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Characterization by high-performance liquid chromatography with diode-array detection coupled to time-of-flight mass spectrometry of the phenolic fraction in a cranberry syrup used to prevent urinary tract diseases, together with a study of its antibacterial activity

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ABSTRACT

The phenolic fraction of a commercial cranberry syrup, which is purported to have good properties for the prevention of urinary diseases, has been thoroughly characterized using HPLC-DAD–TOF-MS. A study of its antibacterial activity has also been carried out. For this purpose a new HPLC-DAD–TOF-MS method using negative and positive ionization modes was developed and it was thus possible to identify 34 different compounds, nine of which have been tentatively characterized for the first time in cranberry syrup. It is also important to highlight that different coumarins in this matrix were also determined, which, to our knowledge, have not been found previously in the cranberry. The phenolic fraction obtained by HPLC-DAD was found to be 5.47 mg/mL. Catechin and procyanidins belonging to flavanols were the family of compounds found at the highest concentrations (2.37 mg/mL); flavonols were at a concentration of 1.91 mg/mL and phenolic-acid derivatives were found at the lowest concentration (0.15 mg/mL). With regard to antibacterial activity, the incubation of *Escherichia coli* with cranberry syrup was found to reduce surface hydrophobicity as a function of the concentration of the extract.

1. Introduction

In recent years there has been ever increasing interest in the presence of certain compounds in foods that are beneficial to human health. In plant-derived foods these naturally occurring compounds form part of the secondary metabolism of many kinds of fruit and vegetable products and are known as phytochemicals. The antioxidant capacity of phytochemicals, as well as their health-promoting and/or disease-preventing properties, are currently the subject of intense study by the scientific community.

Berries, including raspberries, blueberries, black currants, red currants, and cranberries, are a rich source of these dietary antioxidants [1]. The American cranberry (*Vaccinium macrocarpon*) in particular is a rich source of bioactive compounds with antiproliferative, antioxidant [2], anti-inflammatory and antimicrobial properties, which inhibit the growth of pathogenic bacteria such as *Escherichia coli* and *Helicobacter pylori* for example [3,4]. It has traditionally been used in the treatment and prevention of urinary-tract infections in women and also in digestive-tract ailments. The antitumoral properties of cranberries have made them a popular diet component with an eye to the prevention of neoplastic diseases [5]. The phenolic compounds found in cranberries are believed to be the principal ingredients responsible for these beneficial effects. Cranberries are known for their high concentration of anthocyanins, as well as their significant contents of flavonols, flavan-3-ols, tannins (ellagitannins and proanthocyanidins) and phenolic-acid derivatives [6].

One out of two women experience some sort of urinary tract disorder during their lifetime, which often reoccurs. As has been observed, the consumption of cranberries has a significant influence on lowering the incidence of urinary diseases [7,8]. Nowadays therefore, some dietary supplements containing cranberry extracts are being developed.

The aim of this work was to characterize the phenolic fraction contained in cranberry syrup, made up of glucose, sodium benzoate, potassium sorbate and American cranberry (*V. macrocarpon*),

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using HPLC-DAD–TOF-MS, and also to study its antibacterial activity. This study is a preliminary step in our thorough research into the composition of cranberry syrup. The syrup will be then be used for *in vivo* analyses to study the metabolites of these phenolic compounds in urine and evaluate the incidence of urinary disorders in its consumers.

2. Materials and methods

2.1. Materials

Standards of myricetin, *p*-coumaric acid, 7-hydroxycoumarin, and proanthocyanidin A2 were from Extrasynthese (Genay, France). Folin-Ciocalteu phenol reagent was from Fluka, Sigma–Aldrich (Steinheim, Germany). Formic acid and acetonitrile used for preparing mobile phases were from Fluka, Sigma–Aldrich (Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland) respectively. Distilled water with a resistance of 18.2 MΩ was deionized in a Milli-Q system (Millipore, Bedford, MA, USA). Solvents were filtered before use with a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA).

2.2. Sample preparation

200 μL of cranberry syrup, bought in a local pharmacy, was dissolved in 4 mL methanol, vortexed for 2 min in a G560E Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA), filtered with a polytetrafluoroethylene (PTFE) syringe filter (0.2 μm pore size) and injected directly into the HPLC system.

2.3. Measurement of total polyphenols, proanthocyanidins and anthocyanins

To quantify the total phenolic content in cranberry syrup, the Folin-Ciocalteu method was used [9]. The proanthocyanidin (condensed tannins) and total anthocyanin contents were determined according to the vanillin–HCl method [10] and the methods described by Fuleki and Francis respectively [11].

2.4. Chromatographic separation

HPLC analyses were made with an Agilent 1200 series rapid-resolution LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler and a diode-array detector (DAD). Separation was carried out with a Zorbax Eclipse Plus C_{18} analytical column (150 mm × 4.6 mm, 1.8 μm particle size). Gradient elution was conducted using two different programs. Gradient program 1 was used for the MS negative ionization mode consisting of 1% formic acid in water–acetonitrile (90:10, v/v) (phase A) and acetonitrile (phase B) at a constant flow rate of 0.5 mL/min using the following gradient: 0–20 min, linear gradient from 5% B to 20% B; 20–25 min, linear gradient from 20% B to 40% B; 25–30 min, linear gradient from 40% B to 5% B; and 30–35 min, isocratic of 5% B. Subsequently a different chromatographic method (gradient program 2) was used for the MS positive ionization mode. Due to their acid–base equilibrium, anthocyanins need a more acidic pH to be resolved and so the gradient was modified as follows: water–formic acid (90:10, v/v) (phase A) and acetonitrile (phase B) at a constant flow rate of 0.5 mL/min using the following gradient: 0–13 min, linear gradient from 0% B to 20% B; 13–20 min, linear gradient from 20% B to 30% B; 20–25 min, linear gradient from 30% B to 80% B; 25–30 min, linear gradient from 80% B to 0% B; and 30–35 min, isocratic of 0% B. The addition of formic acid gave better results for the ionization of the compounds in positive mode. The injection volume was 10 μL for both gradient elution programs. The two different methods were chosen as they both afforded short analysis times and good chromatographic separations. UV data were collected using DAD set at 280, 320, 360, and 520 nm.

2.5. ESI-TOF-MS conditions

TOF-MS was conducted using a microTOF™ (Bruker Daltonics, Bremen, Germany) orthogonal-accelerated TOF mass spectrometer equipped with an electrospray ionization (ESI) interface. The parameters for analysis were set using both negative and positive ion modes with spectra acquired over a mass range of 50–1000 *m*/*z*. The other optimum values of the ESI-MS parameters were: capillary voltage, 4500 V; dry gas temperature, 190 °C; dry gas flow, 9.0 L/min; nebulizer pressure, 2.0 bar; and spectra rate 1 Hz. The flow delivered into the MS detector from HPLC was split using a flow splitter (1:2) to achieve stable electrospray ionization and obtain reproducible results. The calibrant was a sodium-formate cluster containing 5 mM sodium hydroxide and 0.2% formic acid in water–isopropanol (1:1, v/v), injected at the beginning of each run with a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the interface. All the spectra were calibrated prior to compound identification. All operations were controlled by DataAnalysis 3.4 software (Bruker Daltonik), which provided a list of possible elemental formulas by using the GenerateMolecularFormula™ Editor.

2.6. Assessment of the method

Quantification was made according to the linear calibration curves of standard compounds. Four calibration curves were prepared using the following standards: myricetin, *p*-coumaric acid, 7-hydroxycoumarin and procyanidin A2. The different parameters of each standard compound are summarized in Table 1. All calibration curves show good linearity between different concentrations depending upon the analytes in question. The calibration plots reveal good correlation between peak areas and analyte concentrations, and the regression coefficients were always higher than 0.995. LOD was found to be within the range 0.053–0.233 μg/mL whilst LOQ was within 0.175–0.679 μg/mL.

Intraday and interday precisions were developed to assess the repeatability of the method. A syrup extract was injected ($n = 6$) during the same day (intraday precision) for 3 consecutive days (interday precision, $n = 18$). The relative standard deviations (RSDs) of analysis time and peak area were determined. The intraday repeatability of the peak area, expressed by the RSD, was 1.2%, whereas interday repeatability was 3.8%.

The accuracy of the assay can be determined by the closeness of the test value to the nominal value and was evaluated with

Table 1
Analytical parameters of the method.

Analyte	RSD	LOD (μg/mL)	LOQ (μg/mL)	Calibration range (μg/mL)	Calibration equations	r^2	Accuracy
Myricetin	0.23	0.053	0.175	LOQ-25	$y = 22.852x + 21.117$	0.996	98.7
p-Coumaric acid	0.31	0.204	0.679	LOQ-250	$y = 127.13x + 7.2384$	0.999	99.2
Procyanidin A2	0.36	0.152	0.287	LOQ-100	$y = 5.8648x + 3.8544$	0.997	101.3
7-Hydroxycoumarin	0.27	0.233	0.656	LOQ-50	$y = 37.724x + 12.555$	0.998	100.8

separately prepared individual primary stock solutions, mixtures and working solutions of all standards. It was calculated over the linear dynamic range at three concentration levels: low (LOQ), medium (intermediate concentration value of the linear calibration range), high (highest concentration value of the linear calibration range) via three assays per concentration on different days. The analyte concentrations were calculated from calibration curves and accuracy was calculated by the ratio of this calculated concentration versus the theoretical (spiked) one.

2.7. Bacteria and cultures

Nine strains of uropathogenic *E. coli* (695, 787, 471, 472, 593, 595, 760, 629 and 607) were obtained from patients with acute pyelonephritis, together with 4 strains of *E. coli* from the Spanish Type Culture Collection (CECT): CECT 424 (F- thr- leu- lacY mtl-thi- ara gal ton 2 malA xyl, resistant to phages T1, T2 and T6.); CECT 4076 (Serovar. O157:H7, originally isolated from haemorrhagic colitis); CECT 417 (SupE44[am]. mutant tRNA); and CECT 743 (Serovar. O142 K86B:H6, isolated from children with diarrhoea). To enhance the activity of the Type 1 fimbriae [12] the strains were grown in TSB culture medium at 37 °C for 48 h and then centrifuged at 2000 × *g* for 10 min. The supernatant was then discarded, and the strains resuspended in PBS (pH 7.4). This washing process was performed twice. Finally, the bacterial suspension was adjusted to 10^9 bacteria/mL (OD of 1.0–542 nm). To enhance the activity of the P type fimbriae, the strains were incubated for 16 h on CFA agar [13], extracted from the surface of the agar after washing with 5 mL PBS, and then centrifuged at 2000 × *g* for 10 min.

2.8. Ammonium sulphate aggregation test

The technique used was that described by Lindahl et al. [14]. Briefly, solutions of ammonium sulphate were prepared, with osmolarities ranging from 0.2 M to 4 M, using sodium phosphate as dilutant. Taking 20 μL of bacterial suspension, an equal volume of ammonium sulphate solution was added and then gently mixed. The presence of aggregation was observed after 30 s gentle manual rotation at room temperature over a glass slide, and the lowest concentration of ammonium sulphate that produced visible aggregation was noted. Aggregation with the 4 M solution was interpreted as a hydrophobicity of 0%, whilst aggregation with the 0.2 M solution was interpreted as 95% hydrophobicity.

3. Results and discussion

3.1. Chromatographic profile and compound identification

The base-peak chromatograms (BPC) of a cranberry syrup, obtained using both negative and positive ionization modes, are set out in Fig. 1(a) and (b). The tentatively identified phenolic compounds are summarized in Tables 2 and 3 (negative and positive ionization modes respectively), including retention times, experimental and calculated *m*/*z*, molecular formula, error, sigma values (comparison of theoretical with measured isotope patterns), together with their proposed identities.

Phenolic compounds were successfully separated and identified with a gradient optimized for negative and positive ionization modes. The compounds were identified by interpreting their mass spectra obtained via TOF-MS, taking into account all the data reported in the literature. All these facts were also complemented with the UV spectra provided by DAD, which gave additional information about the family of compounds as far as the absorbance bands are concerned.

3.2. Compounds identified in negative ionization mode

Twenty-seven phenolic compounds were identified in negative ionization mode, including 6 new proposed compounds reported for the first time in the American cranberry (*V. macrocarpon*). Table 2 and Fig 1(a) show the base-peak chromatogram (BPC) in negative mode of an extract of cranberry syrup.

3.2.1. Phenolic-acid derivatives

The first group of peaks migrated between 4.85 and 8.92 min and the compounds were related to the phenolic-acid family. Peak 1 (RT 4.85 min) gave a molecular mass of *m*/*z* 325.0929, which was tentatively identified as coumaroyl-hexose according to the molecular formula provided for its mass and corroborated by its fragment ion at *m*/*z* 163.0406, corresponding to a loss of the sugar moeity (162 Da) [6]. Peak 2 (RT 6.19 min), which showed an ion at *m*/*z* 385.1127, gave a fragment at *m*/*z* 223.0596, corresponding to a loss of sugar moiety; thus, it was identified as sinapoyl-hexose [15]. The ion at RT 6.44 min corresponds to caffeoyl glucose with the precursor and fragment ions at *m*/*z* 341.0888 and 179.0351 respectively, indicating the loss of a sugar moeity. Peak 4 (RT 6.89 min) was assigned to chlorogenic acid [16], showing a fragment at *m*/*z* 191.0558, corresponding to the quinic-acid moiety previously reported. The presence of another isomeric form of coumaroyl-hexose was tentatively identified in peak 5 (RT 7.16 min). Peak 7 (RT 8.92 min) was tentatively identified as canthoside A [17], this apparently being the first time that this compound has been found in the cranberry.

3.2.2. Flavonoids

The HPLC-DAD–TOF-MS analysis of the cranberry syrup extract revealed a total of 20 flavonoids (summarized in Table 2). For most flavonoids, the negative ionization mode provided the highest sensitivity and selectivity [18]. The following flavonols already found in cranberry were confirmed in our sample: myricetin 3-*O*-hexose (peak 9) [19,20], myricetin 3-*O*-arabinoside (peak 14) [21], quercetin 3-*O*-hexose (peak 16) [19], quercetin 3-*O*-xylopyranoside (peak 18) [19], quercetin 3-*O*-arabinopyranoside (peak 19) [19,21], quercetin 3-*O*-arabinofuranoside (peak 20) [19,21], quercetin 3-*O*-rhamnoside (peak 21) [20,21], myricetin (peak 22) [21], methoxyquercetin 3-*O*-galactoside (peak 23) [19] and quercetin (peak 27) [16,21]. As can be see in Table 2, quercetin and derivates with sugar bonds gave the fragment ion at *m*/*z* 301, corresponding to the loss of a sugar moiety.

Three different isomers of A-type procyanidin were identified in cranberry syrup at times 13.35, 14.28, 16.94 min with a *m*/*z* of 575.12 (peaks 11, 13 and 17), showing a typical fragment at *m*/*z* 423. Two B-type procyanidin isomers with a *m*/*z* of 577.13 eluted at 8.00 and 14.15 min (peaks 6 and 12) and their fragments at 425 and 289 were also detected [22]. Peak 8, with a *m*/*z* of 289.0714 and a retention time of 9.65 min, was identified as (+)-catechin.

Dihydroferulic acid 4-*O*-*β*-D-glucuronide (peak 10), cavinin glucoside (peak 15), biochanin A-7-*O*-glucoside (peak 24), prodelphinidin B4 (peak 25) and kaempferol 3-*O*-*β*-D-(6″-*p*-hydroxybenzoyl)-galactopyranoside (peak 26) were identified using mass spectra, UV spectra and the information provided by the GenerateMolecularFormula™ Editor. As far as we know, this is the first time that these compounds have been reported in the cranberry.

Dihydroferulic acid 4-*O*-*β*-D-glucuronide at *m*/*z* 371.0989 presented a fragment at a *m*/*z* of 175.0283, which corresponds to the glucuronide moiety after the fragmentation of the dihydroferulic acid. Cavinium glucoside at *m*/*z* 535.1482 showed a fragment at *m*/*z* 373.0907, indicating the loss of the sugar moiety. Fig. 2 shows the structures of the newly identified compounds in cranberry syrup. Additional unidentified compounds have been included in Table 2

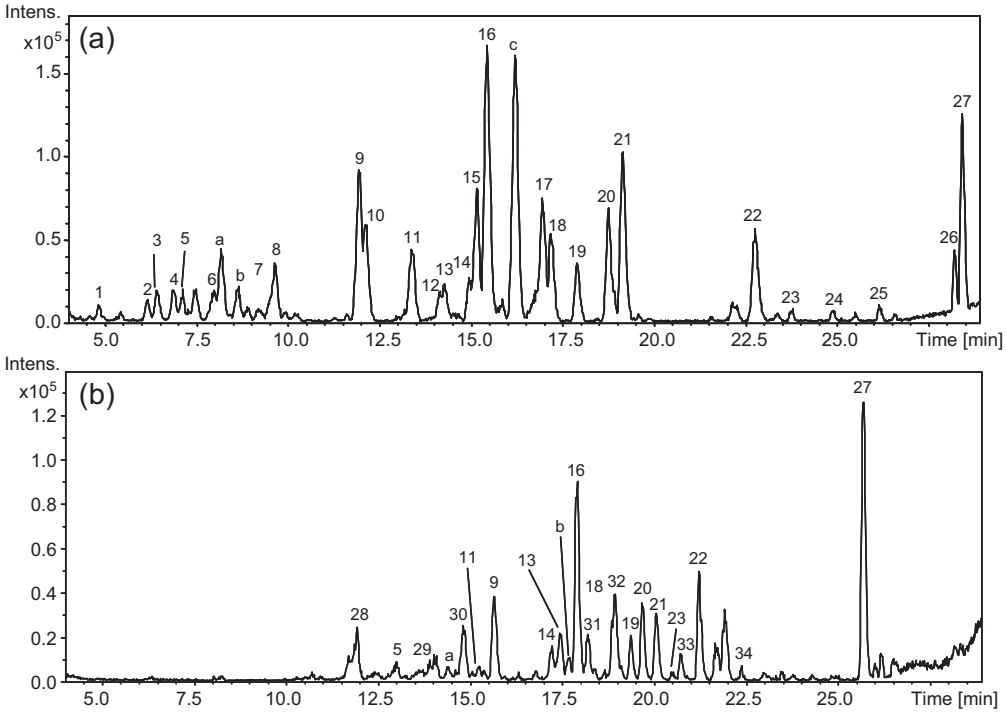

Fig. 1. Chromatographic profiles using the gradient programs: (a) base peak chromatogram (BPC) for the gradient program 1, negative ionization mode; (b) base peak chromatogram (BPC) for the gradient program 2, positive ionization mode.

Table 2
Phenolic compounds in cranberry syrup characterized by HPLC-DAD–TOF-MS in negative ionization mode.

Peak number	Class/phenolic compounds	RT (min)	Selected ion	*m/z* experimental	*m/z* calculated	Fragments	Error (ppm)	Sigma	Molecular formula
	Phenolic acid derivatives								
1	Coumaroyl-hexose	4.85	$[M-H]^-$	325.0929	325.0929	163.0406	0.0	0.0213	$C_{15}H_{18}O_8$
2	Sinapoyl-hexose	6.19	$[M-H]^-$	385.1127	385.1140	223.0596	3.3	0.0176	$C_{17}H_{22}O_{10}$
3	Caffeoyl glucose	6.44	$[M-H]^-$	341.0888	341.0878	179.0351	2.9	0.0107	$C_{15}H_{18}O_9$
4	Chlorogenic acid	6.89	$[M-H]^-$	353.0867	353.0878	191.0558	3.0	0.0561	$C_{16}H_{18}O_9$
5	Coumaroyl-hexose	7.16	$[M-H]^-$	325.0918	325.0929	163.0398	3.4	0.0298	$C_{15}H_{18}O_8$
7	Canthoside A	8.92	$[M-H]^-$	445.1355	445.1351		0.8	0.0238	$C_{19}H_{26}O_{12}$
	Flavonols								
9	Myricetin 3-*O*-hexose	11.94	$[M-H]^-$	479.0835	479.0831	317.0301	0.8	0.0204	$C_{21}H_{20}O_{13}$
10	Dihydroferulic acid 4-*O*-β-D-glucuronide	12.16	$[M-H]^-$	371.0989	371.0984	175.0283	1.5	0.0131	$C_{16}H_{20}O_{10}$
14	Myricetin 3-*O*-arabinoside	14.94	$[M-H]^-$	449.0739	449.0725	317.0286	2.9	0.0217	$C_{20}H_{18}O_{12}$
15	Caviunin glucoside	15.15	$[M-H]^-$	535.1482	535.1457	373.0907	4.7	0.0109	$C_{25}H_{28}O_{13}$
16	Quercetin 3-*O*-hexose	15.44	$[M-H]^-$	463.0894	463.0882	301.0293	2.5	0.0107	$C_{21}H_{20}O_{12}$
18	Quercetin 3-*O*-xylopyranoside	17.14	$[M-H]^-$	433.0784	433.0776	301.0350	1.9	0.0113	$C_{20}H_{18}O_{11}$
19	Quercetin 3-*O*-arabinopyranoside	17.88	$[M-H]^-$	433.0797	433.0776	301.0302	4.8	0.0238	$C_{20}H_{18}O_{11}$
20	Quercetin 3-*O*-arabinofuranoside	18.75	$[M-H]^-$	433.0781	433.0776	301.0332	1.0	0.0132	$C_{20}H_{18}O_{11}$
21	Quercetin 3-*O*-rhamnoside	19.13	$[M-H]^-$	447.0937	447.0933	301.0325	0.9	0.0021	$C_{21}H_{20}O_{11}$
22	Myricetin	22.73	$[M-H]^-$	317.0292	317.0303		3.6	0.0178	$C_{15}H_{10}O_8$
23	Methoxyquercetin 3-*O*-galactoside	23.75	$[M-H]^-$	477.1033	477.1038		1.2	0.0271	$C_{22}H_{22}O_{12}$
26	Kaempferol 3-*O*-β-D-(6″-*p*-*h*ydroxybenzoyl)-galactopyranoside	28.15	$[M-H]^-$	567.1143	567.1144		0.2	0.0104	$C_{28}H_{24}O_{13}$
27	Quercetin	28.36	$[M-H]^-$	301.0337	301.0354		5.5	0.0196	$C_{15}H_{10}O_7$
	Flavanols								
6	Procyanidin B type isomer 1	8.00	$[M-H]^-$	577.1328	577.1351	289.0687	4.1	0.0405	$C_{30}H_{26}O_{12}$
8	(+)-Catechin	9.65	$[M-H]^-$	289.0714	289.0718		1.3	0.0033	$C_{15}H_{14}O_6$
11	Proacyanidin A2 type isomer 1	13.35	$[M-H]^-$	575.1211	575.1195	423.0631	3.0	0.0156	$C_{30}H_{24}O_{12}$
12	Procyanidin B type isomer 2	14.15	$[M-H]^-$	577.1306	577.1351	425.0761	5.5	0.0263	$C_{30}H_{26}O_{12}$
13	Proacyanidin A2 type isomer 2	14.28	$[M-H]^-$	575.1216	575.1195	423.0631	3.7	0.0476	$C_{30}H_{24}O_{12}$
17	Procyanidin A2 type isomer 3	16.94	$[M-H]^-$	575.1218	575.1195	423.0733	4.0	0.0290	$C_{30}H_{24}O_{12}$
25	Prodelphinidin B4	26.11	$[M-H]^-$	609.1238	609.1250		1.9	0.0355	$C_{30}H_{26}O_{14}$
	Isoflavonoids								
24	Biochanin A-7-*O*-glucoside	24.82	$[M-H]^-$	445.1156	445.1140		3.5	0.0284	$C_{22}H_{22}O_{10}$
	Unknown compounds								
a		8.20	$[M-H]^-$	431.1554	431.1559		1.1	0.0138	$C_{19}H_{28}O_{11}$
b		8.67	$[M-H]^-$	431.1923	431.1923		0.1	0.0247	$C_{20}H_{32}O_{10}$
c		16.19	$[M-H]^-$	537.1641	537.1614		5.2	0.0109	$C_{25}H_{30}O_{13}$

Table 3
Phenolic compounds in cranberry syrup characterized by HPLC-DAD–TOF-MS in positive ionization mode.

Peak number	Class/phenolic compounds	RT (min)	Selected ion	*m/z* experimental	*m/z* calculated	Fragments	Error (ppm)	Sigma	Molecular formula
	Phenolic acid derivatives								
5	Coumaroyl-hexose	13.01	$[M+K]^+$	365.0575	365.0633	203.0028	15	0.0225	$C_{15}H_{18}O_8$
29	2-Hydroxybenzoic acid	13.90	$[M+H]^+$	139.0401	139.0390		7.8	0.0113	$C_7H_6O_3$
30	Digallic acid	14.82	$[M+H]^+$	323.0438	323.0398	140.9858	12.4	0.0131	$C_{14}H_{10}O_9$
	Flavonols								
9	Myricetin 3-*O*-hexose	15.66	$[M+H]^+$	481.0973	481.0977	319.0446	0.9	0.0293	$C_{21}H_{20}O_{13}$
14	Mryicetin 3-*O*-arabinoside	17.19	$[M+H]^+$	451.0872	451.0871	319.0439	0.2	0.0352	$C_{20}H_{18}O_{12}$
16	Quercetin 3-*O*-hexose	17.91	$[M+H]^+$	465.1023	465.1028	303.0491	1.0	0.0294	$C_{21}H_{20}O_{12}$
18	Quercetin 3-*O*-xylopiranoside	18.85	$[M+H]^+$	435.0933	435.0922	303. 0485	2.5	0.0351	$C_{20}H_{18}O_{11}$
19	Quercetin 3-*O*-arabinopyranoside	19.33	$[M+H]^+$	435.0936	435.0922	303.0509	3.4	0.0440	$C_{21}H_{18}O_{11}$
20	Quercetin 3-*O*-arabinofuranoside	19.67	$[M+H]^+$	435.0934	435.0922	303.0494	2.7	0.0355	$C_{20}H_{18}O_{11}$
21	Quercetin 3-*O*-rhamnoside	20.04	$[M+H]^+$	449.1063	449.1078	303.0495	3.4	0.0340	$C_{21}H_{20}O_{11}$
23	Methoxyquercetin 3-*O*-galactoside	20.45	$[M+H]^+$	479.1191	479.1184	317.0663	2.1	0.0773	$C_{22}H_{22}O_{12}$
33	Syringetin (3′,5′-*O*-Dimethylmyricetin)	20.69	$[M+H]^+$	347.0765	347.0761		1.1	0.0214	$C_{17}H_{14}O_8$
22	Myricetin	21.19	$[M+H]^+$	319.0445	319.0448		1.2	0.0123	$C_{15}H_{10}O_8$
27	Quercetin	25.66	$[M+H]^+$	303.0500	303.0499		0.2	0.0078	$C_{15}H_{10}O_7$
	Flavanols								
11	Procyanidin A2-type isomer 1	15.20	$[M+H]^+$	577.1342	577.1341	425.0875/287.0542	0.2	0.0713	$C_{30}H_{24}O_{12}$
13	Procyanidin A2-type isomer 2	17.41	$[M+H]^+$	577.1336	577.1341	425.0858/287.0532	0.9	0.0127	$C_{30}H_{24}O_{12}$
	Coumarins								
28	7-Hydroxycoumarin	11.94	$[M+H]^+$	163.0395	163.0390		3.1	0.0168	$C_9H_6O_3$
31	Coumarin	18.21	$[M+H]^+$	147.0447	147.0441		4.6	0.0058	$C_9H_6O_2$
32	Scopoletin	18.90	$[M+H]^+$	193.0415	193.0417	177.0538/147.0446	0.4	0.0271	$C_{10}H_8O_4$
	Anthocyanin								
34	Petunidin	22.34	$[M+H]^+$	317.0629	317.0656		8.5	0.0252	$C_{16}H_{14}O_7$
	Unknown compounds								
a		14.37	$[M+H]^+$	441.1104	441.1086		4.1	0.0258	$C_{30}H_{16}O_4$
b		17.68	$[M+H]^+$	397.1375	397.1341		8.7	0.0248	$C_{15}H_{24}O_{12}$

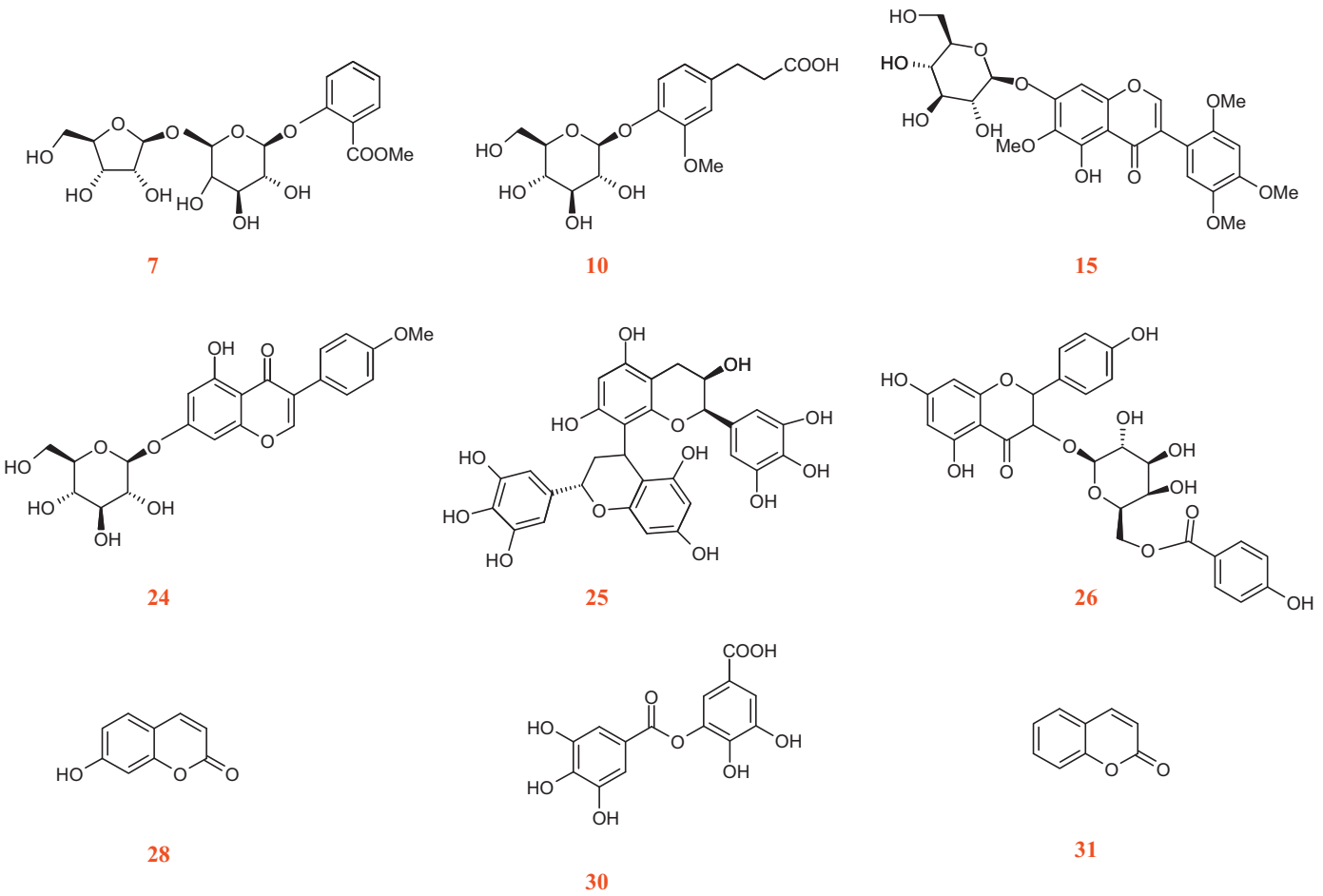

Fig. 2. Structures of new compounds identified in cranberry syrup, (7) canthoside A, (10) dihydroferulic acid 4-*O*-*β*-D-glucuronide, (15) caviunin glucoside, (24) biochanin A-7-*O*-glucoside, (25) prodelphinidin B4, (26) kaempferol 3-*O*-*β*-D-(6″-*p*-hydroxybenzoyl)-galactopyranoside, (28) 7-hydroxycoumarin, (30) digallic acid, and (31) coumarin.

as they form an important part of the polar fraction of cranberry syrup.

3.3. Compounds identified in positive ionization mode

The presence of anthocyanins in cranberries has been reported in the literature, and since anthocyanins have maximum sensitivity in positive mode due to their inherent positive charge [18] the extract was also characterized in positive ionization mode. Fig. 1(b) shows the BPC of an extract of cranberry syrup in positive ionization mode. Thus, by using the proposed method 20 phenolic compounds were identified in positive ionization mode in the cranberry-syrup extract. Despite the fact that several anthocyanidins have been previously described in the cranberry, only petunidin (peak 34) [23] has so far been identified in the syrup.

In addition, several phenolic acids, flavonols, flavanols and hydroxycoumarins were identified in positive ionization mode. It is important to note that some of them were also found in the analysis carried out in negative ionization mode, but most of them could only be identified thanks to this positive ionization mode.

Thus, the phenolic acids identified were: coumaroyl-hexose (peak 5), which had also been identified in the negative mode; 2-hydroxybenzoic acid (peak 29) [24], which presented a *m*/*z* of 139.0401 and digallic acid (peak 30) at *m*/*z* 323.0438, which showed a fragment at *m*/*z* 141, corresponding to the loss of a gallate moiety, thus corroborating its identification [25].

Several flavanols that had already been identified in the negative ionization mode were also detected and identified: myricetin 3-*O*-hexose (peak 9), myricetin 3-*O*-arabinoside (peak 14), quercetin 3-*O*-hexose (peak 16), quercetin 3-*O*-xylopiranoside (peak 18), quercetin 3-*O*-arabinopiranoside (peak 19), quercetin 3-*O*-arabinofuranoside (peak 20), quercetin 3-*O*-rhamnoside (peak 21), methoxyquercetin 3-*O*-galactoside (peak 23); myricetin (peak 22) and quercetin (peak 27). Furthermore, syringetin (peak 33), a flavonoid that could not be detected in negative ionization mode, was also identified. All the quercetin and myricetin-sugar conjugates showed a fragment ion at *m*/*z* 303 and 319 respectively, corresponding to the aglycone of quercetin and myricetin. Moreover, two isomers of Type A procyanidin (peaks 11 and 13 respectively) were also detected in positive ionization mode.

Finally, several coumarins were tentatively identified only in positive ionization mode. Peak 28 presented a *m*/*z* of 163.0395 and was assigned to 7-hydroxycoumarin, as reported in the bibliography [26]. Peak 31, with a *m*/*z* of 147.0447, was tentatively identified as coumarin. Another coumarin, at *m*/*z* 193.0415 and showing fragments at *m*/*z* 177 and 147, was identified as scopoletin, according to Chen et al. [27]. As far as we know, this is the first time that 7-hydroxycoumarin and coumarin have been identified in the cranberry (Fig. 2) by using HPLC-DAD–TOF-MS in the same run.

3.4. Quantification

Quantification was done using the calibration curves shown in Table 1. The calibration curve of myricetin at λ = 280 nm was used to quantify flavonols, whilst phenolic-acid derivatives were quantified with the calibration curve of *p*-coumaric acid at λ = 280 nm. Flavanols were quantified using the curve of procyanidin A2 at λ = 280 nm and coumarins with the calibration curve of 7-hydroxycoumarin at λ = 280 nm. The concentrations of the phenolic compounds identified in cranberry syrup are summarized in Table 4. Thus, the overall phenolic content obtained by HPLC-DAD was found to be 5.47 mg/mL. The family of flavanols (catechin and procyanidins) was found at the highest concentration (2.37 mg/mL) followed by flavonols (1.91 mg/mL). Phenolic-acid derivatives were found at the lowest concentration (0.15 mg/mL).

Table 4
Phenolic compounds in cranberry syrup expressed in μg/mL of syrup (*n* = 5).

Class/phenolic compounds	μg/mL cranberry syrup
Phenolic-acid derivatives	
Coumaroyl-hexose	56.70 ± 2.05
Sinapoyl-hexose	10.72 ± 0.37
Caffeoyl glucose	19.63 ± 0.62
Chlorogenic acid	19.35 ± 0.53
Coumaroyl-hexose	40.18 ± 1.15
Canthoside A	1.61 ± 0.04
2-Hydroxybenzoic acid	49.40 ± 1.45
Gallic acid 3-*O*-gallate	58.36 ± 1.63
Flavonols	
Myricetin 3-*O*-hexoside	125.87 ± 4.87
Dihydro ferulic acid 4-*O*-*β*-D-glucuronide	23.96 ± 1.21
Myricetin 3-*O*-arabinoside	226.63 ± 8.83
Caviunin glucoside	297.30 ± 13.43
Quercetin 3-*O*-hexoside	391.13 ± 19.29
Quercetin 3-*O*-xylopiranoside	68.13 ± 2.37
Quercetin 3-*O*-arabinopyranoside	66.39 ± 2.17
Quercetin 3-*O*-arabinofuranoside	120.53 ± 4.31
Quercetin 3-*O*-rhamnoside	130.51 ± 4.23
Myricetin	114.78 ± 3.79
Methoxyquercetin 3-*O*-galactoside	nq[a]
Kaempferol 3-*O*-*β*-D-(6″-*p*-hydroxybenzoyl)-galactopyranoside	30.70 ± 1.39
Quercetin	303.27 ± 12.81
Syringetin	24.72 ± 0.83
Flavanols	
Procyanidin B type isomer 1	202.84 ± 7.39
(+)-Catechin	374.31 ± 15.43
Proacyanidin A2 type isomer 1	364.03 ± 14.89
Procyanidin B type isomer 2	215.64 ± 9.10
Proacyanidin A2 type isomer 2	356.99 ± 12.88
Procyanidin A2 type isomer 3	644.37 ± 49.71
Prodelphinidin B4	209.98 ± 7.37
Isoflavonoids	
Biochanin A-7-*O*-glucoside	nq
Coumarins	
7-Hydroxycoumarin	246.91 ± 8.77
Coumarin	174.90 ± 6.21
Scopoletin	449.99 ± 19.82
Anthocyanin	
Petunidin	9.59 ± 2.90
Total	5469.35 ± 194.81

[a] Not quantified.

3.5. Total polyphenol, proanthocyanidin and anthocyanin contents

Spectrophotometric methods are normally used to measure phenolic contents so various spectrophotometric assays were made to the syrup extracts. To quantify the total phenolic compounds by the Folin Ciocalteu method, a caffeic acid calibration curve was constructed. The calibration curve showed good linearity between the concentration of caffeic acid and absorbance, as described by the equation $y = 0.0149x - 0.0206$ ($r^2 = 0.995$). The total polyphenol content was 15.26 ± 0.08 mg/mL of cranberry syrup. The total proanthocyanidins expressed as catechin equivalents was 9.9 ± 0.1 mg/mL of cranberry syrup, as described by the equation $y = 0.0098x - 0.0153$ ($r^2 = 0.991$). The anthocyanin content found in cranberry syrup was 1.35 ± 0.04 mg/mL. The results obtained by these spectrophotometric analyses were higher than those obtained by HPLC-DAD. This can be put down to the interference of compounds such as sugars, which can cause an overestimation of the results, and cranberry syrup does in fact, contain a high quantity of glucose.

3.6. Antibacterial activity evaluation

Previous studies [28] have reported the beneficial effect of cranberry syrup in preventing urinary tract infection (UTI) among women, achieving a reduction in the absolute risk of UTI infection compared to placebo treatment. This effect has been explained in terms of the anti-adherent effect of cranberry on *E. coli*. Ferrara et al. [29], in a controlled clinical trial including placebo treatment in children aged over 3 years, showed that cranberry syrup prevents the recurrence of symptomatic UTI.

The first step in the colonisation of the epithelium by *E. coli* is determined by its capacity to adhere to the host cells. Initially, this capacity is determined by the micro-organism's electric surface charge and surface hydrophobicity, and subsequently by other factors such as the formation of diverse types of fimbriae and of specific adhesins. Other authors have shown that fimbriae Types 1, P and S are not essential factors in the adhesion and subsequent colonisation of the urogenital epithelium by *E. coli*. These adhesions may, in general, be considered features of the virulence of extra-intestinal *E. coli*, but they are not essential for *E. coli* to become uropathogenic, all of which accounts for the current research interest in the extent to which cranberry extract may affect the non-specific adherence properties of *E. coli*.

In fact, no differences were observed in the surface hydrophobicity of *E. coli* following its growth in TSB culture medium to enhance the expression of Type 1 fimbriae ($Z = 0.35$; pNS). Nevertheless, the incubation of the bacterial suspension with cranberry syrup at final concentrations of either 1:1000 or 1:100 resulted in significant reductions in surface hydrophobicity, depending upon the concentration of cranberry, both after growth in TSB medium and on CFA agar.

Incubation of *E. coli* with cranberry syrup resulted in a reduction in its surface hydrophobicity and did not depend upon the quantities of Type 1 or Type P fimbriae expressed. In earlier studies [30] it was reported that the incubation of *E. coli* with certain anti-oxidants, such as vitamin E, produced similar reductions in surface hydrophobicity. As far as surface hydrophobicity is concerned, however, the incubation of *E. coli* with 1:1000 dilutions of cranberry extract (which do not affect the haemagglutination mediated by Type P fimbriae) did produce significant reductions in surface hydrophobicity, thus showing that extremely low levels of this extract are capable of modifying the non-specific adherence properties of *E. coli*.

The A-linkage in cranberry procyanidins may represent an important structural feature for anti-adhesive activity in bacteria. It has been demonstrated that Type A cranberry procyanidins hinder the adhesion of P-fimbriated uropathogenic *E. coli* to uroepithelial cells *in vitro* [8] and it has also been found that trimeric proanthocyanidins and Type A dimeric procyanidins are responsible for the anti-adhesive effect of cranberry. The composition of the cranberry syrup used in our study, which was rich in Type A procyanidins, may account for the reductions in surface hydrophobicity.

4. Conclusions

The powerful analytical method HPLC-DAD–TOF-MS was used to characterize a commercial cranberry syrup used to prevent urinary tract diseases. It was possible to identify a total of 34 compounds in the sample in less than 29 min using positive and negative ionization modes. To our knowledge, nine of these compounds are tentatively identified in cranberry for the first time. Different coumarins were also found in cranberry for the first time when analyzing its whole phenolic fraction by HPLC-DAD–TOF-MS. This finding was possible thanks to the mass accuracy and sensitivity provided by TOF-MS. Antibacterial activity was investigated further and it was possible to prove that very low concentrations of cranberry extract have the capacity to modify the non-specific adherence properties of *E. coli*, producing a reduction in surface hydrophobicity.

This study is of great significance for understanding the beneficial effects of cranberry syrup on health. Further analysis will be required to find out more about the activity leading to the preventative function of these compounds.

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