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# A new retention index system for liquid chromatography- mass spectrometry

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# A New Retention Index System for LC-MS

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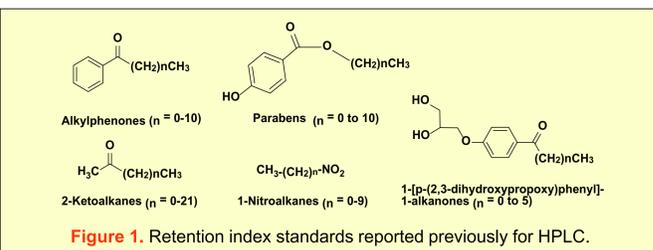
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Measurement Science and Standards

## Introduction

Chromatographic retention times (RTs) are invaluable for the identification of analytes in complex samples. Unfortunately, absolute RTs in liquid chromatography (LC) can be highly variable between different laboratories and instruments. Analysis of chemical reference standards is required with each batch of samples to allow a good match of RTs for conclusive identification. Development of modern LC-MS methods based on techniques like scheduled selected reaction monitoring (SRM) also requires the availability of standards to set retention windows and it is tedious and time consuming to establish a method.

A better way to report and catalog retention data is to use a retention index (RI) system. RI values are determined by measuring analytes' RTs relative to those of a series of homologous reference compounds. Ideally sample and RI standards should be co-injected. Several RI systems (Fig. 1) have been reported for use in LC-UV [1]. However, none of these are well suited to electrospray ionization (ESI) LC-MS as they exhibit low sensitivity. In addition, some of them are not suitable for the low RT range where polar compounds elute.

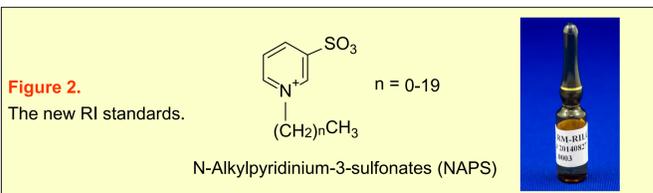


## Project Objectives

The following characteristics were selected for the new standards:

- High sensitivity in both positive and negative ion ESI-MS.
- Common fragment ion to facilitate MS detection.
- A strong UV chromophore for use in LC-UV.
- Overall neutral charge state independent of mobile phase pH.
- A homologous series spanning the widest RPLC gradient run.

The *N*-alkylpyridinium-3-sulfonate (NAPS) standards (Fig. 1) [2] have been designed with two permanently ionized functions (quaternary amine and sulfonate), which enhance detectability in mass spectrometry in both the positive and negative ion modes. They have an overall neutral charge state that will not vary with changes in the mobile phase pH, thus making their RTs less sensitive to pH changes in the mobile phase. The compounds also have a good UV chromophore with an absorbance maximum at 265 nm.

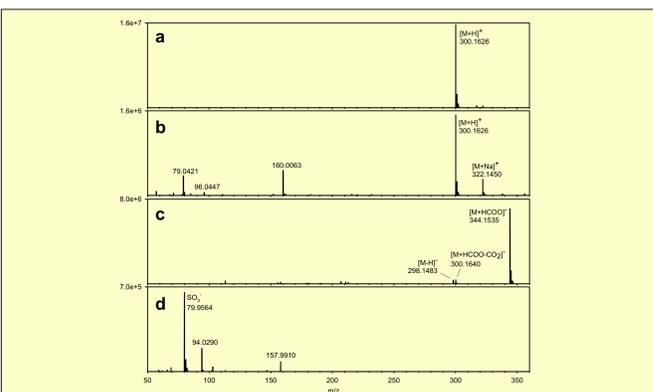


## Experimental

- **Synthesis:** reaction of alkyl halides with pyridinium-3-sulfonate.
- **Preparation of reference material (RM-RILC):** 20 standards at 100  $\mu$ M in  $\text{CH}_3\text{OH}$ ; packaged into flame-sealed glass ampoules.
- **Use of RM:** RM-RILC can be mixed or co-injected with a sample at 1 to 10% of total volume injected.
- **LC-UV:** Agilent 1290 system with DAD at 265 nm.
- **LC-MS:** Sciex QTRAP 4000 with ESI and Thermo Exactive Orbitrap with ESI or APCI sources. SRM used for Sciex; alternating full and HCD scans used for Orbitrap.
- **Columns:** 2 mm id x 5 or 15 cm packed with (a) 2.7  $\mu$ m Poroshell SB-C18; (b) 1.8  $\mu$ m Zorbax SB-C18; (c) 3  $\mu$ m Hypersil BDS-C8; (d) 2.5  $\mu$ m Luna C18(2)-HST; (e) 2.5  $\mu$ m Synergi Hydro RP.
- **Mobile phases:** 0.25 mL/min  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  + 50 mM  $\text{HCOOH}/2$  mM  $\text{HCOONH}_4$  (pH 3). Different pHs and  $\text{CH}_3\text{OH}$  also tested.
- **Curve fitting:** SRS1 Software LLC, <http://www.srs1software.com> (Excel macro)

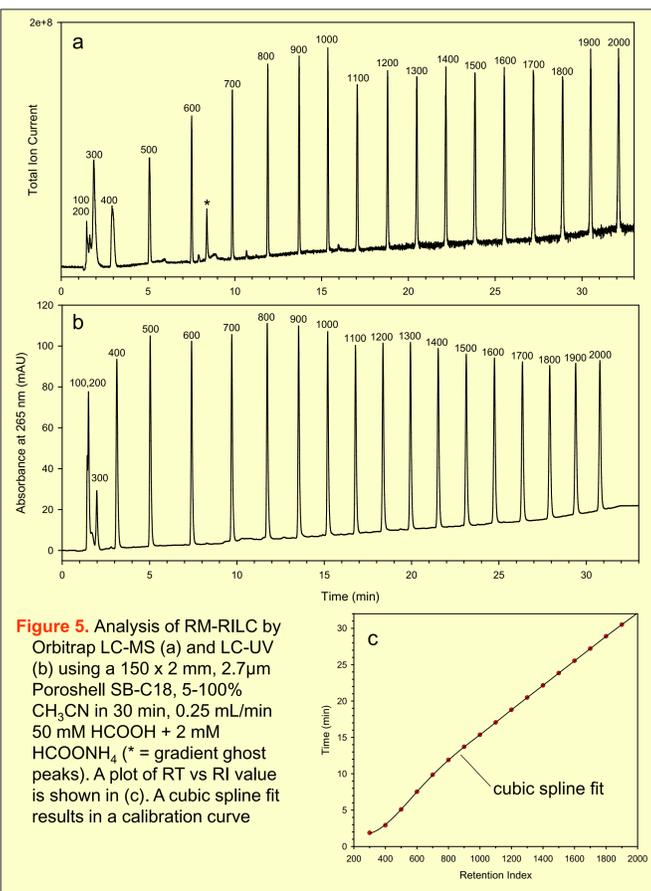
## Mass Spectral Characteristics

- Abundant signals in both positive and negative ion ESI. In positive, the molecule is protonated to form an  $[\text{M}+\text{H}]^+$  ion. In negative, a formate attachment ion,  $[\text{M}+\text{HCOO}]^-$ , is formed.
- Collision-induced dissociation gives common ions at  $m/z$  160 in positive mode and  $m/z$  80 in negative mode. These can be created by using a high orifice potential or a collision cell.



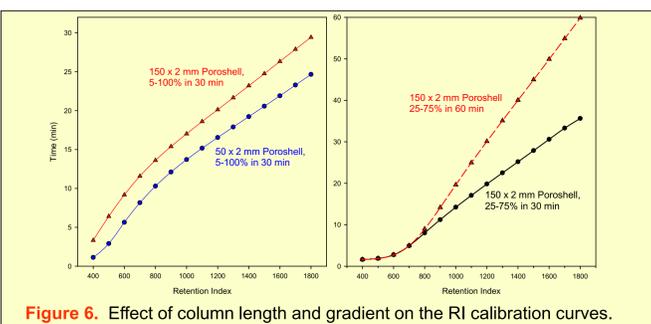
## LC-MS and LC-UV Characteristics

- Excellent behavior in gradient elution reversed phase LC.
- Easily detected by MS or UV absorbance (265 nm). An injection of 10 to 100 pmol is sufficient (0.1 to 1  $\mu$ L of the RM).
- Triple quadrupole analysis can be achieved with SRM using individual transitions or with a high orifice voltage plus a 160 > 160 transition at low collision energy.
- Orbitrap LC-MS analysis best achieved with alternating full scans and HCD scans, the latter allowing extraction of an  $m/z$  160 mass chromatogram.
- A plot of RT vs. RI value (100 x carbon length) and a cubic spline fit results in a calibration curve such as that shown in Fig. 5c.



## Effect of LC Parameters

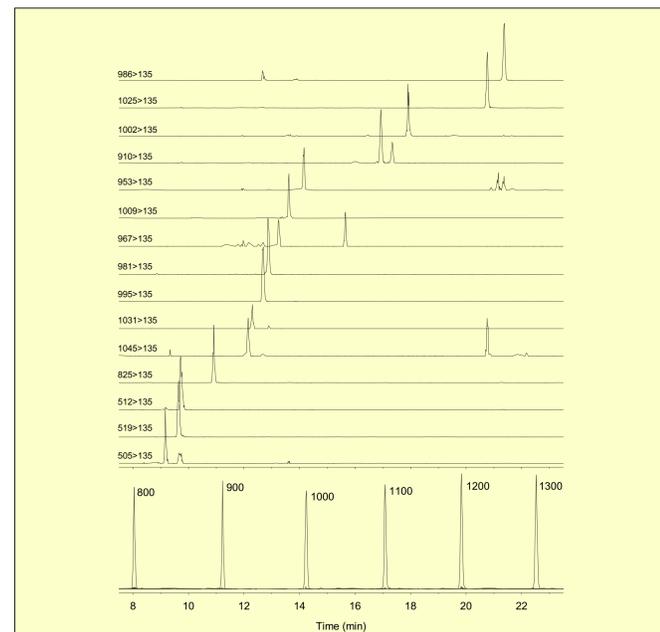
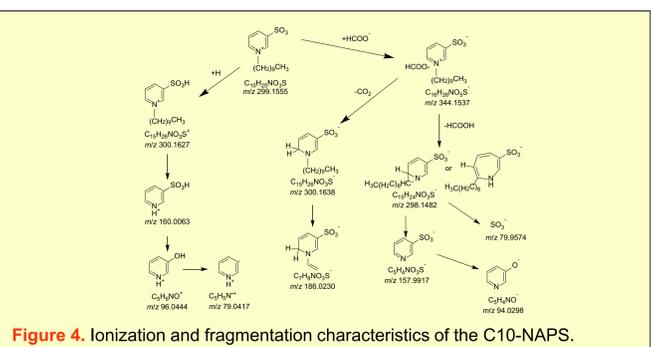
Parameters tested: (a) column length; (b) LC dead volume; (c) flow rate; (d) gradient start/stop % and rate; (e) stationary phase; and (f) mobile phase solvents, buffers and pH. The effects of column length and gradient on the RI calibration curves are shown in Fig. 6. RI values for analytes were very repeatable under each condition. Some variation did occur with a change of conditions, especially with different mobile and stationary phases as expected. A two-point correction using internal standards belonging to a class of analyte can help correct for these shifts (see Table 1).



## Applications

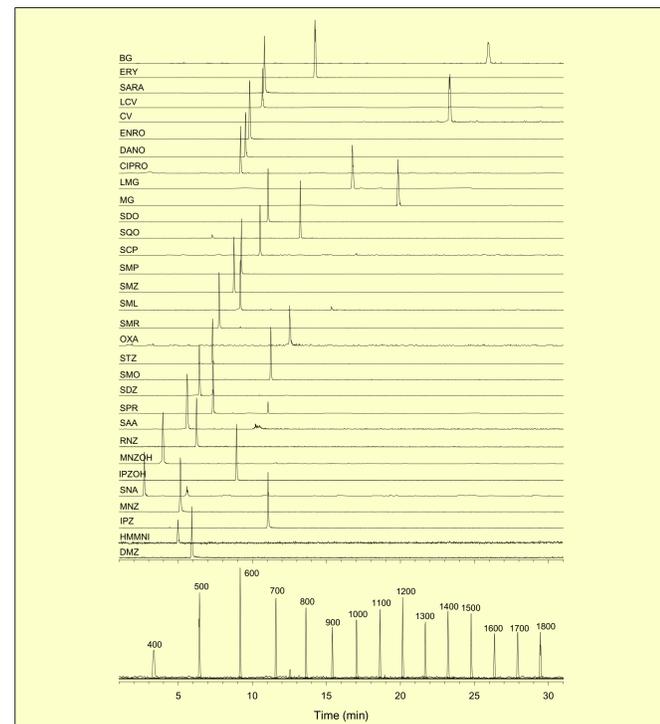
Application of the RI system to microcystins (cyclic peptides produced by cyanobacteria) and an assortment of veterinary drugs used in aquaculture are shown in Figures 7 and 8 and in Tables 1 and 2, respectively. Good repeatability within day and between days was observed.

One interesting application of this data is the prediction of retention time windows for scheduled SRM on different LC-MS systems, where RTs can vary significantly.



**Table 1:** Microcystin RI data from runs with 2 different gradients showing reproducibility. A 2-point correction was applied to the second.

Microcystin Code	Precursor Ion > m/z 135	(a) Short gradient (15-75% 30 min)				(b) Long gradient (15-75% 60 min)				2-point correction	
		Average RT (min)	1 day SD (n=5)	4 day SD (n=5,d=4)	RT (min)	Average RT (min)	1 day SD (n=5)	4 day SD (n=5,d=4)	RI(c)	% Diff. to (a)	
3dm7dmRR	505.8	9.17	832.6	0.4	0.5	11.33	842.3	0.7	1.2	831.6	-0.11
RR	519.8	9.64	847.2	0.3	0.8	12.18	858.4	0.5	1.5	847.2	0.00
7dmRR	512.8	9.72	850.4	0.4	0.7	12.32	862.0	0.3	1.2	850.8	0.04
Nod-R	825.5	10.91	888.7	0.5	0.7	14.29	901.3	0.2	1.1	888.9	0.03
YR	1045.8	12.12	929.3	0.4	0.8	16.75	947.9	0.4	0.8	933.3	0.42
7dmYR	1031.6	12.30	934.1	0.4	0.7	17.01	952.1	0.5	0.8	938.3	0.44
LR	995.6	12.69	946.7	0.1	1.3	17.75	964.9	0.2	1.6	950.7	0.41
7dmL	981.6	12.87	953.3	0.3	1.3	18.19	972.9	0.2	1.9	958.4	0.53
3dm7dmL	967.6	13.24	965.6	0.4	1.2	18.85	984.7	0.5	1.3	969.9	0.43
O-MuL	1009.9	13.61	977.8	0.5	1.3	19.59	997.6	0.4	1.5	982.4	0.46
7dmH	995.6	13.82	985.2	0.8	0.9	19.94	1004.5	0.5	1.4	989.0	0.38
RA	953.8	14.16	995.3	0.6	2.1	20.45	1012.8	0.2	2.3	997.1	0.18
LA	910.6	16.93	1092.5	0.2	2.4	25.63	1119.0	0.4	2.8	1091.0	-0.13
holA	910.6	17.35	1106.8	0.3	2.9	26.39	1124.4	0.2	3.0	1104.8	-0.18
LY	1002.9	17.91	1128.3	0.4	1.8	27.68	1150.4	0.2	1.9	1129.9	0.14
LW	1025.9	20.77	1232.9	0.2	1.8	33.20	1259.1	0.5	1.9	1234.5	0.12
LF	996.8	21.38	1255.1	0.3	2.4	34.25	1280.6	0.4	2.5	1255.1	0.00



**Table 2:** RI data for veterinary drugs used in aquaculture.

Drug Class	Name of Compound	Abbrev.	SRM transition	RI	SD (n = 5)			
Sulfonamides	Sulfanilamide	SNA	173.1 > 92.1	379.7	1.3			
	Sulfacetamide	SAA	215.1 > 156.1	471.9	0.6			
	Sulfadiazine	SDZ	256.1 > 156.1	530.4	0.4			
	Sulfathiazole	STZ	256.1 > 156.1	530.4	0.4			
	Sulfapyridine	SPR	250.1 > 156.1	531.9	0.2			
	Sulfamerazine	SMR	265.1 > 92.1	546.8	0.4			
	Sulfamethazine	SMZ	279.1 > 186.1	554.0	0.3			
	Sulfamethizole	SML	271.1 > 156.1	600.3	0.2			
	Sulfamethoxypyridazine	SMP	281.1 > 126.1	602.9	0.2			
	Sulfachloropyridazine	SCP	285.1 > 156.1	653.9	0.3			
Nitroimidazoles	1-(2-Hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole	MMZ-OH	188.1 > 123.1	419.5	0.5			
	2-Hydroxymethyl-1-methyl-5-nitroimidazole	HMMNI	156.1 > 140.1	453.1	0.2			
	Metronidazole	MNZ	172.1 > 128.1	457.3	0.5			
	Dimetridazole	DMZ	142.1 > 96.1	482.6	0.4			
	Ronidazole	RNZ	201.1 > 55.1	493.3	0.3			
	IPZ-OH	IPZ-OH	186.1 > 108.1	500.6	0.2			
	1-Methyl-2-(2-hydroxyisopropyl)-5-nitroimidazole	IPZ	170.1 > 109.1	678.9	0.4			
	Sulfamethoxazole	SMO	254.1 > 156.1	685.1	0.2			
	Sulfaquinoxaline	SQO	301.1 > 156.1	780.4	0.2			
	Fluoroquinolones	Ciprofloxacin	CIPRO	332.2 > 245.1	601.0	0.2		
Danofloxacin		DANO	358.2 > 96.1	614.1	0.3			
Enrofloxacin		ENRO	360.2 > 316.2	625.2	0.4			
Sarafloxacin		SARA	366.2 > 342.2	666.2	0.3			
Quinolones		Oxolinic acid	OXA	262.1 > 216.1	744.6	0.6		
		Macrolides	Erythromycin	ERY	734.5 > 158.1	833.3	0.6	
			Dyes	Leucocystall Violet	LCV	374.4 > 358.1	860.3	0.6
				Leucomalachite green	LMG	331.1 > 316.1	903.2	0.9
				Malachite Green	MG	329.2 > 313.1	1180.8	1.2
				Crystal Violet	CV	372.4 > 356.1	1406.5	1.1
	Brilliant Green			BG	385.3 > 341.1	1572.6	1.0	

## Conclusions

A unique set of homologous compounds, *N*-alkylpyridine-3-sulfonates, has been prepared for the measurement of retention indices in LC-MS and LC-UV. This RI system should be useful in databases for compound identification. It will also be useful for establishing scheduled SRM windows for LC-MS methods.

## References

1. Smith, R.M., Retention index scales used in high-performance liquid chromatography. J. Chromatography Library 57, 93-144 (1995).
2. Quilliam, M.A., Retention index standards for liquid chromatography, Patent WO2013/134862A1.