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Determination of sterol and fatty alcohols in unsaponifiable matter of *Roystonea regia* fruits oil

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Roystonea regia (Kunth) F. Cook fruit is a good source of oil with possible application in the nutritional and pharmaceutical industries. The chemical constituent of the unsaponifiable matter of this oil, however, has not been studied yet. The aim of this work was to assess the content of sterols and alcohols in this fraction using GC-MS. Samples were submitted to basic hydrolysis with KOH/EtOH solution and then the unsaponifiable fractions extracted with n-hexane, and then alcohols and sterols were analyzed as trimethylsilylether derivatives using cholestane as the internal standard. The average content of the unsaponifiable matter of *R. regia* was 1.3%. The major fraction (66%), was composed of sterols, like ß-sitosterol, stigmasterol, campesterol, 24-methylencycloartanol, Δ5-avenasterol, cycloartanol and cycloartenol, while the fatty alcohol fraction (33.9%) contained $C_{18}OH$-$C_{34}OH$ alcohols.

Key words: *Roystonea regia*, unsaponifiable fraction, GC-MS, sterols, fatty alcohols.

INTRODUCTION

Fruit oils of some plant species, mainly composed of fatty acids (FA), are good sources for nutritional and pharmaceutical purposes (Powel, 2009; USDA, 2009; Bruneton, 2001) and uses that require the proper characterization of their contents. Sterols and fatty alcohols have been reported to be components of the unsaponifiable matter (UM) of fruit oils (Bruneton, 2001). Nevertheless, sterol analysis commonly complement the analysis of these oils, as its pattern (type and relative proportions of sterols) seems to be unique for each oil and allows detect adulterations (Johansson and Croon, 1981; Wretensjo and Kalrberg, 2002), alcohol analysis has been used less frequently (USP 33, 2010).

The isolation and analysis of sterols and fatty alcohols from vegetable oils comprise several techniques like liquid-liquid extraction, ultrasonic extraction, thin layer chromatography, preparative HPLC, GC with flame ionization detector, GC-MS and their combinations (Johansson and Croon, 1981; Wretensjo and Kalrberg, 2002; USP 33, 2010; INA, 2001). The assessment of

such compounds occur in low concentrations, usually as their silyl and acetates derivatives or in free forms by GC-MS (INA, 2001; Reyna et al., 1999; Dutta and Appelqvist, 1996; Jingming et al., 2008), although in this last alternative the absorption onto the chromatographic column might occur (Jingming et al., 2008, Wretensjo and Kalrberg, 2002).

Cuba has a high variety ($\cong$ ninety) species of Arecaceae, where the Cuban royal palm (*Roystonea regia* (Kunth) F. Cook) is the most abundant (Leyva, 2006). *R. regia* fruits, commonly used by farmers to feed hogs, contain seed oil that has been studies and referred to have 0.5% of UM (Stillman and Reed, 1934; Ruebens, 1968). Recently, the oil obtained from the whole-fruit has been employed as starting material in the production of a new active ingredient (D-004) that prevents prostate hyperplasia experimentally induced in rodents (Laguna et al.,2010; Carbajal et al., 2005). Preliminary studies have described general physical and chemical characteristics

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of this oil, such as relative density (0.874 to 0.970 g/ml), refractive index (1.454 to 1.463), saponification (190.0 to 221.8), acid (5.2 to 26.4) and iodine (47.8 to 55.8) indexes. In addition, the total content (74.4 to 91.3%) and individual FA composition of this oil, determined by GC, is as follows: C8:0 and C10:0 (0.1 to 0.9%); C12:0 (17.9 to 27.8%); C14:0 (9.3 to 12.7%); C16:0 (9.3 to 15.0%);C16:1 (0.1 to 0.6%); C18:0 (2.5 to 3.7%); C18:1 (27.3 to 37.3%); C18:2 (12.8 to 17.4%); and C18:3 (0.1 to 0.5%) (Laguna et al., 2010; Rodríguez-Leyes et al., 2007).

Nevertheless, to our knowledge (search up to May 2012) there are no reports of the sterol and fatty alcohols composition of the seed or whole-fruit oil of *R. regia*. The aim of this study, therefore, was to determine the sterol and fatty alcohols in the UM of the whole fruit oil of the Cuban royal palm. Then, this study contributes to the knowledge of the chemical composition minor components of this oil, which could complete its characterization to be used for nutritional and pharmaceutical targets.

MATERIALS AND METHODS

Reagents and dissolutions

β-sitosterol (95%), campesterol (98%), stigmasterol (95%), cholesterol (99%), cholestano (95%), fatty alcohols: $C_{20}OH$-$C_{30}OH$ (98%), and the silylating reagent N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). All solvents, purchased from Merck (Darmstadt, Germany), were of chromatographic or analytical grade.

Dissolutions: Sterols reference solution (SRS): cholesterol (0.03 mg/mL), campesterol and stigmasterol (0.1 mg/mL), and β-sistosterol (0.8 mg/mL), in chloroform. Fatty alcohols reference solution (FARS): $C_{24}OH$ (0.04 mg/mL), $C_{26}OH$ (0.07 mg/mL), $C_{27}OH$ (0.04 mg/mL), $C_{28}OH$ (0.75 mg/mL), and $C_{30}OH$ (0.15 mg/mL) in chloroform. Internal standard solution (ISS): cholestane (5 mg/mL) in chloroform. NaOH aqueous solution (2 mol/L), KOH aqueous solution (2 mol/L) and KOH/ethanol solution (0.5 mol/L). All glassware was silanized with dimethyldichlorosilane in toluene at 5% (Supelco, USA).

Plant material and oil extraction

Ripe fruits of *R. regia* (ca. 1 kg) were collected at the National Botanical Garden of Havana and authenticated by Dr. Angela Leyva (Voucher No. HAJB 84635). *R. regia* fruit oil was obtained in accordance to the procedure described by Rodríguez-Leyes et al., (2007). In brief, the fruits were dried in a well-ventilated area at room temperature for 15 days and ground to a fine powder (10 mesh). Subsequently, 25 grams of powder were extracted in a Soxhlet apparatus for 6 h using n-hexane. Then, the solvent was removed at 60°C under vacuum in a rotary evaporator. Two batches (010409 and 190109) of *R. regia* whole-fruits oil were used for the analyses.

Sample preparation

UM extraction was done by a modification of the procedure of the

Institute for Nutraceutical Advancement (INA, 2001) of USA. Briefly, the oil (5 g), containing 2.0 ml of ISS, was saponified with 50 ml of KOH/ethanol (0.5 mol/L) solution and heated for 1 h. The UM was extracted three times with 60 ml of *n*-hexane in a separator funnel. The combined extract was repeatedly washed with 40 ml of distilled water and with an aqueous KOH solution. The hexanic solution was dried with anhydrous sodium sulfate and filtered through a filter paper. The solvent was removed by evaporation under reduced pressure. The residue was kept at -20°C until the derivatization process; for which 5.0 mg were accurately weighed into a 2 mL vial and 0.14 ml of MSTFA were added, the vial was tightly capped and heated at 80°C for 1 h.

Gas chromatography-mass spectrometry analysis

An Agilent GC 6890N gas chromatograph equipped with a mass selective detector 5975 B inert and a split-splitless injector, in splitless mode, was used (Agilent, Palo Alto, CA, USA). Separations were made on a HP-5Ms fused-silica capillary column (30 m × 0.25 mm), with a film thickness of 0.25 μm D_f (Agilent, Avondale, PA). The injector temperature was 320°C. The temperature program was: 100°C to 200°C at 30°C/min, 200°C to 320°C at 5°C/min and hold 20 min at 320°C. Helium was used as carrier gas at a flow rate of 1 ml/min. Temperatures of interphase, ionization source, and quadrupole were: 300, 230 and 150°C respectively. The ionization energy was 70 eV. The mass spectrum was continuously acquired from 40 to 800 m/z with 3.12 scan /s in full scan mode. The injection volume was 1 μL using the solvent-flush technique.

Compounds were identified by comparing their spectra to those of the Wiley 275 and NIST 11 mass spectra libraries and also by comparison of their relative retention to those of commercial available standards compounds and data from literature (Johansson and Croon, 1981; Wretensjo and Kalrberg, 2002; USP 33, 2010; INA, 2001). Quantification of sterols and alcohols components was performed by the internal standard method (Wretensjo and Kalrberg, 2002; USP 33, 2010; INA, 2001). Mass response factors were determined by means of sterol and fatty alcohols reference solutions with respect to cholestane's ISS. Cholestano, commonly used for quantifying sterols, was chosen as internal standard, whereas TMS derivatives of sterols and fatty alcohols were used for the analyses because they are easy to prepare, thermally stable, have enhanced sensitivity, excellent chromatographic performance, and also provide distinct and selective mass spectra (Wretensjo and Kalrberg, 2002; INA, 2001; Dutta and Appelqvist, 1996; Jingming et al., 2008).

RESULTS AND DISCUSSION

The UM obtained from the two batches (010409 and 190109) of *R. regia* whole-fruits oil were yellow pale solids with characteristic odor. The obtained yields (1.38% and 1.24%, respectively) were higher, as expected, than those reported by Stillman and Reed (1934) and Ruebens (1968) (0.5%) for UM from oil seed, but similar to those of our previous report (Marrero et al., 2011). Figure 1 shows the results of the GC-MS analysis. As can be seen, UM is mainly composed of sterols (66.1%), while fatty alcohols are present in a lower proportion (33.9%), consistent with data of other Arecaceae species (Verleyen et al., 2002; Itoh et al., 1973; Masson et al.,2008), but discrepant from that found in the unsaponifiable fraction of D-004, lipid extract
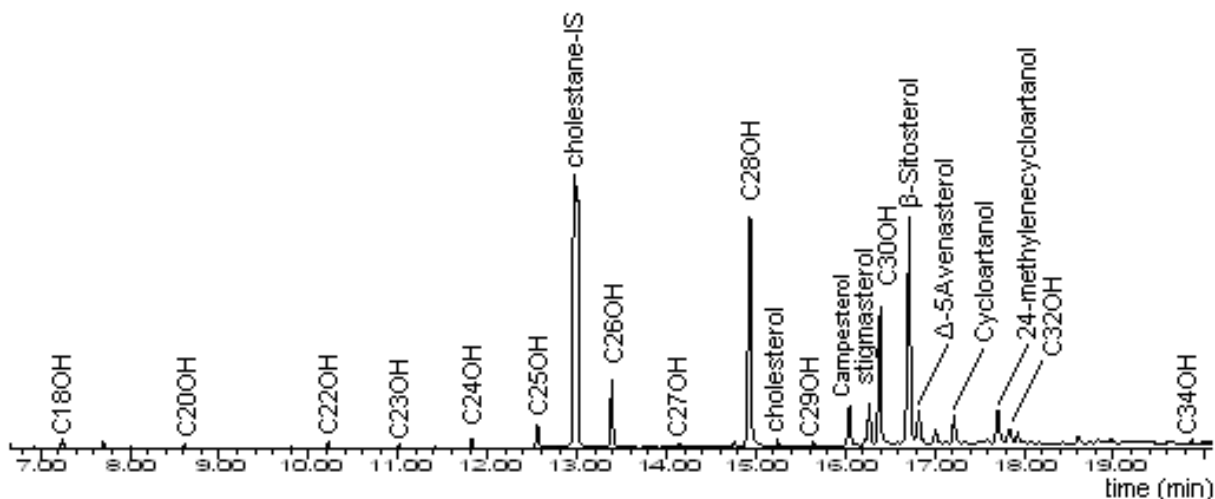
Figure 1. GC-MS partial profile of UM from *Roystonea regia* oil

Table 1. Content of sterols in the unsaponifiable matter of *R. regia* oil (Normalized data).

Esterol	rt (min)	190109	010409	Mean (%)	SD	CV (%)
cholesterol	15.23	0.8	0.5	0.6	0.21	32.5
campesterol	16.05	9.5	8.9	9.2	0.39	4.2
stigmasterol	16.27	9.1	10.2	9.6	0.81	8.4
γ- sitosterol	16.58	0.5	0.5	0.5	0.02	4.4
β-Sitosterol	16.72	50.0	52.4	51.2	1.66	3.3
Δ-5avenasterol	16.82	9.2	8.7	8.9	0.34	3.8
sterol (NI)	17.01	2.0	3.1	2.5	0.78	30.6
cycloartenol	17.13	0.7	0.8	0.7	0.10	13.0
cycloartanol	17.22	8.3	6.7	7.5	1.10	14.7
24-methylene-cycloartanol	17.71	10.2	8.2	9.2	1.45	15.7

NI- Non Identified.

obtained from R. regia fruit after a process of saponification and further n-hexane extraction, in which occurred the opposite (Marrero et al., 2011). This behavior could be attributable to the saponification step during the manufacturing process of D-004. Similar reductions of sterol content in the oil have been previously reported for other oils submitted to refinement process (Kochhar, 1983; Rossell et al., 1983; Wretensjo and Kalrberg, 2002).

Sterols

Sterols which occur in low proportions in vegetable oils, have been widely studied because of their nutritional and pharmaceutical importance (Bruneton, 2001; Johansson and Croon, 1981, USP 33, 2010; INA, 2001).

The analysis of the UM exhibited around 10 compounds with sterol-related structures (Figure 1 and Table 1), β-sitosterol being the predominant, followed by stigmasterol, campesterol, 24-methylenecycloartanol, Δ5-avenasterol and cycloartanol. Also, cholesterol, γ-sitosterol, cycloartenol and another component with a steroid nucleus in its structure (sterol NI) were detected in minor proportions. The sterol composition found in R. regia oil agrees with those of some Arecaceae, where β-sitosterol (50 to 71%), stigmasterol (6 to 14%), campesterol (8 to 23%) and Δ5-avenasterol (0 to 15%) were the major components (Verleyen et al., 2002; Itoh et al., 1973; Masson et al., 2008; Rossell et al., 1983). The main sterols (β-sitosterol, stigmasterol, and campesterol) were easily identified by their spectral similarity to those of the libraries and of their commercial standards, as well as by their relative retention indexes.

Table 2. Content of alcohols in the unsaponifiable matter of *R. regia* oil (Normalized data).

Alcohol	Rt (min)	Oil batch		Mean (%)	SD	CV (%)
		190109	010409			
C18OH	7.25	0.16	0.12	0.14	0.025	18.5
C20OH	8.66	0.09	0.13	0.11	0.027	23.9
C22OH	10.21	0.35	0.33	0.34	0.014	4.2
C23OH	11.01	0.10	0.06	0.08	0.028	36.5
C24OH	11.82	2.53	1.86	2.20	0.478	21.8
C25OH	12.62	0.71	0.38	0.54	0.235	43.4
C26OH	13.39	15.13	12.73	13.93	1.702	12.2
C27OH	14.14	1.67	0.99	1.33	0.480	36.1
C28OH	14.94	54.01	52.92	53.47	0.772	1.4
C29OH	15.65	0.84	0.74	0.79	0.071	9.0
C30OH	16.39	23.04	27.46	25.25	3.124	12.4
C32OH	17.92	1.10	1.93	1.52	0.589	38.8
C34OH	19.92	0.27	0.36	0.31	0.068	21.7

Most identified sterols were Δ5-sterols, which contain a double bound at the position 5 in the steroid skeleton. Also, they contain 28 or 29 carbon atoms, typical of phytosterols. UM was mainly composed by the monounsaturated Δ5-sterols, campesterol and sitosterol and the diunsaturated sterols, stigmasterol and Δ5–avenasterol, common in vegetable oils. Cholesterol was present in lower amounts, which agrees with data of fruit oils of other members of Arecaceae family (0 to 8%) (Itoh et al., 1973; Rossell et al., 1983). One minor unidentified peak between Δ5–avenasterol and cycloartenol, constituting 3% of the fraction, was recorded as "Non Identified". A better structure confirmation of such compound should be complemented combining GC-MS with the nuclear magnetic resonance technique. On the other hand, saturated sterols (stanols) detected in very low concentrations in Cocos nucifera and Elaeis guineensis oils (Dutta and Appelqvist, 1996), were not found in R. regia oil.

As an example of the identification of silylated Δ 5-sterols, we shall discuss the mass spectra of campesterol and β-sitosterol. The most prominent ions in the mass spectra were obtained by loss of trimethylsilyl hydroxide $[(CH_3)_3\text{-Si-OH}]$. This gave ions at M-90, corresponding to the m/z values of 382 and 396 for campesterol and β-sitosterol respectively. Other abundant ions, at m/z 357 for campesterol and m/z 381 for β-sitosterol, were derived from the combined loss of TMSOH and a methyl group (M-90-15). Furthermore, intense peaks at m/z 129 and M-129, typical of sylilated Δ5-sterols, were found. For campesterol, M-129 corresponds to an ion at m/z 343, and for β-sitosterol corresponds to an ion at m/z 357. The spectrum of β-sitosterol showed a molecular ion [M]+ at m/z 486 and the [M-CH₃]+ at m/z 471, due to the loss of the methyl group from the TMS derivative. Similarly, the molecular ion [M]+ of campesterol appeared at m/z 472,

while its [M-CH₃]+ ion was found at m/z 457 (Wretensjo and Kalrberg, 2002; Jingming et al., 2008).

Alcohols

Long chain fatty alcohols, ranging between 18 and 34 carbon atoms, were found in the UM of R. regia oil, but at concentrations lower than those of sterols (Figure1 and Table 2). Thus, the even carbon atoms alcohols dominated the chromatographic profile, where the major compound was the $C_{28}OH$, followed by $C_{30}OH$ and $C_{26}OH$, similar to the major alcohols present in the lipid-sterolic extract of Stylidium repens (USP 33, 2010).

The minor alcohols found in the whole-fruit oil of R. regia were $C_{18}OH$, $C_{20}OH$, $C_{22}OH$, $C_{23}OH$, $C_{24}OH$, $C_{25}OH$, $C_{27}OH$, $C_{29}OH$, $C_{32}OH$ and $C_{34}OH$, some of which ($C_{18}OH$, $C_{23}OH$, $C_{25}OH$ and $C_{29}OH$), which represents the first report. To our knowledge, these compounds in fruit oils extracted from Arecaceae species, probably because the content of fatty alcohols in these oils has received less attention than the identification and quantification of sterols. The identification of these very scarce alcohols was possible in part thanks to the ion extraction technique, where the m/z 103 $[CH_2=O\text{-Si}(CH_3)_3]$ fragment ion, which is specific and characteristic of TMS-ether of alcohols, was used as target ion. The $C_{28}OH$, as well as other alcohols, were identified by its fragment ion M+ -15 (at m/z: 467) and other characteristic fragment ions of TMS derivatives of primary alcohols such as m/z: 75 $(HO\text{-Si}(CH_3)^{2+})$ and 103, as previously mentioned. The M+-15 of the rest of detected alcohols were at m/z: 327 ($C_{18}OH$), 355 ($C_{20}OH$), 383 ($C_{22}OH$), 397 ($C_{23}OH$), 411 ($C_{24}OH$), 425 ($C_{25}OH$), 439 ($C_{26}OH$), 453 ($C_{27}OH$), 481 ($C_{29}OH$), 495 ($C_{30}OH$), 523 ($C_{32}OH$) and 551 ($C_{34}OH$).

Conclusion

The unsaponifiable fraction obtained from the oil of R. regia whole fruit was characterized to our knowledge, for the first time. With the use of a GC-MS method we found that this fraction was mainly composed of sterols and fatty alcohols. Among sterols, the β-sitosterol, stigmasterol, campesterol, 24-methylene-cycloartanol, Δ5-avenasterol and cycloartenol were the predominant; while $C_{28}OH$, $C_{30}OH$ and $C_{26}OH$ were majority in fatty alcohol fraction. These results represent a contribution to the chemical characterization of this oil, and could help for future research on this matter.

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