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Development of an analytical method usefull for the quantification of L-Glu & GlutaMAX during fermentation

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Titre / *Titel*

Development of an analytical method usefull for the quantification of L-Glu & GlutaMAX during fermentation (USP)

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Development of an analytical method useful for the quantification of L-Glu & Glutamax™ during fermentation

Graduate Mayor Mathieu

Bachelor's Thesis
| 2013 |

Degree course
Life Technologies

Field of application
Analytical Chemistry

Supervising professor
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Objectives

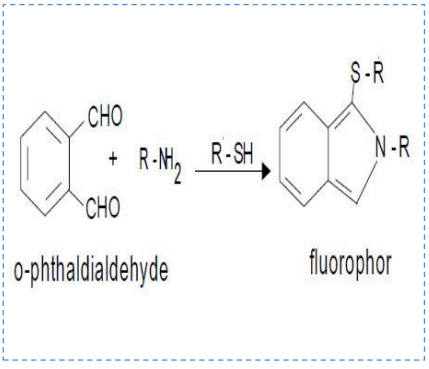
The objectives of this work are to establish time and cost-efficient methods for the quantification of L-Glutamine and Glutamax™ in different mediums containing serums. These methods have to be easy to perform and based on a purpose of daily basis analysis of mammalian cells cultures.

Methods | Experiences | Results

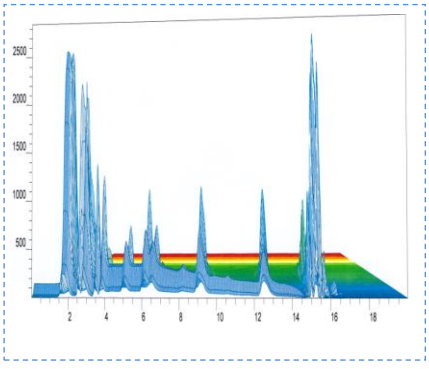
Two methods will be developed in parallel and will allow the monitoring of L-Glutamine and Glutamax™ during bioprocesses. It is really crucial to quantify the amount of L-Glutamine or Glutamax™ which gives information about cells viability and metabolism.

Methods that have been developed allow the quantification of L-Glutamine and Glutamax™ in different media with an iscocratic separation on a RP-HPLC system using a C18 column. The accuracy of both methods is less than +/-10% as well as the reproducibility that is less than 5%. The methods developed are based on a pre-column reaction of derivatization that is controlled automatically by the auto sampler. One of the main parts of these methods development was the optimization of the reaction parameter in order to assure a complete reaction.

A precise SOP for each method will also be developed that will allow people to use these method on daily purpose analysis.



Reaction used for the derivatization of the primary amine of L-Glu and Glutamax™.



One example of the absorbance spectrum of the media SFM4CHO with the serum hyclone SH30548 using Glutamax™ as substrate. The peak of Glutamax™ comes out at 12.4 min

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0. Abbreviations

HPLC: High performance liquid chromatography

LC: Liquid chromatography

HEPT: Theoretical plate height

HPLC-RP: High performance liquid chromatography on reverse phase

SOP: Standard operation procedure

L-Glu: L-Glutamine

ACN: Acetonitrile

MeOH: Methanol

LIF: Laser induced fluorescence

DAD: Diode array detector

UV: Ultraviolet

OPA: ortho-phtalaldehyde

2-MCE: 2-mercaptoethanol

λ : wavelenght [nm]

μ: Flowrate [ml/min]

dp: particles diameter [μm]

w: peak width [min]

R_t: retention time [min]

L_{col}: column length [cm]

R_s: resolution

H: Theoretical plate height [cm]

N: Number of theoretical plates

V_{inj}: injection volume [μl]

σ: Standard deviation from the Gaussian

1. Introduction

L-glutamine is an essential component in cell culture media, being used as a source for the energy production of the cell. It also used for the synthesis of proteins and nucleic acids necessary for the cells growth. However, the L-Glutamine is temperature sensitive and thus degrades spontaneously at 37 °C which is the normal temperature of mammalian cells cultures. The degradation of L-Glutamine does not only result in a loss of energy source but also generates ammonia as by-product. The accumulation of ammonia can be toxic for the cells and can affect the glycosylation and cell viability. This drives to a lower protein production for the cells and can also change the glycosylation pattern.

As an alternative to L-Glutamine, a dipeptide had been developed that is more stable than the simple amino-acid in the same conditions. This molecule is called Glutamax™ and corresponds to the following amino acids sequence: L-Alanyl-L-Glutamine. As mentioned, the considerable advantage of this molecule is its extreme stability in the mammalian cells culture conditions due to its L-glutamine stabilized form. It then prevents the break-down of L-glutamine and thus the formation of ammonia.

Anyways, for each substrate that is chosen, it is really crucial to monitor the amount of L-Glutamine or Glutamax™ which give information about cells viability and metabolism. In order to quantify the ammonia and the L-glutamine, enzymatic kits or automatic devices are often used. However, these kits and automatic devices are really expensive in themselves and also in their maintenances. In contrast to L-Glu, methods to specifically detect the dipeptides of Glutamax™ solutions have not been described so far.

2. Aim and method requirements

The objectives of this work are to establish time and cost efficient methods for the quantification of L-glutamine and Glutamax™. This method has to be easy to perform and based on a purpose of daily basis analysis of the mammalian cells cultures. The methods also have to be according to the method requirements needed from the biotechnical department. The requirements are described in the following points:

- Specificity: the methods have to be specific for L-Glutamine and Glutamax™. Furthermore, it has to be compatible with different medias e.g. serum free and serum containing media.
- Range: the measuring range should lie between 0.1 and 8 mM (0.015 – 1.2 mg/ml for L-glu and 0.0217 – 1,7 mg/ml for glutamax)
- Limit of quantification : limit of quantification should be at least 0.1 mM for each subtracts or lower
- Accuracy: an accuracy (recovery) of +/- 10% is acceptable
- Precision : a precision of +/- 5% (repeatability) is acceptable
- Robustness: the method should run in an easy to perform manner in daily routine circumstances. Easy to perform system suitability testing parameters shall be chosen.

- Linearity: chose the calibration curve to cover the above mentioned range accordingly and based on practical considerations.

3. Theoretical part

In this section, the main keys of methods choices and methods developments will be given. The methods have to be specific to different medium which are presented in the following table:

Table 1: All different mediums containing serums

	Metabolite	
Medias with serum	L-Glu	Glutamax^{tm}
SFM4CHO (Hyclone SH30548)	×	×
CDCHO	×	
DMEM/Ham'F12 (Sigma D6421) with 10% FCS	×	×
DMEM/Ham'F12 (Gibico 10743011) with 10% FCS	×	

As seen below, the analytical method that will be chosen has to be compatible for each media containing each serum presented in the table 1. Each of the media and serum contain a lot of different molecules that can be really big (like for example some DNA residues or some enzymatic molecules). For this reason, the matrix effect has to be study carefully.

3.1. Literature and strategy

Before searching any publications or literature about this area of research, it is always good to think a little bit by him-self. The main analytical instruments which are often present in laboratories are the LC systems. It is therefore a good idea to try to develop methods on it. The second thing is to think about the separation itself. Once again, one of the most popular analytical methods because of its cost and viability is the HPLC-RP. But how it is possible to separate such a little and relatively polar molecule on a system that retains molecule due to non-polar interaction with physiological pH. The solution that comes rapidly to mind is to use a derivatizing agent that can be able to increase the retention on the reversed phase column of the L-Glutamine or Glutamax^{TM}. Therefore, L-Glutamine and Glutamax^{TM} can be detected using a simple RP-HPLC system.

It also appears relatively rapidly that the molecule of OPA is widely used for this purpose. Several publications mentioned the utilization of this reagent for the derivatization of molecules containing primary amines, which is the case of the two molecules of interest. The point of departure was the publication of Jens Olaf Krömer and Michel Fritz (2004) that published about the “In vivo quantification of intracellular amino-acids and intermediates of methionine pathways in *Corynebacterium glutamicum*”. They described a reaction of derivatization using OPA and under certain conditions and at physiological pH and detected with UV at 338 nm.

Then, it is needed to find an adequate column for the separation. As the pH is relatively high, the choice of the column was actually specific. Indeed, at theses pH, most of the rp-columns existing will be affected by this parameter and will result in a considerable deterioration of the column. The next step will be the optimization of the derivatization parameters and conditions. In order to reduce the error relative to this reaction, the 1100 auto sampler of Agilent will be used and will also be a non-negligible part of the method development. Once it is working and the molecules of interest detected using an UV-detector, the matrix effect of all media containing serum will have to be studied in order to make an analytical method specific for all mediums. It requires a working handle sampling and also a proper dilution that avoid all matrix effect.

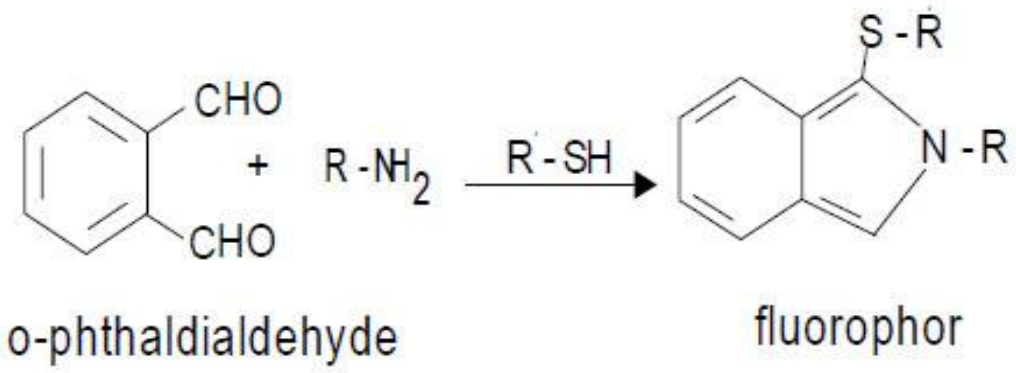

Figure 1 : Reaction of derivatization using OPA and primary amine

4. Experimental part

4.1 Materials

HPLC: Series 1100 Agilent from the laboratory f103

- UV detector : G1315A, Serial : DE91606692
- Auto sampler : G1313A, Serial : DE91609213

HPLC: Series 1100 Agilent from the biotechnical department

- UV detector : G1315A
- Auto sampler : G1313A

pH Meter: Metrohm 654 pH-Meter

Filters 3kDA: Nanostep 3K Omega, Life Science

Micropipettes: from Biohit

All glassware from the laboratory f103

Centrifuge: Hettich, Mikro 200

HPLC Vials of 2 mL

Analytical balance: Metler Toledo, laboratory f103.

Filters 0.45 um: Exapure^{tm}, Syringe Filters PTFE, 0.45µm, 24 mm PTFE membrane

HPLC Column: C18 gravity, Macherey-Nagel, 4,6mm x 150 mm, 3 µm, serial N°: N8090623

4.2 Reagants and safety

Table 2 : Reagents, provenance and safety

Compound name	Formula	Quality [%]	Origin	n° number catalogue	n° CAS	Safety	Notice
Acetonitrile	C2H2N	99.9	Lab-Scan	C73C11X	75-05-8	Xn, F	-
OPA	C8H6O2	99	Sigma	P0657	643-79-8	Corrosive, T	-
MeOH	CH4O	99.9	Lab-Scan	C17C11X	67-56-1	T, F	-
L-Glutamine	C5H10N2O3	99	Sigma	G3126	56-85-9	-	-
MQ Water	H2O	-	-	-	-	-	-
Glutamax^{tm}	C8H15N3O4	-	Invitrogen	A12860	-	-	200 mM solution
2-MCE	C2H6OS	>98	Flukka	63700		B3,D1A,D2B	-
Sodium dihydrogen phosphate	NaH2PO4.H2O		Acros Organics	A0331028	10049-21-5	-	-
Bicine	C6H13NO4	99	Sigma	B3876	150-25-4	-	-

4.3 Sample preparation

The preparation of samples and the sampling itself has to be performed well in order to get acceptable results. Two different methods of preparation and storage of samples are presented here. Both of them are acceptable and will be discussed further. Note here that both preparation of sample containing L-Glutamine or Glutamax^{tm} are the same so there is no consideration about this. The both methods will be discussed later, they are not fundamentally different but they belong to contrasting strategies. Note that all the results that will be presented here result from the method 1 of sampling.

4.3.1 Method 1

Aliquot periodically 1 mL from the culture of mammalian cells, filter it on a 0.45 µm filter (see section 4.1). Store it in the freezer for further analyses. If you use it directly, take 200 µl of the sample and filter it on a 3kDa filters (see section 4.1). In order to do it, run 10 min at 15000 rpm in a centrifuge. Once this is done, add 20 µl of H2O and run another 10 min with the centrifuge. Once it is done, sample 200 µl for further dilutions and analyzes. Again, if the samples are not used directly, store them in the freezer.

4.3.2 Method 2

Aliquot periodically 1 mL from the culture of mammalian cells, filter it on a 0.45 µm filter (see section 4.1). Store it in the freezer for further analyses. If you use it directly, use immediately 200 µl for further analyses. Again, if the samples are not used directly, store them in the freezer.

4.4 HPLC-RP systems

In this part, the main ideas about the systems that have been developed will be given but only about the separation (not the reaction of derivatization) that (both) will be discussed later.

4.4.1 Isocratic

This is basically the best way to have an efficient separation and has the most chance to work for all mediums assuming that the separation (resolution) will be better. Before going any deeper with the description of the method, the following figure explains the strategy that has been used for all isocratic separation that will be presented:

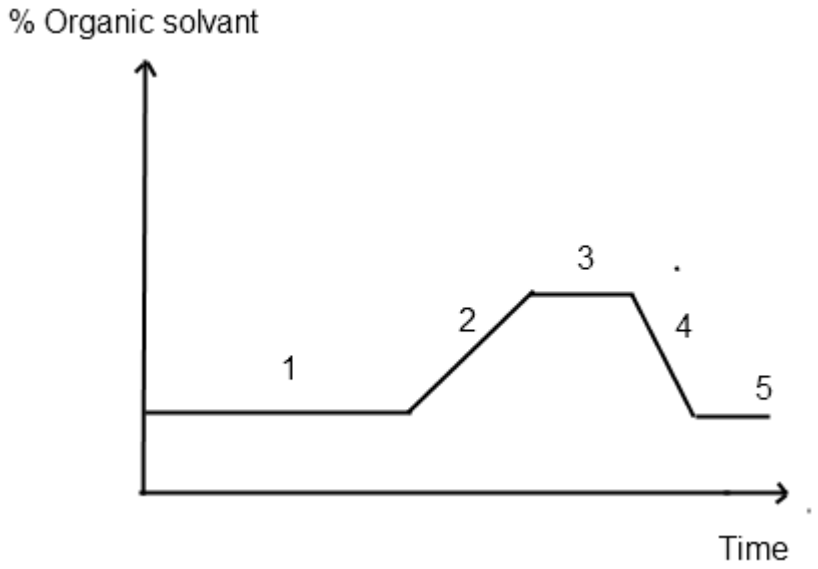

Figure 2: Strategy of the method development with isocratic separation

Once we can see on the figure 2, for the isocratic methods, there is five different parts. They correspond to the following:

1) Isocratic part for the separation of the molecules of interest
2) Augmentation of the percentage of organic solvent in order to empty the column
3) Augmentation of the percentage of organic solvent in order to empty the column
4) Stabilization of the column for the next injection
5) Stabilization of the column for the next injection

Another important thing to notice before go any further is that, two different methods have been developed in parallel for the L-Glutamine and Glutamax™. As Glutamax™ is the most non-polar molecule of interest, once can assume a longer retention time into the rp-column. That's why the percentage of organic solvent has to be increased in the case of Glutamax™. All other parameters remain the same for each method but -by worries of comprehensibility- the details of all methods will still be given.

4.4.1.1 Method 1.1 for l-Glutamine

Column: C18 gravity, Macherey-Nagel, 4,6mm x 150 mm, 3 µm, serial N°: N8090623

HPLC system: Series 1100 Agilent, laboratory f103

- UV detector : G1315A, Serial : DE91606692
- Auto sampler : G1313A, Serial : DE91609213

Wavelength UV detector: 338,4 [nm]

Temperature: 25°C

Eluents: ACN and NaH2PO4 (40mM, pH = 7,8)

Table 3: Eluent composition for method 1.1

Time [min]	%ACN	%Buffer	Flow rate [ml/mn]	max. pressure [bar]
13	14	86	0.8	300
15	60	40	0.8	300
17	60	40	0.8	300
19	14	86	0.8	300

Stop time: 21 min

4.4.1.2 Method 1.2 for Glutamax™

Column: C18 gravity, Macherey-Nagel, 4,6mm x 150 mm, 3 µm, serial N° : N8090623

HPLC system: Series 1100 Agilent, laboratory f103

- UV detector : G1315A, Serial : DE91606692
- Auto sampler : G1313A, Serial : DE91609213

Wavelength UV detector: 338,4 [nm]

Temperature: 25°C

Eluents: ACN and NaH2PO4 (40mM, pH = 7,8)

Table 4: Eluent composition for the method 1.2

Time [min]	%ACN	%Buffer	Flow rate [ml/mn]	max. pressure [bar]
11	19	81	0.8	300
13	60	40	0.8	300
16	60	40	0.8	300
18	19	81	0.8	300

Stop time: 20 min

4.4.1.3 Method 2.1 for L-Glutamine

Column: C18 gravity, Macherey-Nagel, 4,6mm x 150 mm, 3 µm, serial N°: N8090623

HPLC system: Series 1100 Agilent, laboratory f103

- UV detector : G1315A, Serial : DE91606692
- Auto sampler : G1313A, Serial : DE91609213

Wavelength UV detector: 338,4 [nm]

Temperature: 25°C

Eluents: MeOH and NaH2PO4 (40mM, pH = 7,8)

Table 5: Eluent composition for method 2.1

Time [min]	%MeOH	%Buffer	Flow rate [ml/mn]	max. pressure [bar]
13	14	86	0.8	300
15	60	40	0.8	300
17	60	40	0.8	300
19	14	86	0.8	300

Stop time: 21 min

4.4.1.4. Method 2.2 for Glutamax™

Column: C18 gravity, Macherey-Nagel, 4,6mm x 150 mm, 3 µm, serial N°: N8090623

HPLC system: Series 1100 Agilent, laboratory f103

- UV detector : G1315A, Serial : DE91606692
- Auto sampler : G1313A, Serial : DE91609213

Wavelength UV detector: 338,4 [nm]

Temperature: 25°C

Eluents: MeOH and NaH2PO4 (40mM, pH = 7,8)

Table 6: Eluent composition for the method 2.2

Time [min]	%MeOH	%Buffer	Flow rate [ml/mn]	max. pressure [bar]
11	19	81	0.8	300
13	60	40	0.8	300
16	60	40	0.8	300
18	14	81	0.8	300

Stop time: 20 min

4.4.2 Gradient

For the development of a separation using a gradient of organic solvent, the separation will be harder to proceed but the peaks will have a greater height and that will result in a better limit of detection. The method in itself will be faster and that is a really powerful economic argument. As the matrix effect is relatively complicated to handle, only the working methods will be presented here.

4.4.2.1 Method 3.1 for L-Glutamine

Column: C18 gravity, Macherey-Nagel, 4,6mm x 150 mm, 3 µm, serial N°: N8090623

HPLC system: Series 1100 Agilent, laboratory f103

- UV detector : G1315A, Serial : DE91606692
- Auto sampler : G1313A, Serial : DE91609213

Wavelength UV detector: 338,4 [nm]

Temperature: 25°C

Eluents: ACN and NaH2PO4 (40mM, pH = 7,8)

Table 7: Eluent composition for the method 3.1

Time [min]	%ACN	%Buffer	Flowrate [ml/mn]	max. pressure [bar]
8	50	50	0.8	300
10	50	50	0.8	300
12	15	85	0.8	300
14	15	85	0.8	300

Starting eluent composing: 15% ACN and 85% Buffer

Stop time: 14 min

In order to have a better understanding of the evolution of the eluent during time, the following figure will help to visualize it:

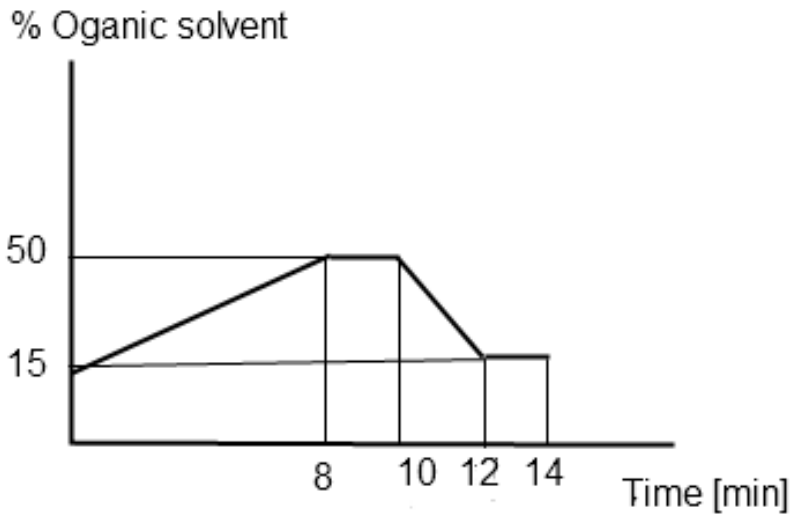

Figure 3: Evolution of the organic solvent for method 3.1

4.4.2.2 Method 3.2 for L-Glu

Column: C18 gravity, Macherey-Nagel, 4,6mm x 150 mm, 3 µm, serial N°: N8090623

HPLC system: Series 1100 Agilent, laboratory f103

- UV detector : G1315A, Serial : DE91606692
- Auto sampler : G1313A, Serial : DE91609213
-

Wavelength UV detector: 338,4 [nm]

Temperature: 25°C

Eluents: ACN and NaH2PO4 (40mM, pH = 7,8)

Table 8: Eluent composition for the method 3.2

Time [min]	%ACN	%Buffer	Flowrate [ml/mn]	max. pressure [bar]
2	15	85	0.8	300
9	60	40	0.8	300
11	60	40	0.8	300
13	15	85	0.8	300

Starting eluent composing: 15% ACN and 85% Buffer

Stop time: 14min

In order to have a better understanding of the evolution of the eluent during time, the following figure will help to visualize it:

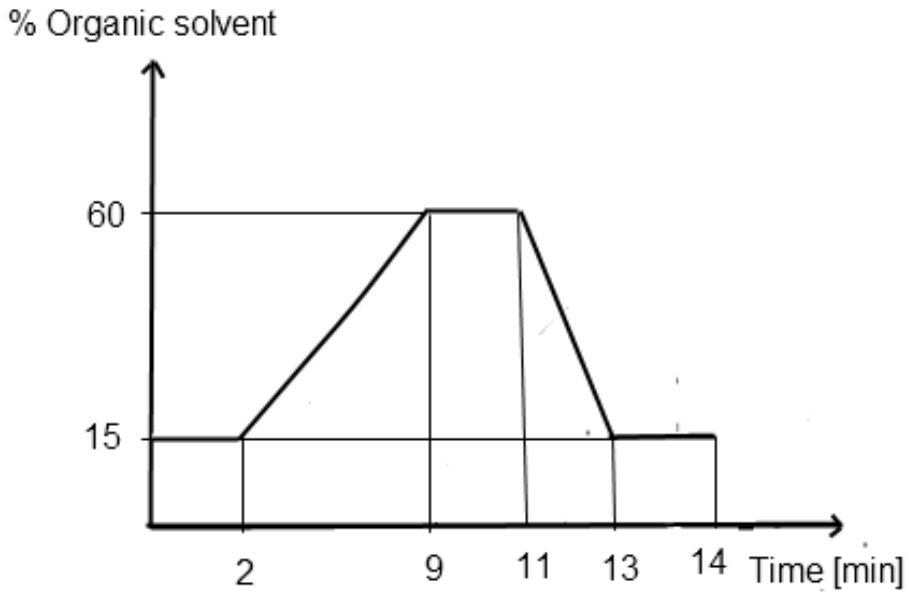

Figure 4: Evolution of the organic solvent for method 3.2

5. Results and discussion

This is the main part of the report, the choice of methods and the method development will be covered here. By worries of comprehensibility, all the steps will be described here in details, but all the specifications about the analytical method itself will only be given in the SOP in appendix.

5.1 Method development

All the methods about the sampling and also about the analytical methods will be covered and discussed here. Once will be able at the end to clearly understand why and which methods are retained in the SOP.

5.1.1 Determination of the sampling method

For the preparation of the sample, it is two different methods described in section 4.3. The only difference between the both methods is the filtration on the 3 kDa filter (see section 4.1) and the dilution after using the centrifuge. As it has been already mentioned earlier, serums as well as samples or medium can contain really big molecules. The main problem with big molecules is that the pre-column used can be relatively quickly blocked. It is the only problem. As the following figure will express, there is no loss in the response of the molecule of interest but only a loss of molecule that cannot go through the pre column. Another interesting thing to explain is the utilization of 20 µl of H2O MQ that will assure the passage of all the molecules of interest. Indeed, it is possible that after the first centrifugation, some of the molecules can be stuck into bigger one. That's why the 20 µl of H2O are used.

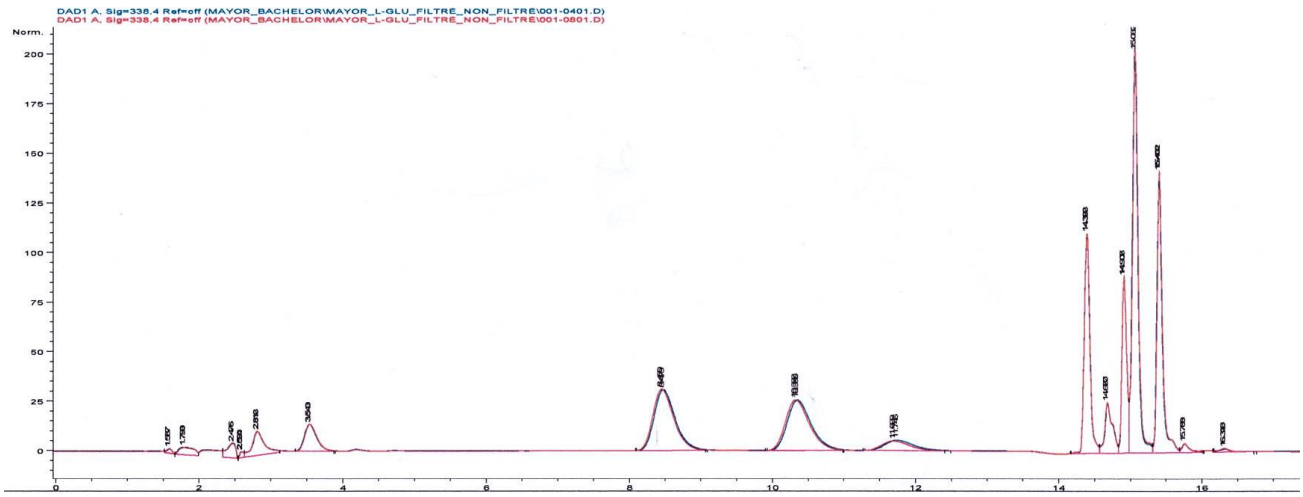

Figure 5: Comparison between the two samples preparation for the SFM4CHO (Gibico 10743011) medium containing L-Glutamine. In red: without filtration on 3kDA filter. In Blue: with filtration on 3kDA.

On the figure 5, once can see no difference in the response of the molecule of interest (~11.8 min). Therefore, the assumption that the big molecules filtered on 3 kDA cannot go through the pre column should be truthful. It is interesting to discuss the two different way of sampling once this assumption is issued.

In the case of method 1 of sampling, the pre column is saved but takes more time for the preparation. On the other hand, method 2 is faster but affects the pre column. At this point, it is interesting to have a little guess:

The price of a pre column is about 100 CHF, and can handle up to twelve injections. So for the method 2, the price of 12 injections is then 100 CHF. It is possible to compare this price to the cost of method 1 for the same amount of injections. As it has been noted, the time needed for the sample preparation is 20 min of centrifuge plus about 7 min for the preparation. Assuming that an operator that can run this method is paid about 60 CHF per hour and the cost of one 3kDA filter is about 5 CHF, it is possible to calculate the price for twelve injections as well. As is it trivial, the calculation will not be given here but only the result: 87 CHF. It appears clearly that the method 1 is cheaper and that is the main argument to choose this method of sampling for the SOP. More details about this method of sampling are given in the SOP in appendix.

5.1.2 Optimization of the OPA reaction

Before go any deeper in the method development of the analytical method itself, it is necessary to discuss the optimization of the reaction of derivatization. In order to optimize the reaction that was mentioned by Jens Olaf Krömer and Michel Fritz (2004) in their publication: “In vivo quantification of intracellular amino-acids and intermediates of methionine pathways in *Corynebacterium glutamicum*”, a fluorescence lector had been used. All different conditions are presented in the following table (on the next page) and also the corresponding figure to the data received.

Table 9: optimization parameters for the OPA reaction

	Bicine + 2ME 5% [ul]	*Glu_1 mg/ml [ul]*	*Bicine 0.5 M [ul]*	*OPA 1,04 mg/0.2 ml [ul]*	*H2O [ul]*	*Somme [ul]*	*Scale down [ul]*
I.1	4	3	6	6	20	39	19.5
	Bicine + 2ME 5% [ul]	Glu_1 mg/ml [ul]	Bicine 1 M [ul]	OPA 1,04 mg/0.2 ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
I.2	4	3	6	6	20	39	19.5
	Bicine + 2ME 5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.1 M [ul]	OPA 1,04 mg/0.2 ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
I.3	4	3	6	6	20	39	19.5
	Bicine + 2ME 5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 1,04 mg/0.2 ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
I.4	6	3	6	6	20	41	20.5
	Bicine + 2ME 0.5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 1,04 mg/0.2 ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
I.5	6	6	6	6	20	44	22
I.5'	4	6	6	6	20	42	21
I.6	4	6	6	6	20	42	21
I.7	6	6	6	6	40	64	32
I.8	4	6	6	6	40	62	31
	Bicine + 2ME 0.5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 1,04 mg/0.2 ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
final 1	4	6	6	6	40	62	31
	Bicine + 2ME 0.5% [ul]	Glu_0,1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 1,04 mg/0.2 ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
final 2	4	6	6	6	40	62	31
	Bicine + 2ME 0.5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 1,04 mg/0.2ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
final 3	4	6	6	6	40	62	31
	Bicine + 2ME 0.5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 0.5 mg/0.2ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
final 4	4	6	6	6	40	62	31
	Bicine + 2ME 0.5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 0.1 mg/0.2ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
final 5	4	6	6	6	40	62	31
	Bicine + 2ME 0.5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA_2,5 mg/0.2ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
final 6	4	6	6	6	40	62	31

As only the results from the final systems will be plotted here, it is interesting to comment this table 9 a little bit. As it can be seen, the experiments I.1, I.2 and I.3 show the effect of different concentration of bicine in the reaction. It appears that a concentration of 0.5 M drives to a better amount of fluorescence. Then, I.4 shows that the concentration of the 2-mercaptoethanol has to be lower than it is in I.1, I.2 and I.3. So, I.5, I.5', I.6, I.7, I.8 are only different concentration of 2-mercaptoethanol using different dilution in order to optimize this parameter.

The following figure will express the final systems that have been retained due to experiments ran before (I.1 to I.8), the system with the more amount of fluorescence will be retained for the derivatization conditions.

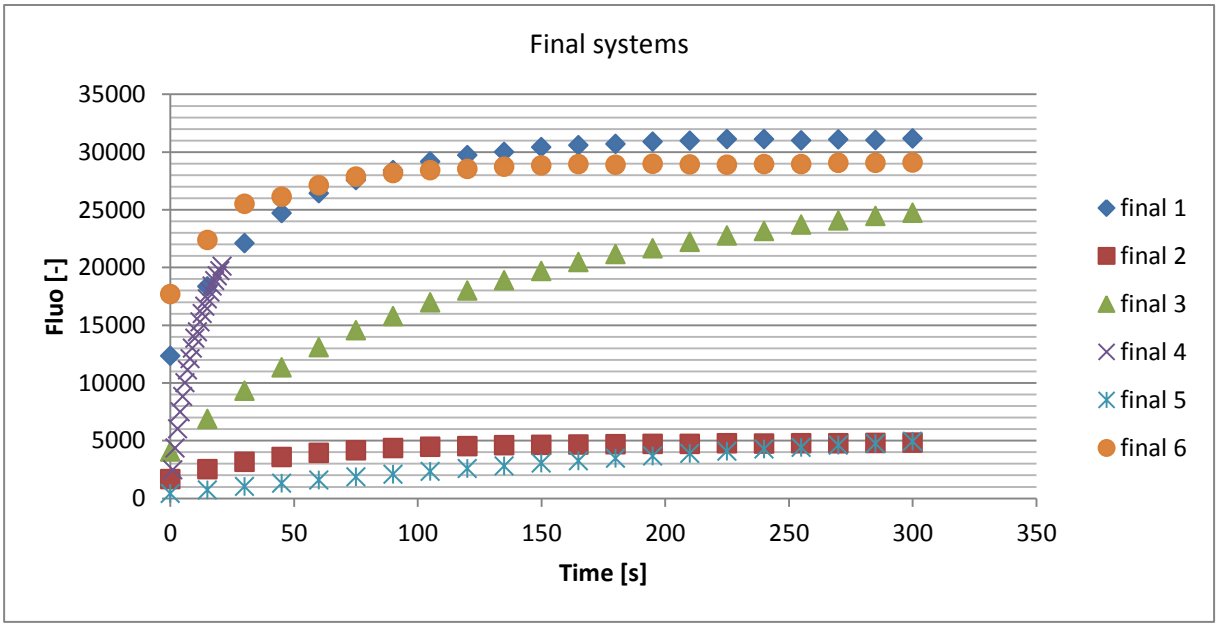

Figure 6: Plot of final systems conditions for the derivatization

The conditions that have been retained are the system final 6. It is visible on the figure 6 that the system final 6 has one of the most amounts of fluorescence. It is also visible that the kinetics of this reaction follows a first order and is really fast. The half time is almost reach during the time that the reagent is put in the box and enters the fluorescence lector. This shows how specific and fast the reaction is. Note that the system called final 4 encountered some troubles that drive to this relatively strange results. Note also that the final 6 parameters drive to a dilution of 10,33 of the sample that will be really important for the further analyzes.

The following table will confirm the data obtained in the table 9. It is actually different HPLC runs with different concentration of OPA used that correspond to systems finals presented in table 9:

L-Glutamine Sig = 338.14 nm					
	Solution stock : 15.98 mg / 10 ml		Area [mAU*s]		
	conc. [mg/ml]	conc. Injection [mg/ml]	OPA 10,47mg/ml	OPA 5,10 mg/ml	OPA 1,06 mg/ml
sol 1	1.598	0.1546	14125.3	12481.56	5296.73
sol 2	0.3995	0.0386	3466.325	3128.086	1570.327
sol 3	0.0998	0.0096	816.402	746.719	383.754
sol 4	0.0249	0.0024	181.637	174.067	60.443
sol 5	0.0062	0.0006	42.996	41.965	22.615

5.1.3 Determination of the wavelength UV detector

In order to define a wavelength for the UV detection, a 3D spectrum of all the absorbance from a sample of SFM4CHO is taken. First of all, this is done in order to confirm that the wavelength used by Jens Olaf Krömer and Michel Fritz (2004) in their publication: "In vivo quantification of intracellular amino-acids and intermediates of methionine pathways in *Corynebacterium glutamicum*" is valuable. It is also done to check the relative purity of the peak, as it was possible to use a DAD detector. The strategy is relatively simple: the 3D spectrum of all the absorbance from 200 to 600 nm will show where the impurity can hide at the elution time of the molecule of interest.

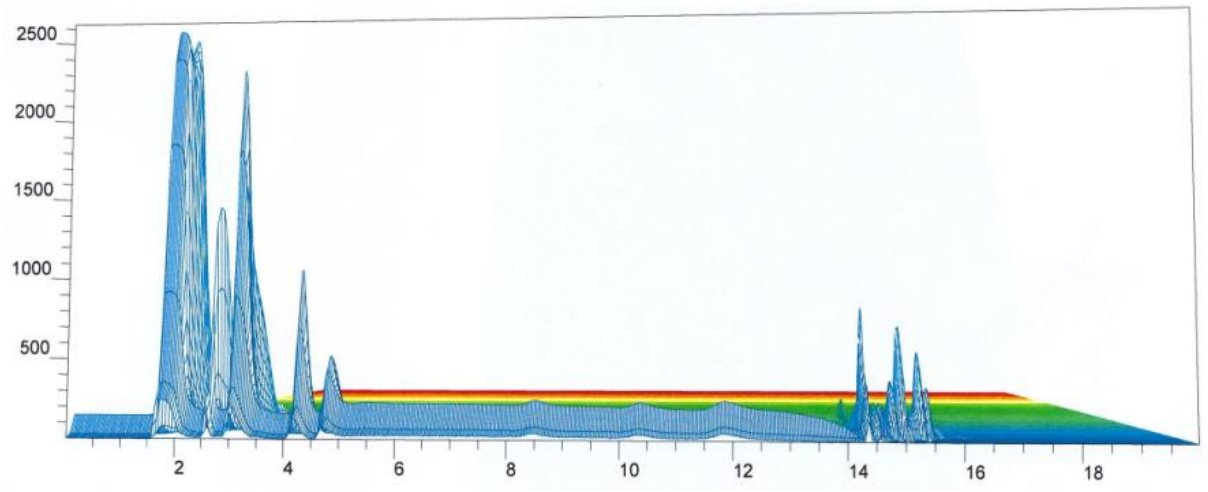

Figure 7: 3D spectrum of 200 to 600 nm of the absorbance for SFM4CHO (Hyclone SH30548) with method 1.1

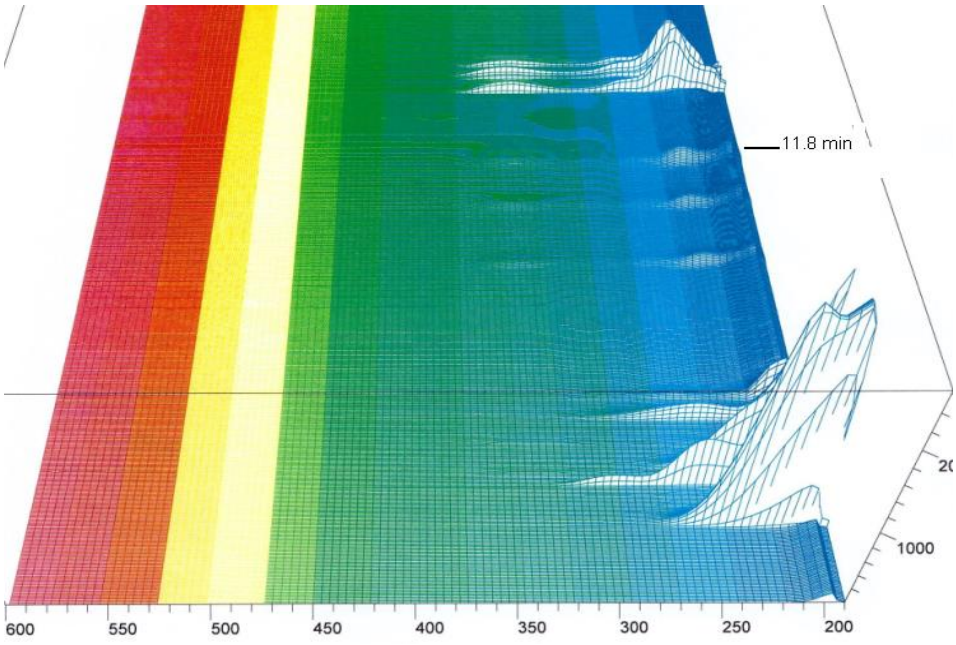

Figure 8: 3D spectrum of 200 to 600 nm of the absorbance for SFM4CHO (Hyclone SH30548) with method 1.1

It is possible to see that the wavelength of 338 is relatively specific and is a good wavelength in order to detect the L-Glutamine (it comes out at about 11.8, figure 8). In the case of the method 1.1 and the L-glutamine, no impurities are detected. It is possible to fix the wavelength at 338 and will empty the chromatogram from all the peaks that come out from 200 to 300 nm.

For the method 1.2, it is almost the same results as it is the same medium and serum that was analyzed. The following figures represent the 3D spectrum of same wavelengths that had been used for figure 7 and 8.

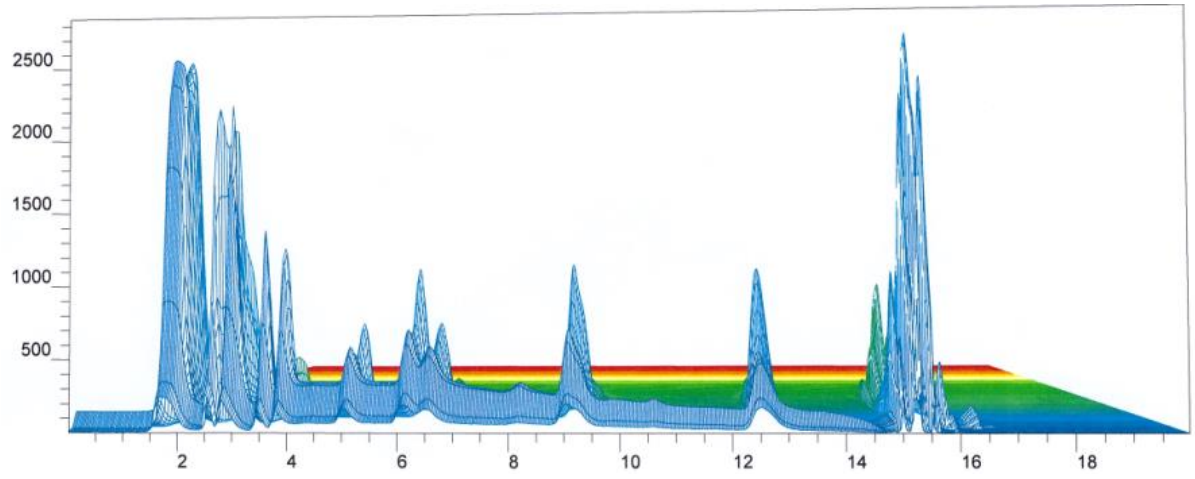

Figure 9: 3D spectrum of 200 to 600 nm of the absorbance for SFM4CHO (Hyclone SH30548) with method 1.2

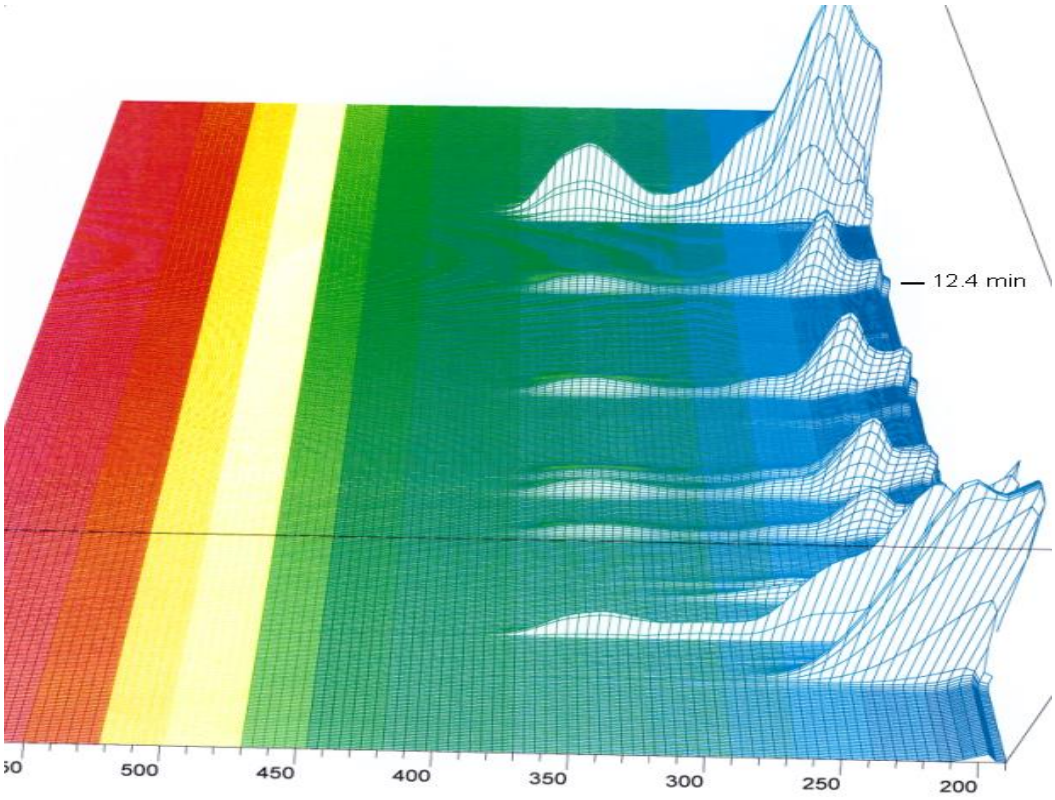

Figure 10: 3D spectrum of 200 to 600 nm of the absorbance for SFM4CHO (Hyclone SH30548) with method 1.2

The molecule of interest is detected at about 12.4 min, as for the figure 7 and 8, it can be seen that the choice of a wavelength of 338 nm is a relatively good choice. And that, for the same reasons that was stated before, in an optic of refining the chromatograms.

5.1.4 Determination of the best separation method

As seen above, several methods had been developed and the next step is to define which of these methods of separation are the most appropriate for the system. All the methods will be described in more details with some chromatogram to illustrate them. Then, the method chosen will be discussed with more precision.

The first two methods that are going to be discussed are the method 2.1 and the method 2.2. As seen above, the only difference with the method 1.1 and 1.2 are the organic solvent that is used. For the case of 1.1 and 1.2, acetonitrile has been used and for the others, it was methanol. Both methods provide a good separation with decent resolutions. The main problem that was encountered was the methanol potential to change significantly the viscosity of the eluent. And this drives to a really strong difference on the pressure during the phase 2, 3 and 4 of the figure 2. As we can see on table 3, 4, 5, 6, 7, 8, the max pressure is 300 Bar. For this reason, and as many experimental problems were encountered about the max pressure, the method 2.1 and 2.2 are not retained. For example, during the phase 3 of figure 2, for a flow rate of 0.8 ml/min, the pressure reached 280 Bar. Hence, these two methods were not retained in the optic of plotting an HEPT experimental curve. Indeed, the assumption that the pressure of higher flow rate with the same conditions would exceed 300 Bar.

In comparison to the methods 2.1 and 2.2, methods 1.1 and 1.2 do not have this problem which is a relatively good point for these methods. It was no differences between the statistics calculated for the both methods but only this problem of viscosity. That's why the statistics of the methods 2.1 and 2.2 as well as their chromatograms will not be given here as they have no real interests. Therefore, methods 2.1 and 2.2 will be eliminated for this purely practical reason.

The following figures present the different chromatograms obtained with all medium and serum for the methods 1.1.

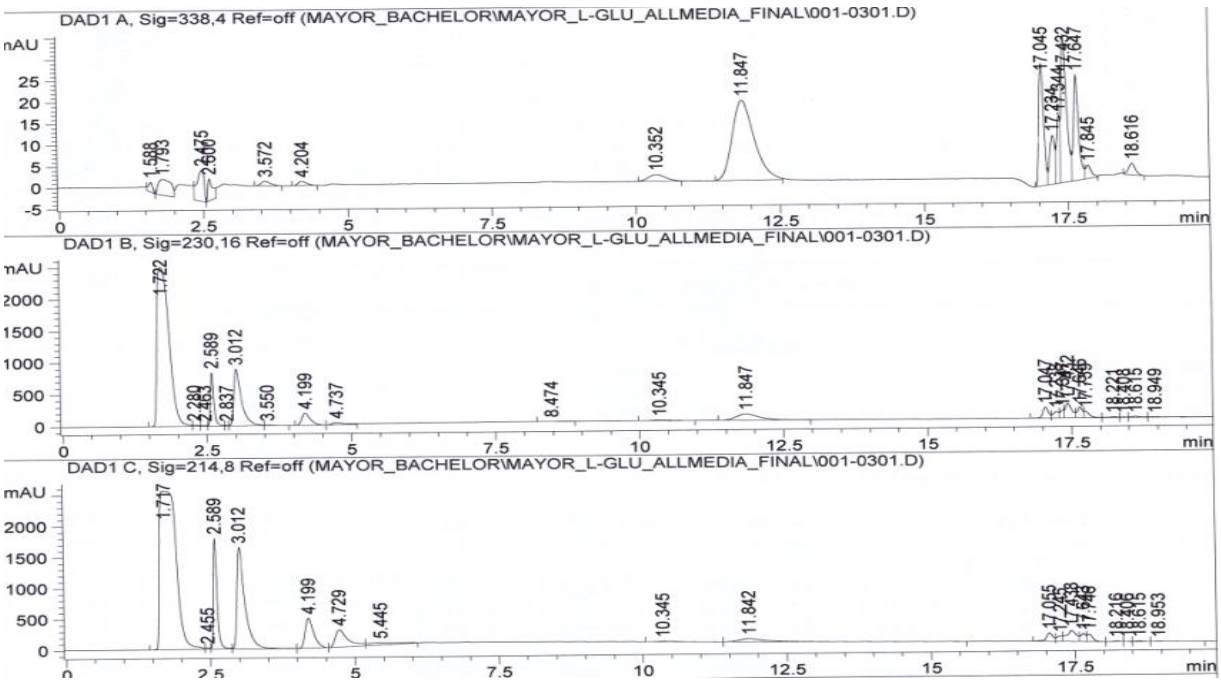

Figure 11: L-Glutamine with DMEM/Ham'F12 (Sigma D6421) with 10% FCS using method 1.1

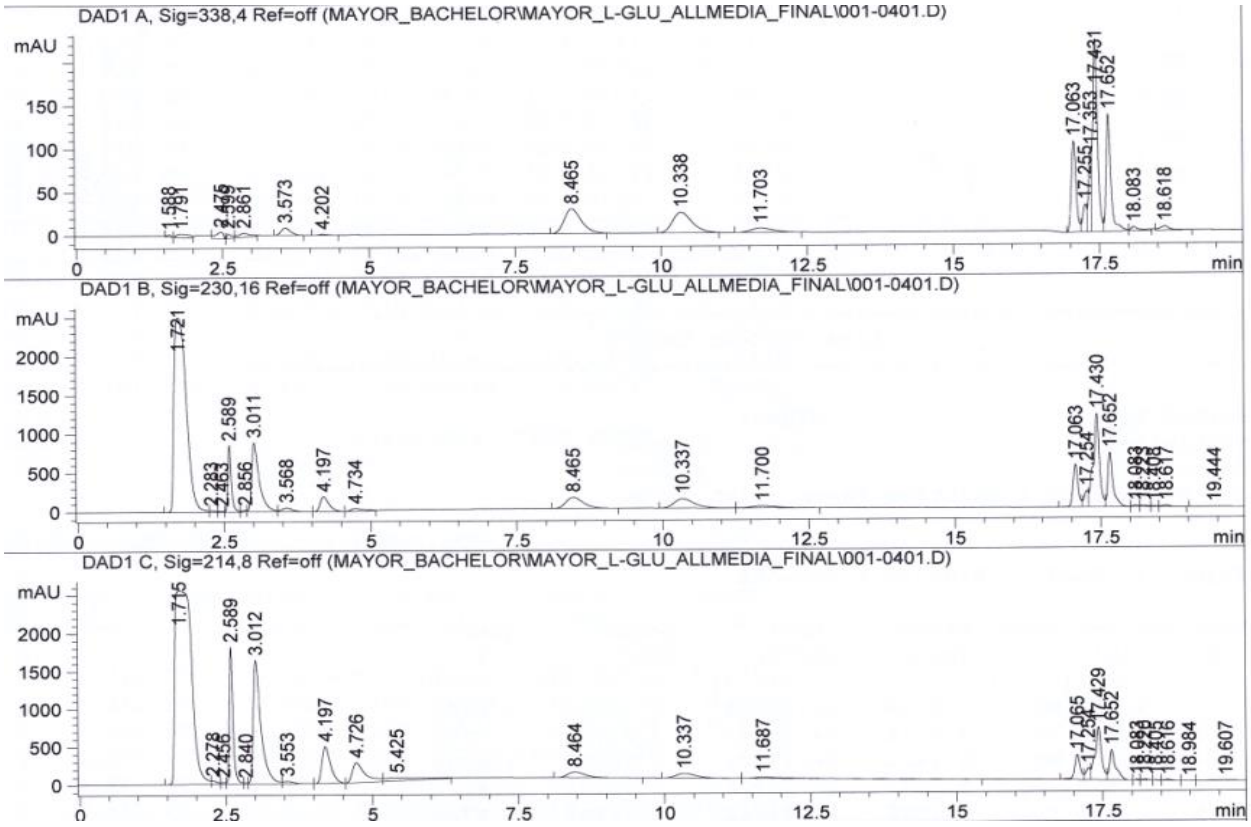

Figure 12: L-Glutamine with CDCHO using method 1.1

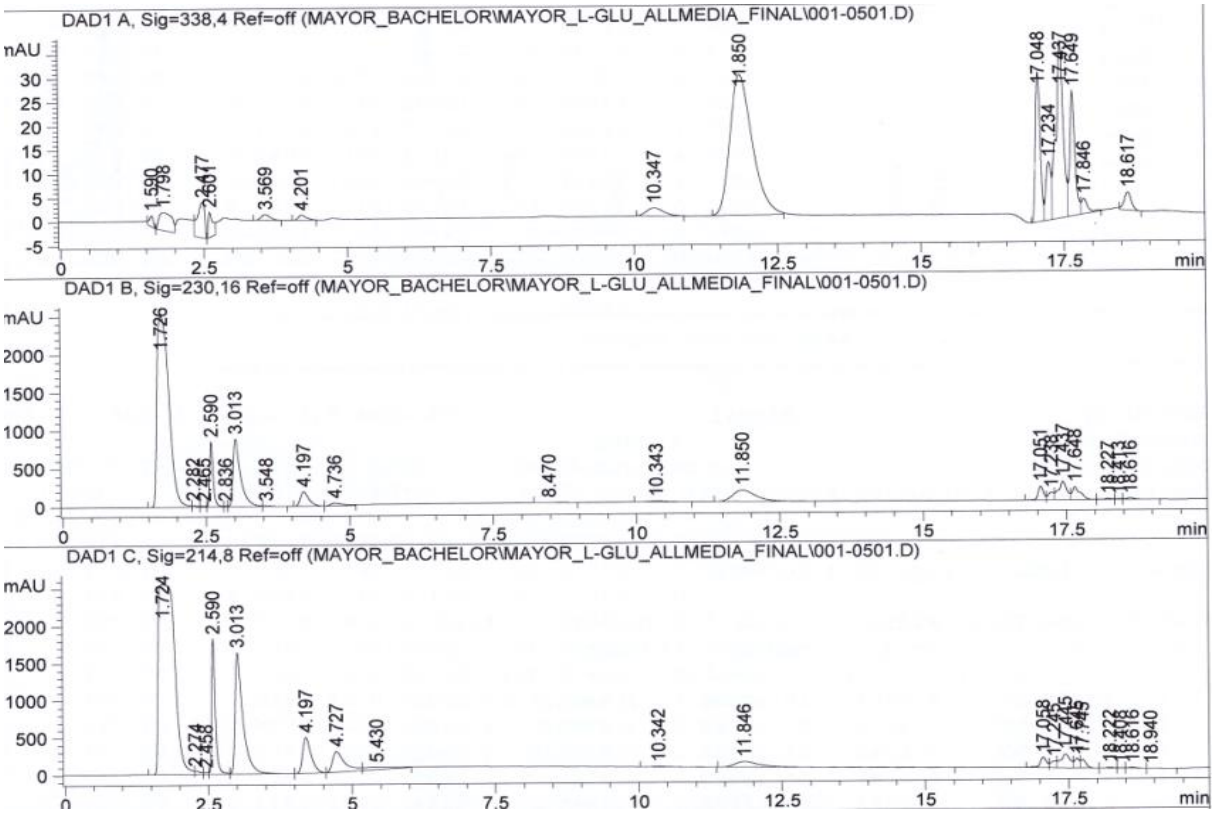

Figure 13: L-Glutamine with DMEM/Ham'F12 (Gibico 10743011) with 10% FCS using method 1.1

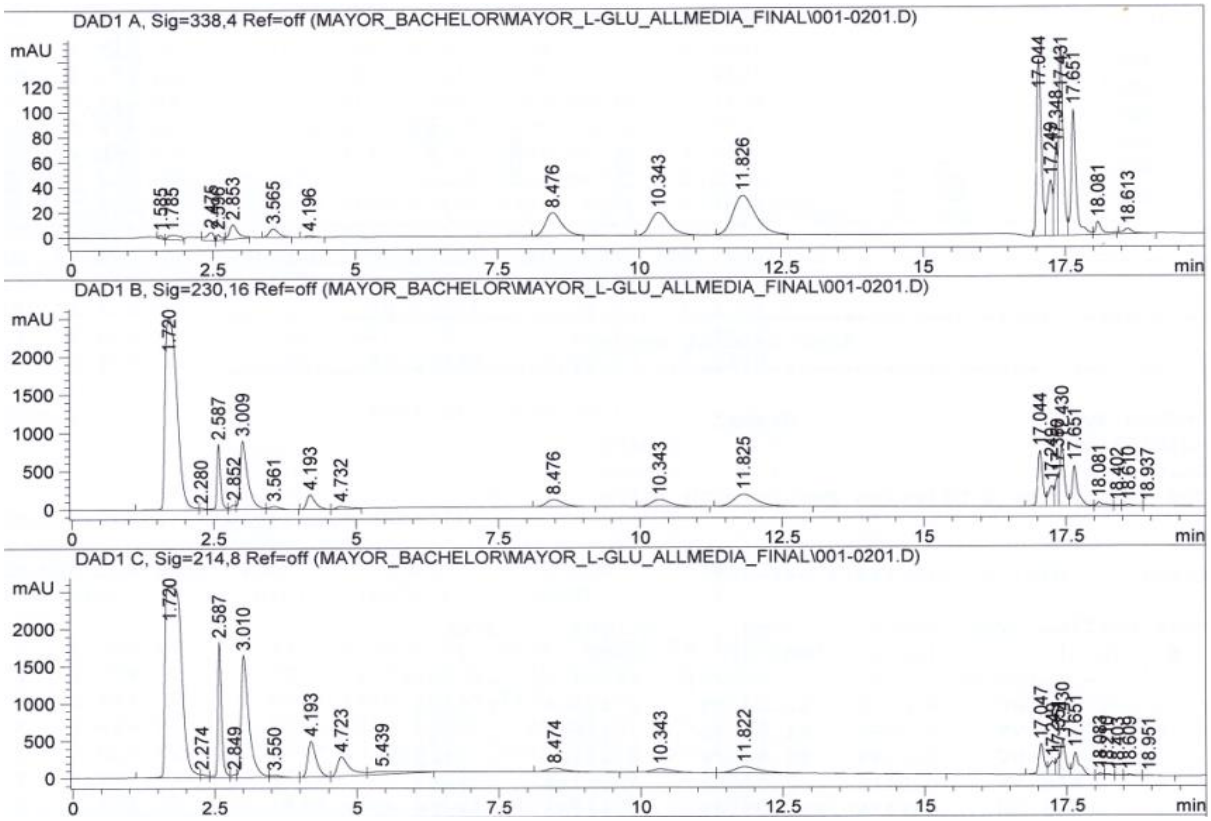

Figure 14: L-Glu tamine with SFM4CHO (Hyclone SH30548) using method 1.1

The retention time and the resolutions for each medium are related in the next table:

Table 10: retention time statistics for all media and resolution from method 1.1

L-Glu_method 1.1	R_t [min]	R_s
DMEM/Ham'F12 (Sigma D6421)	11.847	4.24
CDCHO	11.703	3.57
DMEM/Ham'F12 (Gibico 10743011)	11.85	4.06
SFM4CHO (Hyclone SH30548)	11.826	3.85
Average [min]	11.8065	
SD_t_R [min]	0.06046693	
RSD_t_R[%]	0.51214952	

From a purely experimental point of view, method 1.1 could be validated for all medium with L-glutamine as subtract. The next point will be the optimization of the flow rate with the calculation of the number of theoretical plates (N) that will result in a plot giving the HEPT curve. It will be treated in the further discussion.

The chromatograms for the methods 1.2 for all medium are also given in the following figures:

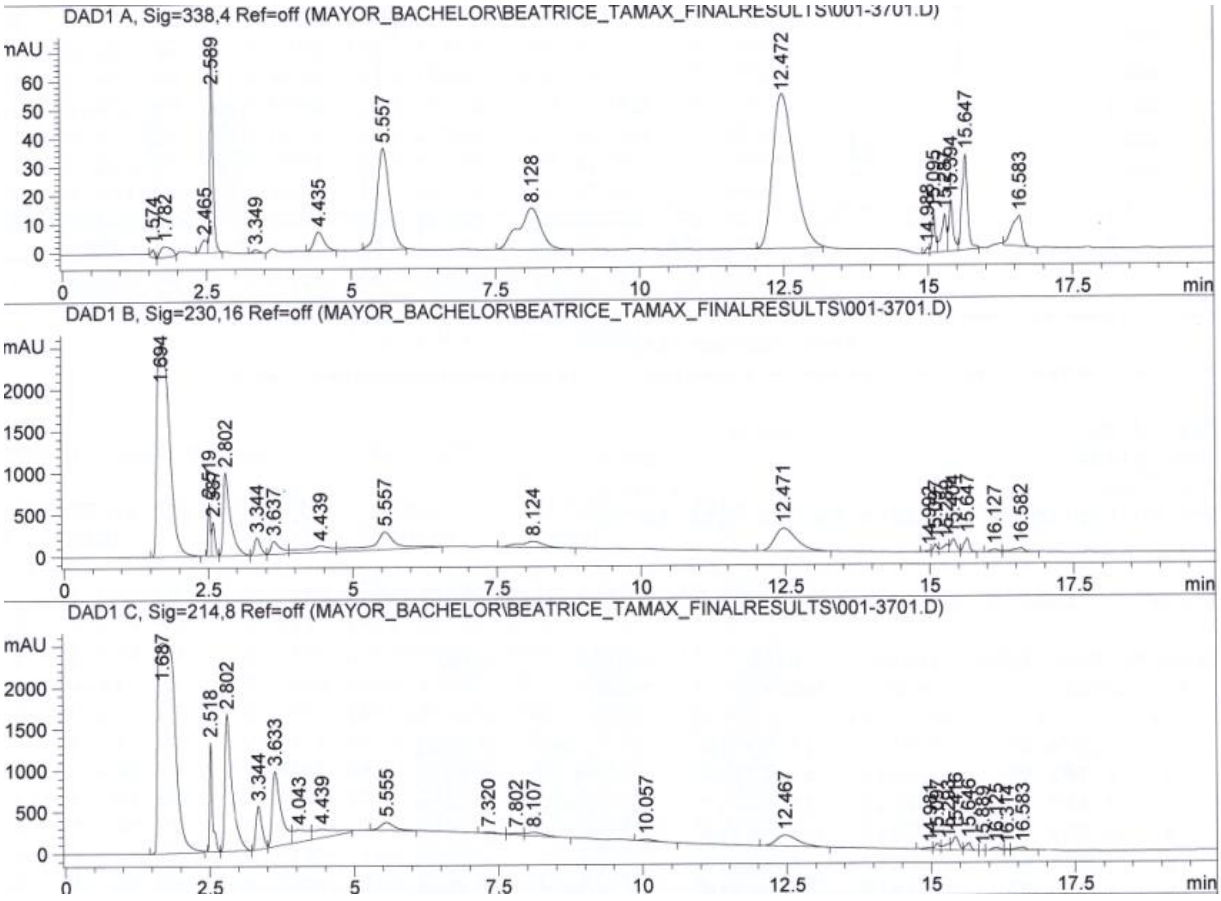

Figure 15: Glutamax^{tm} with DMEM/Ham'F12 (Sigma D6421) using method 1.2

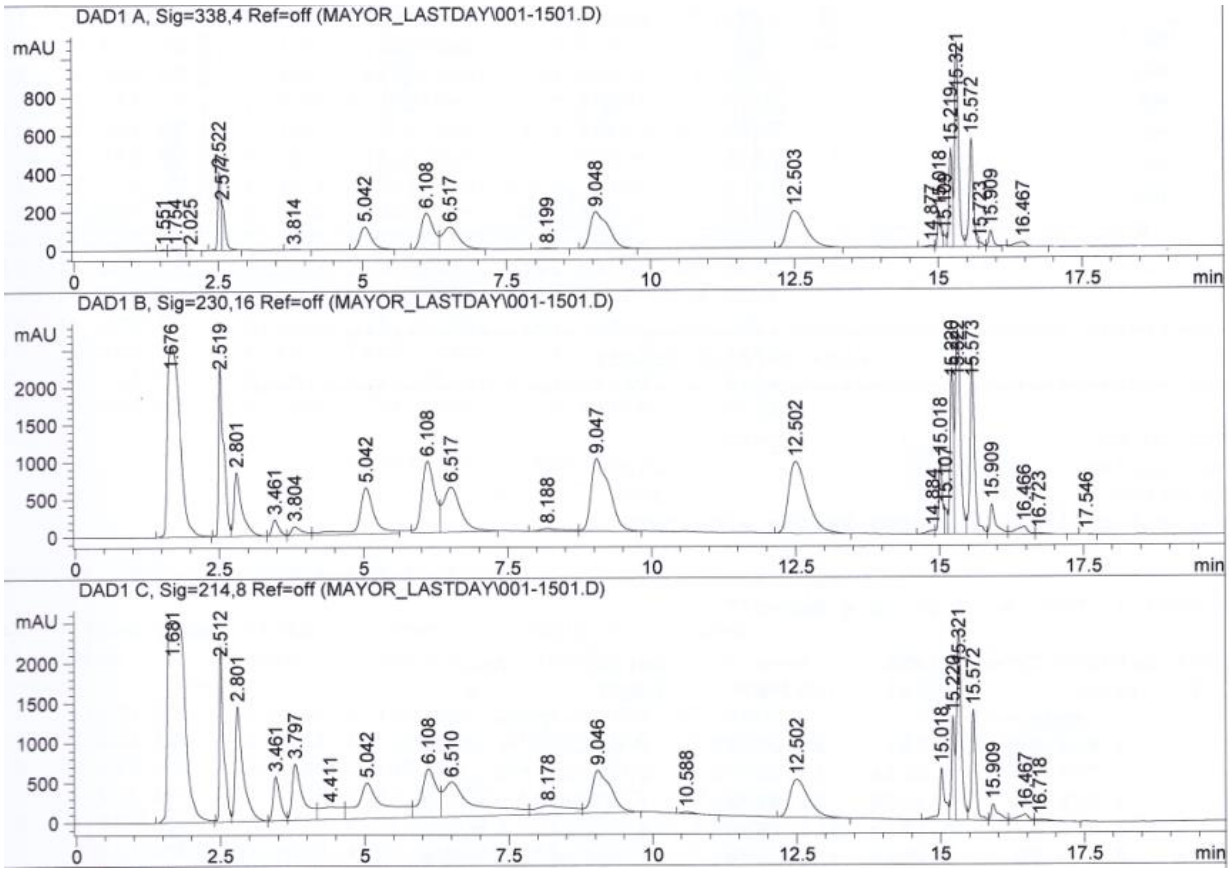

Figure 16: Glutamax^{tm} with SFM4CHO (Hyclone SH30548) using method 1.2

The statistics about retention times for both medium is presented in the following table. Note that the resolution has not been calculated due to the absence of any peaks near the retention time of the molecule of interest in the UV region.

Table 11: retention time statistics for all medium using method 1.2

Glutamax^{tm}_method 1.2	t_R [min]
DMEM/Ham'F12 (Sigma D6421)	12.503
SFM4CHO (Hyclone SH30548)	12.472
Average	12.4875
SD_t_R	0.0155
RSD_t_R[%]	0.12412412

The reason why the organic solvent is not higher is because with a FLD detector, it is possible to detect an impurity as we will see on the next figure:

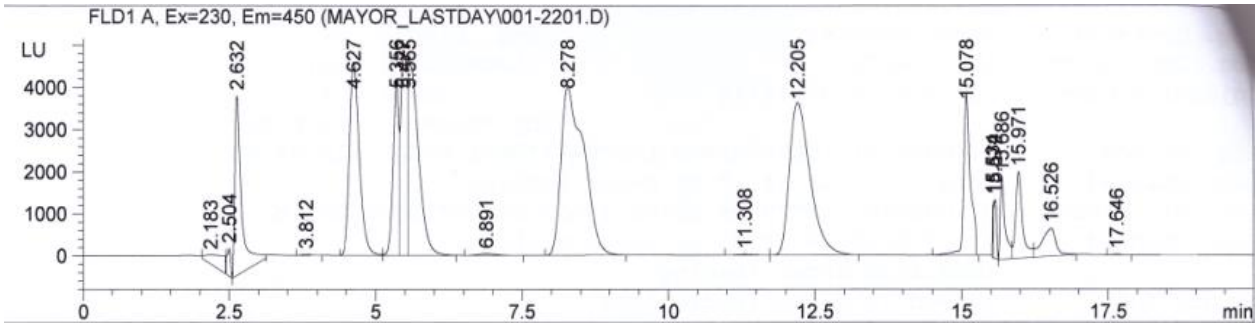

Figure 17: FLD detection (Ex=230, Em=450) of Glutamax^{tm} with SFM4CHO (Hyclone SH30548) using method 1.2

The resolution calculated between these two peaks is 2.6, that is acceptable. It would be also accepted to work with a higher amount of acetonitrile as the impurity peak represents only 0.6% of the peak area of the molecule of interest. By worries of having some changes in the mediums or in the serum used in the future, a safe separation was preferred.

At this point, it is a good to make a recapitulation of what it has been seen until now. We have discussed the difference between the method 1.1 and 1.2 versus the method 2.1 and 2.2. It has appeared that the method 2.1 and 2.2 have some practical problems that drive to the abandon of these two methods even if they present the same quality of separation that 1.1 and 1.2 do have. In order to push the discussion a little bit forward, it is a good time to discuss the two last methods involving a gradient separation.

The method 3.1 as well as the method 3.2 involve a gradient separation which has the effect of refining the peaks and will result in a better detection limit. The problem of this kind of separation is the lower robustness. Assuming the method should be robust and able to adapt on different HPLC, an isocratic separation is preferred. Note that is not the only reason, indeed some change in medium and serum can happen and the method will not be able to adapt. However, this is still interesting and the results about the two methods developed will still be given and discussed briefly. The following figures will present some results using these methods for L-glutamine only.

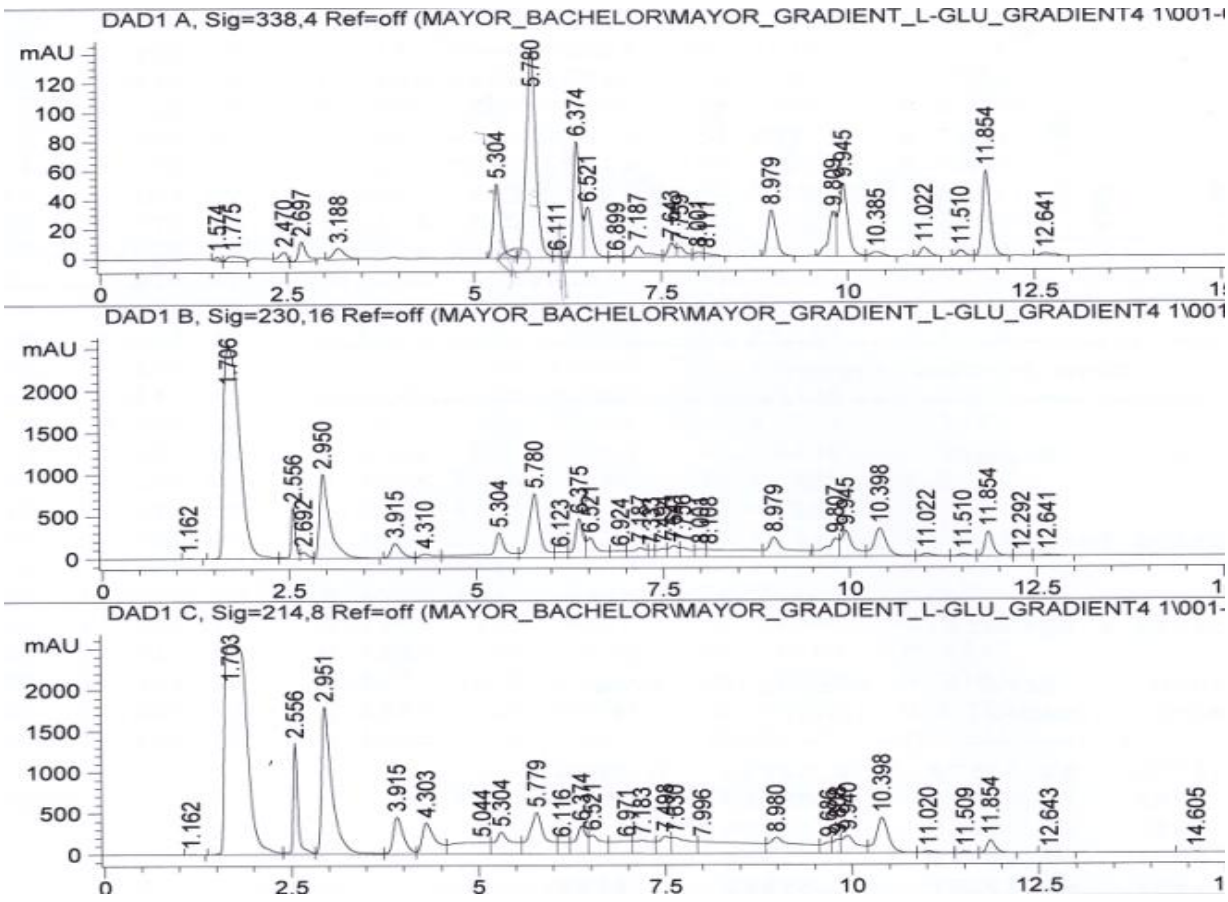

Figure 18: L-Glutamine with SFM4CHO (Hyclone SH30548) using method 3.1

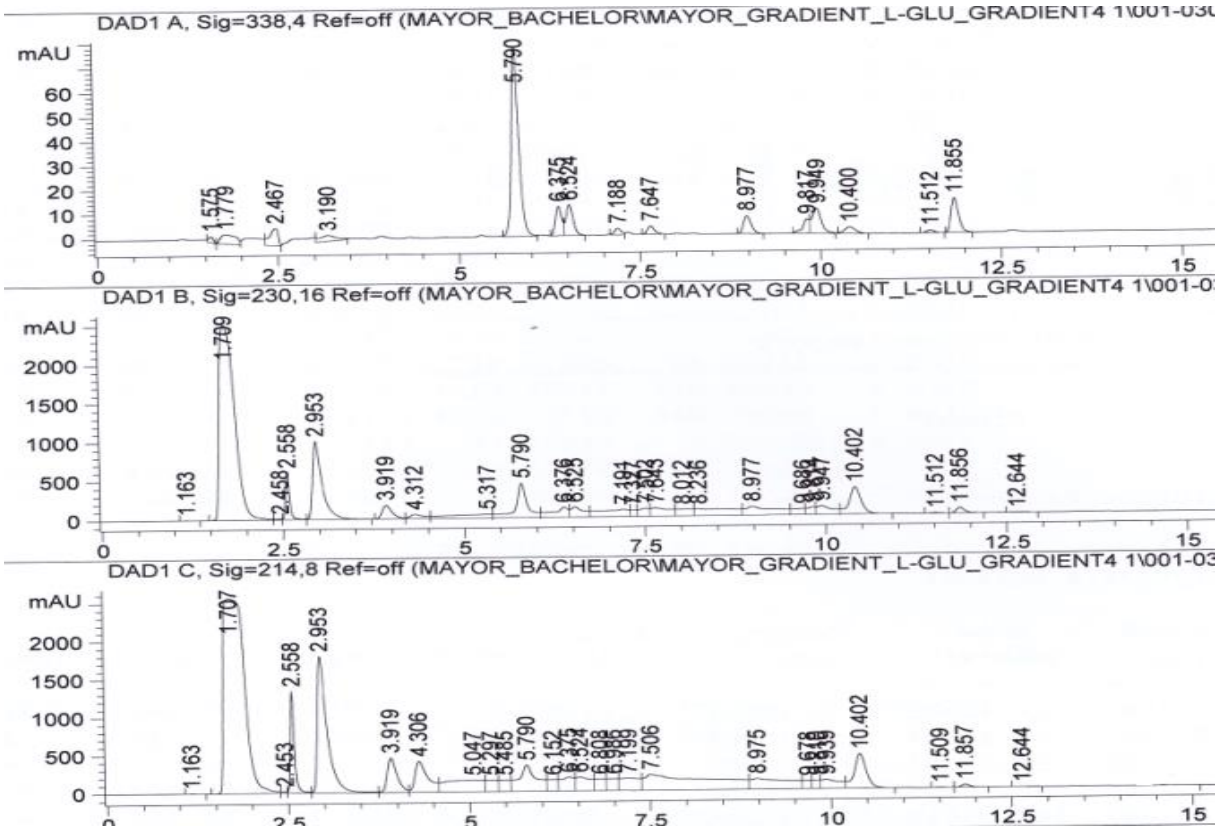

Figure 19: L-Glutamine with DMEM/Ham'F12 (Sigma D6421) with 10% FCS using method 3.1

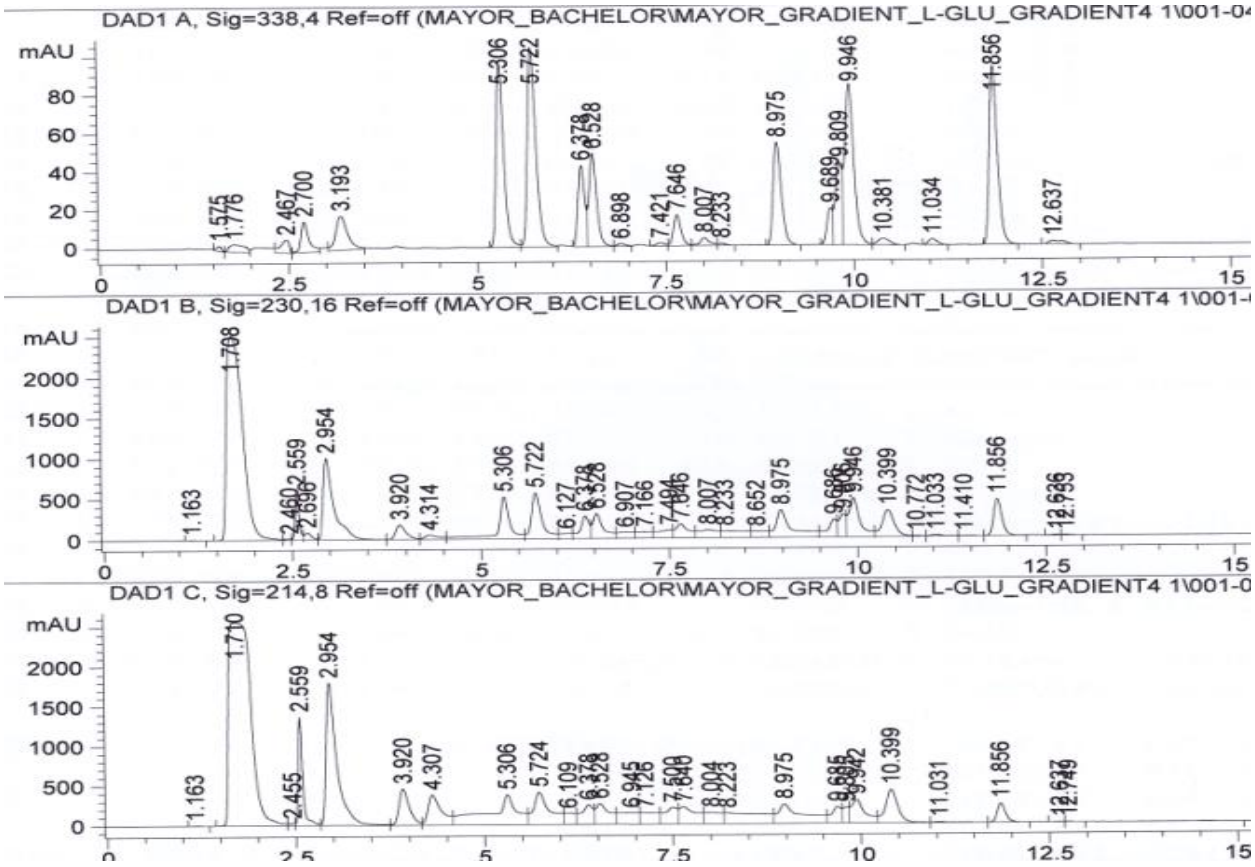

Figure 20: L-Glutamine with CDCHO using method 3.1

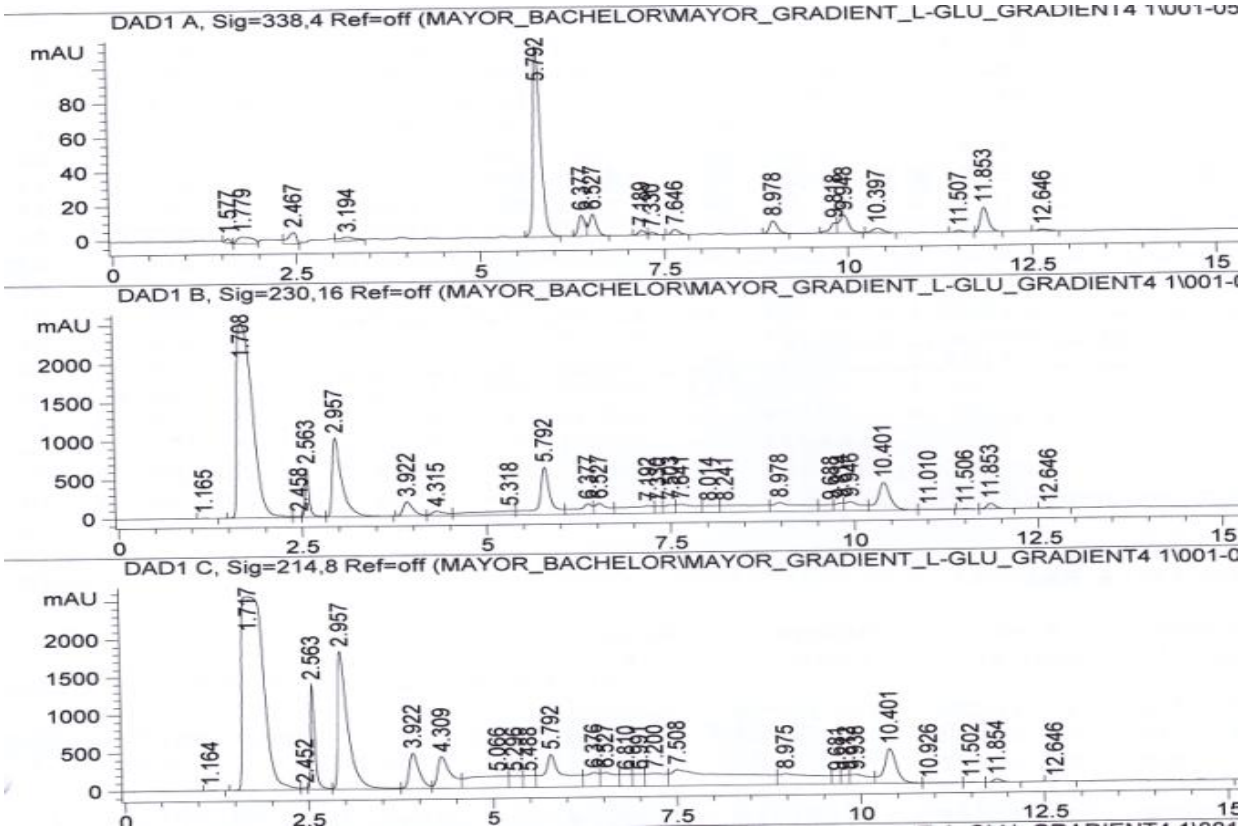

Figure 21: L-Glutamine with DMEM/Ham'F12 (Gibico 10743011) with 10% FCS using method 3.1

The statistics about the retention time and the resolutions for the method 3.1 are presented in the following table:

Table 12: retention time statistics for all media and resolution from method 3.1

L-Glu_method 3.1	R_t	R_s
DMEM/Ham'F12 (Sigma D6421)	5.79	2.36
SFM4CHO (Hyclone SH30548)	5.78	1.49
CDCHO	5.722	1.51
DMEM/Ham'F12 (Gibico 10743011)	5.792	2.32
Average	5.771	
SD_t_R	0.03308575	
RSD_t_R[%]	0.57331047	

As it is possible to see on the table 11, the RSD relative to the retention time is only 0.5%. On the others hand, the resolution calculated for the method 3.1 is still usable but as it has been already said above, any little change in mediums or serums can fast drive into some problems of the separation of the molecules that the method want to separate. However, this method is still performable in an economic optic. But once again, the accent had been put on the separation itself and not on pure statistics and economic pressure.

For the method 3.2, the approach is relatively different. As we can see on the figure 3, it starts with an isocratic mode for 2 min before it goes to a gradient mode. As it is less interesting for the report as one medium does not separate decently, only one example of chromatogram will be given here.

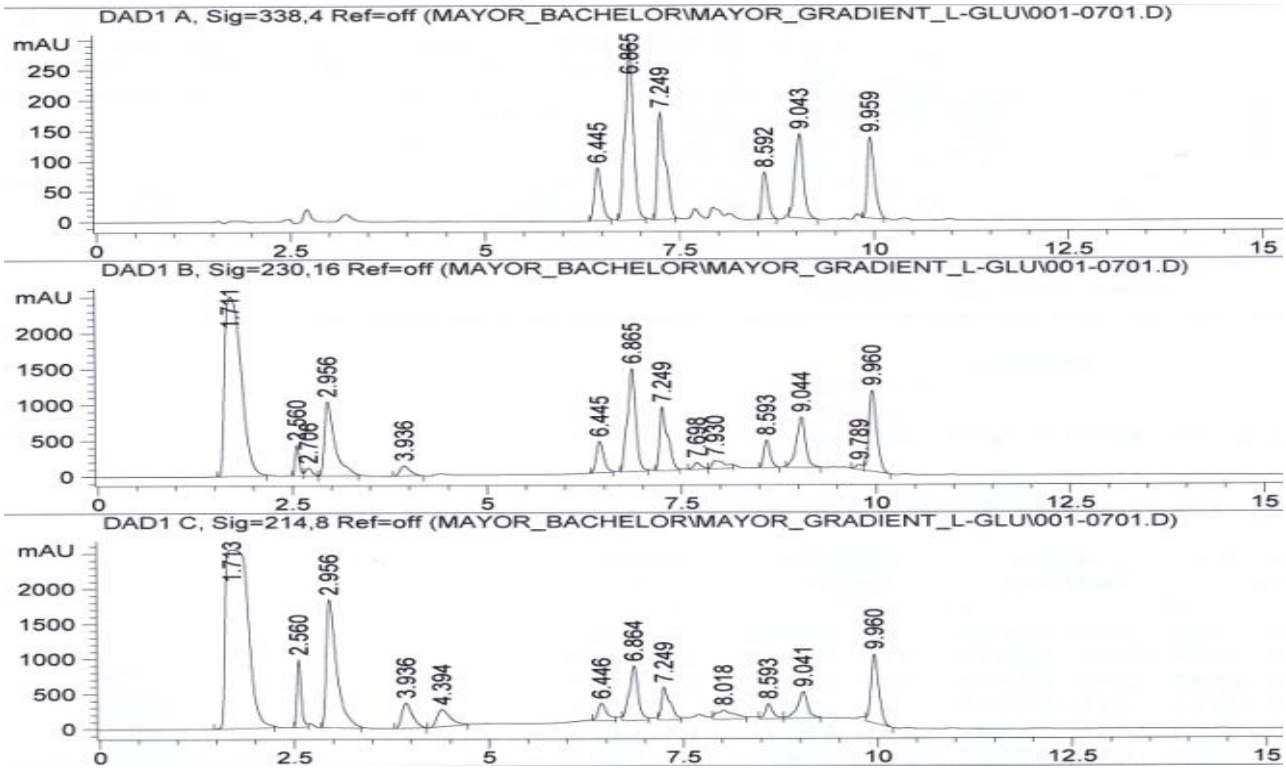

Figure 22: L-Glu with SFM4CHO (Hyclone SH30548) using method 3.2

Once again, all the methods that are presented here are viable. All the statistics are decent and the only argument that will force to make a choice is the quality of the separation. As it had been already said, the method 2.1 and 2.2 are not viable from an experimental point of view. And also from the fact that generally it is not used to work with a higher pressure than 300 Bars. For the method 3.1 and 3.2, their elegance is not enough to fill in the problem that could be encountered with any changes in the matrix. So, the most safe and reproducible choice to make is to retain the method 1.1 and 1.2.

Note also that because of an experimental consideration, the gradient could not go higher than 60% for the simple reason that the buffer could precipitate a bit when it goes more than 60% of organic solvent.

5.1.5 Determination of the experimental HEPT Curve

The final step in order to optimize methods 1.1 and 1.2 that had been developed is to plot the HEPT curve. This curve expresses the theoretical plate height (H) in function of the flow rate and represents the Van Deemter plot that is the sum of three different functions. In order to do this, it is necessary to calculate the number of theoretical plates (N). All is presented in the following figures:

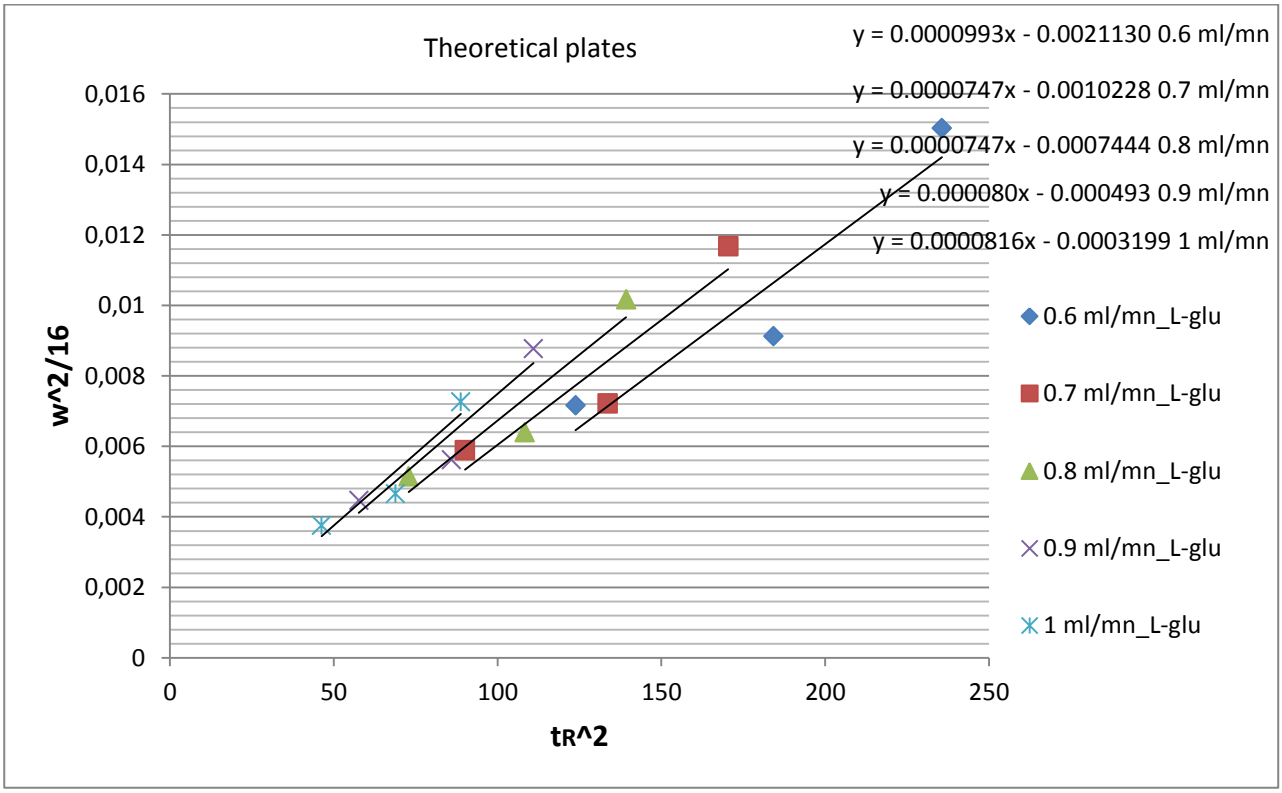

Figure 23: Theoretical plates for method 1.1

Table 13: Number of theoretical plates for method 1.1

Flow rate [ml/min]	N	H [cm]
0.6	10070	0.0014895
0.7	13386	0.0011205
0.8	13210	0.0011205
0.9	12500	0.0012
1	11627	0.00129

Once we know the number of theoretical plates (N), it is possible to plot the HEPT curve and the following figure will express it for the method 1.1.

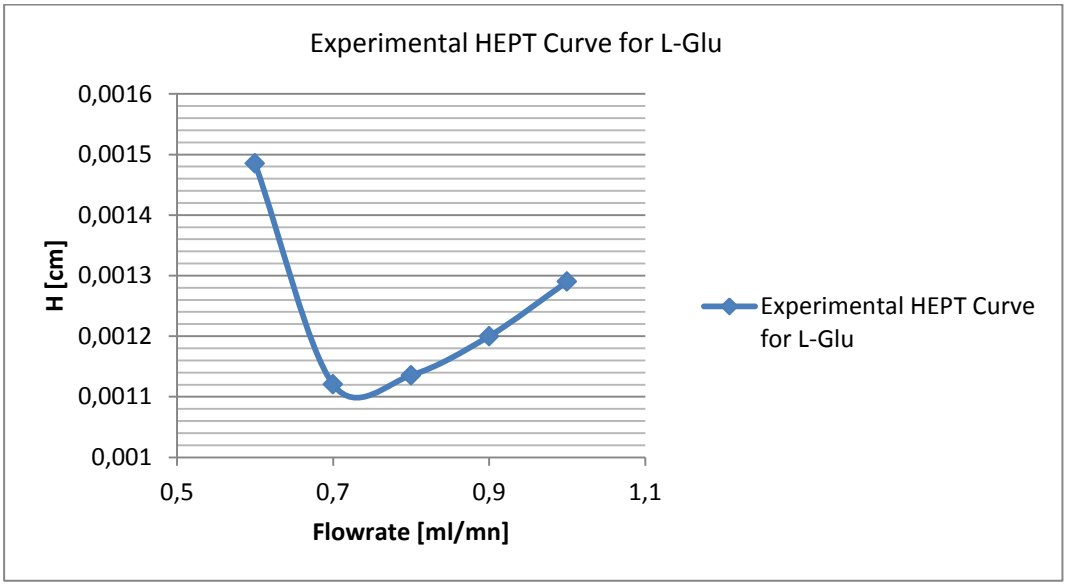

Figure 24: Experimental HEPT Curve for L-Glu with method 1.1

The same calculation has not been done for the method 1.2 because it was not possible to calculate the number of theoretical plates in the same way it was done for the method 1.1. Indeed, as it can be seen on figure 11 and 12, there is no peaks available near the peak of Glutamax™. But the theoretical plates can be calculated for the peak only.

Table 14: Number of theoretical plates for method 1.2

Flowrate [ml/mn]	N	H [cm]
0.6	9710	0.001544
0.7	11846	0.001266
0.8	11533	0.001300
0.9	10875	0.001379
1	10454	0.001434

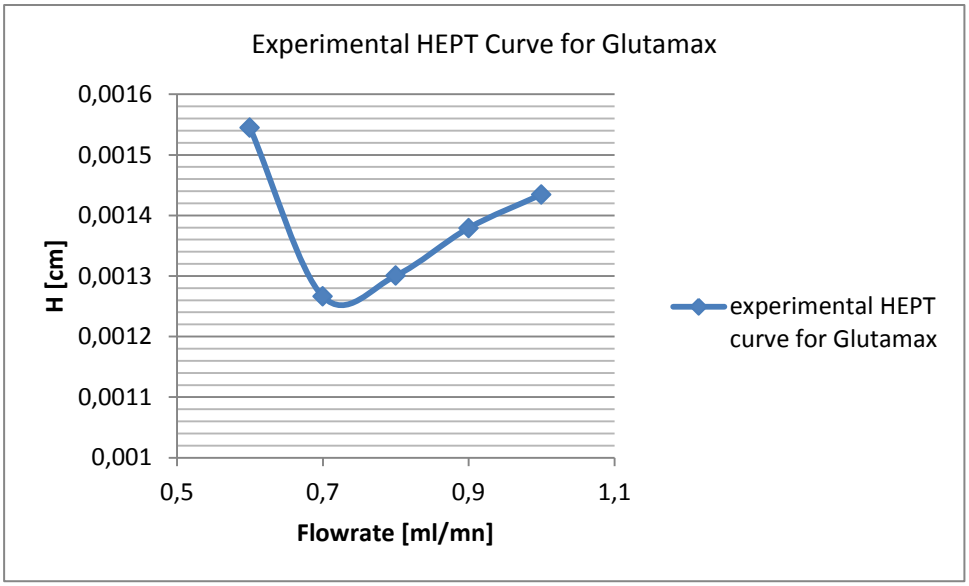

Figure 25: Experimental HEPT curve for L-Glu with method 1.1

We can see on figure 21 and 22 that the optimal region of the curve is between 0,7 and 0,8 ml/mn for the method 1.1 and it is 0,7 for the method 1.2. A flow rate of 0.8 ml/min is then fixed for both methods. It is interesting to think about the parameters that can influence the value of H. As is it relative to the peak shape and width, it is assumable that it will depend on the diameter of the column as well as the diameter of the particles in the column. Its length will also influence it and as it has been shown above, the flow rate will also play a role. More generally it depends of the quality of the column.

5.2 Methods requirements

In this section, only the results from the needed requirements are presented.

5.2.1 Specificity

As it has been already said, the methods 1.1 and 1.2 are specific for each medium containing each serum.

5.2.2 Range

The range that was needed to cover is 0.015 – 1.2 mg/ml for L-glutamine and 0.0217 – 1,7mg/ml for the Glutamax™. Note that the samples are diluted 34.1 times before the injection. Following figures will show examples of calibrations used for method 1.1 and 1.2:

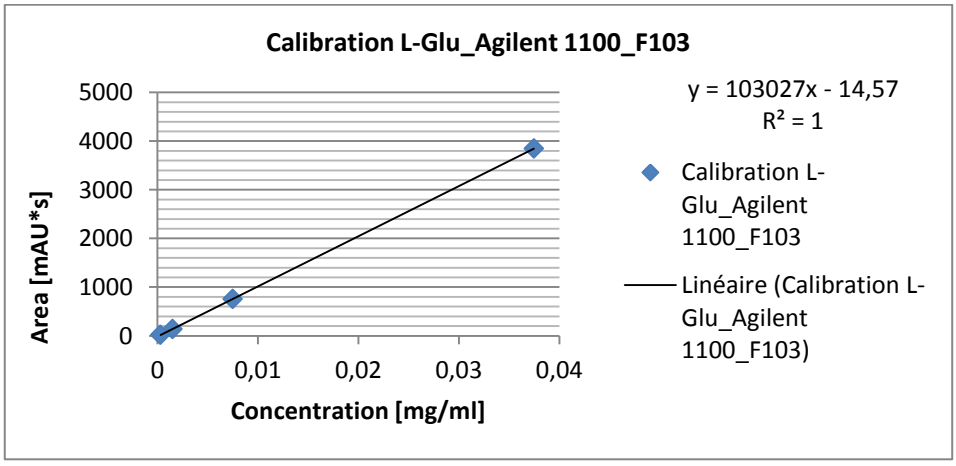

Figure 26: Calibration cruve for L-Glutamine_Agilent 1100_F103 using area.

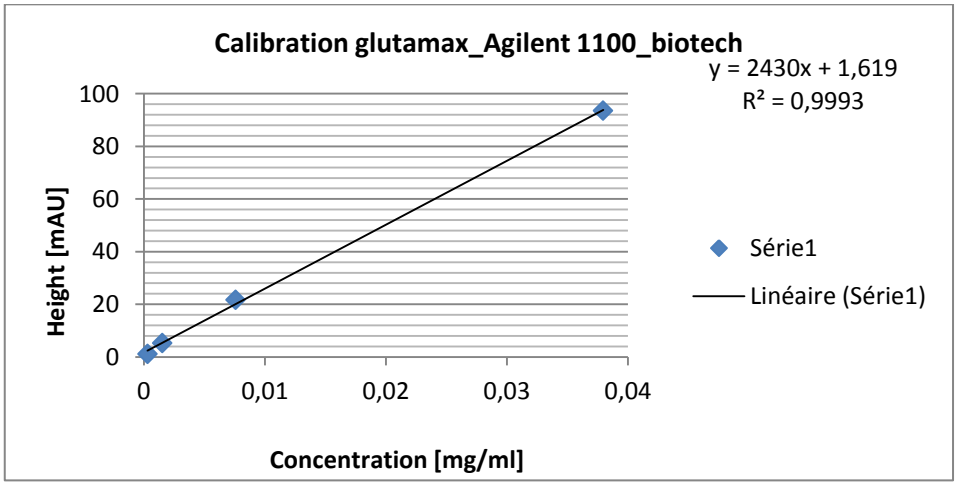

Figure 27: Calibration curve for Glutamax™_Agilent 1100_F103 using height.

5.2.3 Accuracy

For this part, two solutions of known concentrations had been used in order to calculate the recovery. It is acceptable to have an accuracy of +/- 10%.

Table 15: accuracy for method 1.1

Precision L-Glu (n=3)				
concentration : 57,11 mg / 100 ml = 0.5711 mg/ml				
concentration th. After dilution :			0.01842	mg/ml
Area [mAU*s]			Average	
1700.424	1688.57971	1690.5477	1693.183	
concentration calculated :		0.01823	mg/ml	
% recovery		98.97		

Table 16: accuracy for method 1.2

Precision Glutamax^{tm} (n=3)				
solution : 1485 ul / 100 ml. Glutamax^{tm} (MW = 217.22, Conc. = 200 mM).				
Concentration th. after dilution :			0.02081	mg/ml
Area [mAU*s]			Average	
1356.325	1360.32	1361.254	1359.29967	
concentration calculated:		0.02024	mg/ml	
% recovery		97.26		

The recovery calculated for each method is less than +/- 10%. Note that it will be the value used in the SOP by safety.

5.2.4 Precision

Table 17: repeatability for method 1.1

	R_t [min]	**Height [mAU]**	**Area [mAU*s]**
n=1	5.77	1780.067	98.190
n=2	5.88	1797.776	98.528
n=3	5.85	1843.527	98.632
n=4	5.78	1798.979	98.663
n=5	5.66	1774.429	98.744
n=6	5.65	1768.367	98.726
Average	5.77	1793.857	98.580
SD	0.106	27.299	0.206
RSD	1.846	1.521	0.209

There is no relative standard deviation that exceeds 5%. This requirement is also respected.

5.2.5 LOD of the methods

Table 18: LOD for the method 1.1

L-glu DAD Sig = 338.14					
Solution stock : 15.98 mg / 10 ml					
	conc. [mg/ml]	conc. Injection [mg/ml]	mole/litre	Area [mAU*s]	Height [mAU]
solution 1	1.598	0.15465015	0.001058233	14125.3	415.055
solution 2	0.3995	0.038662538	0.000264558	3466.325	115.641
solution 3	0.099875	0.009665634	6.61396E-05	816.402	32.01
solution 4	0.02496875	0.002416409	1.65349E-05	181.637	7.64
solution 5	0.006242188	0.000604102	4.13372E-06	42.996	1.758
solution 6	0.003121094	0.000302051	2.06686E-06	19.842	0.8389
solution 7	0.001560547	0.000151026	1.03343E-06	9.546	0.3856
solution 8	0.000780273	7.55128E-05	5.16715E-07	4.264	0.196
			LOD :	**0.00052**	**mM**

Table 19: LOD for the method 1.2

Glutamax™ DAD Sig = 338.14					
Solution stock : 3450 ul / 100 ml					
	conc. [mg/ml]	conc. Injection [mg/ml]	mole/litre	Area [mAU*s]	Height [mAU]
solution 1	1.498818	0.145051582	0.000667763	9410.545	322.542
solution 2	0.3747045	0.036262896	0.000166941	2456.325	79.654
solution 3	0.093676125	0.009065724	4.17352E-05	559.975	22.401
solution 4	0.023419031	0.002266431	1.04338E-05	130.835	5.658
solution 5	0.005854758	0.000566608	2.60845E-06	30.256	1.335
solution 6	0.002927379	0.000283304	1.30423E-06	17.286	0.623
solution 7	0.001463689	0.000141652	6.52113E-07	7.965	0.245
solution 8	0.000731845	--	--	--	--
			LOQ:	**0.00065**	**mM**

The limit of detection had to be less or equal to 0.1 mM for both methods. It is reached and is about 200 times lower without using an FLD detector. Note that it is also possible to use an FLD detector that will result in a lower LOD.

5.2.6 Linearity

In order to avoid all problems from the matrix effect, a dilution of 34.1 times is performed. It corresponds to the sum of the dilution of all manipulations. This dilution coefficient corresponds of a dilution of 3.3 times for the samples preparation of the samples and 10.333 times for the reaction of derivatization in the syringe of the injector.

5.3 **Comparison between kinetics obtained from two different HPLC**.

In order to illustrate the robustness of the method 1.1 and 1.2, the following section will compare the results from two different experiments run on two different series1100 of agilent.

5.3.1 SFM4CHO media with L-Glu

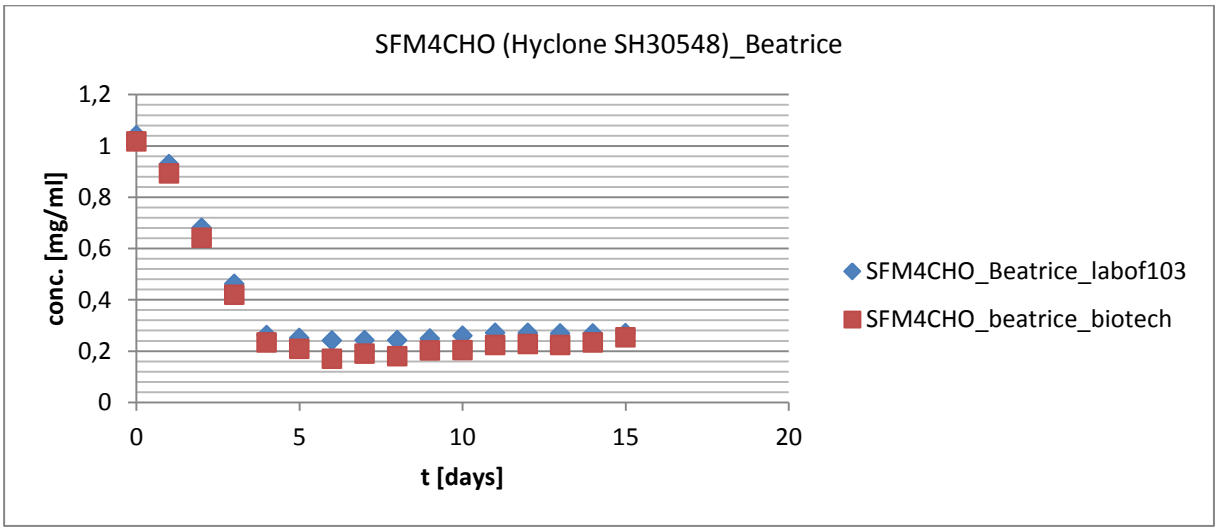

Figure 28: comparison between two same sample series on two different HPLC systems with method 1.1 (Beatrice is the samples name)

Table 20: numerical values of figure 24

	Agilent1100_f103 [mg/ml]	Agilent1100_biotech [mg/ml]
Bea 0	1.041	1.018
Bea 1	0.929	0.892
Bea 2	0.681	0.642
Bea 3	0.462	0.420
Bea 4	0.263	0.233
Bea 5	0.251	0.209
Bea 6	0.241	0.171
Bea 7	0.243	0.190
Bea 8	0.242	0.181
Bea 9	0.249	0.203
Bea 10	0.260	0.204
Bea 11	0.271	0.223
Bea 12	0.271	0.228
Bea 13	0.269	0.223
Bea 14	0.269	0.233

As it can be seen, only the concentration of the same samples using two different HPLC is given here. But it is also interesting to compare the responses for more injections of the same solutions. Sadly, no accuracy test had been done for the second HPLC, but it is possible to compare data about the calibration curve used, as the solutions used come from the stock solution.

Table 21: Statistics about method 1.1 using height and Agilent 1100 from laboratory f103

Series 1100 Agilent from laboratory f103					
Height [mAU]			Average [mAU]	SD [mAU]	RSD [%]
147.365	144.848	145.616	145.943	1.2899	**0.88**
30.649	30.446	31.621	30.905	0.6280	**2.03**
5.7644	5.735	5.622	5.707	0.0751	**1.31**
0.9682	0.97007	0.96552	0.967	0.0022	**0.23**

Table 22: Statistics about method 1.1 using area and Agilent 1100 from laboratory f103

Series 1100 Agilent from laboratory f103					
Area [mAU*s]			Average [mAU*s]	SD [mAU*s]	RSD [%]
3843.33	3843.73	3849.07	3845.376	3.2047	**0.083**
754.4	755.03	756.536	755.322	1.0975	**0.14**
136.8	137.534	134.209	136.181	1.7467	**1.28**
21.614	21.505	21.15609	21.425	0.2392	**1.11**

Table 23: Statistics about method 1.1 using height and Agilent 1100 from biotechnical department

Series 1100 Agilent from biotechnical department					
Height [mAU]			Average [mAU]	SD [mAU]	RSD [%]
104.45	98.36	97.65	100.153	3.7379	**3.73**
19.985	21.446	20.54	20.657	0.7374	**3.57**
3.941	3.915	3.965	3.940	0.0250	**0.63**
0.783	0.822	0.7956	0.800	0.0199	**2.48**

Table 24: Statistics about method 1.1 using area and Agilent 1100 from biotechnical department

Series 1100 Agilent from biotechnical department					
Area [mAU*s]			Average [mAU*s]	SD [mAU*s]	RSD [%]
3244.313	3498.6	3532.365	3425.092	157.467	**4.59**
652.23	651.24	653.545	652.338	1.156	**0.17**
114.565	112.025	111.254	112.614	1.732	**1.53**
17.167	17.622	17.264	17.351	0.239	**1.38**

It appears clearly that for each case, the HPLC used in the laboratory f103 had lower relative standard deviation.

5.3.2 Sigma media with Glutamax™

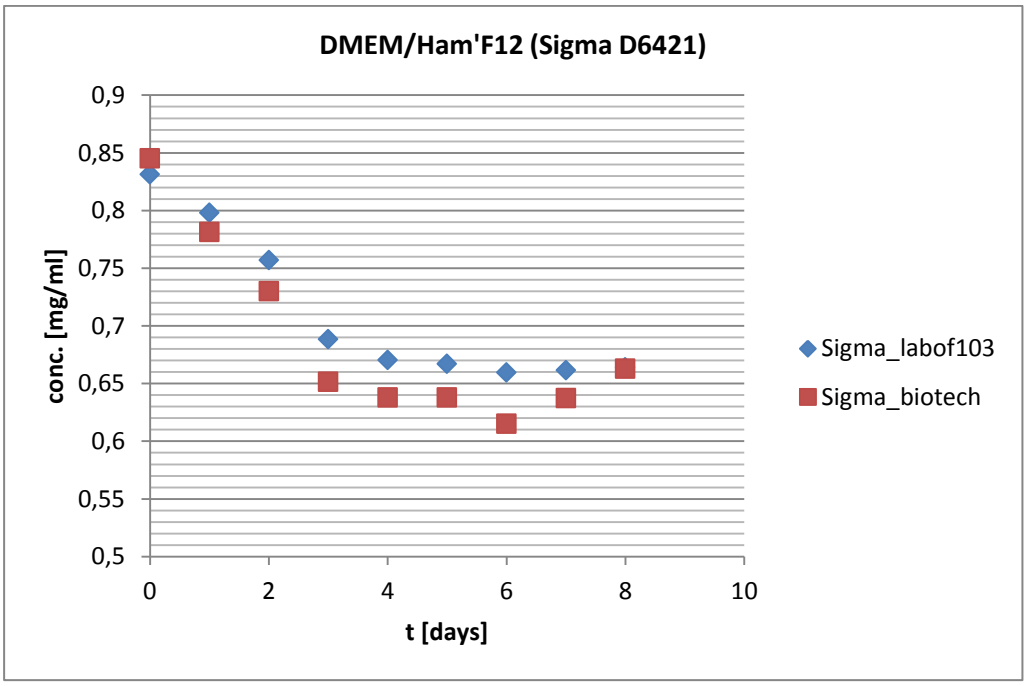

Figure 29: comparison between two same sample series on two different HPLC systems with method 1.2

Table 25: numerical values of figure 25

	Agilent1100_f103 [mg/ml]	Agilent1100_biotech [mg/ml]
Sigma 0	0.831	0.845
Sigma 1	0.798	0.781
Sigma 2	0.757	0.730
Sigma 3	0.688	0.652
Sigma 4	0.671	0.638
Sigma 5	0.667	0.638
Sigma 6	0.660	0.615
Sigma 7	0.662	0.637
Sigma 8	0.664	0.663

As it can be seen, only the concentration of the same samples using two different HPLC is given here. But it is also interesting to compare the responses for more injections of the same solutions. Sadly, no accuracy test had been done for the second HPLC, but it is possible to compare data about the calibration curve used, as the solutions used come from the stock solution.

Table 26: Statistics about method 1.2 using height and Agilent 1100 from laboratory f103

Series 1100 Agilent from laboratory f103					
Height [mAU]			Average [mAU]	SD [mAU]	RSD [%]
98.812	88.265	92.56	93.212	5.304	**5.69**
21.735	21.613	21.965	21.771	0.179	**0.82**
5.1756	5.245	5.365	5.262	0.096	**1.82**
1.122	1.185	1.154	1.154	0.032	**2.73**

Table 27 : Statistics about method 1.2 using area and Agilent 1100 from laboratory f103

Series 1100 Agilent from laboratory f103					
Area [mAU*s]			Average [mAU*s]	SD [mAU*s]	RSD [%]
2506.679	2482.855	2456.581	2482.038	25.059	**1.01**
603.178	598.041	601.256	600.825	2.595	**0.43**
136.924	136.375	136.623	136.641	0.275	**0.20**
27.306	30.703	28.569	28.859	1.717	**5.95**

Table 28: Statistics about method 1.2 using height and Agilent 1100 from biotechnical department

Series 1100 Agilent from biotechnical department					
Height [mAU]			Average [mAU]	SD [mAU]	RSD [%]
53.778	55.434	54.254	54.489	0.853	**1.56**
13.508	14.978	13.965	14.150	0.752	**5.32**
3.799	3.852	3.758	3.803	0.047	**1.24**
0.993	0.956	0.985	0.978	0.019	**1.99**

Table 29: Statistics about method 1.2 using area and Agilent 1100 from biotechnical department

Series 1100 Agilent from biotechnical department					
Area [mAU*s]			Average [mAU*s]	SD [mAU*s]	RSD [%]
1330.834	1425.654	1365.254	1373.914	48.000	**3.49**
315.364	308.654	309.658	311.225	3.619	**1.16**
88.068	82.752	85.598	85.473	2.660	**3.11**
22.049	22.086	22.056	22.064	0.020	**0.09**

It appears clearly that for each case, the HPLC used in the laboratory f103 had lower relative standard deviation. If these result are compared to the tables 21, 22, 23 and 24, once can see that the errors are more important here. It can be explained by the quality of the molecule used. Indeed, for the case of the method 1.2 and Glutamax^{tm} no specification about the purity of molecules was given and therefore it was not possible to guarantee that all the dipeptides are L, L. This can drive to this difference in the response error.

5.4 Others kinetics

The following table will present analyzes of last missing samples:

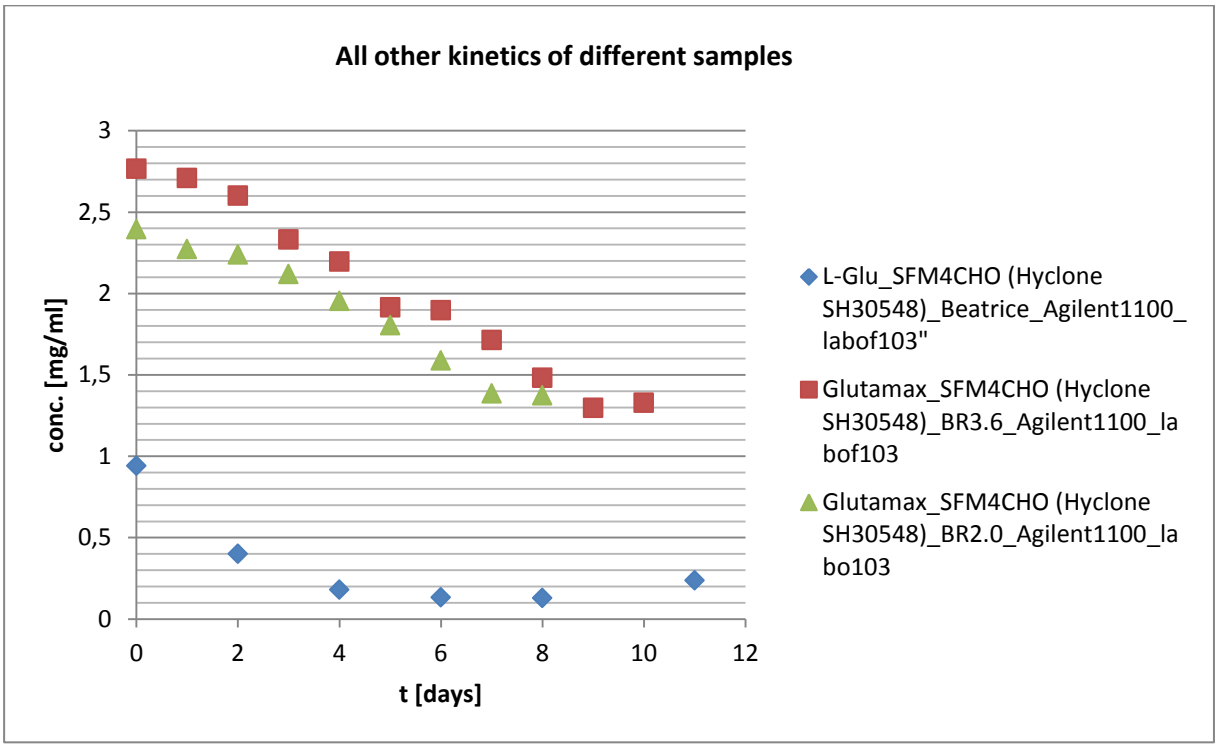

Figure 30: all others kinetics from different samples

In the case of BR3.6 and BR2.0, relatively strange results are calculated. Indeed, the concentration of t_0 found for both experiment are higher than the range imposed in the requirements. But it is still possible to observe the kinetic of the Glutamax^{tm} degradation over the time.

6. Conclusion

This study has developed a brand new way in order to quantify the L-Glutamine and the Glutamax^{tm} in complex matrixes enabling the derivatization pre-injection in the needle of the injector. This method will allow the users to make significant economies because of its cheap and easy to perform manipulations needed. But it will also allow really small error on the reaction of derivatization that would significantly be increased in case of manual manipulation. This was the main key of this study, to accomplish a working method which was economically viable. Indeed, some methods were already usable for the quantification of L-Glutamine but relatively expensive and not so easy to perform as well as their maintenances. In the case of the dipeptide called Glutamax^{tm}, it was actually no direct methods allowing the quantification of this molecule. So it is with a great pleasure that the operator presenting his method and thus, despite the fact that it is two different methods and not only one.

In a personal point of view, this work was really enriching for the operator. It has allowed a personal elaboration of the strategy for the separation in an atmosphere of trust. This is a really important key for the development of an ingenious mind that will be required in the industry. But not only: this work also allows the operator to be familiar with all aspects of the technique of LC- system used for the separation which is priceless. It also allows the understanding of one of the main problem of the analytical work in industry that is the sampling.

7. Prospects

There are several points that can be stated in this part. First of all, if the analyzes have to reach a lower limit of detection, it is possible to work with a FLD detector using the following wavelength of excitation and emission: Ex = 230 nm, Em= 450 nm.

A second point that can be mentioned is the dwell time optimization of the HPLC from the biotechnical department. Indeed, it is significant change in the retention time between the two HPLC used. Note that it can also be the system of eluent splitting that can drive to different retention time. However, since the resolution is always more than 1.5 it would only be some kind of optimization. Note that it is not only for this experiment but more generally for all experiments driven on this HPLC.

What can also be noticed here is the possibility to optimize methods 1.1 and 1.2 to make only one method with both of them. Sadly, this was not possible due to the significant difference in the retention time as well as the matrix effect that is really hard to handle.

8. Acknowledgments

I would like to take the opportunity to thank all people from the laboratory f103 with a special motion for Antoine Fornage that never hesitated to share is extreme experience about analytical instruments. I would like to thank him as well for all advices and the solutions he purposed to me. He is one of the main key of the success of this work. Special mention for Pascal Jacquemettaz that was always able to listen and offer practical solutions. He is also the one that motioned the utilization of the rp-column that can handle a relatively basic pH. I would also thank the apprentice Aurélien Ducrey that supported me for about one month and help me to handle some problems encountered.

The last motion is for my professor in the HES-SO Valais, the Prof. Franka Kalman for his extreme kindness and knowledge that allows me to rapidly understand problems. I would like to thank her also so his availability that permitted to lead this project to a useful end.

9. Literature

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10. Appendix

10.1 Excel tables

- **Calibration_all_systems**

dilution vial : 3x
dilution injection (3ul dans 28ul) : 10,3333 x
dilution centrifugation : 1,1x

Calibration l-Glu_agilent 1100_f102 — ***DAD Sig = 338,4 nm***

solution stock : 127,74 mg / 100 ml = 1,2774 mg/ml

concentration	AREA [mAU*s]			HEIGHT [mAU]			Retention time			Average			SD			RSD		
										Area	Height	Rt	Area	Height	Rt	Area	Height	Rt
0.037460531 mg/ml	3843.33	3843.73	3849.07	147.365	144.848	145.62	11.19	11.58	11.645	3845.3767	145.943	11.47167	3.204767	1.289969	0.246086	0.083341	0.883885	2.145164
0.007492106 mg/ml	754.4	755.03	756.536	30.649	30.446	31.621	11.702	11.855	11.436	755.322	30.90533333	11.66433	1.09753	0.628042	0.212024	0.145306	2.032147	1.817715
0.001498421 mg/ml	136.8	137.534	134.209	5.7644	5.735	5.622	11.97	11.97	12.107	136.181	5.707133333	12.01567	1.74679	0.075179	0.079097	1.282698	1.317278	0.658282
0.000299684 mg/ml	21.614	21.505	21.1561	0.9682	0.97007	0.9655	12.233	12.377	12.292	21.42503	0.96793	12.30067	0.2392	0.002287	0.07239	1.116453	0.236276	0.588506

Calibration l-Glu_agilent 1100_biotech — ***DAD Sig = 338,4 nm***

solution stock : 127,74 mg / 100 ml = 1,2774 mg/ml

concentration	AREA [mAU*s]		HEIGHT [mAU]		Retention time		Average			SD			RSD		
							Area	Height	Rt	Area	Height	Rt	Area	Height	Rt
0.037460531 mg/ml	3244.31	2498.6	105.45	98.36	10.356	10.492	2871.4564	101.905	10.424	527.2985	5.013387	0.096167	18.36345	4.919667	0.922549
0.007492106 mg/ml	652.23	651.24	19.985	21.446	10.447	10.37	651.735	20.7155	10.4085	0.700036	1.033083	0.054447	0.107411	4.987005	0.523103
0.001498421 mg/ml	114.565	112.025	3.941	3.915	10.25	10.386	113.295	3.928	10.318	1.796051	0.018385	0.096167	1.585287	0.468044	0.932027
0.000299684 mg/ml	17.167	17.622	0.783	0.822	10.42	10.386	17.3945	0.8025	10.403	0.321734	0.027577	0.024042	1.849628	3.436407	0.231103

Calibration GlutaMAX_agilent 1100_f102 — ***DAD Sig = 338,4 nm***

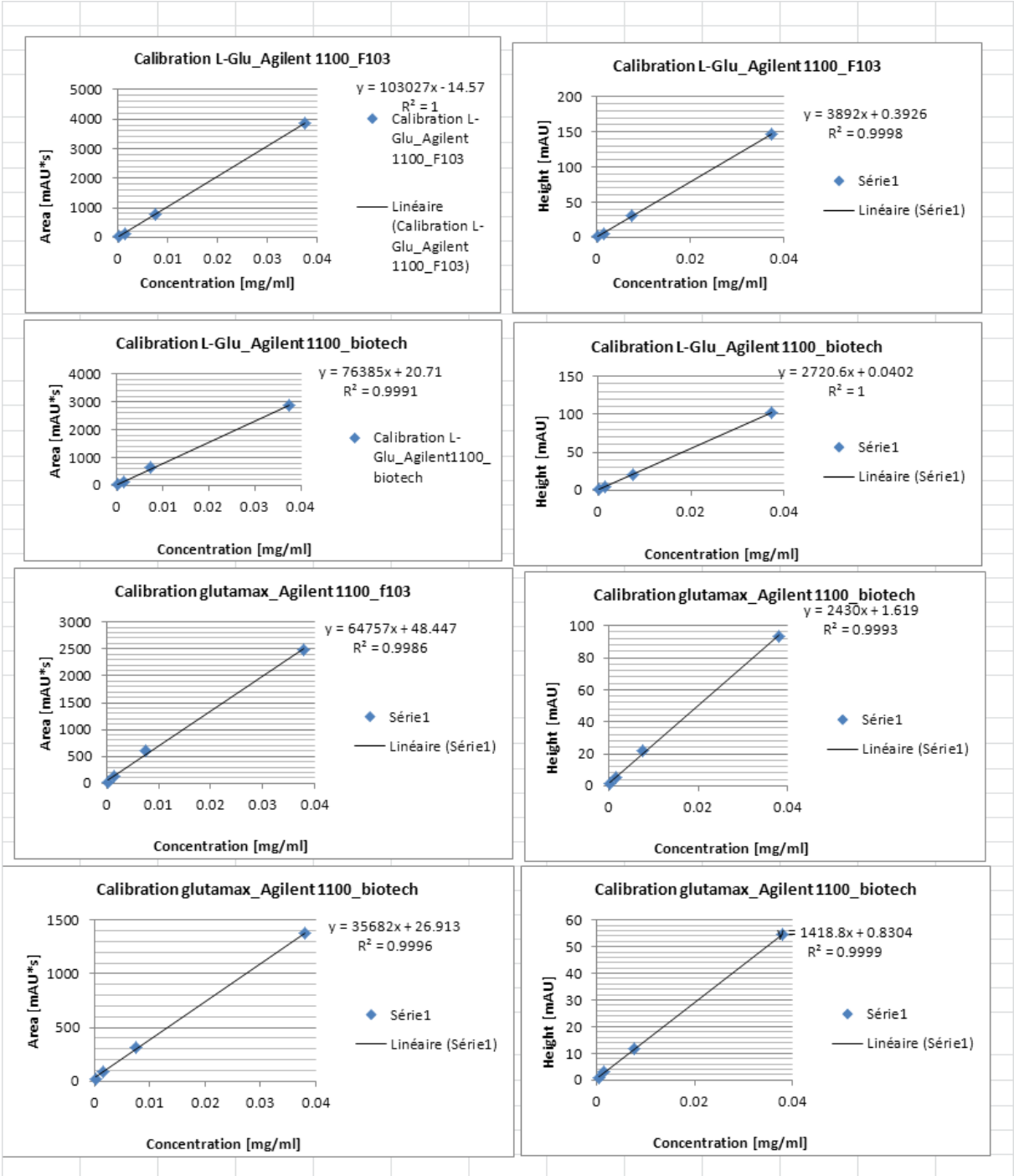
solution stock : 2979 ul / 100 ml. Glutamax(MW = 217.22 , Conc. = 200 mM).

concentration	HEIGHT [mAU]		Retention time		Average			SD			RSD		
					Area	Height	Rt	Area	Height	Rt	Area	Height	Rt
0.037953107 mg/ml	2506.68	2482.855	98.812	88.265	10.894	10.963	2494.7671	93.5385	10.9285	16.84625	7.457855	0.04879	0.675264	7.973033	0.446451
0.007590621 mg/ml	603.178	598.041	21.735	21.613	10.865	11.022	600.6095	21.674	10.9435	3.632408	0.086267	0.111016	0.604787	0.398021	1.014445
0.001518124 mg/ml	136.924	136.375	5.1756	5.245	11.125	10.954	136.6495	5.2103	11.0395	0.388202	0.049073	0.120915	0.284086	0.94185	1.095297
0.000303625 mg/ml	27.306	30.703	1.122	1.185	12.233	12.377	29.0045	1.1535	12.305	2.402042	0.044548	0.101823	8.281617	3.861962	0.827496

Calibration GlutaMAX_agilent 1100_f102 — ***DAD Sig = 338,4 nm***

solution stock : 2979 ul / 100 ml. Glutamax(MW = 217.22 , Conc. = 200 mM).

concentration	HEIGHT [mAU]		Retention time		Average			SD			RSD		
					Area	Height	Rt	Area	Height	Rt	Area	Height	Rt
0.037953107 mg/ml	1330.83	1425.654	53.778	55.434	9.49	9.439	1378.244	54.606	9.4645	67.04786	1.170969	0.036062	4.864731	2.144396	0.381029
0.007590621 mg/ml	315.364	308.654	13.508	14.978	9.511	9.448	312.009	11.985	9.4795	4.744687	1.039447	0.044548	1.520689	8.672899	0.469938
0.001518124 mg/ml	88.068	82.752	3.799	3.852	9.479	9.445	85.41	2.96	9.462	3.75898	0.037477	0.024042	4.4011	1.266103	0.254086
0.000303625 mg/ml	22.049	22.086	0.993	0.956	9.552	9.576	22.0675	0.9745	9.564	0.026163	0.026163	0.016971	0.118559	2.684756	0.177442


Calibration L-Glu_Agilent 1100_F103
y = 103027x - 14.57
R² = 1
Area [mAU*s]
Concentration [mg/ml]
Calibration L-Glu_Agilent 1100_F103
Linéaire (Calibration L-Glu_Agilent 1100_F103)
Calibration L-Glu_Agilent 1100_F103
y = 3892x + 0.3926
R² = 0.9998
Height [mAU]
Concentration [mg/ml]
Série1
Linéaire (Série1)
Calibration L-Glu_Agilent 1100_biotech
y = 76385x + 20.71
R² = 0.9991
Area [mAU*s]
Concentration [mg/ml]
Calibration L-Glu_Agilent1100_biotech
Calibration L-Glu_Agilent 1100_biotech
y = 2720.6x + 0.0402
R² = 1
Height [mAU]
Concentration [mg/ml]
Série1
Linéaire (Série1)
Calibration glutamax_Agilent 1100_f103
y = 64757x + 48.447
R² = 0.9986
Area [mAU*s]
Concentration [mg/ml]
Série1
Linéaire (Série1)
Calibration glutamax_Agilent 1100_biotech
y = 2430x + 1.619
R² = 0.9993
Height [mAU]
Concentration [mg/ml]
Série1
Linéaire (Série1)
Calibration glutamax_Agilent 1100_biotech
y = 35682x + 26.913
R² = 0.9996
Area [mAU*s]
Concentration [mg/ml]
Série1
Linéaire (Série1)
Calibration glutamax_Agilent 1100_biotech
y = 1418.8x + 0.8304
R² = 0.9999
Height [mAU]
Concentration [mg/ml]
Série1
Linéaire (Série1)

- **Precision**

PRECISION : Agilent 1100_labof102

Précision L-Glu (N=3)

concentration : 57,11 mg / 100 ml = 0.5711 mg/ml

concentration th. après dilutions : 0.01842317 mg/ml

Area [mAU*s]			Average
1700.424	1688.57971	1690.5477	1693.18395

concentration calculée : 0.018233331 mg/ml

% recovery **98.97047626**

Précision GlutaMAX (N=3)

solution : 1485 ul / 100 ml. Glutamax(MW = 217.22 , Conc. = 200 mM).

concentration th. après dilutions : 0.02081114 mg/ml

Area [mAU*s]			Average
1356.325	1360.32	1361.254	1359.29967

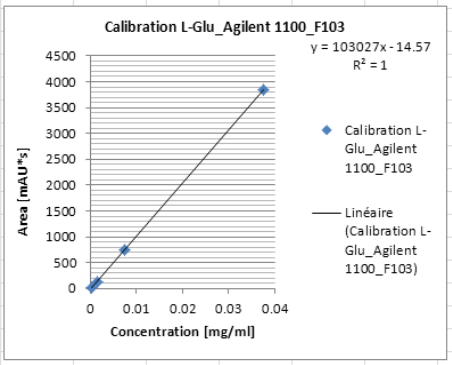
concentration calculée : 0.02024264 mg/ml

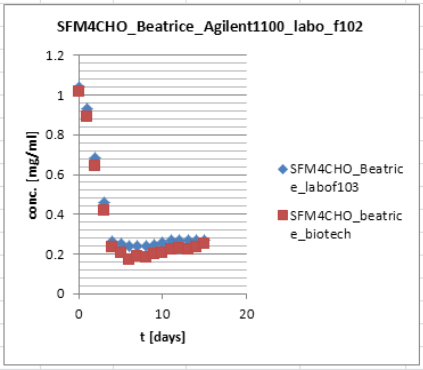
% recovery **97.26827078**

- **SFM4CHO_comparison_l-glu**

DAD Sig = 338,4 nm **Agilent1100_labof103**

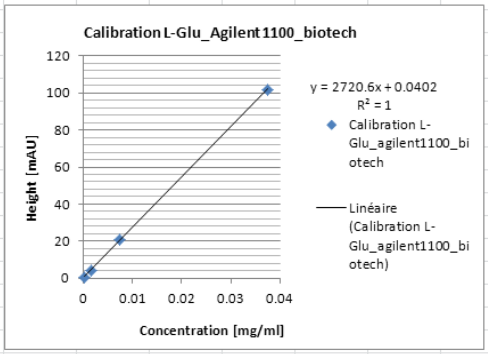
Days	Area	Conc. Injection mg/ml	Conc. Échantillon mg/ml
0	2846.796	0.031	1.041426502
1	2537.279	0.027	0.928774115
2	1856.773	0.020	0.681096116
3	1254.805	0.014	0.462002928
4	707.6252	0.008	0.262850535
5	675.02	0.007	0.250983497
6	647.837	0.007	0.241089931
7	653.25	0.007	0.243060055
8	650.11	0.007	0.241917216
9	669.795	0.007	0.249081798
10	700.124	0.008	0.260120387
11	731.329	0.008	0.271477806
12	730.256	0.008	0.271087276
13	723.481	0.008	0.268621436
14	724.937	0.008	0.269151364
15	725.694	0.008	0.269426883





DAD Sig = 338,4 nm **Agilent1100_biotech**

Days	Height	Conc. Injection mg/ml	Conc. Échantillon mg/ml
0	81.25	0.02984996	1.017555272
1	71.254	0.02617577	0.892305825
2	51.254	0.01882445	0.641706693
3	33.564	0.012322208	0.42005176
4	18.65	0.006840329	0.233179987
5	16.685	0.006118062	0.208558622
6	13.661	0.005006543	0.170668033
7	15.206	0.005574432	0.190026816
8	14.446	0.005295082	0.180504049
9	16.256	0.005960376	0.203183271
10	16.352	0.005995663	0.204386147
11	17.865	0.00655179	0.223343971
12	18.214	0.006680071	0.227716926
13	17.865	0.00655179	0.223343971
14	18.654	0.0068418	0.233230107
15	20.256	0.00743064	0.253303097

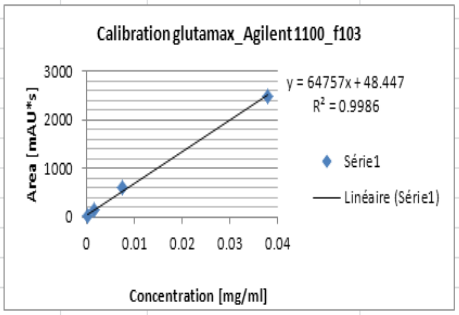


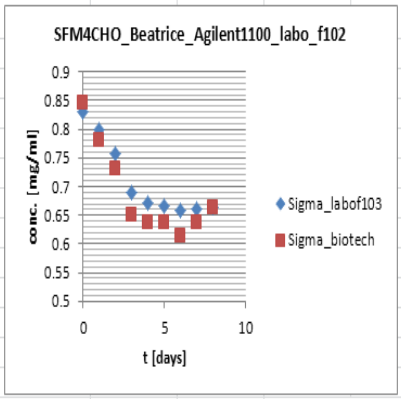
	Agilent1100_f1C	Agilent1100_biotech [mg/ml]
Bea 0	1.041	1.018
Bea 1	0.929	0.892
Bea 2	0.681	0.642
Bea 3	0.462	0.420
Bea 4	0.263	0.233
Bea 5	0.251	0.209
Bea 6	0.241	0.171
Bea 7	0.243	0.190
Bea 8	0.242	0.181
Bea 9	0.249	0.203
Bea 10	0.260	0.204
Bea 11	0.271	0.223
Bea 12	0.271	0.228
Bea 13	0.269	0.223
Bea 14	0.269	0.233

- **Sigma_comparaison_glutamax**

DAD Sig = 338,4 nm **Agilent1100_labof103**

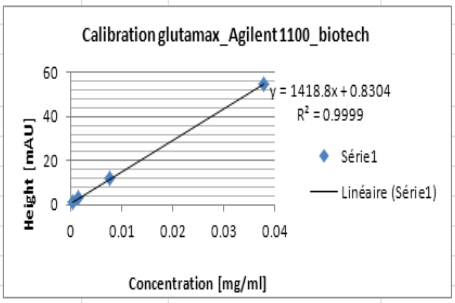
Days	Area	Conc. Injection mg/ml	Conc. Échantillon mg/ml
0	1627.883	0.024390197	0.83143743
1	1564.608	0.023413083	0.79812858
2	1486.856	0.022212409	0.75719883
3	1356.23	0.020195238	0.68843545
4	1322.36	0.019672205	0.67060581
5	1315.65	0.019568587	0.66707357
6	1301.36	0.019347916	0.65955111
7	1305.32	0.019409068	0.66163571
8	1309.9663	0.019480818	0.66408159





DAD Sig = 338,4 nm **Agilent1100_biotech**

Days	Height	Conc. Injection mg/ml	Conc. Échantillon mg/ml
0	37.57	0.024793899	0.84519923
1	34.796	0.022921852	0.78138301
2	32.564	0.021415576	0.73003556
3	29.156	0.01911567	0.65163408
4	28.569	0.01871953	0.63813007
5	28.56	0.018713457	0.63792302
6	27.569	0.018044675	0.61512494
7	28.541	0.018700634	0.63748592
8	29.654	0.019451748	0.66309063



	Agilent1100_	Agilent1100_biotech [mg/ml]
Sigma 0	0.831	0.845
Sigma 1	0.798	0.781
Sigma 2	0.757	0.730
Sigma 3	0.688	0.652
Sigma 4	0.671	0.638
Sigma 5	0.667	0.638
Sigma 6	0.660	0.615
Sigma 7	0.662	0.637
Sigma 8	0.664	0.663

- **Fluorescence data, OPA optimization**

I.1

Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	20.9	20.9	21.1	21	21	20.9	21	20.9	21	21	21	21.1	21	21	21	21.1	21	20.9	21.1	21	21.1
A12	4825	8346	11242	13618	15542	17138	18487	19636	20589	21402	22110	22809	23318	23831	24151	24546	24852	25159	25425	25569	25780

I.2

Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	20.8	20.8	21.2	21	21.3	21.5	21.5	21.20	21.2	21.2	21.5	21	21.5	21.2	21.5	21	21.2	21.1	21.2	21	21.5
A13	4193	7345	9892	11867	13406	14700	15767	16510	17158	17661	18181	18399	18826	19069	19314	19534	19812	19930	20169	20287	20508

I.3

Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	21.3	21.3	21.5	21.2	21.3	21.6	21.1	20.9	21.5	21.2	21.4	21.2	21.2	21.2	21.3	21.6	21.5	21.2	21.2	21.3	21.2
A15	3098	5408	7258	8769	9974	10962	11764	12589	13146	13667	14134	14462	14844	15188	15500	15584	15690	15885	16080	16079	16117

I.4

Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	21.3	21.3	21.2	21.2	21.2	21.2	21.1	21	21.2	21.5	21.1	21.1	21.2	21.2	21.2	21.5	21.2	21.2	21.5	21.5	21.2
A14	3492	6177	8335	10173	11599	12807	13930	14840	15618	16235	16736	17316	17784	18135	18505	18799	19014	19236	19382	19622	19823

I.5

Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	21.7	21.7	21.1	21.3	21.5	21.5	21.3	21.2	21.3	21.3	21.3	21.2	21.6	21.3	21.3	21.5	21.1	21.5	21.5	21.2	21.3
A16	9090	15385	20241	24190	27353	29979	32233	34042	35776	37012	38211	39134	39976	40608	41166	41808	42149	42666	43020	43266	43567

I.5 bis

Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	21.5	21.5	21.5	21.3	21.3	21.2	21.4	21.2	21.5	21.5	21.4	21.3	21.5	21.5	21.3	21.7	21.4	21.5	21.3	21.7	21.7
A17	9819	16489	21651	25769	29061	31887	34119	36340	37873	39164	40559	41451	42513	43132	43819	44378	45081	45486	45623	46259	46578

I.6

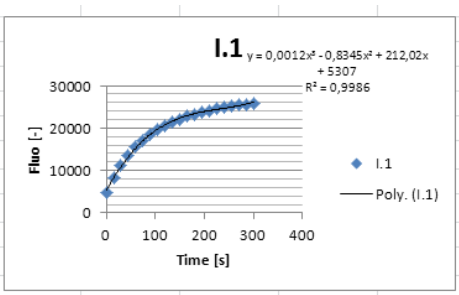
Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	21.7	21.7	21.8	21.7	21.8	21.7	21.6	21.4	21.6	21.7	21.8	21.8	21.7	21.9	21.7	21.7	21.6	21.6	21.7	21.8	21.7
A18	6839	11620	15546	18595	21157	23270	24513	26088	27363	28468	29407	30129	30927	31530	32045	32459	32881	33181	33405	33789	34061

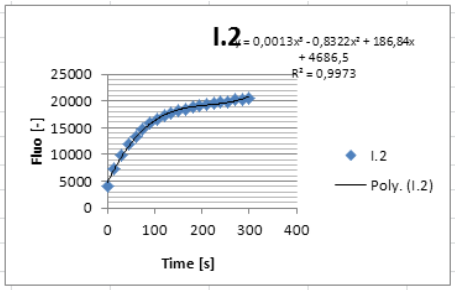
I.7

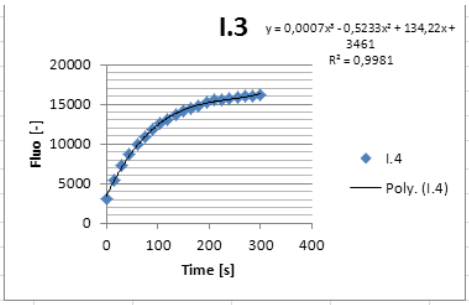
Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	21.9	21.9	21.9	22	21.9	21.9	21.8	21.9	21.7	21.8	21.9	21.8	21.9	21.8	21.9	21.8	21.9	22.1	21.8	21.8	21.9
A19	9086	15353	19445	23237	26361	29086	31256	33154	34680	36254	37411	38373	39449	40186	40947	41613	42038	42661	43149	43554	43760

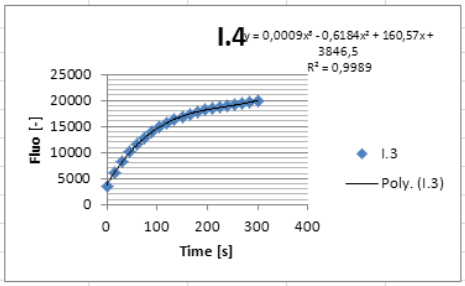
Final_1																					
Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	22.6	22.6	22.5	22.4	22.4	22.3	22.5	22.3	22.6	22.2	22.3	22.6	22.4	22.3	22.6	22.8	22.6	22.4	22.6	22.5	22.4
B4	12331	18367	22103	24714	26422	27561	28418	29170	29734	30010	30424	30578	30713	30896	30970	31126	31121	31010	31089	31031	31182
Final_2																					
Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	22.3	22.3	22.4	22.5	22.6	22.3	22.4	22.5	22.4	22.5	22.6	22.6	22.3	22.6	22.6	22.3	22.6	22.5	22.8	22.5	22.8
B5	1661	2532	3158	3590	3940	4163	4346	4458	4516	4601	4633	4665	4694	4707	4719	4767	4757	4782	4777	4812	4819
Final_3																					
Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	22.7	22.7	22.6	22.9	22.5	22.6	22.9	22.7	22.5	22.9	22.8	22.8	22.9	22.8	22.9	22.6	22.6	22.9	22.5	22.8	22.8
B6	4056	6880	9326	11341	13079	14565	15783	16987	18004	18894	19686	20481	21170	21667	22210	22766	23161	23702	24078	24456	24752
Final_4																					
Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	22.9	22.9	23.1	23	23.1	23.1	23.1	23.1	22.9	22.9	22.9	23.1	23.1	23.1	23.1	23	22.9	23	23	23	23
B7	2460	4356	6032	7497	8810	10018	11112	12081	12996	13876	14420	15296	16006	16640	17272	17861	18379	18870	19262	19717	20115
Final_5																					
Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	23.4	23.4	23.2	23.2	23.2	23.1	23	23.3	23.1	23.2	23	23.2	23.2	23.1	23.1	23.3	23	23.4	23.2	23.2	23.4
B8	426	731	1026	1315	1580	1851	2082	2345	2582	2813	3040	3252	3482	3670	3885	4072	4262	4426	4605	4754	4934
Final_6																					
Cycle Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time [s]	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300
Temp. [°C]	23.3	23.3	23.5	23.4	23.3	23.5	23.4	23.5	23.4	23.4	23.5	23.5	23.2	23.5	23.6	23.5	23.3	23.4	23.5	23.5	23.7
B9	17687	22387	25516	26111	27115	27861	28184	28432	28549	28719	28844	28949	28900	28975	28924	28903	28956	28944	29076	29076	29090

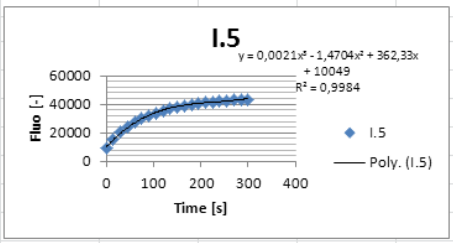
	Bicine + 2ME 5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 1,04 mg/0.2 ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
I.1	4	3	6	6	20	39	19.5
	Bicine + 2ME 5% [ul]	Glu_1 mg/ml [ul]	Bicine 1 M [ul]	OPA 1,04 mg/0.2 ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
I.2	4	3	6	6	20	39	19.5
	Bicine + 2ME 5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.1 M [ul]	OPA 1,04 mg/0.2 ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
I.3	4	3	6	6	20	39	19.5
	Bicine + 2ME 5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 1,04 mg/0.2 ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
I.4	6	3	6	6	20	41	20.5
	Bicine + 2ME 0.5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 1,04 mg/0.2 ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
I.5	6	6	6	6	20	44	22
I.5'	4	6	6	6	20	42	21
I.6	4	6	6	6	20	42	21
I.7	6	6	6	6	40	64	32
I.8	4	6	6	6	40	62	31
	Bicine + 2ME 0.5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 1,04 mg/0.2 ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
final 1	4	6	6	6	40	62	31
	Bicine + 2ME 0.5% [ul]	Glu_0,1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 1,04 mg/0.2 ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
final 2	4	6	6	6	40	62	31
	Bicine + 2ME 0.5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 1,04 mg/0.2ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
final 3	4	6	6	6	40	62	31
	Bicine + 2ME 0.5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 0.5 mg/0.2ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
final 4	4	6	6	6	40	62	31
	Bicine + 2ME 0.5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA 0.1 mg/0.2ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
final 5	4	6	6	6	40	62	31
	Bicine + 2ME 0.5% [ul]	Glu_1 mg/ml [ul]	Bicine 0.5 M [ul]	OPA_2,5 mg/0.2ml [ul]	H2O [ul]	Somme [ul]	Scale down [ul]
final 6	4	6	6	6	40	62	31

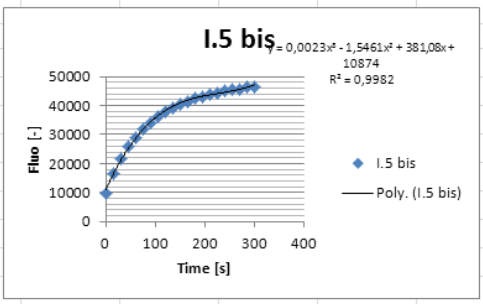

I.1
y = 0,0012x³ - 0,8345x² + 212,02x + 5307
R² = 0,9986
Fluo [-]
Time [s]
I.1
Poly. (I.1)

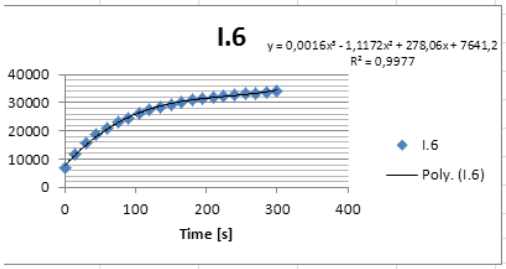

I.2
y = 0,0013x³ - 0,8322x² + 186,84x + 4686,5
R² = 0,9973
Fluo [-]
Time [s]
I.2
Poly. (I.2)

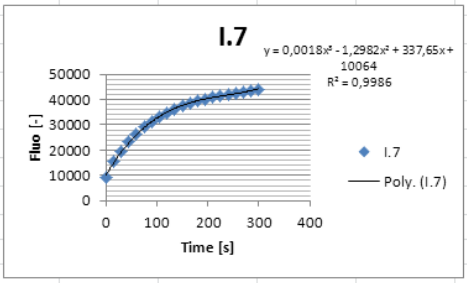

I.3
y = 0,0007x³ - 0,5233x² + 134,22x + 3461
R² = 0,9981
Fluo [-]
Time [s]
I.4
Poly. (I.4)

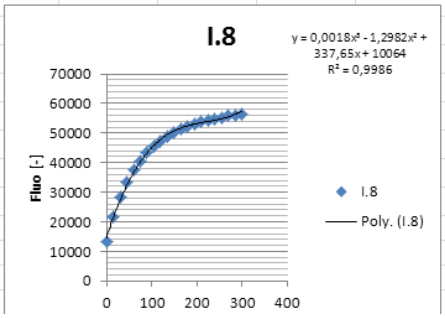

I.4
y = 0,0009x³ - 0,6184x² + 160,57x + 3846,5
R² = 0,9989
Fluo [-]
Time [s]
I.3
Poly. (I.3)

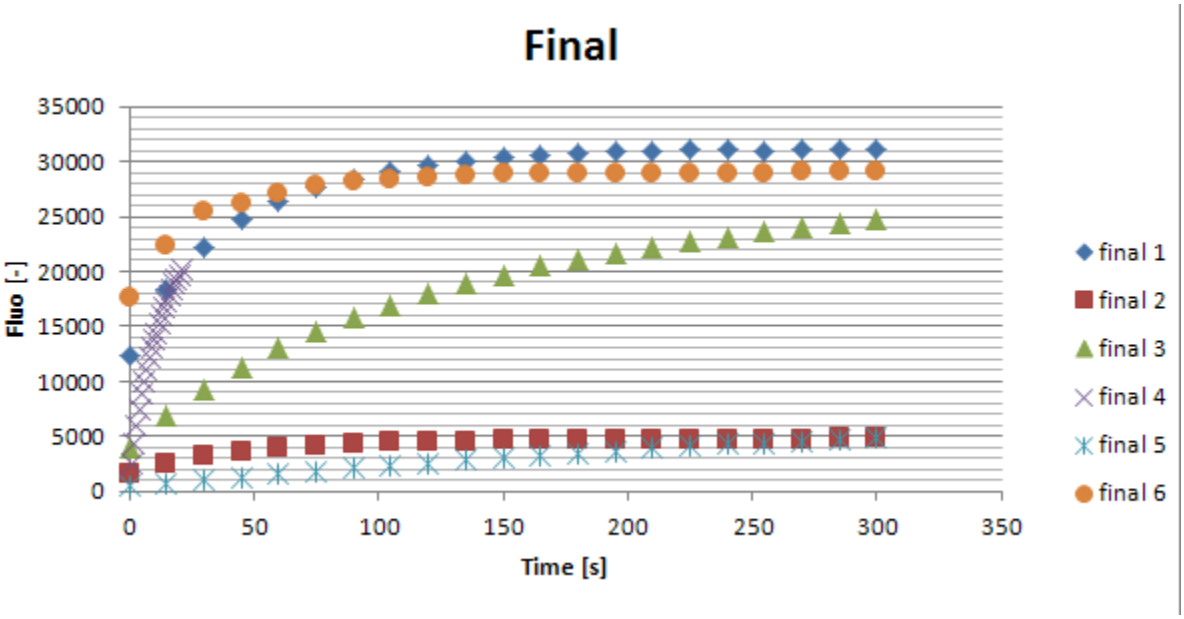

I.5
y = 0,0021x³ - 1,4704x² + 362,33x + 10049
R² = 0,9984
Fluo [-]
Time [s]
I.5
Poly. (I.5)


I.5 bis
y = 0,0023x³ - 1,5461x² + 381,08x + 10874
R² = 0,9982
Fluo [-]
Time [s]
I.5 bis
Poly. (I.5 bis)


I.6
y = 0,0016x³ - 1,1172x² + 278,06x + 7641,2
R² = 0,9977
Fluo [-]
Time [s]
I.6
Poly. (I.6)


I.7
y = 0,0018x³ - 1,2982x² + 337,65x + 10064
R² = 0,9986
Fluo [-]
Time [s]
I.7
Poly. (I.7)


I.8
y = 0,0018x³ - 1,2982x² + 337,65x + 10064
R² = 0,9986
Fluo [-]
Time [s]
I.8
Poly. (I.8)


Final
Fluo [-]
Time [s]
final 1
final 2
final 3
final 4
final 5
final 6

- **LOD/LOQ**

L-glu DAD Sig = 338.14

Solution stock : 15.98 mg / 10 ml

	conc. [mg/ml]	conc. Injection [mg/ml]	mole/litre	Area [mAU*s]	Height [mAU]
solution 1	1.598	0.15465015	0.001058233	14125.3	415.055
solution 2	0.3995	0.038662538	0.000264558	3466.325	115.641
solution 3	0.099875	0.009665634	6.61396E-05	816.402	32.01
solution 4	0.02496875	0.002416409	1.65349E-05	181.637	7.64
solution 5	0.006242188	0.000604102	4.13372E-06	42.996	1.758
solution 6	0.003121094	0.000302051	2.06686E-06	19.842	0.8389
solution 7	0.001560547	0.000151026	1.03343E-06	9.546	0.3856
solution 8	0.000780273	7.55128E-05	5.16715E-07	4.264	0.196
			LOQ :	0.00052	mM

Glutamax DAD Sig = 338.14

Solution stock : 3450 ul / 100 ml

	conc. [mg/ml]	conc. Injection [mg/ml]	mole/litre	Area [mAU*s]	Height [mAU]
solution 1	1.498818	0.145051582	0.000667763	9410.545	322.542
solution 2	0.3747045	0.036262896	0.000166941	2456.325	79.654
solution 3	0.093676125	0.009065724	4.17352E-05	559.975	22.401
solution 4	0.023419031	0.002266431	1.04338E-05	130.835	5.658
solution 5	0.005854758	0.000566608	2.60845E-06	30.256	1.335
solution 6	0.002927379	0.000283304	1.30423E-06	17.286	0.623
solution 7	0.001463689	0.000141652	6.52113E-07	7.965	0.245
solution 8	0.000731845	--	--	--	--
			LOQ:	0.000652113	mM

- **OPA Reaction optimization**

L-glu DAD Sig = 338.14									
Solution stock : 15.98 mg / 10 ml			Area [mAU*s]					Height [mAU]	
	conc. [mg/ml]	conc. Injection [mg/ml]	OPA 10,47mg/ml	OPA 5,10 mg/ml	OPA 1,06 mg/ml		OPA 10,47mg/ml	OPA 5,10 mg/ml	OPA 1,06 mg/ml
sol 1	1.598	0.15465015	14125.3	12481.56	5296.73		415.055	378.254	186.896
sol 2	0.3995	0.038662538	3466.325	3128.086	1570.327		115.641	113.488	60.325
sol 3	0.099875	0.009665634	816.402	746.719	383.754		32.01	28.996	15.815
sol 4	0.02496875	0.002416409	181.637	174.067	60.443		7.64	7.061	7.708
sol 5	0.006242188	0.000604102	42.996	41.965	22.615		1.758	1.695	0.889

- **Reproductibility**

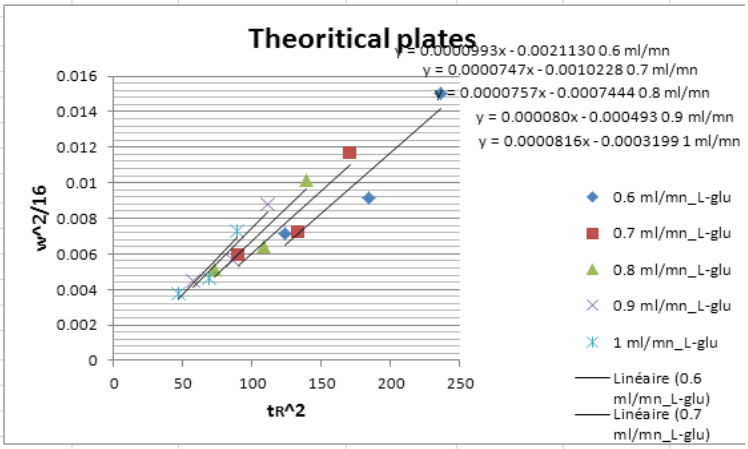
Injection + dérivatisation			
	tR	Height	Area
n=1	5.875	1780.06763	98.1905
n=2	5.881	1797.77661	98.5286
n=3	5.853	1843.52759	98.6323
n=4	5.714	1798.97967	98.6632
n=5	5.664	1774.425	98.7449
n=6	5.659	1768.36755	98.7261
Average	5.77433333	1793.85734	98.5809333
SD	0.10659769	27.2992088	0.20617552
RSD	**1.84606049**	**1.52181604**	**0.20914341**

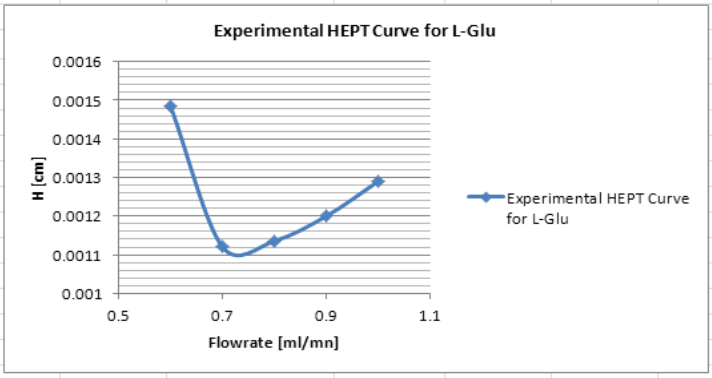
- **L-Glu Flowrate**

Flowrate L-Glu

Flowrate : 0.6 ml/mn				
	t_R [min]	W [min]	$w^2/16$	t_R^2
Subtance 1	11.126	0.3384	0.00715716	123.787876
L-Glu	13.571	0.3821	0.00912503	184.172041
Substance 2	15.351	0.4903	0.01502463	235.653201
Flowrate : 0.7 ml/mn				
	t_R [min]	W [min]	$w^2/16$	t_R^2
Subtance 1	9.488	0.307	0.00589056	90.022144
L-Glu	11.559	0.3398	0.0072165	133.610481
Substance 2	13.055	0.4322	0.0116748	170.433025
Flowrate : 0.8 ml/mn				
	t_R [min]	W [min]	$w^2/16$	t_R^2
Subtance 1	8.536	0.2868	0.00514089	72.863296
L-Glu	10.407	0.32	0.0064	108.305649
Substance 2	11.801	0.4033	0.01016568	139.263601
Flowrate : 0.9 ml/mn				
	t_R [min]	W [min]	$w^2/16$	t_R^2
Subtance 1	7.594	0.2673	0.00446558	57.668836
L-Glu	9.267	0.2997	0.00561376	85.877289
Substance 2	10.534	0.3745	0.00876564	110.965156
Flowrate : 1 ml/mn				
	t_R [min]	W [min]	$w^2/16$	t_R^2
Subtance 1	6.8	0.245	0.00375156	46.24
L-Glu	8.294	0.2728	0.00465124	68.790436
Substance 2	9.422	0.3408	0.00725904	88.774084

Flowrate [ml/min]	N	H [cm]
0.6	10101.0101	0.001485
0.7	13386.88086	0.0011205
0.8	13210.03963	0.0011355
0.9	12500	0.0012
1	11627.90698	0.00129

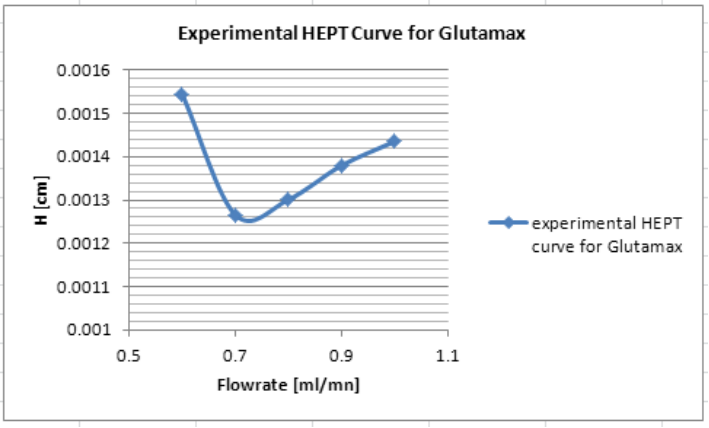




- **Glutamax Flowrate**

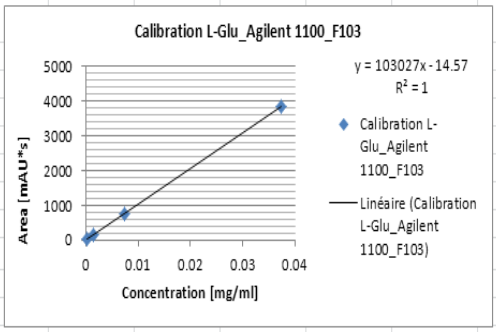
Flowrate Glutamax

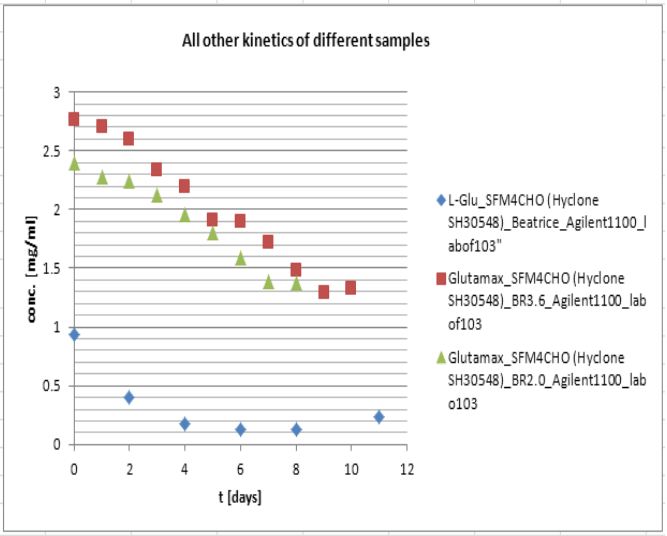
Flowrate	N	H
0.6	9710.18689	0.00154477
0.7	11846.2864	0.00126622
0.8	11533.4331	0.00130057
0.9	10875.3687	0.00137926
1	10454.7946	0.00143475

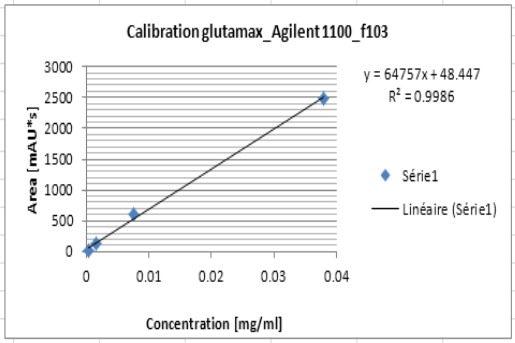


- **All Kinetics**

Agilent1100_labof103	
Conc. Échantillon mg/ml	
0.939972237	
0.399917006	
0.178828696	
0.131696626	
0.12901919	
0.236803852	
Agilent1100_labof103	
Conc. Échantillon mg/ml	
2.765230804	
2.709473545	
2.599857804	
2.331846682	
2.194990063	
1.913049496	
1.89555307	
1.713355348	
1.481751554	
1.295989482	
1.327515898	
Agilent1100_labof103	
Conc. Échantillon mg/ml	
2.393057591	
2.271847579	
2.237563279	
2.119498065	
1.953952927	
1.8038412	
1.587039645	
1.38498137	
1.373159162	







10.2 Formulas

The symbols in the following equations correspond to the description of section 0.

$$R_{s\,B/A} = 2\frac{t_{RB} - t_{RA}}{w_A + w_B} = 1.18\frac{t_{RB} - t_{RA}}{w_{0.5A} + w_{0.5B}} = 0.589\frac{\Delta t_R}{w_{0.5\ av}} = \frac{\Delta t_R}{w_{av}} = \frac{\Delta V_R}{w_{av}}$$

$$N = 16\left(\frac{t_R}{w}\right)^2 = \frac{t_R^2}{\sigma^2} = 5{,}54\left(\frac{t_R}{w_{0.5}}\right)^2$$

$$N = \frac{L}{H} = \frac{Lx}{\sigma^2} = \frac{L^2}{\sigma^2} = \frac{16\ L^2}{w_{base}^2}$$

$$N \approx \frac{41{,}7\ (t_R / w_{0.1})^2}{(b_{0.1} / a_{0.1} + 1{,}25)}$$

$$w = 4\,\sigma$$

HES-SO	Analytical procedure for the quantification of L-Glutamine in different medium containing serums using RP-HPLC with UV detection	SOP Edition : 1
	Domain : CA	

1. Principle of separation

Using a RP-HPLC with UV-Vis detector, this method allows quantifying of the L-Glutamine in different medium containing serum used for the culture of mammalian cells. The requirements of the method are presented in the following points:

- Range: the measuring range should lie between 0.1 and 8 mM (0.015 – 1.2 mg/ml)
- Accuracy: an accuracy (recovery) of +/- 10% is acceptable
- Precision : a precision of +/- 5% (repeatability) is acceptable

2. Area of application

The RP-HPLC method is formulated to provide a good separation of the L-Glutamine in complex matrix. The different mediums and serum are presented in the following table:

Table 30: All different mediums containing serums

	Metabolite
Medias with serum	L-Glu
SFM4CHO (Hyclone SH30548)	×
CDCHO	×
DMEM/Ham'F12 (Sigma D6421) with 10% FCS	×
DMEM/Ham'F12 (Gibico 10743011) with 10% FCS	×

3. Safety and precautions

Standard precautions are required for the handling of chemicals for the following method presented here.

4. Materials and reagants

- HPLC: Series 1100 Agilent
  - UV detector : G1315A
  - Auto sampler : G1313A
- pH Meter: Metrohm 654 pH-Meter
- Filters 3kDA: Nanostep 3K Omega, Life Science
- Micropipette: from Biohit
- Tips: Axigen scientific
- All glassware from the laboratory f103
- Centrifuge: Hettich, Mikro 200
- Vial 2 mL HPLC
- Analytical balance: Metler Toledo, laboratory f103.
- Filter 0.45 um, Exapure^{tm}, Syringe Filters PTFE, 0.45µm, 24 mm PTFE membrane
- HPLC Column: C18 gravity, Macherey-Nagel, 4,6mm x 150 mm, 3 µm, Serial N° : N8090623

Table 31: Reagents, provenance and safety

Compound name	Formula	Quality [%]	Origin	n° number catalogue	n° CAS	Safety	Notice
Acetonitrile	C2H2N	99.9	Lab-Scan	C73C11X	75-05-8	Xn, F	-
OPA	C8H6O2	99	Sigma	P0657	643-79-8	Corrosive, T	-
MeOH	CH4O	99.9	Lab-Scan	C17C11X	67-56-1	T, F	-
L-Glutamine	C5H10N2O3	99	Sigma	G3126	56-85-9	-	-
MQ Water	H2O	-	-	-	-	-	-
Glutamax^{tm}	C8H15N3O4	-	Invitrogen	A12860	-	-	200 mM solution
2-MCE	C2H6OS	>98	Flukka	63700		B3,D1A,D2B	-
Sodium dihydrogen phosphate	NaH2PO4.H2O		Acros Organics	A0331028	10049-21-5	-	-
Bicine	C6H13NO4	99	Sigma	B3876	150-25-4	-	-

5. Analytical procedure

5.1. Sample preparation

Aliquot periodically 1 mL from the culture of mammalian cells, filter it on a 0.45 µm filter (see section 4.). Store it in the freezer for further analyses.

If you use it directly, filter the sample on a 3kDa filter (see section 4.). In order to do it, take 200 µl of the sample and deposit it in the middle of the filter. A first run of 10 min at 15000 rpm in a centrifuge is needed. Then, once this is done, add 20 µl of H2O MQ in the middle of the filter in order to cover the entire surface. Run another 10 min with the centrifuge using the same conditions as before.

Once it is done, take 200 µl of the sample freshly filtered and add 400 µl of H2O in a HPLC vial. Again, if the samples are not used directly, store them in the freezer.

5.2. Solution preparation

- Buffer of NaH2PO4 (40mM, pH = 7,8) :

  1. Weigh exactly about 5.51 g of NaH2PO4.H2O in a 1 L beaker
  2. Fill up with about 800 ml of MQ Water
  3. Adjust the pH to a value of 7.8 ± 0.1 (using a calibrated pH meter, see section 4) under agitation with a solution of 1 M NaOH.
  4. Fill up with H2O MQ to the 1 l mark of the beaker.
  5. Shift the solution in a 1 l bottle.

- Solution of Bicine 0.5 M:

  1. Weigh exactly about 8.15 g of C6H13NO4 in a 100 ml beaker
  2. Fill up with about 80 ml of MQ Water
  3. If needed, Adjust the pH to a value of 8.5 ± 0.1 (using a calibrated pH meter, see section 4) under agitation with a solution of 1M NaOH. (the pka of bicine is 8.3)
  4. Fill up with H2O MQ to the 100 ml mark of the beaker.
  5. Fill up a 2 ml HPLC vial for further utilization.
  6. Stock it for maximum 7 weeks in ambient temperature.

- Solution of 1/45/54 2-MCE (table 2)/MeOH/bicine 0.5M:

  1. In a 100 ml flask, add 54 ml of bicine 0.5M
  2. Then, add 45 ml of MeOH
  3. To finish, add carefully 1 ml of 2-MCE
  4. Mix the solution gently
  5. Stock it for maximum 7 weeks in ambient temperature.

- Solution of OPA:

  1. Weigh exactly about 10 mg of OPA (table 2).
  2. Put it in a HPLC vial of 2 ml.
  3. Add 1 ml of the solution of 1/45/54 2-MCE (table 2)/MeOH/bicine 0.5M prepared before.
  4. Store it ambient temperature for a maximum of three days.

- Solution of calibration:

  1. Weigh about 70 mg of l-Glutamine (see table 2)
  2. Put it in a 100 ml volumetric flask
  3. Fill the volumetric flask to the mark
  4. Take 200 µl with a micropipette (see section 4.) and put it in a 2 ml HPLC vial.
  5. Then, add 460 µl of H2O MQ in the HPLC vial.
  6. The solution in the 100 mL can be conserved up to 4 week in the fridge.

- SST Solution :

  1. Weigh about 50 mg of l-Glutamine (see table 2)
  2. Put it in a 100 ml volumetric flask
  3. Fill the volumetric flask to the mark
  4. Take 200 µl with a micropipette (see section 4.) and put it in a 2 ml HPLC vial.
  5. Then, add 460 µl of H2O MQ in the HPLC vial.
  6. The solution in the 100 mL can be conserved up to 4 week in the fridge.

- Solution of 0.5% 2-MCE

  1. In a 2 ml HPLC vial, add 995 µl of bicine 0.5M
  2. Then, add 5 µl of 2-MCE
  3. Mix the solution gently when the septum is fixed
  4. This solution can be conserved up to 1 week at ambient temperature.

5.3. Method of analysis

5.3.1. Preparation of the auto sampler for the reaction of derivatization:

By worries of comprehensibility, the next figure will help to visualize the explanation that will follow:

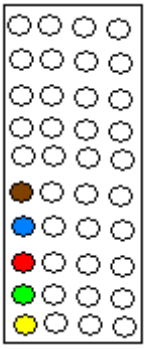


Figure 31: Shematic view of the auto sampler from 1100 series of agilent (not fully designed)

The first colored spot correspond to the first position of the auto sampler and will be crucial to follow the next explanation in order to put the right vial at the right spot.

- In yellow, this corresponds to the first spot of the auto sampler. Put the 2 ml HPLC vial with the solution of 0.5% 2-MCE
- In green, this corresponds to the second spot of the auto sampler. Put the 2 ml HPLC vial with the solution of bicine 0.5 M.
- In red, this corresponds to the third spot of the auto sampler. Put the 2 ml HPLC vial of the solution of OPA.
- For the position 4 and 5 that correspond to the blue and brown spots, put into both spot 2 ml HPLC vial containing H2O MQ.
- All the spot in blank are used to place the sample preparing following point 5.1.

5.3.2. Program for auto sampler injector

As is it the critical part of the method, the following lines as to be followed scrupulously. In order to enter the line code, it is necessary to open the last option (injection programing) of the injector of the agilent software. When it is done, copy the following line into the program:

1. DRAW, 2 µl, vial 1
2. NEEDLE WASH, in vial 5, 2 times
3. DRAW, 3 µl, vial 6 **[This part is variable and have to be increment for each sample if a sequence is done]**
4. NEEDLE WASH, in vial 5, 2 times
5. MIX, max. amount in air, max. speed, 5 times
6. DRAW, 3 µl, vial 2
7. NEEDLE WASH, in vial 5, 2 times

8. MIX, max. amount in air, max. speed, 5 times
9. DRAW, 3 µl, vial 3
10. NEEDLE WASH, in vial 5, 2 times
11. MIX, max. amount in air, max. speed, 5 times
12. WAIT, 0.1 min
13. MIX, max. amount in air, max. speed, 5 times
14. DRAW, 20 µl, vial 4
15. MIX, max. amount in air, max. speed, 5 times
16. MIX, max. amount in air, max. speed, 5 times
17. WAIT, 0.1 min
18. MIX, max. amount in air, max. speed, 5 times
19. MIX, max. amount in air, max. speed, 5 times
20. INJECT

5.3.3. Separation method

- Column: C18 gravity, Macherey-Nagel, 4,6mm x 150 mm, 3 µm, Serial N° : N8090623

- HPLC system: Series 1100 Agilent:

  -UV detector: G1315A
  -Auto sampler: G1313A

- Wavelength UV detector: 338,4 [nm]

- Temperature: 25°C, ambient temperature.

- Eluents: ACN and NaH2PO4 (40mM, pH = 7,8), see table 2 and section 5.2

Table 32: Eluent composition for the separation

Time [min]	%ACN	%Buffer	Flowrate [ml/mn]	max. pressure [bar]
13	14	86	0.8	300
15	60	40	0.8	300
17	60	40	0.8	300
19	14	86	0.8	300

Stop time: 21 min

5.3.4 Analysis progress

The following table will express a good way of using this method showing a sequence that can be used for daily purpose analysis:

Table 33: Example of one sequence injection

	n (number of injection)
SST solution 1	3
Calibration solution 1	3
Sample 1	3
Sample 2	3
Sample 3	3
Sample 4	3
Sample 5	3
Sample 6	3
Sample 7	3
Sample 8	3
Sample 9	3
Sample 10	3
SST Solution 1	3
Calibration solution 1	3

This is an example, assuming the time of one injection is about 30 min (with the time of the derivatization reaction) and there are 42 injections, the total time would be 1260 min that is 21 hours. As the OPA reagent in solution is stable for three days, it can still be used. Whatever, it is always good to use the solution of SST and calibration at the end of the sequence in order to check the reproductibility. The only indications are after about 40 injections is the change the vial 4 and 5 that contains H2O MQ and of course, take care of having enough buffer remaining (a 21 hours sequence corresponds to about 1 L of buffer solution).

6. Results

All the results come from the chromatogram where integrations of signals from UV (338 nm) detector are required. Retention times are also given in the chromatogram report.

7. Calculations

7.1. External calibration

With all data from the calibration solution (see table 3), it is possible to perform an external calibration. The calibration is done using a linear first order equation on excel where the concentration versus area/height of the peak of interest is fitted. For a better understanding, equations are presented and explained:

$$y = ax \quad <=> \quad x = \frac{y}{a} \qquad \text{Eq. 1}$$

y: peak area/height

x: concentration [mg/ml]

a: slope

The following figure will show an example of thus a calibration graphics:

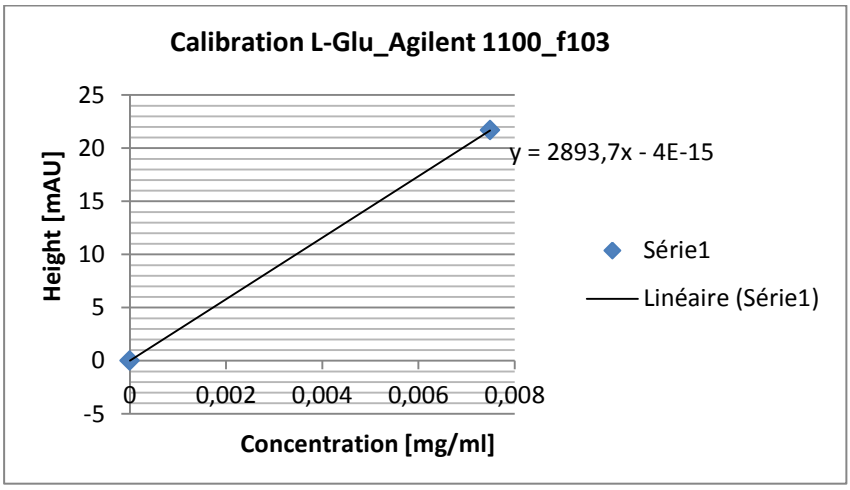


Figure 32: example of a calibration curve using height

There are several important things to notice here. First of all, when you draw this graph you have to take care of all the dilution that equals 34.09 times (1.1*3*10.333). Then you can use the Eq. 1 to calculate the concentration of samples.

7.2. SST Solution

In order to check if the user requirements are respected, there is some calculation needed. As it can be seen in the section 1., there is several user specifications to respect. In order to do that, use the following equation:

$$\frac{\text{conc. calculated}}{\text{conc. known}} \cdot 100 = \%\text{Recovery} \qquad \text{Eq.2}$$

In order to calculate the concentration, Eq.1 has to be used. Then it is possible to use Eq. 2. This equation (Eq.2) will give information about the accuracy. That mean the quality of the method to be the most accurate possible on a known concentration solution. It has to be at least 10% from the exact value. If it is not, the experiment has to be run again.

For the precision (repetability), only take the responses from the SST solution and calculate the standard deviation with the following equation. It has to be less than 5%. If it is not, the experiment has to be run again.

7.2 Retention times

For the retention times, it is necessary to check the dwell time between two instruments that are going to be used. It can change the retention time significally. The only requirement is +/- 0.2 min from the standard deviation from the retention time of the standard and from the samples one.

8. Annexe

8.1. Example of one chromatogram

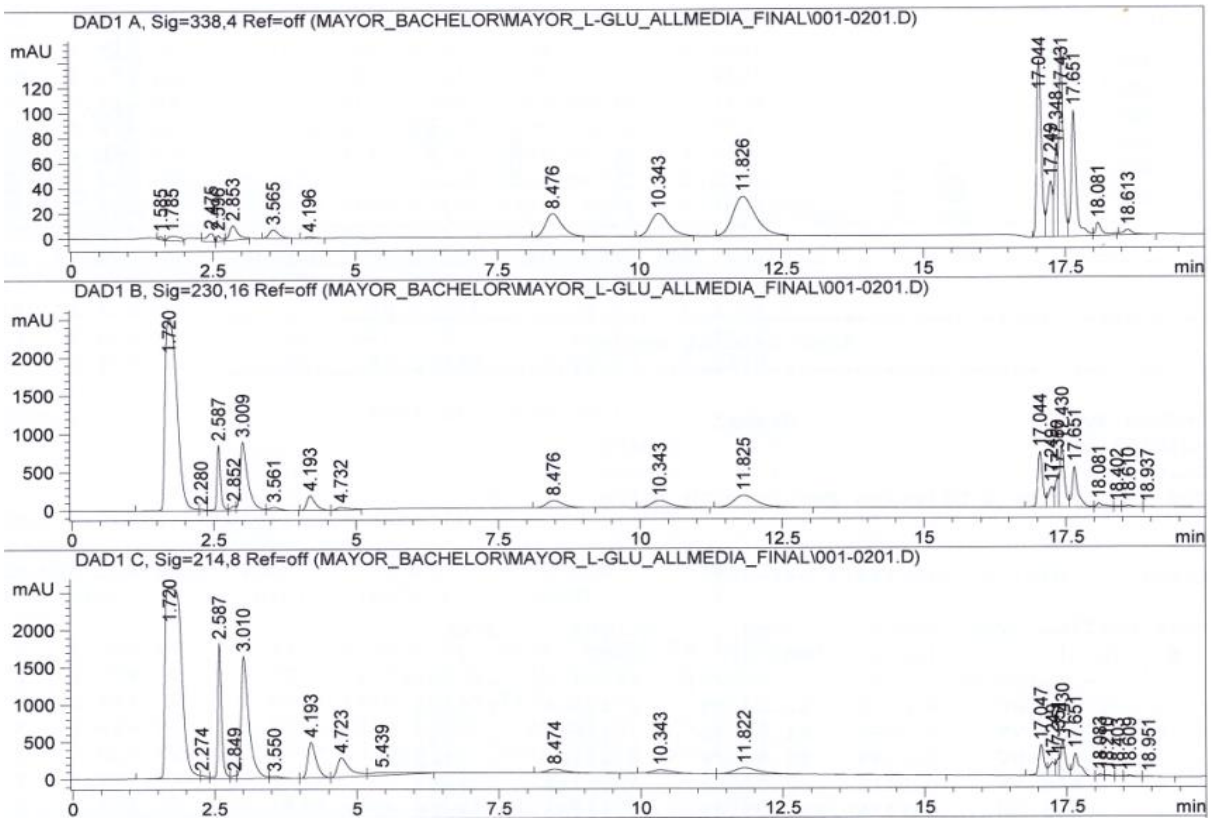


Figure 33: example of a chromatogram from L-Glu with SFM4CHO (Hyclone SH30548) using this method

HES-SO	Analytical procedure for the quantification of Glutamax™ in different medium containing serums using RP-HPLC with UV detection	SOP Edition : 1
	Domain : CA	

0. Principle of separation

Using a RP-HPLC with UV-Vis detector, this method allows quantifying of the L-Glutamine in different medium containing serum used for the culture of mammalian cells. The requirements of the method are presented in the following points:

- Range: the measuring range should lie between 0.1 and 8 mM (0.0217 – 1,7 mg/ml)
- Accuracy: an accuracy (recovery) of +/- 10% is acceptable
- Precision : a precision of +/- 5% (repeatability) is acceptable

1. Area of application

The RP-HPLC method is formulated to provide a good separation of the Glutamax™ in complex matrix. The different mediums and serum are presented in the following table:

Table 34: All different mediums containing serums

	Metabolite
Medias with serum	L-Glu
SFM4CHO (Hyclone SH30548)	×
CDCHO	×
DMEM/Ham'F12 (Sigma D6421) with 10% FCS	×
DMEM/Ham'F12 (Gibico 10743011) with 10% FCS	×

2. Safety and precautions

Standard precautions are required for the handling of chemicals for the following method presented here.

3. Materials and reagants

- HPLC: Series 1100 Agilent
  - UV detector : G1315A
  - Auto sampler : G1313A
- pH Meter: Metrohm 654 pH-Meter
- Filters 3kDA: Nanostep 3K Omega, Life Science
- Micropipette: from Biohit
- Tips: Axigen scientific
- All glassware from the laboratory f103
- Centrifuge: Hettich, Mikro 200
- Vial 2 mL HPLC
- Analytical balance: Metler Toledo, laboratory f103.
- Filter 0.45 um, Exapure^{tm}, Syringe Filters PTFE, 0.45µm, 24 mm PTFE membrane
- HPLC Column: C18 gravity, Macherey-Nagel, 4,6mm x 150 mm, 3 µm, Serial N° : N8090623

Table 35: Reagents, provenance and safety

Compound name	Formula	Quality [%]	Origin	n° number catalogue	n° CAS	Safety	Notice
Acetonitrile	C2H2N	99.9	Lab-Scan	C73C11X	75-05-8	Xn, F	-
OPA	C8H6O2	99	Sigma	P0657	643-79-8	Corrosive, T	-
MeOH	CH4O	99.9	Lab-Scan	C17C11X	67-56-1	T, F	-
L-Glutamine	C5H10N2O3	99	Sigma	G3126	56-85-9	-	-
MQ Water	H2O	-	-	-	-	-	-
Glutamax^{tm}	C8H15N3O4	-	Invitrogen	A12860	-	-	200 mM solution
2-MCE	C2H6OS	>98	Flukka	63700		B3,D1A,D2B	-
Sodium dihydrogen phosphate	NaH2PO4.H2O		Acros Organics	A0331028	10049-21-5	-	-
Bicine	C6H13NO4	99	Sigma	B3876	150-25-4	-	-

4. Analytical procedure

5.1. Sample preparation

Aliquot periodically 1 mL from the culture of mammalian cells, filter it on a 0.45 µm filter (see section 4.). Store it in the freezer for further analyses.

If you use it directly, filter the sample on a 3kDa filter (see section 4.). In order to do it, take 200 µl of the sample and deposit it in the middle of the filter. A first run of 10 min at 15000 rpm in a centrifuge is needed. Then, once this is done, add 20 µl of H2O MQ in the middle of the filter in order to cover the entire surface. Run another 10 min with the centrifuge using the same conditions as before.

Once it is done, take 200 µl of the sample freshly filtered and add 400 µl of H2O in a HPLC vial. Again, if the samples are not used directly, store them in the freezer.

5.2. Solution preparation

- Buffer of NaH2PO4 (40mM, pH = 7,8) :

  1. Weigh exactly about 5.51 g of NaH2PO4.H2O in a 1 L beaker
  2. Fill up with about 800 ml of MQ Water
  3. Adjust the pH to a value of 7.8 ± 0.1 (using a calibrated pH meter, see section 4) under agitation with a solution of 1 M NaOH.
  4. Fill up with H2O MQ to the 1 l mark of the beaker.
  5. Shift the solution in a 1 l bottle.

- Solution of Bicine 0.5 M:

  1. Weigh exactly about 8.15 g of C6H13NO4 in a 100 ml beaker
  2. Fill up with about 80 ml of MQ Water
  3. If needed, Adjust the pH to a value of 8.5 ± 0.1 (using a calibrated pH meter, see section 4) under agitation with a solution of 1M NaOH. (the pka of bicine is 8.3)
  4. Fill up with H2O MQ to the 100 ml mark of the beaker.
  5. Fill up a 2 ml HPLC vial for further utilization.
  6. Stock it for maximum 7 weeks in ambient temperature.

- Solution of 1/45/54 2-MCE (table 2)/MeOH/bicine 0.5M:

  1. In a 100 ml flask, add 54 ml of bicine 0.5M
  2. Then, add 45 ml of MeOH
  3. To finish, add carefully 1 ml of 2-MCE
  4. Mix the solution gently
  5. Stock it for maximum 7 weeks in ambient temperature.

- Solution of OPA:

  1. Weigh exactly about 10 mg of OPA (table 2).
  2. Put it in a HPLC vial of 2 ml.
  3. Add 1 ml of the solution of 1/45/54 2-MCE (table 2)/MeOH/bicine 0.5M prepared before.
  4. Store it ambient temperature for a maximum of three days.

- Solution of calibration:

  1. Weigh about 70 mg of l-Glutamine (see table 2)
  2. Put it in a 100 ml volumetric flask
  3. Fill the volumetric flask to the mark
  4. Take 200 µl with a micropipette (see section 4.) and put it in a 2 ml HPLC vial.
  5. Then, add 460 µl of H2O MQ in the HPLC vial.
  6. The solution in the 100 mL can be conserved up to 4 week in the fridge.

- SST Solution :

  1. Weigh about 50 mg of l-Glutamine (see table 2)
  2. Put it in a 100 ml volumetric flask
  3. Fill the volumetric flask to the mark
  4. Take 200 µl with a micropipette (see section 4.) and put it in a 2 ml HPLC vial.
  5. Then, add 460 µl of H2O MQ in the HPLC vial.
  6. The solution in the 100 mL can be conserved up to 4 week in the fridge.

- Solution of 0.5% 2-MCE

  1. In a 2 ml HPLC vial, add 995 µl of bicine 0.5M
  2. Then, add 5 µl of 2-MCE
  3. Mix the solution gently when the septum is fixed
  4. This solution can be conserved up to 1 week at ambient temperature.

5.3. Method of analysis

5.3.1. Preparation of the auto sampler for the reaction of derivatization:

By worries of comprehensibility, the next figure will help to visualize the explanation that will follow:

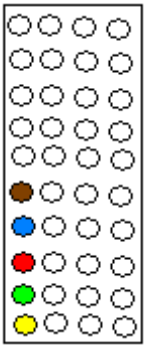


Figure 34: Shematic view of the auto sampler from 1100 series of agilent (not fully designed)

The first colored spot correspond to the first position of the auto sampler and will be crucial to follow the next explanation in order to put the right vial at the right spot.

- In yellow, this corresponds to the first spot of the auto sampler. Put the 2 ml HPLC vial with the solution of 0.5% 2-MCE
- In green, this corresponds to the second spot of the auto sampler. Put the 2 ml HPLC vial with the solution of bicine 0.5 M.
- In red, this corresponds to the third spot of the auto sampler. Put the 2 ml HPLC vial of the solution of OPA.
- For the position 4 and 5 that correspond to the blue and brown spots, put into both spot 2 ml HPLC vial containing H2O MQ.
- All the spot in blank are used to place the sample preparing following point 5.1.

5.3.2. Program for auto sampler injector

As is it the critical part of the method, the following lines as to be followed scrupulously. In order to enter the line code, it is necessary to open the last option (injection programing) of the injector of the agilent software. When it is done, copy the following line into the program:

0. DRAW, 2 µl, vial 1
1. NEEDLE WASH, in vial 5, 2 times
2. DRAW, 3 µl, vial 6 **[This part is variable and have to be increment for each sample if a sequence is done]**
3. NEEDLE WASH, in vial 5, 2 times
4. MIX, max. amount in air, max. speed, 5 times
5. DRAW, 3 µl, vial 2
6. NEEDLE WASH, in vial 5, 2 times

7. MIX, max. amount in air, max. speed, 5 times
8. DRAW, 3 µl, vial 3
9. NEEDLE WASH, in vial 5, 2 times
10. MIX, max. amount in air, max. speed, 5 times
11. WAIT, 0.1 min
12. MIX, max. amount in air, max. speed, 5 times
13. DRAW, 20 µl, vial 4
14. MIX, max. amount in air, max. speed, 5 times
15. MIX, max. amount in air, max. speed, 5 times
16. WAIT, 0.1 min
17. MIX, max. amount in air, max. speed, 5 times
18. MIX, max. amount in air, max. speed, 5 times
19. INJECT

5.3.3. Separation method

- Column: C18 gravity, Macherey-Nagel, 4,6mm x 150 mm, 3 µm, Serial N° : N8090623
- HPLC system: Series 1100 Agilent:

  -UV detector: G1315A
  -Auto sampler: G1313A
- Wavelength UV detector: 338,4 [nm]
- Temperature: 25°C, ambient temperature.
- Eluents: ACN and NaH2PO4 (40mM, pH = 7,8), see table 2 and section 5.2

Table 36: Eluent composition for the separation

Time [min]	%ACN	%Buffer	Flowrate [ml/mn]	max. pressure [bar]
13	14	86	0.8	300
15	60	40	0.8	300
17	60	40	0.8	300
19	14	86	0.8	300

Stop time: 21 min

5.3.4.Analysis progress

The following table will express a good way of using this method showing a sequence that can be used for daily purpose analysis:

Table 37: Example of one sequence injection

	n (number of injection)
SST solution 1	3
Calibration solution 1	3
Sample 1	3
Sample 2	3
Sample 3	3
Sample 4	3
Sample 5	3
Sample 6	3
Sample 7	3
Sample 8	3
Sample 9	3
Sample 10	3
SST Solution 1	3
Calibration solution 1	3

This is an example, assuming the time of one injection is about 30 min (with the time of the derivatization reaction) and there are 42 injections, the total time would be 1260 min that is 21 hours. As the OPA reagent in solution is stable for three days, it can still be used. Whatever, it is always good to use the solution of SST and calibration at the end of the sequence in order to check the reproductibility. The only indications are after about 40 injections is the change the vial 4 and 5 that contains H2O MQ and of course, take care of having enough buffer remaining (a 21 hours sequence corresponds to about 1 L of buffer solution).

6. Results

All the results come from the chromatogram where integrations of signals from UV (338 nm) detector are required. Retention times are also given in the chromatogram report.

7. Calculations

7.1. External calibration

With all data from the calibration solution (see table 3), it is possible to perform an external calibration. The calibration is done using a linear first order equation on excel where the concentration versus area/height of the peak of interest is fitted. For a better understanding, equations are presented and explained:

$$y = ax \quad <=> \quad x = \frac{y}{a} \qquad \text{Eq. 1}$$

y: peak area/height

x: concentration [mg/ml]

a: slope

The following figure will show an example of thus a calibration graphics:

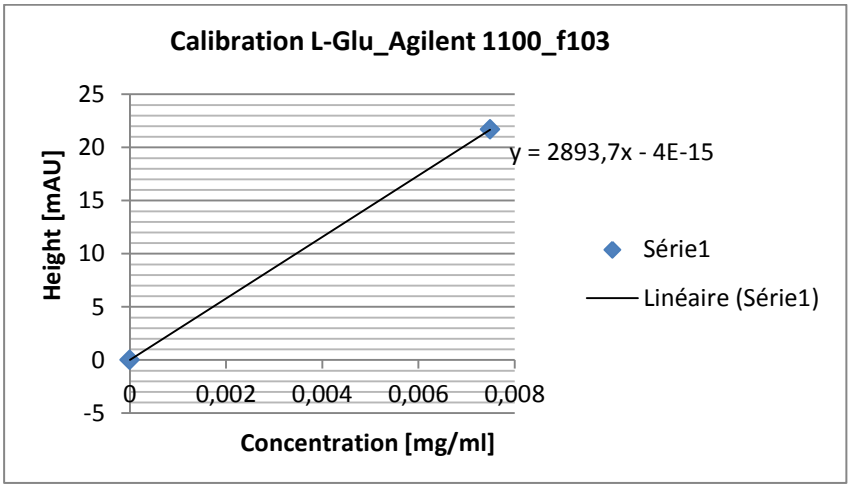


Figure 35: example of a calibration curve using height

There are several important things to notice here. First of all, when you draw this graph you have to take care of all the dilution that equals 34.09 times (1.1*3*10.333). Then you can use the Eq. 1 to calculate the concentration of samples.

7.2. SST Solution

In order to check if the user requirements are respected, there is some calculation needed. As it can be seen in the section 1., there is several user specifications to respect. In order to do that, use the following equation:

$$\frac{\text{conc. calculated}}{\text{conc. known}} \cdot 100 = \%\text{Recovery} \qquad \text{Eq.2}$$

In order to calculate the concentration, Eq.1 has to be used. Then it is possible to use Eq. 2. This equation (Eq.2) will give information about the accuracy. That mean the quality of the method to be the most accurate possible on a known concentration solution. It has to be at least 10% from the exact value. If it is not, the experiment has to be run again.

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7.3. Retention times

For the retention times, it is necessary to check the dwell time between two instruments that are going to be used. It can change the retention time significally. The only requirement is +/- 0.2 min from the standard deviation from the retention time of the standard and from the samples one.

8. Appendix

8.1. Example of one chromatogram

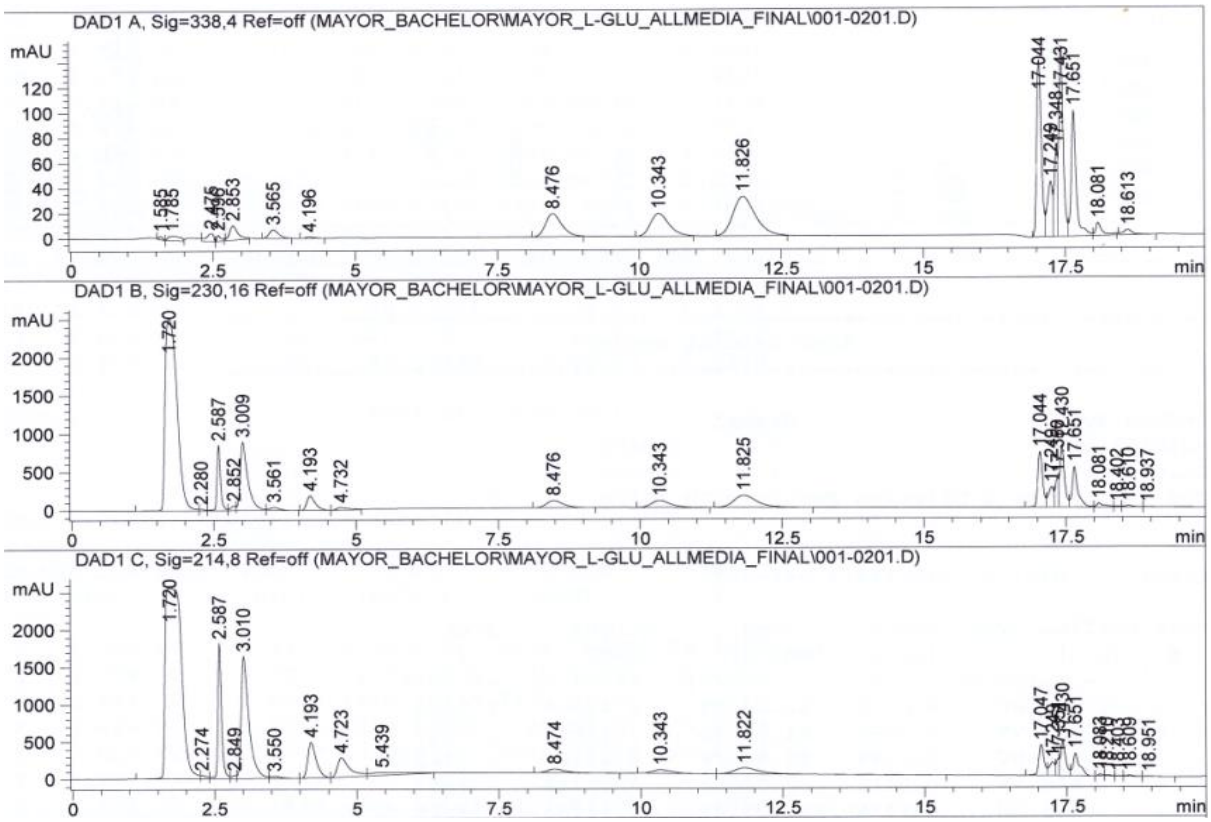

Figure 36: example of a chromatogram from Glutamax with SFM4CHO (Hyclone SH30548) using this method