Isolation, Purification and Identification Of Natural Products

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Mölndal

Isolation Techniques for Natural Products

- Squeezing
- Extraction
- Adsorption
- Centrifugation
- Filtering
- Distillation
- Headspace

General scheme for sample work up

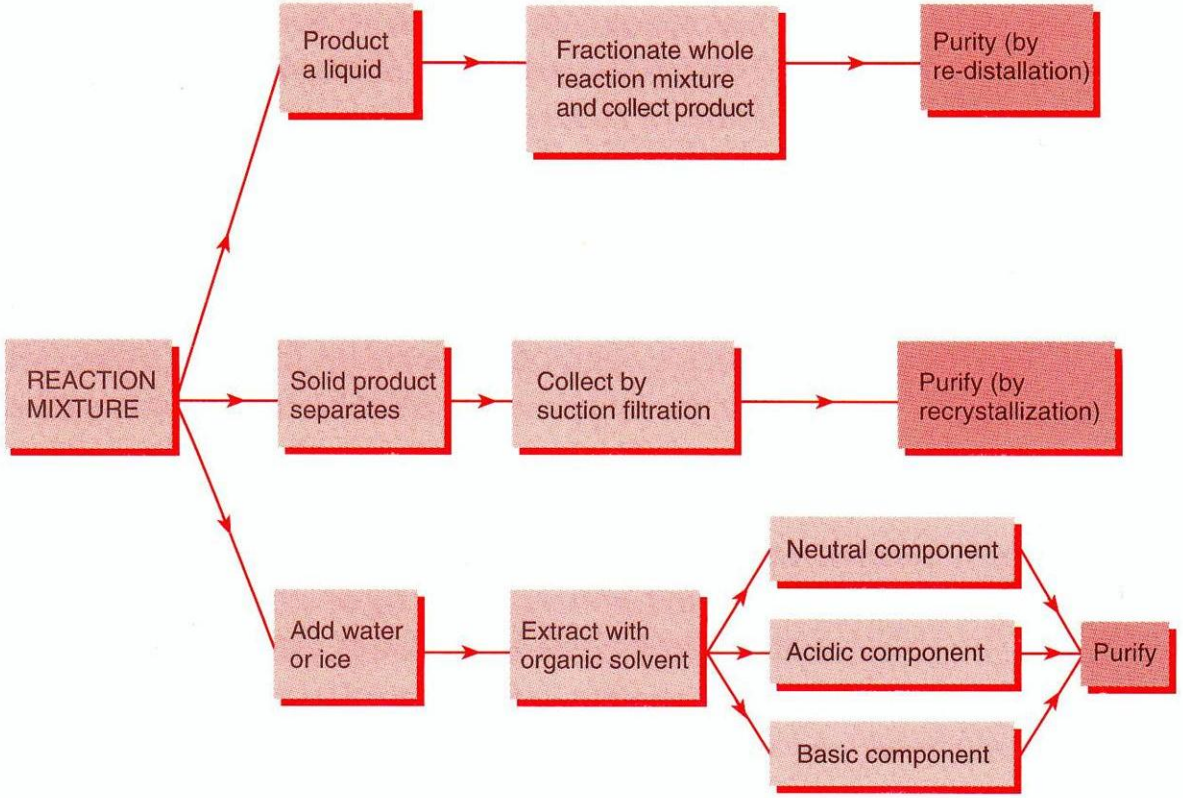
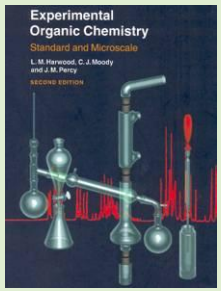


Fig. 3.31 General scheme for reaction work-up.



General outline for the separation of a mixture

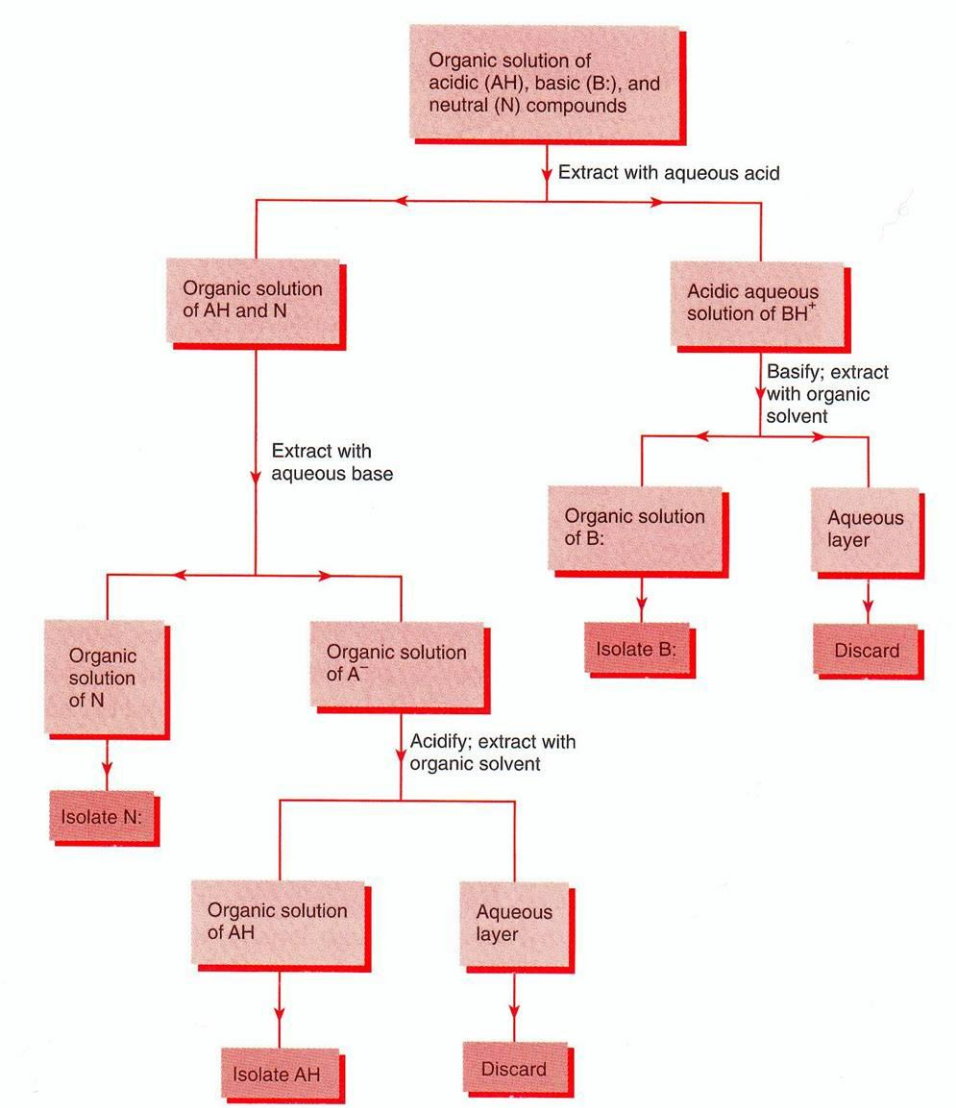


Fig. 3.36 General outline for the separation of acidic (AH), basic (B:) and neutral (N) components of a mixture.

Extraction protocol for purification of a neutral compound

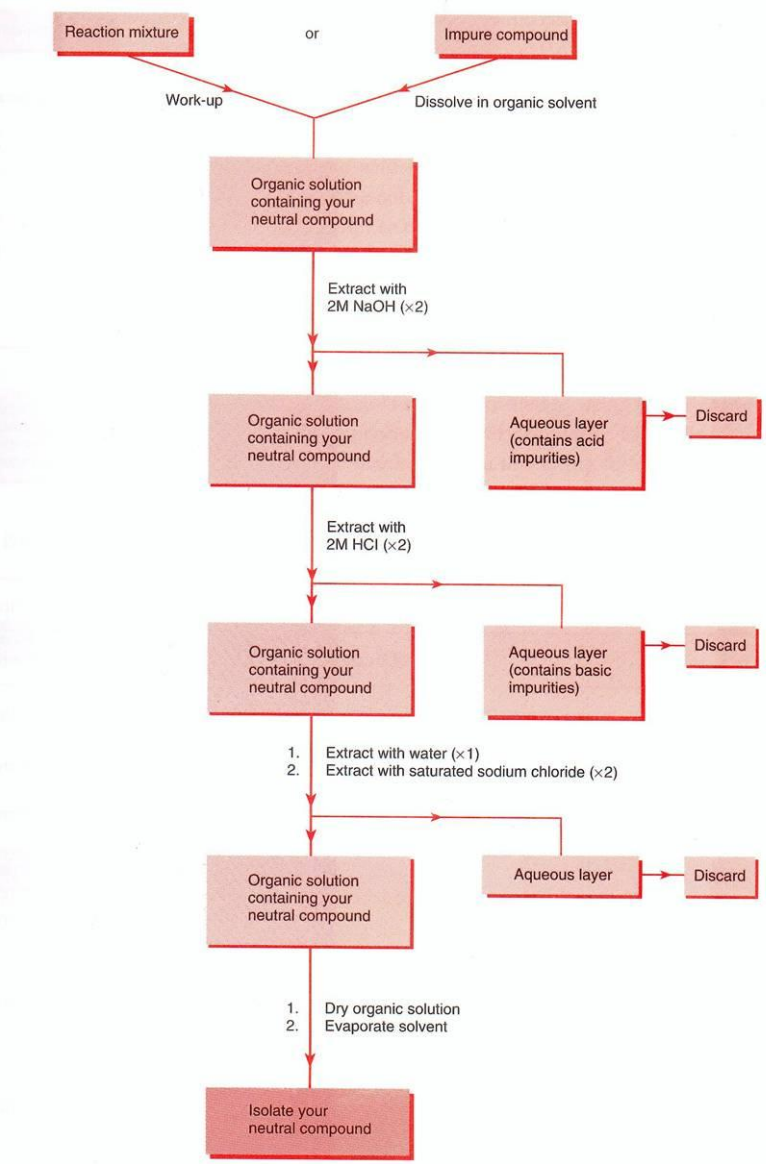


Fig. 3.37 Extraction protocol for the isolation and purification of a neutral organic compound.

Extraction protocols for purification of an **acidic** organic compound

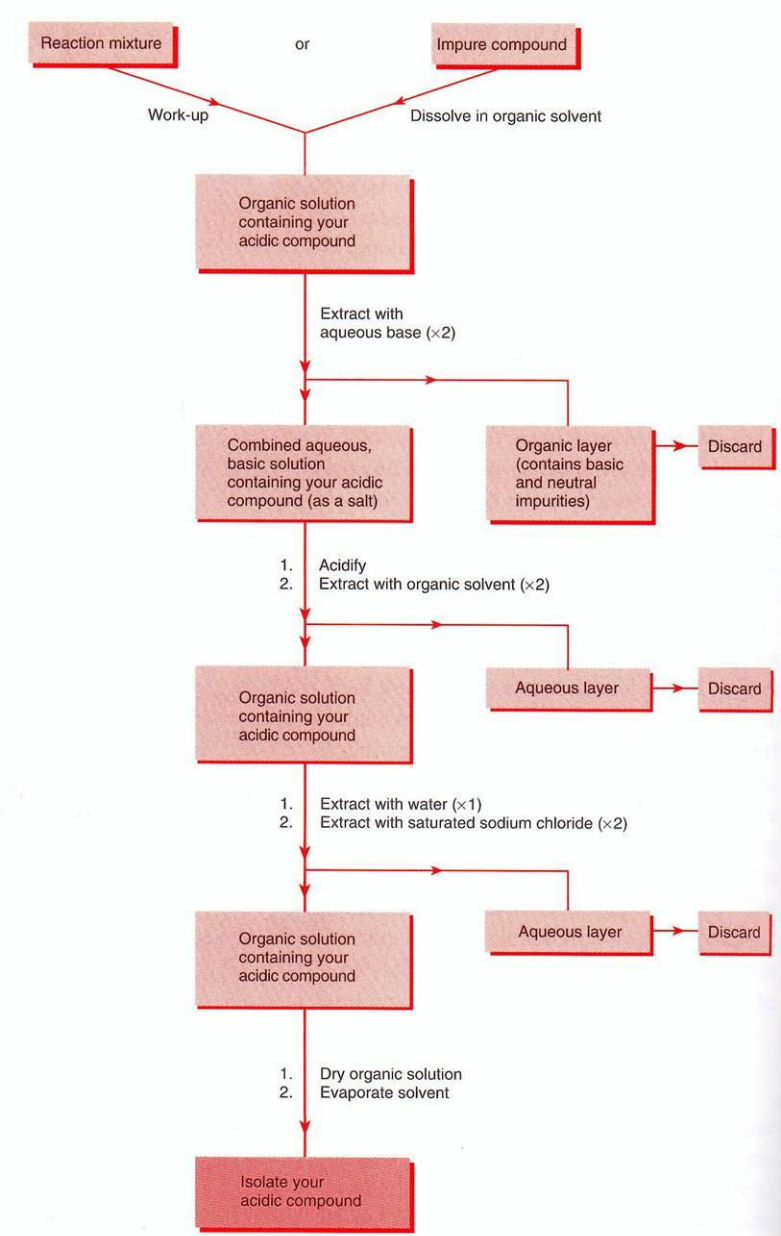


Fig. 3.38 Extraction protocol for the isolation and purification of an acidic organic compound.

Extraction protocols for purification of a **basic** organic compound

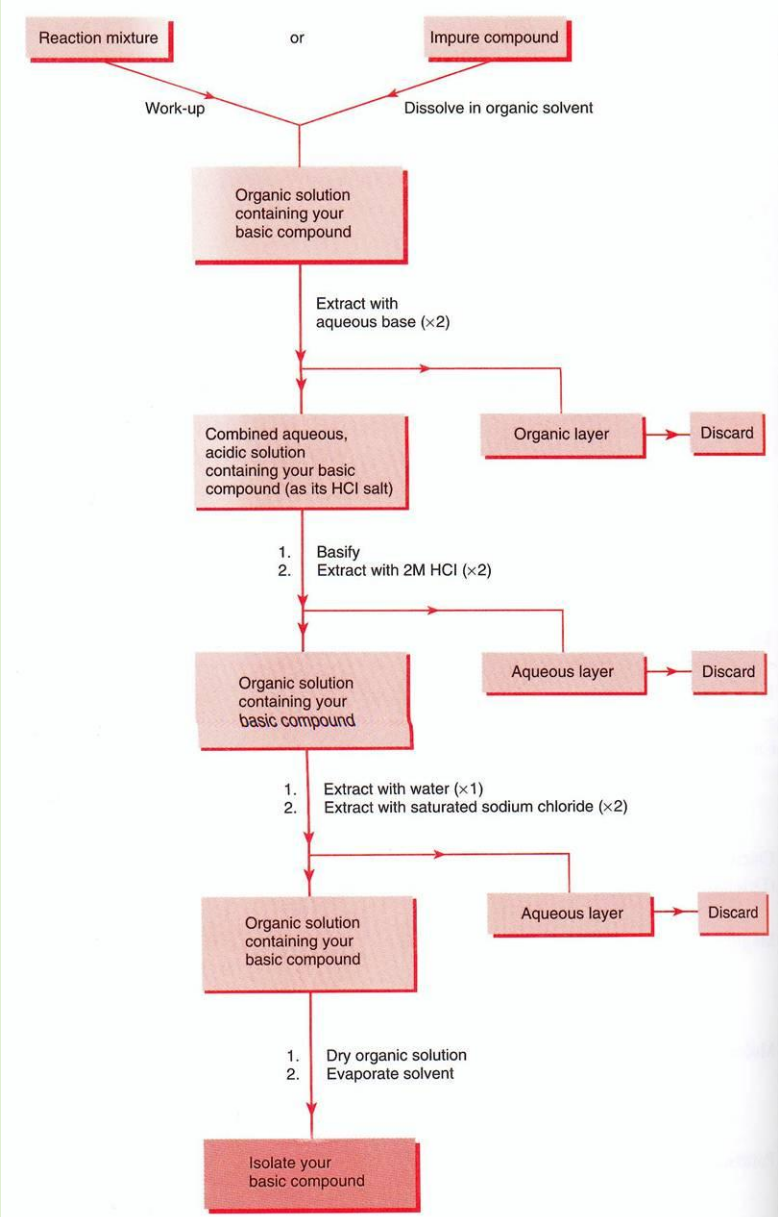


Fig. 3.39 Extraction protocol for the isolation and purification of a basic organic compound.

Purification Techniques for Natural Products

Liquid-liquid Extraction
Soxhlet Extraction
Fractional Distillation
Fractional Distillation under Vacuum
Steam Distillation
Sublimation
Thin layer Chromatography
Liquid Chromatography
Gas Chromatography

Soxhlet Extraction

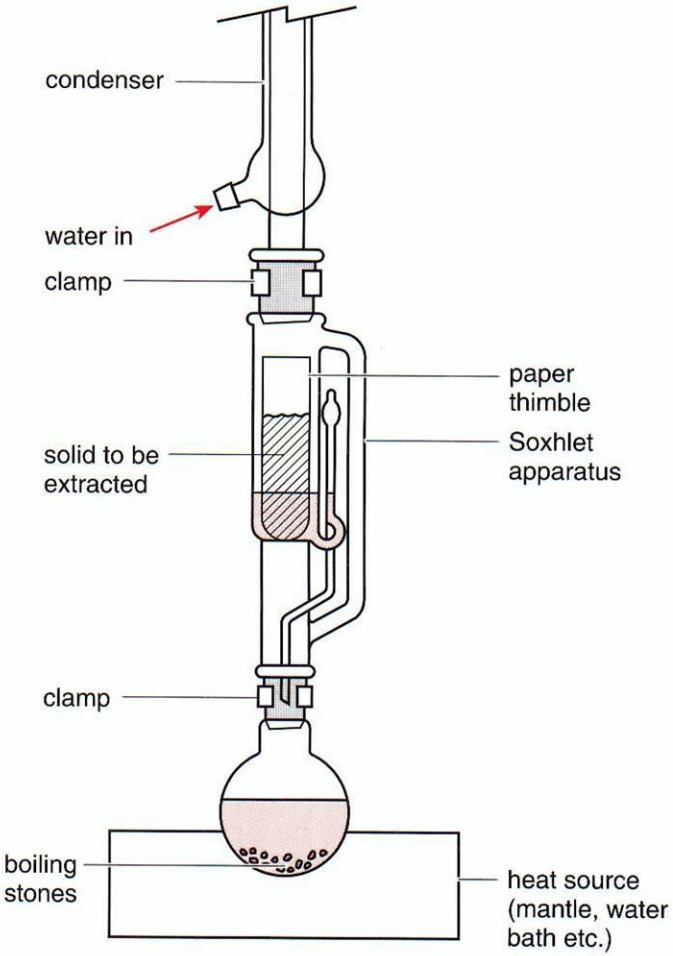


Fig. 3.40 Soxhlet apparatus for the extraction of solids.

Isolation of caffeine from tea leaves using Soxhlet extractor

1. Isolation of caffeine

Place the finely ground tea leaves in the thimble of the Soxhlet extractor and arrange the apparatus for continuous extraction for 1 h with 100 mL ethanol.[1] Transfer the extract to a 1 L round-bottomed flask containing the magnesium oxide and evaporate to dryness on the rotary evaporator, heating with a warm water bath.[2] Extract the solid residue with boiling water (4×50 mL), and filter the slurry with suction whilst hot in each instance. Add 12 mL of 10% sulfuric acid to the filtrate and reduce it to *ca.* one-third of its original volume on the rotary evaporator with heating on a steam or boiling water bath. If a flocculent precipitate forms at this stage, it should be filtered off whilst the solution is still hot and the solution allowed to cool, before extracting four times with 15 mL portions of chloroform. The yellow organic extracts can be decolourized by shaking with a few millilitres of 1% aqueous sodium hydroxide followed by washing with the same volume of water. Remove the solvent on the rotary evaporator and recrystallize the residue of crude caffeine from the minimum quantity of boiling water (<1 mL). Record the weight and mp of your product and obtain the IR spectrum ($CHCl_3$).

Tea leaves

1. Grinding
2. Extracting (ethanol)
3. Evaporation

Solid residue

1. Extracting (water)
2. Filtering
3. Evaporation
4. Extraction (CHCl3)
5. Washing
6. Evaporation
7. Recrystallizing

Distillation

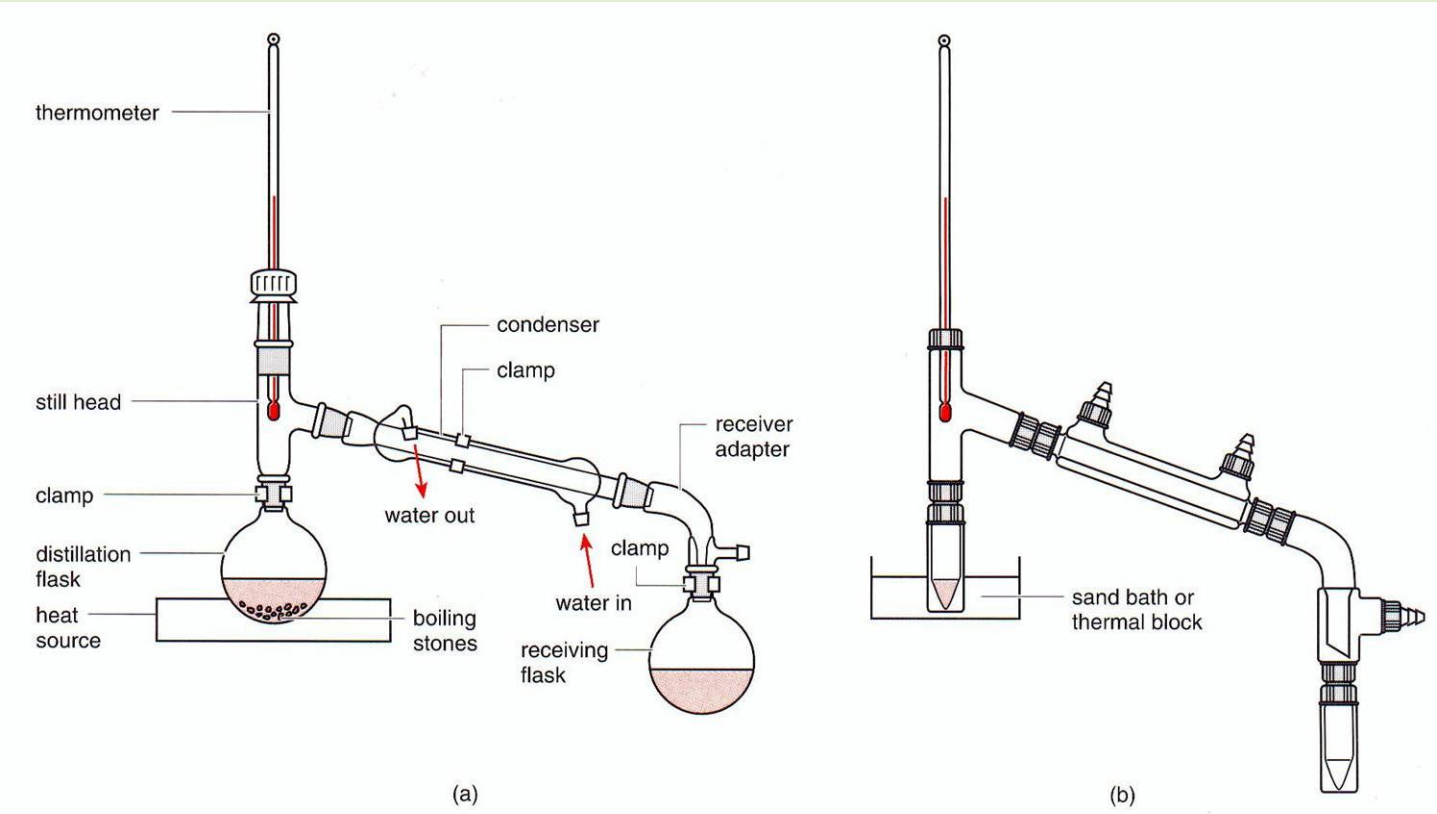


Fig. 3.48 (a) Apparatus for simple distillation. (b) Microscale distillation.

Fractional Distillation

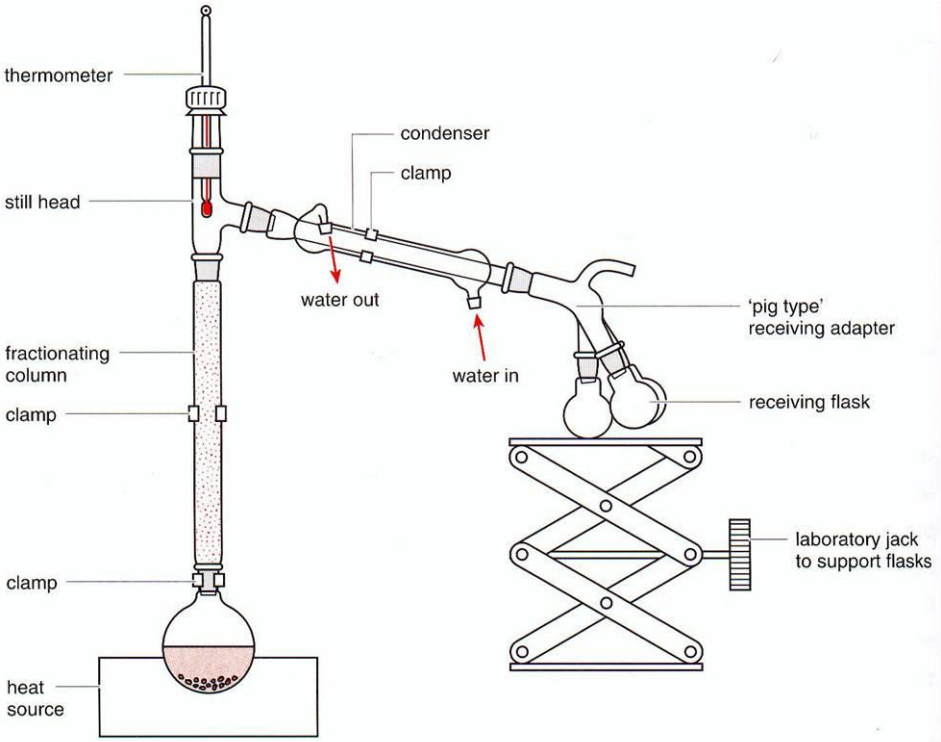


Fig. 3.50 Apparatus for fractional distillation.

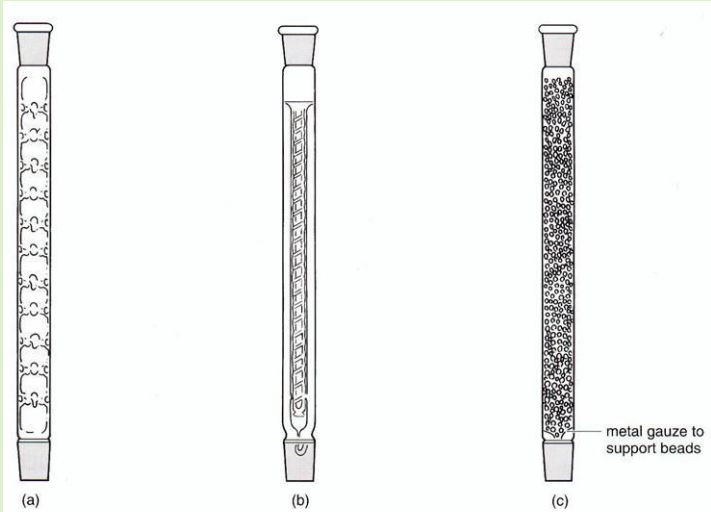


Fig. 3.51 Some types of fractionating columns: (a) Vigreux; (b) Widmer; (c) column packed with glass beads.

Fractional Distillation under Vacuum

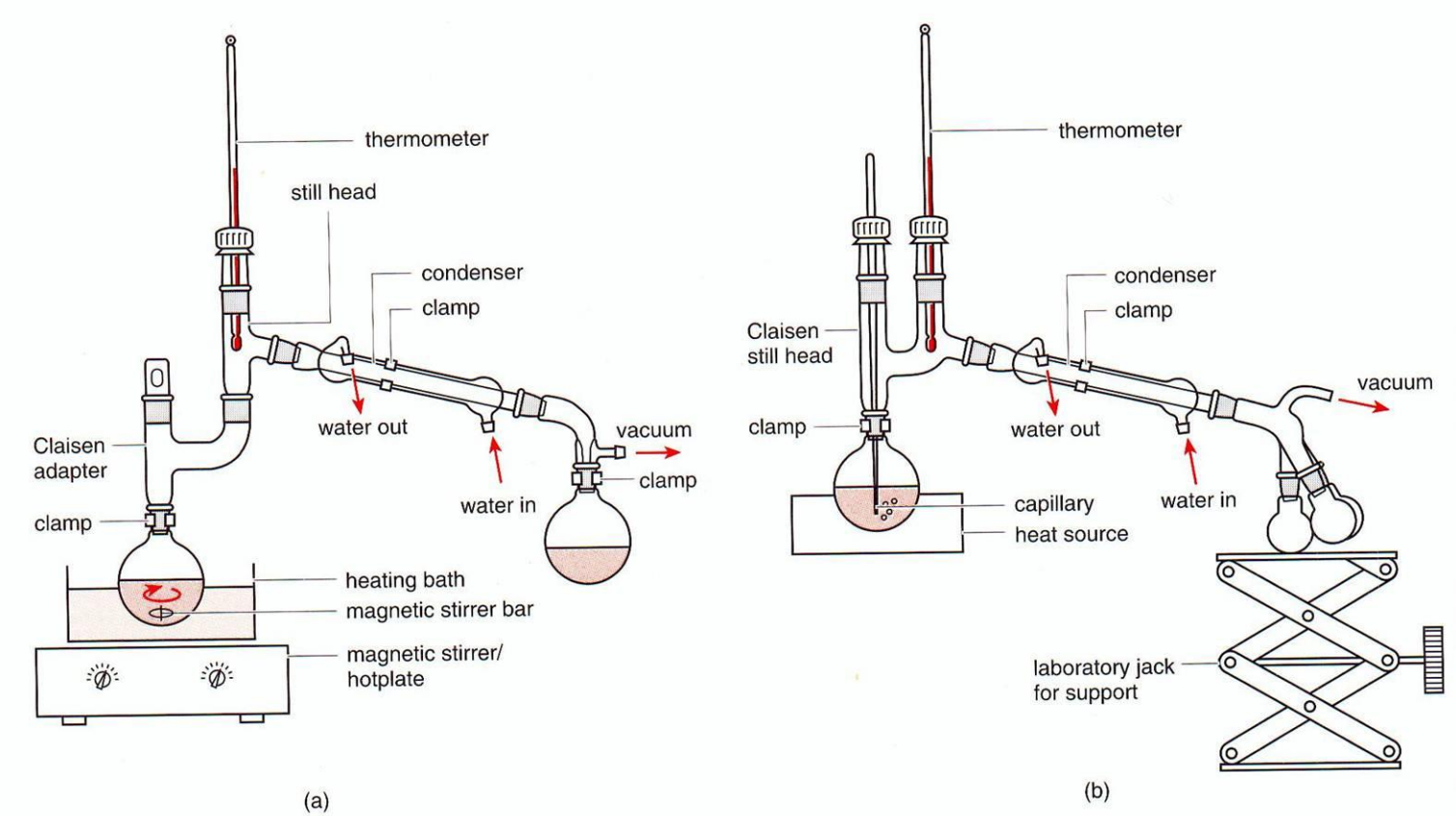


Fig. 3.53 Apparatus for distillation under reduced pressure.

Steam Distillation

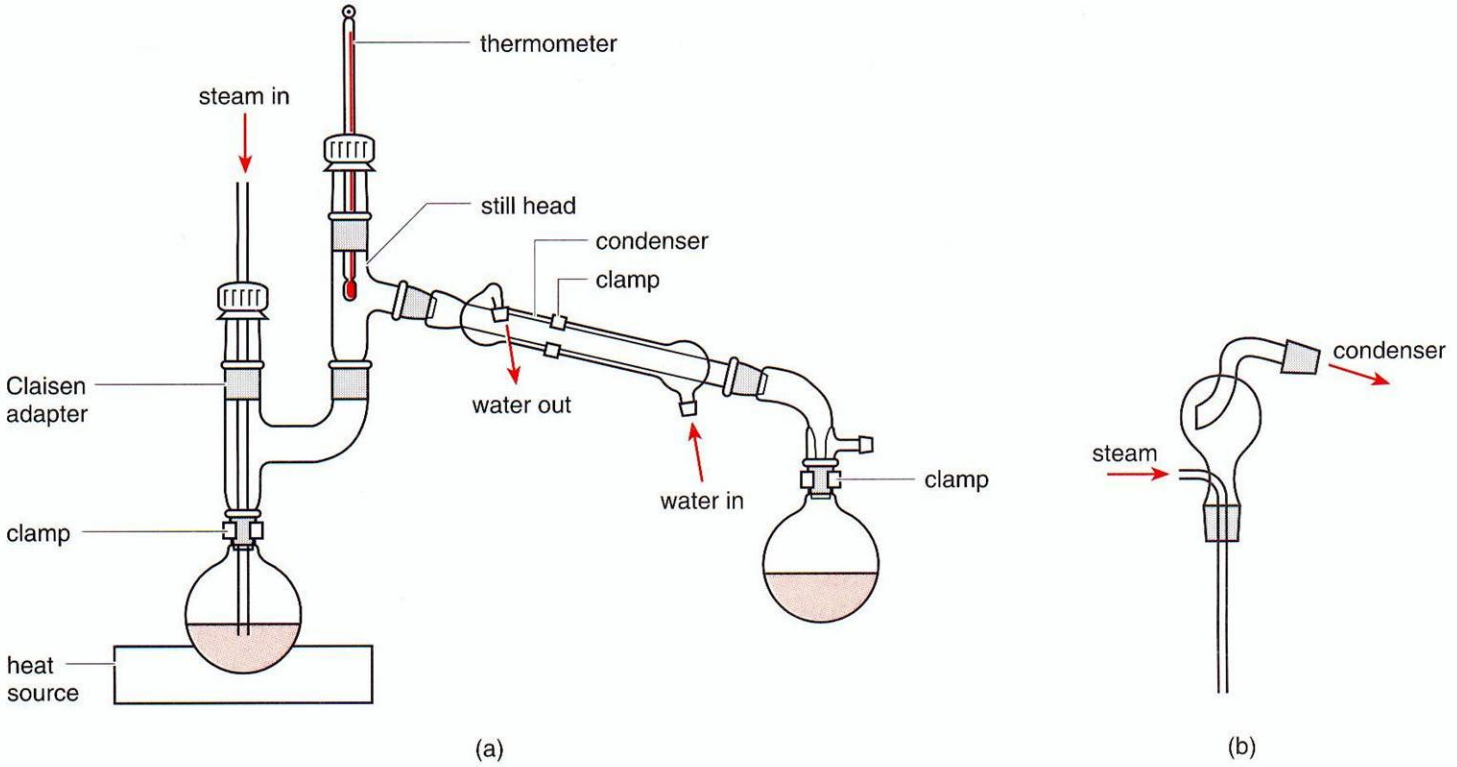


Fig. 3.55 (a) Apparatus for steam distillation; (b) a still head for steam distillation.

Main Chromatographic Techniques

Table 3.9 Main chromatographic techniques.

Stationary phase	Mobile phase	Technique (substances separated)
Solid	Liquid	Adsorption chromatography (wide range of aliphatic and aromatic molecules) Reverse phase chromatography (polar organic molecules) Gel permeation chromatography (macromolecules) Ion exchange chromatography (charged molecules, amino acids)
Liquid	Liquid	Partition chromatography (thermally and acid labile organic molecules)
Liquid	Gas	Gas–liquid chromatography (volatile organic molecules)

Thinlayer Chromatography

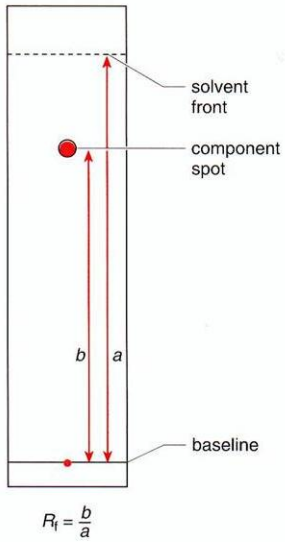


Fig. 3.64 Determination of the retention factor.

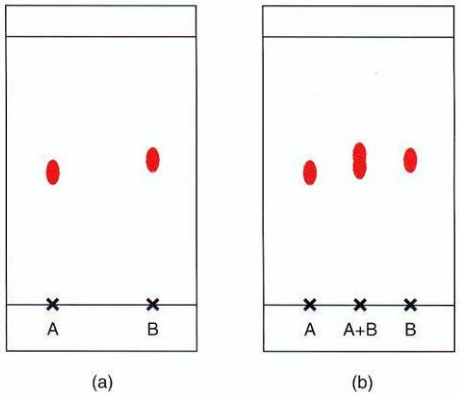


Fig. 3.65 (a) Two compounds having similar R_f values may be indistinguishable, even when run on the same TLC plate. (b) Double spotting shows the typical 'figure of eight' appearance of the two closely running but different compounds.

Thinlayer Chromatography

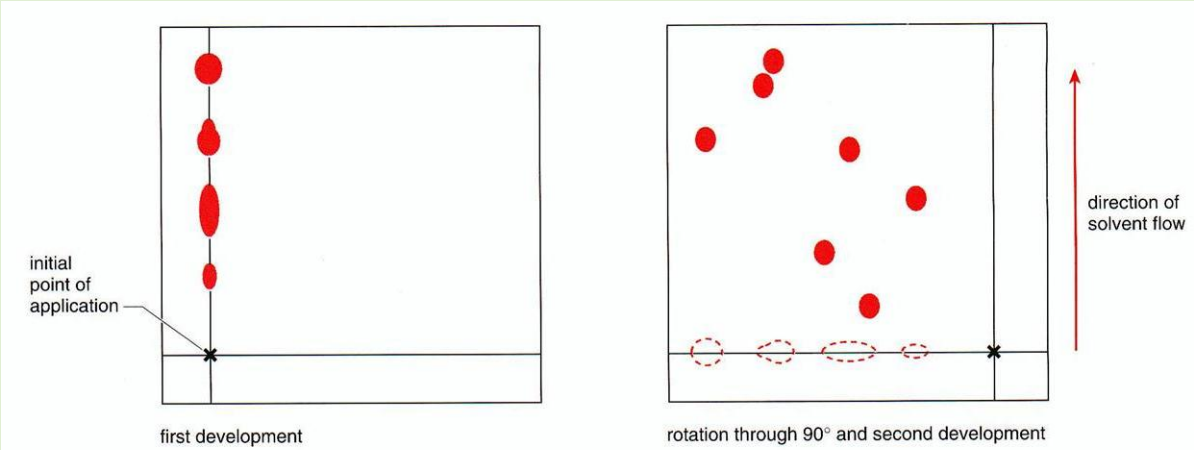


Fig. 3.66 Stages in the development of a two-dimensional chromatogram.

Liquid Chromatography

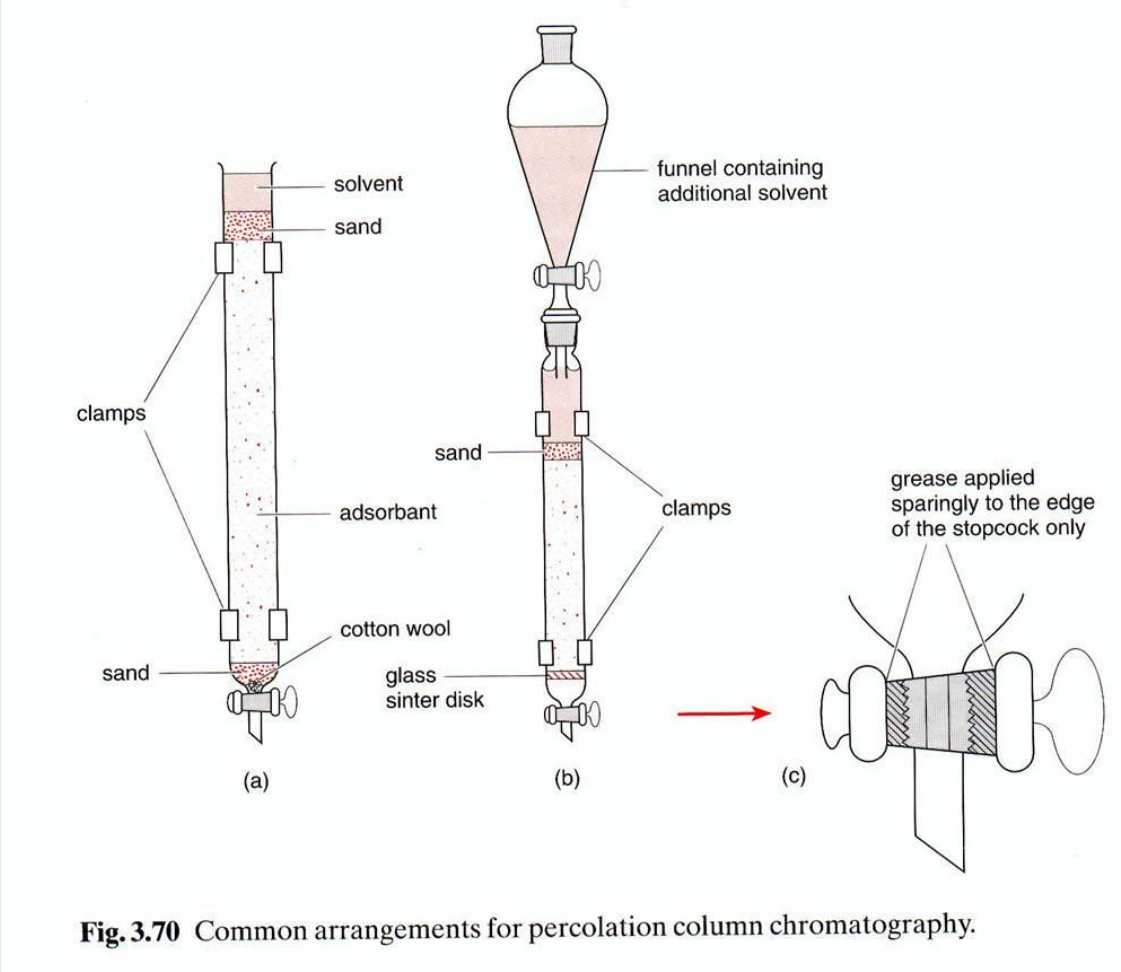
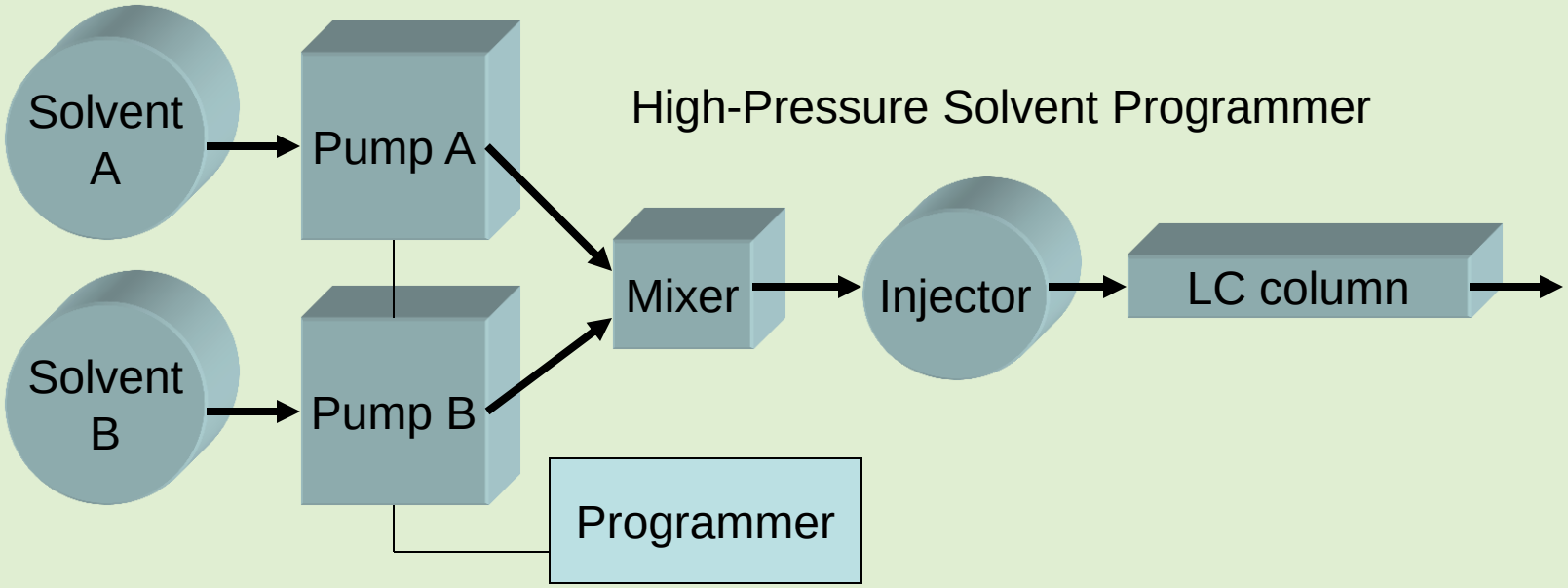
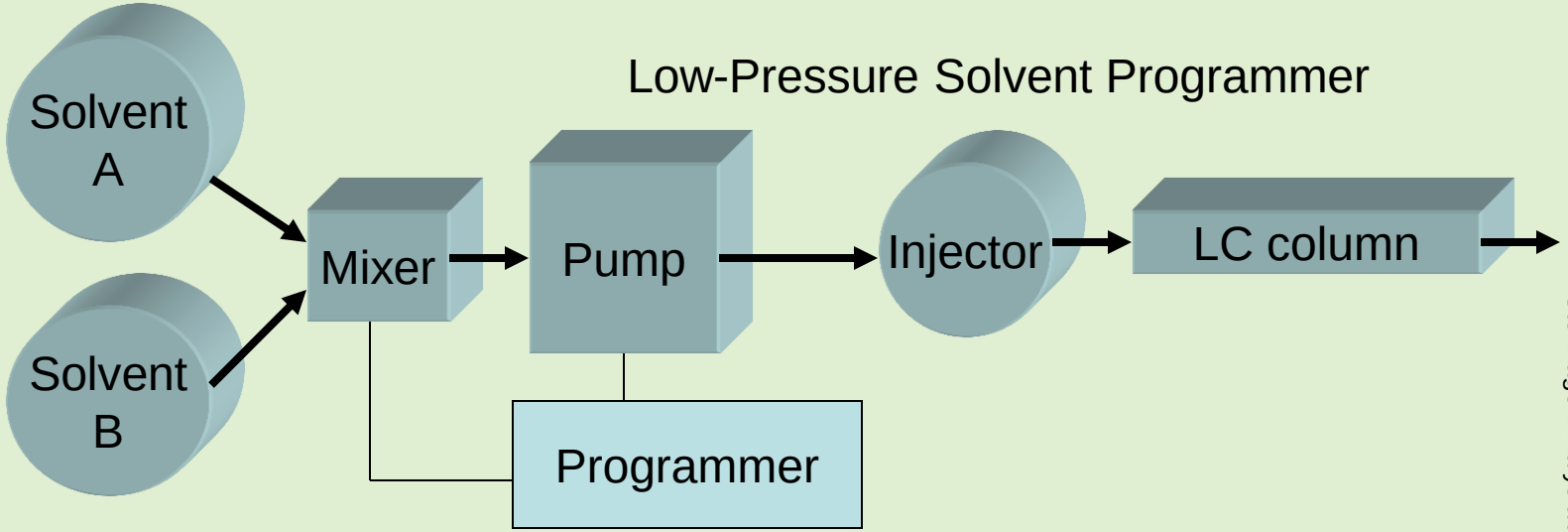


Fig. 3.70 Common arrangements for percolation column chromatography.

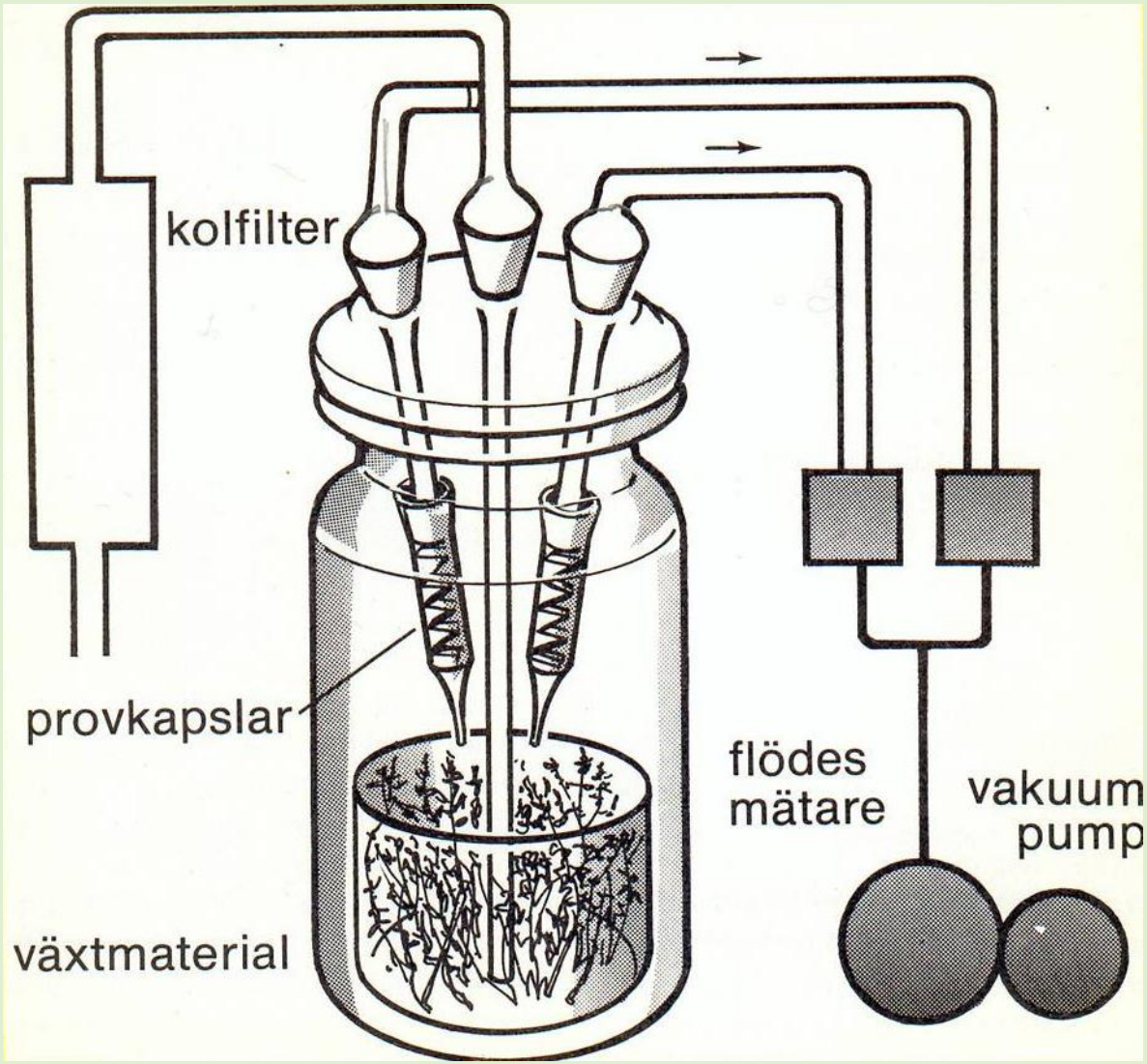
Liquid Chromatography Gradient Elution



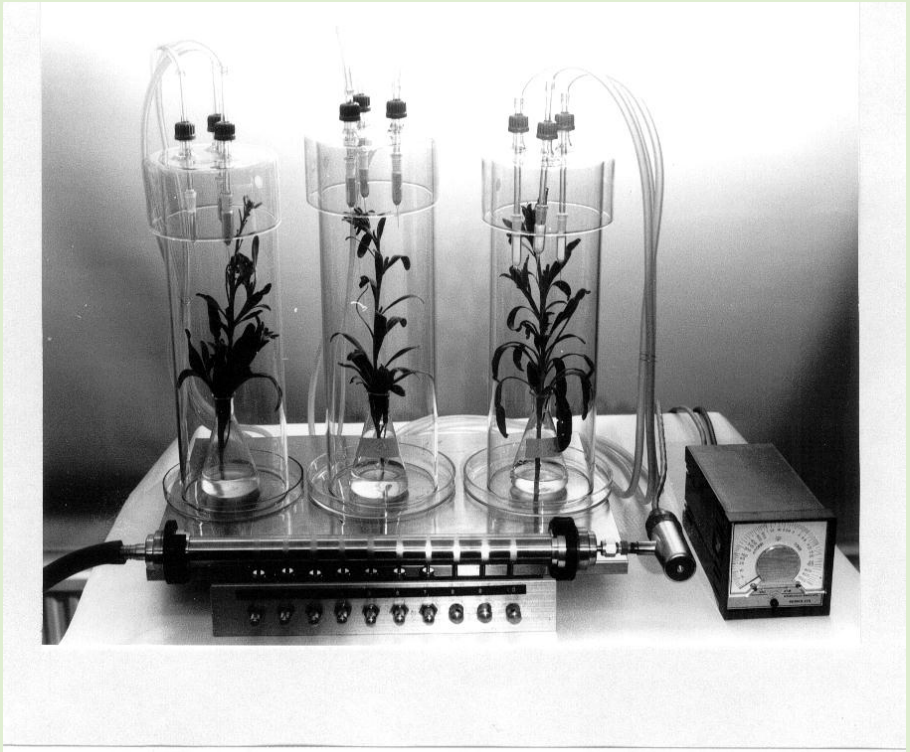


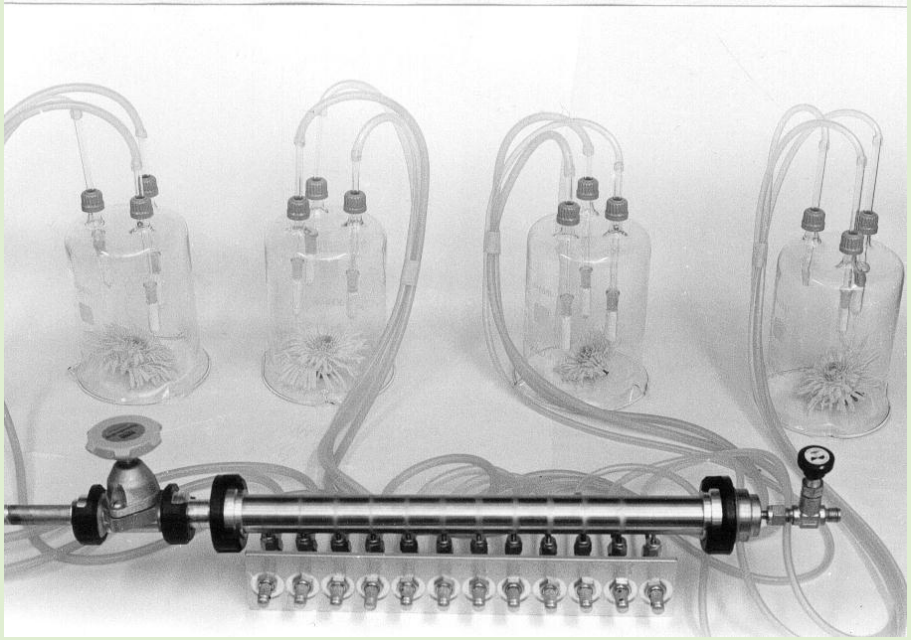

Volatiles from Plants
StenhagenAnalyslab AB

Sampling of Volatiles from Plants

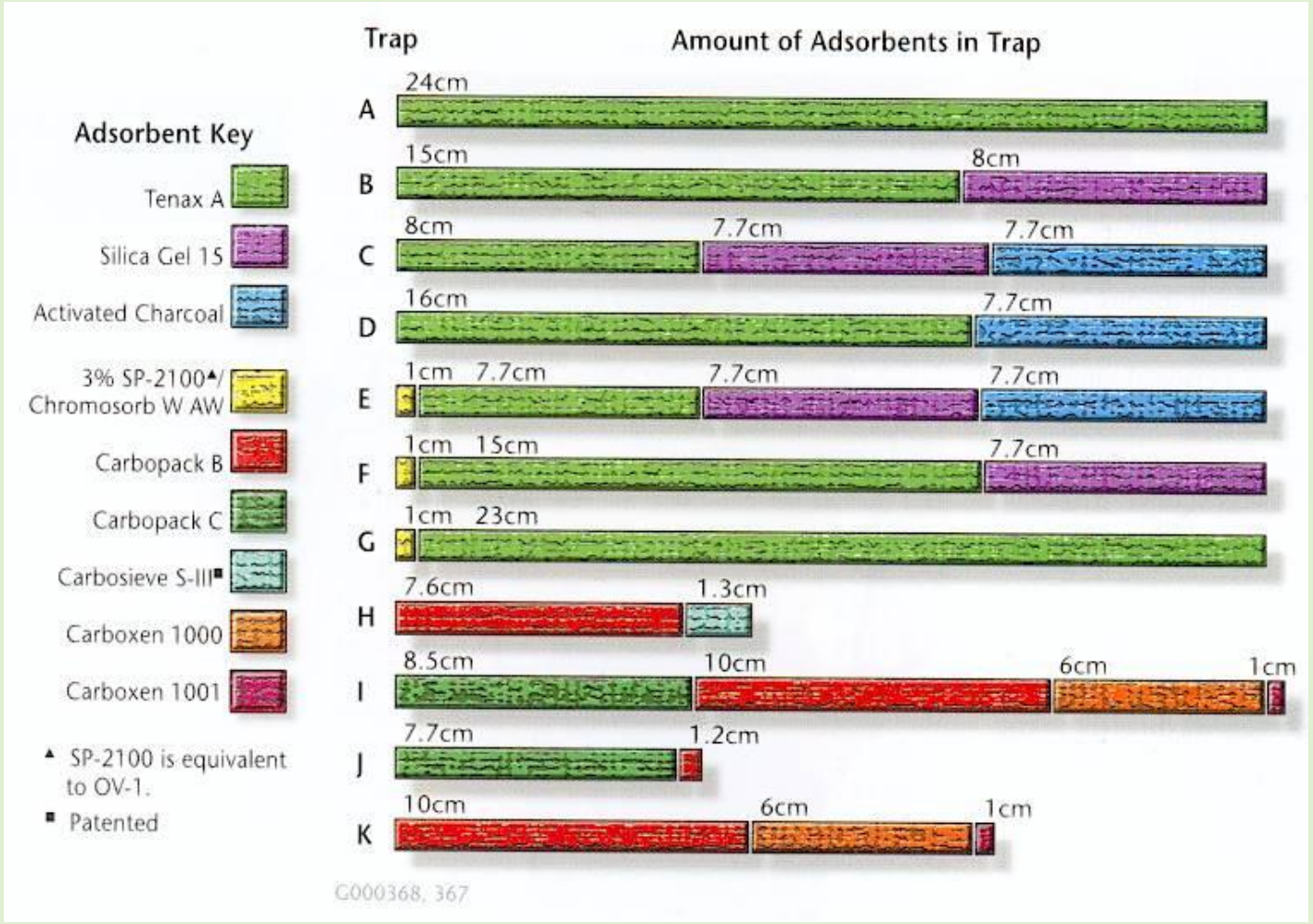


Sampling of Volatiles from Plants





Adsorbent Material used in Trap



Capillary Gas Chromatography

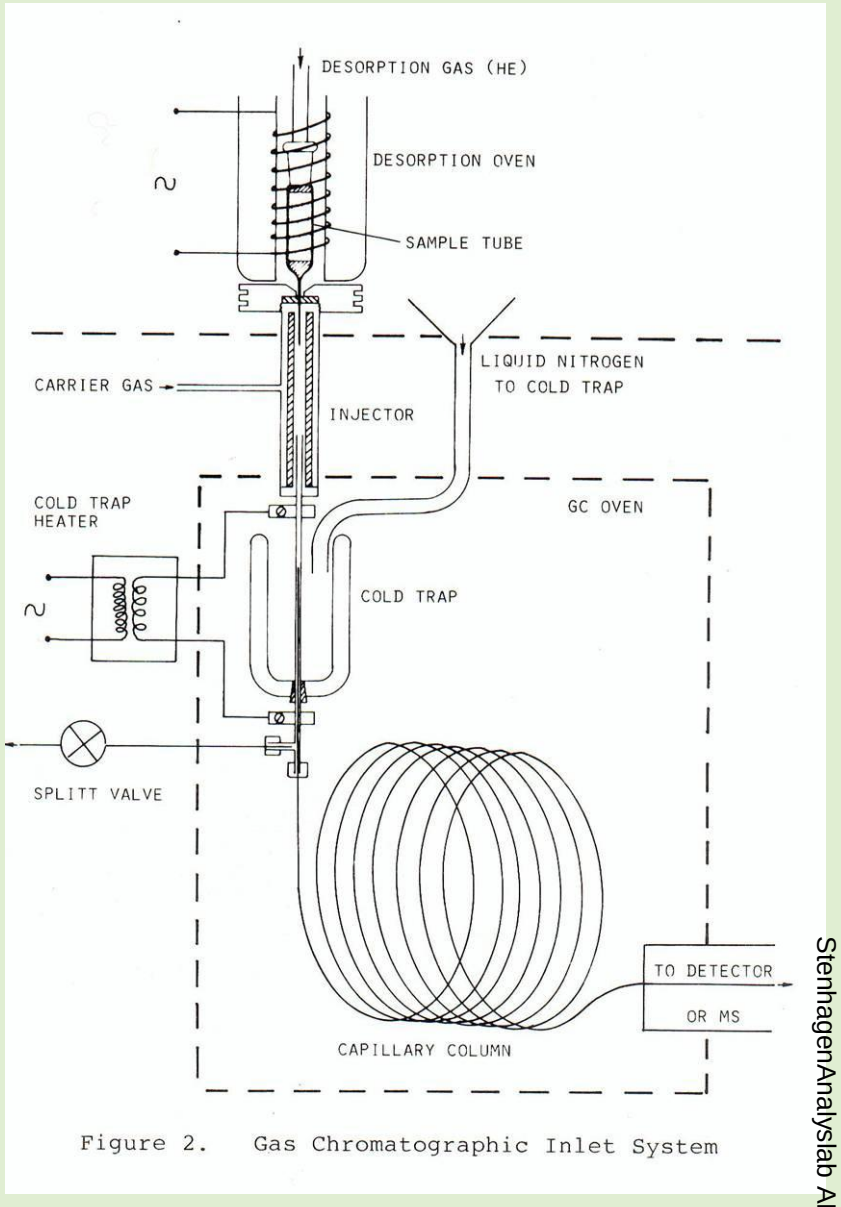


Figure 2. Gas Chromatographic Inlet System

Capillary Gas Chromatography of Volatiles from Tomato Leaves

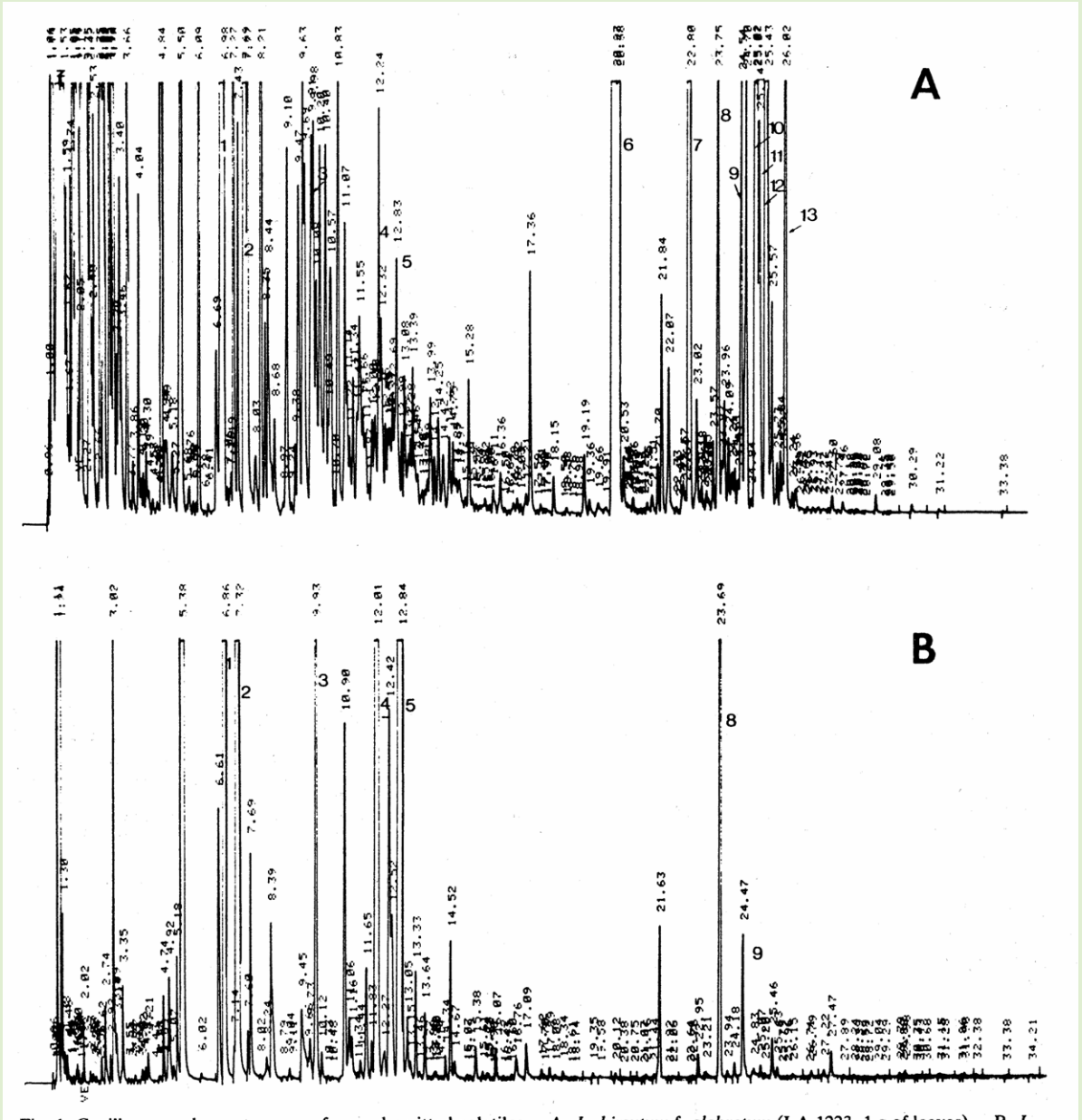


Fig. 1. Capillary gas chromatograms of wound-emitted volatiles. – A. *L. hirsutum* f. *glabratum* (LA 1223, 1 g of leaves). – B. *L. esculentum* (cv Ida, 5 g of leaves). – Numbered peaks: (1) trans-2-hexenal, (2) cis-3-hexenol, (3) α-pinene, (4) α-terpineolene, (5) α-phellandrene, (6) 2-undecanone, (7) α-copaene, (8) β-caryophyllene, (9) humulene, (10) α-curcumene, (11) 2-tridecanone, (12) zingiberene, (13) δ-cadinene. – A fused silica column, i.d. 0.31 mm × 25 m of SE-30, linearly programmed from 30° to 230°C at 5°C/min.

Sampling of Volatiles from Soil

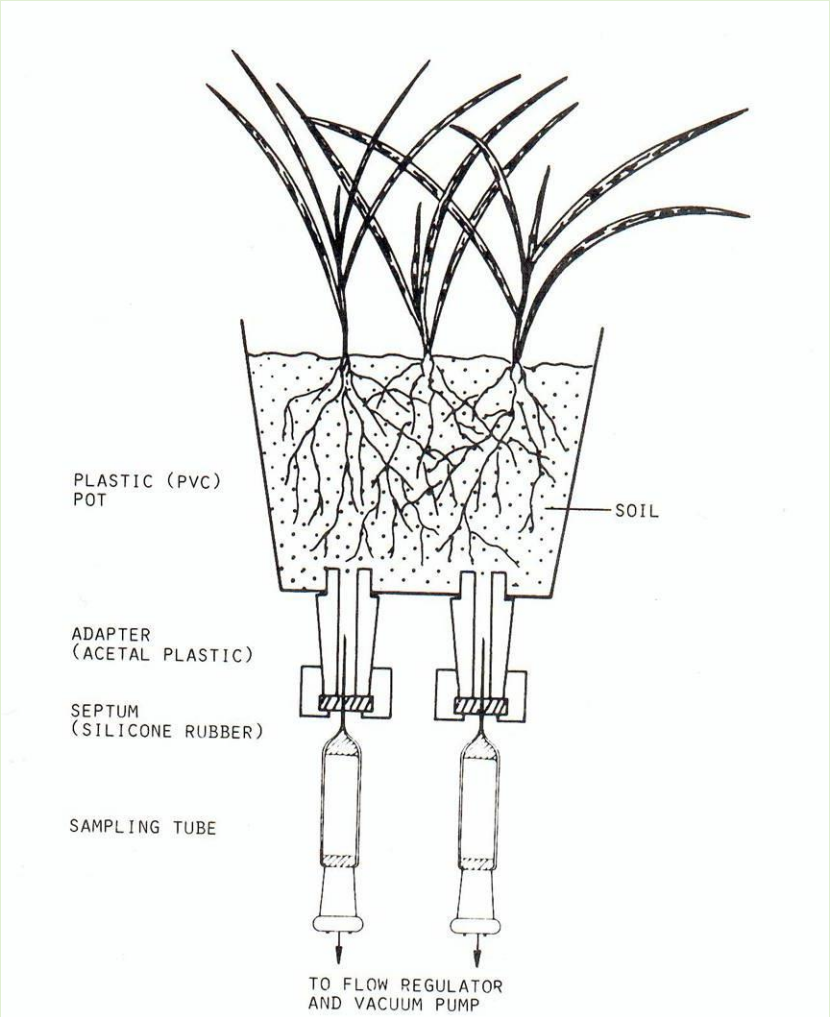


Figure 1. Assembly for Sampling of Volatiles

Capillary Gas Chromatography

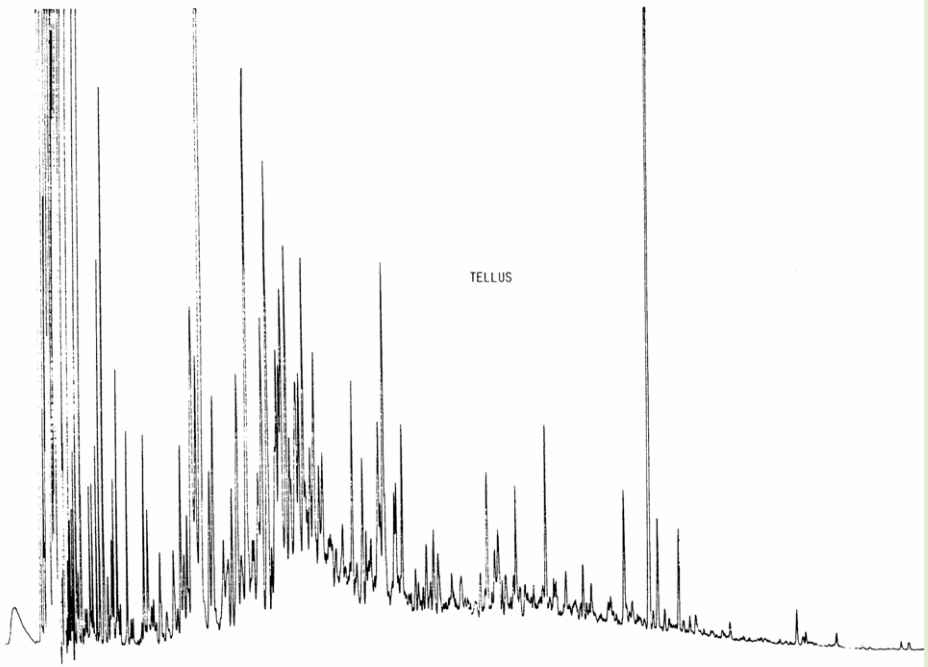


Figure 3. Capillary Gas Chromatogram of Volatiles from Soil. Chromatographic data: adsorption: 4 h on Tenax TA; desorption: 8 min at 200°C; column: fused silica 0.3 mm, 20 m, SE33; temperature programming: 40 to 230°C at 5°C/min; carrier gas: helium (50 cm/s).

Eigenvector projection

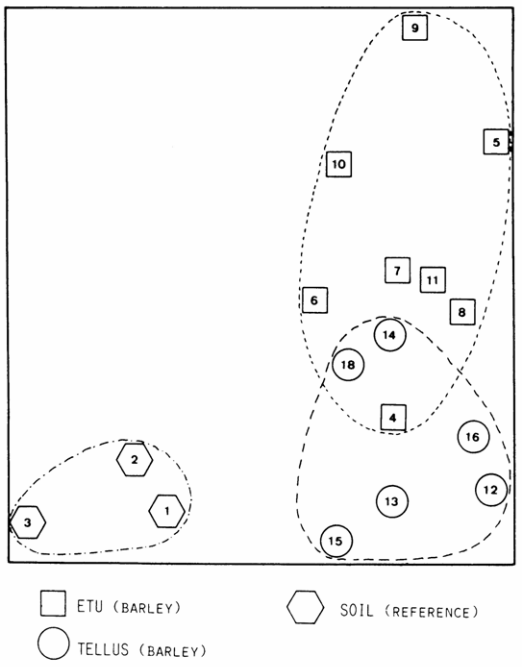
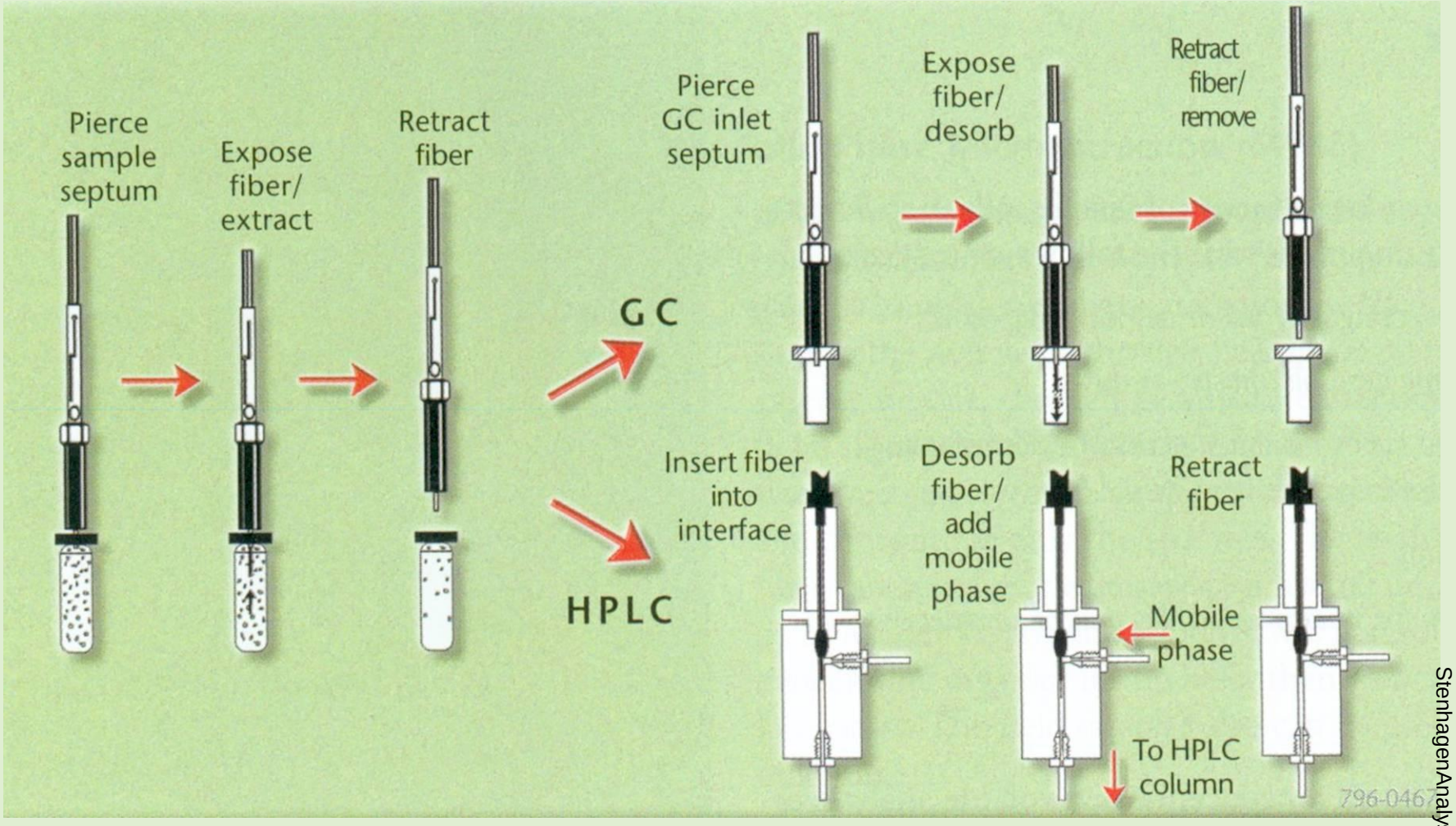


Figure 5. Eigenvector Projection (Principal Vector Plot). A plane is least squares fitted to all the data. This plane constitutes a two-dimensional window into the multi-dimensional measurement space. The projections of the object points down to the plane are visualized in this plot.

Solid Phase Micro Extraction



Solid Phase Micro Extraction

Determine the type of fiber you need according to the molecular weights and polarity of the analytes.

- *Low molecular weight or volatile compounds usually require a 100µm polydimethylsiloxane (PDMS)-coated fiber.*
- *Larger molecular weight or semivolatile compounds are more effectively extracted with a 30µm PDMS fiber or a 7µm PDMS fiber.*
- *To extract very polar analytes from polar samples, use an 85µm polyacrylate-coated fiber.*
- *More volatile polar analytes, such as alcohols or amines, are adsorbed more efficiently and released faster with a 65µm polydimethylsiloxane/divinylbenzene (PDMS/DVB)-coated fiber.*
- *A 60µm PDMS/DVB fiber is a general purpose fiber for HPLC.*
- *For trace-level volatiles analysis, use a 75µm PDMS/Carboxen fiber.*
- *For an expanded range of analytes (C3-C20), use a 50/30 divinylbenzene/Carboxen on PDMS fiber.*

Solid Phase Micro Extraction

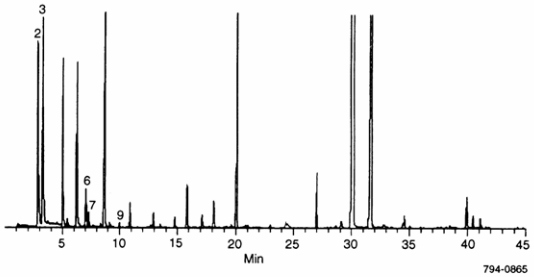
Figure B. Flavor Compounds in Fruit Juice Beverage

SPME: 100µm polydimethylsiloxane phase fiber
Cat. No.: **5-7300**
10 min immersion sampling with stirring
(3mL sample, 0.6g NaCl added) 3 min desorption

GC Column: **Carbowax® 20M-type phase, (Supelco recommendation: SUPELCOWAX 10) 30m x 0.25mm ID, 1µm film**
Cat. No.: **available on request**
Oven: 50°C (2 min) to 220°C at 4°C/min
Carrier: helium, 20cm/sec
Det.: MS (m/z = 35–400)
Inj.: 200°C, splitless (closed 2 min)
(1mm ID injector liner)

1. Dichloromethane
2. Ethyl butyrate
3. Ethyl isovalerate
4. Limonene
5. Ethyl hexanoate
6. Isoamyl butyrate
7. Hexanyl acetate
8. cis-3-Hexenyl acetate
9. Hexanol
10. cis-3-Hexenol
11. cis-3-Hexenyl butyrate
12. Furfural
13. Benzaldehyde
14. Linalool
15. β-Terpineol
16. Butyric acid
17. 2-Methylbutyric acid
18. α-Terpineol
19. Hexanoic acid
20. cis-Methyl cinnamate
21. 1-(2-Furyl)-2-hydroxyethanone
22. Furaneol
23. trans-Methyl cinnamate
24. γ-Decalactone
25. Dodecanoic acid
26. (Hydroxymethyl)furfural

SPME (100µm polydimethylsiloxane)



Solvent Extraction (dichloromethane)

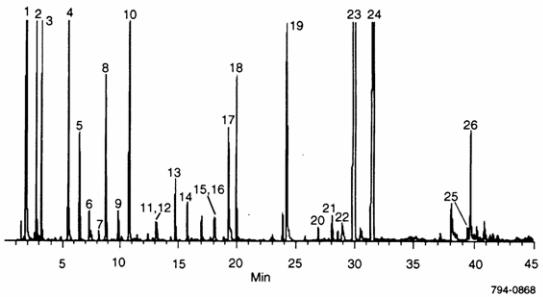


Figure E. Ginger Oil

Sample: 0.5g ginger oil

SPME: 100µm polydimethylsiloxane fiber
Cat. No.: **5-7300**
1 min headspace sampling, 30°C
5 sec desorption, 250°C

GC Column: **β-DEX 120 (modified β-cyclodextrin phase), 30m x 0.25mm ID, 0.25µm film**
Cat. No.: **2-4304**
Oven: 40°C to 220°C at 4°C/min
Carrier: helium, 35cm/sec
Det.: FID, 250°C
Inj.: split (100:1), 250°C

1. Methanol
2. Acetone
3. Isopropanol
4. Ethyl acetate
5. Hexanal
6. 2-Heptanone
7. (±)-α-Pinene
8. β-Myrcene
9. (±)-Camphene
10. (±)-β-Pinene
11. (±)-Limonene
12. (±)-β-Phellandrene
13. (±)-Cineole

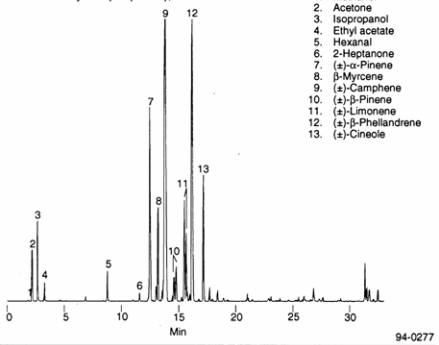


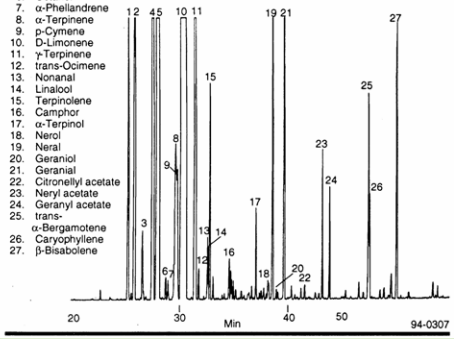
Figure F. Lemon Oil

Sample: 0.5g lemon oil

SPME: 100µm polydimethylsiloxane fiber
Cat. No.: **5-7300**
1 min headspace sampling, 30°C
5 sec desorption, 250°C

GC Column: **SPB-1 (poly[dimethylsiloxane] phase), 100m x 0.25mm ID, 1.0µm film**
Cat. No.: **2-4220**
Oven: 40°C to 220°C at 4°C/min
Carrier: hydrogen, 40cm/sec
Det.: FID, 300°C
Inj.: split (100:1), 250°C

1. α-Thujene
2. α-Pinene
3. Camphene
4. Sabinene
5. β-Myrcene
6. β-Pinene
7. Octanol
8. α-Phellandrene
9. α-Terpinene
10. p-Cymene
11. D-Limonene
12. γ-Terpinene
13. trans-Ocimene
14. Nonanal
15. Linalool
16. Terpinolene
17. Camphor
18. α-Terpinol
19. Nerol
20. Neral
21. Geraniol
22. Geranial
23. Citronellyl acetate
24. Neryl acetate
25. Geranyl acetate
26. trans-α-Bergamotene
27. Caryophyllene
28. β-Bisabolene



Rancid Corn Oil Polyunsaturated oils are susceptible to oxidation over time, and at accelerated rates on exposure to sunlight, elevated temperatures, or metals. Increased levels of volatile compounds formed from oxidation of linoleic acid (particularly pentane, hexanal, and 2-heptenal), linolenic acid (2,4-heptadienal), and oleic acid (octanal and nonanal) are indicators of rancidity in vegetable oils, and headspace analysis is an effective means of detecting these compounds (7–9). The needle on the SPME device will penetrate the foil or plastic seal on a bottle of oil and enable the analyst to sample the headspace in the bottle without changing its composition. Headspace SPME/capillary GC easily enables the analyst to monitor the volatiles of interest (Figure G). Note that butylated hydroxytoluene (BHT) also can be detected through the SPME/GC analysis.

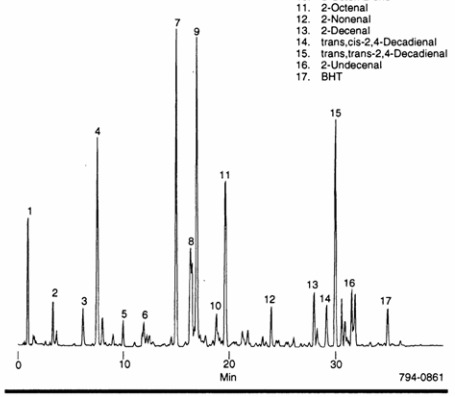
Figure G. Rancid Corn Oil

Sample: 3.0g corn oil

SPME: 100µm polydimethylsiloxane fiber
Cat. No.: **5-7300**
45 min headspace sampling, 40°C
1.5 min desorption, 250°C

GC Column: **SPB-5 (poly[5% diphenyl/95% dimethylsiloxane] phase), 30m x 0.53mm ID, 5.0µm film**
Cat. No.: **2-5347**
Oven: 40°C (5 min) to 220°C at 4°C/min
Carrier: helium, 5mL/min
Det.: FID, 300°C
Inj.: splitless (1 min), 250°C

1. Propane
2. Pentane
3. Pentanol
4. Hexanal
5. 2-Hexenal
6. 2-Heptanone
7. 2-Heptenal
8. 1-Octen-3-ol
9. 2-Pentylfuran
10. 3-Octen-2-one
11. 2-Octenal
12. 2-Nonenal
13. 2-Decenal
14. trans,cis-2,4-Decadienal
15. trans,trans-2,4-Decadienal
16. 2-Undecenal
17. BHT



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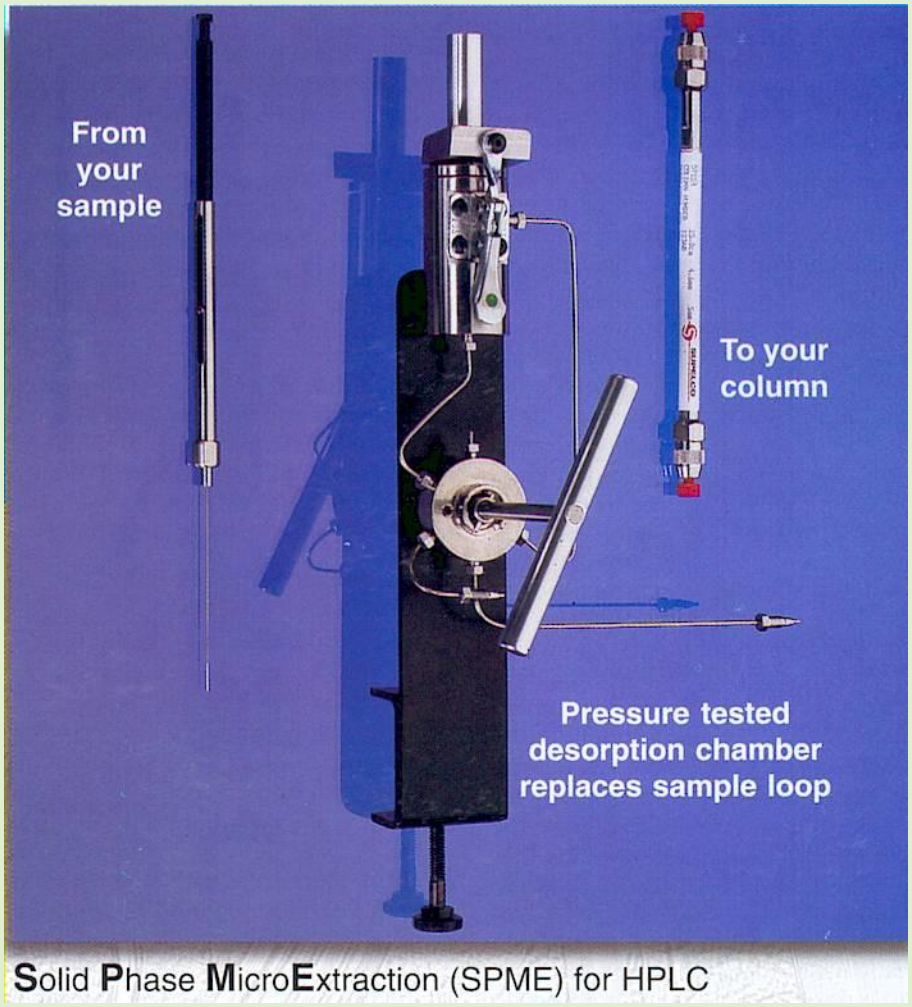
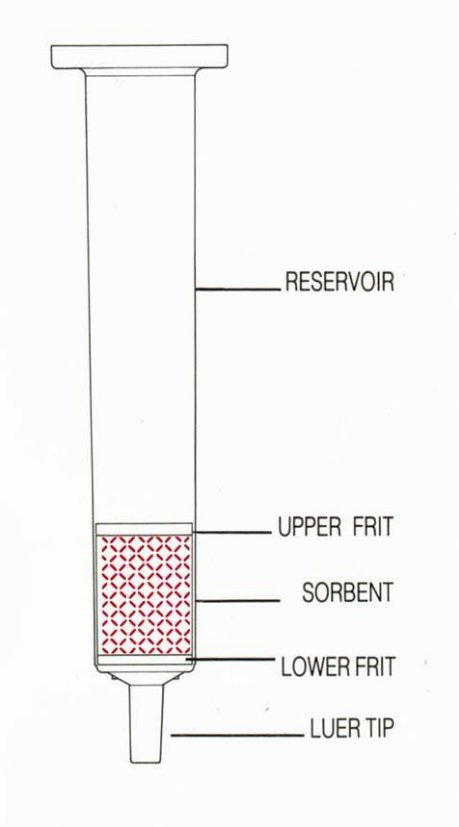
Solid Phase Micro Extraction



Solid Phase Extraction



All the elements in a diagram are color coded as follows:

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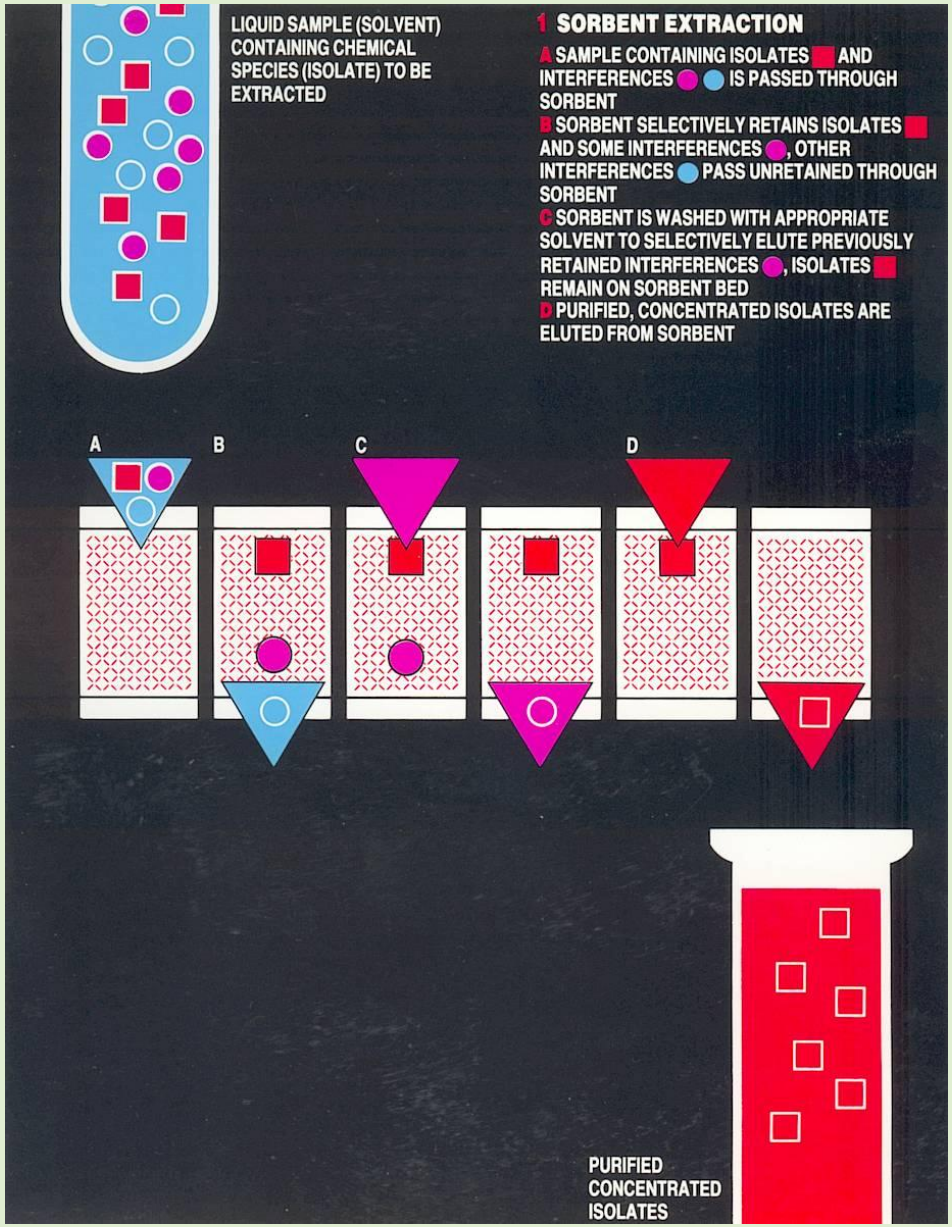
- PALE BLUE is SLIGHTLY POLAR
- PURPLE is INTERMEDIATE POLARITY
- PALE RED is SLIGHTLY NON-POLAR
- RED is NON-POLAR

These colors represent relative polarities in a given diagram, not absolute polarities, e.g., blue is not necessarily as polar as water.



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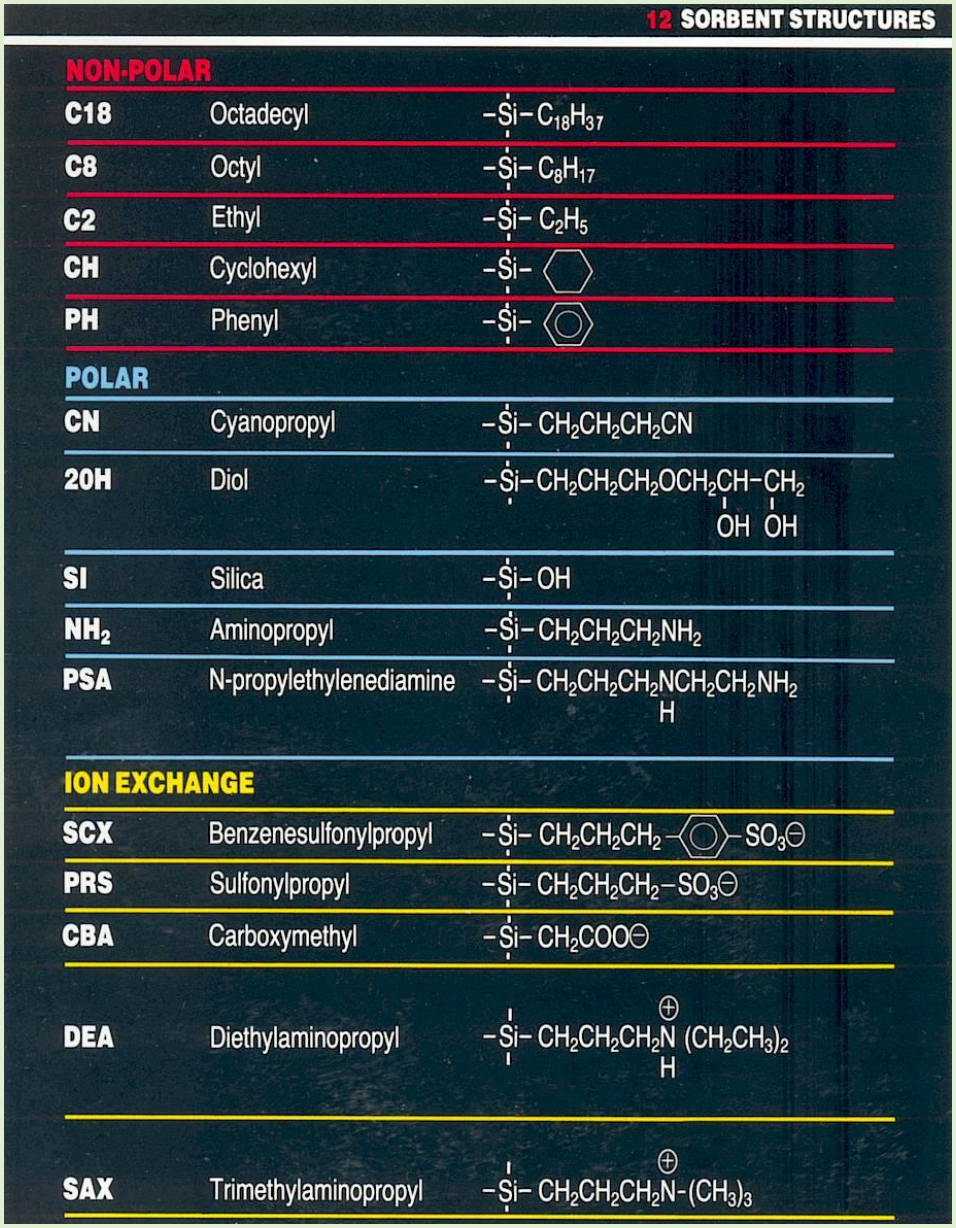
Solid Phase Extraction



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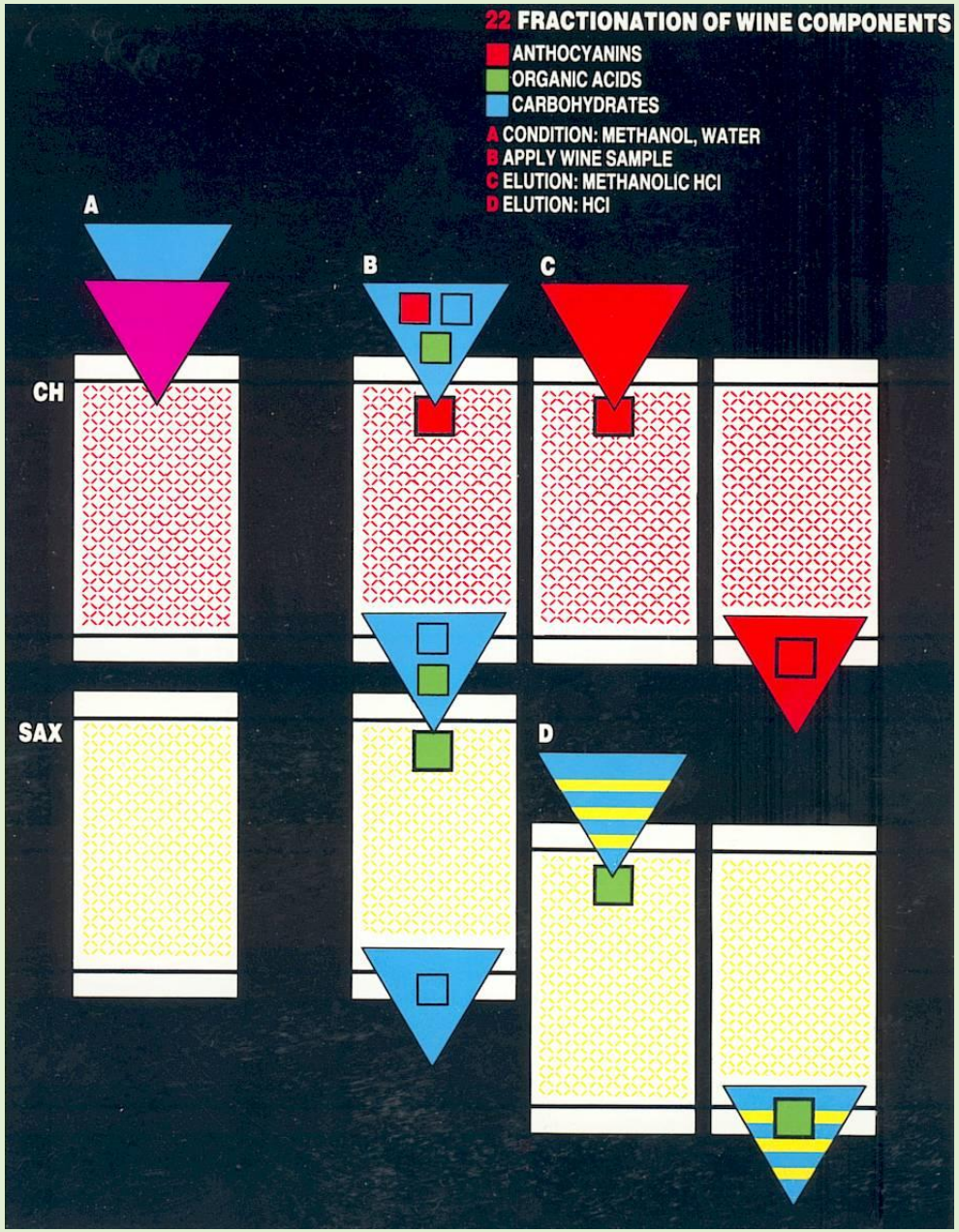
ION EXCHANGE

- YELLOW indicates significant CATIONIC interactions
- GREEN indicates significant ANIONIC interactions
- WATER is BLUE (polar) when it has no ions or is weakly ionic.
- BLUE with a YELLOW stripe is water with significant acidic properties.
- BLUE with a GREEN stripe is water with significant basic properties.



StenhagenAnalyslab AB

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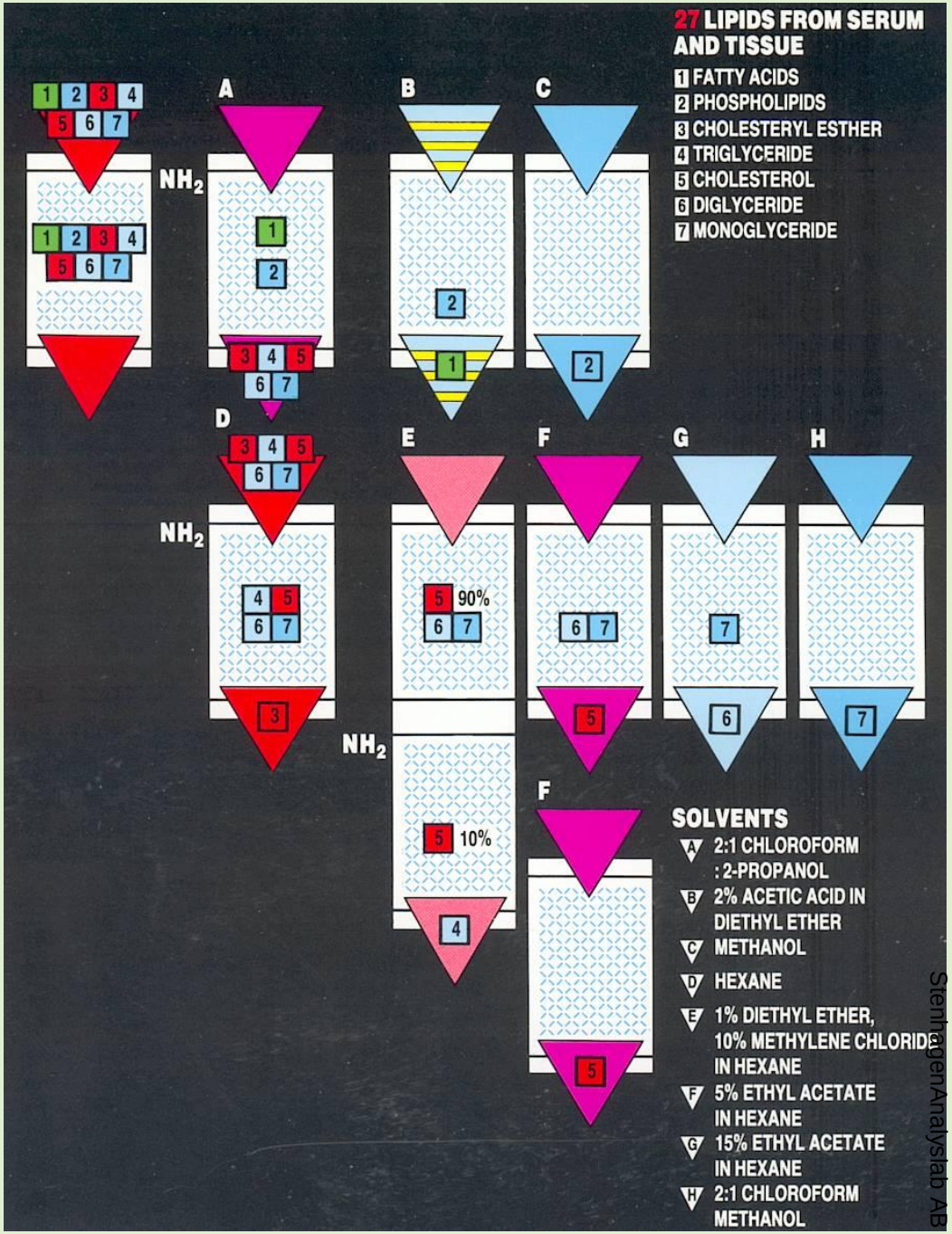
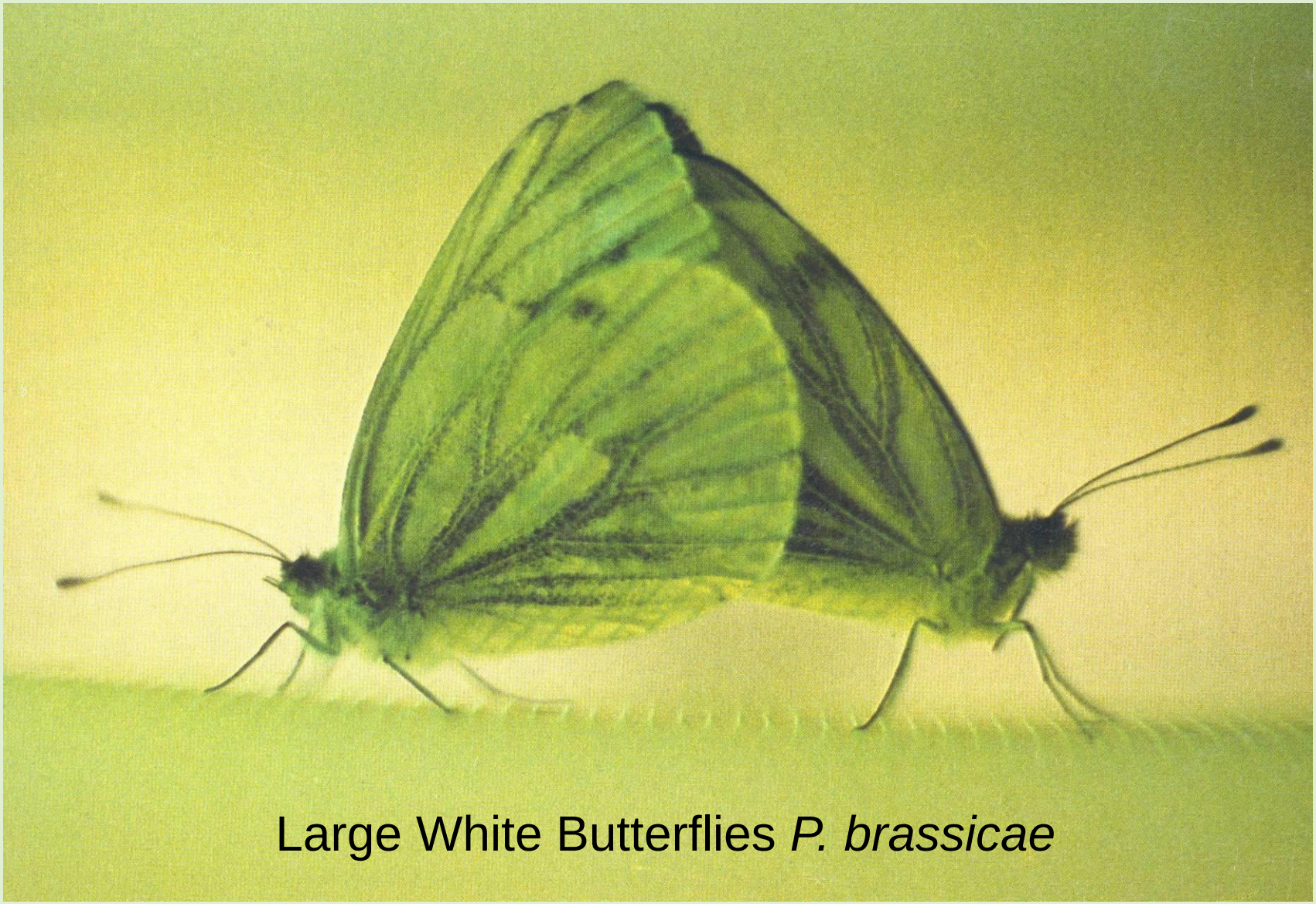
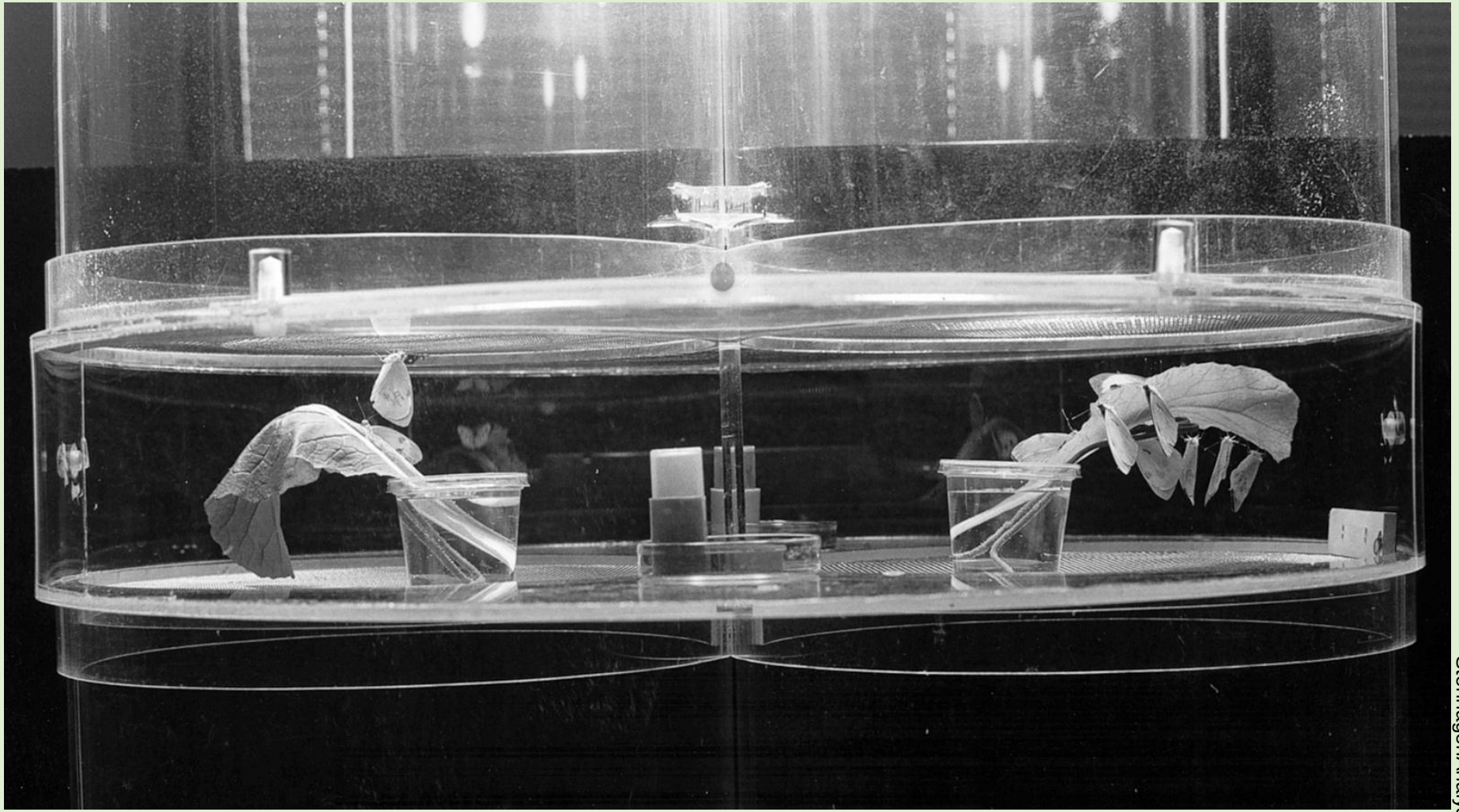




Large White Butterflies *P. brassicae*

Dual Choice chamber



Ovipositing of the Large White Butterflies *P. brassicae*

Cardenolides on Leaf Surface

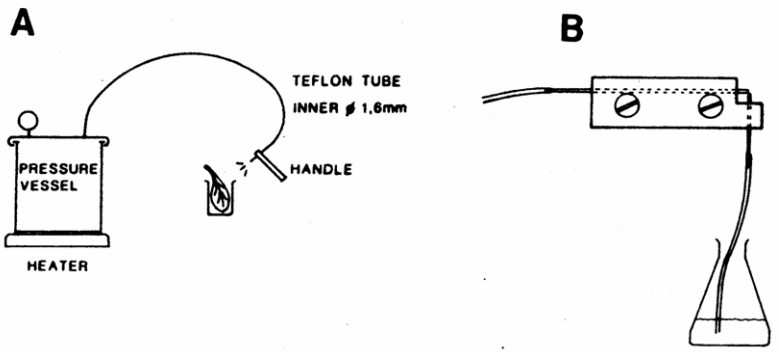


Figure 2. A: The steam spray apparatus used to wash of the surface film of fresh leaves.
B: The spray apparatus used to wash of the surface film with organic solvents.

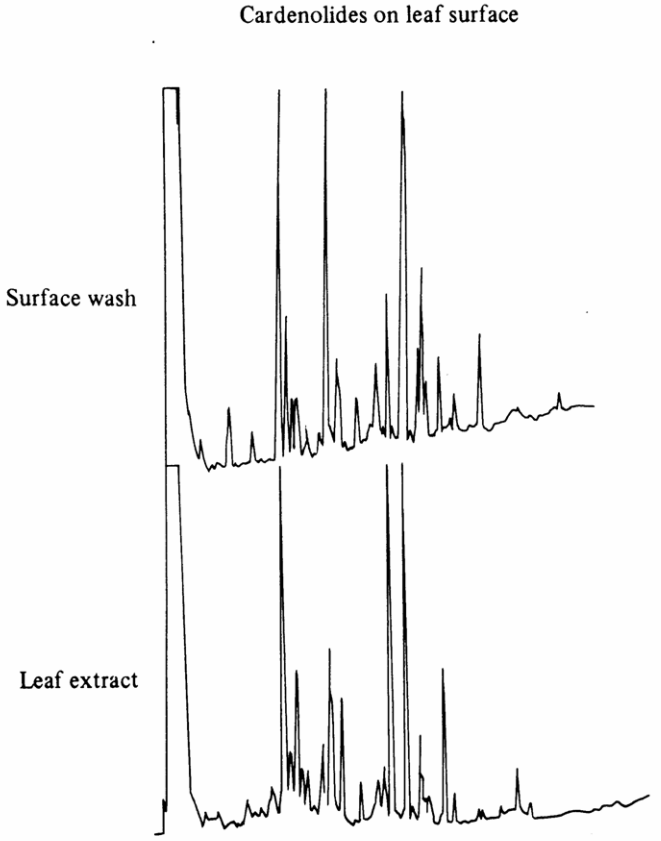


Fig. 1. Comparison of cardenolide contents in leaf extract and on leaf surface of *C. allionii*. Liquid chromatograms of A; 50% methanol–water extract and B; steam wash. Both samples with phenolics removed. Chromatographic column: analytical 15 cm 4.8 mm i.d., packed with 4 μm Novapac C18 (Waters). Flow: 1.0 ml/min. Oven temperature: 60°. Detection: UV adsorption at 220 nm. Mobile phase: gradient from 10 to 40% of methanol in water in 35 min.

Ovipositing of the Large White Butterflies *P. brassicae*

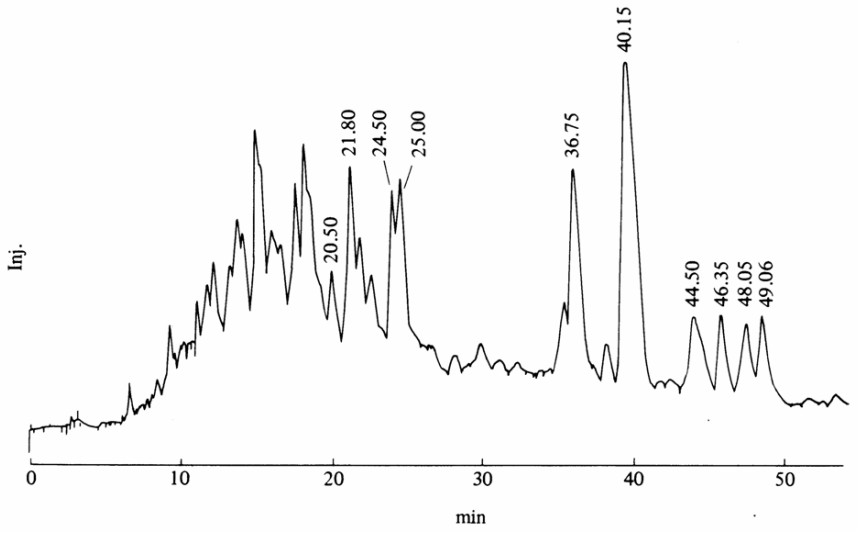


Fig. 2. HPLC of a purified extract obtained from *C.* × *allionii*. Column: 30 cm × 8 mm i.d. 10 μm Polygosil C18. Column temperature: 60C. Mobile phase: methanol–water gradient from 10 to 25% in 5 min followed by 25–45% in 45 min. Detection: UV adsorption at 220 nm. Fourteen cardenolides were isolated from the methanol/water extract of *C.* × *allionii*. Five are of the strophanthidin type and one of the uzarigenin type. The main cardenolide fraction (R_t 40.15 min) does not represent all the activity in the plant extract but it has the strongest deterrent effect on oviposition and was present in large amounts in the leaves of *C. cheiri*, *C.* × *allionii* and *Erysimum scoparium*

Table 6. Ovipositing of Large White butterflies *P. brassicae* in a dual choice chamber.

Test fraction R_t (min)	Test leaves sprayed with fraction (no. of eggs)	Control leaves sprayed with carrier (no. of eggs)	Eggs on test leaves (% of total)
20.50	540	2931	15.6
21.80	208	3710	5.3
24.50	113	2442	4.4
25.00	58	2219	2.5
36.75	371	2431	13.2
40.15	48	3453	1.3
44.50	329	2672	11.0
46.35	167	2660	5.9
48.05	263	3237	7.5
49.06	359	2388	13.1
Sum:	2456	28143	8.0

Sum of data from six (three hr) experiments involving ten females.

Tarsal Contact Chemoreceptory

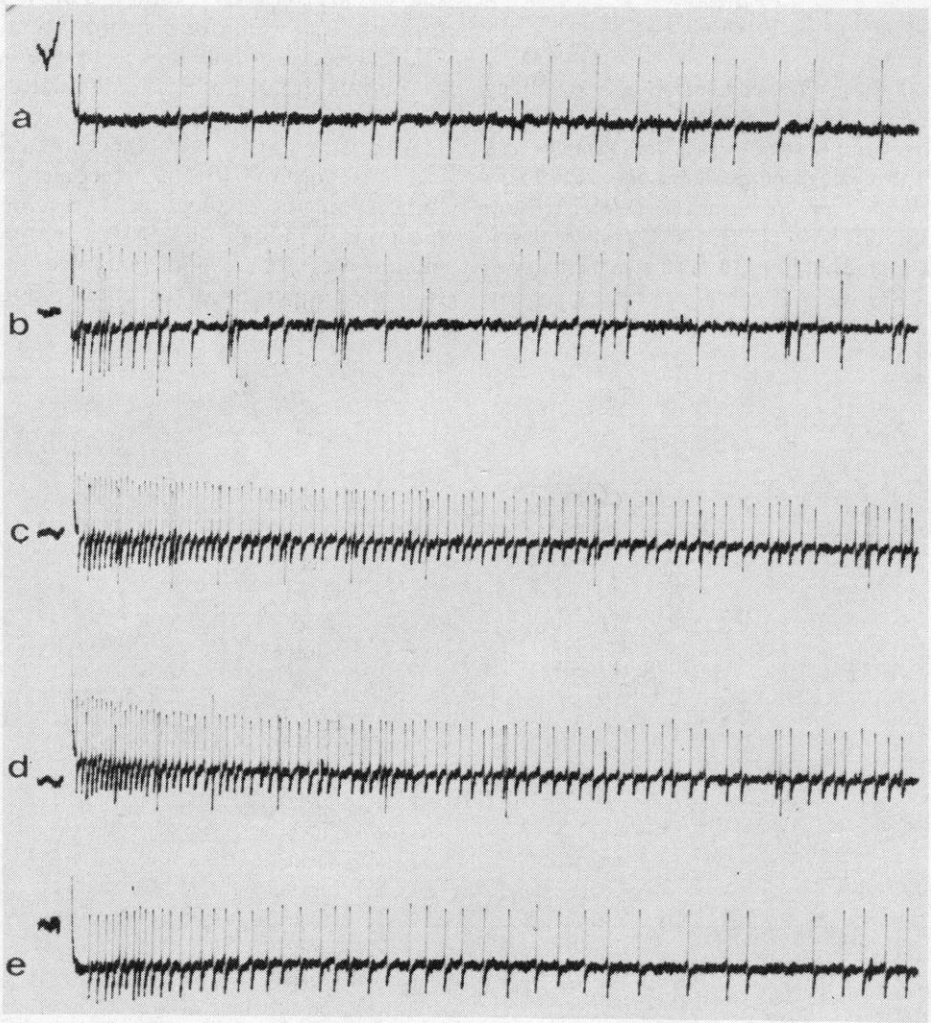
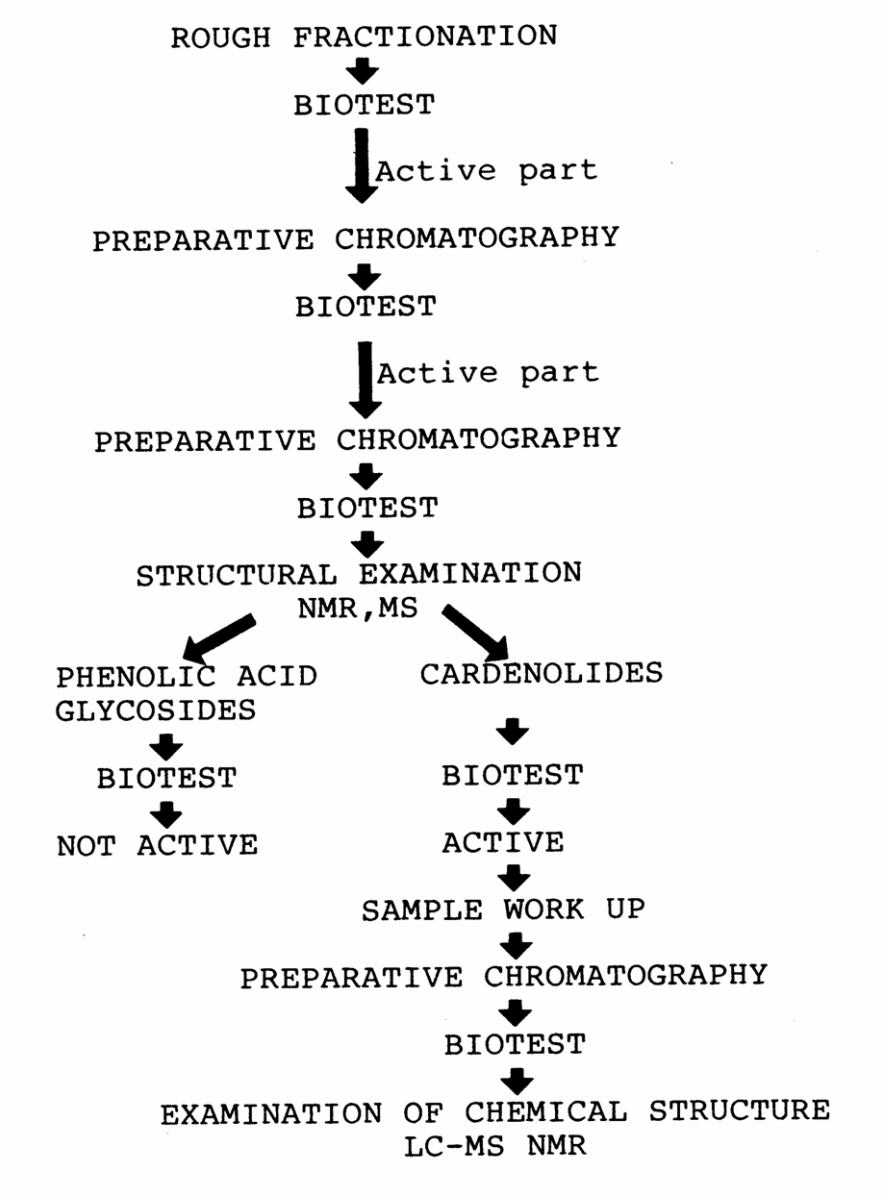


Fig. 3. Neural responses of a tarsal contact chemoreceptory hair of a female of *Pieris brassicae* to different stimuli: (a) 0.01 M NaCl, (b) ODP, eggwash from 500 eggs/ml in water, (c) 0.05% strophanthidin glycoside in 0.01 M NaCl, (d) 0.05% strophanthidin + eggwash from 500 eggs/ml, (e) 0.01% M NaCl after three prior stimulations with strophanthidin. In the latter case the hair was rinsed with aqua dest before stimulation with NaCl. All recordings last one sec.

Protocol for isolation of active component




Identification of
Natural Products
StenhagenAnalyslab AB

Identification Techniques of Natural Products

- **Gas and Liquid Chromatography**
- **Mass Spectrometry**
- UV/Vis Spectrophotometry
- IR Spectrophotometry
- NMR Spectrometry
- Roentgen Diffraction

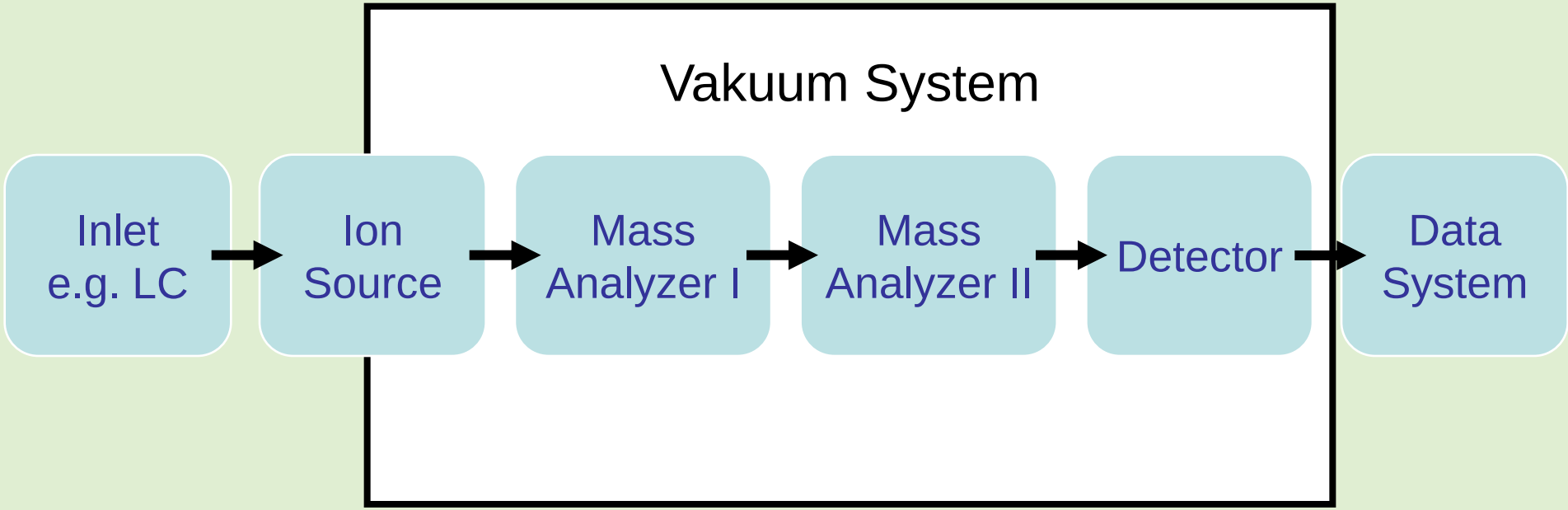
Mass spectrometers separate ions according to their mass to charge (m/z) ratios

1. Quantitative information about samples
2. Qualitative structural information
3. Sensitive for a wide range of compounds
4. Extremely selective

Uses of Mass Spectrometry in Organic and Biological Chemistry

Application	Samples	Methods	Comment
Molecular weight determination	Pure compounds, mixtures	Recognize intact molecular ion in spectrum	Several ionisation methods can be used for confirmation
Molecular formula determination	Usually pure compounds but also mixtures by LC-MS or GC-MS	High accuracy mass measurement on molecular ion	High accuracy alone seldom gives a unique molecular formula
Molecular structure determination	Pure compounds or mixtures by LC-MS, GC-MS, and MS-MS	Spectrum-structure correlations; library comparisons	Confirmation of suspected structures is usual; de novo interpretations rare
Sequence determination	Proteins, other biopolymers	Tandem mass spectrometry (MS-MS)	Sensitive, very rapid and increasingly useful
Isotopic incorporation and fractionation	Naturally and artificially labelled compounds (^{13}C, ^{2}H, ^{18}O, etc.)	Ion abundance measurements	Precise isotope ratio measurements require special instrument
Quantification	Mixtures by LC-MS or GC-MS	Selected ion detection (SIR) or multiple reaction monitoring (MRM)	Sensitive and very selective

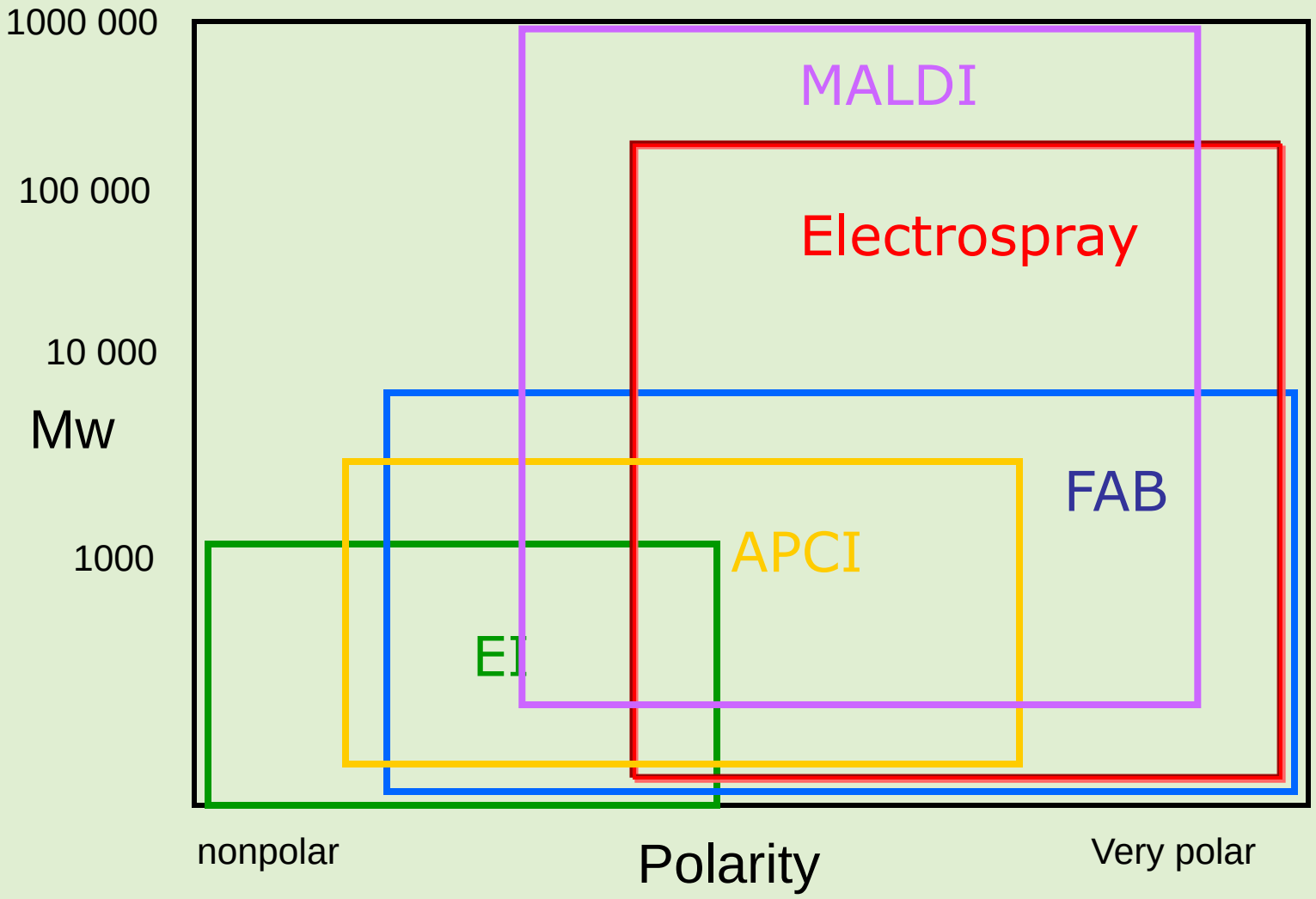
Components of a Mass Spectrometer



Ion Sources

- High vacuum sources
  - Electron Ionization (EI)
  - Chemical Ionization (CI)
  - Field Desorption (FD, FI)
  - Fast atom bombardment (FAB. LSIMS)
  - Matrix-Assisted Laser Desorption (MALDI)
- Atmospheric Pressure Ionization (API)
  - Electro spray Ionization (ESI)
  - Atmospheric Pressure Chemical Ionization (APCI)
  - Atmospheric Pressure Photo ionization (APPI)
  - Atmospheric Matrix-Assisted Laser Desorption

Application range of various ion sources



High Vacuum Sources

- Electron Ionization (EI)
- Chemical Ionization (CI)
- Fast atom bombardment (FAB. LSIMS)
- Matrix-Assisted Laser Desorption (MALDI)

Ion Source for Electron Impact (EI)

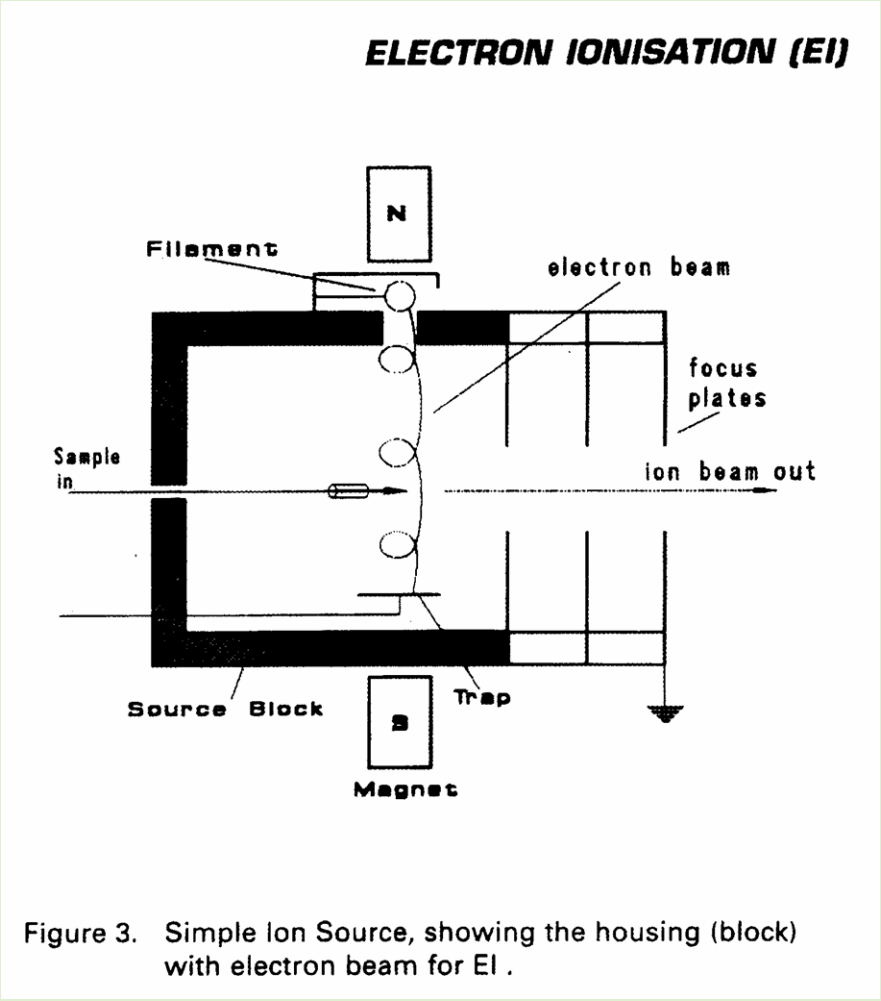
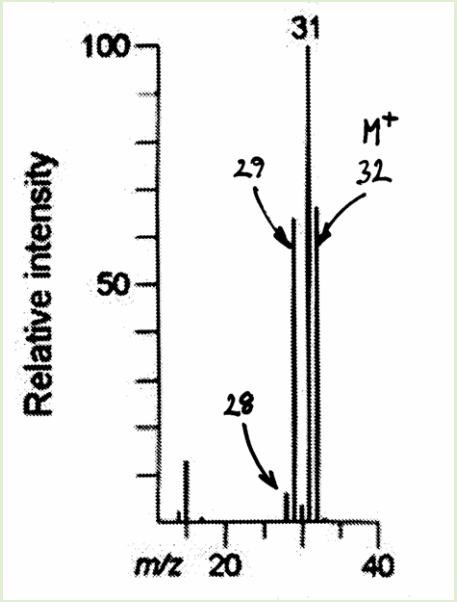
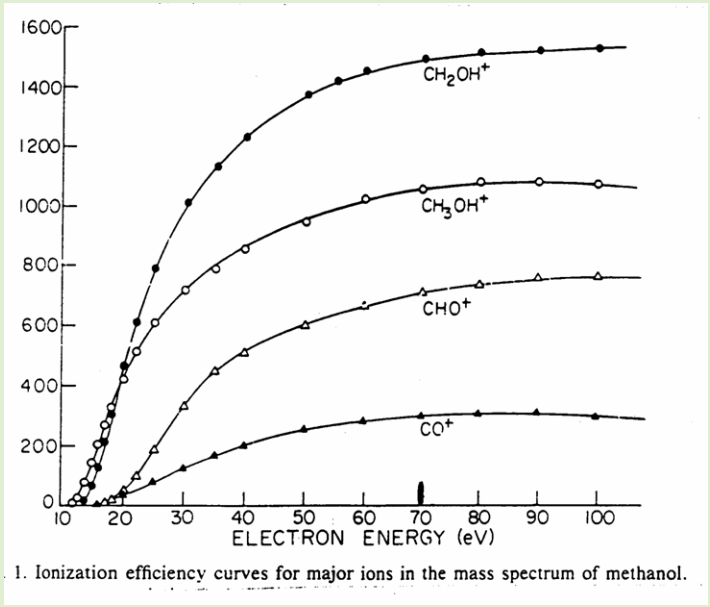


Figure 3. Simple Ion Source, showing the housing (block) with electron beam for EI .

EI mass spectrum of methanol



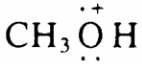


1. Ionization efficiency curves for major ions in the mass spectrum of methanol.

Mass spectra (EI) are routinely obtained at an electron beam energy of 70 eV. The simplest event that occurs is the removal of a single electron from the molecule in the gas phase by an electron of the electrom beam to form the molecular ion, which is a radical cation ($M^{\cdot +}$). For example, methanol forms a molecular ion.

$$CH_3OH + e \longrightarrow \underset{m/z\ 32}{CH_3OH^{\cdot +}} + 2e$$

When the charge can be localized on one particular atom, the charge is shown on that atom.



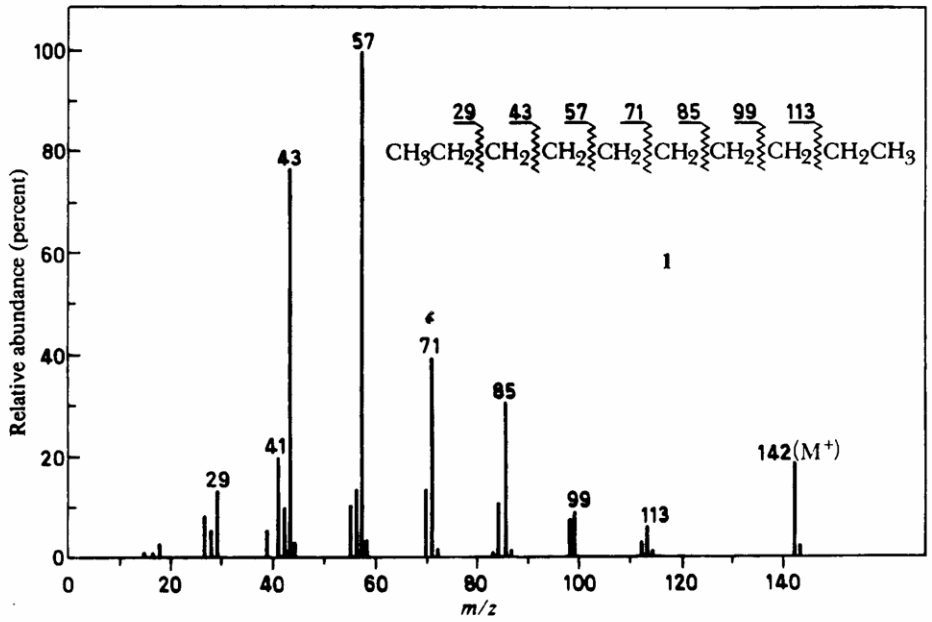
The single dot represents the odd electron. Many of these molecular ions disintegrate in 10^{-10}–10^{-3} s to give, in the simplest case, a positively charged fragment and a radical. A number of fragment ions are thus formed, and each of these can cleave to yield smaller fragments. Again, illustrating with methanol

$$CH_3OH^{\cdot +} \longrightarrow CH_2OH^{+}\ (m/z\ 31) + H^{\cdot}$$

$$CH_3OH^{+} \longrightarrow CH_3^{+}\ (m/z\ 15) + {\cdot}OH$$

$$CH_2OH^{+} \longrightarrow CHO^{+}\ (m/z\ 29) + H_2$$

EI mass spectrum of decan ($C_{10}H_{22}$) Mw 142



$C_4H_9-CH_2:CH_2-C_4H_9 \xrightarrow{-e^-} C_4H_9-CH_2^{+}\cdot CH_2-C_4H_9$

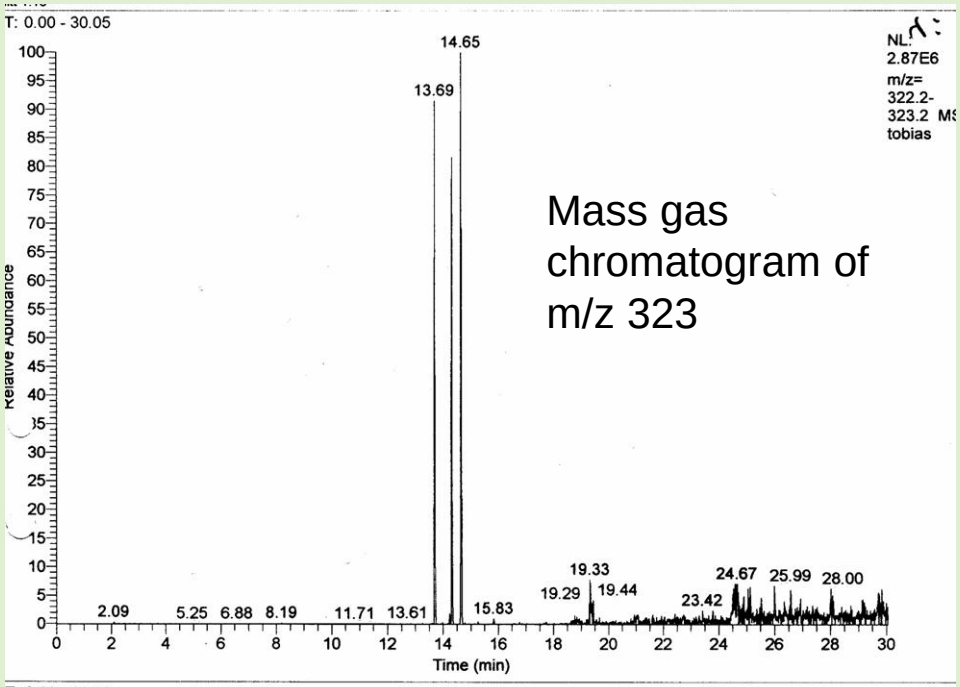
Parent ion 142 m/z

Fragmentering:

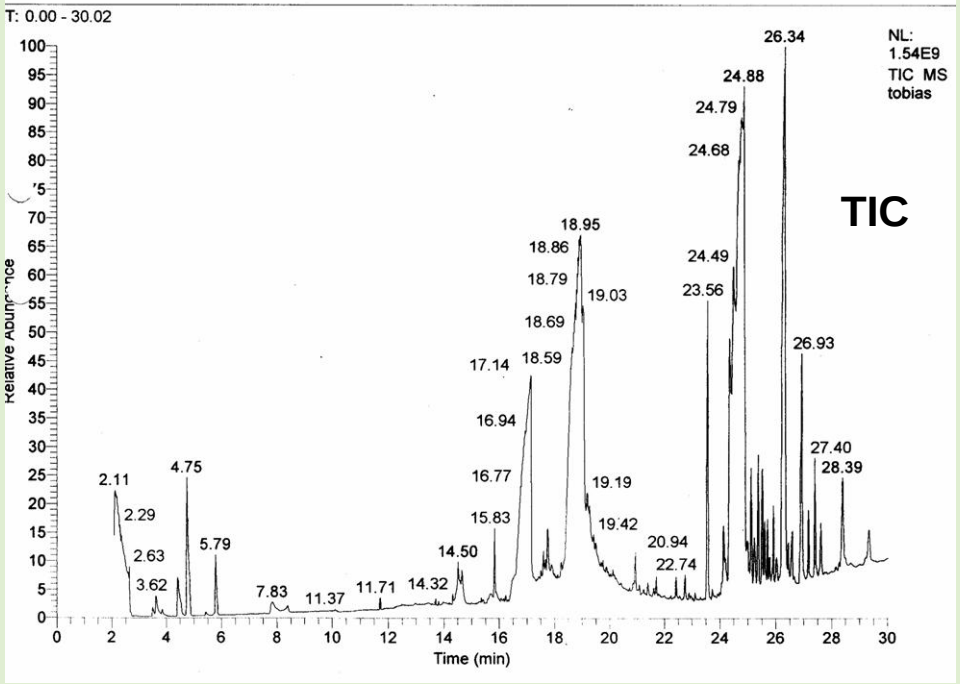
et. $C_4H_9-CH_2^{+}\cdot CH_2-C_4H_9 \rightarrow C_4H_9-CH_2^{+} + \cdot CH_2-C_4H_9$

71 m/z

GC-MS
with EI ionisation



Mass gas chromatogram of m/z 323

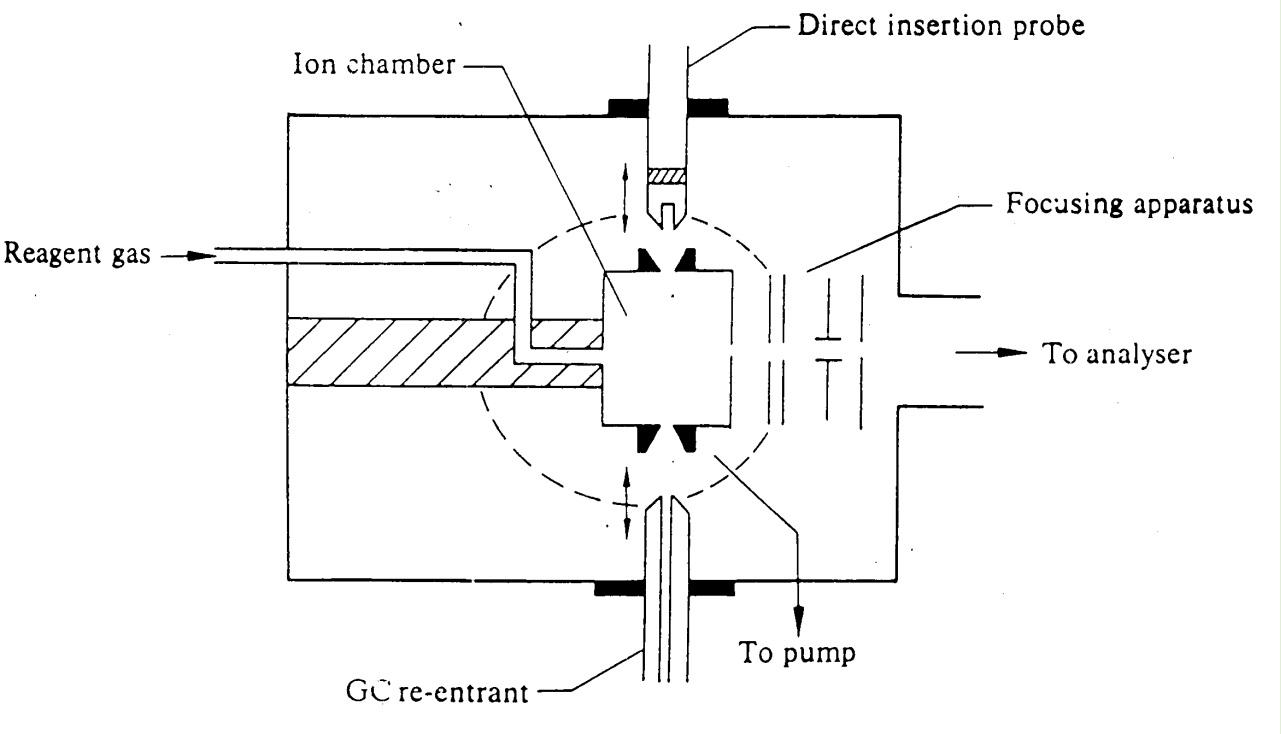


Advantages and Disadvantages of EI

Advantage	Consequence
Reproducible method	Libraries of EI allow compound identification
Extensive fragmentation occurs	Molecular structure information can be deduced
Ionisation efficiency high	Method is sensitive: 1 in 1000 molecules is ionised
Ionisation is non-selective	All vaporized molecules can be ionised

Disadvantage	Consequence
Only positive ions formed	Not ideal for all classes of compounds
Radical cations formed	Rearrangement processes complicate mass spectra
Sample must be volatile	Limited to relative low molecular weight compounds
Ionisation is non-selective	All vaporized molecules contributes to the mass spectrum
Relatively energetic (large interal energy)	Often extensive fragmentation; limits value in molecular weight determination

Chemical ionisation (CI)



CHEMICAL IONISATION (CI)

Reagent gas	Molecular ion	Reactive reagent ion
H_2	$H_2^{\cdot+}$	H_3^+
C_4H_{10}	$C_4H_3^{\cdot+}$	$C_4H_{11}^+$
NH_3	$NH_3^{\cdot+}$	NH_4^+
CH_3OH	$CH_3OH^{\cdot+}$	$CH_3OH_2^+$
NO	$NO^{\cdot+}$	NO^+

Figure 4. Some types of reagent gases and their reactive ions

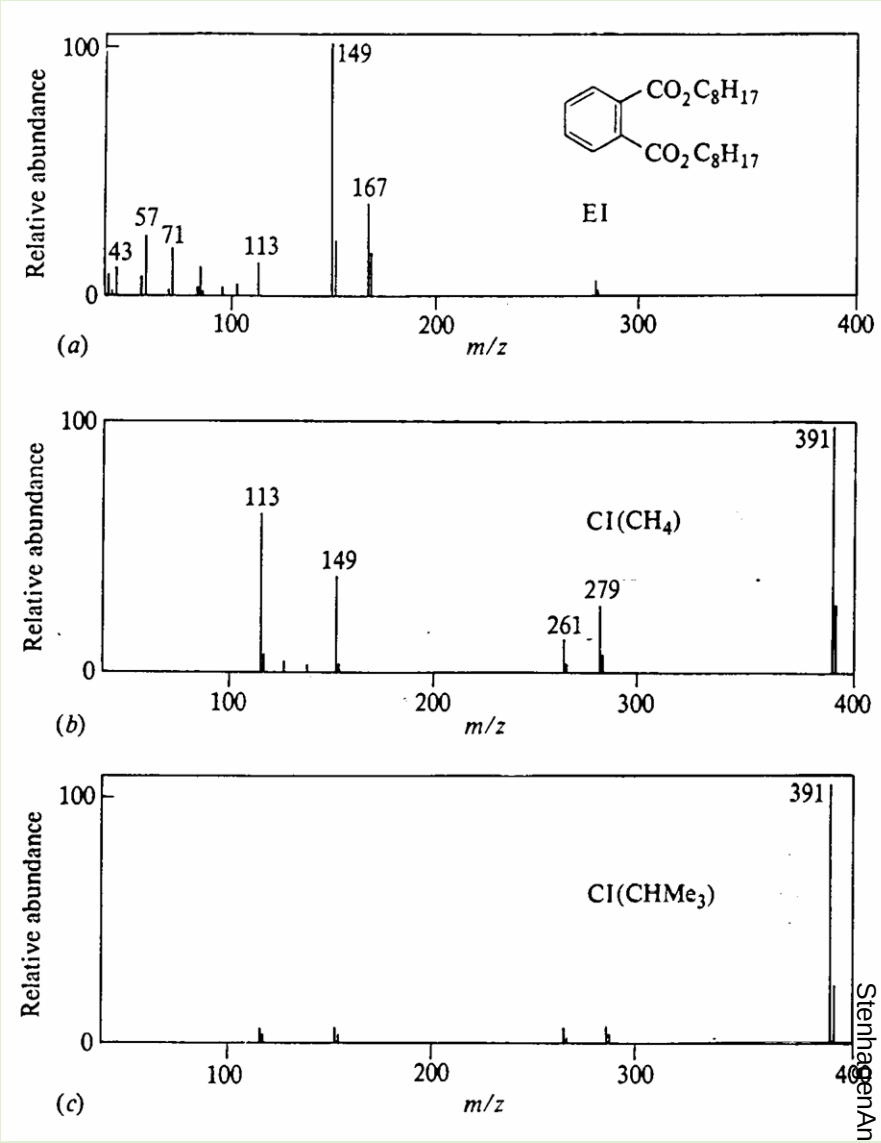
CHEMICAL IONISATION (CI)

$$M + NH_4^+ \rightarrow MH^+ + NH_3 \quad (a)$$

$$M + NH_4^+ \rightarrow M.NH_4^+ \quad (b)$$

$$C_2H_7^+ + M \longrightarrow (M - H)^+ + C_2H_6 + H_2 \quad (c)$$

Figure 5. Typical CI processes in which neutral sample molecules (M) can react with NH_4^+ to give either (a), a protonated ion (MH^+) or (b), an adduct ion (MNH_4^+); the quasimolecular ions are respectively 1 and 18 mass units greater than the true mass (M). In process (C), reagent ions ($C_2H_7^+$) abstract hydrogen, giving a quasimolecular ion 1 mass unit less than M



Fast atom bombardment (FAB. LSIMS)

FAB (Fast Atom Bombardment) och LSIMS (Liquid Secondary Ion Mass Spectrometry)

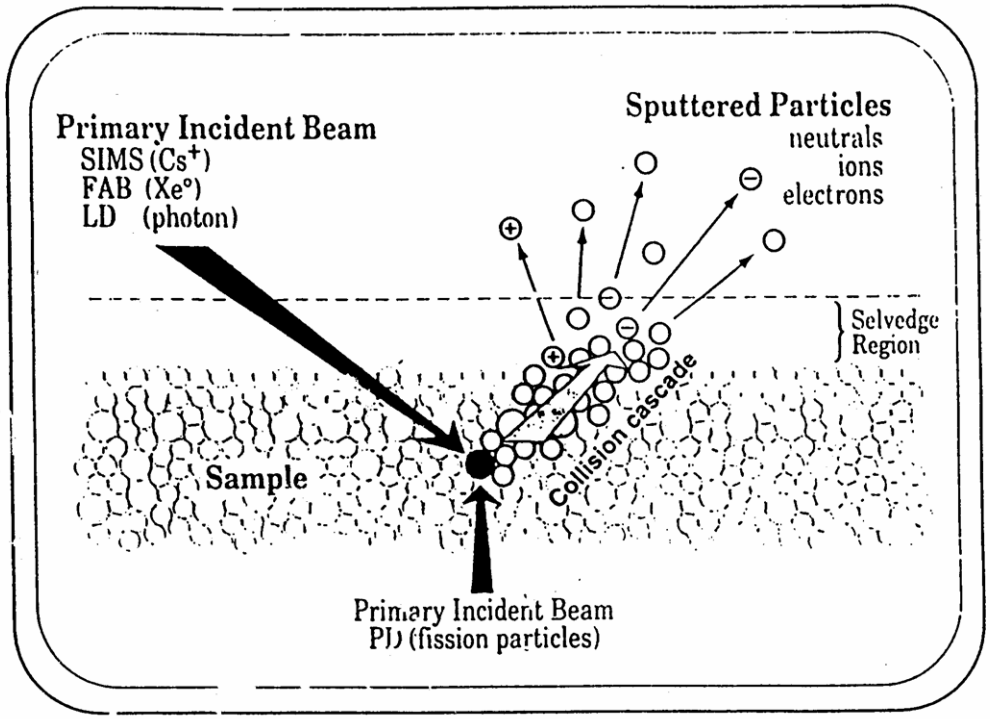


Figure 2. Simple illustration of an instantaneous collision cascade generated as a result of primary particle impact in desorption ionization mass spectrometry.

Fast atom bombardment (FAB. LSIMS)

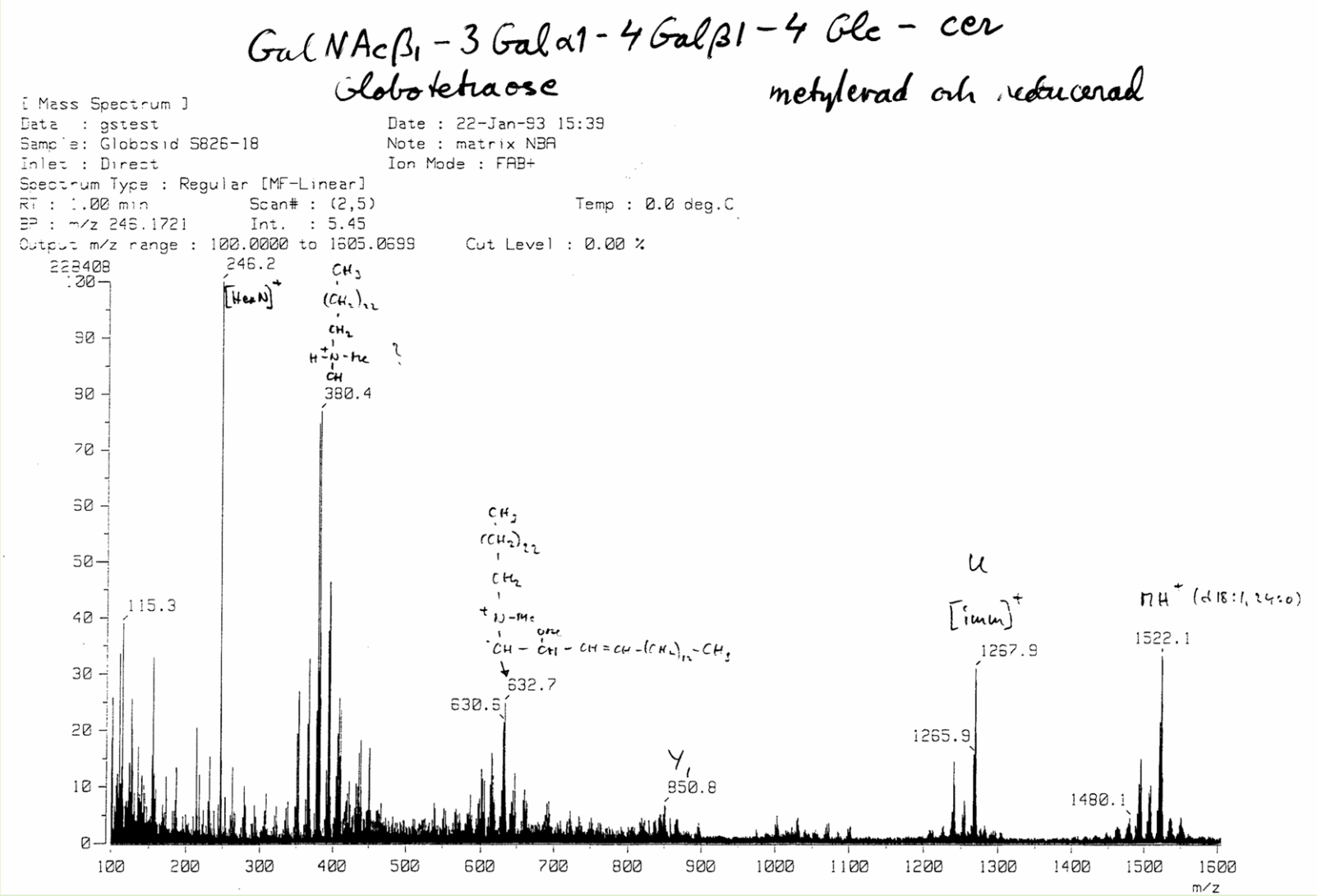
Matrix in FAB/LSIMS

Glycerol	
Thioglycerol	more volatile
Mix glycerol/thioglycerol	
Mix of di-thiothreitol (five parts)/di-thioerythritol (one part)	
m-nitrobenzyl alcohol (m-NBA)	
glycols	
tri-ethylenglycol	
tetra-ethylenglycol	
tri-ethanolamine	also for negative ion
di-ethanolamine	"
2,4-ditertamylphenol	
tetra-methylene-sulphone	
conc. sulphuric acid	
3-aminopropane1,2-diol	negative ion
thiodiethylene glycol (TDEG)	organometallic comp
Mix of TDEG/glycerol	peptide

Problems caused by chemical reactivity:

ex.: m-NBA oxidation to aldehyde under ion bombardment and reaction with analyte amino groups
ex.: formation of formaldehyde from glycerol
ex.: reduction of the analyte such as dyestuffs and peptides in glycerol

Fast atom bombardment (FAB. LSIMS)



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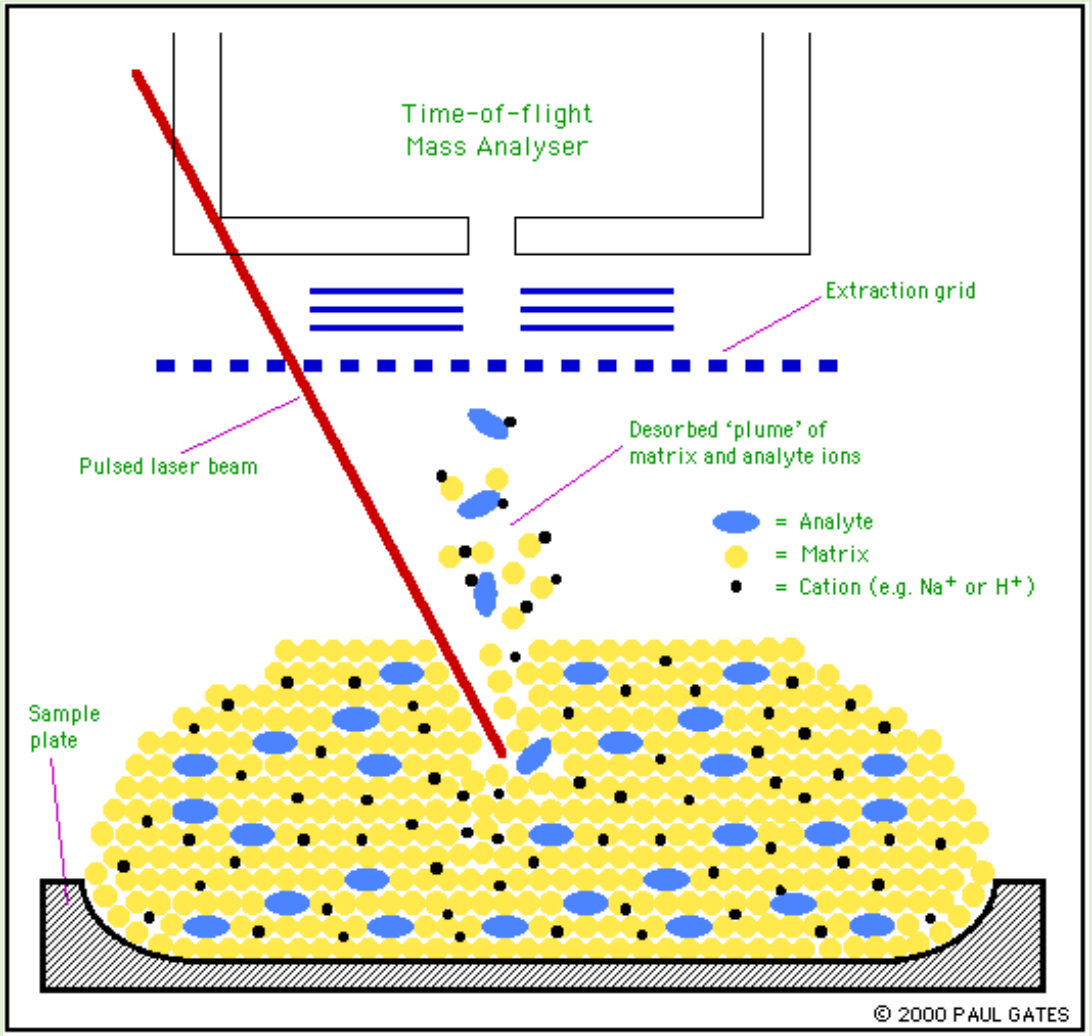
Matrix-assisted Laser Desorption/Ionization



The mechanism of MALDI is not totally understood…:

The Formation of a 'Solid Solution'

The analyte molecules are distributed throughout the matrix so that they are completely isolated from one other. This is necessary if the matrix is to form a homogenous 'solid solution'

Matrix Excitation

Some of the laser energy incident on the solid solution is absorbed by the matrix, causing rapid vibrational excitation, bringing about localized disintegration of the solid solution, forming clusters made up of a single analyte molecule surrounded by neutral and excited matrix molecules. The matrix molecules evaporate away from these clusters to leave the excited analyte molecule.

Analyte Ionization

The analyte molecules can become ionized by simple protonation by the photo-excited matrix, leading to the formation of the typical $[M+X]^+$ type species (where X= H, Li, Na, K, etc.). Some multiply charged species, di- and trimers can also be formed. Negative ions are formed from reactions involving deprotonation of the analyte by the matrix to form $[M-H]^-$ and from interactions with photoelectrons to form the $[M]^{-\circ}$ radical molecular ions.

These ionization reactions occur in the first tens of nanoseconds after irradiance, and within the initial desorbing matrix/analyte cloud.

Applications and Choice of Matrix

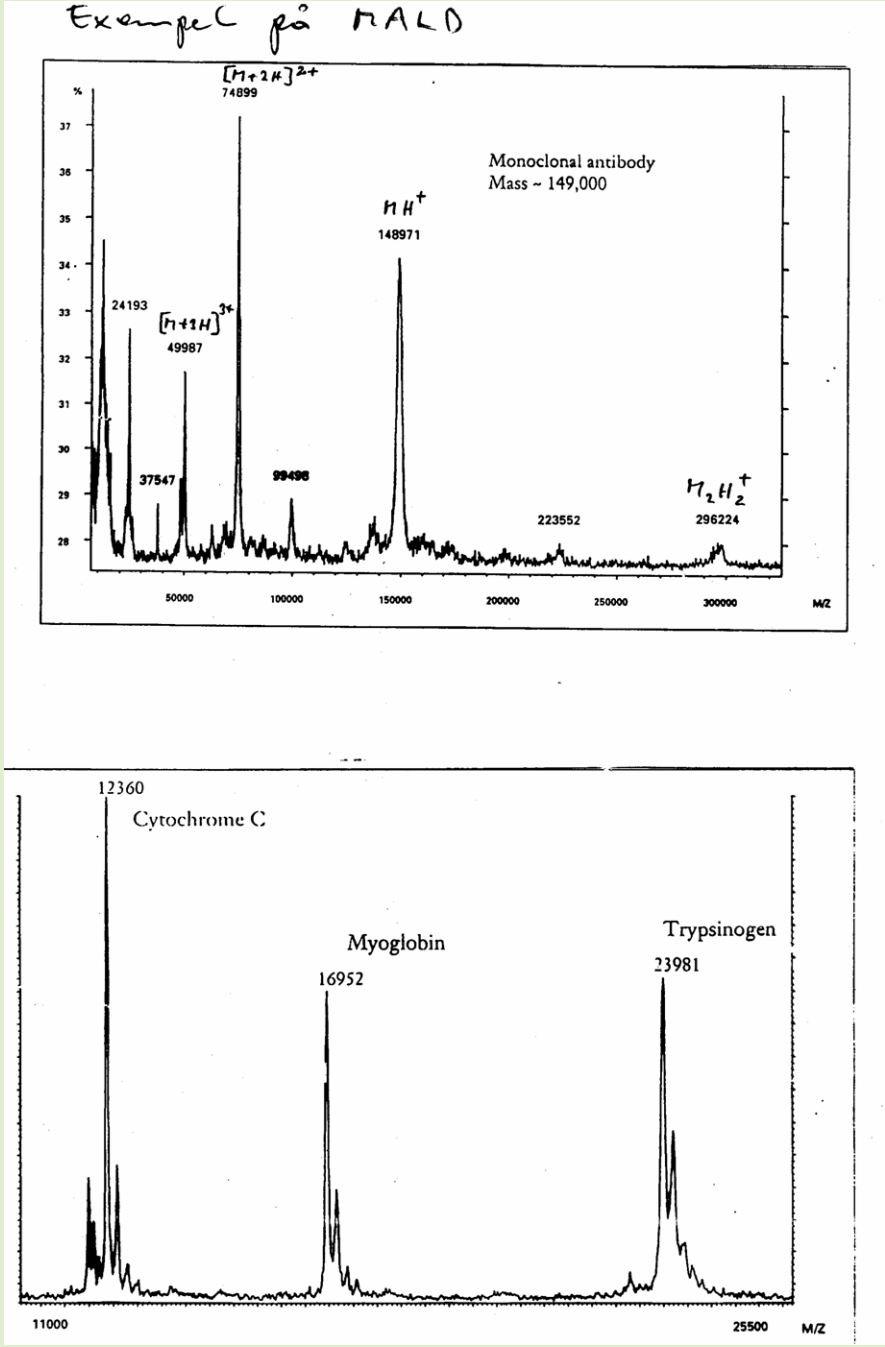
The most important applications of MALDI mass spectrometry are: peptides and proteins, synthetic polymers, oligonucleotides, oligosaccharides, lipids, inorganics.

Numerous matrices have been found for these and other classes of compounds; a summary is given in figure.

Although electrospray ionization (ESI) is somewhat competitive and certainly complementary, MALDI remains the method of choice in several key areas, particularly proteomics. The sensitivity of ESI is reduced by the presence of salts, impurities, and organic buffers which are more easily tolerated by MALDI.

Determined molecule	Abbrev.	Product
Peptide/protein		
Mass < 10 kDa	CHCA	α-Cyano-4-hydroxycinnamic acid
Mass > 10 kDa	SA	Sinapic acid
	HABA	2-(4-Hydroxyphenylazo)benzoic acid
IR-Laser		Succinic acid
UV-Laser		2,6-Dihydroxyacetophenone
UV-Laser		Ferulic acid
UV-Laser		Caffeic acid
Liquid matrix		Glyerol
Liquid matrix		4-Nitroaniline
Oligonucleotide		
Mass < 3.5 kDa	THAP	2,4,6-Trihydroxyacetophenone
Mass > 3.5 kDa	HPA	3-Hydroxypicolinic acid
		Anthranilic acid
		Nicotinic acid
		Salicylamide
Synthetic polymer		
Non-polar	IAA	Trans-3-indoleacrylic acid
	DIT	Dithranol
Polar	DHB	2,5-Dihydroxybenzoic acid
IR-Laser		Succinic acid
Organic molecules		
	DHB	2,5-Dihydroxybenzoic acid
		Isovanillin
Carbohydrates		
	DHB	2,5-Dihydroxybenzoic acid
	CHCA	α-Cyano-4-hydroxycinnamic acid
		3-Aminoquinoline
Acidic	THAP	2,4,6-Trihydroxyacetophenone
Lipids	DIT	Dithranol
Dendrimers		
	SA	Sinapic acid
	DIT	Dithranol
Fullerenes	SA	Sinapic acid
Inorganic molecules	DCTB	T-2-(3-(4-t-Butyl-phenyl)-2-methyl-2-propenylidene)malononitrile
Oligosaccharide		1-Isoquinolinol

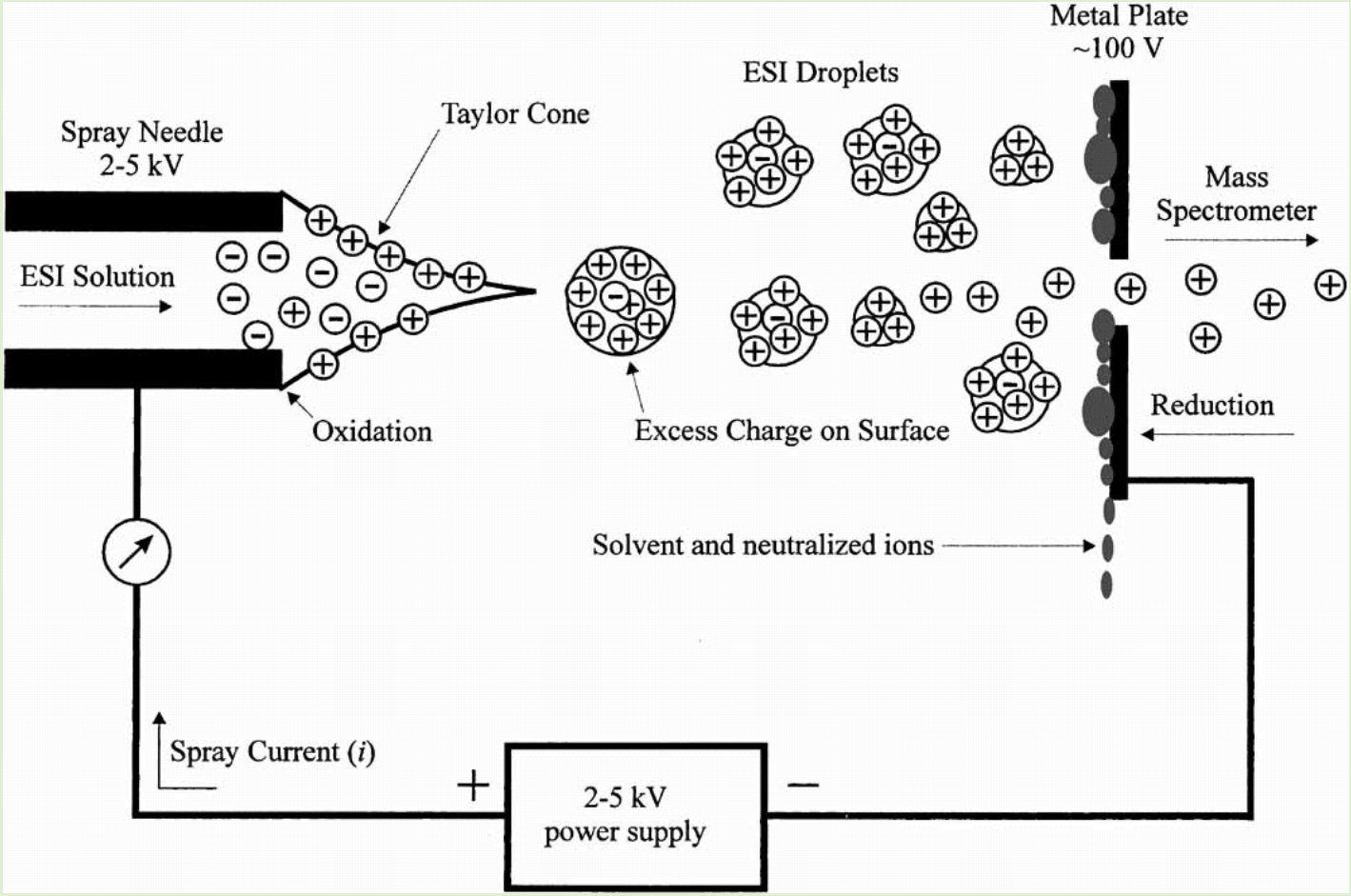
Examples of MALDI mass spectra



Atmospheric Pressure Ionization (API)

Electrospray Ionization (ESI)
Atmospheric Pressure Chemical Ionization (APCI)
Atmospheric Pressure Photo ionization (APPI)
Atmospheric Matrix-Assisted Laser Desorption

Schematic of electrospray ionization process



N B Cech & C G Enke Mass Spectrom Reviews 2001, 20, 362

The major steps in ESI

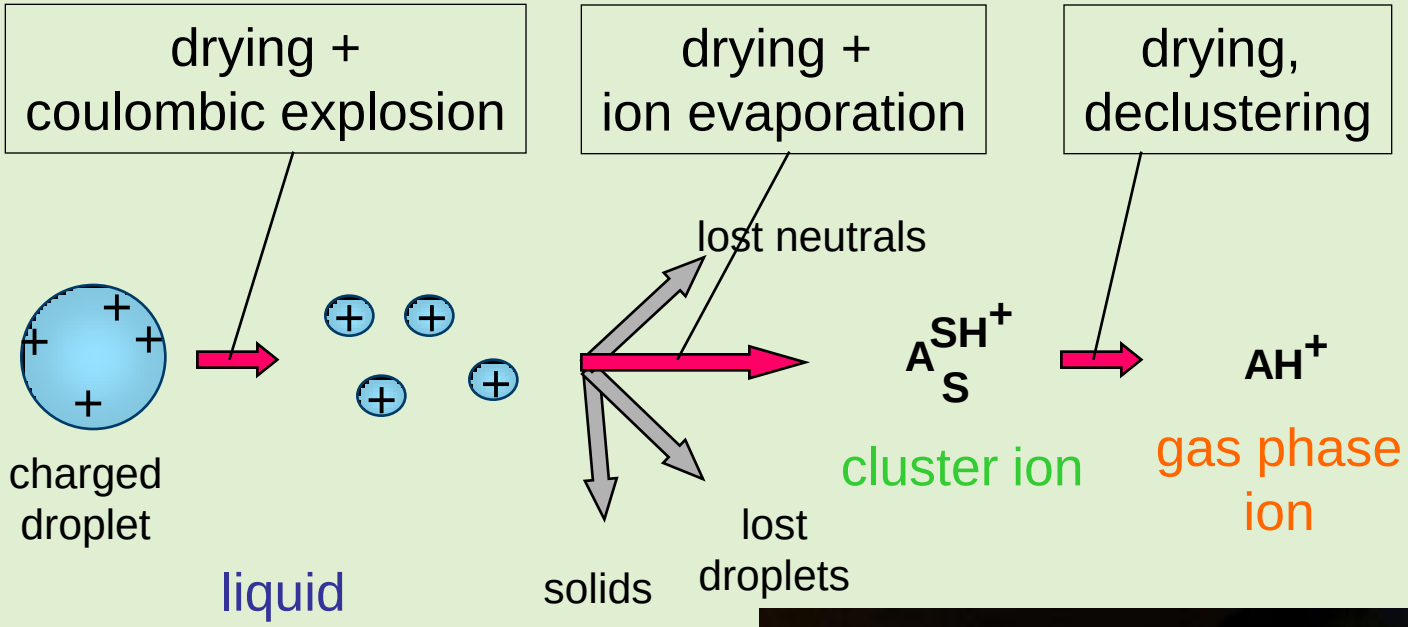
- Formation of a Taylor cone with excess of positive charge on its surface
- Formation of charged droplets
- Shrinkage of droplets through evaporation and columbic fission (droplet disintegration into smaller droplets due to increased charge density)
- Ionization takes place in gas-phase produced by ion evaporation from small highly charged droplets

N B Cech & C G Enke Mass Spectrom Reviews 2001, 20, 362

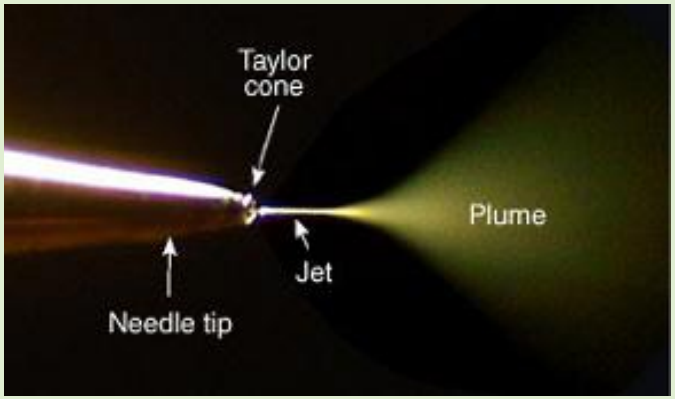
E de Hoffmann & V Stroobant Mass Spectrom: Principles and Applications, 2nd ed, J Wiley & Sons, 1999

Electrospray ionization process

Analyte: A
Solvent: S



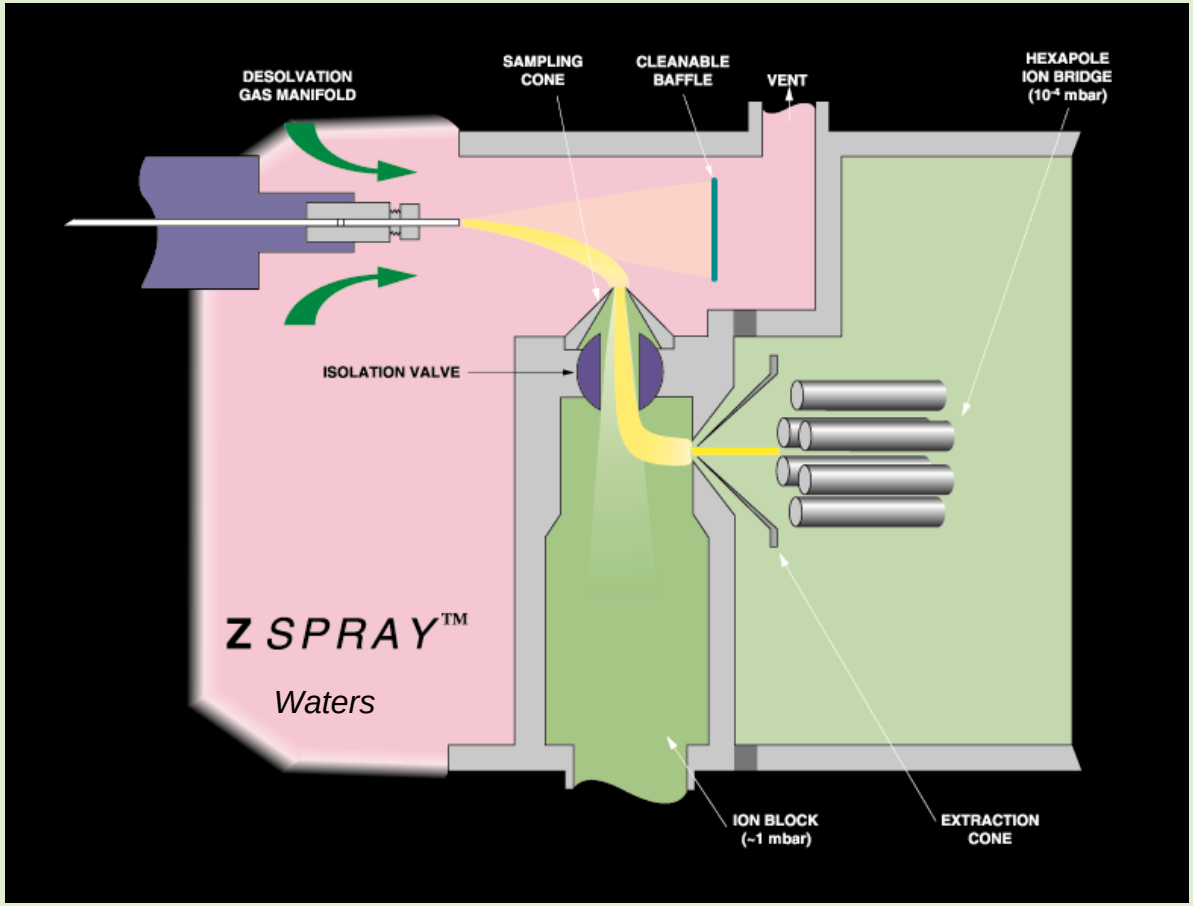
Suppression: Interacts with evaporation or with gas phase ion formation



MA

Orthogonal Spraying

The concept used by most manufactures today



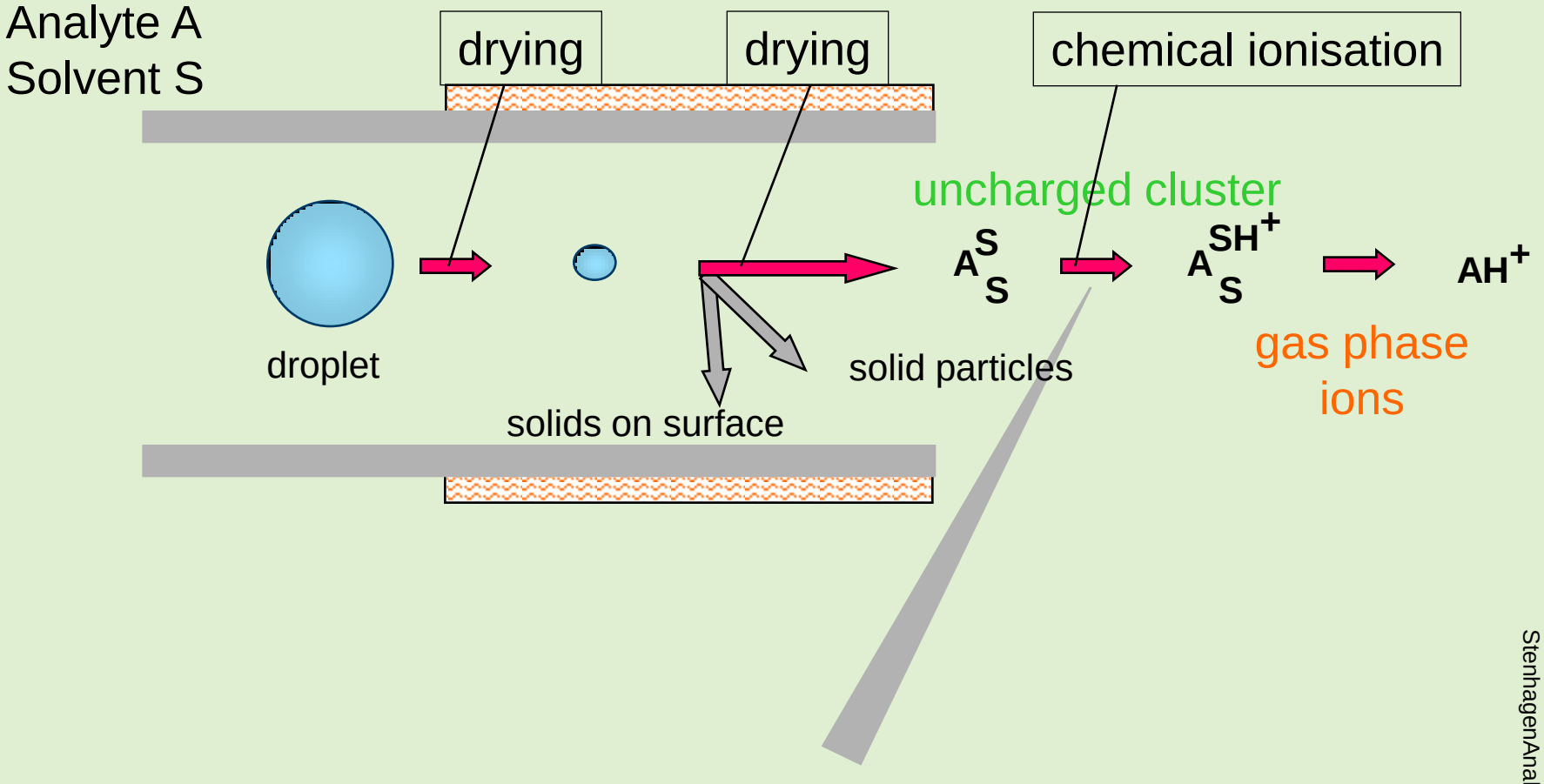
Advantages of ESI

- Suitable to a wide range of polar non-volatile compounds
- Sensitive (low pg amounts)
- Soft ionization technique (gives molecular weight)
- Robust
- Accepts LC flow rates from low nl to over 1 ml/min

Atmospheric Pressure Chemical Ionization APCI

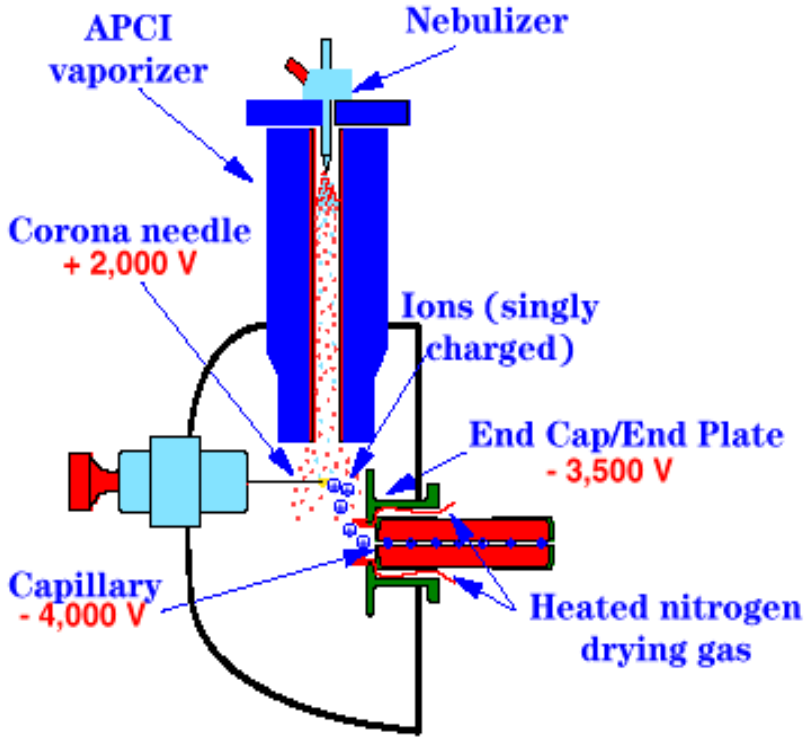
- Mobile phase and analyte are nebulized
- Droplets are vaporized
- Mobile phase molecules are ionized by electrons from the corona discharge
- Analyte molecules are ionized by the mobile phase ions
- Ionization takes place in the gas phase

APCI ionisation process



Atmospheric Pressure Chemical Ionization APCI

APCI Ionization (positive mode)



HEWLETT PACKARD

page 20

Agilent Technologies

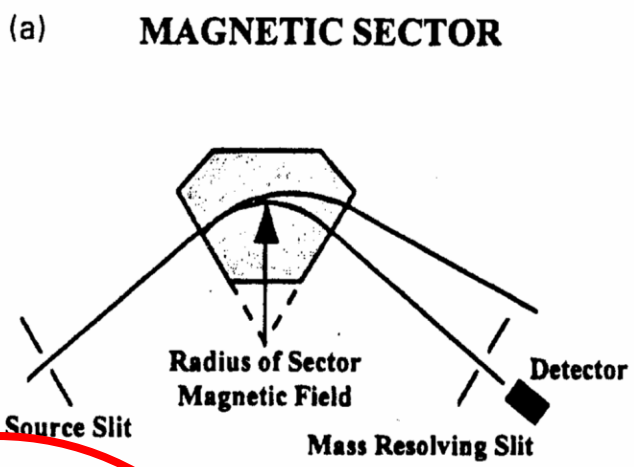
Compatability of API-MS with chromatography

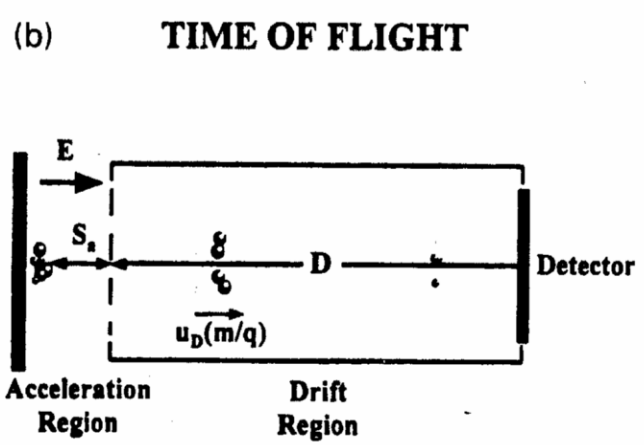
TABLE 2. Compatability of API–MS with Various Chromatographic Modes

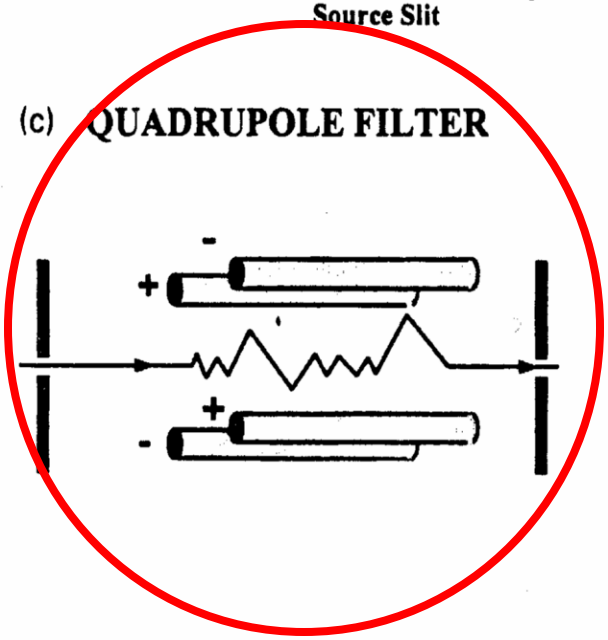
Mode	ES	APCI	Comments
Reversed phase	+++	++	Formation of ions in solution is possible; usually limited sample volatility
Normal phase	+	+++	Ions in solution (nonaqueous miscibility) may be a problem; usually sample is volatile
Size exclusion	+++	+	Buffers to suppress nonexclusion mechanisms may cause problems; most likely sample is not volatile and is a high molecular weight
Ion pair	++	++	Reagent ions may compete for ion-evaporation process; volatility of mobile-phase additive
Ion exchange	+	+	High ionic strength may be a problem; limited volatility of mobile-phase additives
Hydrophobic interaction	+	+	Uses salt gradients to elute biomolecules; salt is not compatible with API–MS
Immunoaffinity	+++	+	Mobile phase often compatible with API–MS, usually nonvolatile sample

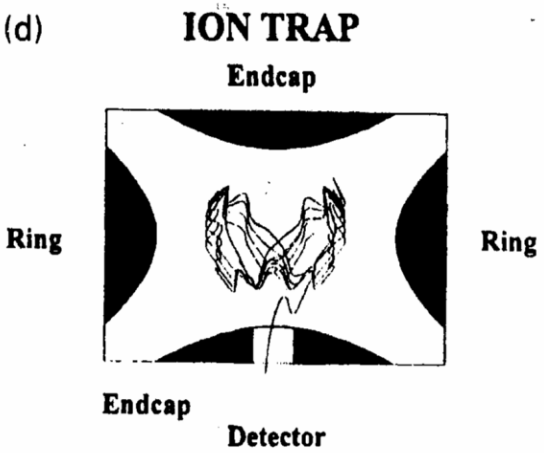
[a] A greater number of plus signs indicates a larger degree of compatibility.

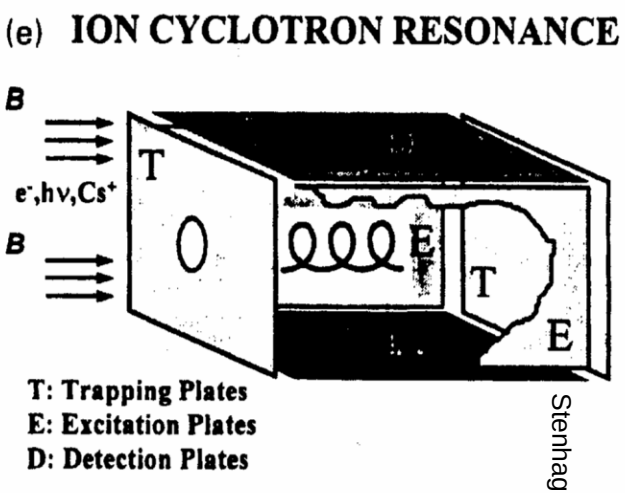
Mass Analyzers



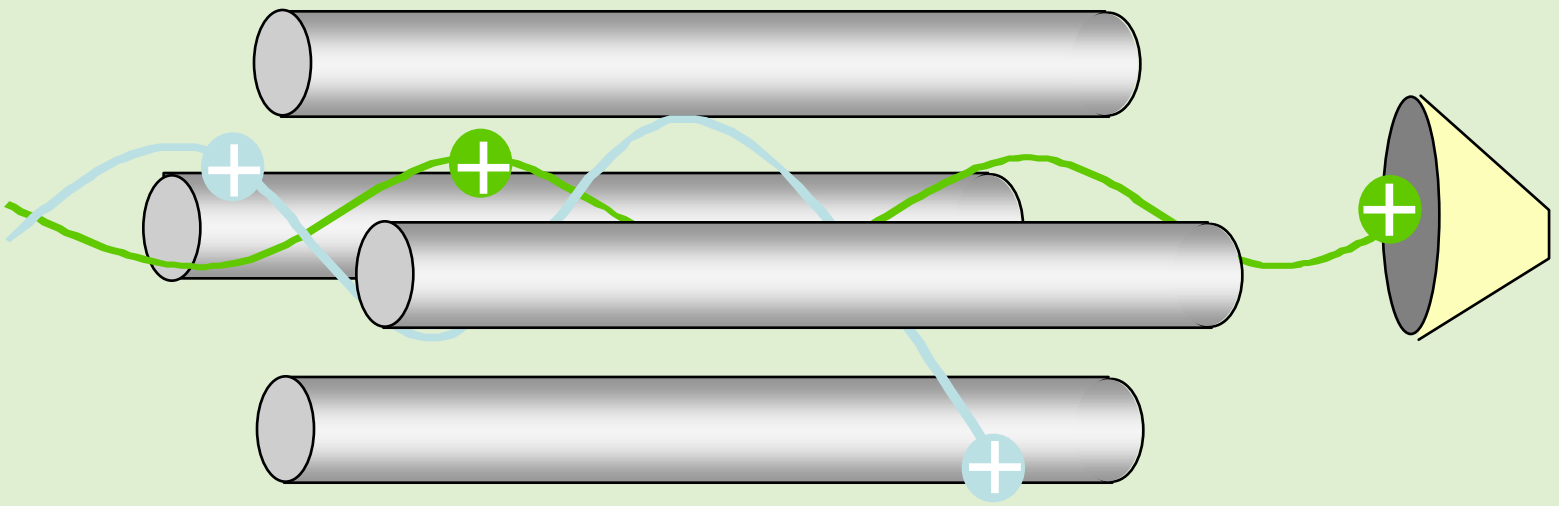






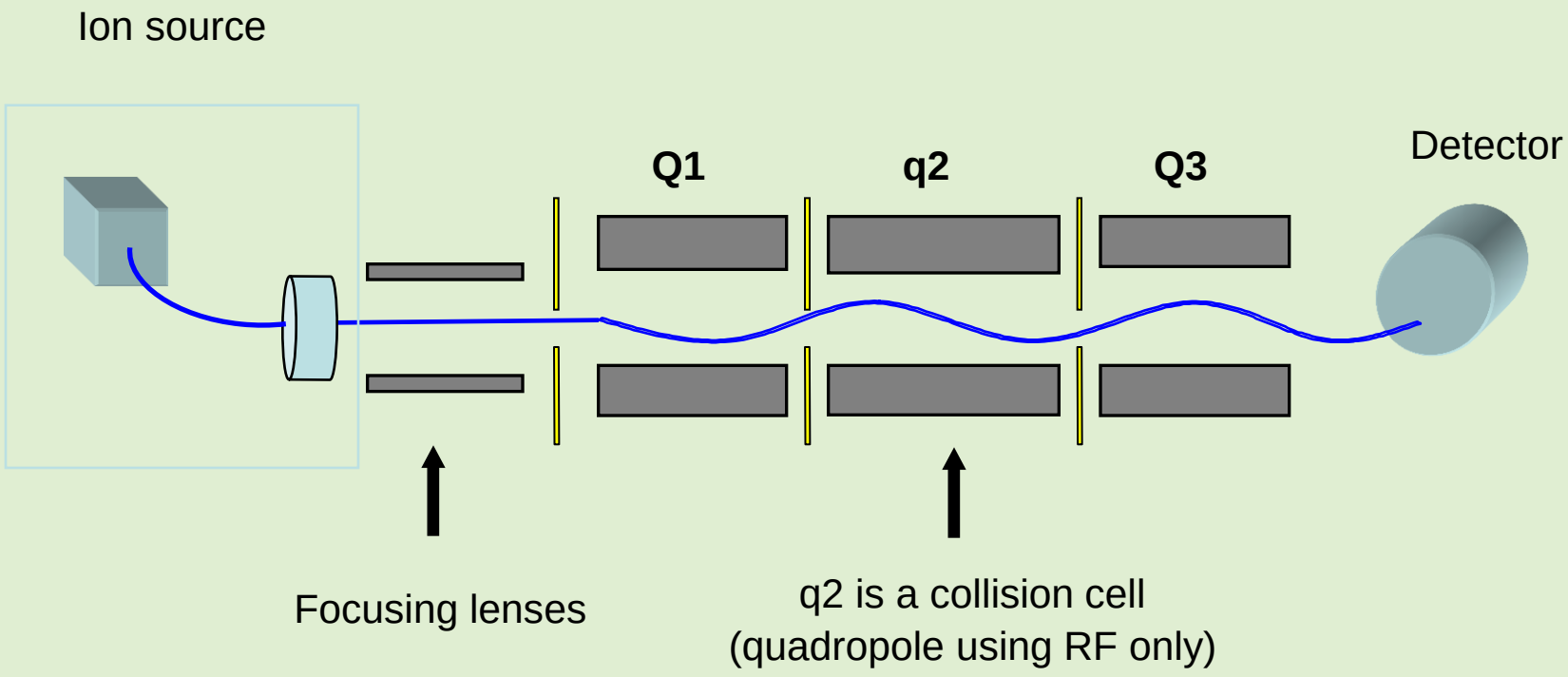


Quadrupole mass filter

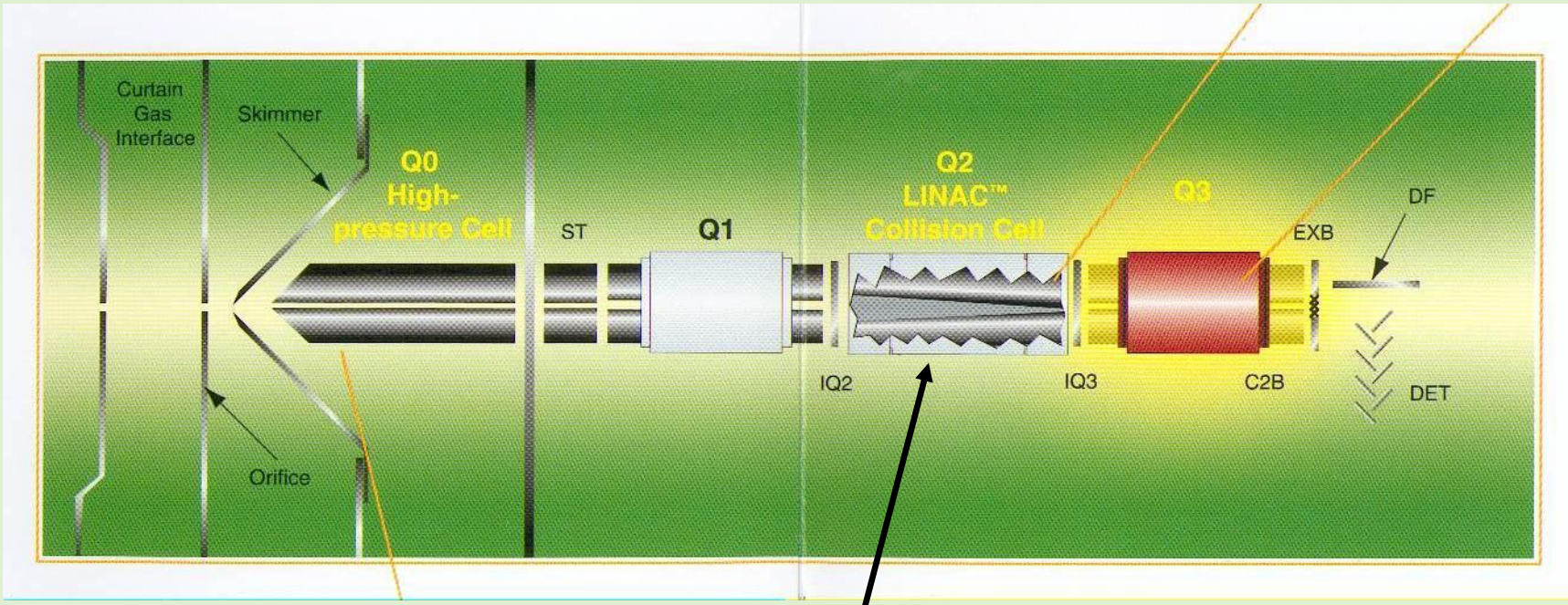


The ion is transmitted along the quadrupole in a stable trajectory Rf field. The ion does not have a stable trajectory and is ejected from the quadrupole.

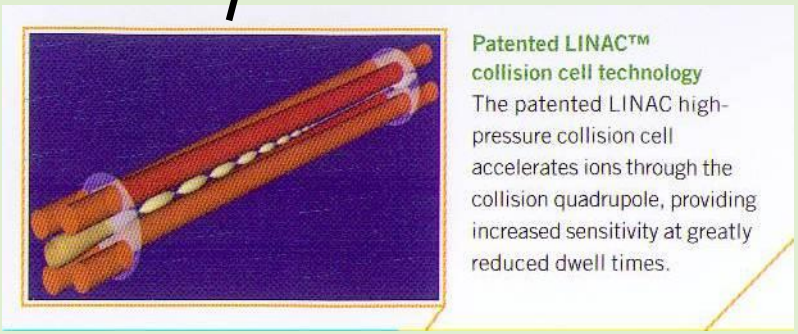
Schematic of a Triple Quadrupole Mass Spectrometer



Triple Quadrupole Mass Spectrometer



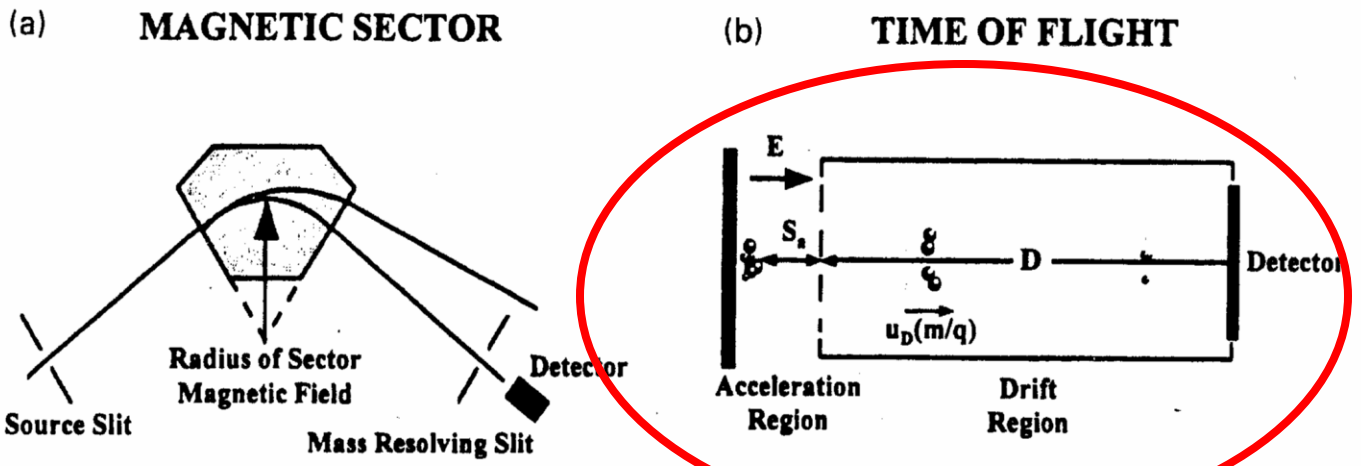
MDS Sciex

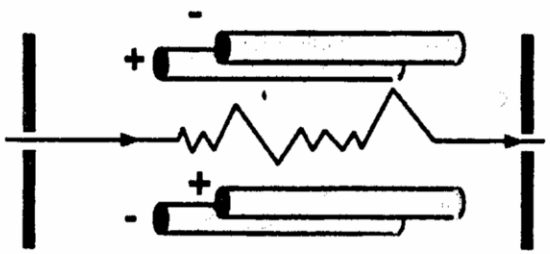


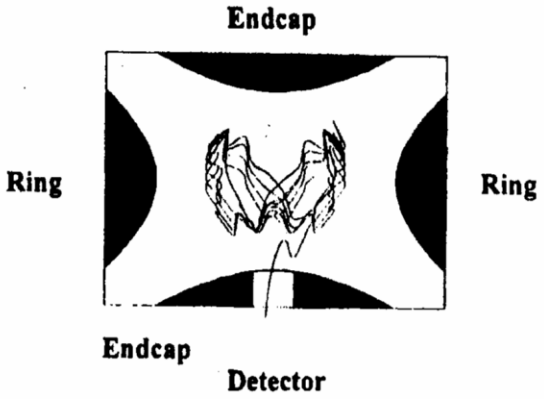
MS/MS-scan functions

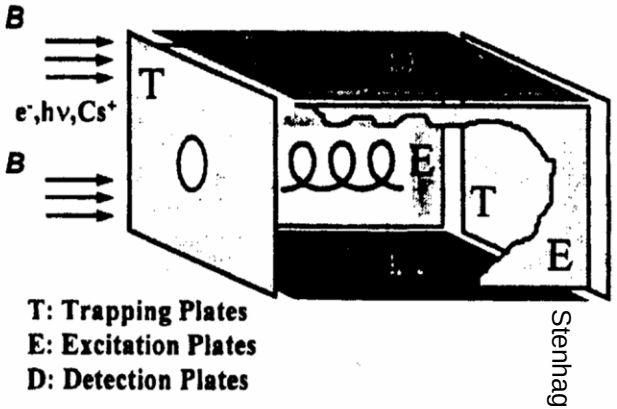
- **Daughter ion scan** – MS1 is set on parent ion mass and MS2 is scanned
- **Parent ion scan** – MS2 is set on daughter ion mass and MS1 is scanned
- **Neutral loss scan** – MS1 and MS2 are linked with an offset of the lost mass and both are scanned synchronized

Mass Analyzers

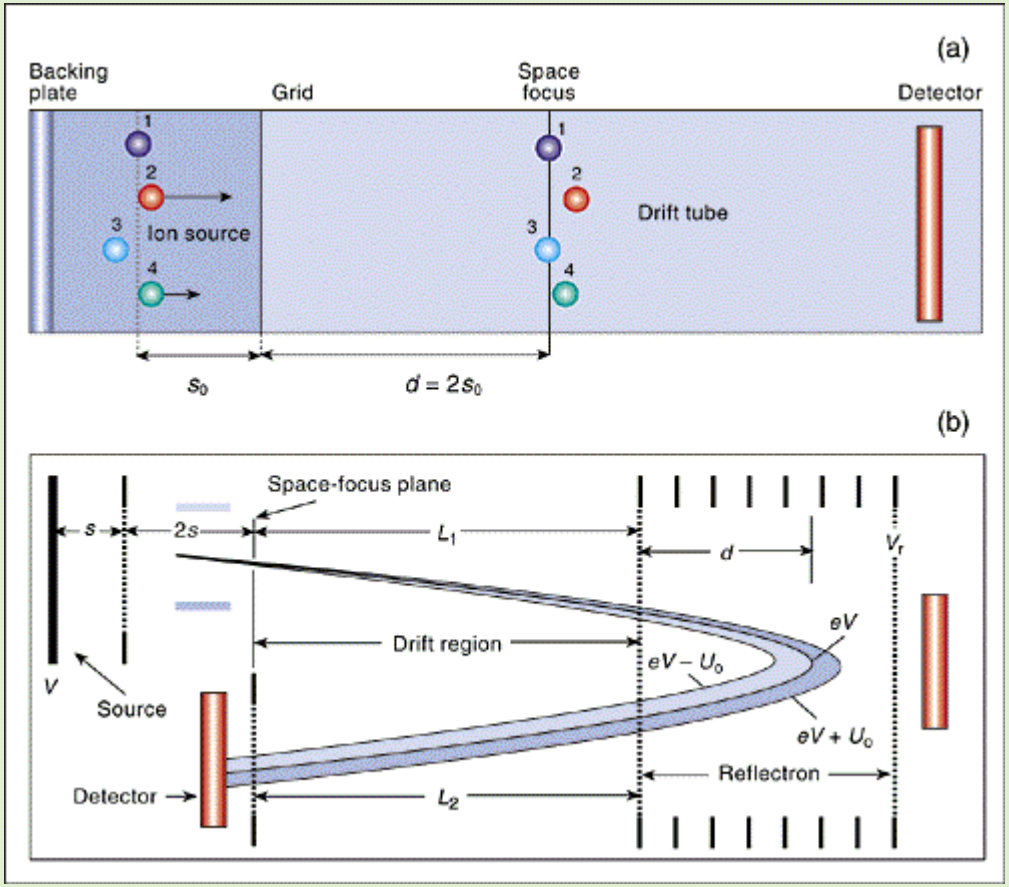








Time-of-flight mass spectrometry (TOF)



R J Cotter Anal Chem 1999, 445A

Linear TOF

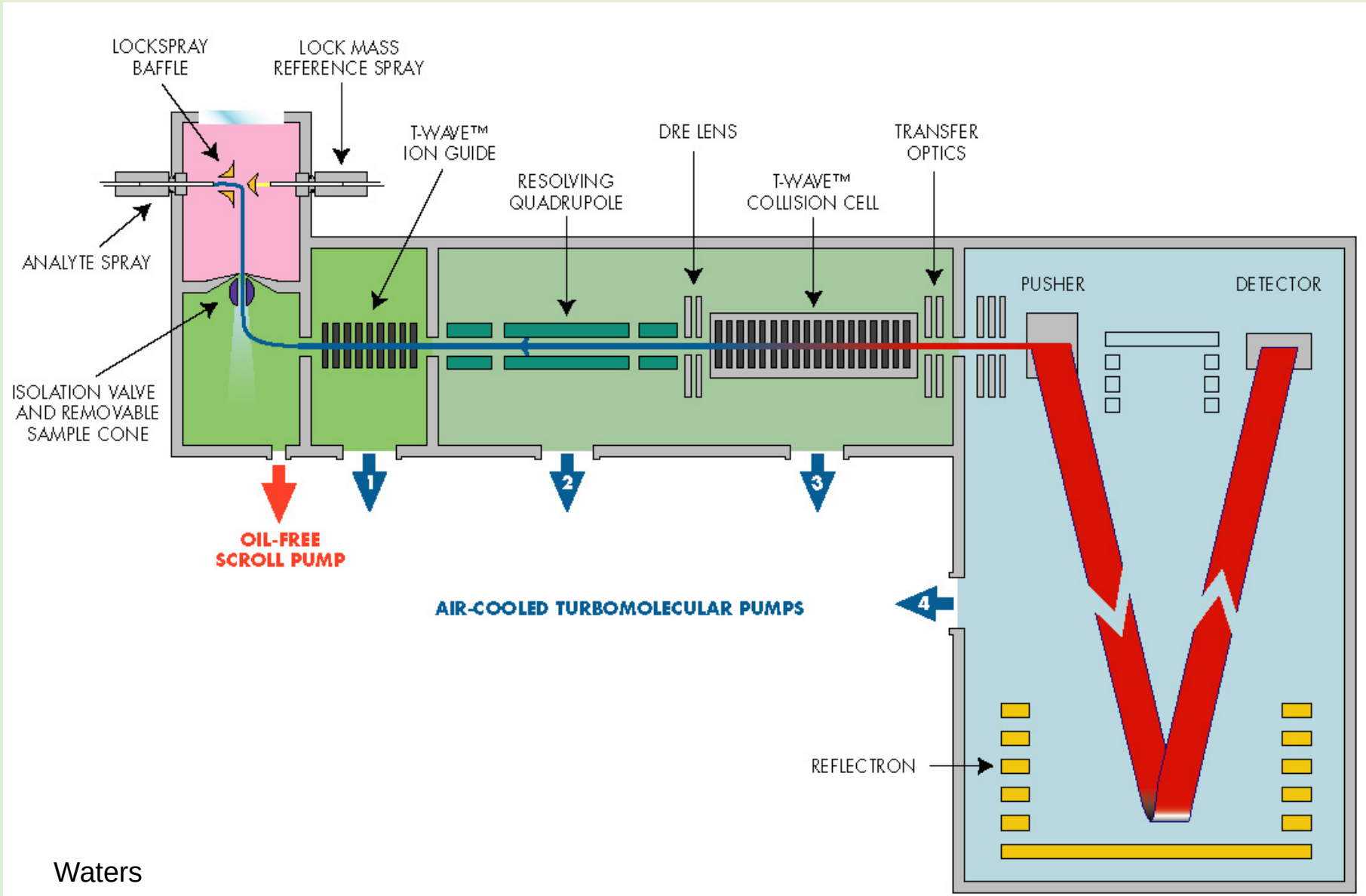
1. Bundles of ions are pulsed down the flight tube.
2. Ions have a velocity relative to their mass.
3. Their arrival time at the detector will be relative to their m/z.

Single-stage reflectron TOF

Time of flight (TOF) mass spectrometer

- High accuracy, 0.005 mass units, 100x better than quadrupole.
- High resolution, peak width 0.05 mass units which is 10x better than a quadrupole.
- High scan speeds
- High sensitivity, though lower than SRM in a quadrupole
- Dynamic range 10^3, about 10-100 times lower than for a triple quadrupole.

Q-Tof Premier ESI schematic



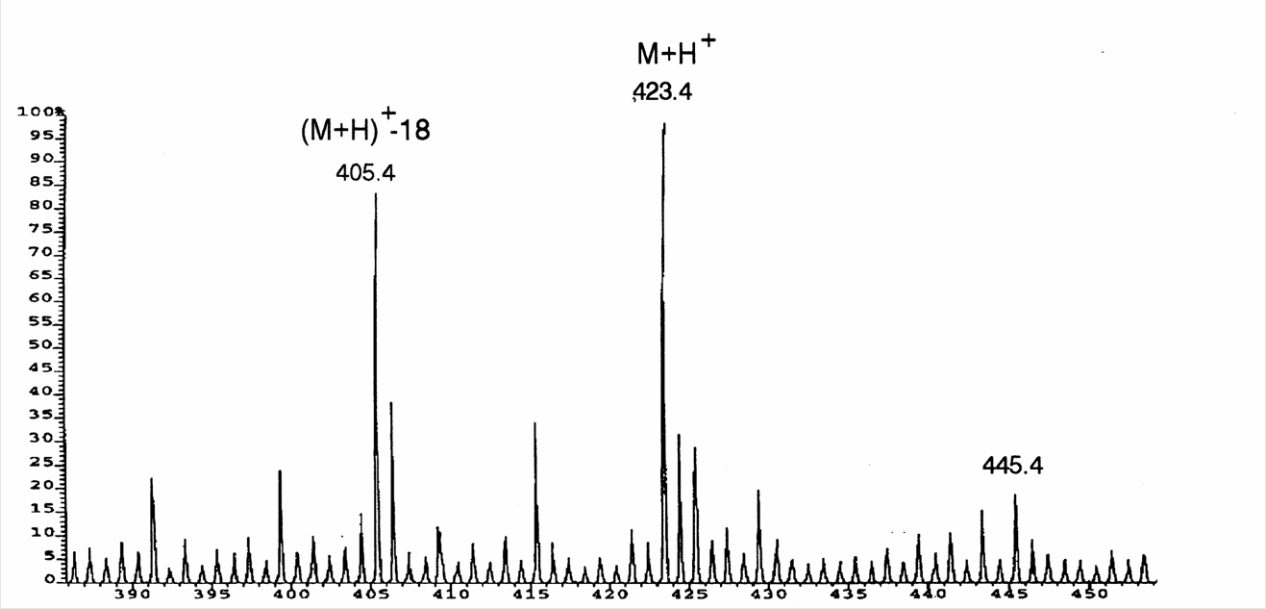
Waters

Attacked plants call the cops

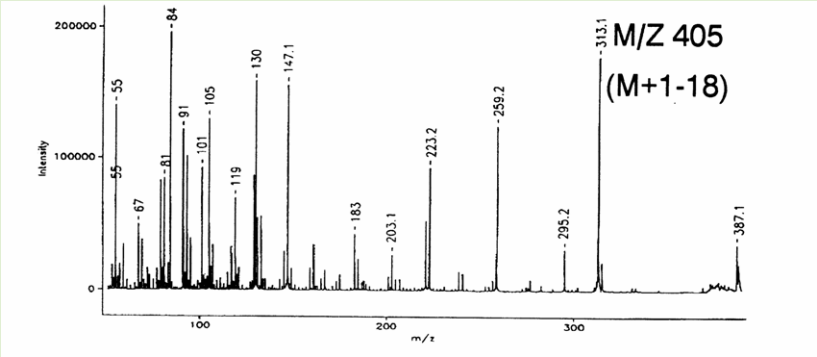
Isolation of An Elicitor of Plant Volatiles from Beet Armyworm Oral Secretion

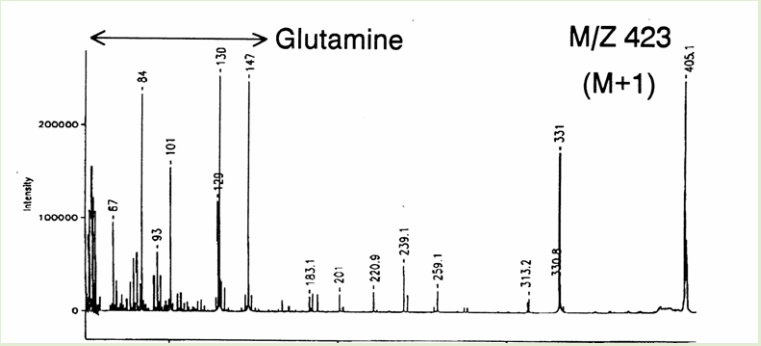
- Crude secretion was centrifuged
- Sterile filtering (0.22µm filter)
- Proteins precipitated and removed
- Solid phase extraction (RP18 column)
- Gradient HPLC (NP C18 column)
- Gradient HPLC (RP ODS column)
- Extraction into organic phase (methylene chloride/acetic acid/water)
- Solid phase extraction (Diol column)
- Isocratic HPLC (RP ODS column)

FAB/MS of active compound



FAB/MSMS Daughter ion mass spectra





Determination of glutamine

Determination of glutamine by adding 10µl aceticanhydride to 100 µl of oral secretion. GC/MS with chemical ionization of the product gave m/z 144 (M+H). GC/MS with EI of the same product gave m/z 143, 84, 56 and 41 and was identified as the methyl ester of pyroglutamate showing the presents of glutamine

Determination of methylester of hydroxy acetic acid

The same GC/MS analyze also show a second peak in the gas chromatogram. The corresponding mass spectrum gave m/z 309 (M) and 291 (M-18) with CI. No molecular ion with EI but M-18 and a fragment pattern corresponding to a straight hydrocarbon

Elemental composition

Elemental Composition

File Edit View Process Help

Single Mass Analysis

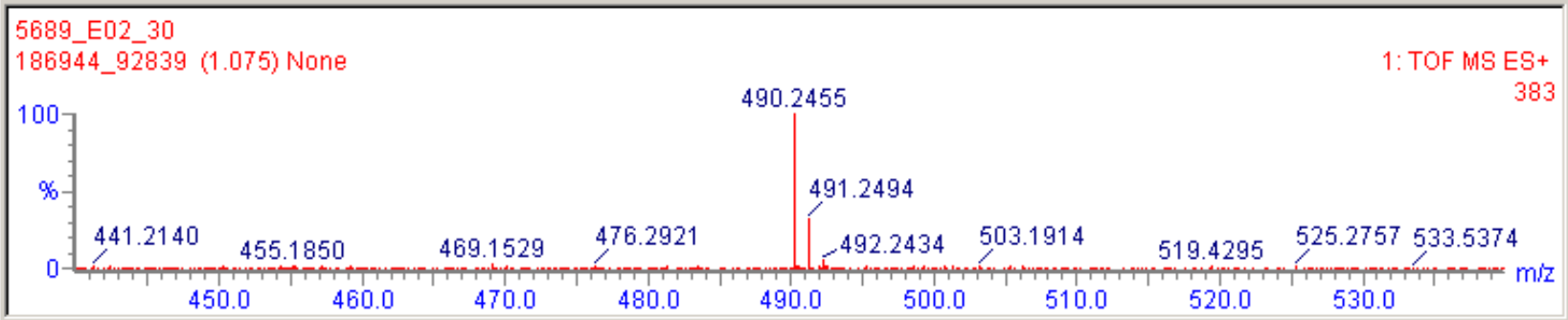
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0

Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions

461 formula(e) evaluated with 10 results within limits (up to 50 closest results for each mass)

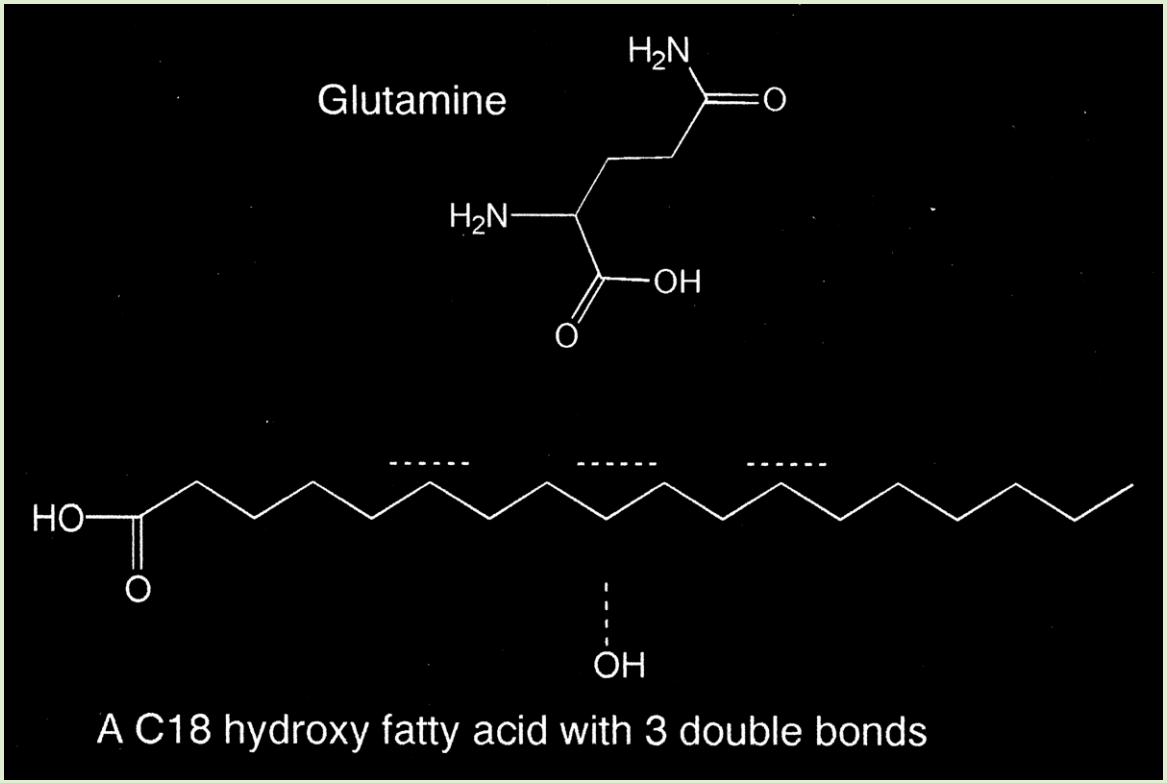
Mass	Calc. Mass	mDa	PPM	DBE	Formula	Score	C	H	N	O
490.2455	490.2454	0.1	0.1	14.5	C27 H32 N5 O4	1	27	32	5	4
	490.2468	-1.3	-2.6	14.0	C29 H34 N2 O5	3	29	34	2	5
	490.2441	1.4	2.9	9.5	C26 H36 N O8	4	26	36	1	8
	490.2441	1.4	2.9	15.0	C25 H30 N8 O3	2	25	30	8	3
	490.2481	-2.6	-5.3	19.0	C30 H30 N6 O	5	30	30	6	1
	490.2427	2.8	5.6	10.0	C24 H34 N4 O7	6	24	34	4	7
	490.2495	-4.0	-8.1	18.5	C32 H32 N3 O2	7	32	32	3	2
	490.2414	4.1	8.3	10.5	C22 H32 N7 O6	8	22	32	7	6
	490.2500	-4.5	-9.1	6.0	C18 H34 N8 O8	10	18	34	8	8
	490.2409	4.6	9.4	23.0	C36 H30 N2	9	36	30	2	



For Help, press F1

StenhagenAnalyslab AB

Accurate mass measurement gave elemental composition $C_{23}H_{38}N_2O_5$. Subtraction of glutamine linked via an ester or amide bond gave $C_{18}H_{30}O_3$ as the elemental composition for the second part of the molecule.



Location of the hydroxyl group. Pyrrolidide derivatives of the fatty acid methyl esters were prepared by dissolving a sample in 10 µl of 1% glacial acetic acid in freshly distilled pyrrolidine and heating to 100°C for 30 min in a sealed tube (Anderson, 1978). The product was cooled to room temperature, 10 µl of CH_2Cl_2 were added, and the product analyzed by GC-MS. This derivative significantly refine the characteristic hydrocarbon MS fragmentation pattern of long chain fatty acids and was originally developed to determine the location of double bonds in the chain. It also resulted in increased intensity of diagnostic ions for the location of hydroxyl groups.

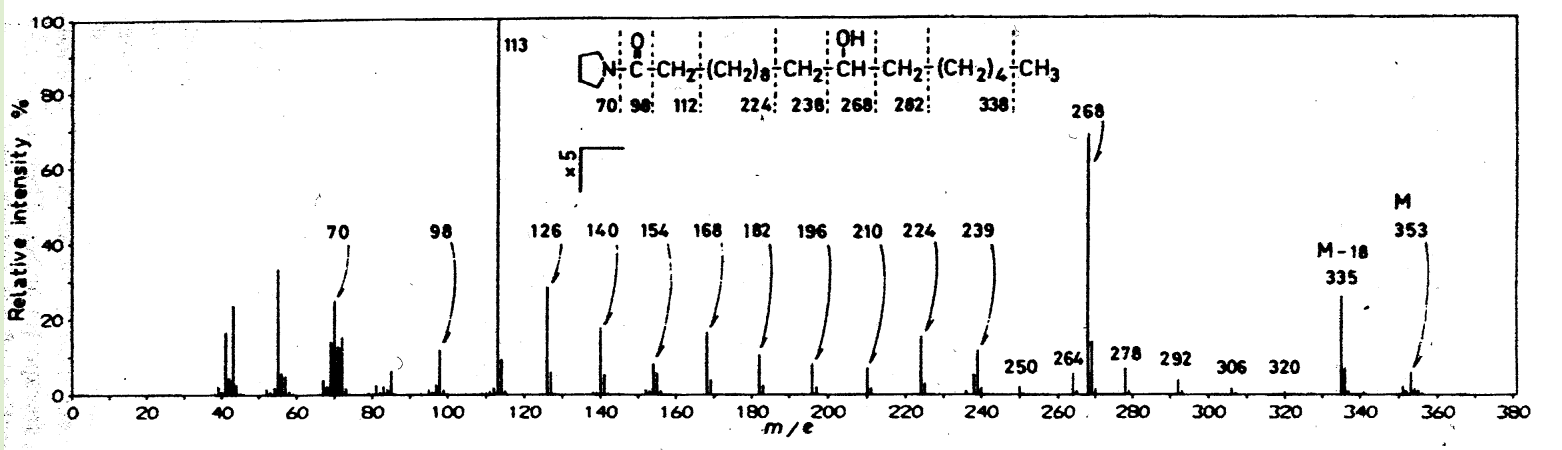


FIG. 20. Mass spectrum of N-12-hydroxy-octadecanoylpyrroline. LKB 9000, 70 eV, direct inlet.

A HPLC method to analyze the acidic components of the BAW oral secretion.

To 1 ml of filter sterilized oral secretion 100 µl acetic acid and 2 ml of CH_2Cl_2 were added. The solution was shaken for 5 min, and the organic phase was evaporated to dryness under vacuum. One milliliter of 50 mM (pH 8) sodium phosphate buffer was added and 10 µl of the solution was analyzed on HPLC with the ODS-AQ S-5 column. UV detection at 200 nm.

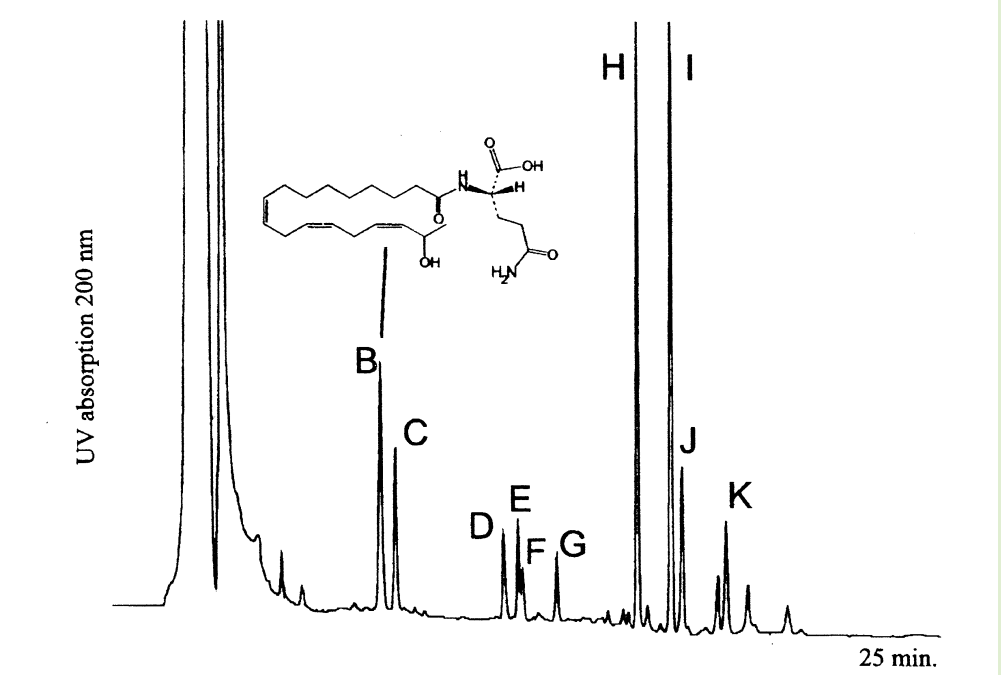


FIG. 3. HPLC separation of acidic components in BAW oral secretion. The YMC ODS-AQ S-5 column (4.6 mm ID × 250 mm long) was eluted at a flow of 1 ml/min with a gradient 40–100% solvent B in A in 10 min and then 100% B for 15 min. A = 0.05% aqueous acid, B = 0.05% acetic acid in acetonitrile. The column eluent was monitored by UV detection at 200 nm. **B**: Volicitin, (*N*-[17-hydroxylinolenoyl]-L-glutamine), **C**: *N*-[17-hydroxylinoleoyl]-L-glutamine, **D**: 17-hydroxylinolenic acid, **E**: *N*-linolenoyl-L-glutamine, **F**: 17-hydroxylinoleic acid, **G**: *N*-linoleoyl-L-glutamine, **H**: linolenic acid, **I**: linoleic acid, **J**: unknown unsaturated C_{18} acid, **K**: oleic acid.

The End