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Proanthocyanidins: Target Compounds as Antibacterial Agents

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Grape seeds accumulate in huge quantities as byproduct during wine production and are therefore a cheap source for pharmacologically active agents. However, studies prove poor antibacterial activity, and results of analyses are sometimes contradictory. The aim of this study was, thus, to determine the antibacterial activity of grape seed extracts with special focus on the chromatographic characterization of active fractions. In the course of these investigations, extraction protocols were optimized so that microwave-assisted extraction (MAE) guaranteed highest preconcentration efficiency. Proanthocyanidins, monomeric flavonoid aglycones, as well as some of their glycosides could be identified within yielded extracts via high-performance liquid chromatography−mass spectrometry (HPLC−MS). By that means the coherence number of possible isomers of procyanidins was approximated by a newly developed equation. As far as antibacterial activity determined via screening tests is concerned, the extracts generally have been found to be positively responsive toward 10 different Gram-positive and Gram-negative bacteria strains. After fractionation of the raw extracts, proanthocyanidins P_2, P_3, P_4 and gallate esters P_2G and P_3G (P = proanthocyanidin consisting of catechin and epicatechin units, n = oligomerization degree, G = gallate ester) were determined as active antibacterial agents toward 10 different pathogens. Only moderate activity was found for monomeric flavonoid fractions.

KEYWORDS: Grape seeds; microwave-assisted extraction; catechin; procyanidin; antibacterial activity

INTRODUCTION

According to the Food and Agriculture Organization of the United Nations (FAO; http://faostat.fao.org), 364 608 tons of grapes were harvested in Austria in 2004, while 2 734 561 hL of wine was produced in the same year (data published by Statistics Austria; http://www.statistik.at). Grape seeds, which are byproduct during wine production, could have a huge potential as nutrition additives due to the fact that ingredients have a positive effect on health (*1, 2*). Grape seeds mainly consist of 35% fibers, 29% nitrogen-free compounds (e.g., polyphenols), 15% lipids, 11% proteins, 3% ash, and 7% water (*3*).

The class of polyphenols, and especially flavonoids, are of great interest due to their antioxidant properties (*2, 4–6*). Monomeric units of catechins, including catechin itself, epicatechin, gallocatechin, and gallate esters thereof, for instance, have been shown to increase plasma antioxidant capacity and the resistance of low-density lipoproteins (LDL) to oxidation (*7*). Related to the content and to the polymerization degree of procyanidins, grape seed extracts were found to possess antitumor activity (*1*). Additionally, procyanidins exhibit potential beneficial effects on the vascular system, including the decrease of LDL and lipid peroxide levels (*7*).

Antibacterial effects of flavonoids extracted from grape seeds are reported in literature (*8–10*). Particularly, ingredients like quercetin and naringenin showed antibacterial activity toward *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, and *Escherichia coli*, whereas catechin and rutin have been reported to rarely possess antibacterial properties (*10*). In addition to flavonoid compounds, the low-polarity fraction containing, e.g., oleic acid, palmitic acid, and stearic acid, which was obtained via supercritical fluid extraction (SFE) from grape

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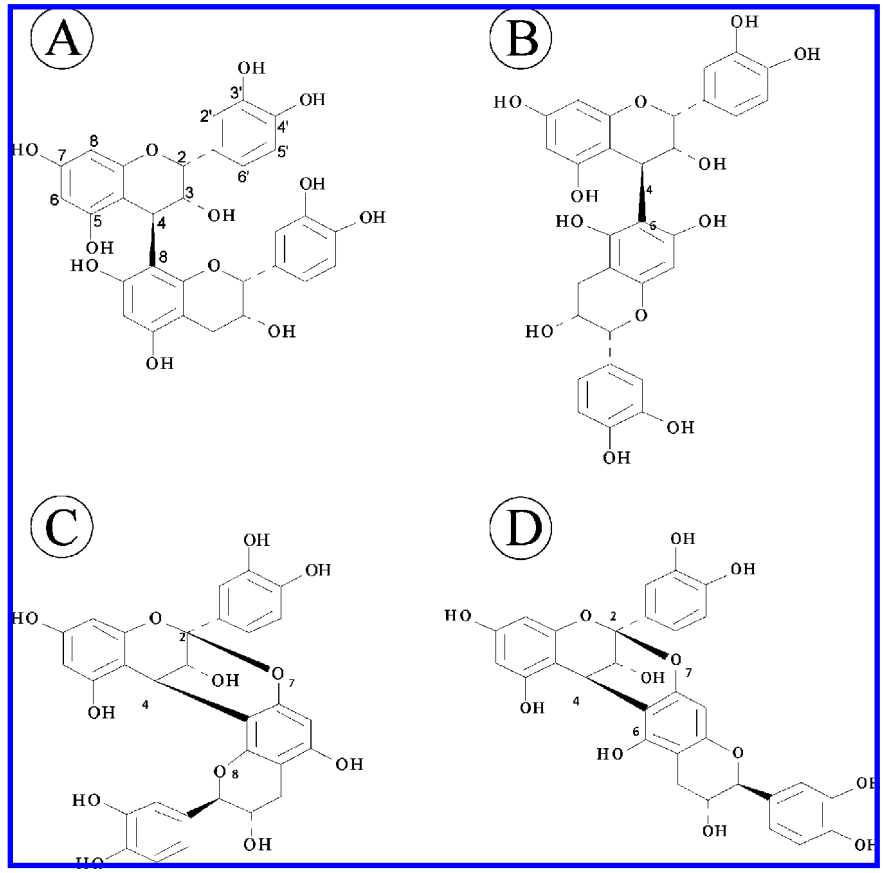

Figure 1. Connectivity of two catechin and epicatechin units, respectively. (**A**) 4−8 connectivity (e.g., procyanidin B2), (**B**) 4−6 connectivity (e.g., procyanidin B5), (**C**) 4−8 and 2−7-O connectivity (e.g., procyanidin A1), (**D**) 4−6 and 2−7-O connectivity (e.g., epicatechin-(2β→O-7, 4β→6)-*ent*-catechin).

seeds, showed high inhibition of growth of *S. coagulans niger*, *Citrobacter freundii*, *E. cloacae*, and *E. coli* (*9*).

Catechin and epicatechin as well as their oligomers have already been identified in different parts of grapes using various methodologies (*11–14*). Prior to high-performance liquid chromatography (HPLC) analysis, Sun et al. (*13*) fractionated proanthocyanidins on Sep-Pak-C_{18} cartridges and hydrolyzed the resulting eluates by use of toluene-α-thiol, which enabled the calculation of the mean degree of polymerization. Together with HPLC with UV detection (*11*), liquid chromatography−mass spectrometry (LC−MS) via atmospheric pressure chemical ionization (APCI) (*14*) and/or electrospray ionization (ESI) (*15*) interfaces has been employed for the evaluation of grape seed extracts. Furthermore, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was introduced as an off-line technique for qualitative characterization (*14*).

Despite the rapid development of analytical separation and of particular detection (MS) methods, the proper identification of oligomeric flavonoid compounds is still challenging. This may be ascribed to the huge number of possible isomers, requiring sophisticated identification tools: Procyanidins, representing a subclass of proanthocyanidins, can be classified into B-type (*16*) and A-type procyanidins, owing to an additional ether linkage. Consisting of catechin and epicatechin units, they can be bound together through four different types of interflavan bonds (*12, 14, 16–18*) (**Figure 1**). By contrast, (epi)gallocatechin units, which contain an additional hydroxyl group at the 5′ position of the B-ring, build up prodelphinidins (*16, 17*). Mixed forms of procyanidins and prodelphinidins can also be formed.

The aim of this work was the determination of antibacterial active agents within grape seed extracts, whereas special focus was put on the preparation of extracts and on the analytical characterization of the yielded fractions. Until now, there is no report published in literature dealing with the identification of antibacterial-active components in grape seeds toward all of these 10 tested pathogens.

MATERIALS AND METHODS

Standards, Chemicals and Microorganisms. All flavonoid standards, (−)-epicatechin (≥90%), gallic acid (min 97%).

(+)-catechin hydrate (min 98%, 1.5 mol H_2O/mol), (−)-epigallocatechin (≥98% HPLC), (−)-epigallocatechin gallate (min 80% HPLC), quercetin dihydrate (min 98% HPLC), and (−)-epicatechin gallate (≥98% HPLC), were from Sigma (St. Louis, MO). Kaempferol (≥96% HPLC) and myricetin (≥95.0% HPLC) were obtained from BioChemika (Fluka, Buchs, Switzerland).

Formic acid, 1-propanol (LiChromasolv), ethanol (absolute), and CASO bouillon were from Merck (Darmstadt, Germany), acetonitrile and methanol (both gradient grade) were from Sigma-Aldrich, and petroleum ether (puriss. p.a.) was from Riedel-de-Haën (Seelze, Germany). Polyamide 6 and acetone (HPLC grade) were obtained from Fluka (Buchs, Switzerland), and Sephadex LH-20 was from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Mueller Hinton agar was purchased from Oxoid Ltd. (Hampshire, United Kingdom). Powdered grape seeds were provided by Bionorica AG (Neumarkt, Germany). Water was purified by an Infinity NanoPure Unit (Barnstead, Boston, MA).

S. aureus (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 13883), *E. coli* (ATCC 25922), *S. epidermidis* (ATCC 12228), and *Enterococcus faecalis* (VRE) (ATCC 19433) were purchased from American Type Culture Collection (ATCC, Manassas, VA); *Streptococcus pyogenes* (DSMZ 20565), *Haemophilus influenzae* (DSMZ 4690), *Enterococcus casilliflavus* (DSMZ 20680),

Table 1. Quantitative Analysis (PDA Detection) of Three Representative Components Extracted by MAE and Aquasolv with Different Solvents[a]

		catechin [mg/g seeds][b]	epicatechin [mg/g seeds][c]	P_2 [mg/g seeds]		
		retention time [min]				
	solvent	5.4	9.4	7.8	11.0	13.4
		(A) Results Obtained in This Work				
microwave	50% methanol	2.44	1.18	0.33	0.41	0.39
	100% methanol	0.80	0.39			
	100% water	1.21	0.75	0.15	0.26	0.16
Aquasolv	50% methanol	0.64	0.40	0.09	0.16	
	100% methanol	0.58	0.31		0.07	
	100% water	0.12	0.05			
		(B) Results Reported in Literature				
Fuleki and Ricardo da Silva *11*		0.21−2.44	0.23−2.84	methanolic extract; quantification via HPLC-UV		
Bakkalbasi et al. *4*		1.21−8.45	0.85−8.93	aqueous extract; quantification via HPLC-PDA		
Yilmaz and Toledo *2*		0.12−3.58	0.96−4.21	methanol/water extract; quantification via HPLC-UV		

[a] Determination of P_2 via catechin calibration curve. [b] $y = 71869797.831x + 6573533.479$, $R^2 = 0.995$. [c] $y = 74731067.549x + 7007386.562$, $R^2 = 0.992$.

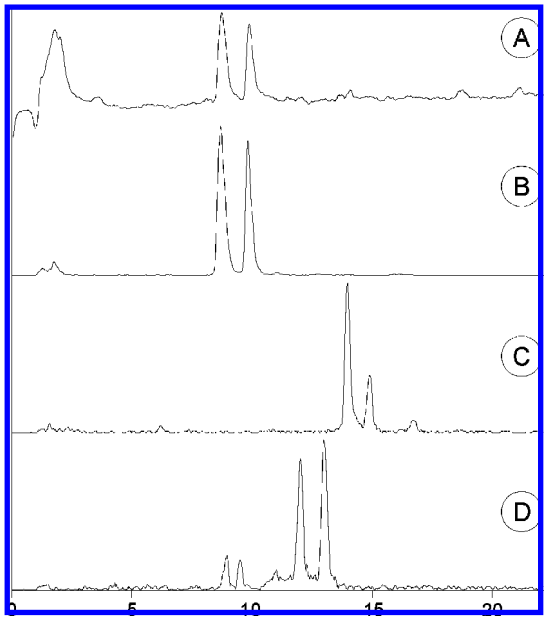

Figure 2. Separation of grape seed extract on HPLC column Prontosil; (**A**) TIC, (**B**) mass range (m.r.) 288.50−289.10; (catechin, epicatechin), (**C**) m.r. 440.5−441.10; (catechin gallate, epicatechin gallate), (**D**) m.r. 456.50−457.10 (gallocatechin gallate, epigallocatechin gallate).

Table 2. Determination of Antibacterial Activity of Crude Grape Seed Extract and of Oligomeric Proanthocyanidins via Spiral Platter (SP)

	crude extract		fraction P3
bacteria	SP 1:20 (420−485 μg/mL)	SP 1:200 (42−49 μg/mL)	SP 1:20 (250 μg/mL)
S. aureus	++	(+)	+++
P. aeruginosa	(+)		(+)
Pneumococcus	++++	+	(+)
St. pyogenes	++++	++	+++
Klebsiella			(+)
E. coli			(+)
H. influenzae	++	(+)	+++
S. epidermidis	++	(+)	+++
En. faecalis (VRE)			+++
En. casilliflavus (VRE)			+

[a] Key: +++ = 10^2 cfu/mL after 4 h; ++ = 10^2 cfu/mL after 8 h; + = 10^3−10^4 cfu/mL after 8 h; (+) = better activity compared to control group.

and *Pneumococcus* (DSMZ 20566) were obtained from the German Collection of Micro-organisms and Cell Cultures (DSMZ, Braunschweig, Germany).

Preparation of Standards. Each standard was dissolved in H_2O/MeOH (50:50, v/v) and immediately frozen at −20 °C, resulting in stock solutions in the range of 0.78−1.19 mg/mL. By dilution, desired working and calibration standards were prepared. External calibration (peak area vs concentration) between 39 μg/mL and 1.19 mg/mL was used for quantification (three concentration points; at least three determinations per standard concentration). Stability of calibration was already shown in our previous investigations (*19*).

For reliability tests of the extraction methods, standard solutions of gallic acid (0.1 mg/mL in 50% H_2O/MeOH and 50% H_2O/1-PrOH, v/v) and quercetin (0.05 mg/mL in 50% H_2O/MeOH and H_2O/1-PrOH, v/v) were prepared.

Sample Preparation. Powdered grape seeds were extracted by Aquasolv and by microwave-assisted extraction (MAE).

Aquasolv Extraction. The Aquasolv system (Berghof, Eningen, Germany) consisted of a boiler, a reactor, two temperature and pressure sensors, two autoclaves, and a Liebig condenser. For extraction, the sample was placed in steel autoclaves within the reactor. After heating the boiler to 120 °C (1.9 bar), steam was transferred to the reactor and to the autoclaves. Between 300 and 700 mg of powdered grape seeds was extracted with 3 mL of H_2O, H_2O/MeOH (50/50; v/v) or pure MeOH for 30 min. After cooling, the samples were centrifuged and the supernatant was frozen at −20 °C.

Microwave-Assisted Extraction. MAE was executed on a MLS-1200 Mega 240 instrument (MLS Ltd., Leutkirch, Germany) consisting of an oven, a degassing unit, and a rotor block (HPR 1000/6M), carrying six lockable Teflon vessels. On the basis of the work done by Hong et al. (*20*), solubilization of analytes was carried out at 150 W for 200 s. Between 600 and 1400 mg of grape seed powder were extracted with 10 mL of H_2O, MeOH, or different H_2O/MeOH mixtures as solvents. After extraction, the samples were centrifuged and the supernatant immediately frozen at −20 °C.

Fractionation of Grape Seed Extracts and Determination of Antibacterial Activities. Grape seed raw extracts were fractionated according to a protocol, published by Svedström and co-workers (*21, 22*): After removing liphophilic compounds, the extract was fractionated on a column, containing polyamide 6 as stationary phase to yield four fractions, (P1−P4) according to their elution order. MeOH and acetone/H_2O (7:3) were used as elution solvents. The residue of the second fraction (P2) was dissolved in EtOH, applied on a Sephadex LH-20 column, and eluted by different volumes of EtOH to give two fractions, S1 and S2. P1 was combined with S1, P3 with S2. P1 + S1 was evaporated and redissolved in H_2O. Afterward this solution was applied on a Sep-Pak-C_{18} cartridge, and target analytes were eluted with H_2O and MeOH. Finally, all obtained fractions were analyzed via LC−MS and further tested on their antibacterial activity. In a second approach approximately 19 mg/mL of pure P1 and approximately 4.5 mg/mL of pure P3 were prepared and analyzed without adding S1 and S2.

The antibacterial activity of grape seeds was tested with selected bacteria strains: *S. aureus* and methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa*, *Pneumococcus* sp., *St. pyogenes*, *Klebsiella*

Table 3. Fragmentation Pattern of Monomers, Their Sugar Derivatives, and Oligomers

compound	mol weight [g/mol]	retention time [min]	fragmentation pattern $[M + H]^+$ mother ion $[M + H]^+$	fragmentation pattern $[M + H]^+$ daughter ion $[M + H]^+$
			Monomers	
catechin	290.27	8.91	**291**: 272.98; 271.16; 248.97; 230.98; 165.00; 151.02; 147.05; 139.08; 123.09	
quercetin	302.24	25.39	**303**: 285.13; 257.14; 247.14; 229.10; 201.07; 165.05; 136.96	
malvidin	331.30	16.30	**331**: 316.08; 299.20; 287.20; 270.06; 242.15;	
			Oligomers	
P_2	578.52	8.72; 12.07	**579**: 426.92; 409.02; 300.97; 290.96; 274.99; 259.09; 247.13	**291**: 289.26; 271.07; 261.06; 243.20; 165.03; 151.06; 139.05; 135.07; 123.03
P_2G	730.62	14.99	**731**: 578.97; 442.88; 426.93; 408.95; 301.21; 288.96	**291**: 288.93; 270.97; 261.00; 248.82; 243.29; 165.01; 151.16; 139.25; 135.03; 126.91; 122.91
P_3	866.77	12.88; 14.28	**867**: 848.82; 714.86; 696.89; 578.87; 576.83; 558.89; 534.94; 426.86; 408.99; 300.93; 288.87	
P_3G	1018.87	13.96; 14.53;	**1019**: 1000.91; 866.92; 730.81; 696.91; 578.82; 558.91; 541.07; 410.96; 408.86; 290.87	
P_4	1154	15.05	**1155**: 1136.72; 1002.85; 984.16; 866.79; 712.81; 578.83; 576.81; 534.99; 409.05; 357.08;	
			Sugar Derivatives of Monomers	
malvidin dihexose		15.97	**655**; 563.08; 492.97; 331.25; 315.18; 287.17;	**331.19**; 315.99; 299.23; 287.06; 269.97; 242.30
malvidin hexose		11.28	**493**; 474.95; 426.36; 340.89; 331.18; 287.29;	**331.18**; 315.23; 299.31; 287.14; 270.10; 267.80; 241.67
malvidin hexose deoxy-hexose		17.02	**639**; 331.22;	**331.24**; 316.02; 299.26; 287.22; 270.03;
(epi)catechin hexose	452.41	6.47	**453**; 435.03; 417.00; 356.94; 300.95; 290.97; 282.99; 272.88; 246.99;	**291.23**; 273.02; 261.23; 231.27; 165.01; 150.99; 147.00; 139.04; 122.95;
quercetin hexose	464.38	16.44	**465**; 374.00; 303.28;	**303.18**; 284.99; 257.12; 229.06; 200.97; 165.13; 148.70; 136.92;
kaempferol hexose	448.38	15.63	**449**; 447.01; 372.96; 358.95; 287.11; 259.18;	**287.16**; 269.96; 259.13; 257.35; 153.03;
quercetin hexose deoxy-hexose	610.52	15.6	**611**; 592.88; 535.05; 464.81; 447.14; 303.23; 287.15;	**303.08**; 285.01; 257.16; 213.16; 200.90

sp., *E. coli*, *H. influenzae*, *S. epidermidis*, vancomycin-resistant *En. faecalis* (VRE), and vancomycin-resistant *En. casilliflavus* (VRE). The test solutions were prepared as follows: 3 mL of extract (in 50% H_2O/MeOH, v/v) was evaporated and dissolved in 3 mL of sterile H_2O. First screenings were performed on Mueller Hinton agar in H_2O and Mueller Hinton agar in H_2O containing 5% mutton blood, using 80 μL of test solution. Each screening was repeated twice. Plates were incubated for 15−18 h at 37 °C. In a second step the antibacterial activity was quantified by the use of a spiral platter (Don Whitley Scientific Limited, Shipley, U.K.). For that purpose, one bacterial colony was suspended in 5 mL of CASO bouillon, incubated for 24 h at 37 °C, washed, and diluted with 0.9% sodium chloride to a concentration of 10^7 cfu/mL. Between 5.9 and 9.7 mg/mL of grape seed extract were diluted 1:2, 1:20, and 1:200 and mixed with the bacterial suspension (1:10 for *Pneumococcus* and *H. influenzae*, 1:100 for all others). Samples were plated after 0, 4, and 8 h and incubated for 15−18 h at 37 °C. A solution of 0.9% sodium chloride was used as positive control.

Separation and Detection. Separation of the crude grape seed extracts for qualitative and quantitative analysis as well as for comparison of different HPLC columns was carried out with the following HPLC systems. Within all three mentioned systems, 5% CH_3CN in H_2O containing 0.05% HCOOH (mobile phase A) and CH_3CN containing 0.05% HCOOH were employed as the mobile phases.

HPLC−ESI-MS for Qualitative Analysis (System 1). For qualitative analysis, an HPLC system was interfaced with an ion trap mass spectrometer (LCQ, Thermo Fisher Scientific, San Jose, CA) via an ESI interface. the HPLC consisted of a Rheos CPS-LC degasser (Flux Instruments AG, Basel, Switzerland), a VICI injection port (E36-230, Valco Instruments Co., Houston, TX), and a Rheos 2000 binary low-pressure gradient pump (Flux Instruments AG). Data were recorded on a PC, using the manufacturer's software package (Janeiro II-SF, version 2.0; Flux Instruments AG). For RP-HPLC a 50 mm × 2.0 mm, 120 Å, 5 μm Prontosil column was used (Bischoff Analysentechnik and -geräte Ldt., Leonberg, Germany). The following binary gradient was employed: zero time conditions were 95% A and 5% B at a flow rate of 175 μL/min. After isocratic conditions for 5 min, B was increased to 45% within 20 min and further to 100% B within 2 min. After 5 min, 100% B mobile phase composition was changed back to starting conditions within 2 min. Separations were carried out at room temperature. A volume of 10 μL of sample was injected for analysis.

Ionization was carried out in positive mode employing 4.5 kV as electrospray voltage. Nitrogen was used as sheath gas. The heated capillary was operated at 190 °C, and capillary voltage was set to 26 V. For low mass range tuning, flavonoid standards were used (epigallocatechin gallate, 0.12 mg/mL in 50% H_2O/MeOH, v/v; quercetin, 0.050 mg/mL in 50% H_2O/MeOH, v/v). System control and data evaluation were done via XCalibur (version 1.3, Thermo Fisher Scientific). LC−ESI-MS was carried out in the full scan mode from *m/z* 50−1500.

HPLC-PDA−MS for Quantitative Analysis (System 2). Quantitative analysis was carried out on an HPLC−MS system from Shimadzu (LCMS 2010, Shimadzu Japan Corp., Kyoto, Japan), consisting of a DGU-14A degasser unit, two LC-10ADvp solvent delivery pumps, an SIL-10ADvp autoinjector, a CTO-10Avp column oven, a PDA, SPD-M10Avp photodiode array detector (PDA), an LCMS-2010 mass spectrometer, an FRC-10A fraction collector, and an SCL-10Avp system controller. Data were recorded on a PC, using the manufacturer's software package (LCMS solutions, version 2.05-H2, LCMS-Post run, ver. 2.05-H2). Separation was carried out on the same column as mentioned for system 1. A binary gradient was used as follows: zero time conditions were 98% A and 2% B at a flow rate of 500 μL/min. After isocratic conditions for 5 min, B was increased to 25% within 25 min, further to 55% B within 10 min, and finally to 100% B within 2 min. After 5 min, 100% B mobile phase composition was changed back to starting conditions within 1 min. Separations were carried out at 30 °C. A volume of 10 μL of sample was injected for analysis. Detection was performed between 200 and 400 nm. Quantification was carried out at 215 nm for catechin, epicatechin, and procyanidin P_2.

Interfacing with the mass spectrometer ionization was performed via an ESI interface employing positive ionization mode. Interface voltage was set to 4.5 kV, and CDL temperature operated at 250

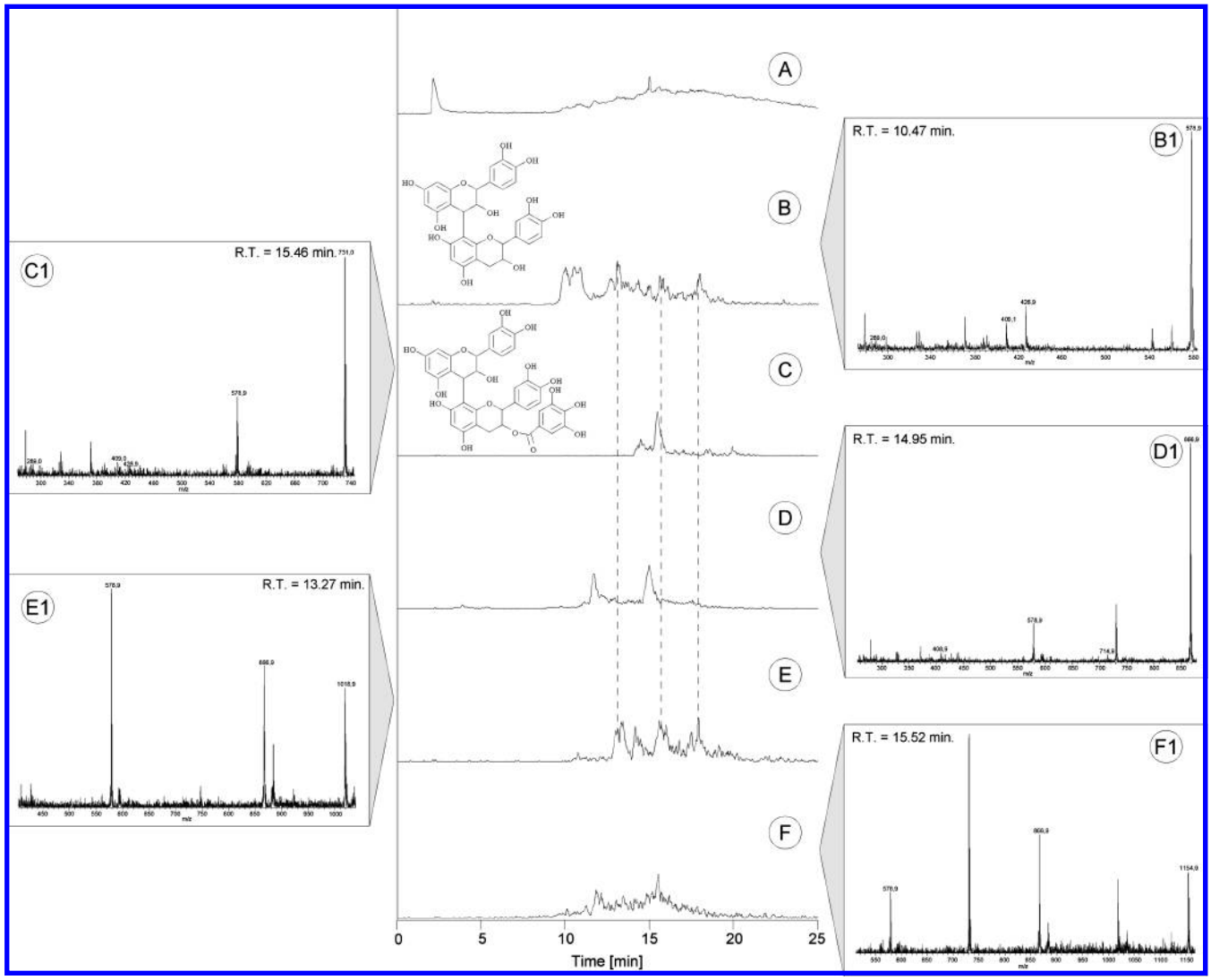

Figure 3. Separation of fraction P3 containing oligomeric units of the grape seed extract on HPLC column Prontosil Eurobond; (**A**) TIC, (**B**) m.r. 578.50−579.10 (P_2), (**C**) 730.50−731.10 (P_2G), (**D**) 866.50−867.10 (P_3), (**E**) 1018.50−1019.10 (P_3G) (**F**) 1154.50−1155.10 (P_4).

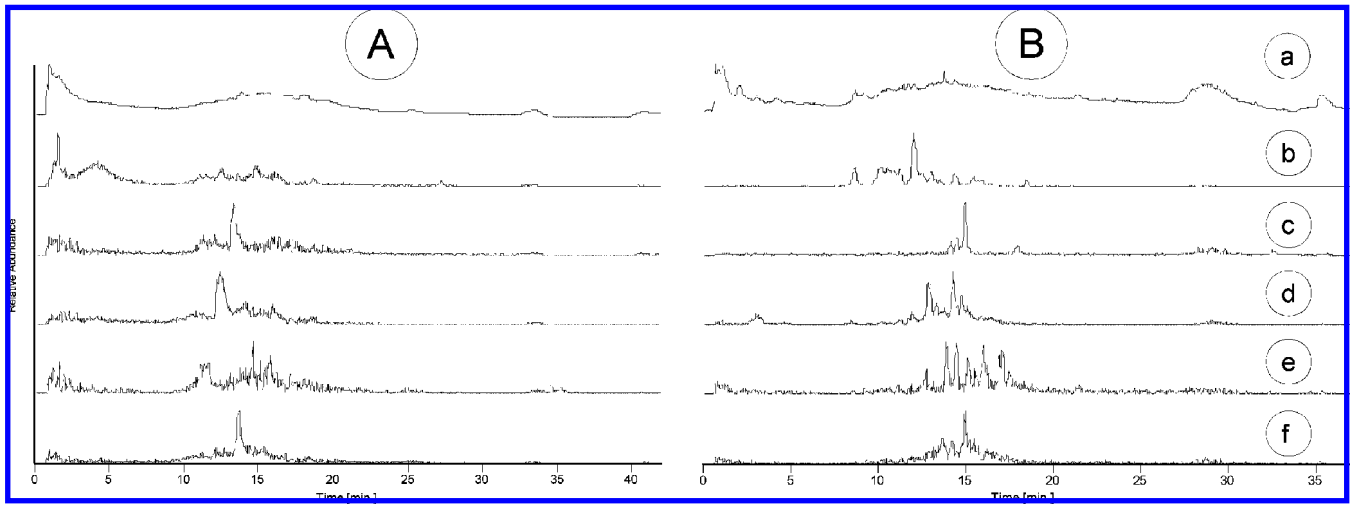

Figure 4. (**A**) Separation of crude grape seed extract by poly(*p*-methylstyrene-*co*-1,2-bis(*p*-vinyl phenyl)ethane. (**B**) grape seed extract separated by HPLC column Prontosil: **a** = TIC, **b** = 578.00−580.00 (P_2), **c** = 730.00−732.00 (P_2G), **d** = 866.00−868.00 (P_3), **e** = 1018.00−1020.00 (P_3G), **f** = 1154.00−1156.00 (P_4).

°C, using a capillary voltage of 25 V. Tuning was performed in accordance to the manufacturer's recommendations using a mixture of different PEG's and raffinose. Nitrogen was produced by a nitrogen generator (N2 LCMS 1, Claind Laboratory Gas Generator, Lenno, Italy). ESI-MS was carried out in the full scan mode between *m/z* 100 and 1500.

RESULTS AND DISCUSSION

The typical workflow in phytochemistry comprises extraction, purification, separation, and identification of analytes. Beside the determination of potential antibacterial agents within grape seeds, one aim of this study is to investigate different extraction technologies in order to gain highly concentrated samples. This was accomplished by the comprehensive comparison of total ion current signals, peak areas of selected mass traces, and by the evaluation of quantitative results of representative components. For that purpose, the HPLC separation system was optimized prior to investigating extraction efficiencies.

Extraction of Grape Seeds: Aquasolv versus MAE. The aim of the extraction is the transfer of analytes to a liquid phase, enabling the determination and/or structural elucidation in a second stage (*23*). Published protocols use distilled water (*4*) or 70% H_2O/MeOH (*2*) as extraction solvents for defatted powdered seeds (*2, 4*), while extraction was performed by vigorous shaking at room temperature. In order to obtain highest yields of solubilized grape seed ingredients, different techniques (MAE and Aquasolv extraction) (*24*) were evaluated, employing different solvents and solvent mixtures. The evaluation criteria of the extraction power were based on the mass spectrometric (MS) total ion current (TIC) signal, on the MS peak areas, and on the quantified LC-PDA results from selected representative components. TIC of Aquasolv and MAE extracts delivered significant differences, i.e., the value of MAE was 3 times higher than that one of Aquasolv. The same tendency was noticed upon comparing MS peak areas of catechin, epicatechin, and quercetin as well as upon quantification of these selected analytes via LC-PDA. **Table 1** gives an overview of the results of the quantitative analysis obtained via LC-PDA. MAE delivered higher yields for all analytes and all investigated solvents. The comparatively low values for Aquasolv raises the following question: Is the low extraction efficiency attributed to the methodology itself, or is it caused by oxidation and degradation processes during the procedure? During the Aquasolv process, samples were extracted at 120 °C at a pressure of approximately 2 bar. This could lead to degradation of heat- and pressure-sensitive analytes. In fact, Moreira da Costa et al. (*25*) described the behavior of quercetin at different stages of temperatures as well as applied atmospheres (air and nitrogen) proving the degradation of 10% quercetin at 116 °C due to loss of water. In order to test the reliability of Aquasolv and MAE, two standard compounds (gallic acid (high polarity) and quercetin (high lipophilicity) were evaluated. In case of MAE, recoveries of gallic acid and quercetin added up to 100% (data not shown). In case of Aquasolv, nearly 100% of gallic acid was recovered, whereas the yield of quercetin was 10−20% lower depending on the employed solvent.

A strong dependency of target analyte quantities on the employed extraction solvent was noticed also within the evaluation of the extraction technique: 50% MeOH proved to be the optimal extraction medium, as no other solvent could deliver comparable yields (**Table 1**): Interestingly, catechin was present at the highest concentrations (2.44 mg/g seeds), followed by epicatechin (1.18 mg/g seeds). This huge difference between catechin and epicatechin values is not described in literature, where data for different grape cultivars are published (catechin, 0.21−8.45 mg/g; epicatechin, 0.23−8.93 mg/g) (*2, 4, 11*). For quantification of oligomeric components like P_2 procyanidins, calibration curves from catechin were employed. The highest amounts were achieved using microwave extraction with 50% methanol, i.e., 0.33, 0.41, and 0.39 mg/g for the three P_2 components.

An interesting phenomenon was noticed when samples were extracted with pure water by Aquasolv: With the use of pure water as extraction solvent, unidentified peaks in the mass range of epicatechin gallate and epigallocatechin gallate were detected. In **Figure 2**, selected ion traces of catechin and epicatechin (**Figure 2B**), epicatechin gallate (**Figure 2C**), and epigallocatechin gallate (**Figure 2D**) of the raw grape seed extract in 100% H_2O are shown. New and unidentified signals were noticed only for epicatechin gallate and epigallocatechin gallate ion traces. An explanation for this phenomenon could be given by partial epimerization. In fact, Wang and Helliwell (*26*) reported epimerization of epicatechin gallate to catechin gallate and epigallocatechin gallate to gallocatechin gallate and vice versa. Epicatechin standards showed higher epimerization rates to the corresponding catechins compared to the reverse direction. These effects took place when temperatures higher than 80 °C were applied for extraction. Suzuki et al. (*27*) studied epimerization of 14 different catechin standards and showed that epicatechin derivatives were converted to catechin derivatives at temperatures of 90 °C, in accordance with Wang and Helliwell (*26*). In this study, grape seeds were extracted at 120 °C for 30 min by Aquasolv.

Determination of Antibacterial Activity. Antibacterial activity of flavonoids in plant extracts has already been described in literature (*28*). Rauha et al. (*10*), for instance, reported high antibacterial activity of quercetin and naringenin. As grape seeds are rich in flavonoids, they could present a promising source for antibacterial agents.

MAE raw extracts from grape seeds as well as the fractions, collected according to the protocol of Svedström et al. (*21*), were tested on their antibacterial activity toward *S. aureus*, *P. aeruginosa*, *St. pneumoniae*, *St. pyogenes*, *Klebsiella* sp., *E. coli*, *H. influenzae*, *S. epidermidis*, *En. faecalis* (VRE), and *En. casilliflavus* (VRE) using screening tests as well as quantitative analysis via a spiral platter. Screening tests, performed with MAE raw extracts without being fractionated, provided some activity against different strains such as *S. aureus*, *P. aeruginosa* (only on blood plates), *St. pneumoniae* (*Pneumococcus*, only on Mueller Hinton plates), *St. pyogenes*, *H. influenzae*, and against *S. epidermidis*. In addition to the raw extracts, fractions obtained according to Svedström et al. (*21*), were considered for evaluation of antibacterial activity. Results clearly proved moderate activity of fraction P1 + S1 and high activity of P3 + S2 (data not shown). Chromatographic investigation of these fractions revealed high content of monomeric catechin and epicatechin units in P1 + S1 and oligomeric units in P3 + S2. However, impurities were also detected in both fractions, i.e., procyanidins in P1 + S1 and monomeric units in P3 + S2. A more detailed investigation of those impurities revealed that the origin of monomeric compounds in P3 as well as oligomeric compounds in P1 is ascribed to the fractions S1 and S2 obtained via Sephadex LH20, which were combined with P1 and P3. In a second approach, pure P1 and P3 fractions were thus subjected to antibacterial activity. Interestingly, fraction P1 containing mainly monomers like catechin and epicatechin, did not show any antibacterial activity except for *P. aeruginosa*. In contrast, fraction P3, containing oligomeric units of catechin and epicatechin, was active against all tested strains of bacteria, even when the oligomeric fraction was approximately 4 times lower in concentration compared to P1. Besides screening tests, quantitative analysis using a spiral platter confirmed the high activity of the crude extract (420−485 μg/mL in 1:20 dilution, 42−49 μg/mL in 1:200 dilution) toward *St. pyogenes* and *Pneumococcus*, as growth inhibition was noticed immediately

after application of the test substance (**Table 2**). High antibacterial activity was also found toward *S. aureus*, *S. epidermidis*, and *H. influenzae* (inhibition was gained after 8 h of application), whereas slight activity was observed toward *P. aeruginosa*. Fraction P3 (250 μg/mL in 1:20 dilution), containing oligomeric procyanidins, was highly active toward *S. aureus*, *St. pyogenes*, *S. epidermidis*, *H. influenzae* and in addition to the crude extracts also toward the vancomycin-resistant *En. faecalis* (VRE). The number of bacterial strains was decreased to $>10^2$ cfu/mL after 4 h. A lower inhibition between 10^3 to 10^4 cfu/mL was gained for *En. casilliflavus* after 8 h of exposure. A slightly higher inhibitory effect was observed against *P. aeruginosa*, *Pneumococcus*, *Klebsiella*, and *E. coli* in comparison to the control medium. Surprisingly the crude extract was more active toward *Pneumococcus* than fraction P3.

Qualitative Analysis of Grape Seeds via HPLC−MS. HPLC−MS investigations of fraction P1 proved the presence of flavonoid monomers like catechin and epicatechin. Besides this, glycosidic monomers (malvidin glycosides, quercetin glycosides, kaempferol glycoside, and one catechin glycoside) were detected in the crude grape seed extract (**Table 3**). As small amounts of malvidin glycoside could be detected and as this component is typically present in the skin of grapes, it cannot be excluded that marginal impurities with parts of the skin were present in the provided sample.

Detailed investigations of fraction P3 proved the occurrence of procyanidins P_2, P_3, and P_4 together with the gallate derivatives P_2G and P_3G. In **Figure 3,** the separation of the oligomeric proanthocyanidins (fraction P3) is shown. Peak assignment of higher oligomers was performed by comparison of fragmentation pattern with the compound of the next lower level (e.g., P_4 with P_3). In **Table 3** fragmentation patterns of oligomeric (epi)catechins are summarized. Even if according to eq 1 a huge number of isomers is possible, only a restricted number of different oligomers could be found.

Interestingly, signals of P_2 are present at the same time as signals of P_2G, P_3, P_3G, and P_4, indicated by vertical lines and by spectra C1−F1 shown in **Figure 3**. This fact could either be explained by partial fragmentation during the ionization process via ESI, which would further increase the number of possible isomers, or by coelution of these analytes. Due to their complex nature, the identification of oligomeric compounds consisting of catechin and epicatechin units is a major challenge. Complexity of procyanidins is caused by different combination possibilities of (±)-catechin and (±)-epicatechin units under inclusion of four different types of feasible interflavan bonds, whereas α and β configurations are automatically predetermined due to the hydroxyl group attached to C-3, always being in trans position to the interflavan bond (**Figure 1**) (*17*). Considering all these facts, eq 1 allows the calculation of the number of theoretically possible combinations for an oligomer consisting of n units of (±)-(epi)catechin. Within this equation we neither considered the frequency of occurrence of single isomers nor the branching of oligomeric and polymeric units.

The number of theoretically possible P_n isomers is determined by

$$P_n = \sum_{m=0}^{n-1} \binom{n-1}{m} 4^{n-m} \times 2^{m+n-1} \qquad (1)$$

where n is the polymerization degree and m is the number of occurring 2-*O*-7 linkages; $0 \leq m \leq n-1$.

A total of 48 different combinations of (epi)catechin are thus theoretically possible to form the oligomer P_2. Even if not all theoretically possible combinations occur in the plant kingdom, identification of oligomers with three or more catechin and epicatechin units is obviously reaching an extreme level of complexity. Haslam (*17*) quotes a much lower number of different possible isomers, e.g., 32 different forms for procyanidin P_3 (*17*). These calculations only referred to two different types of monomers under inclusion of interflavan bonds 4−6 and 4−8. Certainly calculations have to be enlarged to all possible types of monomers particularly since recent literature proves the frequent occurrence of (−)-catechin and (+)-epicatechin, two substances that have not been considered (*29–31*). Concerning interflavan bonds, the same arguments are valid (*29–33*). With regard to the high complexity of the extracts, MS plays a central role and is an indispensable tool for structure elucidation and target characterization.

The direct hyphenation of chromatographic techniques to MS is an issue of particular relevance because the injected sample can be analyzed without sample losses due to splitting. Additionally, low flow rates enable the use of a nanointerface, allowing optimal positioning of the spray capillary and therefore highest sensitivity. Future perspectives will focus on the analysis of procyanidins by μ-HPLC−MS employing novel stationary phases on the basis of *p*-methylstyrene/1,2-bis(*p*-vinylphenyl)-ethane (MS/BVPE) (*34*), as preliminary results proved the performance of the capillaries to be comparable to silica-based HPLC columns (**Figure 4**).

In conclusion, we discuss the determination of the antibacterial activity of grape seed extracts with special focus on the chromatographic characterization of positively matching fractions. Since sample extraction has fundamental influence on concentration of gained analytes, MAE and Aquasolv extraction were evaluated. These led to highest yields for MAE.

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