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RESEARCH ARTICLE

Characterization of Vasoactive Intestinal Polypeptide with LC-MS/MS Method

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SUMMARY

Neuropeptides are peptides used by neurons for communication. Vasoactive intestinal polypeptide (VIP) is a polypeptide belonging to the glucagon/secretin family obtained from the hypothalamus region of the brain. In this study, VIP was analytically characterized and examined using a sensitive Liquid Chromatography-Mass Spectrometry (LC-MS/MS) method. For the analysis of VIP, chromatographic (such as separation column, organic solvent and acid content of the mobile phase, flow rate) and mass spectrometric (observation of precursor ions and fragments of peptides, fragmentation voltage, collision energy) parameters were examined. In the analysis, 799.5 and 770.7 m/z fragment ions were observed, consisting of the 665.9 (M^{5+}) m/z precursor ion for VIP. Moreover, the VIP adsorption problem that emerged during the analyses was investigated, and factors preventing adsorption were tested using LC-MS/MS analyses. Additionally, the effect of albumin solution on the analyses as a barrier to the adsorption of the peptide to the surface was investigated. As a result of these studies, the suitability of the application has been demonstrated in that the masses and fragments of the polycharged species of VIP in the presence of adsorption can be observed with the developed LC-MS/MS method.

Key Words: *Vasoactive intestinal polypeptide (VIP), LC-MS/MS, peptide adsorption.*

Vazoaktif Intestinal Polipeptitlerinin LC-MS/MS Yöntemi ile Karakterizasyonu

ÖZ

Nöropeptitler, nöronlar tarafından iletişim için kullanılan peptitlerdir. Vazoaktif intestinal polipeptid (VIP), beynin hipotalamus bölgesinden elde edilen glukagon/sekretin ailesine ait bir polipeptittir. Bu çalışmada VIP, analitik olarak karakterize edilmiş ve hassas Sıvı Kromatografisi-Kütle Spektrometrisi (LC-MS/MS) yöntemi ile incelenmiştir. VIP analizi için kromatografik (ayırma kolonu, organik çözücü ve hareketli fazın asit içeriği, akış hızı gibi) ve kütle spektrometrik (peptit öncül iyonlarının ve parçalarının gözlenmesi, parçalanma voltajı, çarpışma enerjisi gibi) parametreler incelenmiştir. Analizde VIP için 665.9 (M^{5+}) m/z öncül iyon ve 799.5 ve 770.7 m/z parça iyonları gözlenmiştir. Ayrıca analizler sırasında ortaya çıkan peptidin adsorpsiyon probleminin kaynakları araştırılmış ve LC-MS/MS analizlerinde adsorpsiyonu engelleyen faktörler test edilmiştir. Ek olarak, albümin çözeltisinin peptidin yüzeye adsorpsiyonuna bir bariyer olarak analizler üzerindeki etkisi araştırılmıştır. Bu çalışmalar sonucunda, geliştirilen LC-MS/MS yöntemi ile adsorpsiyon varlığında VIP'nin çoklu yüklenmiş türlerinin kütlelerinin ve parçalarının gözlenebilmesi bakımından uygulamanın uygunluğu gösterilmiştir.

Anahtar Kelimeler: *Vazoaktif intestinal polipeptit (VIP), LC-MS/MS, peptit adsorpsiyonu.*

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INTRODUCTION

Neuropeptides have long been known as chemical signals in the brain. They are small protein-structured substances that function on neural surfaces and are spread by neurons through regular secretion. These are the most diverse class of signaling molecules that have many physiological functions in the brain (Burbach, 2011). Studies have proven that peptides are bioactive compounds throughout the body (Sewald & Jakubke, 2002).

According to the gastroenteropancreatic peptide classification made by Rehfeld (Rehfeld, 1998), vasoactive intestinal polypeptide (VIP) is among the peptides in the secretin group. It is a neuropeptide containing 28 amino acids that is very common in the central and peripheral nervous system (Watanabe, 2016).

VIP is produced in many tissues in the brain, such as the suprachiasmatic nucleus of the hypothalamus, pancreas, and intestine. In addition to its many general functions, it increases glycogenolysis, lowers arterial blood pressure, strengthens the immune system without causing an autoimmune reaction, prevents apoptosis in brain cells, has neuroprotective effects, and has antioxidant properties. Due to these properties, its protective and therapeutic effects point out in a wide variety of pathologies such as septic shock, hemorrhagic shock, ischemia-reperfusion injury, Parkinson's disease, and Alzheimer's disease (Tunçel & Korkmaz, 2010).

When the literature on VIP is reviewed, numerous medical, physiological and pharmacological studies have been exited since it is a neuropeptide. These are studies primarily aimed at the diagnosis and treatment of diseases; however, there are limited analytical studies. It is believed that this might be due to the very low concentrations of neuropeptides in the brain, the challenges faced in peptide analysis (like surface adsorption), and the absence of a method that can effectively analyze them.

Recently, chromatography, isoelectric point-electrophoresis, and their combination methods have been used for peptide analysis. Immunocytochemistry, radioimmunoanalysis (RIA), enzyme-linked immunosorbent analysis (ELISA), and mass spectrometry (MS) are other methods that provide information about the determination and distribution of neuropeptides (Soloviev & Finch, 2005). A summary of the studies in the literature using these and combined methods is given in (Table 1).

Table 1. Research for VIP Analysis in Literature

Analysis Method	Analyte	Sample	Linear Range	LOD	LOQ	References
HPCE	VIP	Rat brain	$1x10^{-6}$-$5x10^{-4}$ M	~10^{-6} M	NA	Soucheleau & Denoroy, 1992
CE-LIF	VIP	Tissue	10-800 pg	5 pg/mL	NA	Phillips, 1998
RIA	VIP and PACAP	-	NA	NA	NA	Wasilewska-Dzubinska et al., 2002
HPLC	Neuropeptide Y and VIP	Gingival Tissue	19-1000 pg/mL	NA	NA	El-Karim et al., 2003
ESI-QTOF-MS	CGRP and VIP	-	1-1000 ng/mL	NA	NA	Abaye et al., 2011
RIA	VIP and PACAP	-	NA	NA	NA	Hansen et al., 2013
HPLC	VIP	-	9-150 µg/mL	360 ng/mL	900 ng/mL	Cui et al., 2013
CE-MS	VIP and PACAP	-	1-200 ng/mL	1 ng/mL	NA	Lock et al., 2015
HPLC	VIP, Glutamate and GABA	-	NA	NA	NA	Korkmaz et al., 2021

*NA: No application

Since neuropeptides exist in extremely low amounts, they are usually analyzed by methods that require high sensitivity, excellent selectivity, and a wide dynamic detection range (Yin et al., 2011). The MS method is indispensable for the identification of the peptide (Baggerman et al., 2004). It is possible to determine the main mass by MS analysis and to observe the masses of fragment ions belonging to this mass by MS/MS. The LC-ESI-MS/MS or LC-MALDI-MS/MS methods are generally used for these studies.

The feature that makes MS analysis of proteins/peptides possible is that their structures are not disrupted by methods such as MALDI/ESI during evaporation/ionization. The ESI method enables multiple charging of the same peptide in an MS spectrum, allowing the use of mass analyzers with a limited m/z range for peptide analysis, and provides more accurate molecular weight calculations through various calculations (Trauger et al., 2002).

There are also several challenges encountered during peptide/protein analysis, as well as factors that limit the analysis. Because of the polar, nonpolar, and charged parts in the structures of these substances, they are adsorbed to silica by hydrogen bonding or ionic interaction (Baker, 1995). Therefore, the choice of materials used during sample preparation is critical. Given that they are generally glass and polypropylene/polyethylene, adsorption may occur depending on these surfaces and vary depending on the material. Additionally, adding organic, or nonionic surfactant to the medium and keeping the medium acidic, basic or neutral helps prevent adsorption.

This study aimed to develop an analytical method that is as sensitive, simple, and fast as possible, to characterize VIP using a highly sensitive technique such as LC-MS/MS, and to refine the analysis method.

MATERIALS AND METHOD

Chemicals

Albumin (>98%), acetonitrile (HPLC purity), formic acid (~98% for LC-MS), methanol (HPLC purity), ultra-pure water, reference standard (≥ 95%) of VIP were purchased from Sigma-Aldrich (Germany), acetic acid (100%) was purchased from Merck (Germany).

Instruments

The analysis column used in LC-MS/MS studies, Ascentis Express Peptides, ES-C18, 100×2.1 mm, 2.7 µm, was obtained from Supelco, Sigma-Aldrich (Germany). Sartorius Stedim Biotech distilled water instrument, Arium Pro VF (Germany); Excellence Plus XP-205 model, Mettler Toledo (Switzerland) analytical balance; Mettler Toledo Seven Compact pH/ion meter S220 (Switzerland) pH meter; 1290 LC system and 6460 Triple Quad MS system Agilent Technologies (USA), Bandelin Electronic RK510 H (Germany) ultrasonic bath and Jeio Tech VM 96 B (Korea) vortex mixer are other instruments used in the studies.

Solvent Selection and Preparation of Stock Solutions

To facilitate the ionization of VIP during the analysis, 1% acetic acid (AcAc) was chosen as the solvent. The referance standart of VIP was dissolved in 1% AcAc to a stock concentration of 0.25 mg/mL and stored at -80°C. Working solutions with concentrations of 1.25, 2.50, and 5.00 µg/mL were prepared from this stock solution. Additionally, 1% AcAc was used as a dilution solution and stored at -18°C.

Chromatographic Parameters

The analytical column used for the separation process is a C18 column with a length of 100 mm, an inner diameter of 2.1 mm, and a particle size of 2.7 µm. It has a pore size of 160 Å to increase the capacity factor of the analyte peak and improve the peak morphology in the analysis of large molecules such as peptides.

When examining the mobile phases used in the literature, they generally include acidic mobile phases, as VIP is basic and will ionize before entering the MS. A 0.2% formic acid-water solution (A) and a 0.2% formic acid-acetonitrile solution (B) were prepared in separate lines, and the experiments were started with this mobile phase. Furthermore, experiments were started with a low flow rate of 0.2 mL/min to ensure that the analyte coming from the column was completely ionized when it entered the ionization chamber and to prevent peptide loss.

Since VIP has a polar structure due to its amino acid content (Watanabe, 2016), it was anticipated that VIP would not be retained for a long time because of the mobile phase and column used, and the analyses were started with the gradient shown in (Table 2). These gradient and flow rate values were used during MS optimization and were later re-evaluated and optimized.

Table 2. Initial Gradient

R_t (min)[a]	A%	B%	Flow Rate (mL/min)
0	90	10	0.2
8	30	70	0.2

[a] Retention Time

In addition, other parameters, such as the injection volume, were set to 2 µL, and the column temperature was set to 30°C.

RESULTS

Optimization of Method

Analyzes launched by examining the parameters of MS. VIP is a large peptide with a molecular weight of 3325.8 g/mol. since scanning can be done up to 3000 m/z with the Triple Quad. mass analyzer, it is not possible to analyze VIP by charging with 1+/1-. However, it can be analyzed with multiple ionization by charging it as 2+, 3+, and 4+.

Moreover, since the pI value of VIP is greater than 11, it will act as a cation in acidic environments, that is, at pH lower than the pI value, so only positive polarity analyses were carried out.

Considering the mass analyzer and the properties of VIP, scanning was first carried out in the 100-3000 m/z range in MS^2 Scan mode. In the source parameters, the gas temperature is set to 300°C, and the gas flow is 5 L/min, the nebulizer pressure is 45 psi, the sheath gas temperature is 350°C, the sheath gas flow is 7 L/min, the capillary voltage is 3500 V, and the nozzle voltage is 500 V. The chromatogram of 5.00 µg/mL VIP obtained after scanning is shown in Figure 1(a) and MS spectrum for 1108.9(M^{3+}), 832.3(M^{4+}), 665.9(M^{5+}), and 554.6(M^{6+}) m/z values is provided in Figure 1(b).

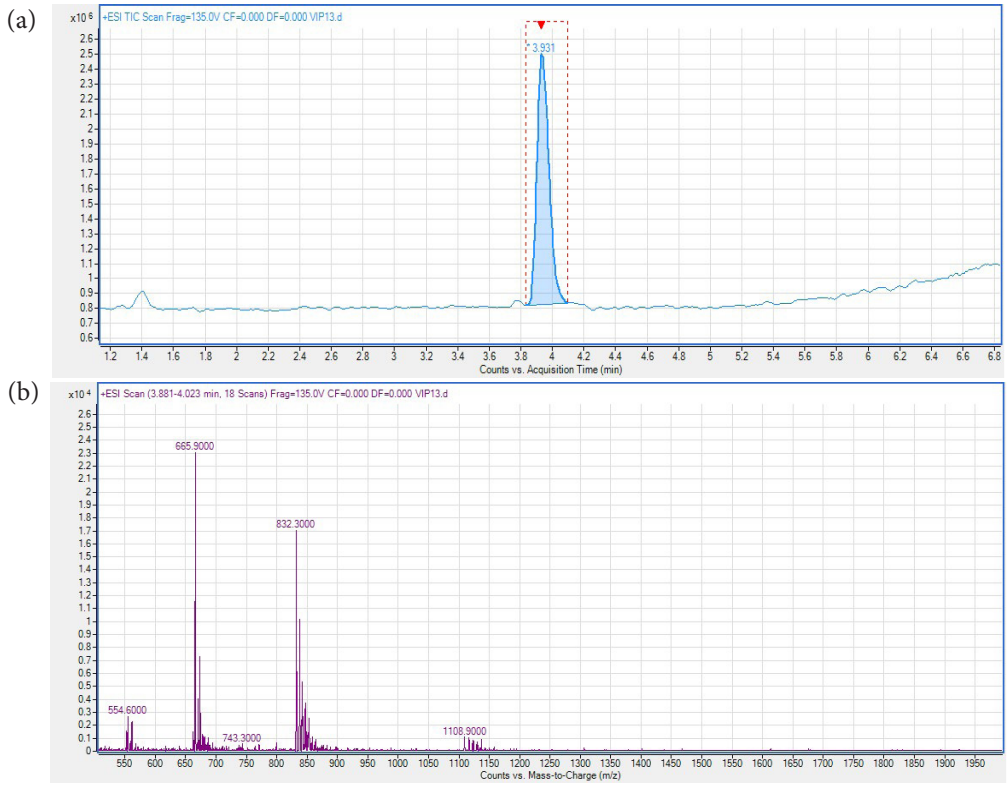

Figure 1. (a) 5.00 µg/mL VIP chromatogram with SCAN mode (b) MS spectrum for 554.6 (M^{6+}), 665.9 (M^{5+}), 832.3 (M^{4+}), 1108.9 (M^{3+}) m/z masses

To increase the selectivity of these masses and to confirm that they belong to VIP, these masses (M^{6+}, M^{5+}, M^{4+}, and M^{3+}) were examined with the selected ion monitoring (SIM) mode known as MS^2 SIM and confirmed by performing analysis with increasing concentrations of 1.25, 2.50 and 5.00 µg/mL VIP.

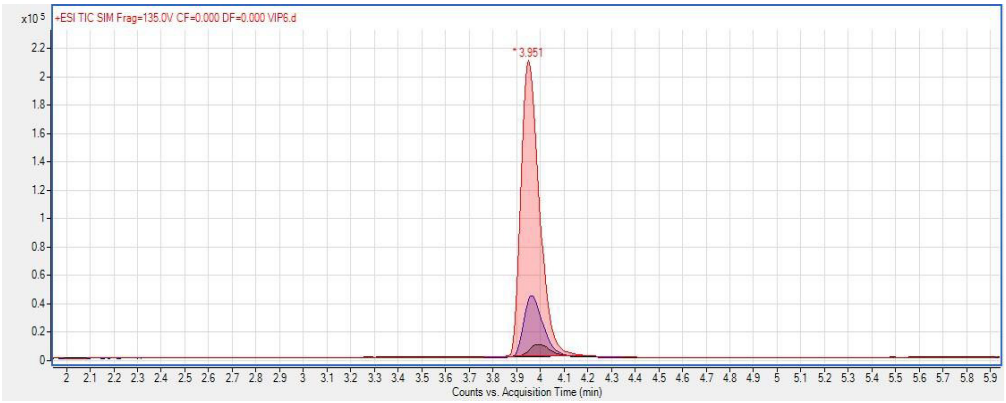

Figure 2. 1.25, 2.50, and 5.00 µg/mL VIP analysis with SIM mode

As seen in (Figure 2), reproducible retention times, the stability of these three masses belonging to VIP in each analysis, and the correlation between concentration and peak areas confirmed that the peak at 3.9 min was VIP.

Since the selected m/z values will be analyzed with fragments in MRM mode in subsequent stages, a fragmentation voltage scan was performed. The fragmentation voltage scan of each m/z value (1109.1, 832.1, and 665.9) was examined between 10-200 V on SIM mode using 2.50 µg/mL VIP solution during the analyses. Differences in peak areas and their corresponding signal-to-noise ratios (SNR) were observed in the result of the fragmentation voltage scan. The peak areas obtained for each mass versus the fragmentation voltage are given graphically (Figure 3).

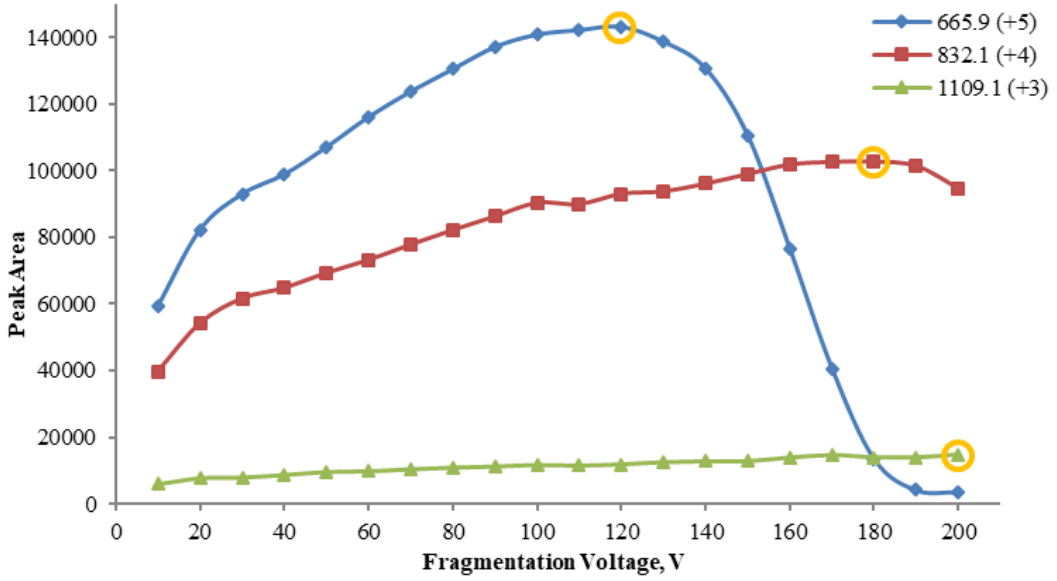

Figure 3. Fragmentation Voltage-Peak Area Graph

As it stands in the graph in Figure 3, the largest peak areas were obtained with fragmentation voltages of 120 V for 665.9 (M^{5+}), 180 V for 832.1 (M^{4+}), and 200 V for 1109.1 (M^{3+}). However, the peak areas obtained from the M^{+3} mass were much lower than those of the M^{4+} and M^{5+} m/z masses, indicating that this mass cannot be used at lower concentrations. Therefore, we focused on 832.1 and 665.9 m/z masses. Repetitive analyses were performed at a fragmentation voltage of 135 V, which is the device's default value, as well as at 180 V for 832.1 and 120 V for 665.9, corresponding to M^{4+} and M^{5+}, respectively, where the largest peak areas were observed. According to the analysis results, evaluations were made on the RSD, and the results are given in (Table 3).

Table 3. Analysis results obtained as the consequence of repetitive analyses at different fragmentation voltages (n=6)

	120 V		**135 V**		**180 V**	
	832.1	**665.9**	**832.1**	**665.9**	**832.1**	**665.9**
	A[a]	**A[a]**	**A[a]**	**A[a]**	**A[a]**	**A[a]**
mean	224085.46	322957.12	231300.72	308692.70	235300.88	35760.87
SD[b]	3426.99	4559.14	5932.42	7302.12	5209.73	1436.86
RSD%[c]	1.53	1.41	2.56	2.37	2.21	4.02

[a] Area, [b] Standard Deviation, [c] Relative Standard Deviation

According to (Table 3), the one with the highest peak area and lowest RSD was 665.9 (M^{5+}) at 120 V fragmentation voltage. Since a higher signal was obtained compared to other m/z masses, it was decided to continue with 665.9 (M^{5+}) and 120 V fragmentation voltage in subsequent analyses.

In the later stages of the study, the multiple reaction monitoring (MRM) mode, which significantly enhances the method's selectivity, was employed. The fragments of the 665.9 (M^{5+}) m/z value of VIP were identified using the Product Ion mode. Fragment scanning was performed in the range of 50-3000 m/z. The reason for scanning in such a wide m/z range is that since the 665.9 m/z mass has a 5+ charge, it is considered the main mass (3328.5 g/mol), and when it is broken down, it is predicted that it may have larger fragments. Fragments of 665.9 m/z were examined with different energies (10, 20, 30, and 40 V) applied to the collision cell.

Although no fragments were formed at low fragmentation energies, such as 10 V, it was observed that small fragments were generated as the applied energy increased. When 20 V was applied, m/z values of 799.4, 770.8, and 132.1 were observed, and these values were also included in the repetitive analyses. The amino acid sequence of VIP and its predicted fragmentation in MS are given as the VIP chromatogram in Figure 4(a) and together with the MS spectrum in Figure 4(b).

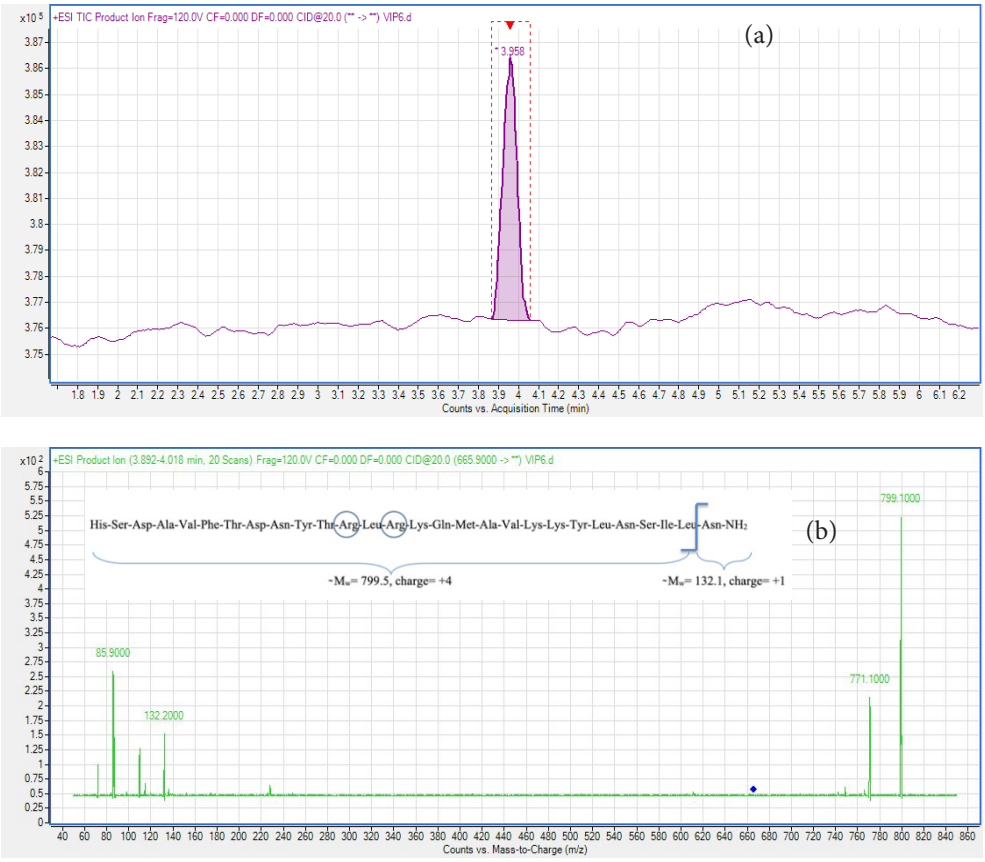

Figure 4. (a) 2.50 µg/mL VIP chromatogram **(b)** Fragments obtained from the analysis of the 665.9 (M^{5+}) m/z using the Product Ion mode and the estimated fragmentation of VIP

Since the amino acid arginine (Arg) in its structure is basic (pKa 13.2), it is estimated that 4+ ionization occurs via Arg, and 1+ ionization occurs via asparagine (Asn) in MS. In this case, VIP fragments were obtained by breaking the Leu-Asn bond, as shown in Figure 4(b). When calculations were made based on the observed masses and predicted charges, it was thought that the 5+ charged mass of 665.9 might have disintegrated into 4+ charged masses of 799.4 and 1+ charged mass of 132.1. As a result of the calculations ((799.4×4) + (132.1×1) = (665.9×5)), the main mass of the VIP was reached. Because of the limited scanning range of the mass analyzer, the 1+ charged mass of VIP (directly 3326.8 m/z mass without a multiply charged species since its molecular weight is 3325.8 Da) could not be observed, and deconvolution could not be performed through the software. However, by verifying the calculations based on this algorithm, the fragments of the 665.9 (M^{5+}) m/z mass were identified as 799.4 and 132.1 m/z.

The scanning range was narrowed to focus on the fragmentation energy, specifically around 20 V. The fragmentation voltage scanning was carried out between 15-20 V with the Product Ion mode; when 16 V fragmentation energy was applied, the maximum areas of the peaks belonging to 799.4 and 132.1 m/z masses were obtained. The 770.7 m/z mass, which has a higher mass amount after the 799.4 m/z mass, is encountered in the spectra. In this case, while the 799.4 m/z mass was chosen for quantitative analysis (as quantifier), the 770.7 m/z mass was selected to qualitatively confirm the VIP (as qualifier). In the following stages, analyses with MRM were carried out on these two masses.

The parameter that affects the dwelling time of the mass coming from the first mass analyzer in the collision cell and accelerates or slows down the mass is the cell accelerator voltage (CAV). Since it varies for each analyte, it is recommended to optimize it. If CAV≥7, the analyte will move very quickly inside the cell, causing ion loss; in contrast, if CAV≤3, as it moves very slowly, it may cause problems in MRM transitions in such a way that it will be subjected to simultaneous fragmentation with the subsequent ion, known as cross-contamination. To examine the effects of this parameter on the fragments of 665.9 m/z, CAV scanning was first performed in Product Ion mode. As a result of these analyses, the change in mass abundance of the fragments was examined and given in (Figure 5).

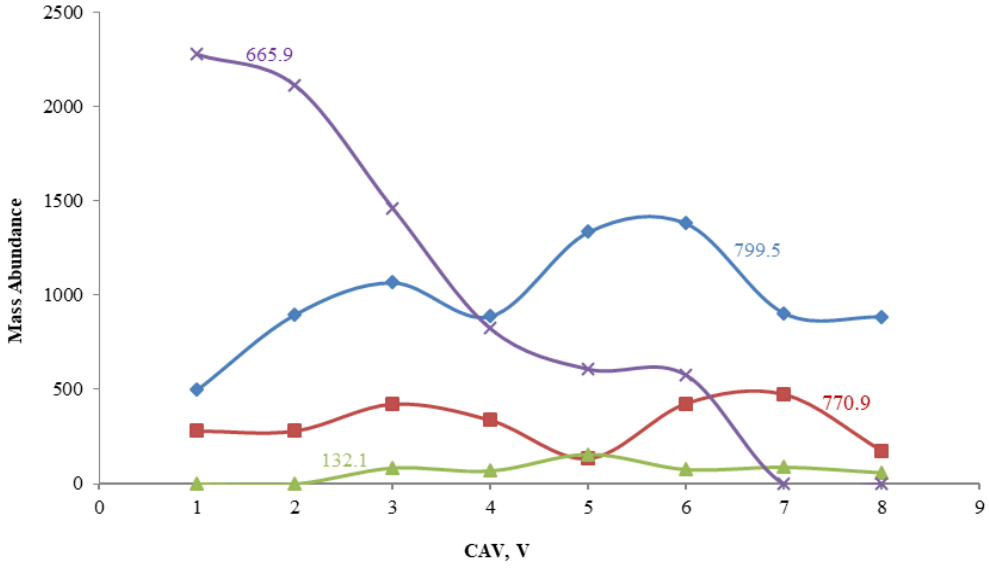

Figure 5. CAV- Fluctuation of mass abundance for precursor and fragment ions

As seen in the graph in Figure 5, fragmentation increased with the rise of CAV, and after a certain point, the 665.9 (M^{5+}) precursor ion was no longer observed when the fragments became abundant in the environment. However, after approximately 6 V, a decrease in the desired masses was observed. The reason for this may be that the 665.9 (M^{5+}) m/z ion moves too quickly in the collision cell, preventing sufficient exposure to fragmentation and resulting in mass loss. Afterward, CAV scanning was performed between 1-8 V with the MRM mode. The relevant graphs, plotted against applied voltage, peak area, and RSD values from repetitive analyses using 2.50 µg/mL VIP, are shown in Figures 6, 7, and 8.

As seen from the graphs of the peak areas of 799.5 m/z, 770.7 m/z, and the ratio of these two masses to each other, although the peak areas are not very high, the point with the lowest RSD% was observed when CAV was set to 5 V. This indicated that by applying 5 V energy, VIP underwent fragmentation at the optimal rate, resulting in stable fragments.

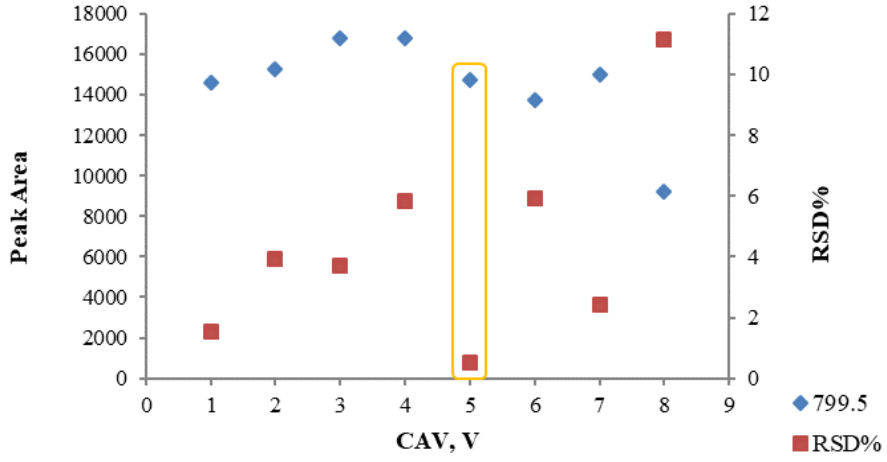

Figure 6. CAV-peak area-RSD graph for 799.5 m/z

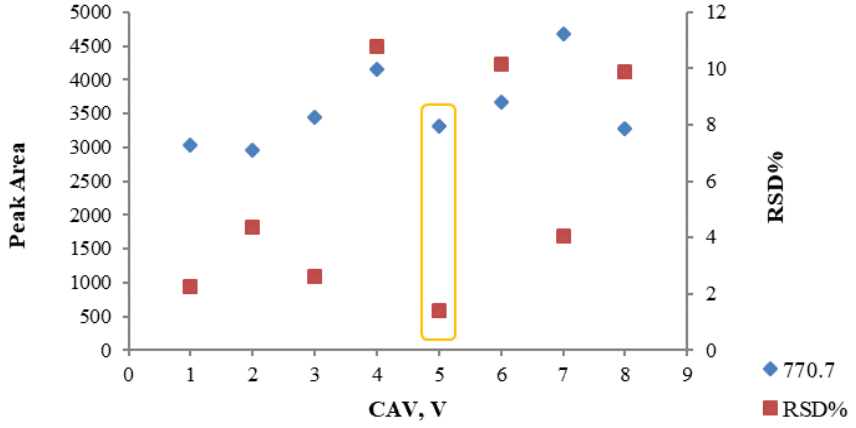

Figure 7. CAV-peak area-RSD graph for 770.7 m/z

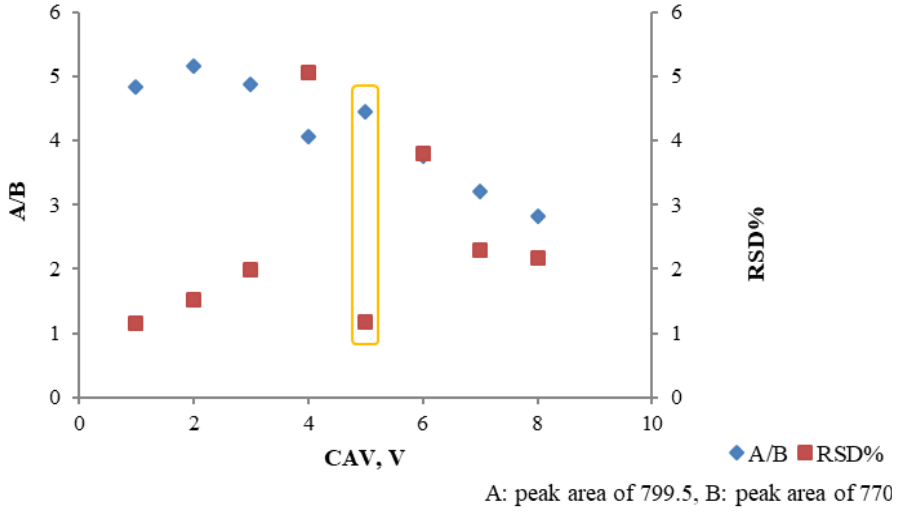

Figure 8. CAV-799.5/770.7 m/z peak area ratio-RSD graph

Before the CAV scanning was performed (when CAV was 7 V), the fragmentation energy had been scanned and found to be 16 V. However, optimizing the CAV to 5 V required slowing down the VIP in the cell and re-scanned the fragmentation energy accordingly. The fragmentation energy was re-scanned between 10-20 V with the Product Ion mode as before. As a result of the analyses carried out in increasingly narrow ranges, the fragments were intensely observed at 18 V. In the analyses performed by applying 18 V fragmentation energy with the MRM mode, larger and more repetitive peak areas were obtained compared to 16 V. Consequently, the analysis continued with the fragmentation energy as 18 V and CAV as 5 V.

After optimizing the MS parameters, the effect of VIP solvent on peak morphology, area and retention time was examined. To observe this difference, 1.25, 2.50, and 5.00 µg/mL VIP solutions were separately prepared in water:ACN (1:1) and 1% AcAc and analysed using the MRM mode. As a result of repetitive analyses, higher and more reproducible peak areas were obtained in the analyses of VIP dissolved in 1% AcAc.

To increase and facilitate the ionization efficiency of VIP in the MS ionization chamber, aqueous (A line), and ACN (B line) solutions containing 0.1, 0.2, 0.3, and 0.4% formic acid (FA) were prepared and a 2.50 µg/mL VIP was analysed in duplicate for each mobile phase separately. As a consequence of the analyses, the peak area and RSD% of VIP analyzed with mobile phase containing different amounts of FA were examined and are given in (Figures 9and 10). Analyzes were performed on MRM mode with 799.5 and 770.7 m/z masses. When these data were evaluated, it was shown that 0.2% FA was most suitable for the VIP peak in terms of high peak area and low RSD.

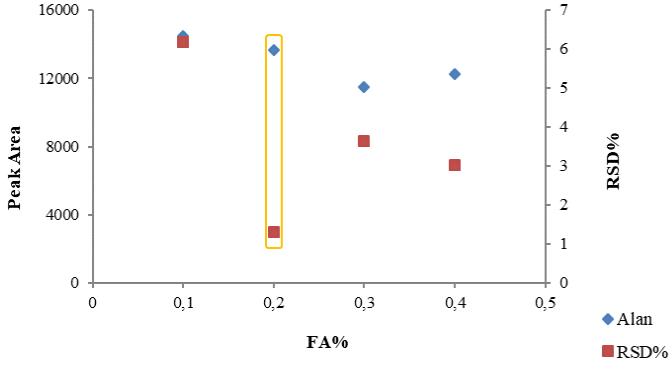

Figure 9. FA%-peak area(n=5)-RSD% graph for 799.5 m/z

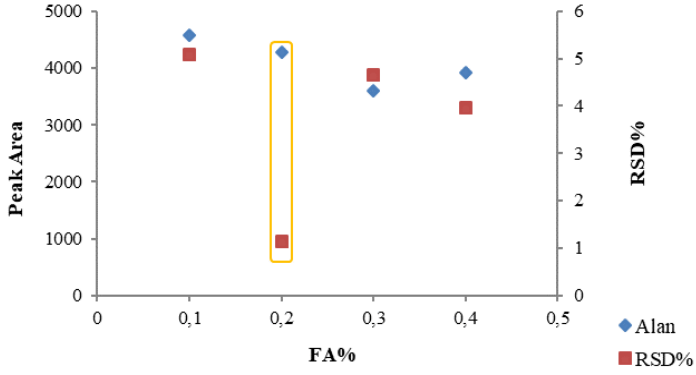

Figure 10. FA%-peak area(n=5)-RSD% graph for 770.7 m/z

To investigate the effect of the pH of the mobile phase containing 0.2% FA (pH 2.45) on the VIP peak, VIP analyses were carried out with two different mobile phases, with the pH of the medium adjusted to 2.94 and 3.15, based on the pK_a value of FA (pK_a 3.8). The analysis results are given in (Table 4), and it was seen that the pH change did not have any positive effect on the symmetry of the VIP peak. For this reason, it was decided not to change the pH of the mobile phase, that is, to continue the studies at approximately pH 2.45.

Table 4. pH Effect on VIP peak

			mean	SD[a]	RSD%[b]
pH= 2.45	**799.5**	**R_t (min)**[c]	3.95	0.00	0.10
		A[d]	13766.77	396.27	2.88
		SNR[e]	10297.42	4248.03	41.25
		S[f]	1.96	0.07	3.68
	770.7	**R_t (min)**[c]	3.94	0.00	0.10
		A[d]	4174.89	82.95	1.99
		SNR[e]	9852.20	5368.70	54.49
		S[f]	2.05	0.14	6.58
pH= 2.94	**799.5**	**R_t (min)**[c]	4.27	0.06	1.31
		A[d]	1029.03	120.81	11.74
		SNR[e]	4488.72	2511.34	55.95
		S[f]	2.17	0.24	11.03
	770.7	**R_t (min)**[c]	4.24	0.01	0.15
		A[d]	327.94	42.88	13.08
		SNR[e]	1412.90	1058.35	74.91
		S[f]	2.30	0.26	11.15
pH= 3.15	**799.5**	**R_t (min)**[c]	4.25	0.01	0.14
		A[d]	483.01	17.36	3.59
		SNR[e]	3217.00	1344.08	41.78
		S[f]	1.78	0.14	7.92
	770.7	**R_t (min)**[c]	4.25	0.01	0.18
		A[d]	148.17	8.84	5.96
		SNR[e]	1210.18	318.36	26.31
		S[f]	1.60	0.13	8.22

[a] Standard deviation, [b] Relative standard deviation, [c] Retention time, [d] Area, [e] Signal noise ratio, [f] Peak symmetry

To investigate the effect of organic solvent polarity in the mobile phase on the VIP peak, ACN and MeOH phases were separately used near the aqueous phase containing 0.2% FA. The effects of ACN and MeOH in terms of ionizing VIP in MS were monitored by MRM mode with 799.5 and 770.7 m/z masses. 2.50 µg/mL VIP solution was used in the analyses, and the results are given in tables (Table 5 and Table 6).

Table 5. Results for 2.50 ***µg***/mL VIP analyzed with mobile phase containing 0.2% FA-ACN (n=5)

	0.2% FA with ACN							
	799.5				770.7			
	R_t (min)[c]	A[d]	SNR[e]	T[f]	R_t (min)[c]	A[d]	SNR[e]	T[f]
Mean	3.99	13667.37	11818.22	2.78	3.98	4277.70	4610.32	2.79
SD[a]	0.00	179.99	5800.85	0.17	0.00	49.09	2633.69	0.11
RSD%[b]	0.00	1.32	49.08	6.23	0.11	1.15	57.13	4.06

[a] Standard deviation, [b] Relative standard deviation, [c] Retention time, [d]Area, [e] Signal Noise Ratio, [f] Tailing factor

Table 6. Results for 2.50 ***µg***/mL VIP analyzed with mobile phase containing 0.2% FA-MeOH (n=5)

	0.2% FA with MeOH							
	799.5				770.7			
	R_t (min)[c]	A[d]	SNR[e]	T[f]	R_t (min)[c]	A[d]	SNR[e]	T[f]
mean	7.45	7414.41	3099.88	3.83	7.44	2270.60	1401.50	3.91
SD[a]	0.02	174.30	1668.05	0.22	0.02	81.73	725.10	0.33
RSD%[b]	0.29	2.35	53.81	5.70	0.33	3.60	51.74	8.48

[a] Standard deviation, [b] Relative standard deviation, [c] Retention time, [d]Area, [e] Signal Noise Ratio, [f] Tailing factor

It can be seen from Tables 5, and 6 that the VIP peak was retained more by the MeOH mobile phase, the peak symmetry was disrupted, the tailing factor increased, and its area decreased. For this reason, it was decided to continue with the mobile phase with ACN.

At the end of the mobile phase optimization, the best VIP peak was obtained under conditions where 0.2% FA-water and 0.2% FA-ACN were used. Therefore, studies were continued with 0.2% FA-water and 0.2% FA-ACN mobile phase.

After optimizing the mobile phase, the flow rate, which is another chromatographic parameter, was checked. VIP was analyzed at different flow rates with 799.5 and 770.7 m/z masses by MRM using 0.2% FA-water and 0.2% FA-ACN mobile phase and 2.50 µg/mL VIP solution. Data obtained at flow rates of 0.15-0.35 mL/min are given in (Tables 7 and 8).

Table 7. Effect of flow rate on 799.5 m/z fragment ion peak (n=3)

		799.5							
Flow rate (mL/min)		R_t (min)[c]	A[d]	SNR[e]	S[f]	T[g]	W[h]	k`[i]	N[j]
0.35	**mean**	3.16	3798.17	3336.30	0.38	2.20	0.48	1.30	7026.67
	SD[a]	0.01	22.49	1022.84	0.02	0.10	0.01	0.00	281.79
	RSD%[b]	0.21	0.59	30.66	4.06	4.55	2.39	0.00	4.01
0.30	**mean**	3.34	4424.45	2930.60	0.40	2.10	0.48	1.40	7514.67
	SD[a]	0.00	38.86	418.22	0.02	0.10	0.00	0.00	206.73
	RSD%[b]	0.10	0.88	14.27	4.33	4.76	0.85	0.00	2.75
0.25	**mean**	3.60	5083.50	3687.20	0.38	2.07	0.53	1.60	7286.67
	SD[a]	0.01	164.05	515.40	0.03	0.06	0.01	0.00	360.22
	RSD%[b]	0.18	3.23	13.98	7.53	2.79	1.95	0.00	4.94
0.20	**mean**	3.98	6218.69	5945.00	0.42	2.03	0.56	1.80	7247.67
	SD[a]	0.00	90.64	3024.66	0.01	0.06	0.01	0.00	130.94
	RSD%[b]	0.10	1.46	50.88	1.39	2.84	1.15	0.00	1.81
0.15	**mean**	4.61	7934.30	20447.83	0.43	1.93	0.65	2.30	6928.67
	SD[a]	0.00	112.72	18867.81	0.02	0.06	0.03	0.00	151.14
	RSD%[b]	0.09	1.42	92.27	3.53	2.99	4.37	0.00	2.18

[a] Standard Deviation, [b] Relative standard deviation, [c] Retention time, [d] Area, [e] Signal noise ratio, [f] Symmetry, [g] Tailing factor, [h] Peak width, [i] Capacity factor, [j] Theoretical plate number

Table 8. Effect of flow rate on 770.7 m/z fragment ion peak (n=3)

		770.7							
Flow rate (mL/min)		**R_t (min)[c]**	**A[d]**	**SNR[e]**	**S[f]**	**T[g]**	**W[h]**	**k'[i]**	**N[j]**
0.35	**mean**	3.15	1171.67	5580.83	0.37	2.27	0.46	1.27	6827.33
	SD[a]	0.01	31.15	2597.53	0.01	0.15	0.01	0.06	168.74
	RSD%[b]	0.32	2.66	46.54	3.09	6.74	1.77	4.56	2.47
0.3	**mean**	3.34	1214.21	7277.03	0.40	2.03	0.46	1.40	7355.00
	SD[a]	0.00	26.72	2594.51	0.03	0.06	0.03	0.00	824.29
	RSD%[b]	0.00	2.20	35.65	6.61	2.84	6.99	0.00	11.21
0.25	**mean**	3.60	1377.41	3031.30	0.41	2.03	0.49	1.60	7616.67
	SD[a]	0.01	7.96	1677.14	0.02	0.06	0.02	0.00	213.88
	RSD%[b]	0.18	0.58	55.33	5.68	2.84	3.53	0.00	2.81
0.2	**mean**	3.98	1753.57	2795.93	0.43	1.97	0.53	1.80	8170.00
	SD[a]	0.00	49.06	1516.71	0.01	0.06	0.02	0.00	534.87
	RSD%[b]	0.10	2.80	54.25	2.66	2.94	3.21	0.00	6.55
0.15	**mean**	4.61	2268.58	5898.47	0.43	2.03	0.67	2.30	6090.00
	SD[a]	0.01	26.96	158.50	0.05	0.15	0.03	0.00	937.33
	RSD%[b]	0.14	1.19	2.69	10.83	7.51	4.20	0.00	15.39

[a] Standard Deviation, [b] Relative standard deviation, [c] Retention time, [d] Area, [e] Signal noise ratio, [f] Symmetry, [g] Tailing factor, [h] Peak width, [i] Capacity factor, [j] Theoretical plate number,

According to the data in (Tables 7 and 8), 799.5 m/z (quantifier) and 770.7 m/z (qualifier) peaks were evaluated according to system suitability parameters. Considering parameters such as peak area, SNR, symmetry, and plate numbers, the optimum flow rate was determined as 0.2 mL/min.

Adsorption Inhibiting Studies for VIP

Peptides can adhere to glass or polypropylene surfaces due to their chemical properties under the environmental conditions in which they are brought into solution. While standard solutions are being prepared, they can be adsorbed on experimental materials such as tubes, pipette tips, and vials. Taking this situation into consideration, the studies were continued by examining the parameters that prevent adsorption.

Albumin (bovine serum albumin, BSA) helps to prevent adsorption by covering the surfaces in contact with the peptide and preventing the interaction. BSA solution (1%) was prepared for the coating process. Materials such as pipette tips, tubes, and vials used in experimental studies were shaken with this albumin solution and dried. Then, standard peptide solutions and working solutions were all prepared using these coated materials. Additionally, BSA was added to the stock peptide solution. VIP stock (0.25 mg/mL) was mixed with 1% BSA solution in a 1:1 ratio and kept in an ultrasonic bath for 1 hour. Working solutions were prepared using this mixture.

At the same time, the effect of the solvent on adsorption was examined. To determine whether it has an inhibitory/reducing effect on adsorption, three different dilutions were prepared: 1% AcAc, 1% AcAc-50% ACN mixture, and 5% AcAc-50% ACN mixture. Three different sets of VIP working solutions (250 and 500 ng/mL) were prepared with these solutions and analyzed respectively by SCAN-SIM-MRM modes. To 500 ng/mL VIP, the peaks belonging to 665.9 m/z mass were evaluated, and the analysis results are presented in table (Table 9). There was no significant difference in the peaks between dilution solutions, and tailing occurred in the VIP peak with all three dilution

solutions. While there was no difference between the retention times and areas of the peaks, the evaluation was made according to SNR. While increasing acidity did not show a significant difference for adsorption, the presence of organic solvent in the environment increased the non-polarity, thus reducing the solubility of polar VIP, preventing its ionization, and stopping it from adhering to the surface, which may be ionic even though it is covered with albumin.

Table 9. Comparison of peaks for 500 ng/mL VIP (665.9 m/z) with different dilution solutions (SIM mode)

VIP	R_t (min)[a]	A[b]	SNR[c]
VIP with %1 AcAc	4.038	160799.98	8863.4
VIP with %1AcAc-%50 ACN	4.015	156932.27	11948.9
VIP with %5AcAc-%50 ACN	4.008	154503.54	11701.6

[a] Retention time, [b] Area, [c] Signal Noise Ratio

The LOD and LOQ values of the developed LC-MS/MS method were calculated using the SNR as 0.14 and 0.42 ng/mL, respectively.

The stability of the VIP standard solution under different storage conditions was also examined during these studies. 500 ng/mL VIP solutions were prepared with the three different dilution solutions above, using VIP solutions in albumin-free and uncoated vials kept at -80°C for 16 months and -20°C for 2 months. These solutions were analysed on SIM mode and comparisons were made based on peaks with 665.9 and 832 m/z masses. While no significant difference was observed between the peaks obtained from different dilution solutions using VIP stored at -80°C for 16 months, it was observed that the peak areas increased as the amount of acid and organic solvent raised in VIP solutions stored at -20°C for 2 months. This indicated that the adsorption at -20°C was greater than at -80°C, and the stability of the peptide was greater at lower temperatures.

DISCUSSION

This study aimed to develop an analytical method for the characterization of VIP using the sensitive and selective LC-MS/MS technique, which will pave the way for the analysis of VIP in biological tissues and fluids.

In LC-MS analyses for VIP, chromatographic and MS parameters were examined. Chromatographically, the mobile phase content was comparatively examined in terms of the amount and type of organic solvent and the amount of acid that influences the ionization of the peptide. In addition, the flow rate was also optimized as it would affect the ionization and ion intensity of the peptide in MS. Chromatographically, the best VIP peak was obtained with a mobile phase containing 0.2% FA-water and 0.2% FA-ACN at a flow rate of 0.2 mL/min. Since it is known that VIP ionizes positively in an acidic environment due to its pI value being greater than 11, MS analyses were carried out in positive mode. Together with the main ion of VIP, the fragmentation voltage and cell accelerator voltage were also optimized, and the multiply charged species obtained due to the fragmentation of the peptide could be observed. When mass 665.9, the 5+ charged ion of VIP, was fragmented by the MRM mode, fragment masses of 799.5 m/z (quantifier) and 770.7 m/z (qualifier) were successfully characterized. The method has become highly selective in the analyses performed using SCAN, SIM, and MRM modes.

Throughout the analysis of VIP, issues such as a decrease in peak areas and the inability to observe VIP masses emerged, which were attributed to peptide adsorption onto surfaces and the instability of VIP. To prevent VIP adsorption to the materials used in experimental studies, they were coated with a 1% BSA solution, which was also added to the stock and working solutions. Furthermore, dilution solutions containing different ratios of acid and organic solvents were prepared for VIP, and their adsorption inhibition powers were compared.

The intense positive charge of VIP, due to its basic Lys and Arg amino acids, causes it to strongly adhere to negatively charged surfaces with which it interacts. However, thanks to a highly sensitive and selective method such as LC-MS/MS and a mass analyzer known as Triple quadrupole, multiply charged masses and fragments for VIP could be observed successfully. However, because of adsorption, the ion intensity of the masses remained very low.

Given the low peptide concentrations in biological samples (such as brain tissue and fluid), different studies can be conducted considering adsorption for VIP analysis. The foundation for methods to be developed was laid in this study. Among the studies reviewed, excluding the CE-LIF method, the LOD and LOQ values of the studies conducted using the LC-MS method, as shown in (Table 1), are approximately 1 ng/mL. The LOD and LOQ values (0.14 and 0.42 ng/mL) of the developed LC-MS/MS method are lower than those reported in these studies. This highlights another contribution of our study to the scientific literature.

It has been demonstrated that the chosen LC-MS/MS method is a unique option for VIP analysis, as it can reach very low levels and perform quantification in biological samples with high accuracy, with an adsorption inhibitor method that does not suppress peptide ions.

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AUTHOR CONTRIBUTION STATEMENT

Concept (SK, ES), Design (SK, ES), Supervision (ES), Resources (SK, ES), Materials (SK, ES), Data Collection and/or Processing (SK), Analysis and/or Interpretation (SK, ES), Literature Search (SK), Writing (SK), Critical Reviews (SK, ES)

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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