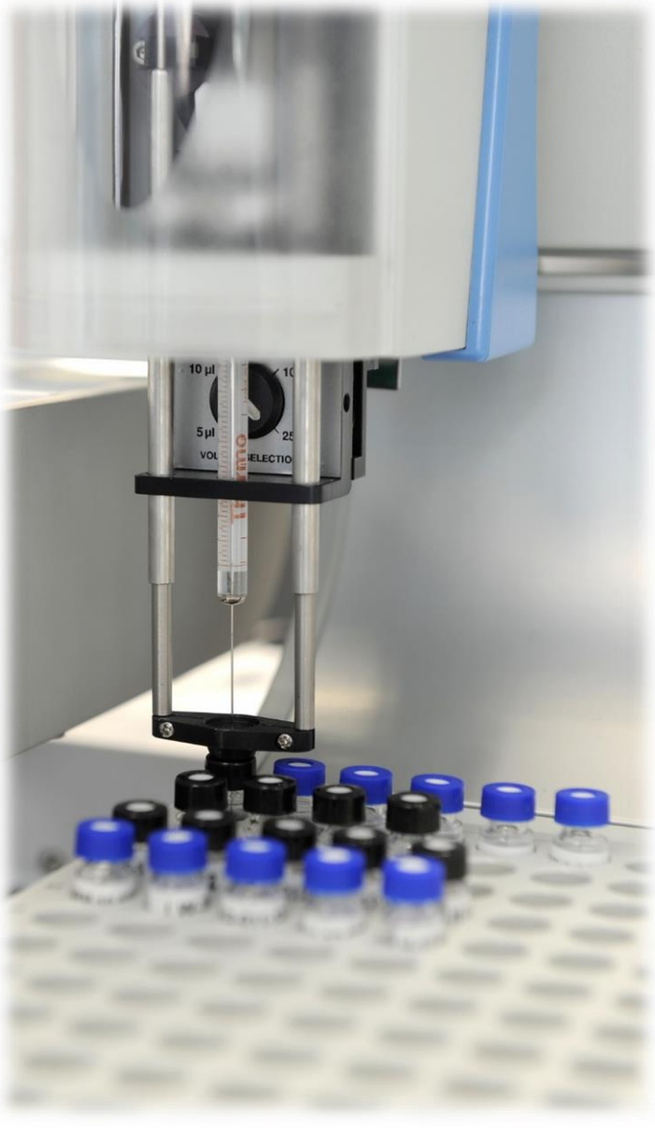
GERMAN FEDERAL INSTITUTE FOR RISK ASSESSMENT

BfR
Bundesinstitut für Risikobewertung

Chromatographic methods for wine authentication

Carsten Fauhl-Hassek

Chromatographic methods



- Quick review:

Based on **the distribution of molecules between two phases**: Mixtures of analytes are dissolved in a **mobile phase** and passed through a system coated or filled with a **stationary phase**. Separation occurs due to different strengths of interaction of the various molecules with the surface of the stationary phase.

- GC, LC with various detectors (FID, UV, MS/MS …)

- Numerous methods in wine (authentication) analysis (see OIV compendium).

2 Examples:

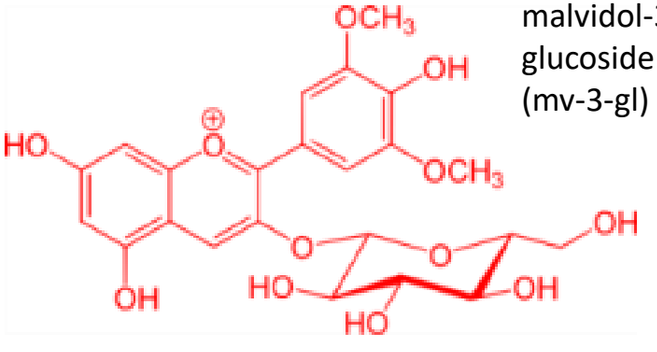
- **Anthocyanins** (HPLC/UV) → Grape variety (red and rosé wines)
- **Cyclic diglycerols & 3-MPD** (GC/MS) → Glycerol adulteration

Anthocyanins in wine

- Phenolic compounds common in fruits and berries
- Antioxidants, give colour to red and rosé wines

- Glucosides (mostly in C3 position). Aglycons are instable in solution
- Also acylated forms (acetylated, coumarylated)

- Anthocyanin composition ± typical for grape variety



malvidol-3-glucoside (mv-3-gl)

cyanidol-3-glucoside (cy-3-gl)

Anthocyanins in wine: OIV method MA-E-AS315-11

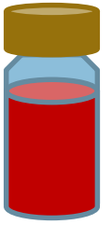
Principle:

- Separation of
  the **five** most important **non acylated anthocyanins** and **four major acylated anthocyanins**

- Analysis of red and rosé wine by **direct separation by HPLC** by using **reverse phase column** with gradient elution by water/formic acid/acetonitrile with **detection at 518 nm**

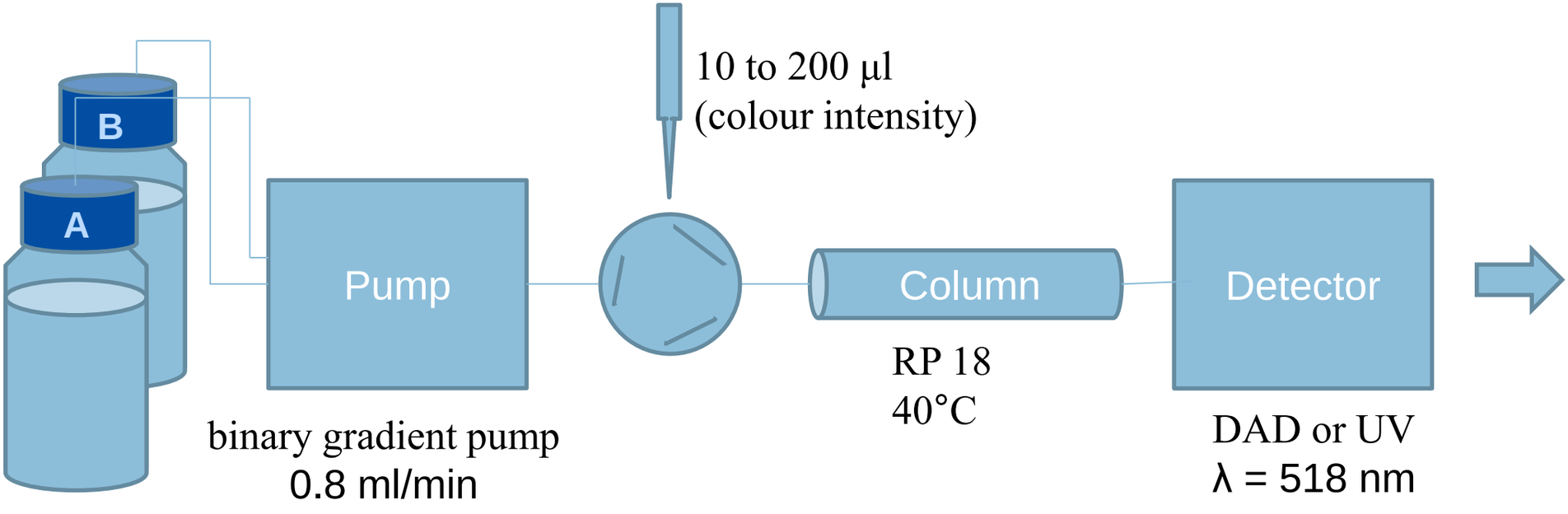
Anthocyanins in wine: OIV method MA-E-AS315-11

Sample preparation:



filter 0.45 µm

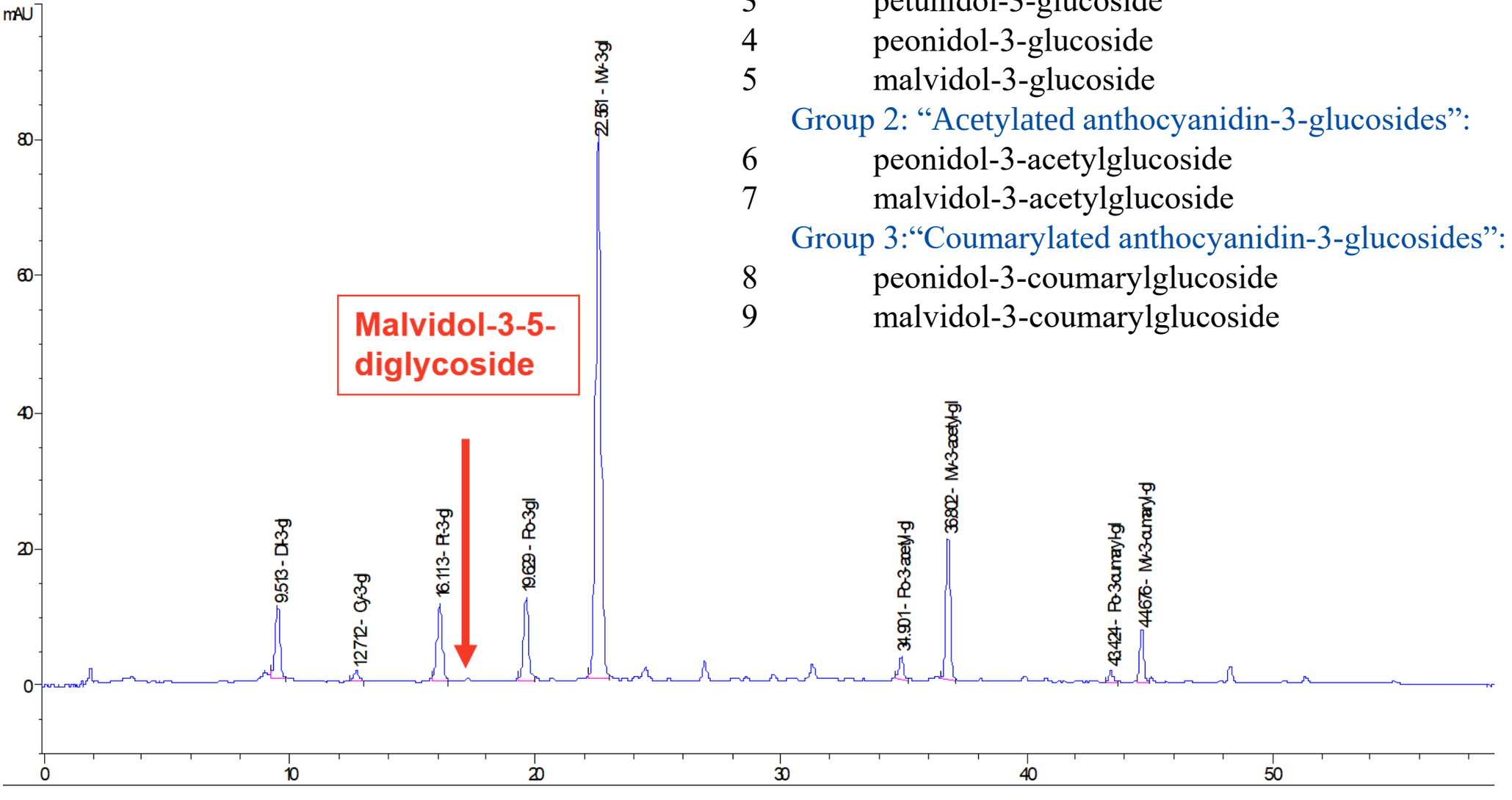
HPLC analysis:



Solvent A: Water/Formic acid/Acetonitrile 87 : 10 : 3 (v/v/v)
Solvent B: Water/Formic acid/Acetonitrile 40 : 10 : 50 (v/v/v)

Anthocyanins in wine: OIV method MA-E-AS315-11

Data analysis: Peak picking



#	Group 1: "Nonacylated anthocyanidin-3-glucosides":
1	delphinidol-3-glucoside
2	cyanidol-3-glucoside
3	petunidol-3-glucoside
4	peonidol-3-glucoside
5	malvidol-3-glucoside
	Group 2: "Acetylated anthocyanidin-3-glucosides":
6	peonidol-3-acetylglucoside
7	malvidol-3-acetylglucoside
	Group 3:"Coumarylated anthocyanidin-3-glucosides":
8	peonidol-3-coumarylglucoside
9	malvidol-3-coumarylglucoside

Anthocyanins in wine: OIV method MA-E-AS315-11

Data analysis: Calculations

- Values are expressed as relative amounts of the sum of the nine anthocyanins in area % : Anthocyanin pattern
- sum of acylated anthocyanins and the ratio of acetylated to coumarylated anthocyanins are calculated if feasible

Ratio acetylated / coumarylated anthocyanins:

$$R = \frac{(6 + 7)}{(8 + 9)}$$

Anthocyanins in wine: Examples

Example 1: Pinot false variety claim

- Pinot Noir, Pinot Meunier, and Pinot Madeleine wines do not contain acetylated anthocyanins

→ Limitations: legal blends (EU wine law: other varieties allowed <15 %*)

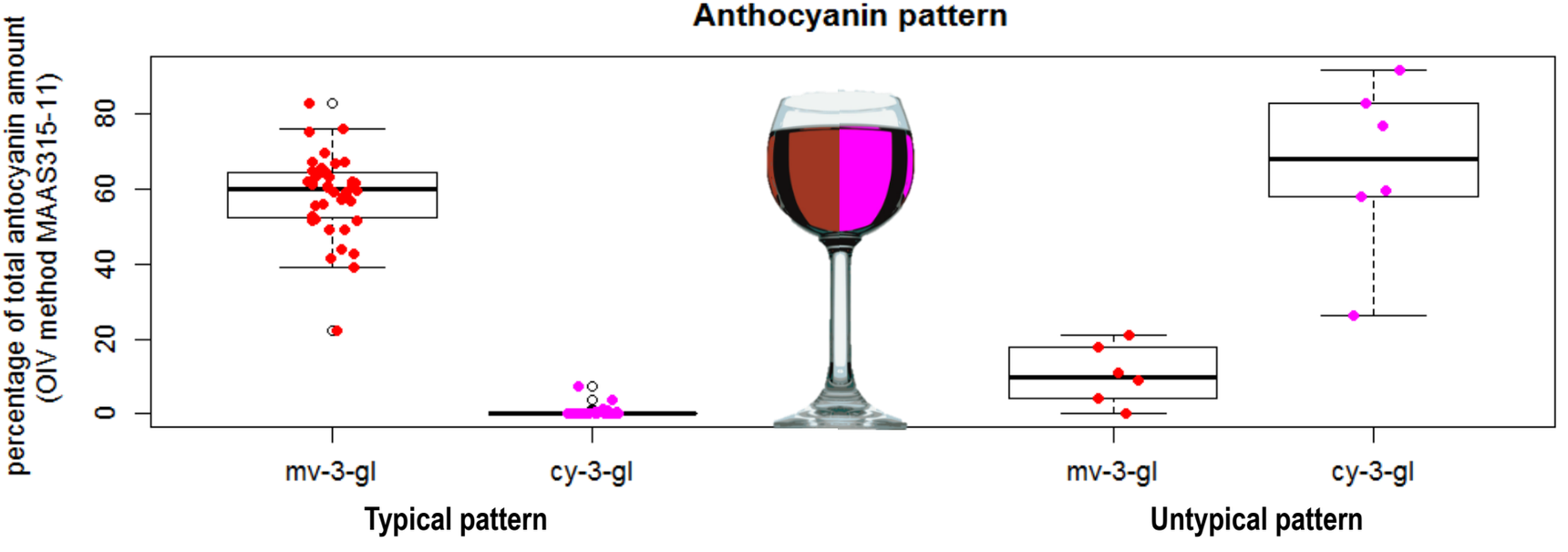
*Commission Delegated Regulation (EU) 2019/33

Anthocyanins in wine: Examples

Example 2: Although anthocyanins were largely degraded (no clear pattern): detection of non-*Vitis vinifera* anthocyanin origin

- Aging processes (oxidation, degradation, polymerisation): Less free or acylated anthocyanins over time, increasingly brownish in colour.

- 6 ECS test wines: **inversed Cyanidin-3-gl/ Malvidin-3-gl ratio**. Contrasting most of the other wines, they were rather pink in colour.



Glycerol addition to wine

- Glycerol has a sweet taste
- It is supposed to contribute to the mouthfeeling

- Natural constituent of wine
- Glycerol 4,8-14 g/l

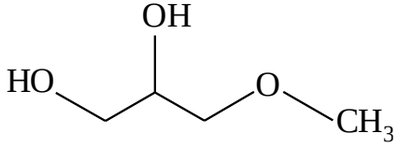
- Methods:
  - wet chemistry, GC, HPLC
  - NMR

- Small additions - 15-30 % of the total glycerol - difficult to detect

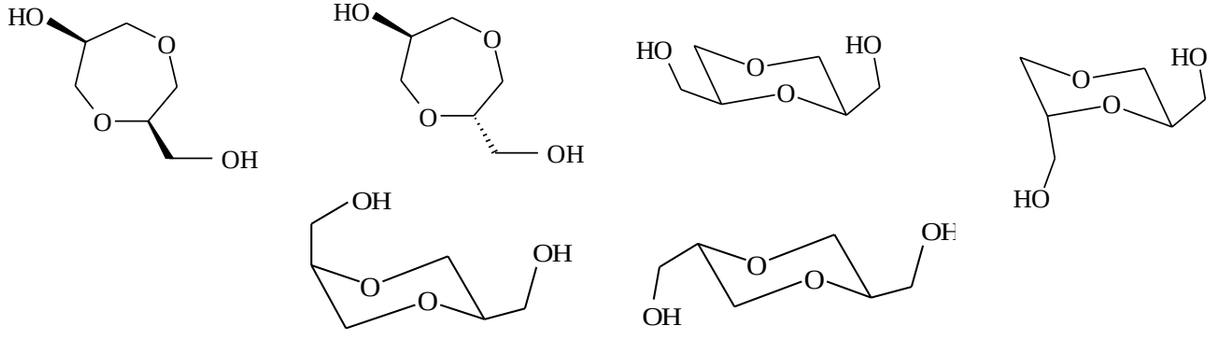
Glycerol addition to wine

- By-products found in technical glycerol (not naturally present in wine)
- Impurities from glycerol synthesis - by fat cleavage (3-MPD)
  - or from petrochemicals (CycDs)

3-Methoxy-propandiol



Cyclic Diglycerols (dioxane, dioxepane)

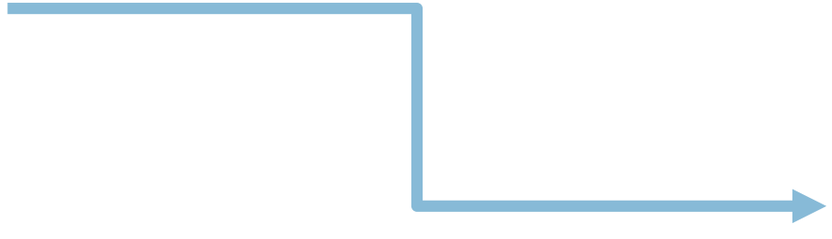


- 1997: 140 of 850 wine samples (mainly German) were “positive” (16 %)
- 1999: 3 of 150 were “positive
- Today: rarely found in European wines – but present in 16 % of wines in ECS project

- OIV-MA-AS315-15 (OENO 11/2007) Type II

Principle OIV-MA-AS315-15 (OENO 11/2007) Type II

- The analytes (3-MPD, 6 cyclic diglycerols) and the internal standard are **salted-out** by addition of K_2CO_3, **and extracted using diethyl ether**.
- Extracts are analyzed **directly by GC-MS** on a polar column
- Detection is then carried out in selected ion monitoring mode
- Quantification is done by **a matrix calibration curve**



Have a look at the OIV method pdf!
We will go through step by step.

OIV-MA-AS315-15 (OENO 11/2007) Type II

Matrix calibration:

- Essential (external calibration is not sufficient)
- Wine free of the analytes is required as blank
- Standards:

Internal standard Butane-1,4 -diol-1,1,2,2,3,3,4,4-$(^2H)_8$
and 3-MPD : commercially available

Cyclic diglycerol mixture: available from BfR

Get yourself organised to manage the pipetting scheme properly

Do not pipette onto the glass joint surface

Table 1. Pipetting scheme of matrix calibration

Matrix calibration level		Spike µl		Volume Wine ml	C Wine µg/L	C Wine mg/L
Blank	IS	-		10	0	0
	3-MPD	-				
	CycDs	-				
ML0	IS	100	S1	10	1000	1.00
	3-MPD	-				
	CycDs	-				
ML1	IS	100	S1	10	1000	1.00
	3-MPD	100	S2		100	0.10
	CycDs	50	S1		500	0.50
ML2	IS	100	S1	10	1000	1.00
	3-MPD	25	S1		250	0.25
	CycDs	100	S1		1000	1.00
ML3	IS	100	S1	10	1000	1.00
	3-MPD	50	S1		500	0.50
	CycDs	20	S0		2000	2.00
ML4	IS	100	S1	10	1000	1.00
	3-MPD	100	S1		1000	1.00
	CycDs	30	S0		3000	3.00
ML5	IS	100	S1	10	1000	1.00
	3-MPD	200	S1		2000	2.00
	CycDs	40	S0		4000	4.00

OIV-MA-AS315-15 (OENO 11/2007) Type II

Adding the salt:

Addition of K_2CO_3

Do not touch the glass joint surface

Shake to dissolve immediately (will get hot!)

Shake well (the salt will not be dissolved completely) and cool down in 20 °C water bath.

OIV-MA-AS315-15 (OENO 11/2007) Type II

Adding the diethyl ether:

- Addition of 1000 µl of diethyl ether

Work exact (quantitative step)

Work under the fume hood

- Shake the mixture by hand or in a vertical-shaking machine for 5 min
- Centrifuge.

OIV-MA-AS315-15 (OENO 11/2007) Type II

Taking off and drying the organic phase:

- Carefully transfer the upper phase to GC vials prepared with molecular sieve

Work under the fume hood

You do not need all of the upper phase (1-1.5 ml), do not disturb the water phase

Close vials immediately

- Keep in the fridge for two hours so that all water is adsorbed by the molecular sieve
- Transfer the liquid into fresh vial: ready for GC analysis

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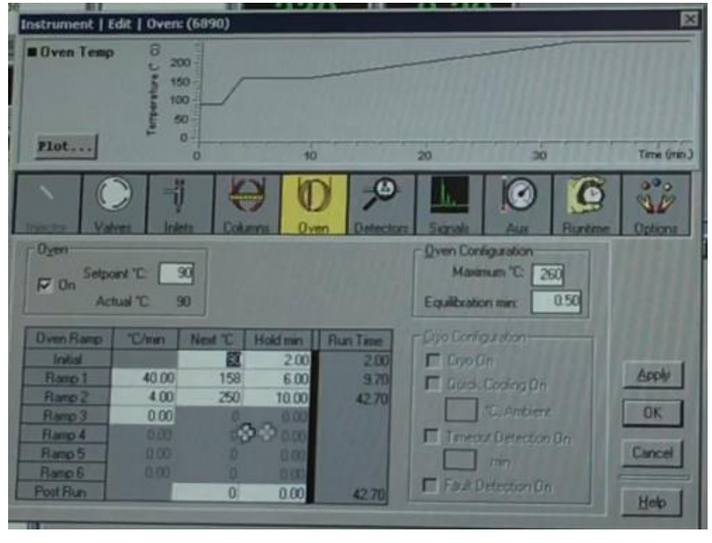
GC/MS analysis:

Typical GC conditions
Gas chromatograph: HP 5890 or equivalent
DB-Wax (J&W) column 60 m, 0.32 mm internal diameter, 0.25 µm film thickness, 2 m capillary containment same dimensions or equivalent
Carrier gas: H2 ,Flow: Pressure 60 k Pa column head
Temperature program:
90° C, 2 min., ramp at 10°C/min. up until 165° C, held for 6 min., ramp at 4° C/min to 250°C, held for 5 min.

Injection temperature: 250° C; Injected volume; 2 µL, splitless for 90 s.



Stated oven program and conditions are examples and need to be optimized

Selected ions:
3-MPD: *m/z* 75, *m/z* 61
IS: *m/z* 78, *m/z* 61
CycDs: *m/z* 57, *m/z* 117

Monitor also *m/z* 91 for the separation of the IS peak from phenylethanol, which also produces a fragment m/z 78.

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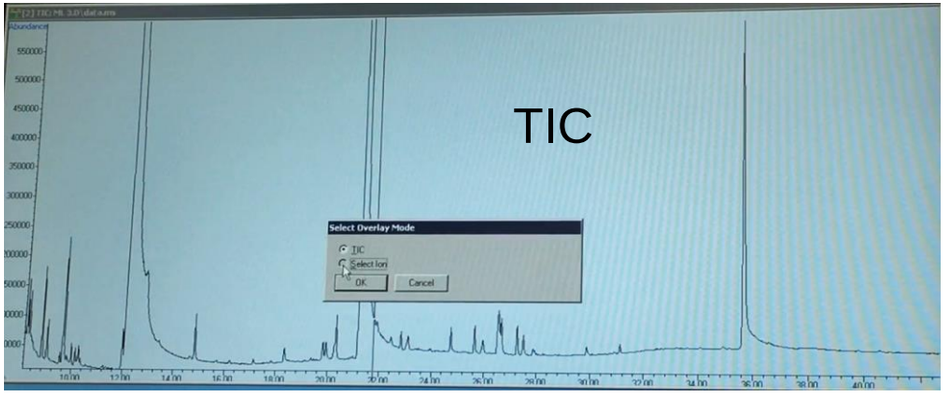
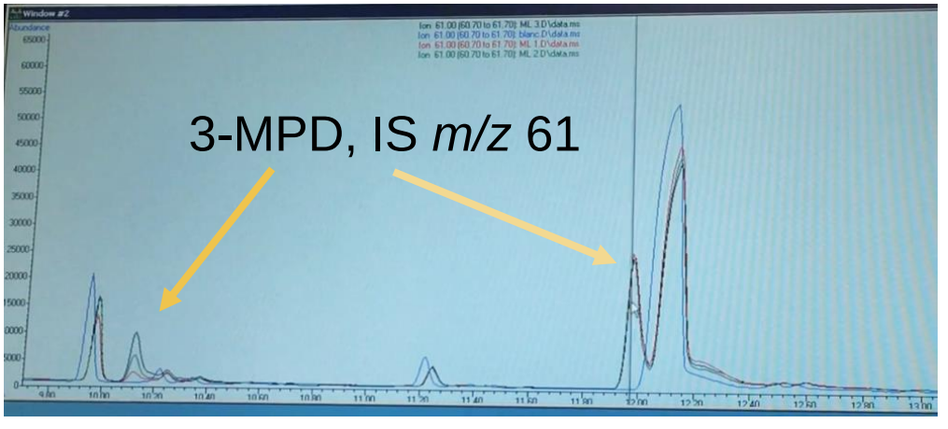
Spectra inspection:

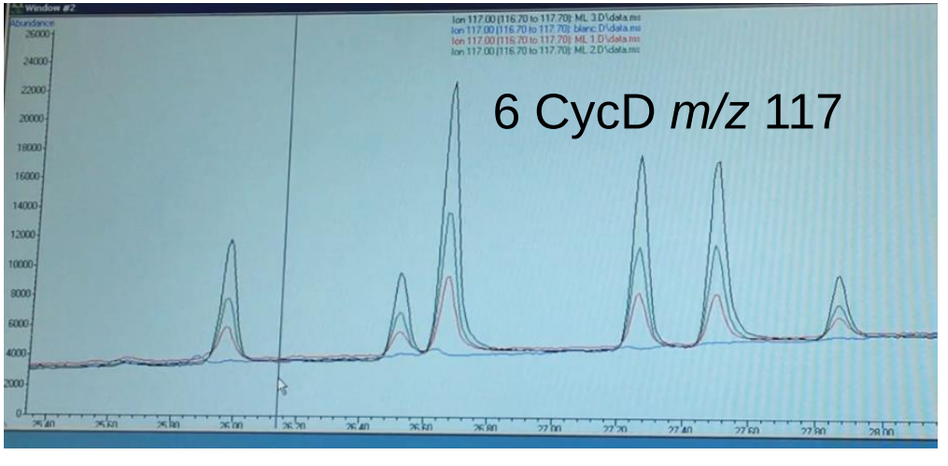
Check Total Ion Chromatogram and Selected ions

Identify signals: select relevant m/z and compare spiked samples with the blank.

Use peak height instead of area if separation of the signals is not excellent







OIV-MA-AS315-15 (OENO 11/2007) Type II

Calculations:

- Linear regression

3-MPD: *m/z* 75 is used for quantification
CycDs: *m/z* 117 is used for quantification → calculate all six peaks first separately and finally the sum of all six peak heights

- Sample calculations based on the calibration function:

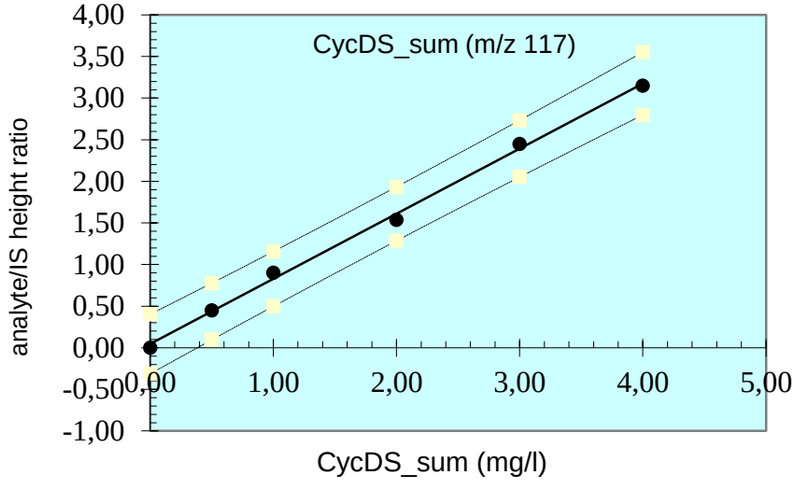
Example data sheet linear regression:

Data sheet for the determination of the linear regression					initial matrix weight	10.0	ml
Analytical form no.		Sum 1-6		concentration/quantity of IS:		1	mg/l
sample. no.	X_i	X_i^2				V^2	$X_i * V$
i				Y_i (Area)			
	mg/l		height	IS	analyte/IS height ratio		
ML 0	0.00	0.0	0	63674	0.00	0.0000	0.000
ML 1	0.50	0.3	16079	35772	0.45	0.2020	0.225
ML 2	1.00	1.0	32202	35764	0.90	0.8107	0.900
ML 3	2.00	4.0	59378	38648	1.54	2.3605	3.073
ML 4	3.00	9.0	95929	39189	2.45	5.9920	7.344
ML 5	4.00	16.0	110386	35069	3.15	9.9079	12.591

total volume of injected solution (in vial):	2	[µl]	
total number of standards (N):	6		
total number of multiple analyses:	1		
correlation coefficient r=	0.9988		
slope:	0.7822	normalised (*IS)=	0.78223458
y-intercept:	0.0447		

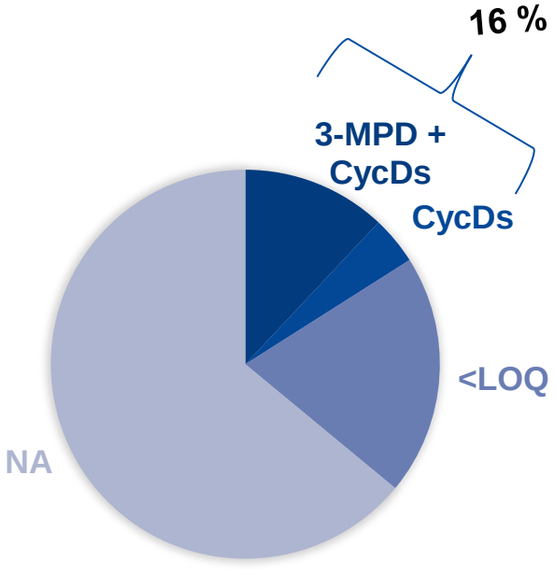
...

Calculation of the samples			further info:	Sum 1-6			
Analytical form no.		Sum 1-6					
sample no.	sample name	X_{added} mg/l	height	IS	ratio Ana/IS	$X_{calculated}$ mg/l	recovery [%]
1	W06492		0	38034	0.00		#DIV/0!
2	W06492		0	35288	0.00		#DIV/0!
3	W03669		25380	33200	0.76	0.92	#DIV/0!
4	W03882		0	41153	0.00	n.d.	#WERT!
5	W03884		9658	36820	0.26	0.28	#DIV/0!
6	W03886		19516	42826	0.46	0.53	#DIV/0!
7	W04202		0	41956	0.00		#DIV/0!
8	W04725		0	30291	0.00		#DIV/0!



Conclusion

- Spot check in China indicated technical glycerol addition **still common adulteration**
- **Straightforward method** for detecting the addition of external glycerol
- Conservative interpretation of very low concentrations: **possible minor entry** via enzyme preparations



ECS wine samples, n=50

Müller, T. M. et al. (2021). *Food additives & contaminants. Part A, 38*(8), 1289–1300. https://doi.org/10.1080/19440049.2021.1916097

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Thank you for your attention

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