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Short communication
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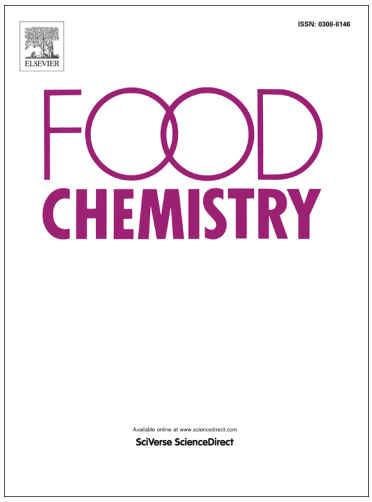

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Novel biologically active principles from spinach, goji and quinoa

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ABSTRACT
Spinach leaves, goji berries and quinoa seeds are claimed to have a great nutraceutical potential due to their high content of compounds providing benefits for human health, such as amino acids, polyunsaturated fatty acids, carotenoids, betaine, vitamins, fibre, minerals and polyphenols. Samples of these plants were extracted with different solvent mixtures (e.g. EtOH, H_2O/EtOH 3:7 and H_2O/EtOH 7:3) and extractions were accomplished using a microwave apparatus. Subsequent UHPLC analysis and photodiode array detection were employed for the quantification of biologically active compounds like 7-isopentenyloxycoumarin, auraptene, umbelliprenin, boropinic acid and 4'-geranyloxyferulic acid. EtOH was found to be the best solvent in terms of extractive yields and the above-mentioned phytochemicals were recorded in the concentration range 2.01 – 49.22 μg/g dry extract. The findings depicted herein revealed that spinach, goji and quinoa are good sources of oxyprenylated umbelliferone and ferulic acid derivatives.

Keywords:

Chenopodium quinoa, *Lycium barbarum*, *Spinacia oleracea*, Nutraceutical, Oxyprenylated phenylpropanoids

1. Introduction

Spinach leaves, goji berries and quinoa seeds are common ingredients of salads and soaps consumed all over the world. The individual ingredients of these food preparations are known to contain several chemicals having well established beneficial effects for human welfare. In particular spinach (*Spinacia oleracea* L., Amaranthaceae) contains bioactive phytochemicals (among which flavonoid glycosides, leuteolin, glycolipids,and coumaric acid derivatives) able to act as effective radicals and reactive oxygen species scavengers, to modulate the expression of genes encoding proteins playing a key role in modulating metabolism, cell proliferation, inflammation and overall anti-oxidant defense of human body, and finally to slow compulsive food intake by promoting the secretion of satiety hormones (Roberts and Moreau 2016). Goji (*Lycium barbarum* L., Solanaceae) berries have been used for centuries in traditional medicine practices in China and now are at disposition worldwide, also in form of food products and supplements like juices, jams, bakery products, energy bars and capsules. They contain mainly polysaccharides, polyphenols and carotenoids able to exert beneficial effects for the prevention of chronic diseases (cancer, atherosclerosis, obesity and diabetes), and to promote weight loss and longevity (Amagase and Farnsworth 2011). Quinoa (*Chenopodium quinoa* Willd, Amaranthaceae) seeds are also classified as "pseudocereals" as they are featured by the same nutritional value as cereals (high content of essential aminoacids and polysaccharides), but without gluten. Furthermore, they contain polyphenols, tocopherols and carotenoids able to lower the risk of oxidative stress related diseases like cancer, cardiovascular diseases, stroke, diabetes and obesity, as well as to exert effective anti-oxidant and anti-inflammatory effects (Tang and Tsao 2017). To date the majority of literature data refer to the properties of the most abundant bioactive compounds and secondary metabolites from spinach, goji and quinoa, while virtually no citations about components contained in low concentrations are available. Prenylation of phenylpropanoids and polyketides is a relatively common metabolic reaction in higher plants and fungi and involves the linkage of an isopentenyl, a terpenyl or a farnesyl chain to an OH moiety and/or to an aromatic carbon atom (Alhassain et al.

2014). Such a biosynthetic step also occurs in plants belonging to the Amaranthaceae and Solanaceae families (Fiorito et al. 2017; Yang et al. 2015). Thus, biosynthetic and chemotaxonomic considerations allow the hypothesis that prenylated secondary metabolites may be additional components of the previously characterized phytochemical pool of spinach, goji and quinoa. Their presence in such plants would be noteworthy if one considers the great pharmacological potential of these natural food products, in the chemoprevention of several acute and chronic syndromes affecting humans, such as cancer, inflammation and neurological disorders (Genovese et al. 2015; Epifano et al. 2015 Okuyama et al. 2016). These components could significantly enhance the

nutraceutical value of the source food plants. In this short communication we wish to describe the extraction and UHPLC analysis and photodiode array (PDA) detection of selected umbelliferone and ferulic acid oxyprenylated derivatives (Figure 1), namely 7-isopentenyloxycoumarin **1**, auraptene **2** and umbelliprenin **3,** boropinic acid **4**, 4'-geranyloxyferulic acid (GOFA) **5** from the title plant species.

2. Materials and methods

2.1. Materials

Ferulic acid, umbelliferone,3-dimethylallyl bromide, geranyl bromide, *trans*, *trans*-farnesyl bromide, dry potassium carbonate were purchased from Sigma-Aldrich (Merck Sigma-Aldrich, Milan, Italy) and used without further purification. 7-isopentenyloxycoumarin **1**, auraptene **2**, umbelliprenin **3,** boropinic acid **4**, GOFA **5,** were chemically synthesized as already reported (Taddeo et al. 2017a) and their purity (> 98.1 %) assessed by HPLC and ^{1}H NMR. All chemicals were stored in a desiccator in presence of P_4O_{10} prior their use. All organic solvents (UHPLC grade) were supplied from Carlo Erba Reagents (DasitGroup-Carlo Erba Reagenti, Milan, Italy). Water HPLC-grade (>18.2 MΩ cm resistivity) was obtained by passage through an Elix 3 and Milli-Q Academic water purification system (Millipore, Bedford, MA, USA). Stock solutions of target analytes at the concentration of 1.0 mg/ml were individually prepared by dissolving 10 mg of each reference powder into 10 ml volumetric flask with methanol. The stock solutions were finally collected to provide one solution. The standard solutions were kept in amber vials at 1-3°C.

2.2. Extraction procedure

S. oleracea leaves, *L. barbarum* berries and *C. quinoa* seeds were purchased from local markets. Voucher specimens (SO-2017-1), (LB-2017-1) and (QS-2017-1) have been stored in the deposit of the laboratory of Chemistry of Natural Compounds at the Department of Pharmacy of the University "G. D'Annunzio" of Chieti-Pescara. All samples have been dried, ground and homogenized prior to extraction experiments. 0.5 g of each plant sample was extracted with 5 ml of each of the following solvent mixture: EtOH, H_2O/EtOH 3:7 and H_2O/EtOH 7:3. Extractions were accomplished using a microwave - assisted procedure. Extractive solutions were evaporated to dryness under vacuum and the resulting solid was re-suspended in MeOH (10 mg of dry extract in 100 µL). An aliquot of 5 µl of this solution was injected into the UHPLC apparatus.

2.3. UHPLC conditions

Analyses were performed on a Waters Ultra Performance Liquid Chromatography system (ACQUITY H-Class). Chromatographic separation was achieved using an Acquity UHPLC® BEH C_{18} (50 x 2.1 mm I.D. 1.7 µm particle size) column protected by a disposable Security Guard C_{18} (4.6 x 2.1 mm I.D.) (Phenomenex, Torrance, CA, USA). The column was thermostated at 25 ± 1°C. All

injected solutions were stored in the auto-sampler at 5° C. The partial loop with needle overfills mode was set up to inject 5 µl. Mobile phases consisted of H_2O / CH_3CN (90/10) containing 0.04% of formic acid (A), CH_3CN containing 0.04% of formic acid (B) and MeOH (C) in gradient elution. A linear gradient elution program as outlined in Table S1 was used. Solvents were filtered before use through a 0.22 µm type GV membrane (Millipore). For quantification purposes, the UV detection was set at 322 nm for each analyte. An example of a UHPLC chromatogram is shown in the Figure S1. Empower v.3 software (Waters) was used for setting-up the analysis and for data management.

2.4. Method validation

The method was validated according to the ICH guidelines using standard mixtures (linearity, limit of detection [LOD] and limit of quantification [LOQ]) and spinach, goji and quinoa extract samples (accuracy and precision). Linearity was calculated in the range 1-100 µg/ml for each analyte. Method precision was tested at three concentration levels (QC_L, QC_M and QC_H) in three replicates for each kind of vegetables extract sample to calculate the percentage of RSD of the determination. Spinach, goji and quinoa extracts spiked with standard solutions at three concentration levels (QC_L, QC_M and QC_H) were used for the determination of the accuracy of the method. Samples were spiked in triplicates and they were run in three triplicates. Selectivity of the method was evaluated comparing standard calibration curves in MeOH with standard calibration curves using the vegetable material extracts containing the lowest concentration of the selected analyte (Kumar et al. 2007).

3. Results and Discussion

3.1. Performance of the analytical methodology

System suitability was assessed by ten replicate analyses of the analytes at a concentration of 5 µg/ml. As reported in Table 1, all retention times, capacity factors, resolutions, theoretical plate numbers and peak asymmetries were within acceptable values. Calibration curves were elaborated

in the range from 1 μg/ml to 100 μg/ml for all analytes. Data were obtained by plotting concentration against the peak area of each standard. Calibration curves were linear over the range tested for all analytes and determination coefficients (r^2) were higher than 0.9990 (Table 2). A linear regression analysis with weighting factors consisted in $1/x^2$ values was applied to describe the relationship between concentration of the analytes and the detector response. LOQ of the method was defined according to International Guidelines ICH Q2 (R1) 2005 as the concentration of the lowest standard on the calibration curve for which (a) the analyte peak is identifiable and discrete, (b) the analyte response is at least ten times the response of the blank sample, and (c) the analyte response is reproducible with a precision less than 20% and trueness greater than 80 – 120%. LOD and LOQ were assessed from the results for three replicates of real samples spiked at different

concentrations, with a signal-to-noise ratio of 3 and 10, respectively. The LOQ and LOD values were equal for all analytes (0.5 μg/ml and 0.3 μg/ml, respectively). Data for intra- and inter-day

precision and accuracy, calculated from the analysis of three batches of LLOQ and QC samples at three different concentrations (LQC, MQC and HQC) in duplicate on the same day, and for five consecutive days, are reported in Table 3. Intra- and inter-day precision (RSD values) did not exceed 6.02 %, while the intra- and inter-day accuracy (BIAS %) values were in the range from -3.52 % to 3.29 %. Thus, according to ICH guidelines, the method has satisfactory accuracy, precision and reproducibility. Non-significant carry over effect (< 0.25%), assessed by injecting two blank samples of each extract followed by two blank samples spiked with the analytes at the LOQ concentration was detected. Recoveries of all the five analytes herein under investigation were > 97.6 % with a good precision (RSD < 2.9 %). Selectivity of the method was evaluated by analyzing blank extract samples. All peaks were well resolved at the baseline and separated from each other and no matrix effect was observed. Values of the selectivity index (α), defined as reported in ICH guidelines (Kumar et al. 2007) were in the range 1.12 – 1.20. Stability of the five analytes were within the range already assessed for the same compounds (Scotti et al. 2018).

3.2. Quantification of oxyprenylated phenylpropanoids in spinach, goji and quinoa extracts

Although several methods for vegetable material extraction are available, in this study we used only the microwave-assisted ones as this largely performed better in terms of yields (around 3- to 16-fold) respect to “classic” maceration (carried out for 96 h) and ultrasound-promoted extraction (data not shown). Results of the quantification of 7-isopentenyloxycoumarin **1**, auraptene **2**,

umbelliprenin **3**, boropinic acid **4**, GOFA **5** in spinach, goji and quinoa extracts are reported in Table 4. Three independent extraction methodologies and analyses were performed to obtain the final calculated concentrations. Data reported in Table 4 clearly demonstrates that performing the extraction step with EtOH was the best effective process in terms of yields of the all secondary metabolites under investigation. The presence of H_2O as co-solvent resulted in a dramatic decrease of these values, and this can be rationalized by the very low hydrophilicity of 7-

isopentenyloxycoumarin **1**, auraptene **2**, umbelliprenin **3**, boropinic acid **4** and GOFA **5**. It is noteworthy also to underline to this concern that we tried to employ also more apolar solvents, like hexane, dichloromethane, acetone and ethyl acetate. However, all attempts resulted in poor yields and more importantly provided extracts for which the analysis and characterization of the above mentioned oxyprenylated phenylpropanoids was harder to achieve due to the presence of contaminants and a great matrix effect (data not shown). Under all the experimental conditions employed, retention times of all analytes in all vegetable extracts matched those obtained from the analyses accomplished with pure chemical standards and reported in Table 1. Other than overlapping of R_ts, the structural assignments was also accomplished by comparison of LC-MS spectra of pure chemical standards with those recorded for diagnostic peaks. As an explicative example, Figure 2 shows the MS spectrum of auraptene **2** obtained by chemical synthesis and that of auraptene extracted from spinach by ultrasound-assisted maceration with EtOH. Our initial hypothesis based on biosynthetic and chemotaxonomic correlations has been confirmed by the presence in all three food plants of at least one of 7-isopentenyloxycoumarin **1**, auraptene **2**, umbelliprenin **3**, boropinic acid **4** and GOFA **5**. Quite surprisingly, only spinach leaves were shown to contain both umbelliferone **1-3** and ferulic acid **4-5** derivatives. Despite the fact that goji berries contain large quantities of ferulic acid (Vulic et al. 2016), its terpenylated derivatives were not detected in the extracts or were recorded in quantities below LOQ. On the other hand, quinoa seeds have not been reported to contain umbelliferone and thus the absence of oxyprenylated coumarins was expected. As with goji, quinoa seeds were also seen to contain high quantities of ferulic acid (Tang et al. 2015), but also in this case its metabolic derivatization is limited to a small degree of geranylation . Quantification and qualitative analysis of oxyprenylated phenylpropanoids, as outlined herein, may also be useful as an additional and alternative criterion to assess the authenticity and genuineness of food plant samples, in the context of industrial production processes, establishment of the nutritional value and chemical fingerprinting in general.

4. Conclusions

This study is part of our ongoing series of investigations aimed at characterizing minor compounds in known plant foods, and related preparations, having potential beneficial effects for human welfare both in terms of prevention and therapy. In this context we developed an easy and effective extractive procedure and UHPLC methodology for the qualitative and quantitative analysis of five main representatives between naturally occurring oxyprenylated phenylpropanoids from ingredients of commonly consumed food plants. The present investigation led to characterize for the first-time in the literature 7-isopentenyloxycoumarin **1**, auraptene **2**, umbelliprenin **3**, boropinic acid **4** and GOFA **5** as additional components of the phytochemical pool of spinach, goji and quinoa. Such secondary metabolites in foodstuffs are nowadays more and more considered for their potential chemopreventive properties, overlapping with those already known for the title food plants. In respect to previous similar studies, we confirmed that food plants belonging to the Amarathaceae and Solanaceae families can be regarded as valuable and alternative sources of oxyprenylated secondary metabolites. The present study may be a stimulus for further similar investigations, considering that such families comprise several other edible vegetables (e.g. beet, silver cockscomb, pigweed, samphire, potato, tomato, eggplant, etc.) and that the UHPLC methodology we set up herein can be very easily, effectively and generally applied to the determination of the phytochemical composition of several other plant matrices.

Conflict of interest

The authors declare no conflict of interest

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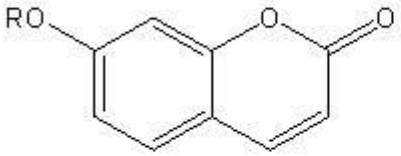
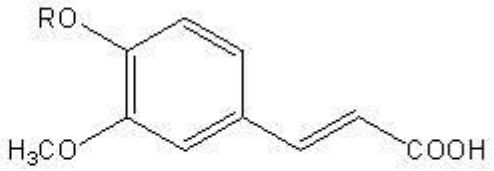
Figure captions
Figure 1: Chemical structures of the oxyprenylated phenylpropanoids under investigation
Figure 2 MS spectra of auraptene **2** obtained by chemical synthesis (A) and extracted from spinach leaves (B)

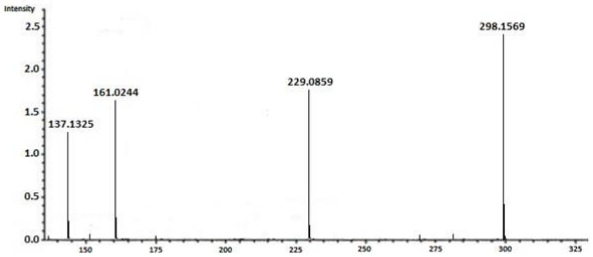


1 R = 3,3-dimethylallyl
2 R = geranyl
3 R = farnesyl



4 R = 3,3-dimethylallyl
5 R = geranyl

(A)



(B)

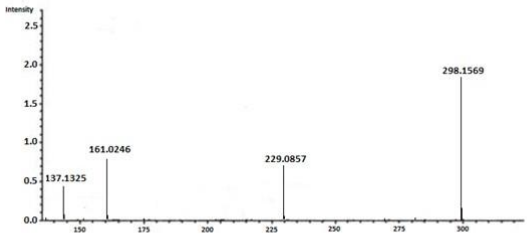


Table 1. System suitability parameters

Compound 1 2 3 4 5
Retention time (min.)[a] 7.3 (0.4 %) 10.5 (0.4 %) 12.2 (0.6 %) 6.7 (0.5 %) 9.3 (0.3 %)
Capacity factor[b] 4.1 6.0 7.4 3.6 5.4
Resolution[c] 1.79 2.83 5.41 2.22 5.23
Theoretical plate 3509 11027 27339 4352 16048 **numbers**
Peak symmetry (10%) 0.7 1.0 1.0 1.0 1.1

[a]Values in brackets represent RSD % of retention time (10)
[b]T_0 was calculated uracil eluted using the same conditions (t_0=1.46) [c]Values represent resolution of adjacent peaks

Table 2. Calibration curves data of the five analytes

Compound* Slope Intercept r^2 LOD LOQ** % Recovery**
1 167358 (154836- 179880) -26600 0.9881 0.5 0.3 100.3 (-186000- 132776)
2 100376 3310 0.9863 0.5 0.3 99.8 (92799- (-93130-99752) 107954)
3 121888 (112150- 131626) -6425 0.9854 0.5 0.3 100.4 (-130400- 117516)
4 181346 (167960- 194733) 25069 0.9869 0.5 0.3 97.6 (-145300- 195443)
5 46568 10810 0.9779 0.5 0.3 99.8 (42599-50536) (-39690-61313)

***1** = 7-Isopentenyloxycoumarin, **2** = Auraptene, **3** = Umbelliprenin, **4** = Boropinic acid, **5** = 4'-Geranyloxyferulic acid, ** expressed as μg/mL

Table 4. Precision and accuracy data (n = 6)

*Data are expressed as mean values from the analysis of three batches of LLOQ and QC samples at three different concentrations (LQC, MQC, and HQC) in duplicate

on the same day and for five consecutive days ****1** = 7-Isopentenyloxycoumarin, **2** = Auraptene, **3** = Umbelliprenin, **4** = Boropinic acid, **5** = 4'-Geranyloxyferulic acid

Table 5. Quantification of analyte in vegetable extracts (Values are means ± standard deviation (*n*=3) expressed as μg/g dry extract).

Compound 1 2 3 4 5**
Precision (%)
Intra-day 2.7-5.2 2.7-4.4 2.5-6.7 3.6-6.8 0.7-5.5
Inter-day 3.1-6.0 3.0-7.1 3.6-7.4 4.1-7.8 1.6-4.2
Accuracy (%)
Intra-day 0.9-2.6 0.7-2.8 1.2-3.2 1.5-3.5 0.9-2.9
Inter-day 2.0-5.1 1.5-3.6 1.9-4.2 1.6-3.7 1.2-2.7

Spinach		
Compound EtOH EtOH /H_2O 7:3 EtOH /H_2O 3:7 *		
1 2.33 ± 0.12 1.94 ± 0.12 0.89 ± 0.03		

2 28.09 ± 1.22	13.12 ± 0.93	3.44 ± 0.11
3 49.22 ± 2.31	18.18 ± 0.82	5.56 ± 0.36
4 4.36 ± 0.22	2.55 ± 0.11	ND
5 21.49 ± 1.11	10.99 ± 0.78	2.33 ± 0.11
Goji		
EtOH	**EtOH /H_2O 7:3**	**EtOH /H_2O 3:7**
1 8.37 ± 0.22	6.12 ± 0.17	3.21 ± 0.09
2 7.91 ± 0.74	5.04 ± 0.33	2.21 ± 0.09
3 8.33 ± 0.44	3.21 ± 0.09	2.12 ± 0.06
4 ND	ND	ND
5 ND	ND	ND
Quinoa		
Compound EtOH	**EtOH /H_2O 7:3**	**EtOH /H_2O 3:7**
1 ND	ND	ND
2 ND	ND	ND
3 ND	ND	ND
4 ND	ND	ND
5 2.01 ± 0.02	0.88 ± 0.02	0.58 ± 0.02

*__1__ = 7- Isopen tenylo xycou marin, **2**= Aurapt ene, **3** = Umbel lipreni n, **4** = Boropi nic acid, **5** = 4'-Geran yloxyf erulic acid

ND = not detecte d or below LOQ 1.

UPLC method set up for the quantification of minor nutraceuticals in spinach, goji, and quinoa 2. Prenyloxyphenylpropanoids have been disclosed for the first time
3. Widening of the nutraceutical pool knowledge in Amaranthaceae and Solanaceae plants