Automated Sample Preparation using the Agilent 7696A WorkBench

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Classical Sample Preparation

- Extraction (food, soil, water, biofluids)
- Concentration of target solutes (enrich X-times)
- Isolate (clean-up, fractionation)
- Transform (derivatization, pyrolysis)

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Also “sample preparation”

- Dissolve / dilute
  - Most used sample preparation in important industries (HPI, pharma)
- Preparation of calibration standards
  - Used in all laboratories, validation and QC

Keywords in “Modern” sample prep

- Reduced solvent consumption
- Miniaturization
- Automation

- Safety (derivatization)

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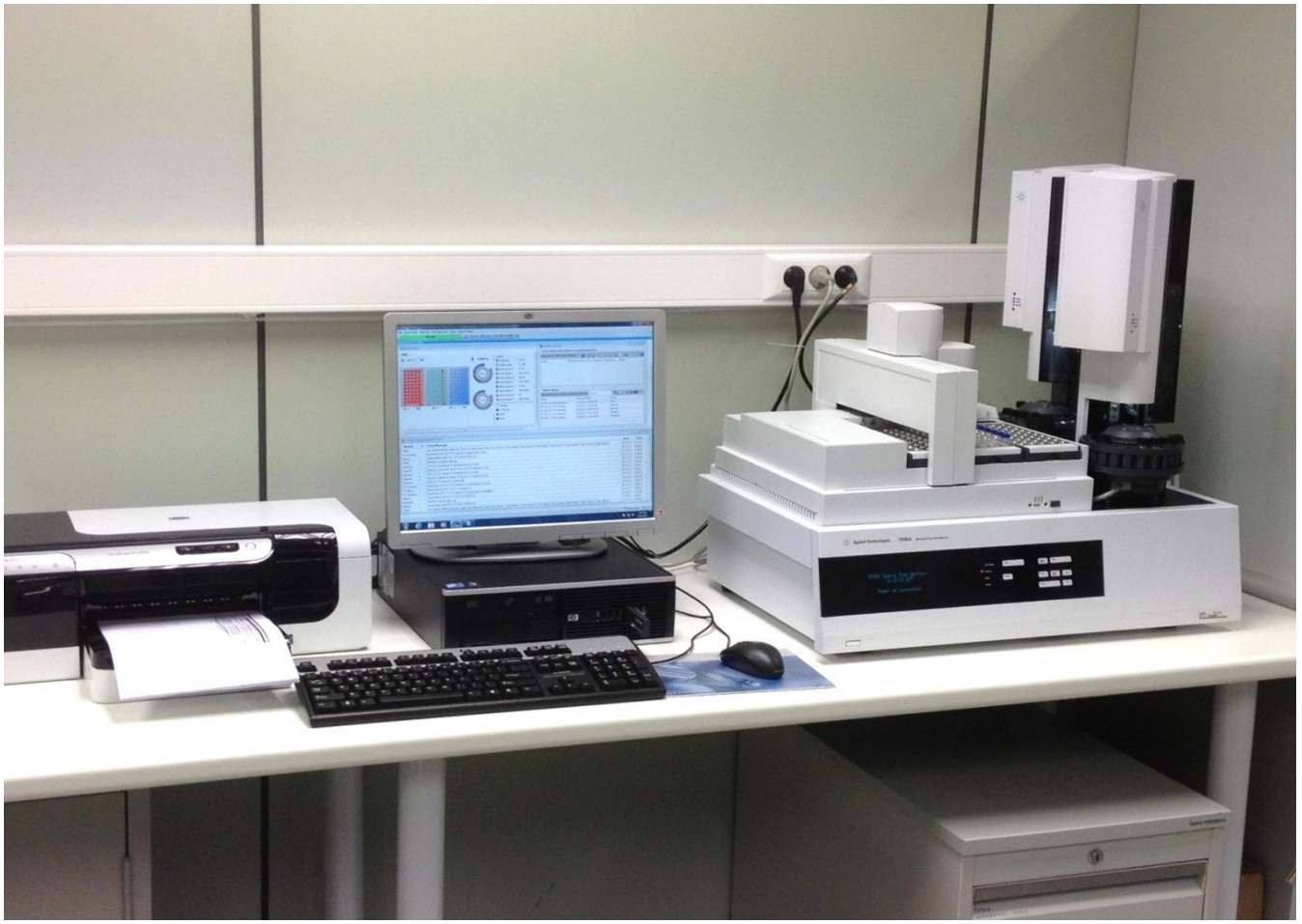
7696A WorkBench
with weighing station option



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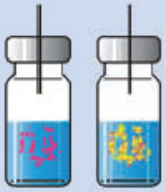
Functionalities

Dilution, aliquoting, and reconstitution

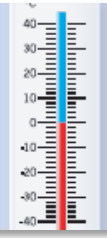
Liquid/liquid extraction



Reagent and standard addition

Bar code reading

Spin-vortex mixing



Flexible sample tray heating and Peltier cooling

2 syringes - 2 mL vials – single vial heater+ tray heating/cooling

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Agilent 7696A WorkBench

- ESP (Easy Sample Prep): icon based programming and resource manager
- Non-batch or batch mode

- Weighing station:
  0.01 mg accuracy

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Installation in Fume Hood

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Bottleneck: Method Translation

- Miniaturization
  - 2 mL vials
  - 2 syringes
  - Limited solvent use
- “Method translation” needed, comparable to translation from wide bore to narrow bore column in GC or from HPLC to UHPLC.
- Advise: first try manually – “simple” methods

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Applications

- Dilution: validation of impurity method in pharma
- Dilution & weighing: routine analysis of active ingredients in eye drops (pharma)
- Derivatization:
  - FAMEs in olive oil characterization (Food)
  - Oximation/silylation (Fiehn) in metabolomics (Life Science)
- Clean-up (drying)
  - Mineral oil (HOI) determination (Enviro)
  - PCBs in waste oil (Enviro)

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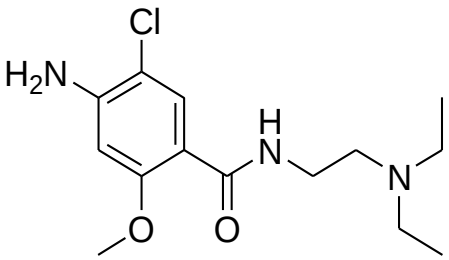
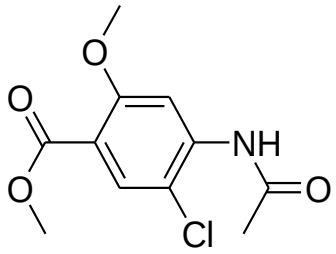
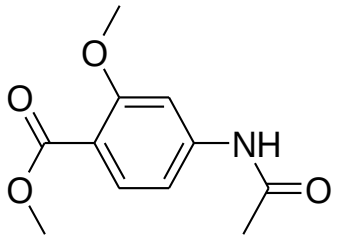
Metoclopramide Purity determination



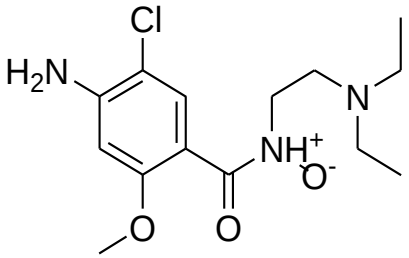
Metoclopramide



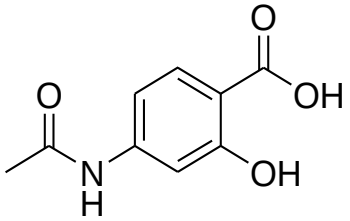
Imp B



Imp D



Imp G



Imp H

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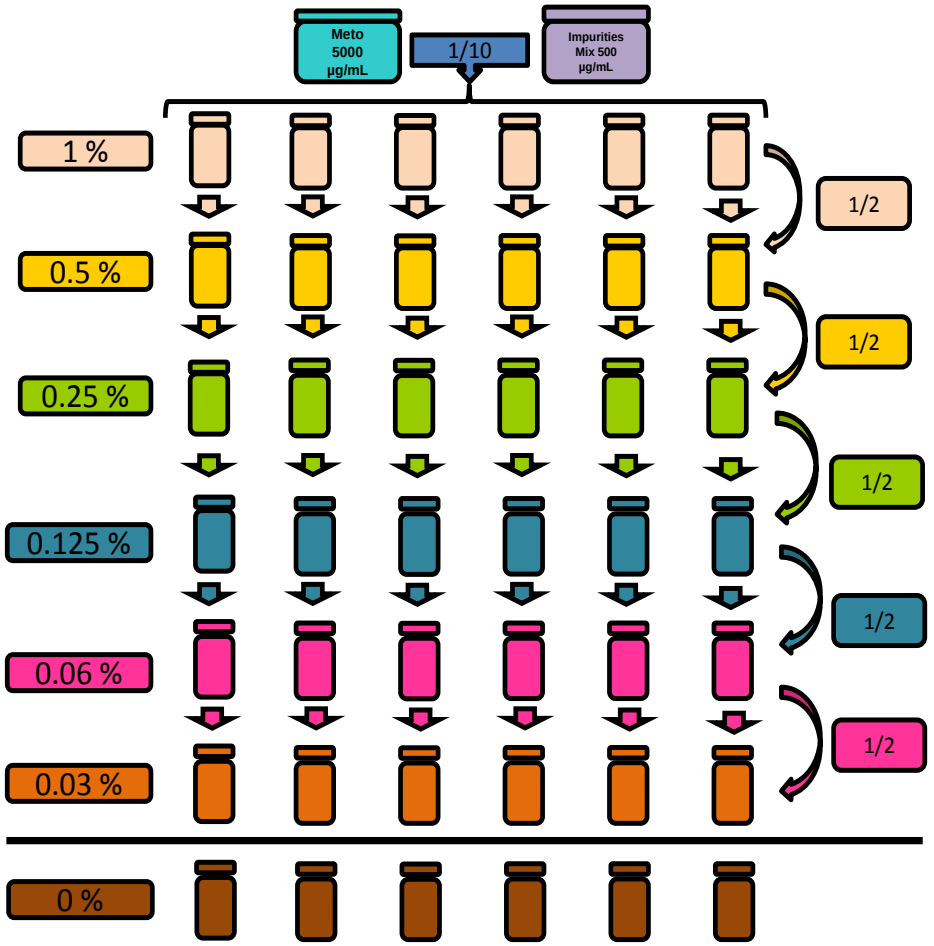
Method validation protocol

- Impurities in matrix (API)
  - Stock API
  - Stock impurities
- Linearity: 4-6 levels
- Repeatability: n=6
- Blank API
- Batch mode



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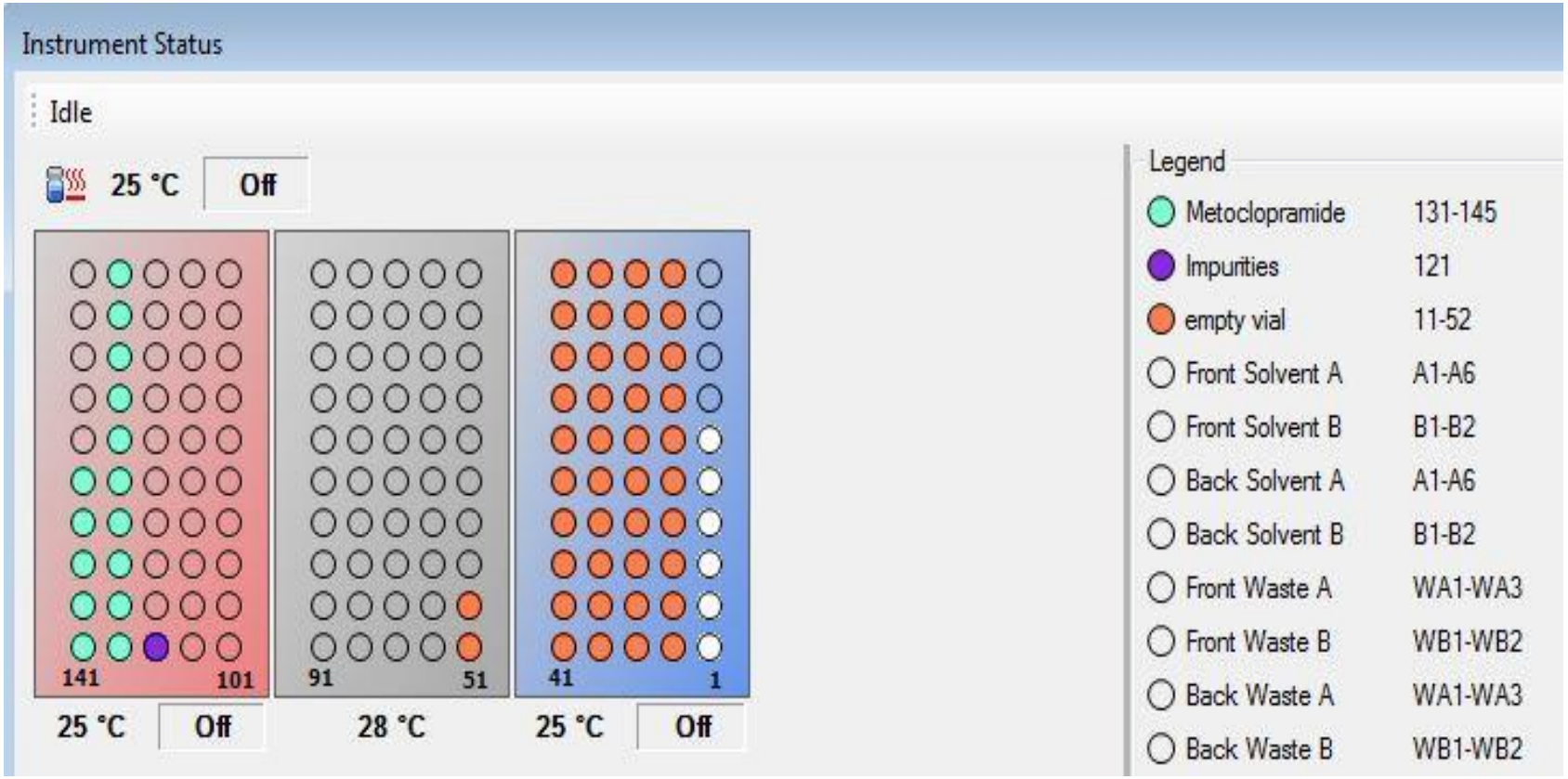
Resource Lay-out



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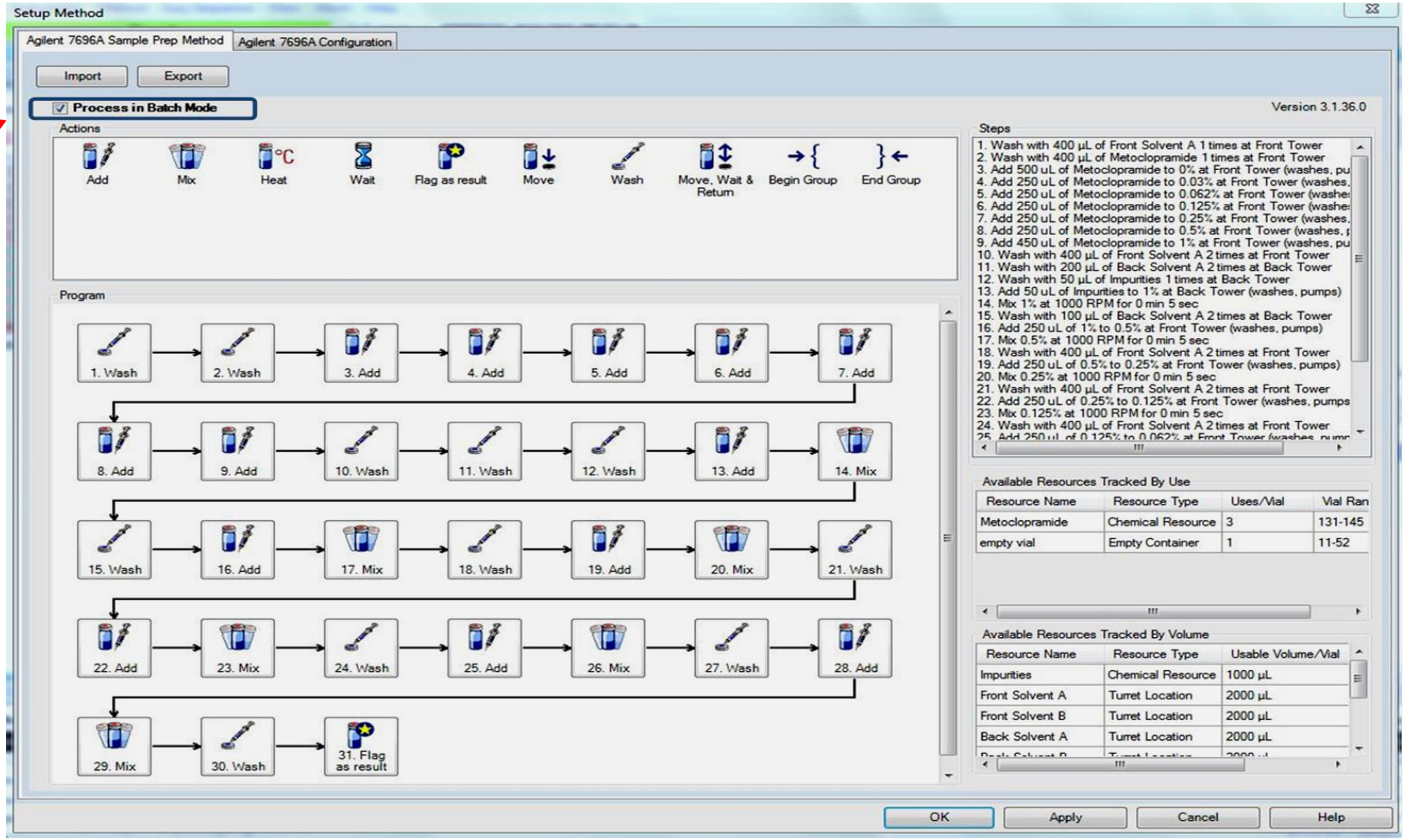
Method Lay-out



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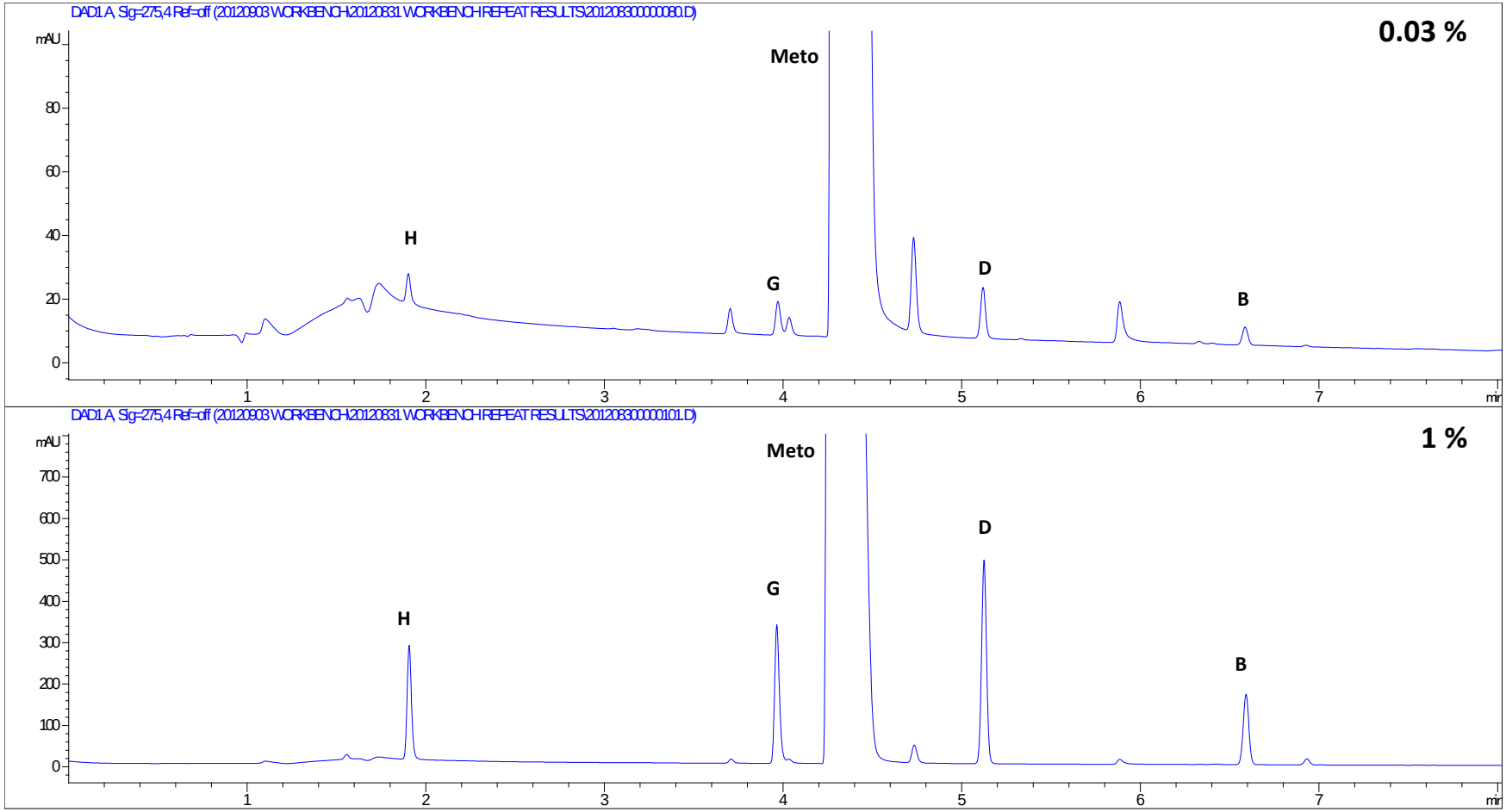
HPLC analysis



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Validation results

Conc % (w,w)	Imp H			
	Area	RSD (%)	Area/Conc	Accuracy (%)
0.03	15.8	1.0	504.5	102.7
0.06	30.6	1.5	490.3	99.8
0.125	62.7	0.9	501.3	102.1
0.25	122.8	0.5	491.2	100.0
0.5	241.0	0.7	482.1	98.1
1	477.9	0.4	477.9	97.3
R^2	0.99999	RSD (A/C) (%)	2.13	

Conc % (w,w)	Imp G			
	Area	RSD (%)	Area/Conc	Accuracy (%)
0.03	20.2	1.1	645.8	100.4
0.06	39.2	0.9	626.6	97.4
0.125	81.3	1.4	650.1	101.1
0.25	161.8	0.7	647.3	100.7
0.5	321.8	1.3	643.5	100.1
1	644.7	0.5	644.7	100.3
R^2	1.00000	RSD (A/C) (%)	1.54	

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Validation results

Conc % (w,w)	Imp D			
	Area	RSD (%)	Area/Conc	Accuracy (%)
0.03	30.9	1.9	988.8	100.01
0.06	60.7	1.8	971.3	98.24
0.125	126.1	1.4	1008.8	102.0
0.25	250.1	0.8	1002.3	101.2
0.5	492.7	1.3	985.4	99.7
1	977.8	0.5	977.8	98.9
R^2	0.99999	RSD (A/C) (%)	1.82	

Conc % (w,w)	Imp B			
	Area	RSD (%)	Area/Conc	Accuracy (%)
0.03	12.3	1.9	391.9	101.8
0.06	23.7	1.9	379.3	98.5
0.125	48.8	1.7	390.3	101.4
0.25	96.5	0.8	386.1	100.3
0.5	191.0	1.3	382.1	99.3
1	379.9	0.5	379.9	98.7
R^2	0.99999	RSD (A/C) (%)	1.86	

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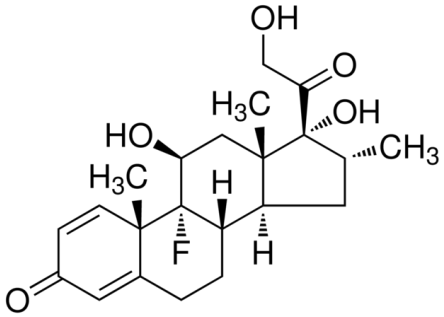
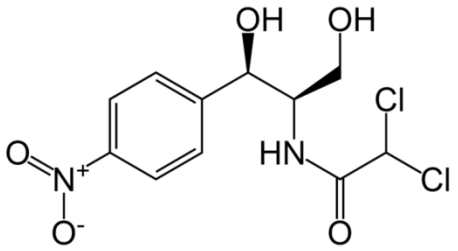
Dexamethasone and Chloramphenicol in Eye-drops

Dexamethasone is one of the most potent corticosteroids; it is 5 -14 times more potent than prednisolone and 25 - 75 times more potent than cortisone and hydrocortisone.



The addition of chloramphenicol, a broad-spectrum antibiotic, to dexamethasone leads to a combination which yields excellent results in inflammation of the anterior uvea (iritis, iridocyclitis)



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Dexamethasone and Chloramphenicol in Eye-drops – USP method

	Vial code	Original Method	
STD solutions		**Preparation Standard solutions**	
			approx
		Preparation Stock solutions	***Conc (µg/mL)***
	DEX Stck	weigh 10 mg dexamethasone in 25 mL vial + add 25 mL water	400
	CLO Stck	weigh 10 mg chloramphenicol in 25 mL vial + add 25 mL water	400
		Preparation Standard solutions	***DEX/CLO***
	LVL1	1 mL Dex Stck + 5 mL CLO Stck in 25 mL water	16/80
	LVL2	2 mL Dex Stck + 10 mL CLO Stck in 25 mL water	32/160
	LVL3	3 mL Dex Stck + 15 mL CLO Stck in 25 mL water	48/240
	LVL4	4 mL Dex Stck + 20 mL CLO Stck in 25 mL water	64/320
SAMPLES		**Preparation Finished product samples**	
	SAM	**1 mL** eye-drop sample + 25 mL water	**40**

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Dexamethasone and Chloramphenicol in Eye-drops – WorkBench method

	Vial code	Workbench method		
STD solutions		**Preparation Standard solutions**		
			approx	
		Preparation Stock solutions	***Conc (µg/mL)***	
	DEX Stck-1	weigh 4 mg dexamethasone in 2 mL vial * + add 1 mL AccN	4000	**WEIGH**
	DEX Stck	150 µL DEX Stck-1 + 1350 µL water (1/10 dilution)	400	**WEIGH**
	CLO Stck-1	weigh 4 mg chloramphenicol in 2 mL vial * + add 1 mL AccN	4000	**WEIGH**
	CLO Stck	150 µL CLO Stck-1 + 1350 µL water (1/10 dilution)	400	**WEIGH**
		Preparation Standard solutions	***DEX/CLO***	
	LVL1	20 µL Dex Stck + 100 µL CLO Stck + add 380 µL water	16/80	
	LVL2	40 µL Dex Stck + 200 µL CLO Stck + add 260 µL water	32/160	
	LVL3	60 µL Dex Stck + 300 µL CLO Stck + add 140 µL water	48/240	
	LVL4	80 µL Dex Stck + 400 µL CLO Stck + add 20 µL water	64/320	
SAMPLES		**Preparation Finished product samples**		
	SAM	**1 eye-drop sample** + 1 mL water	35	**WEIGH**

1 drop = approx 35 µL

* *Manual manipulation while Workbench on hold*

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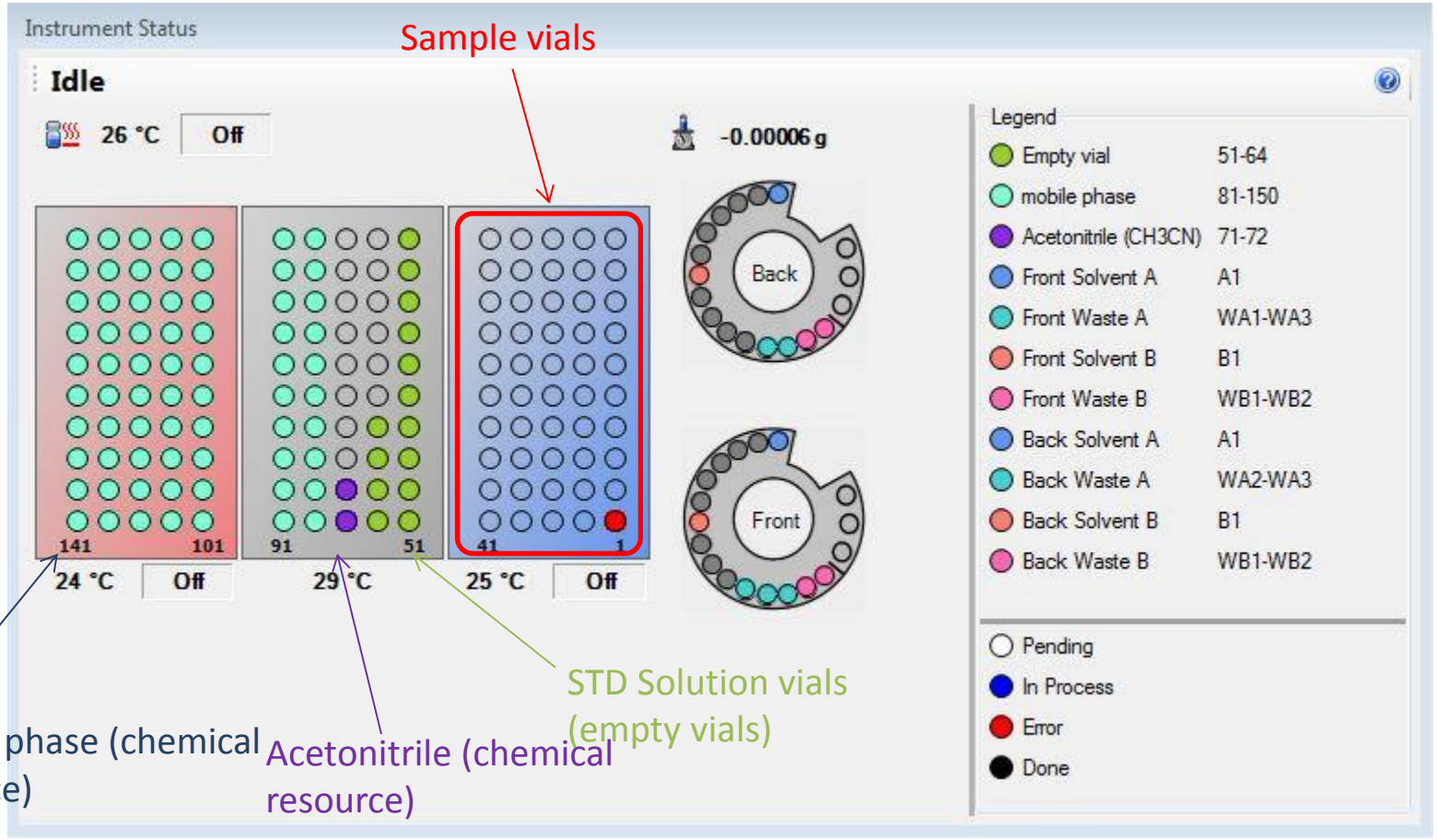
Resource Lay-out



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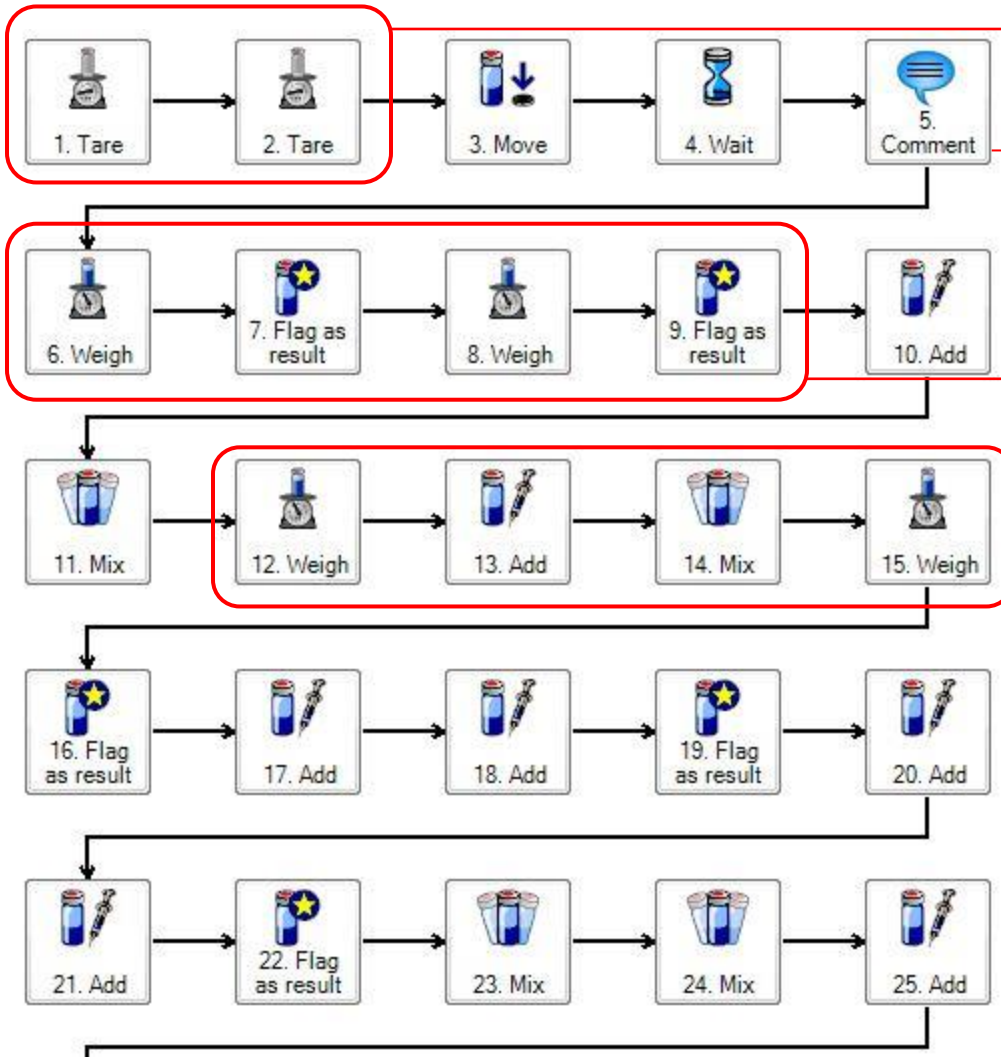
Method – Preparation STD Solutions



Tare DEX stck-1 and CLO stck-1 vials

Press the '**PAUSE**' button on the Workbench and add standard (approx 40 mg). Press '**START**' to resume

Weigh DEX stck-1 and CLO stck-1 vials (accurate standard weighing)

Weigh exact amount of added solvent

.... dilution

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Method – Preparation STD Solutions

1. Tare DEX stck-1
2. Tare CLO stck-1
3. Move vial from CLO stck-1 to Home
4. Wait for 1 min 0 sec
5. Comment: Press PAUSE button on the Workbench to hold the sequence, Add the STD, press START button to resume
6. Weigh DEX stck-1
7. Flag DEX stck-1 as Result.
8. Weigh CLO stck-1
9. Flag CLO stck-1 as Result.
10. Add 1000 uL of Acetonitrile (CH3CN) to DEX stck-1 at Front Tower (washes, pumps)
11. Mix DEX stck-1 at 3000 RPM for 5 min 0 sec
12. Weigh DEX stck-1
13. Add 1000 uL of Acetonitrile (CH3CN) to CLO stck-1 at Front Tower
14. Mix CLO stck-1 at 3000 RPM for 5 min 0 sec
15. Weigh CLO stck-1
16. Flag CLO stck-1 as Result.
17. Add and weigh 180 uL of DEX stck-1 to DEX stck at Front Tower (washes, pumps)
18. Add and weigh 1620 uL of mobile phase to DEX stck at Front Tower (washes, pumps)
19. Flag DEX stck as Result.
20. Add and weigh 180 uL of CLO stck-1 to CLO stck at Front Tower (washes, pumps)
21. Add and weigh 1620 uL of mobile phase to CLO stck at Front Tower (washes, pumps)
22. Flag CLO stck as Result.
23. Mix DEX stck at 2000 RPM for 0 min 20 sec
24. Mix CLO stck at 2000 RPM for 0 min 20 sec
25. Add 20 uL of DEX stck to LVL1-1 at Back Tower (washes, pumps)
26. Add 20 uL of DEX stck to LVL1-2 at Back Tower (washes, pumps)
27. Add 20 uL of DEX stck to LVL1-3 at Back Tower (washes, pumps)
28. Add 20 uL of DEX stck to LVL1-4 at Back Tower (washes, pumps)
29. Add 20 uL of DEX stck to LVL1-5 at Back Tower (washes, pumps)
30. Add 20 uL of DEX stck to LVL1-6 at Back Tower (washes, pumps)
31. Add 40 uL of DEX stck to LVL2 at Back Tower (washes, pumps)
32. Add 60 uL of DEX stck to LVL3 at Back Tower (washes, pumps)
33. Add 80 uL of DEX stck to LVL4 at Back Tower (washes, pumps)
34. Add 100 uL of CLO stck to LVL1-1 at Front Tower (washes, pumps)
35. Add 100 uL of CLO stck to LVL1-2 at Front Tower (washes, pumps)
36. Add 100 uL of CLO stck to LVL1-3 at Front Tower
37. Add 100 uL of CLO stck to LVL1-4 at Front Tower
38. Add 100 uL of CLO stck to LVL1-5 at Front Tower
39. Add 100 uL of CLO stck to LVL1-6 at Front Tower
40. Add 200 uL of CLO stck to LVL2 at Front Tower
41. Add 300 uL of CLO stck to LVL3 at Front Tower
42. Add 400 uL of CLO stck to LVL4 at Front Tower
43. Add 380 uL of mobile phase to LVL1-1 at Front Tower (washes, pumps)
44. Add 380 uL of mobile phase to LVL1-2 at Front Tower (washes, pumps)
45. Add 380 uL of mobile phase to LVL1-3 at Front Tower (washes, pumps)
46. Add 380 uL of mobile phase to LVL1-4 at Front Tower (washes, pumps)
47. Add 380 uL of mobile phase to LVL1-5 at Front Tower (washes, pumps)
48. Add 380 uL of mobile phase to LVL1-6 at Front Tower (washes, pumps)
49. Add 260 uL of mobile phase to LVL2 at Front Tower (washes, pumps)
50. Add 140 uL of mobile phase to LVL3 at Front Tower (washes, pumps)
51. Add 20 uL of mobile phase to LVL4 at Front Tower (washes, pumps)

Add stock solution to all levels, then add dilution solvent: minimum washing and cross-contamination

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Method - Finished Products (Eye-drops)

Add 1 eye-drop

Tare

Weigh

Automatic | Manual | Automatic

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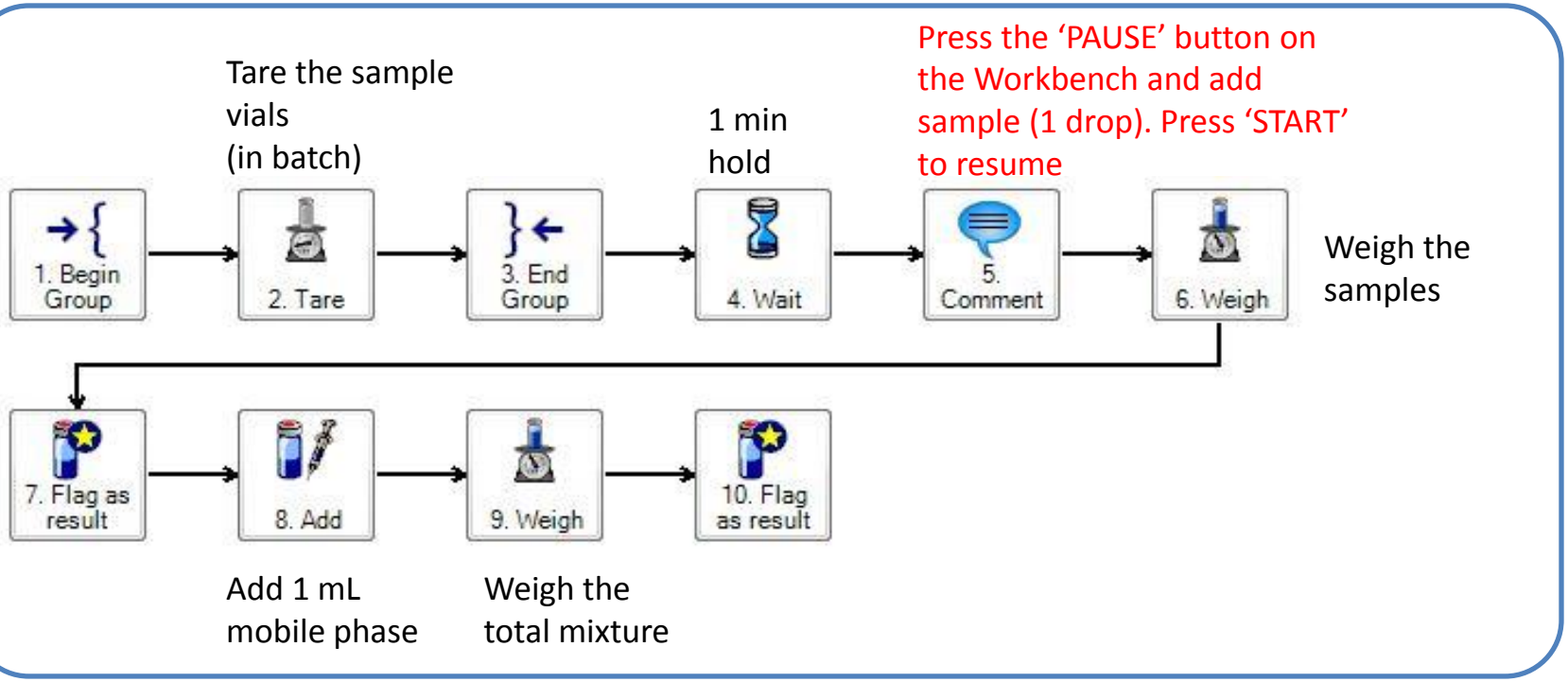
Method - Finished Products (Eye-drops)



1. Begin Group
2. Tare Sample
3. End Group
4. Wait for 1 min 0 sec
5. Comment: Press PAUSE button on the Workbench, press START after adding the sample
6. Weigh Sample
7. Flag Sample as Result.
8. Add 1000 uL of mobile phase to Sample at Front Tower (washes, pumps)
9. Weigh Sample
10. Flag Sample as Result.

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Sequence Report – Samples

WorkBench Sequence Report

Instrument: Instrument 1 [SN CN10460001]

Sequence: DEX CLO-Samples.s
Sequence Data Folder: C:\WorkBench\1\DATA\DEX CLO-Samples 2013-04-03 10-59-57\

Sequence Start: 4/3/2013 11:00 AM
Sequence Finished: 4/3/2013 2:24 PM
Ending status: Completed

Batch Sequence Summary

Sample Info

SAM0001 1
SAM0002 2
SAM0003 3
SAM0004 4
SAM0005 5
SAM0006 6
SAM0007 7
SAM0008 8
SAM0009 9
SAM0010 10
SAM0011 11
SAM0012 12

Batch Steps:
1.Tared 1 (vial 1) Weight = 2.73688 g
2.Tared 2 (vial 2) Weight = 2.70915 g
3.Tared 3 (vial 3) Weight = 2.74612 g
4.Tared 4 (vial 4) Weight = 2.71731 g
5.Tared 5 (vial 5) Weight = 2.75876 g
6.Tared 6 (vial 6) Weight = 2.74328 g
7.Tared 7 (vial 7) Weight = 2.71640 g
8.Tared 8 (vial 8) Weight = 2.76648 g
9.Tared 9 (vial 9) Weight = 2.72094 g
10.Tared 10 (vial 10) Weight = 2.74870 g
11.Tared 11 (vial 11) Weight = 2.71809 g
12.Tared 12 (vial 12) Weight = 2.71568 g
13.Returned all vials to the tray
14.Waited for 00:01:00 (hh:mm:ss)
15.Comment: Press Parking button to hold the sequence, press Parking button after adding the STD to resulme
16.Move vial from Sample to Wait, wait 0 min 1 sec, return
17.Weighed 1 (vial 1) Weight = 2.77038 g, weight change = **0.03350 g**
18.Flagged vial(s) 1 as Result vial(s).
19.Added 1000 µL of mobile phase (vial 51) to 1 (vial 1)
20.Weighed 1 (vial 1) Weight = 3.76947 g, weight change = 1.03259 g
21.Flagged vial(s) 1 as Result vial(s).
22.Weighed 2 (vial 2) Weight = 2.74469 g, weight change = 0.03554 g
23.Flagged vial(s) 2 as Result vial(s).
24.Added 1000 µL of mobile phase (vial 52) to 2 (vial 2)
25.Weighed 2 (vial 2) Weight = 3.74453 g, weight change = 1.03538 g
26.Flagged vial(s) 2 as Result vial(s).
27.Weighed 3 (vial 3) Weight = 2.78379 g, weight change = 0.03767 g
28.Flagged vial(s) 3 as Result vial(s).
29.Added 1000 µL of mobile phase (vial 53) to 3 (vial 3)
30.Weighed 3 (vial 3) Weight = 3.78318 g, weight change = 1.03706 g

Etc.

...

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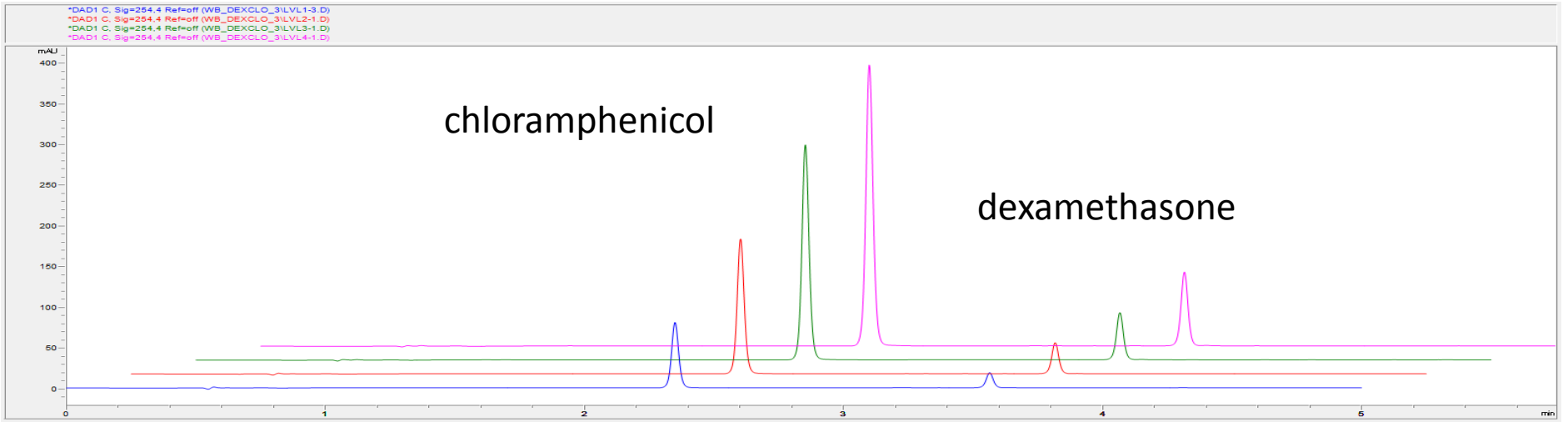
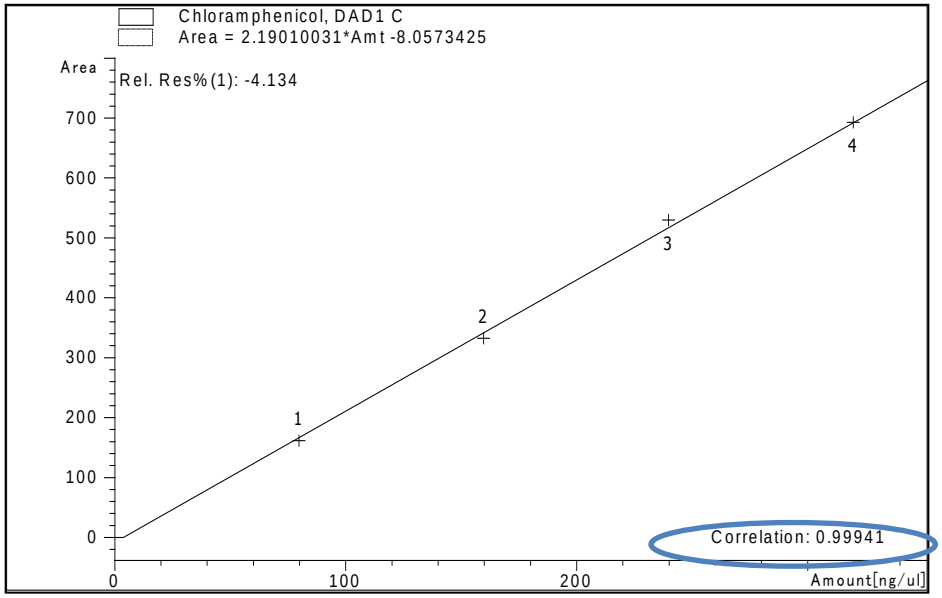
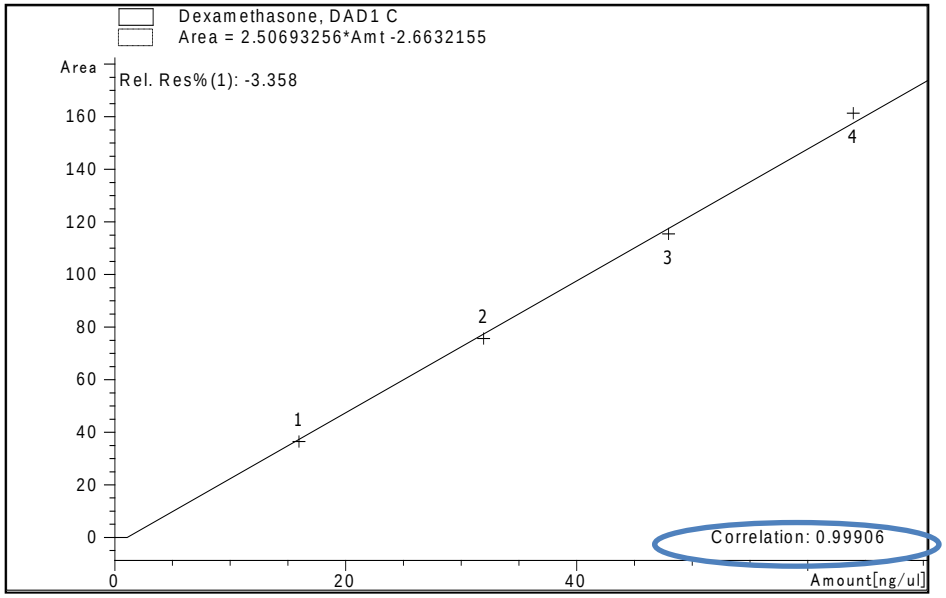
Validation - Linearity







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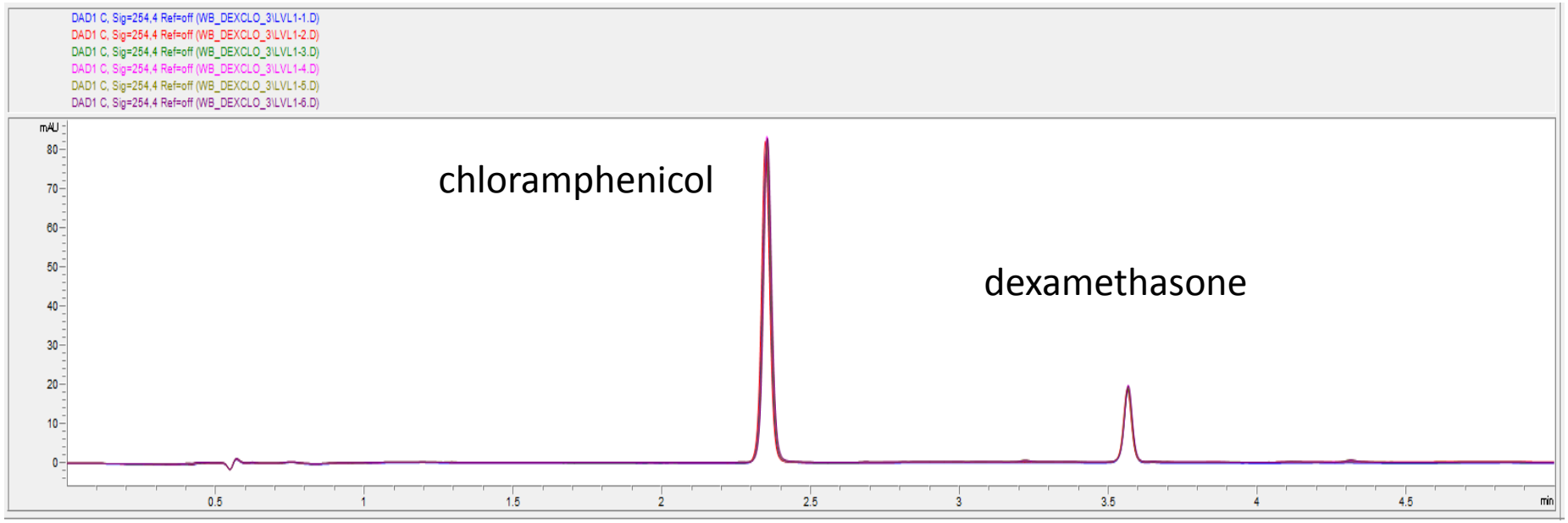
Validation – Repeatability Level 1 (*n*=6)



	RSD (%) – *n=6*
Chloramphenicol	1.5
Dexamethasone	2.7

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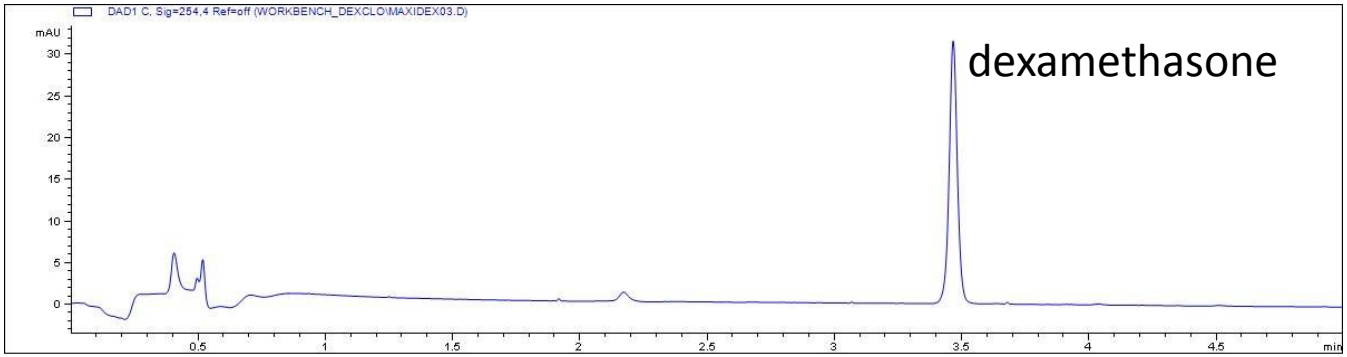
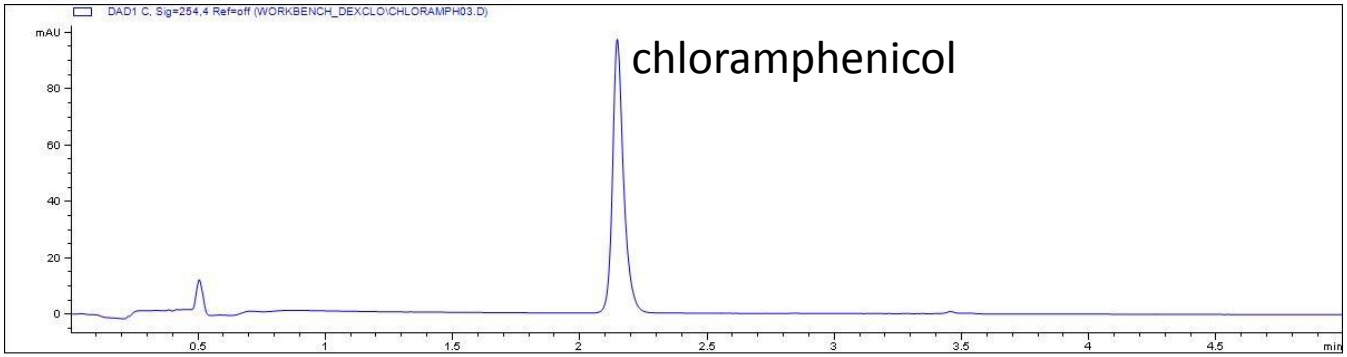
Validation - Samples

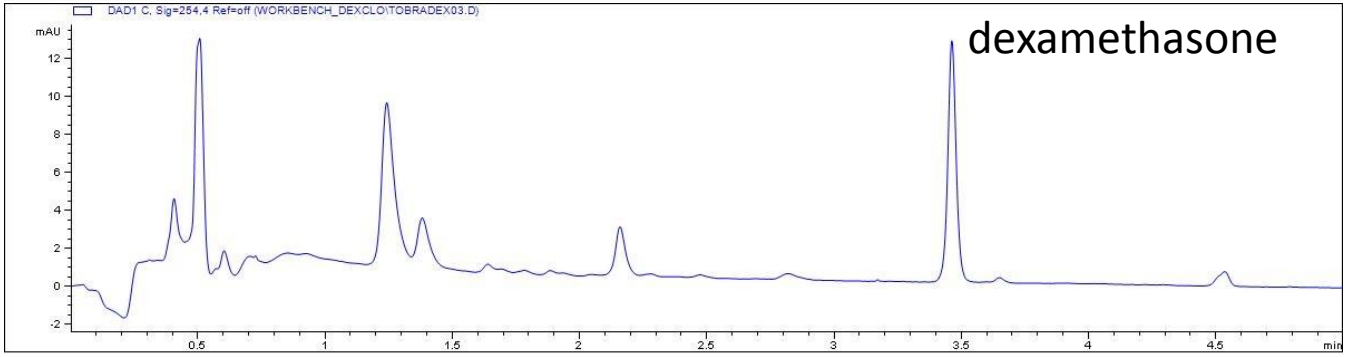
Sample A



Sample B



Sample C



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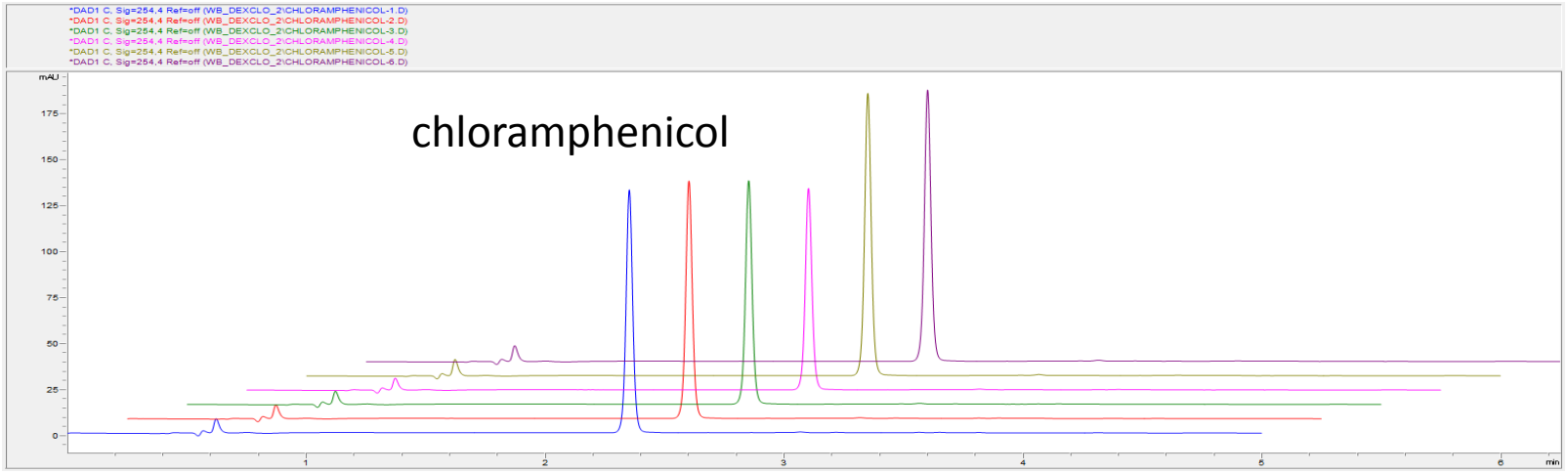
Validation – Repeatability Samples

Chloramphenicol eye-drops



	Caluculated conc (mg/mL)	Sample Weight (mg)	Corrected conc (mg/mL)
SAM-rep01	124	27.72	4.59
SAM-rep02	121	26.95	4.61
SAM-rep03	114	25.41	4.61
SAM-rep04	103	23.07	4.57
SAM-rep05	143	32.58	4.53
SAM-rep06	138	31.27	4.53
		RSD(%)	**0.74**

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Determination of Fatty Acid Methyl Esters in Olive Oil

- 10 mg oil (1 drop) (optional: weighing)
- Add 1 mL heptane
- Add 10 µL 2N KOH in methanol
- 10 min vortex
- Transfer heptane fraction
- Analyse by GC-FID (GC-MS)

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Determination of Fatty Acid methyl Esters in Olive Oil

- RSD % on peak area (corrected for weight)
  - C16:0: 5.5%
  - C18:0: 5.7%
  - C18:1: 5.5%
  - C18:2: 5.9%
- RSD% on relative peak area
  - C16:0: 1.0%
  - C18:0: 1.9%
  - C18:1: 1.0%
  - C18:2: 2.9%

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Metabolomics: oximation + silylation ("Fiehn method")

- Lyophylized sample in 2 mL high recovery vial (extract from plant material, e.g. Arabidopsis) in cooled tray (Tray 1, 5°C)
- Add 70 µL of 20 mg/mL methoxylamine hydrochloride I pyridine
- Heat 60 min – 30°C (Tray 2)
- Add 70 µL MSTFA (+1%TMCS)
- Heat 30 min – 37°C (Tray 3)
- Analyse by GC-MS

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Metabolomics: oximation + silylation ("Fiehn method")

		tR (min)	RSD (%, n=6) area	RSD (%, n=6) relative area
IS	Myristic Acid-d_{27}	16.727	5.78	
1	2-hydroxypyridine	6.519	8.16	3.40
2	phosphoric acid	9.966	6.65	8.15
3	L-threonine 1	10.224	6.42	8.14
4	Fumaric acid	10.940	5.33	1.70
5	Aspartic acid 1	12.002	2.03	4.94
6	D-malic acid	12.794	3.10	3.18
7	L-glutamic acid 1	13.338	10.64	15.02
8	Purine riboside	21.776	8.75	11.06
9	Dehydroascorbic acid 1	16.863	8.96	5.90
10	L- sorbose 2	17.235	9.93	5.79
11	D-mannose 2	17.435	11.10	13.56
12	Lactulose 1	23.867	3.76	6.64
13	Allo-inositol	17.245	5.06	2.92
14	D-(+) trehalose	24.752	8.94	6.72
15	Cellobiose 1	24.444	5.26	7.98
Average			6.87	6.57

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Mineral Oil Analysis (HOI: hydrocarbon oil index)

- Mineral Oil analysis is in top 5 of current environmental analyses (next to VOCs, PAHs, pesticides)
- Analysis is done by GC-FID (replaces old extraction – FTIR analysis)
- Technique is also similar to “TPH” (Total Petroleum Hydrocarbon)

- Ref: ISO 9377-2, ISO 9377-4, EN 14039, DIN H53,…

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What is included in “Mineral Oil” analysis?

- Any mixture of compounds that:

1. are extracted by hydrocarbon solvent (from soil or water)
2. are not adsorbed by Florisil (used for cleanup)
3. elute from an apolar column between decane (C_{10}) and tetracosane (C_{40})

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Typical HOI analytical procedure

1. **Extract**

- Water: extract 1 L water with hexane
- Soil: extract by ASE

2. **Clean-up**

- Florisil (removes polar and semi-polar solutes such as sterols and lipids)

3. **Concentrate to 1 mL (**Kuderna-Danish, N_2 purge)

4. **Analyze extract by "discrimination free" injection, GC-FID, area sum between C_{10} and C_{40}**

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Original Method (ISO/DIN 9377-4)

- 900 mL water, pH adjusted to 2 using HCl
- 80 g $MgSO_4$ added
- 50 mL **solvent** added (pentane or hexane)
- 30 min agitation with a stir bar

➡ **LLE**

- Organic phase transferred; 2 g **Na_2SO_4** added; agitation

➡ **Water removal**

- 2 g activated **Florisil** added and agitation for a few minutes
- Filtration
- **Concentration** down to 1 mL
- Injection (1 µL, splitless, GC-FID)

➡ **Polar compound removal**

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Miniaturization & automation on WorkBench

- 900 mL water, pH adjusted to 2 using HCl
- 80 g $MgSO_4$ added
- 50 mL **solvent** added (pentane or hexane)
- 30 min agitation with a stir bar
- Organic phase **concentrated** to 1 mL and transferred to a 2 mL vial

Manual

- 0.5 mL transferred to a 1.5 mL vial filled with 20 mg Na_2SO_4, agitation
- 0.35 mL transferred to a 1.5 mL vial filled with 30 mg **Florisil**, agitation
- 0.15 mL transferred to a 200 µL insert

•Injection (1 µL, splitless, fast GC-FID)

Automation on Agilent WorkBench

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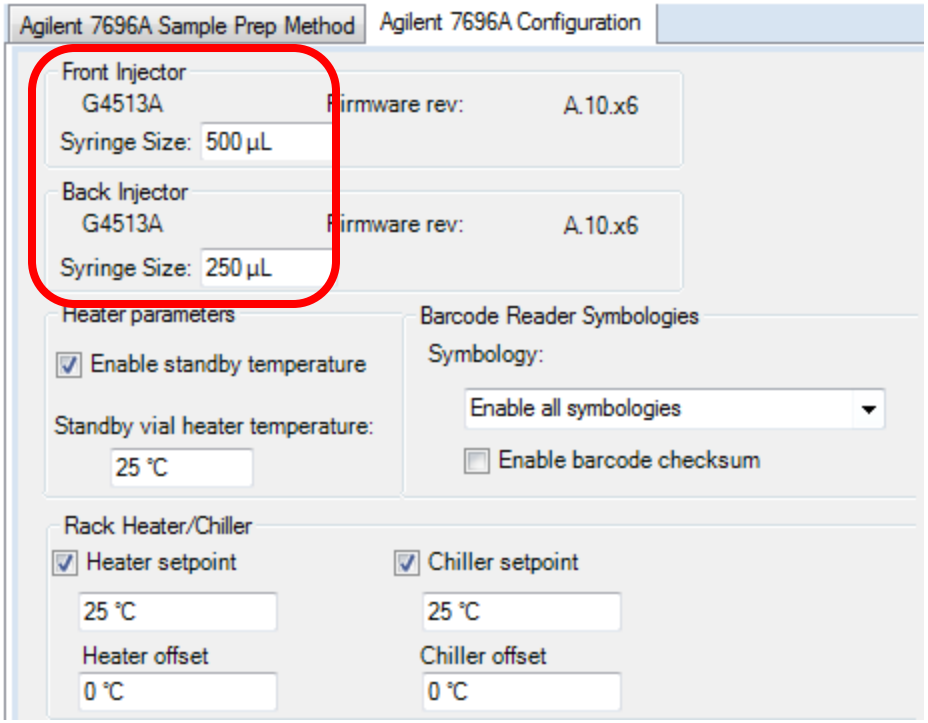
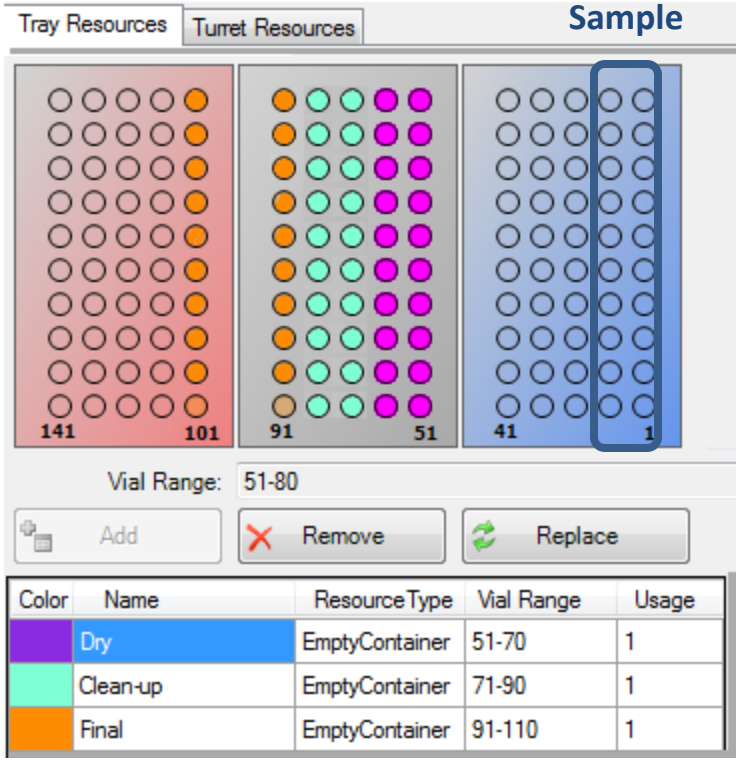
Automated WorkBench Method





All solvent resources filled with hexane (= wash A and B in front & back turrets).

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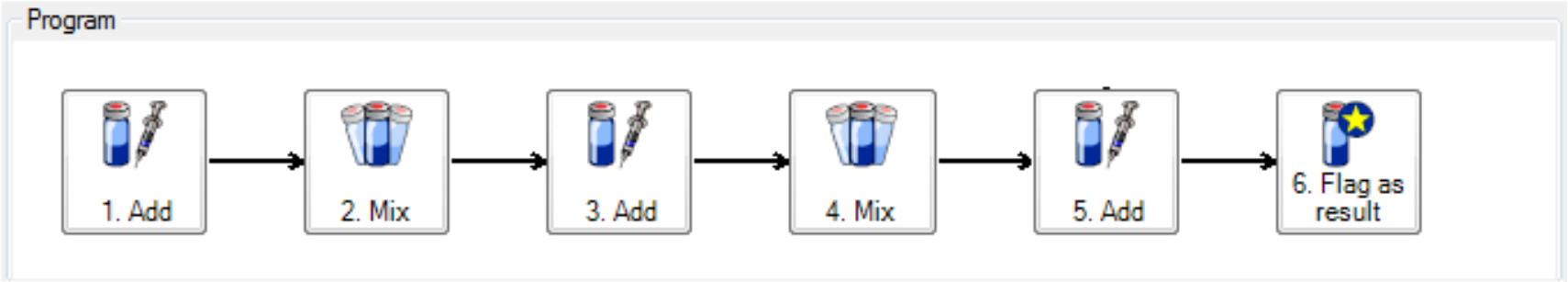
Automated WorkBench Method

1. Add 500 µL of “Sample” (hexane after LLE) to “Dry” at Front Tower
2. Mix “Dry” for 1 min (2000 rpm, bidirectional, 4 s on, 1 s off)
3. Add 350 µL of “Dry” to “Clean-up” at Back Tower
4. Mix “Clean-up” for 1 min (2000 rpm, bidirectional, 4 s on, 1 s off)
5. Add 150 µL of “Clean-up” to ”Final” at Back Tower
6. Flag “Final” as Result



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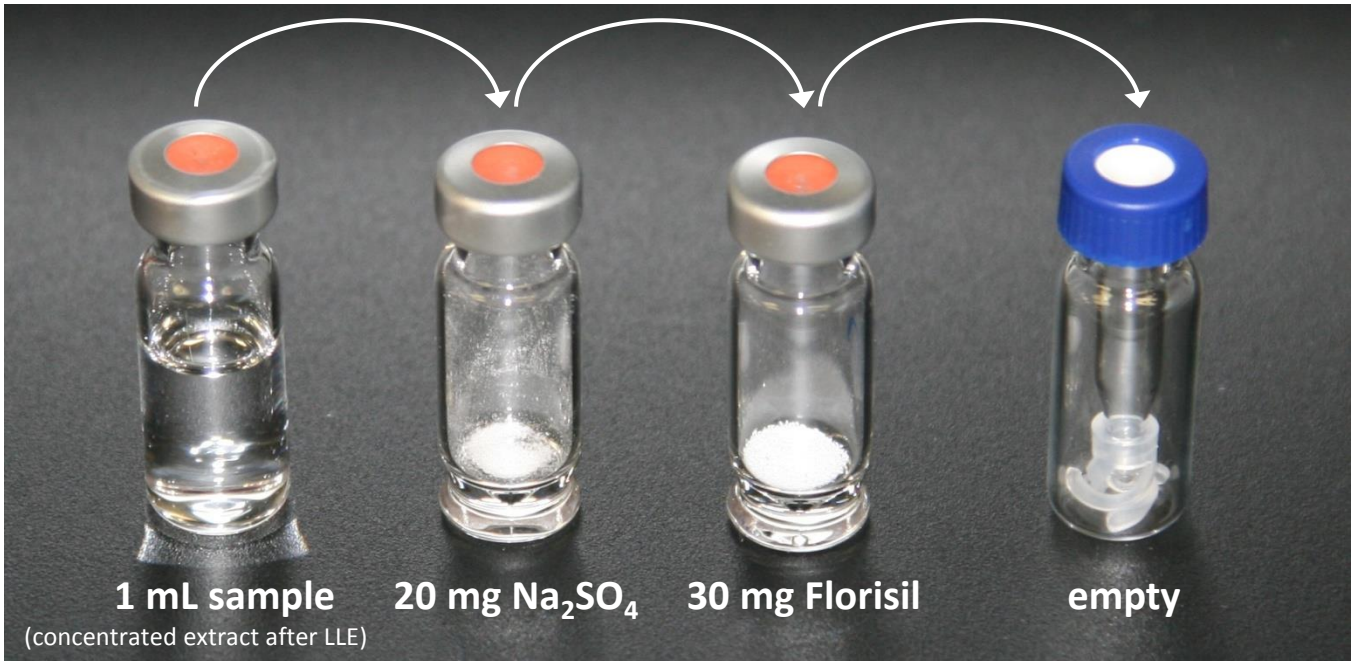
Automated WorkBench Method

Front tower	Back tower	Back tower
Transfer 500 µL and mix	*Transfer 350 µL and mix*	*Transfer 150 µL*



2 mL vial	*1.5 mL high recovery vials*		*2 mL vial with 200 µL insert*
= Sample	**= Dry**	**= Clean-up**	**= Final**

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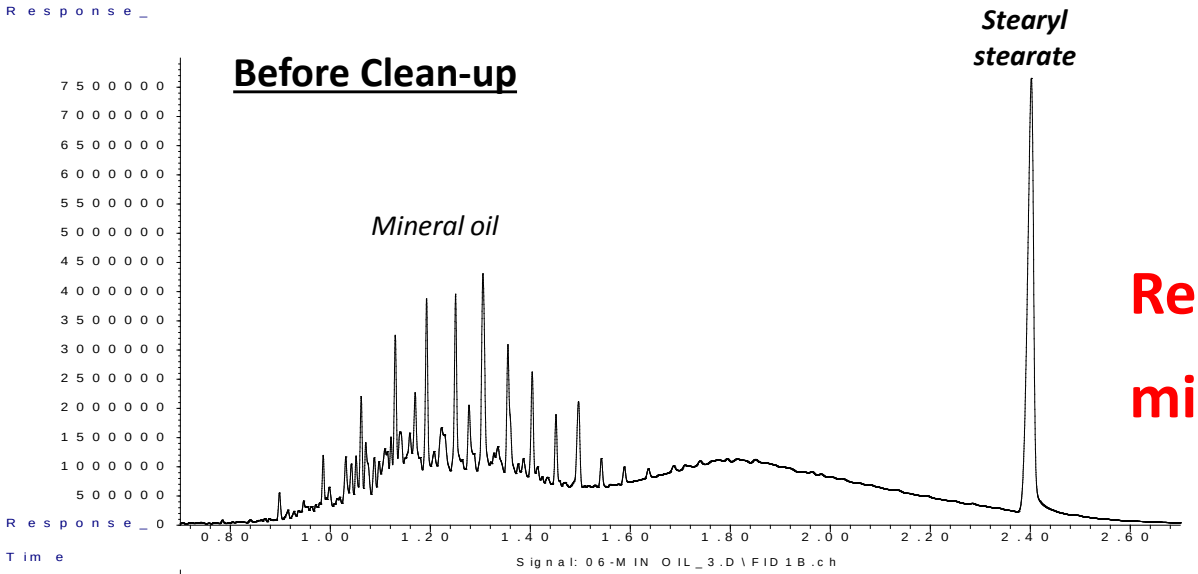
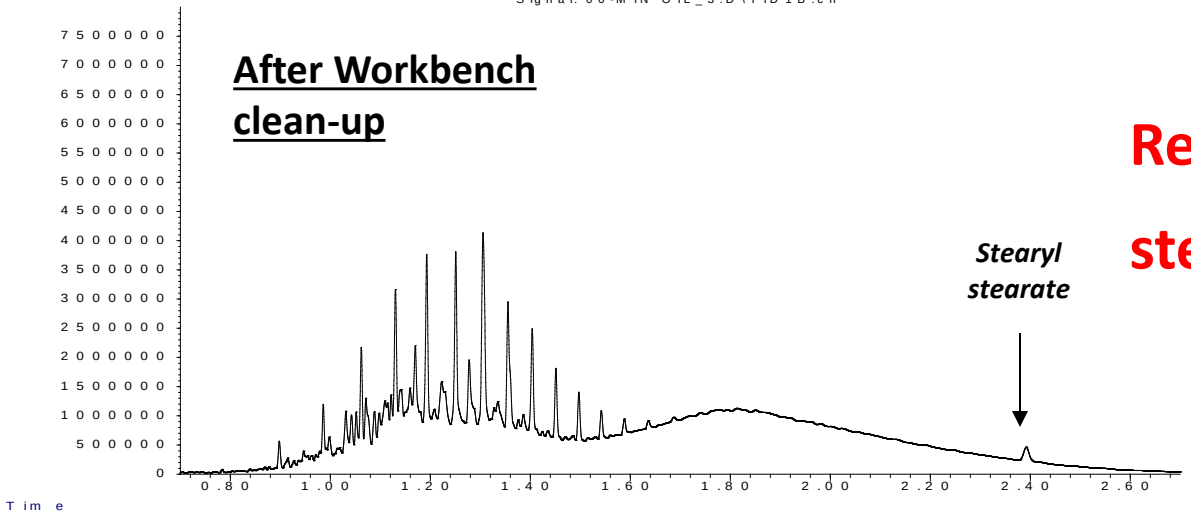
Clean-up efficiency



Recovery

mineral oil > 90%



Recovery

stearyl stearate < 5%

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Repeatability

➡ **Stearyl stearate & n-alkanes mixture (n = 6)**

	Average response	SD	RSD (%)	Recovery (%)
n-decane	58,1	0,54	**0,93**	99,6
n-eicosane	62,7	0,61	**0,97**	99,8
n-tricosane	60,4	0,66	**1,09**	101,1
stearyl stearate	16,0	2,79	na	1,9
n-tetracosane	50,5	1,29	**2,56**	86,4

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PCBs in waste Oil - Original Method

- A 10% dilution of the oil is made in hexane (1 g in 10 mL)
- From this solution, 250 µL was applied to a series-combination of two cartridges:
    - 3 mL cartridge filled with 500 mg of silica treated with H_2SO_4 + 500 mg strong anion exchange resin (“SiH”)
    - 3mL cartridge filled with 500 mg silica.

    (The cartridges preconditioned with hexane)
- The PCBs eluted with 4 mL hexane
- Aliquot of this solution was used for GC/MS analyses.

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PCBs in waste Oil - Automated WorkBench Method

1. Add 50 uL of Sample (10% waste oil in hexane) to “SiH” at Front Tower
2. Add 1350 uL of hexane to” SiH” at Front Tower
3. Add 150 uL of IS (10 ppm OCN in i-C8) to “SiH” at Front Tower
4. Mix “SiH” at 4000 RPM for 5 min 0 sec
5. Wait for 2 min 0 sec
6. Add 1000 uL of “SiH” to SiOH at Front Tower
7. Mix SiOH at 4000 RPM for 5 min 0 sec
8. Wait for 2 min 0 sec
9. Add 200 uL of SiOH to Vial final at Front Tower
10. Flag Vial final as Result

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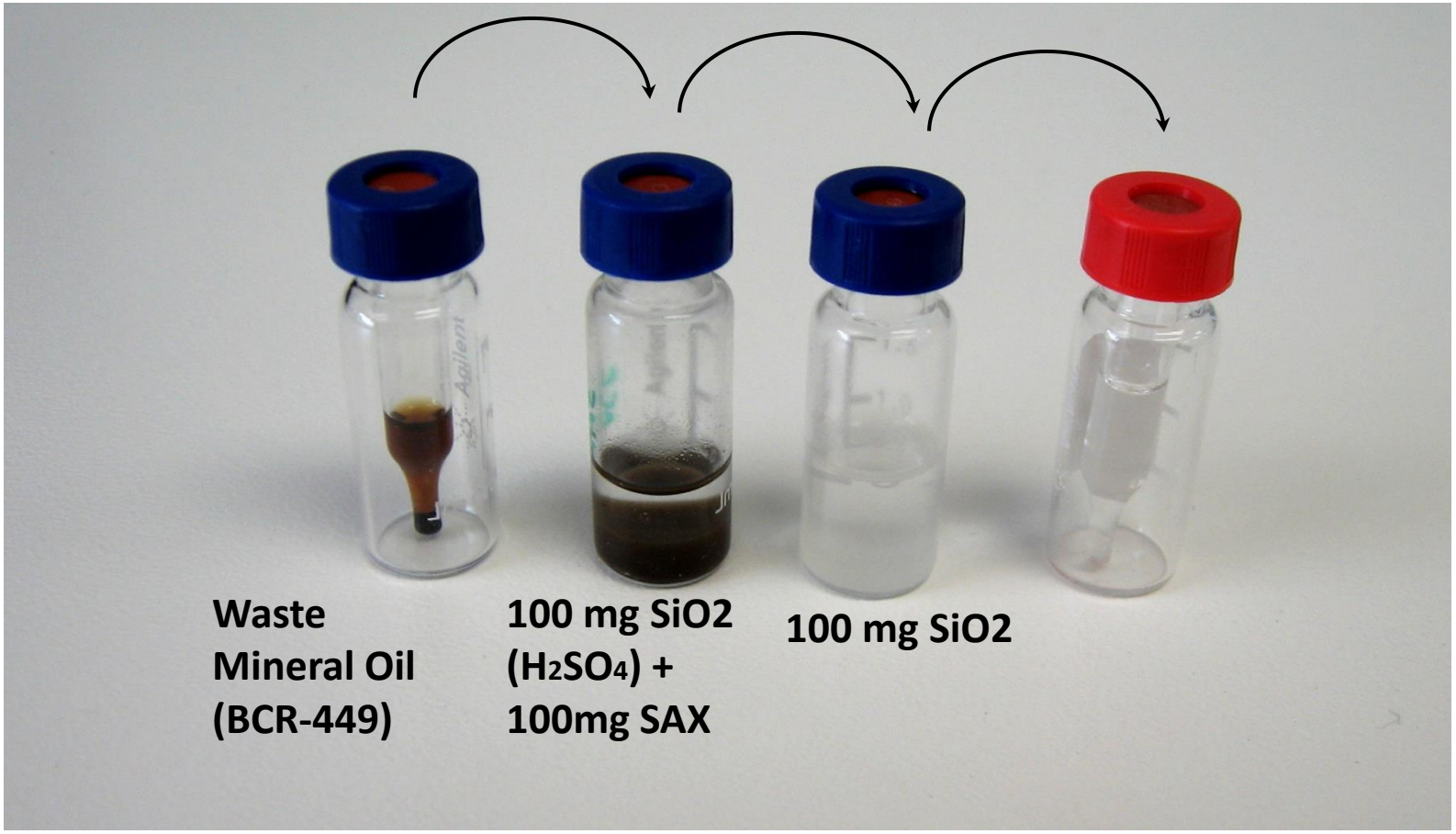
PCBs in waste Oil - Automated WorkBench Method

50 µL Mineral Oil
+ 150 µL IS (OCN)
+ 1350 µL hexane

1000 µL
upper layer

200 µL
upper layer



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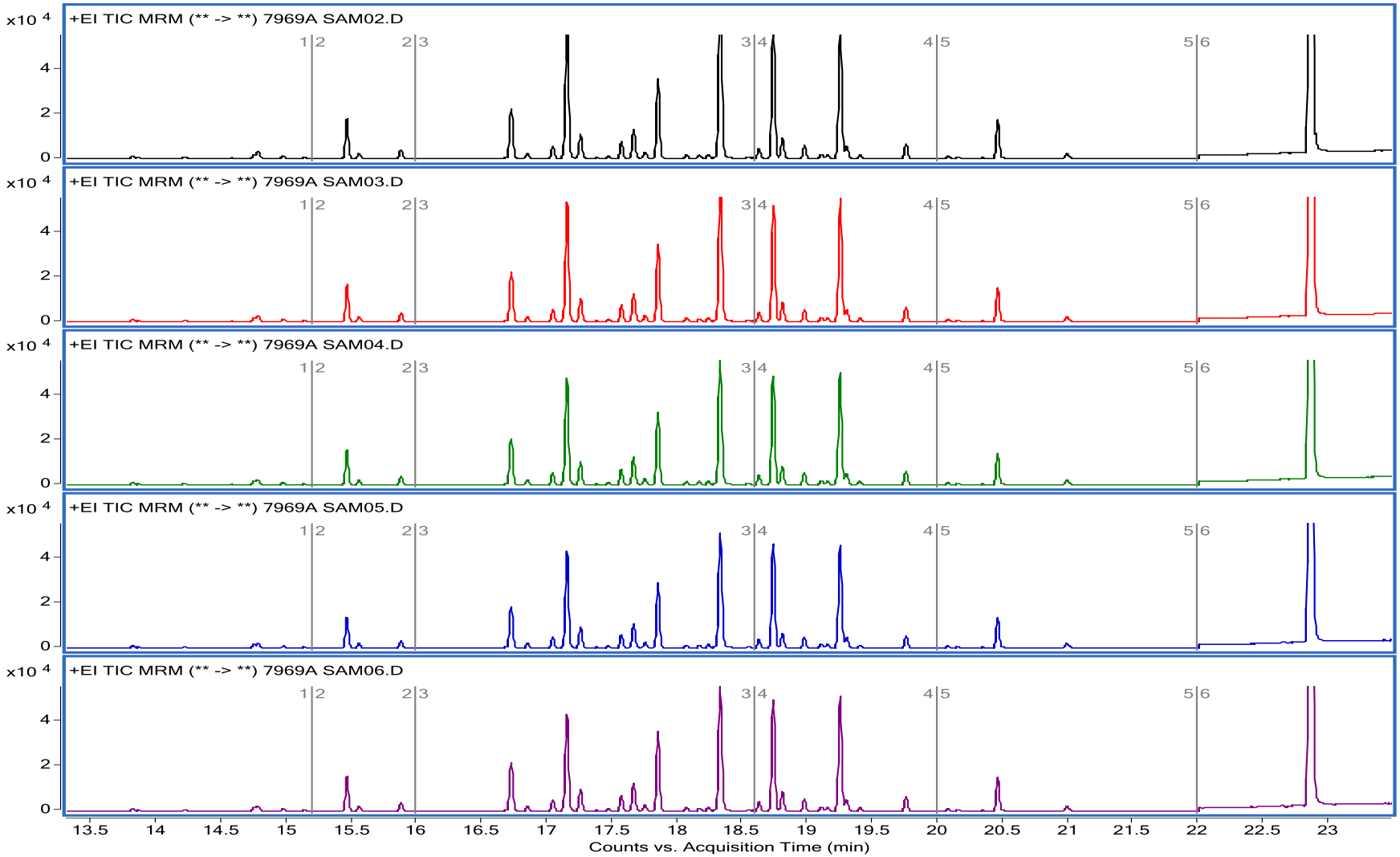
PCBs in waste Oil – GC-MS/MS analysis



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Conclusions

- Agilent 7696A WorkBench offers interesting possibilities for miniaturized sample preparation:
  - (serial) Dilutions for accurate calibration
  - Reduced consumption of API and impurities in pharma method validation and QC
  - Derivatization
  - Miniaturized clean-up (MSPD)

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Q & A

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