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GC-MS Determination of Flavonoids and Phenolic and Benzoic Acids in Human Plasma after Consumption of Cranberry Juice

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A GC-MS method was developed for the determination of various flavonoids and phenolic and benzoic acids in human plasma. The procedure involved the extraction of flavonoids and phenolic and benzoic acids with ethyl acetate, followed by the derivatization of the phenolic and benzoic compounds with BSTFA (*N*,*O*-bis(trimethylsilyl) trifluoroacetamide) + TMCS (trimethylchlorosilane) reagent. The trimethylsilyl derivatives formed were separated and quantitated using GC-MS. Twenty flavonoids and phenolic and benzoic compounds have been well separated in the spiked human plasma without any interference. The average recovery was 79.3%. Several phenolic acids such as *o*-hydroxybenzoic, *p*-hydroxyphenylacetic, 2,3-dihydroxybenzoic, 2,4-dihydroxybenzoic, ferulic, sinapic, and benzoic acid were identified and quantified in human plasma after consumption of a cranberry juice. This developed method provides a simple, specific, and sensitive technique for the simultaneous determination of flavonoids and phenolic and benzoic acids in human plasma and is suitable for bioavailability and pharmacokinetic studies.

KEYWORDS: GC-MS; human plasma; phenolic acids; flavonoids; cranberry juice; antioxidant

INTRODUCTION

Epidemiological studies have shown that consumption of fruits and vegetables rich in phenolic compounds is associated with lower risk of cardiovascular diseases and cancer mortality (*1*−*5*). During the past few decades, the role of phenolics as natural antioxidants and free radical scavengers has attracted considerable public and scientific interest (*6*−*10*). Many separation and analytical techniques have been developed to characterize and quantitate phenolic compounds in fruits, vegetables and other natural plant products (*11*−*27*). The phenolic component of plant foods constitutes a complex mixture. The occurrences of various phenolic compounds and their concentrations in plant foods have been summarized in several reviews (*26*−*28*). As yet, progress in understanding the mechanisms involved in the health effects of phenolics is limited because very little information is available regarding the existence of these compounds in human or animal fluids. Without knowledge about whether specific phenolic compounds are really absorbed in the human intestinal tract and persist in human blood, how their concentrations change over time after consumption of plant foods, and whether the concentrations are at the level that could affect human health, it is challenging to formulate mechanisms for their biological activities and to further harness their pharmaceutical potentials and health benefits.

Few methods have been reported for analysis of flavonoids in humans. Ho et al. (*12*) separated and quantified (+)-catechin in plasma using HPLC with a fluorescence detector. Lee et al. (*13*) used HPLC with a coulometric electrochemical detector (CED) to determine tea catechins. Luthria et al. (*22*) developed a GC-MS method and Natsume (*14*) an LC-MS technique for measurement of plasma catechins. Manach et al. (*15*) investigated the bioavailability of quercetin using HPLC−CED analysis. All of these methods provide good separation and sensitive determination for the target flavonoids. However, most of them are limited to an individual or a single type of flavonoids. Few measurements of phenolic acids in human or animal fluids have been reported. Recent studies have shown that the health benefits exerted by phenolics is due to the combination of phytochemicals (phenolic acids and flavonoids) but not the action of an individual or a single group of flavonoids or phenolic acids alone (*6*, *10*). Thus, development of an analytical method for the simultaneous determination of phenolic acids and flavonoids in human fluids is urgently needed for understanding the bioavailability, mechanism of uptake, and consequences of biotransformation of phenolic compounds after consumption of common foods.

The purpose of the present study was to develop a simple, sensitive, and accurate GC-MS method to simultaneously determine flavonoids and phenolic acids in human plasma and to apply the developed method to monitoring the concentration, and thus the absorption of individual phenolic antioxidants, in human plasma after consumption of cranberry juice. It is expected that the established method may also be used as an identification and quantitation tool for metabolites of these phenolic compounds in different matrixes such as serum, urine,

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and tissues without significant changes in sensitivity and precision. Cranberry juice was chosen in this study because it contains abundant flavonoids and other phenolic compounds, which have been reported to be beneficial to human health (*11*, *18*, *29*).

MATERIALS AND METHODS

Chemicals. Phenolic and benzoic acid standards of *m*-methylbenzoic acid (internal standard), benzoic acid, *o*-hydroxybenzoic acid, *trans*-cinnamic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid, *o*-phthalic acid, 2,3-dihydroxybenzoic acid, vanillic acid, *o*-hydroxycinnamic acid, 2,4-dihydroxybenzoic acid, *p*-coumaric acid, ferulic acid, caffeic acid, sinapic acid, *trans*-resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), (−)-epicatechin (*cis*-3,3′,4′,5,7-pentahydroxyflavan), catechin (*trans*-3,3′,4′,5,7-pentahydroxyflavan), quercetin (3,3′,4′,5,7-pentahydroxyflavone), and myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone) used for this study were obtained from Acros Organics (Springfield, NJ). Methanol, *n*-hexane, and ethyl acetate were obtained from Pharmcoproducts (Brookfield, NJ). Derivatization reagent *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) was purchased from Supelco (Supelco Park, PA).

Samples. Ocean Spray cranberry juice cocktail (containing 27% cranberry juice) was purchased from a local supermarket in Massachusetts and stored at 4 °C until used in this study. Blood samples were drawn from a fasted healthy volunteer before and after drinking 1800 mL Ocean Spray cranberry juice. The blood was collected into Vacutainer K_2EDTA (potassium ethylenediaminetetraacetate) tubes to provide anticoagulation. After centrifugation at 1000*g* for 10 min to sediment the cells, the clear platelet poor plasma (PPP) was collected and stored at −80 °C until analysis.

Extraction of Phenolic Compounds from Plasma and Derivatization. Each plasma sample (300 μL) was extracted three times with 0.5 mL of ethyl acetate by vortexing for 1 min in a 1.5-mL tube after the pH was adjusted to 2.0 by adding 0.5 M HCl. The mixture was then centrifuged for 10 min, and the top layer (ethyl acetate) was removed and pooled in another tube. The ethyl acetate extracts were passed through anhydrous $MgSO_4$ packed in a Pasteur pipet and evaporated to dryness under nitrogen. The calibration standard solution containing 20 phenolic and benzoic compounds was prepared in methanol. The standard solution (100 μL) was pipetted into a 2-mL minivial and dried by a stream of nitrogen. The standard and extracts were derivatized by the addition of 50 μL of the derivatization reagent BSTFA + TMCS and heated at 70 °C for 4 h.

Preparation of Calibration Standards and Curves. The stock standard solutions were prepared by dissolving 20−30 mg of each of 20 phenolic standard compounds in 25 mL of methanol separately. The working standard solutions containing the 20 phenolic compounds were prepared by combining and diluting the individual stock standard solutions to obtain the desired concentrations in the range of 30−50 μg/mL. The working standard mixture was diluted in the ratios 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 (v/v) to obtain the calibration solutions. Calibration curves were constructed by linear regression of the peak-area ratio of individual standard to the internal standard versus the concentration.

Instrumentation. A Hewlett-Pakard (HP) Model GC 5890 Series II gas chromatograph coupled with an HP 5971 series mass selective detector and an HP 7673 GC autosampler was employed for all analyses. Samples were separated on a 30-m × 0.35-mm, 0.25-μm, DB-5 fused silica capillary column (J&W Scientific, Folsom, CA). The column temperature was initially held at 80 °C for 1 min, then the temperature was raised to 220 °C at a rate of 10 °C per min, from 220 to 310 °C at a rate of 20 °C per min and held for 6 min. The total run time was 27.5 min. Ultrahigh purity helium with an inline Alltech oxygen trap was used as carrier gas. The carrier gas was set at 40 psi, column head pressure at 8 psi. Injector temperature was maintained at 280 °C, and the injection volume was 1.0 μL in the splitless mode. The interface temperature was held at 280 °C. Mass spectra were scanned from *m*/*z* 50−650 at a rate of 1.5 scans/s. Electron impact ionization energy was 70 eV.

Identification and Quantification. Identification of phenolic compounds in each plasma sample and cranberry juice was achieved by comparing the gas chromatographic retention times and mass spectra with the authentic standards. In each sample, quantification of these compounds was conducted by relating the peak areas of identified compounds to that of the internal standard, *m*-methylbenzoic acid, at a concentration of 15 μg/mL. All the calibration standards and plasma samples were run in triplicate.

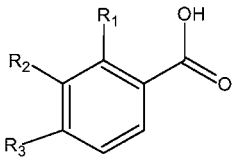
RESULTS AND DISSCUSSION

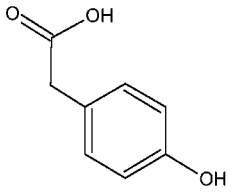
Chromatograpy of the Derivatized Flavonoids and Phenolic and Benzoic Acids in Human Plasma and Cranberry Juice. The chemical structures of the 20 standard compounds studied are given in **Figure 1**. These benzoic and phenolic compounds can be divided into three different structural families: benzoic and phenolic acids, resveratrol, and flavonoids. The multiple hydroxyl functionality on the more complex flavonoids makes their derivatization reaction with BSTFA + TMCS very slow. The original protocol designed for the silylation of phenolic acids does not work well for flavonoid compounds (*11*). After multiple preliminary assays at various derivatization temperatures and times, an increased derivatization temperature and time, 4 h at 70 °C, was chosen, which dramatically improves the derivatization and chromatographic performance. Chromatograms of spiked and sample plasma are presented in **Figure 2**, and retention data for all standards are listed in **Table 1**. Important ions shown in the mass spectra of silylated derivatives of all standard phenolic compounds and internal standard used for identification tests are also presented in **Table 1**. No interfering peaks of endogenous compounds appeared after samples were cleaned-up with ethyl acetate extraction. The phenolic acids, as well as resveratrol and the flavonoids, catechin, (−)-epicatechin, quercetin, and myricetin have been of the most interest to researchers studying the health effects of natural phenolic antioxidants, and they are well resolved from each other in less than 25-min (see **Figure 2A**).

When the developed GC-MS method was applied to the cranberry juice, 16 phenolic compounds, including benzoic and phenolic acids, myricetin, and qucertin were identified, which is in good agreement with published results (*11*, *18*, *24*). *trans*-Resveratrol was also identified by matching GC retention time and mass spectra. However, because the ratio of its peak area and peak area of the internal standard fell outside the applicable range of the calibration curve, no quantitative data have been made on resveratrol. **Figure 3** presents the chromatogram for the separation of phenolic compounds in the cranberry juice.

Recovery. The entire analytical procedure was applied to the blank plasma samples (collected prior to the cranberry juice intake), which were spiked with increasing amounts of the standard mixture (25 μL, 50 μL, 75 μL), containing known amounts of each of 20 standard phenolic compounds. Though there is wide range of polarity of the standard phenolic compounds, the efficiency and consistency of the extraction method was very good. The ethyl acetate extraction achieved at least 63% recovery except for *o*-phthalic acid. The low recovery rates of *o*-phthalic acid (30%) may be due to its low solubility in the organic phase or strong binding with other plasma components. The results are presented in **Table 2** as the mean recovery (amount found as a percentage of the amount added) and its relative standard deviation (RSD) as a percentage of the mean.

Identification and Quantitation of Phenolic and Benzoic Compounds in Plasma. Figure 2, parts **B** and **C**, presents the separation of the phenolic compounds extracted from the plasma samples collected at 45 and 270 min after consumption of





Benzoic acid **(1)** $R_1 = R_2 = R_3 = H$

p-Hydroxyphenylacetic Acid **(6)**

o-Hydroxybenzoic Acid **(2)** $R_1 = OH$, $R_2 = R_3 = H$

m-Hydroxybenzoic Acid **(4)** $R_1 = R_3 = H$, $R_2 = OH$

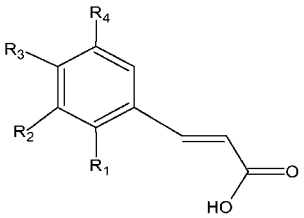
p-Hydroxybenzoic Acid **(5)** $R_1 = R_2 = H$, $R_3 = OH$

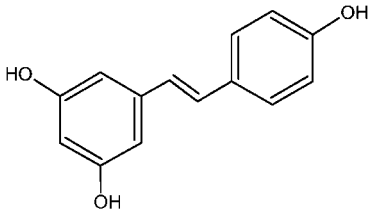
o-Phthalic Acid **(7)** $R_1 = COOH$, $R_2 = R_3 = H$

2,3-Dihydroxybenzoic Acid **(8)** $R_1 = R_2 = OH$, $R_3 = H$

Vanillic Acid **(9)** $R_1 = H$, $R_2 = OCH_3$, $R_3 = OH$

2,4-Dihydroxybenzoic Acid **(11)** $R_1 = R_3 = OH$, $R_2 = H$





trans - Cinnamic Acid **(3)** $R_1 = R_2 = R_3 = R_4 = H$

trans-Resveratrol **(16)**

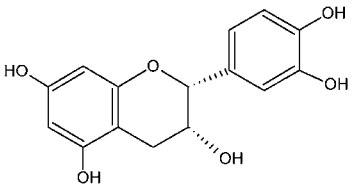
o-Hydroxycinnamic Acid **(10)** $R_1 = OH$ $R_2 = R_3 = R_4 = H$

p-Coumaric Acid **(12)** $R_1 = R_2 = R_4 = H$, $R_3 = OH$

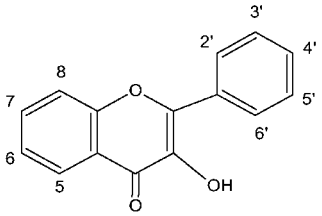
Ferulic Acid **(13)** $R_1 = R_4 = H$, $R_2 = OCH_3$, $R_3 = OH$

Caffeic Acid **(14)** $R_1 = R_4 = H$, $R_2 = R_3 = OH$

Sinapic Acid **(15)** $R_1 = H$, $R_2 = R_4 = OCH_3$, $R_3 = OH$



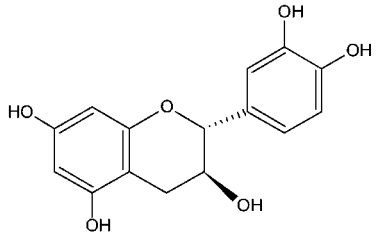
(-)-Epicatechin **(17)**



Flavanoids

Quercetin **(19)** 5=7=3'=4'= OH

Myricetin **(20)** 5=7=3'=4'=5'=OH



Catechin **(18)**

Figure 1. Chemical structures of flavonoids and phenolic and benzoic acids studied.

cranberry juice, respectively. Neither benzoic, phenolic, nor flavonoid compounds were observed in the plasma sample collected before the cranberry consumption. Benzoic acid, *o*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid, 2,3-dihydroxybenzoic acid, and 2,4-dihydroxybenzoic acid were detected in the plasma sample collected at 45 min after the consumption of the cranberry juice. The plasma sample, drawn at 270 min after cranberry juice intake, contained seven phenolic and benzoic acids, which included all five compounds found in the samples collected at 45 min plus ferulic and sinapic acid. It is interesting to note that two phenolic acids, *p*-hydroxyphenylacetic and 2,4-dihydroxybenzoic acid, have been identified in plasma, but not in the juice. These phenolic acids could be the metabolites from some of the combined phenolic constituents of the cranberry juice.

The concentrations of the identified compounds in plasma and cranberry juice were determined by relating their peak areas to that of *m*-methylbenzoic acid as an internal standard. The calibration curves are linear for all flavonoids and phenolic and benzoic acids over the concentration ranges studied, with correlation coefficients $r^2 > 0.993$ except for *p*-coumaric acid ($r^2 = 0.912$) and ferulic acid ($r^2 = 0.926$). Quantitative analysis shows that the concentrations of the identified compounds in cranberry juice were much higher than those of the same compounds in the plasma samples. Benzoic acid was the richest aromatic acid present in the juice (54 μg/mL) and two plasma

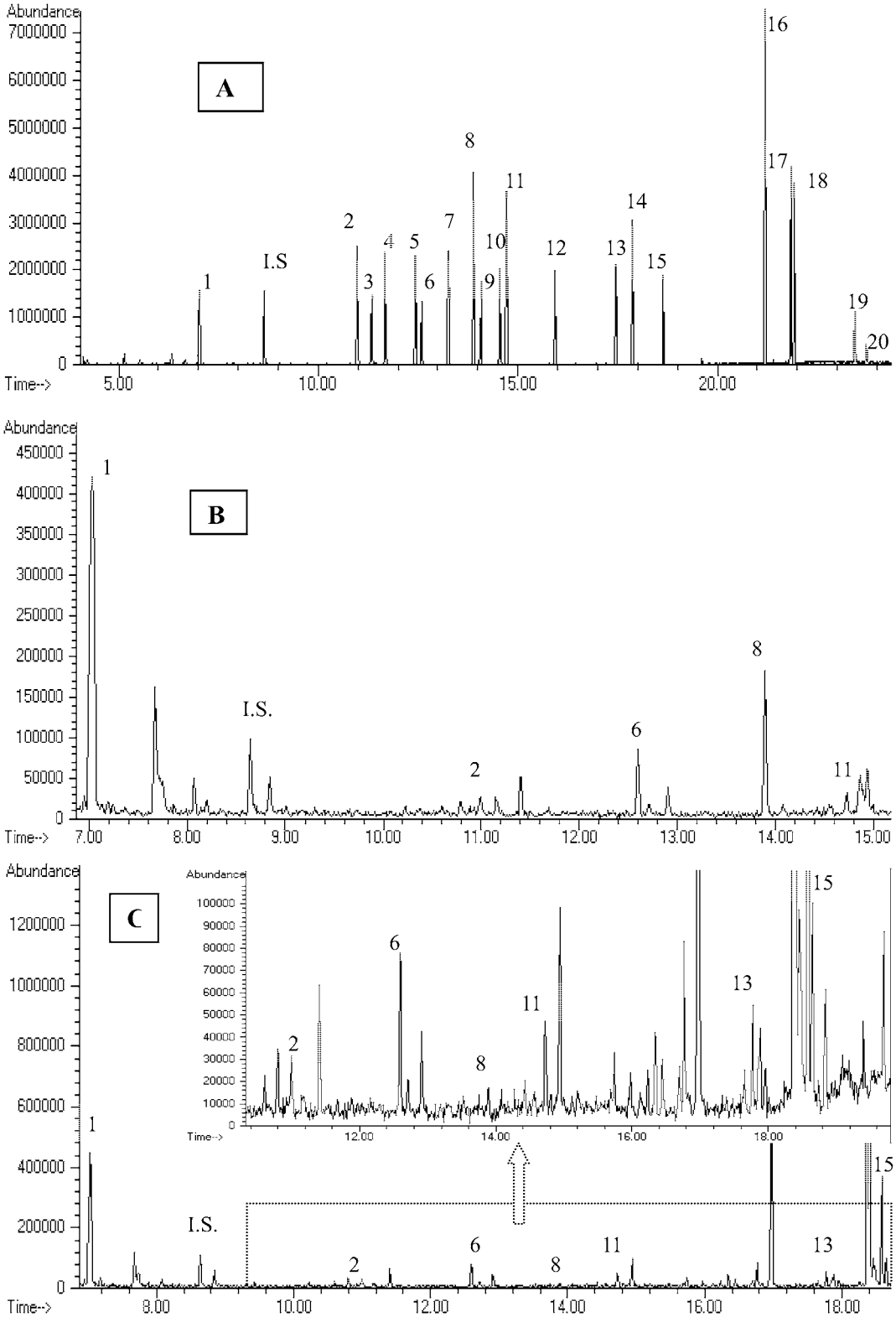


Figure 2. GC chromatogram: (**A**) standard phenolic compounds spiked blank plasma, (**B**) plasma sample collected at 45 min after consumption of cranberry juice, (**C**) plasma sample collected at 270 min after consumption of cranberry juice. Peaks correspond to numbering of structures in **Figure 1**.

samples (4.40 and 3.11 μg/mL) collected after the consumption of cranberry juice. 2,3-dihydroxybenzoic, *p*-hydroxyphenylacetic, *o*-hydroxybenzoic, and 2,4-dihydroxybenzoic acids were also determined in the plasma sample collected at 45 min after cranberry consumption at the concentration of 2.06, 1.40, 0.98, and 0.61 μg/mL, respectively. The results of identification and quantification of compounds of interest in the cranberry juice and all three plasma samples are presented in **Table 3**. This was the first attempt to simultaneously determine both flavonoids and phenolic acids in human fluids. It is not surprising that many of the identified phenolic compounds in the cranberry juice were not detected in the plasma samples. Our previous

Table 1. Retention Times and Characteristic Ions Present in the Mass Spectra of Silylated Derivatives[a] in Standard Solution, Cranberry Juice, and Plasma Samples

standards	retention time (min)	identified ions[b] (*m*/*z*)
1. benzoic acid	7.06	194, 179, 135, 105, 77
2. *o*-hydroxybenzoic acid	11.03	267, 209, 135, 73
3. *trans*-cinnamic acid	11.38	220, 205, 161, 145, 131, 103, 77
4. *m*-hydroxybenzoic acid	11.72	282, 267, 223, 193, 73
5. *p*-hydroxybenzoic acid	12.47	282, 267, 223, 193, 73
6. *p*-hydroxyphenylacetic acid	12.63	296, 281, 252, 179, 164, 147, 73
7. *o*-phthalic acid	13.31	310, 295, 221, 147, 73
8. 2,3-dihydroxybenzoic acid	13.93	355, 193, 137, 73
9. vanillic acid	14.11	312, 297, 267, 253, 223, 193, 126, 73
10. *o*-hydroxycinnamic acid	14.60	308, 293, 147, 73
11. 2,4-dihydroxybenzoic acid	14.75	355, 281, 73
12. *p*-coumaric acid	15.98	308, 293, 249, 219, 179, 73
13. ferulic acid	17.50	338, 323, 308, 249, 219, 146, 73
14. caffeic acid	17.91	396, 381, 219, 73
15. sinapic acid	18.68	368, 353, 338, 249, 207, 161, 73
16. *trans*-resveratrol	21.21	444, 429, 267, 179, 147, 73
17. (–)-epicatechin	21.87	368, 355, 283,179,73
18. catechin	21.96	368, 355, 283,179,73
19. quercetin	23.48	647, 559, 355, 207, 73
20. myricetin	23.79	647, 576, 559, 473, 254, 207, 73
m-methylbenzoic acid (I. S.)	8.66	208, 193, 149, 119, 91

[a] Identified as trimethylsilyl (TMS) derivative. [b] Characteristic peaks of TMS derivatives, which can be used as the identity of the compounds.

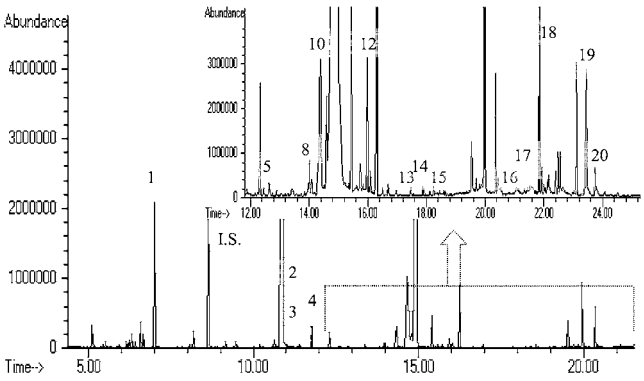


Figure 3. Separation of flavonoids and phenolic and benzoic acids in cranberry juice. Peaks correspond to numbering of structures in **Figure 1**.

studies (*11*, *18*) have indicated that only minor amounts of phenolic and benzoic acids occur in the free state in cranberry fruit. Most of them are present in conjugated forms (over 54−100%). Other studies (*13*−*15*) have further shown that phenolic compounds, particularly flavonoids and resveratrol, when added to blood, become tightly bound to serum proteins and to the membranes of red and white blood cells and platelets, with the possibility that part of the phenolic compounds are internalized. In this study, we have been mainly interested in determination of free forms of flavonoids and phenolic acids in human plasma. However, this developed method is also suitable for the monitoring of total flavonoids and phenolic compounds after a hydrolysis process with an acid, base or enzyme to release the combined phenolics.

The GC-MS method allows simple and simultaneous determination of flavonoids and phenolic and benzoic acids in human plasma and cranberry juice. Sixteen phenolic compounds, including myricetin, qucertin, resveratrol, benzoic and phenolic acids, have been identified in a commercial cranberry juice. Several phenolic and benzoic acids have also been identified and quantitated in human plasma collected after consumption of the cranberry juice without any interference. The reported method is sensitive and specific and has a good repeatability.

Table 2. Extraction Recovery[a] and RSD

standards	avg rec rate (%) ethyl acetate extraction	RSD (%)
benzoic acid	72.22	5.60
o-hydroxybenzoic acid	75.26	2.35
trans-cinnamic acid	86.97	3.29
m-hydroxybenzoic acid	77.51	1.67
p-hydroxybenzoic acid	81.05	1.83
p-hydroxyphenylacetic acid	87.90	2.96
o-phthalic acid	30.45	7.00
2,3-dihydroxybenzoic acid	75.60	1.97
vanillic acid	79.96	4.70
o-hydroxycinnamic acid	81.28	8.00
2,4-dihydroxybenzoic acid	77.26	1.84
p-coumaric acid	98.18	12.27
ferulic acid	87.50	5.81
caffeic acid	87.14	4.43
sinapic acid	88.25	8.77
trans-resveratrol	84.41	20.54
(–)-epicatechin	76.13	10.83
catechin	87.61	6.81
quercetin	87.99	11.91
myricetin	63.90	13.08

[a] All data are means of three independent assays.

Table 3. Concentrations of Identified Phenolic Compounds in Cranberry Juice and Plasma Samples

sample	cranberry juice	plasma (post 45min)	plasma (post 270min)
identified compound	concn (μg/mL)	concn (μg/mL)	concn (μg/mL)
benzoic acid	54.94	4.40	3.11
o-hydroxybenzoic acid	3.11	0.98	0.42
trans-cinnamic acid	0.18	ND	ND
m-hydroxybenzoic acid	0.15	ND	ND
p-hydroxybenzoic acid	0.07	ND	ND
p-hydroxyphenylacetic acid	ND[a]	1.40	1.12
2,3-dihydroxybenzoic acid	2.41	2.06	0.16
o-hydroxycinnamic acid	3.43	ND	ND
2,4-dihydroxybenzoic acid	ND	0.61	0.84
p-coumaric acid	2.63	ND	ND
ferulic acid	1.11	ND	0.31
caffeic acid	0.14	ND	ND
sinapic acid	5.11	ND	1.50
trans-resveratrol	N/A[b]	ND	ND
(–)-epicatechin	5.68	ND	ND
catechin	0.38	ND	ND
quercetin	7.79	ND	ND
myricetin	3.72	ND	ND

[a] ND = not detected. [b] N/A = the concentraction is out of calibration curve range.

It can also be used in the measurement of phenolic antioxidants in other human and animal fluids for their bioavailability and pharmacokinetic studies.

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