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RESEARCH ARTICLE

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# Investigating the detection of the novel doping-relevant peptide kisspeptin-10 in urine using liquid chromatography high-resolution mass spectrometry

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## Abstract

Kisspeptin-10 is a peptide hormone capable of increasing circulating follicle-stimulating hormone, luteinizing hormone and testosterone levels in humans. Clinically, these effects suggest its use as a treatment for infertility. However, its testosterone-increasing effect indicates potential misuse in sports. As such, it is included in the 2024 World Anti-Doping Agency Prohibited List. This work describes the successful validation of an initial testing procedure (screening) and a confirmation procedure for kisspeptin-10 in urine using liquid chromatography–mass spectrometry. Additionally, kisspeptin-10 was incubated in human serum to mimic endogenous metabolism to improve method sensitivity, as previous research had demonstrated a rapid elimination time of only 30 min after injection (in rats). Four metabolites, corresponding to peptide fragments y9, y8, y7 and y5, were found and added to the ITP in full scan mode. A degradation product discovered during early experimentation was found to probably be caused by oxidation of the tryptophan residue into a kynurenine residue. Further research should elucidate the kinetic parameters of the reaction to improve product stability. Using the validated confirmation procedure, a black-market vial of kisspeptin-10 was analysed. The product contained no unexpected impurities, although it appeared to have undergone more degradation than the purchased reference standard.

## 1 | INTRODUCTION

Kisspeptins are a group of peptide products encoded by the KISS-1 gene, originally discovered as metastasis suppressors (Kotani et al., 2001). Further research demonstrated their ability to stimulate the release of the gonadotropins, follicle-stimulating hormone and luteinizing hormone, as well as increasinge circulating testosterone levels in human males (Dhillo et al., 2005; George et al., 2011). The gonadotropin-stimulating effects of kisspeptin-54 and kisspeptin-10, two major kisspeptin isoforms, indicate their potential use in treating infertility (Jayasena et al., 2015). However, as with gonadotropin-releasing hormone itself and its agonist analogues, the testosterone-stimulating effects of kisspeptin provoke potential misuse in sports where increased testosterone levels provide substantial benefit to athletes. As of 2024, the World Anti-Doping Agency (WADA, 2024) has included kisspeptin and its agonist analogues on their Prohibited List under section *S2.2. Peptide hormones and releasing factors*. Of the kisspeptin isoforms, kisspeptin-10 is the smallest peptide capable of activating its target receptor with full affinity and efficacy (Kotani et al., 2001). With a molecular weight below 2 kDa (1.302 kDa), it is considered a small peptide. Detection methods for small peptides, both initial testing procedures (ITPs, screening) and confirmation procedures (CPs), have been described by several research groups (Cuervo et al., 2017; Mazzarino et al., 2015; Okano et al., 2010; Thomas et al., 2016; Timms et al., 2014). Our group described ITP and CP methods using a dilute-and-shoot protocol including direct

  

injection of the sample into a liquid chromatography–mass spectrometry (LC–MS) system (Coppieters et al., 2021; Judák et al., 2017). For the CP, nano-scale LC is used. The goal of this project was to include kisspeptin-10 in these two methods and for the very first time, describe the analysis of kisspeptin-10 in the frame of anti-doping. To improve the kisspeptin-10 detection window, metabolites were sought via serum incubation, as previous research in rats demonstrated rapid elimination after injection (Liu et al., 2013). After intravenous bolus administration of 1 mg/kg bodyweight, kisspeptin-10 became undetectable after just 30 min, indicating the need for targeting degradation products and metabolites.

Although anabolic androgenic steroids make up the bulk of (illegally) sold doping compounds, peptide hormones and proteins also account for a considerable portion, as demonstrated by the Norwegian doping control laboratory in 2015 (Hullstein et al., 2015). Analysis of black-market products confiscated by Norwegian police and custom authorities between 2011 and 2014 revealed that 28% contained peptide hormones or proteins. Further demonstrating the availability of illicit peptide hormones is a simple web search, where the wide array of illicit peptide hormones available for purchase through various online vendors becomes apparent. Among many, vials supposedly containing kisspeptin-10 are displayed. The safety concerns raised by the lack of quality assurance or relevant toxicological data regarding these products are well understood (Kimergård et al., 2014). During this project, a vial of kisspeptin-10 was purchased from such an online vendor and analysed using inhouse developed methods. Of note is the large online availability of specifically kisspeptin-10, whereas other isoforms are mostly available as reference standards at much higher prices.

## 2 | EXPERIMENTAL

### 2.1 | Chemicals and reagents

Kisspeptin-10 (metastin [45–54]), glacial acetic acid (HOAc), LC–MS grade dimethylsulfoxide (DMSO), bovine insulin, [deamino-Cys1, Val4, D-Arg8]-vasopressin (ISTD 1) and trifluoroacetic acid for high-performance LC (HPLC) of purity ≥99% were purchased from Sigma Aldrich (Bornem, Belgium). LC–MS-grade water and LC–MS-grade acetonitrile (ACN) were obtained from J.T. Baker (Deventer, Netherlands). LC–MS-grade formic acid was purchased from Fisher Chemical (Madrid, Spain). Tetrahydrogestrinone was obtained from the National Measurement Institute (Australia). [$^{13}$C,$^{15}$N] growth hormone-releasing peptide-2 (1–3) (ISTD 2) was obtained from the Australian Sports Drug Testing Laboratory (Sydney, Australia) and was synthesized by Auspep (Tullamarine, Australia). Black-market kisspeptin-10 was purchased from a website. Fresh human serum was acquired from a healthy volunteer in the context of a study approved by the Ethical Committee of Ghent University (approval number B6702023000168).

Water containing 10% ACN and 2% HOAc was used as mix A. Mix B was prepared by adding bovine insulin to mix A at a concentration of 1 µg/ml. Tetrahydrogestrinone was added to mix B at 10 ng/ml to create mix C.

### 2.2 | Stock and working solutions

Kisspeptin-10 reference material was dissolved in its original vial using an appropriate volume of mix A to obtain a concentration of 1 mg/ml, considering purity and peptide content. This solution was transferred to a 1.5 ml LoBind polypropylene tube from Eppendorf (Hamburg, Germany) and stored at −80°C. Further dilutions were made using mix B and stored at −20°C. Black-market kisspeptin-10 was dissolved in its original vial with mix A to a concentration of 10 mg/ml and was stored at −20°C. Both internal standards (ISTD 1 and ISTD 2) were combined in mix B to a concentration of 100 ng/ml which was stored in the fridge (2–8°C).

### 2.3 | Sample preparation

Sample preparation is based on previously described protocols (Coppieters et al., 2021; Judák et al., 2017). Briefly, the ITP sample preparation involves diluting 0.5 ml of urine with 480 µl of mix B and adding 20 µl of 100 ng/ml ISTD mix. Diluted samples are centrifuged and injected directly into the LC–MS system. The CP sample preparation is almost identical, with an additional dilution step where, after centrifugation, 25 µl of diluted sample is added to 175 µl of mix C. The 16-fold diluted sample is then injected into the nanoLC–MS system.

### 2.4 | Instrumental

For the ITP, chromatographic separation was performed using a Dionex Ultimate™ 3000 UHPLC system (Thermo Scientific, Bremen, Germany) equipped with a degasser, an Ultimate™ Dual-Gradient Rapid Separation Pump and an autosampler and column compartment thermostated at 10 and 30°C, respectively. Compounds were separated over an Agilent Zorbax RRHD StableBond $C_8$ column (2.1 × 50 mm, 1.8 µm) equipped with an Agilent Zorbax StableBond $C_8$ guard column (2.1 × 5 mm, 1.8 µm). Water and ACN, both containing 1% DMSO and 0.2% formic acid, were used as mobile phases A and B, respectively. The gradient started at 350 µl/min with 1% B for 1 min, after which mobile phase B gradually increased to 40% over 14 min. The flowrate then immediately increased to 500 µl/min with 90% B, held for 1.5 min. Afterwards, mobile phase B decreased rapidly to 1%, followed 1 min later by an immediate decrease of the flowrate to 350 µl/min. This was held for another minute, for a total runtime of 18.5 min. The injection volume was set to 20 µl.

The LC system was coupled with a Q-Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany). The MS was equipped with an electrospray ionization source, set to the following parameters: spray voltage, 4 kV; capillary temperature, 250°C; auxiliary gas heater, 300°C; sheath gas flow rate, 50; auxiliary gas flow rate, 20; S-lens, 50. Both positive full-scan and positive targeted selective ion monitoring mode were used during analysis. The full-scan parameters were set to 70,000 resolution, 300–1200 scan range,

1e6 automatic gain control (AGC) target, and 50 ms maximum ion injection time. The targeted selective ion monitoring parameters were set to 70,000 resolution, 300–1075 scan range, 1e5 AGC target, 50 ms maximum injection time, 2.2 *m/z* isolation window, and 5 msx (multiplexing). Mass extraction was set to 5 ppm.

The CP instrumental configuration, including elution gradient and 10-port switching valve setup, has been fully described in a previously published paper (Coppieters et al., 2021), with only two minor changes. The injection volume and ion source spray voltage were set to 20 µl and 1.9 kV, respectively.

## 2.5 | Validation

Both methods were validated according to WADA guidelines for the validation of ITPs and CPs for non-threshold substances (WADA, 2021) and WADA technical documents (TDs) on the minimum required performance levels (MRPLs) (WADA, 2022) and, for the CP, identification criteria (IDCR) (WADA, 2023). Kisspeptin-10 is not yet included in the latest TD MRPL but a desired limit of identification (LOI) of 2 ng/ml was the goal, like gonadotropin-releasing hormone and analogues as they all fall under section *S2.2.1 Testosterone-stimulating peptides in males* on the WADA Prohibited List (WADA, 2024). Ten different urine samples were retrieved from −20°C storage, covering a pH range of 5.1–8.8 and a density range of 1.002–1.028 g/cm$^3$, measured with an Atago UG-1 digital refractometer.

For the ITP, a signal was deemed acceptable if a clearly distinguishable peak was present with a signal-to-noise ratio greater than three (S/N > 3) and a retention time (RT) like that of the same compound in a reference sample (RT difference <0.3 min). For the CP, a signal was deemed acceptable if it complied with the TD2023 IDCR requirements regarding ion ratio and relative RT (WADA, 2023).

*MRPL compliance* was evaluated by spiking 10 different urines at 0.5× MRPL (1 ng/ml) and at MRPL (2 ng/ml) in two separate batches by two different analysts. The urines were spiked at four additional

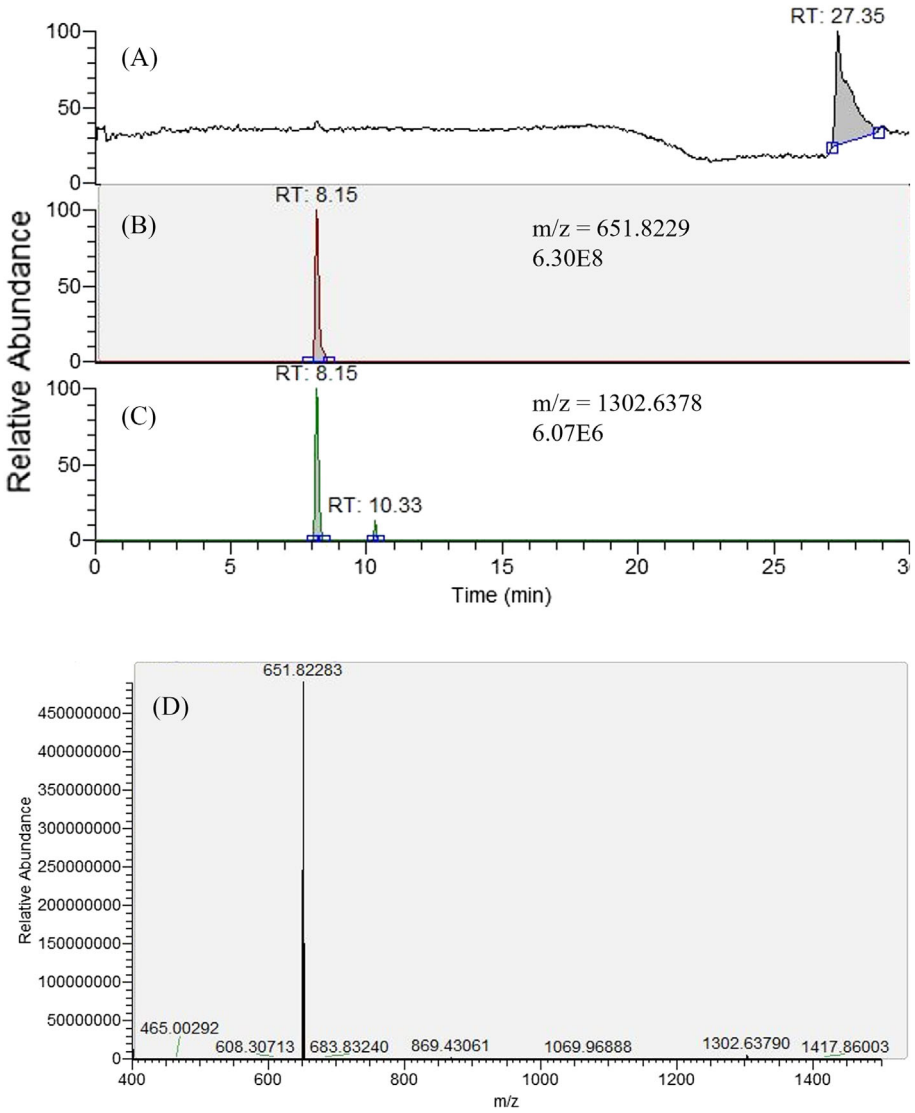


**FIGURE 1** Total ion chromatogram (a) and extracted chromatograms for the double (b) and single (c) charged parent ion after analysis of a kisspeptin-10 reference solution. The mass spectrum of the peak at retention time (RT) = 8.15 is also shown (d). Peak intensities are shown below the *m/z* values.

levels (0.1× MRPL, 0.25× MRPL, 0.75× MRPL, 2× MRPL) to assess the limit of detection (LOD) and LOI for the IPT and CP, respectively. These limits were calculated using a sigmoidal curve model and set at a 95% detection level. *Selectivity* was evaluated by analysing 10 blank urines for interfering signals at the relevant RTs. *Matrix effect* was determined by comparing peak areas in spiked urine samples with spiked blank matrix (mix B) using following formula: Matrix effect (%) = [(peak area urine/peak area blank matrix) − 1] × 100. *Carryover* was assessed by injecting a urine spiked at 4× MRPL (8 ng/ml), followed by a blank (mix A). The extracted samples used to evaluate

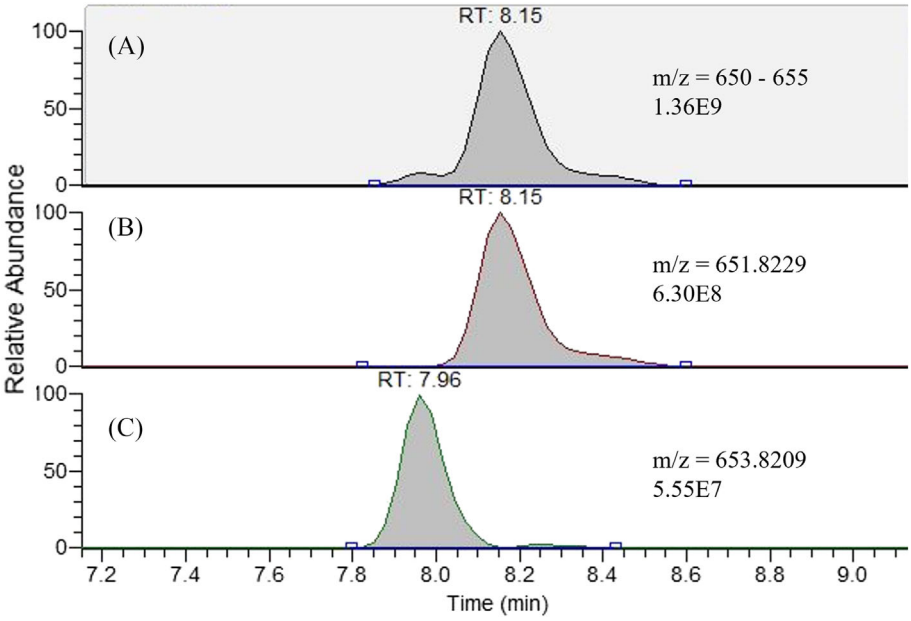


**FIGURE 2** Extracted chromatograms for the *m/z* range 650–655 (a), double charged kisspeptin-10 (b) and an unknown ion (c), after analysis of a kisspeptin-10 reference solution. Peak intensities are shown below the *m/z* values.

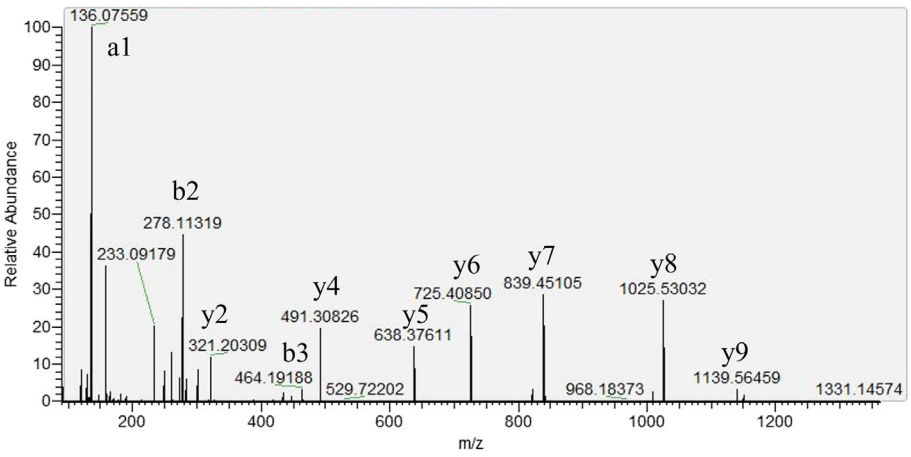


**FIGURE 3** Mass spectrum generated after fragmentation of kisspeptin-10 with collision energy 24. Product ions are labelled with their corresponding peptide fragment.

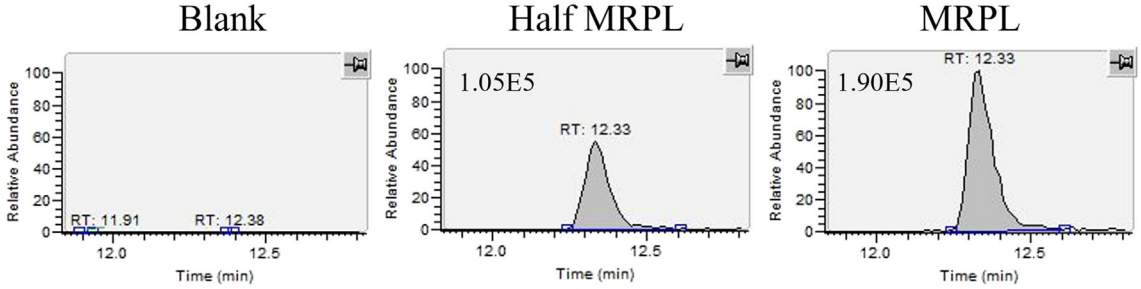


**FIGURE 4** Chromatograms, scaled to the largest peak, showing kisspeptin-10 in validation urine 1 at 0× the minimum required performance level (MRPL), 0.5× MRPL and 1× MRPL. Peak intensities are shown in the upper left corner of each chromatogram (except for the blank urine). RT, Retention time.

MRPL compliance were reinjected after 48 h (for ITP) or 96 h (for CP) in the autosampler at 10°C to assess *sample extract stability*.

## 2.6 | Serum metabolites

As previous *in vivo* research in rats demonstrated the rapid elimination time of kisspeptin-10 after injection (Liu et al., 2013), metabolites were sought to improve method sensitivity and prolong the detection window. To 45 µl of fresh human serum, 5 µl of 10 mg/ml black-market kisspeptin-10 was added. The black-market kisspeptin-10 was chosen over a certified reference standard owing to the lower cost, larger available quantity and demonstrated purity of the product, described in Section 3.5. Aliquots were incubated on a benchtop shaker set to 37°C and 500 rpm for 0, 30, 120 and 18 h. Afterwards, proteins were precipitated with 150 µl of ACN. The tubes were vortexed and the retentate was transferred to a new tube, partially evaporated (only the ACN) in a centrifugal evaporator at 30°C and reconstituted in 950 µl of mix A. Samples were injected into the LC–MS with a mobile phase gradient where the organic phase ramped from 0 to 100% over 20 min.

# 3 | RESULTS AND DISCUSSION

## 3.1 | Initial LC–MS analysis

After receiving the kisspeptin-10 reference standard, it was injected at a high concentration (10 µg/ml) onto the LC–MS system. Maximal chromatographic separation was achieved by employing a mobile phase gradient going from 0 to 100% organic phase over the course of 30 min. Owing to the high background, it was difficult to distinguish a clear peak in the total ion chromatogram. Since small peptides generally form single or double charged ions in positive MS, ion chromatograms for $[M + H]^+$ and $[M + 2H]^{2+}$ were generated. As can be seen in Figure 1, a clear peak can be observed for both ions, although $[M + 2H]^{2+}$ is 100 times more abundant than $[M + H]^+$. Also shown is the mass spectrum of the peak at RT = 8.15, clearly demonstrating the dominant double charged ion. The small peak at RT = 10.33 in Figure 1c was examined and considered an irrelevant interference. The sample was also analysed in negative ionization mode, generating signals ±100 times lower than in positive ionization mode. Also seen in the ion chromatogram was a much smaller peak eluting ±0.2 min earlier, where an ion with $m/z = 653.8209$ was most abundant.

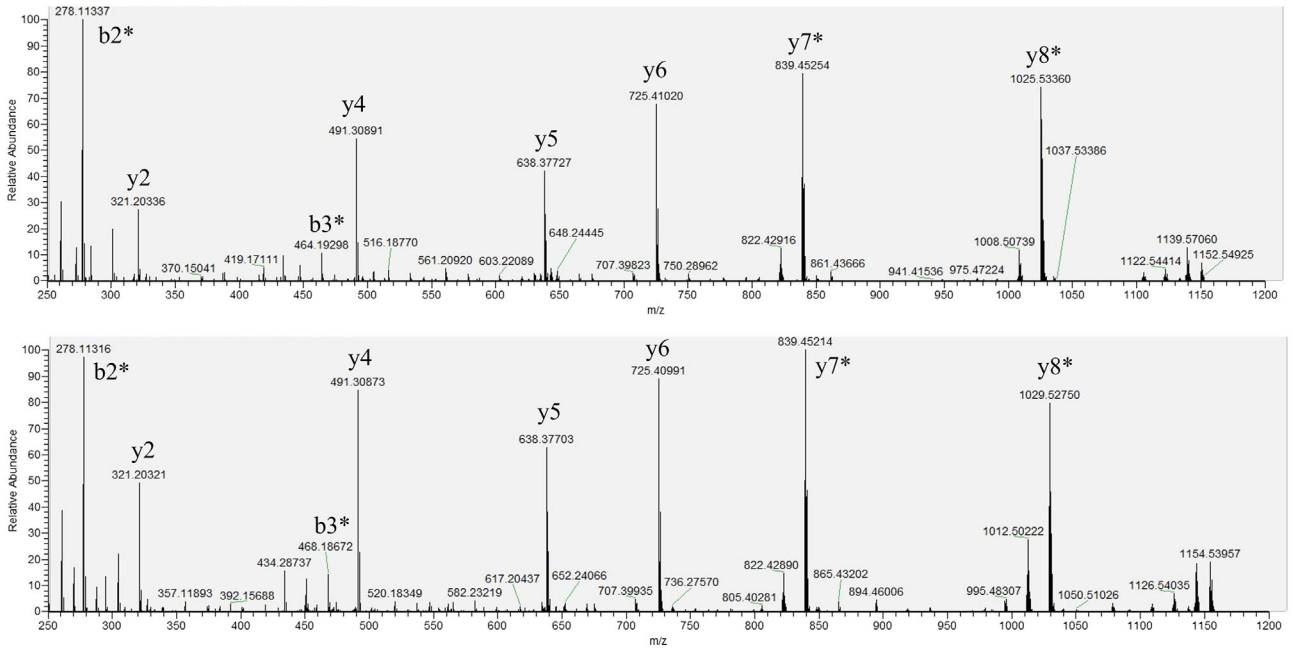


**FIGURE 5** Mass spectrum generated after fragmentation of kisspeptin-10 with collision energy 24. The product ions are labelled with their corresponding peptide fragment. Fragments labelled with an asterisk (*) are used to identify the 4 Da mass shift location, as demonstrated in Figure 6.

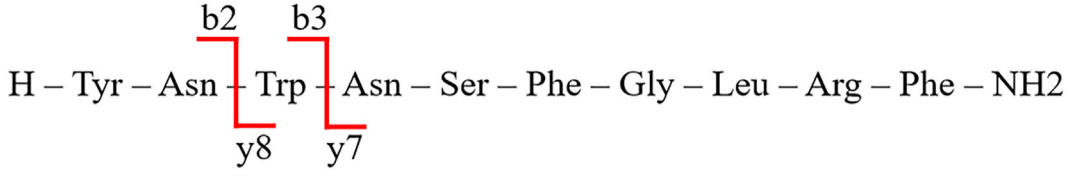


**FIGURE 6** Kisspeptin-10 amino acid sequence. The four indicated fragments are used to identify the 4 Da mass shift location. See Figure 5 for more information.

Extracting both double-charged kisspeptin-10 and this unknown ion, as is shown in Figure 2, demonstrates the small difference in RT and the relatively high abundance of the latter (±9% peak intensity relative to kisspeptin-10). The investigation into this unknown ion is described in Section 3.3.

To identify kisspeptin-10 with the CP, product ions were generated using MS/MS mode. A kisspeptin-10 reference solution was infused directly into the MS, combined with the LC flow. Different collision energies were applied, and the most abundant product ions were considered. Ions corresponding to peptide fragments a1 ($m/z = 136.0756$) and b2 ($m/z = 278.1132$) were selected, with an optimal collision energy of 24. These ions, and other fragments, are indicated on the kisspeptin-10 fragmentation spectrum shown in Figure 3.

## 3.2 | Validation

Kisspeptin-10 was successfully validated for the ITP method. As can be seen in Figure 4, it could be detected at MRPL and half MRPL, showing no interfering peaks at the relevant RT. No carryover effect was present, and it was stable for at least 48 h once extracted. The LOD was determined as 95 pg/ml and an average matrix effect of −38.8% was calculated with a 21% standard deviation.

For kisspeptin-10, product ions a1 ($m/z = 136.0756$) and b2 ($m/z = 278.1332$) were used in the CP. The collision energy was set to 24. It complied with WADA TD IDCR at MRPL and half MRPL, showing no interfering peaks at the relevant RTs. ISTD 1 fragment ion b3 ($m/z = 399.1373$) was used for determining the relative RT criteria according to WADA TD IDCR. No carryover effect was present. Once extracted, after 96 h, kisspeptin-10 no longer complied with WADA TD IDCR at MRPL in one urine and at half MRPL in four urines. Therefore, instrumental analysis should be commenced immediately after sample preparation. The LOI was determined as 302 pg/ml and an average matrix effect of 11.6% was calculated with a high degree of variation (−74.7–131.3%). If kisspeptin-10 were ever to be quantified, it is important to select an appropriate deuterated internal standard (ISTD) to account for this high variance in matrix effect.

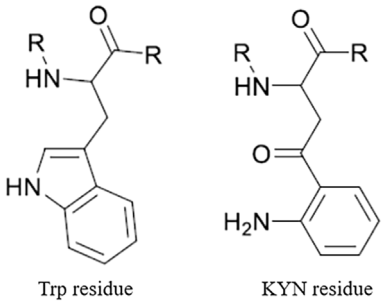


**FIGURE 7** Molecular structure of a tryptophan residue (Trp, left) and a kynurenine residue (KYN, right).

## 3.3 | Kisspeptin-10 degradation product

When analysing the kisspeptin-10 reference standard, surprisingly not only was kisspeptin-10 detected, but also another peak was present in the total ion chromatogram, albeit at a much lower intensity. After reinjecting the sample, left in the autosampler overnight, a relative increase in signal intensity was observed for the unknown ion compared with the parent. Therefore, it was considered as a degradation product. Further investigation revealed a slight difference in elution time and in $m/z$ value (±2). Assuming that the unknown molecule is a modified version of the parent compound, a mass difference of 4 Da is found as both molecules would carry two positive charges (+2). Fragmenting both compounds in MS/MS supports this assumption as the compounds share multiple product ions, whereas others show a 4 Da mass difference, as can be seen in Figure 5. Identification of these ions allows the narrowing down of the location of the mass difference. As can be seen in Figure 6, the 4 Da mass difference is located on the third residue, a tryptophane. A possible explanation for the 4 Da mass shift is given in a review published in 2020, where several tryptophan degradation products are discussed (Bellmaine et al., 2020). When a tryptophan residue oxidizes into a kynurenine (KYN) residue, the molecular mass of the peptide increases by 3.9949 Da. The molecular structure of both residues can be seen in Figure 7.

A controlled degradation study was performed to determine the kinetic parameters of this process. Tubes containing 1 ml 500 ng/ml kisspeptin-10 in mix A were stored under various conditions; at room temperature in darkness, in constant light, on a laboratory bench, in a fridge (2–8°C), in a benchtop shaker at 37°C, in an oven at 50°C, and in an oven at 80°C. To another aliquot, DMSO was added. At various timepoints (0–48 h), 100 µl aliquots were taken and stored at −80°C until analysis. Although there was a significant decrease in parent compound peak area and simultaneous increase in degradation product peak area, the conversion rate appeared similar under all tested conditions. Thus, it is unclear what exactly triggers this conversion,

**TABLE 1** Overview of metabolites found after kisspeptin-10 incubation in fresh human serum.

| Fragment | Elemental composition | Charge state | Monoisotopic mass |
|---|---|---|---|
| y9 | C54H74N16O12 | +2 | 570.2909 |
| y8 | C50H68N14O10 | +2 | 513.2694 |
| y7 | C39H58N12O9 | +2 | 420.2298 |
| y7 | C39H58N12O9 | +1 | 839.4523 |
| y5 | C32H47N9O5 | +2 | 319.6923 |

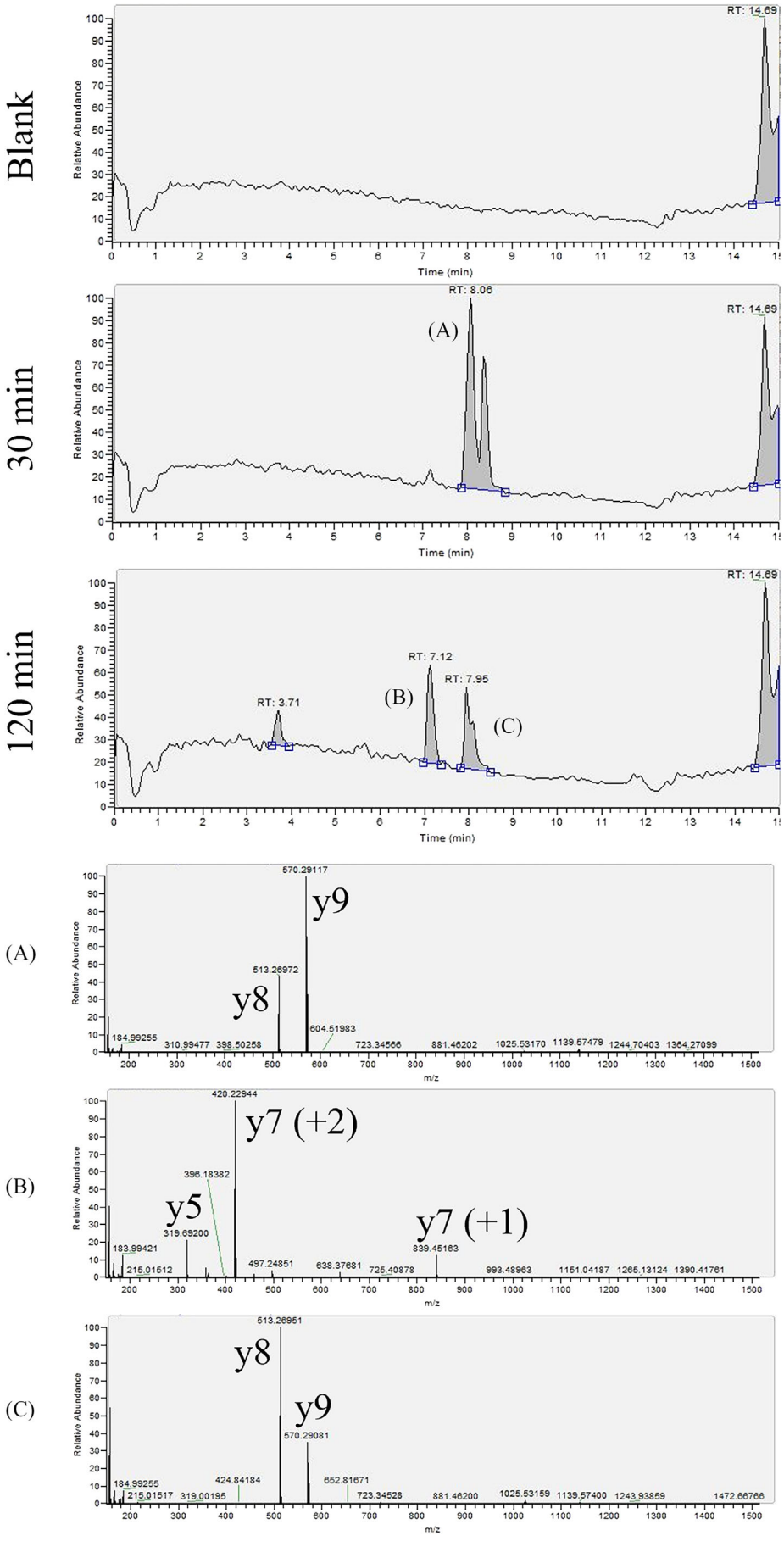

**FIGURE 8** The ion chromatograms ($m/z$ range = 200–1,350) are shown for blank serum, and serum spiked with kisspeptin-10 after 30 and 120 min of incubation. For three peaks (a, b, and c), the mass spectrum is given below. Here, the found metabolites are indicated.

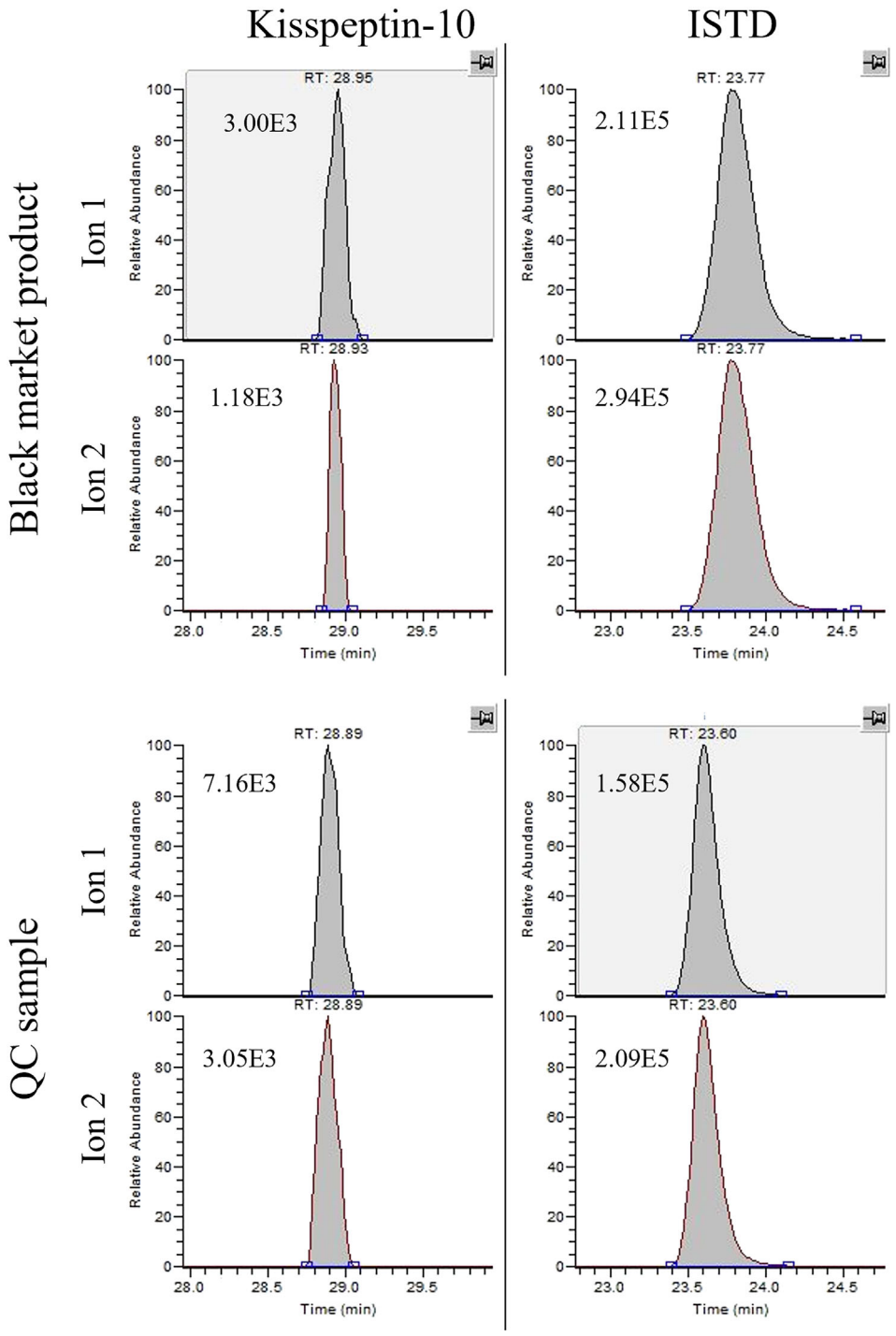


**FIGURE 9** Product ion chromatograms generated by the confirmation procedure (CP) of the black-market kisspeptin-10 sample and a quality control sample (QC, blank urine spiked with kisspeptin-10 at 2 ng/ml). [$^{13}$C,$^{15}$N] growth hormone-releasing peptide-2 (1-3) (ISTD 2) was used as internal standard (ISTD). RT, Retention time.

and whether the oxidation of the tryptophan residue into a KYN residue is what causes the degradation. Further investigation into the cause and kinetics of the degradation should be performed to improve kisspeptin-10 storage conditions and prevent purity loss.

### 3.4 | Serum metabolites

Table 1 gives an overview of the metabolites found through serum incubation. Metabolite y9, an N-terminal tyrosine deleted peptide,

was also discovered by another research group after kisspeptin-10 incubation in rat plasma (Liu et al., 2013). This metabolite was most abundant after 30 min of incubation, whereas y8 and y7 were most abundant after 120 min. These results are demonstrated in Figure 8. Full-scan data of the black-market product was retrospectively analysed for the discovered metabolites to ensure that these were not already present as contaminants. To observe their effectiveness as kisspeptin-10 doping indicators, all fragments (y9, y8, y7 both single and double charged, y5) are monitored in full-scan mode in the ITP. It would be interesting to synthesize these metabolites to be usable as reference standards, like previous research involving TB-500 (Esposito et al., 2012).

### 3.5 | Black-market product

A vial supposedly containing kisspeptin-10, purchased from an online vendor, was confirmed to contain kisspeptin-10 using the validated CP. The product ion chromatograms generated by the CP are shown in Figure 9. When analysing the product in full-scan mode, except for the degradation product with $m/z = 653.8209$ also seen in the reference standard, no impurities or degradation products were seen in the black-market product. The purity of the product was further confirmed by performing proton nuclear magnetic resonance using a Bruker Avance Neo 400 MHz spectrometer (data not shown). Interestingly, the degradation product peak area relative to the kisspeptin-10 peak area was 3 times higher in the black-market product than in the reference standard. As described earlier, this could indicate improper storage (e.g. not frozen) or conversion during the production process. Even though it was transported as a lyophilized powder, perhaps degradation could still take place. Nonetheless, the black-market product contains kisspeptin-10 with no unexpected impurities, at 1/38th the cost of the reference standard.

## 4 | CONCLUSION

This paper describes the successful validation of kisspeptin-10 using inhouse developed ITP and CP methods. Noticed during the initial analysis of the reference standard, a degradation product with $m/z = 653.8209$ was investigated. The 4 Da mass shift was located on the third amino acid residue, a tryptophan, probably caused by an oxidation of this residue into a KYN residue. Further research into the kinetic parameters of this reaction is required to improve stability. After serum incubation, four kisspeptin-10 metabolites were found, corresponding to following peptide fragments: y9, y8, y7 both single and double charged and y5. These will be monitored in full-scan mode to improve method sensitivity. Finally, a black-market kisspeptin-10 vial was confirmed to contain only kisspeptin-10 and the expected degradation product, although more degradation had taken place compared with the reference standard.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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