Comparative Analysis of Concentrations of *trans*- and *cis*-Resveratrol in Must and Wine from Eleven Different Grapevines Grown in Hokkaido, Northern Japan

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Abstract : It has been suggested that resveratrol (RT) has a protective effect against cardiovascular diseases. We analyzed the concentration of RT in must and wine samples made from nine varieties of red and two of white grapes grown in Hokkaido, Japan, by a combination of normal and reverse phase high-performance liquid chromatography. The sum of *trans*- and *cis*-RT concentrations in the must differed significantly among the grapes, ranging from 0.04 to 0.41 mg/L. After alcoholic fermentation, the RT concentrations in the red wines increased to 0.50~2.76 mg/L due to maceration with skin, but those in the white ones scarcely changed. The ratios of *trans*-RT to the *cis*-form in the must samples were in the range of 3.0 to 12.0, but an increasing level of *cis*-RT was usually observed in the wine samples. In red wine samples, after malolactic fermentation, the RT concentrations were found to be increased 1.1- to 1.9-fold compared with those measured at the end of alcoholic fermentation. In contrast, the amounts of piceid (RT glucoside), quantified as the RT equivalent after β -glucosidase digestion, decreased in the wines. It was therefore assumed that the increase in RT might have resulted from the hydrolysis of piceid during malolactic fermentation.

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1 Introduction

Resveratrol (3,5,4'-trihydroxystilbene, RT), isolated from the roots of *Polygonum cuspidatum* ('Kojohkon', an oriental traditional medicine) as one of its pharmacologically active principles, was reported to have lipid-lowering activity (1) and an inhibitory action on cyclooxygenase and lipoxygenase (2). The stilbene compound also possesses antibacterial and antifungal activities (3) as well as antiplatelet aggregation activity (4). RT is originally known to be a precursor of phytoalexins of the *Vitis* species, induced in leaves by the infection of gray mold or abiotic stress (3,5). Since RT

is also synthesized in the skin of grape berries (6,7), physiologically significant amounts of RT are detected in wine (8,9).

As well as other polyphenolics including flavonoids, RT has a strong antioxidant potential, even higher than α -tocopherol as shown in *in vitro* studies (10). Although representative polyphenolics in wine are catechin and epicatechin (11) with striking effects in inhibiting low-density lipoprotein oxidation (12), RT in wine has become the subject of a growing number of studies because of its possible anticarcinogenic and anti-atherogenic effects mainly due to the antioxidant (10) and antiplatelet aggregation activities (4,9). In addition, its anti-tumor effect has been recently reported (13). Many studies on the determination of RT in wines have been carried out (8,14-30), but most were performed on

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commercially available wines from different production areas, vintage years, and technological processes. Moreover, there is a wide variation in the RT content among wines, as reported previously, probably due not only to grape varieties but also to environmental factors in grapevine cultivation (14-16) and to winemaking techniques including aging methods (15,17,18). The choice of variety in particular seems to be primarily important for the RT concentration in wine.

Hokkaido, the northern part of Japan with a relatively cool climate in the summer is taken note as an appropriate district for the culture of grapes. Grapes harvested in Hokkaido are largely used for winemaking, compared with other cultivation areas in Japan. In order to elucidate the varietal differences and characteristics of grapes grown in Tokachi district, Hokkaido, we examined the RT content in the must and wine produced from ten grape cultivars and the wild grape.

In this study, RT (*trans*-RT) together with the *cis*-isomer (*cis*-RT) was determined by high-performance liquid chromatography (HPLC). Besides both the above stilbenes, RT glucoside (resveratrol-3-*O*- β -glucopyranoside, piceid) which is found in grape skins (31), has also been detected in wine (18,22-25,32,33). Since alcoholic yeast and lactic acid bacteria involved in winemaking processes have the potency to produce β -glucosidases (18,33,34), piceid moved from grape skins to wines might be expected to be partially hydrolyzed to

RT during the processes of fermentation and storage, which should be another factor affecting the RT content in wine (18,29,33).

2 Materials and Methods

2.1 Must and Wine Samples

Eight red grape cultivars, Kiyomi (a mutant of Siebel-13053, a French hybrid), Zweigeltrebe, IK 574, IK 567, IK 594, IK 667, IK 612, and IK 3197, and the wild grape (*yama-budou*, a wild grapevine grown in the eastern part of Hokkaido, *Vitis amurensis* RUPR), in addition two white cultivars (Zalagyongye and Morio muskat), were used in the present study. The IK series of IK are hybrids of the wild grape and Kiyomi. The grapes were harvested in vineyards in Tokachi, Hokkaido, Japan, in 1995, except for the wild grape, which was collected from fields in the Tokachi area. After destemming and crushing the grapes, the must from the red grapes was obtained before fermentation by filtration under slightly reduced pressure. Must from the white grapes was sampled after pressing and centrifugation in the winery of the Tokachi-Ikeda Research Institute for Viticulture and Enology. The wine samples collected after alcoholic and malolactic fermentation at the winery were immediately assayed for RT content. General analytical results for the musts and wines tested are shown in Table 1.

Table 1 General Analytical Values of Musts and Wines after Alcoholic Fermentation.

Variety	Must		After alcoholic fermentation			
	pH	Sugar ^a (g/100 mL)	Alc ^b (%, v/v)	Ext ^c (g/100 mL)	pH	TA ^d (g/L)
Kiyomi	3.14	15.0	11.7	3.1	3.3	12.8
Wild grape	2.79	13.7	10.9	3.9	3.0	19.3
Zweigeltrebe	3.03	16.9	12.2	3.4	3.1	8.5
IK 574	2.97	18.7	10.7	4.2	3.3	14.9
IK 567	2.97	19.4	11.4	3.8	3.4	13.4
IK 594	3.04	17.1	11.6	3.9	3.3	14.7
IK 667	2.92	19.2	11.4	4.0	3.3	14.5
IK 612	3.04	15.4	10.6	4.2	3.2	13.4
IK 3197	3.04	19.4	11.1	3.8	3.3	13.6
Zalagyongye	2.99	16.5	11.8	3.6	3.1	10.3
Morio muscat	3.16	16.0	12.0	2.7	3.1	13.8

^aSugar content determined in °Brix.

^bAlcohol.

^cSolid extracts.

^dTotal acidity expressed in tartaric acid equivalent.

2.2 General Analysis of Musts and Wines

The concentrations of sugar, alcohol, solid extracts, total acidity and the pH of musts and wines were analyzed according to the Official Method of the National Tax Administration (35). Total phenols as the gallic acid equivalent were determined by the Folin-Ciocalteu method (36).

2.3 Extraction of RT

Twenty five mL of the must (or wine) was extracted three times by stirring with 15 mL of ethyl acetate and centrifuged to separate the organic phase. The extracts were filtered through Chromatodisk (0.2 μ m, Advantec). The filtrate was concentrated in a rotary evaporator, transferred into a brown test tube, and then evaporated to dryness with nitrogen gas. The residue was dissolved in 750 μ L of chloroform-methanol (2 : 1, v/v), and then an aliquot was subjected to HPLC analysis. The above procedure was done under subdued light to minimize photochemical isomerization of *trans*-RT to the *cis*-form.

2.4 Preparation of Piceid Fraction and Enzymatic Hydrolysis

The piceid fraction in the wine was prepared according to the methods reported by Sato *et al.* (24) and Mattivi (26) with a slight modification. One mL of the wine was neutralized, diluted to 10 mL, and then applied to a C₁₈ Sep-Pak Cartridge (Waters Assoc.), which was first washed with 1/30 M phosphate buffer (pH 7.0) and purged with nitrogen gas to remove the remaining buffer in the cartridge. Piceid and RT were eluted with methanol (30 mL) from the cartridge. The eluates were evaporated to dryness with nitrogen gas. The residue was hydrolyzed with β -glucosidase from sweet almonds (Boehringer Mannheim) in 0.5 M phosphate buffer (pH 5.5) at 37°C for 6 h in a nitrogen atmosphere (31,32). RT in the reaction mixture was extracted with ethyl acetate and analyzed by HPLC. The piceid content was calculated as the RT equivalent by subtracting the RT content in the piceid fraction before the enzymatic hydrolysis.

2.5 HPLC Analysis for the Determination of RT

Standard *trans*-RT was purchased from Sigma Chemical Co. *cis*-RT was prepared from *trans*-RT by UV irradiation (8,20).

Ten μ L of the wine (or must) extracts was first fractionated by normal phase HPLC to obtain the RT-

enriched fraction. The eluate was evaporated to dryness under a stream of nitrogen gas, and the residue was dissolved in 50 μ L of ethanol. Aliquots (40 μ L) were then applied to reverse phase HPLC. Normal phase HPLC was performed with a Shimadzu LC 6A liquid chromatograph equipped with a UV detector and a Superspher Si60 column (4 $\times$ 250 mm, Merck). Dichloromethane-methanol (97 : 3, v/v) was used as the mobile phase at 40°C and a flow rate of 1 mL/min. The system for reverse phase HPLC was as follows : instrument, Tosoh-CCPD with a UV detector ; column, TSK-GEL ODS-80Ts (4.6 $\times$ 250 mm, Tosoh) ; mobile phase, acetonitrile-acetic acid-water (29.9:0.1:70.0, v/v/v) ; flow rate, 1 mL/min. HPLC chromatograms were monitored by UV at 280 nm. Three replicated experiments were carried out for each sample.

The concentration of RT was quantified with a calibration curve of the authentic *trans*-RT. For the calculation of *cis*-RT content with the curve, an appropriate correction factor (1.33, which was based on the absorption ratio at 280 nm between *trans*- and *cis*-RT of 1.00:0.75), was determined by the HPLC analysis of standard *trans*-RT and its *cis*-isomer converted from the *trans* one.

2.6 Thin-Layer Chromatography

Thin-layer chromatography (TLC) was done on plates of silica gel (Wakogel B5, Wako Pure Chemicals) with toluene-ethyl formate-formic acid (5 : 4 : 1, v/v/v) as a developing solvent. Spots were detected with 50% sulfuric acid after charring.

3 Results

3.1 Separation and Identification of RT in Must and Wine Samples

Normal phase HPLC of the ethyl acetate extracts of the must and wine samples showed two peaks with the same elution volumes as those of standard *trans*-RT and its *cis*-form (Fig. 1, left). To separate precisely and identify both the RT peaks, *trans*- and *cis*-RT peaks were combined, concentrated and then re-analyzed by reverse phase HPLC (Fig. 1, right). The UV spectra of *trans*- and *cis*-RT recovered from the reverse phase column were respectively identical with those of the authentic standards (data not shown) (37). Moreover, TLC of the two isomers revealed a single spot with the same *R_f* value as those of the authentic ones, although they had similar mobility (*R_f* : 0.51) on the TLC plate. *trans*- and *cis*-RT in the extracts from the must and wine

Fig.1 Normal and Reverse Phase HPLC Elution Patterns of Ethyl Acetate Extracts of Must and Wine Samples Made from the Wild Grape (Upper) and IK 594 (Lower) Grown in Tokachi, Hokkaido.

trans and *cis* mean *trans*- and *cis*- resveratrol (RT), respectively. The RT fractions, shown by closed arrows, were collected by normal phase HPLC (left side), concentrated, and then injected into reverse phase HPLC (right side). HPLC conditions are described in Material and Methods.

samples were thus confirmed to be completely separated from other compounds by the combination of normal and reverse phase HPLC.

3.2 Concentrations of RT Isomers in the Must

The concentration of *trans*- and *cis*-RT as well as total phenols in the must samples made from nine varieties of grapes for red wine and two for white wine harvested in Tokachi, Hokkaido, are shown in Table 2. Total RT content (the sum of *trans*- and *cis*-RT) ranged from 0.04 to 0.44 mg/L, differing widely among the grapes analyzed. High RT-containing grapes were Kiyomi, IK 567 and the wild grape. The RT content in the must made from white grapes (Zalagyongye and Mario

muskat) was low (0.06 and 0.13 mg/L), but the must from red grapes such as Zweigeltrebe, IK 574, IK 594, IK 667 and IK 3197 was also poor in RT (0.04~0.14 mg/L), so that no distinction between red and white grapes was found on the RT content in must. Soleas *et al.* reported that the concentrations of RT in the skins of some white grape varieties were in the same range or greater than those of many red grape skins analyzed (16).

The concentrations of *cis*-RT in the must were usually lower than those of the *trans*-form, which would be acceptable because *cis*-RT is considered to be isomerized from *trans*-RT by sunlight exposure (20), but ratios of *trans*/*cis* RT were not constant among the grapes, rang-

Table 2 Concentrations of Resveratrol and Total Phenols in Musts and Wines after Alcoholic Fermentation.

No.	Grape variety	Must				Wine				Wine/Must ^a	
		Resveratrol(mg/L)		Total phenols (g/L)		Resveratrol(mg/L)		Total phenols (g/L)		Total resveratrol	Total phenols
		<i>trans</i>	<i>cis</i>	<i>trans/cis ratio</i>	Total	<i>trans</i>	<i>cis</i>	<i>trans/cis ratio</i>	Total		
1	Kiyomi	0.36±0.04	0.08±0.03	4.5	0.44	1.52±0.07	2.19±0.17	0.7	3.71	8.4	1.6
2	Wild grape	0.25±0.07	0.05±0.01	5.0	0.30	0.58±0.10	0.82±0.20	0.7	1.40	4.7	1.2
3	Zweigeltrebe	0.04±0.03	<0.01		0.04	0.24±0.01	0.47±0.07	0.5	0.71	17.8	2.1
4	IK 574	0.06±0.02	0.02±0.01	3.0	0.08	0.65±0.09	0.57±0.15	1.1	1.22	15.3	1.4
5	IK 567	0.27±0.04	0.12±0.05	2.3	0.39	0.77±0.14	0.64±0.05	1.2	1.41	3.6	1.7
6	IK 594	0.10±0.01	0.04±0	2.5	0.14	0.47±0	0.72±0.07	0.7	1.19	8.5	1.9
7	IK 667	0.07±0.01	0.03±0	2.3	0.10	0.81±0.12	0.59±0.04	1.4	1.40	14.0	1.5
8	IK 612	0.16±0.07	0.03±0	5.3	0.19	0.47±0.02	0.60±0.01	0.8	1.07	5.6	1.3
9	IK 3197	0.07±0.02	0.01±0	7.0	0.08	0.37±0.04	0.39±0.05	0.9	0.76	9.5	1.4
10	Zalagyongye	0.12±0.05	0.01±0	12.0	0.13	0.12±0.01	0.09±0.03	1.3	0.21	1.6	0.7
11	Morio muscat	0.05±0.01	0.01±0	5.0	0.06	0.08±0	0.07±0.01	1.1	0.15	2.5	0.8

Results are expressed as means±SD of triplicate analysis. Sample numbers from 1 to 9 are red varieties and the others white ones.

^aRatio of total resveratrol or phenols in wine to that in must.

ing from 3 to 12. Among the grapes tested, IK 567 had the highest *cis*-RT content (0.12 mg/L), and lower *cis*-RT levels were detected in Zweigeltrebe, IK 3197, Zalagyongye, and Morio muskat.

The total phenols in the musts was in the range 0.45~1.75 g/L (Table 2). Of the eleven grape varieties, IK 612 and the wild grape were rich in total phenols (1.75 and 1.70 g/L, respectively); both the grapevines also had relatively high RT levels (0.19 and 0.30 mg/L), but there was no significant correlation found between total RT and phenol in the must by a simple regression analysis.

3.3 Concentrations of RT Isomers in the Wine after Alcoholic Fermentation

The concentrations of RT and total phenols in nine red wines and two white wines measured at the end of alcoholic fermentation are shown in Table 2. Wine samples from Zalagyongye and Morio muskat grapes were produced without maceration. Total RT in the red wines was in the range 0.71 to 3.71 mg/L, which was 4- to 18-fold higher than in the corresponding must because of the further extraction of RT from skin during maceration (8,15,20,27). The differences in RT content among the grape varieties could reflect on the ability to synthesize RT in the berries. On the other hand, the RT concentration in the white wines was lower (0.15 and 0.21 mg/L), since it did not increase substantially during alcoholic fermentation. In all the grapes the proportion of *cis*-RT always increased after alcoholic fermentation, the ratios of *trans/cis* RT isomers in the wine samples being lower than those in the correspond-

ing must (Table 2). This may mean that *trans*-RT extracted from skins was partially converted to the *cis*-isomer during fermentation (21).

When the relationship of the amount of RT in must and wine samples were assayed, there was a moderate correlation ($r=0.768$, $p<0.05$), so that a grape containing a lot of RT in its must may be roughly expected to yield a red wine containing a high RT level. This needs further investigation.

3.4 RT in Red Wine Samples after Malolactic Fermentation

The amounts of both RT isomers in the red wines after malolactic fermentation were found to be 0.80~5.64 mg/L (Table 3), being higher than those measured at the end of alcoholic fermentation (Table 2). It was notable that RT was increased by malolactic fermentation in the eight grape varieties. Pezet & Cuenat also reported that the amount of RT in Gamay wine increased two-fold after malolactic fermentation (29). On the other hand, after the fermentation, the concentrations of total phenols in the red wine significantly decreased, probably due to racking (Tables 2 and 3).

3.5 Determination of Piceid in the Wine

HPLC profiles of the methanol fractions, obtained by solid phase extraction of the red wine produced from Kiyomi are shown in Fig. 2. Increases in *trans*- and *cis*-RT peaks were both recognized in the chromatogram after enzymatic hydrolysis with β -glucosidase (Fig. 2, left), compared with those analyzed before the hydrolysis (Fig. 2, right). This means that two piceid isomers

Table 3 Concentrations of Resveratrol and Total Phenols in Red Wines after Malolactic Fermentation.

Grape variety	Resveratrol(mg/L)			<i>trans/cis</i> ratio	After/Before ^a	Total phenols(g/L)
	<i>trans</i>	<i>cis</i>	Total			
Kiyomi	2.05±0.09	3.59±0.25	5.64	0.6	1.5	1.45±0.05
Zweigeltrebe	0.62±0.01	0.56±0	1.18	1.1	1.7	1.61±0.02
IK 574	0.85±0.10	0.84±0.07	1.69	1.0	1.4	1.32±0.01
IK 567	1.55±0.13	1.12±0.09	2.67	1.4	1.9	1.60±0.01
IK 594	0.77±0.01	1.45±0.07	2.22	0.5	1.9	1.87±0.05
IK 667	0.92±0.03	1.00±0.05	1.92	0.9	1.4	1.72±0.01
IK 612	0.83±0.07	1.13±0.11	1.96	0.7	1.8	1.77±0.02
IK 3197	0.21±0.04	0.59±0.08	0.80	0.4	1.1	1.73±0.02

Results are expressed as means±SD of triplicate analysis.

^aRatio of total resveratrol contents in wines after malolactic fermentation to those before malolactic fermentation measured at the end of alcoholic fermentation.

Fig. 2 Reverse Phase HPLC Elution Patterns of Resveratrol from Piceid Fraction.

The left chromatogram is before and the right one is after hydrolysis with β -glucosidase. Piceid contents were determined as the amounts of resveratrol(RT) by subtracting peak areas of RT before the β -glucosidase treatment from those after the hydrolysis. RT peaks are shown as *trans* and *cis*.

(the glycosides of *trans*- and *cis*-RT) liberated corresponding RT.

The concentrations of piceid (shown as the RT equivalent), calculated from the difference between the RT content measured before and after hydrolysis with β -glucosidase, were 1.8~8.9 mg/L in the four wine sam-

ples measured at the end of alcoholic fermentation (Fig. 3), which was in the range (0.75~9.20 mg/L) of those in the domestic commercial wines analyzed by Sato *et al.* (24). After malolactic fermentation the piceid content showed a notable decrease to a level of 0.9~3.9 mg/L (Fig. 3), indicating that the hydrolysis of piceid to RT had taken place during the fermentation (18).

Fig. 3 Piceid Contents in Wine Samples after Alcoholic and Malolactic Fermentation.

The left and right bars in each grapevine represent the piceid contents, shown as the resveratrol equivalent, in wine samples after alcoholic and malolactic fermentation, respectively.

4 Discussion

RT is a precursor of viniferins (phytoalexin) induced by the infection of pathogenic fungi (3,5). Grapes are generally known to produce a high level of RT under increasing fungal disease stress (38). Hokkaido, the northern part of Japan, experiences a relatively low-temperature, low-humidity climate during the growth stage of grapes (from June to September), which seems to be under the low pressure for gray mold spreading, so that wine made from grapes harvested in Hokkaido would be expected to have a low RT level. In the present study the total RT content (the sum of *trans*- and *cis*-isomers) in red wine samples after alcoholic fermentation was in the range 0.76~3.71 mg/L. The RT levels were consistent with those (0.13~4.91 mg/L), reported by Sato *et al.*, in the commercial red wines made from grapes grown in different places in Japan (24).

Moreover, Sato *et al.* reported that the red wines

made from Zweigeltrebe, grown in Tokachi district, Hokkaido, as well as Pinot noir and Merlot, had a high RT content (24), but our data showed that the red wine made from Zweigeltrebe was poor in RT (0.71 mg/L). This means that environmental factors, in addition to cultivars and enological practices, affected the RT concentration in red wine made by the traditional winemaking process. To clarify the effects of climatic factors, we have analyzed Kiyomi vintages from 1982 to 1992, and the results will be communicated in a separate paper (39).

The present study showed that relative proportions of *cis*-RT in the must usually increased after alcoholic fermentation, suggesting enzymatic isomerization by the yeast used in the fermentation process. It is also possible that the isomerization of *trans*-RT by sunlight exposure takes place in the must as well as in the grape berries during winemaking processes to increase the *cis*-RT level in wine, but Jeandet *et al.* reported that 41% of total RT was the *cis*-form in wine vinted in the dark, whereas *cis*-RT represented 46% in that which was not protected from light (15). This observation indicates that photochemical *trans-cis* isomerization of RT hardly affects the notable amounts of *cis*-RT found in wine.

The RT concentration in the red wine samples after malolactic fermentation was found to be higher than those in the corresponding wines at the end of alcoholic fermentation (Table 3). The same tendency as above was also reported in the case of the wine made from Gamay grapes (29). On the other hand, since the concentration of piceid was shown to decrease (Fig. 3), it was assumed that the increase in RT resulted from the enzymatic hydrolysis of piceid by microorganisms involved in malolactic fermentation. Conversely, Vrhoussek *et al.* reported that malolactic fermentation had less effect on the concentrations of RT and piceid (18). This discrepancy could be attributed to different activities of β -glucosidases produced by lactic acid bacteria (34) responsible for malolactic fermentation. The kinetics of RT and piceid in wines during malolactic fermentation, and the influence of storage on their concentrations will be described in a subsequent paper (39).

We first determined the RT content in healthy grape leaves, but no substantial difference (67~69 μ g/100 g fresh weight) was found between Kiyomi (the largest amount of RT among the red wine samples analyzed) and Zweigeltrebe (poor in RT). The same tendency was also observed in Pinot noir, which was reported to have a high RT content in wine (24), but not in leaves irradiated by UV (40). Moreover, healthy IK 567 leaves

had a higher RT level (192 μ g/100 g leaves) than two other varieties of grape leaves did, so that no direct relationship in RT content was observed between healthy grapevine leaves and the wine made from their grape berries.

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