Evaluation du kit aTRAQ pour le dosage des Acides Aminés Physiologiques par MSMS

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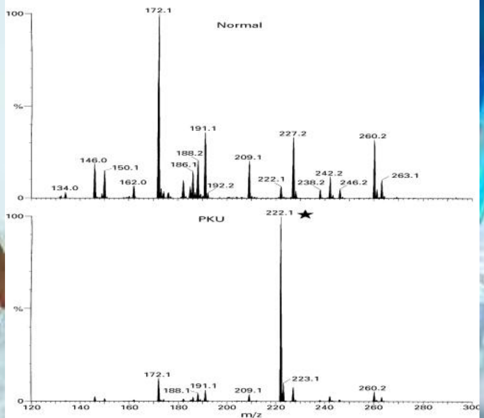
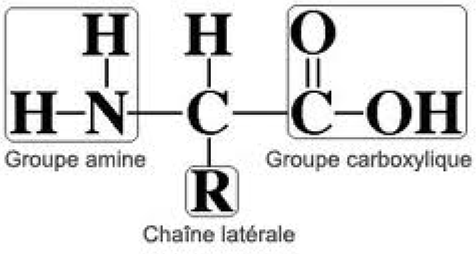


Acides Aminés - Définition

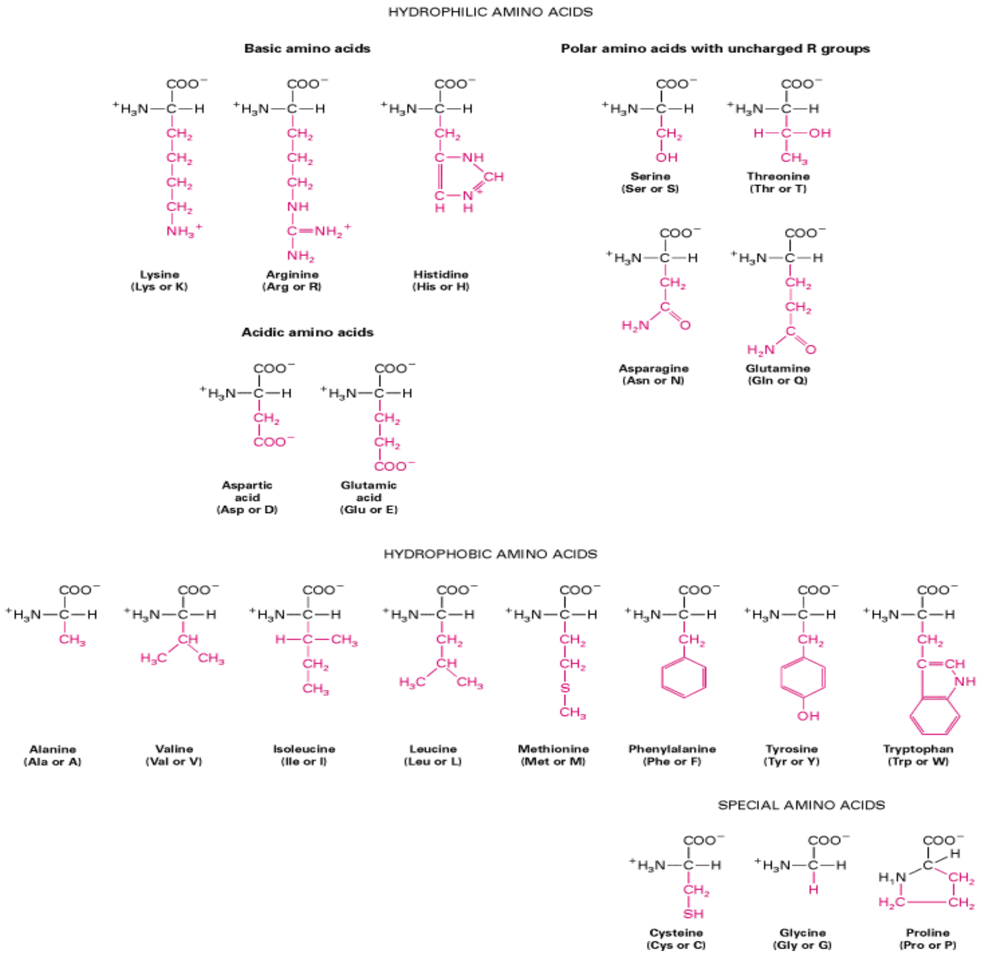
- Composés chimiques possédant deux groupes fonctionnels:
  -COOH et -NH_2



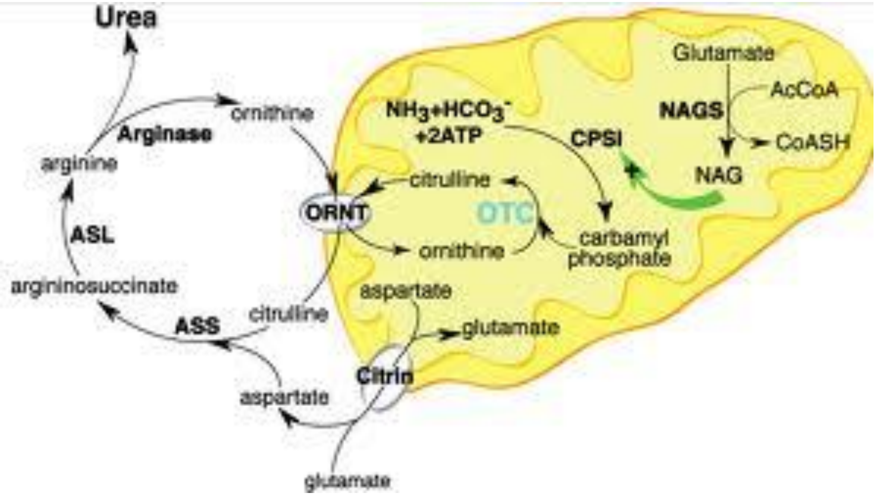
- Rôles
  - 20 AA protéinogéniques (8 essentiels)
  - Intermédiaires métaboliques (ex Cycle de l'Urée)
  - Neurotansmetteurs (ex GABA)
  - Issus de modification post-traductionnelle (ex Hydroxyproline, Methylhistidine,…)

Acides Aminés - Définitions

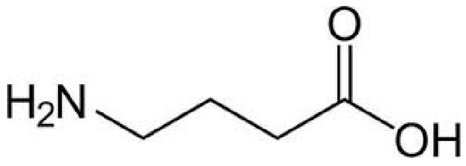
20 AA protéinogéniques



Cycle de l'Urée



GABA



Intérêt du Dosage des AA

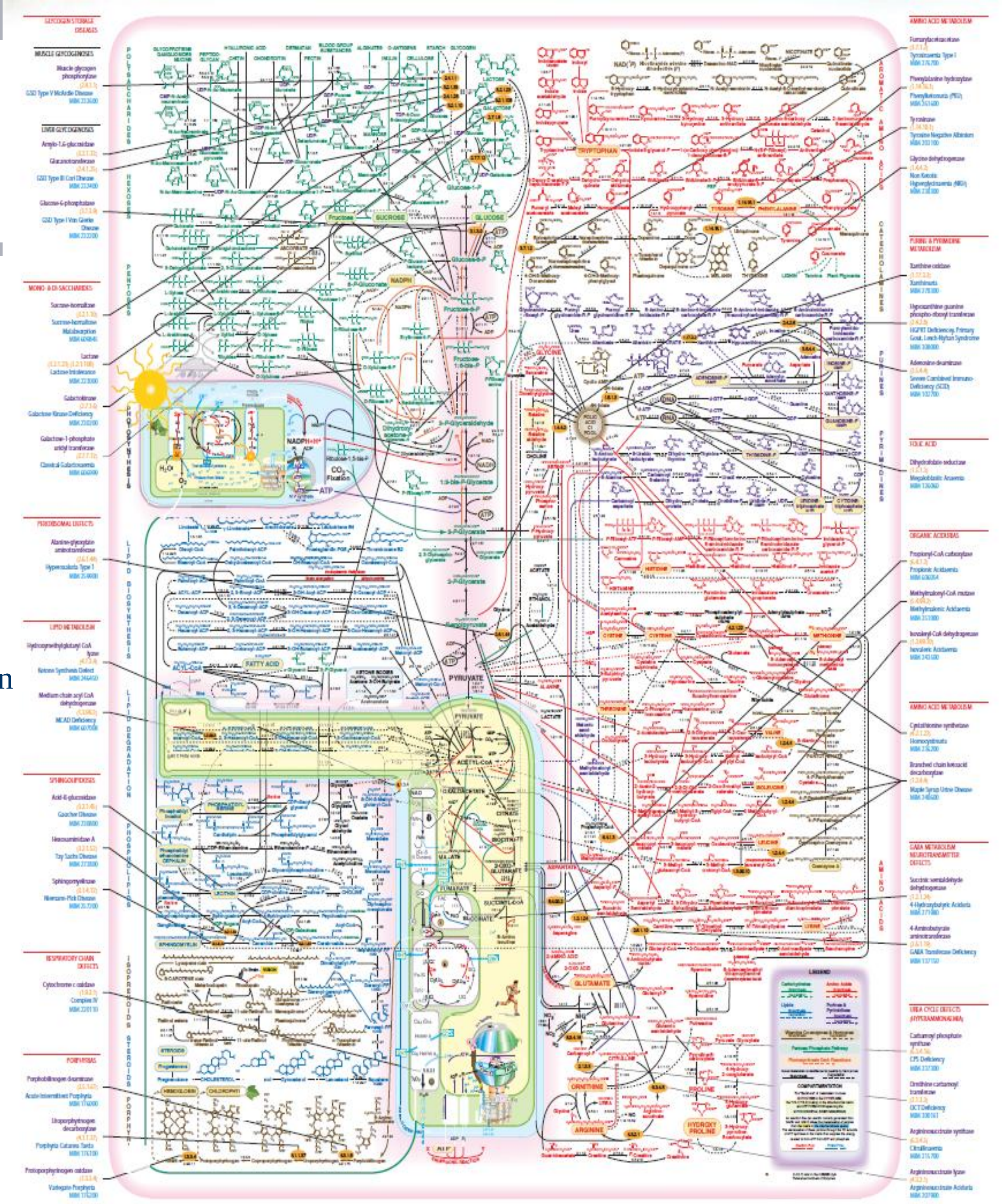
- Erreurs Innées du Métabolisme (= Maladie Métabolique)
  - PCU
  - Leucinose (MSUD)
  - Tyrosinémie (I, II, III, Alkaptonurie)
  - Anomalies du Cycle de l’Urée
- Statut nutritionnel
- Fonction de divers organes
- Marqueurs métabolomiques

Erreurs Innées du Métabolisme

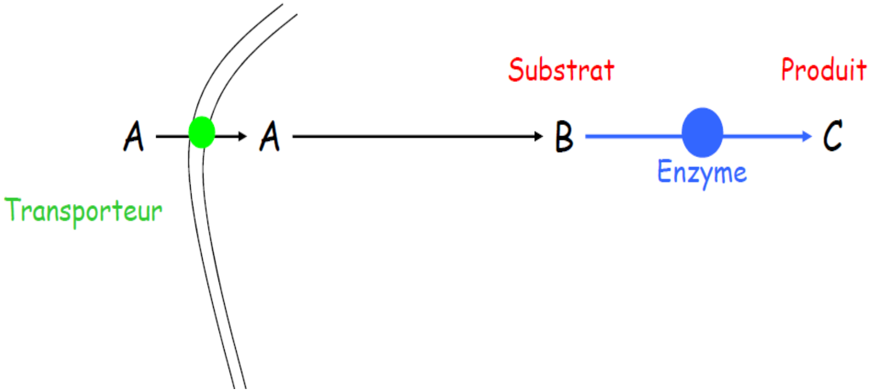
- **Absence (anomalie) d’un enzyme ou d’un cofacteur (1 gène = 1 enzyme)**
- **→ Accumulation d’un métabolite** **(exceptions cystinurie, …)**
  - diminution de la formation du produit de la réaction
  - accumulation du substrat
  - augmentation de la formation d’autres métabolites
  - absence de rétroaction négative
  - échec des mécanismes de transport

- **Classification**

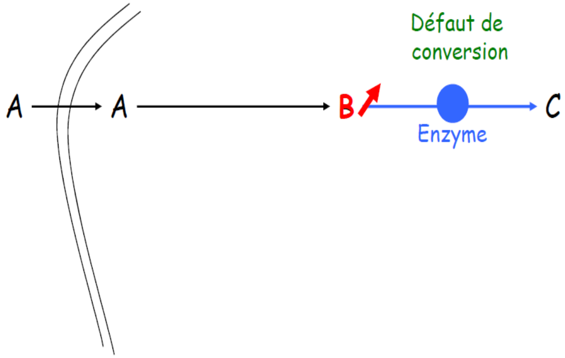
Amino Acid disorders
Organic Acidemias
Urea Cycle disorders
Carbohydrate disorders
Fatty Acid Oxidation disorders
Mitochondrial disorders
Peroxisomal disorders
Lysosomal Storage disorders
Purine and Pyrimidine disorders
Porphyrias
Metal Metabolism disorders
Creatine disorders



Modèle Général de Maladie Métabolique

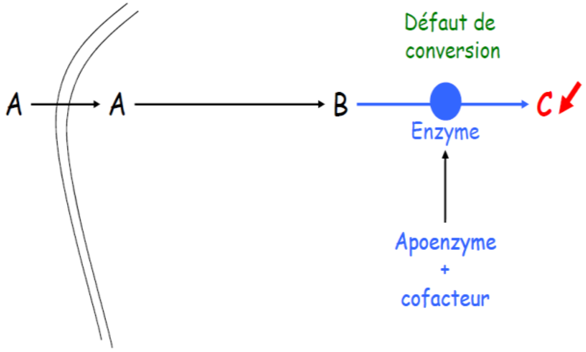


1. Accumulation du substrat



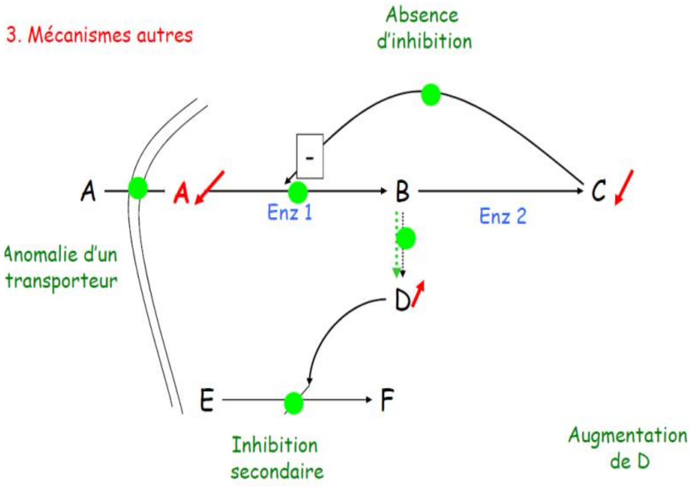
Maladie par intox

2. Déficit du produit en aval



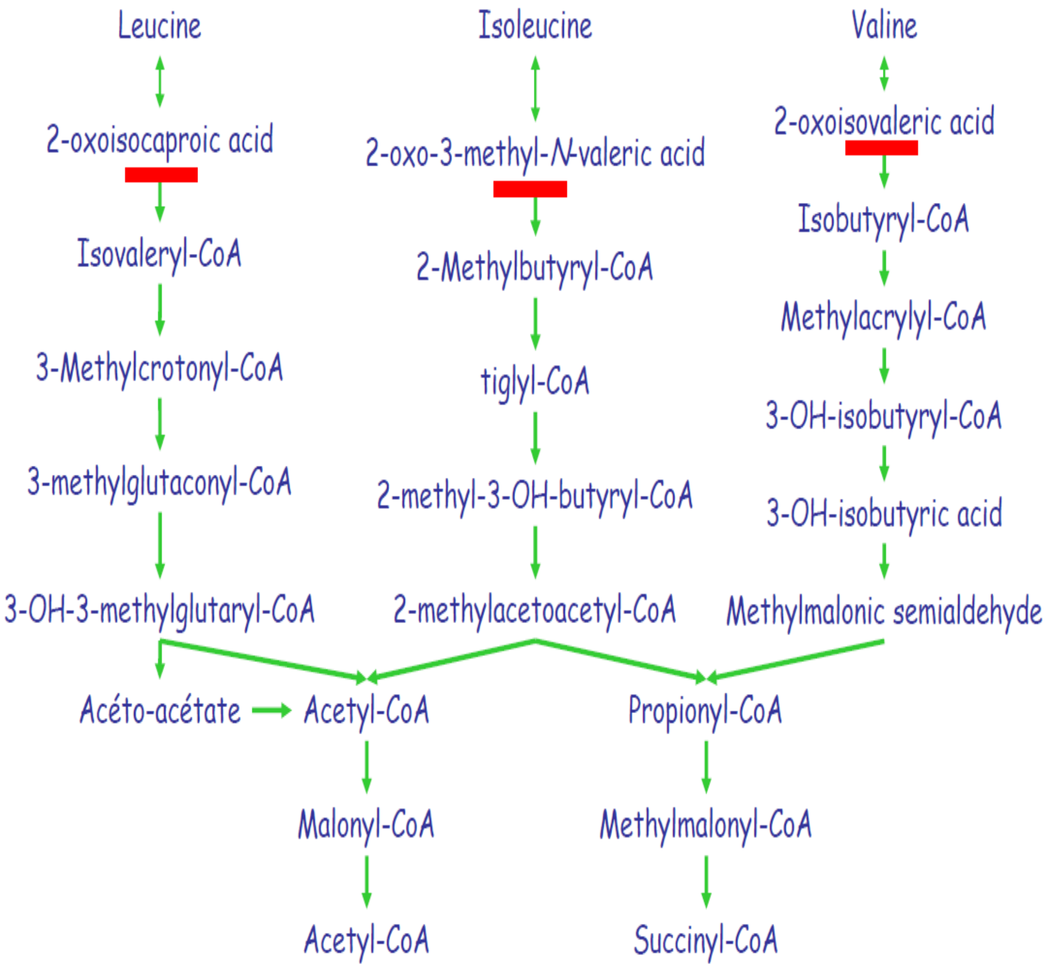
Maladie par déficit

3. Mécanismes autres



Exemples

Maladie des Urines Sirop d'érable



Leucine
2-oxoisocaproic acid
Isovaleryl-CoA
3-Methylcrotonyl-CoA
3-methylglutaconyl-CoA
3-OH-3-methylglutaryl-CoA

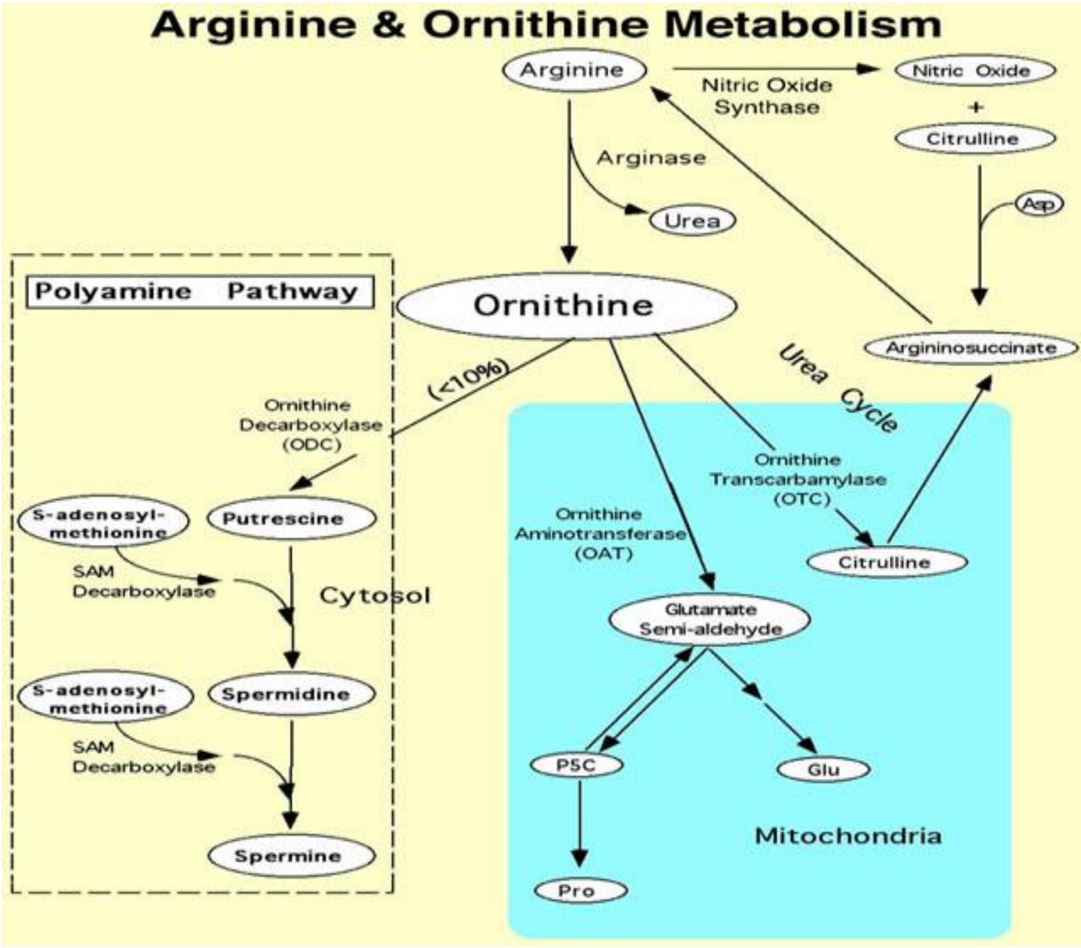
Isoleucine
2-oxo-3-methyl-N-valeric acid
2-Methylbutyryl-CoA
tiglyl-CoA
2-methyl-3-OH-butyryl-CoA
2-methylacetoacetyl-CoA

Valine
2-oxoisovaleric acid
Isobutyryl-CoA
Methylacrylyl-CoA
3-OH-isobutyryl-CoA
3-OH-isobutyric acid
Methylmalonic semialdehyde

Acéto-acétate → Acetyl-CoA
Acetyl-CoA
Malonyl-CoA
Acetyl-CoA

Propionyl-CoA
Methylmalonyl-CoA
Succinyl-CoA

Métabolisme Orn/Arg



Erreurs Innées du Métabolisme

Génétique

- Transmission AR (co-sanguinité fréquente) ou liée au chromosome X (ALD, Hunter,…)
- Pfs transmission AD (porphyries,…)

Clinique

URGENCE MEDICALE

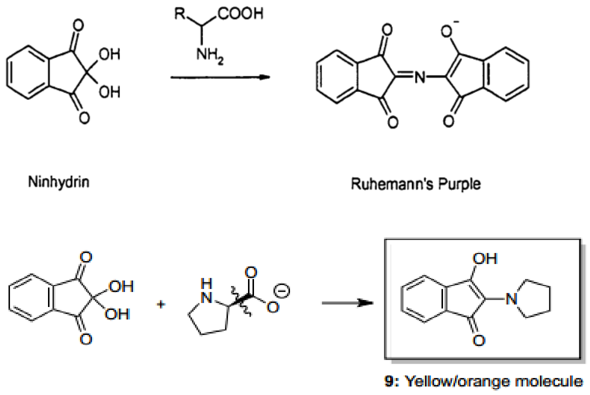
particulièrement chez nouveau-né et jeune enfant (>< formes tardives)

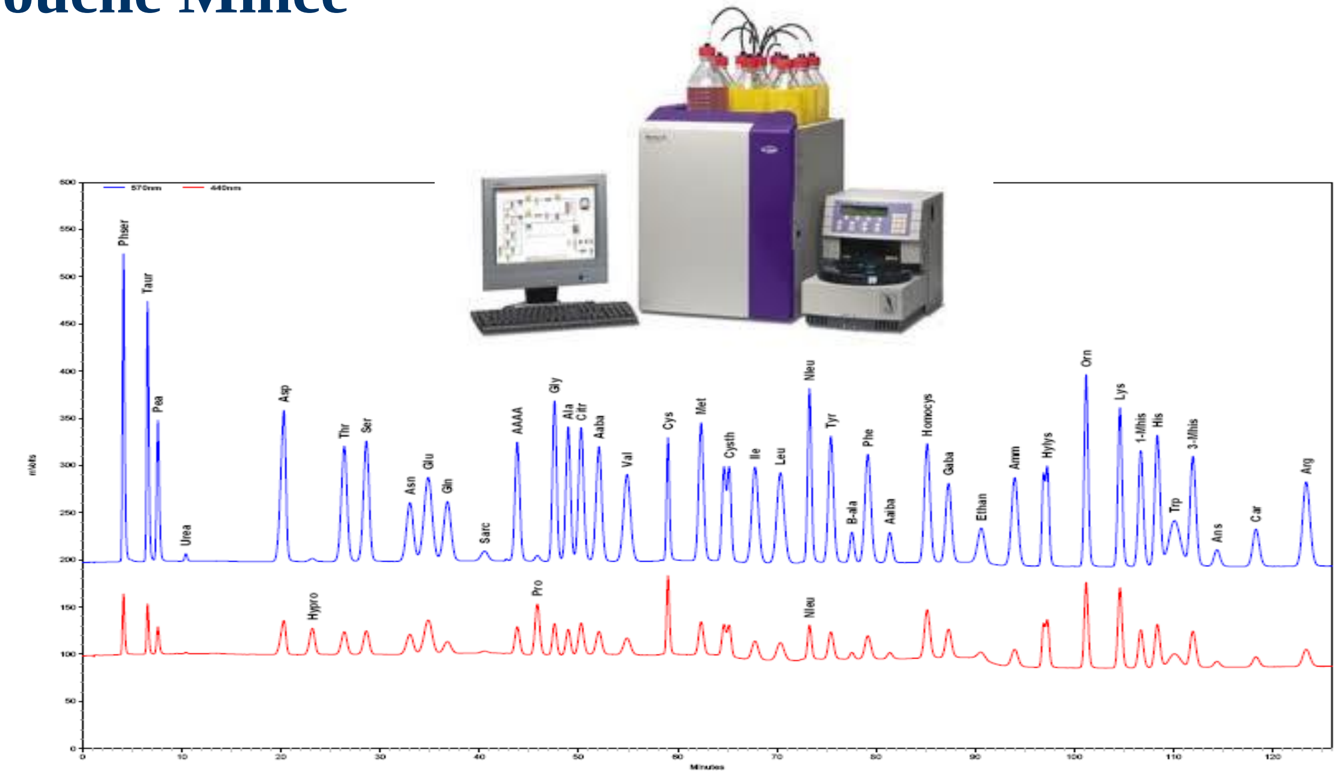
- Symptômes aigus (irritabilité, bradycardie, tachypnée, vomissements, état septique, jaunisse, léthargie, convulsions, alimentation limitée)
- Etat neurologique altéré (ataxie, hypertonie, hypotonie, convulsions, encéphalopathie, coma, retard mental)
- Etat métabolique altéré (hyperglycémie/hypoglycémie, acidose, hyperammoniémie, odeur corporelle inhabituelle)
- Troubles systémiques (hépatiques, oculaires, cutanés, ….).
- Peut être cause de mort subite

Maladies individuellement rares, mais collectivement nombreuses (~1/1400)

Dosage des AA

- ➢ **Chromatographie Couche Mince**
- ➢ **IEC – Ninhydrine**





- ➢ **LCMS - GCMS**

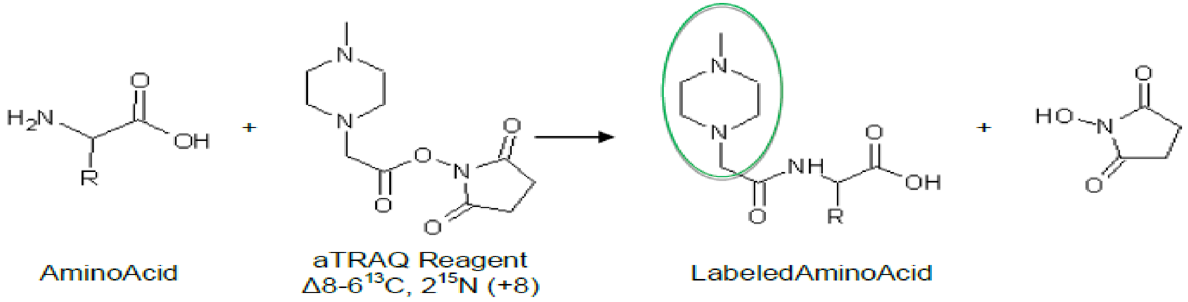
Kit aTRAQ

➢ Identification et Dosage spécifique de 42 AA

- 1-Methylhistidine
- 3-Methylhistidine
- Alpha-Aminoadipate
- Alpha-Aminobutyrate
- Alanine
- Anserine
- Arginine
- Argininosuccinate
- Asparagine
- Aspartate
- Beta-Aminoisobutyrate
- Beta-Alanine
- Carnosine
- Citrulline
- Cystathionine
- Cystine
- Ethanolamine
- Gamma Aminobutyrate
- Glutamine
- Glutamate
- Glycine
- Homocitrulline
- Homocystine
- Histidine
- Hydroxylysine
- Hydroxyproline
- Isoleucine
- Leucine
- Lysine
- Methionine
- Norleucine (IS)
- Norvaline (IS)
- Ornithine
- Phosphoethanolamine
- Phenylalanine
- Proline
- Phosphoserine
- Sarcosine
- Serine
- Taurine
- Threonine
- Tryptophane
- Tyrosine
- Valine

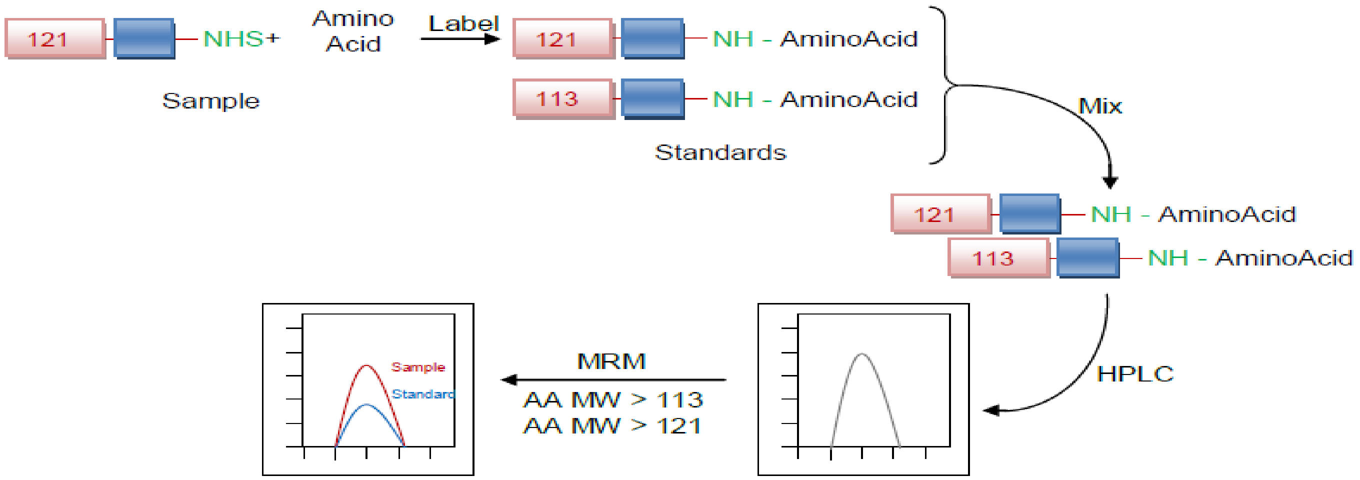
Kit aTRAQ

➢ Principe



Reporter ion
113amu for Internal Standard
121amu for Sample

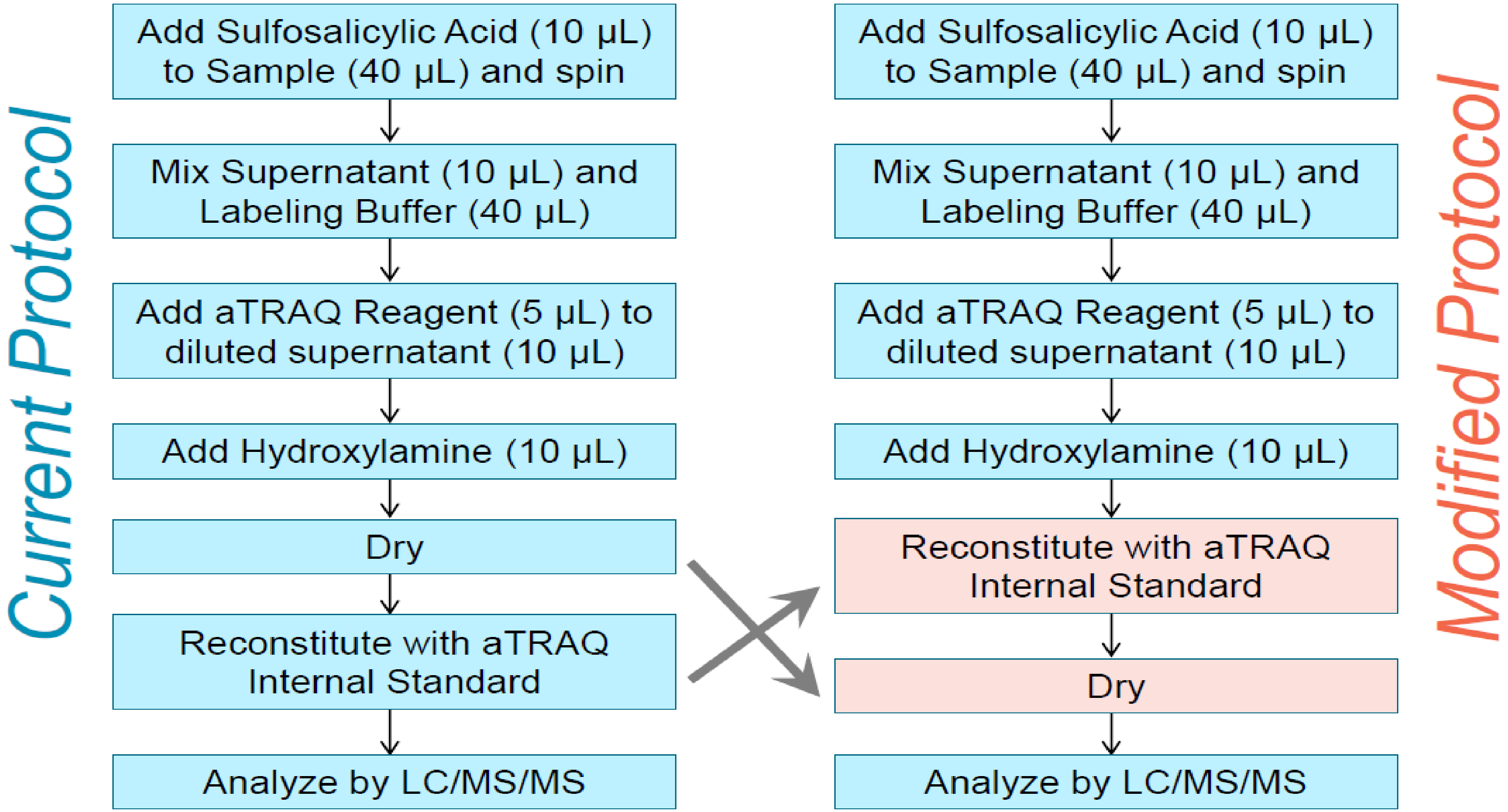
Schema général:



$$Amount_{Sample} = \frac{Area_{Sample}}{Area_{Std}} \times Amount_{Std}$$

Kit aTRAQ

- **Préparation**



Current Protocol

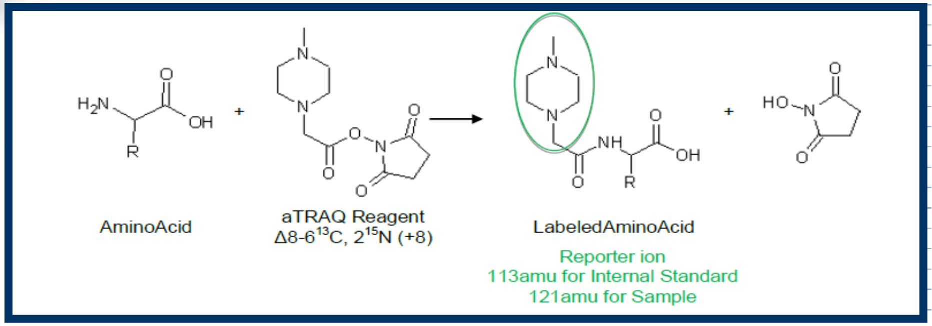
1. Add Sulfosalicylic Acid (10 µL) to Sample (40 µL) and spin
2. Mix Supernatant (10 µL) and Labeling Buffer (40 µL)
3. Add aTRAQ Reagent (5 µL) to diluted supernatant (10 µL)
4. Add Hydroxylamine (10 µL)
5. Dry
6. Reconstitute with aTRAQ Internal Standard
7. Analyze by LC/MS/MS

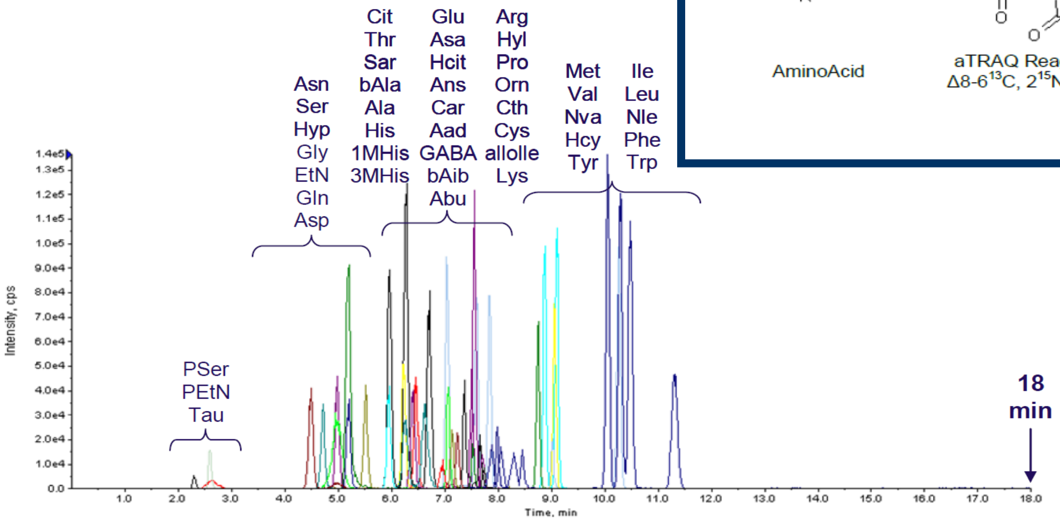
Modified Protocol

1. Add Sulfosalicylic Acid (10 µL) to Sample (40 µL) and spin
2. Mix Supernatant (10 µL) and Labeling Buffer (40 µL)
3. Add aTRAQ Reagent (5 µL) to diluted supernatant (10 µL)
4. Add Hydroxylamine (10 µL)
5. Reconstitute with aTRAQ Internal Standard
6. Dry
7. Analyze by LC/MS/MS

Kit aTRAQ

- Dilution 1/10 - Injection 1 µL (!!!clean !!!)
- Séparation sur colonne C18 → MS





	Q1 (m/z)	Q3 (m/z)	Retention Time (min)
Allo-Ile/uIle/uLeu/uNle	132.1	86.1	5.51
EtN_IS	202.2	113.1	4.07
EtN	210.2	121.1	4.07
Gly_IS	216.1	113.1	3.84
Gly	224.1	121.1	3.84
Sar/bAla/Ala_IS	230.2	113.1	4.58
Sar/bAla/Ala	238.2	121.1	4.58
GABA/bAib/Abu_IS	244.2	113.1	5.54
GABA/bAib/Abu	252.2	121.1	5.54
Ser_IS	246.2	113.1	3.61
Ser	254.2	121.1	3.61
Pro_IS	256.2	113.1	5.73
Pro	264.2	121.1	5.73
Val/Nva_IS	258.2	113.1	6.88
Val/Nva	266.2	121.1	6.88
Thr_IS	260.2	113.1	4.47
Thr	268.2	121.1	4.47
Tau_IS	266.1	113.1	2.83
Tau	274.1	121.1	2.83
Hyp_IS	272.1	113.1	3.76
Hyp	280.1	121.1	3.76
Ile/Leu/Nle_IS	272.2	113.1	8.01
Ile/Leu/Nle	280.2	121.1	8.01
Asn_IS	273.2	113.1	3.57
Asn	281.2	121.1	3.57
Asp_IS	274.2	113.1	3.95
Asp	282.2	121.1	3.95
PEtN_IS	282.1	113.1	2.75
PEtN	290.1	121.1	2.75
Gln_IS	287.2	113.1	3.94
Gln	295.2	121.1	3.94
Glu_IS	288.2	113.1	4.55
Glu	296.2	121.1	4.55
Met_IS	290.1	113.1	6.65
Met	298.1	121.1	6.65
His_IS	296.2	113.1	4.04
His	304.2	121.1	4.04
Aad_IS	302.2	113.1	5.4
Aad	310.2	121.1	5.4
Phe_IS	306.2	113.1	8.04
Phe	314.2	121.1	8.04
1MHis/3MHis_IS	310.2	113.1	4.39
1MHis/3MHis	318.2	121.1	4.39
Arg_IS	315.2	113.1	4.85
Arg	323.2	121.1	4.85
Cit_IS	316.2	113.1	4.42
Cit	324.2	121.1	4.42
Tyr_IS	322.2	113.1	6.98
Tyr	330.2	121.1	6.98
PSer_IS	326.1	113.1	2.46
PSer	334.1	121.1	2.46
Hcit_IS	330.2	113.1	5.3

Evaluation

- Protocole de Validation
  - Enoval

- Définition des Valeurs Normales
  - Plasma - Urine – LCR
  - 3 classes d'âge

- Comparaison de Méthodes
  - IEC >< LCMS

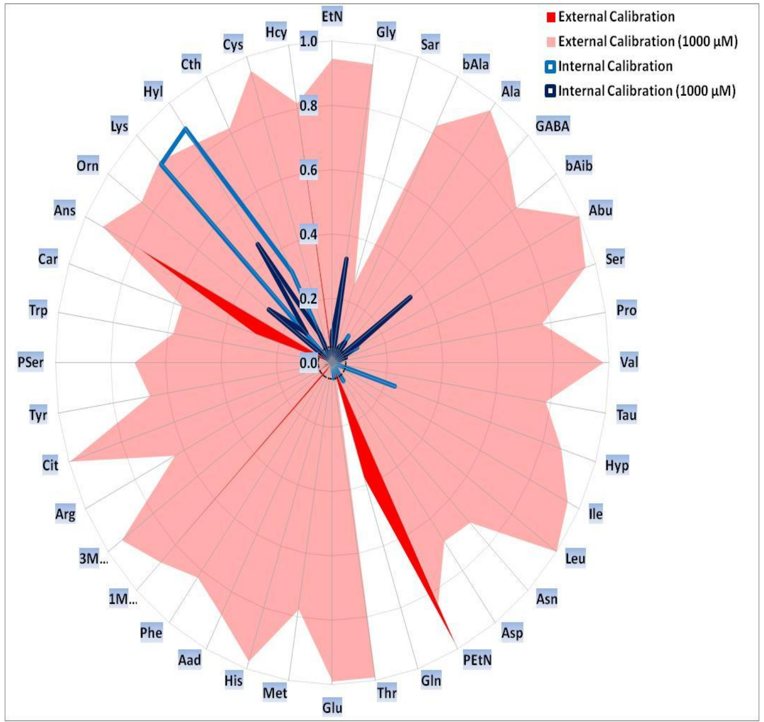
- Evaluation Clinique

Validation

- Comparaison Calibration Interne > < Calibration Externe
  - Calibration Interne: Concentrations IS fournies par AB Sciex
  - Calibration Externe: Mix de Standards Sigma

  - Meilleurs résultats pour calibration externe surtout pour Concentrations élevées

    => Meilleure reproductibilité inter-lot.



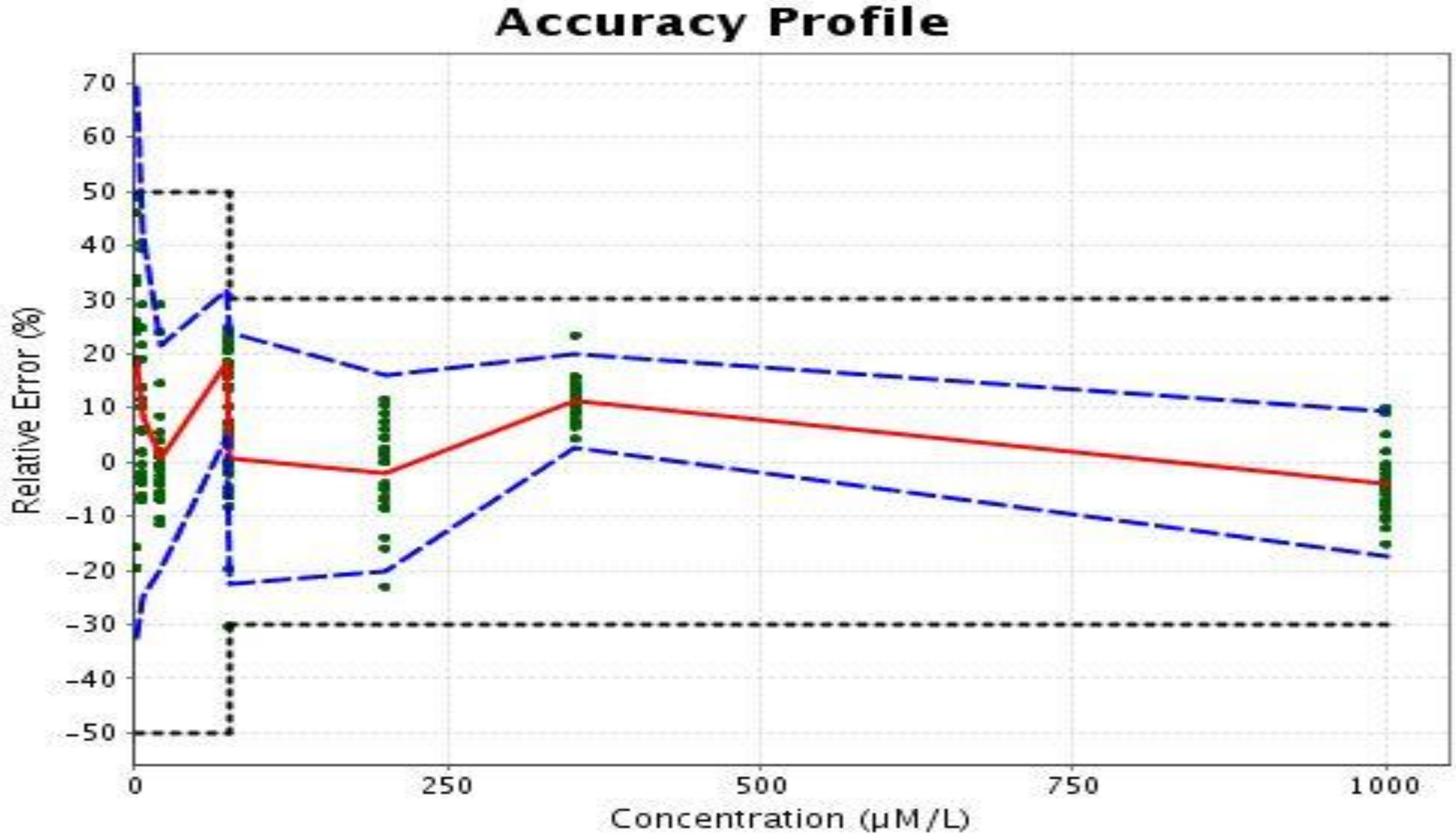
Validation

- Calibration externe en double : 1000-250-100-50-10 µM/L
- Echantillons de validation : 1-1000 µM/L – 5 replicates sur 5 séries

- Limites d'acceptation:
  - <= 75 µM/L: ±50 %
  - >75 µM/L: ±30 %
- Intervalle de tolérance: 95%

Validation

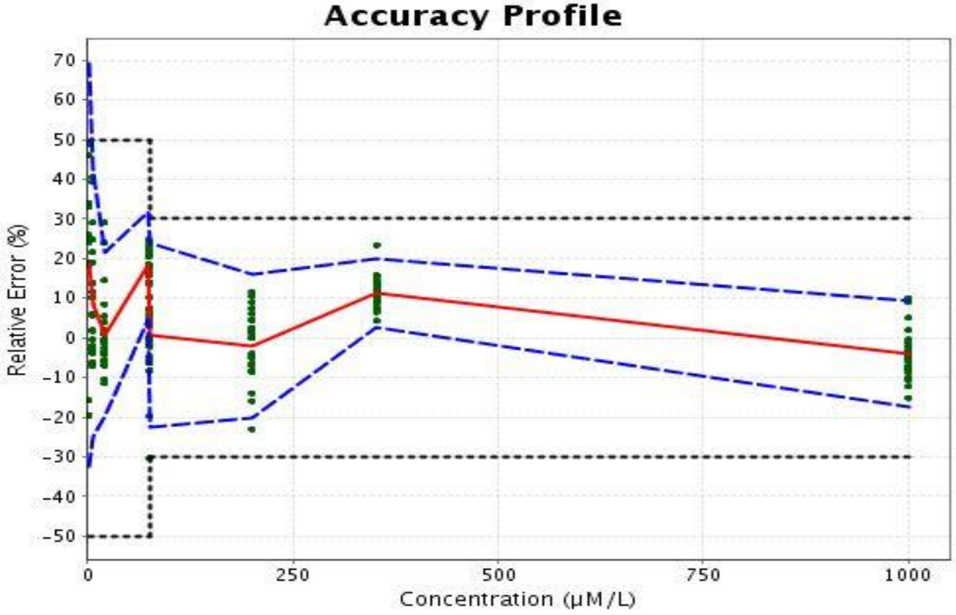
- ➢ **Profil d'exactitude**

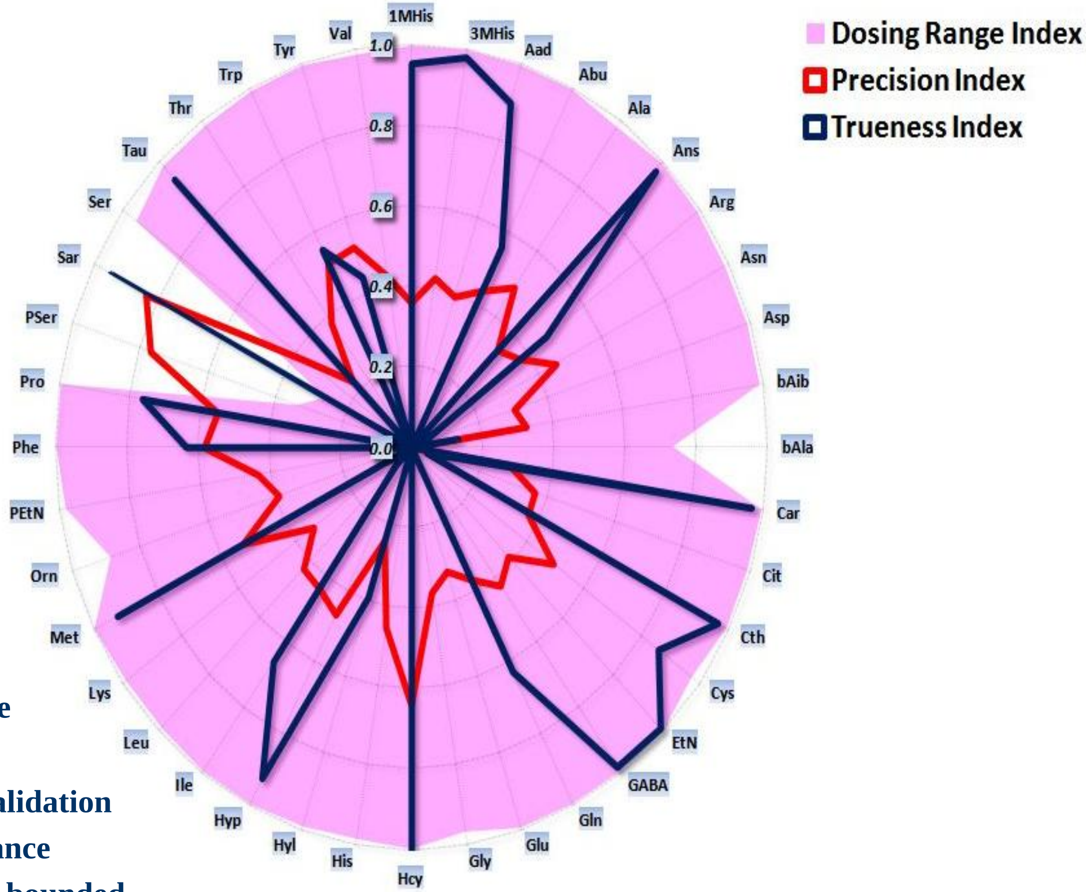


CV

	Concentration Level - 20 µmol/L Cystine - 10 µmol/L		Concentration Level - 200 µmol/L Cystine - 100 µmol/L	
	Intra-Run Coefficient of Variation (%)	Inter-Run Coefficient of Variation (%)	Intra-Run Coefficient of Variation (%)	Inter-Run Coefficient of Variation (%)
1MHis	10.55	11.43	7.88	7.99
3MHis	9.11	9.36	8.54	8.54
Aad	10.93	11.17	8.16	8.16
Abu	8.85	11.48	8.41	9.30
Ala	10.66	11.84	9.42	9.42
Ans	10.78	11.47	7.70	8.31
Arg	11.45	13.11	9.05	9.40
Asn	10.01	10.01	7.27	7.43
Asp	18.72	18.72	6.96	6.96
bAib	11.46	12.19	7.84	7.99
bAla	26.84	41.24	8.38	9.02
Car	12.31	12.31	8.70	8.70
Cit	9.78	10.26	9.14	9.64
Cth	14.54	15.55	8.91	9.44
Cys	11.81	12.94	6.71	9.00
EtN	10.41	4.05	11.65	10.62
GABA	9.82	11.32	7.19	7.19
Gln	10.83	10.85	7.42	7.42
Glu	12.49	13.06	7.15	7.68
Gly	9.85	18.13	10.31	10.31
His	9.61	9.61	10.92	10.92
Hyl	14.30	14.30	10.07	10.36
Hyp	12.14	13.05	3.93	4.39
Ile	12.55	12.55	8.67	8.72
Leu	15.02	15.02	7.79	7.85
Lys	10.73	11.99	9.00	9.00
Met	9.49	10.67	8.09	8.09
Orn	14.47	16.49	7.61	7.72
PEtN	11.51	13.92	7.09	7.25
Phe	9.28	9.71	8.57	8.57
Pro	8.84	9.24	5.82	5.82
PSer	28.71	28.71	10.50	10.50
Sar	10.53	11.25	8.25	8.25
Ser	16.16	18.77	9.06	9.06
Tau	7.23	14.42	3.53	8.36
Thr	9.52	10.24	7.15	8.47
Trp	7.87	9.59	7.54	7.78
Tyr	12.04	14.47	10.76	10.76
Val	10.51	10.91	9.27	9.41

Index





<u>Intervalle de dosage</u>: ratio between the length of interval in which the method is considered as valid and the length of the interval between the highest and lowest concentration levels investigated during the validation

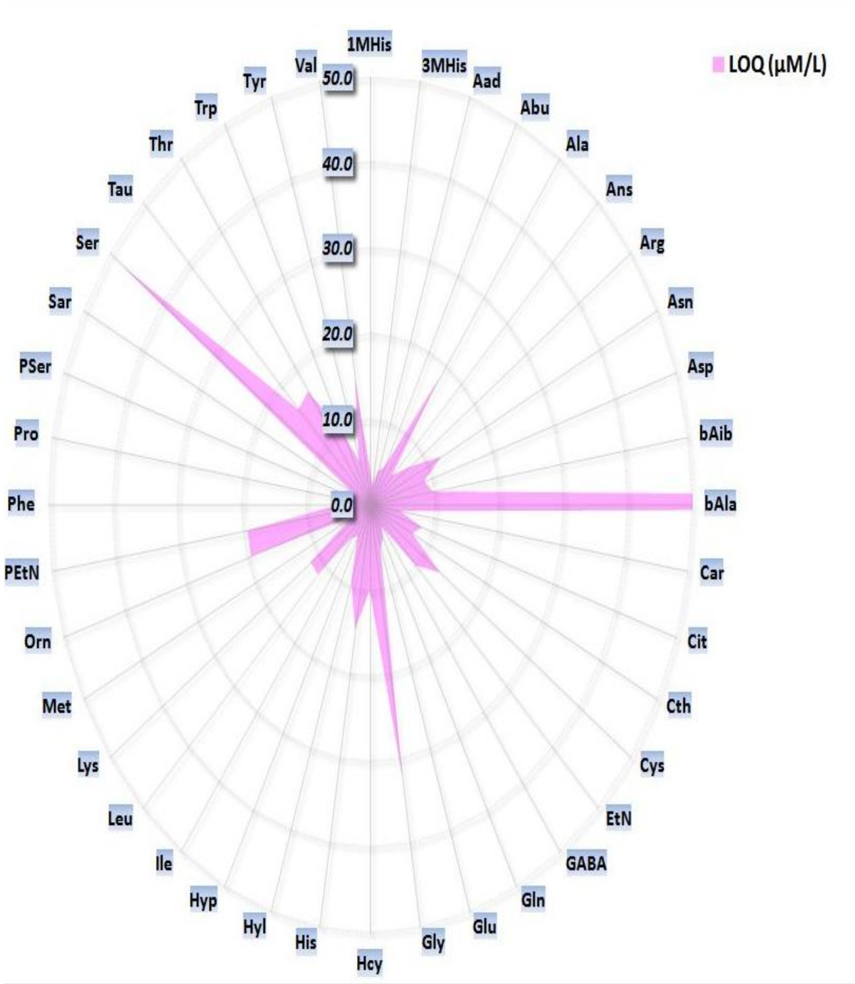
<u>Index de précision</u>: ratio between the area defined within the acceptance limits and the limits of β-expectation tolerance interval, and the area bounded by the upper and lower acceptance limits, for which the method is considered as valid.

<u>Index d’exactitude</u>: 1 minus the ratio between the sum of the square of the observed bias at each concentration level and the sum of the square of the maximum tolerated bias at each concentration level, for which the method is considered as valid

<u>Sar</u>: saturation du détecteur aux hautes [ ]
<u>Pser</u>: reproductible mais pas précis aux hautes [ ]
<u>bAla</u>: peu reproductible aux faibles [ ]

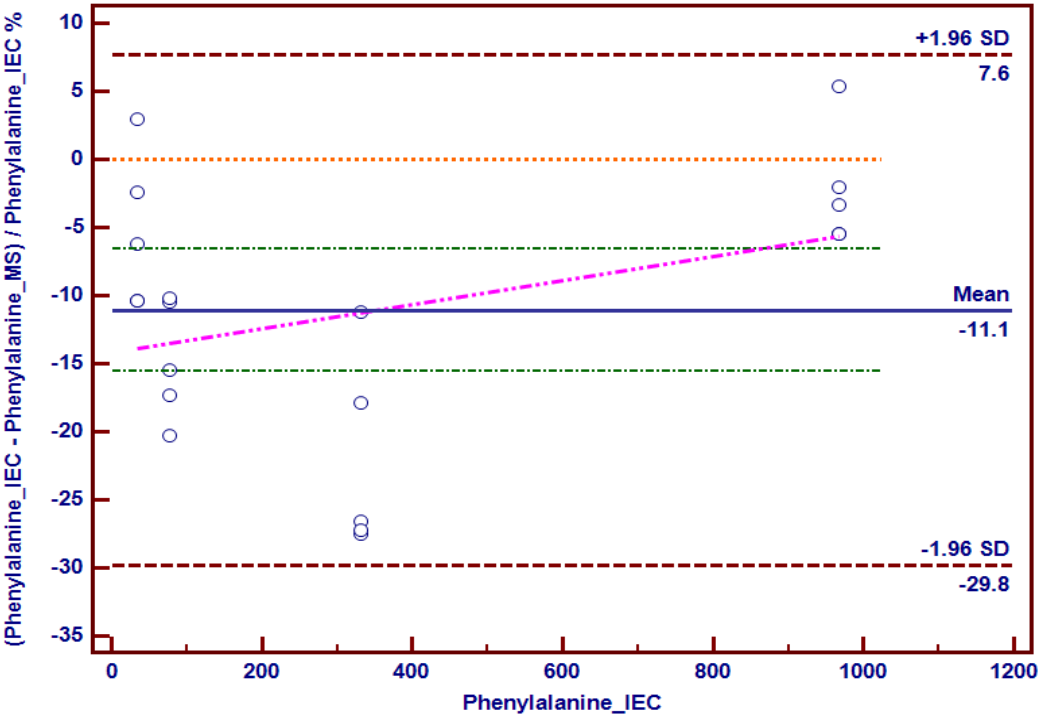
Validation

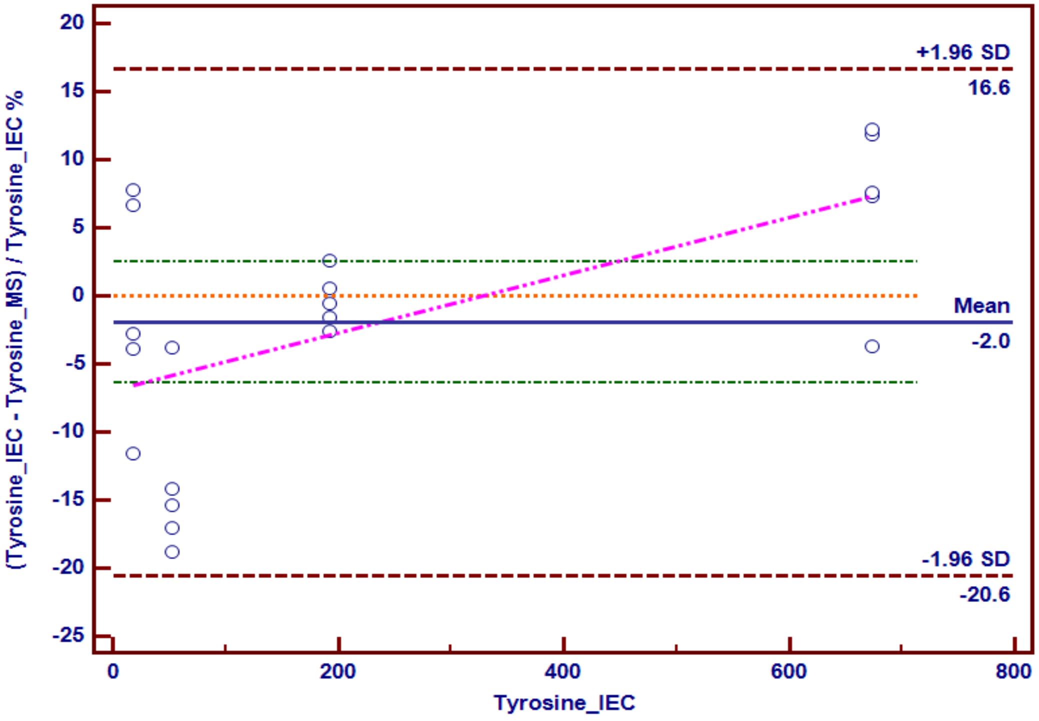
- **LOQ**



Comparaison de Méthodes

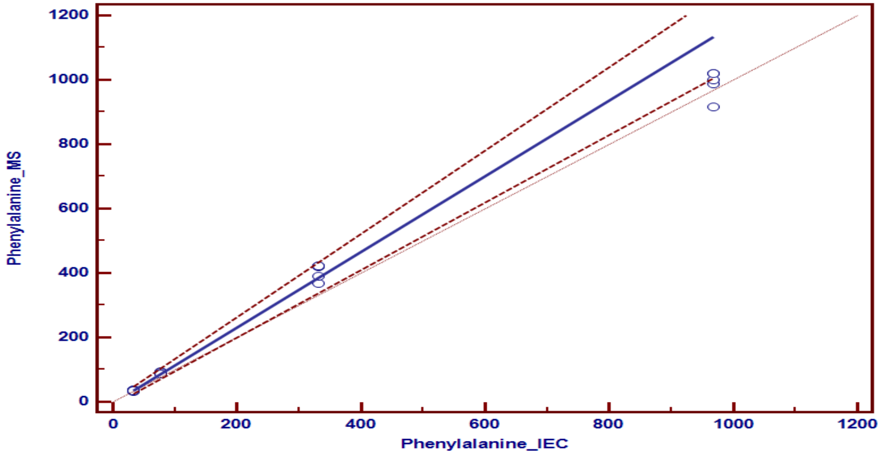
- Echantillons QC externes
  - → Moyenne méthode IEC (> 100 labos) = cible
- Bland & Altman

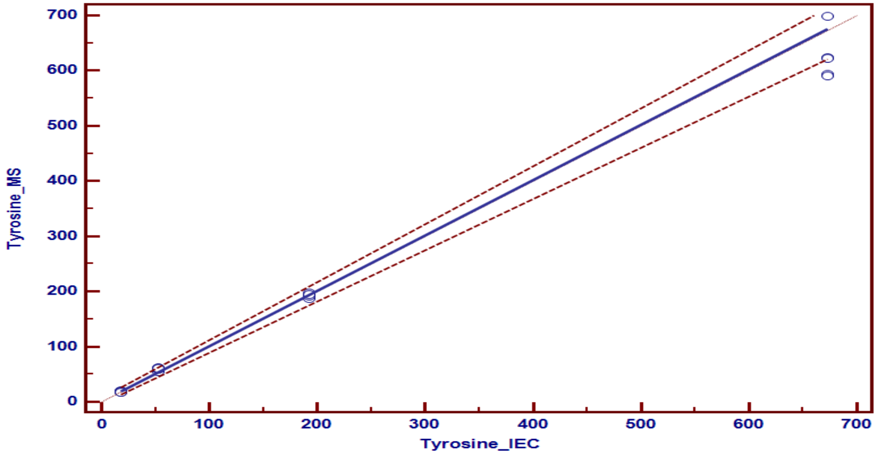




Comparaison de Méthodes

- **Passing & Bablok**





	Slope	Slope 95% CI [a]	Intercept	Intercept 95% CI [a]
3MHis	1.01	0.938 to 1.362	3.19	-3.863 to 5.464
Abu	1.17	1.105 to 1.252	-2.94	-5.209 to 0.857
Ala	1.05	1.016 to 1.085	-7.32	-20.1 to 2.336
Arg	1.06	1.045 to 1.083	-0.92	-5.514 to 1.503
Asn	1.19	1.151 to 1.279	5.70	-5.066 to 9.417
Asp	0.99	0.927 to 1.071	1.06	-2.051 to 2.432
Cit	0.96	0.938 to 1.008	1.88	0.449 to 3.583
Cth	1.12	1.005 to 1.156	0.55	-0.428 to 1.388
Cys	1.08	1.011 to 1.167	-3.07	-6.464 to 0.94
Gln	0.92	0.803 to 1.083	3.72	-33.91 to 9.132
Glu	1.08	1.041 to 1.132	3.38	-2.056 to 6.867
Gly	0.98	0.928 to 1.058	1.28	-15.09 to 1.45
His	1.01	0.98 to 1.071	-0.12	-5.099 to 2.776
Hpro	1.14	1.097 to 1.223	-0.39	-4.912 to 2.063
Ile	1.09	0.995 to 1.188	1.19	-2.022 to 5.713
Leu	1.11	1.001 to 1.094	1.16	-5.852 to 6.11
Lys	1.02	1.001 to 1.094	-4.87	-11.99 to 0.824
Met	1.08	1.072 to 1.173	0.20	-5.297 to 0.879
Orn	1.03	0.986 to 1.097	-5.23	-12.84 to 0.285
Phe	1.18	1.051 to 1.294	-4.46	-10.86 to 3.971
Pro	1.18	1.141 to 1.226	6.21	0.419 to 9.499
Ser	1.15	1.087 to 1.246	1.06	-5.565 to 4.681
Tau	1.08	1.022 to 1.154	-10.95	-21.82 to 2.961
Thr	1.01	0.965 to 1.105	4.28	-4.631 to 9.206
Trp	1.02	0.928 to 1.124	3.40	-5.842 to 2.067
Tyr	1.00	0.927 to 1.05	0.64	-3.441 to 7.371
Val	1.07	1.035 to 1.117	-3.31	-22.83 to 4.374

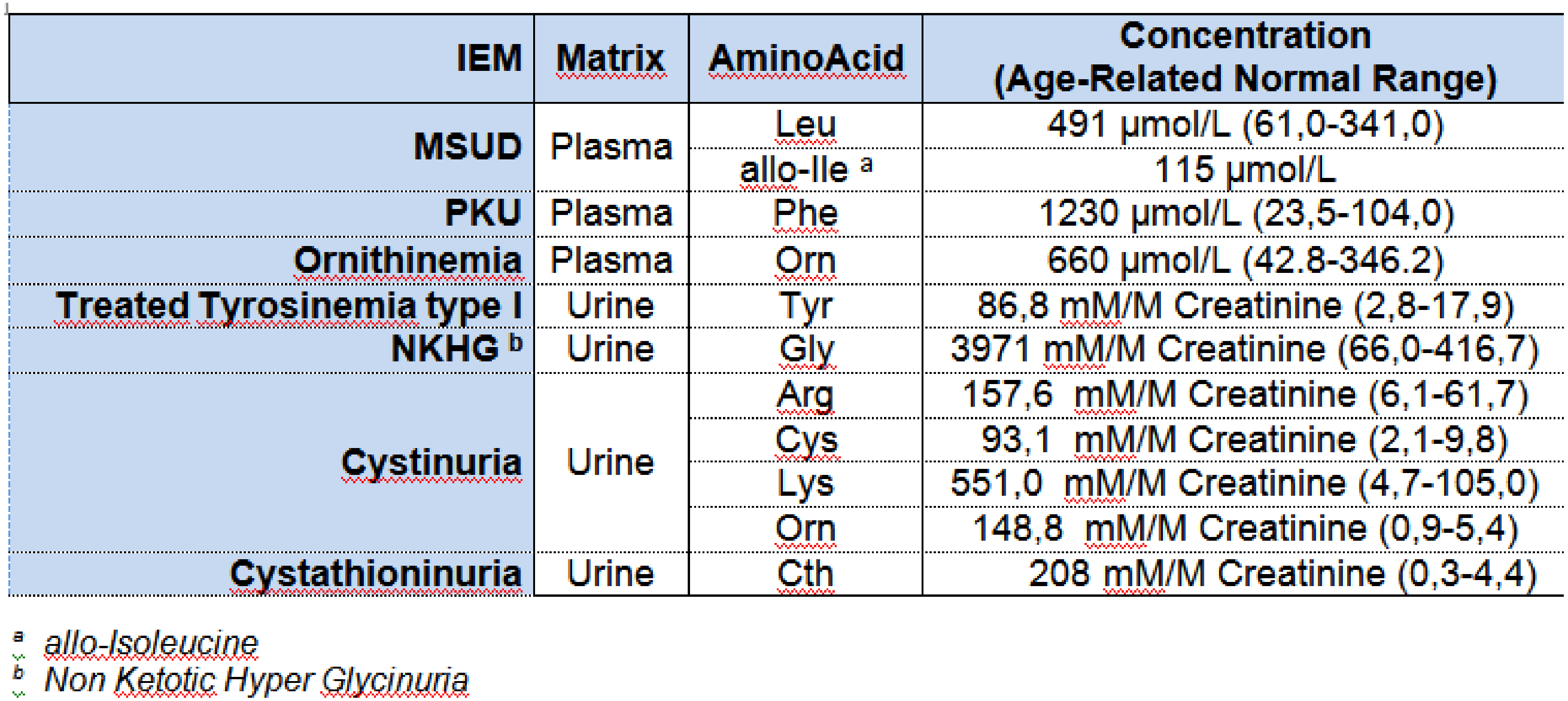
[a] *95% CI = Confidence interval at 95% for slope and intercept respectively*

Valeurs de Référence

- **Plasma – Urine – LCR --- 3 classes d'âge --- 20-30 échantillons**

	Plasma			Urine			CSF
	0 - 2 years *µmol/L*	*2 - 12 years* *µmol/L*	*>= 12 years* *µmol/L*	*0 - 2 years* *mM/M Creat.*	*2 - 12 years* *mM/M Creat.*	*>= 12 years* *mM/M Creat.*	*/* *µmol/L*
1MHis	0.1-3.7	0.2-15.7	0.5-14	2.7-18.9	2.7-208	3.6-137	0-0.9
3MHis	1.4-5.7	1.3-7.1	2.4-8	7.4-37.5	12.5-35.4	10.2-25.7	0.1-0.4
Aad	0.6-1.8	0.3-1.6	0.4-2	0.9-46.3	1.4-13.3	0.7-7.4	0-0.3
Abu	3.1-16.7	7.6-28	4.4-36.3	0.4-7.4	0.6-4.9	0.4-2.4	1.3-4
Ala	122-426	111-518	153-592	32.1-235	16.8-129	7.4-53.8	19-32.5
Ans	0-0.9	0-1.1	0-0.7	0-8.3	0-41.8	0-19.2	0-12
Arg	14.4-164	18.4-102	11.2-128	0.7-43.3	6.1-61.7	1.1-11.6	10.8-27
Asa [a]	0.3-7.8	0-13.7	0-12.5	0-18.2	1.1-4.2	0.5-3	0.4-6.7
Asn	27.1-123	28.4-102	22.7-115	3.3-136	8.4-48	3.5-36.6	6.6-12.1
Asp	6.1-69.4	4.6-60.3	4.4-65	0.8-20.3	0.3-9.4	0.3-4.6	1-11.6
bAib	0.8-31.8	0.5-3.3	0.3-2.9	3-98.1	2.6-107.2	1.5-27.6	0-0.2
bAla	17.2-67.3	15.8-49.9	18.5-60.7	1.5-116	0.3-15.9	0.2-13.7	17-41.4
Car	0-2.2	0-0.4	0-0.5	1.3-62	2-43.1	0.2-12.1	0-0.1
Cit	7.9-33.6	8.4-40.7	13.5-63.3	0.3-18.5	0.3-2.1	0.1-1.7	1.9-20
Cth	0.3-29.9	0.1-1.2	0.1-1.5	0.7-13	0.3-5	0.3-4.4	0.1-0.5
Cys	6.4-124	0.9-39.1	7.3-58.5	2.5-91.7	2.1-9.8	1.7-10	0-1
EtN	3.8-46.6	4.1-21.9	3.8-24.6	12.3-149	21.7-69.3	15.1-34.8	4.7-41.3
GABA	0.2-57.7	0.2-4.7	0.2-6.5	0.2-5.3	0.2-1.3	0.1-0.7	0.2-2.1
Gln	336-691	300-688	296-884	3-357	32-134	15-90	374-836
Glu	38-353	35-288	41.9-236	2.5-42.6	1.3-37.5	0.9-10.7	0.9-12
Gly	113-458	164.7-402	94.9-463	79.2-1057	66-417	43-360	9.3-34
Hcit [a]	0.1-0.7	0-0.8	0.1-0.7	0.3-29.4	1-8.1	0.8-3.9	0.1-0.4
Hcy	0-26.3	0-0.4	0-0.3	0-1.8	0-0.2	0-0.2	0-0.5
His	35-117	34.2-114	35.4-136.2	46-379	34.8-290	18-125	9.2-25.3
Hyl	0.1-110	0.1-86.4	0-103.2	1.1-40.3	0.5-11	0.1-1.7	0.1-2
Hyp	10.8-76.2	8.7-31.9	4.8-34.6	1.4-233	0.4-4.2	0.1-2.3	0.3-1.8
Ile	30.3-129	24.7-94.7	28.3-167.8	1-12	1-4.2	0.2-2.6	3.2-12.9
Leu	44.9-198	44.5-158	61.2-203.8	0.6-21.1	2.1-9.9	0.3-6.6	5-10
Lys	64.5-375	75.5-228	105-253	2-274	4.7-105	1.9-23.5	6.3-28.6
Met	9.4-67.6	9.3-31	9.6-44.4	0.2-5.1	0.5-2.3	0-1.3	1.5-4.2
Orn	19.9-181	23.4-165	42.8-186	1.5-52.8	0.9-5.4	0.6-2.6	4.5-10
PEtN	0-6	0-3.8	0.2-2.3	1.8-28.5	2.5-22.1	0.6-4.9	0.8-5.6
Phe	25.5-131	27.7-95.8	23.5-104	1.4-38.2	4.2-19	1.9-10.1	6.3-13.5
Pro	74.9-276	89.2-286	85.8-327.8	1.9-97.2	0.6-9.5	0.3-5	1.4-5.3
PSer	0-0.4	0-0.4	0-0.4	0-1.2	0-0.3	0-0.2	0-0.3
Sar	0.4-3	0.8-4.7	0.5-2.7	0.2-12.4	0-1.2	0-1.3	0-0.2
Ser	62-253	61.8-230	53.7-216	5.3-302	26.4-90.6	11.1-52.6	25.3-67.1
Tau	34.8-309	34.9-266	30.3-223	6-546	14.6-349	4.5-140	4.5-10.1
Thr	41.7-252	51-167	58.3-206	4.8-171	7.5-40.5	4.5-35	15.3-36.1
Trp	17.1-87.3	15.1-58.4	24.6-85.7	2.2-33.7	4-21.1	2.4-11	1.3-3.2
Tyr	24-154	27.3-92.1	34-101	1.3-76.2	6.2-45.5	2.8-17.9	6.9-17.8
Val	72.6-277	94.8-261	99.2-329	1.2-24.7	3.1-12	1-7.9	8.3-21.5

Evaluation Clinique



IEM	Matrix	AminoAcid	Concentration (Age-Related Normal Range)
MSUD	Plasma	Leu	491 µmol/L (61,0-341,0)
		allo-Ile [a]	115 µmol/L
PKU	Plasma	Phe	1230 µmol/L (23,5-104,0)
Ornithinemia	Plasma	Orn	660 µmol/L (42.8-346.2)
Treated Tyrosinemia type I	Urine	Tyr	86,8 mM/M Creatinine (2,8-17,9)
NKHG [b]	Urine	Gly	3971 mM/M Creatinine (66,0-416,7)
Cystinuria	Urine	Arg	157,6 mM/M Creatinine (6,1-61,7)
		Cys	93,1 mM/M Creatinine (2,1-9,8)
		Lys	551,0 mM/M Creatinine (4,7-105,0)
		Orn	148,8 mM/M Creatinine (0,9-5,4)
Cystathioninuria	Urine	Cth	208 mM/M Creatinine (0,3-4,4)

[a] *allo-Isoleucine*

[b] *Non Ketotic Hyper Glycinuria*

Conclusions - Perspectives

Conclusions

- Méthode conforme aux attentes - Résultats comparables à IEC-ninhydrine – Prix ~identique (hors appareil)
- Avantages:
  - Faible volume d’échantillon (40 µL) – IEC : min 200-400 µL
  - Runtime: 18 minutes – IEC: >150 minutes
  - Appareil non dédié – IEC: appareil dédié avec charges de maintenance spécifiques
- Inconvénients:
  - Procédure analytique longue: extraction, préparation et réintégration fastidieux
  - Certains composés ninhydrine-responsive (ex: pipecolate, sulfocystéine, aspartylglucosamine,…) ne sont pas identifiés par la présente méthode
- Sensibilité de l’instrument => réduction du volume d’injection (1µL) => ↓ souillage
  => combinaisons méthodes ultra-sensibles avec méthodes à haut-débit

Perspectives

- Ajout de transitions MRM à la méthode d’acquisition. Ceci permettrait de détecter certains composés identifiés par la méthode IEC et délaissés par la présente méthode MS.