Freebasing of Peptide Salts and the Removal of Acidic Ion-Pairing Reagents from Fractions after HPLC Purification

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Introduction

Fast, effective removal of acidic ion-pairing reagents from product fractions after HPLC purification is essential for peptides with limited acid stability. VariPure™ IPE devices are ideal as they are simple and inexpensive to use and no additional instrumentation is required.

The use of acids in low concentrations to aid peptide solubility and improve the HPLC separation of peptides is well known. The most commonly used of these ion-pair reagents is 0.1% TFA in mixtures of water and acetonitrile, but acetic acid and formic acid may also be used. When the synthetic peptide is purified using preparative HPLC with such eluent systems, the resulting product fractions will contain the acidic ion-pair reagent. If these fractions are then lyophilized, the acid concentration will increase and the synthetic peptide may undergo hydrolysis or other degradation, thereby compromising purity and recovery. The removal of the acidic ion-pair reagent can be accomplished in several ways, including performing a second HPLC run as a "polishing" step. A simpler way is to use a gravity flow SPE-like device.

Ion-pair extraction – VariPure IPE

Gravity flow SPE is a simple and powerful technique, which can be used in sample preparation both pre- and post-HPLC. VariPure IPE is a specially engineered functional sorbent that will sequester acidic molecules from a wide range of solvent systems. The acid removal is mediated by anion exchange groups on the particle; the counter ions on these groups convert to CO_2 and water, thus providing the entropy drive towards effective freebasing, Figure 1. It is an effective technique that is amenable to high throughput applications and scale-up. The overall process has the following advantages:

- Capacity independent of acetonitrile concentration
- Gravity flow
- Pre-packed cartridges or loose media
- Compatible with all acidic HPLC eluents

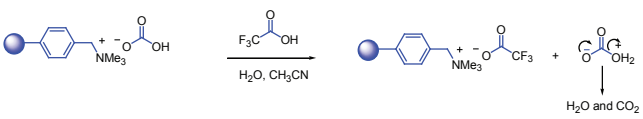


Figure 1. Reaction scheme showing the sequestration of TFA using VariPure IPE.

The removal of TFA from preparative HPLC fractions

The efficacy of removing TFA from HPLC fractions using VariPure IPE was investigated. A series of 0.1% TFA HPLC solutions, varying in percentage of water and acetonitrile, were prepared and 2 mL aliquots passed through a VariPure IPE device. The capacity of the media for TFA removal was determined by measuring the pH of every 2 mL aliquot. When the pH changed from ~7.0 to ~2.5 the medium was considered to be exhausted and the total volume of solution collected was measured. In order to cover a good range of possible HPLC-eluent systems, a series of 0.1% TFA standard solutions were made with 0, 20, 40, 80 and 100% acetonitrile in water. Figure 2 shows a plot of the theoretical volume of 0.1% TFA that can be quenched according to elemental loading of the resin, and the experimental volumes actually quenched. It can be seen that the experimental capacity is reduced slightly from the theoretical maximum when the acetonitrile concentration exceeds 20%. It is recommended that VariPure IPE be used in a two-fold molar excess to ensure complete removal.

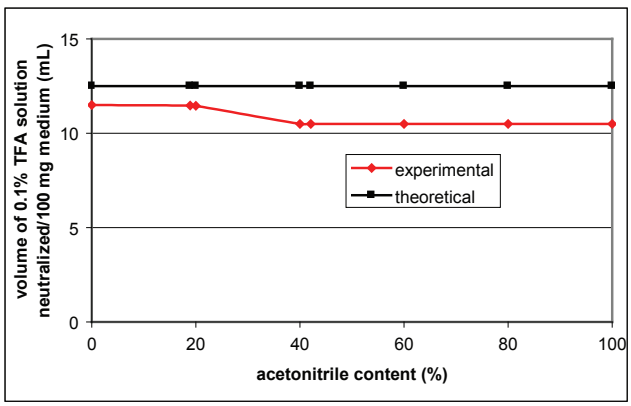


Figure 2. Effect of acetonitrile content on the volume of 0.1% TFA solution neutralized, expressed as mL/100 mg of medium.

Freebase formation of isolated peptides

In some instances it may be necessary to freebase a peptide once it has been isolated, after lyophilization. If an organic acid has been used during purification of the peptide then it will often be isolated as a salt. Peptides often contain

multiple basic sites, which will allow the formation of multiple acid ion-pairs. In order for effective freebasing of such a material, the practical approach is slightly different. Firstly, the peptide needs to be resolvated in an appropriate solvent and then to be passed through the IPE device. The VariPure IPE media has broad solvent compatability, allowing flexibility in solvent selection.

For most applications an acetonitrile:water system can be used, but this is not a pre-requisite. Polar protic solvents such as methanol and ethanol can all be used if preferred, depending on the solubility of isolated peptide salt.

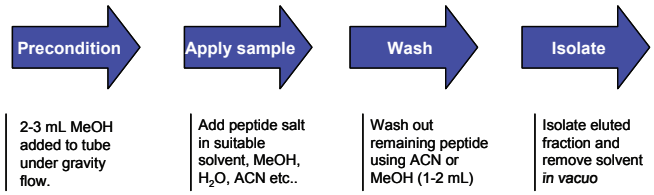


Figure 3. Process flow diagram for TFA removal.

A range of small peptides of varying functionality was isolated as TFA salts, then re-suspended in 50:50 ACN/H_2O and passed through a preconditioned VariPure™ IPE tube using the method described in Figure 3. The overall recoveries of the peptides were good, as shown in Table 1. The ^{19}F NMR spectra showed the complete removal of TFA after treatment. Basic polar and non-polar peptides, and polar acids are all compatible with this method. However non-polar peptide acids, or aspartic acid or glutamic acid containing peptides may experience lower recoveries due to other ion-exchange processes occurring with the media.

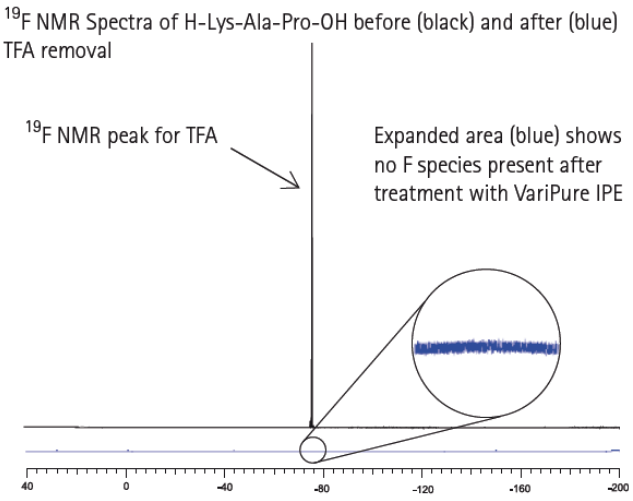
Table 1. Recoveries of peptides after VariPure IPE treatment.

Peptide	Type	Recovery %
H-Lys-Ala-Pro-OH	Basic polar acid	71
H-Lys-Arg-Ser-Arg-OH	Very basic polar acid	90
H-Tyr-Gly-Gly-Phe-Leu-NH_2	Non-polar amide	82
H-Pro-Gln-Arg-Phe-NH_2	Basic polar amide	74
H-Leu-Ala-Val-Phe-Ile-Gly-NH_2	Very non-polar amide	88

Validation of full acid removal: ^{19}F NMR study

The best method to show the full sequestration of TFA from a sample is to use some form of fluorine atom detection. Elemental decomposition analysis would be unsuitable for this type of application, as it would count all fluorine atoms, independent of chemical environment. In our studies we employed ^{19}F NMR as an excellent technique for the determination of acid removal. In the NMR spectra, shown in Figure 4, a sample of H-Lys-Ala-Pro-OH (TFA salt) was analyzed before and after VariPure IPE treatment. The absence of the ^{19}F chemical shift at ~-77.8 ppm in the second analysis was indicative of complete TFA removal.

Figure 4. ^{19}F NMR showing full TFA sequestration post VariPure IPE treatment.



Conclusions

VariPure IPE is highly effective at removing ion-pairing acids such as TFA from organic solutions. The user has the choice of removing ion-pairing reagents from the HPLC fractions or from the isolated peptide. The gravity-flow SPE-type device is very amenable to high throughput applications and scale-up, saving time and resources when compared to techniques requiring more complex instrumentation.

These data represent typical results.
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Application Note SI-02449