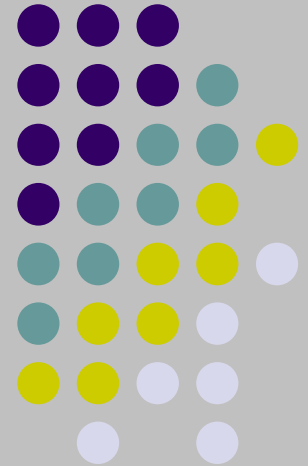


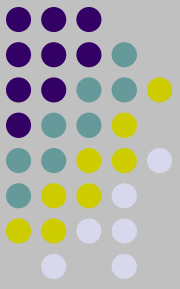
Transferrina carboidrato carente (CDT),
marcatore di abuso cronico di alcol.

Linee Guida e Metodologia di Analisi

Franco Tagliaro e Federica Bortolotti

Dipartimento di Medicina e Sanità Pubblica,
Sezione di Medicina Legale
Università di Verona





OUTLINE

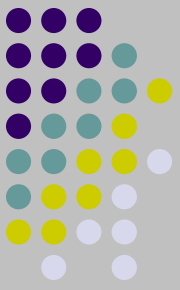
Definizione di CDT

Meccanismo patogenetico di
produzione della CDT

Efficienza diagnostica della CDT

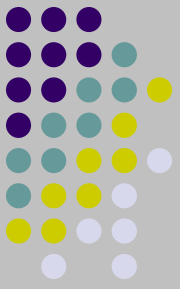
Metodologie d'analisi

Aspetti medico legali

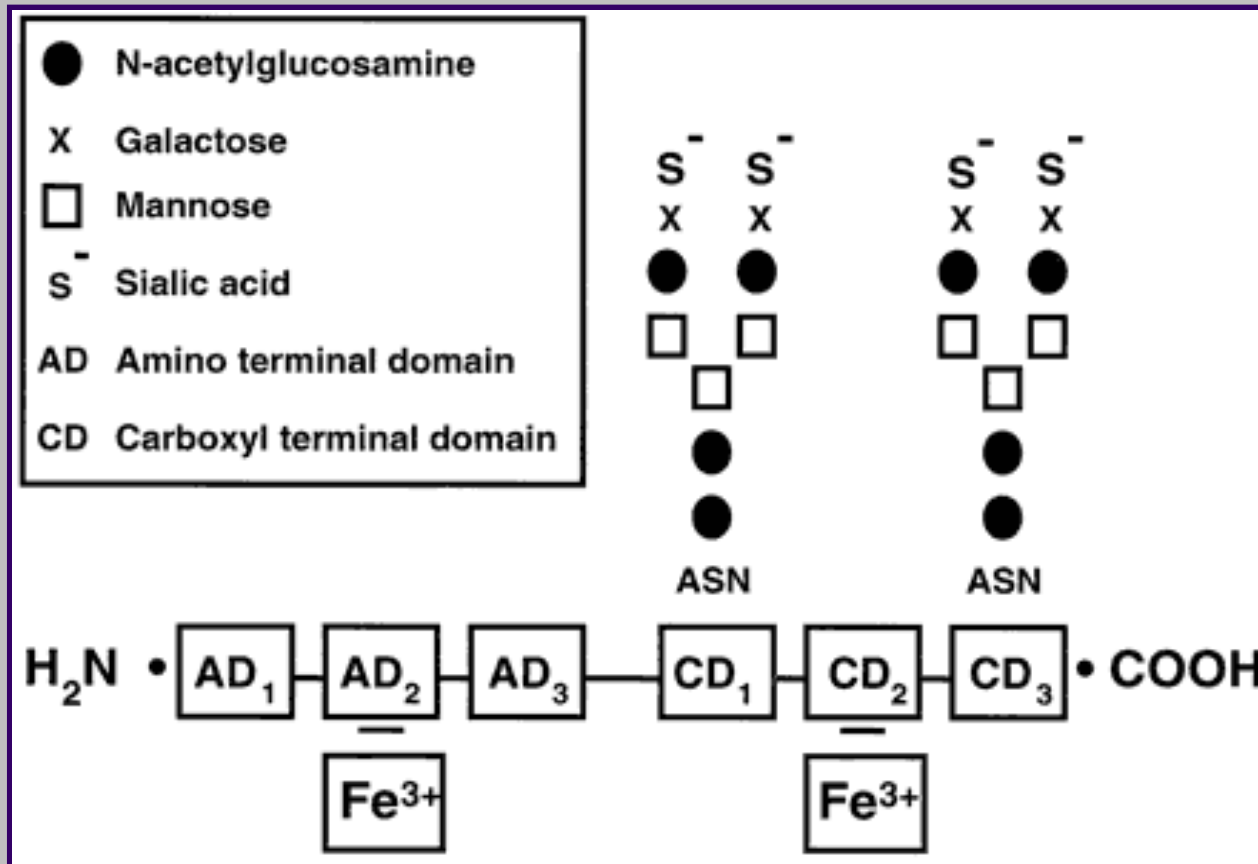


Helena Stibler et al, Abnormal micro-heterogeneity of transferrin in serum and cerebrospinal fluid in alcoholism, Acta Med. Scand, 1978; 204:49-56

Studio condotto sul liquido cerebrospinale di pazienti affetti da degenerazione cerebellare alcolica. Identificazione mediante isoelectro-focusing di isoforme particolari della transferrina caratterizzate da un punto isoelettrico più elevato ($pI > 5.7$) rispetto a quello dell'isoforma maggiore.



Rappresentazione schematica della transferrina (tetrasialo-Tf)

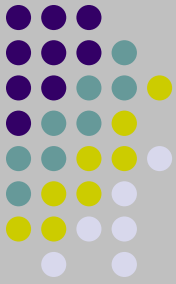


CDT: glicoforme carboidrato-deficienti rispetto alla tetrasialo-Tf

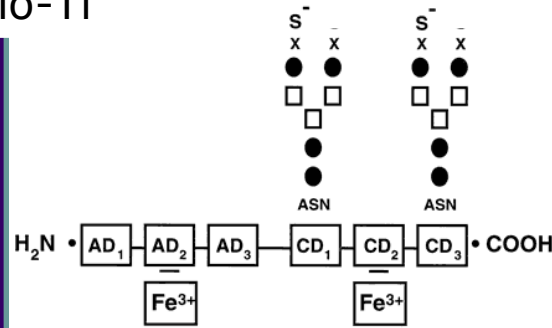
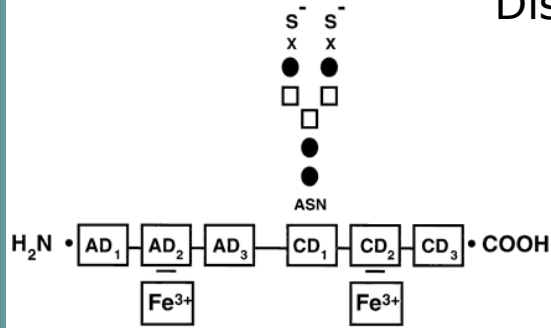
-Disialo-Tf: 1-2%

-Monosialo-Tf: < 0.5%

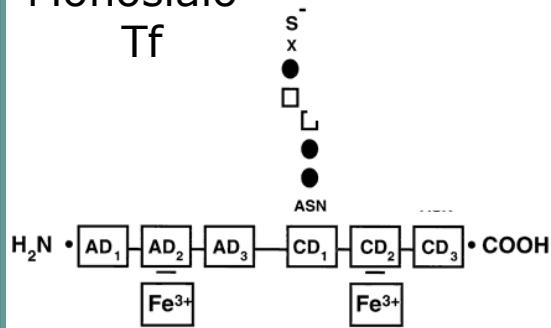
-Asialo-Tf: < 0.5%



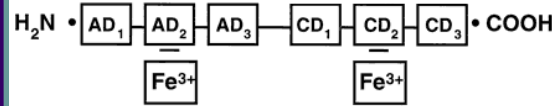
Disialo-Tf



Monosialo-Tf

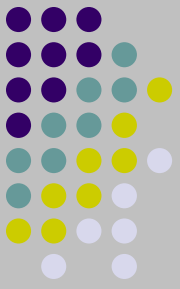


Asialo-Tf



Carbohydrate deficient transferrin

Meccanismo patogenetico di aumento della CDT



H. Stibler, S. Borg. Glycoprotein glycosyltransferase activities in serum in alcohol-abusing patients and healthy controls. Scand J Clin Lab Invest. 51(1991)43-51.

Ridotta attività degli enzimi galattosiltransferasi e N-acetylglucosaminyltransferasi

Xin Y, Lasker JM, Lieber CS.
Serum carbohydrate-deficient transferrin: mechanism of increase after chronic alcohol intake. Hepatology. 22(1995) 1462-1468

Ridotta attività degli enzimi sialil, galattosil e N-acetilglucosaminiltransferasi e aumentata attività della sialidasi.

P. Sillanaukee, N. Strid, J.P. Allen, R.Z. Litten, Possible reasons why heavy drinking increases carbohydrate-deficient transferrin. Alcohol Clin. Exp. Res. 25 (2001) 34-40. Review

Meccanismo multistep coinvolgente sia il trasporto di proteine che l'attività enzimatica

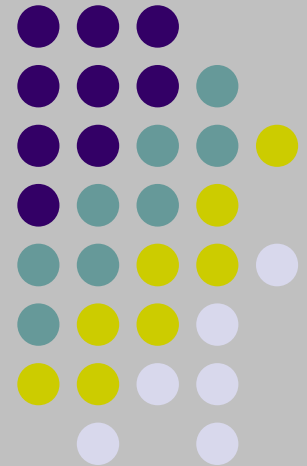
Tempo di "positivizzazione": circa 7 gg
per un consumo alcolico di 60-80 g/die

60-80 g di alcol etilico corrispondono a:

0,5-0,8 L di vino:
5-7 bicchieri

1,5-2 L di birra:
4-6 birre medie

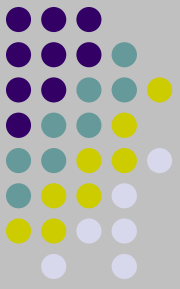
0,15-0,20 L di grappa:
2-4 bicchieri



Emivita: circa 14 giorni

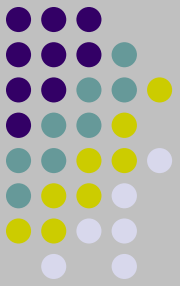
Specificità diagnostica: ~ 100%

Metodi per la determinazione della CDT: excursus storico



isoelectric focusing (I.F.)	[Stibler, 1978]
I.F. + immunofixation	[Stibler, 1980]
I.F. + immunoelectrophoresis	[Vesterberg, 1984]
I.F. + western blotting	[Xin, 1991]
anion exchange chromatography on microcolumn+RIA (or ELISA)	[Stibler, 1986]
HPLC-UV (460 nm)	[Jeppson, 1993]
CE-UV	[Tagliaro, 1998]

Metodi per la determinazione della CDT attualmente in uso



IMMUNOASSAY DIRETTO

Elevata produttività

ELECTROFORESI CAPILLARE

Elevata risoluzione

HPLC

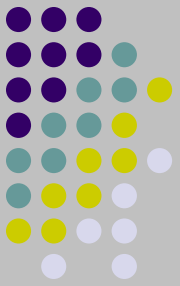
Elevata selettività

LC-MS (!?)

Elevato potere identificativo

ELETTROFORESI CAPILLARE

Separazione delle isoforme della CDT in tampone borato
buffer con rivelazione UV a 200/214 nm

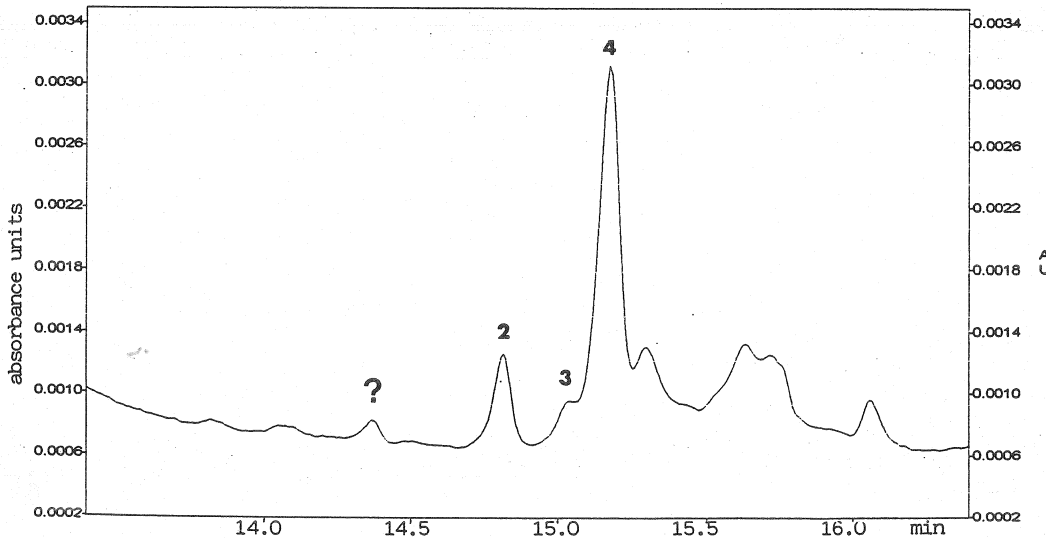
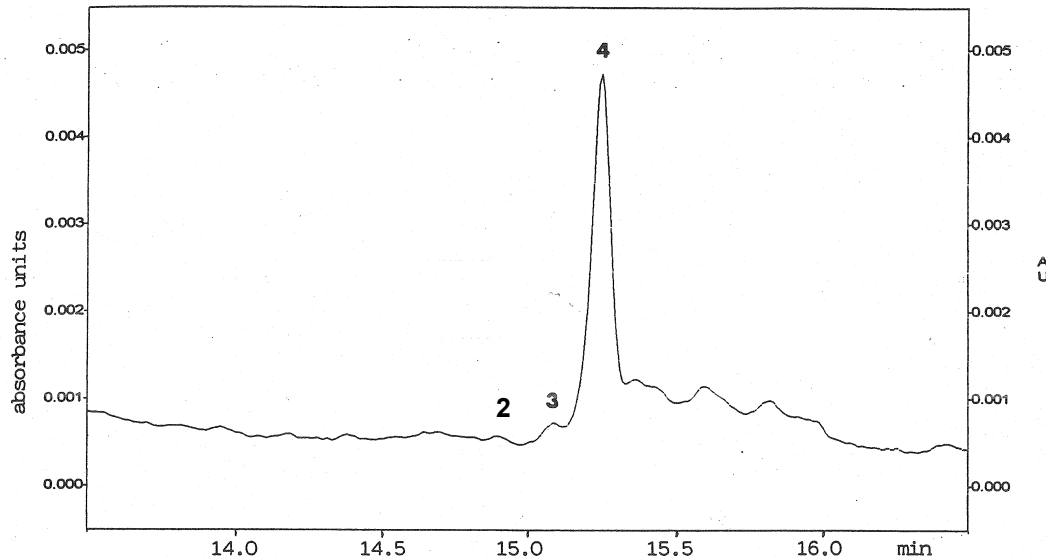


a. Metodi in elettroforesi capillare a singolo capillare impieganti reattivi non commerciali

b. Metodi in elettroforesi capillare a singolo capillare impieganti reattivi commerciali

c. Metodi in elettroforesi multicapillare impieganti reattivi commerciali

2000



Crivellente F et al.

Improved method for carbohydrate-deficient transferrin determination in human serum by capillary zone electrophoresis.

J Chromatogr B 2000; 739:81-93.

ANALYTICAL METHOD

capillary: 57 cm x 20 mm i.d.
uncoated silica

buffer: 100 mM borate pH 8.3, 1.50 mM DAB

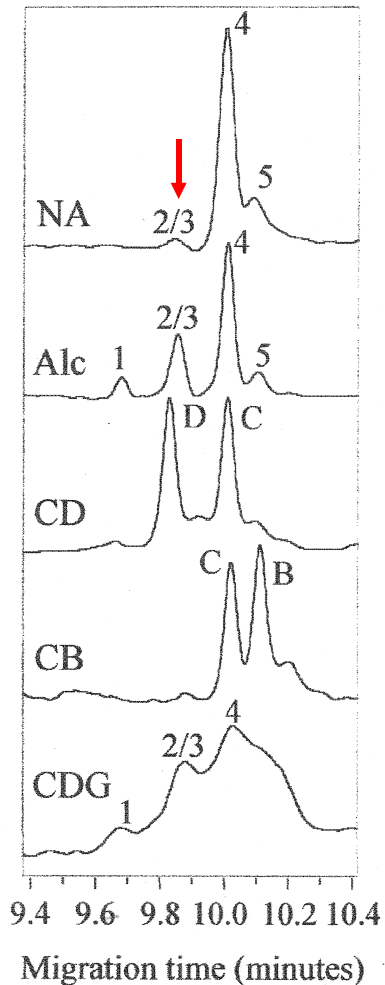
voltage: 20 kV

detection: UV absorbance at 200nm

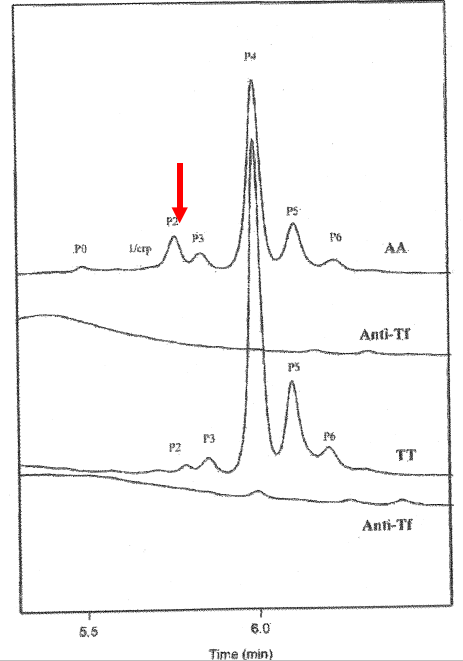
injection: 0.5 psi x 10s

sample prep.: 200 ml serum + 5 ml 50 mM NaHCO₃ + 5 ml 10 mM FeCl₃; sample diluted 1:10 with H₂O

Direct injection of serum



Injection after ferric saturation



Legros FJ et al.

Carbohydrate-deficient transferrin isoforms measured by capillary zone electrophoresis for detection of alcohol abuse.

Clin Chem. 2002;48:2177-2186.

Wuyts B et al.

Determination of carbohydrate-deficient transferrin using capillary zone electrophoresis.

Clin Chem 2001;47:247-255.

2002

ANALYTICAL METHOD

capillary: 47 cm x 50 mm i.d.
uncoated silica

buffer: proprietary reagents
(CEofix® CDT buffer system, Analis,
Namur, Belgium), with a **dynamic**

double coating of the capillary
including a polycation and a
polyanion

voltage: 28 kV

Temperature: 40°C

detection: UV absorbance at 200 nm

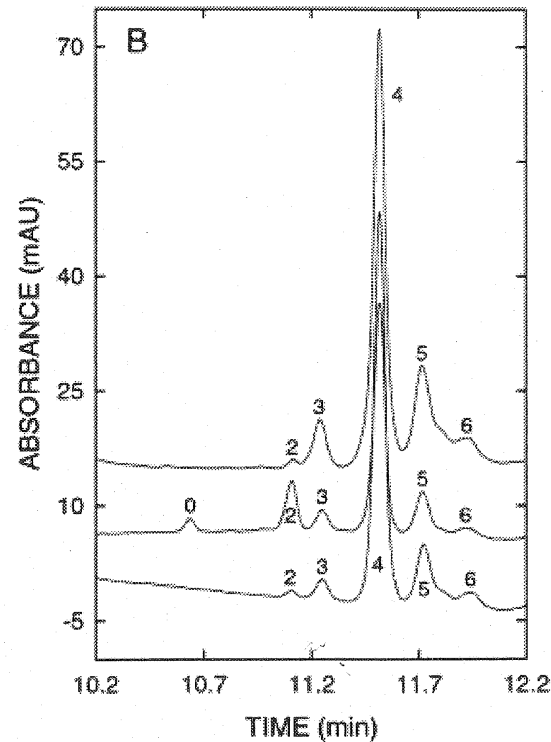
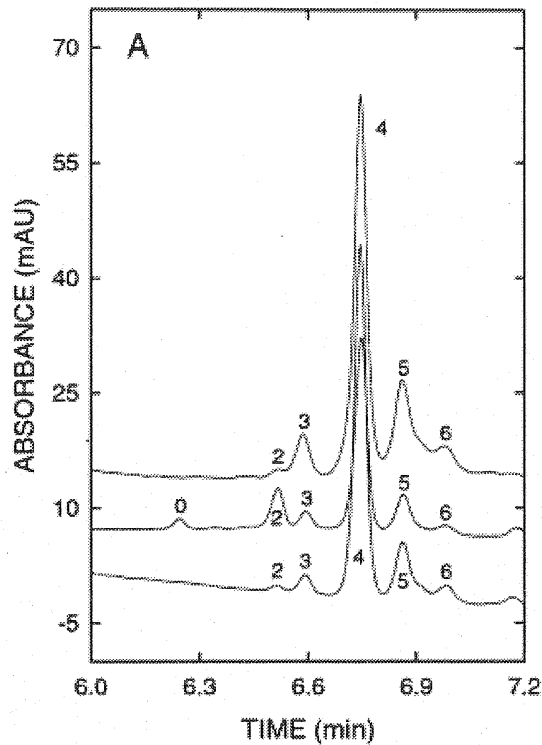
injection: 0.5 psi x 3s

2003

Lanz C and Thormann W.

Capillary zone electrophoresis with a dynamic double coating for analysis of carbohydrate-deficient transferrin in human serum: impact of resolution between disialo- and trisialotransferrin on reference limits.

Electrophoresis 2003; 24: 4272-4281.



ANALYTICAL METHOD

capillary: 47 cm x 50 μ m i.d. uncoated silica

buffer: proprietary reagents (CEofix® CDT buffer system, Analis, Namur, Belgium), with a **dynamic double coating** of the capillary including a polycation and a polyanion

voltage: 20 kV

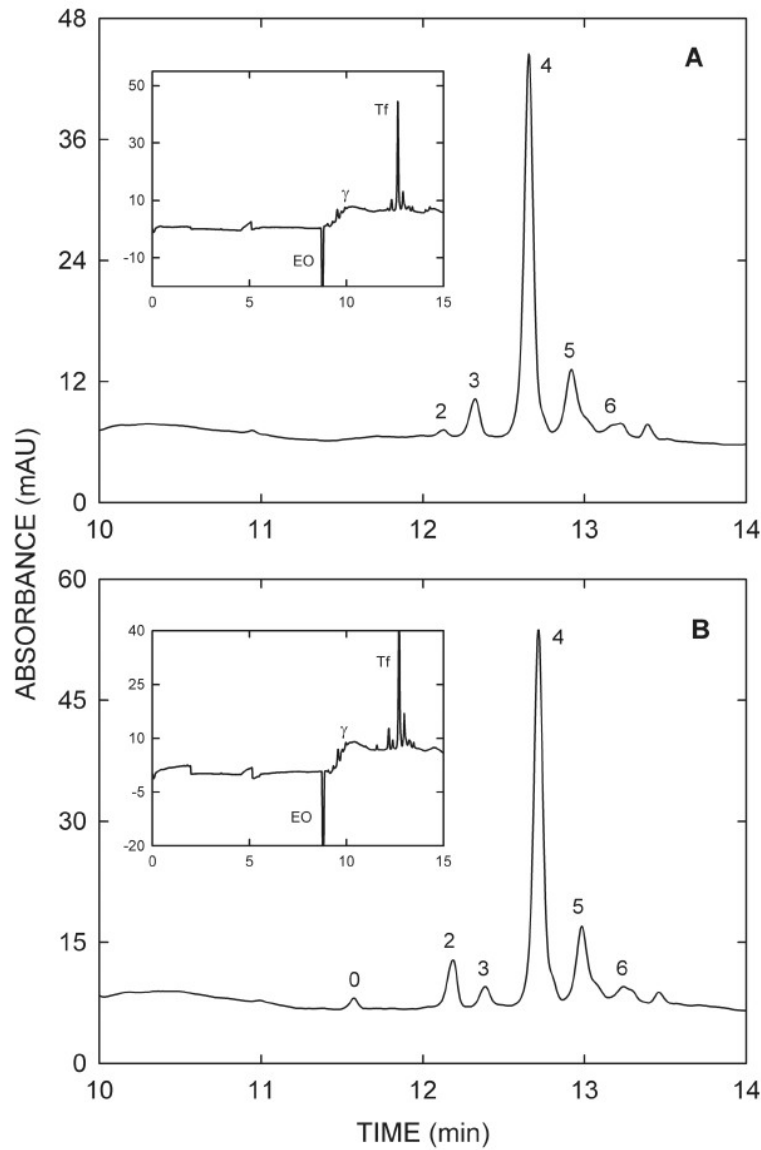
Temperature: 30°C

detection: UV absorbance at 200 nm

injection: 0.5 psi x 3s

R disialo-Tf/trisialo-tf:
1,60

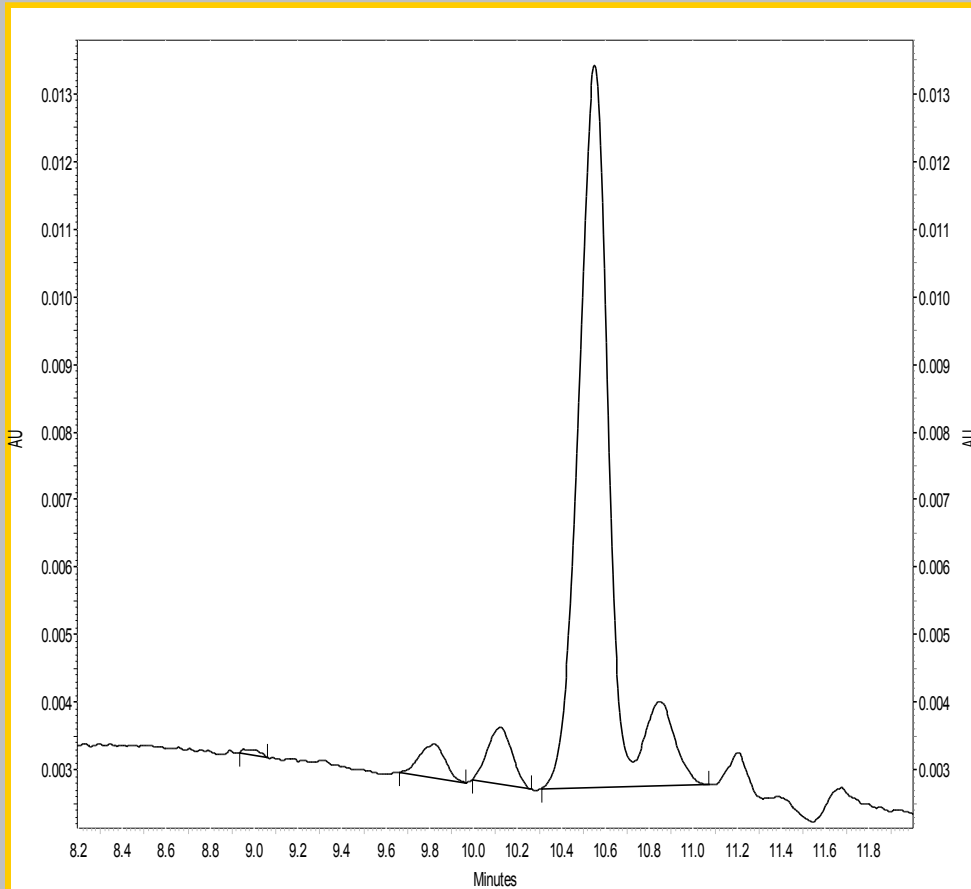
2008



R disialo-Tf/trisialo-tf: 1,69

Marti et al, J Sep Sci 2008

2009



ANALYTICAL METHOD

capillary: 50 cm x 30 μ m i.d.
uncoated silica

buffer: 120 mM boric acid+6 mM
DAB (adjusted to pH 8 with NaOH)

voltage: 20 kV

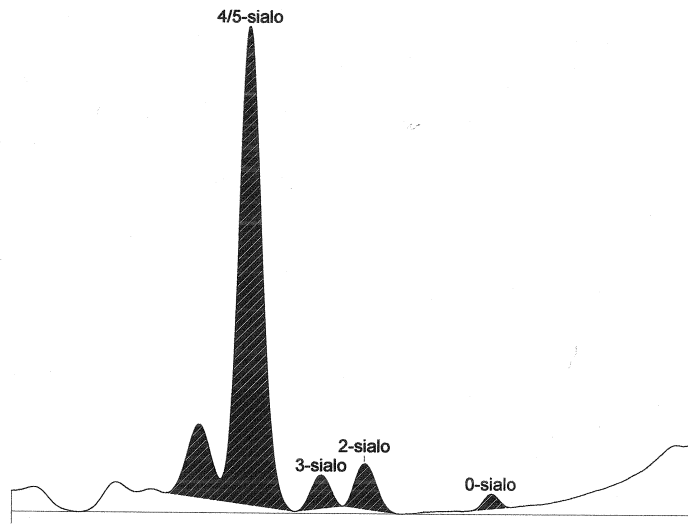
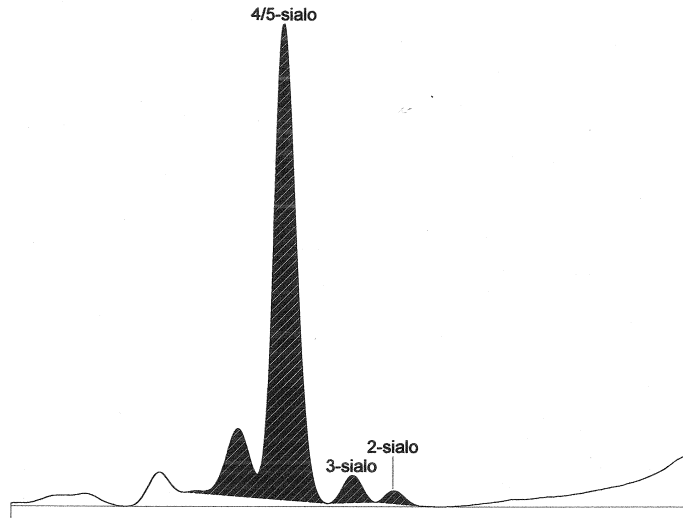
detection: UV absorbance at 200
nm

injection: 0.5 psi x 30s

sample prep.: serum diluted 1:8
with ferric chloride 0.5 mM

R disialo-Tf/trisialo-tf: 1,55

Tagliaro F. et al, work in progress

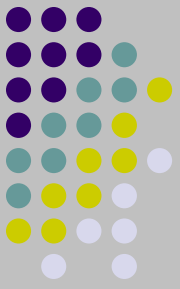


Capillarys 2 (Sebia, Paris):
multicapillary electropherograph
with 8 parallel capillaries.

Commercial kit: Capillarys CDT

R disialo-Tf/trisialo-tf: 1,27

HPLC



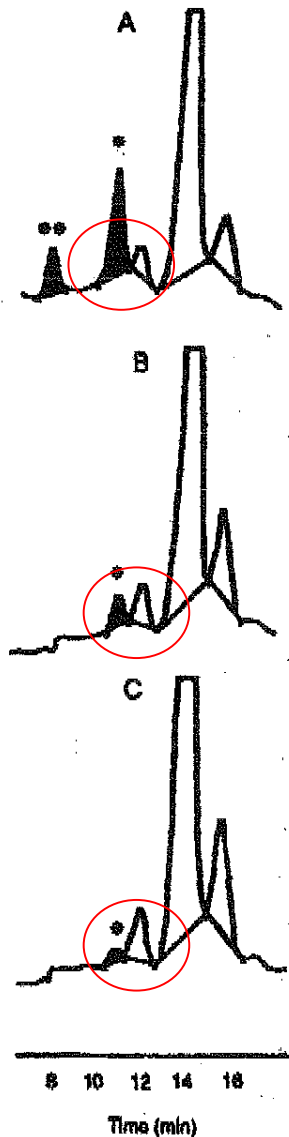
Cromatografia a scambio anionico con rivelazione UV-vis a 460 nm

a. Metodi HPLC impieganti reattivi non
commerciali

b. Metodi HPLC impieganti reattivi
commerciali

1993

J.O. Jeppsson, H. Kristensson, C. Fimiani,
Carbohydrate-deficient transferrin quantitated
by HPLC to determine heavy consumption of
alcohol, Clin. Chem. 39 (1993) 2115-2120.



Column: MonoQ HR 5/5 (Pharmacia)
Mobile phase: A, BIS-TRIS 20 mM adjusted at pH 6.2; B, BIS-TRIS 20 mM + NaCl 350 mM adjusted at pH 6.2; C, NaCl 1 M.
Flow rate: 1 mL/min
Gradient Profile

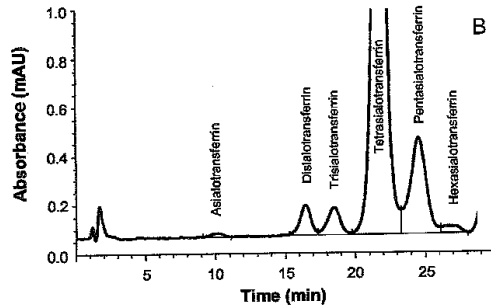
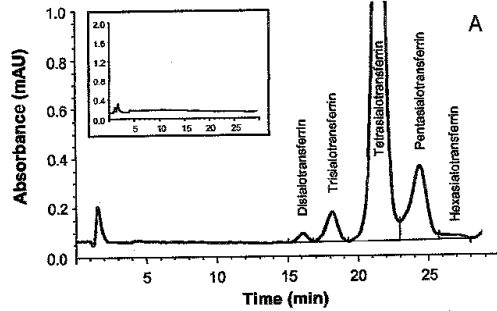
Time	A	B	C
0 min	100	0	0
3.0 min	100	0	0
5.0 min	90	10	0
18.0 min	66	34	0
18.1 min	0	0	100
22.0 min	100	0	0
32.0 min	100	0	0

Detection: UV-Vis at 460 nm

Sample preparation: 1 mL of serum+25 uL sodium carbonate 500 mM+18 uL ferric chloride 10 mM

Results expressed as disialo-Tf/total transferrin x 100

2003



A. Helander, A. Husa, J.O. Jeppsson, Improved HPLC method for carbohydrate-deficient transferrin in serum. Clin. Chem. 49 (**2003**) 1881-1890.

Column: Source 15Q PE 4.6/100 anion exchange chromatography column (Amersham Biosciences)
Mobile phase: A, BIS-TRIS 10 mM adjusted at pH 7.0; B, BIS-TRIS 10 mM + NaCl 500 mM adjusted at pH 6.2; C, BIS-TRIS 10 mM adjusted at pH 6.2; D, NaCl 2 M.
Flow rate: 1 mL/min

Gradient Profile

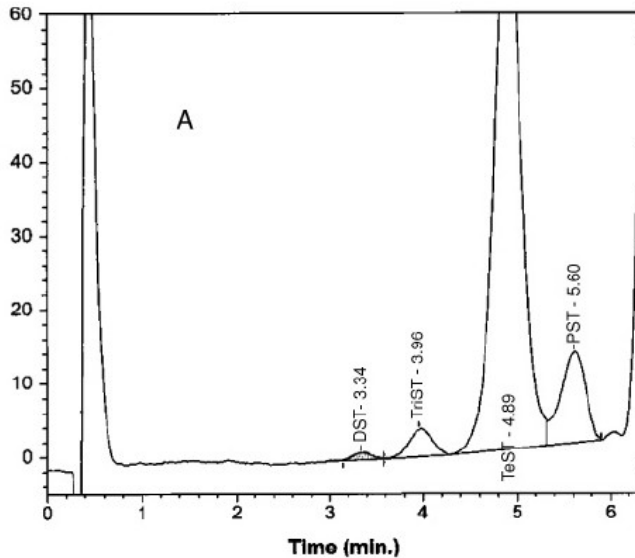
Time	A	B	C	D
0 min	100	0	0	0
1.0 min	100	0	0	0
1.1 min	0	0	100	0
30.0 min	0	20	80	0
30.1 min	0	0	0	100
35.0 min	100	0	0	0
35.50 min	0	100	0	0
37.00 min	0	100	0	0

R disialo-Tf/trisialo-tf:
 $\geq 1,27$

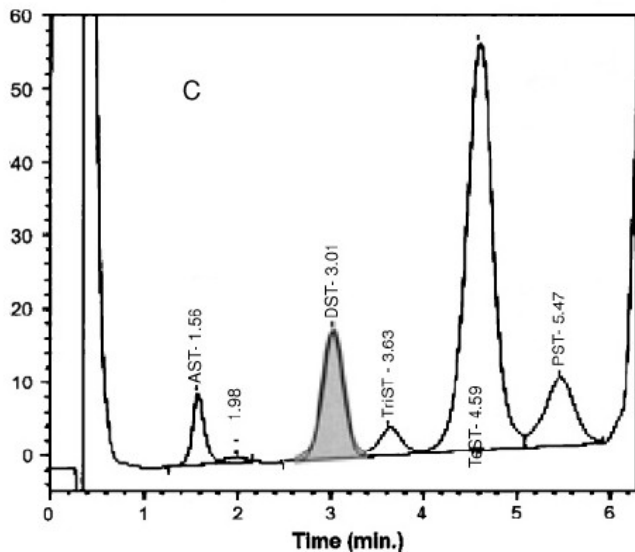
Detection: UV-Vis at 460 nm

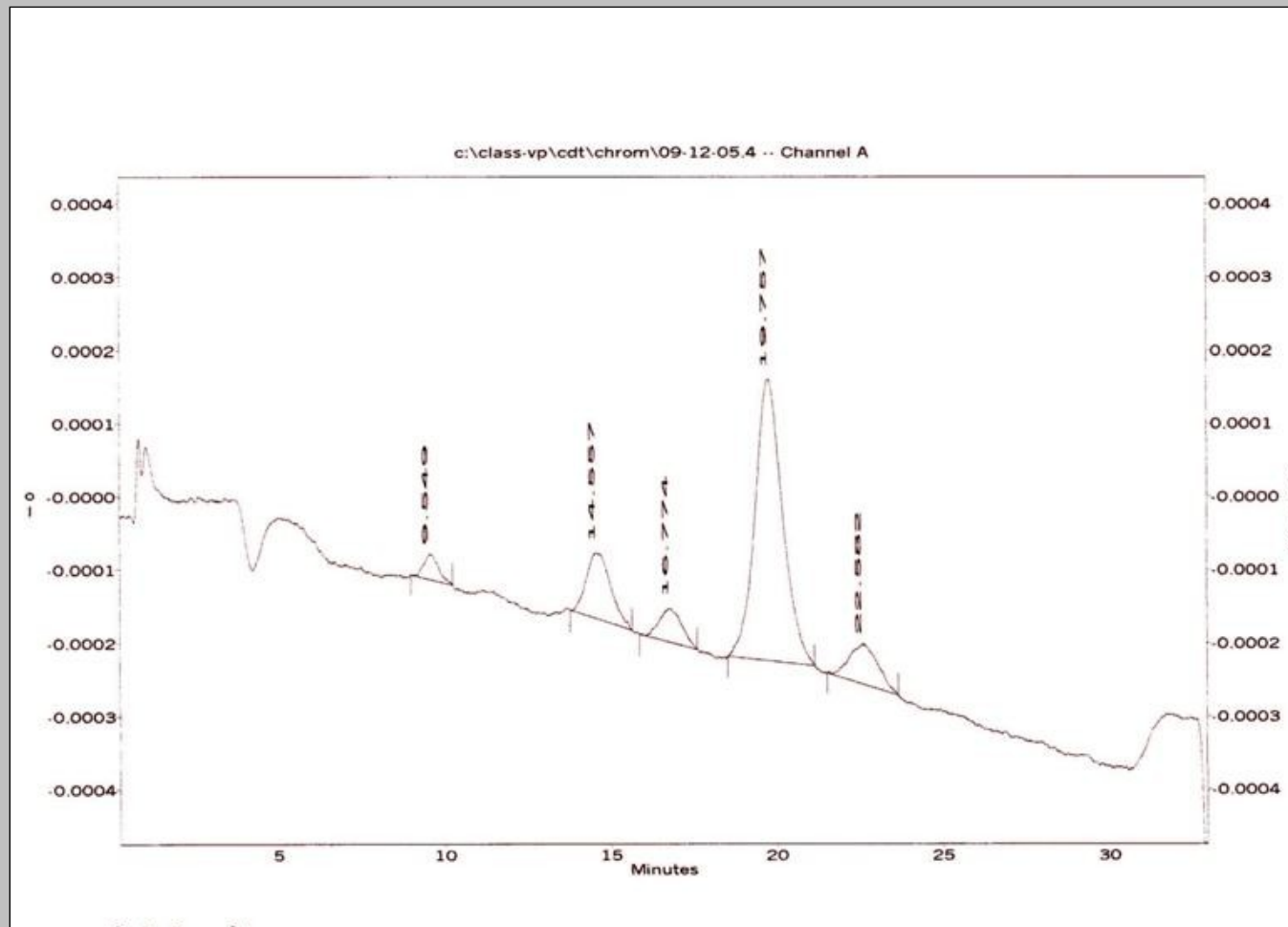
Sample preparation: 100 uL of serum+3 uL sodium carbonate 500 mM+20 uL ferric nitrilotriacetic acid (FeNTA)+20 ul of the dextran sulfate-Calcium chloride

2008



Shellenberg F et al, Alcohol and Alcoholism, 43:569-576
Automated Measurement of Carbohydrate-Deficient Transferrin Using the Bio-Rad %CDT by the HPLC Test on a VariantTM HPLC System: Evaluation and Comparison with Other Routine Procedures





R disialo-Tf/trisialo-tf: 1,65



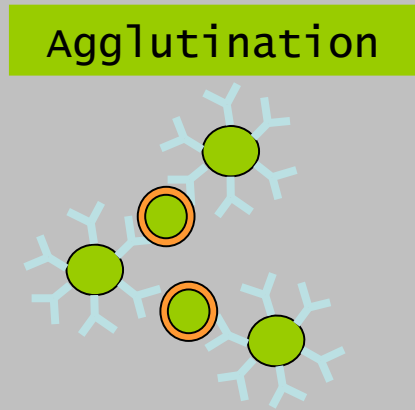
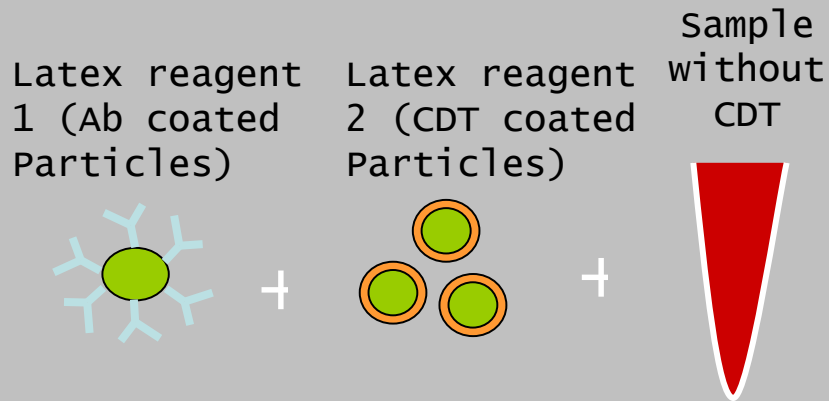
Resolution vs peak area!!!

Table 1. Effect of voltage and temperature on power level, run time, disialo-/trisialo-Tf peak resolution, and peak sizes for a serum with about 8% of trisialo-Tf

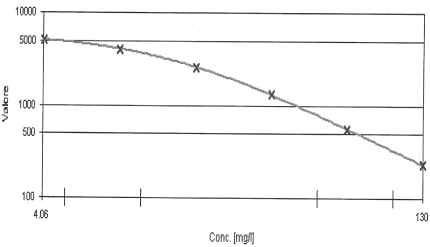
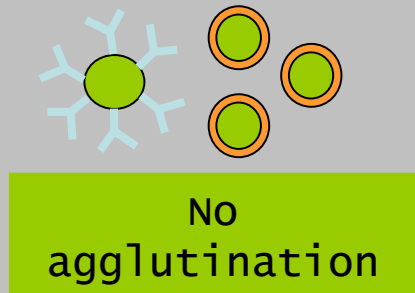
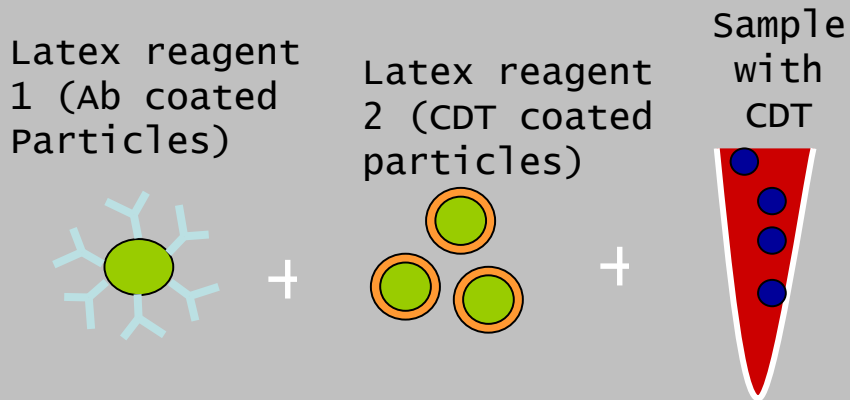
Instrumental conditions					Monitored parameters					
Voltage (kV)	Temp. ^{a)} (°C)	Injection time (s)	Sample plug ^{b)} (%)	Power level (W/m)	Detection time tetrasialo-Tf ^{c)} (min)	Total analysis time ^{d)} (min)	Resolution di-/trisialo-Tf ^{c)}	Peak area of disialo-Tf ^{c)} (area %)	Peak area of trisialo-Tf ^{c)} (area %)	
28.0	40.0	8.0	0.66	1.14	6.61	17.0	1.27	0.505	7.76	
28.0	20.0	12.5	0.67	0.74	9.94	20.6	1.36	0.644	7.98	
20.0	40.0	8.0	0.66	0.57	9.56	20.1	1.31	0.716	7.96	
20.0	30.0	9.5	0.64	0.48	11.34	22.1	1.39	0.708	8.06	
20.0	20.0	12.5	0.67	0.37	14.02	24.8	1.39	0.735	8.14	

Lanz, C., Thormann, W., *Electrophoresis* 2003, 24, 4272–4281.

N Latex CDT method: agglutination inhibition



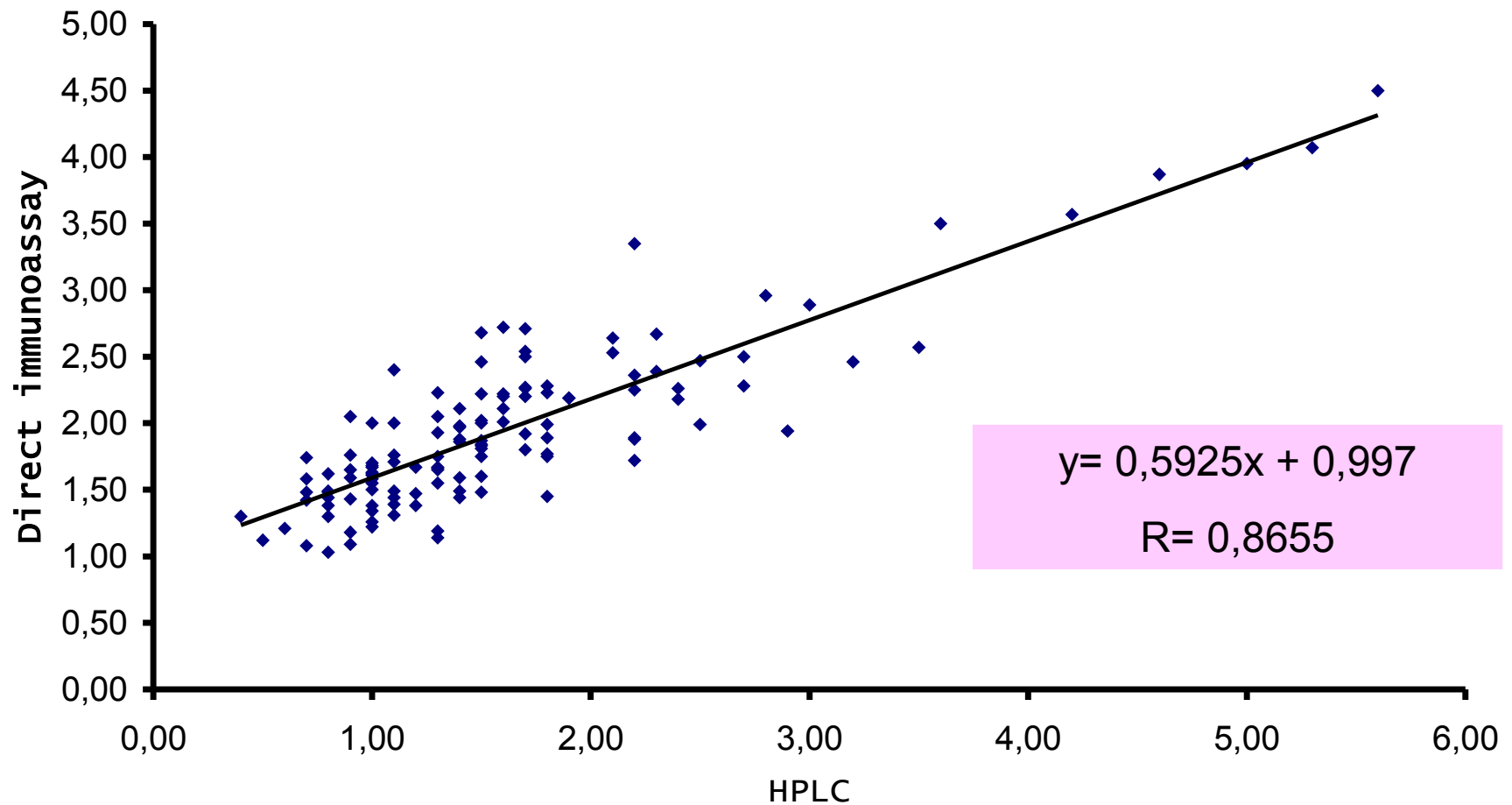
Increase of light scattering



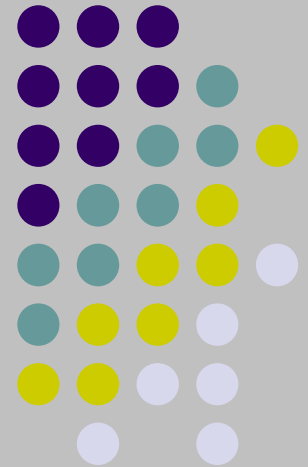
Decrease of light scattering

$$\%CDT = \frac{[CDT]}{[Total Tf]} \times 100$$

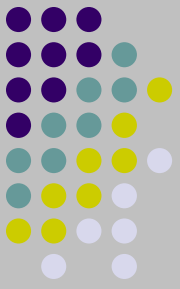
Correlazione tra immunoassay e HPLC (n=150)



Quale è il metodo di scelta per ottenere, se non la "vera CDT", almeno risultati legalmente difensibile?

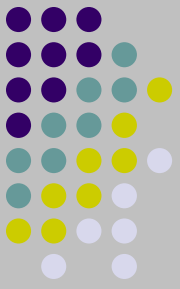


... sulla base delle linee guida della tossicologia forense ,
l'uso di analisi di screening seguite da analisi di
conferma è il METODO DI SCELTA.



"... the **screening method** should be specific, sensitive, reproducible, fast, and ideally can be automated. It should distinguish negative samples from those with borderline or abnormal results to minimize the number of confirmatory analyses ... the **confirmatory method** should be independent from the screening method using a different separation and/or detection principle. The analytical specificity should be higher than that of screening method, and the diagnostic sensitivity should be at least the same..."

T. Arndt and J.Kropf, Clin Chem (2002)



Screening techniques

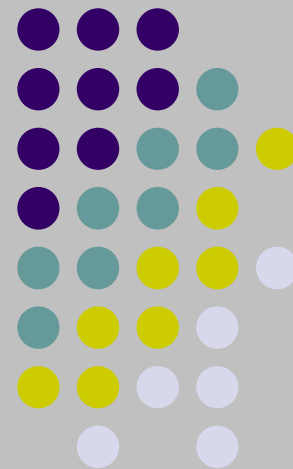
- Immunoassay (group specific antiserum) after CDT extraction
- Immunoassay with CDT specific antiserum
- Multiple channel capillary electrophoresis
- High productivity/low resolution HPLC/capillary electrophoresis

Confirmatory techniques

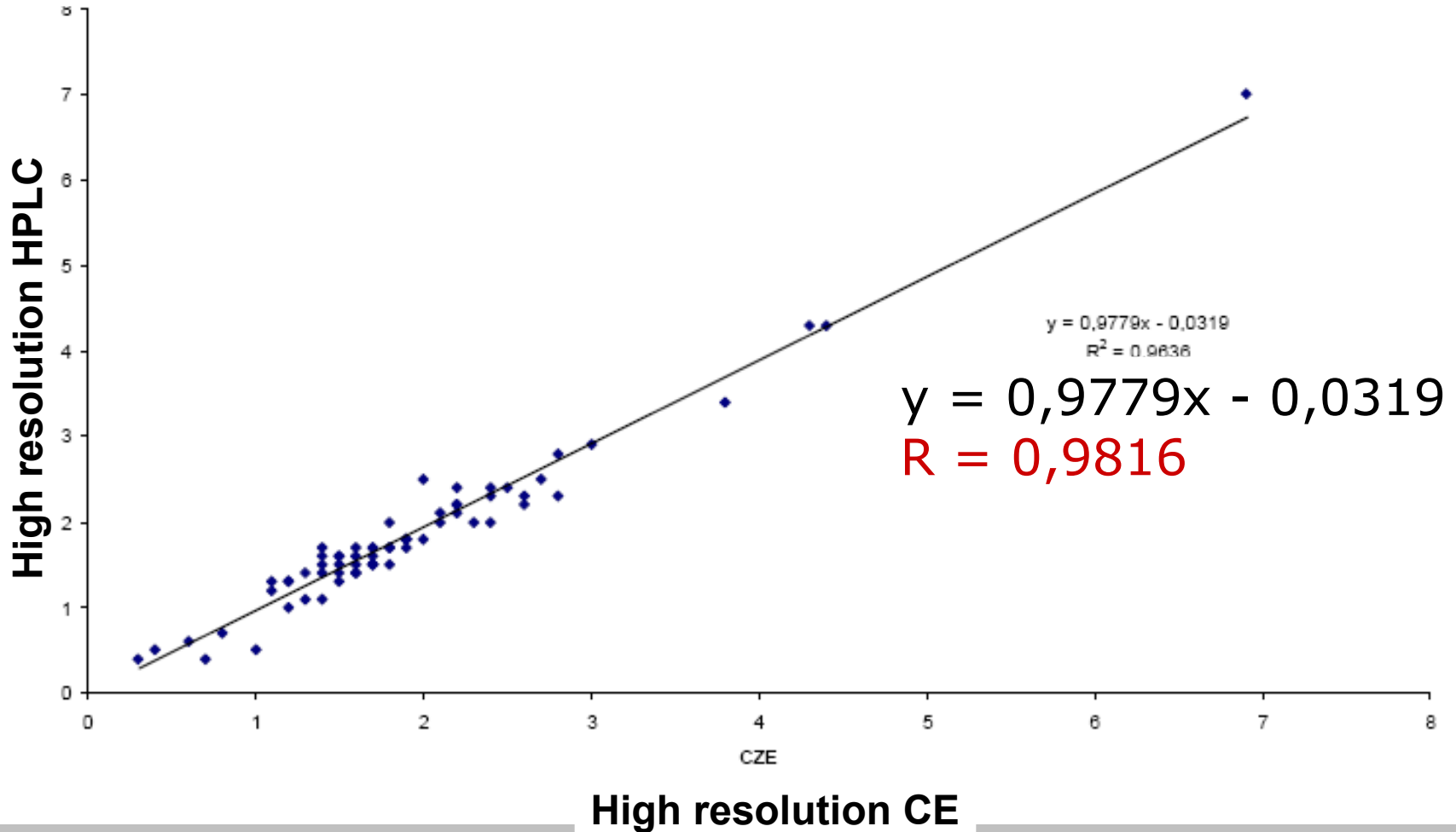
- High resolution ($R \geq 1.5$) gradient HPLC
- High resolution ($R \geq 1.5$) capillary electrophoresis (CE)

E' QUESTO IL METODO DEFINITIVO?

Proposta: concordanza quantitativa
quantitative tra due metodi ad elevata
risoluzione basati su principi chimico-fisici
differenti



High resolution CE vs high resolution HPLC



franco.tagliaro@univr.it

federica.bortolotti@univr.it

F. Bortolotti, G. De Paoli, J. P. Pascali, M.T. Trevisan, M. Floreani, F. Tagliaro. Analysis of Carbohydrate-Deficient Transferrin (CDT): Comparative Evaluation of Turbidimetric Immunoassay (TIA), Capillary Zone Electrophoresis (CZE) and High Performance Liquid Chromatography (HPLC). Clin Chem 51:2368-71 (2005).

F. Bortolotti, G. De Paoli, F. Tagliaro. Carbohydrate-deficient transferrin (CDT) as a marker of alcohol abuse: A critical review of the literature 2001-2005. J Chromatogr B 841, 96-109 (2006).

F. Bortolotti, G. De Paoli, J.P. Pascali, F. Tagliaro. Fully automated analysis of Carbohydrate-Deficient Transferrin (CDT) by using a multicapillary electrophoresis system. Clin Chim Acta 380, 4-7 (2007).