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Use of Innovative (Micro)Extraction Techniques to Characterise *Harpagophytum procumbens* Root and its Commercial Food Supplements

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Abstract

Introduction - For the determination of harpagoside and the wide phenolic pattern in *Harpagophytum procumbens* root and its commercial food supplements, dispersive liquid–liquid microextraction (DLLME), ultrasound-assisted DLLME (UA-DLLME), and sugaring-out liquid–liquid extraction (SULLE) were tested and compared.

Objectives - In order to optimise the extraction efficiency, DLLME and UA-DLLME were performed in different solvents (water and aqueous solutions of glucose, β-cyclodextrin, (2-hydroxypropyl)-β-cyclodextrin, sodium chloride, natural deep eutectic solvent, and ionic liquid).

Material and Methods - The plant material was ground and sieved to obtain a uniform granulometry before extraction. Commercial food supplements, containing *H. procumbens* are commercially available in Italy.

Results - The most effective sodium chloride-aided-DLLME was then optimised and applied for analyses followed by HPLC-PDA. For comparison, microwave-assisted extraction was performed using the same solvents and the best results were obtained using 1% of β-cyclodextrin or 15% of sodium chloride.

Conclusion - All commercial samples respected the *European Pharmacopoeia* monograph for this plant material, showing a harpagoside content ≥ 1.2%.

Keywords: DLLME;Harpagophytum procumbens; IL; NADES; MAE

Introduction

*Harpagophytum procumbens,*also known as Devil's Claw (DC), grows in arid climate conditions in the African continent. For centuries in South Africa and later in Europe, the roots were used as a universal folk remedy for the treatment of several diseases, such as infections, fevers, skin complaints (Grant *et al*., 2007), rheumatis ms (Brien *et al*., 2006) and osteoporosis (Chung *et al*., 2016). The major chemical compounds of *H. procumbens* are iridoid glycosides, sugars, triterpenoids, phytosterols, aromatic acids, and flavonoids (Mncwangi *et al*., 2012). The most representative bioactive component of *H. procumbens* is harpagoside (Supporting Information Section S1), that has been shown to possess anti-inflammatory, analgesic (Haseeb *et al*., 2017), anti-diabetic (Mahomed and Ojewole, 2004), and antioxidant (Grant *et al*., 2009) effects.

According to the *European Pharmacopoeia* (Council of Europe, 2016), harpagoside should be quantified after reflux extraction from *H. procumbens* roots and homoeopathic preparations. This procedure provides reliable extraction of harpagoside, but the main drawbacks are the time-consumption (one hour) and the use of large solvent and plant material amount (0.5 g in 100 mL). In order to reduce the extraction time and to improve its selectivity, microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) techniques were proposed, leading to extraction times being lowered to 5 and 10 min, respectively, and a four-fold reduction in the volume of solvent (Baghdikian *et al*., 2016; Locatelli *et al*.,2017). The development of analytical methods for food supplement control with a reduction of the amount of toxic solvents used, or the replacement with non-toxic ones, without loss of efficacy in the extraction procedure is an important aim for researchers (Płotka-Wasylka *et al*.,2016).

Therefore, liquid phase microextraction methods have shown important innovations for the extraction and pre-concentration of analytes from different matrices. Dispersive liquid–liquid microextraction (DLLME) and its modifications, such as ultrasound-assisted DLLME (UA-DLLME), ionic liquid-based DLLME (IL-DLLME), natural deep eutectic solvent-based DLLME (NADES-DLLME), and sugaring-out assisted liquid–liquid extraction (SULLE) can offer unique benefits such as high pre-concentration factor for the target analytes, low cost, simplicity and combined use with almost every analytical measurement technique (Campillo *et al*., 2017). To the best of our knowledge, most of these procedures have not been developed or applied for the determination of harpagoside in raw plant material and/or in derived commercial products (food supplements).

A large number of microextraction techniques including single drop microextraction, DLLME, and liquid-phase microextraction (LPME) have been reported. Carrying out such techniques can vary widely, but common features are the use of only a small amount of organic solvents

and high sample-to-acceptor volume ratio. The organic phase, which contains the target analyte(s), can be used for quantification by means of different types of instrumental configurations (Yan and Wang, 2013). LPME is usually performed to analyse aqueous solutions. Analysis of solid samples is commonly done in two steps: the solid sample is converted to an aqueous solution using the suitable pretreatment procedure, and then the LPME is applied. Direct analysis of solid samples is somewhat more exceptional than common. Several works have been reported for the determination of phenolic compounds in plant materials and food samples by using DLLME in combination with HPLC-U*V*/*v*is (Hao *et al*., 2012; Yang *et al*., 2017) or GC–MS (Fariña *et al*., 2007).

Replacing hazardous solvents with ionic liquids (ILs) or natural deep eutectic solvents (NADESs) is another important aspect available in DLLME. Depending on the analytical purpose, it is possible to modify the IL properties due to the structure of the cation and the identity of the anion (Tang *et al*., 2012), but high cost and toxicity remain the main disadvantages (Khezeli *et al*., 2016). Recently, NADESs were rapidly developed as new types of green solvents instead of ILs. NADESs are based on primary metabolites, such as organic acids, amino acids and sugars, but limited data are available for their extraction abilities (Craveiro *et al*., 2016; Shishov *et al*., 2017).

The aim of this study is to develop a rapid and reproducible microextraction procedure for the determination of harpagoside in *H. procumbens* root. DLLME and UA-DLLME with auxiliary solvents as solutions of glucose, β-cyclodextrin, (2-hydroxypropyl)-β-cyclodextrin (HP-β-cyclodextrin), sodium chloride, NADESs, and ILs were tested to improve the extraction efficiency. SULLE was also studied as an extraction procedure. MAE, carried out with different solvents, was chosen for comparison. After preliminary tests, the most efficient DLLME and MAE procedures were optimised and applied for the evaluation of harpagoside content and phenolic pattern in roots of *H. procumbens* and in seven commercial food supplements.

Experimental

Chemicals

Chemical standards: gallic acid, (+)-catechin, chlorogenic acid, 4-hydroxybenzoic acid, vanillic acid, (−)-epicatechin, syringic acid, 3-hydroxybenzoic acid, 3-hydroxy-4-methoxybenzaldehyde, *p*-coumaric acid, rutin, sinapinic acid, *t*-ferulic acid, naringin, 2,3-dimethoxybenzoic acid, benzoic acid, *o*-coumaric acid, quercetin, 8-cinnamoyl harpagide (harpagoside), *t*-cinnamic acid, naringenin, and carvacrol (all purity ≥98%) were purchased from Sigma-Aldrich (Milan, Italy).

Solvents [ethyl acetate (≥ 99%), acetonitrile (HPLC-grade), methanol (HPLC-grade), acetic acid (≥ 99%)] were obtained from Carlo Erba Reagents (Milan, Italy). *n*-Hexane (HPLC-grade) and diethyl ether (≥ 99%) were purchased from Sigma-Aldrich (Milan, Italy). Sugars [D(+)-saccharose, D(+)-glucose and D(−)-fructose] were obtained from Carlo Erba Reagents (Milan, Italy). IL (1-butyl-3-methylimidazolium bromide, purity ≥99%) was obtained from Sigma-Aldrich (Milan, Italy). Sodium chloride (≥ 99%) was obtained from Honeywell (Seelze, Germany). NADES (glycolic acid/betaine mixture) was newly synthesised and supplied by the University of Perugia. It was chosen among differently structured novel DESs and NADESs mixtures (Cardellini *et al*., 2014) for its suitable chemical–physical properties (low freezing point and viscosity, absence of aromatic compounds in its composition, low cost and natural source of the molecules forming it). Glycolic acid and trimethylglycine (Betaine) were purchased from Sigma-Aldrich and were used after drying under vacuum over P_2O_5. Ultra-pure water was obtained using a Millipore Milli-Q Plus water treatment system (Millipore Bedford Corp., Bedford, MA). β-Cyclodextrin (≥ 97%) and HP-β-cyclodextrin were purchased from Sigma-Aldrich (Milan, Italy).

Sample preparation

Samples of the dry root of *H. procumbens* were purchased in a local market in Namibia. The plant material was ground to a fine powder using a mixer grinder, passed through a 40 mesh sieve to obtain a uniform granulometry and stored in a vacuum box in the dark at 4°C until use. Commercial food supplements, containing *H. procumbens* plant material or its extracts, were purchased in a pharmacy in Italy.

Microwave-assisted extraction (MAE)

MAE was performed using an automatic Biotage Initiator™ 2.0 (Uppsala, Sweden), characterised by 2.45 GHz high-frequency microwaves and power range 0–300 W. An IR sensor probe controlled the internal vial temperature. After grinding, the dry root (and commercial products) powder (100 mg) was protected from the light and held in a 10-mL vessel for an automatic single-mode microwave reactor. Then, 4 mL of each selected solvent were added to each sample thus obtaining a brown suspension. The sample was sealed and heated by the microwave source at 80°C (±1°C) for 6 min, and then cooled with pressurised air, following a reported procedure previously optimised for the same plant material (Locatelli *et al*., 2017; Ferrante *et al*., 2017). The resulting suspension was filtered through a 0.2 μm syringe filter (Sigma-Aldrich, Milan, Italy) and the extraction solvent stored at −20°C for further analysis.

HPLC analysis

HPLC-PDA analyses were performed according to a validated method reported in the literature (Zengin *et al*., 2016) using an HPLC Waters liquid chromatography (model 600 solvent pump, 2996 PDA). The mobile phase was directly degassed on-line using a Biotech 4CH DEGASI Compact (Onsala, Sweden). Empower v.2 Software (Waters Spa, Milford, MA) was used to collect and analyse data. The analyses were carried out using a gradient elution mode on a C18 reversed-phase column (Prodigy ODS(3), 4.6 mm × 150 mm, 5 µm; Phenomenex, Torrance, CA), thermostated at 30°C (±1°C). The gradient elution was represented by a solution of water–acetonitrile (93:7 *v*/*v* ratio, both with 3% of acetic acid) as initial conditions, and the complete separation was achieved in 60 min (see Supporting Information Section S2for chemical standards chromatograms and for retention times and maximum wavelengths used for quantitative analyses).

Molecular modelling

The molecular structures of harpagoside, sinapinic acid and vanillic acid were obtained from the PubChem database and minimised with OPLS-2005 force field (Kaminski *et al*., 2001; Hornak *et al*., 2006) by Macromodel (MacroModel, version 10.7, Schrödinger, LLC, New York, NY, 2015). The structure of the β-cyclodextrin was extracted from the crystal structure of the Protein Data Bank (PDB) with code 3CGT (Schmidt *et al*., 1998) and was prepared for docking using the Schrodinger preparation wizard protocol that consists in preliminary pre-treatment by adjusting the bond orders, adding hydrogen atoms, refining loop region and energy minimisation. Grid for docking analysis was centred in the centroid of the β-cyclodextrin cavity and generated maintaining the possibility of hydroxyl groups rotation. Molecular docking was carried out using Glide (Glide, version 6.6, Schrödinger, LLC, New York, NY, 2015) SP precision.

Results and discussion

Chemical multicomponent pattern

To evaluate the best extraction methods for harpagoside and phenolic pattern of *H. procumbens* extracts, DLLME, UA-DLLME, SULLE, cyclodextrin-based DLLME (both β-cyclodextrin and HP-β-cyclodextrin), salt-aided DLLME, IL-DLLME, and NADES-DLLME were carried out and compared in terms of harpagoside recovery. The DLLME starting conditions were the following: 50 mg of dry roots and 5 mL of water (or 10% solution of sodium chloride, NADES, IL, glucose or 1% solution of β-cyclodextrin, and HP-β-cyclodextrin) were placed into a 15 mL screw cap plastic centrifugation tube [solid-to-liquid (*w*/*v*) ratio 8.2]. After 30 s of gentle shaking, 600 µL of ethyl acetate (extraction solvent) and 500 µl of acetonitrile (dispersive solvent) were rapidly injected into the test tube. The solution

was vortexed for 30 s and thereafter it was left for 2 min for the complete distribution of the analytes between aqueous and organic phases. In the case of UA-DLLME, the ultrasonication was performed for 10 min. The phase separation was observed after centrifugation for 4 min at 402×*g* (1500 rpm). Subsequently, 350 µL of the extraction solvent was withdrawn from the top layer, transferred into a 2 mL Eppendorf tube, and dried under a gentle stream of nitrogen. The residue was redissolved in 50 µL mobile phase, and 20 µL were subjected to HPLC analysis. Schematic procedure of DLLME is shown in Supporting Information Section S3a.

For SULLE, the procedure was the following: 50 mg of dry roots were placed into 15 mL screw cap plastic centrifugation tube. Then, 1 mL of water and 2 mL of acetonitrile were added. After 30 s of gentle shaking, 1 mL of glucose solution (400 g/L) was rapidly injected [solid-to-liquid (*w*/*v*) ratio 12.5]. For the cloudy solution formation, the mixture was vortexed for 1 min. Phase separation was achieved after centrifugation for 4 min at 402×*g* (1500 rpm), and then 20 µL of the top acetonitrile phase were directly injected into the HPLC system. In comparison with DLLME (11184 µg/g of harpagoside), SULLE showed selective extraction of harpagoside (18793–20034 µg/g) with respect to other phenolic compounds, but the obtained chromatograms showed target analyte peak split in comparison with the standard solution (see Supporting Information Section S4 for the chromatograms that show harpagoside peak split). Probably, the presence of the sugar could influence this behaviour in the chromatogram. To prove those assumptions, three sugars (saccharose, fructose, and glucose) in the concentration range of 400 to 800 g/L were examined. The phase separation was not observed for sucrose and fructose at the concentration level 400 g/L, so the concentration of sugars was two-fold increased. After peaks comparison with fructose, glucose and saccharose, the harpagoside peak split was confirmed. This phenomenon could be attributed to a not complete sample solvent compatibility with the mobile phase used in this validated HPLC procedure, confirmed by the same effect for all tested sugars in SULLE as previously reported by Hawkins and Dolan (2004). The validated procedure required an injection volume of 20 µL and this value did not imply a peak split associated with overloading of the column, as observed for other types of analytes (Liu *et al*., 2008).

Similarly, MAE was chosen for comparison. Previously, the efficacy of MAE was demonstrated with water as the solvent (Locatelli *et al*., 2017) without other salts or modifiers. In the herein reported work the same optimised conditions (solid-to-liquid ratio, temperature, and irradiation time) were further tested by adding different modifiers in order to improve MAE process efficiency. For MAE, 100 mg of dry roots were placed in a 10 mL sealed vessel suitable for an automatic single-mode microwave reactor, and 4 mL of the proper solvent (water, 10% solution of sodium chloride, NADES, IL, glucose or 1% solution of β-cyclodextrin, and HP-β-cyclodextrin) were added [solid-to-liquid (*w*/*v*) ratio 25]. MAE was carried out by microwave irradiation for 6 min at 80°C (±1°C) and then cooling the

sample with pressurised air. Afterwards, 20 µL of the filtered solution (0.2 µm syringe filter, Sigma-Aldrich, Milan, Italy) were directly injected into the HPLC system (Mollica *et al*., 2016). The schematic procedure for MAE is shown in Supporting Information Section S3b. Figure 1 shows that the best extraction of harpagoside was obtained using salt-aided DLLME and MAE with sodium chloride or β-cyclodextrin solution. Accordingly, these three procedures were selected for the optimisation of the extraction conditions.

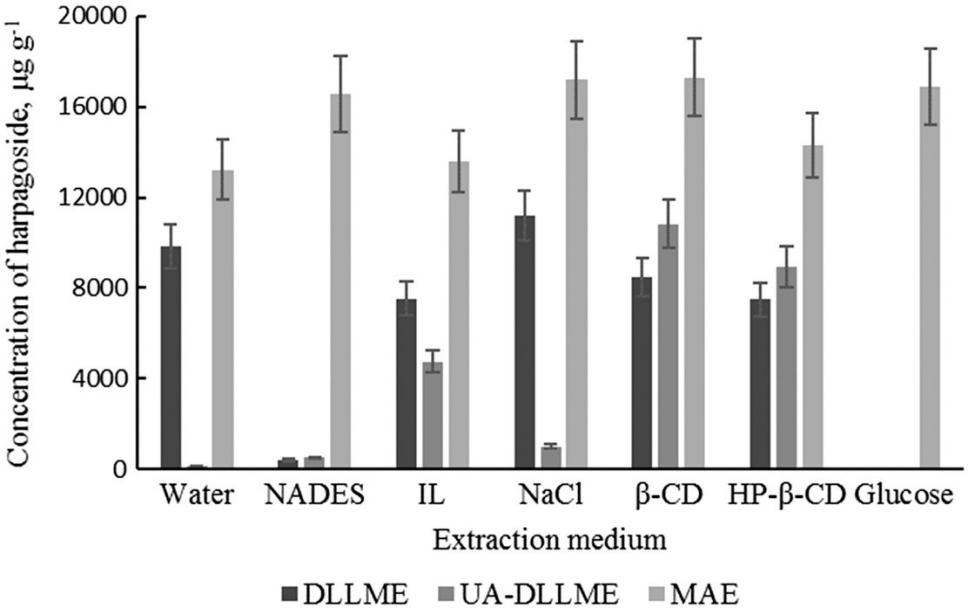

Figure 1. Dispersive liquid–liquid microextraction (DLLME), ultrasound-assisted DLLME (UA-DLLME), and microwave-assisted extraction (MAE) procedures with different extraction solvents [water, natural deep eutectic solvent (NADES), ionic liquid (IL), sodium chloride (NaCl), β-cyclodextrin (β-CD), (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD), glucose]. Error bars represent the standard deviation based on three independent measures. Results are related to *Harpagophytum procumbens* root samples.

Optimisation of salt-aided DLLME

Several experimental parameters could influence the extraction efficiency of a DLLME method: type and volume of extraction and dispersive solvents, concentration of salt, extraction time, and solid/liquid ratio. In DLLME, the type and volume of the extraction solvent have a strong influence on the recovery of the analytes. In this approach, when the extraction solvent showed a higher density than water, the extraction phase withdrawal was difficult due to the presence of solid and inhomogeneous roots structure. Thereby, extraction solvents with lighter density than water (ethyl acetate, *n*-hexane and diethyl ether) were tested to obtain a good extraction efficiency.

n-Hexane showed non-satisfactory results because, owing to its non-polar character, it had low extraction capacity for phenolic compounds. Two more polar solvents, ethyl acetate and

diethyl ether, showed higher efficiency. A 25-fold higher extraction efficiency was reached with the application of ethyl acetate. So, it was selected as extraction solvent for further studies (Supporting Information Section S5a). For the extraction volume optimisation, 100, 200, 500, 600, 800 and 1000 µL were examined, but, unfortunately, the phase separation did not occur with an ethyl acetate volume lower than 600 µL. Thus, 800 and 1000 µL showed approximately similar recovery, but the withdrawn volume was six-fold higher with 1000 µL (Supporting Information Section S5b).

The dispersive solvent should also meet the requirement of good miscibility in both aqueous phase and extraction solvent, then enabling the dispersion of fine droplets of the extractant into the aqueous phase containing the analytes. Based on the earlier considerations, methanol, acetonitrile and acetone were examined. The results showed that the maximum extraction of harpagoside was achieved by using acetonitrile as dispersive solvent (Supporting Information Section S5c). Additionally, to study the effect of dispersive solvent volume, the experiments were carried out using different volumes of acetonitrile (250, 500 and 1000 µL) (Supporting Information Section S5d). A volume of 500 µL of acetonitrile was chosen for the future analysis due to its higher target analyte recovery.

The influence of the solution ionic strength was evaluated by adding 5%, 10%, 15% solution of sodium chloride, while keeping other experimental parameters constant. The results revealed that the maximum harpagoside recovery was obtained by using 10% sodium chloride, whereas no differences were observed on increasing the amount of sodium chloride (Supporting Information Section S5e). Based on the results obtained, 10% solution of sodium chloride was selected for further experiments. In order to increase the extraction efficiency and reduce the extraction time, different intervals for the analyte distribution in extraction phase were checked (1, 2, 5 and 10 min). It was established that the harpagoside extraction efficiency decreased with the extraction time, thus 2 min of distribution time were selected as the optimal value (Supporting Information Section S5f). Finally, the extractions were conducted with a series of different solid/liquid ratios (5:1, 10:1, 20:1, *w/v*). The extraction efficiency reached its maximum with a 10:1 (*w*/*v*) ratio and slightly decreased while increasing the sample weight, probably due to a saturation phenomenon related to other phytochemicals. So, the ratio 10:1 was chosen for further experiments (Supporting Information Section S5g).

From the optimisation studies, the final DLLME conditions to achieve the best performance of extraction of harpagoside consisted of the following steps: (i) amount of plant material – 50 mg, (ii) extraction solvent – 1000 µL of ethyl acetate, (iii) dispersive solvent – 500 µL of acetonitrile, (iv) 10% solution of sodium chloride and extraction time of 2 min, (v) withdrawn volume – 350 µL, dryness under nitrogen atmosphere, then solubilisation of the dry residue with 50 µL of mobile phase.

Optimisation of MAE

For the optimisation of the MAE, different percentages and types of modifiers were evaluated (5%, 10%, 15% solutions of sodium chloride and 0.5%, 1%, 1.5% solutions of β-cyclodextrin) under previously described conditions. Sodium chloride addition increased the harpagoside recovery in the sample solution with respect to reported analyses (Locatelli *et al*., 2017), and improved the extraction efficiency with respect to harpagoside recovery. Conversely, β-cyclodextrin could improve this process due to a direct harpagoside inclusion within its conical cavity, as also demonstrated by a molecular modelling approach. The highest concentration value of harpagoside was obtained using 15% sodium chloride or 1% β-cyclodextrin solution (Supporting Information Section S6).

Molecular docking of β-cyclodextrin/metabolites

To further understand the better recovery of specific metabolites (harpagoside, sinapic acid, and vanillic acid) after MAE, with or without the use of a solution of β-cyclodextrin as solvent, a computational approach was applied. Primarily, the recovery enhancement of harpagoside associated with the use of β-cyclodextrin was rationalised by docking simulations which were carried out with the target kept fixed in the original conformations throughout the docking procedures, apart from the hydroxyl groups. Indeed, it makes sense treating such groups in the β-cyclodextrin structure as flexible (left free to move), allowing them to assume different orientations during the docking to produce the most favourable interactions with ligands. Multiple iso-energetic inclusion modes of the harpagoside within the β-cyclodextrin cavity were obtained (Fig. 2). The glycosidic and cyclopenta[*c*]pyranyl moieties were located at the larger opening of the toroid, exposed to the solvent, and established a wide network of conventional O-H···O hydrogen bonds with the hydroxyl groups of the glucose units placed at the rim. The cinnamoyl moiety was always found to accommodate within the apolar cavity of the oligosaccharide, where it formed C-H···π interactions with the cavity lining. Such interactions involved both the phenyl and the olefinic moieties of the harpagoside.

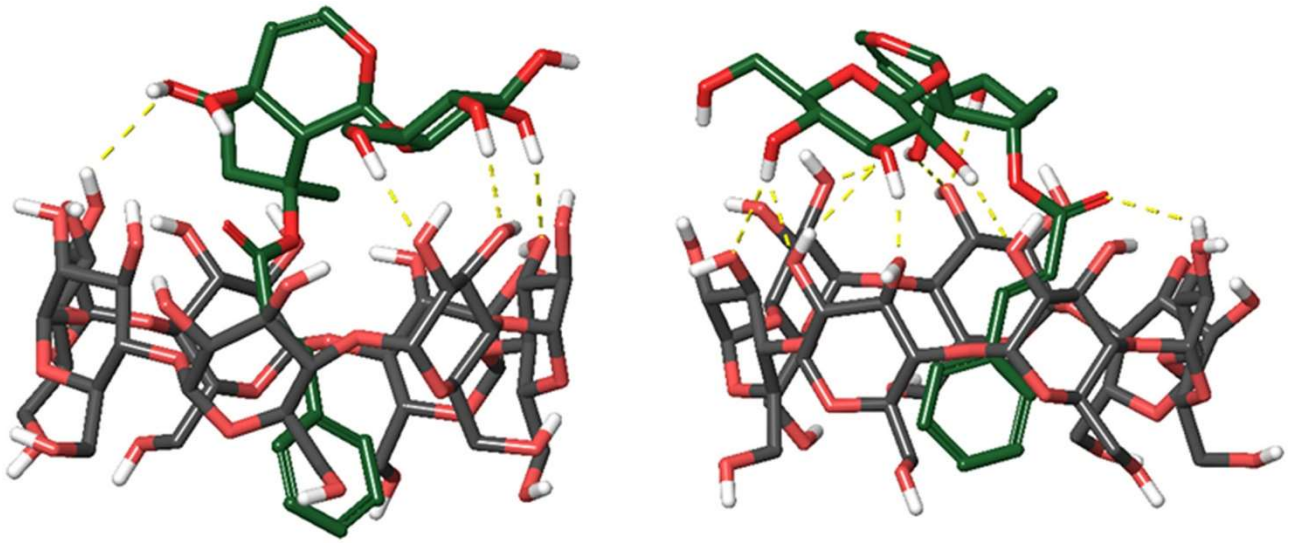

Figure 2. Inclusion complexes of harpagoside within β-cyclodextrin. Hydrogen bonds are represented as yellow dash lines.

Enhancements in the extraction recoveries were also found in the case of vanillic and sinapinic acids. The inclusion complexes of these two carboxylic acids predicted by docking simulations showed a pattern of weak and moderate C-H···O and C-H···π interactions. Moreover, the 4-OH moiety of vanillic acid was engaged in conventional O-H···O interactions with one of the oxygen atoms linking the glucose units of the β-cyclodextrin. Likewise, the O-H···O hydrogen bond involved the carboxylic functions of both acids (Fig. 3). In addition to those pointed out by docking, it is likely that further stabilising interactions might be formed in aqueous solution, involving hydrogen bonds with water molecules. Compared to the poses found for the harpagoside, none of the two carboxylic acids could establish wide hydrogen-bond interactions with the hydroxyl groups at the wide rim of the β-cyclodextrin toroid. It is important to highlight that previous studies also demonstrated that metabolites such as the chlorogenic, vanillic, and sinapinic acids were able to effectively fit and interact with the β-cyclodextrin (Górnas *et al*., 2009; Rajendiran *et al*., 2017; Xiong *et al*., 2014). It is likely that the great predominance of harpagoside into the mixture elicited a β-cyclodextrin saturation, thus interfering with the recovery of other components associated with the use of β-cyclodextrin.

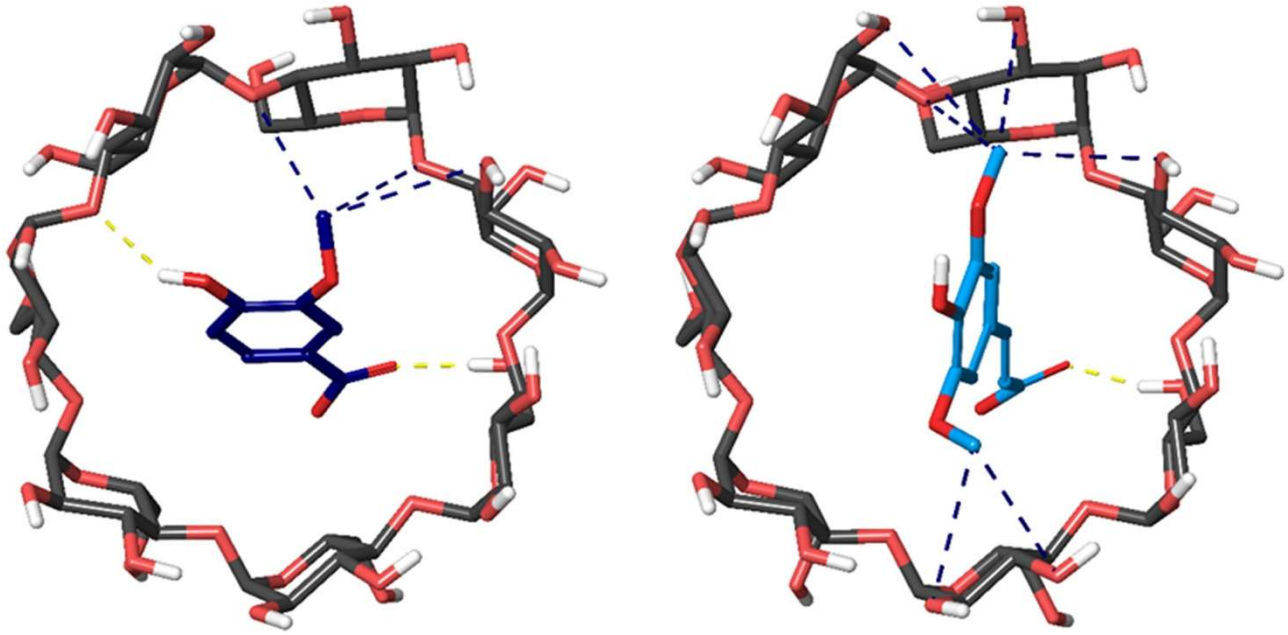

Figure 3. Inclusion complexes of β-cyclodextrin and (a) sinapinic acid and (b) vanillic acid. O–H···O and C–H···O hydrogen bonds are drawn as yellow and blue dashed lines, respectively.

Application to real samples: harpagoside content and phenolic pattern

To evaluate the applicability of the optimised extraction methods, the procedures were then applied to *H. procumbens* root and to its seven food supplements, commercially available in Italy. Table 1 reports the quantitative determination of harpagoside and phenolic pattern of *H. procumbens*-containing samples using salt-aided DLLME, while Tables 2 and 3 show the quantitative results obtained with MAE-sodium chloride and MAE-β-cyclodextrin, respectively. For the standard deviation calculation, the analysis of each sample was performed in triplicate.

Table 1. Concentration of harpagoside and phenolic compounds (in µg/g) in *Harpagophytum procumbens* root (**S1**) and in its commercial food supplements (**S2**–**S8**) extracted by sodium chloride-aided dispersive liquid–liquid microextraction ($n = 3$)

Sample ID#	Roots		Food Supplements													
	S1		**S2**		**S3**		**S4**		**S5**		**S6**		**S7**		**S8**	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
Gallic acid	3.7	1.9			4.7	1.2					25.3	3.6				
Catechin	240.4	33.7	16.2	4.0	125.8	65.4			32.6	37.9			56.1	27.4	20.3	5.8
Chlorogenic acid											139.5	107.0	15.6	2.2		
4-OH benzoic acid	13.5	4.2														
Vanillic acid	54.9	15.5					25.2	11.7			56.0	6.1				
Epicatechin																
Syringic acid	2.3	0.9							58.6	43.4						
3-OH benzoic acid	7.0	0.1									37.7	9.9	18.5	2.0	9.0	3.0
3-OH-4-MeO benzaldehyde	92.3	23.0	15.2	1.1	27.0	7.1	85.7	9.1	15.2	12.0	93.2	9.6				
p-Coumaric acid	1.3	0.7					916.2	360.3	302.4	220.8	2142.4	118.3	154.1	7.7	15.4	3.1
Rutin																
Sinapinic acid	5.1	1.5													4.6	1.0
t-Ferulic acid	2.4	1.5	1.7	0.2	8.8	0.9	16.8	2.9	24.6	18.5	26.6	0.6	1.7	0.3		
Naringin	56.9	20.8	108.9	17.3	266.4	68.4	49.4	9.9	188.3	137.4					22.5	7.3
2,3-DiMeO benzoic acid			242.0	67.7	402.0	70.9	362.9	146.0	96.5	81.7	201.0	7.6	79.3	33.7	38.4	10.5
Benzoic acid	53.3	11.2														
o-Coumaric acid	25.3	8.5											3.5	0.7		
Quercetin																
Harpagoside	8728.6	692.2	2769.7	483.5	8154.3	1759.4	9158.6	878.6	9691.1	7279.6	7658.4	509.2	5844.9	368.2	3344.6	36.0
t-Cinnamic acid	303.9	38.4					4.1	0.8			30.3	3.1				
Naringenin	6.4	2.6			5.2	0.7										
Carvacrol									8.9	6.4	13.6	3.2				
Total (µg/g)	9594.2	775.6	3153.7	569.1	8994.2	1830.3	10618.9	1341.9	10407.4	7793.7	10423.8	712.5	6173.7	317.7	3454.8	23.4
Harpagoside percentage (%, w/w)	0.87	—	1.56	—	6.72	—	8.12	—	7.92	—	3.18	—	4.22	—	7.43	—

Note: Std Dev, standard deviation.

Table 2 Concentration of harpagoside and phenolic compounds (in µg/g) in *Harpagophytum procumbens* root (**S1**) and in its commercial food supplements (**S2–S8**) extracted by microwave-assisted extraction-sodium chloride ($n = 3$)

Sample ID#	Roots		Food supplements													
	S1		S2		S3		S4		S5		S6		S7		S8	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
Gallic acid	52.3	4.4	26.2	0.8	36.7	27.5					27.1	1.5				
Catechin	1311.6	15.6	114.7	42.6					180.6	6.0			170.1	18.2	63.3	9.3
Chlorogenic acid	220.9	20.2			55.7	25.5					172.2	2.1	85.2	4.7		
4-OH benzoic acid																
Vanillic acid	40.0	6.1			81.9	4.1	26.3	3.3	18.9	3.4	43.6	4.8	41.2	0.1	9.2	0.6
Epicatechin																
Syringic acid									50.1	6.5	96.6	1.4				
3-OH benzoic acid	41.9	2.4	172.1	10.2			210.2	14.8			298.7	50.2	90.1	16.9	82.5	6.8
3-OH-4-MeO benzaldehyde	30.2	5.1	18.3	1.3	32.9	18.8	28.6	8.3	12.8	2.2						
p-Coumaric acid	8.7	0.9			6.3	0.1	511.8	96.3	292.9	8.8	837.6	11.1	144.7	18.0	150.2	22.5
Rutin																
Sinapinic acid	26.4	2.4			16.5	2.0									22.9	2.0
t-Ferulic acid	24.9	1.2	3.4	0.6			36.4	2.0	49.0	6.0	29.2	2.0				
Naringin	109.7	10.5	290.4	48.4	19.3	4.0	65.0	27.1	186.5	63.7	109.8	17.6	78.7	16.2	50.5	17.6
2,3-DiMeO benzoic acid	435.7	3.5	76.5	1.4	164.8	14.7	1384.6	510.4	225.5	8.0	649.7	105.5	1650.2	435.2	90.4	39.1
Benzoic acid																
o-Coumaric acid									33.7	1.8			29.9	3.7		
Quercetin																
Harpagoside	17459.0	32.3	6750.1	18.9	12575.2	951.8	15199.6	1432.6	26362.2	681.0	17748.5	299.3	13445.2	271.7	7326.6	231.4
t-Cinnamic acid	165.8	4.6					13.4	6.9			2.2	0.0				
Naringenin	6.2	0.2			6.0	0.7										
Carvacrol	21.4	5.2			33.8	5.9			38.3	1.9	27.1	1.5	26.2	1.8	39.0	21.9
Total (µg/g)	19918.2	89.6	7451.7	76.3	13029.0	1011.8	17454.3	1232.1	27423.0	711.0	20041.7	322.9	15761.5	181.1	7834.7	281.9
Harpagoside percentage (%, w/w)	1.75	—	3.80	—	10.4	—	13.5	—	21.5	—	7.37	—	9.71	—	16.3	—

Note: Std Dev, standard deviation.

Table 3. Concentration of harpagoside and phenolic compounds (in µg/g) in *Harpagophytum procumbens* root (**S1**) and in its commercial food supplements (**S2–S8**) extracted by microwave-assisted extraction-β-cyclodextrin ($n = 3$)

Sample ID#	Roots		Food supplements													
	S1		S2		S3		S4		S5		S6		S7		S8	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
Gallic acid	24.6	11.6			52.6	1.8					23.2	3.2				
Catechin	112.1	27.8	100.4	20.8					119.7	73.6			88.4	4.6		
Chlorogenic acid	227.5	24.0	32.7	13.9	62.8	5.7	76.2	3.8	116.7	27.8	109.1	2.5	54.6	4.9		
4-OH benzoic acid																
Vanillic acid	48.0	3.4	12.1	3.9			24.3	2.2			30.7	1.1			8.6	0.3
Epicatechin																
Syringic acid									14.3	3.1	20.5	2.0				
3-OH benzoic acid	92.7	10.0	607.8	31.4	36.7	4.8	262.4	22.7	63.2	19.0						
3-OH-4-MeO benzaldehyde	23.6	1.1	3.3	0.1					33.7	14.9	15.3	2.1	26.0	32.8		
p-Coumaric acid	589.6	8.4	134.5	28.1	159.3	4.7	553.1	21.5	103.7	49.5	22.2	8.5	35.7	2.1	39.7	42.4
Rutin																
Sinapinic acid					12.1	0.1	11.2	0.4	16.0	0.4						
t-Ferulic acid	21.3	2.1			13.8	7.0	74.7	6.7	28.5	23.1	96.2	1.7				
Naringin	213.3	84.6	59.9	23.8	49.0	27.7	102.6	23.4	365.9	140.6	175.3	2.1	102.2	14.7	47.3	10.3
2,3-DiMeO benzoic acid	875.0	567.7	112.9	28.6	1101.4	293.6	1098.5	272.1	354.6	99.1	937.0	37.2	1936.2	71.3	1845.0	242.2
Benzoic acid					352.1	7.3										
o-Coumaric acid																
Quercetin																
Harpagoside	18034.9	346.9	8230.6	860.4	12016.0	158.6	12672.8	319.4	23006.3	1449.4	15366.0	231.8	12107.2	222.5	6130.2	268.0
t-Cinnamic acid							16.0	1.2			2.6	0.2				
Naringenin	12.1	0.7			25.6	1.2										
Carvacrol	22.6	0.9	25.3	4.3	20.2	0.3			25.9	11.8	24.3	2.3	24.5	2.1	19.5	6.7
Total (µg/g)	20297.2	838.0	9318.3	887.9	13901.5	471.1	14891.9	458.5	24248.4	1607.4	16822.3	203.6	14374.7	235.5	8090.4	545.0
Harpagoside percentage (%, *w/w*)	1.80	—	4.63	—	9.90	—	11.2	—	18.8	—	6.38	—	8.74	—	13.6	—

Note: Std Dev, standard deviation.

Being the main analytical marker for the *H. procumbens* root, as requested by the *European Pharmacopoeia* monograph, among the 22 selected secondary metabolites, harpagoside is the most abundant component disregarding the extraction technique with a concentration ranging from 8729 to 18035 µg/g. The best extraction performances were obtained by using MAE and 1% β-cyclodextrin solution as extraction solvent, as also rationalised by the molecular modelling studies. As regards the phenolic pattern, herein DLLME was firstly applied to *H. procumbens* and provided a more complete phenolic profile with respect to the other technique (MAE with different solvents), even if their quantities were generally lower. This fact could be related to the type and the limited volume of solvent used for this green microextraction procedure. Conversely, MAE exhibited an improved efficiency in terms of total phenolic recovery. The quantification of other secondary metabolites, in addition to harpagoside, took advantage of the important and well-recognised biological activities attributed to this plant material in order to have more exhaustive data about the chemical characterisation of the phytocomplex.

Successively, we applied these three procedures for the comparative analyses of seven commercially available food supplements containing *H. procumbens*. The quantities of harpagoside found in the commercial products (Tables 1-3) resulted in slightly higher values than the declared concentration (percentage *w/w* of dry extract). This discrepancy could be related to the different extraction techniques, processing steps (Cesa *et al*., 2017), and to the fact that all food supplements were characterised by an established content of harpagoside-enriched plant material, but into the formulations was also present an undefined amount of raw plant material in addition to other ingredients. All commercial samples of food supplements respected the *European Pharmacopoeia* monograph that requires, for *H. procumbens*-containing preparations, a harpagoside content higher than or equal to 1.2%. As reported in Tables1-3, the three different extraction techniques registered a similar behaviour observed for the extraction of natural plant material, with DLLME as the least efficient procedure in terms of total recovery, expressed as µg/g. However, all the three extraction procedures coupled to HPLC-PDA confirmed the quality of these food supplements in terms of harpagoside content. Moreover, the total phenolic content in these food supplements ranged from 3154 to 27423 µg/g, for salt-aided DLLME, MAE-β-cyclodextrin, and MAE-sodium chloride.

Based on the achieved results for salt-aided DLLME and MAE, these methods could be successfully applied as rapid and environmentally friendly pre-concentration procedures for the analysis of *H. procumbens* root and its commercial food supplements. These optimised conditions also permitted the reduction of solvent consumption in routine analysis and, coupled with the reported HPLC-PDA instrumental configuration, improved the chemical pattern that could be evaluated in a one-step procedure. Additionally, the possibility to assess a multi-analyte pattern could expand the research studies, related to the fact that

several biological activities were demonstrated not only for the main (and often the most representative) compound, but also for the whole phytocomplex.

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