Supplementary Materials

Chemical profiling of *Limonium vulgare* Mill. using UHPLC-DAD-ESI/MS^n and GC-MS analysis

Benedict Carius [1,2], Helena Silva [3], Artur M. S. Silva [1,*] and Diana C. G. A. Pinto [1,*]

[1]LAQV-REQUIMTE & Department of Chemistry, Campus de Santiago, University of Aveiro, 3810-193 Aveiro, Portugal; benedict.carius@ua.pt
[2] University of Applied Sciences, Department of Chemical Engineering and Biotechnology, 64295 Darmstadt, Germany;
[3] CESAM & Department of Biology, Campus de Santiago, University of Aveiro, 3810-193 Aveiro, Portugal; hsilva@ua.pt
*Correspondence: diana@ua.pt (D.C.G.A.P); artur.silva@ua.pt (A.M.S.S)

Materials and methods

1. Standards and reagents

Several pure compounds were used as standards to ensure the identification of the phytochemicals and to perform the calibration curves for quantification purposes. Tetradecane (99%), hexadecane (99.5%), tetracosane (99%), octadecane (99%), triacontane (99%), 1-monpalmitin (>99%), β-sitosterol (98%), 5α-cholestan-3β-ol (99%), D-mannitol (98%), pentadecan-1-ol (99%) tetradecan-1-ol (98%), sorbitol (99%), D-(+)-galactose (>99%), D-(+)-mannose (>99%), D-(+)-xylose (>99%), D-(−)-ribose (>99.5%), D-fructose (99%), sucrose (>99%), maltose (>98%), stigmasterol (97%), campesterol (95%), ergosterol (98%), rutin (98%), and quercetin (99%) as well as succinic (>98%), citric (>99.5%), palmitic (≥99%) and stearic (99%) acids, were purchased from Sigma-Aldrich (St. Louis, USA). Malonic acid (98%), linoleic acid (≥99%), linoelaidic acid (99%) and glycerol (>99%) were purchased from BDH analytical chemicals (London, UK), D-(-)-cellobiose (>98%), α- and δ-tocopherol (98%) from Merk (Darmstadt, Germany), lactic acid (>98%) and benzoic acid (99%) from Riedel-de-Haën (Seelze, Germany), coumaric acid (>99%) and caffeic acid (99%) were supplied by Acros Organics (Geel, Belgium) and D-(-)-arabinose (>99%) from Fluka (Bucharest, Romania) while, eicosane, docosane, hexatriacontane and paraffin mixtures (C5-C8, C7-C10, C10-C16, C18-C24, C24-C36, C25-C35) were supplied by Supelco Inc. (Bellefonte, USA). Ellagic acid dihydrate (>98%), gallic acid hydrate (>98%), and kaempferol hydrate (>97%) were supplied by TCI (Tokyo, Japan). Finally, isorhamnetin (>99%), apigenin (>99%), chlorogenic acid (>99%), (+)-catechin (>99%), (-)-epicatechin (>99%), luteolin (>99%), apigenin-7-*O*-glucoside (>99%) were supplied by EXTRASYNTHESE (Genay Cedex, France).

2. Extract preparation

For the extraction in hexane, the amount of plant was determined on a precision scale, RADWAG WLC 6/A2 with a precision of d = 0.1 g. In the process V = 10 mL of hexane for each m = 1 g of plant was used. In Table S1 the weights of grinded plants and the amount of hexane are listed. The plant parts were put into a proper flask, a magnetic stirrer was added, and the flasks were placed on a stirring plate. The hexane was added, see volume in Table S1, and the stirring was started at a speed of n = 600 rpm. Due to the issue that some compounds can decompose under light influence, the flasks were covered with aluminium foil in advance. One extraction ran for 48 h and the solvent was changed at least twice until no intensive colour and increase of extraction weight of the solvent occurred.

After an extraction cycle, the solvent was filtered through a paper filter. The filtrate was sampled in a round-bottomed flask and the solvent, hexane, was evaporated in a rotary evaporator. The residues in the filter were added back to its extraction flask to prevent material loss. The extracts were dried until mass consistency before further usage.

Following the extraction with hexane, an extraction with ethanol using a Soxhlet was performed. The plant samples from the first extraction were air-dried and then put into the cartridges, the corresponded volume of ethanol was added (Table S1). Subsequently, the ethanol from all the cycles was evaporated with a rotary evaporator. The extracts were dried until mass

consistence before further usage. The weight of the dried mass from extracting with hexane and ethanol, and the percentage yield are shown in Table S1.

Table S1 Data from the extraction procedure

Part of plant	Dw	Vh	Hew	Pyh	Ve	Eew	Pye
Leaves (L_{Lv})	~80	~800	3.00 ± 0.03	3.74 ± 0.09	~1600	23.62 ± 0.25	29.43 ± 0.39
Flowering state (YF_{Lv})	~26	~260	0.32 ± 0.01	1.14 ± 0.05	~520	5.27 ± 0.13	19.76 ± 0.31
Fruiting state (GF_{Lv})	~160	~1600	7.13 ± 0.09	4.47 ± 0.10	~3200	24.32 ± 0.28	15.03 ± 0.29

Dw = Dry weight of the plant part used in g; Vh = Volume of hexane added in mL; Ve = Volume of ethanol added in mL; Hew = Hexane extract weight in g; Eew = Ethanol extract weight in g; Pyh = Percentage yield of the hexane extract; Pye = Percentage yield of the ethanol extract.

2. Gas chromatography – mass spectrometry analysis

The GC-MS analysis of the hexane extracts was done using a Shimadzu GCMS-QP2010Ultra system equipped with autosampler AOC-20i, ion source: electronic impact High-performance Quadrupole Mass Filter. Separation of the compounds was carried out in a DB-5J&W capillary column (30.0 m in length x 0.25 mm in diameter x 0.25 µm thickness of the film). The spectroscopic detection from the mass spectrometer utilized 0.1 kV electron ionization. Helium was used as a carrier gas with a column flow of 1.18 mL/min. GC-injection temperature was set at 320°C and split ratio of 50 was applied to an injection volume of 1 µL. The mass spectrometer ion source temperature was set at 250°C and the interface temperature at 300°C.

The extracts were weight with approximately m = 20 mg on an analytical scale into a tube. DCM was used as a solvent and tetracosane as an internal standard, added to the tube with 1 mL in total. The extracts were then dissolved in an ultra-sonic bath. For the silylation, 250 µL pyridine, 250 µL BSTFA and 50 µL TMSCl were added. The mixture was maintained in a water bath at 70°C for 45 min being the hydroxyl and the carboxyl groups present in each secondary metabolite converted to trimethylsilyl (TMS) ethers and esters, respectively. Afterwards were injected twice in the GC-MS apparatus. The silylation reagents quantity, the water bath temperature and the reaction time were previously optimized to ensure a total conversion of all compounds with hydroxyl groups into the correspondent TMS derivatives.

The chromatographic conditions were as follows: start time of record at 6.5 min; initial temperature at 90°C, hold for 4.00 min; temperature rate, 16°C/min up to 180°C; temperature rate, 6°C/min up to 250°C; followed by temperature rate, 3°C/min up to 300°C and then hold for 5.00 min.

From the total ion chromatogram, the peaks were identified by comparing their mass spectra with the mass spectra libraries NIST 2014, NIST 2008 and WILEY 2007, and with mass spectra fragmentation published in the literature [Füzfai et al. 2008; Suttiarporn et al. 2015; Golm Metabolome Database 2017]. If possible, it was also compared with the retention time and mass spectra of standard compounds injected in the same chromatographic conditions. Furthermore, identification of some compounds was done using the retention index relative to n-alkanes (C_5-C_{36}) injected in the same chromatographic conditions and using the equation (1). Where z is the number of carbon atoms in the alkane before the unknown compound and Z the number of the longer alkane. The retention time is tr [Nič et al., 2009].

$$I = 100 \cdot [z + (Z - z) \cdot \frac{t_{r(unknown)} - t_{r(z)}}{t_{r(Z)} - t_{r(z)}}] \quad (1)$$

For quantification purposes, four independent replicates of each sample were submitted to silylation procedure and each one injected in duplicate. The internal standard method was applied and the amount of metabolites present was achieved from the calibration curves obtained with the most closed pure standard compounds available or its TMS derivatives (if they have hydroxyl groups). All the injected samples and standards solutions contain a fixed quantity of internal standard (tetracosane). The calibration curves were obtained by injection of at least six different concentrations (5 µg/mL to 1.5 mg/mL) and the detection and quantification limits (LOD and LOQ, respectively) were determined from the parameters of the calibration curves represented in Table S2 (LOD = 3 standard deviation / slope and LOQ = 10 standard deviation / slope). Values of correlation coefficients confirmed linearity of the calibration plots (Table S2). The concentrations of the standards were chosen in order to

guarantee the quantification of each compound in the samples by interpolation in the calibration curve. The results were expressed in mg of compound/g of extract, as mean values ± standard deviation (MV ± SD) of four independent analyses.

Table S2 Linearity (y = mx + b, where y corresponds to the standard peak area / internal standard peak area ratio and x corresponds to the mass of standard/mass of internal standard ratio), LOD and LOQ of pure compounds used as reference

Standard compound	Slope (m)[§]	Intercept (b)[§]	R^2	LOD[§§]	LOQ[§§]
Palmitic acid	0.0021	-0.0002	0.9944	9	21
Linoelaidic acid	0.0028	-0.0786	0.9938	13	42
1-Monopalmitin	0.0083	-0.0009	0.9975	6	17
Glycerol	0.0066	-0.0037	0.9937	9	23
Pentadecan-1-ol	0.0018	0.0140	0.9987	7	24
D-Mannitol	0.0142	-0.0924	0.9998	7	24
Triacontane	0.0050	-0.1859	0.9994	19	62
Eicosane	0.0034	-0.0587	0.9995	19	64
Docosane	0.0032	-0.0804	0.9984	22	75
D-(+)-Maltose	0.0014	-0.0801	0.9998	5	17
D-(+)-Mannose	0.0111	-0.0507	0.9995	7	24
D-(-)-Arabinose	0.0156	-0.2391	0.9980	20	66
D-(+)-Galactose	0.0114	-0.0599	0.9994	9	29
Ergosterol	0.0025	-0.0274	0.9998	13	44
β-Sitosterol	0.0054	-0.0033	0.9983	6	11
α-Tocopherol	0.0038	-0.0028	0.9993	5	14

[§]in area counts mg^{-1}; [§§]in µg/mL

3. Liquid chromatography – mass spectrometry analysis

For the ultrahigh performance liquid chromatography-mass spectrometry (UHPLC-DAD-ESI/MS^n) analysis, 50 mg of each extract were dissolved in methanol (final concentration 5 mg/mL) and the resulting solutions were filtered through a 0.2 mL nylon membrane (Whatman). Three independent analyses were carried out for reproducibility. This technique was performed using a Thermo Scientific Ultimate 3000RSLC (Dionex) equipped with a Dionex UltiMate 3000 RS diode array detector and coupled to a mass spectrometer. The column used was a Thermo Scientific hypersil gold column (Part nº 25002-102130; Dim 100 mm x 2.1 mm; Lot 14913; SN 10518298) with a part size of 1.9 µm and its temperature was maintained at 30°C. The mobile phase was composed of (A) acetonitrile and (B) 0.1% formic acid in water (v/v), both degassed and filtered before use. The flow rate was 0.2 mL/min. For the first 14 min the mobile phase had 5% of (B). Next, the gradient of (B) increased to 40% for 2 min and then to 100% for 7 min. From the 23 min to the end it was set back to 5% (B). The injection volume was 2 µL. UV-vis spectral data were gathered in a range of 250 to 500 nm and the chromatographic profiles were documented at different wave lengths. The mass spectrometer used was an LTQ XL linear ion trap 2D equipped with an orthogonal electrospray ion source (ESI). The equipment was operated in negative-ion mode with electrospray ionization source of 5.00 kV and ESI capillarity temperature of 275°C. The full scan covered a mass range of 50 to 2000 *m/z*. Collision-induced dissociation MS/MS and MS^n experiments were simultaneously acquired for precursor ions.

The identification of individual phenolic compounds by UHPLC-MS was achieved by comparing their retention times, UV-Vis spectra, and MS^n spectra data available on the literature. And also, with the data of reference standards or of the closest available standards, injected under the same UHPLC-MS conditions. The quantification of the individual phenolic compounds in the plant's extract was performed by peak integration at 280 nm, through the external standard method, using the closest reference compounds available. The detection and quantification limits (LOD and LOQ, respectively) were determined from the parameters of the calibration curves represented in Table S3 (LOD = 3 standard deviation/slope and LOQ = 10 standard deviation/slope). The calibration curves were obtained by injection of five known concentrations with variable ranges and the concentrations of the standards were chosen in order to guarantee the quantification of each compound in the samples by interpolation in the calibration curve. Values of correlation coefficients confirmed linearity of the calibration plots (Table S3). The results were expressed in mg of compound/g of dried extract, as mean values ± standard deviation (MV ± SD) of four independent analyses.

Table S3 Linearity (y = mx + b, where y corresponds to the standard peak area and x corresponds to the mass of standard), LOD and LOQ of pure compounds used as reference

Standard compound	Slope (m)[§]	Intercept (b)[§]	R^2	LOD[§§]	LOQ[§§]
Caffeic acid	$1\ 10^7$	-411992	0.9992	10	47
p-Coumaric acid	$4\ 10^6$	-532140	0.9952	14	50
Chlorogenic acid	$2\ 10^7$	-424793	0.9989	12	45
Kaempferol	$4\ 10^6$	-410879	0.9979	9	28
Apigenin	$3\ 10^6$	-210103	0.9991	5	22
Quercetin	$4\ 10^6$	-390882	0.9989	5	27
Rutin	$2\ 10^6$	10080	0.9986	10	34

[§]in area counts/mg; [§§]in µg/mL

8. Statistical analysis

Data Independent replicates of each sample were analysed and each aliquot was injected twice. The presented results are the average of four concordant values obtained for each sample (less than 5% variation between injections of the same aliquot and between aliquots of the same sample) and expressed as mean values ± standard deviation (MV ± SD). One-way analysis of variance (ANOVA) followed by Duncan's multiple-range test were performed using the GraphPad Prism version 7 for Windows (Graphpad Software, Inc.) to compare the results of each independent replicates. A *p*-value lower than 0.0001 was considered statistically significant in all analyses.

9. References

Füzfai, Z.; Boldizsár, I.; Molnar-Perl, I. Characteristic fragmentation patterns of the trimethylsilyl and trimethylsilyl-oxime derivatives of various saccharides as obtained by gas chromatography coupled to ion-trap mass spectrometry. J. Chromatogr. A, 2008, 1177, 183-189

Golm Metabolome Database (GMD). URL http://gmd.mpimp-golm.mpg.de/. Accessed 14/04/2022.

Nič, M.; Jirát, J.; Košata, B.; Jenkins, A.; McNaught, A. IUPAC Compendium of Chemical Terminology, IUPAC, Research Triagle Park, NC, 2009.

Suttiarporn, P.; Chumpolsri, W.; Mahatheeranont, S.; Luangkamin, S.; Teepsawang, S.; Leardkamolkarn, V. Structures of phytosterols and tripernoids with potential anti-cancer activity in bran of black non-glutinous rice. Nutrients, 2015, 7, 1672-1687.