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Development of a Rapid LC-UV Method for the Investigation of Chemical and Metabolic Stability of Resveratrol Oligomers

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Supporting Information

ABSTRACT: Resveratrol, piceatannol, ε-viniferin, r-viniferin, r2-viniferin, and hopeaphenol are naturally occurring polyphenols, associated with potentially beneficial health effects. We developed a rapid liquid chromatography−ultraviolet detection (LC−UV) method, allowing for the simultaneous determination of these six compounds in biological samples in less than 2.5 min with standard LC equipment. Using this method for the assessment of the stability of the six analytes, we demonstrated that all stilbene polyphenols disappear rapidly in Dulbecco's modified Eagle's medium (e.g., half-life of resveratrol of 1 h). In contrast, the tetramer hopeaphenol was stable over the maximum incubation time of 72 h. In incubations with liver microsomes, ε-viniferin was rapidly glucuronidated, although to a lower extent than resveratrol. Hopeaphenol was not glucuronidated at all. Given that glucuronidation is the major metabolic pathway for polyphenols, hopeaphenol might exhibit significantly different pharmacokinetic properties than other polyphenols. When chemical and metabolic stability as well as biological activity of hopeaphenol are taken together, these findings warrant further investigation of this polyphenol.

KEYWORDS: *Rapid LC method, stilbene polyphenols, resveratrol, piceatannol, ε-viniferin, r-viniferin, r2-viniferin, hopeaphenol, polyphenol auto-oxidation*

INTRODUCTION

The polyphenols resveratrol (*trans*-3,5,4′-trihydroxystilbene), piceatannol (*trans*-3′,4′,3,5-tetrahydroxystilbene), ε-viniferin, r-viniferin, r2-viniferin, and hopeaphenol are secondary metabolites occurring in grapes and various plant species, especially in the Vitaceae family but also in the Dipterocarpaceae family.[1−3] The structures of the compounds are depicted in Figure 1. With the exception of r-viniferin and r2-viniferin, all of them have been detected in red wine. For example, the mean stilbene polyphenol concentration in different wines from northern Africa was found to be 26 mg/mL, with a mean of 1.7 mg/L resveratrol, 0.2 mg/L ε-viniferin, and 1.4 mg/L hopeaphenol.[4] In recent years, many studies reported a variety of biological effects exhibited by these substances.[5,6] In particular, resveratrol has been extensively studied and is known to exert antioxidative, cardioprotective, and anticarcinogenic effects.[7] The potential health-promoting effects of piceatannol include antioxidant and cell cycle modulatory activities.[8] Only limited information about the biological activity of the resveratrol oligomers, ε-viniferin, r-viniferin, r2-viniferin, and hopeaphenol is available. Few studies report, aside from antioxidant and anti-inflammatory properties, effects on the growth and proliferation of different tumor cell lines (i.e., leukemia, colon, breast, and prostate cancer cells).[2,5,6] Because of their potency to drastically inhibit the growth of human tumor cell lines, the resveratrol oligomers may represent a new class of natural anticarcinogens.[9] To study these biological effects and their underlying molecular mechanisms in more detail, cell culture experiments are indispensable. However, several studies demonstrate that polyphenols, including resveratrol, rapidly degrade under cell culture conditions.[10,11] Therefore, it is necessary to evaluate the stability of resveratrol oligomers in cell culture medium before biological end points can be further investigated using cell culture systems. For the assessment of the chemical stability, analytical methods are needed, allowing for the quantitation of resveratrol and its oligomers. Numerous methods are described for the quantitation of resveratrol by liquid chromatography (LC),[12,13] but only a few methods for the quantitation of resveratrol oligomers have been published.[1,14,15] Furthermore, these methods have a limited spectrum with regard to the compounds detected; e.g., none of the methods allows the quantitation of r-viniferin and r2-viniferin. For that reason, we developed a rapid LC−ultraviolet detection (UV) method for the fast simultaneous quantitation of resveratrol, piceatannol, ε-viniferin, r-viniferin, r2-viniferin, and hopeaphenol in biological samples. This method was used to assess the chemical stability of resveratrol and its oligomers in cell culture medium under different conditions. As a second application, we employed the developed method to investigate the metabolic stability of the resveratrol oligomers toward conjugation with glucuronic acid *in vitro*.

MATERIALS AND METHODS

Chemicals and Biological Materials. *trans*-Piceatannol (99%) and *trans*-resveratrol (99%) were purchased from Sigma-Aldrich (Schnelldorf, Germany). The dimer ε-viniferin (90%) and the tetramer hopeaphenol (95%) were obtained from Actichem SA (Montauban, France). The two other tetramers, namely, r-viniferin and r2-viniferin, with a purity level of at least 90%, were a kind gift from the laboratory

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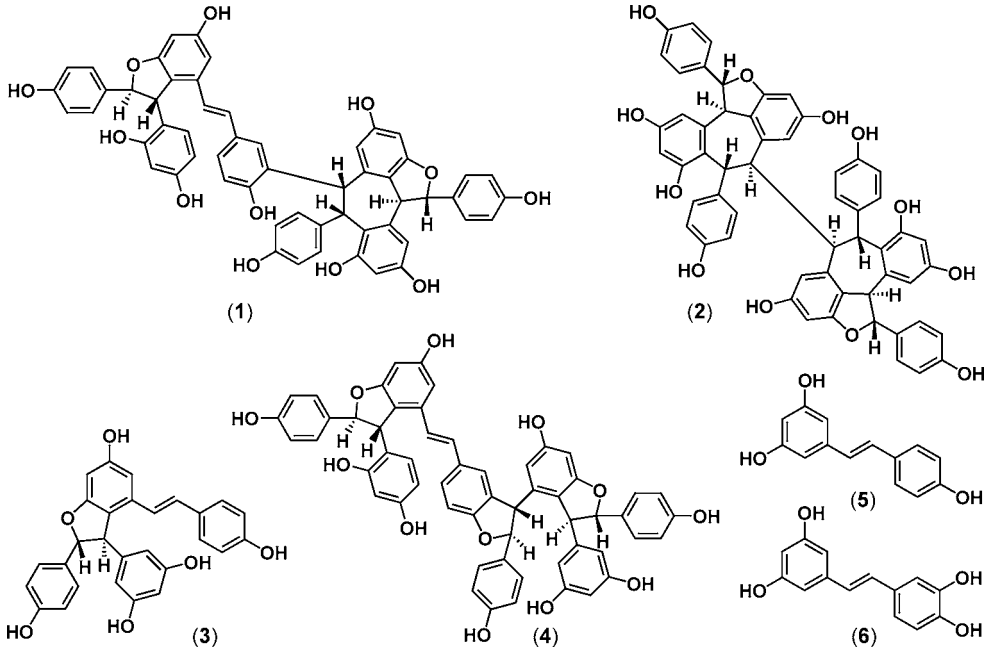

Figure 1. Structures of the analytes: r2-viniferin (**1**), hopeaphenol (**2**), ε-viniferin (**3**), r-viniferin (**4**), *trans*-resveratrol (**5**), and *trans*-piceatannol (**6**). Shown is the conformation as previously reported.

of Dr. Winterhalter of the University of Braunschweig, Germany. The chemical structures of the analytes are displayed in Figure 1. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Biochrom AG (Berlin, Germany), and all other chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany). The ingredients of the DMEM were in accordance with the suggestion of the American Type Culture Collection (ATCC).

Microsomes were obtained from BD Biosciences (Woburn, MA). Preparations of pooled human liver microsomes (HLMs) from 25 mixed gender donors and pooled rat liver microsomes (RLMs) from 150 male Sprague–Dawley rats at a concentration of 20 mg of protein/mL were used. The activity of the microsomal preparations was characterized by monitoring their ability to conjugate the standard UGT substrate 4-(trifluoromethyl)-umbelliferone (TFMU) as previously described.[16] HLMs showed an activity of 56 ± 4 nmol min^{-1} mg^{-1}, and RLMs showed an activity of 71 nmol min^{-1} mg^{-1}.

LC–UV Analysis. LC–UV analysis was performed on an Agilent 1100 system (Waldbronn, Germany). Separation was carried out on a 75 × 4.6 mm inner diameter, 2.7 μm, HALO RP-18 column with "fused core" particles. The analytes (injection volume of 10 μL) were separated by a binary gradient at a flow rate of 2.0 mL/min of 0.1% acetic acid (HOAc) as solvent A and 95:5 acetonitrile (MeCN)/water (v/v) acidified with 0.1% HOAc as solvent B. The following gradient was used: 0.0–0.2 min, isocratic 30% B; 0.2–1.8 min, linear 30–70% B; 1.8–1.9 min, linear 70–100% B; 1.9–2.5 min, isocratic 100% B; and 2.51 min, return to initial conditions of 30% B. The column was reconditioned during the next injection cycle of the autosampler (about 1 min). The analytes were detected by a photodiode array (PDA) detector operating at a detection frequency of 5 Hz with a slit of 4 nm. Piceatannol, hopeaphenol, and r2-viniferin were detected at a wavelength of 283 nm; resveratrol was detected at a wavelength of 305 nm; and the signal of ε-viniferin and r-viniferin was detected at a wavelength of 325 nm.

Quantitation was performed by external calibration of the LC–UV signal of standards using one of the resveratrol oligomers as the internal standard (IS). For the determination of resveratrol, piceatannol, ε-viniferin, and r-viniferin, hopeaphenol (2 μM) was used as the IS. For the quantitation of hopeaphenol and r2-viniferin, the closely eluting ε-viniferin (1 μM) was used as the IS.

For calibration, the polyphenols were sequentially diluted (0.3, 0.5, 1, 2, 3, 5, 8, and 10 μM) in 50:50 MeCN/water containing IS and 0.1% HOAc. The acid was added to ensure the stability of IS and analytes. The analyte/IS area ratios were fitted in a linear way reciprocally weighted by concentration.

Sample preparation was carried out by mixing equal volumes of internal standard solution (4 μM hopeaphenol or 2 μM ε-viniferin in MeCN with 0.2% HOAc) and incubation samples. The resulting mixtures were gently vortexed and centrifuged at 21000 *g* for 10 min at 4 °C. The supernatant was transferred to brown glass vials and immediately analyzed by LC–UV. Recovery rates were determined in DMEM spiked with 3, 5, and 10 μM polyphenols. These samples were analyzed within the same day as the other samples. All analyses were carried out as triplicates, and results are presented as the mean ± standard deviation (SD).

Determination of Chemical Stability during Incubation in Cell Culture Medium. The stability of the stilbene polyphenols was determined in DMEM (pH 7.4) in a polystyrene 96-well plate (Techno Plastic Products, Trasadingen, Switzerland) to mimic cell culture experimental conditions. Each well was filled with 100 μL of polyphenol solution (10 μM) in DMEM, and the plate was incubated in four different conditions: (i) at 4 °C in the dark, (ii) at 23 °C in the dark, (iii) at 37 °C in a cell culture incubator (5% CO_2) in the dark, and (iv) at 23 °C on a laboratory bench at room light (2 m away from a commercial fluorescent tube). In addition, experiments were also carried out at 23 °C in the dark in DMEM adjusted to pH 4.0 with HOAc and pH 9.0 with 1 M tris(hydroxymethyl)aminomethane. After 0.25, 0.5, 1, 3, 6, 9, 24, 48, and 72 h, 75 μL was sampled per well and analyzed by LC–UV. For the unstable piceatannol, sampling was performed additionally after 2.5, 5, 10, 15, 20, 25, 30, and 60 min. To generate *cis* isomers for the characterization of degradation products, the stilbene polyphenols were subjected to daylight from 10 min (piceatannol) to 30 min (resveratrol, ε-viniferin, r-viniferin, and r2-viniferin) as previously described.[17]

Glucuronidation Assays. The glucuronidation assay was carried out as previously described.[16] In brief, resveratrol, ε-viniferin, and hopeaphenol were incubated with microsomes in a total volume of 200 μL of 100 mM potassium phosphate buffer (pH 6.9). In a generic scheme, 10 μL of microsome solution containing 12.5 μg of protein was mixed with 96 μL of buffer and 40 μL of alamethicin solution (125 μg/mL) and placed on ice for 15 min. Alamethicin forms pores in the microsomal membrane and, therefore, increases the substrate accessibility of the uridine 5′-diphosphate-glucuronosyltransferases

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(UGTs).[18] Subsequently, 4 μL of the substrate (20 μM concentration in the assay), 20 μL of magnesium chloride, and 10 μL of the β-glucuronidase inhibitor saccharolactone (both 10 mM concentration in the assay) were added, and the mixture was pre-incubated for 5 min at 37 °C on a heated shaker. The reaction was initiated by the addition of 20 μL of uridine 5′-diphosphoglucuronic acid (UDPGA, 20 mM), and the reaction tubes were incubated for a further 40 min. The reaction was then stopped by the addition of 200 μL of IS solution (4 μM hopeaphenol for ε-viniferin and 2 μM ε-viniferin for hopeaphenol and resveratrol in MeCN acidified with 0.2% HOAc), followed by a centrifugation step and LC−UV analysis. For control incubations, buffer was added instead of UDPGA solution.

RESULTS AND DISCUSSION

LC−UV Method. A new ultrafast LC−UV method was developed, to enable rapid analysis of resveratrol and its analogues and oligomers in biological samples. The separation was carried out on a 2.7 μm "core−shell" particle reversed-phase column. In addition to the advantages of sub-2.7 μm particle size, the not entirely porous stationary phase allows for a more efficient mass transfer between stationary and mobile phases because of a shorter diffusion path in the shell-type particles.[19] The flow rate was set to 2.0 mL/min to compensate for the large void volume of the LC equipment used. As shown in Figure 2, this setup led to a very high chromatographic resolution in an analysis time of less than 2.5 min.

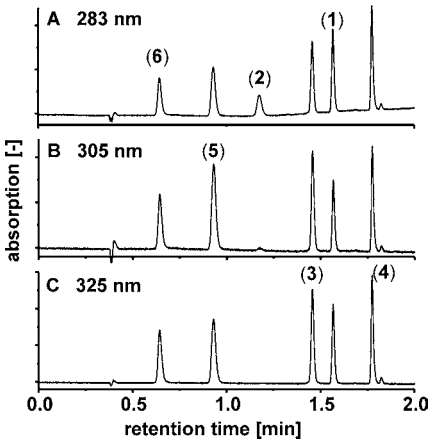

Figure 2. Typical chromatographic separation of a standard solution of resveratrol (**5**), piceatannol (**6**), and four resveratrol oligomers (**1**−**4**). Shown are the chromatograms at (A) 283 nm, (B) 305 nm, and (C) 325 nm used for quantitation of an injection (10 μL) of a 3 μM standard solution.

The mobile-phase gradient was optimized to fully separate analytes from the void volume (22 s, 0.7 mL) of the system, where polar matrix compounds elute. Applying a shallow gradient from an initial 30 to 70% organic solvent in 1.6 min allows for the baseline separation of all six analytes (Figure 2). Piceatannol and resveratrol eluted first in narrow peaks, followed by the tetramer hopeaphenol, which showed a relatively broad peak. Finally, ε-viniferin, r2-viniferin, and r-viniferin eluted in very narrow peaks (for retention times, see Table 1). Thereafter, the column was washed with two void volumes of organic solvent and reconditioned with three void volumes during the autosampler injection cycle of the next sample (about 1 min).

The polyphenols were detected close to their absorbance maxima as determined in the LC solvent using the PDA detector. Piceatannol, hopeaphenol, and r2-viniferin were monitored at a wavelength of 283 nm; resveratrol was monitored at a wavelength of 305 nm; and ε-viniferin and r-viniferin were monitored at a wavelength of 325 nm. With this setup, a limit of detection (LOD) of 0.03 μM (0.3 pmol on the column) was determined for r2-viniferin and r-viniferin eluting in very narrow peaks with a full width at half maximum (fwhm) of around 0.5 s (Table 1). With a broader peak width, the LOD increased to 0.1 μM (1 pmol) for resveratrol and ε-viniferin and 0.3 μM (3 pmol) for hopeaphenol. This sensitivity is almost comparable to a recent LC−UV method for resveratrol oligomers using sub-2 μm particle-filled columns and a high-pressure LC system, leading to LOD values between 5 and 50 μg/mL (0.2−0.55 pmol).[1] With an analysis time of 2.5 min (about 3.5 min total running time), our method is more than twice as fast while only using standard equipment (maximum pressure < 250 bar). Moreover, this method is the first for the quantitative measurement of r-viniferin and r2-viniferin described thus far.

To test the influence of matrix compounds on the separation and detection, DMEM was spiked with the polyphenols and analyzed after mixing with the internal standard and a centrifugation step. For all analytes, the present method shows good accuracy, with a mean recovery rate of 98 ± 6%. Moreover, the method precision was acceptable with an intersample variation of below 6.5% (see the Supporting Information). These results clearly demonstrate that increased analysis speed is not detrimental to analytical performance. With the allowance of the analysis of more than 400 samples in a single day, the developed LC−UV method is ideally suited for stability studies, which require the analysis of large sets of samples.

Chemical Stability of Resveratrol, Analogues, and Oligomers in Cell Culture Medium. The stability of piceatannol, resveratrol, hopeaphenol, ε-viniferin, r-viniferin, and r2-viniferin was analyzed in DMEM, a standard medium

Table 1. Performance of the New LC−UV Method[a]

analyte	retention time (min)[b]	$W_{0.5}$ (s)[c]	N	LOD (μM)[d]	dynamic range (μM)[e]	r^2	slope (μM^{-1})	intercept
piceatannol	0.60 ± 0.03	1.32 ± 0.48	4.2×10^3	0.3	1−10	0.999	0.63	−0.06
resveratrol	0.89 ± 0.03	1.38 ± 0.48	8.2×10^3	0.1	0.3−10	0.999	1.21	−0.02
hopeaphenol	1.11 ± 0.06	1.98 ± 0.84	6.3×10^3	0.3	1−10	0.999	0.49	0.03
ε-viniferin	1.43 ± 0.02	0.78 ± 0.18	6.7×10^4	0.1	0.3−10	0.999	2.56	−0.03
r2-viniferin	1.55 ± 0.01	0.60 ± 0.06	1.3×10^5	0.03	0.1−10	0.998	3.71	−0.10
r-viniferin	1.77 ± 0.03	0.48 ± 0.06	2.5×10^5	0.03	0.1−10	0.994	0.85	0.04

[a]The observed retention times, peak width at half maximum height ($W_{0.5}$), resulting number of theoretical plates (N), limit of detection (LOD), and dynamic range for each analyte are shown. [b]Mean of the 3 μM standard solution over 6 months (n = 6). [c]Injection of 3 μM standard solution (n = 3). [d]Signal/noise ratio ≥ 3:1. [e]The highest concentration tested was 10 μM; linear range ≥ 10 μM.

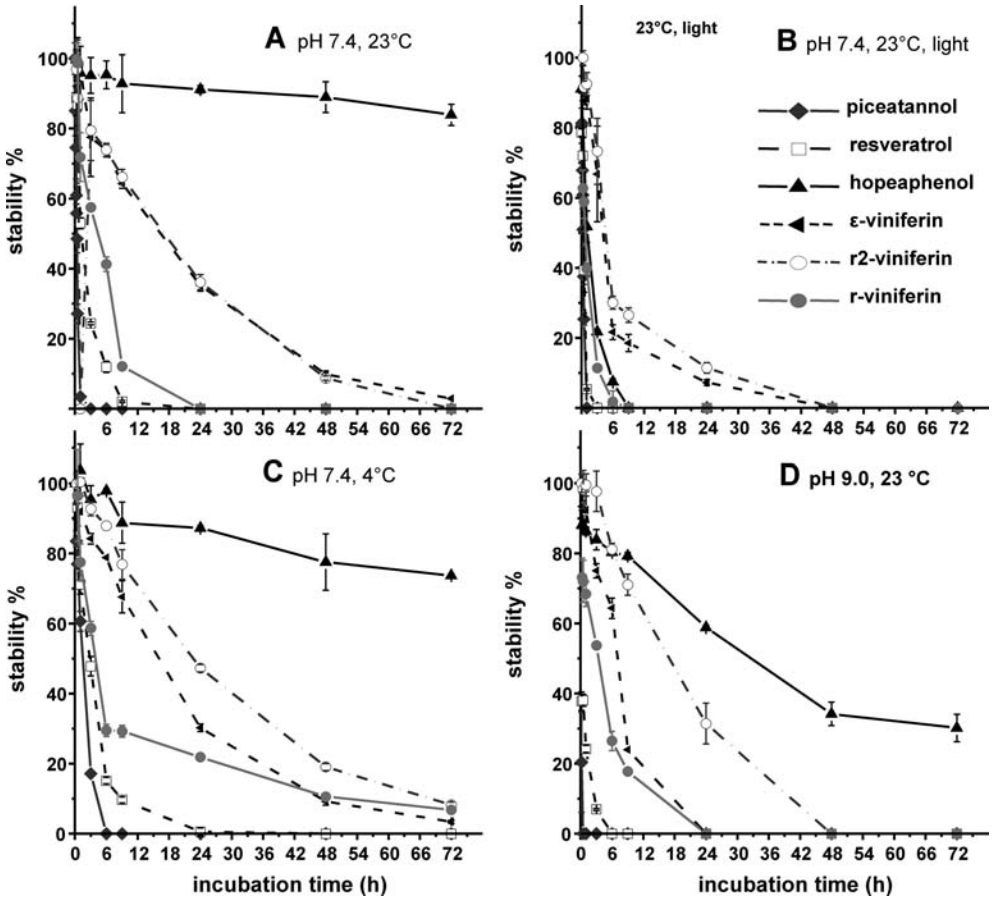

Figure 3. Stability of the six resveratrol oligomers during incubation in DMEM (A) at room temperature (pH 7.4), (B) at room temperature under room light (pH 7.4), (C) at 4 °C (fridge) in the dark (pH 7.4), and (D) in DMEM adjusted to pH 9.0. Shown is the mean ± SD of three incubations.

used to culture various cell lines. The compounds were incubated for up to 72 h under different conditions, and their stability was assessed by LC−UV. As shown in Figure 3A, piceatannol, resveratrol, *ε*-viniferin, r-viniferin, and r2-viniferin were rapidly degraded in the dark at a pH of 7.4 and a temperature of 23 °C. With a half-life ($t_{1/2}$) of about 25 min, piceatannol was the most unstable compound, followed by resveratrol, with a $t_{1/2}$ of about 1 h. Among the oligomers, r-viniferin showed the shortest half-life ($t_{1/2} \sim 5$ h), whereas the tetramer r2-viniferin was as stable as the dimer *ε*-viniferin, with a half-life of about 15 h. In contrast, the concentration of the tetramer hopeaphenol was virtually unchanged. Even after an incubation time of 72 h, 84 ± 3% of the initial concentration were detected. When incubated at a higher temperature of 37 °C in a cell incubator under a 5% CO_2 atmosphere, a similar stability pattern of the compounds was observed ($t_{1/2}$ piceatannol, ∼20 min; $t_{1/2}$ resveratrol, ∼2.5 h; $t_{1/2}$ r-viniferin, ∼5 h; $t_{1/2}$ *ε*-viniferin, ∼19 h; $t_{1/2}$ r2-viniferin, ∼21 h; and $t_{1/2}$ hopeaphenol, >72 h). In accordance with these findings, a decrease in the temperature had only minor effects on the stability of the polyphenols (Figure 3C). Interestingly, the stability of r2-viniferin was markedly increased at low temperatures ($t_{1/2} \sim 24$ h) when compared to the other polyphenols, which disappeared at a similar rate at higher temperatures (Figure 3C). On the basis of these results, one could conclude that the temperature only slightly influences the stability of resveratrol and its analogues and oligomers in DMEM. In contrast to that, the pH strongly influences the stability of the test substances. As already known for many other polyphenolic compounds,[20,21] the stability of piceatannol, resveratrol, *ε*-viniferin, and hopeaphenol decreased at a pH of 9 (Figure 3D). Although hopeaphenol was still the most stable compound among the analytes, with a half-life of about 30 h, it nevertheless was subjected to a significant degradation under alkaline conditions. A decrease in pH to a value of 4.0 increased the stability of all compounds, and ≥90% of the initial concentration of all polyphenols was recovered after 72 h.

A faster loss of the compounds was observed at a pH of 7.4 and a temperature of 23 °C when the DMEM was subjected to room light (fluorescent tube) radiation during incubation. In this case, none of the polyphenols had a half-life of longer than 4 h (Figure 3B), which is a finding that is consistent with previous results showing that *trans*-stilbene derivatives degrade under light.[7,22] It is interesting to note that, under these conditions, r2-viniferin was the most stable compound, followed by hopeaphenol.

Despite the complete disappearance of the peaks of the test compounds during incubations at different conditions in the dark, no new peaks of degradation products could be observed for piceatannol, resveratrol, *ε*-viniferin, and r2-viniferin at the monitored wavelengths (283, 305, and 325 nm). Only r-viniferin gave rise to a new peak at a retention time of 1.56 ± 0.01 min. The area of this peak increased between 0.5 and 9 h during incubation at pH 7.4. At pH 9, the peak already appeared after 0.25 h of incubation. In both cases, longer incubation led to a decrease of the peak area, and after 48 h, it

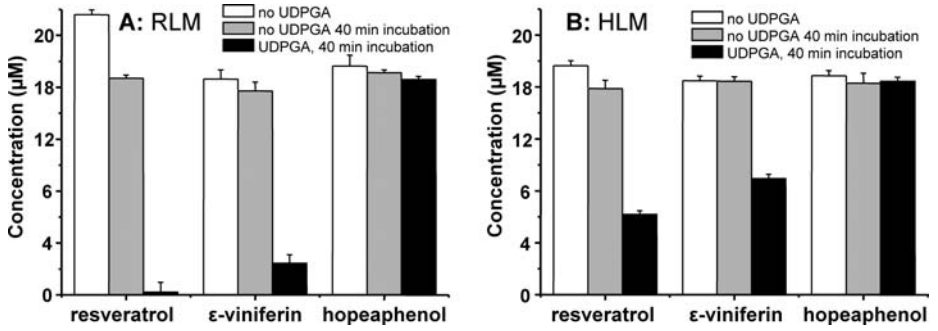

Figure 4. Glucuronidation of resveratrol, ε-viniferin, and hopeaphenol (each 10 μM) by HLMs and RLMs. Shown is the remaining concentration of the substrate after 40 min of incubation with 0.25 mg/mL microsomal protein in the presence and absence of the co-substrate UDPGA. Shown is the mean ± SD of three independent experiments.

disappeared completely, similar to its precursor r-viniferin (more details are presented in the Supporting Information).

It is well-known that *trans*-stilbene polyphenols are converted to their *cis* isomers if subjected to light.[7,22] To generate *cis* isomers, light radiation was used as previously described for resveratrol.[17] All stilbene polyphenols were converted under this radiation, yielding a single product peak in LC−UV. The retention times of the products were as follows: resveratrol, 1.21 ± 0.01 min; piceatannol, 0.82 ± 0.01 min; ε-viniferin, 1.30 ± 0.01 min; and r-viniferin, 1.65 ± 0.01 min. Peaks of these tentatively identified *cis* isomers of resveratrol, ε-viniferin, and r-viniferin were detected during incubation in DMEM in the light but vanished rapidly during further incubation. Thus, it is unlikely that, even under light exposure, the observed disappearance of the polyphenols in DMEM is driven by *cis*−*trans* isomerization.

Recently, Yang et al.[11] reported a complete degradation of resveratrol by auto-oxidative processes at 37 °C in the dark. They showed that, after a 24 h incubation of 200 μM resveratrol in base modified Eagle's medium at 37 °C, 96% of the compound was degraded, with a considerable hydrogen peroxide formation. Consistent with our results, no degradation products could be detected by LC−UV. This may be explained by the complexity of polyphenol auto-oxidation, leading to various products and adducts.[7,10,20,22,23] Among the stilbene monomers, piceatannol is more susceptible to auto-oxidation than resveratrol, which is to be expected for a polyphenol with a catechol moiety.[21] In contrast, dimerization has a stabilizing effect and ε-viniferin shows a significantly slower degradation than its monomer resveratrol (Figure 3). Among the tetramers bearing one *trans*-stilbene moiety (Figure 1), r2-viniferin is significantly more stable toward auto-oxidation than r-viniferin. The stilbene moiety seems to be a key factor for the instability in DMEM. Hopeaphenol, the only substance tested that does not exhibit this structural feature, is stable under physiological conditions (panels A−C of Figure 3). Only prolonged incubation under alkaline conditions (Figure 3D) and light radiation causes a degradation of this compound. When taking this chemical stability into account, hopeaphenol holds a unique place among resveratrol and its oligomers.

Regardless of the nature of the underlying degradation processes in DMEM, the half-life of stilbene polyphenols, particularly that of resveratrol and piceatannol, is short. Therefore, care should be taken in the design of cell culture experiments investigating biological effects of polyphenols using different cell culture media. In particular, the concentration of the stilbene polyphenols should be monitored in the medium throughout the incubation period.

In vivo, considerable amounts of resveratrol are detected as conjugates in serum and urine, suggesting that chemical degradation of stilbene polyphenols seems to be less relevant in the living organism. The difference of the degradation behavior of the test compounds in the cell culture medium used in our study when compared to the *in vivo* situation may be explained by a stabilization of the stilbene polyphenols by extensive protein binding.[7,24] This assumption is substantiated by the finding that only about 50% resveratrol was degraded when incubated for 24 h in DMEM containing 10% fetal calf serum,[10] whereas in our experiments, it disappeared completely.

Metabolic Stability of Resveratrol and Its Oligomers. To date, no information about the metabolism of resveratrol oligomers is available. Glucuronidation is the major metabolic pathway of resveratrol and many other polyphenols. Therefore, we applied the new rapid LC−UV method, to characterize the metabolic stability of ε-viniferin and hopeaphenol, and compare it to that of resveratrol. The polyphenols were incubated with RLMs and HLMs at a substrate concentration of 20 μM. The conjugation rate was determined on the basis of the substrate consumption (Figure 4). At a reduced pH of 6.9, all polyphenols were sufficiently stable over the incubation time of 40 min in phosphate buffer. More than 77 ± 2% resveratrol, 95 ± 2% ε-viniferin, and 97 ± 1% hopeaphenol of the initial amount was recovered in control incubations without UDPGA (Figure 4). As described previously, resveratrol was quickly conjugated by both RLMs and HLMs.[25,26] After 40 min of incubation with RLMs, less than 1% unconjugated substrate remained and HLMs conjugated 65% of resveratrol (35 ± 2% of remaining substrate). The dimer ε-viniferin was also significantly glucuronidated by both RLMs and HLMs, albeit to a lesser extent than resveratrol. After 40 min of incubation with the same amount of microsomal protein, 15 ± 4% RLMs and 54 ± 2% HLMs of ε-viniferin remained unconjugated. To our surprise, the tetramer hopeaphenol was, under identical conditions, not glucuronidated at all. With a recovery rate of the unchanged substrate of 94 ± 1% compared to 97 ± 2% for incubations with RLMs and HLMs, almost the same amount of hopeaphenol was detected as in control incubations without UDPGA (Figure 4).

Despite a high absorption rate, the bioavailability of resveratrol is very low.[27,28] Extensive intestinal and hepatic metabolism of resveratrol by sulfonation and glucuronidation leads to a rapid conjugation of this polyphenol.[7,22,27] For instance, only trace amounts of free resveratrol (<5 ng/mL) are

detectable in the plasma after a dietary relevant single oral dose of 25 mg in humans.[28] Even an ultrahigh single dose of 5 g of resveratrol to human volunteers led only to a plasma concentration of 533 ng/mL (2.5 μM).[29] In contrast to that, the plasma concentration of glucuronide and sulfate conjugates exceeded the concentration of the unchanged compound by a high order of magnitude.[27,28] Although few studies describe a biological activity of the conjugates,[7,22,27] the vast majority of potentially beneficial effects on the regulation of cellular pathways have been exclusively described for the parent compound. Poor oral bioavailability and, thus, a lack of effectiveness are common phenomena described for many biologically active polyphenols.[30] Our finding that hopeaphenol is barely glucuronidated by human and rat liver enzymes sets it clearly apart from most naturally occurring polyphenols. Upon absorption, its slow phase II metabolism might lead to an improved bioavailability in comparison to other stilbene derivatives and polyphenols. However, it is questionable if a compound with a molecular weight of 907 g/mol is efficiently absorbed in mammals after oral intake. Current research at the University of Veterinary Medicine in Hannover, Germany, aims to address these questions.

Except for hopeaphenol, the tested polyphenols disappear rapidly in DMEM under cell culture conditions, probably caused by degradation through auto-oxidation. These findings demonstrate the necessity of cell culture experiments with polyphenols always being accompanied by a monitoring of the stability of the test compounds under assay conditions. Only if the polyphenol is stable for a given incubation time can a reliable correlation between observed biological effects and the substance be deduced. With the new ultrafast LC−UV method, using only basic LC equipment, we developed a tool for the rapid assessment of the stability of resveratrol and its analogues and oligomers.

Our findings show, for the first time, that oligomerization stabilizes stilbene polyphenols in cell culture medium. Among all tested compounds, the tetramer hopeaphenol has a unique stability, with almost all of the initial concentration being recovered after a 72 h incubation period. Our initial investigation of the glucuronidation of the resveratrol oligomers demonstrates that ε-viniferin, similar to resveratrol and many other polyphenols, is rapidly conjugated. Surprisingly, the tetramer hopeaphenol was not glucuronidated by liver UGTs, which might lead to an improved bioavailability upon absorption. If one takes the chemical and metabolic stability as well as the high potency of hopeaphenol upon inhibiting tumor cell growth into account, it is apparent that especially this compound qualifies for further investigation. Current data clearly imply that hopeaphenol could be a promising natural anticarcinogen, which also might contribute to the beneficial effects associated with moderate wine consumption.

■ ASSOCIATED CONTENT

Supporting Information

Recovery rates of the analytes (Table S1 and S2) and chromatogram and formation kinetics of a degradation product of r-viniferin (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DMEM, Dulbecco's modified Eagle's medium; HLM, human liver microsome; RLM, rat liver microsome; UDPGA, uridine 5′-diphosphoglucuronic acid; UGT, uridine 5′-diphosphate-glucuronosyltransferase

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