

Optimization, In-House Validation, and Application of a Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS)-Based Method for the Quantification of Selected Polyphenolic Compounds in Leaves of Grapevine (*Vitis vinifera* L.)

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ABSTRACT: Polyphenols in grapevine can be constitutive or induced, depending upon cultivar, plant organ, and environmental influences. The aim of the presented work was to develop and optimize a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to study the pattern and amount of selected polyphenols in leaves of *Vitis vinifera* L. The method is simple and does not require any sample cleanup. It covers representative metabolites of the structure classes cinnamic acids, flavonoids, and stilbenes and enables the simultaneous separation and quantification of 13 polyphenols within 9 min at concentration levels between 0.1 and 3 $\mu\text{g/g}$. We present the method performance characteristics and its application to the quantification of polyphenols in grapevine leaves of the cultivars Riesling and Pinot noir. A total of 7 of 13 target polyphenols were detected at concentrations above the limits of quantification. Interestingly, instead of the expected *trans*-resveratrol, the investigated leaf samples of both cultivars contained *cis*-resveratrol-3-*O*-glucoside. The measurements also showed that Riesling leaves tended to contain higher concentrations of the selected polyphenols than Pinot noir. In view of its intended future use, the developed method has been shown to be a powerful and fast tool to study polyphenols in grapevine leaves subjected to environmental stress conditions.

KEYWORDS: Grapevine, leaves, polyphenols, LC–MS/MS

INTRODUCTION

The term polyphenols signifies a large and diverse group of chemical compounds that are classified into several structure classes, including, for example, phenolic acids, flavonoids, or tannins. Despite their structural diversity, all polyphenols share a common structure element, which consists of a benzene ring to which more than one hydroxyl group is attached. On the basis of the multitude of naturally existing phenolic compounds, the biological impact is just as manifold and humans make use of polyphenols because of their antiseptic, sun-screening, or disease-preventing properties. One of the most known properties of polyphenols is their antioxidative activity, whereby these compounds are able to scavenge free radicals and positively influence cardiovascular health.¹ In plants, polyphenols are known to have several physiological functions. They are involved in plant–pathogen interactions and defense against pathogenic impact.² Polyphenols that are present prior to an attempted infection of the plant are known as preformed mostly antimicrobial metabolites and are part of a passive resistance mechanism. A mechanism of active resistance is the synthesis, degradation, or metabolism to a different compound in response to the attack of a pathogen.¹ In addition, plants have evolved a variety of mechanisms using polyphenols, including the formation of a protective shield against ultraviolet (UV) radiation by accumulating UV-absorbing phenolic compounds in the leaf epidermis.^{3,4} In *Vitis vinifera*, many studies are published analyzing the effect of polyphenols on human health^{5–7} or reporting the concentration of polyphenols in

grapevine berry-containing foods.^{8–13} Few publications are available using other organs of grapevines,^{14–16} especially leaves.^{17–19}

Several analytical methods using numerous techniques have been developed for the investigation of polyphenols, including UV–Vis spectroscopy,^{15,17} high-performance liquid chromatography (HPLC) coupled with diode array detection (DAD),^{14,18,20} or HPLC coupled with mass spectrometry (LC–MS) or tandem mass spectrometry (LC–MS/MS).^{14,21,22} Analysis by HPLC–DAD is limited by similar or identical absorption maxima of target compounds belonging to the same structural class of polyphenols.²³ Because of its high selectivity, LC–MS/MS with electrospray ionization enables the sensitive and simultaneous detection and identification of a large number of (even co-eluting) compounds from a single chromatogram¹⁶ and is therefore the method of choice.

The focus of the presented study was to develop and optimize a LC–MS/MS method that enables fast and simple sample preparation for the quantitative measurement of selected target polyphenols in grapevine leaves. In consideration of the intended future use of the method for the investigation of the effect of abiotic stress on the profile and concentration of polyphenols, its suitability was demonstrated for standardized grapevine leaves of the cultivars Pinot noir and Riesling.

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MATERIALS AND METHODS

Reagents. Ultrapure water was produced by reverse osmosis and a Milli-Q Plus system (Millipore, Billerica, MA). Methanol (MeOH, LC gradient grade) was purchased from Merck (Darmstadt, Germany), and formic acid (puriss p.a. for MS) was obtained from Fluka (Sigma-Aldrich, St. Louis, MO).

Polyphenolic Standard Solutions. Standards of (+)-catechin (HPLC grade, $\geq 99\%$), (–)-epicatechin (HPLC grade, $\geq 99\%$), (–)-epicatechin gallate (HPLC grade, $\geq 97.5\%$), quercetin (HPLC grade, $\geq 99\%$), quercetin-3-*O*-glucoside (HPLC grade, $\geq 99\%$), kaempferol (HPLC grade, $\geq 99\%$), kaempferol-3-*O*-glucoside (HPLC grade, $\geq 99\%$), caffeic acid (HPLC grade, $\geq 90\%$), 4-coumaric acid (HPLC grade, $\geq 90\%$), *trans*-resveratrol (HPLC grade, $\geq 95\%$), and *trans*-resveratrol-3-*O*-glucoside (HPLC grade, $\geq 95\%$) were obtained from Extrasynthese (Lyon Nord, Genay, France). Ferulic acid (99%) was from ABCR GmbH and Co. KG (Karlsruhe, Germany). Caftaric acid ($\geq 98\%$) was purchased from Phytoflan (Heidelberg, Germany), and anthocyanin cyanidin-3-*O*-glucoside was purchased from Phytolab (Vestenbergsgreuth, Germany). All standards were stored according to the recommendations of the suppliers.

Plant Material. Standardized grapevine plant material came from a greenhouse experiment conducted in April and May 2009. Plants of the cultivars Pinot noir and Riesling have been planted in March 2009 in 3 L pots and were grown in greenhouses until their use in the experiment. Pinot noir was used because of its importance as a model cultivar in scientific studies of grapevine, and Riesling was used to include a white grape cultivar common in Austria. Prior and throughout the experiment, plants were irrigated and fertilized (Ferty 3, Planta, Regensburg, Germany) regularly, assuring the best water and nutrient supply during the study. Samples were taken from seven plants of Pinot noir and Riesling, respectively. Two fully developed leaves per plant were used in this study ($n = 14$ leaf samples per cultivar). After defined time periods (days 0, 4, 7, and 11 of the experiment), leaves were harvested and immediately frozen in liquid nitrogen.

Sampling and Sample Preparation. The samples were stored at $-80\text{ }^{\circ}\text{C}$ until further sample preparation. Leaves were ground for 2 min at 30 Hz in a ball mill using a grinding ball (Retsch, Haan, Germany) and cooled during further preparation. Approximately 500 mg of pulverized sample was accurately weighted and extracted with 5 mL of 0.02% hydrochloric acid (m/v) in 80% aqueous MeOH (v/v) during ultrasonication in ice water for 10 min. The sample suspension was centrifuged at 3750 revolutions per minute (rpm) for 5 min (GS-6 Centrifuge, Beckman Coulter, Inc., Brea, CA), and 4 mL of the supernatants was collected. Re-extraction was performed by adding 4 mL of extraction solvent, 10 min of ultrasonication, and 5 min of centrifugation. A total of 4 mL of the supernatants were collected again, and the two extracts were combined. During method development, two successive re-extraction steps were carried out for determining the optimum number of extraction steps.

Combined extracts were diluted 1+1 with 0.5% aqueous (v/v) formic acid in H_2O , and an aliquot thereof was further diluted 1+19 (v/v) with $\text{H}_2\text{O}/\text{MeOH}/\text{formic acid}$ (59.75:39.75:0.5, v/v/v). These two dilutions of the raw extract [1+1 (v/v) and 1+39 (v/v)] both contained 40% MeOH, and 5 μL aliquots of both dilutions were injected into the LC–MS/MS system.

LC–MS/MS Analysis and Quantification of Polyphenols. Detection and quantification were performed with a QTrap 4000 LC–MS/MS system (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1100 series HPLC system (Agilent, Waldbronn, Germany). Chromatographic separation was performed at $40\text{ }^{\circ}\text{C}$ on a Gemini RP-18 column, 100×2 mm inner diameter, 3 μm particle size (Phenomenex, Torrance, CA) protected with a guard column (Phenomenex, Torrance, CA) packed with the same material. The mobile phase consisted of (A) 0.5% formic

acid in H_2O and (B) 0.5% formic acid in MeOH. Both eluents were degassed online before use. After an initial period of 0.5 min at 20% B, the linear gradient started to reach 90% B after 8 min, followed by a hold time of 3 min, and going back to reach the initial conditions (20% B) after 12 min. The gradient program was followed by column re-equilibration at 20% B for 10 min, resulting in a total run time of 22 min. The flow rate was set at 0.4 mL/min. The column effluent was transferred via a six-port valve (VICI Valco Instruments, Houston, TX) to either the mass spectrometer (between 2 and 12 min) or the waste.

Detection was carried out by ESI–MS/MS in selected reaction monitoring (SRM) mode in negative polarity with the following settings: source temperature, $550\text{ }^{\circ}\text{C}$; curtain gas, 10 psi (69 kPa of maximum 99.5% nitrogen); ion source gas 1 (source heating gas), 50 psi (345 kPa of nitrogen); ion source gas 2 (drying gas), 50 psi (345 kPa of nitrogen); ion spray voltage, -4000 V ; collision gas (nitrogen), high; SRM dwell time, 25 ms; and pause between mass ranges, 5 ms.

The optimization of the analyte-dependent MS/MS parameters was performed via direct infusion of standards (0.8–10 mg/L) dissolved in $\text{MeOH}/\text{H}_2\text{O}$ (1+1, v/v) containing 0.5% formic acid into the mass spectrometer using a 11 Plus syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate of 10 $\mu\text{L}/\text{min}$. Selection of quantifier and qualifier transitions per compound was based on the signal-to-noise ratio for the respective transition.

For quantification of polyphenols in leaf samples, external calibration using standards in pure solvent was applied. Standards were accurately weighted on an analytical balance (M 500 P, Sartorius, Goettingen, Germany) and dissolved in MeOH, and a combined stock solution of all standards was prepared (stored at $-20\text{ }^{\circ}\text{C}$). For external calibration, nine combined working standard solutions were prepared prior to measurements by dilution of the stock solution. The dilutions with $\text{MeOH}/\text{H}_2\text{O}$ (39.75:59.75, v/v) containing 0.5% formic acid resulted in calibration solutions with concentrations of 0.05, 0.08, 0.1, 0.3, 0.5, 0.8, 1.0, 3.0, and 5.0 mg/L for each of the analytes.

Determination of the Water Content of Leaf Samples. For the determination of the water content, separately sampled, frozen leaves were brought to room temperature and the relative water content in percent was determined using a moisture analyzer (MA 51, Sartorius, Goettingen, Germany) as follows:

$$\text{relative water content (\%)} = \left[\frac{(\text{mass}_{\text{beginning}} - \text{mass}_{\text{end}})}{\text{mass}_{\text{beginning}}} \right] \times 100 \quad (1)$$

Evaluation of LC–MS/MS Method Performance. *Evaluation of Extraction Efficiency.* Using authentic leaf samples ($n = 5$), the yield achieved in every extraction step was determined for three replicate extraction steps per leaf sample.

Determination of Matrix Effects/Ion Suppression. Ion suppression and enhancement was evaluated by standard addition experiments. To this end, pulverized grapevine leaf samples were extracted and diluted 1+1 with 0.5% formic acid in H_2O (v/v), and the analyte concentrations were determined using external calibration by standard solutions in pure solvent. Subsequently, undiluted extracts were spiked in three repetitions by adding two combined standard solutions at five concentration levels each. Adding of two standard solutions was chosen to limit the volume of the spiking solution. Afterward, spiked extracts were diluted 1+1 with 0.5% formic acid in H_2O . For quantification of caftaric acid and quercetin-3-*O*-glucoside, the extracts were diluted 1+39 with $\text{H}_2\text{O}/\text{MeOH}/\text{formic acid}$ (59.75:39.75:0.5, v/v/v) prior to spiking. The lowest spiking level corresponded to the amount determined in the non-spiked extracts. The influence of the matrix on the signal intensity was studied in calculating signal suppression and enhancement (SSE) according to ref 24.

$$\text{SSE (\%)} = 100 \times \frac{\text{slope}_{\text{spiked extract}}}{\text{slope}_{\text{liquid standard}}} \quad (2)$$

Table 1. Optimized ESI–MS and ESI–MS/MS Conditions for Negative Ionization Polarity

number	compound	retention time (min) ^a	precursor ion (<i>m/z</i>)	declustering potential (V)	product ions ^b	collision energy (eV)	cell exit potential (V)
1	(+)-catechin	2.45 ± 0.02	289.0 [M – H] [–]	–60	108.9 /122.9	–34/–42	–5/–7
2	caftaric acid	2.50 ± 0.02	310.9 [M – H] [–]	–40	178.9 /149.0	–22/–16	–11/–9
3	cyanidin-3- <i>O</i> -glucoside	3.77 ± 0.12	447.0 [M – H] [–]	–80	284.2 /211.1	–36/–26	–11/–47
4	caffeic acid	4.23 ± 0.02	179.0 [M – H] [–]	–40	135.1/ 133.9	–24/–36	–9/–21
5	(–)-epicatechin	4.59 ± 0.01	289.0 [M – H] [–]	–70	108.8 /122.8	–34/–44	–5/–19
6	(–)-epicatechin gallate	5.69 ± 0.01	441.0 [M – H] [–]	–55	168.9 /124.9	–30/–58	–11/–21
7	4-coumaric acid	5.74 ± 0.01	162.9 [M – H] [–]	–40	118.7 /104.0	–22/–38	–19/–15
8	ferulic acid	6.07 ± 0.01	193.0 [M – H] [–]	–45	134.1 /148.8	–24/–16	–9/–7
9	quercetin-3- <i>O</i> -glucoside	7.00 ± 0.01	463.0 [M – H] [–]	–60	299.8 /271.1	–42/–58	–15/–1
10	<i>trans</i> -resveratrol	7.07 ± 0.011 ^c	226.9 [M – H] [–]	–75	185.1/ 143.1	–28/–36	–13/–9
11	kaempferol-3- <i>O</i> -glucoside	7.45 ± 0.01	447.0 [M – H] [–]	–80	254.9 /226.9	–52/–70	–13/–13
12	quercetin	8.08 ± 0.01	301.1 [M – H] [–]	–55	150.9 /178.8	–30/–24	–1/–7
13	kaempferol	8.63 ± 0.02	285.1 [M – H] [–]	–100	116.8/ 186.9	–70/–42	–5/–21

^a *n* = 63 leaf samples; mean ± standard deviation. ^b In the order of downward final product signal intensities, quantifiers are given in bold (on the basis of the higher signal-to-noise ratio). ^c *n* = 9 leaf samples.

Estimation of Method Detection and Quantification Limits. Instrument limits of detection (LODs) and limits of quantification (LOQs) were estimated from SRM peaks obtained from a chromatogram of standard solutions with a concentration of 0.05 mg/L based on signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively, using the Analyst software. For quercetin, we observed a loss of compound in standard solution; therefore, LOQ and LOD were estimated from the solution with a concentration of 0.3 mg/L. LOD and LOQ for matrix samples were estimated using representative leaf samples. For analyses of results obtained for leaves, a value of LOD/2 and LOQ/2 was assigned to concentrations below the LOD and LOQ, respectively. Peak identification was performed using the automatic function of the Analyst software, version 1.5, with checking every peak detection and integration manually. Linear standard calibration curves for each analyte were constructed by plotting the peak area versus the analyte concentration using a 1/*x* weighted function in Microsoft Excel (Microsoft Office 2007 for Windows, Microsoft Deutschland GmbH, Unterschleißheim, Germany). The slopes and the *y* intercepts of the resulting linear functions were used for calculation of analyte concentrations in the samples. Because the measurement of the complete set of samples investigated in this study spanned 6 days in total, five different calibration functions were used.

Estimation of Method Precision. To monitor the variability of extraction and analysis via LC–MS, quality control samples were prepared from a pooled grapevine (cv. Pinot noir) leaf sample and measured in randomized intervals between the experiment samples (13% of all measured samples). These quality control samples were used to determine the method precision, which was expressed here as the coefficient of variation (CV). For analytes with concentrations below the LOQs, method precision was estimated from linear regression data according to the DIN standard 32645 using the validation software program ValiData.²⁵

RESULTS AND DISCUSSION

Polyphenol Standard Selection. Standard compounds were chosen according to their affiliation to different structure classes of polyphenols and having already been detected in grapevine leaves^{3,15,20} or byproducts.¹⁶ The following substance classes were selected: flavan-3-ols [(+)-catechin, (–)-epicatechin, and (–)-epicatechin gallate], flavonols (quercetin, quercetin-3-*O*-glucoside, kaempferol, and kaempferol-3-*O*-glucoside), hydroxycinnamic acid derivatives (caffeic acid, 4-coumaric acid,

and ferulic acid), the phenolic acid ester caftaric acid, anthocyanins (cyanidin-3-*O*-glucoside), and for the stilbenes, *trans*-resveratrol.

LC–MS/MS Analysis. The chromatographic conditions have been optimized concerning the pH value of the mobile phase and the gradient profile. To improve the separation and peak shapes of the test compounds, different concentrations of formic acid (0.1, 0.27, 0.5, and 1%) were tested. In the literature, the use of higher concentrations (1, 5, and 10%) of acid has been described.^{14,23} In our study, 0.5% formic acid, added to both solvents, resulted in the best performance for the standards in accordance with other studies.²⁶ As the LC stationary phase, the Gemini RP-18 column was chosen because of its wide range of pH stability (pH 1–12). The column temperature was set to 40 °C, as recommended in the literature.^{14,27} Under the applied conditions, a separation of compounds of interest was achieved within 9 min, which resulted in a much lower time needed for analysis compared to other studies, e.g., 71 min,²¹ 24 min,¹⁶ or 21 min.²⁸ Retention times (*t_R*) are listed in Table 1, and a typical chromatogram obtained after injection of a calibration standard solution is shown in Figure 1.

In addition to retention times, Table 1 illustrates optimized MS and MS/MS parameter settings. Initially, MS/MS parameters had been optimized for all analytes in both negative and positive ESI mode. In the positive mode, higher signal intensities of the precursor ion were obtained for (–)-epicatechin-gallate, *trans*-resveratrol, ferulic acid, and cyanidin-3-*O*-glucoside. However, because the signal intensities of the precursor ions of these four compounds in the negative mode enabled quantification at concentration levels typically observed in leaf samples, it was decided to determine all target polyphenols in the negative mode.

Two SRM transitions per analyte were recorded using the deprotonated ion molecules in all cases. For (+)-catechin and (–)-epicatechin, the most intense product ion was observed at *m/z* 245 in accordance with other studies.²⁹ However, because of the low specificity of the loss of CO₂ ($\Delta m/z$ –44) together with the increased noise level of the signal at *m/z* 289 → 245, this transition was not used for quantification of these two compounds. The same was observed for the product ions of (–)-epicatechin gallate (*m/z* 289), quercetin-3-*O*-glucoside (*m/z* 301),

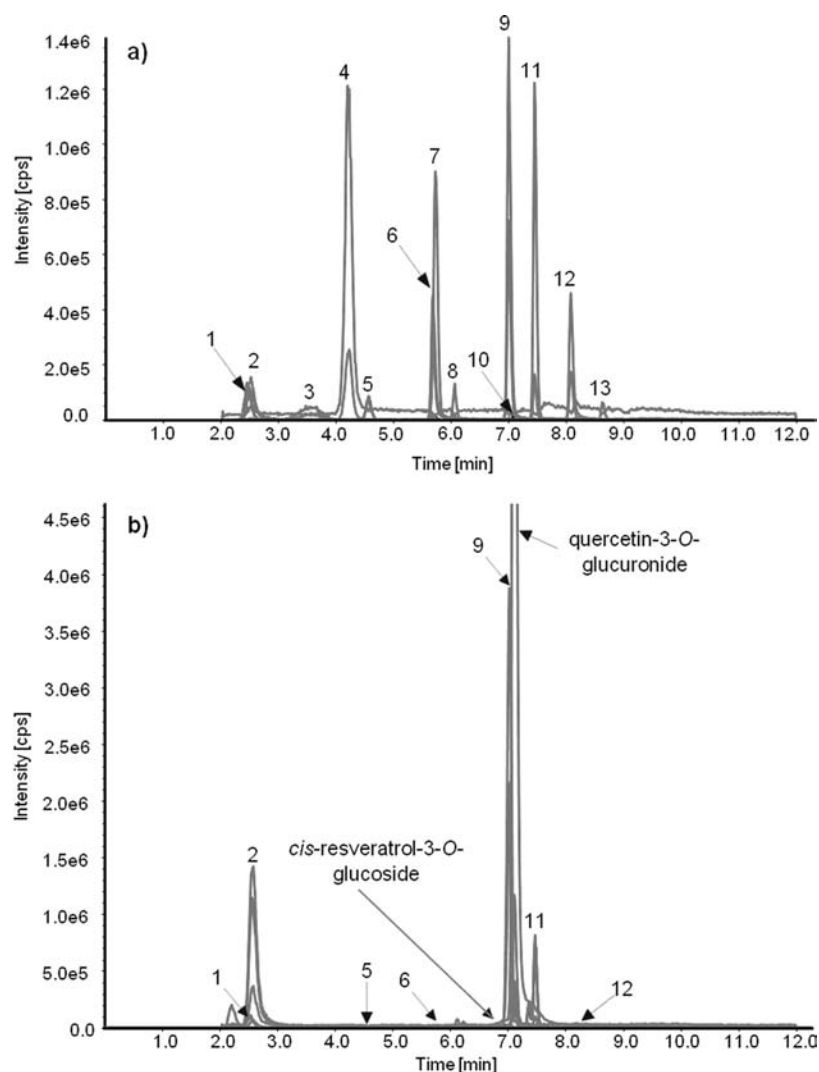


Figure 1. (a) Overlay of SRM chromatograms (quantifier and qualifier SRM transitions) obtained after the measurement of a standard solution (each compound at 1 mg/L). (b) Chromatogram of a Pinot noir leaf. Because of the different concentrations of the target analytes in the leaf samples and diverse MS response factors, not all detected peaks can be illustrated simultaneously. For assignment of individual polyphenols, see Table 1.

and kaempferol-3-*O*-glucoside (m/z 284).^{30,21} The SRM transitions identified with standards of 4-coumaric acid, caffeic acid, caffeic acid, ferulic acid, *trans*-resveratrol, and quercetin are in good agreement with formerly reported values.^{21,29}

Determination of the Water Content. Measurements of relative leaf water contents (76–83%) revealed no significant difference between leaves of different insertion levels ($p = 0.01$; data not shown). For these reasons, data were used on a wet weight basis.

Data Evaluation of *trans*-Resveratrol. When analyzing leaf samples during method development and its application to Pinot noir and Riesling cultivars, we observed that the putative *trans*-resveratrol eluted 0.12 min earlier [$t_R = 6.95 \pm 0.08$ min (standard deviation; $n = 54$)] compared to the standard in pure solvent. To test whether the presence of the matrix caused a shift in the retention time or the sample preparation led to chemical transformation of *trans*-resveratrol, standard addition experiments (fortification of milled leaf powder) were carried out. These resulted in chromatograms showing two distinct chromatographic peaks, with the first corresponding to the compound

detected in the leaf extract and the second originating from the spiked resveratrol standard. Enhanced product ion scans in both positive and negative ionization mode were carried out, resulting in identical MS/MS spectra of the standard in pure solvent and the somewhat earlier eluting peak obtained for the leaf extract.

To test whether the peak, which was observed for leaf samples, corresponds to the *cis* isomer of resveratrol, the *trans*-resveratrol standard was irradiated with UV light (245 nm for 23 h) to create the *cis* form of the compound, as frequently described in the literature (e.g., see ref 31). Injection of the irradiated standard solution showed that the standard had partly been transformed to *cis*-resveratrol, with the latter eluting ca. 0.4 min later than the *trans* isomer. The elution order of both resveratrol isomers corresponds to findings of other studies.^{32,33} Thus, it can be concluded that the leaf samples contained neither *cis*- nor *trans*-resveratrol at detectable levels in contrast to previously published studies.^{34,35}

Full-scan measurements by LC high-resolution MS/MS (LTQ Orbitrap XL, Thermo Fisher Scientific, San Jose, CA) have revealed a MS signal corresponding to a resveratrol-glycoside

Table 2. Relative Extraction Efficiencies (EE) of Two Successive Extractions in a Percentage of the Total Yield after Three Extractions ($n = 5$ Leaf Samples) and SSE Obtained for Spiked Sample Extracts

number	compound	first + second EEs (%)	SSE determined in dilution (v/v)	SSE (%)	measurement dilution (v/v)
1	(+)-catechin	97	1+1	108	1+1
2	caftaric acid	92	1+39	123	1+39
3	cyanidin-3-O-glucoside	100	1+1	109	1+1
4	caffeic acid	74	1+1	104	1+1
5	(-)-epicatechin	88	1+1	112	1+1
6	(-)-epicatechin gallate	68	1+1	121	1+1
7	4-coumaric acid	100	1+1	117	1+1
8	ferulic acid	100	1+1	105	1+1
9	quercetin-3-O-glucoside	97	1+39	116	1+39
11	kaempferol-3-O-glucoside	98	1+1	133	1+39
12	quercetin	68	1+1	118	1+1
13	kaempferol	100	1+1	99	1+1

for the unknown compound ($C_{20}H_{23}O_8$; $[M + H]^+$; theoretical mass, 389.1242; measured mass, 389.1232; relative mass deviation of -2.7 ppm). Because the retention time of the then purchased *trans*-resveratrol-3-O-glucoside standard did not correspond to that of the unknown peak (observed t_R of 6.2 min), the *trans*-resveratrol-3-O-glucoside standard was irradiated, as described above, to produce a mixture of *trans*- and *cis*-resveratrol-3-O-glucoside (*trans*- and *cis*-piceid). The retention time of *cis*-resveratrol-3-O-glucoside matches the unknown compound; therefore, we were able to assign the compound to *cis*-resveratrol-3-O-glucoside, which has also been found in grapevine leaves in previous studies (e.g., see ref 36). For future analyses, the quantification of this compound is aspired.

Evaluation of the Method Performance. *Evaluation of Extraction Efficiency.* Extraction yields of two successive extractions (relative to the sum of all three extractions) of compounds are listed in Table 2. After two extractions, a yield of 68–100% was achieved; therefore, one re-extraction was performed, and the two extracts were combined.

Determination of Matrix Effects/Ion Suppression. Extracts of pulverized grapevine leaves were spiked at five concentration levels by the addition of stock solutions to evaluate the influence of the matrix on the mass spectrometric detection. All analytes were analyzed in a matrix diluted 1+1 (v/v), except caftaric acid and quercetin-3-O-glucoside, which were analyzed in a matrix diluted 1+39 (v/v). Calculated values for SSE (according to eq 2) are shown in Table 2.

The consequences of matrix effects are the over- or underestimation of the actual concentration of analyte present in the sample, affecting both trueness and precision of the analytical method. LC–MS/MS using ESI is a technique prone to various matrix effects, the mechanisms of which have been reviewed (e.g., see ref 36). Because matrix effects are analyte-dependent and highly variable, it is difficult to predict these effects.^{37,38} A SSE value of 100% demonstrates that the signal intensity is not influenced by the matrix. A value of $>100\%$ indicates ionization enhancement, and a value of $<100\%$ indicates ionization suppression.³⁹ As can be seen in Table 2, the SSE was inside the range of $100 \pm 25\%$ for all analytes, except kaempferol-3-O-glucoside, indicating an enhancement caused by the presence of the matrix for this compound. A number of different approaches have been suggested to eliminate or correct for matrix effects in

LC–MS/MS analyses, including sample cleanup, standard addition, matrix-matched standards, internal standards, or changes in chromatographic conditions.⁴⁰ Dilution of extracts and, thus, the concentration of matrix components has been reported to reduce matrix interferences.³⁸ In the case of the investigated leaf samples, we had to adjust the dilution factor of the extracts to 1+39 (v/v) to stay in the linear working range for kaempferol-3-O-glucoside (Table 2). Thus, it can be assumed that the influence of the matrix on the ionization process has been reduced significantly.

Estimation of Method Precision. Precision of the LC–MS/MS steps and the overall method were estimated from repeated measurements of a pooled homogenized leaf sample, which served as a quality control sample and is listed in Table 3 as CVs. Because caffeic acid, 4-coumaric acid, and ferulic acid concentrations were below LOQs, the respective precision of these compounds was estimated from standard addition experiments.

Polyphenols in Leaves of *V. vinifera* L. cv. Pinot Noir and Riesling. Figure 1 presents a chromatogram obtained for a diluted Pinot noir leaf extract. A total of 7 of the chosen 13 polyphenols in this study were detected in the analyzed samples (Figure 2), with caftaric acid showing the highest concentrations, followed by quercetin-3-O-glucoside. The variability of concentrations, observed for individual polyphenols, was found to be characteristic for individual polyphenols: (–)-epicatechin, (–)-epicatechin gallate, quercetin, and kaempferol-3-O-glucoside occurred in narrow concentration ranges, while for (+)-catechin, caftaric acid, and quercetin-3-O-glucoside, the observed concentrations showed higher variations.

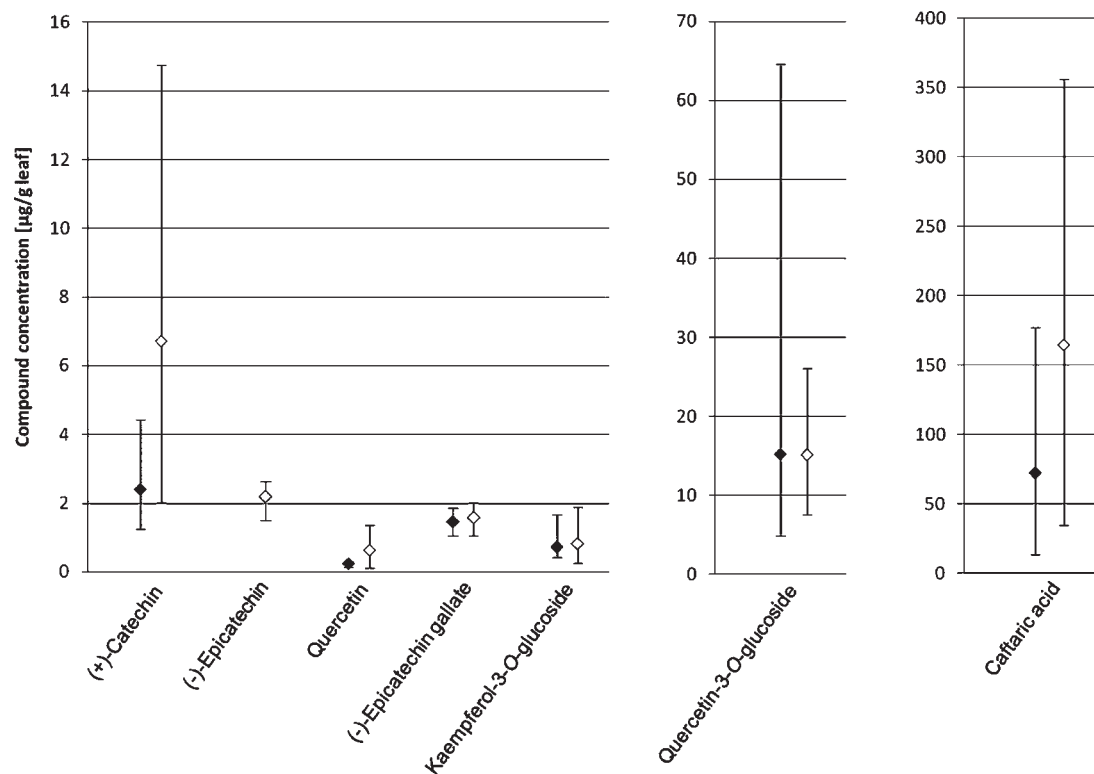
Because the CV of the method ranged at 17% for each of these compounds, the spread of the corresponding measured concentrations can be mainly attributed to biological instead of technical variability. This was also confirmed by the measurement of quality control samples.

Considering the concentration levels per cultivar, the leaves of Riesling tend to contain higher levels of polyphenols than Pinot noir leaves. (–)-Epicatechin was not detected in any of the Pinot noir leaves above the LOQ. Caffeic acid, 4-coumaric acid, ferulic acid, kaempferol, and cyanidin-3-O-glucoside in the leaves of both cultivars were not detected at concentrations above the LOQ.

As already described above, neither *cis*- nor *trans*-resveratrol were detected in the investigated leaf samples, although both have been often described as compounds in leaves³⁵ and grapes.^{41,42}

Table 3. Evaluation of LODs and LOQs in mg/L (1 mg/L Corresponds to 0.033 mg/g of Leaf) and Method Precision at the Determined Concentrations ($n = 10$ Leaf Samples) Expressed as the CV in Percent

number	compound	LOD		LOQ		CV _{method}
		in standard solution	in matrix	in standard solution	in matrix	
1	(+)-catechin	0.007	0.03	0.02	0.1	17
2	caftaric acid	0.019	0.06	0.06	0.2	17
3	cyanidin-3- <i>O</i> -glucoside	0.009	0.05	0.03	0.2	25
4	caffeic acid	0.005	0.01	0.02	0.05	21 ^a
5	(-)-epicatechin	0.01	0.03	0.04	0.09	18
6	(-)-epicatechin gallate	0.004	0.005	0.01	0.02	16
7	4-coumaric acid	0.004	0.005	0.02	0.02	9 ^a
8	ferulic acid	0.002	0.009	0.006	0.03	17 ^a
9	quercetin-3- <i>O</i> -glucoside	0.0003	0.001	0.001	0.003	17
10	<i>trans</i> -resveratrol	0.05		0.2		
11	kaempferol-3- <i>O</i> -glucoside	0.001	0.002	0.002	0.006	15
12	quercetin	0.01	0.02	0.03	0.06	35
13	kaempferol	0.007	0.02	0.02	0.06	26

^a Determined from standard addition experiments.**Figure 2.** Concentration ranges (mean \pm minimum and maximum detected value) of selected polyphenols in leaves of the grapevine cv. Pinot noir (\blacklozenge) and Riesling (\diamond) (in $\mu\text{g/g}$ of leaf; $n = 14$ leaf samples per cultivar).

Instead, *cis*-resveratrol-3-*O*-glucoside was detected in the leaves of both cultivars. Nevertheless, the developed method is suitable to detect *trans*-resveratrol in grapevine leaves.

According to these results and keeping in mind the further application of the developed method for the detection of stress markers, the set of target substances has been chosen well, because seven of the compounds have been directly detected in grapevine leaves. The concentration of acids is known to be under control

of environmental factors,⁴³ and the anthocyanins act as efficient UV-absorbing compounds and are expected to be increased upon the application of UV radiation;⁴⁴ therefore, these substances are not discharged because of their potential use in stress assays.

The largest peak in the SRM chromatograms occurred for the SRM transition of quercetin at $t_R = 7.05$ min and originated from in-source fragmentation of a derivative with higher molecular

mass. This peak was assigned to quercetin-3-O-glucuronide because of enhanced product ion spectra. In accordance with other studies,²¹ MS/MS of the deprotonated molecule resulted in product ion spectra that showed one main peak at m/z 301.0, which corresponds to quercetin after the loss of the glucuronic acid moiety. Enhancing the collision energy to 50 eV already yielded fragments that corresponded to the fragments found in MS/MS spectra of quercetin. Because of its prevalence in the tested Pinot noir and Riesling, we included quercetin-3-O-glucuronide into the set of analytes used for subsequent analyses. Having the standard available, we were able to approve the identity of the analyte, and quercetin-3-O-glucuronide will be quantitatively determined in future experiments.

In conclusion, the developed and optimized LC–MS/MS method is a useful tool to quantify the selected polyphenols in grapevine leaves. The analysis does not need any sample cleanup prior to HPLC injection, while showing suitable extraction efficiencies and low SSE; therefore, the method is a powerful and fast tool for routine analysis as well as scientific studies. The developed method shall be applied to grapevine leaf samples subjected to environmental stress under defined conditions. Moreover, the expansion of the number of target polyphenols as well as the extension to other grapevine organs (e.g., berries and stems) is an option for future research.

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ABBREVIATIONS USED

UV, ultraviolet; DAD, diode array detection; ESI, electrospray ionization; SRM, selected reaction monitoring; CV, coefficient of variation; SSE, signal suppression and enhancement; LOD, limit of detection; LOQ, limit of quantification; t_R , retention time

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