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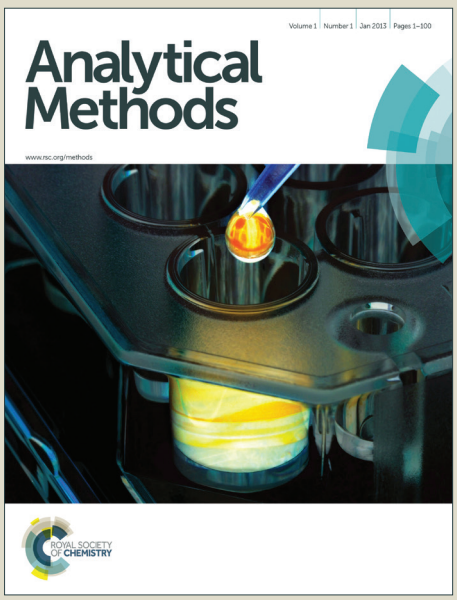

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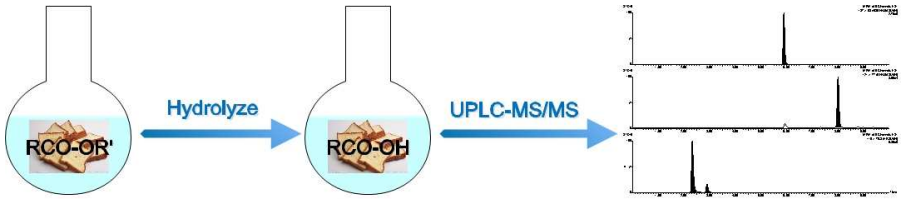

Hydrolysis reaction coupling to UPLC-MS/MS was employed for determination of the total content of four organic acids in food.

Determination of total maleic acid, fumaric acid, *p*-hydroxy benzoic acid and benzoic acid in food by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry

Yiguang Chen, Haiying Luo, Xindong Guo, Yanping Xian, Donghui Luo, Yuluan Wu

Guangzhou Quality Supervision and Testing Institute, Guangzhou, China 510000.

ABSTRACT

Hydrolysis reaction coupling to ultra performance liquid chromatography-triple quadrupole tandem mass spectrometry (UPLC-MS/MS) was applied for the determination of total maleic acid, fumaric acid, *p*-hydroxy benzoic acid and benzoic acid in milk base infant formula, soy base infant formula, beef jerky, starch and cake. Samples were hydrolyzed by sodium hydroxide then acidized. The hydrolysis solution was precipitated by acetonitril. After centrifuged, part of the supernatant was blown to dry by nitrogen gas then dissolved by water for test. The testing solution was separated by a reverse phase column then detected by the triple quadrupole tandem mass spectrometry operated in mutiple-reaction-monitoring mode. Matrix-matched calibrations were used for quantification. Methyl esters of the acids were used to optimize influential parameters of the hydrolysis reaction. The matrix effects of the samples to the four acids were from 45.7% to 157%. Most of the recoveries at two levels of 50 mg/kg and 200 mg/kg were from 74.6% to 129% with the relative standard deviation from 3.5% to 21%. The limits of detection were from 1.0 mg/kg to 10.0 mg/kg. Fifty samples from the local market were tested.

KEYWORDS: hydrolysis reaction, UPLC-MS/MS, food, maleic acid, fumaric acid, *p*-hydroxy benzoic acid, benzoic acid, determination.

INTRODUCTION

Organic acids and their esters are often employed as acidulent or preservative in food industry. Fumaric acid is a non-toxic acidulent widely used as a substitute for tartaric acid or citric acid. In China, the restrictions of it are from 0.3 g/kg to 8.0 g/kg according to different types of food such as desserts, confectionery and carbonated beverage[1]. Maleic acid is the cis-isomer of fumaric acid. Maleic acid has few applications and is not regarded as food additive in China[1]. Benzoic acid or benzoates are primarily used as preservative and corrosion inhibitor. Sodium benzoate and benzoic acid are most suitable for foods, fruit juices and beverages. Benzoic acid also occurs naturally in many plants and animals. *P*-hydroxy benzoic acid and parabens are also low-toxicity preservatives. In Chinese legislation, it can be used in food with restrictions from 0.012 g/kg to 0.5 g/kg (calculated as *p*-hydroxy benzoic acid) [1]. The chemical structures of the four compounds are shown in Figure 1.

For their wide application and easy availability in food manufacture, many methods were developed for the determination of them. Chromatographic methods such as LC-MS/MS [2-9], GC-MS [10-12] and LC [13-15] are the most popular methods while Nuclear Magnetic Resonance [16], fluorescence polarization immunoassay [17], multicomponent spectrophotometric monitoring [18], electrochemical sensor [19], capillary electrophoresis [20], potentiometric sensor [21] and chemometric methods [22] have been set by analytical chemists in recent years.

Some important improvements in the field of extraction and enrichment technology enhanced purification efficiency and decreased the detection limits [23-34]. M. Saraji, et al applied a three-phase hollow-fibre liquid-phase microextraction (HF-LPME) method to determine 7 phenolic acids in fruit juice [23]. M. S. Noorashikin, et al employed beta-cyclodextrin modified ionic liquid to extract parabens from water [27]. Y. G. Zhao, et al

used tetraethylenepentamine-functionalized Fe_3O_4 magnetic polymer (TEPA-MP) as absorbent for cleaning up of nine food additives [28]. B. Delgado provided a new extraction methods based on cationic surfactants [31].

Most of the works mentioned just focused on a series of esters such as parabens, or the acid forms of some organic acids. But both forms of the four acids can play some role in food. This paper provides a method for the determination of the total content of maleic acid, fumaric acid, *p*-hydroxy benzoic acid and benzoic acid. Sodium hydroxide was used to hydrolyze the samples. Methyl esters of the acids were used to optimize the influential parameters of the hydrolysis reaction. Liquid chromatography-triple quadrupole tandem mass spectrometry was employed to separate and detect the four acids. Five food matrixes including milk base infant formula, soy base infant formula, beef jerky, starch and cake were validated for these method. It is an easy and practicle method to determine and screen the total content of the four acids, which can prevent abuse or illegal addition of the acids.

MATERIALS AND METHODS

Chemicals, reagents and materials

Water was from a Pure Lab system, ELGA, Britain; methanol, formic acid, acetonitril, HPLC grade, CNW, Germany; sodium hydroxide, sulfuric acid, ethanol, Analytical reagent, Guangzhou Chemical Reagent Factory, China; maleic acid 99.0%, fumaric acid 99.5%, benzoic acid 99.5%, maleic acid bis-methyl ester 99.0%, fumaic acid bis-methyl ester 99.0%, 4-hydroxy benzoic acid-methyl ester 99.5%, Dr. Ehrenstorfer, Germany; 4-hydroxy benzoic acid 99.5%, methyl benzoate 99.5%, Chem Service, USA.

50 food samples including 25 starch or starch products, 7 milk base formula samples, 1 soy base formula sample, 4 cake samples, 9 bread samples, 4 jerkey were purchased from the local market.

Solutions

1 mol/L NaOH: Dissolved 40 g NaOH in a beaker with water, Transferred to a 1 000 mL volumetric flask. Diluted to volume with water. Stored in a plastic reagent bottle.

2 mol/L H_2SO_4: Dissolved 98g H_2SO_4 in a beaker containing 300 mL water. After cold, transferred to a 500 mL volumetric flask. Diluted to volume with water. Stored in a glass reagent bottle.

Mobile phase A, 0.25‰ formic acid: Dissolved 0.25 mL formic acid in 1 L water.

Mobile phase B, methanol.

Stock solutions 2 000 mg/L: Weighed about 20 mg standards in 10 mL volumetric flasks. Diluted to volume with methanol. Stored at -20℃.

Mix intermediate standard of four acids 200 mg/L: Pipetted 1 mL of the stock solutions of maleic acid, fumaric acid, *p*-hydroxy benzoic acid and benzoic acid in a 10 mL volumetric flask. Diluted to volume with water. Stored at 2℃.

Matrix matched mix standard working solution: Pipetted appropriate volume of mix intermediate standard in 5 mL volumetric flasks. Diluted to volume with negative sample matrix solutions. Prepared when used.

Mix standard of the four methyl esters 200 mg/L: Pipetted 1 mL of the stock solutions of maleic acid bis-methyl ester, fumaric acid bis-methyl ester, 4-hydroxy benzoic acid-methyl ester, methyl benzoate in a 10 mL volumetric flask. Diluted to volume with water. Stored at 2℃.

Instrument

UPLC Column: Waters Acquity UPLC HSS T3, 1.8μm, 100mm×2.1mm id. Waters, USA.UPLC-MS/MS system: Waters Acquity UPLC-Xevo TQ MS, Waters, USA.

Operation conditions

UPLC—Injection volume: 2μL; flow rate: 0.3 ml/min; column temperature: 30℃. Linear gradient: 0.0 min, 5% B; 1.0 min, 5% B; 5.0 min, 50% B; 7.0 min, 50% B; 8.0 min, 5% B; 10.0 min, 5% B.

MS/MS—Ionization mode: negative-ion electro spray ionization (ESI^-); capillary voltage: 1.5kv; source temperature: 130℃; desolvation temperature: 500℃; cone gas flow: 50 L/h; desolvation gas flow: 800 L/h; collision cell pressure: $1.9e^{-3}$ mbar; dwell time: 0.05 s for all analytes. Data acquisition was done in multiple-reaction monitoring (MRM) mode. The mass transitions are shown in Table 1.

Sample preparation

For milk base infant formula, soy base infant formula, beef jerky: Weighed 1.0 g of sample in a plastic centrifuge tube, added 2.0 g 1 mol/L NaOH, added 7.0 g of water, capped the tube, mixed it thoroughly. Then put it in a sonicator, ultrasonic hydrolyzed at 70℃ for 60 min. After cold to room temperature, centrifuged at 15℃ for 5 min at the rate of 8 000 rpm. Afterwards, transferred 2.0 g clear solution to another centrifuge tube, added 110 μL 2 mol/L H_2SO_4, then added 2 mL methanol and made volume to 10 mL with acetonitril. Capped the tube and mixed it thoroughly. Then centrifuged at 15℃ for 5 min at the rate of 8 000 rpm. Transfered 2 mL solution to a test tube and blew it to dry with nitrogen. Added 2 mL water to dissolve the residue. Filtered the solution with a 0.22μm syringe filter for test.

For starch and cake: Weighed 1.0 g of sample in a plastic centrifuge tube, added 2.4 g ethanol, added 2.0 g 1 mol/L NaOH, added 5.6 g water. Capped the tube, mixed it thoroughly. Then put it in a sonicator, ultrasonic hydrolyzed at 70℃ for 60 min. The following procedure was the same with the milk base infant formula.

Method validation

For the validation of hydrolysis of methyl esters of the acids: added 0.25 mL mix standard solution of the methyl ester of the four acids in a centrifuge tube, added vary volume of 1 mol/L NaOH, added ethanol if need, made volume to 2 mL with water. Capped the tube, then put it in a sonicator, ultrasonic hydrolyzed at vary temperature for vary time. After cold to room temperature, added 2 mol/L H_2SO_4 to neutralize the solvent, made volume to 10 mL with water, mixed thoroughly. Transfered 1 mL solution to vial for test.

Milk base infant formula, soy base infant formula, beef jerky, starch, cake were the five matrixes for over spiked experiment. 6 parallel samples at two levels: 50 mg/kg and 200 mg/kg, respectively.

The Matrix effect calculation was as follows:

$$\text{ME（\%）} = \frac{A_m - A_b}{A_s} \times 100$$

Where A_m was the peak area of the analyte in the matrix match standard at the concentrition of 1.0 mg/L, A_b was the peak area of the analyte in the negative sample, and A_s was the peak area of the analyte in the standard at the concention of 1.0 mg/L.

The linearity was measured by using a five level calibration curve in the range 0–5.0 mg/L. The limit of detection was defined as the concentration that yielded a *S/N* ratio of 3, and the limit of quantitation was defined as the concentration that yielded a *S/N* ratio of 10. Accuracy was expressed as percent recovery (Rec., %). Precision was expressed as relative standard deviation (Rsd., %).

RESULTS AND DISCUSSION

UPLC-MS/MS

Polar organic acids have weak retention in reversed-phase columns. We compared a HSS T3 column with a BEH C18 column and found that the former could extend the

retention time for 0.6 min for maleic acid in the same mobile phase condition.

The ionized form of the carboxylic group has strong interaction with the residue silicon hydroxyl group to affect the peak shape and the retention time of the compounds and formic acid is usually added to the mobile phase for reversed phase-LC. But for mass spectroscopy detection in negative-ion electrospray ionization mode, the addition of formic acid lowers the response of the analytes. We had to optimize the amount of formic acid to balance the chromatography performance and the mass spectroscopy sensitivity. Actually, we compared three levels of amount: 0.1‰, 0.25‰ and 0.5‰ of formic acid and found that 0.25‰ was enough to separated fumaric acid and maleic acid. And they also got acceptable peak shapes. The MRM chromatograms are shown in Figure 2. The retention times are shown in Table 2. The retention times and peak shapes of the compounds on the same column showed no obvious change after more than 300 sample injections.

Only one mass transition was chosen for each analyte because all the analysts had just one applicable mass transition during the mass parameter optimization process.

The instrument limits of detection (ILOD, *S/N*=3) of the four compounds were from 0.01 mg/L to 0.1 mg/L. The five-point standard curves showed good linear relationship. The Standard curves, linear ranges, correlation coefficients, detection limits are also shown in Table 2.

Hydrolysis of methyl esters to acids

The experiment was to settle hydrolysis parameters which were enough to hydrolyze the esters totally without degradation of the esters and acids. The parameters which affected the reaction included the concentration of NaOH, the temperature, the reaction time and the assistant device.

For the temperature, we compared the three temperatures 50℃, 60℃ and 70℃ and fixed the other parameters at 0.2 mol/L NaOH-60 min-ultrasonic assisted. The result showed that only 70℃ could get a near 100 percent recovery. The other two temperatures were too low to complete the hydrolysis reaction.

For the concentration of NaOH, we compared the three concentration levels 0.1 mol/L, 0.2 mol/L and 0.5 mol/L and fixed the other parameters at 70℃-60 min-ultrasonic assisted. The result showed that the concentration levels of 0.2 mol/L and 0.5 mol/L were enough to totally hydrolyze the four esters while the concentration level of 0.1 mol/L was just enough to hydrolyze fumaric acid bis-methyl ester and maleic acid bis-methyl ester.

For the reaction time, we compared 15 min, 30 min and 60 min and fixed the other parameters at 70℃-0.2 mol/L NaOH-ultrasonic assisted. 30 min and 60 min could both totally hydrolyze the esters without degradation of them. 15 min got about 91% to 96% recovery.

Finally, we fixed the reaction condition as: 0.2 mol/L NaOH-60 mins-70℃-ultrasonic assisted.

We fixed the reaction condition as: 0.2 mol/L NaOH-60 mins-70℃- ultrasonic assisted when the reaction solution contained 30% (*v/v*) ethanol, although the hydrolysis efficient of 4-hydroxy benzoic acid methyl ester was low. Because the increase of NaOH could enhance the recovery and also cause some starch sample condensation.

The recoveries of the hydrolysis reaction are shown in Table 3. In Table 3, Condition W meant acids as reactants, NaOH concentration was 0.2 mol/L, reacted at 70℃ for 60 min and ultrasonic assisted reaction. Condition X meant methyl esters as reactants. NaOH concentration was 0.2 mol/L, reacted at 70℃ for 60 min and ultrasonic assisted

reaction. Condition Y meant acids as reactants, NaOH concentration was 0.2 mol/L, reacted at 70℃ for 60 min, reaction solution contained 30% ethanol (*v*/*v*) and ultrasonic assisted reaction. Condition Z meant methyl esters as reactants, NaOH concentration was 0.2 mol/L, reacted at 70 ℃ for 60 min, reaction solution contained 30% ethanol (*v*/*v*) and ultrasonic assisted reaction.

Sample preparation

Because the addition of alkali water solution to starch may cause sample condensation, some ethanol was added to the hydrolysis solution to reduced condensation effect for starch sample.

Acetonitril can be used to precipitated protein and starch. We also applied it as a purified method after hydrolysis.

The sample matrixes exhibited moderate signal expressions which were from 45.7% to 157%. The matrix effect of the five matrixes to the four compounds is shown in Table 4. The recoveries of the four acids in the five matrixes are shown in Table 5. In the tables, matrix **A** stands for milk base infant formula, matrix **B** stands for soy base infant formula, matrix **C** stands for beef jerky, matrix **D** stands for starch, matrix **E** stands for cake.

Most of the recoveries were from 74.6% to 129%, with the precision, expresses as relative standard deviation (RSD) from 3.5% to 21%. The chromatograms of spiked starch sample are shown in Figure 3.

From the recovery data we can see that the matrix effects are high at some places. We tried to clean the sample to reduce the matrix effect by solid phase extraction using C18 column and Oasis MAX column, the result showed that using C 18 column got low recoveries of fumaric acid and maleic acid while using Oasis MAX column got interference to fumaic acid. Also we tried other liquid chromatography columns such as

negative ion exchange column and Waters' Fast Fruit juice column with a PDA detector, but their sensitivity and resolution was not good enough to promote them into practice.

Sample test

50 samples were tested by this method. Only one sample was found to contain maleic acid at the concentration of 3.5×10^3 mg/kg. The chromatograms of the positive sample are shown in Figure 4.

CONCLUSION

It's a easy and practical method to screen and determinate the total quantity of maleic acid, fumaric acid, *p*-hydroxy benzoic acid and benzoic acid in food. It has promising application prospect in the field of food quality testing.

ACKNOWLEDGEMENTS

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33 J. He, S. Chen, Y. L. Jiang, Y. Z. Shen, J. Zhu, H. L. Wei, H. X. Zhang, K. Lu, *Journal of Separation Science*, 2012, 35, 308.

34 O. Kritsunankul and J. Jakmunee, *Talanta*, 2011, 84, 1342.

294 Table 1. Mass transitions of the four acids.

Comp.	Precursor m/z	Daughters m/z	Cone voltage V	Collision energy V
1	115	71	15	10
2	115	71	15	10
3	137	93	25	15
4	121	77	25	15

Table 2.The retention time, standard curves, linear ranges, correlation coefficients, ILOD.

Comp.	R.T. (min)	Standard curve	Linear range (mg/L)	r^2	ILOD (mg/L)
1	2.32	y=10745x-153	0.5~10	0.9992	0.03
2	2.90	y=1978x-275	0.5~10	0.9990	0.1
3	5.89	y=35406x+1017	0.5~10	0.9993	0.01
4	8.01	y=593x+5	0.5~10	0.9989	0.1

297 Table 3. The intra-day recoveries and relative standard deviations of the hydrolysis

298 reaction (n=7).

Compound / Condition	**1**		**2**		**3**		**4**	
	Rec., %	Rsd., %	Rec., %	Rsd., %	Rec., %	Rsd., %	Rec., %	Rsd., %
W	101	1.5	101	3.0	100	1.5	97.6	2.5
X	95.0	2.9	96.3	3.1	101	2.4	101	2.3
Y	101	0.9	98.9	1.0	97.4	0.6	98.1	1.4
Z	94.0	1.3	98.5	1.6	60.6	2.0	102	4.9

299 Table 4. Matrix effect of the five matrixes to the four compounds.

Matrix / Comp.	**A**	**B**	**C**	**D**	**E**
1	157	84.0	113.0	71.9	65.2
2	53.6	36.0	94.6	50.6	59.0
3	61.2	47.0	99.3	56.1	64.5
4	122	70.7	103.0	72.2	45.7

300 Table 5. Intra-day accuracy and precision of the four acids in the five matrixes.

Matrix	Conc. mg/kg.	Meleic acid		Fumaric acid		*P*-hydroxy benzoic acid		Benzoic acid	
		Rec.,%	Rsd.,%	Rec.,%	Rsd.,%	Rec.,%	Rsd.,%	Rec.,%	Rsd.,%
A	50	128	4.8	153	5.4	100	14	107	20
	200	103	6.2	121	5.9	94.8	9.0	119	8.3
B	50	135	14	120	12	117	13	96.0	14.6
	200	108	6.9	120	4.9	117	4.0	103	7.3
C	50	90.2	6.8	89.0	8.2	93.4	6.5	86.6	18
	200	99.2	3.5	97.6	4.9	95.7	3.5	97.7	2.4
D	50	102	9.8	102	13	113	7.4	74.6	15
	200	105	6.7	103	9.2	124	6.0	81.0	18
E	50	102	13	129	19	184	17	106	21
	200	108	4.6	108	7.1	111	11	110	9.6

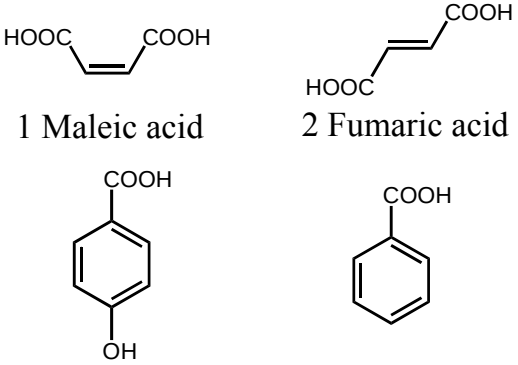
Figure captions

Figure 1. Chemical structures of the four acids.

Figure 2. MRM chromatograms of the four acids.

Figure 3. MRM chromatograms of a spiked starch sample.

Figure 4. MRM chromatograms of a positive starch sample.

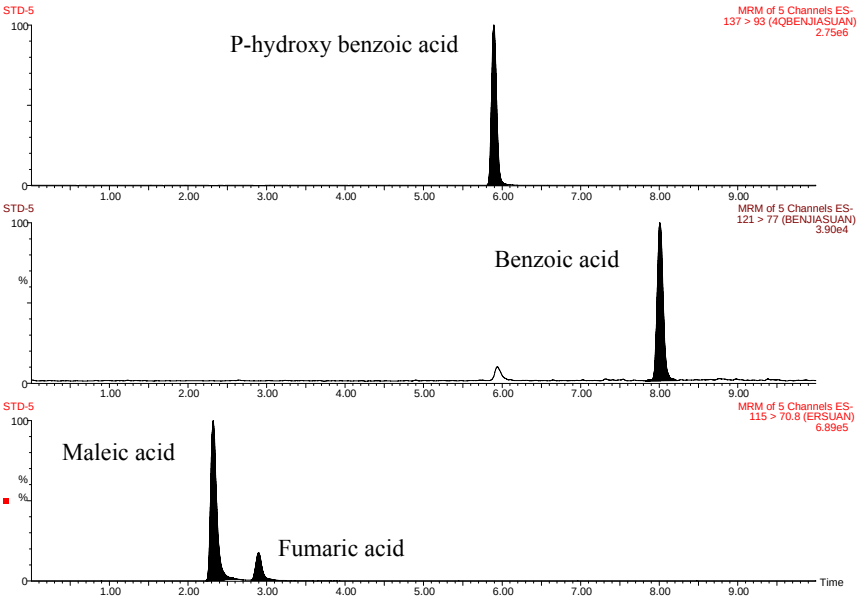


1 Maleic acid 2 Fumaric acid

3 *P*-hydroxy benzoic acid 4 Benzoic acid

306

307 **Figure 1**. Chemical structures of the four acids.



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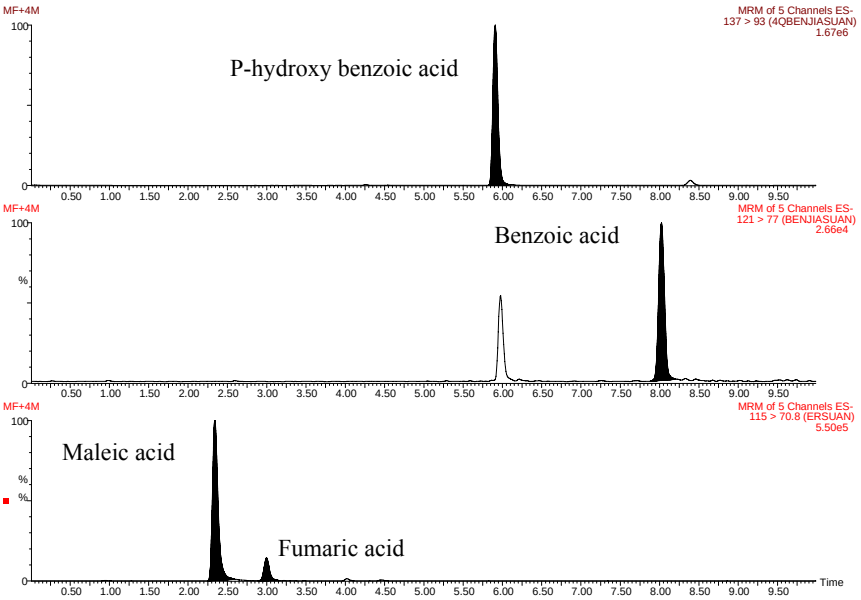

Figure 3. MRM chromatograms of a spiked starch sample.

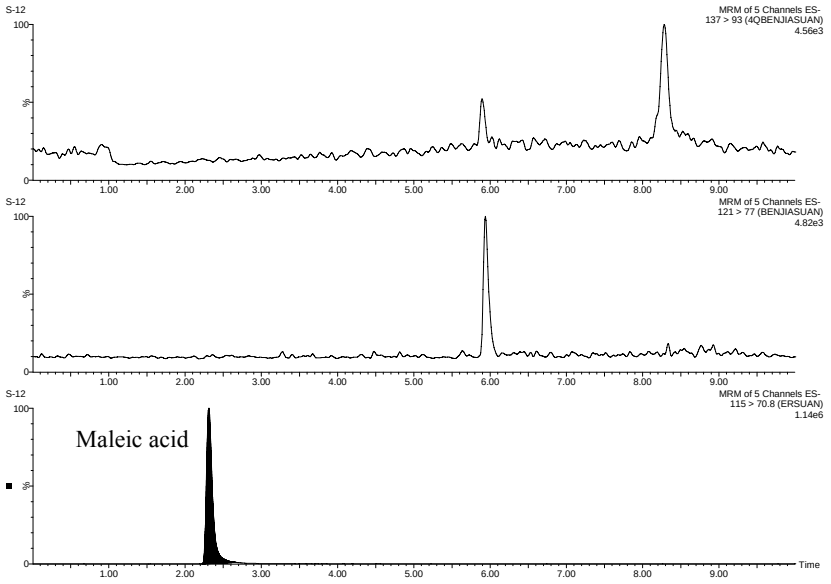

Figure 4. MRM chromatograms of a positive starch sample.