Primary Metabolite Analysis of Plant Material Using a Triple Quadrupole MS Coupled to a Monolith Anion-Exchange Column

CAN 109

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Introduction

A platform using Dionex ion chromatography (IC) coupled to triple quadrupole mass spectrometry (MS) was developed to measure various metabolites in *Brassica napus* seeds. The IC tandem mass spectrometry (IC-MS/MS) technique provides a good tool to identify and quantify metabolites in a complex matrix. The biological pathways include many homologs and structural isomers with the same mass and analog fragmentations, so it is also essential for the analysis to have a good separation of the different compounds. The new IonSwift™ MAX-100 column (1 × 250 mm) provides good peak shape and separation for a broad spectrum of substances. Comparison with the previous product, the IonPac® AS11-HC column (2 × 250 mm), showed a similar separation in a shorter run time and an enhanced sensitivity for several target analytes.

Equipment

Dionex ICS-3000 System including:

- EG Eluent Generator
- DP Dual Pump with Degasser
- AS Autosampler
- DC Detector Compartment with Anion Self-Regenerating Suppressor (ASRS®) 300 2 mm Suppressor

API 4000™ Liquid Chromatography (LC)-MS/MS System (AB SCIEX)

Reagents and Standards

Methanol, chloroform, and other biochemicals were obtained from Sigma-Aldrich Chemie GmbH, (Taufkirchen, Germany) and Roche Diagnostics GmbH (Mannheim, Germany). Water was purified with the Millipore Milli-Q® Plus System.

Preparation of Samples

Homogenize approximately 30 mg frozen material at liquid nitrogen temperature in 2 mL microcentrifuge tubes (Eppendorf, Hamburg, Germany) containing two steel beads (ASK, Korntal-Muenchingen, Germany) by grinding in a TissueLyser (Quiagen GmbH, Hilden, Germany) for 45 s at 1800 strokes.[1] After homogenization, extract the samples with 0.5 mL 1:1 (v/v) methanol/chloroform under the same grinding conditions. Extract water-soluble compounds from the $CHCl_3$ phase by adding 300 µL water, followed by 45 s shaking, and centrifugation at 2000 g for 2 min. Filter the upper aqueous/methanol phase with a Vivaclear centrifugal filter (0.8 µm pore size, SatoriusStedim Biotech, Göttingen, Germany) at 2000 g for 2 min. Re-extract the lower $CHCl_3$ phase with 200 µL water and add the aqueous/methanol phase to the first phase. Evaporate the extract to dryness using a centrifugal vacuum dryer at 20 °C and redissolve in 1 mL water.

Chromatographic Conditions

IonSwift MAX-100, 1 × 250 mm

IonPac AS11-HC, 2 × 250 mm

Flow Rate: 150 µL/min

Inj. Volume: 10 µL (cleaning, 10 µL CH_3OH)

Column Temp.: 40 °C

Table 1. KOH Gradient for the Comparison of MAX-100 and AS11-HC Columns

MAX-100		AS11-HC		Cleaning Run	
Time (min)	KOH (mM)	Time (min)	KOH (mM)	Time (min)	KOH (mM)
0	5	0	5	0	100
10	5	16	5	9	100
16	12	26	12	9.5	5
28	25	41	25	16	5
32	100	48	100		
38	100	67	100		

Electrospray Ionization (ESI)-MS/MS Conditions

Measure the samples in negative mode. Use nitrogen as a curtain gas, nebulizer gas, heater gas, and collision gas. Set the ion spray voltage to -4000 V, the capillary temperature at 450 °C, and the MS parameters as displayed in Table 2.

Table 2. Filtered Masses and Potentials of MS/MS Transitions in Negative Mode

	Q1 Mass	Q3 Mass	DP	EP	CE	CXP
Glycolate	74.8	46.9	-35	-10	-12	-7
Lactate	88.9	42.9	-45	-10	-18	-1
Succinate semialdehyde	100.8	56.9	-45	-10	-14	-1
Pyruvate	86.9	86.9	-30	-10	-14	-3
Trehalose-6-phosphate	420.9	78.9	-85	-10	-60	-5
Glucose-1-phosphate	258.9	79.0	-60	-10	-42	-5
Succinate	116.8	73.1	-45	-10	-16	-1
Malate*	132.8	70.9	-80	-10	-22	-3
Glucose-6-phosphate	258.8	96.8	-50	-10	-22	-5
Glucose-6-phosphate	258.8	198.8	-50	-10	-16	-19
2-Oxoglutarate	144.9	100.7	-55	-10	-12	-17
Fructose-6-phosphate	258.8	78.9	-55	-10	-60	-5
Fructose-6-phosphate	258.8	70.9	-50	-10	-44	-3
Fumarate	114.9	70.9	-40	-10	-12	-11
AMP	345.9	78.8	-75	-10	-66	-5
NADH	331.4	78.8	-40	-10	-72	-3
NADH	331.4	133.8	-40	-10	-28	-11
Oxaloacetate	130.8	86.9	-25	-10	-8	-5
6-Phospho-gluconate	274.9	96.9	-45	-10	-28	-5
3-PGA	184.8	96.9	-35	-10	-20	-15
Citrate*	190.8	87.0	-75	-10	-24	-5
Isocitrate	190.8	72.9	-20	-10	-30	-5
Phosphoenolpyruvate	166.7	78.9	-35	-10	-14	-5
ADP-Glucose	293.4	133.9	-30	-10	-34	-1
ADP-Glucose	293.4	452.8	-30	-10	-12	-13
UDP-Glucose	281.9	111.0	-30	-10	-22	-7
UDP-Glucose	281.9	240.8	-30	-10	-20	-11
Glycerone-phosphate	168.7	78.7	-40	-10	-36	-3
cis-aconitate	172.8	84.8	-35	-10	-18	-15
trans-aconitate	172.8	84.8	-35	-10	-18	-15
ADP	425.8	134.0	-75	-10	-30	-9
ADP	425.9	79.0	-75	-10	-90	-5
Pyrophosphate	177.0	79.0	-35	-10	-36	-5
Fructose-1,6-biphosphate	338.9	240.9	-50	-10	-20	-15
NADPH	371.4	78.8	-45	-10	-68	-10
NADPH	371.4	133.8	-45	-10	-30	-6
UDP	200.9	78.9	-35	-10	-14	-7
UDP	200.9	323.0	-35	-10	-6	-9
ATP	505.9	158.7	-80	-10	-40	-29
UTP	402.9	78.9	-60	-10	-100	-5
UTP	240.9	403.1	-30	-10	-10	-27

DP=Declustering Potential, EP=Entrance Potential, CE=Collision Energy, CXP=Collision Cell Exit Potential.
*Not the optimum potential to reduce sensitivity due to the high concentrations in seeds.

Results and Discussion

In most cases, the IonSwift MAX-100 column showed better sensitivity than the IonPac AS11-HC column (Table 3). The biggest difference between the two columns was seen in pyrophosphate, which had a great signal-to-noise (S/N) ratio on the MAX-100 column. This was significant at low concentration, at which the S/N ratio on the AS11-HC column was below 3. The run time on the AS11-HC column was 30 min longer with the same flow rate. The small inner diameter of the ESI capillary leads to a backpressure of 100 to 110 psi between the 2 mm suppressor and the capillary at a flow rate of 150 µL/min. This value is approximately 75% of the suppressor limit and prevents higher flow rates. For a shorter run time with the AS11-HC column coupled to an ESI-MS/MS, a 4 mm suppressor is recommended.

Oxaloacetate, glycerone-phosphate, and ADP-glucose are not shown in Table 3 due to their instability under IC conditions. Compared to most of the other target analytes with high sensitivity and a low limit of detection, UDP-glucose has a higher limit of detection, possibly due to degradation under IC conditions, even if the calibration showed a good regression factor.

The memory effect of not completely eluted compounds described in the S. Arrivault et al. 2009 study[1] was observed for eight late-eluting compounds (Cit/ICit/c-Aco/t-Aco/ADP/PP/UDP/UTP). A wash run injection of methanol after each sample or standard reduced the problem either completely or to less than 1% (not calculated on the AS11-HC column).

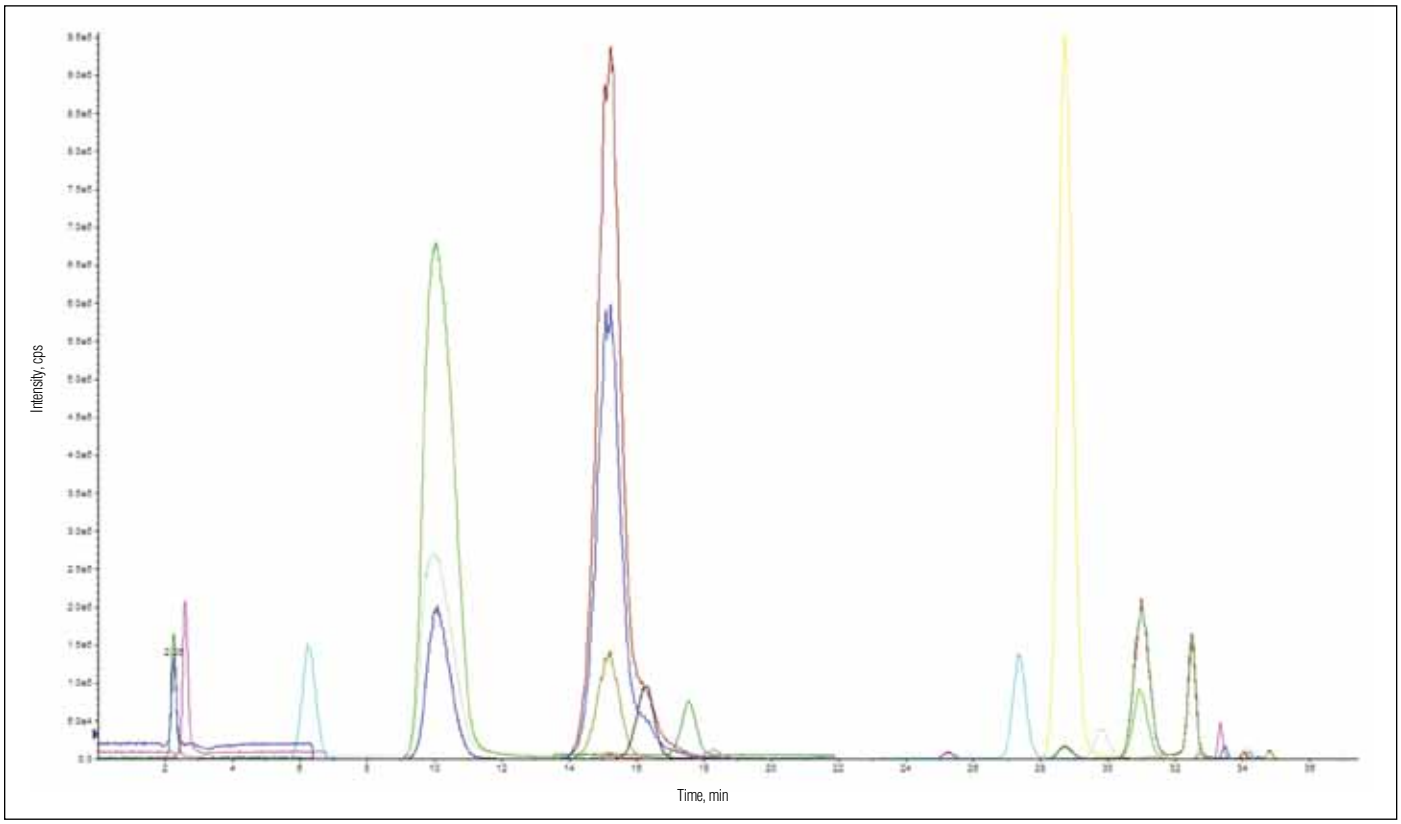
Glucose-6-phosphate (G6P) and Fructose-6-phosphate (F6P) had a retention time gap of 1 min. The high ratio of G6P/F6P in plant material resulted in problems of peak separation. For quantification of G6P and F6P, less intense transitions (259→97/79) were chosen because, for G6P, the 259→199 transition was nearly independent of F6P. The 259→71 transition for F6P had a slightly higher abundance, compared to that for G6P. Nevertheless, F6P was the only peak area that had to be integrated manually.

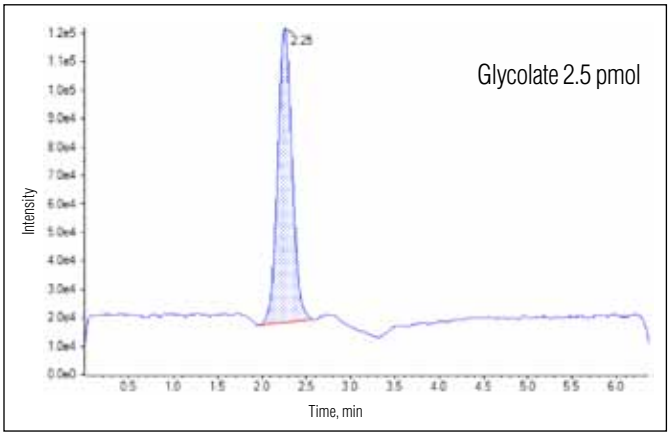
For the isomers citrate/isocitrate, the transitions 191→87 and 191→73 were used because they were absolutely independent of each other. The declustering potential of the major compounds citrate (DP -35 reduced to -75) and malate (DP -40 reduced to -80) were decreased relative to their optimum so that the sensitivity was selectively decreased, thus preventing an overload of the detector and allowing the measurement of the entire set of compounds in one dilution step. This technique allows analysis of concentrations of different compounds over 4 to 5 orders of magnitude in one run (see figures).

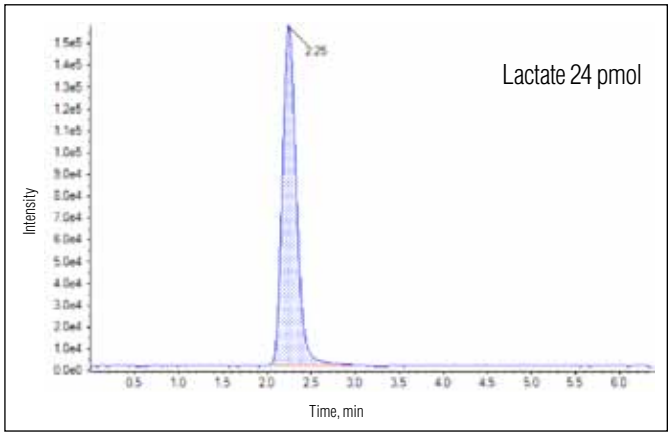
Table 3. Comparison of Signal-to-Noise Ratios on MAX-100 and AS11-HC Columns with a Standard Mix that Reflects the Composition of Plant Samples

Analyte Peak Name	RT IonSwift	Analyte Concentration (pmol)	S/N IonSwift	S/N AS11-HC	r
Glycolate	2.1	0.6	1.5	—	0.9992
Lactate	2.1	2.4	15	3	0.9981
Succinate semialdehyde	2.4	0.3	3	—	0.9972
Pyruvate	2.5	0.8	7	4	0.9970
Trehalose-6-phosphate	5.5	0.012	5	—	0.9947
Glucose-1-phosphate	6.2	0.36	70	10	0.9970
Succinate	10.2	4	50	40	0.9900
Malate*	10.2	16	100	30	0.9993
Glucose-6-phosphate	15.1	2.4	10	10	0.9945
Glucose-6-phosphate	15.1	2.4	20	7	0.9949
2-Oxoglutarate	16.1	1.2	13	15	0.9950
Fructose-6-phosphate	16.2	0.28	4	3	0.9856
Fructose-6-phosphate	16.2	0.28	3	—	0.9862
Fumerate	17.6	0.32	3	—	0.9939
AMP	18.1	0.056	3	—	0.9988
NADH	19.7	0.008	2	—	0.9994
6-Phospho-gluconate	25.2	0.032	6	8	0.9960
3-PGA	27.4	0.24	11	10	0.9955
Citrate*	28.8	20	110	150	0.9973
Isocitrate	29,9	0.24	12	8	0.9954
UDP-Glucose	30.7	3	2.5	—	0.9930
Phosphoenolpyruvate	31.0	0.1	28	30	0.9952
cis-Aconitate	30.9	0.2	25	13	0.9952
trans-Aconitate	32.5	0.12	14	11	0.9932
ADP	32.7	0.08	7	5	0.9957
Pyrophosphate	33.3	0.004	20	2	0.9935
Fructose-1,6-biphosphate	33.4	0.012	3	2	0.9932
NADPH	33.8	0.012	5	—	0.9954
UDP	34.0	0.016	8	4	0.9960
ATP	34.2	0.04	3	—	0.9962
UTP	34.8	0.012	8	2	0.9934

*Citrate and malate have poorer limits of detection than are possible due to a DP that was selected to reduce sensistivity due to the high amounts of these compounds in the sample.


Intensity, cps
Time, min


Glycolate 2.5 pmol
Intensity
Time, min


Lactate 24 pmol
Intensity
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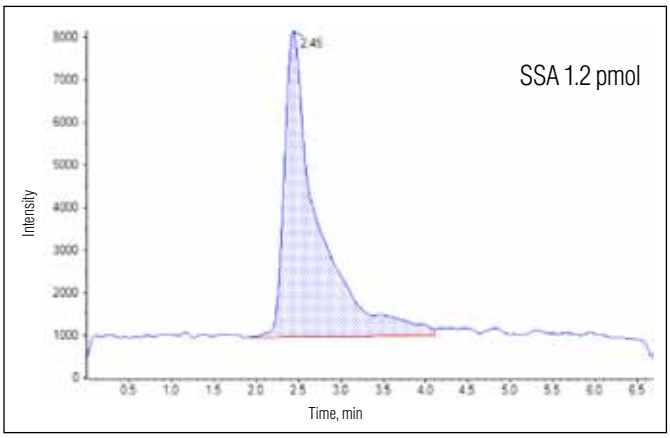
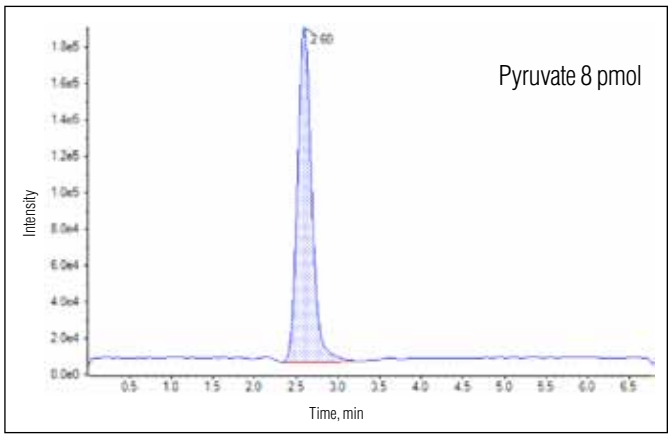
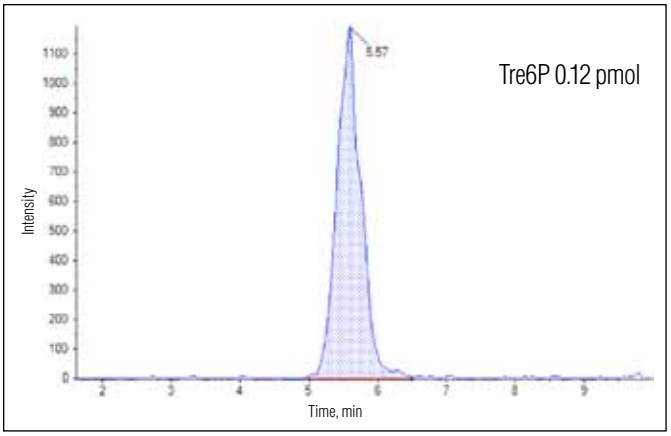
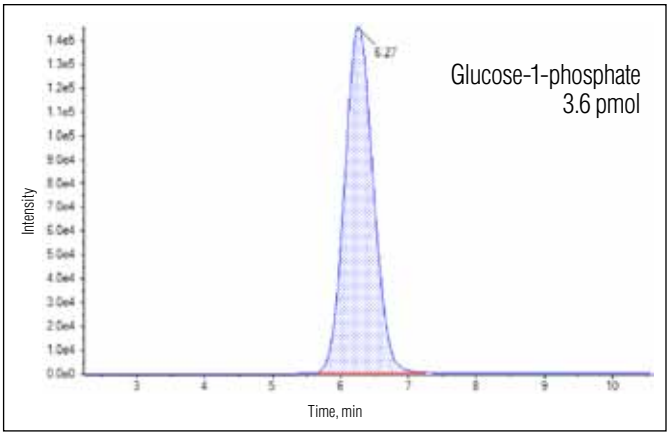
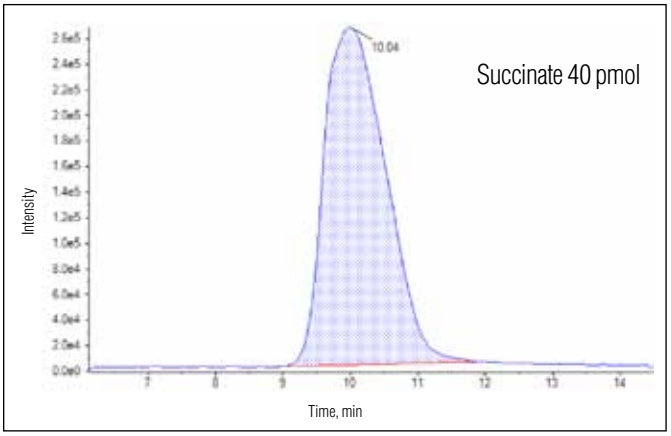
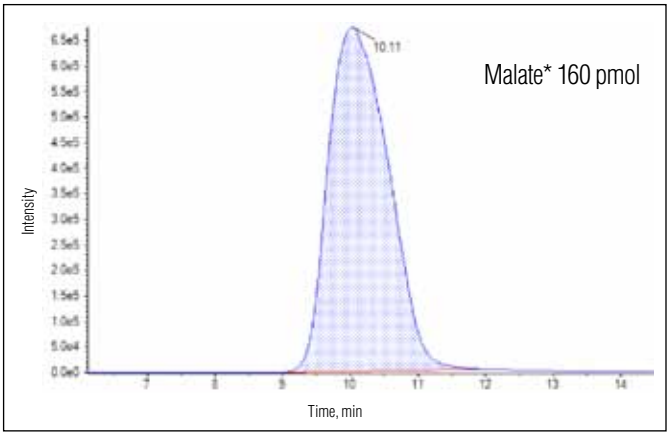

SSA 1.2 pmol
Intensity
Time, min

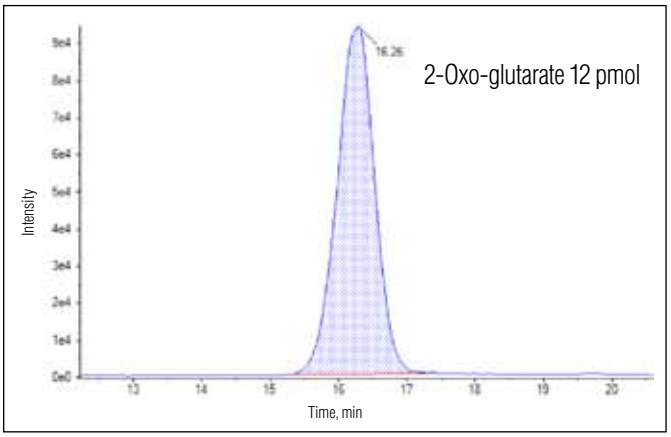

Pyruvate 8 pmol
Intensity
Time, min

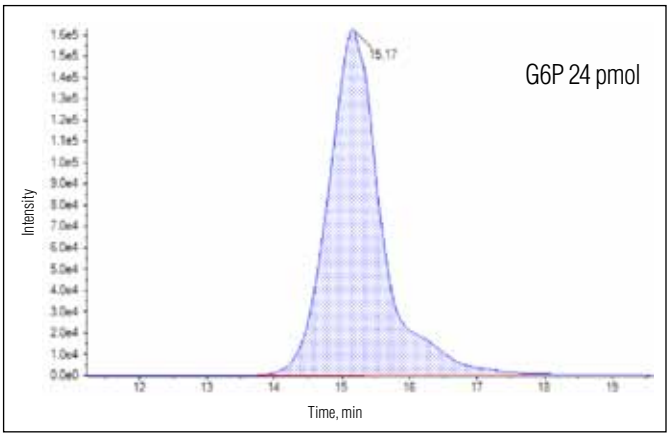


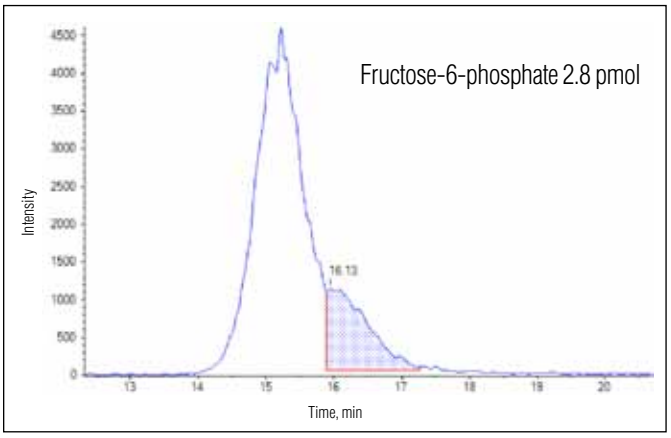


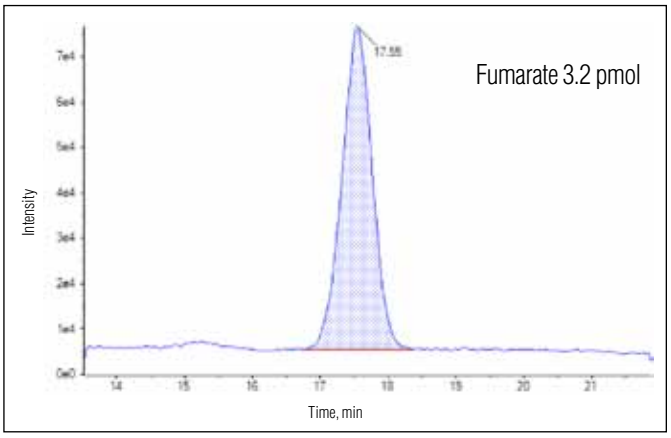




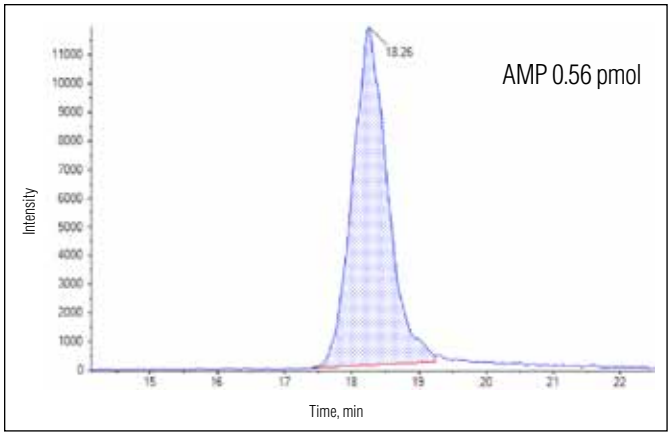


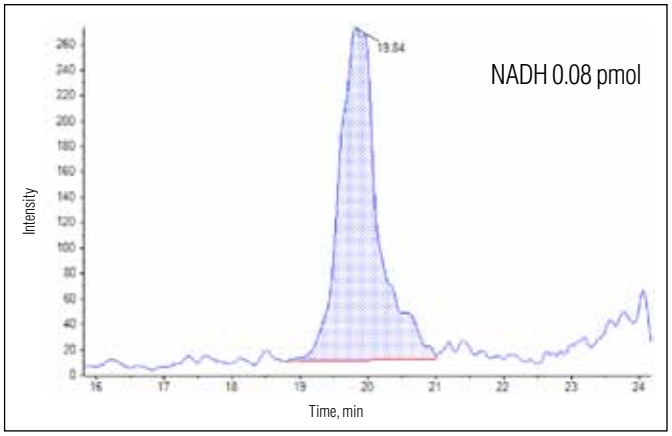


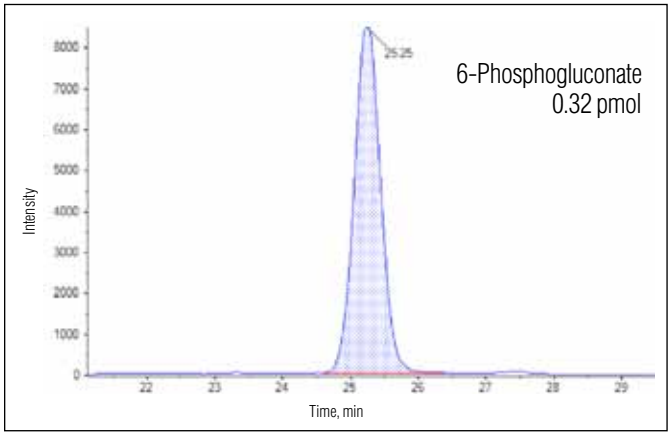


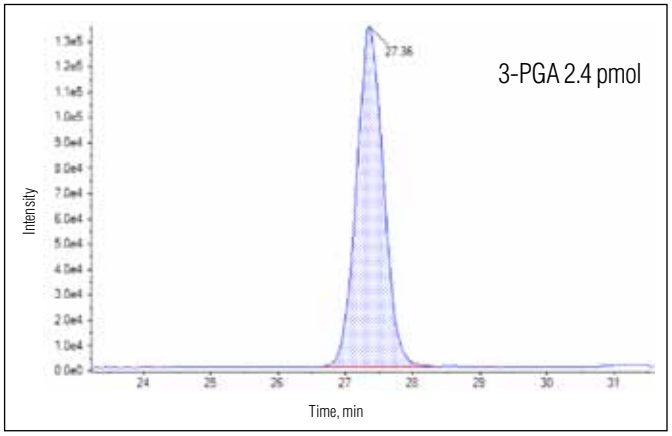


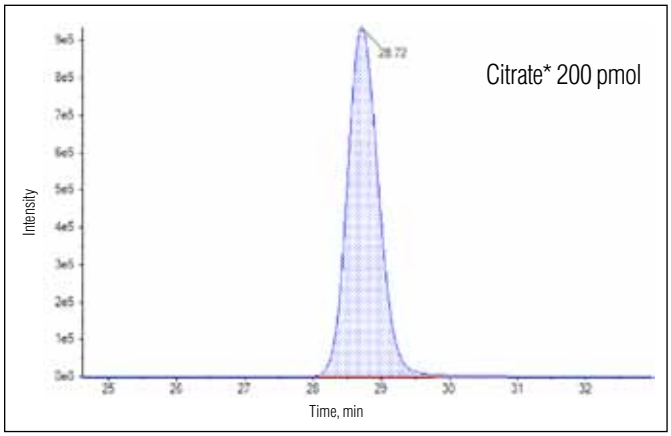
*Malate's limit of detection can be lower with an optimal DP.

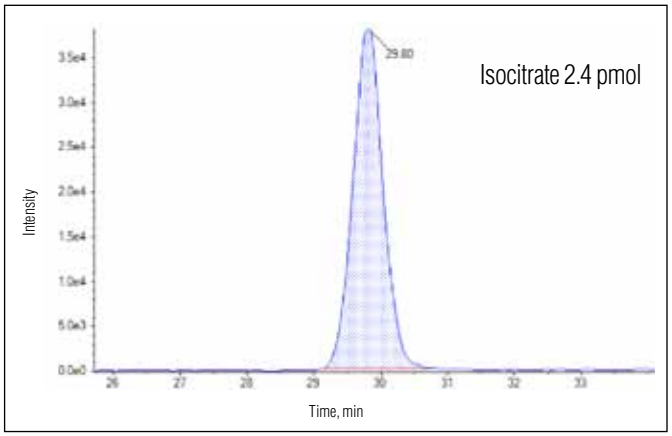


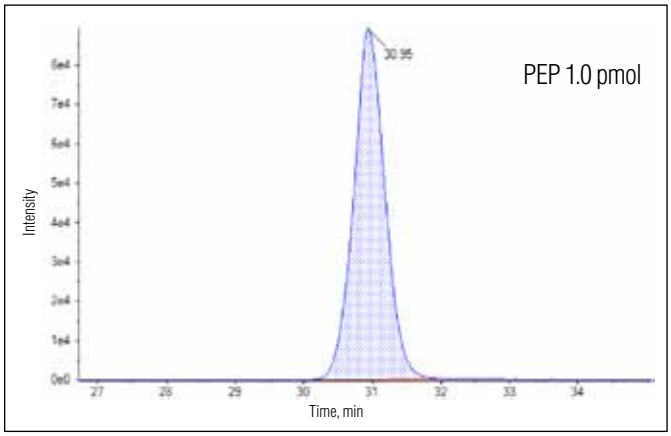


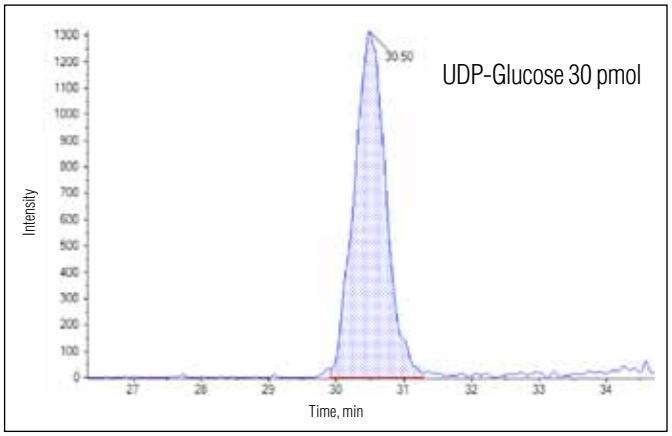




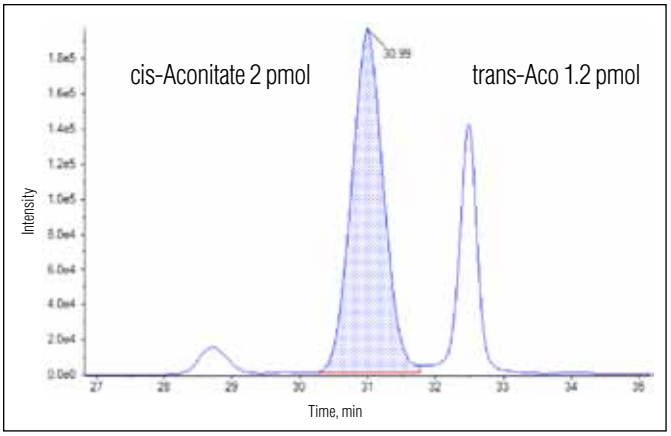


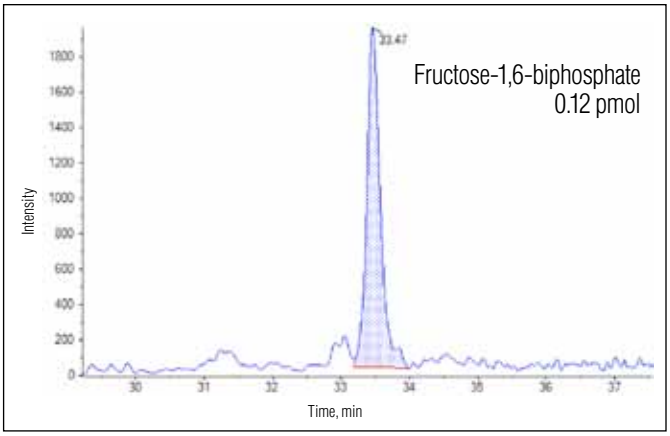


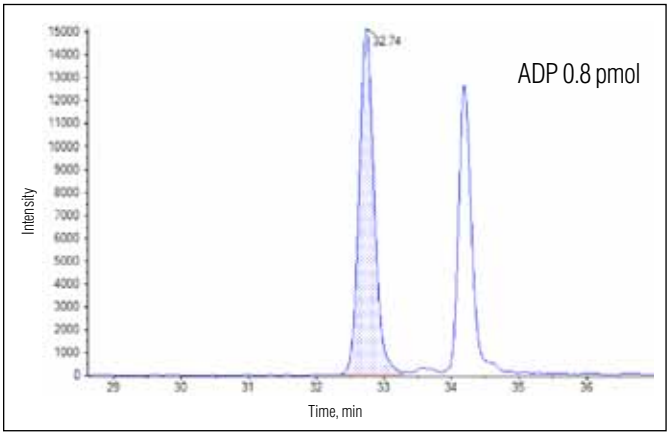


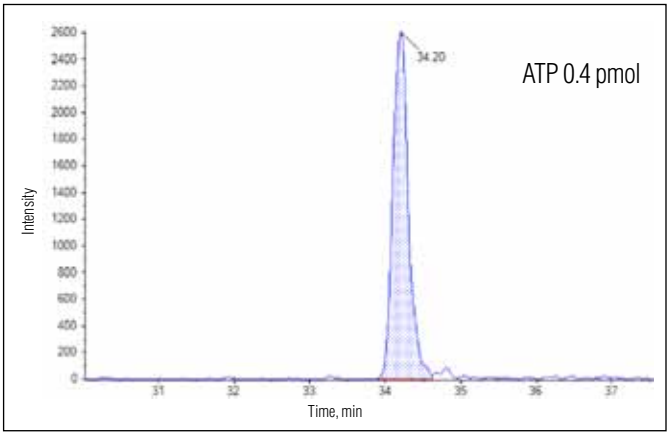


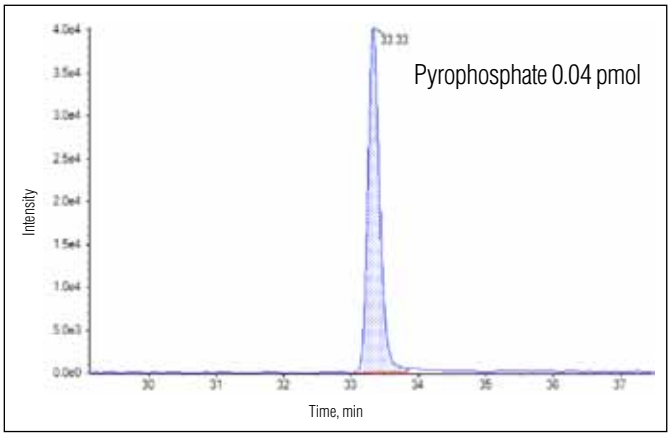
*Citrate's limit of detection can be lower with an optimal DP.

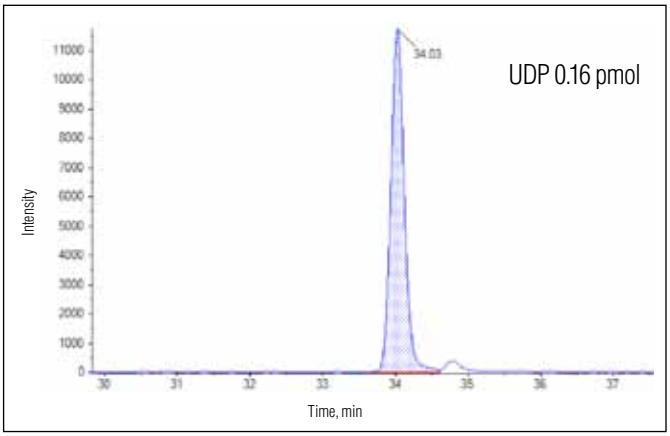


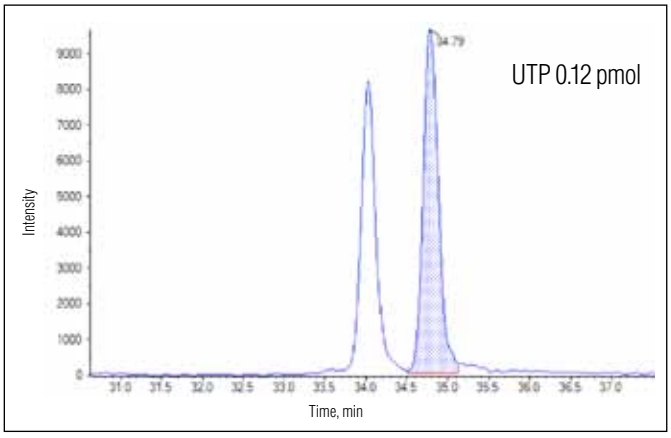












Conclusion

The combination of the 1 mm IonSwift MAX-100 column and the API 4000 ESI-MS/MS system provides a simple, rapid, specific, and sensitive method for the simultaneous detection and quantification of several primary metabolites involved in the TCA cycle and glycolysis pathway. Compared to the previously used IonPac AS11-HC column, the MAX-100 has shorter run times and helps achieve better sensitivity.

Reference

1. Arrivault, S.; Guenther, M.; Ivakov, A.; Feil, R.; Vosloh, D.; van Dongen, J.T.; Sulpice, R.; Stitt, M. The Plant Journal. *Plant Physiol.* **2009**, *59*, 824–839.

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