SUPERHEATED WATER, AN ADVANCED WAY TO MAKE A HEALTHIER EXTRACTION OF MEDITERRANEAN HERBS? A PRACTICAL APPROACH WITH DIOSMIN AND HERBS

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INTRODUCTION

A nutraceutical is usually defined as any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of diseases. In the last few years, there has been an increasing interest towards the search and characterization of new nutraceuticals from natural sources able to provide some additional benefit for human health such as antioxidants, anti-inflammatories, antihypertensives, etc.

The flavone diosmin (5,3´-dihidroxy-4´-methoxyflavone-7-O-rutinoside) shows very interesting functional properties. It has demonstrated effects on platelet aggregation which blocks microcirculation and leads to the corresponding pathologies. The capillary damage includes increase in permeability, extravasations of plasma and blood constituents, leading to the oedematous process. Nowadays, this group of disorders is medically treated with diosmin, hesperidin methylchalcone and hydroxyethylrutosides, which act primarily on the vascular endothelium reducing hyperpermeability and oedema [1]. On the other hand, these flavonoids have a high degree of safety [2, 3].

So far, diosmin has been described as naturally occurring in some citrus and aromatic herbs, being higher the concentration in the latest [4, 5]. Moreover, it is routinely prescribed by doctors against varicous disorders. Furthermore, recently some “in vivo” studies have shown that it may reduce the implantation and invasion index of melanomas, one of the most invasive carcinomas, to a fifth and a half, respectively, when compared with the non-treated groups.

Notwithstanding, and although the flavone diosmin is naturally occurring, it is produced from the flavanone hesperidin through organic synthesis that uses pyridines, chloroform, benzoyl peroxide and iodine, sulphuric acid and caustic soda [6]. The extraction from natural sources may be a suitable alternative to obtain this compound. Unfortunately, it is practically insoluble in most of solvents used in food technology [7].

Extraction using hot water under pressure sufficient to maintain it in the liquid state (so called, subcritical water extraction, SWE), has demonstrated its ability to selectively extract different classes of compounds depending on the temperature used, with the more polar compounds extracted at lower temperatures while the less polar compounds are extracted at higher temperatures. The selectivity of SWE allows for manipulation of the composition of the extracts by changing the operating parameters and it has been used for essential oil isolation [8] as well as for phenol-like compounds extraction [9].

The goal of the present investigation was to screen the flavonic composition of a collection of aromatic Mediterranean herbs, most of them traditionally used as spices or infusions, to select those ones and their anatomic organs with the highest content and to study the selectivity of SWE at several temperatures to extract the flavone diosmin. SWE is proposed in this work as a feasible process to concentrate and isolate this compounds to be used as nutraceuticals or in the pharmaceutical industry.

MATERIAL AND METHODS

Plant Material and Chemicals.

Samples of aromatic herbs were supplied by Herboristeria Murciana, Murcia, España. Samples were dried in forced air oven at 50ºC during 24 h and extracted with DMSO for analytical purposes, while cryogenic grinding of the hyssop dry leaves was performed under carbon dioxide for SWE. The size of the particle (between 250 and 500 μm) was determined by passing the ground plant material through sieves of appropriate size. The whole sample was stored in amber flasks at −20 ◦C until use (a maximum of 2 months). Diosmin and diosmetin standards were supplied by Sigma Sigma–Aldrich (Madrid, Spain), being the rest of chemicals of adequate quality for their use.

Subcritical Water Extraction

To perform the extractions, an Accelerated Solvent Extraction system ASE 200 equipped with a solvent controller unit from Dionex Corporation (Sunnyvale, CA, USA) was used. Extractions were carried out in triplicate using water. Two different extraction procedures have been used depending if individual extractions (at a chosen temperature) or sequential experiments were performed. Individual temperatures considered were 25, 50, 100, 150 and 200 ◦C with 30 min extraction time. Sequential extractions were performed using the same sample considering the following temperatures: 25, 50, 100, 150 and 200 ◦C and 15 min extraction time at each temperature. Previous to each experiment an extraction cell heat-up was carried out for a given time, which changed according to extraction temperature (the heat-up time is automatically fixed by the equipment). Namely 5 min heat-up was used when extraction temperature was set at 50 and 100 ◦C, 7 min at 150 ◦C and 9 min at 200 ◦C. Likewise, all extractions were performed in 11mL extraction cells, containing 750 mg of sample.

The extraction procedure was as follows: (i) sample is loaded into cell, (ii) cell is filled with solvent up to a pressure of 1500 psi, (iii) initial heat-up time is applied, (iv) static extraction with all system valves closed is performed (for 30 or 15 min, depending if individual or sequential extraction was considered), (v) cell is rinsed (with 60% cell volume using extraction solvent), (vi) solvent is purged from cell with N2 gas and (vii) depressurization takes place. Between extractions, a rinse of the complete system was made in order to overcome any extract carry-over. For solvent evaporation a freeze dryer (Freeze Dry System, Model 79480 de Labconco Corp., Kansas City, MO, USA) was employed. The collected extracts were kept protected from light, at 4 ◦C until use.

Analysis

For each extraction, the extraction yield was obtained along with the total phenolic content (according to Folin-Ciocalteau method [10]) and the content in diosmin and diosmetin (measured by HPLC).

For the quantification of flavonoids, an ODS-C18 (250x4.6 mm i.d.) analytical column was used with an average particle size of 5 μm, using water: acetonitrile: methanol: acetic acid (15:2:2:1) as the eluent with a flow rate of 1 ml/min at 30º C. The absorbance change was monitored at 350 nm with a Hewlett Packard mod. HP 1100 UV/Vis diode array detector [11].

Results

A screening on diosmin content in several aromatic herbs was performed, to select the better sources of this compound. For this purpose aerial parts (leaves and flowers), stems, and roots, of *Hyssopus officinalis* L, *Mentha pulegium* L, *Mentha sativa* L, *Mentha piperita* L, *Thymus* sp, *Origanum vulgare* L, *Rosmarinus officinalis* L, *Ocinum basilicum* L, *Coleus* sp, *Lavandula stoechas* L, *Lavandula dentata* L, *Origanum majorana* L, and *Salvia officinalis* L, were analyzed.

In Table 1 the contents in diosmin of those species is shown. As it can be seen, pulegium (*Mentha pulegium* L), hyssop (*Hyssopus officinalis* L) and *Mentha sativa* L show the highest content in diosmin

from all the studied herbs. So far diosmin was only described and quantified in hyssop [4] showing this results, for the first time, other natural sources of diosmin than the previously reported. Although pulegium seems more suitable for extractive purposes, due to its higher content in diosmin, the commercial availability of hyssop determined its election to study the conditions of extraction with SWE.

Table 1.- Diosmin content (expressed as g of diosmin/100 g DW (Dry Weight) ± SE (n=3)) in different parts of several aromatic herbs. ND: Non Detected.

	Aerial parts	**Stems**	**Roots**
Hyssopus officinalis L	3.23 ± 0.3	0.24 ± 0.01	0.01 ± 0.002
Mentha pulegium L	7.08 ± 0.59	1.30 ± 0.08	ND
Mentha sativa L	2.38 ± 0.34	0.19 ±0.03	ND
Mentha piperita L	0.62 ± 0.06	ND	ND
Thymus sp	0.42 ± 0.09	0.11 ± 0.02	ND
Origanum vulgare L	0.2 ± 0.04	0.046 ± 0.005	ND
Rosmarinus officinalis L	0.46 ± 0.05	0.09 ± 0.008	ND

Two different sets of experiments were studied: sequential extractions and individual extractions. When the yields of sequential extractions are compared it is observed that these are higher at room temperature than at temperatures close to 100º C (Fig. 1). Nevertheless, the yield increases again at temperatures above 100º C. These results may be explained by a higher extraction, at room temperature, of polar compounds, leading to an exhausted matrix. Thus the exhausted matrix would have the lowest yields at 50 and 100 ºC. Notwithstanding, the progressive increase in temperature (150 and 200 ºC) might extract the remaining compounds of lower polarity, which were not extracted below 100 ºC when water behaves as expected.

Regarding to the extraction of phenolics, it seems to follow a similar pattern to the above described for yield (data not shown), with a higher extraction at low temperatures and a new increase in the yield over 150 ºC. A similar behaviour, based on polarity, is suggested to explain this phenomenon. Thus, low weight phenols, i.e. hydroxybenzoic and hydroxycinnamic acids, might be extracted below 100 ºC while more complex chemical skeletons and specially those of low polarity, such as flavonoids should explain the phenolic content of extract at higher temperatures, and after the matrix was exhausted by the first sets of extractions.

This explanation is consistent with the observed behaviour of diosmin, and diosmetin during extractions. Thus, diosmin does not behave as yield or total phenolics. In fact, it is not detected when extractions were performed at room temperature (25 ºC), or slightly higher (50 ºC), but its extraction is maximum at temperatures equal to 100 ºC or higher, increasing its content according to the extraction temperature, as shown in Fig 2.

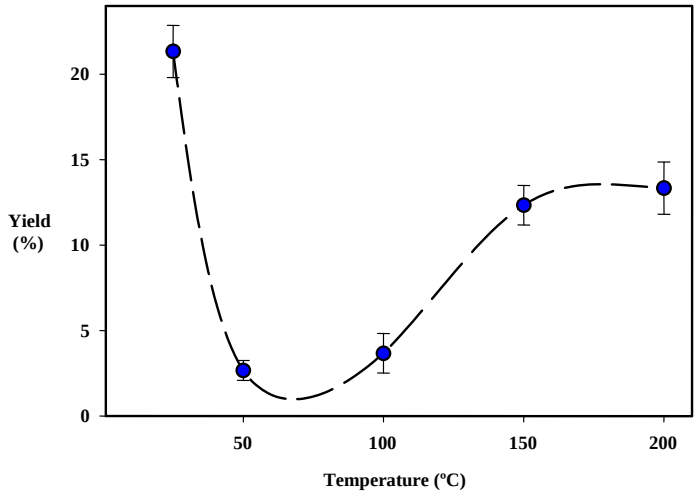

Figure 1.- Yield of extraction versus Temperature (ºC). Data are expressed as percentage of obtained extract with regard to sample. Verticals bars represent ± SE (n=3) when higher than symbols.

The chromatographic analysis of the extracts obtained at the highest temperatures (i.e. 100, 150 and 200 ºC) (Fig. 2) shows a peak with a t_R of 27.3 min, which match with the retention time of the diosmin standard as well as a UV/V absorption spectra and maximum of absorption consistent with this product. The peak number 3 shows a t_R of 38,6 min, which match with the diosmetin standard as well as a UV/V absorption spectra and maximum of absorption consistent with it. On the other hand, the peak number 2 shows a UV/V absorption spectra consistent with those of flavonic skeleton, which are characterized by a maximum of absorption in the band I, ranged between 310-350 nm, and a second band of absorption, band II, between 250 and 280 m [13].

The global behaviour is consistent with the previously observed for phenol-like compound of low polarity [9]. This pattern is characterized by a drop of the eluted products during the first minutes of the chromatography, and therefore more polar, and an increase of those ones eluted lately, less polar, according to an increase of the extraction temperature. This behaviour may be explained by the change of the properties of water as solvent over 100 ºC. Water changes dramatically when its temperature rises because of the breakdown in its structure with temperature. The high degree of association in liquid water causes its dielectric constant to be high (around 80) under ambient conditions, but as temperature rises, this dielectric constant falls down and near its critical point water dielectric constant (ε) drops drastically and water behaves like less polar solvents, i.e. ethanol or methanol-like [12].

This variation on the polarity of the extracted compounds depending on the extraction temperature is explained by a compound class selectivity phenomenon achieved by using subcritical water. This selectivity may exist because extraction depends on solvation of the target compounds in the liquid state of the water. As a polar fluid, water solvates more polar compounds more readily than non-polar compounds. Higher temperatures reduce the polarity of water, thus increasing its ability to solvate non-polar compounds. At the lowest temperature, the more polar compounds, such as some hydroxybenzoic and hydroxycinnamic structures were preferentially extracted. When water was heated up to 200 ◦C, the dielectric constant of water was reduced to values similar to those of methanol or acetonitrile which increased the solubilities of less polar compounds (diosmin and its aglycon form -diosmetin) by several orders of magnitude, resulting in such compounds being the major constituents of these fractions.

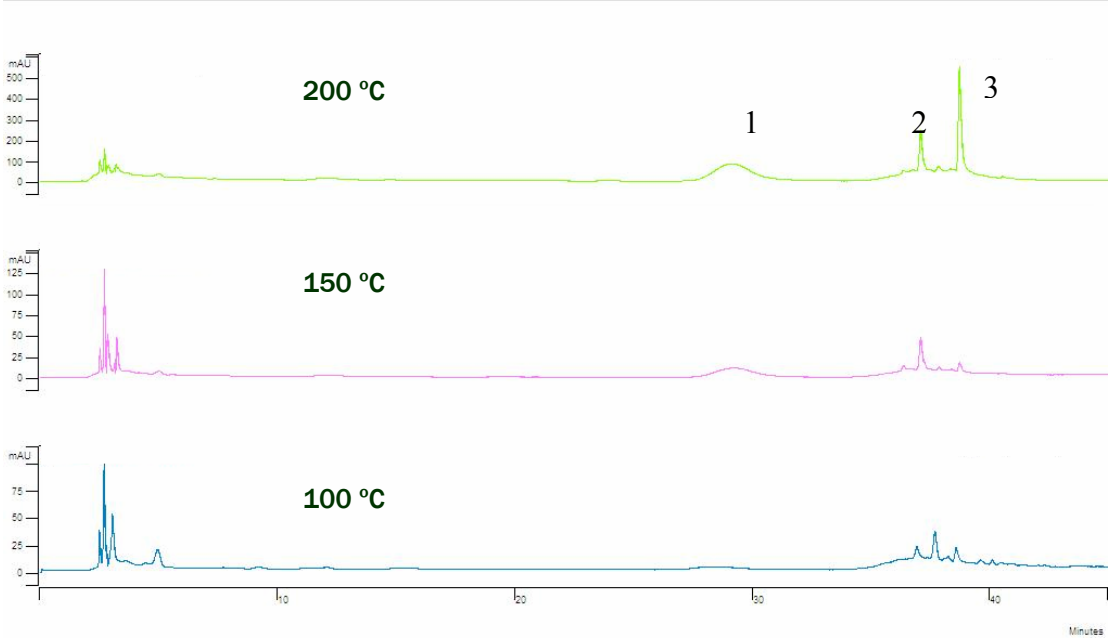

Figure2.- Chromatographic profile (λ=350 nm) of the extracts obtained at 100, 150 and 200 ºC. 1: diosmin, 2: non identified flavone, 3: diosmetin.

Regarding to selectivity, Figure 3 shows an increase on diosmin extraction when temperatures rise over 100 ºC. Thus, the highest amount of diosmin for the sequential extraction was 4,34 g/100 g DW (Dry Weigth) and was obtained at 200 ºC. Furthemore, the total amount of diosmin and diosmetin, the aglycon form that is even less polar, rises up to 9% DW, showing a strong change of selectivity at the highest temperatures that may be explained by the aforementioned change in water polarity. This behaviour is consistent with all the above exposed, and suggest that the optimum conditions are over the region near to the 200 ºC, or even higher, although this last could not confirmed due to the technical limitations of our equipment.

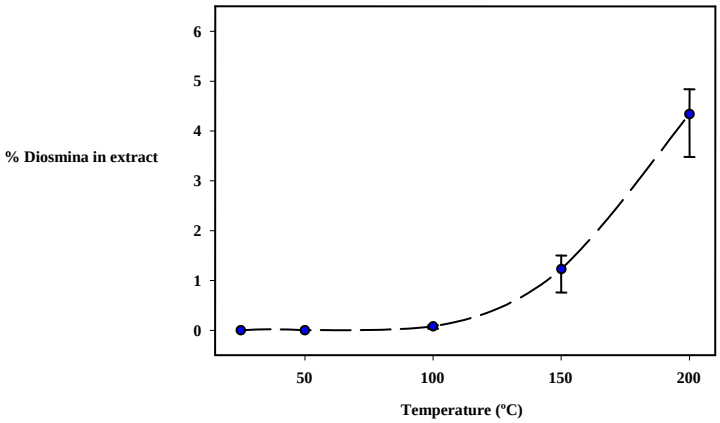

Figure 3.- Selectivity of diosmin extraction (g diosmin/100 g DW) versus temperature. Verticals bars represent ± SE (n=3) when higher than symbols

On the other hand, the aglycon of diosmin, diosmetin, showed a pattern of behaviour similar to diosmin (data not shown). Regarding to individual extractions, yields of diosmin of about 2 g diosmin/100 g DW were achieved, lower to those obtained by sequential extractions. This behaviour might be explained by a previous physical modification of the matrix. Thus, during sequential extractions the matrix underwent a set of rising temperatures during 1 hour, which could have modified the matrix favouring the desorption processes.

CONCLUSION

In the present study we demonstrated the ability of water, at subcritical conditions, to extract flavones of low-medium polarity. Moreover, the possibility of tuning the extraction selectivity of pressurized water by changing its temperature has been demonstrated for the extraction of diosmin compound from hyssop using SWE.

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