
Regular Article

Qualitative and quantitative evaluation of drug and health food products containing red vine leaf extracts on the Japanese market

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Summary

Red vine leaf extracts (RVLEs) have traditionally been used for leg wellness and are now standardized to be used as over-the-counter (OTC) drugs in Europe. In Japan, one brand of RVLE products was recently approved as a direct OTC drug, and RVLEs are still used as ingredients in health food products. Since there is no mandated criterion for the quality of health food products in Japan, the consistent quality and composition of these products are not assured. Here we analyzed OTC drug and health food products containing RVLEs with different lot numbers by LC/MS. Subsequent multivariate analyses clearly indicated that the quality of the health food products was highly variable compared to that of the drug products. Surprisingly, the component contents in the health foods were different even within a same lot in a same brand. The quantitative analyses of flavonols and stilbene derivatives in the drugs and health foods indicated that the concentration of each substance was kept constant in the drugs but not in the health foods. These results strongly indicated that the quality of RVLEs as a whole was not properly controlled in the manufacturing process of health foods. Since RVLE is an active ingredient with pharmaceutical evidences and is used for drugs, the proper regulation for ensuring the consist quality of RVLEs from product to product would be recommended even in the health foods.

Keywords

direct OTC drug, health food product, red vine leaf extract, quality control, LC/MS, principal component analysis
Introduction

Herbal medicines have been used for hundreds of years in Europe as well as Asia. Active ingredients from the western herbal products are standardized by the European Pharmacopoeia in order to be used in over-the-counter (OTC) drugs in European countries, and two of these preparations are now available as direct OTC drugs in the Japanese market. The first consists of aqueous extracts from red vine leaves (*Vitis vinifera* L.), which have traditionally been used for leg wellness in Europe and have recently been approved by the Ministry of Health, Labour and Welfare in Japan. The second type of preparation, red vine leaf extracts (RVLEs), have been also used as ingredients in health food products in Japan. The market of RVLE products for self-medication is now in a complicated situation: one type of RVLE product is a genuine medicine capable of expecting pharmaceutical efficacy, and the other type is RVLE-containing health foods, which are not permitted to indicate any medical efficacy.

In addition, there are concerns over the lack of quality of the health food products. Our continuing research suggests that many health foods obtained in Japan have issues on their origin and disintegration.\textsuperscript{1-15} This would be because the quality control for health food products in Japan is conceptually different from that for drugs; drugs are strictly regulated by the Law for Ensuring the Quality, Efficacy, and Safety of Drugs and Medical Devices (commonly called the Drugs and Medical Devices Law) which requires the same quality, efficacy and safety for a drug at those obtained and reported in clinical trials. In contrast, health foods are regulated by the Food Sanitation Law which merely requires a certain level of quality to ensure the safety of foods.
New regulations for health food products sold in Japan were introduced in April 2015, and Japan’s Consumer Affairs Agency established a third category of voluntary labeling which is known as Food with Functional Claims (FFCs). The use of FFCs allows companies to display specific health benefits on a product’s packaging.\(^1\) Due in part to the affordable process of registering a new FFC, over 200 notifications for FFC products (including western herbal preparations such as ginkgo leaf products) have been submitted since the start of the registration period. With the increasing interest in health in Japan as in other developed countries, self-medication is becoming popular among the Japanese people, and the efficacy of functional food is highly expected. Effective quality control is thus necessary to ensure the functional integrity of western herbal ingredients, even for these ingredients in foods that are marketed as having a specific health-related function or efficacy.

To ensure the efficacy and safety of western herbal products, it is important that (1) the correct original plant species is used, (2) the products are manufactured appropriately, and (3) consistent quality and composition are assured.\(^1,4,15\) In the present study, we used a multivariate statistical approach by LC/MS data to visually evaluate the quality of RVLE-containing health foods compared with OTC drugs. We also quantified the products’ flavonol and stilbene contents, using LC/MS/MS determine the consistency in the component content of RVLE products.
**Experimental**

**Materials**

OTC drugs and health foods containing RVLE extracts were purchased from pharmacies in Japan and from the Japanese market (Table 1). To obtain plural products with different lot numbers, one or two packages of each product were purchased several times during the period December 2013 to June 2015. Hesperidin and monoglucosyl hesperidin were purchased from Nacalai Tesque (Kyoto, Japan) for peak identification. Quercetin 3-O-β-D-glucuronide (Q3GA), quercetin 3-O-β-D-glucoside (Q3G), kaempferol 3-O-β-D-glucoside (K3G), (−)-trans-ε-viniferin and pterostilbene were purchased from Funakoshi (Tokyo), and resveratrol was purchased from Wako (Tokyo) as the reference substances for calibrators (Fig. 1).

**Sample preparation**

The content of the capsule or the tablet containing RVLE 180 mg (one capsule or one tablet) was powdered and extracted with 10 mL of 50% MeOH by sonication for 3 h at room temperature and centrifuged at 12,000 g for 10 min to obtain a supernatant. The supernatant was diluted 20-fold to a final RVLE concentration of 0.9 mg/mL and filtered through a 0.45 μm Ultrafree-MC centrifugal filter unit (Millipore, Bedford, MA, USA) before the LC/MS analyses. The product samples were prepared from every package, and quality control (QC) samples were prepared by pooling equal volumes (50 μL) from each product sample.
Qualitative LC/MS analysis

A 5-μL aliquot of each filtrate was injected onto a Prominence UFLC (Shimadzu, Kyoto, Japan) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA) equipped with an electrospray ionization (ESI) source. Samples were injected three times with the pooled QC injected every 10 sample injections. The LC separations were performed on a Hypersil GOLD column (100 × 2.1 mm i.d., 1.9 μm, Thermo Scientific) at 40°C with a mobile phase consisting of 1% acetonitrile in water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). The gradient started at 0% solvent B and increased linearly to 30% in 25 min, then to 100% in 3 min at a flow rate of 0.2 ml/min.

Mass spectra were obtained in positive ion mode using the following parameters: needle voltage, 4,000 V; capillary temperature, 300°C; target mass resolution, 60,000; m/z range, 100–2,000. The most intense ions in the full-scan mass spectrum were isolated with a 3.5-Da window and fragmented by collision-induced dissociation with a collision energy of 35 V.

The raw LC/MS data were imported to the Progenesis QI software (Nonlinear Dynamics, Durham, NC) for automatic peak detection and alignment using the chromatogram of the QC sample as a reference. All data were normalized to the summed total ion intensity per chromatogram and three-dimensional data matrices were generated consisting of variable ID numbers (retention time-m/z value), sample codes and normalized peak areas. The resulting data matrix of 5,576 peaks was imported into SIMCA-P+ ver. 12 software (Umetrics, Malmo, Sweden) and normalized with Pareto scaling prior to a principal component analysis (PCA). A five-component PCA model resulted accounting for 85% (R2X
= 0.847) of the variation and predicting 80% (Q2X = 0.799). The first two principal components were extracted, and scatter plots were prepared by plotting the score of PC1 vs PC2.

Quantitative LC/MS/MS analysis

A 5-µL aliquot of each filtrate was injected onto an Acquity ultra performance liquid chromatography (UPLC) system coupled to a Xevo TQD mass spectrometer (Waters, Milford, MA) equipped with an ESI source. Samples were injected three times with the pooled QC injected at the beginning and at the end of analysis. LC separations were performed on an Acquity UPLC bridged ethylene hybrid (BEH) C18 column (100×2.1 mm i.d., 1.8 µm, Waters) at 40°C with a mobile phase consisting water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). The gradient started at 5% solvent B and increased linearly to 30% in 20 min, then to 90% within 2 min at a flow rate of 0.2 mL/min.

Tandem mass spectrometry (MS/MS) was carried in positive ion mode using the multiple reaction monitoring (MRM) mode with optimal transitions for each compound. The MS conditions for the MRM transitions were developed using the IntelliStart automatic tuning system (Waters) by infusing stock solutions (0.2 mg/mL) of each referential standard. The optimal MS parameters for the flavonoids were as follows: capillary voltage, 2.7 kV; source temperature, 150°C; desolvation gas (nitrogen gas) temperature, 350°C; desolvation gas flow, 650 L/h. The optimal MS parameters for the stilbenes were as follows: capillary
voltage, 2.8 kV; source temperature, 150°C; desolvation temperature, 300°C; desolvation gas flow, 800 L/h.

The individual cone voltages and collision energies for the target compounds are described in Table 2, and the dwell times were 0.025 s for all transitions. All data were processed using MassLynx and TargetLynx software (Waters). We prepared referential standard solutions of flavonoids, resveratrol, ε-viniferin and pterostilbene at 20, 0.02, 0.001, and 0.00001 μg/mL, respectively, and each content was calculated by peak area versus its concentration.

**Results and Discussion**

*Qualitative analysis of RVLE products*

Product samples were prepared from every package (n= 1 or 2). Nine samples from drug A and four samples each from health foods B and C were obtained in triplicate for the LC/MS analysis and the PCA. In the PCA scores plot (Fig. 2a), samples from drug A concentrated in one cluster (DR), and the drugs and health foods were clearly separated with negative and positive correlations, respectively, for the principal component 1 (PC1, which accounted for 75% of the total variance). The health food samples were widely distributed along PC2 (which accounted for 3.8% of the total variance) on the y-axis.

Surprisingly, the samples from the health foods formed three clusters regardless of the brand or lot: the first group (HF1) was composed of B-7 and C-18 with positive scores on PC2; the second group (HF2) was composed of B-12 and C-17, and the third group (HF3) contained the other batch of B-12, B-7, C-17, C-18 with negative scores on PC2. These
results demonstrated that the ingredients of the health food products were not consistent even within the same lot. To our knowledge, this is the first report to demonstrate the non-uniformity of an ingredient in health foods within the same lot.

We then identified the compounds associated with the separation of drugs and health foods and with variation among the health foods on the loading plots (Fig. 2b). Several hydrophilic compounds with a large molecular mass (RT = 1.57 min, m/z > 950) were negatively correlated with the drug samples (loading on PC1 < −0.1) and were presumed to be sugars because hydrolyzed starch was contained in drug A but not in foods B and C according to the package claims.

On the other hand, some variables that were positively correlated with the health foods (loading on PC1 > 0.09) were identified as deriving from hesperidin and monoglucosylhesperidin (RT = 22.03 min, m/z 663.18 and RT = 21.81 min, m/z 773.25 for [M+H]+, respectively), which are added to foods B and C. Thus, it was clearly indicated that the additives of each product contributed to the separation of drug and health foods. It is quite natural that different chemical components resulting from additives between drugs and health foods were detected by the PCA analysis, and these additives are not strongly linked to the quality and efficacy of RVLE products.

Moreover, we also focused on the compounds associated with the separation of health foods into three clusters along PC2. Variables that were positively correlated with PC2 (> 0.12) and contributed HF1 clustering (RT = 20.73 and 20.21 min, m/z 287.056 and RT = 20.73 min, m/z 471.091) were presumed to be flavonoid glycosides ([M+H]+ = C_{13}H_{11}O_{6} and C_{23}H_{19}O_{11}, respectively) based on their mass spectra. Variables negatively correlated with
PC2 (< -0.12) and contributed HF3 clustering (RT = 26.43 and 27.67 min, m/z 907.277 and RT = 27.89 min, m/z 681.213) were presumed to be resveratrol tetramers ([M+H]⁺ = C₅₆H₄₃O₁₂) and trimer ([M+H]⁺ = C₄₂H₃₃O₉), respectively, in the same way. These results indicated that the contents of real ingredients from vine leaf, flavonoids and resveratrol derivatives, differed in health foods even within the same lot. These differences may have occurred because the extraction and/or mixing process of the health foods were not strictly controlled to provide uniform contents.

Quantitative analysis: Flavonols

Although herbal extracts are considered to be active as a whole, they are generally characterized by the contents of some metabolites as referential substances in order to ensure consistent quality and components in the manufacturing processes. Flavonols as well as their glycosides are very common metabolites widely distributed in plants, and quercetin 3-O-glucuronide (Q3GA), quercetin 3-O-glucoside (Q3G) and kaempferol 3-O-glucoside (K3G) are known to be major metabolites in grapevine.

To evaluate the component consistency of RVLE products, we selected these metabolites as the referential substances and quantified their content using LC/MS/MS with the MRM mode (Fig. 3). The quantitative results indicated that each flavonol concentration from the drug samples was at a nearly constant value within a small margin of error; 13 mg of Q3GA, 14 mg of Q3G and 2 mg of K3G in the consumption per day. In contrast, the health food samples contained 0.6- to 0.8-fold higher amounts of Q3GA, 1.0- to 1.4-fold higher amounts of Q3G, and 1.0- to 1.6-fold higher amounts of K3G compared to the drug samples.
The flavonol contents of the health foods were different in each lot; B-7 contained a 1.25-fold higher amounts of flavonols to B-12 and C-17 contained a 1.16-fold higher amounts of flavonols to C-18.

These results clearly showed that the concentration of major metabolites of grapevine was kept constant in the RVLE drugs but not in the RVLE health foods. Such considerable variations in the component content of RVLE products from lot to lot also strongly indicated that the quality of the whole extract was not controlled during the manufacturing process used for the health foods. Consequently, the pharmacological effects of health food products could differ by each lot even within the same brand.

**Quantitative analysis: Stilbenes**

Stilbene derivatives such as resveratrol, ε-viniferin and pterostilbene are characteristic metabolites for grapevine. They are induced by several biotic stresses, and their contents change depending on the growth environment.\textsuperscript{21,22} Since resveratrol derivatives were identified as inconsistent components from the health foods in the PCA loading plot described above, we quantified resveratrol, ε-viniferin (a resveratrol dimer) and pterostilbene (dimethyl resveratrol) as was done with the three flavonols above (Fig. 4).

The quantitative results showed that each stilbene concentration from the drug samples was at a constant low level, whereas the health food samples contained 2.7- to 5.7-fold higher amounts of resveratrol, 5.0- to 9.3-fold higher amounts of ε-viniferin and 7.9- to 15.0-fold higher amounts of pterostilbene than the drug samples with a relatively large margin of error. These results agree with the positive correlation of resveratrol derivatives to PC1 (> 0.06) on
the loading plots. Several reasons why drug samples contained lower amounts of stilbenes than health foods are considered as follows: 1) the manufacturing process of RVLE for drugs were different from that for health foods to reduce stilbenes, 2) the cultivar of original plants for drugs contained less stilbenes than that for health foods, 3) the original plants for drugs produced little stilbenes as a kind of phytoalexin under an environment without danger of diseases resulting from insects or molds.

Similarly to the findings obtained for flavonols, the stilbene contents from health foods differed by lot: B-12 contained an approx. 1.8-fold higher amounts of stilbenes to B-7, and C-17 contained an approx. 1.1-fold higher amounts of stilbenes to C-18. These results indicated that the RVLE drugs were produced under the proper manufacturing process for uniform quality, whereas the standardization and quality control of the health foods may need to be improved to ensure safety and efficacy at a certain level.

**Conclusion**

We used LC/MS to analyze RVLE-containing OTC drug and health food products with different lot numbers. Subsequent multivariate analyses clearly indicated that lots compared to the drug samples, the quality of the health food products was highly variable even within the same lots. When we quantified flavonols and stilbenes as referential substances for our evaluation of the consistency in quality of the RVLE products, we observed that the concentrations of these referential substances were constant in the drugs but not in the health foods. Our comparison of the variances by means of the F-test revealed that the dispersion of some major ingredients in RLVE was highly significant in the health foods (Table 3). Such
considerable variations in the component contents of RVLE products from lot to lot also strongly indicated that the quality of the whole extracts was not well controlled in the manufacturing process used for the health foods. The present lack of uniform quality in herbal products may well be an impediment to both safety and efficacy, and the lack of consistency of active ingredients from product to product may lead to negative effects on the health of consumers. Since RVLE is an active ingredient with pharmaceutical actions and is used for drugs, proper regulation would be recommended to ensure product-to-product consistency even for the health foods.

Acknowledgments

This work was supported by a grant for ‘Research on the Development of New Drugs’ from the Japan Health Sciences Foundation, a Health and Labour Sciences Research Grant, and a grant for ‘Research on the Development of New Drugs’ from Japan’s Agency for Medical Research and Development (AMED).

Conflict of Interest

The authors declare no conflict of interest.
References


Figure legends

**Fig. 1.** Chemical structures of major flavonols and stilbenes in red vine leaves (*Vitis vinifera*).

**Fig. 2.** Principal component analysis based on the LC/MS data of the RVLE products. PC1 occupies 75% and PC2 3.8% of total variance. (a) Score plot for drug A (squares), health foods B (diamonds) and C (open circles) with triplicated. Samples from drugs and health foods were classified in one DR and three HF clusters, respectively. (b) Corresponding loading plot. The variables that strongly contributed to the class separation are labeled.

**Fig. 3.** Comparison of flavonol contents from package to package.

**Fig. 4.** Contents of resveratrol (a), pterostilbene (b) and ε-viniferin (c) in the drugs and health foods.
Fig. 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_1$</th>
<th>$R_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin 3-O-glucuronide (Q3GA)</td>
<td>Glucuronic acid</td>
<td>H</td>
</tr>
<tr>
<td>Quercetin 3-O-glucoside (Q3G)</td>
<td>Glucose</td>
<td>H</td>
</tr>
<tr>
<td>Kaempferol 3-O-glucoside (K3G)</td>
<td>Glucose</td>
<td>OH</td>
</tr>
</tbody>
</table>

$R = H$  Resveratrol  
$R = \text{CH}_3$  Pterostilbene

$\varepsilon$-Viniferin
Fig. 2a

![Graph showing PCA analysis with PC1 and PC2 axes, clusters labeled DR, HR1, HR2, and HR3.](image-url)
Fig. 4a

![Graph showing the distribution of (mg/day) for different samples labeled A, B, and C. The graph includes error bars indicating variability.](image-url)
Table 1. RVLE samples used

<table>
<thead>
<tr>
<th>Brand ID</th>
<th>Sample ID</th>
<th>Lot No.</th>
<th>Expiration date</th>
<th>Packages</th>
<th>Daily intake</th>
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<tbody>
<tr>
<td>A (OTC drug)</td>
<td>A-59</td>
<td>D0115901</td>
<td>2016.04</td>
<td>2</td>
<td>2 capsules 450 mg (RVLE 360 mg)</td>
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<tr>
<td></td>
<td>A-60</td>
<td>D0116001</td>
<td>2016.04</td>
<td>2</td>
<td></td>
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<tr>
<td></td>
<td>A-53</td>
<td>D0215301</td>
<td>2016.07</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A-54</td>
<td>D0215401</td>
<td>2016.07</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A-33</td>
<td>D0303301</td>
<td>2016.10</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>B (health food)</td>
<td>B-12</td>
<td>12/A</td>
<td>2015.08</td>
<td>2</td>
<td>2 capsules 520 mg (RVLE 360 mg)</td>
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<tr>
<td></td>
<td>B-7</td>
<td>7/A</td>
<td>2016.01</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>C (health food)</td>
<td>C-17</td>
<td></td>
<td>2017.07</td>
<td>2</td>
<td>2 tablets 600 mg (RVLE 360 mg)</td>
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<tr>
<td></td>
<td>C-18</td>
<td></td>
<td>2018.02</td>
<td>2</td>
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### Table 2. LC-MS/MS MRM parameters for flavonoid and stilbene derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT (min)</th>
<th>MRM transitions (m/z)</th>
<th>Cone voltage (V)</th>
<th>Collision energy (eV)</th>
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<tr>
<td><strong>Flavonol derivatives</strong></td>
<td></td>
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<tr>
<td>Quercetin 3-O-glucuronide (Q3GA)</td>
<td>6.62</td>
<td>479.26 &gt; 303.01</td>
<td>68</td>
<td>38</td>
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<tr>
<td>Quercetin 3-O-glucoside (Q3G)</td>
<td>6.68</td>
<td>465.30 &gt; 303.01</td>
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<td>38</td>
</tr>
<tr>
<td>Kaempferol 3-O-glucoside (K3G)</td>
<td>7.52</td>
<td>449.48 &gt; 287.02</td>
<td>74</td>
<td>30</td>
</tr>
<tr>
<td><strong>Stilbene derivatives</strong></td>
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<tr>
<td>Resveratrol</td>
<td>8.81</td>
<td>229.05 &gt; 107.00</td>
<td>40</td>
<td>20</td>
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<tr>
<td>ε-Viniferin</td>
<td>11.26</td>
<td>257.14 &gt; 133.16</td>
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<td>18</td>
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<tr>
<td>Pterostilbene</td>
<td>11.36</td>
<td>455.13 &gt; 107.08</td>
<td>50</td>
<td>36</td>
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</table>
Table 3. Dispersion of flavonol and stilbene contents in drug and health foods

<table>
<thead>
<tr>
<th>Compound</th>
<th>Drug A (n = 9)</th>
<th>Food B (n = 4)</th>
<th>Food C (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average (mg/day)</td>
<td>Variance</td>
<td>Average (mg/day)</td>
</tr>
<tr>
<td>Q3GA</td>
<td>12.7</td>
<td>0.112</td>
<td>89.54</td>
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<tr>
<td>Q3G</td>
<td>14.3</td>
<td>0.124</td>
<td>17.4</td>
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<tr>
<td>K3G</td>
<td>2.29</td>
<td>0.0071</td>
<td>3.13</td>
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<tr>
<td>Resveratrol</td>
<td>0.773</td>
<td>0.00437</td>
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<td>ε-Viniferin</td>
<td>0.0103</td>
<td>0.00000234</td>
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<td>Pterostilbene</td>
<td>0.000233</td>
<td>uncalculated</td>
<td>0.0263</td>
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