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Composition of Phenolic Compounds and Antioxidant Activity of Commercial Aqueous Smoke Flavorings

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The antioxidant activity of 12 aqueous commercial smoke flavorings used in the food industry was determined by two methods: bleaching of the carotenoid crocin and scavenging of the DPPH radical. The reaction with the DPPH radical was evaluated by calculating the effective concentration (EC_{50}) and the antiradical efficiency (AE). A gas chromatography−mass spectrometry method was, moreover, used for the determination of 2-methoxyphenols, 2,6-dimethoxyphenols, and dihydroxybenzenes. The methoxyphenols were extracted from the aqueous smoke by dichloromethane, and also the residue aqueous phase was analyzed to determine the more water-soluble dihydroxybenzenes. The recovery and the repeatability of the method are reported. The total phenolic concentrations of the smoke flavorings showed a wide range, from about 1000 to 25000 mg/kg. Considering the three classes of compounds, the concentrations were about 300–3000 mg/kg for the 2-methoxyphenols, 200–11000 mg/kg for the 2,6-dimethoxyphenols, and 140–10000 mg/kg for the dihydroxybenzenes. The range of the antioxidant activities of the smoke flavorings was wide, reflecting the wide range of the phenolic concentrations. Good correlations were obtained between the total phenolic concentration and the antioxidant activities determined by both the DPPH and crocin assays.

KEYWORDS: Liquid smoke flavorings; antioxidant activity; methoxyphenols; dihydroxybenzenes; crocin; DPPH radical; gas chromatography−mass spectrometry

INTRODUCTION

Smoke flavorings are produced on a large scale and have been applied to a variety of food products, such as meat, fish, and cheese, for more than 40 years. The use of smoke flavorings has several advantages compared to traditional smoking techniques: ease of application, speed, uniformity of the product, reproducibility of the characteristics obtained in the final smoked food, and cleanliness of application as well as environmental protection (*1*). Furthermore, the amount of toxic compounds, mainly polycyclic aromatic hydrocarbons, deriving from the combustion process can be controlled more easily (*1–4*). Smoke flavorings were recently regulated by the European Commission (*5*), which established maximum limits of 10 and 20 μg/kg for the concentrations of benzo[*a*]pyrene and benz[*a*]anthracene, respectively. Smoke flavorings, in both liquid and solid form and of commercial use or laboratory made, have been studied from the point of view of composition (*6–16*), antimicrobial activity (*17–21*), influence on organoleptic properties (*22–24*) and oxidative stability of the smoked foods (*18, 25–27*). The composition of smoke flavorings is very complex and includes compounds belonging to many different chemical classes: aldehydes; ketones; alcohols; acids; esters; furan and pyran derivatives; phenolic derivatives; hydrocarbons; and nitrogen compounds. The phenolic compounds are an important fraction from both qualitative and quantitative points of view. This fraction consists mainly of phenol, 2-methoxyphenol (guaiacol), 2,6-dimethoxyphenol (syringol), and their derivatives and of dihydroxybenzenes originating from the pyrolysis of lignin. The phenolic compounds have been considered to be important contributors to smoke aroma and to the antimicrobial and antioxidant activities in smoked foods. The antioxidant activity of methoxyphenols and other related compounds characteristic of the liquid smoke flavorings have been investigated by some authors (*28–32*). Guillén and Ibargoitia (*9, 11*) reported also the presence of lignin dimers and trimers with potential antioxidant properties after analyzing the brown layer left on the wall of the receptacle containing a liquid smoke flavoring.

The aim of the present work was to correlate the antioxidant activity of smoke flavorings used in the food industry with the phenolic compound concentrations. The antioxidant activity was measured using two methods and a GC-MS method was used to determine the 2-methoxyphenols, 2,6-dimethoxyphenols, and dihydroxybenzenes, based on the analysis of both a dichloromethane extract of the liquid smoke and the aqueous residue.

MATERIALS AND METHODS

Chemicals. All solvents were of analytical grade. 2,2′-Azobis(2-amidinopropane) dihydrochloride (ABAP), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), guaiacol, 4-methylguaiacol, 4-ethylguaiacol,

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4-vinylguaiacol, 4-propylguaiacol, eugenol, isoeugenol (mixture of trans and cis isomers), vanillin, 1-(4′-hydroxy-3′-methoxyphenyl)ethanone (acetovanillone), 1-(4′-hydroxy-3′-methoxyphenyl)-2-propanone (2-propiovanillone), syringol, 4-methylsyringol, 4-allylsyringol, syringaldehyde, 1-(4′-hydroxy-3′,5′-dimethoxyphenyl)ethanone (acetosyringone), catechol, 3-methylcatechol, 4-methylcatechol, 3-methoxycatechol, hydroquinone, Trolox C, zingerone, methyl-4-hydroxybenzoate (methylparaben), and the silylation mixture *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA)/trimethylchlorosilane (TMCS), 99:1, were purchased from Sigma-Aldrich (Milan, Italy).

Samples. The samples were 12 commercial aqueous smoke flavorings used in the food industry. They came from four different manufacturers and were coded from S1 to S12. Regarding the type of wood from which they were made, S1 was from European hardwoods, S5 and S7 were from hickory and other selected hardwoods, S9 was from *Fagus*, *Betula*, and *Quercus*, and S12 was from a hardwood mix. No data were available in the case of the other samples.

GC-MS Analysis. Liquid smokes were analyzed directly or after dilution with water. An aqueous solution (300 μL) of zingerone (1.05 mg/mL) as internal standard and 20 mL of dichloromethane were added to 5 g of sample or to 5 mL of a diluted sample into a 100 mL separatory funnel. The separatory funnel was vigorously shaken for 1 min and allowed to stand until separation of the two phases. The separated organic phase was dried with anhydrous sodium sulfate and analyzed with a Shimadzu 2010 gas chromatograph coupled to a quadrupolar mass spectrometer (Shimadzu QP 2010; Shimadzu, Tokyo, Japan). The analytes were separated on a SPB5 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) (Supelco, Sigma-Aldrich, Milan, Italy). Column temperature was held at 40 °C for 2 min, increased to 200 at 5 °C/min and then to 280 at 15 °C/min, and held for 25 min. Injector, transfer line, and ion source temperatures were 250, 250, and 200 °C, respectively. Injection was in the splitless mode (2 min), and the injection volume was 1.0 μL. Helium was the carrier gas at a flow rate of 0.7 mL/min. Electron impact mass spectra were recorded at 70 eV ionization energy.

The aqueous residue phase, left after the dichloromethane extraction, was transferred into a 50 mL round-bottom flask and evaporated to dryness in a rotary evaporator at 30 °C. The residue was dissolved with 5 mL of methanol, and 300 μL of a methanolic solution of methylparaben (1.09 mg/mL), as internal standard, was added. An aliquot (150 μL) of this solution was reduced to dryness under a nitrogen flow, dissolved in 500 μL of acetonitrile, and silylated with 150 μL of a mixture of BSTFA/TMCS (99:1), before the GC-MS analysis. The GC conditions were the same as for the analysis of the dichloromethane extract except for the column temperature program, which was set at 80 °C for 5 min, increased to 200 at 5 °C/min and then to 280 at 15 °C/min, and held for 25 min.

Liquid smoke components were identified by comparison of their mass spectra and retention times with those of standard compounds or by comparison of the mass spectrum with those of the mass spectra library Wiley 6.

Crocin Bleaching Inhibition Method. Crocin bleaching was measured according to the method reported by Tubaro et al. (*33*). Crocin was extracted from saffron by methyl alcohol, and the concentration of the solution was measured at 443 nm ($\epsilon = 1.33 \times 10^5$ M^{-1} cm^{-1}). The samples were diluted in 10% (v/v) ethanol in water. Different aliquots of the sample and 50 μL of a 0.7 mM methanolic solution of crocin were added to 1200 μL of 10 mM phosphate buffer, pH 7.01, and the volume was adjusted to 2450 μL with 10% (v/v) ethanol in water. The reaction was started by adding 50 μL of a fresh 0.25 M ABAP solution in water. Bleaching reaction rates (V) at five different concentrations of sample and a blank (V_0) were tested at the same time, by following the absorbance decrease at 443 nm and at a temperature of 40 °C, by means of a UV−visible spectrophotometer (Varian Cary 1E) equipped with a thermostable multicell block (Varian, Australia). The ratios V_0/V were plotted as a function of the concentration ratio, [sample]/[crocin], and the slopes calculated by linear regression analysis. The sample concentrations were calculated as pseudo-Trolox C by dividing the weight of the sample by the molecular weight of Trolox C. Reference bleaching rate was determined using Trolox C under the same experimental conditions. The antioxidant capacity was expressed as grams of liquid smoke that have the antioxidant activity of 1 g of Trolox C by dividing the slope of the Trolox C by that of sample. Three replicates were made for each sample.

DPPH Radical Scavenging Method. The method of Brand-Williams et al. (*34*) was used for measuring the DPPH radical scavenging ability of the samples. DPPH was dissolved in methanol at a final concentration of about 6×10^{-5} M. The exact concentration of DPPH was calculated from a calibration curve, $\epsilon = 11870$ M^{-1} cm^{-1} at 515 nm. Different aliquots of the samples were added to 2450 μL of DPPH solution, and the volume was adjusted with methanol to a final value of 2500 μL. Five different concentrations were used for each assay. The decrease of the DPPH radical was followed at 515 nm until the reaction reached a steady state, at a temperature of 25 °C. The percentage of remaining DPPH at the steady state were plotted as a function of the concentration ratio of sample to DPPH to determine the effective concentration (EC_{50}). The time (minutes) needed to reach the steady state for EC_{50} (TEC_{50}) was used to calculate the antiradical efficiency defined as $AE = 1/(EC_{50} \times TEC_{50})$ (*35*). Three replicates were made for each sample.

Statistical Analyses. All of the correlations were obtained by using the program Statistica ver. 6.0 (StatSoft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Optimization of GC-MS Method. The liquid smoke flavorings used in the food industry have compositional characteristics that depend on the type of wood used, the operating conditions of the combustion process, and the other treatments made to the smoke. In the present work, the attention focused on the quantitative determination of the phenolic fraction only and, in particular, of the 2-methoxyphenols, 2,6-dimethoxyphenols, and dihydroxybenzenes, considering these compounds to be principally responsible for an eventual antioxidant activity of the smoke in the smoked foods. The method used in the present work consisted of a single extraction step with dichloromethane, analysis of both the organic phase and the aqueous phase, and the use of two internal standards for the quantitative analysis. A second extraction with 20 mL of dichloromethane did, in fact, not improve substantially the recovery for the derivatives of guaiacol and syringol. Due to the high solubility of the dihydroxybenzenes in the aqueous phase, these compounds and, in particular, catechol and hydroquinone, were not quantitatively extracted by the dichloromethane; therefore, also the analysis of the aqueous phase was necessary for their determination. Guillén et al. (*13, 14*) also analyzed the aqueous phase, after extraction with dichloromethane, in some samples of smoke flavoring.

All of the main characteristic phenolic compounds present in liquid smoke were identified: phenol, methylphenols, dimethylphenols, 2-methoxyphenols (guaiacol and derivatives), 2,6-dimethoxyphenols (syringol and derivatives), and dihydroxybenzenes. Other principal compounds such as 3-methyl-1,2-cyclopentanedione and three furan derivatives were tentatively identified.

The dihydroxybenzenes present in the aqueous phase were analyzed as trimethylsilyl derivatives. The silylation of the hydroxy groups allowed a remarkable improvement of the chromatographic behavior of these compounds and in particular of hydroquinone and methylparaben, which presented chromatographic peaks with a very pronounced tailing, impairing the accuracy of the quantitative analysis. Silylation eliminated peak tailing and, moreover, allowed the separation of 3-methoxycatechol and 3-methylcatechol, which coeluted when analyzed without derivatization.

Quantitative Analysis. Quantitative analysis was carried out with the method of the internal standard. Two internal standards

Table 1. Repeatability ($n = 9$) and Recovery of the Method

compound	repeatability (mg/kg)	recovery[a] (%)
Dichloromethane Extract		
guaiacol	677 ± 31	98 ± 14
4-methylguaiacol	275 ± 13	102 ± 11
4-ethylguaiacol	111 ± 2	101 ± 11
4-propylguaiacol	7.0 ± 0.1	95 ± 7
eugenol	9.7 ± 0.3	97 ± 8
cis-isoeugenol	0.8 ± 0.1	104 ± 13
trans-isoeugenol	0.6 ± 0.1	98 ± 7
vanillin	16.9 ± 0.3	104 ± 2
acetovanillone	12.0 ± 0.2	93 ± 6
2-propiovanillone	11.7 ± 0.7	76 ± 2
syringol	497 ± 22	101 ± 8
4-methylsyringol	116 ± 2	100 ± 5
4-ethylsyringol	74 ± 2	*b*
4-propylsyringol	16.5 ± 0.8	*b*
4-allylsyringol	14.1 ± 0.3	92 ± 3
syringaldehyde	38 ± 1	96 ± 12
acetosyringone	30 ± 1	91 ± 9
catechol	183 ± 19	53 ± 15
3-methoxycatechol	58 ± 5	96 ± 23
3-methylcatechol	57 ± 4	56 ± 9
4-methylcatechol	38 ± 3	81 ± 21
Aqueous Residue		
catechol	95 ± 3	58 ± 6
hydroquinone	24.9 ± 0.6	91 ± 6
3-methoxycatechol	5.0 ± 0.2	11 ± 2
3-methylcatechol	6.8 ± 0.2	10 ± 2
4-methylcatechol	7.8 ± 0.3	25 ± 2

[a] The values are the mean of three determinations ± standard deviation. [b] Standard compound not available.

were used: zingerone for the compounds extracted in dichloromethane and methylparaben for the compounds in the aqueous residue phase. The signal of the extracted molecular ion was used for quantification, because of the partial or total overlapping of some compounds with other sample components, especially in the residual aqueous phase. In the case of 4-ethylsyringol and 4-propylsyringol, for which standards were not available, the relative response factor of the 4-methylsyringol has been used and the quantification was based on the signal of the total ionic current. Moreover, the relative response factor of *trans*-isoeugenol has been used for the corresponding cis isomer. Two calibration curves with linearity ranges between 1.0 and 120 mg/L and between 20 and 200 mg/L were obtained for the methoxyphenols and the hydroxybenzenes in dichloromethane, respectively, whereas a calibration curve with a linearity range between 0.6 and 30 mg/L was obtained for the hydroxybenzenes analyzed as TMS derivatives. The relative response factor of each standard compound was calculated daily during the analyses.

Repeatability and Recovery. The repeatability of the method for the analysis of the extract in dichloromethane and the aqueous phase has been estimated by analyzing a sample of smoke nine times. The results (milligrams per kilogram) are reported in **Table 1**. For the organic as well as for the aqueous residue the repeatability was good, with coefficients of variation lower than 10% except for *trans*-isoeugenol, probably due to its low concentration.

The recoveries were evaluated by analyzing both the dichloromethane and the aqueous residue of a sample of liquid smoke fortified with the standard compounds. The results are reported in **Table 1**. Each value represents the average of three replicates. Recoveries of methoxyphenols and dimethoxyphenols in the dichloromethane extract were practically quantitative with values higher than 90%, except for 2-propiovanillone with a value of 76%. In the case of the dihydroxybenzenes, in the dichloromethane extract, good recoveries were obtained only for 3-methoxycatechol and 4-methylcatechol, whereas low values, 53 and 56%, were attained for catechol and 3-methylcatechol, respectively. Due to the low amount, hydroquinone was not determined in the dichloromethane phase. The recoveries of dihydroxybenzenes were estimated also in the aqueous phase. In this case, the recovery of hydroquinone was almost quantitative (91%), evidencing its high water solubility. Catechol seemed to partition in a similar way between dichloromethane and water, whereas 3-methoxycatechol and 4-methylcatechol showed low recovery in agreement with the high recovery found in the dichloromethane phase. 3-Methylcatechol showed a very low recovery in the aqueous residue and a recovery of only 56% in the organic phase. Considering the sum of the recoveries from the extract in the dichloromethane and in the aqueous residue, good values were obtained for all of the dihydroxybenzenes with the exception of 3-methylcatechol. The results obtained from the recovery test show that for the accurate quantification of catechol and hydroquinone, both the dichloromethane and the aqueous residue should be analyzed.

Phenolic Composition of Liquid Smoke Flavorings. The concentrations (milligrams per kilogram) of the phenolic compounds in the dichloromethane extract and the aqueous residue of 12 liquid smoke flavorings are reported in **Tables 2** and **3**, respectively. **Table 4** shows the concentrations of the 2-methoxyphenols, 2,6-dimethoxyphenols, and dihydroxybenzenes grouped in classes and the total concentration. In **Table 4**, the values are expressed as the sum of both the concentrations in the dichloromethane extract and the aqueous residue. The liquid smokes analyzed showed a wide range of total phenolic compound concentrations from the minimum value of approximately 1000 mg/kg to the maximum of about 25000 mg/kg. Similar wide ranges of concentrations have been reported in the literature for commercial smoke flavoring, both laboratory and pilot-plant scale preparations, although comparison between results should be made with caution because of the different analytical methods and calculation approaches used by the different authors (*6, 9, 16, 17, 19*). Considering the three classes of compounds, the concentrations were about 300–3000 mg/kg for the 2-methoxyphenols, 200–11000 mg/kg for the 2,6-dimethoxyphenols, and 140–11000 mg/kg for the dihydroxybenzenes. Among the 2- and 2,6-dimethoxyphenols, guaiacol and syringol were generally present in a higher amount than the corresponding 4-alkyl derivatives. In the case of dihydroxybenzenes, catechol was the compound present in the highest amount. This wide interval of concentrations can be explained by the type of wood, the smoke generation conditions, and the following treatments on the smoke condensates that determine the characteristics of smoke flavorings and their modes of applications according to the desired organoleptic properties of the final product (*36*). Some smoke flavorings are used pure, whereas others are used only after appropriate dilution. For example, samples S1−S7, which are particularly rich in phenolic compounds, were sprayed on the products after being diluted approximately 10 times with water, according to the user's information. Eight of the 12 smoke samples were characterized by a higher concentration of 2,6-dimethoxyphenols with respect to 2-methoxyphenols. A prevalence of 2,6-dimethoxyphenol derivatives is indicative of the use of hardwoods as starting materials for the preparation of the smoke flavorings. Hardwoods are more frequently used for the production of liquid smoke because their composition gives to the smoked products better organoleptic characteristics. The different relative compositions

Table 2. Phenol Concentrations (Milligrams per Kilogram) in the Dichloromethane Extract of Liquid Smokes[a]

	sample											
compound	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
guaiacol	226 ± 5	675 ± 1	649 ± 56	731 ± 33	466 ± 30	635 ± 30	472 ± 18	134 ± 15	638 ± 13	220 ± 3	677 ± 31	511 ± 36
4-methylguaiacol	173 ± 4	419 ± 6	344 ± 27	374 ± 22	178 ± 11	335 ± 0.8	219 ± 7	82 ± 8	310 ± 8	124 ± 5	275 ± 13	124 ± 8
4-ethylguaiacol	110 ± 5	173 ± 6	155 ± 11	139 ± 13	56.3 ± 0.3	122 ± 2	86.6 ± 1	39 ± 3	108 ± 1	50 ± 3	111 ± 2	35 ± 2
4-propylguaiacol	9.5 ± 0.6	6 ± 1	15 ± 2	11.8 ± 0.7	3 ± 2	9.6 ± 0.2	9.3 ± 0.5	4.5 ± 0.3	13.2 ± 0.4	2.4 ± 0.3	7.0 ± 0.1	2.1 ± 0.1
eugenol	12.5 ± 0.01	28 ± 1	49 ± 3	35 ± 1	12 ± 1	31.7 ± 1.0	29.3 ± 0.5	18 ± 1	9.3 ± 0.1	4.7 ± 0.2	9.7 ± 0.3	6.9 ± 0.1
cis-isoeugenol	7.5 ± 0.2	7 ± 1	24.5 ± 0.8	10.0 ± 0.2	4.8 ± 0.1	19.4 ± 0.1	12.0 ± 0.5	1.7 ± 0.1	2.0 ± 0.4	0.4 ± 0.1	0.8 ± 0.1	0.43 ± 0.03
trans-isoeugenol	12.5 ± 0.7	26 ± 2	28 ± 3	11 ± 2	15 ± 2	45.6 ± 0.7	17 ± 2	2.5 ± 0.2	2.0 ± 0.2	1.6 ± 0.2	0.6 ± 0.1	1.1 ± 0.2
vanillin	86.1 ± 0.1	279 ± 7	108 ± 3	116 ± 3	122 ± 6	86.8 ± 0.9	90 ± 1	30 ± 2	13 ± 3	35 ± 2	16.9 ± 0.3	13.7 ± 0.4
acetovanillone	104 ± 2	392 ± 25	115 ± 6	143 ± 0.1	115 ± 7	102 ± 2	86 ± 4	13.6 ± 0.2	15.0 ± 0.5	50 ± 2	12.0 ± 0.2	11.0 ± 0.1
2-propiovanillone	468 ± 11	988 ± 7	253 ± 2	292 ± 3	235 ± 6	250 ± 1	174 ± 6	20 ± 1	38 ± 6	90 ± 4	11.7 ± 0.7	12.7 ± 0.1
syringol	3582 ± 30	6620 ± 138	1552 ± 52	1749 ± 57	1523 ± 69	1757 ± 6	1162 ± 6	103 ± 5	570 ± 8	1436 ± 54	497 ± 22	119 ± 5
4-methylsyringol	1514 ± 24	1892 ± 42	462 ± 23	277 ± 7	338 ± 11	859 ± 18	237 ± 6	34 ± 1	337 ± 7	548 ± 37	116 ± 2	3.6 ± 0.3
4-ethylsyringol	675 ± 29	696 ± 11	301 ± 22	211 ± 4	188 ± 2	354 ± 4	182 ± 1	28 ± 1	164 ± 16	203 ± 3	74 ± 2	7.4 ± 0.2
4-propylsyringol	97 ± 5	35 ± 2	47 ± 1	35 ± 1	20.2 ± 0.8	46 ± 2	34 ± 1	7.0 ± 0.3	28 ± 3	25.4 ± 0.4	16.5 ± 0.8	2.1 ± 0.1
4-allylsyringol	94 ± 2	232 ± 6	149 ± 5	120 ± 2	71 ± 2	121 ± 1	103 ± 1	18.1 ± 0.1	17 ± 1	22.2 ± 0.7	14.1 ± 0.3	4.0 ± 0.3
syringaldehyde	279 ± 7	827 ± 26	352 ± 4	351 ± 29	410 ± 8	292 ± 23	298 ± 8	55 ± 1	19 ± 3	89 ± 3	38 ± 1	34.0 ± 0.9
acetosyringone	347 ± 12	1102 ± 3	322 ± 4	382 ± 19	342 ± 6	287 ± 17	257 ± 4	18.7 ± 0.2	23 ± 2	119 ± 4	30 ± 1	29.5 ± 0.5
catechol	1326 ± 224	3620 ± 55	1032 ± 12	668 ± 167	510 ± 98	347 ± 7	239 ± 63	43 ± 4	32 ± 7	401 ± 35	183 ± 19	33 ± 3
3-methoxycatechol	1067 ± 120	617 ± 22	228 ± 19	45 ± 3	129 ± 27	382 ± 25	71 ± 15	11.5 ± 0.7	117 ± 11	500 ± 23	58 ± 5	13 ± 5
3-methylcatechol	546 ± 58	892 ± 55	377 ± 11	98 ± 14	84 ± 8	93 ± 14	50 ± 11	19.0 ± 1.0	67 ± 2	182 ± 27	57 ± 4	15.0 ± 0.6
4-methylcatechol	1017 ± 135	1604 ± 50	657 ± 54	211 ± 59	175 ± 10	178 ± 4	132 ± 51	100 ± 3	6.3 ± 0.1	82 ± 3	38 ± 3	17 ± 1
total	11752 ± 298	21128 ± 177	7220 ± 107	6011 ± 194	4996 ± 129	6354 ± 55	3960 ± 87	782 ± 19	2531 ± 29	4185 ± 83	2244 ± 45	994 ± 37

[a] The values are the mean of two determinations ± standard deviation.

Table 3. Phenol Concentrations (Milligrams per Kilogram) in the Aqueous Residue of Liquid Smokes[a]

	compound					
sample	catechol	hydroquinone	3-methoxycatechol	3-methylcatechol	4-methylcatechol	total
S1	1475 ± 181	607 ± 28	166 ± 1	137 ± 5	206 ± 10	2590 ± 183
S2	2518 ± 52	927 ± 34	97 ± 2	183 ± 2	250 ± 11	3976 ± 63
S3	1104 ± 14	386 ± 1	37 ± 1	93.5 ± 0.4	120 ± 1	1741 ± 14
S4	953 ± 64	258 ± 12	9.2 ± 0.1	42 ± 2	48 ± 4	1310 ± 66
S5	770 ± 39	296 ± 9	17.8 ± 0.1	33 ± 1	44 ± 2	1161 ± 40
S6	1026 ± 124	311 ± 23	120 ± 2	108 ± 5	141 ± 10	1707 ± 127
S7	530 ± 4	208 ± 15	12 ± 2	29 ± 2	37.6 ± 0.1	816 ± 15
S8	154 ± 13	3.7 ± 0.2	4.3 ± 0.2	35 ± 2	29 ± 2	226 ± 14
S9	123 ± 10	32 ± 3	21 ± 4	14.2 ± 0.5	22 ± 2	212 ± 11
S10	502 ± 48	172 ± 15	77 ± 5	48 ± 4	86 ± 7	884 ± 51
S11	95 ± 3	24.9 ± 0.6	5.0 ± 0.2	6.8 ± 0.2	7.8 ± 0.3	139 ± 3
S12	45 ± 3	15.2 ± 0.1	0.8 ± 0.05	1.2 ± 0.01	1.6 ± 0.2	64 ± 3

[a] The values are the mean of two determinations ± standard deviation.

Table 4. Phenol Concentrations Grouped for Classes and Antioxidant Activity of Liquid Smokes Obtained by DPPH (AE and EC_{50}) and Crocin Assays

sample	2-methoxyphenols (mg/kg)	2,6-dimethoxyphenols (mg/kg)	dihydroxybenzenes (mg/kg)	total (mg/kg)	EC_{50}[a] (g of smoke/L)/ (g of DPPH/L)	AE × 10^3[a] (g of DPPH/L)/ ((g of smoke/L) × min)	crocin[a] (g of smoke)/ (g of Trolox C)
S1	1209 ± 14	6587 ± 50	6547 ± 346	14342 ± 350	3.2 ± 0.1	2.3 ± 0.1	18.8 ± 1.6
S2	2991 ± 28	11404 ± 147	10709 ± 114	25104 ± 188	1.3 ± 0.1	4.8 ± 0.4	6.0 ± 0.1
S3	1740 ± 64	3186 ± 62	4035 ± 61	8961 ± 108	3.1 ± 0.1	2.1 ± 0.1	15.2 ± 0.7
S4	1863 ± 42	3126 ± 67	2332 ± 189	7321 ± 205	2.9 ± 0.1	2.0 ± 0.1	11.9 ± 0.4
S5	1207 ± 34	2891 ± 71	2059 ± 110	6157 ± 135	4.5 ± 0.1	1.4 ± 0.1	21.5 ± 1.3
S6	1637 ± 30	3716 ± 35	2707 ± 130	8061 ± 138	3.1 ± 0.1	2.2 ± 0.1	22.1 ± 1.2
S7	1195 ± 21	2273 ± 13	1308 ± 85	4776 ± 88	5.0 ± 0.3	1.2 ± 0.1	23.5 ± 1.2
S8	345 ± 18	263 ± 5	399 ± 15	1008 ± 24	25.3 ± 0.3	0.27 ± 0.01	153 ± 12
S9	1149 ± 17	1159 ± 20	435 ± 18	2743 ± 31	14.2 ± 0.2	0.42 ± 0.02	120 ± 4
S10	579 ± 8	2442 ± 66	2049 ± 71	5070 ± 98	8.0 ± 0.7	0.9 ± 0.1	93.8 ± 0.2
S11	1122 ± 34	785 ± 22	476 ± 20	2383 ± 45	27.6 ± 1.1	0.18 ± 0.01	9784 ± 387
S12	717 ± 36	199 ± 5	141 ± 7	1058 ±37	46.8 ± 2.9	0.12 ± 0.01	12766 ± 808

[a] The values are the mean of three determinations ± standard deviation.

of the three classes of phenolic compounds in these smoke samples are probably more due to the combustion temperature and other process parameters than to differences among the botanical species of hardwoods (*31*). These results were in agreement with the available informations on the type of wood used, except for sample S12, for which the higher concentration of 2-methoxyphenols was in contrast with the mixture of hardwoods used as source material for the production of this liquid smoke.

This is probably, to our knowledge, the first report on the concentrations of dihydroxybenzenes in a relatively high number of commercial samples of liquid smoke flavorings.

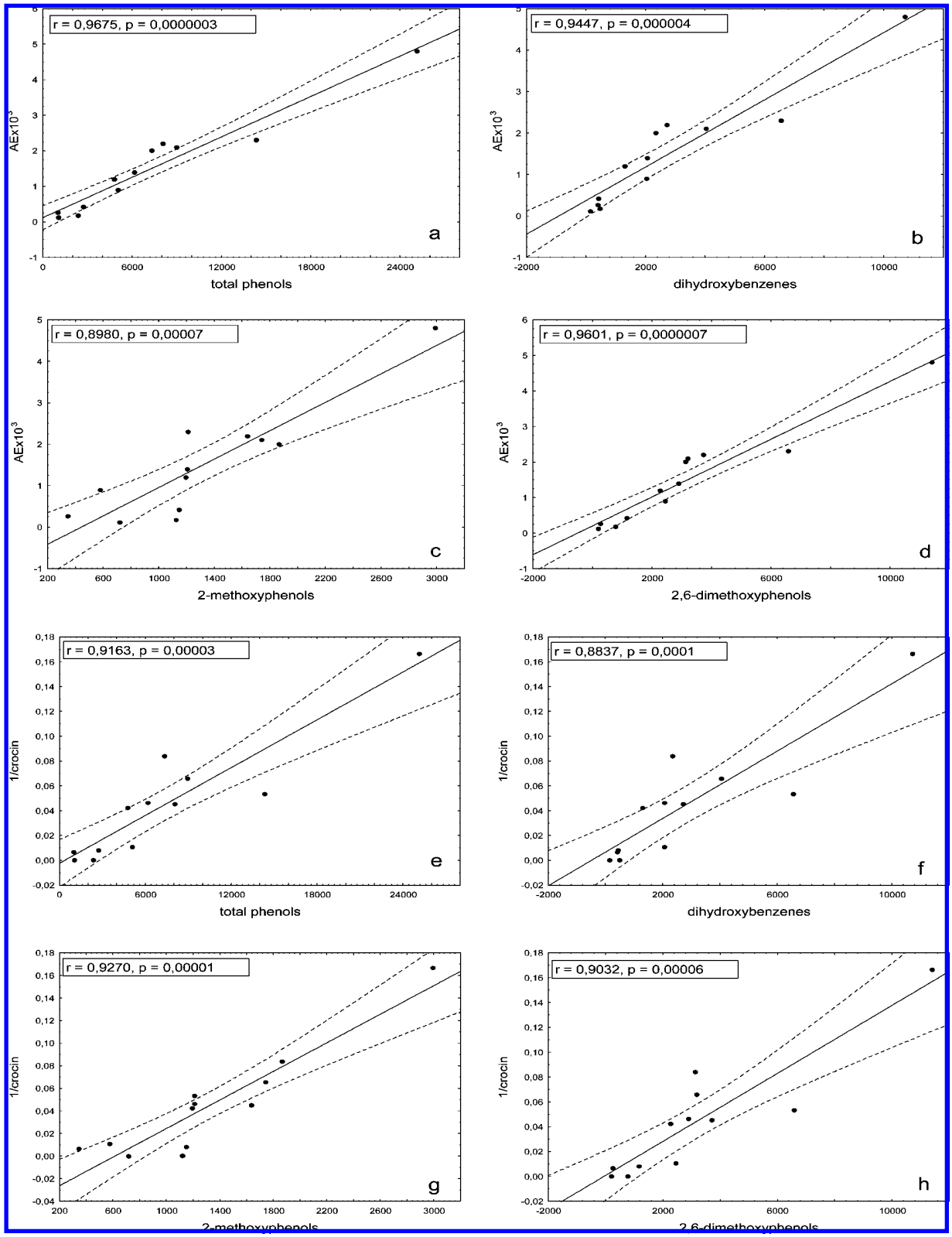

Figure 1. Linear correlations between antioxidant activity and phenolic content.

Antioxidant Activity of Smoke Flavorings. The antioxidant activity of the smoke samples was assessed by two methods, scavenging of the DPPH radical and crocin bleaching inhibition. The results of the DPPH method (**Table 4**) were expressed as EC_{50} and AE. EC_{50} represents the concentration ratio (grams of sample per liter)/(grams of DPPH per liter) necessary to decrease the initial DPPH concentration by 50% at the steady state, whereas AE reflects both the antioxidant power (reflected by the reverse of EC_{50}) and the time needed to reach the steady state (TEC_{50}) (*35*). In the case of the crocin method, the results (**Table 4**) were expressed as grams of liquid smoke that exhibit the same antioxidant activity of 1 g of Trolox C. Lower values correspond to higher antioxidant activities.

The antioxidant activities of the smoke flavorings were very different and, although it is not practicable to analyze the contribution of the single compounds, they can be, in a first approximation, explained by taking into account the total concentrations of the phenolic compounds as well the concen-

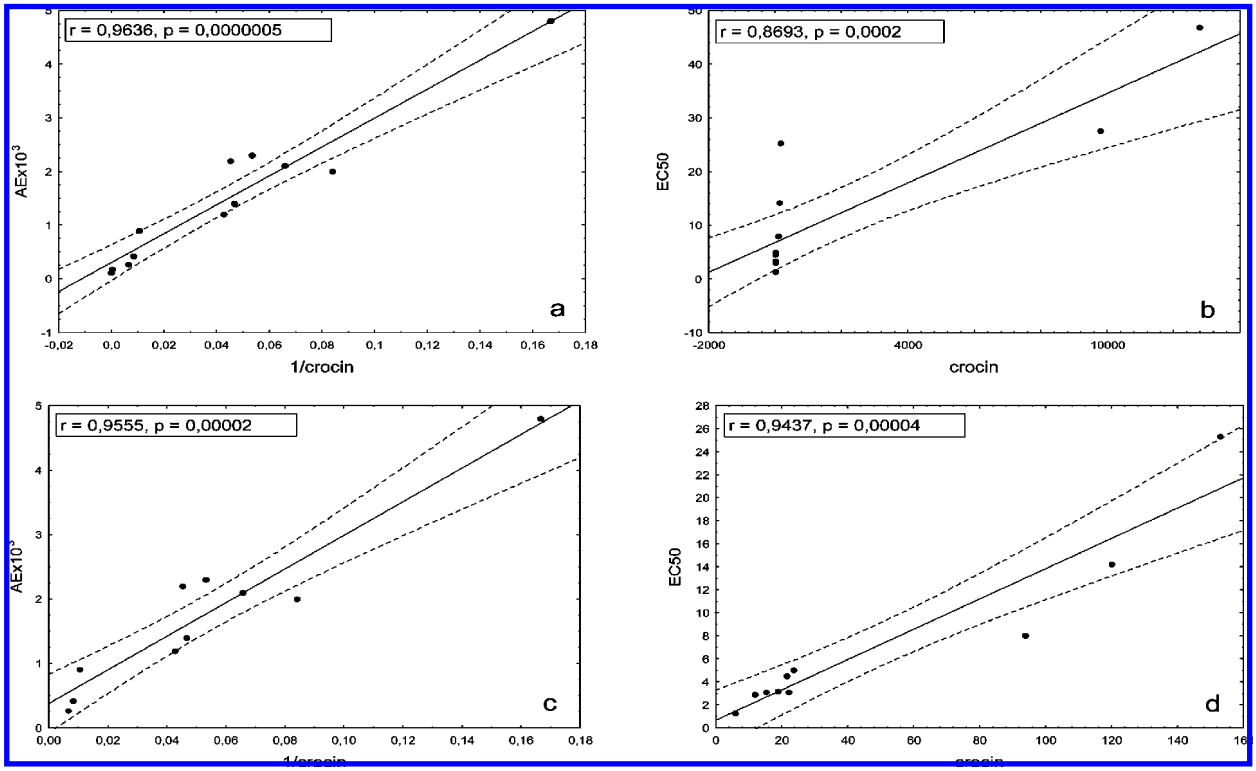

Figure 2. Linear correlations between the antioxidant activities determined by the DPPH (AE and EC_{50}) and crocin methods.

trations of the single classes of compounds: dihydroxybenzenes, 2,6-dimethoxyphenols, and 2-methoxyphenols considered as a whole. On the basis of the structure–activity relationship, the ranking of the antioxidant activity of these classes of compounds is generally in the order dihydroxybenzenes > 2,6-dimethoxyphenols > 2-methoxyphenols depending also on the presence, type, and position of the substituent groups on the benzene ring (*30, 37*). Considering the liquid smokes analyzed, sample S2 with the highest antioxidant activity had also the highest total phenolic concentration and in particular the highest content of dihydroxybenzenes and 2,6-dimethoxyphenols, whereas samples S8, S11, and S12, characterized by the lowest antioxidant activity, with both methods, had also the lowest total phenolic content (**Table 4**). Samples S8 and S12 had practically the same total phenolic content but different antioxidant activity. In particular, the EC_{50} and the AE values of S8 were, respectively, half and twice those of S12. This can be explained by the higher content of dihydroxybenzenes present in S8 with respect to S12. Similar considerations can be drawn also for samples S9 and S11. They had, in fact, a similar total phenolic content but the EC_{50} and the AE values of S9 were, respectively, about half and twice those of S11. The highest content of 2,6-dimethoxyphenols in sample S9 with respect to S11 could partially explain this behavior. Considering the crocin values for the same pairs of samples, the differences between samples S8 and S12 and between S9 and S11 were very high with respect to the corresponding differences between the values of EC_{50} and AE. The very high crocin values and, consequently, the low antioxidant activity of samples S11 and S12 cannot be explained on the basis of the phenolic composition only. Other compounds present in these samples could have interfered with the crocin analysis.

In **Figure 1** are reported the graphic representations of the equations that relate the antioxidant activity of the liquid smokes and the concentrations of the phenolic compounds obtained by GC-MS analysis. Considering the DPPH method, a good correlation ($r = 0.97$) was obtained between AE and total amount of phenols (**Figure 1a**). High correlations ($r = 0.94$ and 0.96) were also obtained between AE and the dihydroxybenzenes and 2,6-dimethoxyphenols, respectively (**Figure 1b**,**d**), whereas a slightly lower correlation coefficient was obtained in the case of 2-methoxyphenols, $r = 0.90$ (**Figure 1c**). The existence of a correlation with all three classes of compounds is not surprising because the trends of the phenolic compound concentrations within the three classes are similar for all of the samples. The highly significant correlation between AE and the total phenolic content indicates that these compounds are probably the more active chemical species in determining the antioxidant activity. A similar behavior, was also obtained by correlating the reciprocal of the parameter EC_{50} ($1/EC_{50}$) and the total phenolic content and the single classes of compounds with correlation coefficients $r = 0.95$, 0.94, 0.92, and 0.92 for total phenols, 2,6-dimethoxyphenols, dihydroxybenzenes, and 2-methoxyphenols, respectively.

Considering the correlation coefficients and the graphic representations of data reported in **Figure 1e**−**h**, the reciprocals of the crocin values were in general less well correlated with the phenolic content with respect the AE values.

Considering the two methods used to determine the antioxidant activity, the results obtained with the DPPH assay expressed as AE were well correlated ($r = 0.96$) with the reciprocal of the crocin values (**Figure 2a**). On the contrary, from the analysis of the graph reported in **Figure 2b**, it is not possible to speak of correlation between EC_{50} and crocin results because of the presence of two distinct groups of data: one group formed by samples S11 and S12 and the other formed by all of the other samples. From the point of view of the crocin method, samples S11 and S12, which were characterized by values 100 times larger, could be considered as a separate group, and no correlation can be made with EC_{50}. The behavior of samples S11 and S12 cannot be rationalized on the basis of the phenolic composition as stated above. The second group formed by the other 10 samples (S1–S10) was, on the contrary, well correlated with EC_{50} ($r = 0.94$) (**Figure 2d**). The graph of the correlation

between AE and the reciprocal of crocin considering only samples S1–S10 is reported in **Figure 2c**. In this case the results were similar to those of **Figure 2a**; the reciprocals of the crocin values of samples S11 and S12 were, in fact, very low and fell in a region of the graph where other points were present.

The DPPH method seems then to better interpret the antioxidant activity of the smoke flavorings as a function of their phenolic content.

The antioxidant activity is the result of the contributions of all phenolic compounds, although considering their relative antioxidant activity and concentration, the dihydroxybenzenes are probably the major contributors. Among the 2-methoxyphenols and 2,6-dimethoxyphenols the main components were guaiacol and syringol, which are characterized by a lower antioxidant activity with respect to their corresponding 4-alkyl derivatives (*32*).

In this work only these classes of phenolic compounds have been investigated. Other phenolic compounds such as phenol, methylphenols, dimethylphenols, and ethylphenols are present in the smoke flavorings, but their antioxidant activities and their total concentrations were low compared to those of the methoxyphenols and dihydroxybenzenes. Among the carbonyl derivatives, the presence of maltol at concentrations of, respectively, 30 and 45.5 mg/kg was reported in a commercial liquid smoke flavoring (*6*) and in a salty smoke flavoring (*8*). This compound has been shown to have antioxidant activity, but its concentration is generally much lower than the total concentration of 2-methoxyphenols, 2,6-dimethoxyphenols, and dihydroxybenzenes; therefore, its contribution to the total antioxidant activity was not considered in this work.

The concentrations of the phenolic compounds in smoked products are strongly affected by the type of smoking process, traditional or by using liquid smoke flavorings, and by many parameters such as smoke production conditions, application technique, temperature, and humidity. Concentrations of total phenolic compounds in the range 0.04–6 mg/100 g in samples of herring and salmon smoked by traditional process and liquid smoke vaporization have been reported by some authors (*23, 38–42*). Moreover, the real antioxidant effect exerted by the phenolic compounds depends, apart from the antioxidant activity and concentration of the single compounds, also on their solubility, which affects their distribution in the food matrix. From this point of view the dihydroxybenzenes have a higher antioxidant activity with respect to methoxyphenols but a lower solubility in the lipid phase of a meat product. Their behavior can be also related to the physical state of the food, for example, in a multiphase system such as an emulsion.

Considering also the composition and the total concentrations of the phenolic compounds in the samples analyzed in this work and a dosage of about 1–3 g of liquid smoke/kg of finished product, as suggested by some manufacturers, in some cases the concentrations of the phenolic compounds seem to be relatively low, and their antioxidant effect is probably secondary with respect to the important organoleptic characteristics imparted by the smoking process. This aspect needs further investigation.

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