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Isolation and identification of phenolic compounds from rum aged in oak barrels by high-speed countercurrent chromatography/high-performance liquid chromatography-diode array detection-electrospray ionization mass spectrometry and screening for antioxidant activity

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ABSTRACT

Beverages, especially wines are well-known to contain a variety of health-beneficial bioactive substances, mainly of phenolic nature which frequently exhibit antioxidant activity. Significant information is available about the separation and identification of polyphenols from some beverages by chromatographic and spectroscopic techniques, but considerably poor is chemical data related to the polyphenolic content in rums. In this paper, a method involving the all-liquid chromatographic technique of high-speed countercurrent chromatography (HSCCC) combined with high-performance liquid chromatography coupled with diode-array detection and electrospray ionization mass spectrometry (HPLC–DAD–ESI-MS^n) has been successfully applied for separation and identification of phenolic compounds in an aged rum. Besides, the phenolic fraction (PF) was assayed for its antioxidant effects using three different free radical *in vitro* assays (DPPH$^\bullet$, $RO_2^\bullet$ and spontaneous lipid peroxidation (LPO) on brain homogenates) and on ferric reducing antioxidant power (FRAP). Results showed that PF potently scavenged DPPH and strongly scavenged peroxyl radicals compared to ascorbic acid and butylated hydroxytoluene (BHT); and almost equally inhibited LPO on brain homogenates subjected to spontaneous LPO when compared to quercetin. Moreover, PF also exhibited strong reducing power. This chemical analysis illustrates the rich array of phenols in the aged rum and represents a rapid and suitable method for the isolation and identification of phenolic compounds from mixtures of considerable complexity, achieving high purity and reproducibility with the use of two separation steps.

1. Introduction

Rum is a fairly tasteless and neutral spirit derived from the fermentation of sugar molasses and sugar cane syrup. Once the alcohol is obtained from the fermentation and distillation processes, it undergoes further processing, such as percolation through carbon filters, aging in oak barrels, and blending, which give the rum particular sensory characteristics [1]. The presence of volatile components, such as alcohols, ethyl acetate, acetic acid, ethyl esters, and non volatile compounds, originating from the raw materials and the fermentation, distillation, and aging processes, is essential to define the beverages composition and, therefore, provide elements for their distinction [2–5].

Despite the extensive works carried out on the assessment of the antioxidant properties of several wines and liquors, few reports are still available about spirits [6–10]. Many types of compounds are transferred from the wood to the product: ellagitannins, lactones, coumarins, polysaccharides, hydrocarbons and fatty acids, terpenes, norisoprenoids, steroids, carotenoids and furan compounds. Volatile phenols and benzoic aldehydes are particularly significant, as they confer important sensorial characteristics on the products [11–15]. Furan and pyran derivatives are compounds with a toasty caramel aroma formed as a consequence of the heat treatment carried out in barrel-making [16]. Hydrolysable tannins (gallotannins and ellagitannins), the main polyphenols released from wood, play a very important role in wine and spirits affinity, and polysaccharides confer astringency and structure and colour

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stabilization to the product [12,17,18]. Besides this, oxygen permeation through the wood favours redox processes and the formation of new and stable anthocyanin and tannin derivatives, with the consequent colour stabilization of red wines, and a loss of astringency [17].

In vivo, reactive oxygen species (ROS) can interact with cellular biomolecules such as DNA, proteins, fatty acids and saccharides causing oxidative damage and subsequent health problems. Hence, ROS scavengers may serve as a possible preventive intervention of free radical mediated diseases [19,20] while they quench free radical reactions. Polyphenols are one of principal compounds related to the benefits of fruits, vegetables, plant tea and wines consumed in the diet due to their antioxidant properties. It is likely that aged rums could exhibit interesting antioxidant properties depending on the content, chemical properties and oxidation degree of phenols extracted from the oak barrels.

In this context, the aim of this work was to study the phenolic composition of rum aged in oak barrels and its antioxidant capacities by three different *in vitro* free radical scavenging assays and by determining its reducing power. We developed a systematic protocol which combines chromatographic and spectroscopic techniques for its fractionation (HSCCC and TLC) and the subsequent identification (HPLC–DAD–ESI-MS^n and NMR).

2. Experimental

2.1. Rum sample

The matured rum (35%, v/v alcohol) under investigation was obtained from a commercial producer in Cuba. It had been produced by stainless-steel column distillation (continuous process) and had been stored in heat charred oak casks for 15 years.

2.2. Reagents

For preparation of the extract, HSCCC separation and TLC, analytical grade solvents *n*-hexane, ethyl acetate, methanol (Fisher Scientific; Loughborough, UK) and nanopure water (Barnstead; Dubuque, IA, USA) were used. Glacial acetic acid, 2,2-diphenyl-2-picrylhydrazyl (DPPH), quercetin, Folin-Ciocalteu reagent, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), butylated hydroxytoluene (BHT), malonaldehyde bis-(dimethyl acetal) (MDA) and *p*-anisaldehyde were purchased from Sigma–Aldrich (Darmstadt, Germany). Thiobarbituric acid and ascorbic acid were from Aplychem (Darmstadt, Germany), whereas 2,2-azobis-2-amidinopropane hydrochloride (AAPH) was obtained from Polyscience (Warington, PA). LC–MS measurements were carried out with MS grade acetonitrile and extra pure formic acid (Acros Organics; Geel, Belgium).

2.3. Extraction and isolation

The aged rum (2.25 l) was evaporated under reduced pressure to remove the alcohol. The remaining aqueous phase was then filtered through a folded filter (Macherey-Nagel, 615¼) and subjected onto a glass column (80 cm × 5.5 cm) filled with Amberlite XAD-7. The column was washed extensively with water to remove sugars, proteins and salts. After elution with methanol the solution was evaporated under reduced pressure and lyophilized to give 1.23 g of crude extract.

2.4. High speed counter-current chromatography

For HSCCC separation of the rum extract, a triple coil "high-speed countercurrent chromatograph" (HSCCC) model CCC-1000 (Pharma-Tech Research Corporation; Baltimore, MD, USA) was used. The HSCCC consists of three preparative coils with a total coil volume of 850 ml. Sample injection was done by a manual sample injection valve with 50 ml loop. Solvents were delivered by a Biotronik HPLC pump BT 3020 (Jasco; Groß-Umstadt, Germany). The UV-absorbance of the eluent was monitored by a Knauer K-2501 UV detector (Berlin, Germany) at the wavelength of 320 nm. The fractions were collected in 4 min intervals into test tubes with a LKB Super Frac 2211 fraction collector (Pharmacia; Bromma, Sweden). The separations were carried out in head to tail mode with a coil speed of 900 rpm and a flow rate of 3 ml/min.

An amount of 800 mg of this extract were separated using high-speed countercurrent chromatography (HSCCC) with *n*-hexane/ethyl acetate/methanol/water + 0.1% trifluoroacetic acid, 1:1:1:1 (v/v/v/v) as solvent system. The crude extract was dissolved in a mixture of upper and lower phase and injected into the HSCCC. After the separation the collected fractions were pooled according to similarities in their TLC profiles and the obtained UV-chromatogram to give six major fractions (F1–F6). The residue remaining on the PTFE column (coil) was ejected with nitrogen to determine the stationary phase retention, which was 67%.

2.5. Thin-layer chromatography

Thin-layer chromatography (TLC) was performed using silica gel 60 F_{254} aluminum sheets (Merck; Darmstadt, Germany). Compounds were visualized by spraying with *p*-anisaldehyde–sulphuric acid–glacial acetic acid spray reagent prepared [21]. The TLC of the fractions from the separation of the XAD-7 extract was performed using chloroform/ethyl acetate/methanol/water, 15:50:35:10 (v/v/v/v).

2.6. HPLC–DAD–ESI-MS^n analysis

The chromatographic analysis of the HSCCC fractions was carried out on a Bruker HCTultra ETD II LC–MS (Bruker Daltonik; Bremen, Germany) with electrospray ionization in the positive and negative mode. The HPLC system consisted of a HP Series 1100 G1312A binary pump, a HP Series 1200 G1329B ALS SL auto sampler and a HP Series 1100 diode array detector (Agilent; Böblingen, Germany). The system was controlled by Compass 1.3 software. As dry gas nitrogen with a gas flow of 10 ml/min (350 °C) was used, the nebulizer was adjusted to 60 psi.

Following parameters were used for the positive mode: capillary (3500 V), end plate (−500 V), capillary exit (−127.0 V), skimmer (−40.0 V), lens 1 (5.0 V) and lens 2 (60.0 V). In the negative mode the values were adjusted as follows: capillary (−3500 V), end plate (−500 V), capillary exit (127.0 V), skimmer (40.0 V), lens 1 (−5.0 V) and lens 2 (−60.0 V).

Separations were performed using a Synergi MAX-RP column (250 mm × 4.6 mm, 4 μm particle size, 80 Å pore size) with a guard column (4.0 mm × 3.0 mm) filled with the same material (Phenomenex; Santa Clara, CA, USA). As mobile phases (A) water/acetonitrile/formic acid 87:3:10 (v/v/v) and (B) water/acetonitrile/formic acid 40:50:10 (v/v/v) with the following gradient were used: 0 min, 6% B; 20 min, 20% B; 35 min, 40% B; 40 min, 60% B; 45 min, 90% B; 55 min, 6% B. The flow rate was set at 0.5 ml/min. Injection volume was set to 20 μl.

2.7. NMR

^{1}H NMR spectra (400.1 MHz), ^{13}C NMR spectra (100.6 MHz) and all 2D experiments (^{1}H–^{1}H COSY and ^{1}H–^{13}C HMQC, HMBC), were recorded in $CDCl_3$ on a Bruker Daltonics DRX-400 Spectrometer (Bruker Biospin; Rheinstetten, Germany). The chemical shifts were referenced to the solvent signals at δ_H = 7.26 ppm and δ_C = 77.16 ppm.

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2.8. In vitro antioxidant activity

2.8.1. Assay of peroxyl radical ($RO_2^{\bullet}$) scavenging effects

The assay was performed according to a modification of the method described previously [22], based on the ability of the hydrophilic radical generator AAPH to generate peroxyl radicals, thereby oxidizing membranes. The capacity of BM-21 by PF to scavenge $RO_2^{\bullet}$ was assessed by determination of its ability to inhibit the extent of oxidation in brain homogenates. To obtain tissue homogenates, male OF-1 mice weighted 20–25 g (CENPALAB, Havana) were slightly anesthetized with ether and euthanatized by cervical traction. Brains were rapidly extracted, rinsed in cold NaCl 0.9% (w/v) and homogenized (1/9, w/v) in ice cold 0.1 M potassium phosphate buffer, pH 7.5 in a Potter–Elvehjem type homogenizer. The homogenate was centrifuged at 4000 × *g* for 20 min at 4 °C, and the resulting supernatant was used for different assays. As brain homogenates were merely used as source of polyunsaturated fatty acids, they were heat-inactivated (60 °C, 1 h) prior to addition to the incubation mixture. Brain homogenate (0.5 mg/ml, final concentration) and AAPH (10 μM final concentration) previously prepared in 0.1 M potassium phosphate buffer, pH 7.5 were incubated in the presence or absence of different concentrations of PF previously dissolved in 50% ethanol. The degree of AAPH-mediated oxidation was measured by thiobarbituric acid reactive substances (TBARS) assay and protein concentration was estimated by a modification of the Lowry procedure [23]. Quercetin was used as standard.

2.8.2. Testing for lipid peroxidation (LPO) in brain homogenates

Effects on spontaneous lipid peroxidation (LPO) in brain homogenates were determined according to Cini et al. [24]. With this aim, samples (1 mg/ml, final concentration) were incubated in 0.1 M potassium phosphate buffer, pH 7.5 at 37 °C for 30 min in the presence or not of PF and BHT. The reaction was stopped by cooling and adding EDTA (1%). The LPO status in samples was monitored through the formation TBARS.

2.8.3. Determination of TBARS

TBARS were determined by a modification of a previously reported procedure [25]. TBARS reagent (acetic acid (20%, v/v, pH 3.5) and TBA (0.8%, w/v) was added to 8.1% (w/v) sodium dodecyl sulphate (SDS), BHT (0.02%, final concentration) and samples and heated to 95 °C for 1 h. Then, samples were cooled (0–4 °C) and *n*-butanol:pyridine (15:1, v/v) was added. The organic layer was taken for measurement of optical density at 534 nm using a UV-1201 spectrophotometer (Shimadzu, Japan). TBARS concentrations were estimated from a standard curve of MDA and reported as nmol MDA/mg protein.

2.8.4. Assay of DPPH scavenging activity

Scavenging ability of PF on DPPH• radical was carried out according to previously reported [26] with minor modifications. Basically, a 60-μM methanolic solution of DPPH (980 μl) prepared daily, was placed in a spectrophotometer cuvette, and different concentrations of PF (3–90 μg/ml) or ascorbic acid (0.5–100 μg/ml) in MeOH (v/v) solution (20 μl) were added. The decrease in absorbance at 515 nm was determined until the reaction plateau step was reached. Methanol was used to zero the spectrophotometer.

2.8.5. Data processing

To evaluate the inhibitory effects of PF, results were expressed as percentage of inhibition which was calculated from the absorbance values of the control and experimental tubes. IC_{50} concentration, that represents the concentration of PF that caused 50% inhibition of the maximal effects, was determined by the percent inhibition *versus* the concentration curves. Every experiment was repeated three times and values represent mean ± SEM.

2.8.6. Ferric reducing/antioxidant power (FRAP) assay

The antioxidant capacity of each sample was estimated according to the procedure described previously [27] with the modification proposed by Pulido et al. [28]. Briefly, 1500 μl of FRAP reagent, prepared freshly and warmed at 37 °C, was mixed with distilled water and 50 μl of test samples, water, or methanol as appropriate for the reagent blank. The FRAP reagent contained 2.5 ml of a 10 mmol/l TPTZ (solution in 40 mmol/l HCl plus 2.5 ml of 20 mmol/l $FeCl_3 \cdot 6H_2O$ and 25 ml of 0.3 mol/l acetate buffer, pH 3.6 [27]. Readings at the absorption maximum (595 nm) were taken. Temperature was maintained at 37 °C and the reaction monitored for up to 30 min. Methanolic solutions of known Fe(II) concentrations in the range of 100–1500 μmol/l [$FeSO_4 \cdot (NH_4)_2SO_4$] were used for calibration. The antioxidant capacity was expressed as μmol of Fe^{2+}/l of sample.

2.8.7. Determination of total phenolic content

The amount of phenol in both samples was determined with Folin-Ciocalteu reagent using a modified method [29]. Briefly, 2.5 ml of 10% Folin-Ciocalteu reagent and 2 ml of Na_2CO_3 (2%, w/v) was added to 0.5 ml of each sample (3 replicates) of sample solution (1 mg/ml). The resulting mixture was incubated at 45 °C with shaking for 15 min. The absorbance of the samples was measured at 765 nm using UV/visible light. Results were expressed as milligrams of gallic acid (0–0.5 mg/ml) dissolved in distilled water.

3. Results and discussion

Separation and fractionation of polyphenols and polyphenol-derived compounds into different sub-fractions are generally a first step for improving their further isolation and identification. CCC is a support-free all-liquid chromatographic technique that is widely used in natural product analysis due to the gentle operation conditions [30]. HSCCC, which is one form of CCC, has been recognized as an efficient preparative technique that yields a highly efficient separation of multigram quantities of samples in several hours and is widely used for separation and purification of various natural and synthetic products [31]. In the present study, a HSCCC system has been applied as the first step for fractionating the aged rum crude extract, from which were obtained six major fractions (F1–F6) according to similarities in their TLC profiles and the obtained UV-chromatogram.

Reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection has been frequently used for the separation and detection of phenolic compounds in complex mixtures [32–35]. The HPLC–DAD separation of F1–F6 recorded at 280 nm using an analytic RP-C_{18} column and mobile phase gradients with water/acetonitrile/formic acid led us to achieve very good peak resolution, which resulted in the isolation of 48 phenolic compounds (cf. HPLC–DAD profiles of F1–F6 in Fig. 1).

MS has been used for unambiguous characterization of phenolic compounds, eliminating artifacts arising from co-eluting compounds with similar UV spectra. An ESI interface may be connected to a HPLC system. ESI is a gentle ionization method generating mainly deprotonated molecules $[M-H]^-$ of the compounds analyzed in the negative ion mode and the final MW assignment is more reliable when it is combined with positive ion mode, which often arise protonated molecules $[M+H]^+$ or other adducts. We have made efforts to determine in our chromatograms as many, both major and minor, compounds as possible. Some compounds were found to be spread over several fractions. The phenolic compounds determined in fractions F1–F6 by LC–MS/MS and partly with DAD

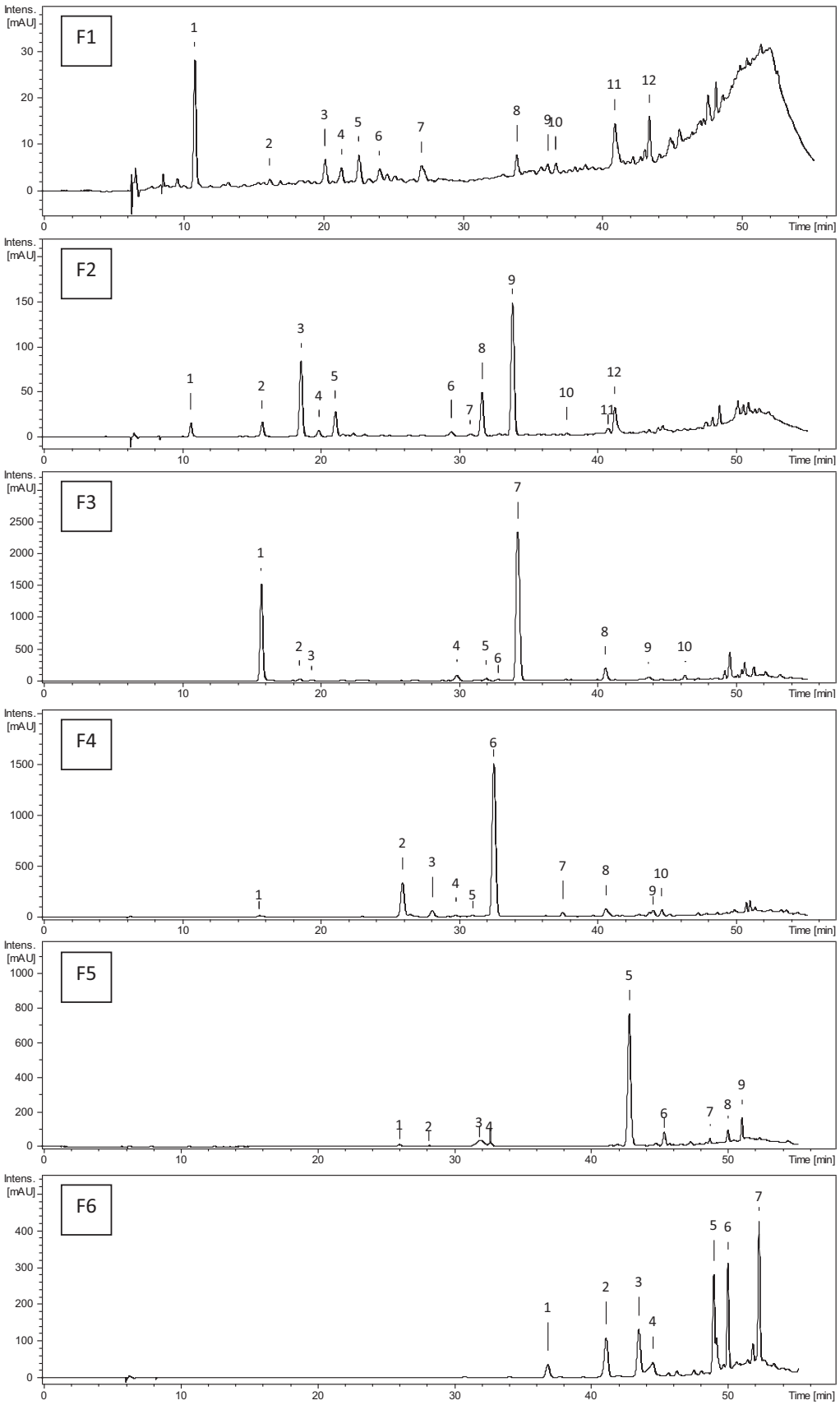

Fig. 1. HPLC–DAD profiles of fractions F1–F6 from rum aged in oak barrels.

Table 1
Spectroscopic and spectrometric data of phenolic compounds from rum aged in oak barrels.

Fraction-peak	t_R (min)	Compound	λ (max)	MW	ESI-MS/MS prominent ions (*m*/*z*)
F1-1	10.8	Gallic acid	270	170	169 $[M-H]^-$, 125
F2-1					
F2-3	18.6	Protocatechuic acid	259, 293	154	153 $[M-H]^-$, 109
F3-2					
F3-3	19.4	*o*-Guaiacol[a]	283, 308	124	123 $[M-H]^-$, 108
F4-2	26.0	Protocatechualdehyde	280, 310	138	137 $[M-H]^-$
F5-1					
F4-3	28.2	*p*-Hydroxybenzoic acid[a]	255	138	137 $[M-H]^-$
F5-2					
F4-4	29.7	*o*-Vanillin[a]	304	152	151 $[M-H]^-$, 136, 108
F3-4	29.8	Methyl protocatechuate	304	168	167 $[M-H]^-$, 109
F2-7	30.8	Peucedanol[a]	326	264	263 $[M-H]^-$, 245, 233, 217, 291, 163
F4-5	31.0	*p*-Vanillin[a]	274	152	151 $[M-H]^-$, 136, 108
F5-3	31.8	Homovanillic acid[a]	290	182	181 $[M-H]^-$, 137
F3-6	32.5	*p*-Vanillic acid	260, 291	168	167 $[M-H]^-$, 152, 123, 108
F4-6					
F5-4					
F2-9	33.8	Syringic acid	275	198	197 $[M-H]^-$, 182, 153, 138, 121
F3-7					
F6-1	36.8	Benzoic acid	283	122	121 $[M-H]^-$
F4-7	37.4	Eudesmic acid[a]	263, 312	212	211 $[M-H]^-$, 167, 152
F2-10	37.4	Kaempferol[a]	365	286	287 $[M+H]^+$, 271, 227
F3-8	40.5	Ethyl gallate	271	198	197 $[M-H]^-$, 169
F4-8					
F2-11	40.7	3-(Carbethoxymethyl)-flavone[a]	320	308	307 $[M-H]^-$, 261, 235
F1-11	40.8	Ellagic acid	365	302	301 $[M-H]^-$, 284, 257, 229, 185
F5-5	42.7	Syringaldehyde	307	182	183 $[M+H]^+$, 155, 123
F6-3	43.4	Ethyl vanillate	282, 318	196	195$[M-H]^-$, 135
F3-9	43.7	Ethyl syringate[a]	280	226	225 $[M-H]^-$, 210, 166
F4-10	44.5	Spigenin[a]	320	270	271 $[M+H]^+$, 243,211,183
F5-7	48.5	Genistein[a]	342	270	269 $[M-H]^-$, 221
F5-8	49.9	Formononetin[a]	272	268	267 $[M-H]^-$, 223
F6-6	49.9	Tectochrysin[a]	294	268	269 $[M+H]^+$, 251, 225
F6-7	52.1	3-Benzyl-7-hydroxy-4-methylcoumarin[a]	297	266	265 $[M-H]^-$, 221

[a] Tentative identification (based on the comparison of UV spectra, t_R and MS with respective literature).

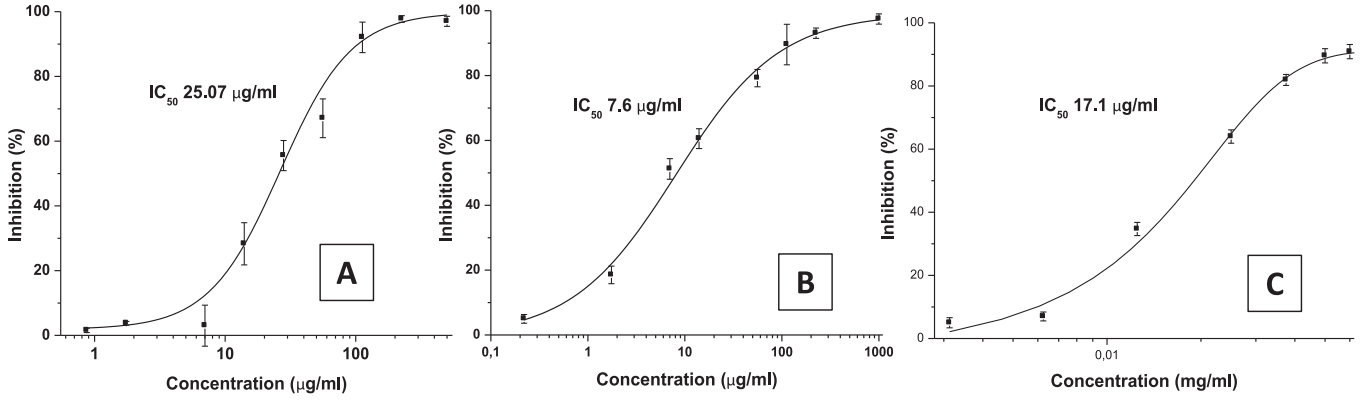

Fig. 2. Effects of polyphenolic fraction from aged rum on three different *in vitro* free radicals. (A) $RO_2^{\bullet}$ radical; (B) LPO assay; (C) DPPH$^{\bullet}$ radical. Values are expressed as mean ± standard deviation ($n = 3$). Quercetin (A), BHT (B) and ascorbic acid (C) were used as the standard. Coefficients of variance were less than 12%.

are presented in Table 1. Besides, the spectroscopic and spectrometric data of the non identified minor components are shown in Table 2.

MS/MS analysis of the chromatograms gave the following results. Low-molecular-weight phenolic compounds, lignin derivatives, were tentatively identified on the basis of their retention time, UV spectra, and MS patterns, as well as taking into account data in related literature [32,36] (Table 1). Among these eluted the benzoic acids like gallic, protocatechuic, 2,3,4,5-tetramethoxybenzoic acid, *p*-hydroxybenzoic, homovanillic, vanillic, syringic, benzoic, and eudesmic acid; the hydroxybenzoic aldehydes protocatechualdehyde, *o*-vanillin, *p*-vanillin, and syringaldehyde; the ethyl esters of ferulic, gallic, vanillic, and syringic acids; and 3,4,5-trimethoxyphenol. Except in the case of 3,4,5-trimethoxyphenol, the respective $[M-H]^-$ quasimolecular ion was the base peak in the MS pattern. The mass spectra of 3,4,5-trimethoxyphenol also gave the fragment ions $[M-CH_3]^-$ and $[M-2CH_3]^-$ at *m*/*z* 168 and 153, respectively. All the acids identified gave the typical anion $[M-H-44]^-$ via loss of a CO_2 group from the carboxylic acid moiety. The fragmentation of *p*-vanillic acid produced an anion radical with *m*/*z* 152 ($[M-H-15]^-$) by losing a CH_3 group from the deprotonated molecular ion. In the methoxylated aldehydes *o*-vanillin and *p*-vanillin, the sequential loss of CH_3 and CO was observed to give fragments at *m*/*z* 136 and 108, respectively. Interestingly, the hydroxycinnamic acid *p*-coumaric, and the hydroxycinnamic aldehydes like coniferylic and sinapylic aldehyde were not detected in the crude extract, although they had been reported in oak wood extracts [37].

Table 2
Spectroscopic and spectrometric data of non identified phenolic compounds from rum aged in oak barrels.

Fraction–peak	t_R (min)	λ (max)	MW	ESI-MS/MS prominent ions (*m*/*z*)
F1-2; F2-2; F3-1;F4-1	16.2	280	220	219, 201, 173
F1-3	20.0	273	222	221 $[M-H]^-$, 203, 185, 173
F2-4				
F2-5	21.0	272	184	183 $[M-H]^-$, 168, 153, 139, 124
F1-4	21.3	269	–	(−) 483, 271, 211, 193
				(+) 237, 221, 214, 133
F1-5	22.6	300	242	(−) 241 $[M-H]^-$, 211, 193
				(+) 265 $[M+Na]^+$, 204
F1-6	23.9	274	378	(−) 377 $[M-H]^-$, 315, 193, 161
				(+) 379 $[M+H]^+$, 363, 343, 167
F1-7	27.0	275	292	(−) 291 $[M-H]^-$, 247
				(+) 293 $[M+H]^+$, 247, 219
F2-6	29.4	310	240	239 $[M-H]^-$, 211, 195
F2-8	31.6	275, 303	298	297 $[M-H]^-$, 253, 209, 165
F3-5	31.9	275, 300	–	(−) 209, 165
				(+) 219, 203, 125
F1-8	33.9	290	–	(−) 297, 207, 163
				(+) 249, 231, 213, 187
F1-9	36.1	260	–	(−) 353, 309, 291
				(+) 567, 295
F1-10	36.6	254	–	(−) 247, 219, 192, 173, 157
				(+) 387,371
F6-2	41.0	280, 308	–	(−) 233, 214
				(+) 420, 181
F2-12	41.2	366	–	(−) 301, 257, 229, 185
F1-12	43.3	277	420	419 $[M-H]^-$, 404, 373
				443 $[M+Na]^+$
F4-9	44.0	340	–	(−) 157, 111
				(+) 519, 271
F6-4	44.4	287	–	293 $[M-H]^+$, 275, 249
F5-6	45.2	298	–	(−) 397, 351
				(+) 425, 405, 366, 335
F3-10	46.2	270	562	(−) 561 $[M-H]^-$
				(+) 563 $[M+H]^+$, 293
F6-5	48.8	338	–	(−) 221, 177, 162
				(+) 179, 161, 147
F5-9	50.9	287, 315	–	(−) 641, 565
				(+) 275, 151

Table 3
Scavenging effects of polyphenol fraction (PF) on three free radicals. Antioxidant effectiveness are expressed as IC_{50} and values represents average of three determinations with ± standard deviation (SD). Quercetin ($RO_2^\bullet$), BHT (LPO) and ascorbic acid ($DPPH^\bullet$) were used as the standards.

Radical specie	Concentration range (μg/ml)	IC_{50} (μg/ml)	Maximum inhibitory effect (%)
Quercetin			
$RO_2^\bullet$	50–0.05	0.72 ± 0.09	92.3 ± 1.7
BHT			
LPO	50–0.2	56.5 ± 1.7	94.2 ± 2.5
Ascorbic acid			
$DPPH^\bullet$	100–0.5	45.0 ± 2.6	95.0 ± 2.7
Polyphenolic fraction			
$RO_2^\bullet$	500–0.85	25.1 ± 0.6	97.7 ± 1.6
LPO	1000–0.2	7.6 ± 1.3	97.4 ± 1.7
$DPPH^\bullet$	90–3	17.1 ± 0.9	91.6 ± 1.9

Following comparison of mass spectra with literature [32], ellagic acid anion (*m*/*z* 301) was characterized by ESI-MS. This fragment anion generates intensive product ions at *m*/*z* 284, 257 and 229.

Only two coumarins were detected, peucedanol and 3-benzyl-7-hydroxy-4-methylcoumarin, characterized by their $[M-H-H_2O]^-$ and $[M-H-CO_2]^-$ anions, respectively.

Five flavonoids were identified distributed in three flavones [spigenin, tectochrysin and 3-(carbethoxymethyl)-flavone]; two isoflavones, genistein and formononetin; and one flavonol, kaempferol. Their mass spectra were according to reported data [34,38,39].

The structures of isolated compounds were confirmed by interpretation of the ^{13}C and ^{1}H NMR experiments and their comparison with reported data. On the other hand, the small quantities of non identified components rendered very difficult their isolation and structure elucidation by NMR spectroscopy. Most of them do not correspond to those of already published polyphenol derivatives and might be new, not previously reported pigmented compounds. Also, the structures of some minor identified components could not be corroborated by NMR and their structure was assigned based on the comparison of their UV, t_R and MS with published data. Consequently, they are represented as a tentative identification in Table 1.

The antioxidant activities of polyphenols are well documented. Normally the phenolic compounds act by scavenging free radicals and thus, they can reduce the lipid peroxidative chain by donating their electron of the hydroxyl and phenoxy groups to the free radicals [40]. Several assay methods have been developed to evaluate the antioxidant activity of a mixture of antioxidant

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compounds. However, since the role of free radicals has been implicated in a large number of diseases, the antioxidant activity of PF was explored by means of several methods that combined different substrates, initiator and appropriate measure of the end-point to ascertain the antioxidant effectiveness in biological systems. Therefore, in the present work we used the DPPH method since it has widely used to measure reduced substances or radical scavengers [41]; the AAPH method that assesses the scavenging ability against $RO_2^{\bullet}$, a radical that is similar to that generated in physiological peroxyl conditions [22]; the inhibition of spontaneous LPO in brain homogenates that also measures the effect on physiologically relevant ROS such as hydroxyl radical [42] and additionally, the reducing power, that measures the presence of reductors that are reported to be terminators of free radical chain reaction and can contribute to limit free radical damage in biological systems [43]. Hence, the measurement of antioxidant capacity of PF in such conditions may have physiological relevance.

Results concerning to the antioxidant capacity of PF and the standard used are shown in Fig. 2 and Table 3. PF showed a strong dose-dependent scavenging effect on $RO_2^{\bullet}$ radical ($IC_{50} = 25.1 \pm 0.6$ μg/ml), where the optimal activity (greater than 95%) was seen at doses of 225 μg/ml as no additional effects were observed by increasing the concentration. It appears that quercetin is a stronger antioxidant than PF using this radical assay. However, PF potently inhibited in a dose-dependent manner LPO when was added to brain homogenates (IC_{50} of 7.6 ± 1.3 μg/ml). This inhibitory action was higher compared to the standard antioxidant BHT (IC_{50} of 56.5 ± 1.7 μg/ml). Furthermore, PF also exhibited a potent dose-dependent scavenging effect on $DPPH^{\bullet}$ and the IC_{50} value was found to be 17.1 ± 0.9 μg/ml, that was stronger than that obtained with ascorbic acid (45.0 ± 2.6 μg/ml). It is worth pointing out that at all the added concentrations (maximum dose assayed 1000 μg/ml) the aged rum exhibited much lower antioxidant activity than PF in all assays used (results not shown). Thus, the higher activity in PF when compared to aged rum may be due to interferences of other compounds present in the rum and suggests that the antioxidant activity is largely due to polyphenols present in aged rum.

Finally, the *in vitro* antioxidant activity of both samples was accessed by their reducing power capacity. These results and those ones obtained by the determination of total phenolic content were generated performing equal dilution for the rum and its phenolic fraction. The reducing power of the aged rum was 2725.0 ± 67.2 μM of Fe^{2+}/l with a phenolic content of 190.6 ± 9.8 mg gallic acid/l, whereas for PF, it was 2394.8 ± 71.7 μM of Fe^{2+}/l and a phenolic content of 119.5 ± 7.7 mg gallic acid/l. Thus, results of the reducing power agree with those observed in the antioxidant assays and suggest again that antioxidant activity of this aged rum is mostly influenced by its phenolic fraction.

4. Conclusions

The proposed fractionation method permits separating rum aged polyphenols into various distinct fractions. It would be a useful and suitable tool to further study the wide range of phenolic compounds present in beverages or natural extracts. Although HSCCC is widely used in natural product separations, to the best of our knowledge, it is the first time that it is applied to the separation of polyphenols in distilled and oak aged beverages, which lets to improve their final isolation and identification in the next separation step. Also, this is the first study which uses HPLC–DAD–MS^n for the isolation and identification of phenolic components in rums. Moreover, it is worth underlining the potent multiple radical scavenger activity and the high content of polyphenols in this rum. However, isolation of the minor unknown molecules present in fractions F1–F6 and their structure elucidation by MS and NMR is undoubtedly a challenging task in the future.

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