Analysis of chemical compounds in different solvent extracts with gas chromatography-mass spectrometry

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ABSTRACT: Objective To compare similarities and differences of different solvents in the extracts of blueberry fruit. **Methods** Compounds in the extracts of Patriot blueberry (*Semen trigonellae*) extracted using different solvents of S1 (100% methanol with 0.1% formic acid), S2 (50% aqueous methanol), S3 (70% acetone, 29.5% water, with 0.5 % acetic acid) or S4 (50% aqueous ethanol) were analyzed and compared by gas chromatography-mass spectrometry (GC-MS) with pre-column derivatization. **Results** Acid, alcohol, phenolic compounds, esters, sugar, ketone, alkane and other compounds were identified from the extracts. The results showed that S4 was the more suitable solvent for extracting the acids and S3 for extracting sugars while S2 was relatively good in extracting ester, phenolic compounds, alkane and others compounds from the fruits. So S2 solution was a better choice than the others for extracting chemical compounds from blueberry. **Conclusion** This study provides a new insight into the needs of different types of blueberry components. Meanwhile, this overall identification of chemical components in blueberry is of great significance to the quality control and effective utilization of its functional compounds.

KEY WORDS: blueberry; polar solvents; chemical compounds; gas chromatography-mass spectrometry

气质联用技术研究爱国者蓝莓不同溶剂提取物

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摘 要: 目的 对比不同溶剂对蓝莓果化合物提取的异同。**方法** 本研究使用 4 种不同的溶剂提取爱国者蓝莓化合物, 分别为: S1(100%甲醇和 0.1%的甲酸), S2(50%甲醇水溶液), S3(70%丙酮、29.5%水、0.5%乙酸)和 S4(50%乙醇), 并结合柱前衍生化-气相色谱质谱联用(GC-MS)技术检测不同溶剂蓝莓提取物。**结果** 研究结果表明, 蓝莓果中含有酸、醇、酚、酯、糖、酮、烷烃等类化合物; 同时研究结果还发现, S4 适合提取酸类化合物, S3 适合提取糖类化合物, S2 相比其他提取溶剂, 能提供更多关于酯类、酚类、烷烃类和其他化合物信息, 因此, S2 是研究蓝莓化合物组分较优的提取剂。**结论** 本实验对于不同类别蓝莓组分的需求提供了研究的新思路, 同时对蓝莓品质控制和功能性成分的高效利用有非常重要的意义。

关键词: 蓝莓; 极性溶剂; 化合物组成; 气质联用技术

基金项目: 北京林业大学科学技术研究项目(2015-01)、林业公益性行业科研专项资助项目(200904014-3)

Fund: Supported by the Science and Technology Research Project of Beijing Forestry University (2015-01) and Forestry Public Welfare Industry Research Special Fund Project (200904014-3)

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1 Introduction

Consumption of blueberry has been claimed to have various nutritional and health promotion functions, including antioxidative properties[1,2], bacteriostatic activities[3], improvement of vision[4]and protection of the central nervous system[5,6]. These functions and bioactivity of blueberry fruits are attributed to the anthocyanins[7] and phenols[8,9] components. Accordingly, the qualitative and quantitative analysis of anthocyanins and polyphenol have attracted considerable public and scientific interests[10,11]. Actually, component species in blueberry such as acids, esters and alkanes, are abundant and diversified, and may also be responsible for some bioactivities[8,12].

After studying all these chemical constituents in blueberry simultaneously, two main factors are considerable. One important procedure to obtain as many as possible chemical components is to select suitable solvents, since the polarity of solvent affects the constitution of extract[13-15]. The most widely used solvents for extracting phenolic compounds are water, ethanol, methanol, acetone, and their water mixtures, with or without acid[16]. It is possible to obtain more compounds from blueberry by using solvents with different polarity. The recovery of phenolic compounds in tropical fruits is dependent on the solvent used in their extraction and its polarity[17]. Ethanol as a weak polar solvent is usually used to extract polyphenols and acids. The methanol solution is more polar than ethanol solution and is able to acquire polar compounds[18]. Acetone-containing extraction mixtures are superior to the ones containing methanol in extraction yield of total phenolic compounds from strawberries. Organic acids such as formic acid are more aggressive solvents than water alone and lead to higher extractability of phytochemicals. Recent studies showed that acidification of distilled water by 5% of formic acid significantly increased its efficiency as an extraction solvent. In compound extraction, the polarity of solutions could be adjusted by altering the ratios between methanol, ethanol, acetone and water [19], which can make up more extraction ways and extract more diversified compounds[14]. The pH of a solvent can also affect the extraction. Diversified solvents can be achieved by adding some volatile acids, such as formic acid and acetic acid. In all these specific types, varying the concentration and pH of selected solvents can solve the problem of extracting of hard-extracted-compounds and acquire plenty of compounds in blueberry[18].

Detection method is also vital to the study. GC-MS is widely used because of its high sensitivity and selectivity on non-target compounds. Compounds can be systematically analyzed to determine the sensitivity. The detection range could be maximized using pre-column derivatization via silanization. Furthermore, the application of hydrolysis steps can counteract the deficiency of detecting high-boiling-point material with GC-MS[20,21].

This work is an attempt to identify the best solvent for the extraction of chemical components from blueberries rather than detecting single species of compound. A simple and practical method is developed to obtain holistic compound information of blueberry based on combination of multiple-solvent extraction with gas chromatography-mass spectrometry (GC-MS)[22,23]. Firstly, chemical constituents were extracted from blueberry via four solutions with different polarities. Secondly, extracts were treated with pre-column derivatization via silanization and analyzed by GC/MS. Finally, corresponding molecular formula was retrieved by removing off [-Si-$(CH_3)_3$] according to the silanization derivatization rules.

2 Materials and Methods

2.1 Samples

The *Patriot* blueberries (*Semen trigonellae*) were cultivated in China and supplied by Dandong Organic Food Company Limited, China. These blueberries were washed with tap water and stored in darkness at -18 ℃ until further processing. Fresh blueberries were collected and sent to Prof. WANG Jian-Zhong at Beijing Forestry University for species confirmation.

2.2 Chemical reagents

Methanol, alcohol, acetone, formic acid, acetic acid, and trifluoroacetic acid were purchased from Fisher Scientific International Inc, (New Jersey, USA). The derivatization reagents N, O-bis (trimethylsilyl) and trifluoroacetamide (BSTFA), with 1% trimethylchlorosilane (TMCS) were purchased from Shanghai Anpel Scientific Instruments Co., Ltd.

2.3 Extraction procedures

Frozen *Patriot* blueberries were thawed at room temperature for 12 h, and then ground into puree and dried at (50±2) ℃ in an air-drying oven for 6 h. After that, 5 gm of dried sample was put in a beaker and extracted three times with 50 mL of each solvent for 6 h.

The solvents were:

S1: 100% methanol with 0.1% formic acid;

S2: 50% aqueous methanol;

S3: acetone: water: acetic acid (70: 29.5: 0.5, *V*:*V*:*V*);

S4: 50% aqueous ethanol.

After that, the extraction solvents were collected in centrifugation tubes. Finally, the mixture was centrifuged at 9000 r/min for 10 min and the supernatant was pooled and transferred to another tube.

2.4 Hydrolysis of the extracts

The primary extracts (0.5 mL) were homogenized with 2 mL of 4 mol/L trifluoroacetic acid (TFA) and heated at 120 ℃ for 2 h in a sealed centrifugal tube (10 mL). The hydrolysate mixture was allowed to cool at room temperature and centrifuged at 9000 r/min for 4 min. Then the sediments were removed and concentrated to a dry sample with a rotary evaporator equipped with a water bath at 35~40 ℃. After that, 20 mL of methanol was added into the dry sample to remove TFA and the liquid was then evaporated in a rotary evaporator at 37 ℃. This step was repeated until the hydrolysate extract reached pH 7 with a volume of 1 mL.

2.5 Derivatization process

For silanization, nitrogen was used to dry hydrolysate extract (1.0 mL), followed by addition of 1 mL of derivatization reagent (BSTFA with 1% TMCS). The mixture was sealed in the reaction vial, sonicated at 60 ℃ for 45 min and heated at 100 ℃ for 1 h. Final solution was stored at 0 ℃ until analysis.

2.6 GC/MS apparatus and analysis

A GC/MS-QP2010A from Japan Shimadzu was used for all analyses. The GC was equipped with a Restek RTX-5 capillary column (0.25 mm×30 m×0.25 μm). The injector temperature was set at 280 ℃, and the injection volume was 1.0 μL in the split mode with a 10:1 split. The column temperature for GC was initially held at 50 ℃, then heated at 6 ℃/min to 250 ℃ and at 15 ℃/min to 280 ℃, and held for 10 min, with a total running time of 55 min. The interface temperature was 250 ℃. The mass spectra were scanned from *m/z* 50 to 500 and started 10 min after the injection. The electron impact ionization (EI) was set at 70 eV.

2.7 Statistic analysis

To investigate the accuracy of the method, three replicates of fresh blueberry samples were extracted and analyzed independently. The data of each repetition were used to obtain RSD. All data were analyzed using the NIST 11.1 software.

3 Results and discussion

3.1 Complete compounds information from the four extracts

After the hydrolysis steps and pre-column derivatization via silanization, the four GC-MS chromatograms of extracts obtained with four solvents with specific polarity and pH were compared. Application of methanol, ethanol, acetone or their water mixture combinations effectively extracted a large amount of phytochemicals from blueberry. The compounds were complicated. However, numbers and distribution of the chemical compounds were different in these four extracts, demonstrating that the differences in chemical compounds were caused by solvents.

To identify particular compounds, each peak representing silane was characterized by comparison with mass spectral library databases. Since “active hydrogens” in –OH, -COOH, $-NH_2$ and so on could be replaced by $[-Si-(CH_3)_3]$ during silylation, we adopted formula restoration of transforming $[-Si-(CH_3)_3]$ to [H]. The statistical data of identified complete compounds in the four extracts are shown in *Table 1*.

As shown in Table 1, the identified compounds in the four extracts were divided into eight types: acids, alcohols, phenolic, esters, sugar, ketone, alkane and other compounds. All these compounds were obtained by using different extraction combinations. Three main components acids, sugars and esters were extracted. The types of chemicals were different among the four extracts. The repeated parts provide us the most important information on compounds of blueberry, while the complementary parts help us choose solvents and conduct profound research on natural plants.

Table 1　Classification of compounds from the four extracts

Types of compound	Number of each compound			
	S1	S2	S3	S4
acids	10	15	11	17
esters	6	7	6	6
sugar	13	14	17	14
alcohol	2	2	2	2
phenolic	1	1	--	1
ketone	1	1	--	1
alkane	--	4	1	2
others	4	4	2	3
total	37	48	39	46

It is difficult to analyze the structures of the compounds with large molecular weight and high boiling point directly by GC-MS. Therefore, hydrolysis was done to transform them into compounds with small molecular weight and low boiling point that are suitable for GC analysis. The amounts of low-boiling-point compounds were speculated to be significantly increased. The basic structure of anthocyanin consists of 15 carbon atoms arranged in three rings (C_6-C_3-C_6), and most of them are linked with sugar. The hydrolysis process may intensify the depolymerization of the anthocyanin chain and liberate some free sugars. This may be one reason why we got more information after hydrolysis.

3.2 Acid compounds from the four extracts

Identified acid compounds in the four extracts are shown in Table 2. The total number of acids was 25, regardless of the solvent groups. In addition, 10, 15, 11 and 17 organic acids were identified and extracted by S1, S2, S3 and S4 respectively, with the largest number of acids identified by S4 (50% ethanol solution). The sensitivity of the four extractions was observed and studied. The acids were divided into four ranges ($<C_5$, C_6, C_7-C_8, $>C_9$) to conclude a histogram (Fig. 2), from which the favorable solvents of different acids can be inferred.

As shown in Fig. 1, S1 was very sensitive in extracting 6-C compounds and S2 did not show obvious differences in the four ranges. Accordingly, S-3 and in particular S-4 were more susceptible to compounds of $< C_5$, and identified 8 low-molecular-weight acids. Most of them are flavor substances and are conjectured to result from the enhanced solvent polarity by using water.

Table 2 Identified acid compounds in the four extracts

Name of acid compound	Formula [a]	S1	S2	S3	S4
oxalic acid	$C_2H_2O_4$			√	√
lactic acid	$C_3H_6O_3$				√
glyceric acid	$C_3H_6O_4$			√	√
malic acid	$C_4H_6O_5$		√	√	√
4-hydroxy-butanoic acid	$C_4H_8O_3$			√	
methylene-succinic acid	$C_5H_6O_4$		√		√
citraconic acid	$C_5H_6O_4$		√	√	√
2-ketoglutaric acid	$C_5H_6O_5$	√	√		√
levulinic acid	$C_5H_8O_3$	√	√	√	√
3-methyl-2-furoic acid	$C_6H_6O_3$	√	√	√	√
5-hydroxymethyl-2-furoic acid	$C_6H_6O_4$		√		
muconic acid	$C_6H_6O_4$	√	√		√
citric acid	$C_6H_8O_7$	√	√	√	√
3-methyl-glutaric acid	$C_6H_{10}O_4$	√			
dimethyl malate	$C_6H_{10}O_5$	√			
hexanoic acid	$C_6H_{12}O_2$		√		
2-hydroxy-4-methyl-pentanoic acid	$C_6H_{12}O_3$	√			
2,6-dihydroxy-benzoic acid	$C_7H_6O_4$		√	√	√
quinic acid	$C_7H_{12}O_6$		√		
2-hydroxy-3-methyl-benzoic acid	$C_8H_8O_3$				√
3-octenoic acid	$C_8H_{14}O_2$		√		
suberic acid	$C_8H_{14}O_4$			√	√
hexadecanoic acid	$C_{16}H_{32}O_2$	√	√	√	√
octadecenoic acid	$C_{18}H_{34}O_2$	√	√		√
nonadecanoic acid	$C_{19}H_{38}O_2$				√
Sum	25	10	15	11	17

[a] compounds were arranged by the number of carbon atoms and hydrogen atoms in ascending order, and were the same with the arrangement in the tables below.

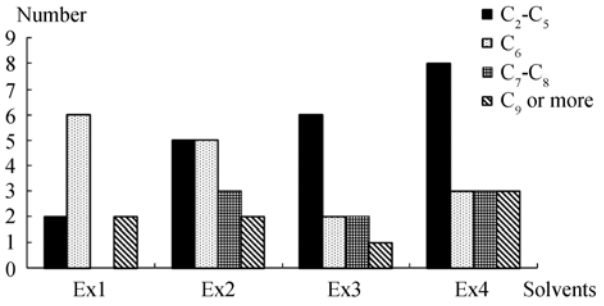

Fig. 1 Acid compounds in the four extracts grouped by the number of carbon atoms

Organic acids are linked to various bioactivities, such as antioxidant and antibacterial effects and benefiting the intestine. Organic acids can also prevent urinary tract infections. The acidification of urine and organic acids in blueberries may be correlated to the antiadhesion of bacteria that cause urinary disorders. In fact, only some of the organic acids have been concerned. Our results offer more aspects about the bioactivities of organic acids in blueberry. In addition, some organic acids such as lactic acid and malic acid are bound to the flavor of blueberries. This method can be used for quantization of flavor substances.

3.3 Sugars from the four extracts

As shown in Table 3, the compound information was sorted in the same way. There were 13, 14, 17 and 14 types of sugars identified by applying S1, S2, S3 and S4 respectively. Extraction with Solvent 3 identified the most types of sugars. We can conclude from Table 3 that most of the identified sugars are pentose, hexose and their disaccharide.

In Fig.2, we can easily find plenty of monosaccharides. Blueberries are abundant with polysaccharides, anthocyanins and ketones, which are macromolecular compounds with many important physiological activities. We can speculate that sugars may be formed from hydrolysis of these three compounds[24,25].

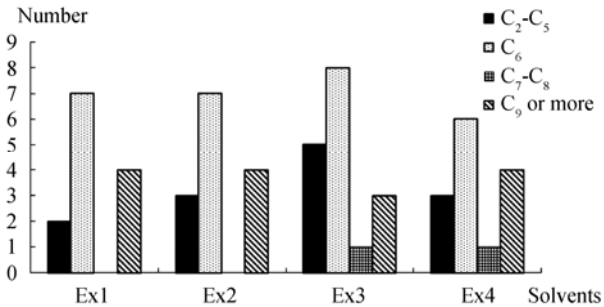

Fig. 2 Sugars grouped by the number of carbon atoms in the four extracts

Furthermore, anthocyanins are usually connected with many types of glycosyl groups, endowing anthocyanins with

more physiological functions. This method can help to identify the glycosyl groups in anthocyanines, which is vital to determine the types of anthocyanins. However, few anthocyanines were detected in this research, which may be attributed to the relatively high molecular weights of anthocyanins. In case of incomplete hydrolysis, the molecules are too large to be detected.

3.4 Ester compounds from the four extracts

Similarly, 6, 7, 7 and 6 esters were identified in the four extracts respectively (Table 4). The four solvents did not show obvious difference in sensitivity for this group of compounds, and the most abundant esters contained six carbon atoms.

Table 3　Identified sugars in the four extracts

Name of sugars	Formula	S1	S2	S3	S4
2-deoxy-ribose	$C_5H_{10}O_4$			√	
arabinose	$C_5H_{10}O_5$	√	√	√	√
xylopyranose	$C_5H_{10}O_5$			√	√
pentopyranose	$C_5H_{10}O_5$			√	
ribose	$C_5H_{10}O_5$		√		
xylose	$C_5H_{10}O_5$	√	√	√	√
fucose	$C_6H_{12}O_5$		√	√	
levoglucosan	$C_6H_{12}O_5$		√		√
2-deoxy-*D*-glucose	$C_6H_{12}O_5$	√	√	√	√
rhamnose	$C_6H_{12}O_5$	√			
6-deoxy-*L*-galactose,	$C_6H_{12}O_5$		√		
tagatose	$C_6H_{12}O_6$		√	√	
allose	$C_6H_{12}O_6$	√		√	√
glucose	$C_6H_{12}O_6$			√	
mannose	$C_6H_{12}O_6$	√		√	√
talose	$C_6H_{12}O_6$	√		√	
hexopyranose	$C_6H_{12}O_6$	√	√		√
sorbose	$C_6H_{12}O_6$	√	√	√	√
methyl glucopyranoside,	$C_7H_{14}O_6$			√	√
lactose	$C_{12}H_{22}O_{11}$	√	√		√
trehalose	$C_{12}H_{22}O_{11}$	√	√	√	√
sucrose	$C_{12}H_{22}O_{11}$	√	√	√	√
glucopyranose,	$C_{12}H_{22}O_{11}$	√	√	√	√
Sum	23	13	14	17	14

Table 4　Ester compounds identified in the four extracts

Name of ester compounds	Formula	Ex1	Ex2	Ex3	Ex4
ribonic acid lactone	$C_5H_8O_5$	√	√	√	√
glucuronic acid lactone	$C_6H_8O_6$	√	√		
malic acid dimethyl ester	$C_6H_{10}O_5$		√		√
gluconic acid lactone	$C_6H_{10}O_6$	√	√	√	√
galactonic acid lactone	$C_6H_{10}O_6$	√		√	√
tartaric acid dimethyl ester	$C_6H_{10}O_6$		√	√	
talonic acid lactone	$C_6H_{10}O_6$			√	
glycero-gulo-heptonicacid,g-lactone;	$C_7H_{12}O_7$	√	√	√	√
methyl-glucopyranoside	$C_7H_{14}O_6$		√		
ethyl gallate	$C_9H_{10}O_5$				√
nonalactone	$C_9H_{16}O_2$	√			
Sum	11	6	7	6	6

Our research was about the esters. Esters usually are in tight relationship with volatile components in plants. Moreover, the richest in the extracts were six-carbon esters (Fig.3).

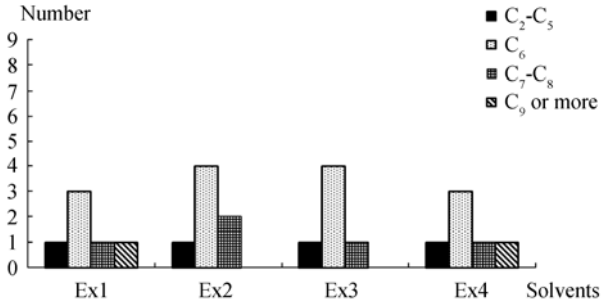

Fig. 3　Ester compounds grouped by the number of carbon atoms in the four extracts

3.5 Other identified compounds

Other identified compounds are shown in Table 5. The esters, phenols and alkanes are less diversified than organic acids or sugars, but they are of vital in plants. Strong scientific evidence shows the positive effects of phenols, such as the antioxidant activity[18]. Moreover, the special flavors of blueberries are attributed to the esters. Besides, alkane is a plant secondary metabolite with antibacterial effect, which is valuable in study at different growing stages[26].

Table 5 Statistical table of other identified compounds in the four extracts

Type	Name	Formula	Ex1	Ex2	Ex3	Ex4
Alcohols	glycerin	$C_3H_8O_3$				√
	propylene glycol	$C_3H_8O_2$				√
	xylitol	$C_7H_{12}O_4$	√	√	√	
	inositol	$C_6H_{12}O_6$	√	√	√	
Sum		4	2	2	2	2
Phenolic	phloroglucinol	$C_6H_6O_3$	√			
	hydroxythiophenol	C_6H_6OS		√		√
Sum		2	1	1	--	1
Ketone	4-methoxy-cyclohexanol	$C_7H_{14}O_2$				√
	1,4,2,5-cyclohexanetetrol	$C_6H_{12}O_4$	√			
	5-hydroxy-2-(hydroxymethyl)-4-pyrone	$C_6H_6O_4$		√		
Sum		3	1	1	--	1
Alkane	1-(1-ethoxyethoxy)-hexane	$C_{10}H_{22}O_2$		√		
	2-(4'-hydroxyphenyl)-2-(4'-methoxyphenyl)propane	$C_{16}H_{18}O_2$		√		
	1,2,4,5-cyclohexanetetrol	$C_6H_{12}O_4$		√	√	√
	dotriacontane	$C_{32}H_{66}$		√		√
Sum		4	--	4	1	2
Others	hydroxyhexanamide	$C_6H_{13}NO_2$		√		
	1,2,5-trimethyl-1h-pyrrole	$C_7H_{11}N$	√			
	2,4-dihydroxy-benzaldehyde	$C_7H_6O_3$			√	
	methylglucoside	$C_7H_{14}O_6$	√		√	
	methyl-xyloside	$C_6H_{12}O_5$	√	√		
	5-(2,4,6-trichlorophenoxymethyl)furane-2-carboxaldehyde	$C_{12}H_7C_{l3}O_3$		√		
	naphthalene,1,2,3,4-tetrahydro-6- methoxy-2-(4-pentylcyclohexyl)-, trans-	$C_{22}H_{34}O$		√		
	methyl arabinopyranoside	$C_6H_{12}O_5$	√			
	6,7-dihydroxy-4-methylcoumarin	$C_{10}H_8O_4$				√
	2-butene-1,4-diol	$C_4H_8O_2$				√
	triphenylphosphine oxide	$C_{18}H_{15}OP$				√
Sum		11	4	4	2	3

4 Discussion

For all the blueberries analyzed, acid, alcohol, phenolic compounds, esters, sugar, ketone, alkane and other compounds were identified. The results showed that S4 was more efficient in extracting the acids compounds while S3 was proved as the best choice to extract sugars. In contrast,

S2 provided the maximum information about esters, phenolic compounds, alkane and others compounds as compared to other extracting combinations. So it was concluded that S2 solution would be a better choice than the others for extraction of chemical compounds when we don't have a specific target. However, when the studies are decided to focus on the acid compounds, S4 will be the better option. And the principle is the same when we use S3 to extract sugar compounds. This study provides a novel strategy for full-scale and rapid confirmation about chemical components in blueberry. This overall identification of chemical components is of great significance to the quality control point of view and to revealing the secrets underlying their effectiveness.

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