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LC-MS/MS Detection of Central Carbon Metabolism using the Collision Fragments Method

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Along with the development of biotechnology, the effects of metabolites on the metabolic cycle and quantitative and qualitative analysis of biological metabolites is attracting more and more attention. We present a set of experiments that detect and quantitate the collision fragments of central carbon metabolism using liquid chromatography mass spectrometry to understand biological metabolites. In order to obtain a suitable method to use liquid chromatography mass spectrometry to detect the collision fragments of the central carbon metabolites, we first set the gradient elution parameters, and then determined whether to join the tributylamine in the sample solution. A good ion peak and a considerable response value were obtained using the gradient elution parameters in Table 2 and adding tributylamine in the sample solution. This was a suitable method for liquid chromatography mass spectrometry detection of the collision fragments of the central carbon metabolism. However, due to limited experimental conditions and time, exploration of other experimental parameters was not discussed in this study. Thus, the results of this experiment were obtained based on these limited experimental conditions.

Key words: Liquid chromatography mass spectrometry; Metabolite; gradient elution; tributylamine; collision fragments.

Thus far, a majority of research has focused on controlling the types of metabolites, but the number of metabolites present in a cell is not very clear. It has been proposed that there are 904 species of metabolites in *Escherichia coli* that can participate in 932 different reactions. [1-2] The number continues to increase, making accurate analysis of metabolites more difficult. Therefore, if there was a method that could analyze hundreds of metabolites at the same time, it would be readily adopted. We performed experiments to find a suitable way to get a good ion peak and higher ion response values in order to analyze the metabolites of the central carbon of collision fragments. Through quantitative analysis, our objective was to measure and characterize the metabolites. LC-MS/MS has several advantages: it is precise; it can analyze several important metabolites at the same time; it is a higher flux quantitative analysis of small compounds; it does not need derivatization; and it can quantitate compounds in a single analysis thereby reducing the manipulation of each sample.

The fragment of the central carbon in our experiment refers to fragment ions or sub ions that can be generated by the ion which has existed in the liquid of the objective metabolite as it has crashed into a medium molecule after it has gained energy from an external force. We can obtain and

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conduct the quality analysis of sub-ions in a mass spectrogram, and we can estimate the structure of the original molecule ion. Thus, this technique is essential for measuring and characterizing metabolites in order to find a suitable method for analyzing metabolite collision fragments of the central carbon.

The development of metabolic flux analysis of LC-MS/MS at detecting metabolites is normally aimed at the central carbon of collision fragments directly. Using LC-MS/MS, one can obtain the data of more fine-grained collision fragments which means it is possible to observe more sub-ions, while the data information obtained can make PPP and TCA more accurate. However, the best development in metabolomics research in recent years has been the detection of specific metabolites in biological fluids such as the circulation system and determining their exact levels[3-6]. At present, metabolite standards for experimentation must be imported into China. For example, tryptophan has a long synthesis metabolism path, weak metabolic flux, complex regulatory mechanism and is difficult to scale into production. Each of the omics platforms is developing rapidly since metabolic engineering was proposed as a new area 20 years ago. Although the metabolomics technology has been applied in metabolic pathways and microbial metabolic engineering, a detailed analysis of the metabolomics of tryptophan production and its analytic strategy has not been reported[7-8].

The extract methods of modern analysis of much of the central metabolic pathway of phosphate metabolic products and the middle metabolites in the TCA cycle have been reported, [9] and have often used GC-MS/MS to detect target metabolites. These modern techniques often need derivatization, while LC-MS/MS does not, and it is possible to cause the denaturation of thermosensitive compounds which are resolved at high temperatures. Therefore, this experiment used LC-MS/MS, quantitative and qualitative analysis, which does not require the quality balance of cell metabolites or knowledge of the energy metabolism and redox force of the target metabolites, which has broken the traditional limitation of quantitative analysis of metabolites[10].

For the vast number of intracellular metabolites, we choose some key metabolites in the TCA cycle and some others in the circulation – 3 types of materials in all. The first type of metabolites was salts, the second type was amino acids, and the third type was acids. There are ten materials in total.

EXPERIMENTAL

Reagents and instruments

6040 triple quadrupole liquid chromatography mass spectrometry; deionizer; sonicator; refrigerator; electronic analytical balance; carbinol; tributylamine; acetic acid; acetonitrile etc.

Research contents and methods

The gradient elution parameter and settings

The process of altering the parent ion to a sub-ion involved mobile phase parameters. Mobile phase can improve the ionization and the different ratios of mobile phases in a gradient elution which can affect the peak times of the metabolites in the chromatograph and mass spectra. Thus, in gradient elution settings, the first thing should be to explicate the polarity of the mobile phase A so that it is greater than the mobile phase B. If the small polar peak time is ahead of time, the polar assembly must be delayed to the peak time, to adjust the gradient elution ratio to a different time. The change of the mobile phase must cause the change of the column pressure, so that the chromatograph and instrument are not damaged by the change of column pressure.

Research on whether to join the tributylamine in the sample solution

Tributylamine has an impact on the chromatograph and mass spectrum. The more unstable the metabolite, the more easier it is to obtain a good peak in the mass spectrum, and the more stable and easier it is to obtain a good peak in the chromatograph. Tributylamine can combined with the ion of detected metabolites to generate a stable complex after it is added, enhancing the stability of the detected metabolites. We can obtain a good peak in the chromatograph after addition of tributylamine. Thus, the results of solutions that contain tributylamine or do not contain tributylamine need to be compared by analyzing the peak and ion response values to determine whether tributylamine needs to be added after ensuring the gradient elution parameters.

Chromatograph condition

a) Demands of the chromatographic column: 150 mm x 2.1 mm x 1.8 mm

n) Mobile phase A: 15 mM acetic acid, 50% (V/V) carbinol

c) Mobile phase B: acetonitrile

Gradient elution parameter settings

The principle of gradient elution parameter settings is that the large polarity of the mixed liquor consists of a sample and a mobile phase which will lay off the peak time. To the contrary, a small polarity will advance the peak time.

Mass spectrum conditions

Capillary temperature: 350°C
Negative Ion Modes
Electrospray voltage (ESI): -2.5KV
Column temperature: 40°C
Sample size: 5ul

Multiple reaction monitoring scanning mode (MRM)

Specified parameters of ten kinds of metabolites in the mass spectrometric detection list in Table 3. [11]

RESULTS AND DISCUSSION

Results of gradient elution parameter settings

In the chromatogram of Fig.1, the abscissa is time, the ordinate is the ion response value, the peak time is 33.913 min, and the ion response value is 1.4×10^3. In the mass spectrogram of Fig.1, the abscissa is m/z, the ordinate is the ion response value, and the ion response value is 5.25×10^1.

In the chromatogram of Fig.2, the abscissa is time, the ordinate is the ion response value, the time of concentration is 34.449 min, and the ion response value is 1.6×10^3. In the mass spectrogram of Fig.2, the abscissa is m/z, the ordinate is the ion response value, and the ion response value is 5.5×10^1.

The result of tributylamine addition to the sample solution

In chromatogram of Fig.3, the abscissa is time, the ordinate is the ion response value, and the ion response value is 3. In the mass spectrogram of Fig.3, the abscissa is m/z, the ordinate is the ion response value, and the ion response value is 4.2×10^1.

In the chromatogramof Fig.4, the abscissa is time, the ordinate is the ion response value, the time of concentration is 32.360 min, and the ion response value is 1.5×10^1. In the mass spectrogramof Fig.4, the abscissa is m/z, the ordinate is the ion response value, and the ion response value is 5.5×10^1.

In the chromatogram of Fig.5, the abscissa is time, the ordinate is the ion response value, and the ion response value is 2. In the mass spectrogram of Fig.5, the abscissa is m/z, the ordinate is the ion response value, and the ion response value is 4.3×10^1.

In the chromatogram of Fig.6, the abscissa is time, the ordinate is the ion response value, the time of concentration is 3.761 min, and the ion

Table 1. Gradient elution parameters

Time (min)	Mobile phase B(%)	Flow rate (ml/min)
0	0	0.2
5	0	0.2
9	2	0.2
12	9	0.175
15	9	0.125
18	25	0.125
19	50	0.075
25	50	0.075
26	0	0.075
32	0	0.2
36	0	0.2

The polarity of the mobile phase A is greater than the mobile phase

Table 2. Gradient elution parameters

Time (min)	Mobile phase B(%)	Flow rate (ml/min)
0	0	0.2
5	0	0.2
9	1	0.2
12	8	0.175
15	8	0.125
18	20	0.125
19	40	0.075
25	40	0.075
26	0	0.075
32	0	0.2
36	0	0.2

The polarity of the mobile phase A is smaller than the mobile phase

Table 3. MS detection parameters of major metabolites set

Metabolite	Collision induced dissociation voltage(eV)	Parent-ion (m/z)	Sub-ion (m/z)
Dipotassium D-Glucose-6-phosphate	135	259	13901690199
Aspartic acid	135	132	71.2088.1
Citric acid	135	191	111.0
D-Frutose-6-phosphate dipotassium salt	135	259	1390169
Pyruvic acid	135	87	43.0
Succitric acid	135	117	73.1
Isocitric acid trisodium salt hydrate	135	191	73.10167.1
Glutamic acid	135	146	84.00102.1
α-ketoglutaric acid	135	145	57.10145.1
Fumaric acid	135	115	71.0

Collision induced dissociation voltage referring to the voltage at capillaries, without optimization using the default in this experiment

response value is 5.8×10^3. In the mass spectrogram of Fig.6, the abscissa is m/z, the ordinate is the ion response value, and the ion response value is 5.5×10^2.

In the chromatogram of Fig.7, the abscissa is time, the ordinate is the ion response value, the time of concentration is 12.040 min, and the ion response value is 2.4×10^3. In the mass spectrogram of Fig.7, the abscissa is m/z, the ordinate is the ion response value, and the ion response value is 9.6×10^1.

In the chromatogram of Fig.8, the abscissa is time, the ordinate is the ion response value, the time of concentration is 12.152 min, and the ion response value is 1.0×10^4. In the mass spectrogram of Fig.8, the abscissa is m/z, the ordinate is the ion response value, and the ion response value is 1.45×10^2.

Result of experiment method application

In the chromatogram of Fig.9, the abscissa is time, the ordinate is the ion response value, the time of concentration is 29.025 min, and the ion

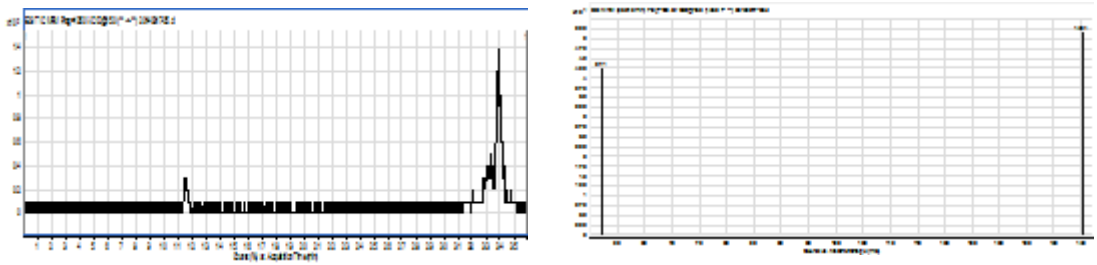

Fig. 1. Results of gradient elution parameters in Table 1(the detected compound is α-ketoglutaric acid)

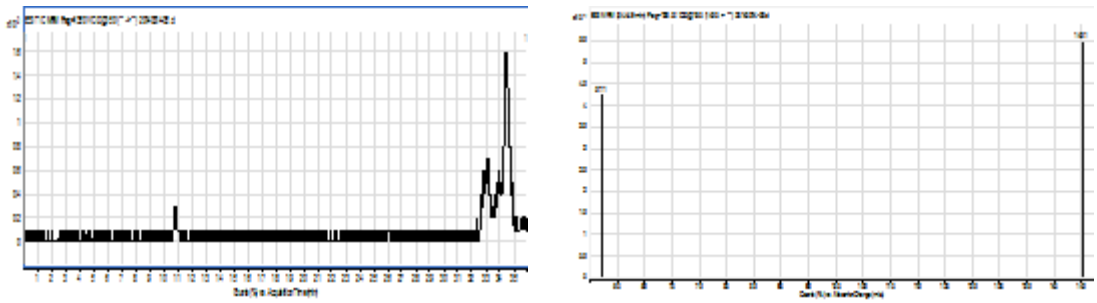

Fig.2. The result of the gradient elution parameters in Table 2 (The detected compound is α-ketoglutaric acid)

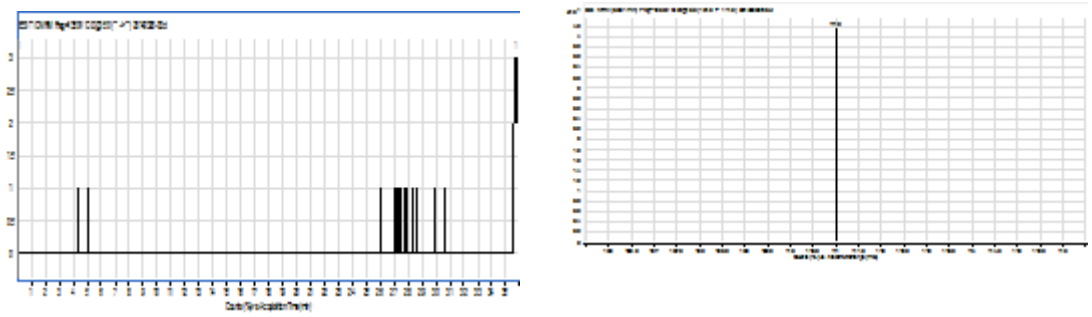

Fig. 3. The result of not adding tributylamine into the citrate sample solution detection

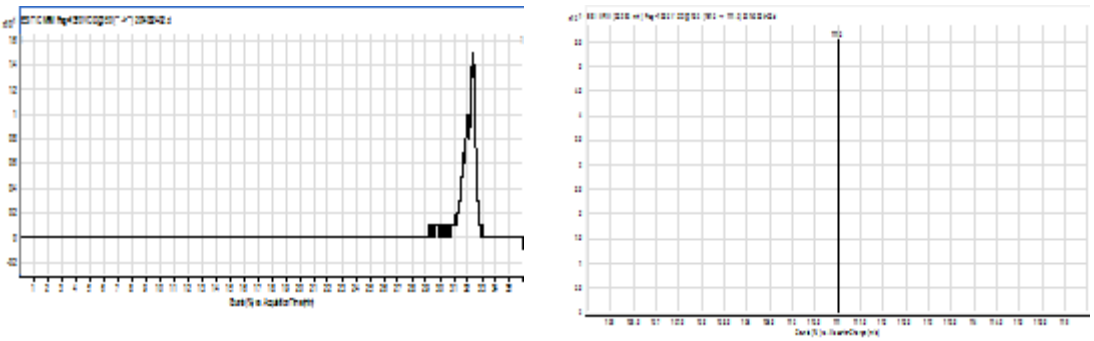

Fig. 4. The result of adding tributylamine into the citrate sample solution detection

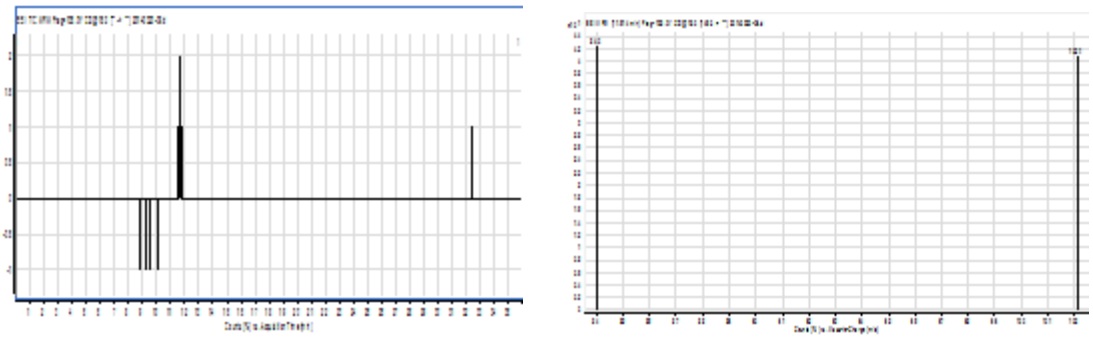

Fig. 5. The result of not adding tributylamine into the glutamate sample solution detection

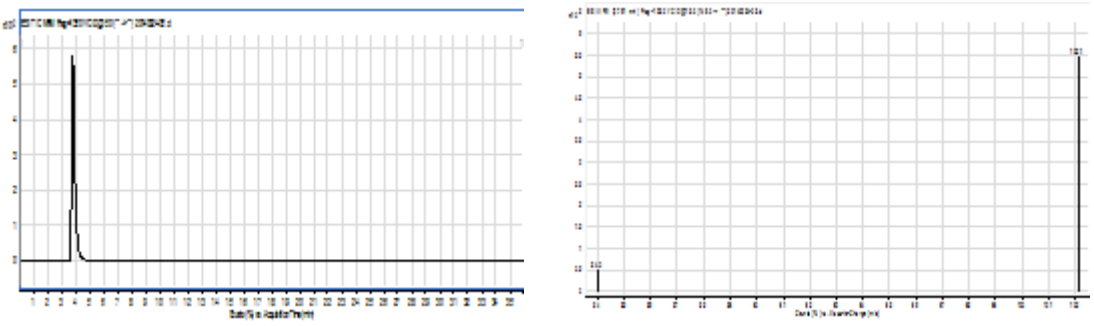

Fig. 6. The result of adding tributylamine into the glutamate sample solution detection

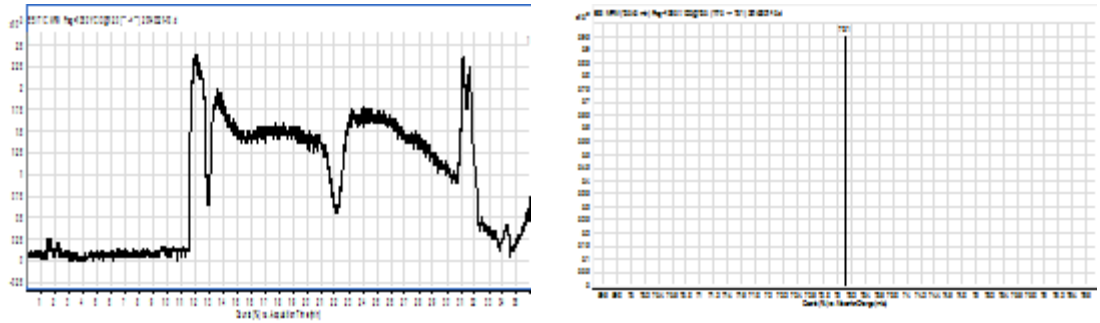

Fig.7. The result of not adding tributylamine into the succinate sample solution detection

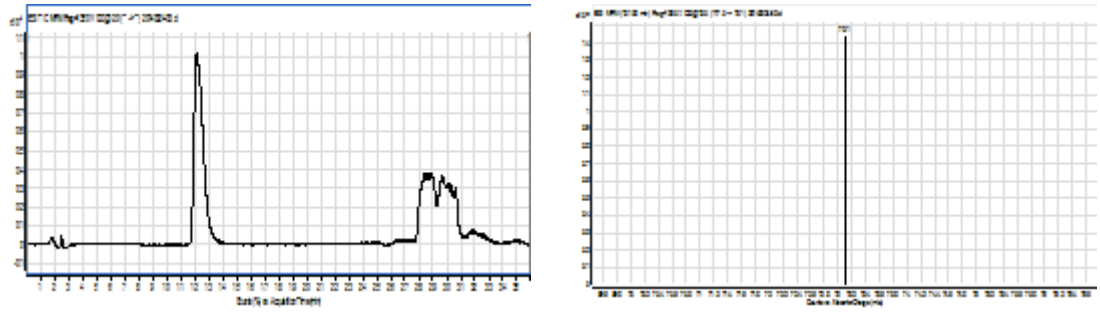

Fig. 8. The result of adding tributylamine into the succinate sample solution detection

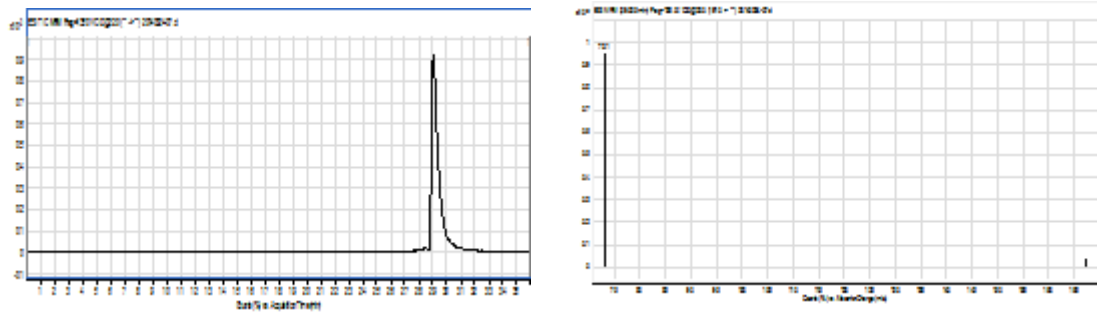

Fig. 9. Detection of isocitric acid trisodium salt hydrate

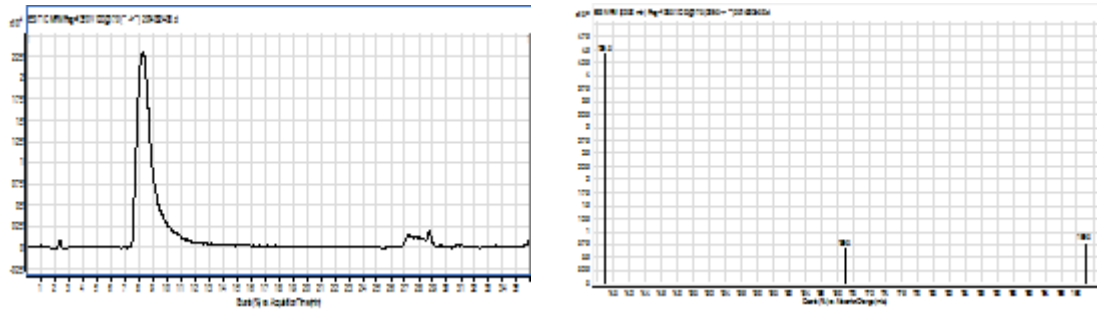

Fig. 10. Detection of dipotassium D-Glucose-6-phosphate

response value is 9.1×10^2. In the mass spectrogram of Fig.9, the abscissa is m/z, the ordinate is the ion response value, and the ion response value is 9.5×10^2.

In the chromatogram of Fig.10, the abscissa is time, the ordinate is the ion response value, the time of concentration is 8.308 min, and the ion response value is 2.25×10^4. In the mass spectrogram of Fig.10, the abscissa is m/z, the ordinate is the ion response value, and the ion response value is 4.4×10^2.

In the chromatogram of Fig.11, the abscissa is time, the ordinate is ion response value, the time of concentration is 8.621 min, and the ion response value is 2.4×10^4. In the mass spectrogram of Fig.11, the abscissa is m/z, the ordinate is ion response value, and the ion response value is 2.7×10^2.

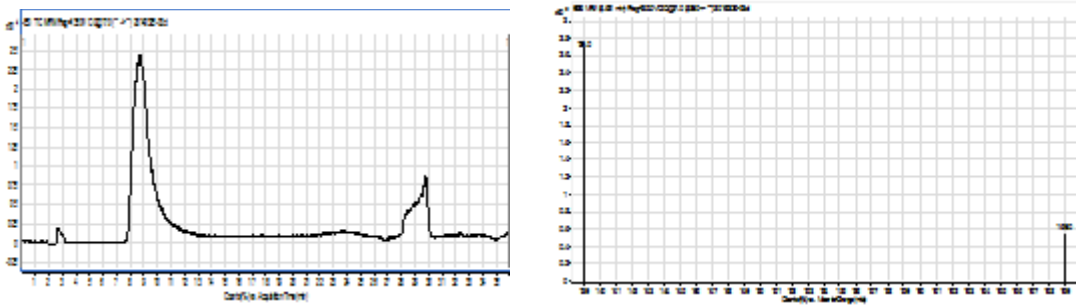

Fig. 11. Detection of D-Frutose-6-phosphate dipotassium salt

DISCUSSION

First, the result from every metabolite is composed of a chromatogram and a mass spectrogram. The sub-ion displayed in the mass spectrogram is the fragment of the collision parent ion. We can obtain more sub-ions after parent ion collision. We can focus on the sub-ion of interest in the mass spectrogram parameters according to our research, and we do not need to show all of the collision sub-ion in the mass spectrogram. Using the selectivity of the Q3 filter, we can focus on the ions of interest and display them in the mass spectrogram. For example, for Fig.11, D-Frutose-6-phosphate dipotassium salt, the sub-ion in mass spectrogram is m/z 139 and m/z 169.

By contrast, the time of the peak is unclear in Fig.1, and the ion response value is smaller than in Fig.2. The peak time will advance when the polarity is smaller, and will continue increasing the ratio of the mobile phase B in the gradient elution which will cause the peak time to be ahead of time. However, this will also cause the column pressure to be too low to enable the sample to inject automatically, thus the ratio of mobile phase B will stop increasing. At 25 min to 26 min in the gradient elution, the apparent change in the mobile phase led to column pressure sustained growth. Therefore, in order to reduce the damage to the column, the gradient elution parameters described in Table 1 were not adopted, while the parameters in Table 2 were considered to be the optimized conditions.

Comparison between Fig. 3-8, we can see that there are no complete peaks in Fig.3, 5. Fig.4 has a complete peak. Fig.6 also has a complete, sharp peak and the ion response value is larger in Fig.5. In Fig.7, there is a complete peak, but the peak tailing phenomenon is very serious. In Fig.8 has a complete peak and there is no trailing phenomenon. Therefore, addition of tributylamine in the sample solution can obtain a good ion peak and a higher ion response value, better than not adding tributylamine.

On the basis of the parameters set in Table 2 and adding tributylamine to the sample, this method is ideal. Using this method to detect isocitric acid trisodium salt hydrate, dipotassium D-Glucose-6-phosphate and D-Frutose-6-phosphate dipotassium salt is effective as indicated in Fig. 9, 11. All of the spectra have a rule peak and a high ion response value, so the gradient elution settings described in Table 2 and the addition of tributylamine in sample standard solution is a good way to obtain good peaks and higher ion response values.

In summary, it was found that a good ion peak and considerable response value can be obtained by using the gradient elution parameters described in Table 2. Addition of tributylamine to the sample solution was a suitable method for liquid chromatography mass spectrometry detection of the collision fragments of the central carbon metabolism. However, due to the limited experimental conditions and time, we did not explore other possible experimental parameters in this study, and the results of this experiment were obtained based on the experimental conditions described herein.

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