likely to be important for female choice.

The two susceptible species, *A. hippocastanum* and *A. turbinata*, belong to the same section of the genus *Aesculus* L., and thus are taxonomically closely

related. They display very similar leaf traits and this might explain why larval performance on these two species was so similar. *A. chinensis* and *A. indica* are also very closely related species, belonging to section *Calothyrsus* of the genus *Aesculus*. *A. indica* had the thickest leaves and the highest level of amino acid content of all five species examined, while *A. chinensis* shared some leaf traits with *A. flava*, which belongs to section *Pavia*. This suggests that not all of the measured leaf traits match the phylogenetic relationship between the *Aesculus* species, and indicates that different species of *Aesculus* might have different mechanisms of resistance to *Cameraria*. This supports the idea of different defensive syndromes in plants (Agrawal & Fishbien, 2006).

All species except *A. hippocastanum* used in this study were novel to *C. ohridella*, and this highlights that indeed preference and performance often decouples when herbivores interact with novel hosts.

It is important to point out that this study assessed chemical traits as total concentrations of carbohydrates, amino acids and phenolics, however, the qualitative chemical profiles of the leaves are also important factors determining plant resistance to herbivores. Therefore, the subsequent chapters of this thesis focus on a more detailed analysis of secondary chemistry profile of the species (e.g. phenolic and saponin content) which could shed light on compounds that could influence the development of *C. ohridella* larvae.

Chapter 4

Hybridisation of *Aesculus hippocastanum* and its effects on leaf traits and susceptibility to *Cameraria ohridella*

4.1 Introduction

Host plant hybridisation is important for understanding mechanisms of plant-herbivore interactions. It provides an opportunity for a herbivore to expand its host range, as hybrids can 'bridge' the genetic gap between actual and potential host species (Floate & Whitham, 1993; Pilson, 1999). Comparing herbivore preference and performance of hybrids with that of the parent species, could help shed light on potential resistance mechanisms (Nahrung *et al*., 2009).

There are four possible outcomes of hybridisation in respect to herbivory: 1) no difference from their parent species, 2) hybrid susceptibility (either through dominance of susceptibility or by the hybrid being more susceptible than either parent species), 3) hybrid resistance (arising through dominance of a resistant parent or from a hybrid which is more resistant than either of the parent species and 4) the hybrid is intermediate between the parent species in terms of herbivory.

Partial dominance can also be expressed, as well as overdominance of susceptibility and resistance (when resistance or susceptibility is greater than that of both parents), (Fritz *et al*., 1997; Fritz *et al*., 1999; Nahrung *et al*., 2009; Cheng *et al*., 2011).

Backcross plants present smaller 'bridges' to herbivores as they contain more of the original host's genome than F_1 hybrids (Floate & Whitham, 1993; Pilson, 1999). When F_1 hybrids are backcrossed with a pure parent, resistance is often found to be intermediate. However, breakdown of resistance or dominance can also take place with backcross hybrids (Fritz *et al*., 1999).

Hybridisation facilitates the transfer of traits between species (Orians, 2000), and thus hybrids often differ from their parent species in morphology, phenology, chemistry and life history, and can also sometimes display novel traits, therefore directly affecting herbivores (Fritz, 1999; Yarnes *et al*., 2008; Cheng *et al*., 2011). Differences in resistance to a herbivore of hybrids and parent species depends on the expression of these various plant resistance traits and their interactions.

Plant secondary metabolites often play a role in the resistance of hybrid plants and also hybridisation can result in the generation of secondary metabolites that are not present in either of the parent species (Cheng *et al*., 2011). In terms of secondary metabolites, hybrids can differ qualitatively (presence or absence of compounds) and quantitatively (concentration of compounds) from their parent species (Orians, 2000).

The quantitative production of secondary plant metabolites is usually dependent on more than one gene with either dominant, over-dominant, recessive, additive or epistatic allelic effects in a locus or between loci (Cheng *et al*., 2011). In F_1 hybrids, secondary plant metabolites could be 1) similar to those found in the two parental taxa, 2) intermediate between the two parents, 3) overexpressed (i.e. found in higher concentrations than in either parent), 4) underexpressed (i.e. found in lower concentrations than is present in either parent), 5) deficient (i.e. absence of compounds which are otherwise present in both parents), 6) novel to both parent species. Usually when either of the parents produces a metabolite, it is found to be in the hybrid as well (Orians, 2000; Cheng *et al*., 2011).

Natural hybridisation is a common phenomenon in the genus *Aesculus* (De Pamphilis & Wyatt, 1990) and hybrids of *Aesculus* vary in their susceptibility to *C. ohridella* (Straw & Tilbury, 2006). Comparing leaf traits and preference and performance of *C. ohridella* on hybrids of *Aesculus hippocastanum* could shed some light on mechanisms of resistance to the miner, and this information could aid plant breeders in producing more resistant cultivars.

The present study investigates the preference and performance of *C. ohridella* and leaf traits similarities of *A. hippocastanum* (main host plant of *C. ohridella*), *A. pavia* (resistant to *C. ohridella*), *A.* × *carnea* (the hybrid between the above two species) and *A.* × *carnea* 'Plantierensis' (the backcross between *A. hippocastanum* and *A.* × *carnea*).

A. pavia is resistant to *C. ohridella* in most cases. However, there are some reports of it being slightly susceptible (Straw & Tilbury, 2006). Botanical garden surveys (Chapter 2) have shown that *A.* × *carnea* is resistant and *A.* × *carnea* 'Plantierensis' is susceptible to the miner. Also, previous studies have shown that even though *C. ohridella* oviposits abundantly on *A.* × *carnea* throughout the vegetative season, the larvae develops slowly and die in the first two larval instars, just after biting into the leaf parenchyma, and thus causing only minimal damage to the leaf tissue of this hybrid (Kukula-Mlynarczyk *et al*., 2004; Kukula-Mlynarczyk *et al*., 2006).

The survey carried out in Chapter 2 investigated only infestation patterns of species and hybrids and not actual larval performance (e.g. larval development and pupal weight). In addition, oviposition preference by females can be properly studied only in an experimental setup, where potential hosts are equally available and accessible to females, which was not the case in the botanical garden survey, where different species and hybrids of *Aesculus* were present at different densities and at various locations.

The objectives of this study were:

1) To investigate how hybridisation of *A. hippocastanum* affects preference and performance of *C. ohridella*.

2) To investigate how hybridisation of *A. hippocastanum* and *A. pavia* affects inheritance of leaf traits.

3) To investigate which leaf traits convey resistance and susceptibility to *C. ohridella*.

4.2 Materials and methods

4.2.1 Study plants

Saplings of *A. hippocastanum*, *A. pavia*, *A.* × *carnea and A.* × *carnea* 'Plantierensis' used in this experiment were obtained from Mallet Court Nursery (Taunton). They were approximately 1 m in height and planted in 30 cm diameter pots.

A. hippocastanum has white flowers, prickly fruits, resinous buds and grows up to 25 meters. *A. pavia* is a shrub native to North America and is usually 3-4 meter tall, however, sometimes it can grow up to 10-12 meters in height. It has smooth fruits, red flowers and non-resinous buds (Upcott, 1936). *A.* × *carnea* is the hybrid between *A. hippocastanum* and *A. pavia*. Its morphology is

intermediate between the parent species, with pink flowers and slightly spiny fruits and less resinous buds than that of *A. hippocastanum*. It is a tree with an average height of about 10-12 meters. *A.* × *carnea* 'Plantierensis' is a backcross between *A.* × *carnea* and *A. hippocastanum* with pink flowers and with intermediate morphology between the two parent species (Upcott, 1936; Straw & Tilbury, 2006). The flowers and leaves of the species and hybrids used in this study are shown on Figure 4.1 below.

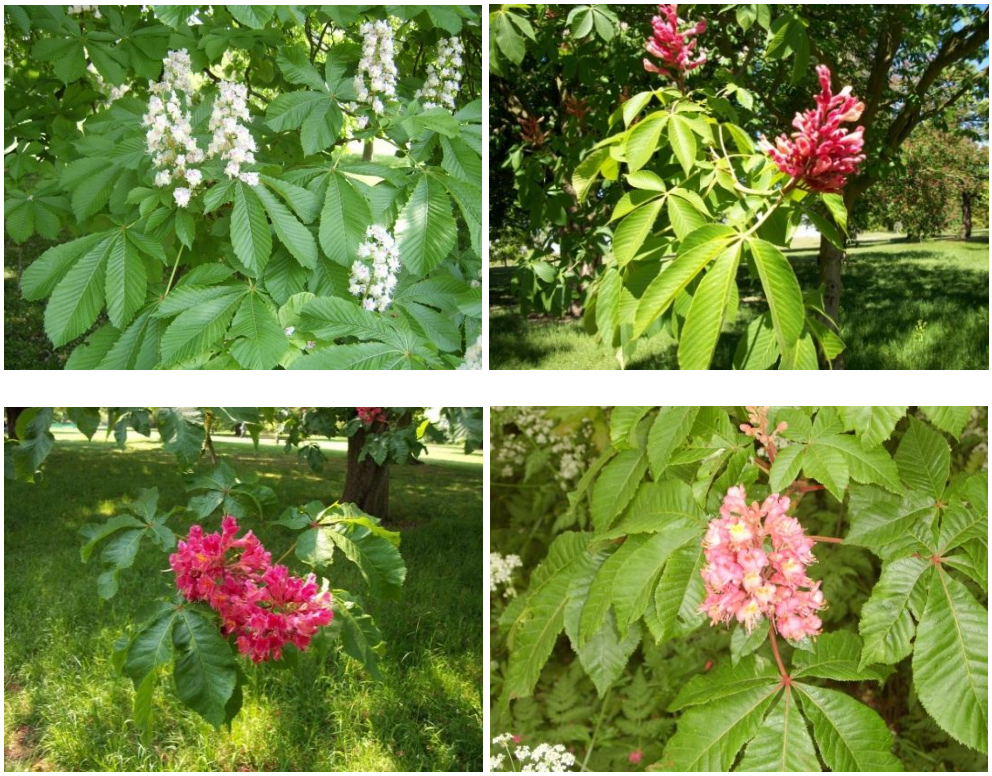


Figure 4.1 Flowers and leaves of *A. hippocastanum* (top left), *A. pavia* (top right), *A.* × *carnea* (bottom left) and *A.* × *carnea* 'Plantierensis' (bottom right)

4.2.2 Oviposition and larval development

The experiment was carried out at Royal Holloway University of London from mid-July to end of August 2011. Ten saplings per species and hybrids were used for the experiment. Ten small greenhouses (approximately 1×2×2 meter) were erected in two rows. One sapling of each species was placed into each greenhouse in such a way that the position of each sapling varied relative to the other saplings from one greenhouse to the other.

Pupae from the first generation of *C. ohridella* were collected from mature *A. hippocastanum* trees in RBG, Kew and kept in cardboard boxes. When adults emerged, they were collected and introduced in each greenhouse (50 individuals per greenhouse). The sex ratio of the adult moths was not known, but it was assumed that both males and females were present among 50 individuals released into each greenhouse. Moths were left to mate and oviposit for 6 days prior to leaf sampling.

Six days after the start of the experiment, 5 middle leaflets were sampled from 5 randomly selected leaves per each sapling (50 leaflets per species) and the number of eggs present on the upper surface of the leaflets was counted using a digital microscope (×400 magnification).

To account for the differences in the leaf size of different species and hybrids of *Aesculus*, average leaf area of each species was measured for 10 leaves per

species using ImageJ software (Abramoff *et al*., 2004) and egg counts were then transformed to egg densities per cm^2 of leaf area.

Mine development started approximately two weeks after oviposition. Mine numbers (>1mm) were counted on each leaf as the larval development progressed. Mine number was then calculated per unit of leaf area.

Successful pupation only occurred on *A. hippocastanum* and *A.* × *carnea* 'Plantierensis'. The first emerging adults were noticed on *A. hippocastanum* ten days earlier than on *A.* × *carnea* 'Plantierensis'. When adults started to emerge from *A.* × *carnea* 'Plantierensis' as well, all mines were dissected and the number of emerged adults, pupae and last instar larvae were counted and compared between *A. hippocastanum* and *A.* × *carnea* 'Plantierensis'. Pupae were sexed as described by Freise & Heitland (1999). Pupal weights of 50 males and 50 females reared from *A. hippocastanum* and *A.* × *carnea* 'Plantierensis' were measured and compared.

4.2.3 Physical characteristics of leaves

Leaf thickness and toughness measurements were carried before adult release. Measurements were taken from all ten saplings of each species, with ten leaves examined per sapling. From each leaf, the middle section of the middle leaflet was chosen for the measurements to standardise the procedure. Three measurements per leaf were taken. Leaf thickness was measured using a digital

micrometer. Leaf toughness was measured using Dial Tension gauge (Mitutoyo).

4.2.4 Water content and Specific Leaf Area (SLA)

Foliar water content and SLA were measured of leaf discs (2 × 2 cm) cut out from 5 leaflets per sapling. Each leaf disc was given a code number and its weight was recorded. The leaf discs were then freeze-dried, and the dry leaf material of each disc was measured once more. Percentage water content [((fresh weight-dry weight) / fresh weight) × 100] and SLA (mg dry plant material/cm^2) was recorded.

4.2.5 Leaf chemical characteristics

Leaf samples (three leaflets) were collected from each sapling prior to the release of *C. ohridella* adults and were immediately frozen (- 20° C). The leaf samples were then lyophilized and homogenized to powder which was later used for the determination of chemical characteristics as described below.

4.2.5.1 Free amino acid analysis

Homogenous plant material (10 mg) per sample was extracted in 80% methanol (1 ml) three times and the pooled extract was centrifuged at 10000 rpm for 10 minutes and the clear solution was then carefully removed with a pipette and taken to dryness and redissolved in 80% methanol (1 ml). The

resulting extract was filtered through a 45 µ nylon filter and this extract was used for the determination of the free amino acid content. The amino acid concentration was determined by the ninhydrin method and analysed by a Tecan (infinite M200) spectrophotometer using Tecan i-control 1.5 software. A calibration curve was prepared using L-glutamine (Sigma Chemical Co.) as the standard and all samples, standards and blanks were prepared and run in triplicate. To 30 µl sample (in 96-well plate) 60 µl double distilled water and 60 µl ninhydrin solution was added and the mixture was heated at 90° C for 10 min. The absorbance of the samples was then measured at 570 nm.

4.2.5.2 Phenolics

Individual phenolics were analysed by LC-MS and quantified by their UV absorbance using standard curves of reference standards. Only compounds with non-overlapping peaks were quantified in this study.

Lyophilised leaf material (10 mg) from each sapling was ground using a pestle and mortar and extracted with 80% methanol at room temperature for 24 hours and filtered through nylon filters (0.45 µm). An aliquot of 10 µl of each extract was used for LC-MS analysis. Analysis was performed using Micromass ZQ spectrometer, controlled by Mass Lynx Software (4.0). ESI-MS conditions were: desolvation temperature 450˚C; nitrogen flow rate 500 L/h; source temperature 120˚C; capillary voltage 3.4 kV; cone voltage 20.0 V; RF lens voltage 0.2 V; *m*/*z* range 250-2000; acquisition time 0.4s; inter scan delay 0.2s.

HPLC separation was achieved using Phenomenex Luna C_{18} column (3 x 150 mm; 5 µm) at 30˚C. The injection volume was 10 µl and elution rate was 0.5 ml/min with the gradient (90:0:10 to 0:90:10, water/methanol/acetonitrile+1 % formic acid from 0-20 min, 0:90:10 from 20-25 min). The column was then equilibrated in the start condition for 10 min. The UV absorbance was monitored between 210-500 nm.

4.2.6 Statistical analyses

For statistical analysis SPSS version 18.0 and R software version 2.15.2 (R Development Core Team, 2012), were used. For oviposition and mine density data the Kruskal-Wallis non-parametric test was used when comparing several groups as distribution of the data was not normal and the Mann-Whitney test was used when comparing two species, with Bonferroni's adjustment. Pupal weights of 50 males and 50 females reared from *A. hippocastanum* and *A.* × *carnea* 'Plantierensis' were compared using independent samples *t*-test.

Leaf traits were compared using ANOVA. All traits were then combined for Principal Component Analysis (PCA) to assess the relationship between the species and hybrids and trait similarities. Hierarchical clustering analysis was used to produce a phenogram of defence trait similarity and to group species by traits and a separate phenogram was generated for grouping species by egg and mine density.

4.3 Results

4.3.1 Oviposition and mine development

C. ohridella laid eggs on all species and hybrids, however, mines were only observed on *A. hippocastanum*, *A.* × *carnea* and *A.* × *carnea* 'Plantierensis'. Overall, there were significant differences in egg and mine densities across the two parent species and hybrids ($H_{(3)}$ = 53.73, p<0.001) and ($H_{(3)}$ = 32.67, p<0.001, respectively). Both egg and mine densities were greatest on *A. hippocastanum* and the backcross *A.* × *carnea* 'Plantierensis' and lowest on *A. pavia* and *A.* × *carnea* (Figure 4.2).

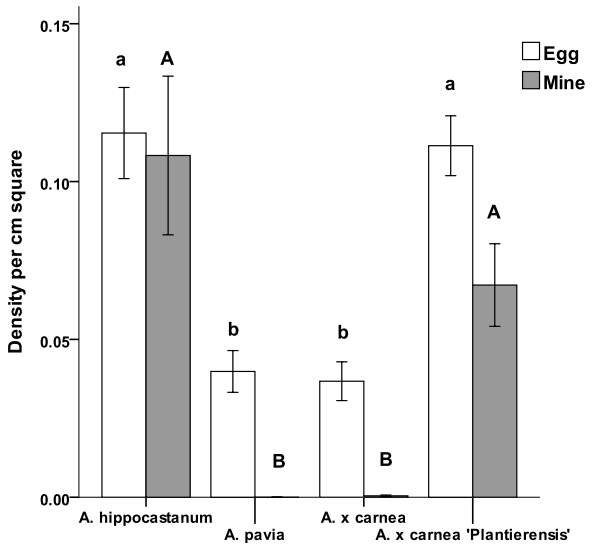


Figure 4.2 Number of eggs and mines per cm^2 on *A. hippocastanum*, *A. pavia*, *A.* × *carnea* and *A.* × *carnea* 'Plantierensis. Small letters indicate significant differences between egg densities of the species and hybrids and capital letters indicate significant differences between mine densities on the different species and hybrids. Error bars represent +/- SE

4.3.2 Larval performance

On *A.* × *carnea* mine development stopped in the early stages and mines never got bigger than 2 mm. Successful larval development and pupation only occurred on *A. hippocastanum* and *A.* × *carnea* 'Plantierensis'. Development time on *A.* × *carnea* 'Plantierensis' was longer compared to that on *A. hippocastanum*.

At the time of mine dissection the percentage of the larval stage was higher on *A.* × *carnea* 'Plantierensis' as compared to *A. hippocastanum* (Figure 4.3). Besides longer development time on the backcross, pupae reared from *A.* × *carnea* 'Plantierensis' were considerably smaller compared to pupae reared from *A. hippocastanum* (Figure 4.4) and weighed less ($p < 0.001$, Figure 4.5).

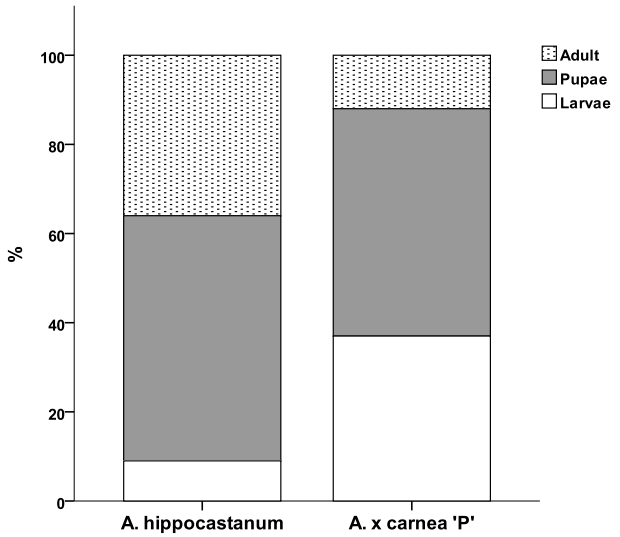


Figure 4.3 Percentage of different development stages of *C. ohridella* on *A. hippocastanum* and *A.* × *carnea* 'Plantierensis' at the time of mine dissection

Figure 4.4 Pupae reared from *A.* × *carnea* 'Plantierensis' (left) and from *A. hippocastanum* (right)

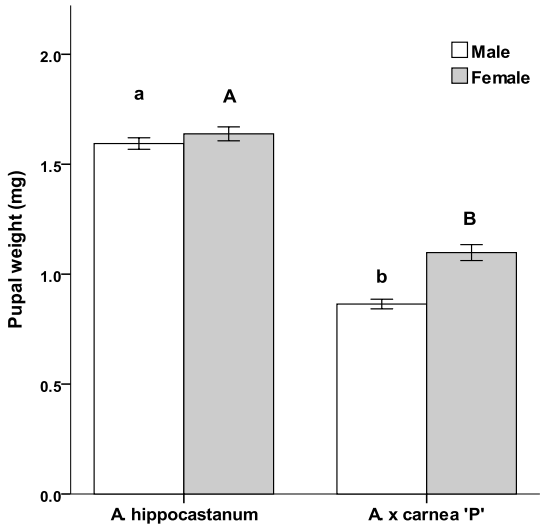


Figure 4.5 Comparison of weights (mg) of pupae reared from *A. hippocastanum* and *A.* × *carnea* 'Plantierensis'. Different letters above the bars indicate significant differences between the two hosts. Small letters indicate significant differences between the weights of male pupae, whereas, capital letters indicate significant differences between the weight of female pupae. Error bars represent +/- SE

4.3.3 Leaf traits

4.3.3.1 Qualitative differences in phenolic composition

Qualitative differences in phenolic composition between the parent species, the hybrid and the backcross are summarised in Table 4.1. Naringenin 7-glucoside and *p*-coumaroylquinic acid were present only in *A. pavia*, but were lacking in *A. hippocastanum* and the hybrids. *A. hippocastanum* also lacks glycosides of rhamnetin which are present in *A. pavia*, *A.* × *carnea* and *A.* × *carnea* 'Plantierensis'. (Typical chromatograms of the saplings are shown in Appendix 2.) The structures of the identified compounds are shown in Figure 4.6.

Table 4.1 Distribution of the detected phenolics in the parent species, hybrids and the backcross. (+) indicates the presence of a compound and (-) indicates the absence of a compound. *A. hippocastanum* (*A. h*), *A.* × *carnea* 'Plantierensis' (*A.* × *c* 'P'), *A.* × *carnea* (*A.* × *c*) and *A. pavia* (*A. p*)

Compounds	***A. h***	***A.* × *c* 'P'**	***A.* × *c***	***A. p***
Chlorogenic acid	+	+	+	+
p-coumaroylquinic acid	-	-	-	+
Flavanol 1	+	+	+	+
Flavanol 2	+	+	+	+
Epicatechin	+	+	+	+
Flavanol 3	+	+	+	+
Flavanol 4	+	+	+	+
Rutin	+	+	+	+
Naringenin 7-glucoside	-	-	-	+
Quercetin 3-*O*-α-arabinofuranoside	+	+	+	+
Quercetin 3-*O*-α-rhamnopyranoside	+	+	+	+
Kaempferol 3-*O*-α-arabinofuranoside	+	+	+	+
Kaempferol 3-*O*-α-rhamnopyranoside	+	+	+	+
Rhamnetin 3-*O*-β-galactoside	-	+	+	+
Rhamnetin 3-*O*-β-glucoside	-	+	+	+
Rhamnetin 3-*O*-α-arabinofuranoside	-	+	+	+
Rhamnetin 3-*O*-α-rhamnopyranoside	-	+	+	+

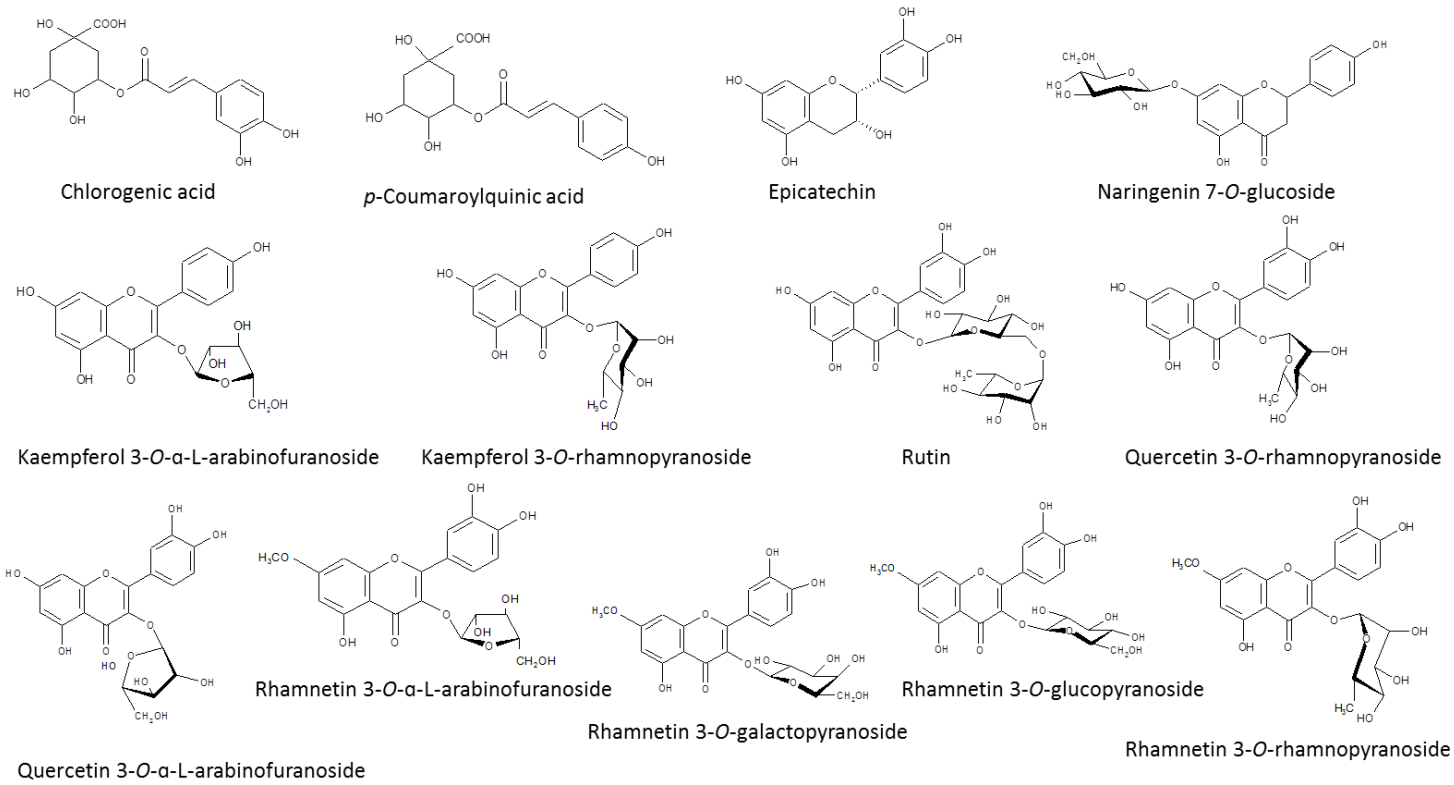
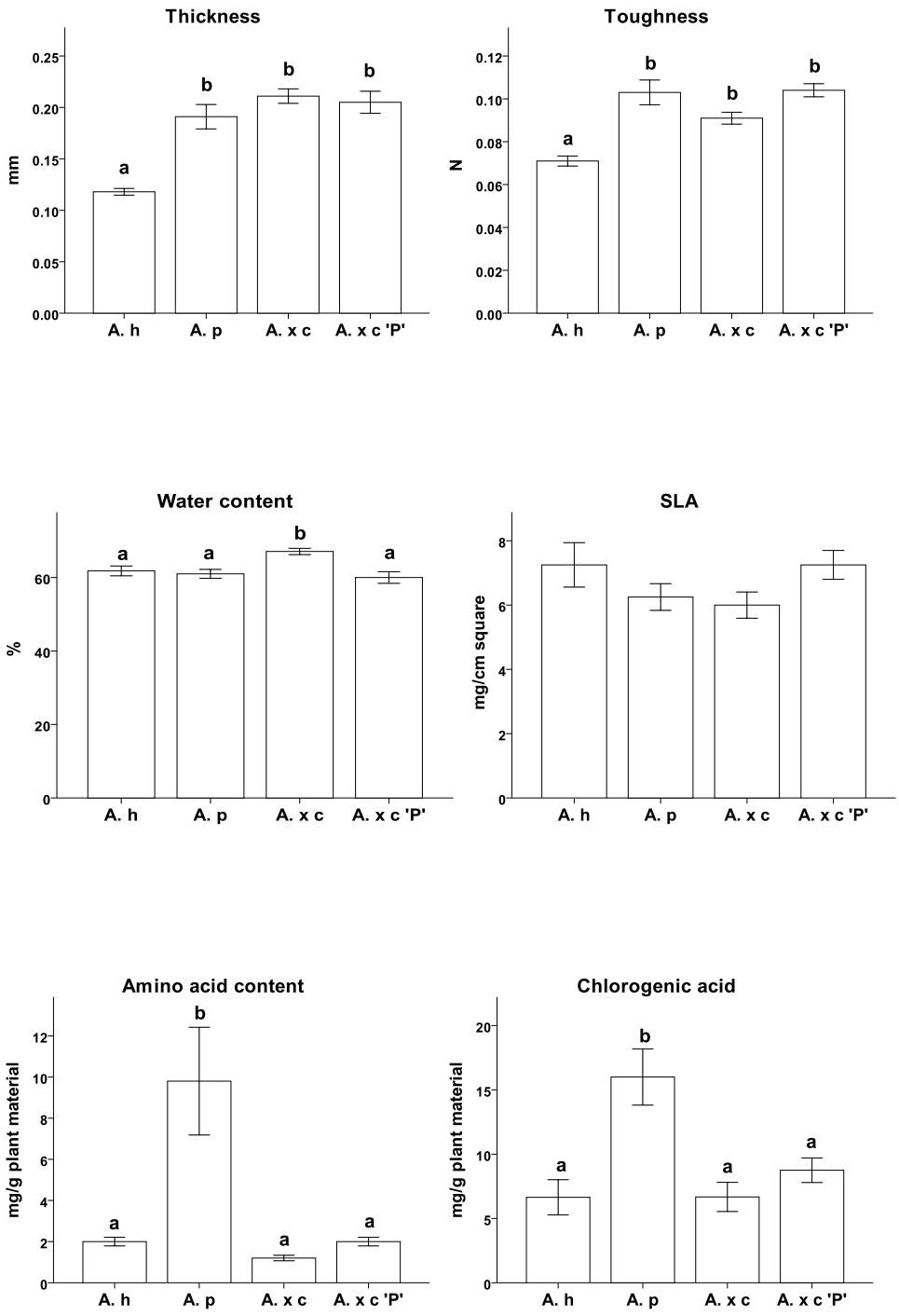


Figure 4.6 Structures of the identified phenolic compounds detected in the 80% MeOH extracts of *A. hippocastanum*, *A. pavia*, *A.* × *carnea* and *A.* × *carnea* 'Plantierenis' saplings

4.3.3.2 Quantitative differences in leaf traits

With the exception of SLA ($F_{3,36}$ = 1.69, p = 0.186) all physical and chemical traits differed significantly among the species and hybrids (Figure 4.7, p< 0.05).



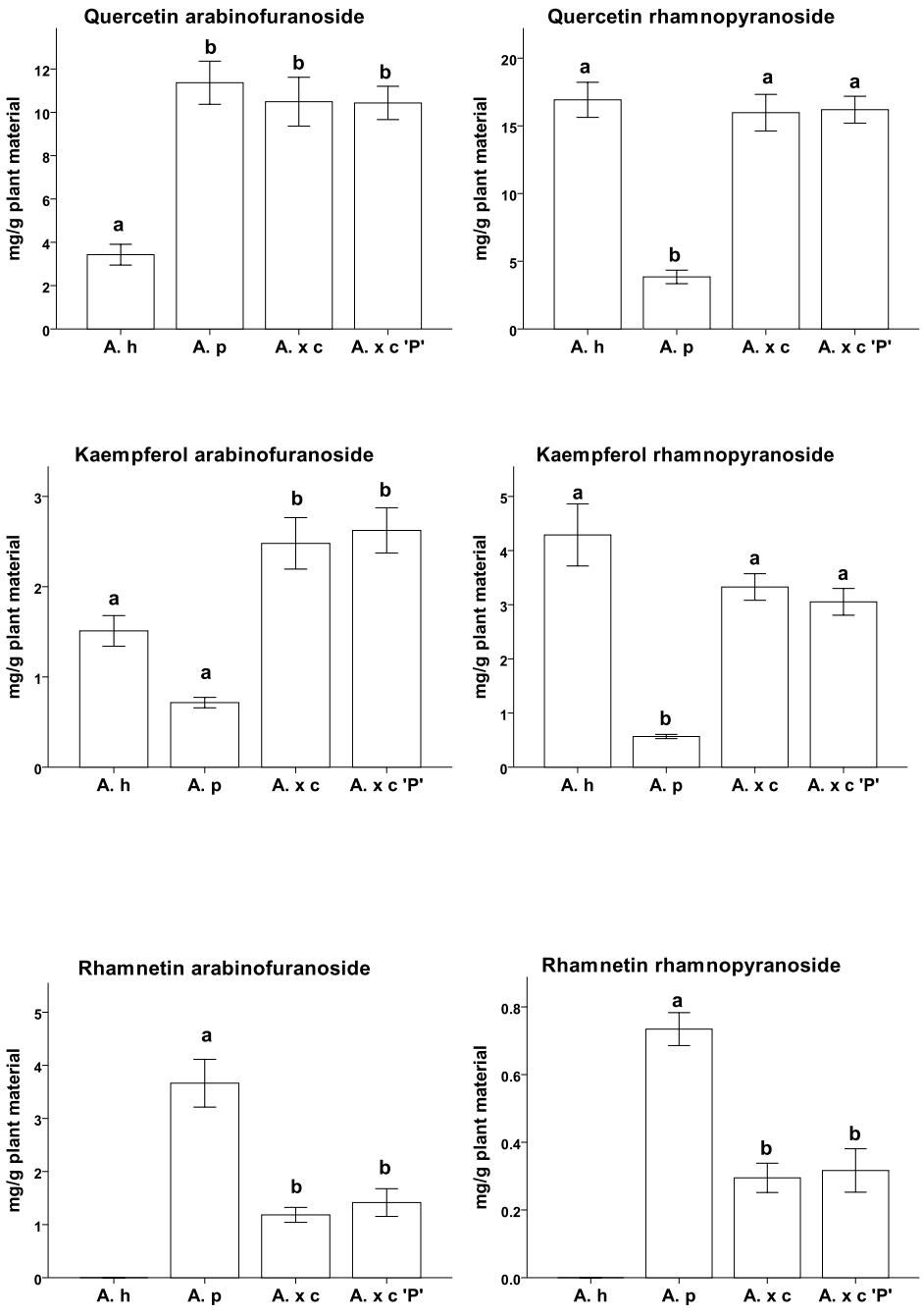


Figure 4.7 Comparison of the different leaf traits between *A. hippocastanum* (A. h), *A. pavia* (A. p), *A.* × *carnea* (A. × c) and *A.* × *carnea* 'Plantierensis' (A. × c 'P'). The letters above the bars represent significant differences among the species. Error bars represent +/- 1SE

Principal component analysis of the hybrids revealed that the first three principal component axes explained 70% of the total variance within the data

(PC1 40%, PC2 19% and PC3 11%, respectively; Figure 4.8). *A. pavia* and *A. hippocastanum* were well separated, while *A.* × *carnea* and *A.* × *carnea* 'Plantierensis' were grouped together.

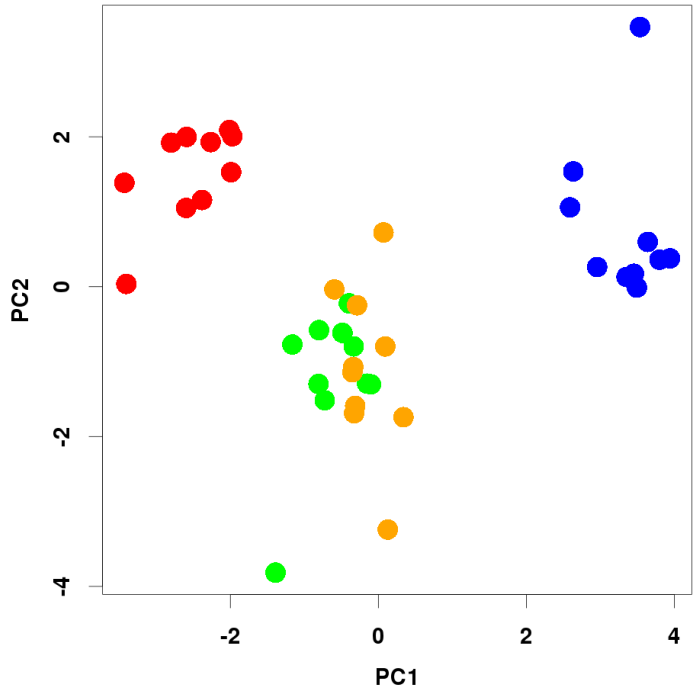


Figure 4.8 Results of PCA analysis of the species and hybrids. The dots represent individual saplings. Blue dots (*A. pavia*), red dots (*A. hippocastanum*), green dots (*A.* × *carnea)* and orange dots (*A.* × *carnea* 'Plantierensis')

The first principal component was dominated by rhamnetin 3-*O*-α-rhamnopyranoside, rhamnetin 3-*O*-α-arabinofuranoside, kaempferol 3-*O*-α-rhamnopyranoside and quercetin 3-*O*-α-rhamnopyranoside (Table 4.2).

Table 4.2 PC loadings of the first three PC axes

Traits	PC1 loading	PC2 loading	PC3 loading
Thickness	0.222	-0.342	0.091
Toughness	0.282	-0.322	0.260
Water content	-0.082	-0.195	-0.623
SLA	-0.078	0.092	0.651
Amino acid	0.271	0.309	-0.015
Chlorogenic acid	0.255	-0.009	0.229
Quercetin arabinoside	0.259	-0.479	0.063
Quercetin rhamnoside	-0.367	-0.296	0.088
Kaempferol arabinoside	-0.203	-0.525	0.060
Kaempferol rhamnoside	-0.391	-0.160	0.003
Rhamnetin arabinoside	0.395	-0.083	-0.154
Rhamnetin rhamnoside	0.410	-0.109	-0.143

Hierarchical cluster analysis revealed the lack of congruence between the phenogram based on egg and mine density and the defence trait phenogram (Figure 4.9). Based on egg and mine densities the backcross *A.* × *carnea* 'Plantierensis' was clustered with the susceptible parent species *A. hippocastanum*, while *A.* × *carnea* was clustered with resistant parent species *A. pavia.* In contrast, based on leaf traits, both the hybrid and the backcross were clustered together with the susceptible *A. hippocastanum*, whereas *A. pavia* shared a few leaf traits in common with the hybrids and *A. hippocastanum*.

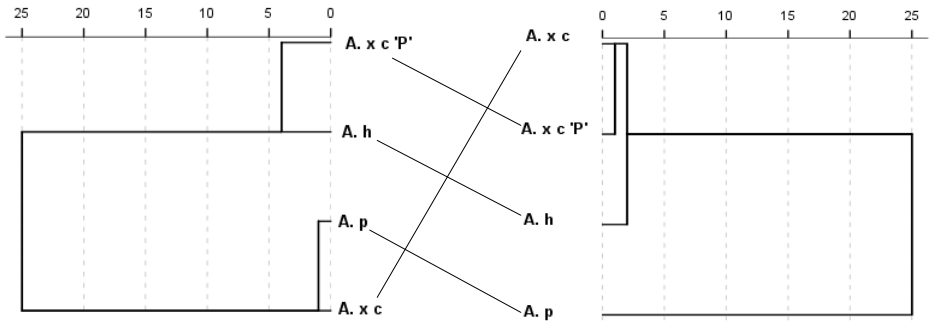


Figure 4.9 Dendrogram using average linkage between species using mean values. The dendrogram on the left groups species by egg and mine density, while the dendrogram on the right groups species by trait similarities. *A.* × *carnea* 'Plantierensis' (A. × c 'P'), *A. hippocastanum* (A. h), *A. pavia* (A. p) and *A.* × *carnea* (A. × c)

4.4 Discussion

Hybridisation between the main host plant of *C. ohridella*, *A. hippocastanum*, and resistant species *A. pavia* had significant effects on both preference and performance of the leaf miner and the leaf chemical and physical traits. In terms of egg and mine density, the hybrid *A.* × *carnea* was almost as resistant to *C. ohridella* as the resistant parent species *A. pavia*. This suggests that resistance to *C. ohridella* is inherited as a dominant trait in the hybrid and agrees with the results of botanical garden surveys (Chapter 2) and previous studies which showed that *A.* × *carnea* does not support full larval development of *C. ohridella* (Kenis *et al*., 2005; Kukula-Mlynarczyk *et al*., 2006; Straw & Tilbury, 2006; Kuldova *et al*., 2007; Péré *et al*., 2010). On the other hand, the backcross *A.* × *carnea* 'Plantierensis' was very similar in terms of susceptibility to the original host *A. hippocastanum*, suggesting that

resistance to the miner is lost when *A.* × *carnea* is backcrossed to *A. hippocastanum*. This breakdown of resistance is commonly observed in backcrosses to the susceptible parents (Fritz *et al*., 1999). However, although the preference of ovipositing females did not differ significantly between the backcross and the original host, the larvae experienced reduced performance, which was indicated by lower pupal weight and slower development. The slower growth and the reduced weight of pupae reared from *A.* × *carnea* 'Plantierensis' could be due to suboptimal nutrients in the ingested plant material or due to the presence of growth inhibitors (Smith, 1989). These inhibitors do not necessarily cause larval mortality but expand the development time and result in reduced size of the insects, therefore, making them more vulnerable to natural enemies and other mortality factors in the field (Farrar and Kennedy, 1989). Prolonged larval development could also be the means of how *C. ohridella* compensates for reduced nutritional content of the leaf tissue. By prolonging the development time, total nutrient intake eventually increases although most probably nutrient intake will still remain in suboptimal levels. For example, nitrogen content of plant material which correlates to protein content is thought to be positively associated with the efficiency of insect growth (Cornelissen & Stiling, 2006). Although reduced pupal weight of *C. ohridella* on *A.* × *carnea* 'Plantierensis' was recorded, adults from these pupae emerged successfully.

In terms of leaf traits, the backcross and the F_1 hybrid *A.* × *carnea* were very similar to each other, with the exception of water content, which was lower in the backcross. Moreover, both backcross and the hybrid were more similar to

A. hippocastanum than to *A. pavia* (cluster diagram), which indicates that the inheritance of most *A. hippocastanum* leaf traits are dominant over *A. pavia*.

The leaves of *A.* × *carnea* 'Plantierensis' were significantly tougher than leaves of *A. hippocastanum* and, as discussed in Chapter 3, leaf toughness can be a mechanical barrier for young larvae of *C. ohridella* and this could partly explain the observed differences in larval performance between the backcross and *A. hippocastanum*. The presence of secondary metabolites in the backcross (such as rhamnetin glycosides) which are absent from the original host of *C. ohridella* might explain the longer larval development time and reduced pupal weight of *C. ohridella* larvae that may not be adapted to these metabolites, however this would require further testing of these compounds in bioassays. The absence of rhamnetin glycosides from *A. hippocastanum* could indicate that *A. hippocastanum* lacks the enzyme 7-*O*-methyltransferase which converts quercetin into rhamnetin.

Traits which are responsible for oviposition and traits which support successful larval development are not necessarily inherited together during hybridisation. In this study, female oviposition choice was not significantly different between the resistant parent species *A. pavia* and the hybrid *A.* × *carnea,* but they were both significantly different from the susceptible *A. hippocastanum* and the backcross *A.* × *carnea* 'Plantierensis', which indicates that traits that elicit oviposition behaviour of *C. ohridella* were probably not inherited by *A.* × *carnea*, but were inherited by the backcross. The same can be said about performance traits as well.

The link between egg and mine density on the one hand, and leaf traits on the other hand, was quite poor (Figure 4.9), as the investigated chemical and physical traits were very similar between the resistant F_1 hybrid *A.* × *carnea* and the susceptible backcross *A.* × *carnea* 'Plantierensis'.

In conclusion, this study did not reveal the main traits responsible for preference and performance of the miner, as the investigated traits were very similar between the resistant F_1 hybrid *A.* × *carnea* and the susceptible backcross *A.* × *carnea* 'Plantierensis'. Further investigations are therefore needed to detect more compounds which may differentiate these two hybrids. Metabolomics could be a good tool for achieving this.

Chapter 5

Distribution of phenolics and saponins in the genus *Aesculus* and their effects on *C. ohridella*

5.1 Introduction

Results of the previous chapters (Chapters 3 and 4) indicate that plant secondary metabolites, such as phenolics, may play a role in the oviposition choice and larval performance of *C. ohridella*. Results in Chapter 4 also suggested that some phenolic compounds may exert more effect on the miner than others. This chapter examines the distribution of two groups of plant secondary compounds – phenolics and saponins – within the genus *Aesculus* and explores the role of the above compounds in resistance to *C. ohridella* using bioassays.

Phenolics are ubiquitous in the plant kingdom (Bernays & Chapman, 1994). They are usually water-soluble, have one or more aromatic rings and one or more hydroxyl groups (Bernays & Chapman, 1994; Harborne, 1998).
Phenolic biosynthesis is illustrated in Figure 5.1.

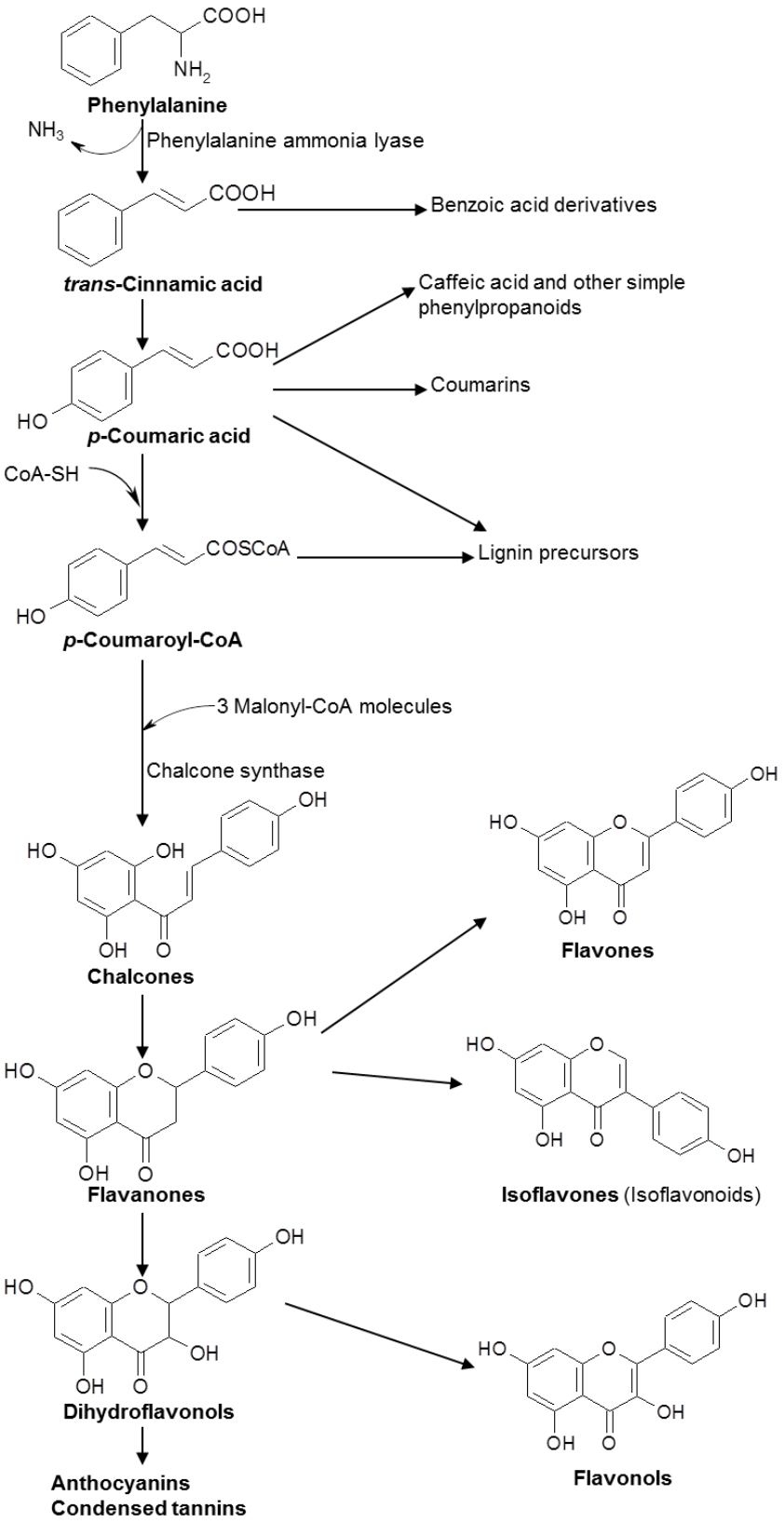


Figure 5.1 Phenolic biosynthesis (Taiz & Zeiger, 2006)

Among the phenolics, flavonoids are the largest group (Harborne, 1998). Flavonoids are ubiquitous and occur in most plant parts such as leaves, roots, wood, bark, pollen, nectar, flowers and seeds. Each plant species produces several different flavonoids and therefore has a unique flavonoid profile (Schoonhoven *et al*., 2005). The basic flavonoid structure is a C_6-C_3-C_6 (Vukics & Guttman, 2010). The flavonoid nucleus is usually linked to a sugar moiety, which forms flavonoid glycosides. These glycosides are more water soluble than the aglycones (Schoonhoven *et al*., 2005). Most flavonoids are stored in the vacuoles (Harborne, 1998; Schoonhoven *et al*., 2005), while some flavonoids can be present in the epicuticular wax on the upper leaf surface (Harborne & Williams, 2000; Schoonhoven *et al*., 2005). Flavonoids absorb in the 280-315 nm region and act like UV filters, thereby protecting plants from tissue damage (Cooper-Driver & Bhattacharya, 1998; Harborne & Williams 2000). Other roles of flavonoids include protection against herbivores (Harborne & Williams, 2000), flower colorants, intracellular and extracellular signalling, pathogen defence (Sirikantaramas *et al*., 2008), and interactions with nitrogen-fixing bacteria and mycorrhiza (Cooper-Driver & Bhattacharya, 1998).

Phenolics can exert both positive (oviposition and phagosimulant) and negative (toxic, deterrent or digestibility-reducing) effects on herbivores. Several flavonoids are known to be used by monophagous or oligophagous insect species to recognise their host plants (Honda, 1995; Simmonds, 2001; Schoonhoven *et al*., 2005). Numerous swallowtail butterflies have been found to require flavonol or flavonol glycosides for stimulation of oviposition

(Honda, 1995; Harborne & Williams, 2000). For example, oviposition by *Luehdorfia japonica* was found to be trigged by isorhamnetin 3-*O*-glucosyl-(1→6)-galactoside-7-*O*-glucoside (Honda, 1995).

Flavonoids in many cases exert varying effects on the feeding behaviour of phytophagous insects. For instance, rutin, a quercetin diglycoside, acts as a feeding stimulant to *Heliothis virescens*, *Schistocera americana*, (Simmonds, 2001), *Schistocera albolineata* and *Melanoplus differentialis* (Simmonds, 2003). Quercetin-3-*O*-glycoside acts as a feeding stimulant on the larvae of *Bombyx mori*, whereas quercetin-3-*O*-rhamnoside is a deterrent and quercetin-3-*O*-rutinoside was found to be inactive (Simmonds, 2003).

The ingested phenolic compounds can also reduce the nutritional value of the plant material for non-adapted herbivores through oxidative processes, which take place in the insect's midgut and create reactive oxygen species (Simmonds, 2003; Salminen & Karonen, 2011). However, even similar compounds can experience different fate in the digestive tract of an insect (Salminen *et al*., 2004b).

Most of the phenolics isolated from the genus *Aesculus* are from the seeds and only a few studies examined the foliar phenolics of this genus (Zhang *et al*., 2010). Many flavonols, flavonones and flavonone-derivates were detected in the seeds of *Aesculus* (Kapusta *et al*., 2007). The most common ones are glycoside of quercetin and kaempferol, proanthocyanidins and polymerised epicatechins (Zhang *et al*., 2010). The fruit and fruit shell of *A. hippocastanum*

are rich in procyanidin trimers (Santos-Buelga *et al*., 1995) and proanthocyanidins (Bate-Smith, 1975).

Phenolics have also been detected in other plant parts in species of *Aesculus*. Seven flavonoid glycosides were isolated and identified from the flowers of *A. hippocastanum;* these compounds were glycosides of kaempferol and quercetin (Dudek-Makuch & Matławaska, 2011). Flavonoid glycosides quercetin 3-*O*-α-L-rhamnopyranoside, quercetin 3-*O*-α-L-arabinopyranoside and isorhamnetin 3-*O*-α-L-arabinoside were detected in the leaves of *A. pavia* (Curir *et al*., 2007; Zhang *et al*., 2010).

Besides phenolics, saponins are another class of secondary metabolites associated with the genus *Aesculus* (Zhang *et al*., 2010). Saponins are widely distributed in the plant kingdom (Oleszek, 2002, Kite *et al*., 2007). They contain a triterpene or steroid aglycone (genin) linked to one or more sugar moieties most commonly to D-glucoronic acid, L-arabinose, D-xylose, L-rhamnose, D-galactose, D-galacturonic acid, D-glucose and D-fructose. Monodesmosidic and bidesmosidic saponins are the most common. Saponins are known to have a wide range of biological activities and are often employed in traditional medicines. They are also known to have piscicidial and insecticidal activities. Many of their biological activities are due to their detergent properties and their interaction with biological membranes (Hostettmann & Marston, 1995). Saponins are also known to negatively affect herbivores (Cui *et al*., 2000).

Research on saponins of the genus *Aesculus* has mostly concentrated on the seeds, with *A. hippocastanum*, *A. indica*, *A. chinensis*, *A. pavia*, *A. turbinata* and *A. glabra* being the most studied species in this respect. Over 80 saponins have been isolated from the genus *Aesculus*, with many of them possessing cytotoxic properties (Zhang & Li, 2007; Zhang *et al*., 2010; Sun *et al*., 2011; Yuan *et al*., 2012), while Assamicin I and II found in the roots of *A. assamica* possess insulin-like activity (Sakurai *et al*., 2002). Saponins from *Aesculus* are classified as polyhydroxylated triterpenoid glycosides, based on four different aglycones such as 24-hydroxy-R_1-barringenol, R_1-barringenol, protoaescigenin and barringtogenol-C (Zhang *et al*., 2010). Saponins present in the leaves of *A. pavia* have been previously shown to have a negative effect on *C. ohridella* (Ferracini *et al*., 2010).

Secondary metabolites such as phenolics and saponins are good chemosystematic markers (Sareedenchai & Zidorn, 2010) and since susceptibility and resistance of the different species of *Aesculus* to *C. ohridella* are linked to phylogeny (Chapter 2), the distribution of saponins and phenolics among the different species could give an indication of possible compounds which determine susceptibility or resistance of a species to *C. ohridella*. Despite the extensive research on saponins in the genus *Aesculus*, no previous study has ever investigated and compared the foliar saponin content of the different species within the genus. Also, there is no comprehensive comparison of the foliar phenolic profile of the different species within the genus *Aesculus*.

In order to test the effects of individual plant secondary metabolites on an insect herbivore, bioassays are normally used, with compounds of interest added to the herbivore's diet. However, it is very challenging to rear internal feeders such as leaf miners on artificial diets and no appropriate method has been developed so far to rear *C. ohridella* artificially. Observations suggest that the removal of *C. ohridella* larvae from mines results in larval mortality within a few hours. This suggests that larvae of *C. ohridella* are only viable within the leaf tissue. Therefore, this study used live or excised leaves rather than artificial diets for the bioassays with *C. ohridella*. Several methods can be used to increase concentrations of particular plant secondary metabolites in plant tissues, e.g. root irrigation, stem brushing, leaf dipping or spraying and petiole feeding. After preliminary experiments with all of the above methods leaf dipping and petiole feeding was found to be the most appropriate methods for the present study. A root irrigation approach requires large volumes of secondary metabolite extracts and compounds can be also metabolised by microorganisms present in the soil. It is difficult to apply liquid extracts using the stem brushing method and compounds applied using the leaf spraying method can accidentally be deposited on non-target leaves when using saplings. Bioassay by petiole uptake using excised leaves however was successfully applied in bioassays with the coffee leaf miner *Leucoptera coffeella* (Magalhães *et al*., 2010).

The aims of this study were:

1) To compare the phenolic and saponin profiles of different species of *Aesculus*.

2) To investigate whether the distribution of these compounds is linked to the phylogeny of the genus.

3) To test the effects of phenolic and saponin fractions of resistant and susceptible species on *C. ohridella* in order to investigate whether phenolics and saponins play a role in resistance to the leaf miner.

5.2 Materials and methods

5.2.1 Plant material for chemical analyses and bioassays

Material for chemical analyses (3 randomly chosen leaves per tree) was collected from twelve species of *Aesculus* at RBG Kew (Table 5.1), in May 2011 and lyophilised. The same trees from three of the above twelve species (*A. hippocastanum*, *A. indica*, and *A. pavia*) were used to obtain crude, phenolic and saponin extracts for the bioassays. *A. hippocastanum* saplings used for bioassays were obtained from Mallet Court Nursery (Taunton).

Table 5.1 Details of *Aesculus* species used for the analysis and their collection reference numbers at RBG Kew. *Aesculus chinensis* was obtained from Mallet Court Nursery (Taunton). Classification scheme of *Aesculus* by Hardin (1957)

Section	Species	Collection reference number
Aesculus	*A. hippocastanum* L.	1969-10423
	A. turbinata Blume	1887-58002
Calothyrsus (Spach) K.Koch	*A. assamica* Griff.	1924-1607
	A. indica (Wall.ex.Camb.) Hook.	1973-14435
	A. wilsonii Rehder	1916-7601
	A. chinensis Bunge	-
	A. californica (Spach) Nutt.	1887-58003
Pavia (Mill) Persoon	*A. pavia* L.	1969-10448
	A. sylvatica Bartarm	1992-804
	A. glabra Willd. var. *glabra*	1925-76901
	A. flava Aiton	1910-65004
Macrothyrsus	*A. parviflora* Walter	1969-10442

5.2.2 Extraction

Lyophilised leaf material (100 mg) of each species was ground using a pestle and mortar, extracted with 80% methanol (5 ml) at room temperature for 24 hours and filtered through nylon filters (0.45µm). An aliquot (10 µl) of each extract was used for LC-MS analysis.

5.2.3 LC-MS analysis

Analyses were performed using a Waters Alliance separation module, connected to photodiode array detector and a Micromass ZQ single quadrupole mass spectrometer, all controlled by Max Lynx Software (v4.0). HPLC

separation was achieved by using Phenomenex Luna C_{18} column (3 × 150 mm; 5 µm) at 30˚ C column temperature. For phenolics the elution rate was 0.5 ml/min using mobile phase gradients 90:0:10 (*t* = 0 min, linear) 0:90:10 (*t* = 25 min) water/methanol/ [acetonitrile + 1% formic acid]. For saponins, the mobile phase gradients used were 90:0:10 (*t* = 0 min, linear) 0:90:10 (*t* = 25 min) water/acetonitrile/[acetonitrile + 1% formic acid]. The column was equilibrated at initial condition for 10 min before injections. The UV absorbance was monitored between 210-500 nm. The ESI-MS conditions were: desolvation temperature 450 °C, nitrogen flow rates 500 l/h for desolvation and 50 l/h at cone; ion source temperature 120 °C; capillary voltage 3.5 kV; cone voltage 20.0V; *m/z* scan range 150-2000; inter scan delay 0.2s. In addition, saponins were also run on LTQ Orbitrap XL spectrometer using Phenomenex Luna C_{18} column (3 × 150 mm; 3 µm) for separation. Two different gradients were used with the elution rate of 0.4 ml/min. In one method the mobile phase was linear gradient of 0:95:5 (*t* = 0-5 min) to 95:0:5 (*t* = 60 min) acetonitrile/water/[acetonitrile + 1% formic acid], followed by an isocratic period of 5 minutes before equilibrating the column in start condition for 15 minutes. In the second method the mobile phase was a (linear) gradients 90:0:10 (*t* = 0 min) to 0:90:10 (*t* = 20 min) water/acetonitrile/[acetonitrile + 1% formic acid], followed by an isocratic period of 5 minutes before returning to the initial condition which was kept for a further 13 minutes.

5.2.4 Compound characterisation

Phenolics detected were identified using a combination of UV absorbance, MS fragmentation pattern and comparison with authentic standards, where available. The identity of the novel rhamnetin glycoside was confirmed by NMR. Although some saponins were isolated and characterised by NMR, this study focused only on the comparison of the presence/absence of the different detected saponins in the different species of *Aesculus*. Therefore, the structures of the detected saponins were not determined in this study. The MS^n spectra of the $[M-H]^-$ ions provided structural information of the molecules. Molecules which possessed the same *m/z* value, retention time and the same fragments in MS/MS were considered to be the same compound.

5.2.5 Bioassays

Bioassays testing the effects of plant extracts on *C. ohridella* oviposition and larval development were carried out in July-October 2012. The crude, phenolic and saponin extracts of *A. indica, A. pavia* and *A. hippocastanum* were tested for biological activity. *A. indica* and *A. pavia* are from sections *Calothyrsus* and *Pavia*, respectively, and are resistant to *C. ohridella*. These species were included into the bioassays to test whether they possess different defence mechanisms against the leaf miner. Extracts of the susceptible *A. hippocastanum* were used to test whether the higher concentrations of phenolics and saponins of the host species have an effect on *C. ohridella* oviposition and larval development. The extracts were prepared as follows:

Crude extracts were obtained by extracting the lyophilised leaf material (~ 65 g) of the above mentioned species in 80% methanol (4 litres) for 24 hours. The obtained extracts were filtered through a filter paper (Whatman No. 1) and concentrated in a rotary evaporator at 45°C. The resulting crude extracts were frozen at -20°C and lyophilised to give dry powder (~21 g). In order to obtain phenolic-rich extracts, the crude extracts (~6 g) were fractionated in multiple runs using reverse phase C_{18} flash column chromatography. The gradient elution was 10:90, 30:70, 50:50, 70:30, 90:10 and 100:0; methanol/water, respectively. Six fractions were collected. The composition of the collected fractions was investigated using LC-MS. Phenolic compounds were detected in fractions 1-4. These fractions were pooled together and concentrated using a rotary evaporator. The concentrated extract was then frozen and lyophilised.

Saponin-rich extracts were prepared by dissolving crude extracts (~10 g) in distilled water and partitioned with *n*-butanol three times (1:1) in a separating funnel. The *n*-butanol layer was dried using a rotary evaporator. The extract was then dissolved in methanol and saponins were precipitated by ice-cold acetone.

5.2.5.1 Leaf dipping experiment

Leaf dipping experiments testing the effects of phenolic and saponin fractions obtained from *A. indica*, *A. pavia* and *A. hippocastanum* were set up in two greenhouses (~ 3 × 4 meter), (Figure 5.2), at Royal Holloway University of London (RHUL). Experiments testing the effect of the phenolic fractions were

carried out from July to August 2012 and experiments testing the effects of the saponin fractions were carried out from late August to early October 2012.

5.2.5.2 Experimental setup of the leaf dipping bioassays

Eight *A. hippocastanum* (~ 1.5 m tall) saplings growing in 30 diameter pots were set up in a greenhouse. 30 leaves on each sapling were randomly marked with one of three different coloured strings (10 leaves per colour). Each of these colours represented a different treatment (Treatment 1: *A. pavia* phenolic extract, Treatment 2: *A. hippocastanum* phenolic extract and Treatment 3: water). Phenolic extracts were prepared (300 mg/l) in distilled water by dissolving the crude freeze-dried phenolic fraction in one litre distilled water and this solution was poured into a plastic container. Leaves were dipped into their designated treatment and the excess solution dripping from the leaves was allowed to drip back into the container. After treatment, leaves were allowed to dry. Saplings were then artificially infested by releasing freshly emerged *C. ohridella* adults (n = 90 per greenhouse). One week after the adult release, one leaflet per leaf (the first leaflet from the middle leaflet on the left side) was removed (10 leaflets per treatment). The number of eggs laid by *C. ohridella* females was counted on the leaflets by using a digital microscope. After the appearance of mines (~ 2 weeks from adult release) leaf dipping treatments were applied once again to test the effect of the treatments on larval development. When the adults started to emerge all leaves were sampled and the number of mines, pupae, larvae and emerged adults was counted.

Figure 5.2 Experiments of leaf dipping bioassay (left) and petiole uptake bioassay (right)

Bioassays testing the effects of *A. indica* phenolics and *A. pavia* and *A. indica* saponin extracts were carried out in a similar manner. Care was taken to ensure that similar sized leaves were chosen in each bioassay and therefore there were slight variations in the number of leaves and number of saplings tested in the different experiments (Table 5.2).

Table 5.2 Summary of the four bioassays of the leaf surface applications to test the effect of extracts applied on *C. ohridella* oviposition and development

Bioassay	Number of saplings used	Number of leaves per treatment	Number of moths released	Treatment applied	Concentration of extract
Bioassay 1	8	10	90	- *A. pavia* phenolic extract - *A. hippocastanum* phenolic extract - distilled water	300 mg/l
Bioassay 2	10	8	106	- *A. indica* phenolic extract - *A. hippocastanum* phenolic extract - distilled water	300 mg/l
Bioassay 3	9	10	100	- *A. pavia* saponin extract - *A. hippocastanum* saponin extract - distilled water	300 mg/l
Bioassay 4	9	10	100	- *A. indica* saponin extract - *A. hippocastanum* saponin extract - distilled water	300 mg/l

5.2.5.3 Petiole uptake experiment to test the effects of *A. pavia* and *A. indica* extracts

Petiole uptake experiments were carried out in late September 2012, using excised leaves obtained from *A. hippocastanum* saplings. The leaf donor saplings were kept protected from *C. ohridella* infestation in a designated greenhouse during the summer. Approximately 3.5 weeks prior to the experiment saplings were artificially infested by introducing freshly emerged *C. ohridella* adults into the greenhouse. Leaves with early instar mines obtained from these saplings were used for the petiole uptake experiment. Care was taken to choose leaves of approximately the same sizes. Excised leaves were then kept with petioles dipped in Eppendorf tubes (Figure 5.2) of their designated treatment (2 ml) for three days. Boards containing the Eppendorf tubes were kept in an airtight plastic container at ~ 20º C. Each board was labelled and represented a treatment and each tube was given a code number. After three days all mines were dissected and checked for larval mortality. The numbers of dead and alive larvae were recorded.

Three petiole uptake experiments were carried out. In the first experiment, seven treatments were applied (crude extract of *A. indica*, phenolic extract of *A. indica*, saponin extract of *A. indica*, crude extract of *A. hippocastanum*, phenolic extract of *A. hippocastanum*, saponin extract of *A. hippocastanum* and distilled water). Each board contained eight excised leaves per treatment and two boards per treatment were used in two separate containers containing seven boards (seven treatments) each. Thus, sixteen leaves per treatment were

used. The extracts were prepared by dissolving lyophilised crude extract, lyophilised phenolic fraction and lyophilised saponin fraction prepared to a concentration of 2 mg/ml in distilled water.

Experiment 2 had the same experimental setup as described above. The treatments used in this experiment were crude extract of *A. pavia*, phenolic extract of *A. pavia*, saponin extract of *A. pavia*, crude extract of *A. hippocastanum*, phenolic extract of *A. hippocastanum*, saponin extract of *A. hippocastanum* and distilled water. The concentration of all extracts was 2 mg/ml.

Experiment 3 tested the effect of 3-*O*-(*E*)-*p*-coumaroylquinic acid on *C. ohridella*, as this acid was found to be present in most of the resistant species of *Aesculus*, but absent from the susceptible ones (Tables 5.2 and 5.3). In this experiment two boards were set up with nine Eppendorf tubes each. Board 1 was the treatment which contained 3-*O*-(*E*)-*p*-coumaroylquinic acid dissolved in distilled water (100µl/ml) and board 2 contained the control (distilled water).

5.2.6 Statistical analyses

Statistical analyses were carried out using SPSS, version 18.0 and R software, version 2.15.2 (R Development Core Team, 2012). Differences between treatments were analysed using analyses of variance (ANOVA) and *t*-test when comparing the treatment with 3-*O*-(*E*)-*p*-coumaroylquinic acid to that of the control. The distribution of phenolics and saponins within the genus *Aesculus*

was analysed with hierarchical cluster analyses, using average linkage. It was carried out using the function 'hclust' in R.

5.3 Results

5.3.1 Qualitative differences in the phenolic profiles of the different species of *Aesculus*

The present study detected 39 phenolic compounds in the different species of *Aesculus* (Table 5.3, Figure 5.3). The detected compounds were phenolic acids, flavonoid glycosides, flavanone glycosides and procyanidins. The detected foliar flavonoid glycosides included *O*-linked mono-, di-, and trisaccharides of the flavonols kaempferol, quercetin, and rhamnetin. In terms of glycosylation pattern, the flavonol glycosides are *O*-linked at C-3, with the exception of Quercetin 3,7-di-*O*-α-rhamnopyranoside, which also has an *O*-linked sugar residue at C-7.

Quercetin 3-*O*-*α*-rhamnopyranosyl-(1→6)-β-glucopyranoside, epicatechin and two unknowns were found to be present in all species. Coumaroylshikimic acids were found only in species belonging to the section *Calothyrsus*. 3-*O*-caffeoylquinic acid, quercetin 3-*O*-β-galactopyranoside, quercetin 3-*O*-β-glucopyranoside, quercetin 3-*O*-*α*-arabinofuranoside, quercetin 3-*O*-*α*-rhamnopyranoside, kaempferol 3-*O*-*α*-arabinofuranoside, kaempferol 3-*O*-α-rhamnopyranoside and the procyanidin trimers were detected in most of the species (Table 5.4). Quercetin 3-*O*-*α*-rhamnopyranosyl(1→2))[*α*-

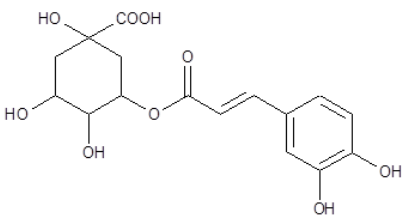
rhamnopyranosyl(1→6)-β-glucopyranoside was found only in *A. californica* whose profile was the most distinct among the species. Species belonging to section *Pavia* had the highest number of flavonoid glycoside compounds detected and it was the only section where quercetin 3-*O*-β-glucopyranosyl(1→2)-β-glucopyranoside, rhamnetin 3-*O*-*α*-rhamnopyranosyl (1→6)-β-glucopyranoside**,** rhamnetin 3-*O*-β-galactoside, rhamnetin 3-*O*-β-glucoside, rhamnetin 3-*O*-*α*-arabinofuranoside and rhamnetin 3-*O*-*α*-rhamnopyranoside were detected. A novel compound rhamnetin 3-*O*-β-glucopyranosyl(1→4)-*α*-rhamnopyranoside was detected in *A. pavia*. (Typical chromatograms of the different species are shown in Appendix 3).

Table 5.3 Characterisation of the phenolics detected in the different species of *Aesculus*.
UV λ_{max} = the UV absorbance of the compound, $[M + H]^+$ = molecular mass of the protonated molecule and $[A + H]^+$ = molecular mass of the protonated aglycone

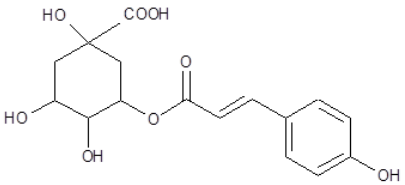
Compound	Retention time	UV λ_{max} (nm)	$[M + H]^+$ (*m/z*)	$[A + H]^+$ (*m/z*)	Name
1	4.0	326	355	-	3-*O*-caffeoylquinic acid
2	5.4	311	339	-	3-*O*-(*E*)-*p*-coumaroylquinic acid
3	6.0	280	579	-	Unknown
4	6.3	284	451	289	Eriodictyol-hexoside (1)
5	6.6	280	865	-	Procyanidin trimer (1)
6	7.0	280	291	-	Epicatechin
7	7.4	288	466	305	Flavanone hexoside (1)
8	7.5	311	339		5-*O*-(*E*)-*p*-coumaroylquinic acid
9	7.6	289	466	305	Flavanone hexoside (2)
10	7.7	280	865	-	Procyanidin trimer (2)
11	8.3	256, 354	627	303	Quercetin 3-*O*-β-glucopyranosyl(1→2)- β-glucopyranoside
12	8.5	256, 354	757	303	Quercetin 3-*O*-α-rhamnopyranosyl(1→2))[α-rhamnopyranosyl(1→6)-β-glucopyranoside
13	8.7	291	437	305	Flavanone pentoside (1)
14	8.9	291	437	305	Flavanone pentoside (2)
15	9.1	280	577	-	Unknown
16	9.4	284	451	289	Eriodictyol-hexoside (2)
17	9.5	266, 384	741	287	Kaempferol 3-*O*-α-rhamnopyranosyl(1→2)[α-rhamnopyranosyl(1→6)-β-glucopyranoside
18	9.6	313	321	-	Coumaroylshikimic acid
19	9.8	256, 349	595	303	Quercetin 3,7-di-*O*-α-rhamnopyranoside
20	10.2	257, 355	611	303	Quercetin 3-*O*-α-rhamnopyranosyl(1→6)-β-glucopyranoside
21	10.4	256, 356	465	303	Quercetin 3-*O*-β-galactopyranoside
22	10.5	312	321	-	5-*O*-*p*-coumaroylshikimic acid
23	10.6	256, 356	465	303	Quercetin 3-*O*-β-glucopyranoside
24	10.8	284, 337	435	273	Naringenin 7-glucoside
25	11.2	257, 356	435	303	Quercetin 3-*O*-α-arabinopyranoside
26	11.4	256, 351	611	303	Quercetin 3-*O*-β-glucopyranosyl(1→4)-α-rhamnopyranoside
27	11.5	266, 348	595	287	Kaempferol 3-*O*-α-rhamnopyranosyl(1→6)-β-glucopyranoside
28	11.5	257, 351	435	303	Quercetin 3-*O*-α-arabinofuranoside
29	11.7	257, 351	449	303	Quercetin 3-*O*-α-rhamnopyranoside
30	12.1	266, 349	419	287	Kaempferol 3-*O*-α-arabinopyranoside
31	12.6	265, 343	595	287	Kaempferol 3-*O*-β-glucopyranosyl(1→4)-α-rhamnopyranoside
32	12.7	266, 347	419	287	Kaempferol 3-*O*-α-arabinofuranoside
33	13.0	265, 343	433	287	Kaempferol 3-*O*-α-rhamnopyranoside
34	13.5	257, 357	625	317	Rhamnetin 3-*O*-α-rhamnopyranosyl(1→6)-β-glucopyranoside
35	13.9	257, 357	479	317	Rhamnetin 3-*O*-β-galactoside
36	13.9	257, 357	479	317	Rhamnetin 3-*O*-β-glucoside
37	14.7	257, 357	449	317	Rhamnetin 3-*O*-α-arabinofuranoside
38	14.9	256, 351	625	317	Rhamnetin 3-*O*-β-glucopyranosyl(1→4)- α-rhamnopyranoside
39	15.3	257, 350	463	317	Rhamnetin 3-*O*-α-rhamnopyranoside

Table 5.4 Distribution of the phenolic compounds detected in the different species of *Aesculus*. '■' indicates the presence of a compound

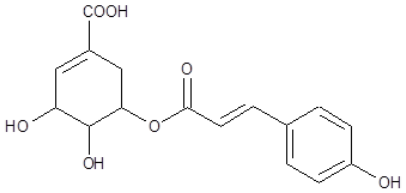
Compound	Section *Aesculus*		Section *Calothyrsus*					Section *Pavia*				Section *Macrothyrsus*
	A. hippocastanum	*A. turbinata*	*A. assamica*	*A. indica*	*A. wilsonii*	*A. chinensis*	*A. californica*	*A. pavia*	*A. sylvatica*	*A. glabra*	*A. flava*	*A. parviflora*
1	■	■	■	■	■	■		■		■	■	■
2			■	■	■	■		■		■	■	■
3	■	■	■	■	■	■	■	■	■	■	■	■
4									■		■	
5	■	■	■	■	■	■			■	■	■	
6	■	■	■	■	■	■	■	■	■	■	■	■
7				■		■						
8			■		■	■						
9				■		■						
10	■	■	■		■			■	■	■	■	■
11								■	■	■	■	
12							■					
13		■										
14				■		■						
15	■	■	■	■	■	■	■	■	■	■	■	■
16											■	
17							■	■	■	■	■	
18			■	■	■	■						
19				■	■							
20	■	■	■	■	■	■	■	■	■	■	■	■
21	■	■						■	■	■	■	
22			■	■	■	■						
23	■	■						■	■	■	■	
24									■		■	
25										■		
26			■			■						
27			■	■			■					
28	■	■			■	■		■	■	■	■	
29	■	■	■	■	■	■		■	■	■	■	■
30										■		
31			■			■						
32	■	■			■	■		■	■	■	■	
33	■	■	■	■	■	■		■	■	■	■	■
34								■	■	■	■	
35								■	■	■	■	
36								■	■	■	■	
37								■	■	■	■	
38								■				
39								■	■	■	■	



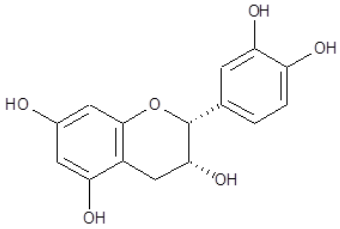
3-*O*-Caffeoylquinic acid



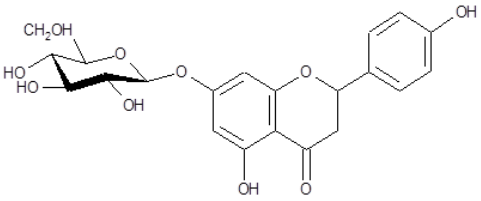
p-Coumaroylquinic acid



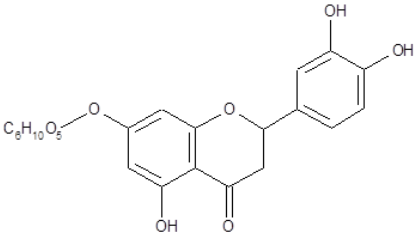
p-Coumaroylshikimic acid



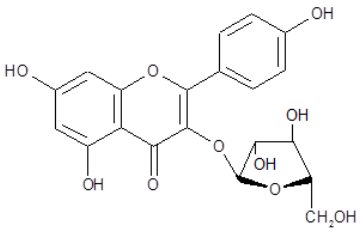
Epicatechin



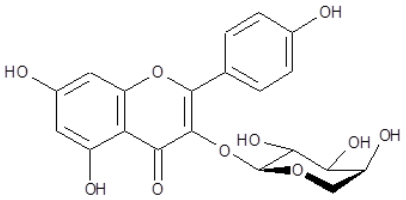
Naringenin 7-*O*-glucoside



Eriodictyol-hexoside



Kaempferol 3-*O*-α-L-arabinofuranoside



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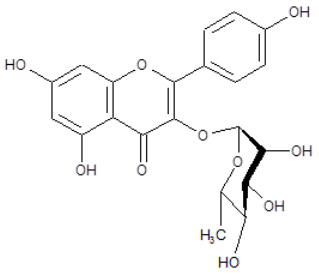
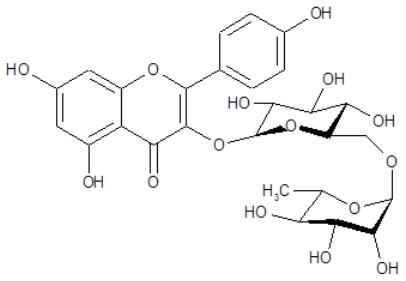
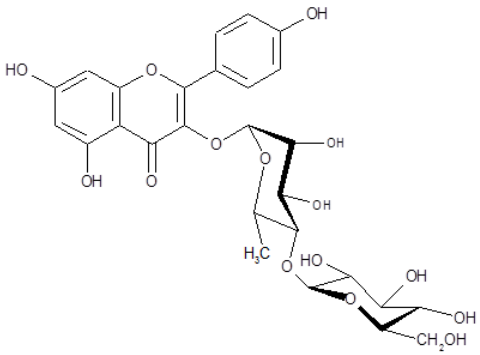


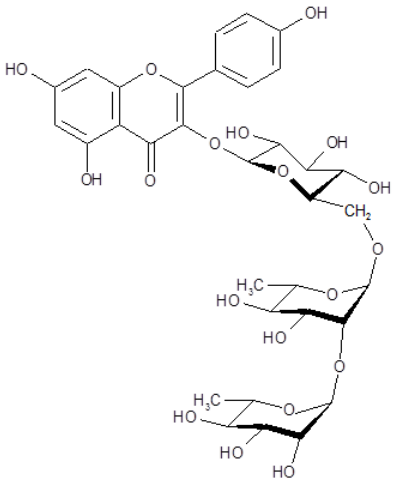
Kaempferol 3-*O*-rhamnopyranoside



Kaempferol 3-*O*-α-rhamnopyranosyl-(1→6)-β-glucopyranoside



Kaempferol 3-*O*-glucopyranosyl-(1→4)-rhamnopyranoside



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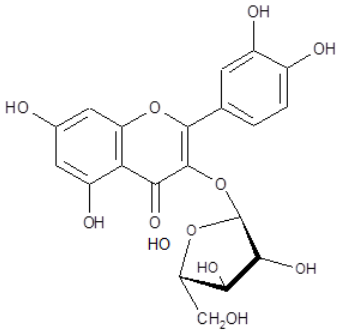
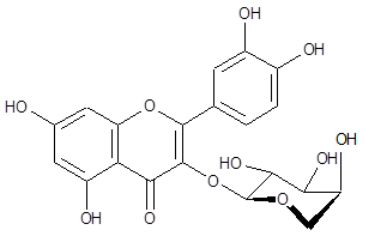
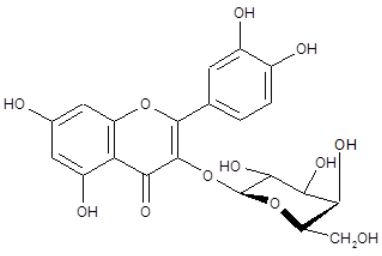


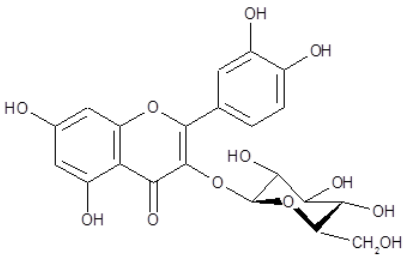
Quercetin 3-*O*-α-L-arabinofuranoside



Quercetin 3-*O*-α-L-arbinopyranoside



Quercetin 3-*O*-galactoside



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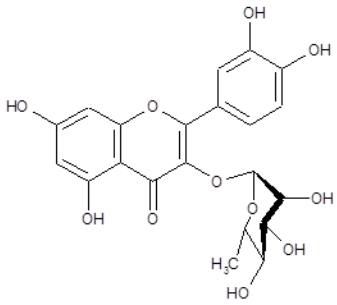

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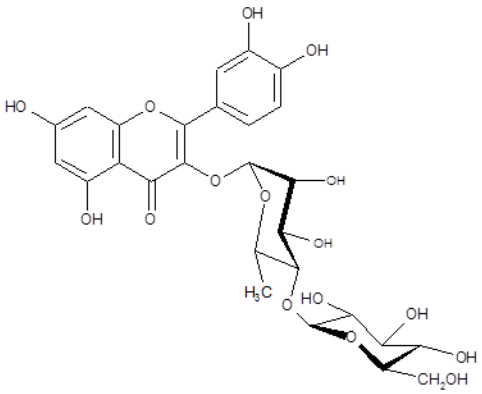
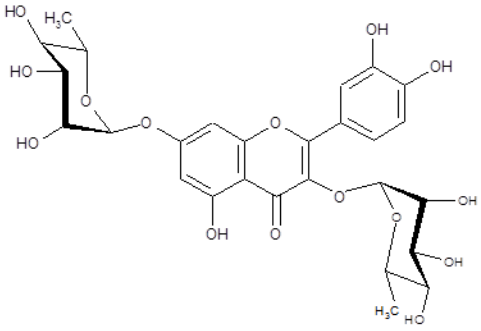
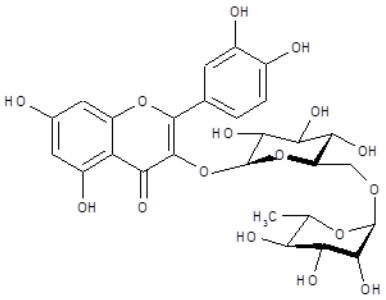


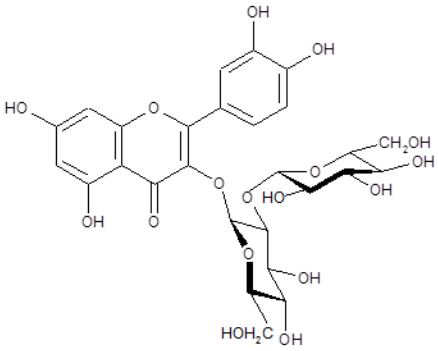
Quercetin 3-*O*-glucopyranosyl-(1→4)-rhamnopyranoside



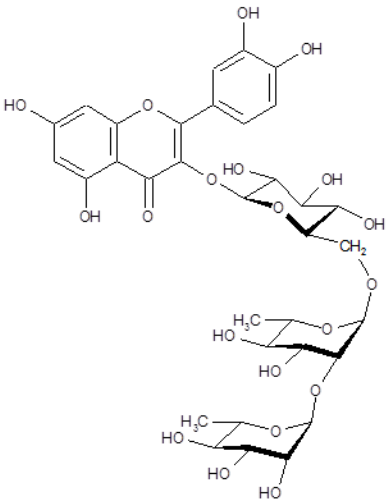
Quercetin 3,7-di-*O*-α-rhamnopyranoside



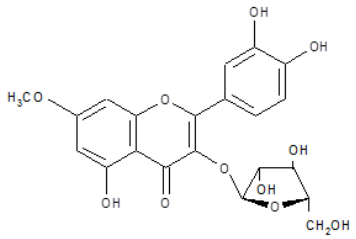
Quercetin 3-*O*-α-rhamnopyranosyl-(1→6)-β-glucopyranoside



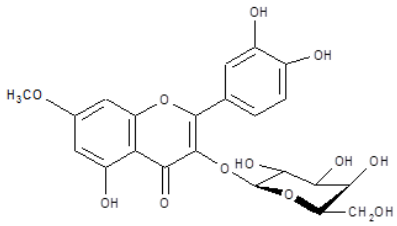
Quercetin 3-*O*-glucopyranosyl-(1→2)-glucopyranoside



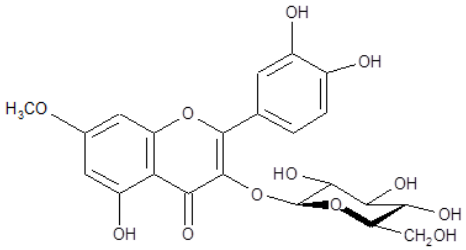
Quercetin 3-*O*-rhamnosyl-(1→2)-rhamnosyl-(1→6)-glucopyranoside



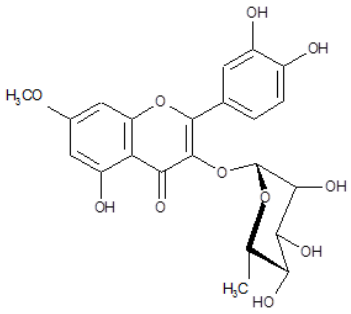
Rhamnetin 3-*O*-α-L-arabinofuranoside



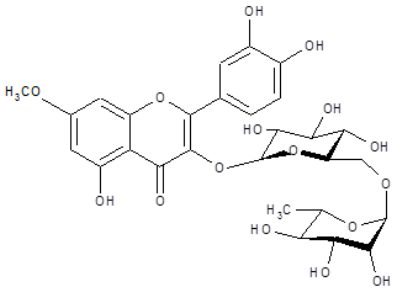
Rhamnetin 3-*O*-galactopyranoside



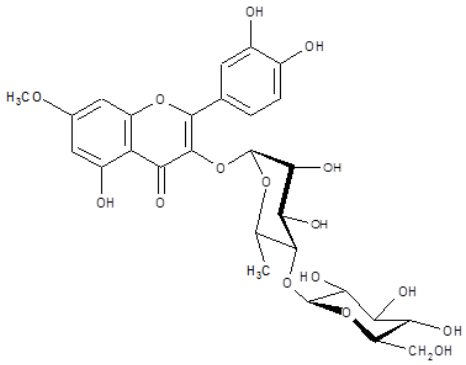
Rhamnetin 3-*O*-glucopyranoside



Rhamnetin 3-*O*-rhamnopyranoside



Rhamnetin 3-*O*-α-rhamnopyranosyl-(1→6)-β-glucopyranoside



Rhamnetin 3-*O*-glucopyranosyl-(1→4)-rhamnopyranoside

Figure 5.3 Structures of the detected phenolics within the genus *Aesculus*

5.3.2 Distribution of the detected phenolic compounds among species in relation to phylogeny of the genus *Aesculus*

Distribution of the phenolics detected in the genus *Aesculus* showed a strong link to phylogeny (Figure 5.4). Hierarchical cluster analysis grouped the two susceptible species from section *Aesculus* (*A. hippocastanum* and *A. turbinata*) together. Species belonging to the section *Calothyrsus* (*A. assamica*, *A. wilsonii*, *A. indica* and *A. chinensis*) were all grouped together, except *A. californica* which had grouped away from the rest of the species in this section. *A. parviflora*, the only representative of the section *Macrothyrsus* had grouped close to the species belonging to section *Aesculus*. All species belonging to the section *Pavia* had grouped close together.

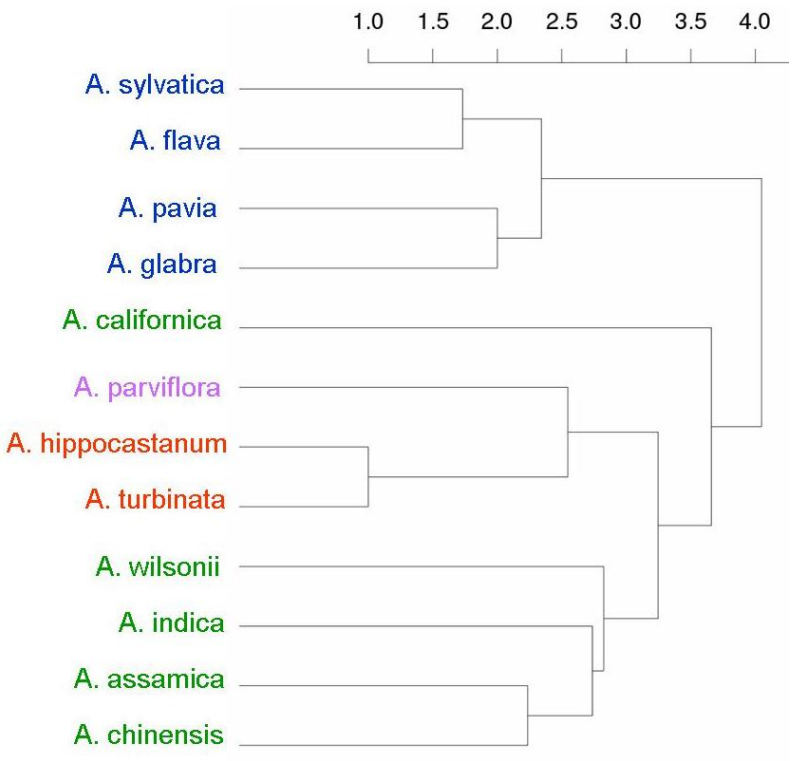


Figure 5.4 Dendrogram of the genus *Aesculus* based on the phenolic profile of the species using average linkage. Different colours indicate the different sections within the genus *Aesculus*; blue = section *Pavia*, green = section *Calothyrsus*, purple = section *Macrothyrsus* and red = section *Aesculus*

5.3.3 Qualitative differences in the saponin profiles of the different species of *Aesculus*

Over 70 saponins were detected in the foliar 80% methanol extract of the different species. The most abundant saponins and their distribution are shown in Table 5.5. Adequate separation of the compounds was achieved by using aqueous acetonitrile gradient on the C_{18} reverse-phase column. In the ES negative ion mode $[M-H]^-$ was the major ion species generated. Similarly to phenolics the distribution of saponins within the genus *Aesculus* also showed a strong phylogenetic link (Figure 5.5). Species of section *Aesculus* were grouped together as well as all species within the section *Pavia*. Species in the section *Calothyrsus* were all grouped together with the exception of *A. californica* the only species within the section of North American origin. *A. parviflora*, the only representative of the section *Macrothyrsus* clustered closely to species in the section *Pavia*. (Typical chromatograms of the different species are shown in Appendix 4).

Table 5.5 Mass spectral data and distribution of the most abundant saponins observed in the LC-MS analyses of the methanolic extracts. Rt = retention time, $[M-H]^-$ = molecular mass of the deprotonated molecule. '■' represents the presence of the molecule in a given species

Compound	Rt (min)	[M-H]- (m/z)	Section *Aesculus*		Section *Calothyrsus*					Section *Pavia*				Section *Macrothyrsus*
			A. hippocastanum	*A. turbinata*	*A. assamica*	*A. indica*	*A. wilsonii*	*A. chinensis*	*A. californica*	*A. pavia*	*A. sylvatica*	*A. glabra*	*A. flava*	*A. parviflora*
1	8.5	611		■										
2	8.5	1224			■	■		■						
3	8.9	715			■	■		■						
4	9.1	1368			■			■						
5	9.3	1222			■									
6	9.4	595		■										
7	9.4	734			■	■	■	■						
8	9.6	1352			■	■		■						
9	9.7	1410					■							
10	9.9	1364							■					
11	10.1	579		■										
12	10.1	1234							■					
13	10.2	1250			■	■	■	■						
14	10.3	579		■										
15	10.3	782			■	■		■	■					■
16	10.5	1072	■											
17	10.5	1581							■					
18	10.5	1250					■							
19	10.6	1434							■					
20	10.6	1110			■	■	■	■						
21	10.7	998	■	■										
22	10.9	1204							■					
23	11.1	1056	■											
24	11.2	1264			■	■	■	■						
25	11.5	780			■	■	■	■						■
26	11.7	1100								■		■		
27	11.8	780												■
28	11.8	958							■					
29	11.9	1100								■		■		
30	11.9	1272							■					
31	12.0	824									■		■	
32	12.3	894		■										
33	12.5	1040	■											
34	12.5	1084										■		
35	12.7	1084								■	■	■	■	
36	12.8	1130							■					
37	13.0	908	■	■										
38	13.0	984							■					
39	13.1	1256							■					

Table 5.5 (continued)

Compound	Rt (min)	[M-H]- (m/z)	Section *Aesculus*		Section *Calothyrsus*					Section *Pavia*				Section *Macrothyrsus*
			A. hippocastanum	*A. turbinata*	*A. assamica*	*A. indica*	*A. wilsonii*	*A. chinensis*	*A. californica*	*A. pavia*	*A. sylvatica*	*A. glabra*	*A. flava*	*A. parviflora*
40	13.1	1304			■	■	■	■						
41	13.4	1228								■			■	
42	13.4	1064							■					
43	13.4	1318			■	■	■	■						
44	13.6	1140									■	■		
45	13.7	1316							■					
46	13.7	1332			■	■	■	■						
47	13.8	1142								■	■	■	■	
48	13.8	1100							■					
49	14.0	722	■	■	■	■	■	■	■	■	■	■	■	■
50	14.1	1142								■			■	
51	14.2	950	■	■										
52	14.4	722	■	■	■	■	■	■	■	■	■	■	■	■
53	14.6	1124								■		■		
54	14.7	950	■											
55	14.8	1126								■		■	■	
56	14.9	978		■								■		
57	15.0	1184	■											
58	15.0	1316			■	■	■							
59	15.0	1228						■						
60	15.1	1126								■			■	
61	15.1	1165							■					
62	15.2	978	■	■										
63	15.2	1168				■		■						
64	15.4	978										■		
65	15.8	958							■					
66	16.2	1020	■	■										
67	16.8	962	■	■										
68	17.3	1048	■	■										
69	17.6	1004	■	■										
70	17.8	1004	■	■										
71	17.9	1014									■			
72	18.8	1032	■											

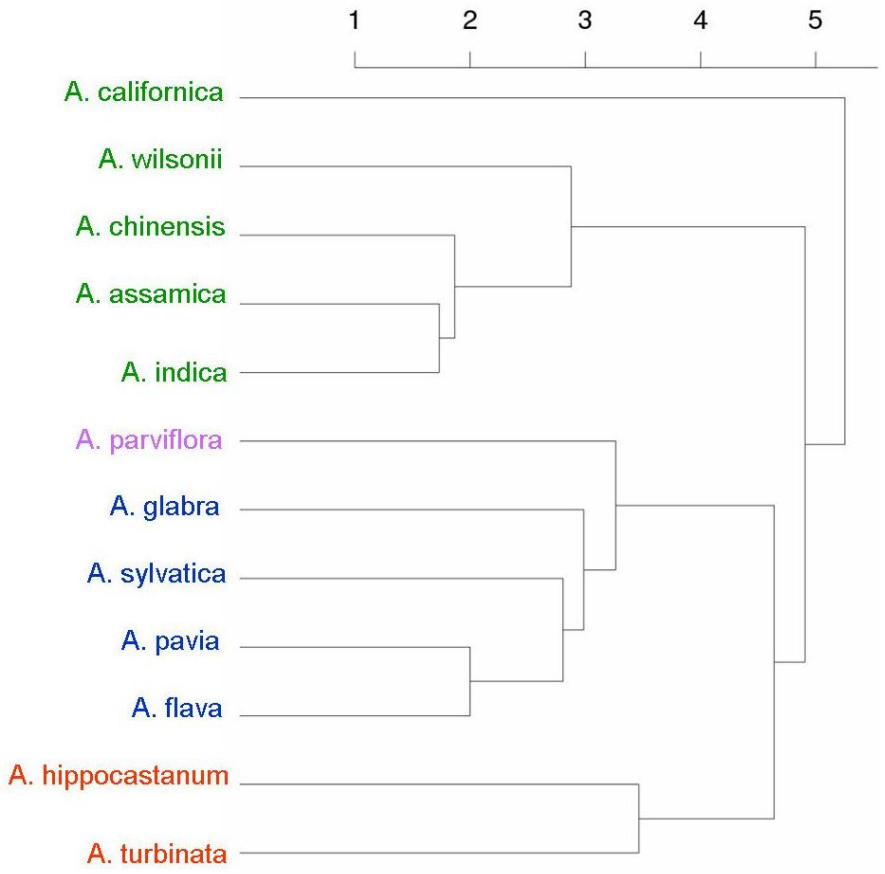


Figure 5.5 Dendrogram of the genus *Aesculus* based on the saponin profile of the species using average linkage. Different colours indicate the different sections within the genus *Aesculus*; blue = section *Pavia*, green = section *Calothyrsus*, purple = section *Macrothyrsus* and red = section *Aesculus*

5.3.4 Novel compounds found in the foliar extracts of *Aesculus*

During the investigation and comparison of the foliar phenolics and saponins within the genus *Aesculus*, some novel compounds were isolated and their structures were elucidated with the aid of NMR (Appendix 5). These compounds were: rhamnetin 3-*O*-β-glucopyranosyl(1→4)-α-rhamnopyranoside (detected in *A. pavia* and isolated from its hybrid *A.* × *carnea*), 9-*O*-angeloyl-4,4´,9-trihydroxy-3,3´-dimethoxy-7,9´-epoxylignan 4´-*O*-β-xylopyranosyl-

(1→4)-β-glucopyranoside (isolated from *A. californica*) and the saponin 21,22-diangeloyl-R_1-barringenol (=12-oleanane-3β-,15α,16α,21β,22α,28-hexol) 3-*O*-β-glucopyranosyl(1→4)[β-arabinofuranosyl(1→3)][β-glucopyranosyl(1→2)]-β-xylopyranoside (isolated from *A. assamica*).

5.3.5 Results of bioassays

5.3.5.1 Leaf surface application of *A. pavia* and *A. hippocastanum* phenolic extracts in bioassay 1

Significantly higher number of eggs was laid by *C. ohridella* females on leaves treated by *A. hippocastanum* phenolic extracts, compared to the *A. pavia* phenolic extract treatment and the control (Figure 5.6A), ($F_{2, 21}$ = 13.20, p< 0.001). There were also significant differences in mine densities between the treatments ($F_{2, 21}$ = 4.21, p = 0.029), with leaves treated with *A. hippocastanum* phenolic extract having higher mine densities than those treated with *A. pavia* extract (Figure 5.6B).

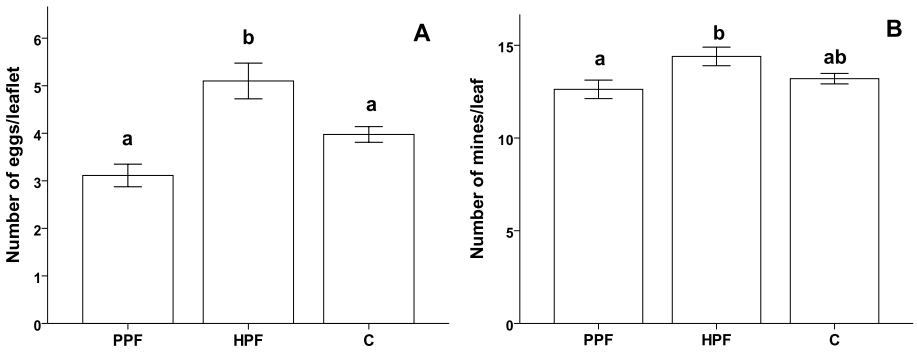


Figure 5.6 Effects of phenolic fraction leaf surface application on *C. ohridella* oviposition (A) and mine density (B) in bioassay 1. PPF – *A. pavia* phenolic fraction, HPF – *A. hippocastanum* phenolic fraction, C - control. The letters above the bars indicate significant differences. Error bar represents +/- SE

5.3.5.2 Leaf surface application of *A. indica* and *A. hippocastanum* phenolic extracts in bioassay 2

Leaves treated with *A. hippocastanum* extracts received more eggs by *C. ohridella* females than those treated by *A. indica* phenolic extracts or water (Figure 5.7A, $F_{2, 27}$ = 10.49, p< 0.001). There were no differences in mine densities between the different treatments ($F_{2, 27}$ = 2.02, p = 0.153) (Figure 5.7B).

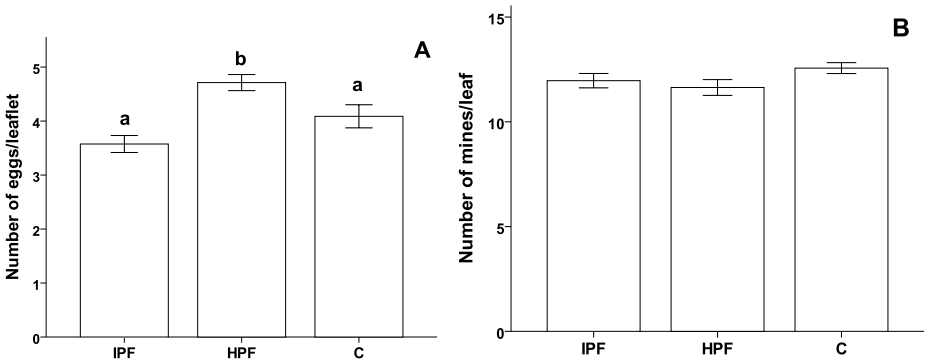


Figure 5.7 Effects of phenolic fraction leaf surface application on *C. ohridella* oviposition (A) and mine density (B) in bioassay 2. IPF – *A. indica* phenolic fraction, HPF – *A. hippocastanum* phenolic fraction, C - control. The letters above the bars indicate significant differences. Error bar represents +/- SE

5.3.5.3 Leaf surface application of *A. pavia* and *A. hippocastanum* saponin extracts in bioassay 3

There were significant differences between the numbers of eggs laid by *C. ohridella* females on the leaves treated by different saponin extracts ($F_{2, 24}$ = 32.33, p< 0.001). Females laid more eggs on leaves treated with water only and there was no difference in egg numbers between leaves treated by *A. pavia*

saponin fraction and leaves treated with *A. hippocastanum* fraction (Figure 5.8A). Despite the differences in egg densities on leaves between treatment and control, there was no significant difference in mine density between the treatments ($F_{2, 24} = 3.06$, $p < 0.065$, Figure 5.8B).

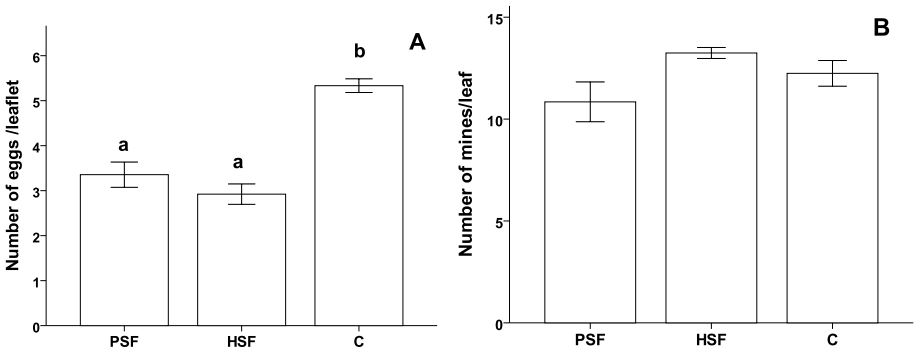


Figure 5.8 Effects of saponin extract leaf surface application on *C. ohridella* oviposition (A) and mine density (B) in bioassay 3. PSF – *A. pavia* saponin fraction, HSF – *A. hippocastanum* saponin fraction, C - control. The letters above the bars indicate significant differences. Error bar represents +/- SE

5.3.5.4 Leaf surface application of *A. indica* and *A. hippocastanum* saponin extracts in bioassay 4

There were significant differences between the numbers of eggs laid by *C. ohridella* females on the leaves treated by the different saponin extracts compared to the control ($F_{2, 24} = 8.95$, $p < 0.01$). Females laid more eggs on leaves treated with water only and there was no difference in egg numbers between leaves treated by *A. indica* saponin fraction and leaves treated with *A. hippocastanum* fraction (Figure 5.9A). The number of mines was significantly lower on leaves treated with *A. indica* saponin fraction (Figure 5.9B).

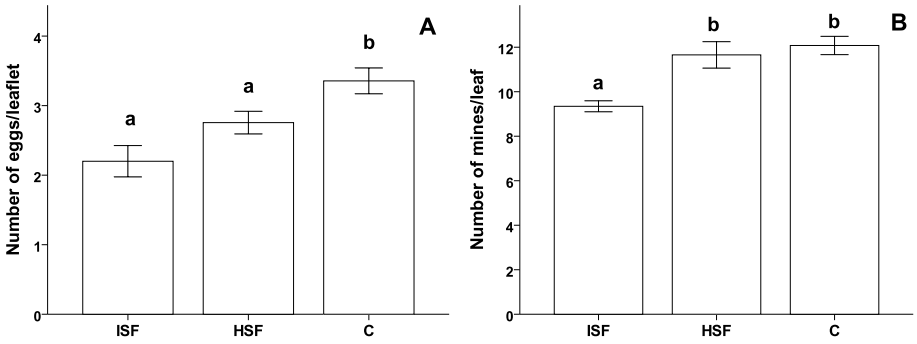


Figure 5.9 Effects of saponin extract leaf surface application on *C. ohridella* oviposition (A) and mine density (B) in bioassay 4. ISF – *A. indica* saponin fraction, HSF – *A. hippocastanum* saponin fraction, C – control. The letters above the bars indicate significant differences. Error bar represents +/- SE

5.3.5.5 Comparison of the different developmental stages of *C. ohridella* between the different treatments in the four leaf surface application bioassays

The distribution of the different larval stages found upon dissection in the four surface application bioassays is shown in Figure 5.10A-D. With the exception of emerged adults in bioassay 3, using *A. pavia* and *A. hippocastanum* saponin extracts as treatment (Figure 5.10C), there were significant differences in the percentage larval, pupal and adult stages between treatments in each bioassay (Table 5.6).

Table 5.6 Differences between treatments of each developmental stage in the four bioassays

	Larvae	Pupae	Emerged
Bioassay 1	$F_{2, 21} = 33.6$, $p < 0.001$	$F_{2, 21} = 18.5$, $p < 0.001$	$F_{2, 21} = 18.5$, $p < 0.001$
Bioassay 2	$F_{2, 27} = 74.9$, $p < 0.001$	$F_{2, 27} = 64.9$, $p < 0.001$	$F_{2, 27} = 46.7$, $p < 0.001$
Bioassay 3	$F_{2, 24} = 28.2$, $p < 0.001$	$F_{2, 24} = 10.5$, $p < 0.001$	$F_{2, 24} = 20.0$, $p = 0.16$
Bioassay 4	$F_{2, 24} = 355.4$ $p < 0.001$	$F_{2, 24} = 92.1$, $p < 0.001$	$F_{2, 24} = 27.8$, $p < 0.001$

In Experiment 1 (Figure 5.10A) larvae developed faster on leaves which were treated with phenolic fractions of *A. pavia* and *A. hippocastanum*, which was indicated by fewer individuals being in the larval stage on the treated leaves. However, when leaves were treated with *A. indica* phenolic fraction (Figure 5.10B), larval development was slower compared to both control and treatment with *A. hippocastanum* phenolic extract. Similarly, more *C. ohridella* were still in larval stage on leaves which were treated with *A. pavia* and *A. indica* saponin fractions compared to both control and leaves treated with *A. hippocastanum* saponin fraction (Figure 5.10C and 5.10D).

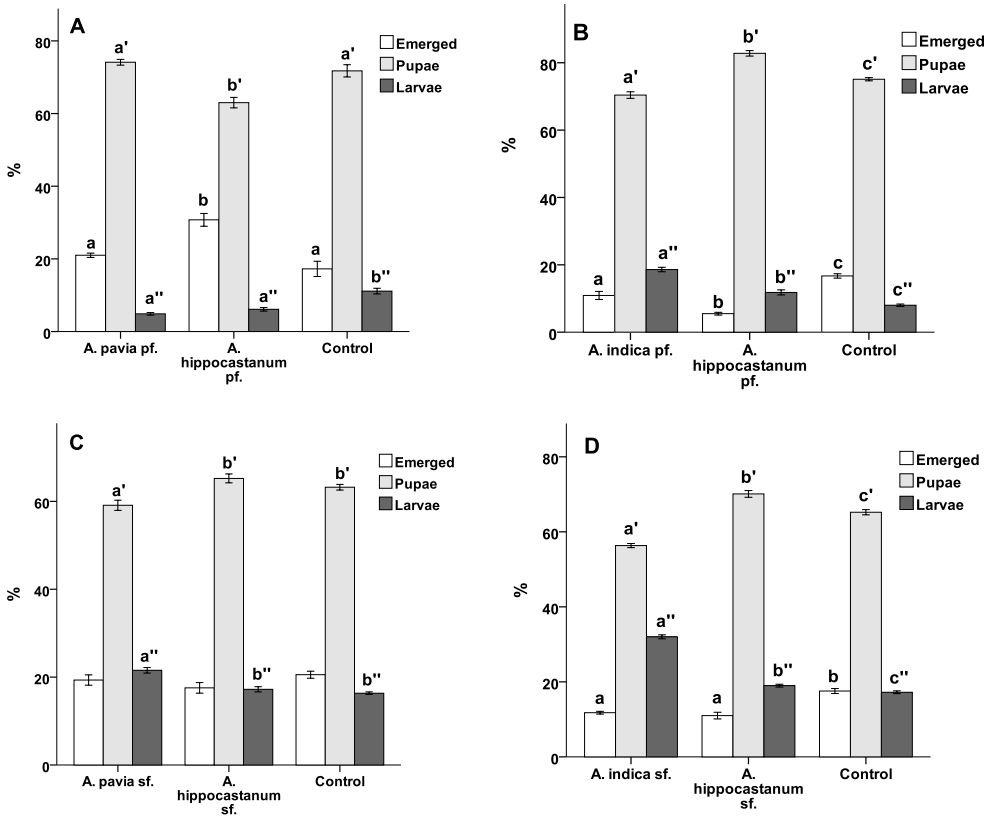


Figure 5.10 Results of the leaf surface application bioassays showing the percentage of the different developmental stages of *C. ohridella*. Bioassay 1 using *A. pavia* and *A. hippocastanum* phenolic fractions (A), bioassay 2 treatment with *A. indica* and *A. hippocastanum* phenolic fraction (B), bioassay 3 treatment with *A. pavia* and *A. hippocastanum* saponin fraction (C), bioassay 4 treatment with *A. indica* and *A. hippocastanum* saponin fraction (D). The letters above the bars indicate significant differences. Error bar represents +/- SE

5.3.5.6 Leaf petiole uptake bioassay 1

Leaf petiole uptake in the first bioassay revealed higher mortality of *C. ohridella* larvae when treated with *A. indica* crude, phenolic and saponin extracts ($F_{6, 105}$ = 78.1, p< 0.001). The most profound difference was found in the treatment of *A. indica* saponin extract where larval mortality was close to 40% (Figure 5.11).

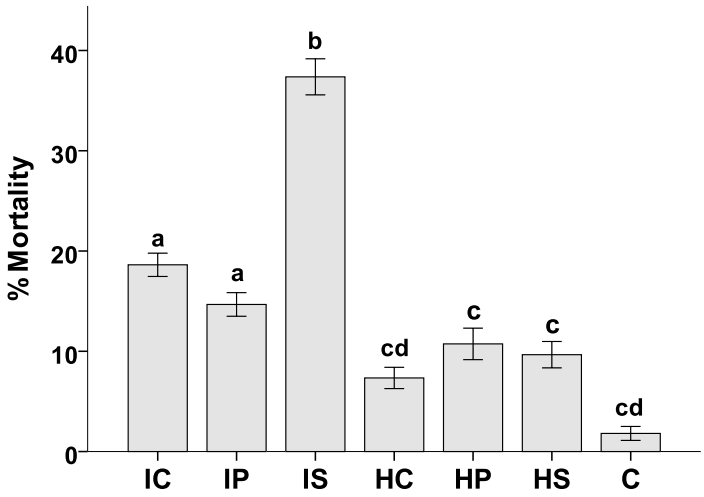


Figure 5.11 Results of the petiole uptake experiment using *A. indica* crude extract (IC), *A. indica* phenolic extract (IP), *A. indica* saponin extract (IS), *A. hippocastanum* crude extract (HC), *A. hippocastanum* phenolic extract (HP), *A. hippocastanum* saponin extract (HS) and water (C). The letters above the bars indicate significant differences. Error bar represents +/- SE

5.3.5.7 Leaf petiole uptake bioassay 2

Leaf petiole uptake bioassay using *A. pavia* and *A. hippocastanum* crude phenolic and saponin extract revealed that although there were significant differences between the treatments ($F_{6, 105}$ = 4.3, p = 0.001), over 80% of larvae were found to be alive upon dissection in each treatment (Figure 5.12).

Leaves treated with *A. hippocastanum* and *A. pavia* phenolic extracts resulted in highest larval mortality.

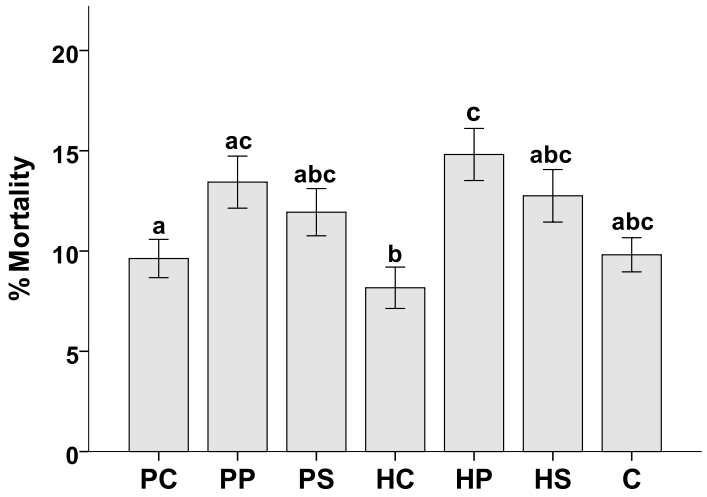


Figure 5.12 Results of the petiole uptake experiment using *A. pavia* crude extract (PC), *A. pavia* phenolic extract (PP), *A. pavia* saponin extract (PS), *A. hippocastanum* crude extract (HC), *A. hippocastanum* phenolic extract (HP), *A.hippocastanum* saponin extract (HS) and water (C). The letters above the bars indicate significant differences. Error bar represents +/- SE

5.3.5.8 Leaf petiole uptake bioassay 3

Dipping leaf petioles in extract of 3-*O*-(*E*)-*p*-coumaroylquinic acid resulted in a significantly higher (over 40%) larval mortality compared to the control ($p < 0.001$, Figure 5.13).

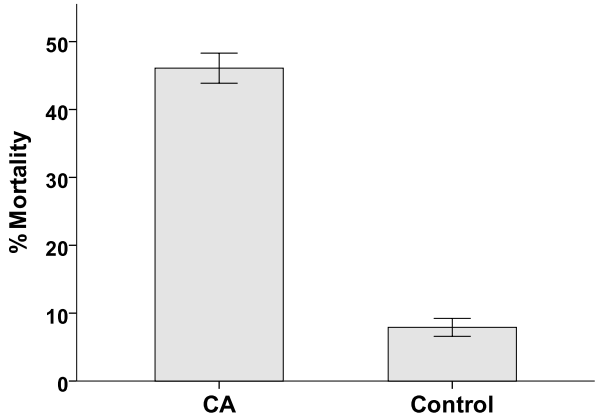


Figure 5.13 Results of petiole uptake bioassay using 3-*O*-(*E*)-*p*-coumaroylquinic acid (CA) as treatment and distilled water (Control) as control. Error bar represents +/- SE

5.4 Discussion

The results of this study highlight that distribution of both phenolic and saponin compounds within the genus *Aesculus* were phylogenetically conserved. Hierarchical clustering of both of these classes of compounds clearly separated each section of *Aesculus* from each other. The dendrogram obtained from this study is very consistent with the phylogeny of the species proposed by Hardin (1957).

Species belonging to the section *Pavia* had the highest number of phenolic compounds present in their leaves, while they possessed fewer saponins, compared to section *Aesculus* and section *Calothyrsus*. This could indicate the presence of different defence syndromes in these two sections, or a trade-off among different types of defences. Interestingly, the lowest number of

phenolics and saponins were detected in the leaves of *A. parviflora*, and yet this species is resistant to *C. ohridella*.

The study also indicates that there is no single phenolic compound solely responsible for resistance to *C. ohridella*, as none of the compounds was consistently absent in the susceptible *A. turbinata* and *A. hippocastanum* and present in all the resistant species. Neither did the study find any individual phenolic compounds which were present only in the susceptible species and absent from the resistant ones.

However, there were several saponins which were only detected in susceptible *A. hippocastanum* and *A. turbinata*, while they were absent from all the remaining species, but once again there were no single saponins which were present in all the resistant species and absent from these two susceptible species. This indicates, as it was hypothesised earlier in previous chapters, that the different resistant species have different resistance mechanisms against *C. ohridella*.

The study detected over 70 foliar saponins within the genus *Aesculus*. This finding is important as most previous studies concentrated on saponins which are present in the seeds and fruit shell of the species (Zhang *et al*., 2010). Many of the previously isolated saponins from the genus *Aesculus* have pharmacological activity, it is very likely that some of the foliar saponins detected in this study could also possess different biological activities and hence could be an important new source for pharmacological studies.

Results obtained from bioassays testing the effects of saponin, phenolic and crude extracts from *A. pavia* and *A. indica* revealed that *C. ohridella* larvae were not affected negatively by phenolics found in the resistant species. Surprisingly, when the *A. hippocastanum* phenolic extract was applied to the leaf surface, it attracted more ovipositing females of *C. ohridella*, indicated by the significantly higher number of eggs on this treatment in both bioassays 1 and 2. This could be due to the fact that some of the extracted phenolics could act as oviposition stimulants for *C. ohridella* females. Even though these stimulants might be present in the phenolic extracts of *A. indica* and *A. pavia*, their attractiveness could be masked by the presence of other compounds such as, for example, 3-*O*-(*E*)-*p*-coumaroylquinic acid in the leaves of *A. indica*, which was found to have a negative effect on *C. ohridella* larvae in this study. Although the application of *A. hippocastanum* phenolic extract resulted in higher egg density, it did not necessarily result in higher number of mines, indicating that some of the eggs laid by the females either did not hatch or larvae died prior to the formation of visible mines. The exact mechanism for this phenomenon is presently not clear.

The saponin fraction applied to the leaf surface significantly reduced the number of eggs oviposited by *C. ohridella*, irrespective of whether the saponin fraction was obtained from the susceptible *A. hippocastanum* or the resistant *A. indica* and *A. pavia*, which indicates that saponins are good oviposition deterrents. Despite deterring oviposition, *A. hippocastanum* and *A. pavia* saponins did not have an effect on the number of mines. Only saponin fractions obtained from *A. indica* resulted in significantly lower number of mines

compared to the controls. However, it is important to note, that saponins are not present on the leaf surface of *Aesculus* leaves in nature, and it is likely that *C. ohridella* females cannot detect them while looking for oviposition sites.

The *A. indica* saponin fraction applied to the leaf surface also resulted in longer larval development time, indicated by the larger proportion of larvae being in the larval state compared to the treatments. The negative effect of *A. indica* saponins was once again confirmed in the petiole uptake experiment, where the treatment resulted in much higher larval mortality than any other treatments. *A. indica* crude extracts (which contained both phenolic and saponin fractions) also resulted in higher larval mortality compared to the controls. The concentration of the actual saponins in the crude extracts is lower than that in the saponin fractions, which indicates that larval mortality is dose-dependent.

The actual concentration of individual compounds found in the plant extracts used in this study could be very low. However, testing plant extracts rather than single compounds provides the opportunity to detect compounds which act synergistically and therefore the combined effect of these compounds could not be detected if compounds were applied individually. Moreover, the qualitative and quantitative content of the plant extracts are representative of what can be found in nature. Therefore, it gives the investigator a better insight into the resistance mechanisms present in the species where the extracts were obtained from.

Overall, the present study showed that the distribution of secondary metabolites within the species is linked to phylogeny. Therefore, the compounds detected in this study are good chemosystematic markers. This study also found that saponins present in *A. indica* and 3-*O*-(*E*)-*p*-coumaroylquinic acid negatively affects the larvae of *C. ohridella*. However, the use of saponins as control agents for *C. ohridella* is not advisable as they are difficult to obtain and their effect is negligible compared to most of the pesticide treatments used for the control of *C. ohridella* (Chapter 1).

Chapter 6

LC-MS-based metabolite profiling of resistant and susceptible species of the genus *Aesculus*

6.1 Introduction

Metabolomics is a useful tool for investigating ecological interactions and finding bioactive compounds in plant-herbivore interactions (Hegeman, 2010), and although the comprehensive coverage of all metabolites at present is not possible, it provides an insight in the metabolic biochemistry of organisms (Bedair & Sumner, 2008). The analysis of the metabolome can be divided into two approaches: (1) the targeted analysis which is the identification and quantification of predefined metabolites and (2) metabolite profiling which is the scanning of all metabolites detectable by the chosen analytical technique (Villas-Bôas *et al*., 2005; Theodoridis *et al*., 2011; Becker *et al*., 2012). The latter technique can be subdivided into metabolic footprinting which investigates extracellular metabolites and metabolic fingerprinting which investigates intracellular metabolites (Villas-Bôas *et al*., 2005). This latter approach is often used to assign organisms to groups based on their metabolomic pattern. Metabolomic approaches can often provide solutions in chemical ecology, where the traditionally employed techniques such as bioassay-guided fractionation fail to reveal the biological activities of compounds, for example, when compounds are unstable or interact

synergistically (Prince & Pohnert, 2010). It is therefore often used in plant biochemistry, characterisation of primary and secondary metabolites, and for understanding physiological and ecological phenomena (Moco *et al*., 2007). Metabolite fingerprinting was successfully employed when comparing susceptible and resistant *Brachypodium distachyon* plants, when interacting with the fungal pathogen *Magnaporthe grisea* and to study the effect of *Manduca sexta* on *Nicotiana attenuate* (Prince & Pohnert, 2010).

Metabolomics studies can detect the presence and absence of metabolites within a group and it can also give quantitative information about compounds. The use of multivariate statistical methods can then discriminate between susceptible and resistant groups and provide a list of candidate molecules which could play a role in susceptibility and resistance.

Although metabolomics is a useful tool in chemical ecology, one drawback is that the detected differences in the metabolites of the different genotypes, phenotypes or groups are not always due to the ecological role in question. Therefore, bioassays are necessary to follow up the findings of such analyses (Prince & Pohnert, 2010).

There are large chemical variations between different plant tissues and a single cell may contain over 5000 metabolites, with differing chemical properties. The detected metabolites will be dependent on the extraction method used for the sample preparation and the analytical platform used for its detection (Moco *et al*., 2007). There are several analytical platforms which can be applied in the

study of metabolomics, such as mass spectrometry (MS), mass spectrometry coupled to liquid chromatography (LC-MS), gas chromatography coupled to mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) or capillary electrophoresis (CE) (Katajamaa & Orešič, 2007). LC-MS is a preferable method for the detection of metabolites present in plant tissues (Villas-Bôas *et al*., 2005). It has high sensitivity, and is used for the identification and quantification of polar and neutral metabolites even though they are present in low concentrations in a complex matrix. Electrospray ionisation (ESI) is one of the most widely used method for the analysis of polar and ionic compounds (Theodoridis *et al*., 2011), and LC-ESI-MS can give better sensitivity than GC-MS when analysing plant metabolites. Moreover, samples do not need to be derivatised prior to analysis (Villas-Bôas *et al*., 2005). The choice of solvents used for metabolite extraction should also be compatible with the instrumentation used for the analysis. Methanol/water solution has been successfully used as an extraction solvent for glycosylated sterols, alkaloids, phenolics and other semi-polar compounds present in plant tissues (Moco *et al*., 2007).

The large amount of data generated by metabolomic studies needs to be processed prior to data analysis. The typical data processing steps are: filtering, peak detection, alignment and normalisation (Katajamaa & Orešič, 2007). The detected metabolites can be identified by studying the accurate mass, fragmentation and isotopic pattern (Moco *et al*., 2007).

Chapter 3 and Chapter 4 revealed that the interaction between *C. ohridella* and its hosts can be influenced by physical traits, such as leaf toughness as well as chemical traits, such as amino acids, phenolics and saponins. However, the previous chapters investigated only predefined leaf traits and the mechanism of interactions between *C. ohridella* and its hosts is not fully resolved. Metabolite profiling could give us further insight into which other, previously not investigated, metabolites could facilitate the mechanisms of resistance and susceptibility of the different species of *Aesculus* towards the leaf miner. Therefore, the aims of this chapter were:

1. To investigate whether susceptible and resistant species and hybrids of *Aesculus* can be differentiated using metabolite profiling.

2. To investigate which compounds may determine resistance and susceptibility towards *C. ohridella* by comparing qualitative and quantitative differences in the metabolites of resistant and susceptible species and hybrids of *Aesculus*.

6.2 Methods and material

6.2.1 Plant material

Leaf material was obtained from mature trees growing in Royal Botanic Gardens, Kew, and from potted saplings growing in experimental greenhouses in Royal Holloway, University of London in May 2011. Species and hybrids used in this study are shown in Table 6.1.

Table 6.1 Species composition of the different data sets used in the study. Numbers in brackets indicate the number of samples from the given species

Data set	Species
Data set 1 (based on Chapter 3)	*A. hippocastanum* sapling (× 10)
	A. turbinata sapling (× 10)
	A. indica sapling (× 10)
	A. chinensis sapling (× 10)
	A. flava sapling (× 10)
Data set 2 (based on Chapter 4)	*A. hippocastanum* sapling (× 10)
	A. pavia sapling (× 10)
	A. × *carnea* sapling(× 10)
	A. × *carnea* 'P' sapling (× 10)
Data set 3 (mature trees RBG Kew)	*A. hippocastanum* (× 6)
	A. turbinata (× 12)
	A. flava (× 9)
	A. pavia (× 3)
	A. indica (× 3)
	A. assamica (× 3)
	A. wilsonii (× 3)
	A. californica (× 12)

6.2.2 Plant extraction

Leaf material from the study species was lyophilised and ground to fine powder using pestle and mortar excluding the leaf veins. The powdered leaf material (100 mg) was extracted with 80% methanol (5 ml) at room

temperature for 24 hours, with occasional shaking. Samples were then centrifuged at (14000 rpm) for 15 min, and aliquots of the supernatants were directly used for analysis by LC-MS (injection volume 10µl).

6.2.3 LC-MS analyses

HPLC separation was performed using Waters 2695 Alliance separation module (Waters, MA, US), with a Luna C_{18} column (3 mm × 150 mm, 5 µm; Phenomenex) at 30 ºC. The mobile phase was a mixture of water, methanol and 1% (v/v) formic acid in acetonitrile, with linear gradient from 90:0:10 (t = 0 min to 0:90:10 (t = 20 min) then isocratic for 5 min, at a flow rate of 0.5 ml/min. The column was equilibrated in the initial condition solvent for 10 min between injections. A ZQ single quadrupole mass spectrometer (Micromass, Manchester, UK), with an electrospray ion source, was used for detection of metabolites. An alternative positive-negative scan mode (1.0 s per scan) was used for *m/z* range 150-2000, with inter-scan delay of 0.2 s. Scanning was continued to R_t = 30 min in order to cover the gradient delay from LC solvent mixer to the column outlet. Capillary voltages were 3.5 kV and 4.0 kV for positive and negative ionisation, respectively. Source (120 ºC and desolvation (450 ºC) temperatures, gas flows (N_2) for desolvation (500 L/h) and at the cone (50L/h), and analyser settings were kept constant throughout experiments. All the instruments were controlled by the MassLynx software (v. 4.0, Micromass). Methanol and acetonitrile were obtained from Fisher Scientific (Leicester, UK). Deionised water was prepared using a Milli-Q Integral system (Millipore, MA, US) and membrane-filtered (0.22 µm) prior to use.

6.2.4 Data processing

Raw data were converted into NetCDF format and were processed by MZmine 2 software (Pluskal *et al*., 2010). Data were centroided and all data points were detected above the intensity level 1.0E3. It was followed by the connection of consecutive m/z values over multiple scans into chromatograms. The software connects these values in the order of their intensity, where the most intense peaks are connected first (Pluskal *et al*., 2010). The minimum time span was 0.1 min, with minimum height of 1.5E3 and m/z tolerance of 1 ppm.

Each chromatogram was then deconvoluted into individual chromatographic peaks with the 'base line' cut-off option where the base line was set at 2.5E4. The 'joint aligner' option was used for peak alignments with m/z tolerance of 5 ppm and retention time tolerance of 0.4 min. The aligned chromatograms were then normalised by the average peak intensity.

6.2.5 Statistical analyses

Statistical analyses were conducted using R software version 2.15.2 (R Development Core team, 2012). Principal component analyses (PCA) were performed to reduce the dimensionality of the data sets and visualise the separation of plant groups according to the detected metabolites. To test for differences in metabolites between resistant and susceptible groups, the Z-factor (Zhang *et al*., 1999) was used to detect variables that discriminate resistant and susceptible classes. It is reflective of the dynamic range of the

mean signals (μ) and standard deviations (σ) of both the resistant and susceptible groups. The μ and σ values for the two classes are obtained separately and the Z-factor is calculated for each detected ion. Compounds with Z-factor higher than -2 were retained.

6.3 Results

Detected peaks after alignment showed a consistency in retention times of the detected compounds in all data sets. Principal component analysis revealed that susceptible species and hybrids always grouped together in all three data sets, indicating chemical similarities among these samples (Figures 6.2 - 6.4). In contrast, resistant species were found to have higher chemical diversity.

6.3.1 Principal Component analysis of data set 1

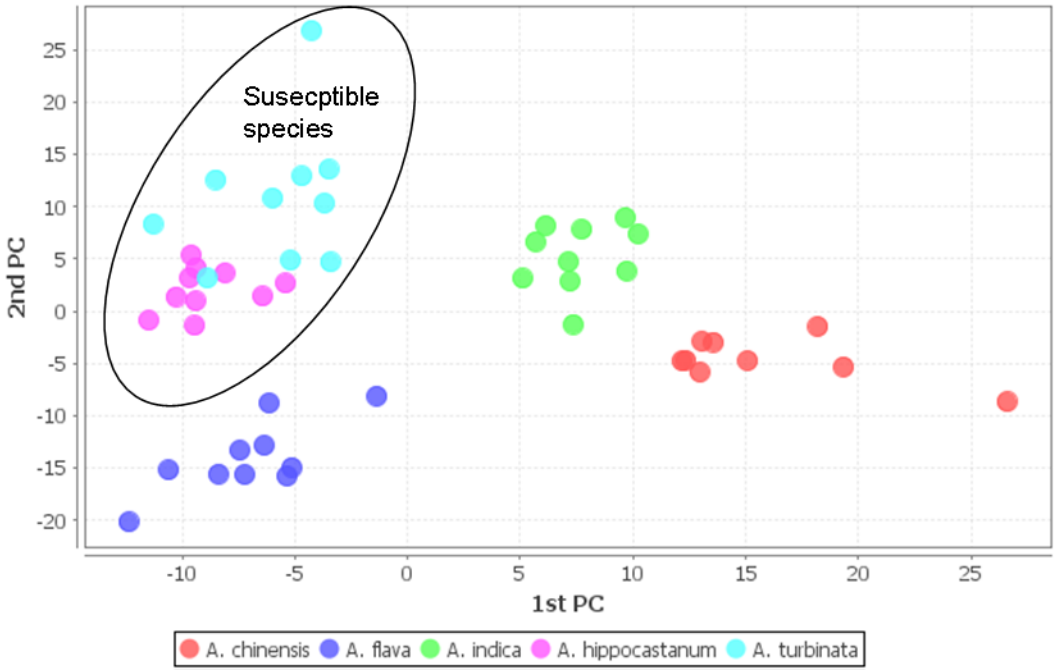


Figure 6.1 Results of PCA analysis of data set 1

Principal component analysis of metabolites of the different species of *Aesculus* used in the greenhouse experiment with saplings in Chapter 3 revealed that the two susceptible species *A. hippocastanum* and *A. turbinata* (both belonging to the section *Aesculus*) are clustered together (Figure 6.1). The resistant species *A. indica* and *A. chinensis* (both belonging to the section *Calothyrsus*) are also grouped together, whereas, the resistant species *A. flava* (section *Pavia*) grouped away from the remainder of the species indicating that this species is chemically different from the other two resistant species, hence, it may possess different chemical traits. The first three principal component explained 40% of the variation within the data set (PC1 19%, PC2 12% and

PC3 9% respectively). The compounds with the highest positive and negative loadings for PC1, 2 and 3 are shown in Table 6.2.

Table 6.2 PCA loadings of data set 1

PC 1		**PC 2**		**PC 3**	
Loading	Compound	Loading	Compound	Loading	Compound
0.5813	Unknown	0.1950	Epicatechin	0.339	Rhamnetin 3-*O*-α-arabinofuranoside
0.3164	Epicatechin	0.1949	Quercetin 3-*O*-α-rhamnopyranoside	0.3111	Rhamnetin 3-*O*-α-rhamnopyranoside
0.1715	Kaempferol 3-*O*-α-rhamnopyranoside	0.1775	Kaempferol 3-*O*-α-rhamnopyranoside	0.3057	Epicatechin
0.1542	Unknown	0.0940	Unknown	0.1865	Quercetin 3-*O*-β-galactopyranoside
0.1240	Flavan 3-ol (2)	0.0794	Procyanidin trimer	0.1723	Quercetin 3-*O*-α-arabinopyranoside
-0.1168	Quercetin 3-*O*-α-arabinofuranoside	-0.1207	5-*O*-*p*-coumaroylshikimic acid	-0.0974	Unknown
-0.1257	5-*O*-*p*-coumaroylshikimic acid	-0.1758	Coumaroylshikimic acid	-0.1674	Quercetin 3-*O*-α-arabinofuranoside
-0.1936	Coumaroylshikimic acid	-0.1944	Unknown	-0.1899	Quercetin 3-*O*-α-rhamnopyranoside
-0.2105	Unknown	-0.2847	3-*O*-(*E*)-*p*-coumaroylquinic acid	-0.2378	3-*O*-caffeoylquinic acid
-0.3893	3-*O*-(*E*)-*p*-coumaroylquinic acid	-0.7475	Unknown	-0.4390	Kaempferol 3-*O*-α-rhamnopyranoside

The compounds which contributed the most to the first 3 principal axes were mainly phenolics and phenolic acids.

6.3.2 Principal component analysis of data set 2

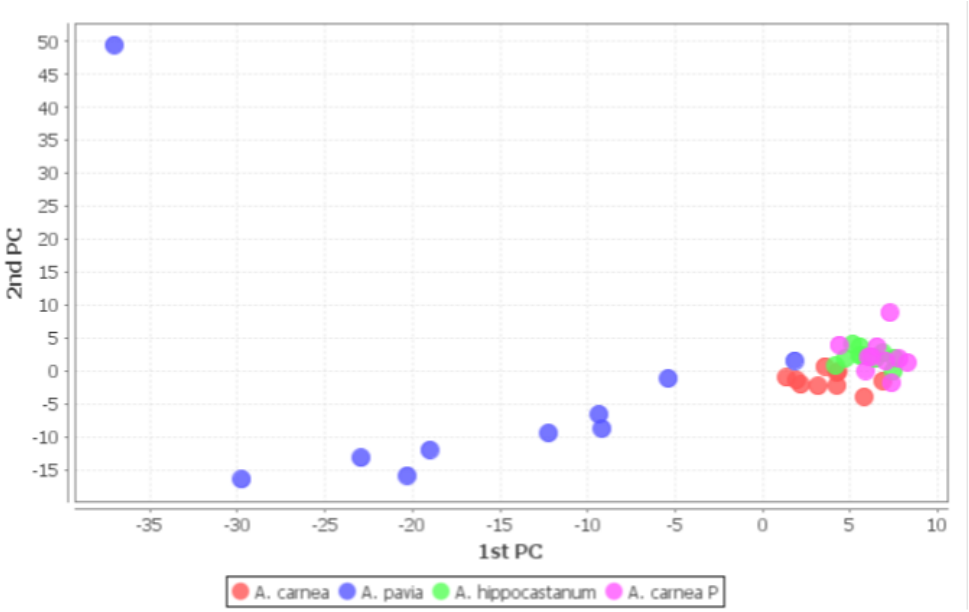


Figure 6.2 Results of PCA analysis of data set 2

In data set 2 the resistant *A. pavia* has grouped away from the susceptible host *A. hippocastanum*, the resistant hybrid *A.* × *carnea* and the susceptible backcross *A.* × *carnea* 'Plantierensis' (Figure 6.2). This indicates that the hybrid and the backcross are chemically more similar to the susceptible parent species *A. hippocastanum* than to *A. pavia*, indicating that the detected chemical traits are inherited from *A. hippocastanum*. This corresponds with the results of the hierarchical cluster analysis based on leaf traits in Chapter 4.

In this data set the first three principal components explained 40% of the total variance (PC1 22%, PC2 13% and PC3 5% respectively). The compounds (Table 6.3) which contributed to most of the variance observed was found to be similar to the compounds which contributed to the loadings of the first three PC axes in data set 1.

Table 6.3 PCA loadings of data set 2

PC 1		PC 2		PC 3	
Loading	Compound	Loading	Compound	Loading	Compound
0.9324	Unknown	0.4441	Quercetin 3-*O*-α-rhamnopyranoside	0.3846	Unknown
0.1118	Kaempferol 3-*O*-α-rhamnopyranoside	0.3117	Quercetin 3-*O*-α-arabinofuranoside	0.3189	Unknown
0.0774	Quercetin 3-*O*-α-rhamnopyranoside	0.2590	Kaempferol 3-*O*-α-rhamnopyranoside	0.2846	Rhamnetin 3-*O*-α-arabinofuranoside
0.0713	Quercetin 3-*O*-α-arabinofuranoside	0.2139	Quercetin 3-*O*-β-glucopyranosyl(1→4)-α-rhamnopyranoside	0.2351	Unknown
0.0619	Unknown	0.2068	Epicatechin	0.2133	Quercetin 3-*O*-β-glucopyranosyl(1→4)-α-rhamnopyranoside
-0.0478	Unknown	-0.1988	Naringenin 7-glucoside	-0.0981	Unknown
-0.0504	Rhamnetin 3-*O*-α-rhamnopyranoside	-0.2006	Quercetin 3-*O*-β-galactopyranoside	-0.1032	Unknown
-0.0573	3-*O*-(*E*)-*p*-coumaroylquinic acid	-0.2031	Rhamnetin 3-O- β-hexoside	-0.1268	Kaempferol 3-*O*-α-rhamnopyranoside
-0.0625	Rhamnetin 3-O- β-hexoside	-0.2142	Rhamnetin 3-*O*-α-arabinofuranoside	-0.1605	3-*O*-caffeoylquinic acid
-0.1330	Rhamnetin 3-*O*-α-arabinofuranoside	-0.2266	3-*O*-(*E*)-*p*-coumaroylquinic acid	-0.2203	Unknown

6.3.3 Principal Component analysis of data set 3

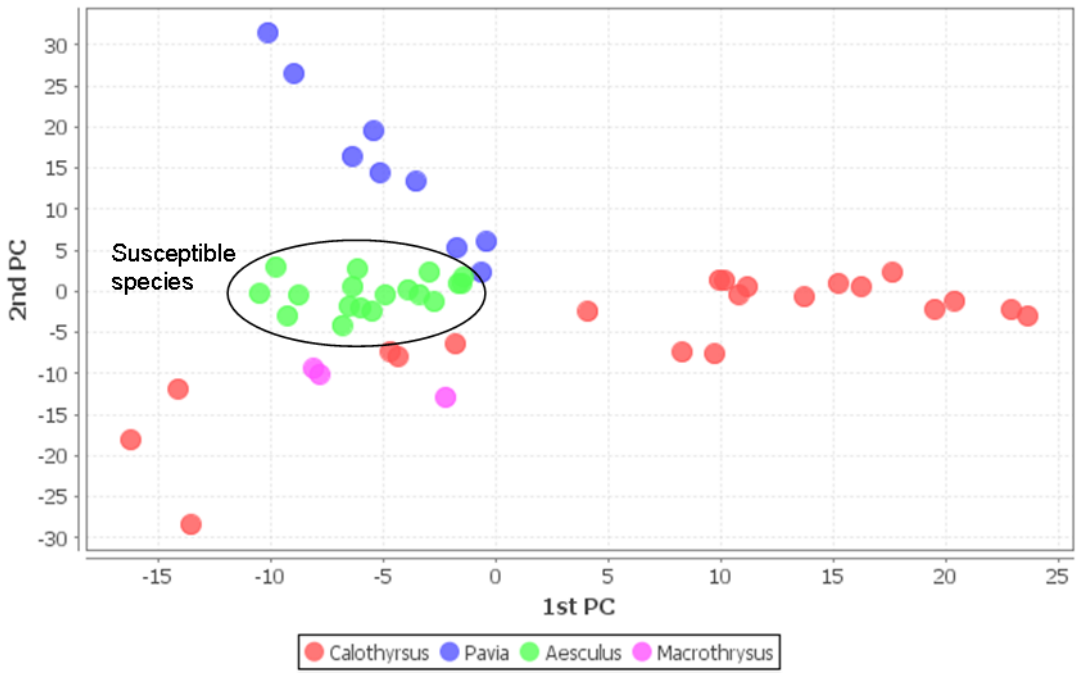


Figure 6.3 Results of PCA analysis of data set 3. The different colours indicate the different sections of *Aesculus*. Species belonging to the sections *Calothyrsus*, *Pavia* and *Macrothyrsus* used in this study are resistant, whereas, species belonging to the section *Aesculus* are susceptible

PCA analysis of mature trees in RBG Kew also showed that the susceptible species tend to group together and the resistant species show more variability among them (Figure 6.3). The first three PC axes in this data set has explained 34% of variance within the data set (PC1 20%, PC2 8% and PC3 6% respectively).

Table 6.4 PCA loadings of data set 3

PC 1		**PC 2**		**PC 3**	
Loading	Compound	Loading	Compound	Loading	Compound
0.4290	Epicatechin	0.2128	Quercetin 3-*O*-α-rhamnopyranoside	0.5820	Unknown
0.3825	Quercetin 3-*O*-α-rhamnopyranoside	0.1863	Coumaroylshikimic acid	0.2316	Flavanone hexoside
0.2311	Unknown	0.1607	5-*O*-*p*-coumaroylshikimic acid	0.1172	3-*O*-caffeoylquinic acid
0.1645	Quercetin 3-*O*-α-arabinofuranoside	0.1515	3-*O*-(*E*)-*p*-coumaroylquinic acid	0.0979	Unknown
0.1459	Procyanidin trimer (1)	0.0927		0.0974	Saponin (1)
-0.0968	Unknown	-0.1649	Quercetin 3-*O*-α-arabinopyranoside	-0.1304	Quercetin 3-*O*-α-rhamnopyranosyl(1→6)-β-glucopyranoside
-0.1053	Kaempferol 3-*O*-α-rhamnopyranosyl(1→6)-β-glucopyranoside	-0.1743	Rhamnetin 3-*O*-α-arabinofuranoside	-0.1782	Unknown
-0.1068	Chlorogenic acid	-0.2006	Unknown	-0.2210	Rhamnetin 3-*O*-α-rhamnopyranoside
-0.1665	Quercetin 3-*O*-α-rhamnopyranosyl(1→2) α-rhamnopyranosyl(1→6)-β-glucopyranoside	-0.2831	Quercetin 3-*O*-α-rhamnopyranosyl(1→6)-β-glucopyranoside	-0.3010	Rhamnetin 3-*O*-α-arabinofuranoside
-0.5022	Quercetin 3-*O*-α-rhamnopyranosyl(1→6)-β-glucopyranoside	-0.6100	Unknown	-0.3198	Epicatechin

The compounds contributing most to the first three PC axes were mainly phenolics and phenolic acids (Table 6.4).

6.3.4 Result of Z-factor analysis

Z-factor analysis of the three data sets revealed that compounds which clearly separated the susceptible and resistant groups were mainly found or were present in higher concentrations in the susceptible groups, with the exception of the hybrid data set (Table 6.5). Compound 1 (*m/z* 183 at retention time 2.4 minutes) was present in data sets 1 and 3.

The only compound which was present in higher concentration in the resistant species was Compound 6 (*m/z* 471.2 at retention time 6.3 minutes) in data set 2. Differences in intensity of the compounds were often tenfold between susceptible and resistant classes.

Table 6.5 Compounds detected with Z-factor > -2 in the different data sets. Numbers in bold indicate that the mean intensity of that compound is higher in the given group. SD is the standard deviation and μ represents the group mean, 'res' stands for resistant and 'sus' stands for susceptible groups. Rt is the retention time and *m/z* is the mass to charge ratio of a given compound

Data set 1 (Greenhouse species)							
	m/z	Rt	Z-factor	μ res	μ sus	SD res	SD sus
Kaempferol 3-*O*-rhamnopyranoside	431.2	12.9	-0.67	12325677	**82003076**	8123617	30705030
Compound 1	183.0	2.5	-0.76	1606980	**16606363**	3236652	5584476
Compound 2	477.2	12.9	-1.41	332006	**7204135**	854858	4663425
Compound 3	367.1	2.5	-1.53	81426	**6115559**	430865	4648068
Data set 2 (Greenhouse hybrids)							
	m/z	Rt	Z-factor	μ res	μ sus	SD res	SD sus
Compound 6	471.2	6.3	-1.65	**9327108**	327611	7098257	839402
Data set 3 (Mature trees)							
	m/z	Rt	Z-factor	μ res	μ sus	SD res	SD sus
Compound 1	183.0	2.5	-1.41	2406	**66266**	8481	42901

6.4 Discussion

Data analyses revealed that *Aesculus* species and hybrids which are susceptible to *C. ohridella* share chemical similarities. Compound 1 is a candidate compound which could play a role in the mechanism of susceptibility of species of *Aesculus* towards *C. ohridella*. This compound had a high Z-factor in two out of three data sets (data sets 1 and 3). In all of these two data sets no candidate compound with a sufficiently high enough Z-factor showed up as a potential candidate in the resistant group which indicates that different mechanisms are present in the chemical defence of the different resistant species and hybrids of *Aesculus* and resistance is not confined to the presence/absence or the higher concentration of a common compound.

In contrast, in the hybrid sapling data set none of the compounds had a sufficiently high enough Z-factor which determines susceptibility. Compound 1 was present in the resistant hybrid *A.* × *carnea*, which otherwise also shows high chemical and trait similarity to its susceptible parent species *A. hippocastanum* as it was revealed from both PCA plot presented in this chapter and trait comparison presented in Chapter 4. This could explain why *C. ohridella* can initiate mines on *A.* × *carnea*, but not on most of the other species and hybrids of *Aesculus.*

Analysis of the hybrid data set also revealed one compound (Compound 6) with a high Z-factor which was present in the resistant parent species *A. pavia* and the resistant hybrid *A.* × *carnea*, but was absent from the susceptible *A.*

hippocastanum and either completely absent or present in low concentrations in some individuals in the susceptible *A.* × *carnea* 'Plantierensis'. This so far is the only one difference found which distinguishes *A.* × *carnea* and *A.* × *carnea* 'Plantierensis' from each other chemically. It is highly likely that Compound 6 plays a role in the mechanism of resistance of *A.* × *carnea* and could also be responsible for the early larval death found both in this study (Chapter 4) and other studies as well. Early larval instars are usually more prone to the adverse effects of secondary metabolites (Zalucki *et al*., 2002).

This study indicates that the different resistant species of *Aesculus* have different defence mechanisms. For example, 3-*O*-(*E*)-*p*-coumaroylquinic acid, which contributed to the first two principal component axes of data set 1 and 2 and to the second principal component axes of data set 3, also showed biological activity against *C. ohridella* in Chapter 5.

On the other hand, low concentration or absence or Compound 1 could also render a species resistant. However, it is very difficult to conduct a bioassay, testing the biological effect of Compound 1, as mines are not even initiated in most of the resistant species and hybrids and thus even if one could introduce Compound 1 into the leaf tissue, larvae most probably would not come into contact with it.

It is extremely difficult to rear leaf miners on artificial diets as leaf miners besides optimal nutrition also require optimal microclimate, which is very difficult to mimic. Therefore, it is yet to be proven whether compounds

detected in this study which differentiated resistant and susceptible species indeed play a biological role in the mechanism of susceptibility to *C. ohridella*. As metabolomic analysis only compares metabolites which are detected with the given instrumentation and obtained from the given extraction method, it is very likely that a lot of other metabolites which were either not extracted or detected could play an important biological role in the defence mechanism of *Aesculus* species against *C. ohridella*. There are several analytical methods which can be used for the direct analysis of metabolites without chromatographic separation such as Direct infusion mass spectrometry (DIMS), Matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS) or Desorption electrospray ionisation (DESI). Some of these techniques require no sample preparation and can be used to analyse plant tissues directly (Bedair & Sumner, 2008). These techniques have the advantage of analysing a much wider range of secondary metabolites and thus can detect compounds which are not detectable or extractable with the technique used in this study.

Chapter 7

General discussion

This chapter summarises the key findings of the thesis in relation to the original aims (section 7.1) and evaluates experimental approaches (section 7.2). This will be followed by suggestions offered for further work (section 7.3), and finally discussion of the implications of the findings for the management of *Cameraria ohridella* (section 7.4).

7.1 Thesis aims and findings

This study was set up to investigate resistance and susceptibility towards *Cameraria ohridella* within the genus *Aesculus* in order to further our understanding about the host plant use of *C. ohridella*. The summary of the approach used to determine whether a species is resistant or susceptible is illustrated in Figure 7.1. Oviposition and infestation patterns of the different species of *Aesculus* in field conditions were investigated in Chapter 2, while oviposition choice and larval development in experimental set ups were examined in Chapters 3 and 4. In addition physical and chemical traits, which may influence oviposition and larval development was discussed in Chapters 3 and 4. The secondary metabolite profiles of the different species and the effects of phenolics and saponins on larval development were investigated in Chapter 5. Finally the metabolic profiles of the different species of *Aesculus* were

compared in order to investigate whether the susceptible and resistant species can be separated from each other based on their metabolic profiles (Chapter 6).

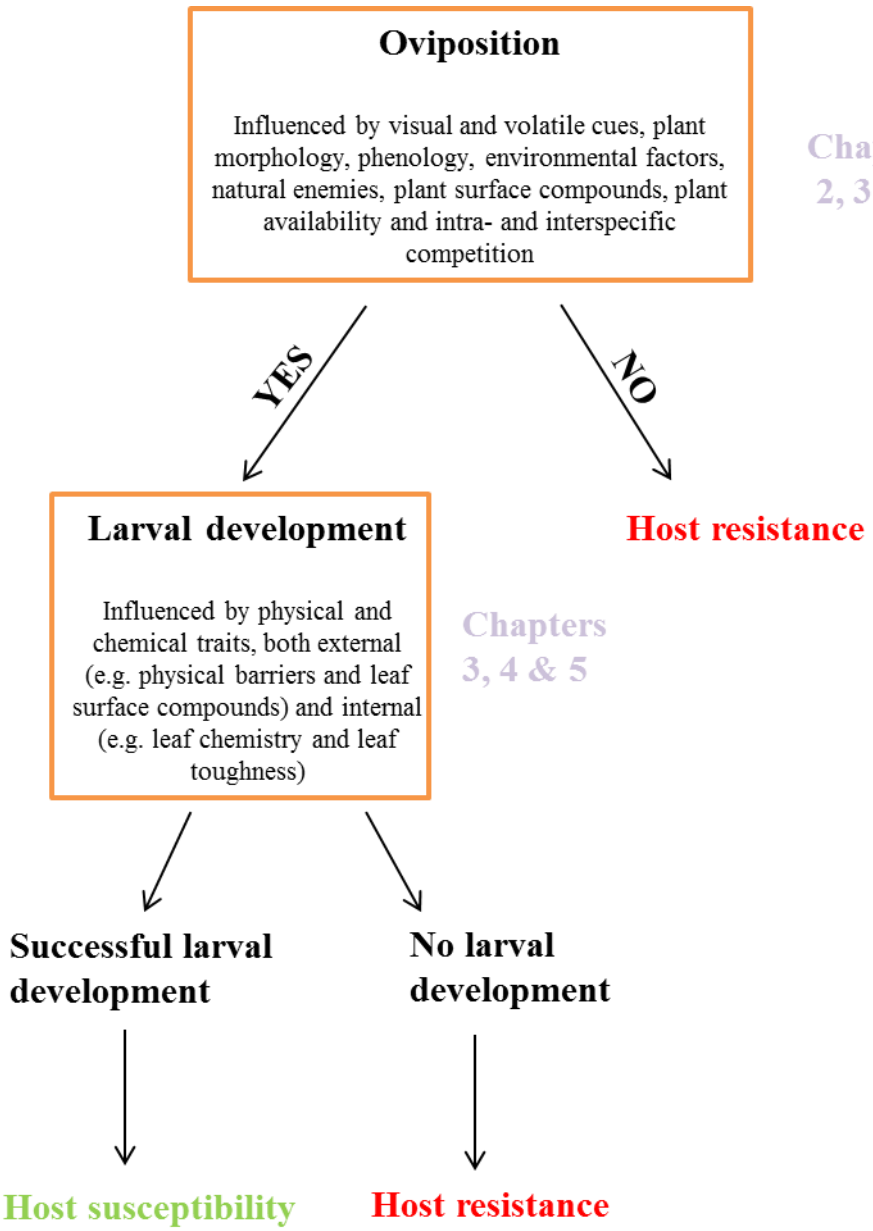


Figure 7.1 Brief outline of the determination of resistance and susceptibility

As leaf miners are sessile herbivores the oviposition choice made by the adult female is one of the main aspects determining whether a species will experience herbivory, as even though a given species would be a good potential host, but not oviposited on by females, would render that species resistant (Figure 7.1). Therefore, this study first set out to investigate in Chapter 2, which species are oviposited on by *C. ohridella* in field condition and in experimental set ups (Chapter 3 and 4).

When females look for oviposition sites the long distance orientation is influenced by visual and volatile cues. Most of the studied *Aesculus* species share similar phenology and morphology, but there are differences in leaf size among the species. Leaf miners generally prefer larger leaves for oviposition as the full larval stage is spent within the leaf tissue (Bultman & Faeth, 1986). However, this was not the case in *C. ohridella* where some of the *Aesculus* species which have large leaves (e.g. *A. indica* and *A. assamica*) received fewer eggs than other species (e.g. *A. flava* and *A. pavia*) whose leaves are smaller in size.

Volatile cues which are another important factor for ovipositing females could also influence adult choice. However, volatiles emitted by the main host *A. hippocastanum* and the resistant hybrid *A.* × *carnea* were found to be rather similar and female antenna responded to identical compounds emitted by both plants in the GC-EAD experiments (Kalinová, *et al*., 2004).

Upon landing, females come into contact with ovipositional cues present on the leaf surface and it is often found that females carry out suitability assessments by 'drumming', in which they assess the leaf surface with their forelegs, therefore comparison of leaf surface chemicals could indicate which compounds may influence the decision making of *C. ohridella* females.

The study found that even though *C. ohridella* showed preference towards some species of *Aesculus* over others, all twelve species of *Aesculus* investigated in this study were oviposited on. Therefore the focus of this study was orientated towards the determination as to why some species can support larval development while others cannot. Among the many factors which could influence larval development leaf physical and chemical characteristics were chosen for the investigation. As results obtained in Chapter 3 and 4 indicated that phenolic compounds may influence host susceptibility; these compounds were chosen for further investigation of their effects on larval development in Chapter 5 along with saponins; a group of secondary metabolites known to have a negative effect on herbivores. Many traits which could also play a role in host susceptibility were not included in this study and would require further investigation to fully understand the factors which determine host suitability. Examples of these are leaf surface compounds, detailed nutrient analysis and analysis of other groups of secondary metabolites.

In Chapter 1 the following questions were formulated which the thesis aimed to address in order to further our understanding about host susceptibility and resistance to *C. ohridella*:

Question 1: Which species and hybrids of *Aesculus* can support larval development and does successful development correlate with high egg densities?

Six out of the eleven investigated species of *Aesculus* (Chapter 2) supported some degree of larval development. These species were *A. hippocastanum*, *A. turbinata*, *A. flava*, *A. pavia*, *A. glabra* and *A. sylvatica*. The most susceptible hosts were *A. hippocastanum* and *A. turbinata*, and all individuals of these species supported full larval development and maintained heavy infestation levels throughout the vegetative season. In contrast, only some individuals of *A. flava*, *A. pavia*, *A. glabra* and *A. sylvatica* supported a degree of larval development, with very low infestation densities (< 2%), which did not increase with subsequent *C. ohridella* generations, as it did on *A. hippocastanum* and *A. turbinata*. Moreover, it was observed that in many cases *C. ohridella* developing on these species did not reach the pupal stage. In the experimental setup (Chapter 3), *A. flava* and *A. pavia* were found to be resistant and did not support full larval development and pupation.

The remaining five species *A. californica*, *A. indica*, *A. wilsonii*, *A. assamica* and *A. parviflora* (Chapter 2) were completely resistant to the miner and did not support any larval development. In the oviposition and larval development

experiment (Chapter 3), no individuals of *A. indica* and *A. chinensis* were able to support larval development of *C. ohridella*. These results are consistent with previous studies (Kenis *et al*., 2005). Infestation levels correlated with high egg densities. *A. hippocastanum* and *A. turbinata* received the highest number of eggs.

Out of the eleven hybrids monitored (Chapter 2) throughout the three year period, only *A.* × *dallimorei* and *A.* × *carnea* 'Plantierensis' were heavily infested, whereas, the other hybrids remained completely mine free. One, *A.* × *bushii* tree was found to be slightly infested (0.5%) in RHS Wisley in 2011.

It is important to note that all hybrids were a cross between species, either within the section *Pavia* or between species from section *Aesculus* and section *Pavia* and no hybrids were available with parent species either from section *Calothyrsus* or *Macrothyrsus*. Species belonging to section *Pavia* may sometimes support some degree of larval development, while all species in section *Aesculus* are good hosts for the leaf miner.

Question 2: How strong is the preference-performance relationship of *C. ohridella* on *A. hippocastanum* and other species of *Aesculus* which are novel to the miner?

The preference-performance of *C. ohridella* was found to be rather weak (Chapters 2, 3 and 4). Although *C. ohridella* laid significantly higher number of eggs on species where the larvae could successfully develop (*A.*

hippocastanum, *A. turbinata* and *A.* × *carnea* 'Plantierensis'), females also laid eggs on all the other species, which do not support larval development. The preference-performance linkage is often weak when herbivores interact with non-native plants (Jaenike, 1990; Thompson & Pellmyr, 1991; Gripenberg *et al*., 2010; Pearse, 2011), or if the herbivore experiences high population densities (Videla *et al*., 2006). These 'oviposition mistakes' can provide a stepping stone for the expansion of the host plant range of *C. ohridella*. Results of Chapter 2 indicated that although this may be a slow process it is already happening as some species in RBG Kew (e.g. *A. pavia*) became slightly infested in 2011, whereas in the preceding two years it was completely resistant.

Question 3: Is preference and performance of *C. ohridella* phylogenetically conserved on the different species of *Aesculus*?

The preference and performance of herbivores is often linked to host phylogeny, when herbivores interact with novel hosts (Rasmann & Agrawal, 2011), and it has been suggested that the resistant/susceptible status of the different species of *Aesculus* is also linked to phylogeny (Straw & Tilbury, 2006). The results obtained in Chapter 2 and 3 indicate that preference and performance of *C. ohridella* is indeed phylogenetically conserved. *A. hippocastanum* and its sister species *A. turbinata*, both belonging to the section *Aesculus*, were found to be highly susceptible, whereas, species belonging to the section *Calothyrsus* (*A. indica*, *A. chinensis*, *A. assamica*, *A. wilsonii* and *A. californica*) and section *Macrothyrsus* (*A. parviflora*) are completely resistant

to the miner. Species belonging to the section *Pavia* (*A. pavia*, *A. sylvatica*, *A. glabra* and *A. flava*) can show minimal damage, therefore, are not completely resistant.

Question 4: Which leaf traits influence oviposition preference and larval performance of *C. ohridella*?

Leaf toughness was the only trait which clearly separated susceptible and resistant species in Chapter 3, and it could be also the trait which influenced the performance of *C. ohridella* larvae developing on *A. carnea* × 'Plantierensis' in Chapter 4. The leaves of the two susceptible species where larval performance was the best (*A. hippocastanum* and *A. turbinata*) had significantly less tough leaves than any other species or hybrids studied in Chapters 3 and 4. Leaf anatomy investigations showed that *A. hippocastanum* has relatively thin outer walls of the epidermical cells and not well developed cuticle layer on the surface and hence provides no sufficient protection against *C. ohridella* (Weryszko-Chmielewska & Haratym, 2011; Weryszko-Chmielewska & Haratym, 2012). As young larvae of *C. ohridella* need to burrow into the leaf tissue, leaf tissue toughness is an important mechanical barrier, which the larvae need to overcome.

The results of this study also showed that oviposition preference of *C. ohridella* is influenced by phenolics. Species with high egg density in Chapter 3 had high concentration of phenolics. Results in Chapter 4 further showed that different phenolic compounds had different effects on egg density.

The resistant species *A. pavia* had significantly higher levels of free amino acids. As individual chemical compounds were not tested separately in bioassays with *C. ohridella*, it is difficult to tease apart the role of individual traits which could account for the resistant and susceptible status of the different species of *Aesculus*. However, it is assumed that usually it is not a single trait which determines resistance and susceptibility, but rather the combination of different traits (Thompson & Pellmyr, 1991; Agrawal & Fishbein, 2006; Agrawal, 2011; Carmona *et al*., 2011). However, it is interesting that the two susceptible species (*A. hippocastanum* and *A. turbinata*) shared trait similarities and clustered closely together in the Principal Component Analysis (Chapter 3). When a herbivore attacks a novel host (*A. turbinata* was a novel host for the miner in Chapter 3), both trait similarity and phylogeny predicts the level of herbivory (Pearse & Hipp, 2009). The results of the Principal Component Analysis in Chapter 3 also suggested that resistant species of *Aesculus* have different defence mechanisms.

Question 5: How does hybridisation of the main host *A. hippocastanum* affect leaf traits and the preference and performance of *C. ohridella*?

Results obtained in Chapter 4 suggested that resistance to *C. ohridella* is inherited as a dominant trait in *A.* × *carnea*. However, the resistance to the miner is lost when *A.* × *carnea* is backcrossed to *A. hippocastanum*. This breakdown of resistance is often observed in backcrosses to the susceptible parent (Fritz *et al*., 1999). The results of Chapter 4 also showed that although

preference of *C. ohridella* did not differ significantly between *A. hippocastanum* and the backcross *A.* × *carnea* 'Plantierensis', *C. ohridella* larvae experienced reduced fitness on the backcross, thus, the performance of the larvae was much better on *A. hippocastanum*.

The results of Chapter 4 indicate that the hybrid *A.* × *carnea* and the backcross *A.* × *carnea* 'Plantierensis' were more similar in terms of traits to the susceptible parent *A. hippocastanum* than to the resistant *A. pavia*. However, there were traits which were inherited from the resistant parent species, such as the presence of rhamnetin glycosides. The results also indicated that traits which influence oviposition and traits which influence larval performance were not inherited together during hybridisation.

Question 6: How do phenolic and saponin profiles differ between species in the genus *Aesculus* and how do these compounds affect the oviposition preference and larval performance of *C. ohridella*? Do the phenolic and saponin profiles of the species show phylogenetic conservatism?

The results of Chapter 5 revealed that both phenolic and saponin distribution within the genus *Aesculus* is linked to phylogeny and different sections within the genus could be clearly separated, based on their phenolic and saponin profiles. Species belonging to the section *Pavia* (*A. flava, A. pavia, A. sylvatica* and *A. glabra*) were the only species where rhamnetin glycosides were detected. The two susceptible species *A. hippocastanum* and *A. turbinata*, which belong to the section *Aesculus*, had very similar phenolic and saponin

profiles. The findings further indicated that there is no single phenolic compound or saponin which is solely responsible for resistance, as there was no single compound which was consistently present in all the resistant species and absent from the susceptible ones. This finding once more indicates that the different resistant species of *Aesculus* defend themselves against *C. ohridella* through different defence mechanisms.

Bioassays testing the effects of phenolic compounds obtained from resistant species *A. pavia* (section *Pavia*) and *A. indica* (section *Calothyrsus*) found that phenolics did not affect *C. ohridella* larvae negatively. The results also indicated that some phenolics found in the susceptible species, *A. hippocastanum*, could act as an oviposition stimulant for *C. ohridella*, as leaves treated with phenolic extracts of *A. hippocastanum* had higher number of eggs. Phenolic compounds present in the host of specialist insects are often found to be oviposition or phagostimulants (Honda, 1995).

Although the study found that saponin fractions applied to leaf surfaces reduced the number of eggs laid by *C. ohridella*, only saponin fraction obtained from *A. indica* affected larval development negatively. This indicates that some saponins may have greater biological activity against *C. ohridella* than others. However, these effects are negligible compared to the pesticide treatments available for the control of *C. ohridella* (Chapter 1).

Question 7: Can metabolomic profiling differentiate resistant and susceptible species and hybrids of *Aesculus*?

Metabolomic profiling (Chapter 6) revealed 2 compounds (1 and 6) that show potential as candidates, which distinguish between susceptible and resistant species and hybrids of *Aesculus*. Compound 1 was present in all susceptible species and absent from species belonging to the resistant sections *Macrothyrsus* (*A. parviflora*) and *Calothyrsus* (*A. indica*, *A. chinensis*, *A. wilsonii*, *A. assamica* and *A. californica*). This compound was also present in species belonging to section *Pavia* which shows varying susceptibility to the miner.

Susceptible species were grouped together in the Principal Component Analysis of the secondary metabolite profiles (Chapter 6), whereas resistant species grouped away from each other, indicating once again that different resistant species may share different resistance mechanisms and susceptible species have similar chemical profiles. The resistant hybrid *A.* × *carnea* grouped together with the susceptible parent *A. hippocastanum*, indicating that they share a similar chemical profile.

So far it was stressed that phylogeny of the genus *Aesculus* predicted suitability for *C. ohridella*. However it is important to point out that species within the section *Pavia* (*A. flava*, *A. glabra*, *A. pavia* and *A. sylvatica*) which show intermediate susceptibility towards *C. ohridella*, also the host of *Cameraria aesculisella*; a leaf miner closely related to *C. ohridella* and the susceptible *A.*

turbinata from section *Aesculus* also hosts *Cameraria* species (Lakatos *et al*., 2004). On the other hand, species belonging to the sections *Calothyrsus* and *Macrothyrsus* which are resistant to the miner do not host any other *Cameraria* species. Therefore, besides host phylogeny, the evolutionary history of *C. ohridella* could also be a determinant factor as to which species it can develop on as it probably did not share coevolutionary history with species belonging to the section *Calothyrsus*.

It has been a central interest of biologist to determine factors which influence the evolution of insect-plant interactions. There are three alternative scenarios which explain the host plant use of mono- and oligophagous insects: 1) parallel cladogenesis; where insects which are associated with their host population might get isolated with host populations which can result in allopatric cospeciation, 2) mediated by secondary compounds; where the herbivore is preadapted to a host whose secondary metabolites are similar to the original host, therefore host shift would occur on chemically similar plants and 3) geographical availability; where herbivores shift to plant species within the geographic distribution of the original host (Becerra & Venable, 1999).

In the case of *C. ohridella* the study showed that host plant use was not related to the geographical origin of the plant species but rather to host phylogeny and chemistry. In the present study it was difficult to tease apart the differences between the influence of phylogeny and host chemistry as both phenolic and saponin profiles of the different species showed a strong correlation to host

phylogeny. However, many studies have concluded that host shifts are more strongly correlated with secondary chemistry than host phylogeny (Becerra & Venable, 1999; Futuyma & Agrawal, 2009). Chemical similarity often determines host suitability as is the case of Brassicaceae specialists also found on Tropaeolaceae, which have cardiac glycosides (Lopez-Vaamonde *et al*., 2003). Also, introduced plant species are first colonised by herbivores which are adapted to chemically similar plants (Futuyma & Agrawal, 2009). Results of Chapter 5 and 6 indicated that the two most susceptible species *A. hippocastanum* and *A. turbinata* were very similar chemically. In overall the study suggested that *C. ohridella* feed on hosts which are chemically similar.

7.2 Advantages and limitations of the experimental approaches adapted in this study

Botanical gardens provided the advantage of investigating *C. ohridella* infestation and oviposition patterns (Chapter 2) in a natural setting under similar field conditions. Further advantage was that infestation patterns could be assessed on mature trees, rather than on saplings. This is important, because most previous studies which investigated the host plant range of *C. ohridella* were carried out using saplings, due to the lack of availability of the different species of *Aesculus* (only *A. hippocastanum* is a European species), and it is known that susceptibility to herbivores can change through ontogeny (Barton & Koricheva, 2010). The fact that the infestation monitoring of *C. ohridella* on the different species was carried out through three consecutive years ensured that the observed infestation patterns are constant in time.

A disadvantage of the botanical garden surveys described in Chapter 2 was that many species of *Aesculus* were represented by only one or a few individuals. Also, different species were located at different distances from infested *A. hippocastanum* trees, which could affect the results. However, it is important to note that individuals of the susceptible *A. turbinata*, *A.* × *dallimorei* and *A.* × *carnea* 'Plantierensis' which were located very far from *A. hippocastanum* trees still experienced high infestation levels.

Aesculus saplings were used in Chapter 3 to test the preference and performance of *C. ohridella* in an experimental setup. This approach ensured equal replicates of each species growing in identical conditions. The saplings used in this study were of approximately same age and height, which reduced the variability within a species and the use of a greenhouse minimised the influence of environmental conditions such as wind, rain, and drought. It also excluded predators, such as birds and arthropods. A further advantage of using this experimental setup is that the approximate time of oviposition by *C. ohridella* females could be recorded and hence the development time of *C. ohridella* on the different species could be compared, unlike under natural field conditions.

One drawback could be that in some cases, herbivores show preferences for a host on which they developed as larvae (Videla *et al*., 2010). In the bioassays conducted in Chapters 3 and 4, *C. ohridella* adults used for the oviposition experiments were reared from *A. hippocastanum* leaves. This could result in preference for *A. hippocastanum* as a host. It was not tested, but would be

interesting to know, whether oviposition choice of *C. ohridella* would be affected if adults were reared from an alternative host, e.g. *A. turbinata* or *A.* × *carnea* 'Plantierensis'.

The different leaf traits were assessed at the time of oviposition (Chapter 3 and 4), thereby ensuring that the results of the physical and chemical leaf characteristics were the true representatives of what *C. ohridella* females perceived. However, this was a disadvantage when testing the performance of *C. ohridella* larvae, which developed several weeks after oviposition as physical and chemical traits are not constant in time. This means that the physical and chemical characteristics experienced by the ovipositing *C. ohridella* adults might be different from the ones that the developing larvae experience. It is also not clear at present whether *C. ohridella* females can detect all the physical and chemical traits investigated. Due to the size of the saplings, it was not possible to remove more leaves from them at different time intervals, as the larval development progressed, because it would then affect the experiment, which aimed to monitor mine number and development. Also, the continuous removal of leaves could evoke induced responses from the saplings, which in turn could affect the larval development. Finally, it is also likely that the presence of *C. ohridella* larvae alters the chemical profiles of the species on which it develops.

Rearing Lepidopteran leaf miner species on artificial diets has not been achieved so far (Body *et al*., 2013); therefore conducting bioassays which could accurately test the biological effects of individual compounds is not

possible at the moment. Although bioassays used in Chapter 5 are generally used for leaf miners, none of them is perfect. It is difficult to get the tested compounds to the target location where *C. ohridella* larvae feed, as the distribution of the compounds within the leaf tissue is not known. Moreover, the larvae have the capacity of avoiding tissues which contain noxious secondary metabolites by changing direction when feeding. It has been previously shown that *C. ohridella* larvae avoid vascular bundles, idioblasts, tannin-containing epidermal cells and oil cells (Weryszko-Chmielewska & Haratym, 2011; Weryszko-Chmielewska & Haratym, 2012). Therefore, it is not known how much of the introduced compounds were ingested by *C. ohridella* larvae. Also the fact that the concentration of the compounds that the larvae ingest is relatively low and the fact that in most cases the applied treatments were mixtures of different compounds, makes quantification a difficult, if not an impossible, process.

There are other potential mechanisms which could influence the host-herbivore interaction of *C. ohridella* that were not tested in this study. Microbial interactions, which facilitate host utilisation, are often observed in Lepidopteran leaf miners. The most commonly known phenomena is the presence of 'green-islands', due to the accumulation of cytokinins which is mediated by bacterial symbionts. Leaf physiological manipulation can also take place on photosynthetically active tissues in order to enhance nutrients, as is the case of the leaf miner *Phyllonorycter blancardella*, which feeds on *Malus domestica* (Body *et al*., 2013). Therefore, it is possible that *C. ohridella* could

have some symbionts which could affect in some ways the microenvironment where the larvae feed.

7.3 Further work

The research conducted in this study opened up many new questions which would be worth pursuing further.

- In order to establish whether the presence of Compound 1 is required for mine initiation by *C. ohridella* larvae, bioassays need to be conducted to test the biological activity of this compound. This could be achieved by using *A. parviflora*, as this species lacks this compound, but has a simple chemical profile compared to the other species of *Aesculus*. It would be interesting to test whether the introduction of Compound 1 into the leaf tissue of *A. parviflora* could result in mine formation. This could be achieved by root irrigation of very small seedlings with high concentrations of Compound 1.

- It would be also useful to investigate other mechanisms that determine resistance and susceptibility. Leaf toughness has been shown as a trait conveying resistance to some species of *Aesculus*. However, *C. ohridella* larvae could still develop on *A.* × *carnea* 'Plantierensis', which has tough leaves, even though larval performance was compromised. In terms of the chemical profiles of the different species of *Aesculus*, it would be useful to expand the range of compounds

investigated in this study. However, there is one main challenge which needs to be addressed first:

It is still not known why *C. ohridella* cannot initiate mines on species belonging to the section *Calothyrsus*. It was shown in Chapter 2 and 3 that *C. ohridella* lays eggs on the species belonging to this section. However, the fate of the eggs is not known. Eggs of *C. ohridella* are ~ 0.3 mm in length and translucent. The newly emerged larvae burrow directly into the leaf tissue. To investigate what happens to the larvae would require monitoring the process through a microscope. However, the drawback is that the time of the egg hatching cannot be predicted (it can take from 10 to 14 days, and in some cases 3 weeks from the time of oviposition) and the light and heat from the microscope can dry the eggs out, and hence destroy them. Therefore, it is not known whether eggs die due to induced chemical defences or the larvae hatch and cannot penetrate through the leaf tissue. Therefore, it remains a possibility that the chemical profile of the resistant species would be suitable for the larvae given that mine initiation could occur.

- Comparison of leaf surface compounds of the different species would be also useful for investigating whether compounds found on the leaf surface could negatively influence the newly hatched larvae. If such compounds are found their affects would need to be investigated using bioassays.

- Results of the present study indicated that different species within the genus *Aesculus* have different resistance mechanism towards the leaf miner. Therefore, hybrids and backcrosses between species belonging to the resistant section *Calothyrsus* and *Macrothyrsus* and species from the susceptible section *Aesculus* would be a good study system to investigate how the altered leaf traits affects susceptibility towards *C. ohridella*, as in the present study only hybrids between species of section *Pavia* and section *Aesculus* were studied.

7.4 Implications of the findings for managing *C. ohridella* invasion

The present study was not aimed to develop a management strategy for the control of *C. ohridella*. However, the findings of this study furthered the understanding of the host plant use of the leaf miner, which could be beneficial in designing new control options.

The success of non-native herbivores in a novel environment is often associated with the escape from natural enemies in their native range (Desurmont *et al*., 2011). Parasitoids can successfully control leaf miner populations. The parasitism rate of *Cameraria niphonica* feeding on *Acer* spp. in Japan is 92% and parasitism rate of *Cameraria* sp., feeding on *A. turbinata* in Japan, is 76% (Kenis *et al*., 2005). Biological control is the most economical option for pest management. Results of Chapters 2 and 3 indicated that the preference and performance of *C. ohridella* on *A. hippocastanum* and *A. turbinata* are very similar and both of these species are equally good hosts. Furthermore, physical and chemical traits investigated in Chapter 3, chemical

and saponin profiles investigated in Chapter 5 and secondary metabolites investigated in Chapter 6 all revealed that these two species are very similar both in physical leaf traits and in leaf chemistry. These two species also share remarkable physical similarities in appearance (e.g. flowers, leaves and tree height) and similar phenology. It has been pointed out in the Introduction that *C. ohridella* lacks specialist parasitoids which could regulate the population density of *C. ohridella*. On the other hand, *A. turbinata* which hosts other *Cameraria* species in its native range (Japan), hosts parasitoids which maintain low levels of *Cameraria* sp. populations. It would be therefore advisable to investigate the parasitoid complex of *Cameraria* species present on *A. turbinata* in Japan, as some of these parasitoids could potentially become an excellent control agent for *C. ohridella* in Europe.

Findings of this study also suggests that *C. ohridella* does not pose a threat to species belonging to the section *Calothyrsus* (*A. indica*, *A. wilsonii*, *A. chinensis*, *A. assamica* and *A. californica*) and it is very unlikely that the leaf miner will switch host to these species in the near future. Therefore, it is recommended that if it is required to replace *A. hippocastanum* trees that species from this section is chosen for ornamental purposes as most of these species are equally attractive to *A. hippocastanum*.

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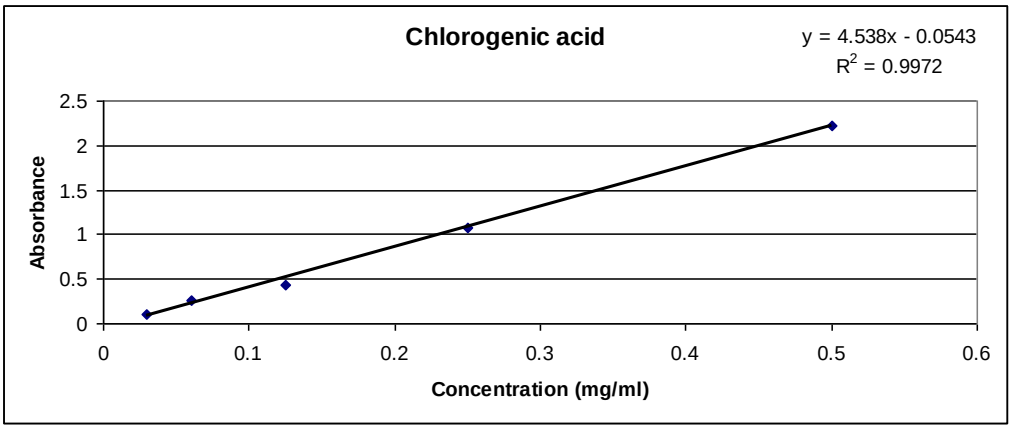
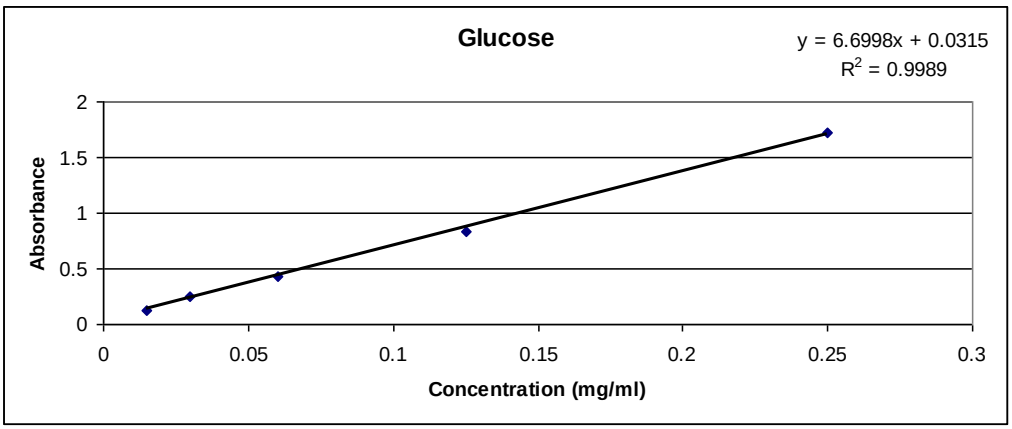
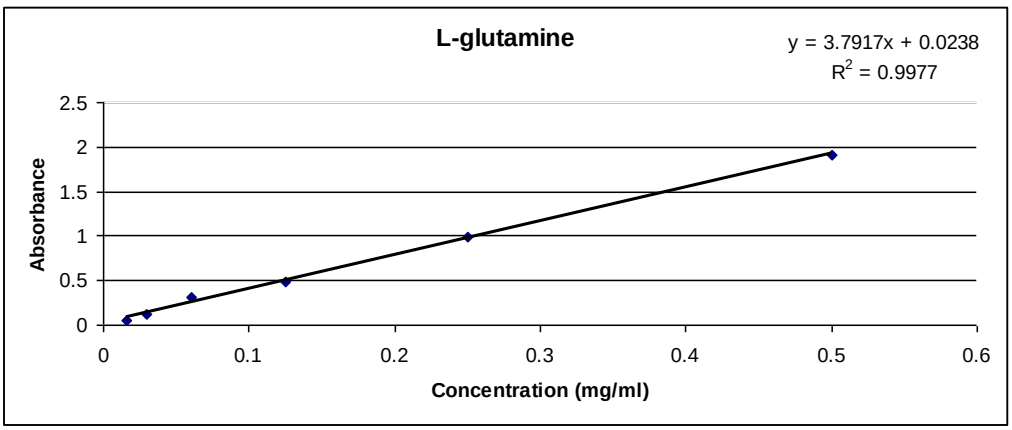
Appendix 1

Standard curves used for the determination of phenolic (chlorogenic acid), carbohydrate (glucose) and free amino acid (L-glutamine) content of the leaf material in Chapter 3



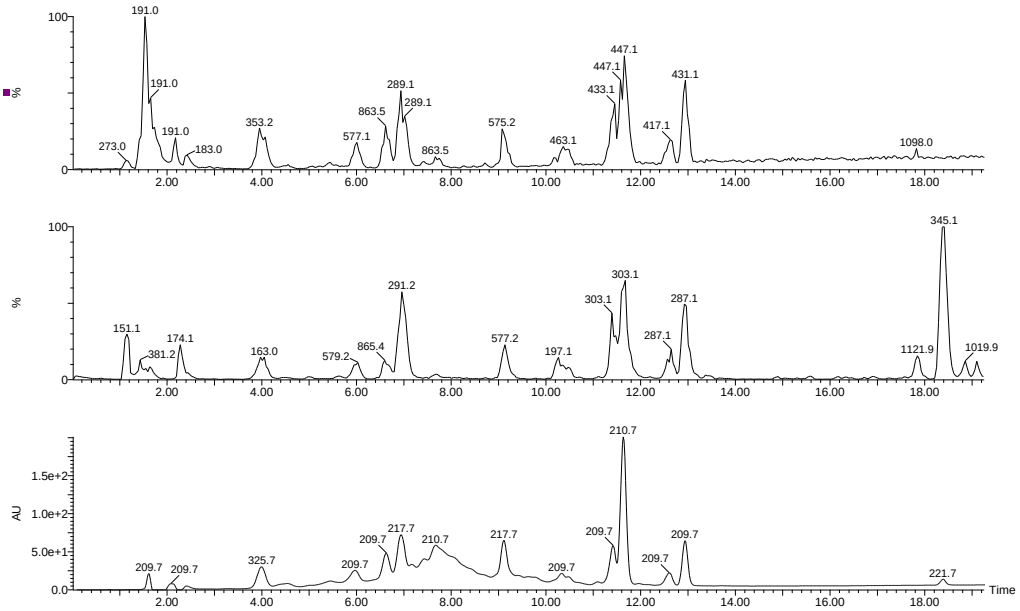




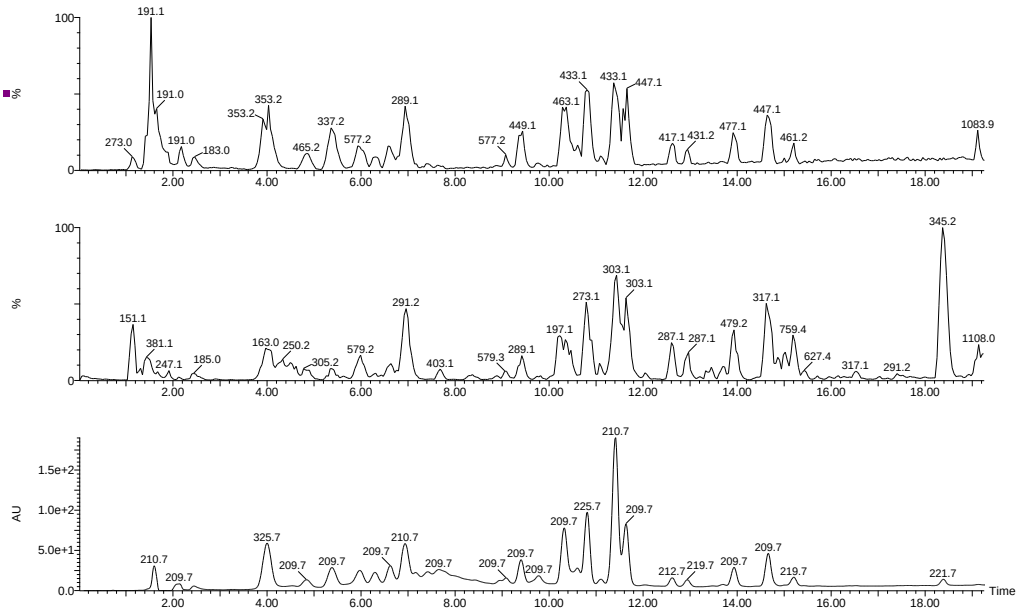
Appendix 2

Typical chromatograms of *A. hippocastanum*, *A. pavia*, *A.* × *carnea* and *A.* × *carnea* 'Plantierensis saplings studied in Chapter 4

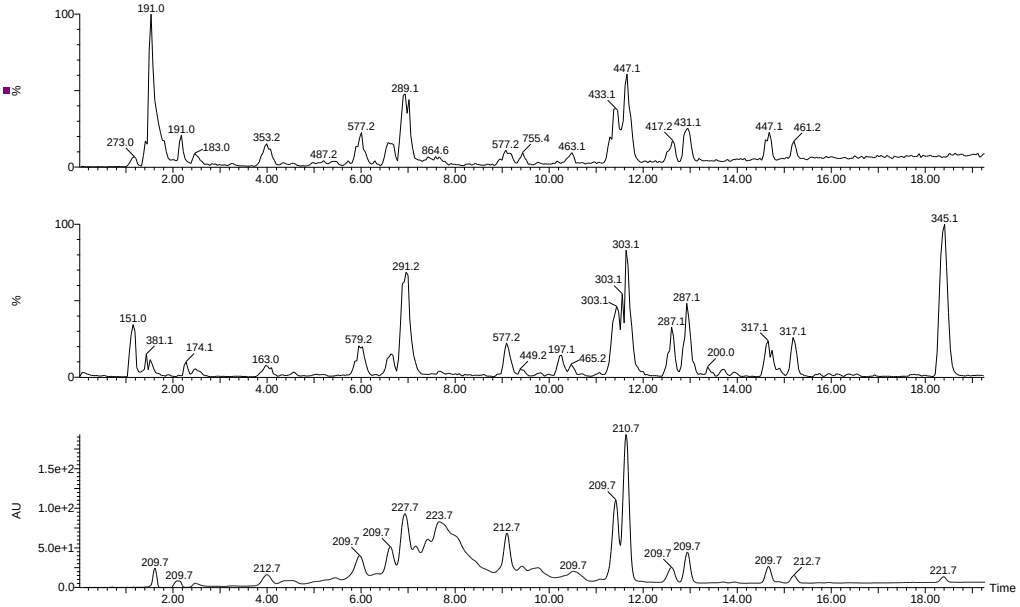
Mobile phase gradients 90:0:10 (t = 0 min, linear) 0:90:10 (t = 25 min) water/methanol/ [acetonitrile + 1% formic acid]



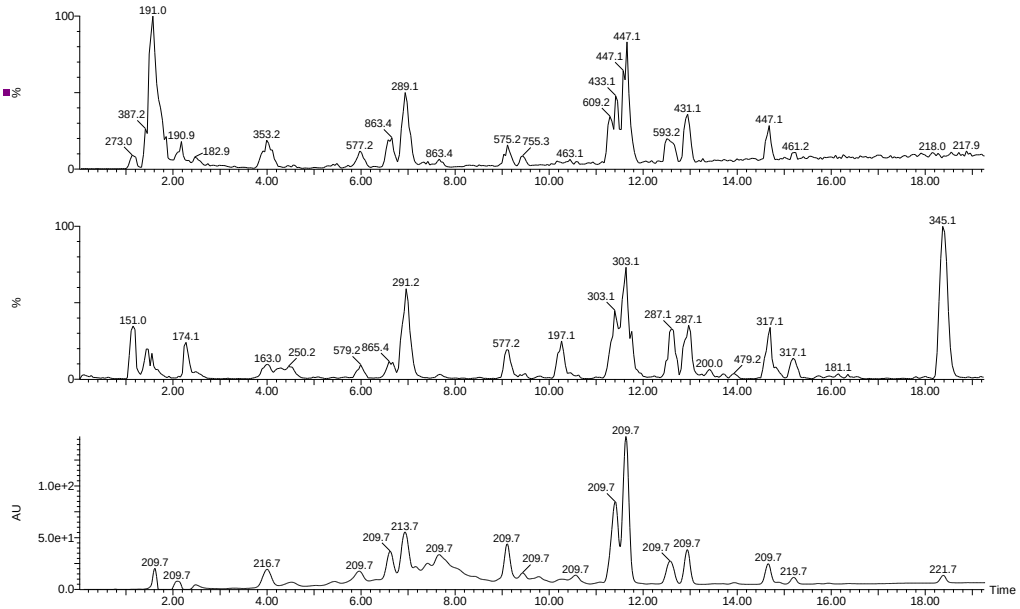
LC-MS chromatogram of *A. hippocastanum* sapling



LC-MS chromatogram of *A. pavia* sapling



LC-MS chromatogram of *A. carnea* sapling

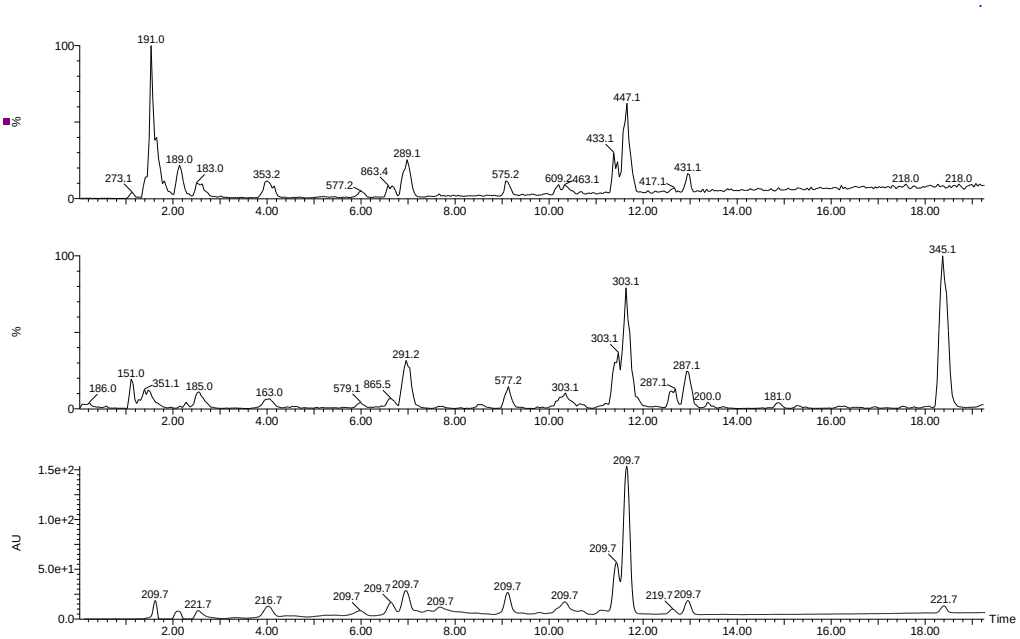


LC-MS chromatogram of *A.* × *carnea* ‘Plantierensis’ sapling

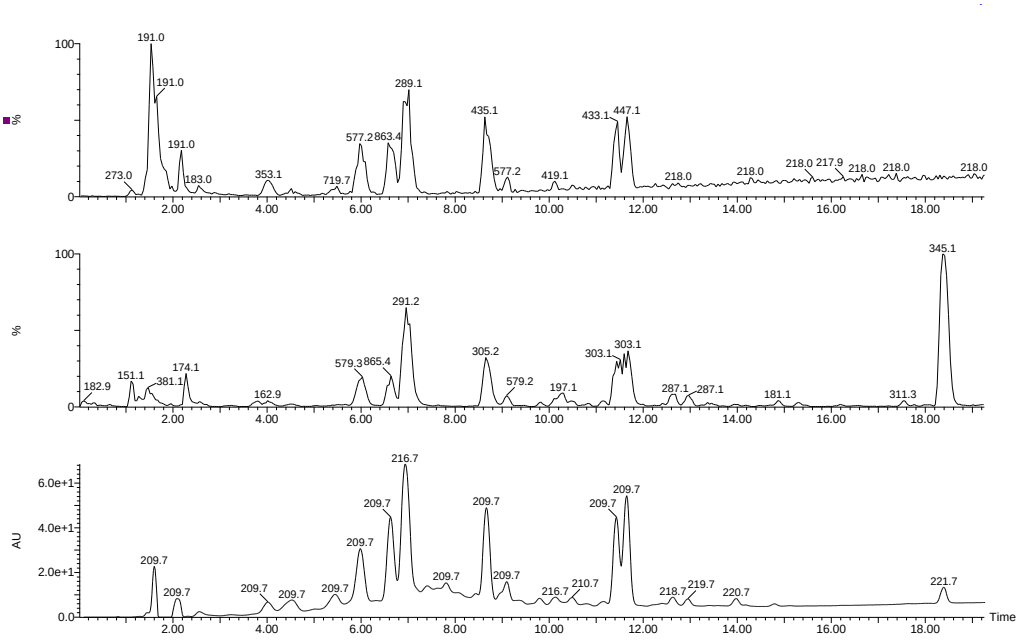
Appendix 3

Typical chromatograms of the different species of *Aesculus* studied in Chapter 5 (phenolics)

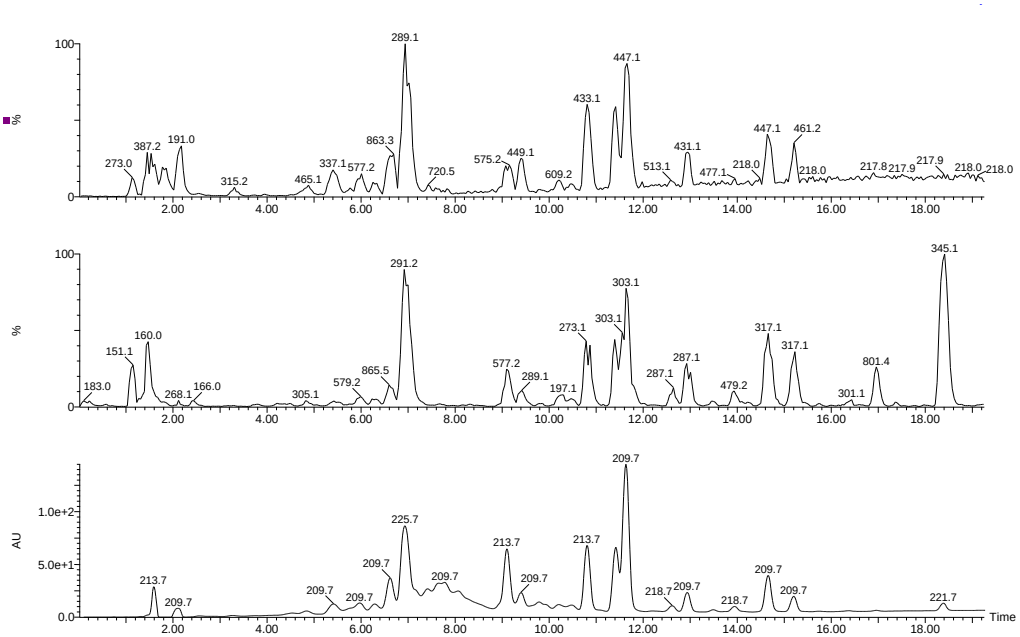
Mobile phase gradients 90:0:10 (t = 0 min, linear) 0:90:10 (t = 25 min) water/methanol/ [acetonitrile + 1% formic acid]



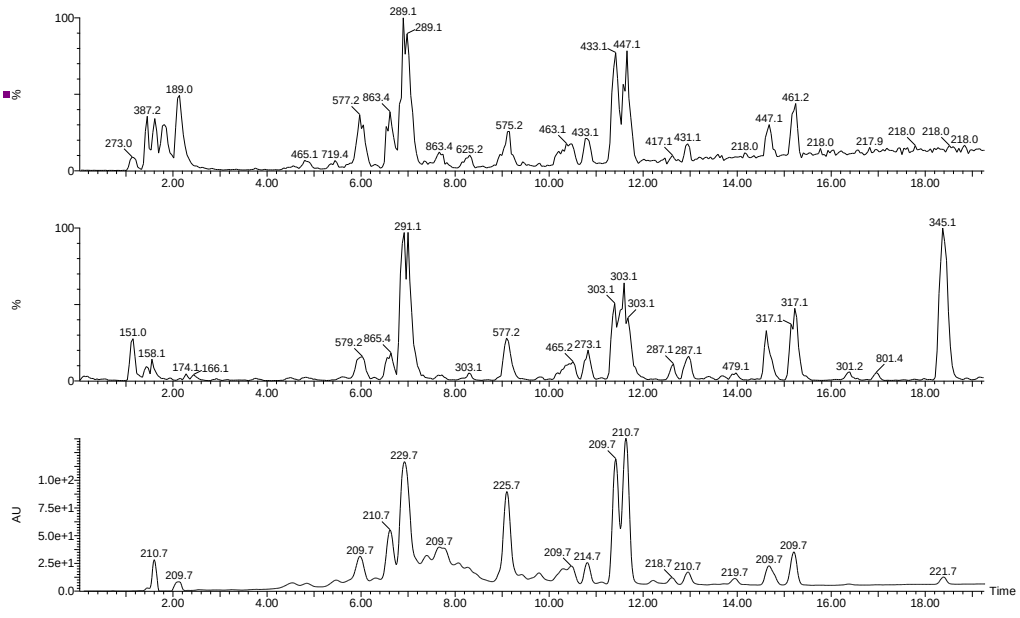
LC-MS chromatogram of *A. hippocastanum* L.



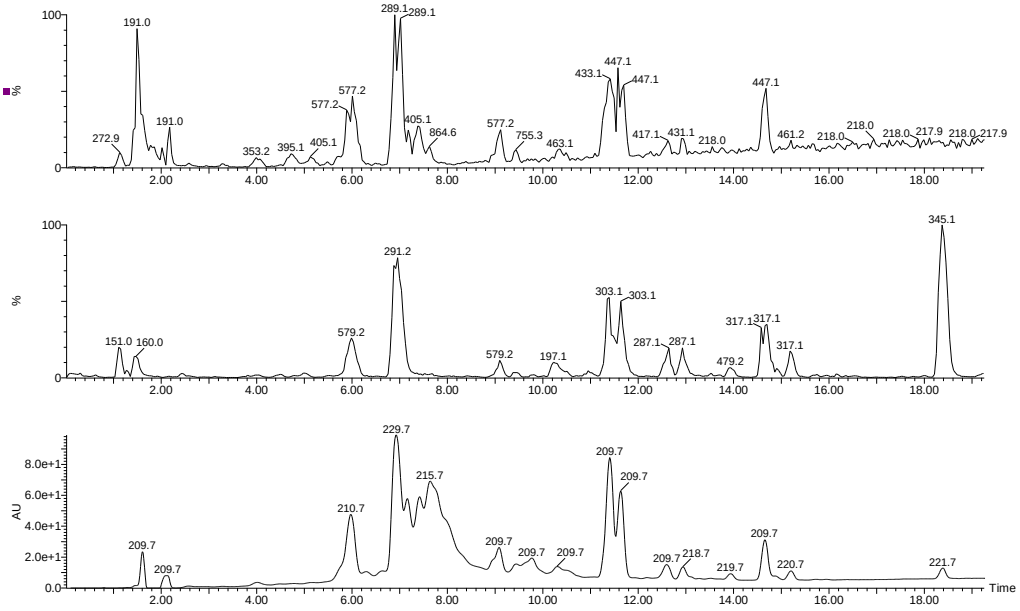
LC-MS chromatogram of *Aesculus turbinata* Blume.



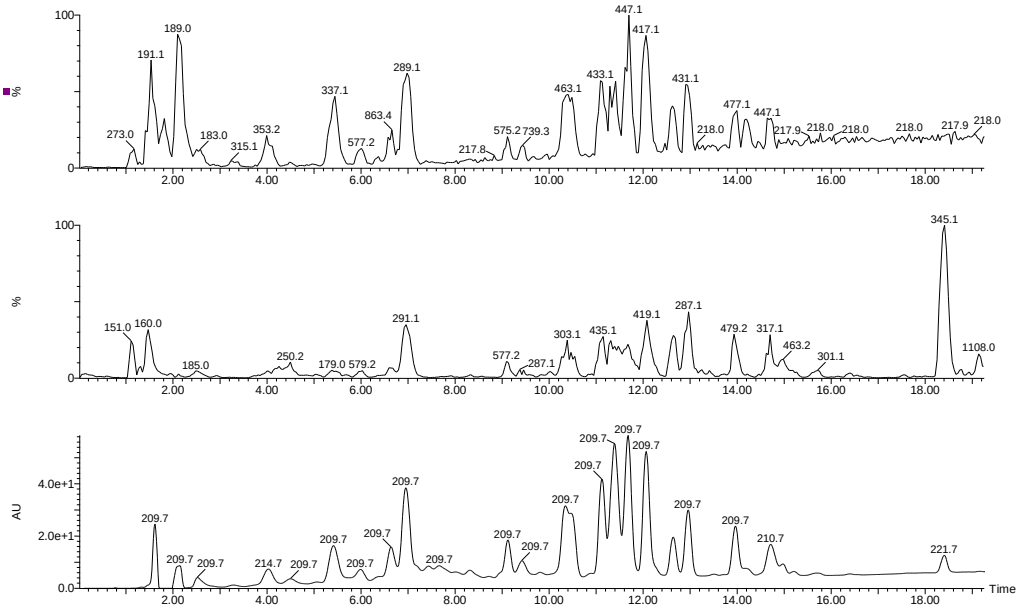
LC-MS chromatogram of *Aesculus flava* Aiton



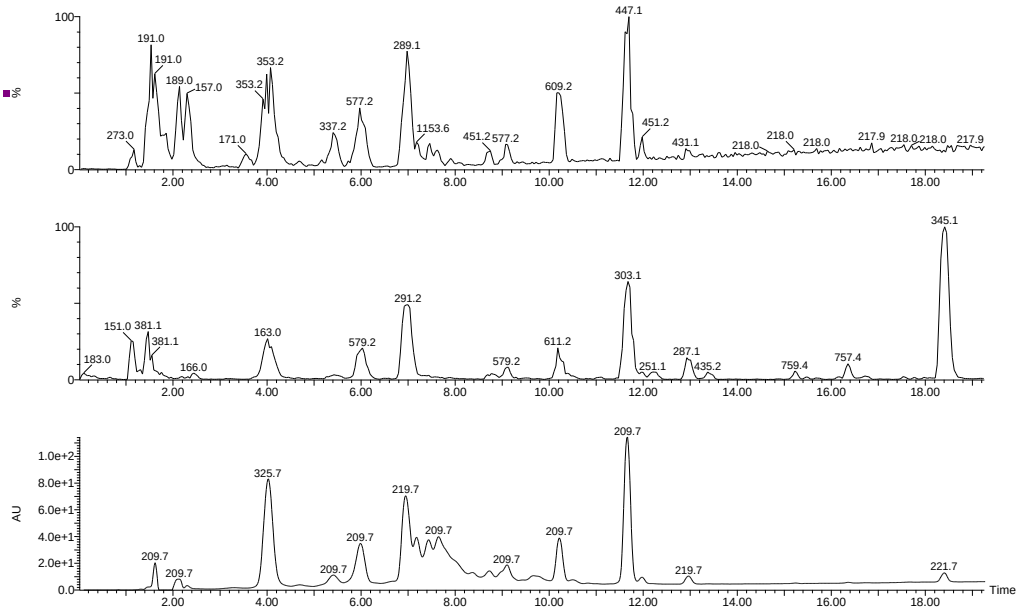
LC-MS chromatogram of *Aesculus sylvatica* Bartram



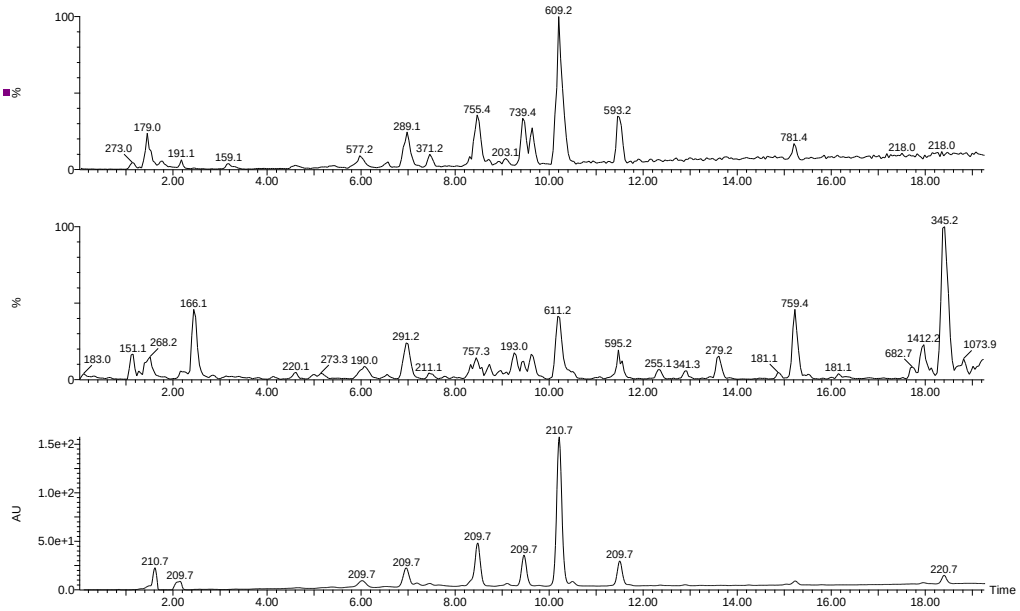
LC-MS chromatogram of *Aesculus pavia* L.



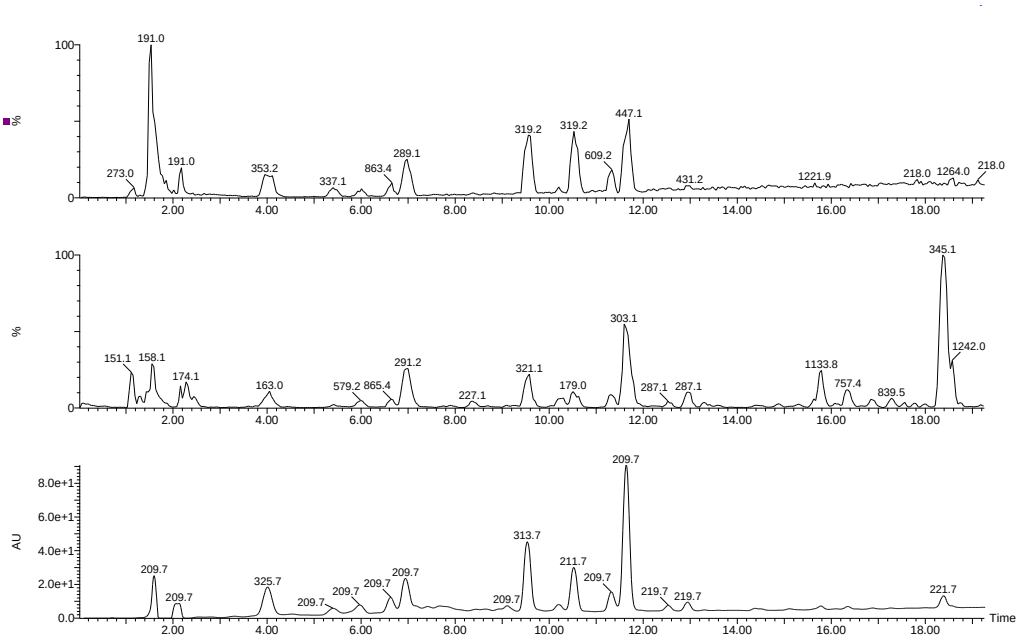
LC-MS chromatogram of *Aesculus glabra* Willd.



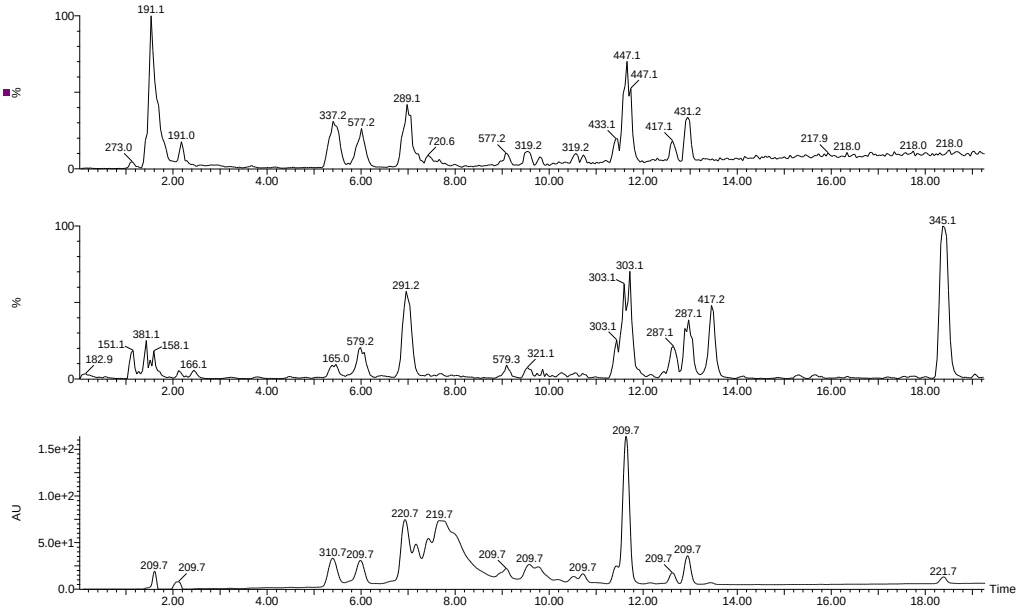
LC-MS chromatogram of *Aesculus parviflora* Walter



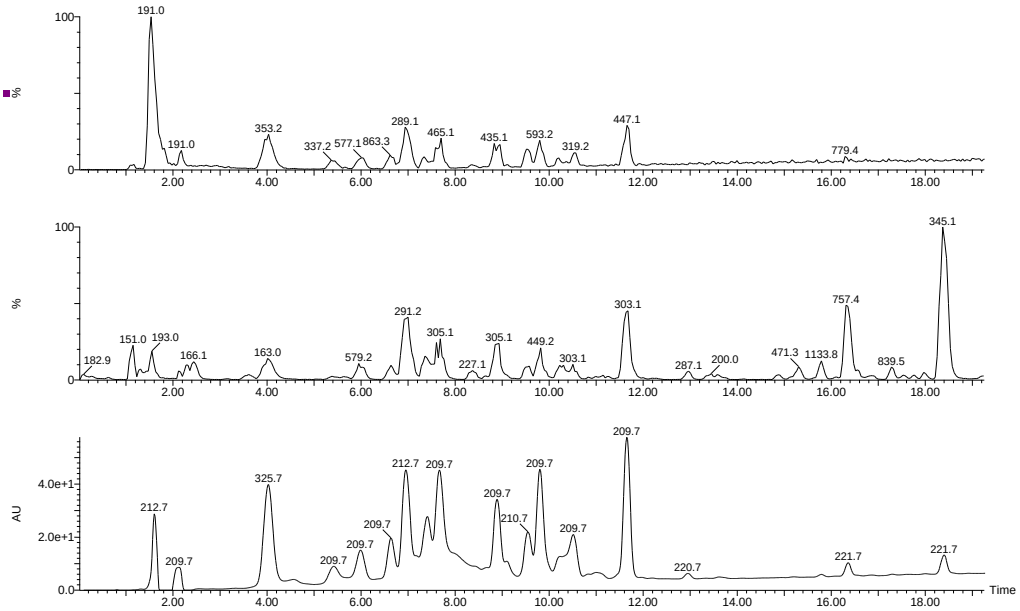
LC-MS chromatogram of *Aesculus californica* (Spach) Nutt.



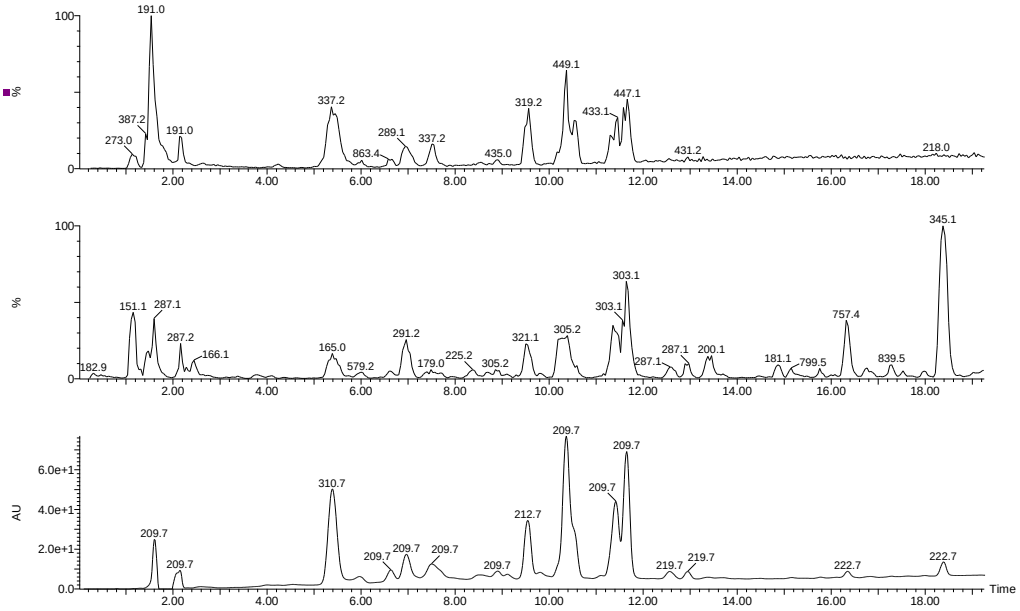
LC-MS chromatogram of *Aesculus assamica* Griff



LC-MS chromatogram of *Aesculus wilsonii* Rehder



LC-MS chromatogram of *Aesculus indica*

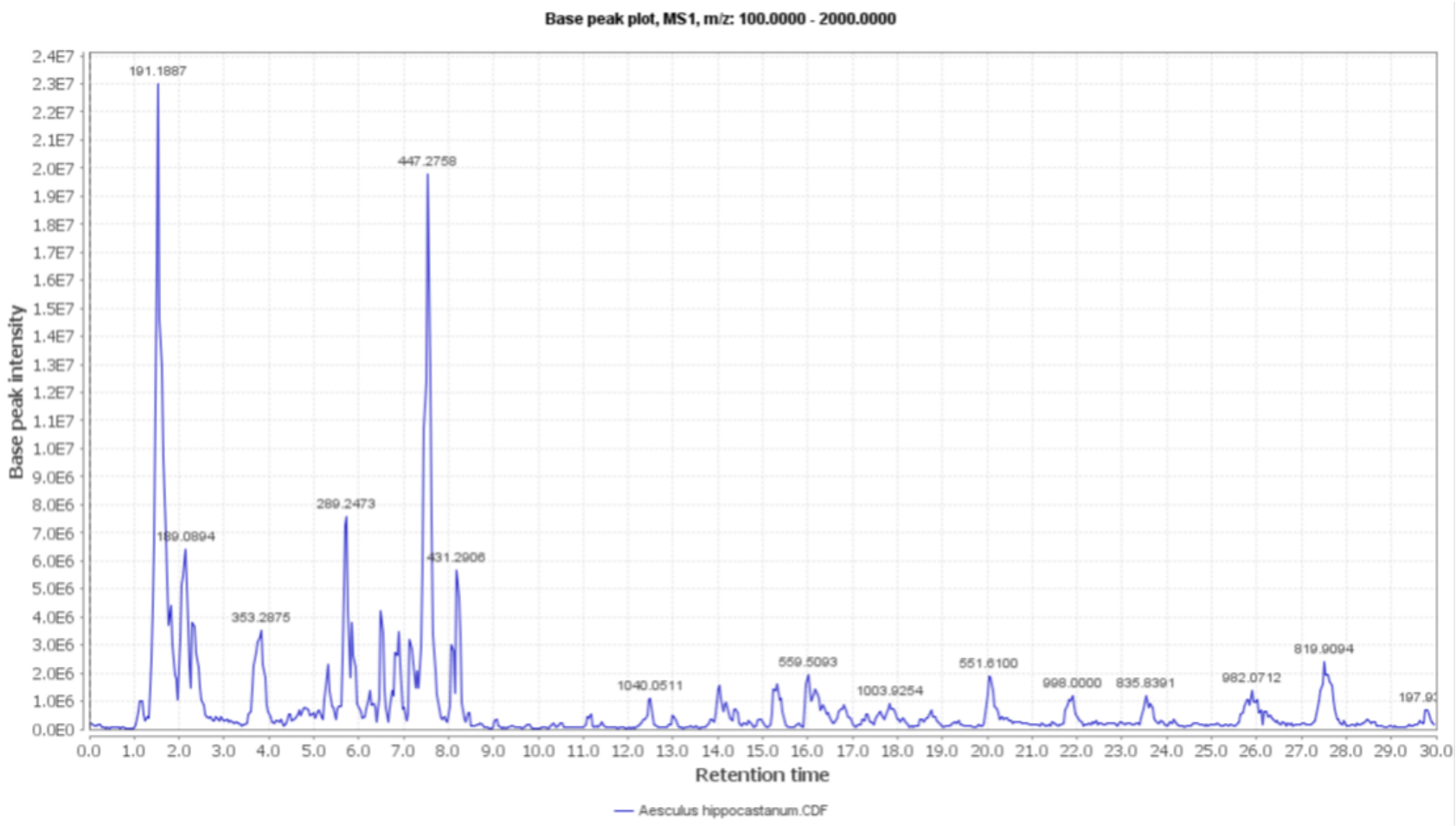


LC-MS chromatogram of *Aesculus chinensis* Bunge

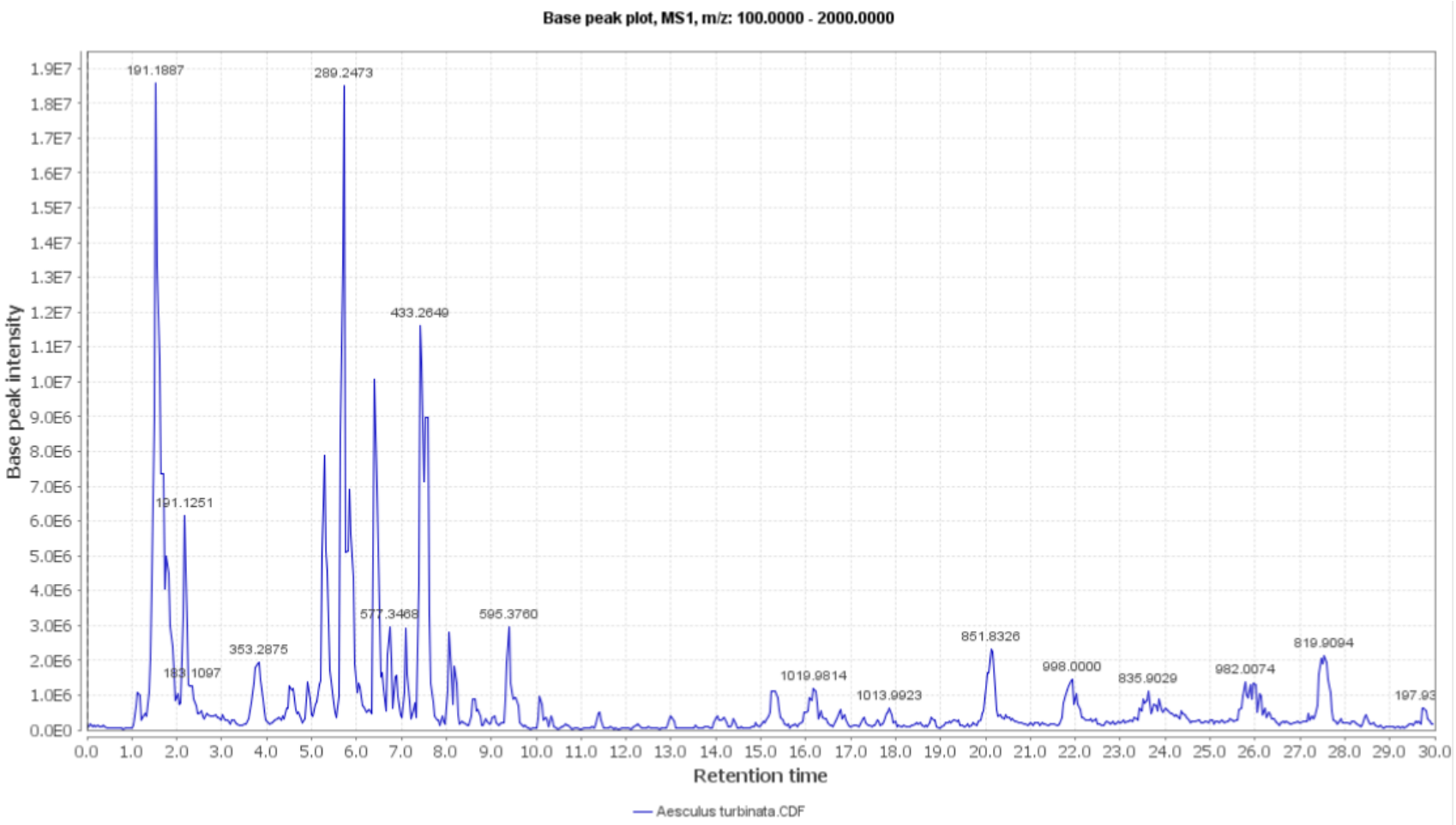
Appendix 4

Typical chromatograms of the different species of *Aesculus* studied in Chapter 5 (saponins)

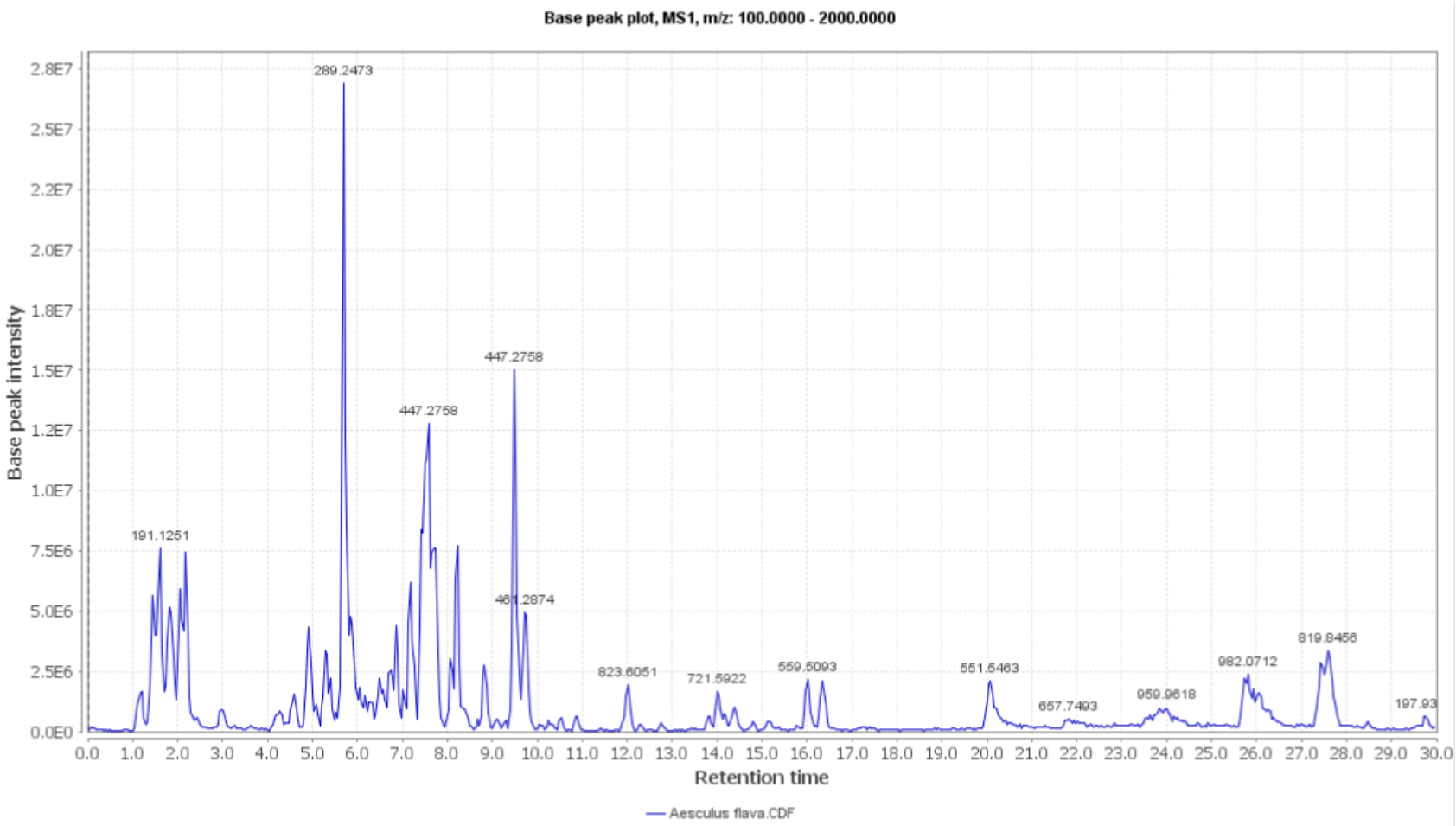
Mobile phase gradient 90:0:10 (t = 0 min, linear) 0:90:10 (t = 25 min) water/acetonitrile/[acetonitrile + 1% formic acid]



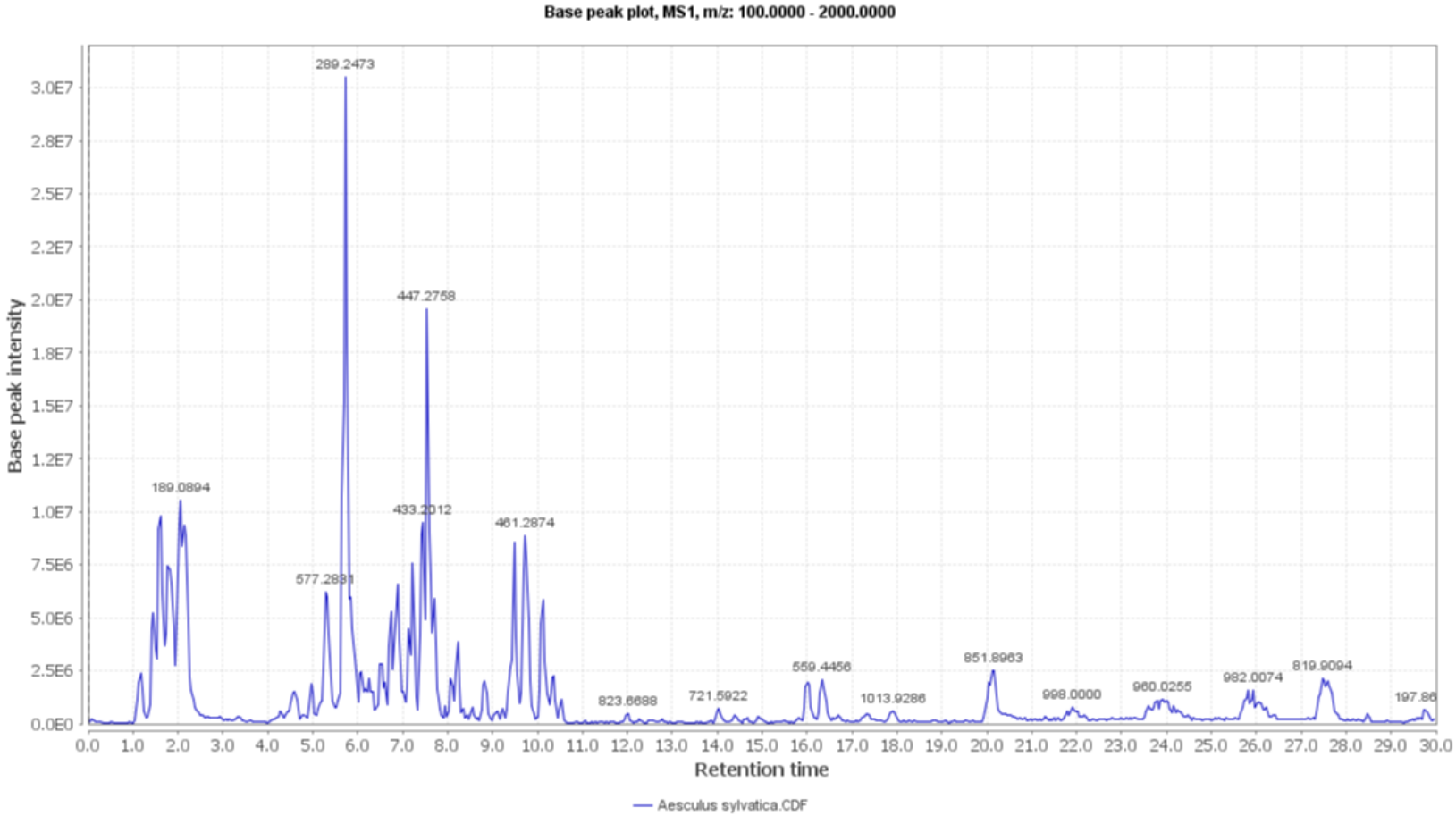
LC-MS chromatogram (ESI⁻) of *Aesculus hippocastanum*



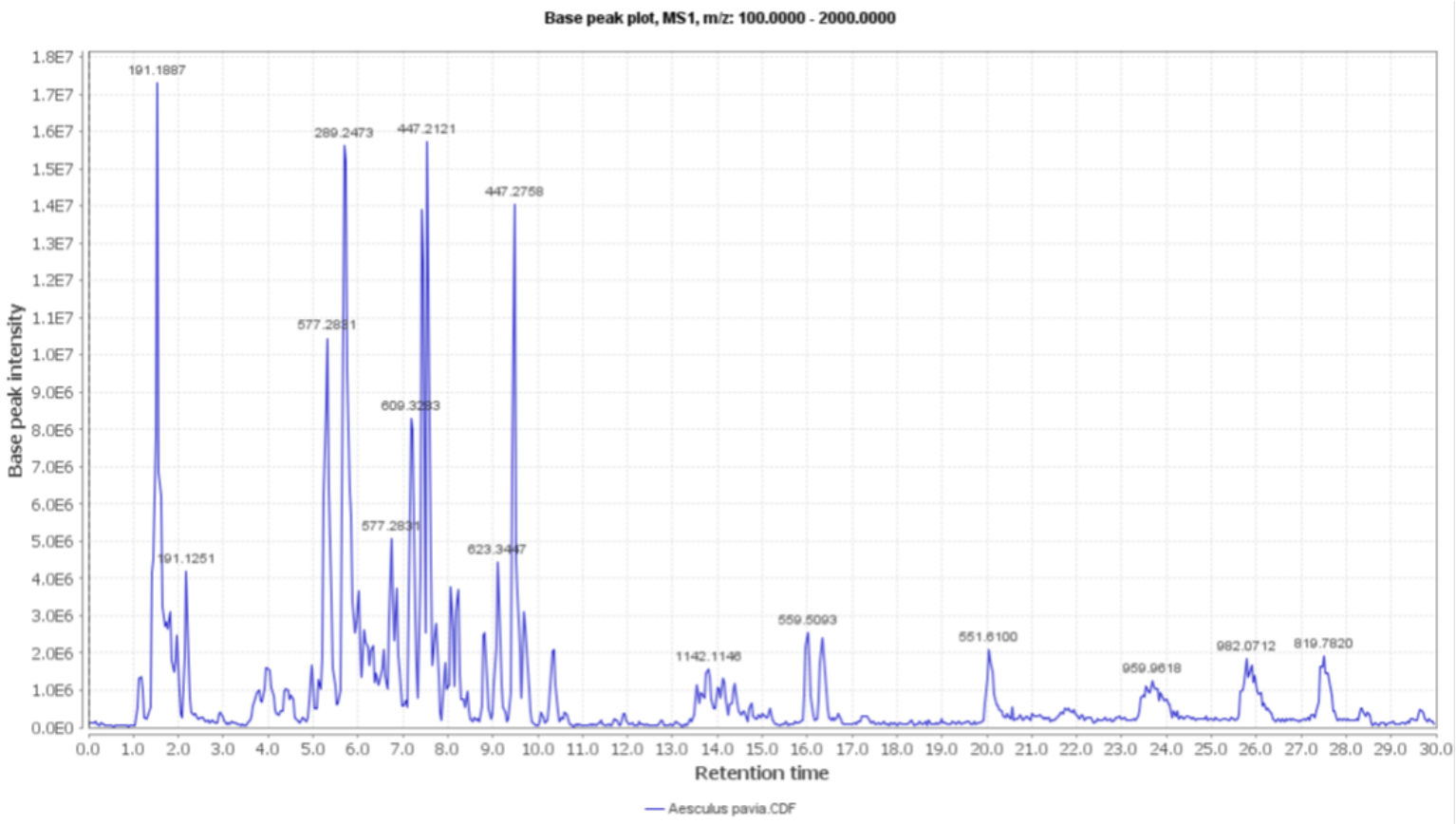
LC-MS chromatogram (ESI^-) of *Aesculus turbinata*



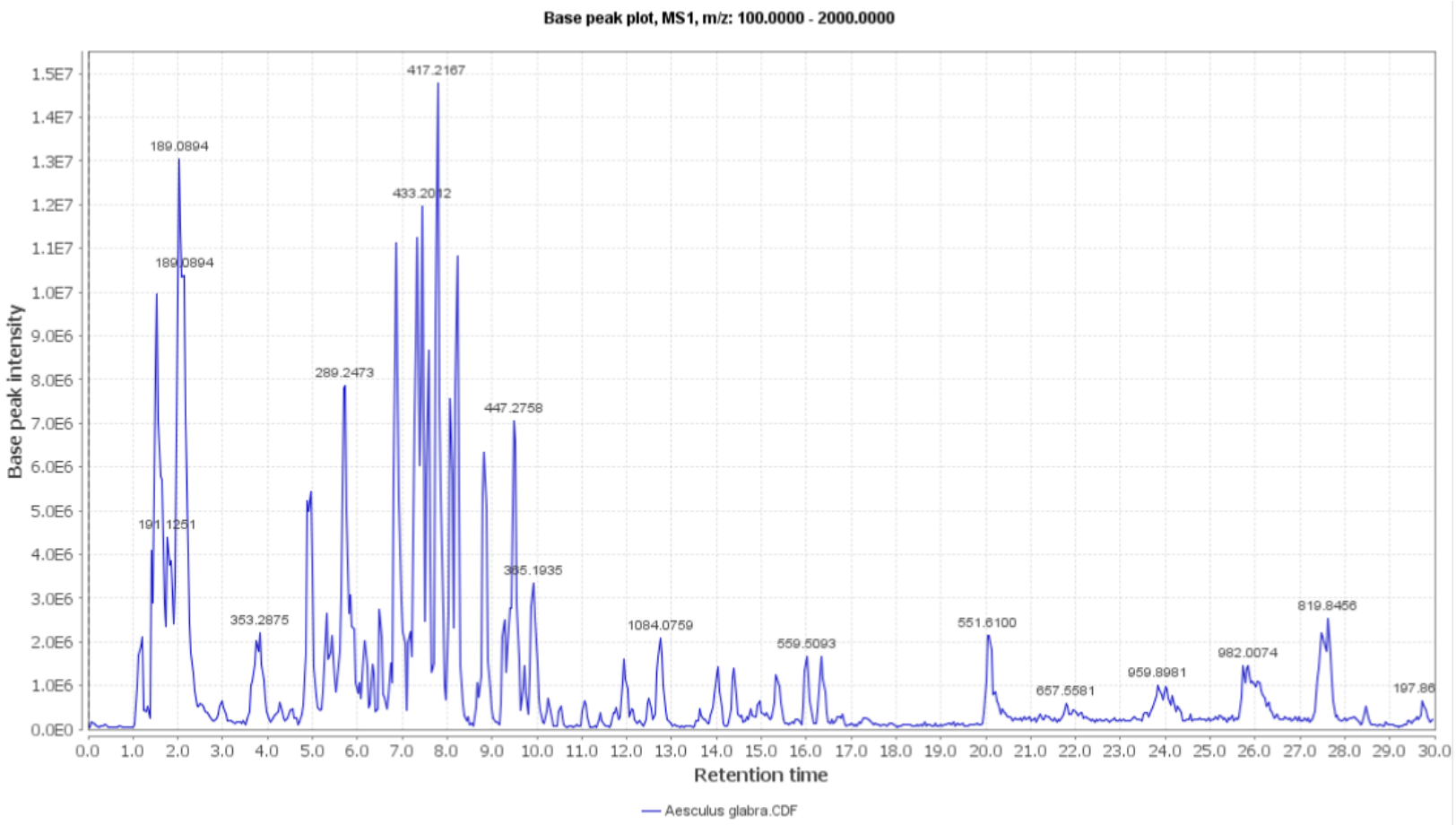
LC-MS chromatogram (ESI$^-$) of *Aesculus flava*



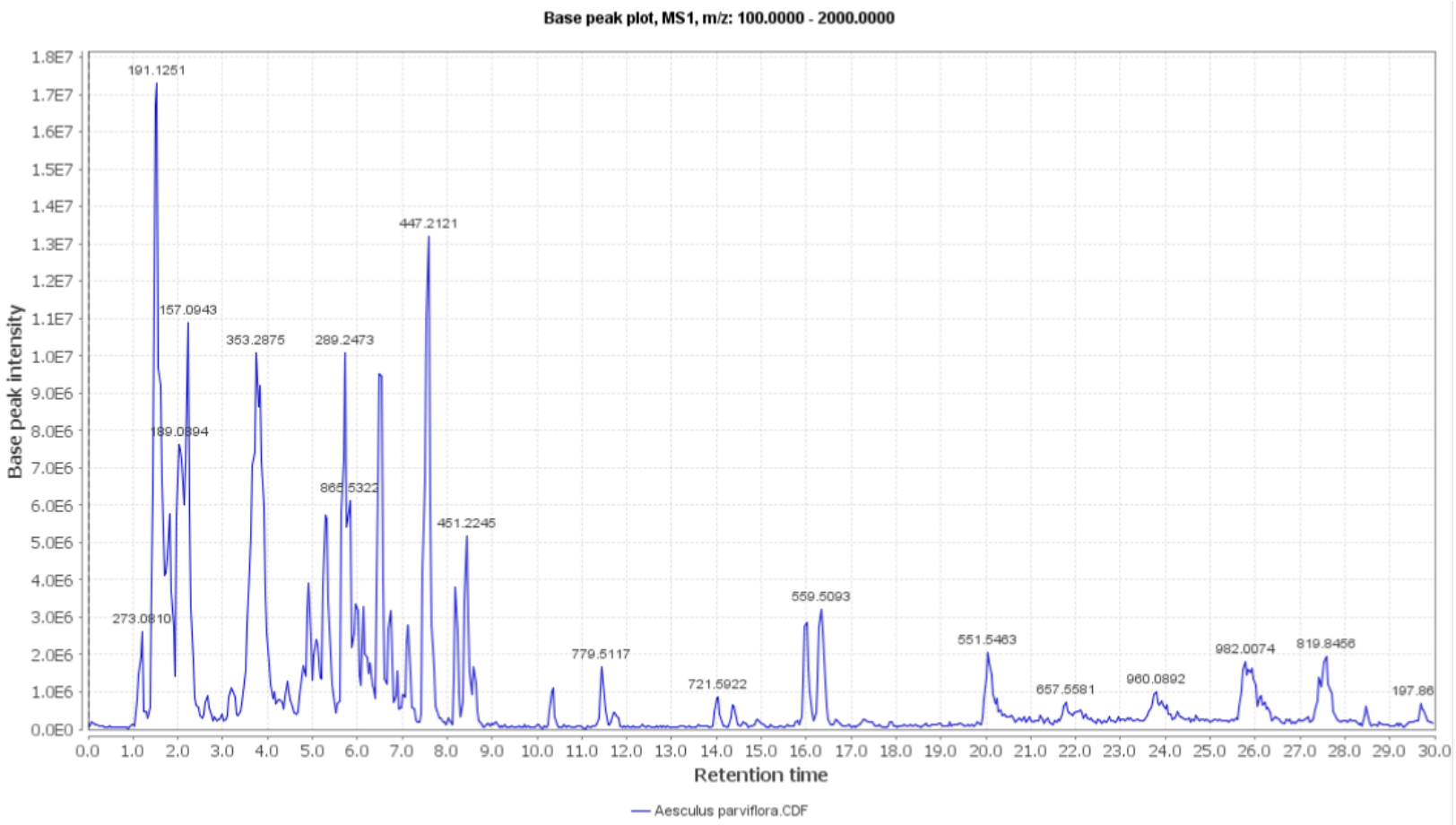
LC-MS chromatogram (ESI^-) of *Aesculus sylvatica*



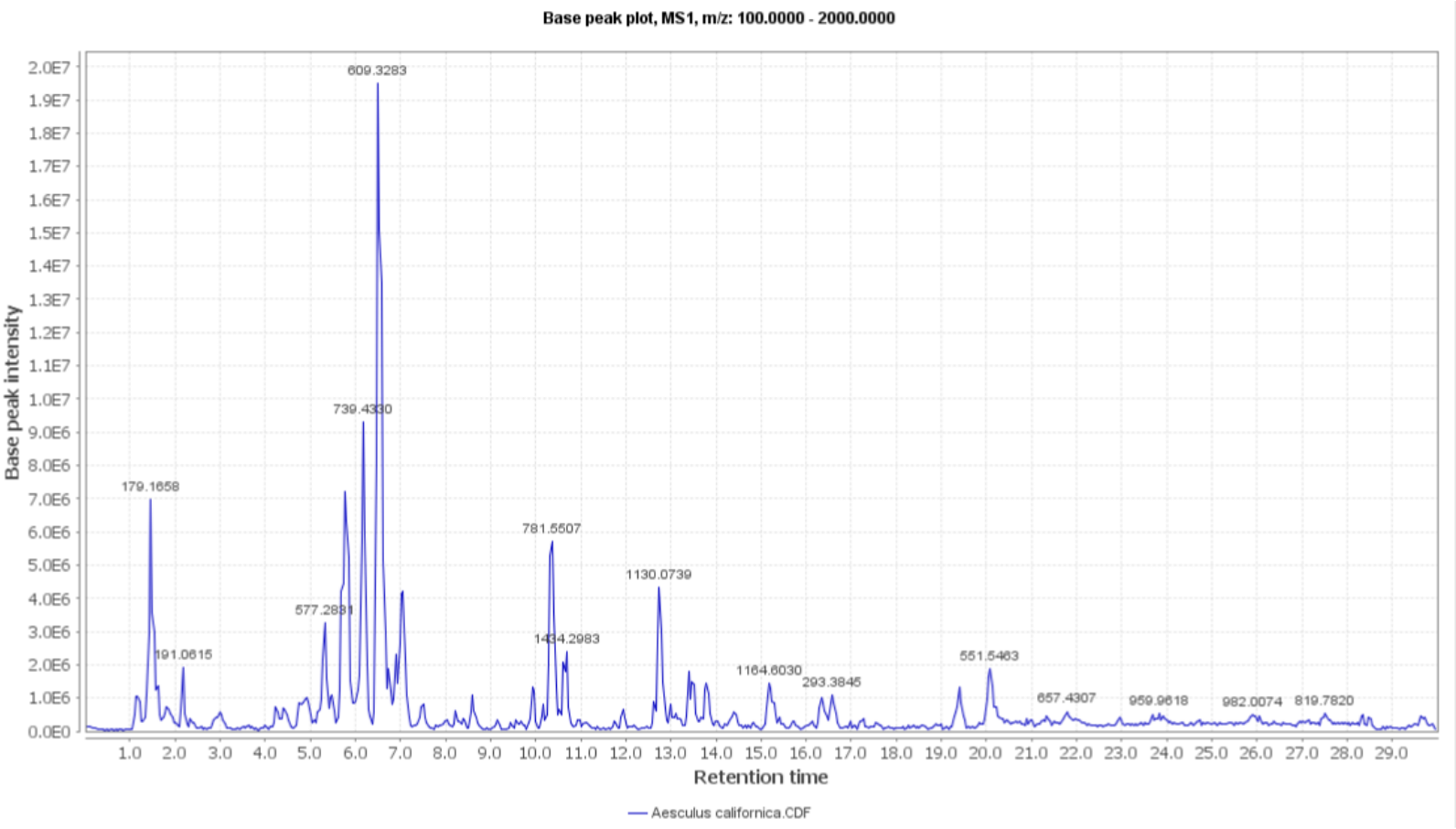
LC-MS chromatogram (ESI⁻) of *Aesculus pavia*



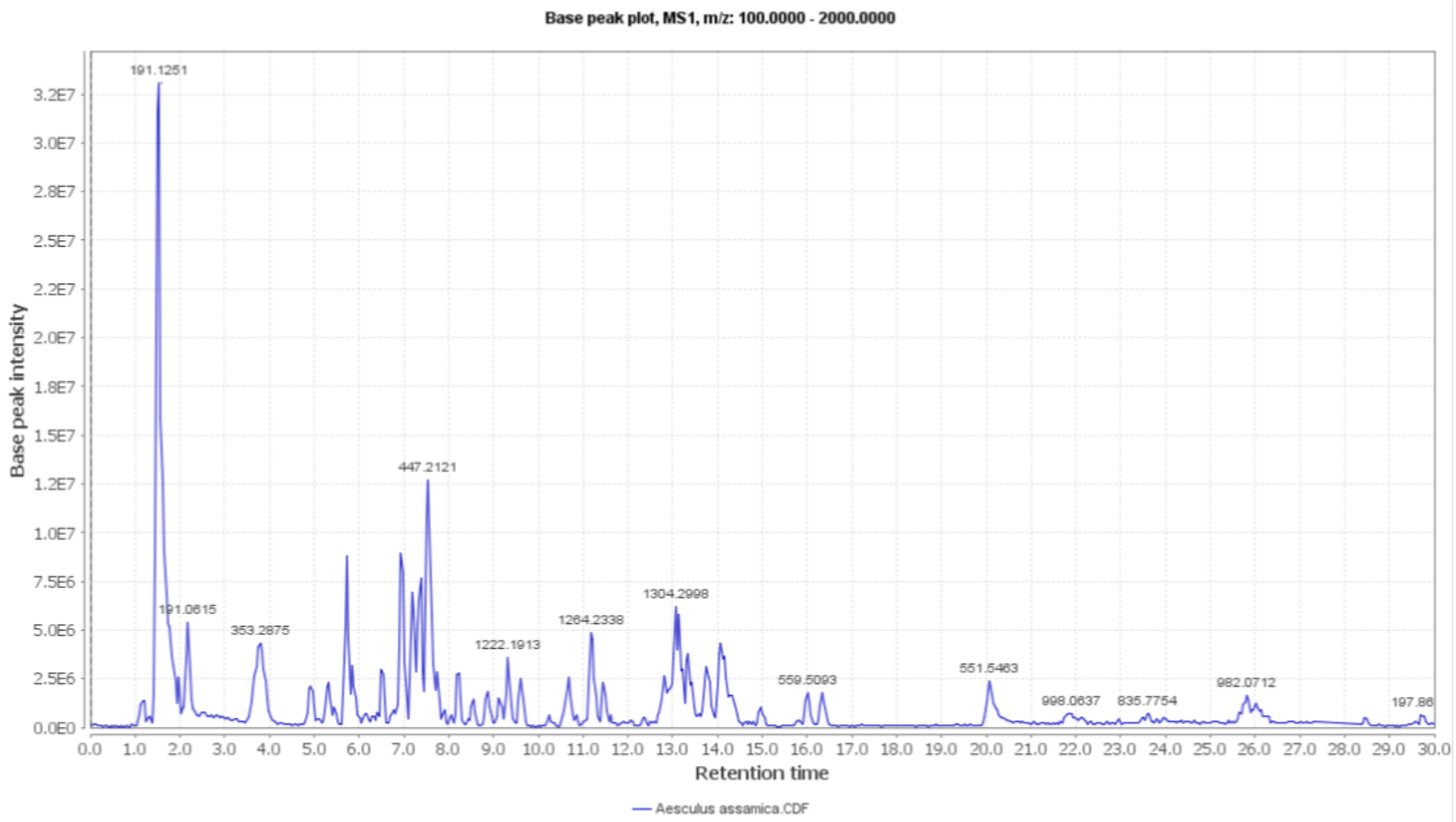
LC-MS chromatogram (ESI^-) of *Aesculus glabra*



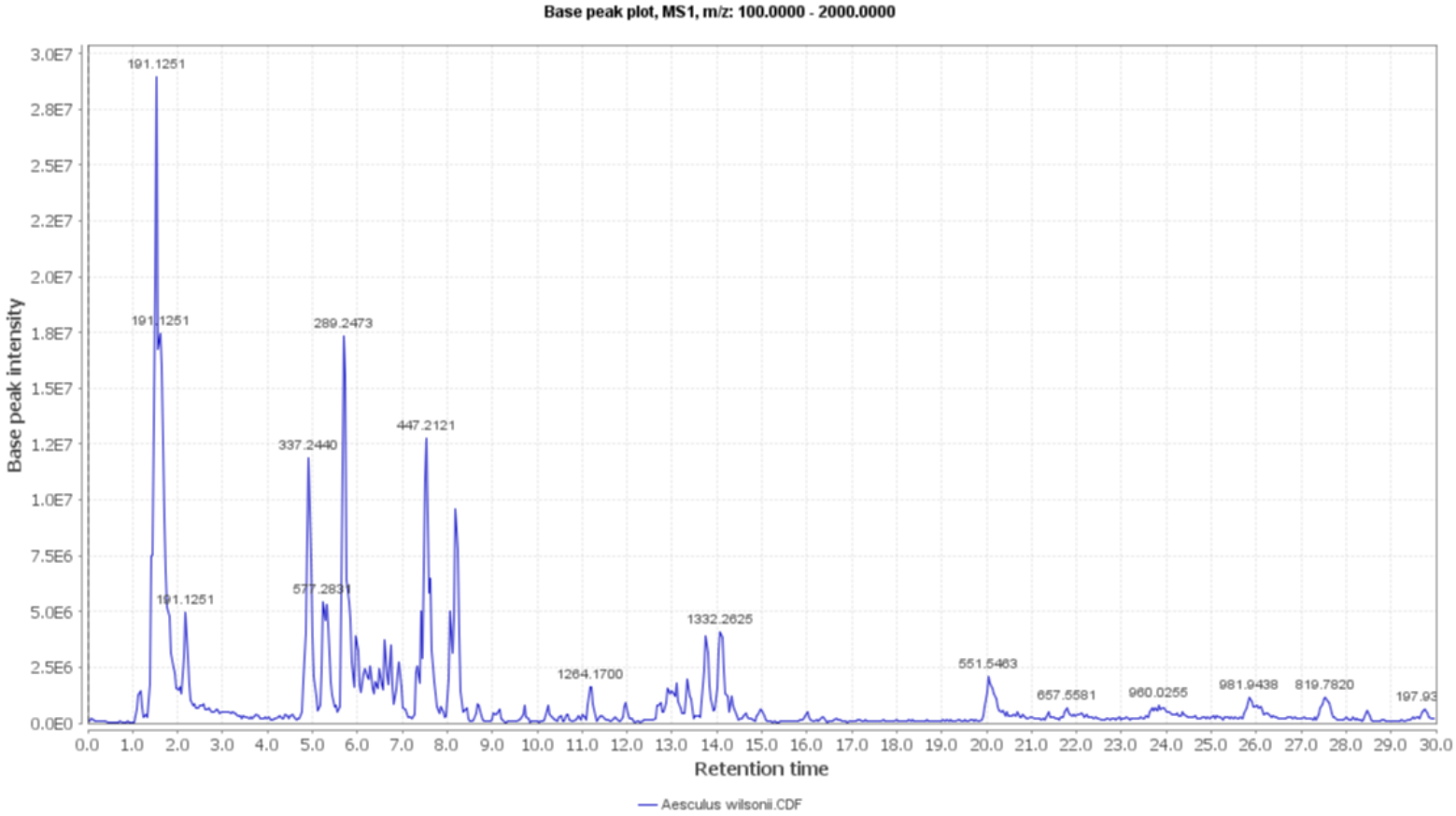
LC-MS chromatogram (ESI$^-$) of *Aesculus parviflora*



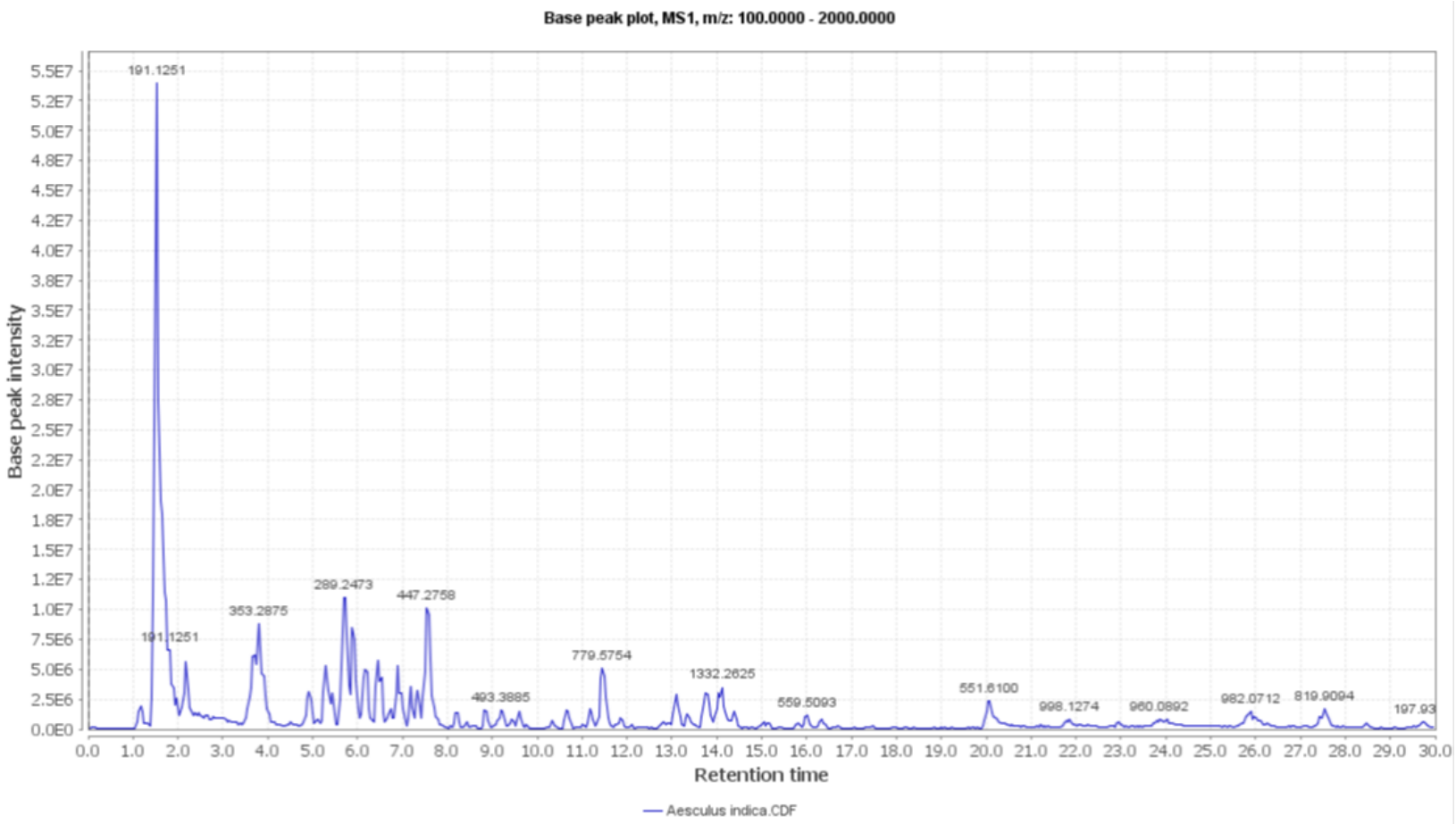
LC-MS chromatogram (ESI$^-$) of *Aesculus californica*



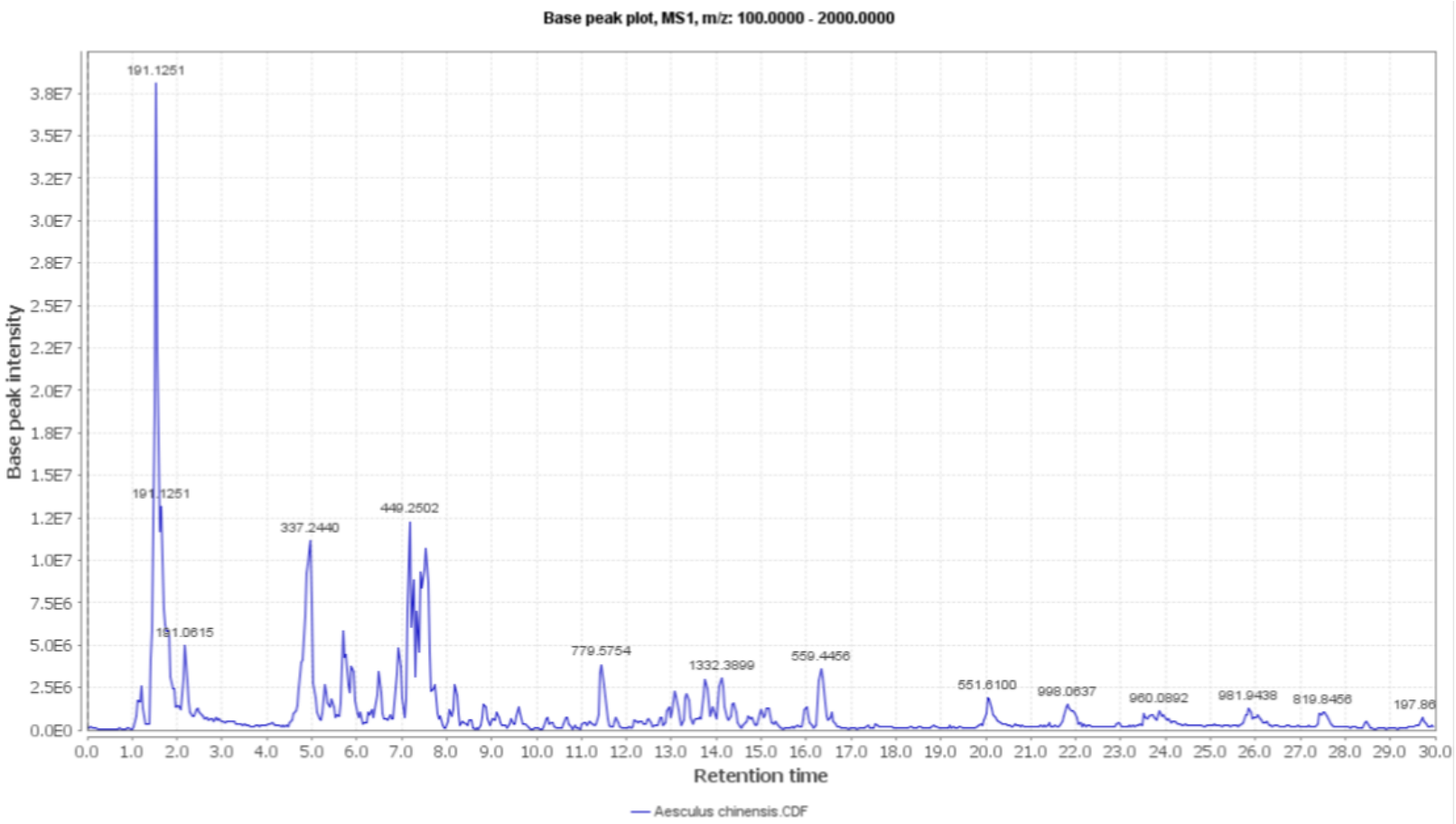
LC-MS chromatogram (ESI$^-$) of *Aesculus assamica*



LC-MS chromatogram (ESI^-) of *Aesculus wilsonii*



LC-MS chromatogram (ESI$^-$) of *Aesculus indica*



LC-MS chromatogram (ESI^-) of *Aesculus chinensis*

Appendix 5

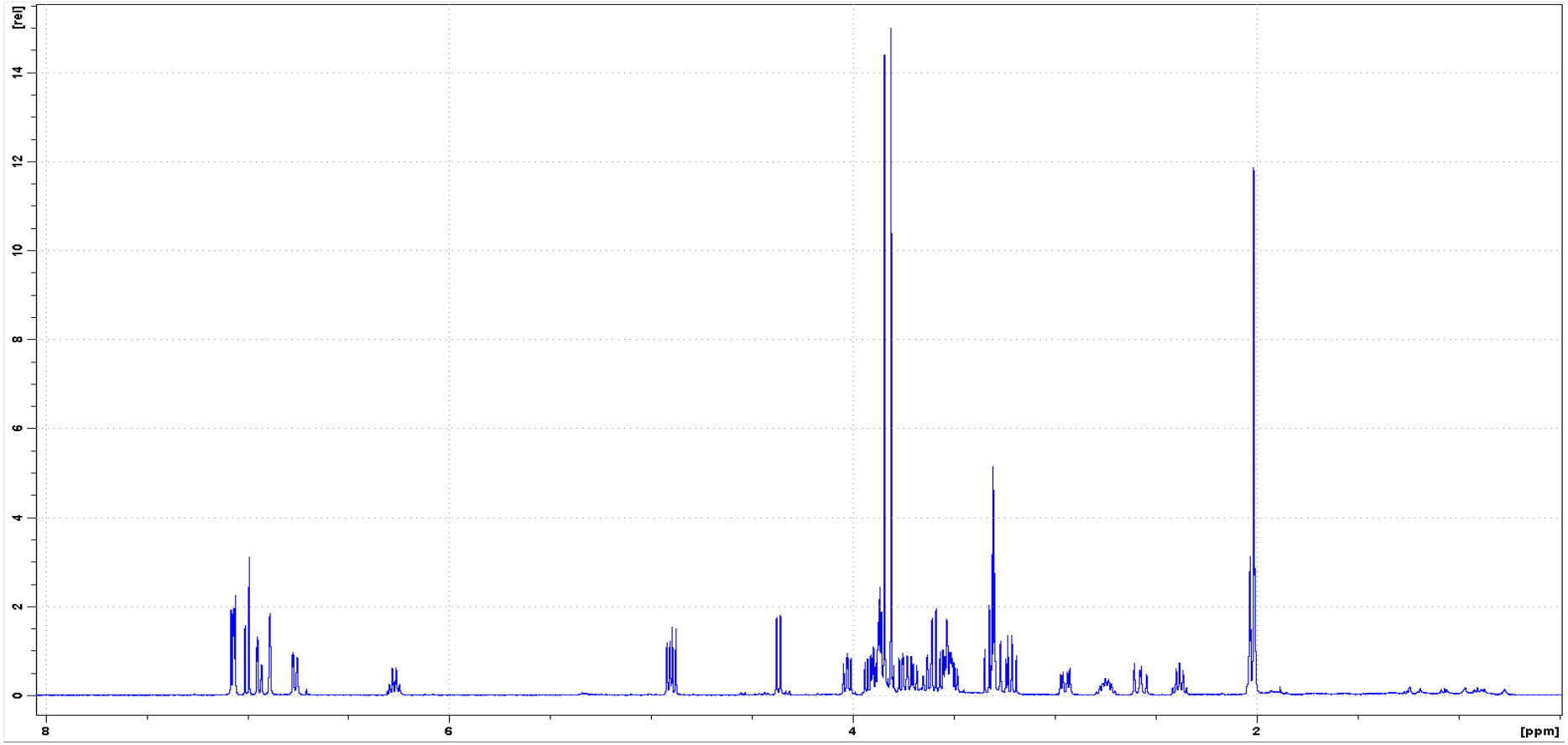
NMR spectra of the isolated novel compounds

9-*O*-angeloyl-4,4´,9-trihydroxy-3,3´-dimethoxy-7,9´-epoxylignan 4´-*O*-β-xylopyranosyl-(1→4)-β-glucopyranoside **(KEW 1237)**

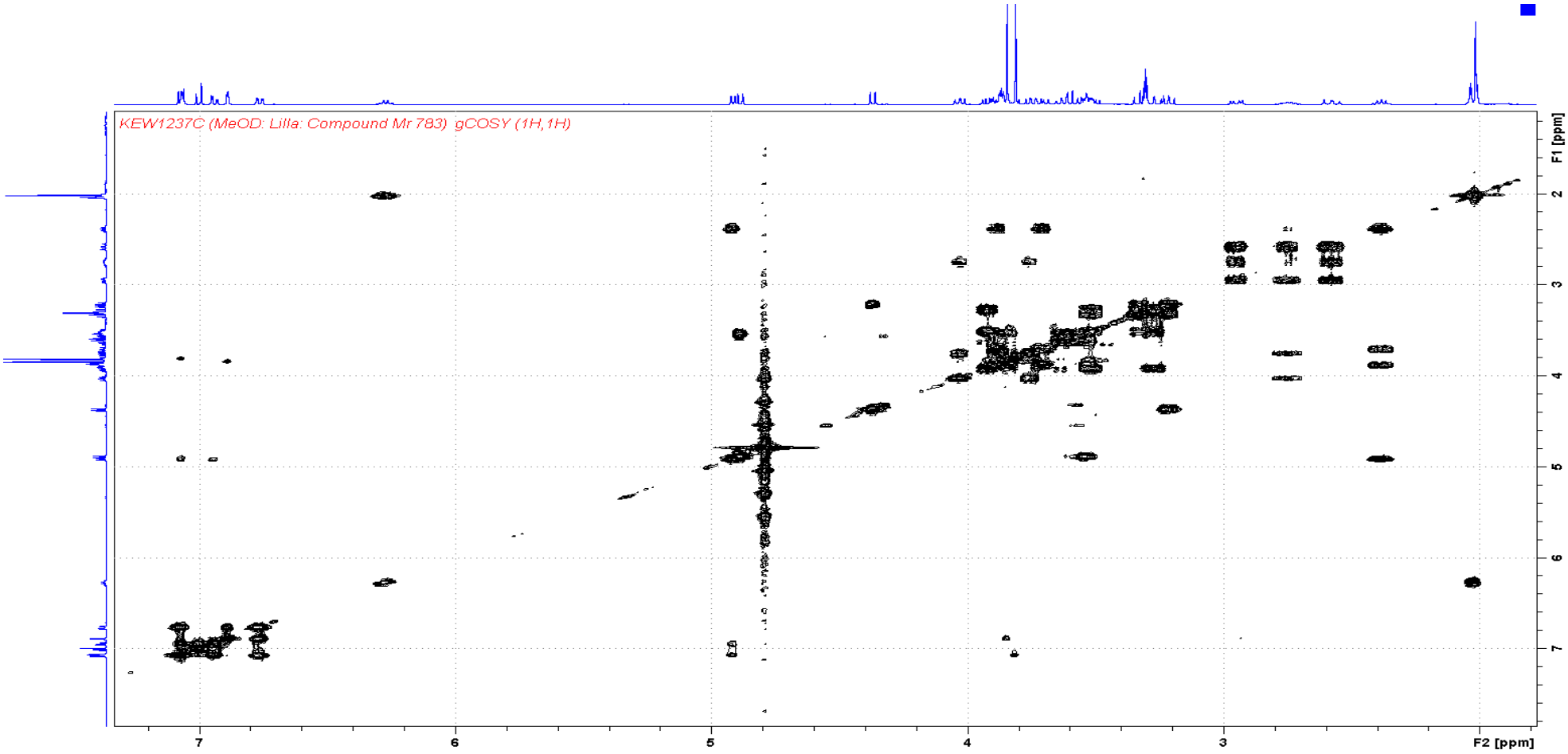
Rhamnetin 3-*O*-β-glucopyranosyl(1→4)-α-rhamnopyranoside **(KEW 1241)**

21,22-diangeloyl-R_1-barringenol (=12-oleanane-3β-,15α,16α,21β,22α,28-hexol) 3-*O*-β-glucopyranosyl(1→4)[β-arabinofuranosyl(1→3)][β-glucopyranosyl(1→2)]-β-xylopyranoside **(KEW 1254)**

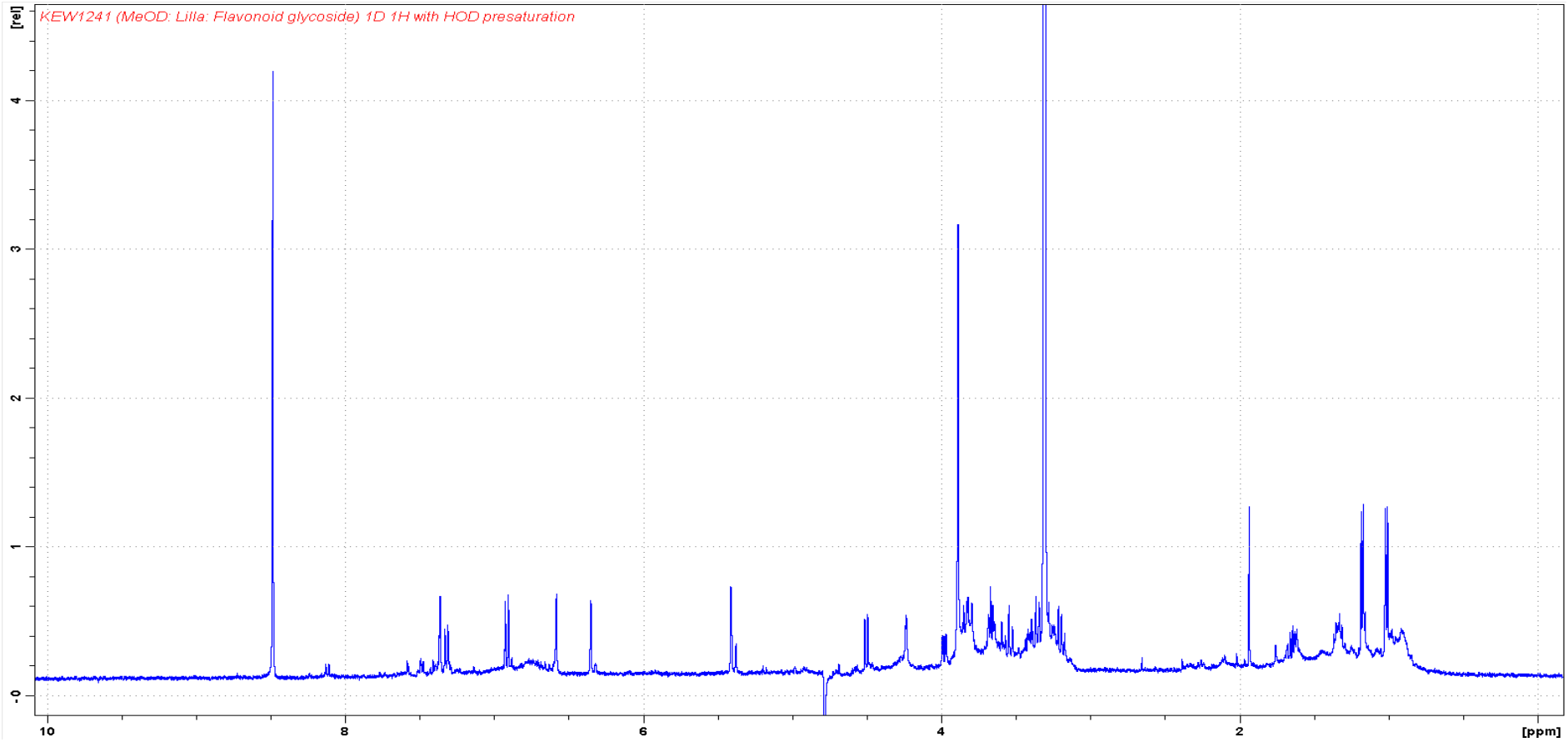
NMR spectra were acquired on either a Bruker 400 (Avance) MHz instrument or a Bruker 700 (Avance II+) MHz instrument equipped with a 5 mm 1H/13C/15N triple-resonance PFG cryoprobe. Standard pulse sequences and parameters were used to obtain one-dimensional ^{1}H, ^{13}C, and site selective NOE, and two-dimensional gradient-enhanced COSY, HSQC, and HMBC spectra.



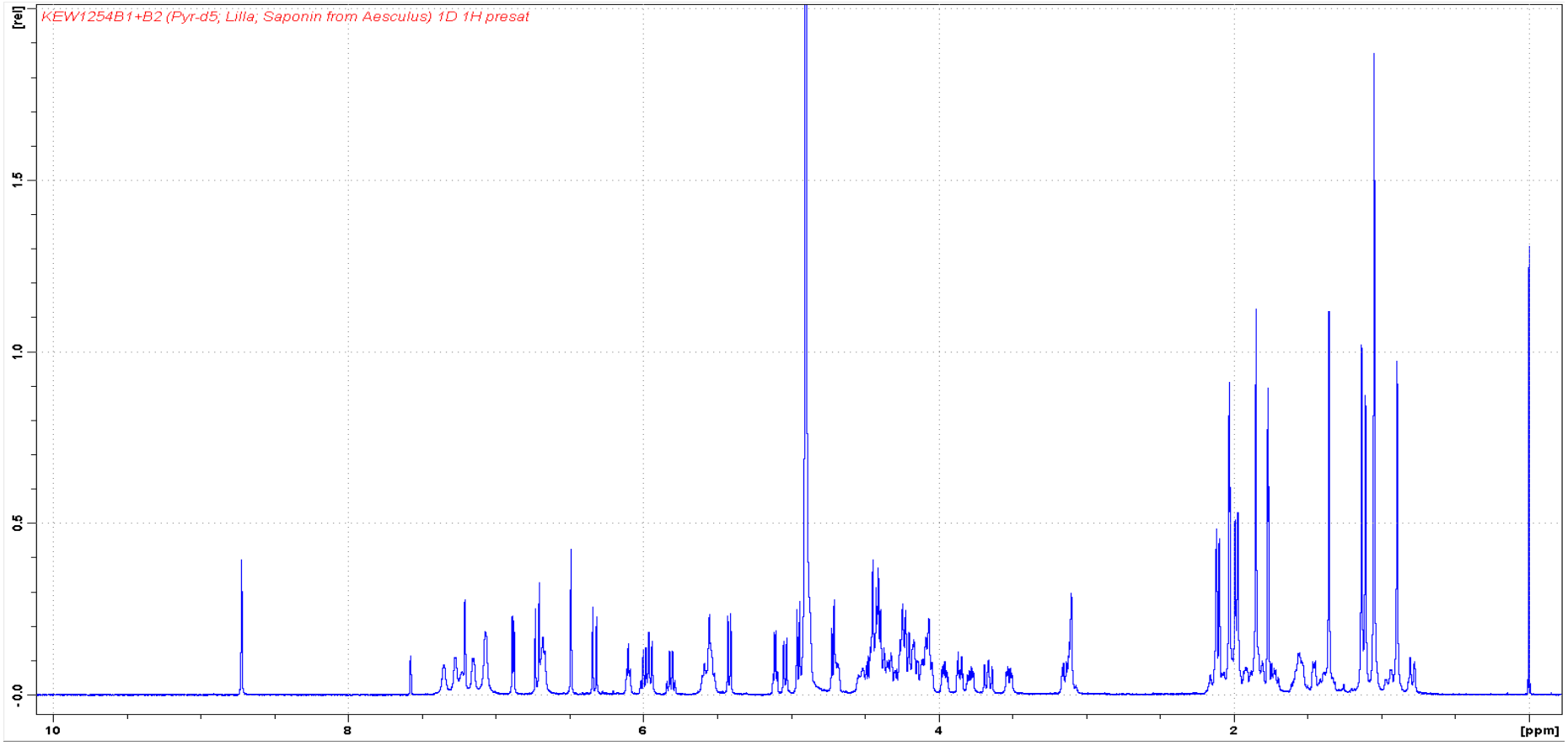
KEW 1237


KEW1237C (MeOD: Lilla: Compound Mr 783) gCOSY (1H,1H)
F1 [ppm]
F2 [ppm]
7
6
5
4
3
2
3
4
5
6
7

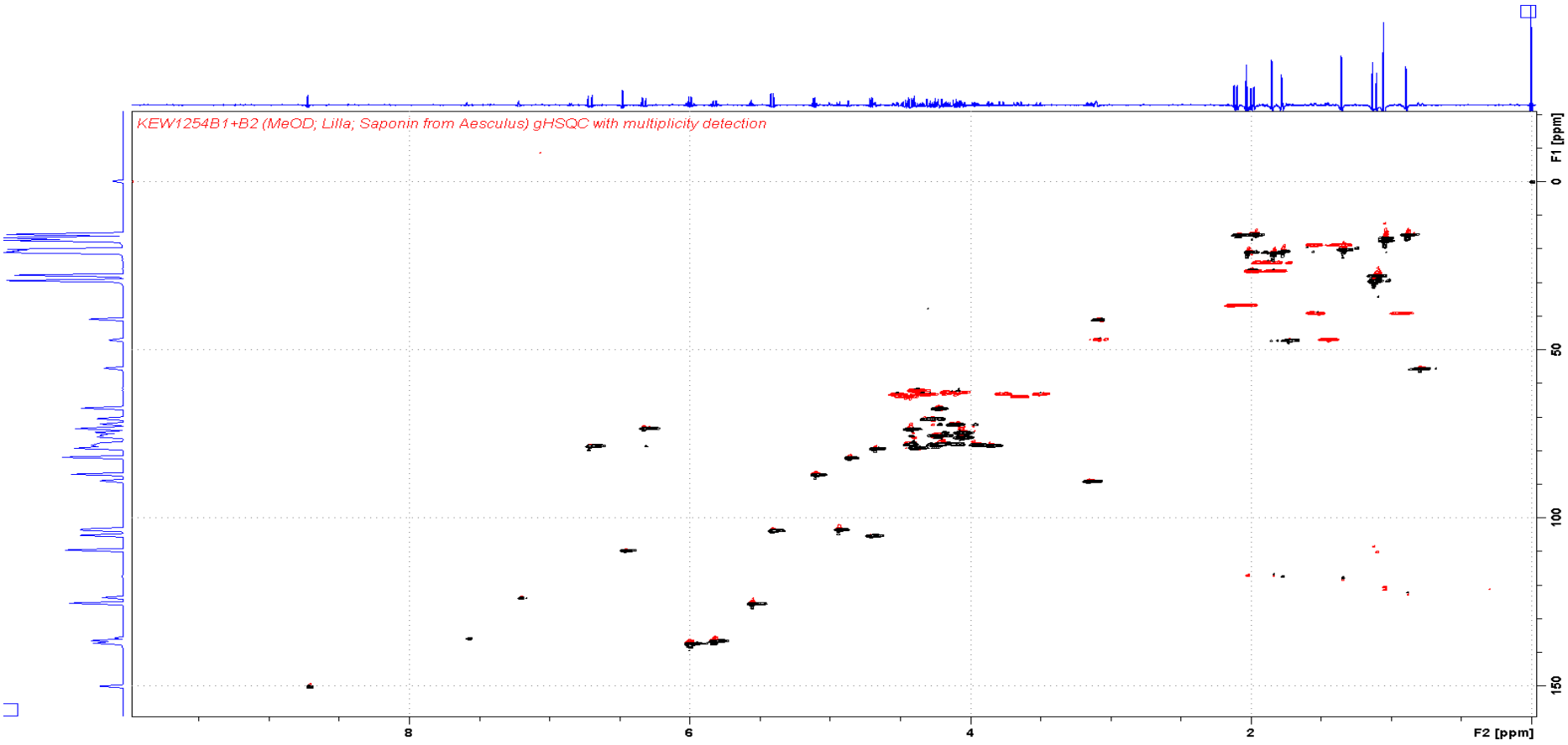
KEW 1237


KEW1241 (MeOD: Lilla: Flavonoid glycoside) 1D 1H with HOD presaturation
[rel]
4
3
2
1
-0
10
8
6
4
2
[ppm]

KEW 1241


KEW1254B1+B2 (Pyr-d5; Lilla; Saponin from Aesculus) 1D 1H presat
[rel]
1.5
1.0
0.5
-0.0
10
8
6
4
2
[ppm]

KEW 1254


KEW1254B1+B2 (MeOD; Lilla; Saponin from Aesculus) gHSQC with multiplicity detection
F1 [ppm]
0
50
100
150
8
6
4
2
F2 [ppm]

KEW 1254