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Advanced Solid Phase Extraction Using Molecularly Imprinted Polymers for the Determination of Quercetin in Red Wine

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Solid phase extraction (SPE) based on molecularly imprinted polymers (MIPs) is a novel approach for sample preparation and preconcentration, gaining increased interest in the fields of environmental, clinical, and food analysis. The first application combining MIPs with SPE for advanced beverage analysis is reported. MIPs for the flavonoid quercetin have been generated, using quercetin as a template molecule in a self-assembly approach and yielding imprinting of 1% of the used template. The MIP achieved a capacity of 0.4 g quercetin per gram polymer and a recovery rate of 98.2%. The application of these synthetic receptors as SPE material for the selective extraction and preconcentration of quercetin from synthetic and red wine samples was investigated. Red wine samples from a French Merlot were directly applied onto the SPE cartridge. The collected fractions were analyzed by high-pressure liquid chromatography. For verification of the obtained results, a similarly prepared nonimprinted polymer and a classical octadecyl silane reversed-phase cartridge were applied as the SPE matrix during control experiments. The MIP enabled the selective extraction of quercetin from a complex matrix, such as red wine, spiked with 8.8 mg per liter quercetin, demonstrating the potential of molecularly imprinted solid phase extraction for rapid, selective, and cost-effective sample pretreatment.

KEYWORDS: Solid phase extraction; molecularly imprinted polymers; flavonoids; quercetin; wine analysis; beverage analysis

INTRODUCTION

Fruit quality, apparent by sensory features such as color, texture, and flavor, is determined to a significant extent by a limited number of flavonoid compounds, which are secondary plant metabolites. Flavonoids, as well as other phenols and related compounds, are also found in finished products, such as wine. They are in part responsible for the color, fragrance, and to some extent for the taste and, therefore, the quality of the wine. Furthermore, positive health effects are attributed to some flavonoids. Flavonols, such as quercetin, have free-radical scavenger properties and are therefore considered as dietary antioxidants. Hence, these compounds are significant for wine production, as they can be used to control the quality of red wine and to determine the varietal origin by quantitative analysis of the flavonoid content. The determination of the free and conjugated content of some flavonoids, such as myricetin and quercetin in red wines of different geographical origins, has already been performed. Accordingly, marked and systemic differences between these wines have been reported (*1*). Over the last years, high-pressure liquid chromatography (HPLC) methods with photodiode array and/or mass spectrometric detection have been used (*2*−*4*) for the determination of quercetin levels in red wine, usually after the hydrolysis of glycosides. Concentration levels were found to be in the range of 5−15 mg L^{-1}. Because of the complexity of the sample matrix, time-consuming sample preparation is necessary, although the direct injection of wine samples during HPLC experiments with subsequent determination of the phenolic compounds has been performed recently (*5*).

A novel approach is presented, by using molecularly imprinted polymers (MIPs) for the cleanup and preconcentration of compounds from a complex matrix, such as wine. During molecular imprinting, cross-linked polymers are formed by free-radical copolymerization of functional monomers with an excess of cross-linker around an analyte that acts as a template. After polymerization, the template is removed by extraction, ideally leaving selective binding sites that are complementary in form and functionality to the imprinted analyte molecules. The functional monomer molecules interact with the template by covalent or noncovalent binding interactions. The main advantage of MIPs over conventional polymers used as separation material is the achievable high selectivity for the target analyte present during the imprinting procedure. Furthermore, MIPs are

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characterized by high mechanical and thermal stability. Summarizing, molecular imprinting enables specific molecular recognition of the imprinted analyte, by forming artificial receptor sites from a synthetic polymer matrix, in analogy to antibody−antigen recognition.

Food analysis using MIPs has been reviewed twice in the past years (*6*, *7*). The potential of MIPs in the agricultural and food sector is emphasized for better quality control of food products by fast, reliable, robust, and cost-effective methods using MIPs. MIPs for pharmaceuticals, food additives, food components and contaminants, herbicides, pesticides, and trace metals have successfully been imprinted. However, many of the reported applications of MIPs in food chemistry demonstrate the potential of the produced MIP without demonstrating the feasibility with real-world samples.

So far, only few molecular imprinting approaches for flavonoid compounds have been reported, for 3-hydroxyflavone (*8*) and recently quercetin (*9*), respectively. A flow-through sensing system based on fluorescence, using the MIP imprinted with 3-hydroxyflavone as a recognition element, was developed. The MIP imprinted with quercetin was used as the sorbent material in solid phase extraction (SPE), to extract a class of compounds, including quercetin and kaempferol, from the hydrolyzate of gingko leaves. The collected fractions were analyzed by HPLC, and the identity of the extracted compounds was confirmed by time-of-flight mass spectrometry. The capacity of the imprinted polymer (IMP) cartridges for quercetin in methanol was 12 μg/g polymer.

In this paper, the synthesized MIPs were first characterized with a two and a five component system, respectively. After characterization, molecularly imprinted solid phase extraction (MISPE) was used for the selective preconcentration of quercetin from red wine prior to chromatographic analysis and compared to results obtained with commonly used reversed octadecyl silane (C_{18}) stationary phases. The collected samples were analyzed with HPLC.

MATERIALS AND METHODS

Chemicals. 3,3′,4′,5,7-Pentahydroxyflavone (quercetin), 2-carbethoxy-5,7-dihydroxy-4-methoxyisoflavone (C-fla), 2′,3,4′,5,7-pentahydroxyflavone (morin), (6-O-α-L-rhamnosyl-β-D-glucoside)-3,3′,4′,5,5′,7-hexahydroxyflavone (rutin), and 3,5,7,3′,4′-pentahydroxyflavane ((+)-catechin) were supplied by Sigma-Aldrich (Steinheim, Germany). Gradient grade acetonitrile, methanol, and acetone were supplied by Merck KGaA (Darmstadt, Germany). Citric acid monohydrate, sodium citrate dihydrate, ethylene glycol dimethacrylate (EDMA), 4-vinylpyridine, and 2,2′-azobisisobutyronitrile (AIBN) were from Sigma-Aldrich.

Preparation of the MIP. The antiquercetin MIP (Fla-IMP) was prepared by bulk polymerization. Quercetin (1 mmol) was dissolved in 15 mL of acetone in a glass tube. The functional monomer 4-vinylpyridine (8 mmol), the cross-linker EDMA (40 mmol), and the initiator AIBN were then added to the solution.

The solution was cooled in an ice bath and purged with nitrogen for 5 min. The degassed prepolymerization mixture was subsequently polymerized by thermal initiation at 60 °C overnight. The same procedure without adding a template was used, to prepare a nonimprinted polymer (Fla-CTL) for control experiments.

The obtained polymer was crushed, then ground for 3 min in a mechanical mortar (Retsch, Haan, Germany), and wet-sieved in acetone until particles with a diameter <25 μm were obtained. The polymer was then sedimented, to eliminate particles <5 μm and for obtaining a uniform particle size. The sedimentation was done in ∼250 mL of acetone; after 1 h, the supernatant was discarded and fresh acetone was added to the precipitated MIP particles. The majority of small particles was separated after 5−6 sedimentations. Finally, the polymer particles were filtrated, washed with methanol, and dried at 45 °C for 24 h.

MISPE. A 50 mg amount of the IMP or the control polymer (CTL), respectively, was packed into 1 mL SPE syringe barrels (Isolute SPE, IST-International Sorbent Technology; Separtis GmbH, Grenzach-Wyhlen, Germany) and capped with fritted polyethylene disks (Isolute accessories, IST) at the top and at the bottom. Prior to each use, the columns were conditioned with the following solvents (in order): methanol−acetic acid (4:1; v/v), methanol, methanol−2 M NaOH (1:1; v/v), methanol−water−acetic acid (18:1:1; v/v/v), methanol, and acetonitrile or water followed by sodium citrate buffer, depending on the application solvent (acetonitrile or sodium citrate buffer containing 15% ethanol and 0.001% Tween 20).

SPE syringe barrels containing 500 mg of C_{18} sorbent were obtained from IST (Mid Glamoran, U.K.) and used for control experiments. The cartridges were conditioned successively with 10 mL of methanol and 10 mL of distilled water.

The extraction was performed using a 12 port vacuum manifold from Alltech (Deerfield, IL). The application and elution solvents were drawn through the syringe barrels into Eppendorf vials, where 500 μL aliquots of the application solvent and 250 μL aliquots of the elution solvents were collected.

Chromatography HPLC−UV/Vis. The collected aliquots of the sample loading and eluting steps from the SPE experiments were analyzed with HPLC, using a column packed with the control polymer (Fla-CTL). The HPLC analysis was performed using a Dionex HPLC (Dionex, Sunnyvale, CA) with a P580 low-pressure mixing pump and an UVD-340S diode array detector with a spectral range from 200 to 600 nm. Acetonitrile containing 10% water and 10% acetic acid was used as the eluent in an isocratic elution with a flow rate of 1 mL min^{-1}. The elutions were performed at room temperature. The injection volume was 20 μL, and the chromatograms were recorded spectrophotometrically at 265 nm.

The red wine sample was also analyzed by direct injection of filtered samples into the HPLC system. Syringe filters with a pore size of 0.45 μm were used (Osmonics) to filter the wine prior to analysis. The HPLC column was a Kromasil C_{18} column (ARC Seibersdorf, Austria) with an Alltech C_{18} precolumn. A linear gradient of the binary eluent, changing the mobile phase from water−acetonitrile (95:5, v/v), adjusted to pH 1.8 with perchloric acid (solvent A), to water−acetonitrile (50:50, v/v), adjusted to pH 1.8 with perchloric acid (solvent B), was used at a flow rate of 0.6 mL min^{-1} (*5*). The linear gradient was also used to analyze some of the collected fractions from the SPE of the red wine, to compare the results with those obtained with the Fla-CTL column.

Capacity of MISPE Columns. The binding capacity of the MISPE column was determined by recovery measurements of quercetin from the imprinted and the CTL. After the columns were conditioned, 8 μg/mL quercetin in acetonitrile was applied in 0.5 mL aliquots onto 1 mL syringe barrels packed with 100 mg imprinted or CTL, respectively. The columns were washed with 2.5 mL of acetonitrile and eluted with 6 mL of methanol−acetic acid (7:1, v/v). The collected aliquots from the washing step (0.5 mL) were directly analyzed by HPLC. The collected aliquots from the elution step (1 mL) were dried under nitrogen and redissolved in 1 mL of the mobile phase. A Kromasil C_{18} column was used in an isocratic elution with water−acetonitrile (1:1, v/v) as the eluent and a flow rate of 1 mL/min.

RESULTS AND DISCUSSION

Optimization of SPE. Successful imprinting of quercetin was confirmed by its application as a stationary phase for HPLC measurements (*10*). The aim of this preliminary work was to determine the maximum capacity of the molecularly imprinted SPE column. Furthermore, proper washing and eluting solvents or solvent mixtures were investigated, allowing a high recovery and maximized separation of quercetin from other analytes contained in a synthetic sample during SPE. The structurally related compound C-fla was selected as a second analyte, to characterize the selectivity of the MIP toward quercetin, since this compound was already applied during the evaluation with HPLC (*10*). Furthermore, morin, rutin, and (+)-catechin were

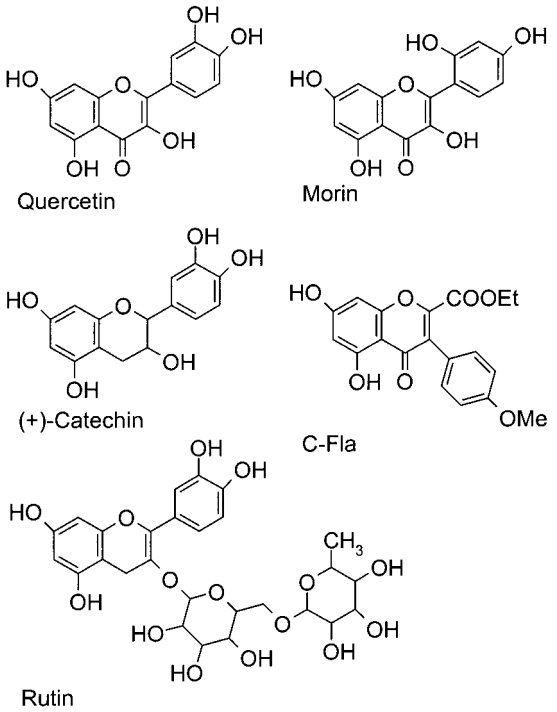

Figure 1. Molecular structure of flavonoids quercetin, morin, (+)-catechin, C-fla, and rutin.

used. The molecular structures of these compounds are given in **Figure 1**.

Better results were obtained when loading the analytes in sodium citrate buffer, rather than in acetic acid buffer. The compounds were therefore applied either in sodium citrate buffer containing 15% ethanol and 0.001% Tween 20 or in acetonitrile.

To determine the column capacity, 192 μg of quercetin in 0.5 mL aliquots of 8 μg/mL acetonitrile was loaded onto the imprinted and CTL columns, respectively. The breakthrough of quercetin from the control column was observed after the application of 64 μg while 104 μg of quercetin could be applied onto the MIP column. The capacity test enables one to determine the extraction performance of the prepared SPE columns. Furthermore, successful imprinting is demonstrated, as the IMP shows selective retention of quercetin as compared to the CTL (**Figure 5**). Quercetin is retained in the control column due to nonspecific interactions with the sorbent material. The imprinted column retains a significantly increased amount of quercetin due to both specific and nonspecific interactions. The recovery was calculated including quercetin determined in acetonitrile after washing the column. Quercetin recovery equals 98.2% from the MIP and 98.8% from the CTL (**Table 1**). Taking into account the amount of nonspecifically bound quercetin, the capacity of the MIP was determined at 40 μg per 100 mg polymer. The capacity achieved by the present imprinting procedure is more than 3 times higher than previously reported in the literature (*9*). The quantitative recovery of quercetin and the high column capacity with the MIP of 0.4 mg/g polymer are optimal prerequisites for the applicability of the synthesized IMP under real-world conditions.

The initial amount of 250 mg of MIP was subsequently reduced to 50 mg. As demonstrated, 50 mg of MIP was enough to quantitatively extract quercetin from 2 mL at a concentration of 9.46 mg L^{-1}.

Application of the Analytes in Sodium Citrate Buffer. Applying the analytes in sodium citrate buffer, the MIP showed a much higher affinity for the target analyte quercetin than for

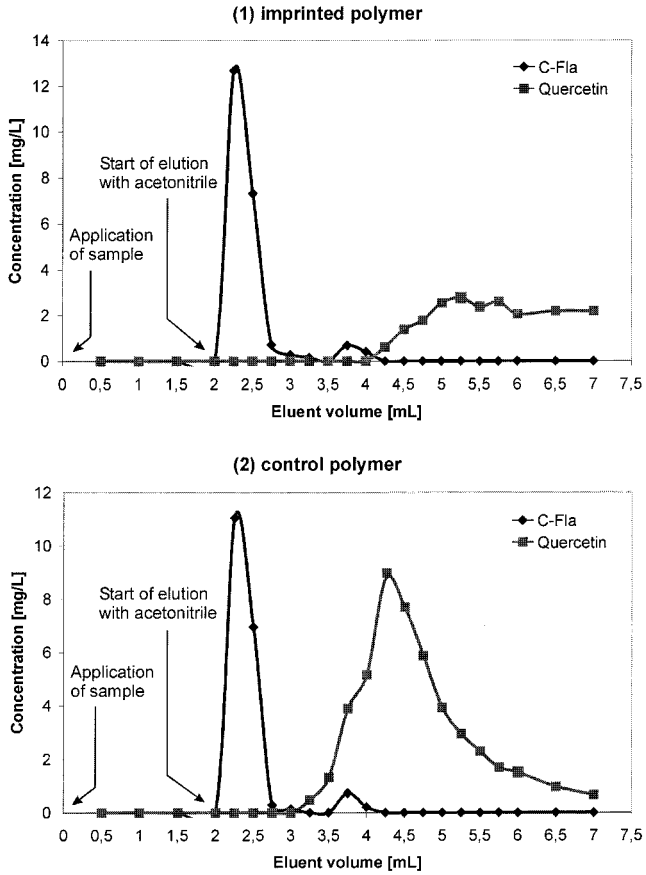

Figure 2. SPE profile for a mixture of C-fla and quercetin: (1) IMP and (2) CTL. The sample was applied in 2 mL of citric acid buffer containing 15% ethanol and 0.001% Tween 20 in 500 μL aliquots (9.66 mg/L quercetin and 3.48 mg/L C-fla) and eluted with 5 mL of acetonitrile after drying the SPE column with a vacuum manifold.

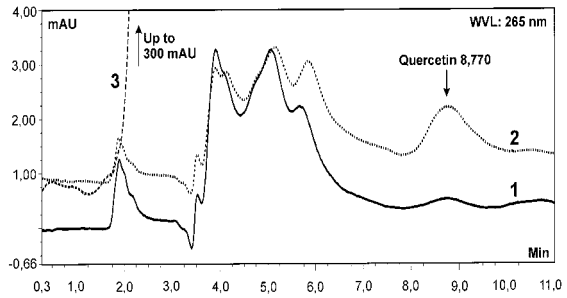

Figure 3. Chromatogram of the SPE fraction from (1) the control and (2) the IMP, collected after 6.5 mL of eluting solvent, as compared to the chromatogram of wine (3) directly injected into HPLC. The red wine sample was eluted with acetonitrile after drying the SPE column with a vacuum manifold. The HPLC analysis was performed with a column packed with the CTL (250 mm × 4.6 mm) and acetonitrile containing 10% water and 10% acetic acid as the mobile phase at a flow rate of 1 mL/min; detection was performed at 265 nm.

the structural analogue C-fla. While there was no difference between the elution behavior of C-fla in the imprinted and in the CTL, there was a marked difference in the elution behavior of quercetin. Quercetin was much stronger retained in the IMP, resulting in a continuous elution when using acetonitrile as the eluent (**Figure 2**). The slow, tailing elution of quercetin with the IMP is caused by the heterogeneity of the binding sites. This effect has been described previously during noncovalent imprinting (*11*−*13*) and can be attributed to the variety of

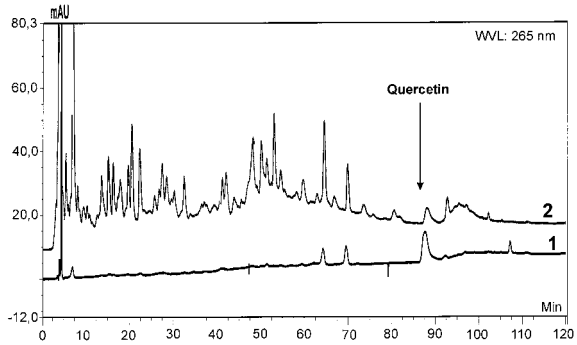

Figure 4. Chromatogram of Merlot registered at 265 nm before (2) and after (1) MISPE; (1) after 1.75 mL of elution with acetonitrile. The HPLC analysis of both samples 1 and 2 was performed using a Kromasil C_{18} column (ARC Seibersdorf, Austria) with a C_{18} Alltech precolumn. A linear gradient of the binary eluent, changing the mobile phase from water–acetonitrile (95:5, v/v), adjusted to pH 1.8 with perchloric acid (solvent A), to water–acetonitrile (50:50, v/v), adjusted to pH 1.8 with perchloric acid (solvent B), was used at a flow rate of 0.6 mL min^{-1}.

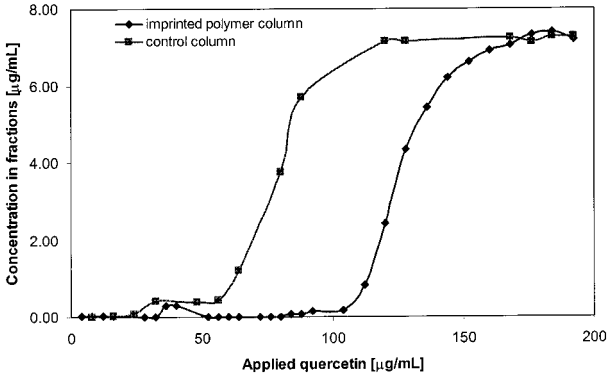

Figure 5. Determination of the column capacity. A total of 21 mL of acetonitrile containing 8 μg/mL quercetin were applied onto 100 mg control and IMP SPE columns, respectively. The collected fractions from the solvent application were analyzed by HPLC with a Kromasil C_{18} column, and the concentration of quercetin was determined. The isocratic elution was performed with a mixture of water and acetonitrile (1:1, v/v) at a flow rate of 1 mL/min; the injection volume was 20 μL.

Table 1. Capacity Test[a]

	amount of quercetin (μg)	
	CTL	IMP
loading	192	192
sorption	64	104
washing	19.15	22.88
elution	44.1	79.2
recovery (μg)	63.25	102.08
recovery (%)	98.8	98.2

[a] Absorbed and recovered amount of quercetin after the washing step (acetonitrile) and the elution step (methanol–acetic acid, 7:1, v/v) from SPE columns packed with 100 mg CTL and IMP, respectively. The collected aliquots were analyzed with HPLC using a Kromasil C_{18} column; the mobile phase was water:acetonitrile (1:1, v/v) with a flow rate of 1 mL/min. The collected aliquots from the elution step (1 mL) were dried under nitrogen and redissolved in 1 mL of the mobile phase.

different binding sites, ranging from very weak interactions to strongly retaining sites. Only 32.1% of the applied quercetin was recovered within a fraction of 5 mL. Quantitative elution (98.3%) of quercetin was obtained when using stronger eluting solvents, such as methanol, containing 15% acetic acid, or acetonitrile, containing 9% water and 1% triethylamine, respectively. These results confirm the potential use and applicability of MIPs imprinted for quercetin for quantitative SPE of aqueous samples.

The A- and C-rings of the flavonoid structure in quercetin and C-fla are identical, but the substituents at the 4′- and 5′-position on the B-ring differ, leading to a marked difference in the three-dimensional structure of both compounds. Therefore, as shape complementarity is not given, the high-affinity binding sites of the MIP are most likely responsible for the difference in the elution behavior of quercetin and C-fla, with the low-affinity binding sites and nonspecific binding sites interacting with both quercetin and C-fla.

Application of the Analytes in Acetonitrile. During MISPE, better results were obtained when using an aprotic solvent for sample application and elution. Ideally, the same solvent is applied, which was used as porogen during the polymerization of the MIP and the CTL, to avoid swelling problems. Acetone was used as porogen, but acetonitrile was chosen as solvent for the application of the analytes. The nonspecific hydrophobic interactions, which reduce the selectivity of the MIP, are considerably reduced in nonpolar acetonitrile, revealing the presence of high-affinity binding sites. The presence of acetic acid, acting as an acidic modifier, was necessary, to achieve quantitative recoveries of quercetin from the MISPE column, but simultaneously resulted in a reduced selectivity. A breakthrough of C-fla was observed when applied in acetonitrile; C-fla was recovered to 100% from both the control and the IMP, while only 15.3% of the applied quercetin was recovered from the imprinted and 51.7% from the CTL, showing a strong binding toward quercetin. When eluting with acetonitrile containing 1% acetic acid, quercetin was recovered to 51.6% from the IMP and to 70.1% from the CTL. Quercetin was retained much longer in the IMP than in the CTL, leading to a better separation of both analytes.

The application of five structurally related compounds quercetin (9.46 mg L^{-1}), C-fla (3.15 mg L^{-1}), morin (9.6 mg L^{-1}), rutin (9.6 mg L^{-1}), and (+)-catechin (8.32 mg L^{-1}) revealed the potential and limitations of the MIP. The elution was started with acetonitrile. C-fla and rutin exposed the same eluting behavior with the imprinted and with the CTL, showing no particular affinity to the IMP. (+)-Catechin had a weak affinity to the IMP. Morin and quercetin showed a strong affinity to the IMP; the complete elution of quercetin and morin had to be enforced by the application of methanol containing 15% acetic acid. The similar molecular structure of the latter three compounds explains the cross-reactivity of (+)-catechin and particularly of morin. The cross-reactivity of structurally related compounds combined with the fact that the compounds of the flavonoid group have similar UV spectra makes the quantification difficult. However, using MIPs instead of nonselective stationary phases at least improves the separation of structurally analogous compounds.

SPE of a Red Wine. After the characterization of the synthesized MIP using synthetic samples containing quercetin and other flavonoids, the polymer was tested as a sorbent material for SPE of quercetin from a complex red wine sample. For this purpose, a Merlot was chosen (1999, Champierre; Vin de pays d'Oc). The wine was spiked with 8.8 mg L^{-1} quercetin. A 2 mL amount of the spiked wine was applied in 500 μL aliquots onto the SPE cartridges, packed with 50 mg of polymer. After the column was dried by a vacuum manifold, the wine sample was eluted with acetonitrile. The fractions from the SPE

and the red wine sample were analyzed with HPLC, using a column packed with the CTL.

The SPE of the wine sample prior to chromatographic analysis enabled one to separate quercetin from the sample matrix. While quercetin had already been eluted from the CTL (1) after 6.5 mL of acetonitrile, it was still released from the IMP (2) and could be detected with a minimum of interferences (**Figure 3**). After direct injection of the wine sample without SPE pretreatment, a very intensive (up to 300 mAU) and broad peak, resulting from overlapping peaks from the many compounds in red wine absorbing in the UV range, made an interpretation impossible or at least very difficult (chromatogram 3 in **Figure 3**). The quercetin peak significantly overlaps with those of the other compounds from the wine sample.

Furthermore, the spiked Merlot was diluted 1:4 with distilled water, and 4 mL was applied onto the C_{18} cartridge. The cartridge was washed with distilled water and dried by vacuum manifold, and the extraction was performed with methanol containing 15% acetic acid. The collected fractions were analyzed in the same way as the fractions from MISPE. It was not possible to separate quercetin from other phenolic extracts of the red wine.

Results of HPLC Investigations for Real Wine Samples. In **Figure 4**, the chromatogram obtained with the 250 μL SPE fraction collected after 1.75 mL of elution with acetonitrile, corresponding to the fraction with the maximum concentration of quercetin, was compared with the chromatogram of the red wine sample directly injected into the HPLC system (binary gradient, C_{18} column). The SPE with the MIP was able to considerably reduce the complexity of the chromatogram and to enrich quercetin from the red wine spiked with 8.8 mg L^{-1} quercetin. The peak corresponding to quercetin appeared between 87.3 and 87.7 min. At 265 nm, the chromatogram obtained from the MISPE fraction contained only five major peaks, with one of them easily identified as quercetin.

The applied binary gradient enabled a sufficient separation of phenolic compounds in red wine for the directly injected sample. However, detection methods such as mass spectrometry and/or multivariate data analysis are required, to identify the present analytes with sufficient certitude. If quercetin is the only analyte of interest, the SPE profile obtained with a control column is sufficient and, with an extraction time of 15 min, much more timesaving than the procedure with a binary gradient and a C_{18} column, which requires 120 min.

Conclusions. MIPs selective for quercetin were prepared and applied as the material for SPE in off-line separations. The MIP showed excellent selectivity toward quercetin and was therefore suitable for the application in SPE. The produced MIP is a low-cost, thermally and mechanically stable material, which offers the advantages of selectivity toward quercetin, enabling an effective sample pretreatment of complex matrixes, such as red wine, and simplifies subsequent chromatographic analysis. A capacity of 0.4 mg quercetin per gram polymer has been achieved, with recovery rates of 98.2%. The presented approach demonstrates the application of MISPE for the analysis of real-world beverage samples for the first time and reveals a substantial potential of this advanced approach soon.

ABBREVIATIONS USED

MIP, molecularly imprinted polymer; HPLC, high-pressure liquid chromatography; SPE, solid phase extraction; MISPE, molecularly imprinted solid phase extraction; IMP, imprinted polymer; CTL, control polymer; EDMA, ethylene glycol dimethacrylate; AIBN, 2,2′-azobisisobutyronitrile; C_{18}, octadecyl silane; C-fla, 2-carbethoxy-5,7-dihydroxy-4-methoxyisoflavone.

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