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Microwave assisted extraction–solid phase extraction for high-efficient and rapid analysis of monosaccharides in plants

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ABSTRACT

Monosaccharides are the fundamental composition units of saccharides which are a common source of energy for metabolism. An effective and simple method consisting of microwave assisted extraction (MAE), solid phase extraction (SPE) and high performance liquid chromatography-refractive index detector (HPLC-RID) was developed for rapid detection of monosaccharides in plants. The MAE was applied to break down the structure of the plant cells and release the monosaccharides, while the SPE procedure was adopted to purify the extract before analysis. Finally, the HPLC-RID was employed to separate and analyze the monosaccharides with amino column. As a result, the extraction time was reduced to 17 min, which was nearly 85 times faster than soxhlet extraction. The recoveries of arabinose, xylose, fructose and glucose were 85.01%, 87.79%, 103.17%, and 101.24%, with excellent relative standard deviations (RSDs) of 1.94%, 1.13%, 0.60% and 1.67%, respectively. The proposed method was demonstrated to be efficient and time-saving, and had been applied to analyze monosaccharides in tobacco and tea successfully.

1. Introduction

Saccharides make up most of the living world around us. Its basic units are monosaccharides, which greatly influence human health in diverse ways [1]. For example, arabinose, xylose, glucose and fructose are four typical kinds of monosaccharides involved in life. Besides, arabinose and xylose are capable of improving human immunity due to their benefits on bifidobacterium growth. Moreover, monosaccharides have a great effect on the taste and smell of plant products, such as tea and tobacco [2,3]. So the detection of monosaccharides becomes very important.

Traditionally, the monosaccharides were detected by GC–MS [4–6] and HPLC–UV/FLD [7–9]. As the monosaccharides have weak UV adsorption and fluorescent response [10], derivatization was usually required prior to analysis [11]. However, the isomers of each monosaccharide were produced during derivatization process [12]. To avoid this problem, refractive index detector was adopted for monosaccharides analysis [13]. Additionally, the complex sample matrix in plants is also challenging to achieve efficient and rapid extraction during sample pretreatment [14–17]. Shih et al. reported a sample pretreatment method with soxhlet extraction for monosaccharides in dry corn stover, the extraction took as long as 24 h [18]. Sharma et al. developed an ultrasonic extraction for the detection of ten saccharides in *Tinospora cordifolia*, which cost 40 min for each sample [19]. Therefore, it is greatly desirable to improve the analytical efficiency and reduce the time cost. It is well-known that MAE is highly efficient in plants pretreatment by decreasing the matrix effect, as only polar molecules can be heated by MAE and then dissolved in extract [20]. The MAE was widely used for the detection of proanthocyanidins, flavonoids, phenolics, drugs and other bioactive compounds [21–25], and so on. However, few reports were focused on developing a MAE strategy for sample pretreatment of monosaccharides. The application of microwave assisted extraction in monosaccharides focused on the hydrolysis of polysaccharides, which took high temperatures and acid reagent to accelerate the polysaccharides hydrolysis [26,27].

Herein, we report an effective and simple method by combination of MAE, SPE and HPLC-RID for rapid detection of monosaccharides from tobacco and tea. To achieve rapid analysis, MAE was conducted to extract monosaccharides from tobacco and tea with

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much lower temperature (60 °C) and relatively mild extraction solvent (water and methanol). The microwave irradiation can make plant cell rupture and accelerate the extraction of monosaccharides. An amino group modified SPE was further used to purify the analytes from complicated sample matrix (Fig. 1). Finally, the purified sample was analyzed by HPLC-RID. Specially, the response surface methodology was applied to optimize the MAE conditions, including temperature, solvent and time. As a result, the method was successfully developed to detect monosaccharides in tobacco and tea.

2. Experimental section

2.1. Chemicals and reagents

The standard analytes arabinose, fructose, glucose, xylose were purchased from TCI (Shanghai, China). Ethanol and acetonitrile were HPLC grades and supplied by J&K Scientific Ltd. (Beijing, China). The plant material of tobacco was obtained from technology center of Yunnan cigarette factory, China. Cigarette samples were bought from market. NH_2-SPE cartridges (500 mg, 6 mL) were obtained from Sigma-Aldrich (Beijing, China).

The stock solutions of glucose, fructose, arabinose and xylose with the concentration of 50 mg/mL were prepared with deionized water and stored at 4 °C. Then, standard solutions (0.5, 1.0, 2.0, 5.0. 10.0 and 20.0 mg/mL) were obtained by serial dilution of the stock solutions with deionized water.

2.2. Apparatus

The MAE WX-4000 equipment was purchased from Shanghai Yiyao Instrument Technology Development Co., Ltd. (Shanghai, China). HPLC-RID system with a binary pump, an auto sampler, a thermostatic oven and a refractive index detector was purchased from Hitachi, Ltd. (Tokyo, Japan). The NH_2 UG 80 S5 column (4.6 mm I.D. × 250 mm, Shiseido CAPCELL PAK) was offered by Shiseido China Co., Ltd. (Beijing, China). Leica DMI 4000 B was provided by Leica Microsystems, Inc. (Wetzlar, Germany).

2.3. Sample pretreatment and detection

The sample was grounded and then sieved by 400 meshes. The grounded sample (500 mg) was added into MAE tank. And the conditions were set as temperature 60 °C, solvent 20 mL (methanol/deionized water, v/v, 72/28), and time 17 min. After MAE, the extract (0.5 mL) was filtered by 0.45 μm water membrane. The analytes were purified by amino group modified SPE with 2 mL methanol. The eluent was then concentrated by nitrogen sweeping method, and redissolved by 500 μL water. The standard procedure of HPLC-RID was performed as follows: (1) 10 μL extracted solutions were injected into HPLC-RID system. (2) To prepare mobile phase, acetonitrile and water were mixed and sonicated to remove air prior to use. (3) The flow rate was 1.0 mL/min and the column chamber temperature was 35 °C, and the detector temperature was maintained at 40 °C. Thus, the chromatograms of analytes were obtained. The treatment conditions for the establishment of the method were shown in Table 1.

2.4. Method validation

The validation of the analytical method was performed by the following parameters: linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision method detection limit (MDL) and method quantification limit (MQL). The calibration curves were evaluated from 0.50 mg/mL to 20.00 mg/mL. The linearity of every standard solution was detected with three parallel experiments. The peak area of each analyte was plotted against the concentrations, and linear correlation coefficient was performed on the resulting curves using the least squares method. The LOD and LOQ were determined with three folds and ten folds of signal-to-noise ratio (S/N), respectively. The intra-day relative standard deviations (RSDs) of samples were detected by triple tests during one day. The inter-day RSDs were calculated from a three-day continuous test. The recoveries were tested to confirm the accuracy with standard addition method by six times. The MDLs and MQLs of each analyte in plants were calculated based on the signal-to-noise (S/N) of 3 and 10 of spiked plant matrix samples, respectively.

3. Results and discussion

3.1. HPLC analysis

Arabinose and xylose are isomers with similar structures, and so are fructose and glucose. Thus, they are difficult to be separated and analyzed simultaneously. Thus, the conditions of HPLC need to be optimized.

The composition of mobile phase was optimized. Acetonitrile/deionized water was chosen as the mobile phase. As shown in Fig. 2, the monosaccharides were separated well by 90% acetonitrile, while the monosaccharides were not separated well by 75% acetonitrile and 80% acetonitrile. It deduced that the polarity of mobile phase was decreased by increasing the ratio of acetonitrile, leading to improving the resolution.

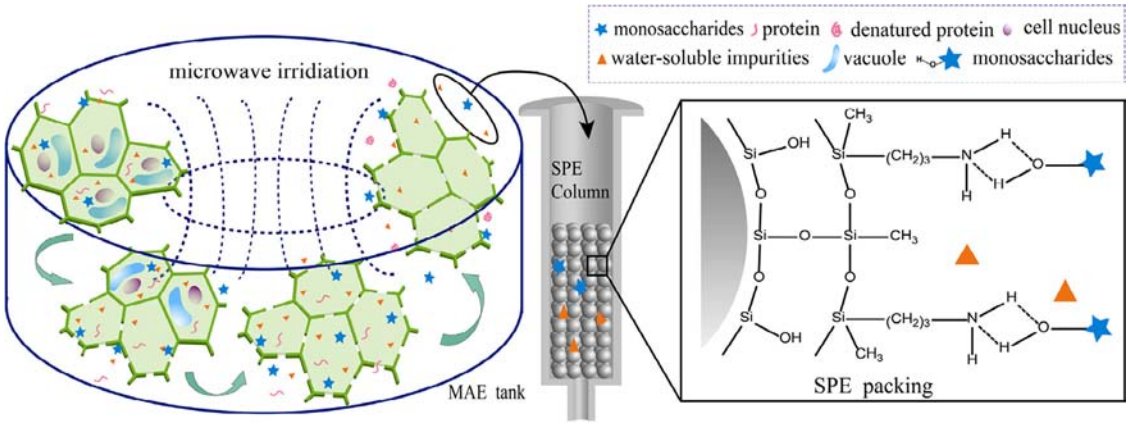

Fig. 1. Schematic diagram of sample pretreatment.

Table 1
Experimental conditions.

HPLC analysis													
Mobile phase (acetonitrile/deionized water, v/v)							75/25		80/20		90/10		
Temperature (°C)							35		35		35		
Detector temperature (°C)							40		40		40		
Flow rate (mL/min)							1.0		1.0		1.0		
SPE conditions													
Elution solvent	Acetonitrile/water		Acetonitrile		Water		Methanol		Methanol		Methanol		Methanol
Volume of solvent (mL)	2		2		2		2		1		2		3
Volume of sample (mL)	0.5		0.5		0.5		0.5		0.5		0.5		0.5
MAE conditions													
T (°C)	80	80	40	40	80	80	40	40	60	60	60	60	60
Extraction solvent (ratio of water, %)	100	60	100	60	80	80	80	80	100	100	60	60	80
Time (min)	15	15	15	15	30	5	30	5	30	5	30	5	15

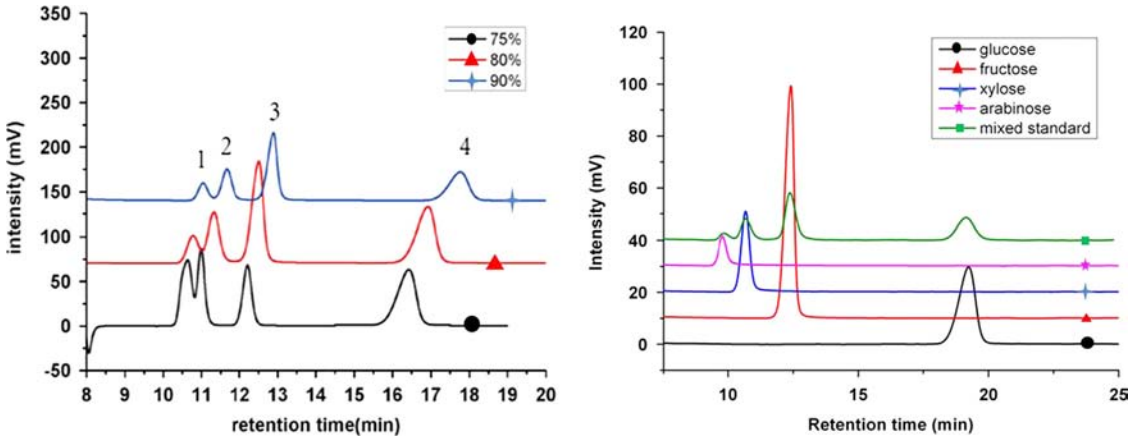

Fig. 2. Separation of standard monosaccharides The left chromatogram is the results of optimization of mobile phase while the right is the separation of single standard and mixed standard monosaccharides (1 arabinose, 2 xylose, 3 fructose, 4 glucose).

The optimal conditions for arabinose, xylose, fructose and glucose were as follows: the mobile phase being acetonitrile/deionized water (90%/10%, v/v) with the flow rate of 1.0 mL/min, and the temperature of column oven was adopted as 35 °C. Single standard sample was tested to identify peaks from mixed samples (Fig. 2).

3.2. MAE process

Parameters influencing MAE process are extraction solution, temperature, and extraction time. For MAE extraction solvent, consideration should be given to the analytes solubility in solvent and the microwave-absorbing properties of the solvent. The common extraction solvents for monosaccharides are water, alcohol (methanol and ethanol), chloroform and their mixtures. Water becomes the first choice of MAE extraction solvent as it has high monosacharide solubility and high dielectric constant. But impurities such as proteins and other small molecules will also be soluble in pure water. Taken into consideration that proteins undergo denaturation and further precipitation in methanol, a hybrid solvent of water and alcohol is used in this work. Besides, the microwave temperature and time also need optimization.

The response surface methodology was adopted for multi-factor optimization of MAE in this paper. Through the establishment of continuous variable surface model, the method evaluates the factors which affect a biological process and their interactions, thus improving extraction efficiency. Since the established complex multidimensional space curved surface is close to the actual situation, the response surface model gradually comes into use [28,29].

The ranges of the MAE factors to be optimized are listed in Table 1. The different extraction solutions with 60%, 80%, and 100% methanol are used; the extraction time are 5 min, 15 min, and 30 min; and the temperatures are 40 °C, 60 °C, and 80 °C. The results and corresponding conditions of optimization are listed in Table 2. Response surface methodology data processing with MATLAB mode was carried out for the optimal condition. The multiple correlation coefficients were verified with binomial fit (greater than 95%). The recovery was taken as the criteria. The extraction conditions for each monosaccharide were calculated, and worked out in MATLAB mode. Arabinose is examined as a model to investigate the mutual influence, and three dimensional surface and contour plot are drawn in Fig. 3. It can be found that there is a highest point on the 3D surface, whose value is 0.865 for recovery prediction on the contour plot. The condition for the value is temperature at 57.6 °C, methanol/deionized water ratio of 72%/28% and extraction time of 15.7 min. Because the optimum extraction conditions of four monosaccharides were slightly different, the extraction conditions were compromised as temperature at 60 °C, methanol/deionized water ratio of 72%/28% and extraction time of 17 min. Then, the experiments were done under the optimal conditions of theory, the recoveries of arabinose with 85.01%, xylose with 87.79%, fructose with 103.17%, and glucose with 101.24% were consistent with the corresponding theoretical recovery of arabinose with 86.5%, xylose with 89.7%, fructose with 104.6%, and glucose with 102.6%, demonstrating that the condition was best for MAE to extract monosaccharides. So the MAE method for monosaccharides sample pretreatment was established with high efficiency (recoveries $>$ 85.0%) and rapid extraction (extraction time 17 min).

To explore the mechanism, images of tobacco leaves with and without MAE treatment were taken with a confocal microscope. As

Table 2
The design and results of MAE optimization.

Nos.	Temperature (°C)	Ratio of water (%)	Time (min)	Recovery for arabinose (%)[a]	Recovery for xylose (%)	Recovery for fructose (%)	Recovery for glucose (%)
1	80	100	15	67.0	73.2	54.2	53.1
2	80	60	15	68.8	82.6	81.8	80.1
3	40	100	15	65.9	64.4	64.8	63.5
4	40	60	15	77.0	77.3	72.8	71.3
5	80	80	30	67.6	75.6	79.3	77.7
6	80	80	5	67.6	74.1	64.1	62.9
7	40	80	30	69.4	75.1	72.5	71.1
8	40	80	5	69.6	67.6	63.5	62.2
9	60	100	30	68.2	65.2	70.9	69.5
10	60	100	5	67.8	62.1	57.0	55.9
11	60	60	30	73.7	77.3	86.2	84.5
12	60	60	5	82.9	81.6	84.4	82.7
13	60	80	15	85.7	87.7	101.7	99.7

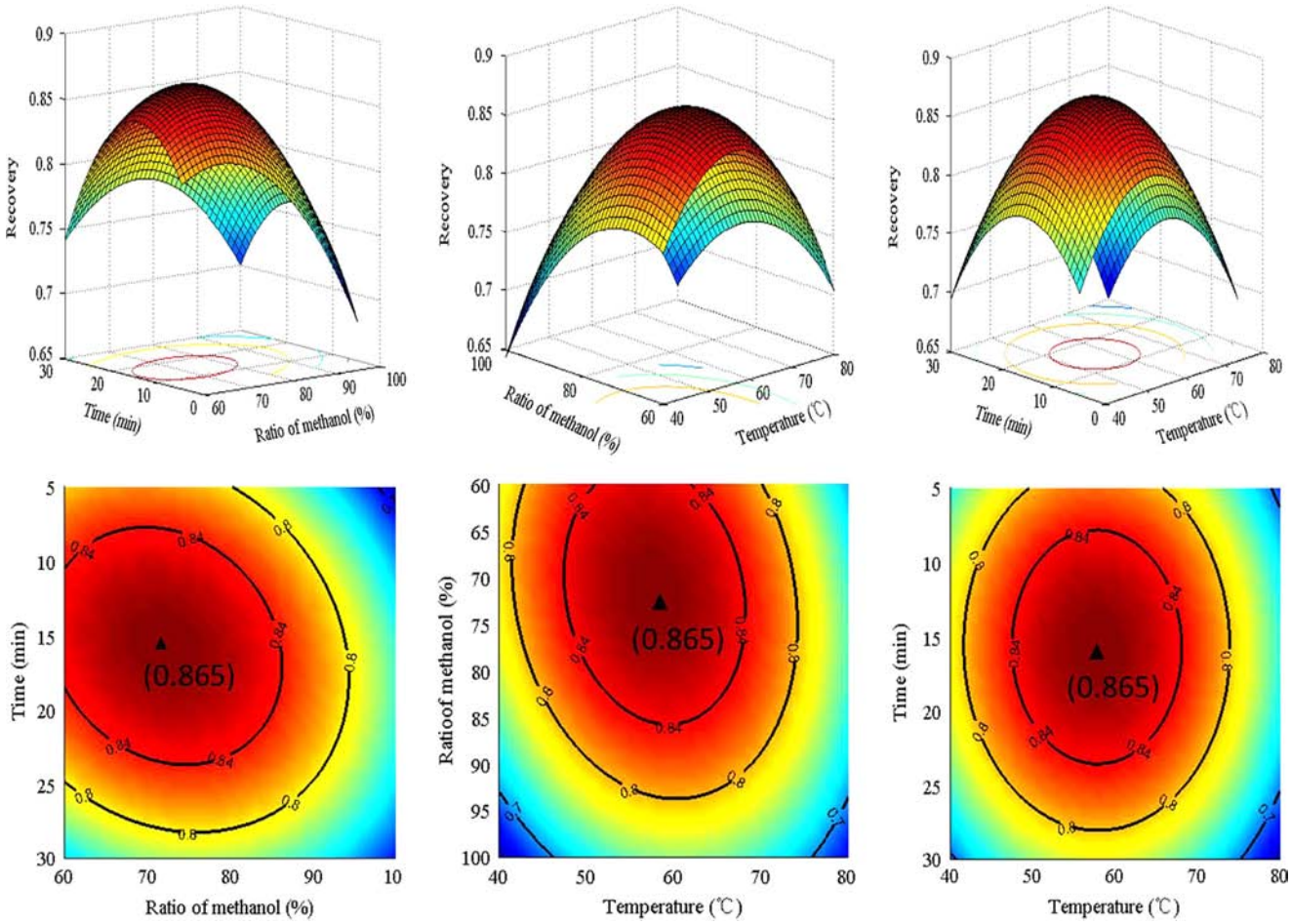

Fig. 3. 3D surface and contour plot for response surface methodology optimizing the mutual effect of MAE conditions (arabinose as model).

shown in Fig. 4, the red shapes are formed by spontaneous fluorescence of chloroplast, while the gray shadows represent protoplasm. Single cells are pointed out by green curves in Fig. 4 respectively. From the confocal images, we can discover that the untreated cell has a good shape and the distribution of chloroplast has clear boundary in Fig. 4A. But after MAE, the cells are broken, and the chloroplasts are distributed without clear boundary any more in Fig. 4B.

The results indicated that microwave irradiation can be well used to break up the plant cells and may be applied to extract cellular substance such as monosaccharides. It can be explained by the following theory. Under microwave irradiation, the intracellular temperature of plants rises sharply, which induces the pressure in cell exceeding the pressure that cell wall can endure. Consequently, plasmatorrhexis occurs, the intracellular components diffuse and dissolve in extraction solvent, in which way the high-efficiency and rapid extraction was achieved.

3.3. Sample preparation by SPE conditions

NH_2-SPE cartridges were further used to purify the monosaccharides after MAE. The choice of a suitable eluent solvent contributed to

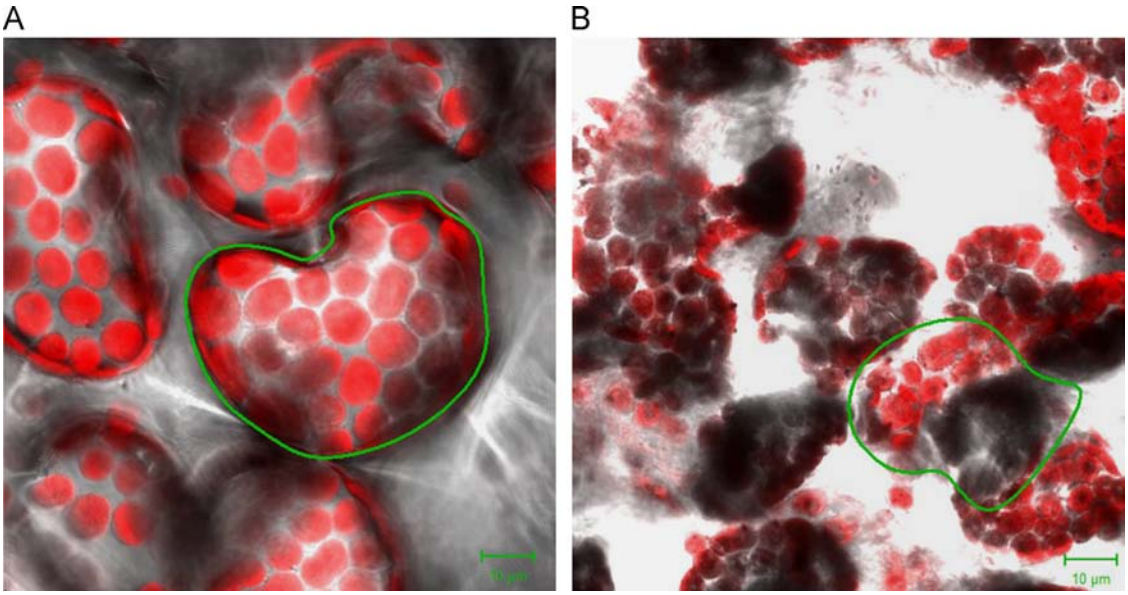

Fig. 4. Fluorescent images of tobacco leaf samples. (A. untreated tobacco leaf sample, B. tobacco leaf sample treated with MAE). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

both selectivity and recovery. Different SPE cartridges were used to purify monosaccharides. The solvent used to extract monosaccharides was chosen by the properties of SPE packings. 2.0 mL of 60% acetonitrile in water containing 0.30% formic acid adjusted to pH 9.0 with ammonia was applied in Supelclean ENVI-Carb SPE cartridges to extract monosaccharides [30]. C_{18} SPE cartridge was eluted with water to purify monosaccharides [31]. Tian et al. investigated the extraction efficiency of different solvents (water, acetonitrile, methanol, ethanol, n-hexane and chloroform) on silica, NH_2 and $PImNH_2$ [32]. The volume of sample used for SPE was 0.5 mL. Taking consideration of the matching of HPLC analysis procedure, acetonitrile, acetonitrile/water, water, and methanol were selected as eluent solvents. Compared the eluent effect of acetonitrile, acetonitrile/water, methanol, and water, As shown in Fig. 5, methanol and water are more suitable for extraction the monosaccharides from the MAE solution because the recoveries are more than 80% by methanol and water extraction, while the recoveries are lower than 60% by acetonitrile extraction. Furthermore, compared with those of acetonitrile/deionized water, it also can be found in Fig. 5 that with the addition of deionized water, the solvent strength of eluent is increased, the recoveries of four monosaccharides (arabinose, xylose, fructose and glucose) are improved from 34.69%, 31.82%, 36.04%, 33.47% to 58.46%, 45.81%, 47.47%, 47.71%. The higher recoveries may be due to high solvent strength of extraction solvent. The higher solvent strength of the solvent is, the easier the monosaccharides get eluted, the higher recoveries are achieved, due to the strong polarity of monosaccharides. Methanol is chosen as the eluent for the next experiments although the recoveries by water elution are slightly higher than that by methanol elution. The reasons are as following. First, deionized water is harder to concentrate with high boiling point and good specific heat capacity. Second, the color of samples eluted with deionized water is deeper than those eluted with methanol, which indicates deionized water will bring out more impurities.

Hereafter, eluent solvent volume was optimized. As shown in Fig. 5, the recoveries are increased with increasing the eluent volume up to 2 mL, the recovery of arabinose and xylose are more than 85.0%; the recovery of fructose and glucose are close to 100.0%. When the eluent volume is up to 3 mL, the recoveries increase little. So the eluent solvent was 2 mL methanol for reducing the consumption and saving the concentration time.

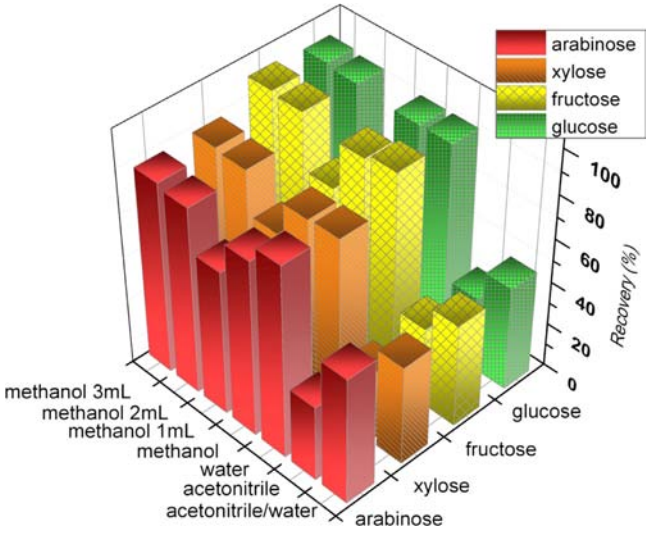

Fig. 5. Optimization of SPE conditions.

Finally, the experiments were carried out six times to investigate the repeatability of SPE method. The relative standard deviations (RSDs) of arabinose, xylose, fructose and glucose were 1.87%, 0.30%, 0.01%, 1.48%, respectively, which demonstrated that 2 mL methanol as eluent was stable and effective for SPE.

3.4. Method validation and real sample analysis

In Table 3, the calibration curves all represent good linearity with linear correlation coefficients in the range of 0.9991–0.9999. The LODs and LOQs of monosaccharides are 0.065–0.117 mg/mL and 0.207–0.358 mg/mL, the MDLs and MQLs are 7.4–13.2 μg/g and 23.0–53.7 μg/g. The recovery of the method was arabinose 85.01%, xylose 87.79%, fructose 103.17%, and glucose 101.24%. And the RSDs were 1.94%, 1.13%, 0.60% and 1.67%, respectively. These results indicated that the established method can be well used to analyze monosaccharides from plants.

Based on the established method, five kinds of tobacco samples and four kinds of tea sample were analyzed. As shown in Fig. 6 and

Table 4, the difference of monosaccharides content in tea and tobacco was larger than that of tobaccos. Five kinds of tobacco samples tested were about the same, which indicated the method was stable and accurate. But the RSDs of fructose content in tobaccos were huge differences. The average of fructose content was 13.17 mg/g. The RSDs for different tobaccos were 1.0%, 2.8%, 9.0%, 26.3% and 0.03%, which indicated tobaccos were different with their kinds and the content of monosaccharides could be used to distinguish the kinds of tobaccos.

Table 3
The linearity, LODs, LOQs, MDLs and MQLs (50 ng/L level) of four monosaccharides.

Compound	RSD% ($n=5$)		r^2	HPLC analysis (mg/mL)		Analysis method (μg/g)	
	Intra-day	Inter-day		LOD	LOQ	MDL	MQL
Arabinose	2.03	2.50	0.9993	0.117	0.358	13.2	39.8
Xylose	1.78	3.90	0.9999	0.113	0.340	12.6	53.7
Fructose	0.60	1.08	0.9991	0.065	0.207	7.4	23.0
Glucose	1.34	3.71	0.9996	0.110	0.334	12.2	37.7

Four different kinds of tea were analyzed. Tea 2 was slimming tea, and honey was added into its formula. Tea 3 was black tea, the fermentation process promoted the hydrolysis of polysaccharides in tea, resulting in the increase of monosaccharides. The monosaccharides in tea can increase the sweetness of tea, and generate substance with flowers smell after reacting with amino acid in hot water. Without fermentation and addition, the contents of monosaccharides in green tea (Tea 1 and Tea 4) were lowest. It indicated that the monosaccharides may be potential used to identify tea types and their baking techniques. The result also demonstrated that the developed method may be well used to analyze monosaccharides in plants.

4. Conclusions

In conclusion, a simple and effective method was successfully established to determine the monosaccharides in tea and tobacco with HPLC-RID. MAE coupled with SPE technique was applied in the pretreatment process. The microwave irradiation impact on cell rupture obtained an effective and rapid extraction to monosaccharides in plants. The SPE purified the monosaccharides with high efficiency.

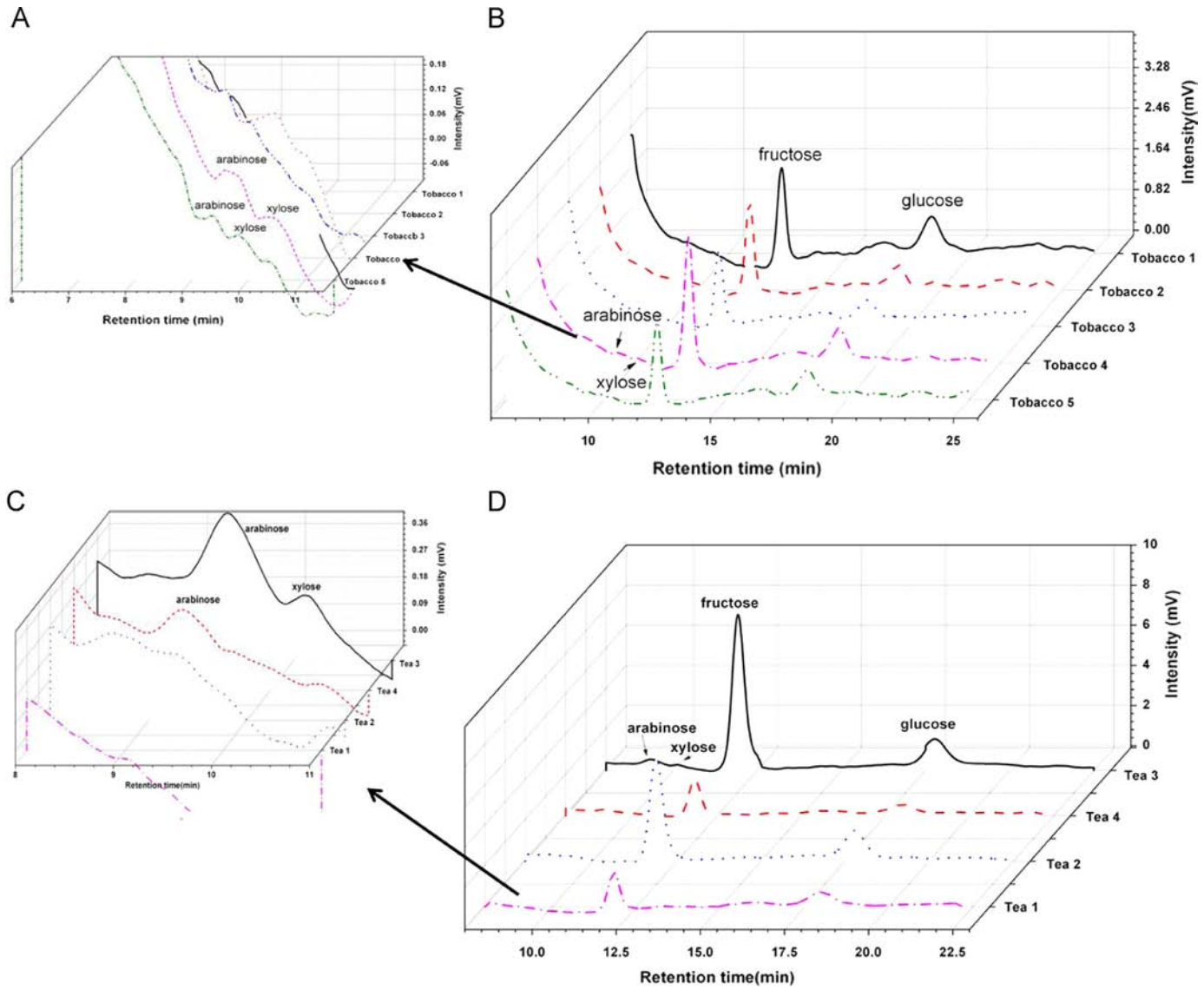

Fig. 6. Chromatograms of monosaccharides from natural plant extract with optimized MAE–SPE process (B, D total chromatograms of monosaccharides for tobacco and tea, A, C enlarged chromatograms of arabinose and xylose for tobacco and tea).

Table 4
Content of monosaccharides detected from tobacco and tea ($n=5$).

Samples	Arabinose (μg/g)[a]	Xylose (μg/g)	Fructose (mg/g)	Glucose (mg/g)
Tobacco 1	ND	ND	12.80 ± 0.07	7.16 ± 0.12
Tobacco 2	ND	ND	12.56 ± 0.07	7.28 ± 0.13
Tobacco 3	ND	ND	11.98 ± 0.06	7.02 ± 0.11
Tobacco 4	12 ± 2	15 ± 3	15.03 ± 0.08	7.49 ± 0.09
Tobacco 5	19 ± 4	11 ± 1	13.19 ± 0.04	7.74 ± 0.08
Tea 1	ND	ND	0.03 ± 0.001	0.03 ± 0.004
Tea 2	ND	ND	1.40 ± 0.01	0.47 ± 0.008
Tea 3	14 ± 3	ND	1.94 ± 0.02	0.38 ± 0.005
Tea 4	ND	ND	0.34 ± 0.002	0.28 ± 0.004

[a] ND means not detected.

The method also is successfully applied in the detection of monosaccharides in tobacco and tea. It might be helpful applied to distinguish kinds of tea or tobacco by the level of monosaccharide and might be helpful in identification of Chinese herbal medicine.

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