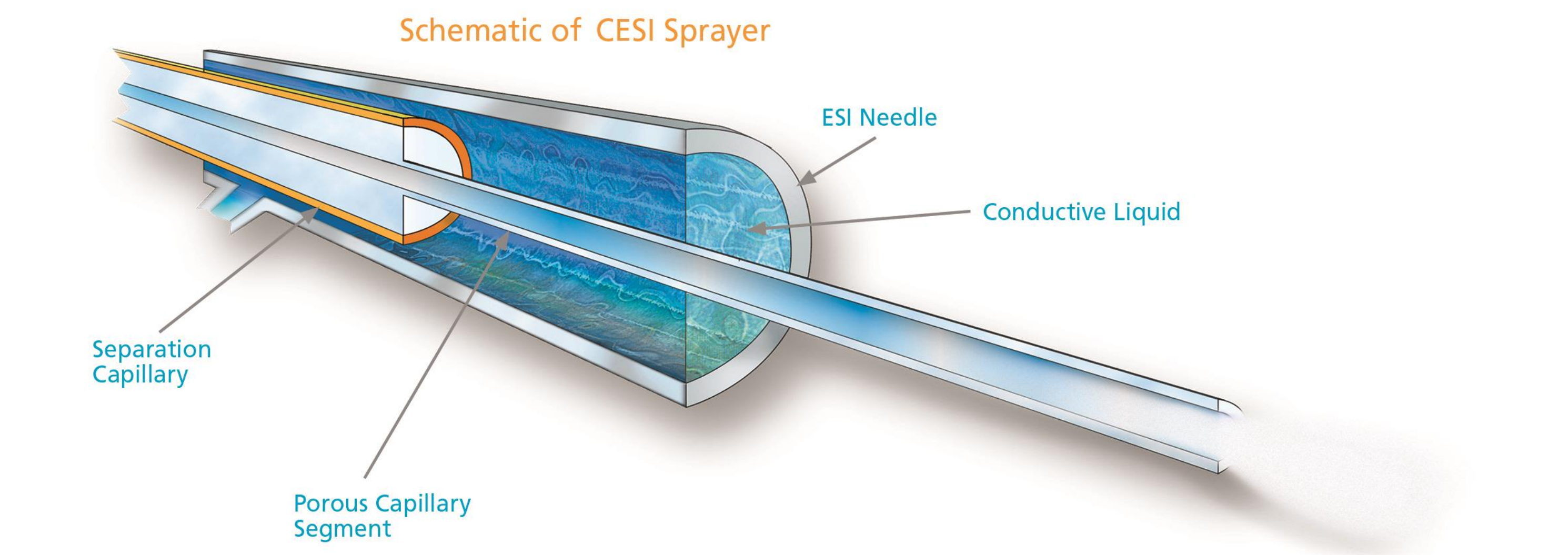


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INTRODUCTION

Standard reverse phase liquid chromatography separates constituents based on their differential interactions with the column stationary phase and an organic mobile phase. CESI-MS is a different separation technique and is the integration of CE and ESI into one dynamic process and provides highly efficient peptide and protein separations based on their size and charge (1) and therefore offers a different selectivity to reverse phase liquid chromatography. The ionization is at the ultralow nanoflow regime (25-100 nL/min) and it uses an open capillary which eliminates some of the challenges of stationary phase based separations such as lack of retention of small polar peptides as well as over retention of larger, basic or hydrophobic peptides.

Vasoactive intestinal peptide (VIP) is an important peptide hormone and a neuropeptide containing 28 amino acid residues and is involved in biological functions such as stimulated contractility in the heart, vasodilation and relaxation of the smooth muscle. Pituitary adenylate cyclase-activating polypeptide also known as PACAP is similar to VIP. One of PACAP effects is to function as a neurotransmitter and neuromodulator. Both peptides have been shown to be important neuropeptides.



The standard methods used to detect these peptides normally involve immunoassay. Although sensitive, cross reactivity is known to affect these type of methods and often this approach is incapable of screening for multiple neuropeptides in a single assay. In closer inspection of the amino acid sequence for these peptides (Figure 1) it can be seen that both contain high levels of basic amino acids which makes them difficult to analyze by conventional LCMS approaches and indeed when this was tried no peak for PACAP was observed (Figure 2). This poster looks at the development of a CESI-MS for the screening for both peptides in a single analysis as an alternative to LCMS or immunoassay methods.

His-Ser-**Asp**-Ala-Val-Phe-Thr-**Asp**-Asn-Tyr-Thr-**Arg**-Leu-**Arg**-Lys-Gln-Met-Ala-Val-**Lys**-**Lys**-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH2

VIP sequence, molecular weight = 3325.8

His-Ser-**Asp**-Gly-Ile-Phe-Thr-**Asp**-Ser-Tyr-Ser-**Arg**-Tyr-**Arg**-Lys-Gln-Met-Ala-Val-**Lys**-**Lys**-Tyr-Leu-Ala-Ala-Val-Leu-Gly-**Lys**-**Arg**-Tyr-**Lys**-Gln-Arg-Val-**Lys**-Asn-**Lys**-NH2

PACAP sequence, molecular weight = 4534.26

Figure 1:- VIP and PACAP amino acid sequence with basic residue in bold and acidic residues in red.

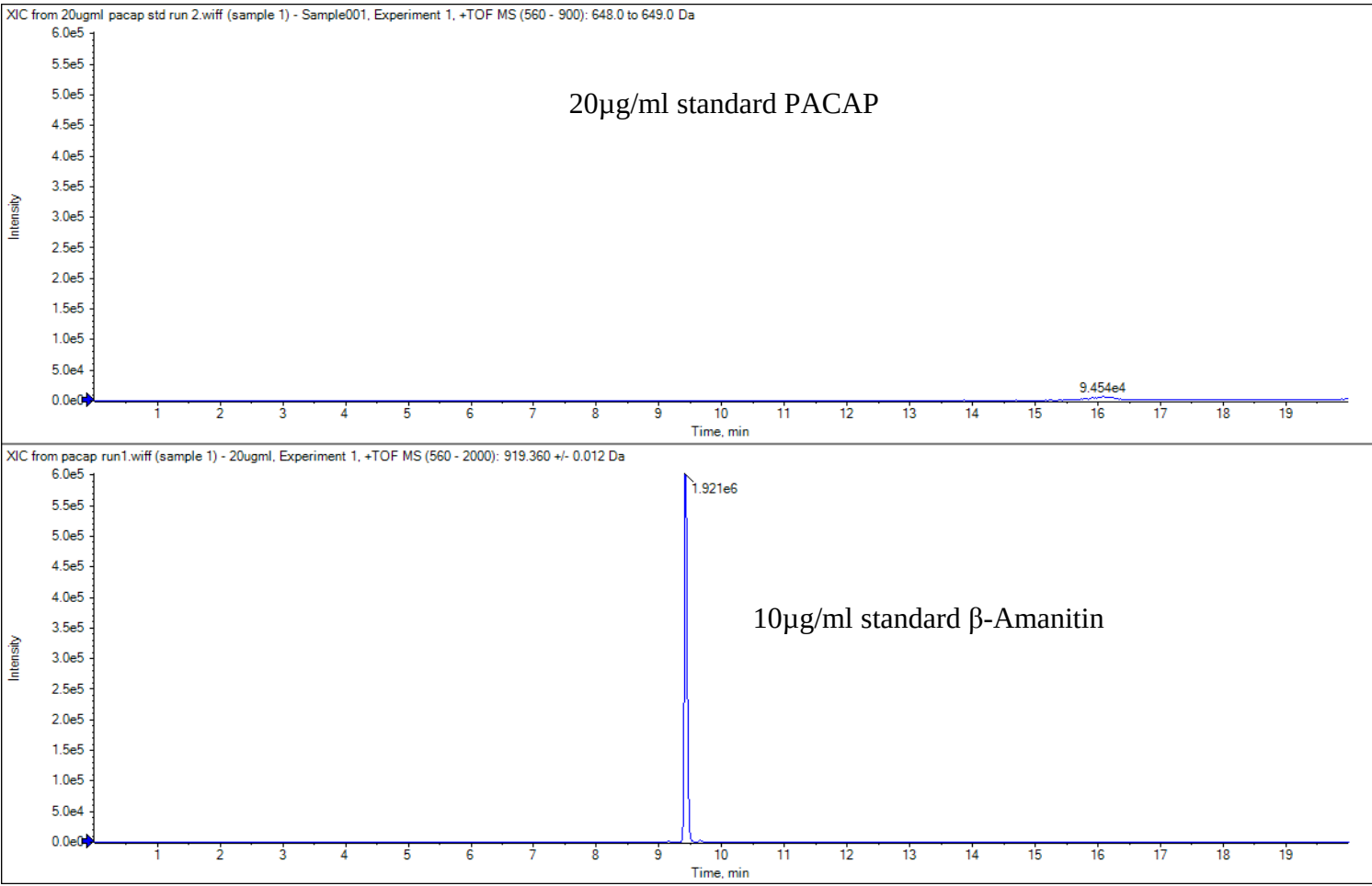


Figure 2:- HPLC-MS analysis for PACAP compared with a standard peptide (Gradient from 5 to 100% acetonitrile).

METHODS

Chemicals: All chemicals were Reagent Grade and were purchased from Sigma Aldrich including standards of VIP and PACAP-38.

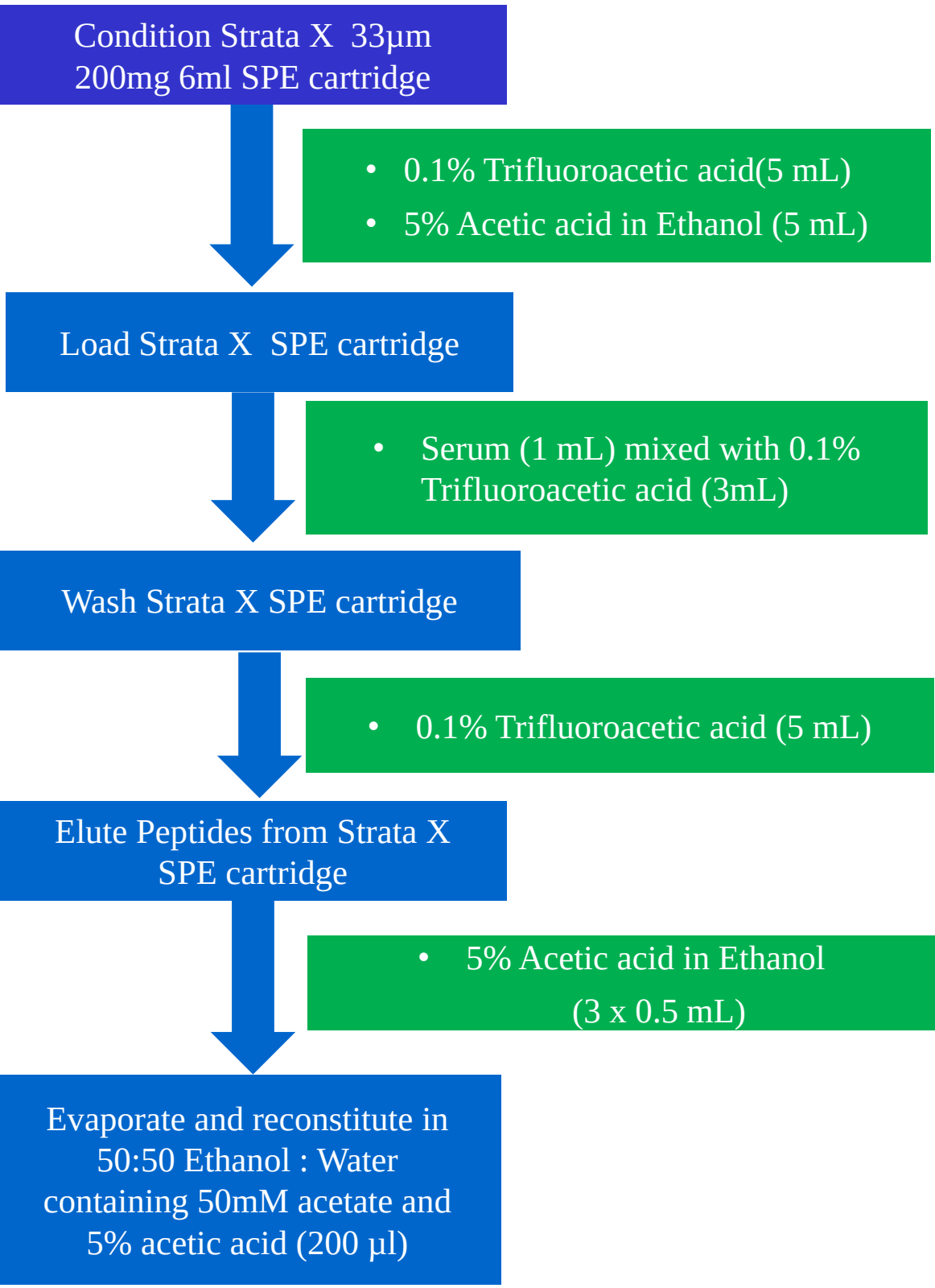


Figure 3:- Sample preparation work flow for serum samples.

Sample Preparation:

Standards were prepared by dissolving the solid peptides into water to a concentration of 0.1mg/ml in the vials supplied by Sigma. These concentrated standards were then serially diluted into a mixture of 45:5:50 Ethanol : Acetic Acid : 100mM acetate. Spiked calf serum was used as a model matrix and prepared using solid phase extraction as shown in Figure 3.

CESI-MS method: The sample was injected by pressure (5 psi, 30 s) onto a 30 µm ID x 91 cm bare-fused-silica capillary (including porous spray tip) housed in an OptiMS CESI cartridge. The capillary was covalently coated with Polyethylenimine (PEI) (2) and thermostatted using recirculating liquid coolant regulated at 25 °C. For this analysis, the SCIEX QTRAP® 5500 mass spectrometer was fitted with the NanoSpray® III source. Gas 1 and 2 were not used and temperature set low (50 °C) as ionization at these very low flow rates occurs by simply applying the ion-spray voltage was set to 1600 V. The curtain gas was set low (5psi) and other MS conditions are shown in Table 1. The MS method was split into 3 periods to cover the CE separation, in the first period and last period (1 minute each) the ionspray voltage was set to zero, the only difference in the MS conditions. The CE separation used the condition shown in Table 2 with a background electrolyte (BGE) of a mixture of 5% Acetic acid : 30% Acetonitrile : 65% Water. The voltage used for the separation was in reverse polarity.

Curtain gas	5psi	Peptide	Q1 Mass	Q3 Mass	CE (V)
Gas 1	0 psi	PACAP	567.6	671.8	23
Gas 2	0 psi	PACAP	567.6	647.7	23
ISV	1600V	VIP	666	663	24
DP	80 V	VIP	666	771.5	27
EP	10 V				
CXP	13 V	Resolution	Q1 Low	Q3 Unit	
Heater	50 C				

Table 1:- MS conditions.

Action	Time (mins)	Pressure (psi)	Direction	Voltage (kV)	Solution	Description
Rinse	1	100	Forward	0	Water	Wash
Rinse	3	50	Forward	0	1 Molar NaCl	Condition
Rinse	0.5	100	Forward	0	Water	Wash
Rinse	2	100	Forward	0	BGE	Capillary Fill
Rinse	0.5	75	Reverse	0	10% Acetic acid	Conductive Fill
Injection	0.5	5	Forward	0	Sample Vial	Injection
Injection	0.5	0.5	Forward	0	BGE	Push
Separation	15	1	Forward	20	BGE	Separation
Voltage	3	5	Forward	1	BGE	Ramp down

Table 2:- CESI separation method.

RESULTS

Analytes are separated in CE based on their mobility (m/z) and elute in HPLC based on hydrophobic interactions with the stationary phase of the column. In CESI-MS the two peptides are well separated due to their size difference, with a migration difference of over 1 minute. Figure 4 shows the low level detection of VIP and PACAP in standards with both peptides easily detected at a 2ng/ml level.

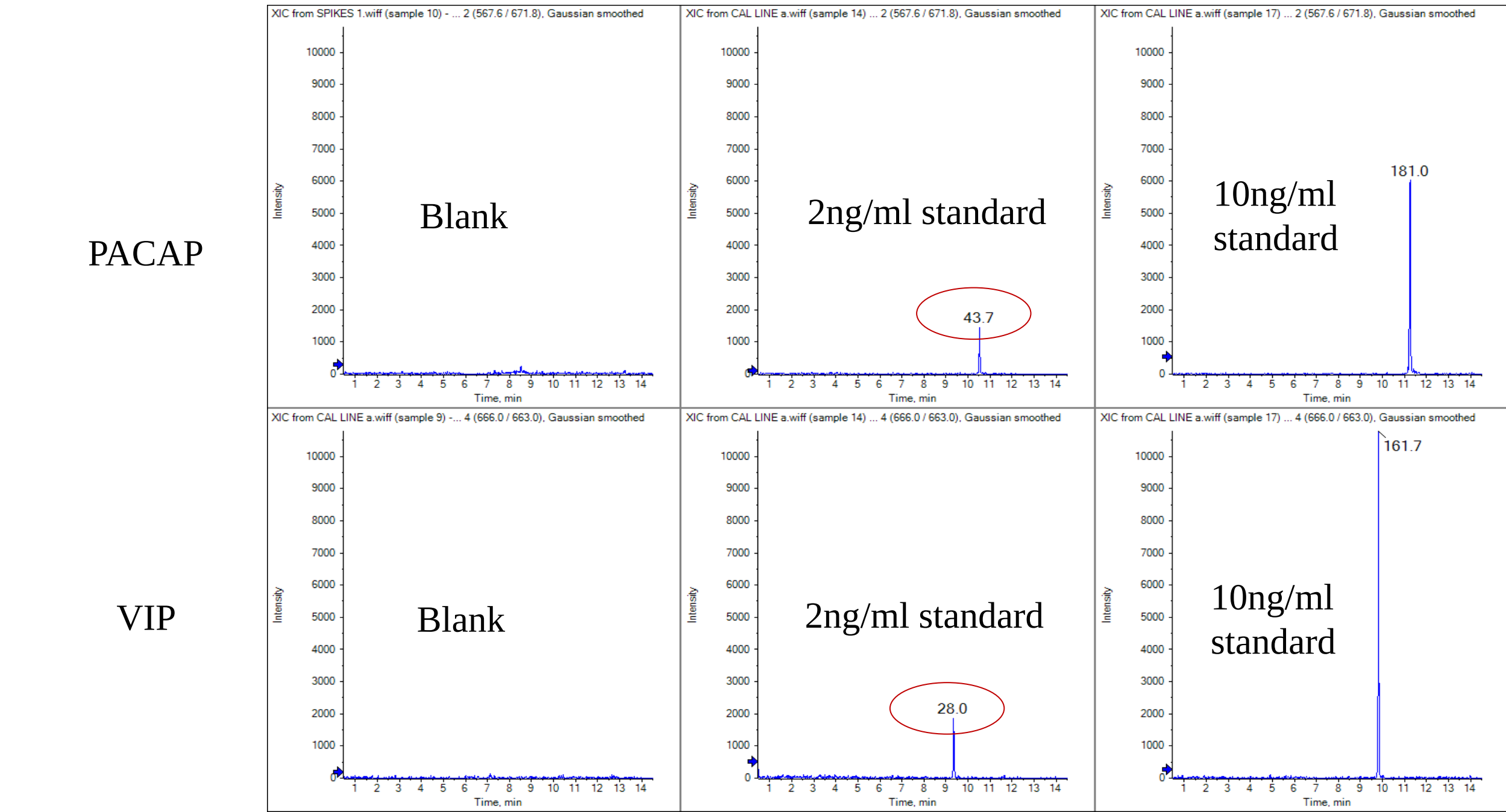


Figure 3:- CESI-MS/MS electropherogram of a solvent blank, 2 ng/ml and 10 ng/ml standards of VIP AND PACAP (the peaks are labelled with signal:noise calculated in Peak View®).

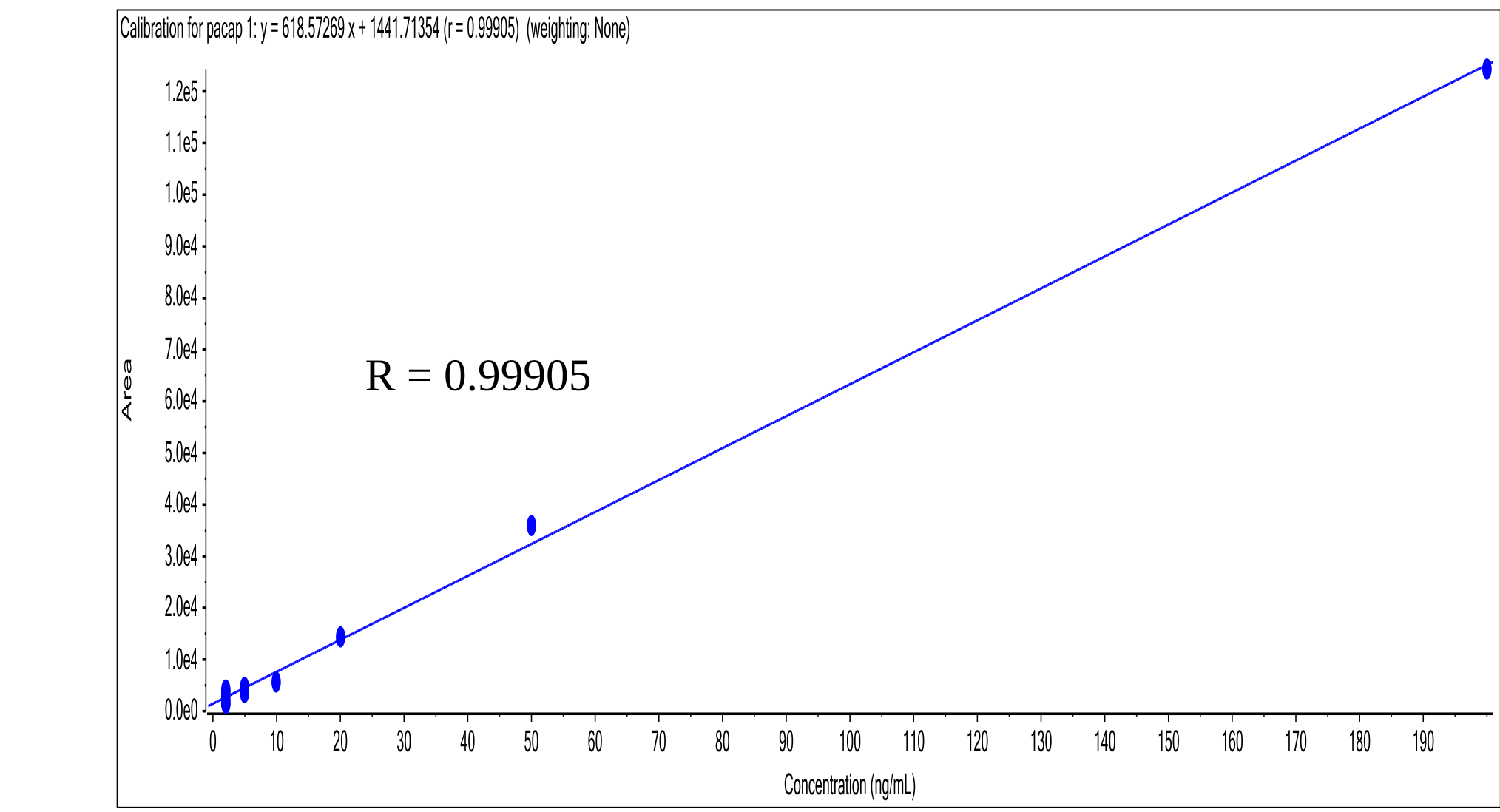


Figure 4:- Calibration line for PACAP. The range for the calibration test was 1 – 200 ng/ml and this was also the range for VIP (data not shown here). This corresponded to 200 fMol/mL - 40 pMol/mL.

The linearity of response was then tested for both VIP and PACAP. An example of the result is shown in Figure 4. It was observed that both peptides gave a linear response over the range 1-200 ng/ml as shown by the R value= 0.99905 displayed in Figure 4 for PACAP. Serum samples were then spiked with PACAP and VIP, extracted and reconstituted. Figure 5 shows the result for the primary MRM for VIP and it clearly shows the detection of VIP at this level in serum. For PACAP again the signal:noise obtained exceeded 100:1 (Figure 6) for the primary MRM and the secondary MRM signal:noise. In these early tests PACAP recoveries, from the SPE protocol, were calculated to be in the range of 80 -110%.

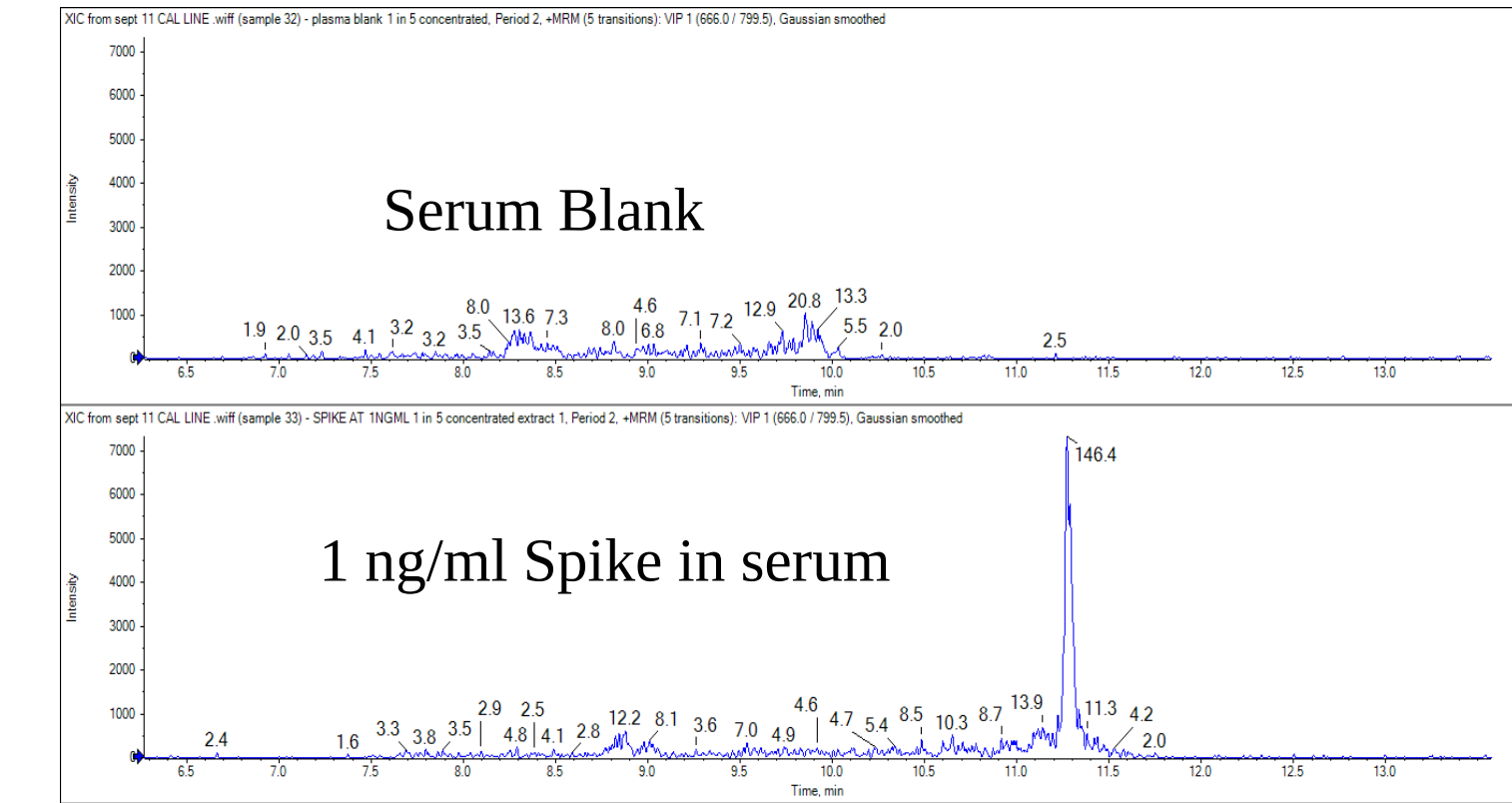


Figure 5:- CESI-MS/MS electropherogram for VIP of a serum blank sample compared with a 1 ng/ml serum spiked sample (the peaks are labelled with signal:noise calculated in Peak View®).

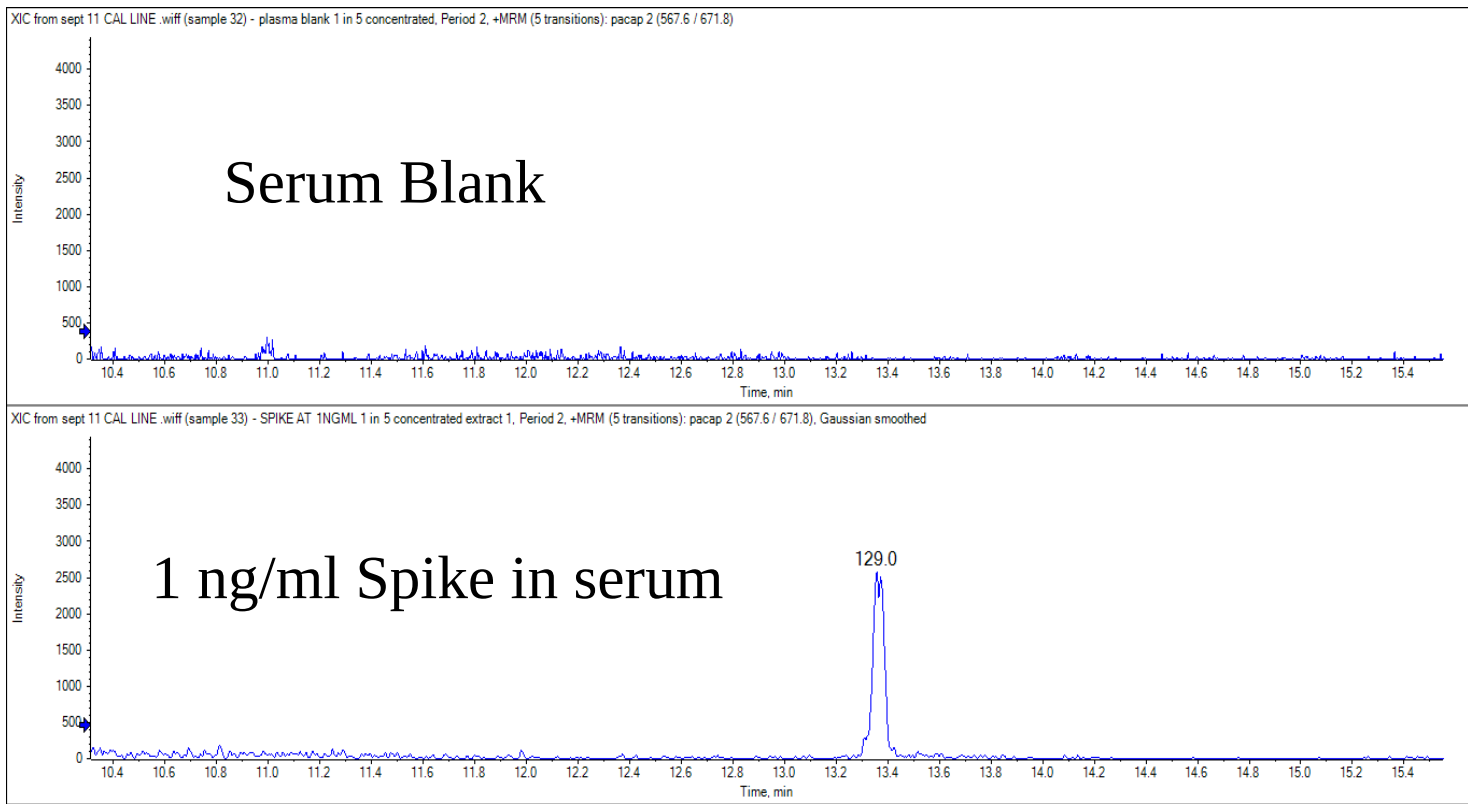


Figure 6:- CESI-MS/MS electropherogram for PACAP of a serum blank sample compared with a 1 ng/ml serum spiked sample (the peaks are labelled with signal:noise calculated in Peak View®).

CONCLUSIONS

In this early study it has been demonstrated that CESI-MS/MS can be used for quantitation and detection of PACAP and VIP in the same method while both of these peptides could not be detected using a conventional reverse phase LCMS analysis.

Using 1mL of plasma and a simple solid phase extraction protocol a 1 ng/mL spike into serum could be detected for both PACAP and VIP with no peak observed in the serum blank. As the signal to noise currently observed is at least 10 fold higher than an expected LOD level and the SPE extraction involved just a 1 in 5 concentration step there is a large room to get lower levels detection for both peptides. This may be done by simple reduction of the volume of the final extract to 50 µl or below.

Finally, this MS based detection method has the potential to be further expanded to include other neuropeptides such as PACAP-27 or PACAP metabolites to further study the role of these peptides in biological systems.

REFERENCES

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