EU Reference Laboratories for Residues of Pesticides
Single Residue Methods

THEMED DAY: SAMPLE SAMPLING- SAMPLE PEREPARATION AND SAMPLE PROCESSING

Pesticides which require special treatment during processing / homogenization and extraction

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Sampling and Sample Preparation

Lot

Take portions from diff. points

Primary Samples

Combine

Bulk Sample (Composite sample)

Reduce (if needed) e.g. using sample divider

Laboratory Sample

Size reduction (segmenting…), remove stones …

Analytical Sample (partly prepared)

Homogenization (using mill/mixer)

Analytical Sample (fully prepared)

Weigh

Analytical Portion

Extraction, Aliquotation, Cleanup …

Final Extract -> Aliquot injected

LABORATORY SAMPLE

Questions

- Is the portion to portion variability acceptable ?
- What losses occur during sample preparation, processing and homogenization?
- What losses occur between homogeneization and start of analysis ?
- How can I minimize those losses?
- What is the impact of homogeneization on extraction yields?
- What measures can I take during extraction to match for defficiencies during milling (large particles)?

Questions

- **Is the portion to portion variability acceptable ?**
- **What losses occur during sample preparation, processing and homogenization?**
- **What losses occur between homogenization and start of analysis ?**
- **How can I minimize those losses?**
- **What is the impact of homogenization on extraction yields?**
- **What measures can I take during extraction to match for deficiencies during milling (breakup large particles)?**

Typical Ambient Milling Approach of wet samples

Cut in segments

Cut into smaller pieces if needed

+ Fill in a bag

Mill Material

Puree like material

Portion

Wait for enough Portions to run a sequence

Extract

Typical Cryogenic Milling Approach

Contact between Peel & Juices (+ bag)

Cut in segments →

Cut into smaller pieces if needed → + Fill in a bag

Freeze-out > 5h (typically over-night) ↓

← Mill Frozen Material

← Add Dry Ice + Remill

Snowlike Material

↑ Portion ↑

Extract

Losses during Sample Preparation/Processing

No single mechanism involved

- **Volatility** ***(e.g. fumigants, biphenyl, dichlorvos)***
- **Chemical reactions (e.g. hydrolysis, oxidation)**

Many Factors play a role:

- pH
- Presence of reactive matrix-components
- Presence of retentive matrix-components (water, lipids)
- Presence of active enzymes
- Presence of oxygen
- Temperature
- Exposition time

Implications

- *True concentration underestimated*
- *MRL-exceedances /misuse may not be detected*

No single mechanism involved

- **Evaporation** ***(e.g. fumigants, biphenyl, dichlorvos)***
- **Chemical reactions** **(e.g. hydrolysis, oxidation)**

Many Factors play a role:

- pH
- Presence of reactive matrix-components
- Presence of retentive matrix-components (water, lipids)
- Presence of active enzymes
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- Temperature
- Exposition time

Implications

- ☹ ***True concentration underestimated***
- ☹ ***MRL-exceedances /misuse may not be detected***

Critical Steps prior to extraction

Ambient Processing

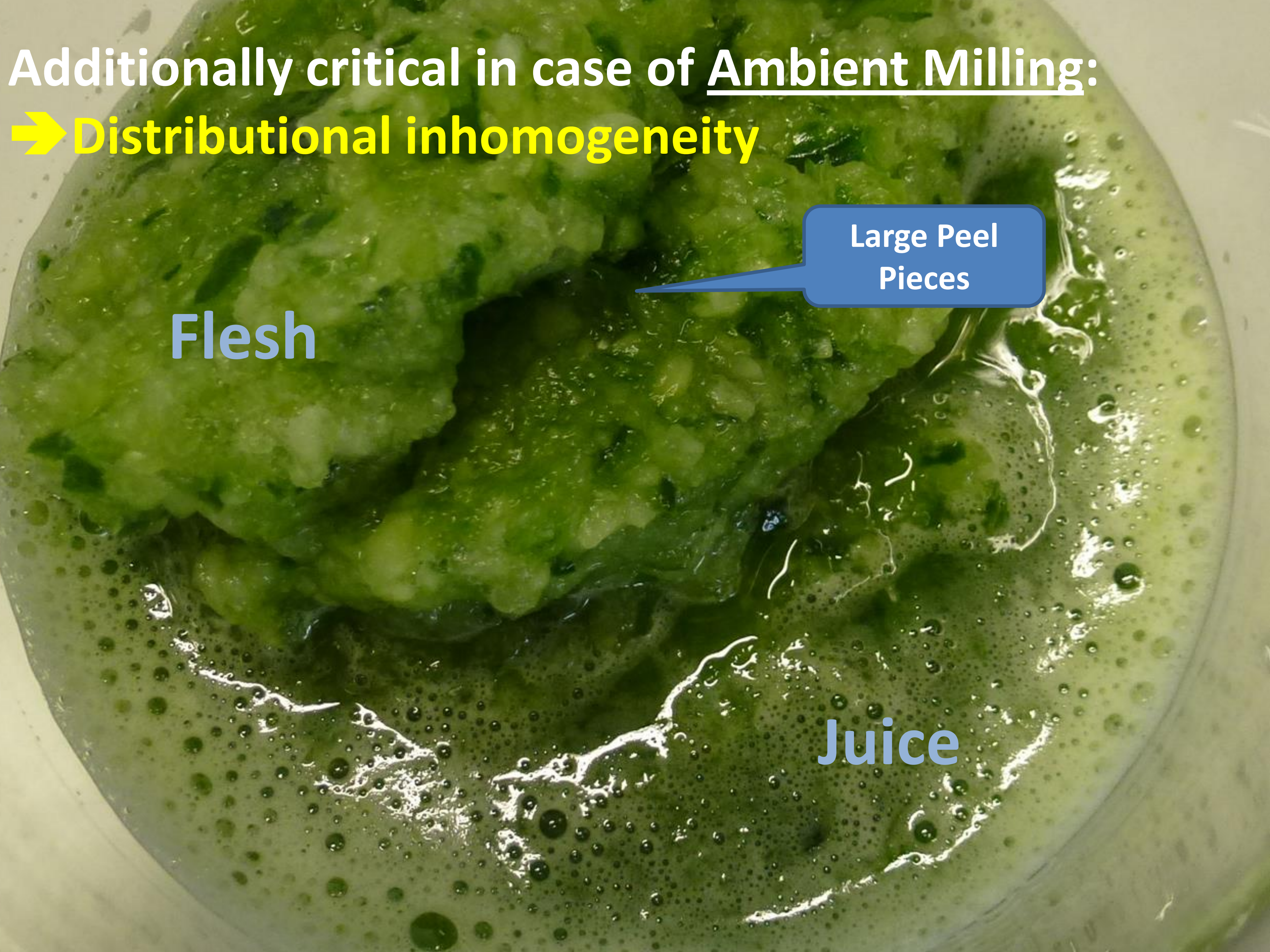
Wait time between homogenization and extraction (could be 1 min but not rarely hours)

Cryogenic Processing

Wait time needed for coarsely cut sample to freeze. During this time contact of pesticides on peel with juices

Additionally critical in case of Ambient Milling:

➔ **Distributional inhomogeneity**




Cryo milling
RT milling

Degradation of pesticides in homogenates at room temperature

	Cucumber			Apples		
	30 min	180 min	Note	30 min	180 min	Note
Ester Saponification (formation of free carboxylic acids)						
Carfentrazone-ethyl	-7%	-22%	Transforms to carfentrazone acid (NA)	-10%	-28%	Transforms to carfentrazone acid (NA)
Clodinafop-propargyl	-21%	-63%	Transforms to clodinafop acid (NA)	-11%	-42%	Transforms to clodinafop acid (NA)
Isoxadifen-ethyl	-4%	-20%	Transforms to isoxadifen acid (NA)	-28%	-56%	Transforms to isoxadifen acid (NA)
Mefenpyr-diethyl	-2%	-4%	Transforms to M. ethyl and M. acid (NA)	-28%	-60%	Transforms to M. ethyl and M. acid (NA)
Pyrafluofen-ethyl	-6%	-16%	Transforms to pyrafluofen acid (NA)	-29%	-58%	Transforms to pyrafluofen acid (NA)
Cinidon-ethyl	-7%	-6%	Transforms to cinidon acid (NA)	-6%	-27%	Transforms to cinidon acid (NA)
Acibenzolar-S-Me	-12%	-45%	Transforms to acibenzolar acid (NA)	NA	NA	
Oxidations of sulfur moieties (formation of oxones, sulfoxides, sulfones)						
Famphur	-21%	-40%	Transforms to oxone (NA)	6	10	Stable
Disulfoton	-6%	-44%	D. Sulfoxide ▲ (26%), D. sulfone (8%), respective oxones NA	-16%	-56%	D. sulfoxide ▲ (49%), D. sulfone (10%), respective oxones (NA)
Demeton-S-methyl	-2%	-5%	Stable	-10%	-35%	Oxydemeton methyl ▲ (24%)
Fenthion	-18%	-33%	Sulfoxide (11%); oxon sulfoxide (24%) ▲	-7%	-29%	F. sulfoxide ▲(26%); oxon sulfoxide(1%)
Ethiofencarb	5%	5%	Stable (-4%)	-11%	-43%	E. sulfoxide ▲ (27%); sulfone (NA)
Terbufos	-1%	-16%	Transforms to T. sulfoxide, sulfone (NA)	-11%	-26%	Transforms to T. sulfoxide, sulfone (NA)
Carboxin	-2%	-4%	Stable	-6%	-32%	Transforms to sulfoxide + oxathiine amide sulfoxide and sulfone (oxicarboxin) (NA)

Degradation of pesticides in homogenates at room temperature

	Cucumber			Apples		
	30 min	180 min	Note	30 min	180 min	Note
Hydrolysis of N-trihalomethylthio Compounds (sensitive at high pH)						
Captan	-63%	-96%	THPI ▲ (not quantified)	-5%	-15%	THPI ▲ (not quantified)
Folpet	-51%	-85%	Phthalimid ▲ (not quantified)	8%	-9%	Phthalimid ▲ (not quantified)
Dichlofluanid	-35%	-87%	DMSA ▲ (not quantified)	-3%	-6%	DMSA ▲ (not quantified)
Tolylfluanid	-22%	-63%	DMST ▲ (not quantified)	3%	-2%	DMST ▲ (not quantified)
Methomyl Pro-pesticides (Alanycarb is acid sensitive; Thiodicarb is base-sensitive)						
Alanycarb	NA	NA		-100%	-100%	Transforms to methomyl (NA)
Thiodicarb	-8%	-34%	Transforms to methomyl (NA)	1%	7%	stable
Thiolcarbamates						
EPTC	-4%	-23%	Transforms to mercaptane (NA)	-4%	-14%	Transforms to mercaptane (NA)
Vernolate	-13%	-28%	Transforms to mercaptane (NA)	-1%	-20%	Transforms to mercaptane (NA)

Degradation of pesticides in homogenates at room temperature

	Cucumber			Apples		
	30 min	180 min	Note	30 min	180 min	Note
Miscellaneous Compounds						
Spirotetramate	-6%	-34%	Formation of Enol ▲ (7% /28%)	3%	3%	stable
TCMTB	-27%	-61%	Hydrolysis to 2-mercaptobenzothiazole (MBT)	5%	4%	stable
Prothioconazole	-28%	-62%	Transforms to P. desthio (NA)	NA	NA	
Profoxydim	-34%	-86%		NA	NA	
Naled (Dibrom)	-29%	-86%	Debrominates to Dichlorvos ▲ (6%/16%) In parallel degradation to Chlorodibromo-acetaldehyde (NA)	-1%	-7%	stable
Chlorothalonil	-22%	-47%	Binding to matrix + degradation (NA)	0%	-4%	stable
Clofentezine	-1%	-42%	Hydrolysis to hydrazide hydrazone (NA) and 2-chlorobenzoic acid (NA)	-7%	17%	Hydrolysis to hydrazide hydrazone (NA) and 2-chlorobenzoic acid (NA)
Pyrifenox 1	-12%	-20%	Hydrolysis of the oxime group, degradation product (NA), Indications of isomerization	14%	31%	indications of isomerization
Pyrifenox 2	-28%	-100%		4%	4%	

Typical Cryogenic Milling Approach

Contact between Peel & Juices (+ bag)

Cut in segments →

Cut into smaller pieces if needed →

+ Fill in a bag

Freeze-out > 5h (typically over-night) ↓

← Mill Frozen Material

← Add Dry Ice

+ Remill

Snowlike Material

↑ Portion ↑

Extract


Wait time until
sample is placed
in freezer
Wait time until
sample gets frozen
Stephan

Experiment on Degradation during Homogenization

Intact Cucumbers **Spiked** (superficially) → **Cut** Coarsely (wheels) → **Processed** in various ways (P1-P7)

P1) Ambient Milling → wait 15 min → Extraction

P2) Ambient Milling → wait 2 h → Extraction

P3) Ambient Milling ***+ AA*** → wait 2 h → Extraction

AA = Ascorbic Acid

P4) Cover with **Dry ice** → wait 15 min → **Cryo-milling** → Extraction

P5) Wait 15 min → Freeze 16h → **Cryo-milling** → Extraction

P6) Wait 15 min → Freeze 16h → **Cryo-milling*+AA*** → Extraction

P7) Wait 15 min (**CONTACT**) → Freeze 16h → **Cryo-milling*+AA*** →Extr.

Experiment on Degradation during Homogenization

#	Sample status during milling	Milling conditions	AA-Added?	Waiting time (before extr.)	RELATIVE YIELDS in % (via ISTD and setting P5 as 100%)					Comments
					Chlorothalonil	Dichlofluanid	Tolylfluanid	Chlozolinate	Dithianon	
P1	Fresh	Ambient	No	15 min	37	2	6	35	27	
P2	Fresh	Ambient	No	120 min	10	0	1	16	11	
P3	Fresh	Ambient	Yes	120 min	47	1	3	36	190	Not acidic enough for Tolylfluanid and Dichlofluanid
P4	Semi-Frozen	Cryo	No	16 h (freezer)	62	18	25	55	90	Fresh sample mixed w. dry ice for 15 min before milling
P5	**Frozen**	**Cryo**	**No**	**16 h (freezer)**	**100**	**100**	**100**	**100**	**100**	**set at 100%**
P6	Frozen	Cryo	Yes	16 h (freezer)	103	112	96	111	272	
P7	Frozen (w. contact)	Cryo	Yes	16 h (freezer)	48	31	52	52	63	**Contact** between peel and juices prior and during freezing

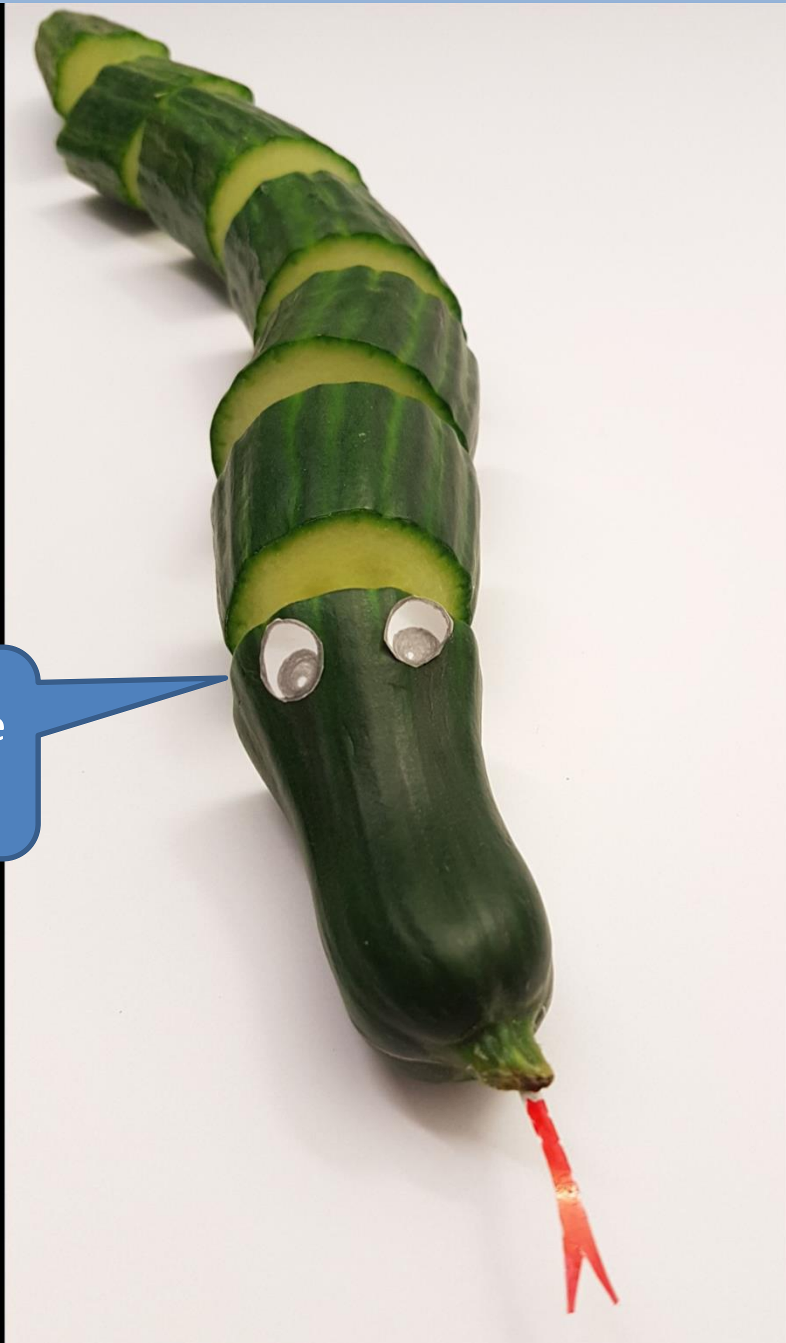
Pesticides spiked superficially on Cucumbers

IS Chlorpyrifos (set at 100%)

- Massive losses during ambient milling (P1-3)
- Losses upon contact between peel & juices (P7) !!
- Oxidations not effectively prevented by cryo-milling

Possible solutions ;-)

Eyes and Tongue are OPTIONAL



Ambient versus Cryogenic Processing and impact of HCl-Addition

CUCUMBER	Tolylfluanid Rec. [%]	DMST formed (expressed as parent)	Rec. SUM [%] (expressed as parent)
Ambient	2%	83%	85%
Ambient +HCl	70%	22%	92%
Cryogenic	77%	25%	102%
Cryogenic + HCl	79%	20%	99%

CUCUMBER	Dichlofluanid Rec. [%]	DMST formed (expressed as parent)	Rec. SUM [%] (expressed as parent)
Ambient	7%	77%	84%
Ambient +HCl	74%	17%	91%
Cryogenic	81%	30%	111%
Cryogenic + HCl	88%	16%	104%

CUCUMBER	Thiodicarb Rec. [%]	Methomyl formed (expressed as parent)	Rec. SUM [%] (expressed as parent)
Ambient	7%	73%	80%
Ambient +HCl	5%	54%	59%
Cryogenic	83%	16%	99%
Cryogenic + HCl	45% (?)	33% (?)	78%

Conclusion:
Cryogenic milling w/o acidication
worked well enough

Cucumbers were spiked superficially prior to processing
After milling all samples were left 30 min standing before analysis

Ambient versus Cryogenic Processing and impact of HCl addition

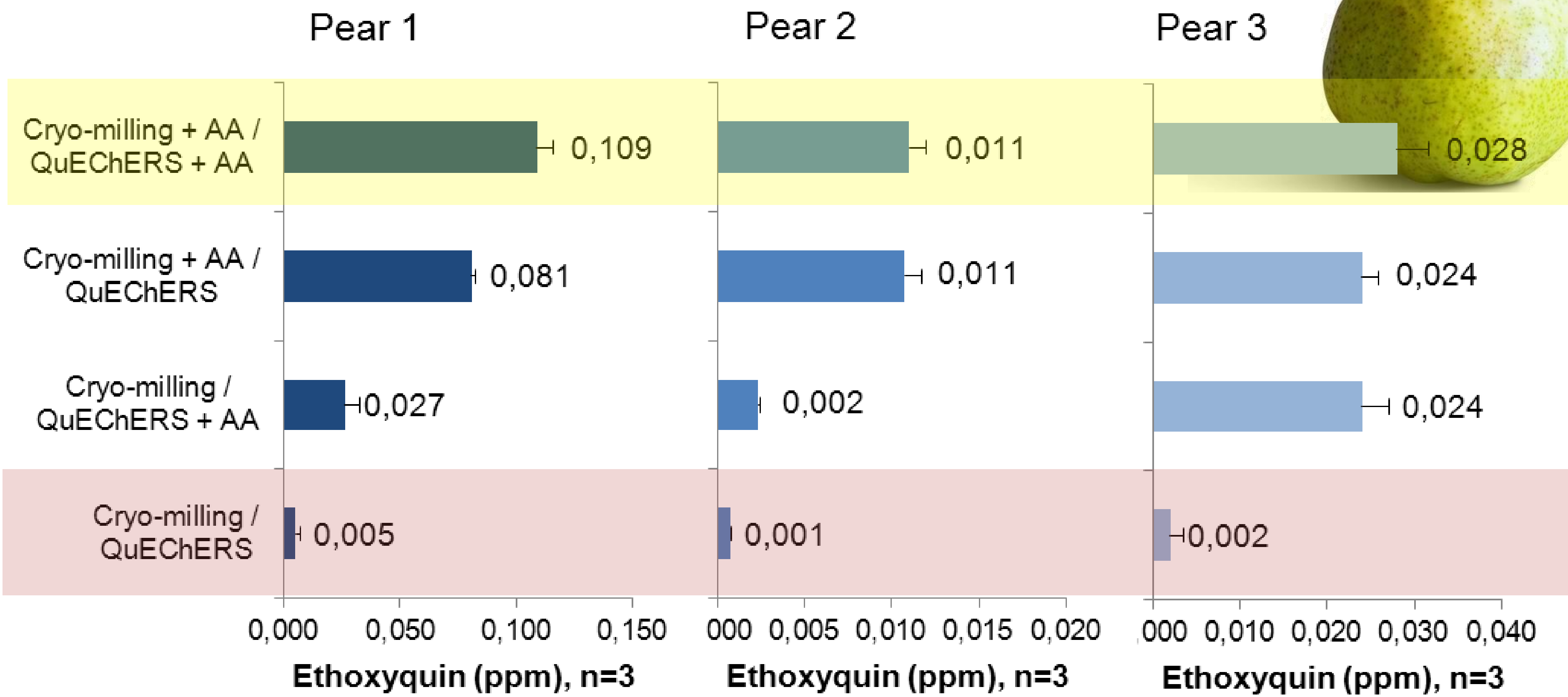
CUCUMBER	Clodinafop-propargyl Rec [%]
Ambient	38%
Ambient +HCl	90%
Cryogenic	97%
Cryogenic + HCl	91%

CUCUMBER	Dithianon Rec [%]
Ambient	4%
Ambient +HCl	109%
Cryogenic	61%
Cryogenic + HCl	93%

Here acidification during milling seems necessary

Ethoxyquin losses during Sample Processing + Extraction

Pears w. incurred EQ (real samples of US-origin)

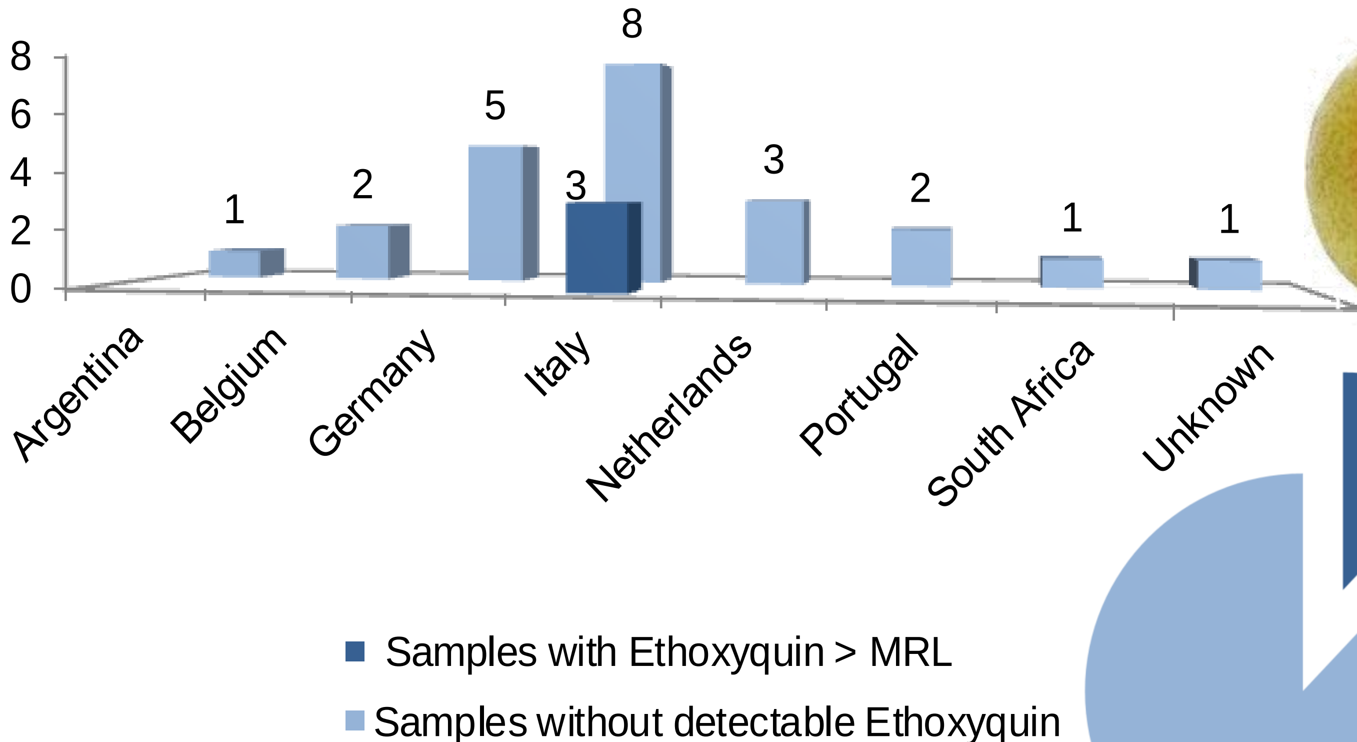


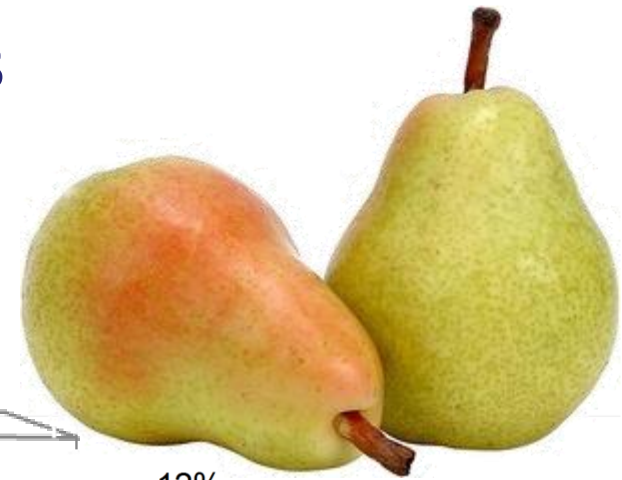
AA-Additions: Milling: 1g /100 g ; Extraction: 0.3 g /10 g

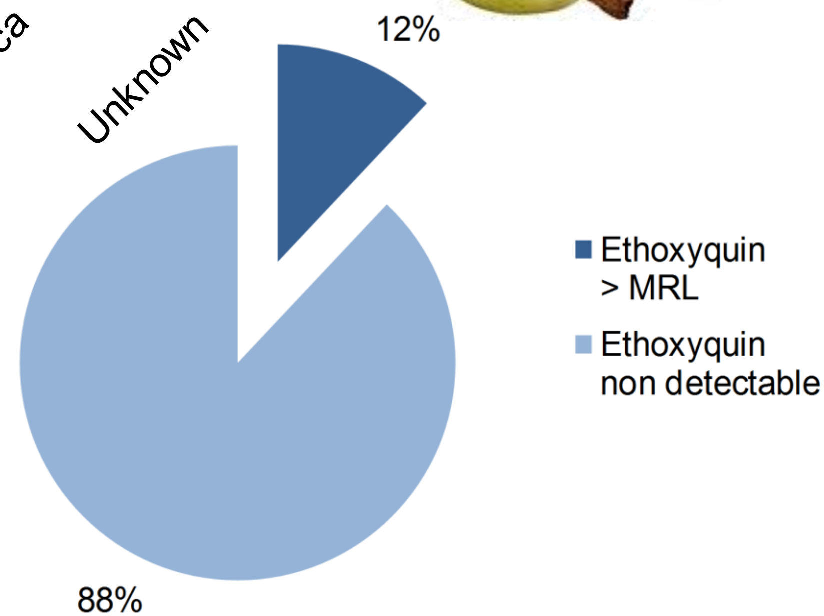
Adding AA during extraction → not enough !!

EQ-protection must already start during homogenization !!!

Ethoxyquin Results in Routine Analysis



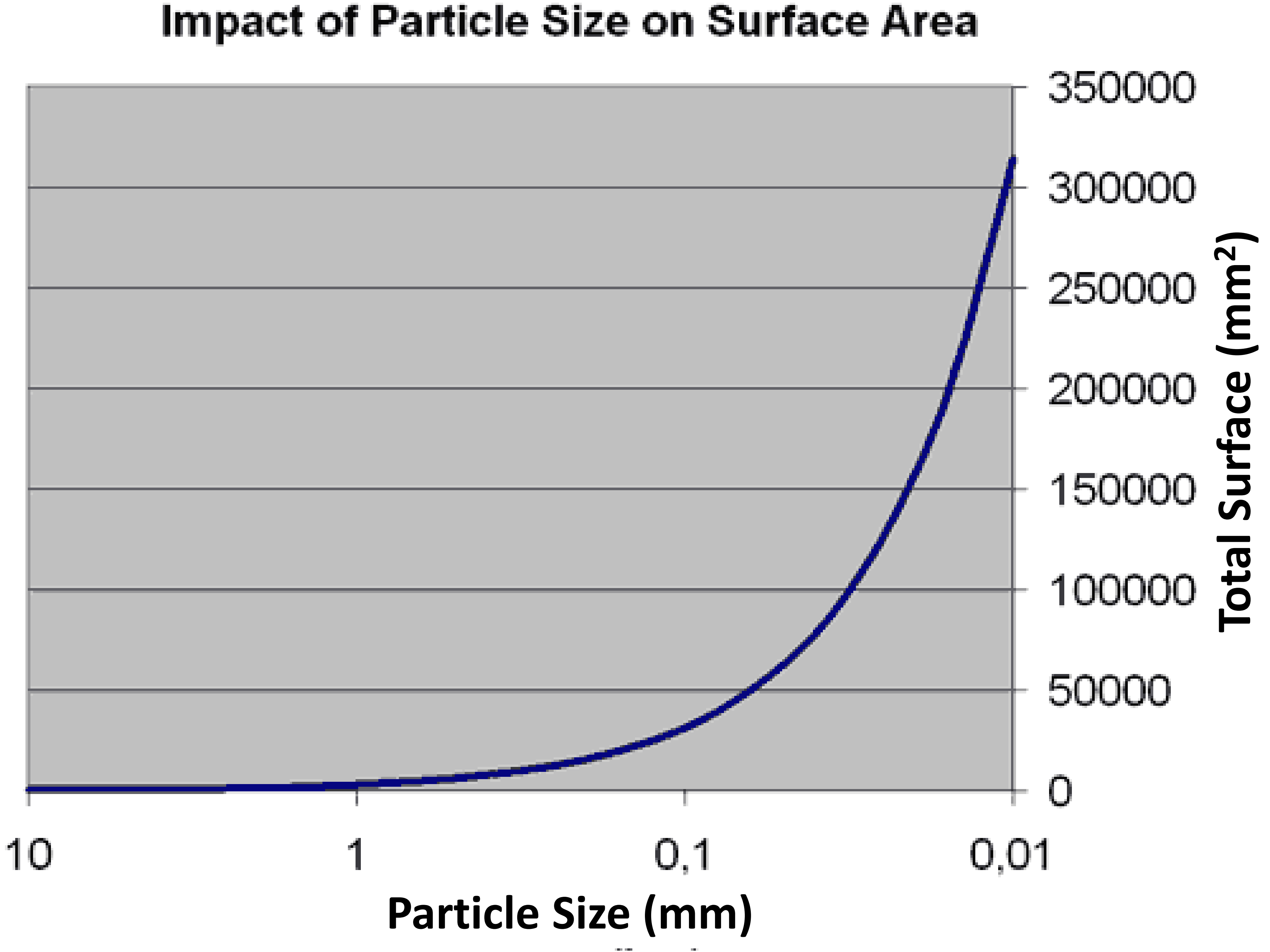




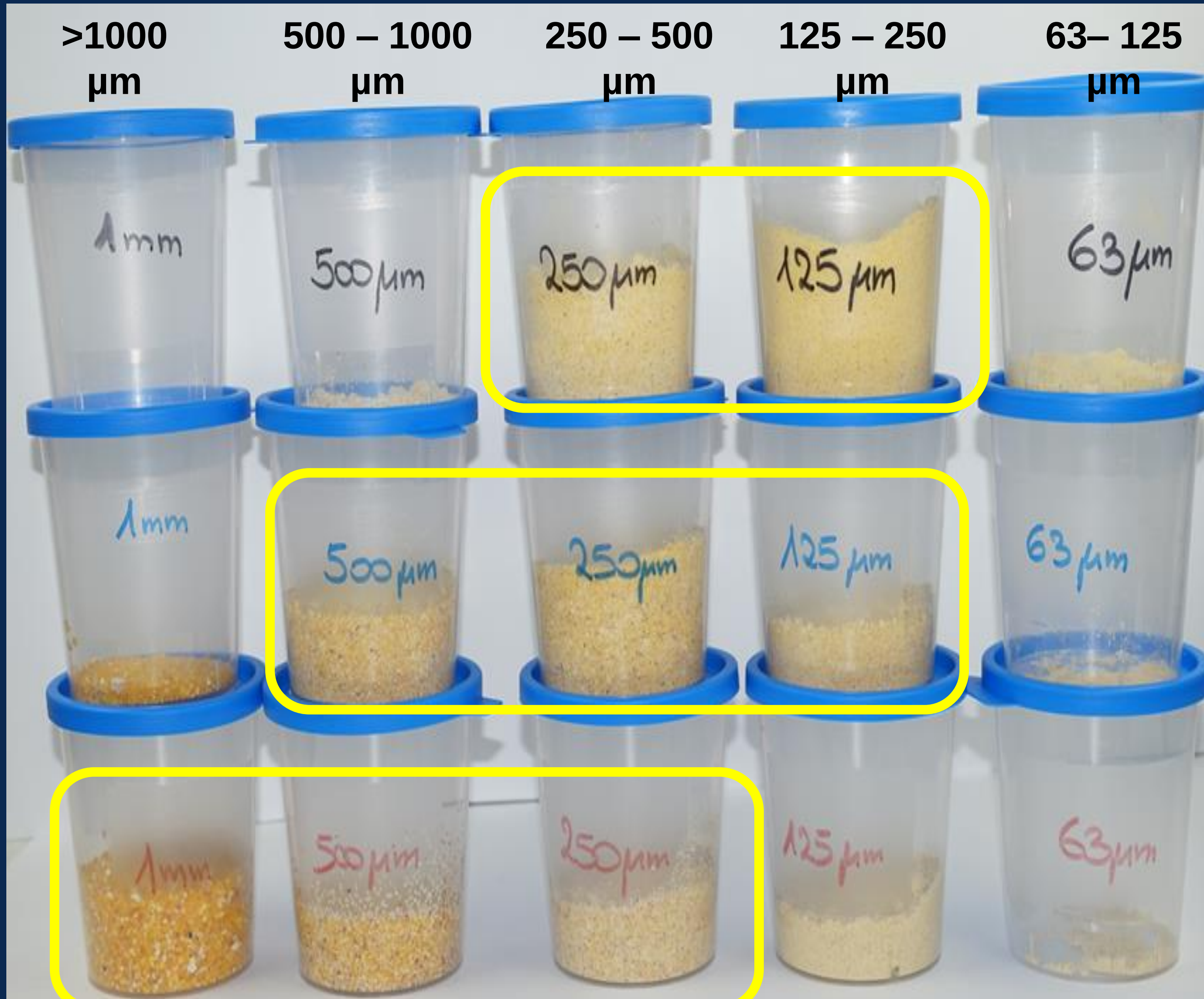
26 samples analyzed

- ►**All cryogenically processed w. AA-addition during milling**
- ► **3 Italian pears >MRL (also illegal use)**

WHAT ABOUT THE IMPACT OF HOMOGENIZATION GRADE ON EXTRACTABILITY?

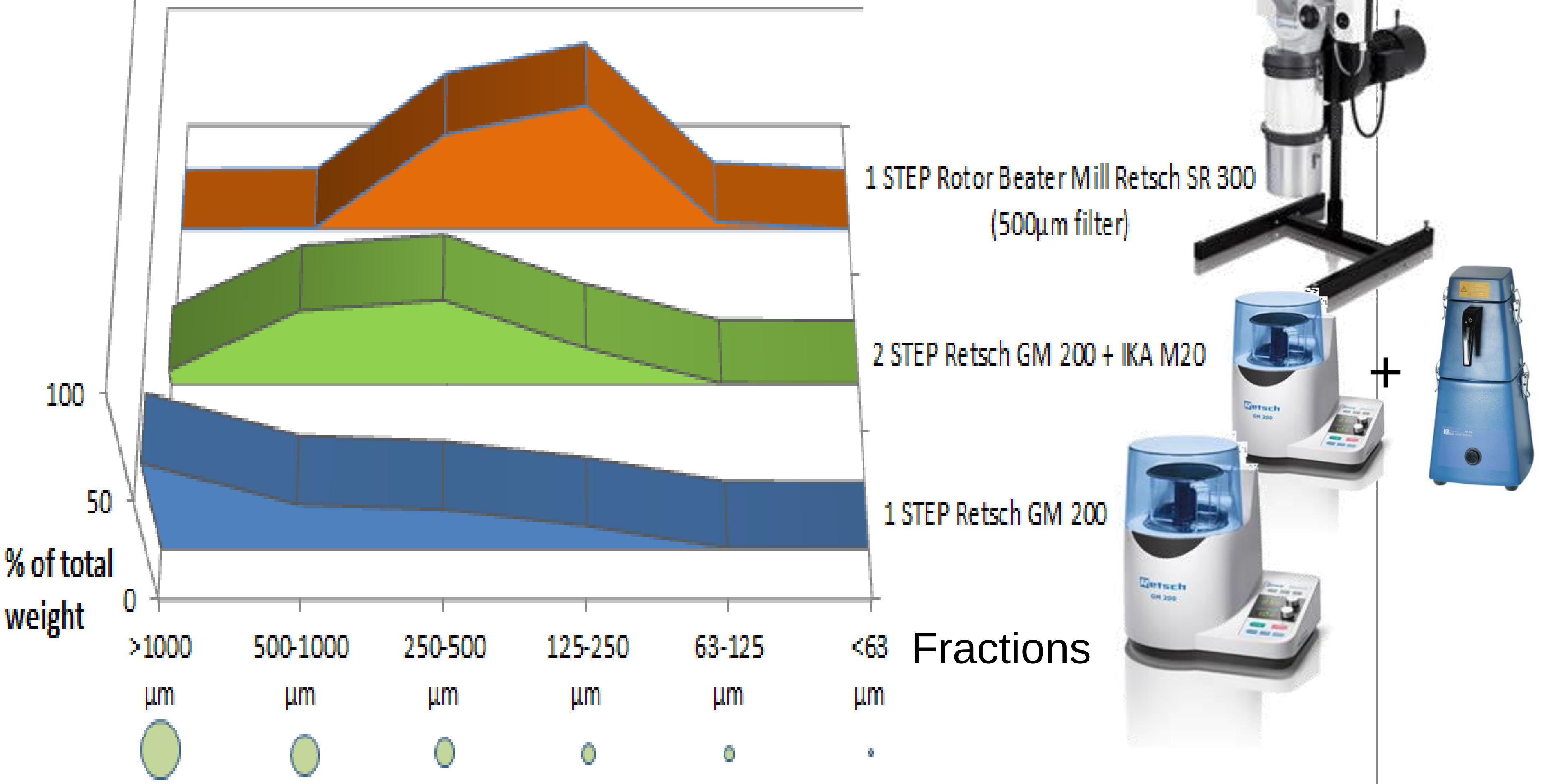

Impact of Particle Size on Surface Area
350000
300000
250000
200000
150000
100000
50000
0
Total Surface (mm2)
10
1
0,1
0,01
Particle Size (mm)

Impact of milling (particle size) on extractability



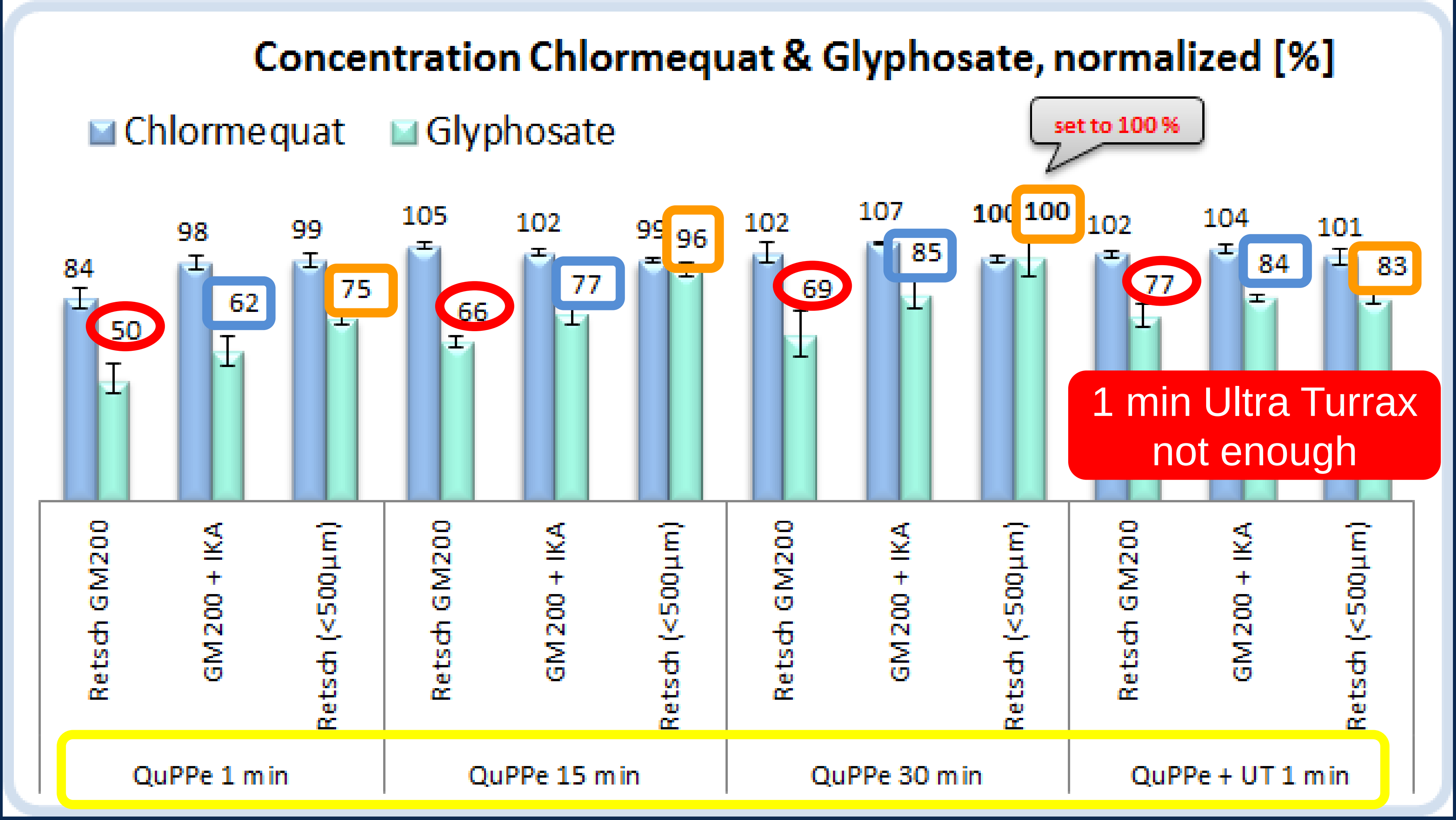
Maize with Incurred Glyphosate milled in different ways

Fractionation by particle size using sieves



Impact of milling on extractability

Maize with incurred Glyphosate and Chlormequat



Pending Experiments: Milling at <250 µm

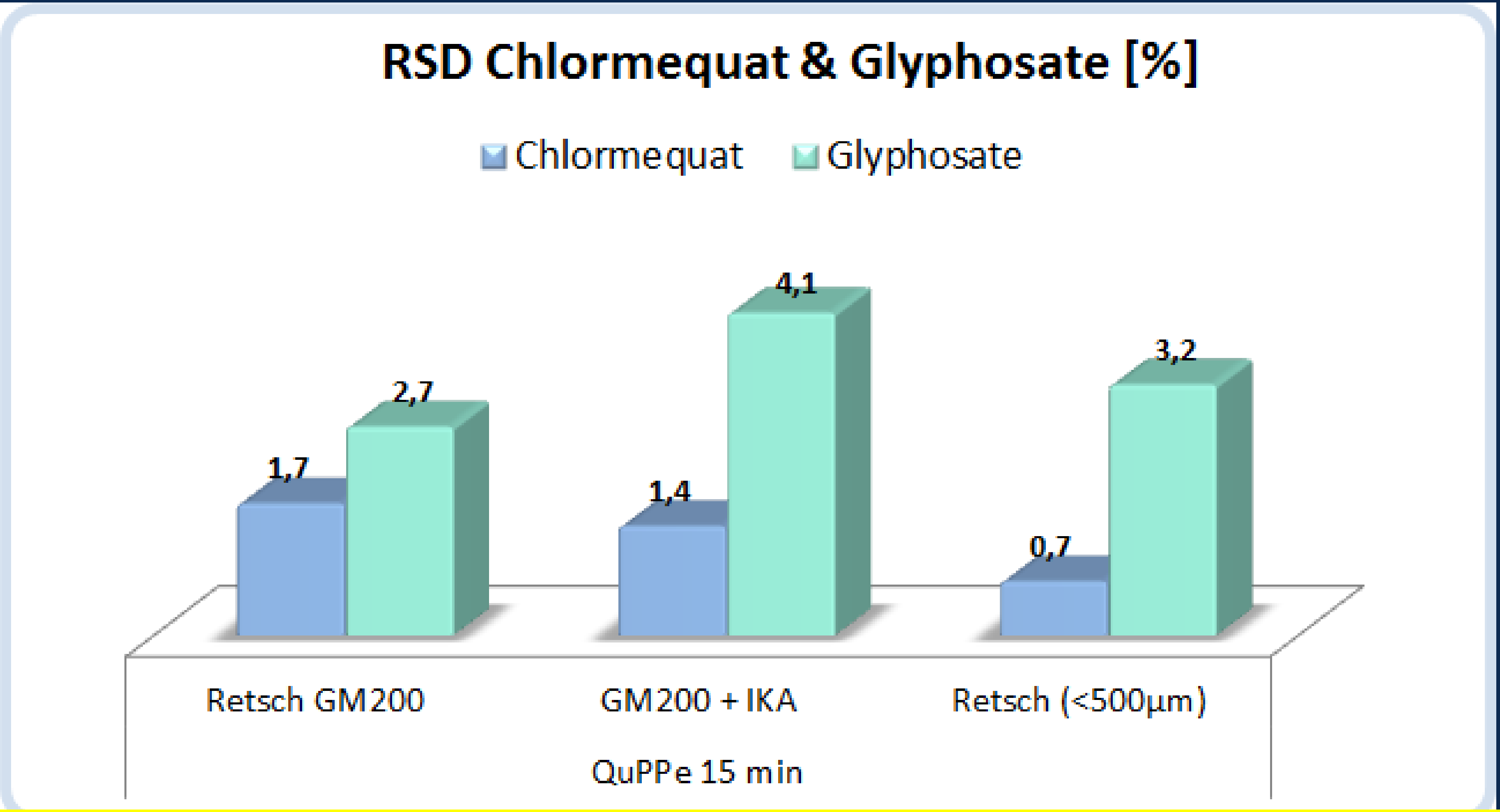
Maize with Incurred Glyphosate (PT-sample)

Extraction mode	Mean Rec [%], (n=3)	RSD [%]
1 min by hand	86	6.7
15 Min mechanical	100	1.1
1 Min Ultra Turrax	105	5.5
15 Min soaking in H2O +1 shaking by hand	103	6.6

Soaking time had the same impact as agitation time
Ultra-Turrax was more effective

Impact of milling on sub-sampling variability

Maize with incurred Glyphosate and Chlormequat



Practically no impact on RSDs

(Obviously enough number of particles of each size present)

WHAT ABOUT THE IMPACT OF HOMOGENIZATION ON VOLATILES ?

QuMFU (Quick Multi-Method for Fumigants)

1. Weigh 10 g of the samples into 50 mL PP tubes
2. Add 10 mL of n-Hexane
3. Add 100 µL of internal standard solution (Chlorobenzene D5, 10µg/mL)
4. Shake tube for 1 minute
5. Centrifuged for 5 min at 4000 rpm
6. Filter through a syringe filter (0.45 µm), if necessary
7. Transfer 1 mL of extract into vials
8. Measure via GC-MS/MS / GC-ECD

Impact of milling on extraction yields of incurred fumigants

Maize fumigated in the lab (in a vacuum desiccator)

Approach	1,2-Dibromo-3-chloro-propane	1,3-Dichloro-propene	Carbon tetra-chloride	p-Dichloro-benzole	p-Nitrochlor-benzol	Tetrachloro-ethan
	Relative recoveries (%)					
Milled **with dry Ice** (ultracentrifugal mill 0.5 mm filter)	**100**	**100**	**100**	**100**	**100**	**100**
Milled **without** dry ice (ultracentrifugal mill 0.5 mm filter) ➔ HEAT	95	80	75	91	120	95
Non-milled (1 min shaking)	39	14	48	6	24	32
Non-milled (15 min shaking)	55	25	74	18	50	47

- Fumigants obviously **diffused inside the corn** becoming **difficult to extract**
- Prolonging **extraction time to 15 min** was helpful but by far **not sufficient**
- **Milling raised extraction yields considerably**
- Milling **without dry-ice** cooling resulted in **moderate losses**

Wheat with <u>Incurred</u> CS_2 (real market sample provided by NRL)

Ultracentrifugal milling (filter 0.5 mm)

Direct Analysis by DTC mth (with SnCl2/HCl, isooctane)

Direct Analysis by DTC mth (with SnCl2/HCl, isooctane)

Simple extraction w. isooctane (NO SnCl2/HCl)

6.3 mg/kg ⟷ 81% ⟷ **7.9 mg/kg**

ND

Milling of cereals does not seem to negatively affect DTC levels (anal. as CS_2).
DTCs endure milling w/o markable CS_2 being formed


Thank you for your attention!