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Analysis of Six Caffeic Acid Derivatives in Sweetpotato Leaves by High-Performance Liquid Chromatography using a Short Column

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Abstract

In 2002, we reported that sweetpotato leaves contained six caffeic acid derivatives, namely, caffeic (CA), chlorogenic (5-*O*-caffeoylquinic acid, ChA), 3,4-di-*O*-caffeoylquinic (3,4-diCQA), 3,5-di-*O*-caffeoylquinic (3,5-diCQA), 4,5-di-*O*-caffeoylquinic (4,5-diCQA), and 3,4,5-tri-*O*-caffeoylquinic (3,4,5-triCQA) acids, which are known to have many physiological functions. A new sweetpotato cultivar 'Suioh' was developed for use of its tops as an edible green by KONARC. It is important to analyze the caffeic acid derivatives efficiently with high-performance liquid chromatography (HPLC) in order to continue developing or selecting new cultivars with higher contents of these compounds in their tops. For this purpose, a short column (4.6 i.d. × 75 mm) packed with small ODS particles (3 μm) was used without replacing the conventional HPLC apparatus and the time for one analysis per sample was decreased to 26 min, from 90 min, which was the analysis time reported in 2002. With the new HPLC conditions, we could quantify the six caffeic acid derivatives in lyophilized leaf samples from 529 sweetpotato cultivars, which compose approximately one-third of the cultivars maintained at KONARC.

Discipline: Food
Additional key words: caffeoylquinic acid, polyphenol

Introduction

Sweetpotato is a very important upland crop in southern Japan. Its roots are consumed and also used for the production of starch, liquor and pigments. However, its tops (leaves, petioles and stems) are not a popular food in Japan. During our search for useful compounds or physiological functionalities in sweetpotato tops, we identified six polyphenols (Fig. 1), namely, caffeic acid (CA), chlorogenic acid (5-*O*-caffeoylquinic acid, ChA), 3,4-di-*O*-caffeoylquinic acid (3,4-diCQA), 3,5-di-*O*-caffeoylquinic acid (3,5-diCQA), 4,5-di-*O*-caffeoylquinic acid (4,5-diCQA), and 3,4,5-tri-*O*-caffeoylquinic acid (3,4,5-triCQA), in the leaves of certain sweetpotato genotypes[5]. Furthermore, we confirmed the antimutagenicity of ChA and the three diCQAs[21]. The antioxidative activities of CA, ChA and diCQAs have been reported by many groups[1,2,19]. These caffeic acid derivatives were also shown to have activities such as protective effects against oxidative stress *in vivo*[11,22] and inhibition of carcinogenesis *in vivo*[9,18]. Mahmood et al. reported that 3,4,5-triCQA

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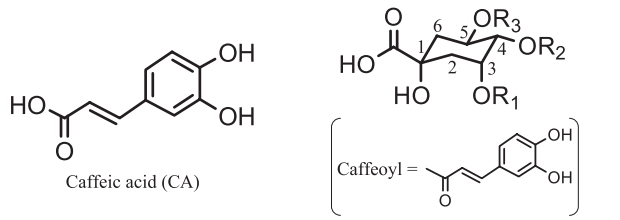
and 3,4-diCQA inhibited human immunodeficiency virus type 1 (HIV-1) replication[7], and Robinson et al. also reported that HIV-1 replication was inhibited by 3,4-, 3,5-, 4,5-, and 1,5-dicafeoylquinic acids[14].

The caffeic acid derivative 3,4,5-triCQA was first isolated from an Asteraceae plant[20]. Compared to monocaffeoyl- and dicaffeoylquinic acids, 3,4,5-triCQA has been found in fewer plants[7,13,15]. However, it was reported that 3,4,5-triCQA inhibited HIV-1 replication to a greater extent than 3,4-diCQA, which exhibited a stronger inhibition than CA[7]. Further, the four dicaffeoylquinic acids, namely, 3,4-, 3,5-, 4,5-, and 1,5-diCQAs, inhibited the replication to a greater extent than ChA and CA[14]. In addition, we found that the order of the antimutagenicity of caffeoylquinic acids was as follows: 3,4,5-triCQA > diCQAs > ChA[21]. To the best of our knowledge, sweetpotato roots are known to contain 3,4-, 3,5- and 4,5-diCQAs[16], while 3,4,5-triCQA is present in its tops but not in the roots. Sweetpotato tops can be harvested six times a year[3,12]. Therefore, sweetpotato tops are excellent sources of useful caffeic acid derivatives.

A new sweetpotato cultivar 'Suioh' was developed for use as an edible green by KONARC[3]. It was reported that the tops of 'Suioh' contained the six derivatives CA, ChA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, and 3,4,5-triCQA[4], and 3,4,5-triCQA, 3,5-diCQA and 4,5-diCQA from propolis were reported to have antihypertensive effects in spontaneously hypertensive rats[8]. Moreover, Ishiguro et al. reported that the six derivatives inhibited angiotensin I-converting enzyme and oral administration of 'Suioh' tops produced an antihypertensive effect in spontaneously hypertensive rats[4].



Chlorogenic acid (5-*O*-caffeoylquinic acid, ChA): R_3 = Caffeoyl

3,4-Di-*O*-caffeoylquinic acid (3,4-diCQA): $R_1 = R_2$ = Caffeoyl, R_3 = H

3,5-Di-*O*-caffeoylquinic acid (3,5-diCQA): $R_1 = R_3$ = Caffeoyl, R_2 = H

4,5-Di-*O*-caffeoylquinic acid (4,5-diCQA): R_1 = H, $R_2 = R_3$ = Caffeoyl

3,4,5-Tri-*O*-caffeoylquinic acid (3,4,5-triCQA): $R_1 = R_2 = R_3$ = Caffeoyl

Fig. 1. Structures of caffeic acid derivatives in sweetpotato tops

The IUPAC numbering system[6] was used in this manuscript.

We found that the total polyphenol content of 'Suioh' leaves, as measured by the Folin-Ciocalteau method, was not in the high range when compared with the total polyphenol contents of the more than 1,000 cultivars tested (unpublished data). The 3,4,5-triCQA content of the whole tops of 'Suioh', as measured by reversed-phase high-performance liquid chromatography (HPLC), was lower than those of some other cultivars[12]. Thus, it may be possible to select or develop new cultivars with higher contents of caffeic acid derivatives than those of 'Suioh'. In KONARC, approximately 1,500 cultivars of sweetpotatoes are maintained. New cultivars are expected to be developed on the same line as 'Suioh', and it is therefore necessary to analyze the chemical components of cultivars with good productivity.

Many investigations have focused on analysis of monocaffeoyl- and dicaffeoylquinic acids rather than 3,4,5-triCQA in plants. In some previous investigations on the biological activities of 3,4,5-triCQA, preparative HPLC procedures were mentioned, but HPLC quantification of 3,4,5-triCQA in plant materials has not been performed[7,13]. During our investigation in 2002, we conducted HPLC using a column of a conventional size (4.6 i.d. × 150 mm, 5-μm particles) and a mobile phase composed of 0.2% (v/v) formic acid and methanol; this was the first report of HPLC analysis of 3,4,5-triCQA where the compound was shown on a chromatogram and the retention time was determined[5]. However, with the conventional HPLC column, the retention time of 3,4,5-triCQA, which was eluted last among the six compounds, CA, ChA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, and 3,4,5-triCQA, was approximately 57 min. Further, a single run lasted as long as 90 min. It is therefore important to decrease the analytical time in order to efficiently investigate the chemical components of a number of sweetpotato cultivars, related products and other crops. With a decrease in analytical time comes the advantage of reduction in the consumption and cost of solvents. In this paper, we describe the contents of caffeic acid derivatives in the leaves of more than 500 sweetpotato cultivars by an improved HPLC technique using a short column (4.6 i.d. × 75 mm); this did not require replacement of the conventional HPLC apparatus to a semi-micro or micro HPLC apparatus.

Materials and methods

1. Reagents

As standards for HPLC analysis, caffeic acid was purchased from Wako Pure Chemical Industries (Osaka, Japan) and chlorogenic, *p*-coumaric and ferulic acids from Sigma (St. Louis, MO, USA). The caffeic acid de-

rivatives 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, and 3,4,5-triCQA were purified by the method reported by Islam et al.[5].

2. Sweetpotato leaf samples

The sweetpotatoes used in this study were grown in greenhouses at the Miyakonojo Branch of KONARC (131°1'E, 31°45'N) in 2005. Seed roots were bedded in nurseries on March 15 and sweetpotato tops were harvested from June 20 to 22. Thirty-centimeter portions from the tips of 5 vines per cultivar were cut, washed with tap water, wiped on paper towels, and then cut into two parts, namely, the leaves and the remaining portion. The leaf samples were placed in plastic bags, frozen and lyophilized. The lyophilized leaf samples were milled to powder and stored at –30°C. The data from the analysis of each cultivar were obtained from a single sample prepared from the leaves of 5 vines.

3. Quantification of caffeic acid derivatives

Eight milliliters of 80% (v/v) ethanol was added to 100 mg of a powder sample in a centrifuge tube with a cap, mixed with a vortex mixer and then allowed to stand overnight at room temperature in the dark. After centrifugation at 2,000 rpm for 5 min, the supernatant was collected and filtered through a membrane filter (DISMIC-13HP, pore size: 0.2 µm; ADVANTEC, Tokyo, Japan). A 5-µl portion of the filtrate was injected into the HPLC system and eluted as described below. The HPLC system consisted of a model DGU-14A degasser, model SIL-10AXL autoinjector, model CTO-10AC column oven, model SPD-M10AVP photodiode array UV-VIS detector equipped with a conventional flow cell of 10 µl intrinsic volume, model CBM-20A system controller, and two model LC-10AT pumps (Shimadzu, Kyoto, Japan). The system was controlled by an LCsolution (version 1.21) workstation (Shimadzu). The column was a Cadenza CD-C18 CD003 (4.6 i.d. × 75 mm, 3-µm particles; Imtakt, Kyoto, Japan). The mobile phase consisted of water containing 0.2% (v/v) formic acid (A) and acetonitrile (B). Elution was performed with the gradient pattern as follows: 2% to 19% B from 0 to 8 min, 19% to 52% B from 8 to 16 min, 52% to 100% B from 16 to 16.01 min, 100% B isocratic from 16.01 to 20 min, 100% to 2% B from 20 to 20.01 min, and 2% B isocratic 20.01 to 26 min. The flow rate was 1 ml/min. The temperature of the column oven was set at 40°C. The caffeic acid derivatives were quantified at 326 nm. Another column as a conventional one was a YMC-Pack ODS-AM AM-302 (4.6 i.d. × 150 mm, 5-µm particles; YMC, Kyoto). The mobile phase for this column consisted of water containing 0.2% (v/v) formic acid (A) and methanol (B). Elution was performed with the gradient pattern as follows: 2% B isocratic from 0 to 15 min, 2% to 45% B from 15 to 50 min, 45% B isocratic from 50 to 65 min, 45% to 100% B from 65 to 65.01 min, 100% B isocratic from 65.01 to 75 min, 100% to 2% B from 75 to 75.01 min, and 2% B isocratic 75.01 to 90 min[5]. The flow rate was 1 ml/min.

Results and discussion

Conventional columns have a length of 150 to 250 mm and an internal diameter of 4.6 mm. The use of columns with a shorter length compared to conventional columns can decrease the analytical time in HPLC analysis[10]. Column efficiency decreases with decreasing column length. However, column efficiency increases as the particle size of the column decreases. Columns packed with 5-µm particles were most widespread until now, and columns packed with smaller particles have become popular in recent times because of their higher resolution. We therefore chose short columns (4.6 i.d. × 75 mm) packed with 3-µm particles in the present study. When columns with an internal diameter of 4.6 mm are used, the intrinsic volume of the detector flow cell and the inner diameter of the connecting tubing in the HPLC apparatus do not need to be changed. Figure 2 shows a chromatogram of an extract of lyophilized leaves of a cultivar obtained with the Cadenza CD-C18 CD003 column. The retention times were 8.5 min for ChA, 8.9 min for CA, 12.9 min for 3,4-diCQA, 13.2 min for 3,5-diCQA, 13.6 min for 4,5-diCQA, and 15.2 min for 3,4,5-triCQA. The retention times of these six compounds were 33.4 min, 32.3 min, 46.7 min, 47.3 min, 50.6 min, and 57.3 min, respectively, in our previous study with a conventional column (YMC-Pack ODS-AM AM-302, 4.6 i.d. × 150 mm, 5-µm particles) and a mobile phase consisting of 0.2% (v/v) formic acid and methanol[5]. A single run lasted for 90 min in our previous study[5], while the cycle time was decreased to 26 min in the present study. Additionally, the volume of the solvent waste decreased to approximately one-third that in the previous study.

An extract of a cultivar 'Tsurusengan' was analyzed to compare the two HPLC conditions, i.e., the conventional column (AM-302) and methanol, with the short column (Cadenza CD-C18 CD003) and acetonitrile. The sample was injected five times under each condition. The contents of the six derivatives in 'Tsurusengan' determined under the two conditions were as follows (milligram per gram of lyophilized leaves [SD]): 1.01 (0.01) and 1.02 (0.01) for CA, 9.78 (0.04) and 9.83 (0.04) for ChA, 3.77 (0.02) and 3.79 (0.02) for 3,4-diCQA, 39.89 (0.23) and 40.04 (0.14) for 3,5-diCQA, 4.39 (0.08) and 4.49 (0.06) for 4,5-diCQA, and 1.15 (0.02) and 1.19 (0.01) for 3,4,5-tri-

CQA, respectively. The analytical values were not significantly different between the two conditions.

The extracts of lyophilized leaf samples of 529 cultivars were analyzed by HPLC with the Cadenza CD-C18 CD003 column. The frequency distribution of the contents of the six caffeic acid derivatives in the 529 cultivars is shown in Fig. 3. Among the 529 cultivars, the content of CA ranged from 0.08 to 3.09 mg; ChA, from 0.17 to 16.01 mg; 3,4-diCQA, from 0.50 to 34.08 mg; 3,5-diCQA, from 1.86 to 56.52 mg; 4,5-diCQA, from 0.69 to 20.73 mg; and 3,4,5-triCQA, from 0.25 to 13.81 mg/g of lyophilized leaves. As shown in the chromatogram in Fig. 2, the cultivar '94TH-18' had the highest 3,4,5-triCQA content among the 529 cultivars tested. The total content of the six compounds among the 529 cultivars ranged from 8.49 to 111.83 mg/g of lyophilized leaves. The caffeic acid derivative 3,5-diCQA was abundant in all cultivars, and this result was consistent with our previous report[5]. 'Suioh' leaves contained 1.37 mg of CA, 5.96 mg of ChA, 6.86 mg of 3,4-diCQA, 32.77 mg of 3,5-diCQA, 7.84 mg of 4,5-diCQA, and 2.22 mg of 3,4,5-triCQA per gram of lyophilized leaves. As shown in Fig. 3, many cultivars had more abundant quantities of all six compounds than 'Suioh', and it may be possible to develop new cultivars following 'Suioh'.

p-Coumaric and ferulic acids were separated under the improved HPLC conditions and their retention times were determined to be 10.9 and 11.9 min, respectively (data not shown). Neither *p*-coumaric acid nor ferulic acid was detected in the leaves of any of the cultivars tested.

Unidentified peaks other than the six above-mentioned caffeic acid derivatives exist in sweetpotato tops as shown in Fig. 2. Liquid chromatography-mass spectrometry (LC-MS) is one of the useful methods for elucidating the structures of such unidentified compounds. Nonvolatile acids such as H_3PO_4 and nonvolatile salts are not suitable for LC-MS analysis. The conditions used in the present study can be employed for LC-MS because there are no nonvolatile chemicals required.

The HPLC conditions were further examined using some commercially available columns and the mobile phase consisting of water containing 0.2% (v/v) formic acid (A) and acetonitrile (B). A separation pattern similar to that in the case of the Cadenza CD-C18 CD003 column was obtained by analysis using an ODS-AM AM12S03-L546WT column (4.6 i.d. × 75 mm, 3-µm particles; YMC, Kyoto, Japan), with the following gradient pattern: 5% to 47% B from 0 to 14 min, 47% to 100% B from 14 to 14.01 min, 100% B isocratic from 14.01 to 19 min, 100% to 5% from 19 to 19.01 min, and 5% B isocratic from 19.01 to 26 min (data not shown). Under these conditions, the retention times of the six compounds were 6.7 min for ChA, 7.2 min for CA, 10.2 min for 3,4-diCQA, 10.4 min for 3,5-diCQA, 10.9 min for 4,5-diCQA, and 12.8 min for 3,4,5-triCQA.

Recently, Tamura et al. reported that 3,4,5-triCQA, which was not found in intact lettuce leaves, was produced in cultured cells derived from plant materials, and the 3,4,5-triCQA amount reached 0.14 mg/g fresh weight[17]. The authors also found that the amount of 3,5-diCQA in cultured cells reached 0.83 mg/g fresh weight, which was 3.8-fold higher than its amount in intact plants[17]. Establishment of techniques to obtain caffeic acid derivatives in large quantities from cultured cells is therefore expected to gain importance. We previously examined the con-

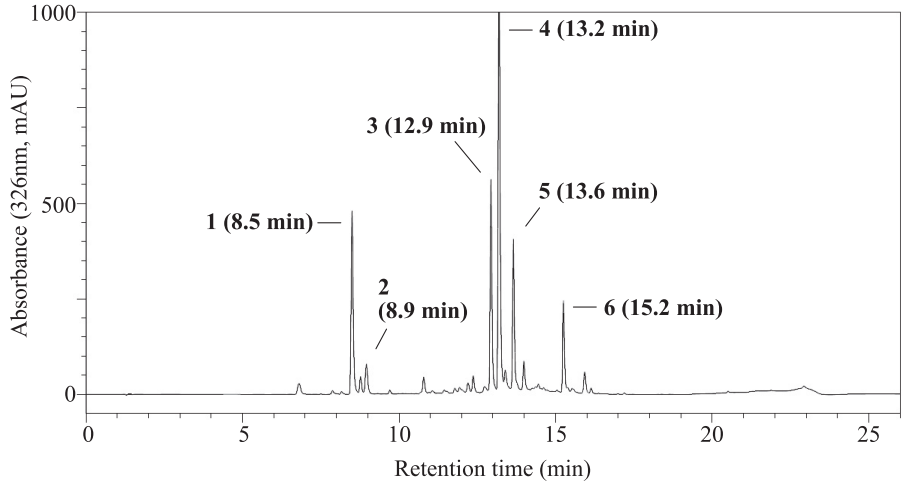


Fig. 2. HPLC chromatogram of an 80% (v/v) ethanol extract of lyophilized leaves of the sweetpotato cultivar '94TH-18'
1: ChA, 2: CA, 3: 3,4-diCQA, 4: 3,5-diCQA, 5: 4,5-diCQA, 6: 3,4,5-triCQA.

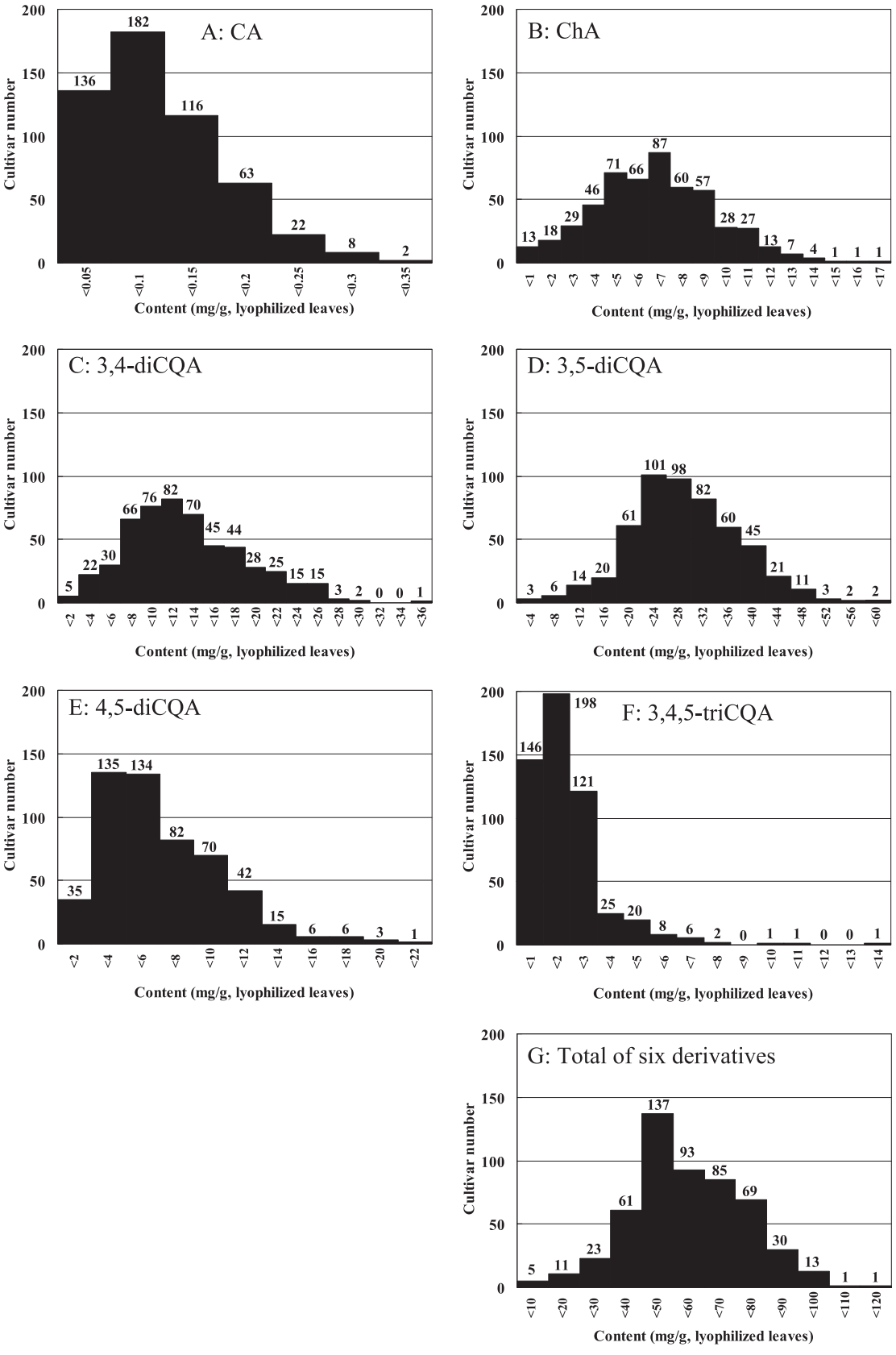


Fig. 3. Frequency distribution of the contents of six caffeic acid derivatives in leaves of 529 sweetpotato cultivars (mg/g lyophilized leaves)

tents of caffeic acid derivatives in sweetpotato tops that were harvested six times periodically from April to October 2002 and oven-dried at 70°C and found that the average content of 3,4,5-triCQA per harvest was > 1 mg/g dry weight for some cultivars. One of the cultivars had a 3,4,5-triCQA concentration of > 2 mg/g dry weight at a certain harvest[12]. Additionally, the 3,4,5-triCQA and 3,5-diCQA contents in the lyophilized leaves of many cultivars exceeded 1.4 and 3.8 mg/g dry weight, respectively, in the present study as shown in Fig. 3. Considering the dry matter contents of sweetpotato tops, the intact tops of this crop may have advantages over cultured cells of lettuce leaves in terms of the 3,4,5-triCQA and 3,5-diCQA concentrations and amount of available raw material. In the future, a large amount of caffeic acid derivatives may be produced by the use of cultured cells derived from intact sweetpotato tops.

At KONARC, approximately 1,500 sweetpotato cultivars are maintained. Analysis of the leaves of cultivars other than those analyzed in the present study is in progress. The present data, i.e., the contents of the six caffeic acid derivatives in each cultivar, were obtained from a single sample. Therefore, it is necessary to obtain data from multiple samples per cultivar. The sweetpotato tops were harvested from morning to afternoon to prepare the leaf samples for analysis in the present study. It is also important to clarify whether there are differences between the contents of each compound in leaves harvested at different times of the day.

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