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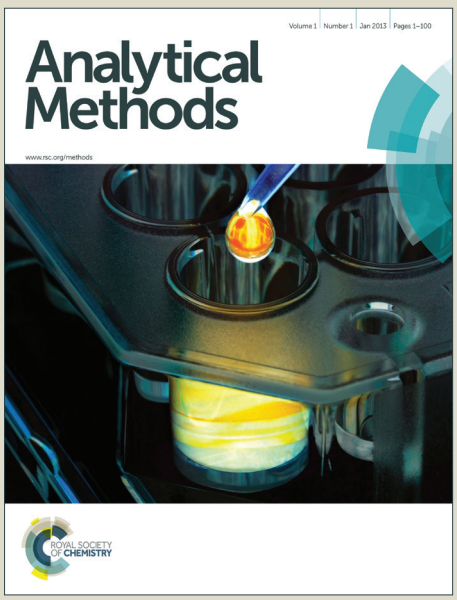

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Rapid and sensitive gas chromatography-triple quadrupole mass spectrometry method for the determination of organic acids in tobacco leaves

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Abstract:

An improved gas chromatography-triple quadrupole mass spectrometry (GC-TriQ-MS) method has been developed to determine organic acids in tobacco leaves. Optimizations of selected reaction monitoring (SRM) scan mode, including the selection of appropriate precursor-product ions and the optimization of collision energy parameters for each acid, were carried out to improve sensitivity and selectivity. Sample preparation was performed by derivatization-free extraction instead of conventional derivatization extraction to shorten the work time and reduce the amount of physical labor. Validation of the method was carried out in terms of linearity, limits of detection (LOD), accuracy, and precision. The calibration line was made over the concentration range from 0.27 to 69.26μg mL^{-1}, and each acid has a selected dosage concentration ranged with a regression coefficient over 0.9975. The LOD was 0.01-0.06μg mL^{-1} and the recovery for most analytes was between 80% -111%, while the relative standard deviation was less than 10%. This method was done without sacrificing the repeatability, reproducibility, and precision compared with previously published methods. The development and validation results discussed in this paper indicate that this method provides a suitable and convenient analytical tool to quantify organic acids in tobacco leaves.

1. Introduction

Tobacco is a very complex matrix which contain thousands of chemical compounds, including organic acids, alcohols, aldehydes, esters, etc, and those

compounds determine the quality and fragrance style of tobaccos[1]. Organic acids including non-volatile, semi-volatile and volatile organic acids and their derivatives are the main components of tobacco flavor, make direct effect on the taste and tactile characteristics of tobacco smoke[2, 3]. Non-volatile acids are mainly citric acid, malic acid and oxalic acid, etc. Their contents are very low, which in total accounted for 3-7%[2] and existed in binding state. Semi-volatile acids mainly are senior fatty acids with more than 10 carbon atoms, including saturated fatty acids and unsaturated fatty acids. Non-volatile and semi-volatile acids affect sensory quality during smoking by regulating the pH value of tobacco and neutralizing alkaloids (especially nicotine) in tobacco smoke[4]. However, some saturated fatty acids may increase the taste of fat and wax, and unsaturated fatty acids, especially linolenic acid and linoleic acid, having a negative impact on flavor. Volatile acids are short-chain fatty acids and some aromatic acids with less than 10 carbon atoms, considered precursors of tobacco flavor[5, 6]. They can directly enter the tobacco smoke during smoking and have obvious effect on flavor. Volatile acids, such as formic acid and acetic acid, are primary components in tobacco, contribute to the offensiveness of smoking. Isovaleric acid, pentanoic acid and benzoic acid can produce the taste of fruit or cream[2]. The so-called acidic components in tobacco generally are volatile and semi-volatile organic acids. The taste and aroma of tobacco products are closely linked with contents of some organic acids, too high would create a spicy hot feeling to the throat[7]. Organic acids are important contributors to tobacco quality. To assess tobacco quality for characterization, it is necessary to develop a fast, sensitive and selective analytical

method that can accurately determine low levels of organic acids in tobacco.

GC-MS[3] has been the most widely used method for organic acids analysis in tobacco and tobacco products due to its rapidity, simplicity, and higher sensitivity[8]. Meanwhile, many other approaches such as, HPLC[7, 9], ion chromatography[10], and capillary isotachophoresis[11] have been used and most of them have excellent resolution and high detection sensitivity. However, all those methods require a laborious and time-consuming derivatization procedure in sample preparation, due to high polarity of organic acids. Under these circumstances, Meng proposed a fast derivatization-free GC-FID method to separate saturated fatty acids[12]. Nevertheless the biggest obstacle in direct quantification of organic acids is to overcome the interference of the chemical background from complex tobacco matrix. Sample matrix effects can lead to poor analyte recoveries and decreased accuracy and precision[13]. GC-MS coupled in the selected ion monitoring (SIM) approach[14] or mass spectrometry/mass spectrometric (MS/MS) methodology[13] was commonly employed for decrease of background interference. Many researchers analyzed the chemical component in complex matrix using GC-MS in single ion monitoring scan mode[2, 15]. Gas chromatography-triple quadrupole mass spectrometry (GC-TriQ-MS) can provide the rapid and accurate analysis of trace components in complex matrix, and avoids the analogues potential interference by monitoring a limited number of precursor-product ion pairs in selected reaction monitoring (SRM) scan mode[16, 17]. This bidimensional mass spectrometric analysis, performed “in time” and “in characteristic ion”, can better improve sensitivity by minimizing matrix interference and strengthening the

signal/noise ratio[18, 19]. These features are well suited for the detection of target analyte in highly complex matrix. *Jiu ai* has directly quantified free saturated fatty acids in tobacco using GC-TriQ-MS by SRM scan mode[20], but his work simply showed one precursor ion 129 m/z for all determined acids. In fact, each acid has specific precursor ion, while different ions correspond to different collision energy. Choose one ion for all acids was not the best choice obviously and had great limitations for the simultaneous determination of short chain, medium chain and long chain acids. When precursor ions were broken into product ions under the optimal collision, a limited number specific precursor-product ion pairs of each organic acid would be better monitored for eliminating background interference and producing good peak shape.

The current study was aimed to find a suitable method to determine organic acids in tobacco. The appropriate precursor-product ions were chosen and the collision energies were optimized for each organic acid, coupling the high sensitivity of gas chromatography-triple quadrupole mass spectrometry with derivatization-free sample preparation. Then, a simplified analytical method for determination of organic acids in tobacco was established.

2. Experimental

2.1. Materials

Three flue-cured tobacco leaves at grade B_2F derive from Hunan province in China were stored in the warehouse of China Tobacco Guangxi Industrial Co.,Ltd.

2.2. Reagents and standard solutions

Twenty organic acids standards (Table 1) used in this study were purchased from ANPEL Scientific Instrument Co.,Ltd.(Shanghai, China) and their purity was higher than 99%. The solvent (dichloromethane) was supplied by Sinopharm Chemical Reagent (Shanghai, China) and its purity was higher than 99.9%. Sodium hydroxide, hydrochloric acid and anhydrous sodium sulfate with purity higher than 99.0% were supplied by Sinopharm Chemical Reagent (Shanghai, China). LC grade water was obtained by purifying demineralized water in a Milli-Q system (Millipore, Bedford,MA, USA).

The stock solution of each organic acid was prepared by dissolving the 20 standard references in dichloromethane at concentrations rang about 1-10 mg mL^{-1}. The standard solution mixture was prepared by diluting the stock solution of each acid in dichloromethane and their concentrations are shown in Table 1. Six calibration solutions were prepared by diluting respectively 25μL, 50μL, 150μL, 250μL, 350μL, 500μL standard solution mixtures to 50mL with dichloromethane and stored in the dark at 0℃ in amber glass vials with Teflon-lined cap.

2.3. Sample preparation

Flue-cured tobacco samples were dried at 25℃ in an oven for 24 h, and then grounded and sieved to fine powder (100 mesh). 1.00g of ground dry tobacco and 10mL of 5% sodium hydroxide solution were placed in a 50mL plastic screw-cap centrifugal tube with stopper. After vortex shocking for 2 minutes and ultrasonication for 20 minutes[20], the mixture was acidified to pH 2～3 with hydrochloric acid. Then

10mL dichloromethane was added to the mixture and again ultrasonicated for 20min to extract organic acids. About 3mL extract solution (the lower solution) were taken and dehydrated with anhydrous sodium sulfate (activated overnight at 20℃). The solution was filtered with a 0.22μm filter membrane and stored in a 1.5 mL screw capped vial for analysis.

2.4. Instruments and chromatographic conditions

The GC-EI-MS/MS analysis was performed on TSQ Quantum XLS system from Thermo Fisher Scientific Inc (USA), which equipped with a triplus autosampler, trace GC Ultra gas chromatograph, TSQ Quantum XLS mass spectrometer, and TR-Waxms column (30m×0.25mm ID, 0.25μm film thickness, Part number: 260×142P). Helium was used as carrier gas, at a constant flow rate of 1mL min^{-1}. Argon with high purity (99.995%) was used as collision gas in mass spectrometers. The injector was operated in PTV splitless mode, with splite flow of 50mL min^{-1} and split rate 10:1. The injection phases temperature program was as follow: 45℃ hold for 1 min, ramp to 60℃ at 14.5℃ min^{-1} keeping 0.5 min for solvent evaporation, then ramp to 250℃ at 8℃ min^{-1} keeping 1 min for target substance transfer into gas state, and then ramp to 270℃ at 14.5℃ min^{-1} keeping 45 min for injection port clearing.

The GC temperature program was as follow: the GC oven temperature was programmed from 60℃ (hold for 2 min) to 110℃ with ramp rate of 10℃ min^{-1}, then ramp to 150℃ at 3℃ min^{-1}, then ramp to 230℃ at 15℃ min^{-1}, held for 40 min. The mass spectrometer was operated in the electron ionization (EI) mode at 70eV. The mass range was scanned from 45 to 350 m/z at 0.2 s/scan for the full-scan mode.

Typically TSQ Quantum XLS mass spectrometers have three quadrupoles named as Q1, Q2, and Q3, refer to them as the precursor mass analyzer, collision cell (ion transmission device), and product mass analyzer, respectively. The SRM scan mode was performed in three stages of analysis. In the first stage of Q1 ions selected by mass analysis are called precursor ions. In the second stage, precursor ions enter Q2, and dissociate into smaller fragment ions by collision-induced dissociation (CID) (interaction with argon collision gas present in the collision cell). Ions formed in Q2 enter Q3 (the product mass analyzer) for the third stage of mass analysis.

For MS/MS, a multi-segment acquisition method, which programmed to the retention time windows of acids, was created to program the sequential EI/MS/MS experiments by applying the selected reaction monitoring (SRM) scan method[13]. The underlying principle of SRM is that the selected set of precursor and product ions contains sufficient information to represent the target compound[21].

2.5. Method validation

Linearity of the developed method was calculated for each acid by fitting a simple linear regression line to the calibrator data, then calculating the correlation coefficient (R^2). The calibration was drawn by the peak area of standard solution which was scan by SRM at the optimized conditions. The calibration lines were obtained using Xcalibur 2.1, Thermo Foundation 1.0, TSQ 2.3 software and also using Microsoft Office Excel 2007. Calibrator concentration was calculated from the calibration line and required to be within 20% of the theoretical target concentration.

The limit of detection (LOD) response method sensitivity was shown by the

minimum detectable amount or the minimum detectable concentration in gas chromatography and calculated by the relative peak area/height, refers to the smallest concentration that the detector can detect from chromatographic peak, it was bigger than 3 times noise[22]. Generally, the LOD of instrument was estimated as $3s_0$, where s_0 was the estimated standard deviation at zero analyte concentration[23]. Standard deviation of intercept of calibration line can be used in computation of LOD and LOQ values by 3σ and 10σ approaches instead of mean blank signal value to the fact that they could be a more accurate estimate of mean blank value[24]. According calibration line of standard substance, to estimate the limit of quantifications (LOQ) of the blank, added analytes concentration with estimates LOQ values to tobacco samples with seven times repeatability, then calculated the value of standard deviation (s_d), The LOD of real method was estimated as 3 s_d[25].

Recovery reflects the accuracy of the method. Recovery was estimated by adding analytes to tobacco samples, and comparing concentration of analytes to those from unspiked samples. It was calculated by following formula[22]:

$$\text{Recovery} = (C_{spiked} - C_{unspiked}) \times 100\% / C_{addition}$$

Where C_{spiked} was the concentration of acids-spiked tobacco extraction, $C_{unspiked}$ was the concentration of unspiked tobacco extraction, $C_{addition}$ was the concentration of standards addition.

The precision of the test results was represented by the relative standard deviation[26], which was the ratio of standard deviation and arithmetic average.

3. Results and discussion

To achieve maximum sensitivity and selectivity, appropriate precursor-product ions of target analytes were selected for qualitative analysis through full scanning standard solution. The optimizations of SRM keep precursor ions broken into product ions under optimal collision condition for eliminating background interference and producing good peak shape. The last and most important, the feasibility of this method was evaluated on tobacco organic acids analysis, and the results of this method was compared with others.

3.1. Qualitation and selection the specific ions of organic acids

The retention time (t_R) of standard substance (Figure.1B) were generally used as analytes identification. From Figure.1A, it was found that the peaks of acetic acid, propionic acid, isobutyric acid, butyric acid, 2-methyl butyric acid, pentanoic acid, caproic acid and heptanoic acid were closer to the baseline. Meanwhile, the peaks of octanoic acid, pelargonic acid, decylic acid, benzoic acid, dodecanoic acid, linoleic acid, linolenic acid appeared as complex and were not separated clearly due to the matrix interference. Therefore, the qualitative and quantitative analysis of organic acids in tobacco samples based on the retention time of standard substance was difficult to perform.

In this study, SRM was carried out minimize matrix interference and improve the S/N ratio by monitoring a limited number of precursor-product ion pairs[16]. In SRM scan mode, the precursor ion collides with a neutral atom or molecule dissociates into smaller fragments in the CID process. The first step of optimization was to choose the appropriate precursor and product ions for each acid. Generally, precursor ion is not

necessarily the molecular ion. Those with high mass-to-charge ratio and high abundant are usually selected as appropriate precursor ions. While those fragments with medium molecular weight and higher relative intensity are usually selected as product ions. Two product ions with a certain mass-to-charge gap between them were chosen in order to improve the accuracy (Table 1).

3.2. Optimization of collision energy parameters

In general, the higher CID efficiency generates higher ion intensity. When the collision energy is higher beyond the optimum value, more collisions take place and more small ions are generated, resulting in weaken CID efficiency and decreased product ion intensity. The product ion intensity also decreases when the pressure is below the optimum value because of fewer collisions. Therefore, it is quite crucial to discover the optimum collision energy to improve the S/N ratio, eliminate background interference and produce good peak shape.

For each acid, optimum collision energy was selected based on Figure.2, corresponding to the maximum of intensities of major product ions. It was found that precursor ions intensity of most acids decreased gradually along with the increased collision energy. Product ions of acetic acid, isobutyric acid, butyric acid, pentanoic acid, heptanoic acid, octanoic acid, pelargonic, podecanoic acid, myristic acid and linolenic acid increased at first and then regularly decreased. The collision energy corresponding to the peak of product ion intensity was selected as the most suitable. However, product ions intensity were not always so regularly changed for some organic acids, for example, 2-furan formic acid with product ion of 55 m/z had two

peaks at 11EV and 15EV, but the precursor ion (112m/z) had sharp peak at 11EV, which indicated the CID efficiency was higher and 11EV was the optimum collision energy. Propionic acid and decylic acid were quite similar. For caproic acid, 2-methyl butyric acid, and benzoic acid, their product ion slowly decreased with increasing collision energy, 8 EV for 59 m/z of the precursor ion of caproic acid and 2-methyl butyric acid and 15 EV for 105 m/z of the product ion of benzoic acid were considered as optimum collision energy, respectively. The change of ion intensity of palmitic acid, stearic acid, oleic acid, and linoleic acid also showed slight fluctuations. According to the higher ion intensity generated by higher CID efficiency, 6 EV for 129 m/z and 7 EV for 115 m/z; 7 EV for 143 m/z and 10 EV for129 m/z; 7 EV for 83 m/z and 10 EV for 55 m/z; 7 EV for 150 m/z and 8 EV for 109 m/z were selected as optimum collision energy for palmitic acid, stearic acid, oleic acid and linoleic acid, respectively.

Figure.1C shows the chromatogram of tobacco sample analyzed through SRM scan at the optimum condition (as described above). It was observed that no interfering peaks were observed and apparent baseline separation for organic acids was obtained, indicating a high selectivity of GC-TriQ-MS used on determination of organic acids without derivatization extraction.

There is special explanation about Figure.1C. In the research process, multi-segments were set due to retention time and the selected specific precursor-product ion pairs for determining twenty organic acids simultaneously. Owing to different segments with different ion pairs, different baselines were

observed. Notably, despite displayed different baseline, it does not affect the qualitative and quantitative analysis of analytes.

3.3. Evaluation of the method

All samples were analyzed using the optimized condition. Quantification was performed by calibration lines for which the concentrations of organic acids in standard mixtures were ranged from 0.27 to 69.26μg mL^{-1}, while each acid had a selected dosage range (Table 2). Calibration lines were generated from the peak area of target analytes. Simple linear regression lines were fitted to the samples data between concentration (Y, μg mL^{-1}) and peak area (X), the correlation coefficient (R^2) were higher than 0.9973 (Table 2). The LOD of this method was 0.01- 0.06μg mL^{-1}.

The accuracy of the method was assessed through recovery assay[27]. Recoveries were analyzed by standard addition method. Compare the concentration differences between the acids-spiked and unspiked samples by adding standard acid mixtures with appropriate level (Table 3). The amounts added were different from each acid according to their different volatileness, and the addition was ranged between 38-81% and 6.7-25% for unspiked amount for volatile acids and semi-volatile acid respectively. The average recovery was calculated from five times replicate determinations. The recovery of organic acids was between 80% and 111% (Table 3), except for acetic acid (72.36%), which lower accuracy could be due to its strong volatility. The recovery of volatile acids ranged from 80.56 to 99.34%, were in range to those obtained by Xiang's method (82.5%-98.3%)[7] and slightly lower than Wang's method (89.5%-99.3%)[2]. Relative standard deviation (RSD) reflects the precision of

the method. For most analytes, the RSD was less than 10% (Table 3), confirming the precision of the method.

3.4. Application to Flue-cured tobacco leaves sample

The concentrations of organic acids in Flue-cured tobacco of B_2F grade were determined by this method under the optimized conditions and shown in Table 4. This proposed derivatization-free method had been compared with previous tobacco research from derivatization methods[2, 7, 9]. This proposed method significantly reduced the analysis time by eliminating the complicated derivatization procedure, and kept higher satisfied accuracy (between 80% and 111%) and precision (less than 10%) simultaneously (Table 3). From Table 4, it was observed that the results from this derivatization-free method were similar to previous tobacco research from derivatization methods. The volatile acids results were consistent with the previous findings[3, 7]. The semi-volatile acids results showed that the palmitic acid was the most abundant saturated fatty acid in flue-cured tobacco, followed by stearic acid, which were in agreement with Jiu reported[20]. Lower levels of palmitic acid 371.8ug g^{-1} and stearic acid 110.4ug g^{-1}, were reported in flue-cured TR Madole tobacco[20], which may be due to the differences between tobacco varieties.

4. Conclusions

In this study, a convenient and sensitive method of gas chromatography-triple quadrupole mass spectrometry (GC-TriQ-MS) coupled with SRM scan mode was established to quantify organic acids in Flue-cured tobacco leaves. During the

measurement of organic acids in tobacco leaves, the appropriate precursor-product ions of each acid were selected, meanwhile the collision energy parameters ranged from 1 to 30eV were optimized to promote sensitivity and selectivity. Sample preparation was performed by derivatization-free extraction. The excellent linearity (>0.9973), detection limits (0.01-0.06μg mL^{-1}), accuracy (80%-111%), and precision (RSD $<10\%$) of this method indicating that it could meet the requirement of quantitative analysis of organic acids in tobacco. Compared with previous methods, this method is more convenient for sample preparation, less matrix background interference and higher sensitivity for analysis of organic acids in tobacco. It was concluded that the method could be applicable for the rapid and sensitive analyze the organic acids content in tobacco.

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Table.1 The concentrations of organic acids in standard solution mixture and their selected ions.

No.	Organic acids	t_R (min)	Concentration (μg mL^{-1})	Precursor-product ions (m/z)
1	Acetic acid	7.54	79.74	60→55,43
2	Propionic acid	8.95	37.63	74→55,45
3	Isobutyric acid	9.53	36.10	73→55,43
4	Butyric acid	11.09	36.52	73→55,43
5	2-methyl butyric acid	12.38	106.73	87→59,45
6	Pentanoic acid	14.72	35.71	73→55,43
7	Caproic acid	18.41	33.11	87→59,45
8	Heptanoic acid	22.03	36.42	101→55,45
9	Octanoic acid	25.51	34.62	115→73,45
10	Pelargonic acid	27.51	33.43	129→59,55
11	Decylic acid	28.74	38.11	143→87,59
12	Benzoic acid	29.57	52.32	122→105,77
13	2-furan formic acid	30.26	179.93	112→95,55
14	Dodecanoic acid	30.82	35.90	171→101,86
15	Myristic acid	33.82	170.11	228→185,115
16	Palmitic acid	37.20	613.80	157→129,115
17	Stearic acid	41.31	608.03	199→143,129
18	Oleic acid	42.02	440.61	111→83,55
19	Linoleic acid	43.46	692.61	163→150,109
20	Linolenic acid	45.71	237.93	278→171,129

Table.2. Method performance data: calibration curve, correlation coefficient (R^2), linear range and limit of detection (LOD)

Organic acids	Calibration curve (μg mL^{-1})	R^2	Linear range (μg mL^{-1})	LOD (μg mL^{-1}) [a]
Acetic acid	Y=2.62E-07*X-1.0741	0.9985	0.33-7.97	0.01
Propionic acid	Y=6.80E-07*X+0.0016	0.9998	0.31-3.76	0.01
Isobutyric acid	Y=3.12E-07*X-0.00001	0.9998	0.30-3.61	0.01
Butyric acid	Y=4.27E-07*X+0.1000	0.9988	0.30-3.65	0.01
2-methyl butyric acid	Y=1.86E-07*X+0.0456	0.9995	0.89-10.67	0.01
Pentanoic acid	Y=3.21E-07*X+0.0680	0.9997	0.29-3.57	0.01
Caproic acid	Y=4.84E-07*X+0.0795	0.9995	0.27-3.31	0.01
Heptanoic acid	Y=1.57E-06*X+0.0731	0.9999	0.30-3.64	0.01
Octanoic acid	Y=1.57E-06*X+0.0512	0.9986	0.29-3.46	0.01
Pelargonic acid	Y=2.67E-07*X+0.0305	0.9975	0.28-3.34	0.01
Decylic acid	Y=9.34E-07*X+0.0930	0.9991	0.31-3.80	0.01
Benzoic acid	Y=3.82E-08*X+0.0843	0.9987	0.29-5.23	0.01
2-furan formic acid	Y=8.72E-06*X-0.0989	0.9986	0.30-17.99	0.01
Dodecanoic acid	Y=1.42E-06*X+0.0926	0.9991	0.30-3.59	0.01
Myristic acid	Y=3.96E-05*X-0.3719	0.9997	0.26-17.01	0.01
Palmitic acid	Y=2.30E-06*X-0.4025	0.9973	2.67-61.38	0.02
Stearic acid	Y=6.21E-06*X-0.4700	0.9999	7.87-60.80	0.06
Oleic acid	Y=6.85E-06*X-0.0612	0.9992	0.88-44.06	0.01
Linoleic acid	Y=4.79E-05*X+0.5632	0.9998	2.66-69.26	0.02
Linolenic acid	Y=5.85E-06*X-0.0412	0.9988	1.52-23.79	0.02

[a]LOD: was estimated by determining tobacco samples with estimated LOQ values added concentration of analytes for seven times repeatability and calculated as 3 times the standard deviation of the peak response.

Table.3. Recovey and relative standard deviation (RSD) of organic acids.

Organic acids	Added ($\mu g\ mL^{-1}$)	Detected ($\mu g\ mL^{-1}$)	±SD ($\mu g\ mL^{-1}$)	Recovery (%)[a]	RSD (%)[b]
Acetic acid	0.00	5.12	0.15	--	2.87
	1.99	6.56	0.60	72.36	9.64
Propionic acid	0.00	0.58	0.03	--	5.17
	0.47	0.99	0.03	87.37	3.03
Isobutyric acid	0.00	1.04	0.02	--	1.44
	0.45	1.44	0.05	88.45	3.26
Butyric acid	0.00	0.33	0.01	--	1.52
	0.46	0.76	0.04	95.92	4.87
2-methyl butyric acid	0.00	7.53	0.11	--	1.51
	1.79	9.31	0.20	99.34	2.13
Pentanoic acid	0.00	0.49	0.01	--	2.04
	0.45	0.85	0.08	80.56	9.29
Caproic acid	0.00	0.52	0.01	--	2.12
	0.41	0.86	0.10	80.90	11.28
Heptanoic acid	0.00	0.27	0.01	--	1.85
	0.23	0.47	0.04	87.95	9.15
Octanoic acid	0.00	0.37	0.01	--	3.78
	0.23	0.57	0.08	86.68	13.33
Pelargonic acid	0.00	0.28	0.01	--	5.00
	0.22	0.48	0.05	89.20	10.63
Decylic acid	0.00	0.20	0.00	--	2.00
	0.23	0.39	0.05	82.51	11.54
Benzoic acid	0.00	3.79	0.03	--	0.71
	1.31	5.02	0.12	93.87	2.47
2-furan formic acid	0.00	12.48	0.25	--	2.03
	1.35	13.62	0.78	84.85	5.71
Dodecanoic acid	0.00	0.46	0.01	--	1.96
	0.85	1.25	0.06	92.48	4.96
Myristic acid	0.00	6.91	0.12	--	1.77
	1.16	7.99	0.14	93.43	1.69
Palmitic acid	0.00	52.55	1.66	--	3.16
	5.34	58.50	2.52	111.47	4.31
Stearic acid	0.00	55.58	0.54	--	0.96
	3.73	59.28	1.44	99.05	2.43
Oleic acid	0.00	6.95	0.71	--	10.27
	5.00	12.28	0.46	106.64	3.71
Linoleic acid	0.00	47.87	1.25	--	2.61
	5.33	53.69	1.75	109.24	3.25
Linolenic acid	0.00	11.84	0.71	--	5.97
	3.00	14.49	0.48	88.42	2.87

[a]Recovery were calculated by $(C_{spiked} - C_{unspiked}) \times 100\% / C_{addition}$; [b]RSD is relative standard deviation (n=5).

Table.4.Concentration and standard deviation (SD) of organic acids in flue-cured tobacco samples

Organic acids	ShaoyangB$_2$F (μg g^{-1})[a]	ChenzhouB$_2$F (μg g^{-1})[a]	LonghuiB$_2$F (μg g^{-1})[a]
Acetic acid	109.13±18.64	153.40±27.35	179.67±28.26
Propionic acid	63.87±12.87	71.59±13.62	64.36±13.04
Isobutyric acid	6.93±0.13	6.13±0.47	4.47±0.96
Butyric acid	2.20±0.44	2.28±0.37	1.71±0.28
2-methyl butyric acid	50.20±5.97	63.87±5.66	44.15±4.83
Pentanoic acid	3.27±0.36	4.09±0.45	3.61±0.54
Caproic acid	3.47±0.07	5.66±0.31	3.59±0.09
Heptanoic acid	1.80±0.05	2.54±0.03	1.83±0.06
Octanoic acid	2.47±0.11	7.63±0.34	3.57±0.16
Pelargonic acid	1.87±0.12	6.79±0.74	2.45±0.02
Decylic acid	1.33±0.11	2.67±0.13	1.31±0.76
Benzoic acid	25.27±4.22	49.62±4.63	30.50±3.91
2-furan formic acid	83.20±11.87	107.20±14.26	99.13±11.68
Dodecanoic acid	3.07±0.32	7.54±1.47	3.42±0.03
Myristic acid	46.07±6.08	56.36±8.66	44.91±8.20
Palmitic acid	370.33±38.24	642.23±40.06	550.36±39.32
Stearic acid	350.53±8.08	533.32±9.37	406.16±9.01
Oleic acid	46.33±10.02	54.38±9.33	60.87±9.81
Linoleic acid	319.13±37.32	623.52±48.01	539.57±48.13
Linolenic acid	78.93±12.21	127.21±12.16	110.00±11.93

a. All values are mean ±SD obtained by five analyses.

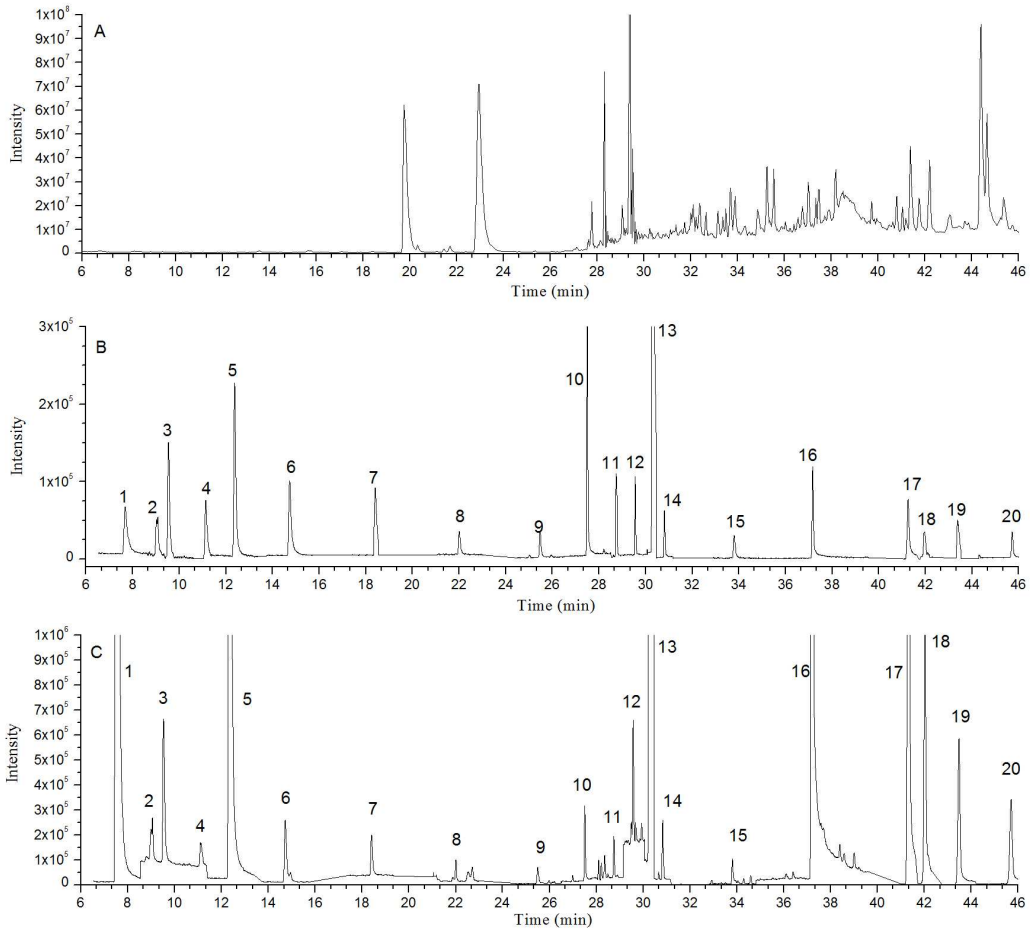
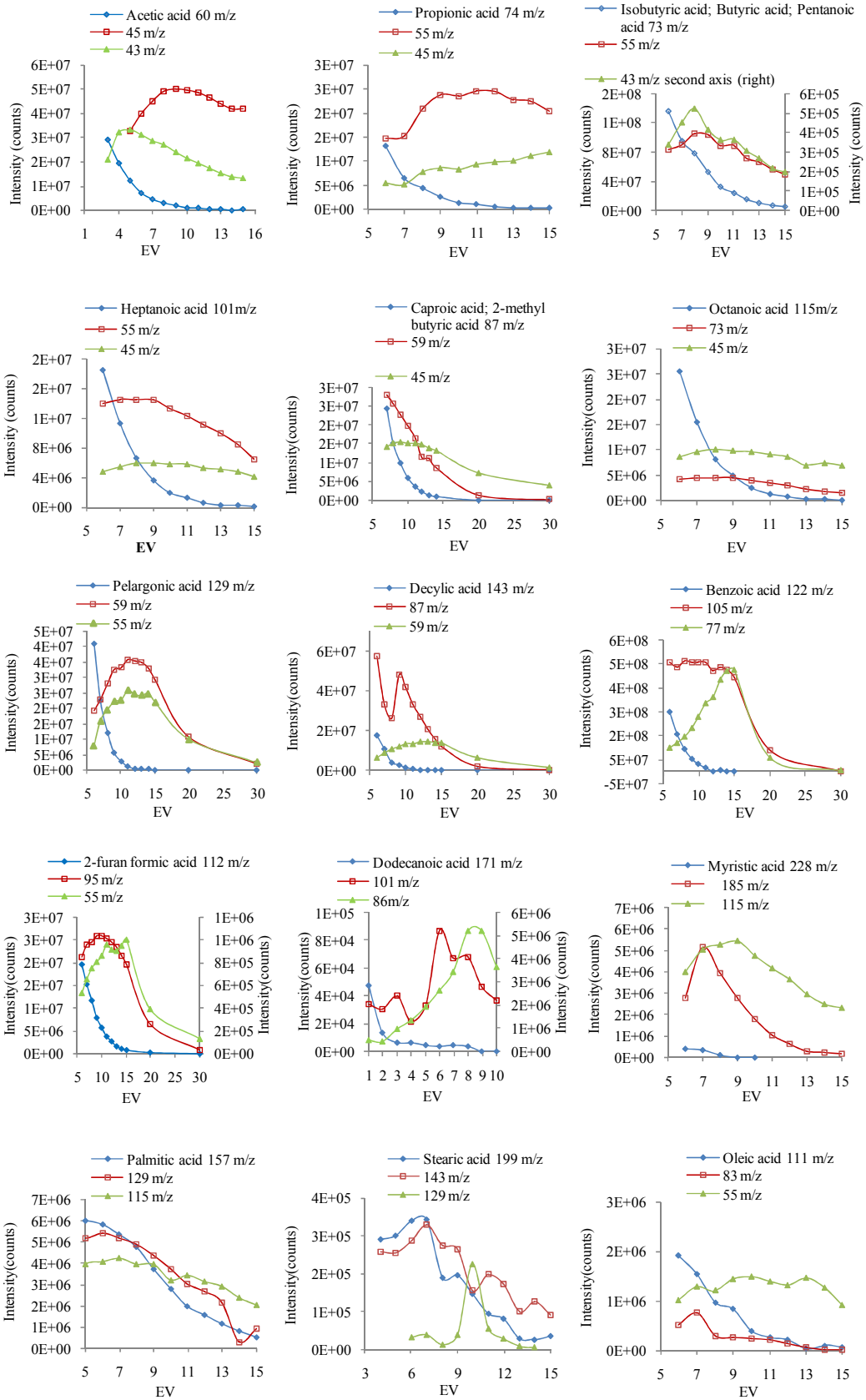


Figure 1. The total ion chromatogram (TIC) of organic acids. A is the TIC of tobacco sample in full scan mode; B is the TIC of mixed standard solution in full scan mode; C is the TIC of tobacco sample in SRM scan mode at the optimum collision enrgy. Organic acids of 1-20 correspond to the code acids in table1.



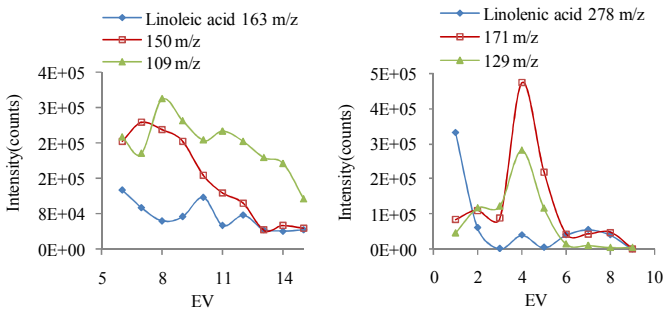


Figure 2. Collision energy optimization of 20 kinds of organic acids. The X-axis represents the collision energy range from 1eV to 30eV, Y-axis represents the intensity at the corresponding collision energy.