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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 $\delta_H$ = 7.26 ppm and $\delta_C$ = 77.16 ppm.