anses

SELECTIVE ANALYSIS OF DITHIOCARBAMATES BY CLASSES:
Overview of the French working group project

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Investigate, evaluate, protect

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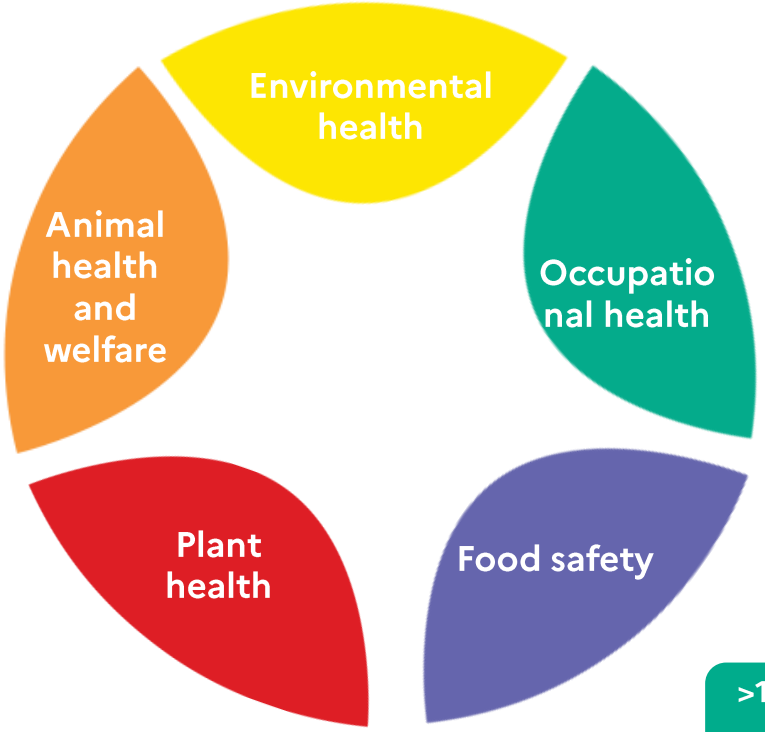
Anses, a « One Health » Agency

French Agency for Food, Environmental and Occupational Health & Safety

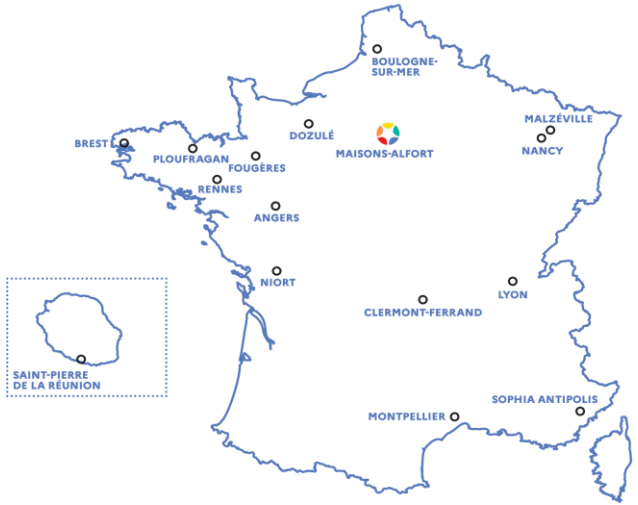


Main missions:

- Assessing health risks
- Producing scientific knowledge
- Vigilance and alerts
- Examining marketing autorisation applications

9 laboratories on **16** sites **1400** employees

>100 national, European or international reference mandates



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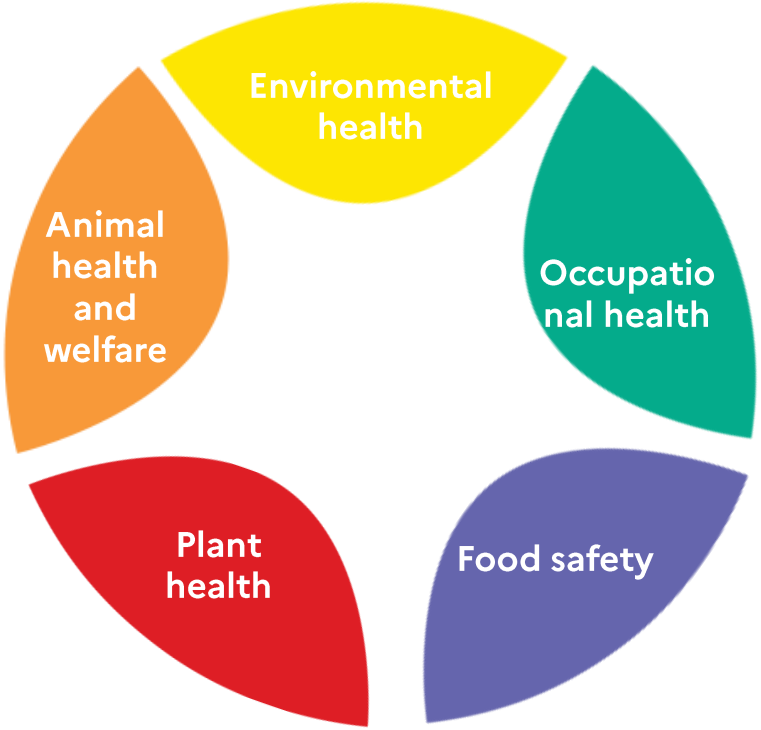


Laboratory for Food Safety

Pesticides and Marine Biotoxins Unit

Pesticides team:

- NRL for AO and SRM (including chlordecone)
- Method development and validation
- PTs organisation
- Scientific/technical advice and support to Ofls
- Scientific advice and support to competent authorities
- Participation to standardization committees
- Official analyses

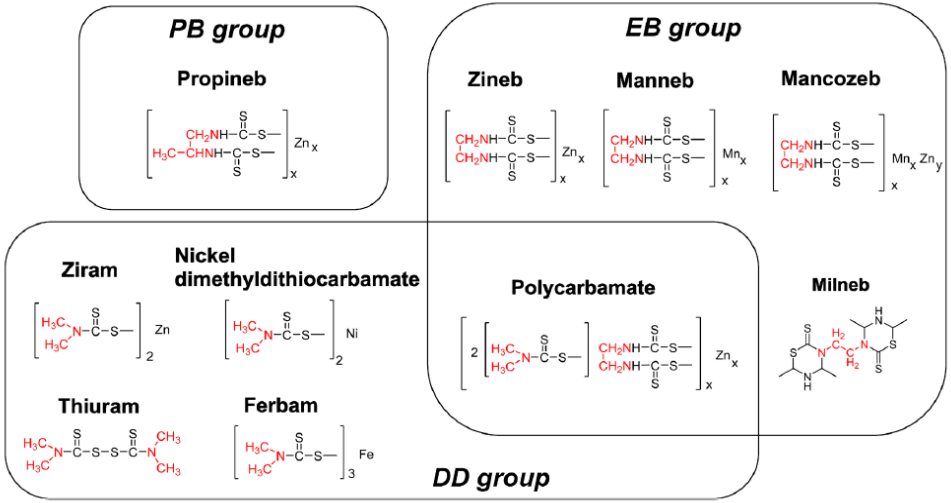
Dithiocarbamates: setting up a working group…

- **Broad-spectrum fungicides** widely used in the past decades

  → among the **most frequently detected pesticides** in the EU

  Residue definition: Dithiocarbamates (dithiocarbamates expressed as CS_2, including maneb, mancozeb, metram, propineb, thiram and ziram)

- **3 main classes of DTCs**:



A rapid sensitive analysis of dithiocarbamates fungicides using modified QuEChERS method and liquid chromatography-tandem mass spectrometry, Kakitani *et al.*, *J. Pestic. Sci.* **42**(4), 145-150 (2017)

… many analytical difficulties

(DTC solubility, stability, complex polymeric structure…)

Several analytical methods and strategies:

- **Reductive cleavage to CS_2**
- Amine-moiety analysis (propineb as propylene diamine)
- Derivatisation methods
- LC-MS/MS detection of the ligands
- Screening approaches of DTC markers (EURL-SRM work, eBIC, pBIC, ETU, PTU)
- …

Dithiocarbamates: setting up a working group…

- **Reductive cleavage to CS_2:** some major drawbacks
  - ➢ **False-positive results** on naturally sulfur containing matrices (*Brassicaceae, Alliaceae*)
  - ➢ **Lack of selectivity**: no identification of the DTC compound(s) involved in a positive result

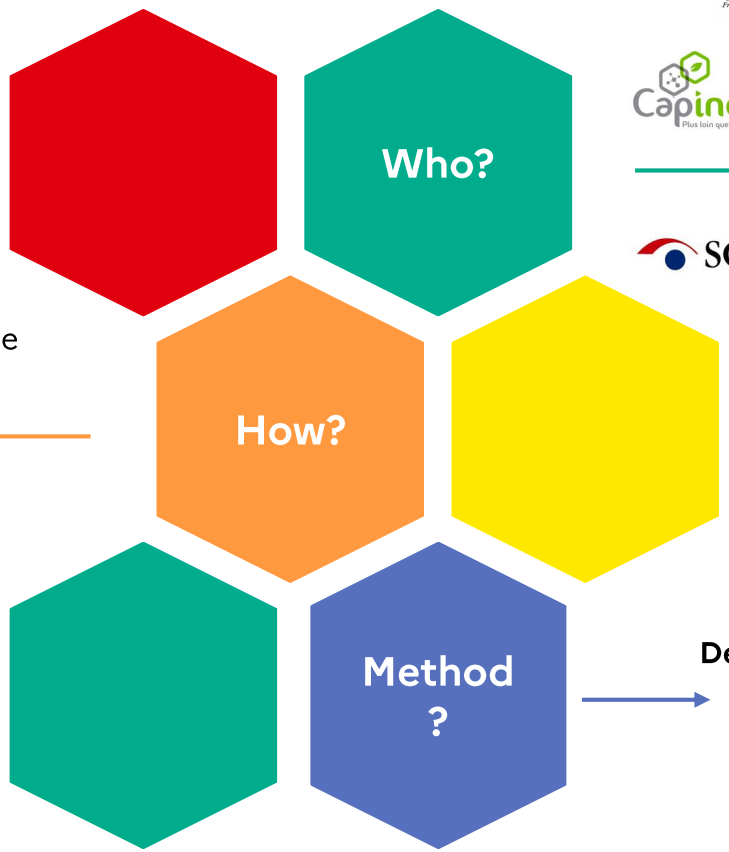
Need for an analytical method more reliable on these matrices which allows the selective and specific quantification of DTCs

→ **EFSA recommendation,** in a context of MRL revisions

→ Emergence of **methylation methods** among some French laboratories

With the support of the French Competent Authority, creation of a national working group in order to assess and improve the reliability of such methods

Dithiocarbamates: setting up a working group…



- Pooling knowledge and experience
- Defining work leads
- Sharing the different actions
- On-line meetings every 3 months

- French NRL-SRM
- all 8 French Ofls
- Anses Laboratory of Hydrology (Nancy)
- the French Directorate General for Food, Ministry of Agriculture

+ Regular feedbacks and exchanges with EURL-SRM

Derivatisation by methylation, Kakitani (2017)

A rapid sensitive analysis of dithiocarbamates fungicides using modified QuEChERS method and liquid chromatography-tandem mass spectrometry, Kakitani *et al.*, *J. Pestic. Sci.* **42**(4), 145-150 (2017)

Methylation method overview

10 g sample

(water addition)

- 100 µL Dithiothreitol 1M
- 1 mL $NaHCO_3$ 1M
- 10 mL acetonitrile
- 50 µL dimethyl sulfate

Mechanical shaking 15 min

QuEChERS « Original » salts addition
Mechanical shaking 10 min
Centrifugation 5 min, 3000g

dSPE clean-up $MgSO_4$/PSA
Vortex 30 s
Centrifugation 3 min, 3000g

Filtration PTFE 0,2 µm

Extract acidification (formic acid)

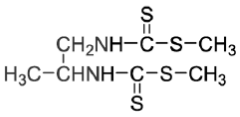
RP-LC-MS/MS

Amounts and volumes can be adapted

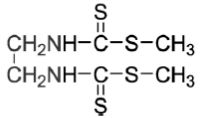
Methylation step

PB → PBMe dimethyl propylenebisdithiocarbamate
EB → EBMe dimethyl ethylenebis(dithiocarbamic acid)
DD → DDMe methyl dimethyldithiocarbamate

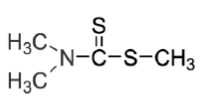
PBMe



EBMe



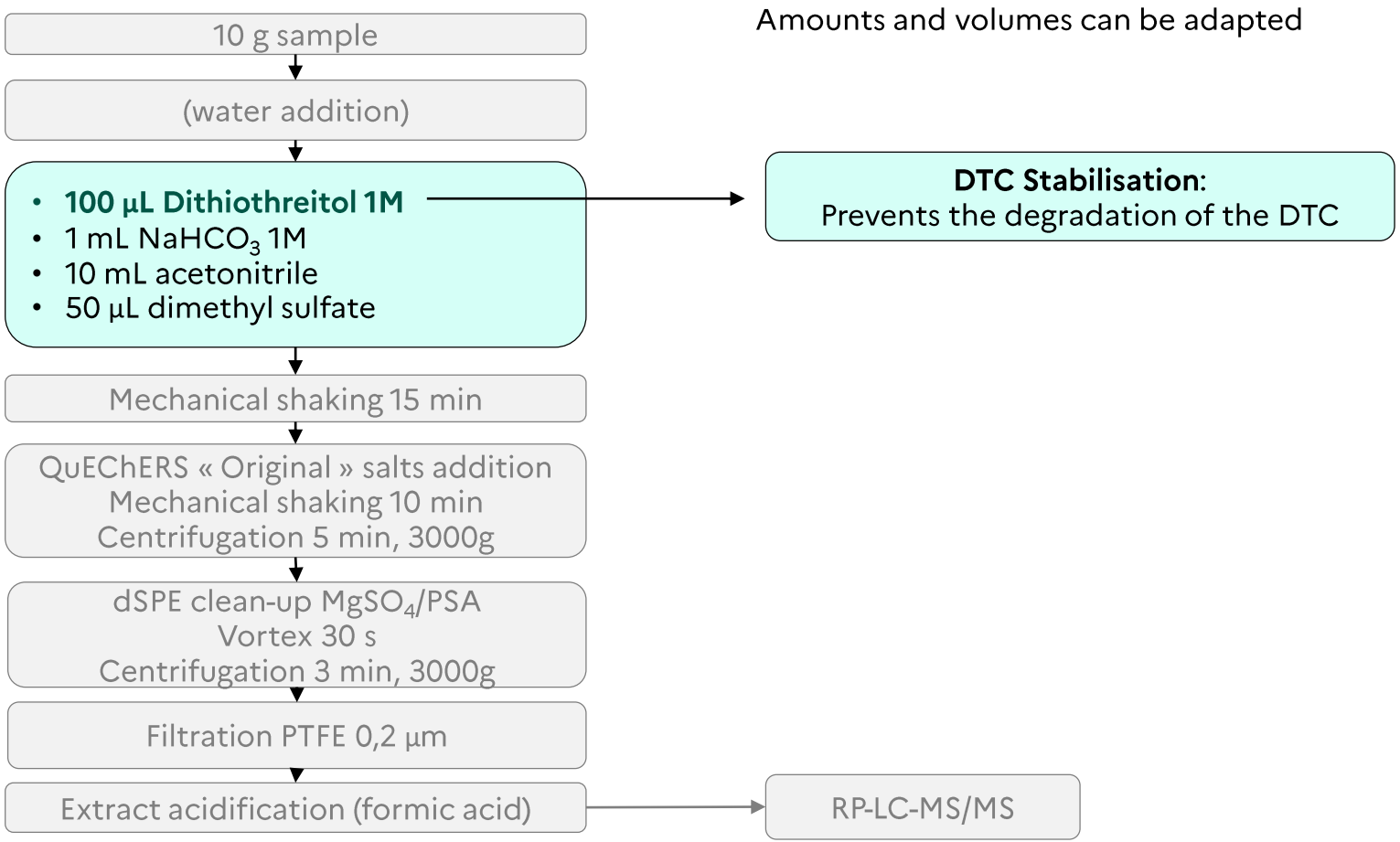
DDMe



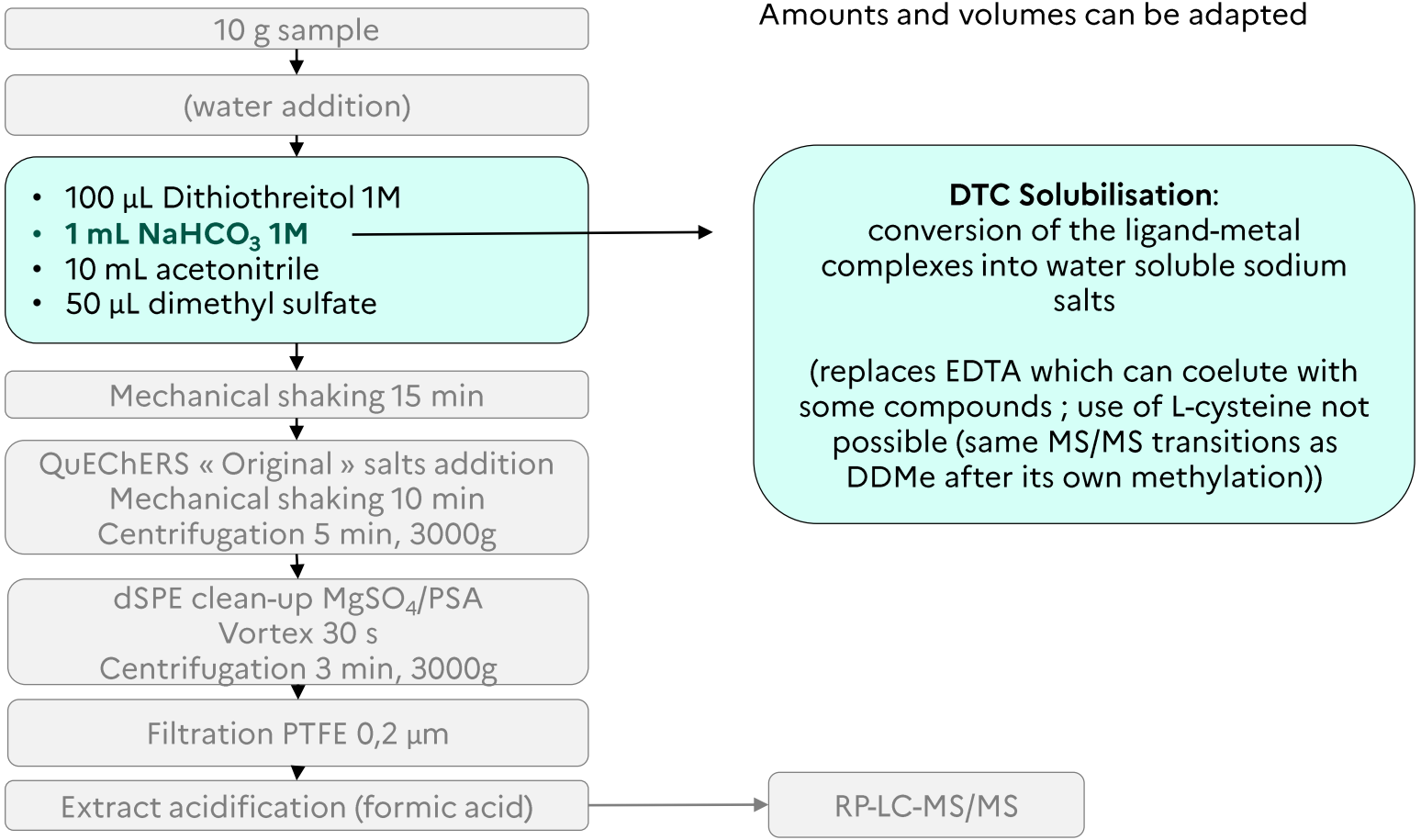
QuEChERS extraction

Results mathematically converted to CS_2 conc. for comparison to MRL

Methylation method overview



Methylation method overview



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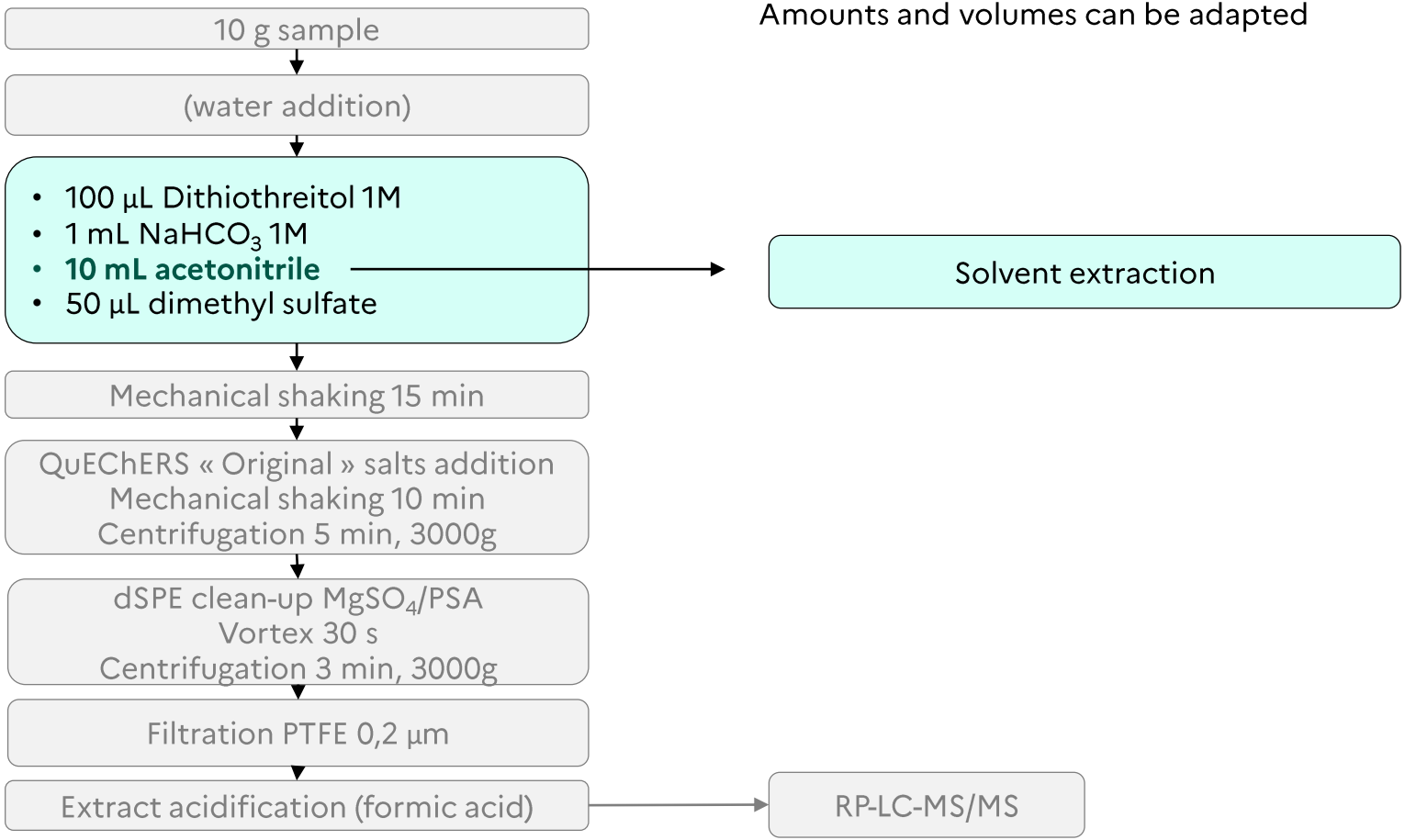

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Filtration PTFE 0,2 µm

Extract acidification (formic acid) → RP-LC-MS/MS

Amounts and volumes can be adapted

Methylation agent

Toxic if swallowed
Causes severe skin burns and eye damage
May cause respiratory irritation
May cause an allergic skin reaction
Fatal if inhaled
Suspected of causing genetic defects
May cause cancer

→ **Use personal protective equipment**:
Wear suitable protective gloves
Wear suitable respiratory protection

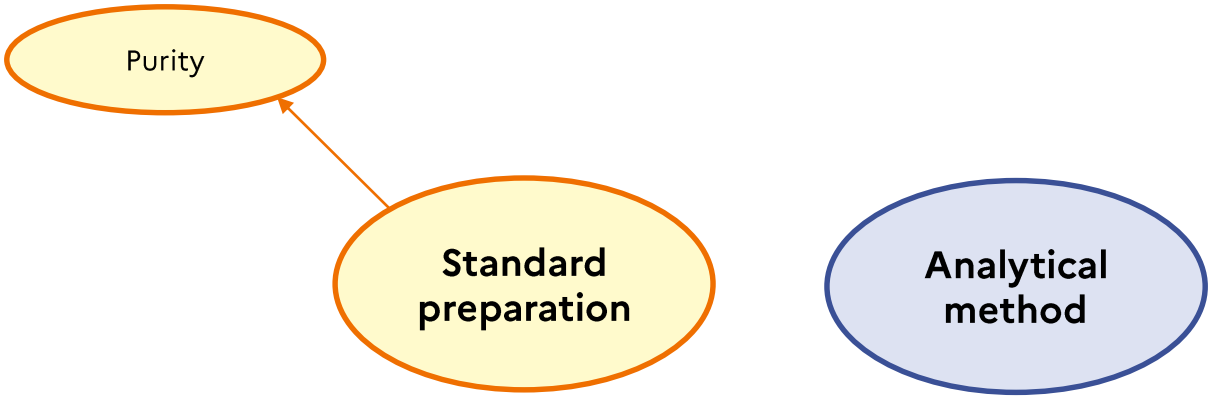
Methylation method overview

Is it routinely manageable?

Occupational exposure values were measured

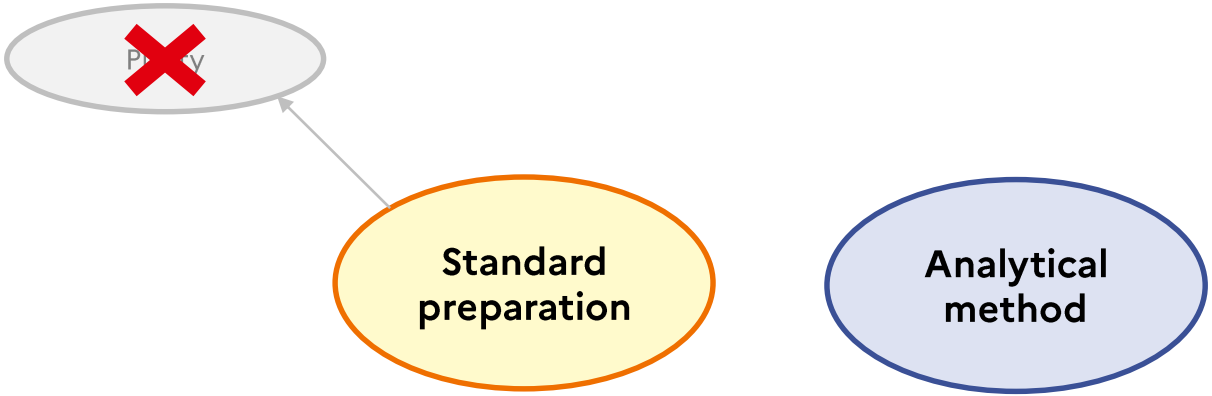
below the limit value → respiratory mask not required <u>in our lab conditions</u> (handling in a fume hood)

Defining the main work leads…



Defining the main work leads…

Cf: EURL-SRM previous work



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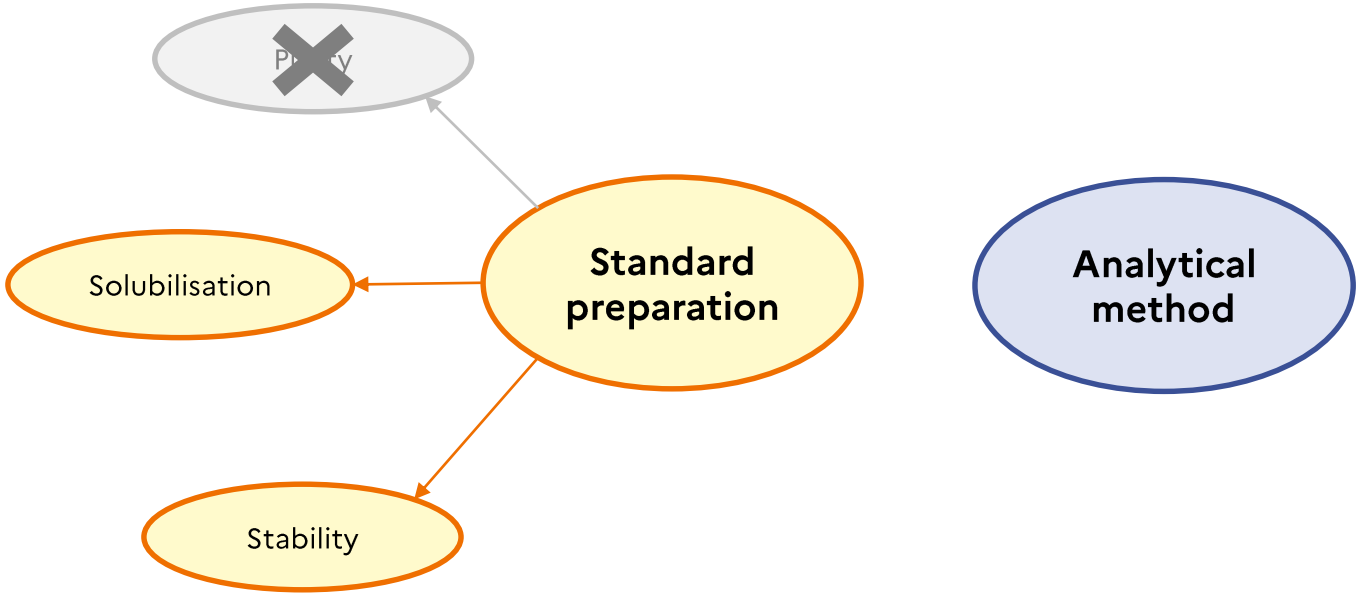
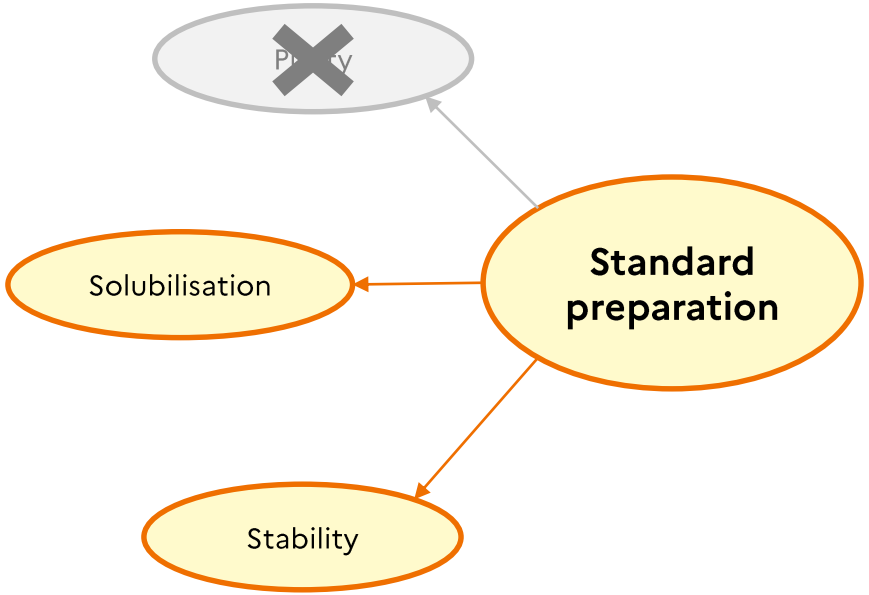


Standard preparation: solubilisation and stability

Compound	Solvent	Conc. (mg/L)	Period of stability (not tested beyond)	Storage conditions
Ferbam	Acetonitrile	100	1 month	+4°C
Mancozeb	L-cystein/EDTA aq. solution (pH 9,6)	100	4 months	+4°C
Maneb	L-cystein/EDTA aq. solution (pH 9,6)	150	1 month	+4°C
Propineb	Dimethylformamide	200	Not stable, to be prepared freshly each day	
Propineb	L-cystein/EDTA aq. solution (pH 9,6)	200	Soluble, no stability data so far, but lower recovery rates obtained after extraction - to be confirmed	
Thiram	Acetonitrile	100	4 months	+4°C
Thiram	Ethanol	100	1 year	-18°C
Zineb	L-cystein/EDTA aq. solution (pH 9,6)	100	15 days	+4°C
Ziram	Acetonitrile	200	1 month	+4°C
Ziram	Ethanol	100	1 year	-18°C
EBMe	Acetonitrile + 0.1% FA	100	2 months	-18°C
PBMe	Acetonitrile + 0.1% FA	100	2 months	-18°C
DDMe	Acetonitrile + 0.1% FA	100	2 months	-18°C

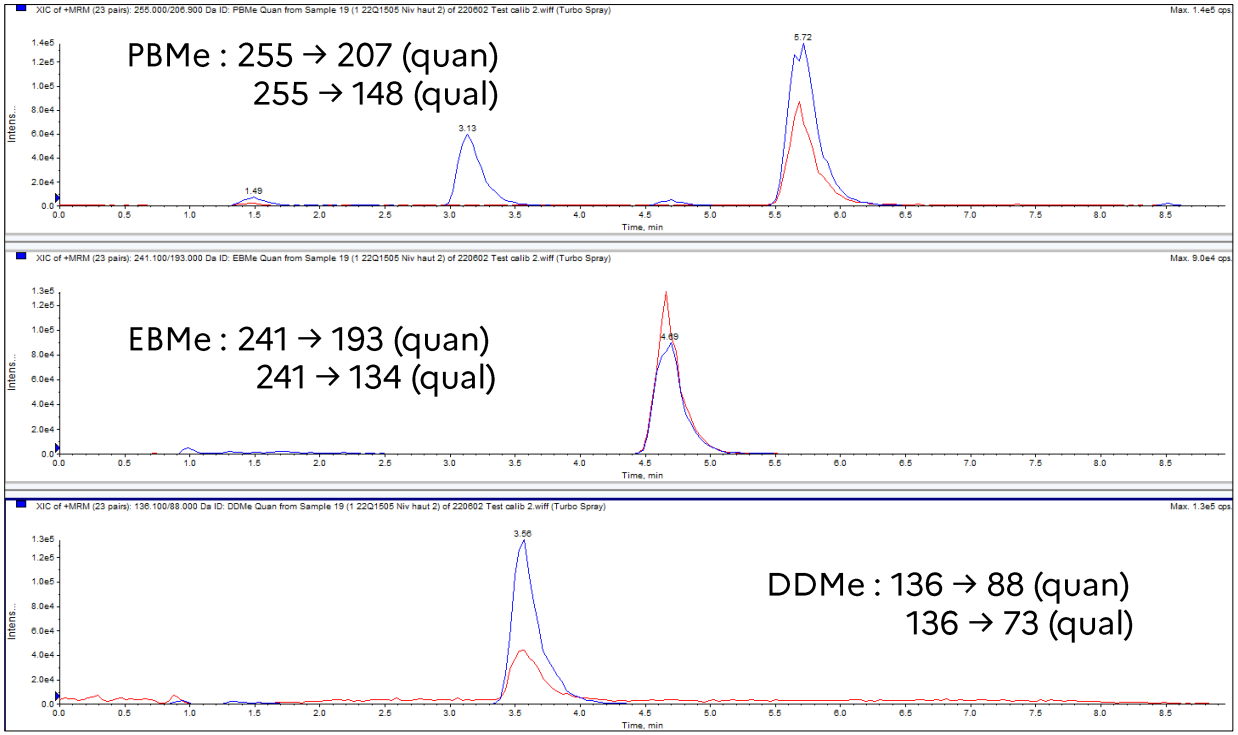
Defining the main work leads…



No LC-MS/MS difficulties identified

Analytical method

LC-MS/MS conditions



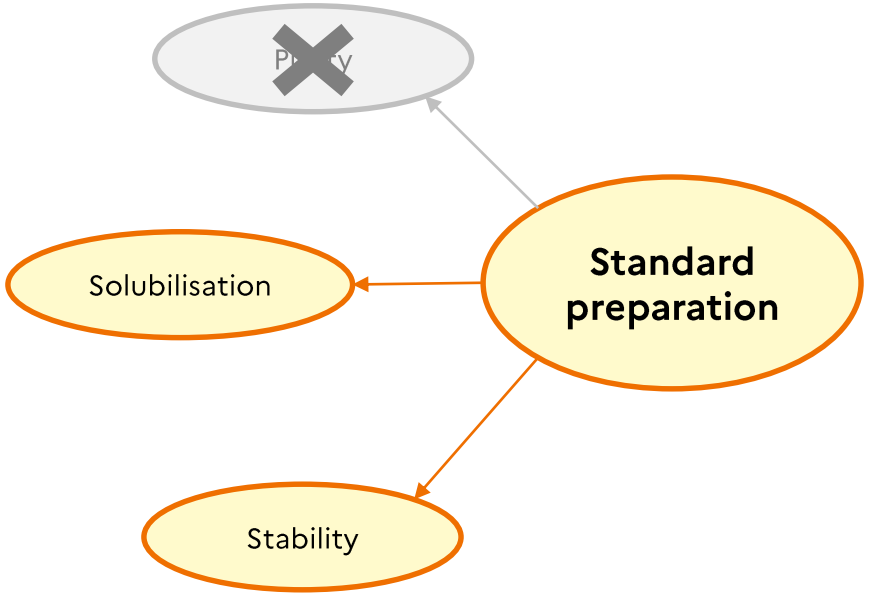
Column: Aqua C18, 2 x 150 mm, 3 µm, 125 Å
Flow rate: 0.4 mL/min
Eluent A : H_2O + 0.1% formic acid (FA)
Eluent B : Acetonitrile

Mode ESI +
Ion voltage : 4200 V
Source temperature : 450°C

Time (min)	%A	%B
0	60	40
1	60	40
6	52	48
8	20	80
10	20	80
12	60	40
15	60	40

Extract of brocoli supplemented at 10 ng g^{-1} of propineb and mancozeb, and 100 ng g^{-1} of thiram

Defining the main work leads…

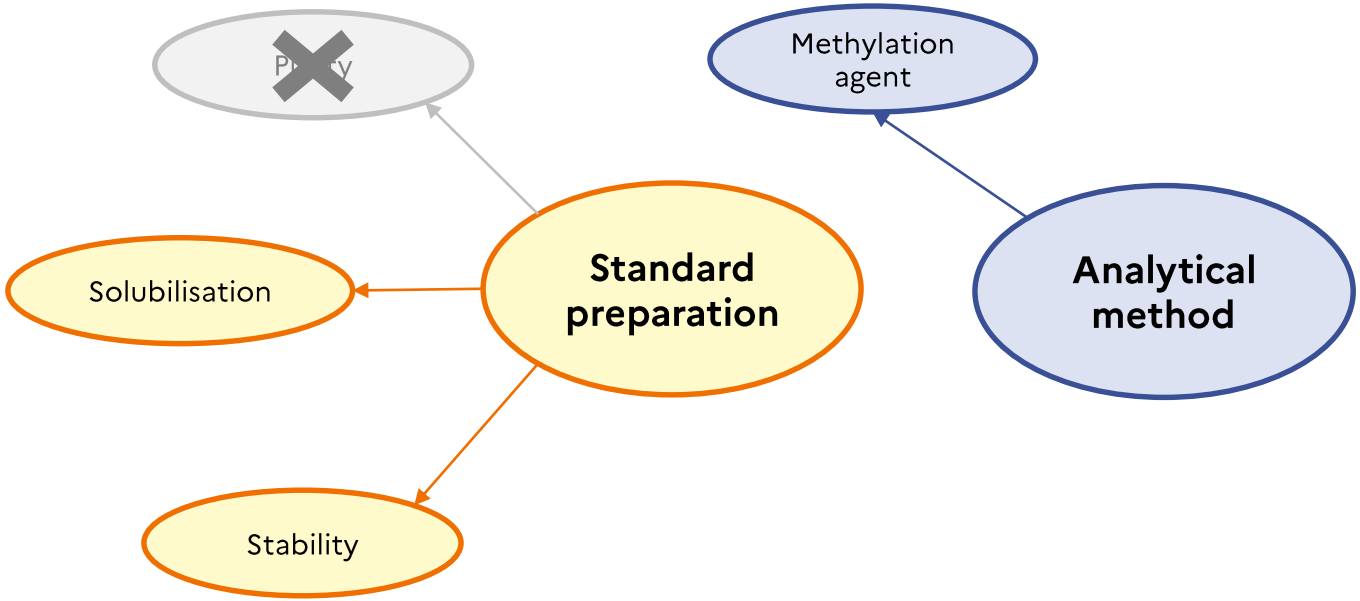


No LC-MS/MS difficulties identified

Analytical method

Methylation/extraction steps

Defining the main work leads…



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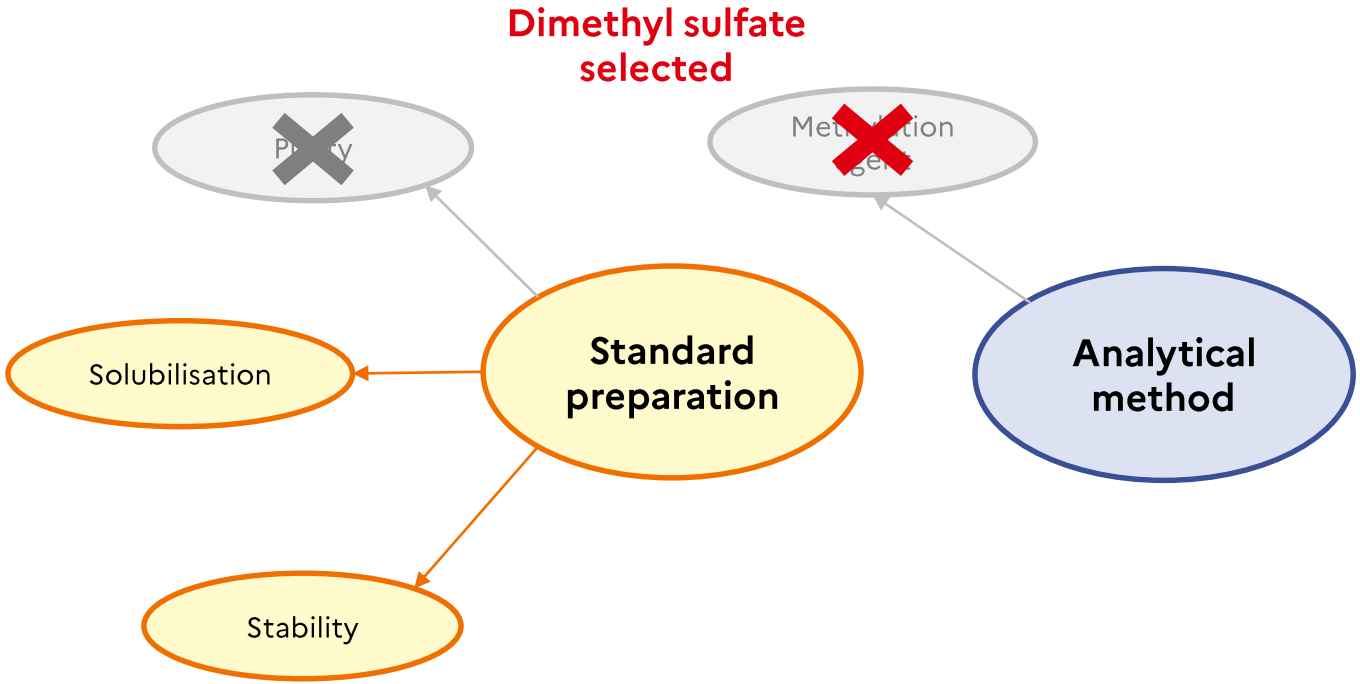

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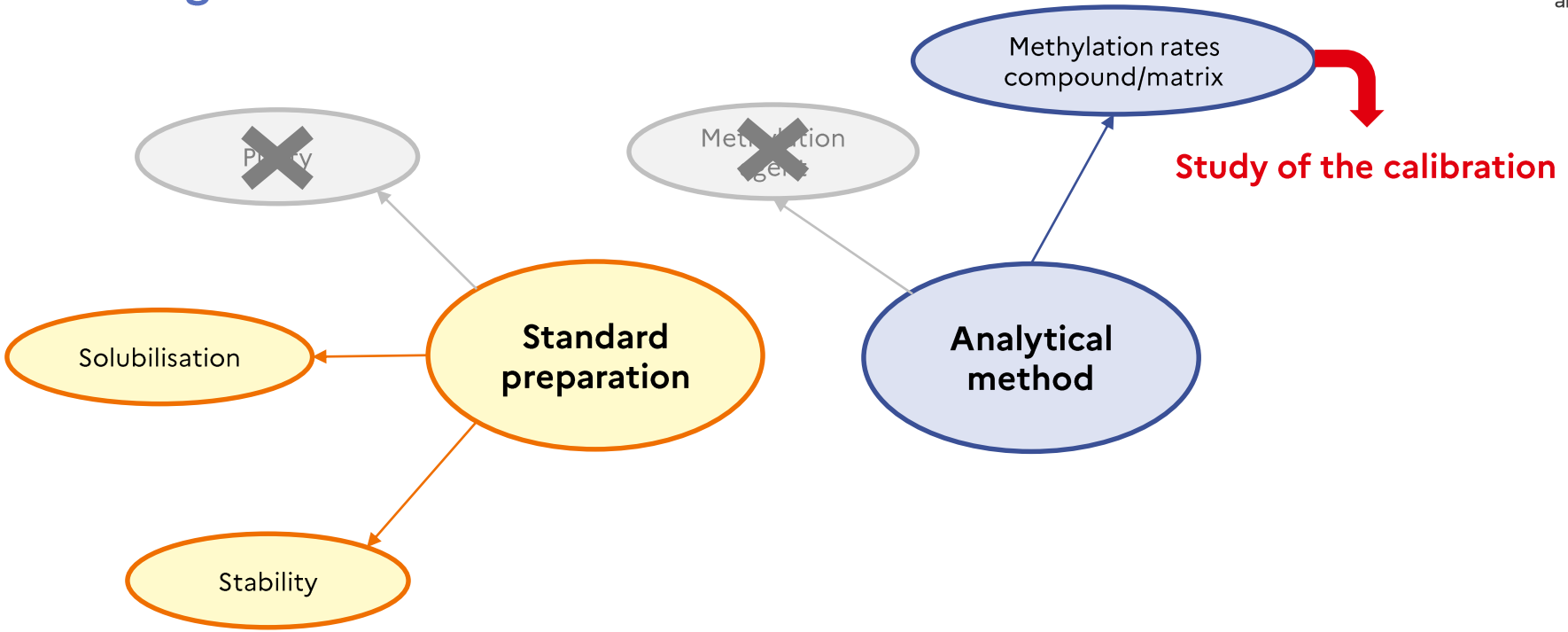


Calibration study

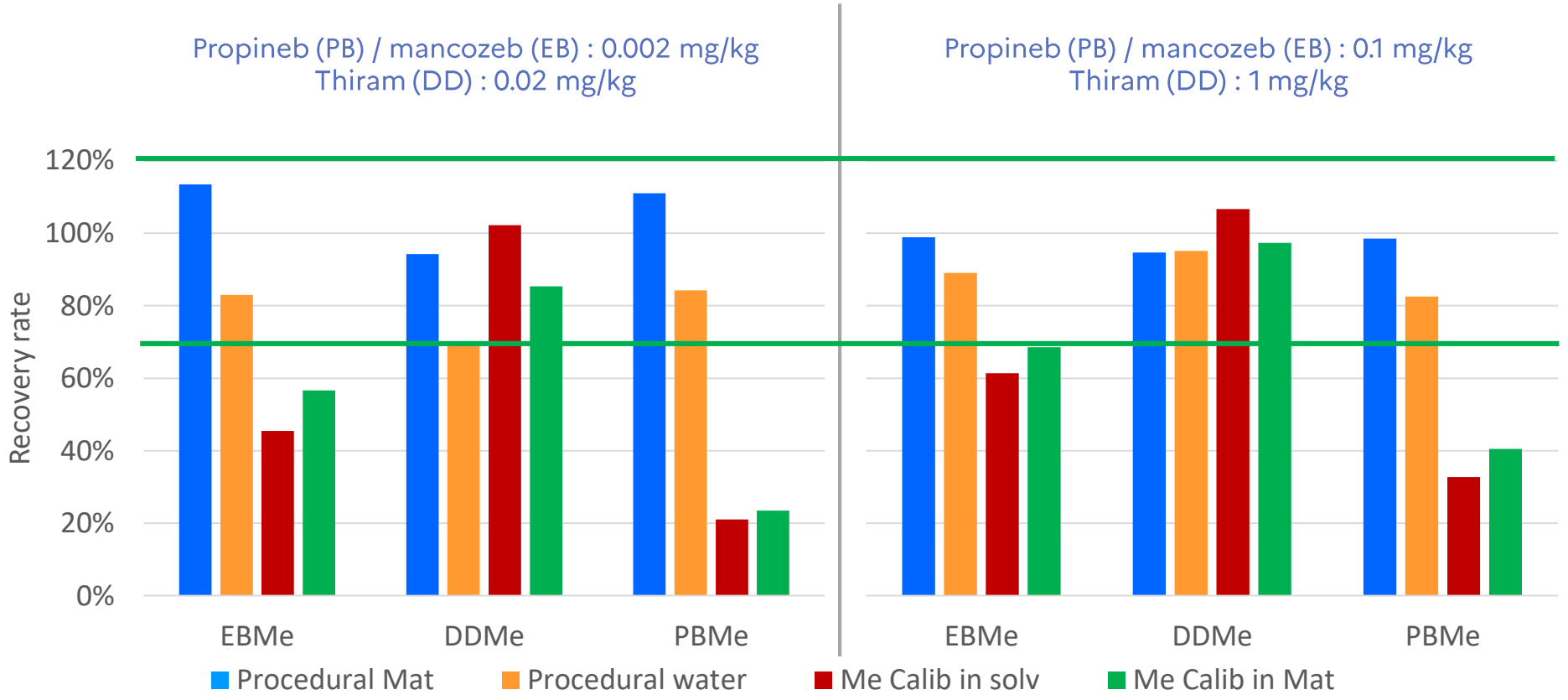
- Lower recovery rates observed on **broccoli** → selected matrix for the tests

- **4** calibration approaches:

  i) Procedural calibration on the matrix test portion (broccoli)
  ii) Procedural calibration on water (no matrix)
  iii) Solvent-based methylated compounds calibration (Acetonitrile + 0.1% FA)
  iv) Matrix-matched methylated compounds calibration (broccoli extract)

- Tested DTCs: **propineb, mancozeb, thiram**

Calibration study



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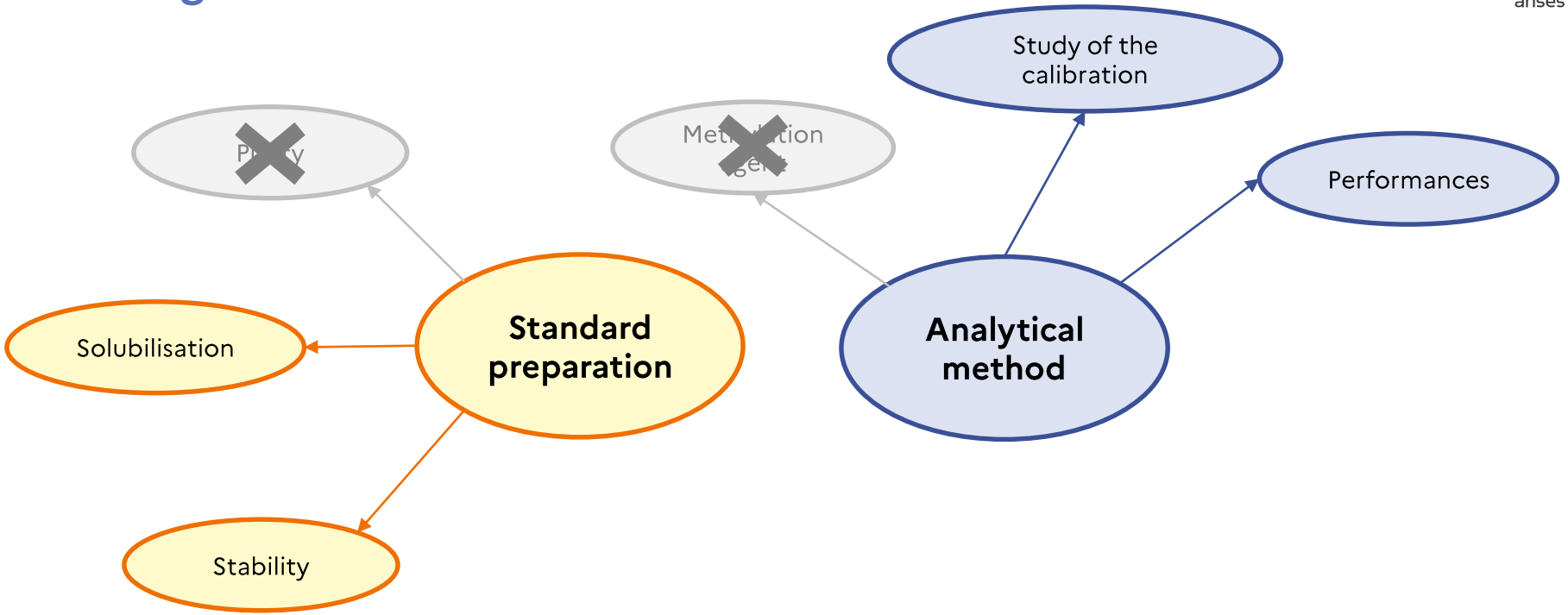


Method performances



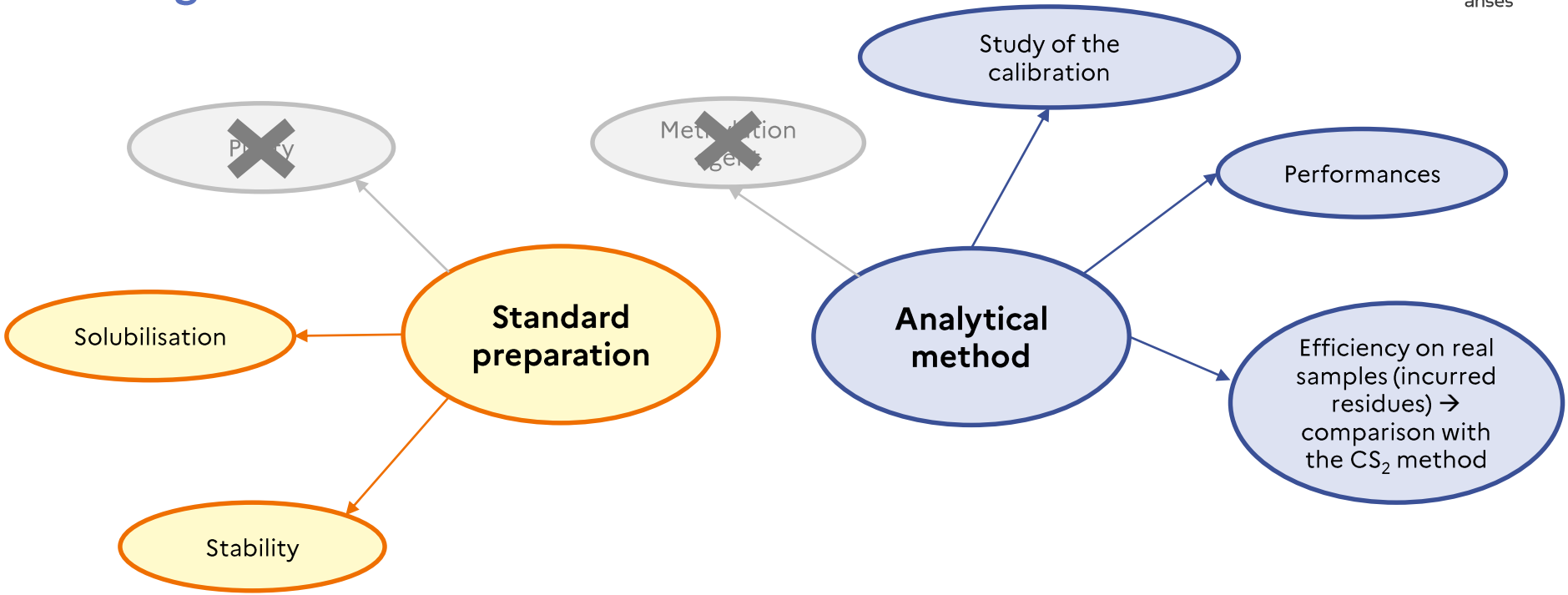

- Validation according to **SANTE/12682/2019 guideline**
- **Matrices**: tomato, onion, broccoli, salad, spinach, turnip, strawberry, kiwi
- 1 series per matrix; 3-5 replicates; 3 concentration levels (**0.002 - 0.8 mg/kg for EB/PB, and 0.006 - 1.2 for DD**)
- **Supplemented DTCs**: maneb (EB), propineb (PB) and ziram (DD)
- **Procedural calibration on water**




Compound	LOQ (mg/kg)	LOD (mg/kg)	Mean recovery	CV_r max	CV_R max	Measurement uncertainty
EB (expr. in CS_2)	0.002	0.0007	84 - 110%	8%	16%	48%
PB (expr. in CS_2)	0.002	0.0007	77 - 84%	10%	11%	32%
DD (expr. in CS_2)	0.006	0.002	81 - 105%	8%	13%	36%

Data obtained by Capinov

Defining the main work leads…



Methods comparison: methylation vs. CS_2

- **Sharing of samples** with CS_2 detected in **official controls**
- **Comparative analyses** performed by Capinov and GIRPA laboratories

  → both methods carried out **by the same lab at the same time**

Matrices with no known CS_2 background

Matrices	Conc. CS_2 (mg/kg)	Conc. methylation (mg/kg) [DTC class]	% Diff
Salads	0.198	0.195 [EB]	-2%
Salads	0.176	0.184 [EB]	5%
Lettuces	0.83	0.93 [EB]	12%
Lettuces	2.23	2.48 [EB]	11%
Baby leaves lettuce	0.71	0.70 [EB]	-1%
Baby leaves lettuce	0.068	0.046 [EB]	-32%
Baby leaves lettuce	0.089	0.113 [EB]	27%
Baby leaves spinach	0.070	< LOQ	-
Grape leaves	0.116	0.090 [EB]	-22%
Corn plants	14.6	14.8 [DD]	1%
Corn plants	0.43	0.50 [DD]	16%
Tomatoes	0.052	0.047 [EB]	-10%

Matrices with known CS_2 background

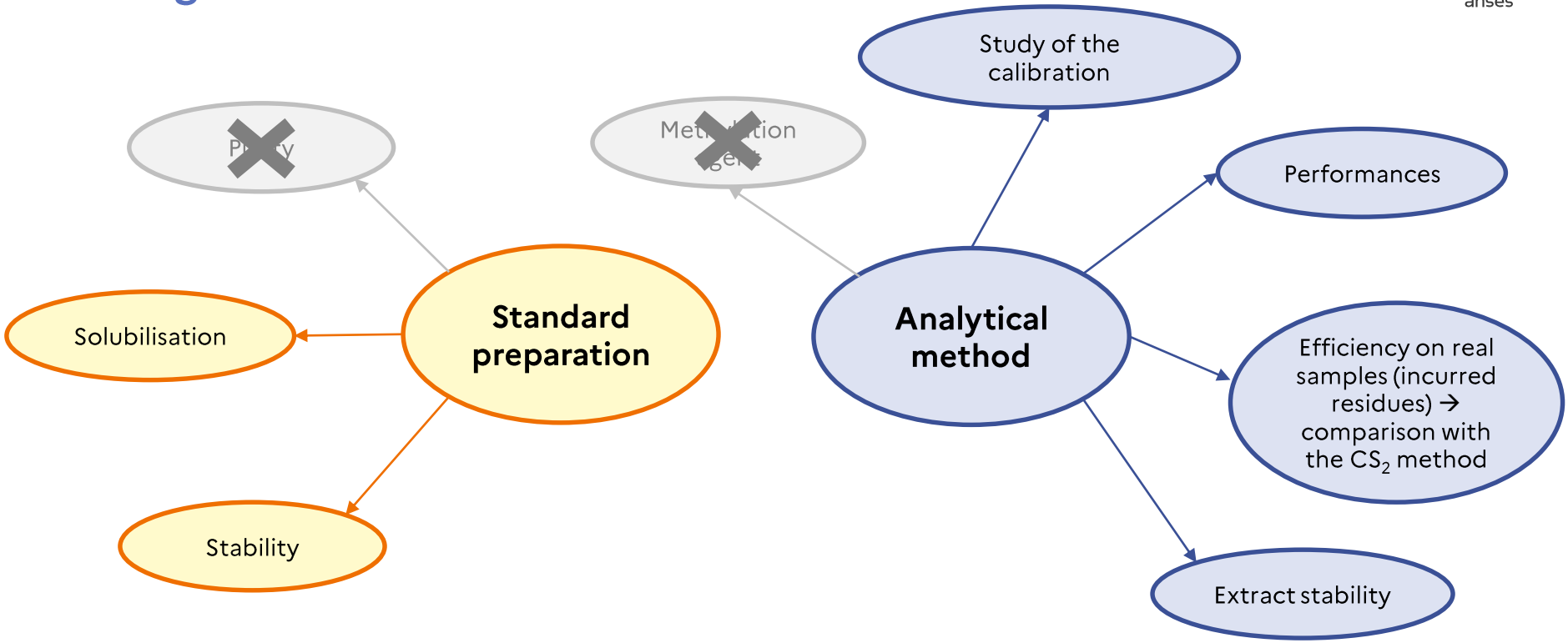
Matrices	Conc. CS_2 (mg/kg)	Conc. methylation (mg/kg) [DTC class]	% Diff
Shallots	0.153	0.143 [EB]	-7%
Onions	0.140	0.102 [EB]	-27%
Onions	0.320	0.282 [DD]	12%
Garlic	< 0.1	0.009 [DD]	-
Kales	0.57	< LOQ	-
Head cabbages	0.225	< LOQ	-
White cabbages	3.342	< LOQ	-
Red cabbages	5.705	< LOQ	-
Black radishes	1.3	< LOQ	-
Black radishes	0.64	< LOQ	-
Turnips	0.42	< LOQ	-
Rucola	8.845	< LOQ	-

Methods comparison: methylation vs. CS_2

- **PT analyses:** similar results and correct identification of the DTC class

Matrices	PT organizer [spiked DTC]	Conc. CS_2 (mg/kg)	Conc. methylation (mg CS_2/kg) [DTC class]	% Diff
Endive	BIPEA – 02/21 [thiram]	1.025	0.783 [DD]	-24%
Apple	BIPEA – 05/22 [thiram]	0.675	0.734 [DD]	9%
Salad	BIPEA – 06/21 [thiram]	0.530	0.648 [DD]	22%
Pear	BIPEA – 02/22 [thiram]	1.066	1.049 [DD]	-2%
Tomato	EUPT-SRM17 02/22 [metiram]	0.188	0.165 [EB]	-12%

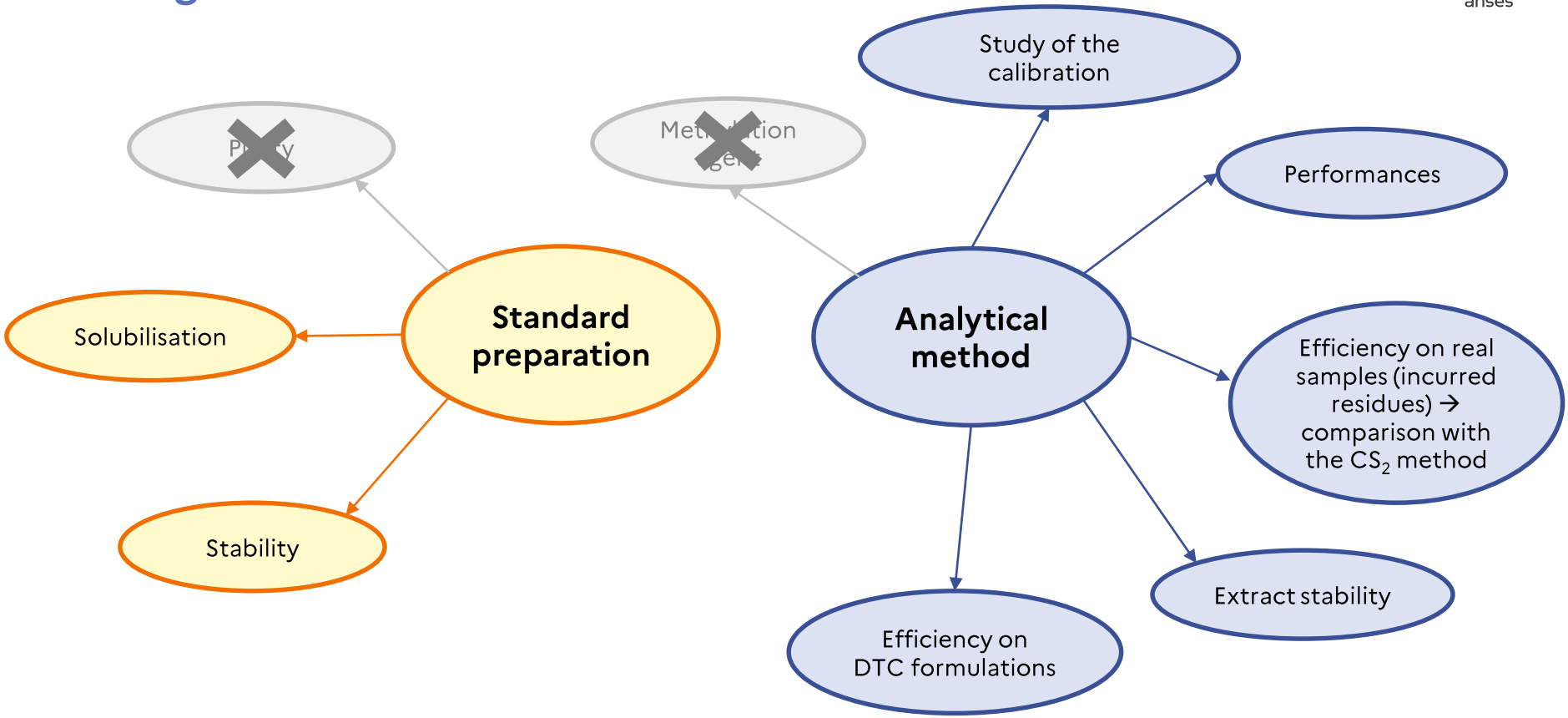
Defining the main work leads…



Extract stability

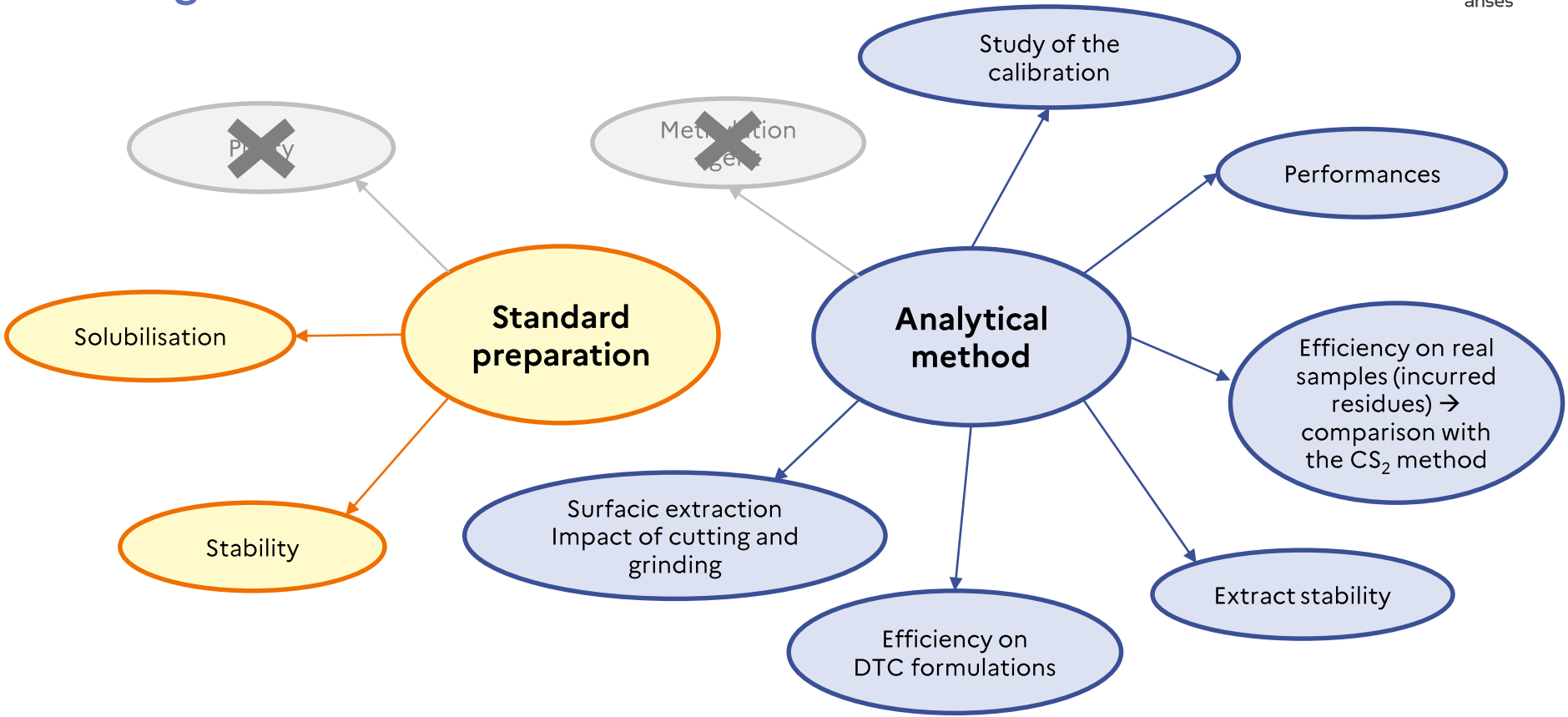
Compound	Tested spiked concentrations (mg/kg)	Period of stability (not tested beyond)	Matrix	Storage conditions
EBMe	0.003 – 0.006 – 1.2	3 months	Tomato	-18°C
PBMe	0.003 – 0.006 – 1.2			
DDMe	0.010 – 0.2 – 2			

Defining the main work leads…



To be done…

Defining the main work leads…



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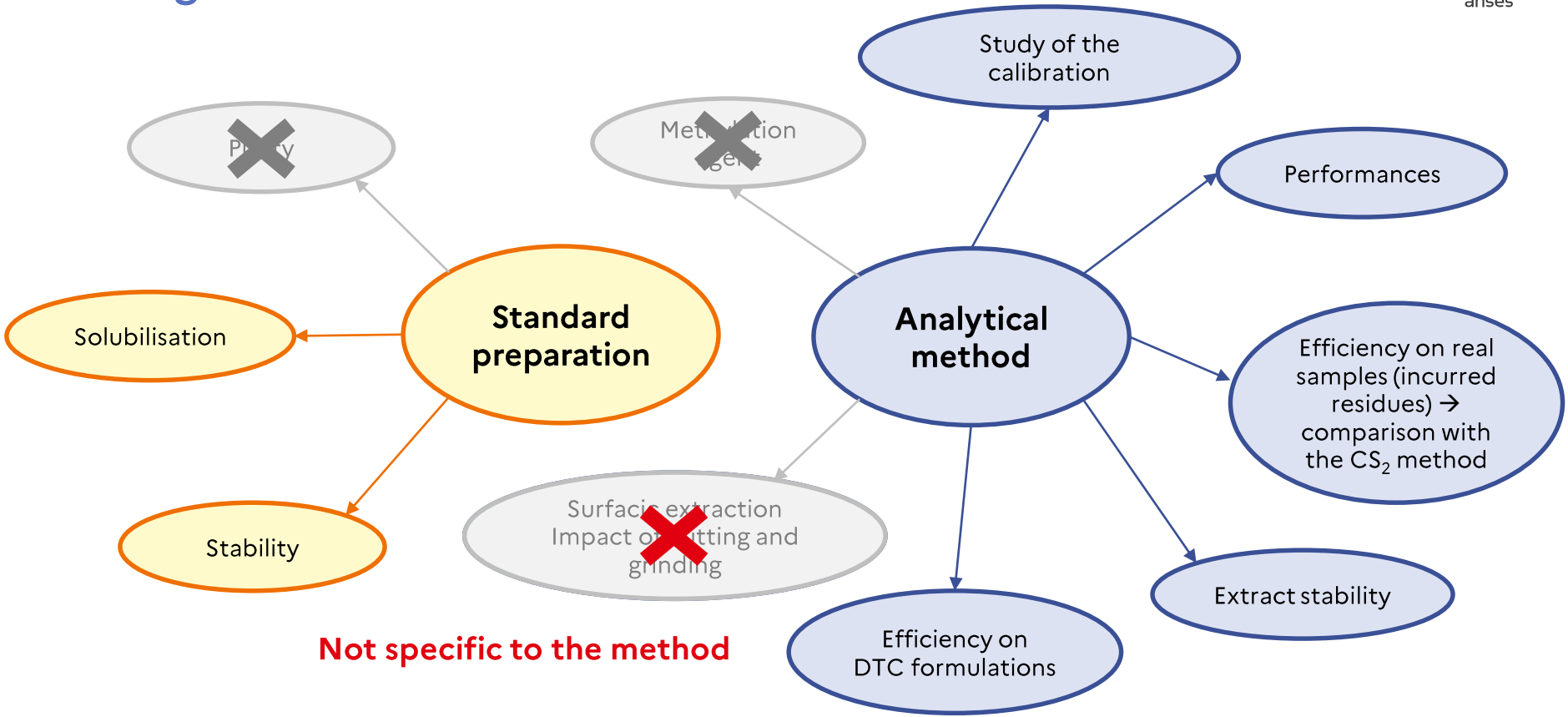

Conclusion and perspectives

- A **fruitful and dynamic** working group
- A **promising** analytical method for DTCs quantification:
  - **Selective**
  - **Specific**
  - **Consistent** with CS_2 results
  - May be suitable for **routine** analyses: simple protocol, procedural calibration on water

Can be used in combination with other approaches on problematic matrices

→ may be useful for supporting the MRL revision process

… but still requires some work to **assess efficiency**

- Test **other individual DTCs (« cross » calibrations)** *(in progress)*
- Test **other relevant matrices**
- Test commercial DTC **formulations**

Acknowledgements

Thanks to Hubert Zipper for valuable discussions

Thank you for your attention