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ARTICLE

Testing and Enhancing the *in Vitro* Bioaccessibility and Bioavailability of *Rosmarinus officinalis* Extracts with a High Level of Antioxidant Abietanes

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An antioxidant-enriched extract (RE) was obtained from rosemary (*Rosmarinus officinalis*) by supercritical fluid extraction to be used as an ingredient to design functional foods. The optimized mixture (42 mg RE g^{-1} sunflower oil) was submitted to *in vitro* digestion and absorption tests (using Caco2 cells) to investigate the effect of these processes on its DPPH scavenging activity and also whether its major abietanes (tricyclic diterpenes) might be bioaccessible and bioavailable. Results indicated that supplementation of the rosemary extract with sunflower oil and lecithin (37 mg g^{-1}) enhanced abietanes micellation (almost 2-fold). *In vitro* digestion of the mixture including RE, sunflower oil, and lecithin reduced 50% the bioaccesibility in terms of antioxidant activity. Bioavailability was 31%. It was evidenced that this activity was not due to the original levels of carnosol, carnosic acid, and methyl carnosate (which only 47% remained after digestion) but due to their derivatives and digestion products.

KEYWORDS: Antioxidant activity; carnosic acid; carnosol; functional food; methyl carnosate; lamiaceae; phenolic diterpenes; rosemary.

INTRODUCTION

Nowadays, many new food products launched in the market as functional foods and claiming a beneficial impact on consumer's health need bioavailability studies of the supplemented bioactive ingredients (*1*). Most of the existing studies are related to the bioavailability of water-soluble compounds due to the difficulties of studying the lipid digestion and absorption. Digestion of exogenous lipids followed a more complex mechanism than that of water−soluble products, since their intestinal absorption requires the formation of dietary mixed micelles. The composition and formation of these micelles is highly dependent on the initial food matrix (*2*).

Digestion is the breaking down of food into smaller components that can be absorbed by the bloodstream and used or excreted. In humans, preparation for digestion begins with the mechanical and chemical digestion in the mouth, where food is chewed and mixed with saliva, breaking down mainly polysaccharides. The stomach continues to break food down mechanically and chemically through the churning of the stomach and mixing with pepsine and some gastric lipases. At this stage, mainly protein and peptide degradation takes place; lipid digestion via acid lipases accounts only for approximately 10−30% of overall triglycerides hydrolysis (*3*). The presence of lipids in the duodenum stimulates the secretion of bile salts, phosphatidylcholine, and cholesterol from the gall bladder and pancreatic fluids (containing pancreatic lipase/colipase, etc.) from the pancreas. Lipases bind at the surface of the oil droplets formed by the gall bladder emulsifiers, hydrolyzing lipids into their digestion products and generating a series of colloidal species, including micelles, mixed micelles, vesicles, and emulsion droplets. Lipophilic compounds will be absorbed by intestinal enterocytes only if they are inside or forming part of the dietary mixed micelles (*4*).

The rosemary (*Rosmarinus officinalis*) abietanes (tricyclic diterpenes) showed antioxidant, antimicrobial, anti-inflammatory, antitumorigenic, and chemopreventive activities (*5*, *6*), which makes them suitable candidates as bioactive ingredients to design functional foods (*7*,*8*). However, they also decrease the hydrophobic interactions between acyl chains in model membranes (*9*), so perhaps they could modify the lipidic profile of the natural dietary mixed micelles. Moreover, these abietanes, in particular carnosic acid and carnosol, are potent inhibitors of the pancreatic lipases, showing half maximal inhibitory concentrations (IC_{50}) of 12 μg mL^{-1} and 4.4 μg mL^{-1} respectively (*10*), suggesting that, during the digestion of those functional foods, these bioactive compounds might not reach the bloodstream, since they could inhibit the dietary mixed micelles formation.

In this work, the bioaccesibility of an antioxidant-rich rosemary extract (obtained by supercritical fluid extraction), containing a high level of abietanes, was investigated using an *in vitro* digestion model. The potential bioavailability of the bioactive

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constituents was estimated by Caco-2 cell absorption tests. The objective of this research was to study the effect of both processes on the abietanes levels and on their antioxidant activities. Recommendations are suggested to formulate functional foods containing rosemary extracts in order to enhance their bioaccessibility and bioavailability.

MATERIALS AND METHODS

Material. Refined sunflower oil (max. 0.2 acidity) and Iberian pork lard were purchased at a local supermarket. Soy lecithin was obtained from Lab. Ynsadiet (Leganes, Spain) in drops containing 500 mg of soy lecithin in soy oil. All the experiments were performed using the same bottle and box.

Rosemary (*Rosmarinus officinalis*) leaves purchased from a herbalist's shop (Murcia, Spain) were dried using a traditional method (*11*), ground under cryogenic carbon dioxide, and stored in amber flasks at −20 °C. Afterward, ground rosemary leaves were submitted to supercritical fluid extraction (SFE) as described by Señoráns et al. (*11*). The extraction cell (with a volume of 285 mL) was filled with 60 g of ground leaves and 75 g of washed sea-sand (Panreac, Spain). The dynamic extraction was carried out on a pilot-scale supercritical fluid extractor (Iberfluid, Spain) at 150 bar and 40 °C, using 7% ethanol as modifier and 50 mL min^{-1} CO_2 flow. The fractionation occurred in two separation vessels (separators) with an independent control of temperature and pressure. The rosemary extract (RE) utilized in this study was the SFE fraction obtained in separator 1 (75 bar and 40 °C) because according to the authors it showed the highest antioxidant activity (*11*).

Reagents. 2,2-Diphenyl-1-picrylhydrazyl (DPPH•), α-tocopherol, pepsin from porcine stomach, pancreatin, and bile salts were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Sodium bicarbonate was obtained from Merck KGaA (Darmstadt, Alemania), and sodium hydroxide, hydrochloric acid, acetonitrile, and methanol were obtained from Panreac (Barcelona, Spain). Carnosic acid was obtained from Alexis Co. (Lausen, Switzerland).

***in Vitro* Digestion.** The rosemary extract (50−450 mg) was mixed with sunflower oil or lard (6−10 g) in several concentrations. α-Tocopherol (0.7 and 2.3 mg g^{-1}) or soy lecithin (18 or 37 mg g^{-1} fat) was also added, depending on the tested formulation.

These preparations were submitted to *in vitro* digestion following a combination of methods proposed by Granado-Lorencio et al. (*12*) and Hornero-Méndez and Mínguez-Mosquera (*13*) which were based on the method previously developed by Miller et al. (*14*). Briefly, the samples (rosemary extract dissolved in the oil or lard with or without lecithin or tocopherol) were mixed with acidified water (pH 2 adjusted with 6 M HCl) until a final weight of 40 g. Then, 3.13 mL of pepsin solution (1.6 g of pepsin in 10 mL of 0.1 M HCl) were added and the mixture was incubated for 1 h (gastric digestion) at 37 °C with slow rotary stirring at 150 rpm (Aerotron, Biogen). Afterward, pH was adjusted to 6 with 1 M $NaHCO_3$ and 10 mL of a pancreatic preparation (40 mg of pancreatin and 250 mg of bile salts in 10 mL of 0.1 M $NaHCO_3$) was added. The mixture pH was again adjusted to 7.5 with 1 N NaOH and stirred and incubated for 2 h (intestinal digestion) under the same conditions as those above-described for gastric digestion.

Isolation of the fraction containing the mixed micelles (generated after the intestinal digestion step) was performed following three procedures: overnight sedimentation (16 h, 37 °C, darkness), low-speed centrifugation (4000 rpm, 40 min, 20 °C) (5810R Eppendorf Iberica, Madrid, Spain), and high-speed centrifugation at 50.000 rpm (183 900*g*) for 35 min at 20 °C in a Centrikon T-1190 ultracentrifuge (Kontron Instruments, Milan, Italy) equipped with a Type 70.1 Ti Rotor (Beckman Coulter Canada Inc., Missisauga, ON, Canada).

After centrifugations or sedimentation, the digested samples were separated into a poorly emulsified oil phase, a highly emulsified aqueous phase, and a precipitated pellet phase. The oily fraction (OF) typically contains dispersed oil including part of the rosemary extract. This fraction should mimic the one that *in vivo* is wasted in faeces or transformed by colonic flora. The aqueous phase is a turbid liquid emulsion because it contains the rosemary extract included in micellar and vesicular structures (MF). This fraction corresponds to the "bioaccesible" fraction, since it can be absorbed by the Caco-2 cells. The pellet contains precipitated insoluble (calcium) soaps of fatty acids liberated during the pancreatic digestion. Since no abietanes were found in this phase, it was not further analyzed. Obtained OF and MF fractions were stored at −20 °C for further analysis, except for the MF isolated using the low-speed centrifugation. This mixed micellar fraction was immediately applied to Caco-2 cell cultures.

Quantification of Dietary Mixed Micelles. First, the amount of mixed micelles was estimated using a light microscope and a Neubauer cell counter chamber (Brand, Germany). The number of micelles mL^{-1} correlated with absorbance at 660 nm (turbidity) follows the equation: Micelles $mL^{-1} = (7 \times 10^7)Abs_{(660nm)} - 1 \times 10^7$ ($r^2 = 0.9904$). Therefore, the mixed micelles' concentration was followed later on by measuring turbidity using a spectrophotometer (Genesys 20, ThermoSpectronic). The dry matter content of the micellar fraction was also calculated by placing the isolated fractions in an oven at 40 °C until constant weight.

Caco-2 Absorption Study. Human colorectal adenocarcinoma cell line Caco-2 (ATCC HTB-37) obtained from the Americam Type Culture Collection (Rockville, MD, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose content (4.5 g L^{-1}) and supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% nonessential amino acids at 37 °C in at humidified atmosphere containing 5% CO_2.

The cytotoxic effect of the mixed micelles fraction on Caco-2 cells was tested using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, according to Mosmann (*15*). Monolayers of Caco-2 cells in 24-multiwell plates were incubated with DMEM containing different concentrations of the mixed micelles fraction for 6 h at 37 °C. Cells were then washed with phosphate buffered saline (PBS), and 0.5 mg mL^{-1} of MTT (Sigma, Spain) was added to each well and incubated for 4 h at 37 °C. Supernatants were discarded and formazan crystals dissolved in an extraction solution (10% sodium dodecyl sulfate in a mixture of dimethyl formamide and water 1:1 v/v, adjusted to pH 4.7 with acetic acid) overnight at 37 °C. Formazan quantification was performed by measuring the optical density at 570 nm using a multiscanner autoreader (Sunrise, Tecan, Germany) with the extraction solution as a blank. The data were plotted as dose−response curves, from which the concentration required to reduce by 50% the number of viable Caco-2 cells (CC_{50}) after 6 h of incubation with the different extracts was obtained.

For absorption assays, cells were seeded onto a twelve-well Transwell clear polyester permeable membrane support (0.4 μm pore size, inserts of 12 mm diameter, Costar, Corning, NY) at a density of 2×10^4 cell per insert. Culture medium was replaced every three days, and cells were allowed to differentiate for 21 days before experiments. The integrity of the cell layer was evaluated by measuring the transepithelial electrical resistance (TEER) (Evon, Sarasota, FL), and cells differentiation was determined by measuring alkaline phosphatase activity (ALP) as described by Bestwick and Milne (*16*) and Dihal et al. (*17*). Only inserts with values above 200 Ω cm^2 and ALP values of 12−14 mUI mg^{-1} protein were utilized.

The isolated MF fractions (60 μL) and a standard solution of carnosic acid in DMSO (1.1 mg mL^{-1}) (12 μL) were added to the apical (upper) compartment of the wells and left incubating during 3 or 6 h. Then, the cell monolayer (extracted as described by Palmgrén et al. (*18*)) and the apical and basolateral (lower) compartments were collected and stored at −20 °C for further analysis.

Abietanes Analysis. A new method was developed to study the major abietanes present in the rosemary extracts and derivative products. Samples obtained after the Caco-2 absorption test (a pool of 12 identical wells) were concentrated in a Sep-pak C-18 cartridge (Waters, Milford, MA) using acetonitrile with 40 mg mL^{-1} butylated hydroxytoluene (BHT) as extraction solvent. Eluted samples were dried under a nitrogen stream and redisolved in DMSO.

Abietanes detection and identification were performed with an HPLC system coupled to a diode array detector (ProStar, Varian). Direct or concentrated samples were injected onto a reversed phase HPLC column (Novapack C18 4 μm, 150 mm × 3.9 mm, Waters) and eluted with a flow rate of 1 mL min^{-1} and a mobile phase containing (A) water/acetic acid (1%) and (B) acetonitrile/acetic acid (1%), following the gradient: from 0 to 30 min, 50 to 100% B.

Mass spectrometry was used in order to confirm the nature of the main antioxidants present in extracts. They were directly infused in a TSQ Quantum (Thermo Scientific, San Jose, CA) triple quadrupole analyzer

via an electrospray interface. The ionization parameters were as follows: spray voltage, 3500 V; sheat gas pressure, 35 psi; capillary temperature, 350 °C; colission gas, Ar (Alphagaz 1 quality, Air Liquide, Madrid Spain); collision pressure, 1.2 psi; collision energy was set depending on the molecule and ranged from 10 to 23 V. Identification of compounds was done using the single reaction monitoring mode (SRM) and full scan in the third quadrupole.

Carnosic acid, carnosol, and methyl-carnosate were tentatively identified on the basis of their retention times, UV−vis spectra, and MS, which were consistent with data previously published by other authors (*11*, *19*, *20*). The quantization of the aforementioned compounds was at 235 nm using a standard curve of carnosic acid and was expressed as mg equivalent to carnosic acid.

The method was validated following ICH guidelines (*21*) and the IUPAC technical report of 2002 (*22*), determining precision, trueness, selectivity, linearity (data not shown), and the limits of detection (LoD) and quantization (LoQ) as more relevant parameters, which were found to be 0.07 and 0.10, 0.08 and 0.11, 0.11 and 0.24 mg L^{-1} for carnosic acid, carnosol, and methyl carnosate, respectively.

Antioxidant Activity. The antioxidant activity was analyzed as the DPPH$^{\bullet}$ scavenging capacity of the different samples and fractions by measuring the disappearance of DPPH$^{\bullet}$ at 515 nm (ε = 12 500 M^{-1} cm^{-1} (*23*)) using a spectrophotometer, according to the procedure of Sánchez-Moreno et al. (*24*).

Specific sample dilutions (25 μL) were mixed with 975 μL of a DPPH$^{\bullet}$ solution (0.06 mmol L^{-1}) in methanol in 1 mL cuvettes and incubated for 180 min at room temperature in darkness. Results were corrected for the effect of pH and $NaHCO_3$ additions on the radical stability and absorbance. Trolox was used as reference standard, and results were expressed as trolox equivalent antioxidant capacity (TEAC) values.

Statistical Analysis. One way analysis of variance (ANOVA) was performed using a Statgraphics Plus 3.1 for Windows software (Statistical Graphics Corporation, Maryland, USA). The mean comparison test used was Fisher's least significant differences procedure. All the presented values are the mean ± SD of three separate experiments.

RESULTS AND DISCUSSION

In vitro digestion models are tools to facilitate the evaluation of the effect of a drastic process such as the human digestion on certain compounds of interest. Although the experimental details of these models differ slightly from author to author, the basic principles of operation are similar. The most controversial step in the digestion of lipid-rich foods is the isolation of the dietary mixed micelles fraction (MF). Some authors preferred the use of high speed centrifuges to separate smaller size mixed micelles (*4*), other proposed overnight sedimentation as more consistent with physiological events such as the estimated transit time through the stomach and small intestine (fed conditions, up to 12−14 h) (*12*). The latter studies (*12*) found no significant differences (depending on the analyzed food sample) between sedimentation and a low-speed centrifugation method. Therefore, the three described methods were compared to determine the best option for the isolation of the mixed micellar fractions obtained after the *in vitro* digestion of the rosemary extract (RE).

The rosemary SFE extract contained carnosic acid, carnosol, methyl-carnosate (respectively 25.81 ± 5.35, 8.91 ± 2.82, and 1.22 ± 0.36 mg equivalents of carnosic acid g^{-1} RE (n = 5)), and other minor diterpenes-derivatives. The presence of the three abietanes was confirmed by their UV spectra, retention times, and mass spectra (**Figure 1**), and they were in concordance with other publications (*11*, *19*, *20*). These compounds were also identified when RE was mixed with sunflower oil (42 mg g^{-1}) prior to the *in vitro* digestion process (see later) and after MF isolation using the three methods. The high speed centrifugation yielded a 10-fold lower micelles concentration than the other two methods. Overnight sedimentation showed slightly lower micelles concentration than low speed centrifugation and higher water content in the oily fraction (30.2%) than the one obtained by low speed

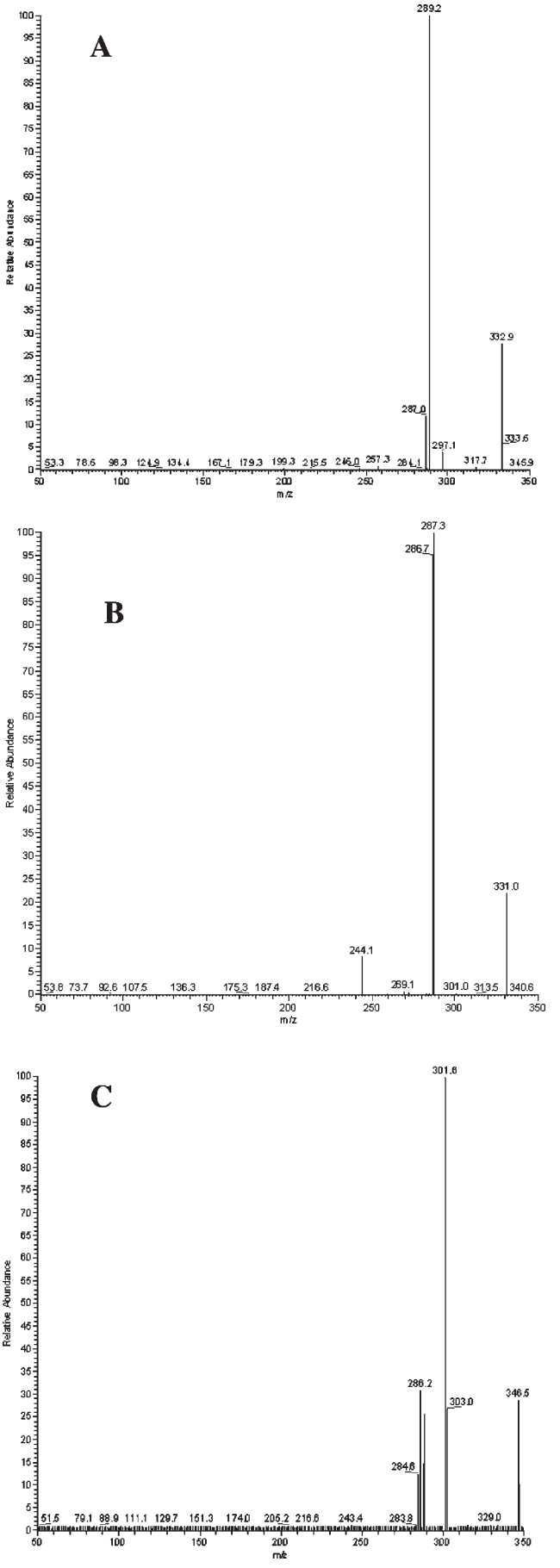

Figure 1. Mass spectra corresponding to the major abietanes present in the rosemary extract: (**A**) carnosic acid; (**B**) carnosol; (**C**) methyl carnosate.

Table 1. Effect of Lecithin Addition to the Sample Including 42 mg RE g^{-1} on the Abietanes Present in the Sample before Digestion and in the Bioaccesible Micellar Phase (MF)[a]

lecithin (mg g^{-1})	digestion step	carnosol (mg g^{-1} dw)	carnosic acid (mg g^{-1} dw)	methyl-carnosate (mg g^{-1} dw)	addition (C + CA + MC)
0	before digestion	1.55 ± 0.10	7.86 ± 1.28	0.36 ± 0.02	9.78
	MF	0.97 ± 0.28	1.49 ± 0.46	0.02 ± 0.03	2.49
	% bioaccesible	**62.59**	**18.98**	**5.67**	**25.41**
18	before digestion	1.68 ± 0.56	5.42 ± 1.44	0.26 ± 0.03	7.36
	MF	1.20 ± 0.28	1.38 ± 1.05	0.09 ± 0.00	2.68
	% bioaccesible	**71.82**	**25.52**	**35.51**	**36.42**
37	before digestion	2.03 ± 0.19	7.23 ± 1.02	0.34 ± 0.03	9.60
	MF	1.79 ± 0.27	2.49 ± 0.01	0.21 ± 0.00	4.49
	% bioaccesible	**87.85**	**34.44**	**60.72**	**46.77**

[a] Values are expressed as mg g^{-1} dw of sample before digestion. C = carnosol, CA = carnosic acid, MC = methyl carnosate.

centrifugation (15.3%). Moreover, the MF abietanes concentrations were significantly different depending on the applied method. After overnight sedimentation, the MF showed 5-fold lower carnosic acid and 1.2-fold lower carnosol content than after low speed centrifugation, suggesting that such a long water exposure at 37 °C was detrimental for these compounds, since both molecules, in particular carnosol, showed very poor stability in the presence of aqueous solutions (*25*). Therefore, the low speed centrifugation was selected to isolate the MF after *in vitro* digestions.

RE Carrier Formulation To Improve Mixed Micelles Formation and Abietane Bioaccesibility. The antioxidant-enriched rosemary extract (RE) utilized for this study was not water-soluble then; after ingestion it should follow a digestive process similar to that for lipidic food. Moreover, rosemary abietanes, particularly carnosic acid and carnosol, are unstable on aqueous solutions (*25*). Therefore, in order to protect these compounds and enhance their bioaccesibility, the RE was dissolved in oil and lard as lipidic carriers. Lipid digestion is dependent on the amount of fat and the type of lipids present in the mixture, since long/short or saturated/unsaturated triglyceride chains highly influence the mixed micelles formation (*3*). Therefore, the *in vitro* digestion of rosemary extract was first evaluated using two types of fatty matrixes as carriers mixed in two different concentrations.

In vitro digestion of both lard or sunflower oil (10 g) followed by micelles isolation by low-speed centrifugation yielded micellar phases containing approximately 2×10^8 micelles mL^{-1}. After the digestion of lard mixed with RE (25 mg g^{-1} fat), 1.5×10^6 micelles mL^{-1} were formed, but when sunflower oil was used as RE carrier (25 mg g^{-1} oil), 1.4×10^8 mL^{-1} was obtained. Moreover, the samples prepared using 6 g of sunflower oil instead of 10 g (but including the same RE concentrations) yielded after digestion similar micellar concentrations but lower OF phase after centrifugation (the unmicellated oily phase), indicating that samples including 10 g of oil were saturated. Therefore, sunflower oil (added as 6 g per sample) was selected as lipidic carrier to dissolve rosemary extract.

Addition of other compounds to the RE−sunflower oil mixture (42 mg of RE g^{-1}) was tested to enhance the bioaccesibility of its antioxidant compounds. α-Tocopherol and soy lecithin were selected, since vitamin E showed a positive effect on the *in vitro* solubilization of several lipophilic drugs (*26*) and lecithin stabilizes lipid-aqueous emulsions (such as the MF).

The sunflower oil utilized for the assays contained 0.7 mg g^{-1} α-tocopherol. Thus, more tocopherol was added to double the concentration and to increase it up to 5-fold. No significant differences were found when it was added up to 1.4 mg g^{-1}. However, results indicated that higher tocopherol addition (3 mg g^{-1}) negatively influenced the mixed micelles formation while lecithin (18 mg g^{-1}) slightly improved it (1.7×10^8 micelles mL^{-1}). After intestinal digestion (before centrifugation), the samples including lecithin were better dispersed than those without the emulsifier. The latter showed oily drops at the surface of the digestates as soon as the stirring was stopped.

Therefore, two different lecithin concentrations were added to the lipidic matrix including rosemary extract (42 mg g^{-1}) to investigate whether they might increase the number of generated micelles and their abietanes content. Results indicated that lecithin not only improved the dispersion of the oily mixture (37 mg g^{-1} lecithin generated MF with 1.9×10^8 micelles mL^{-1}) but also enhanced the bioaccessibility of the three measured abietanes, since addition of 18 or 37 mg g^{-1} lecithin increased respectively 1.4 or 1.8-fold the MF abietanes concentration of samples without the emulsifier (**Table 1**). Particularly interesting was the improvement noticed in the methyl-carnosate bioaccesibility. After *in vitro* digestion and centrifugation, the samples with 37 mg g^{-1} lecithin in their formula included a 10-fold higher amount of methyl-carnosate in their micelles (MF) than those without lecithin. Therefore, formulations for functional foods containing rosemary extracts should include an oily matrix and lecithin as carrier to enhance the abietane bioaccessibility.

Optimization of the RE-Including Formulation. The optimal amount of rosemary extract that might be added to the lipidic carrier (including 37 mg g^{-1} lecithin) was also investigated. No significant difference ($P > 0.05$) was observed on the dry residue resulting from the micellar phase of samples including from 0 up to 75 mg of RE g^{-1} of oil. Additions of 8 and 25 mg of RE g^{-1} showed slightly lower micelles concentration (on average 1.58×10^8 micelles mL^{-1}) than 42 mg of RE g^{-1} (1.92×10^8 micelles mL^{-1}). Additions of higher amounts (58 and 75 mg of RE g^{-1}) showed a slight decrease on mixed micelles formation (1.83×10^8 micelles mL^{-1}) and were not fully dispersed after the intestinal digestion (before centrifugation).

Carnosic acid, carnosol, and methyl-carnosate were also identified in different concentrations when RE was mixed with sunflower oil and lecithin (37 mg g^{-1}) before the *in vitro* digestion (**Figure 2a**). Carnosic acid was the major compound, followed by carnosol. They represented respectively on average 75.3 and 21.5% of the three abietanes when the rosemary extract was mixed as 8, 25, or 42 mg g^{-1} with the lipid carrier. However, if a higher concentration was selected (58 or 75 mg of RE g^{-1}), they represented respectively 70.5 and 26.3%, suggesting that carnosic acid might be partially transformed into carnosol during the mixing process. The higher extract concentrations needed longer time to be completely solubilized in the lipidic matrix, and this might provoke molecular shifting. Methyl carnosate represented, at any of the five selected concentrations, 3.2% of the total diterpenes. Transformation of carnosic acid or carnosol into methyl carnosate is not so spontaneous a reaction as that of carnosic acid into carnosol (*27*).

The gastric digestion process (**Figure 2b**) provoked a general decrease of the abietanes concentrations, probably because of the

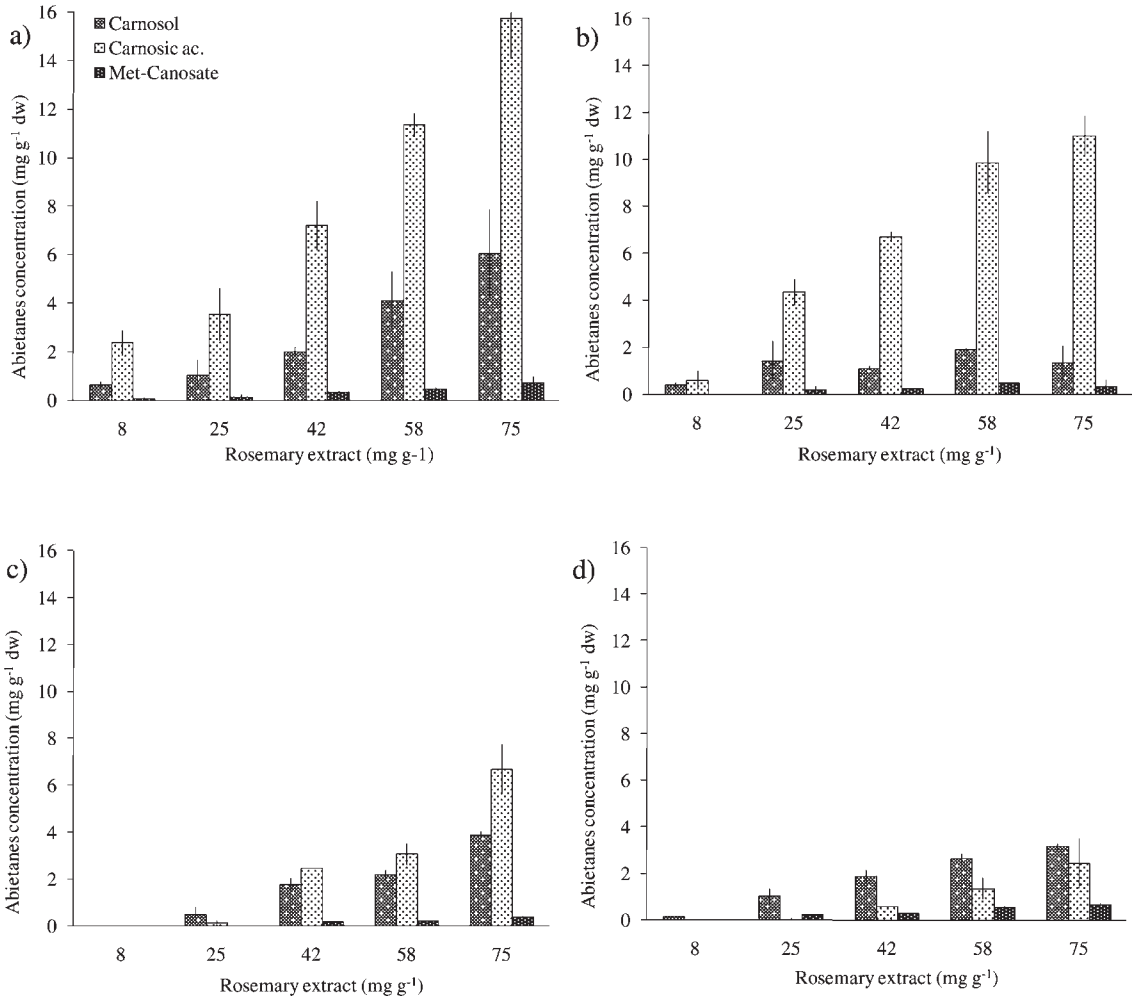

Figure 2. Effect of *in vitro* digestion on the carnosol, carnosic acid, and methyl-carnosate content of samples including lecithin (37 mg g^{-1}) and several RE concentrations. (**a**) Concentrations before digestion, (**b**) concentrations after gastric digestion, (**c**) mixed micellar fraction (MF), and (**d**) oily unmicellated fraction (OF) obtained after intestinal digestion and centrifugation. Values are expressed as mg g^{-1} dw of sample before digestion.

pH reduction, since pepsin degrades only proteins which are in very low quantities in the samples. Moreover, when commercially available carnosic acid (4 mg mL^{-1}) was incubated at 37 °C for 1 h in an acidified methanol solution (pH = 2), a similar degradation (7% of the initial carnosic acid concentration) was observed, confirming the negative effect of low pH on abietanic compounds.

The abietanes reduction provoked by the gastric digestion differed depending on the compound and on the RE amount added to the oil−lecithin mixture. Samples including 58 and 75 mg g^{-1} RE were not completely dispersed after the complete digestion, so perhaps, the gastric digestion could also not be properly performed in these biphasic samples (nonmixable oily drops on an aqueous medium). If only the better dispersed samples were taken into consideration, i.e. sample containing 42 mg g^{-1} RE, a 44.3% carnosol reduction was observed while only 6.8 carnosic acid and 25.4% methyl carnosate reductions were noticed. These results were in concordance with the fact that carnosol is less stable than carnosic acid on water solutions (*25*) and methyl carnosate more stable in oil-in-water emulsions (*28*).

The intestinal digestion provoked more severe changes in the samples than the gastric digestion, since the chromatogram profiles of the MF and OF fractions after intestinal digestion and centrifugation showed many new compounds (**Figure 3**). The abietanes distribution between the fraction containing the mixed micelles and the OF phase was different. Higher carnosic acid levels were found at the micellar phase (**Figure 2c**) while carnosol levels were higher at the OF unmicellated phase (**Figure 2d**). Moreover, carnosic acid levels (addition of both phases) were 57.4% less than the initial concentration before digestion (in the sample including 42 mg of RE g^{-1}) and 54.3% less than those for the previous step (gastric digestion), but carnosol levels increased. The observed decrease in carnosic acid concentration and increase in carnosol concentration could be explained by a molecular shifting of carnosic acid into carnosol, and perhaps other related compounds. To support this explanation, carnosic acid was dissolved in methanol and incubated at 37 °C for 1 h at pH 7.5. A decrease of 26% of the initial carnosic acid concomitantly with formation of 27% carnosol was observed, suggesting that duodenal pH might promote epoxi groups formation, transforming carnosic acid into carnosol, and perhaps other compounds, which seems to be more stable under these conditions. Then, further detailed quantitative evaluation of the effect of the intestinal digestion process was complicated, since molecular transformations and different distributions between the two phases (MF and OF) simultaneously occurred.

The MF obtained after the digestion of 42 mg of RE g^{-1} in sunflower oil with lecithin (**Figure 2c**) contained 87.9% of the carnosol concentration before digestion, 34.4% carnosic acid, and 60.7% methyl-carnosate, indicating that 46.7% of the abietanes quantified before digestion might be bioaccesible (**Figure 4a**). The use of higher RE concentrations (in the oil−lecithin mixture)

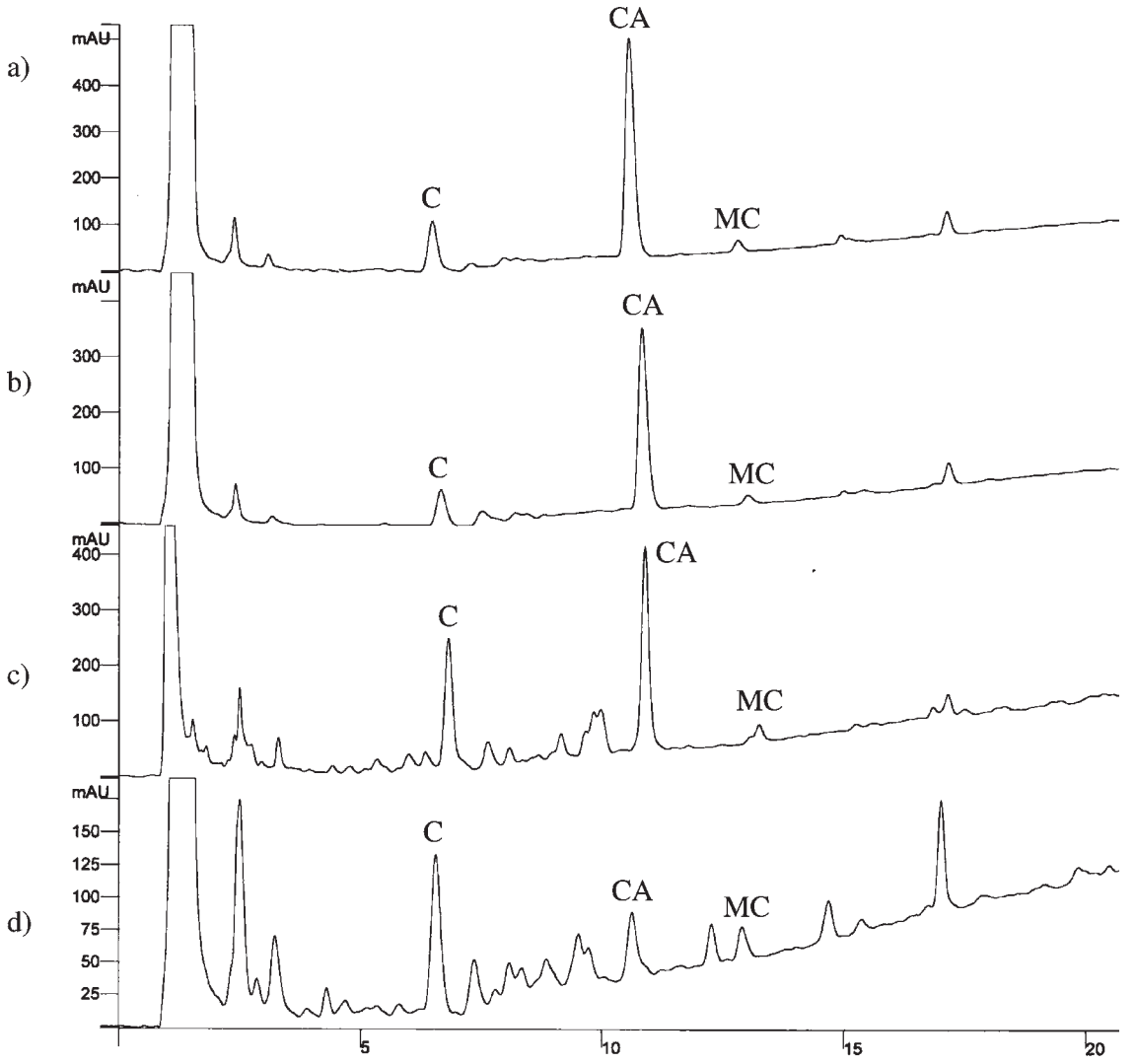

Figure 3. HPLC chromatograms at 235 nm of a sample including lecithin and 42 mg g^{-1} of RE (**a**) before *in vitro* digestion and (**b**) after gastric digestion and after intestinal digestion and centrifugation. (**c**) The fraction containing the mixed micelles (MF) and (**d**) the oily unmicellated phase (OF). C = carnosol, CA = carnosic acid, MC = methyl carnosate.

resulted in higher abietane levels in the bioaccesible fraction up to 48.7% in the sample containing 75 mg of RE g^{-1}. These results suggested that perhaps higher lecithin levels might be required at the initial formula in order to stabilize higher RE concentrations and improve the abietane bioaccessibility.

Similar abietanes concentrations (addition of the three quantified compounds) were found in the OF (**Figure 2d**) obtained from the oil−lecithin mixtures including 42, 58, and 75 mg of RE g^{-1} (respectively 29.2, 28.8, and 27.9% of the abietane concentration before digestions). These results suggested that *in vivo* 28.6% (on average) of the abietane concentration before digestion might be excreted with faeces. Thus, further investigations are necessary to clarify their positive or negative interference with the colonic flora, since carnosic acid showed potent antimicrobial activities (*29*) which might unbalance the microbial colonic ecology.

Effect of *in Vitro* Digestion on RE Antioxidant Properties. The DPPH scavenging capacity of the lipidic matrix including lecithin (37 mg g^{-1}) and rosemary extract (42 mg g^{-1}) was evaluated after each digestion step. RE addition increased 18-fold the antioxidant activity of a control mixture including only sunflower oil and lecithin (**Figure 4b**). The antioxidant values found in the sample before and after the gastric and intestinal digestions did not correlate with its carnosic acid concentration (as reported in other studies (*30*)) or with the addition of the three quantified compounds (**Figure 4a**). After gastric digestion, 55% of the activity found before digestion was lost (calculated after subtraction of the antioxidant activity due to the control sample) while the abietanes reduction was only 15.4%. The different DPPH scavenging capacities of carnosol, carnosic acid, and methyl-carnosate together with the different degradation levels of each individual compound might explain these results; that is, carnosol suffered the highest reduction after gastric digestion (44.3%) and it is the compound with higher antioxidant activity in lipid systems similar to these ones (*31*).

The intestinal digestion produced an increase of the DPPH scavenging capacity of the RE sample. Addition of the antioxidant activities from both OF and MF was higher than the activity before digestion. Particularly high was the antioxidant activity found in the OF. As previously explained, the carnosol concentration in this fraction was higher than that in the sample before *in vitro* digestion, presumably due to the partial transformation of carnosic acid into this compound. Furthermore, the HPLC chromatogram obtained from the analysis of this fraction revealed formation of new compounds that might be tricyclic diterpenes, due to their UV−vis spectral characteristics, which were consistent with those typical of abietanic compounds

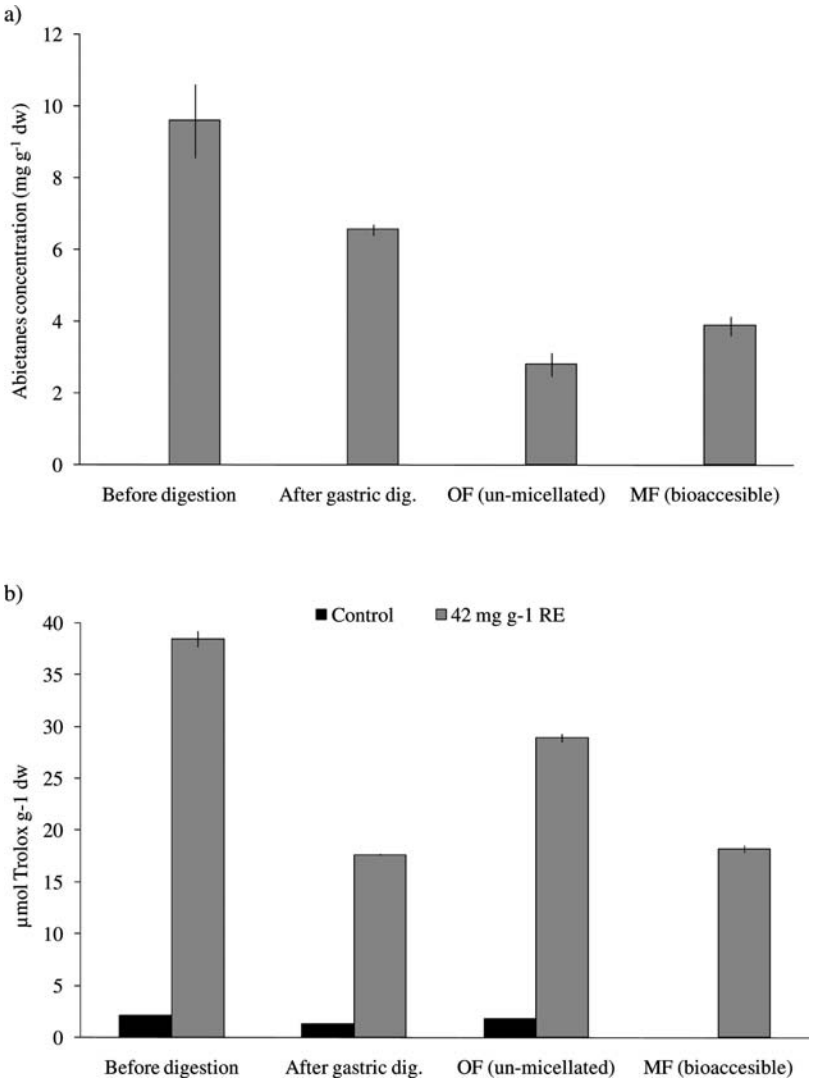

Figure 4. (**a**) Effect of *in vitro* digestion on the concentration of the major abietanes (addition of carnosol, carnosic acid, and methyl-carnosate concentrations) and (**b**) on the DPPH scavenging capacity of a control sample and the sample including 42 mg g^{-1} of RE. Values are expressed as mg g^{-1} and μmol of Trolox g^{-1} dw of sample before digestion.

(**Figure 3d**). Carnosol can be converted into other antioxidants such as rosmanol, epirosmanol, and 7-methyl epirosmanol (*32*,*33*), and carnosic acid in methanol also generates not only carnosol but also other δ- or γ-lactones and methyl carnosate (*20*, *34*); thus, similar changes might occur under the digestion conditions. Thus, the appearance of these (still unidentified) new compounds could certainly modify the OF scavenging capacity. Unfortunately, this fraction corresponded to the one that *in vivo* would be wasted in the colon. In spite of this, still 50% of the antioxidant activity found before digestion was bioaccessible (**Figure 4b**) since the MF also showed DPPH scavenging capacity. As occurred in the OF, this activity might be partially due to carnosol, carnosic acid, and methyl-carsosate but also to other compounds. The MF chromatogram also showed new compounds that were not present in the sample before digestion (**Figure 3c**).

Bioavailability of RE Antioxidant Compounds. The MF was applied to Caco-2 cells to evaluate whether those 46.7% still surviving abietanes after the *in vitro* digestion of the sample including 42 mg of RE g^{-1} were able to pass through the cell monolayer from the apical to the basolateral compartment, indicating that they might be bioavailable (*in vitro*).

First, the MF fractions obtained after the *in vitro* digestion of a control sample (with only sunflower oil and lecithin), a sample including RE, and a solution of carnosic acid in DMSO were evaluated for cytotoxicity on preformed Caco-2 cells monolayers. Results indicated that additions of 60 μL per insert ensured 100% of cell viability after 6 h for both control and RE micellar fractions. Only 12 μL per insert of the prepared carnosic acid solution could be utilized.

The three quantified abietanes together with the new compounds formed during the intestinal digestion were found on the apical compartment (not absorbed) after 3 or 6 h of incubation (the chromatograms were similar to that of **Figure 3c**). Carnosol, carnosic acid, and methyl carnosate were present in lower concentration than initially applied (**Table 2**), but other still unidentified compounds increased to levels even higher than that of carnosic acid (RT = 9−10 min). Those compounds might be degradation products and shifting between abietanes due to the 3 and 6 h of incubation at 37 °C and 5% CO_2 with the cells medium because some of the unidentified compounds still showed UV−vis spectra similar to carnosic acid. Moreover, when only carnosic acid (in DMSO) was applied, a similar reduction was observed and small peaks at retention times similar to those obtained from the RE extract were formed.

No carnosol, carnosic acid, or methyl canosate was detected inside the cells or in the basolateral compartment. Only a small

Table 2. Effect of *in Vitro* Absorption Test on the Carnosol, Carnosic Acid, and Methyl-Carnosate Contents of Micellar Fractions (MF) Obtained after *in Vitro* Digestion of Samples Including 42 mg g^{-1} of RE and Lecithin (37 mg g^{-1})[a]

sample	applied (μg mL^{-1})	not absorbed (μg mL^{-1})	bioavailable (μg mL^{-1})
RE Micellar Fraction			
carnosol	1.12 ± 0.29	0.49 ± 0.25	ND
carnosic acid	2.89 ± 0.85	0.67 ± 0.26	ND
methyl carnosate	0.38 ± 0.26	0.20 ± 0.17	ND
CA in DMSO			
carnosic acid	8.27 ± 1.80	6.18 ± 2.33	0.23 ± 0.12

[a] ND = not detected; CA = carnosic acid.

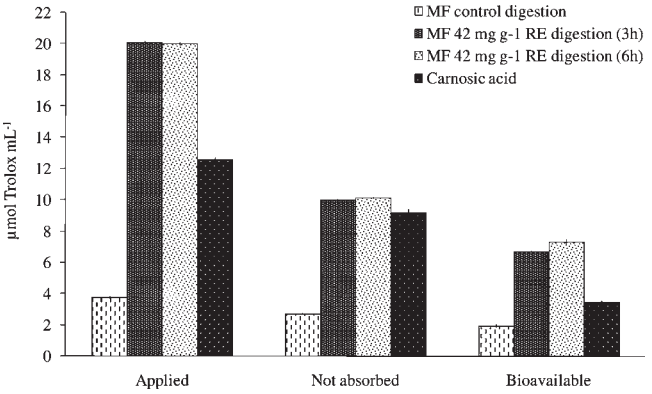

Figure 5. DPPH scavenging activity of the micellar fractions (MF) obtained after digestion of a control sample and a sample with RE (42 mg g^{-1}) and of a carnosic acid solution applied to Caco2 monolayers. DPPH scavenging activity observed at the apical (not absorbed) and basolateral (bioavailable) compartments after 3 and 6 h of incubation of the Caco-2 monolayer in the presence of the micellar phases from both digestions and a carnosic acid solution (8.27 μg mL^{-1}). Values are expressed as μmol of Trolox mL^{-1} of sample before digestion.

amountof those products deriving from the major abietanes was observed at the lower compartment, indicating that the three quantified abietanes were poorly bioavailable and they might pass through the cells in a very low concentration or as derivative molecules. Almost 3-fold higher carnosic acid concentrations were necessary to apply to Caco-2 cells to reach a 2.78% bioavailability (estimated from the results of applying the carnosic acid solution to the cells).

However, the antioxidant activity of applied samples seemed to be distributed between the upper and the lower compartments (**Figure 5**); 45% of the initially detected activity (calculated after subtraction of the antioxidant activity due to the control sample) was not absorbed while 31% (on average) was bioavailable. Longer incubation of the samples with the cells (from 3 to 6 h) did not result in significantly higher antioxidants absorption. The addition of the DPPH scavenging activities from the apical and basolateral compartments was lower than the activity originally found at the applied samples, supporting the possibility of antioxidants degradation during the incubation time. The high antioxidant activity found in the basolateral compartment could not be due to the quantified abietanes but perhaps to their degradation products or derivatives. Moreover, the antioxidant activity of the applied carnosic acid solution was lower than the applied RE micellar fraction, but it contained more carnosic acid (almost double the amount of the three quantified abietanes). A similar effect was observed at the basolateral compartments of the carnosic solution and RE micellar fraction. The 0.23 μg mL^{-1} bioavailable carnosic acid showed lower antioxidant activity than the bioavailable unidentified compounds from the RE micellar fraction, confirming that the antioxidant activity of the latter was not due to carnosol, carnosic acid, and methyl carnosate.

In conclusion, formulas designed to functionalize foods with rosemary SFE extracts should include an oily matrix and lecithin as carrier to enhance their bioaccessibility. In order to set efficient antioxidant doses, it should be taken into account that, if mixed at 42 mg g^{-1} RE and 37 mg g^{-1} lecithin, 50% of the antioxidant activity will be lost in the digestion processes and 31% will be bioavailable. The observed antioxidant activity is not due to the original three major RE abietanes (carnosol, carnosic acid, and methyl carnosate) but due to their derivatives and digestion products. Further studies are needed to identify these generated compounds responsible for the bioavailable antioxidant activities.

ABBREVIATIONS USED

C, carnosol; CA, carnosic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; MIC, micelles; MF, mixed micelles fraction (aqueous phase formed after *in vitro* intestinal digestion and centrifugation); MC, methyl carnosate; OF, oily fraction (nondispersed (oily) phase formed after *in vitro* intestinal digestion and centrifugation); RE, rosemary extract; SFE, supercritical fluid extraction.

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