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DEVELOPMENT OF AN HPTLC METHOD FOR AMINO-ACIDS IDENTIFICATION

HELSINKI 2008

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SUMMARY

- IPSEN GROUP

- AMINO-ACIDS Analysis
  - IPSEN Context and goals
  - Starting point of Bibliography

- IPSEN development on 24 AA
  - Test of pre-derivatisation
  - Test of post-derivatisation
  - IPSEN development
  - Conclusion

- Example on a peptide
  - Identification of amino-acids on a peptide after hydrolysis
  - Detection of amino-acids free in a peptide solution

- CONCLUSIONS

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IPSEN HISTORY

1929 : Creation of the « BEAUFOUR company » by Doctor Beaufour with one medicine product « Romarène® »

1954 : Citrate betaine® on the market

1969 : Creation of the research center «Institut Henri Beaufour »

1970'th : Products of natural origin : Ginkor ®, Tanakan ®, Smecta ®

1980'th : Creation of the Ipsen Foundation for therapeutic research (relationships between scientist and university

1986 : Decapeptyl® launched and open international expansion

1990'th : Somatuline ® with a sustained release form launched in France and UK

Since 2000 : Launch of products in Europe, China, Canada and US

2005 : IPSEN company on Euronext (european Market)

IPSEN GROUP

- 4000 people around the world
- CA 2007 : 920.5 M€
- More than 20 medicine products
- 700 person R&D (20.1%)

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SALES
BY THERAPEUTIC AREA

- **Targeted therapeutic areas 53.6%**
  Oncology **25.5%**
  Endocrinology **14.1%**
  Neuromuscular disorders **14.0%**
- **Primary care 42.7%**
  Gastroenterology **18.7%**
  Cognitive disorders **13.0%**
  Cardiovascular **10.3%**
  Others **0.7%**
- **Drug-related activities 3.7%**
  (active ingredients and raw materials sales)

SALES
BY GEOGRAPHICAL AREA

- **Five major Western European countries 61.3%**
  France **38.5%**
  Italy **7.1%**
  Spain **6%**
  Germany **5.2%**
  United Kingdom **4.5%**
- **Other European countries 22.6%**
- **Rest of the world 16.1%**

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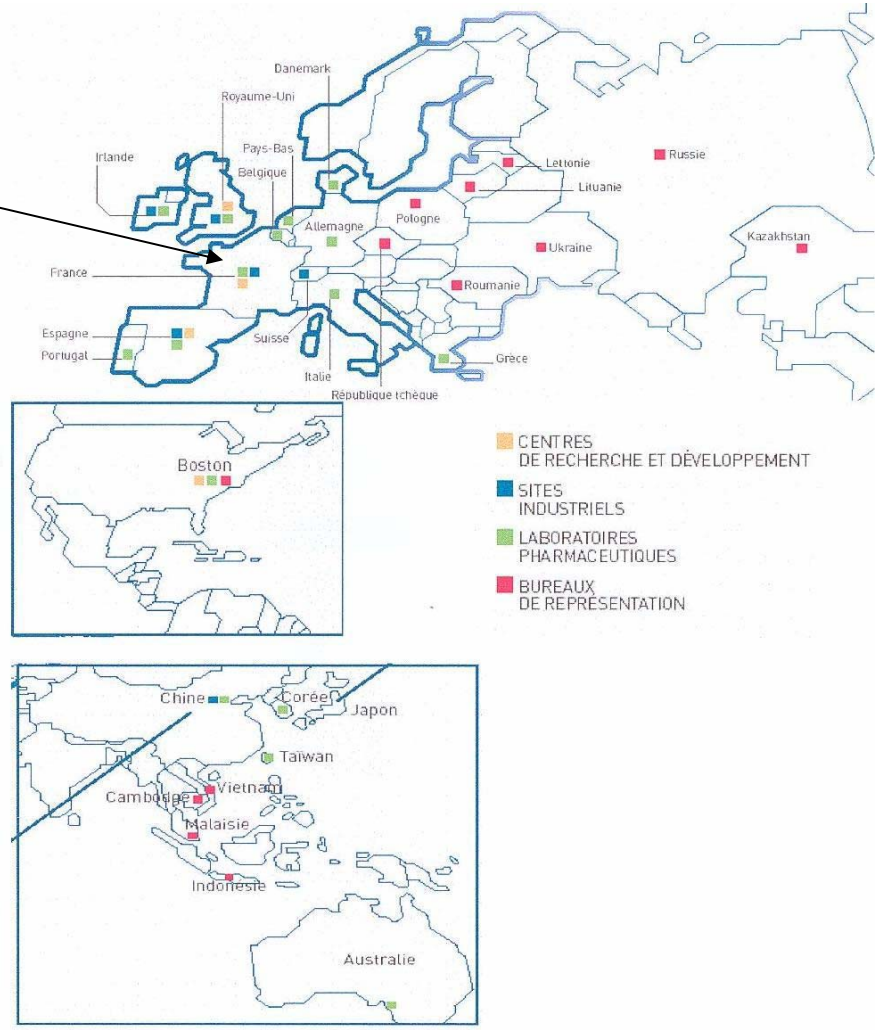
"Institut Henri Beaufour"

Les Ulis (Paris)

API Analytical Development (8 people)

- Mission
  - Development of API method
  - API monograph submission
  - Reference standard of peptide for the Ipsen Group
- Molecules
  - Small molecules
  - Synthetic Peptides



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AMINO-ACIDS ANALYSIS

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AMINO-ACIDS ANALYSIS : Context

WHY ? :

- For registration of a synthetic peptide, an identification test of amino-acids of the chain is mandatory. After an strong hydrolysis, each amino-acid could be identified.
- For a synthetic peptide, the synthesis quality is verified by search of amino-acids free traces.

HOW ?

Ipsen use an amino-acid analyser with post derivatisation with ninhydrin.

This equipment takes time (equilibration, washing..) and cost a lot of money (reagent from Japan, weak equipment).

This method is not flexible regarding the gradient (pb for non natural amino-acids or small molecule linked to the peptide).

AMINO-ACIDS ANALYSIS : Context

WHICH RESULT ?

- HPLC (amino-acids analyser) allows the quantification of each amino-acid after hydrolysis of peptide.

-During the strong hydrolysis (HCl + phenol), some amino-acids are degradated. Only 7 amino acids are stable

➔ The analysis of peptide by HPLC is used to identify natural amino-acids and to give semi-quantitative results.

- For free amino-acids traces, a huge quantity of peptide is injected and this quantity could affect the elution process or the detection.

➔ The LOQ is < 0.03-0.06 %

➔Why don't we try HPTLC with same goals ?

AMINO-ACIDS ANALYSIS : IPSEN goals

IPSEN Goals are :

1) Identification of natural and non natural (D-Bal, Apc, Inp) amino-acids (24)

2) Semi-quantitative results on amino-acids on a peptide analysis after hydrolysis

3) Trace of free amino-acids in peptide solution with LOQ < 0,03-0,06%

Name	AA
Acid amino-Butiric	Abu
Alanine	Ala
Arginine	Arg
Acid Aspartic	Asp
Acid amino-Iso-Butiric	Aib
Cysteine	Cys
Glutamine*	Gln*
Acid Glutamic	Glu
Glycine	Gly
Histidine	His
Isoleucine	Ile
Leucine	Leu
Lysine	Lys
Naphtyl Alanine	Nal
Phenylalanine	Phe
Proline	Pro
Serine	Ser
Threonine	Thr
Tryptophane	Trp
Tyrosine	Tyr
Valine	Val
D-Benzothienyl alanine	D-Bal
amino piperidinyl acid	Apc
Isonipecotic acid	Inp
stable hydrolyse	

Starting point of bibliography

Method	CAMAG	MERCK	
Objective	**Quantification of 8 AA**	HPTLC 2D for protein	Identification of 20 AA
Type of stationary phase	HPTLC silice	HPTLC silice	HPTLC cellulose
Derivatisation	pre= Dansyl Chloride/acétone 16h in dark room	-	
Mobile phase	titriplex3 à pH9/But/Ether (5/10/35) → upper phase	2 But/NH3/pyridine/water (39/10/34/26)	2 But/AA/pyridine/water (30/6/20/24)
Elution time	50min	120min	
Post treatment	Paraffine/n-Hexane (2/5)	**post** = Ninhydrine 0.5%/propan2ol 120°C for 3 mn	
Detection	Fluorescence (313/400nm)	Visible (440nm)	
Publications	« Quantitative determination of amino acids in potatoes Camag, Application notes »	« Detection of amino acids and peptides Michael Schulz/ LSA R&D (Merck) »	

IPSEN DEVELOPMENT

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IPSEN Devlpt on 24 amino-acids

- Test of pre-derivatisation with dansyl chloride : « Camag method »
- Test of post-derivatisation with ninhydrin : « Merck method »
- Conclusion

IPSEN Devlpt : Pre-derivatisation

Method	CAMAG for amino acids on potatoes
Type of stationary phase	HPTLC silica gel
Derivatisation	pre = Dansyl Chloride/acetone (2.5mg/mL)
Mobile phase	titriplex3 à pH9/But/Ether (5/10/35) → upper part of the mixture
Elution time	50min
Post treatment	Paraffine/n-Hexane (2/5)
Detection	Fluorescence (313/400nm)

COMMENTS :

The reaction spend 16h in dark room (quite long)

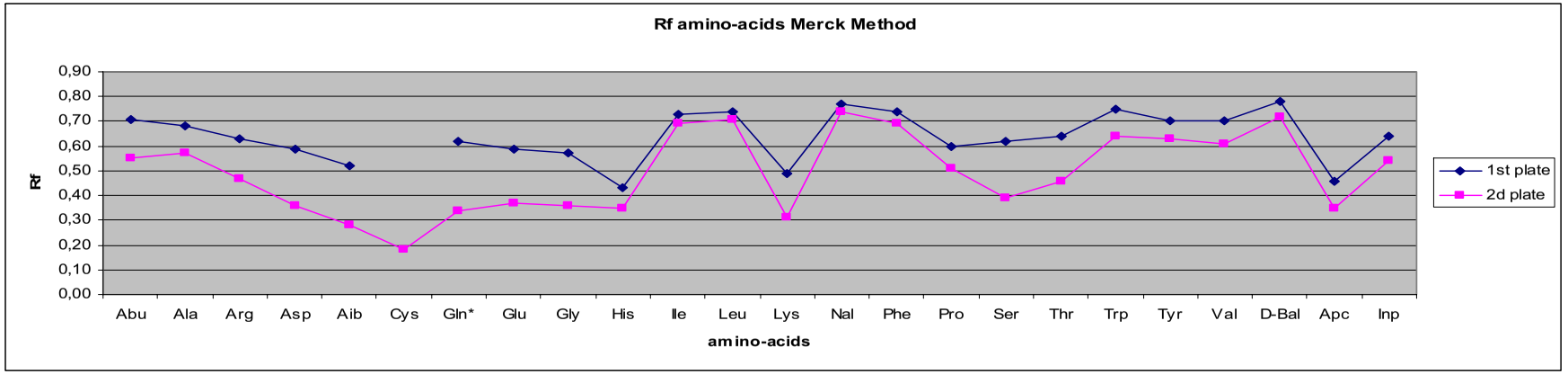
Some amino-acid don't react or co-eluted with this reagent

Dansyl chloride produce a large peak

CONCLUSION → Method not appropriate for our goals

IPSEN Devlpt : « Merck » method

Stationnary phase	Cellulose
Mobile phase	2 Butanol/ Acetic acid/Pyridin/water(30/6/20/24)
Type of migration	Vertical
Detection	Spraying (Ninhydrin/propan2ol (0.5%)



2 plates differents with same method !

=> Due to the flexibility of cellulose on aluminium plate ?

Different amino-acids have same Rf !

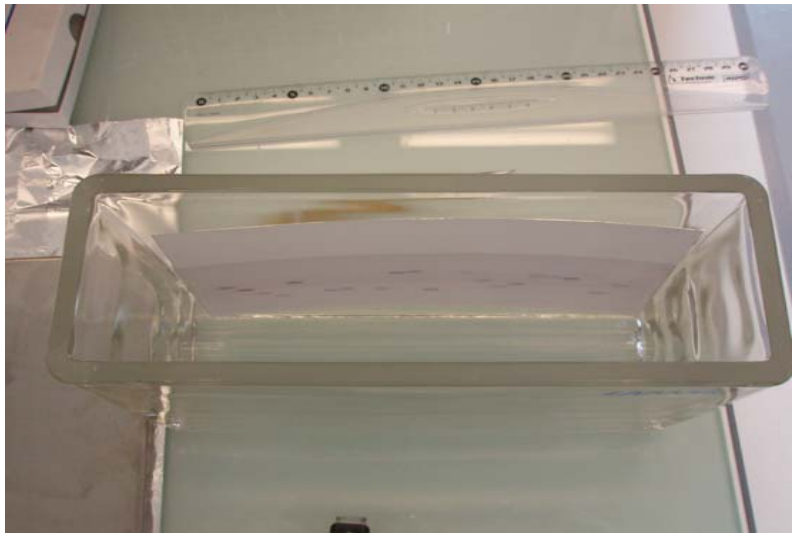
=> Separation is not sufficient for our goals

IPSEN Devlpt : « Merck » method

We decided to test the impact of different parameters :

	Tested parameters
Type of migration	Vertical Horizontal Vertical sandwich
Stationary phase	Cellulose Silice Diol
Mobile phase	2 Butanol/ Acetic acid/Pyridin/water(30/6/20/24) 2 Butanol/NH3/Pyridin/water(39/10/34/26)
Detection (Ninhydrin/propan2ol (0.5%) Fluorescamine	Spraying Dipping Over spot In the mobile phase

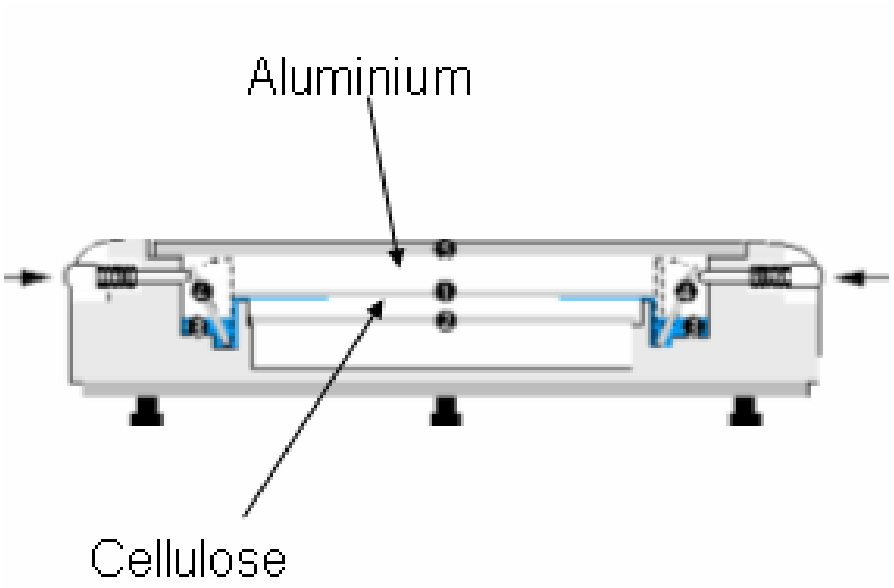
IPSEN Devlpt : Migration type



➔ **For cellulose plate, vertical migration is a real problem (repeatability)**

➔ **Sandwich vertical migration don’t solve the flexibility of the cellulose plate**

IPSEN Devlpt : Migration type



➔ **With horizontal migration, the problem is the aluminium foil : we put the plate upside down and it works !**

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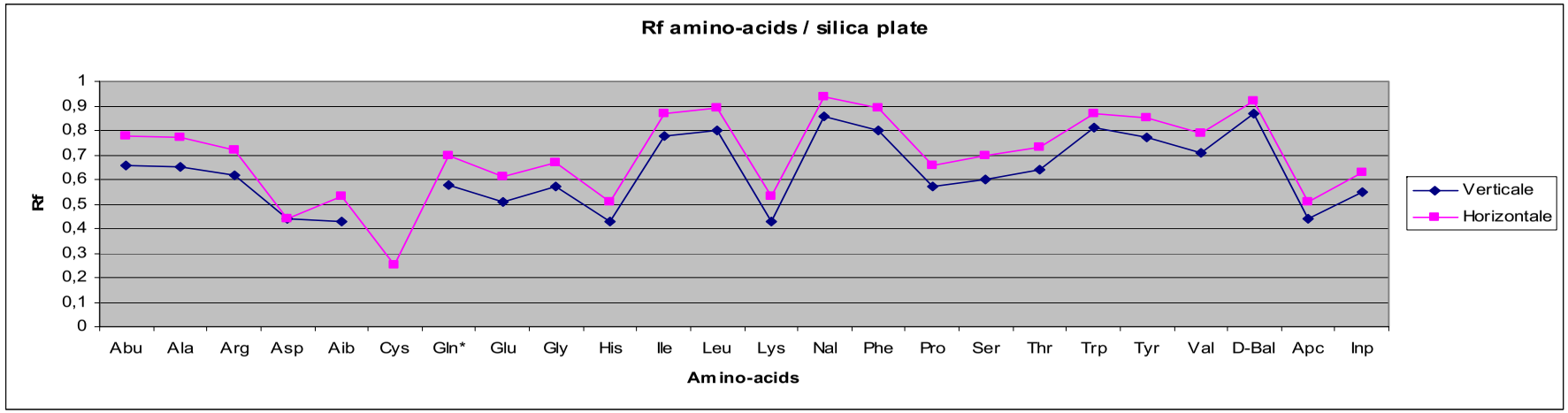
IPSEN Devlpt : Migration type

Stationnary phase	Cellulose, silice and diol
Mobile phase	2 Butanol/ Acetic acid/Pyridine/water (30/6/20/24)
Type of migration	Horizontal, vertical
Detection	Ninhydrine/propan2ol (0.5%)



➔ **Small modification on some amino-acid on the diol plate**

➔ **On silice plate, horizontal migration is more sensitive and allows the identification of cysteine**

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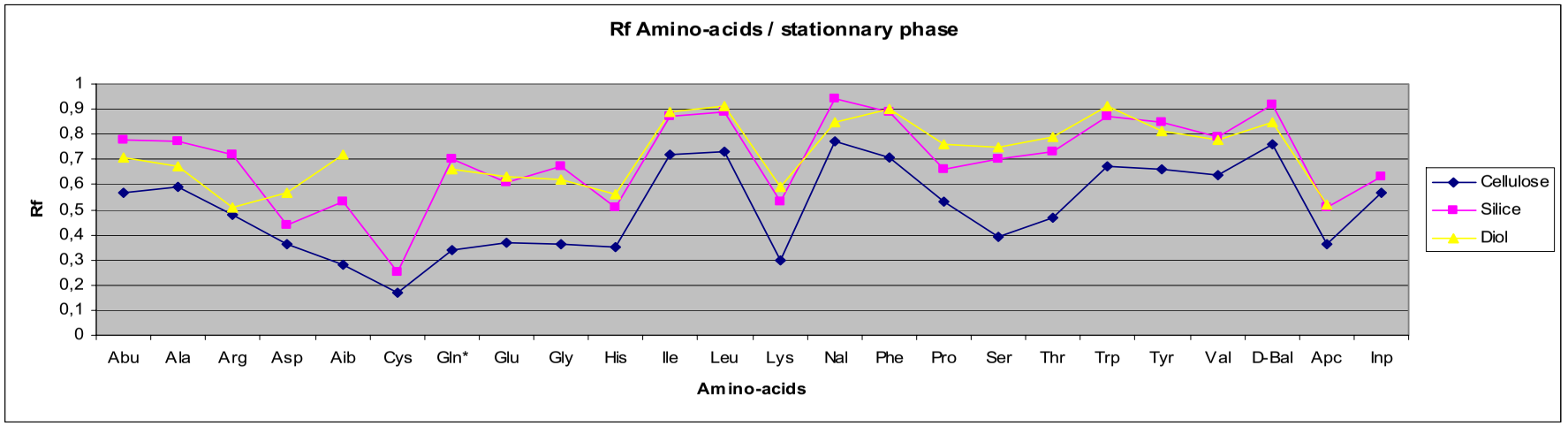
IPSEN Devlpt : Stationnary phase

Stationary phase	Cellulose, silice and diol
Mobile phase	2 Butanol/ Acetic acid/Pyridine/water(30/6/20/24)
Type of migration	Horizontal
Detection	Dipping (Ninhydrine/propan2ol (0.5%)



➔ **The Rf of the amino-acid depend on the plate but the range of the Rf are wider on silice and cellulose than on diol.**

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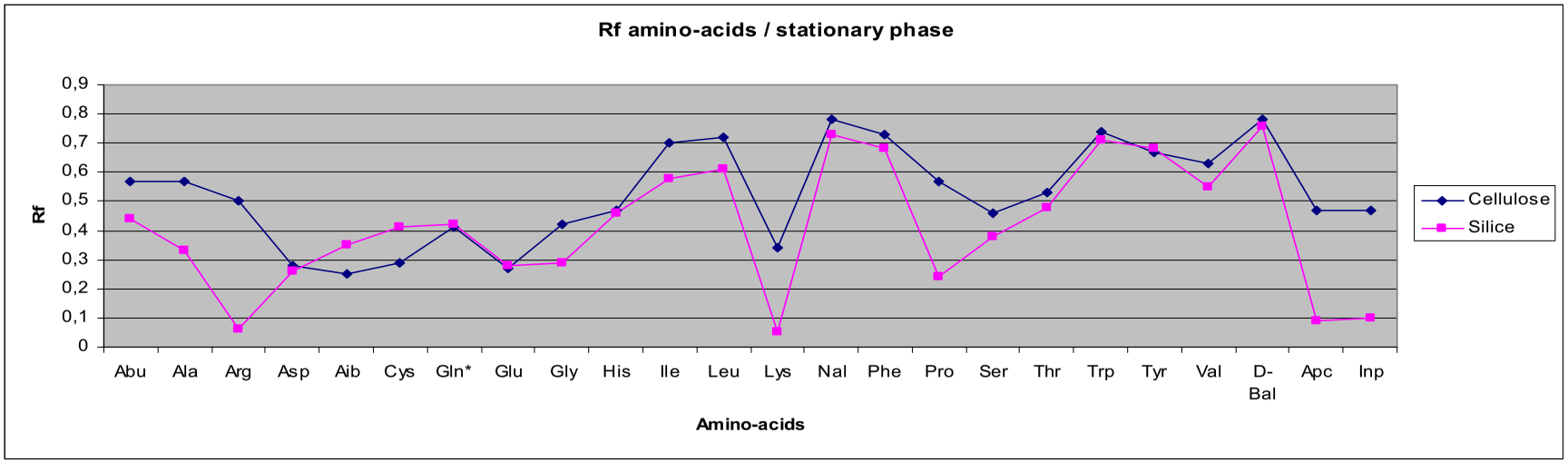
IPSEN Devlpt : Stationnary phase

Stationnary phase	Cellulose and silice
Mobile phase	2 Butanol/NH3/Pyridin/water(39/10/34/26)
Type of migration	Horizontal
Detection	Dipping (Ninhydrine/propan2ol (0.5%)



➔ **The Rf of the amino-acid depend on the plate. The range of the Rf are wider on silice than on cellulose with NH3 mobile phase.**

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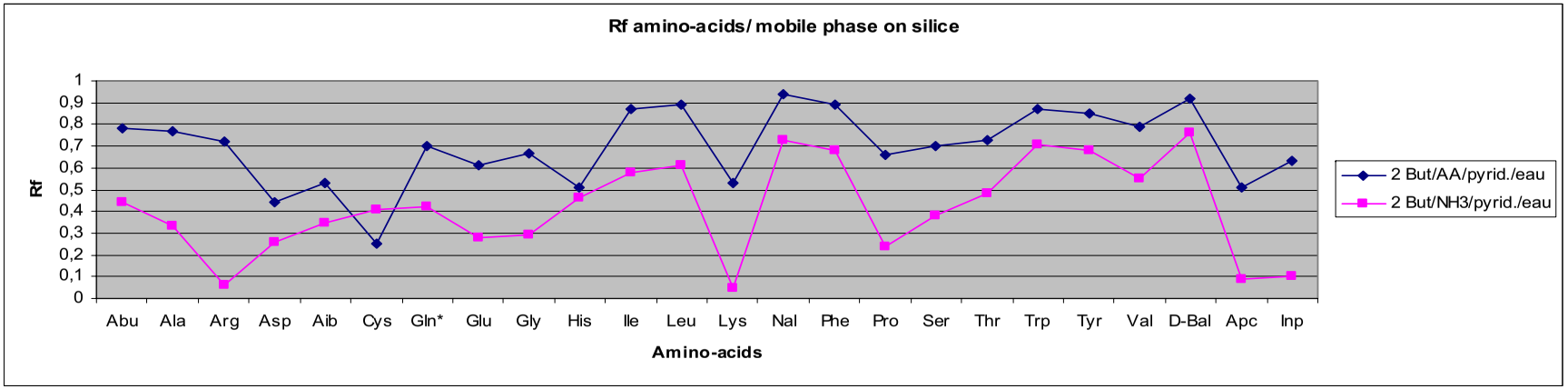
IPSEN Devlpt : Mobile phase

Stationnary phase	Cellulose and silice
Mobile phase	2 Butanol/ Acetic acid/Pyridine/water(30/6/20/24) 2 Butanol/NH3/Pyridine/water(39/10/34/26)
Type of migration	Horizontal
Detection	Ninhydrine/propan2ol (0.5%)



➔ Big impact of mobile phase on silice, only small impact on cellulose plate

➔ With NH3 mobile phase, more diffuse spot than acetic acid phase

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IPSEN Devlpt : Mobile phase

Separation of 24 amino-acids (0.5µg) : Most important plates

➔ **Acetic acid phase is better regarding diffusion / sensitivity**

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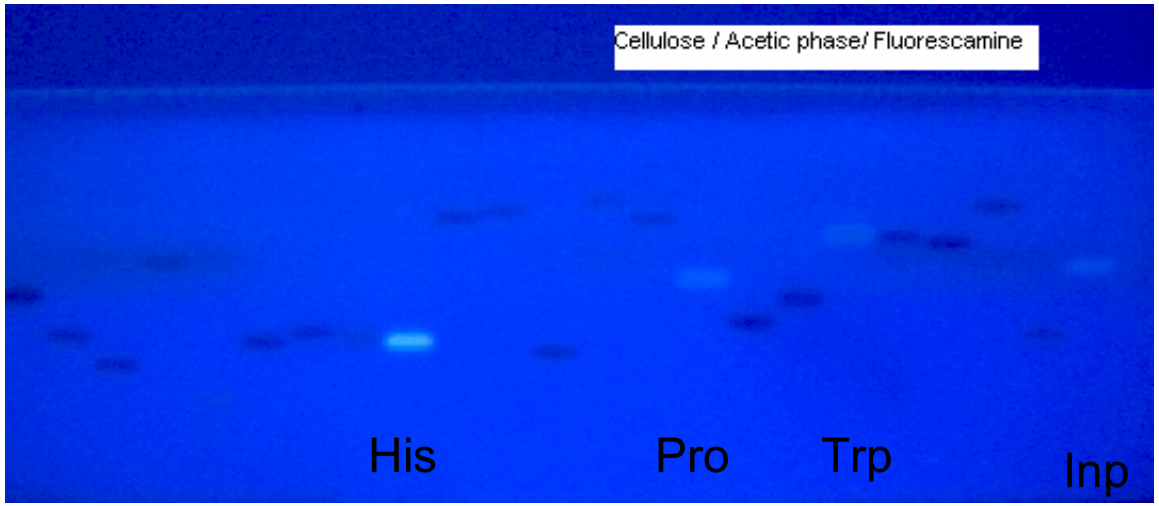
IPSEN Devlpt : Detection type

Stationnary phase	Cellulose
Mobile phase	2 Butanol/ Acetic acid/Pyridine/water (30/6/20/24)
Type of migration	Horizontal
Detection	Ninhydrin/propan2ol (0.5%) Spraying, dipping, over spot and in the mobile phase Fluorescamine (0.5% at pH 9)



➔ **Fluorescamine : less sensitive than ninhydrin except for His, Pro, Trp and Inp**

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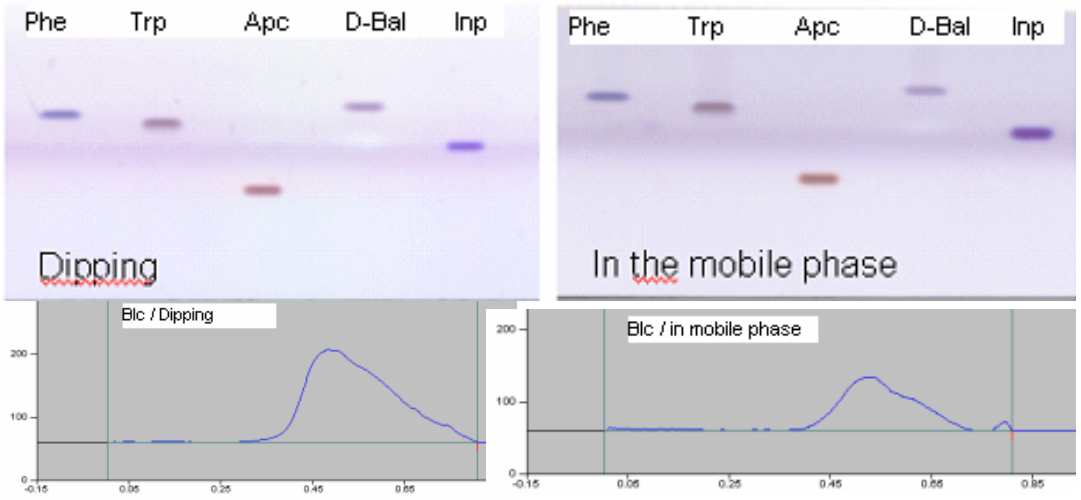
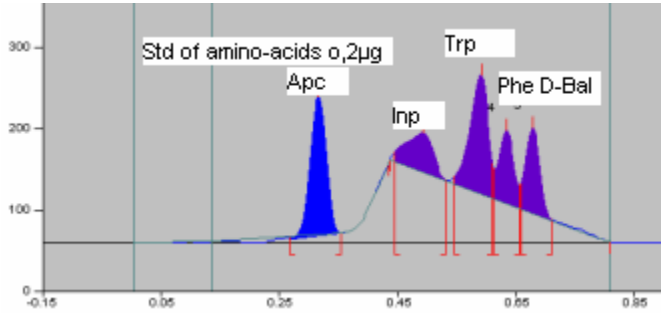
IPSEN Devlpt : Detection type





➔ **The impact of baseline is important regarding the low quantity in the standard**

➔ **Spraying < Dipping < in the mobile phase**

➔**Ninhydrin over spot modify all the selectivity**

➔ **Ninhydrin in the mobile phase is the best and simplier conditions (but not feasible with silice plate)**

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IPSEN Devlpt : CONCLUSIONS

➔**Test of pre-derivatisation with dansyl chloride :** not suitable for the separation of 24 potential amino-acids

➔**Test of post-derivatisation by ninhydrin :** The separation of 24 natural and non natural amino-acids is possible but cannot be performed by only one generic method.

➔ **The Rf table of the 24 amino-acids on 7 different methods allows this comments :**

Acetic acid mobile phase produce less diffusion and is faster than NH3

Horizontal migration upside down is easy to use for flexible cellulose plate and sensitive (<0.5µg)

Ninhydrin in the mobile phase is simple and reproducible but not feasible with silice plate

Fluorescamine is specific for His, Pro, Trp and Inp

➔**The right method depends on the amino-acids mixture**

IPSEN Devlpt : Rf table

Dérivatisatio	post = Ninhydrine 0.5%/propan2ol					post = Ninhydrine 0.5%/propan2o	
Phase Mobil	2 But/AA/pyrid./eau					2 But/NH3/pyrid./eau	
Plaque →	Cellulose	Silice		Diol		Silice	Cellulose
Elution →	Hor 2	Verticale	Horizontale	Verticale	Horizontale	Horizontale	Horizontale
Abu	0,57	0,66	0,78	0,77	0,71	0,44	0,57
Ala	0,59	0,65	0,77	0,75	0,67	0,33	0,57
Arg	0,48	0,62	0,72	0,7	0,51	0,06	0,5
Asp	0,36	0,44	0,44	0,69	0,57	0,26	0,28
Aib	0,28	0,43	0,53	0,59	0,72	0,35	0,25
Cys	0,17		0,25			0,41	0,29
Gln*	0,34	0,58	0,7	0,68	0,66	0,42	0,41
Glu	0,37	0,51	0,61	0,66	0,63	0,28	0,27
Gly	0,36	0,57	0,67	0,65	0,62	0,29	0,42
His	0,35	0,43	0,51	0,53	0,56	0,46	0,47
Ile	0,72	0,78	0,87	0,8	0,89	0,58	0,7
Leu	0,73	0,8	0,89	0,81	0,91	0,61	0,72
Lys	0,3	0,43	0,53	0,56	0,59	0,05	0,34
Nal	0,77	0,86	0,94	0,82	0,85	0,73	0,78
Phe	0,71	0,8	0,89	0,8	0,9	0,68	0,73
Pro	0,53	0,57	0,66	0,68	0,76	0,24	0,57
Ser	0,39	0,6	0,7	0,69	0,75	0,38	0,46
Thr	0,47	0,64	0,73	0,72	0,79	0,48	0,53
Trp	0,67	0,81	0,87	0,8	0,91	0,71	0,74
Tyr	0,66	0,77	0,85	0,79	0,81	0,68	0,67
Val	0,64	0,71	0,79	0,76	0,78	0,55	0,63
D-Bal	0,76	0,87	0,92	0,82	0,85	0,76	0,78
Apc	0,36	0,44	0,51	0,53	0,52	0,09	0,47
Inp	0,57	0,55	0,63	0,68		0,1	0,47

IPSEN Application on peptide

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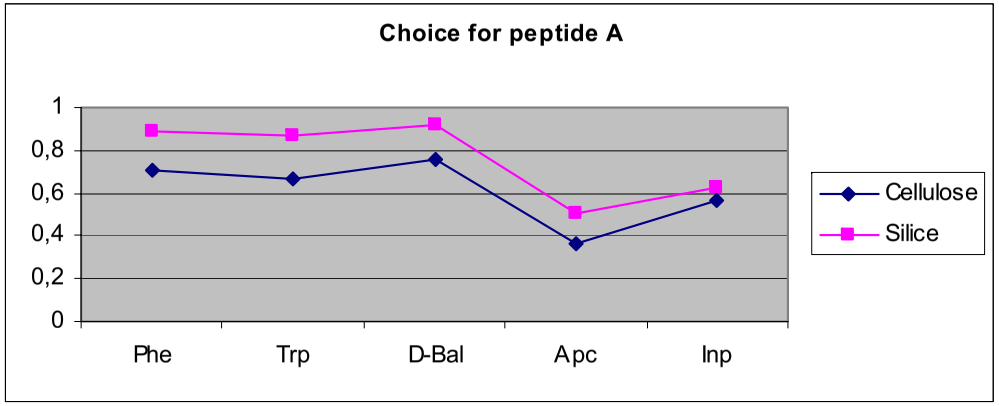
Peptide A

Dérivatisat	post = Ninhydrine 0.5%/propan2ol					post = Ninhydrine 0.5%/propan2ol	
Phase Mob	2 But/AA/pyrid./eau					2 But/NH3/pyrid./eau	
Plaque →	Cellulose	Silice		Diol		Silice	Cellulose
Elution →	Hor 2	Verticale	Horizontale	Verticale	Horizontale	Horizontale	Horizontale
Phe	0,71	0,8"	0,89	0,8"	0,9"	0,68	0,73
Trp	0,67	0,81"	0,87	0,8"	0,91"	0,71	0,74
D-Bal	0,76	0,87	0,92	0,82	0,85	0,76	0,78
Apc	0,36	0,44	0,51	0,53	0,52	0,09"	0,47"
Inp	0,57	0,55	0,63	0,68		0,1"	0,47"



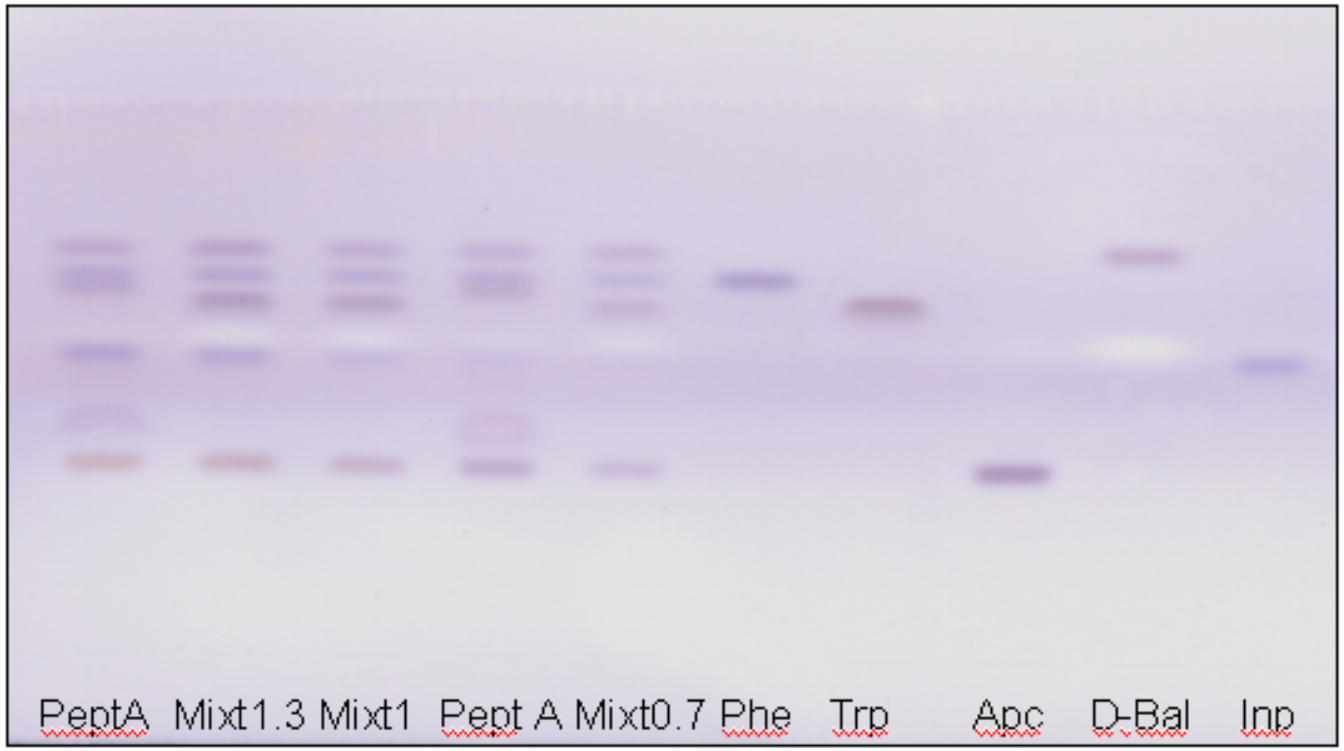
➔ **First choice :**

Cellulose plate

Acetic acid mobile phase

Horizontal migration

Peptide A after hydrolysis



- ➔ **Identification of amino-acids (≈0.15µg) in hydrolysis peptide**
- ➔ **Semi-quantitative results : Mixt 0.7 < pept A < Mixt 1.3**
- ➔ **Achieved goal for peptide A**

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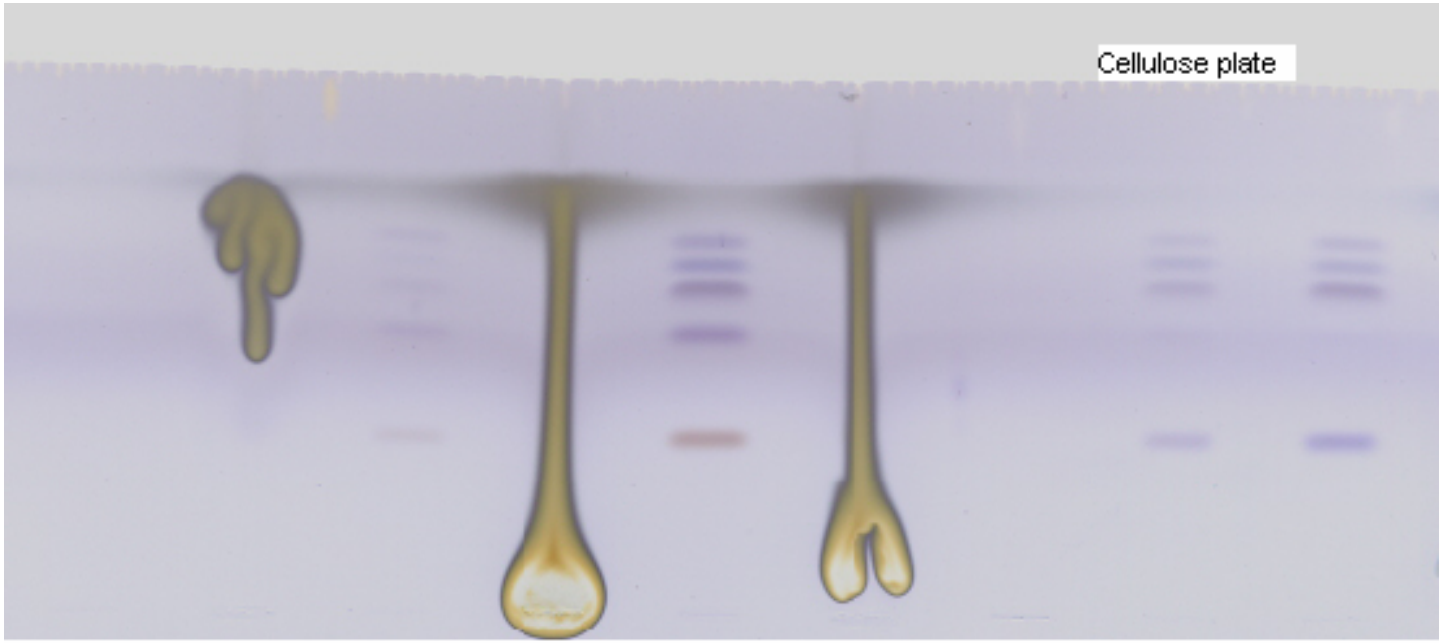
Peptide A : free amino-acids



- ➔ **The LOQ < 0.03-O.O6% is possible if std is compared to 50µl of peptide**
- ➔ **On a cellulose plate, 10µl (= 30mg) of the peptide is too much. On a Proteo Chrom plate, the result is similar**
- ➔ **Limit test (with or without washing) need to be completed**

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CONCLUSION OF THE WORK

Parameter	Goal	Result
Generic method	Obtain a method for separation and identification of our 24 amino-acids (natural and non natural)	Not only one method but choice between 7 methods depending amin-acids mixture. The ninhydrin reagent allows the identification of every amino-acids (<0.5µg) Fluorescamine could be specific for His, Pro, Trp and Inp.
Identification on peptide after hydrolysis	Identification of the amino-acid with semi quantitative result	Identification of the amino-acid with semi quantitative result of peptide A.
Limit test of amino-acid free in the peptide	Less of 0.03 or 0;06% of each amino-acid	Not feasible with external calibration Limit test (with or without washing) need to be completed

CONCLUSION OF THE WORK

- ➔ **2 goals are achieved and the limit test must be tested for free amino-acids**
- ➔ **HPTLC is a simple and quick method for amino-acid identification**

- ➔ **This method will be used for the future registration of peptide A**