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Preformulation Stability of *trans*-Resveratrol and *trans*-Resveratrol Glucoside (Piceid)

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Resveratrol and resveratrol glucoside (piceid) were evaluated in a preformulation stability study. An HPLC assay was used for the analysis of stressed/reference samples. Samples of solid, crystalline material were held under the following conditions: 40 °C/75% RH (both open and protected), ambient fluorescent light (open), 70 °C (open), and exposed using a light cabinet to achieve ICH conditions for UV/fluorescent light. Both compounds were found to be stable out to 3 months for both accelerated and ambient conditions with negligible degradation. Exposure to UV and fluorescent light under ICH conditions did not significantly degrade the solid materials for UV exposure at 3 times the ICH limit and for fluorescent light exposure at 1 times the ICH limit. The results presented demonstrate crystalline resveratrol and piceid are stable solids. No evidence of oxidation of either material by atmospheric oxidants was seen. The data reported may help to clarify widely held beliefs that resveratrol is unstable and extremely sensitive to oxidation/degradation.

INTRODUCTION

Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) is a natural product widely taken as a nutritional supplement. Although often associated with grapes and wine (*1*−*3*), it has been found to occur in peanuts, hop cones, tomatoes, and other plants (*4*−*7*). Structurally, resveratrol is a low molecular weight, phytoalexin, polyphenol, stilbene known to have poor aqueous solubility (*8*). In plants a major form of resveratrol is *trans*-resveratrol-3-*O*-β-D-glucoside, often referred to as piceid or polydatin (**Figure 1**). Glucosides of resveratrol are reported to be a major form of the total resveratrol content of unfermented grape juice and wine (*3*, *9*). Isolated resveratrol and resveratrol glucoside of high purity are available commercially and often originate from Japanese knotweed (*10*). Resveratrol is considered to be a nutritional supplement and is not regulated as a drug in the United States.

Resveratrol has been reported to affect various in vitro, cellular, and animal models. Resveratrol has been found to modulate estrogen receptors α and β (*11*) and inhibit aromatase in an in vitro study of breast cancer cells (*12*) and has been shown to prevent cancer in mice (*13*). Additional studies suggest resveratrol may play a role in inhibiting lipid peroxidation and retarding vascular oxidative stress (*14*−*16*), leading to speculation that it may play a role in the "French paradox" (*17*). It has been suggested that resveratrol may not act directly as an antioxidant but may play a role as a signaling molecule (*18*). Resveratrol affects bacterial phagocytosis (*19*), has been found to inhibit COX-1 (*20*), and may act as a neuroprotective agent through attenuation of β-amyloid induced cell death (*21*). It is known that resveratrol activates SIRT1 (*22*, *23*), suggesting that it may play a role in regulating life spans of eukaryotes.

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The extensive in vitro and in vivo resveratrol literature has been reviewed (*24*, *25*). More recently, Barger et al. presented data that showed through gene expression profiling that low doses of resveratrol may retard aging in mice (*26*). In humans its putative efficacious effects include anticancer and antiaging activities and the ability to slow the progression of cardiovascular disease and diabetes. These purported effects have not been proven in clinical trials.

Resveratrol has poor metabolic stability in humans, with human pharmacokinetic data showing higher levels of sulfate and glucuronide metabolites compared to the parent compound (*27*, *28*). Another aspect of stability is the in vitro solution stability of resveratrol in the absence of enzymes. Solution instability would certainly affect a variety of laboratory assays as well as dosage formulations containing dissolved resveratrol. For example, de Boer et al. suggested for a series of polyphenols that the stability of the substrate in the incubation medium modulated SIRT1 activity. The authors propose that polyphenol oxidation may limit SIRT1 activation (*23*). It is well-known that *trans*-resveratrol is unstable in solution when exposed to light (*29*) and readily isomerizes to the cis isomer (**Figure 1**). To preserve the antioxidant properties of resveratrol, Nam et al. immobilized the molecule on polymeric microspheres and found the antioxidant activity to be preserved for aged samples in ethanolic media (*30*), thus demonstrating a strategy to stabilize resveratrol in solution. Shi et al. has more recently described encapsulation within yeast cells as a technique for stabilizing solid resveratrol (*31*).

Pharmaceutical scientists are particularly concerned with the solid state stability of active pharmaceutical ingredients (APIs) and formulated drugs. As resveratrol isomerizes in solution and is believed to react with various oxidants, it is important to understand how stable solid resveratrol and resveratrol glucoside are under various controlled conditions commonly evaluated in the preformulation stage of product development. This work is of

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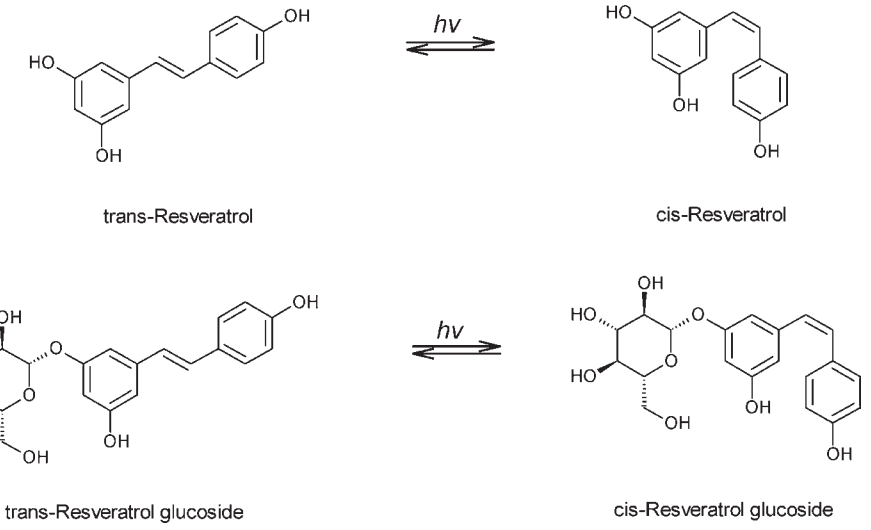

Figure 1. *trans*-Resveratrol (top) and *trans*-resveratrol glucoside (bottom) with the dominant primary photolysis products: *cis*-resveratrol and *cis*-resveratrol glucoside.

Table 1. HPLC Conditions for Stability-Indicating Assay

mobile phase A	water
mobile phase B	methanol
gradient	initial 100% A
	20 min 100% B (linear gradient)
	21−25 100% A (reequilibration)
wavelength	306 nm
flow rate	1.4 mL/min
column temperature	35 °C
sample temperature	ambient
injection volume	10 μL
run time	20 min
nominal concentration (standards and samples)	0.23 mg/mL resveratrol
	0.39 mg/mL resveratrol glucoside

some importance considering that conflicting claims have emerged on the stability of resveratrol. For a discussion of stability issues, see Prokop et al. (*32*). In this study we have evaluated elevated temperature, humidity, and UV and fluorescent light. Light exposure under controlled conditions is an important aspect of product development (*33*).

MATERIALS AND METHODS

High-purity resveratrol and resveratrol glucoside were obtained from an API supplier (Interpharma Praha, Czech Republic) as off-white powders (**Figure 1**). Both compounds originated from Japanese knotweed (*Polygonum cuspidatum*). The bulk material was stored under vacuum, protected from light and elevated humidity. The water and methanol used for chromatography were of HPLC grade (Fisher Scientific). A Hypersil Gold HPLC column (Thermo Scientific, 250 mm × 4.6 mm i.d., 5 μm particle size, part 25005-254630) was used for analysis with no guard column. An Agilent 1100 series HPLC system was utilized with a diode array detector. The HP ChemStation (rev A.10.01) was used for integration and evaluation of the data. Karl Fischer analysis was accomplished using a Metrohm titrater (model KF 784 KFP Titrino) utilizing AquaStar CombiTitrant 1 (EMD). The light cabinet used was an Atlas Suntest XLS+, capable of providing simultaneous UV and fluorescent sample illumination.

HPLC Method. A stability-indicating HPLC method was developed and validated to evaluate resveratrol and resveratrol glucoside. The HPLC conditions are described in **Table 1** with a representative chromatogram in **Figure 2**. The method described is a modification of an HPLC procedure described by Trela (*29*). The method was validated for specificity, linearity, reproducibility (method precision), system precision, baseline stability, limit of quantification, and stability of samples. A summary of the

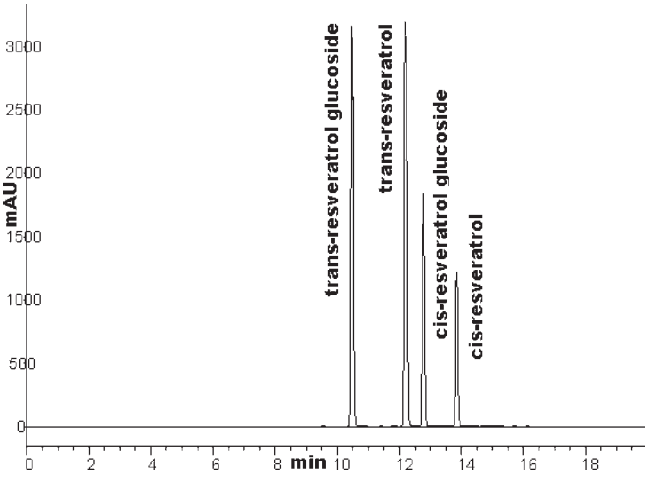

Figure 2. Chromatogram showing resolution of *trans*-resveratrol (RT 12.2 min) and *trans*-resveratrol glucoside (RT 10.5 min) from primary photolysis products (*cis*-resveratrol, RT 13.9 min, and *cis*-resveratrol glucoside, RT 12.8 min). Sample was prepared by exposure of solution (50:50 methanol/water) to UV light.

validation data is presented in **Table 2**. The relatively high nominal concentration (1 mM) was chosen to comply with ICH criteria for impurities (0.05% area compared to the main band of the chromatogram). This corresponds to a reporting threshold of 0.05% for a maximum daily dose of ≤2 g/day (*34*). The linearity of the detector response was evaluated by assessing five standard levels in the range of 80−120% of the nominal concentration (100% level). Triplicate injections were made at each standard level. Statistical analysis was done using JMP software (JMP 8.0, SAS Institute, Inc.). System precision was evaluated by making multiple injections (n = 3) of the same standard at the 100% level. Method precision was determined by assaying three separately prepared sample preparations, each injected singly, against chromatographic standards. The limit of quantification (LOQ) was determined by dilution of chromatographic standards to a concentration level of 0.05% of the 100% level (or nominal concentration). The response factors obtained at the LOQ level were within 5% of the response factors at the nominal concentration. The diluent used for sample/standard preparation was methanol/water, 50:50 (% v/v). Samples were prepared and HPLC vials filled with no filtration. To minimize analyte degradation, samples and standards were freshly prepared and assayed immediately. Precautions were taken to minimize light exposure. In this investigation the autosampler was held at ambient temperature. If available, a chilled

Table 2. Analytical Method Validation Summary[a]

	calibration curve	nominal concn (mg/ML, 100% level)	R^2	precision (%RSD)	LOQ concn (mg/mL)	%RSD at LOQ (n = 3)	S/N ratio at LOQ
trans-resveratrol	$y = 4.23 \times 10^4 x + 0.168 \times 10^4$ std error: m = 1460, y_{int} = 330 range: 80−120% of NC	0.23 (≈1 mM)	0.996	system: 0.2% method: 0.4%	0.00012	7.8	>20:1
trans-resveratrol glucoside	$y = 2.41 \times 10^4 x + 0.165 \times 10^4$ std error: m = 570, y_{int} = 220 range: 80−120% of NC	0.39 (≈1 mM)	0.998	system: 0.1% method: 0.2%	0.0002	7.0	>20:1

[a] LOQ, limit of quantification or 0.05% of nominal concentration (100% level); %RSD, percent relative standard deviation; S/N, signal to noise; NC, nominal concentration.

autosampler could be used to further quench degradation of the samples and standards.

To understand the quantitative relationship between *trans*-resveratrol and the cis form, a solution of resveratrol of known concentration was exposed to a UV/Fl lamp (surface dose rate of 72 W/m^2). The diluent used was methanol/water, 50:50 (% v/v). The resveratrol concentration was 10 mM, which was higher than the final validated method. Consequently, the injection volume was decreased to 1 μL to avoid overloading the column. The diminishment of the main band was evaluated by HPLC for samples withdrawn from the light chamber at 30, 60, 90, and 120 min. The increase in area of the cis degradant peak was also evaluated. The slope of the area versus concentration curve for the resveratrol peak was compared to the slope of the area versus concentration curve for the cis isomer. This procedure allowed for the determination of a relative response factor at the analytical wavelength of the assay. A relative response factor of 3.32 (at 306 nm) for *cis*-resveratrol was determined and used subsequently as part of the assay procedure. This value is in good agreement with measurements made by Trela et al., who determined a relative response factor of 3.426 (at 306 nm) for resveratrol by UV spectroscopy (*29*). The same procedure was used to determine a relative response factor for *cis*-resveratrol glucoside, which was found to be 3.30 at 306 nm.

Stability Study Design. Aliquots of solid resveratrol and resveratrol glucoside were stored at 40 °C/75% RH (open, dark), 40 °C/75% RH (HDPE bottle with foil induction seal), 70 °C (open, ambient humidity, dark), and ambient (open, ambient humidity, laboratory fluorescent light). Samples were evaluated at 1, 2, 4, 6, and 12 weeks. Water content for most stability samples was determined by Karl Fischer. Samples were assayed against a freshly prepared (unexposed) reference standard of resveratrol or resveratrol glucoside at the nominal concentration (100% level). The standards were prepared from material held under vacuum and protected from light and humidity. Sample preparation was done using approximately the same amount of material as for the standards. This ensured that sample concentrations were close to the 100% level. Samples were bracketed by chromatographic standards at the nominal concentration in the sample sequence. Mean standard response factors were used in calculations. The percent weight/weight (%w/w) assay values were determined by the following equation:

$$\%\ \mathrm{w/w} = \frac{\mathrm{concn_{standard}} \times \mathrm{area_{sample}}}{\mathrm{concn_{sample}} \times \mathrm{area_{standard}}} \times 100$$

Concentrations were corrected for water content of samples and standards. Once solutions were prepared, they were assayed immediately to minimize any degradation (conversion to cis).

Light Cabinet Studies. Solid resveratrol and resveratrol glucoside were placed in a thin layer (≈1 mm) in quartz crucibles. A light source combining UV and fluorescent exposure was used. Samples were withdrawn at 2, 4, 6, and 8 h of exposure. The light flux achieved the ICH criteria (*33*) for fluorescent light (1.2 million lx·h) at 8 h. For UV light exposure the criteria of NLT (not less than) 200 W·h/m^2 (*33*) was achieved in 2.6 h with the 8 h period corresponding to ≈3 times the ICH criteria for UV exposure. Additionally, solutions of resveratrol and resveratrol glucoside (methanol/water, 50:50, %v/v) in standard volumetric glassware were exposed to the same radiant exposure as the solid. Aliquots of solution were withdrawn at the same time points as for the solid. Both the resveratrol and resveratrol glucoside solutions were approximately 1 mM. Solid samples were also prepared to correspond to the 100% level of the assay. The diluent used for sample preparation was methanol/water, 50:50 (% v/v). Samples were assayed immediately after sample preparation to minimize any degradation. Samples were assayed against external standards at the nominal concentration prepared from unexposed reference material.

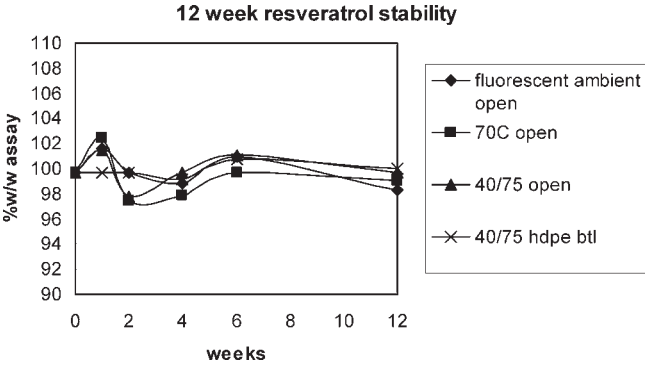

Figure 3. *trans*-Resveratrol assay values expressed on a percent w/w basis. Samples are evaluated against unexposed reference material (held under vacuum, protected from light and humidity).

Thermal Analysis. A Perkin-Elmer Diamond DSC with Pyris software was used for differential scanning calorimetry. Aliquots of 0.6−1.0 mg of resveratrol or resveratrol glucoside were placed in aluminum pans (PE part 0219-0041), crimped, and subjected to a temperature ramp of 25−300 °C at 10 °C/min. DSC thermograms were obtained to compare 3 month stressed samples to unstressed control materials.

X-ray Powder Diffraction. A Siemens D5005 wide-angle diffractometer was used for powder X-ray measurements. Approximately 20−30 mg of sample was packed into a quartz sample holder. The samples were exposed to Cu Kα radiation (1.544 Å) generated at 40 mA/45 kV with a slit arrangement of 1 mm (before), 1 mm (after), empty (after), 0.6 mm (after), and 0.6 mm (after). Materials were typically scanned from 3° to 40° two theta (mode, step; step, 0.048°; dwell, 1.0). Data were processed using MDI Jade version 7.0.

RESULTS AND DISCUSSION

The results of the stability study indicate that the solid resveratrol and solid resveratrol glucoside evaluated are stable pharmaceutical solids. The percent w/w assay compares the sample response factor against the response factor of the unexposed reference material (chromatographic standard). The variability seen in the data is only slightly greater than the method precision and is likely due to some slight difference in the actual versus measured values of water content for both standard and samples. Assay results for *trans*-resveratrol and *trans*-resveratrol glucoside essentially show 100% recovery for all four conditions evaluated for both resveratrol and resveratrol glucoside (**Figures 3** and **4**) with negligible formation of the cis isomer. No trends indicating degradation are seen for either compound. Chromatographic standards were freshly prepared from reference material stored under vacuum, protected from light and elevated humidity.

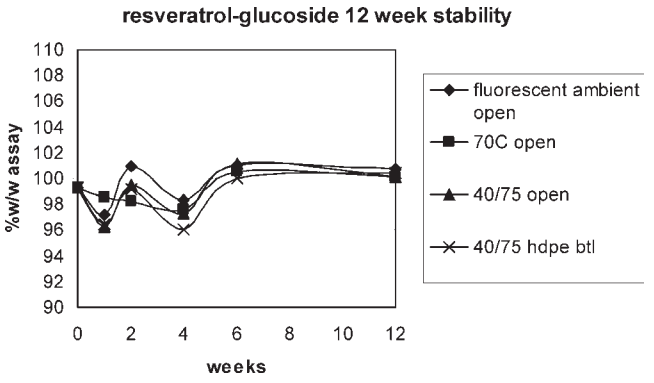

Figure 4. *trans*-Resveratrol glucoside assay values expressed on a percent w/w basis. Samples are evaluated against unexposed reference material (held under vacuum, protected from light and humidity).

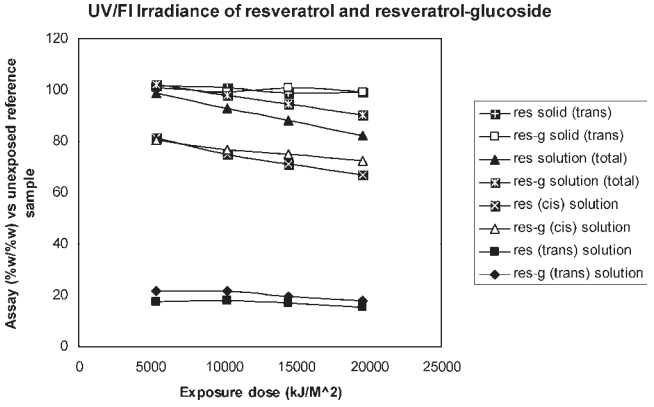

Figure 5. Results of light cabinet study for solid resveratrol, solid resveratrol glucoside, and solutions of resveratrol and resveratrol glucoside. Final time points correspond to 1 times the ICH requirement for fluorescent light and 3 times the requirement of UV light.

Table 3. Area Percent of Main Peak (Parent) versus Total Area for All Peaks in the Same Chromatogram

condition	1 week	2 weeks	4 weeks	6 weeks	12 weeks
resveratrol					
40 °C/75% RH open	99.55	99.50	98.83	99.42	99.70
40 °C/75% RH HDPE bottle	99.58	99.57	98.95	99.47	99.54
70 °C ambient open	99.66	99.58	98.90	99.55	99.62
fluorescent light ambient open	99.54	99.57	98.81	99.57	99.51
resveratrol glucoside					
40 °C/75% RH open	98.98	98.99	98.04	98.54	99.35
40 °C/75% RH HDPE bottle	98.96	99.05	98.25	98.66	99.35
70 °C ambient open	98.96	99.08	98.12	98.76	99.29
fluorescent light ambient open	99.01	99.04	98.16	98.69	99.34

If degradation resulted in reaction products that were not retained on the column (i.e., eluted in the void volume), this method would still show the loss of parent substrate. Water content analysis by Karl Fischer indicates that both resveratrol and piceid are nonhygroscopic. The water content of the resveratrol reference material did not vary significantly during the course of this study (0.1−0.2%). The stressed material had water values from 0.1 to 0.3% with no trends in the data. Control samples of resveratrol glucoside had water at 0.6−1.1%. Stressed samples had water at 0.2−1.1%. Again, no trends were seen in the data.

A related approach that is often used to assess degradation early in product development is area percent of the main (API) band compared to the total area of API and all related peaks in the same chromatogram. This method does not rely on the use of the external chromatographic standard. The area percent values corresponding to **Figures 3** and **4** are reported in **Table 3**. The results agree with the percent w/w assay values. No significant changes in the impurity profiles of solid resveratrol and resveratrol glucoside are seen for the four conditions evaluated. The area percent results also show that formation of the cis isomer is negligible for these experimental conditions and offer evidence that the crystalline orientation of resveratrol consists of the lower energy (trans) isomer.

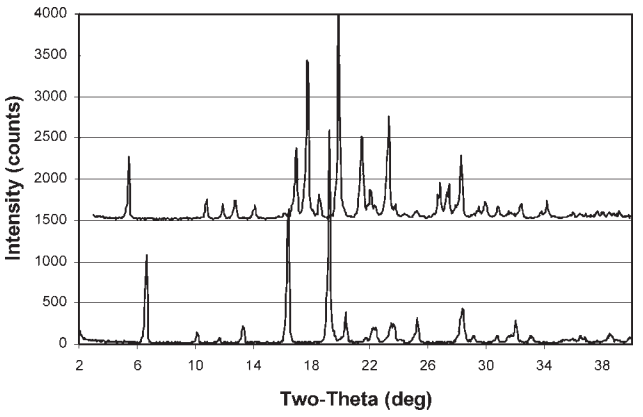

Figure 6. X-ray powder diffractograms of *trans*-resveratrol glucoside (top) and *trans*-resveratrol (bottom).

In our laboratory we have found solutions of resveratrol to be unstable, which is consistent with previous investigations (*3*, *29*). When exposed to laboratory light, the main peak diminishes with a corresponding appearance and increase in the area of a new peak at a longer retention time (**Figure 2**). This new peak is believed to be the cis isomer of resveratrol. The retention behavior, relative response factor obtained, and requirement for light to induce the reaction support our conclusion that the major degradant peak observed (from solution) is the cis isomer. This conclusion is consistent with previous observations (*3*, *29*, *35*, *36*). Resveratrol glucoside exhibits similar behavior with one dominant, new degradant peak appearing when solutions are exposed to laboratory light. Although a quantitative solution kinetics study was not undertaken, the cis degradant peak for both resveratrol and resveratrol glucoside formed somewhat slowly on a time scale of hours to days when exposed to ambient (laboratory) fluorescent light. Trela et al. reported solutions of resveratrol to be stable for days when protected from light (*29*).

It was desirable to understand how solid material would react when exposed to UV light at an irradiance consistent with ICH Q1B requirements (*33*). The results of the light cabinet study (**Figure 5**) show the solid resveratrol and resveratrol glucoside to be stable when exposed to UV and fluorescent light. No significant change is seen in the percent w/w assay values even when the ICH UV criteria is exceeded by ≈3 times. In contrast, the solutions of resveratrol and resveratrol glucoside rapidly formed the cis isomer under the combined UV and fluorescent light exposure. The surface dose rate favors an equilibrium primarily of the cis isomer (for both resveratrol and resveratrol glucoside) under these experimental conditions. Summing both the cis and trans forms accounts for most degradation early in the experiment. At the 8 h point, total amounts (sum of cis and trans forms) of both resveratrol and resveratrol glucoside are at approximately 80% of the initial concentrations (≈1 mM). The loss of the two main peaks and lower percent w/w assay values

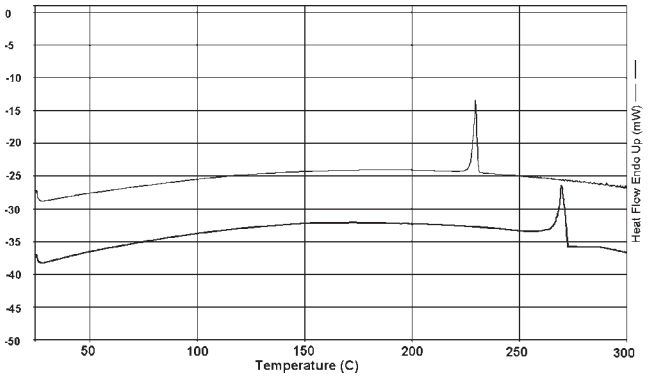

Figure 7. DSC thermograms of *trans*-resveratrol glucoside (top) and *trans*-resveratrol (bottom).

obtained are consistent with the appearance of several unidentified degradant peaks.

Both resveratrol and resveratrol glucoside evaluated in this study are crystalline by X-ray powder diffraction measurements (**Figure 6**) of the reference (unstressed) materials. The 3 month stability samples for the 40 °C/75% RH condition for both compounds were also evaluated by X-ray powder diffraction, and no changes to the diffractograms were noted. DSC was used to evaluate the reference material for both resveratrol and resveratrol glucoside (**Figure 7**). For the resveratrol control sample, a single peak corresponding to melting was observed at 269.5 °C (≈230 J/g) with no other thermal events noted. Resveratrol glucoside was evaluated with the same thermal method and showed a single melting endotherm at 229.6 °C (≈161 J/g). The 3 month stability samples were evaluated by DSC, and similar results were seen for all of the stressed samples. No significant change to the melting point was seen, and no additional thermal transitions were noted. The single, invariant melting endotherm that was observed by DSC is consistent with stable, low-energy, crystalline material.

Overall, our results are in agreement with Prokop et al., who found solid resveratrol and piceid to be stable when stored in glass vials and protected from light (*32*). Typically, in a preformulation study, samples are evaluated open with no packaging. In this case the HDPE bottle with foil induction seal was chosen as this was a less protective package than the stoppered glass vial described in the literature investigation (*32*). As no degradation was reported in the literature study (*32*), it was necessary to understand if a less protective package would be effective in stabilizing the bulk resveratrol powder; thus, the HDPE package was an intermediate level of protection compared to the open dish and closed glass vial of the previously published study (*32*). Results presented here suggest the crystalline, solid resveratrol and piceid evaluated are not particularly sensitive to UV/fluorescent light, elevated humidity, and temperature as well as atmospheric oxidants at ambient concentrations. The formation of viniferins (oxidative dimerization products) is mediated enzymatically (*37*) and is unlikely to occur under our experimental conditions (referring to both solid and solution experiments). When resveratrol and resveratrol glucoside are in solution, we have found the primary degradant to be the cis isomer, consistent with previously published studies. Monsko et al. identified an oxidative product that appeared at a low level in comparison to the *cis*-resveratrol isomer upon irradiation of ethanol/water solutions of *trans*-resveratrol (*36*). This is also consistent with results reported here as several new degradant peaks were seen for solutions exposed to UV/fluorescent light at longer exposure times. In contrast to our results, Shi et al. have recently presented data showing that solid resveratrol is unstable when exposed to light and elevated humidity (*31*).

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