Free Radical-Scavenging Activity of Phenolics by Reversed-Phase TLC

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ABSTRACT: A method was developed to measure the radical-scavenging activity of compounds separated by reversed-phase TLC (RP-TLC) using phenolic acids as model analytes. TLC separation was followed by dipping the plate in a 0.04% (wt/vol) solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol. The compounds possessing radical-scavenging activity were detected as bright yellow bands against a purple background. A video documentation system based on a CCD video camera was used for the detection and quantification of the activity. The developed RP-TLC-DPPH method was compared to the widely used spectrophotometric DPPH assay. The results obtained by the two methods correlated well, apart from syringic acid, ascorbic acid, and *n*-propyl gallate, which proved to be outliers in the regression analyses. The correlation coefficient, after excluding outliers, was $r^2 = 0.923$. The RP-TLC-DPPH method was applied for the measurement of free radical-scavenging activity of rapeseed meal fractions. A total of 10 separated zones with free radical-scavenging activity were detected, with R_f values ranging from 0.04 to 0.85. The results show that the method can be used for the effective fractionation and analysis of potential antioxidative compounds in natural extracts.

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KEY WORDS: 1,1-Diphenyl-2-picrylhydrazyl, free radical scavenging activity, phenolic compounds, reversed-phase thin-layer chromatography.

The oxidation of lipids is the main mechanism responsible for the deterioration of food (1). The oxidation process can be prevented effectively by using free radical-scavenging antioxidants such as BHA, BHT, and propyl gallate. Furthermore, berries, fruits, and vegetables naturally contain many antioxidative compounds, for example, simple phenolics, flavonoids, and tannins.

In addition to the conjugated diene method, which measures the rate of hydroperoxide formation and decomposition in different lipid systems and emulsions (2,3), radical-scavenging assays such as measurement of hydroxyl radical-scavenging activity, superoxide anion radical-scavenging activity, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity (4) have also been used.

DPPH is a stable, water-soluble free radical with an absorption maximum at 517 nm (5). This strong absorption is due to the unpaired electron of the DPPH radical (4). When this radical compound accepts an electron or a hydrogen radical from a radical scavenger, the absorption vanishes and the resulting decolorization is stoichiometric with respect to the number of electrons taken up.

Many variations of the spectrophotometric DPPH method, first developed by Blois (4), are currently used, and this method has attained the status of a routine procedure in the screening of antioxidants (6–10). These methods measure the total DPPH radical-scavenging activity of the extracts or fractions of interest but lack the ability to isolate the activity caused by only one or a few components of the mixture.

TLC combined with DPPH radical detection of antioxidants *in situ* was first introduced in 1967 by Glavind and Holmer (11). The method involved TLC separation of the analyte mixture, followed by detection of DPPH radical scavengers by spraying the TLC plates with 0.01% (wt/vol) DPPH solution in methanol. The plates were evaluated visually, and a quantitative determination of γ-tocopherol was achieved with satisfactory recovery by eluting the antioxidant spots with DPPH reagent. A similar method on normal-phase TLC plates has been used for the screening of antioxidants produced by marine bacteria (12) and antioxidants present in plant extracts (13,14).

The aim of this study was to develop a method that can be used to measure the radical-scavenging activity of antioxidants on a reversed-phase TLC (RP-TLC) plate using phenolic acids and flavonoids (Fig. 1) as model analytes, and to compare their activity to that of known antioxidants such as α-tocopherol and L-ascorbic acid. The spectrophotometric DPPH assay was used as a reference method. The developed RP-TLC-DPPH method was applied for measurement of radical-scavenging fractions of rapeseed meal extract.

EXPERIMENTAL PROCEDURES

Apparatus. A Camag Video Documentation System coupled to a Reprostar 3 Transilluminator cabinet (Camag, Muttenz, Switzerland) was used for imaging the TLC plates. It consisted of a high-resolution 3 × ½″ CCD video camera, model HV-C20 (Hitachi Denshi Ltd., Japan) coupled to a computer equipped with a frame grabber to enable rapid acquisition and processing of the captured images. A built-in CD recorder (CDD3610, Philips, The Netherlands) was used for storing the captured and processed images. The whole system was operated under Video Store 2 (version 2.23) and Video Scan (version 1.01) software. An Epson Stylus Photo color ink jet

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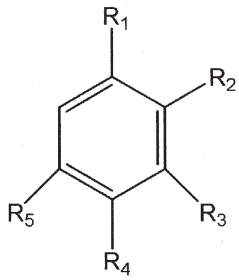

Compound	R_1	R_2	R_3	R_4	R_5	% Inhibition
Hydroxybenzoic acids						
Vanillic acid (HBA1)	COOH	H	OCH_3	OH	H	2.2
Syringic acid (HBA2)	COOH	H	OCH_3	OH	OCH_3	89.4
3-Hydroxybenzoic acid (HBA3)	COOH	H	OH	H	H	2.3
Protocatechuic acid (HBA4)	COOH	H	OH	OH	H	30.0
Gallic acid (HBA5)	COOH	H	OH	OH	OH	64.7
2,3-Dihydroxybenzoic acid (HBA6)	COOH	OH	OH	H	H	38.9
n-Propyl gallate (HBA7)	$COO(CH_2)_2CH_3$	H	OH	OH	OH	90.4
Hydroxycinnamic acids						
Ferulic acid (HCA1)	CH=CHCOOH	H	OCH_3	OH	H	22.8
Sinapic acid (HCA2)	CH=CHCOOH	H	OCH_3	OH	OCH_3	41.4
3-Coumaric acid (HCA3)	CH=CHCOOH	H	OH	H	H	2.5
Caffeic acid (HCA4)	CH=CHCOOH	H	OH	OH	H	46.2
p-Coumaric acid (HCA5)	CH=CHCOOH	H	H	OH	H	2.3
Other phenolics and ascorbic acid (see **B** on page 11)						
Chlorogenic acid (CA)						51.0
Ellagic acid (EA)						63.2
Catechin (C)						54.9
Quercetin (Q)						34.1
Ascorbic acid (AA)						52.6
α-Tocopherol (AT)						53.2

FIG. 1. Structures of compounds studied and their relative radical-scavenging activity.

printer (Seiko Epson Co., Japan) was used for producing illustrative color documents of the images.

A PerkinElmer Lambda 11 UV/vis spectrophotometer was used for the spectrophotometric DPPH assay.

Reagents. Caffeic acid, 3-coumaric acid, 2,3-dihydroxybenzoic acid, ferulic acid, 3-hydroxybenzoic acid, sinapic acid, and vanillic acid were obtained from Extrasynthese S.A. (Genay, France). Catechin, chlorogenic acid, *p*-coumaric acid, ellagic acid, gallic acid, *n*-propyl gallate, protocatechuic acid, quercetin, and syringic acid were purchased from Sigma Chemical Co. (St. Louis, MO), and L-ascorbic acid from Aldrich Chemical Co (Milwaukee, WI). α-Tocopherol was from Merck (Darmstadt, Germany).

DPPH was purchased from Sigma Chemical Co. Methanol (HPLC grade) was obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland), DMSO from Acros Organics (Fairlawn, NJ), *o*-phosphoric acid (analytical grade) from Riedel-de Haën AG (Seelze, Germany), and sulfuric acid (analytical grade) from Merck. Water was purified with an Alpha Q Water Purification System (Millipore Co., Milford, MA).

Extraction of rapeseed meal. Rapeseed meal (0.5 g) obtained from Mildola Ltd. and Raisio Group Ltd., Finland, were extracted with two 10-mL fractions of methanol/water (80:20, vol/vol) at ambient temperature using an UltraTurrax T25 (IKA-Werke GmbH & Co. KG, Staufen, Germany). The extracts were centrifuged for 15 min at 4°C at 3600 × *g* (relative centrifugal force). The clear supernatants were collected and evaporated to dryness on a rotary evaporator. The dry extracts were redissolved in 0.5 mL of methanol and used without further processing for the TLC and spectrophotometric measurements.

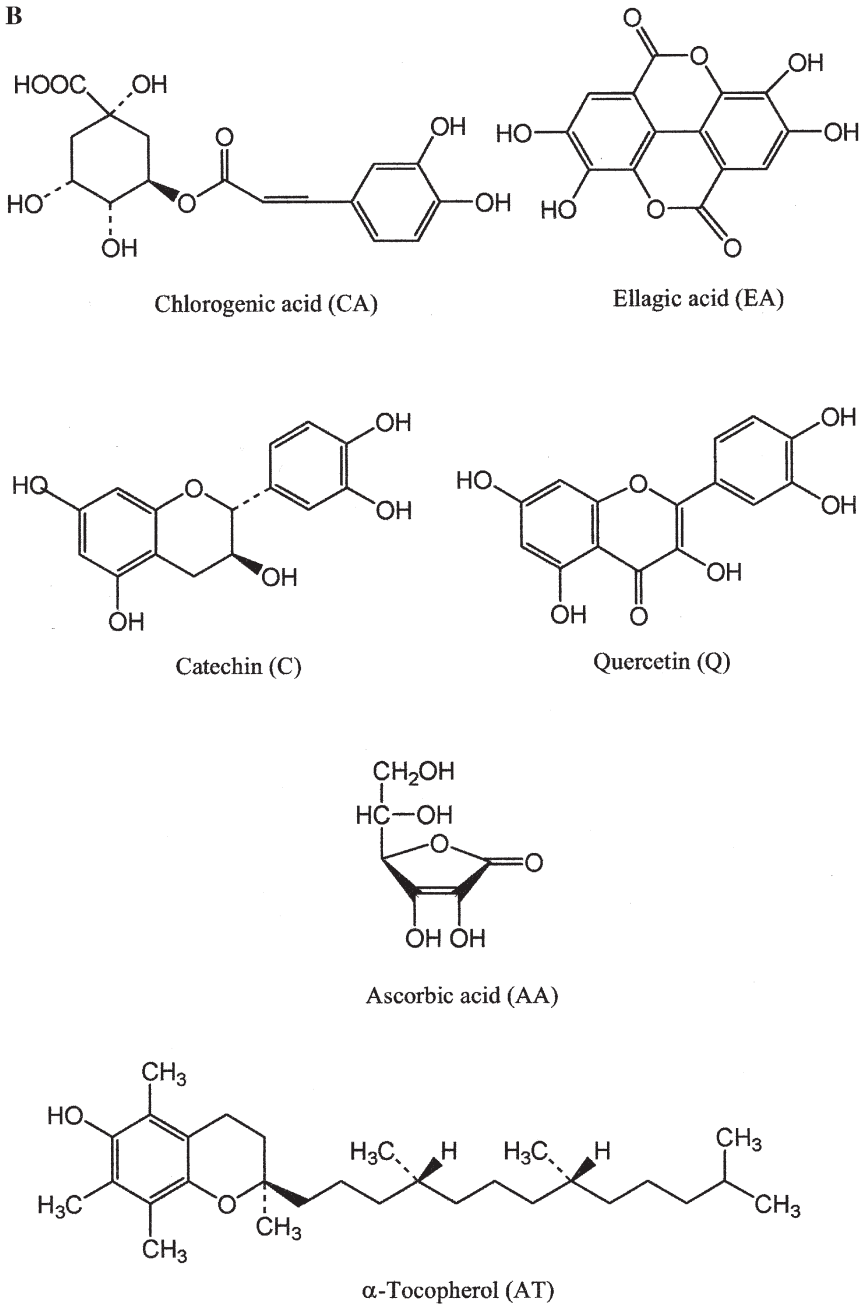

FIG. 1. (continued)

TLC separation. Chromatographic separations were performed on aluminum-backed RP-18 TLC plates (Art. 5559, E. Merck) cut to the size of 10 × 20 cm. The standards were applied as 9-mm-wide bands, 10 mm apart, with a Linomat IV TLC applicator (Camag, Muttenz, Switzerland). The starting position was 12 mm from the side of the plate and 10 mm from the bottom of the plate. The standard solutions were applied in volumes of 1, 2, 3, 4, 6, 8, and 10 µL in random order using an application rate of 15 s/µL. All the standard solutions were prepared to a concentration of 20 mM in methanol, except for ellagic acid, which was dissolved in DMSO. The solvent system used was optimized for the separation of rapeseed meal extract according to the "PRISMA" model (15). The system consisted of methanol and water (55:45, vol/vol) with 1% *o*-phosphoric acid as modifier. The plates were developed in an unsaturated 10 × 20 cm twin-trough chamber (Camag) to a distance of 75 mm at ambient temperature.

Determination of radical-scavenging activity by the TLC-DPPH method. After development, the plates were air-dried for 15 min. The dried plates were then dipped in a 0.04 % (wt/vol) solution of DPPH in methanol using a Chromatogram Immersion Device III (Camag). The speed setting of immersion was 3, and the time for full immersion of the plate was 5 s. The plates were then placed in the center of the imaging cabinet, and the images were obtained under visible light 120 s after dipping.

Determination of radical-scavenging activity by the spectrophotometric DPPH assay. Radical-scavenging activity was measured by adding 50 µL of sample solution to 2.95 mL of

DPPH solution (11.5 mg in 250 mL of methanol) in a cuvette. The absorbance of the solution was then recorded at 517 nm after 5 min. All the measurements were performed in triplicate. Methanol was used as a blank solution and α-tocopherol and L-ascorbic acid as positive controls.

Detection of all the separated compounds by using sulfuric acid reagent. To determine the amount of all the organic compounds present in the extract, the bands were visualized by dipping the plates in 10% (vol/vol) sulfuric acid in methanol, followed by heating at 120°C for 15 min. To obtain darker spots that are more suitable for detection with a CCD video system, the dipping procedure was repeated by using 15% (vol/vol) sulfuric acid in methanol, followed by heating at 180°C for 15 min. After 10 min, images of the plates were captured with the CCD video camera system under visible light.

Statistical analysis. The acquired data were input into Systat® 8.03 (Chicago, IL) for Windows® statistics program in order to perform regression and correlation analyses. Outliers were tested on the basis of the *F* approximation.

RESULTS AND DISCUSSION

The results of the spectrophotometric assay were calculated on the basis of the absorbance values of pure DPPH solution before and 2 min after the addition of a standard. The absorbance value of pure DPPH solution represented 0% inhibition, and the decrease in the absorbance value was converted to percentage value, i.e., the absorbance value of 0.0 at 2 min after the addition of the standard would represent a 100% inhibition. The two most active compounds in this assay were *n*-propyl gallate (HBA7) and syringic acid (HBA2) by a clear margin (Fig. 1). Ellagic acid (EA) and gallic acid (HBA5) formed the next group with significant radical-scavenging activity. Vanillic acid (HBA1), 3-coumaric acid (HCA3), *p*-coumaric acid (HCA5), and 3-hydroxybenzoic acid (HBA3) possessed only very weak radical-scavenging activity.

The difference in the DPPH radical-scavenging activity between *n*-propyl gallate and gallic acid may be due to alkyl esterification that, in a relatively polar solvent such as methanol, could increase the radical-scavenging activity of *n*-propyl gallate compared to that of the more polar gallic acid, as suggested by Porter *et al.* (16). However, Yoshida *et al.* (17) did not find any significant differences between the effects of gallic acid and its methyl, ethyl, *n*-propyl, and *n*-butyl esters on DPPH radical dissolved in methanol. They proposed, based on electron spin resonance measurements, that alkyl gallates in methanol form dimers in the presence of DPPH. As expected, the number of phenolic groups had a marked effect on the radical-scavenging efficiency of the tested compounds: The radical-scavenging activity increased when a second and a third hydroxyl group were introduced into the molecule (1). Our results also confirmed that the addition of one or two methoxy groups at the *ortho* position increased the radical-scavenging effect of monophenols by stabilizing the aryloxyl radical. The effect, however, was not as significant as the addition of one or two hydroxyl groups. Hydroxycinnamic acids showed slightly higher radical-scavenging activities than hydroxybenzoic acids, with the exception of syringic acid (HBA2) and sinapic acid (HCA2), possibly due to the stabilization of the radical by resonance, which is in accordance with previous findings (18).

The original aim of this study was to develop a simple and quick method for measuring the radical-scavenging activity of some known phenolic antioxidants directly from the TLC plate by densitometry. In accordance with the PRISMA model (15), optimization of the solvent system was started using silica gel as the stationary phase. However, a satisfactory separation could not be achieved with normal-phase TLC plates owing to the polar nature of the compounds of interest. Therefore, the system had to be changed to a reversed-phase method. A solvent composition of methanol and water (55:45, vol/vol) with 1% *o*-phosphoric acid as modifier gave sufficient resolution for the extract.

For silica gel plates, the purple background produced by the DPPH radical after spraying the plate with DPPH solution has been proved to be relatively stable, enabling the identification of radical-scavenging activity after a period of 30 min (13,14). A similar result was found in the current study when the silica gel plates were dipped in DPPH solution. However, this was not the case when using RP-TLC plates. The developing color proved to be very unstable and started to fade within approximately 3 min after dipping. The fading background led to poor peak shapes as well as decreased peak heights and areas. Increased interference from the background also made it necessary to use manual integration, thus adding uncertainty to the analysis. Since conventional slit-scanning densitometers operate serially they could not be applied for the quantitative determination of radical-scavenging activity directly from the RP-TLC plate.

The problem of declining intensity of the developing color can, however, be circumvented by using an image analysis system that obtains the image of the whole plate simultaneously with a CCD video camera. Several systems are commercially available, and they have been shown to perform as well as slit-scanning densitometers in terms of accuracy, precision, and robustness (19,20).

Measurement of the radical-scavenging ability of the tested compounds on RP-TLC plates was based on the peak areas produced with a video scanning system 2 min after dipping. Polynomial second-degree calibration equations calculated for the standards were found to give satisfactory correlations between peak area and concentration, with regression coefficients of $r^2 = 0.947$ and 0.996 for protocatechuic acid and caffeic acid, respectively (Table 1).

With the RP-TLC-DPPH method, gallic acid (HBA5), ellagic acid (EA) at 20 nmol, L-ascorbic acid (AA), and 2,3-dihydroxybenzoic acid (HBA6) were the most active DPPH radical scavengers, depending on the amount applied to the plate (Fig. 2). On the other hand, 3-coumaric acid (HCA3), *p*-coumaric acid (HCA5), 3-hydroxybenzoic acid (HBA3), and vanillic acid (HBA1) showed no radical-scavenging activity. With this method gallic acid had a significantly higher radical-scavenging activity than did *n*-propyl gallate (HBA7). The nonpolar nature of the RP-TLC plate may account for this difference, as discussed earlier. The dimethoxylated phe-

TABLE 1
Calibration Equations for the Tested Compounds Obtained by the RP-TLC-DPPH Method[a]

Compound	Range (nmol)	Calibration equation	r^2
HBA1	—	—	—
HBA2	20–200	$y = -1.727x^2 + 761.7x + 19626$	0.993
HBA3	—	—	—
HBA4	20–200	$y = -1.566x^2 + 562.7x + 20747$	0.947
HBA5	20–200	$y = -4.946x^2 + 1954x + 66675$	0.991
HBA6	2–20	$y = -118.1x^2 + 4018x + 23196$	0.979
HBA7	20–200	$y = -1.010x^2 + 548.7x + 36396$	0.989
HCA1	20–200	$y = -2.343x^2 + 821.7x + 10506$	0.995
HCA2	20–200	$y = -1.306x^2 + 641.6x + 53826$	0.985
HCA3	—	—	—
HCA4	20–200	$y = -1.438x^2 + 627.3x + 21280$	0.996
HCA5	—	—	—
CA	20–200	$y = -1.266x^2 + 673.3x + 58551$	0.969
EA	2–20	$y = -109.3x^2 + 5820x + 20110$	0.974
C	2–20	$y = -206.2x^2 + 6876x + 15168$	0.955
Q	2–20	$y = -6.022x^2 + 763.9x + 9100.7$	0.956
AA	2–20	$y = -290.0x^2 + 10660x + 20109$	0.987
AT	20–200	$y = -1.002x^2 + 648.9x + 92962$	0.989

[a]RP-TLC, reversed-phase TLC; DPPH, 1,1-diphenyl-2-picrylhydrazyl; see Figure 1 for other abbreviations.

TABLE 2
Detection Limits for the Tested Compounds Obtained by the RP-TLC-DPPH Method[a]

Compound	Detection limit[b] (nmol)	(ng)
HBA1	ND	ND
HBA2	0.2	40
HBA3	ND	ND
HBA4	2.0	310
HBA5	0.6	100
HBA6	0.6	90
HBA7	0.2	40
HCA1	2.0	390
HCA2	0.6	130
HCA3	ND	ND
HCA4	0.6	110
HCA5	ND	ND
CA	0.6	210
EA	0.2	60
C	0.6	170
Q	0.6	180
AA	2.0	350
AT	1.6	690

[a]See Table 1 and Figure 1 for abbreviations.
[b]Based on a signal-to-noise ratio of 3. ND = not detected.

nolic acids syringic acid (HBA2) and sinapic acid (HCA2), scavenged DPPH radicals more effectively than did the diphenolic acids protocatechuic acid (HBA4) and caffeic acid (HCA4) with this method, which was contrary to the spectrophotometric assay.

Detection limits were determined for all the tested compounds based on a signal-to-noise ratio (S/N) of 3 after dipping the plates in DPPH solution (Table 2). The detection limits were 40 and 690 ng for syringic acid and α-tocopherol, respectively.

The methods were compared by means of regression and correlation analyses. Outliers were tested by F approximation. The two methods seemed to give relatively similar results, although several exceptions were detected. Syringic acid, ascorbic acid, and *n*-propyl gallate were shown to be outliers in the regression analyses ($P > 6/N$, resulting in $r^2 = 0.545$). After excluding these three compounds a value of $r^2 = 0.923$ was obtained.

The method was applied for measuring the free radical-scavenging activity of rapeseed meal fractions separated by RP-TLC. A total of 10 separate bands with free radical-scavenging activity were detected with R_f values ranging from 0.04 to 0.85 (Fig. 3). The most active fraction observed was the one with an R_f value of 0.41, possessing 38% of the total radical-scavenging activity.

Coupling RP-TLC with direct videodensitometric detection by DPPH solution was thus demonstrated to be a suitable

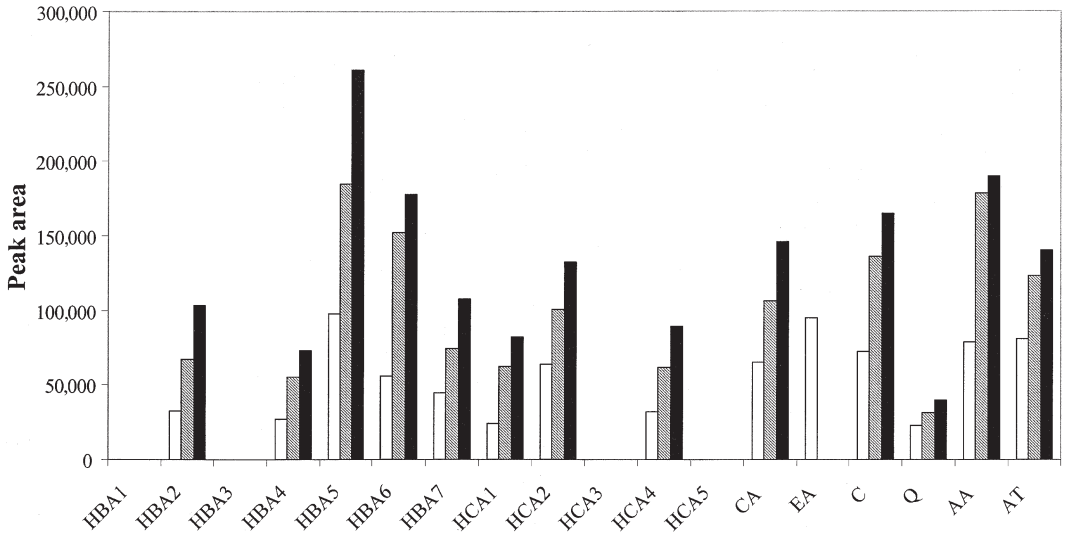

FIG. 2. Peak areas for the tested compounds by the reversed-phase-TLC-1,1-diphenyl-2-picrylhydrazyl (DPPH) method produced with a video scanning system 2 min after dipping the plate in DPPH solution. The amount applied to the TLC plate (left to right) for each compound: 20, 80, and 200 nmol. See Figure 1 for abbreviations.

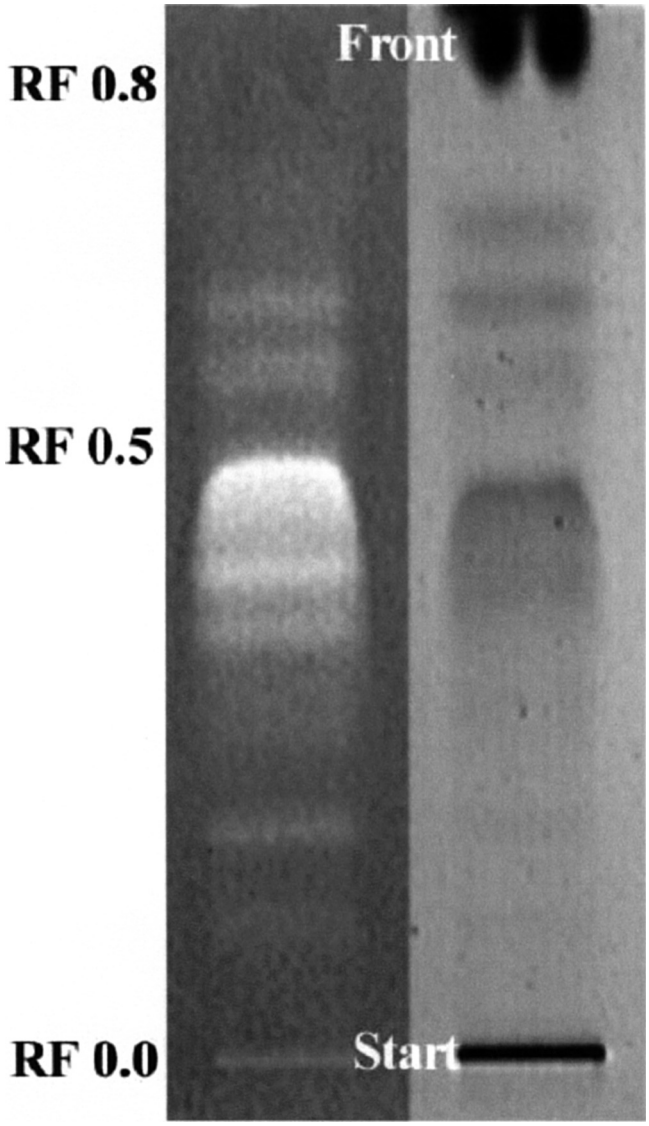

FIG. 3. DPPH radical-scavenging activity of rapeseed meal separated by RP-TLC (left), and the same lane after charring with sulfuric acid reagent (right). See Figure 2 for abbreviation.

method for screening polar antioxidants from plant extracts. One major advantage of the technique is its ability to locate the radical-scavenging activity of a complex mixture, thus enabling detection of the most active antiradical principles. The method is simple and fast, making it suitable for automated systems in screening applications where high throughput and cost efficiency are required.

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