On-line HPLC-DPPH Screening Method for Evaluation of Radical Scavenging Activity of Phenols Extracted from Regional Agricultural Products

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An on-line HPLC-DPPH screening method for phenolic antioxidants in Miyazaki regional agricultural products such as sweet potato leaf, burdock root, carrot leaf, lettuce and lemon balm was applied in order to estimate the contribution ratio of each phenol to total antioxidant activity for those samples. The determination of antioxidants was based on a decrease in the absorbance at 515 nm after postcolumn reaction of HPLC-separated antioxidants with the 2, 2'-diphenyl-1-picrylhydrazyl radical (DPPH.). The on-line method was applied for quantitative analysis of the antioxidants. A linear dependence of negative peak area on concentration of the reference antioxidants was observed. When five kinds of regional agricultural products were applied to the method, several peaks of known phenols were recognized which gave negative peaks corresponding to the DPPH radical scavenging activity. For example, burdock root contained caffeic acid, chlorogenic acid, and dicaffeoylquinic acids and those contributions were 0%, 62% and 38%, respectively. For lettuce, caffeic acid, chlorogenic acid, dicaffeoyltartaric acid and other unknown active components were detected ; those contribution ratios were 0%, 26%, 54%, 8% and 12% respectively. This information should be useful for application of those regional agricultural products to functional materials.

INTRODUCTION

Oxygen and reactive oxygen species (ROS) are among the major sources of primary catalysts that initiate oxidation in vivo and in vitro (1, 2). Oxygen-derived free radicals such as superoxide anion radical and hydroxyl radical are thought to be linked to the onset of various pathological conditions. Many researchers have shown that lipid peroxides and reactive oxygen species are involved in the development of a variety of diseases, including cancer, and also accelerate aging (3-8). A compound might exert antioxidant actions in vivo or in food by inhibiting generation of ROS, or by directly scavenging free radicals (9-12). Antiradical antioxidants act by donating hydrogen atoms to lipid radicals. Radicals obtained from antioxidants with molecular structures such as phenols are stable species and then stop the oxidation chain reaction (4, 13). Furthermore, there is now growing evidence that polyphenols may possess inhibitory effects against cancer (14). Many anticarcinogens are naturally occurring non-nutrients, primarily of plant origin, such as flavones and polyhydroxy compounds (15, 16).

A multitude of natural antioxidants have already been isolated from different kinds of plant materials such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (6, 9, 17). Plants contain a diverse group of phenolic compounds including simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives, and flavonoids. All the phenolic classes have the structural requirements of free radical scavengers and have potential as food antioxidants (18, 19). Factors influencing the antioxidant activity of plant phenolics include position and degree of hydroxylation, polarity, solubility, reducing potential, stability of the phenol to food processing operations, and stability of the phenolic radical (20,

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21). Cuvelier et al. (22) studied the antioxidative efficiency of some phenolic acids, including caffeic acid, ferulic acid, rosmarinic acid, chlorogenic acid, and BHT. Chen et al. (23) investigated the antioxidative potency of some hydroxycinnamic acid compounds and elucidated the relationship between their activities and chemical structures. Koleva et al. (24) published a new rapid online method for screening complex mixtures for radical scavenging components using a methanolic solution of the DPPH stable free radical, and presented an optimized instrumental setup. The greatest benefit of this method is that it is immediately clear which constituent possesses radical scavenging activity.

To the best of our knowledge, there is no information available about the contribution ratio of a phenol compound to the total antioxidant activity present in a sample using an on-line HPLC-DPPH method.

From this purpose, the authors tried to apply the feasible on-line HPLC-DPPH screening method developed by Koleva et al. (24) to some regional agricultural products (such as sweet potato leaf, burdock root, carrot leaf, lettuce and lemon balm) in Miyazaki prefecture and estimate the contribution ratio of the antioxidant activity of a phenol compound which is contained in each sample.

MATERIALS AND METHOD

Materials : The root of burdock (*Arctium lappa* L.), Sweet potato leaf (*Ipomoea batatas* L.), Carrot leaf (*Daucus carota* L.) and Lettuce (*Lactuca sativa* L.) were supplied from Miyazaki Agricultural Experimental Station (Miyazaki, Japan). Lemon Balm (*Melissa Officinalis*) was supplied from Miyazaki herb research center (Miyazaki, Japan). The all samples were washed thoroughly in water, cut into small species and freeze dried (FTS SYSTEM, Dura-Top MP & Dura-Dry MP). The freeze-dried sample was powdered (MRK & RETSCH, EM-1 ; mesh size 0.5 mm). Chlorogenic acid and Caffeic acid were purchased from Sigma Chemicals (St. Louis, MO, USA). Dicaffeoyl Tartaric acid was obtained from ChromaDex Inc. (Santa Ana, CA, USA). Rosmarinic acid was purchased from MP Biomedicals, Inc. (Germany). Gallic acid, formic acid (HPLC grade) and acetic acid were obtained from Nakalai Tesque Inc. (Kyoto, Japan). DPPH (2, 2-diphenyl-1-picrylhydrazyl) and LC grade methanol were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of analytical reagent grade and used without further purification.

Sample preparation : Twenty milliliter (ml) of 80% MeOH was added to freeze-dried sample powder (0.2 g). The slurry was stirred for 10 min at room temperature with a magnetic stirrer for extraction. The solution was filtered with 0.45 μm filter. The filtrate was analyzed by HPLC with proper dilution.

On-line HPLC-DPPH Analysis : An on-line method was described for the detection of radical scavenging components. The on-line HPLC-DPPH method was developed using a methanolic solution of DPPH stable free radical. A scheme of the instrumental setup is given in figure 1. The HPLC separated analytes reacted with the DPPH at a concentration of 10 mg/l in methanol in postcolumn. The flow of the reagent solution was set to 1.0 ml/min and the induced bleaching was detected as a negative peak photometrically at 515 nm. The length of the capillary (15 m) used for the postcolumn reaction was adjusted to achieve a reaction time of 50 seconds. The separation of antioxidative components was carried out by HPLC (JASCO PU-980 intelligent HPLC pump ; JASCO DG-980 3-line degasser ; JASCO CO-1565 intelligent Column Oven) coupled with a photodiode array detector (PDA 195 - 650 nm) (JASCO MD-9109) and an auto-sampler (JASCO AS-950-10), operated by Borwin software. An ODS column (Atlantis dC18 ; 150 × 4.6 mm id ; particle size 3 μm ; waters Corporation, USA) with a guard column containing the same stationary phase was used. The mobile phase consisted of water containing 0.2% (v/v) formic acid (A) and 100% methanol (B). The gradient elution was performed with 2% B from 0-15 min, a linear gradient of 2% to 45% of B from 15 to 50 min, and 45% B from 50 to 65 min. The wavelength of detection was set at 328

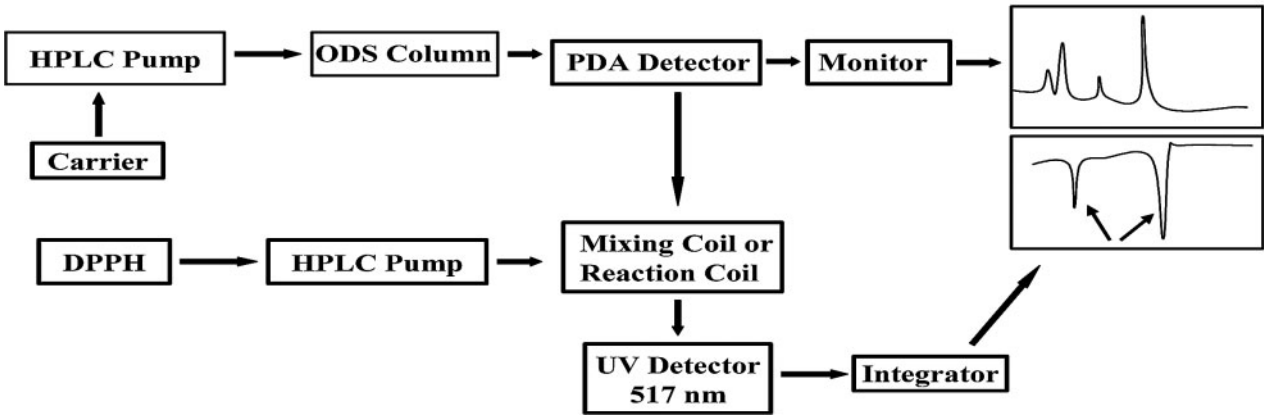

Fig.1. Instrumental setup for the HPLC-DPPH on-line detection of radical scavenging compounds.

nm. The column was temperature controlled at 40 °C. The methanolic extracts were used directly for on-line HPLC analysis. For the stock solution of the standards, the polyphenols（Chlorogenic acid, Caffeic acid, dicaffeoyltartaric acid and rosmarinic acid）were dissolved in 80% methanol at a concentration of 1 mg/ml. The concentrations of the reference substances used for calibration of the HPLC analysis were 0.02, 0.04, 0.06, 0.08 and 0.01 mg/ml.

LC-MS Analysis : Electro-spray mass spectrophotometric analyses were performed in the negative and positive mode using a Finnigan LCQ（Thermo Electron Co.）LC-MS spectrophotometer. All the analyses were performed using electrospray ionization（ESI）with the following settings : Ion-spray voltage : 5 KV ; Capillary voltage : 10 V ; Capillary temperature : 270 °C. The chromatographic conditions were as same as those used for the HPLC analysis. Only flow rate and gradient elution was changed as follows : flow rate 0.5 ml/min and gradient elution : 2% B（0-15 min）, 2-45% B（15-55 min）, 45% B（55-75 min）.
Full-scan data acquisition was performed, scanning from m/z 150 to 1000.

RESULTS AND DISCUSSION

A novel screening method for the antioxidants is achieved by on-line HPLC using the DPPH free radical. It is well known that DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species the absorption disappears（9）. The more rapidly the absorbance decreases, the more potent the antioxidant activity of the compound in terms of hydrogen donating ability（25）. The HPLC separated antioxidants react with the DPPH in postcolumn. The induced bleaching is detected as a decrease in absorbance at 517 nm.

For validation of the on-line HPLC method, the calibration curves of negative peaks at concentration of 10, 20. 40, 60, 80 and 100 ppm of reference phenols（caffeic acid, chlorogenic acid）were obtained. A linear relationship between the negative peak areas and injected sample concentrations was observed for tested phenols（$Y=4.436x-1.08$）. The correlation factors obtained for the relations of all tested radical scavengers were very high（0.998-0.999）. Here reported results are in good agreement with those previously published（26）.

As an example of application to real samples, the determination of antioxidants in extracts from sweet potato leaf, burdock root, carrot leaf, lettuce and lemon balm were carried out with the present on-line HPLC method.

First of all, the radical scavenging activity, total polyphenol and chlorogenic acid contents of burdock root, sweet potato leaf, carrot leaf, and lettuce were measured. These vegetables are respective representatives

of root and leafy vegetables consumed frequently in Japan. Among them, sweet potato leaf had highest radical scavenging activity (290 μmol Trolox eq/g dry) and chlorogenic acid content (9.95 mg/g dry); its radical scavenging activity was 2.5-, 2.7- and 6.5-fold higher than burdock root, carrot leaf and lettuce, respectively. The highest polyphenol was found in sweet potato leaf (64 mg gallic acid eq/g dry). Sweet potato (Suio) leaves have contained highest polyphenol compare to other leafy vegetables.

The antioxidant active component of sweet potato leaf (Fig.2) was determined. Sweet potato leaf contained caffeic acid (trace amount) chlorogenic acid and quinic acid derivatives. Peak 2 was identified as chlorogenic acid with the comparison of authentic marker. In LC-MS spectra peak 2 gave (positive ion peak) m/z at 354 and characteristic fragmentation at m/z 163. The LC-MS chromatogram in the (+)-ve ion mode of both (peak 3 and peak 4) showing m/z 516 and m/z 180 which were dicaffeoylquinic acid and its fragmentation caffeic acid. Yoshimoto et al.(27) reported also dicaffeoylquinic acid, which is present in sweet potato leaf. In sweet potato leaf calculated contribution of chlorogenic acid to the antioxidant activity is 65%. Sweet potato leaf contained 9.95 mg/g (powder) of chlorogenic acid. Therefore the high activity in sweet potato leaf is derived from its high chlorogenic acid content.

The HPLC chromatographic pattern of burdock root is shown in Fig. 3. Among these peaks only 4 peaks (peak 2, peak 3, peak 4 & peak 5) were reacted postcolumn with the DPPH. Peak 1 and peak 2 (Fig. 2.) were identified as caffeic acid and chlorogenic acid by the comparison of authentic marker. The LC-MS chromatogram of peak 2 showed the characteristic fragments at m/z 353 (chlorogenic acid) and 191

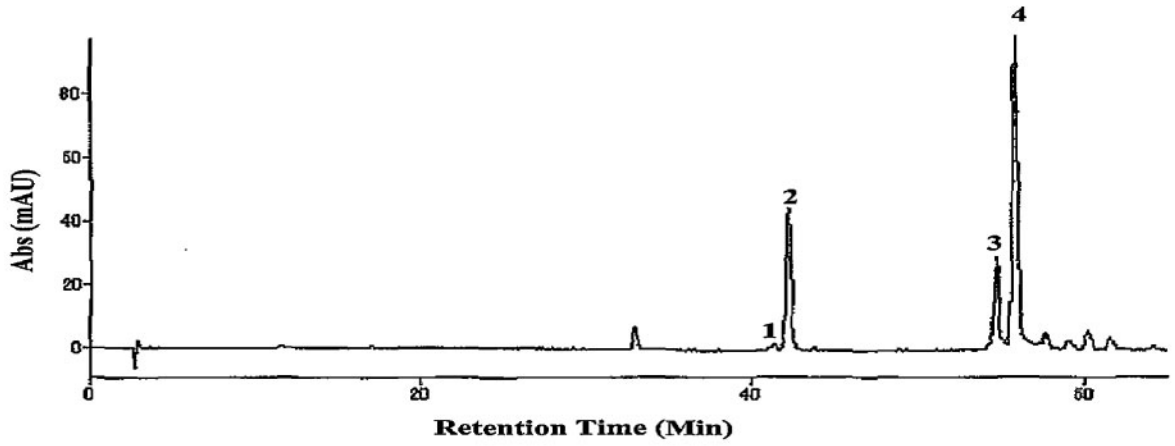

Fig. 2. HPLC Chromatogram of sweet potato leaf (80% methanol extract)

(quinic acid). Peak 3, peak 4 and peak 5 were unidentified quinic acid derivatives with m/z 515, 515 and 630, respectively. Wang et al. (28) reported that burdock root contains chlorogenic acid, 1,5-dicaffeoylquinic acid and 1,5-dicaffeoyl-3-succinylquinic acid, with the predominant polyphenols among 13 kinds of burdock root being chlorogenic acid. We also found the predominant polyphenol in burdock was chlorogenic acid. The content of chlorogenic acid in burdock root was determined. Burdock root contained 3.98 mg/g (powder) chlorogenic acid. The calculated contribution of chlorogenic acid to the antioxidant activity is 62% in burdock root. Therefore, chlorogenic acid is the major antioxidant active component of burdock root. Burdock root have contained very small amount of caffeic acid.

The antioxidant active components of carrot leaf and lettuce were also determined. Both samples contained chlorogenic acid, which was characterized by the comparison of authentic marker and LC-MS. In LC-MS (carrot leaf & lettuce) showed m/z at 353 (chlorogenic acid) and its fragmentation 191 (quinic acid).

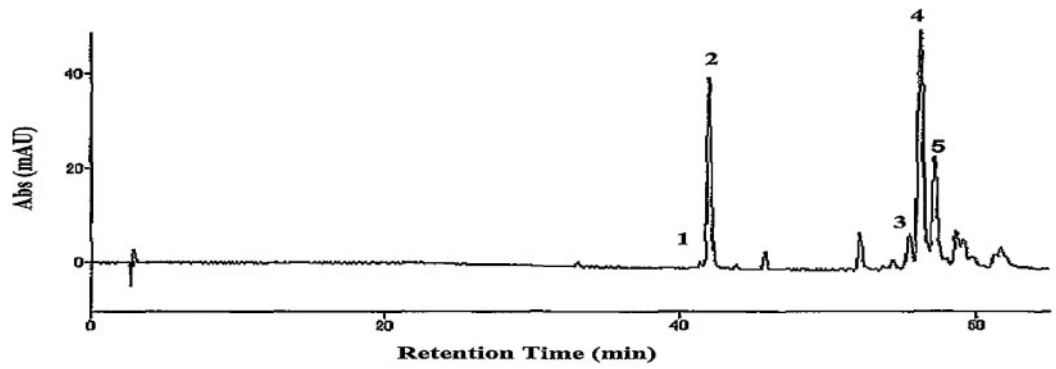

Fig. 3. HPLC Chromatogram of Burdock root（80% methanol extract）.

Carrot leaf contained unidentified two antioxidant components. We also characterized dicaffeoyltartaric acid from lettuce by the comparison of authentic marker. Hisaminato et al.（29）also reported that lettuce contains dicaffeoyltartaric acid. This is the major antioxidant compound in lettuce. This compound showed parent peak（m/z）at 473 by LC-MS. The contribution ratio of chlorogenic acid and other two unidentified antioxidant components in carrot leaf is 58% 32% and 10%, respectively. In lettuce the contribution ratio of chlorogenic acid and dicaffeoyltartaric acid is 26%, 54%, 8% and 12%, respectively.

Figure 4 shows a typical HPLC profile of the lemon balm. The antioxidant active component（only one）of lemon balm was characterized by the comparison of authentic marker and LC-MS. Rosmarinic acid is an important phenolic active compound of lemon balm. In LC-MS base peak was found at（m/z）359（Rosmarinic acid）and its characteristic fragmentation（m/z）at 179（Caffeic acid）. In lemon balm contribution of rosmarinic acid to the antioxidant activity is 98%.

Conclusions :

The proposed screening method using on-line HPLC-DPPH seems to be useful for the detection of antioxidants because of its high sensitivity and ease of handling. The method is advantageous for the determination of individual antioxidants in complex mixtures with simple operation. The method was applied for

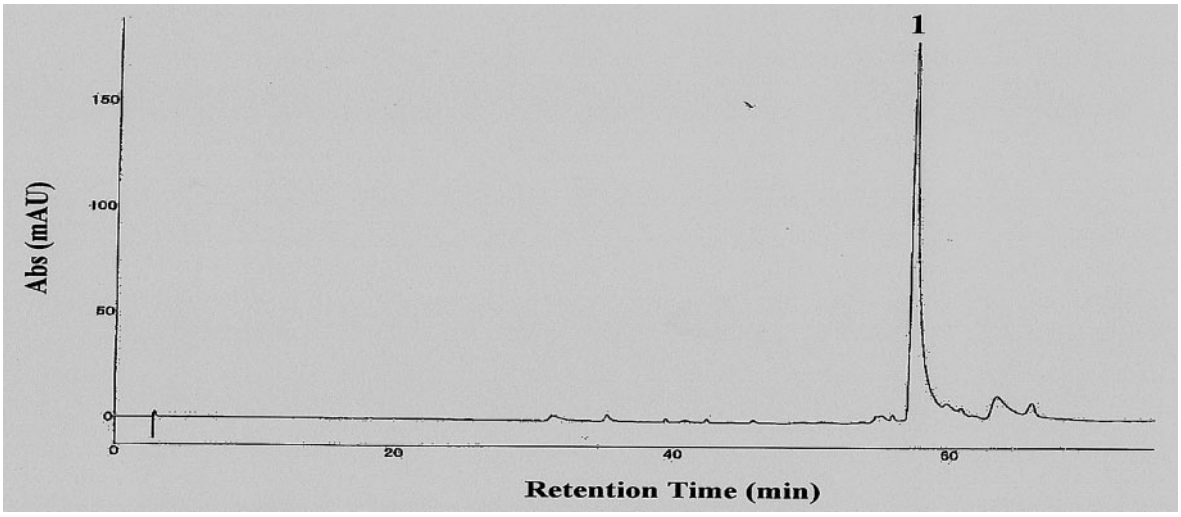

Fig. 4. HPLC Chromatogram of Lemon Balm（80% methanol extract）.

quantitative analysis of the antioxidants. Active phenolic components, caffeic acid, chlorogenic acid, dicaffeoylquinic, dicaffeoyltartaric acid and Rosmarinic acid were identified and characterized from above agricultural products cultivated in Miyazaki Prefecture. Burdock root, sweet potato leaf and carrot leaf possesses significant free radical scavenging activity both DPPH and SOSA method, which was attributed to chlorogenic acid and other antioxidant active components.

The greatest benefit of the method is that, besides the quantification by UV detection, the radical scavenging activity of a single substance can be measured and its contribution to the overall activity of a mixture of antioxidants can be calculated.

The identification of other antioxidant components being conducted in our laboratory.

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