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Separation, Characterization, and Quantitation of Benzoic and Phenolic Antioxidants in American Cranberry Fruit by GC–MS

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A GC−MS method is reported for separation and characterization of widely different amounts of benzoic and phenolic acids as their trimethylsilyl derivatives simultaneously in cranberry. Fifteen benzoic and phenolic acids (benzoic, *o*-hydroxybenzoic, cinnamic, *m*-hydroxybenzoic, *p*-hydroxybenzoic, *p*-hydroxyphenyl acetic, phthalic, 2,3-dihydroxybenzoic, vanillic, *o*-hydroxycinnamic, 2,4-dihydroxybenzoic, *p*-coumaric, ferulic, caffeic, and sinapic acid) were identified in cranberry fruit in their free and bound forms on the basis of GC retention times and simultaneously recorded mass spectra. Except for benzoic, *p*-coumaric, caffeic, ferulic, and sinapic acids, 10 other phenolic acids identified have not been reported in cranberry before. The quantitation of the identified components was based on total ion current (TIC). The experimental results indicated cranberry fruit contains a high content of benzoic and phenolic acids (5.7 g/kg fresh weight) with benzoic acid being the most abundant (4.7 g/kg fresh weight). The next most abundant are *p*-coumaric (0.25 g/kg fresh weight) and sinapic (0.21 g/kg fresh weight) acid. Benzoic and phenolic acids occur mainly in bound forms and only about 10% occurs as free acid.

KEYWORDS: Phenolic acids; benzoic acids; antioxidant; cranberry; fruit; GC−MS

INTRODUCTION

Phenolic compounds are constituents of many fruits and vegetables, and they have attracted a great deal of public and scientific interest because of their potential anticarcinogenic and other health-promoting effects as antioxidants. Recent epidemiological studies have provided convincing evidence that consumption of fruits and vegetables is correlated with reduced incidence of and mortality from cancer, and cardiovascular and neurological diseases (*1*−*6*). The protection that fruits and vegetables provide against these diseases has been attributed to the phenolic and other antioxidant phytonutrients contained in these foods. It is well-known that free radicals cause oxidative damage to nucleic acids, proteins, and lipids. Phenolic and other plant antioxidants can scavenge the harmful free radicals and thus inhibit their oxidative reactions with vital biological molecules (*2*, *7*−*10*).

Interest in the role of phenolic antioxidants in human health has prompted research in the separation and characterization of active phenolic components in various plant-derived foods (*6*, *11*−*15*). Many traditional separation techniques such as paper, thin-layer, and column chromatography, with various solvent systems and spray reagents, have been proposed to separate and identify phenolic compounds (*16*−*18*). Recently, gas−liquid chromatography (GLC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE) methods have been developed (*15*, *19*−*30*). In contrast to GLC methods, HPLC and CE, especially, coupled with photodiode array detection do not require derivatization prior to the quantitative analysis. As such, they have become the most commonly used techniques for qualitative and quantitative analysis of phenolic compounds in plants, including fruits. However, HPLC and CE often do not provide sufficient separating performance, and the UV−Vis spectrum does not supply sufficient identifying power (*23*, *27*, *29*). For these reasons, capillary gas chromatography combined with mass spectrometry (GC−MS) remains the method of choice.

Although previous studies have demonstrated high phenolic content in fruits such as grape, apple, orange, prune ,and berries (*11*, *12*), American cranberry (*Vaccinium macrocarpon*) has received little attention (*27*, *31*). The American cranberry is a prominent agricultural food crop produced in Massachusetts, Wisconsin, Michigan, Canada, New Jersey, Oregon, and Washington. The crop size is approximately 500 million pounds annually, which is processed into three basic categories: fresh (5%); sauce products, concentrate, and various applications (35%); and juice drinks (60%). For many decades, cranberry juice has been widely used, particularly in North America, as a folk remedy to treat urinary tract infections (UTIs) in women (*32*), which has been confirmed by recent clinical and laboratory studies (*33*). Cranberry juice extracts have also been suggested to exhibit anticancer effects and to inhibit the oxidation of low-density lipoprotein in vitro, potentially preventing the development of heart diseases (*34*, *35*). The phenolic components of cranberry juice are believed to be the principal ingredients responsible for these beneficial health effects. Recently, we have

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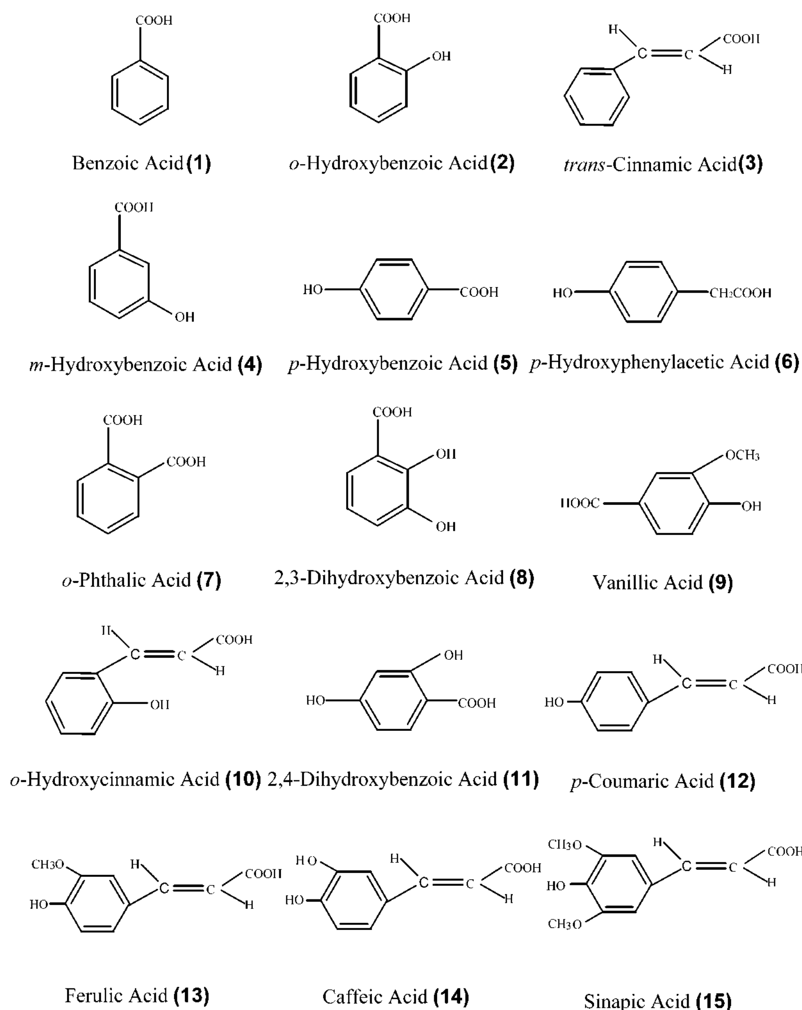

Figure 1. Chemical structures of analyzed benzoic and phenolic acids.

developed HPLC methods for simultaneous determination of flavonoids and phenolic acids in cranberry juice and various Chinese teas (*15*, *27*). The aims of this study were the following: to simplify the extraction and purification process to provide a rapid and effective capillary GC method which could be suitable for routine determinations for phenolic compounds in fruit products; to separate and characterize the potential phenolic antioxidant components of cranberry fruit using GC−MS; and to quantify the level of the identified phenolic antioxidants in cranberry fruit.

MATERIALS AND METHODS

Samples and Reagents. Cranberry fruit (*Vaccinium macrocarpon* Ait., var. Early Black) was obtained from a local supermarket in North Dartmouth, MA and stored at −20 °C until crushed to provide a fresh cranberry juice, which was filtered before extraction.

Standards of *m*-methylbenzoic acid (internal standard) (IS), *o*-hydroxybenzoic acid, cinnamic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenyl acetic acid, phthalic acid, 2,3-dihydroxybenzoic acid, vanillic acid, *o*-hydroxycinnamic acid, 2,4-dihydroxybenzoic acid, *p*-coumaric acid, ferulic acid, caffeic acid, and sinapic acid were purchased from Acros Organics (Springfield, NJ). Benzoic acid was purchased from Baker (Phillipsburg, NJ). Methanol was purchased from Pharmco Products (Brookfield, CT), ethyl ether was from Fisher Chemicals (Fair Lawn, NJ), and ethyl acetate was from Baker (Phillipsburg, NJ). Derivatization reagent BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) + TMCS (trimethylchlorosilane) was purchased from Supelco (Supelco Park, PA).

Extraction of Free Phenolic Acids in Cranberry Juice. Natural cranberry fruit (60 g) was thoroughly ground in distilled−deionized water (200 mL) with an electrical high-speed blender. A 10--mL aliquot of filtrate was acidified by adding 1 N HCl to maintain pH at 2 and extracted with 10 mL of ether twice. The ethereal phase contained free phenolic acids and was extracted with 10 mL of 5% $NaHCO_3$ twice. In this way, the phenolic acids were substantially separated from the other phenolic moieties. The alkali aqueous solution was acidified with 1 N HCl to maintain pH at 2 and extracted again with 10 mL of ether twice. The ethereal extract was dried over anhydrous $MgSO_4$ and evaporated to dryness on a rotary evaporator under reduced pressure at 35 °C. The dry residue was dissolved in 100 μL of freshly distilled pyridine, and 10 μL of the pyridine solution was used for derivatization before GC analysis. For major aromatic components, such as benzoic acid, dilution was made before derivatization.

Extraction of Total Phenolic Acids in Cranberry Juice. An 80-mg amount of ascorbic acid was dissolved in 20 mL of distilled-deionized water in a 100-mL round-bottomed flask. A 20-mL aliquot of filtered cranberry juice containing 6 g of natural cranberry fruits was added. To this solution, 20 mL of 6 N HCl was added slowly within 5 min into the flask. Under an argon atmosphere, the solution was heated at 35 °C for 16 h with a magnetic stirrer. The solution was allowed to cool and then filtered through 0.45 μm membrane. A 15-mL portion of the filtrate was extracted with ethyl acetate and treated with 5% $NaHCO_3$. The ethyl acetate extract was dried over $MgSO_4$ and evaporated at 35 °C as described above in preparation for the derivatization step.

Preparation of Calibration Standards. Four standard solutions each containing 14 target compounds, i.e., *o*-hydroxybenzoic, cinnamic, *m*-hydroxybenzoic, *p*-hydroxybenzoic, *p*-hydroxyphenyl acetic, phthalic, 2,3-dihydroxybenzoic, vanillic, *o*-hydroxycinnamic, 2,4-dihydroxybenzoic, *p*-coumaric, ferulic, caffeic, and sinapic acids, were first prepared in methanol at concentrations of 5, 10, 20, and 50 μg/mL. Benzoic

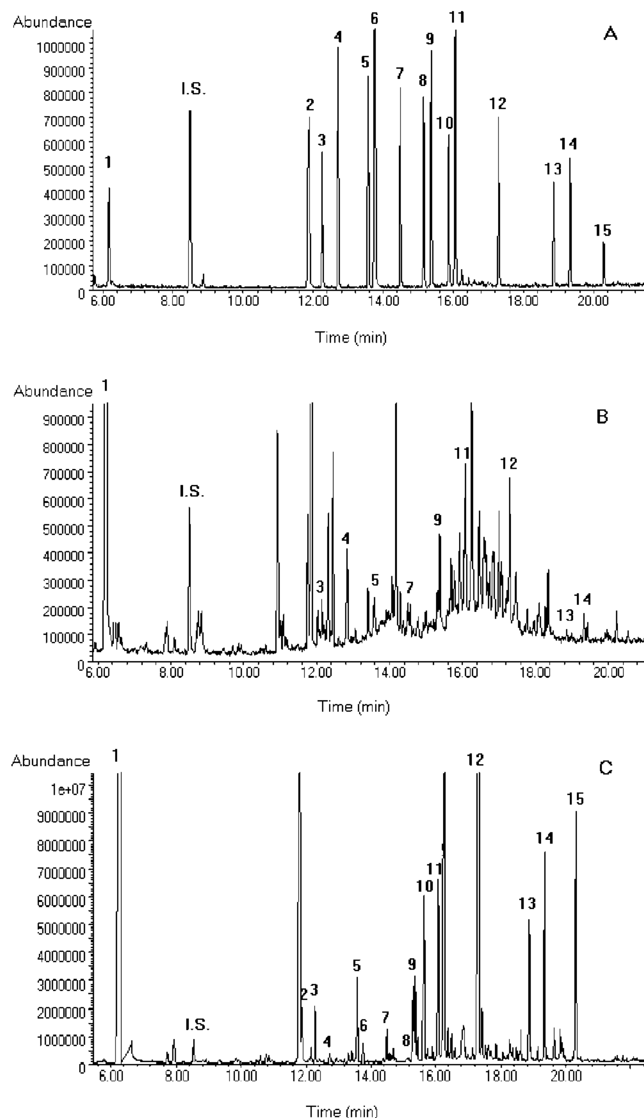

Figure 2. GC chromatogram: A, calibration standards and the internal standard at the concentration of 15 μg/mL; B, separation of the free phenolic acids in cranberry juice; C, separation of the total phenolic acids in cranberry juice. Peaks correspond to numbering of structures in **Figure 1**.

acid standard solutions were prepared in methanol at concentrations of 50, 100, 200, and 500 μg/mL because of its much higher concentration in the cranberry juice. In a separate analysis, 100 μL from each of the four methanolic standard solutions was pipetted into a 2-mL minivial and dried by a stream of nitrogen prior to the derivatization step.

Derivatization. The calibration standards and the cranberry juice samples were derivitized by the addition of 50 μL of pyridine solution of *m*-methylbenzoic acid internal standard at a concentration of 20 μg/mL and 50 μL of the derivatization reagent BSTFA + TMCS with heating at 60 °C silylated in a sealed 2-mL minivial for 30 min. The silylated samples were analyzed by GC−MS. The large excess of BSTFA + TMCS ensured that the derivatization was complete. Care was taken to ensure anhydrous conditions during the preparation and derivatization process because of the high sensitivity of trimethylsilyl (TMS) derivatives toward moisture.

GC−MS Instrumentation and Conditions. GC−MS analysis was carried out on an HP 5890 Series II gas chromatograph coupled with an HP 5971 Series mass selective detector. Samples were separated on a 30 m × 0.35 mm i.d., 0.25-μm film DB-5 fused-silica capillary column (J&W Scientific, Folsom, CA). The column temperature was initially held at 80 °C for 1 min, then programmed to 120 °C at a rate of 5 °C/min, from 120 °C to 240 °C at a rate of 10 °C/min, and from 240 °C to 280 °C at a rate of 20 °C/min, with a final hold time of 5 min. Helium was used as the carrier gas and the column head pressure was maintained at 13.3 psi. Injector temperature was maintained at 280 °C, and the injection volume was 1 μL in the splitless mode. The interface temperature was held at 280 °C. Mass spectra were scanned from m/z 50 to 550 at a rate of 1.5 scans/sec.

Table 1. Retention Times and Important Ions Present in the Mass Spectra of Silylated Benzoic and Phenolic Acid Compounds[a] in Standard Solution, Cranberry Juice, and the Internal Standard by GC−MS

peak	retention time (min)	identified ions[b](m/z)
1	6.18	194, 179(100%), 135, 105, 77
IS	8.47	208, 193(100%), 149, 119, 91
2	11.85	267(100%), 209, 135, 73
3	12.24	220, 205(100%), 161, 145, 131, 103, 77
4	12.71	282, 267(100%), 223, 193, 73
5	13.54	282, 267(100%), 223, 193, 73
6	13.76	296, 281, 252, 179, 164, 147, 73(100%)
7	14.50	310, 295, 221, 147(100%), 73
8	15.13	355(100%), 193, 137, 73
9	15.36	312, 297(100%), 267, 253, 223, 193, 126, 73
10	15.85	308, 293, 147(100%), 73
11	16.04	355(100%), 281, 73
12	17.26	308, 293, 249, 219(100%), 179, 73
13	18.85	338, 323, 308, 249, 219, 146, 73(100%)
14	19.31	396, 381, 219(100%), 73
15	20.27	368(100%), 353, 338, 249, 207, 161, 73

[a] Identified as trimethylsilyl (TMS) derivative. [b] Characteristic peaks of TMS derivatives which can be applied to identify the presence of the compounds.

Identification and Quantitation. The phenolic acids in the cranberry samples were identified by matching retention times and mass spectral data with the calibration standards. All quantitation was performed by the method of internal standardization using *m*-methylbenzoic acid at a concentration of 10 μg/mL. The quantitation of these compounds was based on the ratio of the peak area of the compound of interest over the peak area of the internal standard. All the calibration standards and the cranberry juice samples were run in triplicate.

RESULTS AND DISCUSSION

GC−MS Separation and Identification of Benzoic and Phenolic Compounds in a Standard Mixture and Cranberry Extracts. The structures of phenolic compounds of interest to this investigation are shown in **Figure 1**. All these phenolic acids have relatively low volatility and are not suitable for direct capillary GC analysis. BSFTA + TMCS was chosen as the derivatization reagent to convert analytes into volatile trimethylsilyl derivatives. Prior to employing GC−MS for the determination of phenolic compounds in crude cranberry extracts, the efficacy of the separation and detection of phenolics using the GC−MS technique was tested on a standard mixture of the 15 phenolic acids. The instrument parameters, such as the temperature program and carrier gas flow rate, were optimized to obtain good resolution between all 15 phenolic compounds in a short elution time. The results of this experiment are illustrated in **Figure 2A**, which shows a total ion chromatogram for this analysis. A baseline separation of the 15 phenolic acids and the internal standard was achieved in 28 min using the method described. The identities of the 15 components were established from GC retention times and from recorded mass spectra. The retention data and the mass spectra of the benzoic and phenolic acids studied are listed in **Table 1**.

The separation and quantitation of phenolic compounds in cranberry fruit is difficult because of their chemical complexity. A pre-cleanup and fractionation process prior to chromato-

Table 2. Extraction Recovery (%)[a] and RSD

compound	ethyl ether recovery (%)	ethyl ether RSD (%)	ethyl acetate recovery (%)	ethyl acetate RSD (%)
benzoic acid	32.0	0.621	44.4	6.22
o-hydroxybenzoic acid	79.5	5.28	69.8	4.69
cinnamic acid	61.5	1.68	72.2	0.85
m-hydroxybenzoic acid	70.6	6.81	82.4	6.11
p-hydroxybenzoic acid	76.1	4.92	88.8	0.901
hydroxyphenylacetic acid	47.1	5.30	70.1	7.89
phthalic acid	33.0	1.36	48.2	1.93
2,3-dihydroxybenzoic acid	78.2	7.16	94.6	2.31
vanillic acid	65.3	2.39	92.7	3.83
o-hydroxycinnamic acid	71.5	1.28	59.1	3.12
2,4-dihydroxybenzoic acid	63.2	2.32	82.0	1.12
coumaric acid	29.8	3.17	70.3	2.06
ferulic acid	8.56	0.760	49.7	4.03
caffeic acid	28.5	1.94	48.4	7.82
sinapic acid	44.8	4.91	78.0	5.76

[a] Average of three determinations.

Table 3. Free and Total Content (μg/g) of Benzoic and Phenolic Acids in Fresh Cranberry Fruit

compound	concentration of phenolic acids in free form	concentration of total phenolic acids (free + bound) form	percentage of free phenolic acids
1	481	4741	10.1
2	ND[a]	23.2	NA[b]
3	1.64	20.5	8.00
4	4.18	9.14	45.6
5	4.22	21.6	19.4
6	ND[a]	7.36	NA[b]
7	5.67	15.7	36.1
8	ND[a]	3.16	NA[b]
9	6.98	19.2	36.3
10	ND[a]	89.0	NA[b]
11	8.07	42.5	19.0
12	21.6	253.8	8.51
13	8.17	87.9	9.29
14	3.84	156.4	2.46
15	ND[a]	211.8	NA[b]

[a] Not detected. [b] Not available.

graphic analysis is usually necessary for reliable identification and quantification. In this study, free phenolic acids in cranberry juice were extracted and separated from the bound phenolic compounds with ethyl ether. **Figure 2B** illustrates the separation of the free phenolic acids extracted from fresh cranberry juice. Ten free phenolic acids (benzoic, cinnamic, *m*-hydroxybenzoic, *p*-hydroxybenzoic, phthalic, vanillic, 2,4-dihydroxybenzoic, *p*-coumaric, ferulic, and caffeic acids) were indentified in the ethereal extract of cranberry fruit.

Our previous study has shown that phenolic acids are present in cranberry fruit mainly in combined forms such as glycosides and esters (*27*). To analyze the total phenolic acids, cranberry juice was hydrolyzed with hydrochloric acid under argon using a modified Hertog method (*27*, *36*). Ascorbic acid was added to prevent the oxidation of the phenolic acids. After hydrolysis, the released phenolic acids were extracted and analyzed by GC−MS. The corresponding chromatogram is presented in **Figure 2C**. Besides the 10 compounds found in the unhydrolyzed extract, five other phenolic acids, *o*-hydroxybenzoic, *p*-hydroxyphenyl acetic, 2,3-dihydroxybenzoic, *o*-hydroxycinnamic, and sinapic acid, were identified.

Recovery and Reproducibility Tests. To evaluate the extraction method for phenolic acids, the recovery tests for both free and bound phenolic acids with ether and ethyl acetate, respectively, were performed. Different known amounts of standard compounds were subjected to the entire analytical procedures with the samples in triplicate. The results are given in **Table 2**. The method was reasonably consistent, although poor percentage recoveries were obtained for certain compounds, such as ferulic and caffeic acids. The results also showed that the organic solvents used have different recovery efficiencies for individual phenolic acids due to the variation in their polarities. Ethyl acetate can extract a relatively large amount of phenolic acids. Ethyl ether is a relatively specific solvent for free phenolic acid extraction (*37*). Thus, higher percentage recoveries were achieved for the hydrolyzed samples with ethyl acetate. For example, the ethyl acetate recovered more than 94% of vanillic acid compared to 78% for the ether extraction method. Reproducibility between triplicate extractions was high for both extraction methods with a relative standard deviation of less than 8%.

Quantitative Analysis of Free and Bound Phenolic Acids. The quantitative evaluations of the phenolic components were performed on the basis of the total ion current (TIC) data relative to internal standards. All calibration graphs were linear over the concentration ranges tested with correlation coefficients $r^2 \geq 0.997$, except for sinapic acid with an r^2 value of 0.991.

Results from the quantification of both free and bound benzoic and phenolic acids in cranberry fruit are presented in **Table 3**. To the best of our knowledge, this is the first study to show the comprehensive composition of benzoic and phenolic acids in cranberry fruit in both free and bound (total minus free) forms. Only benzoic, *p*-coumaric, caffeic, ferulic, and sinapic acids have been identified previously in cranberry fruit by liquid chromatography (*38*). Recently, Zheng and Shetty (*39*) have identified bound gallic, chlorogenic, *p*-hydroxybenzoic, and *p*-coumaric acids in cranberry pomace. Our research group (*27*) has recently separated and quantitated benzoic acid and several flavonoids in cranberry juices using an HPLC technique. All other phenolic acids documented here have not been previously reported in cranberry fruit. As expected, only minor amounts of aromatic acids occur in the free state in cranberry fruit. Most benzoic and phenolic acids are present in conjugated forms (over 54% to 100%), which can be liberated by hydrolysis. Benzoic acid was found to be the major aromatic acid present in cranberry fruit at a total concentration of 4.7 g/kg (fresh weight). The results presented here indicate that the content of both free and bound benzoic acid in cranberry is higher than those previously reported (*27*). This may be due to differences in fruit source, ripeness, and length of storage time, as well as differences in the procedures used for obtaining samples. The most abundant cranberry phenolic acids (fresh weight) are *p*-coumaric (0.25 g/kg), sinapic (0.21 g/kg), caffeic (0.16 g/kg), and ferulic acids (0.088 g/kg). *o*-Hydroxycinnamic, 2,4-dihydroxybenzoic, *p*-hydroxybenzoic, *o*-hydroxybenzoic, cinnamic, vanillic, and phthalic acids were present in moderate amounts in cranberry fruit. Minor amounts of other phenolic acids have also been determined as shown in **Table 3**. The total content of benzoic and phenolic acids is over 5.7 g/kg in fresh cranberry fruit, which is much higher than those reported in other common fruits (*11*). Thus, the consumption of cranberry fruit products can represent a significant amount of the total dietary intake of phenolics in many regions in North America. All these benzoic and phenolic acids may serve as good antioxidant and/or antibacterial agents. Most of them have shown interesting activities as inhibitors of mutagenic and carcinogenic processes in vitro. However, the phenolic acids are present predominantly as combined forms

in cranberry and other fruits, and little is known about the fate of most plant phenolic acids after ingestion. Further studies are needed to determine the effect of phenolic acids in cranberry and other plant-derived foods in reducing the risk of cancer and other degenerative diseases. The method described in this report may also aid in the identification and quantification of phenolic acids in human fluids and in other plant-derived foods.

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