QEL, Spices Board

In-house Training – 1:
Analysis of Pesticide Residues

Lecture - 4
Introduction Modern Pesticide Residue Analysis: Part II
24-Jul-23

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Spices Board

Moving on

From last lectures…

What we have already covered:

- General principles of pesticide residue analsys
  - Instrumentation for PRA: LC-MS/MS and GC-MS/MS
  - QuEChERS, chemistry and practice
  - General method development scheme for PRA
  - pH dependency of QuEChERS
  - Origin of matrix effects in GC-MS/MS and LC-MS/MS
  - Matrix effects in spices during PRA

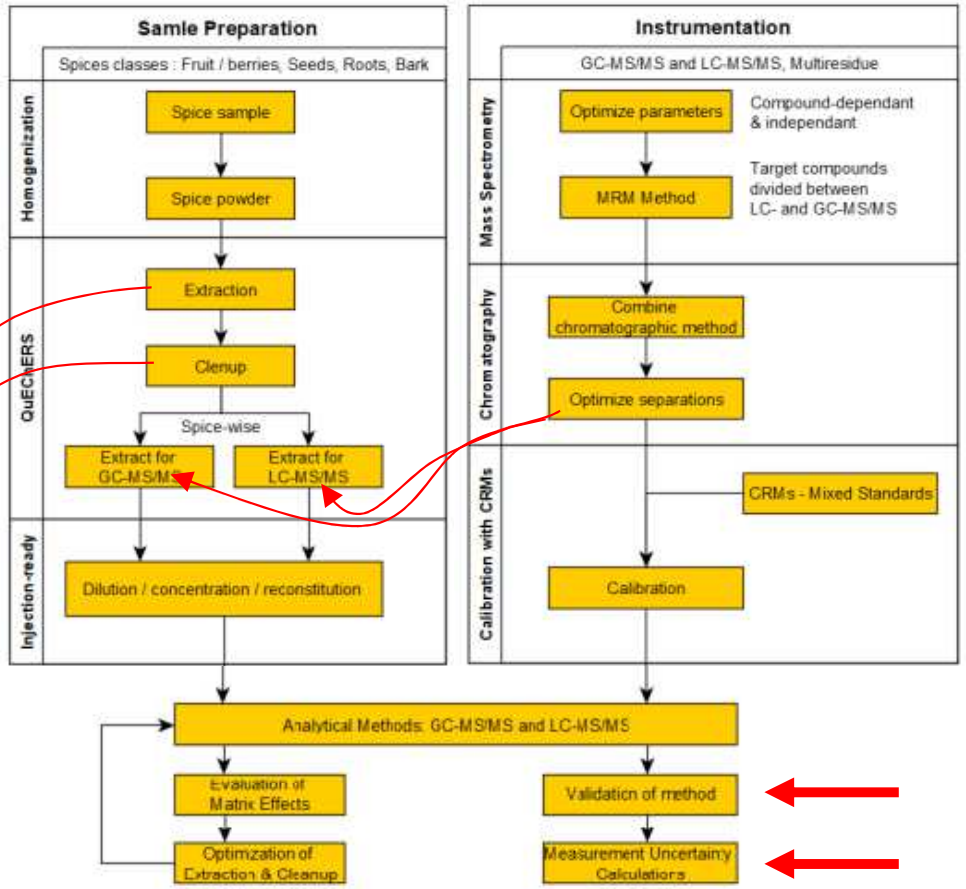
What we will cover today:

- Modern adaptations of QuEChERS
- Optimization of QuEChERS method for different matrices in LC-MS/MS and GC-MS/MS
- CRM Management
- Use of analyte protectants to mitigate matrix effects in GC-MS/MS analysis
- Single residue methods:
  - Dithiocarbamate analysis using GC-MS
  - Ethylene oxide analysis using GC-MS/MS

Method development for PRA

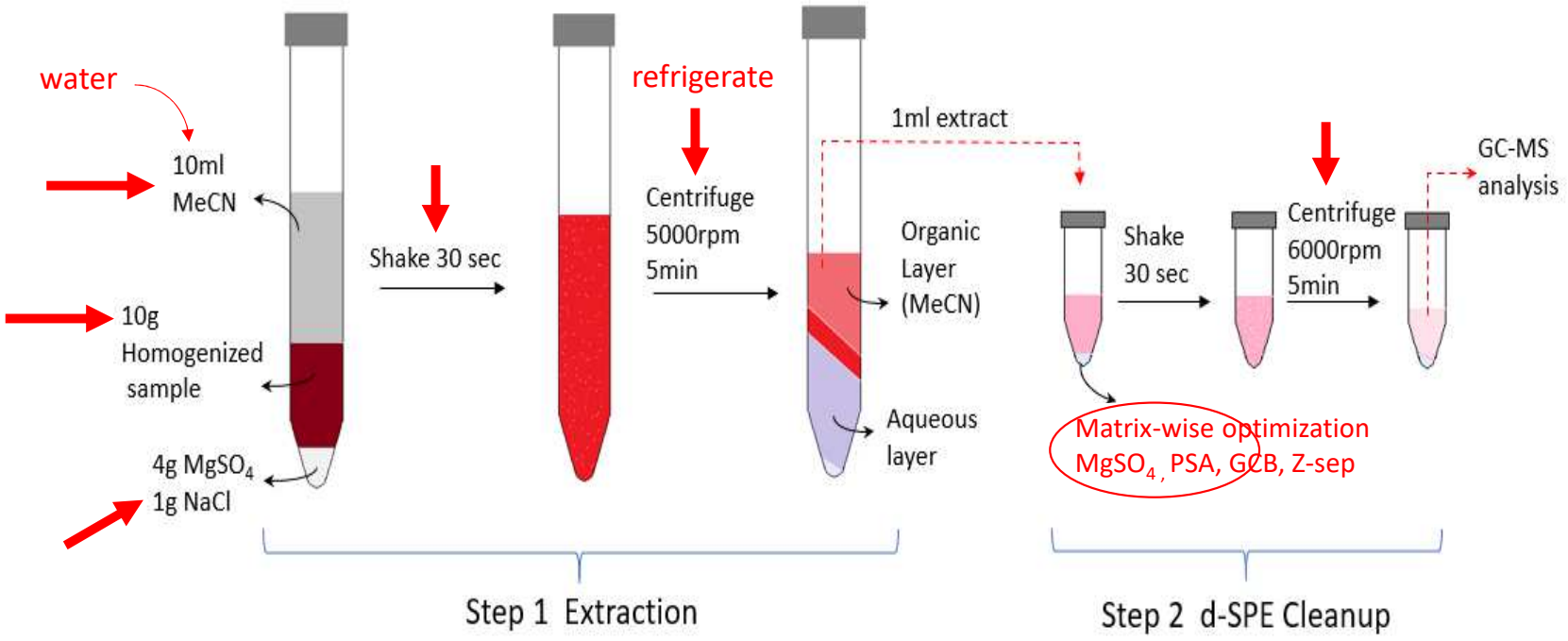
A quick review…

QuEChERS



QuEChERS

A quick review…

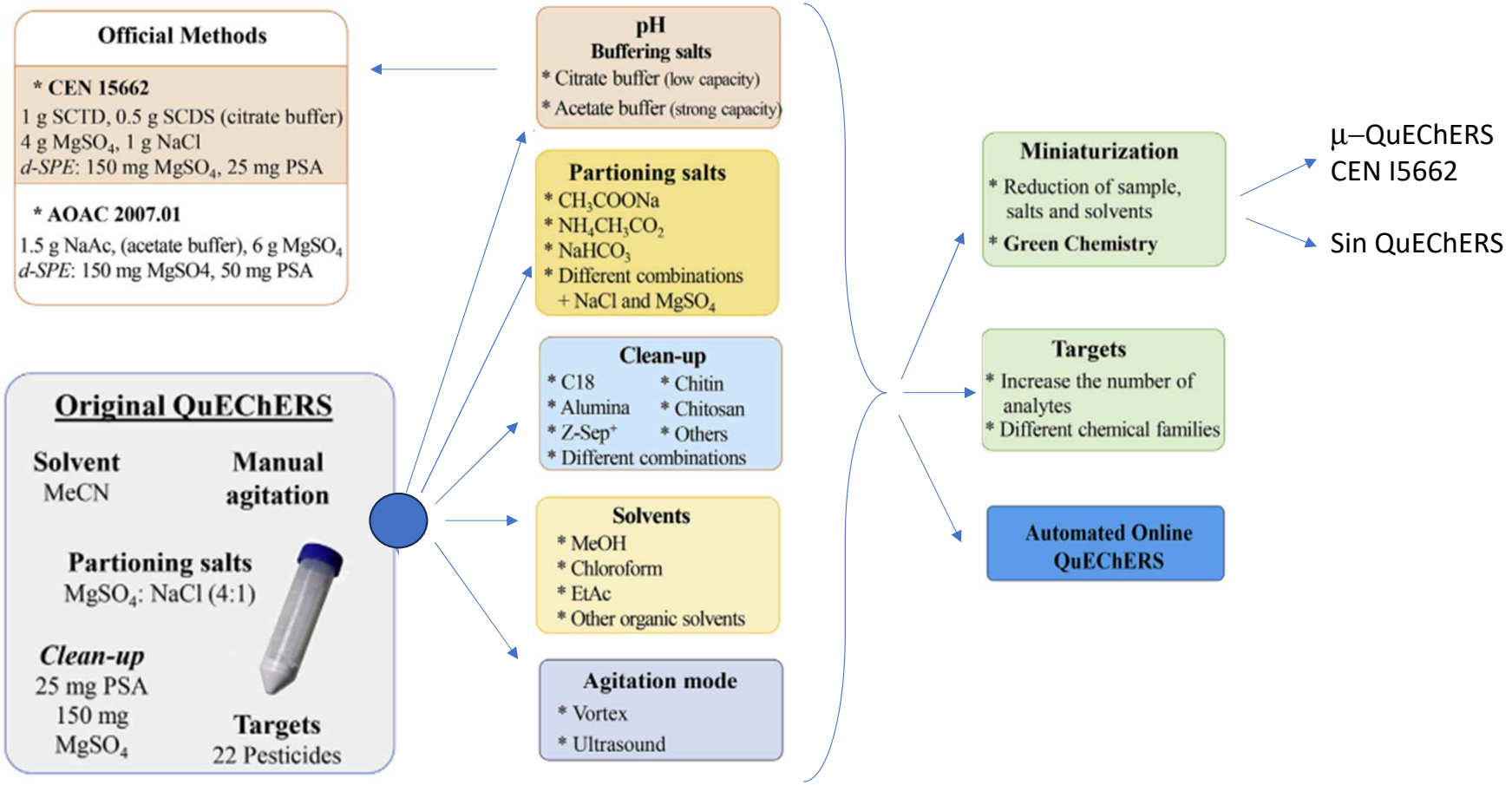


QuEChERS

§ New adaptations

New QuEChERS chemicals

Modern adaptations



QuEChERS chemicals

New adaptations for cleanup

‘Classical’ QuEChERS chemicals

- ↳ PSA - Removes fatty acids, sugars, organic acids, lipids and some pigments
- ↳ C18 - Removes high lipid contents
- ↳ GCB - Removes co-extracted pigments, viz. carotenoids and chlorophyll from highly pigmented matrices (recovery loss in planar pesticides)

Branded Products

Brand	Product	Chemical	Useful for
Agilent	EMR-lipid	proprietary	Enhanced lipid removal (needs added water to activate)
	CarbonX	"	Enhanced chorophyll removal, replacement for GCB. Problems with planar pesticides (HCH)
	ChloroFiltr	"	Enhanced chorophyll removal, replacement for GCB. OK for planar pesticides.
Waters	Z-sep Z-sep+	ZrO_2 and SiO_2 with bound C-18	Removes fats and pigments more efficiently than traditional PSA,

Alumina, florisil®, chitosan and diatomaceous earth: pesticide residues in rice, Cabrera et al. 2016

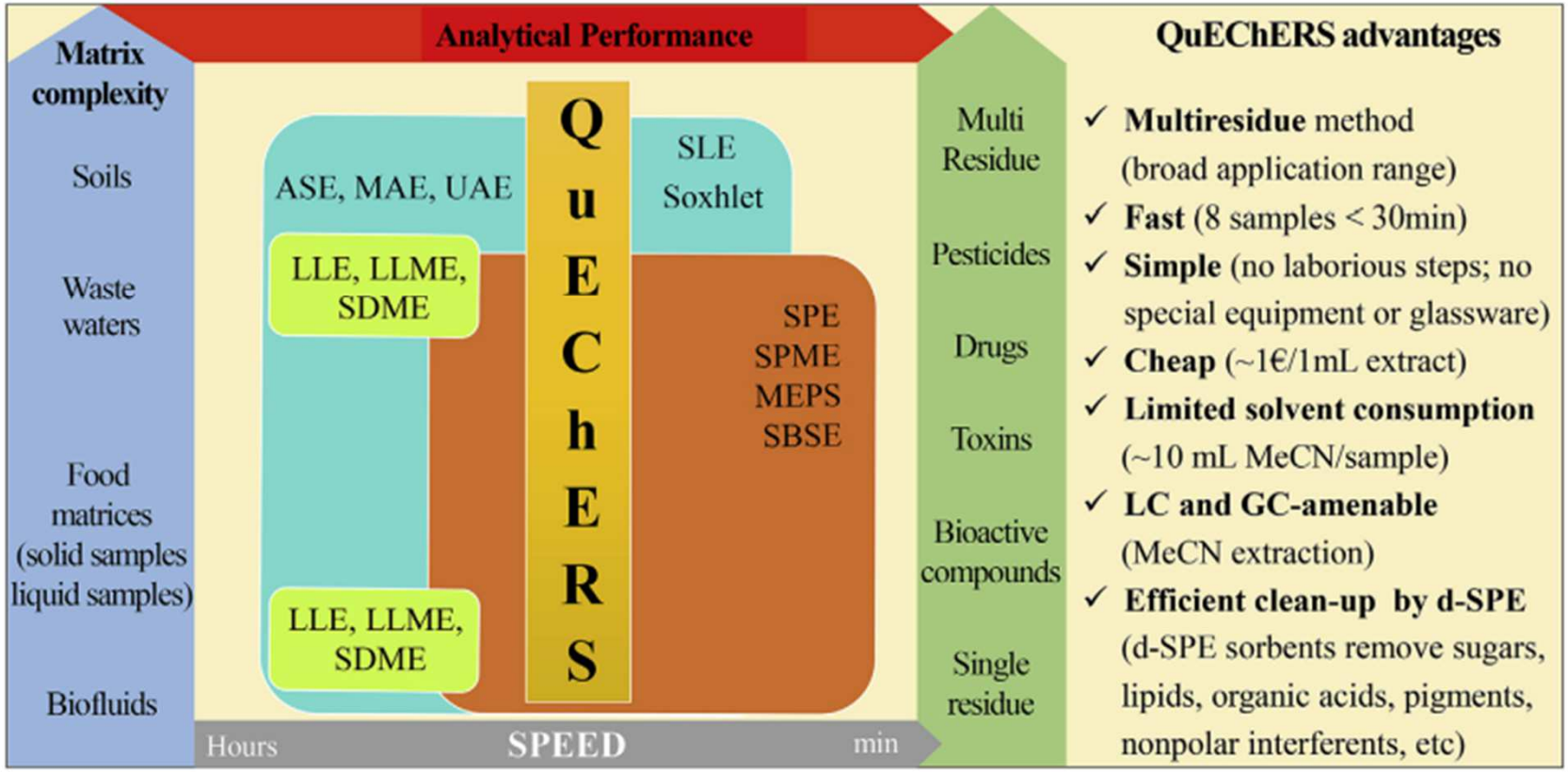
Chitosan as an economic alternative to the C18 sorbent for PRA in milk, Arias et. al. 2018

Optimization in matrix is required!!

QuEChERS

Summing up

Perestrelo *et. al.* 2019



Overview of QuEChERS features and advantages over other extraction methods. Abbreviation: ASE - accelerated solvent extraction, d-SPE - dispersive solid-phase extraction, ıs chromatography, LC - liquid chromatography, LLE - liquid-liquid extraction, LLME - liquid-liquid microextraction, MAE - microwave-assisted extraction, MeCN – aceto- MEPS – microextraction in packed sorbent, SBSE - stir bar sorptive extraction, SDME, SLE - solid-liquid extraction, SPE - solid-phase extraction, Soxhlet – soxhlet extraction, SPME - solid-phase microextraction, USE - ultrasonic solvent extraction.

Method optimization

§ Theory and practice

Optimization vs Validation

Same or different?

Optimization

- A method is being developed…
  - Many variables (steps) are involved (from sample weight to combination of QuEChERS chemicals)
  - We have to choose a combination of variables that give best performance for a matrix
  - How to arrive at the optimum values for different variables?
  - "Multivariate function with a set of constraints" – Chemometrics

Validation

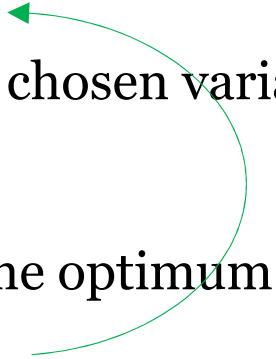
- A method has been optimized. Is it fit for our purpose?
  - Many variables are involved (from sample weight to combination of QuEChERS chemicals)
  - Validation parameters are calculated for the optimized method
  - Compared with standard validation requirements. Pass / fail?
  - If not passed, optimize method further.

Optimization

How?

In Practice:

- Choose one important 'constraint' – e.g. recovery should be minimum 70% with a precision RSD of <10%, n = 5.
- Fix the variable (e.g. sample weight) for the experiment
- Leave the other variables at some base values obtained from literature (e.g. Anastassiades *et. al* 2003, original QueChERS)
- Run the experiment with different values of the chosen variable (e.g sample weight = 1 g, 3 g, 5 g…)
- Calculate the average recovery and precision.
- Fix the sample weight that gave best values as the optimum value.
- Choose the next variable (e.g. moisture added)



Validation

How?

Use the Optimized method to calculate the validation parameters, and compare with a standard requirements

Parameter	Measured as	Performance criterion
Linearity	From a calibration curve of 5 levels, deviation of calculated concentration from true concentration	≤ ± 20 %
Recovery	Average recovery of each spike level analysed, with $n \geq 5$	70-120 %
Repeatability Precision (RSD_r)	Relative standard deviation of each spike level analysed (same analyst, same day, $n \geq 5$)	≤ 20 %
Within-laboratory reproducibility precision (RSD_R)	Relative standard deviation of 3 replicates of each spike level performed on 3 non-consecutive days (different analysts, $n = 9$).	≤ 20 %
Specificity	Response in reagent blank and blank control samples in the same MRM and at the same retention time as the analyte.	≤ 30 % of LOQ
Ruggedness	Relative standard deviation for results obtained from five combinations of three parameters chosen as variables in the optimized method	≤ 20 %
Ion ratio	Quantifier: qualifier ratio in the sample matrix as compared to average of the ion ratios of calibration standards in the same batch	±30%
Retention time (min)	For the quantifying MRM transition, the retention time of the peak in the sample chromatogram as compared to the peak in the standard chromatogram	± 0.1

Method validation is required to ensure that the analytical method is fit for its intended purpose (e.g., assessing compliance of a sample against regulatory limits).

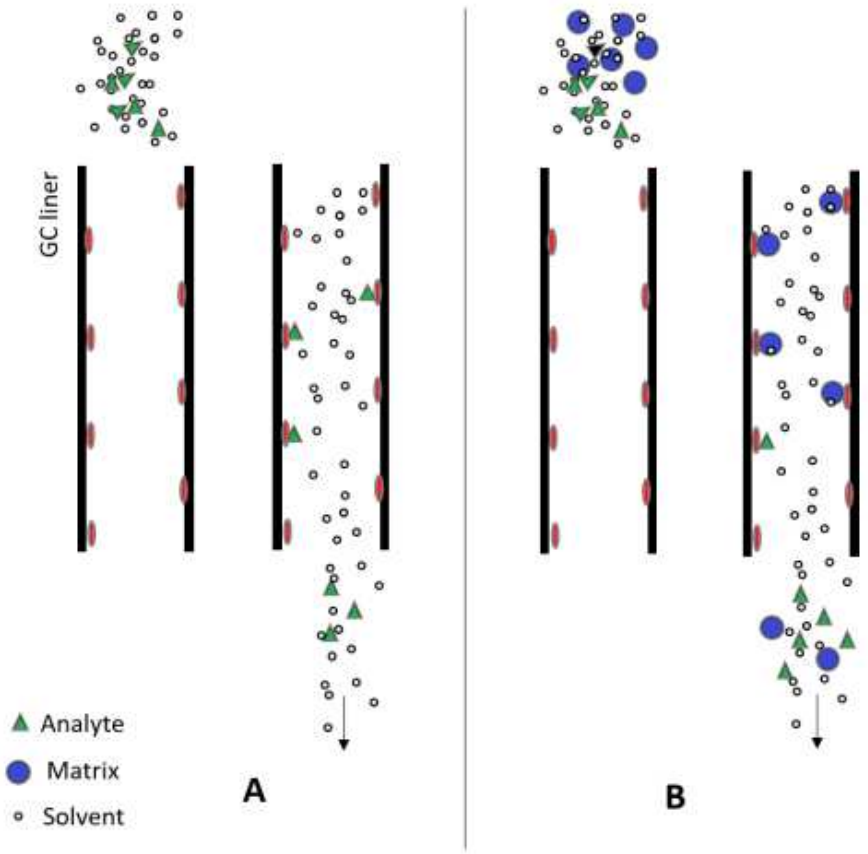
Method validation was conducted as per the internationally accepted EU SANTE 12682/2019 document

Analyte Protectants

§ GC-MS/MS

Origin of Matrix Effect in GC-MS/MS

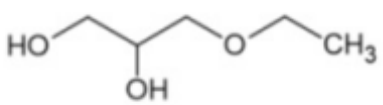
A recap



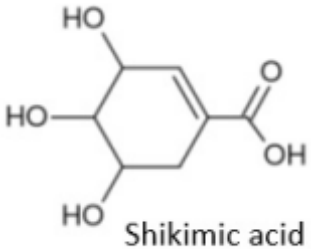
A – Injection of the analyte in solvent, **B** – injection of analyte in a matrix containing interfering coextractives

Analyte Protectants

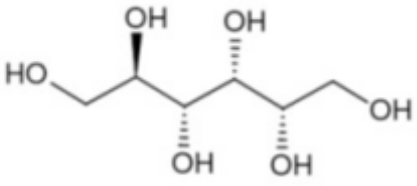
What?



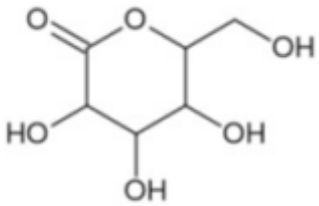
Ethyl glycerol



Shikimic acid



Sorbitol



Gluconolactone

Analyte protectants (AP) are compounds containing multiple hydroxyl groups

When added to solvent-based calibration standards in concentrations much higher than analyte concentrations, AP can mimic the matrix

AP get preferentially adsorbed on the active sites of the injection system inGC, thereby 'protecting' the analytes, and improving response

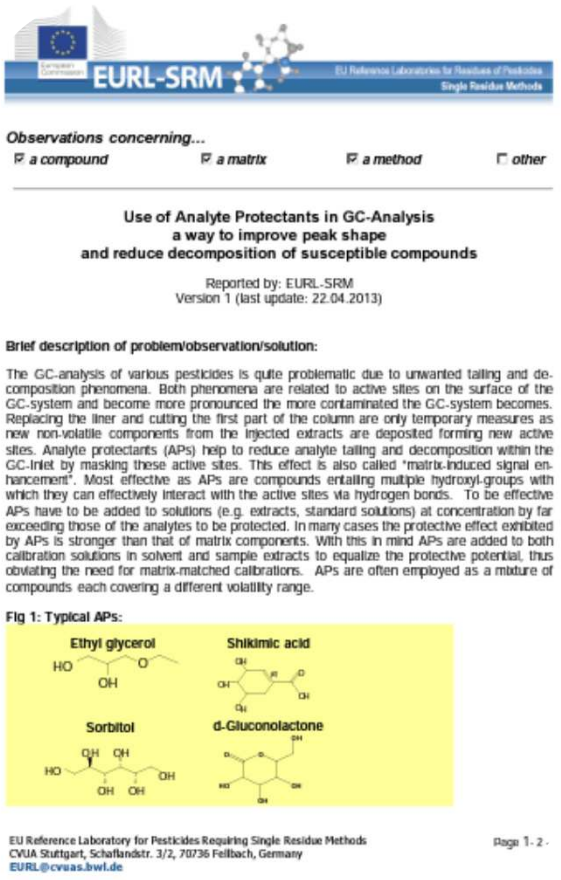
AP should be present at much higher concentrations than the residue concentration expected in the sample

Analyte Protectants

how?

Preparation of AP:
2g of ethylene glycerol,
2ml of gluconolactone,
1 ml each of 50 mg/ml sorbitol and shikimic acid,
diluted to 10 ml
with 60:40 acetonitrile water mixture.

Effective concentrations:
~ 5% each of ethylene glycol and gluoconolactone,
5 mg/ml each of sorbitol and shikimic acid.



EURL-SRM

EU Reference Laboratories for Residues of Pesticides
Single Residue Methods

Observations concerning...

☑ *a compound* ☑ *a matrix* ☑ *a method* ☐ *other*

Use of Analyte Protectants in GC-Analysis
a way to improve peak shape
and reduce decomposition of susceptible compounds

Reported by: EURL-SRM
Version 1 (last update: 22.04.2013)

Brief description of problem/observation/solution:

The GC-analysis of various pesticides is quite problematic due to unwanted tailing and decomposition phenomena. Both phenomena are related to active sites on the surface of the GC-system and become more pronounced the more contaminated the GC-system becomes. Replacing the liner and cutting the first part of the column are only temporary measures as new non-volatile components from the injected extracts are deposited forming new active sites. Analyte protectants (APs) help to reduce analyte tailing and decomposition within the GC-inlet by masking these active sites. This effect is also called "matrix-induced signal enhancement". Most effective as APs are compounds entailing multiple hydroxyl-groups with which they can effectively interact with the active sites via hydrogen bonds. To be effective APs have to be added to solutions (e.g. extracts, standard solutions) at concentration by far exceeding those of the analytes to be protected. In many cases the protective effect exhibited by APs is stronger than that of matrix components. With this in mind APs are added to both calibration solutions in solvent and sample extracts to equalize the protective potential, thus obviating the need for matrix-matched calibrations. APs are often employed as a mixture of compounds each covering a different volatility range.

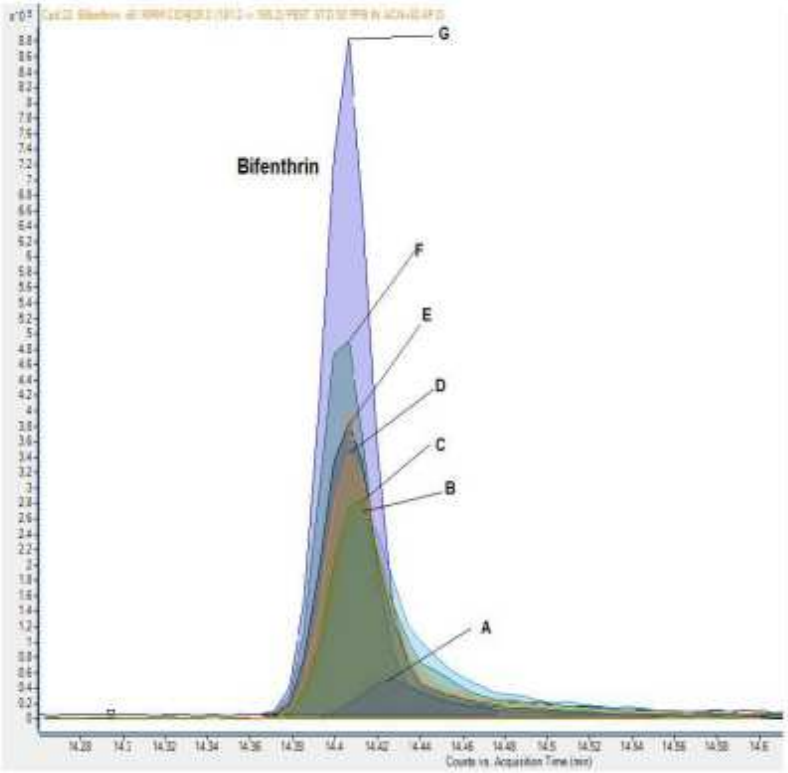
Fig 1: Typical APs:

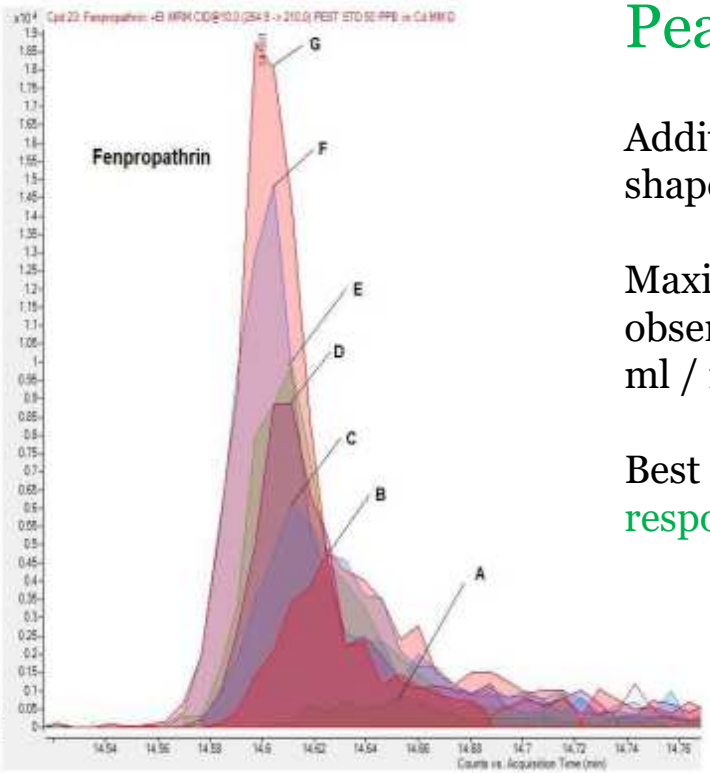
Ethyl glycerol
Shikimic acid
Sorbitol
d-Gluconolactone

Google “EU RL analyte protectants”

Analyte Protectants

Effective?





Analyte concentration 50 µg/kg
A = in solvent, G = in matrix extract
B, C, D, E, F = addition of 10, 20, 30, 50 and 100 µl AP mix

Peak shapes

Addition of AP improves peak shape considerably

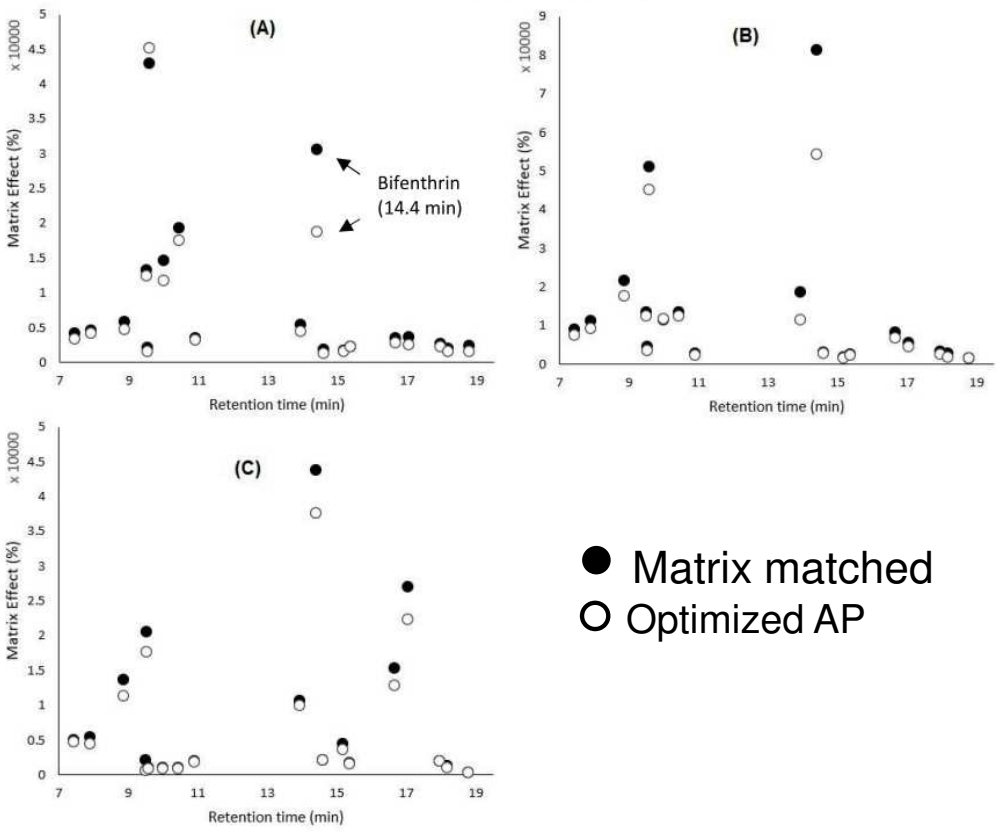
Maximum improvement was observed for an addition of 100 ml / ml extract

Best in the case of low-medium response compounds

Spices Board Data

Analyte Protectants

Effective?



Spices Board Data

Quantitation

Using optimized amunt of AP / ml extract effectively cancelled the matrix effect in spices

Less effective in high-response compounds which showed high matrix enhancement

Analyte Protectants

Precautions

Use AP only in GC-MS/MS analysis, not in LC

Syringe contamination

- AP dissolves best in water or other polar solvents
- It is important to rinse the syringe thoroughly with water or a water-containing polar solvent mix after each injection (set rinse in injection method)
- After 100 injections, remove syringe and clean plunger with water.

Check for interferences

- When using MS full-scan mode, high concentrations of AP may mask the identification of pesticides.

Dithiocarbamates

§ Analysis by GC-MS

Single Residue Analysis (1)

Dithiocarbamates

Application and analysis

Dithiocarbamates (DTC) are broad-spectrum fungicides extensively used in spices

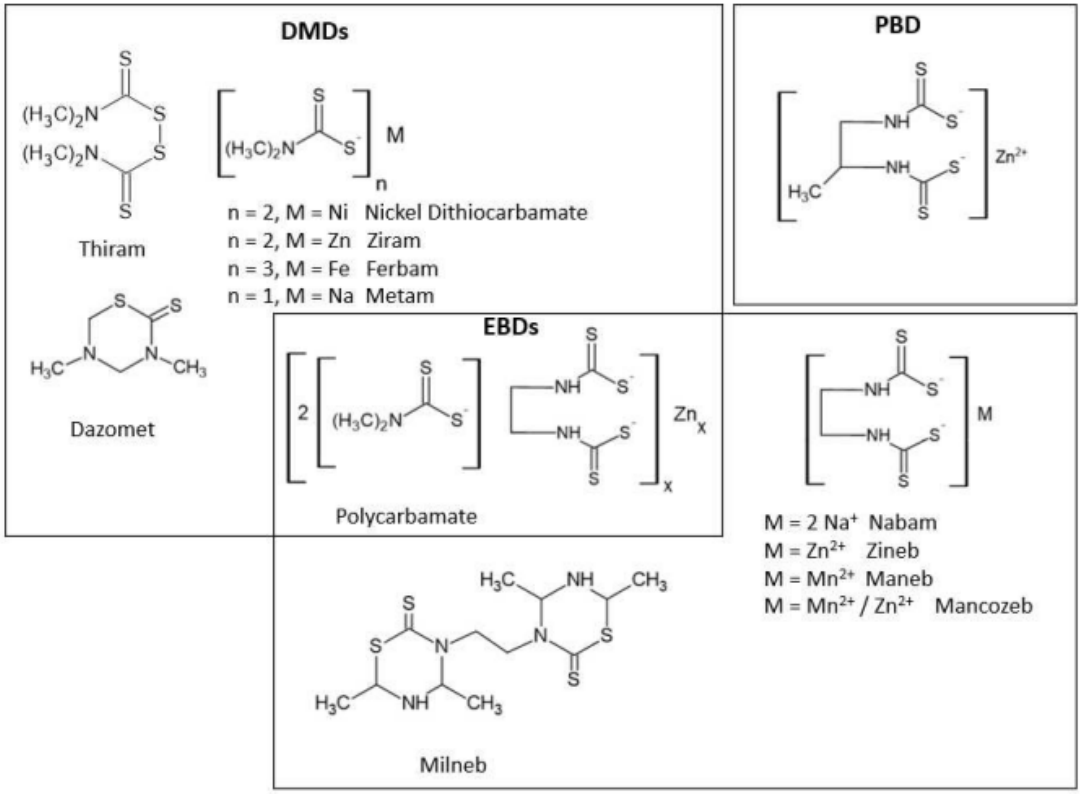
- This class of compounds have complexed metal ions and thus usual sample preparation methods cannot be used
- Important in the case of Cardamom and black pepper (EU and Saudi Arabia have strict limits)

Analysis

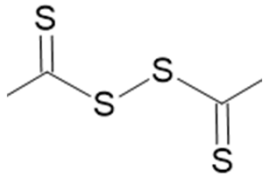
- Sample preparation: DTC in extract is hydrolysed to quantitatively produce CS_2 which is absorbed in isooctane and analysed
- Instrumentation: GC-MS in selected ion monitoring (SIM) mode

Dithiocarbamates

Chemical structures



Conventional methods can't be used because of low solubility of the compounds in organic solvents

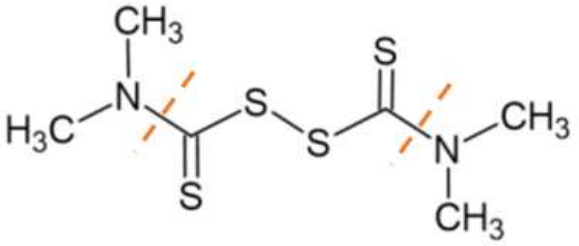


The compounds are hydrolysed to form CS_2 quantitatively which was absorbed into isooctane and analysed in GCMS

Dithiocarbamates

Analysis

- Presence of metal ion in DTC compounds (except in thiram and dazomet) makes conventional extraction analysis difficult
- A mixture of $SnCl_2$ and HCl is used to cleave the DTC compound and produce CS_2
- This CS_2 is absorbed quantitatively in isooctane and analysed in GC-MS



Thiram has to be used in spiking studies, as it is the only water soluble DTC compound.

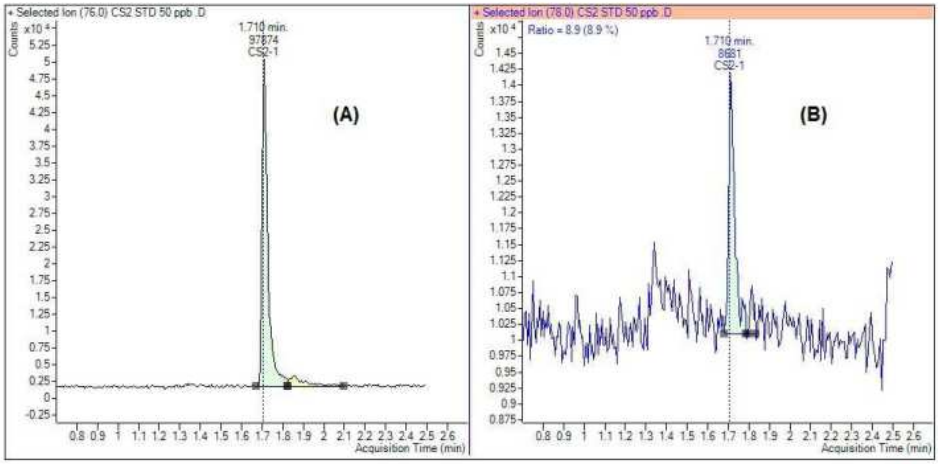
1 mole of thiram = 2 mols of CS_2
Purity of thiram also need to be accounted for

Dithiocarbamates

instrumentation

In selected ion monitoring (SIM) mode,

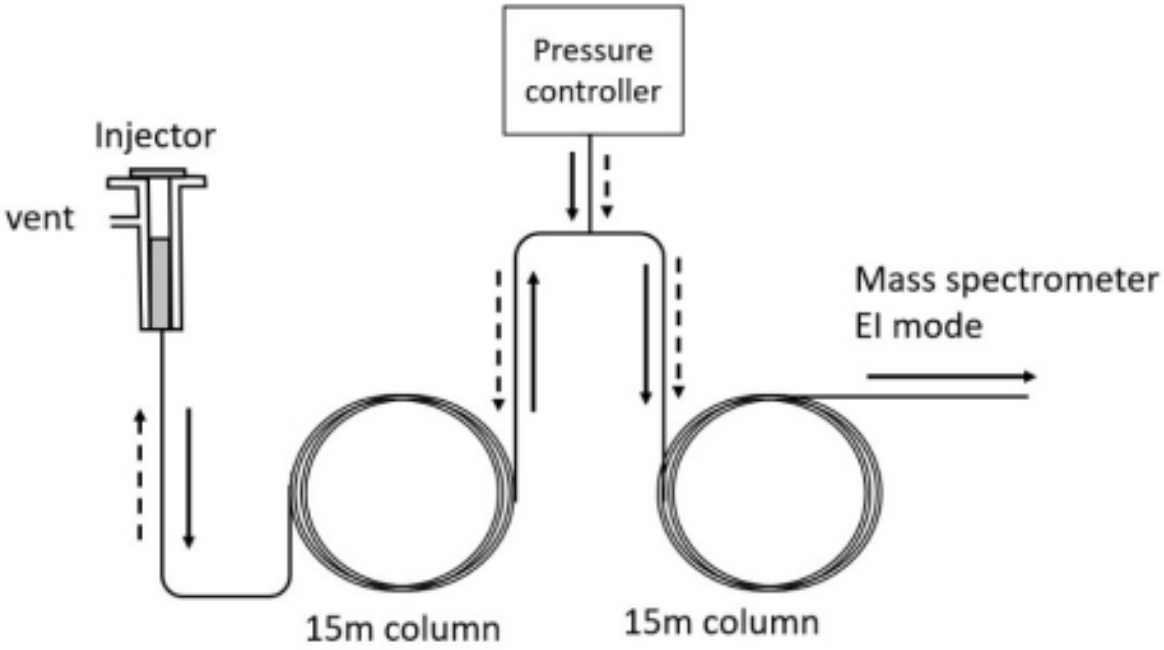
- Ion with *m/z* = 76 was used for quantitation
- Ion with *m/z* = *78*, with intensity of about 9% of the quantifier ion, was used for qualification



(A) m/z = 76, **(B)** m/z = 78, at LOQ concentration of 0.05 mg kg^{-1}

Dithiocarbamates

GC method



Mid-column backflush in GCMS improves precision considerably in DTC analysis

Ethylene Oxide

§ Analysis by GC-MS/MS

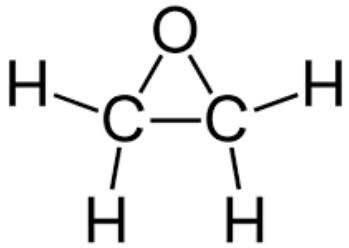
Single Residue Analysis (2)

Ethylene Oxide (ETO)

Why?

Ethylene oxide is a microbial control agent (fumigant) and sterilizing agent

- ↳ Used in spices in India, very effective for control of salmonella



Reported to be a carcinogen in studies conducted in European Union and USA

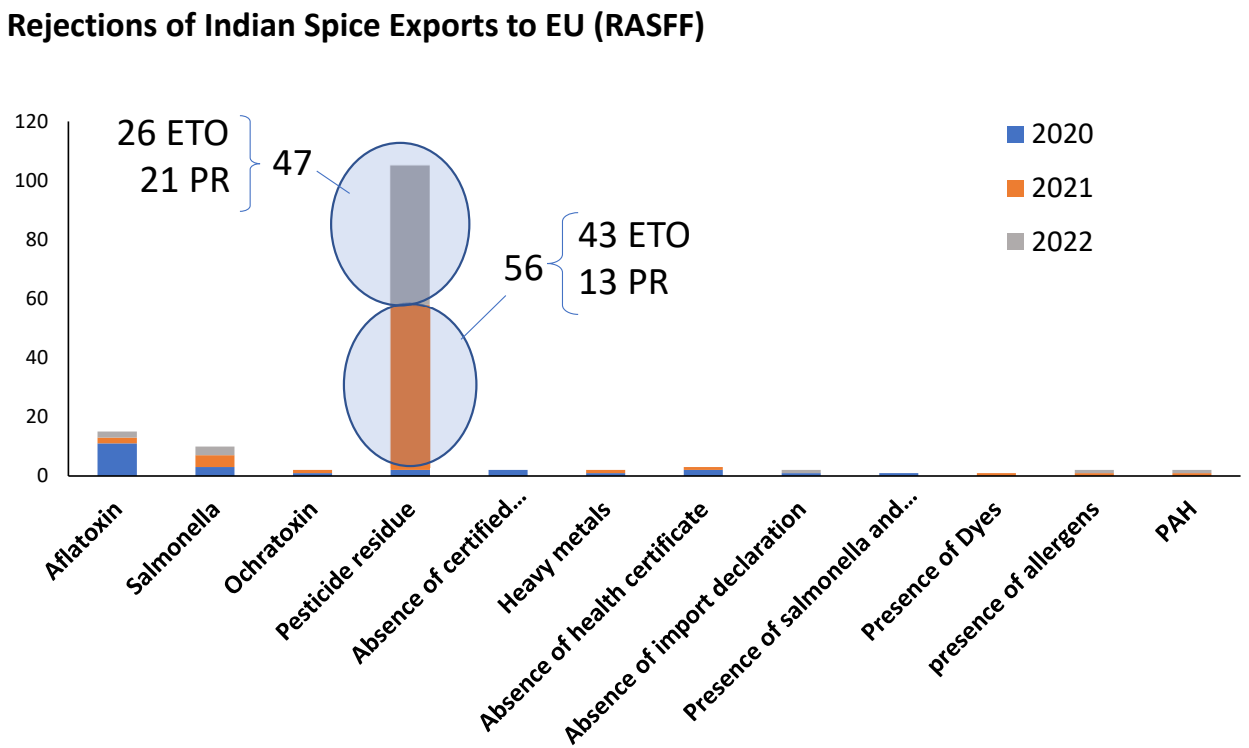
- ↳ Occupational exposure causes lymphoma and leukemia
- ↳ Declared as category 1 carcinogen by the International Agency Research of Cancer (IARC)in 1991

European Union considers ETO as a pesticide, and has MRLs

- ↳ Chilli, ginger MRL is 0.02 mg/kg
- ↳ All other spices 0.1 mg/kg
- ↳ In 2022, EU made health certificate mandatory for spices exported from ↳ India to EU, demonstrating compliance with ETO MRL.

Ethylene Oxide (ETO)

How serious?

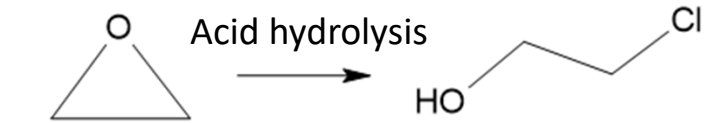


Ethylene Oxide (ETO)

Chemistry

Ethylene oxide is a three membered ring, highly strained and unstable

- ↳ naturally breaks down and forms 2-CE
- ↳ ETO residues can be removed by flushing with CO_2 after treatment, and reduce naturally in time
- ↳ But 2-CE residues are stable and will remain on the treated product



Ethylene oxide

2-chloroethanol

Also called Ethylene chlorhydrin (ECH)

Regulatory limit is for total ETO, i.e. sum of residual concentrations of ETO and 2-CE

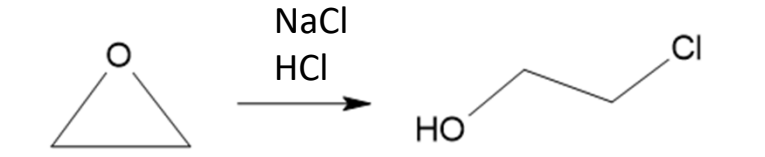
Ethylene Oxide (ETO)

Analysis

ETO can be analysed in two ways

- Chemically converting ETO into 2-CE by hydrolysis (HCl and NaCl), and analysing total 2-CE by GC-MS/MS. In this method, calculation is ETO(sum) = x(2CE) × 0.55

- Analysis of ETO and 2—CE separately using QuEChERS sample preparation followed by GC-MS/MS



Ethylene oxide

2-chloroethanol

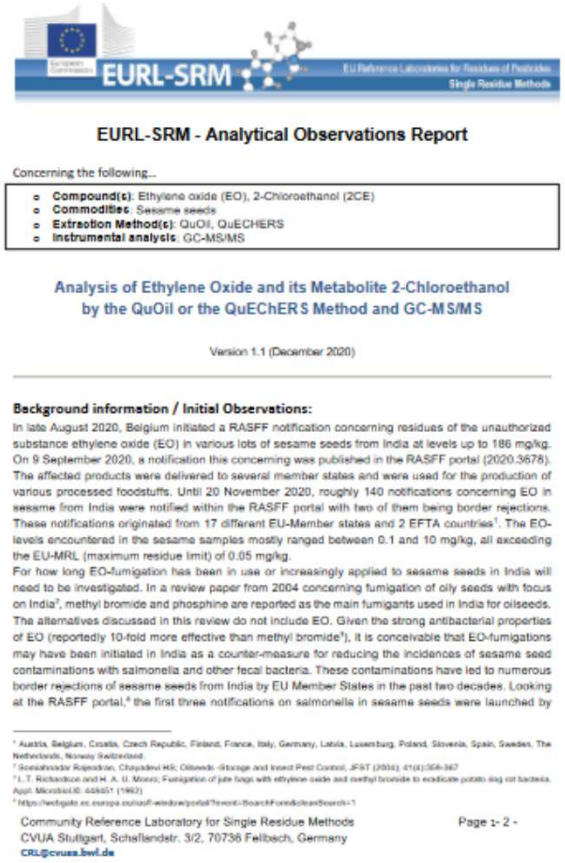
Detecting ETO residues will be an indication of time elapsed after treatment, so EU recommends the QuEChERS method

Ethylene Oxide

QuEChERS (EU method)

For cereals, spices and other dry commodities of low lipid content (based on EN 15662)

- ↳ Weigh 2 g ± 0.02 g of sample homogenates, add 10 mL of acetonitrile, shake 15 min
- ↳ Add the QuEChERS citrate buffer-partitioning salts mixture and shake for 1-2 minutes, centrifuge at >3000 g
- ↳ Dispersive SPE cleanup with C18/PSA/MgSO4 (25/25/150 mg/mL extract)
- ↳ GC-MS/MS analysis



EURL-SRM

EURL-SRM - Analytical Observations Report

Concerning the following...

- **Compound(s)**: Ethylene oxide (EO), 2-Chloroethanol (2CE)
- **Commodities**: Sesame seeds
- **Extraction Method(s)**: QuOil, QuECHERS
- **Instrumental analysis**: GC-MS/MS

Analysis of Ethylene Oxide and its Metabolite 2-Chloroethanol by the QuOil or the QuEChERS Method and GC-MS/MS

Version 1.1 (December 2020)

Background information / Initial Observations:

In late August 2020, Belgium initiated a RASFF notification concerning residues of the unauthorized substance ethylene oxide (EO) in various lots of sesame seeds from India at levels up to 186 mg/kg. On 9 September 2020, a notification this concerning was published in the RASFF portal (2020.3678). The affected products were delivered to several member states and were used for the production of various processed foodstuffs. Until 20 November 2020, roughly 140 notifications concerning EO in sesame from India were notified within the RASFF portal with two of them being border rejections. These notifications originated from 17 different EU-Member states and 2 EFTA countries[1]. The EO-levels encountered in the sesame samples mostly ranged between 0.1 and 10 mg/kg, all exceeding the EU-MRL (maximum residue limit) of 0.05 mg/kg.

For how long EO-fumigation has been in use or increasingly applied to sesame seeds in India will need to be investigated. In a review paper from 2004 concerning fumigation of oily seeds with focus on India[2], methyl bromide and phosphine are reported as the main fumigants used in India for oilseeds. The alternatives discussed in this review do not include EO. Given the strong antibacterial properties of EO (reportedly 10-fold more effective than methyl bromide[3]), it is conceivable that EO-fumigations may have been initiated in India as a counter-measure for reducing the incidences of sesame seed contaminations with salmonella and other fecal bacteria. These contaminations have led to numerous border rejections of sesame seeds from India by EU Member States in the past two decades. Looking at the RASFF portal,[4] the first three notifications on salmonella in sesame seeds were launched by

[1] Austria, Belgium, Croatia, Czech Republic, Finland, France, Italy, Germany, Latvia, Luxemburg, Poland, Slovenia, Spain, Sweden, The Netherlands, Norway Switzerland.

[2] Sivakrishnaiah Rajendran, Chayadevi Hill, Oilseeds: Storage and Insect Pest Control, JFST (2004), 41(4):358-367

[3] L.T. Richardson and M. A. U. Moore, Fumigation of jute bags with ethylene oxide and methyl bromide to eradicate potato ring rot bacteria. Appl. Microbiol.10: 448451 (1962)

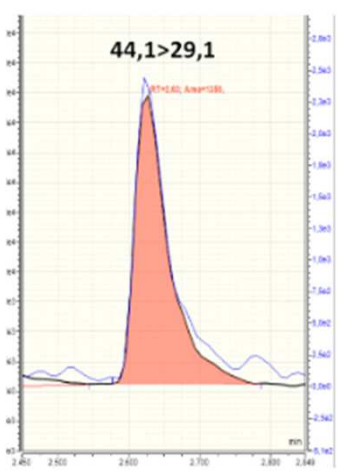
[4] https://webgate.ec.europa.eu/rasff-window/portal/?event=SearchForm&cleanSearch=1

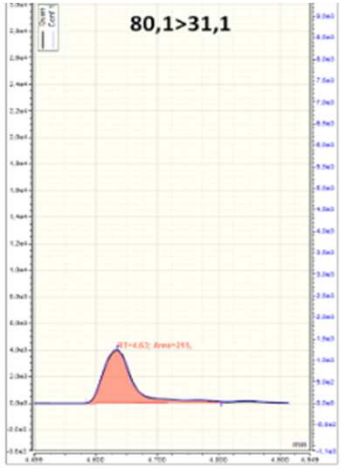
Google “EU RL ethylene oxide”

Ethylene Oxide

Analysis: GC-MS/MS (EU method)

Compound	Transition
Ethlene oxide	44.1 → 14
	44.1 → 28
	44.1 → 29.1 (QN)
2-Chloroethanol	80.1 → 31.1 (QN)
	80.1 → 43
	82.1 → 31.1



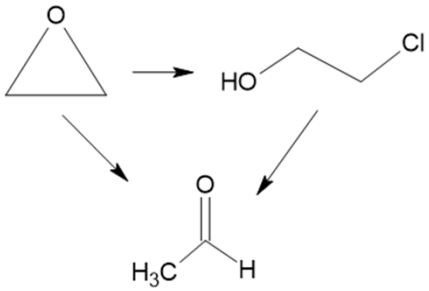


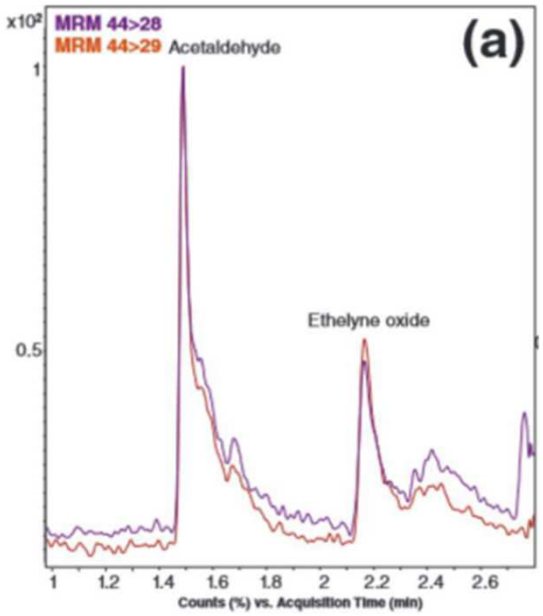
Ethylene Oxide

Issue of acetadehyde

Acetaldehyde can be found naturally in some products (e.g. sesame.
It may also be formed formed at high temperatures from ETO or 2-CE through thermal rearrangement.

- ↳ Share same MRM transitions
- ↳ Chromatographic separation is required





More to learn..

Next session 31st June 2023

- ☑ (1) Introduction to instrumentation: LC-MS/MS, 03-Jul-23
- ☑ (2) Introduction to instrumentation: GC-MS/MS, 10-Jul-23
- ☑ (3) Modern pesticide residue analysis - Introduction, 17-Jul-23
- ☑ (4) Advanced pesticide residue analysis, 24-Jul-23
- 🕒 (5) Method validation: requirements and practice, 31-Jul-23 ⟸
- 🕒 (6) Introduction to measurement uncertainty calculation, 7-Aug-23

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Thank you!

§ Questions?