Automated Large-Volume Injection for Direct Analysis of Alkylphenols and Ethoxylated Alkylphenols by Mixed-Mode Chromatography and Mass Spectrometry

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INTRODUCTION

Alkylphenyl polyethoxylates (APEOs) are among the most commonly used nonionic surfactants, with over 650,000 tons produced annually.[1] APEO degradation products—alkylphenols (APs) and their short chain ethoxylates (mainly mono- and diethoxylates, EO1 and EO2) have been detected and reported in treated wastewater, sewage sludge, and soil. Studies reveal that these substances have varying estrogenic potencies.[2–3] Currently, these compounds are on the US EPA's priority testing list for evaluation of environmental and health effects. Because APEOs with different EO units exhibit different estrogenic potencies,[4] it is important to develop an analytical method to profile and monitor their presence in the environment.

The development of a chromatographic method for separation of APs and APEOs remains a challenge due to their structural complexity. Current methods, including normal-phase liquid chromatography (NPLC) and reversed-phase liquid chromatography (RPLC) separate these compounds by the difference of alkyl groups or EO units.[5–9] The preferred detection technique for APEO analysis is mass spectrometry, due to its sensitivity and selectivity.

Here, a mixed-mode chromatographic method was developed for separation of these compounds. On-line concentration and desalting was fully automated using large-volume injection (50 mL) to increase sample throughput and improve sensitivity. Three mixed-mode columns with different selectivities were evaluated and the results compared, as well as MS and MS/MS instrumentation modes as detection techniques. Parameters affecting chromatographic separation and MS detection were explored and discussed, with each method individually evaluated with respect to linearity, detection limits, precision, and accuracy.

EXPERIMENTAL

Instrumentation

HPLC:	P680 Dual Ternary Pump ASI-100 Autosampler or AS-HV autosampler TCC-100 Thermostatted Column Compartment UVD340U detector
Mass Spectrometer:	MSQ Plus™ single quadrupole mass spectrometer with cone wash TSQ Quantum Access® triple quadrupole mass spectrometer
Software:	Chromeleon® 6.8 SR6 Xcalibur® 2.0 DCMS^{Link}™ for Xcalibur (version 2.0)* for MS/MS analysis

*DCMS^{Link} is a Chromeleon-based software module providing the interface for controlling Dionex chromatography instruments from different mass spectrometer software platforms.

Sample Loading Conditions for AS-HV*

Concentrator:	Acclaim® PA Guard (4.3 × 10 mm, 5 µm)
Injection Volume:	50 mL
Loading Speed:	5 mL/min

* For ASI-100 autosampler, use loop injection and 10 µL injection volume.

Chromatographic Conditions

Analytical Column:	Acclaim Surfactant (2.1 × 150 mm, 5 µm)
Column Temperature:	15 °C
Mobile Phase:	A: Methanol (CH_3OH); B: Buffer (10 mM sodium acetate (NaOAc) for MSQ Plus, 100 mM ammonium acetate (NH_4OAc) for Quantum TSQ); C: water.

Time (min)	*A%*	*B%*	*C%*
0	74	5	21
9	74	5	21
10	94	5	1
18	94	5	1
19	74	5	21

Flow Rate: 0.25 mL/min

Mass Spectrometric Conditions

MSQ Plus

Interface: Electrospray Ionization (ESI)

Detection Mode: Selected Ion Monitoring (SIM), see Table 1 for scan details

Nebulizer Gas: Nitrogen; 80 psi

Probe Temperature: 500 °C

Cone Wash: CH_3CN/H_2O (50/50, v/v) at 50 µL/min

Quantum TSQ

Interface: ESI

Detection Mode: Selected Reaction Monitoring (SRM), see Table 2 for scan details

Capillary Temperature: 270 °C

Needle Voltage: 4000 V

Sheath Gas: 30 units

Auxiliary Gas: 30 for the first segment, 50 for the second segment

Table 1. Scan Functions and Scan Events for MSQ Plus SIM Detection

Name	Adduct	Mass *m/z*	Span *m/z*	Time Range (min)	Dwell Time (sec)	Polarity	Cone Voltage (V)
SIM Group 1							
OPEO1	$[M+Na]^+$	273.2	0.5	4.0–8.5	0.5	Pos.	65
OPEO2	$[M+Na]^+$	317.2	0.5	4.0–8.5	0.5	Pos.	65
SIM Group 2							
NPEO1	$[M+Na]^+$	287.2	0.5	8.0 –12.0	0.5	Pos.	65
NPEO2	$[M+Na]^+$	331.2	0.5	8.0–12.0	0.5	Pos.	65
SIM Group 3							
OP, tert-OP	$[M+CH_3COO]^-$	265.2	0.5	10.0–19.0	0.5	Neg.	65
NP	$[M+CH_3COO]^-$	279.2	0.5	10.0–19.0	0.5	Neg.	65

Table 2. SRM Scan Functions for MS/MS Detection

Scan Segment	Analyte	SRM Transitions			Collision Energy (V)
		Polarity	Parent Ion (*m/z*)	Product Ion (*m/z*)	
1 (0 ~ 12 min)	OPEO1 (Q*)	positive	268.2	113.1	10
	OPEO1 (C**)	positive	268.2	57.3	17
	OPEO2 (Q)	positive	312.2	183.1	11
	OPEO2 (C)	positive	312.2	121.1	22
	NPEO1 (Q)	positive	282.2	127.1	7
	NPEO1 (C)	positive	282.2	85.2	15
	NPEO2 (Q)	positive	326.2	183.1	11
	NPEO2 (C)	positive	326.2	121.1	22
	tert-OP	negative	205.1	133.1	20
2 (12 ~ 20 min)	4-OP	negative	205.1	106.1	21
	4-NP	negative	219.2	106.1	21

* Quantitative SRM ; ** Confirmative SRM.

Standard Preparation

IGEPAL CA-210 and IGEPAL CO-210 were purchased from Sigma-Aldrich. OPEO1, OPEO2, NPEO1 and NPEO2 were chromatographically fractionated in our laboratory using an Acclaim WAX-1 column.[10] Concentrations of collected fractions were calculated based on relative UV peak areas.

N-octylphenol, tert-octylphenol, and n-nonylphenol were purchased from Sigma-Aldrich and dissolved in CH_3CN to 500 µg/mL. Structures are shown in Figure 1.

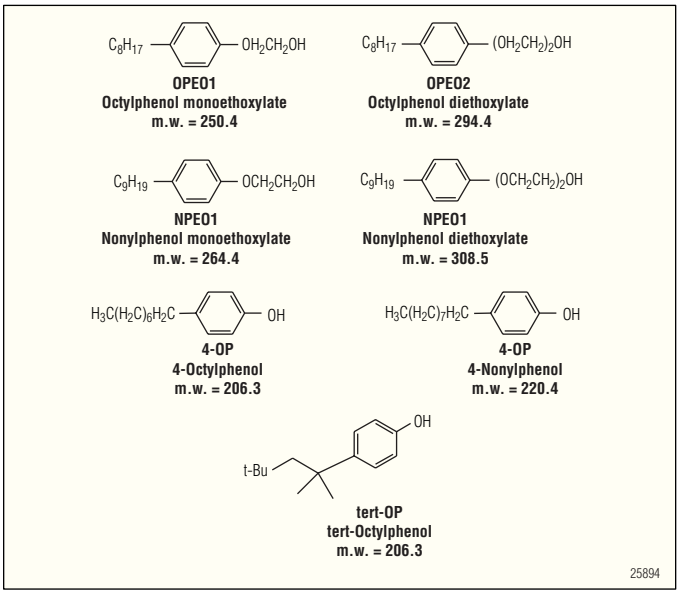

Figure 1. Structures of studied analytes.

Calibration standards were prepared in mobile phase the following concentration levels: 2000, 1000, 500, 200, 100, 50, 20, 10, and 5 ng/mL (ppb). Each level contains seven analytes: OPEO1, OPEO2, NPEO1, NPEO2, 4-OP, tert-OP, and 4-NP.

RESULT AND DISCUSSION

Chromatography

Among the three column candidates, the Acclaim Surfactant and Acclaim Mixed-Mode WAX-1 possessed the capability of separating APEO EO short oligomers. To achieve the goal of total resolution of APEO EO oligomers and APEO groups, i.e., OPEOs and NPEOs, these columns were compared with respect to their group separation efficiency. As shown in Figure 2, the Acclaim Surfactant column demonstrated the ability to differentiate APEOs by the EO units as well as alkyl group difference.

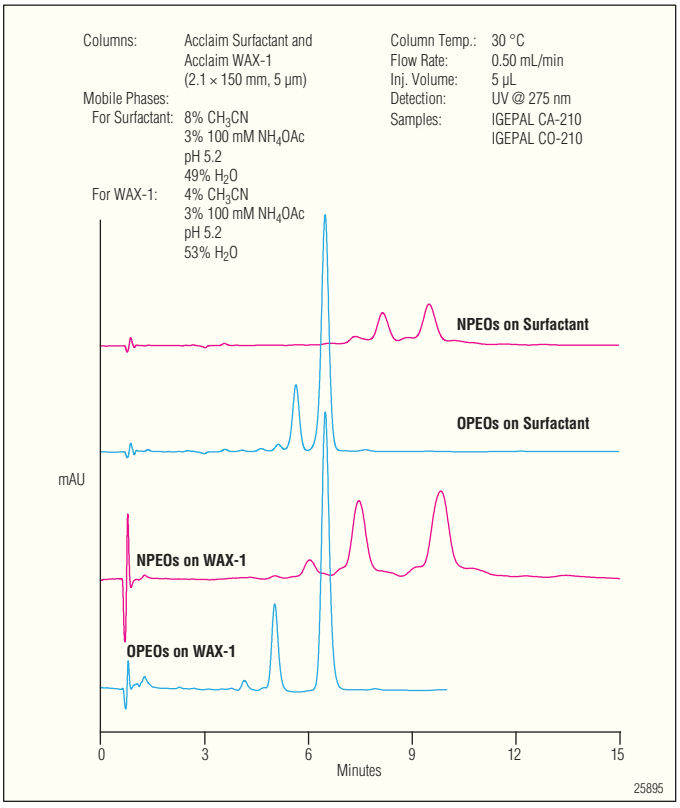

Figure 2. Resolution comparison of WAX-1 and Surfactant columns for APEO groups.

Column temperature was a key parameter for fine tuning resolution and adjusting run time. Seven temperatures from 20–50 °C were examined to determine optimum conditions (acetonitrile was used in the mobile phase). Results are shown in Table 3. Three resolutions (Rs) were measured to evaluate overall method resolution: Rs_{OPEO2} represents the resolution for EO oligomers; Rs_{OPEO1} represents the resolution for oligomer groups; Rs_{NPEO1} measures the separation of APEOs and AP. Oligomers with the same alkyl chain but different EO units were better resolved using low temperatures.

Table 3. Effects of Column Operating Temperature

Temperature (°C)	Chromatography Time* (min)	Resolution OPEO2	Resolution OPEO1	Resolution NPEO1
20	19.5	2.75	2.82	2.43
25	17.8	2.64	2.92	2.24
30	16.3	2.55	3.02	2.41
35	14.7	2.40	3.06	1.90
40	13.5	2.26	3.08	1.70
45	12.1	2.12	3.09	1.53
50	11.3	2.06	3.09	1.45

* Minimum time required to elute last peak (4-NP). Mobile phase: 52% CH_3CN, 5% 100 mM NH_4OAc, 43% H_2O; flow rate: 0.5 mL/min

Temperature dependence for the separation of APs and APEOs followed the same trend, with lower temperature yielding better resolution. However, separation of oligomer groups (OPEOs and NPEOs) showed the opposite trend: OPEOs and NPEOs were better resolved with elevated temperature. Column temperature was examined further using methanol as an organic modifier for MS detection and 15 °C was selected as the optimum condition to achieve total separation.

A mobile phase with higher organic content is favorable for MS sensitivity. The methanol-containing mobile phase showed significantly improved MS response for APEO1.[10] Therefore, methanol was used for HPLC-MS analysis.

Method Performance with Single Quadrupole MS Instrumentation

Mass Spectrometry with SIM Detection

When coupling MS detection to chromatography, ESI is the preferred ionization interface for APEO analysis. ESI showed better sensitivity and specificity for a wider range of oligomers.[11] To improve detection limits using MS SIM detection, 0.5 mM sodium acetate was added to the mobile phase to form sodium adducts. Because sodium acetate is nonvolatile, salt condensation was observed after operating for a short period of time. To ensure the long term stability of MS detection, the optional cone wash feature was activated with a wash stream of 50 µL/min methanol/water (50/50, v/v). Significant improvement of system stability was observed when employing the cone wash (5.5% decrease in intensity with cone wash compared with 46% decrease without cone wash after 6 runs.)

Linearity and Method Detection Limits

The linearity of this method was evaluated by three replicate injections of calibration standards. The method detection limit (MDL) for each analyte was calculated by the equation:

$MDL = t_{99\%} \bullet S_{(n=7)}$ Where t is Student's t at 99% confidence intervals ($t_{99\%,\ n=7} = 3.143$) and S is the standard deviation. The standard deviation was obtained from seven replicate injections of a calibration standard at 50 ppb for OPEOs and 100 ppb for NPEO2 and APs (a calibration standard at 1000 ppb was used to obtain the standard deviation for NPEO1 due to its low response). The results for linearity and MDLs are summarized in Table 4. When using adducts (sodium adducts for APEOs and acetate adducts for phenols) as quantitative ions, linear responses were observed in the range from low ppb to 2000 ppb with correlation coefficients greater than 0.99. The calculated MDLs vary from 7.0 (OPEO2) to 75.6 ppb (NPEO1).

Precision and Accuracy

Method precision and accuracy were calculated from seven replicated injections of standards at different concentrations based on their MS response. Precision is shown by RSD from the replicate analyses, and accuracy was calculated by (observed amount)/(specified amount) • 100%. Method precision for each analyte is less than 10% with the highest bias of 9.2% for OPEO1 (Table 4). Accuracy measurements were within 10% bias for each analyte except for NPEO1 which was 38% higher than the specified amount.

Table 4. Linearity, Precision, Accuracy, and Method Detection Limits Using MS SIM

Analyte	MS Adduct	Linearity			Precision (RSD, n = 7)	Accuracy (%)	MDL (pg)
		From (ppb)	To (ppb)	R^2			
OPEO1	$[M+Na]^+$	10	2000	0.999	9.2	96.9	290[a]
OPEO2	$[M+Na]^+$	5	1000	0.999	5.8	94.2	182[a]
NPEO1	$[M+Na]^+$	200	2000	0.996	2.4	138	1536[b]
NPEO2	$[M+Na]^+$	10	2000	0.995	2.2	96.9	142[c]
tert-OP	$[M+CH_3COO]^-$	50	2000	0.998	3.5	90.5	222[c]
4-OP	$[M+CH_3COO]^-$	50	2000	0.994	4.5	97.2	282[c]
4-NP	$[M+CH_3COO]^-$	10	2000	0.997	3.6	104	224[c]

[a]Calculated from 7 replicate injections of a 50 ppb standard.
[b]Calculated from 7 replicate injections of a 1000 ppb standard.
[c]Calculated from 7 replicate injections of a 100 ppb standard.

Method Performance with Triple Quadrupole MS/MS Instrumentation

Mass Spectrometry with SRM Detection

An ESI interface and methanol-containing mobile phase were also used for MS/MS SRM detection. NH_4OAc was used to buffer the mobile phase instead of NaOAc because analyte-sodium adducts did not show strong fragment ions with SRM conditions. Two SRM transitions were used for quantification and confirmation. The SRM scan details are shown in Table 2.

Calibration Range and Method Detection Limits

Coefficient of determination (R^2) measured greater than 0.99 for each analyte from low calibration levels (2–100 ppb) to 2000 ppb. MDLs were calculated by the same means as for MS SIM detection, and details are shown in Table 5. Lower detection limits can be achieved using MS/MS SRM compared with MS SIM detection. The SRM chromatograms of APs and APEOs are shown in Figure 3 with the injection amount of 2 ng of each analyte.

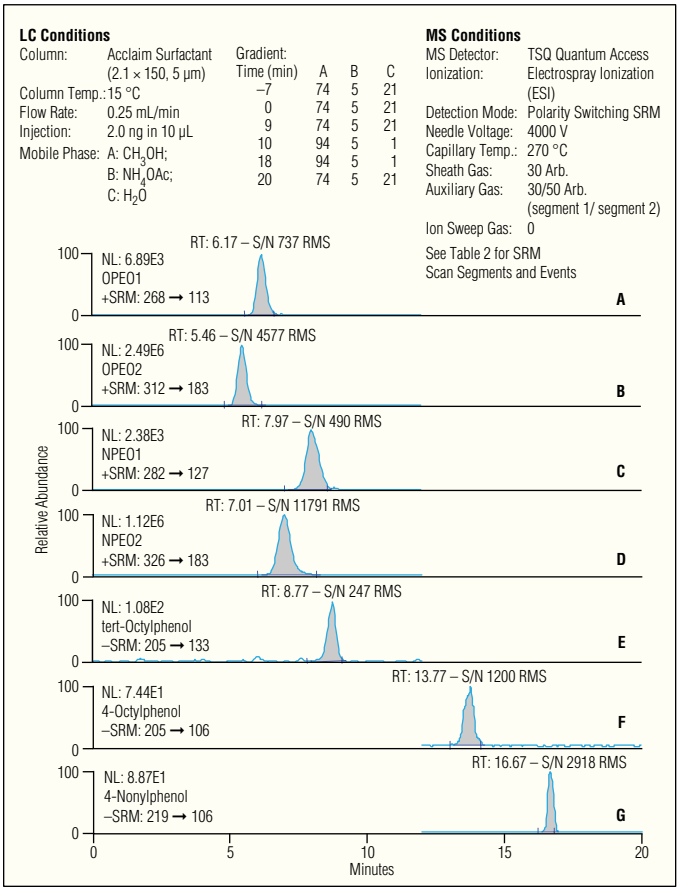

Figure 3. SRM Chromatograms of alkylphenols and alkylphenol ethoxylates. Reconstructed SRM chromatograms A–G show APs and APEOs with 2 ng injection.

Table 5. Calibration Range and Coefficient of Determination Using MS/MS SRM						
Analyte	**SRM**	**Calibration Range (ppb)**		**R^2**	**Fitting Function**	**Weighting**
		From	**To**			
OPEO1	268 → 113	50	2000	0.9972	Linear	1/X
	268 → 57	50	2000	0.9946	Linear	1/X
OPEO2	312 → 183	2	2000	0.9976	Quadratic	1/X
	312 → 121	2	2000	0.9976	Quadratic	1/X
NPEO1	282 → 127	50	2000	0.9936	Linear	1/X
	282 → 85	50	2000	0.9946	Linear	1/X
NPEO2	326 → 183	2	2000	0.9977	Quadratic	1/X
	236 → 121	2	2000	0.9976	Quadratic	1/X
tert-OP	205 → 133	100	2000	0.9917	Linear	1/X
4-OP	205 → 106	50	2000	0.9959	Linear	1/X
4-NP	219 → 106	50	2000	0.9938	Linear	1/X

Precision and Accuracy

Method precision and accuracy were measured at three levels: 10, 200, and 2000 ppb. Calculations were performed by the same means as for MSQ SIM detection, and results are shown in Table 6. Accuracy varies from 86% to 103%, and RSDs range from 1.44% to 19.5%.

Table 6. Precision and Accuracy Using MS/MS SRM Detection					
Analyte	**Level (ppb)**	**Mean (ppb)**	**Accuracy (%)**	**RSD**	**MDL (pg)**
OPEO1	10	ND	N/A	N/A	270
	200	205	103	4.30	
	2000	1899	95.0	5.25	
OPEO2	10	8.91	89.1	2.75	8.64
	200	196	98.0	1.44	
	2000	1756	87.8	1.91	
NPEO1	10	ND	N/A	N/A	402
	200	203	102	6.39	
	2000	1852	92.6	4.21	
NPEO2	10	9.88	98.8	19.5	61.3
	200	187	93.5	2.11	
	2000	1812	90.6	2.21	
tert-OP	10	ND	N/A	N/A	562
	200	212	106	8.94	
	2000	2003	100.2	3.60	
4-OP	10	ND	N/A	N/A	527
	200	172	86.0	8.38	
	2000	1978	98.9	3.51	
4-NP	10	ND	N/A	N/A	100
	200	204	102.0	15.90	
	2000	2016	100.8	4.61	

Automation of Large Volume Injection for Direct Analysis of Water Samples

To improve the method detection limits and to reduce intensive sample preparationin the laboratory, an automated large-volume injection method was employed for direct analysis of these analytes using the AS-HV autosampler. The system schematic is shown in Figure 4. The water sample is loaded into a 50 mL sample loop by the AS-HV then delivered by the second system pump unit through a concentrator column (Acclaim PA, 4.6 ×1 0 mm, 5 µm) using DI water as the mobile phase. The analytes trapped on the concentrator column were back-eluted and separated on the analytical column (Acclaim Surfactant, 2.1 × 150 mm, 5 µm), and detected by UV and MS detectors. Automation was achieved through Chromeleon software.

Detection limits were significantly improved by using large-volume injections. Direct analysis of water samples for APs and APEOs can be performed using this method.

Method specificity was evaluated by analyzing 50 mL treated DI water (organic residues were removed by passing DI water through an analytical PA column (4.6 × 150 mm, 5 µm) as a system blank. No measurable peaks were observed at the specific retention times for target analytes except for NPEO2 (23% peak area of NPEO2 at 10 ppt).

Carryover was examined by analyzing a system blank sample after injection of a calibration standard at the highest concentration, i.e., 2000 ppt. No carryover peaks were observed except for NPEO2 whose peak area was 106% of NPEO2 in a blank sample. Therefore, carryover was deemed to be negligible when using large-volume injection for direct analysis.

The coefficient of determinations (R^2) achieved were greater than 0.99 for each analyte from low levels (OPEO1: 10 ppt; OPEO2: 5 ppt; NPEO1: 20 ppt; NPEO2: 10 ppt; t-OP: 100 ppt; 4-OP: 200 ppt; 4-NP: 500 ppt) to 2000 ppt. MDLs were measured by the statistical method mentioned in prior sections with $n \geq 6$. MDLs for APEOs were measured at low ppt levels (OPEO1: 10.45 ppt; OPEO2: 6.79 ppt; NPEO1: 8.20 ppt; NPEO2: 5.40 ppt), tert-OP at 178 ppt and 4-OP at 104 ppt. (MDL for 4-NP was not measured due to low response.)

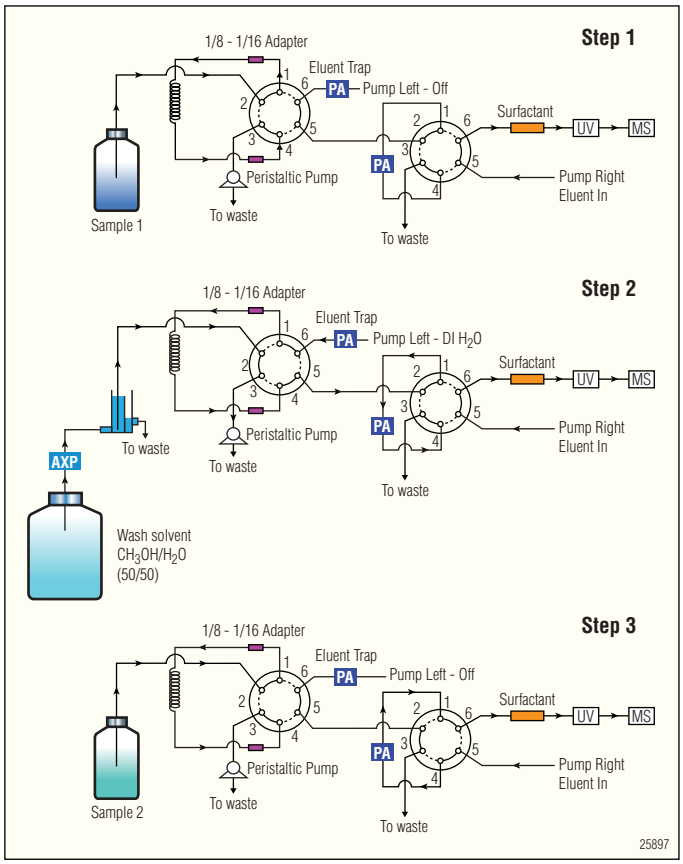

Figure 4. System schematic.

CONCLUSION

This study produced methods for profiling degradation products of ethoxylated alkylphenols in water samples. Using a large-volume injection, automated direct analysis of APs and APEOs was demonstrated without labor-intensive sample preparation.

Total separation of seven target analytes was achieved and selective, sensitive detection was achieved using mass spectrometric detection. Detection limits and specificity were improved over MS SIM using MS/MS SRM detection, and additional confirmation information can be obtained by introducing a second SRM transition.

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