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Isolation of ß-CTX, a molecule implicated in the follow-up of the treatment of osteoporosis

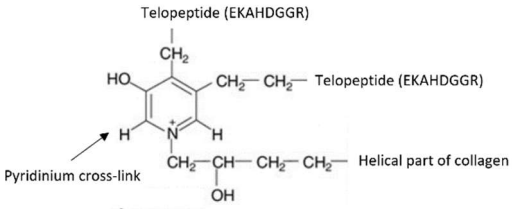

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TABLE OF CONTENT

ABBREVIATIONS

ACN	Acetonitrile
AF	Amonium Formate
ß-CTX	Beta C-terminal telopeptide
BMD	Bone Marrow Density
BSA	Bovine Serum Albumin
DMSO	Dimethyl Sulfoxide
DXA	Dual-energy x-ray absorptiometry
ECLIA	ElectroChemiLuminescence ImmunoAssays
ESI	ElectroSpray Ionisation
ELISA	Enzyme Linked ImmunoSorbent Assay
FA	Formic Acid
FRAX	Fracture risk assessment
HPLC	High Performance Liquid Chromatography
IDS	ImmunoDiagnostic System
IOF-IFCC	International Osteoporosis Foundation- International Federation of Clinical Chemistry
MS	Mass Spectrometry
m/z ratio	Mass to charge ratio
MeOH	Methanol
MRM	Multiple Reaction Monitoring
PBS	Phosphate Buffer Saline
PCFS	Post-Columnal Flow Splitting
SD	Standard Deviation
TOF	Time-Of-Fly
TQ	Triple Quadrupole
UPLC	Ultra-high Pressure Liquid Chromatography
2D	Two dimensions

ABSTRACT

As the population is becoming older, osteoporosis is more and more important worldwide. It affects 200 million women and costs enormously to society. Also, the treatment is not always effective, and the compliance is low. Adverse effects are really important and the absorption of molecules for bisphosphonates, which is the most common therapy, is less than 2%. A follow-up of the treatment is thus necessary, as it is not correctly taken. This follow-up is based on bone formation and resorption markers. In the present work, the focus will be realised on ß-CTX, a degradation product of type 1 collagen.

Immunoassays analysing the concentration of ß-CTX in biological samples gave different concentrations when the analysis was realised on a same sample. The work here was part of a larger one which consisted of creating a commutable standard to harmonize every immunoassay. The project was separated in different parts. The first one was the isolation of ß-CTX by liquid chromatography, followed by a characterization by mass spectrometry. Indeed, the structure was still unknown. Finally, the creation of an internal standard and a commutable product was planned. This work describes the isolation part.

The isolation was an important part of the work. Indeed, the more the sample was separated, the more ß-CTX was pure and the more the characterization was realised easily. Different parameters were tested, such as the addition of ammonium formate, formic acid and DMSO in phases. These were really efficient for the separation. Also, the gradient was optimized, as it was elongated to allow a better separation of the peaks. Finally, the injection solvent was modified, with the removing of the acetonitrile and the addition of DMSO.

All these modifications of parameters on the method are described in detail. They lead to a good separation of the peaks and it was thus possible to pass to the second part of the project which was the characterization.

1. Introduction

1.1 Osteoporosis

Osteoporosis is defined as a systemic skeletal disease whose main characteristics are low bone mass and microarchitectural deterioration of bone tissue. Decreased bone mass and worsened microarchitecture lead to a consequent increase in bone fragility and susceptibility to fracture as represented in Figure 1[1]. These fractures can lead to complications in patients and have a significant cost on society. Currently, the most common fractures are hip, vertebral and wrist fractures. These may generate clinical consequences. Hip fractures, for example, are the most devastating ones. Indeed, one out of five patients dies in the year after the facture, less than one third recovers completely and one out of four enters for the first time in a nursing home. One year after the fracture, 40% of the patients are still unable to walk independently. However, hip fractures are not the only ones, vertebral fractures are also important to consider as they will increase the morbidity and mortality of patients. One out of four women suffering from postmenopausal osteoporosis underwent a vertebral fracture which may lead to vertebral deformity[2]. The other types of fractures remain common even if the clinical consequences are less significant regarding life quality of the osteoporotic patients.

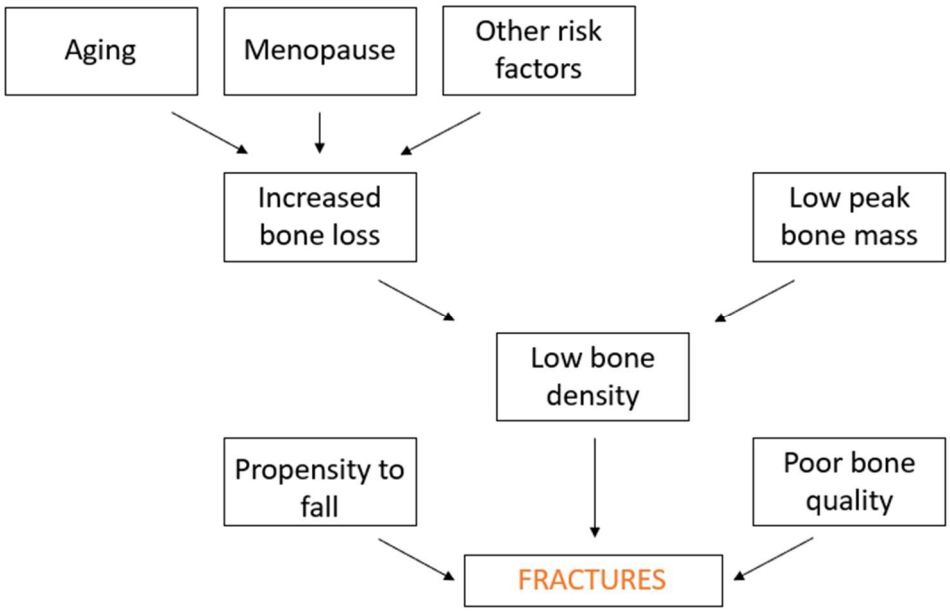

Figure 1 Pathogenesis of osteoporotic fractures, inspired from the review of the Nancy et al. about epidemiology, aetiology, and diagnosis of osteoporosis [3]

Nowadays, more than 200 million women worldwide are suffering from osteoporosis. This number will increase in the future with the aging of the population – including developing countries – as bone density decreases with age.

Regarding the economic burden, osteoporosis currently costs more to the society than breast cancer, stroke, diabetes or chronic lung diseases.

In conclusion, osteoporosis is a highly important disease to be treated and it should not be underestimated as it will become more and more present in the future [3].

1.1.1 Etiology

As said previously, osteoporosis is characterized by deteriorated bone micro-architecture and decreased bone density. Those clinical signs are caused by an imbalance between the bone formation and resorption. Bone resorption is increased due to several factors such as ageing, menopause or the intake of drugs including glucocorticoids. Decrease of bone mineral density (BMD) can be associated with different factors:

- Medical factors: malabsorption syndromes, inflammatory bowel disease, …
- Behavioural factors: smoking, physical activity, …
- Nutritionary factors: calcium intake, …
- Genetic factors[3,4]

Different categories of risk factors are represented: risk factors of fall, which can be subdivided in endogenous and exogenous factors and risk factors for osteoporosis which may lead to bone fragility. These risk factors will then increase the risk of fracture [5].

1.1.2 Diagnosis

The diagnosis of osteoporosis is based on the BMD assessment, which is measured as an amount of bone mass per unit volume. The gold standard method to assess the BMD is dual-energy x-ray absorptiometry (DXA). The measurement of BMD is essential as it will allow the assessment of a Z-score and a T-score. Z-score is defined as the number of standard deviations (SD) between an individual and the mean value expected for age and sex. The T-score can be defined as the number of SD between an individual and the mean value expected in young healthy individuals as represented in Figure 2[6]. The cut-off point to diagnose osteoporosis is a T-score of 2.5 SDs below the mean BMD of a healthy female population [5].

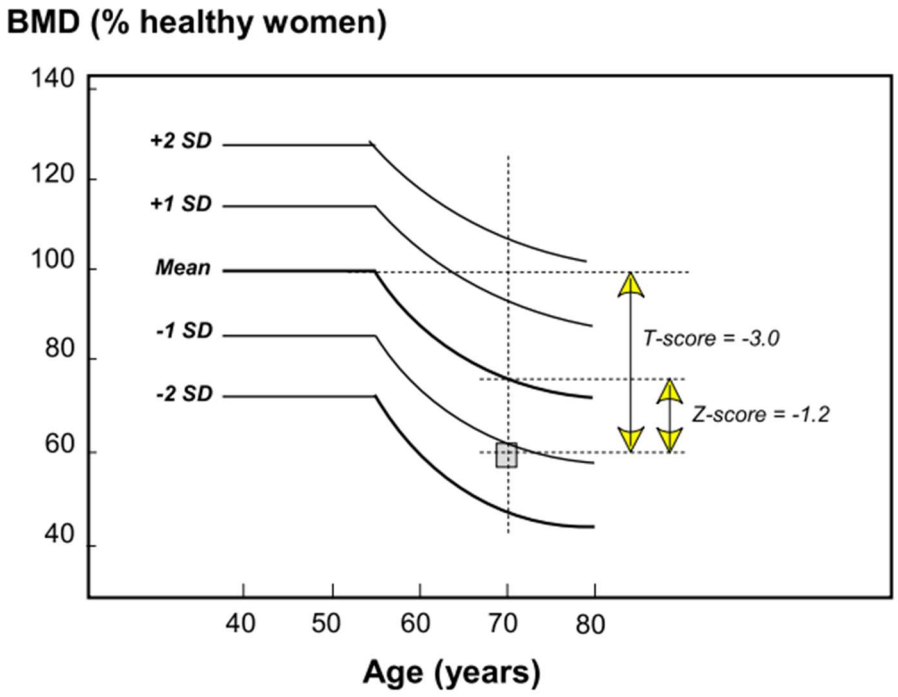

Figure 2 Graph plotting the mean BMD with SD intervals as a function of women age and the derivation of Z-scores and T-scores from BMD[5]

Bone turnover biomarkers are not used for the diagnosis of osteoporosis. Indeed, only 20% of postmenopausal women diagnosed as osteoporotic by DXA had serum ß-CTX concentrations corresponding to osteoporosis [4].

FRAX (fracture risk assessment) is another diagnostic tool commonly used to predict the likelihood of fracture in 10 years. The tool is based on an algorithm that takes into account some risk factors namely age and sex, as well as the medical background. The BMD is not strictly necessary for the FRAX algorithm, it is only an option that can be provided [7].

1.1.3 Treatment

Different types of treatments were developed for osteoporosis, such as oestrogen, SERMs, denosumab or teriparatide, but the most often prescribed one remains bisphosphonates.

1.1.3.1 Bisphosphonates

Bisphosphonates are the most efficient treatments for osteoporosis. Indeed, they lead to a decrease up to 70% and 50% in vertebral and hip fractures, respectively [8].

Different types of bisphosphonates exist: Alendronate, Risedronate and Zoledronate. The most commonly prescribed treatment is oral Alendronate because of its prize and effect on fracture prevention. If the patient does not respond to the treatment, oral Risedronate will be prescribed. In case of severe osteoporosis, Zoledronate will be advised. However, a day of hospitalization is necessary. For all these treatments, a glomerular filtration rate above 30ml/min is necessary [9,10].

Serious adverse effects were described, even if they rarely occur: atypical femur fractures and osteonecrosis of the jaw. Inflammatory reactions, such as conjunctivitis, uveitis, iritis, episcleritis, scleritis or keratitis, or atrial fibrillation may also occur. Moreover, the efficacy after 5 years of treatment remains unproven [9].

Another well-known problem of bisphosphonate is the administration method. Indeed, the administration protocol remains hard to effectively follow for patients and absorption of the molecules does not exceed 2%[9].

Side-effects, combined with the complicated and ineffective administration protocol, lead to low compliance. Due to all of those factors, a follow-up protocol was developed to assess the response-to-treatment and the compliance of the patients.

1.1.4 Follow-up

Several markers were developed and recognized by the International Osteoporosis Foundation-International Federation of Clinical Chemistry (IOF–IFCC) Bone Marker Standards Working Group for the assessment of the bone turn over:

- Markers of bone formation: bone-specific alkaline phosphatase, Procollagen type I C propeptide
- Markers of bone resorption: carboxy-terminal cross-linking telopeptide of type I collagen, pyridinoline, carboxy-terminal crosslinking telopeptide of type I collagen [11].

An algorithm proposed by European Calcified Tissue Society and IOF is available to help in the follow-up of the treatment, as can be seen in Figure 3.

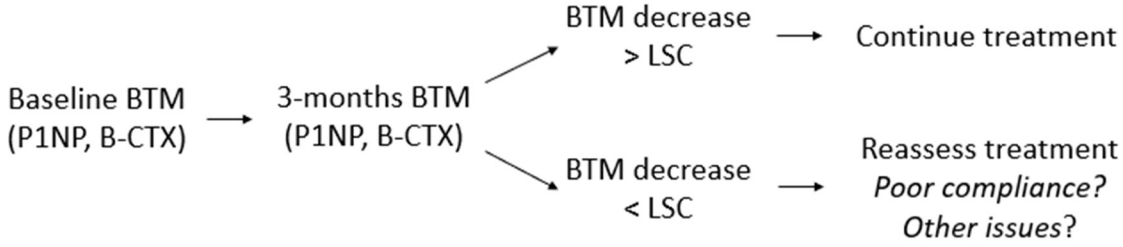

Figure 3 Algorithm used for the follow-up of the treatment

1.1.5 Limitations

1.1.5.1 Immunoassays

Three commercially available immunoassays were developed to assess Beta C-terminal telopeptide (ß-CTX) concentrations:

- IDS-Crosslaps Enzyme Linked ImmunoSorbent Assay (ELISA)
- IDS-iSYS CTX-I Crosslap
- Automated chemiluminescence immunoassays (ECLIA) B-Crosslap by Cobas (Roche)

Immunoassays are based on antibodies directed against an octapeptide present in ß-CTX. The octapeptide is composed of eight amino acids which are: E-K-A-H-D-G-G-R.

1.1.5.1.1 Crosslaps ELISA

Crosslaps ELISA is a manual immunoassay realised in microwell plates.

The method used is a sandwich method. Indeed, as represented in Figure 4, a first antibody, coated in a well, is used to capture the octapeptide. A second antibody is used for the detection. Indeed, antibodies are coated with biotin. The biotin binds a streptavidin combined with horseradish peroxidase (HRPO). HRPO will react with a revelation solution, and the colorimetry will be assessed to give the exact concentration of the sample.

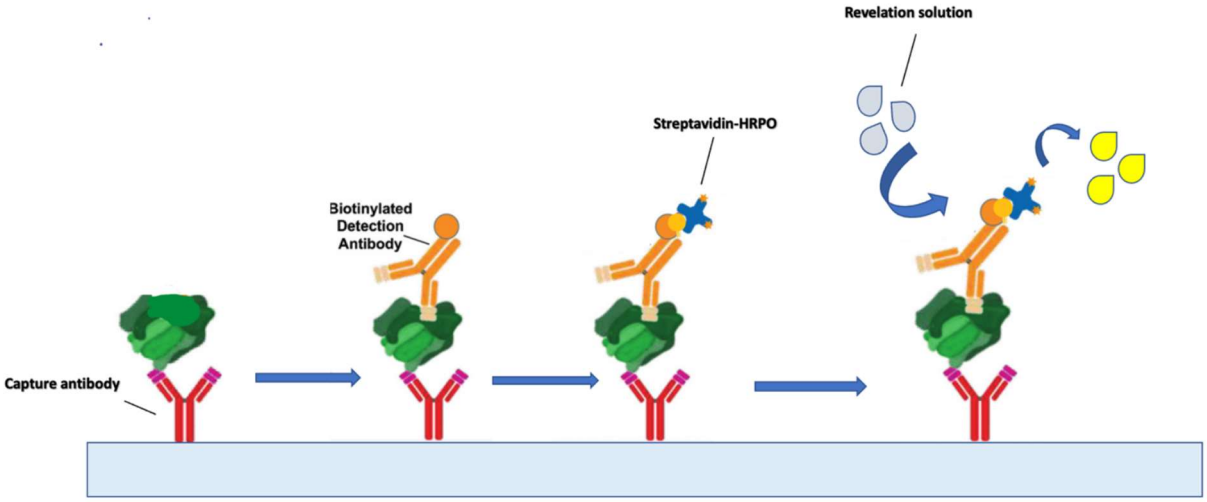

Figure 4 Cross-laps ELISA

1.1.5.1.2 IDS-iSYS CTX Crosslaps

The IDS-iSYS CTX Crosslaps is also based on a sandwich method. In this case, the first antibody is conjugated with a biotin, and the second one with acridinium. The antibody combined with the biotin interacts with streptavidin associated with magnetic particles. A wash is necessary to suppress excess antibodies and solvent. After that, an activation reagent is used for the interaction with the acridinium and produces a chemiluminescence that will be detected by a highly sensitive photomultiplier. It is represented in Figure 5.

1.1.5.1.3 ECLIA Cobas

ECLIA is used on Cobas immunoanalyzers.

The principle is the same as IDS-iSYS CTX Crosslaps, with a sandwich-method. However, differences exist between the different methodologies. Indeed, IDS-iSYS CTX Crosslaps uses acridinium conjugated antibodies while ECLIA Cobas uses ruthenium labelled antibodies. Certain reagents are also different. It is represented in Figure 5.

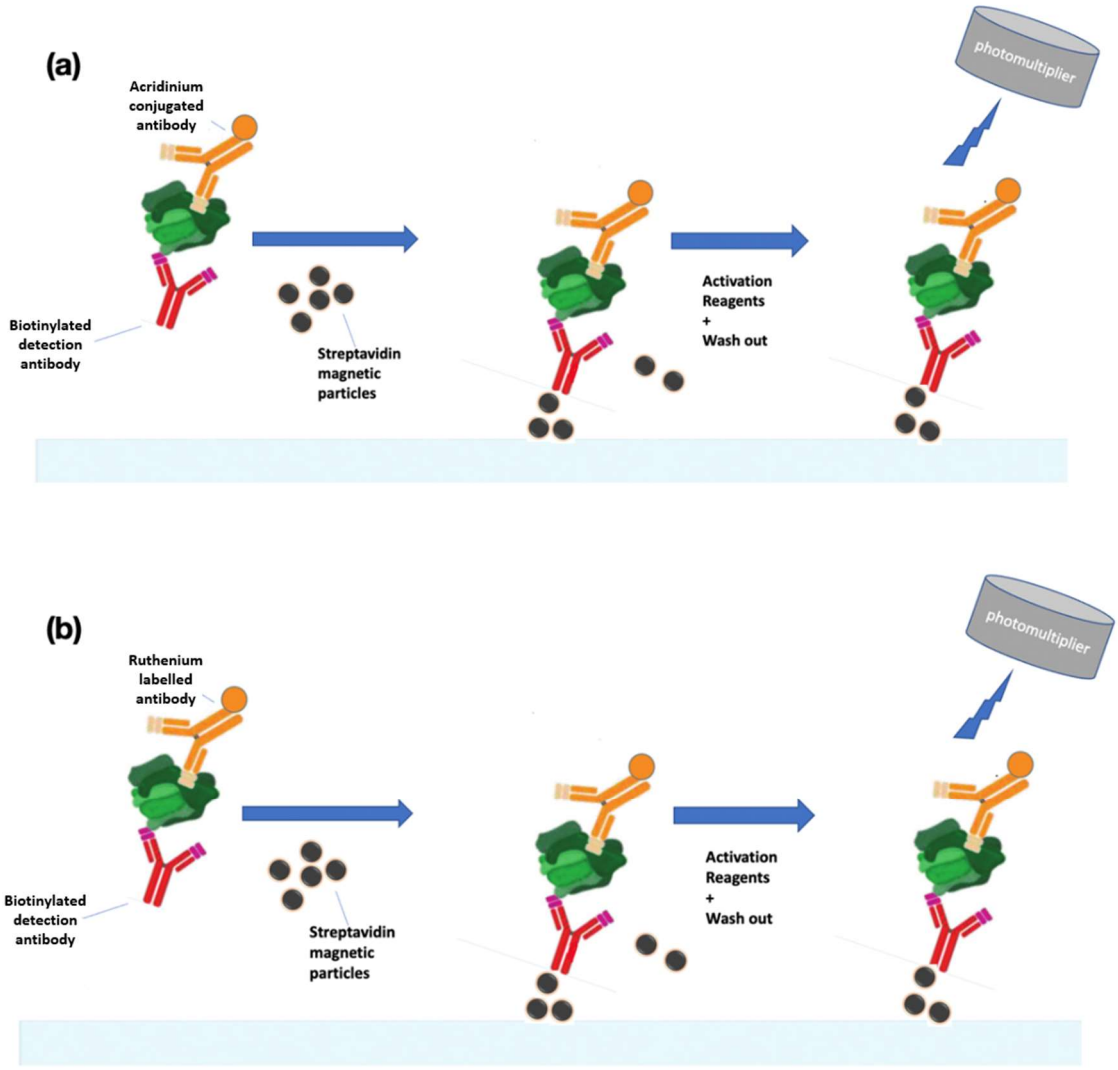

Figure 5 (a) *IDS-iSYS CTX Crosslaps (b)* ECLIA Cobas

1.1.5.2 Passing-Bablok plot

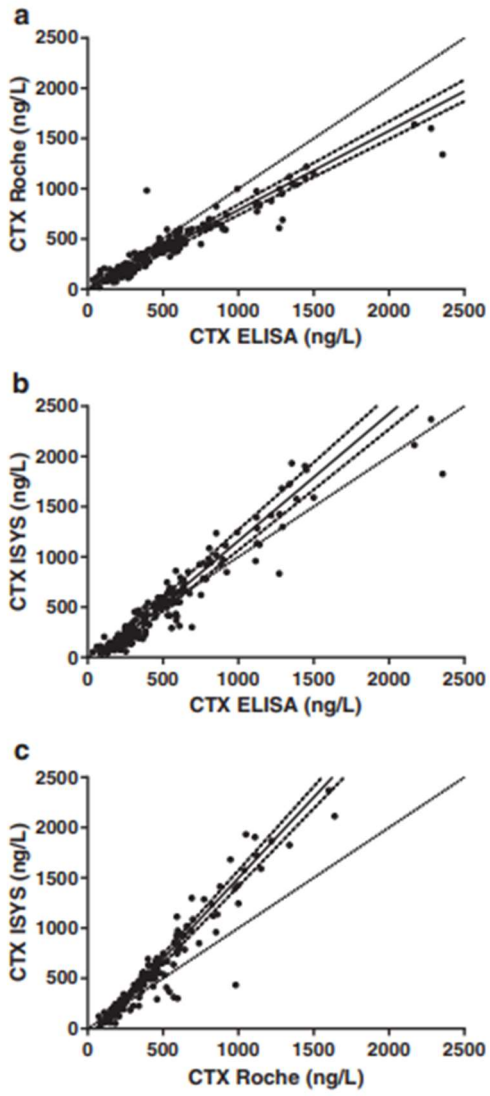

Figure 6 Passing-bablock plot of the different immunoassays[12]

A Passing-Bablok test was realised to compare the three methods, as shown in Figure 6.The results indicated that concentrations obtained with the different kits were statistically significantly different, which means that for a same sample, different concentration will be provided depending on the immunoassay kit. Thus, the analysis showed important lack of standardization in the currently available methods for the quantification of ß-CTX[12].

The structure and sequence of ß-CTX remain unknown, which makes the standardization process difficult.

1.2 ß-CTX

1.2.1 Collagen

ß-CTX is a degradation product of the type I collagen. The latter is a triple helix made up of two α1-chains and one α2-chain. The chains have a helical section thanks to the repetition, in their primary structure, of three amino acids: Gly-X-Y in which Y is often a proline or a hydroxyproline increasing the molecule stability while X can be either a proline or lysine.

The helical section is flanked by non-helical parts called telopeptides as represented in Figure 7 [13]. Telopeptides are not characterized by the Gly-X-Y structure which is why they do not form triple helixes. They represent 2% of the total molecule and they are essential to fibril formation [14]. Aspartic acid in the telopeptide can be isomerized in α-form or ß-form.

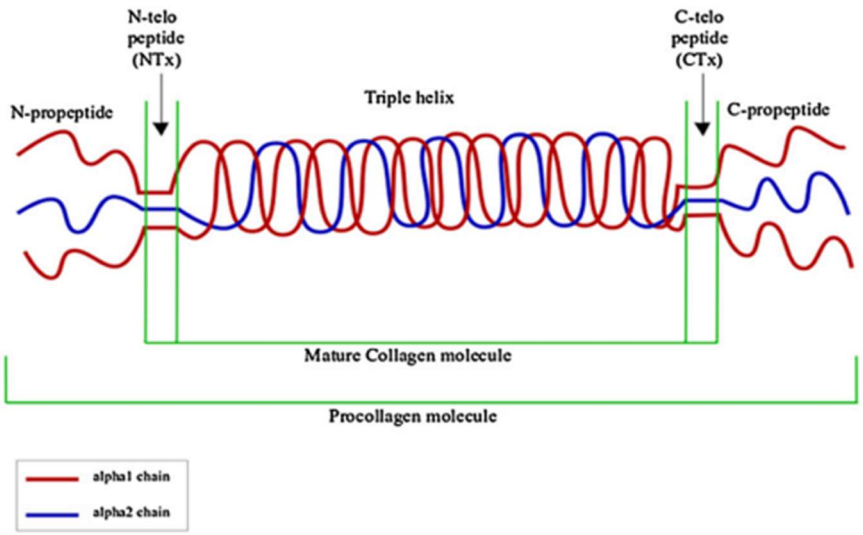

Figure 7 Structure of the collagen[13]

1.2.2 Cross-links

Triple helix structures are stabilized by covalent links. These covalent links are called cross-links and can be either divalent or trivalent cross-links.

Regarding divalent cross-links, they are less stable and can easily be reduced [13]. The first step of the cross-link's formation is the aldehydation of a lysine or hydroxylysine on a helical section by the lysin oxidase to obtain an allysine and hydroxyallysine, as shown on Figure 8. In bone tissues, divalent cross-links are mostly formed from hydroxylysine in contrast with soft tissues which display more lysin-based cross-links. Once formed, hydroxyallysine will be combined with a lysine or an hydroxylysine from another collagen molecule telopeptide, leading to the formation of a divalent cross-link. Divalent cross-links can then maturate into trivalent ones by adding an aldehydated lysine or hydroxylysine from another telopeptide of the same collagen molecule [13,14]. Thus, trivalent cross-links bind two collagen proteins together[13,14]. However, it is important to note that depending on the last bound residue, the crosslinks will be different. If the last residue involved in the cross-links is an allysine, the cross-links core will be a pyridinoline while if last residue is an hydroxyallysine, it will form a pyrrole [13]. Depending on the nature of the secondly bound residue, the trivalent cross-link will be either deoxypyridinoline (DPL) or deoxypyrrololine (DPD), as shown in Figure 9.

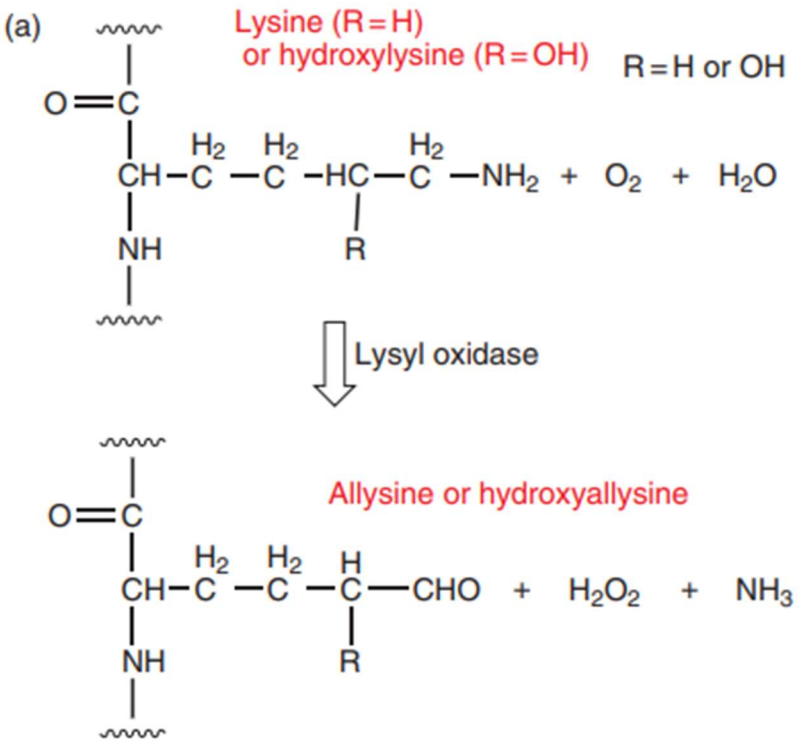

Figure 8 Oxidation of lysine and hydroxylysine in allysine and hydroxyallysine[15]

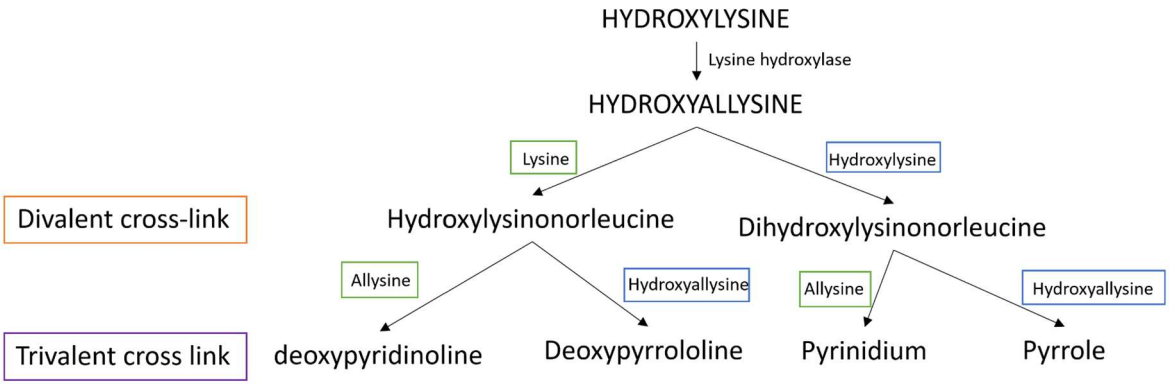

Figure 9 Different trivalent bounds following their components, inspired by 1 and 9

1.2.3 Structure

ß-CTX is a collagen degradation product, made up of two telopeptides sections and a helical bound together by a pyridinoline cross-links. Indeed, during bone resorption, ß-CTX is cleaved by cathepsin K and then enter the bloodstream [14]. As pyridinoline cross-link always occurs at the same lysin/hydroxylysine residue, the group of Fledelius et al. were able to partially characterize the section of the telopeptide which was involved in the cross-link [16]. An octapeptide was characterized: EKAHDGGR. Thus, the octapeptide must be present twice in ß-CTX as two telopeptides are involved in the cross-links while the third peptide comes from the helix and is thus helical, as displayed in Figure 10. The antibodies used in the immunoassay's kits were produced by ImmunoDiagnostic System (IDS) and target the octapeptide.

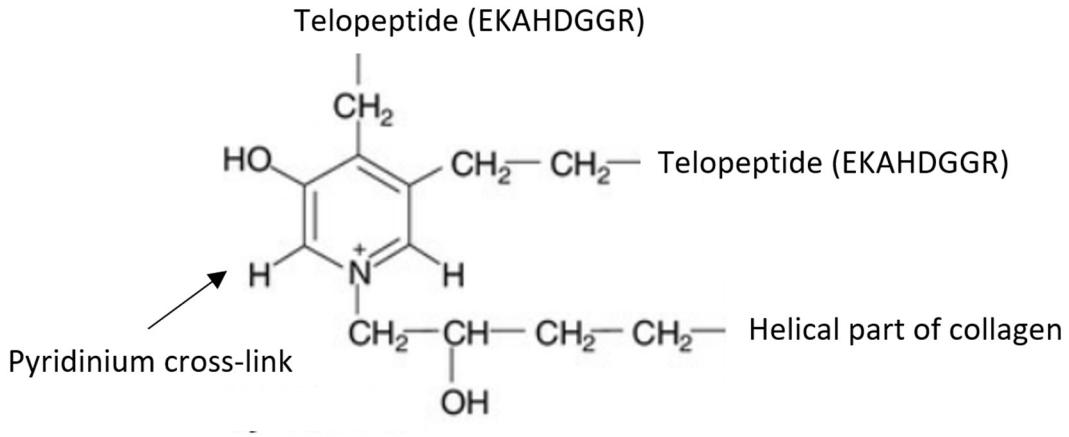

Figure 10 Different amino acids composing the telopeptide, and the ones that are implicated in cross-links. The 19th amino acid, the aspartic acid is implicated in the isomerization

Figure 11 represents the different amino acids composing the telopeptide, and the ones that are implicated in cross-links. The 19th amino acid, the aspartic acid is implicated in the isomerization.

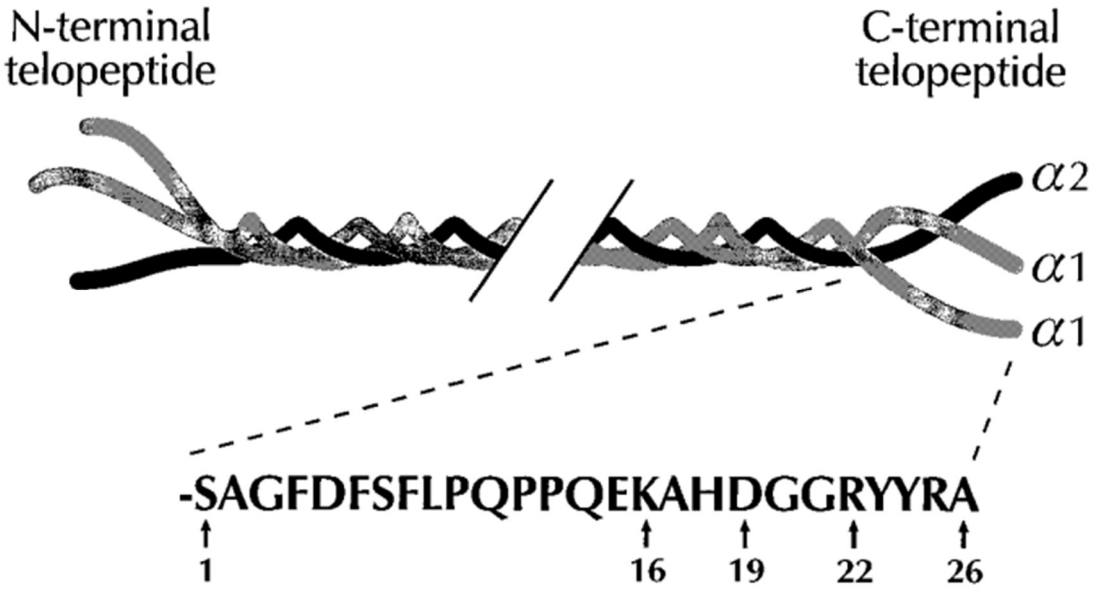

Figure 11 Collagen with the amino acids involved in the crosslinks[16]

As ß-CTX is made up of three peptides linked by a pyridinoline cross-link, different isomers may coexist. Indeed, aspartic acid present in the octapeptide can be α-isomer or ß-isomer but also L-isomer or D-isomer. Thus, four different isomers (i.e., L-α-isomer, D-α-isomer, L-ß-isomer and D-ß-isomer) are present in different percentages as represented in Figure 12. The native peptide isomer is a L-α-isomer. The three other isomers are age-related isomers [17].

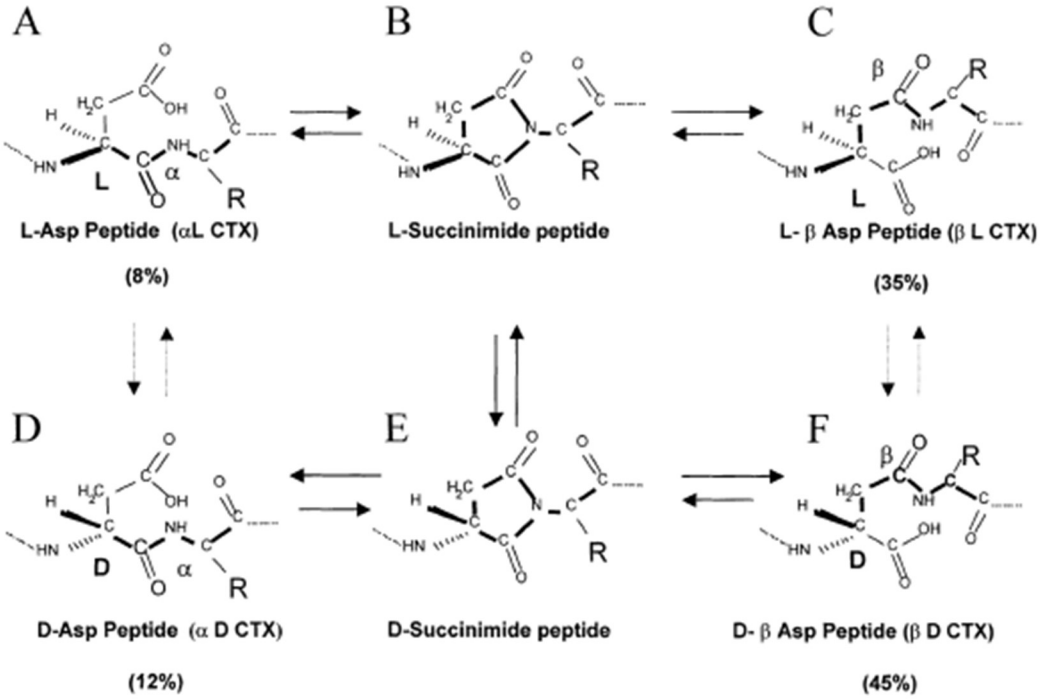

Figure 12 Different types of isoforms of CTX[17]

1.3 High Pressure Liquid Chromatography (HPLC)

1.3.1 Principles

HPLC is a separation technique in which different compounds of a solution are separated depending on their interactions with the mobile and the stationary phase.

Complex instrumentations are necessary for the correct functioning of HPLC system, as represented in Figure 13.

Solvent reservoirs, mostly bottles made of glass, contain mobile phases. Several solvent reservoirs can be used in order to create a gradient with a mobile phase A, often water and additives, and a mobile phase B, a mixture of organic solvents and additives. The gradient allows a quicker elution of compounds with widely different physicochemical properties within the same run. The reservoirs are connected to the pumps by a tubing system. A degassing module prevents air bubbles in the mobile phase and thus, in the tubing system providing a good pressure stability.

Pumps are used to pump a specified flow of mobile phase and the sample through the analytical column. Injection system introduces a defined volume of the sample in the tubing system thanks to an injection valve. The sample is mixed with the mobile phase before going through the column.

The analytical column, maintained in an oven at a defined and steady temperature, separates the compounds of the sample depending on their affinity for the stationary and the mobile phase. Different types of chemistry are available for the stationary phases such as C18 which is the most common chemistry used for the reverse phase chromatography.

Once separated, the sample compounds are analysed and detected in order to obtain a chromatogram. Different detectors are available, such as mass spectrometry or UV detector [18].

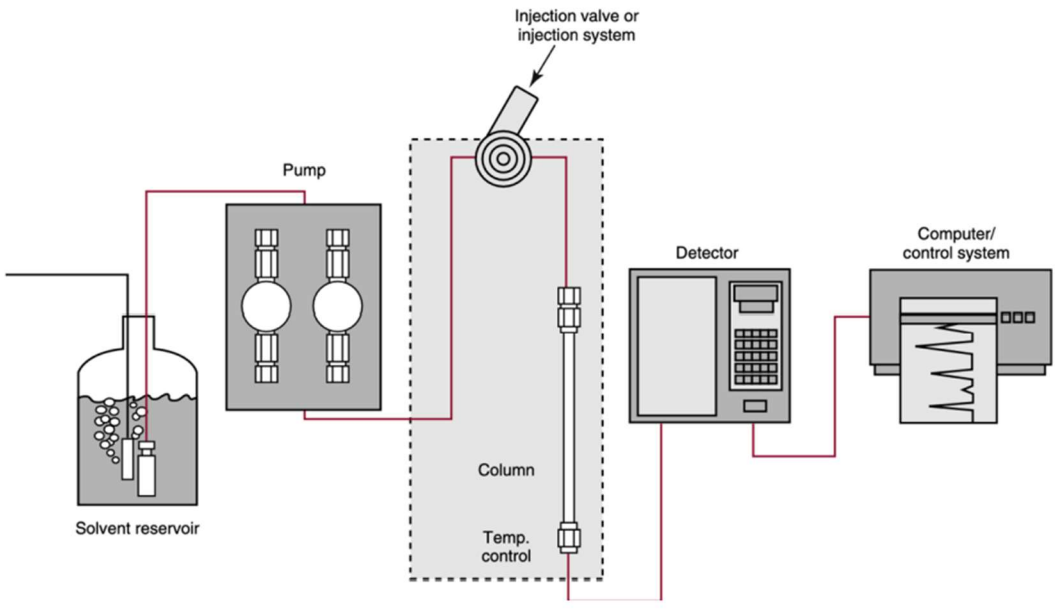

Figure 13 Instrumentation of HPLC

1.3.2 Parameters

1.3.2.1 Efficiency

The efficiency is measured as the capacity of a chromatographic system to make a peak thinner at his base as represented in Figure 14. To measure the efficiency, the height equivalent of a theoretical plate (EHPL or H) is used. The equation is:

$$H = L/N$$

Where H is the height equivalent of a theorical plate, L is the length of the column and N is the number of theoretical plates.

The number of theoretical plates is represented by the number of peaks width that can fit in a column. It is divided by the length of the column as the longer the column is, the higher the number of theoretical plates will be.

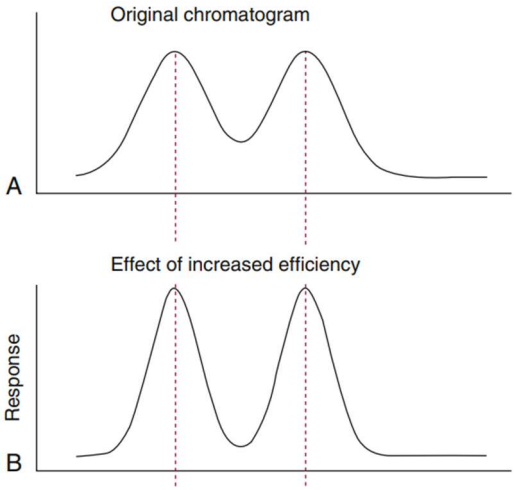

Figure 14 Representation of the effect of an increased efficiency

1.3.2.2 Resolution and peak capacity

Resolution is a relative measurement of separation between two different peaks. Indeed, it is based on the retention time and the width of the corresponding peaks. Often, it is considered that two peaks are completely separated if the resolution is about 1.50. Two peaks are correctly separated if the resolution is higher than 1 as shown in Figure 15.

The peak capacity is defined as the number of different peaks (corresponding to different components of the sample) that can be theoretically seen on a chromatograph.

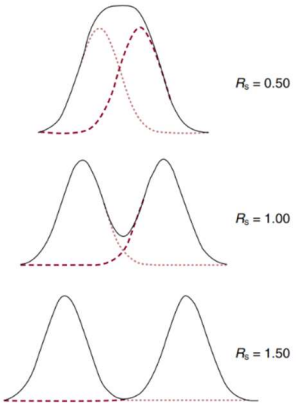

Figure 15 Resolution of two peaks

1.3.2.3 Selectivity

Selectivity represents the affinity of a sample compound for the stationary and mobile phases. It is represented by a retention factor, that is measured k = (TR-TM)/TM, where TR is the retention time and TM the void time. Higher selectivity leads thus to higher separation.

1.3.3 Ultra-High Pressure Liquid Chromatography (UHPLC)

UHPLC is a liquid chromatography using columns packed with sub-2 µm particles. Because of the size particles, the back pressures are higher.

The Van Deemter equation is useful to determine the efficiency of the chromatographic system. It is defined as:

$$H = A + \frac{B}{\mu} + C\mu$$

H = plate height = efficiency

A = Eddy diffusion constant

B = Longitudinal diffusion constant

C = Mass transfer kinetic constant

µ = Linear velocity

The A term (Eddy diffusion) is mainly determined by the size of particles and by how the chromatographic bed is packed. The uniformity of the flow is also important.

The B term (longitudinal diffusion) can be described as the dilution of the compounds in the mobile phase occurring during their transit in the column and tubing system. It decreases when the velocity of the mobile phase increases.

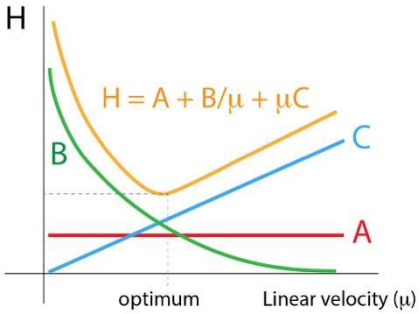

Figure 16 Representation of the Van Deemter equation

"The C term (mass transfer) depends on the interaction of the compounds with the internal surface of the particle pores. The mass transfer increases with the velocity of the mobile phase and the size of the particles.

The three parameters and their best combinations are represented in Figure 16.

As said before, the diminution of the size of the particles in UHPLC technology leads to a reduction of the Eddy's diffusion and the mass transfer. Thus, UPHLC is a highly effective technique as the analysis is faster while keeping the same efficiency [19].

1.4 Mass spectrometry

1.4.1 Overview

Mass spectrometry (MS) is an analytical technique widely used for the detection, characterisation and quantitation of biomolecules such as peptides and proteins.

First, analytes are ionised by an ion source. Secondly, these ions can be separated or selected depending on their mass to charge ratio (*m/z* ratio) in one or multiple mass analyser(s). Finally, the detection of ions is represented by a mass spectrum where ion intensities are plotted as a function of the *m/z* as schematized in Figure 17 and Figure 18.

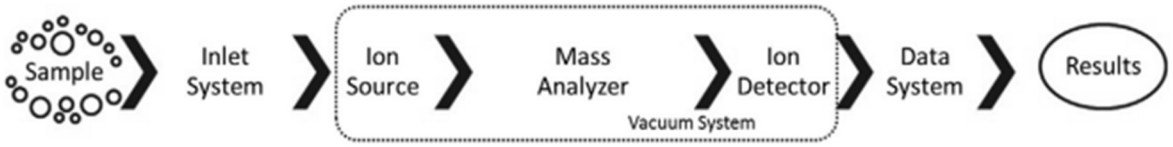

Figure 17 Representation of the different step of a mass spectrometer

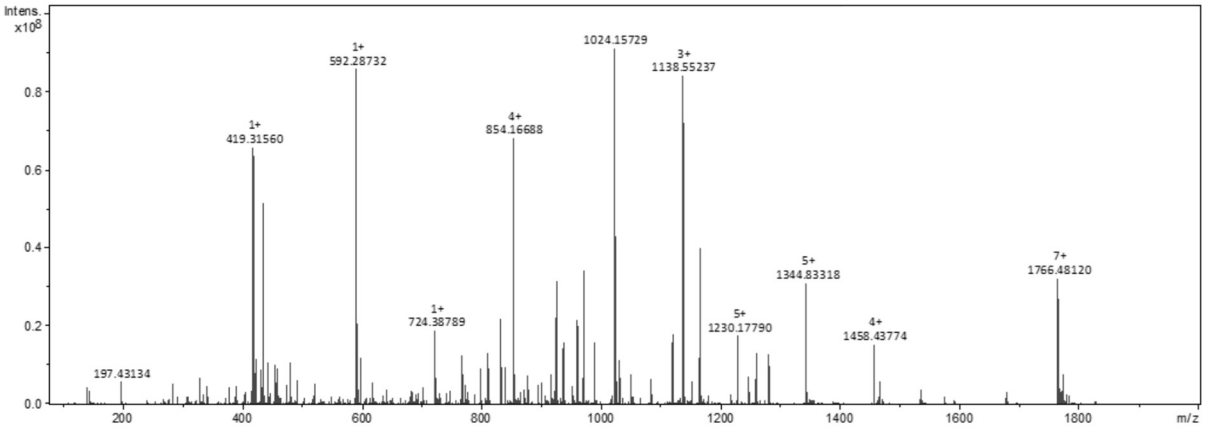

Figure 18 Representation of a mass spectra[31]

1.4.2 Ion source

As the analytes have to be ionized to be analysed and detected, the ion source is an essential component of the mass spectrometer. Electrospray ionization (ESI), represented in Figure 19 is commonly used for the ionization of proteins and peptides as it leads to multiply charged analytes. Moreover, ESI is considered as a soft ionization meaning that the structure of the analyte will be more easily preserved [20].

The flow coming from the inlet method enters the capillary and undergoes high potential difference. This high potential difference leads to the ionisation in solution of the analytes. When the flow exits the capillary, small droplets containing the analyte are shaped and form the spray. Thanks to the gas and the heat, the droplets evaporate until the Rayleigh limit is reached meaning that the surface tension can no longer sustain the charges. Columbic explosion then occurs leading to the formation of smaller droplets. The process is repeated until the solvent is fully evaporated, releasing the charged analyte. This analyte can be mono- or multi- charged [21].

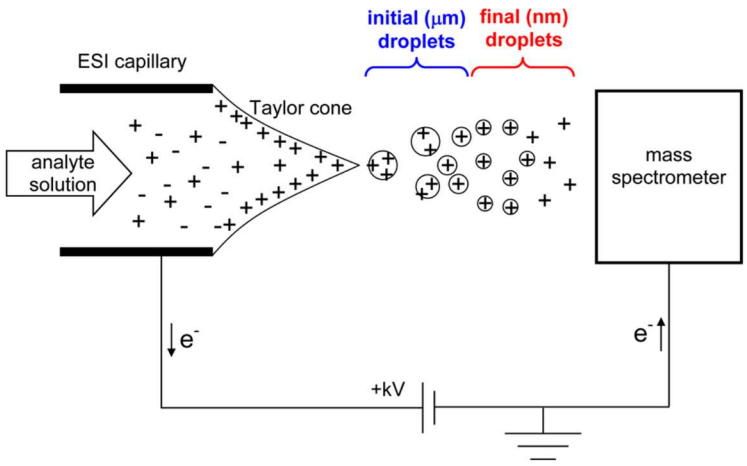

Figure 19 Representation an ESI ion source [22]

1.4.3 Mass analysers

The mass analyser is localized after the ionisation source. Different mass analyzers exist, such as quadrupole and time of flight (TOF).

1.4.3.1 Quadrupole

The most commonly used analyzer for the analysis and quantitation of proteins and peptides is the quadrupole. It is composed of four metallic parallelly disposed rods. Two opposite rods are electrically connected. Only the ions characterized by a specified *m/z* ratio have a stable trajectory and reach the detector. Thus, the quadrupole mainly acts as a *m/z* filter [23,24]. Limitations are lower resolution and limited mass range leading to complications for the analysis of larger molecules. A quadrupole is represented in Figure 20.

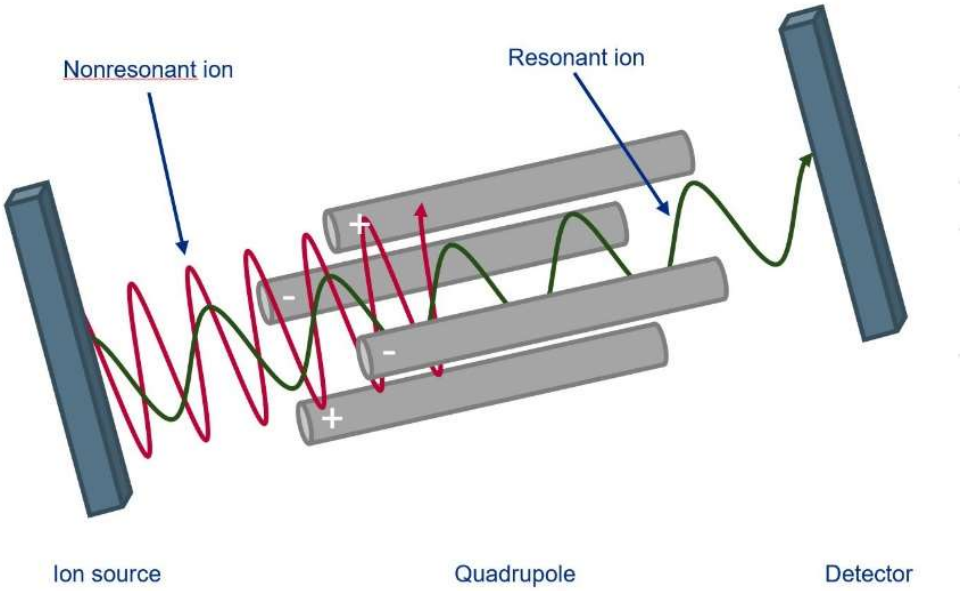

Figure 20 Representation of a quadrupole[a]

1.4.4 Experiment

1.4.3.1 Triple quadrupole

A triple quadrupole (TQ) instrument consists of three successively disposed quadrupoles. However, the second quadrupole serves as collision cell. Different types of analysis can be realised on a TQ, such as Q1 scan for example. Q1 scan is an analysis in which all ions will go through the quadrupoles while the collision cell will not be effective, as represented in Figure 21. The purpose of this experiment is to find the *m/z* ratio of the parent ions in order to define the transitions. Transitions are a couples of the *m/z* ratios of a parent ion and a daughter ion. Each compound can be characterized by multiple transitions.

The product ion analysis is an experiment during which the first quadrupole will select a precise *m/z* ratio while the third one will let everything pass through after fragmentation. The goal is to find the *m/z* ratio of the daughter ions still with the aim of finding the transitions.

TQ are essentially used for quantitation, in this case, the multiple reaction monitoring (MRM) is used. During MRM, several transitions are monitored. In fine, the first and third quadrupole select a m/z ratio while the second one fragments.

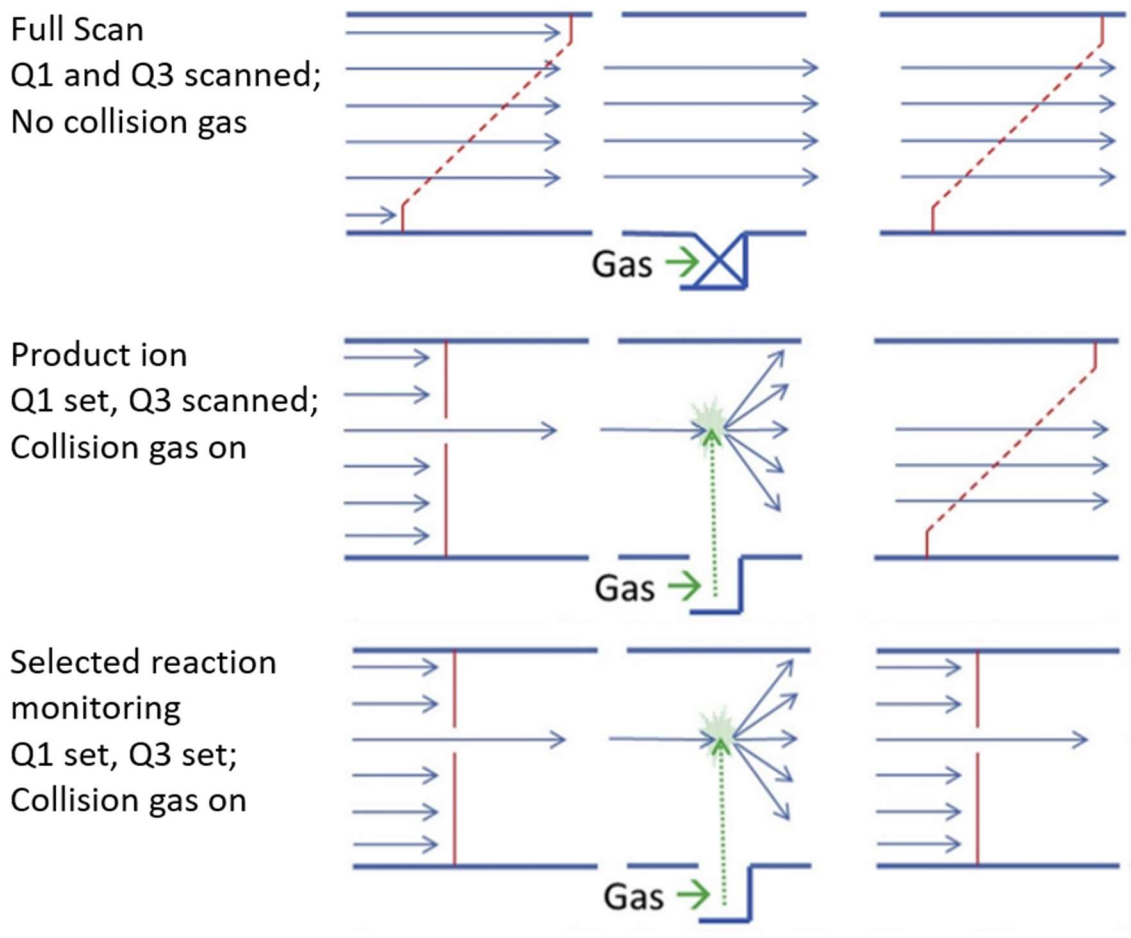

Figure 21 Different modes of a triple quadrupole: (1) Full scan (2) Product ion (3) Selected reaction monitoring

2. Objectives/Strategy

2.1 Objectives

The goal of the present work was to develop a preparative liquid chromatography in order to isolate ß-CTX from other components of the matrix.

This work is part of a larger project aiming the production of a commutable standard which will allow the standardisation of immunoassays. Indeed, as ß-CTX structure remains unknown, an isolation by preparative liquid chromatography followed by a characterisation of the molecule by High Resolution Mass Spectrometry (HR-MS) is needed. After the identification of the structure, a LC-MS/MS method will be developed in order to quantitate accurately in plasma.

2.2 Strategy

The strategy is explained in Figure 22. First, a preparative liquid chromatography coupled to post-column flow injection will be realised. The plate containing the fractions will be then evaporated for solvent exchange to ensure compatibility with the immunoassays and reconstituted with 150 µl of PBS. Reconstituted fractions will then be assessed for their concentration of ß-CTX and elution profiles will be drawn in order to identify retention times. Highly concentrated fractions will undergo analysis by HR-MS. The accurate mass and primary structure of ß-CTX will be defined by HR-MS. Once the structure defined, synthesis of native and internal standards will be ordered. With these standards, a LC-MS/MS method will be developed and validated. Finally, human plasma samples will be quantified by LC-MS/MS and sent to manufacturer for standardisation.

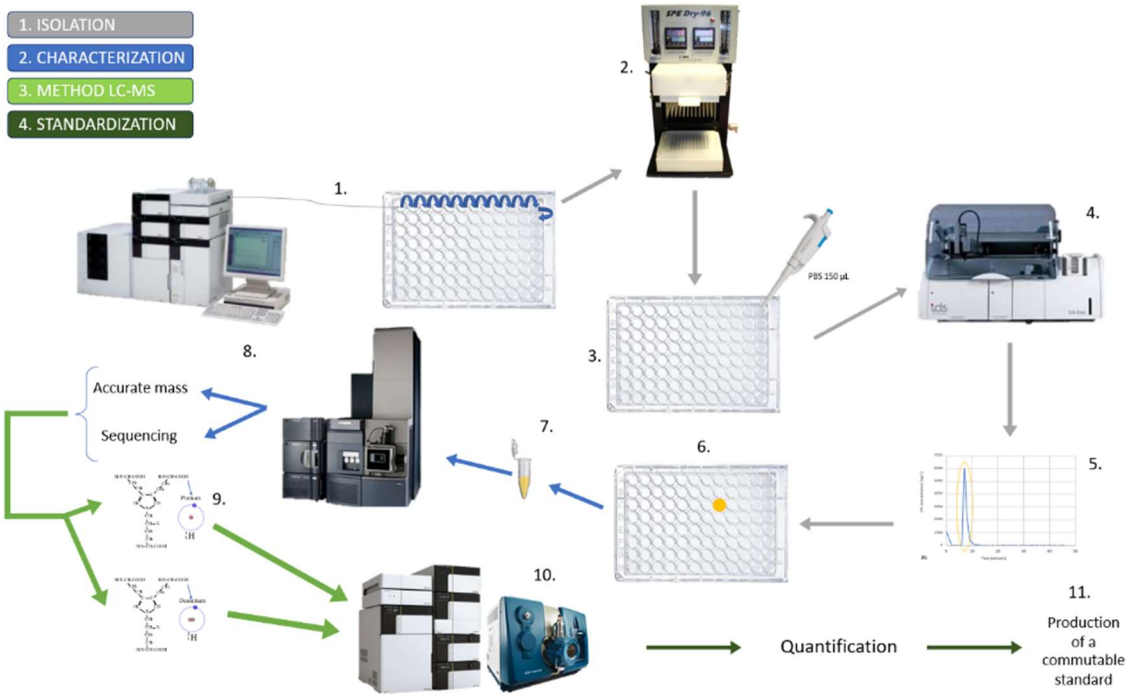

Figure 22 Description of the method (1) HPLC coupled to post-column flow splitting (2) Evaporation (3) Reconstitution (4) Immunoassay (5) Elution profile (6) Identification of the retention time (7) Concentration of ß-CTX (8) Mass spectrometry (9) Synthesis of internal and native standards (10) Development and validation of a LC-MS/MS method (11) Production of commutable standards

3. Material and methods

3.1 Reagents and instruments

3.1.1 Reagents

- Zinc sulfate heptahydrate ReagentPlus® ≥99.0% (CAS: 1446-20-0), Ammonium Formate (CAS: 540-69-2) and Bovine Serum Albumin (BSA) heat shock fraction, pH 7 were provided by SigmaAldrich (Saint Louis, Missouri, US)
- "Top dose" concentrated urine was provided by IDS immunodiagnostic systems Holdings Ltd (Boldon, UK)
- LC-MS Formic acid (CAS: 64-18-6) was provided by Fisher Chemicals (Loughborough, UK)
- PBS buffer powder was provided by Immuno Concepts (Sacramento, US)
- IDS-iSYS CTX-I (CrossLaps®) Assay (IDS immunodiagnostic systems Holdings Ltd (Boldon, UK)
- LC-MS Grade Water (CAS: 7732-18-5), LC-MS Grade Acetonitrile (CAS: 75-05-8) and LC-MS Grade Methanol (CAS: 67-56-1) were provided by Biosolve BV (Valkenswaard, The Netherlands)

3.1.2 Instruments

- Nexera X2 UPLC (Shimadzu, Kyoto, JP)
    - 3 binary pumps LC-30AD
    - Temperature control for samples and column CTO-20AC
- Sciex QTRAP 6500 (Framingham, MA, US)
- SPE Dry 96 Biotage (Uppsala, SE)
- MicroStar Let Hamilton (Manitowoc, US)
- IDS-iSYS Multi-Discipline Automated System (IDS im munodiagnostic systems Holdings Ltd, Boldon, UK)
- Centrifuge 5415R (Eppendorf AG, Hamburg, DE)
- Concentrator SpeedDry RVC 2-25 CH plus (Christ, Osterod am Harz, DE)
- Evaporator Ankersmit Techne DRI.BLOCK DB.3 (Techne, Staffordshire, UK)
- XSelect PREMIER peptide HSS T3 100 Å 2.5 µm 2.1 x 150mm (waters Milford, MA, US)
- ThermoMixer® C (Eppendorf AG, Hamburg, DE)

3.2 Methods

3.2.1 Protein precipitation

First, 200uL of sample were pipetted in 2mL Eppendorfs. 50µl of H_2O, $ZnSO_4$ 10% (w/v) were added to the sample before being vortexed. 100µl of ACN were then added to the mix prior to homogenisation. This step was realised in a thermomixer at 2000 rpm for 20 minutes at 4°C. After homogenisation, samples were centrifugated at 4000 rpm for 10 minutes. Supernatant was transferred in Protein LoBind 96 deep-wells Plate from Eppendorf. Supernatants were evaporated until dry under heated nitrogen flow and reconstituted with 100 µl H_2O, 5% ACN, 0.2% FA, 0.1% BSA.

3.2.2 Liquid Chromatography

The chromatographic separation was achieved on the column XSelect PREMIER Peptide HSS T3 100A 2.5um 2.1 x 150mm. Optimized mobile phases were constituted of H_2O, 0.2% FA, 10 mM AF for Phase A and 90% ACN, 10% methanol, 0.2% FA, 10mM AF for phase B. The oven temperature, the injection volume and the flow rate were respectively, 40°C, 50µl and 0.5 ml/min. The gradient started at 0% phase B. After 30 seconds, percentage of phase B increased to reach 19.8% in nine minutes. A purge of 1 minute at 90% phase B was then applied, followed by a 4-minutes equilibration at 0% phase B.

4. Results and discussion

4.1 Previous work

First, the column ACQUITY UPLC peptide BEH C18 130 Å 1.7 µm 100 mm, 2.1 mm from Waters was operated using mobile phases constituted of water, 0.2% formic acid (FA) for phase A and acetonitrile (ACN), 0.2% FA for phase B. The column with a C-18 chemistry was chosen as it is the most common chemistry for the analysis of proteins and peptides. The gradient consisted of a slope going from 5% phase B to 100% phase B in 45 minutes followed by a 10-minutes purge. Post-columnal flow splittings (PCFS) were performed and the ß-CTX concentration of each fraction was assessed by immunoassay. Different experiments were performed to optimize HPLC parameters, however, ß-CTX could not be retained by the column.

After these results, more polar stationary phases such as Luna Omega 1.6 µm polar C18 100 Å 100 x 2.1 mm and Luna C8 30 100 Å 50 x 2 mm from Phenomenex and Kinetex 2.6 µm PFP 100 Å 100 x 2.1 mm from Phenomenex were tested.

No retention was obtained. However, these experiments allowed the identification of a characteristic of ß-CTX which was its hydrophilicity.

The approach was thus changed. Instead of changing the stationary phase, the initial conditions regarding the mobile phase were changed. The gradient of the mobile phase, beginning at 5% phase B, was decreased to 0% of phase B.

4.2 Workflow

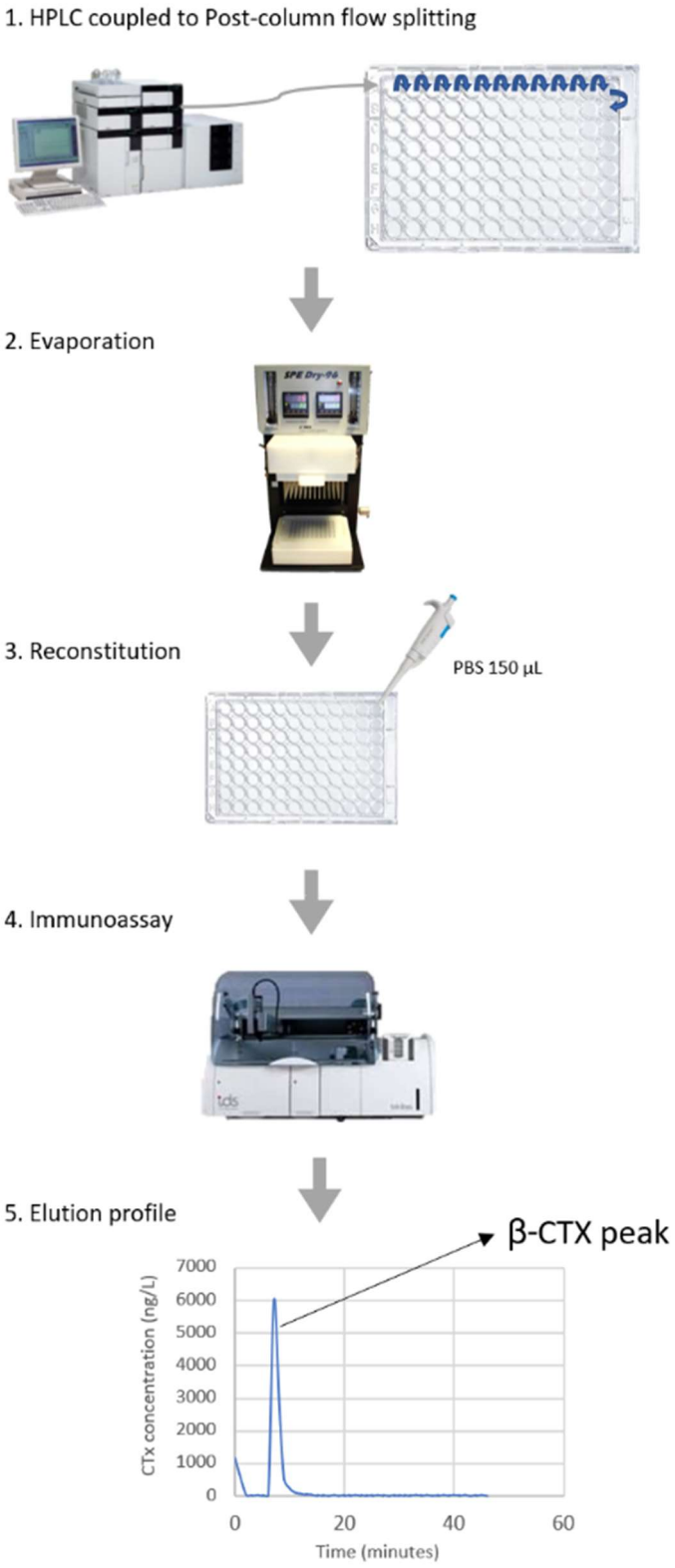

Figure 23 Workflow, constituted of (1) a HPLC coupled to post-columnal flow splitting, followed by an (2) evaporation and then (3) a reconstitution. Finally, (4) immunoassays are used for the analysis of the concentration of each well and this allows the creation

As the final aim of the work is to characterize ß-CTX, optimization of the LC is necessary to isolate the compound prior to its characterisation. Indeed, as the compound remains uncharacterized, no standard is available and thus, analysis by tandem mass spectrometry is impossible. PCFS coupled to immunoassay detection was realised to optimize the liquid chromatography (LC) parameters, in order to get better peak shape and a higher isolation. PCFS was realised for each optimization parameter.

The workflow, represented in Figure 23, is divided in 5 steps. The first one is the PCFS performed on an ultra-high pressure liquid chromatography (UPLC), during which fractions are collected at regular intervals throughout the analysis. Then, solvent exchange, including evaporation and reconstitution, is realised to ensure compatibility with the immunoassay. Reconstituted fractions were then assessed by immunoassay. Finally, elution profiles are produced with the data obtained from the immunoassay.

4.3 Optimization of the preparative liquid chromatography parameters

4.3.1 Addition of Bovine Serum Albumin (BSA) in injection solution

EXPERIMENTAL DESIGN

1. Sample

The sample was a top-dose sample provided by IDS. This sample consists of concentrated and treated urine. The top-dose has been precipitated and evaporated. After the evaporation, samples had to be reconstituted. Reconstitution was realised with two different conditions:

1) Condition BSA-: H_2O, 5% ACN, 0.2% FA
2) Condition BSA+: H_2O, 5% ACN, 0.2% FA, 0.1% BSA

ACN was added to the reconstitution solution in order to increase the solubility of the proteins while FA was added to increase ionisation. Adding 0.1% BSA to solutions is well-known to prevent adsorption to the plastic containers. Moreover, BSA tends to coat the free silanols of the column leading to decreased secondary interactions. Indeed, BSA has an isoelectric point of 4.7-4.9 which means that in acidic conditions, it will be charged positively. The positive charge present on BSA will interact with the negative ones of the free silanols.

2. Liquid chromatography parameters

Regarding the column, the XSelect PREMIER peptide HSS T3 100 Å 2.5 µm 2.1 x 150 mm was chosen for its compatibility with 100% aqueous medium. The limited number of free silanols of this column should lead to a sharper peak shape and thus, a higher peak capacity. The column was heated at 40°C in the oven. The flow rate was 0.5 mL/min and the injection volume was 50 µL.

During PCFS, fractions were collected every minute and the run lasted 45 minutes following a continuous gradient.

The mobile phase was composed of:

1. Phase A: H_2O, FA 0.2%
2. Phase B: ACN, FA 0.2%

The first tested condition was BSA+.

After PCFS, fractions were evaporated under heated nitrogen flow until dry. After evaporation, they were reconstituted with 150 µL of phosphate buffer saline (PBS), shaken for 15 minutes and sonicated for another 15 minutes. They were then transferred in 2 mL Sarstedts and assessed for their ß-CTX concentration by immunoassays.

RESULTS

The elution profile corresponding to the BSA+ condition, represented in blue in Figure 24, has slightly shifted to the right. Indeed, the peak eluted at 5 minutes while, in the profile corresponding to the BSA- condition, the peak eluted at 4 minutes. It is more interesting to have a later retention time as salts, which lead to ion suppression, elute mostly at the beginning of the run.

Regarding the peak shape, the width of the basis of the peak was smaller in the elution profile corresponding to the condition BSA+ than in the profile of the condition BSA-, meaning that the tailing was decreased.

Those results led to the conclusion that the addition of 0.1% BSA to the injection solvent was advantageous to the LC [25].

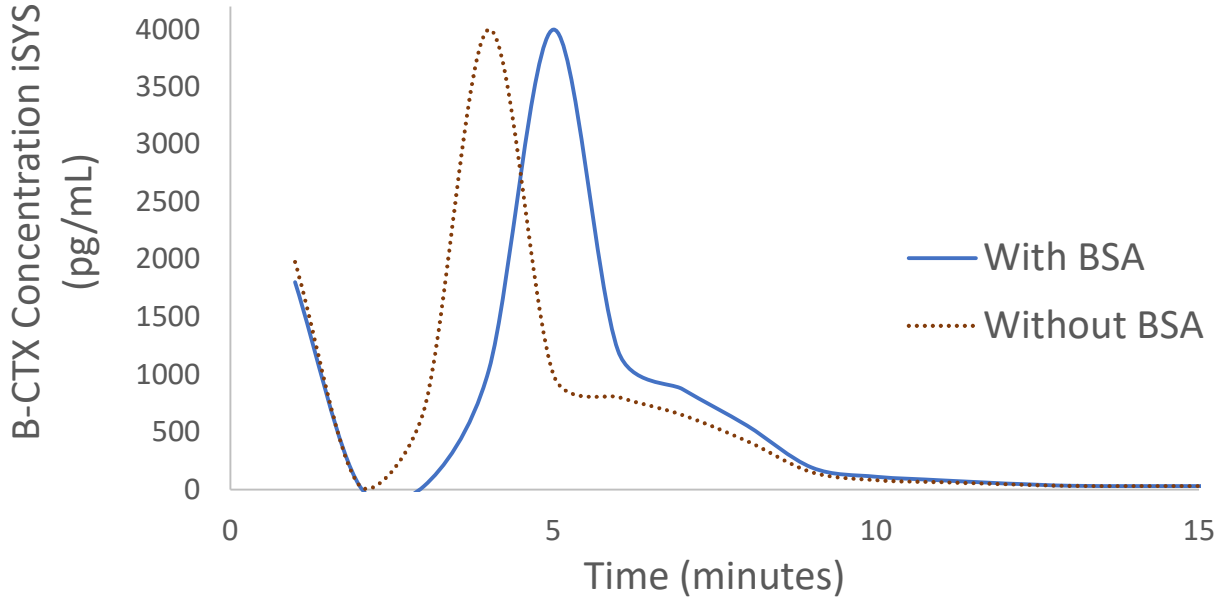

Figure 24 ß-CTX concentration following the time and the condition. The blue line is the elution profile with BSA while the dotted brown line is in absence of BSA.

DISCUSSION

The improved peak shape can be explained by the presence of the BSA as it decreases the secondary interactions between ß-CTX and the column.

A loss of ß-CTX can be observed at the beginning of the run. This could be due to a column overload or that the interactions between the stationary phase and the compound of interest are not stable enough.

Problems, such as the tailing, are remaining. Tailing is a phenomenon due to interactions with free silanols which affects mainly the polar and basic compounds[18,26]. Different solutions are possible:

- Endcapping of the stationary phase
- Lower the mobile phase pH
- Addition of mobile phase additives

As the column is already endcapped, it would be interesting to focus on the composition of mobile phases. As formic acid is already added to the mobile phases, the addition of supplemental mobile phase additives should be explored. Indeed, extremely low pH leads to short column lifetime. The addition of ammonium formate to mobile phases containing formic acid was studied in Johnson *et al*, 2013 [27]. It is said that the combination of formic acid and ammonium formate provides an equivalent ionic strength to the one observed for the trifluoroacetic acid which is well-known for its ion suppression phenomenon. The addition of ammonium formate should also increase the separation of the peptides and decrease the tailing.

4.3.2 Addition of ammonium formate in mobile phases

EXPERIMENTAL DESIGN

The purpose of this experiment is to assess the impact of ammonium formate (AF) on the peak shape.

1. Sample

Precipitated top-dose sample was evaporated and reconstituted with H_2O, 5% ACN, 0.2% FA, 0.1% BSA.

2. Liquid chromatography parameters

Two sets of mobile phases were prepared:

Condition 1:

Phase A: H_2O, 0.2% FA

Phase B: ACN, 0.2% FA

Condition 2:

Phase A: H_2O, 0.2% FA, 10 mM AF

Phase B: 90% ACN, 10% methanol, 0.2% FA, 10mM AF

Methanol (MeOH) needs to be added in phase B of the condition 2 in order to solubilize AF.

The flow rate, the oven temperature, the injection volume and the gradient were maintained through the different experiments.

A post-columnal flow splitting was realised and fractions were collected every minute during 45 minutes. Fractions were evaporated, reconstituted with 150 µL PBS and assessed by immunoassay.

RESULTS

The elution profiles obtained for both of the conditions are represented in Figure 25. The elution profile corresponding to the condition 2 (FA and AF) displayed a peak with a reduced peak tailing. Regarding the retention time, it shifted to the right. The retention time of the peak in the condition 1, was 4 minutes while in condition 2, the retention time is 7 minutes. The loss of ß-CTX seemed decreased in the elution profile of condition 2. However, this loss remains an issue to fix.

Given those results, it was decided that the mobile phases of the second condition will be used in the following experiments.

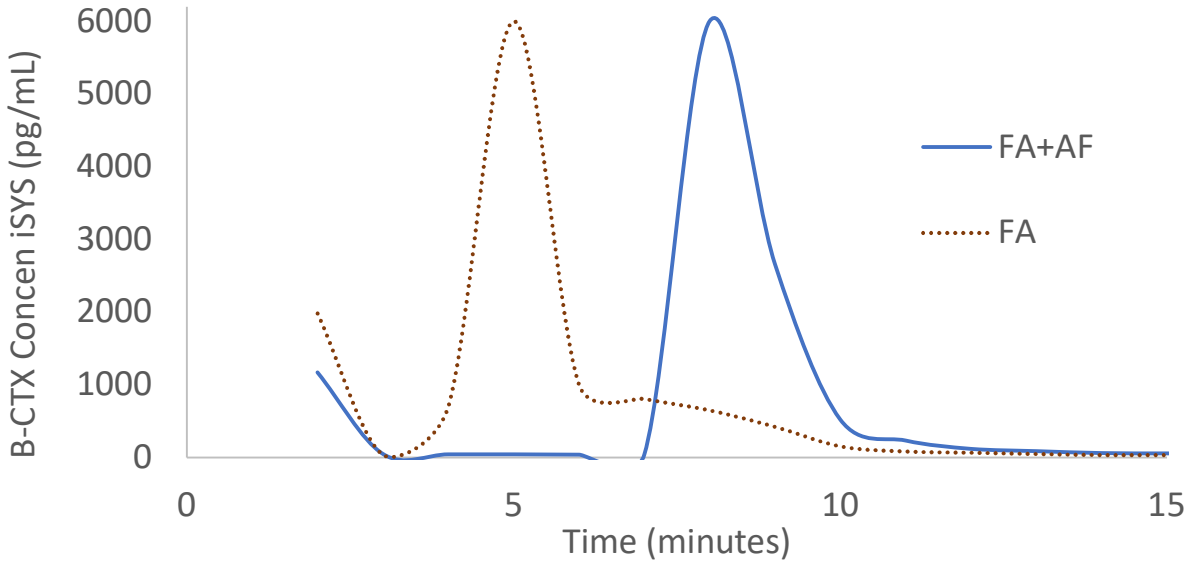

Figure 25 ß-CTX concentration following the time and the presence of ammonium formate (AF). The blue line is the elution profile with AF while the dotted brown line is in absence of AF.

DISCUSSION

One of the great advantages of FA is its compatibility with mass spectrometry in contrast with trifluoroacetic acid (TFA) which is prone to ion suppression. FA is thus widely used even if its ion strength is lower than TFA. However, adding AF to the composition of mobile phases has been proven to increase ion strength of the FA by nearly four times [28]. Also, another advantage of the AF is the increase of the sample load tolerance[28]. The combination of both these modificators increases the peak shape and decreases the band-broadening leading to the resolution obtained by the TFA. It will thus increase the separation of the compounds and, the stability of the peaks. It will therefore decrease the tailing[28].

Regarding the loss of ß-CTX, the addition of a step at the beginning of the gradient could help to strengthen the interactions between the stationary phase and the analyte. Diluting the sample could decrease the loss of analyte at the beginning of the run if the loss is due to overloading.

4.3.3 Addition of a step in the gradient

The aim of the experiment is to determine if a 30-seconds step may stabilize the interactions between the sample and the column in order to decrease the loss of analyte at the beginning of the gradient.

EXPERIMENTAL DESIGN

1. Sample

50 µL of precipitated top-dose reconstituted in H_2O, 5% ACN, 0.2% FA, 0.1% BSA were injected.

2. Liquid chromatography parameters

A step of 30 seconds or 60 seconds – respectively condition 1 and condition 2 – was added at the beginning of the gradient. The gradient was shortened while its slope remains the same as the analyte elutes in the ten first minutes.

Regarding mobile phases, phase A was H_2O, 0.1% FA, 0.1 Mm AF while phase B was ACN, 10% MeOH, 0.1% FA, 1% AF. Injection volume, oven temperature and flow rate remained unchanged.

Fractions were collected every 30 seconds. They were then evaporated and reconstituted with 150 µL of PBS. Fractions were assessed for their ß-CTX concentration by immunoassay.

RESULTS

Differences were notable between the elution profiles corresponding to condition 1 and 2, as represented in Figure 26. In condition 1, two main peaks were present. It appeared that the loss of ß-CTX at the beginning of the run was a different peak eluting as soon as the percentage of phase B increased. This observation can also be done for the condition 2 elution profile.

In the condition 2 elution profile, two different peaks were notable at 1 minute and 1 minute 15.

Regarding the peak shape of the second peak of both elution profiles, the low peak shape could be due to massive tailing or the presence of a second peak which is not fully separated.

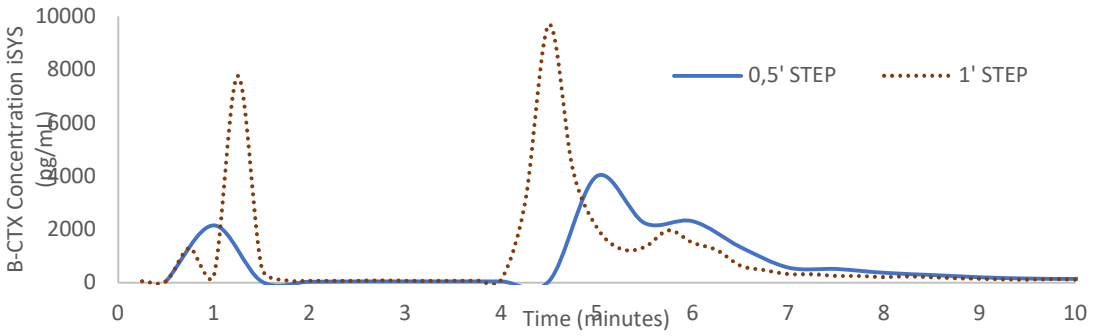

Figure 26 Elution profiles of the comparison between a step of 30 seconds (blue curve) and 1 minute (brown curve)

DISCUSSION

The observation of the elution profile corresponding to condition 2 led to the hypothesis that the two different peaks at 0.75 and 1.25 minutes may be due to the fact that the same molecule eluted once in isocratic mode once in a gradient mode. Gradient mode elution provided higher resolution and better peak shape than isocratic mode thus the gradient mode is preferable. For this reason, a step of 30 seconds will be applied for the upcoming experiments.

Regarding the peak shape of the second peak, several hypotheses could be suggested:

- Tailing which could be due to:
    - Connexions not tightly screwed in the tubing
    - LC parameters not fully optimized
- Another peak, corresponding to another form of ß-CTX or a cross reactivity, not fully separated

On the condition 1, we can see that there are two to three different peaks. These peaks can be explained by different hypothesis:

- Peaks are different isomers/forms of ß-CTX
- The ß-CTX molecules are retained in different ways by the column, leading to different retention times.
- It can also be due to a cross-reaction

4.4 Identification of the peaks present on the elution profile

4.4.1 Identification of the most abundant peaks

In order to define the nature of those peaks, an off-line nonorthogonal two dimensions (2D) LC-LC separation coupled to PCFS was performed.

EXPERIMENTAL DESIGN

1. Sample

The samples used were top-dose samples provided by IDS as explained above.

2. Off-line nonorthogonal 2D LC-LC

The need of highly concentrated samples was the limitation of experiment. To obtain high concentrations, 6 steps of LC-PCFS were realised. Fractions were collected every 15 seconds and were then pooled accordingly. Pooled fractions were then evaporated and reconstituted in 150 µL of PBS in order to be immunoassayed. Highly concentrated fractions were then evaporated and reconstituted with 100 µL of H_2O, 5% ACN, 0.2% FA, 0.1% BSA before undergoing a second run of LC-PCFS. The experiment was realised in duplicate for each selected fraction. After the LC-PCFS, collected fractions were evaporated until dry and reconstituted with 150 µL of PBS prior to ß-CTX concentration determination by immunoassay. The workflow is represented in Figure 27.

Regarding the other LC parameters, no changes were done.

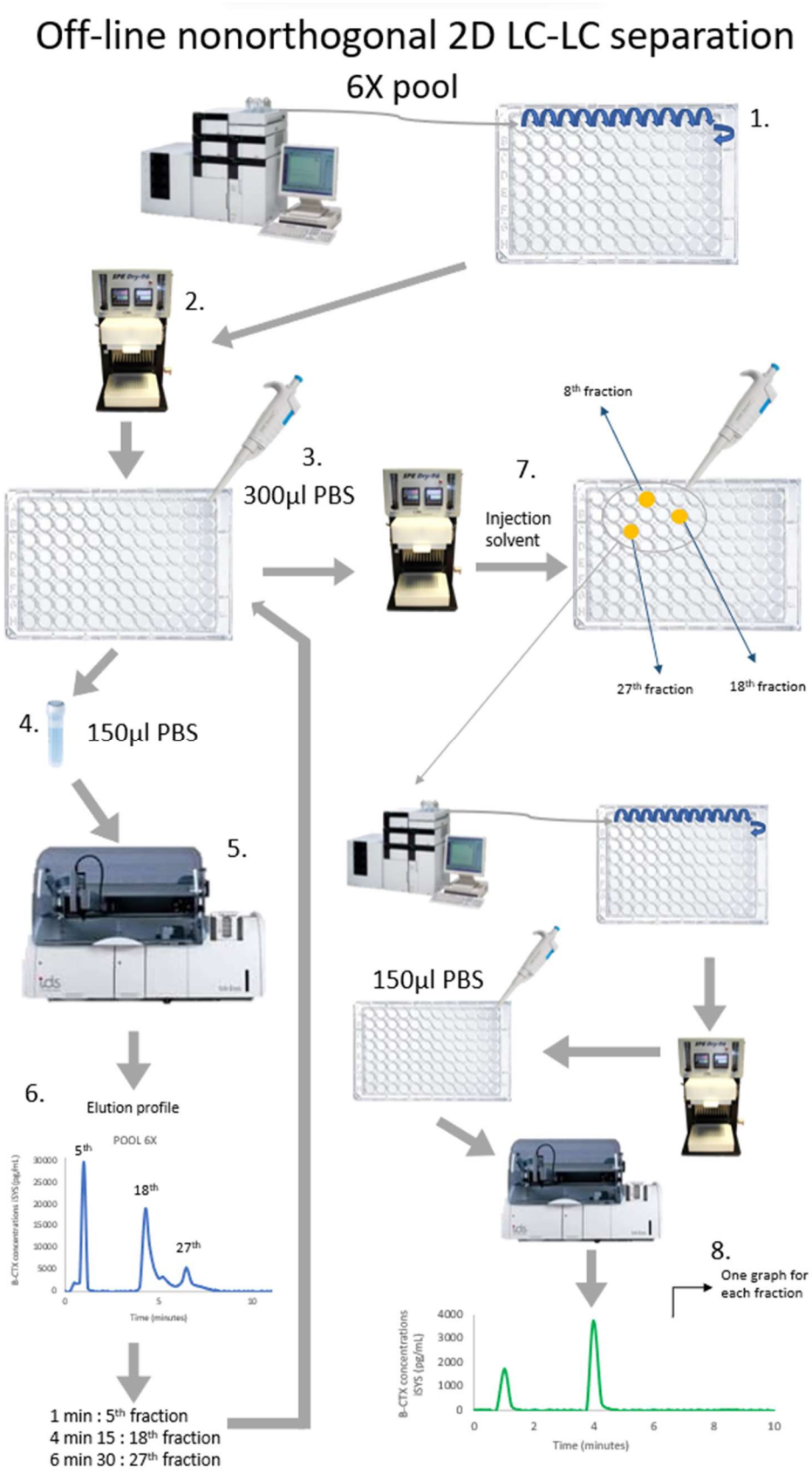

Figure 27 **Workflow The splitting will be done every 15 seconds in a 96-wells plate (1). The plate containing pooled fractions was evaporated (2) and reconstituted with 300 µl of PBS (3). 150 µL of pooled fractions were transferred into cupula (4) and immunoassayed (5) in order to obtain an elution profile and determine the retention time of ß-CTX (6). The remaining volumes of pooled fractions were evaporated and put in injection solvent (7). A second PCFS was done to create an elution profile of the selected fractions (8).**

RESULTS

The elution profile corresponding to the pooled fractions is depicted in Figure 28. Three main peaks at 1minute15, 4minutes30 and 6minutes45 were notable. These peaks correspond to fractions 5, 18 and 27. They were then selected for the second run of LC-PCFS.

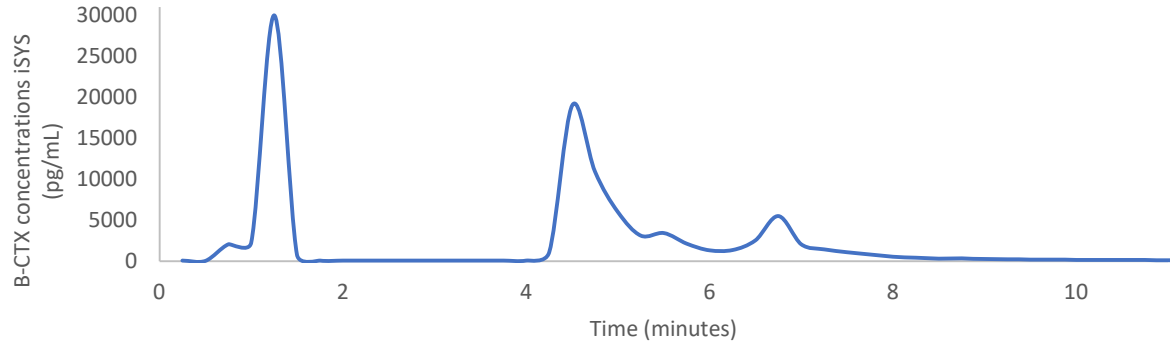

Figure 28 Elution profile of ß-CTX concentration following the time, done six times in a row and pooled together

Elution profiles obtained from fraction 5 (Figure 29) both displayed two peaks: one at 1minute15 for both elution profiles and one at 4minutes15 for the first run, and one at 4minutes45 for the second run.

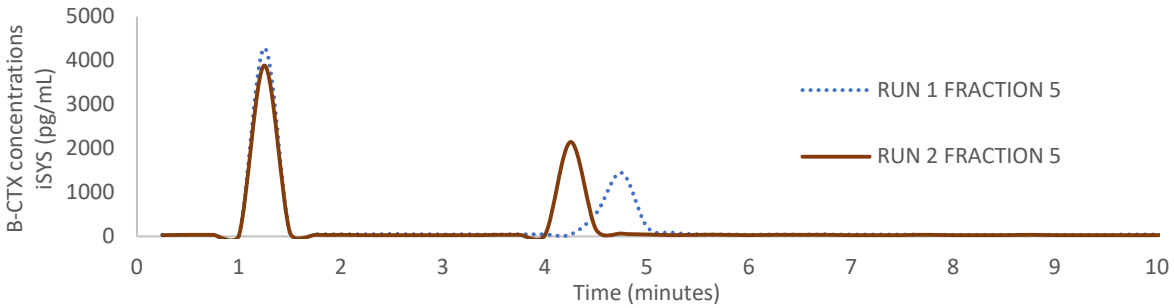

Figure 29 Elution profile of the fraction 5, realized in duplicate (Brown line is Run 1 and blue line is Run 2)

Regarding the elution profiles obtained from fraction 18 Figure 30), the one corresponding to the first run displayed peaks at 1minute15 and 6minutes30 while the elution profile of the second run perfectly overlayed the one obtained for the second run of the fraction 5 LC-PCFS in terms of retention time.

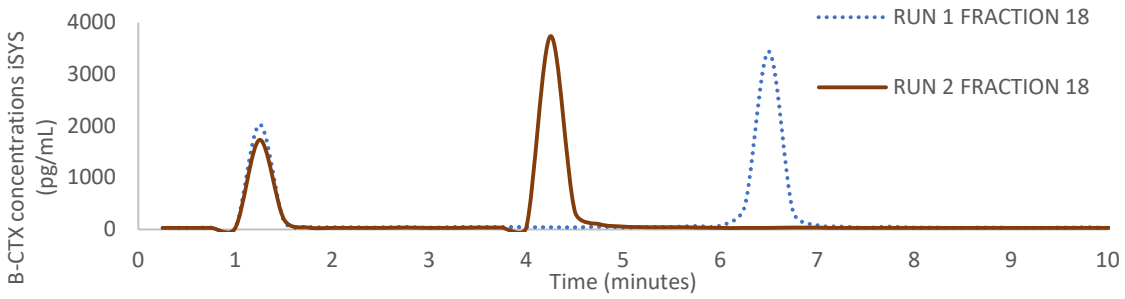

Figure 30 Elution profile of the fraction 18, realized in duplicate (Brown line is Run 1 and blue line is Run 2)

The first run of the LC-PCFS of the fraction 27 (Figure 31) corresponding to the second run also overlayed the elution profiles of the first run of the two first conditions.

Finally, the elution profile corresponding to the first run of the LC-PCFS of the fraction 27 displayed only one peak at 1minute while the elution profile corresponding to the second run perfectly overlayed the ones obtained for the second run of fraction 5 and 18.

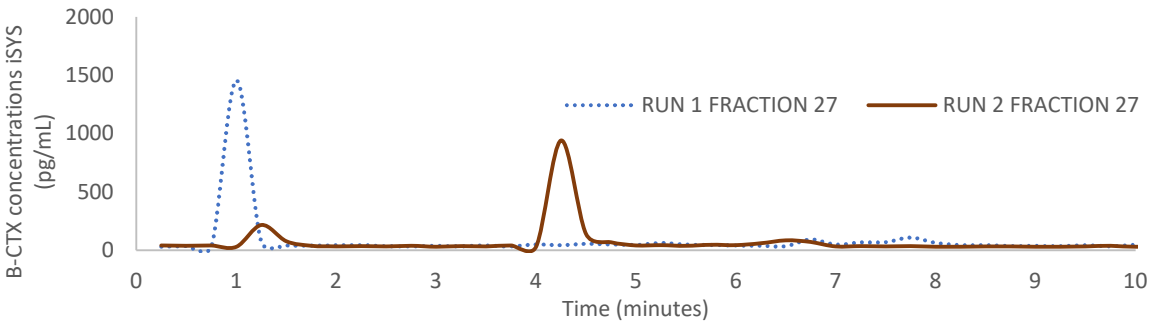

Figure 31 Elution profile of the fraction 27, realized in duplicate (Brown line is Run 1 and blue line is Run 2)

It is important to note that a similar pattern can be observed in the elution profiles of second runs corresponding as displayed in Figure 32.

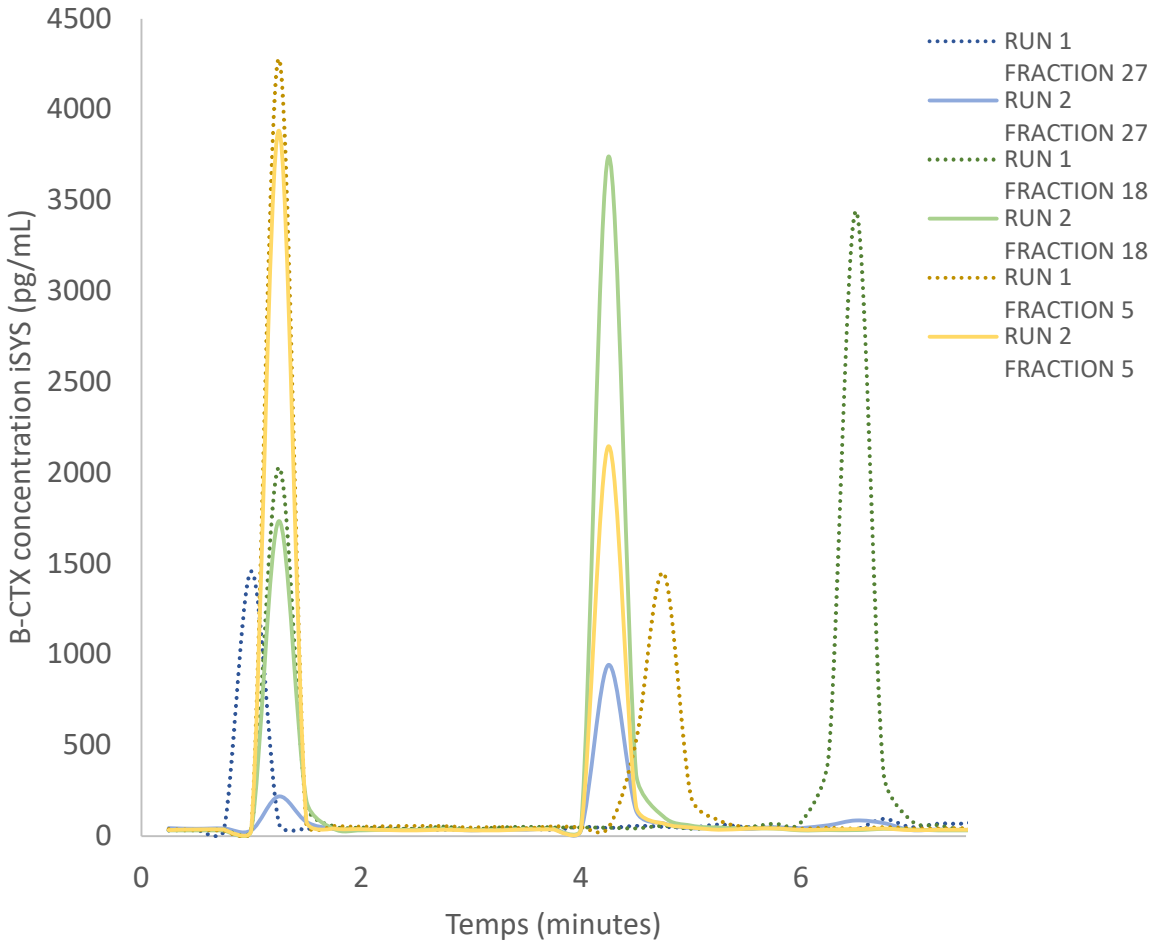

Figure 32 Combined elution profiles of each run, realised in duplicate. Both runs of the same fraction have the same colour (blue for fraction 5, green for fraction 18 and yellow for fraction 27). Dotted lines are Run 1, solid lines are Run 2

DISCUSSION

As said above, the interesting thing to highlight is that the elution profiles obtained for each second run, both peaks retention time were perfectly reproducible in contrast with elution profiles obtained for the first runs of each fraction which were not reproducible. This observation brought to light lack of reproducibility of the LC which is corrected after one injection. This led to the hypothesis that if the fraction 27 was highly concentrated, it may be due to the column which was not correctly equilibrated. Indeed, none of the obtained elution profiles displayed three peaks. In the light of those results, it can be stated that two peaks are eluting during the preparative LC and not three.

Different hypothesis can be elaborated regarding these two peaks. As fractions 5 and 18 both provided peaks at 1.15 and 4.15 minutes, it can likely be concluded that it is not due to a cross-reactivity. Two main hypotheses can be proposed:

- Two different retention mechanisms
- Two isomers of ß-CTX that are existing in equilibrium

It is important to note that none of the two most abundant peaks did not display a tailing in the elution profiles obtained for the second step of the off-line nonorthogonal 2D LC-LC separation in contrast with the elution profile corresponding to the first step of the experiment. Thus, it could be hypothesized that the tailing may be due to different molecules than the one(s) represented by the two most abundant peaks. It would therefore be interesting to analyse the fractions corresponding to the tailing.

4.4.2 Identification of the less abundant peak

EXPERIMENTAL DESIGN

1. Sample

The samples used were top-dose samples provided by IDS as explained above.

2. LC parameters

The need of highly concentrated samples was again a limitation for this experiment. To obtain high concentrations, 10 steps of LC-PCFS were realised in a row. Fractions were collected every 15 seconds and pooled accordingly. Eppendorfs were then divided in two different plates, the first one was evaporated and reconstituted in 150 µL of PBS. Each fraction was immunoassayed and fractions with a ß-CTX concentrations above 1000 pg/mL were selected. The second plate was then evaporated and reconstituted with 100 µL of H_2O, 5% ACN, 0.2% FA, 0.1% BSA. Selected fractions then underwent a second run of LC-PCFS. The experiment was realised in duplicate for each selected fraction. After the LC-PCFS, collected fractions were evaporated until dry and reconstituted with 150 µL of PBS prior to ß-CTX concentration determination by immunoassay. The workflow is represented in Figure 33.

Regarding the other LC parameters, no changes were realised.

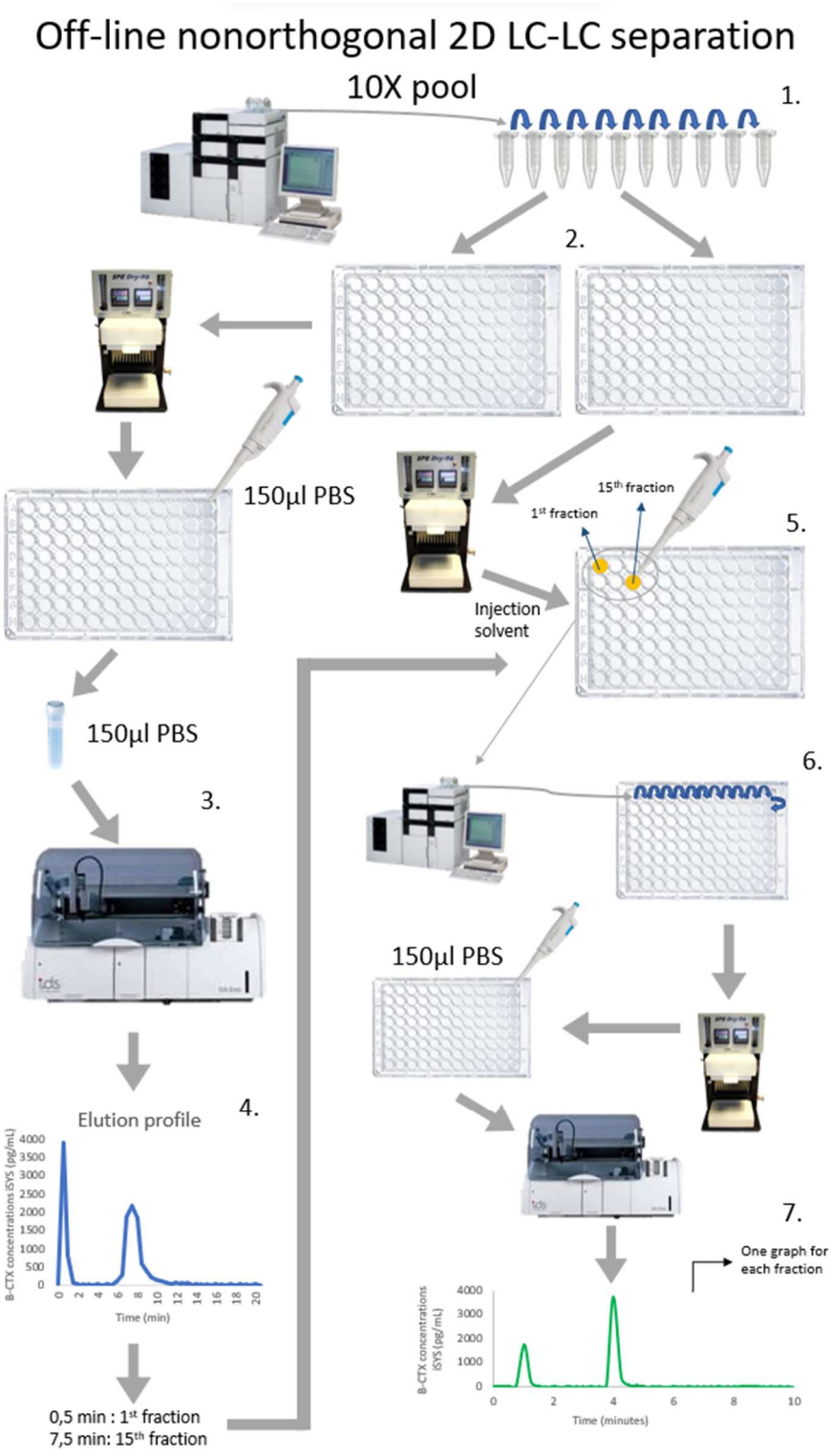

Figure 33 Off-line non-orthogonal 2D LC-LC separation pool 10X 10 runs were first realised, and put in Eppendorfs (1). The content of the Eppendorfs was then separated in two different 96-well-plates (2). The first one was reconstituted with 150µl of PBS, then immunoassayed (3), to give an elution profile containing different peaks (4). The second plates, that have been reconstituted with injection solvent (5), were used to selected the fractions giving a peak on the elution profile, and realising a second PCFS on those fractions (6). Each fraction gave an elution profile that could be analysed (7).

RESULTS

In the first step of the off-line nonorthogonal 2D LC-LC separation, peaks at 1.15 and 4.15 were present as expected. Ten fractions had a ß-concentration above 1000 pg/mL : fractions 3,4,5,17,18,19,20,21,22 and

23. Elution profile is represented in Figure 34. An LC-PCFS experiment was realised, in duplicate, on these fractions.

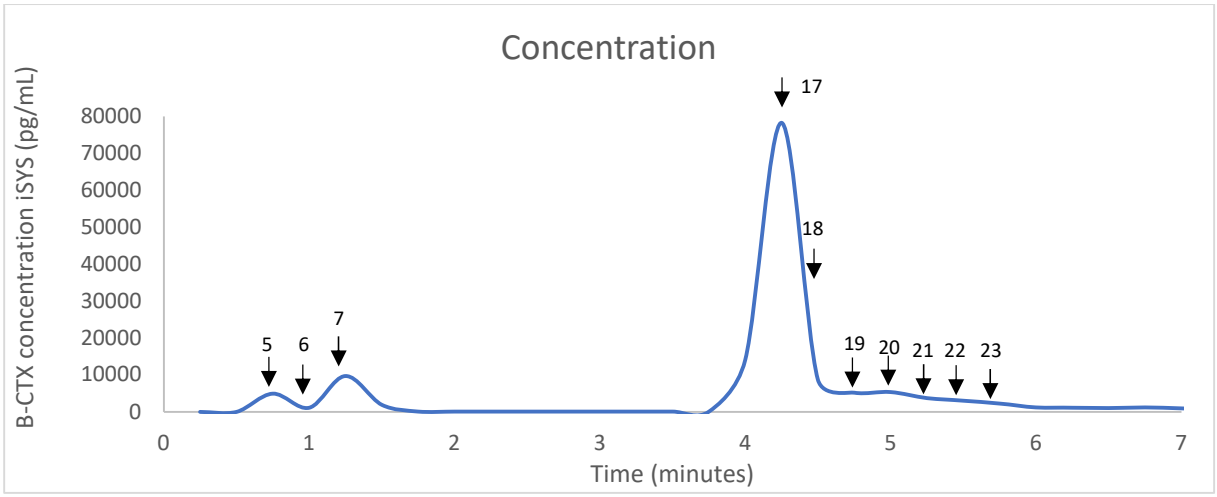

Figure 34 Elution profile representing the first step of the off-line nonorthogonal 2D LC-LC separation pool 10X

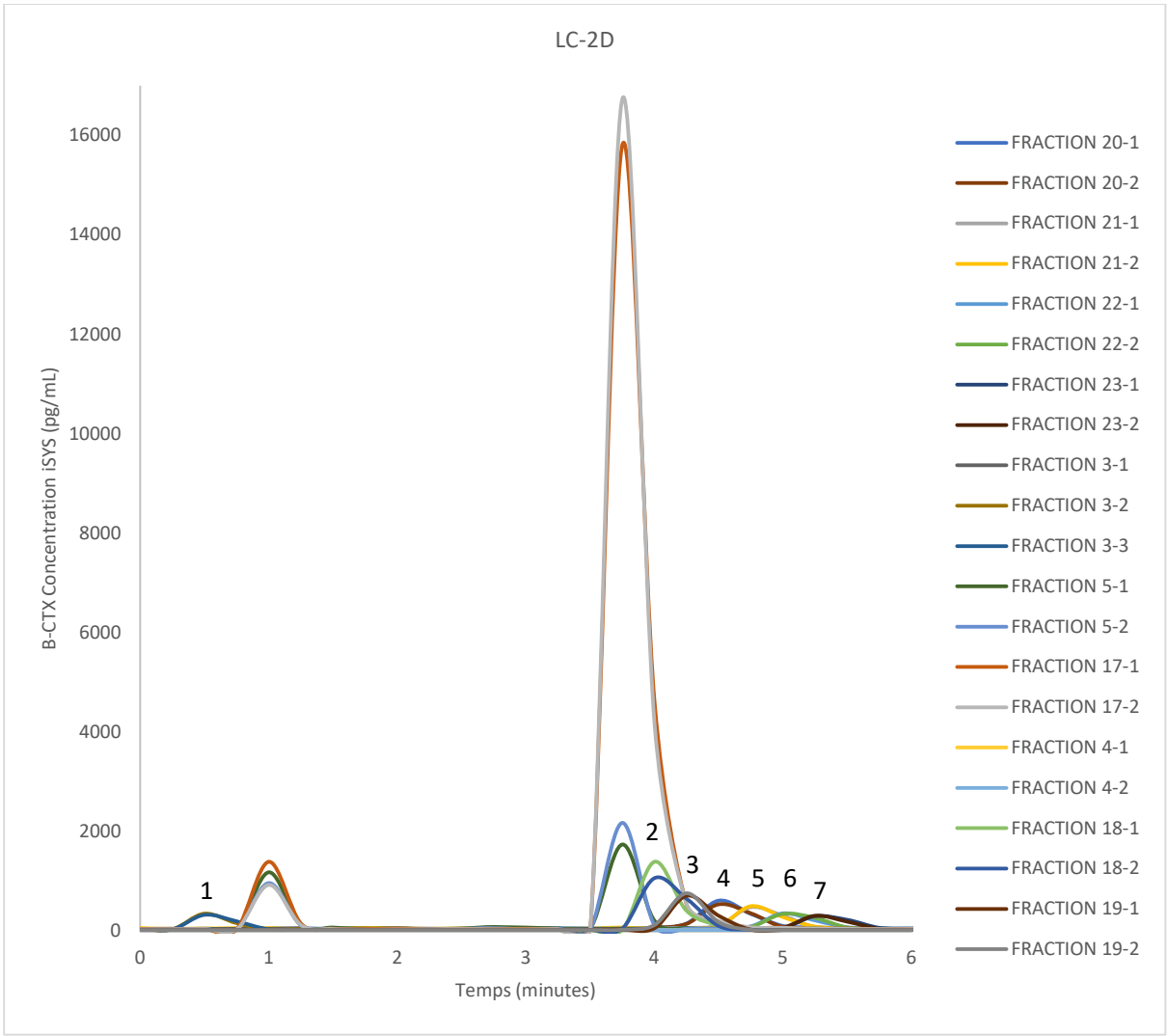

Figure 35 Elution profile of ß-CTX concentration depending on the time, compilation of all the obtained fraction, made in duplicate

Regarding both main peaks, the results were the same as previously. However, in the light of those results, it can be concluded none of the most abundant peaks displayed tailing as thought previously. Indeed, six independent peaks (2-7, Figure 35) can be observed after the most abundant peak. One peak (1) was also observed before the second most abundant peak.

DISCUSSION

The less abundant peaks represent other molecules than the ones represented by both the most abundant peaks as their retention times are different. However, as the retention time remains close, it can be thought that the differences between all the molecules remain small. It can be hypothesized that post-translational modifications such as glycation and glycosylation, which are well known in collagen, are present on the molecule of ß-CTX, leading to multiple peaks such as peak 1-7.

On the basis of the overall results, there is a need for higher separative power in order to separate as much as possible all the different forms of ß-CTX.

4.5 Optimization of the chromatographic resolution

4.5.1 Injection solvent Optimization

EXPERIMENTAL DESIGN

1. Sample

Urine had first been precipitated following the protocol explained above. However, two different reconstitution/injection solutions were used to reconstitute the samples:

- H_2O, 5% ACN, 0.2% FA, 0.1% BSA
- H_2O, 0.2% FA, 0.1% BSA

The purpose was to fully match the initial condition of the LC in order to increase the quality of the LC.

2. Liquid chromatography parameters

Regarding mobile phases, no changes were realized. The flow rate, the oven temperature and the injection volume were the same as in the first experiment. The gradient that was applied was the same as the experiment 4.3.3. The workflow followed was the same as the one explained in 2.2.

RESULTS

Both different elution profiles present in Figure 36 were similar. However, small differences remained. Indeed, the peak of the elution profile corresponding to the second condition (H_2O, 0.2% FA, 0.1% BSA) was sharper and the tailing was decreased.

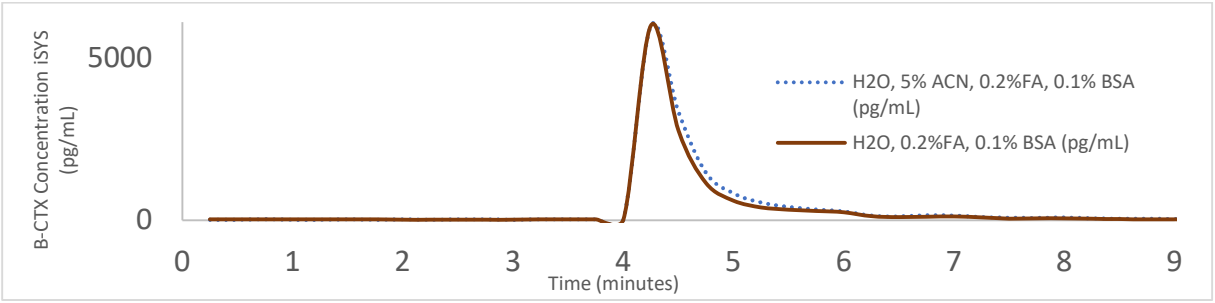

Figure 36 Elution profile of the injection solvent optimization, blue line is the first condition (H_2O, 5% can, 0.2% FA, 0.1% BSA) and brown line is the new condition (H_2O, 0.2% FA, 0.1% BSA)

DISCUSSION

H_2O, 5% ACN, 0.2% FA, 0.1% BSA was used until now as ACN is well-known to increase the solubility of proteins/peptides. This experience however proved that ß-CTX is actually soluble in a 100% aqueous medium.

As peak shape was a bit thinner with the reconstitution/injection solvent H_2O, 0.2% FA, 0.1% BSA, it was decided to keep this injection solvent for next experiments.

4.5.2 Optimization of the focusing

EXPERIMENTAL DESIGN

1. Sample

The samples used were top-dose samples provided by IDS as explained above. The injection solvent was the condition 2 of the previous experiment (H_2O, 0.2% FA, 0.1% BSA) as it displayed a better peak shape.

2. Liquid chromatography parameters

Except for the gradient, all the liquid chromatography parameters remained the same.

In this experiment, two gradients were tested:

1) 30 seconds focusing
2) 2 minutes focusing

Focusing tend to provide a good separation of the peaks as it strengthens the interaction between the column and the molecules. As the purpose of this optimization is to separate all the forms of ß-CTX, it is really useful in this situation.

RESULTS

In Figure 37, the elution profile corresponding to the 2-minutes focusing gradient displayed a retention time shift of more than 1 minute, which was expected. The separative power seemed also higher in this elution profile. Indeed, now, it has been established that what was thought to be tailing was multiple molecules recognized as ß-CTX.

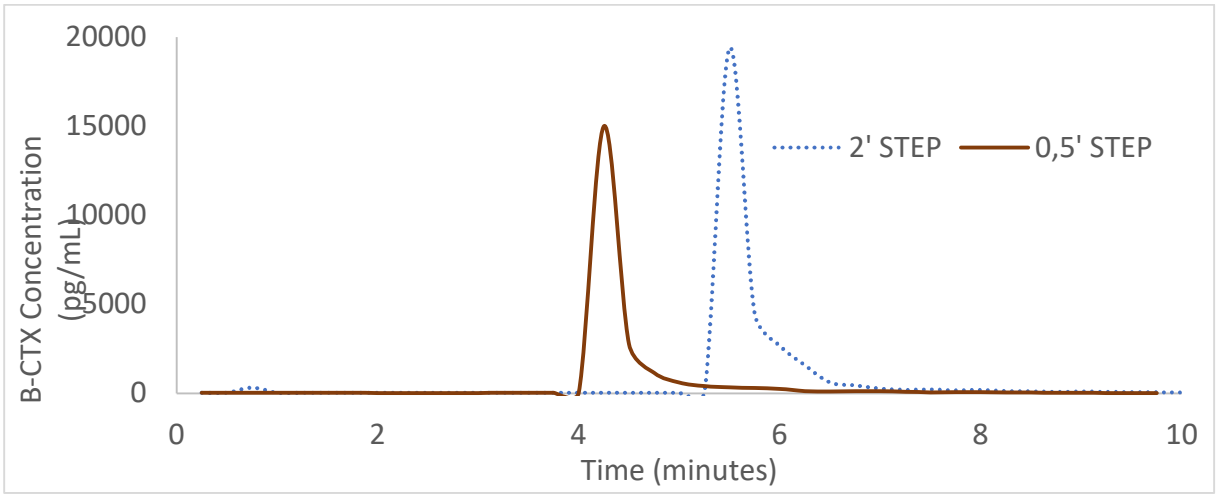

Figure 37 Elution profile comparing a step of 30 seconds (brown curve) and a step of 2 minutes (blue curve)

DISCUSSION

The longer tailing in the elution profile corresponding to the 2-minutes focusing may be due to a better separation of the different molecules recognized by the CLIA as ß-CTX. However, the differences remain small thus other optimizations are needed.

4.5.3 Addition of 5% Dimethyl sulfoxide (DMSO) in mobile phases

As explained above, ß-CTX contains a portion of the helical part of type I collagen. Thus, it would be interesting to use a denaturant agent such as DMSO to remove the secondary structure in order to facilitate the interaction between the stationary phase and the target molecules.

1. Sample

As previously, the samples consist of precipitated top-dose reconstituted with H_2O, 0.2% FA, 0.1% BSA.

2. LC parameters

Two different sets of mobile phases were tested:

Set 1:

Phase A: H_2O, 0.2% FA, 10 mM AF

Phase B: 90% ACN, 10% MeOH, 0.2% FA, 10mM AF

Set 2:

Phase A: H_2O, 5% DMSO, 0.2% FA, 10 mM AF

Phase B: ACN, 10% MeOH, 5% DMSO, 0.2% FA, 10 mM AF

The flow rate, oven temperature and injection volume were the same as in the first experiment. The gradient that was applied was the same as the experiment 4.3.3.

RESULTS

Four peaks were observable in the elution profiles obtained from the second set of mobile phases. As could be seen on Figure 38, the first one was at 45sec, the second one, the most abundant, eluted at 1min30. Other less abundant peaks eluted at 2min15 and 3min15. Pattern obtained in elution profiles in both of the conditions is observable. In the elution profile corresponding to the set of mobile phases with 5%DMSO, the pattern is visible between 1min and 3min30. In the elution profile corresponding to the mobile phases without DMSO, the same pattern is visible from 3min45 to 6min in addition to a peak at 1min15.

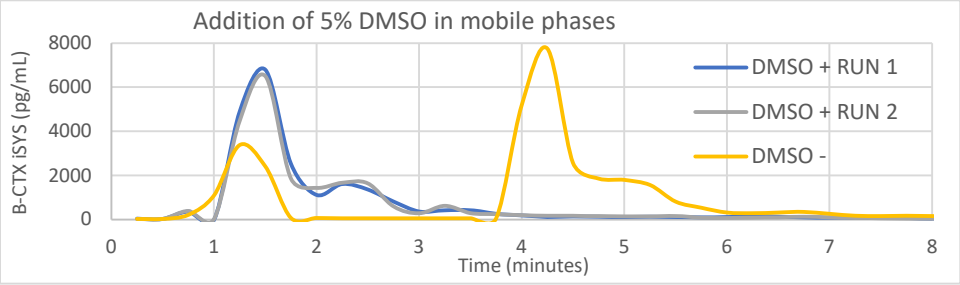

Figure 38 Elution profile comparing the IDS Top Dose with or without DMSO in the phases

DISCUSSION

In the previous experiment, both the most abundant peaks were said to be isomers or the same molecule with different retention mechanisms. Considering that the second most abundant peak is not present in the elution profiles corresponding to the DMSO mobile phases, it can be hypothesised that the addition of DMSO in the mobile phases led to the disappearance of the second mechanism of retention. Indeed, DMSO is well-known for its denaturant action and its compatibility with mass spectrometry. This denaturant property can be due to a strong H-bond accepting property of the sulfoxide, leading to a break of the hydrogen bounds in proteins. With the suppression of H-bond, the secondary and tertiary structures, such as the α-helixes which are highly present in collagen, disappear which could explain the disappearance of the second retention mechanism. [29,30]

Regarding the following experiments, it would be interesting to increase the separative power of the LC. Different parameters could be interesting:

- Mobile phases composition
- Slope of the gradient

4.5.4 Comparison of gradient

EXPERIMENTAL DESIGN

1. Sample

The samples used in this experiment was prepared the same as in previous experiments.

2. LC parameters

The mobile phases were H_2O, 5% DMSO, 0.2% FA, 10 mM AF and ACN, 10% MeOH, 5% DMSO, 0.2% FA, 10 mM AF. Three gradients (gradient 20%B, 10%B and isocratic) were designed, as represented in Figure 39, and tested. The flow rate, the oven temperature and the injection volume were the same as in the first experiment.

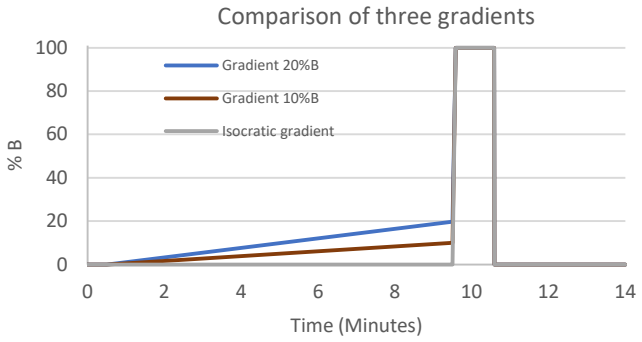

Figure 39 Representation of three gradients: 20% B (Blue), 10% B (Brown) and isocratic (Grey)

RESULTS

As represented in Figure 40, all obtained elution profiles displayed at least two peaks eluting almost at the same time, at 45 seconds and 1minute15. A third peak which is badly separated from the second peak is visible in all the elution profiles. However, the last peak, observable between 3min45 and 5min45 in the elution profile corresponding to the gradients 20%B and 10%B, was absent in the elution profile corresponding to the isocratic gradient. As separation is better with gradient 10%B, this gradient was chosen to continue the development.

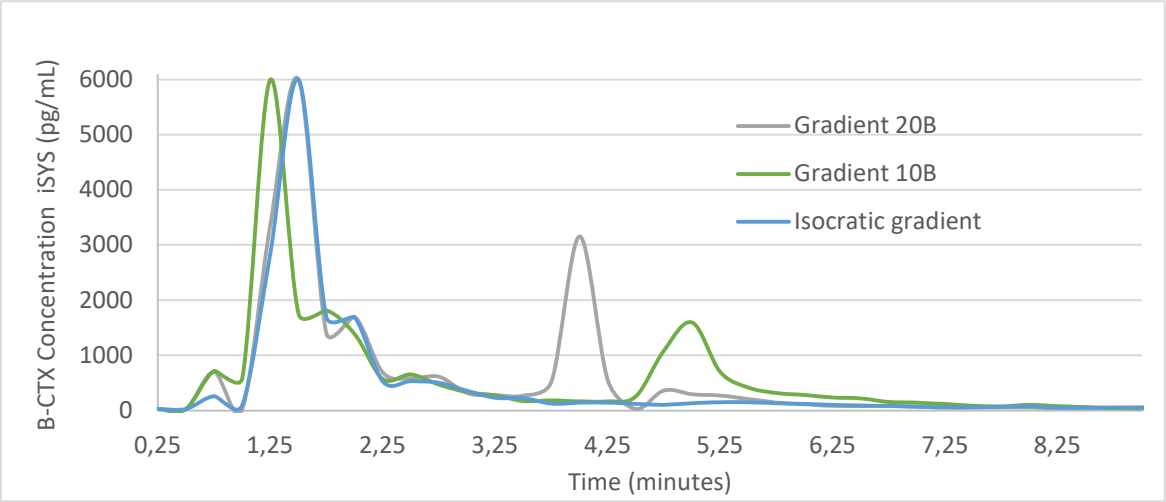

Figure 40 Elution profile of ß-CTX concentration iSYS (pg/m) in function of the time, comparing three gradients: 10% phase B (brown), 20% phase B (grey) and isocratic (blue)

DISCUSSION

Regarding the missing peak in the elution profile corresponding to the isocratic gradient, it can be explained by the fact that these molecules are not eluting in 100 aqueous medium in contrast with the molecules which eluted between 0min30 and 3min. This is logical knowing that the higher the retention time is, the more unipolar the molecule is.

4.5.5 Comparison of mobile phases 0.2% FA, 0.5% FA

EXPERIMENTAL DESIGN

1. Sample

As previsouly, the samples consist of precipitated top-dose reconstituted with H_2O, 0.2% FA, 0.1% BSA.

2. LC parameters

Regarding mobile phases, two sets were prepared:

Set 1:

Phase A: H_2O, 5% DMSO, 0.2% FA, 10 mM AF

Phase B: 90% ACN,10% MeOH, 5%DMSO, 0.2% FA, 10mM AF

Set 2:

Phase A: H_2O, 5% DMSO, 0.5% FA, 10 mM AF

Phase B: ACN, 10% MeOH, 5% DMSO, 0.5% FA, 10 mM AF

Flow rate, oven temperature and injection volume were the same as in the first experiment. The gradient that was applied was here the gradient 10%B, as selected in the previous experiment.

RESULTS

As can be seen on Figure 41, a peak was present at 5min on both of the elution profiles. Regarding the first part of the profiles, it can be seen that separation was decreased between the peaks which eluted between 0.5min and 3min30 in the elution profile corresponding to the set 2.

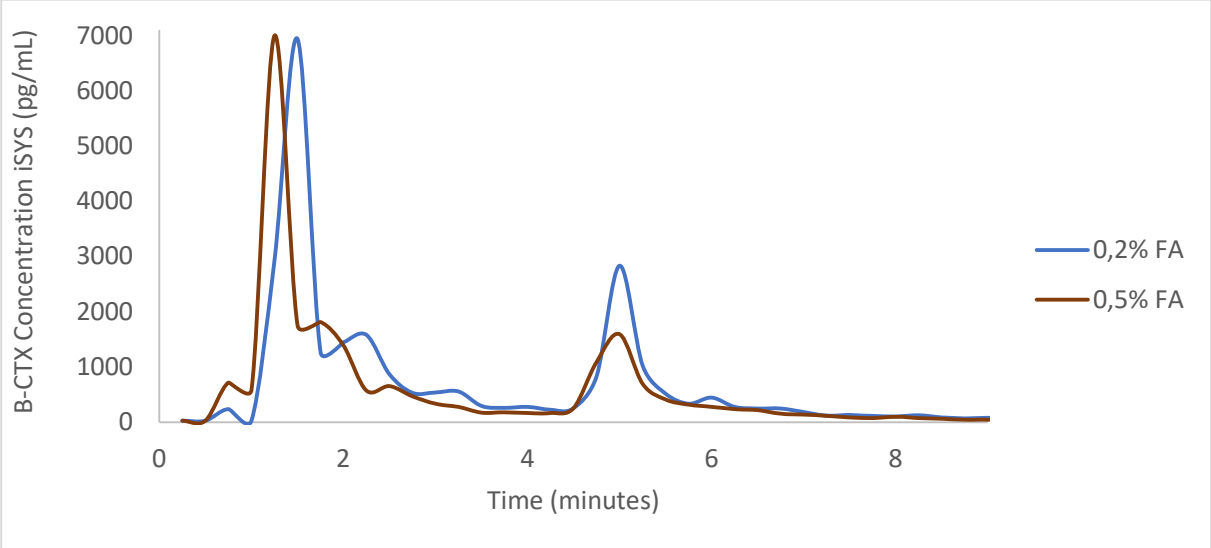

Figure 41 Elution profile of the ß-CTX concentration (pg/mL) in function of the time (minutes), with two conditions of injection solvent : 0.2% FA (blue) and 0.5% FA (brown)

DISCUSSION

FA is a well-known additive used to reduce the tailing and thus increase the separation. However, in this case, the increased percentage of FA decreased the separation. It was thus decided to keep the set of solvent with 0.2% FA.

4.5.6 Comparison of injection solvent: with or without 5% DMSO

EXPERIMENTAL DESIGN

1. Sample

Urine had first been precipitated following the protein precipitation protocol described above. Samples were then evaporated and reconstituted with two different injection solvents:

- H_2O, 0.2% FA, 0.1% BSA
- H_2O, 0.2% FA, 0.1% BSA, 5% DMSO

2. LC parameters

Regarding mobile phases and gradient, the ones selected from the previous experiment were used. The flow rate, the oven temperature and the injection volume were the same as in the first experiment.

RESULTS

On Figure 42, both of the elution profiles displayed a different pattern. Indeed, four different peaks were visible in the elution profile corresponding to the injection solvent without DMSO while five were visible in the second elution profile. The separation was thus higher with the second injection solvent. However, as the peak pattern remained different, peak association between both elution profiles is impossible.

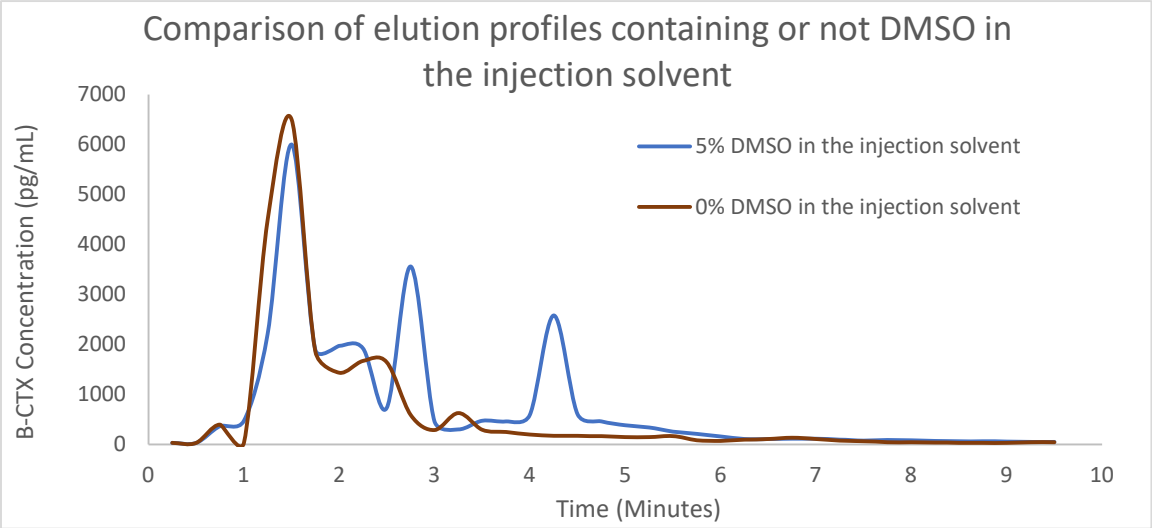

Figure 42 Elution profile comparing the presence (blue curve) or not (brown curve) of 5% DMSO in the injection solvent

DISCUSSION

DMSO was added in the sample to assure a complete interaction with proteins in order to optimize the unfolding. Indeed, if DMSO is only present in the mobile phases, the unfolding may not be completely achieved before the proteins enter the column. The appearance of new peaks could be thus due to a full unfolding.

4.6 System check

A system check has been realized before every experiment to ensure the good functioning of the LC and the mass spectrometer. Indeed, if the system check provides satisfying and reproductible results, it means that the machine is behaving properly. Usually, the system check consists of a clean solution of the target analyte, however, as ß-CTX remains uncharacterized, an octapeptide – EKAHDGGR –, which is the only known part of ß-CTX will be used. Different steps were necessary to develop the system check MS/MS method: Q1 Scan, ion product experiment and MRM experiment.

4.6.1 Q1 Scan

The aim of the Q1 scan is the determination of the m/z ratio of the molecular ions. Clean sample of the octapeptide at a concentration of 1 ug/mL was directly injected. During a Q1 scan experiment, both quadrupoles and the collision cell are “open”. The analyte can be multiply charged, providing multiple m/z ratios and consequently, several peaks on the graph as displayed in Figure 43. Two abundant peaks were selected: one at 290.6 m/z, one at 435.1 m/z. The ion with the m/z ratio of 290.9 Da corresponded to the octapeptide (870 Da) charged three times, while the ion with the m/z ratio of 435.1 Da corresponded the octapeptide charged twice.

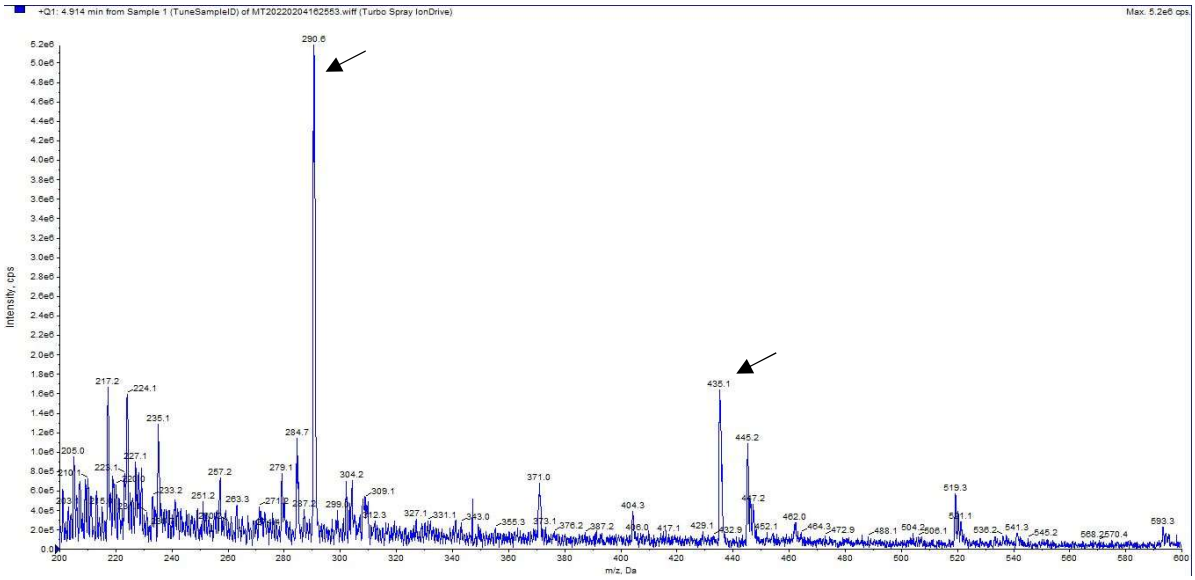

Figure 43 Q1 scan of the octapeptide

4.6.2 Ion product experiment

Ion product experiment was used to determine the m/z ratios of the product ions. The same solution as previously was directly injected in the mass spectrometer. The first quadrupole was set to select m/z ratio of 290.6 Da and then, 435.1 Da. These ions were then fragmented in the collision cell while the last quadrupole was set in scan mode.

4.6.3 Optimization of compound parameters

Compound parameters, namely: declustering potential, exit potential, entrance potential and collision energy, were optimized thanks to a ramp in MRM mode. Results are reported in Table 1:

Table 1 Optimization of compound parameters

ID	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	DP (volts)	EP (Volts)	CE (Volts)	CXP (Volts)
H_2O+3	290.7	284.6	150	50.000	10.000	11.000	15.000
y_4 +1	290.7	404.1	150	50.000	10.000	19.000	15.000
y_6 +2	290.7	306.9	150	50.000	10.000	15.000	15.000
y_3 +1	435.5	289.2	150	50.000	10.000	35.000	15.000
y_4 +1	435.5	404.2	150	50.000	10.000	32.000	15.000
-H_2O +2	435.5	426.3	150	50.000	10.000	24.000	15.000
y_5 +1	435.5	541.4	150	50.000	10.000	28.000	15.000
Y_6 +1	435.5	612.4	150	50.000	10.000	26.000	15.000

DP = Declustering potential EP= exit potential CE = Collision energy CXP = cell exit potential

4.6.4 Optimization of source parameters

Source parameters were optimized using ramps during a post-columnal injection. The set-up is represented in Figure 44. Optimized source parameters are reported in Table 2.

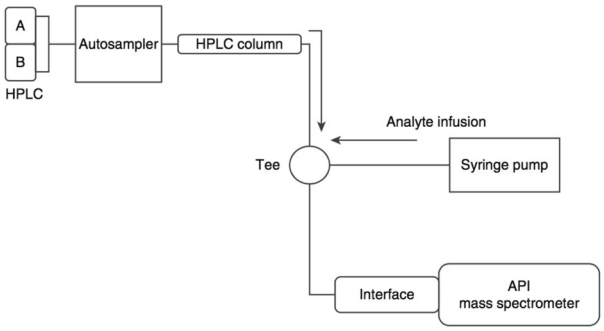

Figure 44 post-columnal injection

Table 2 Optimized source parameters

Curtain gas	20
Collision gas	Low
IonSpray Voltage	4500
Temperature	650
Ion Source Gas 1	50
Ion Source Gas 2	50

4.6.5 Liquid chromatography parameters

The parameters were the same as the ones used during the first experiment. The octapeptide eluted at 1.6 minute. The intensity of the peak was 14330. Chromatogram is represented in Figure 45. As can be seen, the peak shape is good, proving that LC works correctly.

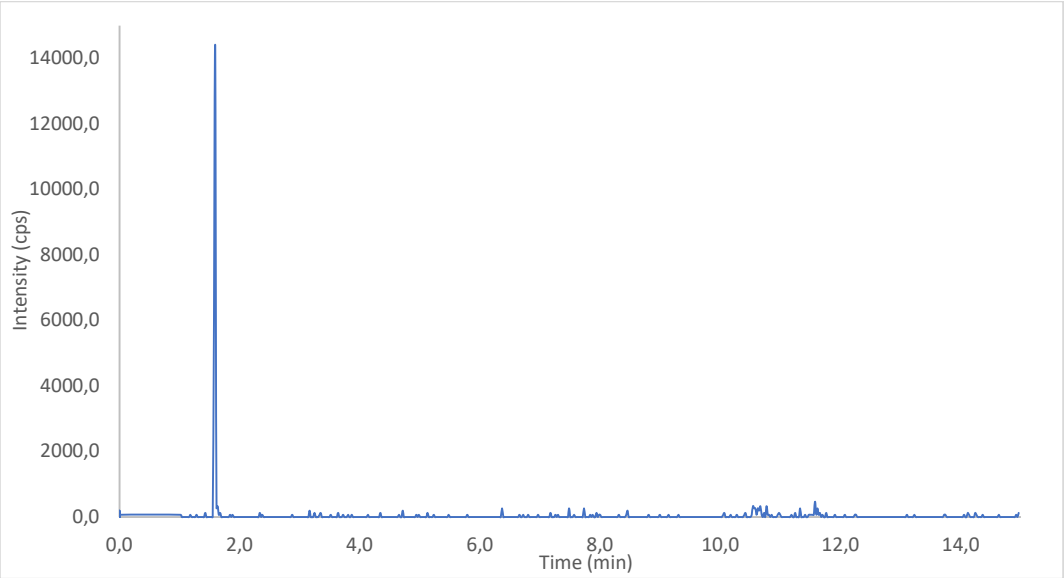

Figure 45 Chromatograph of the octapeptide: intensity following the time

5. Conclusion and perspectives

5.1 Conclusion

The final goal of the work was to develop a preparative LC in order to obtain a good separation of ß-CTX from the other component of the matrix. Different parameters needed to be optimized to achieve a good separation: injection solvent, mobile phases and gradient. However, the presence of different retention mechanisms or/and isomers or/and PTMs made the process more complicated. Although good results were obtained, the complete separation of the different peaks present on the elution profile was not reached. However, it was decided to keep the parameters of the last experiment and to start the characterization process.

5.2 Perspectives

Even if no modification will be added to the preparative liquid chromatography protocol, several experiments are left to be done before the characterization process.

5.2.1 Comparison between different kits and matrices

As said in the introduction, three different kits are commercially available for the quantification of ß-CTX:

- Crosslaps Enzyme Linked ImmunoSorbent Assay (IDS)
- iSYS CTX-I Crosslap (IDS)
- Automated chemiluminescence immunoassays (ECLIA) ß-Crosslap by Cobas (Roche)

In this work, fractions obtained by LC-PCFS were quantified by the first one. However, as those kits are not based on the same antibodies and detection system, it could thus be interesting to quantify each fraction with the three kits to obtain three elution profile. The purpose would be to study the differences between the elution profile and which form of ß-CTX is recognized by which kit.

For this experiment, it would be interesting to performed LC-PCFS on a pool of plasma, a pool of serum and a pool of urine to identify which form is present in which matrix.

5.2.2 Gel electrophoresis

It would also be interesting to realize a gel electrophoresis in order to separate each component of a fraction depending on its size and charge. This experiment could lead to a good assessment of the purity of the fractions and could estimate the mass of the different components.

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Abstract

As the population is becoming older, osteoporosis is more and more important worldwide. It affects 200 million women and costs enormously to society. Also, the treatment is not always effective, and the compliance is low. Adverse effects are really important and the absorption of molecules for bisphosphonates, which is the most common therapy, is less than 2%. A follow-up of the treatment is thus necessary, as it is not correctly taken. This follow-up is based on bone formation and resorption markers. In the present work, the focus will be realised on ß-CTX, a degradation product of type 1 collagen.

Immunoassays analysing the concentration of ß-CTX in biological samples gave different concentrations when the analysis was realised on a same sample. The work here was part of a larger one which consisted of creating a commutable standard to harmonize every immunoassay. The project was separated in different parts. The first one was the isolation of ß-CTX by liquid chromatography, followed by a characterization by mass spectrometry. Indeed, the structure was still unknown. Finally, the creation of an internal standard and a commutable product was planned. This work describes the isolation part.

The isolation was an important part of the work. Indeed, the more the sample was separated, the more ß-CTX was pure and the more the characterization was realised easily. Different parameters were tested, such as the addition of ammonium formate, formic acid and DMSO in phases. These were really efficient for the separation. Also, the gradient was optimized, as it was elongated to allow a better separation of the peaks. Finally, the injection solvent was modified, with the removing of the acetonitrile and the addition of DMSO.

All these modifications of parameters on the method are described in detail. They lead to a good separation of the peaks and it was thus possible to pass to the second part of the project which was the characterization.

Résumé

Avec le vieillissement de la population, l'ostéoporose est de plus en plus importante dans le monde. Elle touche 200 millions de femmes et coûte énormément à la société. De plus, le traitement n'est pas toujours efficace et l'observance est faible. Les effets indésirables sont vraiment importants et l'absorption des bisphosphonates, qui est la thérapie la plus courante, est inférieure à 2 %. Un suivi du traitement est donc nécessaire, car il n'est pas correctement pris. Ce suivi est basé sur des marqueurs de formation et de résorption osseuses. Dans ce travail, l'accent sera mis sur ß-CTX, un produit de dégradation du collagène de type 1.

Des dosages immunologiques analysant la concentration de ß-CTX dans des échantillons biologiques ont donné des concentrations différentes lorsque l'analyse était réalisée sur un même échantillon. Le travail ici faisait partie d'un travail plus vaste qui consistait à créer une norme commutable pour harmoniser chaque immuno-dosage. Le projet a été séparé en différentes parties. La première était l'isolement de ß-CTX par chromatographie liquide, suivi d'une caractérisation par spectrométrie de masse. En effet, la structure était encore inconnue. Enfin, la création d'un étalon interne et d'un produit commutable était prévue. Ce travail décrit la partie « isolation ».

L'isolation était une partie importante du travail. En effet, plus l'échantillon était séparé, plus ß-CTX était pur et plus la caractérisation se faisait facilement. Différents paramètres ont été testés, tels que l'ajout de formate d'ammonium, d'acide formique et de DMSO en phases. Ceux-ci étaient vraiment efficaces pour la séparation. De plus, le gradient a été optimisé, car il a été allongé pour permettre une meilleure séparation des pics. Enfin, le solvant d'injection a été modifié, avec l'élimination de l'acétonitrile et l'ajout de DMSO.

Toutes ces modifications de paramètres sur la méthode sont décrites en détail. Ils conduisent à une bonne séparation des pics et il a ainsi été possible de passer à la seconde partie du projet qui était la caractérisation.