Multi-residue analysis of pesticides in bee bread and pollen

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LAYOUT

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- **Context and objectives**
- **Method development**
  - Constraints and targeted pesticides
  - Analytics
  - Extraction protocol
  - Summary of method performance
- **Occurrence of pesticides in pollen and bee bread**
  - Sampling
  - Quantitative results
  - Potential links with bees mortality
- **Conclusions and perspectives**

CONTEXT AND OBJECTIVES

- Pesticides used in fields can potentially contaminate pollen collected by bees and bee bread (fermented pollen)
- High losses in honey bee colonies have already been correlated with exposure to some pesticides and occasionally with some kinds of crops

Question: Can the active ingredients of pesticides used in these crops be found in pollen collected by bees or stored pollen (bee bread)?

Objectives

Develop an analytical method for the quantification of pesticides in pollen and bee bread

Investigate potential relationships between the presence of pesticides in these matrices and the honey bee colony losses in winter

METHOD DEVELOPMENT

Constraints and targeted pesticides

- **Constraints**
  - Trace contaminations
  - Difficult matrices (waxes)
  - Limited amounts of samples
  - Wide spectrum of potential contaminants

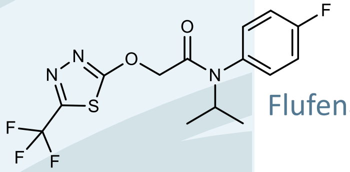
→

Sensitive and reliable method
Multi-residue method
Combination of analytical techniques
Versatile extraction procedure

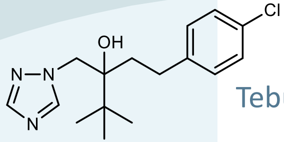
- **Targeted pesticides**
  - Insecticides, Herbicides, Fungicides
  - Used in crops around bee colonies or by beekeepers
  - Authorized, restricted or banned
  - Known degradation products

METHOD DEVELOPMENT

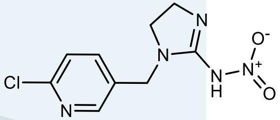
Constraints and targeted pesticides



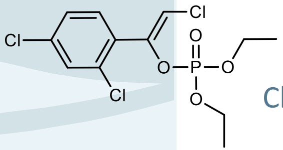
Flufenacet



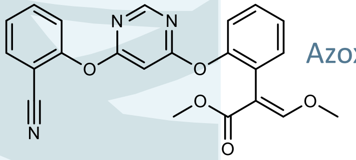
Tebuconazole



Imidacloprid



Chlorfenvinphos



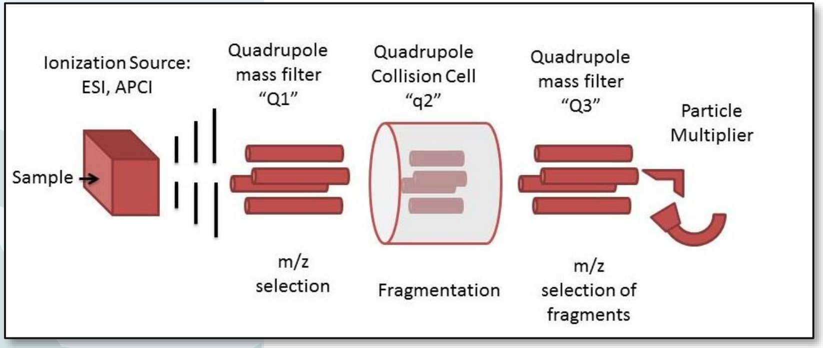
Azoxystrobin

Chemical family	Number of analytes	Activity
Anilides	15	Fungicides, Herbicides
Carbamates	15	Fungicides, Herbicides, Insecticides
Chlorotriazines	7	Herbicides
Conazoles	14	Fungicides
Neonicotinoids	5	Insecticides
Organochlorines	5	Fungicides, Insecticides
Organophosphorus	14	Insecticides
Phenylureas	4	Herbicides
Pyrethroids	7	Insecticides
Strobilurins	7	Fungicides, Insecticides
Sulfonylureas	7	Herbicides
Others	12	Fungicides, Herbicides

METHOD DEVELOPMENT

Analytics

- **Combination of analytical techniques**
  - Gas Chromatography and Liquid Chromatography
  - Detection by Tandem Mass Spectrometry in positive and negative modes
  - One extract, 3 analyses



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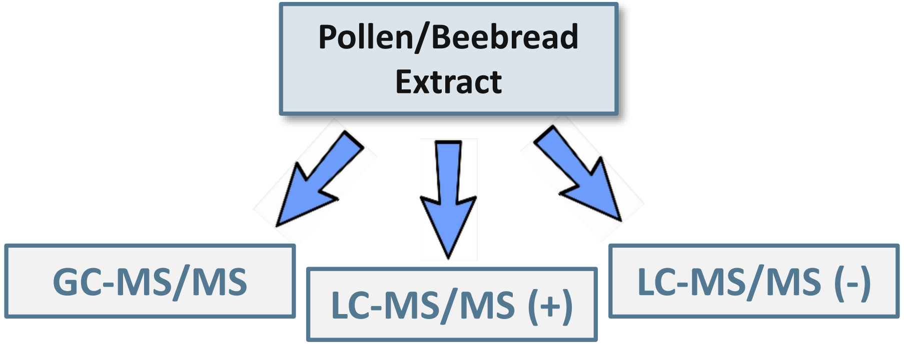
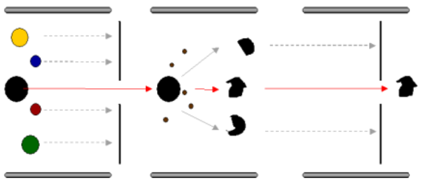
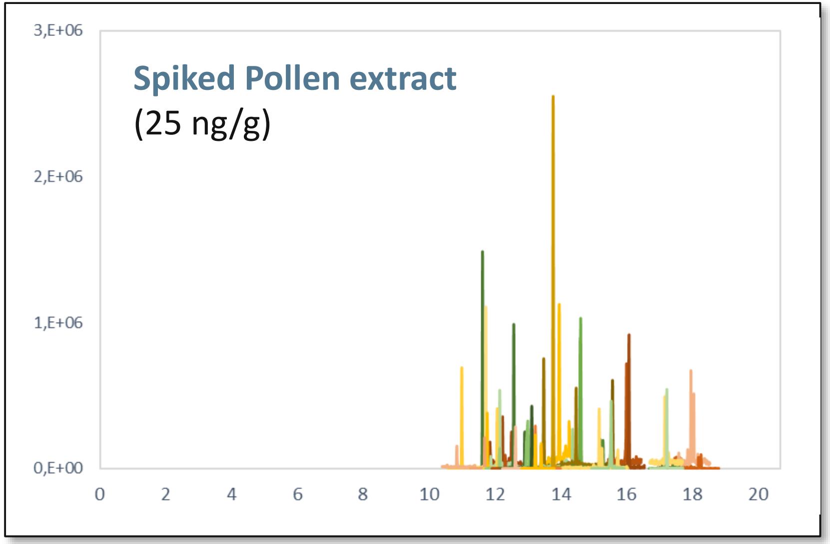


METHOD DEVELOPMENT

Analytics

- **Gas Chromatography – Tandem Mass Spectrometry**
  - Detection by Multiple Reaction Monitoring (MRM)
  - Large-Volume Injection (10 µL)
  - Matrix-matched calibration (Pollen extracts)
  - 34 compounds, 5 internal standards





METHOD DEVELOPMENT

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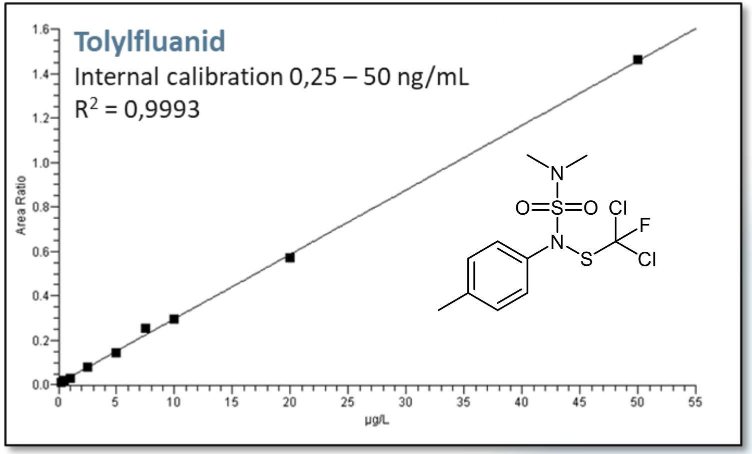
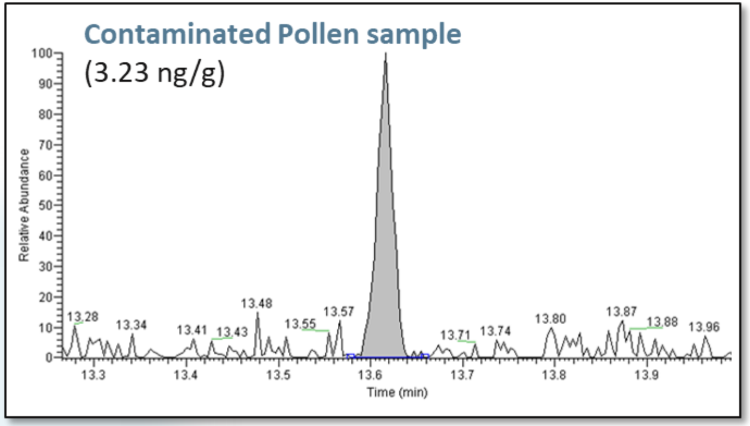
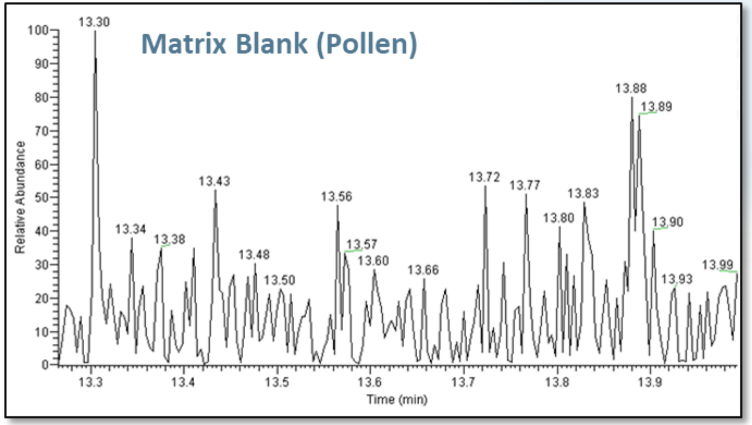
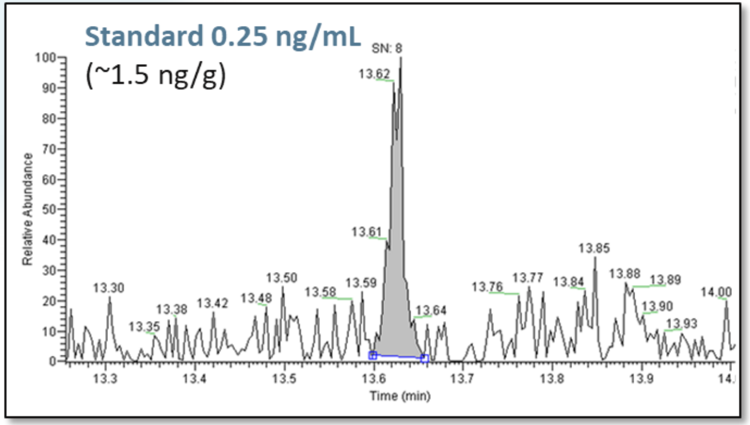




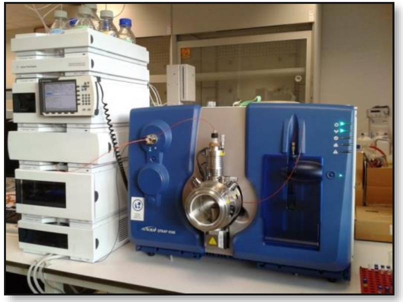


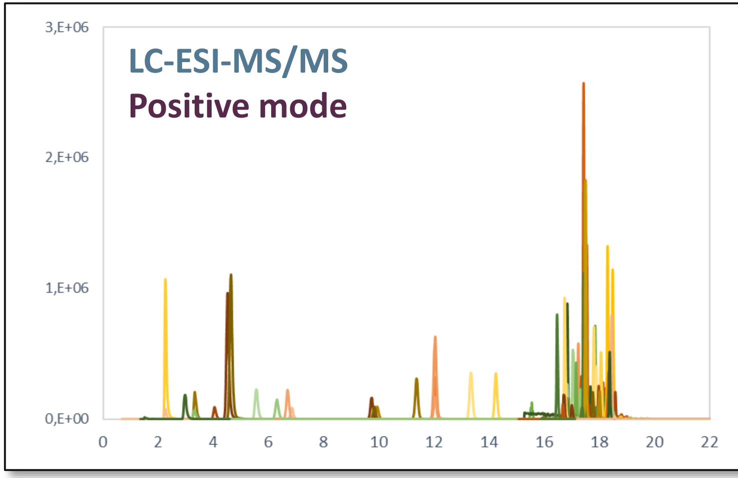


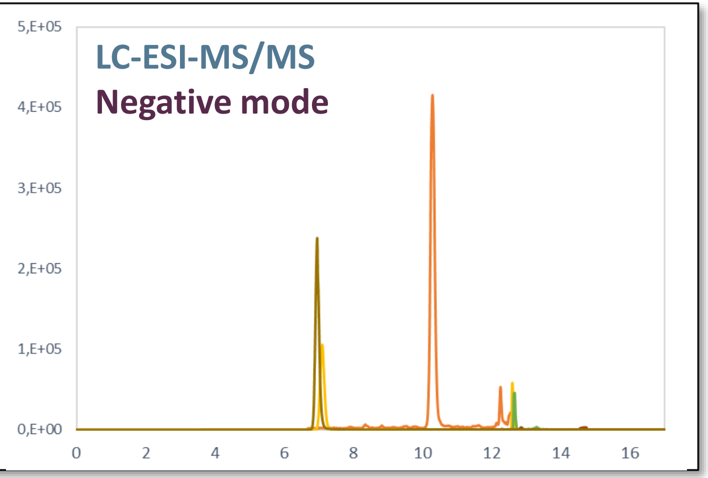
METHOD DEVELOPMENT

Analytics

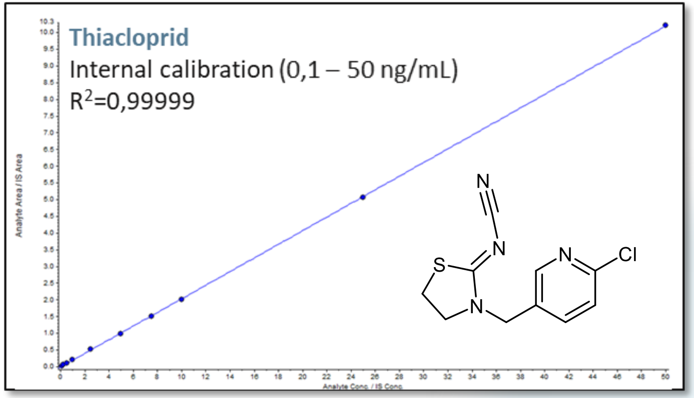
- **Liquid Chromatography – Tandem MS**
  - Detection by Multiple Reaction Monitoring (MRM)
  - Reverse-Phase LC
  - Electrospray Ionisation (+/-)
  - Positive mode: 73 compounds, 6 internal standards
  - Negative mode: 5 compounds, 1 internal standard

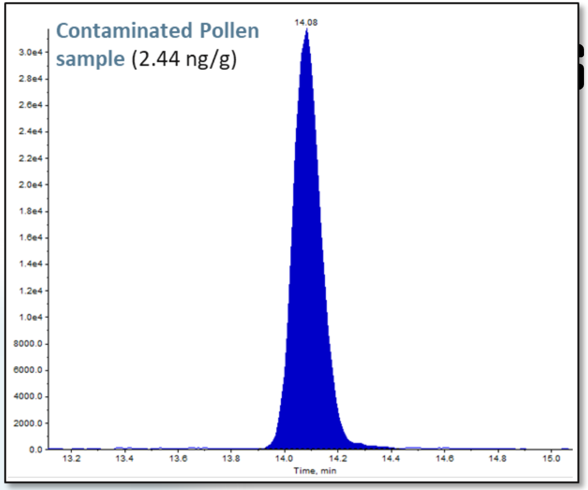


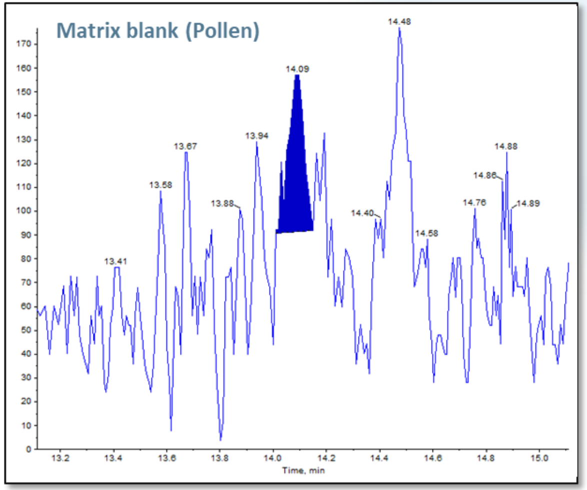


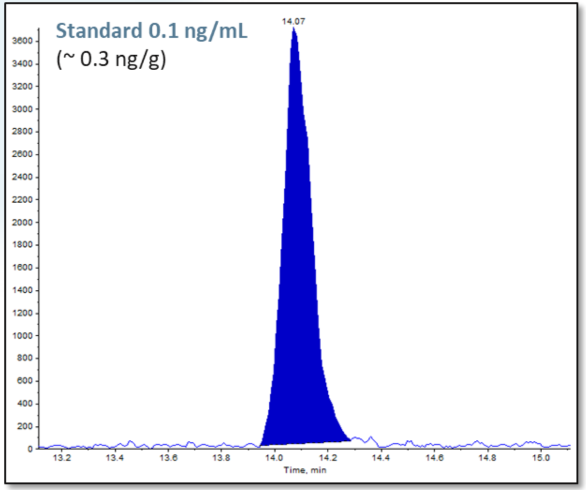


METHOD DEVELOPMENT









METHOD DEVELOPMENT

Sample preparation and extraction

- **Sample preparation**
  - Pollen: grinding, homogenization
  - Bee bread: needs to be isolated from waxes, dead insects, honey and hive frames

→ Freezing using liquid Nitrogen
Sieving (2 mm)
Manual removal of honey drops

METHOD DEVELOPMENT

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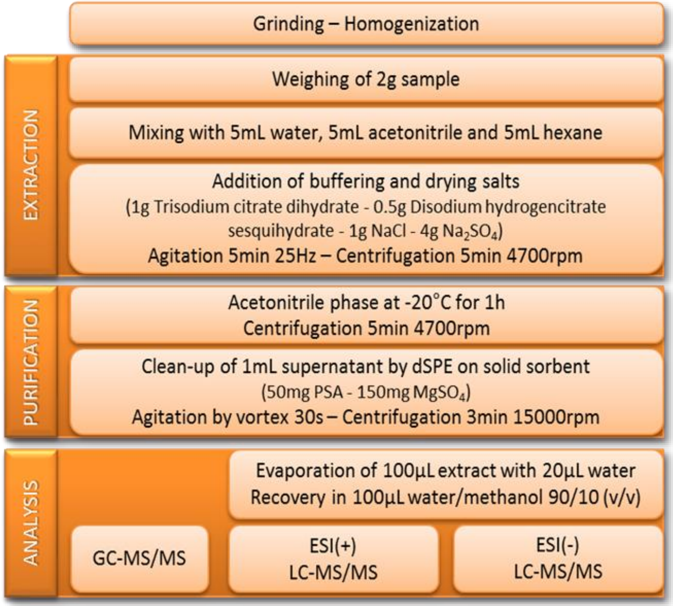
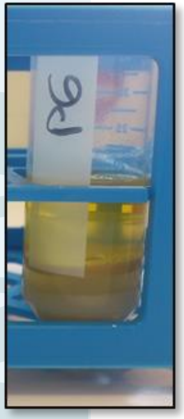
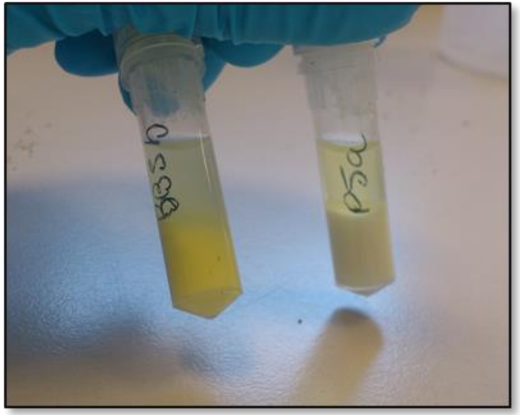
Sample preparation and extraction

- **Extraction**
  - Objectives: High and/or stable recoveries for most compounds, removal of matrix interferences
  - Adapted from the QuEChERS method(s)
  - Solvent extraction followed by dSPE cleanup
  - Addition of n-hexane to remove residual waxes
  - Protocol optimized for pollen and bee bread







METHOD DEVELOPMENT

Summary - Method performance

- Detection of 112 pesticides and degradation products at trace level
- One single sample extracted and analysed on 3 methods
- Reduced amount of sample needed: ~2 g per replicate
- Reduced risk of false positives:
    - ✓ 2 MRM channels per target compound
    - ✓ Overlap between methods
- Limits of quantification (LoQs):
    - ✓ Variable depending on the matrix and the target compound
    - ✓ From 0.16 to 18.4 ng/g, generally around 1 ng/g

Occurrence of pesticides in pollen and Beebread

Project BeeFirst (2011-2013)

Effects of agricultural structures and beekeeping practices on honeybee colony losses in winter

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PESTICIDES IN POLLEN AND BEEBREAD

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Sample collection

- Samples taken by local beekeepers
- 85 bee bread samples
- 154 pollen samples with sufficient mass (≥ 2g)
- 19 colonies from 5 apiaries
- Maximum of 4 colonies per location
- Time frame
  - 2011-2013: Collection of data and samples
  - 2013-2016: Analysis of samples and data
  - 2017/18: Publication of results

PESTICIDES IN POLLEN AND BEEBREAD

Results

- Compounds NOT found in pollen samples

Acetamiprid	Cyfluthrin	Imazalil	Picoxystrobin
Alachlor	Cypermethrin	Iodosulfuron-methyl	Pirimiphos-ethyl
Aldicarb	DEA (Desethylatrazine)	Ioxynil	Pirimiphos-methyl
Aldicarb-sulfone	Deltamethrin	Iprodione	Procymidone
Aldicarb-sulfoxide	DIA (Deisopropylatrazine)	Malathion	Propachlor
Amidosulfuron	Diazinon	Mefenacet	Propanil
Aminocarb	Diethofencarb	Mesosulfuron-methyl	Propiconazole
Asulam	Dimethachlor	Methabenzthiazuron	Propoxur
Atrazine	Dimethoate	Methamidophos	Pyraclostrobin
Benalaxyl	Epoxiconazole	Methomyl	Quintozene
Bromopropylate	Ethion	Methoxychlor	Simazine
Carbaryl	Fenbuconazole	Metobromuron	Sulfosulfuron
Carbofuran	Fenvalerate	Metolachlor-OXA	Tetramethrin
Carbofuran-3-hydroxy	Fluoxastrobin	Monolinuron	Thiabendazole
Chlordane-alpha	Fluquinconazole	Omethoate	Tolylfluanid
Chlordane-gamma	Flutolanil	Oxamyl	Triadimefon
Chlorpyrifos-methyl	Fonofos	Parathion	Tribenuron-methyl
Chlortoluron	Furalaxyl	Pentachlorothioanisole	Vinclozolin
Cyanazine	Hexazinon	Phosalone	

75 compounds were not found in any pollen sample

PESTICIDES IN POLLEN AND BEEBREAD

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Results

- **Compounds NOT found in bee bread samples**

Acetamiprid	Cyproconazole	Iprodione	Permethrin-trans
Alachlor	DCBA (2,6-Dichlorobenzamide)	Malathion	Phosalone
Aldicarb	DEA (Desethylatrazine)	MCPA (2-Methyl-4-	Picoxystrobin
Aldicarb-sulfone	DIA (Deisopropylatrazine)	chlorophenoxyacetic acid)	Piperonyl-butoxide
Aldicarb-sulfoxide	Diazinon	Mefenacet	Pirimiphos-ethyl
Amidosulfuron	Diethofencarb	Mesosulfuron-methyl	Prochloraz
Aminocarb	Diflufenican	Methabenzthiazuron	Procymidone
Asulam	Dimethachlor	Methamidophos	Propachlor
Benalaxyl	Dimethoate	Methomyl	Propanil
Bromopropylate	Ethion	Methoxychlor	Propiconazole
Carbaryl	Fenbuconazole	Metobromuron	Propoxur
Carbofuran	Fenvalerate	Metolachlor	Quintozene
Carbofuran-3-hydroxy	Fluoxastrobin	Metolachlor-OXA	Simazine
Chlordane-alpha	Fluquinconazole	Monolinuron	Sulfosulfuron
Chlordane-gamma	Flutolanil	Myclobutanil	Tetramethrin
Chlorpropham	Fonofos	Nicosulfuron	Thiabendazole
Chlorpyrifos	Furalaxyl	Omethoate	Thifensulfuron-methyl
Chlorpyrifos-methyl	Hexazinon	Oxamyl	Tolylfluanid
Cyanazine	Imazalil	Parathion	Triadimefon
Cyfluthrin	Iodosulfuron-methyl	Pentachlorothioanisole	Tribenuron-methyl
Cypermethrin	Ioxynil	Permethrin-cis	Vinclozolin

83 compounds were not found in any bee bread sample

PESTICIDES IN POLLEN AND BEEBREAD

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Results

- Compounds found in pollen samples

- Thiacloprid restricted in 2017
- Neonicotinoids banned in 2013
- Metazaclor restricted in 2015

Rückstände im Pollen (N=154) Wirkstoff	Wirkspektrum	Stoffgruppe	MoA-Gruppe	MIN (ng/g)	MAX (ng/g)	∅ (ng/g)	# Positive Proben	Positive Proben (%)
Thiacloprid	Insektizid	Neonikotinoid	4A	< 0.36	133.54	5.256	45	29.4
Permethrin-cis	Insektizid, Akarizid	Pyrethroide	3A	< 1.19	39.71	1.551	18	11.8
Permethrin-trans	Insektizid, Akarizid	Pyrethroide	3A	< 2.24	46.81	1.603	16	10.5
Azoxystrobin	Fungizid	Methoxy-Acrylat	C3	< 0.29	22.77	0.585	14	9.2
Clothianidin	Insektizid	Neonikotinoid	4A	< 0.31	1.40	0.059	12	7.8
DCBA (2,6-Dichlorobenzamide)	Herbizid	Metab. v. Dichlobenil	-	< 0.35	36.74	0.351	12	7.8
Metazachlor	Herbizid	Chloroacetamide	K3	< 0.28	7.57	0.176	12	7.8
Methiocarb	Insektizid, Akarizid	Carbamate	1A	< 0.74	39.32	0.578	11	7.2
Difenoconazole	Fungizid	Triazol	G1	< 0.78	12.29	0.292	10	6.5
Trifloxystrobin	Fungizid	Oximino-acetates	C3	< 0.38	173.23	2.043	10	6.5
Fenhexamid	Fungizid	Hydroxyanilid	G3	< 0.99	224.41	1.745	9	5.9
Carbendazim	Fungizid	Benzimidazole	B1	< 0.34	19.95	0.203	8	5.2
Imidacloprid	Insektizid	Neonikotinoid	4A	< 0.30	0.79	0.029	7	4.6
Tebuconazole	Fungizid	Triazoles	G1	< 2.10	10.54	0.255	7	4.6
Metalaxyl	Fungizid	Acylalanin	A1	< 0.22	3.83	0.065	6	3.9
Flufenacet	Herbizid	Oxyacetamide	K3	< 0.63	2.76	0.053	5	3.3
Metolachlor	Herbizid	Chloroacetamide	K3	< 0.60	10.93	0.167	5	3.3
Penconazole	Fungizid	Triazole	G1	< 0.62	4.71	0.069	5	3.3
Bromoxynil	Herbizid	Nitrile	C3	< 0.67	3.78	0.057	4	2.6
Chlorfenvinphos	Insektizid, Akarizid	Organophosphate	1B	< 0.53	22.11	0.227	4	2.6
DET (Desethylterbutylazine)	Herbizid	Unklassifiziert	N/A	< 1.57	4.13	0.061	4	2.6
Diflufenican	Herbizid	Pyridinecarboxamide	F1	< 0.16	3.86	0.041	4	2.6
MCPA (2-Methyl-4-chlorophenoxyacetic acid)	Herbizid	Phenoxy-carboxylic-acid	O	< 7.79	64.98	0.890	4	2.6
Nicosulfuron	Herbizid	Sulfonylurea	B	< 3.01	61.20	0.770	4	2.6
Thiamethoxam	Insektizid	Neonikotinoid	4A	< 0.28	0.84	0.017	4	2.6
Bentazone	Herbizid	Thiadiazine	C3	< 0.34	0.70	0.012	3	2.0
Chlorpyrifos	Insektizid	Organophosphate	1B	< 0.92	3.68	0.045	3	2.0
Flusilazole	Fungizid	Triazol	G1	< 0.58	79.84	0.562	3	2.0

PESTICIDES IN POLLEN AND BEEBREAD

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Results

- Compounds found in bee bread samples

- Thiacloprid restricted in 2017
- Neonicotinoids banned in 2013
- Metazaclor restricted in 2015

Rückstände im Bienenbrot (N=85) Wirkstoff	Wirkspektrum	Stoffgruppe	MoA-Gruppe	MIN	MAX (ng/g)	∅	# Positive Proben	Positive Proben (%)
Thiacloprid	Insektizid	Neonikotinoid	4A	< 0.38	149.38	13.11	52	61.2
Chlorfenvinphos	Insektizid, Akarizid	Organophosphate	1B	< 0.36	265.93	8.74	40	47.1
Tebuconazole	Fungizid	Triazoles	G1	< 1.34	21.45	2.05	22	25.9
Methiocarb	Insektizid, Akarizid	Carbamate	1A	< 0.48	5.26	0.17	10	11.8
Flufenacet	Herbizid	Oxyacetamide	K3	< 0.42	2.03	0.08	8	9.4
Azoxystrobin	Fungizid	Methoxy-Acrylat	C3	< 0.24	57.39	1.13	7	8.2
Fenhexamid	Fungizid	Hydroxyanilid	G3	< 0.62	25.17	2.78	6	7.1
Trifloxystrobin	Fungizid	Oximino-acetates	C3	< 0.30	16.87	1.46	6	7.1
Bentazone	Herbizid	Thiadiazine	C3	< 0.30	6.43	0.12	5	5.9
Carbendazim	Fungizid	Benzimidazole	B1	< 0.37	4.21	0.04	5	5.9
Clothianidin	Insektizid	Neonikotinoid	4A	< 0.32	1.60	0.03	5	5.9
Pyraclostrobin	Fungizid	Methoxy-Carbamat	C3	< 0.33	8.17	0.12	5	5.9
Deltamethrin	Insektizid	Pyrethroide	3A	< 0.34	6.59	0.13	4	4.7
Imidacloprid	Insektizid	Neonikotinoid	4A	< 0.32	1.17	0.01	4	4.7
Metalaxyl	Fungizid	Acylalanin	A1	< 0.28	7.52	0.45	3	3.5
Metazachlor	Herbizid	Chloroacetamide	K3	< 0.25	1.75	0.04	3	3.5
Bromoxynil	Herbizid	Nitrile	C3	< 0.71	2.09	0.02	2	2.4
Chlortoluron	Herbizid	Anilides/anilines	?	< 0.37	3.00	0.05	2	2.4
Kresoxim-methyl	Fungizid	Oximino-acetates	C3	< 1.24	54.66	0.64	2	2.4
Pirimiphos-methyl	Insektizid	Organophosphate	1B	< 0.23	0.42	0.01	2	2.4
Atrazine	Herbizid	Triazine	C1	< 0.35	1.80	0.02	1	1.2
DET (Desethylterbutylazine)	Herbizid	Unklassifiziert	N/A	< 1.43	2.78	0.03	1	1.2
Difenoconazole	Fungizid	Triazol	G1	< 0.45	7.81	0.09	1	1.2
Epoxiconazole	Fungizid	Triazol	G1	< 0.57	1.46	0.02	1	1.2
Ethofumesate	Herbizid	Benzofuran	N	< 10.00	2.40	0.24	1	1.2
Fenpropathrin	Insektizid	Pyrethroide	3A	< 0.97	2.65	0.03	1	1.2
Flusilazole	Fungizid	Triazol	G1	< 0.33	0.52	0.01	1	1.2
Penconazole	Fungizid	Triazole	G1	< 0.75	1.93	0.02	1	1.2
Thiamethoxam	Insektizid	Neonikotinoid	4A	< 0.30	0.44	0.01	1	1.2

PESTICIDES IN POLLEN AND BEEBREAD

Comparison with MRLs

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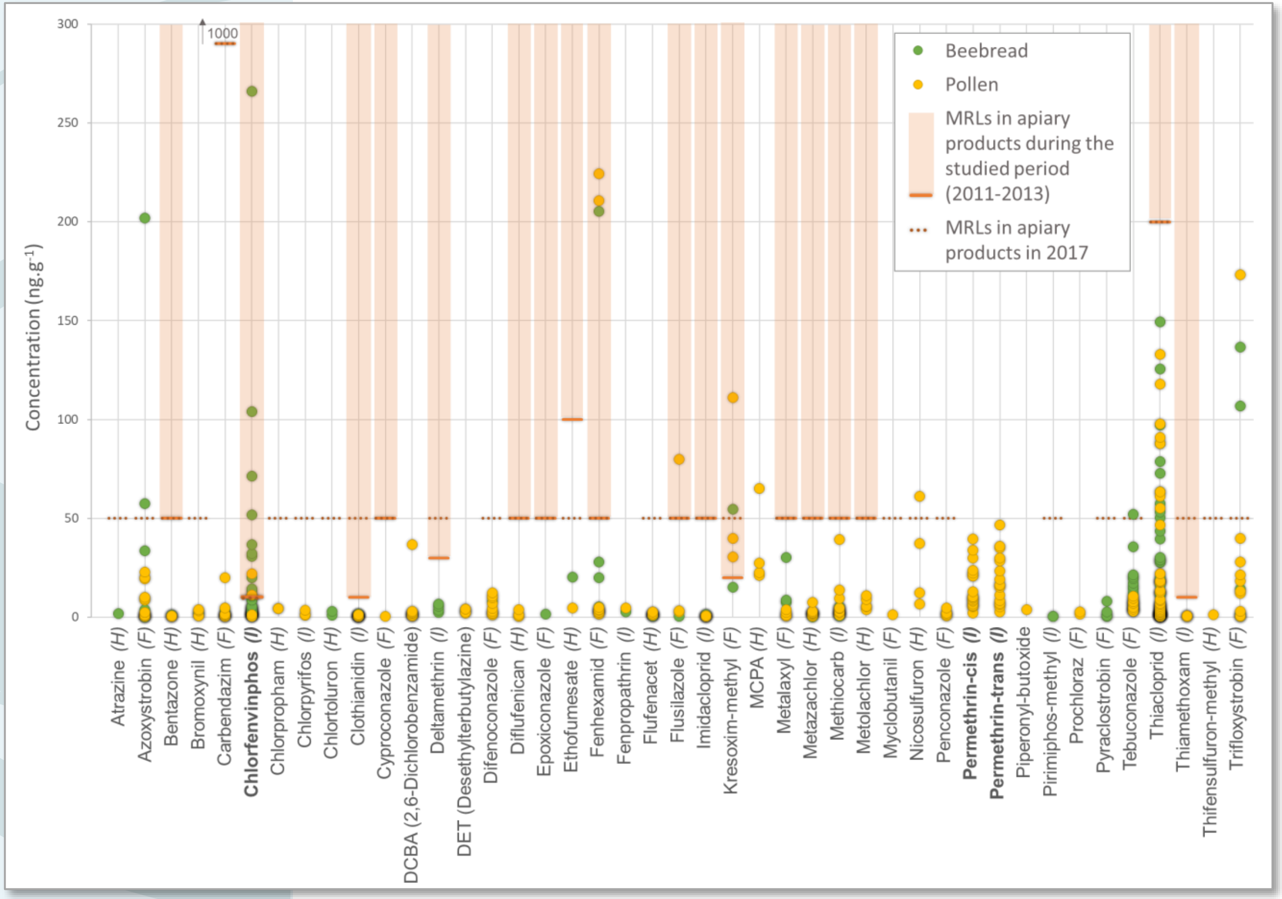


In **pollen**, 7 concentrations (4,5%) exceeded the current maximum levels for apiary products:

- Chlorfenvinphos (2x)
- Flusilazole (1x)
- Fenhexamid (1x)
- Kresoxim-methyl (1x)
- Nicosulfuron (1x)
- Trifloxystrobin (1x)

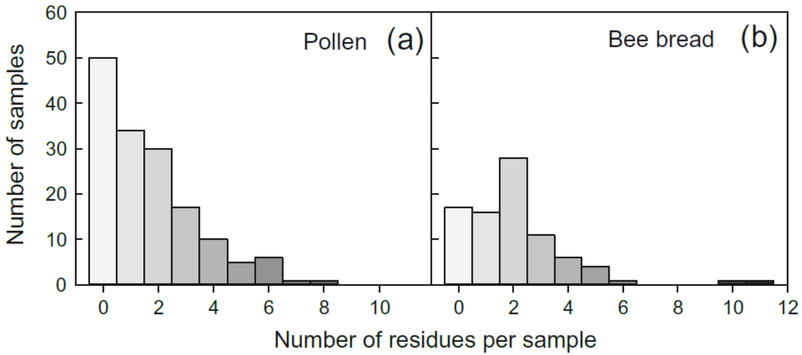
In **beebread**, maximum levels were exceeded 11 times (12,9%):

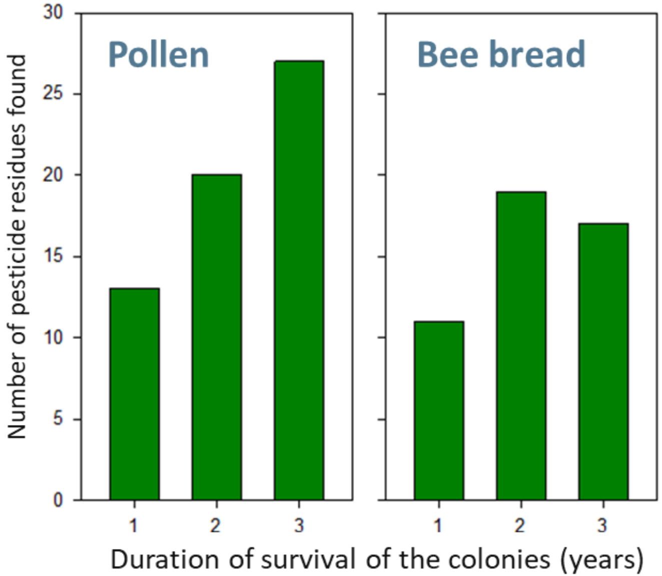
- Azoxystrobin (1x)
- Chlorfenvinphos (9x)
- Kresoxim-methyl (1x)

PESTICIDES IN POLLEN AND BEEBREAD

Potential link with bees mortality

- **Effect of multiple contaminations**





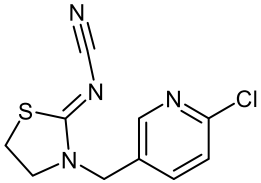
- Reduced number of multiple contaminations
- No direct effect on bees mortality
- Excessive sensitivity?

PESTICIDES IN POLLEN AND BEEBREAD

Potential links with bees mortality

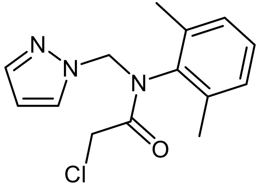
- **Alternative approach: 3-years mortality vs. highest concentration**

  - For most of the pesticides: no relationship (neither alone nor in combination) with honey bee colony losses
  - Thiacloprid and Metazachlor seem to contribute to the reduction of colony lifetime



Thiacloprid

Formula	$C_{10}H_9ClN_4S$
Activity	Insecticide
Family	Neonicotinoid



Metazachlor

Formula	$C_{14}H_{16}ClN_3O$
Activity	Herbicide
Family	Chloroacetanilide

PESTICIDES IN POLLEN AND BEEBREAD

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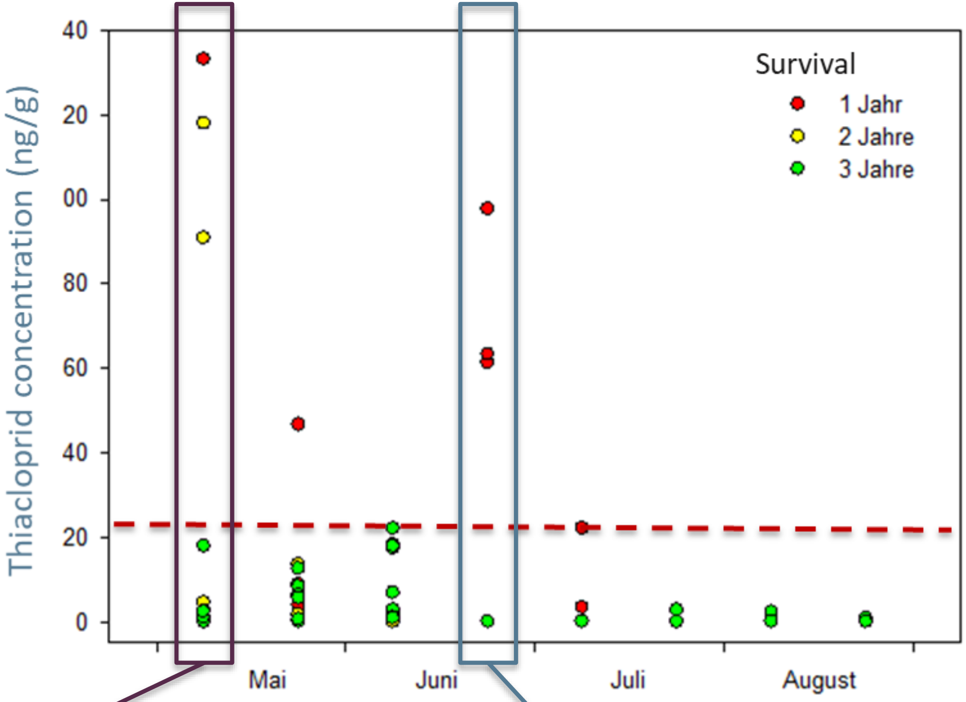
Potential linkage with colonies mortality

- **Time course**
  - Bees exposed to pollen with more than 23 $ng.g^{-1}$ of Thiacloprid did not survive the studied period of three years
  - The same trend is observed with concentrations in Metazachlor higher than 2.8 $ng.g^{-1}$



Pest control in rapeseed

Aphid control in horticultural crops?

CONCLUSIONS AND PERSPECTIVES

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- **Method**
  - The combination of 3 analytical methods allows the determination of trace amounts of 112 pesticides in pollen and bee bread
  - One single extraction protocol, requiring only 2 g of starting material
  - The method has recently been adapted and applied to honey
  - New pesticides have been included in the method

- **Pesticides in pollen and bee bread**
  - Pesticides from different families were found in the samples, sometimes in concentrations exceeding the former MRLs
  - No correlation was found between the colony mortality and the presence of individual or combined pesticides, except for Thiacloprid and Metazachlor
  - Since the use of Metazachlor was restricted in 2015, concentrations are probably lower today than in 2011-2013
  - Following the publication of these results, the use of Thiacloprid was restricted in 2017 in Luxembourg

CONCLUSIONS AND PERSPECTIVES

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- **Need more information?**
  - All tables available on :

    https://agriculture.public.lu/dam-assets/pictures/actualites/ma/2017/conference-beefirst-vhs/BeeFirst-Pestizidr%C3%BCckst%C3%A4nde-2011-2013.pdf

  - Beyer, Lenouvel, Guignard, Eickermann, Clermont, Kraus, Hoffmann, *Environmental Science and Pollution Research* (2018)

Environmental Science and Pollution Research (2018) 25:32163–32177
https://doi.org/10.1007/s11356-018-3187-4

RESEARCH ARTICLE

CrossMark

Pesticide residue profiles in bee bread and pollen samples and the survival of honeybee colonies—a case study from Luxembourg

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