

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

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SOLAPHID project (WALEO 2)

Funding from the Walloon Region (2006-2011)

*“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?

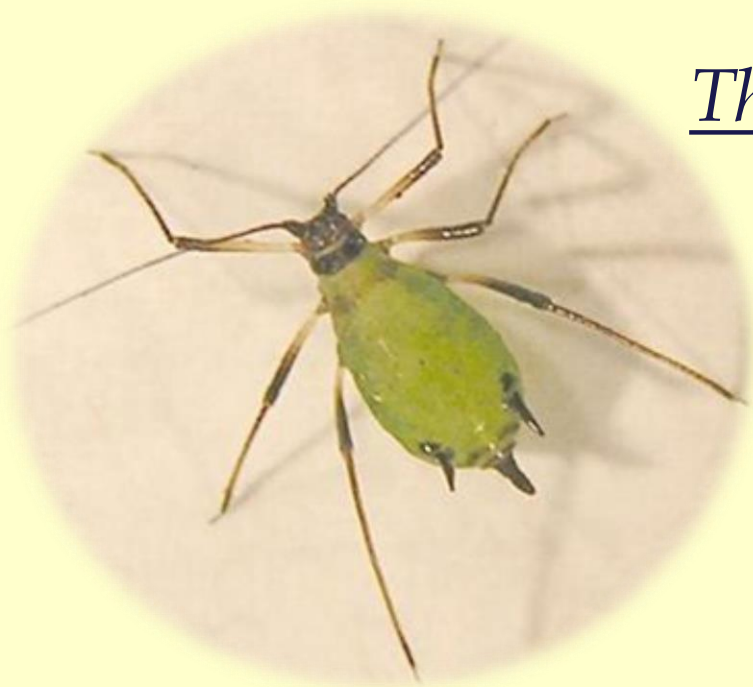
How to purify semiochemicals?

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

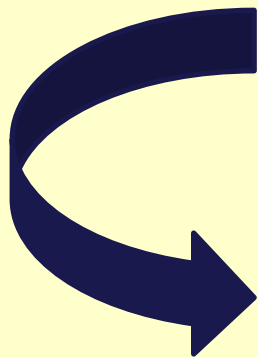
- resistance of pest insects
- non species-specific
- unsafe for environment and human health

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

1st level

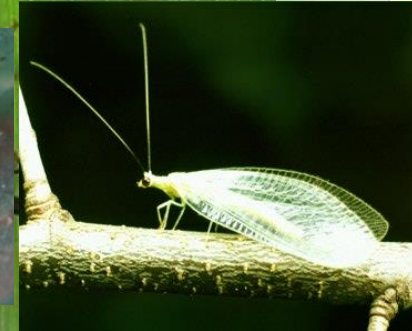
2nd level

3rd level

Host plant

Aphids

Predators



Parasitoids



➔ *Chemical communication : semiochemicals*

Semiochemicals

Plant – insect – insect chemical communication signals

Intraspecific
interactions

Pheromones

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

Interspecific
interactions

Allelochemicals

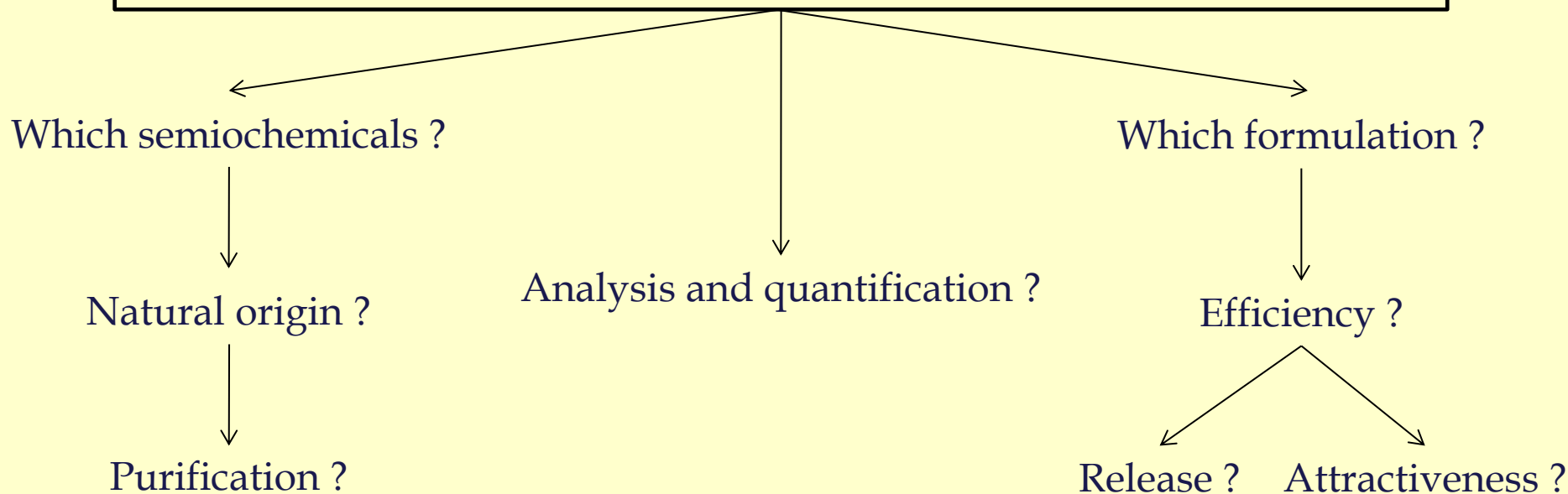
- allomones: + emitting species
- kairomones: +receptor species
- synomones: + emitting, + receptor

*A same molecule can act as a pheromone and
as an allelochemical substance*

Objective

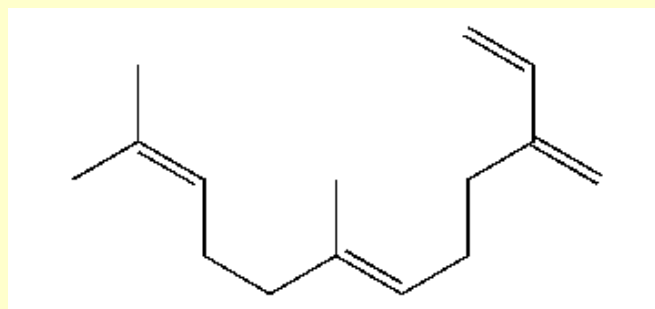
Global objective

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



The choice of semiochemicals and their natural origin

E-β-farnesene



Sesquiterpene (C₁₅H₂₄)



- Aphid alarm pheromone ¹
- Kairomone: attraction of aphid predators (*Episyrphus balteatus* De Geer)²⁻⁴ and aphid parasitoids (*Aphidius ervi* Haliday) ⁵⁻⁶

¹ Bowers et al., 1972

² Francis et al., 2005

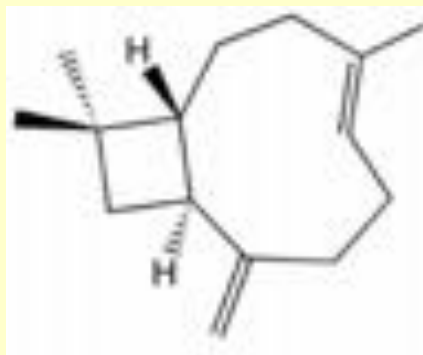
³ Verheggen et al., 2008

⁴ Verheggen et al., 2009

⁵ Du et al., 1998

⁶ Powell et al., 2003

E- β -caryophyllene



Sesquiterpene (C₁₅H₂₄)

- Reducer of aphid reproduction³
- Attractive towards aphid parasitoids (*A. ervi* Haliday)⁴

¹ Tomova et al., 2005

² Sasso et al., 2009

Natural matrix for sesquiterpenes

➔ Essential oils

- *Matricaria chamomilla* L. (Asteraceae): *E*- β -farnesene
- *Nepeta cataria* L. (Lamiaceae): *E*- β -caryophyllene



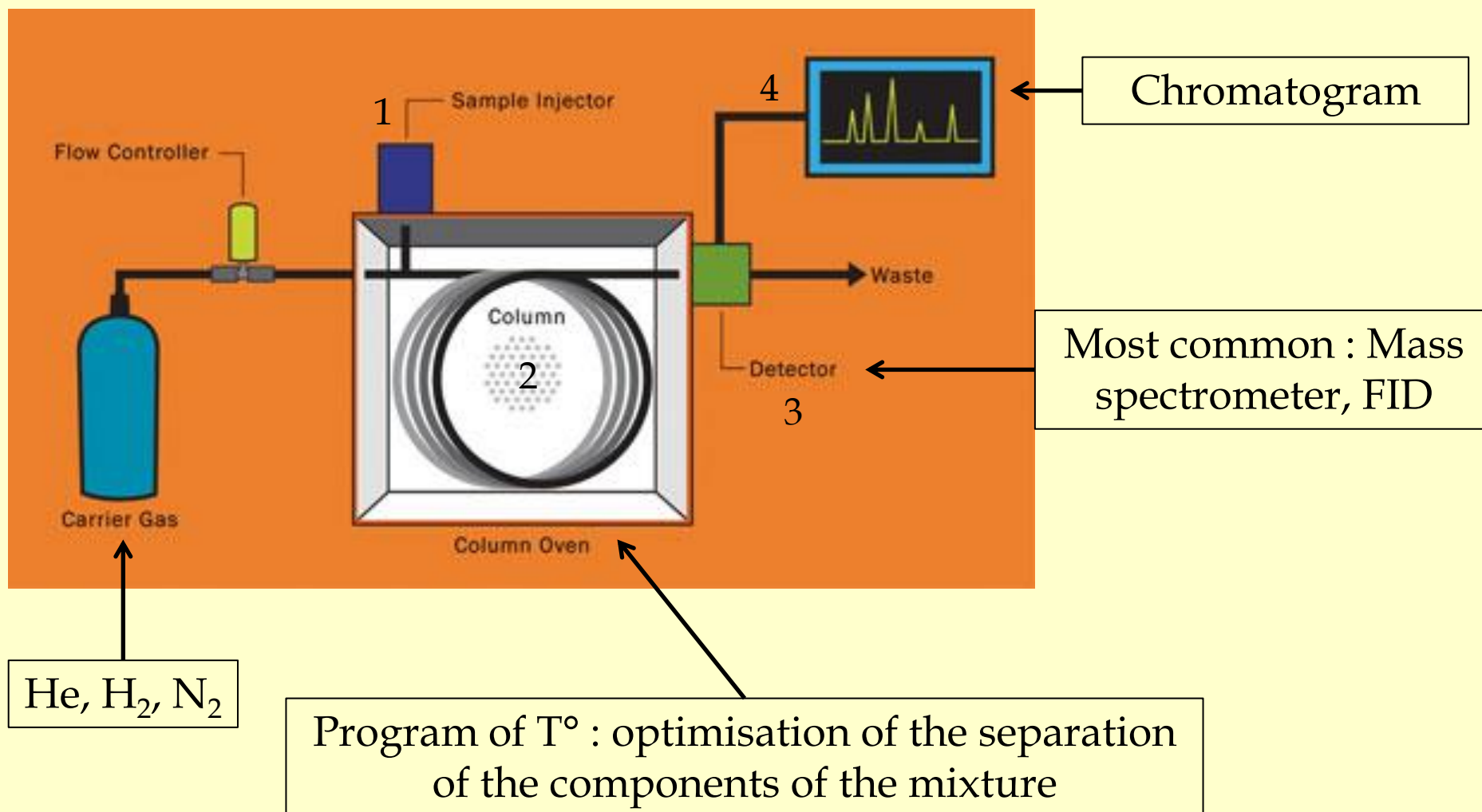
Essential oil characterisation : Gas chromatography

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

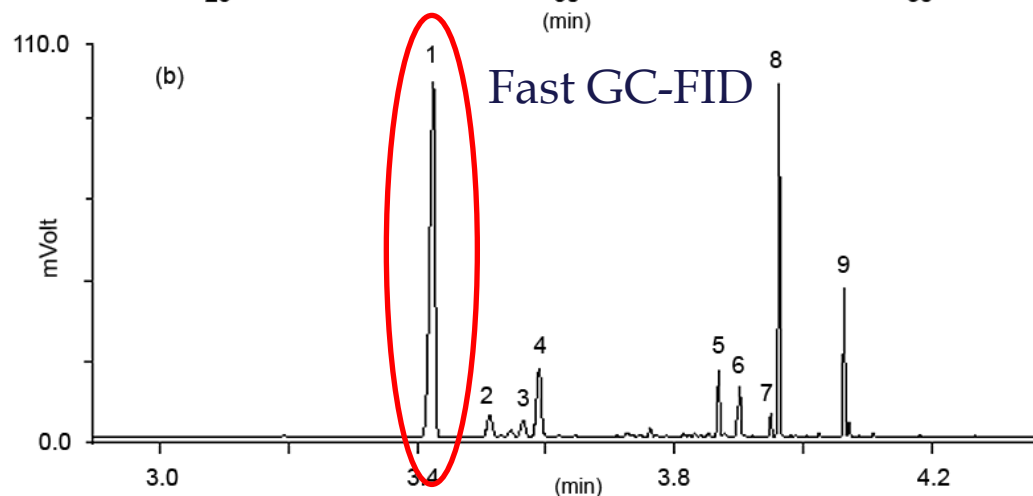
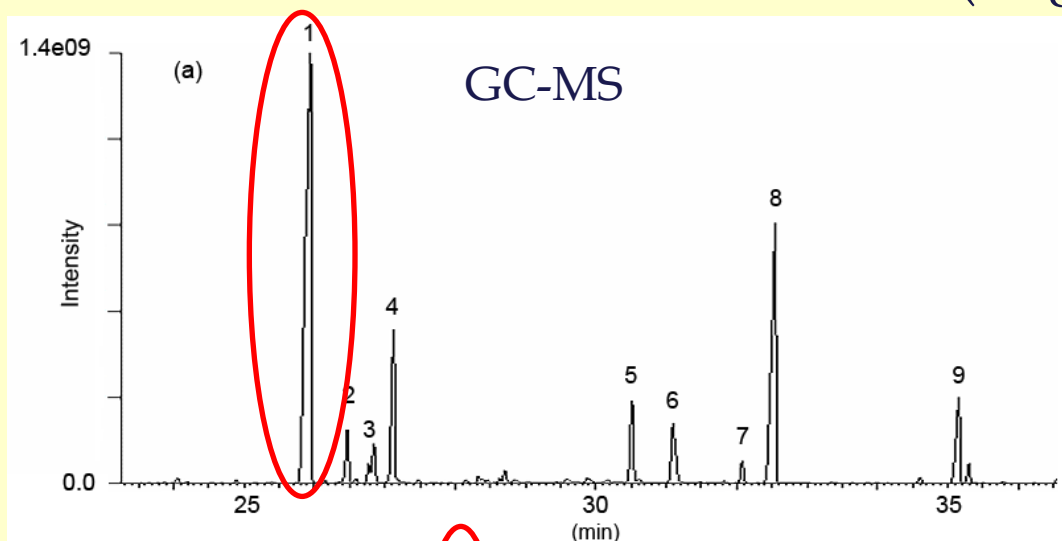
- mixture : gas (headspace or vaporisation of a liquid)
- stationary phase : liquid or polymer in capillary column
- mobile phase : gas (inert carrier)

Essential oil characterisation : Gas chromatography



Essential oil characterisation

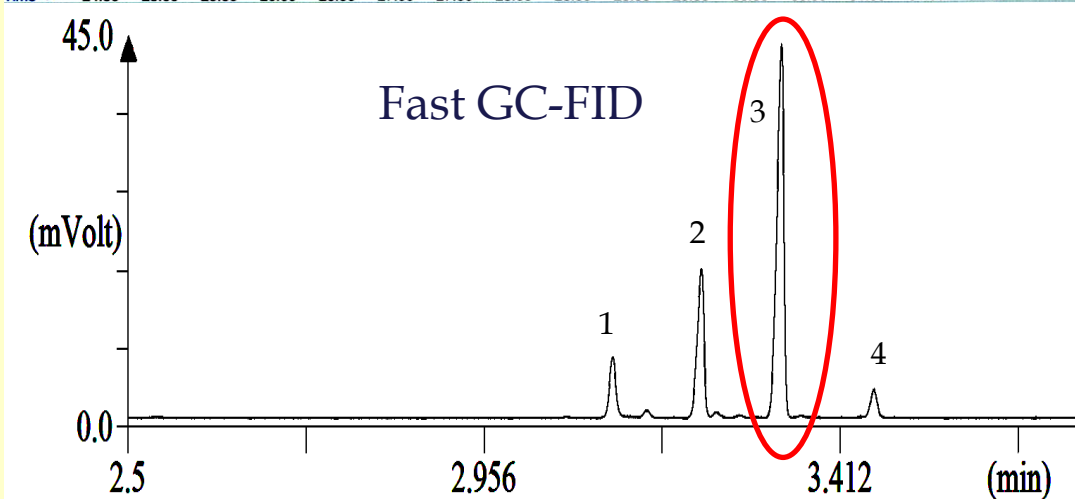
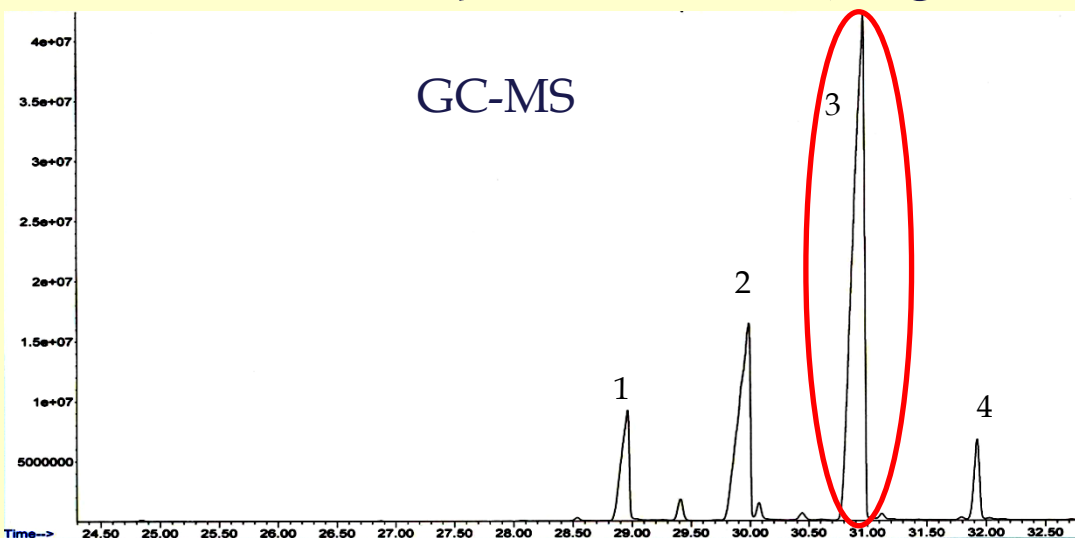
Matricaria chamomilla L. (originated from Nepal)



N°	Major compounds	Retention index	%
1	E-β-farnesene	1456	42,6
2	Germacrene D	1478	2,9
3	bicyclogermacrene	1494	1,9
4	(E,E)-α-farnesene	1506	8,3
5	α-bisabolol oxide B	1649	4,4
6	α-bisabolone oxide A	1673	4,5
7	Chamazulene	1715	1,1
8	α-bisabolol oxide A	1735	21,1
9	Cis-ene-yne-dicycloether	1802	5,9

Essential oil characterisation

Nepeta cataria L. (originated from Canada)



N°	Major compounds	Retention index	%
1	(Z,E)-nepetalactone	1353	8.4 %
2	(E,Z)-nepetalactone	1377	22.5 %
3	E-β-caryophyllene	1415	58.9 %
4	α -humulene	1465	3.9 %

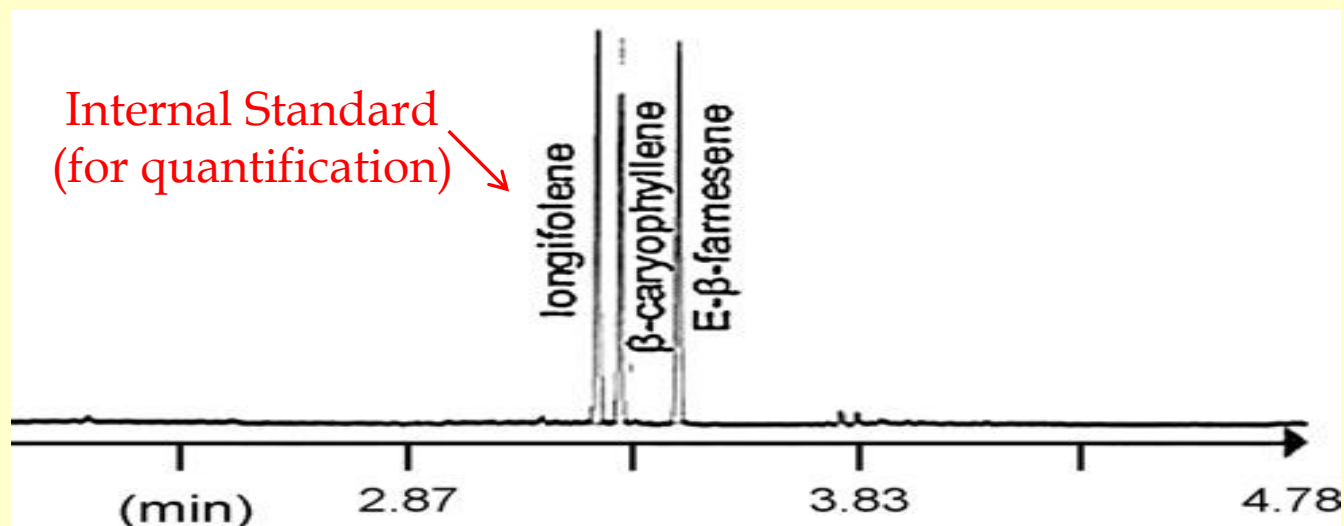
How to analyse and quantify semiochemicals ?

Heuskin S.et al., 2009, J. Chrom. A, 1216, 2768-2775

Heuskin S.et al., 2010, J. Pharm. Biomed. Anal., 53, 962-972

Ultra fast GC-FID analyses

Optimised analytical method



→ Good resolution of peaks in less than 5 min:

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

$$R_s = 1,65 > 1,5 \rightarrow \text{OK}$$



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

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

λ = 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

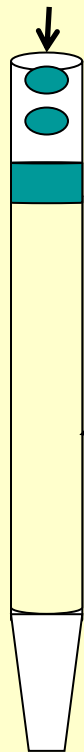
How to purify semiochemicals from essential oils ?

Purification of components : chromatographic techniques

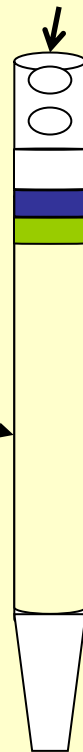
Solid-Liquid chromatography

Essential oil

Solvent of elution



Silica gel



1. Essential oil in
the head of the
column

2. Beginning of the
elution with solvent



3. Elution process



Semiochemical
of interest



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

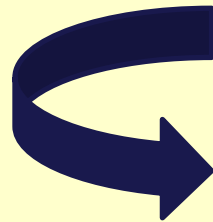
➔ Evaporation of solvent of elution

Choice of the solvent of elution

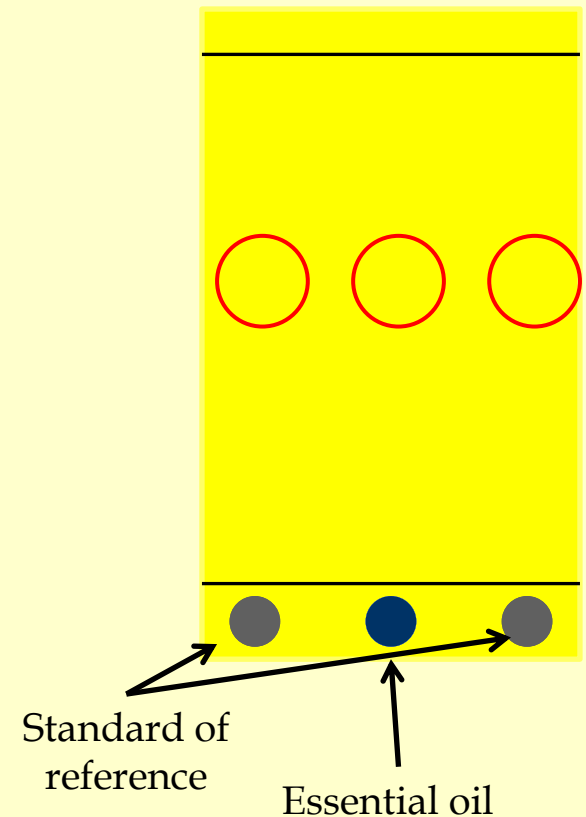
By thin layer chromatography

→ 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

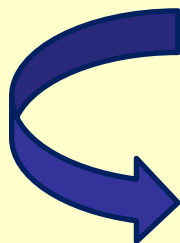


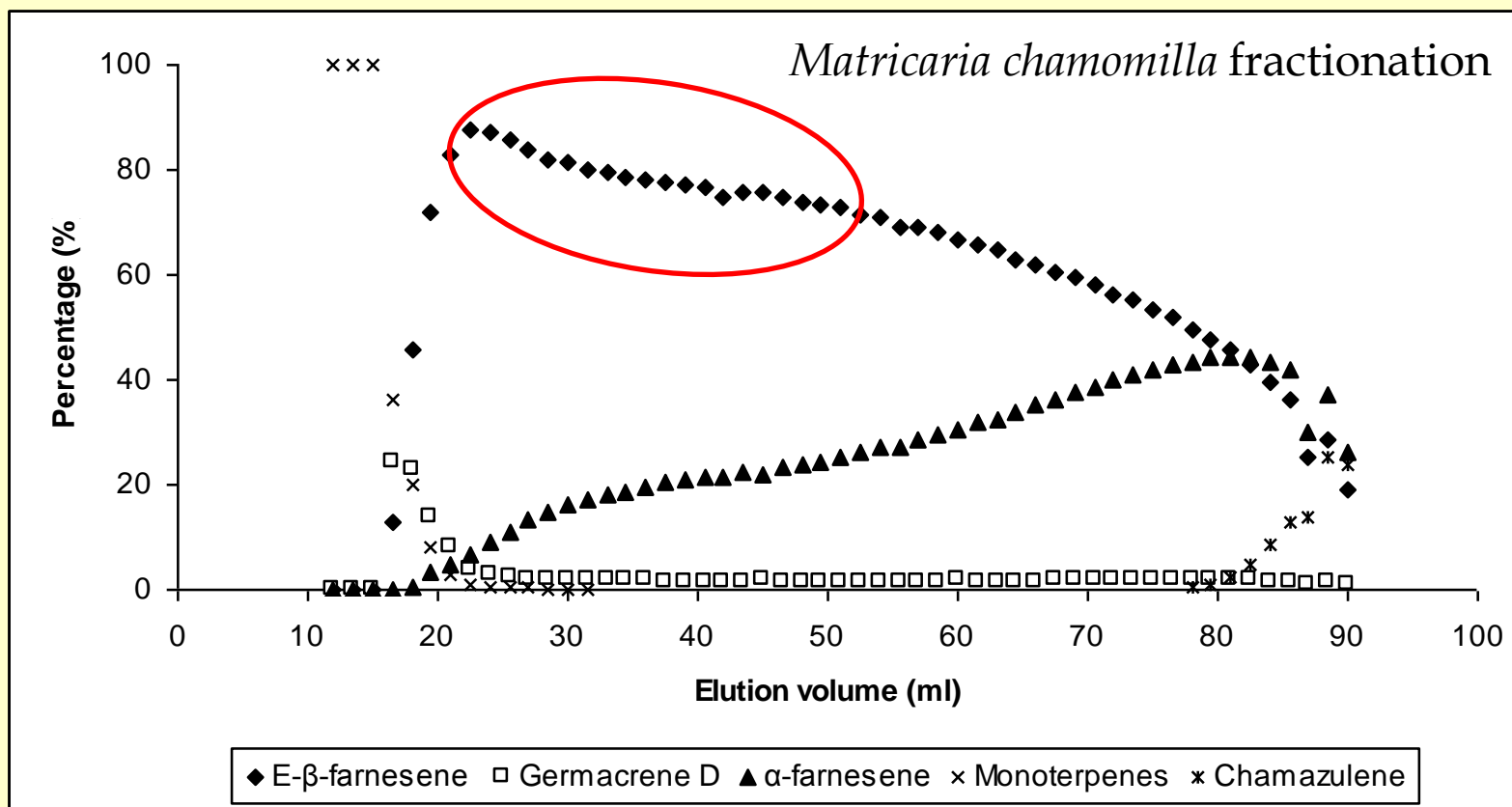
Collection of fractions (1.5 ml)



Fast GC analysis

Dilution





Elution volume (ml)	% EBF	% Germacrene D	% E,E-α-farnesene	% monoterpenes	% chamazulene
0 - 10,5 (F0)	0	0	0	0	0
10,5 - 16,5 (F1)	0	0	0	100	0
16,5 - 22,5 (F2)	0 - 82	7,8 - 26	3 - 5	47 - 2	0
22,5 - 51 (F3)	86,3 - 76	4 - 1,4	5,7 - 22	0	0
51 - 72 (F4)	72 - 56	1,4 - 1,6	22 - 33	0	0
72 - 90 (F5)	55 - 33	1,6	33 - 41	0	0,5 - 16

Essential oil fractionation

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

	Water bath	Büchi evaporator at atmospheric pressure	Büchi evaporator under vacuum
Mean	98.73 %	96.30 %	92.47 %
SD	0.35 %	0.94 %	3.43 %
RSD (%)	0.36 %	0.98 %	3.71 %
Time	More than 4h.	30 min.	10 min.

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



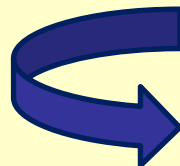
Elution with n-pentane under pressure ($N_2 = 0.5$ bar)



Collection of concentrated fraction + solvent evaporation



Dilution



Fast GC analysis

→ *Solvent-free purified semiochemicals*



Essential oil fractionation

Flash chromatography

→ *Highly purified semiochemicals*

Matricaria chamomilla

Compounds	Purity
Sum of monoterpenes	1.3 %
E-β-farnesene	84.0 %
Germacrene D	1.4 %
Bicyclogermacrene	1.4 %
(E,E)- α -farnesene	11.9 %

Nepeta cataria (Canada)

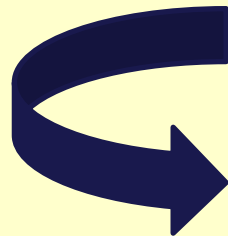
Compounds	Purity
Sum of monoterpenes	1.5 %
β-caryophyllene	97.4 %
α -humulene	1.1 %



How to formulate semiochemicals?

Formulation criteria

- Natural and biodegradable matrix
- Protection of semiochemicals over time $><$ oxidation
- Sufficient release rate of semiochemicals over time
- Attractive towards aphid predators and/or parasitoids



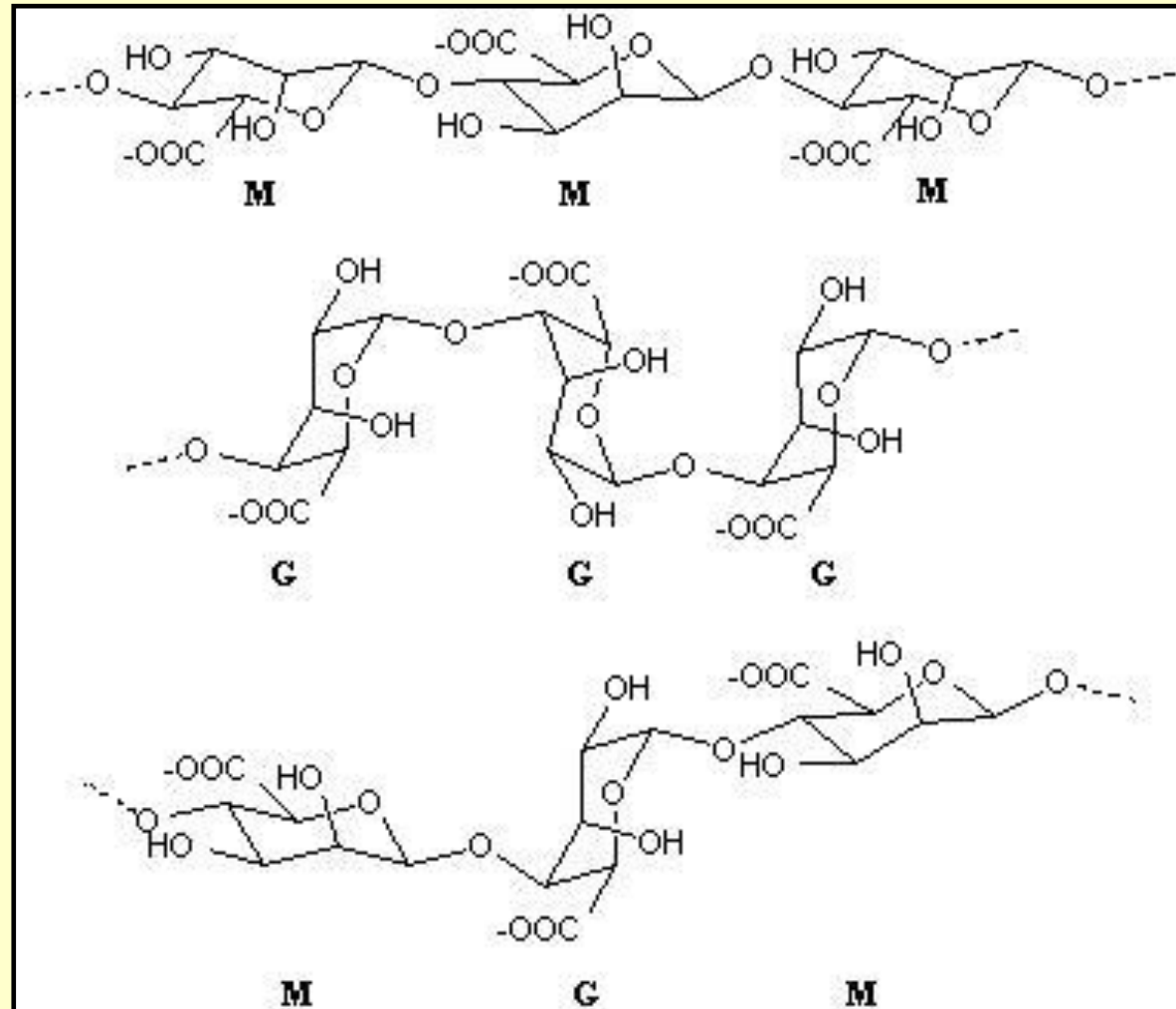
Alginate gel beads

Alginate

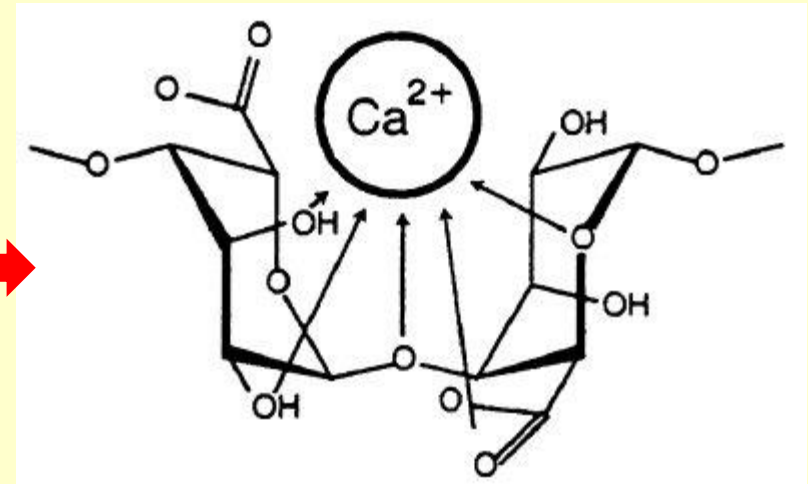
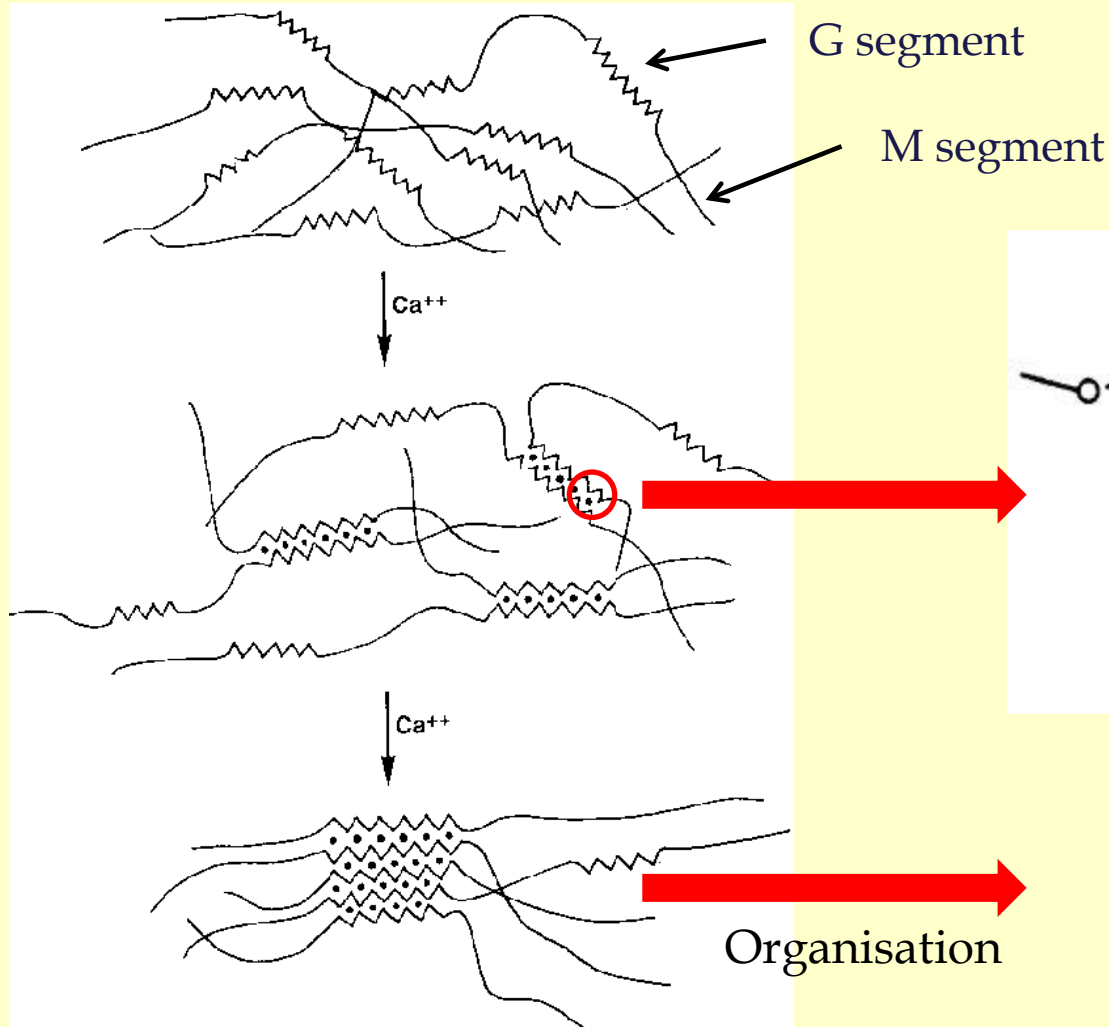
β -D-mannuronate (M)
 (Poly M segment)

α -L-guluronate (G)
 (Poly G segment)

Poly MG segment



Gelling process of alginate



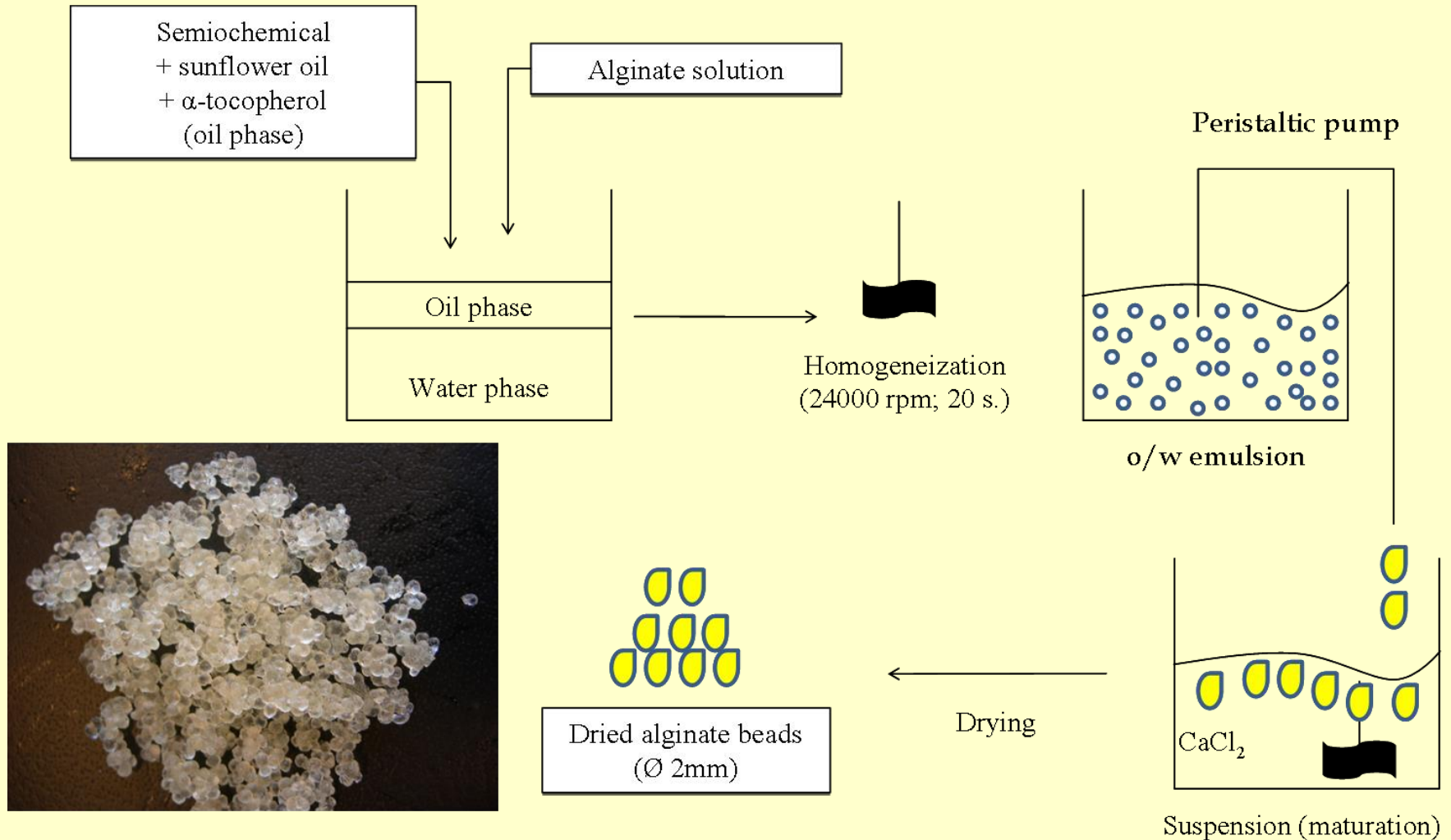
« Egg-box » structure

Formulation of alginate bead

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

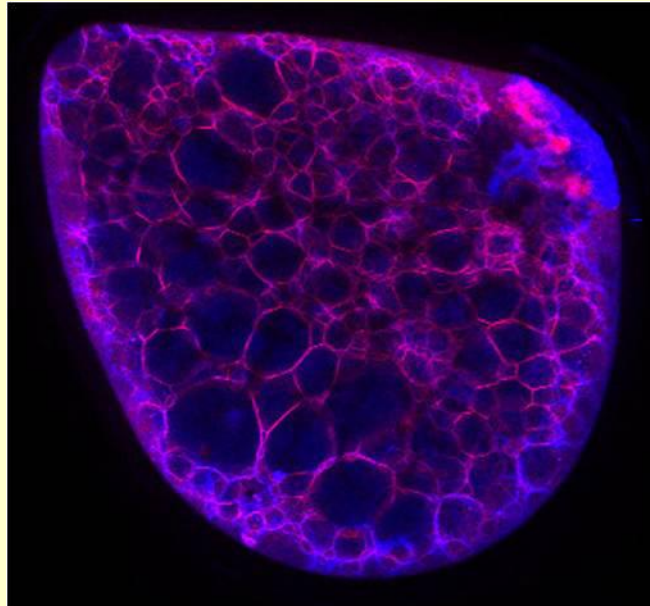
- Type of alginate (M/G – molar mass)
- Alginate concentration
- Type of cross-linker ion
- Cross-linker ion concentration
- Maturation time

Formulation of alginate bead



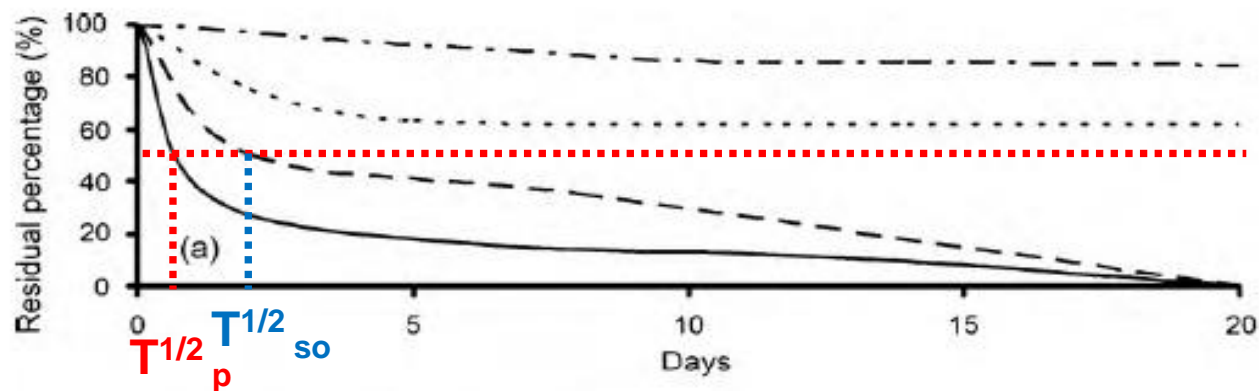
Characterisation of alginate bead

« Semiochemical – oil » dispersion in the alginate network

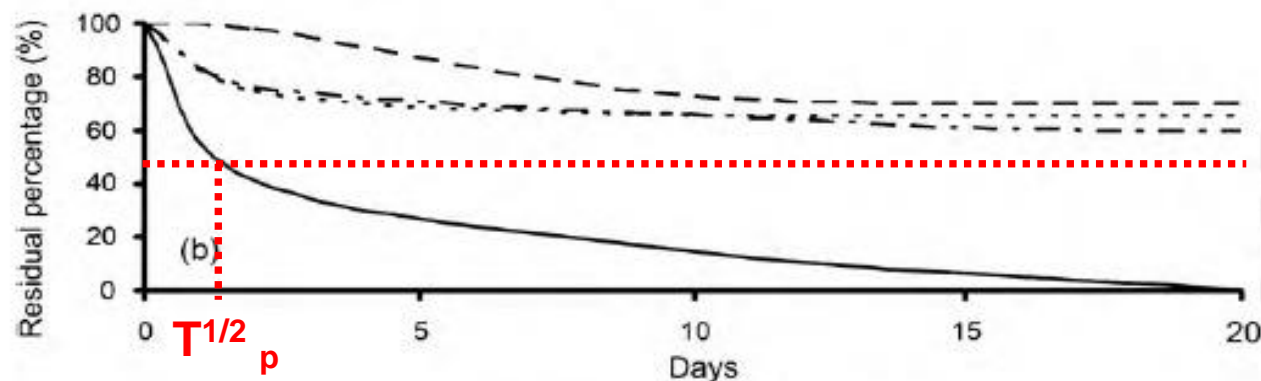


CLSM imaging of a dried ($A_w=0.42$) *E*- β -farnesene alginate bead

Protection efficiency of beads towards sesquiterpenes



E- β -farnesene



E- β -caryophyllene

- Alginate gel beads without alpha-tocopherol
- ... Alginate gel beads with alpha-tocopherol
- - - Sunflower oil formulation
- Purified compounds non formulated

Is the formulation efficient...

... in terms of semiochemical release ?

Volatile collection system

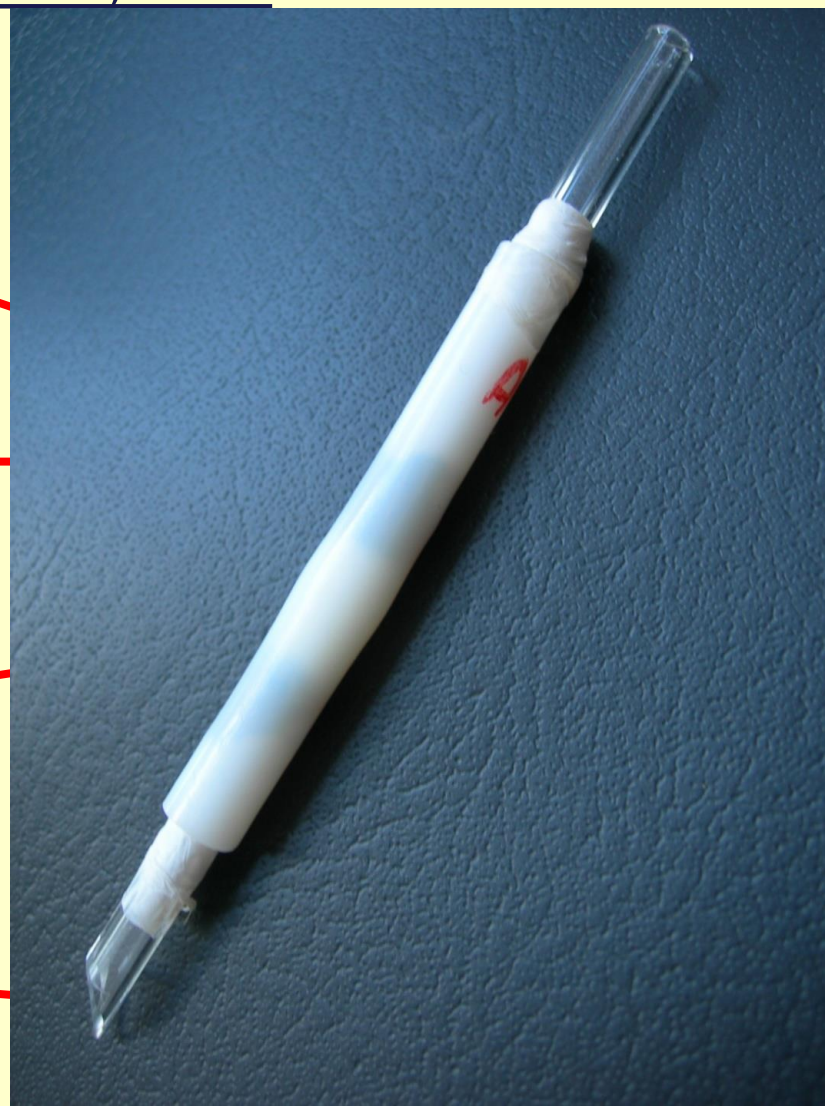
Activated charcoal filter

Adsorbent (HayeSep Q) cartridge

→ Solvent elution + IS quantification (Fast GC)

Teflon box with
semiochemical alginate beads

Pump

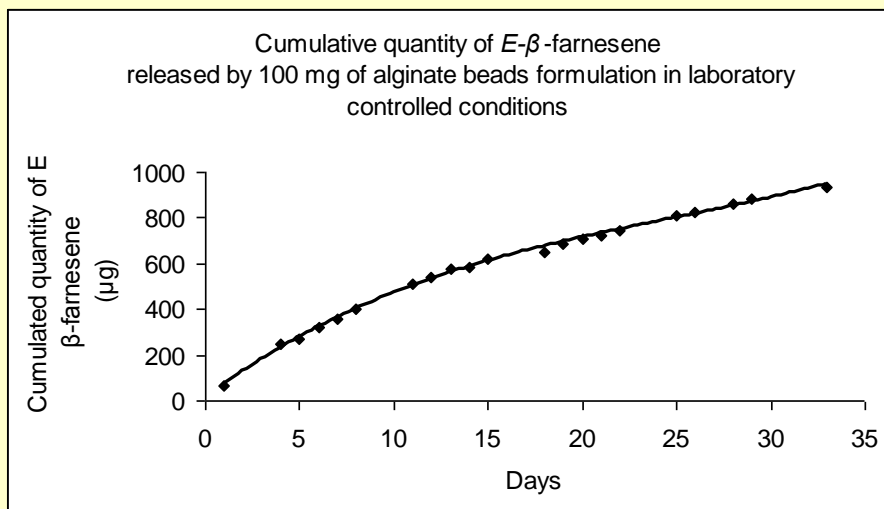


Volatile collection system

Specifications and performances

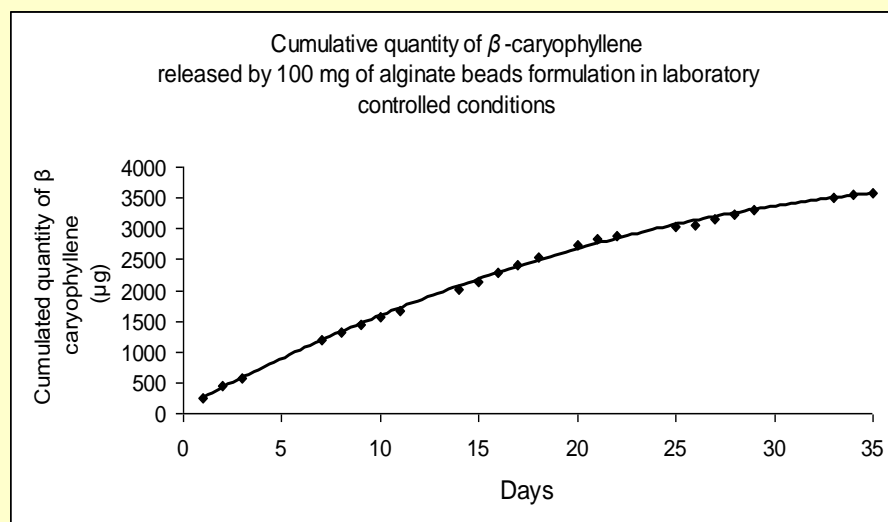
- Boxes and tubing in Teflon >< adsorption of semiochemicals
- Activated charcoal filters: air purification
- Sampling + security cartridges → breakthrough
- Total volume of eluting solvent: 4 × 250 µL n-hexane/cartridge
- Mean recovery of elution: 94.5 % ± 4.2 %

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

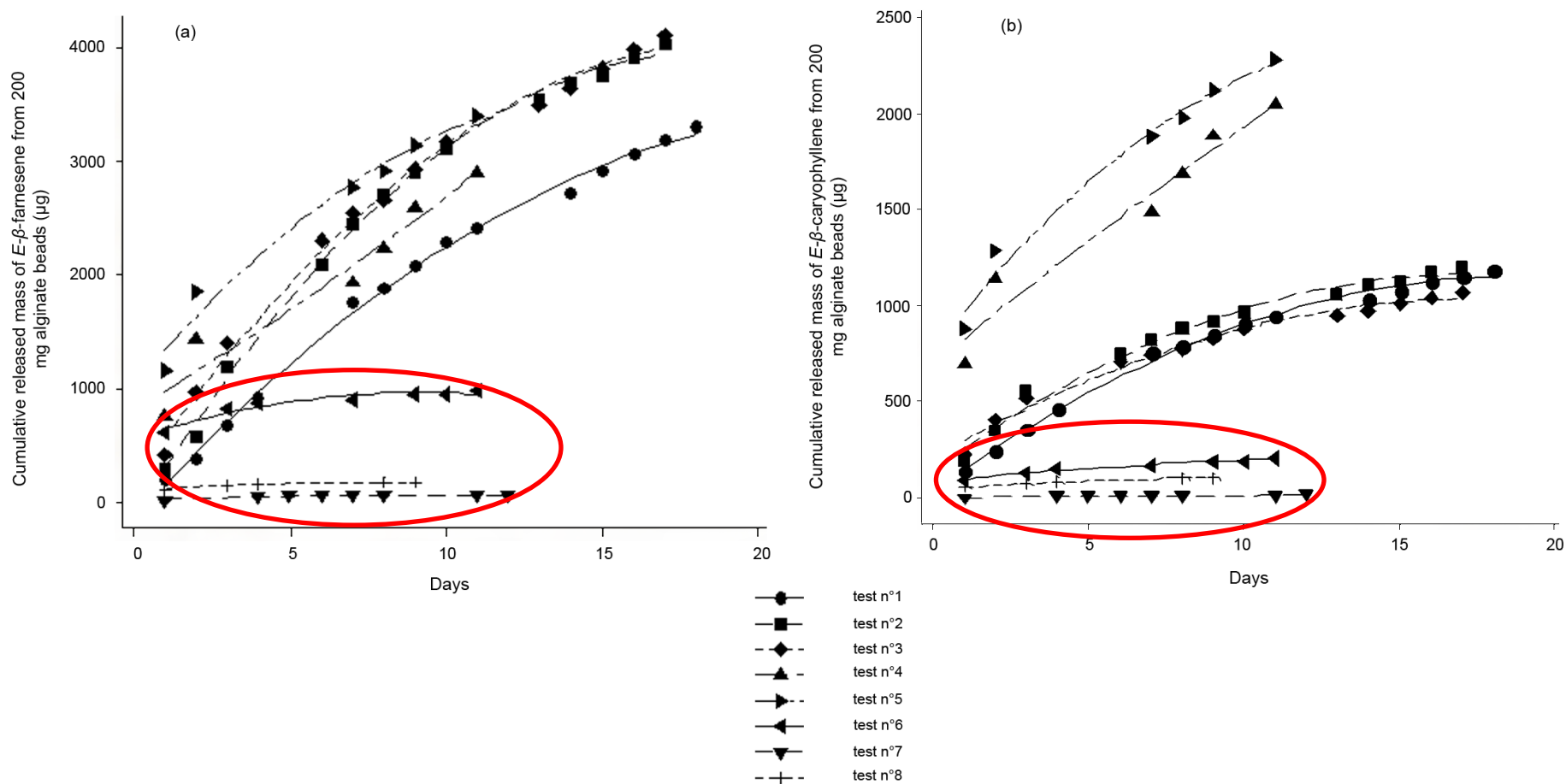
Experimental test	Relative humidity (%)	Airflow (L/min)	Temperature (°C)
N° 1	25	0.05	20
N° 2	25	0.50	20
N° 3	25	1.00	20
N° 4	75	0.50	20
N° 5	75	0.50	40
N° 6	85	0.50	20
N° 7	90	0.50	20
N° 8	100	0.50	20

Semiochemical diffusion coefficient estimation

Diffusion in a sphere (Cranck, 1975):

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

- M_t (μg): cumulative mass of semiochemical released at time t
- M_∞ (μg): cumulative mass of semiochemical released at time ∞ (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 test	Relative humidity (%)	Airflow (L/min)	Temperature (°C)	Diffusion coefficient for <i>E</i> -β-farnesene (m ² /s)	Diffusion coefficient for <i>E</i> -β-caryophyllene (m ² /s)
N° 1	25	0.05	20	1.98 * 10 ⁻¹⁴	1.35 * 10 ⁻¹⁵
N° 2	25	0.50	20	3.40 * 10 ⁻¹⁴	1.57 * 10 ⁻¹⁵
N° 3	25	1.00	20	3.71 * 10 ⁻¹⁴	1.23 * 10 ⁻¹⁵
N° 4	75	0.50	20	1.23 * 10 ⁻¹⁴	7.39 * 10 ⁻¹⁵
N° 5	75	0.50	40	2.12 * 10 ⁻¹⁴	1.03 * 10 ⁻¹⁴
N° 6	85	0.50	20	1.56 * 10 ⁻¹⁵	1.33 * 10 ⁻³²
N° 7	90	0.50	20	6.15 * 10 ⁻³³	8.26 * 10 ⁻³³
N° 8	100	0.50	20	1.03 * 10 ⁻³²	9.93 * 10 ⁻³¹

Influence of abiotic factors on semiochemical diffusion

- Most limiting factor: relative humidity $\geq 85\%$
- Influence of temperature
- Weak influence of air flow

Improvement of the research

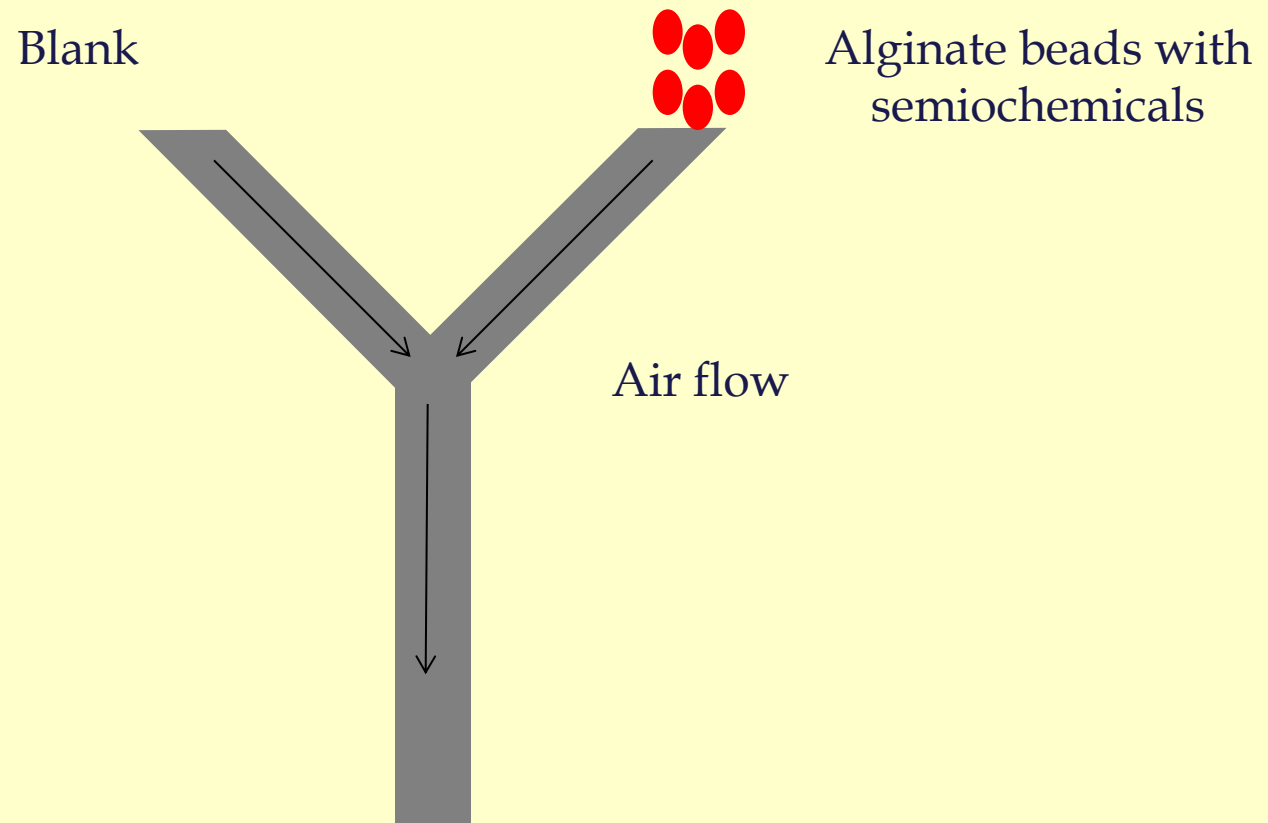
- Box-Behnken experimental design (3 factors in 3 levels)
 - Water sorption / desorption isotherms on alginate beads
 - Evolution of bead diameter with A_w
- ➔ Confirmation of the preliminary results

TFE F. Daems (2011), GxABT, ULG

Is the formulation efficient...

... in terms of biological control devices ?

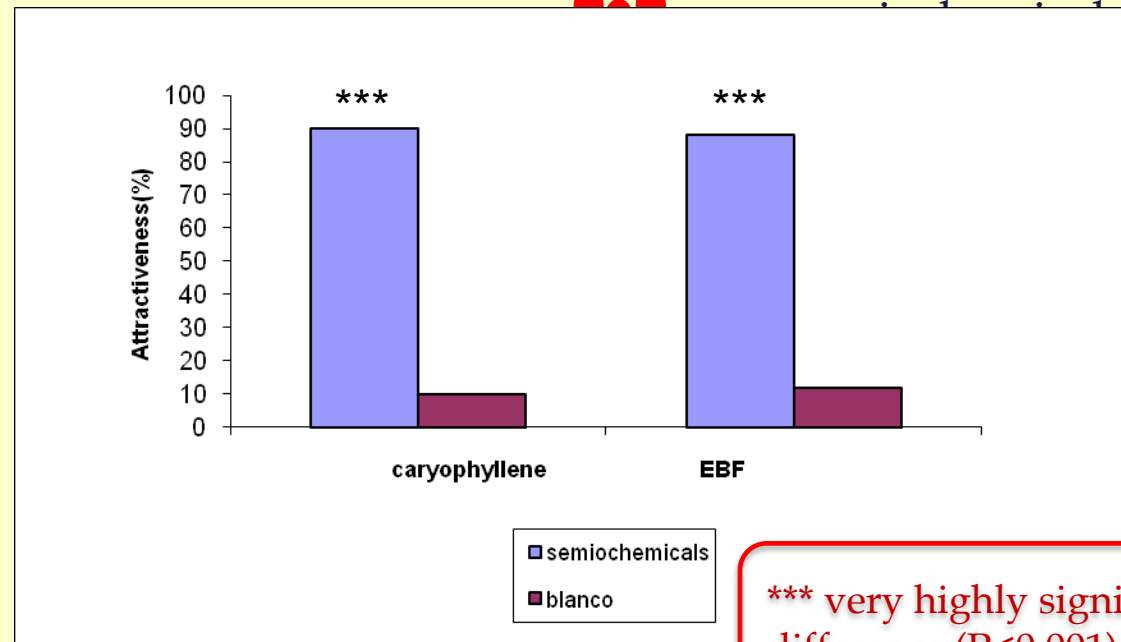
*On parasitoids (*Aphidius ervi*): 2-way olfactometer*



On parasitoids (*Aphidius ervi*): 2-way olfactometer

Blank

Alginate beads with



N = 30

On Syrphidae: on-field experiments

- 3 crops: beet, horse bean, winter wheat
- *E*- β -farnesene, *E*- β -caryophyllene and blank alginate beads
- 1 latin square design per crop

On Syrphidae: on-field experiments



Dunnnett 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