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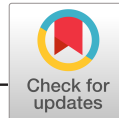
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# Simplified quantification of insulin, its synthetic analogs and C-peptide in human plasma by means of LC-HRMS

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**Abstract**

The quantification of peptide hormones by means of liquid chromatography (LC) coupled to mass spectrometry (MS) or other techniques (e.g. immunoassays) has been a challenging task in modern analytical chemistry. Especially for insulin, its synthetic analogs, and C-peptide, reliable determinations are urgently needed due to their diagnostic value in the management of diabetes and insulin resistance and because of the illicit use of insulin as a performance-enhancing agent in professional sports or as an effective toxin in forensic toxicology. The concomitant measurement of C-peptide and insulin offers an established tool for the diagnostic workup of hypoglycemia (endogenous vs. exogenous hyperinsulinemia), characterizing hepatic insulin clearance, and the assessment of beta-cell function (insulin secretion). Thus, the present approach offers the possibility to determine human insulin and its synthetic analogs (lispro, glulisine, aspart, glargine metabolite, degludec, detemir, porcine, and bovine) and C-peptide simultaneously after sample preparation utilizing protein precipitation and a mixed-mode cation-exchange solid-phase extraction, and subsequent detection by LC-high resolution MS. The method was fully validated regarding the following parameters: specificity, limit of detection (0.2 ng/mL), limit of quantification (0.6 ng/mL), recovery (40–90%), accuracy (78–128%), linearity, precision (< 21%), carry over, robustness, and matrix effects. The proof-of-concept was shown by analyzing authentic plasma samples from adults with class II obesity and prediabetes collected in the course of an oral glucose tolerance test. All sample preparation steps were controlled by two stable isotope-labeled internal standards, namely [<sup>2</sup>H<sub>10</sub>] Leu<sub>B6, B11, B15, B17</sub>-insulin, and [<sup>13</sup>C<sub>6</sub>] Leu<sub>26, 30</sub> C-peptide.

**KEYWORDS**

high resolution mass spectrometry, insulin analogs, mixed mode solid phase extraction, plasma

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## 1 | INTRODUCTION

Insulin is an endogenous peptide hormone having important physiological roles in glucose regulation as well as in energy and anabolic metabolism. C-peptide is produced in equal amounts to insulin from the same single-chain prohormone (proinsulin) and is the best measure of endogenous insulin secretion.<sup>1</sup> While C-peptide is a single-chain linear peptide consisting of 31 amino acids, the structure of insulin is significantly more complex. It consists of two chains ( $\alpha + \beta$ -chain) with three disulfide bridges and contains a total of 51 amino acids (21 in the  $\alpha$ -chain, 30 in the  $\beta$ -chain). Table 1 shows the amino acid sequence of the two hormones. By equimolar production and release from the pancreas, the concentration of insulin and C-peptide commonly correlates, although their plasma half-lives are different. C-peptide is physiologically largely inert, does not undergo hepatic first-pass extraction and therefore persists longer in the circulation, while insulin exhibits a half-life of only a few minutes.<sup>2</sup> The absolute plasma concentrations for both peptide hormones are in the lower ng/mL range depending on the blood glucose level, secretory capacity of the pancreatic beta-cell, and insulin sensitivity.<sup>3</sup> Analytically, these low concentrations in the co-existence of highly abundant plasma proteins (albumin, IgGs, etc.) represent the particular challenge in quantitative analysis.<sup>4</sup> Existing liquid chromatography/mass spectrometry-based assays mainly use immunoaffinity-assisted extraction or other sophisticated isolation techniques for the target peptides from the matrix.<sup>4-9</sup> Other assays using solid phase extraction strongly focus on insulins (without C-peptide) only.<sup>10-12</sup> The immune-enriched sample aliquots are of highly purified quality, enabling the injection into nano-scale liquid chromatographs without the risk of blocking due to residual matrix components. The accomplished specificity of these assays were surpassing, but in terms of quantification, several issues may arise: Due to the limited capacity of the antibodies, the linear range of antibody-based assays is restricted and in the case of co-existing anti-insulin antibodies, which were occasionally described in patients with diabetes receiving exogenous insulin therapy, the extraction by means of techniques employing immunoaffinity interaction is potentially affected.<sup>5</sup> Thus, a solid-phase extraction based assay was developed offering an alternative approach. Herewith, the aforementioned issues influencing the quantitative result interpretation for insulin resp. C-peptide analysis should be avoided. Within the present approach we aimed to determine human insulin and its synthetic analogs (lispro, glulisine, aspart, glargine metabolite, degludec, detemir, porcine, and bovine) simultaneously with C-peptide after sample preparation utilizing a protein precipitation and a mixed-mode cation-exchange solid-phase extraction and subsequent detection by liquid chromatography-high resolution mass spectrometry (LC-HRMS). The method enables the quantification of insulin and C-peptide in physiological non-fasting states with concentrations  $>0.6$  ng/mL. The main characteristics of the target analytes are summarized in Table 1. Two stable isotope-labeled internal standards [ $^2\text{H}_{10}$ ] Leu<sub>B6, B11, B15, B17</sub>-Insulin (human) and [ $^{13}\text{C}_6$ ]

Leu<sub>26, 30</sub>-C-peptide (human) were used to control all sample preparation steps.

## 2 | EXPERIMENTAL

Methanol, acetonitrile, glacial acetic acid, dimethylsulfoxide, formic acid, bovine plasma (EDTA), ammonium hydroxide, porcine insulin, bovine insulin, and [ $^{13}\text{C}_6$ ] Leu<sub>26, 30</sub>-C-peptide (human) were purchased from Sigma (Schnellendorf, Germany). Recombinant human insulin was supplied by Aventis (Kansas City, MO). Solid-phase extraction cartridges OASIS MCX (30 mg, 3 mL) were bought from Waters (Eschborn, Germany). All aqueous buffers and solutions were prepared in MilliQ water. Recombinant human insulin was from Aventis (Frankfurt, Germany). The labeled insulin standard [ $^2\text{H}_{10}$ ] Leu<sub>B6, B11, B15, B17</sub>-Insulin (human) was purchased from PeptaNova (Sandhausen, Germany). For all dilution steps and preparation of aqueous solutions, ultrapure water of MilliQ-quality was used. Insulin analogs lispro (Humalog<sup>®</sup>), aspart (Novolog<sup>®</sup>), glulisine (Apidra<sup>®</sup>), detemir (Levemir<sup>®</sup>), and insulin degludec (Tresiba<sup>®</sup>) were supplied by Eli Lilly (Indianapolis, IN), Novo Nordisk (Princeton, NJ), and Aventis (Kansas City, MO), respectively. The glargine metabolite (DesB31-32 glargine) was obtained from IBA (Warsaw, Poland). This metabolite was shown as the main metabolite of insulin glargine.<sup>13</sup>

### 2.1 | Blood samples

EDTA-plasma samples were collected from healthy volunteers five male, five female; without any known medication prior to sampling, centrifuged and stored frozen until analysis. Additionally, a blood sample from a patient with type 1 diabetes using basal insulin detemir (10 IU/d) was analyzed. Another set of samples was obtained from two adults with class II obesity and pre-diabetes undergoing a 75 g oral glucose tolerance test with frequent blood sampling at time points – 15, 9, 10, 20, 30, 60, 90, 120, and 180 min (ongoing clinical trial, NCT03880162). Written consent from the volunteers and ethical approval from the local ethical committee of the German Sport University (Cologne) was obtained for the study.

### 2.2 | Liquid chromatography

Chromatographic separation of the target analytes was conducted by high performance liquid chromatography (Thermo Vanquish, Bremen, Germany). Solvent buffers A (aqueous solution of 0.1% formic acid) and B (acetonitrile with 1% DMSO and 0.1% formic acid) were used for generating the gradient. As the analytical column, a Poroshell C8 3  $\times$  50 mm (Agilent, Karlsruhe, Germany) was utilized and the flow was set to 400  $\mu\text{L}/\text{min}$ . The gradient started at 99% A and decreased to 40% A within 8 min. In the next 2 min, the gradient decreased further to 20% A for cleaning. Finally, the system was re-equilibrated for 4 min at initial conditions. The resulting overall run time was 14 min, and the injection volume was 20  $\mu\text{L}$ .

**TABLE 1** Main characteristics of all target analytes. Modifications in the amino acid sequence and most abundant product ions are in **bold**. (\*Stable isotope labeled amino acids)

Peptide	Amino acid sequence	Monoisotopic mass (Da)	Precursor (m/z)	Dominant charge state	Product ions	Retention time (min)
Human insulin	GIVEQCCTSI <sup>15</sup> SLYQLENYCN - FVNQHL <sup>13</sup> CGSHLVEALYLVCGERGFF <sup>10</sup> YTPK <sup>1</sup>	5803.6	1452/1162	4+/5+	<b>226, 219, 143, 345</b>	7.03
Insulin aspart	GIVEQCCTSI <sup>15</sup> SLYQLENYCN - FVNQHL <sup>13</sup> CGSHLVEALYLVCGERGFF <sup>10</sup> YTDK <sup>1</sup>	5821.6	1457/1166	4+/5+	<b>226, 219, 248, 464</b>	7.00
Insulin glulisine	GIVEQCCTSI <sup>15</sup> SLYQLENYCN - FVKQHL <sup>13</sup> CGSHLVEALYLVCGERGFF <sup>10</sup> YTPET	5818.6	1456/1166	4+/5+	<b>227, 346, 199</b>	6.96
Insulin lispro	GIVEQCCTSI <sup>15</sup> SLYQLENYCN - FVNQHL <sup>13</sup> CGSHLVEALYLVCGERGFF <sup>10</sup> YTKPT	5803.6	1452/1162	4+/5+	<b>217, 230</b>	7.00
Insulin glargine met	GIVEQCCTSI <sup>15</sup> SLYQLENYCGG - FVNQHL <sup>13</sup> CGSHLVEALYLVCGERGFF <sup>10</sup> YTPK <sup>1</sup>	5746.6	1438/1151	4+/5+	<b>226, 219</b>	7.08
Insulin degludec	GIVEQCCTSI <sup>15</sup> SLYQLENYCN - FVNQHL <sup>13</sup> CGSHLVEALYLVCGERGFF <sup>10</sup> YTPK- $\gamma$ -L-Glu-Pal	6099.8	1527	4+	<b>641, 244</b>	8.30
Insulin detemir	GIVEQCCTSI <sup>15</sup> SLYQLENYCN - FVNQHL <sup>13</sup> CGSHLVEALYLVCGERGFF <sup>10</sup> YTPK-Myr	5912.8	1489	4+	<b>454, 357</b>	9.16
Porcine insulin	GIVEQCCTSI <sup>15</sup> SLYQLENYCN - FVNQHL <sup>13</sup> CGSHLVEALYLVCGERGFF <sup>10</sup> YTPKA	5773.6	1445/1156	4+/5+	<b>226, 315</b>	7.04
Bovine insulin	GIVEQCASVCSLYQLENYCN - FVNQHL <sup>13</sup> CGSHLVEALYLVCGERGFF <sup>10</sup> YTPKA	5729.6	1433/1147	4+/5+	<b>226, 315</b>	6.98
C-peptide	EAEDLQVQVEI <sup>1</sup> GGPGAGSLQPLA <sup>31</sup> EGSL <sup>32</sup> Q	3018.5	1510	2+	<b>927, 260, 785</b>	7.30
Labeled insulin	GIVEQCCTSI <sup>15</sup> SLYQLENYCN - FVNQHL <sup>13</sup> CGSHL <sup>14</sup> VEAL <sup>15</sup> YL <sup>16</sup> VCGERGFF <sup>10</sup> YTPK <sup>1</sup>	5843.9	1462/1170	4+/5+	<b>226, 219, 143, 345</b>	7.01
Labeled C-peptide	EAEDLQVQVEI <sup>1</sup> GGPGAGSLQPLA <sup>31</sup> EGSL <sup>32</sup> Q	3030.6	1516	2+	<b>939, 266, 785</b>	7.29

## 2.3 | Mass spectrometry

A Q Exactive HFX high resolution mass spectrometer (Thermo, Bremen, Germany) equipped with a heated electrospray ion source was used for the analysis of the target peptides. The instrument was operated in positive ionization mode acquiring data in full scan mode ( $m/z = 400\text{--}2000$ , resolution 60 000 FWHM) and targeted single ion monitoring with data dependent MS2 by means of an inclusion list. Targeted single ion monitoring experiments (tSIM) were performed for the multiply protonated precursors of the target peptides and multiplexed four times with an isolation width in the quadrupole of 3 Da. The data dependent MS2 experiments were acquired with a resolution of 15 000 FWHM. As an alternative to the tSIM experiments, product ion experiments (PRM) were also performed for the respective five- or four-fold protonated precursors. The corresponding diagnostic product ions are listed in Table 1. The instrument was calibrated according to the manufacturer's recommendations using a calibration mixture (consisting of caffeine, the tetrapeptide MRFA, and Ultramark). The gas supply consisted of nitrogen ( $N_2$ -generator, CMC, Eschborn, Germany) and was used for all ion source gases and collision gas. Ionization in positive mode was accomplished at a voltage of 2.5 kV, and the temperature of the transfer capillary was adjusted to 550°C. The main characteristics of the target analytes are summarized in Table 1.

## 2.4 | Sample preparation mixed-cation exchange

An ice-cold mixture of 600  $\mu\text{L}$  of acetonitrile/methanol (1:1) was added to 250  $\mu\text{L}$  of plasma in an Eppendorf tube (not low bind). After a short vortexing, the tube was centrifuged for 10 min at  $17000 \times g$ , and the supernatant was transferred to a new 2 mL Eppendorf tube. To this tube, 1.4 mL of acetic acid (1%) was added and vortexed. Solid-phase extraction was performed with mixed-mode cation-exchange cartridges (Waters MCX, 3 mL, 30 mg) which were preconditioned with 1 mL of methanol and 1 mL of water prior to loading with the supernatant from the 2 mL Eppendorf tube. The samples were washed with 2 mL of water and 2 mL of methanol (acidified with freshly prepared 2% of acetic acid). Finally, the sample was eluted to a new 1.5 mL Eppendorf tube with 1.2 mL of a mixture of methanol/ammonium hydroxide solution (5:1), and the volume was reduced to near dryness in a vacuum centrifuge. The dried residue was dissolved in 80  $\mu\text{L}$  of aqueous acetic acid (1%), and 20  $\mu\text{L}$  was injected into the LC-MS system.

The external calibration curve for quantification was prepared using commercial bovine EDTA plasma. This plasma was fortified with all insulin analogs and C-peptide at 0, 0.5, 1, 2, 5, 10, and 20 ng/mL. Aqueous dilutions from the respective stock solutions (1–14 mg/mL) were not stable and were prepared freshly in Eppendorf protein-low bind tubes just prior to fortifying.

## 2.5 | Validation

The validation of the assay was performed for quantitative purposes including the parameter specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, recovery, carry-over, matrix effects, and robustness.

### 2.5.1 | Specificity

The specificity of the method was demonstrated by 10 blank samples (EDTA plasma) from 10 healthy volunteers (5 male, 5 female), who were proven not to have received exogenous insulin. The chromatograms were monitored for interfering signals at the respective retention times.

### 2.5.2 | Linearity

The linearity and the working range of the method were determined by a blank matrix spiked with 0, 0.5, 1, 2, 5, 8, 10, and 20 ng/mL of human insulin, all insulin analogs, and C-peptide. The blank matrix was bovine plasma (EDTA), which was previously tested for the absence of all target analytes inclusive of bovine insulin. The linear approximation was calculated with the respective peak area ratios to the corresponding internal standard.

### 2.5.3 | Precision

The precision or variation coefficient of the method was demonstrated by spiking blank samples at 1 and 5 ng/mL. The precision was computed under consideration of the peak area ratios to the internal standard. This experiment was performed on two different days by different persons (intermediate precision).

### 2.5.4 | Accuracy

In order to enable quantitative interpretations, the correctness of the method was verified by determining possible systematic errors. For this purpose, five spiked blank samples were quantified at three concentration levels (1, 3, and 8 ng/mL) using an external calibration curve in bovine plasma. Quantification was performed considering the peak area ratios using the internal standards and the external calibration curve. This was followed by the measured value with the target value.

### 2.5.5 | Recovery

In order to characterize the losses of analytes during processing, the recovery was determined at 10 ng/mL. For this purpose, six negative

control samples were spiked before processing and six further samples were spiked after processing shortly before injection into the LC-MS. The comparison of the results using the peak area ratios to the internal standard provided information on the recovery of the method.

### 2.5.6 | Carry-over

A possible carry-over of analyte from one sample to the next was investigated by injecting a negative control sample after a spiked sample from the upper working range (20 ng/mL) and inspecting the respective chromatograms for possible carry-over signals.

### 2.5.7 | Matrix effect

The comparison of the signal intensities of five processed negative control samples that were subsequently spiked with all target analytes with a standard sample that did not contain any matrix was used to determine the matrix effects. This strategy is described in detail elsewhere.<sup>14</sup>

### 2.5.8 | Robustness

Fortified samples (at 10 ng/mL) of EDTA-, citrate-, heparin-plasma as well as serum (two samples each) were prepared and analyzed as described above. All these samples were evaluated for potential additional interfering signals at the respective retention times.

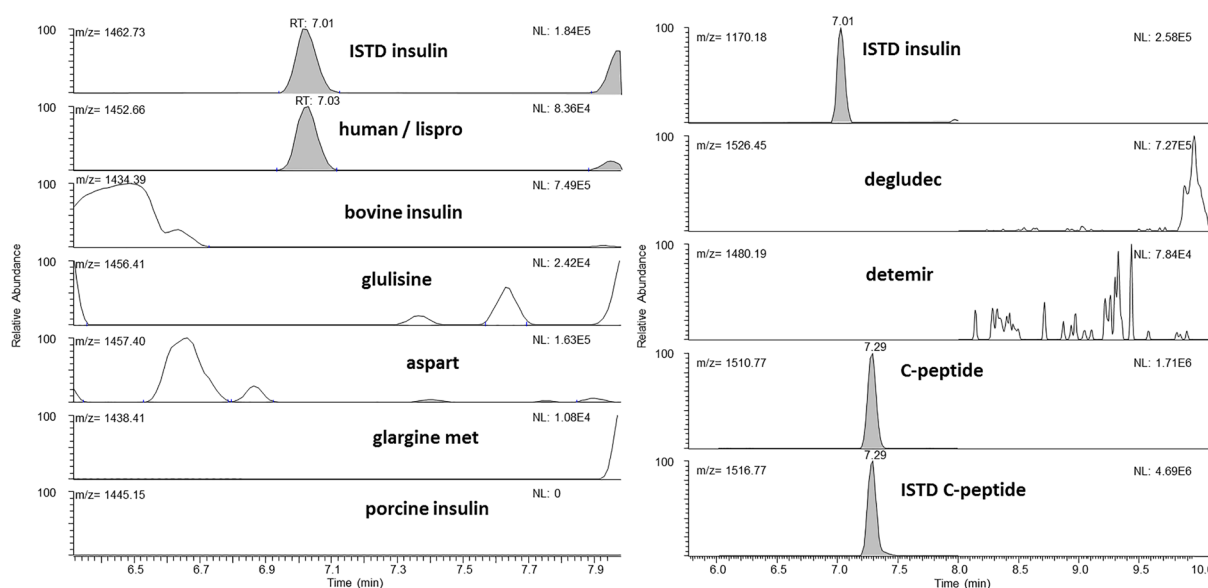
## 3 | RESULTS

### 3.1 | Liquid chromatography/mass spectrometry

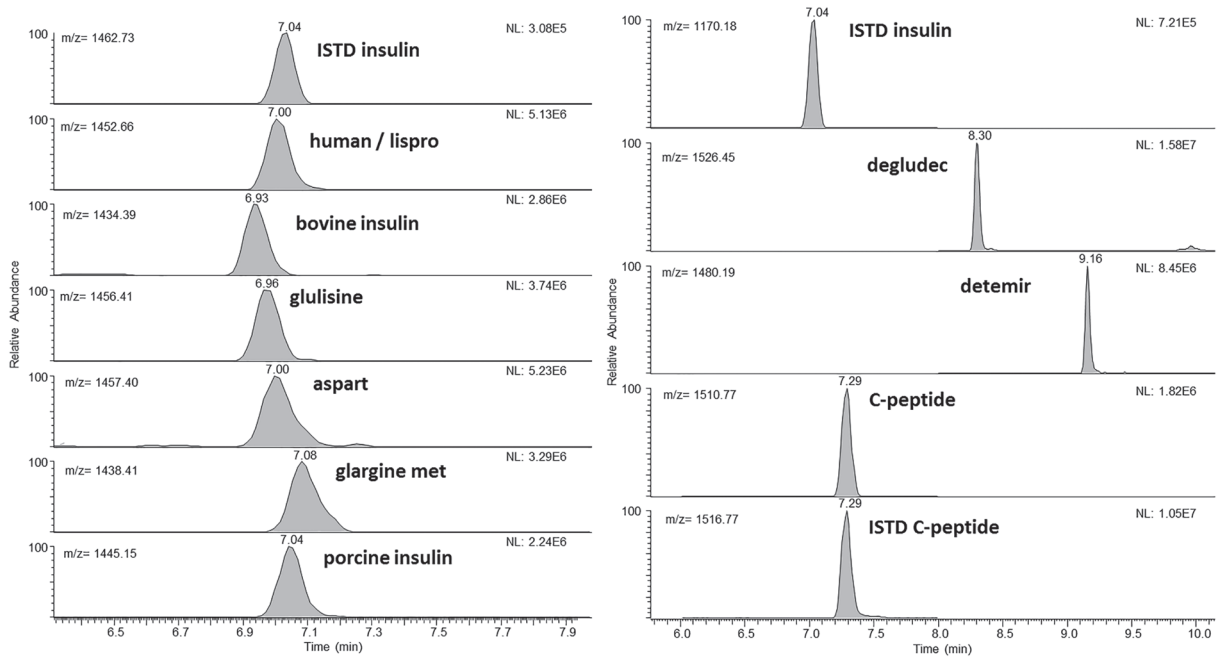
Within the 14 min analytical run, all target analytes were detected resulting in acceptable peak shapes. Figure 1 shows an example of a blank sample with endogenous human insulin and C-peptide only (Figure 1) and a fortified sample at 0.6 ng/mL (Figure 2). As expected, the synthetic analogs comprising a fatty acid (insulin degludec 8.3 min and insulin detemir 9.2 min) eluted significantly later. Noteworthy, although all other insulins exhibited similar retention times, the minor retention time shifts are reproducible and characteristic. This was already shown in former studies.<sup>15</sup> In addition to the diagnostic ion traces in the respective SIM experiments, data dependent MS/MS spectra supported the detection and confirmed the identification of the insulins. This is especially true for the differentiation of human insulin and insulin lispro, which present identical molecular masses. Here the monitoring of the MS/MS experiment at  $m/z$  1162 or 1452 is crucial for all samples. As described earlier, human insulin shows a diagnostic product ion at  $m/z$  226 ( $\gamma_3\text{-}\gamma_1$ ), insulin lispro yields an ion at  $m/z$  217 as the most abundant product ion.<sup>16</sup>

### 3.2 | Validation

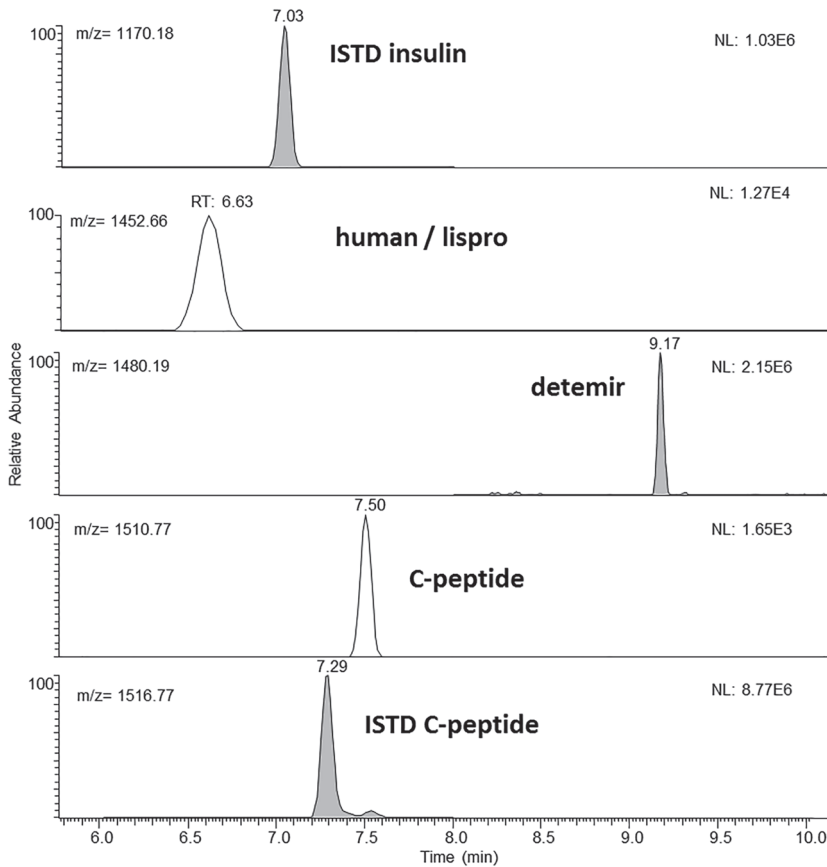
Full method validation was performed for quantitative analysis. The main results are summarized in Table 1. The verification of the specificity demonstrated, when measuring plasma samples of healthy volunteers, that the method showed no interfering signals in the ion traces of the individual analytes at the corresponding



**FIGURE 1** Extracted ion chromatograms of a blank sample from a healthy volunteer showing signals for endogenous human insulin and C-peptide only



**FIGURE 2** Extracted ion chromatograms of a human blank sample fortified at the LOQ with 0.6 ng/mL (except 1 ng/mL for detemir) for all insulin analogs. C-peptide is endogenous and was not fortified



**FIGURE 3** Extracted ion chromatograms of a post administration sample from a patient suffering from diabetes mellitus (type I) with a regular treatment of insulin detemir. Besides the detection of insulin detemir at 9.17 min, no endogenous C-peptide as well as no endogenous human insulin was detected in this sample

retention times (Table 2). Only human insulin and C-peptide were detectable at endogenous levels. The limits of detection ranged at approximately 0.2 ng/mL for all target analytes (only the LODs for insulin detemir and insulin degludec were estimated at 0.5 ng/mL), which is sufficient to monitor normal to non-fasting levels in blood. In six out of six fortified blank samples at 0.2 ng/mL (0.5 ng/mL), the target analytes were detected with a signal-to-noise (S/N) > 3. At the limit of quantification (1 ng/mL), the S/N was > 9 and the coefficient of variation (six-fold determination of fortified blank sample pool) for all target analytes was better than 21%. Within the aimed working range (0–10 ng/mL), linear approximation shows acceptable results with a coefficient of correlation  $r > 0.985$ . The accuracy indicates no systematic bias for the assay with results ranging from 76% to 128% at 1 ng/mL and between 77% and 108% at 5 ng/mL. The recoveries ranged at about 50% (38–57%) for all insulins and at 92% for C-peptide. Under the chosen chromatographic conditions and within the working range (up to 20 ng/mL), no carry-over was observed. Ion suppression or enhancement effects for different blank samples were monitored and resulted in considerable variation of signal intensity at approximately 60–120%. While these effects are ideally compensated for by labeled ISTDs for human insulin and C-peptide, this is also true (to a lesser extent) for all insulins, which elute at about 7 min. Here, a sub-optimal ISTD-compensation is obtained for the late eluting insulin detemir/degludec, yielding significantly higher coefficients of variation (precision), lower coefficients of correlation, and inferior accuracies. The analysis of different sample matrices (EDTA, heparin, citrate or serum) for robustness showed that in principle any of these matrices can be used. The signal intensities showed comparable results for all of these fortified samples.

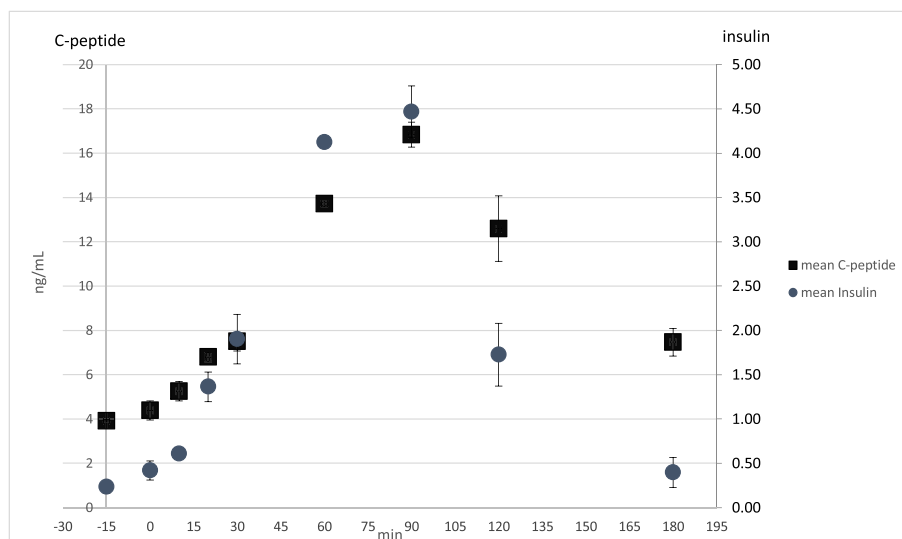
### 3.3 | Proof of concept

In Figure 3, extracted ion chromatograms of a post administration blood sample are shown, which indicate the proof of concept of the method under authentic conditions. These obtained results are in accordance with the patient's daily injection dose of the synthetic insulin analog detemir. Additionally, plasma samples from two adults with class II obesity and prediabetes undergoing an oral glucose tolerance test (oGTT) were measured. Insulin and C-peptide concentrations were calculated with an external calibration curve in bovine plasma at 0, 0.5, 1, 2, 5, and 10 ng/mL for human insulin and 0, 1, 2, 10, and 20 ng/mL for C-peptide. Figure 4 shows the insulin and C-peptide concentrations in ng/mL starting from –15 to 180 min after oGTT for two adults with class II obesity and prediabetes. The basal insulin levels start at 0.2 ng/mL, peak after 90 min at approximately 4 ng/mL, and decrease to 0.5 ng/mL after 180 min. C-peptide starts at about 4 ng/mL, increases to approx. 16 ng/mL after 90 min before decreasing to 6 ng/mL at 180 min. The results presented here are in good accordance with the established data.<sup>17</sup>

**TABLE 2** Main validation results for all target analytes

	Specificity	LOD (ng/mL)	LOQ (ng/mL)	Linearity		0–20 ng/mL		Precision	Accuracy	Recovery	Carry over	Matrix effect
				Intercept	Slope	1 ng/mL (%)	5 ng/mL (%)					
Human insulin	Ok	0.2	0.6	0.2249	0.4786	3.7	4.9	3.7	94.8	57	< 1%	80–120
Insulin aspart	Ok	0.2	0.6	0.0144	0.2988	8.4	6.1	8.4	84.7	48	< 1%	80–120
Insulin glulisine	Ok	0.2	0.6	–0.0384	0.3806	13.0	6.1	13.0	91.6	46	< 1%	80–120
Insulin lispro	Ok	0.2	0.6	–0.0284	0.2613	7.9	6.0	7.9	94.8	42	< 1%	80–120
Insulin glargine metabolite	Ok	0.2	0.6	–0.0853	0.3483	10.7	4.8	10.7	82.3	57	< 1%	80–120
Insulin degludec	Ok	0.5	1.0	0.0056	0.0345	20.6	8.0	20.6	127.7	48	< 1%	80–120
Insulin detemir	Ok	0.5	1.0	–0.008	0.0063	7.3	18.5	7.3	80.9	38	< 1%	60–120
Porcine insulin	Ok	0.2	0.6	0.0483	0.2261	15.9	12.0	15.9	76.0	57	< 1%	80–120
Bovine insulin	Ok	0.2	0.6	–0.0116	0.2538	14.6	6.3	14.6	92.3	51	< 1%	80–120
C-peptide	Ok	0.2	0.6	–0.004	0.0336	13.3	3.6	13.3	94.0	92	< 1%	80–120





**FIGURE 4** Insulin and C-peptide concentrations with standard deviations measured from EDTA plasma samples after oral glucose tolerance test from two volunteers

## 4 | CONCLUSION

Although the obtained results are very promising, there are limitations that must be taken into account in order to avoid misinterpretations and false expectations. For example, it is known that hemolyzed samples (plasma or serum) lead to major problems, which are caused on the one hand by the considerable disturbance of ionization (matrix effects) and on the other hand by the previously described degradation of insulin in the presence of hemoglobin.<sup>18</sup>

Another aspect is the modest specificity of the sample preparation, which in principle enriches all peptidic analytes more or less well from the blood sample. Here, antibody-based sample preparations have considerable advantages and should always be performed additionally to confirm the qualitative presence of the respective insulin.<sup>19,20</sup> The present approach offers advantages in terms of lower costs and simplicity. Furthermore, the aforementioned incompatibility of the method with nano-scale LC systems limits the accomplished sensitivity, which would also be necessary to comprehensively cover fasting states (< 0.2 ng/mL) of insulin in the circulation.

Finally, the quantification performance for insulin detemir and insulin degludec is significantly lower, which is most likely due to the impaired ISTD-compensation for these late eluting acylated synthetic analogs. This was also seen in earlier studies.<sup>5</sup> Whether the yielded results are still in the acceptable range, strongly depends on the aimed requirements. Considering the mentioned limitations, a comparison of this method with earlier published assays showed that the present method is to some extent less sensitive, but by using high resolution mass spectrometry the qualitative (and quantitative) result interpretation is very strong.<sup>5,11,12,21,22</sup> Additionally, the simultaneous determination of C-peptide in the same assay enables a more comprehensive result.

In summary, the method shown here can be used reliably for the quantification of insulin and its synthetic or animal analogs and C-peptide in blood samples (plasma and serum). In comparison with

earlier methods, this simplified sample preparation approach allows for a faster and straight-forward testing method, which shows an improved upper dynamic range in comparison with antibody-based procedures, especially at concentrations in moderate ng/mL levels.

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