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Dear Editor,
Please find enclosed the manuscript (original full research article) entitled:

"Polyphenols from artichoke heads (*Cynara cardunculus* (L.) subsp. *scolymus* Hayek): *in vitro* bio-accessibility, intestinal uptake and bioavailability.

by: Isabella D'Antuono, Antonella Garbetta, Vito Linsalata, Fiorenza Minervini, and Angela Cardinali

The paper deals with the assessment of the potential for bioavailability of the artichoke polyphenols using both *in vitro* digestion and Caco-2 human intestinal cell models. The results obtained showed that all the artichoke polyphenols were stable and bioaccessible. Some isomerization products were identified for both mono and dicaffeoylquinic acids, probably derived by the mild alkaline condition occurred during the *in vitro* intestinal digestion. Moreover, the differentiated Caco-2 monolayer were used in order to simulate absorption and bioavailability. All the artichoke polyphenols were absorbed with a maximum after 30 min with an efficiency of 0.16%, according to the poor absorption of dietary polyphenols. Some compounds probably derived from cellular metabolism on caffeoylquinic acids, were detected in the basolateral side. Apigenin-7-O-glucoside was absorbed and transported through the Caco-2 monolayer with a P_{app} value of 2.29×10^{-5} cm/sec, at 60 min. Although the data obtained with this model of simulated *in vitro* gastro-intestinal digestion coupled with Caco-2 uptake, cannot be directly extrapolated to human *in vivo* conditions, could be useful tool for investigating mechanistic effects, such as the release from food matrix, impacting polyphenols bio-accessibility and bioavailability.

We hope that the manuscript could be considered for publication in the ***Food and Function***, if suitable**.**

Bari, December, 16, 2014

My best regards,
Angela Cardinali

Sede: Via Amendola, 122/O – 70126 Bari (Italy); Tel. 080 5929359, Fax 080 5929374
Sezioni: Lecce (Tel. 0832 420000), Milano (Tel. 02 50316685), Sassari (Tel. 079 233466), Torino (Tel. 011 6709230)

Polyphenols from artichoke heads (*Cynara cardunculus* (L.) subsp. *scolymus* Hayek): *in vitro* bio-accessibility, intestinal uptake and bioavailability.

Isabella D'Antuono[a], Antonella Garbetta[a], Vito Linsalata[a], Fiorenza Minervini[a], Angela Cardinali[a]*.

[a] Institute of Sciences of Food Production (ISPA), CNR, Via G. Amendola, 122/O, 70126 Bari, Italy

Corresponding author at: Institute of Sciences of Food Production, Via Amendola 122/O, 70126 Bari, Italy. Telephone: +39 080 5929303, Fax: +39 080 5929374.

E-mail address: angela.cardinali@ispa.cnr.it (A. Cardinali)

Abstract

Artichoke is a rich source of health promoting compounds such as polyphenols, important for their pharmaceutical and nutritional properties. In this study, the potential for bioavailability of the artichoke polyphenols was estimated by using both *in vitro* digestion and Caco-2 human intestinal cell models. *In vitro* digestive recoveries (bio-accessibility) were found to be 55.8% for total artichoke phenolics and in particular 70.0% for chlorogenic acid (CGA), 41.3% for 3,5-O-dicaffeoylquinic acid (3,5 diCQA), and 50.3% for 1,5-O-dicaffeoylquinic acid (1,5 diCQA), highlighting potential sensitivity of these compounds to gastric and small intestinal digestive conditions. Uptake of artichoke polyphenols was rapid with peak accumulation occurring after 30 min with an efficiency of 0.16%, according to the poor absorption of dietary polyphenols. Some compounds, such as coumaric acid, caffeic acid (CAA) and CAA derivatives, were also detected in the basolateral side assuming an extra and intracellular esterases activities on CGA. Only apigenin-7-O-glucoside was absorbed and transported through the Caco-2 monolayer demonstrating its bioavailability in the extent of 1.15% at 60 min. In addition, permeability coefficient (P_{app}=2.29 x 10^{-5} cm/sec), involving apical to basolateral transport of polyphenols, was calculated to facilitate estimation of absorption and transport through Caco-2 monolayer. Finally, the mono and dicaffeoylquic acids present in artichoke heads, exert an antioxidant activity on human low density lipoprotein (LDL) system correlated to their chemical structure. In conclusion, the utilized *in vitro* models, although not fully responding to the morphological and physiological features of human *in vivo* conditions, could be a useful tool for investigating mechanistic effects occurring in a food matrix.

Keywords: Artichoke Polyphenols, *in vitro* digestion, Bioavailability, Permeability Coefficient

Introduction

Artichoke (*Cynara cardunculus* (L.) subsp. scolymus Hayek) represents an important component of Mediterranean diet and a good source of health-promoting compounds, such as phenolics, inulin, fibres and minerals.[1,2] The main compounds present in artichoke heads are caffeic acid derivatives, in particular a wide range of caffeoylquinic acids with CGA as the most abundant of them. In addition, other compounds present in small amount, such as glycosides of apigenin and luteolin and different cyanidin caffeoylglucoside, have been identified in artichoke tissues.[1,3,4]

The pharmaceutical properties of artichoke polyphenols are widely studied and attributed to many activities, such as hepatoprotective, anticarcinogenic, antioxidative, antibacterial, anti-HIV, bile-expelling, and diuretic.[1] Others studies, already performed, have found evidence for antioxidative properties of artichoke leaf extracts against hydroperoxide-induced oxidative stress in cultured rat hepatocytes.[5] In addition, the artichoke extract has shown to inhibit cholesterol biosynthesis and to protect LDL from *in vitro* oxidation.[6-8] Nevertheless, to achieve any health properties, the polyphenols must be bioavailable, effectively absorbed from the gut into the circulation, and delivered to the target tissues where can exert their beneficial effects.[9,10]

.The bioavailability (the fraction of a nutrient or compound ingested that, through the systemic circulation, reaches specific sites) is dependent upon the digestive stability of compound, its release from the food matrix (referred as bio-accessibility), and the efficiency of its transepithelial passage.[11] After the release from the food matrix, the bio-accessible polyphenols must be presented to the brush-border of the small intestine in such a state that they can be absorbed into the enterocyte by passive diffusion or by active transport systems.[12] Passive paracellular diffusion, overcoming the tight junctions, may also occur, but this is not normally a major route of ingress, probably because most polyphenols are too hydrophilic to penetrate the gut wall.[12, 13] The polyphenols bioavailability differs greatly from a polyphenol to another, and the most abundant in our diet is not necessarily the better bioavailable[14] their bioavailability is considered to be low, not exceeding the plasma

concentrations of 10 μM. Furthermore, the polyphenols low absorption can be attributed to glucuronidation and sulphation of free hydroxyl groups present in the chemical structures of the different compounds.[14,15] However, a part of their low bioavailability, dietary polyphenols, after a meal rich in vegetables and fruit, may be present in the gastro-intestinal (GI) lumen, at much greater concentrations where they can play an important role in protecting from oxidative damage and in delaying the development of stomach, colon and rectal cancer.[16]

Although little is known about the *in vivo* bioavailability and digestive modification of artichoke polyphenols,[17,18] the *in vitro* digestive models were used to predict, in a simplified manner, the polyphenols behavior in simulated digestive processes of GI tract . As reported by some authors, these models can provide important information on the stability and putative modifications of interest compounds under GI conditions.[19] In this paper, the authors have investigated on the composition, antioxidant activity, and stability of polyphenols present in artichoke infusion after GI digestion, reporting the high stability of the identified flavonoids.[19] Furthermore, a recent study performed by our group, have evaluated the influence of gastro-intestinal digestion on antioxidant effect of artichoke polyphenols showing that, *in vitro* digestion did not modify the antioxidant activity of artichoke polyphenols, except for 1,5 diCQA.[20] On the other hand, many studies are already performed on bioavailability of pure standards, such as CGA, showing its high stability also to the extreme gastric conditions. The CGA could be absorbed even in the stomach, in fact it was identified in both the gastric vein and aorta in its intact form.[21] Instead, its bioavailability and metabolism were mainly dependent by gut microflora.[22,23]

This study aims to generate insight into the digestive stability and bio-accessibility of the major classes of polyphenols present in artichoke heads, using the *in vitro* digestion model. The influence of some chemical (pH, temperature and bile salts) and biological (gastric and pancreatic enzymes) GI conditions on the artichoke polyphenols modifications, were investigated. Moreover, the intestinal absorption (as predictors of bioavailability) was performed using Caco-2 cell line model.

Permeability coefficient, involving apical to basolateral transport of polyphenols, was calculated to facilitate estimation of absorption and transport through Caco-2 monolayer.

Material and Methods

Materials

Artichoke heads were supplied from a local market and stored at 4 °C until used. Extraction and chromatography solvents, methanol (MeOH), glacial acetic acid (AcOH), ethanol (EtOH), ethyl acetate (EtOAc), were HPLC certified. Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (PBS), L-glutamine 200 mM, antibiotic and antimycotic solution, non essential amino acid solution, bovine serum albumin Cohn V fraction fatty acid depleted (BSA) were purchased from Sigma Aldrich (Milan, Italy). Caco-2 (HTB-37) cell line was purchased from IZSLER (Brescia, Italy). Foetal bovine serum (FBS) was purchased from Gibco (Milan, Italy). All enzymes and bile salts used *in vitro* digestion were obtained from Sigma Aldrich (St. Louis, Mo., U.S.A.). The 1,5 diCQA and 3,5 diCQA were supplied by PhytoLab GmbH & Co. KG (Dutendorfer Str. 5-7, 91487 Vestenbergsgreuth Germany). CGA, CAA, and LDL from human plasma, were purchased from Sigma Aldrich, Milan, Italy.

Artichoke polyphenols extraction

The polyphenolic fraction presents in artichoke was extracted by using water, the solvent that better simulate the extraction process in the digestive system. In particular, 4.5 g of blanched heads (5 min, 100°C, ascorbic acid 0.5% in H_2O) of artichoke were extracted by refluxing for 60 min at 100 °C with 50 mL of H_2O containing 0.5% of ascorbic acid. Then, the aqueous solution was recovered and further extracted for additional 30 min with 50 mL of the same solution. The extracts were pooled, filtered at 0.45 μm and utilized for HPLC analysis. In order to avoid the cytotoxicity of the solvent for uptake experiments, an hydroalcoholic extract was obtained homogenizing 4.5 g of fresh

112 artichoke head and refluxing at 100°C (twice for 1 h and 30 min) with 50 mL of methanol/water
113 (50:50, v/v). After filtration through a Whatman 1 filter paper, methanolic extracts were concentrated
114 under vacuum, filtered at 0.45 μm, and then analysed by HPLC.
115
116 **HPLC Analysis**
117 Analytical-scale HPLC analyses of the artichoke extracts were performed employed Thermo
118 Scientific HPLC spectra System equipped with a P2000 gradient pump, a SCM 1000 membrane
119 degasser, an UV6000LP UV/Vis DAD, an AS3000 autosampler, and ChromQuest 4.1 software. The
120 UV–Vis absorption chromatogram was detected at 325 nm. Separation was performed by gradient
121 elution on a 4.6 × 250 mm reverse phase Luna C-18 (5 μm) column (Phenomenex Torrance,
122 California, USA). The elution was performed using methanol (eluent A) and water/acetic acid 95:5
123 (eluent B) following the method of Lattanzio.[24] The gradient profile was: 85–60% B (0–25 min),
124 60% B (25–30 min), 60–37% B (30–45 min), 37% B (45–47 min), 37–0% B (47–52 min). The flow
125 rate was 1 mL/min. Samples were applied to the column by means of a 25 μL loop valve.
126 Polyphenols compounds were identified by retention time and spectra of the pure standard. Results
127 were expressed as μg/mL.
128
129 **LDL oxidation *in vitro* assay**
130 LDL oxidation was measured by monitoring the formation of hexanal, which is the major end
131 product of lipid peroxidation. LDL in PBS dispersion was diluted to a concentration of 1 mg of
132 protein/mL. The production of hexanal was monitored by headspace, following the method of
133 Teissedre *et al*.[25] with some modifications. Briefly, in 10 mL vial were added 50 μL of LDL samples,
134 $CuSO_4$ (80 μM) solution and PBS to reach the volume of 4 mL, finally the vial was sealed and
135 incubated for 2 h (propagation phase) at 37 °C, in order to determine the production of hexanal
136 formed in the control. At the same time, various concentrations of polyphenols from artichoke (2-20

μg/mL), CAA (0.2-1.5 μg/mL), and CGA (0.3-7.0 μg/mL) were tested for their antioxidant activity. The hexanal formation was determined using Gas Chromatography (Varian CP3800) equipped with a flame ionization detector. Hexanal was separated by a ZB-Wax-Plus fused silica Capillary column (30m x 0.32 mm i.d., 0.5 μm film thickness, Zebron Phenomenex Inc. Torrance, CA U.S.A) and helium was the carrier gas. GC conditions were as follows: injector temperature, 180 °C; detector temperature, 200°C; oven program, held at 40 °C for 2 min, increased at 20 °C/min to 140 °C, and then held for 1 min. The results, obtained after replicate analyses, were expressed as percent of relative inhibition:

$$(\%\ \mathrm{In}) = [(C - S)/C] \times 100$$

where *C* was the amount of hexanal formed in the control and *S* was the amount of hexanal formed in the sample.

***In vitro* gastro-intestinal digestion**

Artichoke heads were subjected to gastric and pancreatic digestion, following the method of Versantvoort *et al.* [26] Before to start, all the simulated digestive juices are heated to 37 °C for 2 h. Artichoke head was blanched for 5 min at 100 °C in H_2O containing 0.5% of ascorbic acid, and homogenized in a laboratory blender for 1 min to simulate mastication. Homogenized samples (4.5 g) were transferred to a centrifuge tube and 6 mL of simulated saliva fluid, containing α-amylase, mucin and several organic and inorganic salts at pH 6.8 ± 0.2 were added. The solution was incubated at 37 °C and rotated head-over-heels (55 rpm at 37 °C) (Rotator Type L2, Labinco BV, Netherlands) for 5 min. Then, 12 mL of simulated gastric juice was added, and the mixture was rotated head-over-heels for 2 h. The gastric pH was 1.5 ± 0.5. Finally, 12 mL of duodenal juice and 6 mL of bile, were added and the pH adjusted at 6.5 ± 0.5, and the mixture was rotated for another 2 h. Using the head-over-heels rotation in each steps of digestion, a gentle but thorough mixing of the matrix with the digestive juices was achieved, simulating the peristaltic movement. At the end of the

in vitro digestion process, the samples were centrifuged for 10 min at 2,900 *xg* and an aliquot of the supernatant (chyme) was recovered for the assessment of the bio-accessibility. During all the digestive process, different aliquots of samples in the different steps (salivary, gastric and duodenal) were recovered in order to determine the polyphenols stability. Three independent experiments were performed in duplicate.

The bio-accessibility of polyphenols, defined as the fraction of external dose released from its matrix in the GI tract, was calculated as follows:

$$\text{Bioaccessibility (\%)} = (CF/CI) \times 100$$

Where CF is the amount of polyphenols present in the digesta (chyme) and CI is the initial amount of polyphenols.

Intestinal bioavailability of polyphenols by using Caco-2 human cell line

To assess the potential intestinal absorption of artichoke polyphenols, experiments were carried out using the Caco-2 human intestinal cell line, following the method described by Failla *et al.* [27] with some modifications. Briefly, Caco-2 cells were seeded at 1.2×10^5 cells/mL in cell culture inserts for 6 well plates with polyethylene terephtalate (PET) track-etched membranes (pore size 0.4 µm, growth area 4.2 cm^2, Falcon, BD), pretreated with poly-L-lysine (50 µg/mL), in complete DMEM, with 4.5 g/L glucose supplemented with 10% foetal bovine serum, 1% L-glutamine, 1% antibiotic and antimycotic solution, 1% non essential amino acid solution at 37 °C in a humidified atmosphere containing 5% CO_2. The basolateral compartment was filled with 3 mL of complete DMEM. Cells monolayers were cultured for 21 days in order to obtain a full differentiated cells and media from apical and basolateral compartment were replaced twice a week. The integrity of the cells monolayer was evaluated by transepithelial electrical resistance (TEER) measurements using a volt-ohm meter (Millicel ERS-2, Millipore, Italy). TEER values were expressed as Ω/cm^2. Only Caco-2 monolayers showing TEER values higher than 700 Ω/cm^2 were used for *in vitro* experiments. The absorption

experiments were performed following protocol described by Neilson *et al.*[28] with some modifications. Briefly, monolayers were first washed with 2 mL PBS (pH 5.5), and then 2 mL of DMEM phenol red free containing 100 μg/mL methanolic artichoke extract were applied to each well. Cells were then incubated at 37 °C for 30, 60, 90 and 120 min. Following incubation, media of apical and basolateral compartments were aspirated and stored at -80 °C before the HPLC analysis. The monolayers were washed first with PBS (pH 5.5), then with 2 mL of 0.1% fatty acid free bovine albumin (w/v) in PBS (pH 5.5) and, finally, cells were scraped from the plate into 1 mL of cold PBS (pH 5.5), collected and stored at −80 °C under N_2 until analysis. Protein values for cell monolayers were determined by BIO-RAD protein assay method.[29] Artichoke polyphenols from sonicated Caco-2 cells and basolateral solutions, were extracted with 3 mL of EtOAc (0.01% BHT). Extraction was repeated a total of 3 times and EtOAc layers were pooled, dried under vacuum, and resolubilized in 200 μL mobile phase for HPLC analysis.[30]

Permeability coefficients

Values obtained from HPLC analysis were used to calculate the permeability coefficients (P_{app}, cm/s) that represents the apical-to-basolateral transport rate of polyphenols across the epithelial barrier and it was calculated from the following equation:

$$P_{app} = (dC/dt)\ V/(C_0A)$$

where dC/dt is the appearance rate of polyphenols in the receiver compartment at different time of incubations (30, 60, 90, and 120 min); V is the volume of the receiver compartment (3 cm^3); C_0 is the initial concentration in the donor compartment and A is the exposed area of the tissue (4.2 cm^2).[31]

Statistical analysis

Statistical analyses were performed using the SigmaPlot 11.0 software (SigmaPlot™ Exact Graphs and Data Analysis, Systat Software, San Jose, CA, USA). The *t*-Student test was used for statistical

analysis of the bio-accessibility and performed comparing the values in the three steps of GI digestion (salivary phase *vs* gastric phase, salivary phase *vs* intestinal phase, gastric phase *vs* intestinal phase). Values of $p < 0.05$ were considered as significant difference. In addition, the 50% of inhibitory concentration (IC_{50}) of LDL oxidation was determined using sigmoidal fitting of the concentration–response curve by SigmaPlot 11.0 software.

Results and Discussion

Artichoke phenolic profile and antioxidant activity

In this study the phenolic fraction of artichoke head after aqueous extraction was analyzed and characterized before *in vitro* GI digestion. The HPLC analysis shows the presence of eleven peaks that represent the main phenolic compounds present in artichoke heads (Fig. 1A). The concentrations of the identified polyphenols were shown in Table 1. The most abundant constituents are: CGA, 3,5 diCQA, and 1,5 diCQA. It should be stressed that, despite the concentrations of phenolics can vary with the physiological stage of the plant material, these three compounds are always the constituents quantitatively more representative of the phenolic fraction of artichoke head (70-80%).[1] In addition, as reported by the same authors,[1] a flavonoid glycoside compound, apigenin-7-O-glucoside, was present.

Furthermore, the antioxidative properties of artichoke extract and of two phenolic compounds (CAA and CGA) were investigated on human LDL *in vitro* system. The two standards were used in order to understand the antioxidant contribution of CGA (the main compound present in artichoke) and of CAA (present as caffeoyl group in both mono- and di-caffeoylquinic acids). In particular, in the LDL system, the formation of hexanal (one of the end-products of lipid oxidation) was determined at the propagation phase (2 h). This phase was detected by oxidizing LDL with copper, without antioxidants presence, at different time (1-4 h), and was equivalent at the time of maximum

hexanal production.[32] The polyphenol concentrations tested ranged from 2-20 μg/mL for artichoke extract, 0.18-1.44 μg/mL for CAA, and 0.35-7 μg/mL for CGA. The concentrations used, for each compound, were selected on the dose-response curve for the hexanal production ranging from 2 to 99% of inhibition. The IC_{50} values showed that CAA (0.28 μg/mL) was the most active against LDL oxidation, followed by CGA (1 μg/mL) and artichoke extract (6 μg/mL).

This different antioxidant capacity exhibited by the samples could be related to their chemical structures and to the possible synergic/antagonistic effect occurred in the complex mixture, such as artichoke head. In fact, as reported by other authors,[33,34] the antioxidant activity is strongly dependent on the number of hydroxy groups present in the molecule, and has a tendency to decrease with the esterification of them: this can explain the higher antioxidant activity of CAA respect to CGA.[33] Regarding the possible synergic/antagonistic effect, it was reported that the interaction among molecules could influence the hydrogen donating ability, mechanism underlying antioxidant activity. For this reason, and in this experimental conditions, the complex mixture of polyphenols (mono and dicaffeoylquinic acids) present in artichoke extract, could influence differently, the expected antioxidant response.[34] In fact, by the comparison of IC_{50} value of CGA standard (1 μg/mL) with IC_{50} value of artichoke extract (6 μg/mL), that is containing 2.2 μg/mL of CGA (Table 1), could be supposed an antagonistic effect among polyphenols, when they are present in a complex mixture.. The activity of artichoke extract against LDL oxidation inhibition, was already demonstrated by other authors on artichoke leaves extract and with overall lower efficacy.[7,8]

In vitro bio-accessibility of artichoke heads after simulated digestion

In order to determine the bio-accessibility of polyphenols from artichoke heads, the *in vitro* GI digestion process was performed. This model, applying physiologically based conditions, i.e. chemical composition of digestive fluids, pH, and residence time typical for each compartment,

simulates the digestion process in the GI tract in humans. The procedure follows the three-steps of digestive process (mouth, stomach and small intestine) in order to evaluate the stability and bio-accessibility of artichoke polyphenols. The influence of GI digestion on total phenolics was reported, as percentage of bio-accessibility, in Table 2 and the phenolic profile was showed in Fig. 1B. After *in vitro* gastro intestinal digestion, the phenolic profile of artichoke remains qualitatively unchanged, showing the same relative abundance among constituents (Table 1), in agreement with a previous study.[20] As observed in Table 2 , the amount of released phenolics increased stepwise from mouth to intestine. In particular, after simulated salivary condition and mastication, the total polyphenols bio-accessibility was 27.2%, instead the bio-accessibility of the most abundant phenolics present in artichoke heads was 35.2% for CGA, 19.7% for 3,5 diCQA, and 25.3% for 1,5 diCQA.

After two hours of gastric digestion, the amount of the recovered total polyphenols was 35.7% of bio-accessibility, indicating that, despite of the strong acidic conditions, the gastric environment significantly improved the polyphenols extraction.[35] In particular, CGA and 4-O-caffeoylquinic acid (4 CQA) seem to be more stable to the gastric condition with a bio-accessibility of 51.7% and 64.5%, respectively, while other monocaffeoylquinic acids such as 1-O-caffeoylquinic acid (1 CQA), 3-O-caffeoylquinic acid (3 CQA) significantly increased in the gastric phase. Otherwise, the acid environment mainly affected the 3,5 and 1,5 diCQAs, with a significant low bio-accessibility of 9% and 8.3%, respectively. In addition, in this phase the 1,3-O-dicaffeoylquinic acid (cynarin) and apigenin 7-O-glucoside, were not detectable.

Successively, the transition from the acidic gastric to the mild alkaline intestinal environment caused a significant increase in the total polyphenols recovery, with a 55.8% of bio-accessibility for the total polyphenols and in particular with 70.0% for CGA, 41.3% for 3,5 diCQA and 50.3% for 1,5 diCQA (Table 2). Particularly interesting is the two main diCQAs behavior that, after have suffered a significant ($p<0.05$) reduction in gastric phase, their concentrations increased of about 5-6 times, indicating that intestinal conditions favored their extraction from the plant matrix, and that the

compounds are quite stable. In addition, HPLC analysis of the duodenal juice showed, similarly to the mouth phase, the presence of cynarin and apigenin 7-O-glucoside. Moreover, 4 CQA, cynarin and 1,4 diCQA were detected in higher amount respect to the aqueous extract, probably derived from isomerization processes of the other mono and diCQAs, favored by pH and more suitable conditions for their extraction.

For better understand the behavior toward mono and diCQAs in intestinal compartment, standard solutions of CGA and of two main diCQAs (1,5 and 3,5) were individually submitted to a *in vitro* digestion process, using the same method. The HPLC analysis of intestinal digesta highlighted the presence of compounds absent in the starting solution, derived from isomerization processes (Table 3). In particular, after digestion, CGA was stable for 48.1% and spectral analysis and retention times allowed the recognition of 8% of 4 CQA, with a total loss of 44.3%. This result is in agreement with the data published by other authors where the CGA overcome the extreme gastric conditions without modifications,[36, 21] but with about 52% of total degradations at the end of intestinal digestion process.[36] This behavior was also found for other polyphenols such as ferulic acid, gallic acid and rutin,[37,38,39] that are degraded in the mild alkaline environment of the intestine.

Regarding the two diCQAs stability, after GI digestion the 1,5 diCQA is quite stable with a loss of 34.6% and some isomerization products such as cynarin (6%) and 1,4 diCQA (10%). Instead, the 3,5 diCQA digestion gives a loss of 45.7% but a higher isomerization effect with the presence of 4,5 diCQA (16%) and 3,4 diCQA (12%), (Table 3). Although no many study are at the moment available on the dicaffeoylquinic acids stability, our results are in agreement with the data published by Bermudez-Soto *et al.*[40] that have attributed the presence of caffeoylquinic isomers to mild alkaline conditions occurred during the *in vitro* intestinal digestion. The instability of artichoke polyphenols to gastrointestinal conditions is similar to that reported for other compounds including catechins,[41] quercetin,[42] resveratrol,[38] and anthocyanins.[43]

Caco-2 accumulation, bioavailability and P_{app} coefficient

To validate that artichoke polyphenols were indeed absorbable and bioavailable, the intestinal uptake was performed by incubating differentiated Caco-2 cells with media containing artichoke methanolic extract (100 µg/mL) from 30 min to 2 h. The cellular uptake of polyphenols was determined by HPLC-DAD after EtOAc extraction of cellular pellets. The results obtained showed that the incubation time influenced the polyphenols uptake (Fig. 2). In particular, the maximum of absorption (0.71 ng/µg proteins) was reached after 30 min and the polyphenols absorbed from Caco-2 monolayer were CGA, 1,4 diCQA, 3,5 diCQA, and 1,5 diCQA, with the latter two as the most abundant (respectively 0.22 ng/µg proteins, and 0.30 ng/µg proteins).

After 60 min, besides to the previous identified compounds, in the cells were recovered: 1 CQA, 3 CQA, cynarin,, 4,5 diCQA, 3,4 diCQA. The total cellular absorption was 0.58 ng/µg proteins. Particularly interesting is the absence of apigenin-7-O-glucoside and the presence, as traces, of cumaric and caffeic acids, probably derived by a cellular metabolism of CGA. Since other authors[22] have attributed the presence of coumaric acid to the gut microflora metabolism of CGA, cellular involvement cannot be excluded.[44,45] After 90 min, the total absorption was similar to 60 min (0.54 ng/µg proteins) and all the identified compounds were quantitatively and qualitatively recognized, a part of 1 CQA, 3 CQA that were not detected in Caco2 monolayer. At 120 min, the total recovery (0.54 ng/µg proteins) and the phenolic composition were similar to the previous incubation time. In particular, at this time, coumaric acid was not detected, while some caffeic acid derivatives, probably derived from cellular metabolism activity, were ~~detected~~ in a very low amount (0.03 ng/µg proteins). The results obtained for the uptake permit to speculate about a time-dependent saturation effect, that, as reported by other authors, permit to speculate about the presence of a primary or secondary active or facilitated transport mechanism[46]. The efficiency of absorption was about 0.16% (30 min), showing that the mono and dicaffeoylquinic acids were poorly absorbed in

cell line system. These results are in agreement with other studies on CGA[23] and with the absorbance efficiency of other polyphenols such as catechins and phenilpropanoids.[47-49, 30] The presence of a pool of polyphenols in intestinal cells can support the possible protective effect that these compounds may have against oxidative stimuli.

The basolateral side, that simulated the blood plasma compartment, was analyzed in the aim to have insight of polyphenols transport and bioavailability. The results obtained showed the presence of many unidentified caffeic acid derivatives, and apigenin-7-O-glucoside. In particular, no one of the identified mono and dicaffeoylquinic acids was present in the basolateral side, but only hydroxycinnamic acids (CAA and coumaric acid) at very low concentrations, 110.5 pg/μL and 37 pg/μL, respectively. In addition, the transport was time–dependent with the presence of coumaric acid and CAA already after 30 min, whereas CAA derivatives and apigenin-7-O-glucoside became visible only after 60 min. The amount of CAA derivatives increased up to 90 min for diminishing until the end of the experiments (120 min), while coumaric acid was not detected already after 60 min. The presence of coumaric acid, CAA and CAA derivatives in the basolateral side, permit to speculate a metabolism activity occurred on intestinal ephitelium. In fact, some evidence have supported the hypothesis that CGA hydrolysis, and its metabolites release could begin in the small intestine that, in this study, is simulated by Caco-2 cells. Indeed, is reported that enterocyte-like differentiated Caco-2 cells have extra- and intracellular esterases able to de-esterify hydroxycinnamate and diferulate esters that could be responsible to the metabolism.[44,45]

Interesting results are related to the apigenin-7-O-glucoside that was recovered in the basolateral side simulating its bioavailability. The higher concentration of apigenin-7-O-glucoside was recovered after 60 min then, its amount decreased after 90 min, reaching the lower value at 120 min. In particular the percentage of bioavailability (respect to the original amount in the apical side) was 1.15%, 0.34%, and 0.2%, after 60, 90 and 120 min, respectively.

In the aim to have another tool for the prediction of absorption of apigenin-7-O-glucoside, the values obtained on its transport were used to calculate the P_{app} that represents the apical-to-basolateral transport rate across the epithelial barrier, normally used for drugs.[50] The coefficient calculation was performed at 60, 90 and 120 min, being the apigenin-7-O-glucoside absent at 30 min. The maximum of permeability was reached after 60 min, with a P_{app} value of 2.29 x 10^{-5} cm/sec, followed by 90 min (P_{app} = 0.46 x 10^{-5} cm/sec), and 120 min (P_{app} = 0.20 x 10^{-5} cm/sec). These results showed that apigenin-7-O-glucoside is time-dependent absorbed and transported through the Caco-2 monolayer with high rate at shorter incubation time. The results obtained are in agreement with the data presented by other authors [51,14] that found the same value for apigenin-7-O-glucoside. Furthermore, the authors, have highlighted that the apigenin aglycone had absorptive permeabilities, in Caco-2 model, at least 5 times higher respect to the corresponding glucoside, indicating that the latter compounds are poorly absorbed.[50,12] In addition, although the Caco-2 system lacks the morphological and physiological features of intestine, our *in vitro* results (P_{app} = 2.29 x 10^{-5} cm/sec) resulted similar to the results obtained with *in vivo* permeability of apigenin-7-O-glucoside (P_{eff} = 1.4 × 10^{-5}).[52] Many studies have demonstrated that apigenin-7-O-glucoside possesses significantly higher anti-proliferative and anticancer activity compared to other glucoside derivatives.[53,54] The capacity to predict the absorption and the bioavailability of this compound is an important point in the study of health benefit of artichoke heads.

Conclusions

In conclusion, the simulated GI conditions not particularly affect the stability and bio-accessibility of the eleven identified polyphenols in artichoke heads. Some isomerization products were identified for both CGA and diCQAs probably derived by the mild alkaline condition occurred during the *in vitro* intestinal digestion. In addition, the differentiated Caco-2 monolayer were used in order to simulate absorption and bioavailability. All the artichoke polyphenols were absorbed with a maximum after 30

min with an efficiency of 0.16%, according to the poor absorption of dietary polyphenols. Some compounds, such as coumaric acid, CAA, and CAA derivatives, were also detected in the basolateral side hypothizing an extra- and intracellular esterases activities on artichoke caffeoylquinic acids. Only apigenin-7-O-glucoside was instead absorbed and transported through the Caco-2 monolayer with a P_{app} value of 2.29 x 10^{-5} cm/sec, at 60 min. Although the data obtained with this model of simulated *in vitro* GI digestion coupled with Caco-2 uptake, cannot be directly extrapolated to human *in vivo* conditions, could be useful tool for investigating mechanistic effects, such as the release from food matrix, impacting polyphenols bio-accessibility and bioavailability.

Competing interests

The authors declare no competing financial interest.

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References

1. V. Lattanzio, P. A. Kroon, V. Linsalata, and A. Cardinali, *J. Funct. Foods*, 2009, **1**, 131–144.
2. T.V. Orlovskaya, I. L. Luneva, and V. A. Chelombitko, *Chem. Nat. Compd.*, 2007, **43**, 239-240.
3. S. Aubert and C. Foury, in *Studi sul Carciofo*, ed V. Marzi and V. Lattanzio, Industrie Grafiche Laterza, Bari, 1981, pp. 57–76.
4. V. Lattanzio, A. Cardinali, D. Di Venere, V. Linsalata, and S. Palmieri, *Food Chem.,*1994, **50**,1-7.
5. R. Gebhardt, *Toxicol. Appl. Pharmacol.*, 1997, **144**, 279–286.
6. Z. Küskü-Kiraz, G. Mehmetçik, S. Dogru-Abbasoglu and M. Uysal, *Phytother. Res*., 2010 **24**, 565–570.
7. J. E. Brown, and C. A. Rice-Evans, *Free Radical Res.* 1998, **29**, 247–255.
8. D. Zapolska-Downar, A. Zapolski-Downar, M. Naruszewicz, A. Siennicka, B. Krasnodebska and B KolCodziej, *Life Sci.,* 2002, **71,** 2897–2908.
9. T. K. McGhie, G. D. Ainge, L. E. Barnett, J.M. Cooney and D. J. Jensen, *J. Agric. Food Chem.*, 2003, **51**, 4539.4548.
10. M. D'Archivio, C. Filesi, R. Varì, Scazzocchio B, and R. Masella, *Int. J. Mol. Sci.* 2010; **11**, 1321–1342.
11. U. Gawlik-Dziki, M. Swieca, M. Sułkowski, D. Dziki, B. Baraniak, J. Czyz, *Food Chem. Tox.* 2013, **57**, 154–160.
12. W. Stahl, H. van den Berg, J. Arthur, A. Bast, J. Dainty, R. M. Faulks, C. Gartner, G. Haenen, P. Hollman, B. Holst, F.J. Kelly, M. C. Polidori, C. Rice-Evans, S. Southon, T. van Vliet, J. Viña-Ribes, G. Williamson, and S. B. Astley, 2002, *Mol. Aspects Med.* **23** 39–100
13. C. Manach, A. Scalbert, C. Morand, C. Rémésy, and L. Jiménez, *Am. J. Clin. Nutr.*, 2004, **79**, 727–747.
14. C. Manach, G. Williamson, C. Morand, A. Scalbert, and C. Rémésy, *Am. J. Clin. Nutr.*, 2005, **81**, 230S–242S.

15. Z. Teng, C. Yuan, F. Zhang, M. Huan, *W.Cao, Li Kangch*u, et al. *PLoS One* 2012, **7**, e29647, http://dx.doi.org/10.1371/journal.pone.0029647
16. B. Halliwell, K Zhao and M Whiteman, *Free Radic Res.,* 2000 **33**, 819-830
17. E. Azzini, R. Bugianesi, F. Romano, D. Di Venere, S. Miccadei, A. Durazzo, M. S. Foddai, G. Catasta, V. Linsalata and G. Maiani, *Br. J. Nutr.,* 2007, **97**, 963–969.
18. S.M. Wittemer, M. Plocha, T. Windeck, S.C. Muller, B. Drewelow, H.,Derendorf and M. Veit, *Phytomedicine*, 2005, **12**, 28–38.
19. P.L. Falé, C. Ferreira, A.M. Rodrigues, P. Cleto, P.J.A. Madeira, M.H. Florêncio, F.N. Frazão and M.L. Serralheiro, *J. Med. Plants Res.*, 2013, **7**, 1370-1378.
20. A. Garbetta, I. Capotorto, A. Cardinali, I. D'Antuono, V. Linsalata, F. Pizzi and F. Minervini, *J. Funct. Foods.*, 2014 **10**, 456–464.
21. S. Lafay, A. Gil-Izquierdo, C. Manach, C. Morand, C. Besson and A. Scalbert, *J. Nutr.*, 2006, **136**, 1192–1197.
22. M.P. Gonthier, M.A. Verny, C. Besson, C. Rémésy and A. Scalbert, *J. Nutr*., 2003, **133**, 1853–1859.
23. C. Dupas, A. Marsset Baglieri, C. Ordonaud, D. Tomé and M.N. Maillard, *Mol. Nutr. Food Res.*, 2006, **50**:1053–60.
24. V. Lattanzio, *J. Chromatogr. A*, 1982, **250**, 143-148.
25. P. L. Teissedre, E. N. Frankel, A. L. Waterhouse, H. Peleg and J. B. German, *J. Sci. Food Agric.*, 1996, **70**, 55-61.
26. C.H.M. Versantvoort, A. G. Oomen, E. Van de Kamp, C.J.M. Rompelberg and A.J.A.M. Sips, *Food Chem. Toxicol.*, 2005, **43**, 31-40.
27. M.L. Failla, C. Chitchumronchokchai, M.G. Ferruzzi, S.R. Goltz and W.W. Campbell, *Food Funct*., 2014, **5**, 1101-1112.

28. A.P. Neilson, B.J. Song, T.N. Sapper, J.A. Bomser and M.G. Ferruzzi, *Nutr. Res.,* 2010, **30**, 327-40.

29. M.M. Bradford, *Anal. Biochem*. 1976, **72**, 248-253.

30. A. Cardinali, V. Linsalata, V. Lattanzio and M. G. Ferruzzi, *J. Food Sci.*, 2011, **76**, H48–H54.

31. A. Cardinali, F. Rotondo, F. Minervini, V. Linsalata, I. D'Antuono, L. Debellis and M.G. Ferruzzi, *Food Res. Int.*, 2013, **54**, 132-138.

32. A. Cardinali, S. Pati, F.Minervini, I. D'Antuono, V. Linsalata and V. Lattanzio, *J. Agric. Food Chem*., 2012, **60**, 1822−1829.

33. M. Nardini, M. D'Aquino, G. Tomassi, V. Gentili, M. Di Felice and C. Scaccini *Free Radic. Biol. Med.*, 1995, **19**, 541–552.

34. A. S. Meyer, M. Heinonen and E. N. Frankel, *Food Chem*. 1998, **61**, 71-75.

35. J. Bouayed, L. Hoffmann and T. Bohn, *Food Chem*., 2011, **128**, 14–21.

36. J. Bouayed, H. Deußer, L. Hoffmann and T. Bohn, *Food Chem.,* 2012 **131**, 1466–1472.

37. Z. Zhao, Y. Egashira and H. Sanada, *J Nutr.* 2004;**134**, 3083–3088.

38. D. Tagliazucchi, E. Verzelloni, D. Bertolini, and A. Conte, *Food Chem.,* 2010, **120** 599–606.

39. M. Matsumoto, N. Matsukawa, H. Mineo, H. Chiji and H. Hara, *Biosci Biotechnol Biochem*. 2004, **68**, 1929–1934.

40. M.J. Bermùdez-Soto, F.A. Tomàs-Barberàn and M.T. Garcìa-Conesa, *Food Chem.* 2007 **102**: 865–874.

41. R.J. Green, A.S. Murphy, B. Schulz, B.A. Watkins and M.G. Ferruzzi, *Mol. Nutr. Food Res.* 2007, **51**, 1152–1162.

42. J. Boyer, D. Brown and R.H. Liu, *Nutr. J.* 2005, **4**, 1–15.

43. G.J. McDougall, S. Fyffe, P. Dobson and D. Stewart, *Phytochemistry*, 2005, **66**, 2540–2548.

44. S. M. Kern, R. N. Bennett, P. W. Needs, F. A. Mellon, et al.. *J. Agric. Food Chem*., 2003, **51**, 7884–7891.

45. M. F. Andreasen, P. A. Kroon, G. Williamson and M. T. Garcia-Conesa, *J. Agric. Food Chem.*, 2001, **49**, 5679–5684.

46. L. Ziberna, S. Fornasaro, J. Cvorovic, F. Tramer and S. Passamonti, in *Polyphenols in Human Health and Disease*, ed. R.R.Watson, V.R. Preedy, S. Zibadi, Accademic Press Publications Elsevier, London, 2014, vol. 1, ch. **37**, pp. 489-51.

47. J. B. Vaidyanathan, and T. Walle, *J. Pharmacol. Exp. Ther.*, 2003, **307**, 745–752.

48. A. P. Neilson, J. C. George, E. M. Janle, R. D. Mattes, R. Rudolf, N. V. Matusheski, et al. *J. Agric. Food Chem.,* 2009, **57**, 9418–9426.

49. C. M. Peters, R. J. Green, E. M. Janle and M.G. Ferruzzi, *Food Res. Int.*, 2010, **43**, 95–102.

50. V. Rozehnal, D. Nakai, U. Hoepner, T. Fischer, E. Kamiyama, M., Takahashi, et al., *Eur. J. Pharm., Sci.,* 2012, **46**, 367–373.

51. Y Liu and M. Hu, *Drug Metab. Dispos.* 2002, **30**, 370-377.

52. S. Waldmann, M. Almukainzi, N. A. Bou-Chacra, G. L. Amidon, B. J. Lee, J. Feng, and R. Löbenberg, *Mol. Pharm.*, 2012, **9**, 815-822.

53. J.K. Srivastava and S. Gupta, *J. Agric. Food Chem*., 2007, **55**, 9470–9478.

54. J.K. Srivastava and S. Gupta, *Mol. Cell. Pharmacol*., 2009, **1**, 138.

Table 1.
Phenolic contents determined in artichoke heads after aqueous extraction and after simulated *in vitro* digestion, by HPLC DAD analyses.
Data were expressed as mg/100g of artichoke and represent means +/- SD (n= 6 independent experiments).

Phenolics	**Artichoke Extract**	**Intestinal digesta**
1-O- caffeoylquinic acid	23.9 ± 5.0	6.9 ± 2.1
3-O- caffeoylquinic acid	6.3 ± 0.6	6.3 ± 2.8
Chlorogenic acid	287.0 ± 64.4	183.5 ± 24.2
4-O- caffeoylquinic acid	8.3 ± 0.6	13.1 ± 3.2
cynarin	3.2 ± 0.9	4.7 ± 1.6
1,4-O-dicaffeoylquinic acid	9.5 ± 3.4	7.9 ± 1.6
4,5-O-dicaffeoylquinic acid	15.1 ± 7.3	10.2 ± 2.4
3,5-O-dicaffeoylquinic acid	182.6 ± 10.2	76.4 ± 5.6
1,5-O-dicaffeoylquinic acid	208.1 ± 39.8	97.5 ± 6.2
3,4-O-dicaffeoylquinic acid	29.7 ± 10.0	12.3 ± 2.4
apigenin-7-O-glucoside	12.1 ± 7.0	4.5 ± 1.3
Total phenolics	785.8 ± 12.4	423.3 ± 38.5

Table 2. Bio-accessible individual polyphenolics in the saliva, gastric and intestinal phases as determined from globe artichoke after simulated gastrointestinal digestion. Data are expressed as % and represent means +/- SD (n= 6 independent experiments).

Compounds	Bioaccessibility (%)		
	Saliva phase	Gastric phase	Intestinal phase
1-O- caffeoylquinic acid	16.9 ± 2.9 [a]	22.7 ± 1.2 [b]	30.3 ± 5.0 [c]
3-O- caffeoylquinic acid	21.8 ± 1.9 [a]	30.2 ± 5.3 [b]	87.3 ± 22.5 [c]
Chlorogenic acid	35.2 ± 3.2 [a]	51.7 ± 18.0 [ab]	70.0 ± 10.0 [b]
4-O- caffeoylquinic acid	60.2 ± 12.2 [a]	64.5 ± 10.6 [a]	159.8 ± 23.5 [b]
cynarin	46.5 ± 12.5 [a]	-	171.2 ± 22.5 [b]
1,4-O-dicaffeoylquinic acid	45.2 ± 15.7 [a]	14.9 ± 2.0 [b]	104.2 ± 37.3 [c]
4,5-O-dicaffeoylquinic acid	20.5 ± 3.4 [a]	14.4 ± 2.3 [b]	93.4 ± 24.9 [c]
3,5-O-dicaffeoylquinic acid	19.7 ± 2.8 [a]	9.0 ± 1.4 [b]	41.3 ± 2.7 [c]
1,5-O-dicaffeoylquinic acid	25.3 ± 3.3 [a]	8.3 ± 1.4 [b]	50.3 ± 6.9 [c]
3,4-O-dicaffeoylquinic acid	16.6 ± 4.4 [a]	6.2 ± 0.9 [b]	47.4 ± 14.0 [c]
apigenin-7-O-glucoside	28.9 ± 11.0	-	49.9 ± 16.0
Total bio-accessibility	27.2 ± 2.7 [a]	35.7 ± 2.5 [b]	55.8 ± 8.4 [c]

Table 3. Stability of pure phenolic compounds individually subjected to *in vitro* gastrointestinal digestion.

Phenolics	**Std solutions** (μg/mL)	**Intestinal digesta** (μg/mL)
Chlorogenic acid	100	48.1 ± 6.3
4-O- caffeoylquinic acid	-	7.6 ± 0.6
1,5-O-dicaffeoylquinic acid	100	49.6 ± 0.7
cynarin	-	6.2 ± 0.4
1,4-O-dicaffeoylquinic acid	-	9.6 ± 0.2
3,5-O-dicaffeoylquinic acid	100	25.8 ± 7.7
4,5-O-dicaffeoylquinic acid	-	16.4 ± 0.4
3,4-O-dicaffeoylquinic acid	-	12.1 ± 1.4

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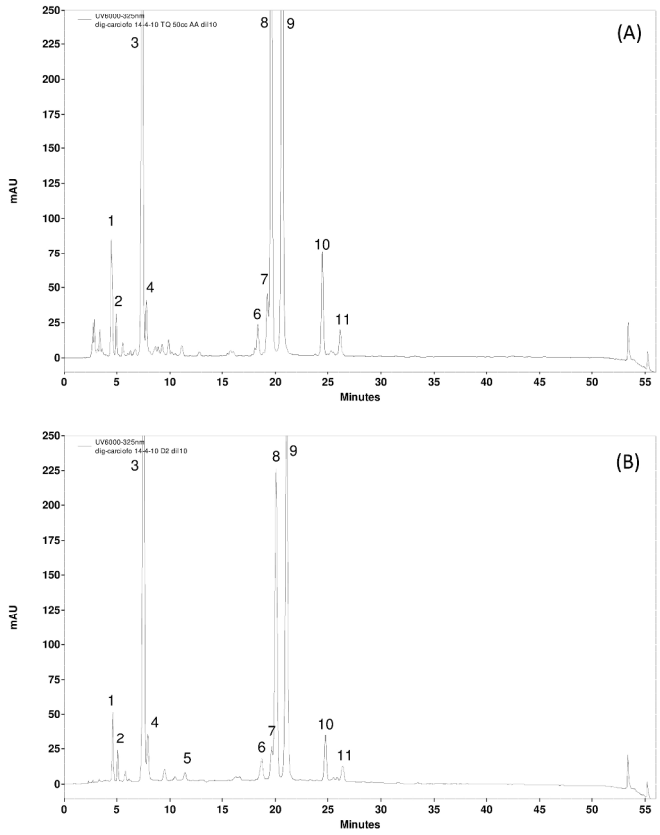

Fig. 1. HPLC chromatograms of artichoke heads. A) Aqueous extraction B) Simulated in vitro gastrointestinal digestion. (1=1 CQA, 2=3 CQA, 3=CGA, 4=4 CQA, 5=cynarin, 6=1,4 diCQA, 7= 4,5 diCQA, 8=3,5 diCQA, 9=1,5 diCQA, 10=3,4 diCQA, 11=apigenin-7-O-glucoside)
297x420mm (300 x 300 DPI)

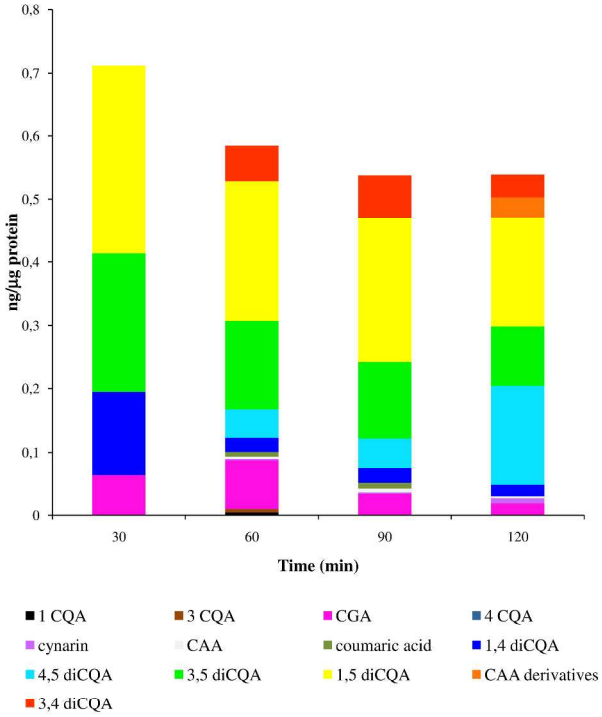

Fig. 2. Uptake of total artichoke polyphenols (100 μg/mL) by Caco-2 human intestinal cells at different times of incubation. Experiments were conducted as described in Materials and Methods. Data represents mean ± DS of mean for n=3.
297x420mm (300 x 300 DPI)