Pharmaceutical Research for Quality Evaluation and Characterization of Foods and Natural Products

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In Japan, existing food additives are those included in the List of Existing Food Additives specified in the Supplementary Provisions to the Law Concerning Amendments to the Food Sanitation Law and Nutrition Improvement Law. Most of the currently available food additives are natural extracts containing various ingredients. However, the characteristic and active components of existing food additives are not always properly defined due to poor characterization of the constituents of the respective raw materials. For that reason, the characteristic components of existing food additives from natural extracts have been evaluated using various methods and reported. Here we review examples of our research on the characterization of marker constituents of existing food additives from natural products.

Key words existing food additive; mousouchiku extract; gentian root extract; grape skin extract; sage

1. Introduction

In Japan, existing food additives are those included in the List of Existing Food Additives in Japan specified in the Supplementary Provisions to the Law Concerning Amendments to the Food Sanitation Law and Nutrition Improvement Law. Additives of natural origin were widely used before the revision of the Food Sanitation Law in 1995. Natural additives were marked as on the List of Food Additives Other than the First Edition of Chemical Synthetic Products in 1989 and full labeling was implemented in 1991. After a major revision of the Food Sanitation Law in 1995, the designation system was applied to natural additives. Currently, existing additives that have been approved for use as a transitional measure with the exception of the Food Sanitation Law in 1995. Natural additives were investigated the constituents of mousouchiku extract to obtain the chemical data requisite for the development of standardized specifications. Commercial mousouchiku extract (commercial product A, EtOH extract of P. heterocyca) was separated by column chromatography to isolate 13 compounds: propiothene 4′-O-primeveroside (1); 5-hydroxymethyl-2-furfural (2); 4-hydroxybenzoic acid (3); trans-p-coumaric acid (4); transferulic acid (5); N,N′-diferuloylputrescine (6); 4′-hydroxypropionphenone (7); β-arbutin (8); tachioside (9); isotachioside (10); 3,4′-dihydroxypropioiphopnon 3-O-glucoside (11); koabarudse (12); and (+)-llyonesinol 9′-O-glucoside (13) (Fig. 1). Among them, 1 was a newly reported compound.4)

To estimate the distribution of phenolic compounds in mousouchiku extract, commercial product A was analyzed by reversed-phase HPLC (Fig. 2) with authentic original samples, and compound 13 was found to be the major ingredient. Other commercial products B–F were analyzed under the same conditions, and the peak of 2,6-dimethoxy-1,4-benzoquinone (peak DB), defined in the official list as the main constituent, was not detected among the major ingredients. Therefore, the characteristic constituent of food additives should be clearly defined based on scientific data. Compound 13 was
also detected in commercial products E and F; therefore, it is suggested that this compound is a characteristic phenolic compound in mousouchiku extract.

3. Gentian Root Extract

Gentian root extract is defined as “an additive obtained from the root and rhizome of Gentiana lutea L. (Gentianaceae) in the List of Existing Food Additives in Japan.” Concerning the origin, manufacturing method, and essential qualities of this additive, the official list states: “This additive is prepared from the root and rhizome of G. lutea using extraction with water or EtOH.” Its active constituents are gentiopicroside and amarogentin. The root and rhizome of G. lutea constitute the crude drug “Gentianae Radix,” which is used as an herbal stomachic worldwide. Gentianae Radix is also listed as an official drug in the Japanese Pharmacopoeia. Phytochemical studies of this drug revealed the presence of bitter secoiridoid glycosides, including gentiopicroside, swertiamarin, and amarogentin, as well as xanthones, phenolic acids, flavonoids, and triterpenoids. Thus, although it is known that the bitterness of gentian root extract is attributable to the presence of gentiopicroside and amarogentin, the bittering agent is not well characterized.

We identified 17 constituents of this extract: loganic acid 7-(2'-hydroxy-3'-O-β-D-glucopyranosyl)benzoate (14); 5-hydroxymethyl-2-furfural (15); furan-2-carboxylic acid (16); 2,3-dihydroxybenzoic acid (17); gentiopicroside (18); isovitexin (19); gentisin 7-O-primeveroside (20); isogentisin 3-O-primeveroside (21); a mixture of gentisin (22) and isogentisin (23); vanillic acid (24); loganic acid (25); sweroside (26); 6'-O-glucosylgentiopicroside (27); swartiajaposide D (28); anofinic acid (29); and 2-methoxylanofinic acid (30) (Fig. 3). Among them, 14 was reported as a new compound.

The HPLC chromatogram of the gentian root extract is shown in Fig. 4. Compound 18 was detected as the major component, but amarogentin, which is reported to be the other main constituent in the Food Additives List, was not isolated. The bitter principles of the gentian root extract were attributed...
to two secoiridoid glucosides, compound 18 and amarogenin.\(^\text{16}\) Amarogenin was reported to be one of the main bitter components of gentian,\(^\text{25}\) although its content decreases over the cultivation period. The amarogenin content in materials cultivated for more than 5 years was between 0.2–0.4 mg/g, but the content of amarogenin in the commercial products of gentian varies markedly. In contrast, the content of compound 18 in commercial products was reported to be 30–40 mg/g with low variability.\(^\text{16}\) Another study reported the isolation of amarogenin from fresh raw materials.\(^\text{7}\) Therefore, the detection of compound 18 and amarogenin is influenced by the status of raw materials. The detection of both or either of these two ingredients is suitable for evaluating the quality of gentian root extract as a food additive.

4. Grape Skin Extract

Grape skin extract is defined as an additive obtained from the skin of American grapes or grapes, with the polyphenol as its main constituent in the List of Existing Food Additives in Japan.\(^\text{1}\) Concerning the origin, manufacturing method, and essential qualities of these additives, the official list states: “This additive is prepared from the pressed lees of the skin of Koshu, Chardonnay, or Riesling grape and all Vitis labrusca or V. vinifera varieties, by extraction with EtOH at lukewarm or room temperature.” Their skins have been reported to be composed of polyphenols such as anthocyanins.\(^\text{17–22}\) Although the polyphenols from whole grapes have been exhaustively investigated, there is little detailed information on the chemical constituents of grape skin extract products.

We investigated and reported the constituents of grape skin extract to accumulate the necessary chemical data for the development of standardized specifications.\(^\text{23}\) To characterize the constituents, the extract was analyzed using HPLC, and the HPLC chromatogram of an aqueous methanol solution of grape skin extract is shown in Fig. 5. The extract was separated by repeated column chromatography, and 12 constituents were identified: tryptamine (31); luteolifavan (32); procyanidin B-1 (33); (+)-catechin (34); vanillic acid (35); procyanidin B-2 (36); syringic acid (37); (-)-epicatechin (38); ethyl gallate (39); myricetin 3-O-glucoside (40); quercetin 3-O-glucuronide (41); and quercetin (42) (Fig. 5). In addition, grape skin anthocyanins were detected by HPLC, with malvidin 3-O-glucoside (43) as the major component, together with 3-O-glucosides (44–47) of delphinidin, cyanidin, petunidin, and peonidin (Fig. 6). A fraction of condensed tannins, which correspond to only a broad peak, and not the other sharp peaks, was obtained.\(^\text{13}\) C-NMR analysis indicated the trend of oligomeric B-type proanthocyanidins and that fraction was also subjected to gel permeation chromatography analysis of the molecular weight distribution. A calibration curve based on retention times correlated with the molecular weight of a polystyrene standard and thus allowed the assignment of the number and weight-averaged molecular weights of the fraction concerned as 5999.6 and 21287.7, respectively. Proanthocyanidins have been reported to be the main constituents of grape skin.\(^\text{24}\) However, the proanthocyanidin content of grape skin extract has not been fully investigated. There are limited possibilities for the analysis of grape skin extract products. Colorimetric analysis with vanillin-sulfuric acid of the proanthocyanidins in the extract was performed to provide a simple method for quantifying these components. The proanthocyanidin content of two commercial grape skin extract products was 62.1 and 60.7%, respectively, as catechin equivalents.

5. Comparison of Constituents by Sage Extract Preparation Method

Existing food additives are those in which active components are not always defined due to poor characterization of the ingredients in the respective raw materials. Food additives are prepared according to the procedures described in the List of Existing Food Additives.\(^\text{1}\) In many cases, several preparation procedures are described for a single food additive. For example, antioxidant extracts of sage can be prepared by extraction from the leaves of Salvia officinalis L. using water, EtOH, or n-hexane. It was suggested that the ingredients extracted by these methods show different effectiveness, while scientific evidence for such a distinction has been lacking until now. Therefore, investigating the appropriate preparation method for food additives should facilitate their proper use. In this review, constituent distributions of sage ingredients, extracted using the different preparation methods described in the list are summarized.\(^\text{25}\)

HPLC chromatograms of the EtOH, 50% EtOH, n-hexane, and water extracts of sage are shown in Figs. 7(a–d). To characterize the HPLC peaks of these extracts, the 80% EtOH extract was prepared and separated into n-hexane-, ethyl acetate-, and water-soluble fractions, as shown in Figs. 7(e–h). After separation and purification of each fraction, six compounds, i.e., vicenin-2 (48), luteolin 7-O-beta-D-glucoside (49), scutellarein (50), rosmarinic acid (51), cirsimaritin (52), and salvigenin (53), were identified based on the spectral data (Fig. 8). Based on comparison with the isolated standards, the main peaks in the HPLC chromatograms of the 50% EtOH and water extracts were identified as compounds 49–51 (Figs. 7(a, d)). These peaks were also detected in the EtOH extract, although they were lower than those in the 50% EtOH and water extracts [Fig. 7(b)]. However, these peaks were not detected in the n-hexane extract [Fig. 7(c)].

To compare the differences in antioxidative activities de-
Depending on the extraction conditions, the radical scavenging activities of the EtOH, 50% aqueous EtOH, n-hexane, and water extracts were assessed using oxygen radical absorbance capacity (ORAC) values as indicators. As shown in Fig. 9(a), the 50% aqueous EtOH extract was demonstrated to exhibit the highest antioxidative activity, followed by the water extract. Among the ORAC values for the n-hexane, ethyl acetate, and water fractions of the 80% EtOH extract, the ethyl acetate fraction mainly including compound 51 had the highest antioxidative activity, followed by the water fraction. The ORAC value of compound 51 was higher than that of epigallocatechin gallate, which is a typical tea catechin, as shown in Fig. 9(b). On the other hand, the n-hexane fraction, which indicated the presence of diterpenes such as carnosol and carnosic acid and the absence of compound 51, had low antioxidative activity. Thus, it was suggested that the extraction of the main ingredients of sage can be improved by using different preparation methods.

Fig. 3. Structures of Compounds 14–30 Identified from Gentian Root Extract and Amarogentin (AG)
Reproduced from ref. 15.
Fig. 4. HPLC Chromatograms of Gentian Root Extracts

Numbers on chromatograms correspond to the compound number. The conditions are as follows: column, YMC-pack ODS AQ-3C2 (5 µm, 150 × 2.0 mm i.d.) (YMC Co., Ltd., Kyoto, Japan); mobile phase, solvent A was 10 mM H₃PO₄-10 mM KH₂PO₄ (1:1) and solvent B was MeOH (0–30 min, 0–50% B in A; 30–50 min, 50–60% B in A); column temperature, 40 °C; flow-rate, 0.25 mL/min; detection, 254 nm. Reproduced from ref. 15.

Fig. 5. HPLC Chromatogram and Structures of Compounds 31–42 Identified from Grape Skin Extract

Numbers on chromatograms correspond to compound numbers. The conditions were: column, Cosmosil 5C18-II (5 µm, 150 × 2.1 mm i.d.) (Nacali Tesque, Inc., Kyoto, Japan); mobile phase, solvent A = water (including 0.1% formic acid) and solvent B = acetonitrile (0–30 min, 0–50% B in A; 30–35 min, 50–85% B in A; 35–40 min, 85% B in A; 40–50 min, 85–90% B in A; 50–55 min, 90–100% B in A; 55–60 min, 100% B in A); column temperature, 40 °C; flow rate, 0.3 mL/min; detection, 280 nm. Reproduced with permission from ref. 23.

Fig. 6. HPLC Chromatogram and Structures of Compounds 43–47 Identified from Grape Skin Extract

Numbers on chromatograms correspond to compound numbers. The conditions were: column, L-column ODS (5 µm, 150 × 2.1 mm i.d.) (Chemicals Evaluation and Research Institute, Tokyo, Japan); mobile phase, solvent A = 5% acetic acid and solvent B = acetonitrile (0–30 min, 0–50% B in A; 30–35 min, 50–85% B in A; 35–40 min, 85% B in A; 40–50 min, 85–90% B in A; 50–55 min, 90–100% B in A; 55–60 min, 100% B in A); column temperature, 40 °C; flow rate, 0.3 mL/min; detection, 520 nm. Reproduced with permission from ref. 23.

Fig. 7. HPLC Chromatograms of Extracts Prepared from Sage

(a) 50% EtOH extract; (b) EtOH extract; (c) n-hexane extract; (d) water extract; (e) 80% EtOH extract; (f) 80% EtOH extract–n-hexane fraction; (g) 80% EtOH extract–ethyl acetate fraction; (h) 80% EtOH extract–water fraction. Numbers on chromatograms correspond to compound numbers. The conditions were: column, L-column ODS (5 µm, 150 × 2.1 mm i.d.) (Chemicals Evaluation and Research Institute); mobile phase, solvent A = 5% acetic acid and solvent B = acetonitrile (0–30 min, 0–50% B in A; 30–35 min, 50–85% B in A; 35–40 min, 85% B in A; 40–50 min, 85–90%, 50–85% B in A; 50–55 min, 90–100% B in A; 55–60 min, 100% B); column temperature, 40 °C; flow rate, 0.3 mL/min; detection, 280 nm. Reproduced with permission from ref. 25.
6. Conclusion

Many food existing additives are extracts from natural products and composed of multiple constituents. Therefore, it is often difficult to set standards for their components. This review summarized examples of our research on the characterization of marker constituents of mousouchiku extract, gentian root extract, grape skin extract, and sage, as these are existing food additives from natural products. It is expected that clarification of the active ingredients and component composition of existing additive products will lead to the establishment of analytical methods and standards to determine their content regulations. We hope that the results of our research will contribute to the proper use of existing additives.

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Conflict of Interest The authors declare no conflict of interest.

References
1) Ministry of Health, Labour and Welfare of Japan, “Notification No. 120,” Apr. 16, 1996.
2) Sugimoto N., JAFAN, 35, 207–221 (2015).
3) Nishina A., Hasegawa K., Uchibori T., Seino H., Osawa T., J. Agric. Food Chem., 39, 266–269 (1991).
4) Yoshimura M., Ochi K., Sekiya H., Tamai E., Maki J., Tada A., Sugimoto N., Akiyama H., Amakura Y., Chem. Pharm. Bull., 65, 878–882 (2017).
5) Niio Y., Yamazaki T., Nakajima Y., Yamamoto T., Ando H., Hirai Y., Torizuka K., Ida Y., J. Nat. Med., 60, 82–88 (2006).
6) Ministry of Health, Labour and Welfare, Japan, “The Japanese Pharmacopoeia,” 17th ed., 2016.
7) Ando H., Hirai Y., Fujii M., Hori Y., Fukushima M., Niio Y., Nakajima Y., Sibata T., Torizuka K., Ida Y., J. Nat. Med., 61, 269–279 (2007).
8) Inoue H., Ueda S., Nakamura Y., Chem. Pharm. Bull., 18, 1856–1865 (1970).
9) Korte F., Chem. Ber., 88, 704–707 (1955).
10) Hayashi T., Yamagishi T., Phytochemistry, 27, 3696–3699 (1988).
11) Atkinson J. E., Gupta P., Lewis J. R., Tetrahedron, 25, 1507–1511 (1969).
12) Yamada S., Kakuda R., Yaoita Y., Kikuchi M., Nat. Med., 59, 189–192 (2005).
13) Kakuda R., Machida K., Yaoita Y., Kikuchi M., Kikuchi M., Chem. Pharm. Bull., 51, 885–887 (2003).
14) Toriumi Y., Kakuda R., Kikuchi M., Yaoita Y., Kikuchi M., Chem. Pharm. Bull., 51, 89–91 (2005).
15) Amakura Y., Yoshimura M., Morimoto S., Yoshida T., Tada A., Ito Y., Yamazaki T., Sugimoto N., Akiyama H., Chem. Pharm. Bull., 64, 78–82 (2016).
16) Hayashi T., Minamizama Y., Miura T., Yamagishi T., Kaneshima H., Report of the Hokkaido Institute of Public Health, 40, 103–106 (2015).
17) Polovka M., Šťavíková L., Hohnová B., Karásek P., Roth M., J. Chromatography A, 1217, 7990–8000 (2016).
18) Lago-Yanzela E. S., Du-Silva R., Gomes E., García-Ramero E., Hernandez-Gutierrez L., J. Agric. Food Chem., 59, 13146–13146 (2011).
19) Zhang Y., Jayaprakasam B., Seeram N. P., Olson L. K., Dewitt D., Nair M. G., J. Agric. Food Chem., 52, 228–233 (2004).

Fig. 8. Structures of Compounds 48–53 Identified from Sage

Fig. 9. ORAC Values
(a) Extracts and fractions prepared from sage; (b) rosmarinic acid (51) and epigallocatechin gallate (EGCG). Reproduced with permission from ref. 25.
20) Chafer A., Pascual-Marti M. C., Salvador A., Berna A., *J. Sep. Sci.*, **28**, 2050–2056 (2005).

21) Moteki H., Hibasami H., Ohakawa S., Katsuzaki H., Imai K., Ishii Y., Nakagawa M., Komiya T., *Food Sci. Technol. Res.*, **7**, 131–134 (2001).

22) Kennedy J. A., Hayasaka Y., Vidal S., Waters E. J., Jones G. P., *J. Agric. Food Chem.*, **49**, 5348–5355 (2001).

23) Amakura Y., Yoshimura M., Yoshida T., Tada A., Ito Y., Yamazaki T., Sugimoto N., Akiyama H., *Jpn. J. Food Chem. Safety*, **22**, 108–114 (2015).

24) Chira K., Schmauch G., Saucier C., Fabre S., Tessedre P.-L., *J. Agric. Food Chem.*, **57**, 545–553 (2009).

25) Amakura Y., Yoshimura M., Yoshimura A., Yoshida T., *Jpn. J. Food Chem. Safety*, **18**, 25–34 (2011).