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Flavored Waters: Influence of Ingredients on Antioxidant Capacity and Terpenoid Profile by HS-SPME/GC-MS

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ABSTRACT: The antioxidant profiles of 39 water samples (29 flavored waters based on 10 natural waters) and 6 flavors used in their formulation (furnished by producers) were determined. Total phenol and flavonoid contents, reducing power, and DPPH radical scavenging activity were the optical techniques implemented and included in the referred profile. Flavor extracts were analyzed by HS-SPME/GC-MS to obtain the qualitative and quantitative profiles of the volatile fraction of essential oils. Results pointed out a higher reducing power (0.14−11.8 mg of gallic acid/L) and radical scavenging activity (0.29−211.5 mg Trolox/L) of flavored waters compared with the corresponding natural ones, an interesting fact concerning human health. Bioactive compounds, such as polyphenols, were present in all samples (0.5−359 mg of gallic acid/L), whereas flavonoids were not present either in flavored waters or in flavors. The major components of flavor extracts were monoterpenes, such as citral, α-limonene, carveol, and α-terpineol.

KEYWORDS: total antioxidant capacity, flavored water, essential oils, total phenols and flavonoids contents, radical scavenging activity, reducing power, HS-SPME/GC-MS

INTRODUCTION

Reactive oxygen species (ROS) are continuously produced in all living beings, especially in higher organisms, as a result of normal cellular metabolism, phagocytises, inflammation, and exogenous factors such as ionizing radiations and xenobiotics.[1] ROS can induce cell damage by reacting with biomolecules (proteins, lipids) and cause serious lesions in the DNA molecule,[2] such as strand breaks, DNA−protein cross-linking, and base-free sites.[3] The mammalian body has certain endogenous antioxidant defense mechanisms to combat and reduce oxidative damage such as enzymatic systems, and exogenous antioxidant systems, such as as vitamins, minerals, and proteins. Antioxidants, which can inhibit or delay the oxidation of a substrate in a chain reaction, therefore, appear to be very important in the prevention of many diseases.[4] Foodstuffs constitute an excellent exogenous source of natural antioxidants. It is known that vegetables, fruits, wholegrain, and some beverages (tea, juice, wine) are rich in antioxidants and bioactive compounds. Examples of antioxidants present in food are vitamins (particularly C and E), phenolic compounds (flavonoids, catechins, flavones, flavonols, anthocyanins), and carotenoids including β-carotene.[5] A healthy diet should provide an adequate and continuous supply of these antioxidants. Other antioxidants, such as ubiquinol and thiol compounds, produced in small amounts by the organism, can be obtained in higher amounts by dietary supplements.[6] Consequently, interest is increasing in new effective natural antioxidants as well as in the chemical and biochemical characterization of foodstuffs and beverages to evaluate them with regard to their antioxidant profiles.

To answer consumers' preferences, the food industry has applied several technical improvements to plain water. Today, a significant part of commercialized water is in flavored formulation. Flavors, juices, bioactive compounds, preservatives, and/or sweeteners are added to water, providing a product with singular tastes and smells appreciated by consumers.

Flavors (or essential oils) from fruits contain 85−99% of volatile and 1−15% of nonvolatile compounds. Volatile constituents are a mixture of monoterpenes and sesquiterpenes,[7] being flavonoids present in the nonvolatile fraction.[8] Terpenes and flavonoids present antioxidant and antiradical properties[9] and can be transferred to water samples if flavors/aromas extracts are used. Therefore, drinking this type of beverage can improve the daily intake of antioxidants, contributing to the exogenous protective system. However, there are no reports concerning the antioxidant properties of these waters, although their macro- and micromineral compositions are known.[10,11] These properties will be a new source of information for consumer's about the advantages/disadvantages on the consumption of these beverages.

Antioxidant capacity determination is not an easy task to perform. Several factors (substrates, conditions, analytical methods, and concentrations) can affect the estimated values, and it is difficult to measure each antioxidant component separately and/or the interactions among different antioxidant components in the samples.[4] Total antioxidant capacity measures can be classified in two groups: assays based on the inhibition of human low-density lipoprotein oxidation or those based on oxygen free radical scavenging ability. Current In vitro methods for antioxidant efficacy evaluation have as a basic principle the oxidation

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inhibition of a suitable substrate. After oxidation of the substrate, under standard conditions, the extent of the reaction is determined at a fixed time point or over the range that is characteristic of the generated free radical.[3] UV−vis spectrophotometric, chemiluminescence, fluorometric,[4] and chromatographic methods[12] can be used to do that.

In the present study, four optical methods were applied to evaluate the antioxidant profile of 39 mineral and spring, natural and flavored water samples, and 6 flavors/aromas used in their formulation. This was carried out by means of the total phenol content (TPC), total flavonoid content (TFC), reducing power, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity (RSA). The volatile fractions of the flavor extracts were isolated by headspace solid-phase microextraction (SPME) and analyzed by gas chromatography−mass spectrometry (GC-MS).

MATERIALS AND METHODS

Chemicals. Gallic acid, (−)-epicatechin, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble analogue of vitamin E) standards were from Sigma-Aldrich or Fluka. Folin−Ciocalteu reagent and DPPH were obtained from Sigma-Aldrich. All of these chemicals, of the highest quality available (95−99%), were used without purification. Other compounds of analytical grade, such as sodium carbonate, sodium nitrite, aluminum chloride, sodium hydroxide, ethanol, and sodium acetate (0.1 mol/L, pH 4.3), were from Merck. All solvents used were of HPLC grade. Standard antioxidant solutions were prepared daily and stored in the dark at 4 °C when not in use. Water used was ultrapure (18.2 MΩ/cm), obtained from a Millipore Simplicity 185 system. For spectrophotometric measurements a Shimadzu 160-A spectrophotometer was used.

Sample Preparation. Mineral water arises from a geologically and physically protected underground source, characterized by constant levels and relative proportions of minerals and trace elements at the source. Spring water derives from an underground formation from which water flows naturally to the surface at an identified location.

Thirty-nine water samples, corresponding to 10 different brands, acquired in supermarkets in northern Portugal and stored in the dark at 4 °C were analyzed. Each brand (still or sparkling, mineral or spring water) had different flavors and aromas. Natural waters of each brand were used as control. Sonication was used to eliminate gas from sparkling water samples.

Table 1 summarizes the nutrient information on the labels, taking into account its different composition in gas, flavor, vitamins, preservatives, acidifying regulators, and sweeteners.

Six flavors or concentrate extracts (lime, tangerine, strawberry, lemon, apple, and gooseberry) used in the formulation of some water brands, and provided by producers, were also analyzed. As expected, these flavors had no description about its chemical composition.

TPC Determination. TPC values of flavors and flavored waters were determined by a colorimetric assay based on procedures described by Singleton and Rossi[13] with some modification. Folin−Ciocalteu reagent and the reduced phenols produced a stable blue product at the end of reaction. The reaction mixture (20 μL of sample, 1.58 mL of ultrapure water, and 100 μL of Folin−Ciocalteu reagent) was sonicated for 30 s. After this, it was added to 300 μL of 7% Na_2CO_3, and the mixture was incubated for 10 min at 50 °C. Factor dilutions of 10 times on the mother standard antioxidant gallic acid (GA) were carried out to obtain a calibration curve ranging from 0 to 5.00 mg of GA/L of water. Quantifications were carried out in triplicate, and the absorbance was measured at 760 nm.

TFC Determination. TFC was determined by a colorimetric assay based on the formation of flavonoid−aluminum compound.[14] One milliliter of flavored water was mixed with 4 mL of ultrapure water and 300 μL of 5% $NaNO_2$ solution. After 5 min, 300 μL of 10% $AlCl_3$ solution was added. After 6 min, 2 mL of 1 mol/L NaOH and 2.4 mL of ultrapure water were added. The solution was mixed well, and the absorbance of a pink color was read at 510 nm. (−)-Epicatechin was used to plot the standard curve ranging from 0 to 66.26 mg/L, and the results of TFC were expressed as milligrams of epicatechin per liter of water. All measurements were carried out in triplicate.

Reducing Power Assay. Reducing power was determined according to the method of Oyaizu.[15] One milliliter of sample was mixed with 2.5 mL of 0.2 mol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. This mixture was incubated for 20 min at 50 °C, and then 2.5 mL of 10% trichloroacetic acid (w/v) was added and centrifuged at 1000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm. The calibration curve was prepared with GA solutions ranging from 0 to 19.6 mg/L, and the results are given as milligrams of GA per liter of water.

DPPH Radical Scavenging Activity. RSA of samples against the stable nitrogen radical $DPPH^•$ was determined spectrophotometrically at 517 nm.[16] $DPPH^•$ free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors, such as an antioxidant. In this technique, samples (200 μL) were mixed with 2.80 mL of 1.86×10^{-4} mol/L ethanolic solution of $DPPH^•$. The mixture, vigorously shaken, was left to stand for 15 min in the dark (until stable absorption values). Lower absorbance values of the reactive mixture indicated higher free radical scavenging activity. The calibration curve was prepared with Trolox solutions ranging from 0 to 19.6 mg/L, and the results are given as milligrams of Trolox per liter of water.

Validation of the Optical Methodologies. Calibration standards were daily prepared, and all samples were determined in triplicate. The methods were validated by linear range, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy. LOD and LOQ were defined, respectively, as 3 and 10 times the standard deviation of 10 blank signals divided by the slope of the calibration plot.[17] Precision was calculated by intraday and interday determinations of standard solutions and expressed by relative standard deviations (RSD). For intraday evaluation, each concentration was assessed by five measurements, three times during a working day. The interday precision measurements were made over 1 week. Accuracy and reproducibility were checked by recovery (REC), relative error (RE), and RSD. All results were expressed as the mean ± standard deviation.

Flavor/Fragrance Extraction by Headspace SPME and Detection by GC-MS. Extraction of fragrances was carried out by SPME using a 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber (Supelco, Inc., Bellefonte, PA), for all experiments. This fiber was selected according to the best results for the extraction of fruit volatiles.[18,19] Fibers were conditioned for 30 min at 250 °C before use.

For each extraction 2 g of flavor and 0.5 g of NaCl (to inhibit enzymatic reactions and to favor the transfer of the analytes from the aqueous solution to the headspace) or 50 μL of extract were transferred into a 10 mL Teflon-lined septum cap vial equipped with a Teflon-coated magnetic bar. To favor the transfer of the analytes from the aqueous solution to the headspace, the solution was stirred (200 rpm) at 70 °C. The PDMS/DVB fiber was used to extract the nonpolar volatile compounds (in the headspace). The fiber was exposed to the sample headspace for 20 min at 70 °C. The fiber was then removed and introduced into the injector port of the GC-MS for desorption at 250 °C for 3 min, in the splitless mode.

The separation and detection of the analytes was achieved using a GC-MS system (Agilent Technologies, USA) with a GC 6850 coupled to a 595C VL MSD mass selective detector, with a silica capillary column (30 m × 0.32 mm i.d.; df, 0.25 μm) covered with 5% phenyl/95% dimethylpolysiloxane (DB-5 ms, Agilent-J&W Scientific), kept at 30 °C

Table 1. Label Information in the Evaluated Bottled Flavored Waters

brand	sample	flavor	juice	vitamin	preservatives	acidifying regulators	sweeteners	other ingredients
					Still Water			
A	1	lemon			potassium sorbate, sodium benzoate	citric acid, sodium citrate	acesulfame-K	fiber (1%), wheat dextrin (0.1%)
mineral	2	mango			potassium sorbate, sodium benzoate	citric acid, sodium citrate	acesulfame-K	fiber (1%), wheat dextrin (0.1%)
	3	strawberry			potassium sorbate, sodium benzoate	citric acid, sodium citrate	acesulfame-K	fiber (1%), wheat dextrin (0.1%)
	4	natural						
B	5	pineapple/orange	apple		potassium sorbate, sodium benzoate	citric acid, aspartame	acesulfame-K	calcium lactate
spring	6	lemon	apple	niacin, pantothenic acid B_6, folic acid biotin, B_{12}	potassium sorbate, sodium benzoate	citric acid	acesulfame-K, aspartame	
	7	natural						
C	8	lemon/magnesium	fruit	(mg/100 mL): B_3 (2.7), B_5 (0.9), B_6 (0.3), B_8 (0.022), B_9 (0.03), B_{12} (1.5×10^{-4})	potassium sorbate, dimethyl dicarbonate	citric acid		magnesium carbonate, ginseng
mineral	9	apple/white tea	fruit	(mg/100 mL): B_3 (2.7), B_5 (0.9), B_6 (0.3), B_8 (0.022), B_9 (0.03), B_{12} (1.5×10^{-4})	potassium sorbate	citric acid		malic acid calcium lactate
	10	pineapple/fiber	fruit		potassium sorbate, dimethyl dicarbonate	citric acid		wheat dextrin (0.9%), L-carnitine (200 mg/L)
	11	natural						
D	12	apple			dimethyl dicarbonate, sodium benzoate	citric acid	sucralose, acesulfame-K	
mineral	13	orange/peach			dimethyl dicarbonate, sodium benzoate	citric acid	sucralose, acesulfame-K	
	14	lemon			dimethyl dicarbonate, sodium benzoate	citric acid	sucralose, acesulfame-K	
	15	natural						
					Sparkling Water			
E	16	lemon	lemon	(mg/100 mL): B_3 (2.7), B_{12} (0.15)	potassium sorbate, sodium benzoate	citric acid, sodium citrate	acesulfame-K, aspartame	carbon dioxide
mineral	17	orange/raspberry	orange raspberry	(mg/100 mL): B_3 (2.7), B_{12} (0.15)	potassium sorbate, sodium benzoate	citric acid, sodium citrate	acesulfame-K, aspartame	carbon dioxide

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Table 1. Continued

brand	sample	flavor	juice	vitamin	preservatives	acidifying regulators	sweeteners	other ingredients
added gas	18	peach/pineapple	peach pineaapple	(mg/100 mL): B_3 (2.7), B_{12} (0.15)	potassium sorbate, sodium benzoate	citric acid, sodium citrate	acesulfame-K, aspartame	carbon dioxide
	19	guava/lime	guava/lime	(mg/100 mL): B_3 (2.7), B_{12} (0.15)	potassium sorbate, sodium benzoate	citric acid, sodium citrate	acesulfame-K, aspartame	carbon dioxide
	20	natural						
F	21	lemon/green tea	fruit, lemon, apple			citric acid	green tea	
mineral	22	raspberry/ginseng	raspberry, apple, pear			citric acid	ginseng	
natural gas	23	peach/white tea	fruit, peach, apple, pear			citric acid	white tea	
	24	mango/ginkgo beloba	mango, apple, pear				Ginkgo biloba	
	25	melon/mint	fruit, melon, apple, pear			citric acid	mint	
	26	natural						
G	27	lemon		C (12 mg/250 mL)	potassium sorbate	citric acid	acesulfam-K, sucralose	
mineral	28	lime						
added gas	29	apple		C (12 mg/250 mL)	potassium sorbate	citric acid	acesulfame-K, sucralose	
	30	peach		C (12 mg/250 mL)	potassium sorbate	citric acid	acesulfame-K, sucralose	
	31	natural						
H	32	lemon	lemon, apple	C (30 mg/100 mL)	sodium benzoate, potassium sorbate	citric acid	aspartame	
mineral natural gas	33	natural						
I	34	lemon	lemon, apple		sodium benzoate	citric acid	aspartame	
mineral	35	green apple	apple		sodium benzoate	citric acid	sucralose	
natural gas	36	strawberry	apple, strawberry		sodium benzoate	citric acid	aspartame	
	37	natural						
J	38	lemon	lemon		potassium sorbate, sodium benzoate	citric acid sodium citrate	aspartame, acesulfame-K	
spring added gas	39	natural						

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Table 2. Calibration Curves, Limit Values, Precision, and Accuracy Obtained in the Determination of Antioxidant Activity Assays

parameter	TPC (mg of gallic acid/L)	TFC (mg of epicatechin/L)	reducing power (mg of gallic acid/L)	DPPH scavenging activity (mg of Trolox/L)
linear concentration (μg/L)	0−5.0	0−66.2	0−19.6	0−19.6
slope (Abs mg/L)	$7.34 \pm 0.10 (\times 10^{-2})$	$3.52 \pm 0.03 (\times 10^{-2})$	$2.74 \pm 0.07 (\times 10^{-1})$	$-6.76 \pm 0.2 (\times 10^{-2})$
intercept (Abs)	$-9.24 \pm 0.40 (\times 10^{-4})$	$1.67 \pm 0.80 (\times 10^{-2})$	$-2.65 \pm 0.5 (\times 10^{-2})$	1.30 ± 0.02
correlation coefficient (n = 5)	0.999	0.999	0.998	0.997
LOD (mg of standard/L)	3.22×10^{-2}	1.00×10^{-1}	5.43×10^{-3}	2.84×10^{-2}
LOQ (mg of standard/L)	1.07×10^{-1}	3.33×10^{-1}	1.81×10^{-2}	9.48×10^{-2}
intraday studies[a]				
added (μg/L)	5.0	6.0	10.0	10.0
found (μg/L)	4.8	5.8	11.1	9.3
REC[b] (%)	95.0	96.7	111.0	93.0
RE[c] (%)	- 5.0	−3.3	11.0	−7.0
RSD[d] (%)	3.2	4.6	2.1	6.9
interday studies [e]				
added (μg/L)	5.0	6.0	10.0	10.0
found (μg/L)	5.2	5.6	9.5	9.8
REC (%)	104.0	93.3	95.0	98.0
RE (%)	4.0	−6.7	−5.0	−2.0
RSD (%)	4.0	5.8	6.3	7.4

[a] Average of three measurements, three times during a day. [b] REC, recovery. [c] RE, relative error. [d] RSD, relative standard deviation. [e] Average of five measurements over a week.

for 3 min, and then ramped to 300 °C at 8 °C/min and held at the final temperature for 4 min. The splitless injection (3 min) was achieved with an injector temperature at 250 °C. Helium was the carrier gas used at flow of 1.0 mL/min. Ion source, quadrupole, and transference line were kept at 230, 150, and 280 °C, respectively. MS spectra were obtained by electronic impact (EI) at 70 eV and collected at the rate of 1 scan/s over an m/z range of 35−400, and using MSD ChemStation E.02.00493 software (Agilent Technologies, USA). Identification of the individual components was performed by comparing their mass spectra with the standards and spectral libraries of GC-MS (NIST 98 and Wiley 275), enabling the detection of some minor components and identification of compounds that arise from incompletely resolved chromatographic peaks.

For each compound, quantitation was performed by measuring the corresponding peak area of the total ion chromatogram and expressed as relative (percent) areas by normalization.

RESULTS AND DISCUSSION

Descriptive Statistics. Table 1 represents the labeled nutrient information in flavored waters. About 38% of water samples are still and 62% sparkling (11 water samples with added gas). Labels indicate the presence of several compounds added for technological purposes, with biological activity (flavors, juice fruit, and vitamins). Inevitably, these waters also need other ingredients, without positive relationship with well-being and health, but necessary to ensure the quality desired for producers and consumers and for the safety of the product. This is the case of preservatives, acidifying regulators, and sweeteners.

Twelve different flavors were present in flavored waters: lemon (10 samples); mango, strawberry, lime, and raspberry (2 samples each); pineapple, apple, and orange (3 samples each); peach (4 samples); guava, melon, and green apple (1 sample each). Lemon is the predominant flavor, present in all water brands (A−J; 10 samples). Seventeen flavored water samples had only one flavor, and 12 samples had a combination of two flavors. About 50% of the samples have fruit juices or concentrates. Only flavored brands A, D, and G do not report the addition of this type of ingredient.

Eleven samples, according to the label, have in their composition vitamins of the B complex (7 samples) and C (4 samples). It is important to remember that vitamin C is an antioxidant with protection capacity against oxidative stress, being also a cofactor in several vital enzymatic reactions. Other bioactive compounds (ginseng, L-carnitine, white and green tea, and *Ginkgo biloba*) are present in some samples from different brands. Green tea contains numerous components with antioxidant activity, such as polyphenols (catechins, epicatechin, epigallocatechin) and vitamins.[20] Ginseng is an herbal medicine with antioxidant and anti-inflammatory activities and *G. biloba* is rich in phenolic and flavonoid compounds.

Forty-nine percent of samples contain sweeteners. There are water samples with only one (acesulfame-K, sucralose, or aspartame) and with two sweeteners in association (acesulfame-K and aspartame; acesulfame-K and sucralose). The most used was acesulfame-K (present in 14 samples), followed by aspartame (10 samples). It is interesting to note that, in general, the samples from the same brand have the same sweetener, the exception being brand I that uses different sweeteners for different flavors. Brands C and F do not have sweeteners, providing more energetic products, of 9−13 and 19 kcal/100 mL, respectively (sweetened samples ranged from 0.4 to 4 kcal/100 mL).

Each sample contains a single preservative (potassium sorbate or sodium benzoate) or the association of two (potassium sorbate and sodium benzoate; potassium sorbate and dimethyl dicarbonate; sodium benzoate and dimethyl dicarbonate). From this discussion, different behaviors and antioxidant values among the samples in the study are expected.

Method Validation. Table 2 presents the results obtained in the validation procedures of the applied methodologies (TPC,

Table 3. TPC, Reducing Power, and DPPH RSA Determined in Flavors and Flavored and Natural Waters

brand	sample	TPC (mg of GA/L)	reducing power (mg of GA/L)	DPPH (mg of Trolox/L)
flavors	tangerine	116.70 ± 1.50	10.21 ± 0.09	51.21 ± 0.35
	lime	359.30 ± 0.60	11.03 ± 0.07	78.51 ± 0.23
	strawberry	15.34 ± 0.10	11.12 ± 0.04	213.53 ± 2.50
	lemon	380.20 ± 0.09	10.71 ± 0.08	38.90 ± 0.42
	gooseberry	8.53 ± 0.04	11.80 ± 0.04	211.53 ± 4.60
	apple	37.15 ± 0.03	10.62 ± 0.03	54.43 ± 0.29
A	1, lemon	4.68 ± 0.05	2.61 ± 0.11	14.71 ± 0.04
	2, mango	7.72 ± 0.10	3.68 ± 0.09	13.16 ± 0.05
	3, strawberry	9.26 ± 0.03	3.91 ± 0.08	12.27 ± 0.04
	4, natural	nd[a]	nd	0.76 ± 0.03
B	5, pineapple/orange	18.30 ± 0.09	6.01 ± 0.42	13.49 ± 0.32
	6, lemon	17.62 ± 0.020	5.45 ± 0.14	16.26 ± 0.15
	7, natural	nd	nd	0.62 ± 0.03
C	8, lemon/magnesium	24.44 ± 0.20	8.48 ± 0.20	15.71 ± 0.09
	9, apple/white tea	28.10 ± 0.03	8.20 ± 0.15	16.49 ± 0.03
	10, pineapple/fiber	11.40 ± 0.05	4.56 ± 0.08	8.05 ± 0.45
	11, natural	nd	nd	0.89 ± 0.04
D	12, apple	0.54 ± 0.03	3.31 ± 0.03	46.55 ± 0.45
	13, orange/peach	nd	2.79 ± 0.06	44.11 ± 0.07
	14, lemon	nd	3.07 ± 0.07	44.56 ± 0.04
	15, natural	nd	nd	0.41 ± 0.02
E	16, lemon	2.24 ± 0.11	0.28 ± 0.05	12.38 ± 0.25
	17, orange/raspberry	6.18 ± 0.05	1.07 ± 0.02	16.49 ± 0.05
	18, peach/pineapple	1.51 ± 0.02	0.14 ± 0.03	15.27 ± 0.10
	19, guava/lime	8.57 ± 0.03	5.45 ± 0.04	14.71 ± 0.04
	20, natural	nd	nd	0.76 ± 0.02
F	21, lemon/green tea	39.70 ± 0.10	9.64 ± 0.03	41.45 ± 0.27
	22, raspberry/ginseng	37.90 ± 0.08	13.78 ± 0.05	48.66 ± 0.32
	23, peach/white tea	29.20 ± 0.15	8.29 ± 0.02	42.45 ± 0.04
	24, mango/*Ginkgo biloba*	36.50 ± 0.02	10.52 ± 0.04	45.89 ± 0.37
	25, melon/mint	19.70 ± 0.04	10.11 ± 0.04	41.56 ± 0.05
	26, natural	nd	nd	0.29 ± 0.05
G	27, lemon	0.29 ± 0.02	nd	38.23 ± 0.06
	28, lime	1.75 ± 0.04	nd	38.79 ± 0.17
	29, apple	284.0 ± 2.3	154.04 ± 0.26	268.89 ± 2.45
	30, peach	147.0 ± 1.3	nd	133.87 ± 1.35
	31, natural	nd	nd	0.42 ± 0.06
H	32, lemon	7.23 ± 0.05	3.77 ± 0.03	44.78 ± 0.48
	33, natural	nd	nd	0.31 ± 0.05
I	34, lemon	4.31 ± 0.03	5.91 ± 0.04	43.67 ± 0.28
	35, green apple	4.92 ± 0.06	4.00 ± 0.07	54.21 ± 0.03
	36, strawberry	5.89 ± 0.03	6.10 ± 0.38	42.67 ± 0.06
	37, natural	0.07 ± 0.03	nd	0.27 ± 0.13
J	38, lemon	1.88 ± 0.02	4.33 ± 0.29	41.89 ± 0.04
	39, natural	nd	nd	0.38 ± 0.08

[a] nd, not detected.

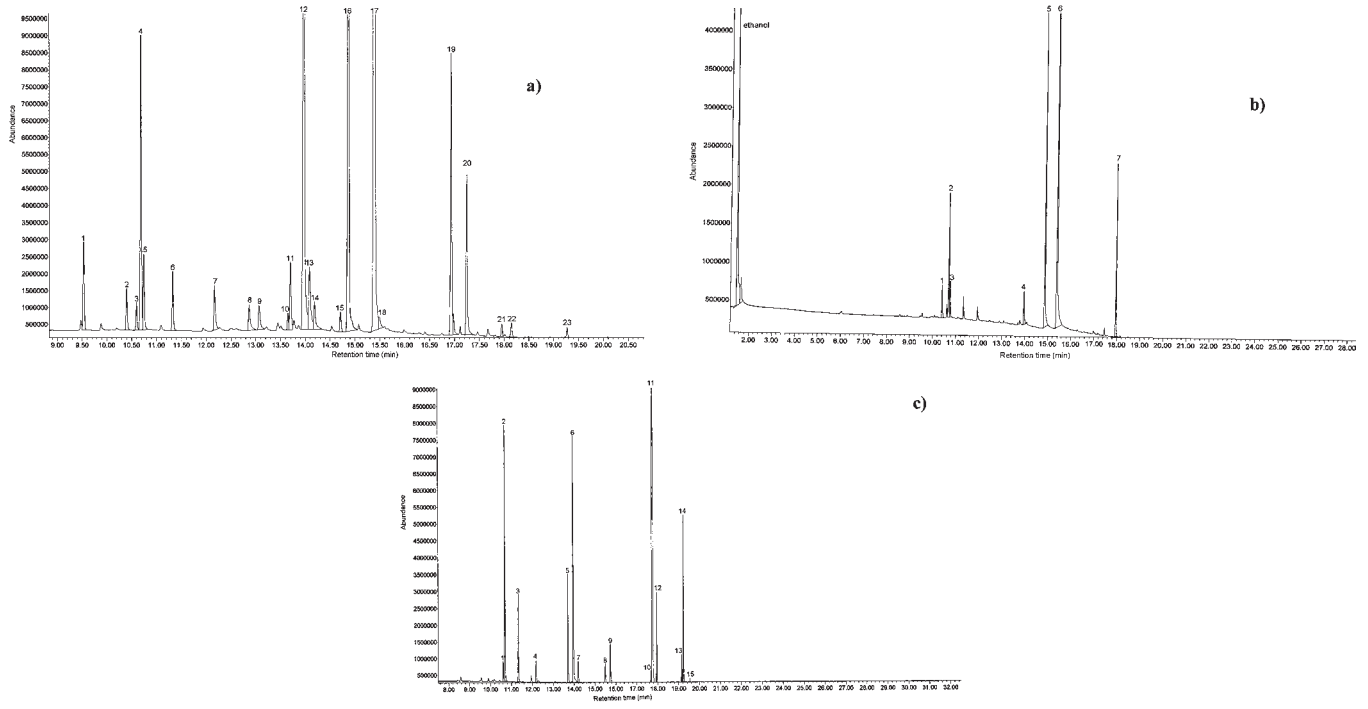

Figure 1. GC-MS chromatograms of flavor extracts: (a) lime; (b) lemon; (c) tangerine.

TFC, reducing power, DPPH RSA). Linearity ranges from 0 to 5.0 mg of GA/L in TPC, from 0 to 66.2 mg of epicatechin/L in TFC, and from 0 to 19.6 mg of GA/L and Trolox/L in reducing power and DPPH RSA methods, respectively. LOD values ranged from 5.43×10^{-3} (reducing power) to 1.00×10^{-1} (TFC) mg of standard antioxidant/L, and LOQ values ranged from 1.81×10^{-2} to 3.33×10^{-1} mg of standard antioxidant/L.

Precision and accuracy values are also shown in Table 2. RSD values ranged from 2.1 (intraday studies) to 7.4 (interday studies) and confirmed the high precision of the methods. REC and RE values assessed the accuracy of the results. RE were always <11.0%, and recovery trials ranged from 93 to 111%, confirming the accuracy of the implemented methods.

Determination of TPC and TFC. Recently, bottled flavored waters have become popular, and the consumption of flavored waters is globally increasing, including in Portugal. In the first half of 2010, 6.08 million liters of this kind of water was consumed by the Portuguese population. Considering the emergent market of this kind of beverage, it is important to deepen the knowledge of the antioxidant capacity of these beverages. The method used to determine TPC has been extensively applied in plants and beverages. Phenolic and flavonoid compounds, correlated with antioxidant activity, seem to have an important role in stabilizing lipid oxidation. Generally, the antioxidant mechanism of phenolic compounds is inactivating lipid free radicals and preventing decomposition of hydroperoxides into free radicals. This is the case of fruits and beverages in relation to their phenolic compounds.[5] Therefore, in this research TPC and TFC were evaluated in 6 flavors used in flavored water formulation and in 39 water samples commercialized in northern Portugal. However, TPC determination should always be considered as an indicative value instead of an accurate measure of phenolic compounds. This method should be aware of possible interferences (reducing sugars and some amino acids) that can overestimate evaluated amounts. On the other hand, it is difficult to measure all phenolic molecules individually. Nevertheless, this assay is a simple, sensitive, and precise technique.[4] Table 3 presents TPC and TFC values obtained in samples.

With regard to TPC, only natural waters (without added ingredients) and two samples of flavored waters (13 and 14) present "not detected" values. As an exception, sample 37 (natural water) presents trace TPC levels (0.07 mg of GA/L). TPC values ranged from 8.5 (gooseberry) to 380.2 (lemon) mg of GA/L in flavors and from 0.29 (sample 27) to 284 mg of GA/L (sample 29) in flavored waters. Comparing flavor TPC values, the highest contents are from citrus fruits such as tangerine, lime, and lemon, respectively, 117, 359, and 380 mg of GA/L. These values are similar to those obtained by other authors in juices of citrus fruits,[5] but less than those found in other studies with beverages containing milk and fruits of the same kind used in this work.[21] Gooseberry, in contrast to what was expected, presented the lowest levels. This flavor is different from Indian gooseberry, described by Mayachiew and Devahastin,[22] as rich in TPC (290 mg of GA/g extract). Calixto and Goñi[23] reported the TPC of beverages (coffee, tea, and red wine) and fruits. The TPC values were higher than the TPC values obtained in this work and ranged from 76 mg of GA/100 mL in beverages to 538 mg of GA/100 g in dry fruit.

With regard to flavored waters and their TPC contents, the lowest value (0.29 mg of GA/L, lemon flavor, sample 27) and the highest value (284 mg of GA/L, apple flavor, sample 29) were determined in samples from the same brand (G). This information can be important for consumers because they generally correlate brands with similar behaviors. In this case, flavored waters from the same brand can be distinct. According to Table 3 and the values presented, brand G is unique, having significant differences.

The addition of bioactive compounds such as tea (samples 9, 21, and 23), ginseng (sample 22), and *G. biloba* (sample 24) seems

Table 4. Chemical Composition of the Volatile Fraction of Lime Flavor

peak	retention time (min)	compound	MW	m/z	Relative content[a] (%)
		Monoterpenes			
1	9.53	(−)-β-pinene	136	93; 41; 91	2.17
2	10.40	1,4-Cineole	154	111; 43; 71	1.06
3	10.60	*o*-cymene	134	119; 134	0.67
4	10.69	α-limonene	136	68; 67; 93	7.45
5	10.74	eucalyptol (1,8-cineole)	154	43; 81; 108	1.87
6	11.33	γ-terpinene	136	93; 91	1.43
7	12.17	linalyl butyrate	224	93; 43; 41	1.27
8	12.87	3-carene	136	93; 91; 79	0.95
9	13.07	*p*-menth-8-en-2-ol (1,6-dihydrocarveol)	154	93; 107; 121; 136	0.86
10	13.65	n-octanal dimethyl acetal	174	75; 71; 41	0.38
11	13.70	1-terpinen-4-ol	154	71; 93; 111	1.90
12	13.98	α-terpineol	154	59; 93; 121	19.34
13	14.09	6-isopropylidene-1-methylbicyclo[3.1.0]hexane	136	121; 93; 136	2.43
14	14.19	L-isopulegol	154	41; 67; 69; 81; 55	1.06
15	14.71	geranyl isovalerate	238	85;: 43; 57; 41; 69	0.52
16	14.87	carveol (*p*-mentha-1,8-dien-6-ol)	152	119; 91; 134	17.13
17	15.40	citral (geranial)	152	69; 41; 84	27.12
18	15.49	*cis*-*p*-menth-2,8-dienol	152	91; 134; 43; 119; 134	0.47
19	16.94	*cis*-geraniol	154	69; 41; 93	6.74
20	17.25	β-myrcene	136	41; 69; 93; 39; 27	4.19
		Sesquiterpenes			
21	17.96	β-caryophyllene	204	41; 69; 93; 133; 79	0.30
22	18.15	α-bergamotene	204	93; 41; 119; 91	0.38
23	19.27	β-bisabolene	204	69; 41; 93	0.31

[a] Relative content was calculated from area ratio.

Table 5. Chemical Composition of the Volatile Fraction of Lemon Flavor

peak	retention time (min)	compound	MW	*m*/*z*	relative content[a] (%)
		Monoterpenes			
1	10.40	*cis*-β-terpineol	154	43; 71	2.55
2	10.69	α-limonene (*p*-mentha-1,8-diene)	136	68; 67; 93	8.57
3	10.75	eucaplyptol (1,8-cineole)	154	43; 81; 108	2.95
4	13.96	α-terpineol (*p*-menth-1,8-dien-6-ol)	154	59; 93; 121	2.92
5	14.85	carveol (*p*-mentha-1,8-dien-6-ol)	152	119; 91; 134	27.09
6	15.37	citral (geranial)	152	69; 41; 84	44.32
		Sesquiterpenes			
7	17.95	β-caryophyllene	204	41; 69; 93; 133; 79	11.59

[a] Relative content was calculated from area ratio.

to increase TPC contents of the flavored waters. These samples presented values ranging from 28.1 to 39.7 mg of GA/L, the highest ones excluding samples 29 and 30. It is important to remember that according to the label information, these samples (29 and 30) have vitamin C as an added ingredient (12 mg/250 mL), a compound related with antioxidant properties. Nevertheless, the presence of the vitamin, referred to on the label, does not always imply high TPC levels. This is the case for samples 27 and 32, with added vitamin, which have very different TPC values lower than those previously mentioned. Sometimes, the expectation that samples with bioactive compounds have a dual phenolic protective effect is not true.

Samples from brand D presented the lowest TPC values (from not detected to 0.54 mg of GA/L). According to the label information, these samples have only flavors in its formulation. It is possible to speculate whether this is a synthetic substance without the complexity of vegetable/fruit extracts, namely, without phenolic compounds. Another approach can be the use of trace amounts without influence in values of the parameters in appreciation.

All brands have water flavored with lemon. TPC values have extreme discrepancies, ranging from not detected (sample 14) to 17.62 mg of GA/L (sample 6) and higher in association with magnesium (24.44 mg of GA/L, sample 8) or green tea (39.70 mg of GA/L, sample 21). It is also interesting to verify that waters

Table 6. Chemical Composition of the Volatile Fraction of Tangerine Flavor

peak	retention time (min)	compound	MW	*m*/*z*	relative content[a] (%)
		Monoterpenes			
1	10.61	β-cymene	134	119; 91	0.89
2	10.69	α-limonene (*p*-mentha-1,8-diene)	136	68; 67; 93	11.78
3	11.33	γ-terpinene (*p*-mentha-1,4-diene)	136	93; 91	3.76
4	12.17	linalyl butyrate	224	93; 43; 41	1.01
5	13.70	4-terpineol (*p*-menth-1-en-4-ol; 1-terpenen-4-ol)	154	71; 93; 111	4.74
6	13.95	α-terpineol (*p*-menth-1-en-8-ol)	154	59; 93; 121	11.38
7	14.19	*n*-decanal	156	41; 43; 57	1.01
8	15.49	*p*-mentha-1,8-dien-7-al ((−)-perillaldehyde))	150	68; 79	1.22
9	15.73	carvacrol (*p*-cymen-2-ol)	150	135; 150	2.29
10	17.67	*n*-dodecanal	184	41; 57; 55	0.71
11	17.76	methyl aminobenzoate	165	165; 105	48.75
		Sesquiterpenes			
12	17.96	β-caryophyllene	204	41; 69; 93; 133; 79	3.84
13	19.14	α-selinene	204	108; 204; 93	1.54
14	19.22	α-farnesene	204	41; 93	6.65
15	19.53	β-cadinene	204	161; 204; 134	0.44

[a] Relative content was calculated from area ratio.

flavored with lemon generally presented the lowest TPC values compared with other flavors, the exception being the examples referred to above with magnesium and green tea. This is especially important taking into account that lemon flavor was the richest in TPC. The use of more diluted extract, due to its strong taste, can be a possible explanation for the obtained results.

In the case of lime flavor, the second most rich in TPC, is only present in sample 28. However, this flavor, being slightly poorer in TPC compared with lemon, is present at levels 6-fold higher in flavored waters from the same brand.

With regard to flavors and their TPC contents, it is important to note that TPC values from red fruits (strawberry and gooseberry) were the lowest. Taking into account its antioxidant power, it can be speculated that the mechanism does not involve phenolic compounds. However, when used as ingredients, they provide water samples the highest TPC values compared with other samples of the same brand. This is the case of sample 3 in brand A and sample 36 in brand I.

From the studied samples, labels do not reveal the presence of gooseberry. Two samples (17 and 22) have raspberry as an ingredient, being the second richest considering all samples of the brands.

Unfortunately, the labels of the samples evaluated do not declare tangerine flavor in their compositions. Probably it is used in combination with other flavors, in minimal amounts, and not declared in the final list of ingredients. The same explanation can be proposed for gooseberry flavor.

With regard to TFC, all flavors and flavored water samples had no flavonoids, in detectable amounts, in their composition. These results are consistent with those obtained by Tabard and collaborators.[24] Using the same optical technique used in this work, these authors did not find flavonoids in apple, grape, or vegetable juices. However, those authors found a high TFC level in red wine. On the other hand, flavors (essential oils) contain about 1−15% of nonvolatile components when flavonoids are included.[8] Therefore, flavonoids are present in small or not detected amounts in flavors. When quantified by a colorimetric method, and after dilution in water, it is difficult to detect them.

Reducing Power Assay. In reducing power determination, the yellow color of the solution changes to various shades of green and blue, depending on the compounds present in the solution. The presence of antioxidants causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form.

Table 3 presents the values of reducing power from flavors and flavored waters. As expected, flavors presented higher values than flavored waters due to their higher concentrations of bioactive compounds. Nevertheless, samples from brand F present similar values, and one sample from brand G (sample 29) presents values 15-fold higher than those in flavors.

With regard to flavors, reducing powers are very similar (from 10.2 to 11.8 mg of GA/L). No correlation among the different evaluated parameters was verified. As referred to above, flavors presented very different TPC values. It is interesting to remember that the lowest TPC value (gooseberry) corresponds to the highest value in reducing power determination. Some studies indicated a high reducing power activity in wild fruits.[25]

All natural waters and three flavored water samples from the same brand (27, 28, and 30) presented values of this parameter below the LOD. It should be noted that samples 27 and 30 have vitamin C as an added ingredient and sample 30 presented the second highest content in TPC. Inversely, sample 29, with apple flavor, had the highest value in TPC and reducing capacity and had also vitamin C as an added ingredient. The highest reducing power values were obtained (like in TPC) in flavored waters with bioactive compounds (tea, ginseng, and *G. biloba*) ranging from 8.3 to 13.8 mg of GA/L.

It is verified that samples from the same brand had similar values, except for brand C (sample 10, without addition of bioactive compounds) and brand E (sample 19 with a value 5-fold higher than the other samples). This behavior occurred also in TPC values, sample 19 being also the richest in these compounds.

From a general point of view and except for brand F (with values similar to flavors) and brand G, as referred to above, all brands can be grouped into two sets: A, D, E, H, and J with lower

values ranging from 3 to 4 mg of GA/L; and B, C, and I with relatively higher values near 6−7 mg of GA/L.

DPPH RSA. DPPH RSA is a technique based on the reduction of the DPPH radical in the presence of a hydrogen-donating antioxidant. A DPPH solution, freshly prepared, exhibits a deep purple color with maximum absorption at 517 nm. This color disappears in the presence of an antioxidant, because antioxidant molecules can quench DPPH free radicals and convert them into a colorless product. Hence, the more rapidly the absorbance decreases, the more potent is the antioxidant. Table 2 presents RSA values obtained with water samples and flavors. According to the previously discussed parameters, flavors presented, in general, higher RSA values than flavored waters, except some samples of brand G (samples 29 and 30) with higher values than some flavors. Flavor RSA values ranged from 39 (lemon) to 214 (strawberry) mg of Trolox/L. The highest RSA values were determined in strawberry and gooseberry flavors (214 and 212 mg of Trolox/L), which presented the lowest values of TPC (15 and 9 mg of GA/L). Choi and collaborators[26] reported the RSAs of 34 kinds of citrus essential oils and their components by HPLC, showing that all essential oils have scavenging effects on DPPH ranging from 5.4 to 172 mg of Trolox equiv/mL.

As with other parameters (TPC and reducing power), flavored waters with bioactive compounds (tea, ginseng, and *G. biloba*) have increased RSA values, demonstrating the dual effect of radical scavenging of these bioactive compounds. Further global comparisons are difficult to establish due to the fact that different standards are used in the several analytical methods described.

GC-MS Analysis of Flavors/Fragances. Six flavors were evaluated with regard to antioxidant activity, but only citrus flavors (lime, lemon, and tangerine) were analyzed by GC-MS.

Flavors (essential oils) are volatile and complex natural mixtures characterized by a strong odor, which can contain about 20−60 components at quite different concentrations. Terpenes and terpenoids constituted the main group of compounds with other aromatic and aliphatic constituents, all characterized by low molecular weight.[7] Volatile compound profiles were obtained by HS-SPME using a PDMS/DVB fiber and analyzed by GC-MS. Figure 1 shows the chromatograms of citrus flavors (lime, lemon, and tangerine). The characterization of individual components was performed with mass spectrometry (MS). Qualitative and quantitative composition of the citrus flavors (lime, lemon, and tangerine), obtained by comparison of mass spectra data, and library data are listed in Tables 4−6. A total of 28 terpenes were identified: 22 monoterpenes and 6 sesquiterpenes. Terpenes are a combination of several 5-carbon-base (C_5) units called isoprenes. The main terpenes are monoterpenes (C_{10}) and sesquiterpenes (C_{15}). A terpene containing oxygen is called a terpenoid. The monoterpenes identified and present in the flavors can be classified as (i) acyclic (β-myrcene) (ii) monocyclic (α-limonene, γ- terpinene, o-cymene; β-cymene); (iii) bicyclic (6-isopropylidene-1-methylbicyclo[3.1.0]hexane, (−)-β-pinene, 3-carene); (iv) terpenoid alcohol acyclic (cis-geraniol); (v) terpenoid alcohol monocyclic (1,6-dihydrocarveol, 1-terpinen-4-ol, α-terpineol, L-isopulegol, carveol, *cis-p*-menth-2,8-dienol, *cis*-β-terpineol); (vi) terpenoid aldehyde (geranial, *p*-mentha-1,8-dien-7-al); (vii) terpenoid ester (linalyl butyrate); (viii) terpenoid ether (1,4-cineole, 1,8-cineole); and (ix) terpenoid phenol (carvacrol). The sesquiterpenes are classified as (x) acyclic (α-farnesene); (xi) monocyclic (β-bisabolene); and (xii) bicyclic (β-caryophyllene, α-bergamotene, α-selenene, and β-cadinene).

For the lime flavor the mass spectral data revealed that monoterpenes represented >99% of the volatile fraction. Citral (27.12%) was the major ingredient followed by α-terpineol (19.34%), carveol (17.13%), and α-Limonene (7.45%). The sesquiterpenes β-bisabolene (0.31%), β-caryophyllene (0.30%), and α-bergamotene (0.38%) were in minor quantities.

In lemon flavor, the volatile fraction extracted was represented by 88.41% of monoterpenes and 11.59% of sesquiterpenes. The major ingredients were the terpenes citral (44.32%), carveol (27.09%), and α-limonene (8.57%) and the sesquiterpene β-caryophyllene (11.59%). With regard to the tangerine flavor, methyl aminobonzoate (48.75%), α- limonene (11.78%) and α-terpineol (11.38%) were the major compounds followed by the sesquiterpene α-farnesene (6.65%). With regard to the tangerine flavor, α-limonene (11.78%) and α-terpineol (11.38%) were the major compounds followed by the sesquiterpene α-farnesene (6.65%).

By comparison of the obtained results with those of the literature, several analogies can be pointed out. According to studies carried out by several authors, the essential oil obtained from citrus fruit (orange, lemon, bergamot, grapefruit) had a similar composition to that described in this study, considering only the analysis of the most volatile fraction of the essence.[9,27−29] Some authors reported that the major ingredients present in essential oils from citrus fruit (orange and lemon) is limonene[9,28] followed by α- and β-pinenes and γ-terpinene.[28] However, Caccioni and collaborators[27] reported that lemon oil collected in February showed the highest content of oxygenated compounds, two geraniol−geranial and nerol−neral couples being the main compounds. Thus, the analysis and extraction of the compounds in flavors can change in quality and quantity with seasonal variation, ripeness, soil composition, and geographical region.[7,8] Almost authors agree that monoterpenes make up 97% of the citrus oil composition, with alcohol, aldehydes, and esters being the lowest percentage components ranging from 1.8 to 2.2%.[29] Flavonoids are another group of components that are present in citrus flavors, making up the nonvolatile part of the oils.[8] Indeed, antimicrobial, antifungal, antioxidant, and radical scavenging properties have been reported for flavors (essential oils) and fruits.[9] Di Vaio and collaborators[9] reported that the peel ethanol extract from lemon presented antioxidant activity and high radical scavenging power, suggesting that lemon essential oils and their related flavor components may contribute to preventing oxidation in foods and inhibit lipid oxidation. Other studies reported by Crowell[30] revealed that terpenoids such as carveol and limonene present in plant essential oils are effective in treating breast, liver, and/or other cancers.

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