

Peptide Chromatography Report

Diane Cripps
Prepared for Phoenix S&T
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I. Problems to be Solved

Phoenix S&T had the following chromatography issues that needed to be resolved in order to produce data for upcoming ASMS posters and for other needs such as quality control of columns:

- A. Verification of pump system performance
- B. Ability to produce peptide separations

II. Selection of Peptides

Some peptides for use as standards were selected in view of cost and suitability for ease of LC-MS analysis. Factors to be considered for the latter are:

A. Ease of ionization

Peptides containing one or two lysine or arginine residues are more amenable to positive ion mass spectrometry since these residues are positively charged at acidic pH.

B. Peptide MW

Peptides in a mass range of 800-3000 amu are easily seen in the mass range of the ion trap, since when doubly charged (usual for peptides) they are in the range of ~400-1500.

Small peptides (such as MRFA) may be observed when singly charged but most are usually seen as doubly- or triply-charged species. Since each positive charge results from the addition of a proton, the mass of the peptide also increases by 1 amu for each charge.

The following peptides were selected based on the above criteria.

Table 1. Table of peptides

Name	Sequence	Mass	[MH+2] ²⁺	[MH+3] ³⁺	nmoles/mg
α-Endorphin	YGGFMTSEKSQTPLVT	1744.82	872.4	582.6	573
β-Endorphin (h)	YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE	3462.81	1732.4	1155.3	289
γ-Endorphin	YGGFMTSEKSQTPLVTL	1857.90	930.0	620.3	538
Bradykinin1-9	RPPGFSPFR	1059.55	530.8	n/a	944
Bradykinin2-9	PPGFSPFR	903.44	452.7	n/a	1,110

Notes:

- 1) (Lysine (K) and arginine (R) residues are in bold)
- 2) m/z for doubly-charged peptide: $(MW + 2)/2$
- 3) m/z for triply-charged peptide $(MW + 3)/3$
- 4) Although beta-endorphin is larger than the mass range indicated above, it was surmised that charge states 3 or above might be readily achieved due to the large number of lysine residues.

The peptides were obtained in vials of 1 mg each, the endorphin peptides from American Peptide and the bradykinin peptides from Sigma.

Note: To obtain the mass of a peptide, go to ionsource.com, and click "Peptide Mass Calculator" from the "Card File" menu at that site. Enter the sequence of the peptide in the new screen that appears, and click "Calculate". A popup screen will appear with the average and monoisotopic masses of the peptide (for MRFA, these are 523.63 and 523.24, respectively). For a smaller peptide, the monoisotopic mass is usually produces the major ion in the isotopic envelope for the peptide. For large peptides, the probability that there is a ^{13}C in the peptide starts to approach the point where the major ion will be an amu larger than the calculated monoisotopic mass, etc. The ionsource.com site has some other handy information, such as the accurate masses of elements and amino acid residues. I have used these to calculate the accurate masses of peptides for high mass accuracy calibration, for example.

III. Handling and Preparation of Peptides

A. Handling of peptides and preparation of stock solutions

Peptides easily degrade at room temperature and in acidic solution, whereas they will be fairly stable frozen in a buffer solution. Therefore, stock solutions were prepared in ammonium bicarbonate buffer and frozen for future use. After peptide solutions are diluted for use with mass spectrometry using an acidic buffer (A or B), they will last for a period of days in an ordinary freezer, but will eventually degrade.

Each peptide (1 mg) was dissolved in 250 μL of 100 mM ammonium bicarbonate, which is a suitable buffer for use with mass spectrometry since it contains no metal ions. This produced a stock solution of 4 $\mu\text{g}/\mu\text{L}$ (4 mg/mL), which was then divided into 25 μL aliquots and frozen. (It was expected that there would be 10 aliquots of each but sometimes only 7 or 8 resulted, perhaps because of pipettor performance.)

Note: Preparation of ammonium bicarbonate buffer

Vendor: Sigma
 Product Number: A6141
 MW: 79.06 g mol^{-1}

To make 50 mL of buffer, weigh 0.3953 g of ammonium bicarbonate and dissolve in HPLC water:

$$\frac{(0.3953 \text{ g} / 79.06 \text{ g mol}^{-1})}{(50 \text{ mL})} = 100 \text{ mM}$$

The ammonium bicarbonate buffer can be stored in the refrigerator.

B. Preparation of dilute peptide solutions

Several serial dilutions of each peptide were prepared from the stock solution for use in peptide analysis by HPLC-MS.

Dil 1: Initial dilution of stock for use in preparing further dilutions. One 25 μL aliquot of stock peptide was brought to 1000 μL with solvent A (0.1% formic acid), by adding 975 μL solvent A, resulting in a solution of 0.1 $\mu\text{g}/\mu\text{L}$.

Dil 2: For use in testing the chromatography of each peptide separately. A second dilution was prepared by taking 10 μL of the first dilution and adding solvent A to obtain a concentration of about 1 $\text{pmol}/\mu\text{L}$. Since the sample loop has a volume of 5 μL , this would result in injecting about 5 pmole onto the column.

Dil 3: For use in preparing very dilute peptide solutions. A one-tenth dilution of Dil 1 was prepared by diluting 10 μL of Dil 1 with 90 μL of solvent A.

The 25 μL aliquots of stock peptide solutions can always be used to prepare fresh dilutions when needed, when there is any doubt that the peptides in a dilute solution may have degraded.

Table 2: Stock and dilute solutions of peptides

	MRFA	Brad. 1-9	Brad. 2-9	α-Endo.	β-Endo.*	γ-Endo.
Stock:	1 mg/300 μL 6.37 $\text{nmol}/\mu\text{L}$	1 mg/250 μL 3.78 $\text{nmol}/\mu\text{L}$	1 mg/250 μL 4.44 $\text{nmol}/\mu\text{L}$	1 mg/250 μL 2.29 $\text{nmol}/\mu\text{L}$	-----	1 mg/250 μL 2.15 $\text{nmol}/\mu\text{L}$
Dil 1	10 μL stock + 975 μL A: 63.7 $\text{pmol}/\mu\text{L}$	25 μL stock + 975 μL A: 94.4 $\text{pmol}/\mu\text{L}$	25 μL stock + 975 μL A: 111 $\text{pmol}/\mu\text{L}$	25 μL stock + 975 μL A: 57.3 $\text{pmol}/\mu\text{L}$	----	25 μL stock + 975 μL A: 53.8 $\text{pmol}/\mu\text{L}$
Dil 2 (test peptide)	-----	10 μL Dil 1 + 890 μL A: 1.05 $\text{pmol}/\mu\text{L}$	10 μL Dil 1 + 990 μL A: 1.11 $\text{pmol}/\mu\text{L}$	10 μL Dil 1 + 490 μL A: 1.15 $\text{pmol}/\mu\text{L}$	-----	10 μL Dil 1 + 490 μL A: 1.076 $\text{pmol}/\mu\text{L}$
Dil 3 (1/10 Dil 1)	10 μL Dil 1 + 90 μL A: 6.37 $\text{pmol}/\mu\text{L}$	10 μL Dil 1 + 90 μL A: 9.44 $\text{pmol}/\mu\text{L}$	10 μL Dil 1 + 90 μL A: 11.1 $\text{pmol}/\mu\text{L}$	10 μL Dil 1 + 90 μL A: 5.73 $\text{pmol}/\mu\text{L}$	-----	10 μL Dil 1 + 90 μL A: 5.38 $\text{pmol}/\mu\text{L}$

Notes:

* Not yet available upon writing this report.

The MRFA solutions were prepared using the stock MRFA solution made for calibration.

A glass syringe was used for addition of solvent A, because of its acidity, but a pipet tip would probably not cause any real problem. Some users are concerned that degradation of the pipet tip by the acid will result in the addition of unwanted polymers into the analysis.

Sample calculations (bradykinin 1-9):

A. Stock:

$$(1 \times 10^{-3} \text{ g} / 1059.55 \text{ g mol}^{-1}) / (250 \times 10^{-6} \text{ L}) = 3.78 \times 10^{-3} \text{ M} \\ = 3.78 \text{ mM} = 3.78 \text{ nmol}/\mu\text{L}$$

B. Dil. 1:

$$(25 \mu\text{L} \times 3.78 \text{ nmol}/\mu\text{L}) / 1000 \mu\text{L} = 0.0945 \text{ nmol}/\mu\text{L} = 94.5 \text{ pmol}/\mu\text{L}$$

C. Dil 2:

$$(10 \mu\text{L} \times 94.5 \text{ pmol}/\mu\text{L}) / 900 \mu\text{L} = 1.05 \text{ pmol}/\mu\text{L}$$

D. Dil. 3:

$$(10 \mu\text{L} \times 94.5 \text{ pmol}/\mu\text{L}) / 100 \mu\text{L} = 9.45 \text{ pmol}/\mu\text{L}$$

Notes:

- 1) Since sample loop volumes are typically in the μL range, and the amounts of peptides injected are typically in the picomole range (1×10^{-12} mol), concentration units of pmol/ μL are handy. For reference, pmol/ μL = uM and nmol/ μL = mM.)
- 2) For a 15 cm x 75 μm C18 column, 1 picomole (1×10^{-12} mole) of protein is on the upper edge of the amount that can be used without overloading the column. It was estimated that 5 pmol would be suitable for the 15 cm x 150 μm column to be used for the analyses.

C. Preparation of mixtures of peptides for testing peptide separation

To test the chromatography of all the peptides together, a mixture of four peptides (the bradykinin peptides plus alpha- and gamma-endorphin) was prepared using the first dilution of each peptide. The resulting mixture would load about 5 pmol of each peptide onto the column (with a 5 μL sample loop).

Table 3: Mixture of peptides for 5 pmol on-column

Peptide	Dil 1 concentration	μL added	Final concentration	Moles on-column (5 μL loop)
Bradykinin 1-9	94.4 pmol/ μL	12	1.13 pmol/ μL	5.65
Bradykinin 2-9	111 pmol/ μL	10	1.11 pmol/ μL	5.55
α-Endorphin	57.3 pmol/ μL	20	1.146 pmol/ μL	5.73
γ-Endorphin	53.8 pmol/ μL	20	1.076 pmol/ μL	5.38

To obtain a final volume of 1000 μL the above amounts of each peptide were added to ~938 μL of solvent A.

Since the peak shapes indicated that 5 pmol of peptide was somewhat overloading the column, a peptide mixture was prepared that would produce about $\frac{1}{2}$ pmol/peptide (500 fmol/peptide) on-column with a 5 μL sample loop. For this mixture MRFA (MW = 523.2), was also included since previous results showed that it eluted in a separate peak before the other four.

Table 4: Mixture of peptides for 0.5 pmol on-column

Peptide	Dil 3 concentration	μL added	Final concentration	Moles on-column (5 μL loop)
Bradykinin 1-9	9.44 pmol/ μL	12	0.113 pmol/ μL	0.566 pmol
Bradykinin 2-9	11.1 pmol/ μL	10	0.111 pmol/ μL	0.555 pmol
α-Endorphin	5.73 pmol/ μL	20	0.115 pmol/ μL	0.573 pmol
γ-Endorphin	5.38 pmol/ μL	20	0.108 pmol/ μL	0.538 pmol
MRFA	6.37 pmol/ μL	17	0.108 pmol/ μL	0.541 pmol

To obtain the final volume, the above amounts of peptides were added to $\sim 921 \mu\text{L}$ of solvent A.

IV. Chromatography: Gradient Method Development

A. Testing chromatography of each peptide separately

To determine the m/z values (charge states) observed for each peptide and to approximate the percentage of solvent B at which it elutes, each peptide was analyzed using an HPLC method with 30 minutes of equilibration at 10% solvent B followed by a 10 minute load period and a 30 minute gradient from 10-90% B. (Petide_test080415_01.mts) The following results were obtained.

Table 5: Retention times and % solvent B of peptides in 10-90%B gradient

Peptide	R.T. (min)	%B at R.T.
Bradykinin 1-9	26.29	$\sim 53\%$
Bradykinin 2-9	26.86	$\sim 55\%$
α-Endorphin	31.33	$\sim 64\%$
γ-Endorphin	34.47	$\sim 75\%$

B: Gradient method for peptide separation

Based on the elution information obtained by running each peptide separately, a gradient method was developed that might serve to separate the peptides, with 30 minutes of equilibration at 10% B, followed by a load step and gradient as follows:

RUN TIME	Solvent B
0-10 minutes (load step)	10%
10-15 minutes	10-40%
15-35 minutes	40-70%
35-40 minutes	70-90%
40-50 minutes	90%

(The flow rate was 500 nL/min. throughout.)

Name: Peptide_Test080415_03.mts

This method proved to reproducibly separate the four peptides, as shown in the following table of retention times and the following chromatogram.

Table 6: Peptide retention times and m/z values observed in gradient method

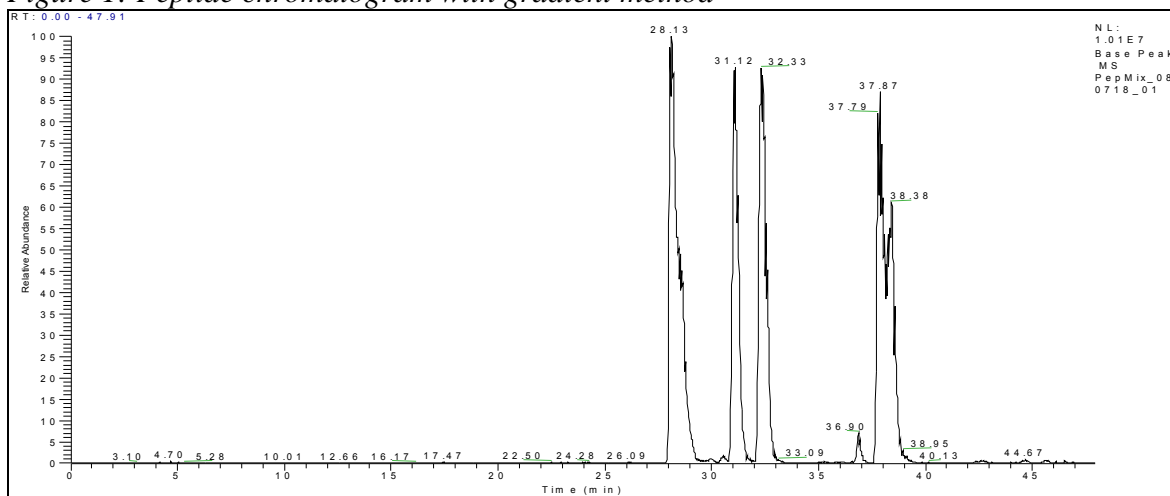
Name	R.T.				Charge	m/z
	Run 1	Run 2	Run 3	Run 4		
α-Endorphin	33.60	33.29	33.67	32.33	2	873.9
β-Endorphin *	-	-	-	-	-	-
γ-Endorphin	37.31	38.17	n/a	37.87	2	930.4
Bradykinin1-9	28.69	29.61	28.86	28.13	2	531.2
Bradykinin2-9	31.76	31.61	31.89	31.12	2	453.2
MRFA			26.47		1	523.9

Notes:

Due to a bubble problem, there was no peak for gamma-endorphin in the third run. The MRFA was observed inadvertently, when switching to a syringe that had been used for calibration. The retention times are not exactly comparable, since the collection of mass spec data was sometimes triggered by hand, etc.

* Not yet available as of writing this report.

Figure 1: Peptide chromatogram with gradient method



File name: PepMix_080718_01.raw

The four large peaks, in order of elution, are bradykinin1-9, bradykinin 2-9, alpha-endorphin and beta-endorphin. The fourth, smaller peak represents the oxidized form of beta-endorphin.

Note: To obtain a good image of a chromatogram or spectrum from Xcalibur Qual Browser, first select the cell (so the “thumbtack” for that cell is green, then go to the Edit pulldown menu and select “Copy Cell”. This will put the image into the computer’s copy/paste buffer. Then do a “control V” or select “Paste” in a word processor program, and the image will be pasted into the document. This produces a better image than screen capture.

C. Percentages of oxidized endorphin peptides

Based on our calibration experience, there was concern that peptides containing methionine residues may be oxidized at the spray nozzle. The areas of peaks representing oxidized and unoxidized endorphin peptides were determined to see if a significant percentage of oxidized peptides were present.

Table 7: Percentages of oxidized endorphin peptides

Peptide	Non-oxidized peak area	Oxidized peak area	Percentage oxidized
α -Endorphin	219293661	2786728	1.3%
γ -Endorphin	327708611	7765457	2.3%

These peak areas were obtained from file PepMix_080718_01.raw using 7 points boxcar smoothing and the Genesis peak algorithm.

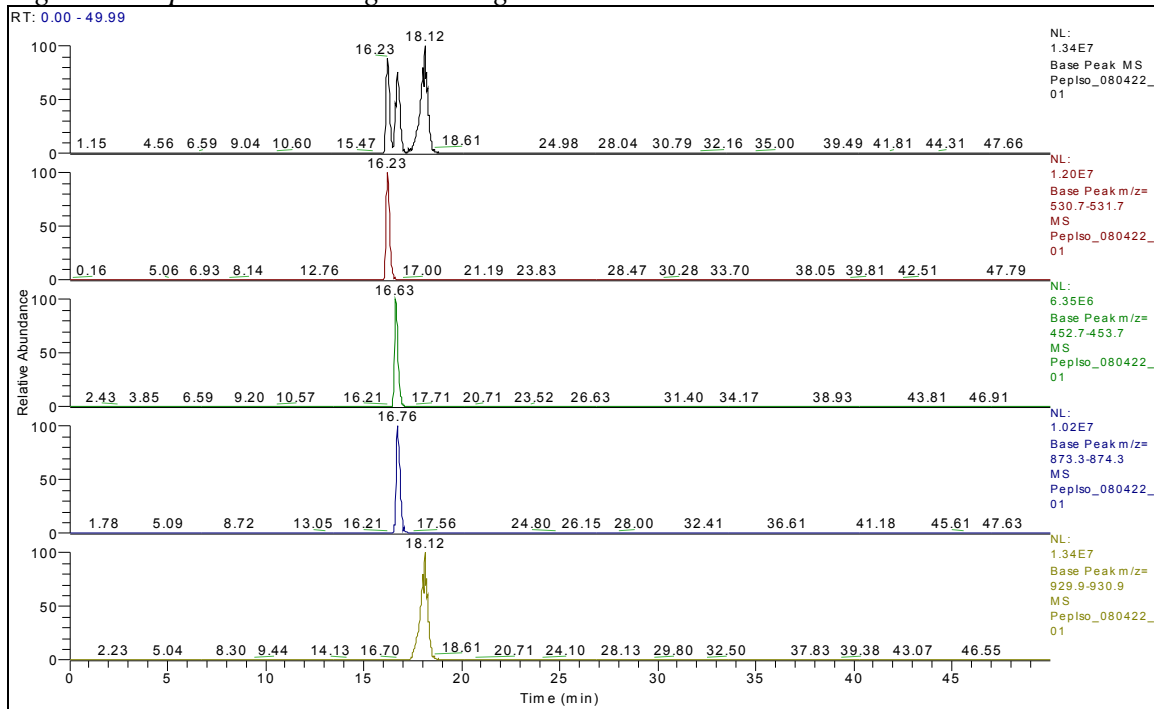
The percentages were obtained by dividing the area of the oxidized peak by the sum of the oxidized and non-oxidized peaks and multiplying by 100.

It is normal to see a small percentage of oxidized methionine residues in any peptide sample; therefore, there does not appear to be any untoward level of peptide oxidation.

V. Chromatography: Isocratic Method Development

Based on the approximate percentages of solvent B at which the various peptides eluted (Table 5), an initial guess was made that the peptides might be separated on an isocratic method of 60% B. The following chromatogram resulted from the use of this method, where the first 10 minutes are the load time:

Figure 2: Peptide chromatogram using isocratic method



Method name: Peptide_Test080415_04.mts

File: PepIso_080422_01.raw

The top window shows the overall base peak chromatogram, while the 2nd through 5th windows represent the peaks for Bradykinin1-9, bradykinin 2-9, alpha-endorphin and beta-endorphin, respectively.

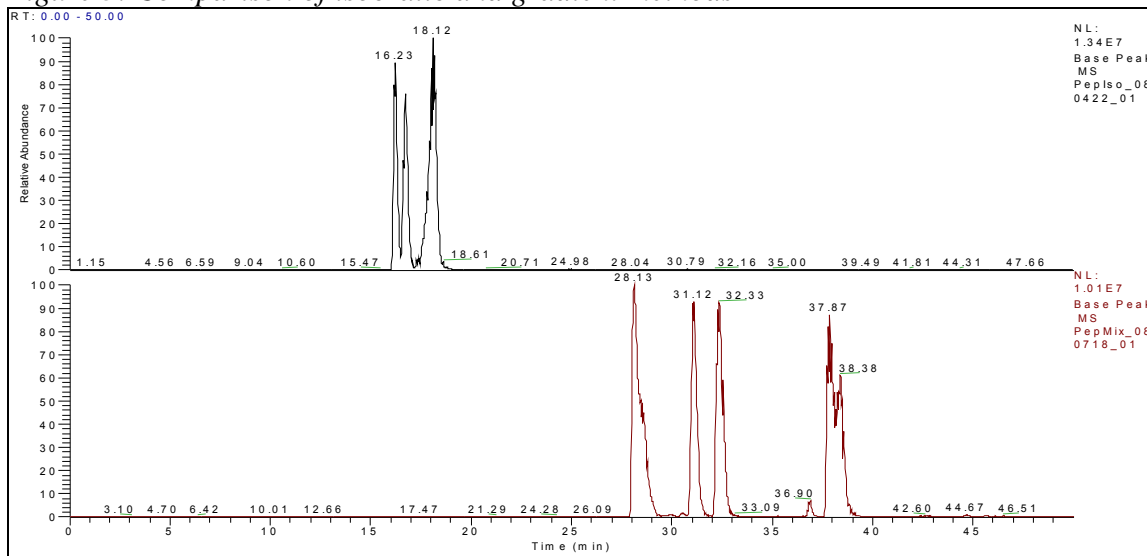
The chromatogram and the following table of retention times show that the method succeeded in producing three distinct peaks.

Table 8: Retention times of peptides in isocratic method:

Name	R.T.	m/z
α-Endorphin	16.76	873.8
β-Endorphin *	-	-
γ-Endorphin	18.12	930.4
Bradykinin1-9	16.23	531.2
Bradykinin2-9	16.63	453.2

For comparison, the elution times of the gradient method and the isocratic method are compared in the following figure:

Figure 3: Comparison of isocratic and gradient methods



The top window shows the base peak chromatogram for the isocratic method, while the bottom window shows the base peak chromatogram for the gradient method. Both include an initial 10-minute load period. Files:

PepIso_080422_01.raw, PepMix_080718_01.raw

This comparison indicates that for a three-nozzle method, overlapping elution could be achieved by starting one isocratic method at ~12 minutes into the gradient method, and starting a second isocratic method at ~24 minutes into the gradient method.

VI. Issues Encountered

A number of issues were encountered during the development of the peptide methods, as enumerated below:

A. Spray problems

Frequent spray problems (bubbling) were encountered at first, which turned out to be due to a leaky capillary junction between the column and the nozzle.

B. Pipettor accuracy/reproducibility issues

Some inconsistencies were encountered in measurement using the pipettors. For example, a 250 uL aliquot did not necessarily result in 10 25 uL aliquots using a smaller-volume pipettor.

C. Conversion dynode turning off

For some reason, the conversion dynode appears to turn off when running an instrument method using Xcalibur (when opening Tune following a run, it is off). I plan to investigate this problem and get back to you on possible solutions.

D. Lack of low-mass ions

Following the calibration of the LCQ Advantage, it appears that the normal high-signal-level solvent noise ions up to about 300 m/z no longer appear. I plan to investigate this problem and get back to you about possible solutions. One possibility is to re-calibrate making sure that the conversion dynode is on during calibration, in case the problem was caused by calibration being performed while it was accidentally turned off.

VII. Conclusion

Some basic peptide chromatography methods were developed that would meet the initial needs of Phoenix S&T. These methods show that the XtremeSimple HPLC system is working. The four peptides that were tested are separable on a gradient method, and so can be used for column QC testing. The isocratic method, combined with the gradient method, should suffice for an experiment combining two isocratic LCs with a gradient method for use in the ASMS poster. Some mass spectrometer issues were encountered, however, that need further investigation.

Appendix:

Peptide chromatography usually involves injecting femtomole to picomole amounts of analytes; I have even read of zeptomole levels being detected in some papers. The following table may prove useful.

Prefixes for decimal multiples of units and parts of units

Factor	in full	in words	SI prefix	SI symbol
1.0E+24	large 1 000 000 000 000 000 000 000 000	septillion	yotta	Y
1.0E+21	1 000 000 000 000 000 000 000 000	sextillion	zetta	Z
1.0E+18	1 000 000 000 000 000 000 000	quintillion	exa	E
1.0E+15	1 000 000 000 000 000 000	quadrillion	peta	P
1.0E+12	1 000 000 000 000 000	trillion	tera	T
1.0E+9	1 000 000 000	billion	giga	G
1.0E+6	1 000 000	million	mega	M
1.0E+3	1 000	thousand	kilo	k
1.0E+2	100	hundred	hecto	h
1.0E+1	10	ten	deka	da
1.0E 0	ref. 1	initial value	one	-
1.0E-1	0.1	tenth	deci	d
1.0E-2	0.01	hundredth	centi	c
1.0E-3	0.001	thousandth	milli	m
1.0E-6	0.000 001	millionth	micro	μ
1.0E-9	0.000 000 001	billionth	nano	n
1.0E-12	0.000 000 000 001	trillionth	pico	p
1.0E-15	0.000 000 000 000 001	quadrillionth	femto	f
1.0E-18	0.000 000 000 000 000 001	quintillionth	atto	a
1.0E-21	0.000 000 000 000 000 000 001	sextillionth	zepto	z
1.0E-24	small 0.000 000 000 000 000 000 000 001	septillionth	yocto	y

From: <http://www.sengpielaudio.com/ConvPrefe.htm>

With an LTQ mass spectrometer, which is much more sensitive than an LCQ, it is easily possible to detect femtomoles of peptides.

Calculation of tubing lengths for hand-made sample loops

The upchurch.com site has a handy “Conversion Tools” page under “Tech Center” pull-down menu. This has a conversion tool by which you can calculate the length of tubing you need to make your own sample loops, for example.