Development of semiochemical slow-release formulations as biological control devices against aphids

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SOLAPHID project (WALEO 2)

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“Biotechnologies related to the industrial production of insects used in integrated pest management”

5 teams:

ECOL (UCL), CIFA (UCL), CWBI (ULG), ENTO (GxABT), CA-CGO (GxABT)
Summary

General introduction

Objective

The choice of semiochemicals and their origin

How to analyse and quantify semiochemicals?

How to purify semiochemicals?

How to formulate semiochemicals?

Is the formulation efficient?

Conclusions and perspectives
General introduction
The aphid problem

Damages to crops: virus and disease transmitter

Economical and agricultural problem

Pesticide control is limited
- resistance of pest insects
- non species-specific
- unsafe for environment and human health

Biological control
Biological control as pest management strategy

“The use of natural enemies to reduce the damage caused by a pest population”

Attraction of aphid natural enemies
**Aphid tritrophic system**

1\(^{st}\) level  
Host plant

2\(^{nd}\) level  
Aphids

3\(^{rd}\) level  
Predators

Parasitoids

→ *Chemical communication: semiochemicals*
Semiochemicals
Plant – insect – insect chemical communication signals

Intraspecific interactions

Pheromones
- alarm
- sex
- aggregation
- trail
- host marking
- ...

Interspecific interactions

Allelochemicals
- allomones: + emitting species
- kairomones: + receptor species
- synomones: + emitting, + receptor

A same molecule can act as a pheromone and as an allelochemical substance
Objective
Global objective

To develop natural semiochemical slow-release formulations as biological control devices attractive towards aphid natural enemies

Which semiochemicals?  
- Natural origin?  
  - Purification?

Which formulation?  
- Analysis and quantification?

Efficiency?  
- Release?  
  - Attractiveness?
The choice of semiochemicals and their natural origin
**E-β-farnesene**

- **Sesquiterpene (C\textsubscript{15}H\textsubscript{24})**

- Aphid alarm pheromone \(^1\)

- Kairomone: attraction of aphid predators (*Episyrphus balteatus De Geer*)\(^2\)-\(^4\) and aphid parasitoids (*Aphidius ervi* Haliday) \(^5\)-\(^6\)

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\(^1\) Bowers et al., 1972

\(^2\) Francis et al., 2005

\(^3\) Verheggen et al., 2008

\(^4\) Verheggen et al., 2009

\(^5\) Du et al., 1998

\(^6\) Powell et al., 2003
**E-β-caryophyllene**

Sesquiterpene ($C_{15}H_{24}$)

- Reducer of aphid reproduction\textsuperscript{3}
- Attractive towards aphid parasitoids (A. ervi Haliday)\textsuperscript{4}

\textsuperscript{1} Tomova et al., 2005
\textsuperscript{2} Sasso et al., 2009
Natural matrix for sesquiterpenes

- *Matricaria chamomilla* L. (Asteraceae): *E*-β-farnesene

- *Nepeta cataria* L. (Lamiaceae): *E*-β-caryophyllene
**Essential oil characterisation: Gas chromatography**

*Chromatography*: a technique for separating the components of a mixture (liquid or gas) on the basis of differences in their affinity for a stationary (solid or liquid) and a mobile phase (liquid or gas)

*Gas chromatography*

- mixture: gas (headspace or vaporisation of a liquid)
- stationary phase: liquid or polymer in capillary column
- mobile phase: gas (inert carrier)
Essential oil characterisation: Gas chromatography

Program of $T^\circ$: optimisation of the separation of the components of the mixture

Most common: Mass spectrometer, FID

Chromatogram

He, $H_2$, $N_2$
Essential oil characterisation

Matricaria chamomilla L. (originated from Nepal)

GC-MS

Fast GC-FID

<table>
<thead>
<tr>
<th>( \text{No} )</th>
<th><strong>Major compounds</strong></th>
<th><strong>Retention index</strong></th>
<th><strong>%</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E-( \beta )-farnesene</td>
<td>1456</td>
<td>42.6</td>
</tr>
<tr>
<td>2</td>
<td>Germacrene D</td>
<td>1478</td>
<td>2.9</td>
</tr>
<tr>
<td>3</td>
<td>bicyclogermacrene</td>
<td>1494</td>
<td>1.9</td>
</tr>
<tr>
<td>4</td>
<td>(E,E)-( \alpha )-farnesene</td>
<td>1506</td>
<td>8.3</td>
</tr>
<tr>
<td>5</td>
<td>( \alpha )-bisabolol oxide B</td>
<td>1649</td>
<td>4.4</td>
</tr>
<tr>
<td>6</td>
<td>( \alpha )-bisabolone oxide A</td>
<td>1673</td>
<td>4.5</td>
</tr>
<tr>
<td>7</td>
<td>Chamazulene</td>
<td>1715</td>
<td>1.1</td>
</tr>
<tr>
<td>8</td>
<td>( \alpha )-bisabolol oxide A</td>
<td>1735</td>
<td>21.1</td>
</tr>
<tr>
<td>9</td>
<td>Cis-ene-yne-dicycloether</td>
<td>1802</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Essential oil characterisation

*Nepeta cataria* L. (originated from Canada)

<table>
<thead>
<tr>
<th>№</th>
<th>Major compounds</th>
<th>Retention index</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(Z,E)-nepetalactone</td>
<td>1353</td>
<td>8.4 %</td>
</tr>
<tr>
<td>2</td>
<td>(E,Z)-nepetalactone</td>
<td>1377</td>
<td>22.5 %</td>
</tr>
<tr>
<td>3</td>
<td>E-β-caryophyllene</td>
<td>1415</td>
<td>58.9 %</td>
</tr>
<tr>
<td>4</td>
<td>α-humulene</td>
<td>1465</td>
<td>3.9 %</td>
</tr>
</tbody>
</table>
How to analyse and quantify semiochemicals?

Heuskin S. et al., 2009, J. Chrom. A, 1216, 2768-2775
Ultra fast GC-FID analyses

Optimised analytical method

→ Good resolution of peaks in less than 5 min:

\[ R_s = 2(t_{R-E-\beta-caryophyllene} - t_{R-longifolene})/(W_{longifolene} - W_{E-\beta-caryophyllene}) \]

\[ R_s = 1.65 > 1.5 \rightarrow OK \]
Analytical validation

Objective of an analytical method for quantification:

*To be able to quantify the more precisely the routine samples*

\[ x_i \leftrightarrow \mu_T \]

Results \quad True \ value
Analytical validation

Objective of a validation:

To give to the laboratory the guarantees that the results are within acceptance limits.

\[ |x_i - \mu_T| < \lambda \]

Bias

\( \lambda = \text{acceptance limits} \)
Analytical validations

1. « Classical » validation

ISO 5725, GLP standard operating procedures:
criteria validated 1 by 1

2. « Accuracy profile » validation

Guidelines of the SFSTP:
Total error concept: combination of systematic and random errors

Accuracy = Trueness + Precision
How to purify semiochemicals from essential oils?
Purification of components: chromatographic techniques

Solid-Liquid chromatography

1. Essential oil in the head of the column
2. Beginning of the elution with solvent
3. Elution process
4. Collection of the semiochemical of interest
Purification of components: chromatographic techniques

Solid-Liquid chromatography

- Mixture: liquid – essential oil
- Stationary phase: solid – silicagel
- Mobile phase: liquid – solvent of elution

Goal: To obtain highly purified semiochemicals without solvent

→ Evaporation of solvent of elution
Choice of the solvent of elution

By thin layer chromatography

→ Choice of solvent based on:

- Best separation of compounds on silica
- Importance of solvent boiling point

N-pentane (36°C)
Essential oil fractionation

By liquid column chromatography

Preliminary tests

Small scale liquid column chromatography

1 ml essential oil deposited on 11 g dried silicagel

Elution with n-pentane

Collection of fractions (1.5 ml)

Fast GC analysis

Dilution
Matricaria chamomilla fractionation

<table>
<thead>
<tr>
<th>Elution volume (ml)</th>
<th>% EBF</th>
<th>% Germacrene D</th>
<th>% E,E-α-farnesene</th>
<th>% monoterpenes</th>
<th>% chamazulene</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 10,5 (F0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10,5 - 16,5 (F1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>16,5 - 22,5 (F2)</td>
<td>0 - 82</td>
<td>7,8 - 26</td>
<td>3 - 5</td>
<td>47 - 2</td>
<td>0</td>
</tr>
<tr>
<td>22,5 – 51 (F3)</td>
<td>86,3 - 76</td>
<td>4 - 1,4</td>
<td>5,7 - 22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>51 – 72 (F4)</td>
<td>72 - 56</td>
<td>1,4 - 1,6</td>
<td>22 - 33</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>72 – 90 (F5)</td>
<td>55 - 33</td>
<td>1,6</td>
<td>33 - 41</td>
<td>0</td>
<td>0,5 - 16</td>
</tr>
</tbody>
</table>
Essential oil fractionation

Solvent evaporation at 40°C: recoveries of E-β-farnesene

<table>
<thead>
<tr>
<th>Method</th>
<th>Water bath</th>
<th>Büchi evaporator at atmospheric pressure</th>
<th>Büchi evaporator under vacuum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>98.73 %</td>
<td>96.30 %</td>
<td>92.47 %</td>
</tr>
<tr>
<td>SD</td>
<td>0.35 %</td>
<td>0.94 %</td>
<td>3.43 %</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>0.36 %</td>
<td>0.98 %</td>
<td>3.71 %</td>
</tr>
<tr>
<td>Time</td>
<td>More than 4h.</td>
<td>30 min.</td>
<td>10 min.</td>
</tr>
</tbody>
</table>

Compromise between analyte recovery and evaporation time
Essential oil fractionation

Flash chromatography: higher scale under pressure

⇒ Reduced time

10 ml essential oil deposited on 110 g dried silicagel

Elution with n-pentane under pressure (N₂ = 0.5 bar)

Collection of concentrated fraction + solvent evaporation

Dilution

Fast GC analysis

⇒ Solvent-free purified semiochemicals
# Essential oil fractionation

## Flash chromatography

- **Highly purified semiochemicals**

### Matricaria chamomilla

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum of monoterpenes</td>
<td>1.3 %</td>
</tr>
<tr>
<td>E-β-farnesene</td>
<td>84.0 %</td>
</tr>
<tr>
<td>Germacrene D</td>
<td>1.4 %</td>
</tr>
<tr>
<td>Bicyclogermacrene</td>
<td>1.4 %</td>
</tr>
<tr>
<td>(E,E)-α-farnesene</td>
<td>11.9 %</td>
</tr>
</tbody>
</table>

### Nepeta cataria (Canada)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum of monoterpenes</td>
<td>1.5 %</td>
</tr>
<tr>
<td>β-caryophyllene</td>
<td>97.4 %</td>
</tr>
<tr>
<td>α-humulene</td>
<td>1.1 %</td>
</tr>
</tbody>
</table>
How to formulate semiochemicals?

Heuskin et al., 2012, Pest Manag. Sci., 68, 127-136
Formulation criteria

- Natural and biodegradable matrix
- Protection of semiochemicals over time (not oxidation)
- Sufficient release rate of semiochemicals over time
- Attractive towards aphid predators and/or parasitoids

Alginate gel beads
**Alginate**

- **β-D-mannuronate (M)**
  - (Poly M segment)

- **α-L-guluronate (G)**
  - (Poly G segment)

Poly MG segment
Gelling process of alginate

G segment

M segment

organisation

« Egg-box » structure
Formulation of alginate bead

Formulation optimisation in terms of semiochemical encapsulation capacity and texturometry, considering:

- Type of alginate (M/G – molar mass)
- Alginate concentration
- Type of cross-linker ion
- Cross-linker ion concentration
- Maturation time

For details: see Heuskin et al., 2012, Pest Management Science, 68, 127-136
Formulation of alginate bead

Semiochemical + sunflower oil + α-tocopherol (oil phase) → Alginate solution → Oil phase → Water phase → Homogenization (24000 rpm; 20 s.) → o/w emulsion → Dried alginate beads (Ø 2mm) → Drying → CaCl₂ Suspension (maturation)
Characterisation of alginate bead

« Semiochemical – oil » dispersion in the alginate network

CLSM imaging of a dried (Aw=0.42) E-β-farnesene alginate bead
Protection efficiency of beads towards sesquiterpenes

- **E-β-farnesene**
- **E-β-caryophyllene**

Heuskin S. et al., JPBA, 2010, 53, 962-972
Is the formulation efficient...
... in terms of semiochemical release?
Volatile collection system

Activated charcoal filter

Adsorbent (HayeSep Q) cartridge

Solvent elution + IS quantification (Fast GC)

Teflon box with semiochemical alginate beads

Pump
Volatile collection system

Specifications and performances

- Boxes and tubing in Teflon => adsorption of semiochemicals
- Activated charcoal filters: air purification
- Sampling + security cartridges → breakthrough
- Total volume of eluting solvent: 4 x 250 µL n-hexane/cartridge
- Mean recovery of elution: 94.5 % ± 4.2 %
Release rate of semiochemicals

Laboratory controlled conditions:
- Temperature: 20°C
- Relative humidity: 65%
- Air flow: 0.5 L/min
Influence of abiotic factors on semiochemical diffusion

Temperature – Relative humidity – Air flow

Preliminary experiments

<table>
<thead>
<tr>
<th>Experimental test</th>
<th>Relative humidity (%)</th>
<th>Airflow (L/min)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N° 1</td>
<td>25</td>
<td>0.05</td>
<td>20</td>
</tr>
<tr>
<td>N° 2</td>
<td>25</td>
<td>0.50</td>
<td>20</td>
</tr>
<tr>
<td>N° 3</td>
<td>25</td>
<td>1.00</td>
<td>20</td>
</tr>
<tr>
<td>N° 4</td>
<td>75</td>
<td>0.50</td>
<td>20</td>
</tr>
<tr>
<td>N° 5</td>
<td>75</td>
<td>0.50</td>
<td>40</td>
</tr>
<tr>
<td>N° 6</td>
<td>85</td>
<td>0.50</td>
<td>20</td>
</tr>
<tr>
<td>N° 7</td>
<td>90</td>
<td>0.50</td>
<td>20</td>
</tr>
<tr>
<td>N° 8</td>
<td>100</td>
<td>0.50</td>
<td>20</td>
</tr>
</tbody>
</table>
**Semiochemical diffusion coefficient estimation**

Diffusion in a sphere (Cranck, 1975):

\[
\frac{M_t}{M_\infty} = 1 - 6 \sum_{n=1}^{\infty} \frac{1}{n^2} \exp\left(-Dn^2 \pi^2 t / a^2\right)
\]

- \(M_t\) (µg): cumulative mass of semiochemical released at time \(t\)
- \(M_\infty\) (µg): cumulative mass of semiochemical released at time \(\infty\) (supposed to be the total quantity of volatile in the bead at time \(t=0\))
- \(a\) (m): radius of one bead
- \(t\) (s): diffusion time
- \(n\): number of terms in the sum
- \(D\) (m²/s): effective diffusion coefficient of semiochemical
## Experimental Data

<table>
<thead>
<tr>
<th>Experimental test</th>
<th>Relative humidity (%)</th>
<th>Airflow (L/min)</th>
<th>Temperature (°C)</th>
<th>Diffusion coefficient for E-β-farnesene (m²/s)</th>
<th>Diffusion coefficient for E-β-caryophyllene (m²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N° 1</td>
<td>25</td>
<td>0.05</td>
<td>20</td>
<td>1.98 * 10⁻¹⁴</td>
<td>1.35 * 10⁻¹⁵</td>
</tr>
<tr>
<td>N° 2</td>
<td>25</td>
<td>0.50</td>
<td>20</td>
<td>3.40 * 10⁻¹⁴</td>
<td>1.57 * 10⁻¹⁵</td>
</tr>
<tr>
<td>N° 3</td>
<td>25</td>
<td>1.00</td>
<td>20</td>
<td>3.71 * 10⁻¹⁴</td>
<td>1.23 * 10⁻¹⁵</td>
</tr>
<tr>
<td>N° 4</td>
<td>75</td>
<td>0.50</td>
<td>20</td>
<td>1.23 * 10⁻¹⁴</td>
<td>7.39 * 10⁻¹⁵</td>
</tr>
<tr>
<td>N° 5</td>
<td>75</td>
<td>0.50</td>
<td>40</td>
<td>2.12 * 10⁻¹⁴</td>
<td>1.03 * 10⁻¹⁴</td>
</tr>
<tr>
<td>N° 6</td>
<td>85</td>
<td>0.50</td>
<td>20</td>
<td>1.56 * 10⁻¹⁵</td>
<td>1.33 * 10⁻³²</td>
</tr>
<tr>
<td>N° 7</td>
<td>90</td>
<td>0.50</td>
<td>20</td>
<td>6.15 * 10⁻³³</td>
<td>8.26 * 10⁻³³</td>
</tr>
<tr>
<td>N° 8</td>
<td>100</td>
<td>0.50</td>
<td>20</td>
<td>1.03 * 10⁻³²</td>
<td>9.93 * 10⁻³¹</td>
</tr>
</tbody>
</table>
Influence of abiotic factors on semiochemical diffusion

- Most limiting factor: relative humidity ≥ 85%
- Influence of temperature
- Weak influence of air flow
Improvement of the research

- Box-Behnken experimental design (3 factors in 3 levels)
- Water sorption / desorption isotherms on alginate beads
- Evolution of bead diameter with $A_w$

→ Confirmation of the preliminary results

TFE F. Daems (2011), GxABT, ULG
Is the formulation efficient...
... in terms of biological control devices?
On parasitoids (Aphidius ervi): 2-way olfactometer

Blank

Alginate beads with semiochemicals

Air flow

On parasitoids (Aphidius ervi): 2-way olfactometer

Blank

Alginate beads with semiochemicals

*** very highly significant difference (P<0.001)

N = 30
On Syrphidae: on-field experiments

- 3 crops: beet, horse bean, winter wheat
- \( E-\beta \)-farnesene, \( E-\beta \)-caryophyllene and blank alginate beads
- 1 latin square design per crop
On Syrphidae: on-field experiments

Dunnett Test (95%): comparison of attractiveness between semiochemical beads and blank

- **E-β-Farnesene**: P-value = 0.0200 (< 0.05) * significant difference
- **E-β-Caryophyllene**: P-value = 0.0064 (< 0.01) ** highly significant difference
Conclusions and perspectives
Conclusions

1. How to analyse and quantify semiochemicals?
   ➔ Ultra Fast GC method validated

2. How to purify semiochemicals?
   ➔ Flash Chromatography: molecules at high purity

3. How to formulate semiochemicals?
   ➔ Alginate gel beads: formulation optimised and characterised

4. Is the formulation efficient?
   ➔ In terms of release... YES
   ➔ In terms of biological control device... YES
**Perspectives or improvements for the research**

- Time of degradation and microbiological study of alginate beads outdoors
- Field experiments: maximal distance of attraction; maintaining beneficial insects on field
- At larger scale:
  - automated flash chromatography + solvent recycling
  - automated alginate bead production system
- Encapsulation of other molecules useful in chemical ecology
Thank you for your attention