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Phenolic compound profile of selected vegetables frequently consumed by African Americans in the southeast United States

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Abstract

The phenolic composition of vegetables commonly consumed by African Americans in the southeast United States was analyzed with HPLC–MS. The vegetable samples included collard greens, mustard greens, kale, okra, sweet potato greens, green onion, butter beans, butter peas, purple hull peas, rutabagas, eggplant, and purslane. Five compounds out of total 29 peaks detected from the 12 samples – caffeic acid, ferulic acid, quercetin, kaempferol, and isorhamnetin – were identified. No gallic acid, *p*-coumaric acid, myricetin, luteolin, apigenin, hesperetin, naringenin, or flavanols was detected. The major flavonoids were isorhamnetin, quercetin and kaempferol. Isorhamnetin was found in kale, mustard greens, and purslane. The content ranged from 2.8 to 23.6 mg/100 g fresh edible part. Quercetin was found in collard greens, mustard greens, kale, okra, sweet potato greens, purple hull peas, and purslane. The content ranged from 1.3 to 31.8 mg/100 g with the highest content in kale and lowest content in purslane. Kaempferol was found in collard greens, mustard greens, kale, sweet potato greens, green onion, and purslane. The content ranged from 1.1 to 90.5 mg/100 g. Caffeic acid was only found in sweet potato greens. Ferulic acid was found in collard greens, mustard greens, kale, okra, purple hull peas, and purslane. Although some peaks were found in eggplant, butter beans, butter peas and rutabagas, these peaks were not identified due to lack of reference compound and no flavonoid or phenolic acid was quantified in these samples. The results suggest that these indigenous vegetables among African Americans are good sources of the phenolic compounds, which can be useful for the prevention of cardiovascular and other chronic diseases.
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Keywords: Phenolic compound; Vegetables; HPLC–MS; African Americans

1. Introduction

Phenolic compounds are widely distributed in fruits and vegetables. Epidemiological studies have indicated that regular consumption of foods rich in phenolic compounds (fruits, vegetables, whole grain cereals, red wine, tea) is associated with reduced risk of cardiovascular diseases, neuro-degenerative diseases, and certain cancers (Hung et al., 2004; Halliwell, 1994). Phenolic compounds are thought to deliver health benefits by several mechanisms, including: (1) free radical scavenging; (2) protection and regeneration of other dietary antioxidants (i.e. vitamin E); and (3) chelating of pro-oxidant metal ions. The species and levels of phenolic compounds vary dramatically among vegetables. Phenolic compounds with different structures or levels are likely to have different functional properties. Therefore, it is important to analyze the composition of phenolic compounds in vegetables before their health promoting properties can be adequately studied.

Analysis of phenolic compounds in plant samples is difficult because of the great variety of their structure and the lack of appropriate standards. Phenolic compounds exist mainly as esters or glycosylated forms in plant (Rommel & Wrolstad, 1993). The sugar moiety and its binding position have many variations. It has become an accepted practice to hydrolyze the glycosides into aglycones before further analysis. The hydrolysis treatment simplifies the peak identification process (Mattila, Astola, & Kumpulainen, 2000).

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In the past decades, extensive analytical methods have been developed to separate and determine phenolic compounds in various plant samples. The techniques include thin-layer chromatography (Kader, Rovel, Girardin, & Metche, 1996), gas chromatography (GC) (Zuo, Wang, & Zhan, 2002; Fiamegos, Nanos, Vervoort, & Stalikas, 2004; Saitta, Curto, Salvo, Bella, & Dugo, 2002), high-performance liquid chromatography (HPLC) (Seeram, Lee, Scheuller, & Heber, 2005; Maatta, Kamal-Eldin, & Torronen, 2003; Mattila & Kumpulainen, 2002; Soong & Barlow, 2005), and capillary electrophoresis (Deng et al., 1998; Kronholma et al., 2004). Capillary gas chromatography equipped with mass spectrometry (GC–MS) is able to provide sufficient separating capacity and good peak identification power, but the analyte requires derivatization prior to analysis. The derivatization process often causes the loss of analyte, creates artifacts, and normally is not applicable to the conjugated form of phenolic compounds. For the purpose of separation and quantification of individual phenolic compounds, HPLC is most frequently used because of its high-separation capacity and relative simplicity. It does not require sample derivatization prior to analysis.

Collard greens, mustard greens, kale, okra, sweet potato greens, green onion, butter beans, butter peas, purple hull peas, rutabagas, eggplant, and purslane are commonly consumed and considered as traditional foods by African Americans. During a recent Tuskegee University Nutrition Outreach Program (TUNOP), daily food diaries showed that while African Americans do not consume the recommended five servings of fruits and vegetables daily, they do consistently eat green leafy vegetables, peas, and beans that were evaluated in this study. However, chemical characterization and health benefits of these traditional vegetables have not been adequately studied. Published data on the phenolic compounds in these vegetables are limited (Hertog, Hollman, & Katan, 1992; Chu, Chang, & Hsu, 2000; Justesen, Knuthsen, & Leth, 1998; Lugasi & Hovari, 2000). Comprehensive food composition data for flavonoids and phenolic acids in these African Americans commonly consumed vegetables are also lacking from the USDA National Nutrient Database (USDA, 2003). Characterization of the bioactive phenolic composition of these traditional African American foods is necessary for the evaluation of these vegetables' consumption on human health, especially in the African-American population. This characterization can be translated into culturally appropriate health messages for promoting increased consumption of these vegetables, with the goal of improving nutritional status and reducing the risk of diet-related diseases among African Americans. One example of the diet-related diseases is cardiovascular diseases, which are found more prevalent in African Americans than in other ethnic groups in the United States. In 2002, the prevalence was 41.1% for African-American males and 44.7% for African-American females, whereas the prevalence of cardiovascular diseases in the general population was 34.2% (American Heart Association, 2006). We expect that increasing the consumption of vegetables rich in phenolic compounds is able to reduce the risk of cardiovascular diseases among African Americans.

The objective of this study was to characterize the phenolic composition of vegetables commonly consumed by African Americans. An acid-catalyzed hydrolysis process and methanolic extraction were employed to liberate and extract phenolic compounds from samples. The phenolic compounds were then separated, identified or quantified using HPLC–MS.

2. Materials and methods

2.1. Reagents and standards

The standard compounds, including gallic acid, *p*-coumaric acid, caffeic acid, ferulic acid, isoferulic acid, flavonols (quercetin, kaempferol, myricetin, isorhamnetin), flavones (luteolin, apigenin), flavanones (hesperetin, naringenin), and flavanols (catechin, epicatechin, epicatechin gallate), were purchased from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid, HPLC grade methanol, acetonitrile, water, and acetic acid were from Fisher Chemicals (Fair Lawn, NJ). Isoferulic acid was used as internal standard (Nardini et al., 2002).

2.2. Sample collection

Twelve vegetables commonly consumed by African Americans (collard greens, mustard greens, kale, okra, green onion, butter beans, butter peas, purple hull peas, rutabagas, eggplant, sweet potato greens, and purslane) were selected. Each vegetable (500 g) was collected in its fresh state at three locations: local grocery stores, local farms, or the local farmers market in Tuskegee, AL. At the time when the samples were collected, we were only able to obtain one variety for most of the samples. So no variety differences were studied. After collection, the vegetables were stored in ice and brought to the laboratory within 2 h. The vegetables were washed and blotted dry, and non-edible parts were removed. For beans, the pods were removed and only the seeds were analyzed. Eggplant and rutabagas were chopped. All samples were immediately stored at −20 °C until they were freeze-dried within 2 weeks.

2.3. Sample preparation

Samples were freeze-dried for 48 h with a Virtis Genesis 25SL freeze-dryer (Virtis Company, Gardiner, NY), ground to pass a 60 mesh sieve, and stored in tight-capped bottle in a −80 °C deep freezer until analyzed. One gram sample was weighed into a 60 mL test tube with screw cap. An aliquot of 40 mL 80% methanol, 10 mL 6 M HCl and 80 mg ascorbic acid were added. The tube was flushed with nitrogen for 30 s, sealed tightly, and incubated in a water bath at 90 °C for 2 h. After acid hydrolysis, the tube

was allowed to cool, brought up to 50 mL with methanol, sonicated for 10 min, and centrifuged at 4000 g for 10 min. Five mL of supernatant was dried with a rotary evaporator. The residual was re-dissolved in 5 mL methanol. Approximately 3 mL was filtered through a Millex®-GN 0.2 μm syringe filter (Millipore, Bedford, MA). The filtrate was mixed with a constant level of internal standard (isoferulic acid, 50 μg/mL) prior to HPLC analysis.

2.4. Recovery test of extraction procedure

The standard mixture containing known amount of each of the 15 standard compounds was added to a tube and subjected to the same acid hydrolysis treatment as the samples. After hydrolysis treatment, the standard solution was mixed with 50 μg/mL of internal standard, then analyzed on HPLC–MS. Recovery rate was calculated as the percent ratio between the amount found after treatment and the amount added as standard.

2.5. HPLC procedure

The HPLC–MS system was composed of Shimadzu LC-20AD HPLC system, Shimadzu SPD-20AV UV–vis detector, and Shimadzu 2010EV mass spectrometer fitted with an electro-spray interface (Shimadzu, Kyoto, Japan). The column was an Alltech Prevail C-18 (Alltech, Deerfield, IL), 150 mm × 4.6 mm. The mobile phase was 0.05% (v/v) aqueous acetic acid (phase A) and 0.05% acetic acid in 80% acetonitrile + 20% methanol (phase B) at a flow rate of 0.8 mL/min. The linear gradient of phase B was 5% for the first 2 min, increased from 5% to 40% from 2 min to 48 min, maintained at 40% from 48 min to 57 min, and decreased from 40% to 5% from 57 min to 57.1 min. Finally, isocratic elution with 5% phase B was maintained until 65 min. UV–vis detector wavelength was set at 280 nm. Mass spectra were acquired in negative ion mode. Ion was scanned from *m*/*z* 150–600 with scan speed 1000 amu/s. Nebulizing gas flow was 1.5 L/min. Drying gas pressure was 0.1 MPa.

2.6. Calculations and statistical analysis

Peak identification was performed by comparison of the retention time and mass spectrum with respective reference compounds. Tentative identification of peaks for which standard compounds were not available was obtained by comparing their elution order and molecular ions ($M–H^-$) with the data from the literature. The quantification was calculated using the internal and external standard method. Quantification of individual flavonoids was performed using the peak area of identified compounds relative to the peak area of the internal standard. The recovery rates were not applied in the quantitative calculation. Data analysis was performed using SPSS (SPSS for Windows, Version 10.0.5., 1999, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Effect of acid hydrolysis on the recovery of phenolic compounds

Several hydrolysis conditions have been reported (Rommel & Wrolstad, 1993; Chen, Zuo, & Deng, 2001; Hertog, Hollman, & Venema, 1992). Among these conditions, hydrochloric acid hydrolysis is the most commonly used method. The hydrolysis condition used in this study was based on the studies by Mattila et al. (2000) and Hertog, Hollman and Venema (1992). This condition has been proven to be effective in releasing the aglycone from the conjugated phenolic compounds. The effectiveness was also verified by our study. After hydrolysis, no significant peaks of glycosides (*m*/*z* higher than 400) were found. This indicated that most of the glycosides were hydrolyzed to aglycones. On the other hand, acid hydrolysis at high temperature (90 °C) might cause the degradation of the phenolic compounds. In the study by Hertog, Hollman and Katan (1992), the recovery rate of quercetin, kaempferol, myricetin, apigenin, and luteolin ranged from 77% to 106%, but the recovery rate of flavanols (catechin, epicatechin, and epicatechin gallate) and phenolic acids was not tested in their study. Our results showed that the recovery rate of each compound after acid hydrolysis varied dramatically (Table 1). The recovery rate of flavonols (quercetin, kaempferol, myricetin, isorhamnetin) and flavones (luteolin, apigenin) was from 76.1% to 98.6%. These data were in agreement with the study by Hertog, Hollman and Katan (1992). The recovery rate of these compounds was acceptable for quantification purpose. However, the recovery rate of gallic acid, *p*-coumaric acid, caffeic acid, ferulic acid, and flavanones (hesperetin, naringenin) was quite low (from 9.7% to 33.8%). Due to the low recovery rate, these compounds were not quantified in this study. Only the identification of these compounds was conducted. Flavanols (catechin, epicatechin,

Table 1
Recovery rate of phenolic compound standards after hydrochloric acid hydrolysis

Standard compounds	Recovery rate (%)
Gallic acid	22.1
Catechin	0.0
Caffeic acid	9.7
Epicatechin	0.0
p-Coumaric acid	10.8
Epicatechin gallate	0.0
Ferulic acid	10.3
Myricetin	93.8
Quercetin	83.7
Luteolin	98.6
Naringenin	29.8
Hesperetin	33.8
Apigenin	92.6
Kaempferol	78.3
Isorhamnetin	76.1

epicatechin gallate) were almost completely degraded. Recovery rate of flavanols was 0%. These findings indicated that the hydrolysis condition used in our study was not applicable to the quantitative analysis of phenolic acids, flavanones, and flavanols. Suitable methods for these compounds need to be developed in future work. Nevertheless, previous studies reported that flavanols were mainly found in tea rather than in vegetables (Naczk & Shahidi, 2004; Nishitani & Sagesaka, 2004), so the low recovery rate for flavanols would not be problematic for this study because they were most likely not present in our samples. Fig. 1 showed the chromatograph of standard mixtures before and after acid hydrolysis. The peaks of degradative products can be found in Fig. 1(b). The peaks with *m*/*z* value of 183, 193 (eluted after the internal standard), 177, 449, and 479, which were not found before hydrolysis in Fig. 1(a), can be regarded as degradative products of the standard mixture used in this study. However, the chemistry underlying those degradation reactions needs to be further investigated.

3.2. Identification and quantification of phenolic acids and flavonoids

To analyze individual phenolic compounds, HPLC is often coupled with UV–vis detector or mass spectrometer. However, UV–vis detector cannot differentiate the co-eluting compounds with similar UV–vis spectra. The co-eluting

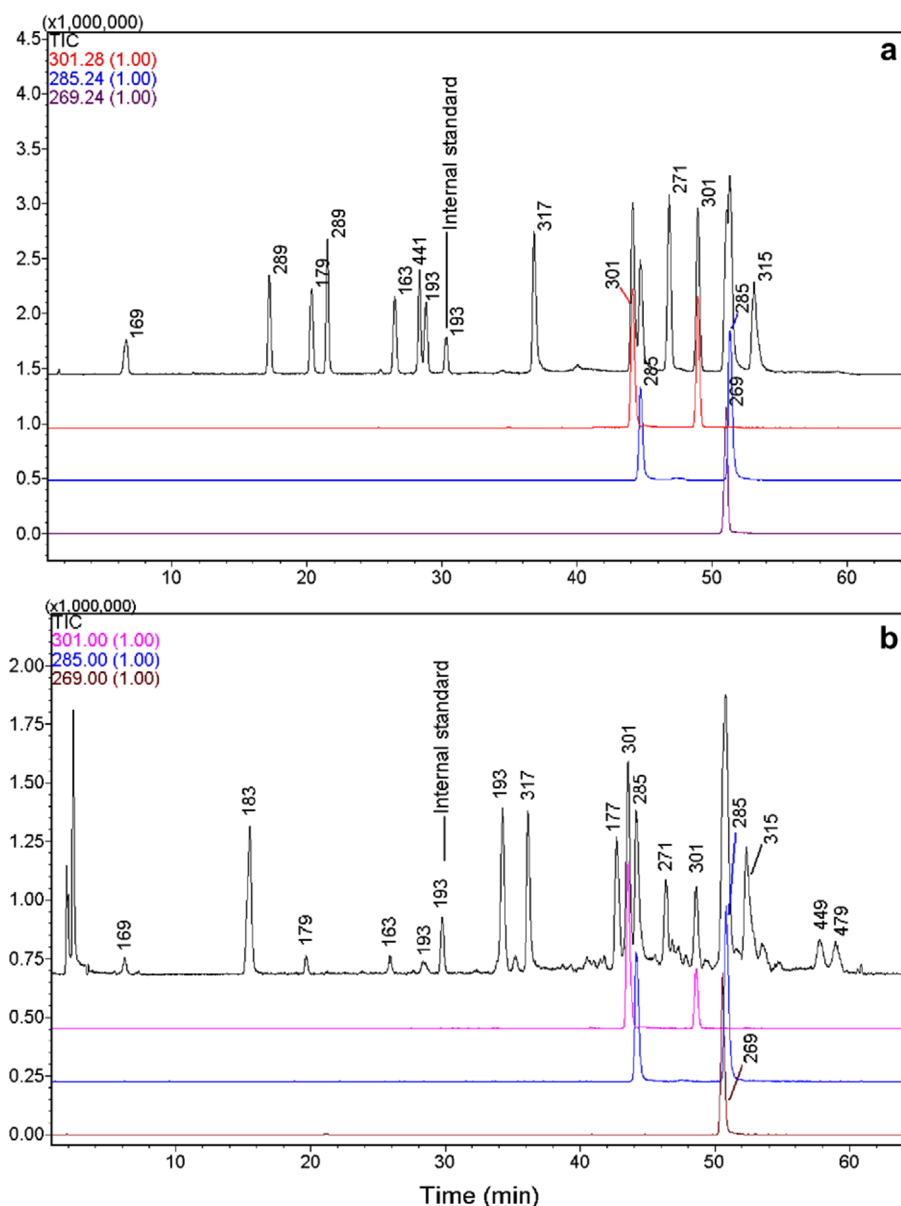

Fig. 1. HPLC–MS chromatograms of standard mixture (a) and standard mixture after acid hydrolysis (b). The peak top number was *m*/*z* value. Identification of peaks (in the order of elution): 169, gallic acid; 289, catechin; 179, caffeic acid; 289, epicatechin; 163, *p*-coumaric acid; 441, epicatechin gallate; 193, ferulic acid; 193, isoferulic acid (internal standard); 317, myricetin; 301, quercetin; 285, luteolin; 271, naringenin; 301, hesperetin; 269, apigenin; 285, kaempferol; 315, isorhamnetin.

phenomena will lead to overestimation of certain components and missing of other components. HPLC coupled with mass spectrometry provides good peak identification power. The mass spectrum combined with the match of retention time with reference compounds can provide information to identify the peaks unambiguously. The overestimation of concentration caused by co-elution can be eliminated by the selected ion monitor mode. The HPLC–MS chromatograph of the standard mixture is shown in Fig. 1(a). The peaks were sharp, symmetrical and generally separated from each other. Baseline separation was not achieved between apigenin and kaempferol. Quercetin was also partially co-eluted with luteolin. Our preliminary studies indicated that changing the composition of mobile phase B (e.g., increasing the concentration of acetonitrile) led to the baseline separation of apigenin and kaempferol, but simultaneously the separation between quercetin and luteolin became worse. This co-elution of peaks would not be a problem if selected ions were monitored. As shown in Fig. 1(a), the co-eluted components could be differentiated by monitoring the corresponding m/z value. Furthermore, our results indicated that the above co-eluting compounds did not coexist in our samples. Hence, the mobile phase B composition was optimized as 80% acetonitrile and 20% methanol.

Among all the 12 samples, more than 29 peaks were found. Due to the lack of availability of standard compounds, only five of them (caffeic acid, ferulic acid, quercetin, kaempferol, isorhamnetin) were identified. No gallic acid, *p*-coumaric acid, myricetin, luteolin, apigenin, hesperetin, naringenin, or flavanols was detected. The results are reported in Tables 2, 3, and Fig. 2. The major flavonoids found in the samples were isorhamnetin, quercetin and kaempferol. Isorhamnetin was found in kale, mustard greens, and purslane. The content ranged from 2.8 to 23.6 mg/100 g. Quercetin was found in collard greens, mustard greens, kale, okra, sweet potato greens, purple hull peas, and purslane. The content ranged from 1.3 to 31.8 mg/100 g, with the highest content in kale and the lowest content in purslane. Kaempferol was found in collard greens, mustard greens, kale, sweet potato greens, green onion, and purslane. The content ranged from 1.1 to 90.5 mg/100 g. Due to their low recovery rate, the identified phenolic acids, caffeic acid and ferulic acid, were not quantified in our samples. Although some peaks were

Table 2
Identification of phenolic compounds in vegetables by their HPLC–MS data

Peak #	t_R (min)[a]	m/z	Peak identification
1	9.7	219	Unknown
2	11.9	219	Unknown
3	17.2	183	Unknown
4	17.8	249	Unknown
4a	19.3	189	Unknown
5	19.9	367	3-*O*-Feruloylquinic acid (tentative)[b]
6	21.1	399	Unknown
7	21.4	179	Caffeic acid[c]
8	23.3	265	Unknown
9	23.8	367	4-*O*-Feruloylquinic acid (tentative)[b]
9a	25.8	223	Unknown
10	26.3	349	Unknown
11	26.8	367	5-*O*-Feruloylquinic acid (tentative)[b]
12	27.6	263	Unknown
13	29.4	193	Ferulic acid[c]
14	31.5	193	Isoferulic acid (internal standard)
15	33.4	279	Unknown
16	35.5	193	*trans*-Ferulic acid (tentative)[b]
17	35.6	231	Unknown
18	37.4	387	Unknown
19	38.1	447	Unknown
20	40.2	264	Unknown
20a	40.6	354	Unknown
21	43.2	475	Unknown
22	44.3	177	Unknown
23	45.6	301	Quercetin[c]
24	50.9	367	Unknown
25	53.2	285	Kaempferol[c]
26	55.1	315	Isorhamnetin[c]

[a] Retention time.
[b] Tentative identification was obtained by comparing their elution order and molecular ions ($M–H^-$) with the literature data.
[c] Peak was identified by matching the retention time and m/z value with reference compound.

Table 3
Flavonoids content in selected vegetables[a]

Vegetables	Latin name	Isorhamnetin	Quercetin	Kaempferol
Collard greens	*Brassica oleracea var. viridis*	nd[b]	12.4 ± 3.2	43.3 ± 12.5
Mustard greens	*Brassica juncea*	16.2 ± 8.6	8.8 ± 4.5	38.3 ± 17.6
Kale	*Brassica oleracea var. acephala*	23.6 ± 3.4	31.8 ± 5.7	90.5 ± 12.5
Okra	*Hibiscus esculentus* L.	nd	11.1 ± 2.8	nd
Sweet potato greens	*Ipomea batatas*	nd	27.9 ± 6.8	5.0 ± 0.0
Purple hull peas	*Vigna unguiculata*	nd	5.5 ±0.8	nd
Green onion	*Allium fistulosum*	nd	nd	4.8 ± 0.2
Butter beans	*Phaseolus lunatus*	nd	nd	nd
Butter peas	*Phaseolus coccineus* L.	nd	nd	nd
Rutabagas	*Brassica napus napobrassica*	nd	nd	nd
Eggplant	*Solanum melongena*	nd	nd	nd
Purslane	*Portulaca oleraceae*	2.8 ± 0.4	1.3 ± 0.7	1.1 ± 0.8

[a] The results are expressed as mean ± standard deviation. Data are the means of three determinations. The unit of content is mg/100 g fresh edible part.
[b] Not detected.

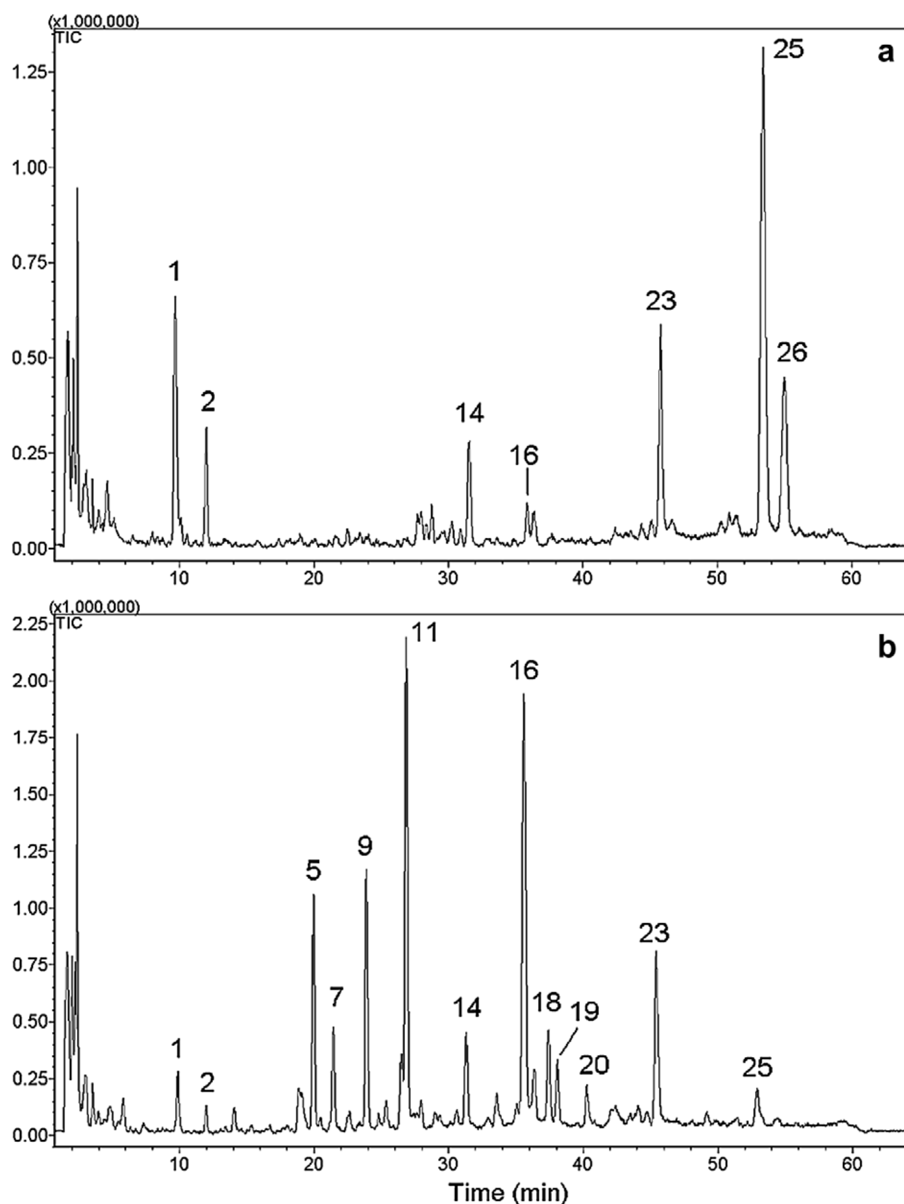

Fig. 2. The representative HPLC–MS chromatograms of vegetable samples: (a) kale; (b) sweet potato greens. Refer to Table 2 for the identification of each numbered peak.

found in eggplant, butter beans, butter peas and rutabagas, these peaks were not identified due to the lack of a reference compound. No flavonoids or phenolic acids were quantified in these samples.

Among our 12 samples, previous studies (Hertog, Hollman, & Katan, 1992; Chu et al., 2000; Justesen et al., 1998; Lugasi & Hovari, 2000) identified the flavonoid content only in kale, sweet potato greens, and rutabagas. The data on the flavonoid composition in the vegetables collected in our study is also generally lacking in the USDA database for the flavonoids content (USDA, 2003). The contents of quercetin and kaempferol in kale (31.8 mg/100 g and 90.5 mg/100 g, respectively) were higher than the values obtained by Hertog, Hollman and Katan (1992) and Justesen et al. (1998). Furthermore, no isorhamnetin was reported in their studies. In contrast, we found isorhamnetin in kale, mustard green, and purslane. Apigenin, luteolin, and myricetin were reported in sweet potato greens and rutabagas in previous studies (Chu et al., 2000; Justesen et al., 1998; Lugasi & Hovari, 2000), but none of these three flavonoids was detected in our samples.

Based on their elution order and *m*/*z* value, some peaks were tentatively identified. Three significant peaks (peak 5, 9, and 11) in Fig. 2(b), which were tentatively identified as 3-*O*-feruloylquinic acid, 4-*O*-feruloylquinic acid, and 5-*O*-feruloylquinic acid respectively, were found in sweet potato greens. Peak 11 was also found in eggplant. The molecular weight of these three compounds is 368 and their chemical structure was shown in Fig. 3. The result indicated that the acid hydrolysis condition can cleave the glycosidic bond in conjugated phenolic compound, but might not be able to cleave the ester bond between quinic acid and ferulic acid.

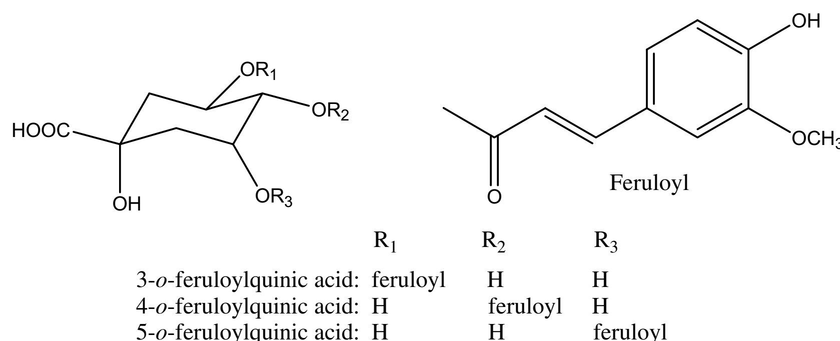

Fig. 3. Chemical structure of feruloylquinic acid tentatively identified in sweet potato greens.

Previous studies also found feruloylquinic acid in loquat and coffee beans (Ding, Chachin, Ueda, Imahori, & Wang, 2001; Iwai, Kishimoto, Kakino, Mochida, & Fujita, 2004; Clifford & Ramirez-Martinez, 1991), but no feruloylquinic acid was reported in sweet potato greens. Islam et al. (2002) identified six phenolic compounds in sweet potato greens of different genotypes. The highest level was 3,5-di-*O*-caffeoylquinic acid, with a content of 1529 mg/100 g dry powder, followed by 4,5-di-*O*-caffeoylquinic acid, with a content of 706 mg/100 g dry powder, and 3,4-di-*O*-caffeoylquinic acid, with a content of 277 mg/100 g dry powder. These three phenolic acids have similar structures, with molecular weights of 516. Nevertheless, these compounds were not found in our study. Although many peaks remained unidentified in our samples, these peaks could provide useful information to comprise a particular chromatographic pattern of each vegetable, the so-called chromatographic fingerprint.

4. Conclusion

Among all the 12 samples, more than 29 components were found. Five compounds – caffeic acid, ferulic acid, quercetin, kaempferol, and isorhamnetin – were identified. The major flavonoids were isorhamnetin, quercetin and kaempferol. Isorhamnetin was found in kale, mustard greens, and purslane. The content ranged from 2.8 to 23.6 mg/100 g. Quercetin was found in collard greens, mustard greens, kale, okra, sweet potato greens, purple hull peas, and purslane. The content ranged from 1.3 to 31.8 mg/100 g with the highest content in kale and lowest content in purslane. Kaempferol was found in collard greens, mustard greens, kale, sweet potato greens, green onion, and purslane. The content ranged from 1.1 to 90.5 mg/100 g. Caffeic acid was only found in sweet potato greens. Ferulic acid was found in collard greens, mustard greens, kale, okra, purple hull peas, and purslane. Although some peaks were found in eggplant, butter beans, butter peas and rutabagas, these peaks were not identified due to the lack of a reference compound. The results suggest that these indigenous vegetables among African Americans are good sources of the phenolic compounds, which can be useful for the prevention of cardiovascular and other chronic diseases.

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