Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

**Faculty of Landscape Planning,
Horticulture and Agricultural Sciences**

Fakulteten för landskapsplanering, trädgårds- och jordbruksvetenskap

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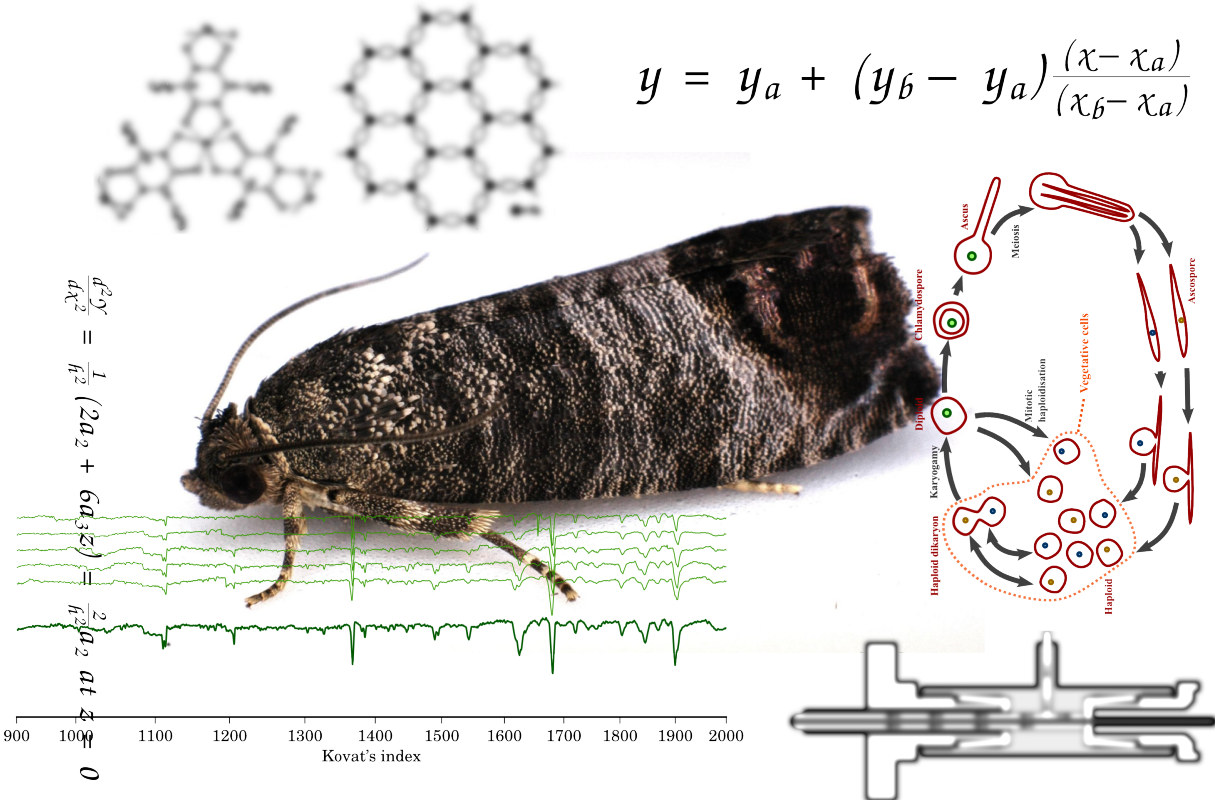

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Abstract

Insect-yeast interactions have been known for decades but are poorly understood. To investigate the codling moth (*Cydia pomonella*) olfactory connection to yeasts, volatiles from two ascomycete yeasts, *Metschnikowia pulcherrima* and *Metschnikowia andauensis*, were analysed using gas chromatography combined with electroantennographic detection (GC-EAD). To be able to detect minute, but possibly behaviourally important responses to volatiles , multiple signals were averaged using a novel time synchronisation technique. Antennally active compounds were identified with GC-MS. Utilizing a wavelet-based peak separation and spectral enhancement algorithm, compounds in otherwise undetectable amounts could be identified. Results showed multiple responses to both yeasts indicating the ability of the moth to detect yeast synthesised volatile compounds.

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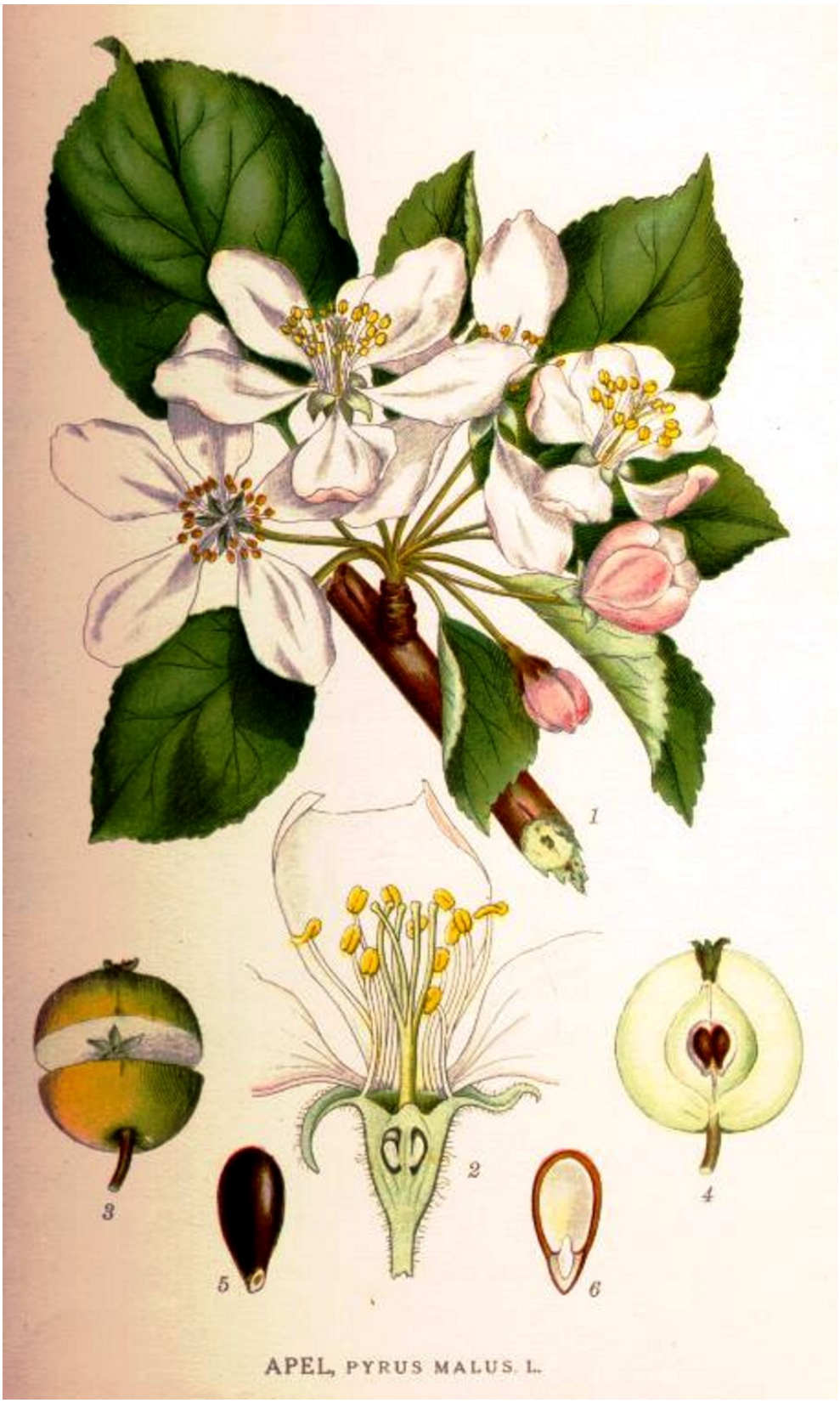


Figure 1: *M. domestica*, plate 283. (Lindman, 1917)

1 Introduction

Figure 2: Codling moth, *Cydia pomonella*, imago ♀. Photograph by author

1.1 Codling moth

The codling moth, *Cydia (Laspeyresia) pomonella* L., is a major pest in apple orchards around the world. Though apple, *Malus domestica* (Fig. 1), is the preferred host, *C. pomonella* can attack other members of the *Roceaceae* family such as pear (*Pyrus communis*) and almond (*Prunus dulcis*). Outside the *R.* family, walnut (*Julglans regia*) is a known host. The codling moth overwinter as larvae in the ground, under organic matter such as leaves or under tree bark. In early spring, larvae pupate and emerge as imagines (Fig. 2) after 3-4 weeks. During dusk, calling females, sitting in the host canopy, attract searching males with a sex pheromone (Barnes et al., 1966). Within 24 hours of mating, the female lays 30-50 eggs (Ferro et al., 1975). Early in season, eggs are laid on the upper side of corymb leaves or other leaves close to the inflorescence. Later, eggs are to a lesser degree also laid on fruit and wood(MacLellan, 1962) . Hatching occurs after 5-20 days after which larvae search, using exploratory bites, for suitable fruit. Upon acceptance, the larvae bore into fruits where they feed on seed and pericarp. Generally only one larva is found in each fruit. After maturation which take 3-4 weeks, the larvae leave the fruit to enter diapause or, in warmer climates where more than one generation per season is possible, to pupate.

One component of the female sex pheromone, codlemone ((E,E)-8,10-Dodecadien-1-ol) (Roelofs et al., 1971), has for more than a decade been used in traps for monitoring of pest population . Monitoring has made it possible to reduce the use of insecticides, as unnecessary spraying thereby can be avoided (Witzgall et al., 2010).

Codlemone has also successfully been used for mating disruption where males are confused, obstructing their location of mating partners. As a population control technique of codling moth, mating disruption has worked well at low and medium population densities (Witzgall et al., 2008). However, a major drawback of this method is that female sex pheromone mainly effect male behaviour. Gravid females, either flying in from neighbouring orchards or females mated with non-confused males, can still lay eggs and cause a loss in production.

Female moths find and evaluate oviposition sites using a combination of visual, olfactory and gustatory cues. Green leaf volatiles such as α-farnesene, β-caryophyllene as well as fruit volatiles, of which many are esters, are thought to play important roles in the female location of suitable oviposition sites [Variu refs incl. Bengtsson et al., ansebo etc]. Host acceptance compounds include sugars and sugar alcohols (Lombarkia and Derridj, 2002).

The apple is however not only an apple. Micro-organisms, particularly fungi, colonise the plant surface and intracellular space (Lindow and Brandl, 2003), making the plant in essance, its own ecosystem. Previous work have identified *Metschnikowia* yeasts on flowers (Davenport, 1976; Gimenez-Jurado et al., 2003), leaves (Slávi kov á et al., 2007), fruit surface and in larval galleries of codling moth infested fruits (Kurtzman and Droby, 2001; Xue et al., 2006). The yeasts have shown to play an important role in the defence against infectious and necrotic microorganisms, protecting the fruit as well as the larva (Curtis et al., 1996; Bergin et al., 2006). The yeast metabolites also serve as a nutrient source for the developing larva (Tasin et al., 2011).

The olfactory and gustatory relationships between moth, plant and yeast are poorly understood. It is however likely that the female moth uses not only volatiles and surface compounds from host plants, but also from micro-organisms, when finding and evaluating her oviposition site. With more knowledge about the volatiles involved in this process, it may be possible to create traps, or other means of pest control, directly targeted on the female moth.

1.2 The *Metschnikowia* yeasts

The actomycete yeast clade *Metschnikowia (Monospora)* (Metschnikoff 1884) Kamienski 1899 is characterized by the ability to form ascus, containing one or two needle-shaped haploid ascospores (Metschnikoff, 1884; Miller et al., 1967).

Metschnikowia is currently divided into three main groups of species: one aquatic group of small-spore forming species containing the genus type species, *Metschnikowia bicuspidata* - a pathogen on *Daphnia* ; a second group of terrestrial small-spore forming species found in insects, flowers and fruits; lastly, a third heterogeneous group compromised of, mainly tropical, large-spore forming species (Naumov, 2011).

Only perfect species, with proven complete sexual cycle, are given the genus name *Metschnikowia*, several para- or non-sexual closely related species and strains exists in the clade, sometimes with same epithet as the perfect species to indicate close relationship, ex. *Chlamydozyma pulcherrima*, *Chlamydozyma reukaufii*.

In the complete sexual cycle , vegetative haploid yeast cells can fuse with the opposite mating type creating a dikaryon, not necessarily within the same species, and through karyogamy create diploid zygotes, The zygotes then form chlamydospores, later developing into asci in which, through meiosis, haploid ascospores are formed. The ascospores then completes the cycle after germination into vegetative haploid cells. An alternate, para-

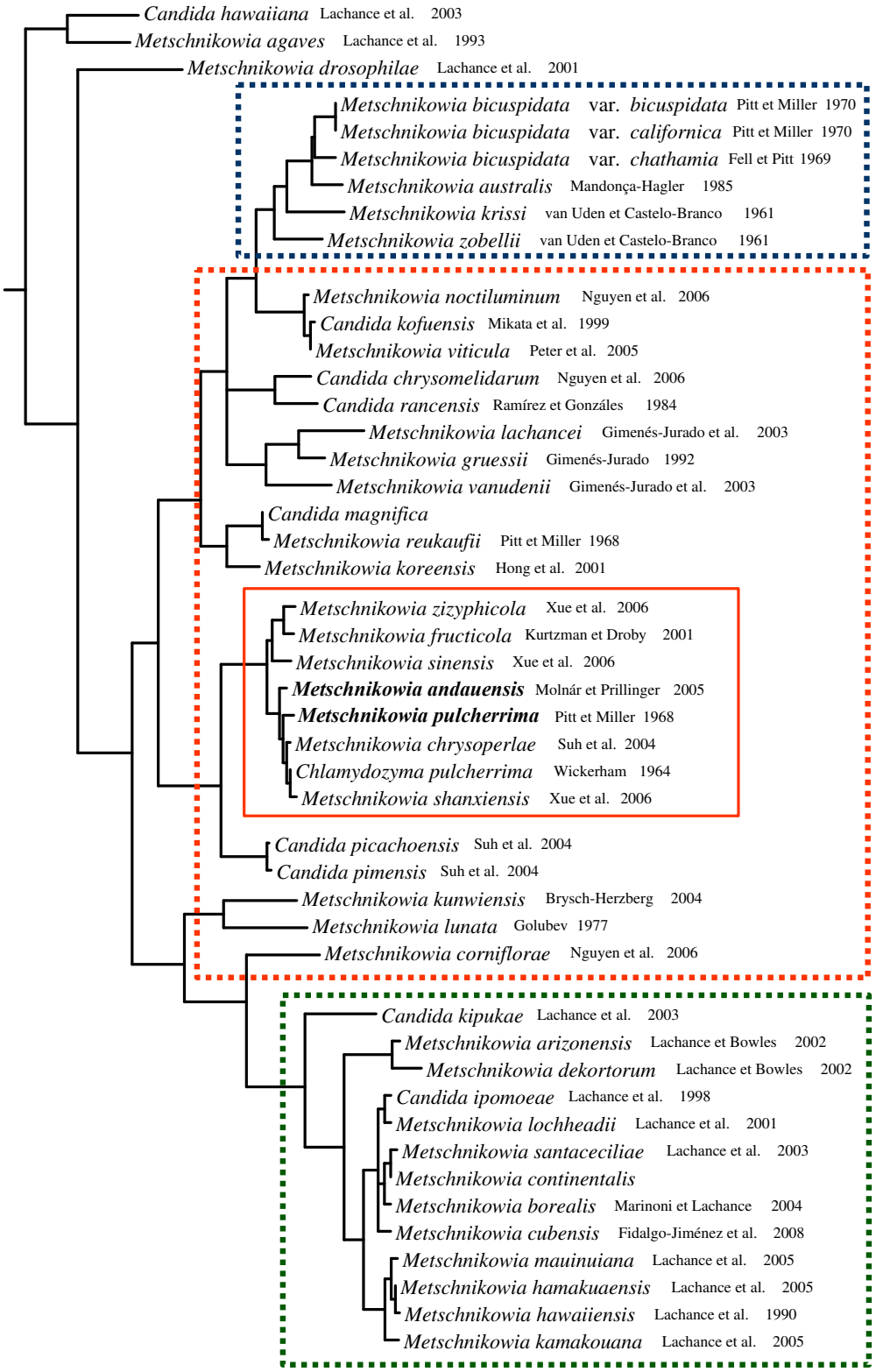


Figure 3: Phylogeny of the *Metschnikowia* clade. Analysis of sequences of D1/D2 domains of 26S rRNA, after Nguyen et al. 2006 and Naumov 2011. Upper dotted box in blue - Small-spored aquatic group; Center dotted box in orange - Small-spored terrestial group; Lower dotted box in green - Large-spore forming group; Center solid box in orange - *M. pulcherrima* and its closely related sibling species

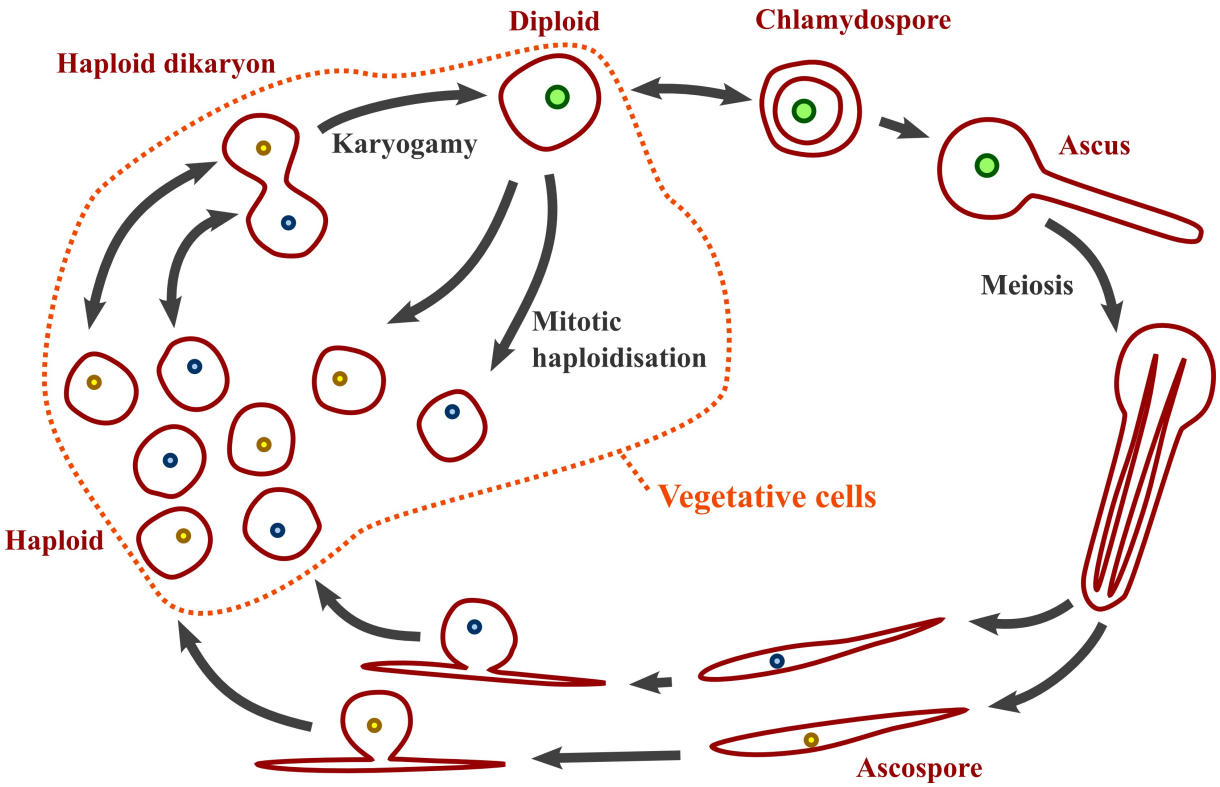


Figure 4: Life cycle of *Metschnikowia*, adapted from Naumov 2011 and Pitt and Miller 1968

sexual path is also possible; Diploid cells may undergo mitotic haploidisation, during which genetic information through cross-overs is exchanged, forming haploid cells (Pitt and Miller, 1968; Naumov, 2011) (Fig. 4).

The yeasts of interest in this study are *M. pulcherrima* and its closely related sibling species *Metschnikowia andauensis*. Both species are capable of fermenting or assimilating a wide range of mono- and disaccharides. Neither can however assimilate or produce starch (Molnár and Prillinger, 2005). Another characteristic of these species is the capability to produce the red pigment pulcherrimin (Beijerinck, 1918) (Fig. 5). When synthesised, the yeast depletes the close environment of iron. This process is believed to be one of the important anti-microbial effects of these yeasts (Sipiczki, 2006).

1.3 Electrophysiology

Electroantennographic detection is very useful way of detecting compounds eliciting a relatively strong response such as pheromones. Many host and oviposition cues have also been identified using this method. A drawback is however that behaviourally important compounds do not necessarily elicit strong antennal responses (Omura et al., 2000). This could be due to a low number of responding olfactory neurons or low receptor affinity. As with all measurement, there is also an inherent noise problem potentially masking these weak responses. Another issue is the occurrence of false responses originating as mechanical vibrations through air or equipment, muscular contractions or antennal movement. Multiple repetitions and EAD signal averaging has been used previously to overcome this problem. Some available GC-EAD software also contain basic implementation of signal averaging.

Ideally, given the same sample and parameters, gas chromatograms produced using the

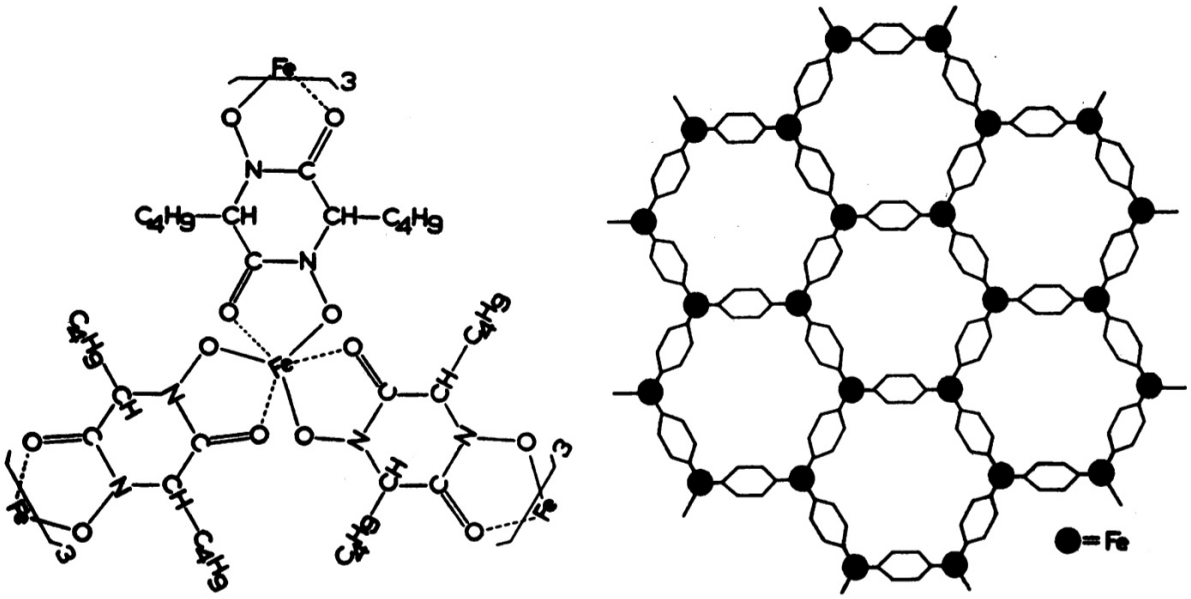


Figure 5: The red pigment pulcherrimin. The pigment is a complex formed of units of pulcherriminic acid and iron ions. Left: unit configuration; right: molecular pattern. From Kluyver et al. 1953

same column would produce identical results. In reality, variations in injection volume, temperature, pressure, column age and condition may cause changes to retention times of eluted compounds. Consequently, antennal responses will be occurring with varying time between recordings, making averaging of the signal traces difficult as the responses potentially cancel each other out. A possible solution to this problem is presented in (2.2).

1.4 Objective and research question

The main objective in this master thesis are to, by using established techniques such as GC-EAD and GC-MS, and to improve upon data treatment methods, show biological activity in the codling moth antenna to yeast-produced volatiles and to, where possible, identify some of these volatile compounds.

Formal hypotheses:

H^1_{NULL}	The antennae do not respond to volatile compounds in extracts
H^1_1	The antennae do respond to only a few volatiles in extracts
H^1_2	The antennae do respond to many volatiles in extracts

H^1_{NULL} may be rejected in favour of H^1_1 or H^1_2 if consistent antennal responses to volatiles in extracts can be shown.

Premise	H^1_{NULL} was rejected in favour of H^1_2
H^2_{NULL}	Responses are not yeast dependant
H^2_1	One or more responses are yeast dependant

H^2_{NULL} may be rejected in favour of H^2_1 if responses exclusive to yeast extracts can be found.

2 Methodology

2.1 Material

2.1.1 Insects

Paper fiber board rolls containing pupated codling moth (USDA-ARS, Wapato, WA, USA) were moisturised and placed in cubic acrylic cages with $\frac{1}{3}$m side. Cages were kept at 22℃ with 16 hours photo-period. Plastic petri-dishes, each with a dental cotton roll stuck through a 10mm hole in the lid, were filled with tap water supplying moths water access. Cages were moisturised daily and in case of adult moth emergence, pupae rolls were moved to fresh cages, facilitating dated adults.

2.1.2 Yeast extracts

Yeast isolates of *M. pulcherrima* and *M. andauensis* in exponential growth phase were inoculated into Infors HT Multifors (Infors HT, Bottmingen, Switzerland) fermentation reactors (Fig. 6) with working volume of 0.8 l, containing synthetic minimal media (Merico et al., 2007) and anti-foaming agent (Antifoam 204, Sigma, A6426). Temperature were kept at 25℃, pH was automatically adjusted to 5.0, Dissolved oxygen to 30% of air saturation. Airflow was kept at 0.5 $\mathrm{l\,min^{-1}}$ (Rozpędowska et al., 2011). After 24 hours of fermentation, efflux air were collected for 20 hours onto air filters (Super Q, 80/100 mesh; Alltech, Deerfield, IL, USA). Filters were eluted with 600 µl of hexane into glass vials.

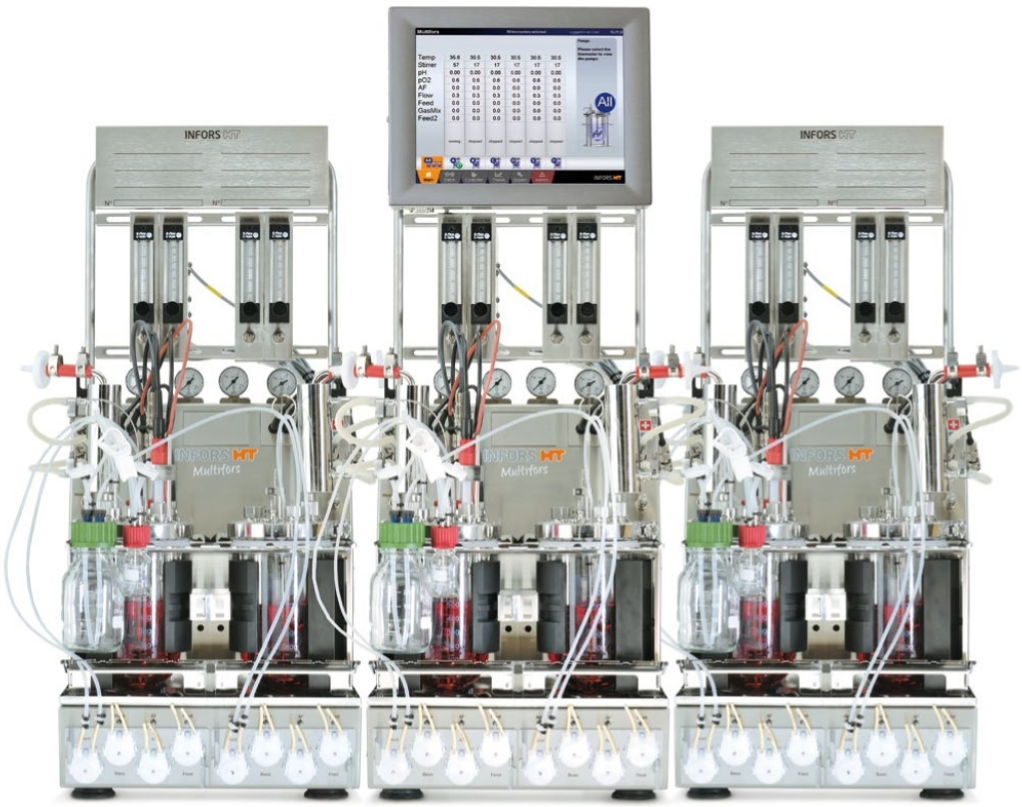


Figure 6: Multifors fermentation reactors. Image from company flyer.

2.2 Electrophysiology

Gas chromatography combined with electroantennographic detection (GC-EAD) were performed using a gas chromatograph (6890 GC, Agilent Technologies, Palo Alto, CA, USA) interfaced with equipment measuring electric potential of codling moth antennae subjected to column efflux stimuli. Extracts were injected (2µl) in split-less mode. The injector was open for 30s at 225℃. A fused silica polar column (DB-Wax, J&W Scientific, Folsom, CA, USA) was used used with hydrogen as mobile phase at constant flow of 42 $\mathrm{cm\,s^{-1}}$. The GC oven was programmed from 35℃, (holding 4min), to 225℃ at a rate of $8℃\,\mathrm{min^{-1}}$. The end of the column was connected to a four-way low dead volume connector (3D/2, Gerstel, Mülheim an der Ruhr, Germany) where nitrogen at 4.0 psi was introduced. From one of the remaining connectors, a deactivated column leading to the GC Flame Ionisation Detector (FID), was connected. Another deactivated column, from the remaining connector, was led through a transfer line heater (ODP-2, Gerstel) programmed to follow oven temperature, ending in an angled glass tube having a flow of purified and humidified air at $50\mathrm{cm\,s^{-1}}$. At the end of the glass tube, codling moth antennae from 2 to 3 day old mated females were connected between two glass electrodes, containing Beadle-Ephrussi ringer solution. One of the electrodes was connected to measurement ground, the other was connected to a high input impedance operational amplifier (EAG Kombi-Probe, Ockenfels SYNTECH GmbH, Kirchzarten, Germany). The amplifier was connected to a analog to digital converter (IDAC-2, Ockenfels SYNTECH GmbH) interfaced with a personal computer equipped with acquisition software (GcEad/2011, open source, http://gcead.sourceforge.net/).

A custom software was written in Python (van Rossum, 1990–) to facilitate data conversion, alignment, filtration, averaging and plotting. The numpy/scipy library (Jones et al., 2001–) was used for array manipulation routines. PySide (Lizardo et al., 2011–) for reading of the binary EAD files. Matplotlib (Barrett et al., 2005) was used for visualization of the data.

2.2.1 Signal extraction

FID data files (.D directories) were converted to Net CDF format by using the batch export function in Agilent mass spectroscopy software [REF agilent]. The open specification CDF format was selected as more meta-data such as sample name, description and time-stamp are retained compared to regular text (ASCII/CSV) export. Files were then read into the program using the netcdf_file class in the scipy.io module.

The open source version of the Syntech GC-EAD software is written as a Qt application and utilises the Qt data storage mechanisms. The EAD data files (.EAD files) could therefore be read directly into the program using the QIODevice and QDataStream classes in the PySide.QtCore module. The EAD files contained no time index information. Sampling rate was estimated to 100.04 Hz, making it possible to create a needed time index for alignment.

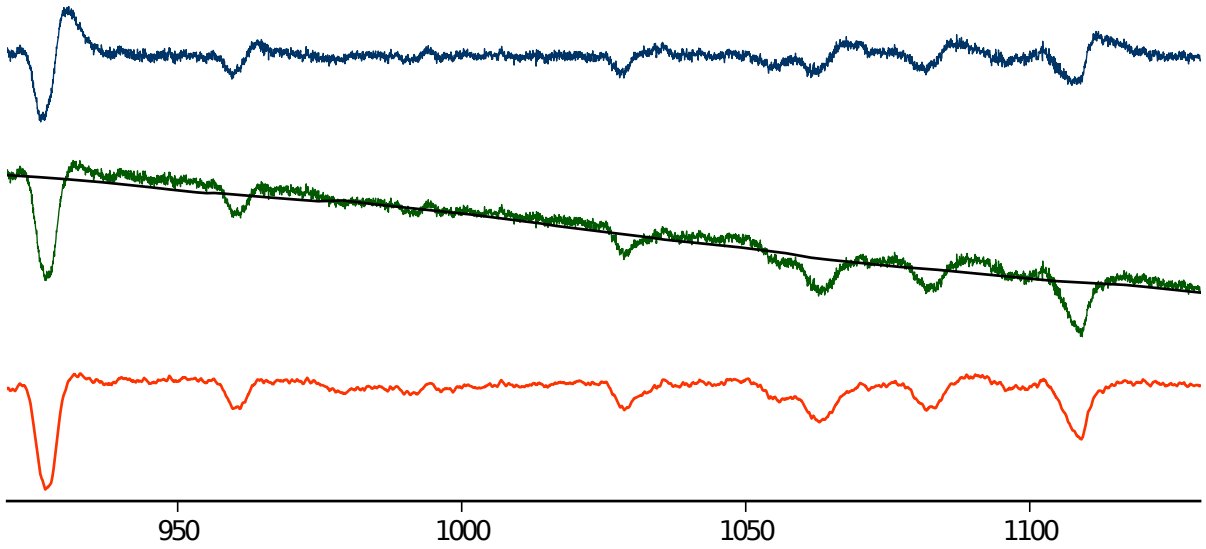


Figure 7: EAD signal restoration and filtering. Original signal (top, blue) is subjected to a reverse highpass filter creating restored signal (middle, green). A wideband filter is applied approximating a baseline (overlaid, black). A narrowband filter is applied to restored signal and the approximated baseline subtraced (bottom, orange).

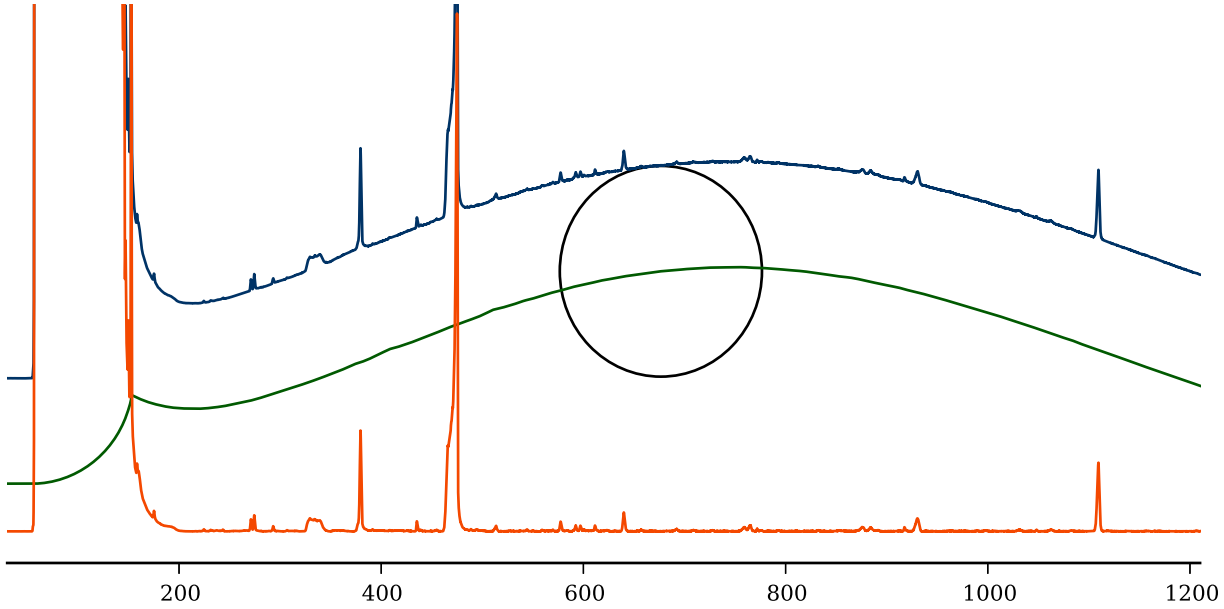


Figure 8: FID baseline correction using "Rolling ball" technique (Kneen and Annegarn, 1996). An imagined ball (black) is rolling on the underside of the original signal (blue). The path it follows (green) plus the radius describe the baseline that is subsequently subtracted from the signal, resulting in an adjusted signal (orange)

2.2.2 Signal filtering

The Syntech IDAC-2 has a hardware high-pass filter forcing the EAD signal back to zero. Without the filter, the signal would often drift outside of the recording range. However,

a few drawbacks occur using this filter: Low frequency responses are potentially reduced or removed from the signal; response maxima are shifted in time as the filtered signal is a first-order derivative; large responses may mask smaller overlapping responses; shape of response is distorted. To counter this, a reverse high-pass filter algorithm was applied to the recorded data restoring the unfiltered signals. A wideband, symmetric first degree polynomial Savitzky-Golay (Savitzky and Golay, 1964), filter was then applied to the EAD signals approximating baselines. The approximations were then subtracted from the signals and then filtered with a narrow band filter of same type (Fig. 7).

Baseline correction of FID data were done using "Rolling ball" method (Fig. 8).

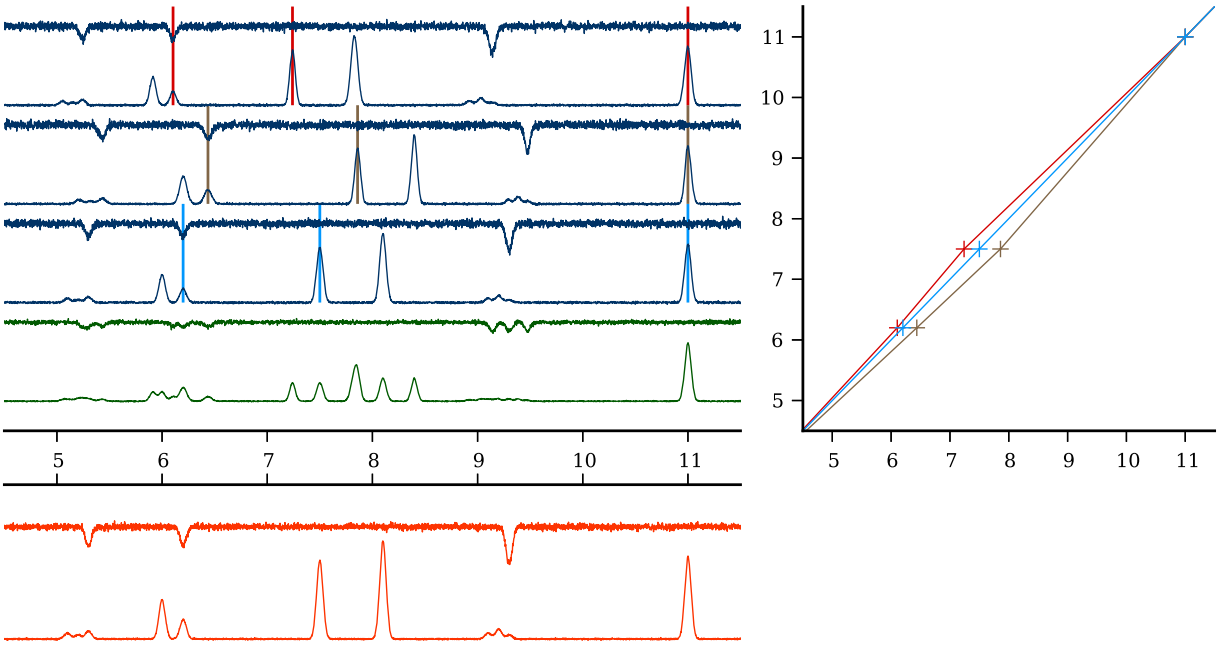


Figure 9: Signal alignment. FID and EAD signals (dark blue) without alignment yields unsatisfactory averages (green). After time transformation functions (right) are applied, the results are improved (orange). Simple offset could not solve the problem. To create the transformation functions, retention time of prominent peaks are interpolated against a reference recording.

2.2.3 Signal alignment

As the GC-EAD recording software suffered from program instability, often causing program termination when initiating the GC, recordings were started before sample injection. EAD and FID signals from the EAD recordings were manually offset to match FID signals as recorded by the GC software. FID signal from the EAD software could subsequently be discarded as noise, recording resolution and range of this data were unsatisfactory. Retention times of prominent and size consistent chromatogram peaks were selected for each sample. The retention times were then interpolated, using concatenated linear interpolation, against matching peaks in GC-MS total ion chromatograms, creating time transformation functions for each recording matching the timebase of GC-MS analysis. After application of these functions on the time indices, averages of the antennal responses were calculated (Fig. 9).

2.3 Chemical analysis

Extracts were analysed using a quadropole GC-MS (6890/5975 GC-MS, Agilent Technologies). Extracts were injected (2µl in splitless mode. The injector was open for 30s at 225℃. A polar column (DB-Wax, J&W Scientific) and a semi-polar column (HP-5MS, Agilent Technologies) were used with helium as mobile phase at constant flow of 35cm s^{-1}. The GC oven was programmed from 30℃, (holding 3min), to 225℃ at a rate of 8℃ min^{-1}. Transfer line was set to follow oven temperature. The detector was operating in negative mode (electron ionisation at 70eV).

2.3.1 MS spectra enhancement

Ion chromatograms from GC-MS analysis were extracted from exported CDF datafiles as per 2.2.1. Continuous Wavelet Transform (CWT) were applied to each individual ion channel using Ricker wavelet (negative normalized second derivative of a Gaussian function). The second scales' real part of these transforms were selected, negative numbers were set to zero and the signal then saved into new CDF files (Fig. 10). The new files could be imported into the Agilent MS software and the spectra of the now separated peaks could be matched against library and Kovat's retention index.

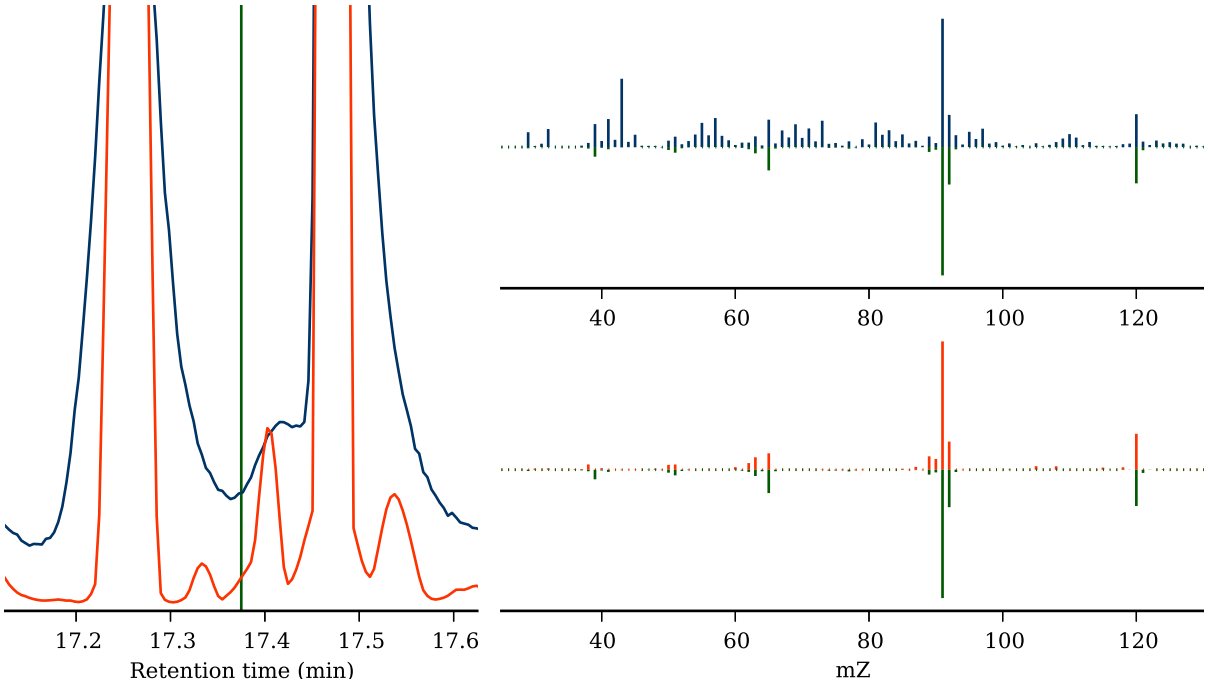


Figure 10: Peak separation in GC-MS analysis. Figure shows effect of GC-MS filtration algorithm on data. Identified compound was Phenyl acetealdehyde. Left: Partial total ion chromatograms. Unfiltered spectra (blue), filtered spectra (orange), spectra selection time (green vertical line). Top right: Unfiltered spectra versus reference spectra (NIST MS Number 235513). Lower right: Filtered spectra versus reference spectra.

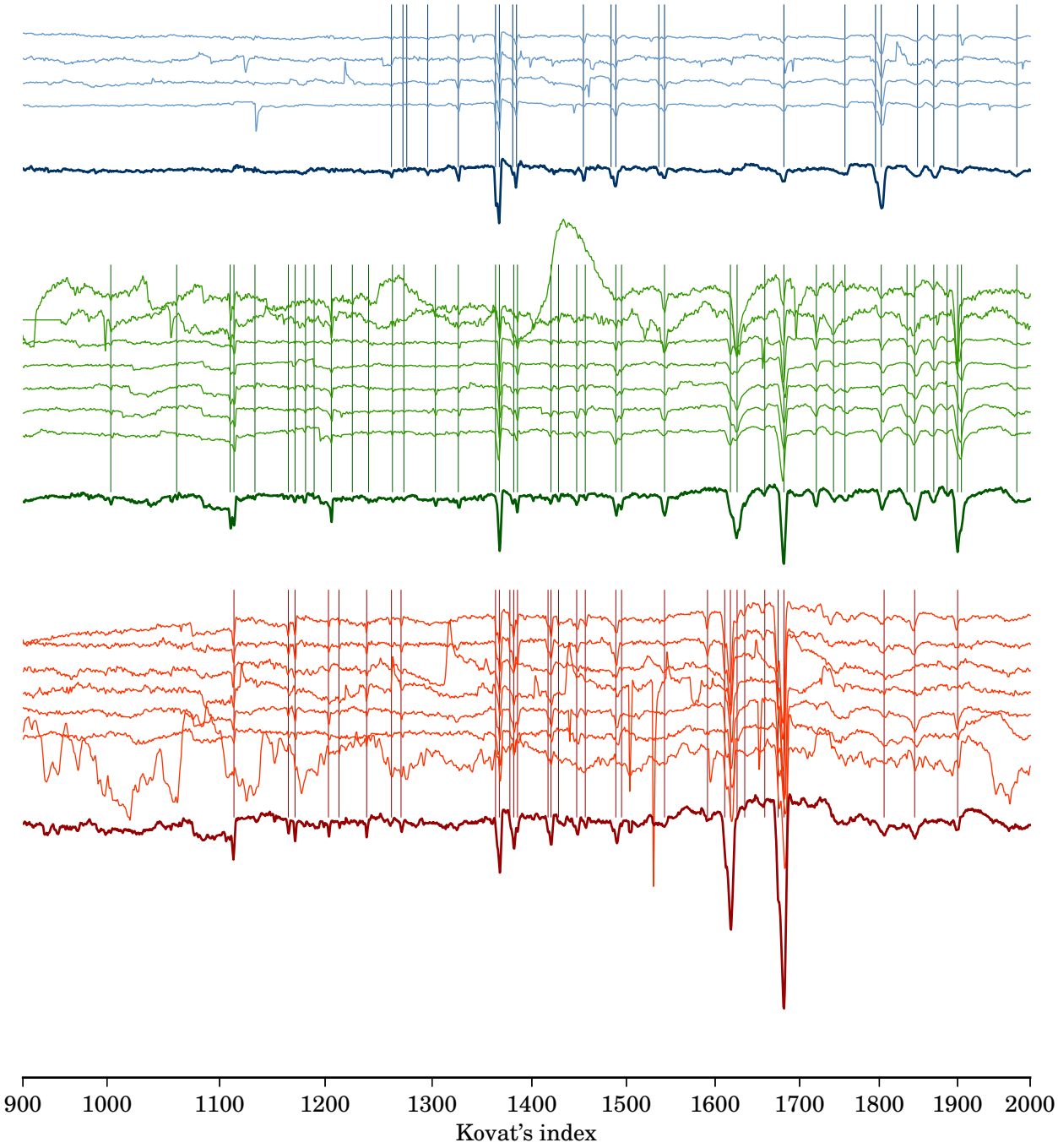
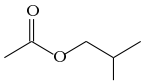
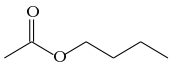
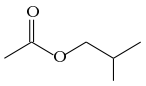
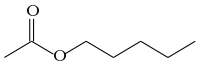
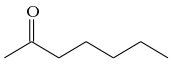
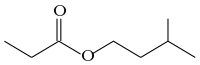
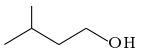
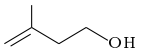
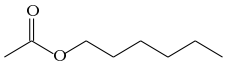
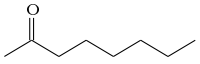
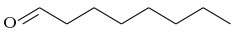
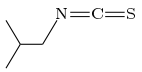
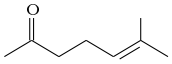
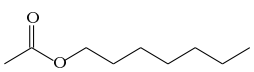


Figure 11: Codling moth antennal responses to fermentation volatiles - EAD traces; blues - control; greens - *M. andauensis*; reds - *M. pulcherrima*. Thin horizontal lines show individual recordings. Thicker lines show windsorised mean at 25%. Vertical lines mark responses.

3 Results

Visual inspection of EAD responses showed 23 responses to the control, 45 responses to the *M. andauensis* extract and 33 responses to the *M. pulcherrima* extract (Fig. 11). Matching FID peaks to MS peaks proved difficult, even when aligning GC-EAD to GC-MS. For many of the responses a matching MS peak/spectra could therefore not be found. Also, a number of responses with aligned spectra did not have good matches in compound libraries. Several unknown compounds could however be identified as some kind of esters (Tab. 1).

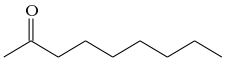
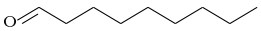
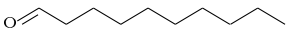
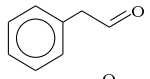
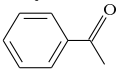
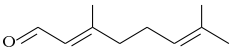
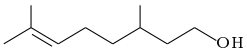
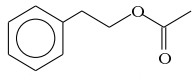
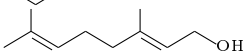
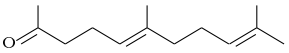
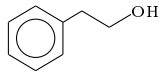
Table 1: Antennal responses to yeast volatile extracts

Compound	K.I.	ctrl	*Metschnikowia* *and.*	*pul.*	CAS	Structure
isobutyl acetate	**1003**		•		**110-19-0**	
butyl acetate	1062		•		123-86-4	
unknown	*1110*		•			
isoamyl acetate	**1114**		•	•	**110-19-0**	
unknown ester	*1133*		•			
amyl acetate	**1165**		•	•	**628-63-7**	
2-heptanone	**1171**		•	•	**110-43-0**	
isoamyl propionate	1181		•		105-68-0	
unknown ester	*1190*		•			
isoamyl alcohol	1206		•	•	123-51-3	
unknown ester	*1215*		•	•		
unknown	*1225*		•			
isoprenol	**1241**		•	•	**763-32-6**	
hexyl acetate	1263	•	•	•	142-92-7	
2-octanone	1274	•	•	•	111-13-7	
octanal	1276	•			124-13-0	
unknown	*1296*	•				
isobutyl isothiocyanate	1303		•		591-82-2	
sulcatone	**1326**	•	•		**110-93-0**	
unknown	*1364*	•	•	•		
heptyl acetate †	**1367**	•	•	•	112-06-1	

Antennal responses to yeast volatile extracts. Bullet (•) mark response to sample. Bold rows indicate that compound spectra, retention time and antennal activity were confirmed using synthetic compounds. †: internal standard

Continued on next page...

Table 1- continued from previous page

Compound	K.I.	ctrl	*Metschnikowia and.*	*Metschnikowia pul.*	CAS	Structure
unknown	*1377*			•		
2-nonanone	**1382**	•	•	•	**821-55-6**	
nonanal	**1386**	•	•	•	**124-19-6**	
unknown	*1416*			•		
unkown ester	*1420*		•	•		
unknown	*1428*		•	•		
unknown	*1447*		•	•		
unknown	*1457*	•	•	•		
unknown	*1483*	•		•		
unknown	*1489*	•	•	•		
decanal	1495		•	•	112-31-2	
unknown	*1537*	•				
unknown	*1543*	•	•	•		
unknown	*1591*			•		
unknown	*1611*			•		
unknown ester	*1618*		•	•		
unknown	*1626*		•	•		
phenyl actaldehyde	**1629**	•	•		**122-78-1**	
acetophenone	1635		•	•	98-86-2	
unknown	*1659*		•	•		
unknown	*1675*			•		
unknown	*1681*	•	•	•		
geranial	1721		•		141-27-5	
unknown	*1743*		•			
citronellol	**1757**	•	•		**106-22-9**	
unknown	*1795*	•				
2-phenylethyl acetate	**1802**	•	•	•	**103-45-7**	
geraniol	1835		•		106-24-1	
geranylacetone	**1845**	•	•	•	**3796-70-1**	
unknown	*1870*	•	•			
unknown	*1887*		•			
2-phenyl ethanol	**1900**	•	•	•	**60-12-8**	
unknown	*1905*		•			
unknown ester	*1982*	•	•			

Antennal responses to yeast volatile extracts. Bullet (•) mark response to sample. Bold rows indicate that compound spectra, retention time and antennal activity were confirmed using synthetic compounds. †: internal standard

4 Discussion

4.1 Method assessment

No formal simulation was done to properly test the performance of self-developed data treatment methods. It is however safe to say that without the alignment and averaging techniques it would have been difficult to assuredly pinpoint more than some ten responses. In this experiment only seven recordings per yeast extract and four recordings for the control were used; Increasing these numbers would result in a higher signal to noise ratio and results at a higher fidelity. Doubling the signal to noise ratio however, would require some 25 recordings as it increases with the square root of the number of recordings (Van Drongelen, 2007).

There is still a lot of room for improvement of the averaging methods to maximise data utilisation, as averages in this thesis were made using simple Windsorised means. Other techniques, possibly involving variance and co-variance estimation, could be used to create confidence intervals making it possible to test responses statistically. Another angle that should be considered is the separation of the signal into its components (response, non-random and random noise) by utilising least-square fitting or wavelet decomposition methods. This could possibly include filtering and detection methods based on bandwidth and EAD-FID covariance as described by Slone and Sullivan (2007). Another opportunity emerging, if responses can be accurately measured, is the possibility for response quantification and correlation to stimuli amount.

GC-MS spectra enhancement were done using continuous wavelet transforms. The method was far from perfect but proved fairly robust and spectra library matches scored generally higher compared to unfiltered spectra, especially for small chromatogram peaks. Other techniques such as those used by the AMDIS software (Stein, 1999), may yield better spectra enhancement and may also facilitate accurate quantification of compounds but it requires an experienced user and some measure of parameter estimation. Another software developed more recently is the BinBase system which includes automatic deconvolution, annotation and a mass spectral database aimed at metabolomics (Skogerson et al., 2011). This system shows great promise and should be considered in future work to reduce the workload of identifying leagues of compounds in many different samples.

Many GC-EAD responses occurred in the control extract as well as yeast extracts. It should be mentioned that responses to control were generally weaker, and that a matching GC-FID or GC-MS peak could sometimes not be found in the control but were found in yeast extracts. A possible explanation for responses to the control is the use of silicon tubing in the fermentation reactors. An earlier fermentation, using the same reactor, could have produced compounds solving into the silicon tubing. Even after autoclaving, the tubing could release minute amounts of antennaly active compounds onto absorption columns. This suspicion is strengthened by the fact that early eluding active compounds were exclusive to yeast volatile samples. Isoamyl alcohol and isoprenol have for example both a boiling point of around 130 ℃, close to autoclave temperature. The next two eluding active compounds showing activity, both in control and yeast extracts, hexyl acetate and 2-octanone have a boiling point of 172 ℃ and 174℃ respectively, well above that of the autoclave. Future collections should therefore not be done from fermentors unless absence of contaminants can be guaranteed.

The use of synthetic minimal media and liquid phase fermentation during collection may reflect yeast metabolism and lifestyle in floral nectar or in fruit pericarp. However, when the yeast grows on the plant surface, carbohydrates as well nutrients such as nitrogen

are less abundant compared to synthetic media. This is likely to effect yeast metabolism and its volatile profile. Also, in a liquid, emitted compounds first have to solve into the liquid phase before leaving it to be collected/detected. This may give a bias towards water soluble compounds.

To show attraction to yeast volatiles comparable to a natural situation, effort should be made to mimic natural conditions. Yeasts inoculated on sterilised fruit or on the leaves of sterile micro-propagated plants compared to an equivalent control would better approximate conditions found *in vivo*. If a completely synthetic environment is preferred, yeasts could be grown on a somewhat porous, inert material and small amounts of weak nutrient solution applied. Both these suggestions may not produce enough compounds for identification but could be used for behavioural experiments. Sugars and sugar alcohols on leaf and fruit surface of apple are suspected of playing an important part of the codling moth gustatory host assessment. Sorbitol, which is almost exclusively produced by species within the *Rosaceae* family, is likely a key part in this (Lombarkia and Derridj, 2002, 2008). These sugars and related compounds could serve as a guideline for nutrient solutions simulating leaf surface chemistry.

4.2 Ecology

The results of GC-EAD experiments showed a large number of responses to yeast volatile extracts, in particular to the *M. andauensis* extract. H^1_{NULL} is therefore safely rejected in favour of H^1_2. Many responses to yeast extracts without equivalent responses in the control were observed. H^2_{NULL} is therefore rejected in favour of H^2_1. This means that the codling moth can detect volatiles synthesised by the yeasts.

Among the active compounds, several esters, associated with ripe fruit odours, were identified. Several phenolic compounds, such as 2-phenylethanol, were identified. These types of compounds are mainly released from apple flowers (Omata et al., 1990; Bengtsson et al., 2001) and could serve as cues thereof.

Another interesting compound found in the extract is isothiocyanate compound. Both phenols and isothiocyanates have anti-microbial effects. The yeast could, with these compounds together with other anti-microbial compounds such as pulcherrimin, mentioned in the introduction, create a toxic or otherwise inhospitable environment which would likely not effect the egg development but would protect it from pathogenic micro-organisms. This would give the moth an early advantage and could potentially explain why leaves are the preferred oviposition site. This is however depending on whether the larva is inoculated by yeast from the oviposition site or if the adult female deposits the yeasts with her eggs.

4.3 Conclusion

We can safely conclude the female moth can detect yeast volatiles. Previous studies on other insects have shown insect attraction to yeasts (Guerenstein et al., 1995; Nout and Bartelt, 1998). Ongoing unpublished data suggest the codling moth is also attracted to yeast volatiles.

To further understand the ecological relationships between host, yeast and insect it is necessary to investigate whether the moth acts as a vector for the yeasts or if yeast colonisation of the host plant are by other means. Synergistic effects between yeast and plant volatiles should also be investigated as either one on its own may be insufficient as

attractants.

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