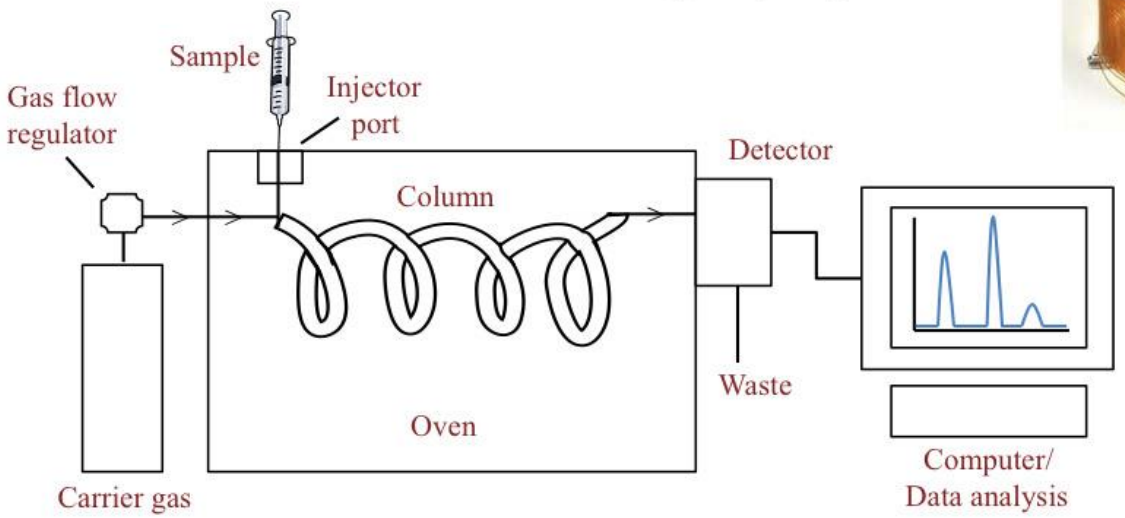
A folyadékkromatogáfia alapjai
Klinikai folyadékkromatográfiás vizsgálatok

Dr. Karvaly Gellért Balázs

Semmelweis Egyetem Laboratóriumi Medicina Intézet

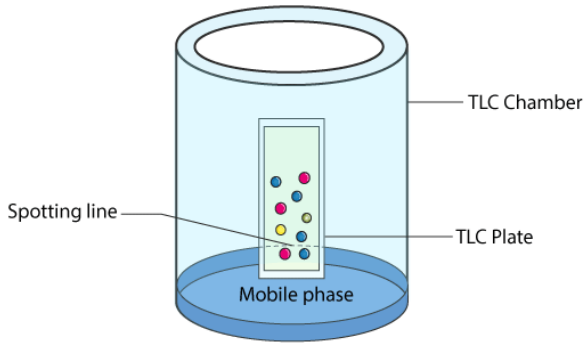
Tömegspektrometriai és Elválasztástechnikai Laboratórium

Gas Chromatography

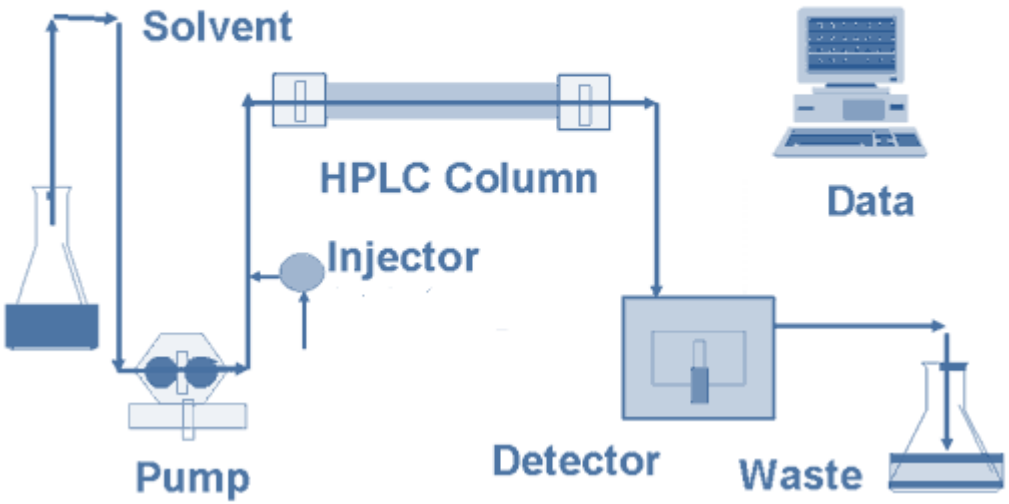


THIN LAYER CHROMATOGRAPHY

BYJU'S The Learning App



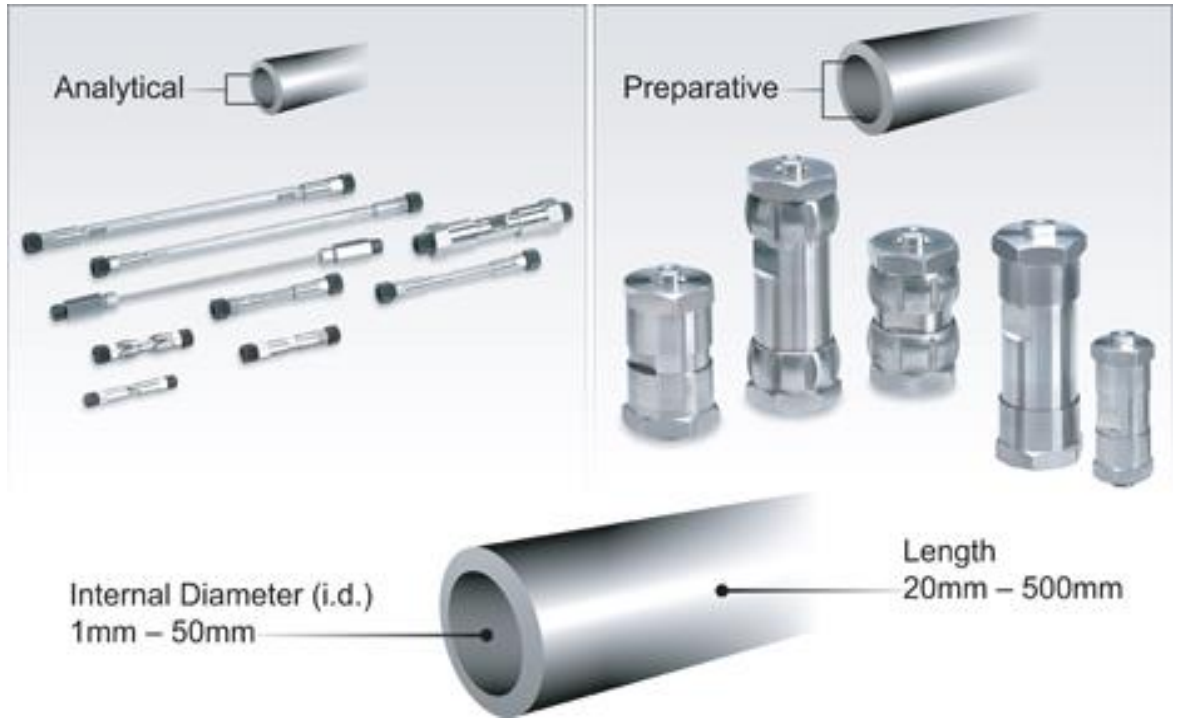
HPLC System

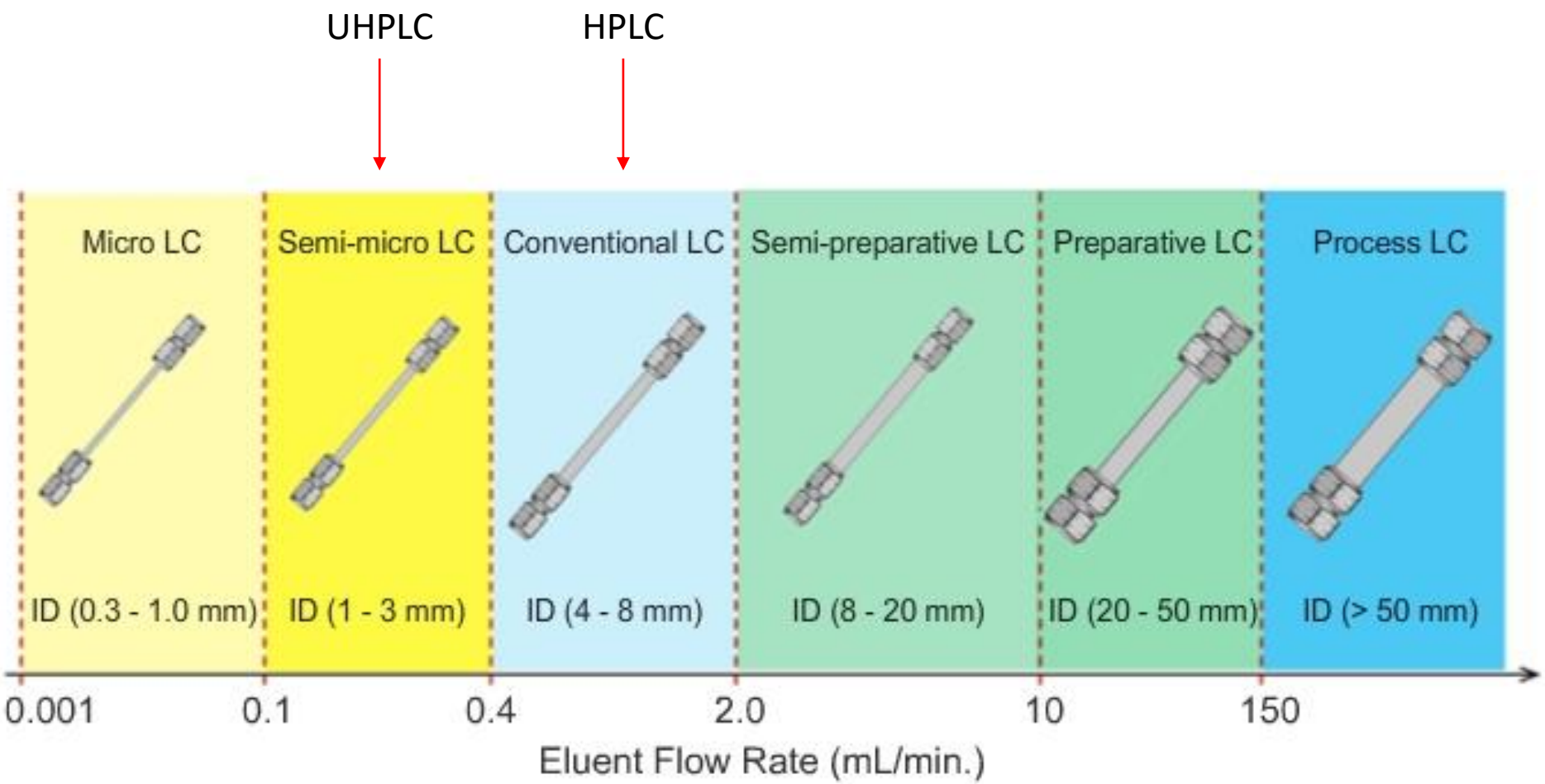


HPLC dimensions

www.waters.com

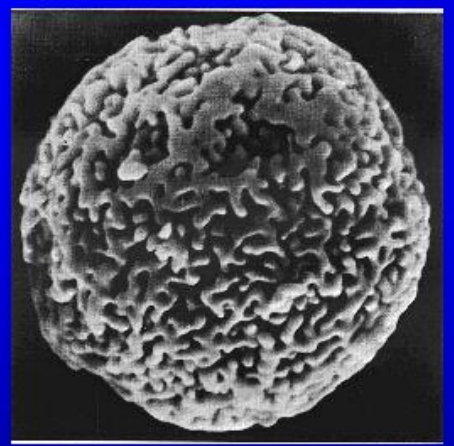
column ID	analytical	semi-prep	preparative	process
1-8 mm	X			
10-40 mm		X		
50-100 mm			X	
>100 mm				X
particle size (µm):	0.1-10	5-15	15-100	100+



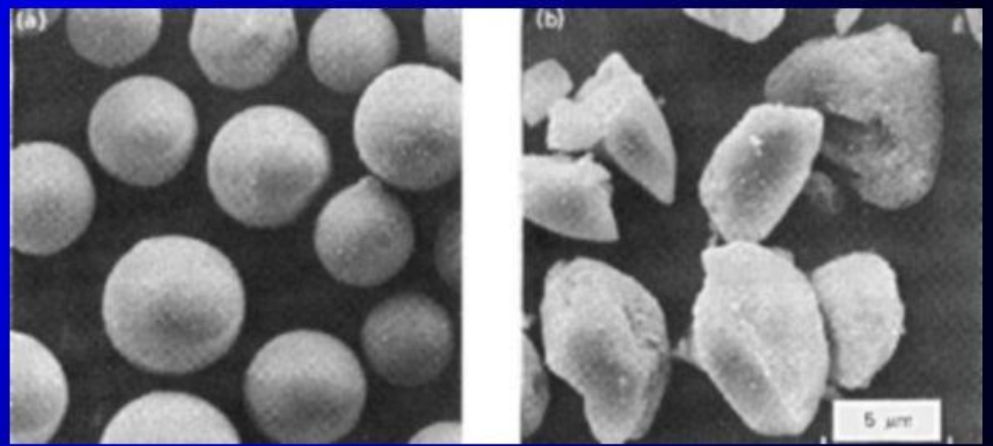

UHPLC
HPLC
Micro LC
Semi-micro LC
Conventional LC
Semi-preparative LC
Preparative LC
Process LC
ID (0.3 - 1.0 mm)
ID (1 - 3 mm)
ID (4 - 8 mm)
ID (8 - 20 mm)
ID (20 - 50 mm)
ID (> 50 mm)
0.001
0.1
0.4
2.0
10
150
Eluent Flow Rate (mL/min.)

The Most Popular Particle: Silica

- Different morphology for different applications:

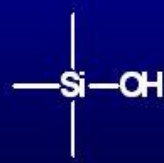


Macroporous spherical silica particle. [K.K.Unger, Porous silica, Elsevier, 1979]

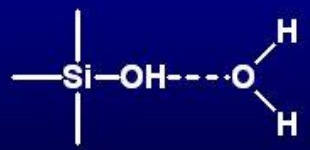


Electron microphotograph of spherical and irregular silica particles. [W.R.Melander, C.Horvath, Reversed-Phase Chromatography, in HPLC Advances and Perspectives, V2, Academic Press, 1980]

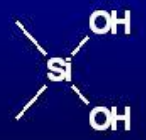
- Different chemistry:



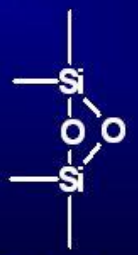
Free Silanol



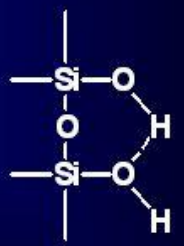
Adsorbed Water



Geminal Silanol



Dehydrated Oxide Siloxane



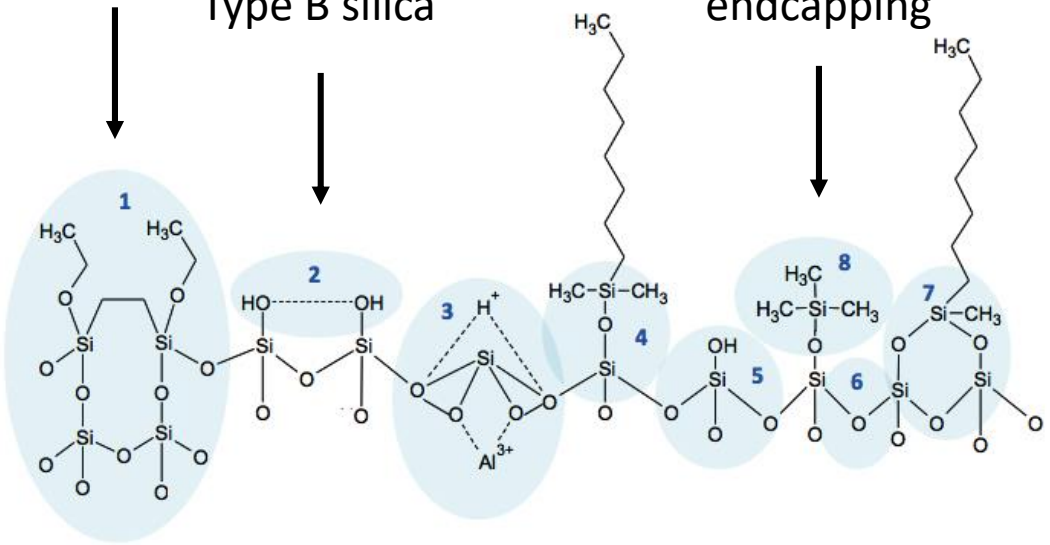
Bound and Reactive Silanols

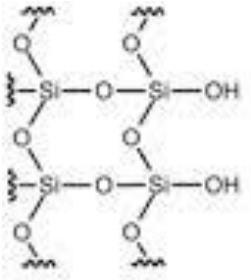
Silica-based stationary phases

hybrid silica

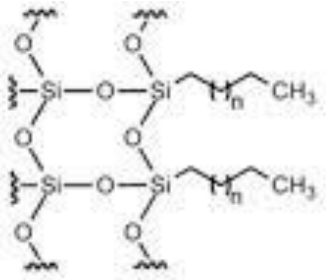
Type B silica

endcapping

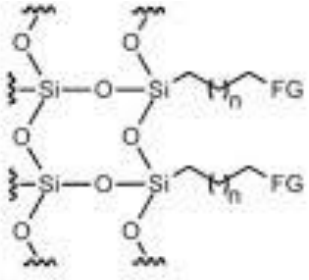




Normal phase silica



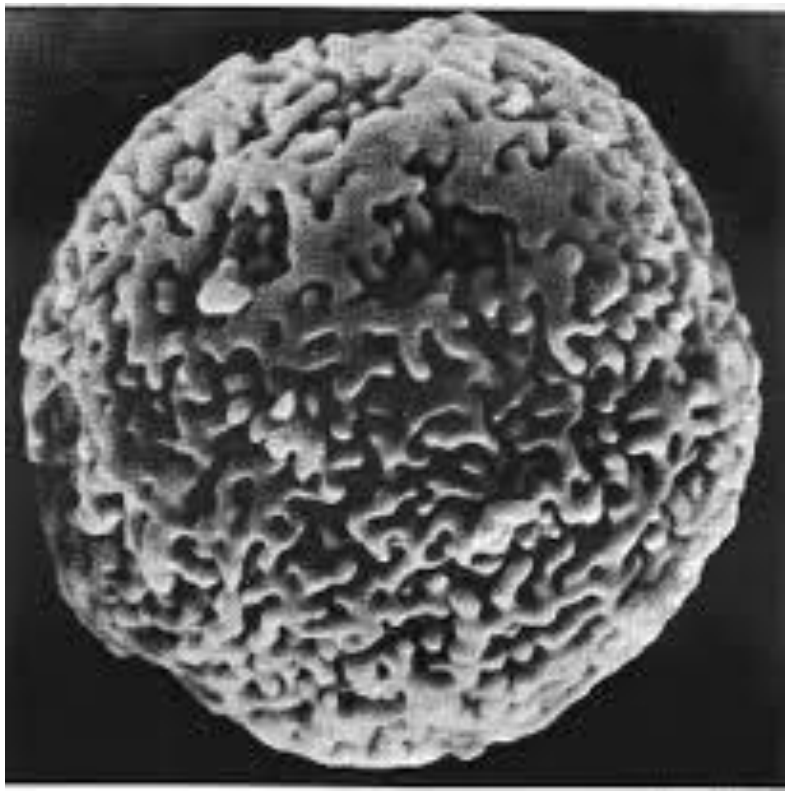
Reversed phase silica



Functionalized silica

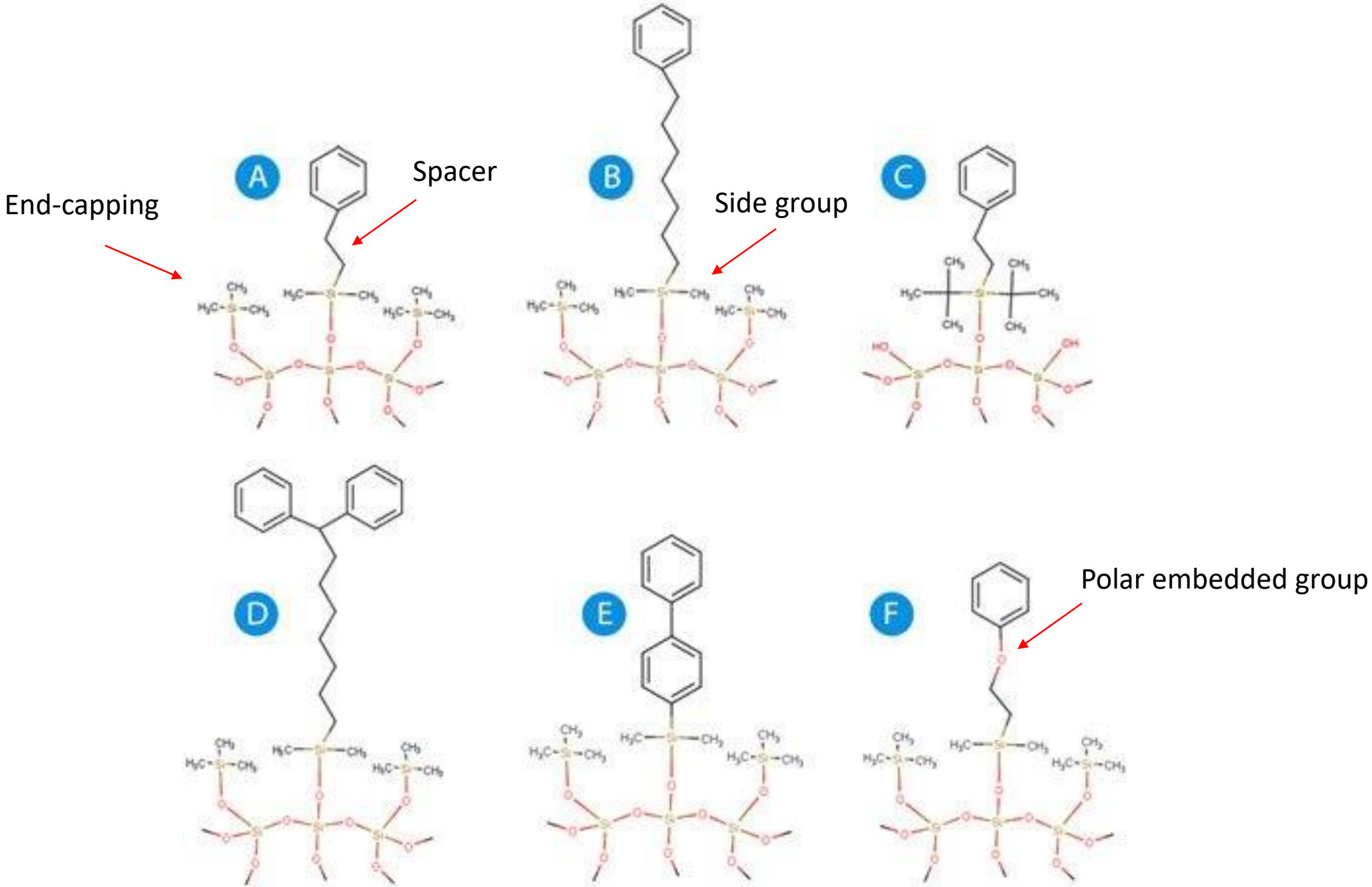
FG = $-NH_2$
$-CN$

d_p = 1-10 µm



density of –OH groups: 8-9 µmol/m^2

surface coverage of bonded phase: 40-50% of silanol groups


End-capping
A
Spacer
B
Side group
C
D
E
F
Polar embedded group

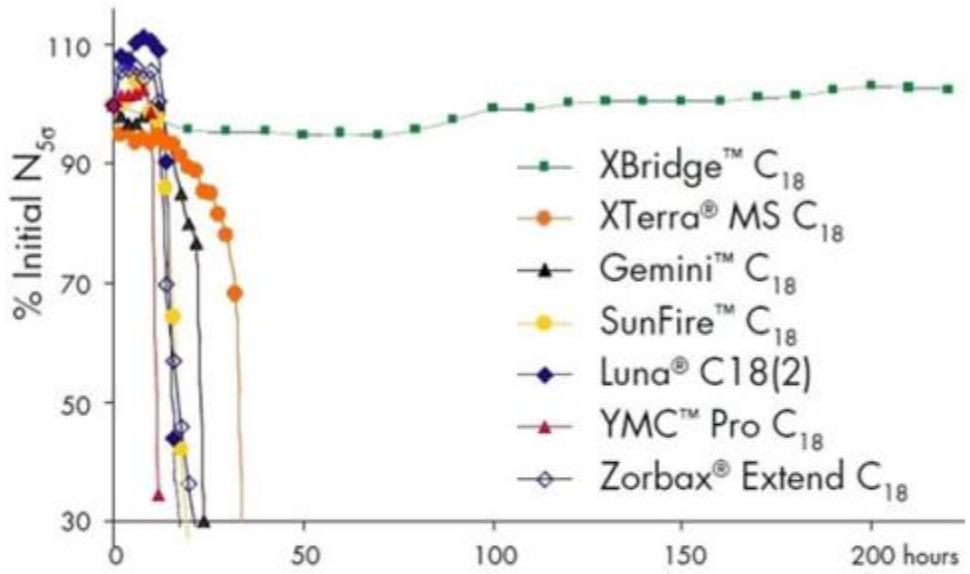


Figure 1. Stationary phase stability: efficiency loss as a function of time of purge with an aggressive mobile phase (analyte: acenaphthene; mobile phase: triethylamine, pH 10; 50 mM at 50°C). Figure reprinted with permission from reference (16).

phase as determined by gradient elution chromatography using acetonitrile/ammonium carbonate (pH 10, 10 mM) at 40°C for 8,000 min (Figure 3) (21). Phenomenex evaluated its hybrid sta-

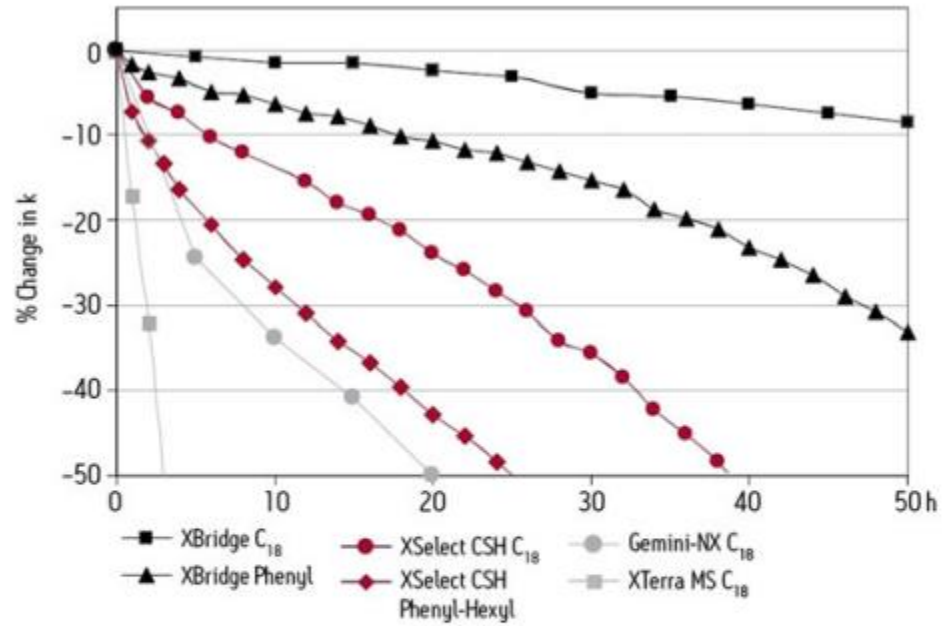
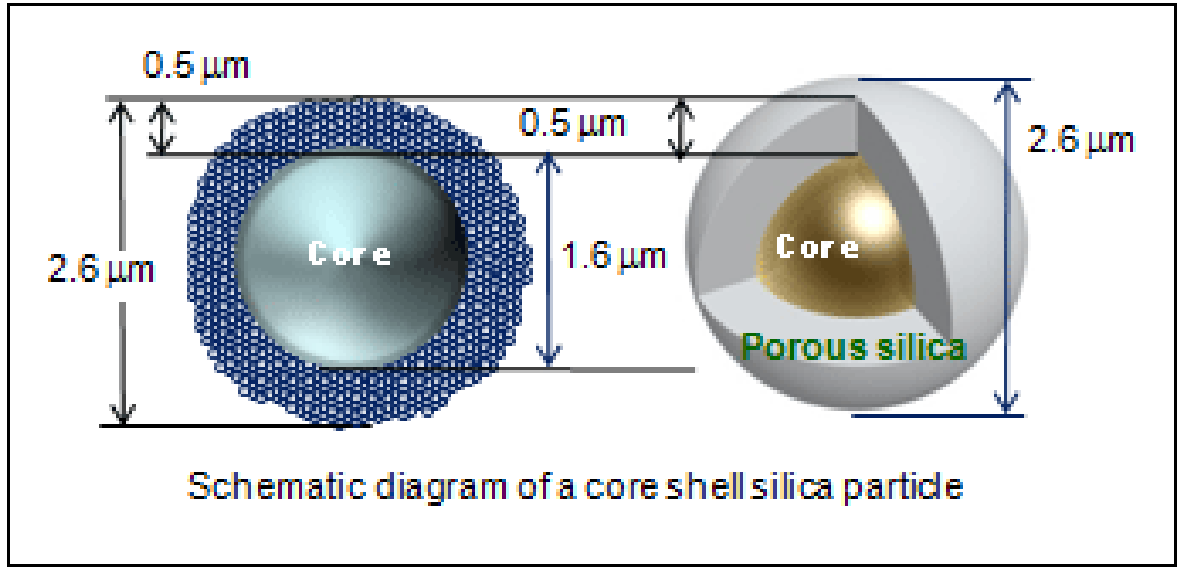
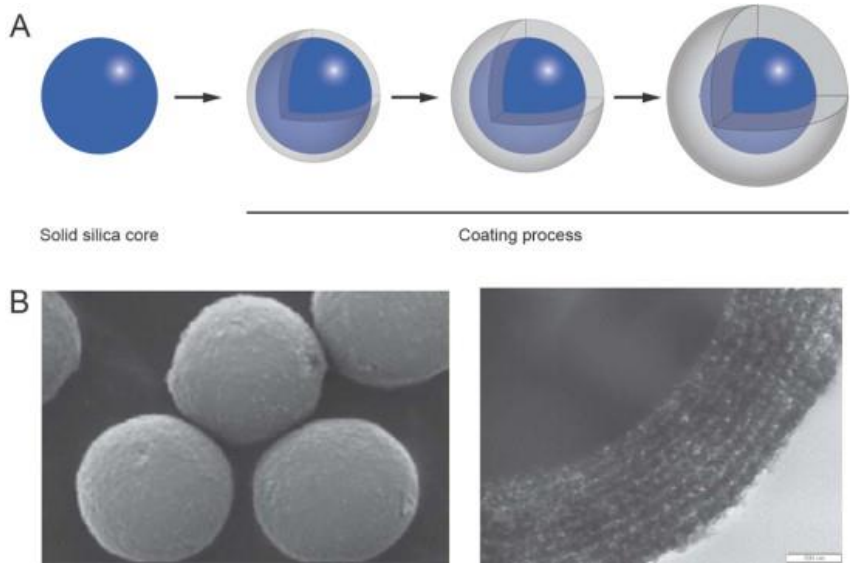


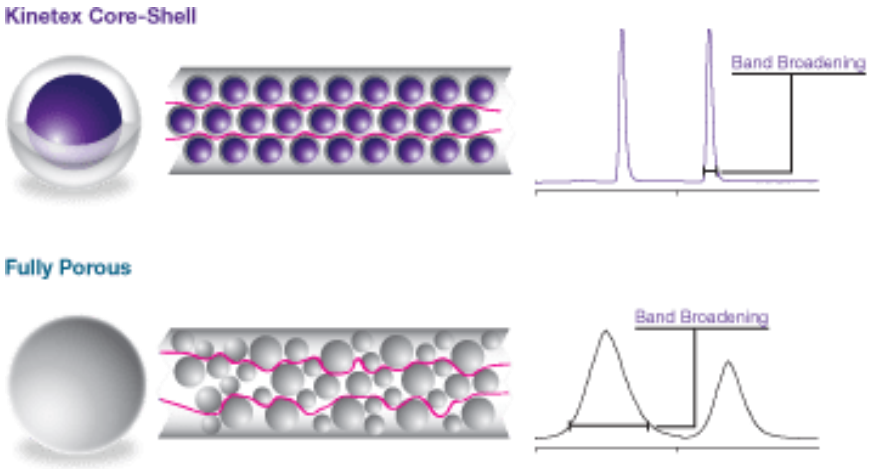
Figure 2. Results of accelerated base stability testing for six stationary phases, showing percent change of retention factor (*k*) for decanophenone versus exposure time (h) to aqueous sodium hydroxide (20 mM; pH 12.3) at 50°C. The stationary phases were purged at 0.85 mL/min for 1.8 h, washed for 10 min at 0.43 mL/min. Mobile phase: acetonitrile/water, 50:50 v/v. Columns: XTerra MS C18 (50 × 3 mm), Gemini C18 and Xbridge C18 (50 × 4.6); all other columns 30 × 3 mm. Figure reprinted with permission from reference (17).

Borges EM, Volmer DA. J Chrom Sci 2015;53:1107-22.



Schematic diagram of a core shell silica particle



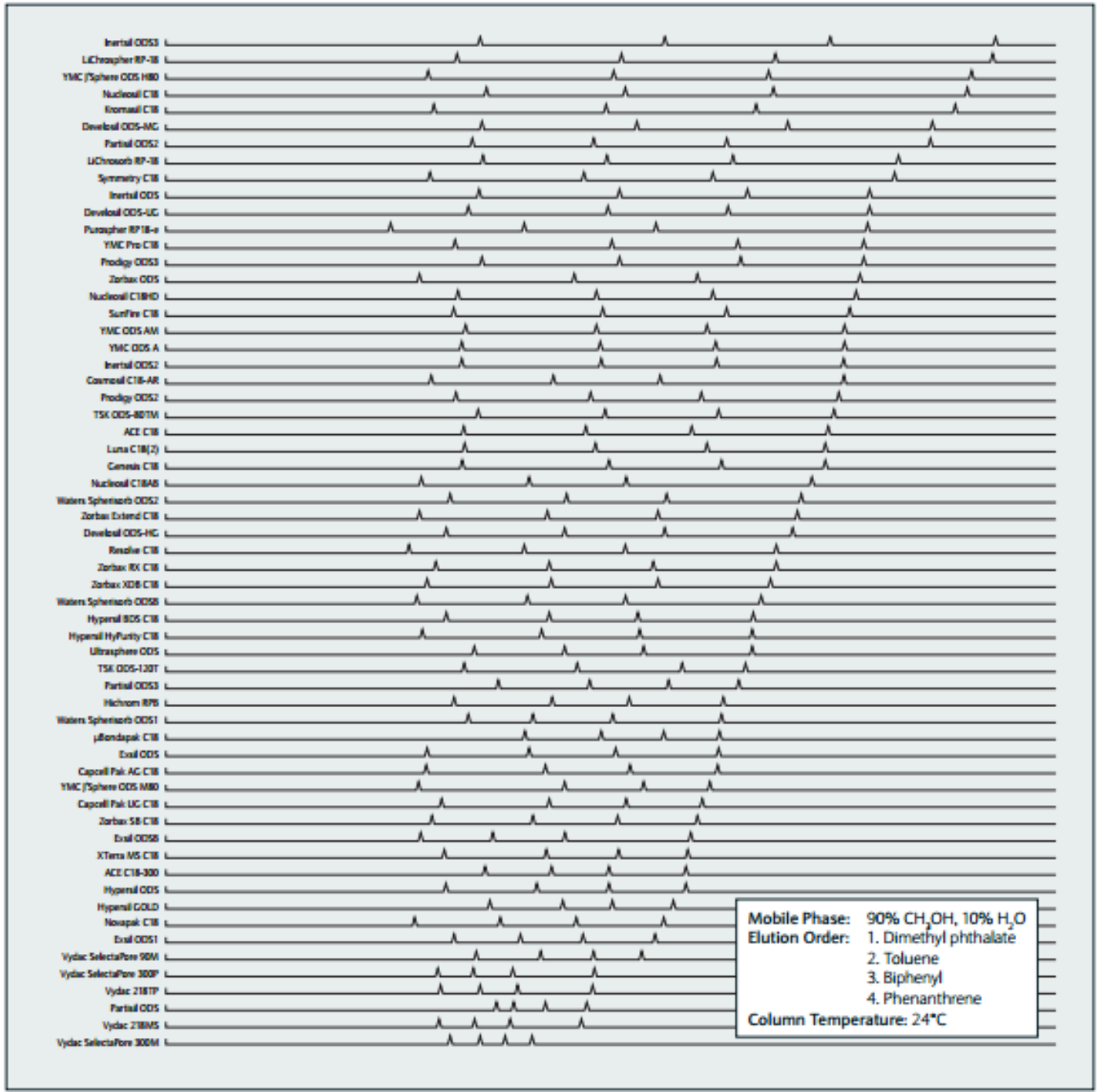


Fully Porous		Kinetex Core-Shell	Average Efficiency Gain with Kinetex*
5 µm	vs	5 µm	90 % Higher
3 µm	vs	2.6 µm	85 % Higher
1.7 µm	vs	1.7 µm	20 % Higher
1.7 µm	vs	1.3 µm	50 % Higher

* May not be representative of all separations.

Comparison Guide to C18 reversed phase HPLC columns. 4th ed. MacMod Analytical, 2008.
http://www.mac-mod.com/pdf/technical-report/036-ColumnComparisonGuide.pdf

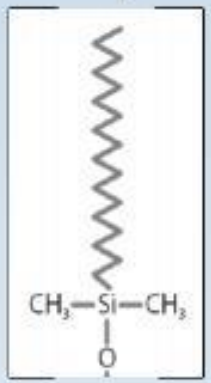
C18 Phases Compared According to Relative Hydrophobicity



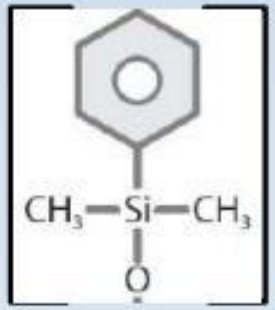
Common Reversed Phase Stationary Phase Ligands

Non Polar — Polar

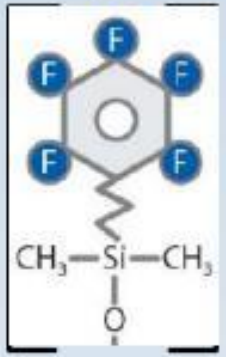
normal phase separations



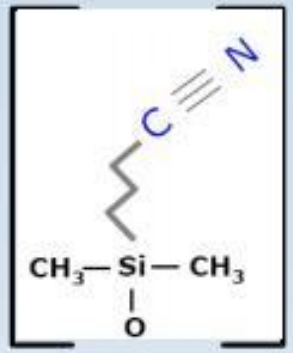
Alkyl



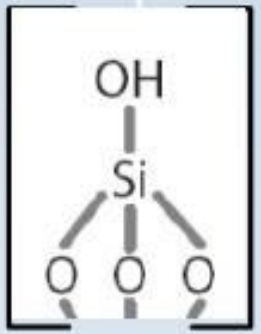
Phenyl



Fluoro



Cyano



Silica

Dispersive — **Pi-Pi Interactions** — **Electrostatic / Dipole** — **H Bonding**

reversed phase separations

RESTEK Chromatography Products
www.restek.com

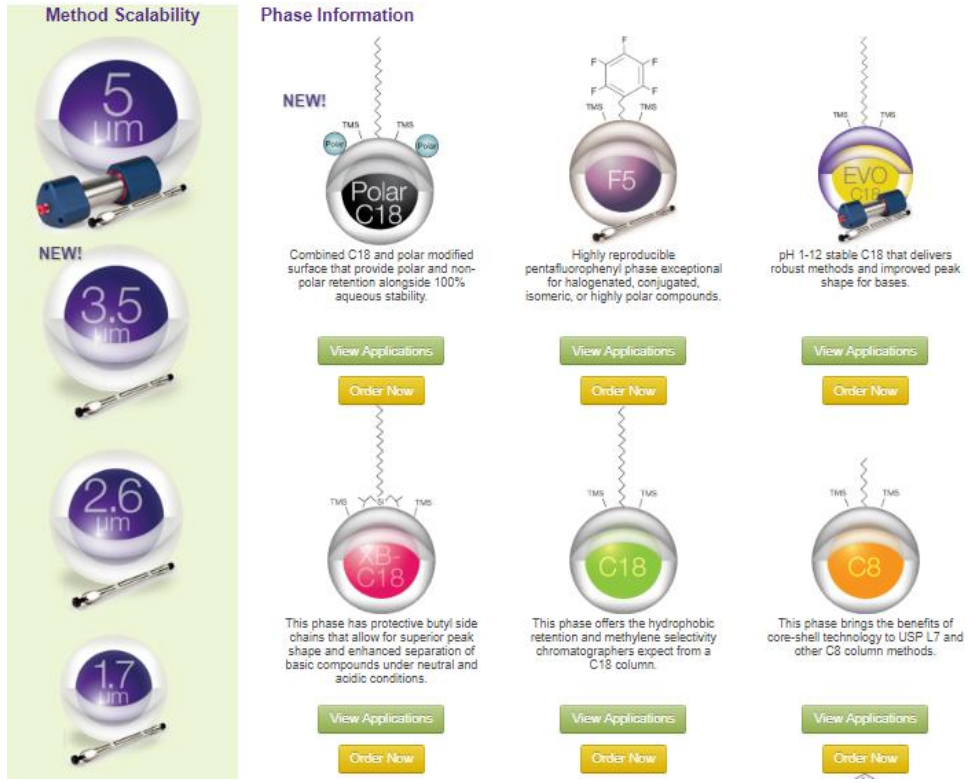
Choosing the mobile phase

- **The choice of mobile phase(s) depends on a number of factors:**
  - Type of separation (reversed phase/HILIC/normal phase/ion chromatography)
  - Type of detector
  - Chemical properties of the analyte: polarity, pKa, presence of aromatic rings and heteroatoms, steric properties

- **Basic types of HPLC separations:**
  - Isocratic: no change in mobile phase
    - No equilibration time
    - Lower solvent consumption
    - Fewer sources of error
    - Lower risk of losing reproducibility
  - Gradient: programmed changes in mobile phase compositions
    - Shorter runs
    - Longer column lifetime
    - Requires fast re-equilibration of detector → not used with electrochemical detection

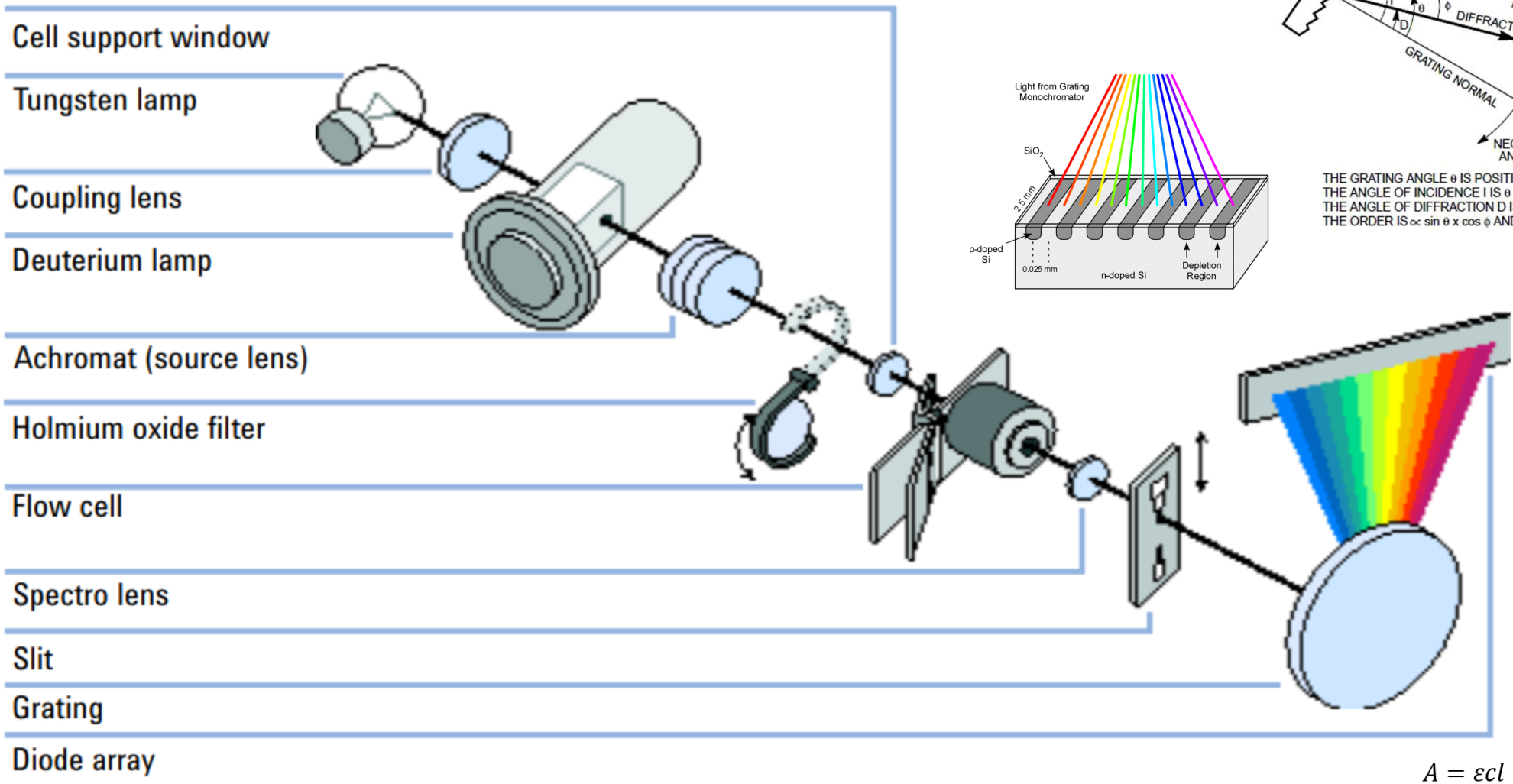
What can the chromatographer do to enhance selectivity, specificity and sensitivity?

- select the instrument configuration
- select the stationary phase
- select the mobile phases
- select the separation settings
  - analytical column dimensions
  - column space temperature
  - mobile phase gradient program
  - mobile phase flow rate
- select the detection settings



Classification of detectors

- Bulk-property detectors: refractive index
- Analyte specific detectors: absorbance, fluorescence, electrochemical, conductivity, radioactivity
- Mobile phase modification detectors: evaporative light scattering, corona discharge
- Hyphenated techniques: mass spectrometry, infrared spectroscopy, nuclear magnetic resonance spectroscopy



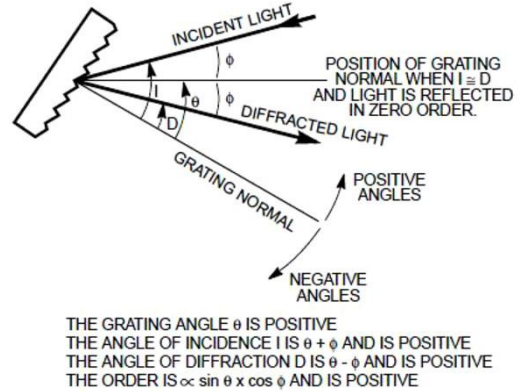
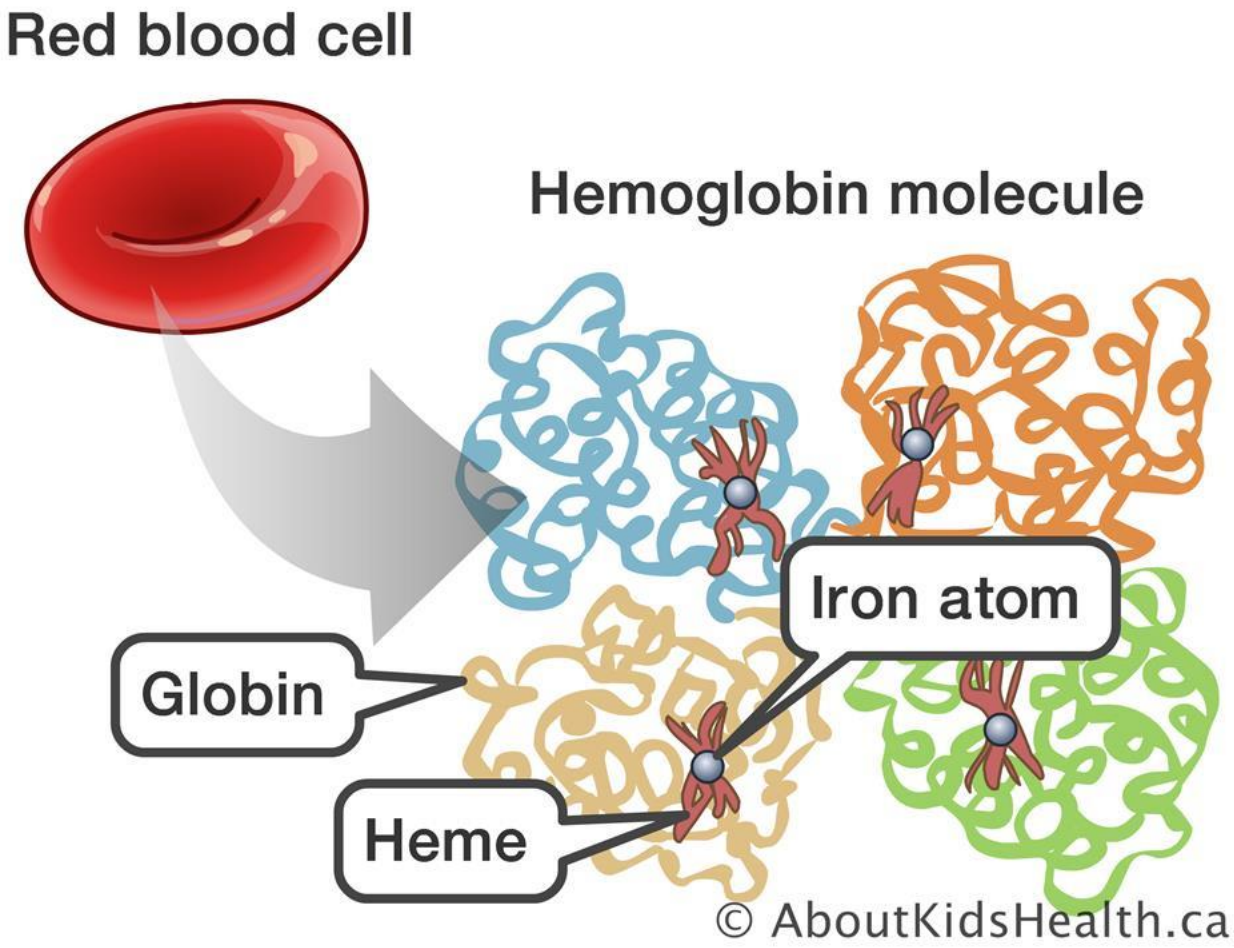


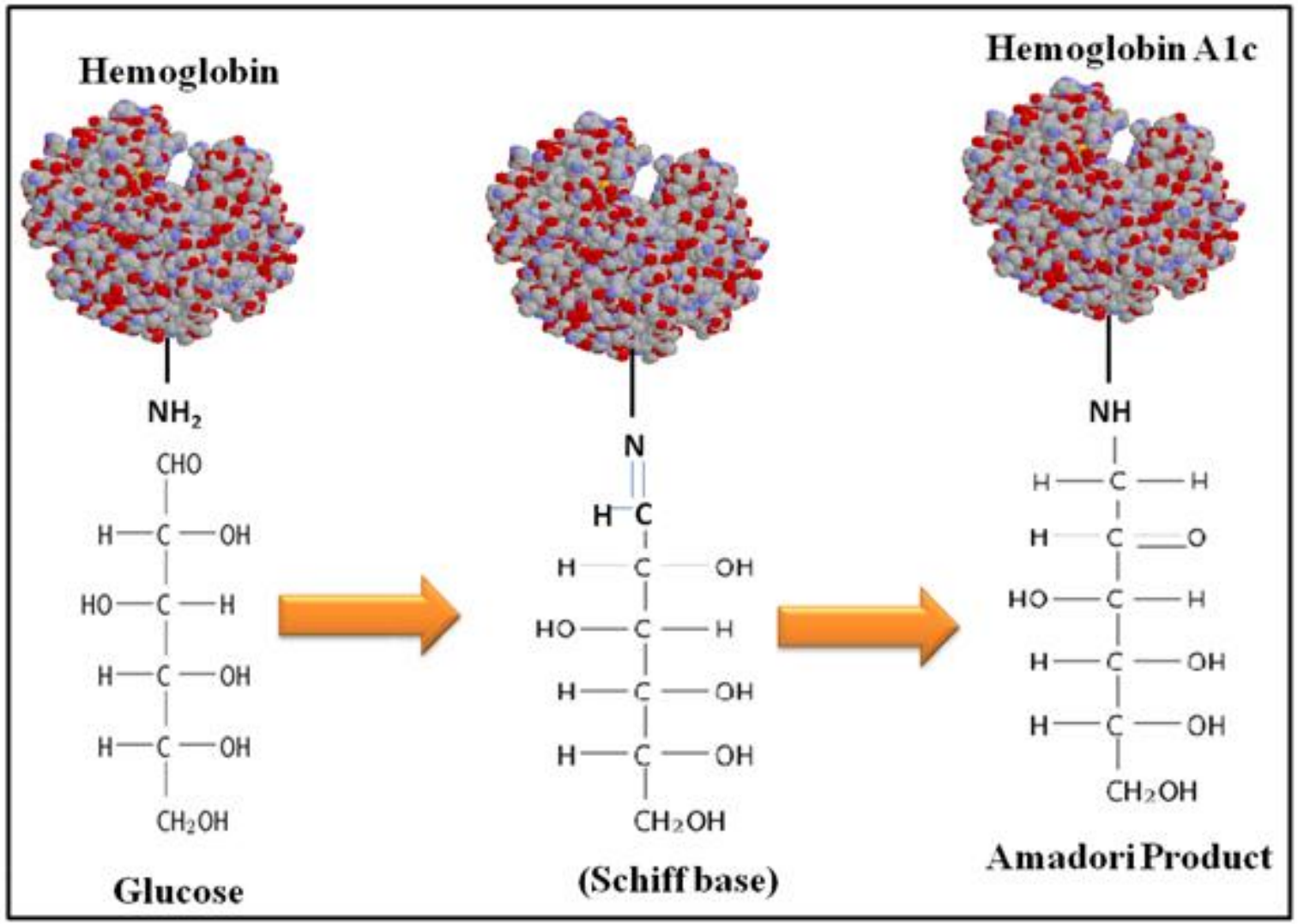
Figure 3 Optical System of the Detector

$$A = \varepsilon c l$$

A	Absorbance	
ε	Molar absorption coefficient	$M^{-1}cm^{-1}$
c	Molar concentration	M
l	optical path length	cm



- Makes up 96% of the dried content of RBC's
- HbA (>95%): α (α_1 and α_2) and β chains ($\alpha_2\beta_2$)
- A2 (1.5-3.5%): $\alpha_2\delta_2$
- F: $\alpha_2\gamma_2$
- Several pathological variants exist


Hemoglobin
NH2
CHO
H—C—OH
HO—C—H
H—C—OH
H—C—OH
CH2OH
Glucose
N
H—C
H—C—OH
HO—C—H
H—C—OH
H—C—OH
CH2OH
(Schiff base)
Hemoglobin A1c
NH
H—C—H
H—C—O
HO—C—H
H—C—OH
H—C—OH
CH2OH
Amadori Product

HbA1c	mmol/mol	%
Normal	Below 42 mmol/mol	Below 6.0%
Prediabetes	42 to 47 mmol/mol	6.0% to 6.4%
Diabetes	48 mmol/mol or over	6.5% or over

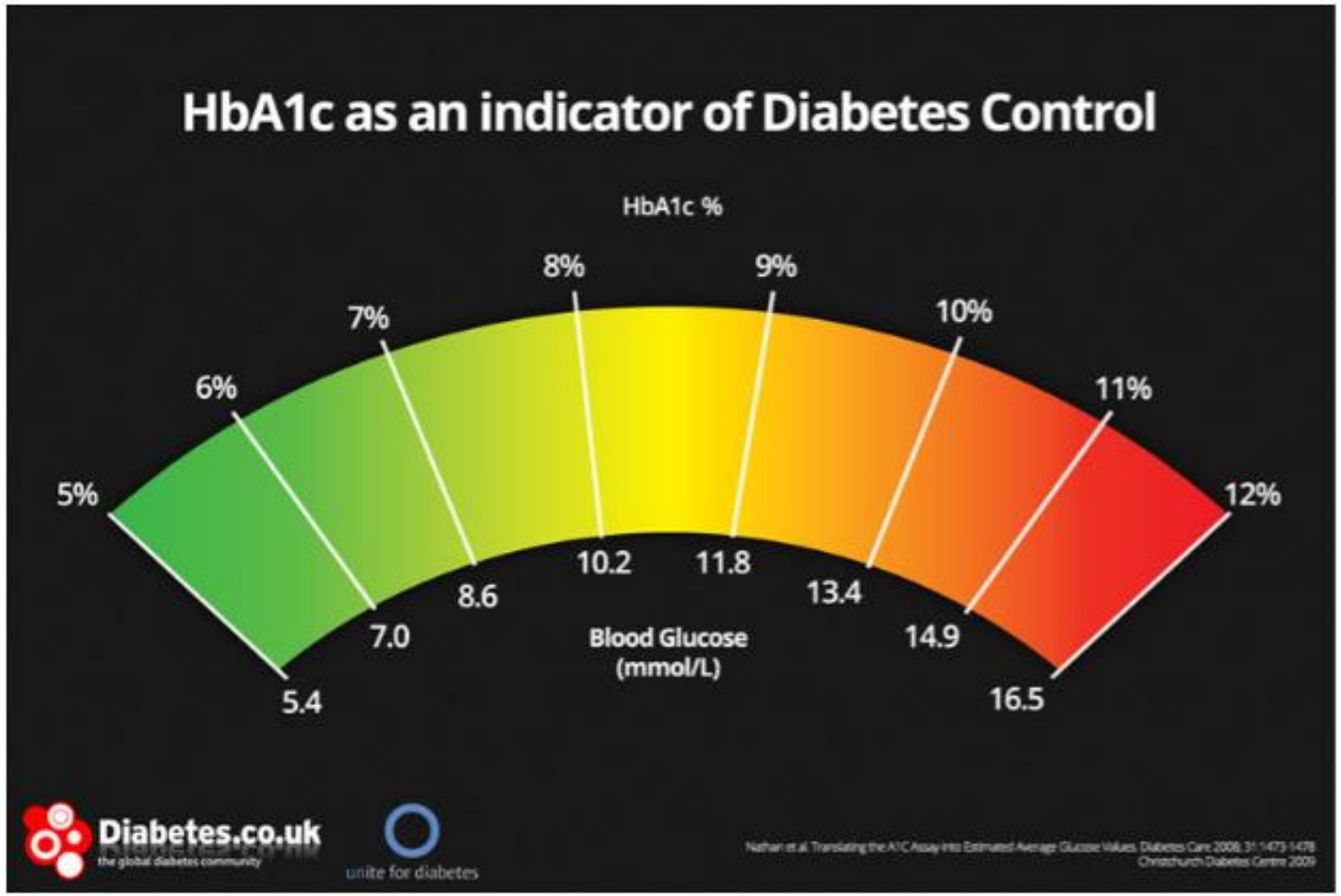


Table 4 The relationships between the hemoglobin A_{1c} units as % values to the mmol/mol values are presented at different levels of hemoglobin A_{1c}

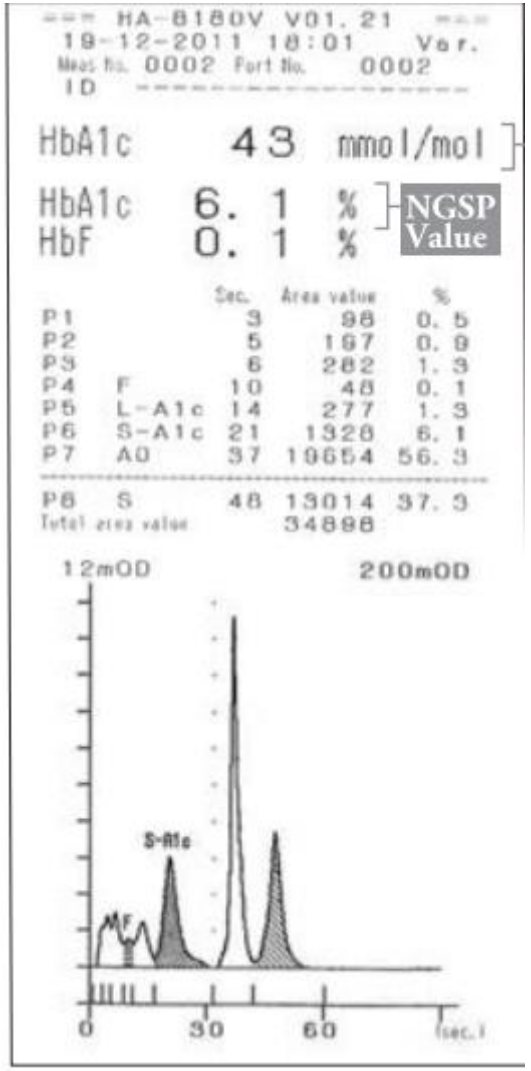
HbA_{1c}	HbA_{1c} (%)	HbA_{1c} (mmol/mol)
Reference limits	4.0-6.0	20-42
Diagnosis limit	6.5	48
Treatment limits, adults	7.0	53
children < 6 yr	7.5	69
children 6-12 yr	8.0	64
children 13-19 yr	8.5	58
Poor diabetic balance	9.0	75
Very poor diabetic balance	12.0	108

Applications of glycated haemoglobin measurements

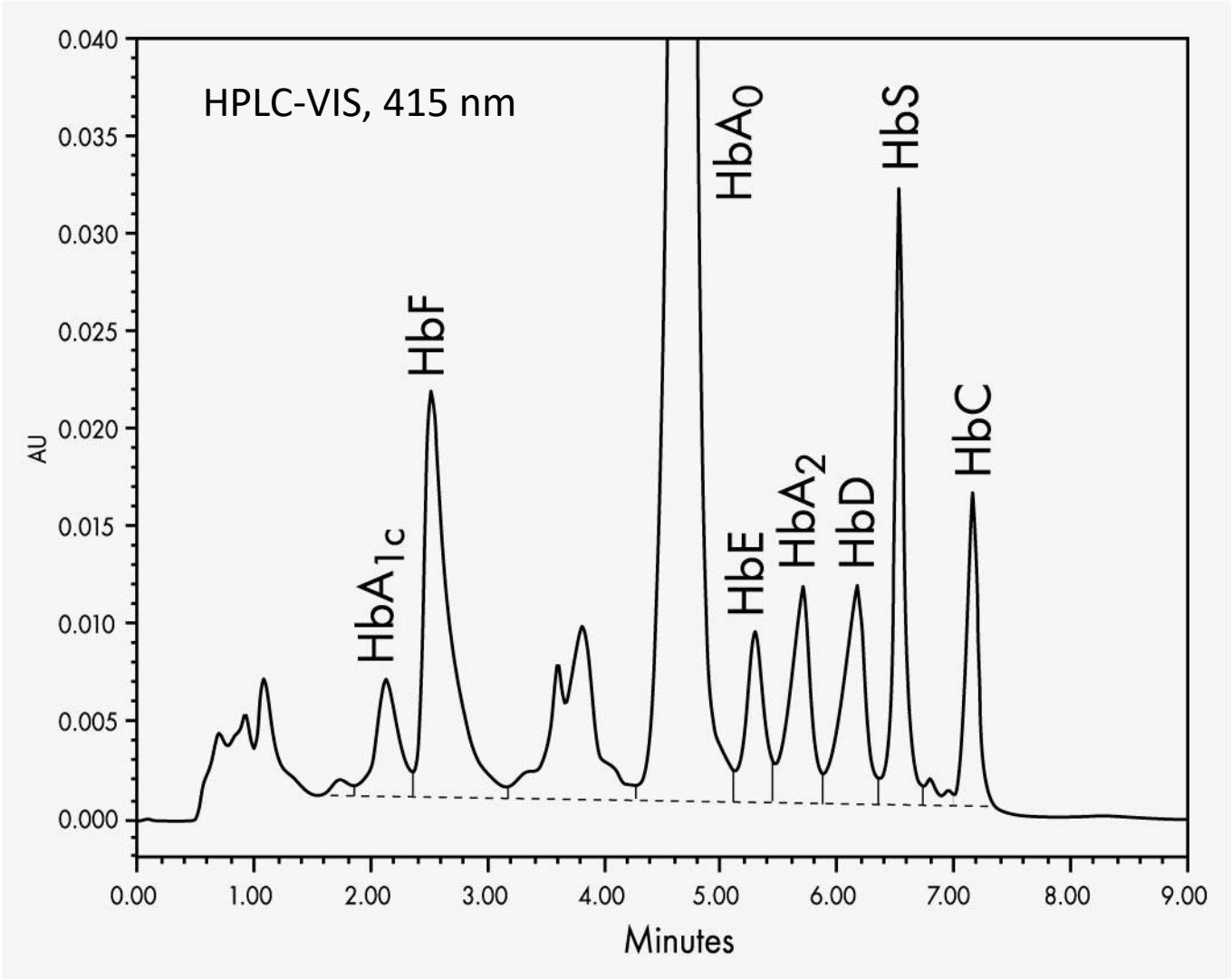
- Diagnosis of diabetes
- Monitoring the progress of therapy
- Results correlate with average blood glucose levels in past 6-12 weeks

Lab technology for evaluating HbA1c

- HPLC-VIS (420/500 nm)
- Separation by cation exchange chromatography
- Sample: whole blood, EDTA-coated tube
- Fully automated
- Run time: 48-90 s



http://www.arkray.eu/english/products/laboratory/habalc/ha-8180.html



https://www.chromsystems.com/products/hemoglobin-testing/hemoglobin-variants-hplc-15330.html

Pharmacokinetic-pharmacodynamic indices of antibiotics

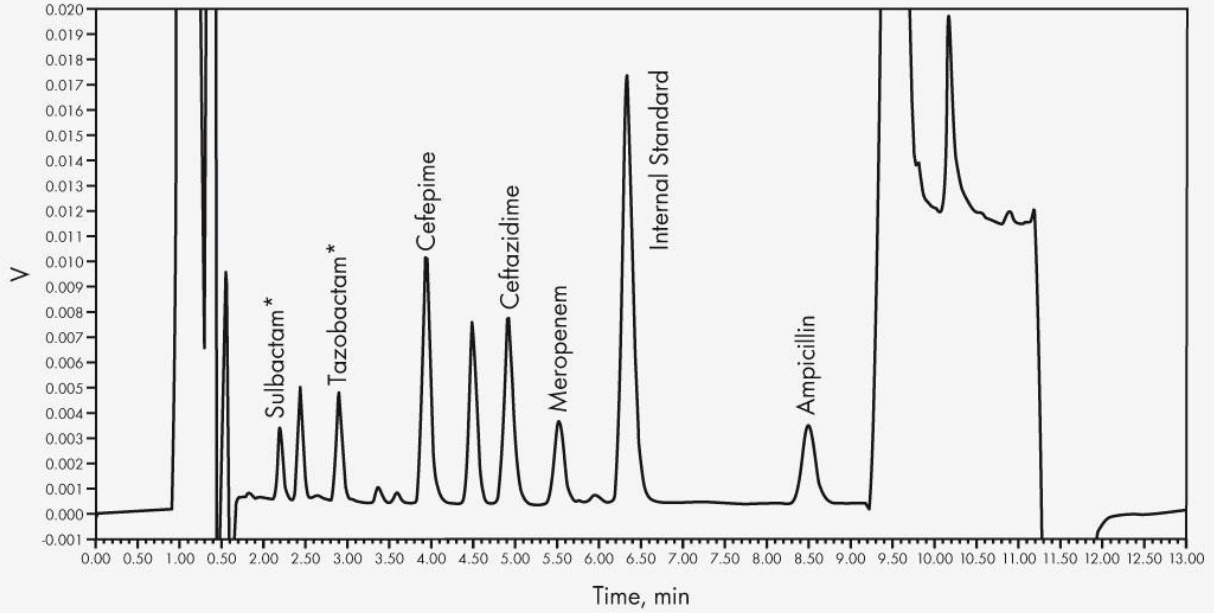
Pharmacodynamic index	$f\ T_{>MIC}$	C_{max}/MIC	AUC_{0-24}/MIC
Antimicrobials	β-Lactams Carbapenems Linezolid Erythromycin Clarithromycin Lincosamides	Aminoglycosides Metronidazole Fluoroquinolones Telithromycin Daptomycin	Fluoroquinolones Aminoglycosides Azithromycin Tetracyclines Glycopeptides Tigecycline Linezolid

Roberts JA et al. Brit J Clin Pharmacol 2011;73:27.

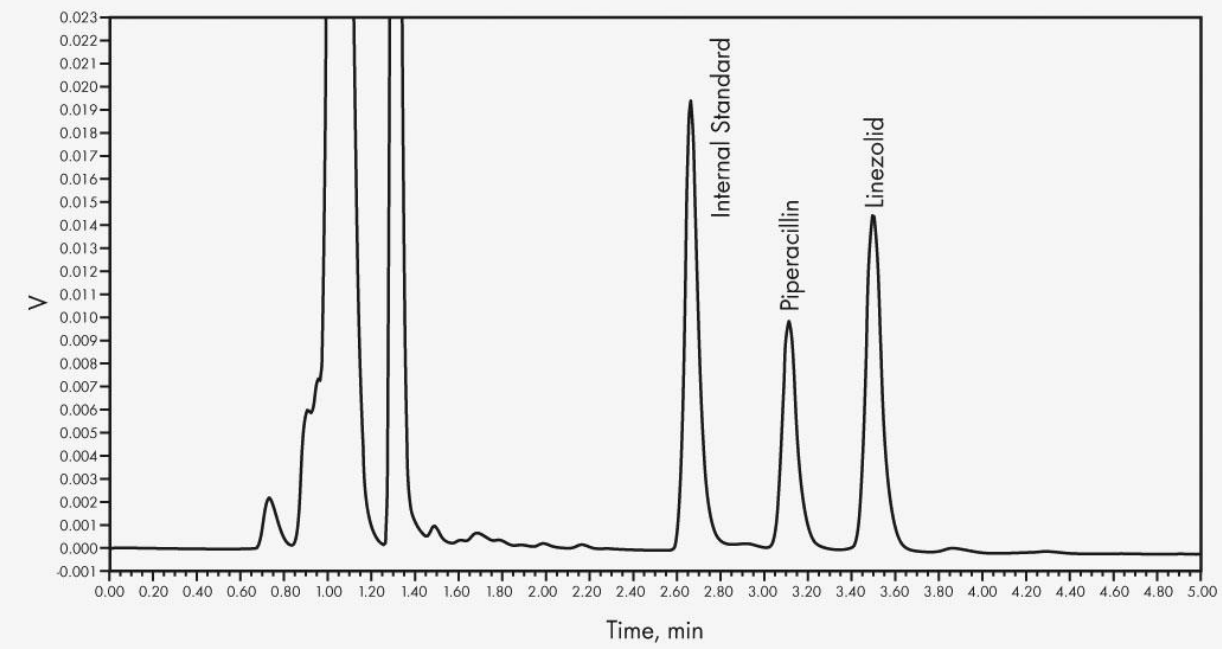
Assaying 6 antibiotics and 2 β-lactamase inhibitors in serum using reversed-phase HPLC with UV detection

- Analytes: ampicillin, cefepime, ceftazidime, linezolid, meropenem, piperacillin, sulbactam, tazobactam
- Preanalytical considerations:
  - Analytes are unstable
  - Serum should be separated, 100 µL mixed with 20 µL solution of preservative
  - Store sample frozen
- HPLC conditions:
  - 2 groups of analytes
    - Group 1: gradient run (13 min)
      - Detection at 210 nm: ampicillin, sulbactam, tazobactam
      - Detection at 290 nm: cefepime, ceftazidime, meropenem
    - Group 2: isocratic run (5 min)
      - Detection at 255 nm: linezolid, piperacillin
  - Column temperature: 22 °C

https://www.chromsystems.com/antibiotics-in-serum-plasma-61000.html

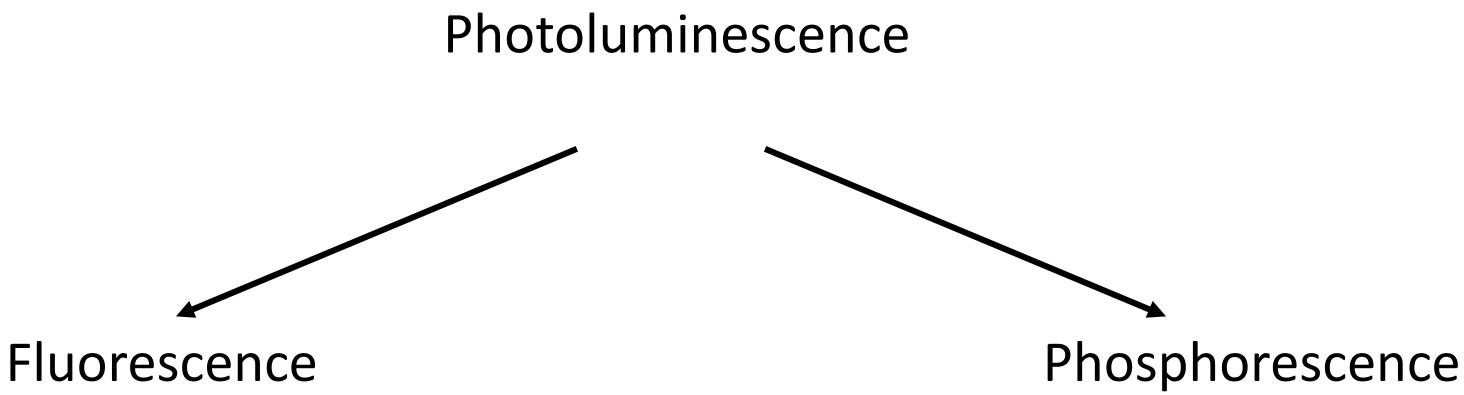


Group 1
Gradient run
210/290 nm

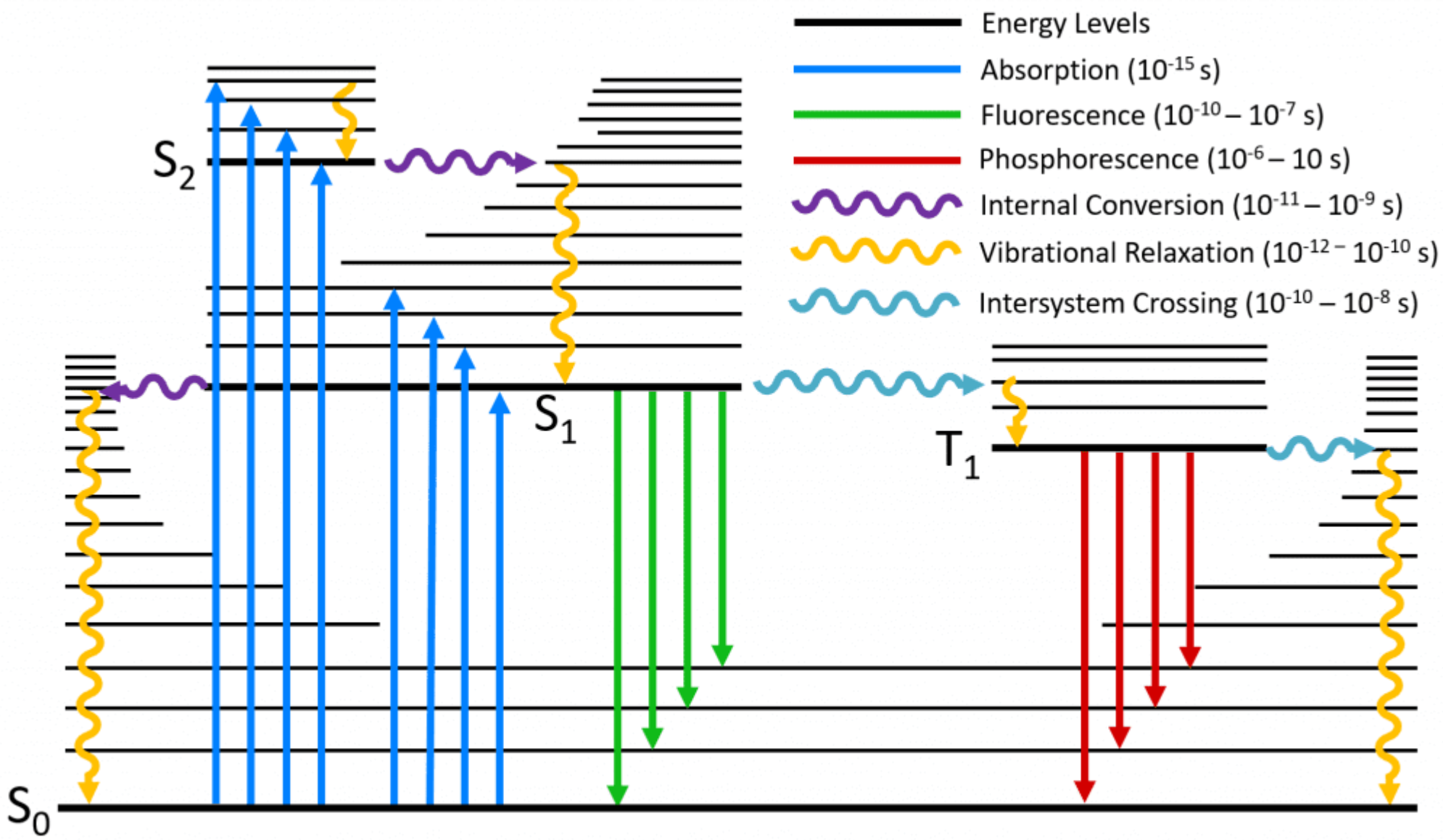


Group 2
Isocratic run
255 nm

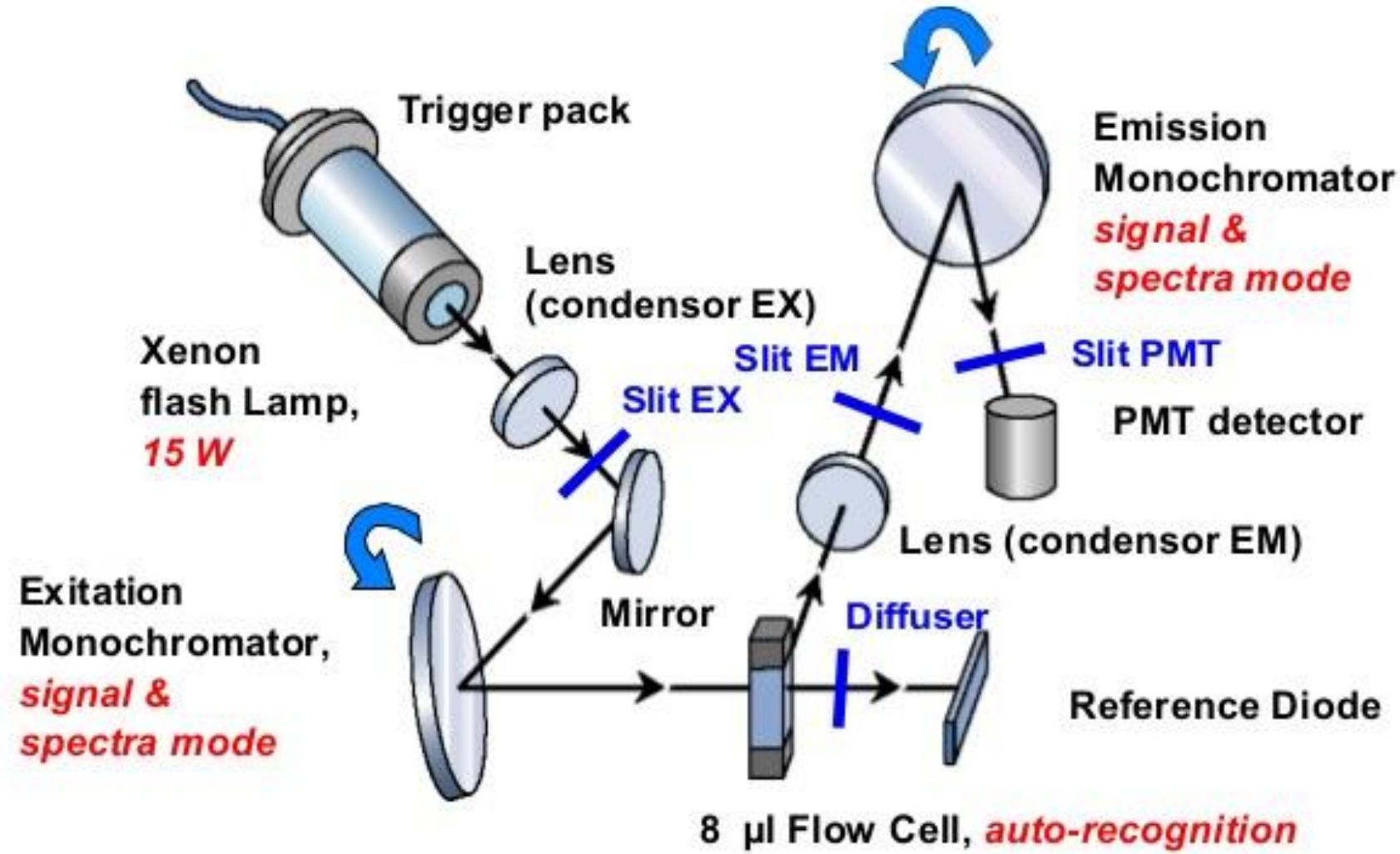
Fluorescence detection


Photoluminescence
Fluorescence
Phosphorescence

Jablonski diagram



Fluorescence Detection



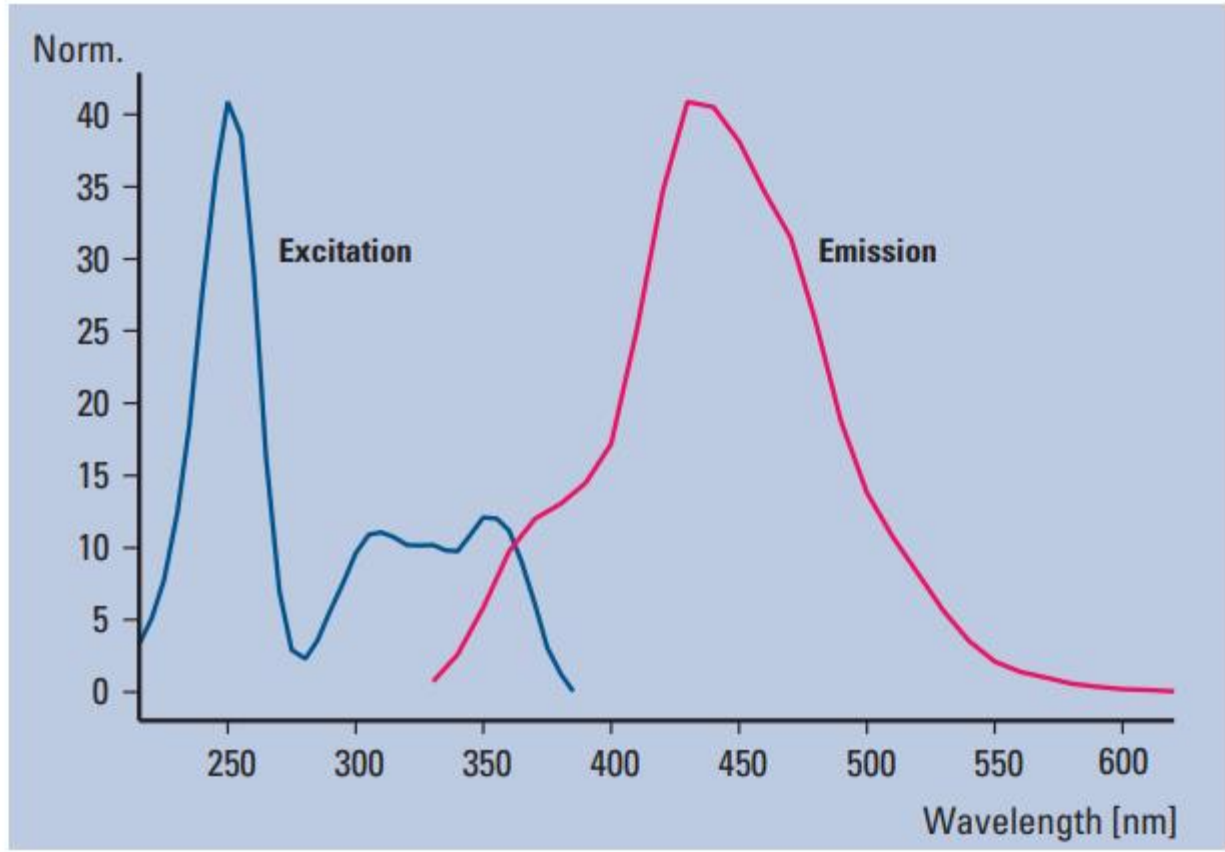
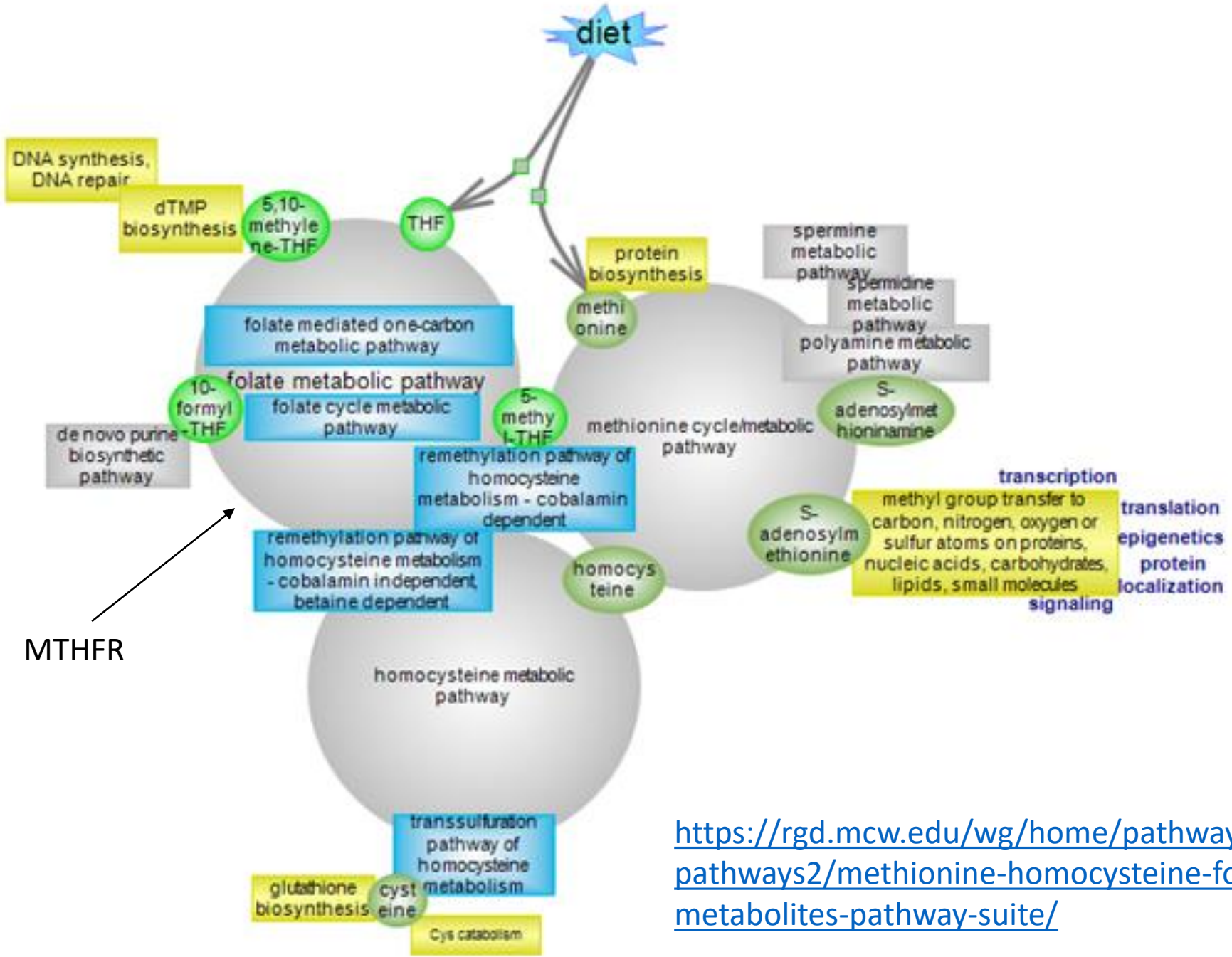


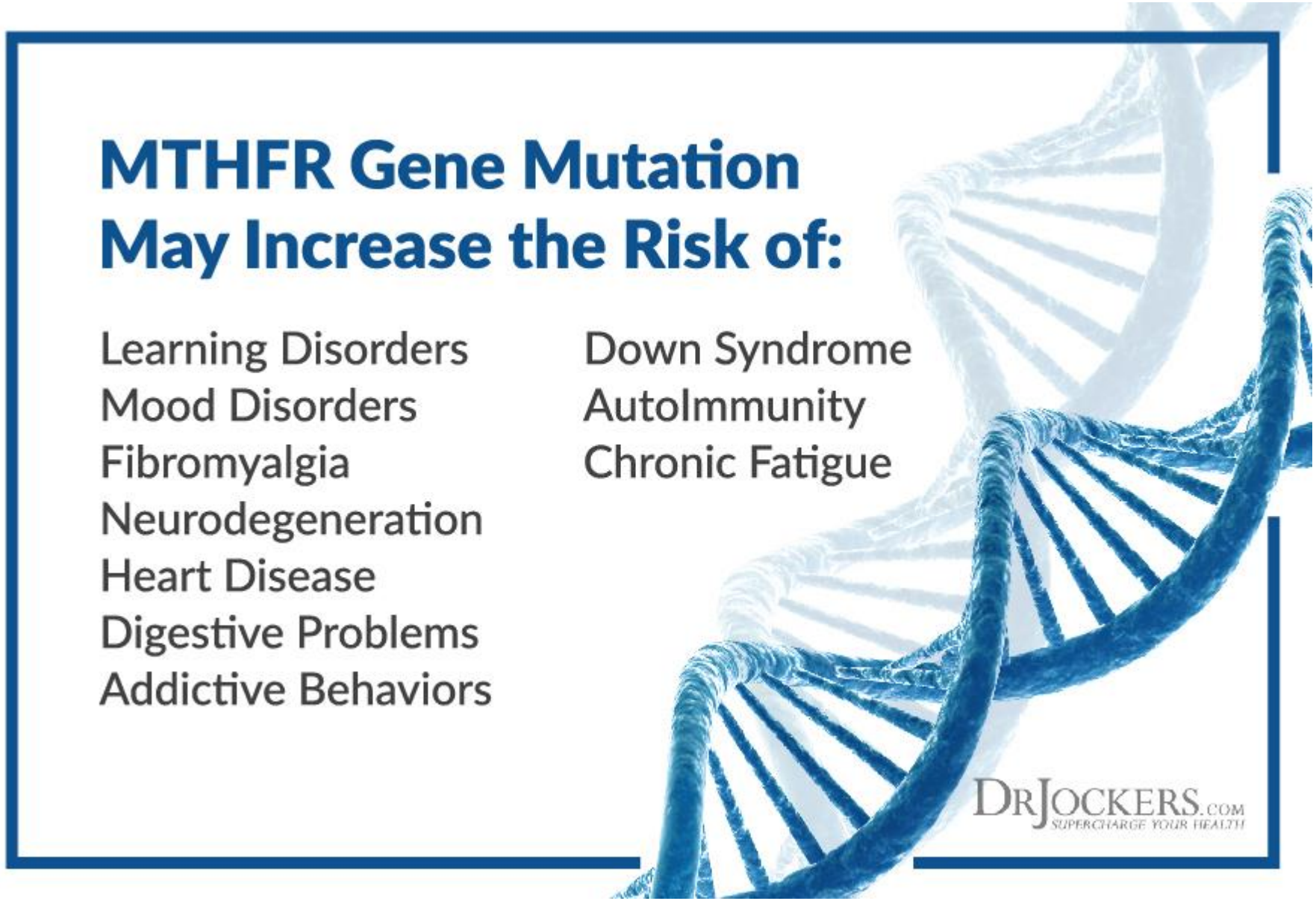
Figure 5
Excitation and emission spectra of quinidine
Excitation spectrum with emission at 440 nm, emission spectrum with excitation at 250 nm of 1 µg/ml quinidine.
Detector settings: step size 5 nm, PMT 12, Response time 4 s.

Clinical laboratory parameters frequently assayed using HPLC with fluorescence detection

Homocysteine



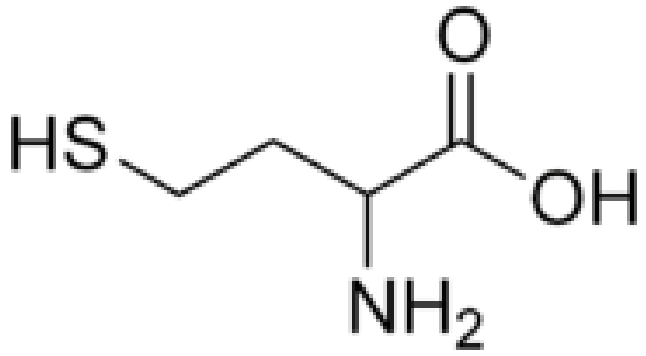
https://rgd.mcw.edu/wg/home/pathway2/molecular-pathways2/methionine-homocysteine-folate-and-related-metabolites-pathway-suite/



http://myhealthmaven.com/mthfr-gene-mutation-impact-health/

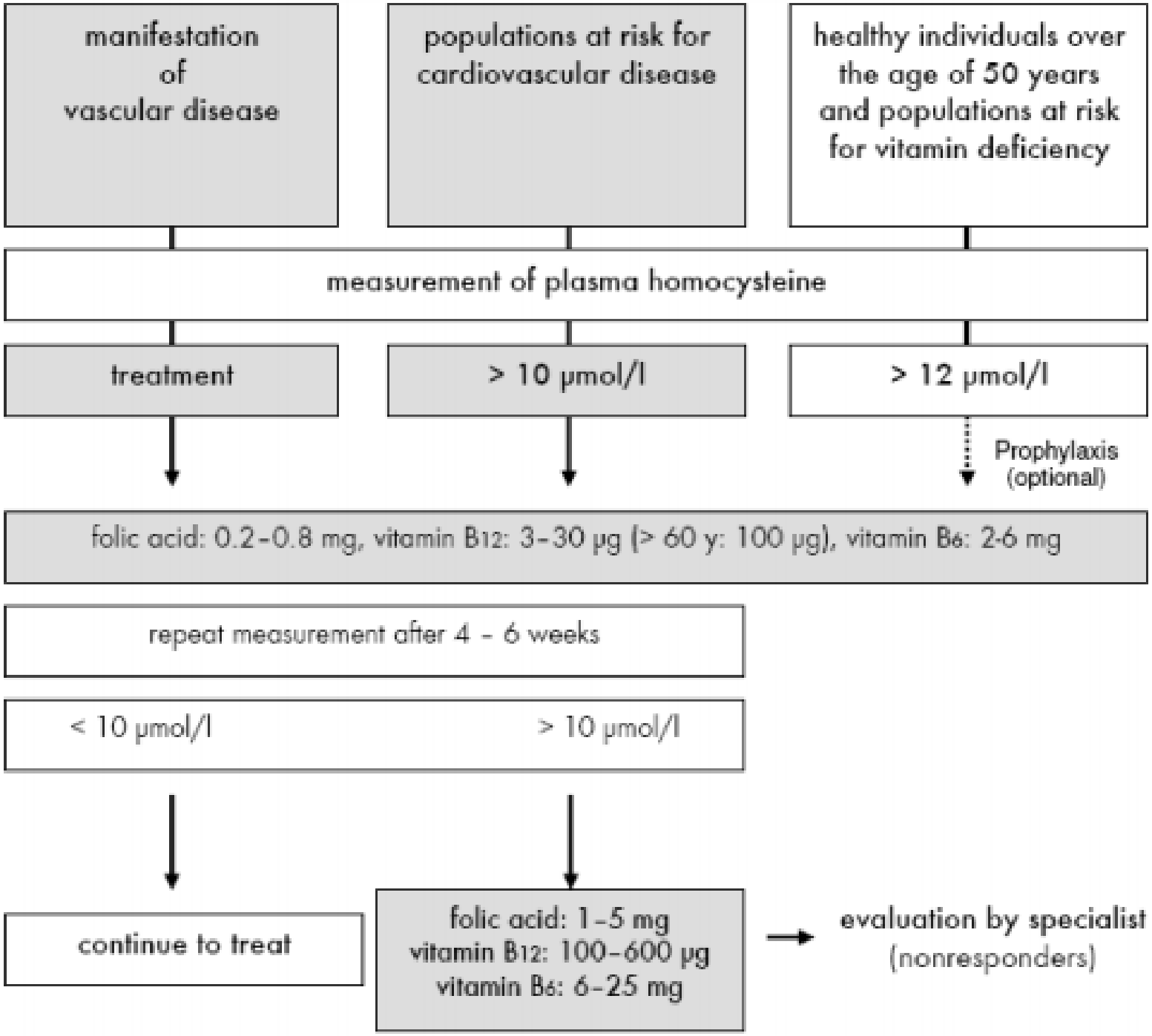
Homocysteine: clinical lab details

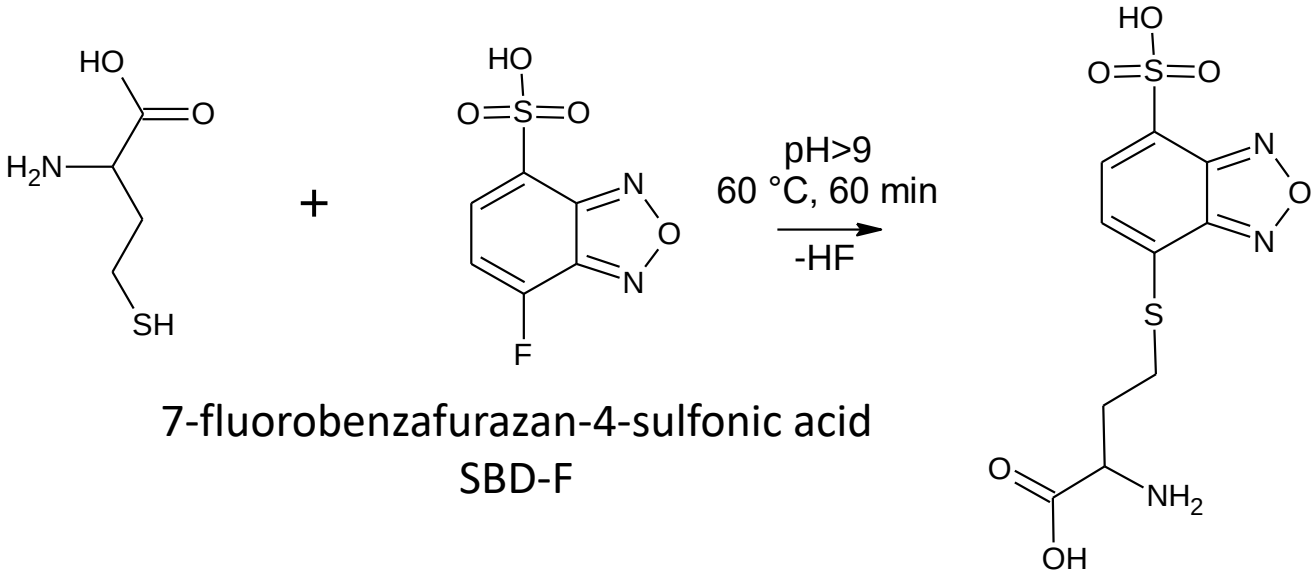
- specimen: plasma (EDTA, heparinized)
- specimen stability: ambient, 1 h. Refrigerated: 1 week. Frozen: 3 months
- 0-30 y: 4.6-8.1 µmol/l
- 30-59 y: males – 6.3-11.2 µmol/l, females – 4.0-7.9 µmol/l
- >59 y: 5.8-11.9 µmol/l



Pathology of homocysteine

- plasma and urine levels elevated in homocystinuria
- plasma levels elevated in Vitamin B-12 deficiency, vitamin B-6 deficiency, folic acid deficiency
- plasma levels may be elevated in hypothyroidism, impaired kidney function, SLE
- drugs can increase homocysteine levels: methotrexate, carbamazepine


manifestation of vascular disease
populations at risk for cardiovascular disease
healthy individuals over the age of 50 years and populations at risk for vitamin deficiency
measurement of plasma homocysteine
treatment
> 10 µmol/l
> 12 µmol/l
Prophylaxis (optional)
folic acid: 0.2–0.8 mg, vitamin B12: 3–30 µg (> 60 y: 100 µg), vitamin B6: 2-6 mg
repeat measurement after 4 – 6 weeks
< 10 µmol/l
> 10 µmol/l
continue to treat
folic acid: 1–5 mg
vitamin B12: 100–600 µg
vitamin B6: 6–25 mg
evaluation by specialist
(nonresponders)

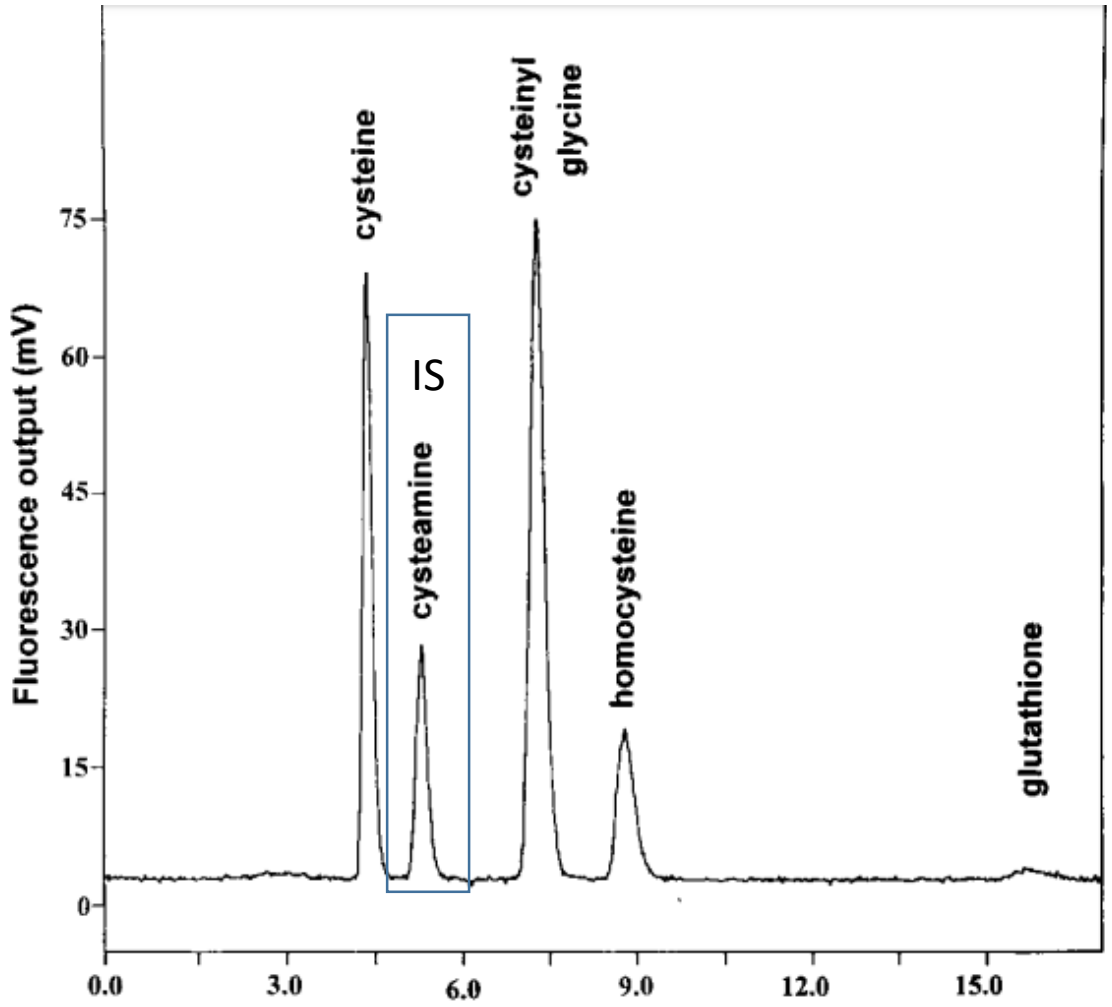


7-fluorobenzafurazan-4-sulfonic acid
SBD-F

Sample volume: 150 µL

1. Liberation: 100 mL/L tri-n-butylphosphine in DMF 4 °C/30 min
2. Deproteinization: 150 µL TCA solution (0.1 g/mL)
3. Derivatization

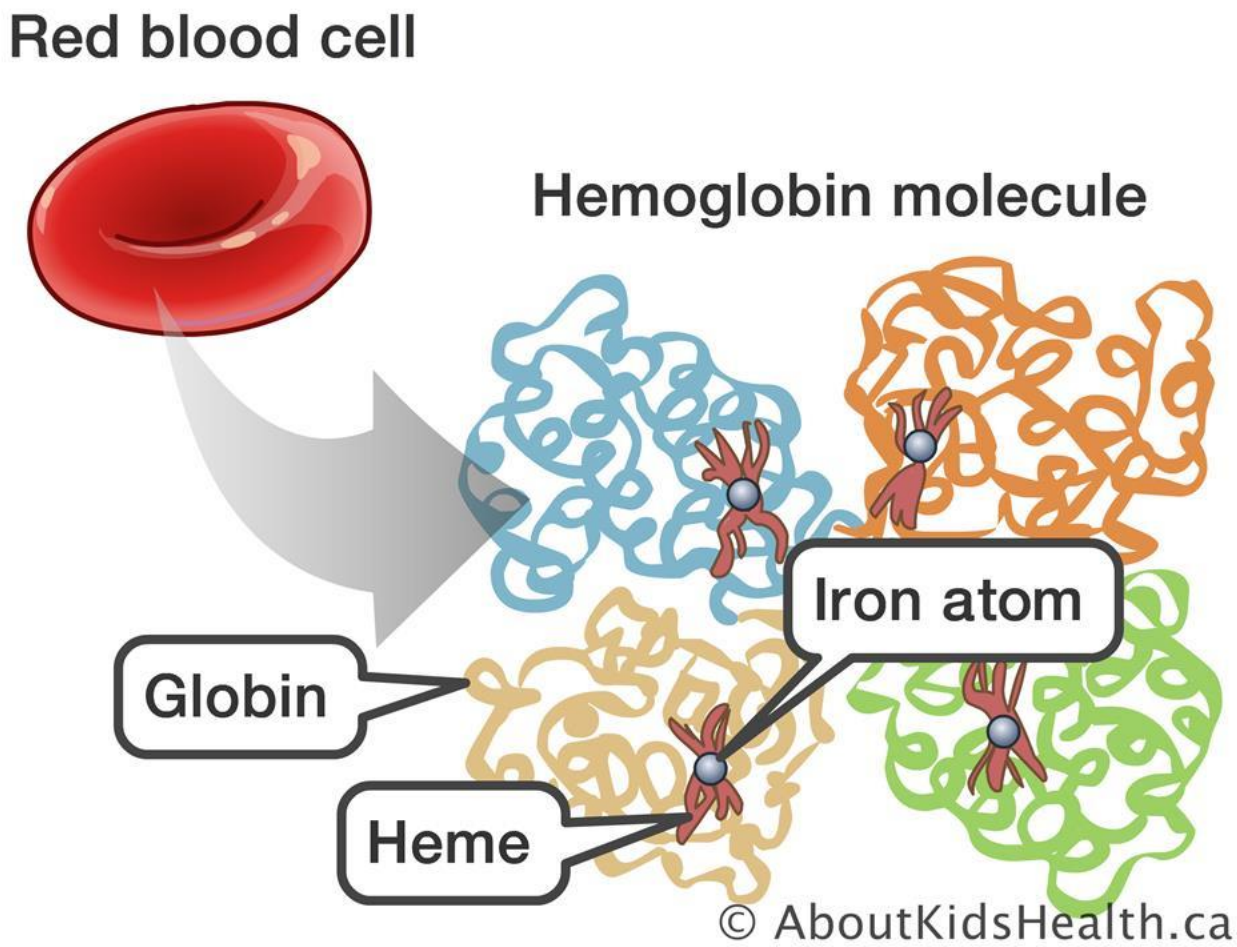
SP: C18 250x4x6 mm, 5 µm (ambient)
MP: 0.1 M KH_2PO4 (pH=2.0):acetonitrile 96:4
FR: 0.8 mL/min
EX: 385 nm
EM: 515 nm

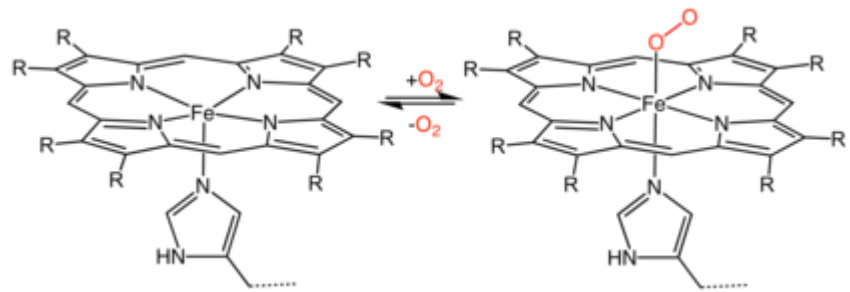


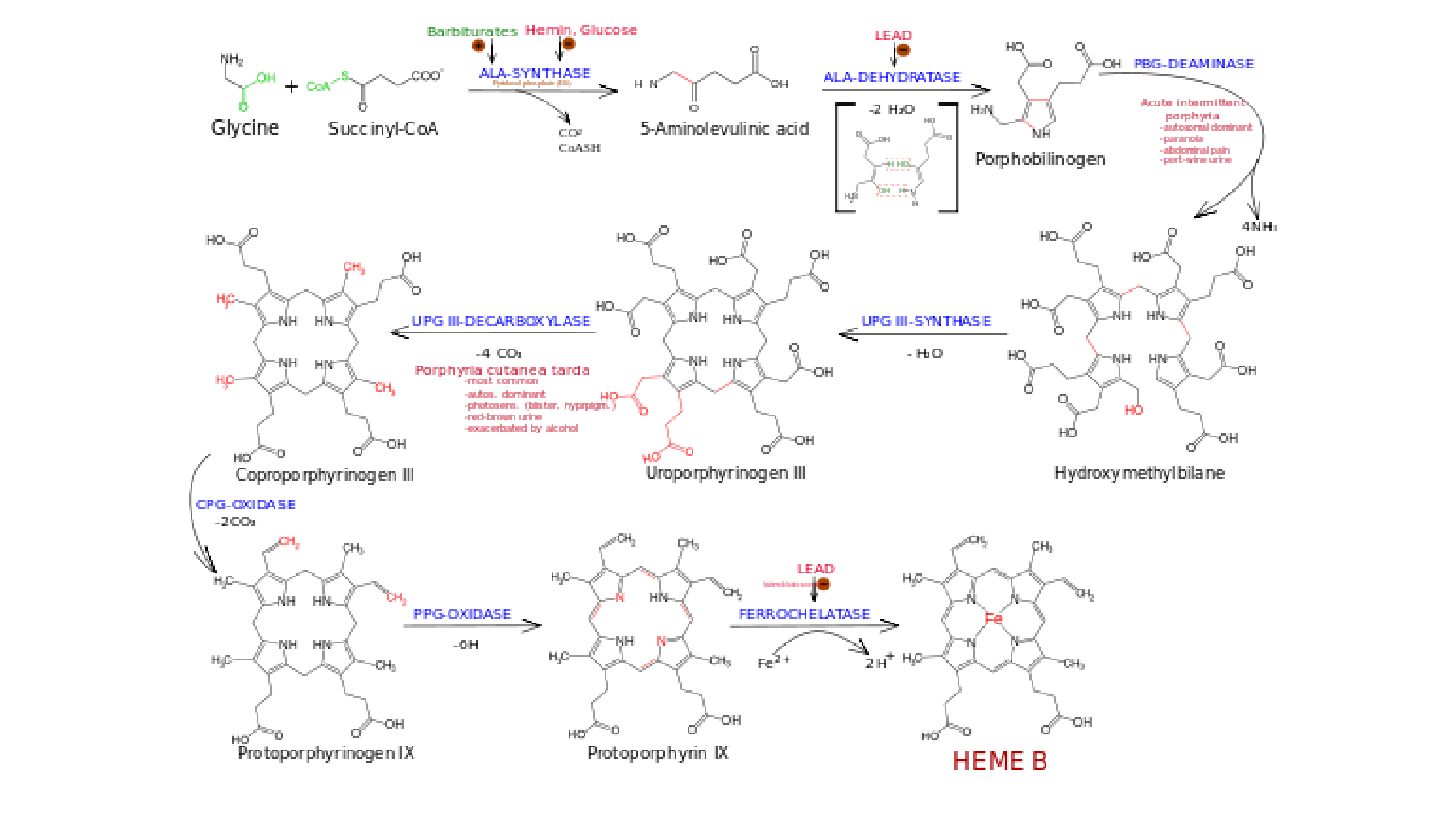
Kuo K et al. Clin Chem 1997;43:1653.

Clinical laboratory parameters frequently assayed using HPLC with fluorescence detection

Porphyrins


Red blood cell
Hemoglobin molecule
Iron atom
Globin
Heme
© AboutKidsHealth.ca


+O2
-O2
Fe
HN


Barbiturates
Hemin, Glucose
ALA-SYNTHASE
Glycine
Succinyl-CoA
CO2
CoASH
5-Aminolevulinic acid
LEAD
ALA-DEHYDRATASE
-2 H2O
Porphobilinogen
PBG-DEAMINASE
Acute intermittent porphyria
-autosomal dominant
-paranoia
-abdominal pain
-port-wine urine
4NH3
UPG III-DECARBOXYLASE
-4 CO2
Porphyria cutanea tarda
-most common
-autos. dominant
-photosens. (blister, hyperpigm.)
-red-brown urine
-exacerbated by alcohol
UPG III-SYNTHASE
- H2O
Coproporphyrinogen III
Uroporphyrinogen III
Hydroxymethylbilane
CPG-OXIDASE
-2CO2
PPG-OXIDASE
-6H
Protoporphyrinogen IX
Protoporphyrin IX
LEAD
FERROCHELATASE
Fe2+
2 H+
HEME B

Types of porphyrias

Acute porphyrias	Chronic porphyrias
Aminolevulinic acid dehydratase deficiency (ALAD)	X-linked dominant protoporphyria (XLPDD)
Acute intermittent porphyria (AIP)	Congenital erythropoietic porphyria (CEP)
Hereditary coproporphyria (HCP)	Porphyria cutanea tarda (PCT)
Porphyria variegata (VP)	Erythropoietic protoporphyria (EPP)

Signs and symptoms of acute porphyria may include:

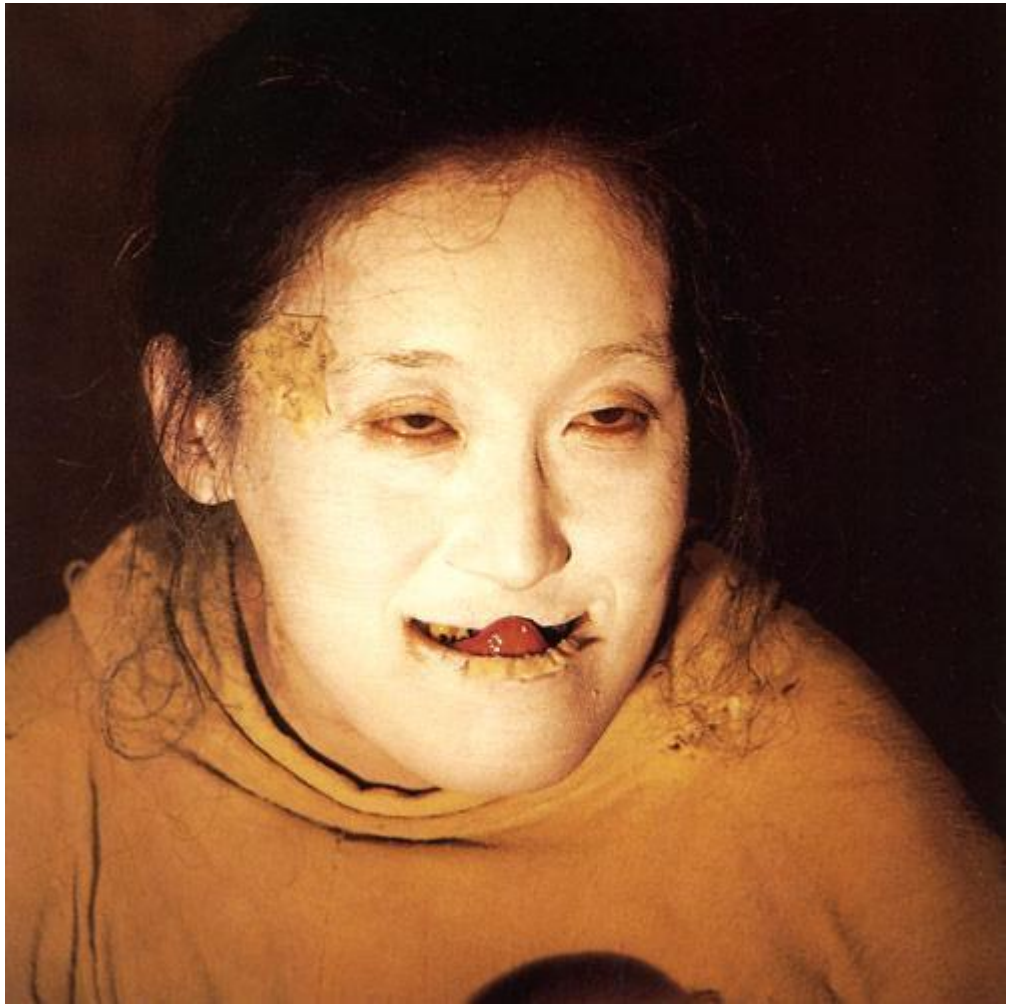
- Severe abdominal pain
- Pain in your chest, legs or back
- Constipation or diarrhea
- Nausea and vomiting
- Muscle pain, tingling, numbness, weakness or paralysis
- Red or brown urine
- Mental changes, such as anxiety, confusion, hallucinations, disorientation or paranoia
- Breathing problems
- Urination problems
- Rapid or irregular heartbeats you can feel (palpitations)
- High blood pressure
- Seizures

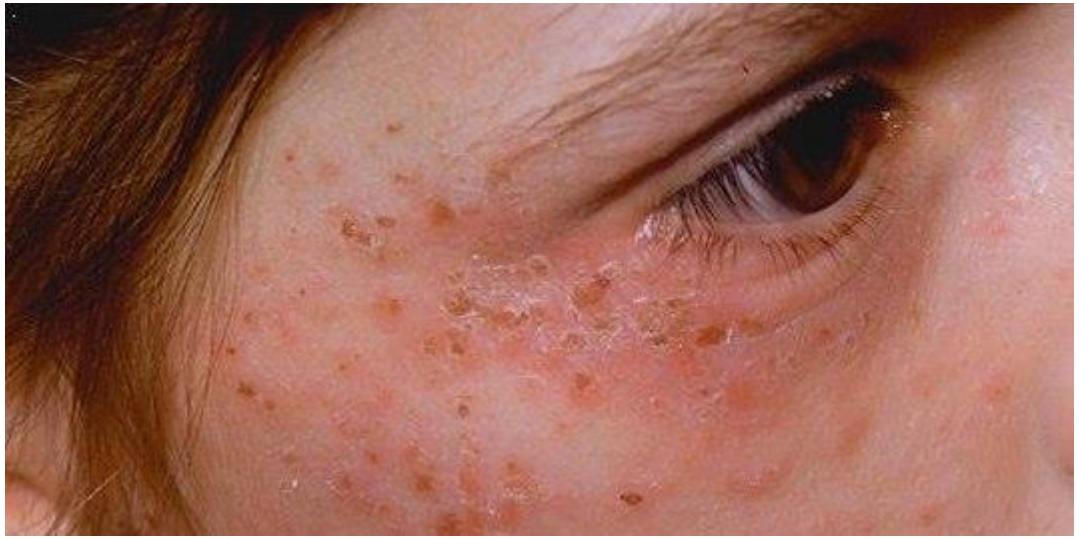
Cutaneous porphyrias

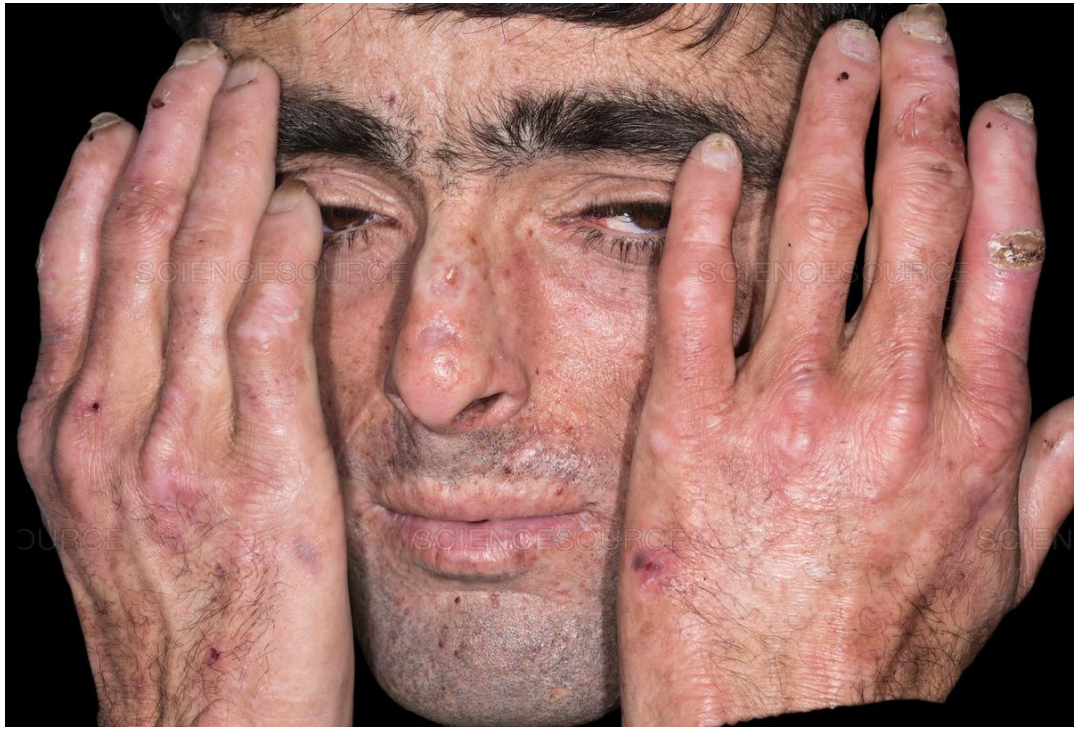
Cutaneous porphyrias include forms of the disease that cause skin symptoms as a result of sensitivity to sunlight, but these forms don't usually affect your nervous system. Porphyria cutanea tarda (PCT) is the most common type of all the porphyrias.

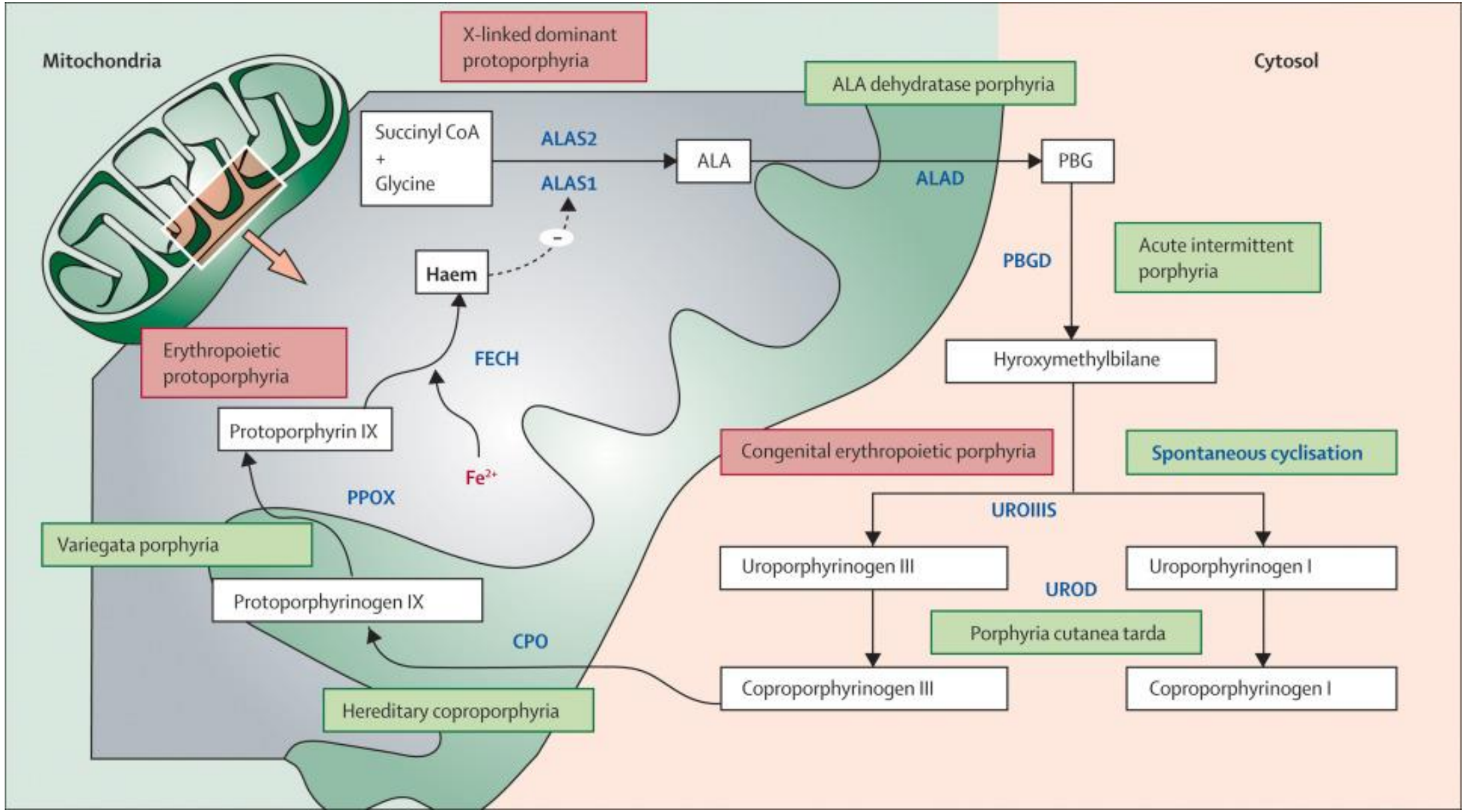
As a result of sun exposure, you may experience:

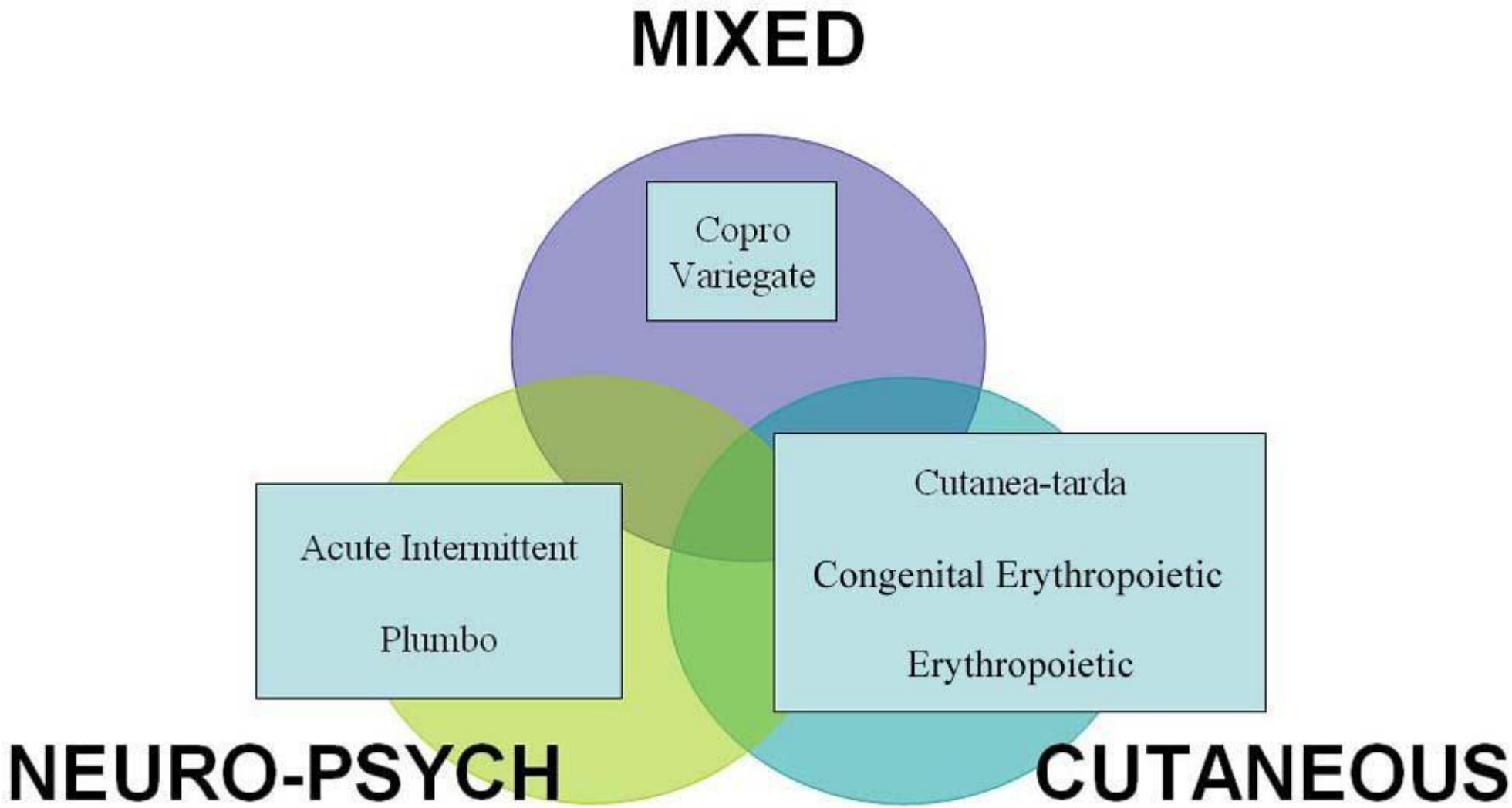
- Sensitivity to the sun and sometimes artificial light, causing burning pain
- Sudden painful skin redness (erythema) and swelling (edema
- Blisters on exposed skin, usually the hands, arms and face
- Fragile thin skin with changes in skin color (pigment)
- Itching
- Excessive hair growth in affected areas
- Red or brown urine








Mitochondria
Cytosol
X-linked dominant protoporphyria
ALA dehydratase porphyria
Succinyl CoA + Glycine
ALAS2
ALAS1
ALA
ALAD
PBG
Haem
PBGD
Acute intermittent porphyria
Erythropoietic protoporphyria
FECH
Hyroxymethylbilane
Protoporphyrin IX
Fe2+
Congenital erythropoietic porphyria
Spontaneous cyclisation
PPOX
UROIIIS
Variegata porphyria
Uroporphyrinogen III
Uroporphyrinogen I
Protoporphyrinogen IX
UROD
Porphyria cutanea tarda
CPO
Coproporphyrinogen III
Coproporphyrinogen I
Hereditary coproporphyria



https://emedicine.medscape.com/article/1389981-overview

EX: 400 nm (Soret-band)
EM: 620 nm

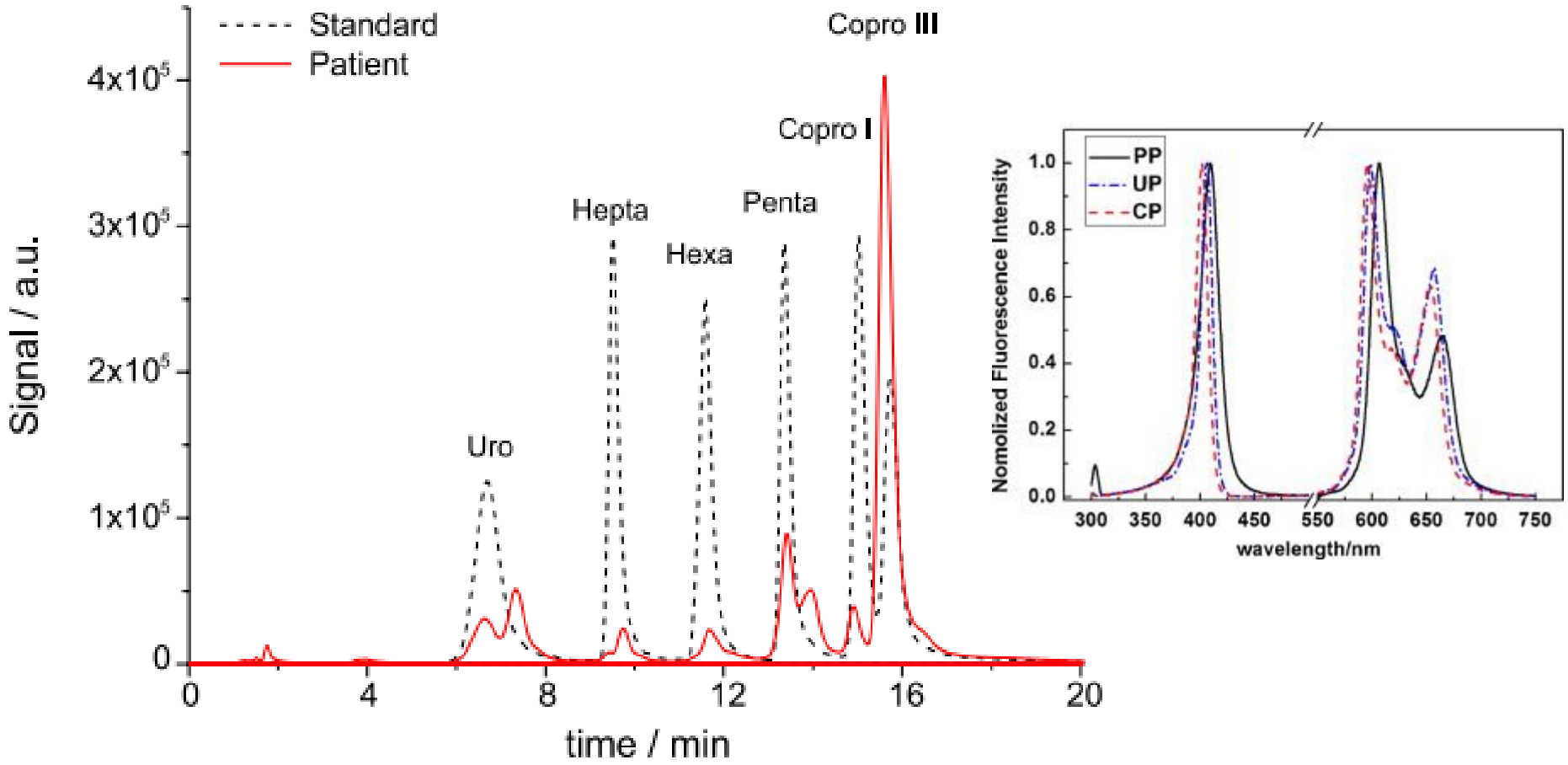
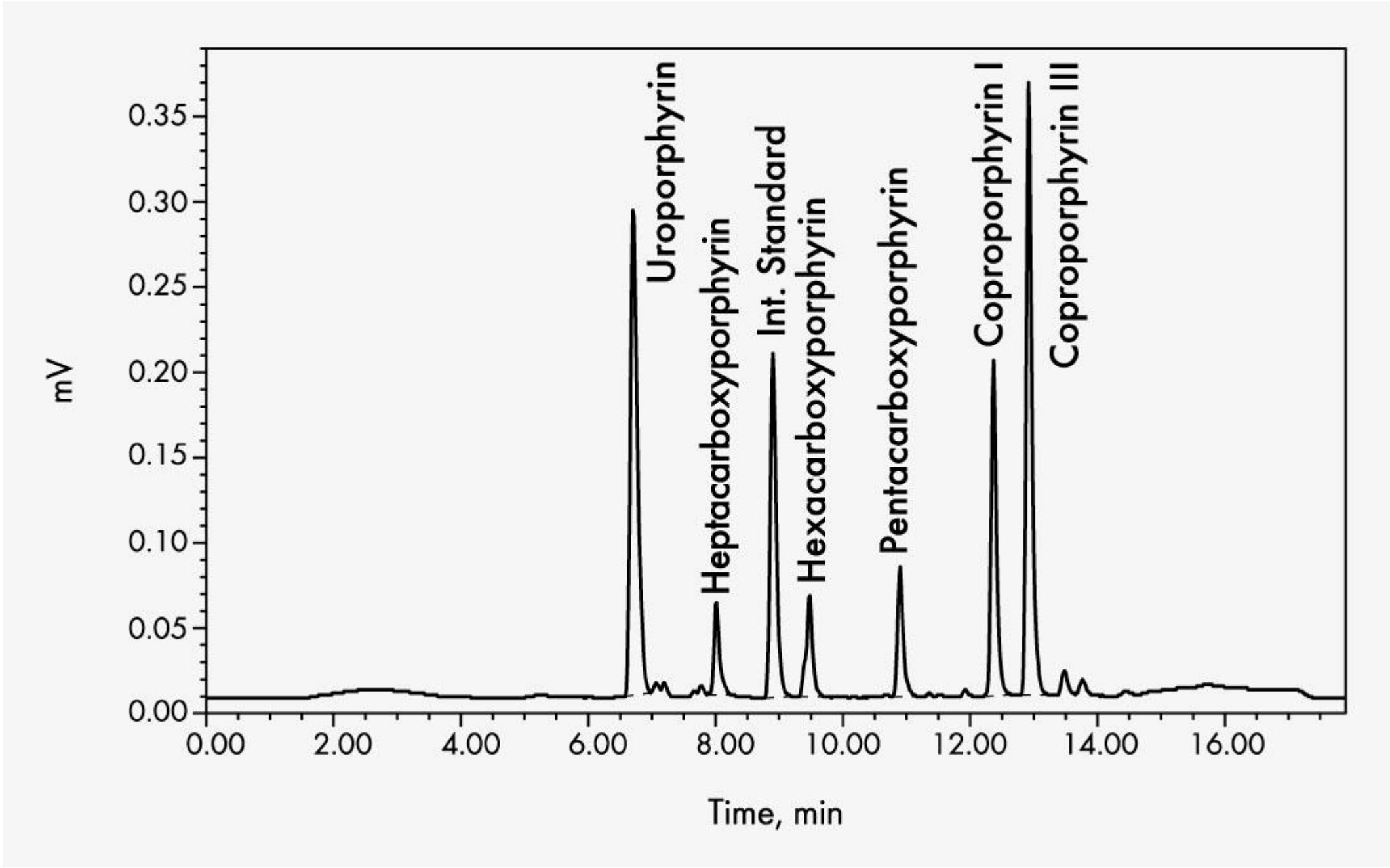


Figure 2. Comparative HPLC/fluorescence traces obtained for a standard mixture of porphyrins (dashed line) and the urine of a porphyric patient (solid line). Eluent A: 1.0 mol L^{-1} ammonium acetate buffer containing 10% acetonitrile at pH 5.7. Eluent B: methanol:acetonitrile (9:1, v/v). Patient 2 was clinically diagnosed as an acute intermittent porphyria carrier. Porphyrin concentration was 500 nmol L^{-1}.

Cypriani MGSC et al. J Braz Chem Soc 2014;25:1918.


Uroporphyrin
Heptacarboxyporphyrin
Int. Standard
Hexacarboxyporphyrin
Pentacarboxyporphyrin
Coproporphyrin I
Coproporphyrin III
mV
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
Time, min

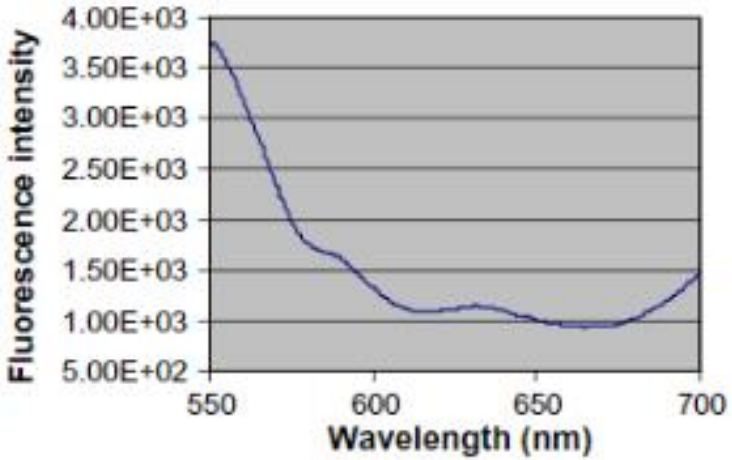


Figure 3 Florescence emission spectrum of normal plasma: no porphyrin like emission is observed. (The nonzero baseline is due to scattered exciting light).

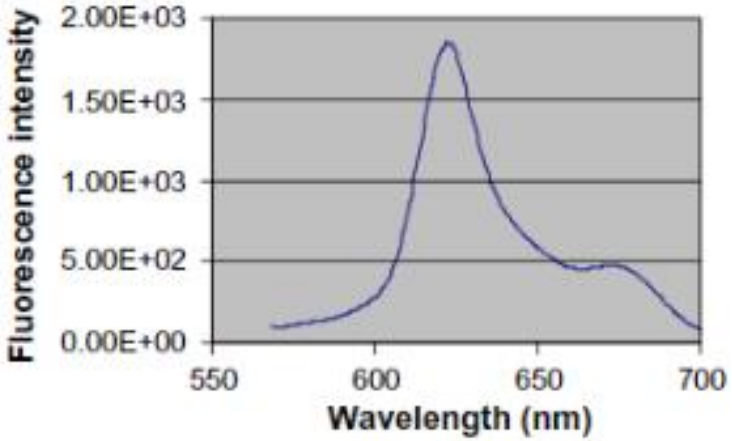


Figure 4 Florescence emission spectrum of plasma of VP patient: excitation, 400 nm and 625 indicates fluorescence emission maximum of 625 nm.

Fateen E et al. Egypt J Med Hum Genet 2011;12:49.

Table 2. Second-line Biochemical Testing for Acute Porphyrias: Laboratory Findings to Differentiate Between AIP, HCP and VP.

Acute Porphyria	HMBS activity in RBCs	Urine PBG	Urine ALA	Urine porphyrins	Fecal porphyrins	Plasma porphyrins
AIP	Decreased in ~90% of cases	elevated	elevated	Markedly increased; mostly uroporphyrin	Normal or slightly increased	Normal or slightly increased
HCP	normal	elevated	elevated	Markedly increased; mostly coproporphyrin	Markedly increased; mostly coproporphyrin III	Normal or slightly increased
VP	normal	elevated	elevated	Markedly increased; mostly coproporphyrin III	Markedly increased; mostly protoporphyrin	Markedly increased; Fluorescence peak (at neutral pH) at ~626nm
ADP	normal	normal	elevated	Markedly increased; mostly coproporphyrin III	Normal or slightly increased	Normal or slightly increased

J Clin Pathol. 2014 Jan;67(1):60-5. doi: 10.1136/jclinpath-2012-201367. Epub 2013 Aug 1.

Urinary excretion of porphyrins, porphobilinogen and δ-aminolaevulinic acid following an attack of acute intermittent porphyria.

Marsden JT[1], Rees DC.

Author information

Abstract

BACKGROUND AND OBJECTIVES: The porphyrias are a group of rare, mainly inherited, diseases caused by a deficiency of one of the enzymes of the haem biosynthesis pathway. The biochemical hallmark of an acute attack is an increase in urine porphobilinogen (PBG), together with an increase in urinary excretion of δ-aminolaevulinic acid (ALA) and total urine porphyrins (TUP). In patients with acute intermittent porphyria (AIP) the concentrations of the porphyrin precursors are thought to remain elevated for many years following an acute attack, although this has not been well documented.

METHODS: We measured urine ALA, PBG and TUP excretion in 20 patients with AIP following an attack of acute porphyria over a time period of 3 months to 23 years after their last documented acute attack.

RESULTS: We showed that urinary concentrations of all metabolites remain elevated for many years. The urinary half life of TUP was 5.3 years, ALA 7.7 years and PBG 10.6 years. Even after 20 years, PBG concentrations remained elevated above the normal range.

CONCLUSIONS: Our study highlights the difficulties of using urinary analysis for diagnosing recurrent attacks, and also raises important questions about the pathophysiology of the condition.

KEYWORDS: Inherited Pathology; Laboratory Tests; Metabolism

PMID: 23908454 DOI: 10.1136/jclinpath-2012-201367

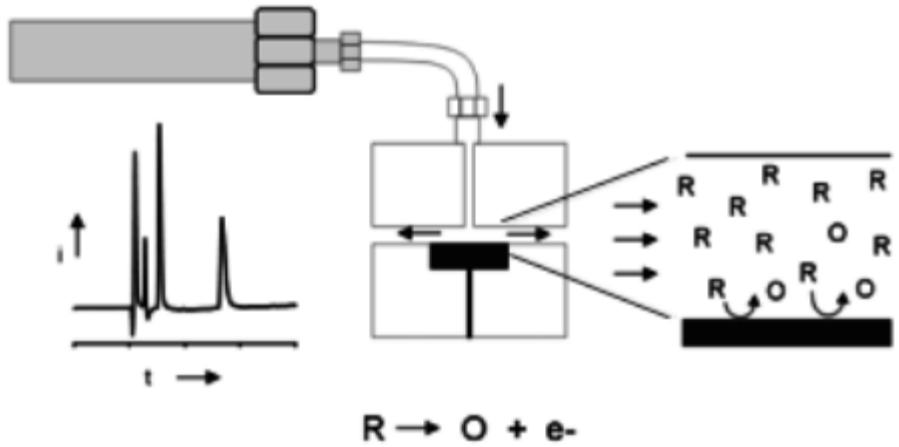
[Indexed for MEDLINE]

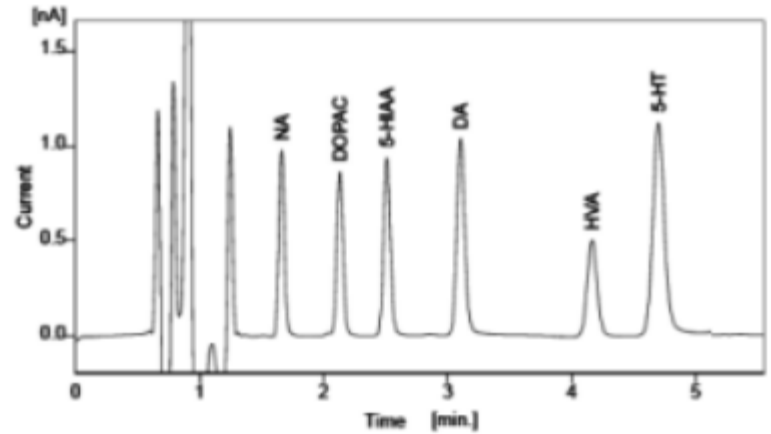
How to approach porphyrin tests

- Urine: dilute and shoot
- Faeces: extract with organic solvent, concentrate+dilute with aqueous buffer
- Blood: separate red blood cells and add water for hemolysis

Electrochemical detection

Amperometric, coulometric and voltammetric detection modes





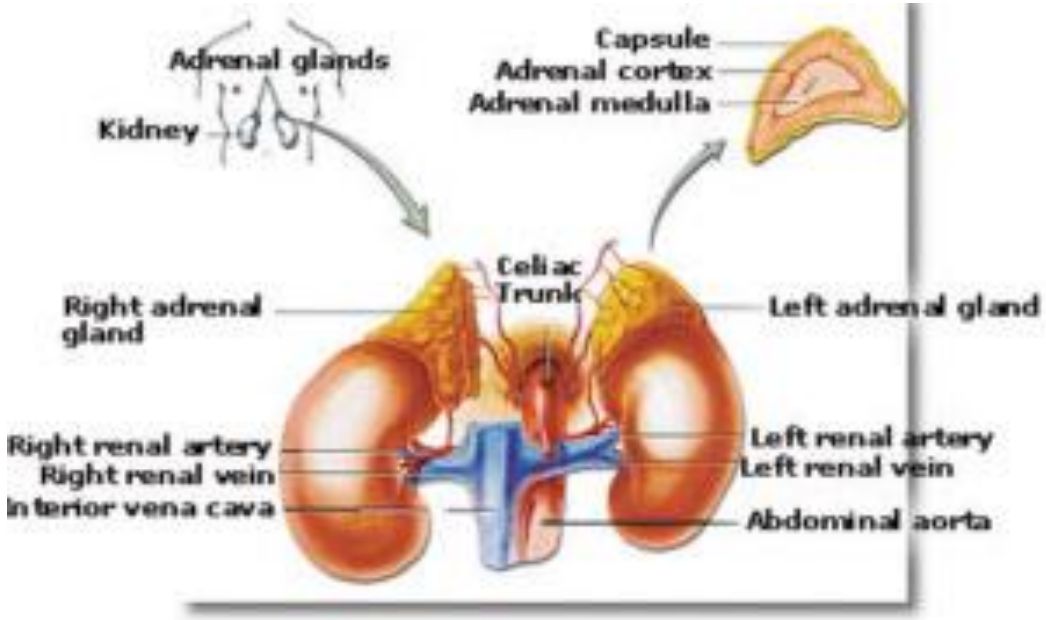
A sample is introduced in HPLC and separated on the chromatographic column. The column is connected to an ECD cell, an electrochemical sensor where a reaction takes place at an electrode. Electrochemically active substances that elute from the column undergo an electrochemical reaction, electrons are transferred resulting in an electrical current.

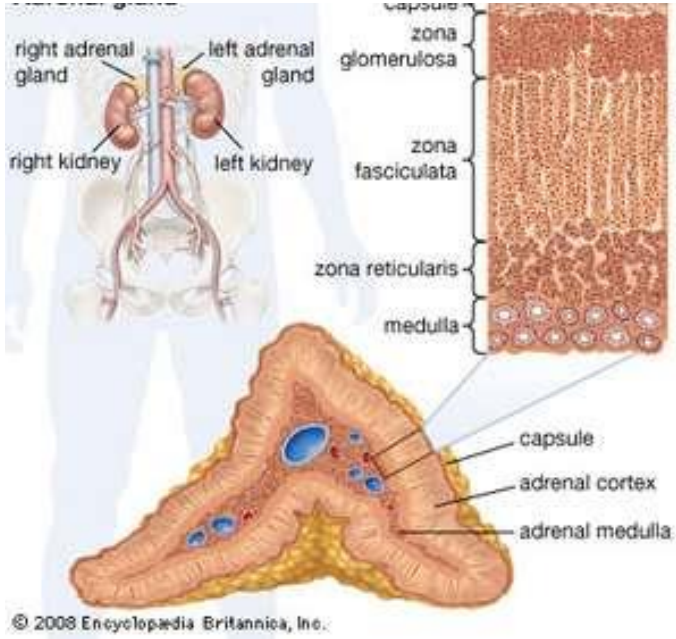
https://www2.chemistry.msu.edu/courses/cem837/Electrochemical%20Detection.pdf

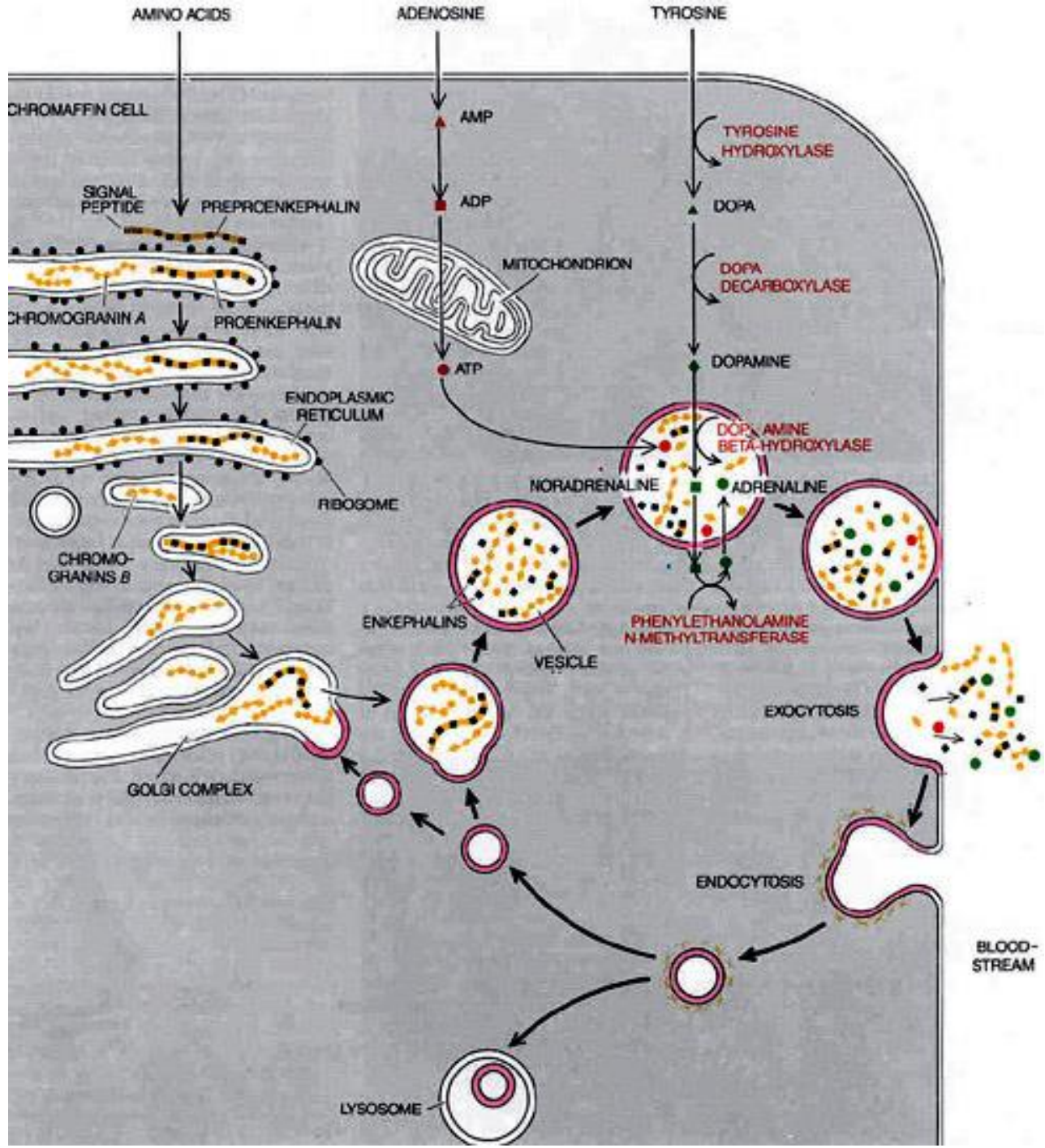
Pros and cons of using HPLC-ECD systems

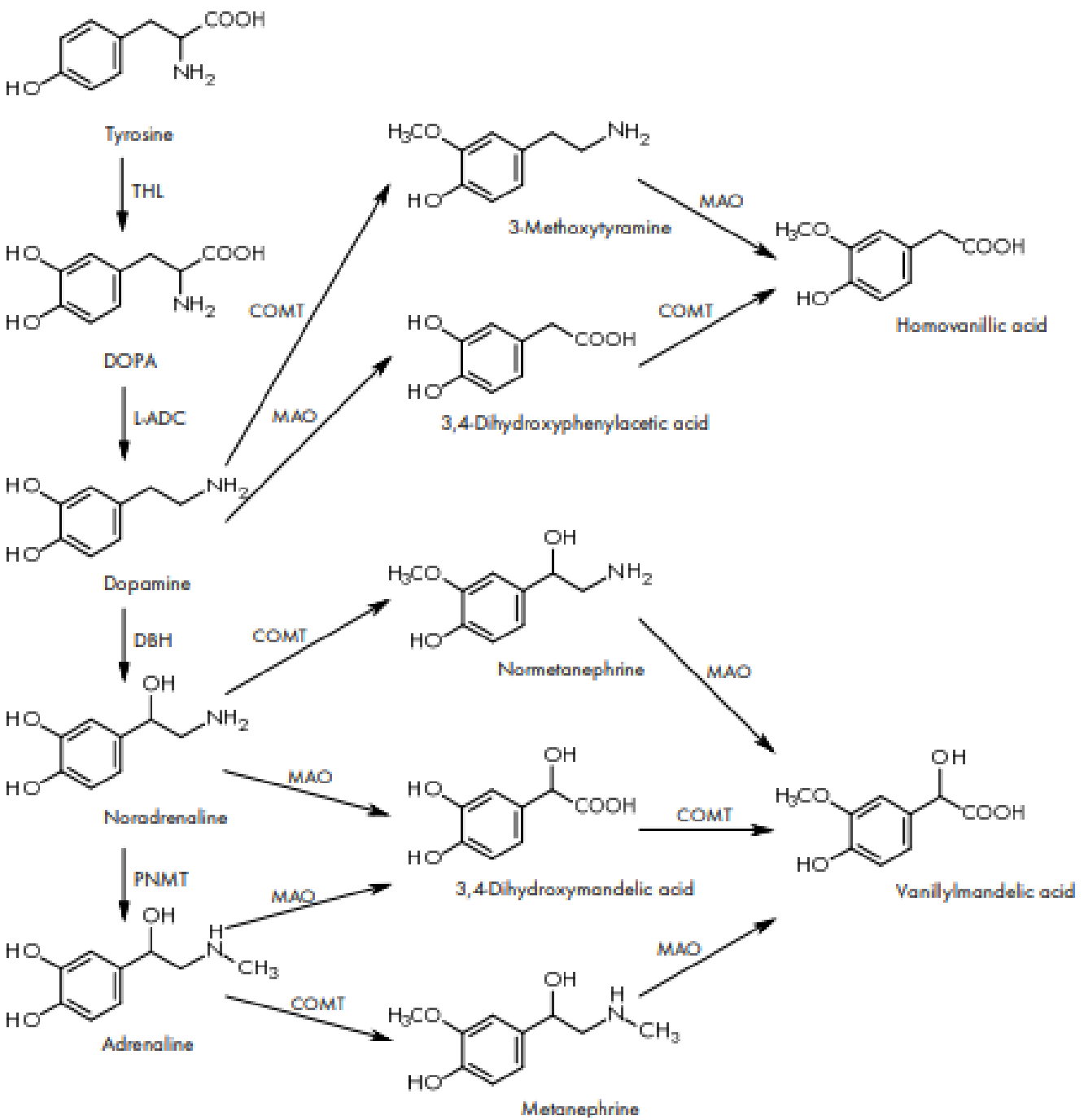
- **Advantages:**
  - High selectivity
  - MS-level sensitivity
  - Electrochemical cell is enduring and maintainable by user

- **Disadvantages:**
  - Only isocratic methods can be used efficiently
  - No opportunity to obtain structural information on analytes
  - Sensitive to chemical contaminations, requires user maintenance


Adrenal glands
Kidney
Capsule
Adrenal cortex
Adrenal medulla
Right adrenal gland
Left adrenal gland
Right renal artery
Right renal vein
Left renal artery
Left renal vein
Abdominal aorta

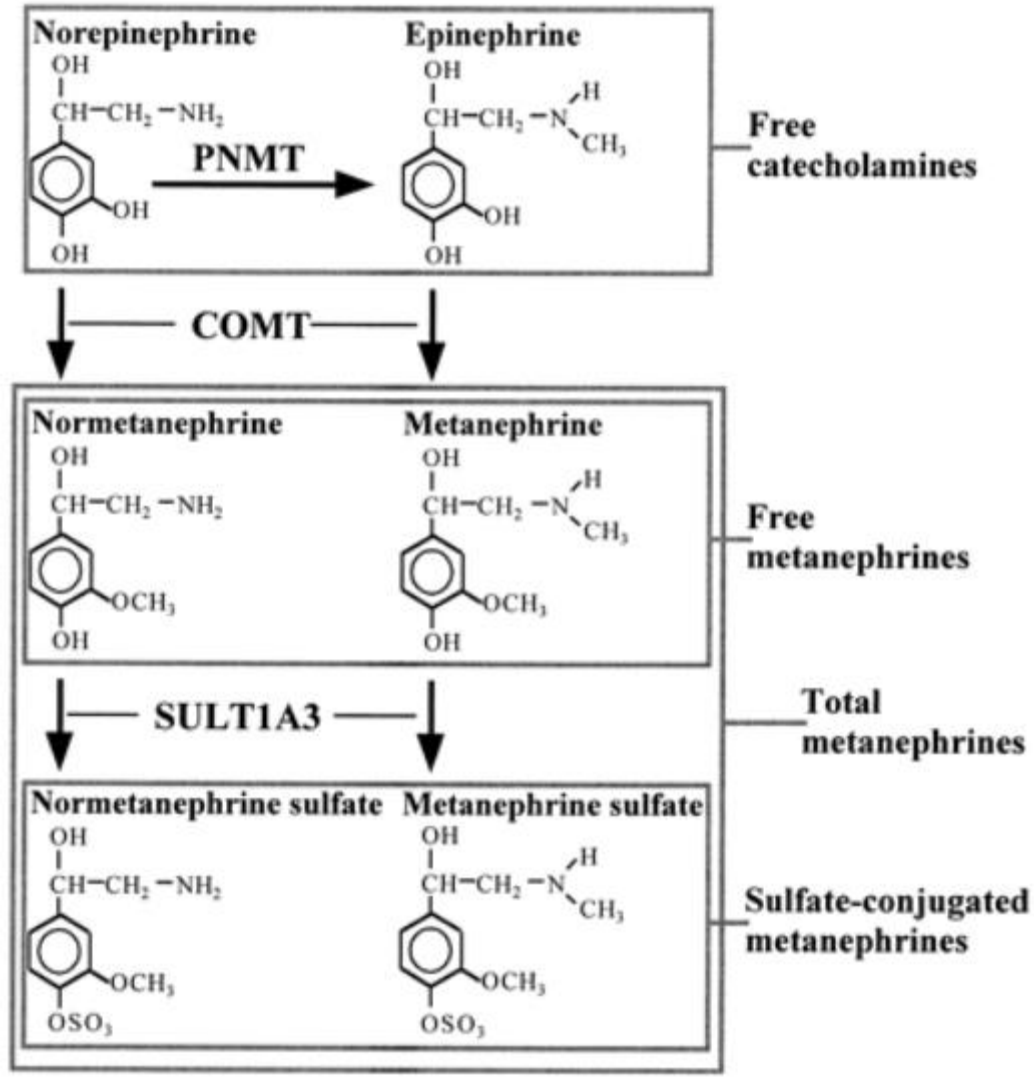

right adrenal gland
left adrenal gland
right kidney
left kidney
zona glomerulosa
zona fasciculata
zona reticularis
medulla
capsule
adrenal cortex
adrenal medulla
© 2008 Encyclopædia Britannica, Inc.


AMINO ACIDS
ADENOSINE
TYROSINE
CHROMAFFIN CELL
AMP
ADP
ATP
TYROSINE HYDROXYLASE
DOPA
DOPA DECARBOXYLASE
DOPAMINE
SIGNAL PEPTIDE
PREPROENKEPHALIN
PROENKEPHALIN
CHROMOGRANIN A
MITOCHONDRION
ENDOPLASMIC RETICULUM
RIBOSOME
DOPAMINE BETA-HYDROXYLASE
NORADRENALINE
ADRENALINE
CHROMO-GRANINS B
ENKEPHALINS
VESICLE
PHENYLETHANOLAMINE N-METHYLTRANSFERASE
EXOCYTOSIS
GOLGI COMPLEX
ENDOCYTOSIS
BLOOD-STREAM
LYSOSOME


Tyrosine
THL
DOPA
LADC
Dopamine
DBH
Noradrenaline
PNMT
Adrenaline
COMT
MAO
3-Methoxytyramine
3,4-Dihydroxyphenylacetic acid
Homovanillic acid
Normetanephrine
3,4-Dihydroxymandelic acid
Vanillylmandelic acid
Metanephrine

Chromaffin cell tumors

- account for about 0.2-0.6% of hypertension cases
- genetic disposition or sporadic
- 10-17% of tumors are malignant
- phaeochromocytoma (80-85%), paraganglioma (15-20%)
- neuroblastoma: neural crest origin, most common malignancy at 0-1 y


Norepinephrine
Epinephrine
PNMT
Free catecholamines
COMT
Normetanephrine
Metanephrine
Free metanephrines
SULT1A3
Total metanephrines
Normetanephrine sulfate
Metanephrine sulfate
Sulfate-conjugated metanephrines

Diagnostic workup of catecholamine producing chromaffin cell tumors

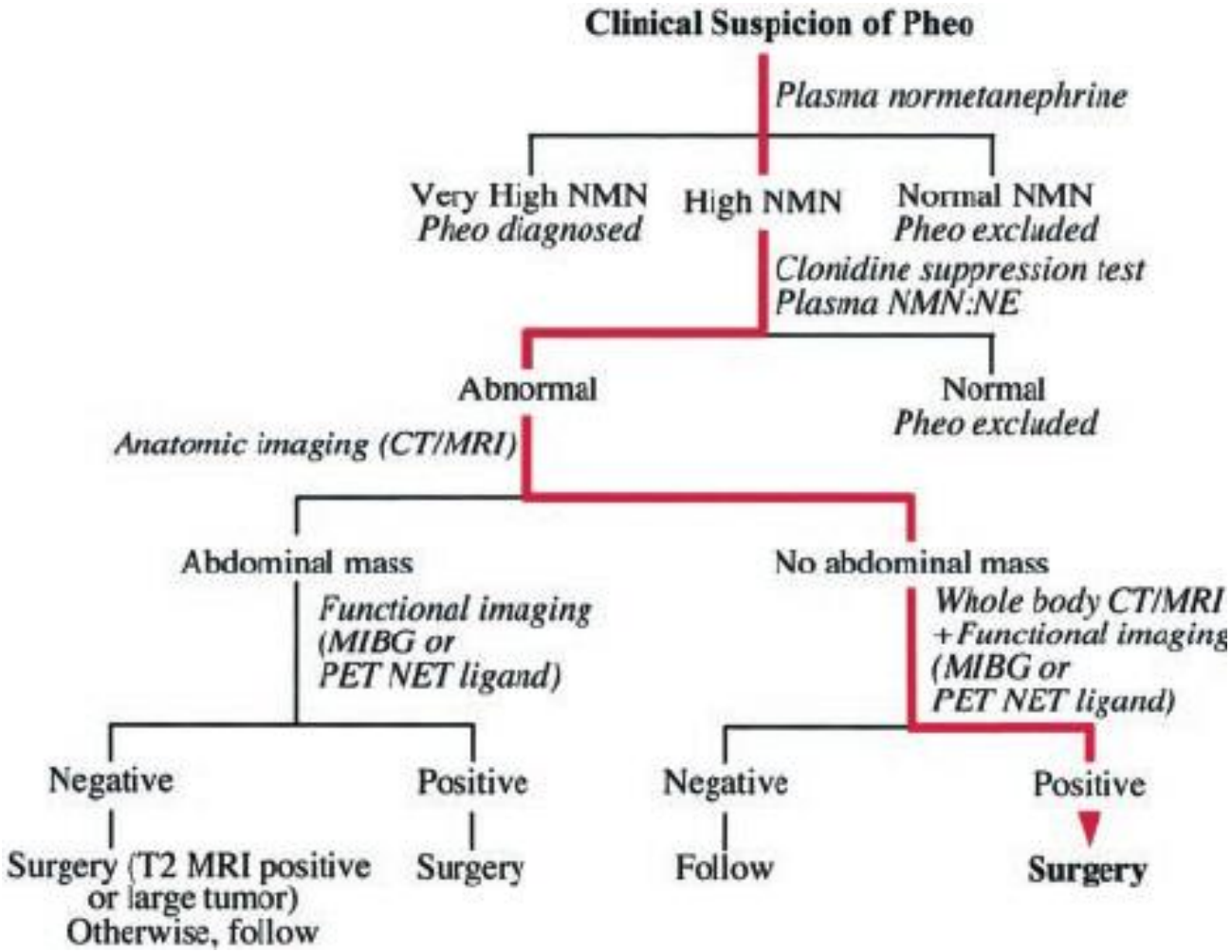


Table 1. Plasma concentrations (medians and ranges) and urinary outputs of catecholamine *O*-methylated metabolites in the reference population and patients with and without PPGLs.

Test panel	Reference population[a]	No PPGL	PPGL
Plasma free metabolites	n = 586[a]	n = 1820	n = 236
Normetanephrine, pg/mL	62 (18–201)	67 (11–365)	642 (45–25 444)
Metanephrine, pg/mL	29 (5–89)	30 (0.2–145)	120 (5–6889)
Methoxytyramine, pg/mL	4.9 (1.4–17.6)	4.8 (0.4–36.7)	14.1 (0.6–11 444)
Urine free metabolites	n = 580[a]	n = 1756[b]	n = 226[b]
Normetanephrine, μg/day	21 (4–100)	22 (1–170)	229 (9–3478)
Metanephrine, μg/day	18 (2–61)	16 (0.2–172)	64 (1–3547)
Methoxytyramine, μg/day	33 (4–136)	34 (2–212)	50 (8–3202)
Urine deconjugated metabolites	n = 581[a]	n = 1757[b]	n = 226[b]
Normetanephrine, μg/day	189 (41–803)	212 (26–2678)	1239 (172–21 850)
Metanephrine, μg/day	105 (17–446)	108 (1–991)	419 (9–14 946)
Methoxytyramine, μg/day	188 (52–2185)	197 (20–2990)	323 (58–13 031)

[a] Inclusion of the reference population in the table is to provide a comparison with patients without PPGLs. Specified ranges do not indicate the reference intervals that were used, which are supplied in the online Data Supplement. Among the 590 subjects of the reference population, measurements of plasma concentrations and urinary outputs of metabolites were not possible in up to 10 patients.

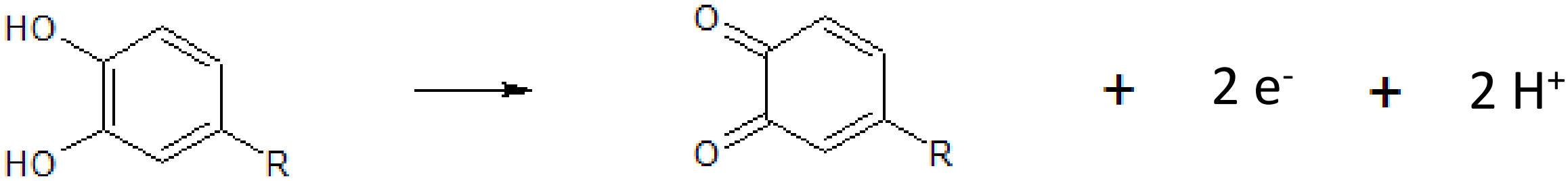
[b] Urinary measurements were not possible in up to 64 of the 1820 patients without PPGLs and 10 patients with PPGLs. To convert pg/mL to pmol/L and μg/day to nmol/day, divide values for normetanephrine, metanephrine, and methoxytyramine by 0.1832, 0.1972, and 0.1672, respectively.

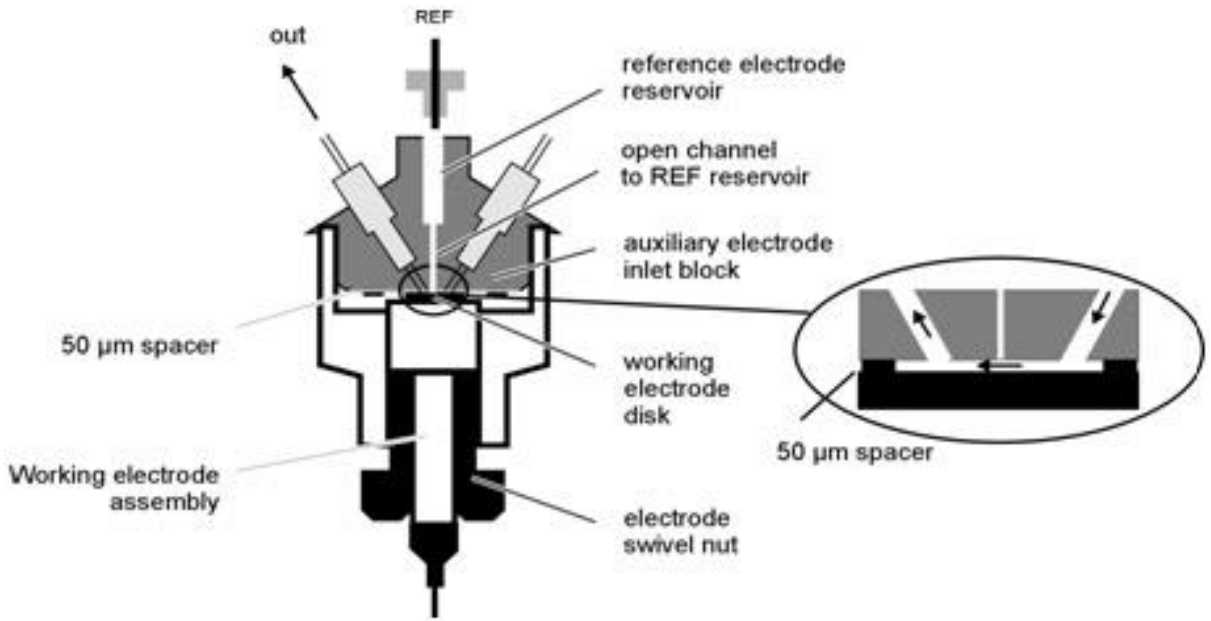
Eisenhofer G et al. Clin Chem 2018;64:1646.

The prerequisite of conducting efficient biochemical tests is a precise preanalytical workflow

- **Plasma tests**:
  - correct collection tube
  - seated vs supine sampling
  - sampling environment (patient stress)
  - specimen storage and transport

- **Urine tests:**
  - collected vs random urine
  - pH control
  - specimen storage and transport


HO
HO
R
O
O
R
+ 2 e- + 2 H+


out
REF
reference electrode reservoir
open channel to REF reservoir
auxiliary electrode inlet block
50 µm spacer
working electrode disk
50 µm spacer
Working electrode assembly
electrode swivel nut

SUMMARY OF RECOMMENDATIONS

1.0 Biochemical Testing for Diagnosis of Pheochromocytoma and Paraganglioma (PPGL)

1.1 We recommend that initial biochemical testing for PPGLs should include measurements of plasma free metanephrines or urinary fractionated metanephrines. (**1**|⊕⊕⊕⊕)

1.2 We suggest using liquid chromatography with mass spectrometric or electrochemical detection methods rather than other laboratory methods to establish a biochemical diagnosis of PPGL. (**2**|⊕⊕◯◯)

1.3 For measurements of plasma metanephrines, we suggest drawing blood with the patient in the supine position and use of reference intervals established in the same position. (**2**|⊕⊕◯◯)

1.4 We recommend that all patients with positive test results should receive appropriate follow-up according to the extent of increased values and clinical presentation. (**1**|⊕⊕◯◯)

Weak recommendation

Low quality evidence

Lenders JWM et al. J Clin Endocrin Metab 2014;99:1915.

Catecholamines:
epinephrine, norepinephrine, dopamine

urine: free

- extraction using cation exchange
- WE: 550 mV
- analysis time: <5 min

plasma: free

- extraction using aluminum oxide
- WE: 550 mV
- SP: ambient
- analysis time: <20 min

Metanephrines:
metanephrine, normetanephrine, 3-methoxytyramine

urine: free or total

- hydrolysis of conjugated fraction (cc acid, 95 °C, 30 min)
- extraction using cation exchange
- WE: 750 mV
- analysis time: <10 min

plasma: free

- extraction using cation exchange
- SP: ambient
- analysis time: <5 min

End products:
vanilmandelic acid, homovanillic acid

urine: free

- extraction using anion exchange
- WE: 700-850 mV
- SP: ambient
- analysis time: <20 min

Electrochemical detection: amperometric
Working electrode: glassy carbon
Reference electrode: Ag/AgCl

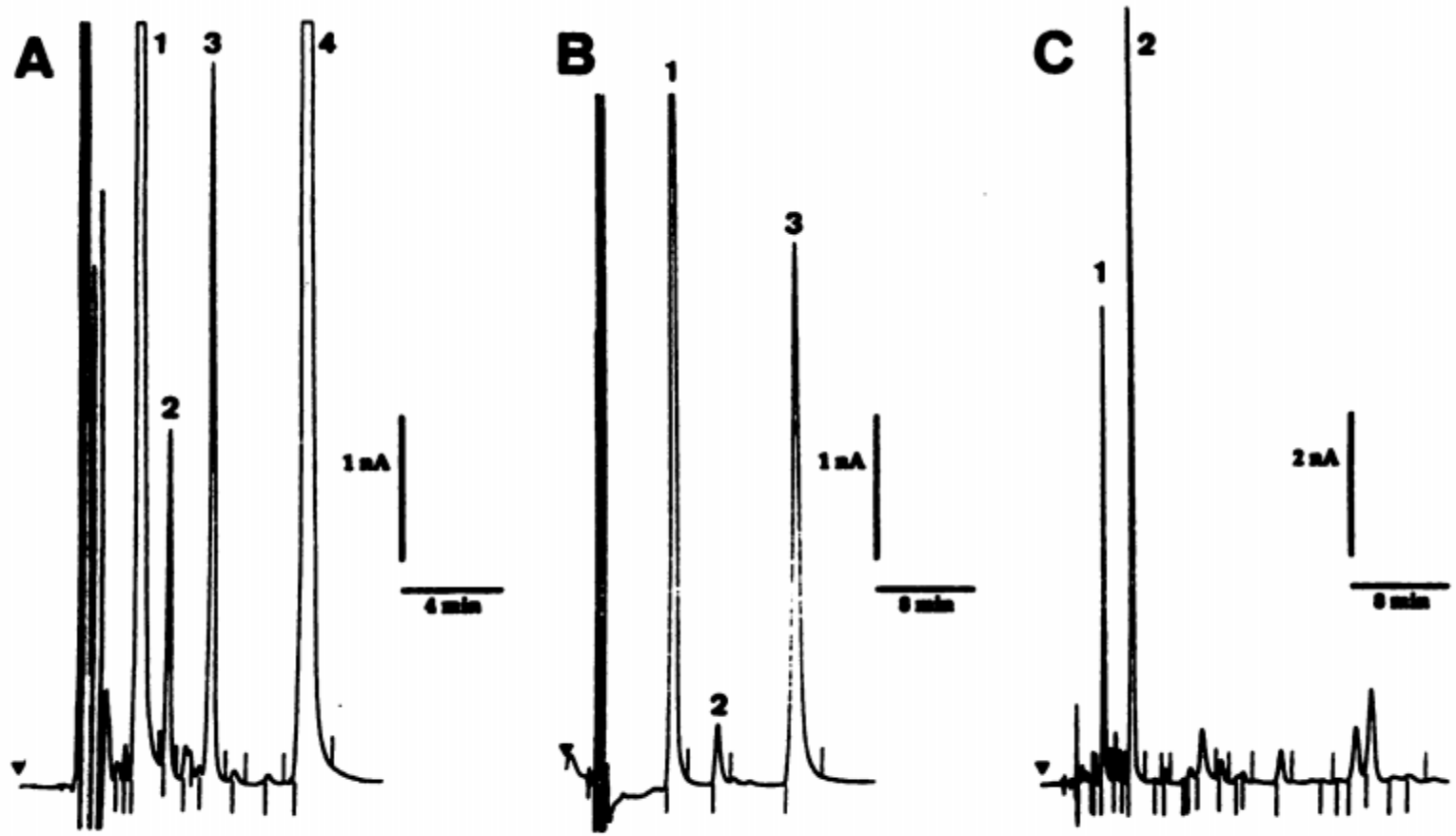
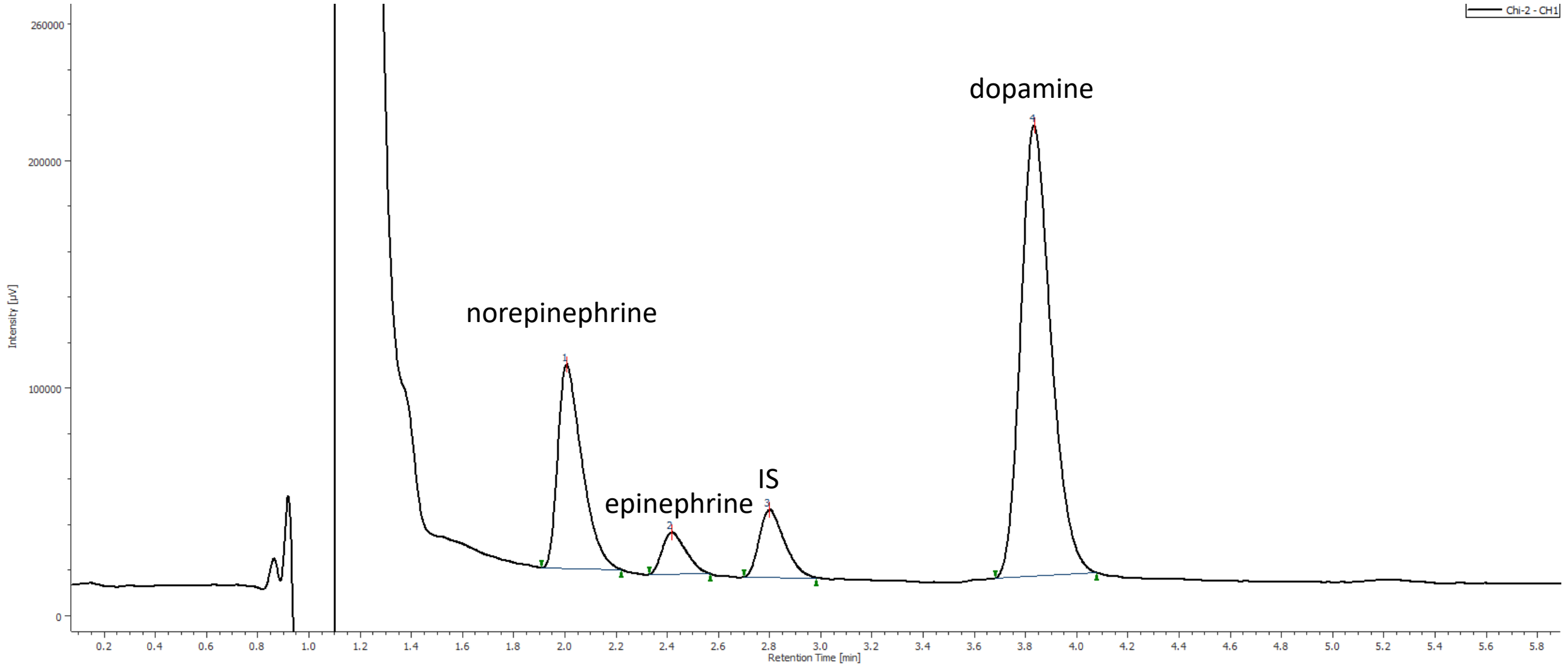
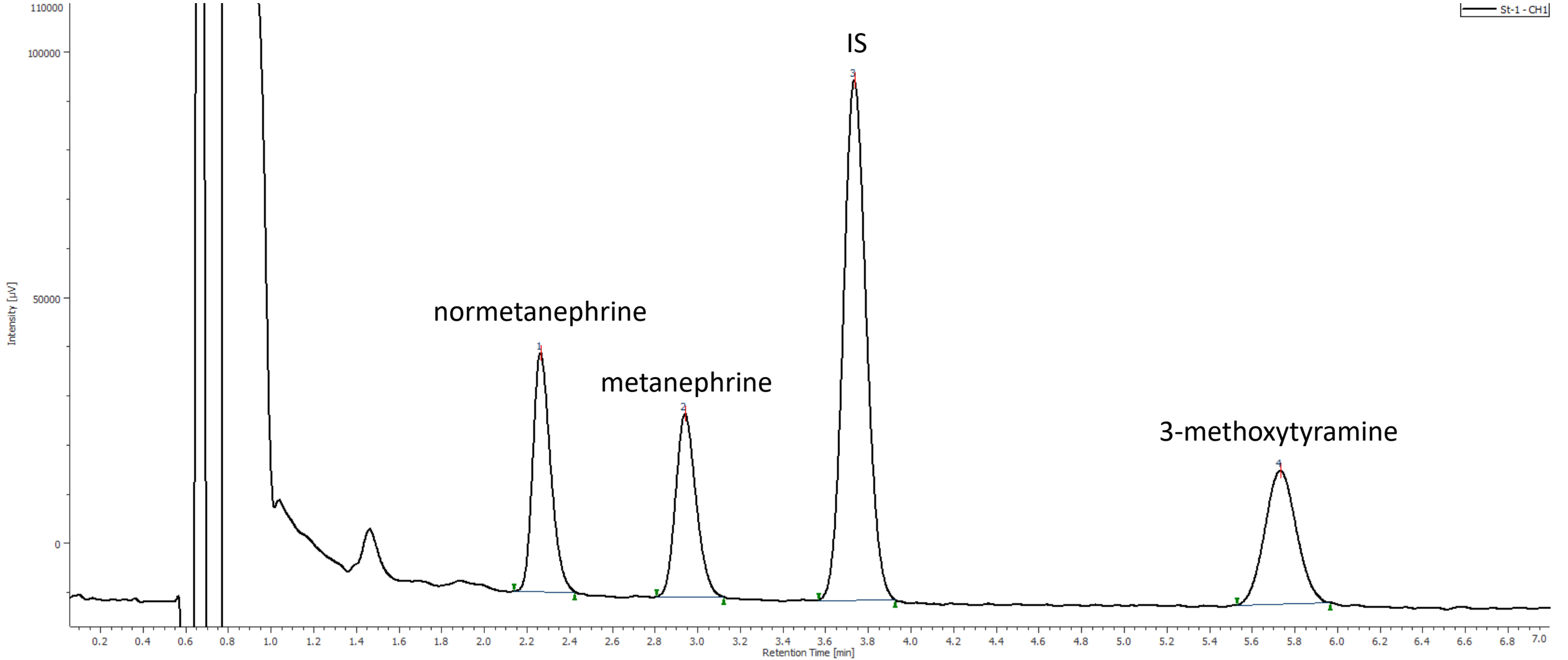
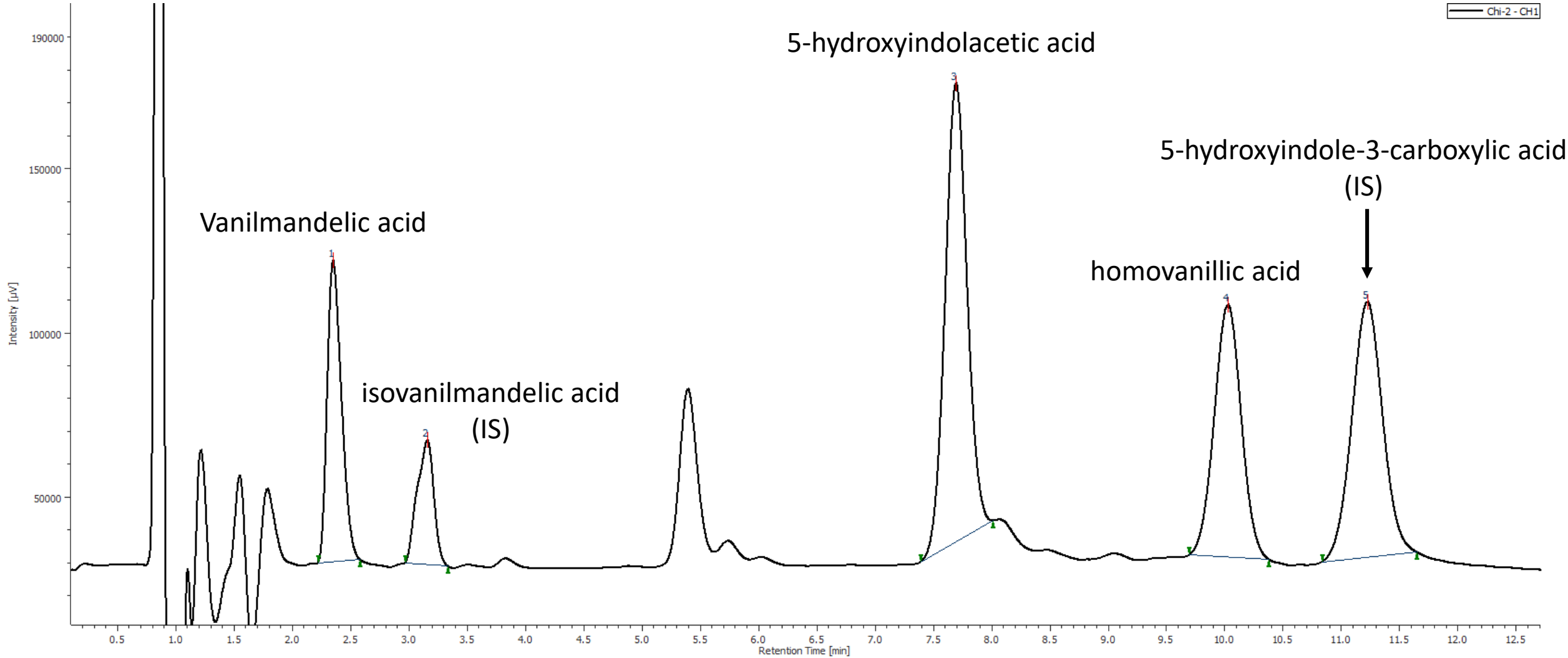


Fig. 1. Chromatograms of urinary catecholamines (A), metanephrines (B), and VMA (C) from a patient with a predominantly NE-secreting pheochromocytoma (no. 8).

A: (*1*) norepinephrine, 5.1 μmol/L; (*2*) epinephrine, 0.13 μmol/L; (*3*) dihydroxybenzylamine (internal standard); (*4*) dopamine. *B*: (*1*) normetanephrine, 19 μmol/L; (*2*) metanephrine, 1.0 μmol/L; (*3*) MHBA (internal standard). *C*: (*1*) VMA, 49 μmol/L; (*2*) iso-VMA (internal standard). The 24-h urinary volume was 1.65 L.


Chi-2 - CH1
dopamine
norepinephrine
epinephrine
IS
1
2
3
4
Intensity [µV]
260000
200000
100000
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
5.2
5.4
5.6
5.8
Retention Time [min]


St-1 - CH1
110000
100000
50000
0
Intensity [µV]
normetanephrine
metanephrine
IS
3-methoxytyramine
1
2
3
4
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6 4.8 5.0 5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6 6.8 7.0
Retention Time [min]


Chi-2 - CH1
5-hydroxyindolacetic acid
Vanilmandelic acid
isovanilmandelic acid
(IS)
homovanillic acid
5-hydroxyindole-3-carboxylic acid
(IS)
Intensity [µV]
Retention Time [min]