A design of experiments concept for the minimization of nonspecific peptide adsorption in the mass spectrometric determination of substance P and related hemokinin-1

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Substance P and hemokinin-1 were predominantly examined by immunoassays with their limitation to differentiate appropriately between both peptides. The use of liquid chromatography coupled with tandem mass spectrometry is a promising, highly selective alternative. Adsorption processes have been identified in preliminary experiments to play a crucial role in the loss of mass spectrometry intensity of both peptides. Therefore, a design of experiments concept was created to minimize nonspecific peptide adsorption. For this purpose, the most critical influencing parameters—(1) the composition of the injection solvent as well as (2) the most suitable container material—were systematically and concordantly investigated. The addition of modifiers, such as formic acid, dimethyl sulfoxide, and organic solvents, to the injection solvent led to a substantial gain of intensity of substance P and hemokinin-1 compared to the start gradient as an injection solvent. Furthermore, the systematic investigation underlined the high impact of the container material, demonstrating polypropylene as the most favorable material. A conjoint injection solvent optimum was found to determine both peptides simultaneously by the conduction of a sweet-spot analysis. The experimental design substantially reduced nonspecific peptide adsorption and enabled the simultaneous and selective determination of endogenous substance P and hemokinin-1 plasma levels.

KEYWORDS adsorption, design of experiments, hemokinin, mass spectrometry, substance P

1 INTRODUCTION

The neurokinin-1 (NK-1) receptor is highly involved in various physiological and pathological processes, like inflammation, pain, emesis, immune response, and tumor progression [1,2]. Its effects are predominantly mediated by the endogenous tachykinins substance P (SP) and hemokinin-1 (HK-1), playing an emerging role in the etiology and progress of inflammatory diseases [3]. Firstly described as a neurotransmitter in the central nervous system, SP recently gained focus as an immune and inflammatory modulator with elevated blood levels in a wide range of preclinical studies. The profound knowledge of the pathomechanism of SP in cell and animal models consequently led to an interest...
TABLE 1 Overview of the amino acid sequence of substance P and hemokinin-1 in the one-letter code and their physicochemical characteristics

|                | Substance P       | Hemokinin-1       |
|----------------|-------------------|-------------------|
| Amino acid     | RPKPQQFFGLM-NH2   | TGKASQFFGLM-NH2   |
| sequence*      |                   |                   |
| Molecular weight [g/mol] | 1347.63   | 1185.43   |
| GRAVY index (Hydrophobicity) | −0.70   | 0.31   |
| Isoelectric point (GenScript©) | 11.66   | 8.86   |
| Charge state at neutral pH | +2   | +1   |

*Identical primary amino acid sequence of SP and HK-1 are marked in bold letters

2 | MATERIAL AND METHODS

2.1 | Chemicals and materials

SP and HK-1 were purchased from Sigma-Aldrich (Darmstadt, Germany) as lyophilized acetic salts in HPLC grade with a purity of 98% for SP and 99% for HK-1. As recommended by the supplier, stock solutions of 1 mg/mL SP and 0.5 mg/mL HK-1 were prepared in water fortified with 0.1% formic acid (FA) and stored in 100 µL polypropylene aliquots at −80°C until analysis. Water (MS grade) was supplied by Honeywell (Erkrath, Germany); ACN, methanol (MeOH), DMSO, FA (all HPLC grade) were acquired from VWR chemicals (Langenfeld, Germany); polypropylene tubes (1.5 mL) were obtained from Eppendorf (Hamburg, Germany) and Sarstedt (Nuembrecht, Germany) in regular and protein low binding quality; glass vials (1 mL) were supplied by Waters (Eschborn, Germany) in regular and deactivated glass quality; regular pipetting tips were acquired from Sarstedt.

2.2 | Instrumentation

LC–MS/MS was performed on an Agilent 1200 series binary pump system (Waldbronn, Germany) coupled to an AB SCIEX API 4000 triple quadrupole mass spectrometer (Darmstadt, Germany). Sample handling was executed by a CTC Analytics HTS PAL system (Zwingen, Switzerland).

2.2.1 | Chromatographic conditions

Samples were injected in full loop mode (20-µL injection volume). SP and HK-1 were separated onto a Waters XSelect CSH™ C18 column (3.5 µm, 3.0 × 150 mm) at an oven temperature of 60°C using a gradient of mobile phase A (water:DMSO:FA, 98.9:1:0.1, v/v/v) and mobile phase B (MeOH:DMSO:FA, 98.9:1:0.1, v/v/v). The gradient started with 5% of mobile phase B for 3 min and was linearly changed to 25% mobile phase B from 3 to 5.5 min. It was followed by a further change to 98% mobile phase B in 0.5 min. The gradient was kept at 98% mobile phase B until another 4 min elapsed before being reduced to 5% of mobile phase B again. The total
TABLE 2  Mass spectrometric settings of substance P and hemokinin-1. V: volt; m/z: mass to charge ratio; MRM: multiple reaction monitoring

|                 | Substance P | Hemokinin-1 |
|-----------------|-------------|-------------|
| Declustering potential [V] | 86          | 107         |
| Entrance potential [V]  | 7           | 10          |
| MRM transitions [m/z]   | 674.6 → 600.5 (quantifier) | 674.6 → 254.2 (qualifier) | 1185.7 → 1037.7 (quantifier) | 1185.7 → 287.1 (qualifier) |
| Collision energy [V]   | 33          | 37          | 57          | 77          |
| Collision exit potential [V] | 16         | 20          | 32          | 22          |

runtime from injection to injection was 13 min. The flow rate was set to 400 µL/min.

2.2.2  | MS

The detection of both peptides was performed in positive mode, utilizing ESI. Nitrogen was used as collision and curtain gas. Collision gas was set to 10 psi, curtain gas to 25 psi, nebulizer gas to 55 psi, heater gas to 75 psi, and ion spray voltage to 5500 V. The temperature in the SCIEX Turbo V™ ion source was held at 550°C. The dwell times for both peptides were set to 125 ms. Detection was executed in multiple reaction monitoring mode using ion transitions at 674.6 m/z to 600.5 m/z for SP and 1185.7 m/z to 1037.7 m/z for HK-1. The multiple reaction monitoring transitions and ion source parameters were optimized during the LC–MS/MS method development by flow injection analysis (Table 2). Data acquisition was performed using Analyst© (SCIEX, version 1.6.2) and Multiquant© (SCIEX, version 3.0.2).

2.3  | DoE

In preliminary experiments [not published], the composition of the injection solvent and the utilized container material had a critical impact on the MS intensity of SP and HK-1, leading to inappropriate limits of detection. Therefore, both critical parameters were jointly investigated in the DoE. MODDE Pro© (MKS Instruments AB, Malmoe, Sweden, version 12.0) was used for planning and analyzing the DoE. Partial least squares regression was applied to generate the model.

2.3.1  | D-optimal design

A D-optimal design as a quadratic model was selected to find the optimal composition of the injection solvent and the most suitable container material. The purpose of the D-optimal design is the minimizing of the generalized variance of the parameter estimates (here: composition of the injection solvent and most suitable container material) [21]. The D-optimal design is a computer-aided design, which is – in contrast to factorial or fractional designs - able to handle constraints in the factor settings of the here-presented model (see therefore the restrictions in factor settings in section 2.3.2). Different to a full factorial design, whereby each combination has to be investigated, a D-optimal design needs only a limited number of experiments, and the results for the omitted experiments were statistically calculated as predicted peak areas. Thus, the D-optimal design allowed for the investigation of a larger selection of process and mixture factors in a limited number of experiments [here: n = 42]. Since the simple addition of an internal standard, which would had mask any increase of mass signal as the internal standard would also be affected by nonspecific peptide adsorption, appeared not reasonable, the possible variability due to electrospray ionization or MS detection was addressed by two aspects: First, each experiment was independently prepared in triplicate and all experiments ran in random order spread over multiple days to calculate the repeatability of each peptide. Second, the center point of the D-optimal design, measured in nonaplicate, ran also in random order, was included to ensure the inter-day repeatability. SP and HK-1 were separately investigated to avoid possible adsorption interactions of the two peptides, resulting in a total of 270 runs for both peptides. The responses were set to the peak areas of SP and HK-1, as the aim of this investigation was the maximization of the MS intensity.

A cross-validation of the obtained model was performed based on the predictive error sum of squares value. The predictive error sum of squares value was expressed as Q², which was reserved as a parameter for the goodness of prediction. The predefined requirement was a value of at least 0.5 for Q² to generate an appropriate model with a good predictive power [22]. Furthermore, the difference between R² and Q² should be less than 0.3 for a reliable model.

2.3.2  | Investigated parameters

Composition of the injection solvent

Four quantitative parameters (MeOH, ACN, DMSO, and FA) considered as additives to the injection solvent were investigated in the DoE, based on their ability to improve the solubility (organic solvents, DMSO) [23] and to reduce ionic interactions (FA) [20]. The investigated ranges started from the absence of each component (0%) and were limited to 10% of FA, 75% of DMSO, and 50% of either MeOH or ACN. The resulting injection solvent consisted of a composition of a maximum of three additives to water, whereby the water
fraction in the injection solvent was set to a minimum of 25% to guarantee sufficient solubility of SP. The organic fraction was restricted to 50% to avoid breakthroughs and peak distortions in the reversed phase chromatography, while the FA fraction was limited to 10% to evade corrosive damages in the sensible parts of the HPLC system. The addition of TFA to the injection solvent was abandoned, as TFA led to strong ion suppression in preliminary experiments.

**Consumables**

Furthermore, the variable composition of the injection solvent was conjointly examined in different container materials. Polypropylene tubes from two suppliers, each in distinct quality (regular and protein low binding) and regular glass vials were included in the DoE to investigate the adsorption affinity of SP and HK-1 to the material surfaces. Additionally, the benefit of using deactivated glass vials was examined and compared to the best performing container material of the DoE approach.

### 2.3.3 Preparation of experimental solutions

For analysis, freshly thawed stock solutions were consequently diluted in a generic mixture of 2% FA added to equal parts of ACN and water (50:50 v/v) as recommended to avoid non-specific peptide adsorption [12]. An amount of 10 µL of either 0.1 µg/mL SP solution or 1 µg/mL HK-1 solution was evaporated to dryness in the investigated test containers for 10 min at 40°C and 300 rpm under a gentle steam of nitrogen. The residual was reconstituted in 1 mL of the experimental solution to obtain a final concentration of 1 ng/mL SP and 10 ng/mL HK-1 and was stored on ice until same-day measurement by LC–MS/MS.

### 2.3.4 Sweet-spot and setpoint analysis

A sweet spot was created to find the optimal composition of the injection solvent, which depended on the best performing container material for SP and HK-1, when measuring both peptides simultaneously. Especially in simultaneous determination of compounds with opposite physicochemical properties, the sweet-spot analysis allows to identify the intersecting set for optimal quantification in bioanalysis. The focus in the sweet-spot analysis lied on the predicted peak area of SP, as reported endogenous blood levels were much lower than blood levels of HK-1 [24]. The sweet spot was defined as injection solvent composition, in which a maximally accepted deviation to the maximal predicted peak area of both SP and HK-1 must not exceed the predefined limits (conjoint optimal conditions for both peptides). Therefore, the predefined acceptable limits to the maximal predicted peak area were set to −5% for SP and −15% for HK-1.

To confirm the accuracy of the sweet-spot as conjoint optimal injection solvent composition, a minimal-risk analysis was performed. The minimal-risk analysis bases on the Monte-Carlo simulation and indicates the probability of failing the desired peak area, which is predicted by the model. By taking into account the response specifications, the standard error of the model and the factor precision, the predicted peak areas were simulated one million times and the probability of failure was determined. In the here-presented model, failure was defined as a loss of more than 15% of the maximal predicted peak area for both peptides. This requirement was derived from regulatory bioanalytical recommendations [25]. The area with a probability of failure of less than 0.5% (5000 defects per million simulations) was defined as the design space, involving the robust setpoint.

### 2.4 Application of the experimental design

As one of the objectives of this study was the determination of endogenous plasma levels of both peptides, the chromatographic method was also optimized to facilitate the reduction of the LOD of both peptides and to enable the determination of endogenous peptide levels. First, 5% instead of 1% DMSO were added as supercharger to both mobile phases to improve the electro spray ionization. Second, 50-µL instead of 20-µL injection volume was utilized to increase the signal intensity during the applicability experiments. Under these conditions, the benefit of using the optimized injection solvent composition in the most suitable container material was further evaluated in human plasma samples, as the presence of endogenous plasma matrix components could also affect adsorption processes. The degradation of the endogenous peptides was induced at ambient temperatures to generate blank plasma (donated by one male volunteer) [6]. Afterwards, 300 µL of fresh blank plasma was spiked with 1 ng/mL SP and HK-1. As endogenous SP plasma levels, determined by immunoassays, were found in the picomolar range (median: 217.8 pg/mL in healthy children \( n = 80 \) [26]; mean ± SD: 116.5 ± 20.5 pg/mL in healthy adults \( n = 110 \) [27]), blank plasma samples were further spiked with 100 pg/mL SP and HK-1, as low-concentrated samples are more critically affected by adsorption processes.

Spiked plasma samples were purified with a customized SPE protocol using a mix-mode weak anion exchanger (Oasis WAX μelution, Waters). SP and HK-1 were eluted by 125 µL of MeOH fortified with 10% FA, evaporated to dryness at 35°C and 300 RPM under nitrogen, and were reconstituted in both (A) the optimized solvent composition in the most suitable container and (B) the start gradient as injection solvent (93.9% water, 5% MeOH, 1% DMSO, 0.1% FA) in regular tubes. The mean observed peak areas of SP and HK-1 in settings A and B \( n = 3 \) at different concentration levels (1 ng/mL and 100 pg/mL) were compared to evaluate the benefit of the experimental design in human plasma samples.
3 | RESULTS AND DISCUSSION

3.1 | Model fit and goodness of prediction

The goodness of fit ($R^2$) was determined by an observed-versus-predicted peak area plot, resulting in an $R^2$ of 0.92 for SP and 0.88 for HK-1 (Figure 1). The goodness of prediction is expressed by the $Q^2$ value and amounted to 0.68 for SP and 0.73 for HK-1, fulfilling the predefined requirements for a predictive model.

As the addition of an internal standard was not reasonable, each replicate of an experiment ran in a random order spread over five days to ensure the robustness of the analytical setting. The calculated repeatability—based on the variance of the replicates over the whole study period—amounted to 92% and 91.9% for SP and HK-1, respectively. Moreover, the evaluation of the center point demonstrated robust mass signals (mean ± SD: 2545.6 ± 518.5 counts per second (cps) for SP and 2317.9 ± 310.4 cps for HK-1 (each peptide; $n = 9$)), indicating stable and repeatable analytical conditions during the experimental conduct.

3.2 | Optimization of the injection solvent composition

3.2.1 | Impact of the type of organic solvent

The gain of intensity did not only depend on the percentage but, moreover, on the type of organic solvent. Regardless of the container material, the predicted peak areas for SP and HK-1 generally profited from adding organic solvents to the injection solvent, whereby predominantly adding 25% MeOH led to substantial increases of predicted peak areas (+69.1% for SP and +69.4% for HK-1) compared to the absence of organic solvent. With a higher fraction of 50% MeOH, the increase of intensity was not as pronounced compared to adding 25% MeOH (+5.7% for SP and +0.1% for HK-1). Contrarily, by adding 25% ACN to the injection solvent, the predicted peak areas were increased by 27.8% for SP and by 63.2% for HK-1 compared to the absence of organic solvent. Furthermore, by adding 50% ACN to the injection solvent, the predicted peak areas were decreased by 20.3% for SP and 17.4% for HK-1 compared to the addition of 25% ACN. Therefore, ACN was classified as inferior in this setting for both compounds of interest. SP, as a hydrophilic peptide, seems to be more soluble in MeOH as a polar protic solvent, leading to less nonspecific adsorption. For HK-1, as a hydrophobic peptide, the benefit of adding MeOH or ACN to the injection solvent was almost equivalent.

3.2.2 | Impact of dimethyl sulfoxide

The predicted peak areas of the tachykinins benefited from distinct maximal fractions of DMSO (45% for SP, 60% for HK-1). The conjoint investigation of the impact of DMSO and the used container material led to substantial differences in benefit of adding DMSO to the injection solvent composition. In regular polypropylene tubes an increase of 91.8% for the predicted peak area of SP and 63.9% for the predicted peak area of HK-1 was achieved. In contrast, the predicted peak areas of SP and HK-1 were increased by 34.8% and 36.9% respectively, using protein low binding polypropylene tubes. In glass vials the benefit of adding DMSO was pronounced for the predicted peak area of SP (+209.6%), whereby the benefit for the predicted peak area of HK-1 was limited (+26.0%).

As a strong solubilizer in the injection solvent, DMSO plays a critical role of minimizing nonspecific adsorption of the tachykinins to the container material (Figure 2). The substantial differences between the investigated container materials led to the assumption that the principal function of DMSO in the injection solvent was the reduction of nonspecific peptide adsorption by improving the peptide solubility. Coherently, the reduction of nonspecific peptide adsorption.

**FIGURE 1** Goodness of fit plot showing observed versus predicted peak areas of substance P (blue) and hemokinin-1 (red) with the identity line (solid red line) and the regression lines (dashed black lines); cps: counts per second; SE: standard error
by DMSO was previously shown for digested peptides, which underline the here presented results [23]. On the other hand, the possible impact of DMSO on the ESI cannot be ruled out but seems to be inferior with regard to the identified high variability in distinct container materials (+209.6% in glass vials compared to +34.8% in protein low binding polypropylene for the predicted peak area of SP). If DMSO primarily acted as a super charger for ESI, less extent of variability between the different materials would have been expected. Due to the potential of DMSO to oxidize methionine residuals of both peptides, the use of DMSO as injection solvent composition requires critical evaluation. However, no degradation during short-term investigations over 12 h was observed under the here presented experiments.

### 3.2.3 | Impact of FA

The addition of FA to the injection solvent was one of the essential ways to optimize the signal intensity of both peptides (Figure 2). Regardless of the container material, the addition of 10% FA to the injection solvent resulted in a maximum gain of the predicted peak area of SP (+85.8%). The maximum of the predicted peak area of HK-1 was achieved by the addition of 8.5% FA (+58.0%). Contrary to SP, a higher FA fraction showed no further benefit for the gain of the MS intensity of HK-1.

The observed peak areas in regular polypropylene and glass containers were reduced by more than 97% for SP in the absence of FA (mean ± SD: 16.8 ± 29 cps in pure aqueous injection solvent versus 634.3 ± 140.1 cps in pure aqueous solvents fortified with 10% FA (n = 9)) and by more than 80% for HK-1 in absence of FA (mean ± SD: 203.8 ± 106.4 cps in pure aqueous injection solvent versus 1088.9 ± 206.0 cps in pure aqueous solvents fortified with 10% FA (n = 9)). In contrast, the comparison in protein low binding polypropylene led to an increase of 48.2% of observed peak area of SP (mean ± SD: 2612.3 ± 577.3 cps in pure aqueous solvents fortified with 10% FA versus 1763.2 ± 144.4 cps in pure aqueous injection solvent (n = 6)) and 76.8% of observed peak area of HK-1 (mean ± SD: 1991 ± 274.8 cps in pure aqueous solvents fortified with 10% FA versus 1126.4 ± 263.2 cps in pure aqueous injection solvent (n = 6)), indicating fewer nonspecific peptide adsorption of SP and HK-1 in aqueous solvents using protein low binding polypropylene tubes.

Under acid conditions, the affinity of the positively charged tachykinins to negatively charged glassware was lower than in neutral solutions probably due to the saturation of the free silanol groups by protons. Moreover, the building of a more soluble FA cluster with SP and HK-1 seemed to avoid further adsorption processes. As an acid building a cluster with positively charged molecules, FA has a high potential to reduce nonspecific adsorption. The results of the experimental design suggested that more hydrophilic peptides like SP profit from a higher fraction of FA to adsorb less to the material surfaces.

Although TFA is often used in chromatographic separations due to the strong cluster building, it also leads to strong ion suppression in MS applications [28]. In preliminary experiments, the addition of 0.25% TFA to the injection solvent led to a substantial decrease of peak area of SP (~72.2%). Moreover, the ion suppression was not only observed in runs with TFA added to the injection solvent, but also in consecutive runs without TFA added to the injection solvent. Since a gain of intensity was the principal objective of the experimental design, the use of TFA was abolished in the DoE to avoid extensive chromatographic wash procedures between each run.

### 3.3 | Impact of the container material

Besides the selection of modifiers as well as the optimal composition of the injection solvent, the container material used played an essential role in the accurate measurement of SP and HK-1 (Figure 3).

#### 3.3.1 | Glass vials
As expected from the literature [16], regular glass vials were unsuitable for measuring SP sensitively. It is most likely that the two positive charges of arginine and lysine in the amino acid sequence of SP showed high affinity to the untreated negatively charged silanol groups of glassware, resulting in a substantial loss of predicted peak area (~71.6% compared to regular polypropylene tubes). HK-1, as a more hydrophobic and single-charged peptide, tolerated regular glassware better, which was principally suitable for the determination of HK-1 (+10.8% compared to regular polypropylene tubes) (Figure 3C).
Deactivated glass vials promised less adsorption of positively charged peptides compared to regular glassware by capping the free negatively charged silanol groups of glass. Therefore, the use of deactivated glass vials was independently investigated by comparing to untreated glass vials and regular polypropylene tubes both in the optimized injection solvent in triplicates. The peak areas of SP substantially benefited from the use of deactivated glass vials compared to untreated glass vials (+66%). Nevertheless, the mean observed peak area in deactivated glass vials were lower with a higher standard deviation than in regular polypropylene tubes (mean ± SD: 4358.7 ± 797.3 cps versus 5174.7 ± 234.8 cps (n = 3)). On the other side, the observed peak areas of HK-1 showed bare differences in deactivated glass vials compared to regular polypropylene tubes (mean ± SD: 3410 ± 462 cps versus 3519.3 ± 136.3 cps (n = 3)). In contrast to regular and deactivated glass vials, regular polypropylene tubes were the most suitable material to measure SP accurately and sensitively.

### 3.3.3 Protein low binding polypropylene tubes

Protein low binding polypropylene tubes promise fewer adsorption of peptides and proteins based on a distinct manufacturing method resulting in high-density, fine-pored polypropylene with a minimized hydrophobic interaction potential. Protein low binding polypropylene tubes from both suppliers showed the most considerable advantage in pure aqueous solutions compared to regular polypropylene tubes (+329.7% and +115.1% for SP and HK-1, respectively). As nonspecific peptide adsorption was minimized in protein low binding tubes, there was no further profit in the addition of MeOH to the injection solvent. The predicted peak areas of HK-1 profited from a maximum of 30% MeOH using protein low binding tubes, whereby the advantage was not as substantial as that in regular polypropylene tubes (+35.2% in protein low binding polypropylene versus +81.3% in regular polypropylene). The predicted peak areas of both peptides in protein low binding polypropylene tubes were not much affected by the composition of the injection solvent as compared to regular polypropylene tubes and were constant over a considerable range of variable compositions of injection solvent (Figure 3A). Contrary to regular polypropylene tubes, in protein low binding tubes, a maximum of the predicted peak area of SP was predominantly achieved in an injection solvent composition of equal parts of DMSO and water fortified with 10% FA. For HK-1, differences in predicted peak areas were not distinctive in protein low binding compared to regular polypropylene tubes.
3.4 | Sweet-spot and setpoint analysis

Focusing on the maximum gain of intensity for SP and HK-1, the sweet-spot analysis was performed to evaluate the optimal composition of the injection solvent when measuring both peptides simultaneously. The minimal-risk analysis was executed to confirm the sweet-spot, resulting in the first design space including a robust setpoint of injection solvent composition of 50% MeOH, 15% DMSO, 25% water, and 10% FA in regular polypropylene tubes (Figure 4A).

As protein low binding tubes were substantially advantageous in non-organic compositions of injection solvents, an alternative robust setpoint was included to evaluate their benefit compared to regular polypropylene tubes. On the basis of the alternative setpoint, a separate minimal-risk analysis was executed resulting in the second design space with an injection solvent composition consisting of 45% DMSO, 45% water, and 10% FA in protein low binding polypropylene tubes (Figure 4B).

The mean values of the observed peak areas of the investigated tachykinins in triplicates dissolved in (A) the start gradient (93.9% water, 5% MeOH, 1% DMSO, 0.1% FA), (B) the optimized solvent in regular polypropylene tubes, and (C) the optimized solvent in protein low binding polypropylene tubes were compared to evaluate the benefit of the DoE. Predominantly, the observed peak areas of SP profited from the optimization and resulted in an approximately 135% higher intensity and lower standard deviation in both optimized injection solvents (mean ± SD: (A) 2203.5 ± 748.4 cps versus (B) 5174.7 ± 234.7 cps and (C) 4926.7 ± 426.0 cps (n = 3)).

For HK-1, a gain of intensity of 33% was achieved (mean ± SD: (A) 2628.0 ± 216.4 cps versus (B) 3519.3 ± 136.3 cps, and (C) 3456 ± 72.3 cps (n = 3)). The differences in the hydrophobicity and charge state seemed to be crucial for the effect of optimization. SP, as a more hydrophilic peptide, was affected by higher adsorption processes in unmodified injection solvents due to stronger ionic interactions to the material surfaces than HK-1. There were no substantial differences in performance identified by comparing both optimized injection solvents in protein low binding and regular polypropylene tubes.

As both robust setpoints were equivalent regarding the observed peak areas, the use of the optimized solvent in regular polypropylene tubes (50% MeOH, 15% DMSO, 25% water, 10% FA) was abandoned in further experiments to avoid breakthroughs using a 50-µL injection volume due to high organic fraction.

The sensitivity of the method was investigated under the optimized conditions, which were obtained by the DoE. Thus, a dilution series of SP and HK-1 was prepared in the optimized solvent composition (45% DMSO, 45% water, 10% FA) in protein low binding polypropylene tubes and was measured in triplicate. LOQ of 20 pg/mL for SP and of 40 pg/mL for HK-1 in compliance with regulatory guidelines [25] were achieved by using 50-µL injection volume.

3.5 | Application in human plasma samples

Analogically to the neat solution, the observed peak areas of 1 ng/mL SP—spiked in human plasma samples—predominantly benefited from (A) the optimized solvent in protein low binding (45% DMSO, 45% water, 10% FA) resulting in an increase of intensity of 74% compared to (B) the start gradient as the injection solvent in regular tubes (93.9% water, 5% MeOH, 1% DMSO, 0.1% FA) (mean ± SD: (A) 20 943.3 ± 2630.9 cps versus (B) 11 983.0 ± 1835.3 cps (n = 3)). The substantial increase of signal intensity between neat solution and spiked plasma (2203.5 cps versus 11 983 cps) based on the combined effects of all identified optimization potentials during the here presented investigations (50-µL injection volume and optimized chromatographic conditions (e.g. 5% DMSO)). The comparison of the observed peak areas of 1 ng/mL HK-1, spiked in human plasma samples, resulted in an increased intensity of 15% (mean ± SD: (A) 3510.4 ± 775.4 cps versus (B) 3051.3 ± 216.9 cps (n = 3)). Plasma matrix components may also reduce adsorption processes by likely concurring with the investigated tachykinins, resulting in a lower
FIGURE 5 Representative stacked chromatogram of substance P (blue) and hemokinin-1 (red) under optimal conditions in human plasma. Plasma was spiked with 100 pg/mL of each peptide (solid lines). For comparison, the start gradient composition in standard containers is presented in dashed lines and non-spiked plasma in dotted lines; cps: counts per second

The benefit of observed peak areas compared to the results in the neat solution (Section 3.4) No substantial matrix effect was observed in the here presented experiments.

In physiological relevant endogenous levels (100 pg/mL [26,27], the comparison of the setpoint composition to the start gradient as an injection solvent resulted in an increase of intensity of 107.5% for SP (mean ± SD: (A) 2433.8 ± 616.1 cps versus (B) 1172.8 ± 162 cps (n = 3)) and enabled the determination of 100 pg/mL HK-1 (mean ± SD: (A) 378.4 ± 100.4 cps versus (B) 177.4 ± 94 cps (signal to noise < 10:1) (n = 3)) in human plasma samples (Figure 5). As expected, due to the more pronounced adsorption effects in low-concentrated samples, the setpoint composition was essential for the sensitive determination of endogenous tachykinin concentrations.

3.6 | Reliability of determined substance P levels

SP plasma levels were significantly distinct between diseased and healthy subjects, and therefore could be a potential biomarker to support a diagnostic or monitor the progress of pathological events. However, measured SP plasma levels showed high variability between clinical studies [8]. Variability was known to be caused by distinct analytical methods and different sampling procedures. The results of the DoE further indicated that adsorption processes account for another reason for the high variability of SP plasma levels. The used container materials possessed a high impact on the adsorption extent of SP and were rarely mentioned in investigated SP plasma levels. Predominantly, the use of regular glassware in sampling and measuring may result in significantly lower reported SP levels. Therefore, for accurate determination of SP, the pronounced adsorption behavior has to be concerned.

Furthermore, with the discovery of HK-1 in 2000, a tachykinin with a similar amino acid sequence (match of 63.6%) targeting the same receptor as SP, more specific analytical methods than immunoassays are required to differentiate accurately between SP and HK-1 levels. The role of both peptides has to be re-evaluated, and therefore the selective and simultaneous determination is a crucial step to obtain non-biased concentrations of SP and HK-1 for a better insight into the complex tachykinin system.

4 | CONCLUDING REMARKS

Nonspecific peptide adsorption as a major reason for a lack of MS intensity of SP and HK-1 was successfully addressed by the presented DoE concept. The results should encourage other scientists working with peptides to implement concepts to investigate and minimize unspecific peptide adsorption for the sensitive determination of low abundant peptides by LC–MS. For the here investigated peptides, a high extent of DMSO and FA substantially reduced adsorption processes, resulting in an injection solvent composition of 45% DMSO, 45% water and 10% FA. By combining the aforementioned solvent composition with the most suitable container material (protein low binding polypropylene), low picomolar concentrations of SP (20 pg/mL) and of HK-1 (40 pg/mL) were detectable. Thus, the experimental design facilitated the simultaneous and sensitive determination of both peptides in human plasma samples by LC-MS/MS to overcome the current limitations by immunoassay determination.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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