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

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SOLAPHID project (WALEO 2)

Funding from the Belgium Walloon Region

(2006-2011)

"Biotechnologies related to the industrial production of insects used in biological control"

5 teams : chemistry – formulation – entomology – chemical ecology - industrial production

Summary

General introduction

Objective

The choice of semiochemicals and their origin

How to analyse and quantify semiochemicals?

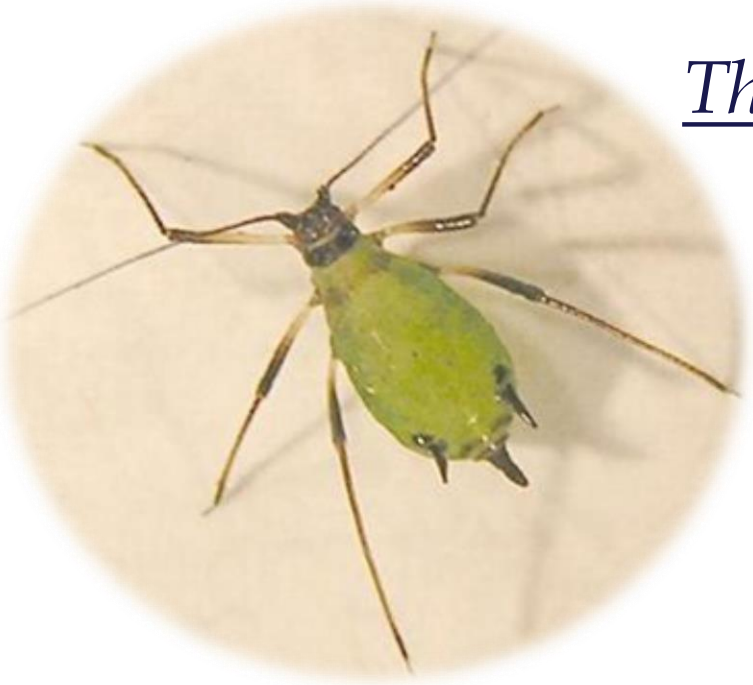
How to purify semiochemicals?

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

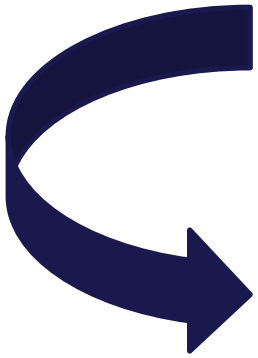
- resistance of pest insects
- non species-specific
- unsafe for environment and human health

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

Host plant

2nd level

Aphids

3rd level

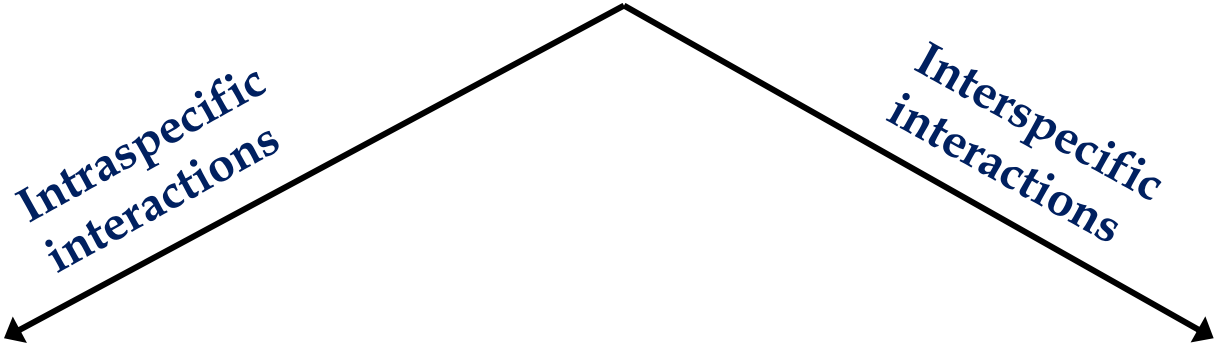
Predators

Parasitoids

➔ *Chemical communication : semiochemicals*

Semiochemicals

Plant – insect – insect chemical communication signals



Pheromones

- alarm
- sex
- aggregation
- trail
- host marking
- …

Allelochemicals

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

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

Objective

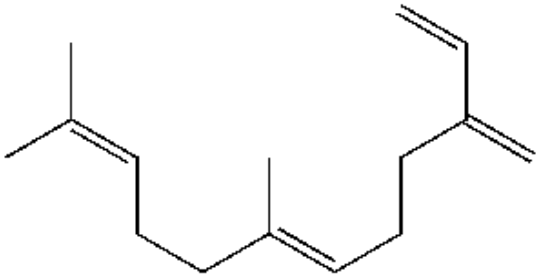
Global objective

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

- Which semiochemicals ?
  - Natural origin ?
    - Purification ?
- Analysis and quantification ?
- Which formulation ?
  - Efficiency ?
    - Release ?
    - Attractiveness ?

The choice of semiochemicals and their natural origin

E-β-farnesene



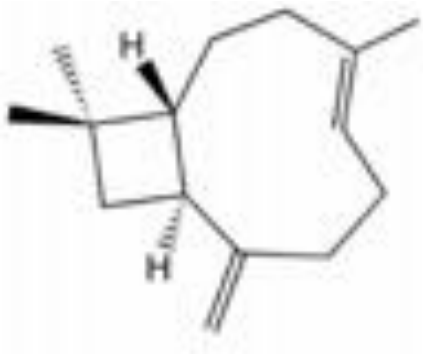
Sesquiterpene ($C_{15}H_{24}$)

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

- Reducer of aphid reproduction[1]
- Attractive towards aphid parasitoids (*A. ervi* Haliday)[2]

[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

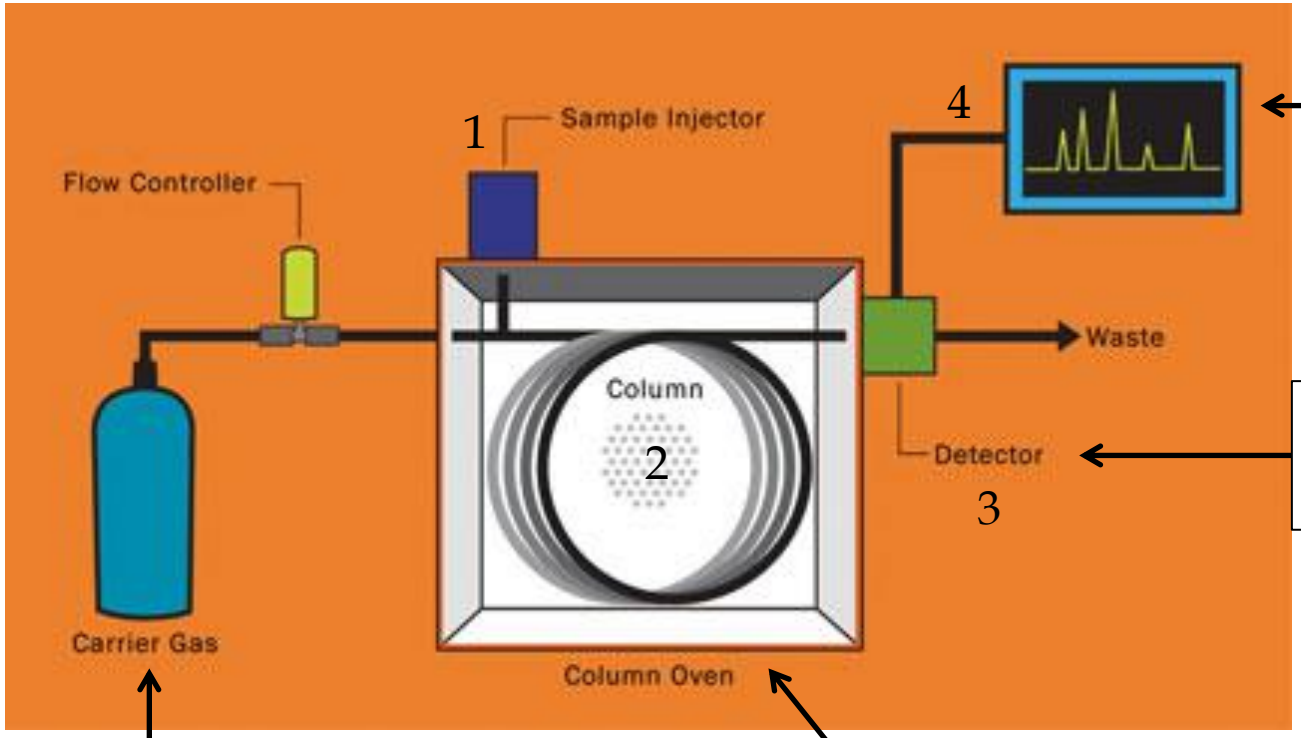
Essential oil characterisation : Gas chromatography

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



Chromatogram

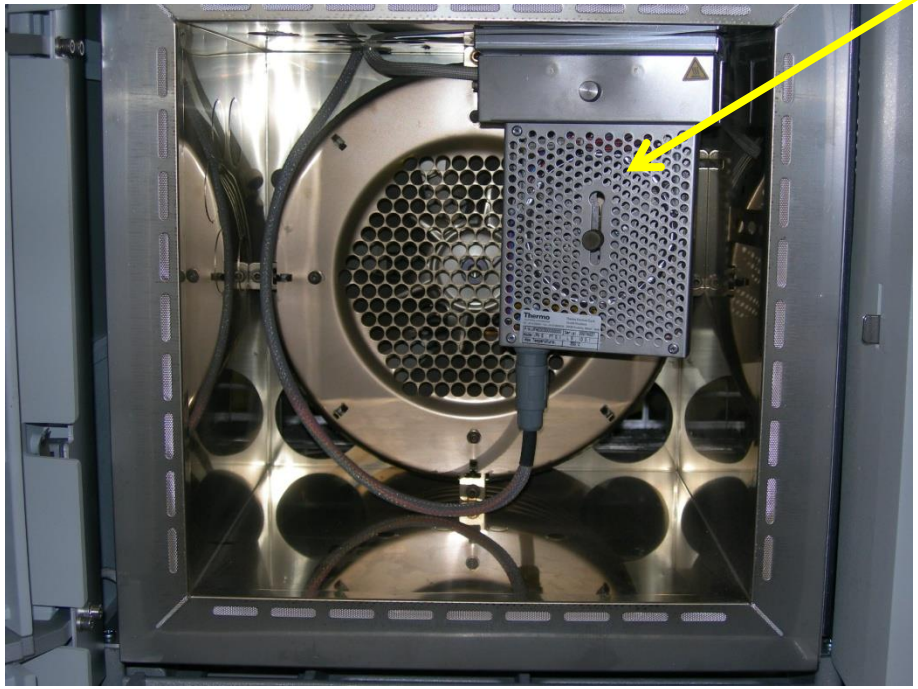
Most common : Mass spectrometer, FID

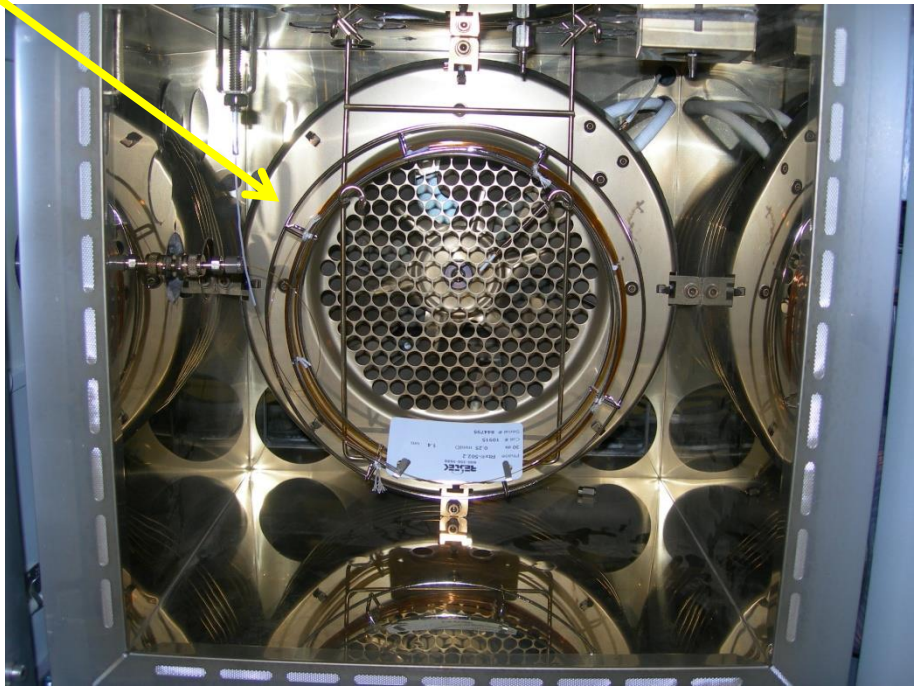
He, H_2 or N_2

Program of T° : optimisation of the separation of the components of the mixture

Gas chromatography : Ultra Fast GC >< Classic GC

column





Ultra Fast GC

Classic GC

Gas chromatography : Ultra Fast GC >< Classic GC

- Ramp of T° : 100 – 1200° C/min
- Column : 2 – 5 m, 0.1 mm ID

➔ **Time for 1 analysis < 5 min**

Ultra Fast GC

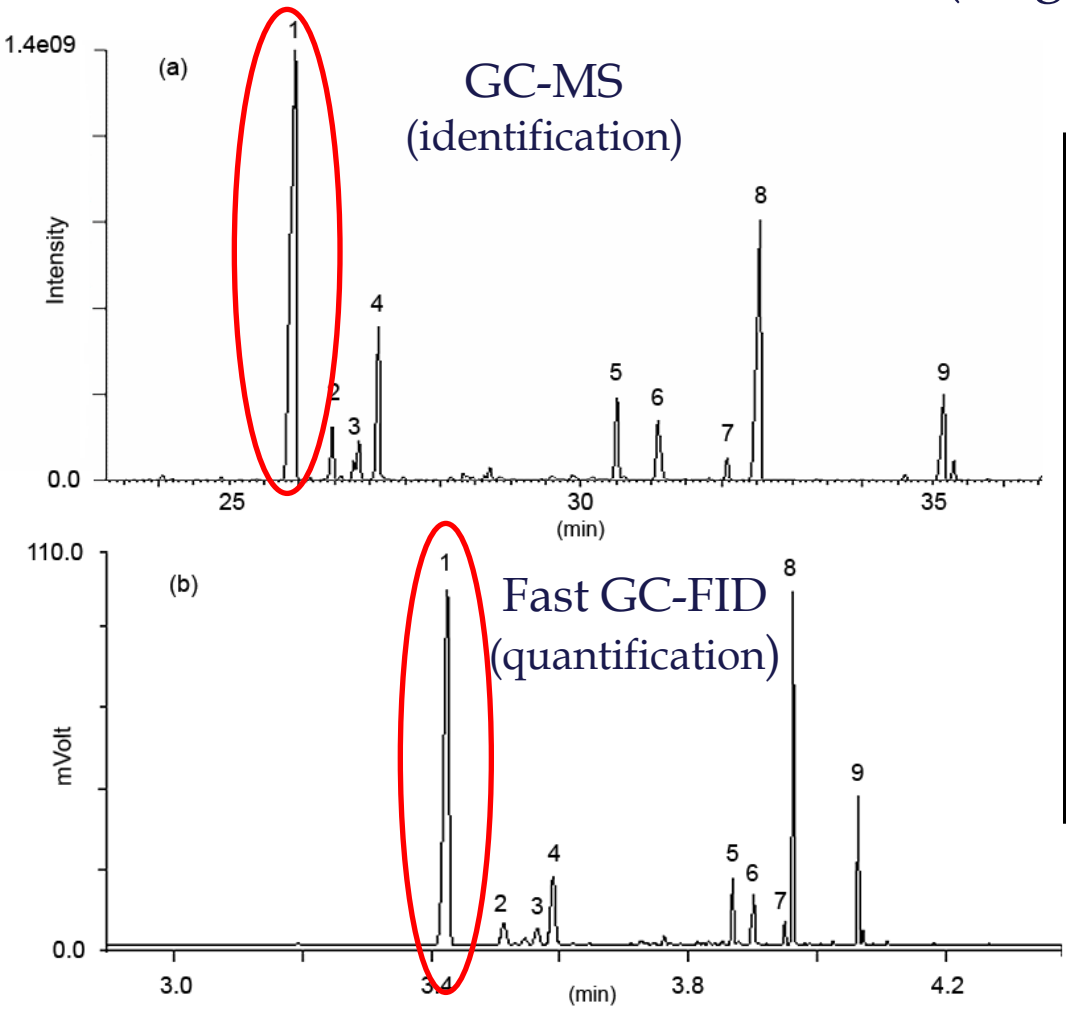
- Ramp of T° : 10-30° C/min
- Column : 10 – 30 m, 0.25-0.32 mm ID

➔ **Time for 1 analysis > 35 min**

Classic GC

Essential oil characterisation

Matricaria chamomilla L. (originated from Nepal)

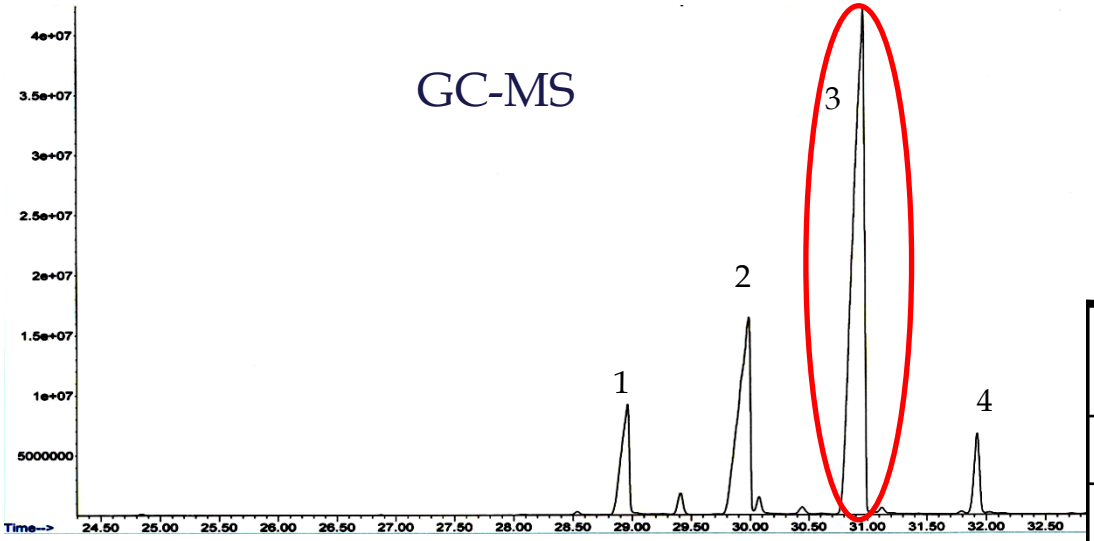


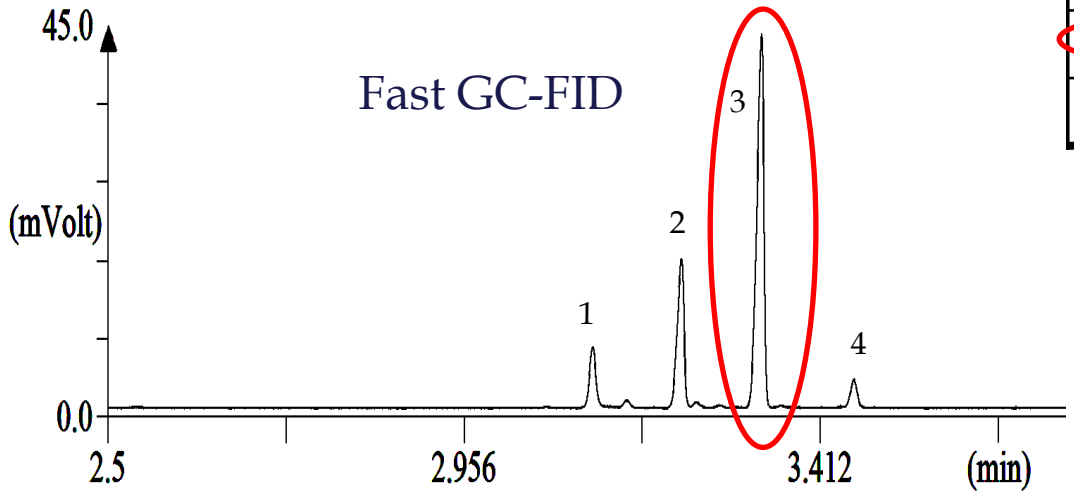
N°	Major compounds	Retention index	%
1	E-β-farnesene	1456	42.6
2	Germacrene D	1478	2.9
3	bicyclogermacrene	1494	1.9
4	(E,E)-α-farnesene	1506	8.3
5	α-bisabolol oxide B	1649	4.4
6	α-bisabolone oxide A	1673	4.5
7	Chamazulene	1715	1.1
8	α-bisabolol oxide A	1735	21.1
9	Cis-ene-yne-dicycloether	1802	5.9

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

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

Quantification of semiochemicals: various steps

1. Quantification with internal standard
2. Optimisation of analytical method: resolution of compounds
3. Validation of analytical method:

    - calibration curve

    - evaluation of validation criteria according to norms

Quantification with internal standard

Why an internal standard?

- to avoid the problem of variation of injected volume in GC with autosampler

How to add an internal standard?

- in reference solutions to construct calibration curve : the same concentration of IS in all the levels of concentration of analytes

- in routine samples at a known concentration

Quantification with internal standard

Which internal standard?

- Compound of the same family than the analytes
- Retention time of IS close to the rentention time of analytes
- Response factor close to 1:

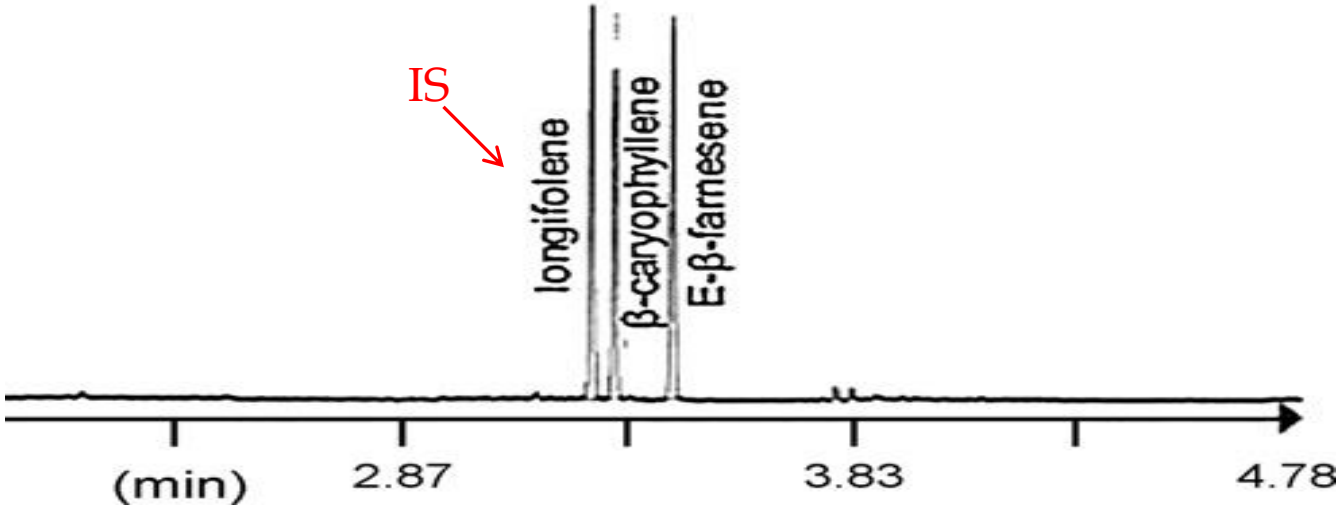
$$F = (Area_A.Conc_{IS} / Area_{IS}.Conc_A)$$

- Not naturally present in the routine sample

➔ Here : IS = longifolene

Optimisation of the analytical method

Ultra Fast GC analysis



➔ Good resolution of peaks in less than 5 min.

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

$R_s = 1{,}65 > 1{,}5$ ➔ 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 garantees that the results are within acceptance limits

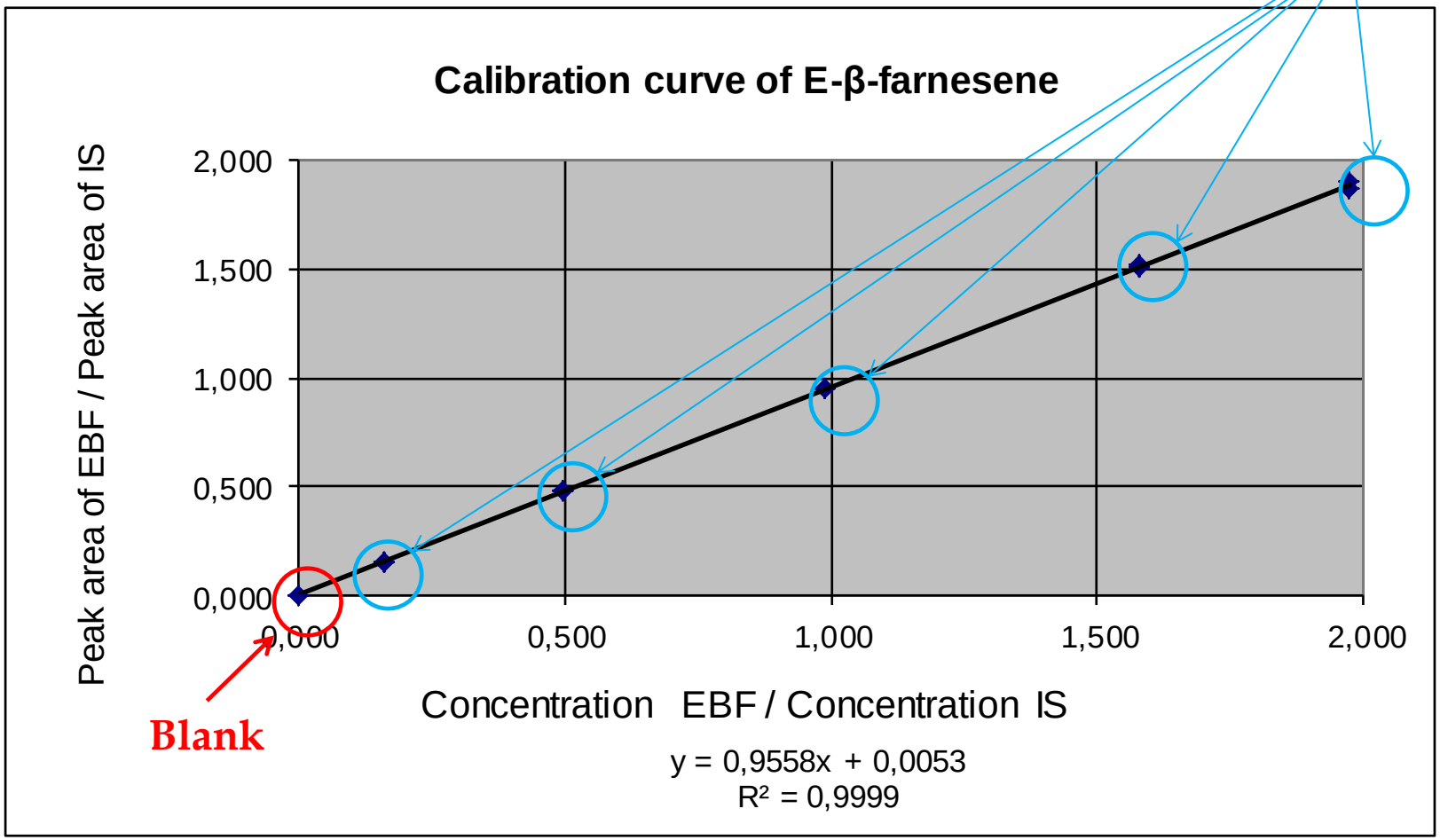
$$|x_i - \mu_T| < \lambda$$

Bias

λ = acceptance limits

Calibration curve

5 concentrations * 3 replicates



Analytical validations

1. « Classic » 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

**SFSTP= Société Française des Sciences et Techniques Pharmaceutiques*

1. « Classic » validation

	E-β-Farnesene		β-Caryophyllene		
Range (µg/µl)	0.008–0.100	0.080–1.000	0.008–0.100	0.080–1.000	
Equation of the calibration curve	$y = 0.9592x - 0.0028$	$y = 0.9558x + 0.0053$	$y = 0.8381x + 0.0030$	$y = 0.8408x + 0.0056$	
Linearity					
r^2	0.9998	0.9999	0.9998	0.9999	> 0.996
Reduced residual (Grubb's test)	2.668	1.866	2.147	1.880	< 2.75
Accuracy of calibration curves (%)[a]	99.19	99.86	99.77	99.90	90 <x< 110
Internal standard	Longifolene	Longifolene	Longifolene	Longifolene	
LOD (pg)	2.38	2.40	1.79	0.74	
LOQ (pg)	4.76	4.80	3.58	1.48	

[a] Bias (%) between the measured value and the theoretical value.

1. « Classic » validation

Precision of the method

	E-β-Farnesene		β-Caryophyllene	
Concentration (µg/µl)	0.050	0.500	0.050	0.500
Repeatability (RSD, %)	1.16	0.70	0.43	0.12
Reproducibility (RSD, %)	3.00	2.82	0.89	0.81

RSD % < 8%

RSD % < 6%

RSD % < 16%

RSD % < 12%

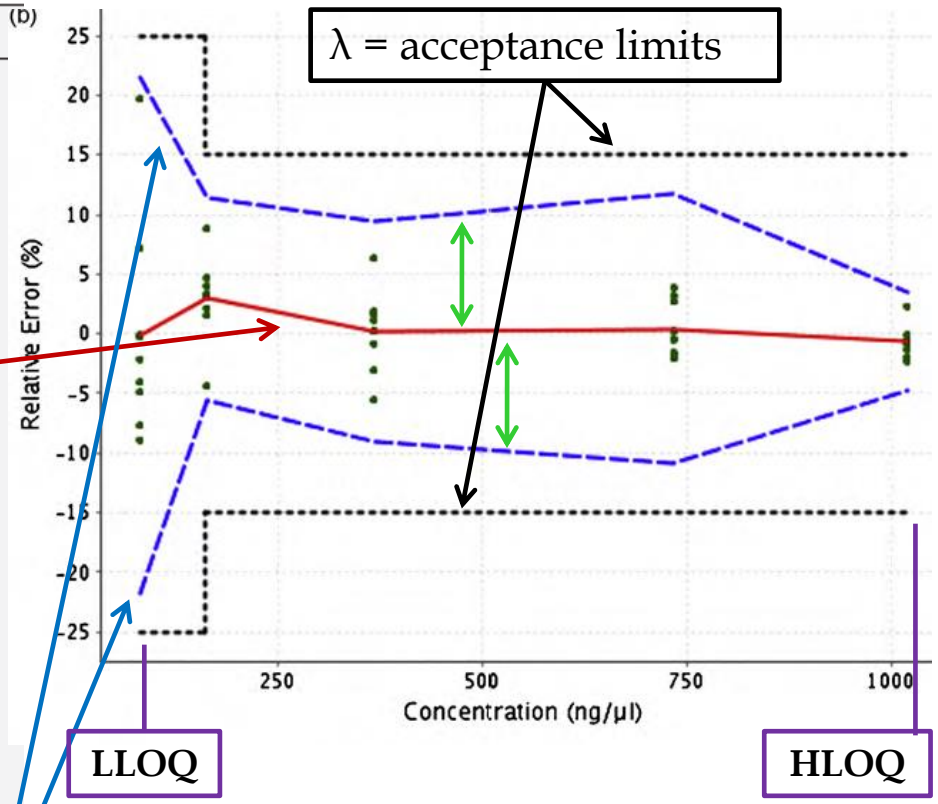
2. Accuracy profile validation

Trueness - Bias – Systematic error

Precision – Repeatability + Intermediate precision – Random error

Accuracy - Trueness + Precision – Total error

	E-β-Farnesene		
Range (ng µl^{-1})	81.6–1019.7		
Response function ($m = 3$, $n = 3$)			
	Series 1	Series 2	Series 3
Slope	0.0089	0.0091	0.0089
Trueness ($n = 3$, $p = 3$)			
Concentration levels	Absolute bias (ng µl^{-1})	Relative bias (%)	
1	−0.1	−0.1	
2	4.8	2.9	
3	0.8	0.2	
4	2.9	0.4	
5	−6.8	−0.7	
Precision ($n = 3$, $p = 3$)			
Concentration levels	Repeatability (RSD, %)	Intermediate precision (RSD, %)	
1	8.8	8.8	
2	3.4	3.4	
3	2.9	3.4	
4	0.8	2.6	
5	1.0	1.4	
Accuracy ($n = 3$, $p = 3$, $\beta = 0.95$)			
Concentration levels	β-Expectation tolerance limits (ng µl^{-1})	β-Expectation tolerance limits (%)	
1	[63.9–99.1]	[−21.7 to 21.4]	
2	[154.2–181.7]	[−5.5 to 11.4]	
3	[333.9–401.9]	[−9.0 to 9.5]	
4	[654.3–820.0]	[−10.9 to 11.7]	
5	[970.5–1055.0]	[−4.8 to 3.5]	
Linearity ($n = 3$, $m = 5$, $p = 3$), $N = 45$			
Range (ng µl^{-1})	81.6–1019.7		
Slope	0.9928		
Intercept	3.7050		
r^2	0.9989		
Lower LOQ (ng µl^{-1})	81.6		
Lower LOD (ng µl^{-1})	40.8		



Accuracy profile

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r^2	0.9989		
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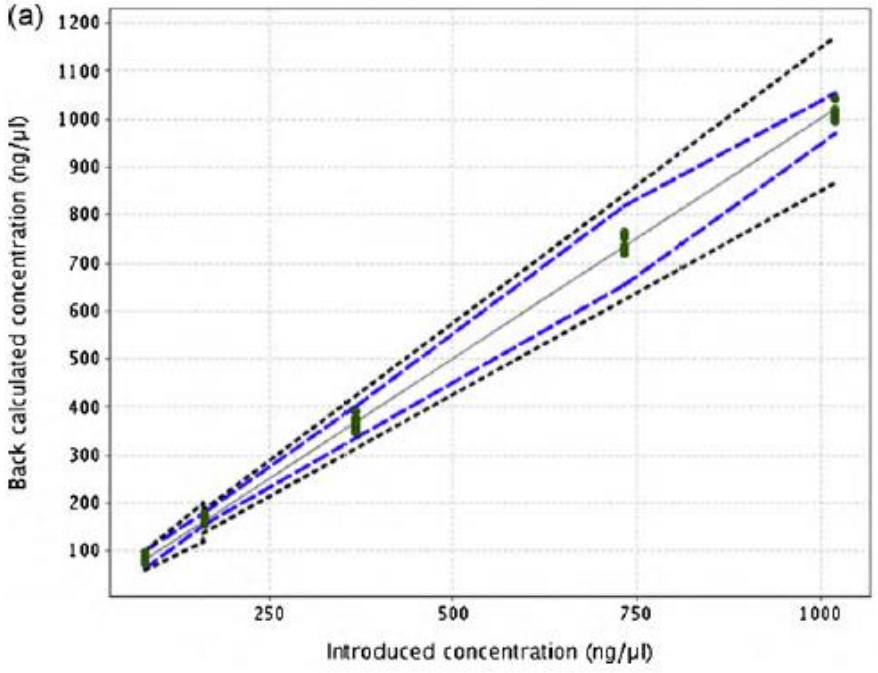
Linearity of the method



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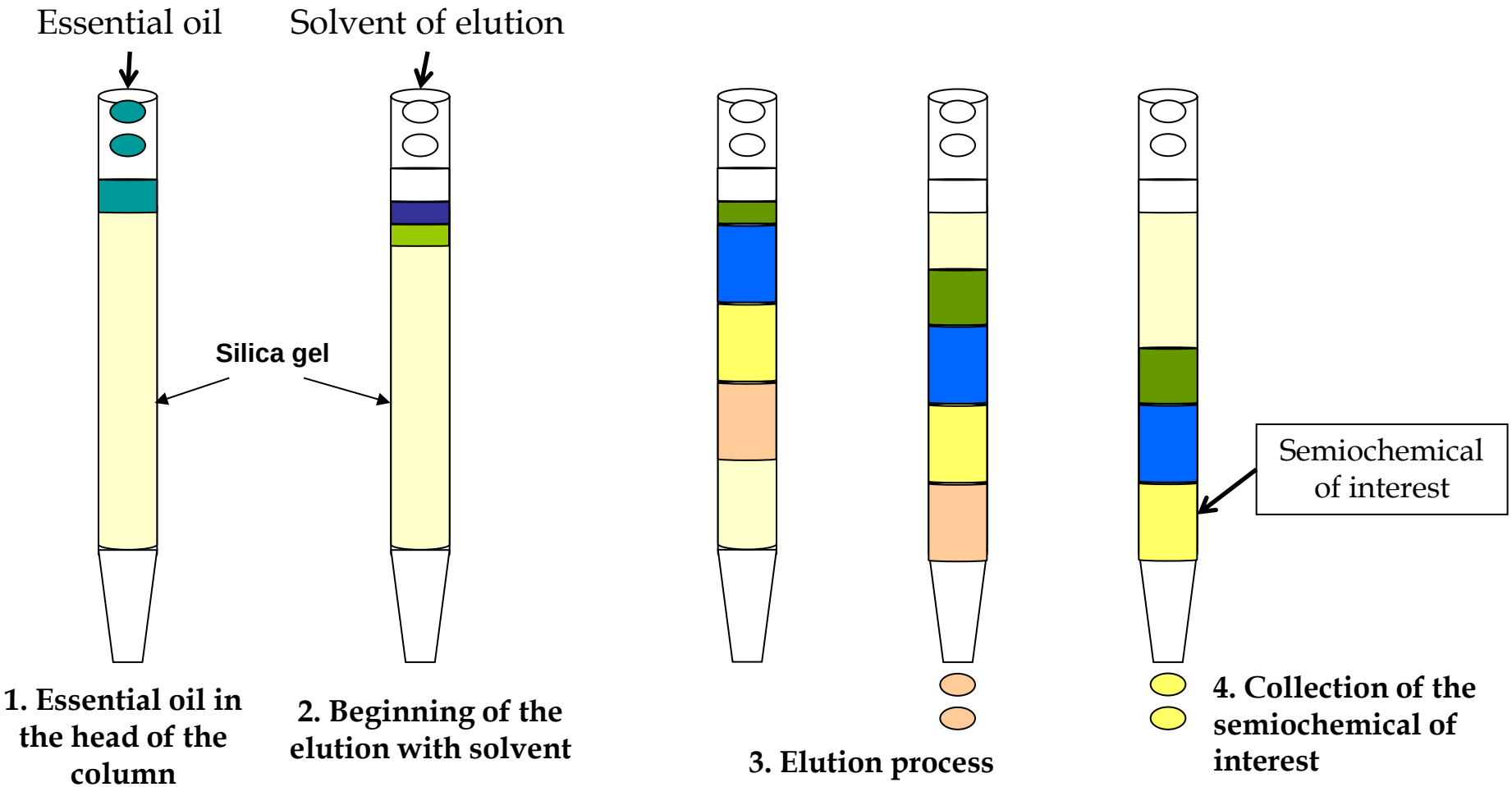
How to purify semiochemicals from essential oils ?

Purification of components : chromatographic techniques

Solid-Liquid chromatography



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

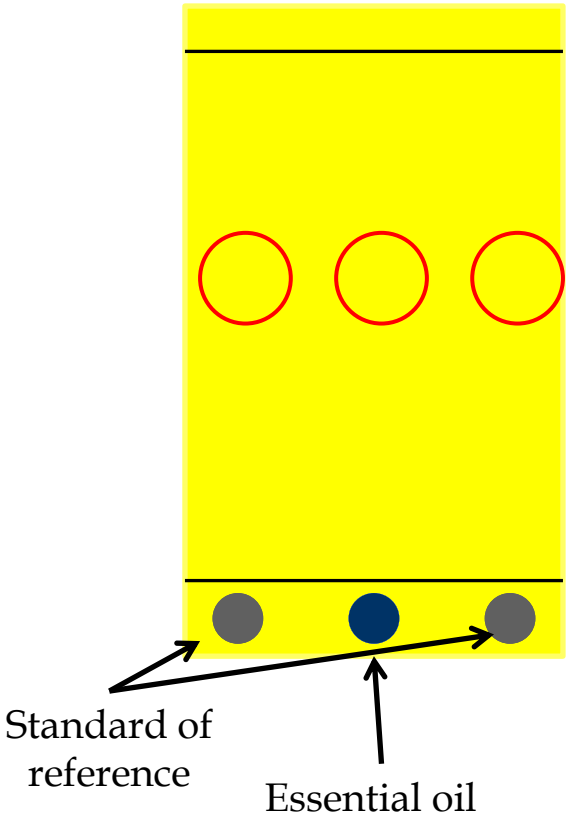
By thin layer chromatography

➔ *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

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Elution with n-pentane

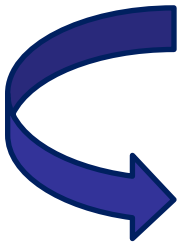
↓

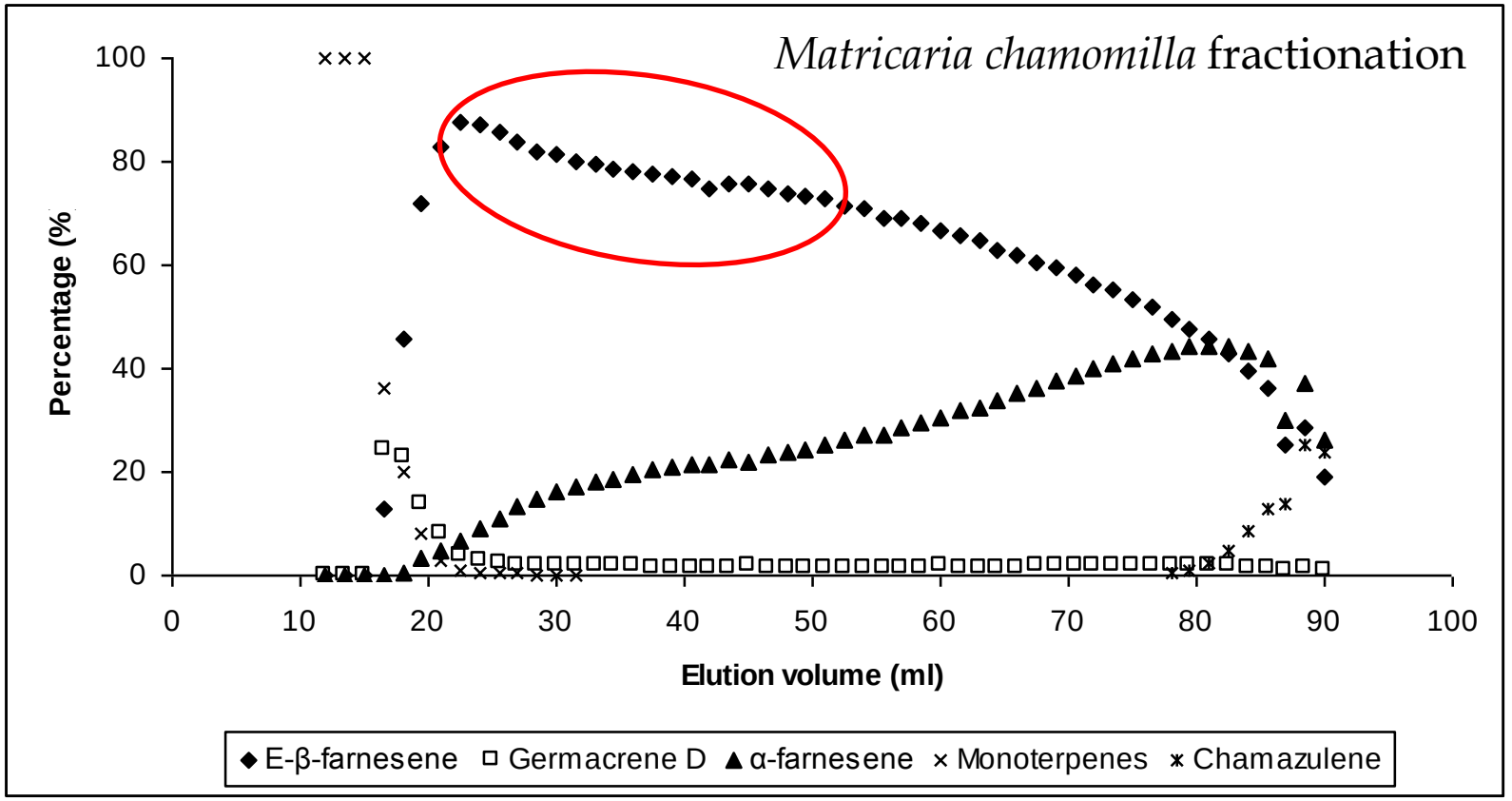
Collection of fractions (1.5 ml)

↓

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

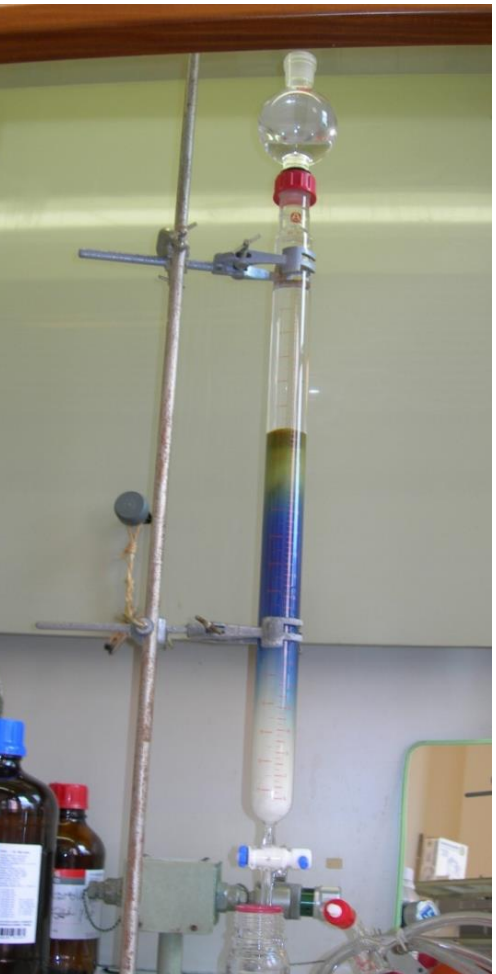
Essential oil fractionation

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)

↓

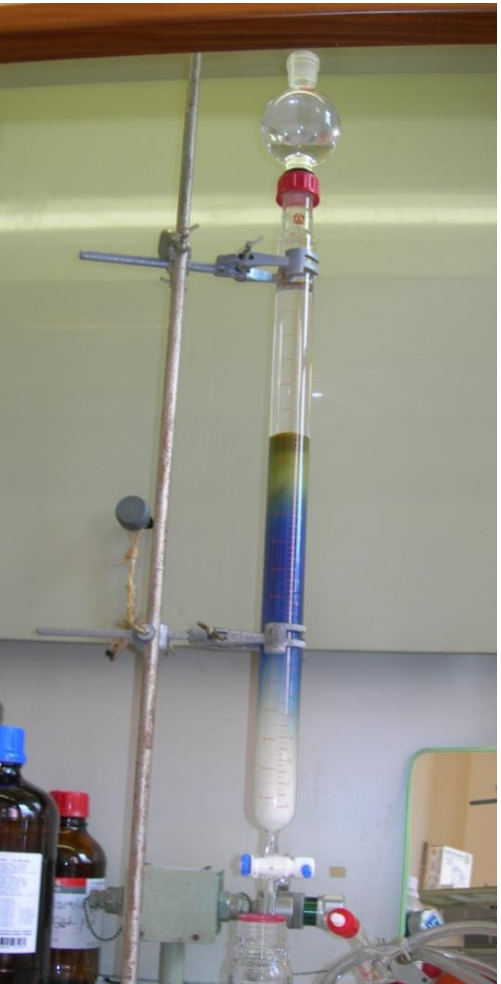
Collection of concentrated fraction + solvent evaporation

↓

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

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

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 >< oxidation
- Sufficient release rate of semiochemicals over time
- Attractive towards aphid predators and/or parasitoids

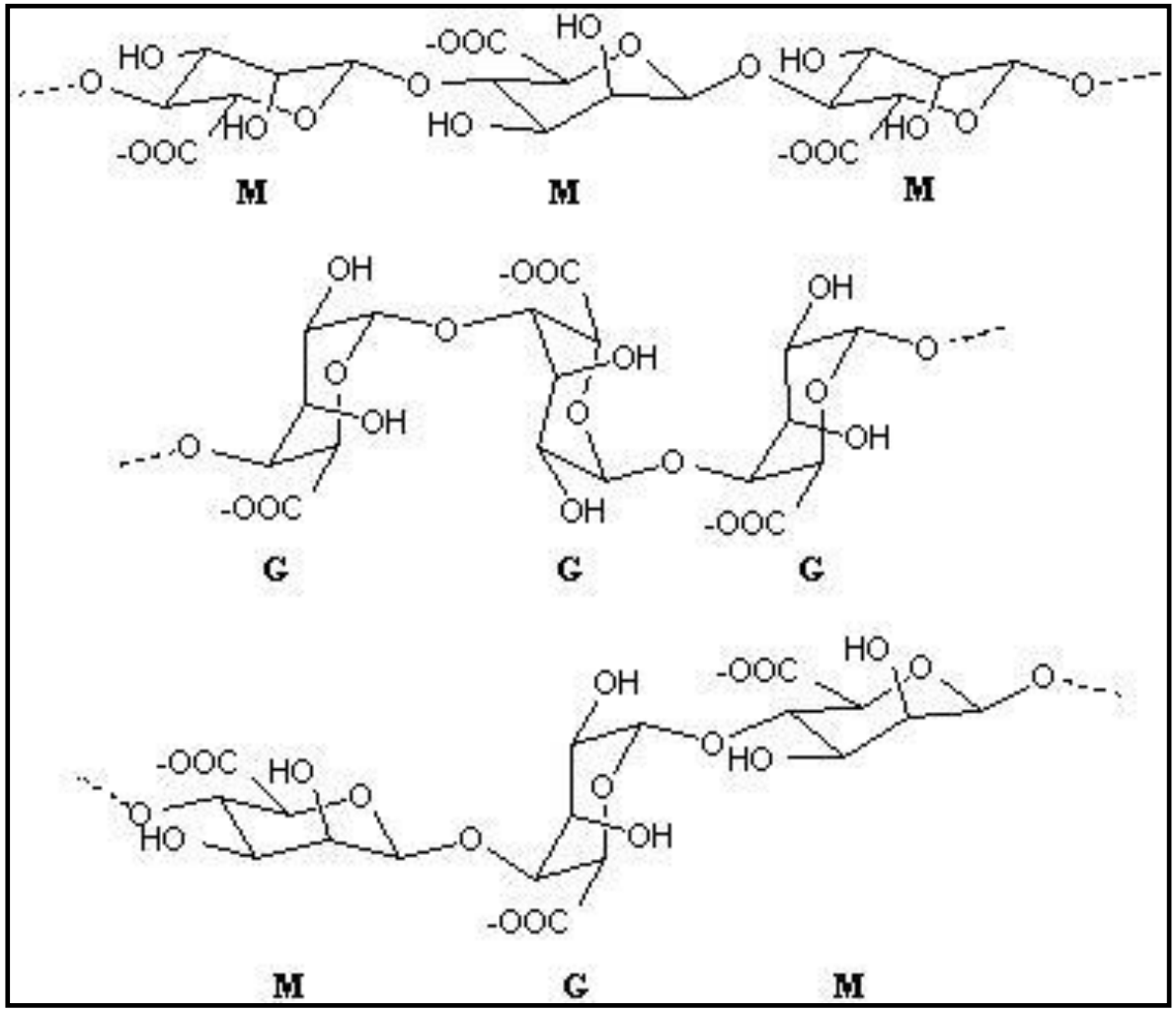
Alginate gel beads

Alginate

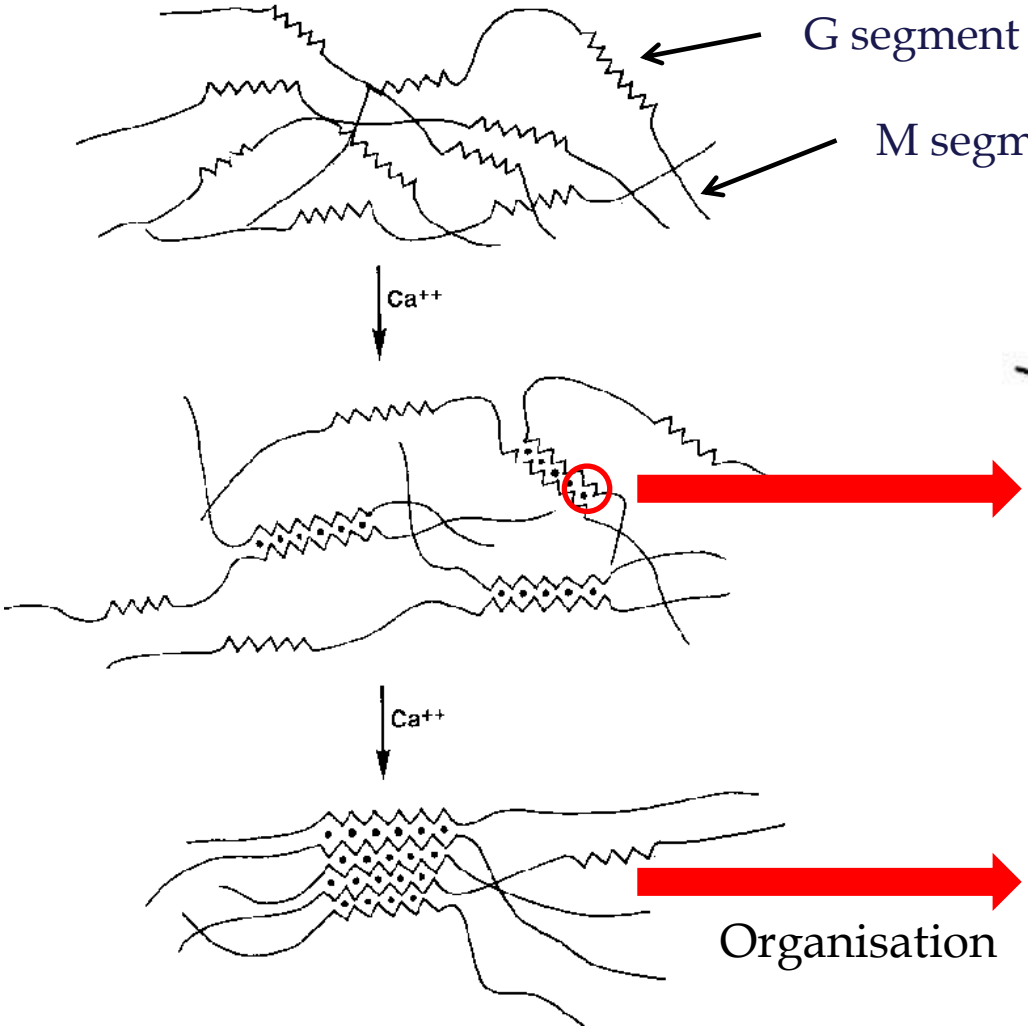
β-D-mannuronate (M)
(Poly M segment)

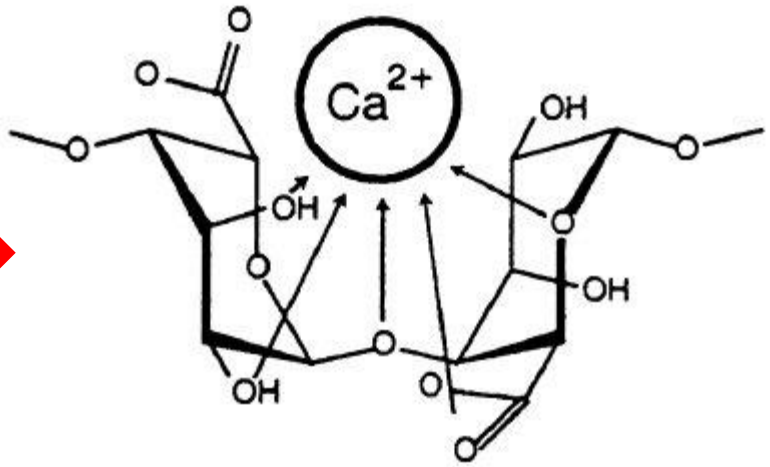
a-L-guluronate (G)
(Poly G segment)

Poly MG segment



Gelling process of alginate





« Egg-box » structure

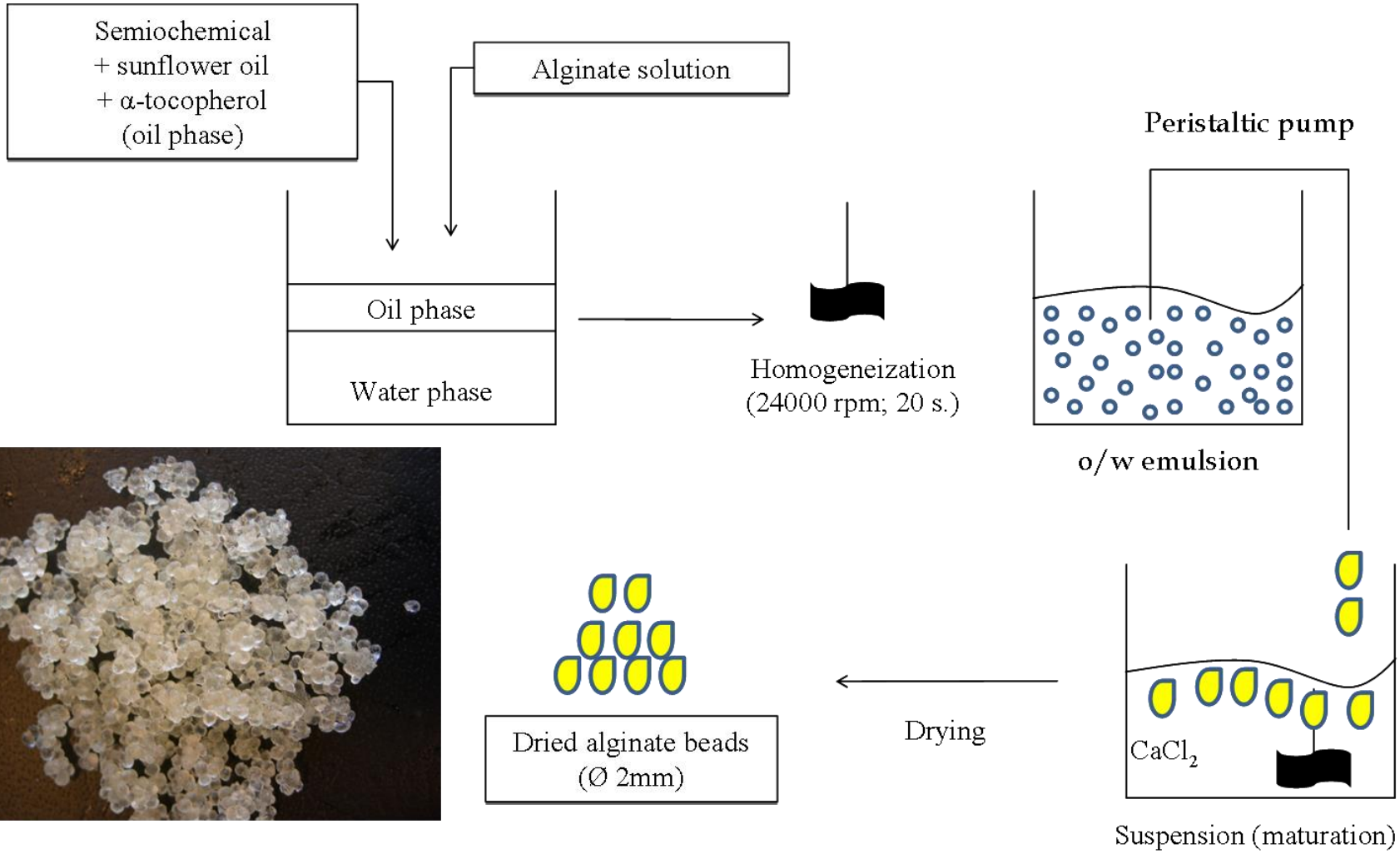
Formulation of alginate bead

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

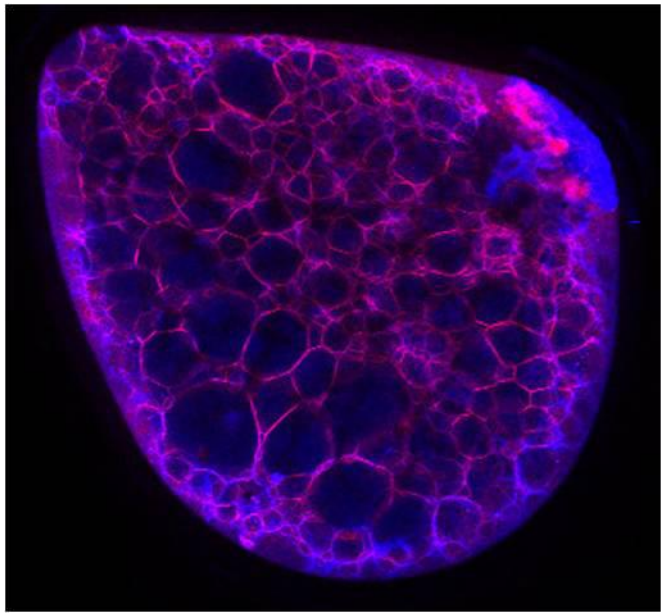
For details : see Heuskin et al., 2012, Pest Management Science, 68, 127-136

Formulation of alginate bead



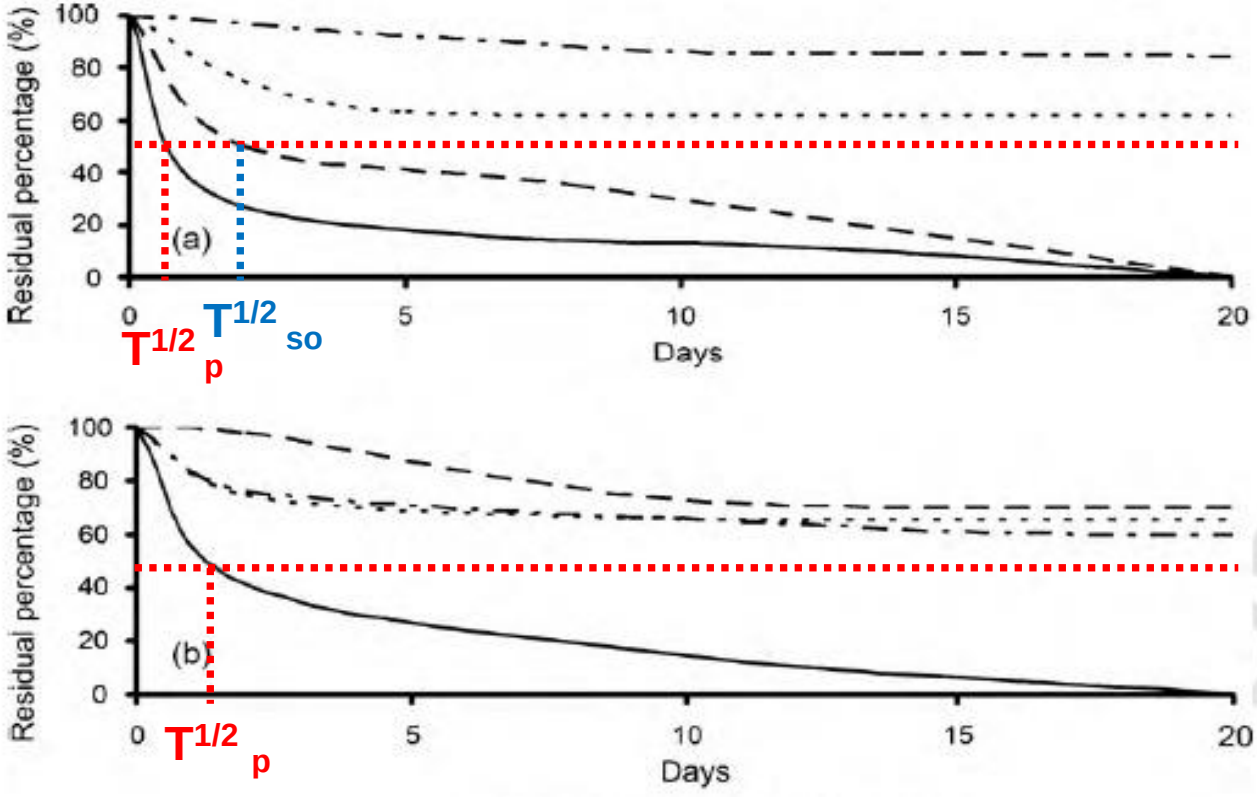
Characterisation of alginate bead

« 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

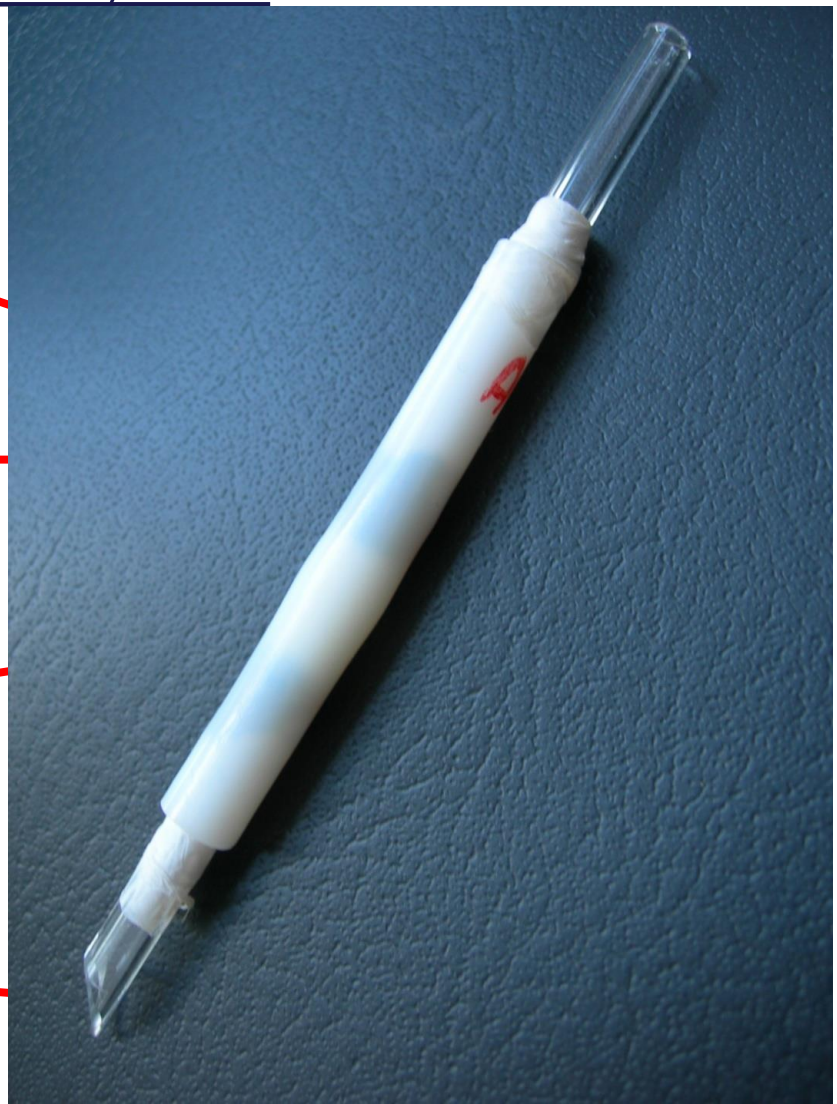
Activated charcoal filter

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

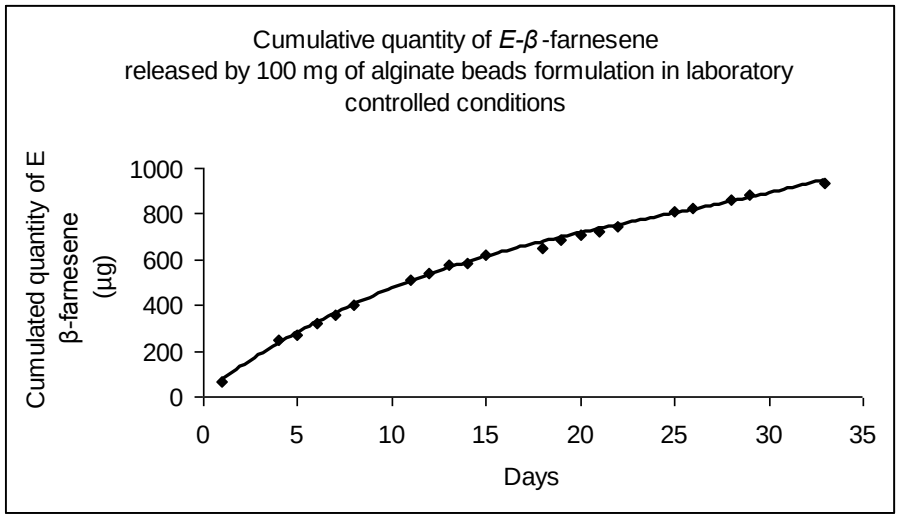
- Activated charcoal filters: air purification

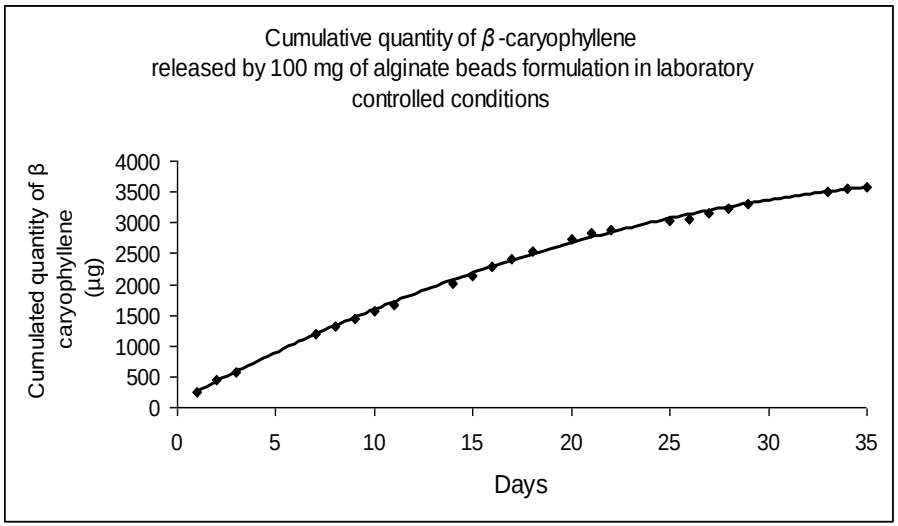
- Sampling + security cartridges ➔ breakthrough

- 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

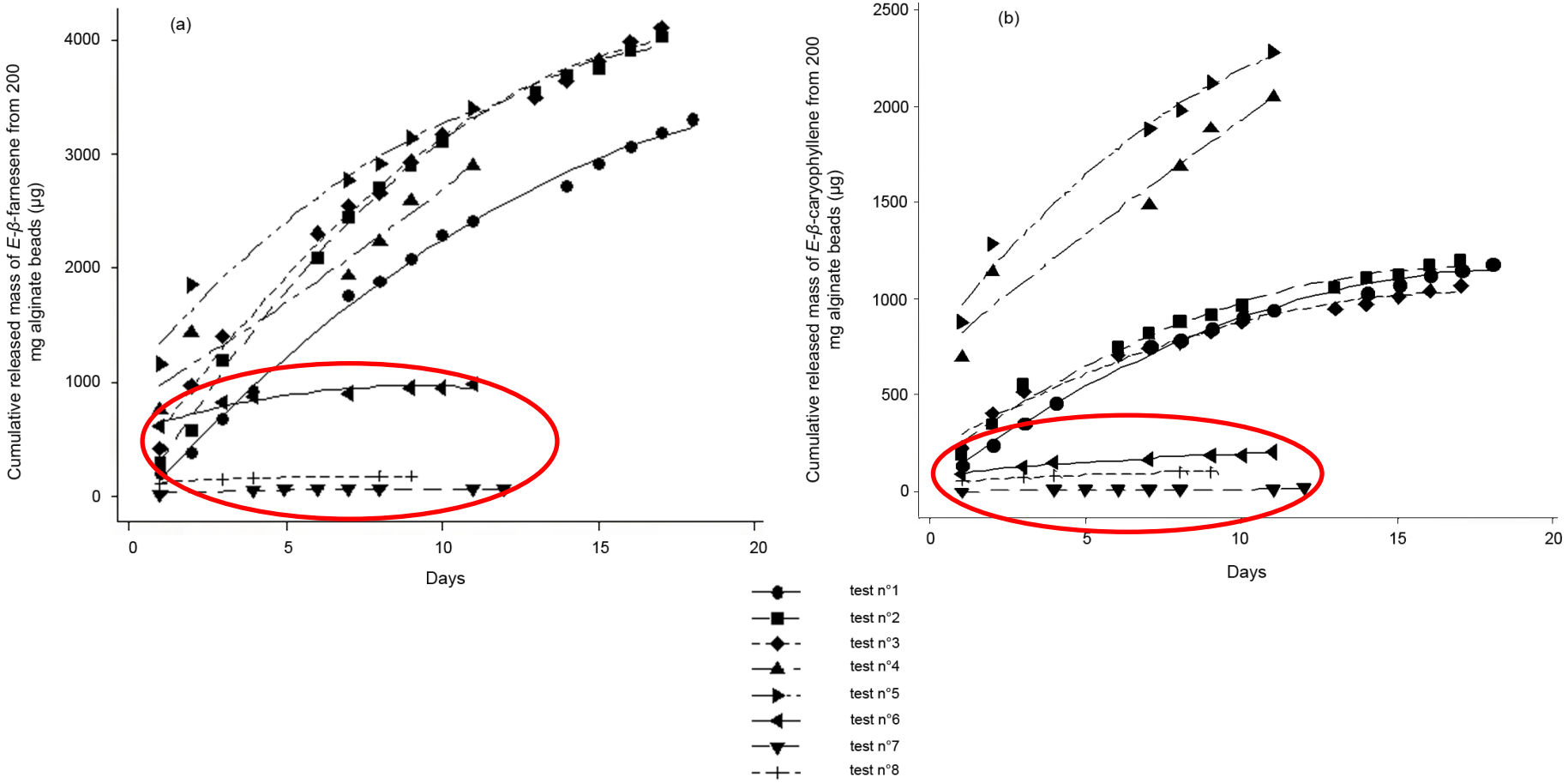
Experimental test	Relative humidity (%)	Airflow (L/min)	Temperature (°C)
N° 1	25	0.05	20
N° 2	25	0.50	20
N° 3	25	1.00	20
N° 4	75	0.50	20
N° 5	75	0.50	40
N° 6	85	0.50	20
N° 7	90	0.50	20
N° 8	100	0.50	20

Semiochemical diffusion coefficient estimation

Diffusion in a sphere (Cranck, 1975):

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

- 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 *E*-*β*-farnesene (m^2/s)	Diffusion coefficient for *E*- *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 ≥ 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

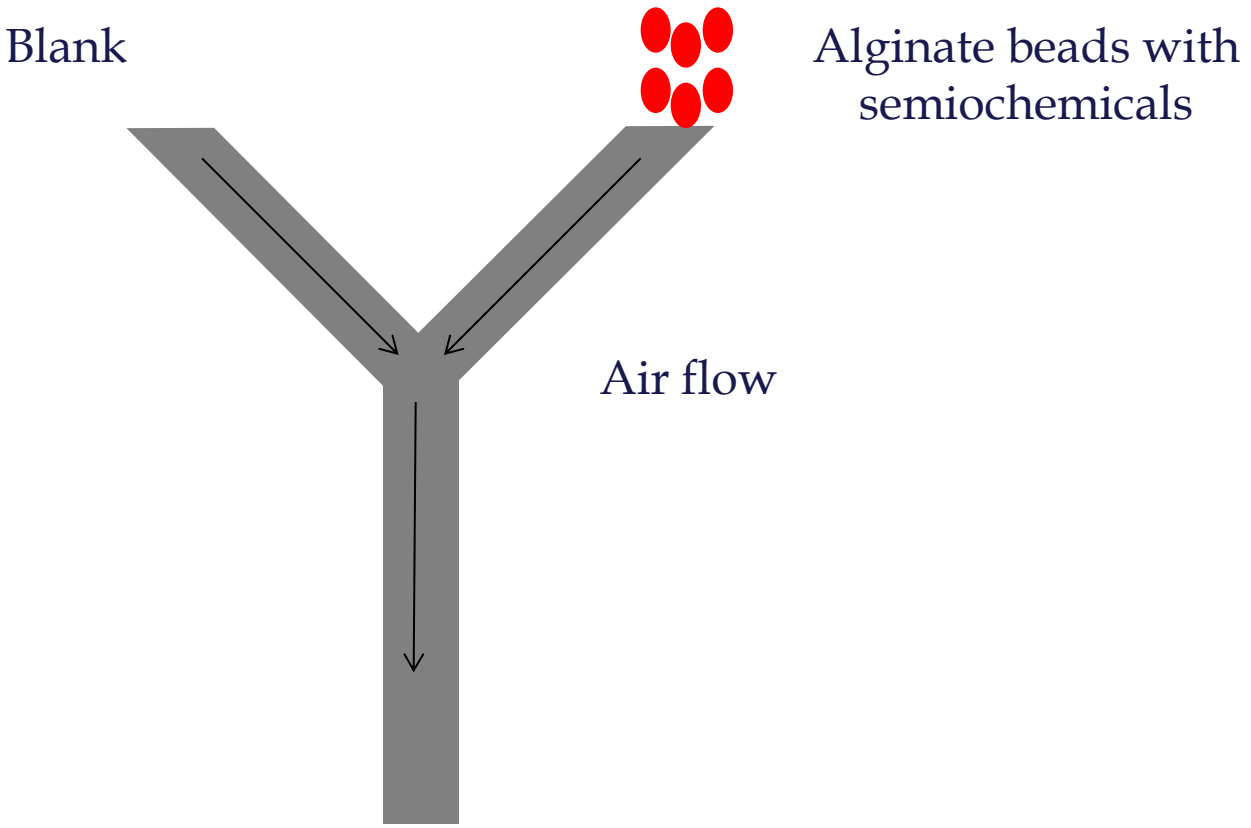
➔ Confirmation of the preliminary results

TFE *F. Daems* (2011), GxABT, ULG

Is the formulation efficient…

… in terms of attractiveness of beneficial insects?

On parasitoids (Aphidius ervi): 2-way olfactometer

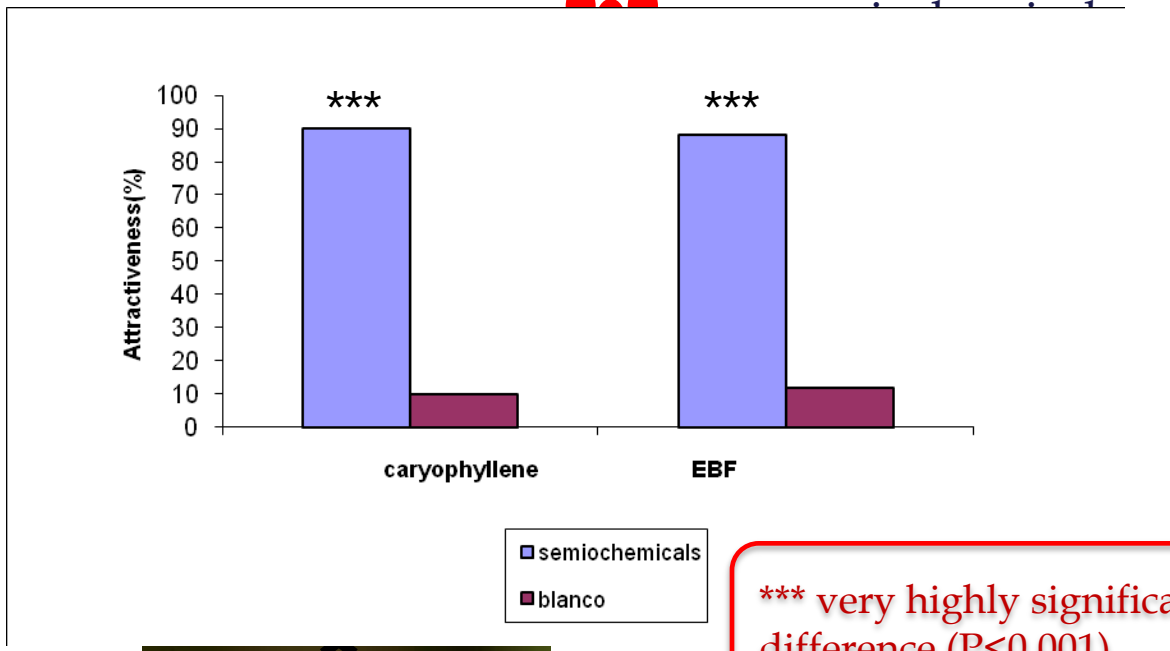


Heuskin S.et al., 2012, Pest Manag. Sci., 68, 127-136

On parasitoids (Aphidius ervi): 2-way olfactometer

Blank

Alginate beads with



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

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

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 of 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 system

  ➔ automated alginate bead production system

- Encapsulation of other molecules useful in chemical ecology

Thank you for your attention