Formation of 5-(hydroxymethyl)furfural During Subcritical Water Extraction of Natural Matrices. Is It Bioactivity-Relevant?

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ABSTRACT

Subcritical water extraction (SWE) has demonstrated a great potential for the extraction of bioactive compounds from natural matrices. The application of high extraction temperatures and pressures, while keeping the water in the liquid state, allows the attainment of high extraction yields in fast extraction processes.
Although the applications of SWE to obtain bioactives from natural matrices are increasing, the studies dealing with the processes that might take place during the extraction are still scarce. Recently, we demonstrated how under SWE conditions not only the solubility of the analytes might be improved but also neoformed antioxidants can be obtained. Indeed, it was observed how the occurrence of reactions like Maillard and caramelization reactions during SWE resulted on the formation of neoantioxidants. This fact can be advantageous although the possible toxicity of the compounds formed during these non-enzymatic reactions should be carefully considered.
Formerly, we observed that during the extraction of olive tree leaves at high extraction temperatures (200ºC), more active extracts in terms of antioxidant and in-vitro antiproliferative activities were obtained. Chemical characterization of those extracts revealed not only the presence of phenolic bioactives but also 5-(hydroxymethyl)furfural (HMF), a compound related to Maillard reaction. For this reason, in this work, a more exhaustive study has been devised to observe the influence of the temperature in the formation of HMF in SWE extracts of olive leaves. The final aim was to determine the relevance of the HMF present on different SWE extracts in terms of bioactivity. Thus, extracts obtained at different extraction temperatures were produced (50, 75, 100, 125, 150, 175 and 200 ºC) and chemically characterized by LC-MS. HMF was quantified in the extracts by HPLC-DAD. Moreover, the antioxidant activity, the amount of total phenols (Folin method) as well as the anticancer activity of both, extracts and HMF standard, were determined.

INTRODUCTION

Subcritical water extraction is a relatively new advanced extraction technique that is gaining increasing attention nowadays for the extraction and recovery of bioactive compounds from different natural matrices [1]. SWE is based on the extraction with hot water at temperatures below its critical point while maintaining high pressures in order to keep the water in the liquid state during the whole extraction process. These conditions change some physic-chemical properties of water, producing some effects on its solvent-abilities; the increase on the temperature of liquid water will produce a series of effects, including an improved mass transfer as a result of the increment of the solubility of the compounds present on the matrix being extracted as well as a decrease on the surface tension of water that allows its better penetration into the sample matrix. Moreover, the mass transfer kinetics will be also favored by the disruption of intermolecular forces (i.e., van der Waals forces, hydrogen bonds ad dipole attractions) in the sample matrix.
However, the most important parameter of the increment of liquid water temperature is the weakening of hydrogen bonds, resulting in a lower dielectric constant (ε) [2], and thus, the use of SWE could be an alternative to the use of non-polar organic solvents in some applications. From a green chemistry perspective, to avoid the use of organic solvents will provide with an additional advantage for the extraction of highly to medium polarity compounds.

The potential of SWE to obtain bioactive compounds from natural matrices has been already shown in several applications to different matrices. Besides, we recently demonstrated the possibility of attaining newly-formed

antioxidant compounds during a SWE procedure both from glycation model systems as well as from natural real samples [3,4]. These neoantioxidants derived from reactions occurred under the extraction conditions, such as Maillard reaction, caramelization and/or thermooxidation. This possibility could further increase the interest on SWE considering that this technique would be capable not only of recovering the naturally present antioxidants from a natural sample but also to allow the generation of new antioxidant compounds during the same SWE process. This fact could partially explain the increase on antioxidant capacity observed from natural extracts attained at very high temperatures compared to other obtained at milder conditions [5].
However, caution must be taken in any case, as more studies are needed to assess the safety of the obtained extracts. Recently, we observed the formation of 5-hydroxymethylfurfural (HMF) during SWE processes from olive leaves at very high temperatures, while this compound was not found in extracts obtained at lower temperatures [6]. HMF is an intermediate compound that can be formed through Maillard and/or caramelization reactions and, therefore, is broadly generated during food processing and also during cooking, being commonly found in carbohydrate-rich foods such as honey, marmalade, bread and cereal-based foods, among others. Consequently, this compound is consumed in high amounts in diet. However, the presence of HMF has generated some debate as this compound has been demonstrated to be cytotoxic at high concentrations as well as responsible for other effects. Thus, the containing of HMF in foods has generated some safety concerns.
In this work, the formation of HMF during SWE was studied using olive leaves as model natural sample. Different extraction temperatures were tested and the antioxidant and anticancer activities of the generated extracts were studied. Besides, the HMF present in those extracts was quantified and its influence on the formerly detected bioactivities was assessed.

MATERIALS AND METHODS

Samples
Olive tree leaves (variety Hojiblanca) generated as by-products from the olive oil industry were dried and provided by Oleoestepa (Sevilla, Spain). The leaves were dried following a traditional procedure as follows: the olive leaves (once they were separated from the rest of plant materials) were covered to avoid direct light and left ventilated at ambient temperature to remove humidity for ca. 50 days, depending on the ambient conditions of the season. Before extraction, cryogenic grinding of the sample was performed under liquid nitrogen. The samples were stored protected from light at4 °C until their use.

Subcritical water extraction (SWE)
SWE extractions of olive leaves were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller. In order to avoid any possible oxidation effect and to remove the dissolved oxygen, Milli-Q water was sonicated for 15 min prior to use. Extractions were performed at seven extraction temperatures (50, 75, 100, 125, 150, 175 and 200 °C) whereas the static extraction time was maintained for 20 min. An extraction cell heating-up step was applied for a given time prior to any extraction. The warming up time changed depending on the extraction temperature (i.e., 5 min when the extraction temperature ranged from 50 to 100 °C, and 6, 7, 8 or 9 min if the extraction temperature was 125, 150, 175 or 200 °C, respectively). All extractions were done using 11 mL extraction cells at 1500 psi, containing 2 g of sample mixed homogeneously with 3 g of sea sand to prevent clogging of the system. The extracts obtained were protected from light and lyophilized using a freeze-dryer (Virtis Unitop 400 SL, Gardiner, NY, USA).

Antioxidant activity DPPH radical scavenging assay
The DPPH radical scavenging method employed to determine the antioxidant capacity of the extract was based on a procedure previously applied [6]. Briefly, a solution was prepared dissolving 23.5 mg of DPPH in 100 mL of methanol. This stock solution was further diluted 1:10 with methanol. Both solutions were stored at 4 °C until use. Different concentrations of extracts were tested (from 0.125 to 1.0 mg/mL). 25 μL of these extract solutions was added to 975 μL of DPPH diluted solution to complete the final reaction medium (1 mL). After 4 h at room temperature, 300 μL of the mixture was transferred into a well of the microplate, and the absorbance was measured at 516 nm in a microplate spectrophotometer reader (BioTek Instruments, Winooski, VT, USA). DPPH–methanol solution was used as a reference sample. The DPPH concentration remaining in the reaction medium was calculated from a calibration curve. The percentage of remaining DPPH against the extract concentration was then plotted to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% or EC_{50}. Therefore, the lower the EC_{50} value, the higher the antioxidant capacity. Measurements were done, at least, by triplicate.

Determination of total phenols

Total phenols were estimated in the obtained SWE extracts as gallic acid equivalents (GAE), expressed as mg gallic acid/g extract according to the Folin–Ciocalteu assay. The total volume of reaction mixture was miniaturized to 1 mL. 600 μL of water and 10 μL of sample were mixed, to which 50 μL of undiluted Folin–Ciocalteu reagent was subsequently added. After 1 min, 150 μL of 2% (w/v) Na2CO3 was added and the volume was made up to 1.0 mL with water. After 2 h of incubation at 25 °C, 300 μL of the mixture was transferred into a well of the microplate. The absorbance was measured at 760 nm in a microplate spectrophotometer reader (BioTek) and compared to the gallic acid calibration curve (0.025–2 mg/mL) elaborated in the same manner. Data were presented as the average of duplicate analyses.

In-vitro activity against different cancer cells

Cell proliferation in the presence of HMF at concentrations ranging from 1 to 300 μg/mL for 72 h, was determined using 96-well plates by the colorimetric tetrazolium (MTT) assay as follows: the MTT reagent was added and incubated for 3 h at 37 °C in humidified 5% CO2/air atmosphere. After the incubation, the media were aspirated and 200 μL of DMSO was added to each well to dissolve the formazan product by shaking for 30 min. Then, the absorbance at 570 nm was measured in a microplate reader (Anthos 2001 Labtec Instruments GmbH, Wals, Austria). Control and cell viability in the presence of the olive extracts or HMF was measured by counting total and non-viable cells with ADAM Cell Counter (Digital-Bio, Korea) technology. Harvested cells were washed and diluted in PBS to a final concentration of 5×104–4×106 cells/mL. These cells were stained with T solution (total cells) containing PBS Triton 0.5% with propidium iodide (25×10−3 μg/mL), and N solution (non-viable cells), which is composed of the fluorescent dye and PBS (both stain solutions were provided by the manufacturer). Then, the cells were counted separately by measuring the fluorescence at 617 nm.

Cell cycle analyses were performed by flow cytometry (FCM) in an Epics XL instrument (Beckman Coulter, Miami, FL, USA) equipped with an Argon laser at 488 nm, as follows: cells were centrifuged and washed with cold 10 mM phosphate buffer pH 7.4, supplemented with 2.7 mM KCl and 137 mM NaCl (PBS) and centrifuged again. The pelleted cells were resuspended in 75% of cold ethanol, fixed for 1 h at −20 °C, centrifuged and resuspended in 0.5 mL of PBS supplemented with 0.5% Triton X-100 and 0.05% RNase A. Then, cells were incubated for 30 min at room temperature, stained with propidium iodide and analyzed in terms of distribution of cellular DNA content. The apoptosis induced by treatment of the cells with olive extracts or HMF was measured by FCM by determining the amount of apoptotic cells in the sub-G1 phase. Flow cytometry data analysis was made upon gating the cells to eliminate dead cells and debris. A total of 105 cells were measured during each sample analysis.

Liquid chromatography analysis

For the quantification of HMF an Agilent 1100 Liquid Chromatograph equipped with a DAD (Agilent Technologies, Palo Alto, CA, USA) and an ACE C18 column (150 mm×4.6 mm, 3 μm particle size, Advanced Chromatography Technologies, Aberdeen, Scotland) was used. The mobile phase was a mixture of ACN (A) and water (0.1% formic acid, B) eluted according to the following gradient: 0 min, 95% B; 5 min, 95% B; 50 min, 40% B; 53 min, 5% B; 57 min, 5% B; 60 min, 95% B. Flow rate was 0.4 mL/min, the injection volume was 10 μL, and detection was at 280 nm. For the calibration curve, five standard solutions from 0.5 to 50 μg/mL were prepared by appropriate dilution with water from a HMF stock solution (1 mg/mL).

The characterization of the obtained extracts was performed by LC–MS. The instrument employed was an Agilent 1200 liquid chromatograph (Agilent, Santa Clara, CA, USA) equipped with a DAD and autosampler, directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) via an electrospray interface. To carry out the analyses, a Hypersil C18-AR column (150 mm×4.6 mm, d.p. 3 μm) (Thermo Scientific, San Jose, CA) was employed using as mobile phases ACN (A) and water (0.1% formic acid, B) eluted according to the following gradient: 0 min, 95% B; 5 min, 95% B; 50 min, 40% B; 53 min, 5% B; 57 min, 5% B; 60 min, 95% B. The optimum flow rate was 0.4 mL/min while the injection volume was 10 μL. The diode array detector recorded the spectra from 200 to 550 nm. MS was operated under ESI negative ionization mode using the following parameters: dry temperature, 350 °C; dry gas flow, 9 L/min; nebulizer gas pressure, 40 psi; capillary voltage, 3500 V. The instrument acquired data in the range m/z 90–1200.

RESULTS AND DISCUSSION

In **Table 1** the antioxidant capacity values together with the total amount of phenols are shown for the different extraction conditions.

Table 1. SWE extraction temperatures tested, antioxidant capacity (DPPH radical scavenging assay, EC_{50}), and total phenols amount (Folin assay of the obtained extracts from olive leaves. Superscripts indicate differences not statistically significant ($p > 0.05$).

Extraction Temperature (ºC)	EC_{50} (μg/ml)	Total Phenols (mg gallic acid/g extract)	Extraction yield (%)	HMF concentration (μg/mg extract)
50	44.17 ± 4.91	45.376 ± 2.674	15.0	N.D.
75	29.35 ± 1.44	53.324 ± 1.306	18.7	N.D.
100	25.71 ± 1.68[1]	63.873 ± 1.904[1]	22.2	N.D.
125	24.17 ± 1.30[1]	67.068 ± 0.928[1,2]	27.6	0.022 ± 0.001
150	25.08 ± 0.69[1]	66.474 ± 1.581[1,2]	34.7	0.501 ± 0.001
175	21.87 ± 0.68	71.302 ± 4.381[2]	39.2	3.056 ± 0.011
200	19.85 ± 0.51	77.840 ± 3.462	39.3	3.169 ± 0.010

Besides, these extracts were also characterized in terms of the total extraction yield (%, w/w) produced at each extraction conditions and HMF was quantified in the attained olive leaves extracts. As it can be observed, the extraction yield clearly increased with the temperature up to 175 °C, with values ranging from 15.0% to ca. 40%. Maximum values were obtained at 175 and 200 °C. HMF was not detected in those SWE extracts obtained at temperatures lower than 100 °C. However, an increase in the extraction temperature (from125 to 200 °C) gave rise to the formation of HMF, so that the higher concentrations of this compound were obtained using the highest extraction temperatures, reaching up to 3.169 μg/mg extract when the extraction temperature was 200 °C.

The chemical characterization of the extracts was completed using a previously developed method [6] based on LC–MS. Besides the already commented differences on the amounts of HMF detected, other phenolic compounds also differed among the extracts, both, quantitatively and qualitatively. An example of the chromatogram obtained for the extract produced at 200 ºC can be appreciated in **Figure 1**. Besides, in that Figure, the UV-Vis and MS spectra of the peak identified as HMF are shown (compound 1). Among the main identified compounds, hydroxytyrosol (compounds 2), oleoside (compound 3), luteolin-7-glucoside (compound 11) and oleuropein (compound 16) are pointed out. These phenolic compounds are well known for possessing good antioxidant activity.

Regarding the antioxidant capacity, it is observed that increased with the increasing of the extraction temperature. The values obtained for the extracts obtained at 100, 125 and 150 °C did not differ statistically ($p>0.05$). Next, the antioxidant capacity of the HMF commercial standard was studied in order to know to which extent the HMF present on the extracts could have an influence on the determined antioxidant capacity. Several concentration of HMF were assayed, namely from 0.4 to 3.18 μg/mL HMF (that is, the concentrations in which this compound was determined in the extracts. No antioxidant activity was observed for the HMF standard at these concentrations. Thus, from these results it can be concluded that the HMF present in the olive leaves' extracts obtained using SWE at 125, 150, 175 and 200 °C did not influence the antioxidant capacity of these extracts. Moreover, higher concentrations of HMF were tested in order to gain more insight about the possible antioxidant activity of this compound. Concentrations from 5.0 μg/mL up to 1000 μg/mL, were tested. No antioxidant activity was observed at any of these concentrations, confirming the null effect of the HMF on the overall antioxidant capacity of the SWE extracts.

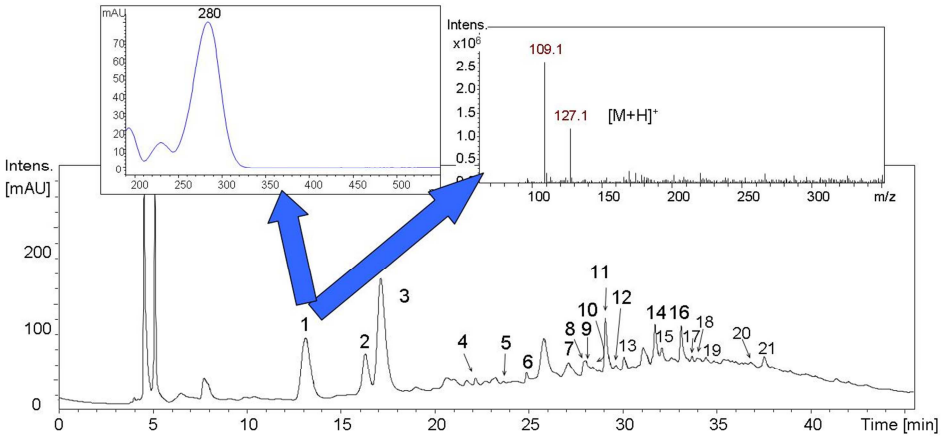

Figure 1. Chromatogram (280 nm) obtained from the LC-DAD-MS analysis of the olive leaves extracts obtained at 200 ºC and UV-Vis and MS spectra of HMF (peak 1). Peak identification: 1, HMF; 2, hydroxytyrosol; 3, oleoside; 4, coumaroyl derivative; 5, elenolic acid-glucoside; 6, luteolin-glucoside, 7, rutin; 8, luteolin-rutinoside; 9, 10-hydroxy-oleuropein; 10, verbascoside; 11, luteolin-7-glucoside; 12, oleuropein-diglucoside; 13, apigenin-glucoside; 14, luteolin-glucoside; 15, oleuropein-diglucoside; 16, oleuropein; 17, oleuropein isomer; 18, oleuroside; 19, oleuropein-derivative; 20, ligstroside; 21, luteolin.

Once the null influence of HMF on the overall antioxidant capacity of the olive leaves' extracts was assessed, the possible relevance of this compound on the antiproliferative activity presented by these natural extracts was studied. To do that, two different human colon adenocarcinoma cell culture models were employed, namely HT-29 and SW-480 cells. Results are shown in **Table 2**.

Table 2. Cell proliferation (%) values of human colon adenocarcinoma cells obtained at different HMF concentrations by the MTT assay or with 300 µg/mL of SWE olive leaves' extracts.

HMF (µg/mL)	HT-29 proliferation (%)	SW-480 proliferation (%)
Control	100	100
1.26	88.1 ± 1.3	87.5 ± 6.2
2.52	85.9 ± 0.1	100 ± 0.1
3.15	91.1 ± 2.0	96.9 ± 4.7
3.78	92.8 ± 0,0	99.3 ± 0.1
4.41	90.9 ± 0.1	ND
5.04	95.1 ± 4,9	94.4 ± 7.3
5.67	99.1 ± 0.1	ND
6.30	87.5 ± 0.1	82.1 ± 8.5
9.45	97.7 ± 0.1	92.2 ± 0.1
12.6	99.2 ± 0.1	73.9 ± 0.0
300	67.7 ± 2.4	68. 5 ± 4.8
SWE 200 ºC	64.5 ± 5.4	80.2 ± 6.5
SWE 50 ºC	57.2 ± 6.8	69.1 ± 13. 8

HT-29 cells were more sensitive to the presence of the compounds found in the studied extracts. It is important to remark that HMF should not have any influence on these results, firstly considering that the most active extract did not contain HMF, and secondly bearing in mind that the HMF amount present on the extract obtained at 200 °C under the conditions employed in this experiment (i.e., 300 µg/mL) was only 0.95 µg/mL, far less than those concentrations tested in the first set of experiments.

CONCLUSIONS

This study demonstrates how HMF is produced in SWE olive leaves extracts at higher concentrations. As expected, the HMF concentration was higher at the highest tested temperatures. However, after appropriate antioxidant capacity and anti-proliferative assays, it was concluded that the HMF found in those extracts did not have any influence on the bioactivities described for the obtained natural extracts. Consequently, the entire mentioned activities could be assigned to the presence of natural bioactive components in those extracts, such as phenolic compounds.

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