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# On-Line HPLC-DPPH Screening Method for Evaluation of Radical Scavenging Phenols Extracted from Apples (*Malus domestica* L.)

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An on-line HPLC-DPPH screening method for phenolic antioxidants in apple methanol/water (80:20, v/v) extracts was applied. The determination of antioxidants was based on a decrease in absorbance at 515 nm after postcolumn reaction of HPLC-separated antioxidants with the 2,2′-diphenyl-1-picrylhydrazyl radicals (DPPH•). Each of the antioxidants separated by the HPLC column was observed as a negative peak corresponding to its antioxidative activity. The on-line method was applied for quantitative analysis of the antioxidants. A linear dependence of negative peak area on concentration of the reference antioxidants was observed. For validation of the on-line method the limit of detection, LOD ($\mu$g/mL), and the limit of quantification, LOQ ($\mu$g/mL), of the phenolic compounds were determined. Comparison of the UV and DPPH radical quenching chromatograms with authentic compounds identified catechin, chlorogenic acid, caffeic acid, epicatechin, and phloridzin in the apple cultivars (Lobo, Golden Delicious, and Boskoop), and the distribution of total antioxidant activity was calculated.

**KEYWORDS: Radical scavenger activity; on-line HPLC-DPPH; phenols; apple extracts; *Malus domestica* L.**

## INTRODUCTION

Oxygen and reactive oxygen species (ROS) are among the major sources of primary catalysts that initiate oxidation in vivo and in vitro (*1*, *2*). Oxygen-derived free radicals such as superoxide anion radical ($O_2^{-\bullet}$) and hydroxyl radical ($^{\bullet}OH$) are thought to be linked to the onset of various pathological conditions. Many researchers have shown that lipid peroxides and reactive oxygen species are involved in the development of a variety of diseases, including cancer, and also accelerate aging (*3*−*8*). A compound might exert antioxidant actions in vivo or in food by inhibiting generation of ROS, or by directly scavenging free radicals (*9*−*12*). Antiradical antioxidants act by donating hydrogen atoms to lipid radicals. Radicals obtained from antioxidants with molecular structures such as phenols are stable species and will then stop the oxidation chain reaction (*4*, *13*). Furthermore, there is now growing evidence that polyphenols may possess inhibitory effects against cancer (*14*). Many anticarcinogens are naturally occurring non-nutrients, primarily of plant origin, such as flavones and polyhydroxy compounds (*15*, *16*).

A multitude of natural antioxidants have already been isolated from different kinds of plant materials such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (*6*, *9*, *17*). Plants contain a diverse group of phenolic compounds including simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives, and flavonoids. All the phenolic classes have the structural requirements of free radical scavengers and have potential as food antioxidants (*18*, *19*). Factors influencing the antioxidant activity of plant phenolics include position and degree of hydroxylation, polarity, solubility, reducing potential, stability of the phenol to food processing operations, and stability of the phenolic radical (*20*, *21*). Catechins are a group of flavonoids that have attracted much attention because of their relatively high antioxidant capacity, reducing a variety of toxic effects caused by reactive oxygen (*14*). Cuvelier et al. (*22*) studied the antioxidative efficiency of some phenolic acids, including caffeic acid, ferulic acid, rosmarinic acid, chlorogenic acid, and BHT. Chen et al. (*23*) investigated the antioxidative potency of some hydroxycinnamic acid compounds and elucidated the relationship between their activities and chemical structures.

The antioxidant potential of individual substances is thus being actively investigated, and several methods, including the use of a chemiluminescent (CL) reaction, or a reaction with a stable radical species such as 2,2′-diphenyl-1-picrylhydrazyl (DPPH), or 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$), etc., have been developed as sensitive assess (*24*−*29*). However, it is difficult to quantitatively assay the antioxidant activity because of the short lifetimes of these radicals. Additionally, the activity of natural antioxidants often decreases

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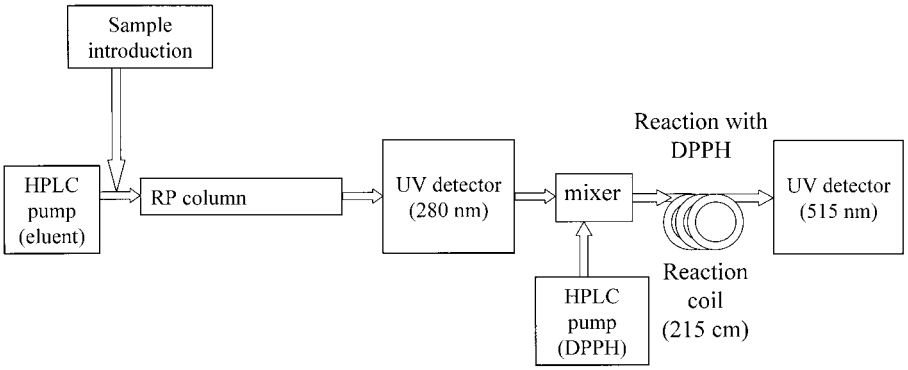


**Figure 1.** Instrumental setup for the HPLC analysis of radical scavenging compounds using an on-line reaction with DPPH.

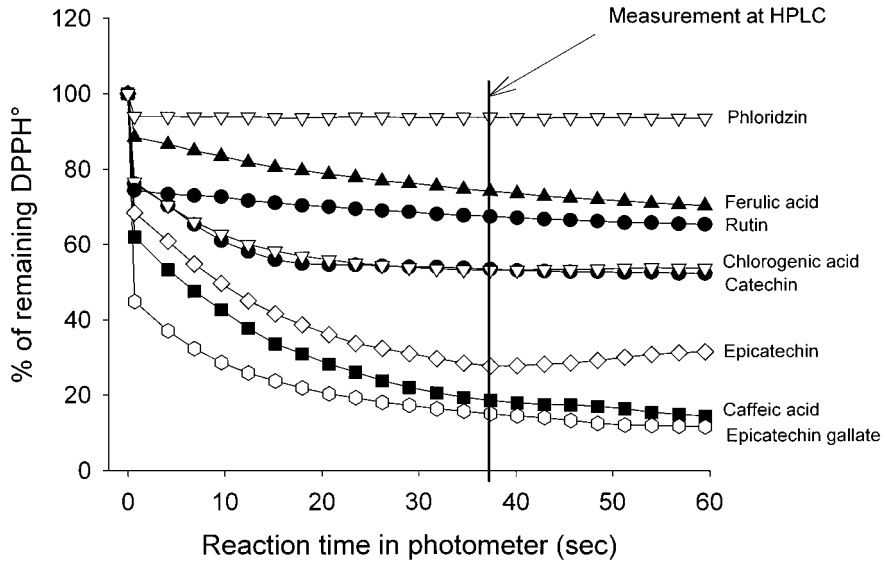


**Figure 2.** Kinetics of the reaction of DPPH with phenols during the first 60 s.

during their isolation and purification due to their decomposition. A method combining separation of antioxidants and activity evaluation would present a major advantage for such investigations. Reports by Dapkevicius et al. (*30*) and Ogawa et al. (*3*) concern on-line detection of HPLC antioxidative eluates by means of chemiluminescence. Although very sensitive, these methods require a special instrumental setup and expensive and unstable reagents. Koleva et al. (*31*) published a new rapid on-line method for screening complex mixtures for radical scavenging components using a methanolic solution of the DPPH stable free radical, and presented an optimized instrumental setup. The greatest benefit of the method is that, besides the quantification by UV detection, the radical scavenging activity of a single substance can be measured, and its contribution to the overall activity of a mixture of antioxidants can be calculated. Thus, it is no longer necessary to purify every single constituent for off-line assays, leading to very significant reductions of costs and time to obtain results (*31*). However, reports concerning on-line separation and antioxidant activity assessment, as well as quantitative analysis of radical scavenging compounds in complex samples, are scarce.

The aim of the present study is development of a screening method for radical scavenging compounds in food samples (apples), based on a postcolumn reaction of the antioxidant with the DPPH radical.

## MATERIALS AND METHODS

**Materials.** The solvents used were of analytical grade and purchased from Merck (Darmstadt, Germany). All chemicals were commercially available, chosen of the highest purity, and used as received. 2,2′-Diphenyl-1-picrylhydrazyl (DPPH•), caffeic acid, ferulic acid, chlorogenic acid, catechin, epicatechin, epicatechin gallate, phloridzin, and rutin were purchased from Sigma-Aldrich (Taufkirchen, Germany). DPPH• solutions were freshly prepared in methanol every day and kept protected from light.

**Preparation of Apple Extracts.** The apple cultivars (Lobo, Golden Delicious, and Boskoop) harvested in Austria in the 2000 season were obtained from a local market. To extract soluble phenolic compounds from fruits (*32*) 100 mg of the freeze-dried and homogenized apple material was extracted with 8 mL of a methanol/water mixture (80:20, v/v) at room temperature by using an ultrasonic bath for 10 min and a vibromixer for 5 min. The mixtures were centrifuged (Z 320 HERMLE Labor Technic, Germany) at 3400 $min^{-1}$ for 5 min and filtered through paper filter MN 615 (Macherey-Nagel, Düren, Germany). The resulting liquid extracts were stored in the dark at −20 °C under nitrogen until analysis for less than 1week. During the storage no change in the analytes was observed.

The methanolic apple extracts were used directly for the spectrophotometric assay and HPLC analysis.

**Scavenging Effect on DPPH Radicals in Photometer.** This method was originally published by Gadow et al. (*26*). The time course of the radical scavenging ability of the tested phenolic compounds and apple extracts was measured in terms of hydrogen donating or radical scavenging ability, using the DPPH radical. Methanolic solution (50 $\mu$L) of the phenolic compounds at a concentration of 200 $\mu$g/mL and 50 $\mu$L of apple extracts at 50 $\mu$g extracted substances/mL was placed in 1-cm cuvettes (Greiner, Labortechnik), and 2 mL of methanolic solution of DPPH ($6 \times 10^{-5}$M) was added. The decrease in absorbance at 515 nm was determined continuosly with data aquisition at 2-s intervals with a spectrophotometer (Varian, Cary 50 (Mulgrave, Australia)) for 1 min. The percent inhibition of the DPPH radical by

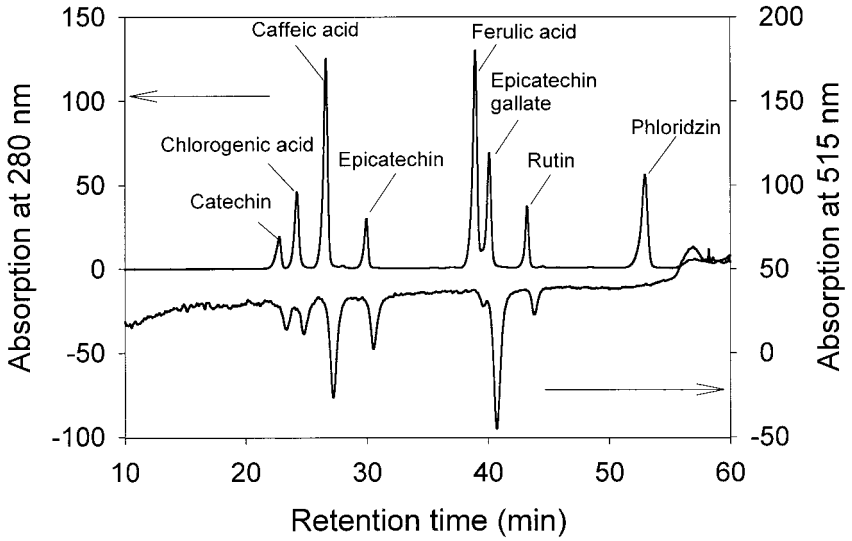


**Figure 3.** UV and DPPH radical quenching chromatograms of phenolic standard substances.

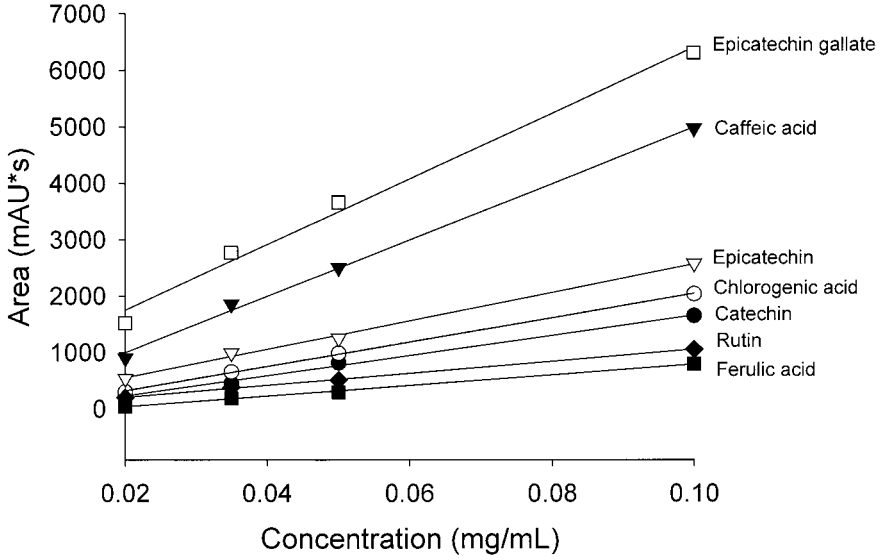


**Figure 4.** Linear dependence of negative peak area on concentration of the phenols.

the polyphenols and apple extracts was calculated according to the formula

$$\% \text{ inhibition} = [(A_{C(0)} - A_{A(t)})/A_{C(0)}] \times 100$$

where $A_{C(0)}$ is the absorbance of the control at $t = 0$ min and $A_{A(t)}$ is the absorbance of the reaction solution at $t = 1$ min.

**On-Line HPLC-DPPH Analysis.** An on-line method was described for detection of radical scavenging components (*31*). The on-line HPLC-DPPH method was developed using a methanolic solution of DPPH stable free radical. A scheme of the instrumental setup is given in **Figure 1**. The HPLC-separated analytes reacted postcolumn with the DPPH at a concentration of 50 mg/L in methanol. The flow of the reagent solution was set to 0.4 mL/min, and the induced bleaching was detected as a negative peak photometrically at 515 nm. The length of the capillary (215 cm) used for the postcolumn reaction was adjusted to achieve a reaction time of 0.6 min. The separation of antioxidative components was carried out by HPLC: Hewlett-Packard series 1100 VW detector model G1314A, consisting of a model G1311A quat pump and a model G1329A thermostatted autosampler; LiChroCart RP-18 column (5 $\mu$m, 250 $\times$ 3 mm); gradient elution at 0.6 mL/min with gradient program (0−40 min 2−20% B, 40−50 min 20% B, 50−55 min 20−80% B, 55−60 min 80% B) with 2% acetic acid in water as solvent A and acetonitrile as solvent B; UV detection was carried out at 280 nm.

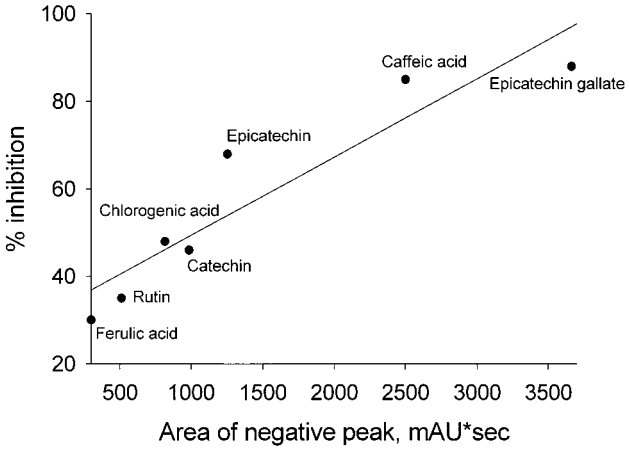


**Figure 5.** Correlation of antioxidant activity measured by a photometric and HPLC assay using the reduction of the DPPH radical.

The methanolic extracts were used directly for on-line HPLC analysis. For the stock solution of the standards, the polyphenols (chlorogenic acid, caffeic acid, ferulic acid, catechin, epicatechin, epicatechin gallate, rutin, and phloridzin) were dissolved in methanol at a concentration of 1 mg/mL. The concentrations of reference

**Table 1.** Limits of Detection (LOD) and Limits of Quantification (LOQ) of Phenolic Standards in the On-line HPLC-DPPH System

| | UV detection at 280 nm | | | VIS detection at 515 nm | |
|---|---|---|---|---|---|
| tested compound | RT min | LOD[a] $\mu$g/mL | LOQ[a] $\mu$g/mL | RT min | LOD[a] $\mu$g/mL |
| catechin | 22.5 | 0.91 | 3.0 | 23.1 | 10 |
| chlorogenic acid | 24.0 | 0.17 | 0.55 | 24.6 | 7.5 |
| caffeic acid | 26.4 | 0.05 | 0.17 | 27.0 | 1.9 |
| epicatechin | 29.9 | 0.23 | 0.77 | 30.5 | 3.1 |
| ferulic acid | 39.0 | 0.86 | 2.9 | 39.6 | 7.7 |
| epicatechin gallate | 40.2 | 0.06 | 0.20 | 40.8 | 1.1 |
| rutin | 43.3 | 0.11 | 0.38 | 43.9 | 4.3 |
| phloridzin | 53.2 | 0.16 | 0.53 | | |

[a] Injection volume 10 $\mu$L.

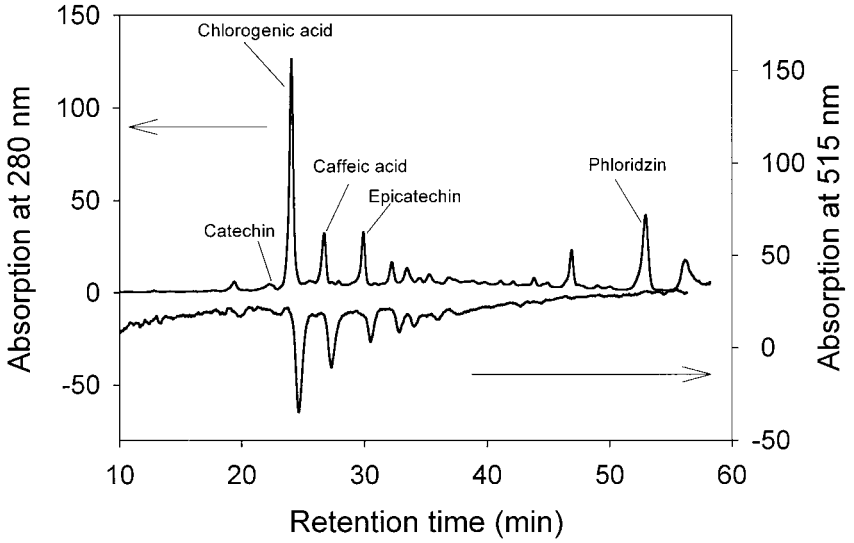


**Figure 6.** Typical UV and DPPH radical quenching chromatograms of an apple extract (Boskoop).

**Table 2.** Total Antioxidant Activity of the Apple Extracts Obtained by the HPLC-DPPH Method and by the Photometric Measurements

| | | % of total negative peak area of main identified antioxidants in HPLC | | | |
|---|---|---|---|---|---|
| apple sample | % inhibition in photometer[a] | catechin | chlorogenic acid | caffeic acid | epicatechin |
| Boskoop | 27 | 4 | 39 | 22 | 12 |
| Golden Delicious | 6 | 3 | 27 | 23 | 18 |
| Lobo | 2 | | 40 | 12 | 26 |

[a] Calculated on the basis of Gadow et al. (*26*).

**Table 3.** Phenols Content (mg/100 g, fresh weight) in Freeze Dried Apples as Determined with HPLC–UV

| | amount of phenolic compounds, mg/100 g f.w. | | | | |
|---|---|---|---|---|---|
| apple sample | catechin | chlorogenic acid | caffeic acid | epicatechin | phloridzin |
| Boskoop | 15.7 | 137 | 14.2 | 56.0 | 43.5 |
| Golden Delicious | 10.0 | 56.5 | 9.0 | 43.7 | 11.4 |
| Lobo | 6.1 | 24.3 | 1.8 | 9.6 | 24.7 |

substances used for calibration of the HPLC analysis were 0.01, 0.02, 0.035, 0.05, and 0.1 mg/mL.

## RESULTS AND DISCUSSIONS

A novel screening method for antioxidants is achieved by on-line HPLC using the DPPH free radical. It is well-known that DPPH$^\bullet$ absorbs at 515 nm, but upon reduction by an antioxidant or a radical species the absorption disappears (*9*). The more rapidly the absorbance decreases, the more potent the antioxidant activity of the compound in terms of hydrogen donating ability (*26*). The HPLC-separated antioxidants react postcolumn with the DPPH. The induced bleaching is detected as a decrease in absorbance at 515 nm.

Additionally for on-line analysis the reaction kinetics of reference phenols with DPPH$^\bullet$ was measured with a spectrophotometer by using the DPPH free radical method. The different kinetic behavior of antioxidants is a crucial point in the evaluation of the antiradical activity, as the reaction time in the HPLC analysis is fixed at 0.6 min because of the fixed length of the reaction capillary. Each antioxidant was tested at a concentration of 5 $\mu$g antioxidant/mL DPPH$^\bullet$ and the percentage of remaining DPPH$^\bullet$ was determined (**Figure 2**). Epicatechin

gallate, caffeic acid, and epicatechin show very rapid reactions during the first minute. The reactions of catechin, chlorogenic acid, rutin, and ferulic acid were significantly slower. It is necessary to note that catechin and chlorogenic acid showed the same kinetic behaviors. From the phenolic compounds tested only phloridzin showed no reaction with DPPH•.

For validation of the on-line HPLC method, the calibration curves of negative peaks at concentrations of 0.01, 0.02, 0.035, 0.05, and 0.1 mg/mL of reference phenols were obtained. Simultaneously obtained UV and DPPH radical quenching chromatograms using gradient elution of the standard phenolic compounds mixture consisting of catechin, chlorogenic acid, caffeic acid, epicatechin, ferulic acid, epicatechin gallate, rutin, and phloridzin at a concentration of 0.05 mg/mL for each substance are presented in **Figure 3**. Using the on-line method for analysis of the phenolic compounds, negative peaks were observed for all compounds except phloridzin. Phloridzin was the only compound in the mixture which did not possess any radical scavenging ability.

The different antioxidant kinetic behaviors for these tested compounds were assayed photometrically, and the results were compared with those of the HPLC−DPPH method. A linear relationship between the negative peak areas and injected concentrations was observed for all compounds. The correlation is shown in **Figure 4**. The correlation factors obtained for the relations of all tested radical scavengers were very high ($R^2$ = 0.9909−0.9997). Antioxidative activity of the substances can be calculated from **Figure 4** as the slope of the regression line. It is highest for epicatechin gallate and decreases in the following order: epicatechin gallate > caffeic acid > epicatechin > chlorogenic acid > catechin > rutin > ferulic acid. In our experiments, epicatechin gallate was the strongest of the three catechins. Ogawa et al. (*3*) reported a stronger antiradical activity of epicatechin compared to catechin using liquid chromatography with chemiluminescence detection. The hydroxycinnamic acids showed similar results as reported by Chen et al. (*23*) and Gadow et al. (*26*) who also used DPPH• for their assays. The antioxidative activity of each substance is reflected by the increase of the peak area after the postcolumn reaction with increased concentrations. The results obtained by HPLC are comparable to the data obtained by photometric measurements. A linear correlation ($R^2$ = 0.8633) of antioxidant activity measured by the two assays is shown in **Figure 5**. It can also be seen in **Figure 5** that epicatechin gallate and caffeic acid (rapid kinetics) are the most effective antioxidants in both experiments. Ferulic acid and rutin (slow kinetics) were not so effective antioxidants.

For validation of the on-line method, the limits of detection (LOD) and the limits of quantification (LOQ) of the phenolic compounds were determined on the basis of the signal-to-noise ratio (S/N) of 3 and 10, respectively, by use of the HP Chemstation software (Rev. A.06.03 [509]) (**Table 1**). However, UV absorption is more sensitive (LOD = 0.05−0.91 $\mu$g/mL) than the DPPH reaction (LOD = 1.1−10 $\mu$g/mL) and therefore better suited for the quantification of single substances. Comparison of the LOD of the studied compounds as determined by this method with the data published by Koleva et al. (*31*) gives similar results.

Also, according to these studies (**Figure 4** and **Table 1**), we can assert that the reaction kinetic with DPPH• is one important factor among several others influencing minimum detectable amounts. The rapid reaction of epicatechin gallate and caffeic acid led to lower limits of detection: 1.1 and 1.9 $\mu$g/mL, respectively.

As an example of application to real samples, the determination of antioxidants in extracts from three different apple cultivars (Lobo, Golden Delicious, and Boskoop) was carried out with the presented on-line HPLC-DPPH method. It is known that the main phenolic constituents of apples are phenolic acids, catechins, procyanidins, and flavonol glycosides. In addition, phloridzin is present in seeds and peel. The concentration and relative proportions vary from one cultivar to another and in relation to fruit maturity. The compounds are involved in enzymatic browning and haze formation during juice manufacture (*32*).

The typical UV and DPPH radical quenching chromatograms of the Boskoop apple extract are shown in **Figure 6**. From the comparison of the chromatograms of authentic compounds, the peaks with retention times at 22.4, 24.0, 26.7, 29.9, and 52.9 min in the UV chromatogram, and at 23.0, 24.6, 27.3, and 30.5 min (for phloridzin no signal is obtained) in the DPPH chromatogram were identified as catechin, chlorogenic acid, caffeic acid, epicatechin, and phloridzin, respectively. The chromatograms show that antioxidants from apples can be identified. The total antioxidant activity of the apple extracts is split up after HPLC-separation (**Table 2**). The amounts of the identified phenols in the three apple cultivars were calculated from UV chromatograms (**Table 3**). In the three cultivars chlorogenic acid was the most abundant antioxidant and thus contributed most to the antioxidant activity of the extracts, with 27−40% of the total antioxidant activity (**Table 2**).

## CONCLUSIONS

The proposed screening method using on-line HPLC-DPPH seems to be useful for the detection of antioxidants because of its high sensitivity and ease of handling. The method is advantageous for the sensitive determination of individual antioxidants in complex matrixes (apple) with simple operation. The method was applied for quantitative analysis of the antioxidants. A linear dependence of negative peak area on concentration of the antioxidants was observed. The antioxidative activity of each substance is reflected by the increase of the peak area after the postcolumn reaction with increased concentration. However, UV absorption is more sensitive and therefore better suited for the quantification of single substances. Catechin, chlorogenic acid, caffeic acid, epicatechin, and phloridzin were identified in the tested apple cultivars (Lobo, Golden Delicious, and Boskoop), and the distribution of total antioxidant activity of the apple extracts was calculated from DPPH chromatograms. Chlorogenic acid was the main antioxidant in all three cultivars.

The greatest benefit of the method is that, besides the quantification by UV detection, the radical scavenging activity of a single substance can be measured and its contribution to the overall activity of a mixture of antioxidants can be calculated.

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