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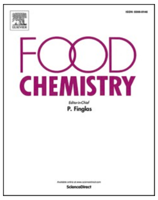

Microwave hydrodiffusion and gravity: A green extraction technology for phenylethyl isothiocyanate from watercress by-products

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ABSTRACT

Watercress is a cruciferous vegetable and the primary natural source of phenylethyl isothiocyanate (PEITC), a powerful health-promoting agent. Conventional extraction of PEITC relies on harmful solvents, which limits sustainability. This study introduces a novel, solvent-free method—microwave hydrodiffusion and gravity (MHG)—to extract PEITC from watercress by-products (WCBP). Results showed that freezing WCBP is fundamental for effective PEITC extraction *via* MHG. Using a three-level factorial experimental design, optimal MHG extraction conditions were determined (microwave power: 1.91 W g^{-1}; extraction time: 29 min), yielding a PEITC content of 1818.26 μg g^{-1} WCBP (dry basis), along with extracts rich in polyphenols and possessing antioxidant properties. This work demonstrates that MHG is a sustainable method for extracting isothiocyanates, supporting a zero-waste approach since the clean residue from the extraction process can be reused to obtain new ingredients, thus enhancing resource efficiency and promoting a circular economy in the agro-food industry.

1. Introduction

Microgreens are novel foods rich in bioactive compounds and can be grown easily and sustainably. Among the most widespread crops, cruciferous vegetables (*Brassicaceae*) are particularly noteworthy due to the vast evidence of their beneficial effects on human health (Alloggia et al., 2023). These benefits are attributed to its nutritional composition (low in fat and high in vitamins, minerals, and fibre) and the presence of phenolic compounds, carotenoids, vitamins, and most importantly, glucosinolates and their main breakdown products – isothiocyanates (Fusari et al., 2020).

Isothiocyanates (R-N=C=S; ITCs) are primary products formed through the enzymatic breakdown of glucosinolates, catalysed either by plant myrosinase (β-thioglucoside glucohydrolase, EC 3.2.1.147) or by myrosinase produced by human gut microbiota (Karanikolopoulou et al., 2021; Sikorska-Zimny & Beneduce, 2021). According to epidemiological data, consuming cruciferous vegetables and ITCs reduced the risk of developing non-communicable diseases such as cardiovascular disorders, diabetes, and cancer (N. Li et al., 2022). There is evidence that ITCs are potent modulators of enzyme activity involved in phases I and II detoxification pathway, as well as regulators of the cell cycle, angiogenesis, and epigenetic effects (Ağagündüz et al., 2022; Esteve, 2020; Mitsiogianni et al., 2019; Park et al., 2017; Soundararajan & Kim, 2018; Sundaram et al., 2022). Following their modulation of detoxification pathways, ITCs reduce oxidative stress by enhancing Nrf2-dependent antioxidant responses and mitigating inflammation by inhibiting NF-κB activity (Burčul et al., 2018; Caglayan et al., 2019; Zhu et al., 2013).

Despite the great diversity among *Brassicaceae*, *Nasturtium officinale* (watercress) is known as one of the most potent antioxidant cruciferous vegetables (Fusari et al., 2020; Pinela et al., 2020). Watercress is the

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Abbreviations: ABTS, ABTS radical scavenging assay; DB, Dry basis; FW, Fresh weight; GAE, Gallic acid equivalent; ITC, Isothiocyanate; MHG, Microwave hydrodiffusion and gravity; ORAC, Oxygen radical absorbance capacity assay; PEITC, Phenylethyl isothiocyanate; PLF, Purple liquid fraction; RSM, Response surface methodology; SEM, Scanning electron microscopy; TE, Trolox equivalent; TPC, Total phenolic content; WCBP, Watercress by-products; WLF, White liquid fraction.

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richest natural source of glucosinolate gluconasturtiin, which, on hydrolysis, produces phenylethyl isothiocyanate (PEITC), one of the most important ITCs (Coscueta et al., 2022). PEITC exhibits antioxidant and anti-inflammatory (Coscueta et al., 2022; Dayalan Naidu et al., 2018; Moon & Kim, 2012), bactericidal (Abrantes et al., 2024), and anticarcinogenic effects (Gupta et al., 2014), among other benefits. Recent reviews suggest that PEITC could be used as a natural nutraceutical or as an adjuvant to treat various disorders due to its chemopreventive action and health-promoting effects (Coscueta et al., 2022; N. Li et al., 2022; Mitsiogianni et al., 2019; Palliyaguru et al., 2018).

Nowadays, there is a growing emphasis on sustainable practices and a higher demand for natural products. As a result, it is crucial to develop innovative techniques for efficiently extracting PEITC while minimising the environmental impact of the process. Moreover, the methods used should be as safe for potential human use. However, research on green extraction for ITCs is still limited. The few sustainable methods reported for PEITC include pressurised fluid extraction (Rodrigues et al., 2016) and aqueous micellar systems optimised in our previous work (Coscueta et al., 2020). However, these innovative methods can be expensive and challenging to scale, highlighting the need for a more cost-effective and environmentally friendly option for obtaining PEITC from cruciferous vegetables.

A promising alternative is microwave hydrodiffusion and gravity (MHG), a recent solvent-free extraction created by Vian et al. (2008) to extract essential oils. Currently, there is considerable research interest in MHG across various fields, including the recovery of bioactive ingredients from agro-food resources. This includes a focus on extracting polyphenols, producing juices and using pretreatment methods such as dehydration and bleaching (Farias et al., 2021; Ferrati et al., 2024; Moraes et al., 2022; Moro et al., 2021; Singh Chouhan et al., 2020). The MHG technique is based on the *in situ* dielectric heating of water in plant cells using microwave irradiation, which increases pressure within the cells, leading to their rupture and the release of cellular content through hydrodiffusion (extraction and diffusion). Then, the crude extract (*in situ* water and metabolites) is collected from plant material due to gravity (Vian et al., 2008). MHG is eco-friendly, economically beneficial, and offers numerous advantages, including avoiding costs and risks of large volumes of solvent, facilitating scaling-up, greater purity of the extract, and reducing extraction time from hours to minutes (Chemat et al., 2019).

Optimising the extraction process for its application is crucial to achieving better and more consistent results. As MHG does not use solvents, parameters such as microwave power and extraction time play a critical role in extraction efficiency (Singh Chouhan et al., 2019b). Therefore, using a statistical tool such as response surface methodology (RSM) is crucial for systematically defining optimal extraction conditions.

To our knowledge, no information has been reported on using MHG to extract PEITC from the watercress. Is MHG suitable for PEITC extraction? What are the variables that most affect the extractive process? What variables most significantly influence extraction conditions? This study aims to answer these questions by investigating the effects of microwave power and extraction time on MHG efficiency for extracting PEITC from watercress by-products (WCBP). Additionally, it examines the total phenolic content (TPC) and its antioxidant activity. This approach utilises WCBP and a green extraction process, aligning with Goal 12 of The Agenda 2030 for Sustainable Development, which focuses on promoting sustainable consumption and production patterns.

2. Materials and methods

2.1. Chemicals

2,2′-azino-bis(3-ethylbenzothiazoline-6- sulphonic acid) (ABTS) diammonium salt, D-glucose, 2,2-diphenyl-1-picrylhydrazyl, sodium carbonate, 2,2′-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH, 97 %), fluorescein (F-6377), 6-hydroxy-2,5,7,8-tetra-methylbroman-2-carboxylic acid (Trolox, 97 %, EC 258–422-8), gallic acid, potassium dihydrogen phosphate (KH_2PO_4), phosphate buffer saline tablet, glutaraldehyde, as well as the solvents used for HPLC analysis (formic acid, hexane, and methanol), were all purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Folin-Ciocalteu's reagent from Merck (Darmstadt, German). Standard PEITC (HPLC ≥99 %) was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Ultrapure water was used to prepare all the solutions.

2.2. Raw materials

Fresh sprigs of WCBP, resulting from the washing process and the selection of plant material for direct consumption, were gently supplied by the food processing company Vitacress Portugal SA (Odemira, Portugal). When received, the watercress used for the extraction process was stored at −18 °C until further use (unless it was used fresh).

2.3. Determination of moisture content

The moisture content of the WCBP was determined by drying the watercress (2 g) in the oven at 105 °C for 24 h until it was at a constant weight (AOAC, 1990). The results were expressed as a percentage of fresh weight (FW).

2.4. MHG extraction

The extraction procedures for MHG used in this study are described in the flowsheet of Fig. 1.

The MHG was carried out in an advanced microwave extraction system (ETHOS X, Milestone, Milan, Italy) consisting of a microwave reactor of 2.45 GHz, with a maximum delivering power of 1800 W, accomplished by the presence of two magnetrons of 900 W each and an infrared sensor to monitor the temperature. The extraction procedures were performed using a medium glass reactor (Pyrex, Charleroi, PA, USA) closed with a glass cover. The MHG system was configured using the "Flavor's setup." In the MHG procedure performed at atmospheric pressure, 250 g of randomly selected frozen WCBP (which had been frozen for at least 24 h) was heated using fixed microwave power and time settings, without adding any solvent or water, according to the conditions determined by a three-level factorial experimental design, presented in **Tables 1 and 2**, as described in Section 2.9 and Section 3.1.2. The microwave irradiation heated the *in situ* water in WCBP, causing the diffusion of the molecules of interest to the outside plant tissues. Due to the earth's gravity, the crude extract (*in situ* water, frozen water, and metabolites) naturally dropped out of the microwave reactor into the condenser outside the reactor that continuously cooled the extract at 8 °C by a Chiller (Smart H150—2100S, Labtech S.r.l., Sorisole, Italy). The crude extract from each extraction run was collected in a flask, weighted and stored at −80 °C and used for further analysis. A fibre optic sensor monitored the temperature during the experiments, and the final temperature was recorded for each run.

2.5. Extraction yield

The percentage yields of crude extract (total and for each liquid fraction) were calculated by comparing the weight of each collected fraction for each assay with the original weight of the plant material. The results were expressed as a percentage of FW. The extraction yield was calculated as given in eq. (1).

$$Extraction\ yield\ (\%) = \frac{weight\ of\ the\ collected\ liquid\ fraction\ (g)}{weight\ of\ the\ raw\ material\ (g)} \times 100\% \tag{1}$$

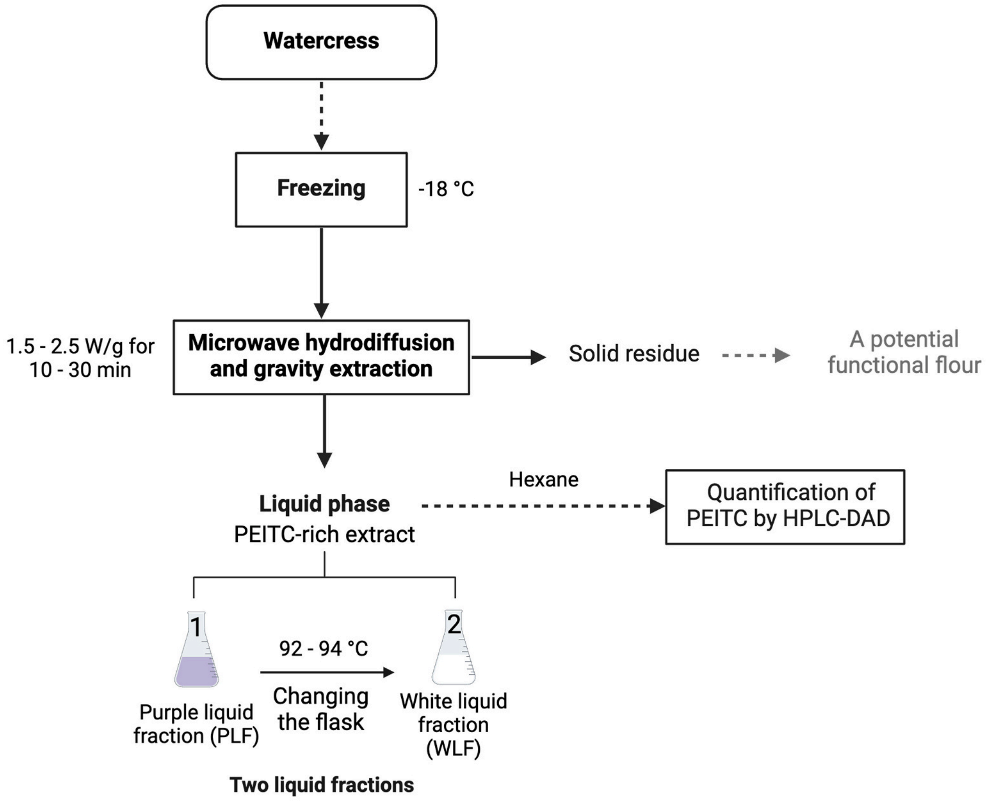

Fig. 1. Microwave hydrodiffusion and gravity (MHG): a schematic diagram of the phenylethyl isothiocyanate (PEITC) extraction protocol.

Table 1
Independent factors, respective levels and responses evaluated on MHG process optimisation.

Factors	Levels		
Symbol, independent variables, unit	**–1**	**0**	**+1**
X_A: Microwave irradiation power (W g^{-1})	1.5	2.0	2.5
X_B: Extraction time (min)	10	20	30
Response	**Units**		
Final temperature	°C		
Extraction yield	% (g extract 100 g^{-1} WCBP FW)		
PEITC content	μg PEITC g^{-1} WCBP DB		
Total phenolic content	mg GAE g^{-1} WCBP DB		
ABTS scavenging	μmol TE g^{-1} WCBP DB		
ORAC assay	μmol TE g^{-1} WCBP DB		

Abbreviations: MHG – microwave hydrodiffusion and gravity, PEITC – phenylethyl isothiocyanate, TPC – total phenolic content, WCBP – watercress by-products, FW – fresh weight, DB – dry basis, GAE - gallic acid equivalent, TE – trolox equivalents.

2.6. *Identification and quantification of PEITC by HPLC*

PEITC was quantified by high-performance liquid chromatography (HPLC-DAD). Prior to quantification, a **selective analytical extraction step** was performed using n-hexane in order to isolate PEITC from the crude aqueous MHG extract. For this, 5 mL of each MHG extract was mixed vigorously with 5 mL of n-hexane for 2 h at 200 rpm and 37 °C using an Orbital Shaker MaxQ 6000 (Thermo Fisher Scientific Inc., Waltham, USA). The mixture was then centrifuged at 4193 ×*g* for 10 min. This process was carried out due to the greater affinity of PEITC for the organic phase, allowing it to be isolated. Then, 4 mL of the organic phase was evaporated till dryness in an RVC 2 − 18 speed-vacuum evaporator (Christ, Osterode am Harz, Germany) at 40 °C. The resulting residue was dissolved in 1 mL methanol. Finally, an HPLC-DAD analysis was performed to identify and quantify PEITC. The separation was carried out by applying the operating conditions described by Coscueta et al. (2020), using a Waters e2695 separation module system interfaced with a photodiode array UV/Vis detector (PDA 190–600 nm) (Waters, Milford, CT, USA). Detection was performed at 245 nm, and data acquisition and analysis were accomplished using Software Empower 3. The quantification was performed using a pure standard calibration curve (127–12,860 μM), and the results were expressed in micrograms of PEITC per g of WCBP dry basis (DB) (μg PEITC g^{-1} WCBP DB).

2.7. *Quantification of total phenolic content (TPC)*

The TPC of MHG extracts was determined by the Folin–Ciocalteu method, following the procedure reported by Coscueta et al. (2018), with some modifications. Briefly, 100 μL of Folin–Ciocalteu reagent 20 % (*v*/v) was added to 30 μL of extract, followed by 100 μL of sodium carbonate (7.4 % (m/v)) and allowed to react in the dark at room temperature (23 °C) for 1 h. After that, the absorbance was measured at 765 nm with a Multidetection plate reader (Synergy H1, BioTek, Winooski, VT, USA) in a 96-well microplate (Nunc™, Thermo Fisher Scientific Inc., Waltham, USA). Gallic acid was used as a calibration curve standard (0.01–0.125 mg mL^{-1}), and the results were expressed in milligrams of gallic acid equivalent (GAE) per g of WCBP dry basis (mg GAE g^{-1} WCBP DB).

2.8. *Antioxidant activity*

The MHG extracts described in Section 2.4 were used to assess the antioxidant activity. This evaluation was conducted using ABTS radical scavenging assay (ABTS) and oxygen radical absorbance capacity assay (ORAC), following the procedures described by Coscueta et al. (2020). The calibration curves of ABTS and ORAC methods were performed with Trolox (25–175 μM and 10–80 μM, respectively). The results were expressed in micromoles of trolox equivalents (TE) per g of WCBP dry

Table 2
Three-level factorial experimental design for two factors and six responses.

Run	Factors		Responses										
	Natural values						PLF	Total (PLF + WLF)					
							WLF						
	X_A	X_B	Temp.	Extraction yield		PEITC content[a]		TPC[b]		ABTS[b]		ORAC[b]	
1	2	20	93	14	**34**	246 ± 18	**610 ± 31****	2.16 ± 0.03	**2.59 ± 0.07**	10.54 ± 0.21	**12.65 ± 0.39**	28.34 ± 1.31	**34.40 ± 0.75**
				20		364 ± 13		0.43 ± 0.03		2.11 ± 0.52		6.16 ± 0.56	
2	2.5	20	96	12	**61**	319 ± 20	**538 ± 32****	2.17 ± 0.03	**2.94 ± 0.05**	11.07 ± 0.37	**16.16 ± 0.68**	30.15 ± 0.27	**40.26 ± 0.37**
				48		219 ± 12		0.76 ± 0.02		5.09 ± 0.38		10.11 ± 0.64	
3*	2	20	96	13	**44**	217 ± 15	**1263 ± 58**	2.10 ± 0.01	**3.06 ± 0.04**	10.50 ± 0.16	**15.73 ± 0.15**	30.69 ± 0.11	**45.22 ± 0.17**
				31		1046 ± 43		0.96 ± 0.04		5.23 ± 0.07		14.54 ± 0.29	
4	2	30	97	11	**81**	239 ± 15	**1985 ± 119**	1.92 ± 0.02	**3.08 ± 0.08**	8.44 ± 0.24	**16.06 ± 0.37**	27.61 ± 0.18	**42.54 ± 0.09**
				70		1746 ± 104		1.16 ± 0.06		7.62 ± 0.17		14.93 ± 0.26	
5	1.5	30	93	16	**42**	169 ± 12	**1128 ± 47**	2.70 ± 0.02	**3.04 ± 0.03****	13.23 ± 0.50	**15.56 ± 0.45****	40.55 ± 0.66	**45.90 ± 0.75****
				26		959 ± 35		0.34 ± 0.01		2.33 ± 0.06		5.35 ± 0.09	
6*	2	20	95	14	**43**	244 ± 15	**1479 ± 65**	2.38 ± 0.03	**3.02 ± 0.05**	11.25 ± 0.30	**15.46 ± 0.07**	34.75 ± 0.09	**44.66 ± 0.23**
				29		1236 ± 50		0.64 ± 0.01		4.21 ± 0.23		9.91 ± 0.14	
7	1.5	10	94	8	**8**	85 ± 6	**85 ± 6**	1.39 ± 0.02	**1.39 ± 0.02**	5.65 ± 0.18	**5.65 ± 0.18**	19.03 ± 0.30	**19.03 ± 0.30**
				nd		nd		nd		nd		nd	
8	2.5	10	94	16	**22**	438 ± 26	**549 ± 34**	2.39 ± 0.06	**2.83 ± 0.08**	11.18 ± 0.46	**13.37 ± 0.51**	33.72 ± 0.72	**39.95 ± 0.69**
				6		111 ± 8		0.44 ± 0.02		2.19 ± 0.05		6.23 ± 0.04	
9	2	10	94	16	**16**	723 ± 22	**723 ± 22**	2.14 ± 0.01	**2.14 ± 0.01**	10.84 ± 0.46	**10.84 ± 0.39**	31.63 ± 0.31	**31.63 ± 0.31**
				nd		nd		nd		nd		nd	
10	2.5	30	109	11	**88**	198 ± 11	**1461 ± 100**	1.55 ± 0.02	**2.69 ± 0.05**	6.96 ± 0.34	**14.55 ± 0.80****	23.81 ± 0.08	**37.18 ± 1.07**
				77		1262 ± 90		1.14 ± 0.07		7.59 ± 0.48		13.37 ± 0.99	
11*	2	20	94	13	**40**	216 ± 14	**1343 ± 59**	1.76 ± 0.01	**3.08 ± 0.08**	8.45 ± 0.17	**16.06 ± 0.37**	23.61 ± 0.21	**34.68 ± 0.28**
				27		1128 ± 45		0.71 ± 0.02		4.21 ± 0.08		11.07 ± 0.07	
12	1.5	20	94	18	**28**	151 ± 11	**360 ± 26****	2.71 ± 0.08	**3.19 ± 0.08**	12.54 ± 0.30	**15.02 ± 0.38**	37.59 ± 0.56	**45.47 ± 0.71****
				10		209 ± 15		0.48 ± 0.01		2.47 ± 0.08		7.88 ± 0.15	
13*	2	20	94	16	**43**	141 ± 11	**1266 ± 53**	2.30 ± 0.06	**3.16 ± 0.09**	11.06 ± 0.38	**15.54 ± 0.33**	30.66 ± 0.44	**42.67 ± 0.68**
				27		1125 ± 42		0.86 ± 0.03		4.49 ± 0.20		12.02 ± 0.23	
14	2.5	20	97	10	**59**	322 ± 10	**1536 ± 75**	1.42 ± 0.03	**2.57 ± 0.01**	6.74 ± 0.07	**13.35 ± 0.41**	19. 62 ± 0.14	**37.63 ± 0.01**
				49		1214 ± 65		1.15 ± 0.01		6.61 ± 0.34		18.01 ± 0.16	
15	2	20	96	15	**42**	360 ± 23	**1276 ± 52**	2.08 ± 0.03	**2.94 ± 0.03**	8.77 ± 0.23	**13.48 ± 0.28**	30.87 ± 0.37	**45.41 ± 0.10**
				27		916 ± 34		0.86 ± 0.01		4.71 ± 0.27		14.54 ± 0.26	
16	2	30	97	17	**73**	343 ± 23	**1271 ± 83**	2.64 ± 0.04	**3.25 ± 0.05**	11. 69 ± 0.45	**15.62 ± 0.46**	36.11 ± 0.19	**45.08 ± 0.21**
				56		929 ± 60		0.60 ± 0.01		3.63 ± 0.01		8.97 ± 0.02	
17	1.5	30	93	12	**35**	40 ± 4	**1115 ± 45**	2.08 ± 0.01	**2.71 ± 0.02****	9.96 ± 0.14	**13.55 ± 0.06**	29.10 ± 0.40	**37.92 ± 0.85****
				23		1075 ± 40		0.62 ± 0.02		3.60 ± 0.11		8.81 ± 0.45	
18*	2	20	93	12	**37**	273 ± 15	**1001 ± 46**	1.59 ± 0.00	**2.31 ± 0.02**	7.34 ± 0.27	**10.93 ± 0.26****	22.28 ± 0.22	**33.99 ± 0.00**

(*continued on next page*)

Table 2 (*continued*)

Run	Factors			Responses									
	Natural values			PLF				Total (PLF + WLF)					
				WLF									
	X_A	X_B	Temp.	Extraction yield		PEITC content[a]		TPC[b]		ABTS[b]		ORAC[b]	
				25		417 ± 32		0.73 ± 0.02		3.59 ± 0.18		11.70 ± 0.22	
19	1.5	10	93	9	**9**	89 ± 6	**89± 6**	1.30 ± 0.01	**1.39 ± 0.01**	5.39 ± 0.22	**5.39 ± 0.22**	17.35 ± 0.34	**17.35 ± 0.34**
				nd		nd		nd		nd		nd	
20	2.5	10	94	15	**22**	168 ± 10	**423 ± 19**	2.28 ± 0.04	**2.77 ± 0.03**	11.64 ± 0.42	**14.45 ± 0.44****	30.59 ± 0.40	**37.04 ± 0.20**
				7		254 ± 9		0.49 ± 0.01		2.81 ± 0.06		6.45 ± 0.19	
21	2	10	94	14	**14**	180 ± 12	**180 ± 12**	2.05 ± 0.04	**2.05 ± 0.04**	9.69 ± 1.06	**9.69 ± 1.06**	28.27 ± 0.62	**28.27 ± 0.62**
				nd		nd		nd		nd		nd	
22	2.5	30	111	16	**85**	319 ± 19	**1542± 77**	2.16 ± 0.02	**3.21 ± 0.05****	18.73 ± 0.46	**25.24 ± 0.54****	28.01 ± 0.90	**40.49 ± 1.42**
				69		1224 ± 57		1.05 ± 0.02		6.51 ± 0.21		12.48 ± 0.53	
23*	2	20	95	14	**36**	264 ± 16	**1292 ± 53**	2.15 ± 0.04	**2.55 ± 0.05**	11.07 ± 0.12	**13.36 ± 0.13**	28.81 ± 0.31	**34.08 ± 0.35**
				22		1028 ± 37		0.40 ± 0.01		2.30 ± 0.16		5.27 ± 0.05	
24	1.5	20	93	16	**23**	99 ± 8	**154 ± 13****	2.48 ± 0.05	**2.91 ± 0.06**	12.49 ± 0.21	**14.83 ± 0.25**	28.75 ± 0.26	**34.15 ± 0.42**
				7		56 ± 5		0.43 ± 0.01		2.34 ± 0.09		5.40 ± 0.16	

Abbreviations: XA - Microwave irradiation power per gram of frozen watercress by-products (WCBP) used for extraction (W g^{-1}), XB - Extraction time (min), (PLF) – purple liquid fraction, (WLF) – white liquid fraction, nd – not detected. Temp. – Final Temperature of MHG process (°C), Extraction yield of each liquid fraction and total crude extract (% of fresh weight - g/100 g FW), PEITC – phenylethyl isothiocyanate content of each liquid fraction and total crude extract (μg g^{-1} WCBP DB), TPC – total phenolic content of each liquid fraction and total crude extract (mg GAE g^{-1} WCBP DB), ABTS scavenging (μmol TE g^{-1} WCBP DB) of each liquid fraction and total crude extract and ORAC assay of each liquid fraction and total crude extract (μmol TE g^{-1} WCBP DB).
*Central point. **The experiment was removed from the statistical model to improve its fit.
[a] Each value was expressed as mean ± standard deviation ($n = 2$). [b] Each value was expressed as mean ± standard deviation ($n = 3$).

basis (μmol TE g^{-1} WCBP DB).

2.9. Design of experiment and statistical analysis

2.9.1. Preliminary tests

Preliminary experiments were performed to define the experimental domain of the variables to be studied. The experiments involved testing fresh and frozen (at −18 °C) edible parts of watercress with a microwave power of 2 W g^{-1} for 20 min to confirm the identification and quantification of PEITC on extracts.

2.9.2. MHG experimental design

The optimisation of experimental conditions was carried out using a three-level (3^2) factorial experimental design. Two factors were considered in the three-level response surface design: microwave irradiation power (X_A; 1.5–2.5 W g^{-1}) and extraction time (X_B; 10–30 min). The responses were studied at three levels coded as −1 (lowest level), 0 (central level), and +1 (highest level) (Table 1). The experimental designs were performed using Design-Expert & Stat-Ease 360 V22 (Stat-Ease, Inc. Minneapolis, USA). The design resulted in 24 runs of experiments according to the $3^n + 3$ design in 2 base blocks on successive days, which includes 9 factorial runs and 3 central runs (Table 2).

For this proposed experimental design, six selected responses (Y) were evaluated: the final temperature of the extraction process, extraction yield, PEITC content, TPC, scavenge capacity of ABTS free radicals and ORAC assay (Table 1). The responses were fitted to two-factor interaction (Eq. (2)) or quadratic (Eq. (3)) regression models acquired by the response surface methodology (RSM).

$$Y = \beta_0 + \beta_A X_A + \beta_B X_B + \beta_{A,B} X_A X_B + \varepsilon \tag{2}$$

$$Y = \beta_0 + \beta_A X_A + \beta_B X_B + \beta_{A,B} X_A X_B + \beta_{A,A} X_A^2 + \beta_{B,B} X_B^2 + \varepsilon \tag{3}$$

where Y is the measured response; β_0 is the intercept (constant); β_i are the coefficients associated with linear effects ($\beta_A - \beta_B$); $\beta_{i,j}$ the coefficient associated with interaction ($\beta_{A,B}$); $\beta_{i,i}$ the coefficients associated with quadratic effects ($\beta_{A,A} - \beta_{B,B}$); and ε is the (random) error.

2.9.3. Statistical analysis

The ANOVA test was applied to assess the quality of the regression models. The significance of the effects of each experimental factor in the model was estimated for each analysed response ($p < 0.05$). The adequacy of the regression models was determined by checking the calculation of the coefficient of determination (R^2), predicted coefficient of determination (predicted R^2) and if the difference between the predicted R^2 and the adjusted R is less than 0.2, which validates that the model is fitting the data and can be reliably used to interpolate. Moreover, the adequate precision was checked, and if it was greater than 4, then the model had a strong enough signal to be used for optimisation. The lack of fit value was also considered insignificant ($p \geq 0.05$). The normality, independence and randomness of residues were also confirmed. This statistical analysis was carried out using Design-Expert & Stat-Ease 360 Version 22.

Statistical analysis was also done using GraphPad Prism 10.2.1 Software (GraphPad Software Inc., San Diego, CA, USA). The *t*-test for independent samples was employed to compare the extracts from the MHG method with fresh and frozen, with $p \leq 0.05$ considered statistically significant. The analysis of both conditions was performed in triplicate (Section 3.2).

2.9.4. Multi-criterion optimisation

Regression models that presented adequacy and quality (with statistical significance, $p < 0.05$) and a good explanation of the data variability (R^2 and adjusted R^2 should be more than 0.7 and without lack of fit, $p \geq 0.05$) were used to perform a multi-criterion optimisation based on Derringer's desirability function to the results of the experimental design, expressing the desirability of responses value on a scale of 0–1 (Derringer & Suich, 1980). For optimisation, the criterion chosen for all responses was maximisation, except for the final temperature of the extraction process. This response was targeted at a temperature of 97 °C to prevent the residue from burning in the MHG reactor, with the importance of (+++). To maximise the remaining responses, greater importance was given to the PEITC content (+++++), total phenolic compounds (++++), antioxidant activity (++++), and extraction yield (+++).

2.10. Microstructure

The fresh and frozen watercress leaf microstructure was analysed by scanning electron microscopy (SEM). The samples were subjected to fixation, dehydration, and drying steps according to Marçal et al. (2024). First, the watercress leaf from each condition (fresh and frozen) was carefully cut transversely with a scalpel. Next, the samples were chemically stabilised by immersion in glutaraldehyde fixation solution 0.1 M phosphate-buffered saline (2.5:97.5, *v*/v) for 90 h. Then, the samples were rinsed four times with ultrapure water and dehydrated by immersion in an ascending gradient of ethanolic solution (30, 50, 70, 80, 90, 100 %) for 1 h each. The samples were submerged in 100 % ethanol overnight and dried in a Nova Sterilis Nova Genesis Supercritical CO_2 reactor at 40 °C, 100 bar, for 45 min. Finally, the dried leaves were placed on top of the observation pin with the cross-section placed vertically (the cut was visible from above) and coated with gold/palladium as described by Almeida et al. (2022). Their microstructure was viewed using a Phenom XL G2 desktop scanning electron microscope (Thermo Fisher Scientific Inc., Waltham, USA) at an accelerating voltage of 15 kV. Observations were performed, and micrographs were taken using the secondary electron detector.

3. Results and discussion

3.1. Optimisation of MHG extraction

3.1.1. Preliminary extractions and MHG technique critical factors

During preliminary tests, the only quantitative response evaluated was the PEITC content, as this study focuses on obtaining an extract rich in PEITC using the MHG technique. Since freezing is commonly used for food preservation and is known to open plant cells, thereby facilitating the extraction of soluble molecules, we tested the extraction process using both fresh and frozen watercress. We found that we could not extract PEITC from the watercress using the MHG method without first freezing the vegetable. Therefore, all extractions were carried out with frozen watercress to ensure the PEITC extraction. Moreover, the preliminary analysis revealed that frozen watercress under MHG produced two distinct extract fractions: a coloured fraction and a colourless/whiteness fraction. When the extraction temperature reached 92–94 °C, the crude extract changed colour from purple (purple liquid fraction; PLF) to colourless/white (white liquid fraction; WLF). These two fractions are of particular interest, as they may contain different bioactive

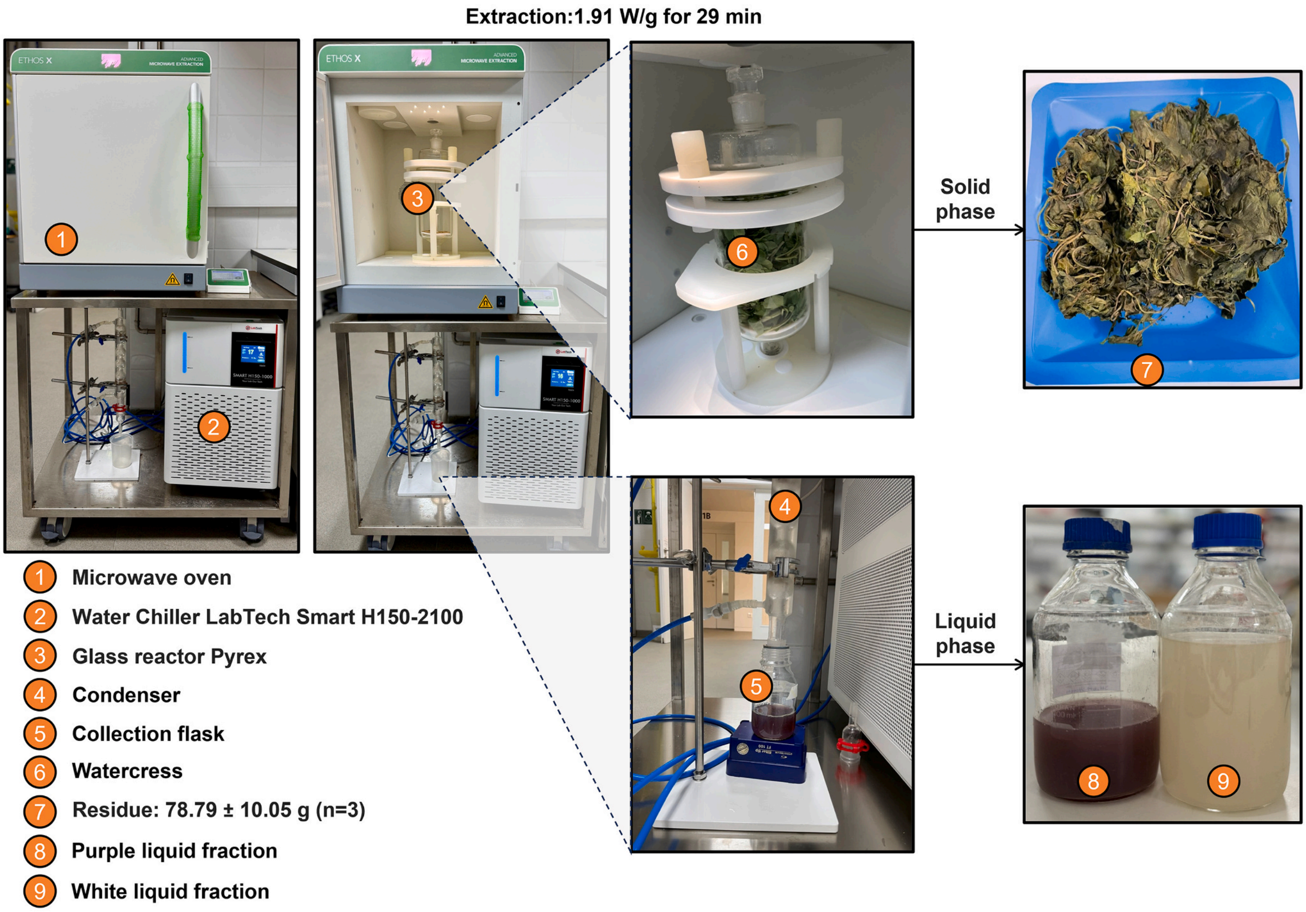

Fig. 2. Equipment for microwave and gravity hydrodiffusion (Milestone, ETHOS X, Sorisole, Italy), liquid fractions and watercress residue resulting from the extraction process.

compounds, with the PLF potentially being richer in PEITC and other phenolic compounds, while the WLF could offer insights into other beneficial properties. Each liquid fraction was collected separately (Fig. 2). Nonetheless, for the extraction optimisation process, as reported in Section 3.7, the responses of the crude extract were analysed with the total extract (PLF + WLF), which combined both the PLF and the WLF. This approach allowed for a comprehensive evaluation of the overall extraction efficiency and bioactive compound yield. Regarding the PEITC content, the preliminary results showed that at 2 W g^{-1} for 20 min, we obtained a total of 1128 ± 305 μg g^{-1} WCBP DB ($n = 3$; PLF: 232 ± 23 μg g^{-1} WCBP DB; WLF: 896 ± 312 μg g^{-1} WCBP DB).

The efficient performance of MHG for phytochemical extraction depends on three critical factors: microwave power, irradiation time, and the moisture content of the plant material (Singh Chouhan et al., 2019a). These factors play a crucial role in maximising the yield and quality of the extracted bioactive compounds. Therefore, the extraction conditions vary depending on the plant material structure, moisture content, extraction power and time, and the aim of the study. The selection of optimal conditions is closely tied to the specific objectives the researchers aim to achieve, such as maximising extraction yield, isolating a particular type of compound or antioxidant content, and drying efficiency (Moraes et al., 2022). Combining high microwave power with a long extraction time can negatively affect the quality of the crude extract by causing the loss of compounds due to excessive heating. Besides, it can also burn the residue, rendering it unsuitable for reuse after the extraction process. This opportunity to use the biological matrix for any biorefinery is one of the advantages that MHG offers. Otherwise, lower microwave power irradiation may not be enough to induce the rupture of cells and consequently not promote an efficient extraction (Singh Chouhan et al., 2019b). Therefore, an optimum balance between microwave power and time must be required to obtain maximum yield for the compounds of interest.

Based on our preliminary extractions and other reported studies on MHG extraction, we selected the factor levels with the understanding that powers lower than 1.5 W g^{-1} are inefficient for the complete extractions of compounds, resulting in a smaller extract volume. On the other hand, powers higher than 2.5 W g^{-1} can damage the target analytes (Moro et al., 2021), compromising the quality of the extract. Regarding the extraction time, MHG is known for its efficiency, with typical extraction times ranging from 6.5 to 20 min to obtain bioactive compounds such as phenolic compounds and essential oil (Moraes et al., 2022; Vian et al., 2008). In this study, 20 min was selected as the central level, as it was found to be sufficient for optimal extraction without causing damage to the residue in the preliminary tests.

Additionally, moisture in the raw material is a critical factor in the success of MHG (Benmoussa et al., 2018). The moisture level is recommended to be 20–100 %. Following this idea, watercress, with its high moisture content of over 90 %, is an ideal matrix for applying the MHG extraction method. The moisture content observed in the WCBP used in this study was 91.880 ± 0.004 % ($n = 5$), as expected and reported by Pinela et al. (2020).

Why did we use watercress by-products? This microgreen grows easily in hydroponic systems and is cultivated worldwide, including in the UK, USA, New Zealand, China, Spain, and Portugal (Hibbert et al., 2023). Although watercress is a fresh-cut edible product, waste is expected during production, processing, and transportation. Thus, with the growing interest in improving resource efficiency by utilising side streams of the agro-food chain, WCBP offers an excellent opportunity to obtain PEITC and promote sustainable agro-food industrial practices by reducing food waste.

3.1.2. *MHG experimental design analysis*

In this study, we employed a three-level factorial experimental design (described in Section 2.9) to develop, for the first time, an optimised MHG model for PEITC extraction. Hence, the experimental design consisted of 24 runs in 2 base blocks on successive days, including 9 factorial and 3 central runs. Our multifactorial design spotlighted two influential factors (independent variables): microwave irradiation power and extraction time. Table 2 displays the experimental design matrix with the responses obtained for each assay. We focused on six responses: the final temperature, extraction yield, PEITC content as an efficiency indicator for the process, TPC, and ABTS and ORAC for antioxidant activity. While this study focused on extracting PEITC through MHG, recent studies suggested that combining phytochemicals may be more effective in treating and preventing cancer and other major chronic diseases compared to using isolated compounds (Rodrigues et al., 2016). We hypothesise that watercress PEITC-rich extract with a high content of phenolic compounds can effectively prevent or treat diseases caused by oxidative stress, such as inflammation and cancer. This approach leverages the potential synergistic effects of multiple bioactive compounds, enhancing their overall therapeutic impact. This is because a high content of phenolic compounds is closely linked with a high antioxidant activity, which is essential in sectors such as food and health to fight against oxidative stress (Martins et al., 2016). For these reasons, we included TPC and antioxidant activity in our responses.

According to the model adequacy-output (Table 1**S,** Supplementary Material), the two-factor interaction model (2FI) showed significance for the extraction yield, phenolic compound extraction, and antioxidant activity by ORAC assay. On the other hand, the quadratic model was significant for the final temperature of the process, PEITC content, and antioxidant activity by ABTS scavenging activity assay. In addition, the 2FI and the quadratic models' "adjusted R^2" and "predicted R^2" values were higher than 0.7. The adequacy of the models' fitness showed a strong correlation between the predicted and actual values (Fig. 3 **B**, **D**, **F**, **H**, **J**, **L**, "Predicted vs Actual" graphs). The mathematical models were statistically acceptable for all six responses due to the significant regression level and non-significant lack of fit. This indicates that the model was precise enough to predict suitable responses. The interaction terms of the model $X_A X_B$, the coefficients of the intercept, linear (X_A,X_B), and quadratic $\left(X_A^2, X_B^2\right)$ were determined by linear regression. The effects of the linear, 2IF or quadratic factors on the response were tested for significance using analysis of variance (ANOVA). Further details about the statistical analysis for the tested models and the model's equation coefficients are provided in the Supplementary Material (Table 1S and Table 2S).

3.1.3. *Statistical analysis of the experimental results*

In this study, we used a linear regression model to find significant variables affecting the extraction of PEITC and other meaningful responses, such as final temperature, extraction yield, TPC, and antioxidant activity (obtained by ABTS and ORAC assays). Table 3 shows the responses and the formulae for the response model coefficients, F-values, *p*-values, R^2, adjusted R^2, and predicted R^2.

With an F-value of 25.51 and $p < 0.0001$, the model for the final temperature was statistically significant, indicating that the results were not due to random chance. This suggests that the factors influencing the final temperature have a meaningful impact on the extraction process. Though an Adequate Precision ratio of 16.143, considerably above the criterion of 4, demonstrated its great predictive potential, the non-significant lack of fit ($p = 0.1121$) indicated a good match between the model and the data. Reiterating the model's dependability, the modified R^2 result of 0.8478 matched the predicted R^2 of 0.7356. The coefficients showed that microwave power and extraction time and their interaction strongly influenced the final temperature, with extraction time having a noticeable quadratic effect. As shown in the 3D surface plot (Fig. 3C), temperatures were observed as both microwave power and extraction time increased.

We observed that the temperature changes during the MHG process were similar to those reported by Périno et al. (2016). Briefly, the process had three phases of temperature evolution: (i) a rapid heating phase, where the core temperature reaches 90–100 °C. The duration of

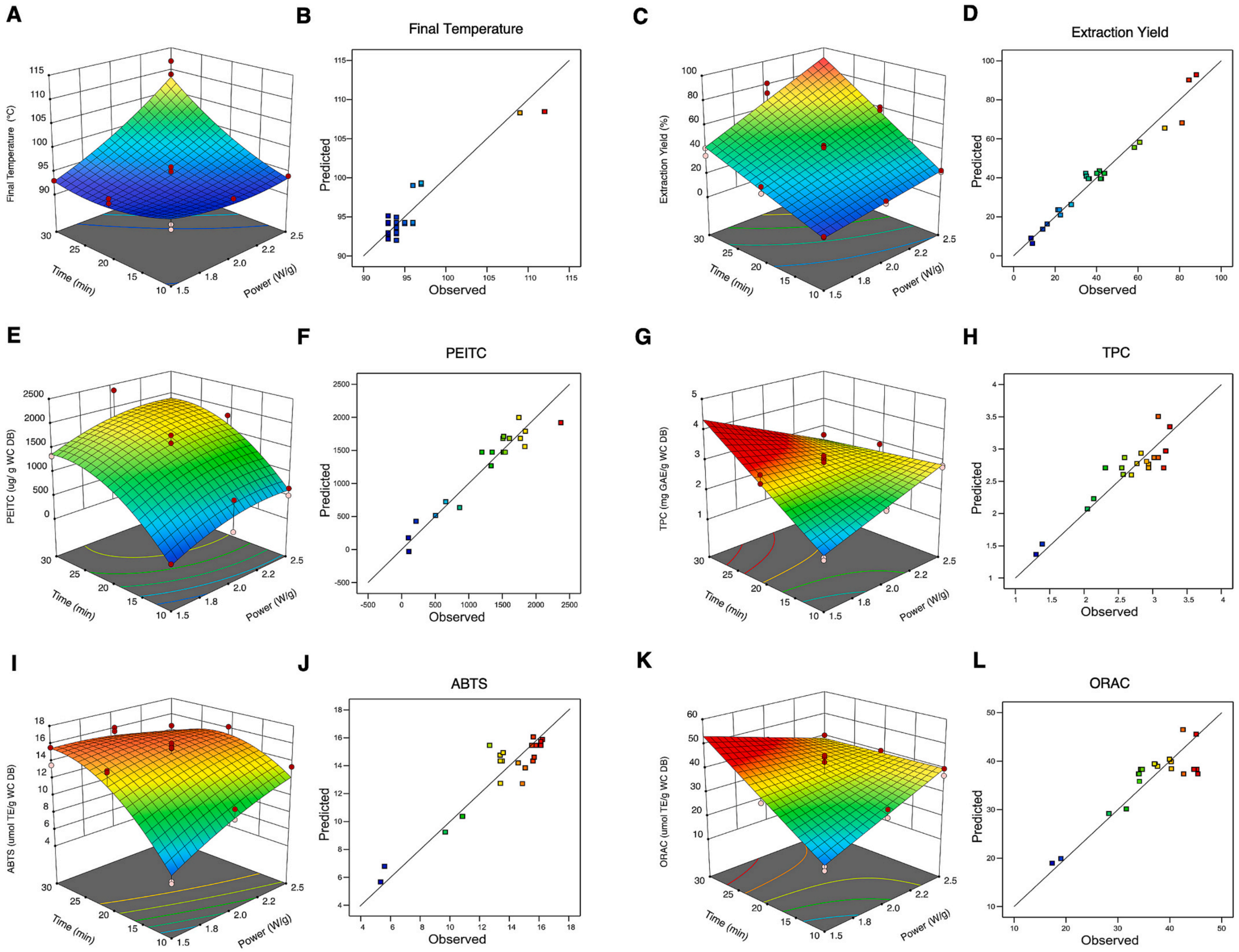

Fig. 3. Optimisation of phenylethyl isothiocyanate (PEITC) using microwave hydrodiffusion and gravity as an extraction method. Response surface models are estimated based on two experimental factors (A, C, E, G, I, K). Observed *versus* predicted values for all responses (B, D, F, H, J, L). Abbreviations: TPC – total phenolic content, ABTS – ABTS radical scavenging assay, ORAC – oxygen radical absorbance capacity assay.

this phase varies depending on the microwave power applied. The temperature increases rapidly at higher powers and reaches higher values in a shorter time; (ii) the evaporation phase, where heating causes the cells' water to move out downwards with gravity; (iii) the burning phase of plant residue, where the temperature rises again. This critical phase indicates the extraction process's end, preventing residue combustion.

However, due to the delicate structure of the watercress matrix and the freezing pretreatment, water droplets formed before reaching a temperature range of 90–100 °C preventing the completion of the first phase of the extraction process. Generally, the water was released after reaching a temperature of around 50 °C for all tested power settings, and the induction phase (where the temperature reached 90–100 °C) occurred after approximately 10 min.

With an F-value of 165.45 and $p < 0.0001$, the model proved relatively strong for extraction yield, excluding the possibility of random error. Substantial predictive accuracy was shown by the tight alignment between the predicted R^2 (0.9389) and adjusted R^2 (0.9389) values; the non-significant lack of fit ($p = 0.2243$) validated the dependability of the model even further. With a value of 38.9813, much over the established threshold, the Adequate Precision ratio highlighted an excellent signal-to-noise ratio. The coefficients affected the extraction yield for both microwave power and extraction time, as well as their interaction term. Increasing microwave power and extraction time led to higher temperatures, as depicted in the 3D surface plot (Fig. 3C). With its statistical and practically significant nature, the model proved to be a helpful instrument for maximising the extraction yield of bioactive molecules.

The extraction yield can be improved by increasing the irradiation power and temperature. Higher power and temperature can cause cell rupture, allowing for more efficient water displacement from plant tissue. However, it was essential to define the maximum allowable temperature based on the thermostability of solutes and to ensure that it does not cause any damage to the residues (Pérez et al., 2014). As previously mentioned, the moisture content of the WCBP used was, on average, 91.880 %. Thus, in 250 g of the plant material, we estimated that up to 229.7 g of water was present, which translates to approximately 230 mL of crude extract, representing 100 % of the "*in situ* water," at a power setting of 2.0–2.5 W g^{-1} (500–625 W) for about 20–30 min. However, to prevent the sample from burning and degrading the bioactive compounds, none of the extraction runs reached the total water content of 230 mL. In cases where extremely high power was used for an extended period, such as 2.5 W g^{-1} (625 W) for 30 min, the crude extract's average volume was 216 ± 6 mL ($n = 2$), but the watercress core started to burn. Therefore, it was generally observed that for long extraction times (≥ 30 min), the power should be less than 2.5 W g^{-1}. However, at lower power (1.5 W g^{-1}) and shorter times (10 min), the

Table 3

ANOVA and regression coefficients of the polynomial models for the response variables.

Factor	Coefficient	SS[a]	DF[b]	MS[c]	F-value	*p*-value	R^2	Adjusted R^2	Predicted R^2	CV %	Adequate Precision Ratio
Final temperature (°C)											
Model		454.58	5	90.92	25.51	< 0.0001	0.8824	0.8478	0.7356	1.97	16.1432
Intercept	−5.25500										
Linear											
$\mathbf{X_A}$	−2.82500	147.00	1	147.00	41.25	< 0.0001					
$\mathbf{X_B}$	−0.884167	120.33	1	120.33	33.77	< 0.0001					
Interaction											
$\mathbf{X_A X_B}$	1.73825	144.50	1	144.50	40.55	< 0.0001					
Quadratic											
$\mathbf{X_A^2}$		10.08	1	10.08	2.83	0.1108					
$\mathbf{X_B^2}$		18.75	1	18.75	5.26	0.0348					
Residual		60.58	17	3.56							
Pure error		10.00	6	1.67							
Lack of fit		50.58	11	4.60	2.76	0.1121					
Total		515.33	23								
Extraction Yield (%)											
Model		11,729.02	3	3909.67	165.45	< 0.0001	0.9631	0.9573	0.9389	11.87	38.9813
Intercept	137.37500										
Linear											
$\mathbf{X_A}$	−32.00000	3060.49	1	3060.49	129.51	< 0.0001					
$\mathbf{X_B}$	−2.13333	8064.23	1	8064.23	341.26	< 0.0001					
Interaction											
$\mathbf{X_A X_B}$	0.850000	604.30	1	604.30	25.57	< 0.0001					
Residual	5.50000	448.98	19	23.63							
Pure error	0.018750	88.35	6	14.72							
Lack of fit		360.64	13	27.74	1.88	0.2243					
Total		12,221.91	23								
PEITC content (μg PEITC g^{-1} WCBP DB)											
Model		6.975E+06	5	1.395E+06	27.46	< 0.0001	0.9135	0.8803	0.7848	17.97	15.1987
Intercept	−5372.42536										
Linear											
$\mathbf{X_A}$	3492.63868	6.177E+05	1	6.177E+05	12.16	0.0040					
$\mathbf{X_B}$	227.91792	4.950E+06	1	4.950E+06	97.45	< 0.0001					
Interaction											
$\mathbf{X_A X_B}$	−1.08725	236.42	1	236.42	0.0047	0.9466					
Quadratic											
$\mathbf{X_A^2}$	−734.04574	1.156E+05	1	1.156E+05	2.28	0.1554					
$\mathbf{X_B^2}$	−4.03796	5.343E+05	1	5.343E+05	10.52	0.006					
Residual		6.603E+05	13	50,794.95							
Pure error		1.169E+05	5	23,384.04							
Lack of fit		5.434E+05	8	67,929.77	2.90	0.1277					
Total		7.727E+06	19								
TPC (mg GAE g^{-1} WCBP DB)											
Model		4.97	3	1.66	27.74	< 0.0001	0.8387	0.8085	0.7525	9.19	17.9405
Intercept	−4.52765										
Linear											
$\mathbf{X_A}$	3.02108	0.0678	1	0.0678	1.14	0.3023					
$\mathbf{X_B}$	0.386111	3.17	1	3.17	53.11	< 0.0001					
Interaction											
$\mathbf{X_A X_B}$	−0.161206	2.33	1	2.33	38.96	< 0.0001					
Residual		0.9550	16	0.0597							
Pure error		0.6003	6	0.0355							
Lack of fit		0.3547	10	0.1000	0.3545	0.9288					
Total		6.04	20								
ABTS (μmol TE g^{-1} WCBP DB)											
Model		164.70	5	32.94	18.45	< 0.0001	0.8682	0.8211	0.7098	9.95	13.3233
Intercept	−29.01030										
Linear											
$\mathbf{X_A}$	19.28857	9.32	1	9.32	5.22	0.0385					
$\mathbf{X_B}$	1.99132	65.74	1	65.74	36.81	< 0.0001					
Interaction											
$\mathbf{X_A X_B}$	−0.386071	20.87	1	20.87	11.69	0.0042					
Quadratic											
$\mathbf{X_A^2}$	−2.38786	1.73	1	1.73	0.9695	0.3415					
$\mathbf{X_B^2}$	−0.023845	27.62	1	27.62	15.47	0.0015					
Residual		25.00	14	1.79							
Pure error		10.39	5	2.08							
Lack of fit		14.61	9	1.62		0.6486					
Total		196.73	20								
ORAC (μmol TE g^{-1} WCBP DB)											
Model		924.06	3	308.02	18.09	< 0.0001	0.7723	0.7296	0.6746	11.32	13.6765
Intercept	−54.19291										
Linear											
$\mathbf{X_A}$	37.84801	15.86	1	15.86	0.9312	0.3489					

(*continued on next page*)

Table 3 (*continued*)

Factor	Coefficient	SS[a]	DF[b]	MS[c]	F-value	*p*-value	R^2	Adjusted R^2	Predicted R^2	CV %	Adequate Precision Ratio
$\mathbf{X_B}$	4.29008	513.15	1	513.15	30.13	< 0.0001					
Interaction											
$\mathbf{X_A X_B}$	−1.73618	326.66	1	326.66	19.18	0.0005					
Residual		272.47	16	17.03							
Pure error		212.21	6	35.37							
Lack of fit		60.26	10	6.03	0.1704	0.9926					
Total		1201.34	20								

Abbreviations: (XA) - Microwave irradiation power per gram of frozen WCBP used for extraction (W g^{-1}), (XB) - Extraction time (minutes), (SS) - sum of squares, (DF) - degrees of freedom, (MS) - mean squares, (CV) – coefficient variation.
$p < 0.05$ was considered significant. The Adequate Precision Ratio should be greater than 4.

extraction was not sufficient to extract most of the water, only around 96 ± 11 % (n = 2). For a power set between 2 and 2.5 W g^{-1} for 30 min, the cycle resulted in more than 193 ± 15 mL (n = 2) of water depletion from the matrices, leaving a dry residue. Powers around 2 W g^{-1} were more suitable for extracting the *in situ* water without causing any damage to the residue.

The model for PEITC content was highly significant, as indicated by an F-value of 27.46 and $p < 0.0001$. The non-significant lack of fit ($p = 0.1277$) suggested a good fit; an Adequate Precision ratio of 15.199 strengthened the model's predictive power. Further demonstrating the dependability of the model were the nearly matched adjusted R^2 (0.8803) and predicted R^2 (0.7848). The coefficients indicated that PEITC content was much impacted by microwave power, extraction time, and the quadratic term for extraction time. Particularly in the 20–30 min range, the 3D surface plot (Fig. 3E) showed that extraction time had the most critical influence on PEITC levels independent of microwave power. As shown in Table 2, the water-based liquid fraction (WLF) had the most outstanding PEITC content.

During the MHG process, the extraction of compounds was visibly inhomogeneous, leading to variations in the extract composition. This variability may be attributed to differences in heat distribution and the interaction between the plant material and microwave energy during the extraction. The diffusion of different molecules occurs at different times and depends on their specific characteristics, including their localisation within the matrix and solubility (Cendres et al., 2014; Moraes et al., 2022). Cendres et al. (2014) studied the diffusion of fruit compounds during microwave hydrodiffusion extraction. The authors reported that the extraction process initially results in a high concentration of sugar, acids, and total polyphenols, followed by the extraction of specific polyphenols, especially anthocyanins and hydrophilic biomolecules such as carotenoids and volatile compounds (such as benzaldehyde), which are more retained on the cell walls. The study also concluded that high power density can lead to more matrix destructuring, which is beneficial for extracting hydrophobic compounds. According to our study, the PLF was obtained during the extraction induction phase and ended with the appearance of the first visible white drops of the extract. This typically occurs about 10 min after the start of extraction and when the temperature reaches 92–94 °C. This indicates that the PLF had a higher concentration of soluble compounds easily hydrodiffused from the WCBP.

On the other hand, the WLF had a higher content of hydrophobic compounds. This is consistent with the higher content of PEITC found in WLF (Table 2) since PEITC is a compound with considerable hydrophobicity (logP = 3.47) (Coscueta et al., 2022). PEITC was primarily extracted after the induction phase when particles are carried along at high flow rates and with the fragmentation of watercress cells. We hypothesise that the opalescent appearance of the white fraction is due to the formation of a microemulsion, as PEITC droplets disperse in the aqueous solution, leading to increased turbidity. PEITC can remain stably suspended due to the presence of natural emulsifiers, including proteins, phospholipids from cell membranes, and other surface-active compounds found in watercress (McClements & Gumus, 2016). Although PEITC is present in lower concentrations compared to water, it is sufficient for detection and quantification without visible phase separation. Additionally, in the purple fraction, we observe similar white microemulsions in suspension, which arise from the onset of the induction phase prior to changing the collected flask. This also accounts for the detection and quantification of PEITC in the first fraction collected.

The extraction process presents several key aspects that are useful for developing new products and tailoring the chemical properties of the extracts. These findings provide insights into producing extracts rich in bioactive compounds by generating distinct liquid fractions with varying bioactive compositions. This can be achieved by carefully adjusting extraction time and temperature, enabling the customisation of extracts for specific applications. For instance, PLF contained a higher TPC, while WFL was richer in PEITC. Interestingly, WFL had a characteristic sulfurous aroma, typical of PEITC, resulting from the higher content of this compound in the extract. Furthermore, these extracts rich in specific bioactives are an excellent opportunity to consume beneficial compounds as they are concentrated and can be easily added to foods and beverages. Specifically, WLF has potential health benefits as PEITC is an effective antioxidant, antimicrobial, and cancer chemopreventive agent.

With an F-value of 27.74 and $p < 0.0001$, the model was likewise rather highly significant regarding the TPC. Though an Adequate Precision ratio of 17.9941 further supported its predictive usefulness, the non-significant lack of fit ($p = 0.9288$) suggested a good model fit. Predictive solid powers of the model were shown by the closely matched values of the adjusted R^2 (0.8085) and predicted R^2 (0.7525). The coefficients showed that TPC was much influenced by the relationship between microwave power and extraction time, as well as by extraction time itself. Consistent with results from other studies, the 3D surface plot (Fig. 3G) showed that extending extraction time raised TPC while excessive microwave power had a negative effect. High power can thus degrade phenolic compounds. On the other hand, increasing power energy does not always positively impact TPC; in fact, higher power values can lead to a decrease in TPC. This aligns with findings from other studies using different matrices, which report that excessive power can degrade phenolic compounds, thereby reducing their concentration in the extract (Moro et al., 2021; Périno et al., 2016; Rodríguez-Seoane et al., 2019).

The microwave powers employed are related to the raising of the temperature, which influences the content of polyphenols. On the one hand, the increase in temperature is necessary to heat the *in situ* water, destroy the vegetable cells where the phenolic compounds are, and transfer them to the outside of the matrix (Moraes et al., 2022). On the other hand, thermally induced phenolic degradation occurs at high temperatures (80–110 °C), leading to decomposition and epimerisation (Afoakwah et al., 2023). Using the Folin-Ciocalteu method, as expected, the TPC were highest at the beginning of extraction in PLF, as shown in Table 2. This is because most of the total polyphenols are released early during MHG extraction and may remain stable up to 100 °C for short periods (Pérez et al., 2014).

Regarding the TPC, the observed results, namely the 3D surface plot (Fig. 3G), align with findings from other studies indicating that combined moderate microwave with more extended extraction time is more effective for obtaining phenolic compounds (Jokić et al., 2012). To evaluate the potential use of liquid fractions, as further work, it is essential to characterise the phenolic profile of extracts using HPLC and verify how that profile is affected by the MHG method. Thus, MHG extraction is a promising method for recovering phenolic compounds from watercress that could provide various health benefits when incorporated into the human diet.

The ABTS model was statistically significant for antioxidant activity with an F-value of 18.45 and $p < 0.0001$. While an Adequate Precision ratio of 13.323 proved the predictive strength, the non-significant lack of fit ($p = 0.6486$) supported the model's fit. Closely aligned and validating the dependability of the model were the adjusted R^2 (0.8211) and the predicted R^2 (0.7098). According to the coefficients, ABTS values were influenced by microwave power, extraction time, their interaction, and quadratic terms. The 3D surface map (Fig. 3I) shows that ABTS activity rose with increasing extraction duration, particularly between 10 and 25 min, somewhat offset by changing power levels.

With an F-value of 18.09 and $p < 0.0001$, the model was likewise robust for ORAC. The Adequate Precision ratio of 13.677 was above the threshold, so verifying the model's predictive capacity and the lack of fit was not significant ($p = 0.9926$). The correctness of the model was supported by the near alignment of the adjusted R^2 (0.7296) with the predicted R^2 (0.6746). The coefficients indicate that extraction time and its interaction with microwave power greatly affected ORAC values; the surface plot (Fig. 3K) shows a linear increase in ORAC activity as extraction time and microwave power rose. As ABTS found, the most important consideration was extraction time. With a statistically significant ($p = 0.0001$) model, the extract's ability to neutralise peroxyl radicals highlights its capacity to minimise antioxidant activity during extraction, helping to highlight NF-κB activation's potential to help lower inflammation through this reliable framework (Moro et al., 2021).

Concerning antioxidant activity, it is worth noting that despite being reported as an antioxidant compound, pure PEITC presented low antioxidant activity (ORAC), as demonstrated by Coscueta et al. (2020) and Rodrigues et al. (2016), not directly acting on radicals. Instead, PEITC can act as an antioxidant by Nrf2 activation, increasing the activities of several antioxidant enzymes, particularly the induction of phase II enzymes of the detoxification pathway, displaying a marked effect in boosting the total antioxidant activity of the cells, thereby significantly reducing the endogenous level of reactive oxygen species (Chikara et al., 2018; Coscueta et al., 2022; Dayalan Naidu et al., 2018). Additionally, microwave hydrodiffusion is a method that can extract several micronutrients and phytochemicals. Phenolic compounds in fruits and vegetables are highly reactive and are known for their antioxidant activity due to their structure's hydroxyl substitution ability, enabling them to combat free radicals effectively (Zeng et al., 2023). Therefore, MHG extracts provide antioxidant action through different mechanisms by synergistically combining the properties of PEITC and phenolic compounds.

3.1.4. *Optimised MHG extraction and model validation*

We used a multi-criterion optimisation desirability function as a comprehensive tool to evaluate the combined performance of multiple responses. This approach was crucial for identifying the most favourable extraction conditions for bioactive compounds. This desirability index, ranging from 0 to 1, indicates how well the conditions align with the defined constraints and objectives, with higher values representing more optimal outcomes. The prediction for the optimal conditions identified a microwave power of 1.91 W g^{-1} and an extraction time of 29 min, providing detailed insights into the expected responses. To validate the predictive model, we conducted four experiments using these optimal conditions, summarising results in Table 4.

The predicted values supported the choice of these optimal conditions, yielding the highest desirability score of 0.871, demonstrating the model's robustness and reliability. Except for the extraction yield, the observed validation and predicted values showed no significant difference ($p > 0.05$), affirming the model's precision and acceptability. Notably, the extraction yield (70 ± 6 %) significantly exceeded the predicted value (60 ± 5 %) but remained within the upper range of the 95 % prediction interval, demonstrating effective performance.

Table 4
Desirability values for an optimised model for PEITC.

Factors	**Factors values**		**Levels**
	Optimum	**Low**	**High**
X_A: Microwave irradiation power (W g^{-1})	1.91	1.50	2.50
X_B: Extraction time (min)	29	10	30
	Optimal desirability: 0.871		
		Values	
Response	**Optimal predicted**	**Observed**	**95 % Prediction interval**
Final temperature	97.3 ± 1.9 ^{a}	97.2 ± 0.5 ^{a}	94.7–100.0
Extraction yield	60 ± 5 ^{a}	70 ± 6 ^{b}	54–66
PEITC content	1774 ± 225 ^{a}	1818 ± 376 ^{a}	1444–2105
Total phenolic content	3.51 ± 0.24 ^{a}	3.63 ± 0.50 ^{a}	3.14–3.87
ABTS scavenging	15.43 ± 1.33 ^{a}	14.26 ± 1.96 ^{a}	13.49–17.37
ORAC assay	46.34 ± 4.13 ^{a}	40.88 ± 6.51 ^{a}	39.89–52.79

Abbreviations: Final Temperature of MHG process (°C), Extraction yield of total crude extract (% of fresh weight), PEITC – phenylethyl isothiocyanate content of total crude extract (μg g^{-1} WCBP DB), Total phenolic content (TPC) of total crude extract (mg GAE g^{-1} WCBP DB), ABTS scavenging (μmol TE g^{-1} WCBP DB) of total crude extract and ORAC assay of total crude extract (μmol TE g^{-1} WCBP DB).

Each value was expressed as mean ± standard deviation ($n = 4$). Values in the same row with the same superscript letter are not significantly different from each other ($p < 0.05$).

The core focus of the study, the PEITC content, was predicted to be 1774 ± 153 μg g^{-1} WCBP DB, while the value obtained from the validation experiments was 1819 ± 225 μg g^{-1} WCBP DB. This result fell within the 95 % prediction interval (1444 to 2105 μg g^{-1} WCBP DB), indicating reasonable reliability in the extraction of this bioactive compound. The broader 99 % prediction interval suggests that extreme conditions could lead to significant variability, highlighting the importance of precise control over extraction parameters to maintain consistency.

These results, particularly concerning the PEITC content, support the conclusion that the model provides accurate and reliable predictions, laying a solid foundation for future scaling and potential commercial applications. The narrow confidence intervals across most parameters underscore the effectiveness of the optimisation approach used in this study.

3.2. *Freezing: The key novelty to obtaining PEITC*

We previously performed a fresh watercress test extraction but did not detect PEITC. Therefore, we concluded that freezing the plant matrix before extraction is crucial for obtaining PEITC. As a result, all subsequent extractions were carried out with frozen WCBP. Bringing the material to room temperature is generally recommended to allow efficient use of microwave energy. However, in this study, watercress was kept frozen in the glass reactor to avoid any loss of bioactive compounds in the thawed water and maximise the extraction of the compounds of interest.

Moreover, our findings revealed that using frozen watercress for MHG extraction resulted in a significantly higher content of phenolic compounds ($p = 0.0013$) and substantially greater antioxidant activity (ABTS $p = 0.0041$; ORAC $p = 0.0004$) under optimum conditions compared to the fresh matrix (Fig. 1S, Supplementary Material). In particular, fresh watercress exhibited a TPC of 0.71 ± 0.02 mg GAE g^{-1}

WCBP DB, five times lower than frozen WCBP. Similarly, the ABTS assay (6.14 ± 0.08 mg μmol TE g^{-1} WCBP DB) and the ORAC assay (8.25 ± 0.25 mg μmol TE g^{-1} WCBP DB) of fresh WCBP were observed to be two and five times lower, respectively, compared to frozen WCBP.

During freezing, water in cellular foods crystallises, forming ice crystals that disrupt cell membranes and walls, thereby damaging the food's microstructure (D. Li et al., 2018). To investigate this phenomenon, we used SEM to assess the effect of freezing on the watercress during the extraction process (Fig. 4). These morphological alterations are evident in the SEM micrographs, where there is a decrease in uniformity of structure and enlargement or even rupture of pores, indicating the deformation of the cell walls can be observed in the frozen samples, particularly in the spongy mesophyll region. In contrast, fresh tissue retains a more compact and organized structure. The white arrows in the images highlight these structural differences, supporting the hypothesis that freezing enhances tissue permeability and facilitates the mass transfer of intracellular compounds. Similar findings were reported by Marçal et al. (2024), who observed tissue damage caused by ice crystal growth during the freezing storage of mango peels.

Although freezing damages the internal structure of the fresh matrix, this destruction improves water diffusion, which can potentially increase the extractability of bioactive compounds such as phenolic compounds (Moraes et al., 2022). Indeed, studies have shown that using frozen fruit along with microwave hydrodiffusion as an extraction method can result in better yields of over 30 % due to the combination of freezing and microwave heating processes destructures the cellular structures, resulting in improved extraction (Cendres et al., 2014). Our results concerning extracting phenolic compounds and antioxidant activity are consistent with these findings.

Regarding the extraction of PEITC, we questioned: why freezing is essential for the successful extraction of PEITC using MHG? Glucosinolates, which are stable and relatively nonreactive compounds, are located in vacuoles in plant tissue and are isolated from myrosinase in distinct cellular compartments; however, when the cells are physically disrupted, such as freezing, myrosinase comes into contact with glucosinolates during thawing. As a result, myrosinase catalysed the hydrolysis of glucosinolates into various bioactive compounds, namely ITCs (Coscueta et al., 2022; Theunis et al., 2022). As previously discussed, the production of ITCs is influenced by various factors, and it is important to emphasize that the variation lies in the production of PEITC itself rather than the extraction process. This distinction highlights the importance of understanding the biochemical pathways and conditions affecting PEITC formation. Thus, in this study, by freezing the watercress, a physical process, we provide a simple, economical option and a favourable environment to promote the enzymatic hydrolysis of gluconasturtiin into PEITC. This process makes PEITC available and more accessible to extract through the MHG method. The results of our research offer valuable insights into improving the process of extracting ITCs from watercress. This knowledge can prove beneficial for industries producing watercress-based products, as freezing is a commonly used method for long-term storage. Considering this, the frozen matrix is prepared for extracting ITCs and other bioactive compounds with economic and health benefits using the method outlined in our work.

3.3. *Can MHG be a sustainable solution for extracting PEITC from watercress?*

ITC-rich extracts are typically obtained from plants using a solid-liquid extraction process that involves organic solvents such as hexane, dichloromethane, and chloroform. This method involves several steps, including pretreating the plant material (drying, grinding, and hydrolysing glucosinolates) and post-treating the liquid extract (filtration and concentration) (Karanikolopoulou et al., 2021). Although this process is simple, it is time-consuming, energy-intensive, and uses large amounts of water (a scarce resource) or harmful solvents to humans and the environment (Chemat et al., 2019). Despite this, there is limited research on obtaining PEITC from watercress. Previous studies that used n-hexane as a solvent reported PEITC amounts ranging from 1682 to 3346 μg g^{-1} watercress DB (Coscueta et al., 2020; Kyriakou et al., 2022; Rodrigues et al., 2016). These differences in PEITC amounts extracted are mainly due to the plant's conditions, such as temperature, incubation time, pH, and the presence of additives during the enzymatic hydrolysis of glucosinolates as pretreatment. Moreover, environmental factors, including cultivation and climatic conditions, plant stage and age, storage and processing, also impact the production of ITCs (Coscueta et al., 2020; Kyriakou et al., 2022). Considering this, preliminary pretreatment of the plant material to facilitate PEITC production by the action of endogenous myrosinase is a critical step in ensuring successful extraction. This preparation optimises the availability of PEITC for subsequent processing. This statement is also valid for non-conventional techniques with a more sustainable approach reported in the literature, such as pressurised fluid extraction with supercritical CO_2 as a solvent or using aqueous micellar systems. Rodrigues et al. (2016) extracted PEITC from watercress using supercritical CO_2, resulting in 4783 μg g^{-1} WC DB. In previous work, we also demonstrated the effectiveness of using micellar systems of Genapol X-080 (2887 μg g^{-1} WCBP DB) and Tergitol 15-S-7 (2971 μg g^{-1} WCBP DB) to isolate PEITC-enriched fractions from WCBP (Coscueta et al., 2020). These selective methods have a low environmental impact and are exceptionally cost-effective, but the pretreatment process significantly influences both. This means that an additional step in the extraction process can be pretty time-consuming, as it requires drying the material, careful control of temperature, pH balance, selection of the maceration solution (water or buffer), and incubation time (1 to 4 h).

In the present work, we have developed a new, eco-friendly methodology for extracting PEITC from watercress that is more straightforward and cost-effective than conventional methods. The optimal conditions yielded a value of 1818 ± 225 μg g^{-1} WCBP DB. Our results indicate that the MHG approach is highly effective in extracting PEITC. It is important to note that the actual yield may vary depending on the biological matrix, a complex system strongly influenced by various factors.

The MHG method stands out from other methods as it does not require the conventional pretreatment step of drying and maceration of the plant material. Instead, it is a single-stage process that requires less than 30 min of irradiation to achieve satisfactory results. The 30 min of the MHG process does not include the freezing time for the matrix (which can be applied as a storage method for the vegetable). Even so, this MHG method has a considerably shorter time extraction than other methods, reducing costs and providing better energy utilisation by decreasing the number of steps in the process chain. Furthermore, the MHG method does not use any solvents (hazardous organic and/or green solvents), which eliminates the need for solvent evaporation during extract purification. This is relevant because organic solvents can harm human health and cause environmental pollution. Besides, the eco-friendly features of the MHG method are also reflected in its reduced CO_2 emissions compared to conventional extraction methods (Singh Chouhan et al., 2019b). Additionally, currently, the producers of natural extracts must consider not only the production with high yields and quality but also the safety of human health, the protection of the environment, and the preservation of biodiversity. Considering this, the proposed method is clean, fitting in the principles of "green extraction," which is based on designing extraction processes with reduced energy consumption, using alternative solvents and renewable natural products, ensuring a safe and high-quality product (Chemat et al., 2019).

MHG provides an excellent opportunity to reuse post-extraction residue, as it is not exposed to any chemical substance (Singh Chouhan et al., 2019b). After liquid extraction, complex carbohydrates, fibres, proteins, minerals, as well as other nutritional and bioactive compounds, such as phenolic compounds and vitamins, can be extracted from the residue. Additionally, this residue can be processed to obtain gluten-free flour. These products can be used as potential ingredients in

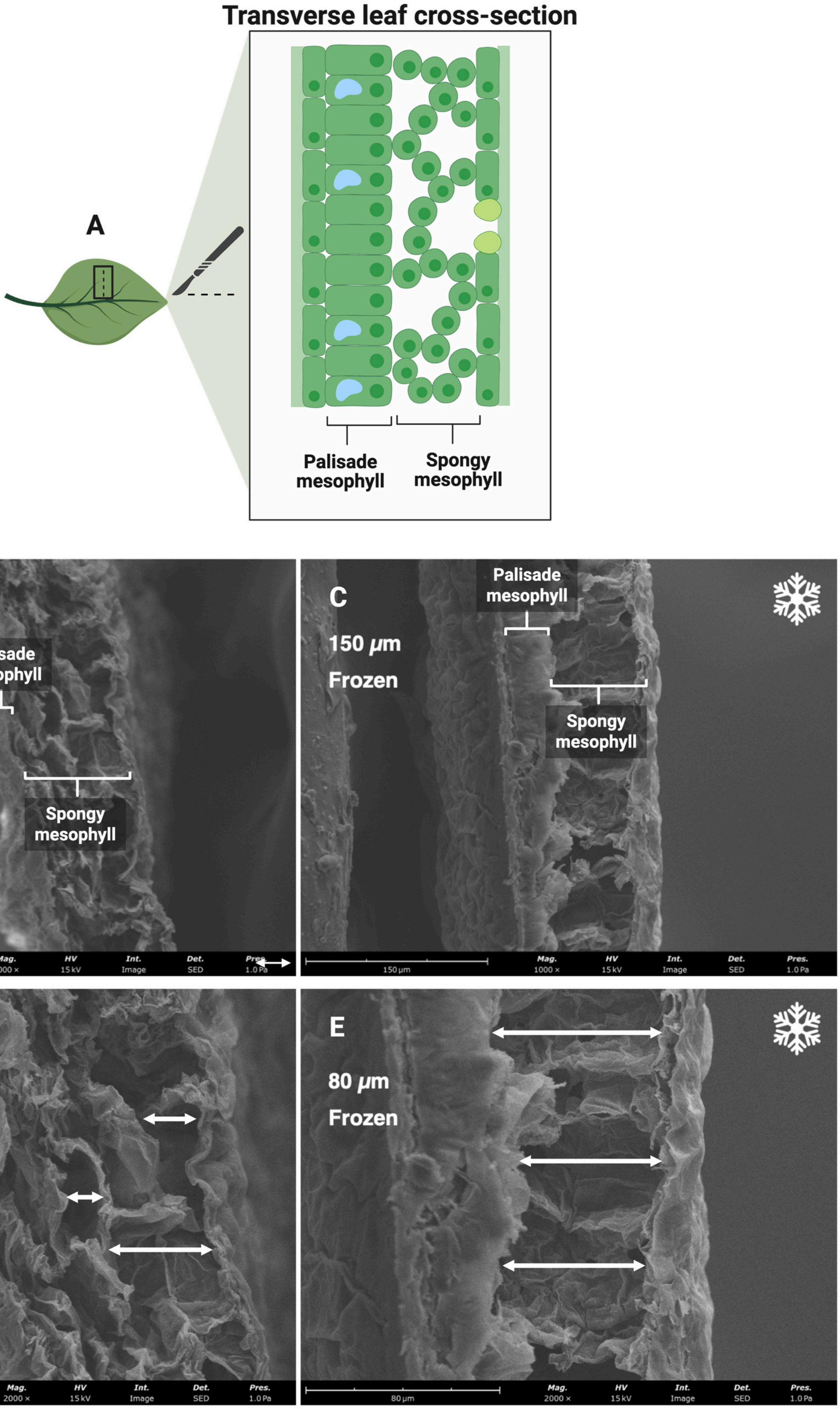

Fig. 4. Microstructure of watercress by-product leaves in fresh (micrographs B and D) and frozen (micrographs C and E) conditions, observed through scanning electron microscopy (SEM). Image A represents the transverse cross-section of the leaf and highlights the palisade and spongy mesophyll layers. The scale bars in the micrographs represent 150 μm (B and C) and 80 μm (D and E).

the food industry. Therefore, MHG's approach ensures the use of plant residue under a zero-waste strategy, which contributes to preserving biodiversity.

Several studies have been conducted to assess the effectiveness of microwaves in inactivating microorganisms in various food products. The results revealed no evidence of any pathogenic bacteria having unique resistance to microwave heating (Bozkurt-Cekmer & Davidson, 2017). Following this idea, the products obtained through MHG, including extracts and post-extraction residue, appear safe from a microbiological perspective and can be used in products intended for human consumption. Our investigation assessed the microbiological quality of watercress extracts and residue after MHG extraction (**Text 1S** and Table 3S, Supplementary Material). Our findings indicated the absence of pathogenic vegetative microorganisms in the residue, except mesophilic bacteria in extracts (at an acceptable level). According to Moraes et al. (2020), 10 min of microwave radiation can eliminate microorganisms. Based on this information, the extraction residue can be used in other foods or incorporated into a biorefinery system, aligning with the fourth principle of green extraction, which emphasises co-product production rather than waste (Chemat et al., 2019).

Overall, the MHG method offers time, energy, cost advantages, and microbiological quality of products. It is a promising alternative to conventional extraction methods and even the sustainable approaches mentioned before to obtain PEITC-enrich extracts.

4. Conclusion

For the first time, we demonstrated with an optimised approach that MHG can be a viable alternative for obtaining PEITC from watercress. Compared to conventional solid-liquid extraction methods for ITCs, this approach eliminates the need for organic solvents or extensive pre-treatment of plant material to ensure the production of ITCs. Our findings indicate that pre-freezing is fundamental in obtaining PEITC through MHG. Moreover, the freezing of watercress enhances the extraction of bioactives. The outcomes of this study suggest that using MHG to extract ITCs is a rapid, sustainable method that also supports a low-waste strategy by utilising the residue resulting from the extraction process.

Future work should focus on scaling up studies to adapt and optimise MHG extraction for the food industry. The research should focus on thoroughly understanding the functional properties, absorption, shelf-life, and safety of the bioactive compounds found in the extracts for potentially developing valuable products for human consumption. This study highlights the potential of exploring natural sources for PEITC as a substitute for synthetic alternatives, with potential applications in the food and nutraceutical industries. Additional refining steps could purify the compound for applications in pharmaceutical and cosmetic products.

Therefore, these findings lay the groundwork for future studies focused on improving extraction parameters to boost yields of specific bioactive compounds. Considering the health benefits of ITCs, optimising their extraction has broader implications for functional foods and therapeutic product development.

CRediT authorship contribution statement

Ana Sofia Sousa: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sérgio Sousa:** Methodology, Investigation, Formal analysis. **Inês V. Silva:** Methodology, Investigation, Formal analysis. **Celso A. Reis:** Writing – review & editing, Validation, Supervision. **Ezequiel R. Coscueta:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition. **Maria Manuela Pintado:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Grammarly in order to improve readability and language in the final edition. After using this tool/service, the authors reviewed and edited the content as needed and takefull responsibility for the content of the publication.

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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.

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Patents

Green method for obtaining isothiocyanates from cruciferous vegetables. World Intellectual Property Organization. Patent No. WO 2025/078970 A1. 17 April 2025.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.144551.

Data availability

Data shared in Appendix

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