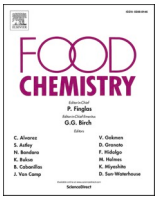
Food Chemistry 384 (2022) 132462

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Sequential steps of the incorporation of bioactive plant extracts from wild Italian *Plantago coronopus* L. and *Cichorium intybus* L. leaves in fresh egg pasta

Costanza Ceccanti [a,b], Tiane C. Finimundy [a], Bruno Melgar [a], Carla Pereira [a], Isabel C.F. R. Ferreira [a], Lillian Barros [a,*]

[a] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal
[b] Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto, 80-56124 Pisa, Italy

ARTICLE INFO

Keywords:
Wild plants
Cichorium intybus L.
Plantago coronopus L.
Enriched pasta
Nutraceutical properties
Functional food

ABSTRACT

The application of bioactive extracts from *Cichorium intybus* L. and *Plantago coronopus* L. species were incorporated as a functional ingredient in fresh egg pasta (Fettuccine). In that sense, a pasta making procedure was accessed using different concentrations of the plant extracts (0.25–0.63 mg/g), drying times (20–420 min) and drying temperatures (40–90 °C; only for *P. coronopus* enriched pasta), to screen an optimal factor selection in the pasta making procedure and to enhance the bioactive properties of the final product. In the chemical characterisation of the plant extracts, twenty-five phenolic compounds were tentatively identified (twenty compounds belonging to phenolic acid and phenylpropanoid classes and five belonging to the flavonoid sub-class) and a strong synergy between the plant extract concentration and the drying time was showed. The analysed antioxidant properties were enhanced by the phenolic compounds of the extracts and a new functional food with higher bioactive quality was developed.

1. Introduction

Consumers are increasingly aware of the strong relationship between diet and health *status,* and, for this reason, they have changed food preferences and have increasingly oriented towards functional foods. Nowadays, wild edible plants represent new functional foods, and their nutraceutical properties are even more studied (Ceccanti, Landi, Benvenuti, Pardossi, & Guidi, 2018; Pinela, Carvalho, & Ferreira, 2017). Among the vast number of wild edible plant species with functional properties, *Plantago coronopus* L. (Plantaginaceae) and *Cichorium intybus* L. (Asteraceae) are commonly used as vegetables in Balkan traditional cuisine and their leaves are salad ingredients in France and Italy (Pereira et al., 2017).

C. intybus, also known as chicory, is well known for their richness in phenolic compounds with antioxidant properties, namely, coumarins such as aesculetin, esculin, chicoriin A-B-C, scopoletin and 6,7-dihydroxycoumarin (Street, Sidana, & Prinsloo, 2013), hydroxycinnamic acids, vitamins, phenolic acids (caffeic and chlorogenic acid derivatives such as chicoric acid and neochlorogenic acid), flavonoids, and sesquiterpenes lactones (Heimler, Isolani, Vignolini, & Romani, 2009; Saybel, Rendyuk, Dargaeva, Nikolaev, & Khobrakova, 2020).

P. coronopus, also known as buck's-horn plantain, is mainly known for their content in phenylpropanoids such as verbascoside and plantamajoside (Ravn & Brimer, 1988).

Due to these aspects, *C. intybus* leaves are traditionally used as antidiabetic, antibacterial, anti-inflammatory, diuretic, digestive and protector by other disorders (Saybel et al., 2020; Street et al., 2013), while *P. coronopus* leaves are used for their high radical scavenging and cytotoxic activity (Galvez, Martın-Cordero, Lopez-Lazaro, Cortes, & Ayuso, 2003; Pereira, et al., 2017).

The use of these species as functional ingredients to enrich common food may be a new step in wild edible plat species utility. The enrichment of pasta with functional ingredients as well as the addition of different plant species such as nettle (Marchetti et al., 2018) has already been studied from a nutraceutical point of view as well as from a technological and sensory point of view (Bustos, Paesani, Quiroga, & León, 2019; Vimercati et al., 2020). However, different concentrations of plant extract and different steps in the pasta making procedure have

* Corresponding author.
E-mail address: lillian@ipb.pt (L. Barros).

https://doi.org/10.1016/j.foodchem.2022.132462
Received 7 October 2021; Received in revised form 10 February 2022; Accepted 12 February 2022
Available online 16 February 2022

marginally been tested from the nutraceutical point of view. For example, the drying process is considered the most critical step in the pasta manufacturing process since the used temperature highly influences the product's quality characteristics (Vimercati et al., 2020). On the contrary, no information is available about applying different drying times to the pasta making process, using a standard temperature (for example, 40 °C).

In this study, a new functional food wants to be proposed, applying phenolic compounds (i.e., phenolic acids such as chicoric acid and caffeoylquinic acids and phenylpropanoids such as verbascoside, plantamajoside and, specifically, flavonoids as kaempferol, quercetin and apigenin derivatives) and antioxidant properties (i.e., inhibition of lipid peroxidation and antihaemolytic activity) found in *C. intybus* and *P. coronopus* extract to egg pasta. Different extract concentrations, different drying times and different drying temperatures during the pasta making procedure were also evaluated. This step was necessary to obtain a screening analysis and to arrive to an optimal factor selection in the pasta making procedure to enhance the bioactive properties of the final product.

2. Materials and methods

2.1. Plant material

Wild *C. intybus* and *P. coronopus* leaves were randomly collected in the Monte Serra area (43°44′39.6″N 10°31′53.39″E, Pisa, Italy) during the vegetative stage of plants in spring 2019. Leaves with uniform colour, dimensions, and texture were sampled with a cut approximately 5 cm above the soil and transported in the laboratory within 1 h. Three polled samples were prepared with the leaves collected from 20 individual plants to have significant samples of the whole plant population for a correct statistical analysis. Each sample was dried at 60 °C using a laboratory electric thermostatic oven (Memmert GmbH+ Co. KG Universal Oven UN30, Schwabach, Germany) until plant material reached a constant weight.

2.2. Wild plant species extracts preparation

The dried plant material was ground and sieve in a 20 μm mesh and kept in a desiccator, protected from light and humidity. The extraction process to obtain the useful extract to incorporate in the egg pasta was performed according to Bessada, Barreira, Barros, Ferreira, & Oliveira (2016). An amount (1 g) of the dried plant powder was stirred with 30 mL of ethanol–water solution (80:20, *v/v*) for 60 min at room temperature. This procedure was carried out in triplicate. The obtained extracts were filtered through Whatman paper No. 4 filters and evaporated under reduced pressure (Büchi R-210, rotary evaporator, Flawil, Switzerland) at 100 rpm and 40 °C until ethanol was entirely removed. Then, the residual aqueous phase of each sample was frozen, lyophilised (Feee-Zone 4.5, Labconco, Kansas City, MO, USA) obtaining a range between 0.2 and 0.4 g of lyophilized material, and stored in a dry room until further analyses. Then, each extract was re-dissolved in the aqueous solution to obtain different concentrations to test in the pasta (5, 2.5, 1.25, 0.625, 0.3125 mg/mL), searching the best combination between palatability of pasta and enrichment in bioactive compounds.

2.3. Fresh pasta making procedure and cooking

Traditional fresh pasta, namely Fetuccine, was prepared with wheat flour, eggs and different concentrations of *C. intybus* and *P. coronopus* plant extracts (range of 0.25–0.63 mg per g of pasta).

The egg pasta recipe was as follows: 3 medium eggs were added to 300 g wheat flour, and the mixture was subjected to a hand-blending for 6 min to obtain a homogeneous dough. The dough was divided by hand for each extract concentrations into appropriate sizes (20 g) and laminated to a final pasta thickness of 2 mm using a home pasta lamination machine (MARCATO Atlas 150 Pasta Machine). In particular, each sample was passed 7 times at 4.8 mm of thickness and 1 time at 4.2, 3.6, 3.0, 2.5, and 2-mm. Laminated pasta sheets were cut in the same equipment, using a 2-mm wide cutting roll, to obtain the Fettuccine shaped pasta (Fig. 1). 10 g of pasta was separated to analyse the quality of different drying times (range of 20–420 min) and different drying temperatures (range of 40–90 °C; only for *P. coronopus* enriched pasta). Then, samples of 10 g of fresh pasta were submitted to cooking in 170 mL boiling distilled water for 10 min (*al dente point*), if applicable. The pasta samples were kept lyophilized until analysis. All the analyses were carried out in triplicate. The different extract concentrations, drying times and temperatures used in the present experiment were evaluated in terms of palatability (texture, taste and flavour) through a preliminary internal panel test among researchers (50% males and 50% females) of the Mountain Research Center, Bragança, Portugal, as trained panellists (data not shown).

2.4. Analysis of phenolic compounds

The phenolic compounds were determined according to Bessada et al. (2016). The extraction of bioactive compounds from cooked and uncooked enriched pasta was carried out according to the description reported in section 2.2. The samples were dissolved in an aqueous ethanol solution (80% v/v) to a final concentration of 10 mg/mL and filtered through 0.22 μm disposable filters. The analyses of the plant extracts or the pasta samples were performed by using a Dionex Ultimate 3000 (Dionex Ultimate 3000 UPLC and Linear Ion Trap LQT XL, Thermo Scientific, San Jose, CA, USA) ultra-performance liquid chromatographic equipment coupled to a diode array detector and an electrospray ionisation mass spectrometry detector (HPLC-DAD-ESI/MS) working in negative mode. For analysis, a Waters Spherisorb S3 ODS-2 reverse phase C18 column (4.6 × 150 mm, 3 μm; Waters, Milford, MA, USA) and an elution gradient using as mobile phase formic acid/water (0.1%) and acetonitrile recorded at 280, 320 and 370 nm as preferred wavelengths, as previously described by Bessada et al. (2016). The acquisition and the processing of data were achieved with the Xcalibur® data system (Thermo Scientific, San Jose, CA, USA). For the identification of the compounds, the data obtained (UV–Vis spectra, and mass spectra) were compared with data available in the literature and, when available, standards (Extrasynthèse, Genay, France) were used to identify individual phenolic compounds. In cases where no standard compound was available, the quantification was performed using the calibration curve of a compound within the same phenolic group. The quantification of the identified compounds was based on the calibration curves of authentic standards which were protocatechuic acid ($y = 214168x + 27102$, $R^2 = 0.999$; LOD = 0.14 μg/mL; LOQ = 0.52 μg/mL), sinapic acid ($y = 197337x + 30036$; $R^2 = 0.999$; LOD = 0.17 μg/mL; LOQ = 1.22 μg/mL), chlorogenic acid ($y = 168823x - 161172$; $R^2 = 0.999$; LOD = 0.20 μg/mL; LOQ = 0.68 μg/mL), caffeic acid ($y = 388345x + 406369$, $R^2 = 0.994$; LOD = 0.78 μg/mL; LOQ = 1.97 μg/mL); verbascoside ($y = 301950x + 6966.7$, $R^2 = 0.999$; LOD = 0.68 μg/mL; LOQ = 1.61 μg/mL), quercetin-3-*O*-glucoside ($y = 34843x - 160173$; $R^2 = 0.999$; LOD = 0.21 μg/mL; LOQ = 0.71 μg/mL); kaempferol-3-*O*-glucoside ($y = 11117x + 30861$, $R^2 = 0.999$; LOD 0.15 μg/mL; LOQ 0.41 μg/mL), apigenin-7-*O*-glucoside ($y = 10683x - 45794$; $R^2 = 0.999$; LOD = 0.10 μg/mL; LOQ = 0.53 μg/mL). The results were presented as mg per g of extract obtained by the re-extraction from uncooked/cooked pasta.

2.5. Screening analysis for optimal factor selection in the pasta making procedure

In order to maximise response outputs while decreasing experimental runs, a double screening analysis was performed (one for each plant extract). After a careful selection of experimental factors through previous experiments and literature data, factors selected were X_1:

Fig. 1. Fettuccine-enriched with *Cichorium intybus* extract. A. fresh (no extract); B. 0.25 mg/g; C. 0.50 mg/g, and D. 0.63 mg/g.

drying time (20–420 min), X_2: drying temperature (40–82.5 °C), X_3: extract concentration, and categorical factor X_4: cooking ((–1) uncooked and (+1) cooked), analysing 3 responses: Y_1: Total Phenolic Acids (TPA), Y_2: Total Flavonoids (TF), and Y_3: Total Phenolic Compounds (TPC). In the first analysis, *C. intybus* was analysed for factors X_1 (20, 120 & 420 min), X_3 (0.25, 0.5 & 0.63 mg/mL) and X_4, with a fixed value of 40 °C for X_2. For the second analysis on *P. coronopus* and the first screening results, X_3 was fixed to 0.5 mg/mL. A 2^2 factorial design was employed on the X_1 factor, reducing the range of values to 70 and 170 min and extending the X_2: factor with a low value (-1) of 52.5 and a high value (+1) of 87.5 °C, fixing the categorical factor to (-1) for convenience.

2.6. Antioxidant properties

2.6.1. Thiobarbituric acid reactive substances (TBARS) assay

The *in vitro* antioxidant activity was evaluated by applying TBARS assay previously described by Svobodova et al. (2017), using the above-prepared chicory or pasta hydroethanolic extracts. Briefly, the TBARS formation inhibition was evaluated in porcine brain homogenates. The colour intensity of the malondialdehyde–thiobarbituric acid (MDA–TBA) complex formed during heating at 80 °C for 20 min was measured at 532 nm using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and the inhibition ratio calculated using the following equation: [(A – B)/A] × 100%, where A and B correspond to the absorbance of the control and the sample solution, respectively. Trolox was used as a positive control. Results were expressed as half maximal effective concentration (EC_{50}) values (mg/mL), the sample concentrations providing 50% of inhibition activity.

2.6.2. Antihaemolytic activity (OxHLIA) assay

The *in vitro* antihaemolytic activity was evaluated by the OxHLIA assay following Petropoulos et al. (2019) methodology. An erythrocyte solution (2.8%, *v/v*; 200 μL) prepared in phosphate-buffered saline (PBS, pH 7.4) was mixed with 400 μL of either extract solution (30–1000 μg/mL in PBS), trolox (7.81–250 μg/mL in PBS), PBS (control), or water (for complete haemolysis). After pre-incubation at 37 °C for 10 min with shaking, 200 μL of 2.2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 160 mM in PBS) were added, and the optical density was measured at 690 nm every ~10 min in the same microplate reader used for TBARS assay until complete haemolysis. The results were expressed as half maximal inhibition concentration (IC_{50}) values (μg/mL) at a Δt of 60 min.

2.7. Statistical analysis

Data are the mean ± standard deviation (SD) of three replicates in each assay. Pasta data were analysed by two-way ANOVA and *t*-test for *C. intybus* extract and ANOVA within the 2^2 factorial design in the Response Surface Methodology (RSM) for *P. coronopus* extract using Design expert 12.0.1. (Stat-Ease, Inc., Minneapolis, MN, USA) software. Variance homogeneity (homoscedasticity) was verified with Bartlett's test. Means were separated by Fisher's Least Significant Difference (LSD) *post-hoc* test ($p = 0.05$).

3. Results and discussion

3.1. Phenolic compounds fingerprinting

Table 1 showed data (λmax in the visible region, molecular ion, and main fragment ions observed in MS^2) obtained by HPLC-DAD-ESI/MS analysis, regarding phenolic compounds tentatively identification and quantification, compounds characteristic of wild plant extracts and enriched pasta under investigation. An exemplification phenolic profile is shown in Fig. S2. Firstly, a fingerprinting of wild *C. intybus* and *P. coronopus* leaves was performed to verify the quality and the quantity of the potential enrichment compounds to the pasta due to this wild species nutraceutical properties and bioactive compounds. Additionally, the nutraceutical characterisation of the extracts and the best-selected conditions for cooked and uncooked experimental runs are reported in Fig. 2. The phenolic composition data resulted in agreement with results reported by other authors (Petropoulos et al., 2017; Savo, Salomone, Mattoni, & Tofani, 2019). Twenty-five phenolic compounds were detected, from which twenty were classified as phenolic acids (hydroxycinnamic acids, phenylethanoids and phenylpropanoids) and five as flavonoid glycoside derivatives (flavonols, flavones and flavan-3-ols) (Table 1). In the *C. intybus* extract, peak 2 ($[M-H]^-$ at m/z 353), 20 and 23 ($[M-H]^-$ at m/z 515) were identified according to their mass spectra and UV–vis characteristics as 5-*O*-caffeoylquinic acid (chlorogenic acid; commercial standard), 3,5-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid, respectively, taking into account the findings reported by Clifford, Johnston, Knight, & Kuhnert (2003). These compounds have been already reported by previously in *C. spinosum* and *C. intybus* (Papetti, Maietta, Corana, Marrubini, & Gazzani, 2017; Petropoulos, Fernandes, Barros, & Ferreira, 2018). Peak 3 ($[M-H]^-$ at m/z 385), was tentatively identified as sinapic acid hexoside. Peaks 5 ($[M-H]^-$ at m/z 179) and 7 ($[M-H]^-$ at m/z 341) were identified as caffeic acid and caffeic acid hexoside ($[M-H-162]^-$, loss of hexosyl moiety), being these identifications based on the typical fragmentation pattern of the commercial standard, caffeic acid, releasing a MS^2 fragment at m/z 179, 135. Similarly, the pseudomolecular ion of compound 17 ($[M-H]^-$ at m/z 469), released a MS^2 fragment at m/z 179 ($[M-H-290]^-$, loss of hexosyl and methylglutarate moieties, respectively), being coherent with caffeoyl-hexoside-methylglutarate. These

Table 1
Wavelengths of maximum absorption in the visible region (λmax), and mass spectral data of the identified phenolic compounds and nutraceutical quality in the wild plant extracts and cooked and uncooked pasta.

Peak	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative of identification	*C. intybus*			*P. coronopus*	
					Extract	cooked Pasta*	uncooked Pasta*	Extract	Uncooked Pasta**
1	295	497	335(100), 221(32), 163 (16),153(25)	Protocatechuic acid derivative	nd	nd	nd	1.71 ± 0.01	1.00 ± 0.03
2	325sh278	353	191(100), 179(18)	5-*O*-Caffeoylquinic acid	14.12 ± 0.1	3.5 ± 0.1	2.42 ± 0.2	nd	nd
3	272	385	223(100),207(40),179(2),161 (19),153(36)	Sinapic acid hexoside	4.8 ± 0.2	nd	nd	nd	nd
4	288sh325	801	639(100), 477(33), 315 (60),179(23), 161(17)	Plantamajoside hexoside	nd	nd	nd	1.66 ± 0.02	3.2 ± 0.2
5	324sh271	179	135(100)	Caffeic acid	0.84 ± 0.05	0.38 ± 0.02	0.364 ± 0.004	nd	nd
6	289sh328	639	621(100), 622(28), 529(19)	Plantamajoside	nd	nd	nd	0.91 ± 0.01	0.38 ± 0.01
7	326	341	179(100)	Caffeic acid hexoside	0.63 ± 0.03	0.204 ± 0.005	0.186 ± 0.002	nd	nd
8	289sh328	639	621(100), 622(32), 529(18)	Plantamajoside isomer 1	nd	nd	nd	1.57 ± 0.1	0.559 ± 0.01
9	328	473	311(100)	Cichoric acid	36 ± 2	0.49 ± 0.02	0.45 ± 0.02	nd	nd
10	290sh331	785	623(100)	Rossicaside A	nd	nd	nd	0.37 ± 0.01	0.498 ± 0.02
11	288sh327	639	621(100), 622(29), 529(21)	Plantamajoside isomer 2	nd	nd	nd	3.0 ± 0.1	0.45 ± 0.02
12	325	623	461(100)	Verbascoside	5.2 ± 0.1	1.13 ± 0.05	1.4 ± 0.1	151 ± 6	23 ± 2
13	302, sh329	769	623(100)	6′-*O*-Caffeoyl verbascoside	nd	nd	nd	9.6 ± 0.2	4.7 ± 0.1
14	347	477	301(100)	Quercetin-3-*O*-glucuronide	0.81 ± 0.02	1.3 ± 0.1	1.526 ± 0.003	nd	nd
15	328	623	461(100), 315(5), 161(2)	Isoverbascoside	nd	nd	nd	12.2 ± 0.5	7.3 ± 0.5
16	333	469	307(4), 179(100),161(7), 135 (8)	Caffeoyl-hexoside-methylglutarate	2.7 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	nd	nd
17	328	623	461(100), 315(6), 161(3)	Verbascoside isomer 1	nd	nd	nd	18 ± 1	5.8 ± 0.2
18	335	549	505(35), 301(100)	Quercetin-*O*-malonyl-glucoside	0.87 ± 0.04	nd	nd	nd	nd
19	277sh332	515	353(100),191(85),179 (42),173(11),135(16)	3,5-*O*-Dicaffeoylquinic acid	4.7 ± 0.1	4.0 ± 0.2	3.9 ± 0.1	nd	nd
20	328	623	461(100), 315(4). 161(2)	Verbascoside isomer 2	nd	nd	nd	4.2 ± 0.2	2.9 ± 0.1
21	336	461	285(100)	Kaempferol-*O*-glucuronide	0.68 ± 0	1.35 ± 0.03	1.55 ± 0.01	nd	nd
22	278sh330	515	353(81),191(18),179(45),173 (100),135(54)	4,5-*O*-Dicaffeoylquinic acid	4.9 ± 0.1	1.53 ± 0.04	1.3 ± 0.1	nd	nd
23	351	445	269(100)	Apigenin-*O*-glucuronide	1.9 ± 0.3	3.5 ± 0.2	4.26 ± 0.11	nd	nd
24	342	533	489(71), 285(100)	Kaempferol-*O*-malonyl-hexoside	0.81 ± 0.03	1.08 ± 0.05	1.00 ± 0.01	nd	nd
25	328	623	461(100), 315(6), 161(4)	Verbascoside isomer 3	nd	nd	nd	4.3 ± 0.3	3.0 ± 0.1
				Total Phenolic Acids	74 ± 2	13 ± 0.5	11.96 ± 0.03	208 ± 4	53 ± 2
				Total Flavonoids	5.1 ± 0.3	7.3 ± 0.3	8.3 ± 0.1	nd	nd
				Total Phenolic Compounds	79 ± 3	20 ± 1	20.3 ± 0.1	208 ± 4	53 ± 2
			Analytical Determination	TBARS IC_{50} (mg/mL)	0.150 ± 0.001	1.7 ± 0.1	0.37 ± 0.05	0.26 ± 0.02	0.24 ± 0.01
				OxHLIA IC_{50} (μg/mL) Δ*t* = 30 min	94 ± 4	18 ± 1	1.8 ± 0.2	63 ± 3	27 ± 2

The best condition: *extract concentration: 0.63 mg/g dw, drying temperature: 40 °C; drying time: 120 min; and **extract concentration: 0.5 mg/g dw, drying temperature: 52.5 °C, driyng temperature: 170 min. nd. not detected. Calibration curves: protocatechuic acid (y = 214168x + 27102, R^2 = 0.999; LOD = 0.14 μg/mL; LOQ = 0.52 μg/mL; peak 1), sinapic acid (y = 197337x + 30036; R^2 = 0.999; LOD = 0.17 μg/ml; LOQ = 1.22 μg/ml; peak 3), chlorogenic acid (y = 168823x − 161172; R^2 = 0.999; LOD = 0.20 μg/mL; LOQ = 0.68 μg/mL; peaks 2, 20 and 23), caffeic acid (y = 388345x + 406369, R^2 = 0.994; LOD = 0.78 μg/mL; LOQ = 1.97 μg/mL; peaks 4–11, 14, and 16); verbascoside (y = 301950x + 6966.7, R^2 = 0.999; LOD = 0.68 μg/mL; LOQ = 1.61 μg/mL; peaks 12, 14, 16, 18, 21 and 26), quercetin-3-*O*-glucoside (y = 34843x – 160173; R^2 = 0.999; LOD = 0.21 μg/mL; LOQ = 0.71 μg/mL; peaks 15 and 19); kaempferol-3-*O*-glucoside (y = 11117x + 30861, R^2 = 0.999; LOD 0.15 μg/mL; LOQ 0.41 μg/mL; peaks 22 and 25), apigenin-7-*O*-glucoside (y = 10683x − 45794; R^2 = 0.999; LOD = 0.10 μg/mL; LOQ = 0.53 μg/mL; peak 25).

compounds were previously reported in different species, such as sorghum (Kang, Price, Ashton, Tapsell, & Johnson, 2016) or potato (Narváez-Cuenca, Vincken, & Gruppen, 2012). Peak 9 ($[M-H]^-$ at *m/z* 473) presented a fragmentation pattern that allowed assigning it as chicoric acid (dicaffeoyltartaric acid). Indeed, chicoric acid has already been reported in many studies in *C. intybus* (Carazzone, Mascherpa, Gazzani, & Papetti, 2013; Sinkovič et al., 2015).

Peaks 15, 19, 22, and 25 were identified as flavonols and they were constantly reported in the phenolic profile of *Cichorium* sp. by other authors (Heimler et al., 2009; Papetti et al., 2017; Petropoulos et al., 2018). Compounds 15 and 19 presented pseudomolecular ions at $[M-H]^-$ at *m/z* 477 and 549, respectively, releasing a common MS^2 fragment at *m/z* 301 ($[M-H-176]^-$ and ($[M-H-248]^-$, loss of glucuronyl and malonyl-hexoside moieties, respectively) and were tentatively

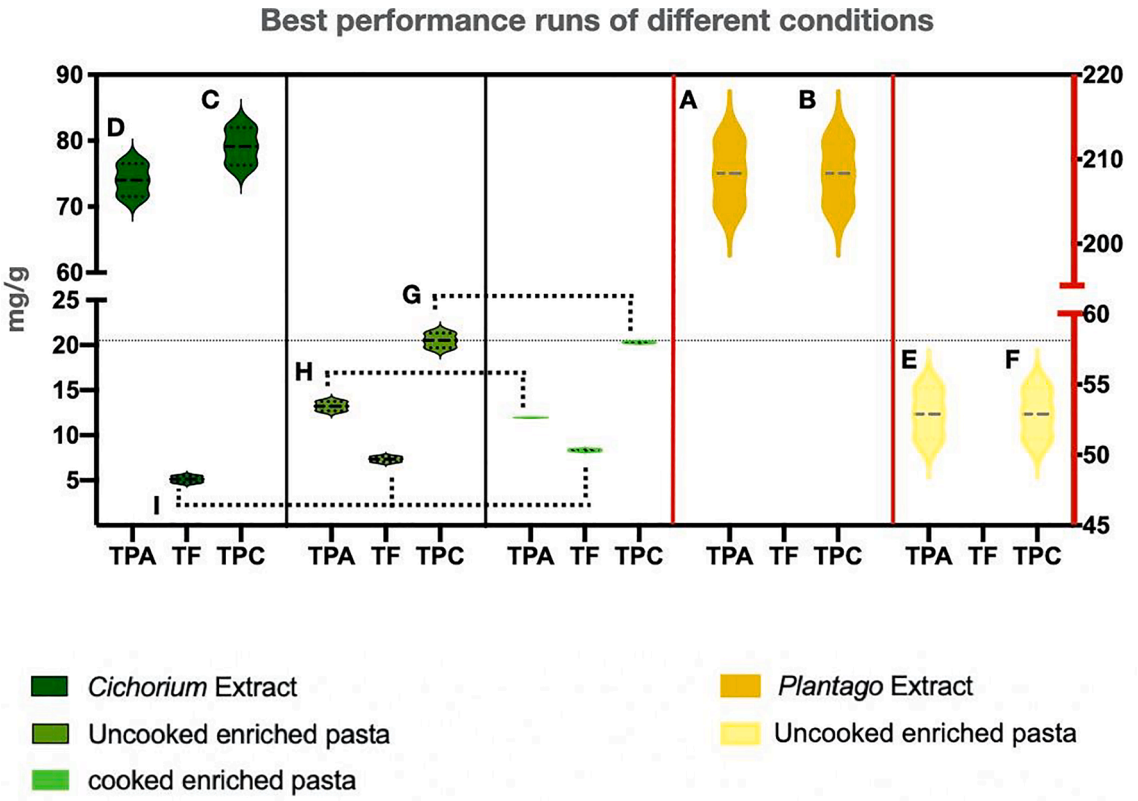


Fig. 2. Total phenolic acids (TPA), Total flavonoids (TF) and Total phenolic compounds (TPC) of *Cichorium intybus* extract, and selected uncooked and cooked enriched pasta, followed by *Plantago coronopus* extract and selected uncooked enriched pasta. Each value is the mean ± SD of three replicates, whilst presence of different uppercase letters in the legend boxes denotes significant differences for $p < 0.001$ following one-way ANOVA using the TPA, TF and TPC groups concentration as the variability factors.

identified as quercetin-3-*O*-glucuronide (compared with standard) and quercetin-*O*-malonyl-hexoside, respectively. Similarly, the pseudomolecular ion of compounds 22 ($[M-H]^-$ at *m/z* 461) and 25 ($[M-H]^-$ at *m/z* 533), released an MS^2 fragment at *m/z* 285 ($[M-H-176]^-$ and $[M-H-248]^-$, loss of glucuronyl and malonyl-hexoside moieties, respectively), were coherent with kaempferol-*O*-glucuronide and kaempferol-*O*-malonyl-hexoside, respectively. These compounds were previously identified in chicory by Heimler et al. (2009). Peak 24 ($[M-H]^-$ at *m/z* 445) was assigned as a flavone, and it was identified as apigenin-*O*-glucuronide.

Regarding the phenolic profile of the *P. coronopus*, no compounds from the flavonoid class were identified. Peak 1 was tentatively identified as protocatechuic acid derivative. Peaks 4 ($[M-H]^-$ at *m/z* 811) 6, 8 and 11 ($[M-H]^-$ at *m/z* 639), were identified as plantamajoside, a phenylethanoid glycoside, and its isomers, except peak 4, which lost a hexosyl moiety ($[M-H-162]^-$), being tentatively identified as plantamajoside hexoside. These compounds are prevalent in *Plantago* species (Zubair, Widén, Renvert, & Rumpunen, 2019). Peaks 12, 14, 16, 18, 21 and 23 ($[M-H]^-$ at *m/z* 623) were identified as verbascoside and its isomers, except peak 14 ($[M-H]^-$ at *m/z* 769) that loss of caffeoyl moiety ($[M-H-144]^-$), being tentatively identified as 6′-*O*-caffeoyl verbascoside. According to Henn et al. (2019), verbascoside is a major compound found in leaves of *P. australis* Lam. Gonçalves, Grevenstuk, Martins, & Romano (2015) reported a high verbascoside content also in *P. algarbiensis* Samp. and *P. lagopus* L.

Similar results were reported regarding phenolic compounds between *C. intybus* enriched cooked and uncooked pasta. Indeed, the gluten from the wheat flour in pasta provides an intense protein network, minimizing leaching during pasta cooking (Alamprese et al., 2017). Moreover, some flavonoids showed a higher affinity with the pasta, as with quercetin-3-*O*-glucuronide, kaempferol-*O*-glucuronide, apigenin-*O*-glucuronide and 3,5-*O*-dicaffeoylquinic acid, present in *C. intybus* as also the phenylpropanoids present in *P. coronopus*, when compared with the other phenolic compounds identified in the plant extracts. This aspect could be due to the interaction of chemical components of the egg-pasta such as the gluten as already reported or the starch present in the wheat flour, able to link with the glycosidic phenolic compounds, inhibiting the release of the glucose in the human body and making the phenolic compounds more bioavailable for intestinal absorption (Ayua et al., 2021; Coe, Clegg, Armengol, & Ryan, 2013; Törrönen et al., 2013). These results are confirmed by the antioxidant activity assays, showing higher inhibition of lipid peroxidation and antihemolytic activity of plant extract enriched pasta than the control pasta, as reported in the next paragraph and in Table 1.

3.2. Antioxidant properties of wild plant species and enriched pasta

Table 1 reports the results of TBARS and OxHLIA assay of *C. intybus* and *P. coronopus* plant extracts and enriched pasta samples. *C. intybus* extract showed IC_{50} values of 0.150 ± 0.001 mg/mL and 94 ± 4 μg/mL for TBARS and OxHLIA, respectively. Petropoulos et al. (2017) analysed two stages of growth of *C. spinosum* L. and they reported a range of 0.454–0.163 mg/mL of EC_{50} values providing 50% inhibition of lipid peroxidation, confirming our findings. Regarding *P. coronopus* extract, IC_{50} results for TBARS and OxHLIA were 0.26 ± 0.02 mg/mL and 63 ± 3 μg/mL, respectively. The TBARS assay of enriched pasta (Table 1, Fig. S1 and Table S1 in Supplementary material) reported similar results to the OxHLIA assay. Curiously, cooked pasta showed higher inhibition of lipid peroxidation and antihaemolytic activity when compared with uncooked pasta (Table 1). Probably, this may be due to phenolic compounds esterified to other molecules such as fatty acids, making them more insoluble (Melini, Melini, & Acquistucci, 2020).

3.3. C. intybus screening analysis

Fig. 3 summarises the experimental data obtained from the first screening analysis where graphs are clustered in two images, on top are the results obtained from the uncooked enriched pasta with *C. intybus* and, at the bottom, the cooked enriched pasta. Every cluster includes TPC and the two main classes TPA and TF in their different drying times and separated into three blocks where each conglomerate represents the

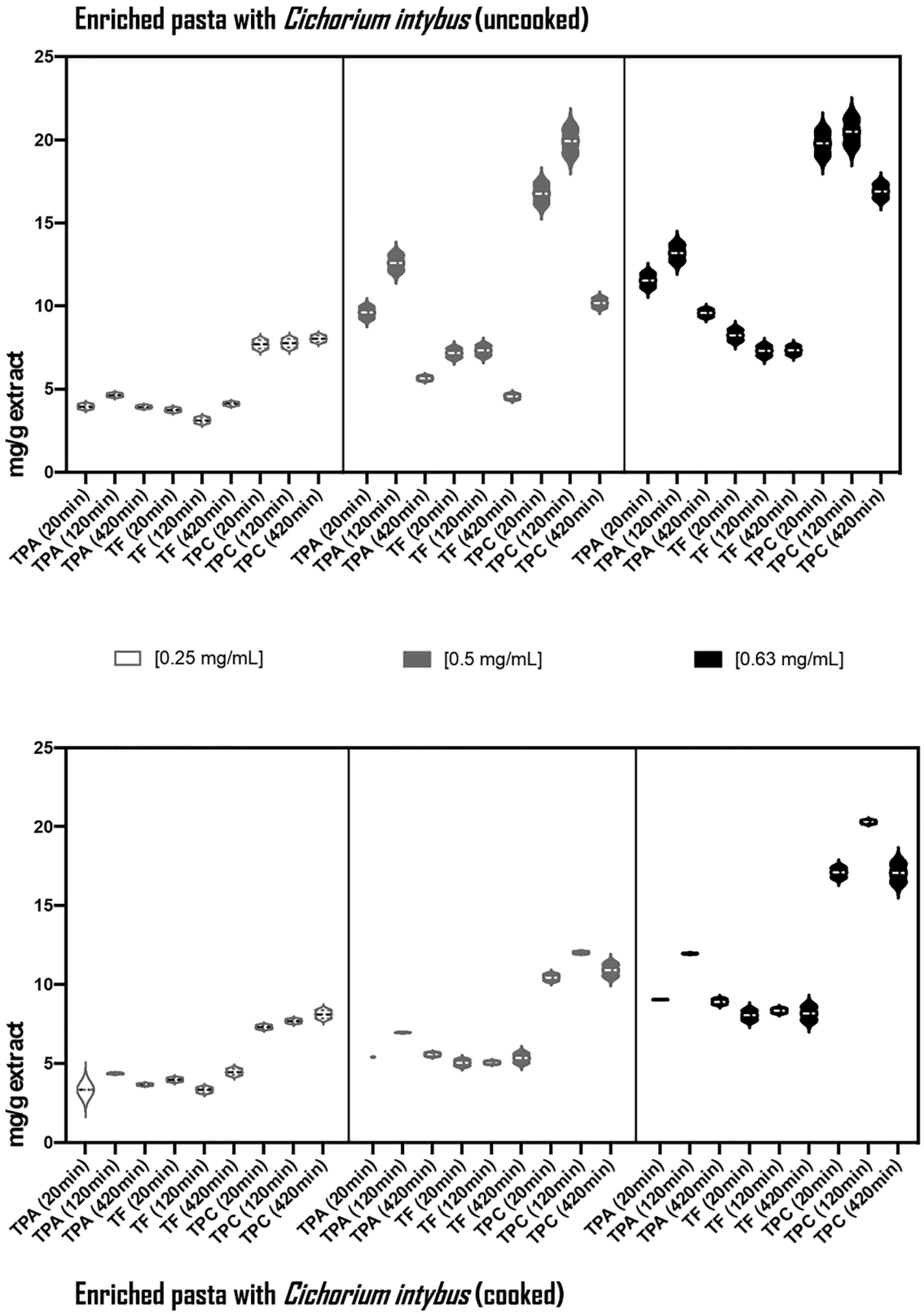


Fig. 3. Total phenolic acids (TPA), total flavonoids (TF) and total phenolic content (TPC) of fresh pasta enriched with chicory extract and the three different drying times (20 min, 120 min and 420 min) as the variability factors. Each value is the mean ± SD of three replicates.

different incorporated extract concentrations.

Along with Fig. 2, the displayed data provide an extensive understanding of their behaviour, outstanding the positive and unexpected result of categorical factor "cooked" due to the lack of significant differences. However, with a global analysis of the results, including bioactive activity and the *P. coronopus* results, the structural matrix created between the carbohydrates and starch of the wheat flour and the proteins of the eggs provides an excellent fixation of the bioactive molecules. Moreover, considering the starch of the wheat flour inside the pasta, the interactions between amylases and phenolic compounds may significantly contribute to the nutritional quality of the enriched pasta among the starch-enzyme-phenolics three-component system (Zhu, 2015). Indeed, this system could slow-release glucose from starch in humans, reducing/preventing the occurrence of various diseases (Aleixandre, Gil, Sineiro, & Rosell, 2022; Coe et al., 2013). These results are presented in Fig. 2 comparing uncooked-cooked enriched pasta, where the amount of TPA, TF and TF displayed the same behaviour.

Besides, related to concentration and drying times, first, we can also observe the same trends with a slight increase of available compounds (Fig. 3) for the concentration gradient which was expected, whilst for the time employed, an inverse "*V*" effect is displayed most of the times with the longer time, decreasing the availability of the bioactive compounds, which could be explained due to an extended exposure of these compounds to heating and oxidating air conditions. Therefore, those results guide us for the next experiment, fixing the middle-value for the concentration factor and focus on values around the median value of time employed in this first screening analysis.

3.4. *P. coronopus* factorial screening analysis

After a first approach to understand the responses given by the pasta-enriched with *C. intybus* extract, a second and complementary factorial 2^2 analysis was designed adjusting the drying times and increasing the temperatures of drying in order to model a prediction related to these 2 factors, since energetic tall have to be considered.

Table 1 and Fig. 2 displays a summary of the data obtained for *P. coronopus,* whilst in Fig. 4, a specific exhibition of factorial interpretation was compiled. The decision to cluster the plant's data was to

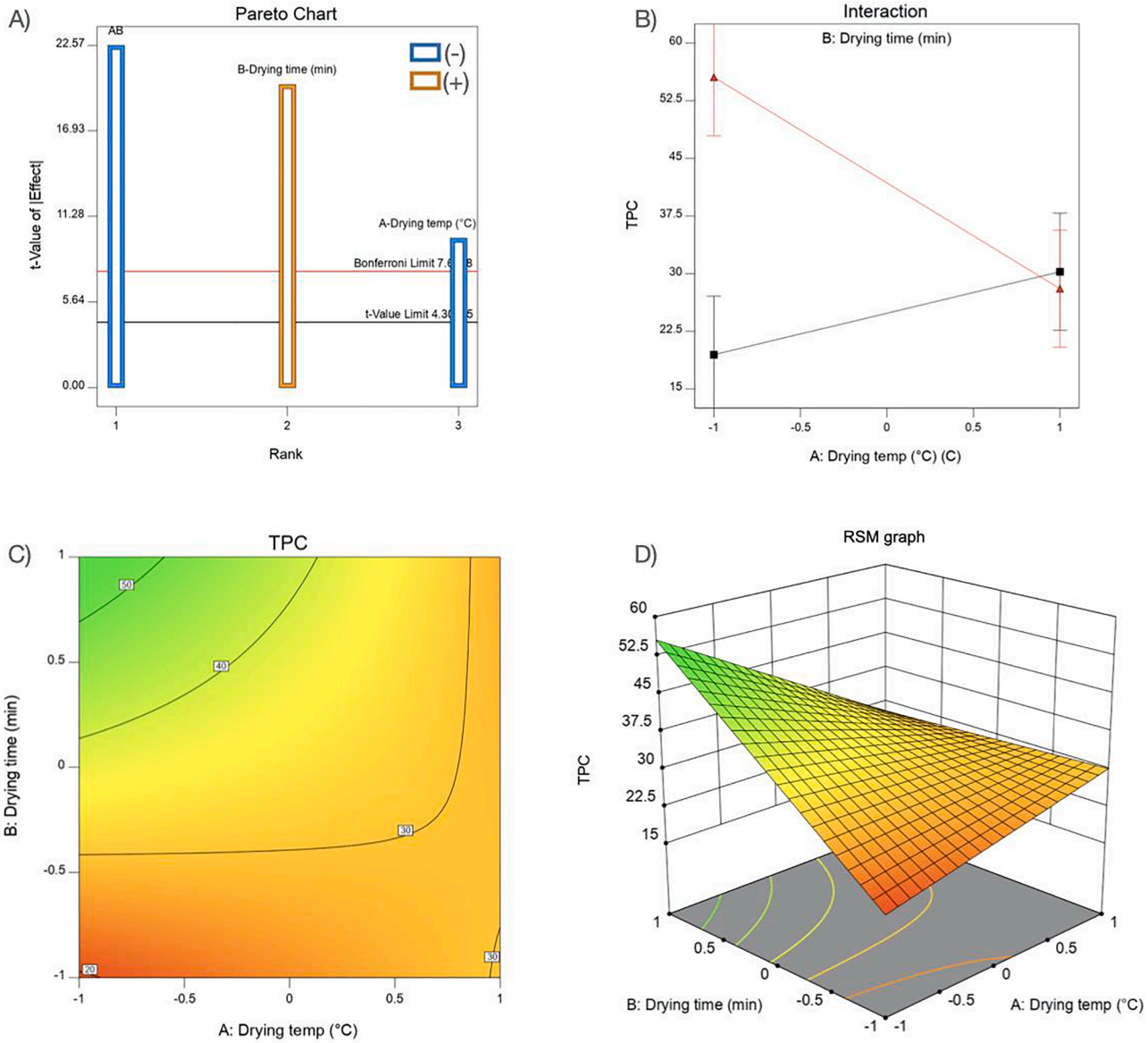


Fig. 4. 2^2 factorial design graphs from the enriched pasta with *P. coronopus*. TPC presented in mg/g of dried pasta, A) Pareto Chart that shows the magnitudes of the effect represented by the size, and coloured blue is employed for a negative effect while orange was used for a positive one, B) interaction of single effects, C) and D) two- and three-dimensional representation of data for optimal range spotting.

understand better their complementary profile of bioactive compounds, as shown in Fig. 2, where *P. coronopus* displayed a concentration of TPA almost 3-fold higher than *C. intybus* and, at the same time, shown the absence of TF compounds.

According to the responses obtained in the factorial design presented in Fig. 4, only TPC is presented for the sole reason of being analogous to TPA due to the absence of TF. Also, the strong interaction between drying temperature and time, despite the low Bonferroni limit value of drying temperature or its insignificant statistical value in the ANOVA test, indicates us the importance of this effect, even though the range analysed has proved the contrary (Fig. 4A and 4B).

Following the order in the contour plot, Fig. 4C unveils the best range for incorporation within our selected factors and ranges; the contour plot is the bidimensional representation of the RSM graph (Fig. 4D), but both factors complement each other, highlighting in green the best region that provides higher availability of bioactive molecules, which could almost increase 2-fold higher than the point detected at low temperature and low time. In this case, we are focusing on a target area around higher drying times (+1 = 170 min) and lower temperatures (–1 = 52.5 °C).

A longer time follows the results shown in the first screening analysis since longer times of 420 min were employed. Lower temperature is in agreement with the literature, where some of the bioactive molecules have proved to exhibit thermolabile behaviour (Alara, Abdurahman, & Ukaegbu, 2021). However, these results could be misunderstood with interpretations such as higher dehydration of sample higher concentration of bioactive molecules. However, as stated in the material and methods section, samples were frozen and freeze-dried right after each treatment run was performed to stop any reaction happening and end up with the same moisture content in each sample analysed. Consequently, according to Goetz & Koehler (2005), egg proteins start denaturing around 60 °C and finally, over 80 °C, proteins cross-link and solidify. Hatanaka, Yamauchi, Kobayashi, & Muro (2009) observed that the egg proteins, in the presence of phenolic compounds, could create stronger bonds, independently to the temperature. Since our lower temperature is below the critical temperature (56 °C) of denaturation of albumen (Tan, Zhong, & Langrish, 2020), albumen could form stronger bonds with the bioactive molecules, also due to the high viscosity of the matrix dependent to the employed low temperature. At the same time, the longer times could allow that this interaction between albumen and phenolic compounds may be formed in an extended manner and act as an encapsulant-like for the bioactive molecules. In contrast, a higher temperature could modify this matrix array, exposing the molecules negatively to higher temperatures.

Nevertheless, it is worth noting that this response revealed an important belief that has to be explored further, employing more experimental runs but also with the help of microscopic images to understand the matrix arrays forms within the egg pasta and the extracts. Meanwhile, it has been identified essential factors, especially X_1 and X_2, showing the relevance of their interactions and the behaviour after cooking principally in the class of compounds studied and their bioactivities.

4. Conclusions

The present work analysed the nutraceutical quality of fresh egg pasta when enriched with wild edible plants. Wild plant species *C. intybus* and *P. coronopus* extracts were previously analysed for their bioactive compounds and nutraceutical properties. Then, a pasta-making procedure was realised using different extract concentrations, different drying times and temperatures of pasta, and the categorical cooking factor to stimulate and guide the pasta making process. Results showed that chicoric acid is the peculiar phenolic compound of the *C. intybus* extract, and verbascoside is the major phenolic acid of the *P. coronopus* extract. However, flavonoids rearranged with different glycosidic molecules were also found in the enriched pasta, i.e., apigenin-*O*-glucuronide, kaempferol-*O*-malonyl-hexoside and kaempferol-*O*-glucuronide. Different drying times showed significant differences in the pasta making procedure, whilst the temperature was not significant, although a strong interaction of these factors was exposed. Thus, considering the drying temperature as an essential factor is fundamental. The extract concentration seems to be a positive linear effect. Cooking did not show significant differences due to a protective matrix composed of the egg proteins and starch at mild temperatures. These results indicated that plant extracts could be used as a functional food product due to the significant antioxidant capacity and phenolic profile preserved after the incorporation process in typical pasta. Relevant factors have already been established, given that implementation of an experimental design provides more expansive knowledge. However, further research will be performed to optimise the incorporation of natural extracts in the pasta making process, possibly navigating within our selected factors and levels.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) and FEDER under Programme PT2020 for financial support to CIMO (UID/AGR/00690/2020); the national funding by FCT, P.I., through the institutional scientific employment program-contract for L. Barros, and C. Pereira contracts and for B. Melgar (NORTE-06-3559-FSE-000188) contracts through the individual program-contract. This work is supported by: European Investment Funds by FEDER/COMPETE/POCI–Operacional Competitiveness and Internationalization Programme, under Project POCI-01-0145-FEDER-006958 and National Funds by FCT – Portuguese Foundation for Science and Technology, under the project UID/AGR/04033/2013.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132462.

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