sciensano

healthy all life long

Development, validation and application of multi-class methods for the analysis of food additives by LC-MS/MS

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Based on a project funded by

Health
Food Chain Safety
Environment

Spotlight: Food additives – status quo on chemical analysis and European regulations
Berlin, 27th of November 2024

Our next
20 minutes

01

The WHY and the HOW?

The strategy

03

Tools used for the validation process
The Horwitz paradox

Ultimate test

02

Setting-up the methods
The funnel effect

No pain no gain

04

Implementation examples
Wrapping-up

This is real

THE TARGET

WHAT IS ON THE MARKET?

Label study in 2020 of food products in the Belgian market from the 6 major supermarkets

Market coverage of around 80%

36 601 unique food products (not taking into account fresh produce)

240 **FAs were IDENTIFIED**

44% **Contained at least 1 FA**

THE IDEA

1 food product

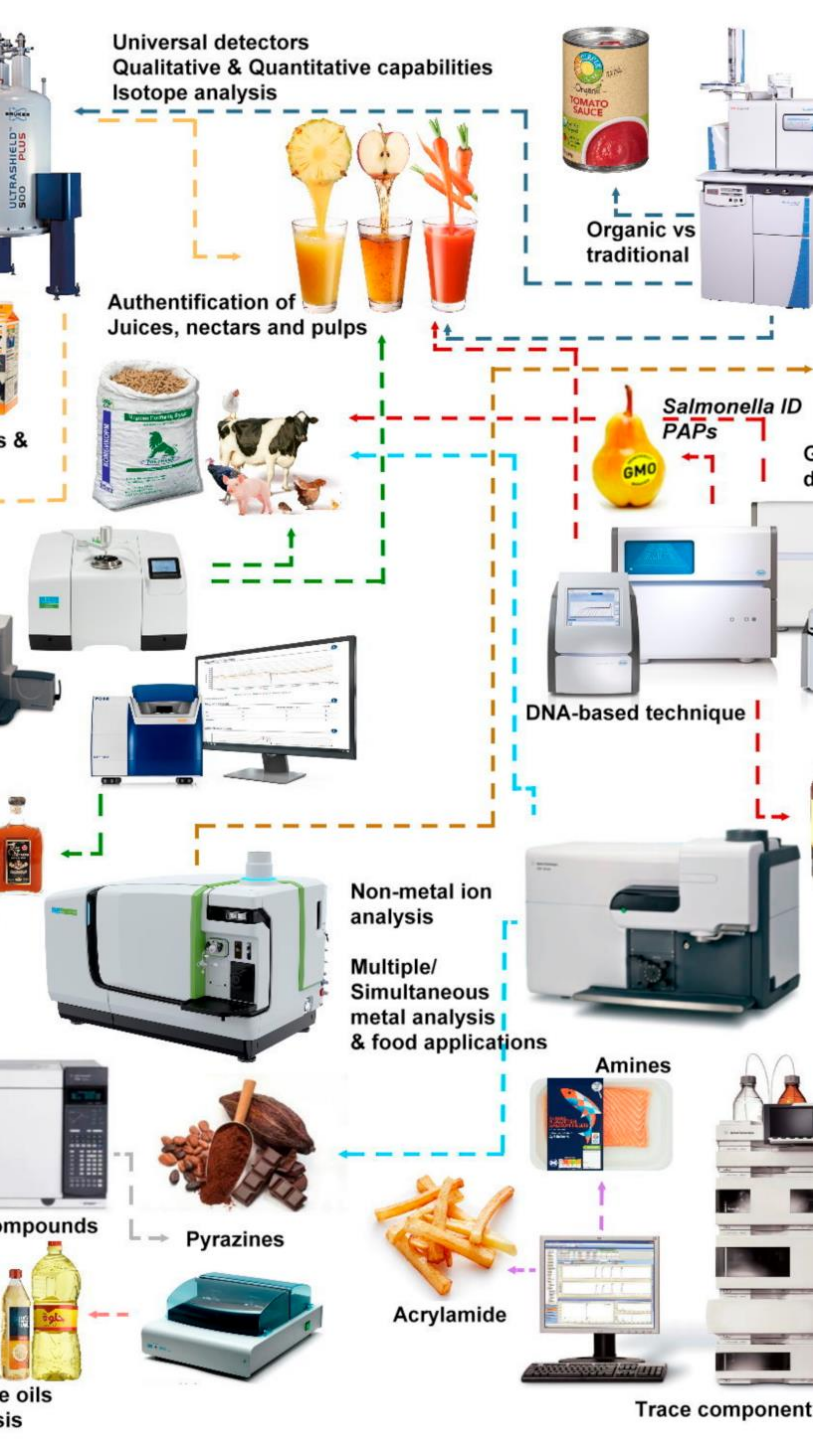
Various FAs

1 analytical method

THE IDEA

1 food product Various FAs 1 analytical method

Where to start????

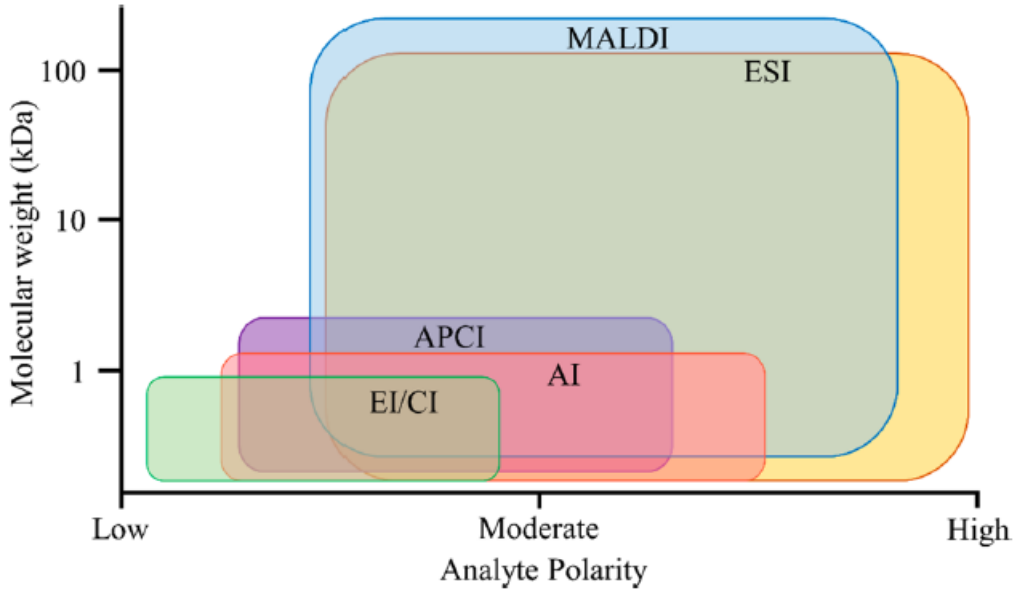


START AT THE END

Detection then separation

Mass spectrometry: as an universal detector

Liquid chromatography: UHPLC for fast separation



Authorised

NON-authorized

substances

COLOURS

ANTIOXIDANTS

SWEETENERS

PRESERVATIVES

MPLs

FCS

Our next
20 minutes

01

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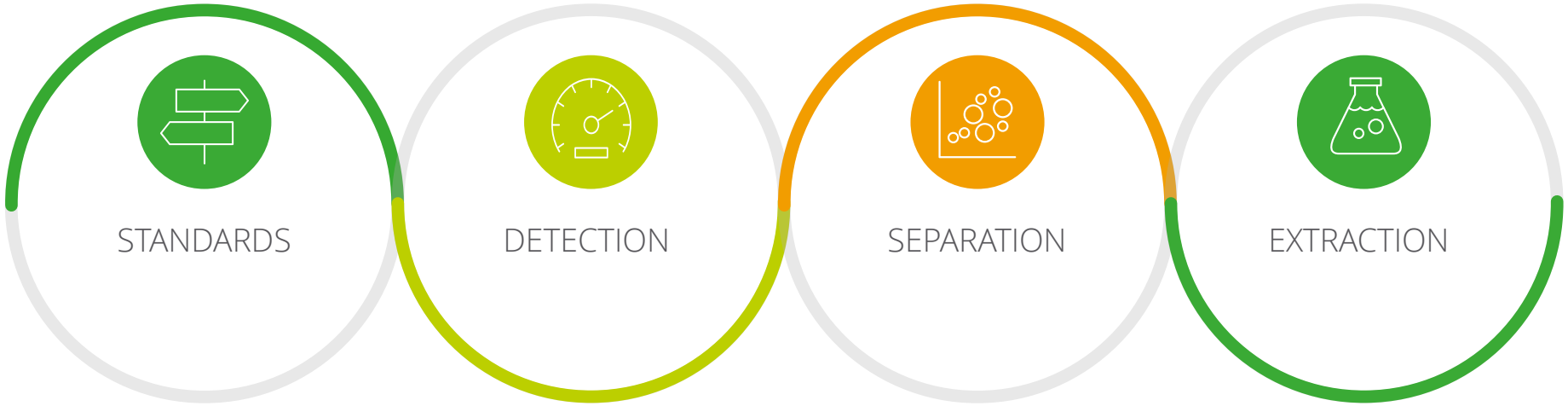
The funnel effect

No pain no gain

Start from the end of the analytical process

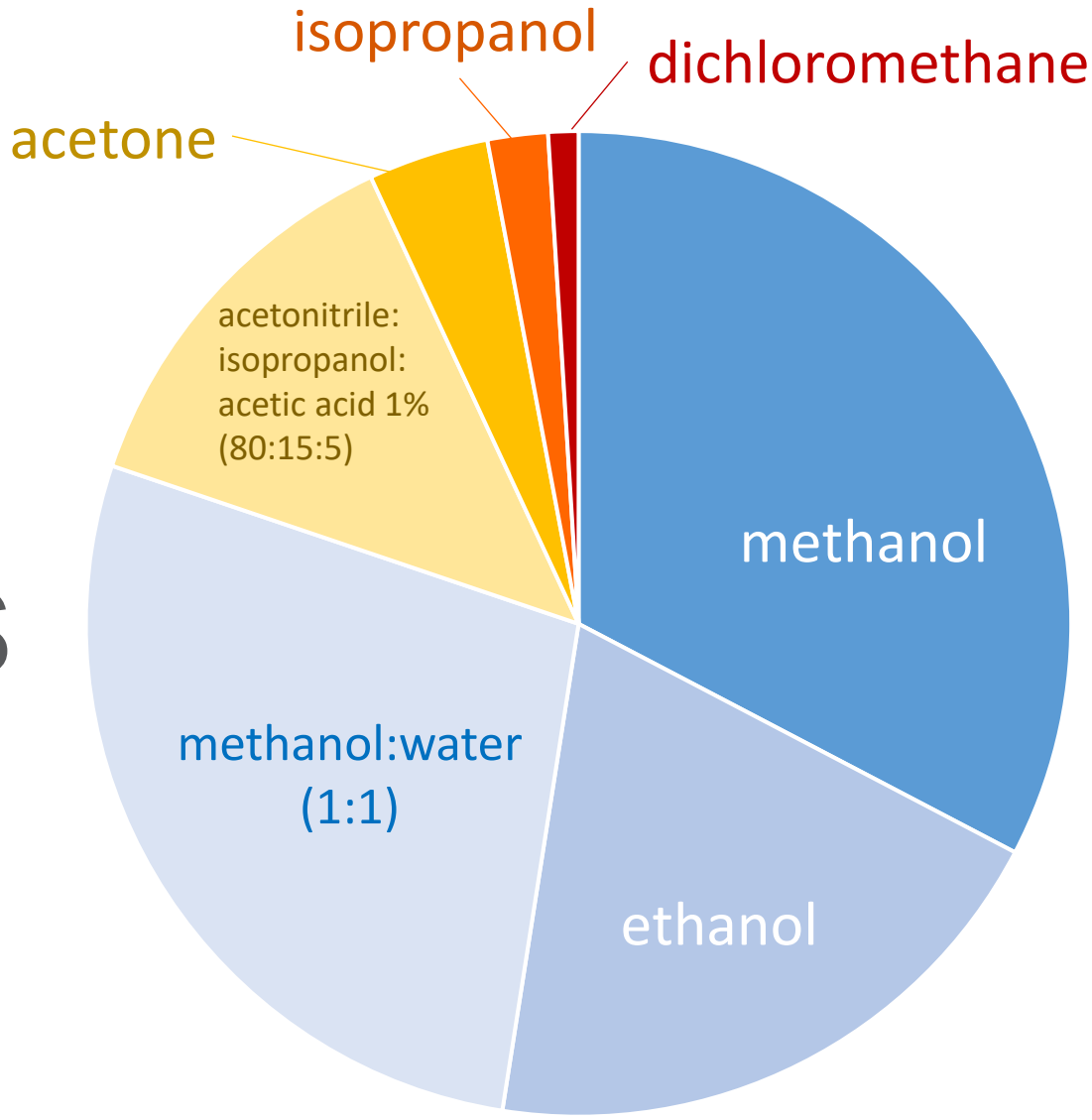


FROM STOCK SOLUTIONS to WORKING MIXES

How to mix substances with various stability properties, different instrumental response, environmental requirements?

Solubilisation tests

95 substances



Daily laboratory work

MS response

The “MS-sensitive” Mix (e.g. hydroxybenzoates)

The “non-sensitive” Mix (e.g. Tartazine)

Stability

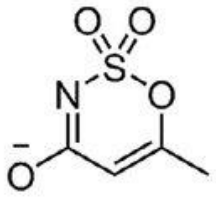
Indigotine has to be handled separately

Sweeteners solutions are more stable at 4°C

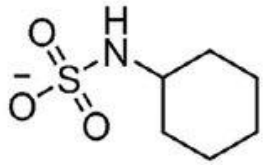
THE DETECTION

MASS SPECTROMETRY

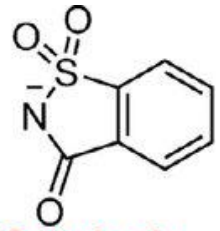
Easy like Sweeteners



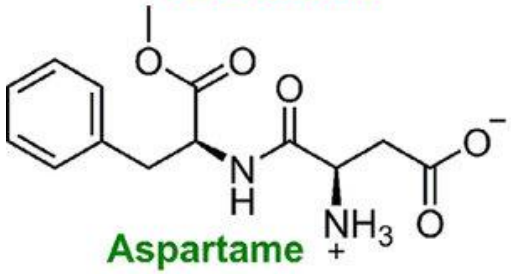
Acesulfame



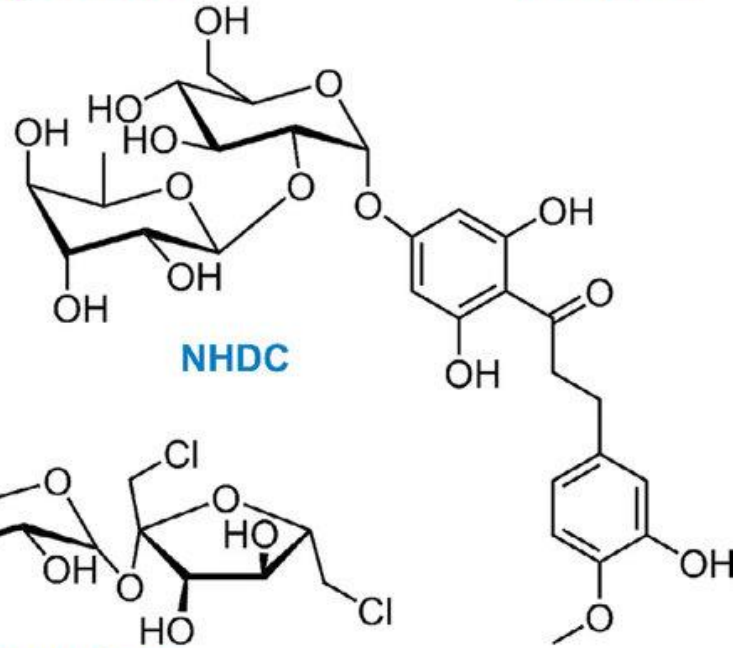
Cyclamate



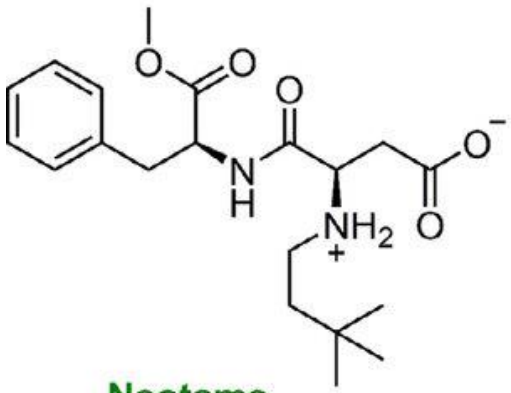
Saccharin



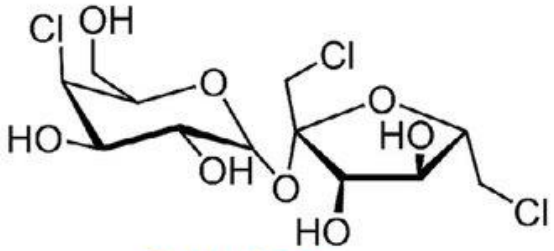
Aspartame



NHDC

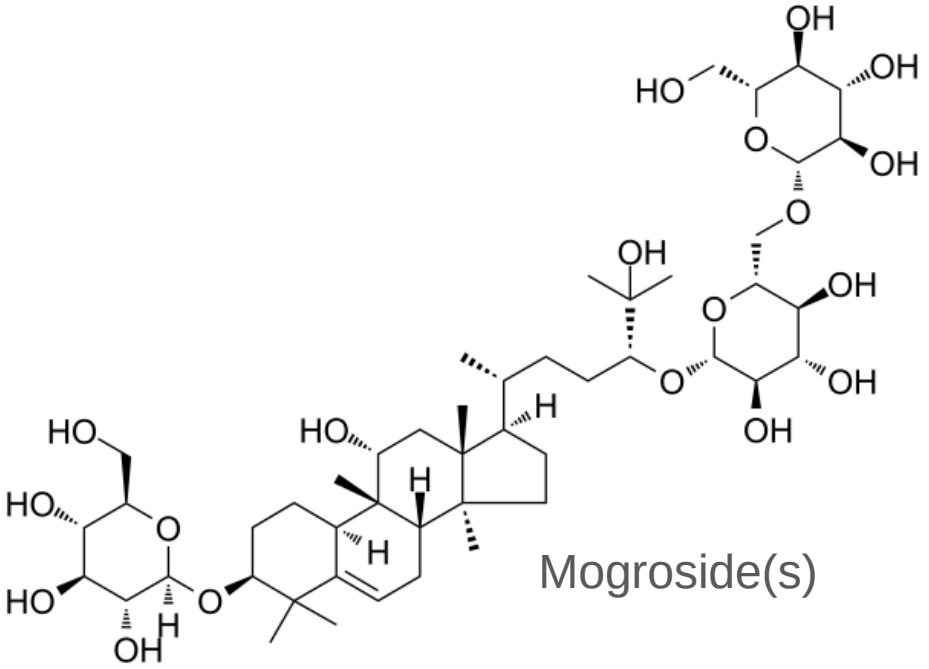


Neotame



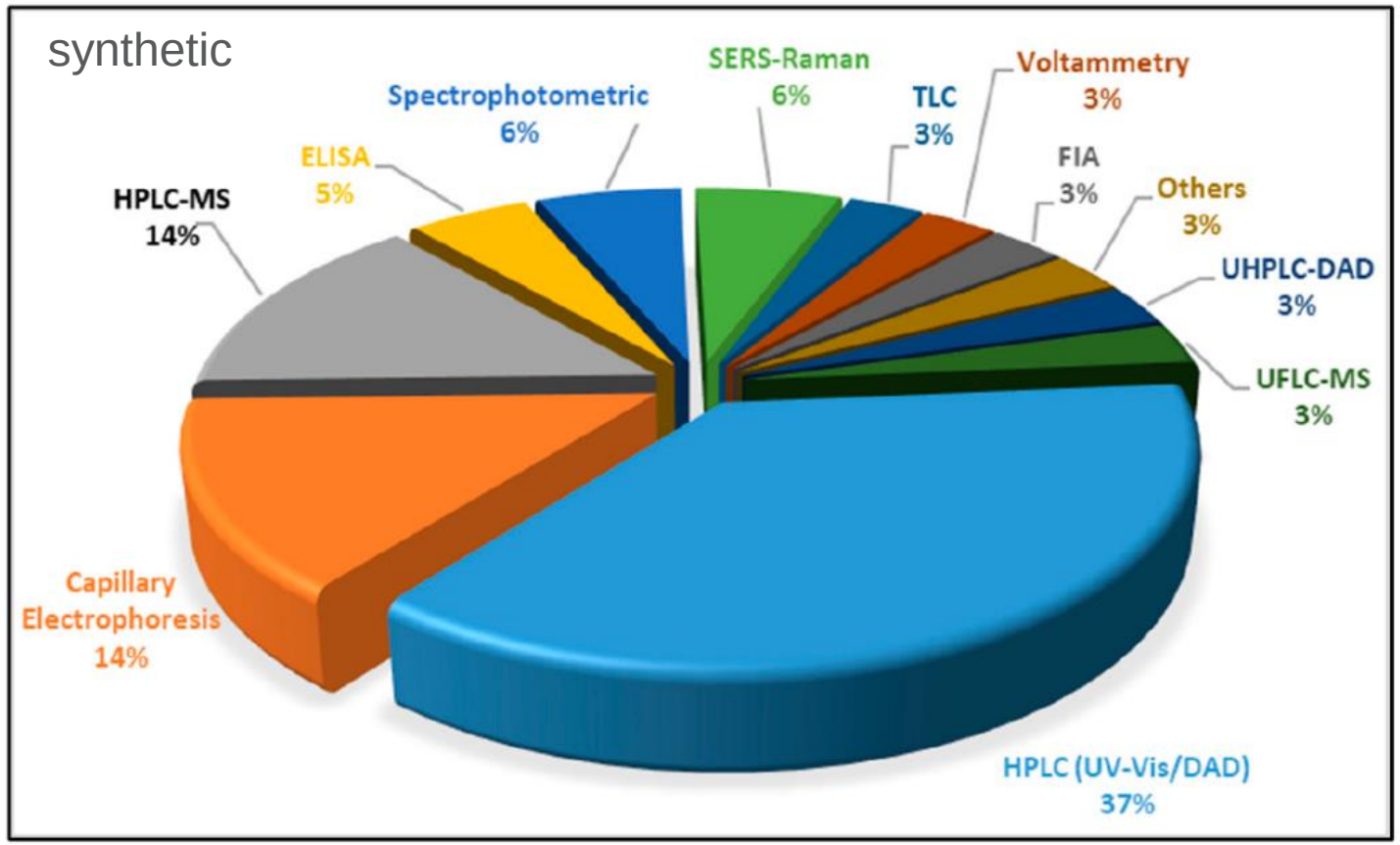
Sucralose

MS/MS transitions are very easy to find by solution infusion!



Mogroside(s)

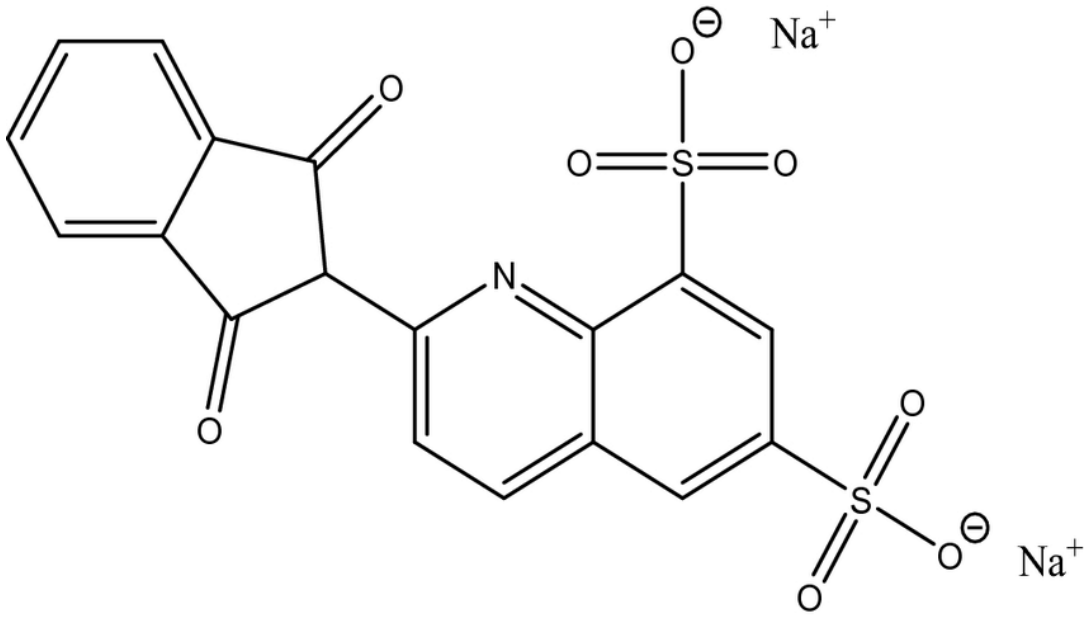
Terrible like Colours



m/z in some cases
corresponds to a loss of 1 Na+,
Or 2 Na+
or no losses!

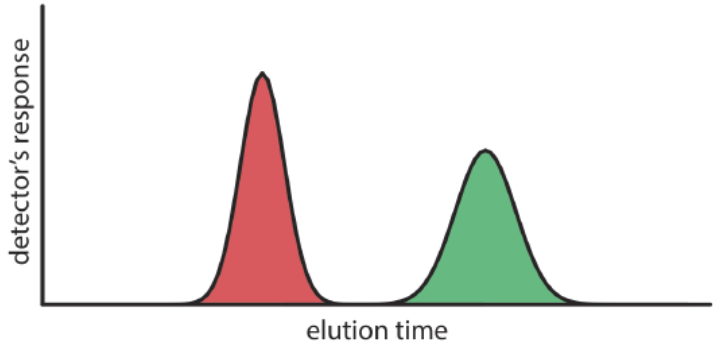
Published in 2020!

Terrible like Colours



Quinoline Yellow

2 chromatographic peaks



3.6 min charge = 2
MS/MS transition 215 > 80

4.3 min single charge
MS/MS transition 432 > 352

Related issues…

1 MS/MS transition

2 compounds

- Quinoline Yellow E 104
- Mogroside III-E

Low ES ionisation efficiency

4 compounds:

- TBHQ E 319
- BHA E 320
- BHT E 321

synthetic phenolic antioxidants

- Brilliant Black BN E 151

Background noise – Low MW (< 192 Da)

7 compounds: *small organic acids*

- Sorbic acid E 200
- Benzoic acid E 210
- Lactic acid E 270
- Malic acid E 296
- Fumaric acid E 297
- Propionic acid E 280
- Citric acid E 300

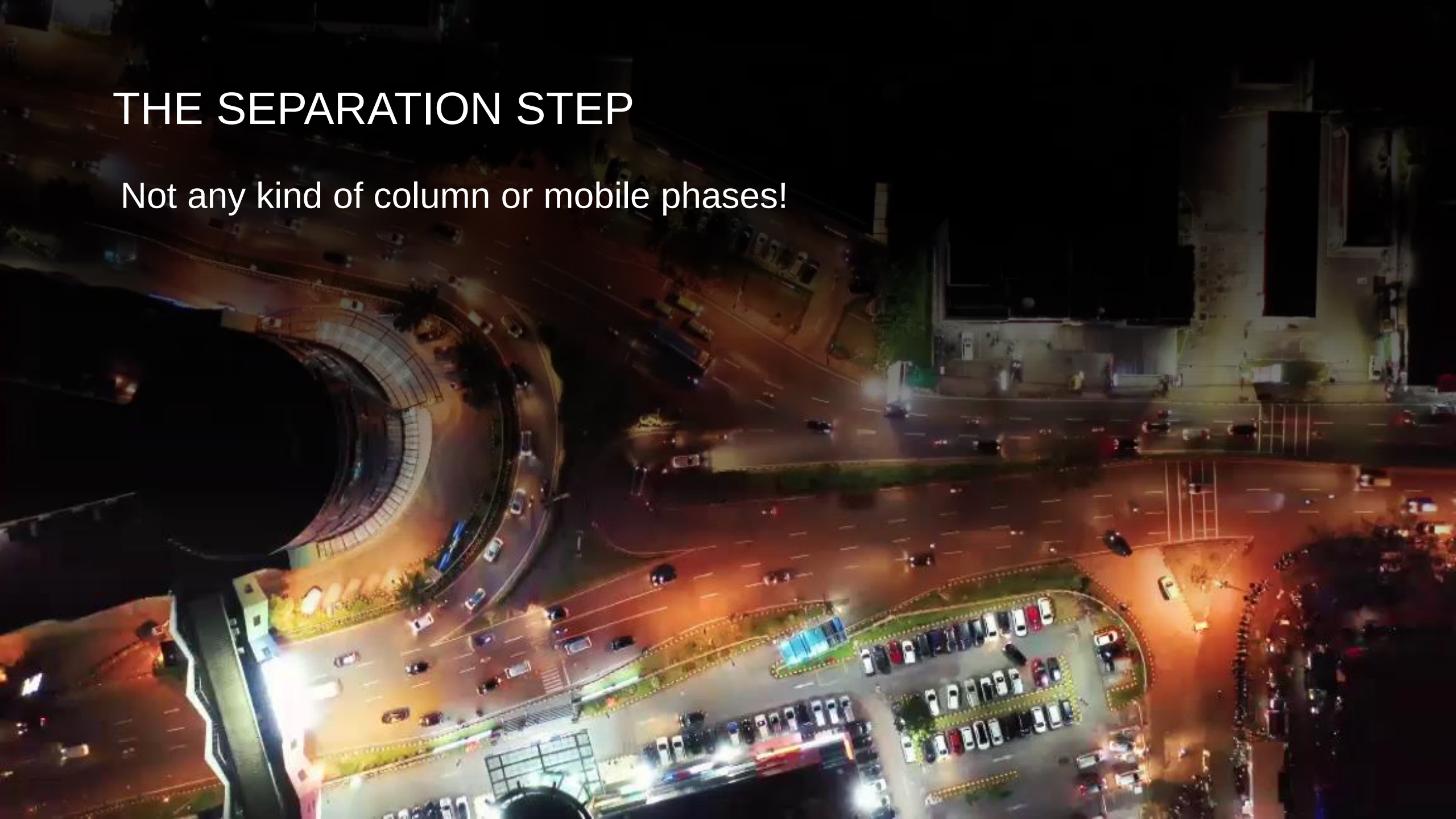
Low stock solution stability

1 compound:

- Erythorbic acid E 315

THE SEPARATION STEP

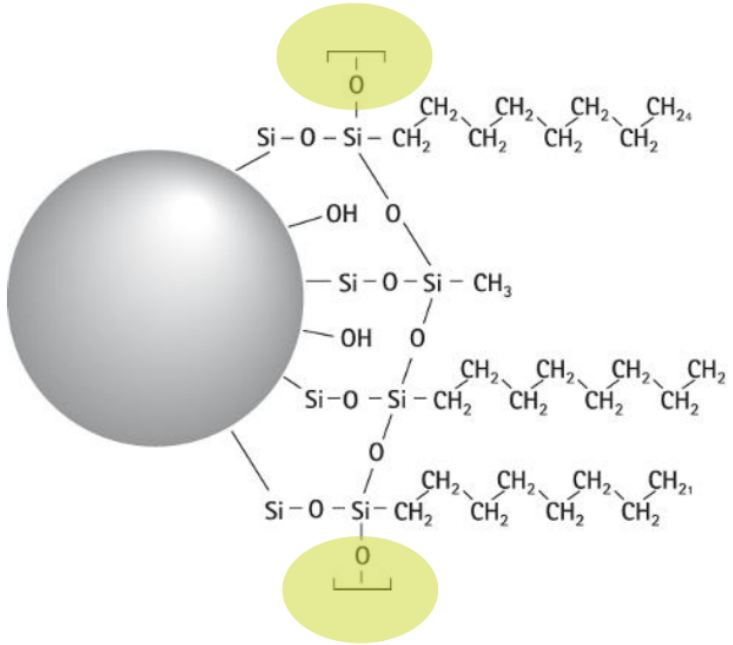
Not any kind of column or mobile phases!



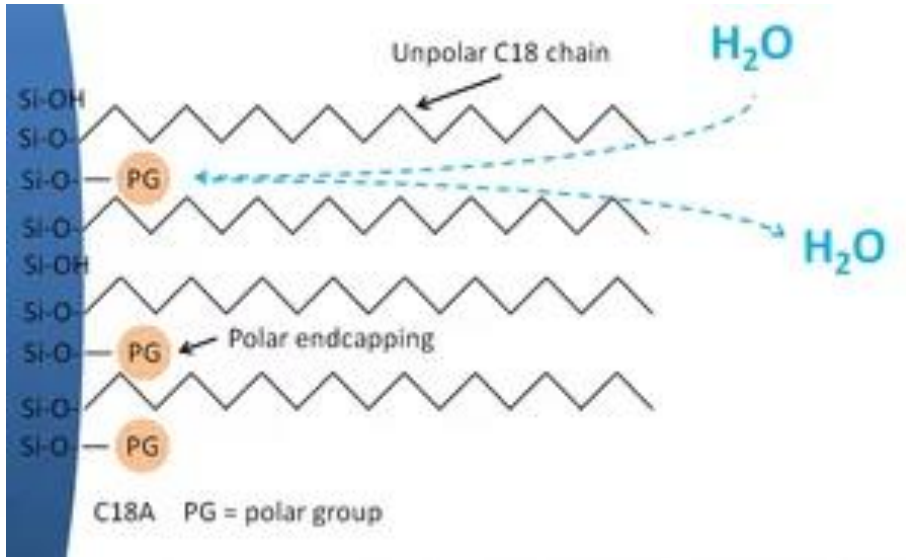
Not all C18 columns are created equal!

Specifications: endcap, carbon load, polar interactions

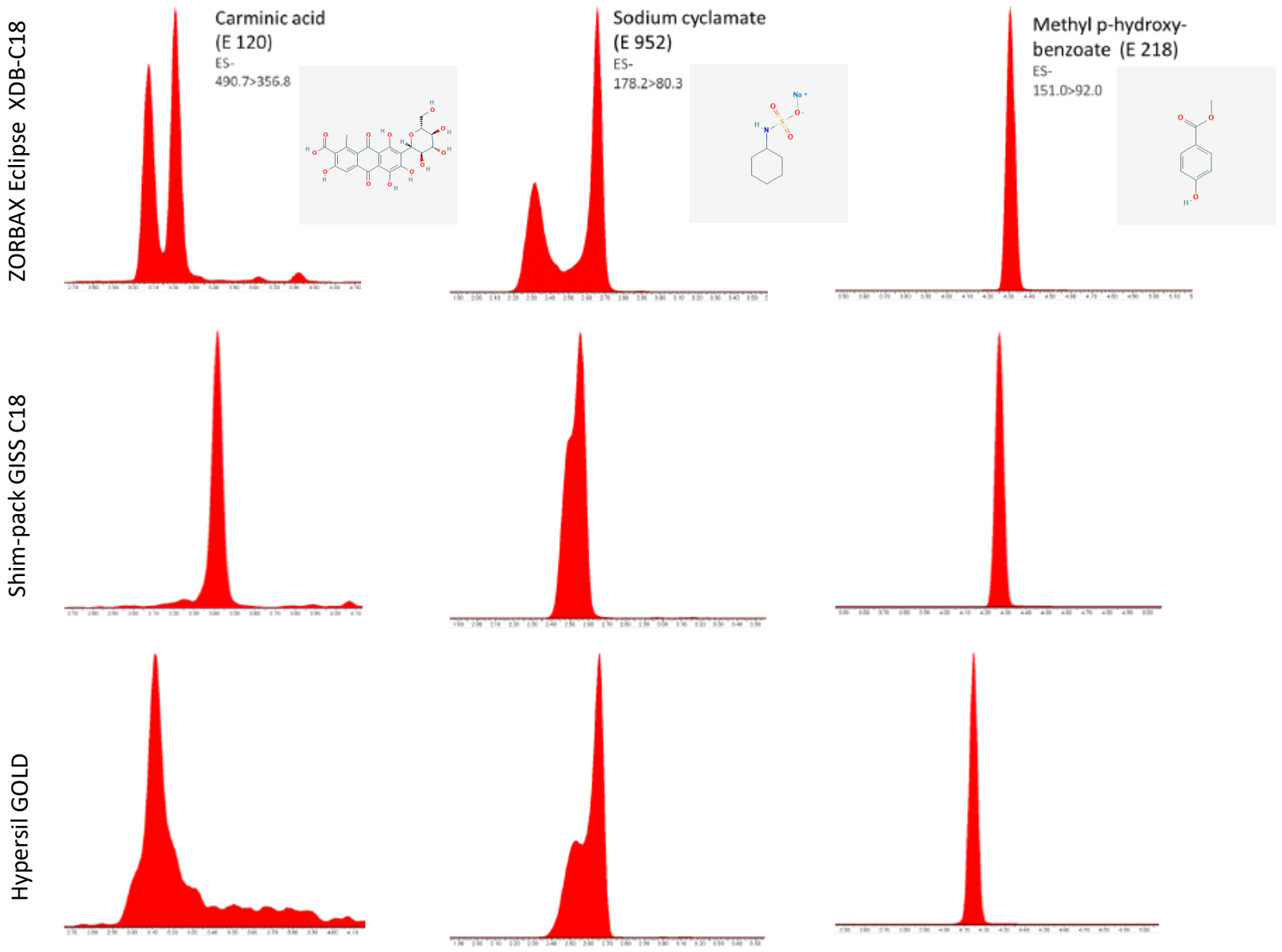
more **carbon load** usually means more retention for nonpolar compounds



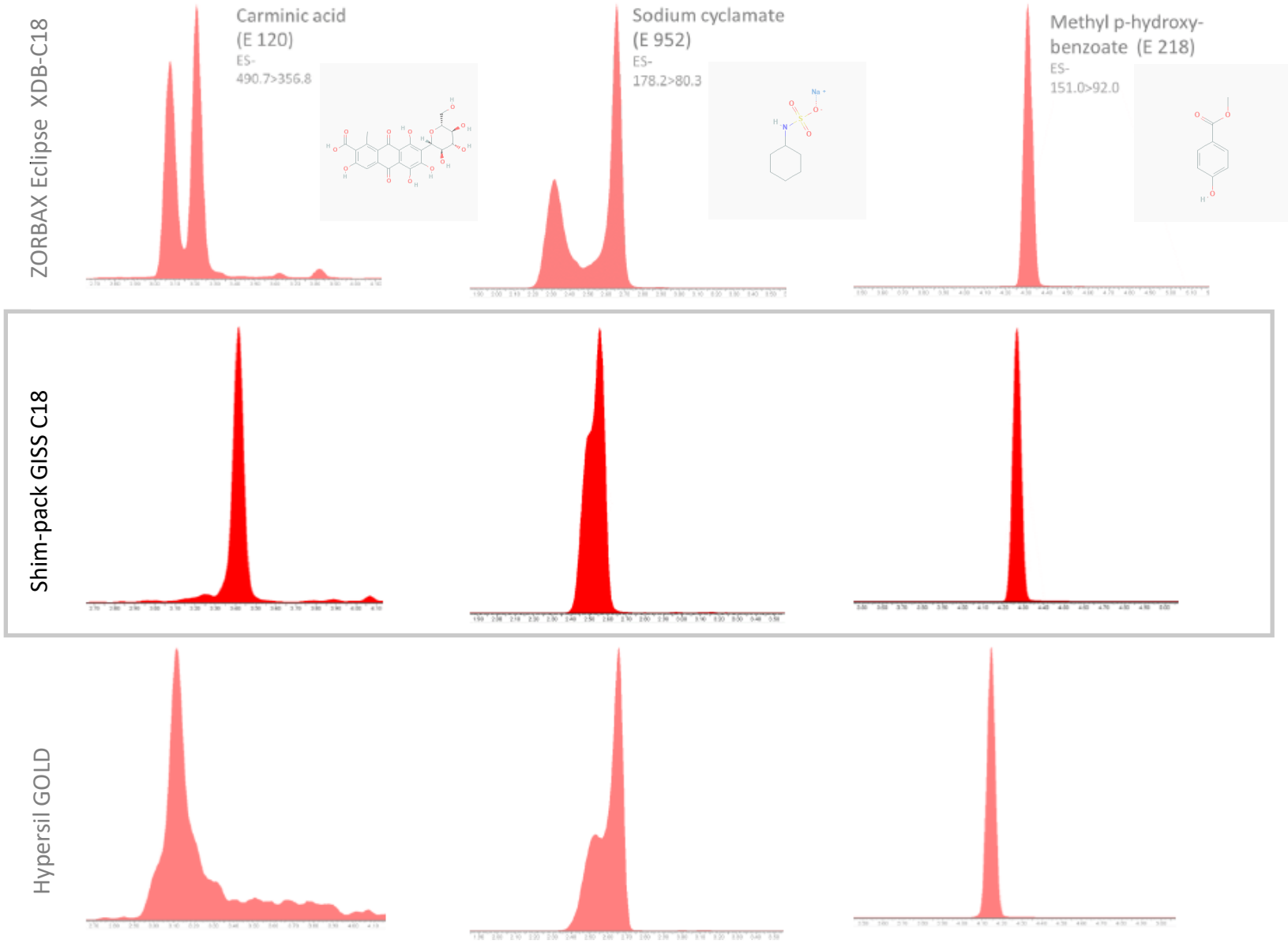
in some circumstances, endcapping may be **GOOD**, in others endcapping may **not make a difference**



Column choice



Column choice



Mobile phases: to be or not to be eccentric?

Favour the ionisation process: Neg and Pos

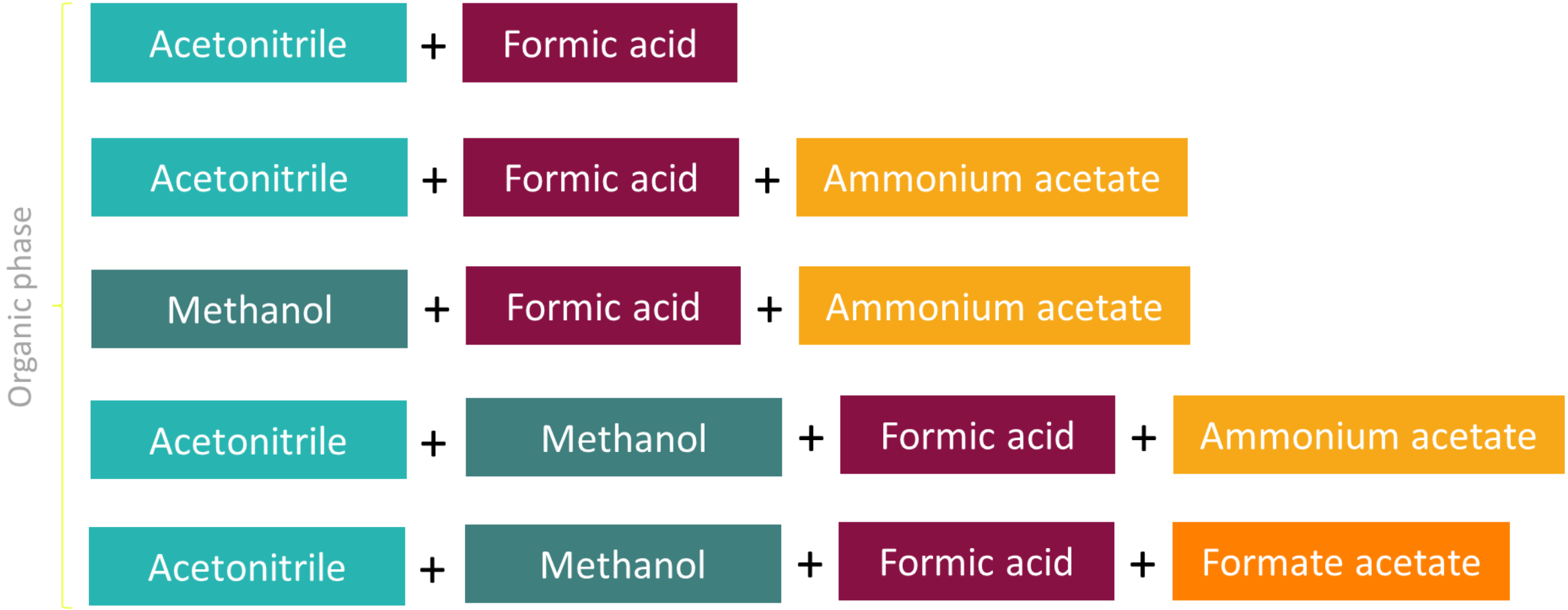
Keep the pressure under control

Compatibility with the injection solvents

Balance good distribution, leave some space for new compounds:

- 12 minutes in positive mode
- 10 minutes in negative mode

Mobile phases: To be or not to be eccentric?



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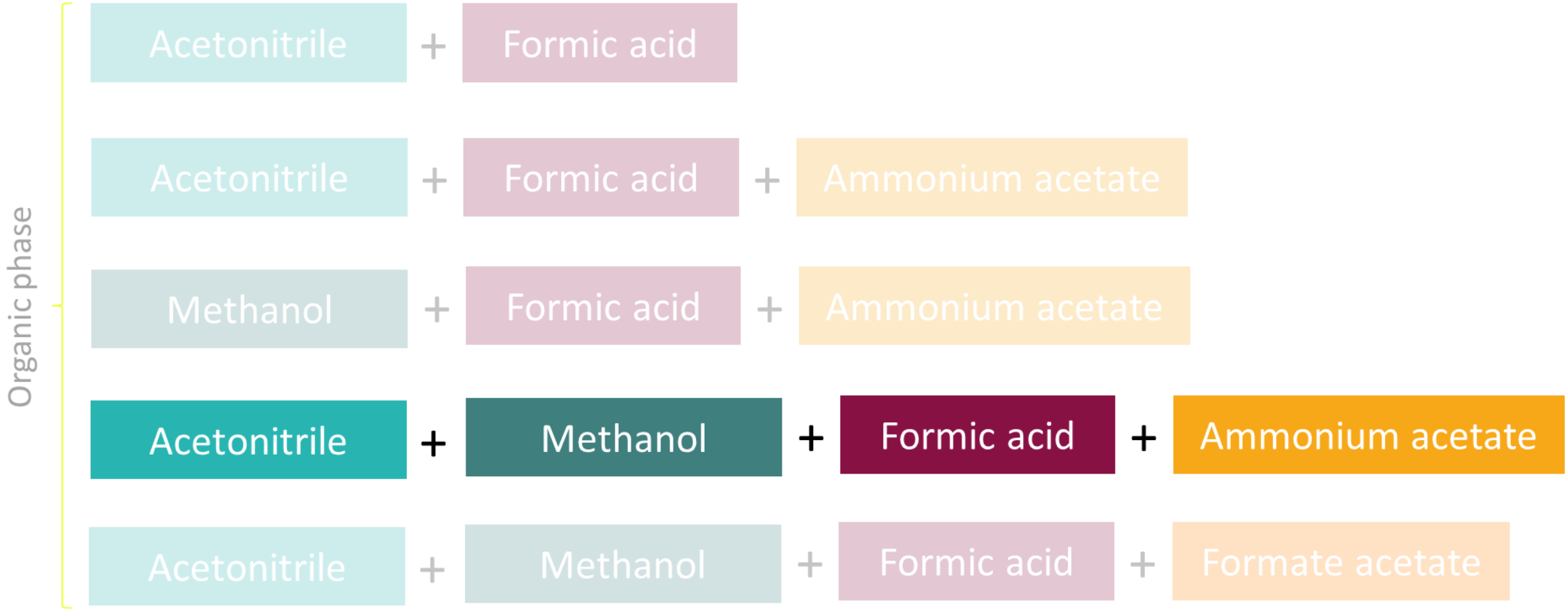
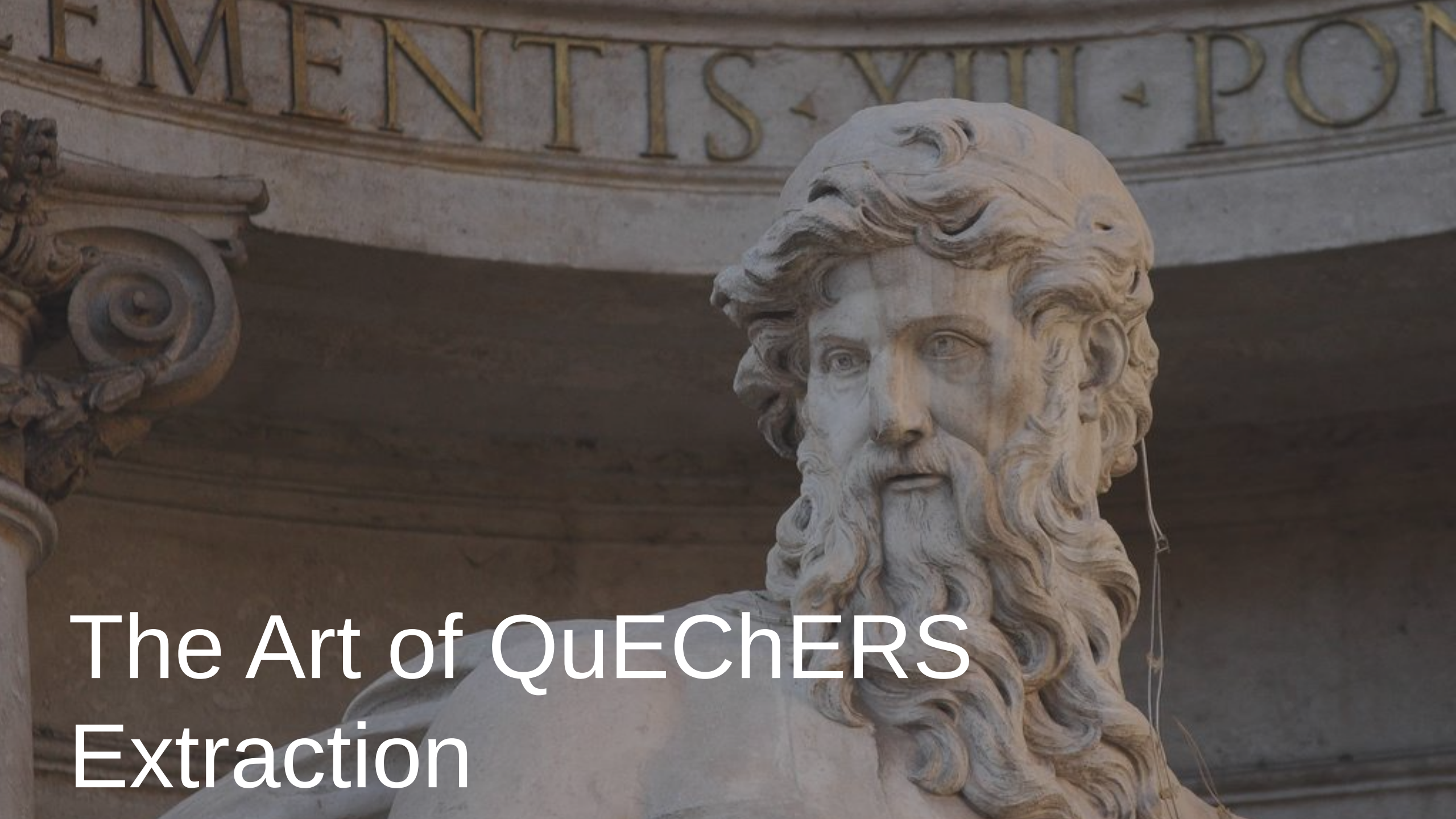


THE EXTRACTION STEP

Selective (trap the targeted fish), Robust (food matrices), and if possible fast!



The Art of QuEChERS Extraction

The Art of QuEChERS Extraction: 2 STEPS

Generic as QuEChERS: STEP 1

EXTRACTION:
Add Acetonitrile

SALTING-OUT: add salts and shake

CENTRIFUGE
Aliquot of supernatant

EXTRACTION STEP

Generic as QuEChERS: STEP 2

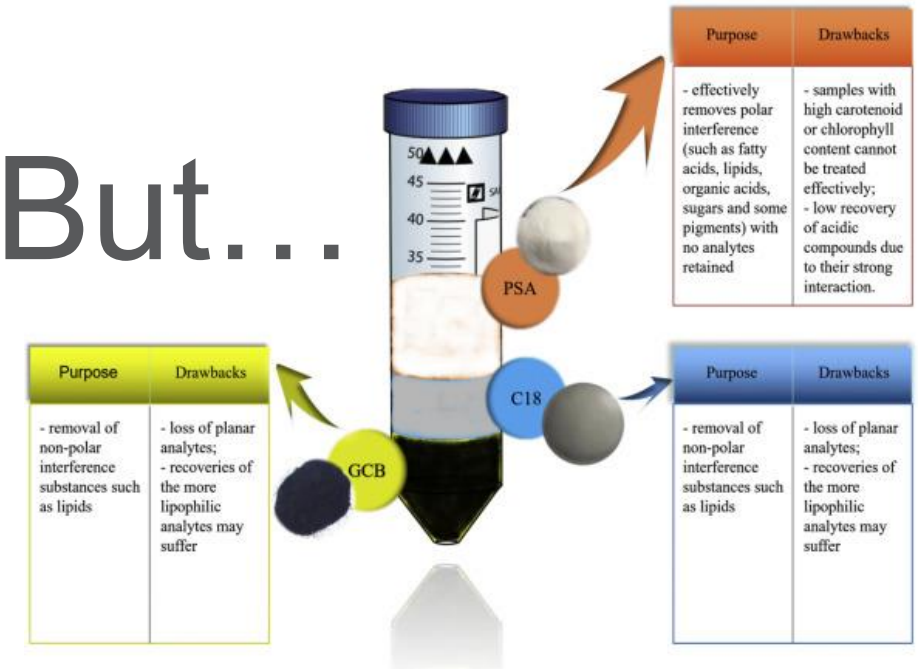
CLEAN-UP

d-SPE: **add sorbents and salts Shake**

CENTRIFUGE Aliquot of supernatant

Dispersive sorbents

But…



Purpose	Drawbacks
- effectively removes polar interference (such as fatty acids, lipids, organic acids, sugars and some pigments) with no analytes retained	- samples with high carotenoid or chlorophyll content cannot be treated effectively; - low recovery of acidic compounds due to their strong interaction.

Purpose	Drawbacks
- removal of non-polar interference substances such as lipids	- loss of planar analytes; - recoveries of the more lipophilic analytes may suffer

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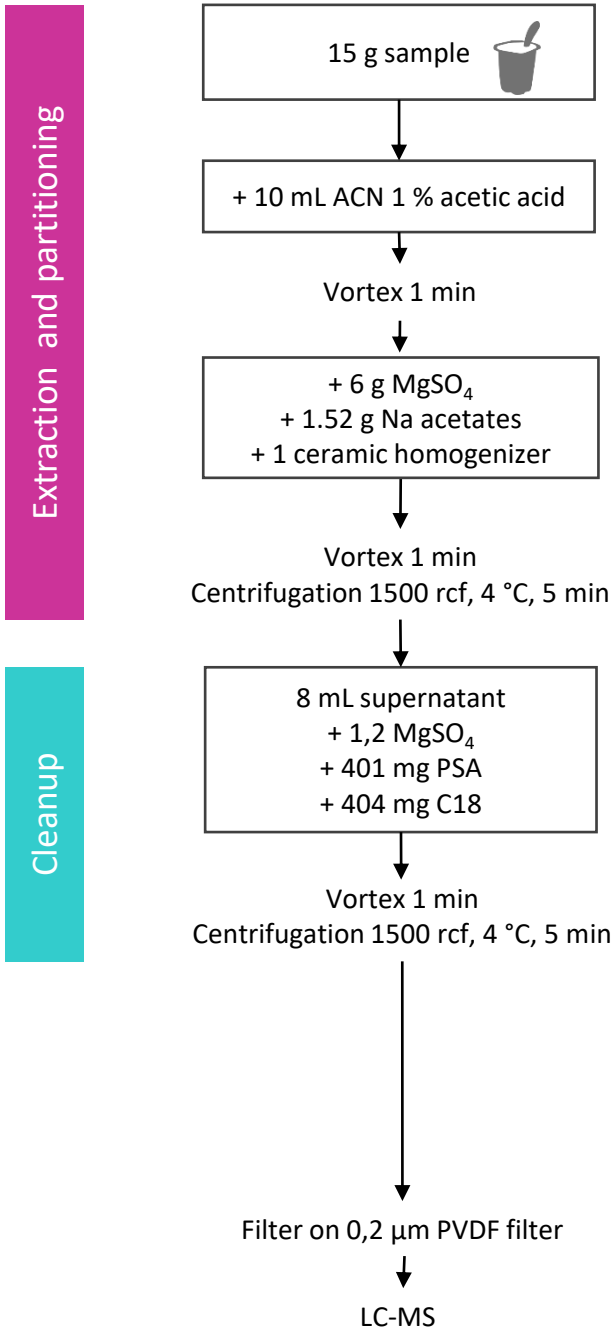
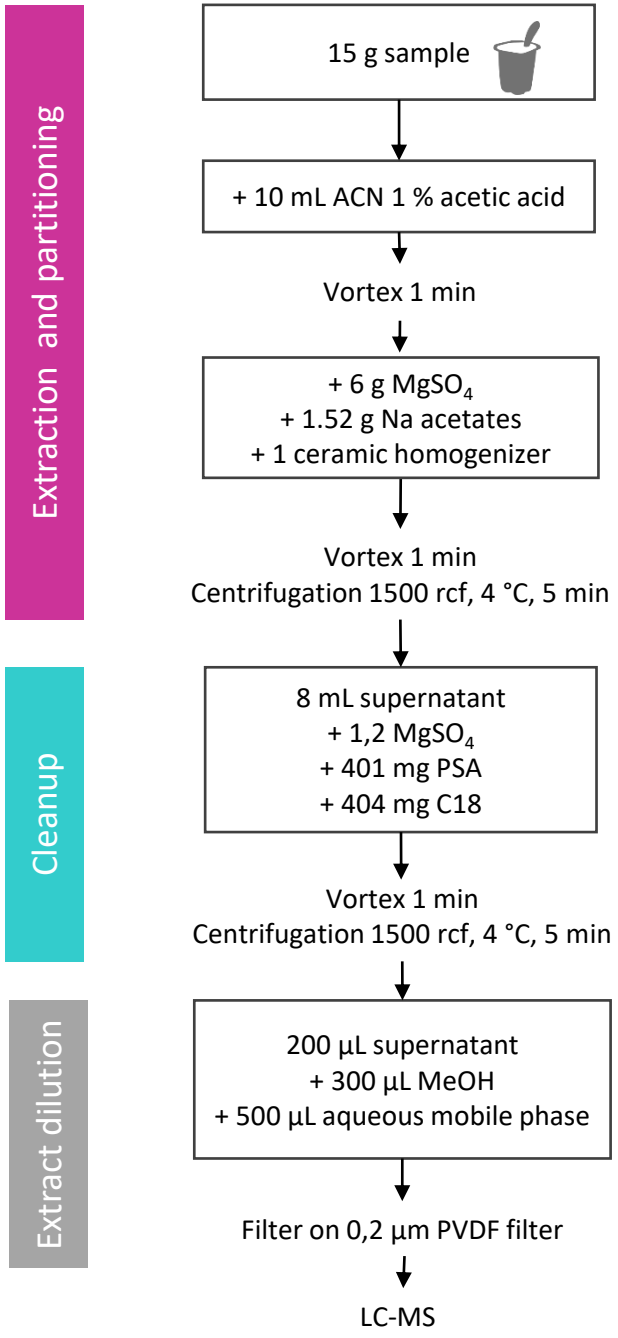
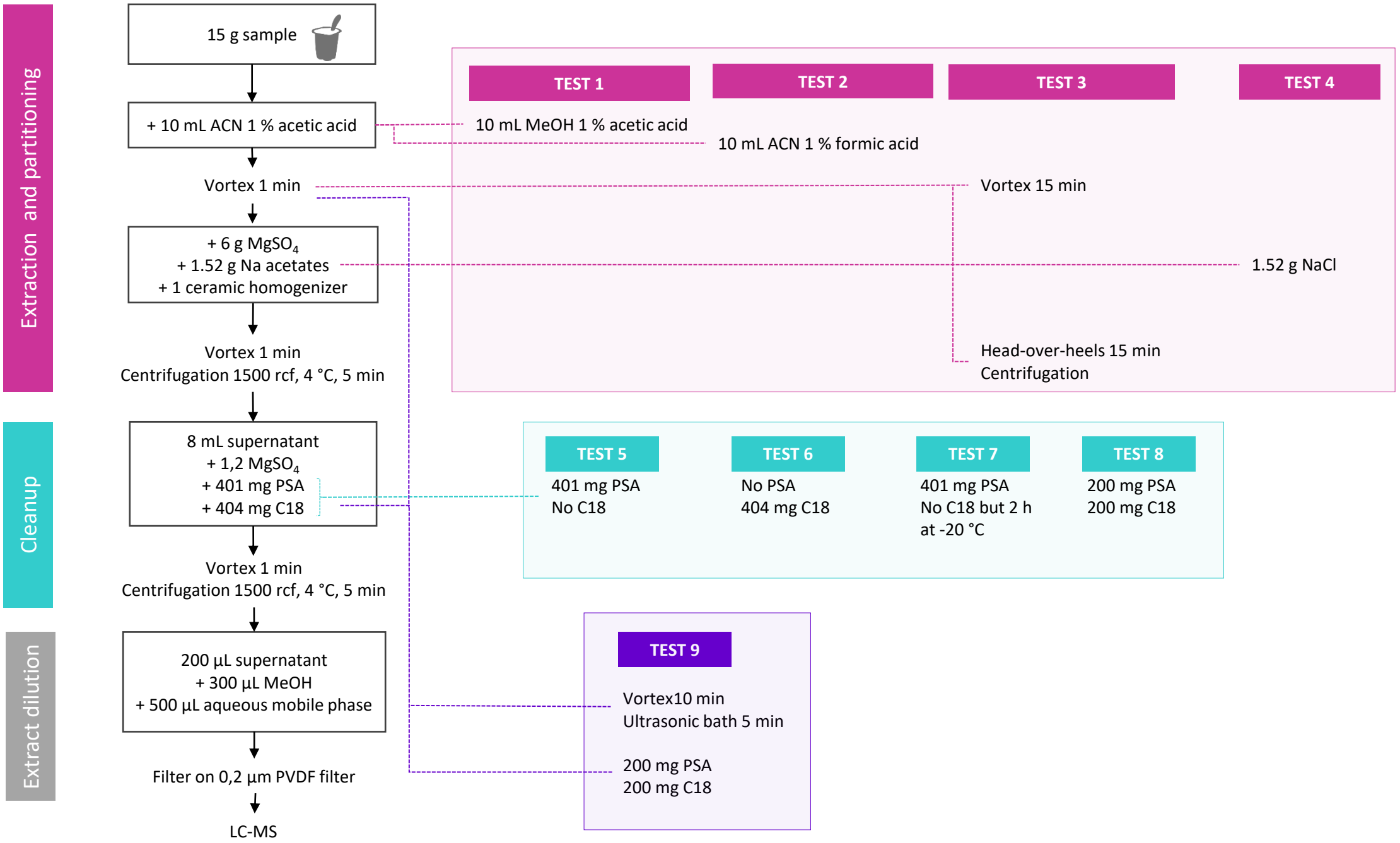
- removal of non-polar interference substances such as lipids	- loss of planar analytes; - recoveries of the more lipophilic analytes may suffer

QuEChERS extraction

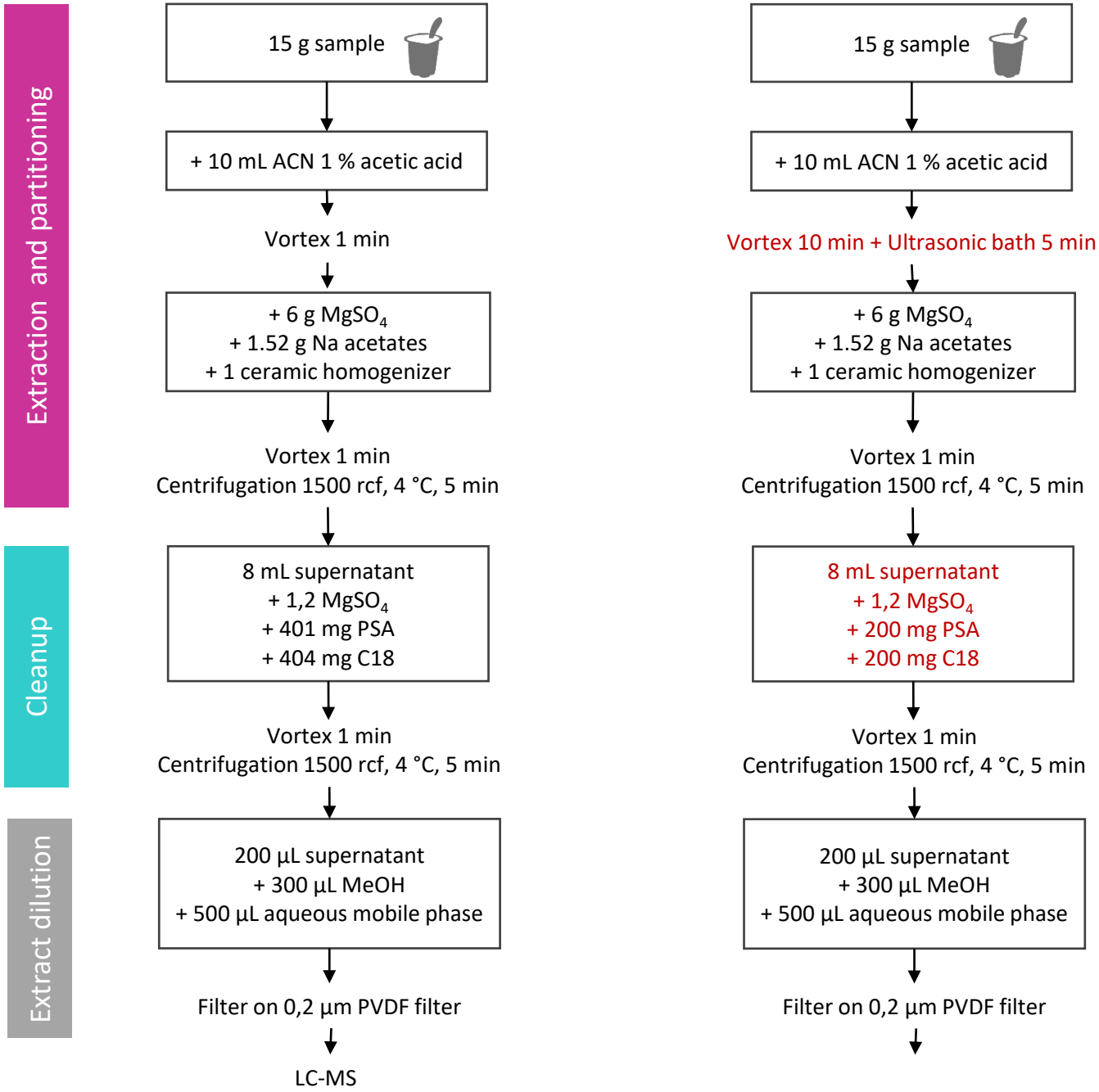


QuEChERS extraction




Extraction and partitioning
Cleanup
Extract dilution
15 g sample
+ 10 mL ACN 1 % acetic acid
Vortex 1 min
+ 6 g MgSO4
+ 1.52 g Na acetates
+ 1 ceramic homogenizer
Vortex 1 min
Centrifugation 1500 rcf, 4 °C, 5 min
8 mL supernatant
+ 1,2 MgSO4
+ 401 mg PSA
+ 404 mg C18
Vortex 1 min
Centrifugation 1500 rcf, 4 °C, 5 min
200 µL supernatant
+ 300 µL MeOH
+ 500 µL aqueous mobile phase
Filter on 0,2 µm PVDF filter
LC-MS
TEST 1
TEST 2
TEST 3
TEST 4
10 mL MeOH 1 % acetic acid
10 mL ACN 1 % formic acid
Vortex 15 min
1.52 g NaCl
Head-over-heels 15 min
Centrifugation
TEST 5
401 mg PSA
No C18
TEST 6
No PSA
404 mg C18
TEST 7
401 mg PSA
No C18 but 2 h
at -20 °C
TEST 8
200 mg PSA
200 mg C18
TEST 9
Vortex10 min
Ultrasonic bath 5 min
200 mg PSA
200 mg C18

QuEChERS extraction



Development and adjustments

Sample take

Water
added to sample take

dSPE quantities
for clean-up

QuEChERS extraction adjustements

tests in the lab

Reference

Food matrice		Content for 100 g	Content in sample take	Adjustement protocol
Yoghurt 	Water	80 g	12 g	/
	Fat	3,2 g	480 mg	200 mg C18
	Carbohyd.	13,2 g	1980 mg	200 mg PSA
Pickles 	Water	94 g	9 g	+ 2 mL water
	Fat	0,2 g	50 mg	200 mg C18
	Carbohyd.	3,6 g	360 mg	50 mg PSA
Mayonaise 	Water	17 g	0,5 g	+ 9 mL water
	Fat	81 g	2430 mg	400 mg C18
	Carbohyd.	0 g	0 mg	0 mg PSA

Something is still off!

Recoveries << 50%

Colours

Sunset Yellow FCF (E 110)
Carminic acid (E 120)
Carmoisine (E 122)
Amaranth (E 123)
Ponceau 4R (E 124)
Erythrosine (E 217)
Allura Red AC (E 129)
Brilliant Blue FCF (E 133)

Fast Yellow AB
Yellow 2G
Acid Red 2G
Ponceau SX
Ponceau 6R
Fast Green FCF

Sweeteners

Aspartame (E 951)
Cyclamates (E 952)
Saccharin (E 954)
Alitame
Glycyrrhizin
Mogroside III-E
Mogroside IV
Mogroside V

Preserv. & antiox.

Natamycin (E 235)
Rosmarinic acid
Dehydroacetic acid

Something is still off!

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Polar compounds !

Something is still off!

Also problems for polar pesticides extraction…

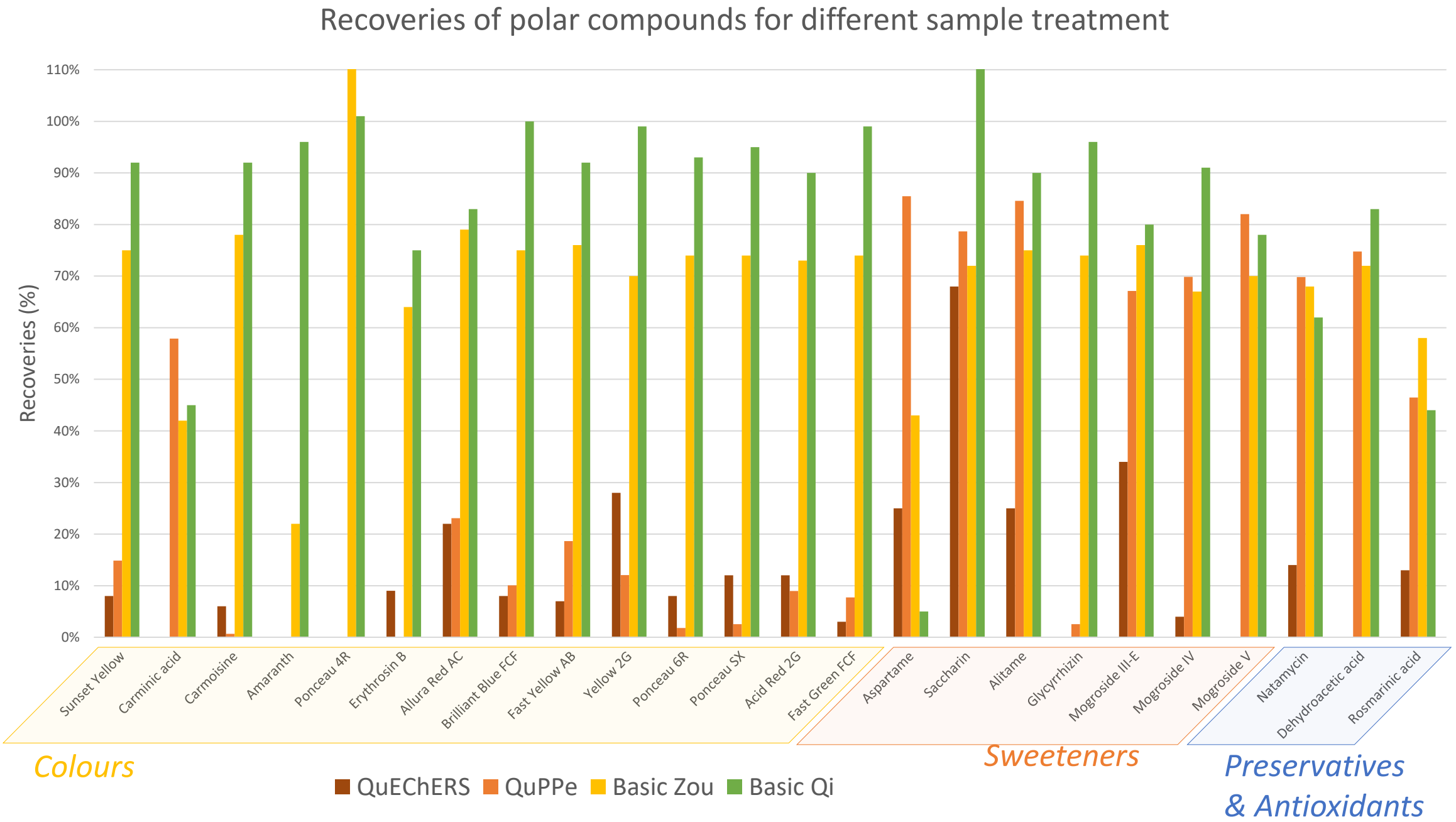
Quick **P**olar **Pe**sticides Method (QuPPe)

Extraction solvent: methanol 1 % formic acid

Different « basified methanol extraction » methods published

Extraction solvent ethanol-ammonia-water (80:1:19, V/V/V) (Zou *et al.*, 2013)

methanol-ammonia-water (80:2:18, v/v/v) (Qi *et al.*, 2015)


Recoveries of polar compounds for different sample treatment
Recoveries (%)
110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Sunset Yellow
Carminic acid
Carmoisine
Amaranth
Ponceau 4R
Erythrosin B
Allura Red AC
Brilliant Blue FCF
Fast Yellow AB
Yellow 2G
Ponceau 6R
Ponceau SX
Acid Red 2G
Fast Green FCF
Aspartame
Saccharin
Alitame
Glycyrrhizin
Mogroside III-E
Mogroside IV
Mogroside V
Natamycin
Dehydroacetic acid
Rosmarinic acid
Colours
Sweeteners
Preservatives & Antioxidants
QuEChERS
QuPPe
Basic Zou
Basic Qi

Basified methanol extraction: ajustements

Basified methanol extraction: ajustements

2 g

20 or **200** mg C18
No PSA!

depending on matrix fat content

Basified methanol extraction: ajustements

2 g

20 or **200** mg C18
No PSA!

depending on matrix fat content

For cereal based products

Very low recoveries (H_2O in centrifuged pellet)
→ Hydratation before extraction with solvent mix

+ 2 mL water

Not reproducible results
→ Data treatment without IS

DATA PROCESSING

Quantification and screening for 2 extraction methods

QuEChERS

	E 100 Curcumin
	E 104 Quinoline Yellow
	E 155 Brown HT
	E 160b Bixin
	E 955 Sucralose
	E 959 Neohesperidin DC
	E 961 Neotame
	E 969 Advantame
	E 214 Ethyl p-hydroxybenzoate
	E 218 Methyl p-hydroxybenzoate
	E 310 Propyl gallate
	E 392 Carnosol
	E 392 Carnosic acid
	- Caffeine

Basified methanol

	E 100 Riboflavin
	E 102 Tartrazine
	E 110 Sunset Yellow FCF
	E 120 Carminic acid
	E 122 Carmoisine
	E 123 Amaranth
	E 124 Ponceau 4R
	E 127 Erythrosine
	E 129 Allura Red AC
	E 131 Patent Blue V
	E 132 Indigotine
	E 133 Brilliant Blue FCF
	E 142 Green S
	E 950 Acesulfame K
	E 951 Aspartame
	E 952 Cyclamic acid & salts
	E 954 Saccharin & salts
	E 959 Neohesperidin DC
	E 235 Natamycin

The screening substances

Lowest screening detection determined during 1st validation

- ≠ for each compound
- Matrix dependent

Included in the QC/QA checks

1 MS/MS transition monitored

50	compounds
37	colours
6	sweeteners
7	antioxidants & preservatives

QuEChERS

Auramine O	Basic Red 9
Sudan Yellow (Butter Yellow)	Citrus Red 2
Fast Garnet GBC	Disperse Red 1
Disperse Yellow 3	Rhodamine B
Sudan Orange G	Malachite Green
Basic Orange 2 (Chrysoidine)	Leucomalachite Green
Disperse Orange 3	Sudan Blue 2
Disperse Orange 11	Methylene Blue
Disperse Orange 37	Victoria Pure Blue
Sudan 1	Crystal Violet
Sudan 2	Dulcin
Sudan 3	Heptyl paraben
Sudan 4 + Sudan Red B	Propyl paraben
Sudan Red 7B	Ethyl gallate
Sudan Red G	Octyl gallate
Para Red	Lauryl gallate
Toluidine Red	Ethoxyquin

Basic methanol

Fast Yellow AB
Acid Yellow 36 (Metanil Yellow)
Orange 2
Ponceau 6R (Acid Red 44)
Ponceau SX
Acid Red 2G
Acid Red 52
Fast Green FCF
4-amino carminic acid
Alitame
Glycyrrhizin
Mogroside V
Mogroside III-E
Mogroside IV
Rosmarinic acid

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The WHY and the HOW?

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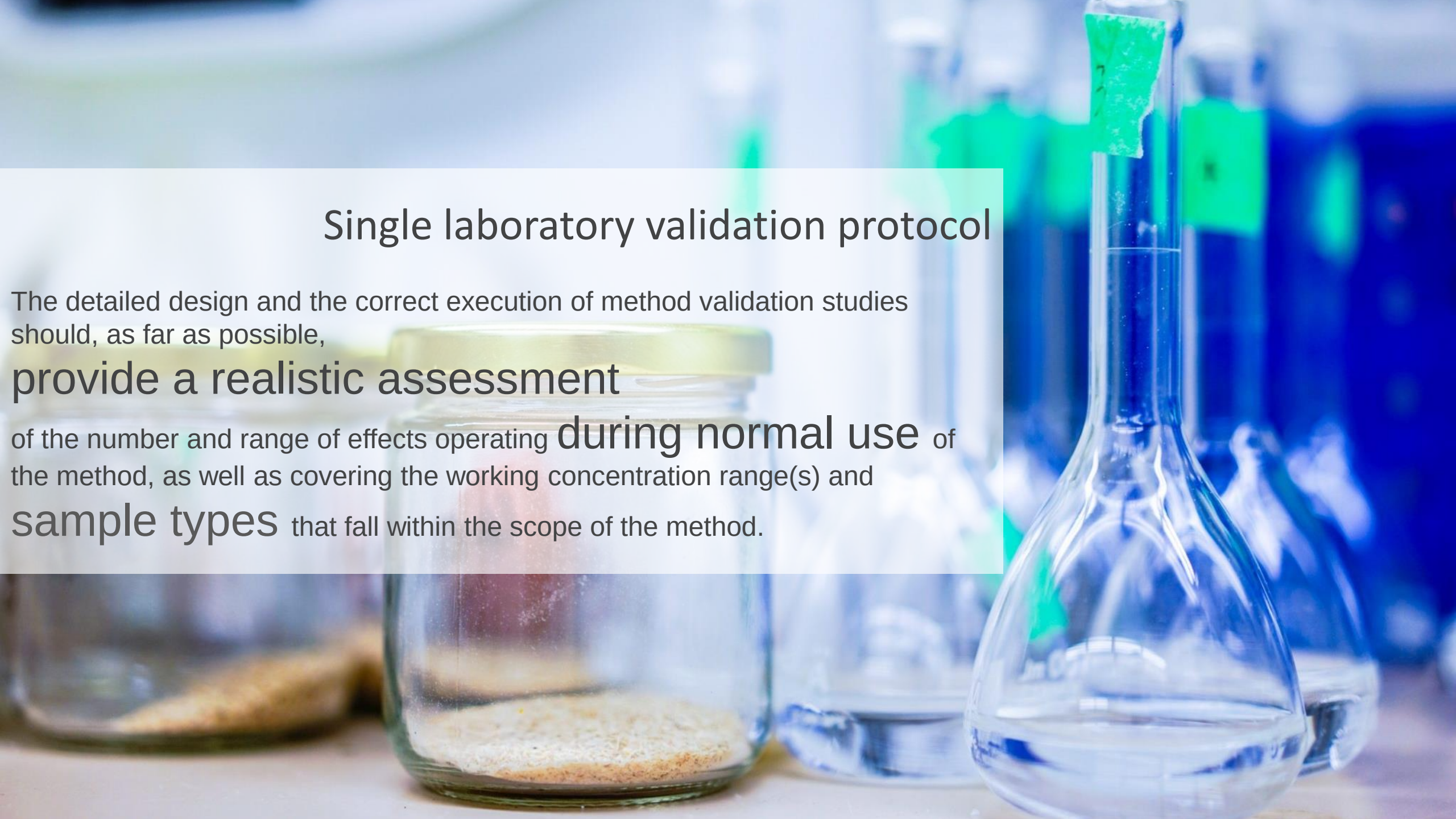
Ultimate test

METHOD PERFORMANCE



Single laboratory validation protocol

The detailed design and the correct execution of method validation studies should, as far as possible,

provide a realistic assessment

of the number and range of effects operating during normal use of the method, as well as covering the working concentration range(s) and

sample types that fall within the scope of the method.

Food Categories 1333/2008	
1	Dairy products and analogues
2	Fats and oils and fat and oil emulsions
3	Edible ices
4	Fruit and vegetables
5	Confectionery
6	Cereals and cereal products
7	Bakery wares
8	Meat
9	Fish and fisheries products
10	Eggs and egg products
11	Sugars, syrups, honey and table-top sweeteners
12	Salts, spices, soups, sauces, salads and protein products
13	Foods intended for particular nutritional uses
14	Beverages
15	Ready-to-eat savouries and snacks
16	Desserts
17	Food supplements
18	Processed foods

Food Categories 1333/2008		Matrices selected for the validation of the method
1	Dairy products and analogues	merged validation with edible ices
2	Fats and oils and fat and oil emulsions	spreads
3	Edible ices	*full fat ice cream*
4	Fruit and vegetables	canned vegetables and fruit mix
5	Confectionery	Sweets
6	Cereals and cereal products	breakfast cereals
7	Bakery wares	Waffles with chocolate
8	Meat	canned sausages
9	Fish and fisheries products	
10	Eggs and egg products	
11	Sugars, syrups, honey and table-top sweeteners	
12	Salts, spices, soups, sauces, salads and protein products	mayonnaise
13	Foods intended for particular nutritional uses	
14	Beverages	Flavoured drinks
15	Ready-to-eat savouries and snacks	potato chips
16	Desserts	Flan
17	Food supplements	
18	Processed foods	lasagne

Validation – Protocol

Selectivity
- Blank matrix

LOD & LOQ
- LOQ: S/N = min. 10
  Lowest point of calibration linearity curve of concentration
- LOD: S/N = 3

Linearity & working range
- Min. 6 concentration levels
- Fixed working range for all substances

Accuracy

- **Precision**
  - Repeatability
  - Reproducibility

  3 repetitions,
  3 concentrations (low, high and out of curve),
  repeated on 3 days:
  - Day 1 : MATRIX 1
  - Day 2 : MATRIX 2
  - Day 3 : MATRIX 3
- **Trueness**
  - Bias

  From recovery (because no CRM)

Full validation work load

Spikes at 3 concentration levels

- medium
- high
- very high (dilution required)

3 repetitions

3 days

1 day of validation	1 full validation	
12	36	samples to extract (blanks comprised)
30	90	extracts injections (calibration curve, BP, spikes)
12	36	hours of injection (ES+ and ES-)

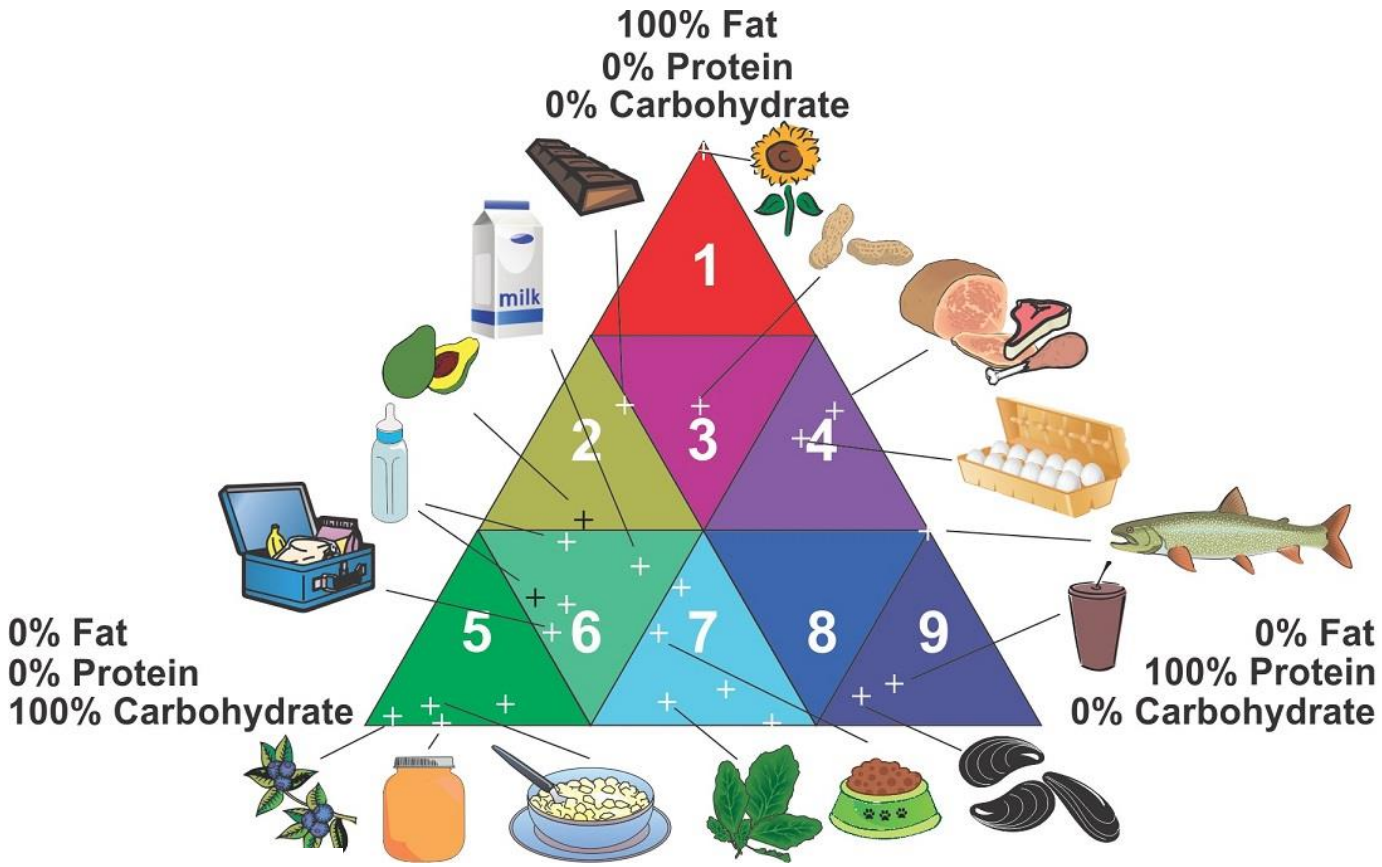
+ data treatment!

X 12

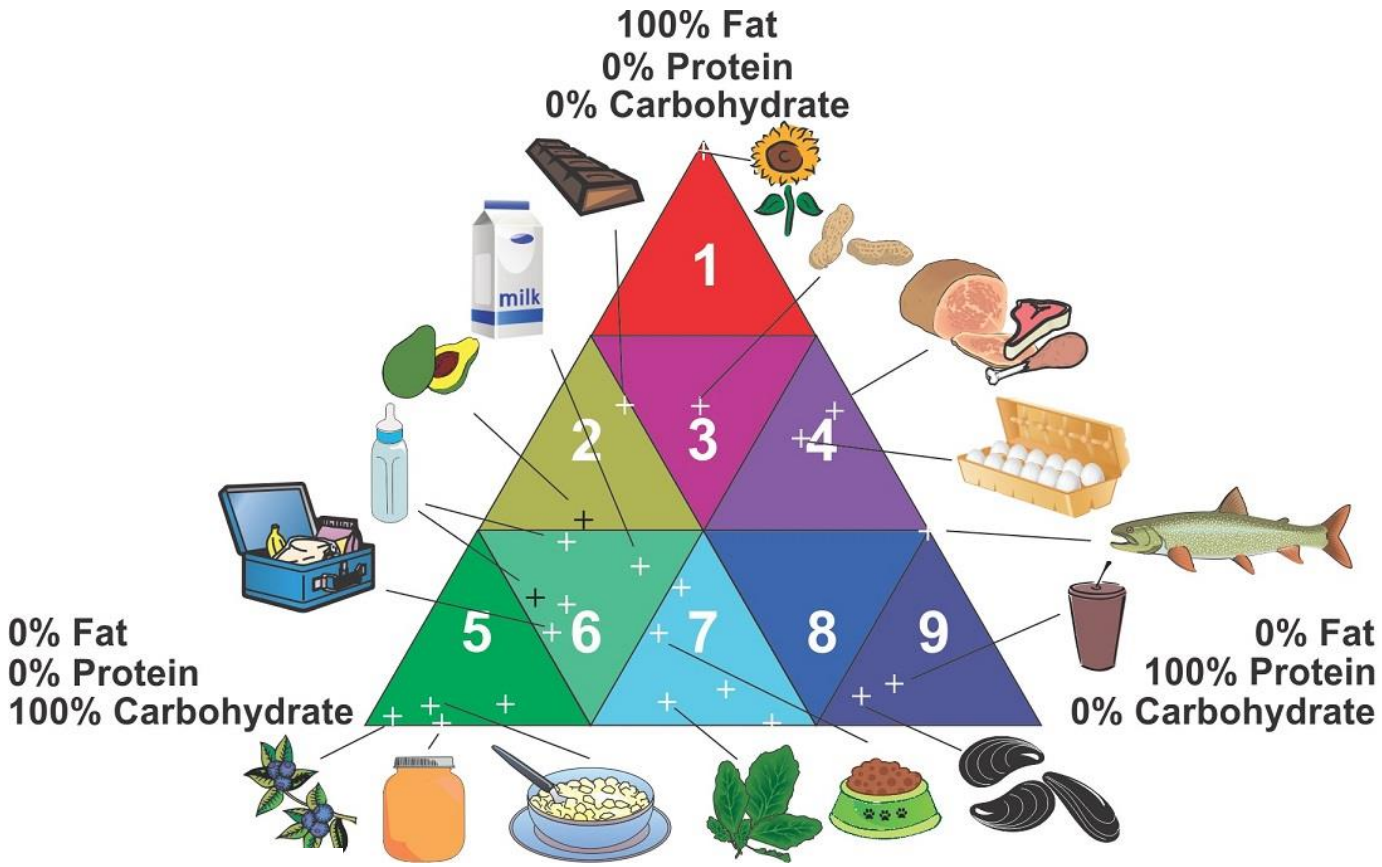

Tool 1

Not all matrices are created equal!

AOAC matrix clusters



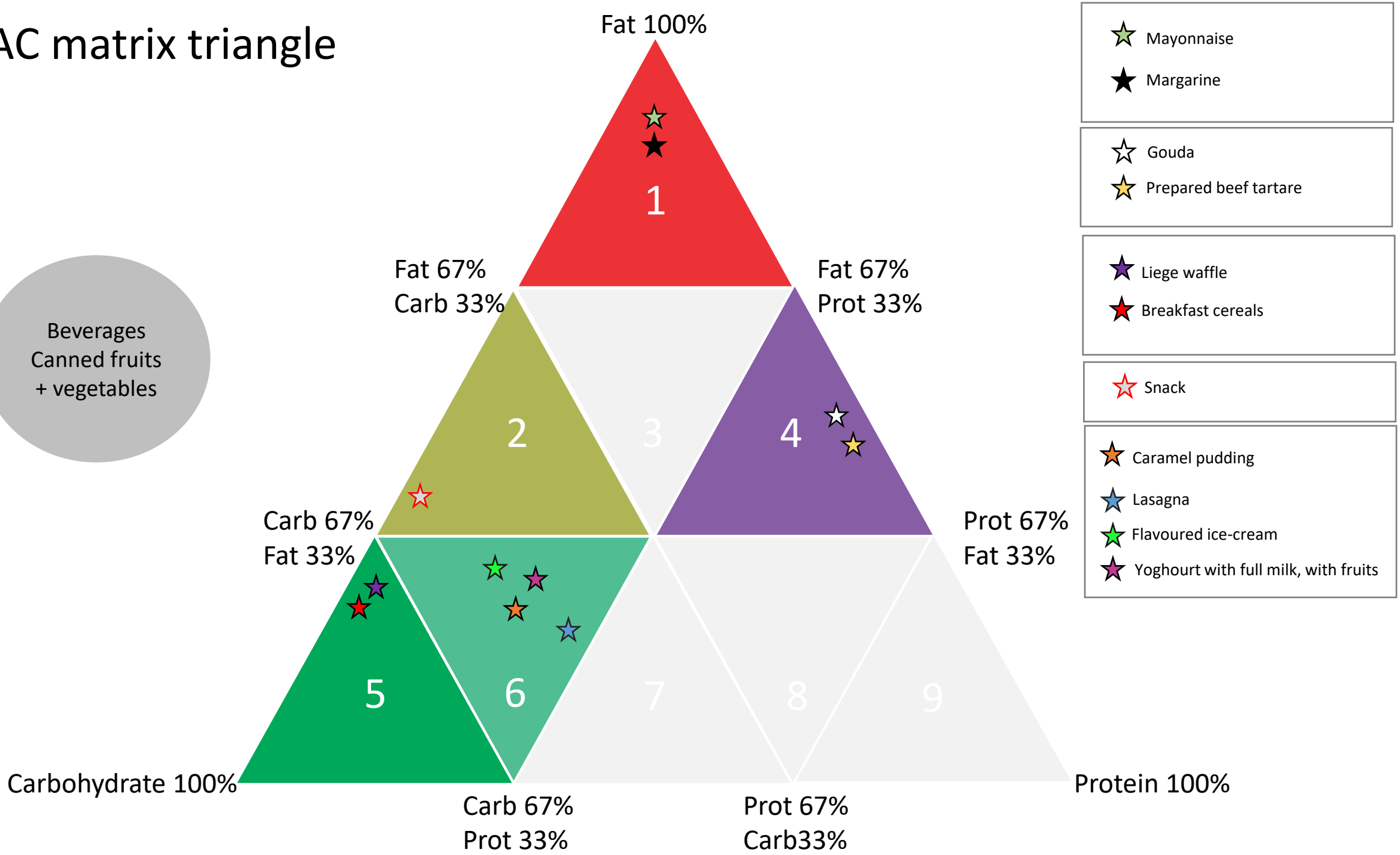
Not all matrices are created equal!



What about items with high water content?

Beverages
Canned fruits
+ vegetables

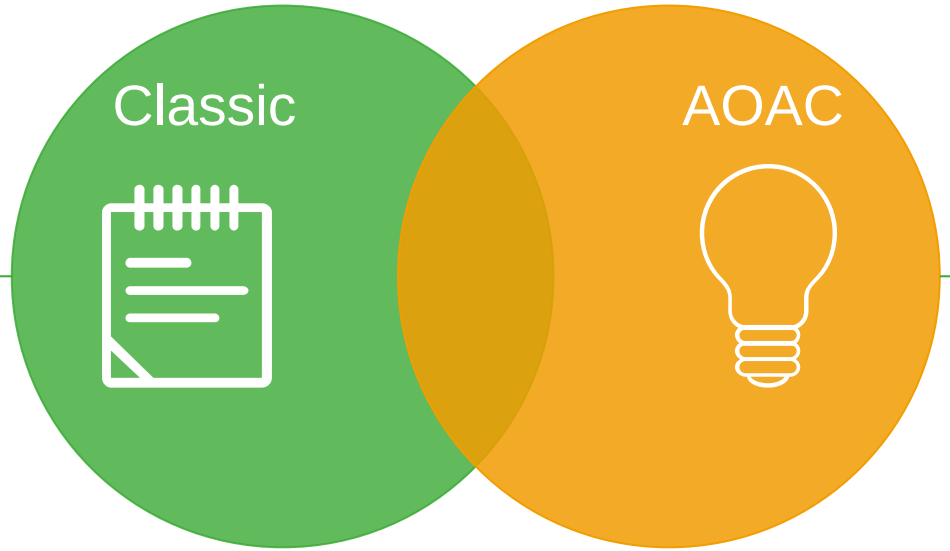
AOAC matrix triangle



Food Categories 1333/2008		Matrices selected for the validation of the method	AOAC triangle number
1	Dairy products and analogues	merged validation with edible ices	4+6
2	Fats and oils and fat and oil emulsions	spreads	1
3	Edible ices	*full fat ice cream*	6
4	Fruit and vegetables	canned vegetables and fruit mix	“Circle”
5	Confectionery	Sweets	“Circle”
6	Cereals and cereal products	breakfast cereals	5
7	Bakery wares	Waffles with chocolate	5
8	Meat	canned sausages	4
9	Fish and fisheries products		
10	Eggs and egg products		
11	Sugars, syrups, honey and table-top sweeteners		
12	Salts, spices, soups, sauces, salads and protein products	mayonnaise	1
13	Foods intended for particular nutritional uses		
14	Beverages	Flavoured drinks	“Circle”
15	Ready-to-eat savouries and snacks	potato chips	2
16	Desserts	Flan	6
17	Food supplements		
18	Processed foods	lasagne	6

Cutting in HALF!

12 full validations
Considering
the classic selection of
matrix groups



6 full validations ☺
for the same matrix
coverage:
Using the AOAC groups
3 ≠ foods
per matrix group

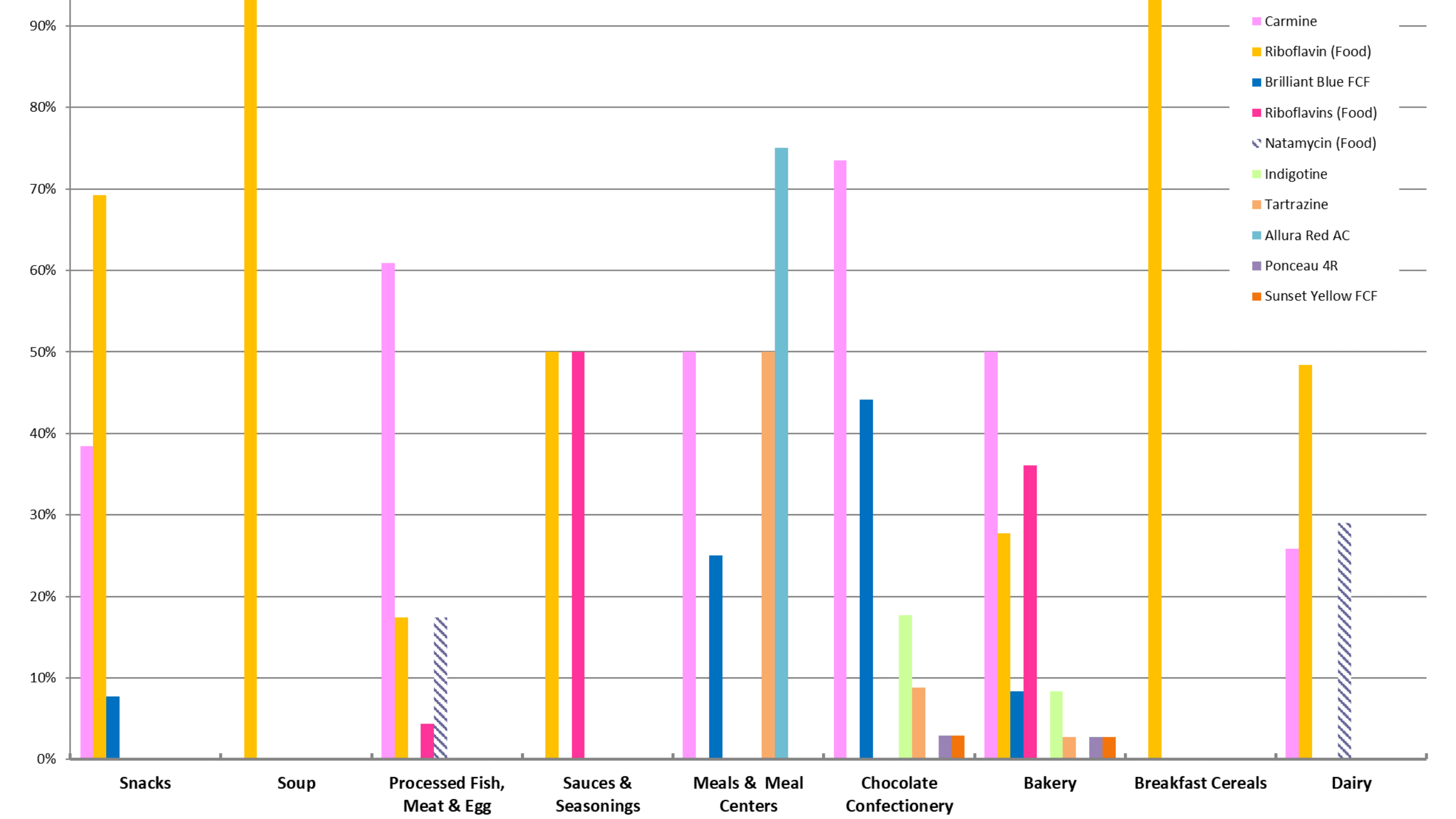

Tool 2

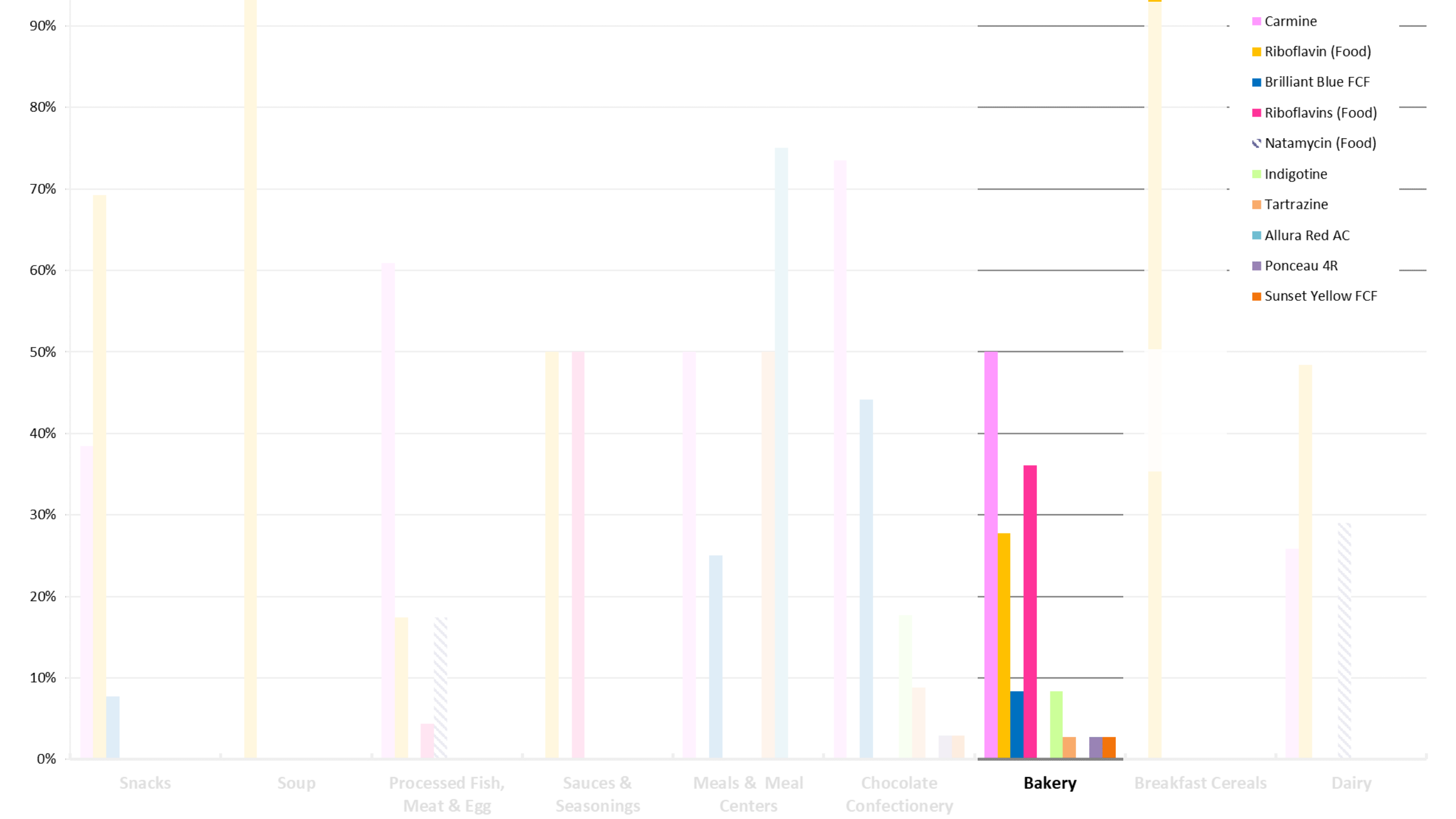
This database is an online database, which observes product introductions in consumer packaged goods marketed worldwide.

The GNPD contains data of EU food markets since 1996 and currently
25 of its 28 member countries and Norway are represented in the GNPD.10

New foods are regularly added to the database.

Scope for the Methanol Basic Method
All food categories


90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Carmine
Riboflavin (Food)
Brilliant Blue FCF
Riboflavins (Food)
Natamycin (Food)
Indigotine
Tartrazine
Allura Red AC
Ponceau 4R
Sunset Yellow FCF
Snacks
Soup
Processed Fish, Meat & Egg
Sauces & Seasonings
Meals & Meal Centers
Chocolate Confectionery
Bakery
Breakfast Cereals
Dairy


90%
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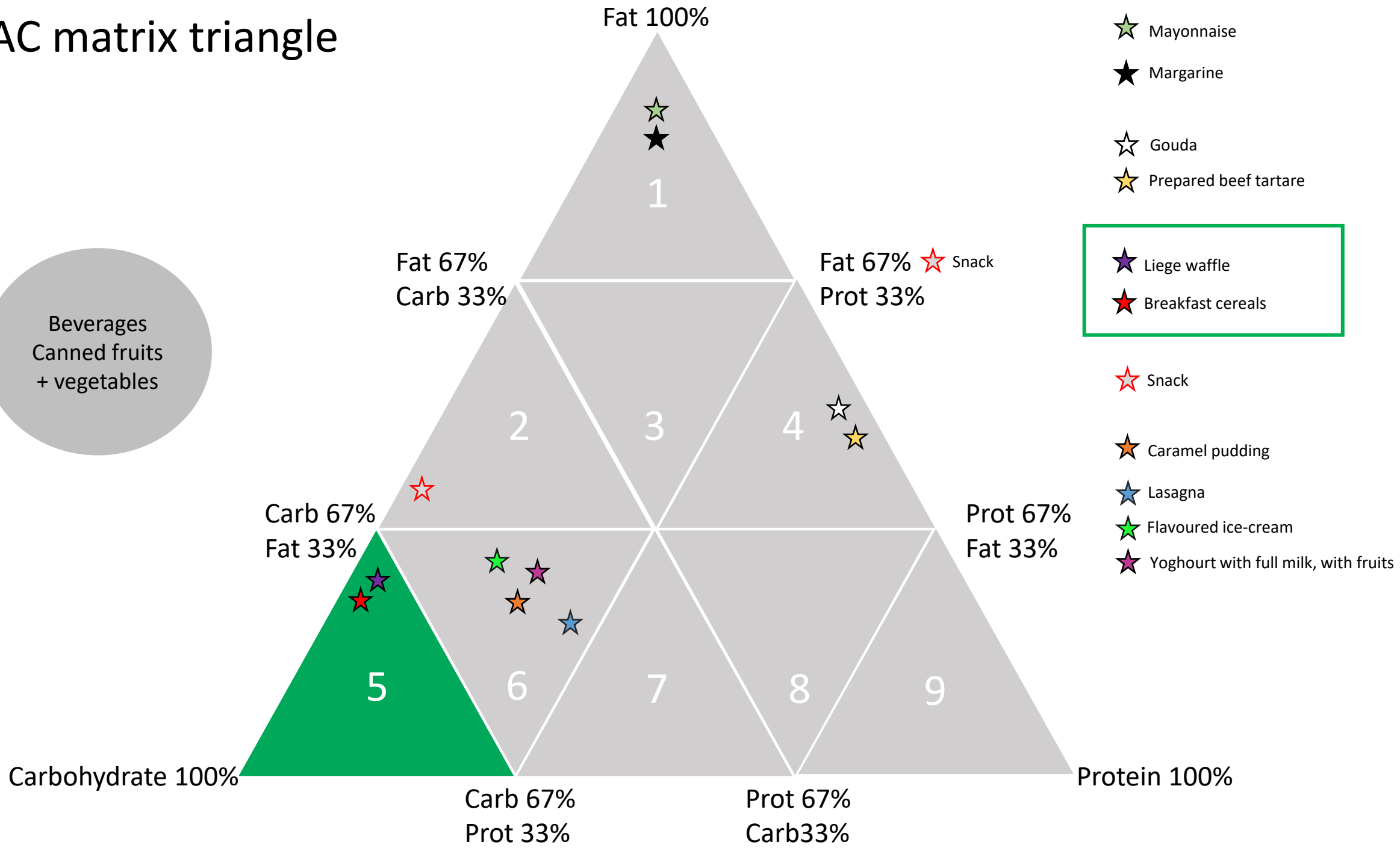
Allura Red AC
Ponceau 4R
Sunset Yellow FCF
Snacks
Soup
Processed Fish, Meat & Egg
Sauces & Seasonings
Meals & Meal Centers
Chocolate Confectionery
Bakery
Breakfast Cereals
Dairy

AOAC matrix triangle



Validation – Results

NOK for	
Riboflavin E 100	Bad reproducibilty and repeatability Recoveries of 200 % Naturally present in milk, meat, etc
Brown HT E 155	Calibration curve very unstable!
Neohesperidin DC E 959	Low sensitivity
Carnosic acid (E 392)	Depending on the matrix Low stability of the compound (stability: degradation after 1 day in the autosampler)
Carminic acid E 120 Green S E 142 Aspartame E 951 Natamycin E235	Reproducibility above Horwitz criteria!!!

E 100	Curcumin (Q)
E 102	Tartrazine (B)
E 104	Quinoline Yellow (Q)
E 110	Sunset Yellow FCF (B)
E 120	Carminic acid (B) *
E 122	Carmoisine (B)
E 123	Amaranth (B)
E 124	Ponceau 4R (B)
E 127	Erythrosine (B)
E 129	Allura Red AC (B)
E 131	Patent Blue V (B)
E 132	Indigotine (B)
E 133	Brilliant Blue FCF (B)
E 142	Green S (B) *
E 160b	Bixin (Q)

E 950	Acesulfame K (B)
E 951	Aspartame (B) *
E 952	Cyclamic acid & salts (B)
E 954	Saccharin & salts (B)
E 955	Sucralose (Q)
E 961	Neotame (Q)
E 969	Advantame (Q)
E 214	Ethyl p-hydroxybenzoate (Q)
E 218	Methyl p-hydroxybenzoate (Q)
E 235	Natamycin (B) *
E 310	Propyl gallate (Q)
E 392	Carnosol (Q)

27	additives
15	colours
7	sweeteners
5	antioxidants &
+ 1	caffeine

+ 43 banned substances (screening)

Under within-laboratory reproducibility conditions

Horwitz paradox!

Medium level = 1.3 mg/kg

High level = 10 mg/kg

Very high level = 20 mg/kg

Mass fraction	CV (%)
≥ 10 µg/kg to 100 µg/kg	20
> 100 µg/kg to 1 000 µg/kg	15
≥ 1 000 µg/kg	10

Our next
20 minutes

01

The WHY and the HOW?

The strategy

03

Tools used for the validation process
The Horwitz paradox

Ultimate test

02

Setting-up the methods
The funnel effect

No pain no gain

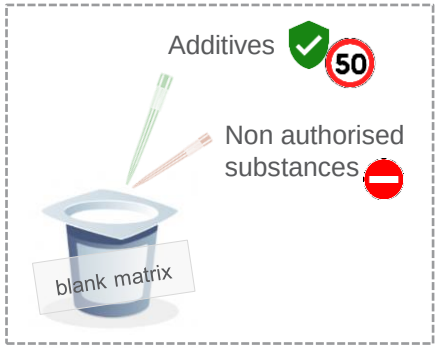
04

Implementation examples
Wrapping-up

This is real

Sample analysis – Protocol

Batch
of samples



Quality control sample

Blank matrix

Procedural blank

QuEChERS

Basified methanol

50 Quantification of FA

Screening of non
authorised substances

Dairies

Yoghurts

n = 5

Liquid dairies

n = 5

Edible ices

n = 5

Dairies Analytical results

- **Methyl p-hydroxybenzoate E 218** (1 ice cream)

- **Riboflavin E 101** in all samples, naturally in milk
- **Caffeine** in all samples containing chocolate, naturally present

Concentrations too high for calibration with dilutions 100x

- **4-amino carminic acid** (1 yoghurt, containing carminic acid E 120)

Non-alcoholic beverages

n = 22

Non-alcoholic beverages Analytical results

- **Acesulfame K E 950** detected while not mentioned (1 lemonade, 1 energy drink)
- **Saccharin E 954** detected while not mentioned (1 lemonade)
- **Carnosol** and **carnosic acid (E 392)** detected while not mentioned (2 energy drinks)

- **Orange II** detected (3 non alcoholic panache - cocktails)

Processed foods

Italian dishes

n = 10

European, Asian dishes

n = 15

Processed foods (italian dishes)

n = 10

No additives

« spices », « herbs »

- **Carnosic acid** & **carnosol (E 392)** (naturally present in rosemary and other herbs) (7 samples)
- **Ethyl p-hydroxybenzoate E 214** (1 frozen lasagna)

- **Ethyl gallate** (1 lasagna)

2 samples with no additive detected

Processed foods (european, asian dishes)


n = 15

No additives

« spices », « herbs », curcuma

- **Curcumin E 100** (naturally present in curcuma) (7 samples)
- **Carnosic acid** & **carnosol (E 392)** (naturally present in rosemary and other herbs) (5 samples)

6 samples with no additive detected

- **Natamycin E 235** (1 Tikka Massala chicken)
- **Caffeine** (1 dehydrated noodles)
- **Acesulfame K E 950** (1 curry chicken salad)
- **Ethyl E 214** & **Methyl p-hydroxybenzoate E 218** (1 Nasi Goreng)

- **Propyl paraben** (1 quiche)
- **Dulcin** (1 beef carbonnade)
- **Glycyrrhizin** (naturally present in liquorice, mentioned on the label) (1 Tikka Massala chicken)

Lets’ wrap up with numbers…

123 food products
from different food categories were analysed

16 products contained additives not mentioned on label (13 %)

processed foods samples not included,
ingredients potentially naturally present not included
(ex: ribloflavin, caffeine).

7 different substances not authorised as food additives were detected in **12** products (10 %)

ingredients potentially naturally present not included
(ex: rosmarinic acid),
degradation product of carminic acid not included.

Take-home message

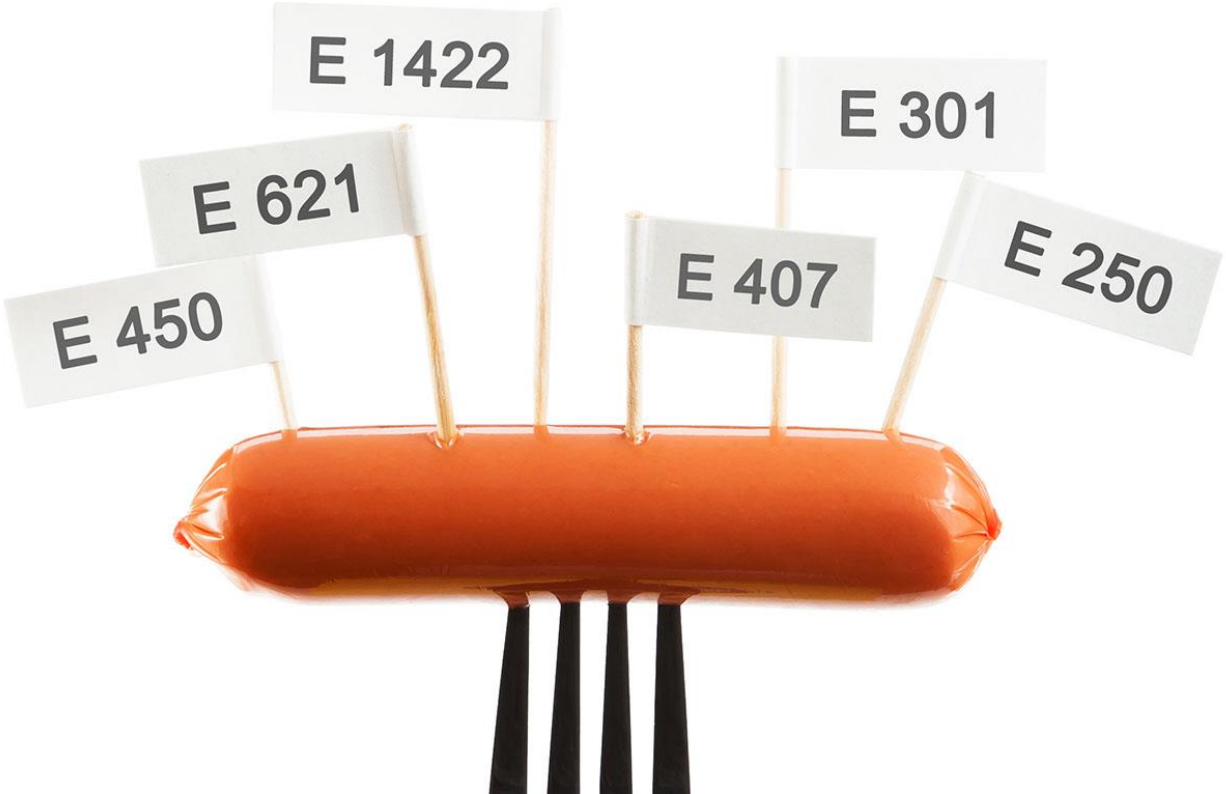
Every step
of the analytical process
requires
some level of compromise

Validation and
Data processes
Is time-consuming!

Expertise
is a key factor, and
a learning curve!

Versatile methods
-Scope & matrix
-Accreditation

Next steps:
-Scope extension
-Validation criteria
-Banned substances



Questions?

☺ Thank you to the artists for the beautiful pictures