Vysokoúčinná kapalinová chromatografie

Petr Kozlík
Katedra analytické chemie
e-mail: kozlik@natur.cuni.cz

Aplikace HPLC

Analýza složek životního prostředí

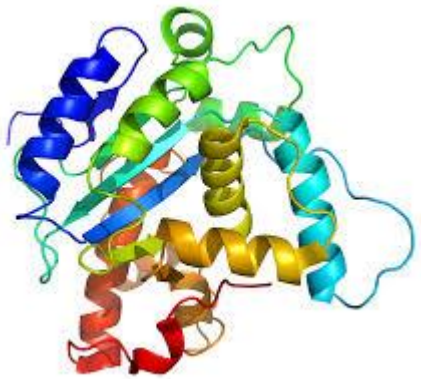
Toxikologie

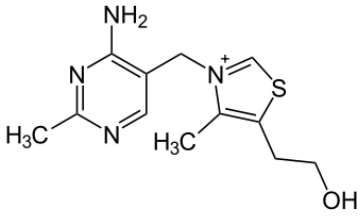
Potravinářská analýza

Farmaceutická analýza

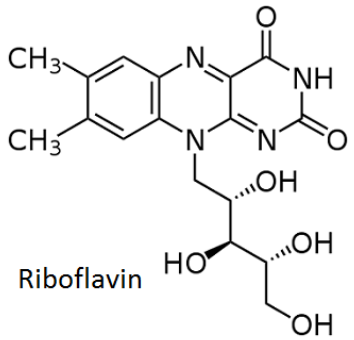
Aplikace HPLC

- Aminokyseliny
- Peptidy
- Proteiny
- Lipidy
- Sacharidy
- Steroidy
- Vitamíny
- Barviva
- Léčiva
- Drogy
- Metabolity

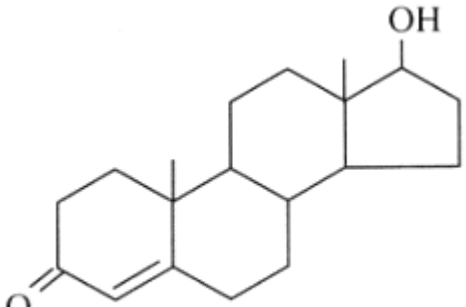




thiamine

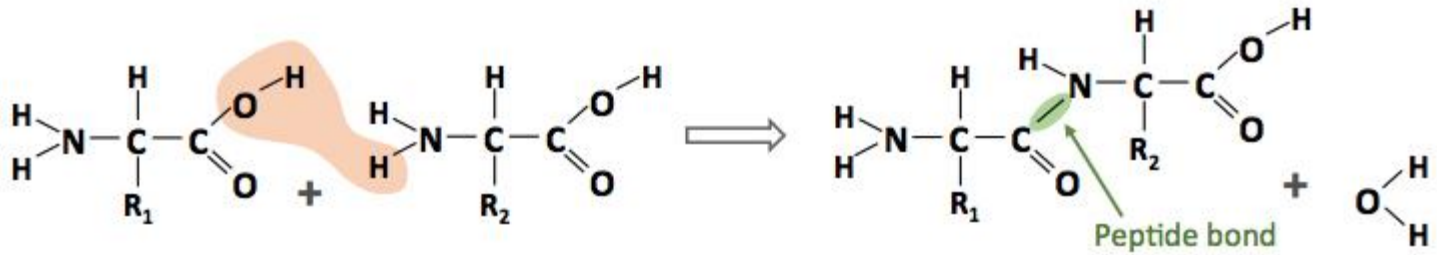


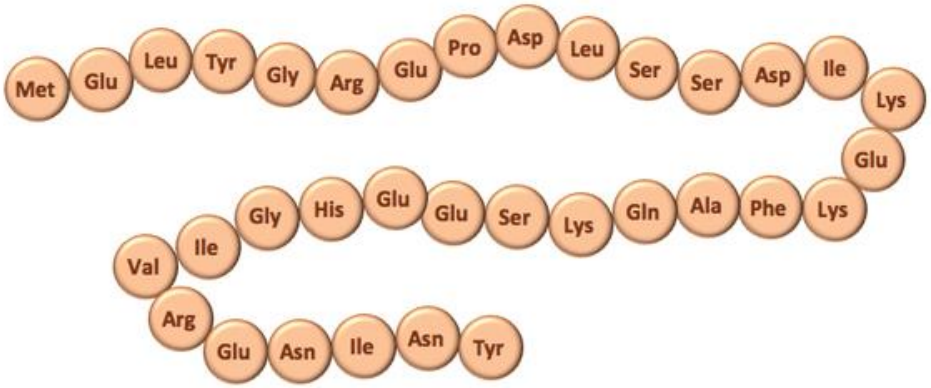
Riboflavin

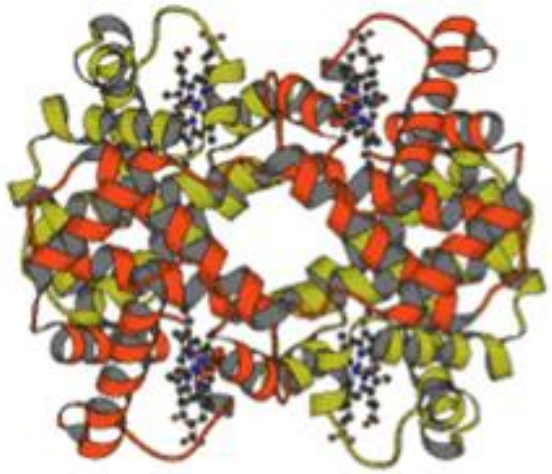


Testosterone

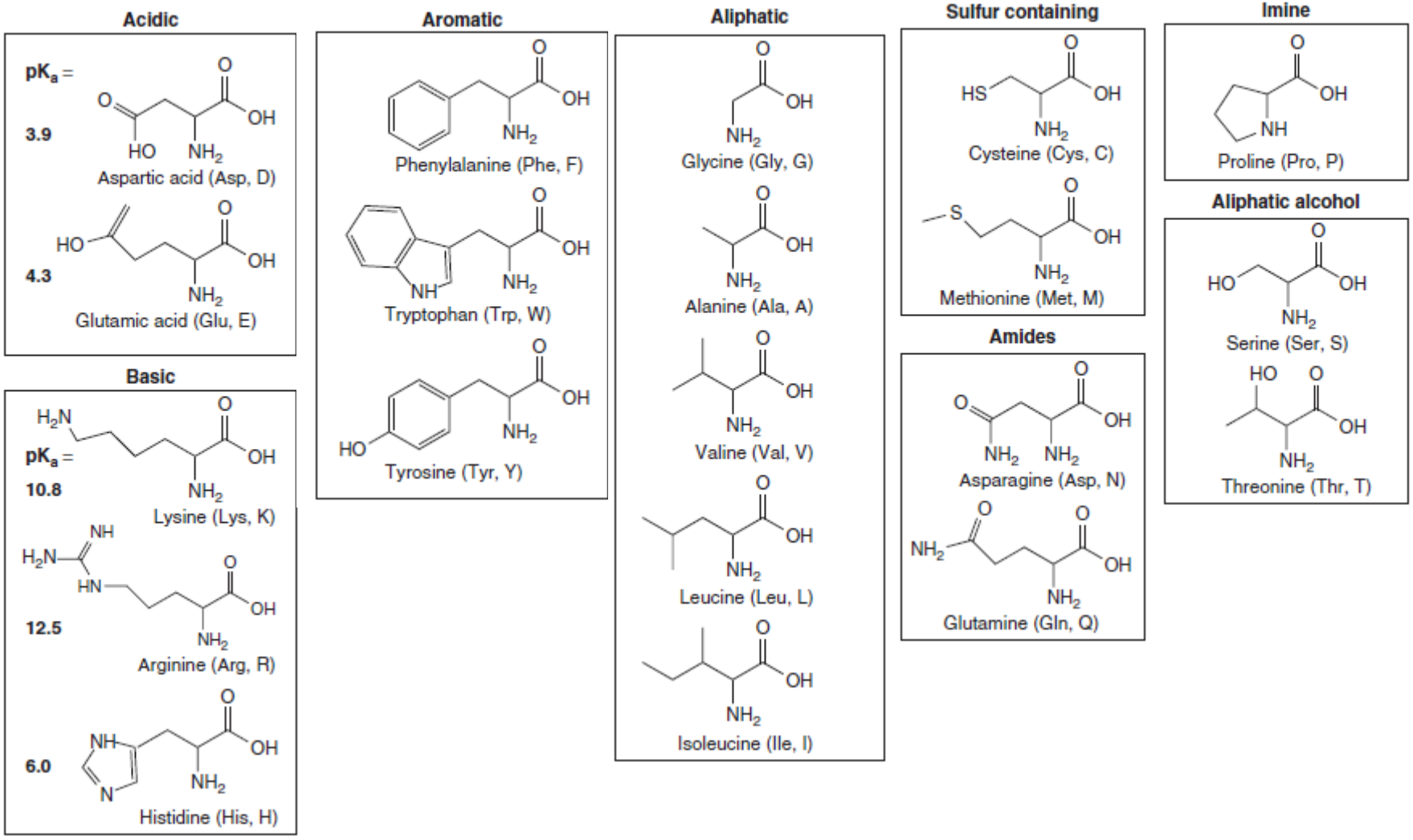
HPLC proteinu, peptidu a aminokyselin







Analýza aminokyselin



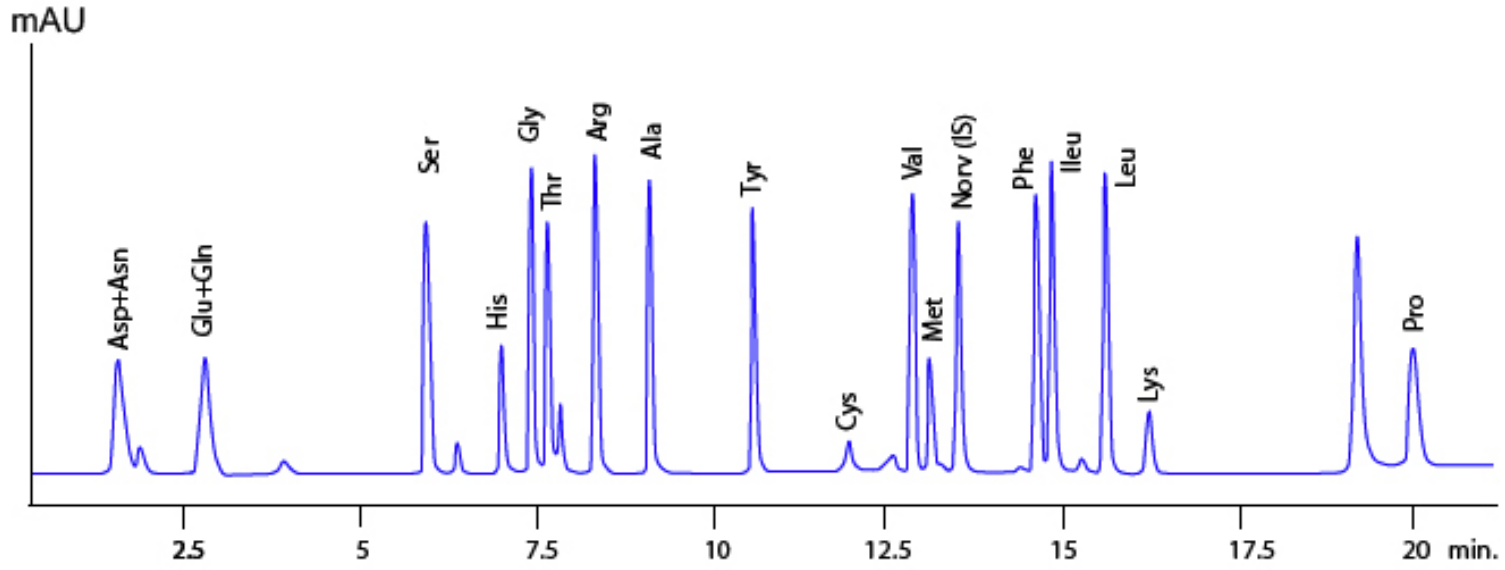
Analýza aminokyselin

Hydrolýza – 6M HCl po dobu 16 hodin

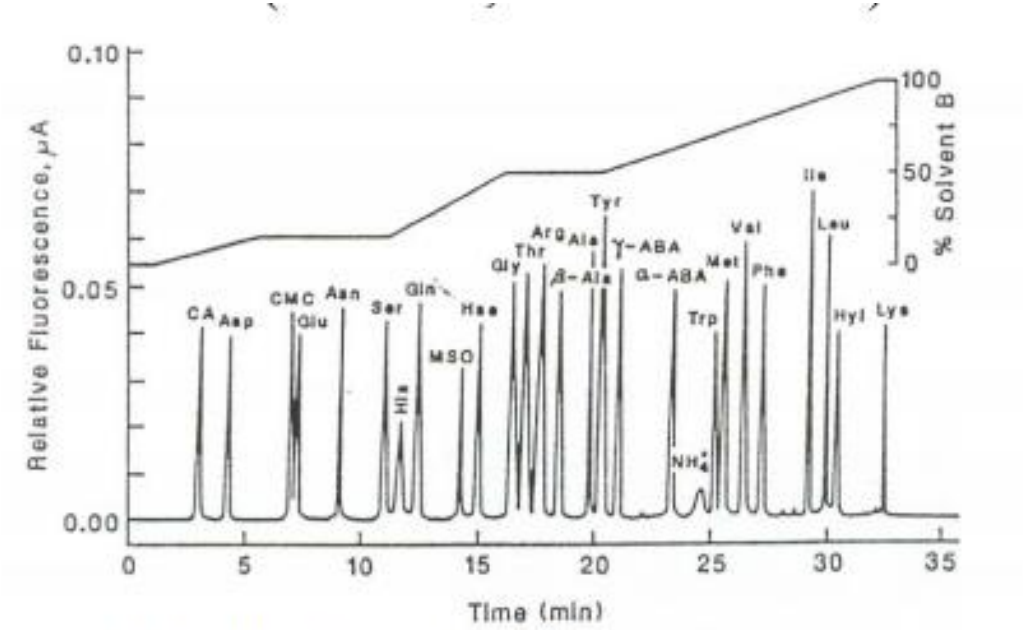
Derivatizace – stabilní UV/fluoro značka – OPA (o-phtaldehyd) nebo FMOC (fluorenylmethyloxycarbonyl)

Separace – RP, C18, gradient ACN/pufr

Detekce – UV nebo fluorescenční



Analýza aminokyselin



RP-HPLC směsi derivátů aminokyselin s OPA a ME
kolona C_{18}, 5μm, 250×4,6 mm, předkolona C_{18}, 40μm, 80×4,6 mm
mobilní fáze A: THF+ MeOH+ 0,05M NaOAc (pH 5,9) 1+19+80
mobilní fáze B: MeOH + 0,05M NaOAc (pH 5,9) 80+20
průtok 1,7 ml/min
fluorescenční detekce
nástřik 5 pmol (aminokyseliny), NH_3 (20 pmol)

Analýza peptidů

Určení pořadí AMK

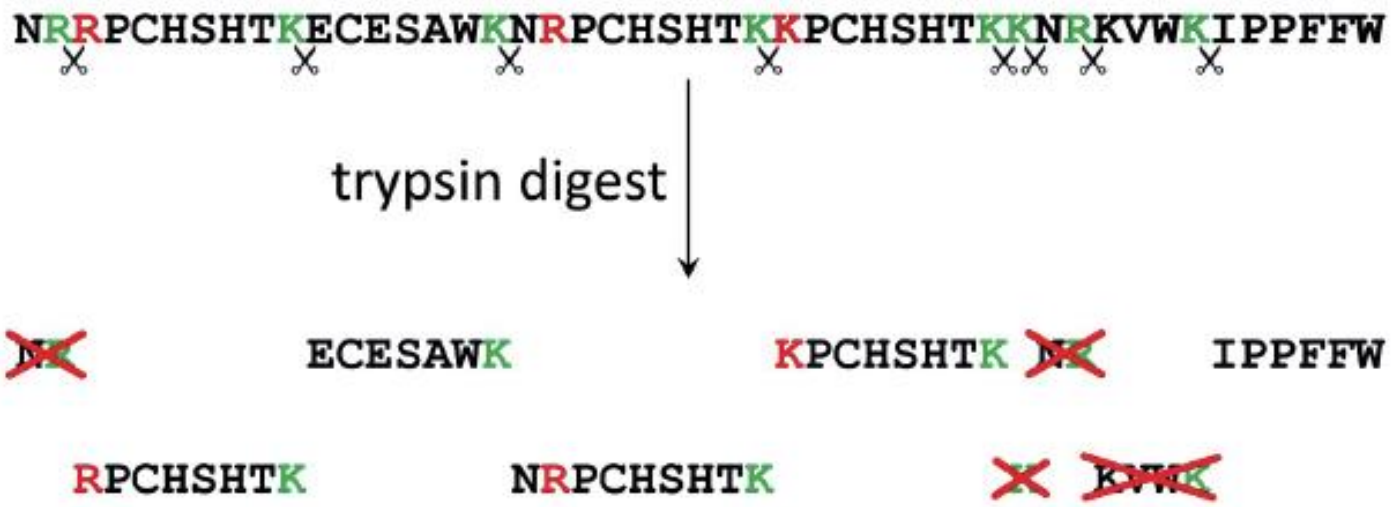
Odhalení modifikací AMK

Používá se bottom up přístup: enzymatické štěpení

Enzyme	Site of Cleavage
Trypsin	Lys, Arg (C)
Chymotrypsin	Phe, Trp, Tyr (C)
Asp-N-protease	Asp, Glu (C)
Pepsin	Leu, Phe, Trp, Tyr (N)
Elastase	Ala, Gly, Ser (C)
Cyanogen bromide	Met (C)
Endoproteinase Lys C	Lys (C)

Analýza peptidů

Redukce – surfaktant + DTT – dithiotreitol
Alkylace: IAA – 2-iodoacetamid
Enzymatické štěpení – trypsin – štěpí za Lys (K) a Arg (R)



Analýza peptidů

RP HPLC

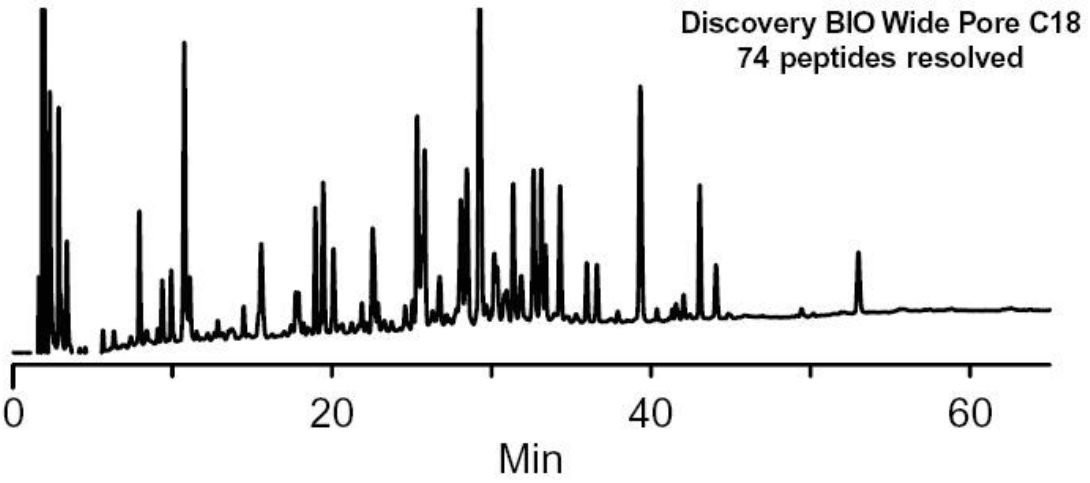
Stacionární fáze: C18

Gradientová eluce: ACN/kys. mravenčí; TFA

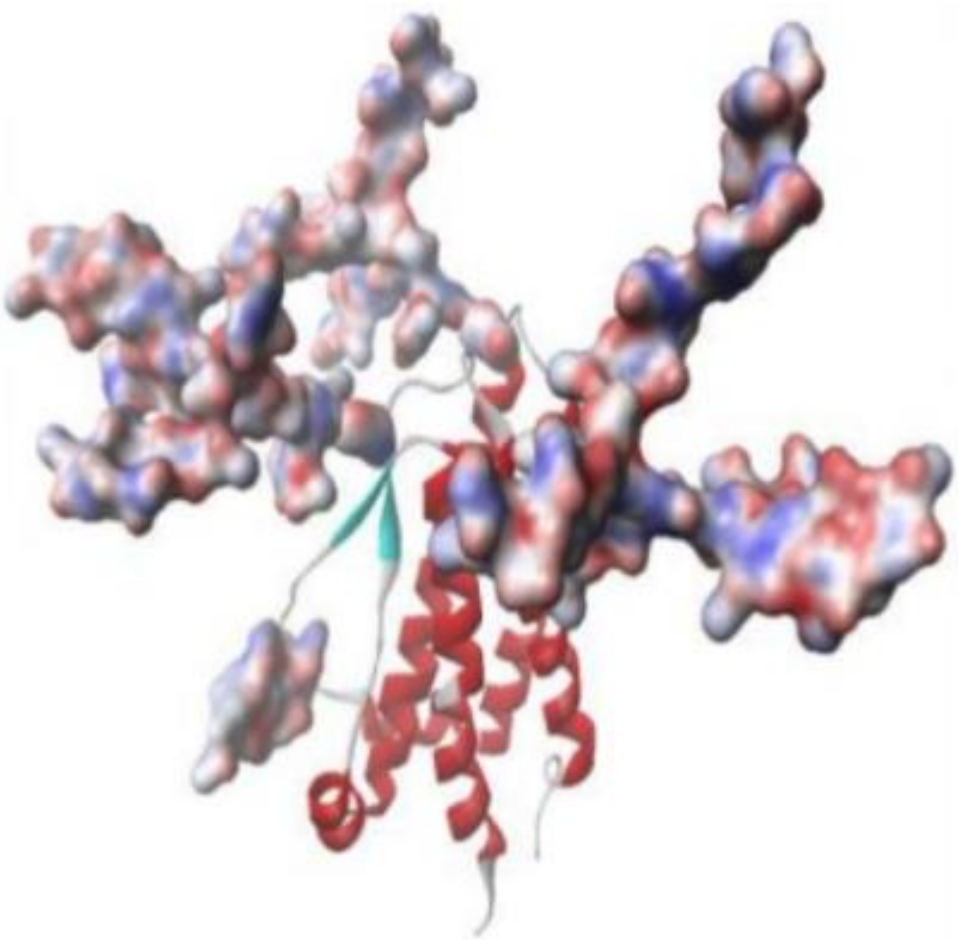
Detekce: MS/MS, UV při nízkých vlnových délkách

Tryptic Digest of Carboxymethylated Apohemoglobin on a Discovery Wide Pore C18

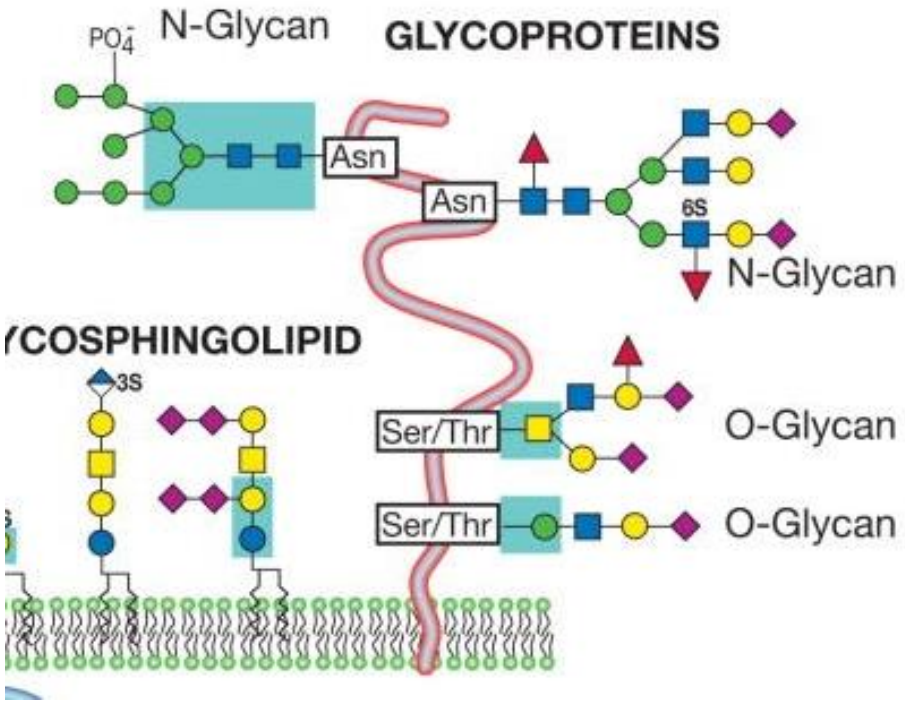
Column:	Discovery BIO Wide Pore C18, 15cm x 4.6mm, 5µm
Mobile Phase:	(A) 95:5, (0.1% TFA in water):(0.1% TFA in CH_3CN);
	(B) 50:50, (0.1% TFA in water):(0.1% TFA in CH_3CN)
Flow Rate:	1.0mL/min
Temp.:	30°C
Detection:	215nm
Injection:	50µL carboxymethylated apohemoglobin tryptic digest in 50mM NH_4HCO_3
Gradient:	0-100%B in 65 min

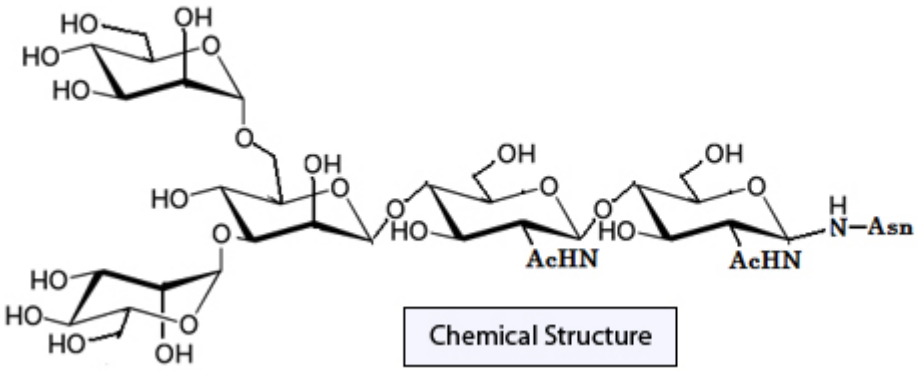


Analýza glycopeptidů



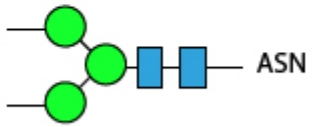
Analýza glycopeptidů



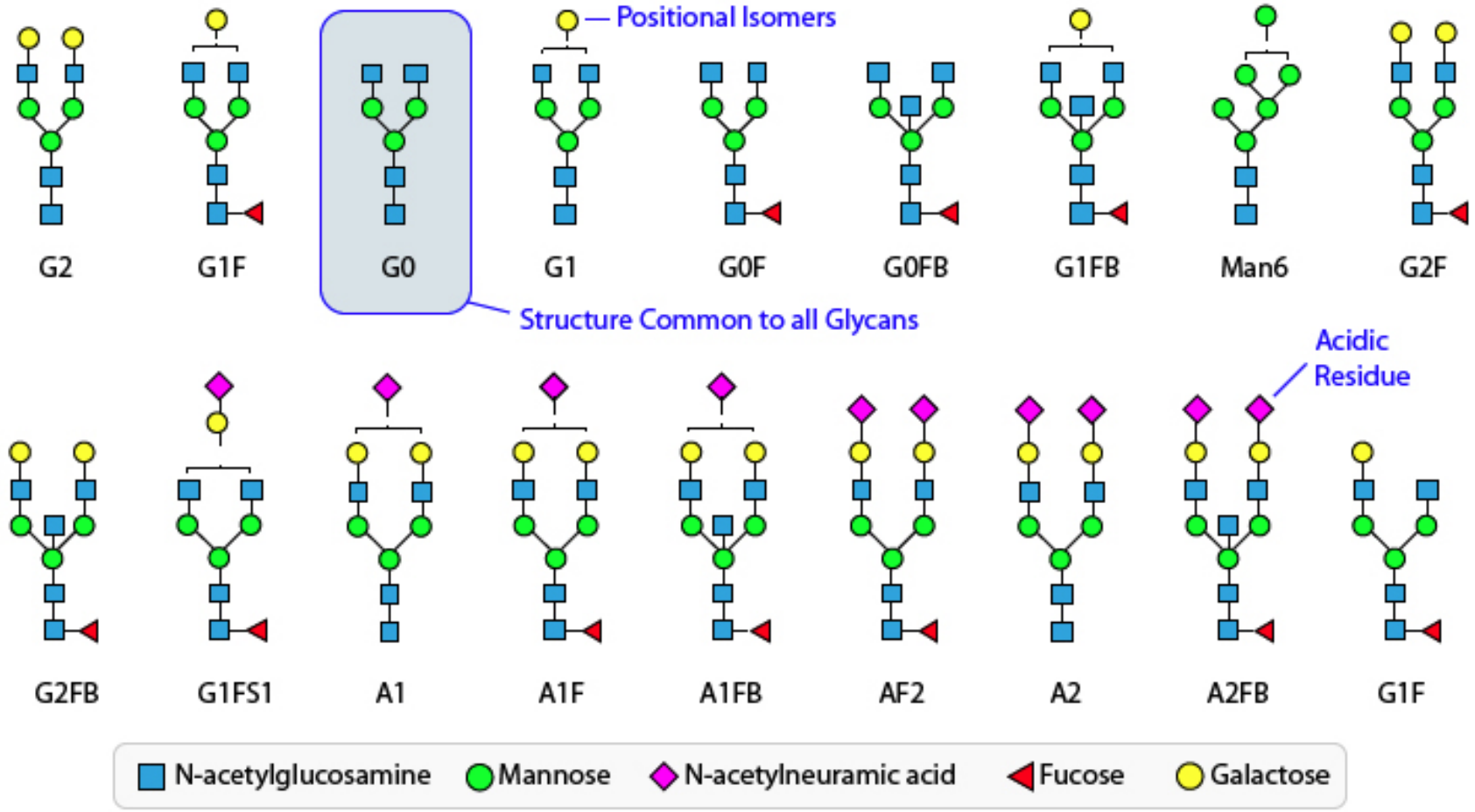


Chemical Structure

Cartoon Structure

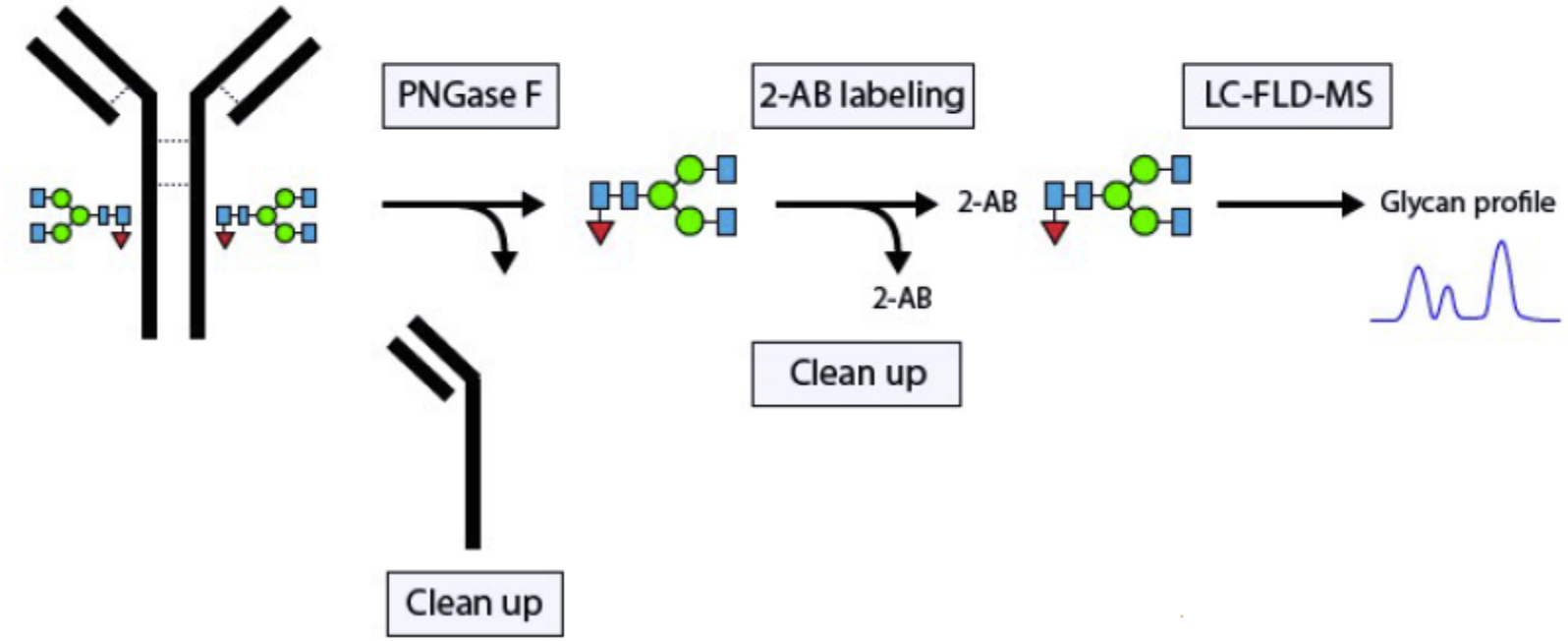


Analýza glycopeptidů



Analýza glycopeptidů

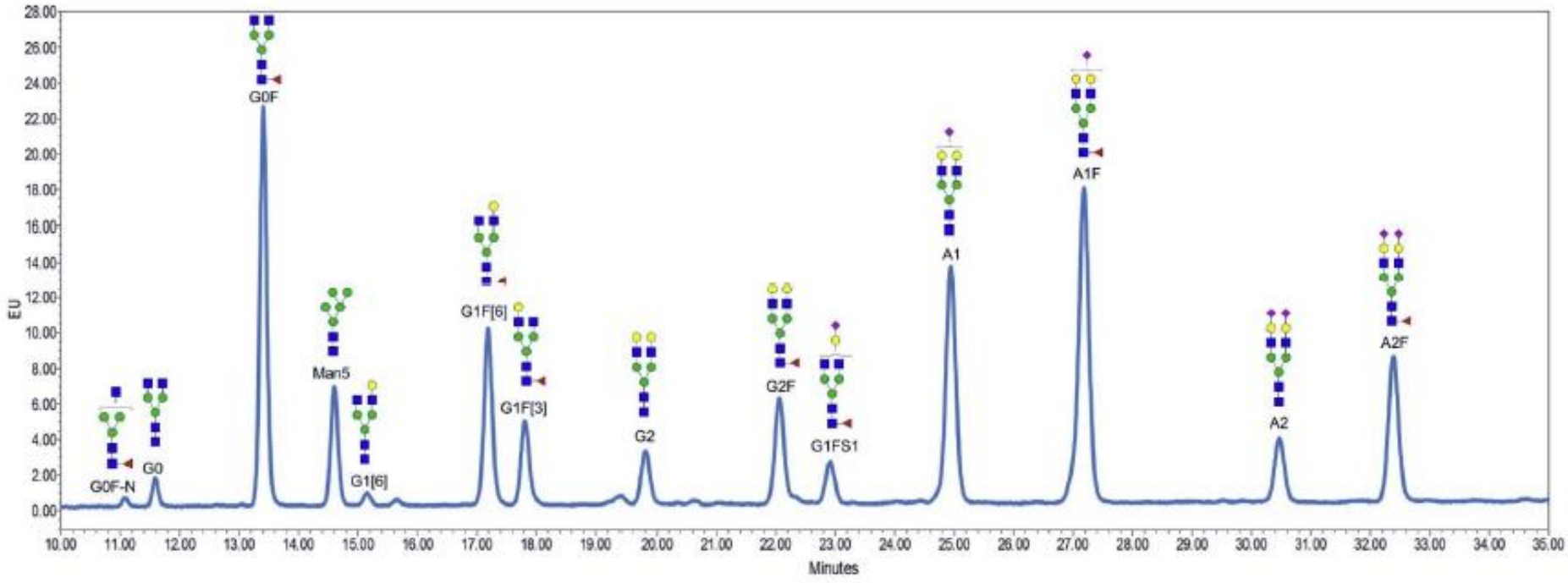
- redukce DTT, alkylace IAA
- enzymatické štěpení PNGasa F: 37 °C, 18 hod, pouze N-glykany!
- přečištění HILIC-SPE
- značení 1-aminobenzamidem (reduktivní aminace, 65 °C, 3 hod)
- alternativou je prokainamid



Analýza glycopeptidů

Instrument:	(U)HPLC
Mobile Phase A:	100 mM ammonium formate pH 4.4
Column:	150 mm L x 2.1 mm ID x < 2 µm FPP Amide
Mobile Phase B:	Acetonitrile
Flow Rate:	400 µL/min
Gradient:	0-25 min. 80-60%B 25-30 min. 60-20%B~
Column Temperature:	55 °C
Injection Vol:	5 µL
Sample solvent:	1:1 v/v Water: Acetonitrile
FLD Settings*:	Excitation = 260 nm, Emission = 430 nm

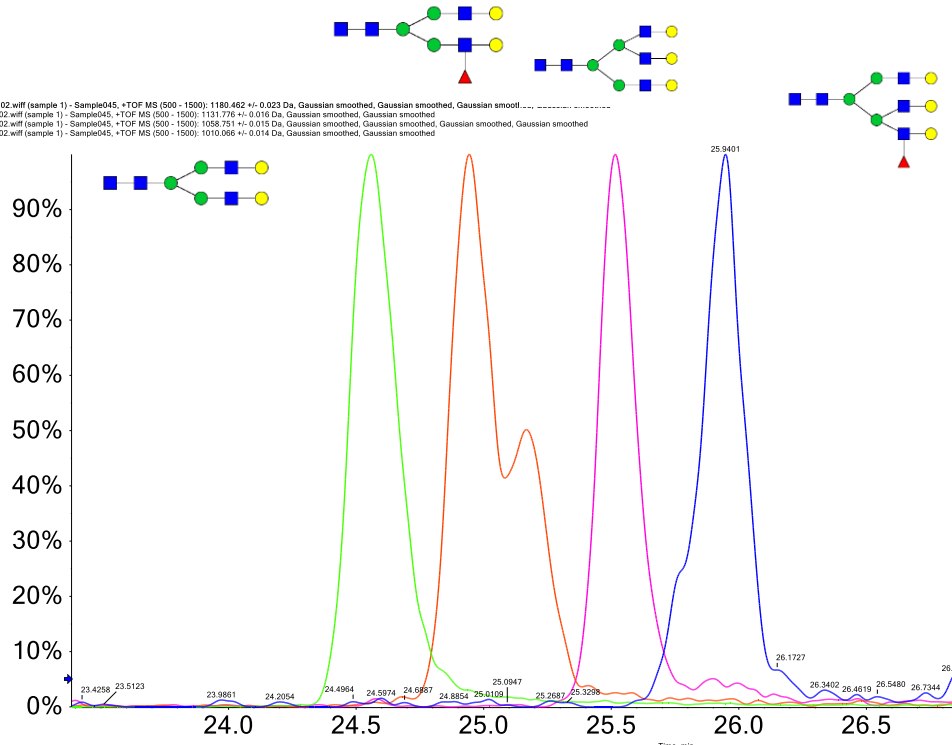
Analýza glycopeptidů

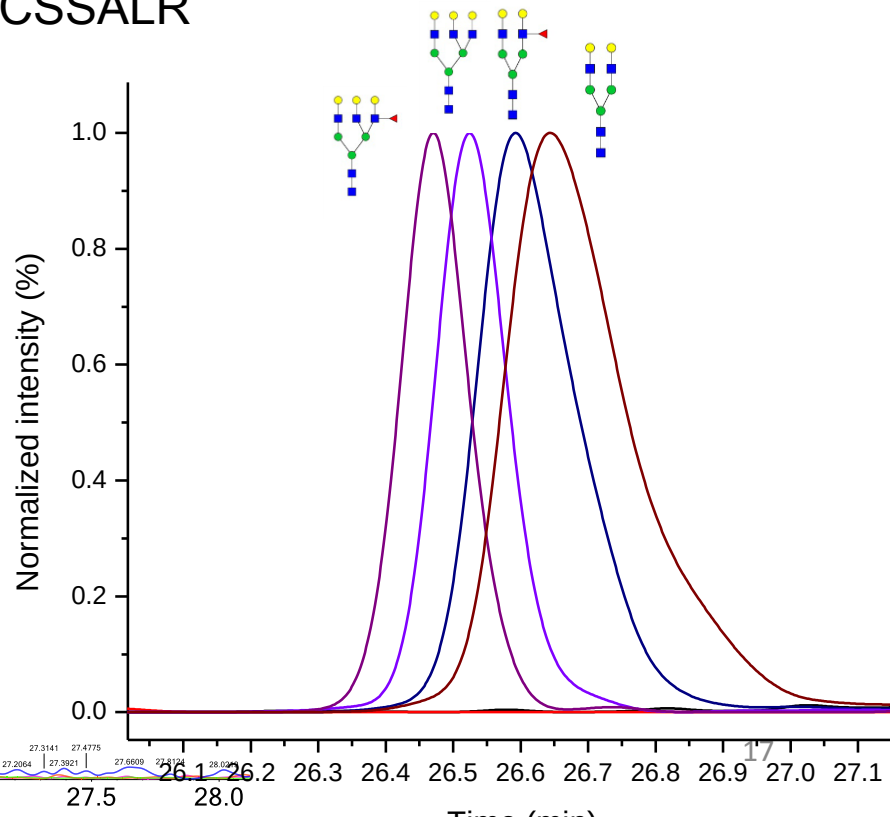


Analýza glycopeptidů

Instrument	Eksigent cHiPLC+5600TripleToF	Acquity+6600TripleToF
Column	HALO Hilic, **2.7 µm**; 0.075 x 150 mm	Peptide BEH C18; **1.7 µm**; 0.075 x 150 mm
A	2% Acetonitrile with 0.1% HCOOH	2% Acetonitrile with 0.1% HCOOH
B	0.1% HCOOH in Acetonitrile	0.1% HCOOH in Acetonitrile
Sample volume (nL)	300	1000
Flow rate (nL/min)	500	300
Column temperature (°C)	30	40
Gradient %B (min)	90-50-50-90-90 (0-45-45-46-60)	1-43-99-99-1-1 (0-60-65-75-77-97)
Slope (%/min)	1	0.7

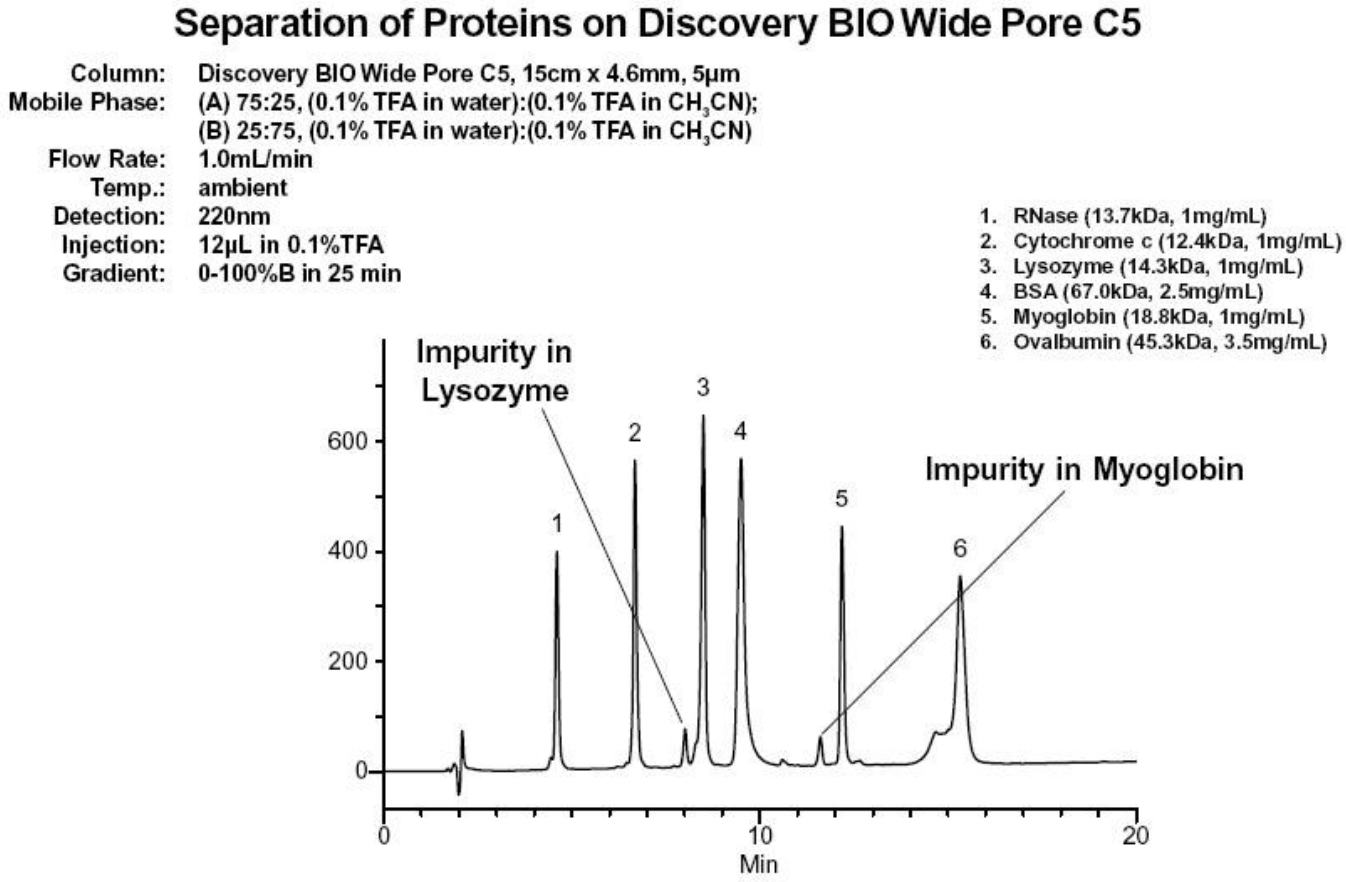
SWPAVGNCSSALR





Analýza proteinů

Proteiny lze separovat na základě celkové hydrofobicity, velikosti molekul a počtu nabitých skupin. Nejčastěji využívanými separačními systémy jsou: reverzní chromatografie, vylučovací (size-exclusion, gelová) chromatografie a iontově výměnná chromatografie.



Analýza proteinů

HIC – hydrofobní interakční chromatografie

- **stacionární fáze:** C4, C8, propylalkylamid
  100 x 4,6 mm, 5 µm, 1000 Å
- **mobilní fáze:** $(NH_4)_2SO_4$ + 0,1 M fosforečnanový pufr pH 7
  0,1 M fosforečnanový pufr pH 7
- **gradientová eluce:** 0 – 100% pufru
- **průtok:** 1 mL/min
- **teplota:** 25 °C (nedenaturující)
- **detekce:** UV, FLD

SEC – size exlusion chromatography

- **stacionární fáze:** silikagel nebo organický polymer (PMA)
  300 nebo 150 x 4,6 mm, 3 nebo 5 µm
- **mobilní fáze:** vodná, vysoká koncentrace soli
  150 mM fosfátový pufr pH 7 ± 100 mM NaCl
- **isokratická eluce:** 30 – 60 min: 0,1 – 0,4 mL/min
- **teplota** 20 – 30 °C
- **detekce:** UV, FLD, RI

Analýza proteinů

IEX – iontově výměnná chromatografie

- **stacionární fáze:** **CAX**
- **mobilní fáze:** **vodná, vysoká koncentrace soli**
- **gradientová eluce:** **pH, koncentrace soli**
- **detekce:** **non-MS**

Analýza lipidů

Rozdělení lipidů

Jednoduché

- triacylglyceroly
- vosky
- ceramidy
- nerozpustné ve vodě
- rozpustné v lipofilních rozpouštědlech

Složené

- glycerofosfolipidy
- sfingofosfolipidy
- sfingoglykolipidy

Analýza lipidů

Normální chromatografie – nepolární třída lipidů, separace na základě polarity

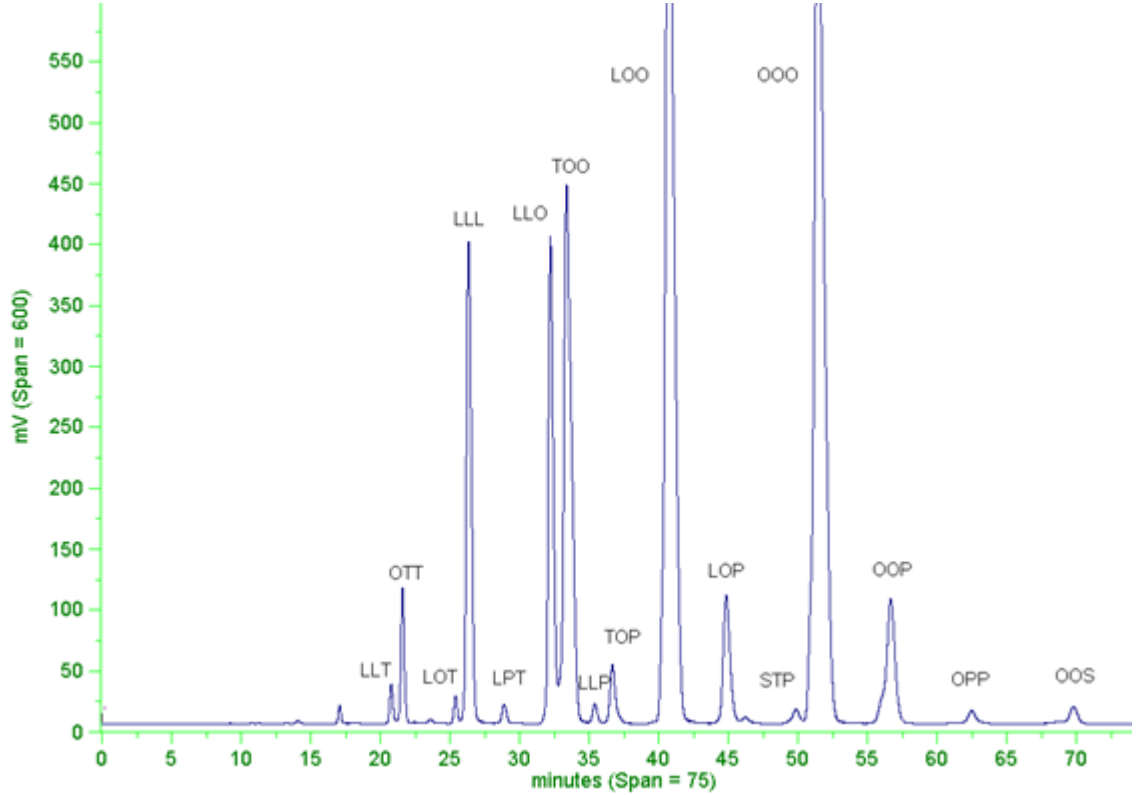
Reverzní chromatografie – délka acylového řetězce, množství a pozice dvojných vazeb

HILIC – separace na základě polarity a elektrostatických interakcí

Argentační chromatografie – tvorba slabých reverzibilních komplexů iontů stříbra a pí elektronů dvojných vazeb

Analýza lipidů

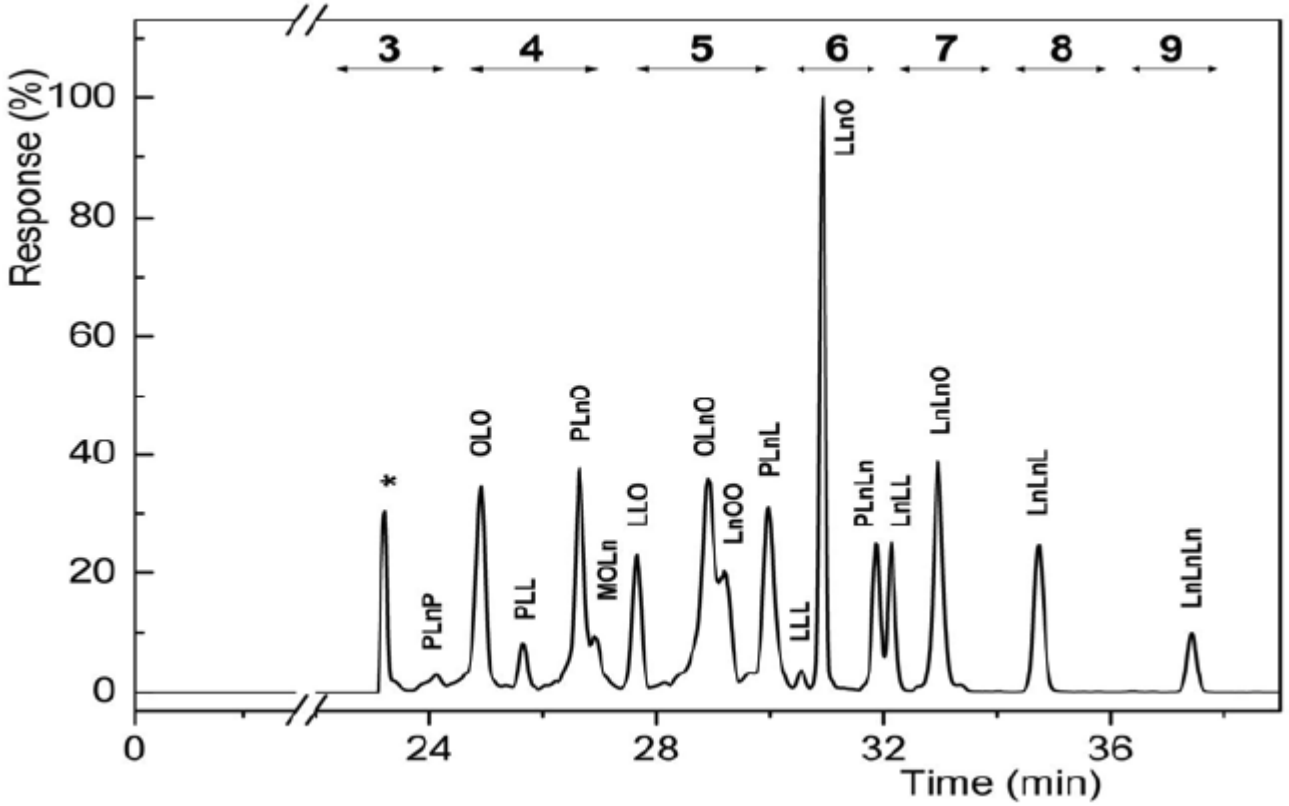
Reverzní chromatografie



Separation of triacylglycerols from a rapeseed oil by reversed-phase HPLC (Spherisorb ODS2; 250 × 4.6 mm column) with a gradient of acetone into acetonitrile (1 mL/min) and evaporative light-scattering detection (Abbreviations: P = palmitate, S = stearate, O = oleate, L = linoleate, T = triene (linolenate).

Analýza lipidů

Argentační chromatografie

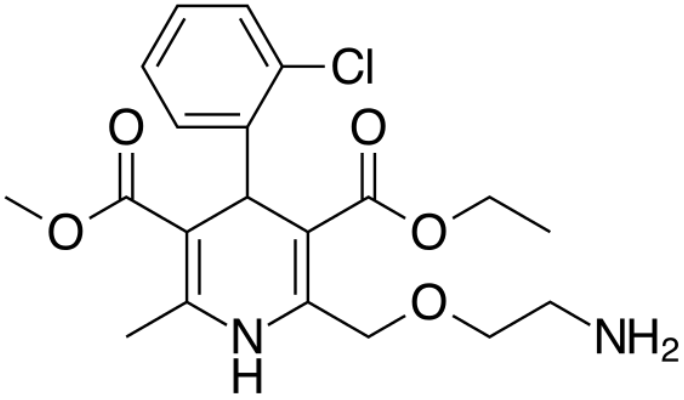


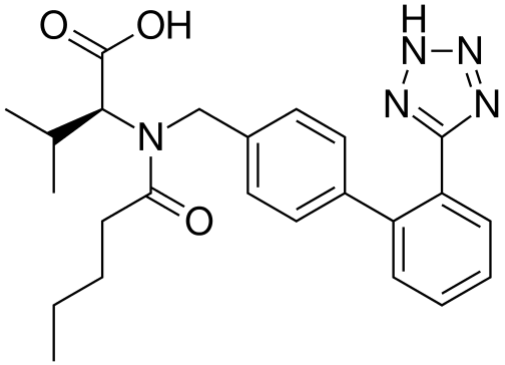
HPLC podmínky: kolona Valco ChromSpher 5 Lipids (250x4.6 mm, 5 mm) fáze: hexan (A), hexan/2-propanol/acetonitril 30:9:1, v/v/v (B) . Gradientový program: 100 % A 10 min, lineárně do 18 % B ve 32 min, do 35 % ve 37 min a do 100 % of B ve 40 min. Isokraticky 100 % of B do 41 min. Průtok 1ml/min. Pokolonový přídavek 100 mM octanu amonného v 2-propanol-vodě (9:1, v/v). APCI MS detekce full scan m/z 150-1200, 400°C.

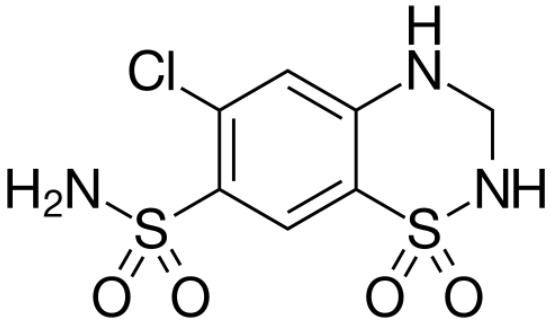
Farmaceutická analýza

Amlodipine besylate, valsartan a hydrochlorothiazid

Léčba vysokého krevního tlaku





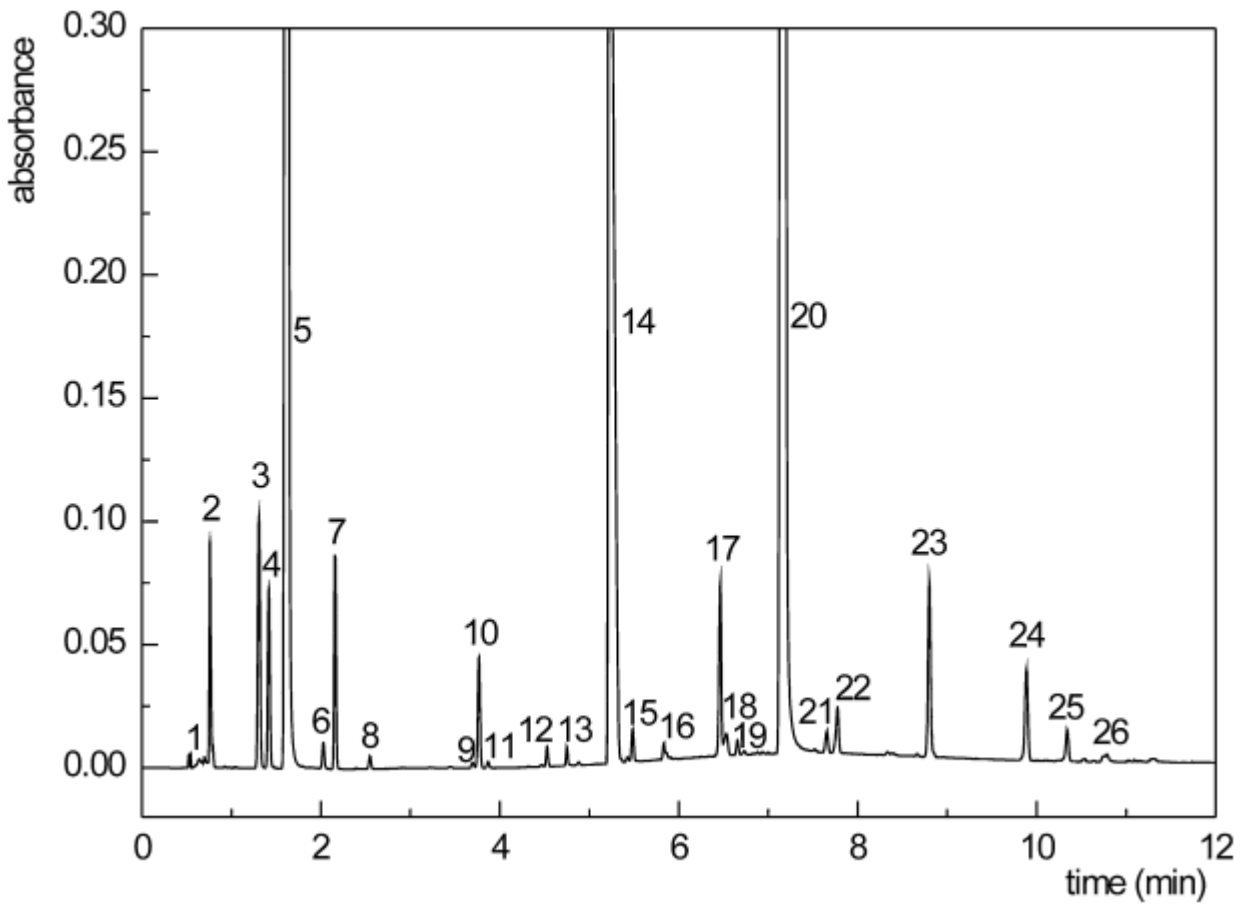


Valsartan – ovlivňuje renin-angiotensinový systém

Hydrochlorothiazid – diuretikum

Amlodipin – ovlivňuje vápenaté kanály

Amlodipine besylate, valsartan a hydrochlorothiazid

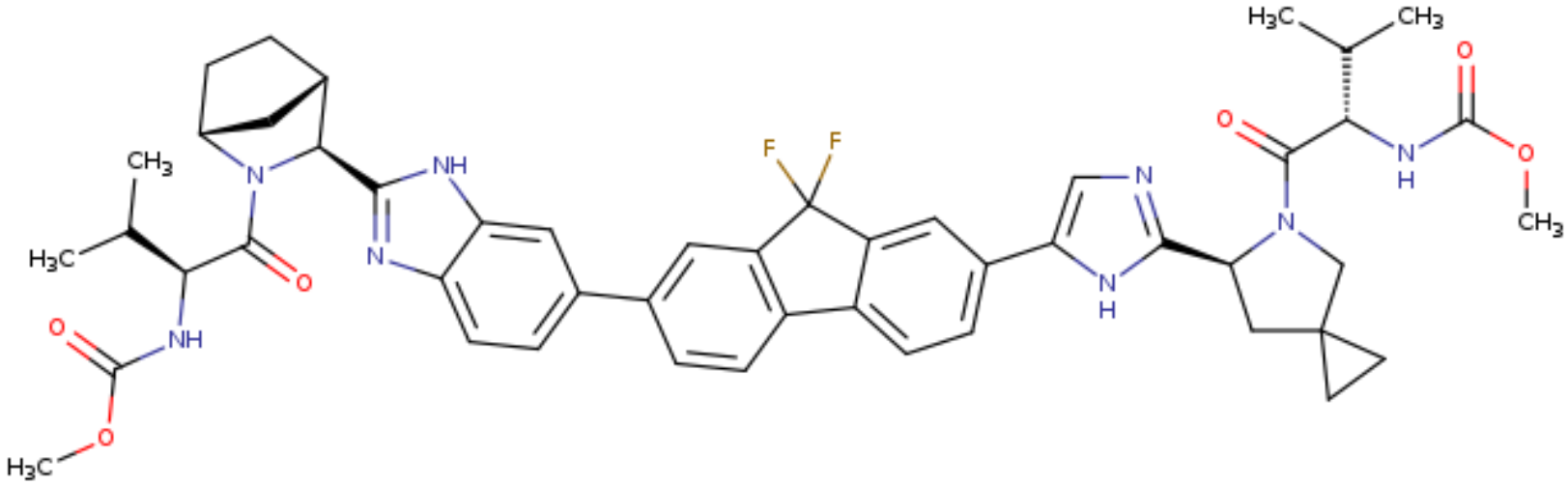


Chromatogram of the sample solution of formulated tablets spiked with impurities at . Evaluated at 225 nm. Peaks: (1) solvent peaks; (2) besylate; (3) impurity B (HCTZ); (4) impurity A (HCTZ**); (5) hydrochlorothiazide;** (6) unknown impurity RRT 1.25 (HCTZ); (7) impurity 09 (VALS); (8) unknown impurity RRT 1.57 (HCTZ); (9) minor unknown impurity of hydrochlorothiazide; (10) impurity C (HCTZ); (11) unknown impurity RRT 0.54 (VALS); (12) impurity D (AMLO); (13) impurity F (AMLO); (**14) amlodipine**; (15) unknown impurity RRT 0.77 (VALS);(16)impurity E (AMLO);(17)impurity C (VALS); (18) unknown impurity RRT 0.91 (VALS); (19) minor unknown impurity of valsartan; **(20) valsartan**; (21) impurity B (AMLO); (22) impurity G (AMLO); (23) impurity 07 (VALS);(24)impurity B (VALS);(25)impurity A (AMLO);(26) solvent peaks. HCTZ – hydrochlorothiazide, AMLO – amlodipine, VALS – valsartan.

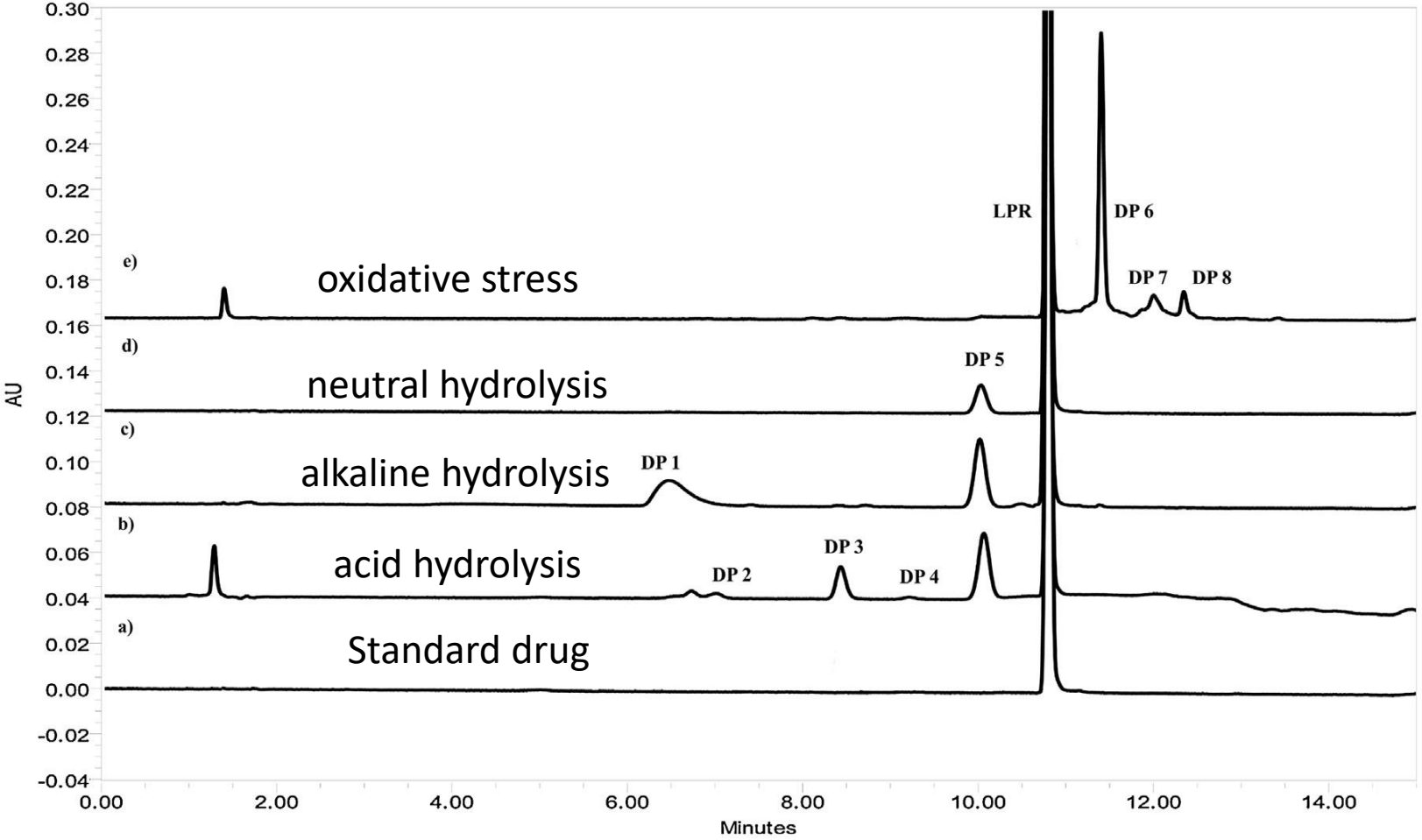
SF: Zorbax Eclipse Plus C8 RRHD, 100 mm × 3.0 mm, 1.8 µm
MF: A – 10mM $NH_4H_2PO_4$ pH 2.5 B: ACN
Gradient:[(min)/% B] 0/15, 11/75, 12/75, 12.5/15 a 15/15
Průtok: 0.8 mL/min; Nástřik 2µL; Teplota kolony: 30 °C; Detekce: PDA

Lidespavir

Léčba proti žloutence C



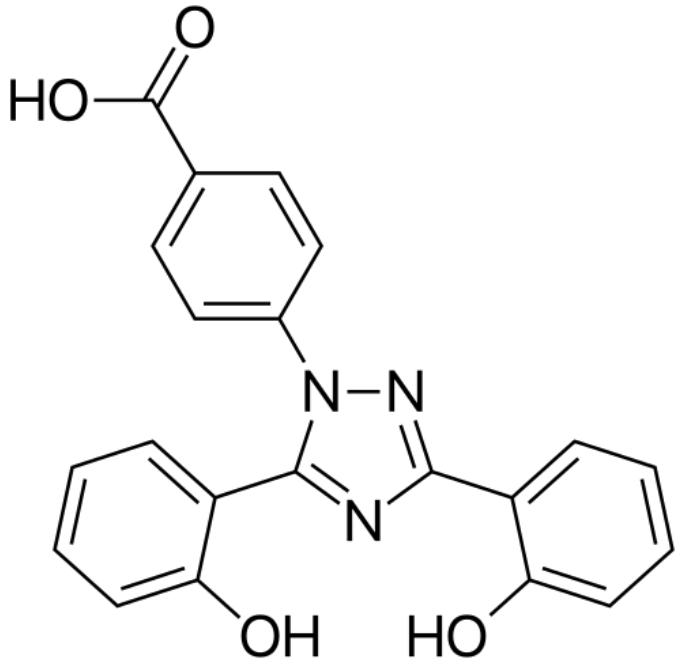
Lidespavir



SF: C18, 150 mm × 4.6 mm, 5 µm
MF: A – 0.1% HCOOH B: ACN
Gradient:[(min)/% B] 0/25, 3/25, 8/35, 11/80, 13/80, 14/25 a 15/25
Průtok: 1 mL/min; Nástřik 20µL; Teplota kolony: 30 °C; Detekce: PDA

Deferasirox

Chelatační činidlo pro ionty železa (použití při dlouhodobých krevních transfuzích)



Deferasirox

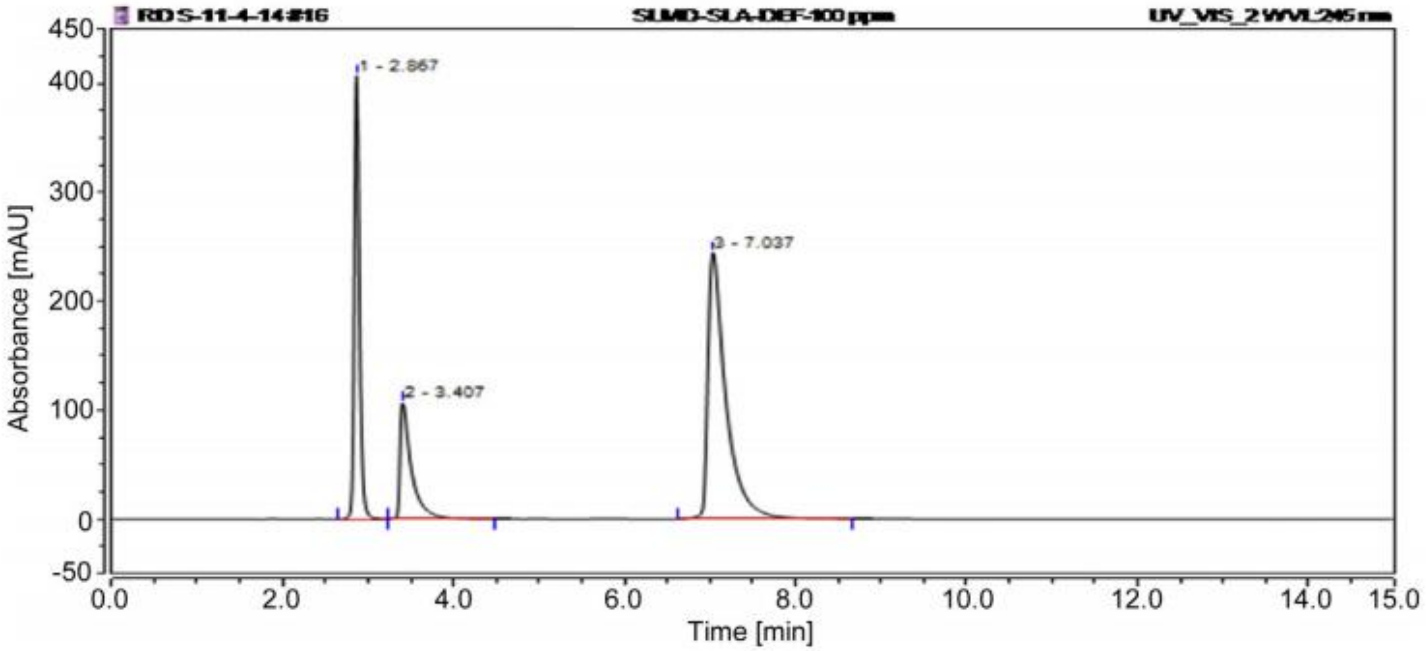


Fig. 2. Optimized chromatogram of salicylamide, salicylic acid and deferasirox.

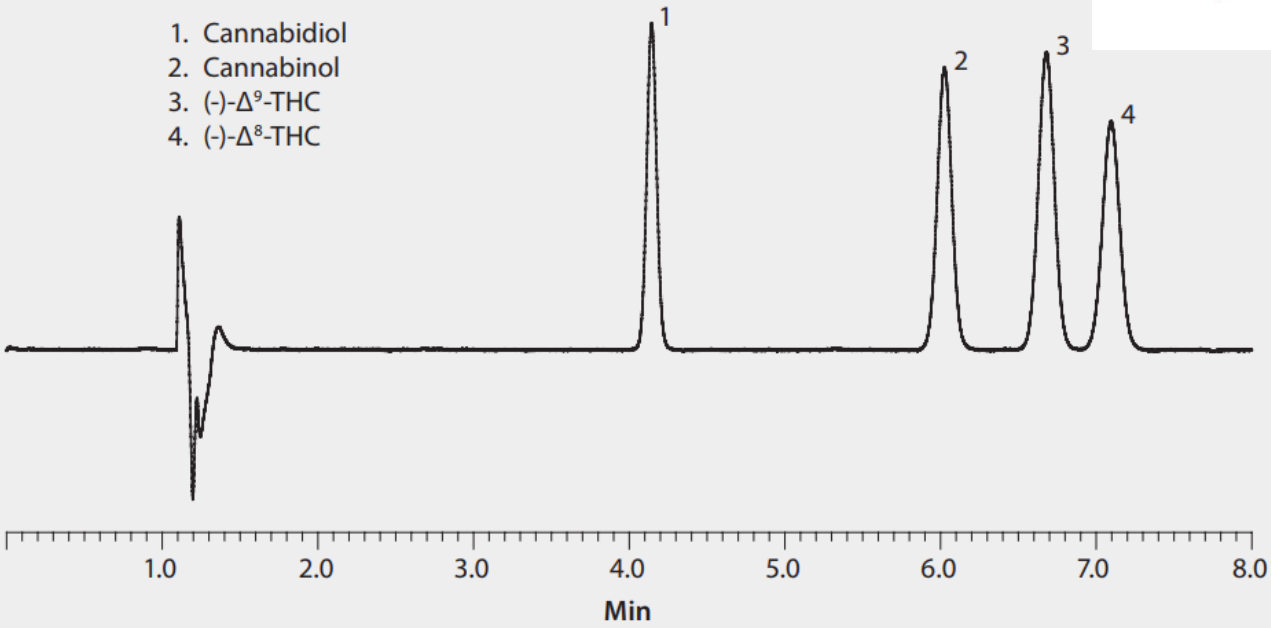
Parameter	Optimized condition
Flow rate	1.0 mL/min
Mobile phase	40:60 v/v (buffer: ACN)
Buffer pH	Potassium phosphate buffer pH 3.2 adjusted by OPA
Wavelength	245 nm
Injection volume	10 µl
Run time	15 min
Column and column oven temperature	30 °C

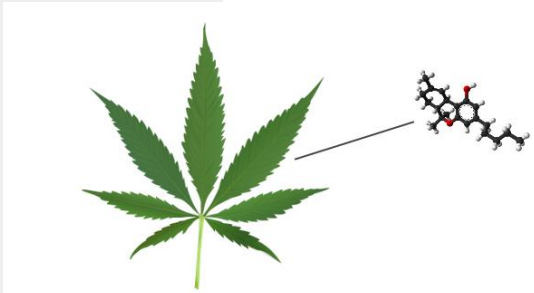
Toxikologická analýza

Figure 1. HPLC Analysis of Cannabinoids using Ascentis® Express RP-Amide

column: Ascentis Express RP-Amide, 15 cm × 4.6 mm I.D., 5 µm particles (50774-U)
mobile phase: 5 mM ammonium acetate (pH 4.5 with acetic acid) in 20:80, acetonitrile: water
flow rate: 1.0 mL/min
pressure: 1450 psi (100 bar)
column temp.: 35 °C
detector: UV, 214 nm
injection: 5 µL

1. Cannabidiol
2. Cannabinol
3. (-)-Δ^9-THC
4. (-)-Δ^8-THC





Toxikologická analýza

Analýza ephedrinů v rámci dopingového testování

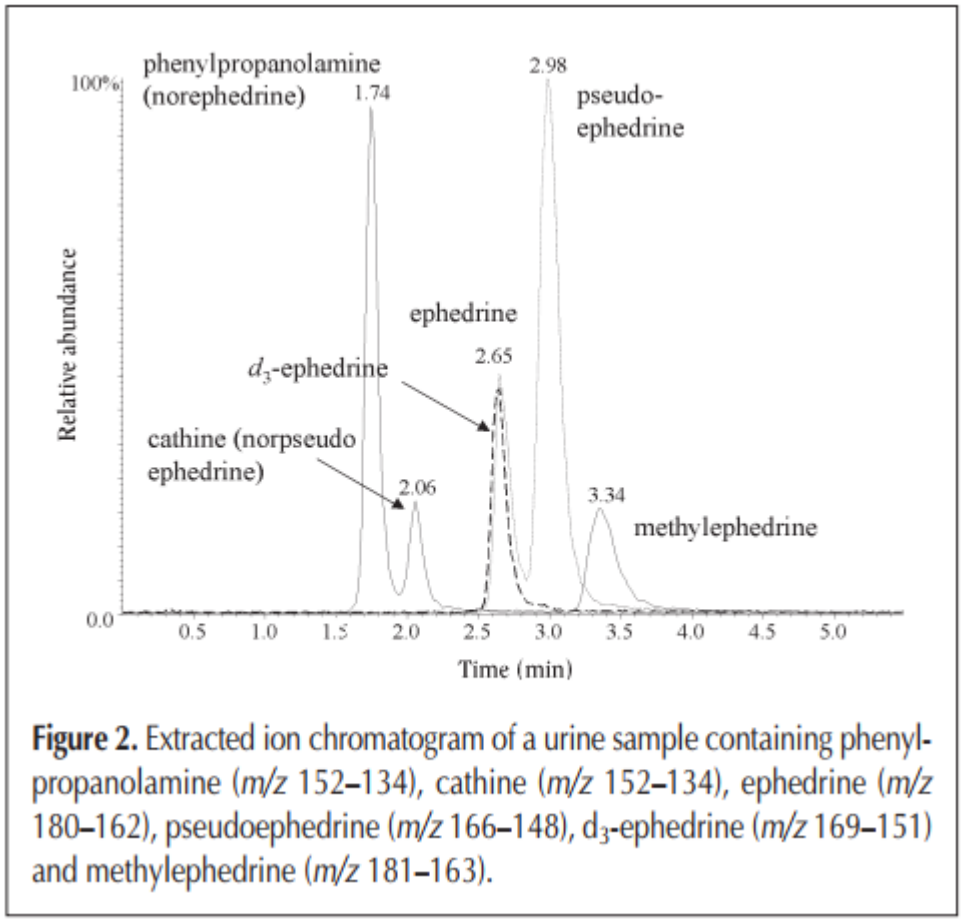
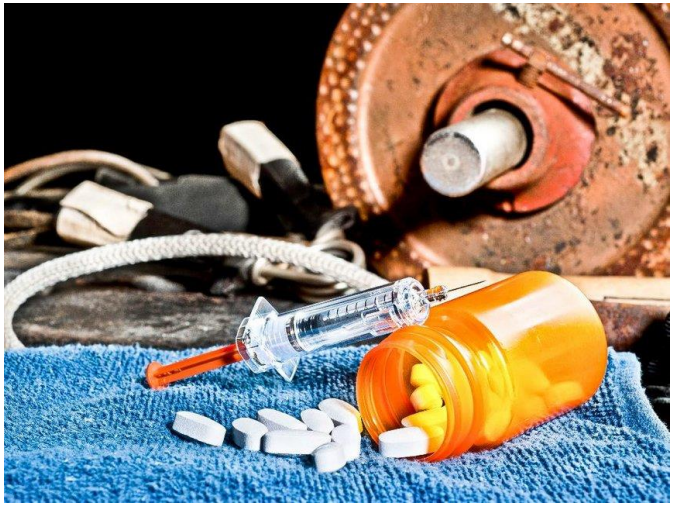


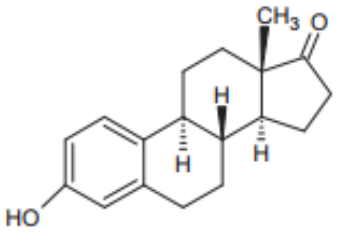
Figure 2. Extracted ion chromatogram of a urine sample containing phenylpropanolamine (*m/z* 152–134), cathine (*m/z* 152–134), ephedrine (*m/z* 180–162), pseudoephedrine (*m/z* 166–148), d_3-ephedrine (*m/z* 169–151) and methylephedrine (*m/z* 181–163).



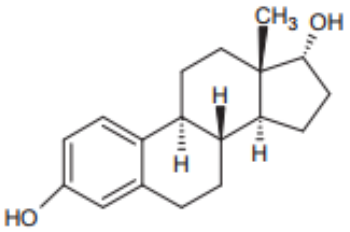
Environmentální analýza

Stanovení estrogenních látek ve vodách

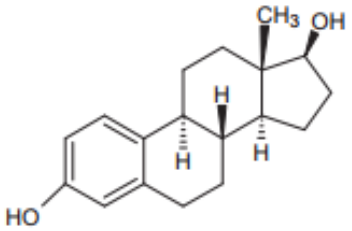
estrone (E1)



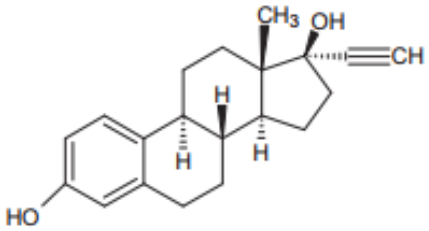
17α-estradiol (αE2)



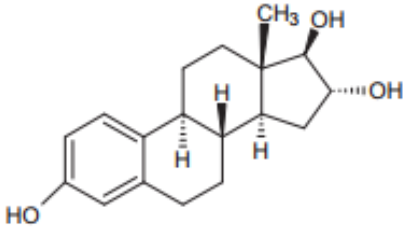
17β-estradiol (βE2)

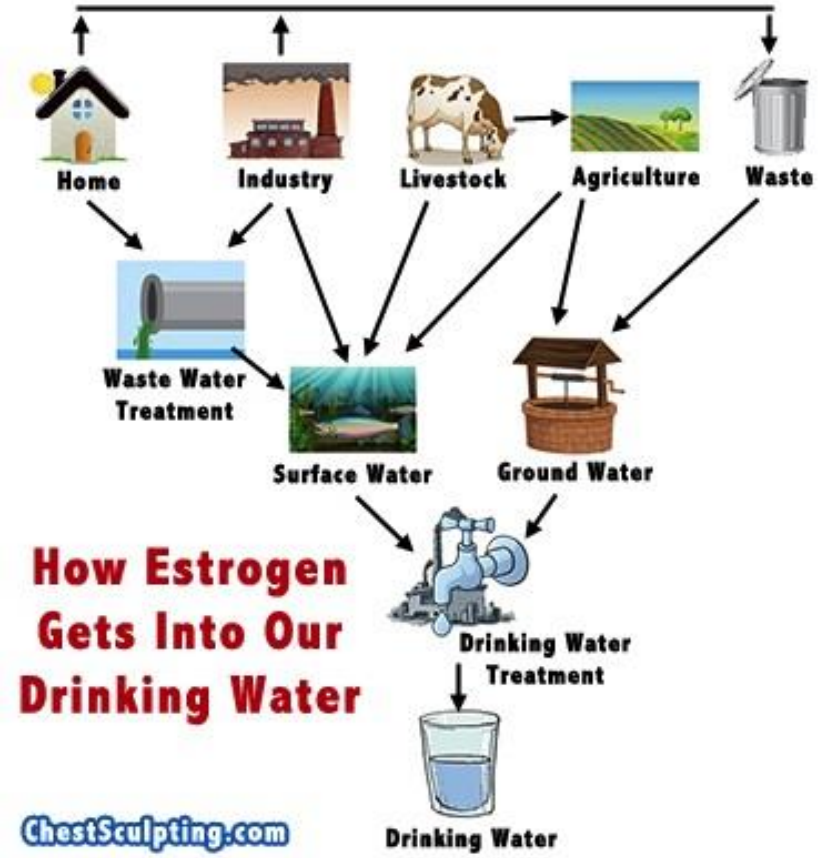


17α-ethynylestradiol (EE2)

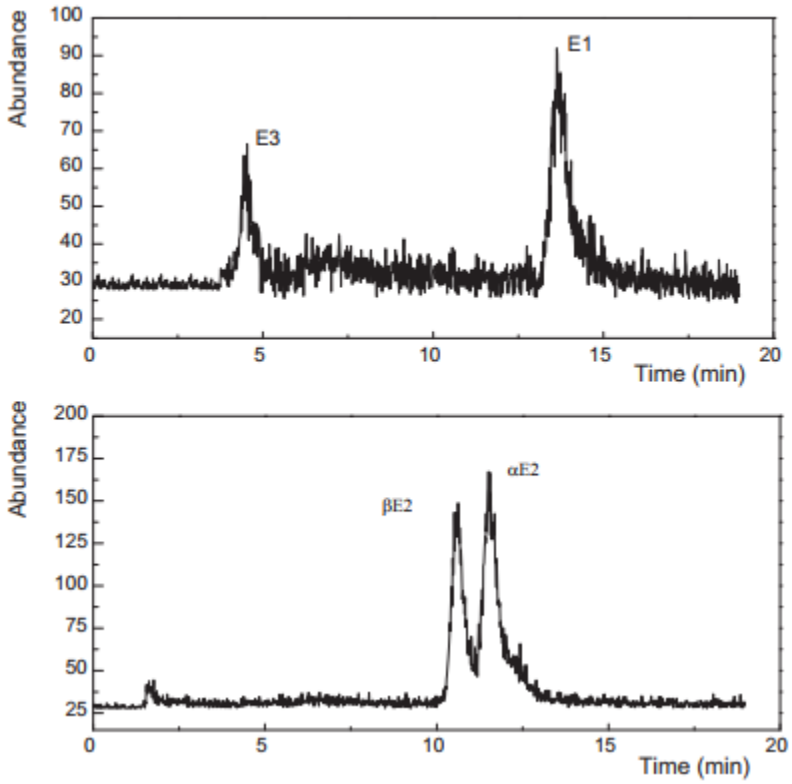


estriol (E3)





Stanovení estrogenních látek ve vodách



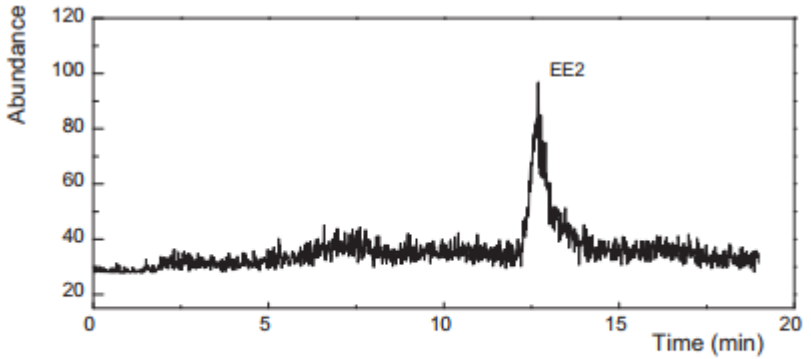


Fig. 2. MRM chromatograms of estrogens obtained for spiked river water (50 ng/L, Uhlava, Klatovy) under the optimized preconcentration and gradient elution conditions (see Sections 3.2 and 3.3, respectively).

SF: Zorbax SB C18, 150 mm × 0.5 mm, 5 µm
MF: A – 0.1% HCOOH B: ACN
Gradient:[(min)/% B] 0/26, 3/38, 5/38, 8/42, 16/42, 17/26 a 19/26
Průtok: 18 µL/min; Nástřik 2µL; Teplota kolony: 30 °C; Detekce: MS/MS