Formulation development and analytical evaluation of α-CGRP 8-37

for a potential subcutaneous psoriasis therapy

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A potential subcutaneous psoriasis therapy

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Making a fragile peptide measurable and usable as an injectable drug

Psoriasis is described as a skin disease, the immune system and sensory nerves help shape the disease process. Calcitonin gene-related peptide, shortened to CGRP, is one of the nerve-derived signaling molecules that is linked to skin inflammation. A shortened fragment of the peptide, called CGRP 8-37, can block some signaling caused by the whole version of CGRP. That makes it interesting as a research molecule and as a possible starting point for a future treatment strategy.

Before a peptide like CGRP 8-37 can become a realistic medicine, two practical questions need to be answered. First, can the peptide be measured, detected and observed in a reliable manner? Second, can it be formulated in a liquid that stays stable and works for injection under the skin? This thesis focused on those two questions.

The development part tested how CGRP 8-37 behaved after being dissolved in different liquid vehicles and observed what happened when they were stored over time, regarding stability. The peptide did not behave like a table salt or sugar dissolved in water. Its apparent stability depended on concentration, the vehicle used to dissolve the peptide and contact with container surfaces that the samples came into contact with, such as storage vials. Low-concentration samples often lost measurable signal more strongly than higher-concentration samples. This led to an experiment where a protein called BSA was used to coat the inside of the storage vials, acting as a barrier between the peptide and the glass vial. The goal was to see if the peptide was getting stuck to the vials and therefore not getting detected when the samples were getting measured. In theory this matters more with lower concentration samples since only a set amount of peptide can get stuck to the containers before there is no more space for the peptide to stick to, for higher concentration samples this amount could be neglectable, but for smaller concentration samples most of the peptide could get stuck to the container vials. The results were that samples stored in BSA-coated vials gave higher signal in several conditions, which points toward that the peptide is sticking to glass or vial surfaces as one source of peptide loss.

The analytical part used HPLC, a machine that can be used to separate and quantify chemicals. For formulation samples and LC-MS, a machine that can separate and quantify like the HPLC, but can also identify chemicals, for blood-matrix experiments. HPLC worked as a practical tool for formulation and stability screening. In pilot experiments, CGRP and CGRP 8-37 signals changed rapidly after blood contact. In one experiment, signal decreased strongly between 30 seconds and 10 minutes. In another, an inhibitor-containing preparation gave unexpectedly high CGRP 8-37 signal while CGRP stayed low. These results do not yet give reliable concentrations in blood. They however show why blood sampling needs tighter control before volunteer or patient samples are analyzed.

The main conclusion is that CGRP 8-37 development is dependent on analytical control. A stable formulation cannot be judged if the method loses peptide to surfaces or sample preparation. Blood levels cannot be interpreted if the peptide changes during handling. The next research step should combine rapid blood quenching, internal standards for LC-MS, pH and osmolality testing, and long-term formulation stability studies.

Að gera viðkvæmt peptíð mælanlegt og nothæft sem sprautulyf

Psóríasis er lýst sem húðsjúkdómi, en ónæmiskerfið og skyntaugar taka einnig þátt í sjúkdómsferlinu. Calcitonin gene related peptide, skammstafað CGRP, er eitt af þeim boðefnum sem taugakerfið getur losað, en það getur haft áhrif á æðar, húð og ónæmissvör líkamans. CGRP 8-37 er minkuð útgáfa af CGRP og getur hindrað CGRP-tengdar boðleiðir. Þess vegna er það áhugavert í rannsóknum á sjúkdómum þar sem taugar, bólga og húðvefur tengjast.

Áður en peptíð eins og CGRP 8-37 getur orðið að raunhæfum stungulyfjakosti þarf að komast að gera tvennt. Í fyrsta lagi þarf að vera hægt að mæla peptíðið með áreiðanlegum hætti. Í öðru lagi þarf að leysa það upp í vökva sem helst stöðugur við geymslu og við meðhöndlun. Oft getur verið erfitt að vinna með peptíð, þar sem þau geta brotnað niður, loðað við gler eða plast, myndað agnir eða fallið út úr lausn.

Í fyrsta hluta verkefnisins var skoðað hvernig CGRP 8-37 hegðaði sér eftir að það var leyst upp í mismunandi vökvum og geymt yfir tíma. Peptíðið hegðaði sér ekki eins og borðsalt eða sykur í vatni. Stöðugleiki þess virtist ráðast af styrk, leysivökva og snertingu við yfirborð í ílátum. Sýni með lágan styrk töpuðu oft mælanlegu merki hlutfallslega meira en sýni með hærri styrk. Sýni sem voru geymd í glösum húðuðum með BSA, próteini sem getur minnkað hlutfall peptíða sem festast við yfirborð á glers og tilraunaglasa, með því að búa til filmu á milli glasins og peptíðins. Magn peptíða sem festist við yfirborð skiptir yfirleitt meira máli í sýnum með minni styrk, þar sem aðeins ákveðið magn peptíðsins getur fest sig við yfirborðið áður en ekkert pláss er til staðar fyrir fleyri peptíð að festa sig. Í sýnum með hærri styrk getur þessi yfirborðsfesting haft hverfandi áhrif, en í sýnum með veikari styrk getur það gerst að allt peptíðið festist við yfirborðið. Niðurstöðurnar voru að í sumum tilvikum var hærra mælanlegt magn þegar mæliglös voru húðuð með BSA. Það bendir til þess að hluti tapsins geti stafað af því að peptíðið festist við ílátið frekar en að það brotni aðeins niður.

Í greiningarhluta verkefnisins var HPLC, mælitæki sem getur aðskilið og magngreint efni, notað fyrir mótunar- og stöðugleikasýni. LC-MS, mælitæki sem getur aðskilið og magngreint eins og HPLC en einnig borið kennsl á efni, var notað fyrir tilraunir með blóðsýni. HPLC reyndist gagnlegt tæki til að fylgjast með mótunar- og stöðugleikasýnum. Í forprófunum með blóðsýni reyndist hins vegar erfitt að mæla CGRP og CGRP 8-37 með fullnægjandi áreiðanleika. Blóð er flókið sýni. Ensím geta brotið niður peptíð eftir sýnatöku og mismunandi meðhöndlun sýna getur breytt því magni sem mælist. Þess vegna ætti að líta á blóðtilraunirnar í þessu verkefni sem aðferðarþróun, en ekki sem endanlega mælingu á peptíðmagni í fólki.

Meginniðurstaðan er sú að þróun á CGRP 8-37 byggir á góðri greiningarstjórnun. Ekki er hægt að meta stöðuga lyfjaformúleringu ef mæliaðferðin tapar peptíðinu í yfirborð, sýnameðhöndlun eða undirbúningi. Það er ekki heldur hægt að draga ályktanir um magn peptíðs í blóði fyrr en aðferðin nær betri stjórn á niðurbroti við sýnameðhöndlun og endurheimt. Næstu skref ættu því að vera skýr: bæta blóðsýnameðhöndlun, þróa LC-MS aðferð með innri staðli og rannsaka betur sýrustig, osmósuþéttni, síun og geymslustöðugleika fyrir áframhaldandi þróun CGRP 8-37.

Abstract

α-CGRP 8-37 is a peptide fragment of calcitonin gene-related peptide and is commonly used as a CGRP antagonist in pharmacological research. CGRP signaling has been discussed in relation to neuroimmune mechanisms in psoriasis, but a practical development of α-CGRP 8-37 requires analytical methods and formulation strategies that can handle peptide instability, adsorption, and matrix effects. The aim of this thesis was to develop and evaluate analytical and formulation approaches for α-CGRP 8-37, with emphasis on subcutaneous formulation relevance and pilot blood-matrix method development.

An HPLC method with UV detection at 214 nm was used for formulation and stability samples. LC-MS was used for pilot blood-matrix experiments. Formulation studies evaluated α-CGRP 8-37 in aqueous and excipient-containing vehicles at different peptide concentrations during room-temperature storage. An adsorption experiment compared apparent peptide recovery in BSA-coated and non-coated vials. Blood-matrix experiments tested dilution and protein-removal approaches, including direct dilution into aqueous formic acid, inhibitor-containing formic acid, and organic solvent, with and without SPE cleanup.

The formulation data showed concentration-dependent differences in apparent stability. At day 41, 10 and 20 mg/mL samples retained mean signals of 55.0% and 58.2% across tested conditions, while 2 mg/mL samples retained a mean signal of 15.3%, with several conditions becoming undetectable. BSA-coated vials increased apparent recovery in several low-concentration samples, supporting adsorption as a likely contributor to peptide loss. In the first whole-blood LC-MS pilot, CGRP and CGRP 8-37 signals for 30 second old samples were approximately 19.1% and 24.5% of blank-spiked controls, and signals decreased by 84.6% and 79.5%, respectively, from 30 seconds to 10 min. In the SPE-based experiment, enzyme inhibitor containing unspiked samples gave a high CGRP 8-37 signal while CGRP remained low. Likely indicating that sample preparation chemistry strongly affected the measured peptide signal.

The HPLC method was suitable for formulation screening, while the current blood-matrix LC-MS workflow was not ready for quantitative volunteer or patient analysis. The blood results should be interpreted as method development findings rather than biological concentration data. Future work should prioritize internal standards, controlled quenching immediately after blood draw, recovery studies, matrix-factor assessment, pH and osmolality testing, and longer formulation stability studies.

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1. Background

Psoriasis is a chronic immune-mediated disease that affects the skin and, in some patients, the joints. The disease involves keratinocyte hyperproliferation, inflammatory cell infiltration, vascular changes, and cytokine driven feedback between skin cells and immune cells [1,2]. Modern treatment has improved disease control, but psoriasis still remains an intersting disease context for studying neuroimmune signaling because sensory nerves, neuropeptides, and inflammatory pathways interact in the skin [3,4].

Calcitonin gene-related peptide, CGRP, is a 37 amino acid neuropeptide produced from the calcitonin gene family. It is best known as a potent vasodilator and as a major target in migraine pharmacology, but it also participates in sensory nerve signaling and neurogenic inflammation [5]. Human α-CGRP 8-37 is a shortened peptide lacking the first seven amino acids of α-CGRP. This shortening removes agonist activity at the CGRP receptor, while preserving antagonist activity at CGRP-responsive receptors [6,7].

A previous thesis at the University of Iceland studied the relation between CGRP, CGRP 8-37, and psoriasis. That work reported that CGRP and CGRP 8-37 were measurable in blood from healthy individuals, but it also stated that the methodology required improvement due to variability and quality-control limitations [8]. The current thesis builds on the biological motivation of that work but shifts the focus toward pharmaceutical development. The main question is not whether CGRP 8-37 has biological relevance in psoriasis. The question is however whether CGRP 8-37 can be measured and formulated with enough reliability to support further biological studies.

Peptide drug development often fails for various reasons, peptides can degrade, adsorb to container surfaces, precipitate, aggregate, or generate inconsistent analytical responses in biological matrices. These problems matter more for subcutaneous formulations because the dose volume is limited and the product must remain physically and chemically acceptable during handling, storage, and injection [9,10].

This thesis therefore treats formulation, analytical quantification, and pilot blood-matrix sample preparation as one cohesive development problem. The formulation work asks how CGRP 8-37 behaves in simple vehicles and excipient systems. The HPLC work tests formulation screening. The blood-matrix work tests whether LC-MS sample preparation can preserve and detect CGRP and CGRP 8-37 in whole blood. Together, these experiments shed light on the present development barriers, and the next practical research steps.

2. Aim of the thesis

The aim of this thesis was to develop and evaluate analytical and formulation strategies for α-CGRP 8-37 under conditions relevant to subcutaneous formulation development and future blood-matrix quantification.

The specific objectives were to:

- develop and apply an HPLC method suitable for monitoring CGRP 8-37 in formulation and stability samples.
- evaluate the stability of CGRP 8-37 in selected aqueous and excipient-containing vehicles at different peptide concentrations.
- investigate whether adsorption to vial surfaces contributes to apparent peptide loss.
- perform pilot LC-MS blood-matrix experiments to identify major sample-preparation limitations for CGRP and CGRP 8-37.
- define the most important analytical and formulation steps needed before volunteer or patient samples are analyzed.

3. Theoretical background

The theoretical background of this thesis focuses on the concepts needed to interpret the experiments. The project does not test psoriasis biology directly and does not aim to establish clinical efficacy. It addresses a prior step in drug development: whether α-CGRP 8-37 can be measured and handled in a way that supports formulation development and future biological studies. The relevant theory therefore connects to four main areas: the biological reason for studying CGRP 8-37, peptide instability in solution, formulation constraints for subcutaneous administration, and analytical challenges in peptide quantification [1-12,19-24].

3.1. Biological rationale for CGRP 8-37

Calcitonin gene-related peptide, CGRP, is a 37 amino acid neuropeptide expressed mainly in sensory neurons. It is widely recognized as a potent vasodilator and as a mediator in nociception and neurogenic inflammation [5]. CGRP belongs to the calcitonin peptide family, together with calcitonin, amylin, adrenomedullin, and adrenomedullin 2 [5,6]. These peptides act through receptors formed from calcitonin receptor or calcitonin receptor-like receptor combined with receptor activity-modifying proteins, called RAMPs [6]. The main canonical CGRP receptor consists of calcitonin receptor-like receptor and RAMP1, although receptor pharmacology is more complex when calcitonin receptor and other RAMP combinations are present [5,6]. This receptor complexity matters because CGRP-related peptides are often discussed as if they act through a single receptor in all tissues, that assumption is too simple for a formulation or bioanalytical thesis that uses CGRP biology as context [5-7].

CGRP 8-37 is an N-terminally truncated fragment of CGRP. Removal of the first seven amino acids removes the receptor-activating domain while preserving much of the receptor-binding region, which makes CGRP 8-37 a commonly used peptide antagonist in pharmacological experiments [5,6,28]. It is best understood as a reference compound for probing CGRP-dependent signaling rather than as a clinically established drug [6,28]. That distinction is important for this thesis. A compound that works as an antagonist in cell culture still needs to pass several practical development steps before it can be treated as a realistic therapeutic candidate [9,11]. That includes analytical control, stability, suitable excipients, manufacturability, and a dosage form that fits the intended route [9,11,23,24].

Psoriasis provides the biological context for the project. Psoriasis is a chronic immune-mediated skin disease involving abnormal keratinocyte proliferation, immune-cell infiltration, vascular changes, and cytokine-driven feedback between immune cells and the epidermis [1,2]. The IL-23 and IL-17 axis is central in current models of plaque psoriasis. Inflammatory dendritic cells release IL-23 and IL-12, which support IL-17-producing T cells, Th1 cells, and Th22 cells. These cells produce cytokines such as IL-17, interferon γ, TNF, and IL-22, which act on keratinocytes and amplify skin inflammation [1]. This framework showcases why psoriasis is not just a disorder of keratinocyte growth. It is an integrated skin immune disease.

Sensory nerves add another layer to this system. Several reviews describe neurogenic components in psoriasis, including altered nerve distribution and changes in neuropeptides such as CGRP, substance P, and vasoactive intestinal peptide [3,4]. CGRP released from sensory nerve endings can affect vascular tone, immune-cell behavior, and local inflammatory responses [5,7]. The literature does not support that

CGRP is either pro-inflammatory or anti-inflammatory. CGRP has been described with both inflammation-promoting and inflammation-dampening effects depending on tissue and disease context [5,7]. CGRP effects are depended on tissue, receptor expression, cell type, disease context, and concentration [5-7]. In psoriasis-related development, this uncertainty calls for careful framing [1,3,4]. CGRP 8-37 is biologically interesting because it links sensory-neuronal signaling to immune and vascular mechanisms [3], but formulation and analytical work must be solved before patient-facing conclusions can be drawn.

Previous work at the University of Iceland examined CGRP and CGRP 8-37 in relation to psoriasis and reported that both peptides were measurable in blood from healthy individuals. That work also stated that the measurement method needed improvement before stronger conclusions could be drawn [8]. This thesis builds from that methodological gap rather than repeating the earlier biological study. The aim is to support future studies by developing practical analytical and formulation knowledge for α-CGRP 8-37. The biological rationale gives the project direction; the experimental work tests the physical and analytical feasibility behind that direction [9,11].

3.2. Peptide developability and instability

Peptides occupy a space between small molecules and proteins. They often show high target specificity and biological potency, but they can and do often suffer from short biological half-life, limited oral absorption, enzymatic degradation, chemical instability, poor solubility in some media, adsorption to surfaces, and aggregation [9,11]. The developability of a peptide therefore depends on more than receptor pharmacology. A candidate that works in an assay can still fail because it cannot be stored, filtered, injected, recovered from a sample, or quantified reproducibly. This thesis treats those practical limits as scientific data rather than as side problems [9,11].

CGRP 8-37 is a short peptide fragment. Compared to larger proteins, short peptides often lack a rigid tertiary structure and therefore expose more side chains directly to solvent [9,11]. This exposure increases sensitivity to pH, ionic strength, organic solvent composition, temperature, oxygen, light, and container surfaces [10,11,16]. A peptide solution can lose apparent potency through true chemical degradation, physical aggregation, precipitation, adsorption to a vial, adsorption to a pipette tip, or analytical signal suppression. These mechanisms can occur at the same time [10,11,16]. An HPLC assay that only measures soluble peptide in the sampled liquid cannot automatically distinguish all of them; method specificity, sample recovery, and matrix effects must be considered separately [21,23,24].

Chemical instability changes the covalent structure of the peptide. In aqueous peptide formulations, important chemical routes include peptide backbone hydrolysis, deamidation, oxidation, isomerization, racemization, β-elimination, and disulfide exchange when cysteine residues are present [11]. CGRP 8-37 lacks the N-terminal disulfide ring present in full-length CGRP, so disulfide exchange is less relevant than for the intact 37 amino acid peptide. Other degradation routes remain relevant. The CGRP 8-37 sequence contains residues such as asparagine and phenylalanine, and these residues can influence degradation susceptibility depending on neighboring residues and formulation conditions. Sequence-based predictions are useful, but they do not replace experimental stability data [11,23,24].

pH is one of the most important formulation variables for peptide stability. Acidic and basic conditions can catalyze different degradation pathways, and many peptides show a pH range where degradation slows [11]. The best pH for stability is not necessarily the

best pH for injection comfort. This creates a development tradeoff. A formulation scientist often must select a pH that keeps degradation low while remaining acceptable for subcutaneous administration. Buffer selection also matters. Buffers control pH, but buffer species and buffer strength can influence local tolerability, ionic strength, solubility, and degradation rate [11,17].

Temperature and time determine the practical relevance of degradation. A small degradation rate can be acceptable for short sample handling but unacceptable during long-term storage. A peptide that is stable for hours in the autosampler can still fail after weeks at room temperature. The stability experiment in this thesis is therefore formulation-relevant rather than purely analytical. It asks whether dissolved α-CGRP 8-37 remains detectable over time in selected vehicles and concentrations under room-temperature storage. The answer is not expected to be universal. It is expected to identify conditions that deserve further testing under controlled pH, osmolality, and refrigerated storage [9-11,23,24].

Enzymatic degradation adds a different problem in biological matrices. Blood contains proteases and peptidases that can cleave peptides after sampling. For a labile peptide, the measured concentration may depend more on pre-analytical handling than on the true in vivo concentration [19,20,22]. If enzyme inhibitors or denaturing solvents are added after a delay, they stop or slow the state that exists at that moment; they do not reconstruct the original concentration. This distinction is central to the blood-matrix experiments in this thesis. A time-course experiment where inhibitors are added at each timepoint measures the combined effect of blood contact, sample timing, and quench chemistry. It does not by itself provide a clean biological half-life [20,22].

Chemical degradation, enzymatic degradation, adsorption, and aggregation can all appear as a loss of chromatographic peak area. This should therefore be interpereted with caution. A lower peak area can mean the peptide degraded into fragments. It can mean the peptide adsorbed to glass. It can mean the peptide precipitated [10,13,14,19]. It can mean extraction recovery changed. It can mean ionization in the mass spectrometer changed. A higher peak area after a delay can also occur if a larger precursor or bound pool releases or transforms into the monitored analyte. Peak area becomes concentration only after calibration, recovery, matrix effect, stability, and specificity have been addressed [21-24].

3.3. Physical instability: aggregation, precipitation, and adsorption

Physical instability changes the physical state or distribution of the peptide without necessarily changing covalent structure. For peptide formulations, the most relevant physical processes are aggregation, precipitation, gel formation, adsorption to surfaces, and interfacial accumulation [10,13,14]. These processes matter for three reasons. They can reduce the amount of soluble active peptide. They can make dosing inaccurate. They can create quality and safety concerns, especially for injectable products. Aggregates and particles in biopharmaceutical preparations are also discussed as potential contributors to immunogenicity risk, although the relationship between aggregate type and immune response is complex [15].

Aggregation occurs when peptide molecules associate into larger species. The driving forces include hydrophobic interactions, electrostatic interactions, and changes in secondary structure [10,13,14]. Aggregation depends on the peptide sequence, concentration, pH, ionic strength, temperature, mechanical stress, and excipients. A

condition that stabilizes one peptide can destabilize another. This is why formulation screening remains necessary even when general rules are available [9-11].

Ionic strength can strongly affect peptide solubility and aggregation. Salts screen electrostatic repulsion and alter water's properties. In some cases, salt can improve the solubility or reduce the adsorption. In other cases, it promotes salting out, aggregation, or gel-like behavior [10,14,16]. For an injectable product, sodium chloride is tempting because it is familiar and useful for tonicity. Familiarity is not evidence of compatibility. Internal Nepsone formulation work previously showed that 0.9% sodium chloride was associated with solid or gel-like behavior for α-CGRP 8-37 under some conditions [12]. That observation gives a strong reason to test saline-containing and non-saline vehicles carefully in this thesis.

Precipitation and gel formation are especially important for subcutaneous development. A precipitated or gelled sample does not deliver a controlled dose through a syringe or needle. It can clog filters, retain peptide during sterile filtration, and create uncertainty about the actual concentration in the injected liquid [17,18]. Filtration can make the sample appear clean while retaining a large fraction of the peptide on the filter [16,27]. A clear filtrate is not proof that the original formulation was stable. It only proves that the material passing through the filter was clear under that test condition [23,27].

Adsorption is a separate source of apparent loss. Peptides and proteins can adsorb to glass, plastic, pipette tips, vial inserts, membranes, and air-liquid interfaces [16]. Low-concentration samples are particularly vulnerable because the absolute surface area relative to dissolved peptide is high. A small amount of adsorption can remove a large fraction of peptide from solution. This matters both for formulation experiments and analytical standards. If standards adsorb differently from samples, calibration becomes unreliable. If aged samples are repeatedly transferred to HPLC vials, each transfer adds another surface contact [16,25].

The adsorption literature contains a useful warning for this thesis. Kristensen et al. showed that cationic peptides can be rapidly lost from solution through adsorption to common glass and plastic containers, with low concentration and high surface-to-volume ratio increasing the problem [16]. CGRP 8-37 is not identical to the peptides in that study, so the study cannot be copied as a quantitative prediction. It still supports the experimental logic of testing BSA-coated vials and low-binding handling conditions. Surface adsorption is a plausible explanation whenever apparent peptide concentration decreases without a clear degradation peak.

BSA coating can be interpreted in two ways, depending on the experimental protocol. If BSA is used to coat the vial and then rinsed away, the experiment mainly tests surface passivation. If BSA remains in the sample, the experiment also introduces a soluble protein that can bind surfaces, occupy interfaces, and possibly interact with the peptide [25,26]. Those mechanisms are not equivalent. For this thesis, the BSA protocol must therefore be described precisely. The result can only be interpreted correctly if the reader knows whether the BSA acted as a surface treatment, a residual excipient, or both [23,24].

Physical instability complicates analytical interpretation. A stability assay based on HPLC peak area detects the peptide fraction that remains soluble, sampled, injected, and chromatographically resolved [23,24]. It does not automatically quantify material stuck to the container wall, retained in a gel, or removed during centrifugation and filtration. This is limits of the HPLC method [23,24]. The thesis therefore uses cautious wording such as apparent recovery and remaining detectable peptide when the mechanism has

not been isolated. That prevents the formulation error of calling every decrease degradation.

3.4. Formulation constraints for subcutaneous peptide administration

Subcutaneous administration is attractive for chronic therapy because it can support outpatient treatment, self-administration, and lower clinical burden compared with intravenous administration [17,18]. It also places strict demands on the formulation. The product must be injectable, tolerable, physically stable, chemically stable, sterile or sterilizable, compatible with the container closure system, and accurate at the intended dose. For a peptide candidate, the route is not a label added at the end of development. It shapes the concentration, excipient, pH, osmolality, viscosity, and filtration strategy from the beginning [9,11,17,18].

Injection volume limits force concentration decisions. Subcutaneous injection volumes around 1 to 1.5 mL are often treated as conventional upper limits for comfortable administration, while larger volumes require specific justification and device or tolerability assessment [17,18]. If the required dose is high, the formulation concentration must increase. Higher concentration often worsens aggregation, viscosity, adsorption behavior, and filtration performance [10,13,14]. A low concentration may look stable in an assay but require too large an injection volume. A high concentration may fit the dose volume but create gelation or particle risks. The formulation target must therefore be selected with both stability and delivery in mind.

pH and osmolality affect both stability and local tolerability. Usach et al. reviewed factors influencing pain at subcutaneous injection sites and identified injection volume, osmolality, viscosity, pH, injection speed, and excipients as relevant variables [17]. Ideally, injectable formulations are close to isotonic conditions, around 300 mOsm/kg, and strongly hypertonic solutions increase concern for pain and tissue irritation [17]. A pH near physiological values is often better tolerated, but many peptides are more stable under mildly acidic conditions [11,17]. This tradeoff is directly relevant to α-CGRP 8-37 because internal prior formulation work indicated pH shifts after API addition and suggested that acetate-buffered conditions around pH 4.7 may be worth exploring [12].

Excipients are selected to solve specific problems. Sugars and polyols such as trehalose, sucrose, mannitol, glycerol, and propylene glycol are often investigated because they can alter preferential hydration, viscosity, water activity, tonicity, and physical stability [11]. Trehalose and sucrose are common stabilizing sugars for proteins and peptides, but their effects depend on the molecule and the concentration [11,14]. Mannitol can support tonicity and is widely used in parenteral systems, but crystallization and concentration effects need attention. Glycerol and propylene glycol can change solvent properties and viscosity. These excipients should therefore be treated as formulation variables with both potential benefits and limitations [9,11].

Buffers stabilize pH but also add formulation risk. Increasing buffer strength improves pH control after API addition, but buffer concentration can influence injection pain, ionic strength, and peptide behavior [17]. Acetate buffers are common in injectable formulations and are compatible with mildly acidic pH ranges. For α-CGRP 8-37, acetate-buffered pH around 4.7 is relevant because earlier internal work suggested that low-strength buffers shifted after API addition and that pH control needed improvement [12]. The thesis data should therefore be read as pre formulation screening rather than a final formulation. A final product would still require controlled pH, osmolality, sterility, filter compatibility, container compatibility, and longer storage studies.

Surfactants can reduce interfacial adsorption and agitation-induced aggregation, but they must be used cautiously. Non-ionic surfactants such as polysorbates are common in biologic formulations, yet they can degrade, interact with proteins or peptides, and complicate analytical interpretation [14,15]. Cyclodextrins, co-solvents, amino acids, and salts can also improve one property while worsening another. For example, an excipient that improves solubility may create chromatographic interference or increase viscosity. Internal prior screening reported that PEG 400 and SBE-β-CD were unsuitable for α-CGRP 8-37 under tested conditions because solid or gel-like structures formed [12]. That does not ban those excipients in every possible formulation, but it argues against them as first-line choices in the present thesis.

Sterile filtration is a practical gate for injectable development. A solution that passes chemical assay but loses substantial peptide during filtration has not solved the formulation problem [27]. Loss during filtration can arise from adsorption to the membrane, retention of aggregates, or gel retention. This matters for α-CGRP 8-37 because dose accuracy depends on the peptide remaining in the filtrate, not merely dissolving before the filter step. Filterability should therefore be discussed together with stability and appearance. A formulation candidate should remain clear, assay close to target concentration, avoid excessive particle formation, and pass through a relevant filter without large peptide loss [10,15,17,27].

3.5. Analytical control of peptide formulation samples

Analytical control is the foundation of formulation development. Without a method that measures the peptide reproducibly, every formulation conclusion becomes suspect [23,24]. HPLC with UV detection is a practical first-line method for peptide formulation samples because peptides absorb strongly around 214 nm through the peptide bond. This wavelength is sensitive for peptide detection but not highly selective [29]. Other sample components, degradation products, or excipients can also absorb in this region. Chromatographic separation and peak identity therefore matter [23,24,29]. A clean retention time and consistent peak shape are more informative than a raw absorbance signal alone [23,24].

HPLC is well suited for formulation screening when the peptide concentration is high enough and the matrix is simple. In this thesis, formulation samples contain defined vehicles rather than whole blood. That makes HPLC useful for calibration, assay, and stability screening. The method can compare apparent peptide remaining across time, vehicle, and concentration. It can also reveal additional peaks when degradation products or interfering excipient peaks appear [23,24,29]. The method is less suitable for endogenous blood quantification, where peptide concentrations are lower, proteins and salts are abundant, and specificity requirements are higher [19,20,22].

Calibration defines the quantitative range of an HPLC method. A calibration curve should cover the expected sample concentrations after dilution and should be prepared in a solvent or matrix that behaves like the samples. If standards are prepared in clean solvent while samples contain excipients that alter peak shape or recovery, the assay may become biased [21-23]. Replicate standards, quality controls, and repeat injections help separate instrumental variation from sample behavior. For this thesis, calibration data should be shown because it supports the claim that the HPLC method was suitable for formulation and stability samples [23,24]. Raw chromatograms belong in the appendix unless they show a central result.

The analytical readout must match the question. For chemical stability, peak purity and degradation products matter [11,23,24]. For formulation screening, apparent soluble

recovery and visual appearance matter. For adsorption, the difference between coated and uncoated containers matters. For injectability, pH, osmolality, viscosity, particles, and filtration loss matter [15,17,18]. HPLC alone does not answer all these questions. It answers a narrower but necessary question: how much detectable soluble peptide appears under the selected assay conditions [23,24].

The use of glass vials and repeated transfers deserves attention. Samples in the stability experiment were stored in glass vials, gently mixed, transferred to glass HPLC vials, and sampled by the autosampler needle. Each of those steps introduces contact with surfaces [16,25]. For a peptide with possible adsorption liability, sample handling becomes part of the experiment. It is not enough to state the storage medium. The container, transfer procedure, and injection setup must be reported because they influence the amount of peptide that reaches the detector [23,24]

3.6. LC-MS bioanalysis in blood matrices

LC-MS provides higher selectivity than UV-HPLC and is better suited for peptide analysis in biological matrices. Peptide bioanalysis by LC-MS or LC-MS/MS can target defined mass-to-charge signals and distinguish analytes from many background components [19,20]. This advantage does not make the method automatically quantitative. Blood and plasma matrices contain proteins, lipids, salts, phospholipids, metabolites, endogenous peptides, and enzymes. These components can suppress or enhance ionization, contaminate the column, interfere with peaks, bind the analyte, and change extraction recovery [19-22].

Matrix effect is one of the central problems in LC-MS bioanalysis. Matuszewski et al. described matrix effect as a key source of altered analyte response in HPLC-MS/MS bioanalytical methods and provided strategies for assessing it [21]. ICH M10 defines matrix effect as an alteration of analyte response due to interfering, often unidentified components in the sample matrix, and recommends evaluation across independent matrix sources during validation [22]. This matters for α-CGRP 8-37 because an apparent difference between blood samples can reflect biology, enzymatic processing, extraction recovery, adsorption, or ionization differences. Without matrix assessment and internal standards, interpretation must remain cautious [19-22].

Recovery is different from matrix effect. Recovery describes how much analyte survives sample preparation and extraction. Matrix effect describes how remaining matrix components alter detector response. A sample can have high recovery but severe ion suppression. It can also have low recovery but little ion suppression [21,22]. Solid-phase extraction is often used to remove matrix components and concentrate the analyte, but SPE introduces its own variables: sorbent chemistry, loading solvent, wash strength, elution solvent, dilution, and adsorption to the plate or collection well. For peptide analytes, charge state, hydrophobicity, and solvent composition all influence recovery [19,20, 22].

Internal standards are especially important in peptide LC-MS. An ideal internal standard resembles the analyte, experiences the same extraction loss and ionization effects, and remains distinguishable in the mass spectrometer. Stable-isotope labelled analogues are often preferred [19-22]. Without an internal standard, injection-to-injection variation, extraction loss, and matrix effect are harder to separate [19-22]. The blood-matrix experiments in this thesis should therefore be framed as pilot method development. They can identify failure modes and promising handling conditions. They should not be presented as validated quantification of endogenous peptide concentration.

Pre-analytical stability is another requirement for blood peptide measurement [20,22]. A blood sample begins changing as soon as it is drawn. Proteases can degrade endogenous and spiked peptides. Cellular components can release or bind compounds. Acid, organic solvent, cooling, centrifugation, enzyme inhibitors, and SPE timing all influence the measured result [19-22]. If a peptide signal increases after a waiting period, several explanations remain possible: conversion from a precursor, release from a bound state, altered extraction recovery, reduced ion suppression, carryover, or integration error [20-22]. A single experiment cannot rank these possibilities with confidence. A good thesis acknowledges this uncertainty rather than decorating it.

The enzyme inhibitor cocktail used in this project targeted cysteine proteases, serine proteases, and metalloproteases. Such cocktails can reduce enzymatic activity, but they do not guarantee complete stabilization [19,20,22]. Inhibitors have different solubilities, mechanisms, and compatibility with acid or organic solvent. They may also affect extraction, ionization, or peptide binding. If inhibitors appear to preserve CGRP 8-37 but not CGRP, that observation should be treated as a method-development finding rather than immediate biological evidence [19-22].

The SPE experiment adds a useful methodological layer because it compares sample dilution vehicles and SPE handling. The protocol used methanol conditioning, 0.1% formic acid equilibration, sample loading, water washes, and elution with 50% acetonitrile containing 0.1% formic acid. The experimental design included formic acid, formic acid with enzyme inhibitors, and acetonitrile/ethanol dilution conditions, with spiked and unspiked samples. The additional dilution of the organic-solvent condition must be corrected during interpretation. The setup is not a validation study, but it directly addresses sample preparation, which is the main bottleneck in blood-matrix analysis.

A validated bioanalytical method would require more than detection. It would require selectivity, calibration performance, accuracy, precision, recovery, matrix effect assessment, carryover assessment, dilution integrity, stability during sample collection and processing, autosampler stability, and suitable quality controls [22]. The current thesis does not need to meet all validation criteria because it is positioned as method development. It should still use the language of validation correctly. Detection means the analyte signal is observed. Quantification means the signal is converted to concentration using a fit-for-purpose calibration and sample preparation method. Validation means the method has been shown to perform within defined limits for its intended use. These words are not synonyms.

The theoretical conclusion is straightforward. α-CGRP 8-37 development is limited by the same problems that often limit peptide drugs: aqueous instability, surface loss, aggregation risk, injection-route constraints, and difficult bioanalysis. These issues are not secondary to the biological question. They determine whether the biological question can be tested reliably. The experiments in this thesis therefore belong together: HPLC establishes practical analytical control for formulation samples; formulation and adsorption studies test how the peptide behaves in candidate vehicles and containers; pilot LC-MS blood experiments expose the limits of current biological-matrix handling. The shared theme is developability.

4. Materials and Methods

4.1. Materials

The active pharmaceutical ingredient used for formulation and HPLC experiments was α-CGRP 8-37 human acetate salt with a C-terminal amide. The material was supplied by Vivotecnia, and the analytical data sheet identified the peptide as Bachem product number 4050232, lot 1000099175. The material was stored at -20 °C, with an allowed variation of ±5 °C. The API had undergone four freeze-drying cycles before use, API information can be seen in table 4.1.

The analytical data sheet reported a white powder with no visible impurities. The listed molecular formula was C139H230N44O38, with an average peptide molecular mass of 3125.6 Da and a monoisotopic mass of 3123.7 u by ESI-MS. HPLC purity was 96.0%, with total HPLC impurities of 4.0%. The largest listed impurity was 0.82% at RRT 1.045. The elemental analysis assay found 80.2%. Water content by Karl Fischer was 3.0%, acetic acid content was 16.3%, and TFA content was below 0.015%.

Nominal formulation concentrations in this thesis refer to weighed peptide acetate powder unless explicitly stated otherwise. For assay-corrected calculations, the weighed mass should be corrected for the 80.2% as-is assay. Thus, 1.00 mg of assay-corrected peptide corresponds to approximately 1.25 mg of the supplied powder.

For HPLC calibration, a 1.0 mg/mL CGRP 8-37 stock solution was prepared fresh in 70:30 Milli-Q water:acetonitrile containing 0.1% TFA. Calibration standards were prepared in the same solvent system. The calibration range was 0.0125 to 1.0 mg/mL. Samples were usually diluted 1:10 before injection in the same solvent system; samples with nominal peptide concentration of 20 mg/mL were normally diluted 1:20.

Table 4.1. CGRP 8-37 API information used for formulation and HPLC experiments.

Parameter	Value
Peptide	α-CGRP 8-37 human acetate salt, C-terminal amide
Supplier	Vivotecnia; analytical sheet identified Bachem product number 4050232
Lot number	1000099175
HPLC purity	96.0%
As-is assay	80.2%
Water content	3.0% by Karl Fischer
Acetic acid content	16.3%
TFA content	<0.015%
Peptide molecular mass	3125.6 Da average molecular mass
Storage	-20 °C ±5 °C

4.2. HPLC method for formulation and stability samples

Formulation and stability samples were analyzed by reversed-phase HPLC with UV detection. The system was a Thermo Scientific Vanquish-type HPLC/UHPLC system controlled through Chromeleon 7. The system consisted of a quaternary pump, split sampler, diode array detector, and thermostatted column compartment. Separation was performed using a Waters ACQUITY UPLC Peptide BEH C18 column, 300 Å, 1.7 µm, 2.1 × 100 mm. The column compartment was maintained at 40 °C. Method parameters can be seen in table 4.2.

The flow rate was 0.300 mL/min and the injection volume was 5 µL. UV data were collected at 214, 226, and 246 nm. Quantification was based on the peptide peak area at

214 nm. The diode array detector used a wide slit, a data collection rate of 5.0 Hz, response time of 1.000 s, and peak width of 0.100 min. The autosampler temperature control was off.
Mobile phase A was Milli-Q water, mobile phase B was methanol, mobile phase C was 90:10 acetonitrile:Milli-Q water containing 0.1% TFA, and mobile phase D was Milli-Q water containing 0.1% TFA. The gradient can be seen in table 4.3.
Calibration curves were prepared in Excel by plotting peak area against standard concentration. The slope and intercept were calculated using the SLOPE and INTERCEPT functions. The R^2 value was obtained from the calibration plot for each run. The calibration range was 0.0125 to 1.0 mg/mL. R^2 values were 0.9995, 0.9995, 0.9960, and 0.9999 for the stability runs on days 3, 7, 21, and 41, respectively. The day 15 stability run was excluded from interpretation because the HPLC performance was anomalous on that measurement day. For the adsorption runs, R^2 values were 0.9997, 0.9999, and 0.9997 on 14 April, 22 April, and 4 May, respectively. The ACN/EtOH supporting experiment had $R^2 = 0.9886$ on 28 April.
Most samples were injected twice. Duplicate injections are marked as #1 and #2 in the data sheets. The average peak area of accepted injections was used for calculations. If one injection failed, the accepted injection was used alone. Peak assignment was therefore based on the corresponding standard chromatograms in each run rather than on a fixed retention time alone.

Table 4.2. HPLC method parameters for formulation and stability samples.

Parameter	Value
Software	Chromeleon 7
System	Thermo Scientific Vanquish-type HPLC/UHPLC system
Column	Waters ACQUITY UPLC Peptide BEH C18, 300 Å, 1.7 µm, 2.1 × 100 mm
Column part number	186003686
Column temperature	40 °C
Flow rate	0.300 mL/min
Injection volume	5 µL
Detection	Diode array detector
Quantification wavelength	214 nm
Additional UV channels	226 nm and 246 nm
Calibration range	0.0125 to 1.0 mg/mL
Typical sample dilution	1:10; 1:20 for 20 mg/mL samples
Module model codes	VF-P20-A pump; VF-A10-A split sampler; VF-D11-A diode array detector; VH-C10-A column compartment

Table 4.3. HPLC gradient used for CGRP 8-37 analysis.

Time (min)	Flow (mL/min)	%B MeOH	%C ACN phase	%D aqueous 0.1% TFA
0.0	0.300	0	30	70
1.0	0.300	0	30	70
4.0	0.300	0	50	50
5.0	0.300	0	50	50
6.0	0.300	0	30	70
10.0	0.300	0	30	70

4.3. Stability experiment

CGRP 8-37 was dissolved in selected vehicles at nominal peptide concentrations of 2, 10, and 20 mg/mL. Vehicles included water, glycerol-containing solutions, propylene glycol-containing solutions, trehalose-containing solutions, and sodium chloride-containing solutions. Samples were stored at room temperature in glass vials. At each timepoint, the aged sample was gently mixed and transferred to a cleaned glass HPLC vial before analysis. The stability timepoints used for interpretation were 3, 7, 21, and 41 days. A day 15 measurement was excluded because the HPLC behaved anomalously on that day. Peptide signal was expressed as the fraction remaining relative to the original measured concentration. An owerview of the parameters used can be seen in table 4.4.

Values reported as zero represent samples where no detectable peptide peak was observed. Samples with spreadsheet errors or division errors were treated as failed analytical entries rather than true zero values.

During storage, CGRP 8-37 dissolved in 0.5% NaCl showed visible instability. The solution gradually developed a pink color, and small spherical gel-like structures, approximately 1 mm in diameter, were observed at the bottom of the vials when the samples were 11 days old. Similar but weaker color changes were observed in other NaCl containing samples. These observations were recorded as physical instability and interpreted separately from chromatographic peak area.

Table 4.4. Stability experiment overview.

Parameter	**Description**
Storage temperature	Room temperature
Container during storage	Glass vials
Container during HPLC analysis	Glass HPLC vials
Mixing before analysis	Gentle shaking
Detection	UV at 214 nm
Timepoints	3, 7, 15, 21, and 41 days
Concentrations	2, 10, and 20 mg/mL

4.4. Adsorption experiment

An adsorption experiment was added to test whether surface binding contributed to apparent peptide loss. CGRP 8-37 samples were prepared in matched glass vials with silicone caps. For each formulation and concentration, one vial was sham-treated, and one vial was BSA-coated. The only intended difference between the matched pair was the surface treatment of the 10 mL glass vial.

For BSA-coated vials, a fresh 1% w/v BSA solution was prepared in Milli-Q water. One milliliter of the BSA solution was added to each 10 mL glass vial. The vials were capped and gently rotated or inverted to wet the full inner wall, then incubated for 30

min at room temperature. The BSA solution was removed completely. Each vial was rinsed once with 1.0 mL Milli-Q water, drained, and air-dried for approximately 15 min until no visible droplets remained. No heat drying was used. The coated vials were used the same day.
For sham-treated control vials, 1.0 mL Milli-Q water was added to each vial. The vials were capped, gently rotated or inverted for 30 min at room temperature, emptied, rinsed once with 1.0 mL Milli-Q water, drained, and air-dried using the same procedure as the BSA-coated vials. This controlled for wetting, rinsing, and drying. The protocol can be seen in table 4.5.
The intended role of BSA was surface passivation. Free liquid BSA was removed before sample addition, although trace desorbed BSA could still enter the samples over time. CGRP 8-37 formulations were prepared directly in the treated 10 mL vials to avoid adding an extra adsorption surface before the experiment. At each timepoint, the vial was gently mixed, an aliquot was withdrawn, and the aliquot was diluted 1:10 into the HPLC solvent, consisting of 70:30 Milli-Q water: acetonitrile containing 0.1% TFA. The diluted samples were transferred to standard HPLC vials before injection.

Table 4.5. BSA coating protocol for the adsorption experiment.

Parameter	**Method**
Vial type	10 mL glass vial with silicone cap; brand not recorded
BSA concentration	1% w/v
Coating solution	BSA in Milli-Q water
Coating volume	1.0 mL per 10 mL vial
Coating time	30 min
Coating temperature	Room temperature, approximately 20 to 25 °C
Rinse step	Once with 1.0 mL Milli-Q water
Drying	Brief air-drying for approximately 15 min, until no visible droplets remained
Residual BSA	Adsorbed BSA layer intended to remain; free liquid BSA removed
Sample preparation	Formulations prepared directly in treated vials
Sampling	Gentle mixing, aliquot withdrawal, 1:10 dilution into HPLC solvent

No BSA-coated vehicle blank was included. After samples had lost detectable peptide signal, BSA-coated and sham-treated chromatograms did not show an obvious difference at 214 nm under the tested conditions. Possible UV background from trace residual BSA therefore could not be fully excluded, but it was not observed as a dominant chromatographic feature in these late samples.

4.5. ACN/EtOH solvent behavior experiment

A 70:30 (v/v) acetonitrile: ethanol solvent mixture was prepared and diluted with water to obtain nominal water contents of 0, 10, 20, 30, 40, and 50% (v/v). For each condition, 396 µL of solvent mixture was transferred to a 0.5 mL LoBind microcentrifuge tube and spiked with 4 µL of standard solution at either 1.0 or 0.1 mg/mL, corresponding to a 100-fold dilution and final standard concentrations of 10 or 1 µg/mL. The experiment was used as supporting data to assess whether organic solvent composition strongly changed peptide response.

4.6. Initial whole-blood LC-MS pilot experiment

The first whole-blood pilot experiment tested whether CGRP and CGRP 8-37 signals remained detectable after short contact with whole blood. Samples were prepared by combining 980 µL whole blood with 10 µL CGRP stock solution and 10 µL CGRP 8-37 stock solution. After either immediate handling or 10 min waiting time, 100 µL of the blood mixture was transferred into 900 µL of 50:50 ethanol: water. Samples were centrifuged at 4 °C and 4000 rpm, corresponding to approximately 2000 rcf, for 10 min. A 100 µL aliquot of supernatant was transferred into 900 µL of 0.1% formic acid before LC-MS analysis. Blood and solvent blanks were included, with spiked versions used to compare response and sample-preparation loss.

No SPE was used in the initial whole-blood LC-MS experiment, and no internal standard was included. The experiment was interpreted qualitatively and semi-quantitatively.

4.7. Time-course blood experiment

The time-course experiment was designed to estimate time-dependent signal behavior of CGRP and CGRP 8-37 in whole blood. Blood was obtained from two donors. Three vials were taken from each donor. Samples A, B, and C came from one donor, and samples D, E, and F came from the second donor. The vials were drawn 2.5 min apart, starting with A and ending with F.

Subsamples were taken at 0, 0.5, 2.5, 5, 10, 15, 20, and 30 min. For each timepoint, 100 µL blood was transferred into 900 µL of 50:50 ethanol: water. Two versions of the quench solution were used: one without enzyme inhibitors and one containing enzyme inhibitors. The inhibitor solution was prepared by dissolving E-64, leupeptin, antipain dihydrochloride, chymostatin, N-ethylmaleimide, and 1,10-phenanthroline in 50 mL of 50:50 ethanol: water. Nominal inhibitor concentrations in the quench solution were 40 µg/mL E-64, 20 µg/mL leupeptin, 10 µg/mL antipain, 20 µg/mL chymostatin, 10 µg/mL N-ethylmaleimide, and 20 µg/mL 1,10-phenanthroline. After mixing 100 µL blood with 900 µL quench solution, the nominal final concentrations were 90% of those values.

Because inhibitors were added only when the subsample reached its planned timepoint, the inhibitor condition did not test immediate prevention of blood-vial peptide processing. It tested the effect of adding inhibitors at the end of each blood-contact interval.

4.8. SPE-based blood sample preparation experiment

The SPE experiment tested different dilution vehicles before LC-MS analysis. Sample A used 0.1% formic acid. Sample B used 0.1% formic acid containing the enzyme inhibitor cocktail. Sample C used 70% acetonitrile and 30% ethanol. The plus sign indicated spiking with 10 µL CGRP and 10 µL CGRP 8-37, both from 1 mg/mL stock solutions, before blood was introduced. Blood samples were prepared at time 0 min and 10 min in duplicate. Samples C and C+ received an additional dilution step after centrifugation: 50 µL sample was diluted into 950 µL 0.1% formic acid, producing 400-fold total dilution. Samples A, A+, B, and B+ had 100-fold total dilution. Comparisons involving C and C+ were corrected for this extra dilution.

All samples were centrifuged at 4 °C and 4000 rpm, corresponding to approximately 2000 rcf, for 10 min before SPE. Samples were stored on ice until cleanup. SPE wells were conditioned with 600 µL methanol, equilibrated with 600 µL 0.1% formic acid,

loaded with 510 µL sample or standard, washed three times with 600 µL water, and eluted with 75 µL 50% acetonitrile containing 0.1% formic acid. The eluates were analyzed by LC-MS.

4.9. LC-MS method parameters

Two LC-MS setups were used for the blood-matrix method-development experiments. The whole-blood and time course experiments were analyzed using an Evosep One inlet coupled to a Waters Xevo TQ Absolute triple quadrupole mass spectrometer (XEVO-TQAbs#WDA0521). The Evosep inlet method was EV1109_SPD_100, reported as 100 SPD(RC), with a run time of 12.7 min. The acquisition report did not provide the full Evosep chromatographic gradient or a conventional LC injection volume, so those details were not used for interpretation. These experiments were therefore treated as pilot signal-monitoring experiments rather than validated quantitative assays.
The SPE experiment was analyzed using a Waters Acquity UPLC system coupled to a Waters Xevo TQ-XS triple quadrupole mass spectrometer (XEVO-TQXS#WBA0294). Separation was performed on a Waters ACQUITY UPLC Peptide BEH C18 column, 300 Å, 1.7 µm, 2.1 × 100 mm. Mobile phase A was water containing 0.1% formic acid, and mobile phase B was acetonitrile containing 0.1% formic acid. The flow rate was 0.500 mL/min, the injection volume was 2.0 µL, the column temperature was 40 °C, and the sample temperature was 10 °C. The method run time was 4.0 min. Flow was directed to the MS during the 1.0 to 2.0 min window according to the timed flow-state events in the acquisition method. An overwiev of the blood matrix experimetns can be seen in table 4.6.

Table 4.6. LC-MS overview for the blood-matrix experiments.

Experiment	**LC or inlet system**	**Mass spectrometer**	**Purpose**	**Key limitation**
Whole-blood pilot	Evosep One, EV1109_SPD_100, 100 SPD(RC)	Waters Xevo TQ Absolute	Initial signal and short blood-contact test	No SPE, no internal standard, qualitative interpretation
Time-course experiment	Evosep One, EV1109_SPD_100, 100 SPD(RC)	Waters Xevo TQ Absolute	Time-dependent signal behavior	Quench added at each timepoint, no internal standard, not a clean half-life study
SPE experiment	Waters Acquity UPLC	Waters Xevo TQ-XS	SPE cleanup and dilution-vehicle comparison	No internal standard and incomplete validation

For the SPE method, the gradient used a short reversed-phase cleanup and elution program. The gradient is listed in Table 4.7.

Table 4.7. LC-MS gradient used for the SPE experiment.

Time (min)	**Flow rate (mL/min)**	**%A water + 0.1% FA**	**%B acetonitrile + 0.1% FA**	**Curve**
0.00	0.500	85	15	Initial

1.40	0.500	70	30	6
1.80	0.500	70	30	6
1.90	0.500	10	90	6
2.90	0.500	10	90	6
3.00	0.500	85	15	6
4.00	0.500	85	15	6

Both LC-MS workflows monitored CGRP 8-37 and full-length CGRP using positive electrospray MRM. CGRP 8-37 was monitored using precursor m/z 625.80 and product ions m/z 460.35, 508.03, 552.31, 654.50, 736.21, 741.06, 802.36, and 840.16. Full-length CGRP was monitored using precursor m/z 758.74 and product ions m/z 460.45, 919.86, 1061.50, and 1134.30. Collision energies were 15 eV for CGRP 8-37 transitions and 23 eV for CGRP transitions. Dwell times were 0.013 s in the initial whole blood and time-course experiments and 0.019 s in the SPE method. The transition table used tuned cone voltage settings; the general source cone setting was 30 V.
For the initial whole-blood and time-course Evosep method, the source used positive electrospray with nano-lockspray, capillary voltage 3.00 kV, source temperature 80 °C, collision gas flow 0.18 mL/min, and nebuliser gas flow 7 bar. For the SPE method, the source used positive electrospray with capillary voltage 3.20 kV, source temperature 150 °C, desolvation temperature 500 °C, cone gas flow 150 L/h, desolvation gas flow 1000 L/h, collision gas flow 0.15 mL/min, and nebuliser gas pressure 7 bar. In all LC-MS runs, SIR/MRM chromatogram spike removal was enabled and smoothing was off. This can be viewed in table 4.8.

Table 4.8. MRM transitions used for CGRP 8-37 and CGRP.

Analyte	**Precursor m/z**	**Product ions m/z**	**Collision energy (eV)**	**Dwell time**
CGRP 8-37	625.80	460.35, 508.03, 552.31, 654.50, 736.21, 741.06, 802.36, 840.16	15	0.013 s in whole-blood and time-course; 0.019 s in SPE
CGRP	758.74	460.45, 919.86, 1061.50, 1134.30	23	0.013 s in whole-blood and time-course; 0.019 s in SPE

4.10. Data treatment

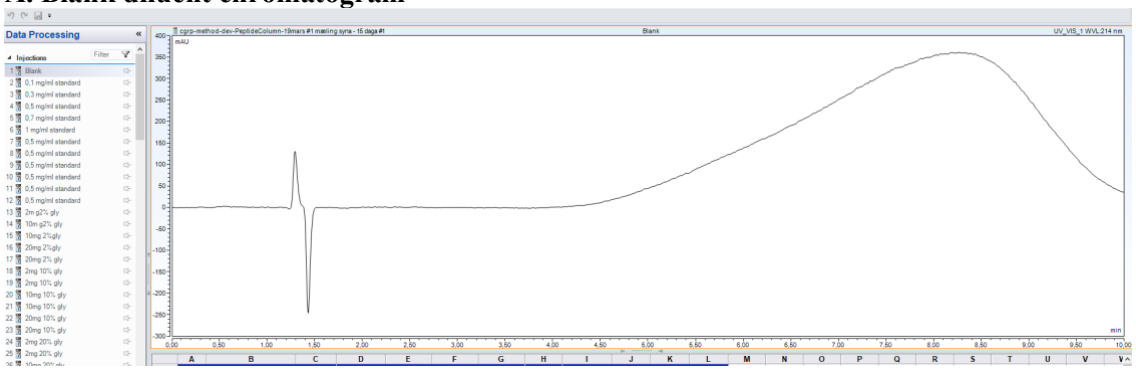
HPLC stability data were expressed as relative remaining signal. For blood-matrix LC-MS experiments, area values were used because the pilot workflows were not validated and no internal standard was included. In the SPE experiment, the Refined sheet values were used. C and C+ values were corrected by a factor of four when compared with A and B due to the additional dilution step. Very small A and C peaks were interpreted with caution based on analytical review.
The initial whole-blood pilot was interpreted qualitatively. The comparison between solvent-spiked and blank-spiked samples was used to assess whether matrix effect dominated the signal. The comparison between blank-spiked samples and early blood-contact samples was used as a rough estimate of recovery or signal loss, but not as a validated recovery calculation because the blank samples were prepared later than the first blood samples.

5. Results

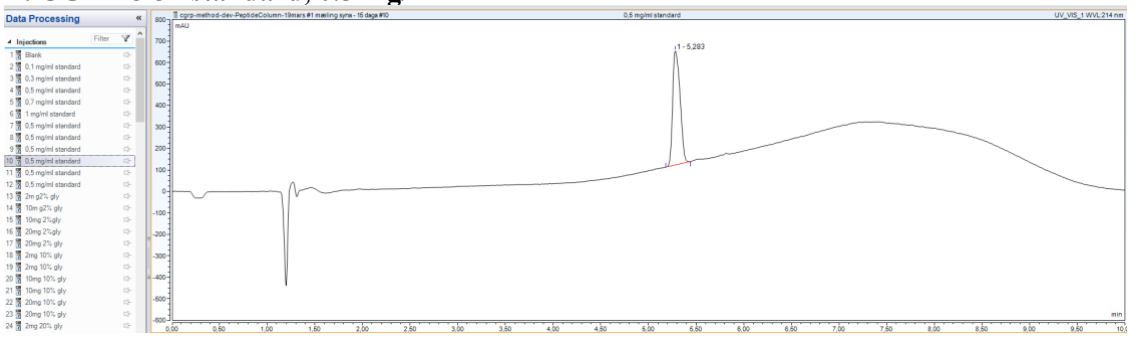
5.1. HPLC method performance

The HPLC method was first evaluated as the main analytical tool for formulation and stability samples. Representative chromatograms can be seen in figure 5.1. The calibration range covered the diluted formulation samples, and calibration curves were prepared separately for each measurement day. The method was considered suitable for formulation screening because the calibration curves were linear across the relevant concentration range and the peptide peak could be identified from standards in each run. Calibration statistics can be seen in table 5.1.

A. Blank diluent chromatogram



B. CGRP 8-37 standard, 0.5 mg/mL



C. Formulation sample, 10 mg/mL CGRP 8-37 in 5% trehalose

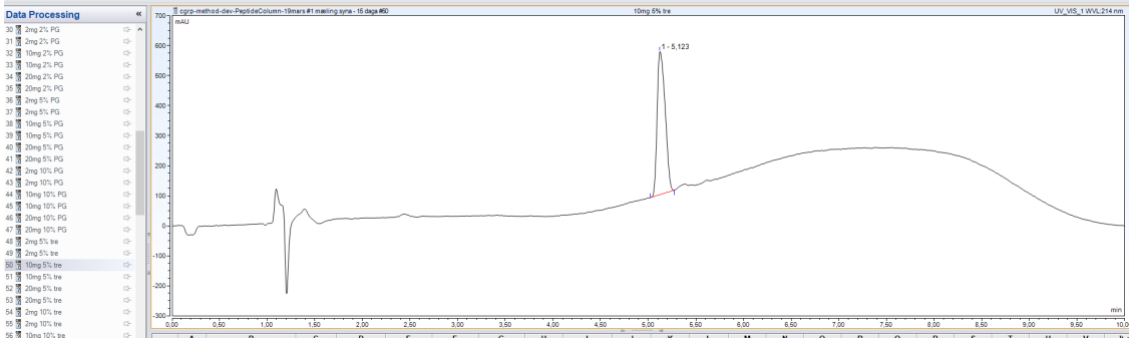


Figure 5.1. Representative HPLC chromatograms at 214 nm: blank diluent, 0.5 mg/mL CGRP 8-37 standard, and a 10 mg/mL CGRP 8-37 formulation sample in 5% trehalose.

Table 5.1. Same-day HPLC calibration performance for stability, adsorption, and supporting solvent experiments. Calibration was performed by plotting peak area at 214 nm against nominal standard concentration.

Experiment	Run/day	R^2	Calibration range
Stability	Day 3	0,9995	0.0125-1.0 mg/mL
Stability	Day 7	0,9995	0.0125-1.0 mg/mL
Stability	Day 21	0,9960	0.0125-1.0 mg/mL
Stability	Day 41	0,9999	0.0125-1.0 mg/mL
Adsorption	14 Apr	0,9997	0.0125-1.0 mg/mL
Adsorption	22 Apr	0,9999	0.0125-1.0 mg/mL
Adsorption	5 May	0,9997	0.0125-1.0 mg/mL
ACN/EtOH side experiment	28 Apr	0,9886	0.0125-1.0 mg/mL

Because the column was old and retention time shifted during the project, retention time alone was not used as the only identification criterion. Instead, each run was interpreted relative to the calibration standards analyzed during the same run.

5.2. Stability of CGRP 8-37 in formulation vehicles

The stability experiment compared the remaining detectable CGRP 8-37, seen in figure 5.2, signal after storage at room temperature in selected formulation vehicles, a comparison can be seen in figure 5.3. Results are reported as apparent remaining peptide, because a decrease in HPLC peak area can reflect chemical degradation, precipitation, adsorption, or incomplete sampling.

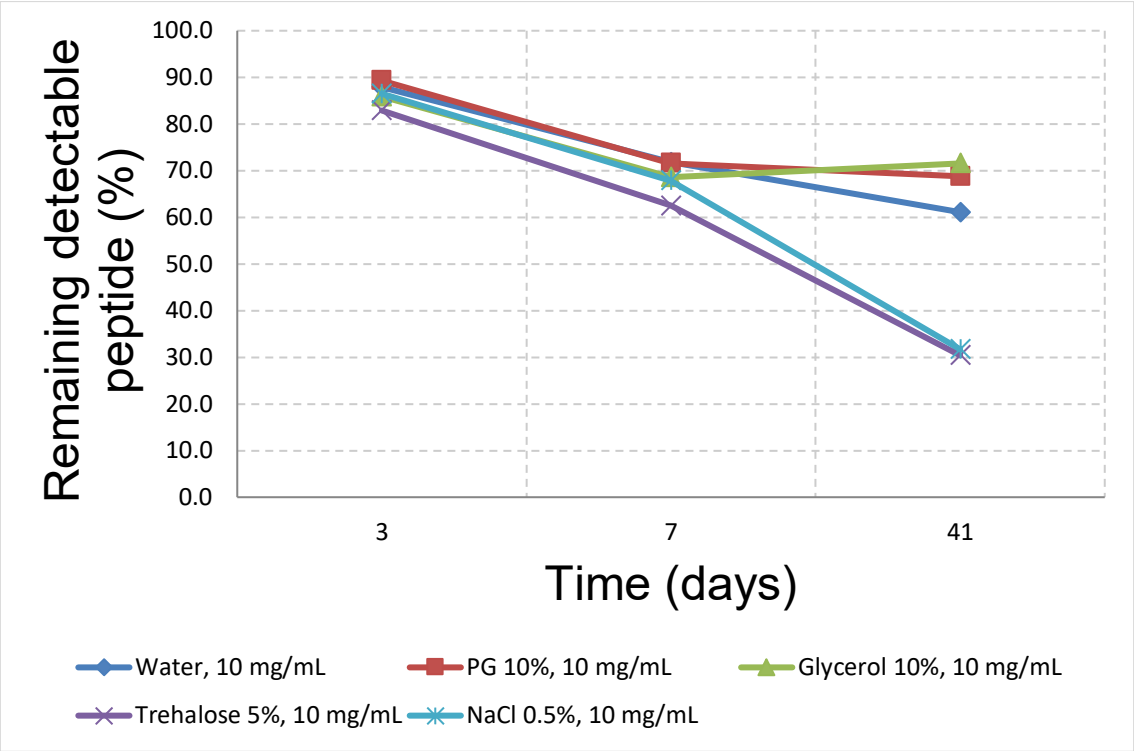


Figure 5.2. Remaining detectable CGRP 8-37 in selected 10 mg/mL formulation samples stored at room temperature and analyzed by HPLC at 214 nm.

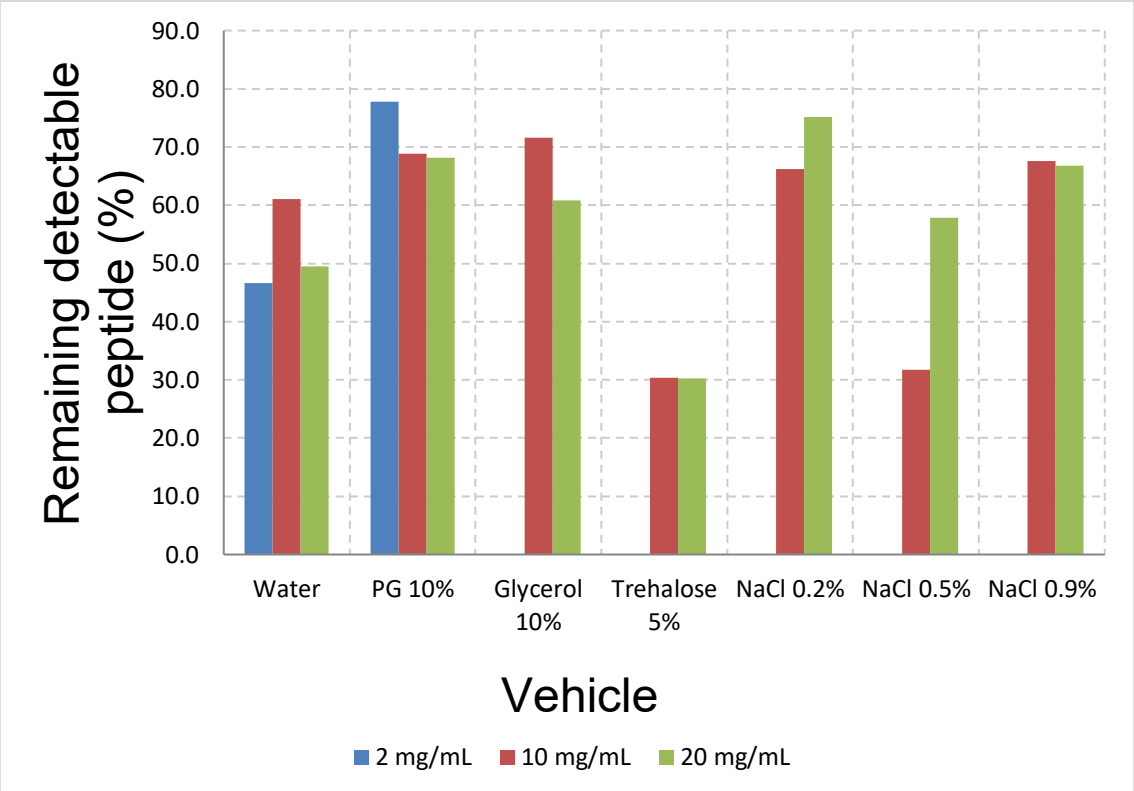


Figure 5.3. Remaining detectable CGRP 8-37 after 41 days at room temperature. Values are based on HPLC peak area relative to the nominal starting concentration.

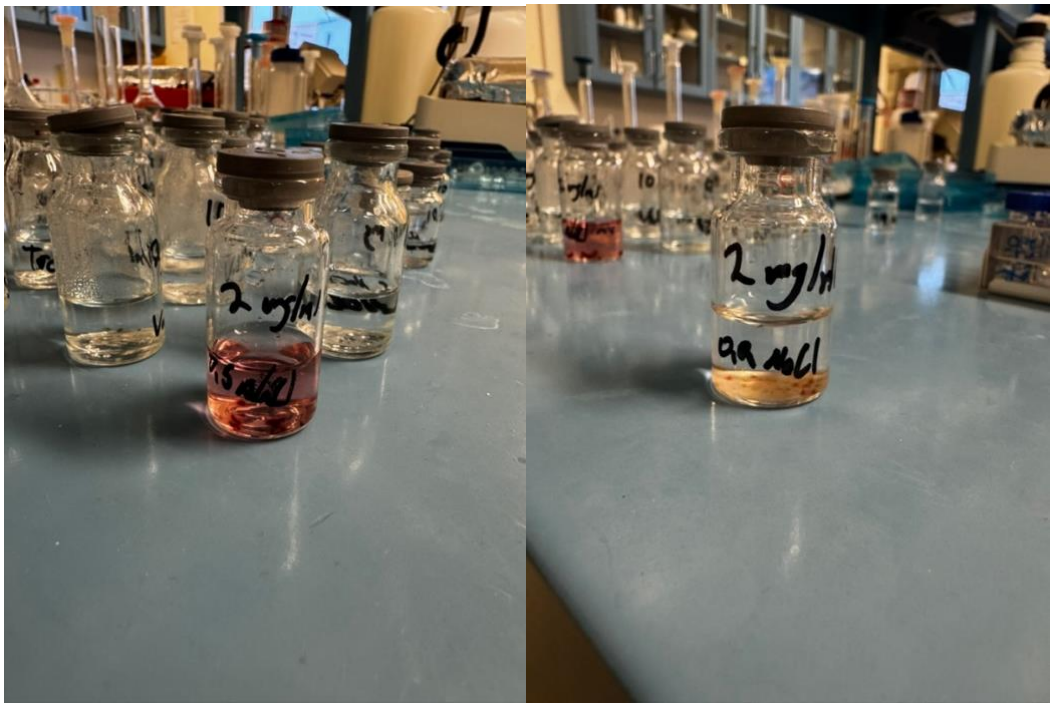


Figure 5.4. Visual instability in NaCl-containing CGRP 8-37 samples after storage. The 0.5% NaCl sample developed a pink color and gel-like spherical material, the 0.9% NaCl sample showed weaker but similar visible changes.

The NaCl samples, especially the 0,5% concentration, see figure 5.4, showed visible physical instability during storage, with pink color formation and small spherical gel-like structures after approximately 11 days. These same visibly changed samples also showed loss of detectable peptide signal by HPLC. Similar but weaker color changes were observed in other NaCl-containing samples. This observation should be described together with the HPLC data because visual instability affects dose uniformity and filterability even if part of the peptide remains detectable by HPLC.
pH and osmolality were not measured in the present thesis. The formulation results should therefore be interpreted as preformulation stability and handling data rather than as evidence for a final subcutaneous formulation.

5.3. Adsorption and surface passivation

The adsorption experiment compared apparent peptide recovery in sham-treated glass vials and BSA-coated glass vials on day 0 (14 April), day 7 (22 April), and day 14 (5 May), see figure 5.5. Each sample was measured twice by HPLC on each measurement day. At each timepoint, sample was taken from the coated or non-coated glass storage vial, diluted into an HPLC vial, and analyzed.

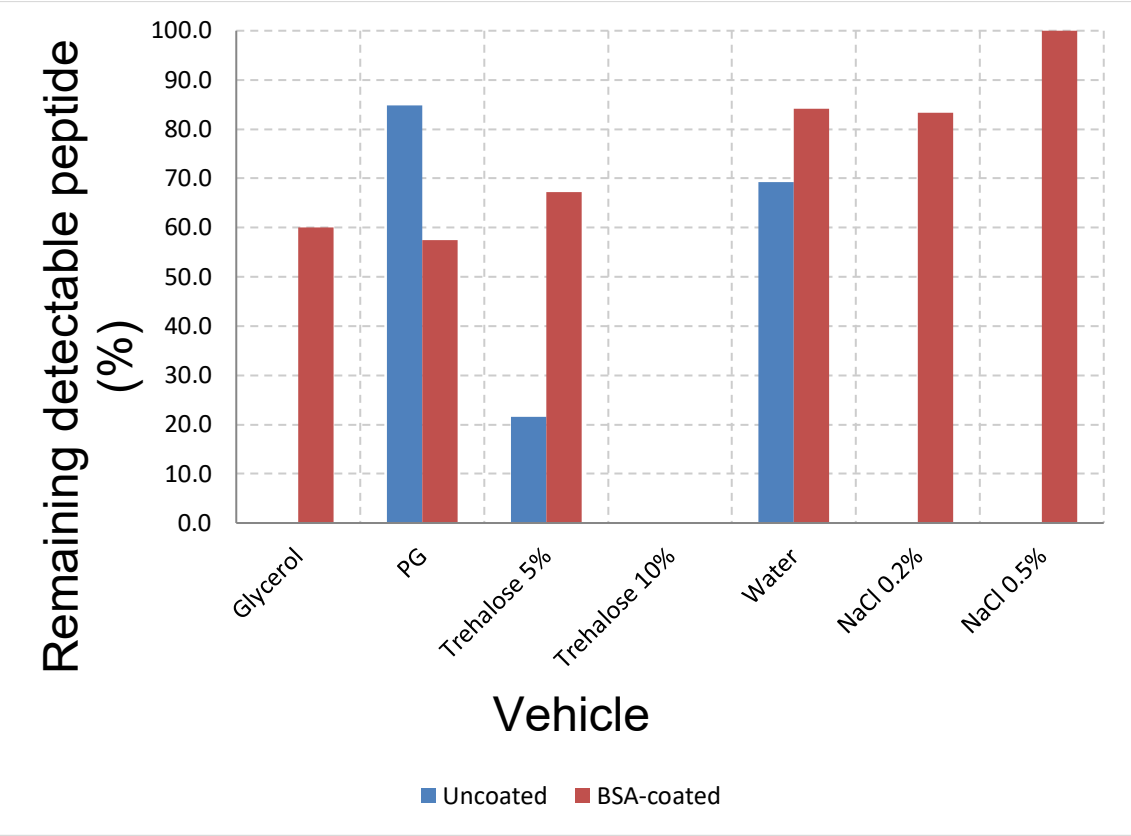


Figure 5.5. Apparent CGRP 8-37 recovery in uncoated and BSA-coated glass vials after storage after 14 days.

The comparison in figure 5.5 shows that BSA coating reduced apparent loss in some vehicles, while propylene glycol and water performed well without a consistent BSA advantage.

Table 5.2. Selected adsorption endpoint results at 0.5 mg/mL after storage after 14 days. Values are apparent remaining peptide (%) in uncoated and BSA-coated glass vials.

Vehicle	Uncoated day 14(%)	BSA-coated on day 14(%)	BSA effect (%)
Glycerol	0,0	60,0	60,0
PG	84,8	57,5	-27,3
Trehalose 5%	21,6	67,2	45,6
Trehalose 10%	0,0	0,0	0,0
Water	69,2	84,2	15,0
NaCl 0.2%	0,0	83,4	83,4
NaCl 0.5%	0,0	100,0	100,0

Table 5.3. Selected adsorption endpoint results at 0.5 mg/mL after storage after 14 days. Values are apparent remaining peptideconcentration in uncoated and BSA-coated glass vials

Vehicle	Uncoated day 14 mg/ml	BSA-coated on day 14 mg/ml	BSA effect mg/ml
Glycerol	0,00	0,29	0,29
PG	0,42	0,29	-0,13
Trehalose 5%	0,11	0,33	0,22
Trehalose 10%	0,00	0,00	0,00
Water	0,35	0,42	0,07
NaCl 0.2%	0,00	0,42	0,42
NaCl 0.5%	0,00	0,50	0,50

The adsorption data showed that BSA coating did not produce a universal improvement across all vehicles, see table 5.2 and 5.3. Water and propylene glycol gave the most favorable apparent recovery profiles in the adsorption dataset. At 0.5 mg/mL, water retained 69% detectable peptide without BSA and 84% with BSA on day 14 while propylene glycol retained 85% without BSA and 57% with BSA. At 2 mg/mL, water and propylene glycol remained around 76 to 93% on day 14 across coated and uncoated conditions.

BSA coating was most informative where uncoated samples showed strong apparent loss. For example, 0.5 mg/mL glycerol showed no detectable peptide without BSA AFTER 14 days, while the BSA-coated pair retained 60%. Trehalose 5% also showed improved apparent recovery with BSA at 0.5 mg/mL on day 14, 67% with BSA compared with 22% without BSA. In contrast, trehalose 10% performed poorly at low and intermediate concentrations, with no detectable peptide at 0.2 and 0.5 mg/mL by day 14 both with and without BSA.

NaCl-containing samples showed mixed adsorption behavior. In 0.2% NaCl, BSA coating improved the 0.5 and 2 mg/mL samples on day 14, 83% and 88%, respectively, compared with 0% and 49% without BSA. In 0.5% NaCl, uncoated 2 mg/mL samples dropped to 1% detectable peptide by day 14, while the BSA-coated pair retained 89%; however, earlier values for this BSA pair were not available because of calculation errors. These results support adsorption as one contributor to apparent loss, but they also show that vehicle-dependent instability remained important.

5.4. Supporting solvent behavior experiment

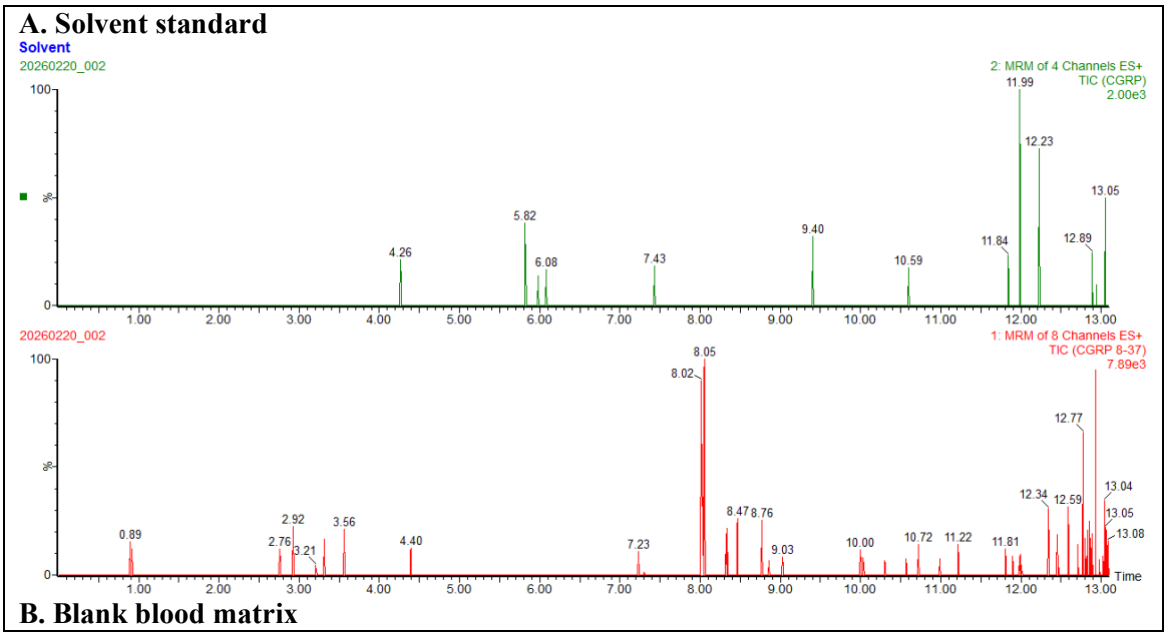
The ACN/EtOH solvent experiment did not identify a solvent composition that clearly improved peptide recovery, but it supported the broader conclusion that organic solvent alone does not solve apparent peptide loss. It was therefore treated as a supporting experiment rather than as a central formulation result.

5.5. Pilot blood-matrix method development

The blood-matrix experiments were treated as pilot method development rather than validated quantification. The initial whole-blood experiment tested short whole-blood contact without SPE. The time-course experiment tested time-dependent signal behavior in whole blood. The last LC-MS experiment introduced SPE and compared different dilution vehicles before LC-MS analysis, an overview can be seen in table 5.4.

Table 5.4. Overview of the blood-matrix LC-MS method-development experiments and their main interpretation limits.

Experiment	Purpose	Samples	Key issue
20 Feb whole-blood LC-MS pilot	Estimate matrix effect, recovery and time-dependent signal loss	Spiked whole-blood samples at 30 s and 10 min	No internal standard; no SPE; recovery estimate qualitative
20 Mar PK time-course	Attempted half-life measurement	Two donors, three vials each; samples A-F; timepoints TIME_1-TIME_7; with and without inhibitors	Not suitable for half-life calculation; standards and timing limited interpretation
16 Apr SPE experiment	Compare dilution/quench vehicles and SPE preparation	A/A+, B/B+, C/C+; 0 and 10 min	A and C peaks small; C has extra 4x dilution correction



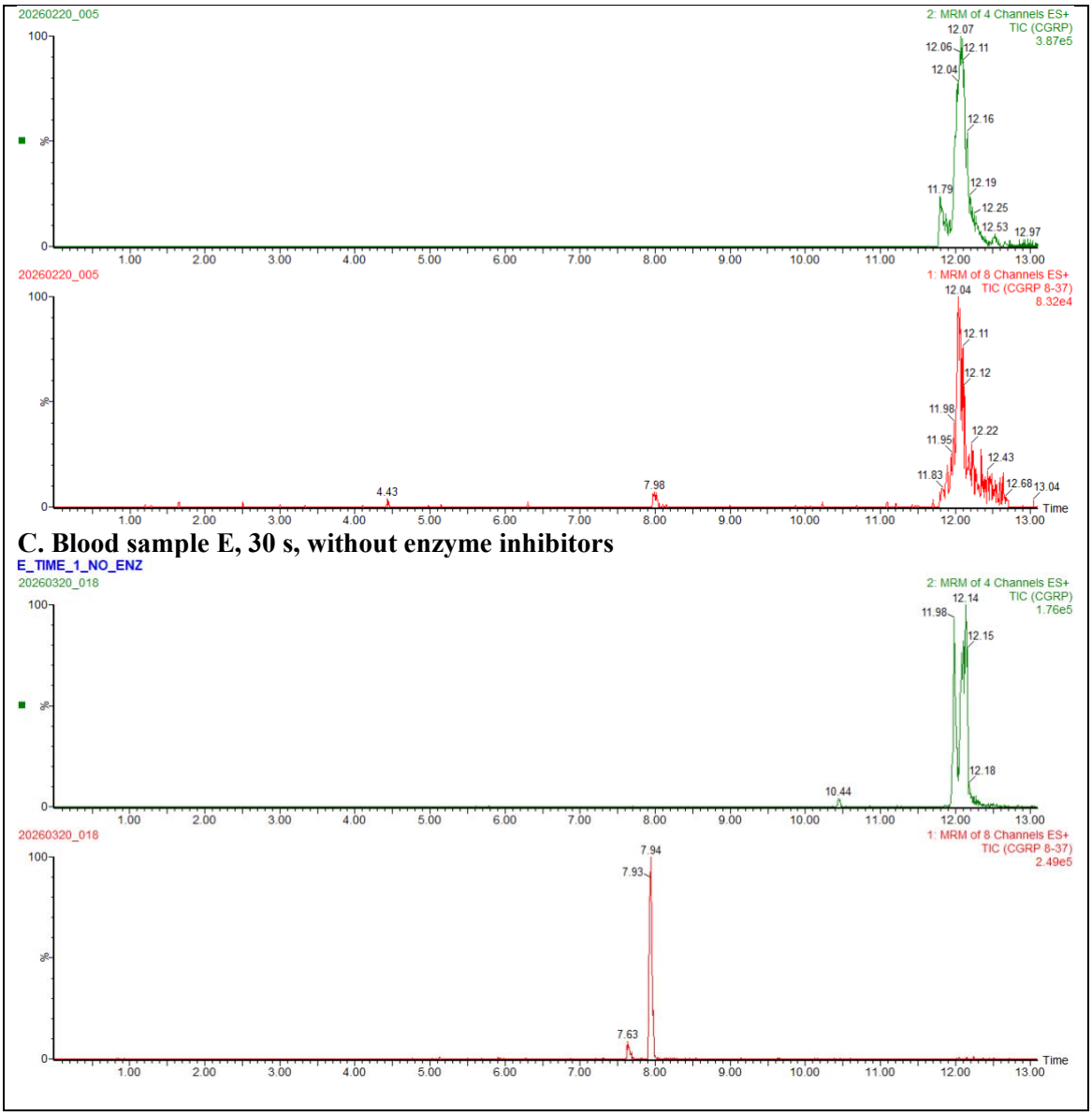


Figure 5.6. Representative LC-MS chromatograms from the pilot blood-matrix experiments: solvent standard, blank blood matrix, and blood sample E after 30 s contact without enzyme inhibitors.

The blood data should not be presented as endogenous concentration data. The correct conclusion is that detectable signal was possible, but the workflow showed major variability caused by blood contact time, quench chemistry, dilution, extraction, and lack of an internal standard, see figure 5.6.

6. Discussion

6.1. Overall interpretation

The central outcome of this thesis is a development map for α-CGRP 8-37 rather than a finished drug product or a validated blood assay. The strongest experimental information came from the formulation, adsorption, and HPLC work. Together, these data show that α-CGRP 8-37 behavior depends on vehicle, concentration, container surface, and handling conditions. The adsorption results add a key point to this interpretation: apparent peptide loss was not only a question of chemical degradation. Surface interaction and sampling history also affected the detectable peptide signal.

This framing matters because the project started from a biologically interesting peptide, but biological interest alone does not make a peptide developable. The work therefore fits better as early formulation and analytical development than as a psoriasis efficacy study. The correct question is whether the peptide can be dissolved, stored, sampled, quantified, and handled reproducibly enough to support later biological work.

6.2. HPLC suitability and analytical limitations

The HPLC method was suitable for comparative formulation screening. Same-day calibration curves covered the diluted sample concentrations, and the R^2 values were high across the stability and adsorption runs. The method therefore supported comparisons between vehicles, concentrations, and timepoints. It should not be described as fully validated. Accuracy, precision, robustness, specificity, and system suitability were not evaluated according to a formal validation plan.

The age of the HPLC column caused retention-time drift during the project. This limited direct comparison of retention behavior between runs. The effect was managed by assigning peaks relative to standards analyzed in the same run and by using same-day calibration curves. This approach was adequate for screening but would not be sufficient for a locked analytical method intended for routine quality control.

The API assay correction also matters. The supplied peptide acetate material had an as-is assay of 80.2%, meaning weighed powder mass did not equal free peptide mass. This affects interpretation of nominal concentrations and becomes especially important when comparing formulation concentration, assay recovery, and future dose calculations. For the present work, nominal concentrations should be read as prepared experimental concentrations, not as final clinical dose strengths.

6.3. Formulation stability and NaCl incompatibility

The stability data indicate that formulation vehicle and concentration strongly influenced the remaining detectable peptide. Low-concentration samples were more difficult to interpret because small absolute peptide losses can produce large relative changes, and several low-concentration conditions eventually gave no detectable peak. Higher-concentration samples gave more stable analytical behavior and were more useful for comparing vehicles.

The NaCl-containing samples provided the clearest practical warning. The 0.5% NaCl samples developed pink coloration and small gel-like spheres during storage, and the visually changed samples also lost detectable peptide by HPLC. This supports the conclusion that NaCl-containing vehicles were incompatible with α-CGRP 8-37 under the tested conditions. The mechanism was not determined, but could potentially be due to chemical, electrostatic interactions, or formation of aggregate, but further research is

needed. The observation is still important because visible gel formation is enough to make a formulation unsuitable for controlled injection without further investigation.
The result is consistent with internal prior formulation work, where sodium chloride was associated with solid or gel-like behavior for α-CGRP 8-37 under some conditions [12]. The present thesis does not prove that all saline-containing formulations are impossible. It shows that NaCl is a high-risk variable for this peptide and should not be treated as a neutral tonicity agent without specific compatibility testing.
pH and osmolality were not measured in this thesis. This limits the formulation conclusions. The results can support preformulation screening and selection of conditions for further testing, but they cannot establish subcutaneous suitability. A route-compatible formulation would still require pH, osmolality, visual appearance, particle behavior, sterile filtration recovery, and longer-term storage assessment.

6.4. Adsorption and surface passivation

The adsorption study showed that surface interaction contributed to apparent peptide loss, but the effect was not uniform. BSA coating improved apparent recovery in some conditions, especially where uncoated samples lost signal strongly. Examples include 0.5 mg/mL glycerol and 0.5 mg/mL trehalose 5%, where BSA-coated vials retained detectable peptide on 5 May while the matched uncoated samples showed major loss. This supports adsorption as a plausible contributor to the low-concentration losses.
The data also show that BSA coating was not a universal solution. Water and propylene glycol were the best-performing vehicles in the adsorption dataset, and BSA did not consistently improve propylene glycol samples. Trehalose 10% performed poorly at low and intermediate concentrations even with BSA. These patterns suggest that adsorption, vehicle-dependent instability, and possibly concentration-dependent sampling effects were occurring together.
Because no BSA-coated vehicle blank was included, residual BSA-related UV background cannot be fully excluded. Late chromatograms from samples where peptide had degraded did not show an obvious difference between BSA-coated and blank chromatograms, so BSA did not appear to dominate the 214 nm chromatographic background under those conditions. Still, BSA coating should be interpreted as a diagnostic surface-passivation tool, not as a final formulation strategy.

6.5. Blood-matrix method development

The blood-matrix experiments should be described as pilot method development. The current LC-MS workflows detected peptide signal, but they did not provide a reliable method for endogenous quantification. The whole-blood and time-course experiments showed that blood contact time, quench timing, enzyme inhibitors, dilution, and lack of internal standard all affected interpretation. The SPE experiment improved the sample-preparation structure, but it also showed that dilution vehicle and extraction chemistry strongly influenced the measured signal.
The inhibitor-containing condition in the SPE experiment produced a high apparent CGRP 8-37 signal in unspiked blood while CGRP did not show the same behavior. This is interesting, but it should not be treated as biological evidence for selective endogenous CGRP 8-37 preservation. It may reflect differential recovery, conversion, adsorption, ionization, or an interference near the monitored signal. Without an internal standard and validation experiments, the conservative interpretation is that the workflow remains under development.

The blood experiments did not provide reliable endogenous concentrations, but they helped identify where the current workflow fails. It shows that future patient or volunteer sampling should wait until quench timing, internal standard correction, matrix effect, recovery, and pre-analytical stability have been controlled. Running biological samples before that would mainly generate numbers with false confidence.

6.6. Overall limitations

The main limitations are the lack of full HPLC validation, retention-time drift from the old column, absence of pH and osmolality measurements, no internal standard in the blood-matrix experiments, no BSA-coated vehicle blank, and limited mechanistic separation between degradation, precipitation, adsorption, and sampling loss. These limitations do not invalidate the thesis. They define its scope: early development screening and method troubleshooting.

The project still provides a coherent development picture. HPLC can support formulation screening. NaCl-containing vehicles are high risk. Water and propylene glycol performed best in the adsorption dataset. BSA coating showed that surface passivation can change apparent recovery. Blood-matrix LC-MS requires further method development before biological conclusions can be drawn.

7. Conclusion

This thesis developed and applied an HPLC-based workflow for screening α-CGRP 8-37 in formulation-relevant samples. The method was suitable for comparative stability and adsorption experiments, although it was not fully validated and was affected by retention-time drift from an old column.

The formulation experiments showed that α-CGRP 8-37 behavior depended on vehicle, concentration, and container surface. NaCl-containing vehicles were unsuitable under the tested conditions because they showed visible physical instability, including pink coloration and gel-like sphere formation, and the visibly changed samples also lost detectable peptide signal by HPLC.

The adsorption experiment showed that surface passivation with BSA changed apparent peptide recovery in several conditions, supporting adsorption as a contributor to apparent loss. The effect was not universal. Water and propylene glycol performed best in the adsorption dataset, while trehalose 10% performed poorly at low and intermediate concentrations. Further formulation experiments should focus on pH and osmolality studies, and further long term stability research with the promising vehicles. The possibility of electrostatic interactions, and aggregation should also be investigated.

The blood-matrix experiments identified important pre-analytical and analytical problems but did not establish validated quantification of endogenous CGRP 8-37. The blood data should therefore be interpreted as pilot method development. The next development stage should focus more on validating a LC-MS method, with an internal standard, rapid quenching, recovery and matrix-effect assessment. Formulation characterization also needs to be completed, before studies involving patients.

It can be concluded that α-CGRP 8-37 can be dissolved, stored, sampled, quantified and handled reproducibly enough to continue and support further biological work, regarding further formulation and method development. The blood matrix and LC-MS workflow need further validation and method development before reliable biological research and patient related studies are performed.

8. Future work

8.1. LC-MS bioanalysis

The first priority should be development of a validated or fit-for-purpose LC-MS method for α-CGRP 8-37 in blood or plasma. The method should include a suitable internal standard, preferably a stable-isotope labelled analogue or another closely matched peptide standard. The internal standard should be added before sample preparation so that extraction loss, adsorption, and ionization variation can be corrected.
The blood workflow should then be rebuilt around immediate quenching. Future experiments should compare acidification, organic solvent precipitation, enzyme inhibitors, cooling, centrifugation timing, and SPE timing using controlled spike experiments. A repeat time-course should add the quench solution at fixed and well-defined times and should include quality-control samples, recovery assessment, matrix-effect assessment, dilution integrity, carryover assessment, and short-term stability testing.

8.2. Formulation characterization

The formulation work should continue with pH and osmolality measurements. These measurements are required before any formulation can be discussed as a realistic subcutaneous candidate. The most promising vehicles from the present adsorption dataset, especially water and propylene glycol-containing conditions, should be compared with selected sugar-containing formulations under controlled pH conditions.
The effects of electrostatic interactions should also be measured with combined experimental approaches, such as binding affinity across varying salt and pH conditions, and computational modeling focusing on charge distribution and free energy. To prevent aggregation, increase long term stability, investigate effects on viscosity in regards to injections.
Longer-term stability should be tested at refrigerated storage, room temperature, and elevated stress conditions. The study should use predefined acceptance criteria for appearance, assay, impurity profile, and remaining detectable peptide. Orthogonal particle analysis, such as DLS or microscopy, should be used to support the HPLC and visual observations.

8.3. Container compatibility and filtration

Container effects should be studied directly. Low-binding vials, ordinary glass vials, BSA-passivated glass vials, and relevant clinical container materials should be compared using the same peptide concentration and vehicle. This would separate vehicle-driven instability from surface-driven loss more cleanly than the current screening experiment.
Sterile filtration recovery should also be tested. Each candidate formulation should be assayed before and after filtration through a relevant membrane. The retained material should be considered when possible, because a clear filtrate does not prove that the original formulation was stable or that the dose passed through the filter.

8.4. Development path

Future psoriasis-related biological sampling should remain development-focused until the analytical and formulation problems are solved. Patient or volunteer sampling

should only be performed after the blood-matrix method has demonstrated sufficient specificity, recovery, matrix control, and pre-analytical stability. The next practical step is to strengthen analytical and formulation studies.

Disclosure of AI:

Artificial intelligence was used to aid with the writing of this thesis. AI was used to get feedback on the structure and language of the thesis.

References

1. Lowes MA, Suárez-Fariñas M, Krueger JG. Immunology of psoriasis. Annu Rev Immunol. 2014;32:227-255. doi:10.1146/annurev-immunol-032713-120225.
2. Boehncke WH, Schön MP. Psoriasis. Lancet. 2015;386(9997):983-994. doi:10.1016/S0140-6736(14)61909-7.
3. Saraceno R, Kleyn CE, Terenghi G, Griffiths CEM. The role of neuropeptides in psoriasis. Br J Dermatol. 2006;155(5):876-882. doi:10.1111/j.1365-2133.2006.07518.x.
4. Zhang J, Zhao S, Xing X, Shang L. Effects of neuropeptides on dendritic cells in the pathogenesis of psoriasis. J Inflamm Res. 2023;16:35-43. doi:10.2147/JIR.S397079.
5. Russell FA, King R, Smillie SJ, Kodji X, Brain SD. Calcitonin gene-related peptide: physiology and pathophysiology. Physiol Rev. 2014;94(4):1099-1142. doi:10.1152/physrev.00034.2013.
6. Hay DL, Garelja ML, Poyner DR, Walker CS. Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review 25. Br J Pharmacol. 2018;175(1):3-17. doi:10.1111/bph.14075.
7. Bonura A, Brunelli N, Marcosano M, Iaccarino G, Fofi L, Vernieri F, Altamura C. Calcitonin gene-related peptide systemic effects: embracing the complexity of its biological roles. Int J Mol Sci. 2023;24(18):13979. doi:10.3390/ijms241813979.
8. Björnsdóttir S. The relationship of calcitonin gene-related peptide (CGRP) and CGRP(8-37) to psoriasis. MSc thesis. University of Iceland; 2018.
9. Bak A, Leung D, Barrett SE, Forster S, Minnihan EC, Leithead AW, Cunningham J, Toussaint N, Crocker LS. Physicochemical and formulation developability assessment for therapeutic peptide delivery: a primer. AAPS J. 2015;17(1):144-155. doi:10.1208/s12248-014-9688-2.
10. Zapadka KL, Becher FJ, Gomes dos Santos AL, Jackson SE. Factors affecting the physical stability (aggregation) of peptide therapeutics. Interface Focus. 2017;7(6):20170030. doi:10.1098/rsfs.2017.0030.
11. Nugrahadi PP, Hinrichs WLJ, Frijlink HW, Schöneich C, Avanti C. Designing formulation strategies for enhanced stability of therapeutic peptides in aqueous solutions: a review. Pharmaceutics. 2023;15(3):935. doi:10.3390/pharmaceutics15030935.
12. Nepsone. NEPS-P24-323: α-CGRP 8-37 F&F formulation and manufacturability testing. Internal formulation development summary; 2025.
13. Roberts CJ. Therapeutic protein aggregation: mechanisms, design, and control. Trends Biotechnol. 2014;32(7):372-380. doi:10.1016/j.tibtech.2014.05.005.
14. Wang W, Nema S, Teagarden D. Protein aggregation: pathways and influencing factors. Int J Pharm. 2010;390(2):89-99. doi:10.1016/j.ijpharm.2010.02.025.
15. Jiskoot W, Randolph TW, Volkin DB, Middaugh CR, Schöneich C, Winter G, Friess W, Crommelin DJA, Carpenter JF. Protein instability and immunogenicity: roadblocks to clinical application of injectable protein delivery systems for sustained release. J Pharm Sci. 2012;101(3):946-954. doi:10.1002/jps.23018.
16. Kristensen K, Henriksen JR, Andresen TL. Adsorption of cationic peptides to solid surfaces of glass and plastic. PLoS One. 2015;10(5):e0122419. doi:10.1371/journal.pone.0122419.
17. Usach I, Martinez R, Festini T, Peris JE. Subcutaneous injection of drugs: literature review of factors influencing pain sensation at the injection site. Adv Ther. 2019;36(11):2986-2996. doi:10.1007/s12325-019-01101-6.

18. Mathaes R, Koulov A, Joerg S, Mahler HC. Subcutaneous injection volume of biopharmaceuticals: pushing the boundaries. J Pharm Sci. 2016;105(8):2255-2259. doi:10.1016/j.xphs.2016.05.029.
19. Ewles M, Goodwin L. Bioanalytical approaches to analyzing peptides and proteins by LC-MS/MS. Bioanalysis. 2011;3(12):1379-1397. doi:10.4155/bio.11.112.
20. van den Broek I, Sparidans RW, Schellens JHM, Beijnen JH. Quantitative bioanalysis of peptides by liquid chromatography coupled to tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2008;872(1-2):1-22. doi:10.1016/j.jchromb.2008.07.021.
21. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem. 2003;75(13):3019-3030. doi:10.1021/ac020361s.
22. International Council for Harmonisation. ICH M10 guideline on bioanalytical method validation and study sample analysis. Step 5. 2022.
23. International Council for Harmonisation. ICH Q2(R2) guideline on validation of analytical procedures. Step 4 version. 2023.
24. International Council for Harmonisation. ICH Q14 guideline on analytical procedure development. Step 4 version. 2023.
25. Suelter CH, DeLuca M. How to prevent losses of protein by adsorption to glass and plastic. Anal Biochem. 1983;135(1):112-119. doi:10.1016/0003-2697(83)90738-8.
26. Park JH, Sut TN, Jackman JA, Ferhan AR, Yoon BK, Cho NJ. Controlling adsorption and passivation properties of bovine serum albumin on silica surfaces by ionic strength modulation and cross-linking. Phys Chem Chem Phys. 2017;19(13):8854-8865. doi:10.1039/C7CP01310H.
27. van den Oetelaar PJM, Mentink IJ, Brinks GJ. Loss of peptides and proteins upon sterile filtration due to adsorption to membrane filters. Drug Dev Ind Pharm. 1989;15(1):97-106. doi:10.3109/03639048909040198.
28. IUPHAR/BPS Guide to Pharmacology. α-CGRP-(8-37) (human). Ligand ID 700. Accessed May 15, 2026.
29. Kuipers BJH, Gruppen H. Prediction of molar extinction coefficients of proteins and peptides using UV absorption of the constituent amino acids at 214 nm to enable quantitative reverse phase high-performance liquid chromatography-mass spectrometry analysis. J Agric Food Chem. 2007;55(14):5445-5451. doi:10.1021/jf070337l.

Appendix A - Stability experiment supporting data

This appendix contains the full stability dataset, the dilution factors used for HPLC sample preparation, representative stability chromatograms, and calibration curves. Day 15 stability data and the corresponding calibration curve were excluded because the HPLC performance was anomalous on that measurement day.

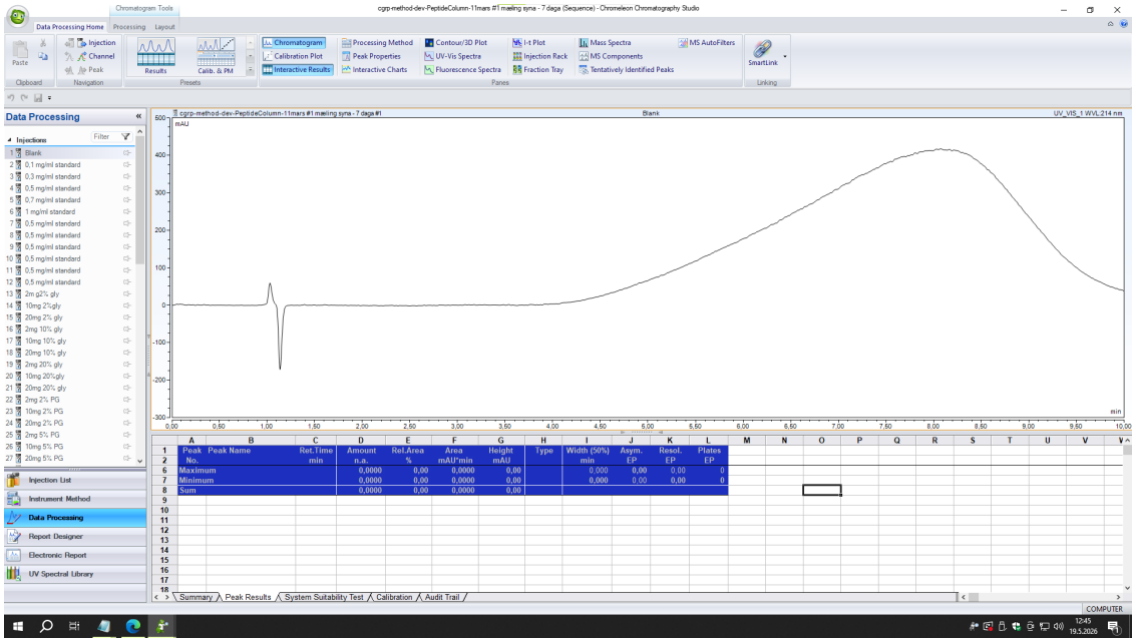


Figure A1. Blank diluent chromatogram from the HPLC method. No CGRP 8-37 peak is expected in the peptide retention region.

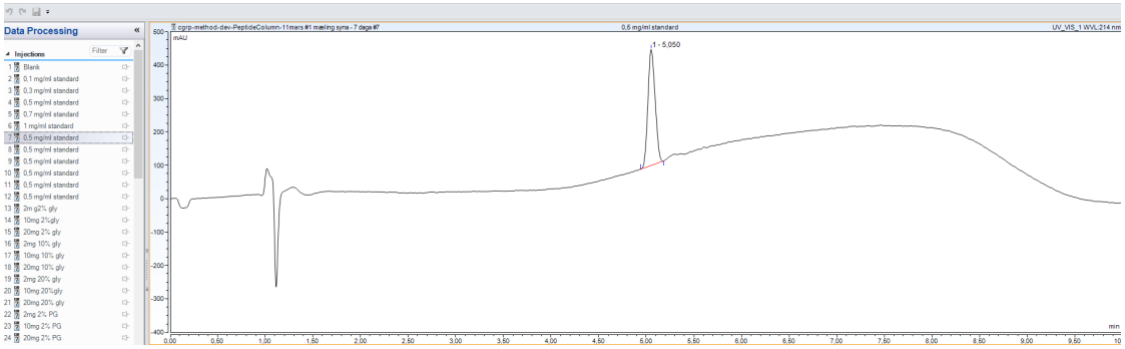


Figure A2. Calibration standard chromatogram for 0.5 mg/mL CGRP 8-37 at 214 nm.

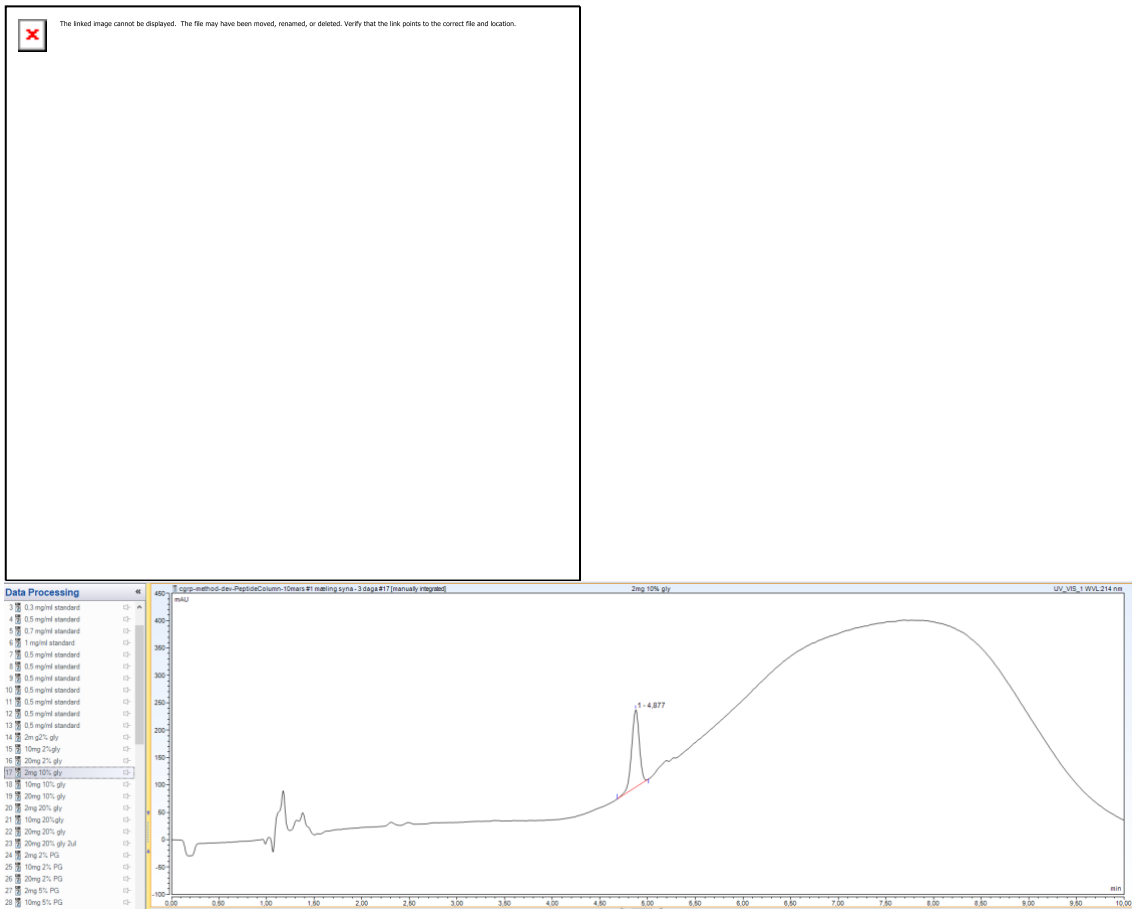


Figure A3. Fresh formulation sample chromatogram for 2 mg/mL CGRP 8-37 in 10% glycerol.

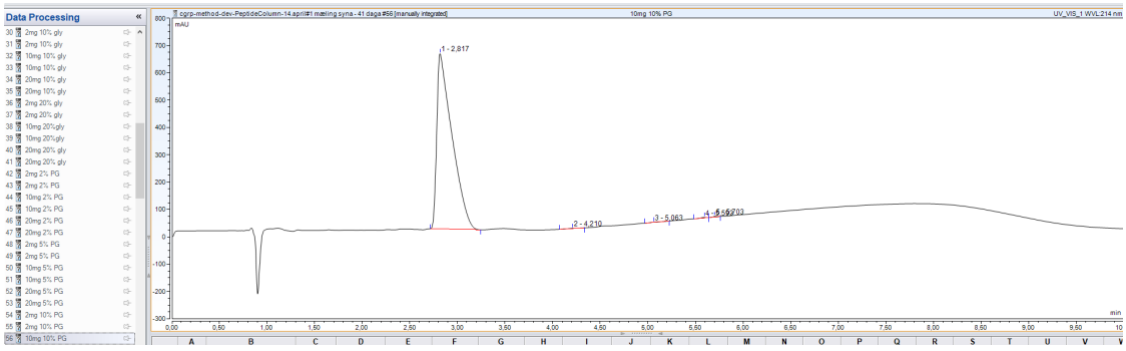


Figure A4. Aged stable sample chromatogram for 10 mg/mL CGRP 8-37 in 10% propylene glycol.

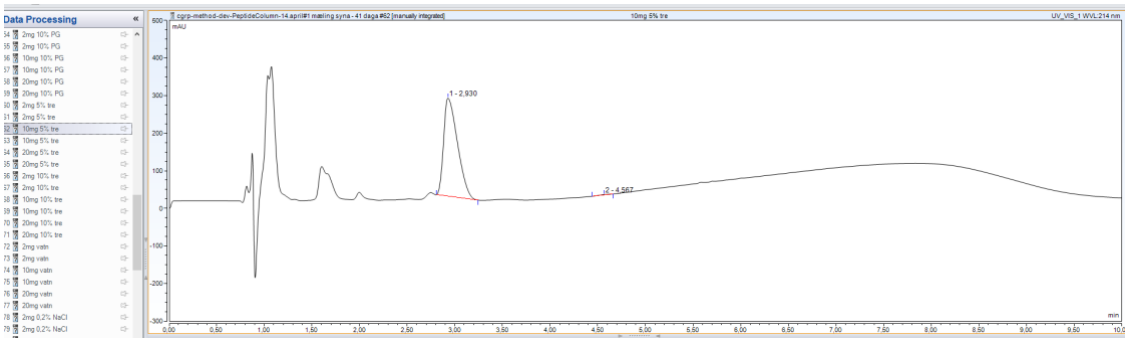


Figure A5. Aged sample chromatogram for 10 mg/mL CGRP 8-37 in 5% trehalose, showing strong loss of detectable peptide signal.

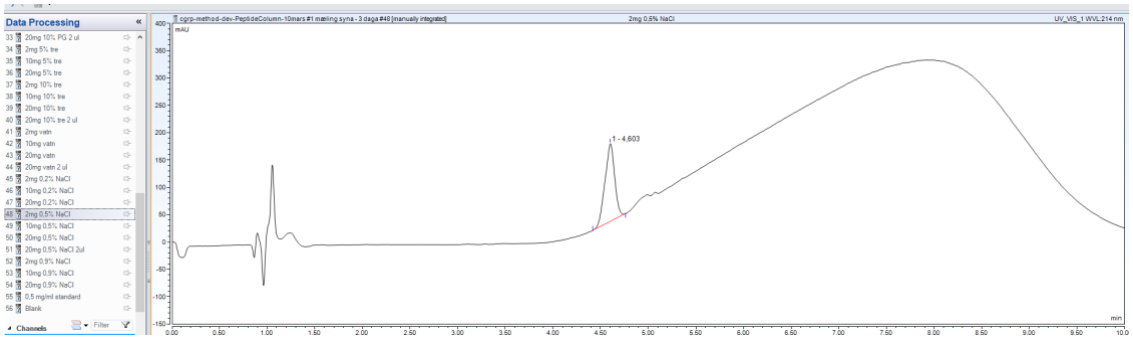


Figure A6. Chromatogram for 2 mg/mL CGRP 8-37 in 0.5% NaCl before visible instability was observed.

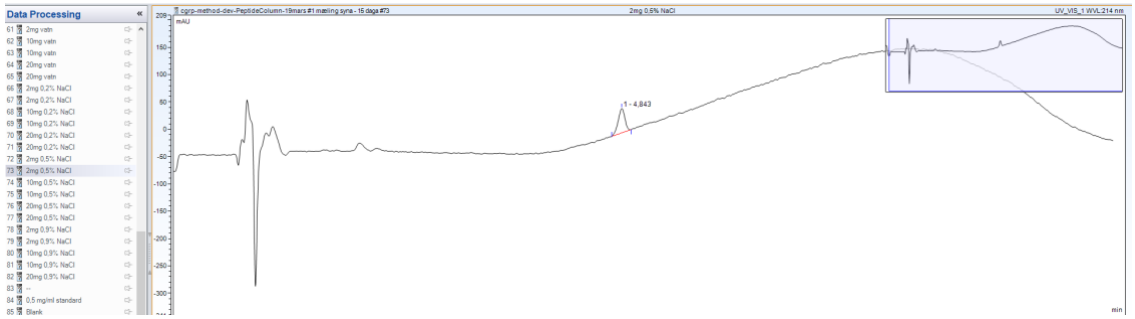


Figure A7. Chromatogram for 2 mg/mL CGRP 8-37 in 0.5% NaCl after visible pink coloration and gel-like material formation.

Table A1. Full stability summary. Values are remaining detectable CGRP 8-37 (%). The excluded day 15 dataset is not shown.

Sample	Day 3 (%)	Day 7 (%)	Day 41 (%)
2 mg/ml 2% glycerol	88,0	62,6	22,5
10 mg/ml 2% glycerol	90,3	64,8	48,5
20 mg/ml 2% glycerol	85,6	61,3	31,6
2 mg/ml 10% glycerol	68,5	47,7	0,0
10 mg/ml 10% glycerol	85,9	68,6	71,6

20 mg/ml 10% glycerol	86,7	67,7	60,8
2 mg/ml 20% glycerol	64,5	39,4	0,0
10 mg/ml 20% glycerol	91,0	68,1	71,8
20 mg/ml 20% glycerol	84,4	80,1	72,7
2 mg/ml 2% PG	85,4	77,3	0,0
10 mg/ml 2% PG	91,4	64,1	51,8
20 mg/ml 2% PG	86,4	65,0	49,0
2 mg/ml 5% PG	90,0	83,2	37,2
10 mg/ml 5% PG	92,7	68,5	31,7
20 mg/ml 5% PG	89,5	71,5	65,3
2 mg/ml 10% PG	89,8	77,4	77,8
10 mg/ml 10% PG	89,3	71,6	68,8
20 mg/ml 10% PG	87,2	64,9	68,2
2 mg/ml 5% trehalose	91,9	64,1	0,0
10 mg/ml 5% trehalose	82,9	62,5	30,4
20 mg/ml 5% trehalose	88,5	64,9	30,2
2 mg/ml 10% trehalose	73,1	60,6	0,0
10 mg/ml 10% trehalose	82,9	63,9	59,4
20 mg/ml 10% trehalose	85,5	65,1	71,4
2 mg/ml water	100,6	84,5	46,6
10 mg/ml water	87,9	71,8	61,1
20 mg/ml water	82,0	65,2	49,5
2 mg/ml 0,2% NaCl	86,2	65,3	0,0
10 mg/ml 0,2% NaCl	84,3	68,1	66,2
20 mg/ml 0,2% NaCl	75,5	71,6	75,1
2 mg/ml 0,5% NaCl	88,6	73,8	0,0
10 mg/ml 0,5% NaCl	86,5	67,9	31,7
20 mg/ml 0,5% NaCl	87,9	67,0	57,8
2 mg/ml 0,9% NaCl	94,2	81,3	0,0
10 mg/ml 0,9% NaCl	88,2	69,0	67,6
20 mg/ml 0,9% NaCl	92,7	67,6	66,8

A value of 0% indicates that no detectable CGRP 8-37 peak was observed under the applied HPLC conditions. Failed analytical entries or spreadsheet errors were not treated as true zero values.

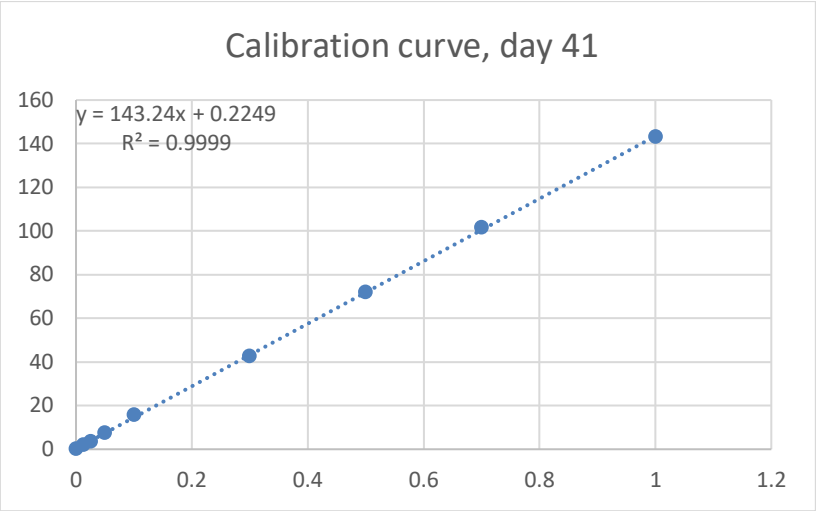


Figure A8. HPLC calibration curve used for the day 41 stability measurements.

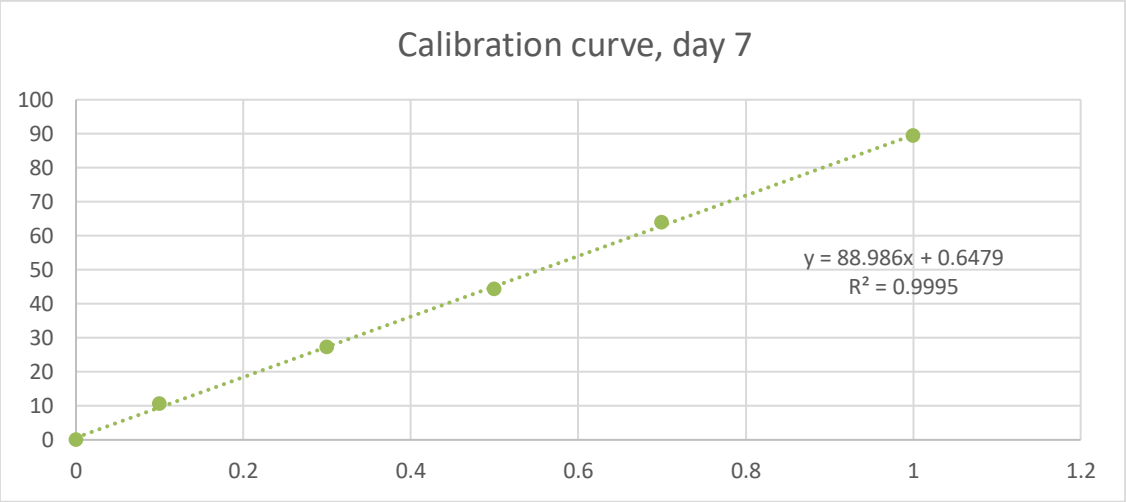


Figure A9. HPLC calibration curve used for the day 7 stability measurements.

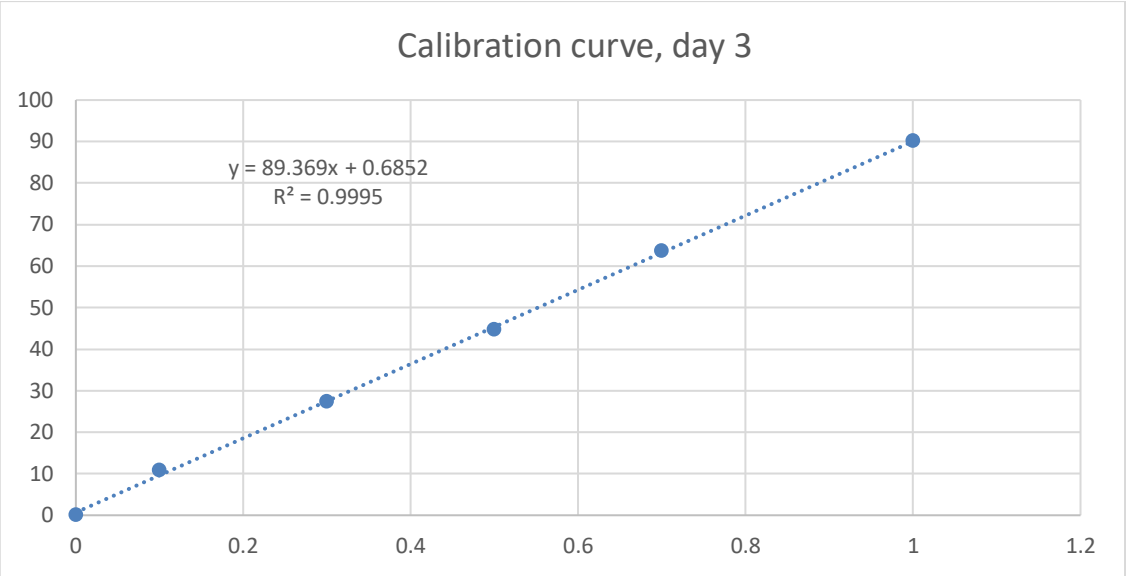


Figure A10. HPLC calibration curve used for the day 3 stability measurements.

Table A2. Dilution factors used before HPLC analysis of formulation samples.

Sample concentration, mg/ml	Dilution factor
2	10x
10	10x
20	20x

Appendix B - Adsorption experiment supporting data

Table B1. Full adsorption dataset. Values are calculated apparent remaining peptide (%) from duplicate HPLC measurements on each measurement day. Empty cells indicate failed measurements or calculation errors.

Sample	14 Apr (%)	22 Apr (%)	5 May (%)
0.2 mg/ml glycerol	39,1	1,3	0,0
0.2 mg/ml glycerol BSA	70,6	47,3	0,0
0.5 mg/ml glycerol	51,1	35,6	0,0
0.5 mg/ml glycerol BSA	72,2	70,4	60,0
2 mg/ml glycerol			105,7
2 mg/ml glycerol BSA	75,7	75,9	76,4
0.2 mg/ml PG	60,2	62,3	0,0
0.2 mg/ml PG BSA	62,1	62,9	0,0
0.5 mg/ml PG	87,5	90,9	84,8
0.5 mg/ml PG BSA	70,3	78,5	57,5
2 mg/ml PG	76,0	87,8	76,0
2 mg/ml PG BSA	76,8	86,5	75,8
0.2 mg/ml trehalose 5%	68,9	56,2	23,7
0.2 mg/ml trehalose 5% BSA	108,4	108,2	94,3
0.5 mg/ml trehalose 5%	55,8	47,0	21,6
0.5 mg/ml trehalose 5% BSA		65,1	67,2
2 mg/ml trehalose 5%	82,3	90,7	88,1
2 mg/ml trehalose 5% BSA	73,2	80,8	81,0
0.2 mg/ml trehalose 10%	34,1	1,3	0,0
0.2 mg/ml trehalose 10% BSA	37,7	1,3	0,0
0.5 mg/ml trehalose 10%	45,6	3,3	0,0
0.5 mg/ml trehalose 10% BSA	56,3	3,9	0,0
2 mg/ml trehalose 10%	72,4	38,6	17,2
2 mg/ml trehalose 10% BSA	77,5	58,8	39,8
0.2 mg/ml water	64,7	7,8	0,0
0.2 mg/ml water BSA	47,6	60,1	0,0
0.5 mg/ml water	64,5	77,0	69,2
0.5 mg/ml water BSA	70,4	90,9	84,2
2 mg/ml water	76,8	87,4	88,2
2 mg/ml water BSA	78,6	93,0	92,8
0.2 mg/ml 0.2NaCl	59,5	44,2	0,0
0.2 mg/ml 0.2NaCl BSA	96,6	94,4	0,0
0.5 mg/ml 0.2NaCl	79,4	52,5	0,0
0.5 mg/ml 0.2NaCl BSA	84,3	89,1	83,4
2 mg/ml 0.2NaCl	70,9	68,7	49,3
2 mg/ml 0.2NaCl BSA	81,0	87,7	88,3

0.2 mg/ml 0.5 NaCl	60,1	65,9	0,0
0.2 mg/ml 0.5 NaCl BSA	72,2	87,5	77,3
0.5 mg/ml 0.5 NaCl	81,7	87,8	0,0
0.5 mg/ml 0.5 NaCl BSA	96,3	103,7	100,0
2 mg/ml 0.5 NaCl	83,2	72,8	1,1
2 mg/ml 0.5% NaCl BSA			88,9

All adsorption samples in Table B1 were diluted tenfold before HPLC analysis.

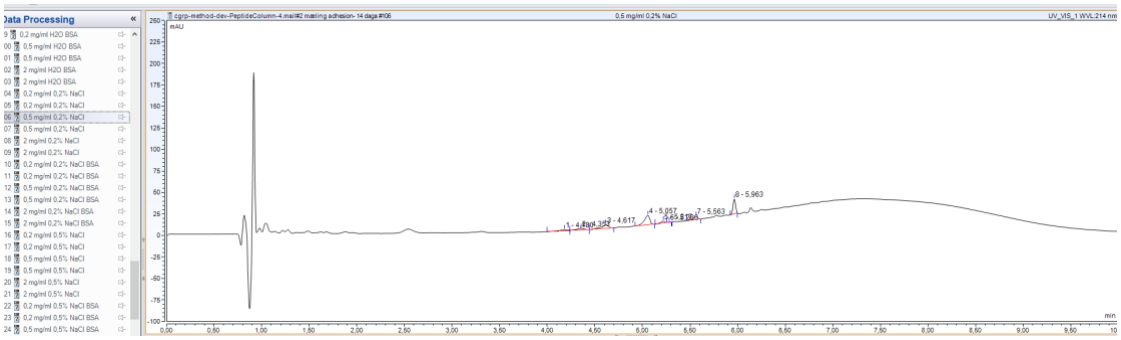


Figure B1. Adsorption experiment chromatogram for 0.5 mg/mL CGRP 8-37 in 0.2% NaCl stored in an uncoated glass vial.

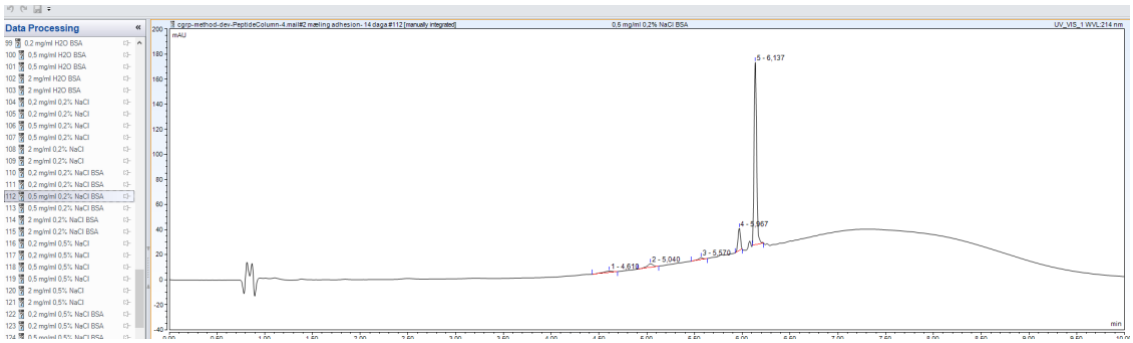


Figure B2. Matched adsorption experiment chromatogram for 0.5 mg/mL CGRP 8-37 in 0.2% NaCl stored in a BSA-coated glass vial.

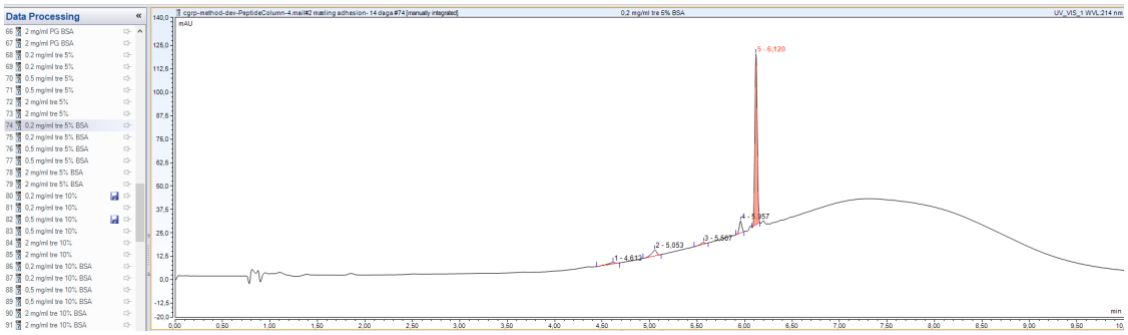


Figure B3. Adsorption experiment chromatogram for a condition where BSA coating improved apparent recovery: 0.2 mg/mL CGRP 8-37 in 5% trehalose.

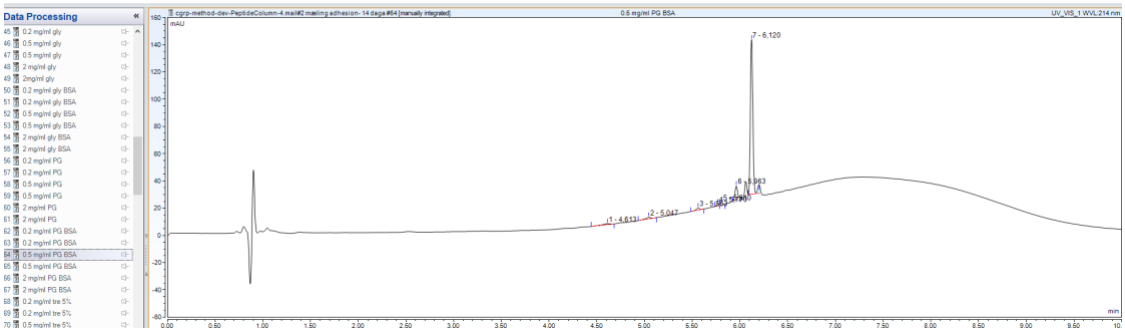


Figure B4. Adsorption experiment chromatogram for 0.5 mg/mL CGRP 8-37 in propylene glycol with BSA coating, representing a mixed BSA effect.

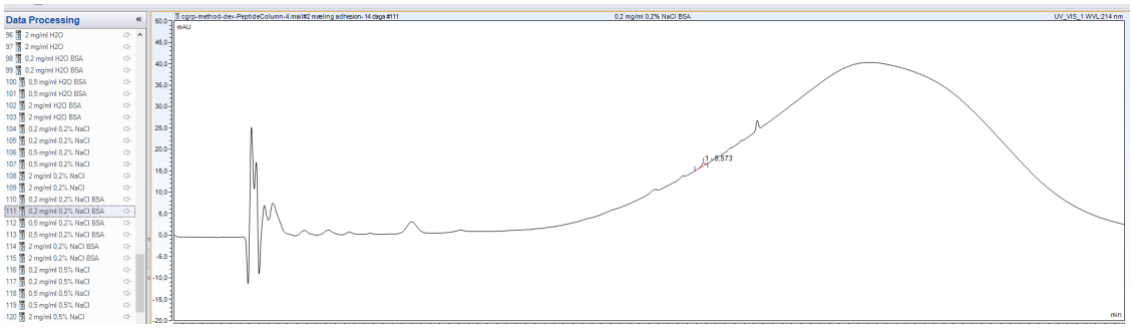


Figure B5. Late timepoint adsorption experiment chromatogram after apparent peptide loss for 0.2 mg/mL CGRP 8-37 in 0.2% NaCl with BSA coating.

Appendix C - Blood-matrix raw LC-MS outputs

Table C1. Results from the SPE experiment. Corrected mean areas include the fourfold dilution correction for C and C+ samples.

Peptide	Condition	Vehicle	Spike	Time (min)	Raw mean area	Correction factor	Corrected mean area	n
CGRP 8-37	A+	0.1% FA	Spiked	0	885944	1	885944	2
CGRP 8-37	A+	0.1% FA	Spiked	10	1173504	1	1173504	2
CGRP 8-37	B+	0.1% FA + inhibitors	Spiked	0	718252	1	718252	2
CGRP 8-37	B+	0.1% FA + inhibitors	Spiked	10	964038	1	964038	2
CGRP 8-37	C+	70% ACN/30% EtOH	Spiked	0	7424	4	29694	2
CGRP 8-37	C+	70% ACN/30% EtOH	Spiked	10	6898	4	27590	2
CGRP 8-37	A	0.1% FA	Unspiked	0	184	1	184	2
CGRP 8-37	A	0.1% FA	Unspiked	10	86	1	86	2
CGRP 8-37	B	0.1% FA + inhibitors	Unspiked	0	11238	1	11238	2
CGRP 8-37	B	0.1% FA + inhibitors	Unspiked	10	9472	1	9472	2
CGRP 8-37	C	70% ACN/30% EtOH	Unspiked	0	102	4	410	2
CGRP 8-37	C	70% ACN/30% EtOH	Unspiked	10	84	4	334	2
CGRP	A+	0.1% FA	Spiked	0	430262	1	430262	2
CGRP	A+	0.1% FA	Spiked	10	570750	1	570750	2
CGRP	B+	0.1% FA + inhibitors	Spiked	0	425936	1	425936	2
CGRP	B+	0.1% FA + inhibitors	Spiked	10	561208	1	561208	2
CGRP	C+	70% ACN/30% EtOH	Spiked	0	1676	4	6704	2
CGRP	C+	70%	Spiked	10	2196	4	8782	2

		ACN/30% EtOH						
CGRP	A	0.1% FA	Unspiked	0	92	1	92	2
CGRP	A	0.1% FA	Unspiked	10	44	1	44	2
CGRP	B	0.1% FA + inhibitors	Unspiked	0	152	1	152	2
CGRP	B	0.1% FA + inhibitors	Unspiked	10	122	1	122	2
CGRP	C	70% ACN/30% EtOH	Unspiked	0	58	4	230	2
CGRP	C	70% ACN/30% EtOH	Unspiked	10	38	4	150	2

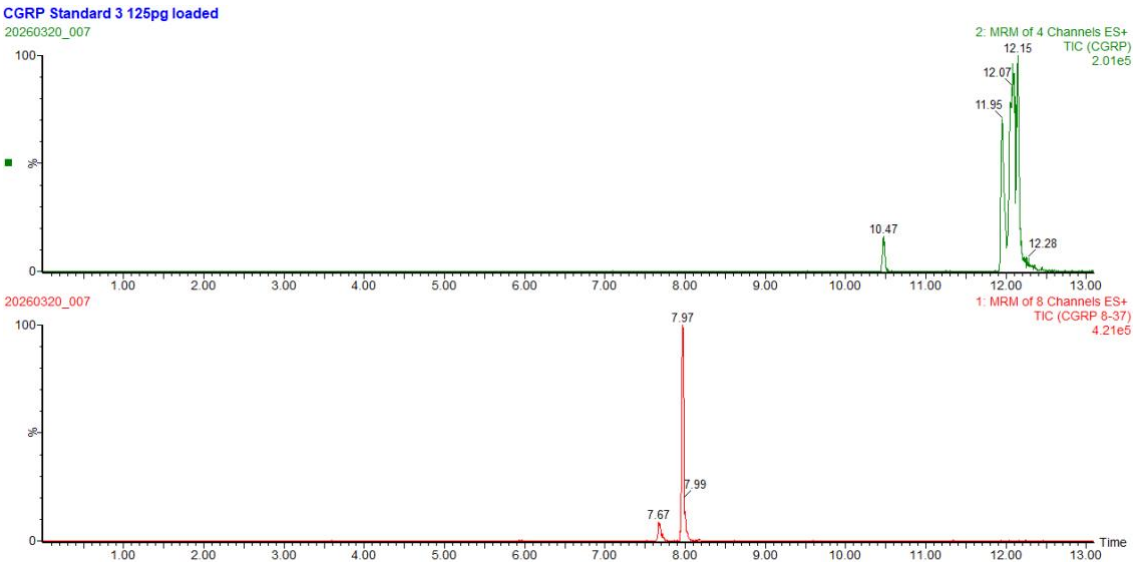


Figure C1. LC-MS chromatogram for the solvent-spiked CGRP/CGRP 8-37 standard, 125 pg loaded.

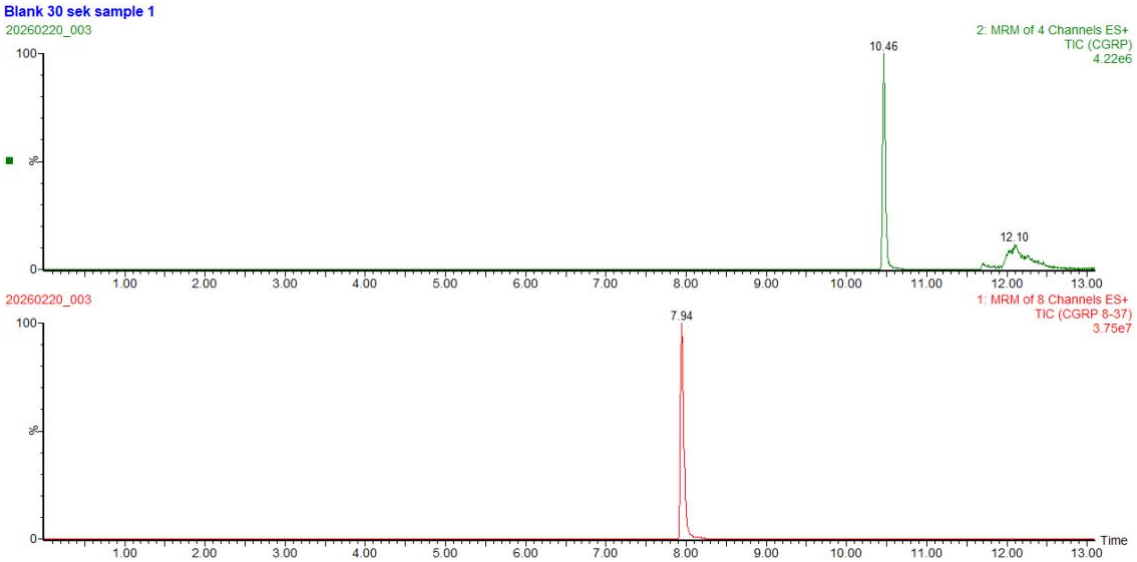


Figure C2. LC-MS chromatogram for the pilot blood-spiked sample after 30 s blood contact.

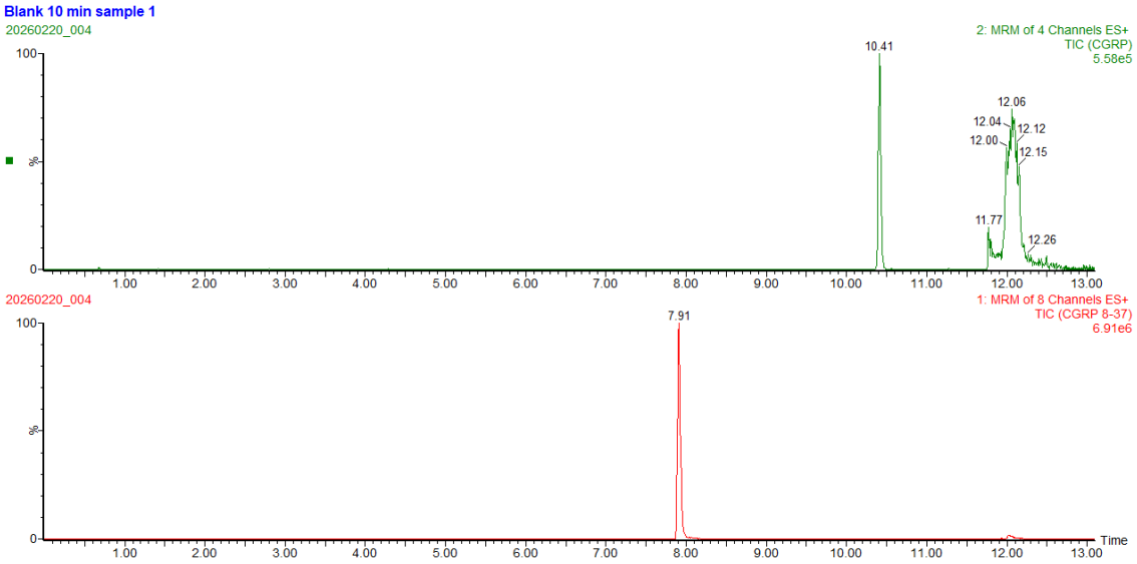


Figure C3. LC-MS chromatogram for the pilot blood-spiked sample after 10 min blood contact.

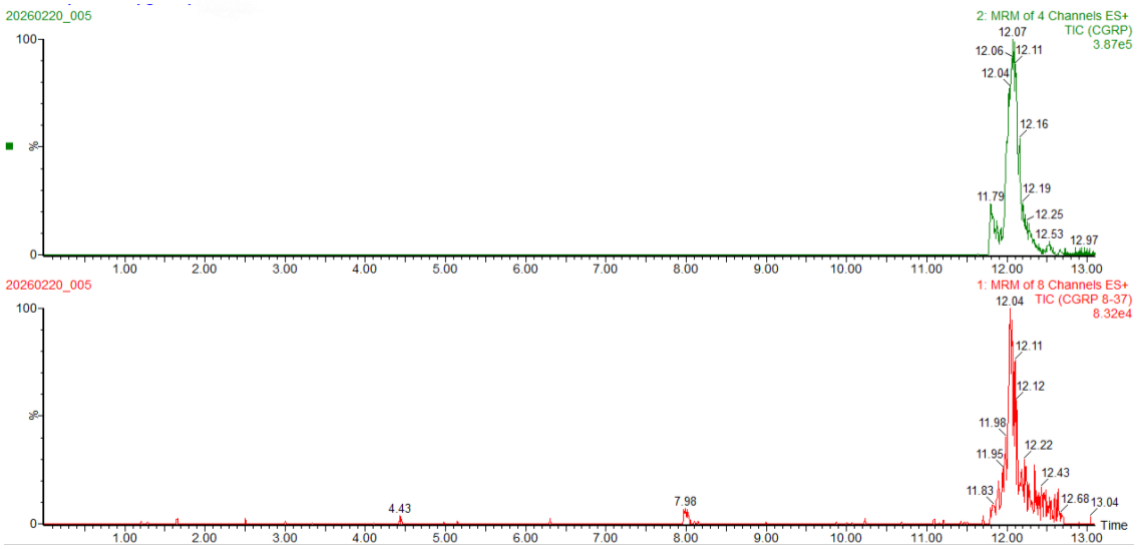


Figure C4. LC-MS chromatogram for the blank blood matrix sample.

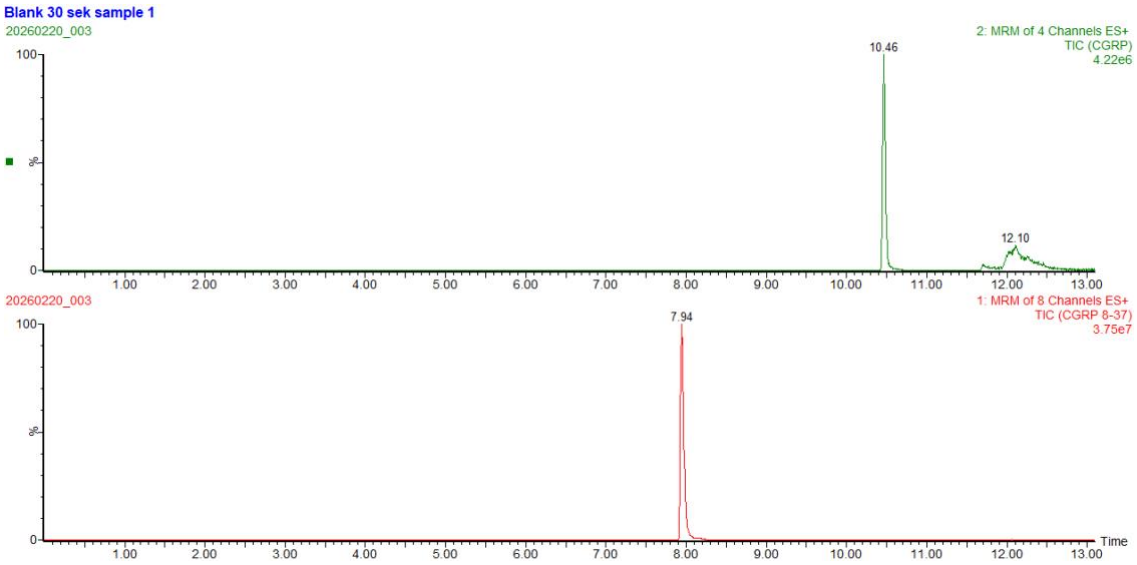


Figure C5. LC-MS chromatogram for the pilot blank blood sample after 30 s handling.

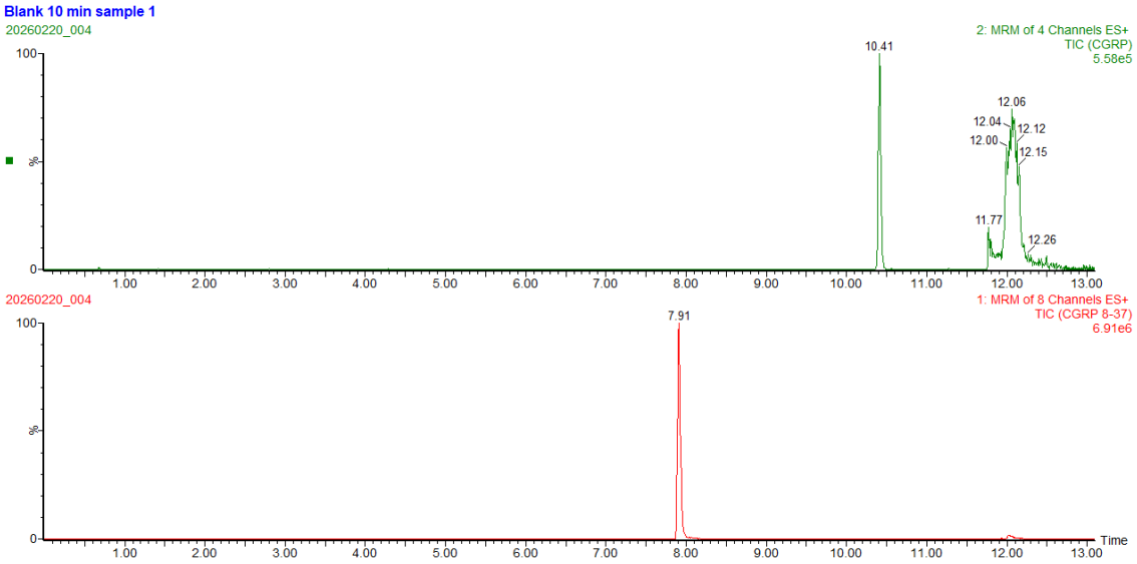


Figure C6. LC-MS chromatogram for the pilot blank blood sample after 10 min handling.

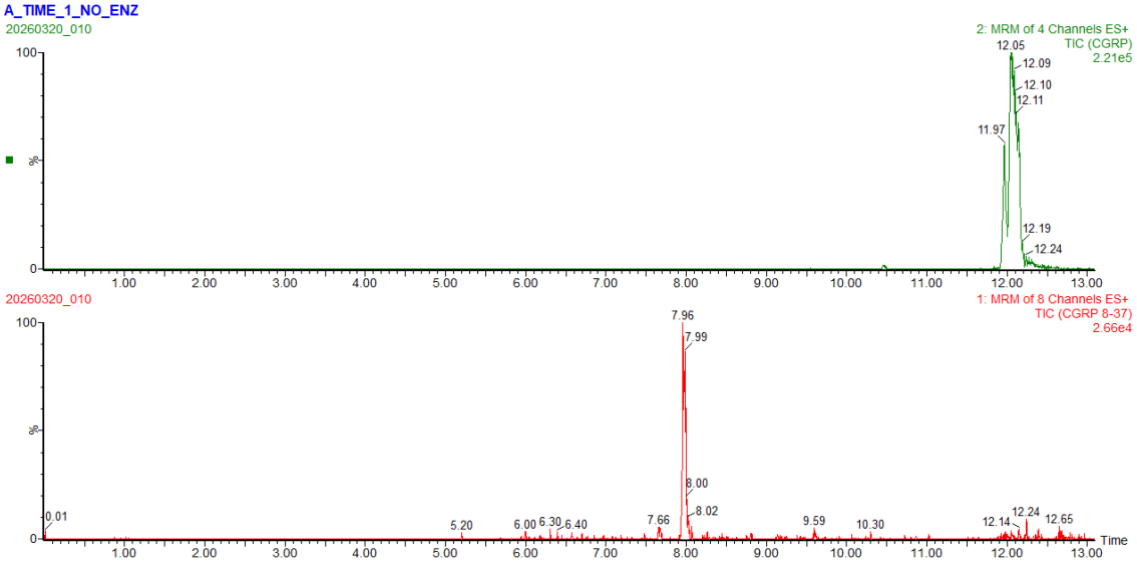


Figure C7. Time-course LC-MS chromatogram for an early blood sample without enzyme inhibitors.

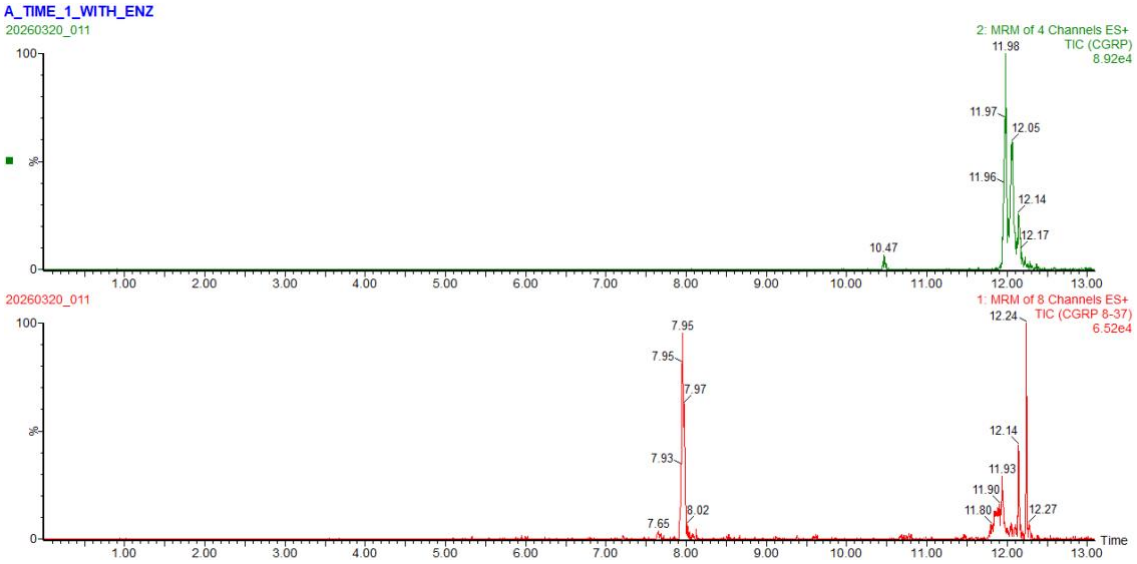


Figure C8. Time-course LC-MS chromatogram for an early blood sample with enzyme inhibitors added at the timepoint.

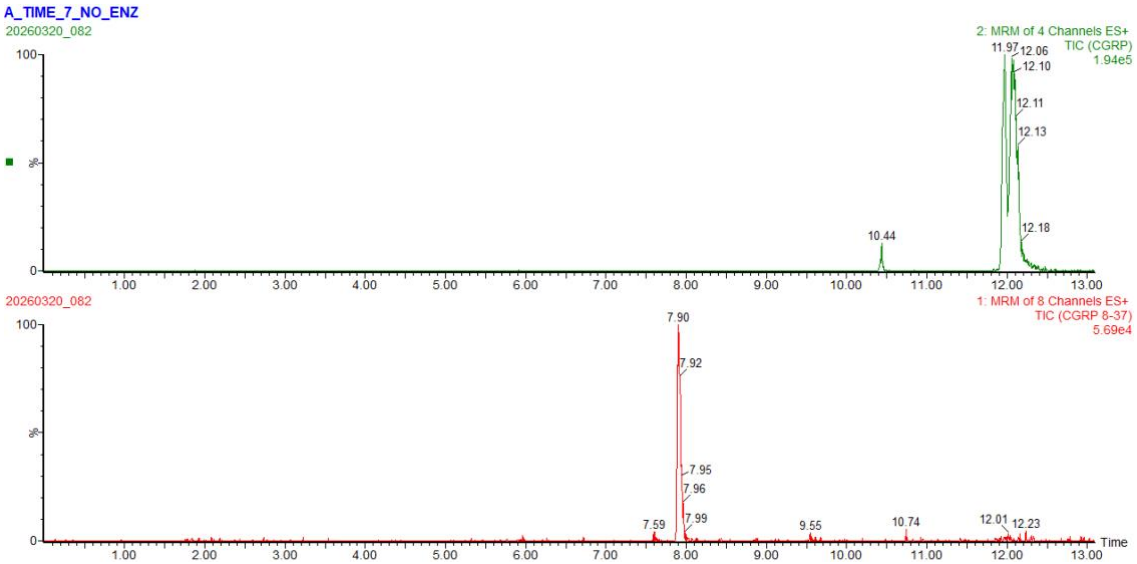


Figure C9. Time-course LC-MS chromatogram for a late blood sample without enzyme inhibitors.

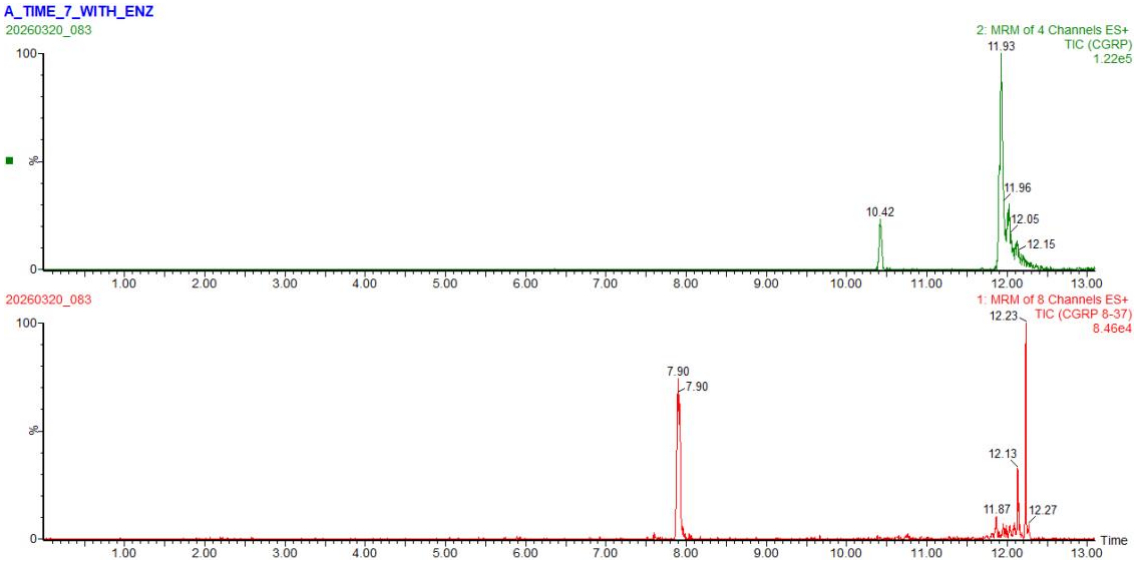


Figure C10. Time-course LC-MS chromatogram for a late blood sample with enzyme inhibitors added at the timepoint.

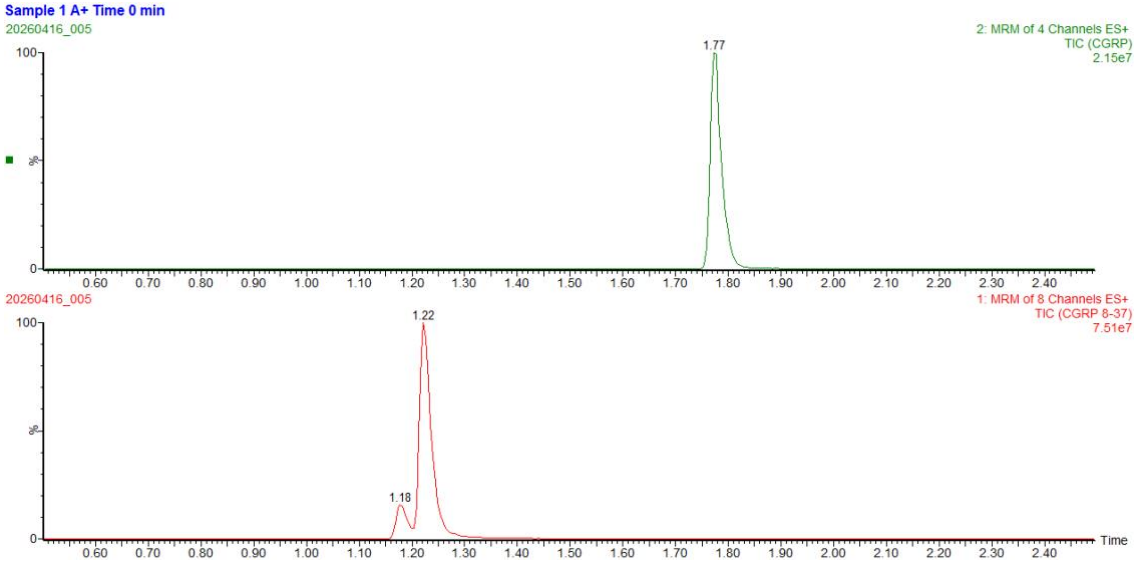


Figure C11. SPE LC-MS chromatogram for sample A+ at 0 min.

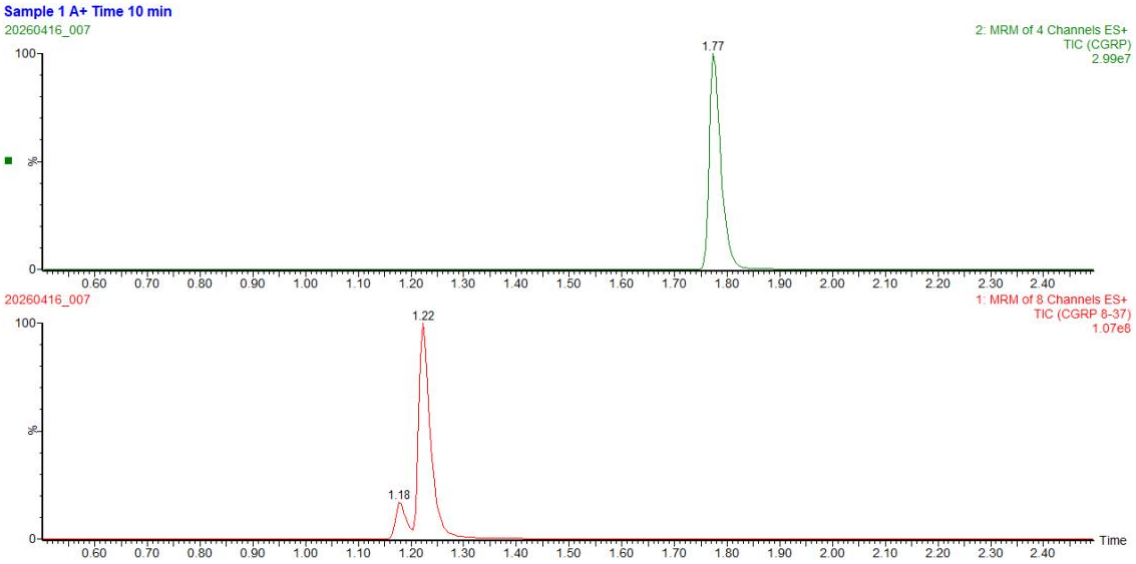


Figure C12. SPE LC-MS chromatogram for sample A+ at 10 min.

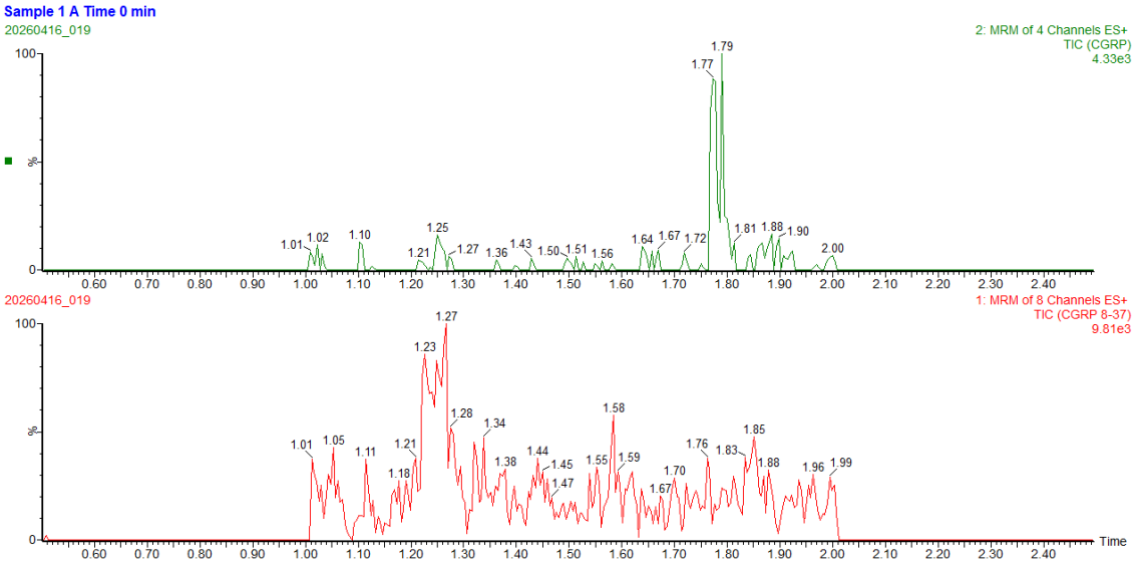


Figure C13. SPE LC-MS chromatogram for unspiked sample A at 0 min.

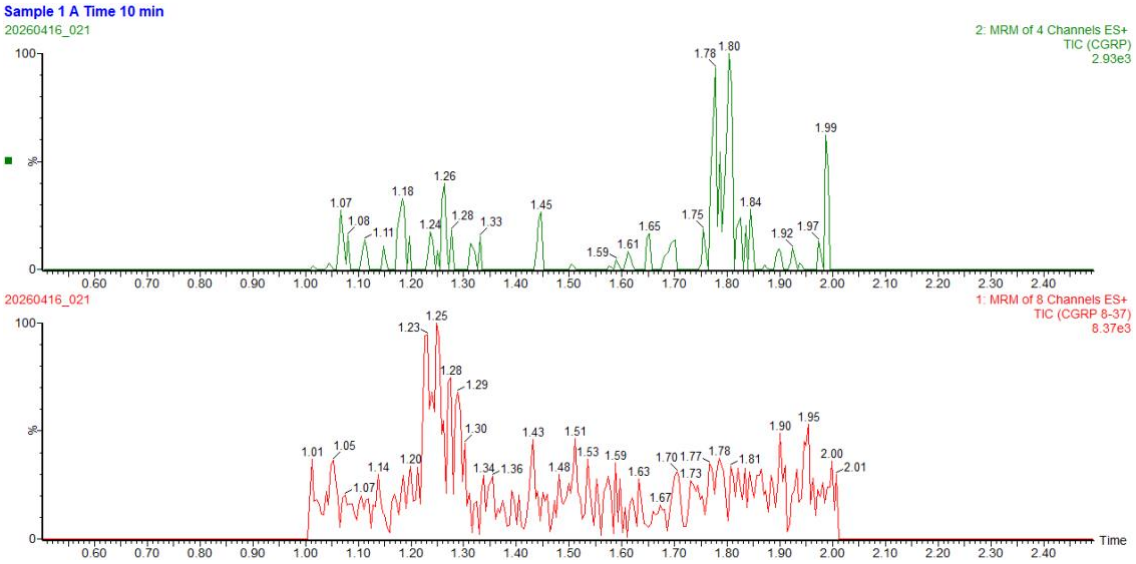


Figure C14. SPE LC-MS chromatogram for unspiked sample A at 10 min.

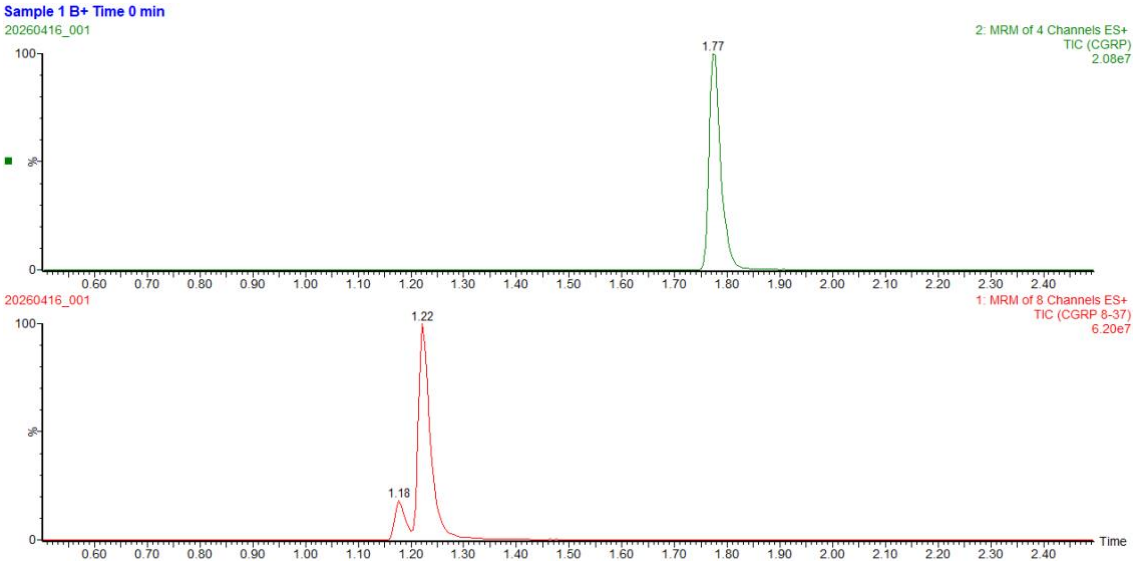


Figure C15. SPE LC-MS chromatogram for sample B+ at 0 min.

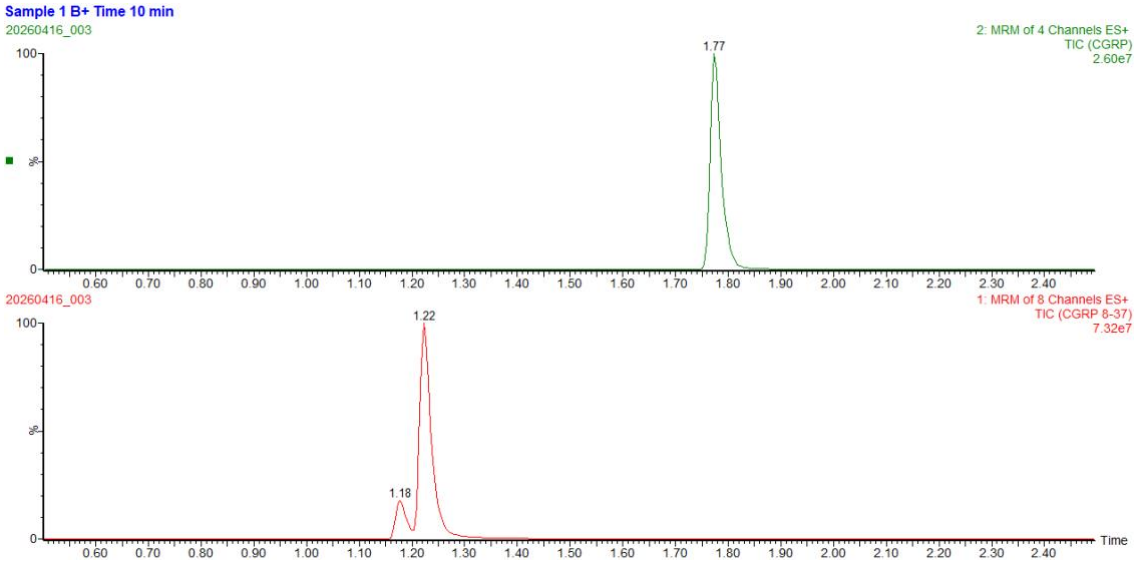


Figure C16. SPE LC-MS chromatogram for sample B+ at 10 min.

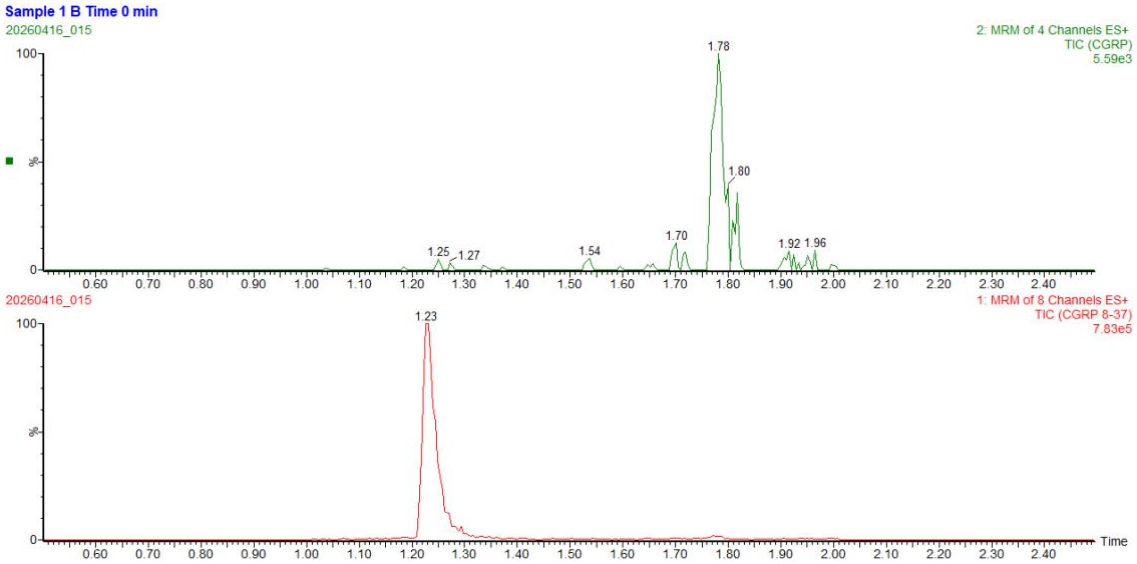


Figure C17. SPE LC-MS chromatogram for unspiked sample B at 0 min.

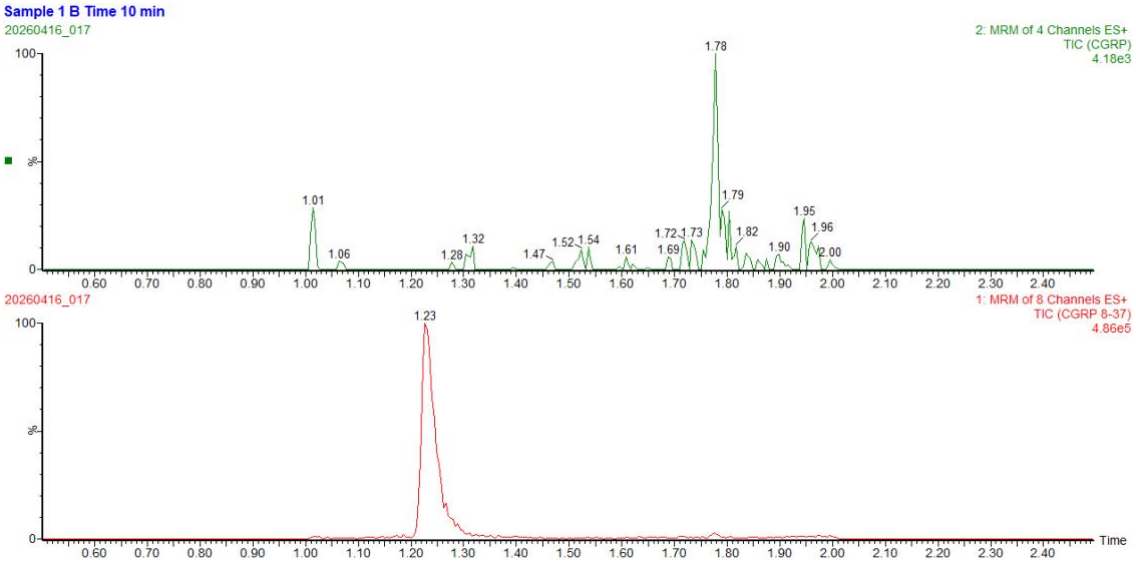


Figure C18. SPE LC-MS chromatogram for unspiked sample B at 10 min.

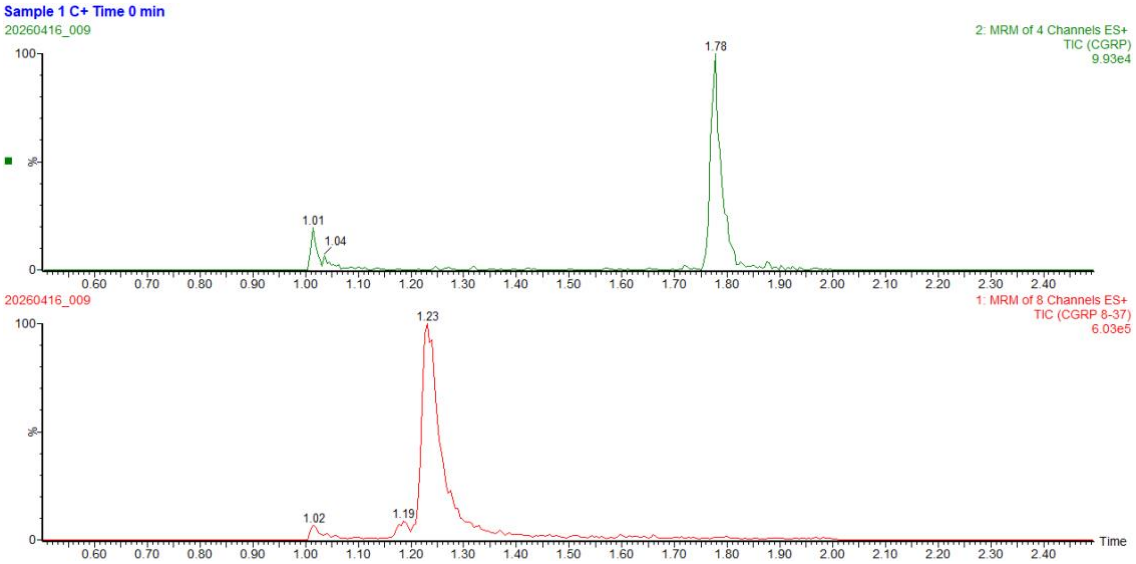


Figure C19. SPE LC-MS chromatogram for sample C+ at 0 min.

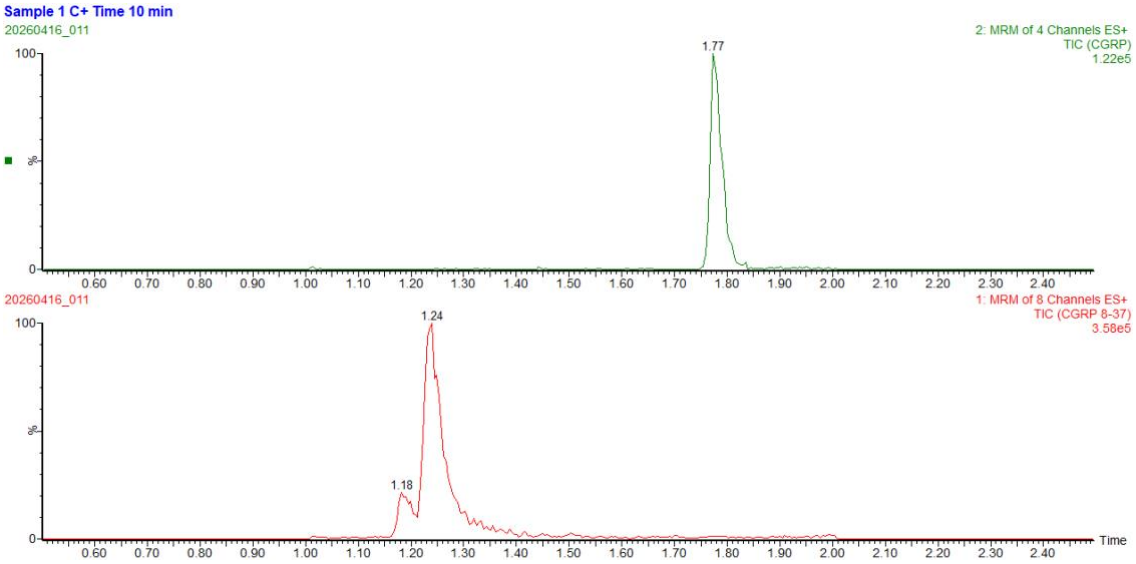


Figure C20. SPE LC-MS chromatogram for sample C+ at 10 min.

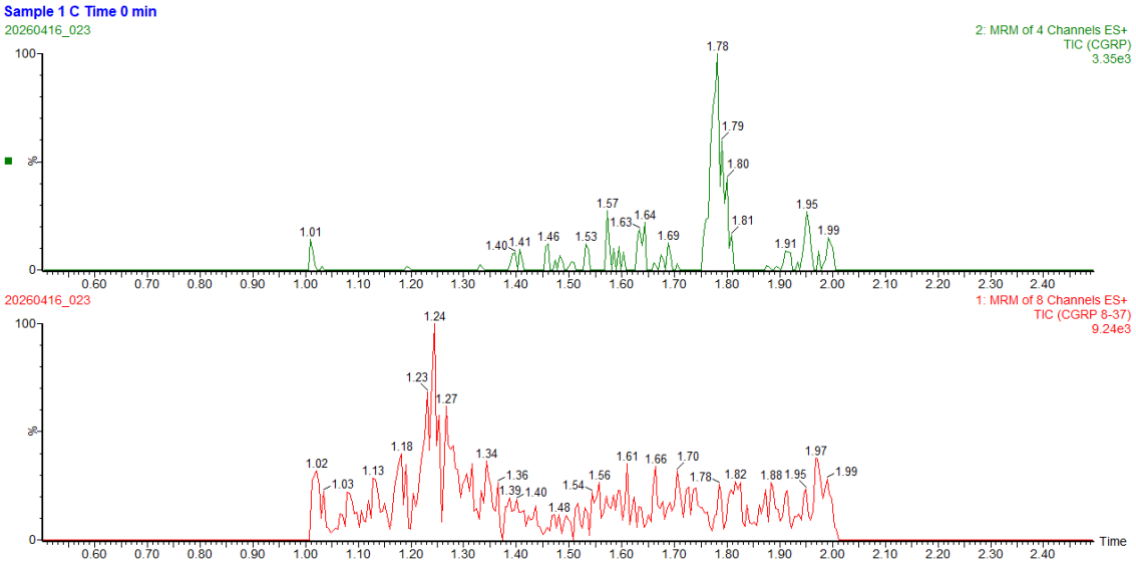


Figure C21. SPE LC-MS chromatogram for unspiked sample C at 0 min.

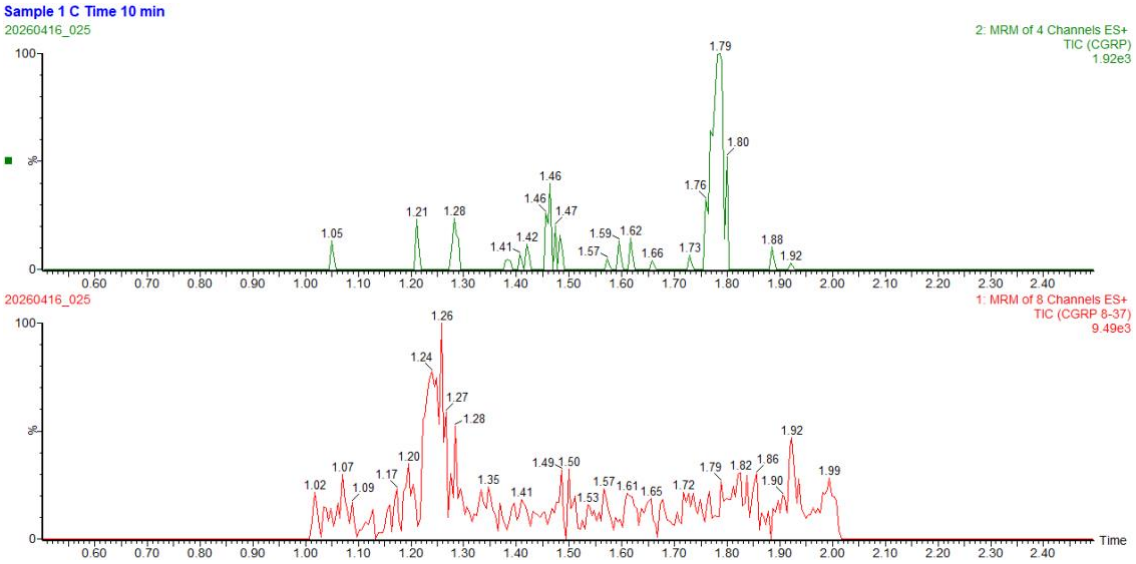


Figure C22. SPE LC-MS chromatogram for unspiked sample C at 10 min.

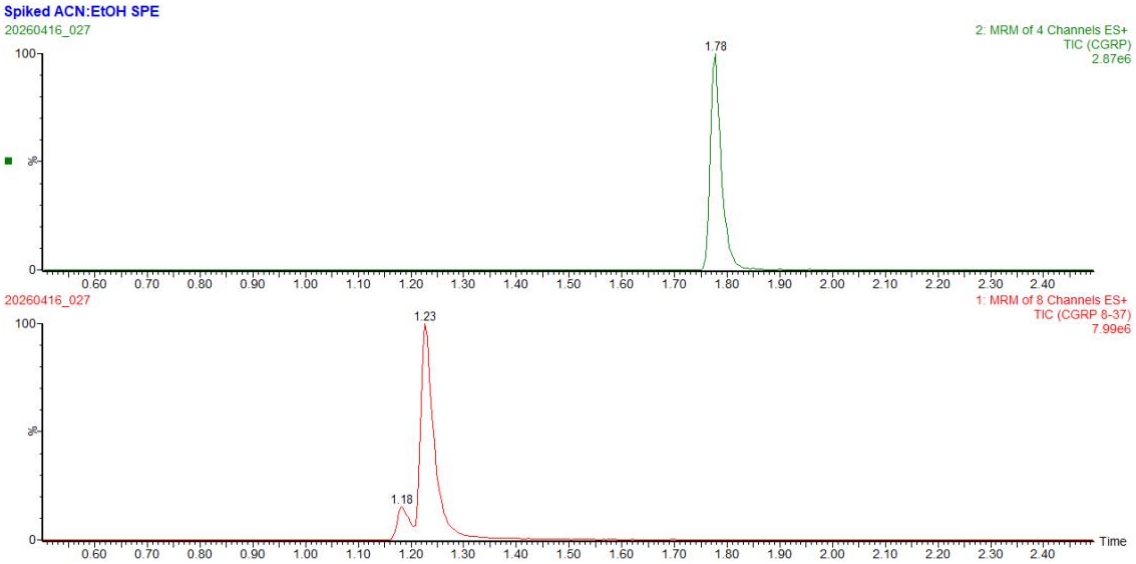


Figure C23. SPE LC-MS chromatogram for the spiked ACN:EtOH solvent control.

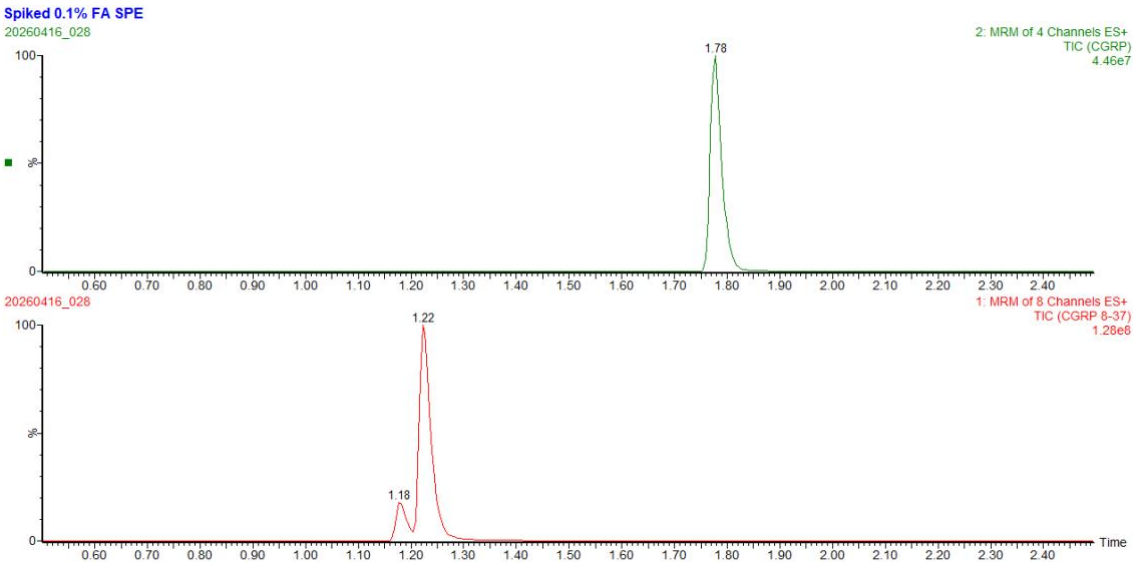


Figure C24. SPE LC-MS chromatogram for the spiked 0.1% formic acid solvent control.

LC-MS method details are summarized in Chapter 4. The chromatograms in Appendix C are representative raw outputs from pilot method-development experiments and should not be interpreted as validated endogenous quantification.