FORMATION AND RELEVANCE OF 5-HYDROXYMETHYLFURFURAL IN BIOACTIVE SUBCRITICAL WATER EXTRACTS FROM OLIVE LEAVES.

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ABSTRACT.

Although subcritical water extraction (SWE) has already shown its great potential for the attainment of natural bioactive extracts, concerns still remain on possible unexpected reactions that can arise during the extraction process, usually taking place at high pressure and temperature. It is already well-known that different components might be formed during the SWE extraction protocol due e.g. to Maillard reaction, which can improve the bioactivity of the obtained extracts. On the other hand, the formation of other compounds derived from these reactions, such as 5-hydroxymethylfurfural (HMF), has raised some concerns, mainly related to its safety. In this work, the formation of HMF during subcritical water extraction, at different conditions, from olive leaves has been monitored by using liquid chromatography with mass spectrometry (LC-MS) and diode array detection (LC-DAD). The possible influence of this compound in the overall antioxidant and antiproliferative activities against colon cancer cells has been also studied. Results showed an increase of HMF formation when increasing the extraction temperature, being the maximum concentration achieved at 200 ºC (3.17 μg HMF/mg extract); nevertheless, the HMF contained in the olive leave extracts did not influence the antioxidant capacity or the antiproliferative activity of the natural extracts, thus demonstrating the safety of the SWE process.

Keywords: 5-hydroxymethylfurfural, HMF, subcritical water extraction, SWE, antiproliferative, antioxidant, Maillard reaction

1. INTRODUCTION.

Subcritical water extraction (SWE) has recently emerged as a new possibility for the green extraction of bioactive compounds from natural matrices, particularly, to be used in the food industry (Mendiola, Herrero, Cifuentes & Ibáñez, 2007). This advanced extraction technique takes advantage of the particular properties attained when liquid water is heated to high temperatures. Indeed, 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. The high temperature employed not only improves the mass transfer kinetics during the extraction, increasing the solubility of analytes and decreasing the viscosity of water, but also produces physicochemical changes in water that will directly influence its ability as extracting solvent (Ong, Cheong & Goh, 2006). In fact, the most important parameter in this kind of extractions is the dielectric constant of water. This value measures the polarity of water and varies in great extent according to changes in the temperature if water is maintained in liquid state. Although the dielectric constant of water is around 80 at room temperature, this value might be decreased to around 30 at 250 ºC, which is a similar value to that presented by some organic solvents such as ethanol or methanol (Wiboonsirikul & Adachi, 2008). Thus, SWE could potentially be employed as an alternative to solvent extraction in some applications.

The potential of SWE to extract bioactive compounds from natural matrices has been already demonstrated using different matrices such as plants or algae (Herrero, Cifuentes & Ibáñez, 2006; Mendiola et al., 2007). In fact, SWE antioxidant extracts have been obtained from rosemary (Herrero, Plaza, Cifuentes & Ibáñez, 2010), sea buckthorn (Kumar, Dutta, Prasad & Misra, 2011), rice bran (Fabian, Tran.Thi, Kasim & Ju, 2010), bitter melon (Budrat & Shotipruk, 2009), canola meal (Hassas-Roudsari,

Chang, Pegg & Tyler, 2009), citrus pomaces (Kim, Nagaoka, Ishida, Hasegawa, Kitagawa & Lee, 2009) or grape pomace (Monrad, Howard, King, Srinivas & Mauromoustakos, 2010), among others. Moreover, target compounds possessing other activities have been also extracted by SWE. Recently, the possibility to obtain antioxidant compounds in SWE extracts not naturally present in the original matrix has been studied. In this regard, it has been demonstrated, both in model systems as well as in real samples, that depending on the particular chemical composition of the natural matrix, neoformed antioxidants derived from reactions such as Maillard or caramelization reactions might be produced during SWE processes (Plaza, Amigo-Benavent, del Castillo, Ibáñez & Herrero, 2010a; Plaza, Amigo-Benavent, del Castillo, Ibáñez & Herrero, 2010b). This fact could increase the interest on the bioactive extracts obtained, although it would also require focusing on the safety of the complex extracts attained. Besides, this observation could also partially explain the increase on the antioxidant activity of natural extracts obtained at very high temperatures, compared to others attained at milder conditions (Rodríguez-Meizoso, Jaime, Santoyo, Señorans, Cifuentes & Ibáñez, 2010).

In a previous work, we identified the presence of 5-hydroxymethylfurfural (HMF) in SWE extracts from olive leaves obtained at high temperature (Herrero, Temirzoda, Segura-Carretero, Quirantes, Plaza & Ibáñez, 2011), although the extent of its formation was not studied in detail. HMF is an intermediate compound that can be formed through Maillard and/or caramelization reactions (Durling, Busk & Hellman, 2009) 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 (Teixido, Santos, Pignou & Galceran, 2006). For this reason, HMF has been sometimes selected as a marker of thermal treatments in the food

industryDifferent studies have estimated the consumption and daily intake of this compound up to levels of more than 100 mg per person (Janzowski, Glaab, Samini, Schlatter & Eisenbrand, 2000), although other authors have calculated a mean consumption og 10 mg/day (Rufián-Henares & de la Cueva, 2008). The precise amount of HMF ingested might be highly dependent on the food-related habits (Arribas-Lorenzo & Morales, 2010). In any case, its presence in food has generated concerns on its safety and toxicology. At high concentrations, HMF has demonstrated to be cytotoxic as well as responsible for irritation to the eyes and upper respiratory tract, among other effects (Morales, 2008). Nevertheless, up to now, it is widely regarded as a compound that does not pose a serious risk to human health (Abraham, Gurtler, Berg, Heinemeter, Lampen & Appel, 2011), even if some of its possible metabolites have been identified as genotoxic (Capuano & Fogliano, 2011; Glatt, Schneider & Liu, 2005).

Consequently, it is of great interest to gain insight on the formation of HMF during SWE processes from natural matrices, as well as its possible (positive or negative) bioactivity. For this reason, in this work, the formation of HMF during subcritical water extraction was investigated using olive leaves as natural matrix. Different extraction temperatures were tested and the antioxidant and anticancer activities of the extracts were studied to determine to which extent the HMF generated during the extraction process was responsible for extracts' bioactivities. To the best of our knowledge, this is the first work in which the formation and activities of HMF during SWE processes is monitored.

2. MATERIALS AND METHODS.

2.1. Samples and Chemicals.

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 at 4ºC until their use.

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 95% purity), ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), gallic acid, potassium persulfate, NaCl, KCl and HMF were obtained from Sigma–Aldrich (Madrid, Spain), ethanol from VWR BDH Prolabo (Madrid, Spain) and methanol from Panreac Quimica (Barcelona, Spain). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) and formic acid were provided by Fluka Chemie AG (Buchs, Switzerland). Folin-Ciocalteau phenol reagent and sodium carbonate (Na_2CO_3) were acquired from Merck (Darmstadt, Germany). Acetonitrile (ACN) was obtained from LabScan (Gliwice, Poland). The water used was Milli-Q Water (Millipore, Billerica, MA, USA).

DMEM, fetal bovine serum, antibiotics (penicillin/streptomycin) and PBS were supplied by PAA Laboratories GmbH (Pasching, Austria). Triton-X-100, MTT and propidium iodide were from Sigma-Aldrich (Steinheim, Germany). RNase A was acquired from SERVA Electrophoresis GmbH (Heidelberg, Germany).

2.2. 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 ºC) 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 ºC, and 6, 7, 8 or 9 min if the extraction temperature was 125, 150, 175 or 200 ºC, 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).

2.3. Antioxidant Activity assays.

2.3.1. DPPH radical scavenging assay.

The DPPH radical scavenging method employed to determine the antioxidant capacity of the extract was based on a procedure described by Brand-Williams, Cuvelier & Berset (1995). 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 extracts solutions were 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 were 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.

2.3.2. Trolox equivalent antioxidant capacity (TEAC) assay.

Trolox equivalent antioxidant capacity (TEAC) assay was performed as described by Re, Pellegrini, Proteggent, Pannala & Rice-Evans (1999), with some modifications. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The aqueous $ABTS^{\bullet+}$ solution was diluted with 5 mM phosphate buffer (pH 7.4) to an absorbance of 0.70 (± 0.02) at 734 nm. 10 μL of sample (different concentrations) were added to 1 mL of diluted $ABTS^{\bullet+}$ radical solution. After 50 min at 30 °C, 300 μL of the mixture were transferred into a well of the microplate, and the absorbance was measured at 734 nm in a microplate spectrophotometer reader (BioTek Instruments, Winooski, VT, USA). Trolox was used as reference standard and results were expressed as TEAC values (mmol Trolox/g extract). These values were obtained from at least four different concentrations of each extract tested in the assay giving a linear response between 20-80 % of the blank absorbance. All analyses were done at least in triplicate.

2.4. 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-Ciocalteau assay

(Kosar, Dorman & Hiltunen, 2005). 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) Na_2CO_3 were 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 were 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.

2.5. In vitro activity against different cancer cells.

2.5.1. Cells and Culture conditions

Human colorectal adenocarcinoma HT-29 and SW-480 cells were grown in DMEM supplemented with 5% heat-inactivated fetal calf serum, 2 mM of L-glutamine, 50 U/ml of penicillin G and 50 µg/ml of streptomycin, at 37 ºC in humidified atmosphere and 5 % CO_2.

2.5.2. Cell proliferation and cell viability assays

Cell proliferation in the presence of HMF at concentrations ranging from 1-300 µg/mL for 72 h, was determined using 96-well plates by the colorimetric tetrazolium (MTT) assay described by Mosmann (1983) as follows: the MTT reagent was added and incubated for 3 hours at 37ºC in humidified 5% CO_2/air atmosphere. After the incubation, the media were aspirated and 200 µL of DMSO were 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 $5x10^4$-$4x10^6$ cells/ml. These cells were stained with T solution (total cells) containing PBS-Triton 0.5% with propidium iodide ($25x10^{-3}$ 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.

2.5.3. Cell cycle analysis and apoptosis

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 1h at –20 ºC, 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 as previously described (Castro-Galache et al., 2003).

240 Flow cytometry data analysis was made upon gating the cells to eliminate dead cells
241 and debris. A total of 10^5 cells were measured during each sample analysis.
242
243 **2.6. Quantification of HMF by LC-DAD.**
244 Analyses were performed with an Agilent 1100 Liquid Chromatograph equipped with a
245 DAD (Agilent Technologies, Palo Alto, CA, USA) and an ACE C_{18} column (150 mm x
246 4.6 mm, 3 μm particle size, Advanced Chromatography Technologies, Aberdeen,
247 Scotland). The mobile phase was a mixture of ACN (A) and water (0.1% formic acid,
248 B) eluted according to the following gradient: 0 min, 95% B; 5 min, 95% B; 50 min,
249 40% B; 53 min, 5% B; 57 min, 5% B; 60 min, 95% B. Flow rate was 0.4 ml/min, the
250 injection volume was 10 μL, and detection was at 280 nm. For the calibration curve,
251 five standard solutions from 0.5 to 50 μg/mL were prepared by appropriate dilution with
252 water from a HMF stock solution (1 mg/ml).
253
254 **2.7. LC-MS characterization of the olive leaves SWE extracts.**
255 To chemically characterize the SWE extracts obtained at the different temperatures
256 tested, a LC-MS method previously developed was used (Herrero et al., 2011). The
257 instrument employed was an Agilent 1200 liquid chromatograph (Agilent, Santa Clara,
258 CA, USA) equipped with a DAD and autosampler, directly coupled to an ion trap mass
259 spectrometer (Agilent ion trap 6320) via an electrospray interface. To carry out the
260 analyses, a Hypersil C_{18}-AR column (150 mm×4.6 mm, d.p. 3 μm) (Thermo Scientific,
261 San Jose, CA) was employed using as mobile phases ACN (A) and water (0.1% formic
262 acid, B) eluted according to the following gradient: 0 min, 95% B; 5 min, 95% B; 50
263 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. On the other hand, the MS was operated under ESI negative ionization mode using the following parameters: dry temperature, 350 ºC; 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.

2.8. Statistical analysis.

IBM SPSS Statistics software v.19 was employed for data elaboration and statistical analysis using a level of significance set at 95 %. One-way analysis of variance (ANOVA), together with least significant differences (LSD) model, was employed to assess differences among extracts. Differences were considered statistically significant if $p < 0.05$.

3. RESULTS AND DISCUSSION.

3.1. SWE extraction of olive leaves and determination of HMF.

In a previous work from our group, HMF was identified in bioactive SWE extracts obtained at 200 ºC from olive oil leaves (Herrero et al., 2011). Although this extract was the most active in terms of antioxidant activity, the contribution of HMF to the final bioactivity was not clear. As mentioned, the goal of the present work was to study the formation of HMF under subcritical water extraction conditions and thus, different extraction temperatures, from 50 to 200 ºC in 25 ºC steps, were tested. However, according to previous reports conducted in our lab (Herrero et al., 2011) both the extraction time and pressure were always maintained constant at 20 min and 1500 psi, respectively, considering their low statistical influence on the extraction process.Chemical and functional characterization of the extracts was also carried out in

289 order to find any possible correlation between the presence of HMF and the observed
290 activities.
291 Once the SWE extracts from olive leaves were collected, the amount of HMF was
292 determined by LC-DAD. Figure 1 shows the UV-Vis and MS spectra of HMF along
293 with its chemical structure. Calibration curve was obtained with a regression coefficient
294 equal to 0.999, demonstrating the good possibilities for the quantitative determination of
295 HMF. Limits of detection (LOD) and limits of quantification (LOQ) of 13.7 and 45.8
296 ng/mL were achieved, respectively(calculated as the minimum analyte concentration
297 giving a S/N ratio equal to 3 for LOD or 10 for LOQ). Table 1 shows the extraction
298 yields (defined as the percentage of dried extract obtained from the original sample
299 used) attained after the SWE extraction of olive leaves, as well as the concentration of
300 HMF in the extracts. As it can be observed, the extraction yield clearly increased with
301 the temperature up to 175 ºC, with values ranging from 15.0 % to ca. 40 %. Maximum
302 values were obtained at 175 and 200 ºC. As it can be also observed in Table 1, HMF
303 was not detected in those SWE extracts obtained at temperatures lower than 100 ºC.
304 However, an increase in the extraction temperature (from 125 to 200 ºC) gave rise to the
305 formation of HMF, so that the higher concentrations of this compound were obtained
306 using the highest extraction temperatures, reaching up to 3.169 μg/mg extract when the
307 extraction temperature was 200 ºC. These results are in agreement with previous work
308 carried out in our laboratory, which indicated the occurrence of Maillard reaction during
309 SWE processes at high extraction temperatures (Plaza et al., 2010a; Plaza et al., 2010b).
310 As expected, the amount of HMF in the extracts was significantly increased ($p < 0.05$)
311 with the temperature, indicating a higher extent of Maillard reaction at the maximum
312 tested temperature.

The chemical characterization of the extracts was completed using a previously developed method (Herrero et al., 2011) 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. Comparing the extracts obtained at 50 ºC and at 200 ºC, it could be observed (see Figure 2 and Table 2 for compounds identification) how the extracts obtained at 200 ºC were the most complex in terms of number of compounds. In fact, some compounds present at 200 ºC were not detected at 50 ºC, such as elenolic acid glucoside (compound 5), rutin (compound 7), luteolin-rutinoside (compound 8), verbascoside (compound 10). Nevertheless, among the compounds present on both extracts, some of them were detected in significantly higher amount on the SWE extract obtained at 200 ºC, as it can be seen for hydroxytyrosol and oleoside (see Figure 2), among others. Besides, interestingly, the least polar identified compounds (compounds 18-21), most notably luteolin, were only detected in the extract obtained at 200 ºC. This observation is in agreement with the fact that at higher temperatures the dielectric constant of water decreases allowing a better solubilization of less polar compounds.

Once the presence and concentration of HMF was confirmed in the SWE extracts from olive leaves, their functional characterization was carried out, paying special attention to the possible correlation between amount of HMF and observed bioactivity.

3.2. Functional characterization of the obtained extracts.

The functional characterization of the extracts was based on the determination of their antioxidant and antiproliferative activity. Firstly, the determination of the antioxidant capacity of the extracts was determined.

3.2.1. Antioxidant capacity measurements.

Two different methods, DPPH-radical scavenging method and trolox-equivalents antioxidant capacity assay (TEAC) were employed to determine the total antioxidant capacity of the attained extracts. The obtained results are summarized in Table 1. The DPPH method provided the EC_{50} value, usually defined as the amount of antioxidant needed to reduce to the half the initial radical concentration. Thus, the lowest the EC_{50}, the highest the antioxidant capacity. Consequently, it can be observed how the antioxidant capacity of the extracts was increased with the increasing of the extraction temperature. As it can be also observed in Table 1, the values obtained for the extracts obtained at 100, 125 and 150 ºC did not differ statistically ($p > 0.05$). These results were further confirmed using the TEAC assay that provided a similar trend. To know to which extent the HMF present on the extracts could have an influence on the determined antioxidant capacity, different solutions of HMF standard were also included in the determinations, at concentrations similar to those found in the extracts obtained at 175 and 200 ºC at their corresponding tested concentrations (from 0.4 to 3.18 μg/mL HMF). No antioxidant activity was observed for the HMF standard at these concentrations (data not shown). Thus, from these results it can be inferred that the HMF present in the olive leaves' extracts obtained using SWE at 125, 150, 175 and 200 ºC did not influence the antioxidant capacity of these extracts. To further study the possible antioxidant effect of HMF, higher concentrations were tested in both methods, from 5.0 μg/mL up to 1000 μg/mL, corresponding the latter to a concentration two orders of magnitude higher than the maximum concentration of HMF found in the SWE extract obtained at 200 ºC. 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.

Once the influence of the HMF on the overall antioxidant capacity of the extracts could be effectively discarded, the amount of total phenols present on the extracts was determined. This value, obtained through the use of the Folin method, offers an estimation of the amount of phenolic compounds present on the extracts that could potentially be responsible for their antioxidant capacity. The results obtained from these determinations are summarized in Table 1. As it can be observed, the amount of total phenols, expressed as mg gallic acid equivalents/g extract, increased with the extraction temperature. Nevertheless, the values obtained for the extracts obtained at 100, 125 and 150 ºC were very similar, as it happened with the antioxidant capacity. In fact, a clear positive correlation (R^2 = 0.867) was appreciated between antioxidant capacity measured using the DPPH method and the amount of total phenols found, thus, suggesting that the antioxidant capacity of the extracts could be most probably correlated with the phenolic compounds present. Moreover, the differences among extracts were not only quantitative but also qualitative, as it has been already mentioned. In fact, the presence of different compounds (see Section 3.1.), even in minor amounts, could have an important influence on the antioxidant activity determined. Besides, the presence of other compounds derived from Maillard and caramelization reactions form at high temperatures which could potentially increase the overall antioxidant activity cannot be discarded.

3.2.2. Antiproliferative activity.

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

388 cells. Considering the amount of HMF formed in the olive leaves' extracts, a first set of
389 experiments was carried out using increasing concentrations of HMF (from 1 to 13
390 μg/ml) in order to see its influence on the cell cultures growth at a concentration range
391 in the same order of magnitude to that found in the extracts (Table 3). As it can be
392 observed, by using the MTT assay, it could be assessed that the proliferation of the two
393 cell lines employed was not affected regardless of the HMF amount used within the
394 concentration range studied.
395 In order to study in more detail the possible effect of the HMF, two olive leaves extracts
396 were considered as models, namely, the extracts obtained at 50 and 200 ºC. In this way,
397 the possible antiproliferative activities of these extracts could be compared including a
398 HMF-free extract (the one obtained at 50 ºC) and the extract which possessed the
399 highest level of HMF (obtained at 200 ºC). Both extracts showed a modest
400 antiproliferative effect when added to the cell cultures at a concentration of 300 μg/ml,
401 as it can be observed in Table 4. Under these conditions, the proliferation of the HT-29
402 cells was reduced to 64.5 and 57.2 % for the extracts obtained at 200 and 50 ºC,
403 respectively, whereas the proliferation of SW-480 cells was less inhibited, to 80.2 and
404 69.1 %, respectively. Thus, the HT-29 cells were more sensitive to the presence of the
405 compounds found in the studied extracts. It is important to remark that HMF should not
406 have any influence on these results, firstly considering that the most active extract did
407 not contain HMF, and secondly bearing in mind that the HMF amount present on the
408 extract obtained at 200 ºC under the conditions employed in this experiment (i.e., 300
409 μg/ml) was only 0.95 μg/ml, far less than those concentrations tested in the first set of
410 experiments (see Table 3). A higher concentration (300 μg/ml) of HMF was also tested
411 to test the antiproliferative activity associated to this compound at extremely high
412 concentrations. As can be observed from the results presented on Table 4, at this

concentration, the HMF inhibited the proliferation of both cells in a similar extent than the extract obtained at 50 ºC. Thus, a relatively modest antiproliferative activity could be associated to HMF, although exceptionally high concentrations of this compound should be employed to achieve this effect. It is important to remark that this concentration is 300-times greater than the maximum amount of HMF obtained in the SWE extracts from olive leaves. Therefore, from the data shown in Table 4, it can be deduced that the compounds identified in the extracts, mainly phenolic compounds (see Table 2 and Figure 2), could be most probably responsible for the antiproliferative activity of the extracts.

In order to analyze more in deep the mechanisms behind the inhibition of the cell proliferation exerted by the SWE extracts and HMF at 300 μg/ml, the study of possible cytotoxic effects on the colon cancer cells was carried out. For this purpose, HT-29 and SW-480 cells were incubated for 48 h in the presence of SWE extracts or HMF, at the indicated concentration, and cell viability was determined. The results indicated that whereas HT-29 cells were not affected by the treatments (cell viability values were close to 95 % with respect to control cells), SW-480 cells displayed cell survival values ranging 70% and 80% after treatments with SWE extracts and HMF, respectively. Consistent with these cell viability values, distribution of cellular DNA by FCM analysis in the HT-29 cells indicated the absence of apoptosis induction by treatment with HMF or SWE extracts (data not shown). On the contrary, as it can be observed in Figure 3, HMF and SWE extracts induced a level of apoptosis close to 35% in the case of SW-480 cells, as determined by the cell population content obtained in the sub-G1 phase corresponding to the histograms showing the distribution of cellular DNA.

4. CONCLUSIONS.

In this study, it has been clearly shown for the first time how HMF is produced during SWE extraction processes at high temperatures from olive leaves, being the formation rate maximum at the highest tested extraction temperature (200 ºC). Nevertheless, from the data collected it seems clear that the presence of this compound does not have influence either on the overall antioxidant capacity of the obtained extracts or on their antiproliferative activity against colorectal adenocarcinoma HT-29 and SW-480 cells. Consequently, the entire mentioned activities could be assigned to the presence of natural bioactive components in those extracts, such as phenolic compounds.

ACKNOWLEDGEMENTS.

This work has been financed by AGL2008-05108-C03-01 and AGL2011-29857-C03-01 (Ministerio de Educacion y Ciencia), CSD2007-00063 FUN-CFOOD (Programa CONSOLIDER-INGENIO 2010) and by ALIBIRD, S2009/AGR-1469 (Comunidad de Madrid) projects. M.H. would like to thank MICINN for a “Ramón y Cajal” research contract. M.C.P. thanks MICINN for her “Juan de la Cierva” contract.

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FIGURE LEGENDS.

Figure 1. 5-Hydroxymethylfurfural (HMF) UV-Vis (A) and MS (B) spectra and chemical structure of HMF.

Figure 2. Chromatograms (280 nm) obtained from the LC-DAD-MS analysis of the olive leaves extracts obtained at 50 (A) and 200 ºC (B). For peak identification see Table 2.

Figure 3. Representative histograms of the distribution of cellular DNA in SW-480 cells obtained by FCM: (A) control of untreated SW-480 cells; Histograms B, C and D correspond to SW-480 cells treated with 300 µg/mL for 48 h with: pure HMF (B); olive leave extract obtained at 200 ºC (C) and olive leave extract obtained at 50 ºC (D). Arrows indicate the presence of the apoptotic sub-G1 cell population.

Table 1. SWE extraction conditions, extraction yield (%), antioxidant capacity (DPPH radical scavenging assay, EC_{50}, and trolox-equivalents antioxidant capacity assay, TEAC), total phenols amount (Folin assay) and HMF concentration of the obtained extracts from olive leaves. Superscripts indicate differences not statistically significant ($p > 0.05$) among the extracts. All the data given as mean ± standard deviation of, at least, three replicates.

Extraction Temperature (ºC)	Time (min)	Extraction yield (%)	EC_{50} (μg/ml)	TEAC (mmol/g)	Total Phenols (mg gallic acid/g extract)	HMF concentration (μg/mg extract)
50	20	15.0	44.17 ± 4.91	0.997 ± 0.009	45.376 ± 2.674	N.D.
75	20	18.7	29.35 ± 1.44	1.122 ± 0.016	53.324 ± 1.306	N.D.
100	20	22.2	25.71 ± 1.68[a]	1.314 ± 0.007[a]	63.873 ± 1.904[a]	N.D.
125	20	27.6	24.17 ± 1.30[a]	1.364 ± 0.000[a]	67.068 ± 0.928[a,b]	0.022 ± 0.001
150	20	34.7	25.08 ± 0.69[a]	1.408 ± 0.030[a]	66.474 ± 1.581[a,b]	0.501 ± 0.001
175	20	39.2	21.87 ± 0.68	1.992 ± 0.012	71.302 ± 4.381[b]	3.056 ± 0.011
200	20	39.3	19.85 ± 0.51	2.301 ± 0.015	77.840 ± 3.462	3.169 ± 0.010

570 **Table 2.** Phenolic compounds tentatively identified in the SWE extracts from olive
571 leaves.
572

ID	Tr (min)	Identification	UV-Vis max (nm)	$[M-H]^-$	Main fragments
1	12.1	5-Hydroxymethylfurfural [a]	283	127.1[b]	109.1, 93.1[b]
2	16.7	Hydroxytyrosol [a]	278	153.9	123.8
3	17.4	Oleoside	176	389.1	226.8, 182.9, 164.9, 121.1
4	22.5	Coumaroyl derivative	295s, 310	491.2	325.1, 162.9
5	24.0	Elenolic acid-glucoside	296, 321	403.1	371.1, 222.9, 179.0
6	25.1	Luteolin-diglucoside	331	609.5	447.2, 285.3
7	27.5	Rutin	340	609.4	300.9, 179.1
8	28.1	Luteolin-rutinoside	340	593.2	285.0
9	28.2	10-hydroxy-oleuropein	280	555.4	403.2, 393.4, 323.3, 291.0
10	28.5	Verbascoside	290, 325	623.5	461.2, 315.1
11	29.3	Luteolin-7-glucoside [a]	346	447.6	284.9
12	29.8	Oleuropein-diglucoside	280	701.4	539.4, 377.4, 307.2, 275.3
13	30.3	Apigenin-rutinoside	335	577.7	269.0
14	32.1	Luteolin-glucoside	336	447.8	284.9
15	32.7	Oleuropein-diglucoside	280	701.4	539.2, 377.1, 307.1, 275.0
16	33.3	Oleuropein [a]	280	539.3	377.2, 307.4, 275.7
17	33.9	Oleuropein isomer	280	539.3	377.1, 307.2, 275.6
18	34.1	Oleuroside	280	539.4	377.2, 307.2, 275.4
19	35.1	Oleuropein derivative	280	601.6	539.2, 377.1, 307.1, 275.2
20	36.1	Ligstroside	280, 320s	523.4	361.1, 291.1, 259.4
21	38.0	Luteolin	344	285.7	

573 [a] identification corroborated using commercial standards;

574 [b] Parent and fragment ions detected in ESI positive ionization mode; s, shoulder.

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576

577 **Table 3**. Cell proliferation (%) values[a] of human colon adenocarcinoma cells different
578 HMF concentrations.
579

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

580 [a] Values are expressed as the mean of at least three independent experiments ± SEM.
581 ND: Not determined
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583
584

585 **Table 4**. Cell proliferation (%) values[a] upon incubation of human colon
586 adenocarcinoma cells with 300 µg/mL of SWE olive leaves' extracts or HMF.
587

Extract	HT-29 proliferation (%)	SW-480 proliferation (%)
Control	100	100
HMF	67.7 ± 2.4	68. 5 ± 4.8
200 ºC	64.5 ± 5.4	80.2 ± 6.5
50 ºC	57.2 ± 6.8	69.1 ± 13. 8

588 [a] Values are expressed as the mean of five independent experiments ± SD.
589
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