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Non-destructive quantification of peptide trifluoroacetates[1]

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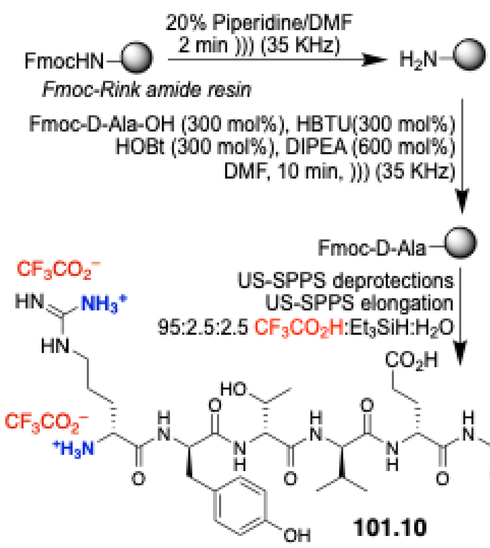
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Abstract

Trifluoroacetic acid (TFA) is widely used in peptide science but can influence conformation, cytotoxicity, and IR spectroscopic analysis. Quantification of TFA often relies on accurate weighing of peptide salts. A rapid, non-destructive NMR method for quantifying TFA in peptides without weighing is now described using correlation of ^{1}H and ^{19}F integrations with fluorinated alcohols as internal standards in D_2O.

TFA in peptide science and medicine

Trifluoroacetic acid (TFA) is central in peptide synthesis, purification and biological analysis.[2]

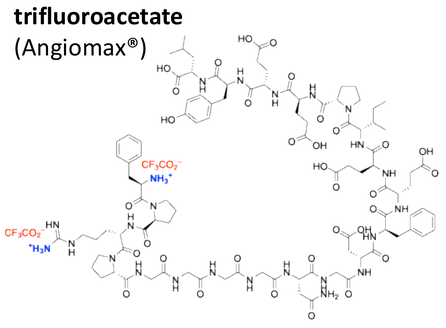


Versatile and volatile, TFA is key to Fmoc/*t*-Bu peptide synthesis,[2] as illustrated by the synthesis of allosteric interleukin-1 receptor modulator **101.10** (H-D-Arg-D-Tyr-D-Thr-D-Val-D-Glu-D-Leu-D-Ala-NH_2) using ultra-sound solid phase peptide synthesis (US-SPPS).[3-4]

A per- and polyfluoroalkyl substance (PFAS), TFA is a regulated environmental toxin,[5] and in trace amounts, may alter peptide conformation, cytotoxicity, and pharmacological profile.[6]

Common in biological studies,[7] two peptide trifluoroacetates are U.S. Food and Drug Administration (FDA) approved: Bivalirudin TFA (Angiomax®) for treating thrombotic disorders;[8] Corticorelin ovine TFA (Acthrel®) for pituitary–adrenal function evaluation.[9]

Bivlirudin trifluoroacetate (Angiomax®)

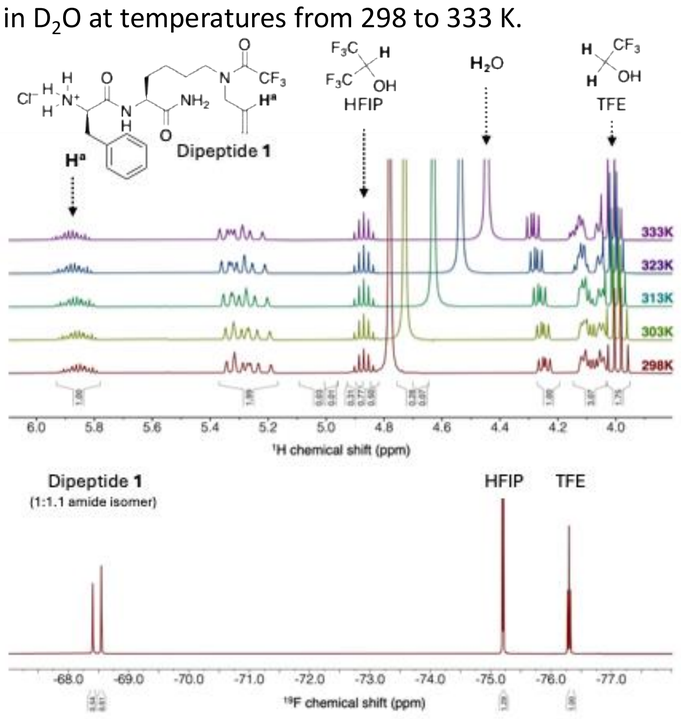


Issues in peptide TFA quantification

Commonly used methods for TFA quantification are often destructive and require accurate sample weighing: e.g., FT-IR,[10] liquid-phase chromatography,[11] gas chromatography,[12] ion chromatography,[13] and capillary electrophoresis (CE).[14]

Benchmarking HFIP and TFE as NMR probes

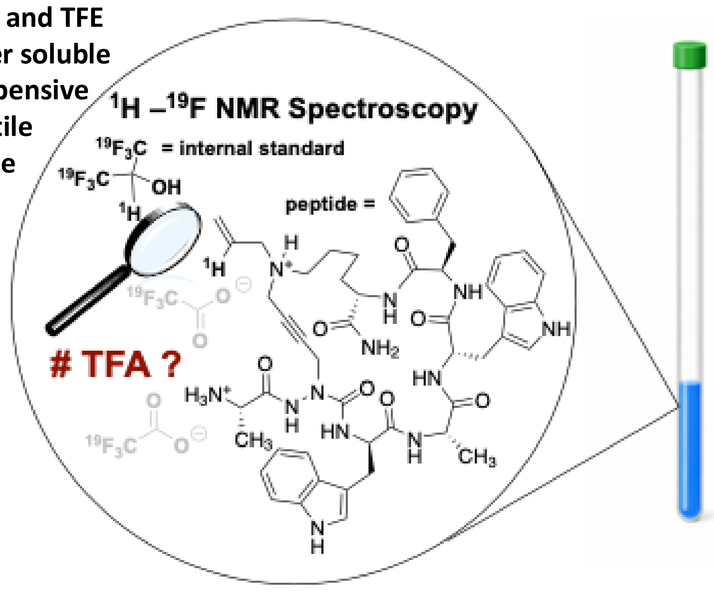
Dipeptide **1** [HCl•H-D-Phe-Lys(Tfa,allyl)-NH_2] is used to benchmark hexafluoroisopropanol (HFIP) and trifluoroethanol (TFE) in D_2O at temperatures from 298 to 333 K.



TFA quantification by NMR Correlation

Solution : Peptide to TFA ratios are quantified using nuclear resonance magnetic (NMR) spectroscopy and correlation of ^{1}H and ^{19}F signal integrations employing internal standards: hexafluoroisopropanol (HFIP) and trifluoroethanol (TFE). Without signal overlap, TFA equivalents are calculated from $^{1}H/^{19}F$ integration ratios (Eq. 1, Method A).

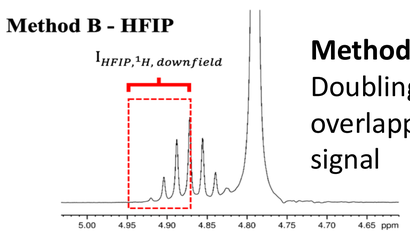
HFIP and TFE
- ✓ water soluble
- ✓ inexpensive
- ✓ volatile
- ✓ stable



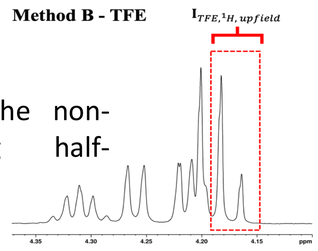
$$Eq.\,1:\ TFA\ equivalents = \frac{^{1}H\ probe}{^{1}H\ peptide} \Big/ \frac{^{19}F\ probe}{^{19}F\ peptide}$$

Integration correction strategies

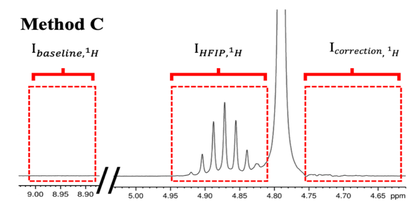
^{19}F signals are readily integrated.
^{1}H quantification is affected by temperature and peak overlap.
Correction methods can overcome signal overlap.



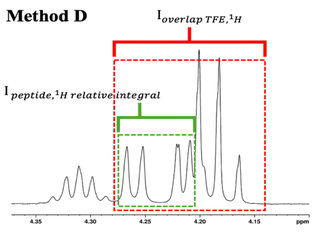
Method B: Doubling the non-overlapping half-signal



Method C: HFIP integral correction using H_2O symmetry

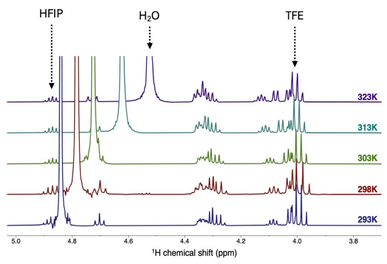


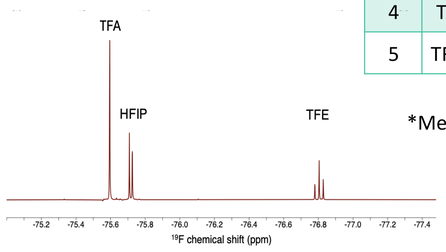
Method D: TFE integral correction by peptide integral subtraction



Studies of temperature and equiv. of standard

Influences of temperature (293–323 K) and internal standard equivalents on TFA/peptide ratio were studied using the TFA salt of **101.10** (**1**, H-rytvela-NH_2).





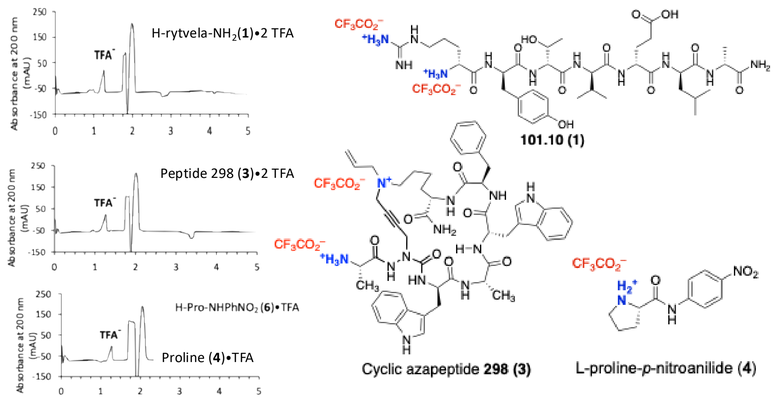
Entry	Standard (equiv.)	Temp. (K)	Method		
			A	B	C/D*
1	HFIP (1)	298	1.86	1.87	**1.96**
2	HFIP (5)		1.90	1.91	**2.04**
3	HFIP (1)	323	**1.97**	1.96	1.33
4	TFE (3)	298	2.45	2.15	**2.02**
5	TFE (14)		2.23	2.11	**2.05**

*Methods C and D were respectively used with HFIP and TFE

Higher internal standard equivalents (5 equiv. for HFIP and 14 equiv. for TFE) improved quantification accuracy.
For HFIP, Method A at **323 K provided the highest accuracy**, likely due to the displacement of the H_2O peak in D_2O.

Comparable accuracy vs CE method

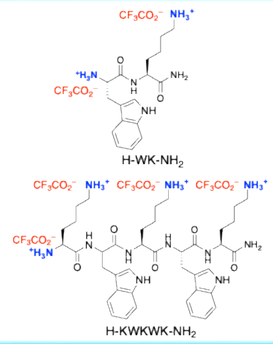
Benchmarked against the previously reported capillary electrophoresis (CE) method, NMR correlation spectroscopy shows comparable accuracy with differences arising from relative signal-based quantification versus weight-based absolute quantification.[14]



TFA Salt	Expected # TFA	TFA # by CE	TFA # by NMR (HFIP) Method C at 298 K	TFA # by NMR (TFE) Method A/D at 298 K
101.10•2 TFA	2	1.48	1.96	2.02 (D)
298 •2 TFA	2	1.43	2.04	2.04 (A)
Proline **4**•TFA	1	**0.99**	**0.99**	**0.94 (A)**

Quantification of peptides with multiple TFAs

Positive charge is abundant in antimicrobial peptides.[11] Model Lys/Trp peptides H-WK-NH_2 and H-KWKWK-NH_2 were made by US-SPPS and assessed using Method B with HFIP at 313 K to have 2.19 and 4.20 TFA equivalents, respectively.



Conclusions and Perspectives

- ✓ Non-destructive NMR correlation quantifies TFA in peptides effectively.
- ✓ HFIP and TFE proved reliable internal standards.
- ✓ Accuracy is contingent on equivalents of internal standards, temperature, and integration method.
- ➢ Applicable to diverse therapeutic peptides without sample destruction.

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Acknowledgements

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