UNIVERSITÉ DE STRASBOURG

Formation CE-MS :

Couplage électrophorèse capillaire-spectrométrie de masse (CE-MS)

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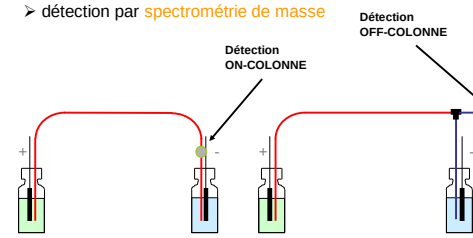
DETECTION

LES PLUS COURANTS:

- détection UV
- détection par fluorescence
- détection par spectrométrie de masse

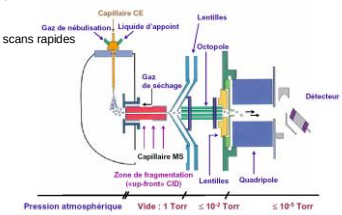


OFF-COLONNE

- Détection par spectrométrie de masse

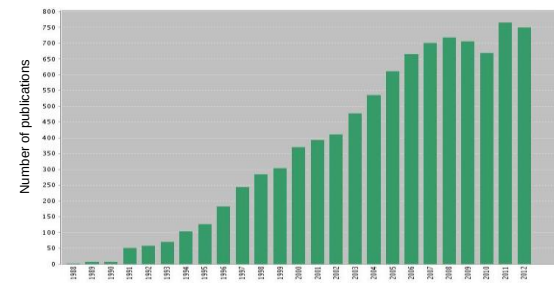
nécessite de concevoir une interface adaptée

- assurer le maintien du champ électrique
- diminuer les effets d'aspiration
- utiliser des analyseurs permettant des scans rapides



Interface basée sur le mode ESI/MS
appliquée aux sels d'ammonium, amines, dipeptides
ex : pour les ions simples, LOD = 10 amol

CE–MS Coupling



Web of science℠ search using term "capillary electrophoresis and mass spectrometry"

Couplage CE-MS

Advantages CE-MS →
- Bonne efficacité
- Ultra-low flow rate
- Selectivité
- Sensibilité
- Information de structure

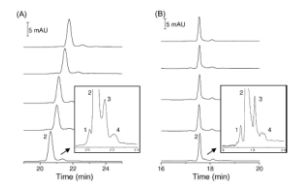
Inconvénients CE-MS →
- Faible volume d'échatillon (haute concentration)
- Compatibilité du BGE avec la MS
- Adsorption protéine surface interne
- **Difficulté pour maintenir le courant**

Les réponses aux limitations

Adsorption des protéines à la surface interne du capillaire

- Greffage des parois internes du capillaire.
  - ✓Covalent (idéal pour MS)
  - ✓Dynamique (compatible MS)

Exemple



Tampon Tris-Phosphate 400 mM pH 8,5, 30 kV

Répétition d'analyse CE-UV d'une solution d'hormone de croissance (3mg/mL).

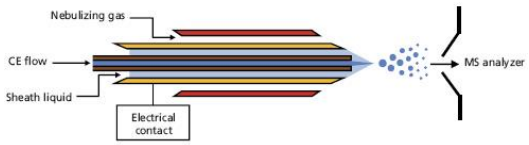
(A) Capillaire Viège
- Baisse de l'eof
- Baisse de la résolution

(B) Capillaire greffé PB-PVS
- RSD=0,68% (n=5)

Catai et al. J. Chrom. B., 2006, 852, 160-166

Interface Sheath liquid CE-ESI-MS

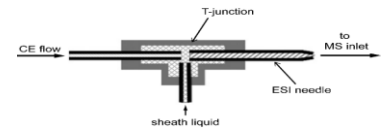
- Interface à liquide additionnel « Sheath liquid » CE-MS la plus communément utilisée
  - *Initially developed by Richard D. Smith group (Olivares et al., Anal. Chem.* ***1987****, 59, 1230-1232)*



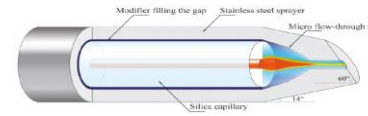
Haselberg et al., LCGC North America ***2012****, 30 (6), 504-525*

- Sheath liquid induces analytes dilution (~ 4 µl/min) → Significantly reduced sensitivity

Interface à liquide de jonction « junction liquid »



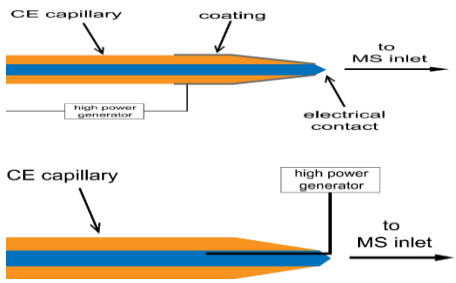
Maxwell, E.J. et al. Analytica Chimica Acta, 2008. **627**(1): p. 25-33.



Zhong, X. et al. Analytical Chemistry, 2011. **83**(12): p. 4916-4923.

- Débit > 300 nL/min → Réduction de la sensibilité
  Développement instrumentale

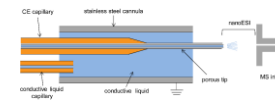
Interface sans liquide additionnel « Sheathliquid »



- Débit < 100 nL/min → Augmentation de la sensibilité
  Modification du capillaire

Interface CESI-MS

- CESI-MS allows to be operated using nano flowrates
  - Favorable to ESI ionization



- CESI-MS showed improved sensitivity compared to sheath liquid interface
  - *Faserl et al., Anal. Chem.* ***2011****, 83, 7297-7305*
  - *Busnel et al., Anal. Chem.* ***2010****, 82, 9476-9483*

Diagram and picture of the CESI interface

Optimisation du couplage CE-MS

CE-ESI-MS Coupling

Advantages of CE-MS

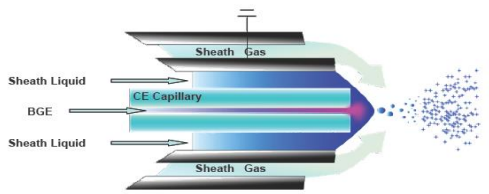
- Great efficiency
- Ultra-low flow rate
- Selectivity
- Sensitivity
- Structural information

Les réponses aux limitations

436 Anal. Chem. 1988, 60, 436–441

Capillary Zone Electrophoresis–Mass Spectrometry Using an Electrospray Ionization Interface

Richard D. Smith,* José A. Olivares,[1] Nhung T. Nguyen, and Harold R. Udseth
Chemical Methods and Separations Group, Chemical Sciences Department, Pacific Northwest Laboratory, Richland Washington 99352



Jusqu'à présent, l'interface « sheathliquid » a été la plus utilisée

CE-ESI-MS Coupling

CE is a miniaturized technique performing ultra-low flow rates

Decreasing the flow allows for increased sensitivity in the ESI-MS[1]

"Ultra-low flow" CESI-MS

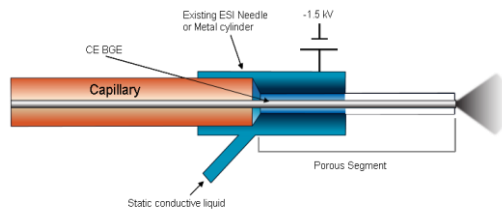
[1]Wilm, Mann International Journal of Mass Spectrometry 1994, 136, 167–180

Flow rates comparison

Separation Technology	Column Diameter	Flow-rate (nL/min)
High flow LC-MS	2.1 – 4.6 mm	200,000 – 2,000,000
Microbore LC-MS	1 mm	50,000-200,000
Microflow LC-MS	0.3-0.5 mm	2,000-50,000
CE-MS	50-100 µm	2,000 – 4,000
Nanoflow LC-MS	50-200 µm	100-1500
CESI-MS	**30 µm**	**< 30**

CESI allows performing real nano flowrates

CESI Interface



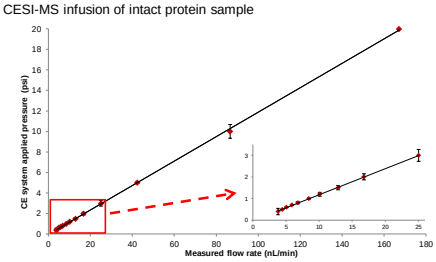
30 µm ID separation capillary with outlet portion etched by HF, provides electrical contact

Originally developed by M. Moini at U. of Texas and further developed by Beckman Coulter Inc.

What are the accessible flow rates?

CESI Interface Achievable Flow rates

- CESI-MS infusion of intact protein sample

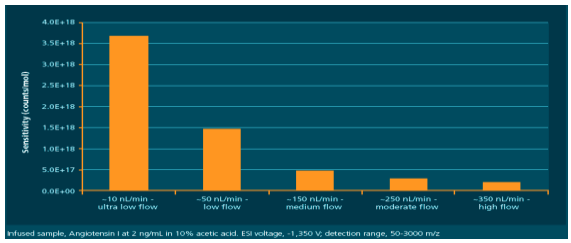


Conditions : Myoglobin 1 µM (in 10% acetic acid), Flow rates 3, 7 - 170 nL/min, Capillary voltage: -1400V, Investigated m/z : 848,94

Spray could be obtained using flow rate as low as 4 nL/min

Gahoual et al, Analytical and Bioanalytical Chemistry 2014, 406 (4), 1029-1038

Influence of Flowrates on Sensitivity



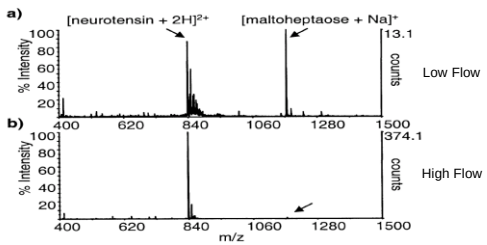
Evolution of the peak intensity of Angiotensin I (a) and detection sensitivity as a function of the flow rate (b) Experimental conditions: capillary electrophoresis; bare fused silica capillary with a porous tip, total length 88.5 cm×30 µm.d.×150 µm.o.d.; Infused sample, Angiotensin I at 2 ng/mL in 10% acetic acid. Mass spectrometry; capillary voltage,-1350 V; detection range, 50-3000m/z;

Decreasing the flowrate from 350 to around 10 nL/min, sensitivity increased by a factor of 20

Busnel et al, Analytical Chemistry 2010, 82, 9476-9483

Decrease of the Ion Suppression Phenomenon at Very Low Flow Rates?

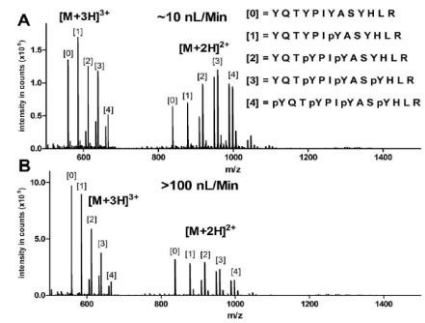
Importance of Flow Rates in ESI-MS: Ion Suppression



"Analyte suppression is practically absent at minimal flow rates below 20 nL/min"

Schmidt, Karas, Dulcks , J Am Soc Mass Spectrom 2003, 14, 492–500

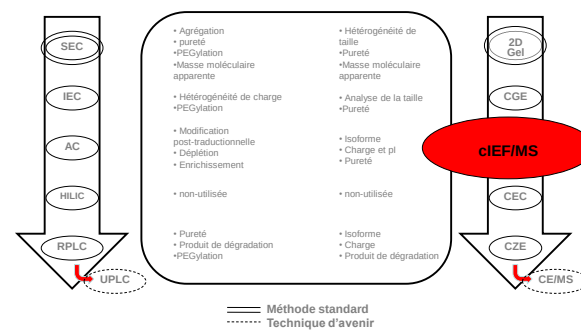
Infusion Pattern as a Function of Infusion Flow Rates



Heemskerk et al, Analytical Chemistry 2012, 84, 4552-4559

Applications

Applications

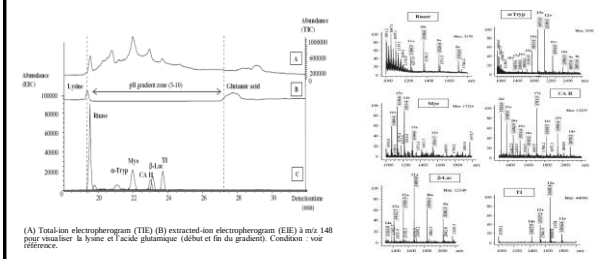


Staub et al, J. of Pharm. Biomed. Anal., 2011, 55, 810-822

Le couplage cIEF-ESI/MS

- Séparation suivant le point isoélectrique des protéines
- Nature de l'électrolyte support :
  - ✓ Anolyte : Acide formique 50 mM
  - ✓ Catholyte : Ammoniac 100 mM
  - ✓ Ampholyte support
- Focalisation sous champs puis Mobilisation sous champs et pression
- Grand champ d'applications :
  - Séparation d'isoformes
  - Détermination de PTMs (Glycosysation, phosphorylation…)
  - Analyse des produits de dégradation
  - Etc…

Séparation de 6 protéines modèles par CIEF-ESI/MS



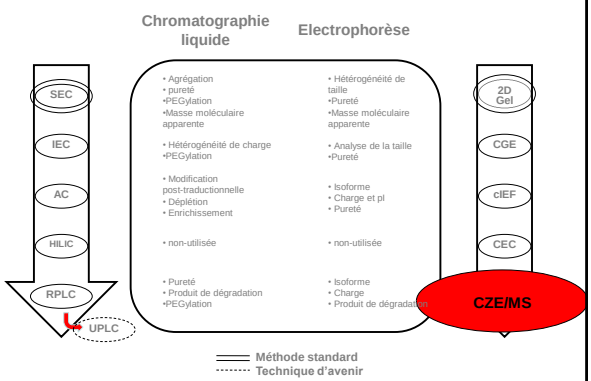
(A) Total-ion electropherogram (TIE) (B) extracted-ion electropherogram (EIE) à m/z 148 pour visualiser la lysine et l'acide glutamique (début et fin du gradient). Condition : voir référence.

- Milieu glycérol/eau
  - → **Solubilisation, pas EOF**
- Optimisation de la concentration d'ampholyte
  - → **Minimum de suppression d'ion**
- Injection de 40% du volume du capillaire
  - → **Focalisation optimale**

→
- Détermination du pI
- Caractérisation
- Pureté

Mokaddem et al, Electrophoresis, 2009, 30, 4040-4048

Applications



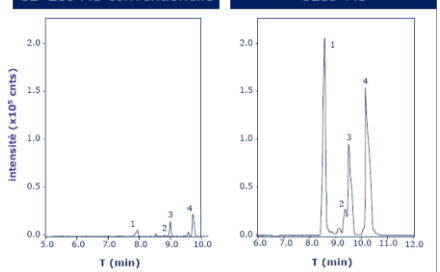
Staub et al, J. of Pharm. Biomed. Anal., 2011, 55, 810-822

Le couplage CZE-ESI/MS

- Séparation suivant des différences de mobilités électrophorétiques
- Nature de l'électrolyte support :
  - ✓ Volatile
  - ✓ Peu concentré en sel
  - ✓ Compatible pour des études en non dénaturant
- Efficacité inversement proportionnelle au coefficient de diffusion des molécules.
- Grand champ d'applications :
  - Séparation d'isoformes
  - Détermination de PTMs (Glycolsylation, phosphorlation…)
  - Analyse des produits de dégradation
  - Détermination de constante de stabilité

Comparaison CE/MS et CESI/MS

proteines (50 µg/mL par proteine)
1. insuline
2. anhydrase carbonique
3. ribonuclease A
4. lysozyme



Haselberg et al, J. Chrom., 2010, 1217, 7605-7611

Comparaison CE/MS et CESI/MS

limites de détection (nM)		
protéine	CESI-MS	CE-ESI-MS
insuline	1.3	106
anhydrase carbonique	0.58	79
ribonuclease A	0.62	33
lysozyme	0.50	41

- Limites de détection sub-nM
- 50-135 X de sensibilité

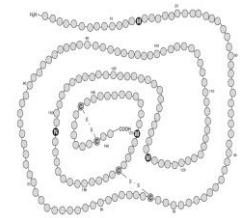
	RSDs, n=15		linéarité
protéine	T de migration	peak area	R²
insuline	0.63%	8.5%	0.999
anhydrase carbonique	0.61%	6.3%	0.989
ribonuclease A	0.68%	8.4%	0.992
lysozyme	0.74%	7.0%	0.997

- **Faible limite de détection**
- **Répétabilité sur les temps de migration**
- **Bonne linéarité**

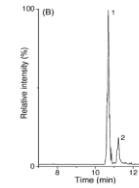
Analyse d'hormone de croissance (hGH)
par CE-ESI/MS

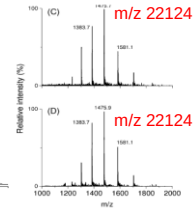
Analyse d'hormone de croissance (hGH) par CE-ESI/MS

hGH



Structure de la hGH, M : méthionine , N: asparagine, C,: cysctéine





Etude de rhGH (recombinant), Tampon ammonium formate pH 8,5

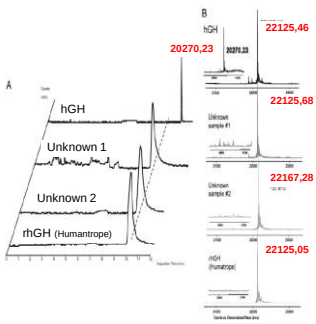
- Déamidation de l'asparagine
- Oxydation des méthionines

- Hypothèse de déamidation
- Manque de résolution en MS

Catai et al. J. Chrom. B., 2006, 852, 160-166

Analyse d'hGH par CE-ESI/MS

Différentiation entre hormone endogène et recombinante (rhGH)



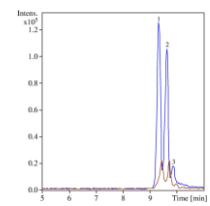
hGH et rhGH à 200 µg/mL, Tampon ammonium formate pH 8,5, 20% ACN

- Présence de l'isoforme à 20kDa pour hGH
- Absence de l'isoforme à 20 kDa pour rhGH
- Echantillons inconnus = rhGH :
- LOD déterminé à 50µg/mL
- Possibilité de diagnostique

Staub et al. Electrophoresis, 2010, 31, 388-395

Analyse d'hGH par CE-ESI/MS

Résolution isotopique de protéines intactes



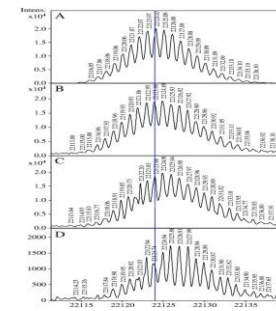
CE-ESI-TOF/MS de rhGH. Tampon ammonium hydrogeno carbonate pH 8,5,

A : Simulation de la distribution isotopique de la rhGH

B : rhGH intact

C : rhGH monodéamidée

D : rhGH didéamidée

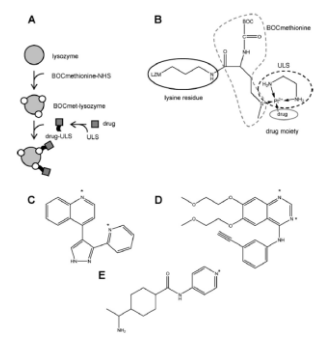


Haute résolution pour des protéines intacts (30kDa) avec TOF MS

Taichrib et al. J. of. Proteomics, 2011, 74, 958-966

Caractérisation de « drug-lysozyme » conjugués
par CESI-TOF/MS

Caractérisation de « drug-lysozyme » conjugués par CESI-TOF/MS



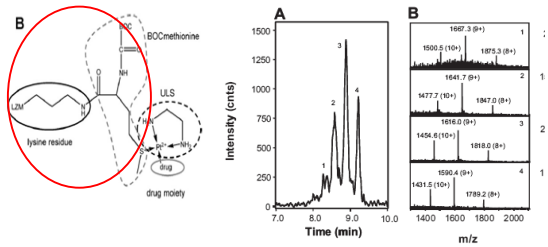
(A)Voie de synthèse drug-LZM conjugué (B) Représentation schématique de drug-ULS-BOCmet-LZM conjugué (C-E) structures moléculaires de kinase inhibitor LY364947, erlotinib et Y27632. Asterisks = potentiel site de complexation

- traitement BOC-L-methionine hydroxysuccinimide ester (BOCmet-NHS) :
  - ✓introduction d'un groupement sulfure pour une coordination au platine
- LZM est une protéine basic pI 11
  - ✓Greffage positif au polyéthylèneimine
- BGE, acide acétique 100 mM pH=3,1, 5% isopropanol

Haselberg et al, Anal. Chim. Acta, 2011, 698, 77-83

Application

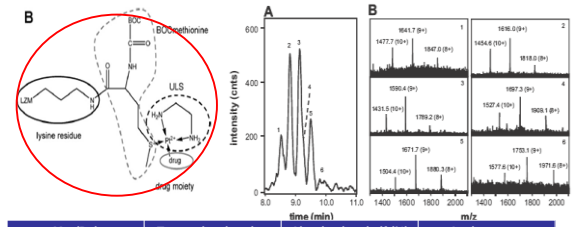
Caractérisation de « drug-lysozyme » conjugués par CESI-TOF/MS



Mw (Da)	Temps de migration (min)	Aire de pic relatif (%)	Assignement
14994.3	8.25 ± 0.03	5.9 ± 0.5	$BOCmet_3$-LZM
14766.1	8.44 ± 0.07	28.9 ± 1.5	$BOCmet_2$-LZM
14535.1	8.69 ± 0.08	37.4 ± 2.8	$BOCmet_1$-LZM
14304.9	9.08 ± 0.08	27.8 ± 1.3	LZM

Application

Caractérisation de « drug-lysozyme » conjugués par CESI-TOF/MS

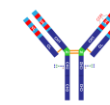


Mw (Da)	Temps de migration (min)	Aire de pic relatif (%)	Assignement
14766.2	8.50 ± 0.01	10.7 ± 0.3	$BOCmet_2$-LZM
14535.0	8.82 ± 0.01	21.7 ± 1.1	$BOCmet_1$-LZM
14304.5	9.14 ± 0.01	28.2 ± 0.8	LZM
15033.7	9.47 ± 0.02	20.2 ± 1.0	$(drug\text{-}ULS)_1$-$BOCmet_1$-LZM
15264.2	9.18 ± 0.03	15.5 ± 0.3	$(drug\text{-}ULS)_1$-$BOCmet_2$-LZM
15764.5	9.89 ± 0.06	2.6 ± 0.2	$(drug\text{-}ULS)_2$-$BOCmet_2$-LZM

Complete primary structure of monoclonal antibodies in a single analysis using CESI-MS/MS

mAbs characterization by CESI-MS/MS

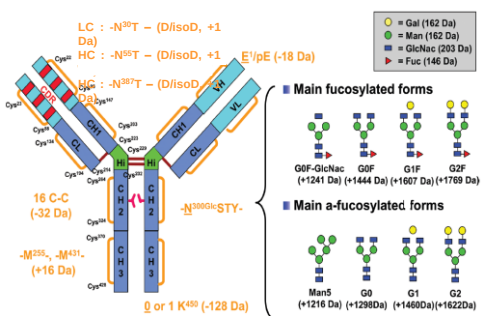
- Monoclonal antibodies (mAbs) have been introduced as therapeutic treatment since 1986
  - 43 marketed mAbs in 2014
  - more than 30 mAbs in clinical trial phase III
- mAbs specificity for its antigen opens new avenues for therapeutic treatments
  - oncology
  - autoimmune diseases
  - Transplant rejection prevention



- mAbs are complex and heterogeneous glycoproteins representing a challenge to analytical sciences
  - Characterization on different level of the mAbs
  - Necessity of precise and high throughput characterization

*Zhang Z. et al., Mass Spec. Rev., **2009** (28), 147-176*

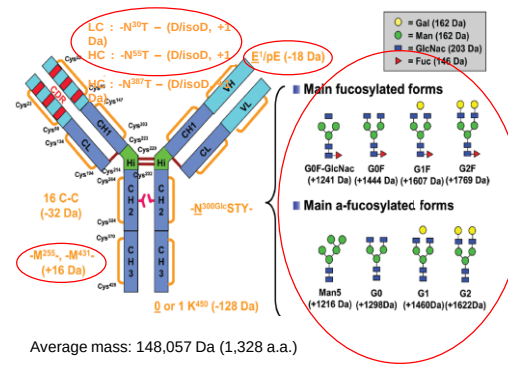
Trastuzumab (Herceptin)



Average mass: 148,057 Da (1,328 a.a.)

A. Beck et al., Anal. Chem. 2012, 84, 4637-4646

Trastuzumab (Herceptin)

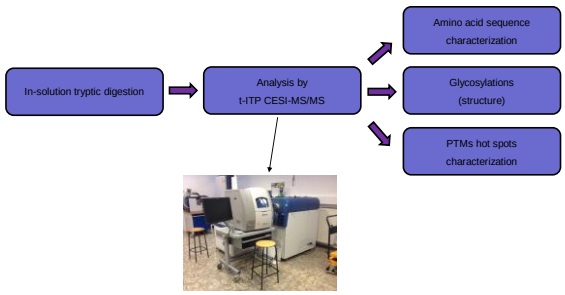


Average mass: 148,057 Da (1,328 a.a.)

A. Beck et al., Anal. Chem. 2012, 84, 4637-4646

mAbs characterization workflow

- Primary structure characterization workflow based on bottom-up proteomics strategy



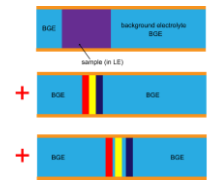
CESI8000 coupled to 5600 TripleTOF MS

Transient isotachophoresis (t-ITP)

- Several improvements have been implemented during the development of the CESI-MS/MS methodology

Transient isotachophoresis implemented to the method

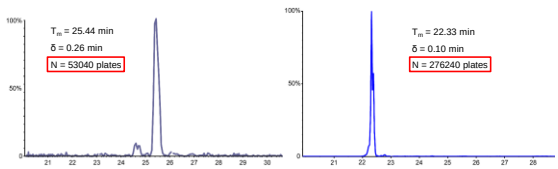
- Transient isotachophoresis (t-ITP) is a preconcentration method in CZE to increase sample volume injection likewise separation efficiency



leading electrolyte (LE) is ammonium acetate

*Larsson M. et al, Electrophoresis **2000**, 21, 2859-2865*

Transient isotachophoresis (t-ITP)



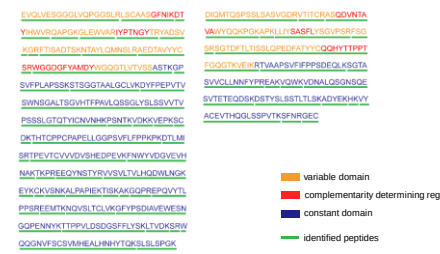
Peptide **HT07** separated in conventional CZE

Peptide **HT07** separated using t-ITP CZE

- Injection up to 25% of capillary volume without losing separation efficiency (2% in conventional CZE)
- Increases separation efficiency compared to equivalent CZE conditions

Amino acid sequence characterization

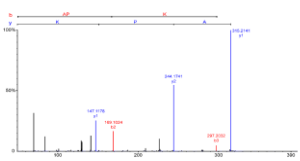
- MS/MS amino acid sequence characterization (trastuzumab)



100% sequence coverage achieved in a single injection through **only purely tryptic unmodified peptides**

*Gahoual R. et al., Anal. Chem., **2014** (86), 9074-9081*

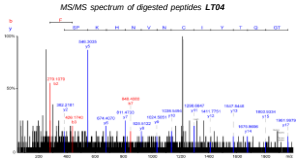
Amino acid sequence characterization



*MS/MS spectrum of digested peptides **LT04***

APK

(m/z 315.2039 ; 2+)



*MS/MS spectrum of digested peptides **HT15***

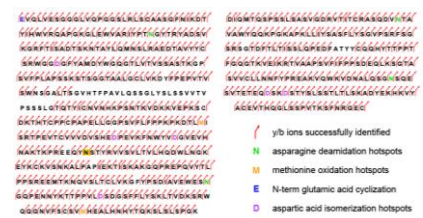
DYFPEPVTVSWNSGALTSGVHTFPAVLQS SGLYSLSSVVTVPSSSLGTQTYICNVNHKP SNTK

(63 amino acids ; m/z 1119.898 ; 6+)

Implementation of CE allows separation and successful detection of a larger variety of peptides

Amino acid sequence characterization

MS/MS amino acid sequence characterization (trastuzumab)



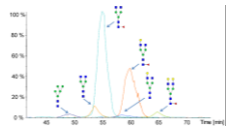
Systematically > 70 % of y/b ions could be retrieved **(> 90% for trastuzumab)**

*Gahoual R. et al., Anal. Chem., **2014** (86), 9074-9081*

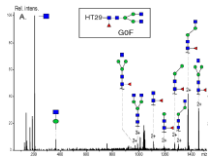
Glycosylations characterization

- mAbs glycosylations are characterized simultaneously using the same CESI-MS/MS data

CE induces separation of glycopeptides → limiting competition during ESI ionization



- MS/MS spectra exhibited fragmentation of the glycosylation

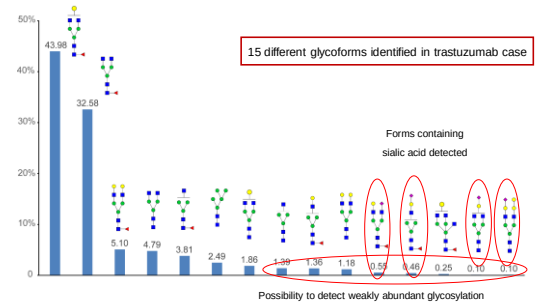


Glycosylation structural characterization obtained from the CESI-MS/MS data

*Gahoual R. et al., mAbs, **2013** (5), 479-490*

Glycosylations characterization

- Glycopeptides MS signal intensity used to estimate glycoforms relative abundances

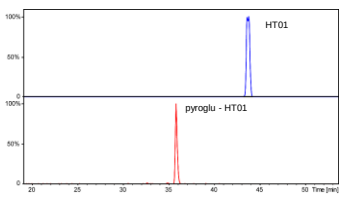


15 different glycoforms identified in trastuzumab case

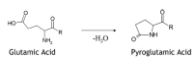
*Gahoual R. et al., Anal. Chem., **2014** (86), 9074-9081*

PTMs hot spots characterization

N-terminal glutamic acid cyclization characterization



Extracted ion electropherograms of peptides HT01 and modified HT01

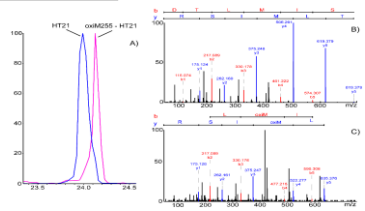


- CE mechanism separates of peptide with N-terminal glutamic acid cyclization from the unmodified peptide

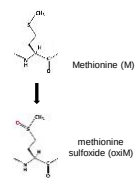
Results suggest partial modification of sample → Favorable conditions to estimate sample modification level

PTMs hot spots characterization

Methionine oxidation



EIEs and MS/MS spectra of peptides HT21 (intact and modified)



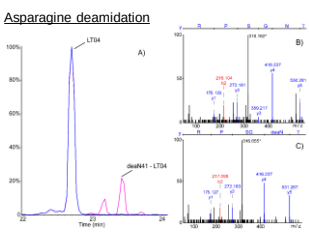
- Methionine oxidation causes peptide mass shift (+15.99 Da) leading to the separation of the modified peptide in CZE

confirmed by MS/MS spectra

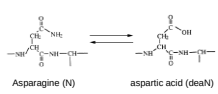
*Gahoual R. et al., Anal. Chem., **2014** (86), 9074-9081*

PTMs hot spots characterization

Asparagine deamidation



EIEs and MS/MS spectra of peptides LT04 (intact and modified)



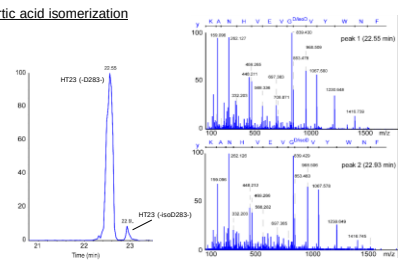
- Deamidation (+ 0.98 Da) involves mobility change in CZE enabling the separation of the unmodified peptide

CE separation of deamidated peptides eases the identification of the modification by MS

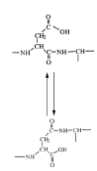
*Gahoual R. et al., Anal. Chem., **2014** (86), 9074-9081*

PTMs hot spots characterization

Aspartic acid isomerization



EIEs and MS/MS spectra of peptides HT23 (intact and modified)



CE separation prior to MS analysis allows in this particular case to include aspartic acid isomerization in the overall characterization workflow

*Gahoual R. et al., Anal. Chem., **2014** (86), 9074-9081*

mAbs characterization by CESI-MS/MS

	Trastuzumab	Cetuximab	mab in-dev #1	mab in-dev #2
sequence coverage	100%	100%	100%	100%
% MS2 y/b ions	> 90%	> 70 %	> 90%	> 70%
identified glycosylations	15	15	10	16
other PTMs hotspots				
glutamic acid cyclization	1 / 1	1 / 1	1 / 1	1 / 1
methionine oxidation	2 / 2	0 / 0	2 / 2	0 / 0
asparagine deamidation	4 / 4	4 / 4	2 / 2	4 / 4
aspartic acid isomerization	6 / 6	2 / 2	3 / 3	2 / 2

Results summary obtained with the t-ITP CESI-MS/MS method

The t-ITP CESI-MS/MS method developed demonstrated its robustness on different samples including technical replicates in each case

Gahoual R. et al., Anal. Chem., ***2014*** (86), 9074-9081

Conclusion

- Single injection (200 fmol) of mAbs digest by t-ITP CESI-MS/MS →
  - 100% amino acid sequence characterization
  - 15 glycoforms characterization
  - All PTMs hot spots characterization
- CESI-MS system and conditions provide an excellent ESI ionization yield →
  - High sensitivity
  - Positive impact on MS/MS spectra
  - Isomers separation without sample teatment

mAbs biosimilarity assessment

- As several mAbs patent are ending in the next few months/years, other companies should have the possibility to commercialize « unprotected » mAbs
- mAbs complexity and production process (cell line selection) makes it nearly impossible to produce strictly the same product as the innovator company
- FDA and EMA are introducing guidelines to help biopharma companies to determine the key features needed for a biosimilarity between two products in term of structure, PK and PD => reducing clinical trials

guidelines → biosimilars approval

1st case

trastuzumab vs. candidate biosimilar

Herceptin trastuzumab

Amino acid sequence similarity

- Complete sequence coverage obtained for trastuzumab
- Biosimilar candidate sequence could be successfully identified except HC **K**[217]

Suggesting an amino acid substitution between the two samples

Gahoual R. et al., mAbs ***2014****, in press*

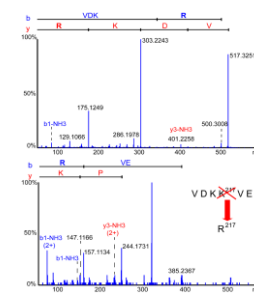
Amino acid sequence similarity

Interpretation of unidentified MS/MS spectra

Unambiguous characterization of the amino acid substitution of biosimilar candidate

V D K **R**[217] V E P K

rejected candidate

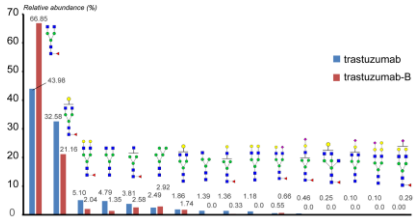


CESI-MS/MS spectra of trastuzumab biosimilar candidate

Gahoual R. et al., mAbs ***2014****, in press*

Glycoforms characterization

- Glycosylation distribution evaluated for each sample using CESI-MS/MS data



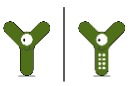
Identification of a significant number of glycoforms → Minor differences of glycoforms could be distinguished

*Gahoual R. et al., mAbs **2014**, in press*

2nd case

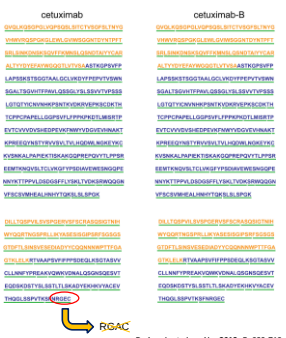
cetuximab vs. candidate biosimilar

ERBITUX
Cetuximab



Amino acid sequence similarity

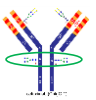
- A single analysis of each sample sufficient to conclude on the complete similarity regarding AA sequence
- Complete sequence coverage is obtained through peptides without miscleavages or PTMs
- CESI-MS/MS enabled to confirm an error, recently reported in the litterature

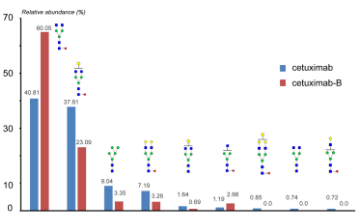


*D. Ayoub et al., mAbs **2013**, 5, 699-710*

Glycoforms characterization

- Fc/2 glycosylation site characterization





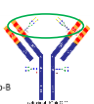
Cetuximab possess two different N-glycosylation sites

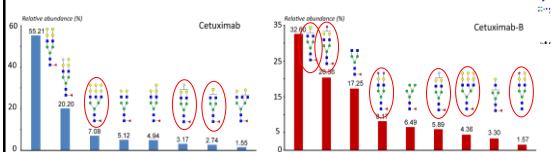
Significant number of glycans could be characterized

Heterogenous glycoforms could be identified → Difference in glycoforms distribution could be observed

Glycoforms characterization

- Fd glycosylation site characterization





Glycoforms exhibited by the candidate biosimilar are significantly different from cetuximab

- *30 % of glycans contains N-acetylneuraminic acid*
- *Capped gal-α1,3-gal glycans significantly reduced*

→ Rejected as biosimilar

Biosimilarity assessment conclusion

- CESI-MS/MS enabled biosimilarity assessment regarding primary structure and PTMs

Trastuzumab vs. Biosimilar	Cetuximab vs. Biosimilar
1 amino acids difference	Complete sequence similarity
Minor differences in glycoforms relative abundances	Glycoforms expressed significantly different
No significant differences in modification levels	No significant differences in modification levels

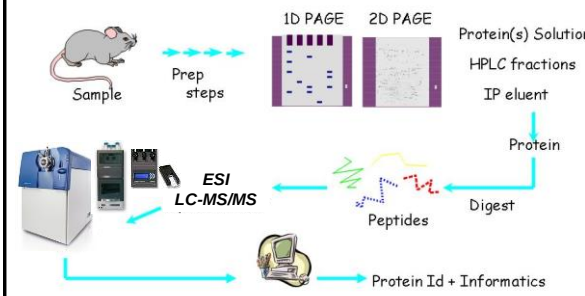
Summary of the biosimilarity assessment by I-ITP CESI-MS/MS

→ Distinction of significant difference between innovator and candidate

- CESI-MS/MS allowed to conclude in each case on the biosimilarity assessment

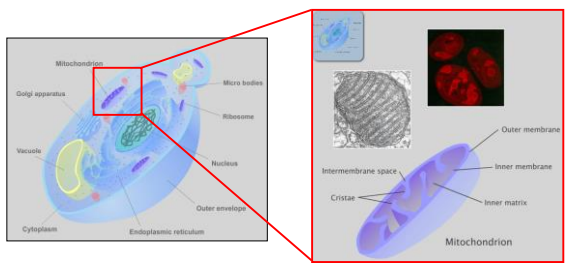
Characterization of yeast mitochondrial proteome through CESI-MS workflow

The Proteomic Approach



Steady increase in complexity of samples

Challenge to analytical sciences



Yeast mitochondrial Gln tRNA(Gln) is generated by a GatFAB-mediated transamidation pathway involving Arc1p-controlled subcellular sorting of cytosolic GluRS. *Genes & development*, Becker et al., 2009

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Content of the Study

- **Assessment of capabilities of CESI-MS/MS for the analysis of 100 ng yeast mitochondrial digest**
- **NanoLC-MS/MS is performed in parallel as a reference method**

In the same conditions

- **Using a classical protocol (60 min gradient)**
- **Same sample condition: 100 ng mitochondrial yeast digest**

- **CESI-MS/MS**

Bare Fused Silica Capillary (90 cm*30µm i.d.)
BGE: 1% formic acid (pH 2.1)
Sample: yeast mitochondrial digest sample 1 µg/µL (100 ng injected)
Voltage: 15 kV
Current: 2 to 4 µA
Distance between capillary tip and MS: 3 to 8 mm
Flowrate: <10 nL/min

- **MS Conditions**

AB SCIEX TripleTOF 5600 System, positive mode, capillary voltage - 1750V, Curtain gas 5, T=75°C, GS1= 0
Mass range: 150-1250 (TOF-MS), 100-2000 (TOF-MS/MS)
IDA Top 20 (duty cycle 2.0 sec, mean spectra rate 12.1 Hz)

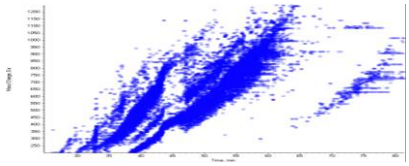
- **NanoLC-MS/MS**

Eksigent nanoLC 2D plus system with cHiPLC System
Pre column: ChromXPC18-CL (200 µm, 0.5 mm, 3 µm, 120Å)
Column: ChromXPC18-CL (75 µm, 15 cm, 3µm, 120Å)
Gradient: started at 5% B. The concentration of solvent B was increased linearly from 4% to 40% during 50 min and from 50% to 100% during 1 min (solvent A, 0.1% formic acid; solvent B, 0.1% formic acid in 100% acetonitrile)
Sample: mitochondrial digest sample 1 µg/µL (100 ng injected)
Flowrate: 300 nL/min

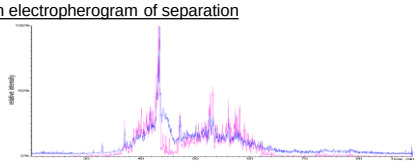
- **MS Conditions**

AB SCIEX TripleTOF 5600 System, positive mode, capillary voltage 2300 V, Curtain gas 22, T=150°C, GS1= 5
Mass range: 150-1250 (TOF-MS), 100-2000 (TOF-MS/MS)
IDA Top 20 (duty cycle 1.6 sec, mean spectra rate 12.1 Hz)

- MS/MS heat map



- Total ion electropherogram of separation



- Identification results

100 ng of yeast mitochondrial digest injected in CESI-MS/MS

Paragon™ Database Search Algorithm in ProteinPilot™ Software

- Decoy database automatically generated
- False Discovery Rate < 1%

Peptides in Group

Con...	Conf	Sequence	Modifications	Cleavages	ΔMass
2.00	99	AAGEAEPVPEETLQTEPST...		cleaved V-...	0.0008
2.00	99	AAGEAEPVPEETLQTEPST...		cleaved V-...	0.0006
2.00	99	ADTVFLAPGLSAITIDAUF		cleaved A-...	-0.0053
2.00	99	ADTGIAMEGATDAAR			-0.0001
2.00	99	ALGVAR			-0.0025

2774 unique peptides identified

341 proteins identified

- Identification results

100 ng of mitochondrial yeast digest injected in NanoLC-MS/MS

Paragon Database Search Algorithm in ProteinPilot Software

- Decoy database automatically generated
- False Discovery Rate < 1%

Peptides in Group

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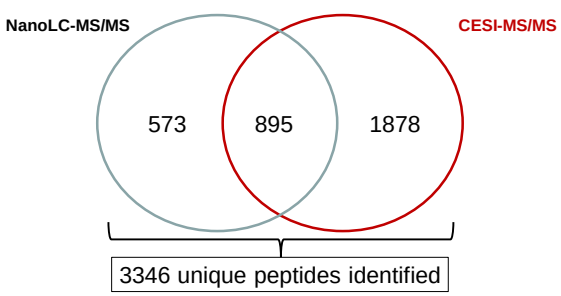
2.00	99	AAGEAEPVPEETLQTEPST...		cleaved V-...	0.0006
2.00	99	ADTVFLAPGLSAITIDAUF		cleaved A-...	-0.0053
2.00	99	ADTGIAMEGATDAAR			-0.0001
2.00	99	ALGVAR			-0.0025

1468 unique peptides identified

310 proteins identified

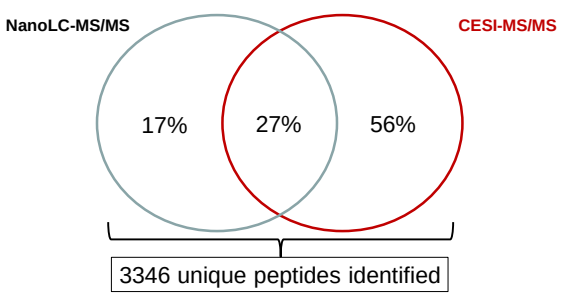
Peptide identifications

- Peptide identifications (in terms of unique peptides)



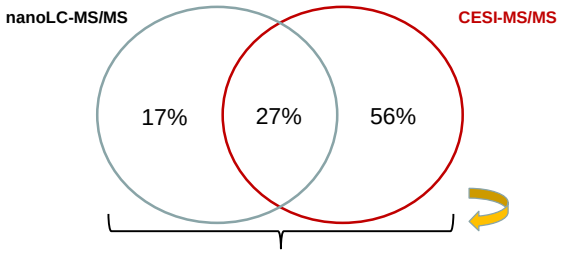
Same sample (yeast mitochondrial digest 1 µg/µL (100 ng injected))

- Peptide identifications (in terms of unique peptides)



Same sample (yeast mitochondrial digest 1 µg/µL (100 ng injected))

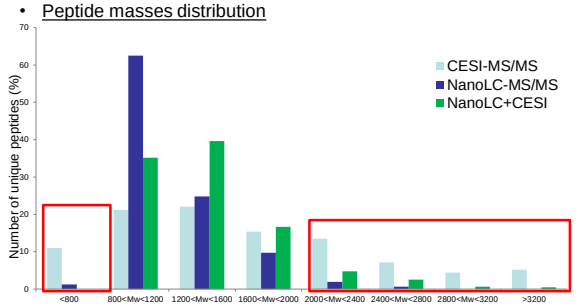
- Peptide identifications (in terms of unique peptides)



CESI-MS/MS allowed the detection of additional peptides

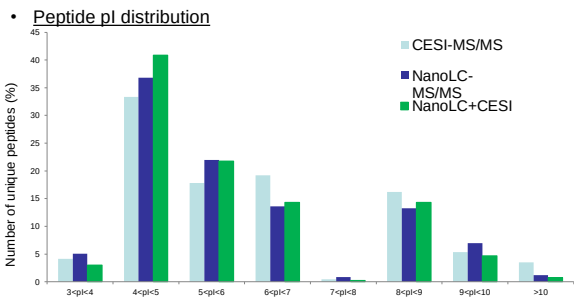
Same sample (yeast mitochondrial digest 1 µg/µL (100 ng injected))

- Peptide masses distribution



CESI-MS was capable of analyzing small and very large peptides under same experimental conditions

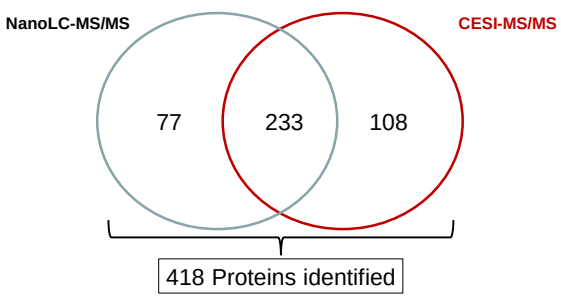
- Peptide pI distribution



CESI-MS was capable of analyzing acidic peptides and basic peptides under same experimental conditions

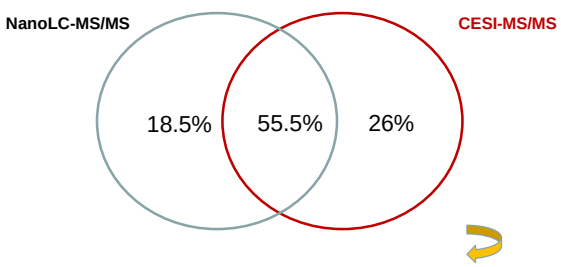
Protein identifications

- Protein identifications



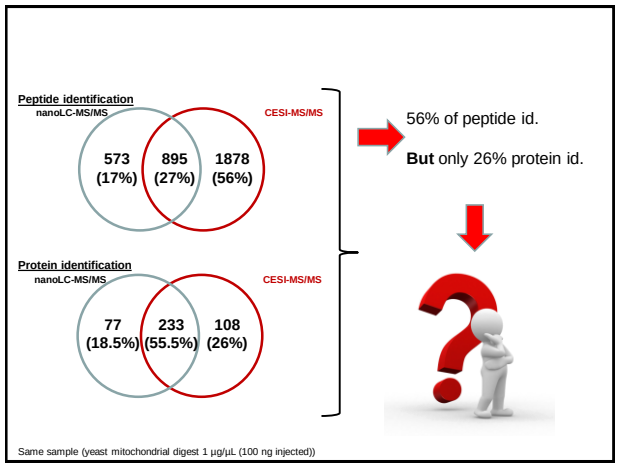
Same sample (yeast mitochondrial digest 1 µg/µL (100 ng injected))

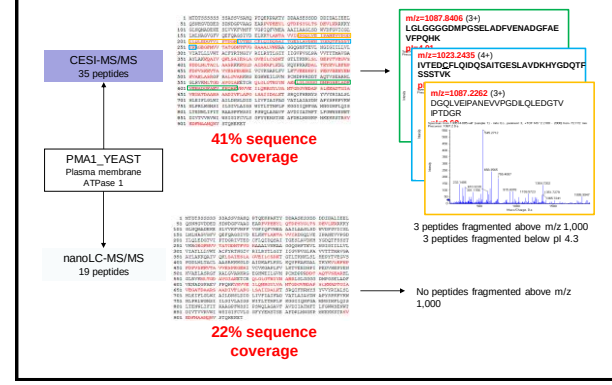
- Protein identifications



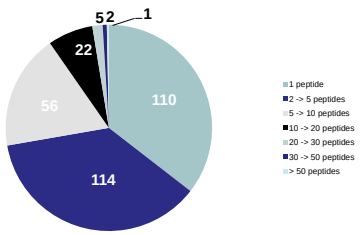
CESI-MS/MS allowed to detect additional proteins

Same sample (yeast mitochondrial digest 1 µg/µL (100 ng injected))

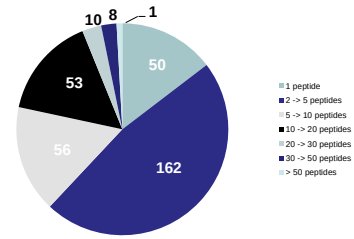




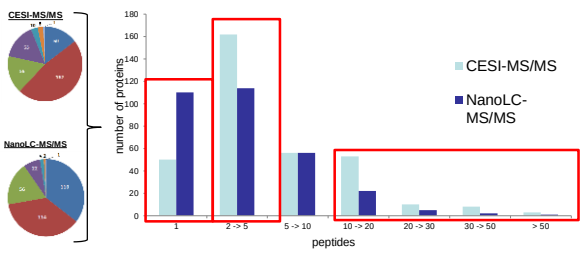
- NanoLC-MS/MS



- CESI-MS/MS



- Protein identification distribution



Using CESI-MS proteins could be identified with more confidence due to additional identified peptides

Conclusion

- **CESI-MS/MS generated 2774 peptide IDs corresponding to 341 proteins IDs from 100 ng mitochondrial yeast digest injected**
- **CESI-MS was capable of analyzing small and large peptides under same experimental conditions**
- **CESI-MS protein could be identified with more confidence due to additional identified peptides**

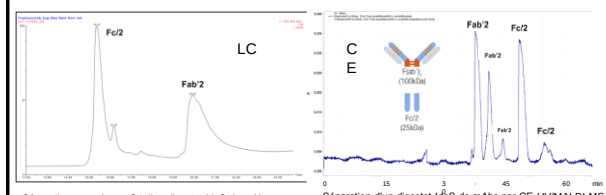
Couplage CE-MALDI-MS

Couplage CE/MS

INTERET

Electrophorèse capillaire (CE)

- Principe de séparation (Electrocinétique)
- **Bonne efficacité**



Séparation sur colonne C4 d'un digestat IdeS de mAbs par LC-MS

Séparation d'un digestat IdeS de mAbs par CE-UV/MALDI-MS

Couplage CE/MS

INTERET

Electrophorèse capillaire (CE)

- Principe de séparation (Electrocinétique)
- **Bonne efficacité**

MS

- Sélectivité / Précision (qq ppm)
- Sensibilité (fmol-Amol)

DIFFICULTES

- Maintien du courant
- Capacité de chargement du capillaire (qq nL)
- Sels Détergents

⇨ **MALDI- MS plus de tolérance aux sels**

SOMMAIRE

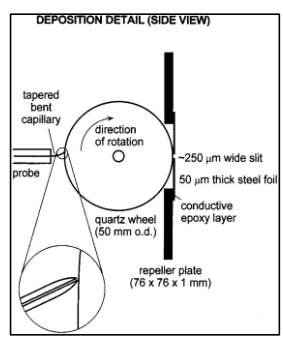
1. Le couplage CE/MALDI –MS
2. Mise au point et évaluation du CE-UV/MALDI-MS
3. Mise au point de l'analyse Top DOWN par CE-UV/MALDI-MS

Couplage CE/MALDI-MS Direct

Preisler, Foret, Karger
Anal.Chem. 1998, 70, 5278-5287

Dépot sur un disque tournant ou sur une boule en rotation

Bonne sensibilité mais difficile à mettre en place et très faible robustesse

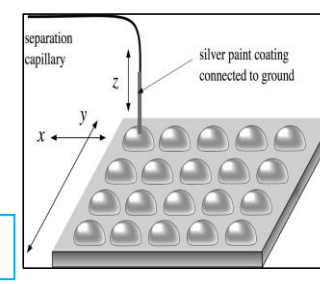


Couplage indirect
Collecteur de fraction sans liquide additionnel

Busnel, Josserand, Lion, Girault
***Anal. Chem.* 2009, *81*, 3867-3872**

Dépot sur plaque MALDI Sans liquide additionnel Capillaire conducteur

✓ **Bonne sensibilité**
✓ **Bonne efficacité**
✓**Miniaturisation**



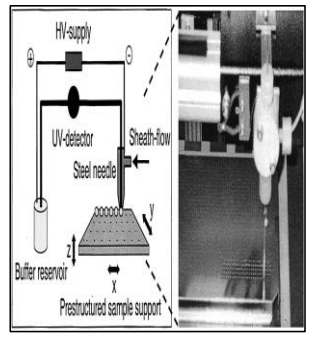
Couplage indirect

Collecteur de fraction avec liquide additionnel

Johnson, Bergquist, Ekman, Nordhoff, Schürenberg, Klöppel, Müller, Lehrach, Gobom
***Anal. Chem.* 2001, *73*, 1670-1675**

Dépot sur plaque MALDI avec liquide additionnel

- ✓ **Bonne sensibilité**
- ✓ **Bonne efficacité**
- ✓ **Bonne robustesse**
- ✓ **Capillaire fixe**



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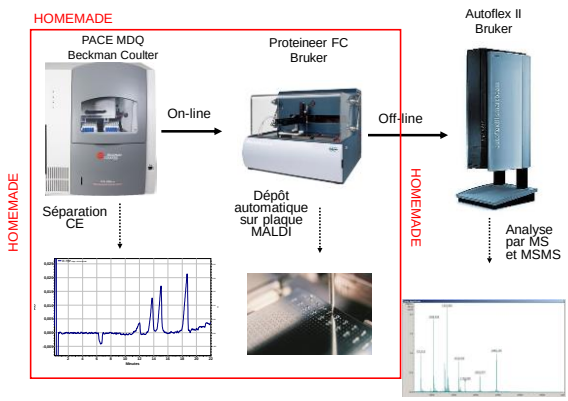
Couplage CE/MALDI-MS indirect



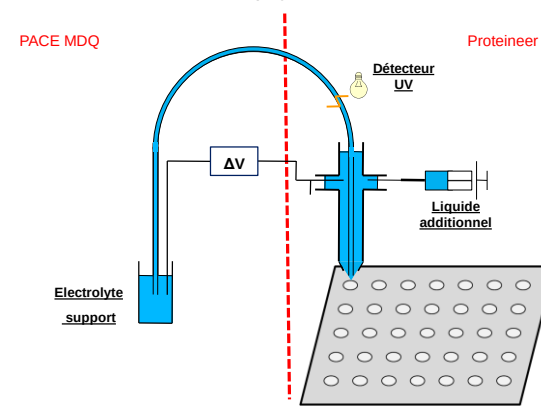
Couplage indirect

Collecteur de fraction avec liquide additionnel

Couplage indirect

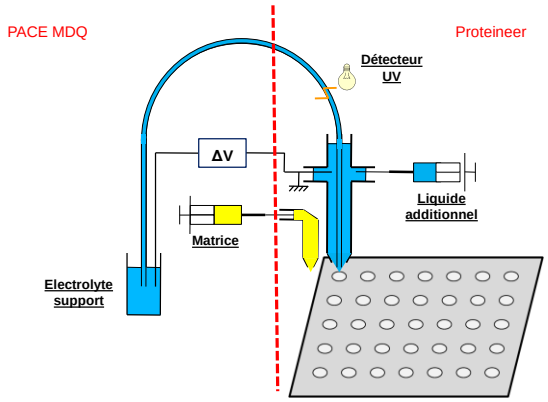
Collecteur de fraction avec liquide additionnel

Schéma du couplage CE-UV/MALDI-MS



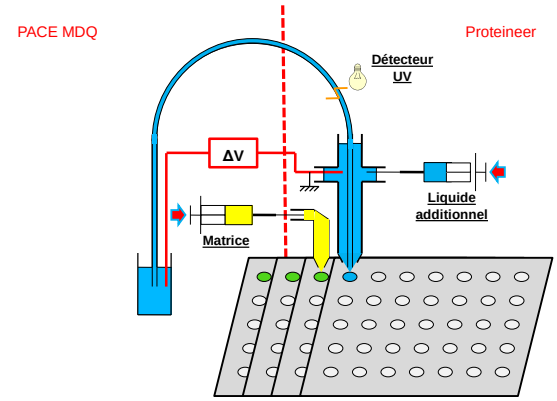
M. Biacchi et al. *Electrophoresis* **2014**, online available

Schéma du couplage CE-UV/MALDI-MS



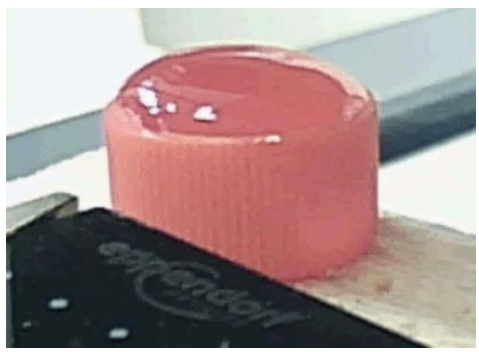
M. Biacchi et al. *Electrophoresis* **2014**, online available

Schéma du couplage CE-UV/MALDI-MS



M. Biacchi et al. *Electrophoresis* **2014**, online available

Couplage indirect
Collecteur de fraction avec liquide additionnel



Analyse de peptides ou de protéines entières

Analyse de peptides ou de protéines entières

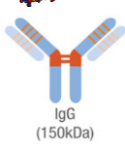
Différentes stratégies d 'analyses

Digestion

Protéines Entières
mAbs Entiers

Peptides

Sous unités protéiques :
• mAbs



10 kDa – 70 kDa
150 kDa

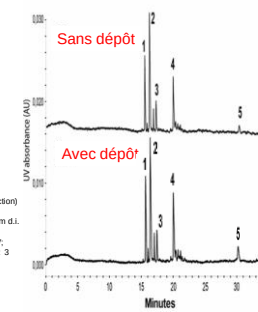
Quelques
Da- kDa

25 kDa – 100 KDa

Protéines entières de 10 kDa – 70 kDa

Protéines modèles :

- 1 Cytochrome C
- 2 Lysozyme
- 3 Myoglobine
- 4 Ribonucléase A
- 5 α-lactoglobuline



UV Electropherogram without and with fraction collection) of a five protein sample by **CE-UV/MALDI-MS**.
Experimental conditions: HPC coated capillary, 50 µm d.i. x 60 cm (detection cell, 60 cm); BGE: 83.3mM ionic strength ammonium acetate (pH 4.0); Voltage: 20 kV; Temperature: 25°C; UV Detection: 200 nm; Injection: 3 kV, 8 min

Pas d'influence du processus de dépôt

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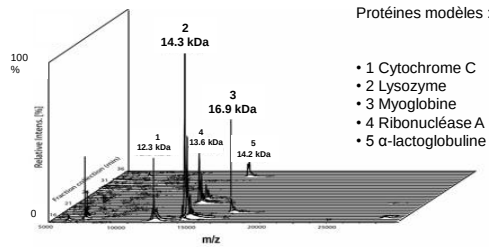
Protéines entières de 10 kDa – 70 kDa



Protéines modèles :

- 1 Cytochrome C
- 2 Lysozyme
- 3 Myoglobine
- 4 Ribonucléase A
- 5 α-lactoglobuline

CE-MALDI mass spectra of 5 proteins sample. Experimental conditions: HPC coated capillary, 50 µm d.i. x 60 cm (detection cell, 60 cm); BGE: 83 mM ionic strength ammonium acetate (pH 4.0); Voltage: 20 kV; Temperature: 25°C; UV Detection: 200 nm; Injection: 3 kV, 8 min

Respect de l'ordre de séparation
Respect des masses
Absence de contamination

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Application à un mélange de variants de mAbs entiers

Structure de l'anticorps monoclonal (mabs)

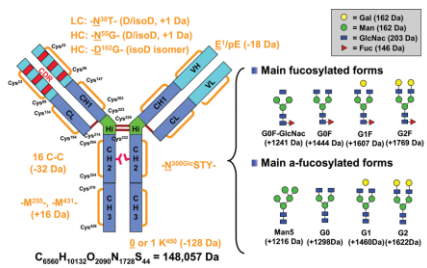
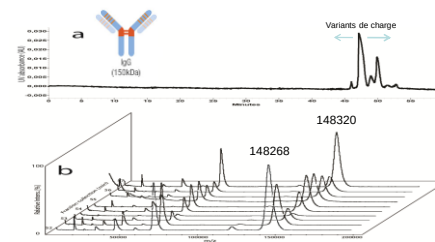


Figure fournie par le Centre immunologie Pierre Fabre et Dr Alain Beck

Structure complexe de haut poids moléculaire
Nombreuses micro hétérogénéités
Présence de plusieurs isoformes

Isoformes d'anticorps monoclonaux 150 kDa



Électrophérogramme UV (a) and Spectre MALDI-MS correspondant à chaques fractions (b) de variant de mAb entiers par CE-UV/MALDI-MS.
Experimental conditions: HPC coated capillary, 50 µm d.i. x 80 cm (detection cell, 70 cm); BGE: 400 mM EACA-acetic acid, 0.05% m/v, pH 5.7; Voltage: 30 kV; Temperature: 25°C; UV Detection: 200 nm; Injection: 2 psi, 30 s; Sample: mAb at 4 g/L

1ère séparation CE-MS d'isoformes d'anticorps monoclonaux entiers

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Evaluation du système : Peptidomique

Mélange de peptides

B Caséine
Carbonic anhydrase II
Cytochrome C
αLactogloguline
Lysozyme
Albumine bovine
Myoglobine
Insuline
Ribonucléase A

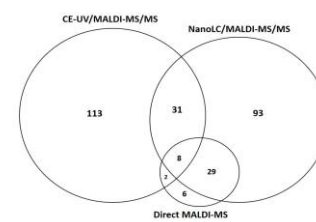

Diagramme de Venn representant la comparaison des peptides identifés par CE/MALDI-MS/MS, NanoLC/MALDI-MS/MS et dépot direct MALDI-MS

Singularité des techniques
Complémentarité des méthodes

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Mélange de peptides

	CE-UV/MALDI-MS/MS		NanoLC/MALDI-MS/MS		Direct MALDI-MS		Combination of the three deposition modes	
	identified peptides	Sequence coverage (%)	identified peptides	Sequence coverage (%)	identified peptides	Sequence coverage (%)	Identified peptides	Sequence coverage (%)
βCas	3	22.3	4	20.5	3	14.7	7	34.4
CAII	20	58.8	11	34.2	5	37.7	28	89.6
Cyt C	13	58.1	7	43.8	1	10.5	16	74.3
αLac	9	40.8	12	51.4	1	7.0	18	83.5
Lys	28	79.6	24	83.0	12	58.5	41	85.7
BSA	55	73.0	68	77.3	13	23.6	113	90.1
Myo	15	85.1	6	44.2	0	0	19	85.1
Ins	1	58.8	4	100	2	43.1	8	100
RNase A	10	100	25	93.5	8	54.4	32	100
	Total of id. pep.	AverageSeq. Cov. (%)	Total of id. pep.	AverageSeq. Cov. (%)	Total of id. pep.	AverageSeq. Cov. (%)	Total of id. pep.	AverageSeq. Cov. (%)
	154	64.1	161	60.8	45	27.7	279	75.9

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Mélange de peptides

Combination of the three deposition modes	
Identified peptides	Sequence coverage (%)
7	34.4
28	89.6
16	74.3
18	83.5
41	85.7
113	90.1
19	85.1
8	100
32	100
Total of id. pep.	AverageSeq. Cov. (%)
279	75.9

CE + LC + Dépôt Direct
=
Augmentation significative % recouvrement

Complémentarité des méthodes

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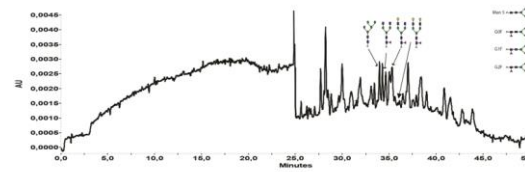
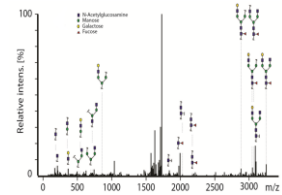
Application à un digestat d'anticorps monoclonaux

Digestat de mAbs



UV Electropherogram corresponding to the analysis by **CE-UV/MALDI-MS/MS** of mAbtryptic digest



- **92% pour la Chaine Lourde (HC)**
- **100% pour la chaine légère (LC)**
- **92 uniques peptides identifiés**
- **4 N-Glycosylation majeures de mAb**

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Conclusion

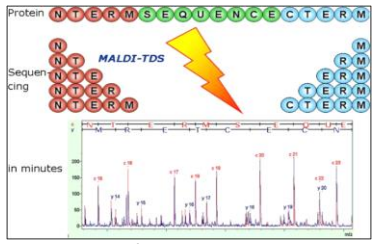
Développement d'une nouvelle interface CE-UV/MALDI MS

- Modification de 2 appareils commerciaux
  - ➢ Automatisation, Répétable et Robustesse
- Evaluation du système
  - Mélange des protéines entières jusqu'a 150 kDa
  - Digestat de proteines
  - ➢ Complémentarité à la LC-MS

SOMMAIRE

1. Le couplage CE/MALDI –MS
2. Mise au point et évaluation du CE-UV/MALDI-MS
3. Mise au point de l'analyse Top DOWN par CE-UV/MALDI-MS

L'analyse Top Down par MALDI-MS



Fragmentation ISD : sequence en AA

- Nécessité de concentration suffisante (pmol)
- Nécessité de séparer les protéines

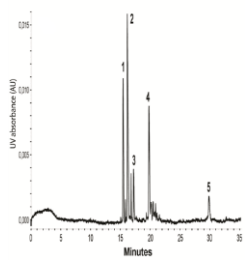
⇨ **Collecte et Enrichissement par CE/MALDI-MS**

Evaluation du système sur un mélange de protéines de référence

Séparation, Enrichissement, Top-Down d'un mélange de 5 protéines

Protéines modèles :

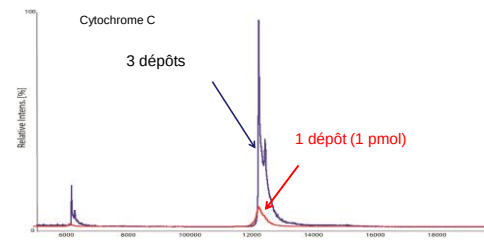
- 1 Cytochrome C
- 2 Lysozyme
- 3 Myoglobine
- 4 Ribonucléase A
- 5 α-lactoglobuline



Conditions : Injection : 5kV 8 min ; tampon : 83 mM de force ionique d'une solution d'acétate d'ammonium à pH=4 ; Ltot= 60 cm, ld= 53 cm, Φ = 50 µm ; greffage : HPC, UV à 200 nm, 25°C. (1) 40 nM Cyt c, (2) 40 nM Lys, (3) 50 nMMyo, (4) 50 nMRnase A and (5) 120 nM αLac dans l'eau

⇨ **Répétabilité RSD < 2% sur temps de migration**
Enrichissement directement sur plaque MALDI

Séparation, Enrichissement, Top-Down d'un mélange de 5 protéines

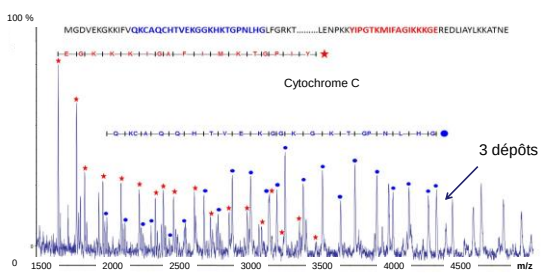


Superposition des spectres de masse du cytochrome C à 1 (Rouge) et 3 (Bleu) répétitions ; Puissance laser : 71% Matrice : 2,5-dihydroxybenzoïque (DHB) (saturé) dans du TA30 (0.1%TFA, 70% eau et 30% acétonitrile).

Absence d'effet mémoire
Augmentation du signal en fonction du nombre du dépôt

⇨ **Enrichissement**

Séparation, Enrichissement, Top-Down d'un mélange de 5 protéines



Identification de 39 résidus correspondant à la séquence de la Cytochrome C

caractérisation Top Down de protéine par CE/MALDI-MS/MS