Analysing the METABOLOME

1. Metabolite Extraction
2. Metabolite Separation
3. Metabolite detection (with or without separation)
4. Data analysis

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EXTRACTION

Each group of metabolites will have an optimal extraction method
(no single solution)

Stopping the enzymatic activity!!

Metabolite Extraction

- Liquid phase extraction

  Grind sample, extract with solvent
- Liquid : Liquid extraction

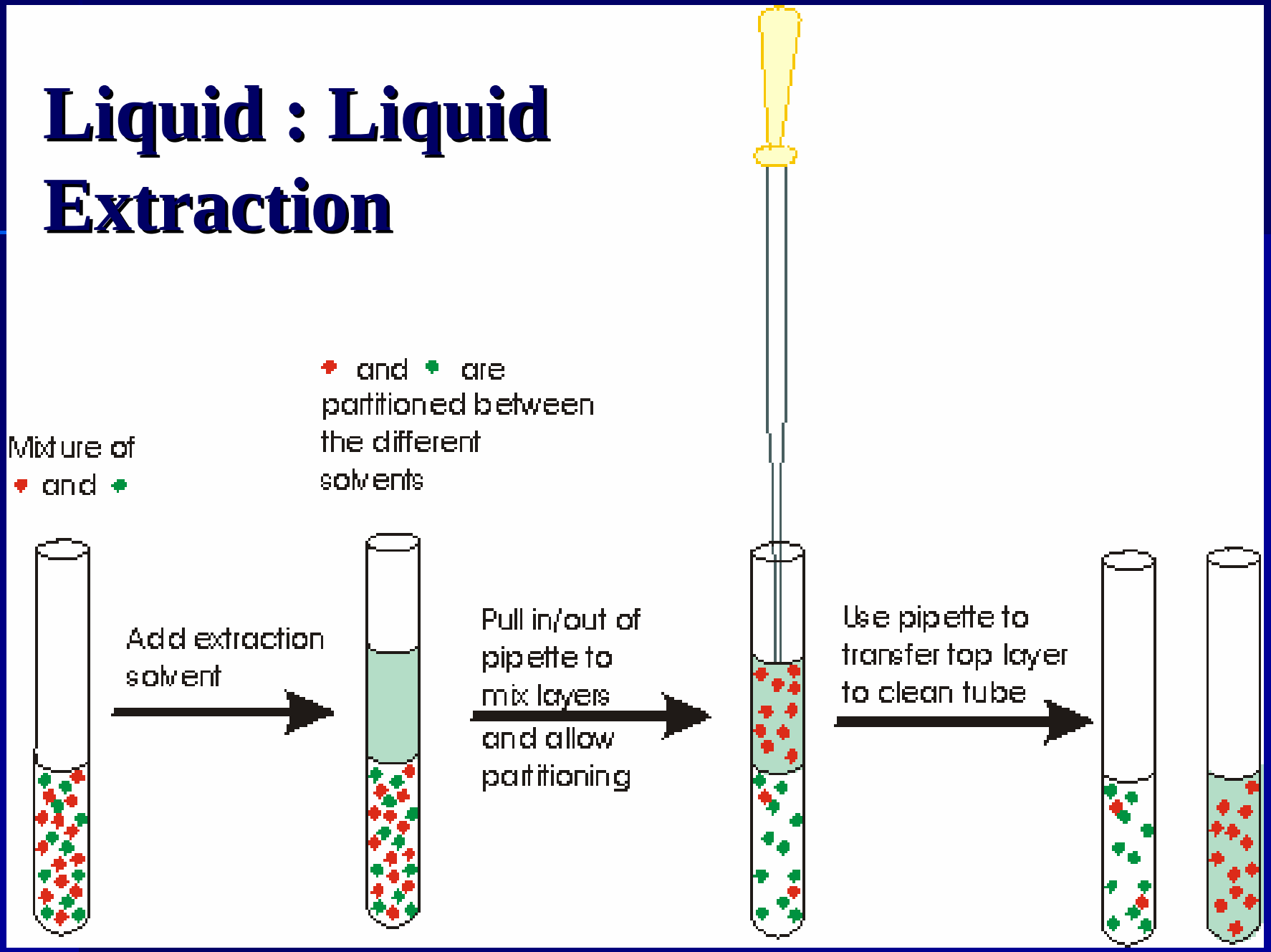
  Take liquid extract, extract with another solvent
- Solid : Liquid Extraction

  Take liquid extract, extract with solid phase material

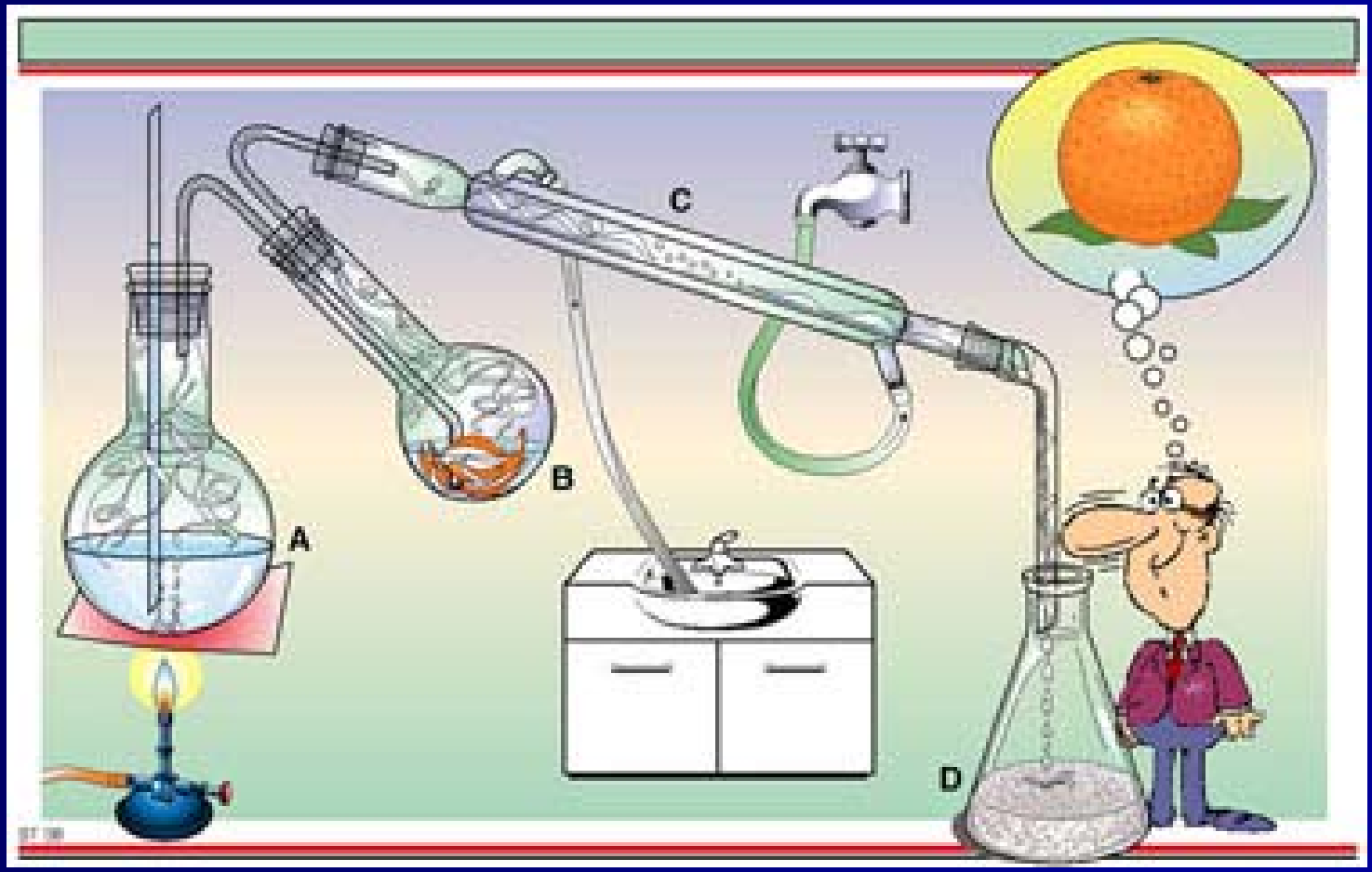
Volatile Metabolite Extraction

- Steam distillation
- Headspace
- Headspace & solid phase extraction (Trapping)
- Solid phase micro-extraction (SPME)

Liquid : Liquid Extraction

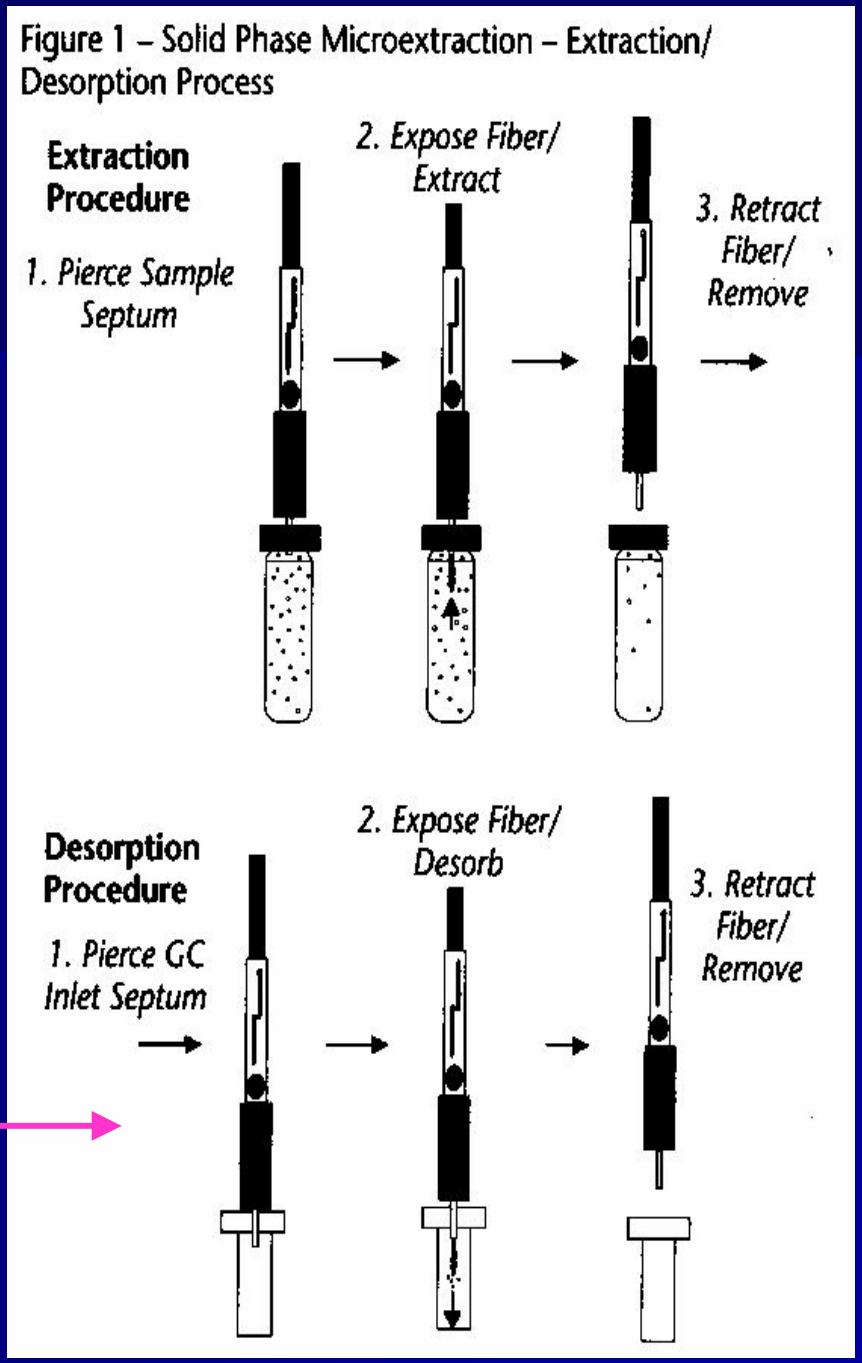


Volatiles (Essential oils) Steam Distillation



Solid Phase Micro Extraction (SPME)

To GC-MS injection port



Headspace & solid phase extraction (Trapping)

Measuring Headspace Volatiles Emitted by Arabidopsis

Headspace & solid phase extraction (Trapping)

Measuring Headspace Volatiles Emitted by Roses

Analysing the METABOLOME

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Metabolite detection as part of

Metabolome Analyses Technologies

Detection methods (MS, direct/flow injection)

Separation /detection methods (LC-MS)

Separation /combination of detection methods (LC-NMR-MS)

Metabolite detection

Metabolome Analyses Technologies

- Infrared spectroscopy (IR)
- Nuclear magnetic resonance (NMR)
- Mass spectrometry (MS)
- Thin layer chromatography (TLC)
- High performance liquid chromatography (HPLC) equipped with different kinds of detectors: UV or photodiode array (PDA), fluorescent, electrochemical, etc.
- Capillary electrophoresis (CE) coupled to different detectors: UV, laser induced fluorescent (LIF), mass spectrometer (MS or MSMS), etc.
- Gas chromatography (GC) coupled to different detectors: MS or MSMS, FID
- Liquid chromatography tandem mass spectrometry (LC/MS or LC/MS/MS)
- Fourier transform ion cyclotron mass spectrometry (FTMS)
- HPLC coupled to NMR detection (LC/NMR)
- HPLC coupled to NMR and MS detectors (LC/NMR/MS)

Metabolite Detection

No single solution! most used systems are:

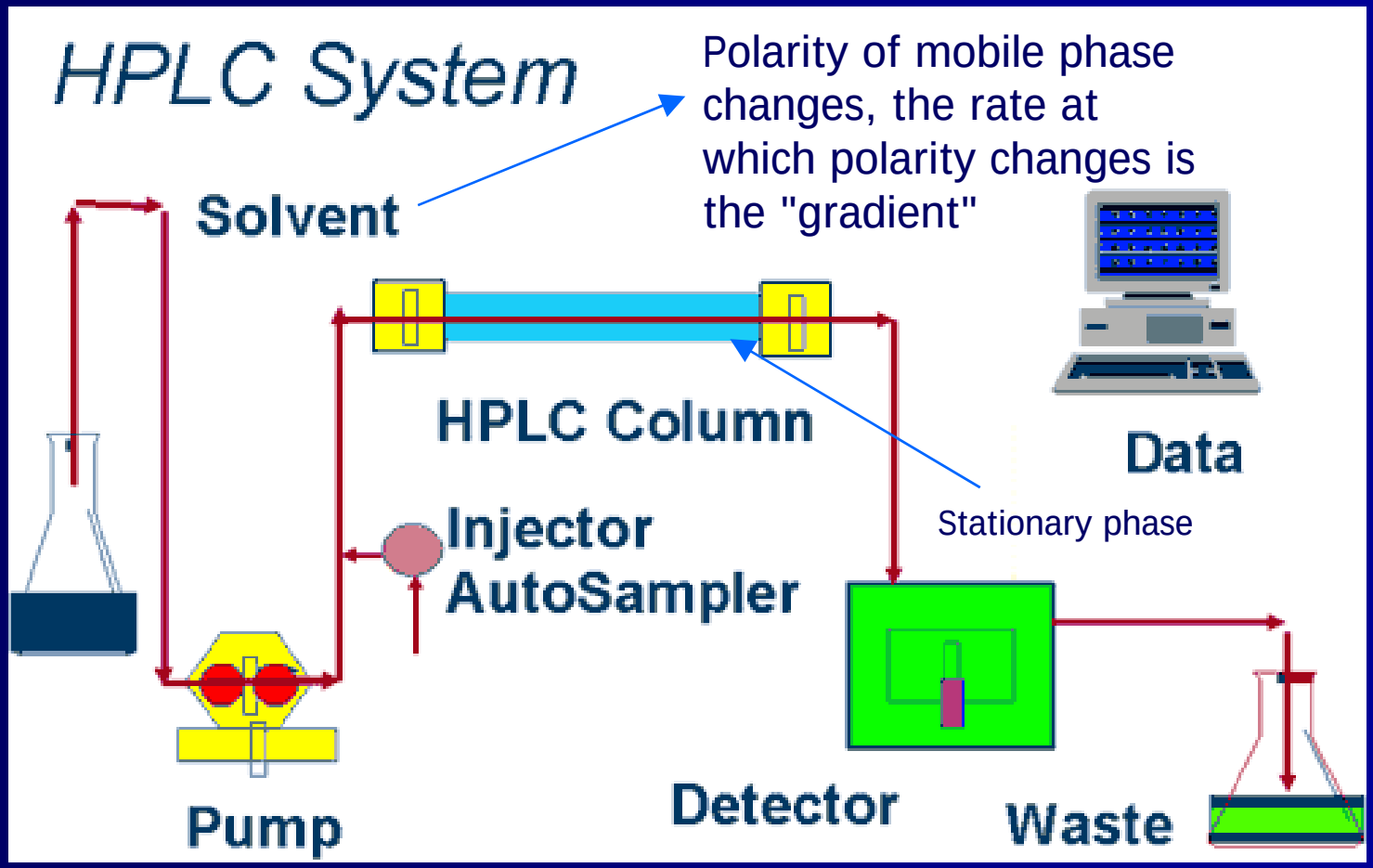
- GC-MS: Naturally volatile or made volatile (any organic- flavors, sugars, lipids, acids)
- HPLC- Chromatography + detector

  For example UV-detector (phenolics) or MS
- NMR – Any Compound containing hydrogen
- LC-MS/NMR- compounds that are not well characterized by other methods. Structure elucidation capacity

Separation Methods

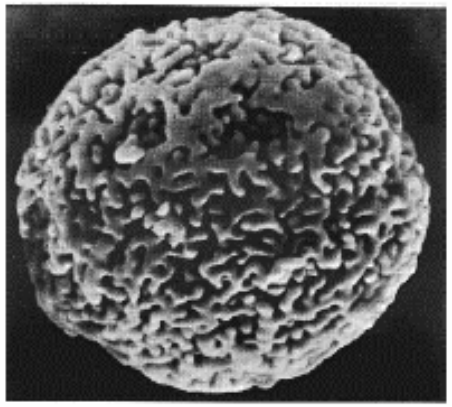
1. Thin layer chromatography
2. High Performance Liquid Chromatography (HPLC)
3. Gas chromatography (GC)
4. Capillary electrophoresis (CE)

HPLC Separation



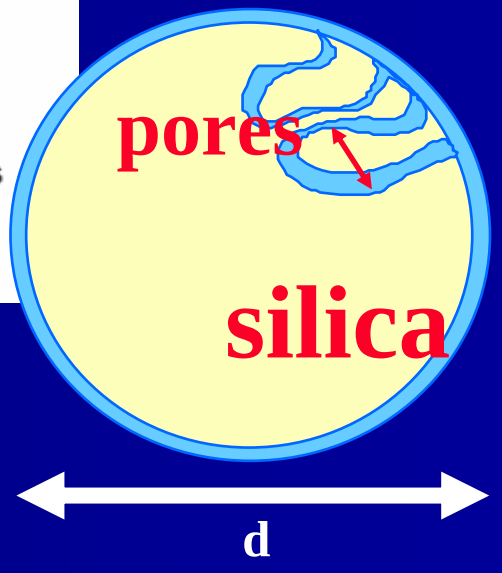
Stationary Phase in the HPLC Column

Pore size, shape and distribution



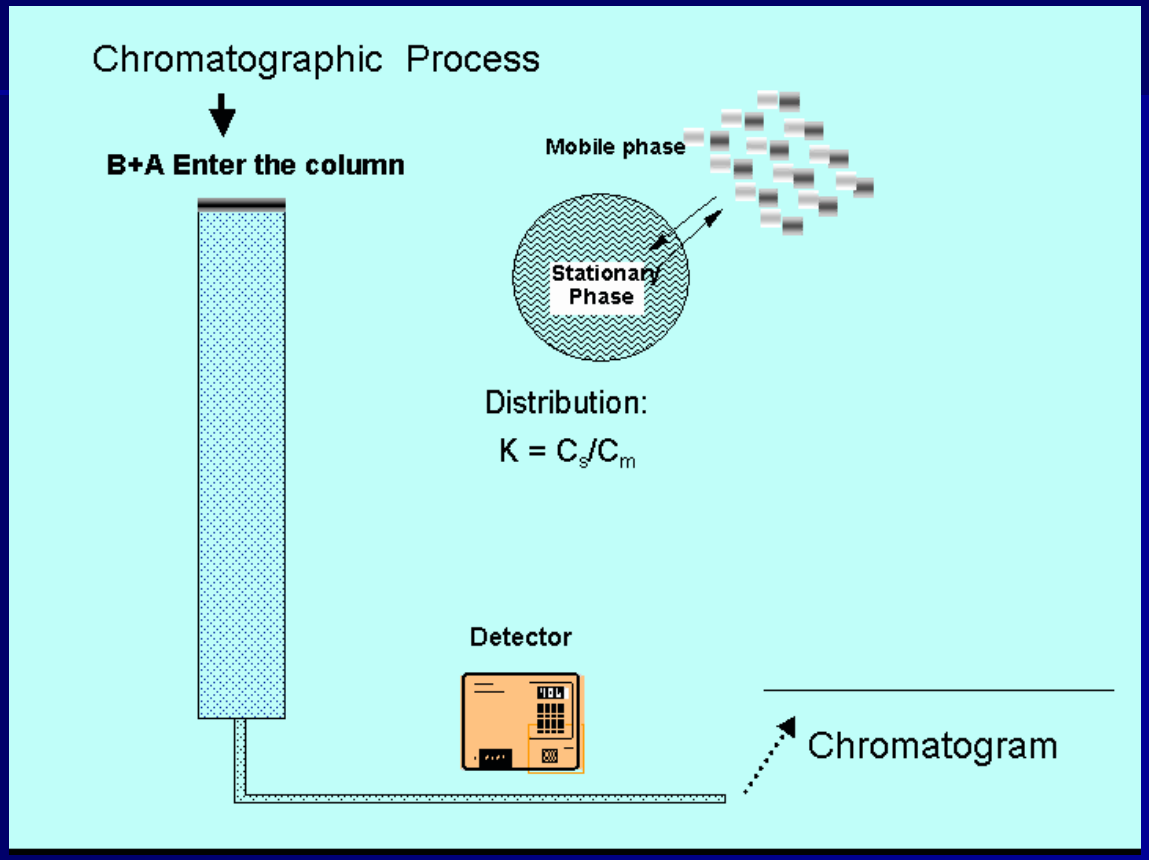
- Macroporous spherical silica particle. [K.K.Unger, Porous silica, Elsevier, 1979]

Pore size defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface. This is especially important because the ratio of the outer particle surface to its inner one is about 1:1000. The surface molecular interaction mainly occurs on the inner particle surface.



Analytical HPLC –
3, 5, 10 µm particle size

Stationary Phase and Mobile Phase in HPLC



As the analytes pass through the column they interact between the two phases--mobile and stationary--at different rates.
The difference in rates is primarily due to different polarities for the analytes.

HPLC- Normal & Reverse Phase

Normal Phase Chromatography:

Stationary Phase- polar and Mobile phase- non-polar

Least polar analyte elutes first

Reverse Phase Chromatography:

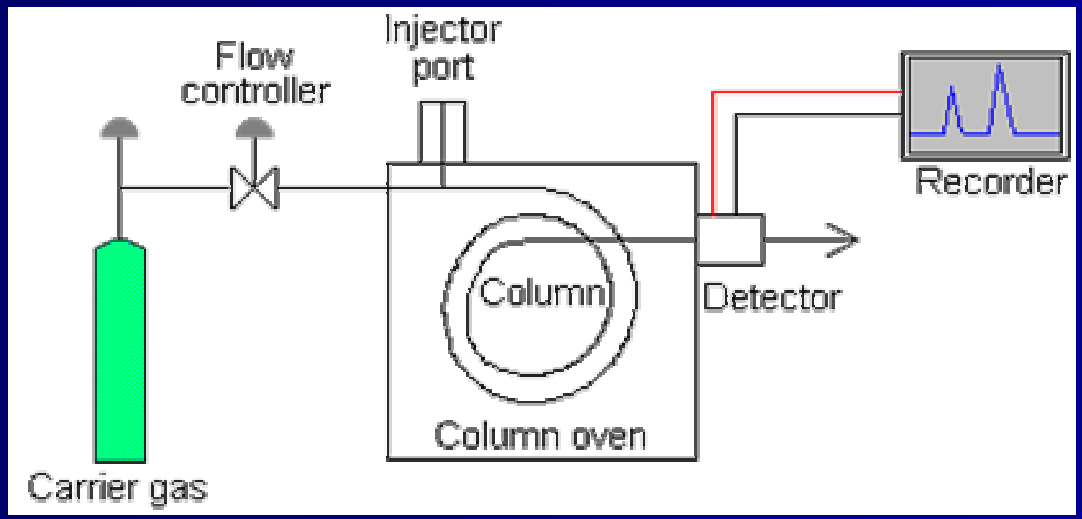
Stationary Phase- non-polar and Mobile phase-polar

Most polar analyte elutes first

Separation Methods

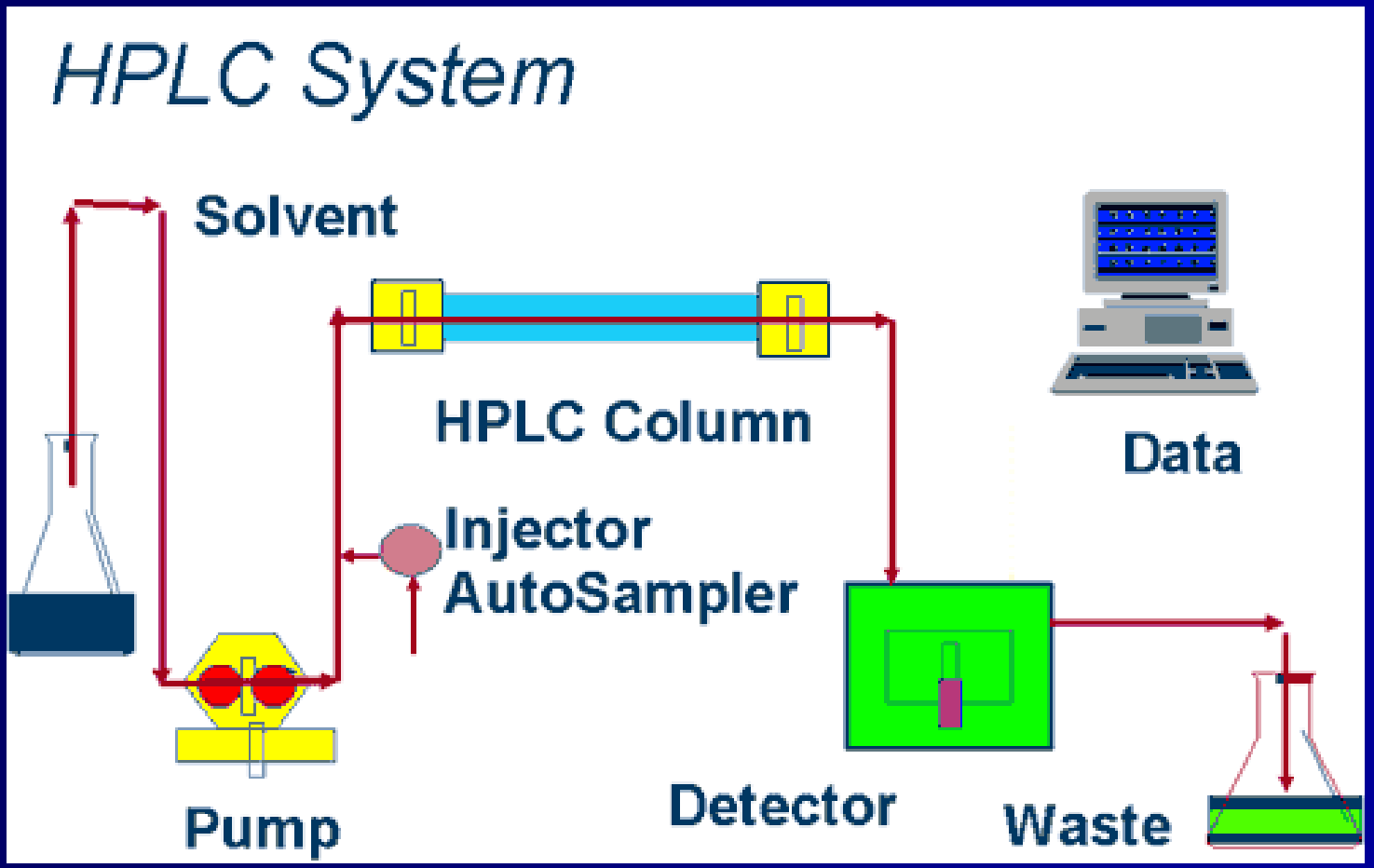
1. Thin layer chromatography
2. High Performance Liquid Chromatography (HPLC)
3. Gas chromatography (GC)
4. Capillary electrophoresis (CE)

3. Gas Chromatography (GC)



- The sample is vaporized in the injection port
- Sample injected to the head of the chromatographic column
- The sample transported through the column (in a heated oven) by the flow of inert, gaseous mobile phase
- Separation according to boiling points of compounds

The HPLC Instrument & Detectors



Detectors for HPLC

1. UV/VIS:
   - Fixed wavelength
   - Variable wavelength
   - Diode array
2. Refractive index
3. Fluorescence
4. Conductivity
5. Antioxidant
6. Evaporative light scattering
7. Electrochemical
8. Mass Spectrometer

Fixed Wavelength Absorbance (320nm)

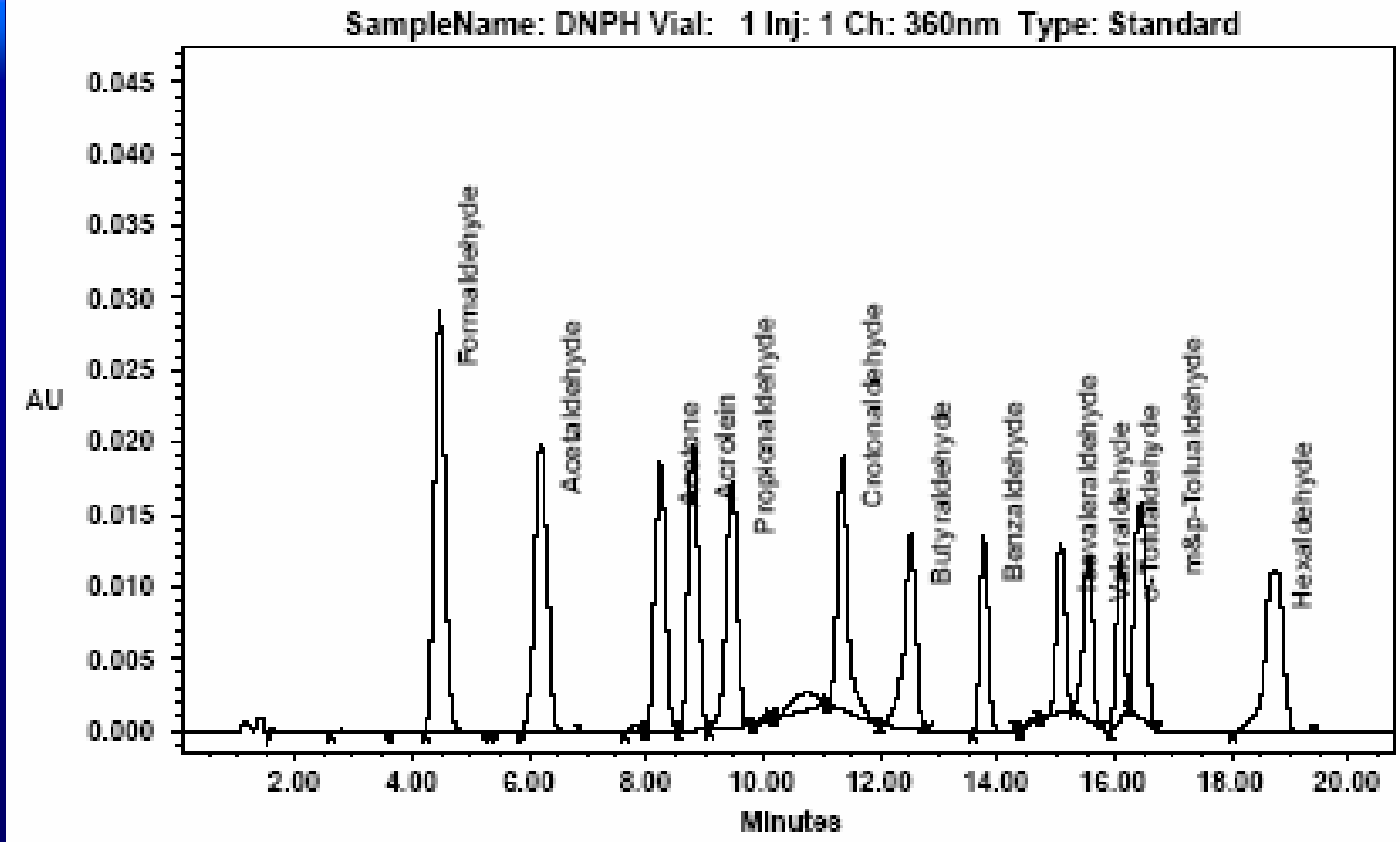
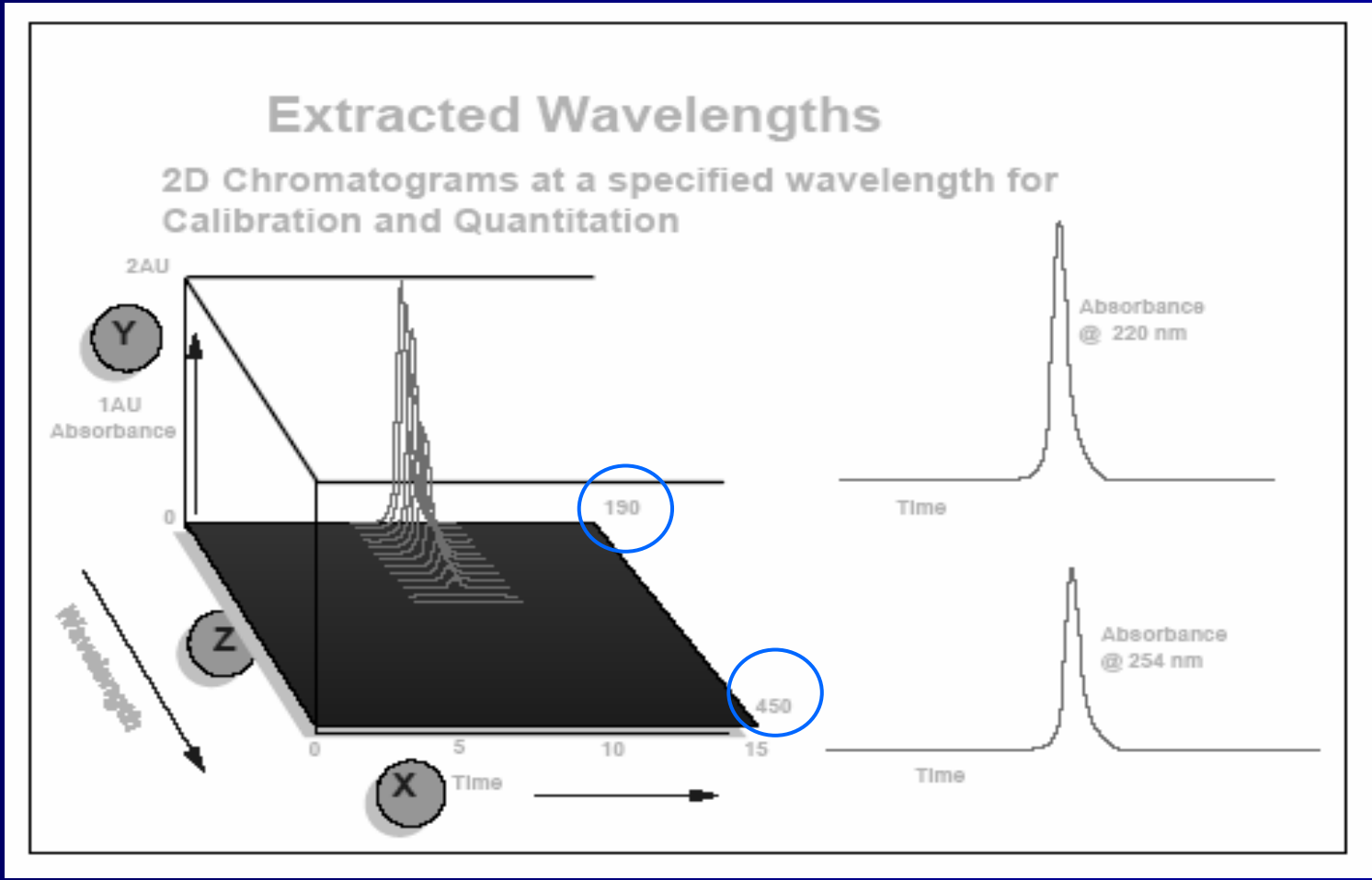
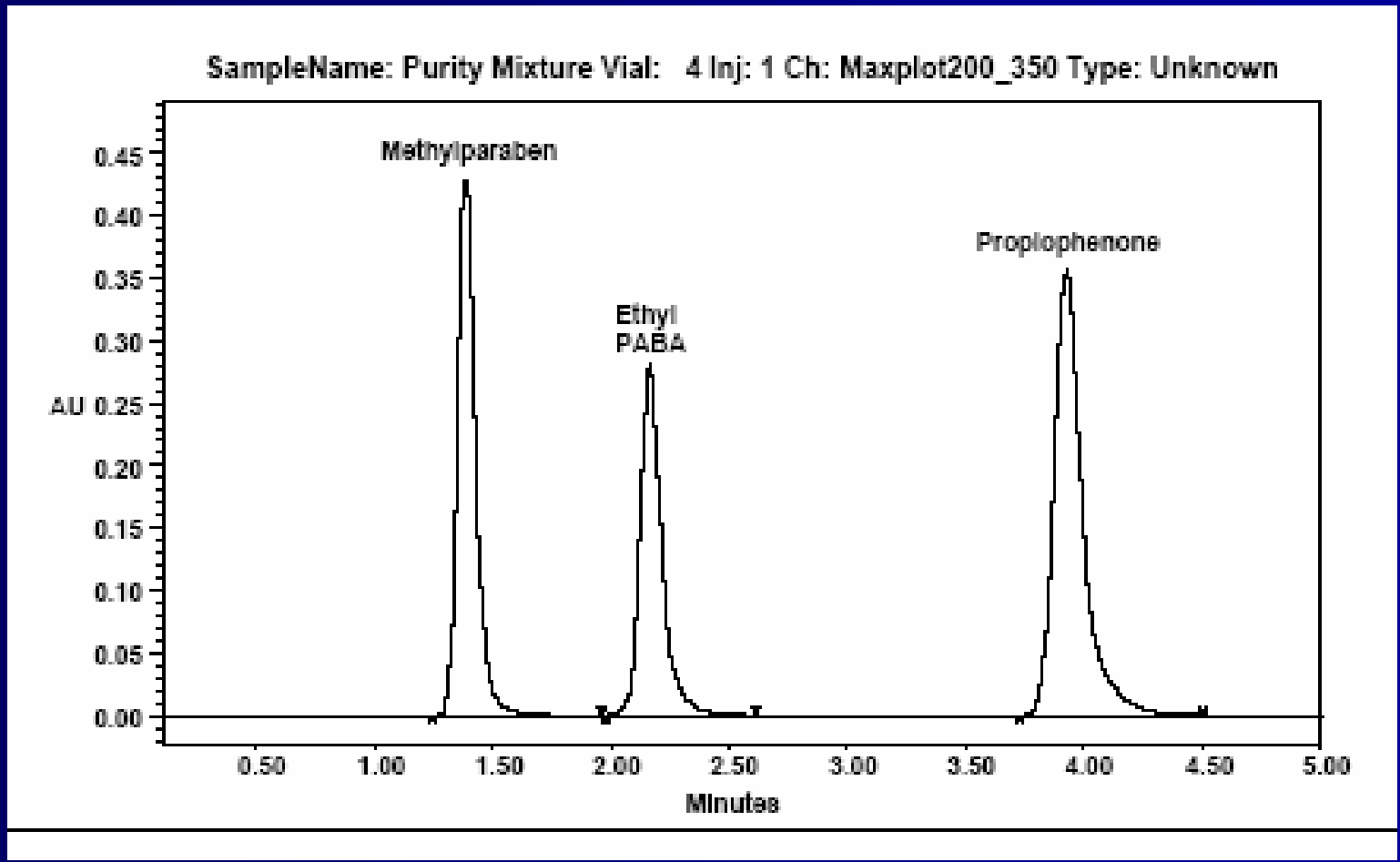


Photo Diode Array (PDA) Detector



PDA Detector and Visualization with the MaxPlot Option



Detectors

1. UV/VIS:
- Fixed wavelength
- Variable wavelength
- Diode array
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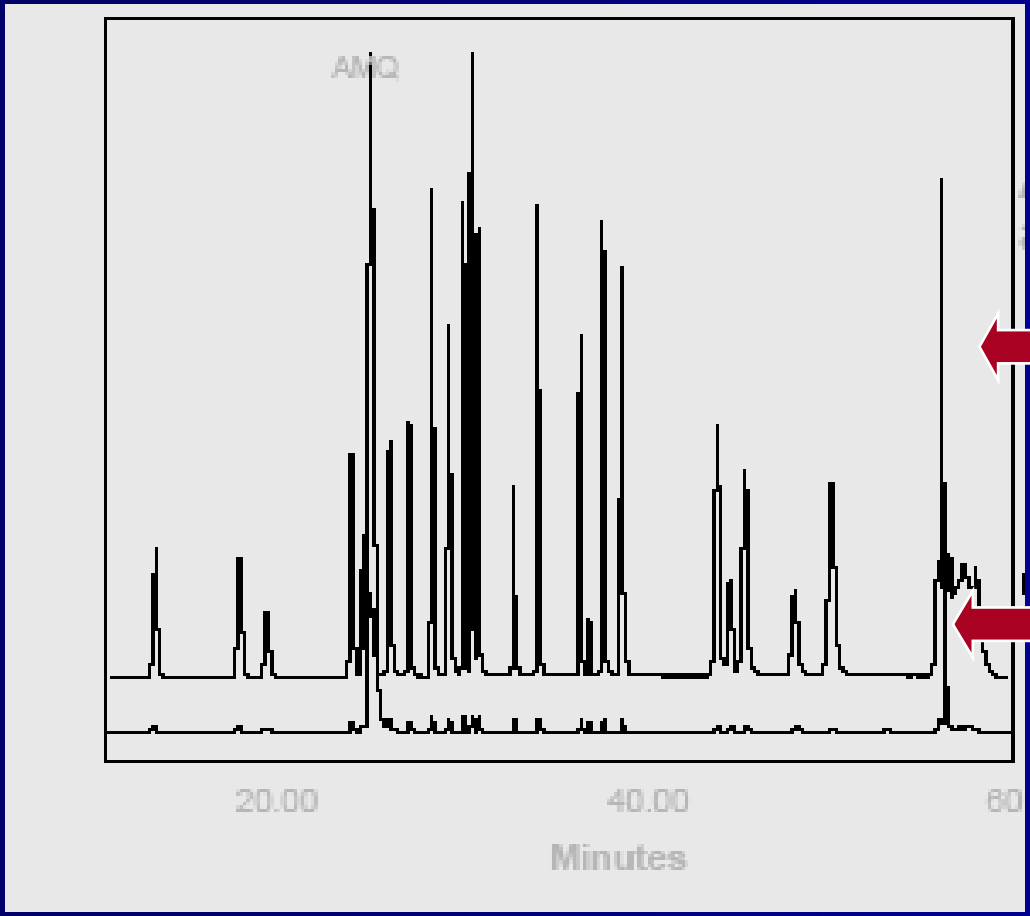
Fluorescence vs. UV Detection



Fluorescence
Excitation at 250 nm
Emission at 395 nm

UV
Absorbance 254nm

GC Detectors (except MS)

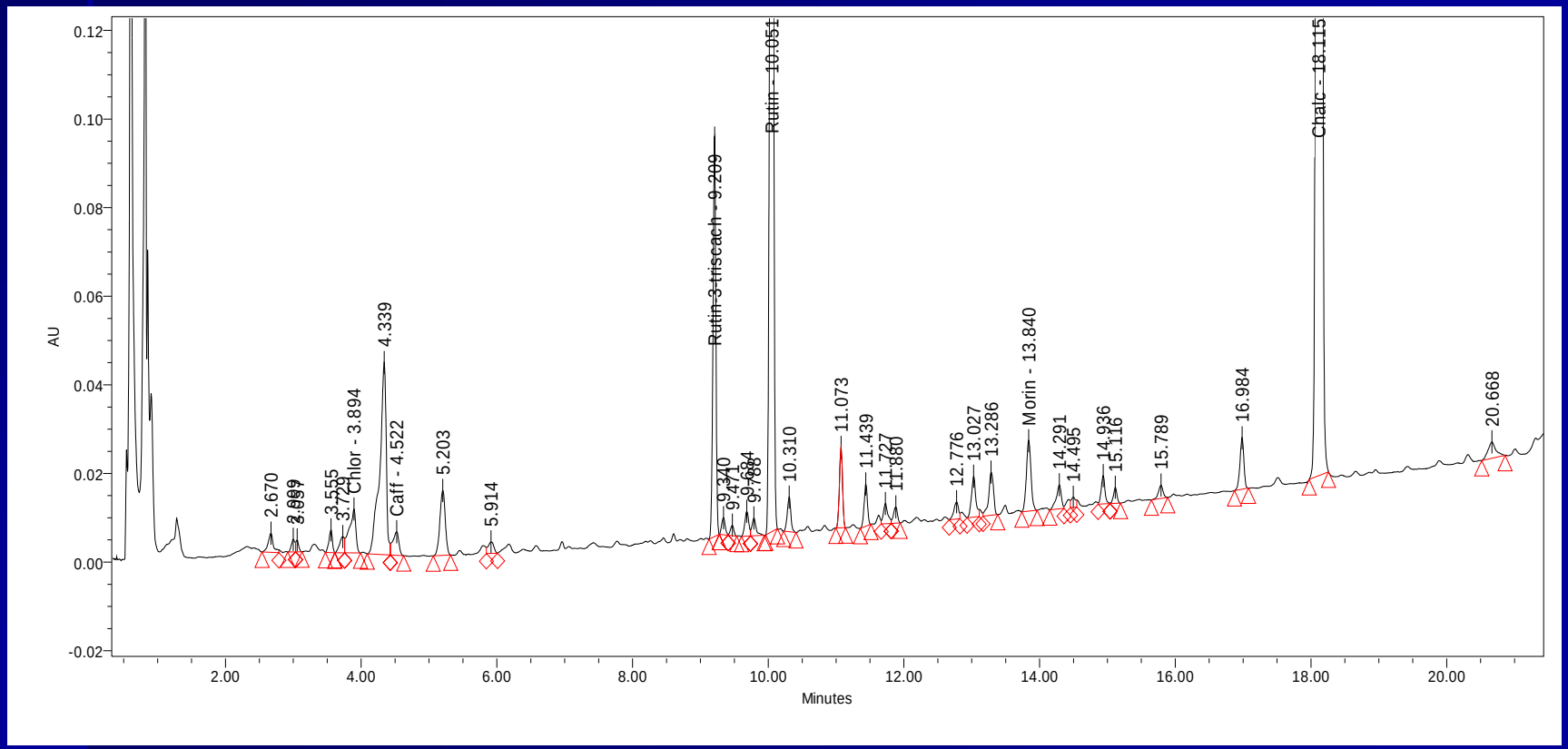
Detector	Selectivity
Flame ionization (FID)	Most organic cpds.
Thermal conductivity (TCD)	Universal
Electron capture (ECD)	Halides, nitrates, nitriles, peroxides, anhydrides, organometallics
Nitrogen-phosphorus	Nitrogen, phosphorus
Flame photometric (FPD)	Sulphur, phosphorus, tin, boron, arsenic, germanium, selenium, chromium
Photo-ionization (PID)	Aliphatics, aromatics, ketones, esters, aldehydes, amines, heterocyclics, organosulphurs, some organometallics
Hall electrolytic conductivity	Halide, nitrogen, nitrosamine, sulphur

About the Mass Spectrometer Detector

In the next class

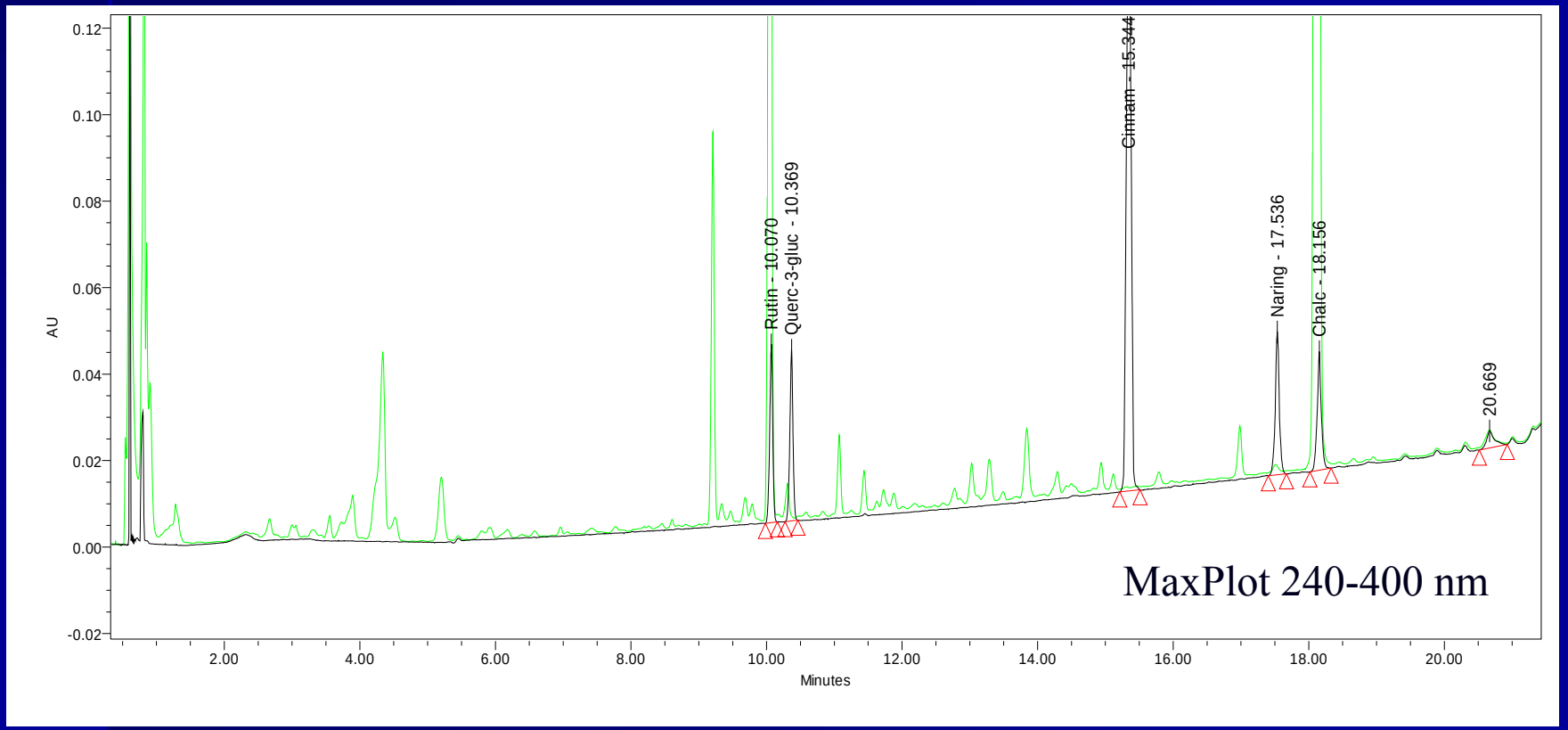
HPLC Chromatogram of a Tomato Sample

Tomato, WT, peel, MaxPlot 240-400 nm

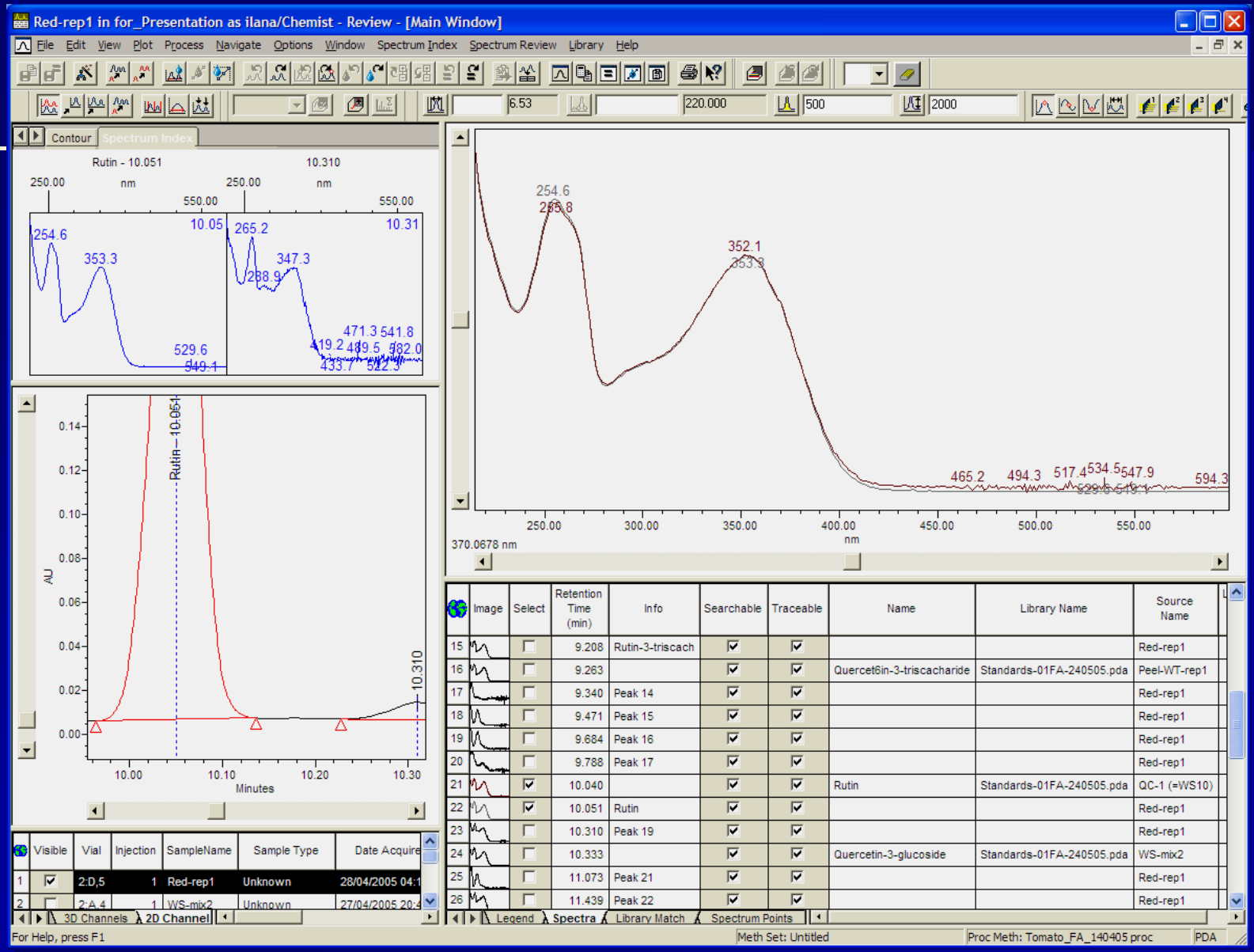


Peaks assignment: Comparison of sample chromatogram with the known standards

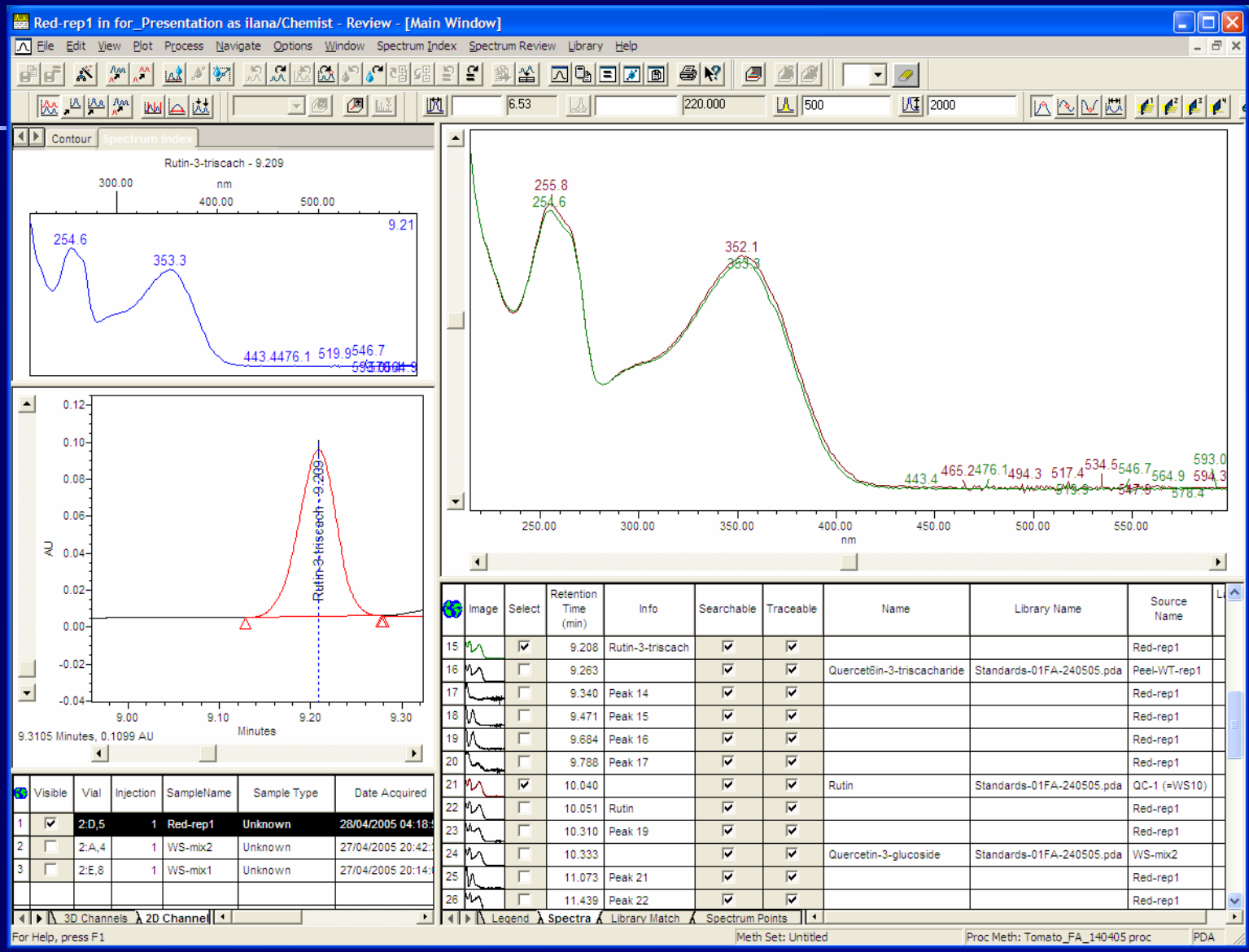
1. Comparison of Retention times (RT)



2. Comparison of UV Spectra



2. Comparison of UV Spectra



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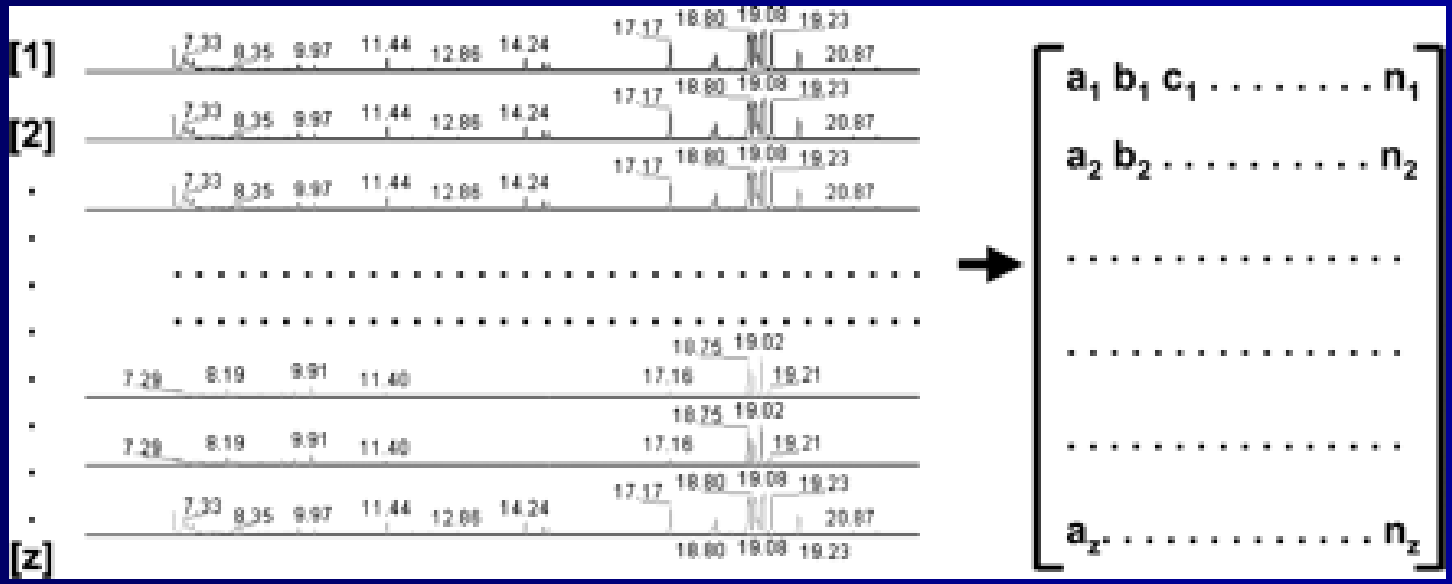
3. **Data analysis (HPLC-PDA only)**

Data analysis

The data transformation required in profiling techniques such as GC/MS and LC/MS.

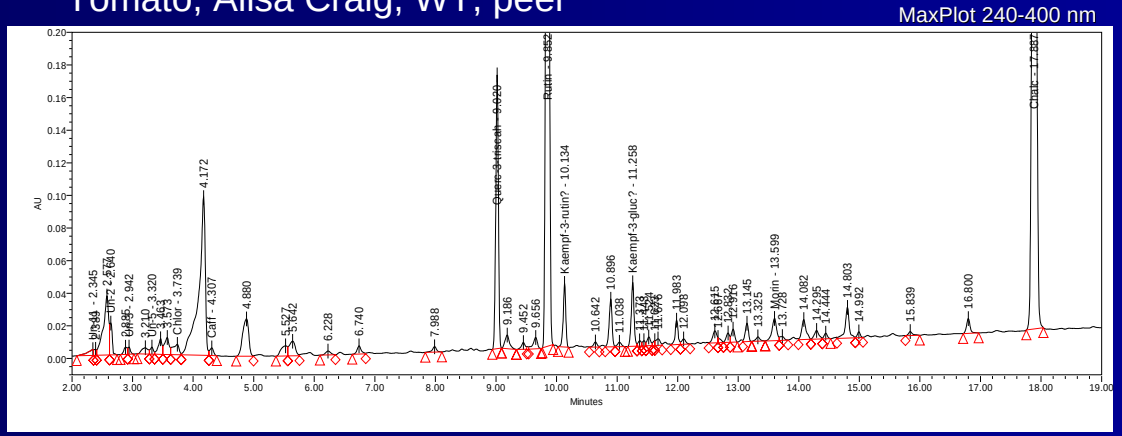


Areas of mass chromatographic peaks corresponding to components (a,b,c...n) are entered into a peak table for each sample chromatogram (1,2,3...z).

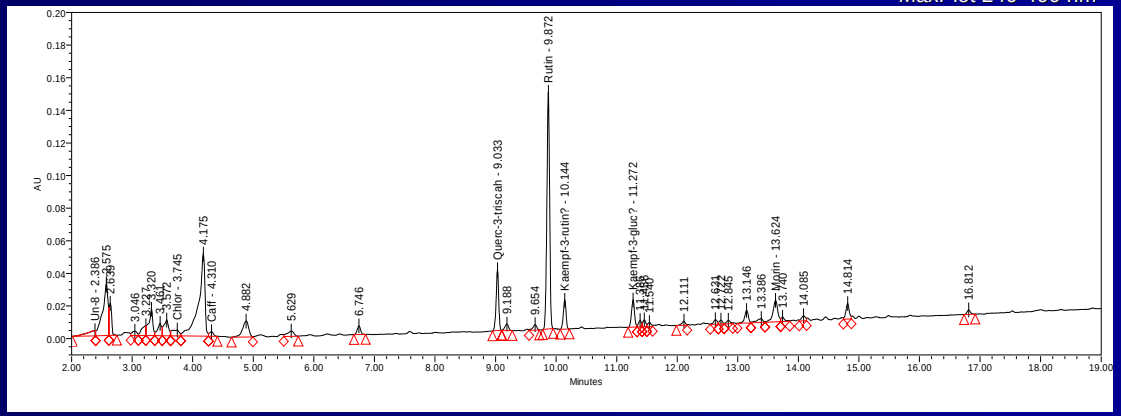
Data analysis

Metabolite profiling of Tomato samples

Tomato, Ailsa Craig, WT, peel



Tomato, mutant LA 3189, peel



Data analysis

Peak table for WT and mutant samples, replicate injections

SampleName	Peel-WT-rep1	Peel-WT-rep2	Peel-LA#3-rep2	Peel-LA#3-rep1	Peel-LA#1-rep2	Peel-LA#1-rep1	Peel-WT	Peel-LA#7-rep2	Peel-LA#7-rep1	Peel-LA#4-rep2
Un-1	241419	206868	127397	127465	283709	288794	223706	135812	143342	198087
Un-2	58658	49553	44465	45298	102208	96992	56025	35551	41020	55688
Un-3a		11983	11980	11853	33604	28021	17871			
Un-3	15697	13955			16356	13766	12349			
Un-4a			45896	51075	46460	41609		48528	62768	14663
Un-4	23317	21423	9951	14101	22116		27942	39957	32262	28838
Un-5	20978	18301	110211	106722	91241	81591	15828	122351	148823	74525
Un-6	40369	34817	44531	45493	34699	31952	41978	32308	36773	35221
Un-7	59121	52742	54175	56498	62357	62883	56481	43456	51682	52655
Chlor	60635	51580	50031	47574	55521	56685	46114	25864	32777	29494
Un-8a			39871	42790	26326	25153		57171	58947	
Un-8	797014	750225	431987	435713	550682	570399	739248	422014	431370	374172
Caff	31757	28552	18032	20774	27292	28168	20320	10940	16075	12492
Un-9	154463	130710	118495	117701	96152	103453	160836	88943	87544	69428
Un-10	46934	44525	14103	12741	18646	13982	46447			
Un-11	58382	51794	32383	33504	42987	46055	63893	36171	37916	24898
Un-12	24969	22427	28086	24816	23448	29831	24283	21412	21020	20592
Un-12a			10784	10907						
Un-13	15962	14911					16454			
Querc-3-triscah	548496	490093	142452	143017	231519	247238	506375	138289	132110	108476
Un-14	40676	33057	34559	27633	18420	19195	43531	25382	23390	16342
Un-15	19185	18774	10124	9608			18593			
Un-16	32835	30307	26264	26374	21456	22488	34726	23371	22987	15531
Rutin	2165740	1855442	586954	584920	1023246	1112867	2161457	500042	483482	459848
Kaempf-3-rutin?	115876	100613	54579	54070	70633	75203	114859	48936	46941	55266
Un-17			19119	16196						
Un-18	11641	12457					12891			
Un-19	100923	94039					98629			
Un-20	12273	11041					12377			
Kaempf-3-gluc?	123120	101469	62389	60959	96890	108029	125141	54871	53133	57656
Un-21	12150	10519			14970	15960	12123	13275	12882	13679
Un-22	11670	11517	14896	15341	12646	13589	10048	13895	13273	14338
Un-23	23697	21827			14099	14825	21655	9729	9304	9320
Un-24	19321	17837			8832	9591	20225			
Un-25	56180	48832					50890			
Un-26	17586	16376	11359	11052		11554	15299	9623	11407	9931
Un-27	36367	31995	21220	21936	10728	11290	35381	12254	10414	11770
Un-28	12688	11200	13503	14015	6914	6982	9336	9234	8182	8315
Un-29	26325	23456	15359	14310	23423	24290	22341			8964
Un-30	35669	30426					30896			
Un-31	45809	39438	63621	63139	41221	43591	41085	40248	37798	32968
Un-32	17508	12101	13800	13874	18755	18933	11717	17368	16111	16724
Un-32a			12553	12805			12095	11454	11099	11572
Morin	78144	73707	75296	76038	78599	78383	71043	77880	76128	72394
Un-33	63320	54971	29735	29592	16903	18412	61414	20785	23672	14215
Un-34	30660	25598	15932	13710			28092	11134		
Un-35	15954	13386	10221	9951	11424	12546	12983	11196	11283	
Un- 36	92129	84365	51525	52151	46150	47945	86399	41251	41093	37133
Un-37	14063	12546		7681			12198			
Un-38	42542	38626	15852	15838	17312	18458	37353	9360	10311	10419
Chalc	2535919	2230976					2370837	21394	20105	

Visit to the GC-MS and HPLC lab