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Certificate of Analysis

Reference Material

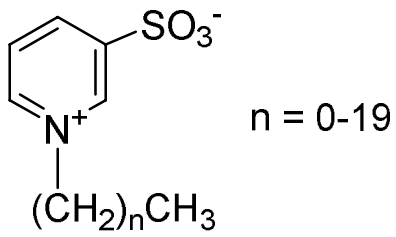
RM-RILC (Lot# 20140827)

Reference material for measurement of liquid chromatography retention indices

RM-RILC is a reference material (RM) designed for measurement of liquid chromatography (LC) retention indices (RIs) based on *N*-alkylpyridinium-3-sulfonates (NAPS) [1]. RM-RILC is a solution of 20 homologous NAPS compounds, in methanol with ~1% water, with RI values defined as 100 x (n +1). The RI values range from 100 to 2000.

Table 1: Concentrations of *N*-alkylpyridinium-3-sulfonates (NAPS) in RM-RILC.

Compound	µmol/L (15-30 °C)
NAPS n = 0-2	45 ± 5
NAPS n = 3-19	100 ± 10



***N*-alkylpyridinium-3-sulfonate (NAPS) standards**

Period of validity: 3 years from date of sale
Storage conditions: ~ +4 °C

Canada

Intended Use

Analytical methods based on liquid chromatography (LC) separation followed by ultraviolet absorbance (UV) or mass spectrometry (MS) detection are used for analysis of a wide range of substances. The identification of compounds present in samples frequently includes a match of chromatographic retention time of authentic chemical standards with those of putative compounds observed in a sample. However, the absolute retention times of analytes can be highly variable between different laboratories and instruments. Analysis of reference standards with each batch of samples increases the analytical workload and cost. In many cases, reference standards are not commercially available for all compounds of interest. A useful way to evaluate and report retention time data is through the use of "retention index (RI)" values [2]. In this procedure, a series of homologous reference compounds is co-injected with the sample. An interpolation of analyte retention times into a fitted curve of the plot of retention time versus RI value for the reference compounds produces a RI value for each analyte. Several different RI systems have been reported for use in LC-UV analysis [3] but the chemical standards used in those systems have a number of limitations such as poor response in LC-MS, incomplete coverage of the retention time range, or lack of commercial availability.

RM-RILC is intended for the measurement of RIs of various compounds in gradient elution reversed phase LC systems using MS or UV detection. This is accomplished by measuring the retention times of analytes in samples relative to the retention times of the NAPS standards. RM-RILC is also useful for predicting the retention time of analytes based on previously documented RI values and for testing analytical instrument performance for system suitability.

Instructions for Storage and Use

To ensure the stability of RM-RILC, ampoules should be stored in the dark at +4 °C. It has been observed that some of the longer chain NAPS compounds can precipitate or adsorb on glass surfaces if stored in a freezer.

Prior to opening, each ampoule should be equilibrated to room temperature for a period no less than 2 h and the contents mixed thoroughly. The ampoule should be opened at the pre-scored mark. Once an ampoule has been opened, the contents should be transferred to a screw-cap vial for further use and storage.

An aliquot of RM-RILC can be mixed or co-injected with samples. Alternatively, it can be run separately by itself each day if the instrument has good retention time reproducibility. The concentration of RM-RILC to use or the volume to inject depends on the internal diameter of the LC column and the sensitivity of the instrument. Typically, with a method using a 2 mm i.d. column and a moderately sensitive LC-MS system, only 0.1 to 0.5 μL needs to be injected for a good signal. Note that large injections of neat RM-RILC can cause band broadening of the early eluting NAPS compounds due to the high methanol content of the RM. For co-injection with the sample, a volume ratio of RM to sample of 1:5 to 1:25 should be suitable.

The NAPS standards [1] in RM-RILC have been designed with two permanently ionized functions (quaternary amine and sulfonate), which enhance detectability in LC-MS in both positive and negative ion modes. The NAPS standards also have a good UV chromophore with an absorbance maximum at 265 nm. In positive ion electrospray ionization MS, the NAPS molecules are protonated to form $[M+H]^+$ ions (Figure 1a). In negative ion mode, a formate attachment ion, $[M+HCOO]^-$, is formed (Figure 1c). Collision-induced dissociation gives common ions at *m/z* 160 and 79 in positive mode and *m/z* 80 in negative mode (Figures 1b and 1d). These can be created by using either a high orifice potential or MS/MS in a collision cell. The proposed fragmentation mechanisms are shown in Figure 2. Analysis can be conducted using selected ion monitoring or selected reaction monitoring using individual ion masses or mass transitions, respectively (Table 2). Alternatively, a high orifice voltage plus an *m/z* 160 → 160

transition at low collision energy may be used. Full scan MS methods, with or without alternating fragmentation scans, can also be used.
The NAPS standards show excellent chromatographic behavior in gradient elution reversed phase LC covering elution from 100% aqueous to 100% organic, thus allowing the measurement of RIs of very polar to very lipophilic compounds. Figure 3 shows the analysis of RM-RILC by LC-UV and LC-MS, respectively. The NAPS standards have an overall neutral charge state that will not vary significantly with changes in the mobile phase pH, so their retention times are not sensitive to mobile phase pH changes.

A plot of retention time vs. RI value (100 x carbon length) and a linear interpolation between points or a cubic spline fit results in a calibration curve such as that shown in Figure 4. The shape and slope of this line will vary depending on the LC pump, column length, mobile phase flow rate and the gradient conditions.

Preparation of RM-RILC

The NAPS standards were prepared by reacting a series of n-alkyl halides with 3-pyridinesulfonic acid, followed by purification with C18-silica column chromatography or solid phase extraction depending on the polarity of the NAPS. The structures of the synthesized compounds were confirmed by LC with high resolution-mass spectrometry (HRMS). Each NAPS gave the correct accurate $[M+H]^+$ and fragment ion masses within 2 ppm of calculated. The structures of a sub-set of the NAPS compounds were verified by proton nuclear magnetic resonance spectroscopy.

The RM-RILC solution was prepared by mixing stock solutions of the 20 individual NAPS standards and diluting in filtered (0.2 µm) and degassed methanol with approximately 1% water. The final RM solution was dispensed into glass ampoules pre-filled with argon and then immediately flame-sealed. Each ampoule contains approximately 0.75 mL of solution.

Analytical Methods and Value Assignment

Concentration information values were assigned to RM-RILC through a combination of data from quantitative NMR [4] on a sub-set of the NAPS compounds and by LC-UVD-CAD measurements on the final RM.

Homogeneity

A representative number of RM-RILC ampoules (n = 17) were selected from across the fill series and NAPS response was measured using LC-UVD. Results were evaluated using ANOVA and no significant heterogeneity was detected.

Stability

Stability studies have demonstrated good long-term stability of RM-RILC solutions stored in sealed ampoules at +4, +20 and +40 °C, with no detectable decomposition observed over a period of 1 year within the limits of uncertainty of the analytical method (LC-UVD).

Safety Instructions

The solution of NAPS compounds (RM-RILC) should be handled with due care using appropriate personal protective equipment. Inhalation and ingestion of methanol is harmful. Only qualified personnel should handle the solution and appropriate disposal methods should be used. Heavy gloves and eye protection should be used when opening the ampoule in the event the glass shatters. A safety data sheet (SDS) is available for RM-RILC.

Period of Validity

If stored unopened at the recommended storage conditions (~+4°C and in the dark), the assigned concentration of RM-RILC is valid for 3 years from the date of sale**.**

References

1. Quilliam MA, *Retention index standards for liquid chromatography*. US Patents 9,594,063 B2 (Mar. 14, 2017) and 10,228,356 B2 (Mar. 12, 2019). Quilliam MA, *Retention index standards for liquid chromatography*. Patent: US9,594,063 B2, Mar. 14, 2017.

2. Babushok VI (2015). Chromatographic retention indices in identification of chemical compounds. *TRAC - Trend Anal Chem* 69: 98-104.

3. Smith RM (1995). Retention index scales used in high-performance liquid chromatography. *J Chromatogr Libr* 57: 93-144.

4. Burton IW, Quilliam MA, Walter JA (2005). Quantitative ^{1}H NMR with external standards: use in preparation of calibration solutions for algal toxins and other natural products. *Anal Chem* 77: 3123-3131.

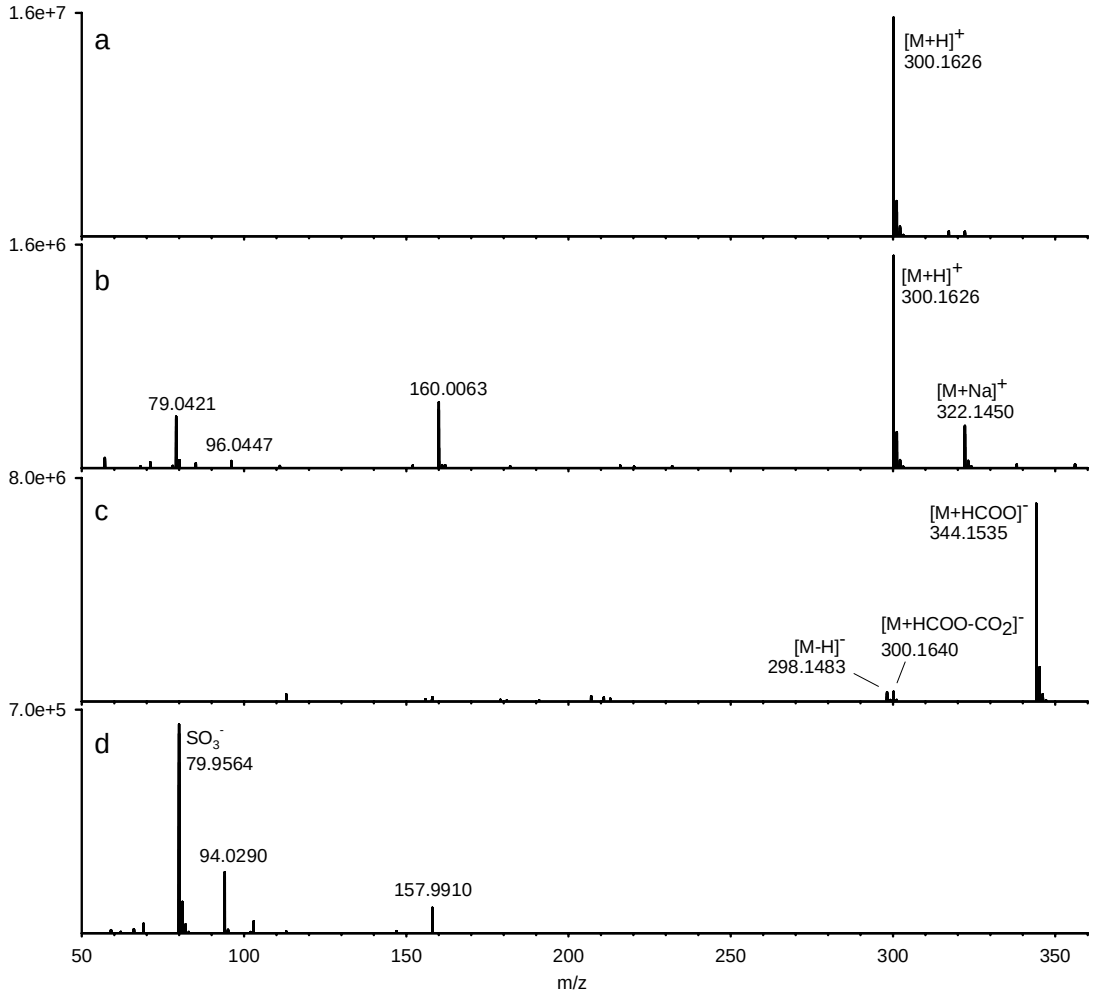


Figure 1: Positive (a, b) and negative (c, d) mass spectra of the C10-NAPS acquired on an Exactive Orbitrap MS without fragmentation (a, c) or fragmentation with HCD (b, d).

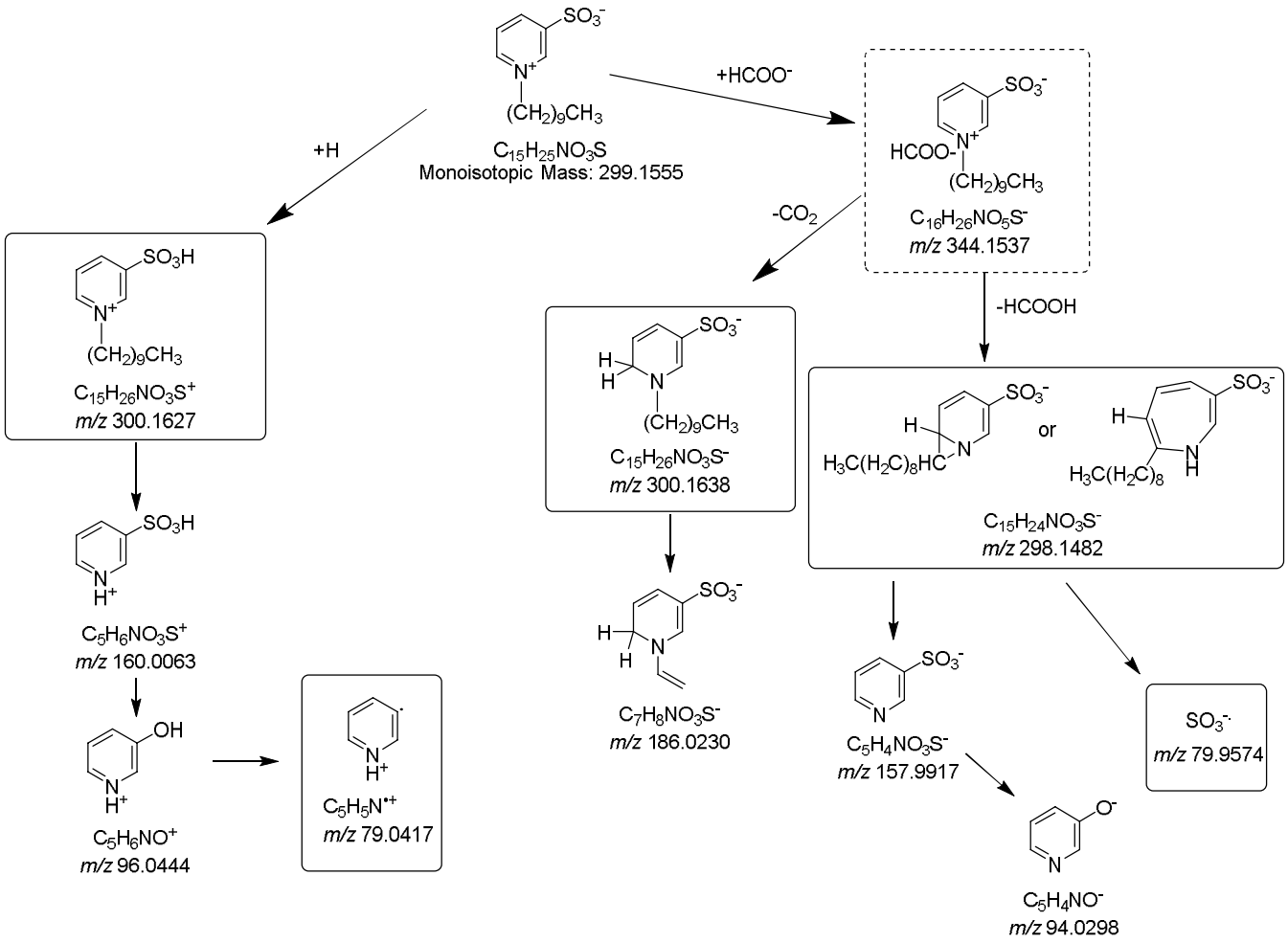


Figure 2: Ionization and fragmentation characteristics of the C10-NAPS.

Table 2: Mass spectral data for the NAPS standards.

		Elemental Composition						Positive Ion *m/z* values				Negative Ion *m/z* values				
RI Value	n	C	H	N	O	S	MW	$[M+Na]^+$	$[M+H]^+$	PosFrag1	PosFrag2	$[M+HCOO]^-$	$[M+H]^-$	$[M-H]^-$	NegFrag1	NegFrag2
100	0	6	7	1	3	1	173.19	196.0039	**174.0219**	92.0495		**218.0129**	174.0230	172.0074	157.9917	79.9574
200	1	7	9	1	3	1	187.22	210.0195	**188.0376**	160.0063	79.0170	**232.0285**	188.0387	186.0230	157.9917	79.9574
300	2	8	11	1	3	1	201.25	224.0352	**202.0532**	160.0063	79.0170	**246.0442**	202.0543	200.0387	157.9917	79.9574
400	3	9	13	1	3	1	215.27	238.0508	**216.0689**	160.0063	79.0170	**260.0598**	216.0700	214.0543	157.9917	79.9574
500	4	10	15	1	3	1	229.30	252.0665	**230.0845**	160.0063	79.0170	**274.0755**	230.0856	228.0700	157.9917	79.9574
600	5	11	17	1	3	1	243.32	266.0821	**244.1002**	160.0063	79.0170	**288.0911**	244.1013	242.0856	157.9917	79.9574
700	6	12	19	1	3	1	257.35	280.0978	**258.1158**	160.0063	79.0170	**302.1068**	258.1169	256.1013	157.9917	79.9574
800	7	13	21	1	3	1	271.38	294.1134	**272.1315**	160.0063	79.0170	**316.1224**	272.1326	270.1169	157.9917	79.9574
900	8	14	23	1	3	1	285.40	308.1291	**286.1471**	160.0063	79.0170	**330.1381**	286.1482	284.1326	157.9917	79.9574
1000	9	15	25	1	3	1	299.43	322.1447	**300.1628**	160.0063	79.0170	**344.1537**	300.1639	298.1482	157.9917	79.9574
1100	10	16	27	1	3	1	313.46	336.1604	**314.1784**	160.0063	79.0170	**358.1694**	314.1795	312.1639	157.9917	79.9574
1200	11	17	29	1	3	1	327.48	350.1760	**328.1941**	160.0063	79.0170	**372.1850**	328.1952	326.1795	157.9917	79.9574
1300	12	18	31	1	3	1	341.51	364.1917	**342.2097**	160.0063	79.0170	**386.2007**	342.2108	340.1952	157.9917	79.9574
1400	13	19	33	1	3	1	355.54	378.2073	**356.2254**	160.0063	79.0170	**400.2163**	356.2265	354.2108	157.9917	79.9574
1500	14	20	35	1	3	1	369.56	392.2230	**370.2410**	160.0063	79.0170	**414.2320**	370.2421	368.2265	157.9917	79.9574
1600	15	21	37	1	3	1	383.59	406.2386	**384.2567**	160.0063	79.0170	**428.2476**	384.2578	382.2421	157.9917	79.9574
1700	16	22	39	1	3	1	397.62	420.2543	**398.2723**	160.0063	79.0170	**442.2633**	398.2734	396.2578	157.9917	79.9574
1800	17	23	41	1	3	1	411.64	434.2699	**412.2880**	160.0063	79.0170	**456.2789**	412.2891	410.2734	157.9917	79.9574
1900	18	24	43	1	3	1	425.67	448.2856	**426.3036**	160.0063	79.0170	**470.2946**	426.3047	424.2891	157.9917	79.9574
2000	19	25	45	1	3	1	439.70	462.3012	**440.3193**	160.0063	79.0170	**484.3102**	440.3204	438.3047	157.9917	79.9574

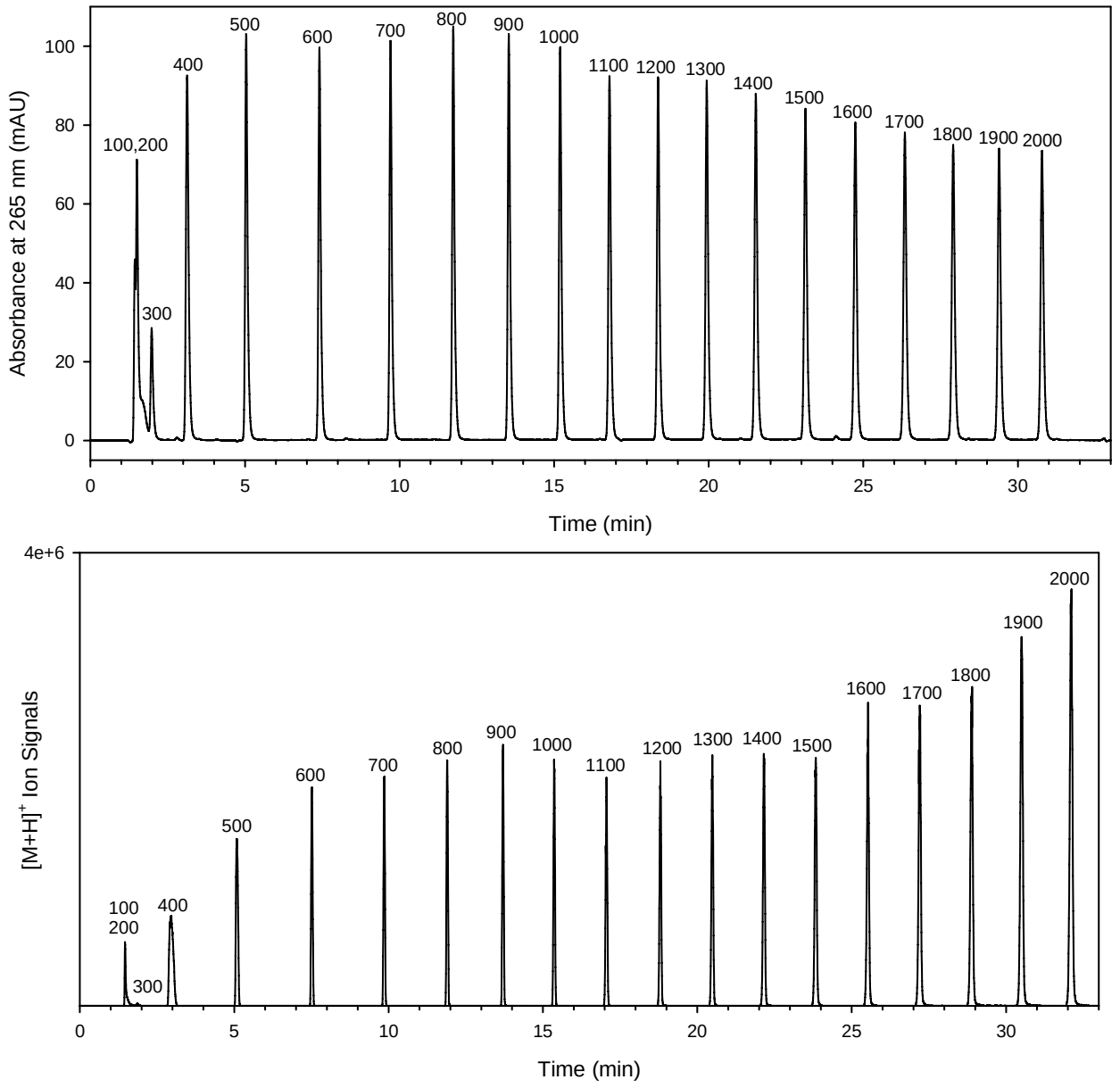


Figure 3: LC-UV (top) and LC-MS (bottom) analysis of RM-RILC. Peaks are labelled with the RI values of the 20 NAPS standards. LC conditions: 2.7 µm Poroshell 120Å SB-C18 column (150 × 2 mm); solvent A = H_2O with 50 mM HCOOH, 2 mM $HCOONH_4$; solvent B = CH_3CN with 50 mM HCOOH, 2 mM $HCOONH_4$; gradient = 5-100% B in 30 min; flow = 0.25 mL/min.

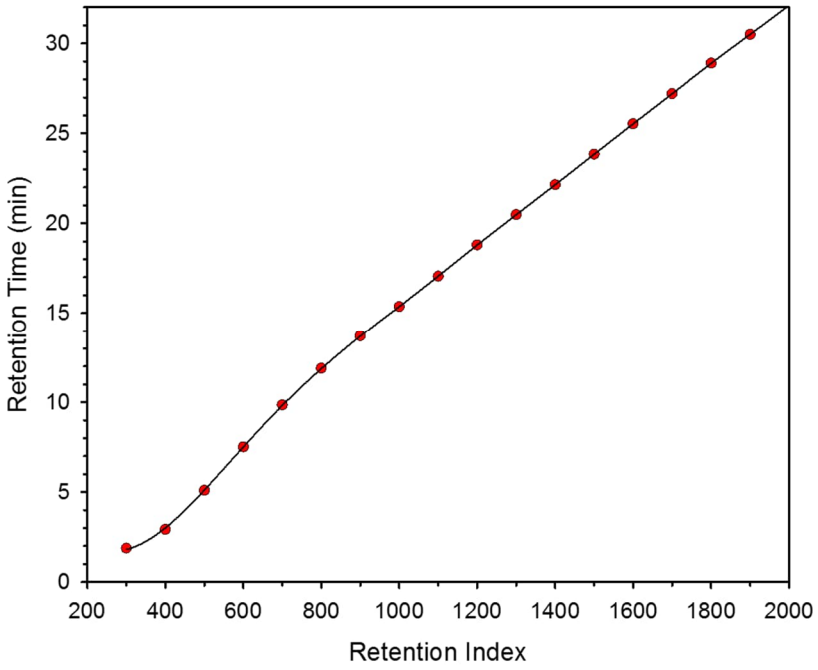


Figure 4: A plot of retention times versus RI values for NAPS standards. A linear interpolation or a cubic spline fit (shown here) results in a calibration curve for determining the RI values of analytes.

Acknowledgements

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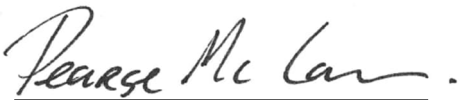
Approved by: 

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