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OPTIMISATION OF THE ENZYME-ASSISTED EXTRACTION OF POLYPHENOLS FROM SAFFRON (*CROCUS SATIVUS* L.) TEPALS

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ABSTRACT

Background. Saffron tepals are a by-product from the processing of *Crocus sativus* flowers, whose dried stigmas (saffron spice) are widely used both in the food and health sectors. Saffron tepals are rich in polyphenols, particularly flavonol glycosides and anthocyanins, which are considered to be potent antioxidants. Enzyme-assisted extraction of polyphenols offers several advantages in comparison to conventional methods. The present study evaluated the efficiency of enzyme-assisted extraction as a green technology for recovery of polyphenols from saffron tepals.

Materials and methods. The by-product was obtained from a saffron producer in the region of Kozani, Greece. A simplex-centroid design was applied to select the enzyme preparations mixture required for aqueous extraction of polyphenolic antioxidants from dried saffron tepals. In addition, the parameters of enzymatic hydrolysis, enzyme dose and incubation time were optimised using response surface methodology.

Results. The binary combination, comprising cellulolytic and hemicellulolytic preparations (1:1), led to the highest yield of total polyphenols (30.9 g GAE/kg saffron tepals) and total anthocyanins (2.0 g CGE/kg saffron tepals), reaching 45% and 38% higher values, respectively, as compared to the control sample (without enzymatic treatment). The experimental data regarding optimisation of the extraction conditions were fitted to second-degree regression models (R^2 = 0.85–0.98).

Conclusion. The newly developed process may be applied as an environmentally friendly alternative to conventional organic solvent extraction, thus supporting valorisation of the saffron industry by-product. The polyphenols recovered could be used as biologically active substances or as natural food ingredients, replacing synthetic additives.

Keywords: plant by-product, antioxidants, green technology, enzyme preparation

INTRODUCTION

Plant by-products originating from the agri-food industry have been demonstrated to contain large amounts of valuable compounds that could be recovered and used as biologically active substances or as natural food ingredients, replacing synthetic additives (Schieber, 2017).

Saffron, *Crocus sativus* L., is systematically cultivated in Greece, Iran, Spain, Morocco and other countries for the use of its dried stigmas (saffron spice) in foods both for their intensive flavouring and colouring properties (Carmona et al., 2007). In addition, stigmas

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have also been shown to possess pharmacological properties, including activity on the nervous and cardiovascular systems and antidepressant, anxiolytic and antineoplastic effects (Bagur et al., 2018; Moratalla-López et al., 2019).

Since approximately 150 000 flowers are needed to produce 1 kg of saffron spice (Goli et al., 2012), representing only 7% of the total weight of the flower (Serrano-Díaz et al., 2012), a large amount of by-product (mainly tepals) is generated. The presence of carbohydrates, proteins, dietary fiber and minerals (Serrano-Díaz et al., 2013), alkaloids and steroids (Kumar et al., 2011), carotenoid derivates (Goupy et al., 2013) and monoterpenoids (Li et al., 2004) has been found in the tepals of *Crocus sativus*. Moreover, saffron tepals are rich in polyphenols, particularly flavonol glycosides and anthocyanins, which are considered to be potent antioxidants (Termentzi and Kokkalou, 2008; Tuberoso et al., 2016).

Kaempferol glycosides are the major flavonols in *C. sativus* tepals, the main compound being kaempferol 3-*O*-sophoroside (Goupy et al., 2013; Lakka et al., 2019). Using LC-MS, Goupy et al. (2013) identified delphinidin 3,7-*O*-diglucoside as the main anthocyanin, while Lakka et al. (2019) reported delphinidin 3,5-di-*O*-glucoside as the major compound.

Besides organic solvent extraction of polyphenols and anthocyanins from saffron tepals (Da Porto and Natolino, 2018; Mahdavee Khazaei et al., 2016), several green technologies, such as subcritical water extraction (Ahmadian-Kouchaksaraie et al., 2016) and use of a deep eutectic solvent (Lakka et al., 2019), have been proposed. To date, to the best of our knowledge, there is only one study (Lotfi et al., 2015) on enzyme-assisted extraction dealing solely with the recovery of anthocyanins.

Enzyme-assisted extraction of polyphenols offers several advantages in comparison to conventional methods: no environmental hazards due to the usage of flammable or volatile solvents; mild reaction conditions; processes requiring fewer steps, while also potentially lowering production costs by replacing multiple installations (Gligor et al., 2019). Commercially available enzyme preparations contain multiple activities which act on the plant cell wall, with different enzyme mixtures optimised for different substrates (Gama et al., 2015).

As there are no tailor-made enzyme mixtures for saffron tepal processing, the present study investigated combinations of preparations which together would contain the enzymes required for cell wall degradation. In addition, the parameters of enzymatic hydrolysis, enzyme dose and incubation time were optimised for recovery of total polyphenols and total anthocyanins using response surface methodology.

MATERIALS AND METHODS

Plant material

Saffron (*Crocus sativus* L.) tepals, harvest year 2019, were obtained from a producer in the region of Kozani, Greece. The tepals were dried at room temperature (25–27°C) for one week before final hot air drying (60°C, 1 h). Dried saffron tepals were stored in plastic bags at room temperature until used. The moisture content of the dried tepals was 9.4% (w/w).

Chemicals

For analytical purposes, the following reagents were used: DPPH [2,2-diphenyl-1-picrylhydrazyl] and Trolox [(+/−)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid] (Sigma-Aldrich, Steinheim, Germany); TPTZ [2,4,6-tripyridyl-s-triazine] and gallic acid monohydrate (Fluka, Buchs, Switzerland); Folin-Ciocalteau's reagent (Merck, Darmstadt, Germany). All the other reagents and solvents used were of analytical grade.

Enzyme preparations

The following commercial enzyme preparations were used: pectinolytic preparation Pectinex Ultra Color and cellulolytic preparation Celluclast BG (both form Novozymes A/S, Bagsvaerd, Denmark); hemicellulolytic preparation Xylanase AN (Biovet JSC, Peshtera, Bulgaria).

Enzyme-assisted extraction

Finely ground (particle size <700 μm) saffron tepals were mixed with water (10:1, v/w), acidified (pH 4.0) with HCl, and left for 1 h for rehydration at 25°C. After pH adjustment (pH 4.0), the suspension (100.0 g) was placed in a 50°C water bath (W3, VEB MLW Prüfgeräte-Werk Medingen, Sitz Freital, Germany) for 20 min before 10 mL of an acidified water solution

of enzyme preparation was added. After incubation at 50°C, the sample was placed in a boiling water bath for 10 min to inactivate the enzymes, then immediately cooled in an ice bath and centrifuged (4200 g × 15 min, 25°C). The supernatant obtained was filtered through a paper filter and weighed to determine the extract yield.

Sample preparation

An aliquot (5 mL) of filtered extract was transferred into a 50 mL volumetric flask using 40 ml of acidified (0.1% HCl) methanol. After sitting for 24 h at 10°C, the flask was filled up to the mark with acidified methanol and filtered through a paper filter. Samples were prepared in triplicate.

Phytochemical analyses

All measurements were performed with a Helios Omega UV-Vis spectrophotometer equipped with VISION-*lite* software (all from Thermo Fisher Scientific Inc., Waltham, MA, USA) using 1 cm pathlength cuvettes.

The content of total polyphenols (TPP) was determined using the method of Singleton and Rossi (1965) in the following modification: an appropriately diluted sample solution (0.1 mL) was mixed with 0.5 mL of FC-reagent (diluted with distilled water 1:4, v/v) and 1.5 mL of sodium carbonate solution (7.5%, w/v) and the volume was made up to 10 mL with distilled water; the mixture was incubated for 2 h at room temperature before the absorbance was measured at 750 nm. The results were presented as equivalents of gallic acid (GAE).

The amount of total monomeric anthocyanins (TMA) was determined using the pH-differential method (Giusti and Wrolstad, 2001). The sample solution was diluted in parallel with buffer pH 1.0 (0.025 M potassium chloride) and buffer pH 4.5 (0.4 M sodium acetate). After 1 h of incubation at room temperature, the absorbance was measured at 520 and 700 nm. The results were calculated using a molar extinction coefficient of 26 900 L/mol·cm and molecular weight of 449.2 g/mol (Moyer et al., 2002) and expressed as equivalents of cyanidin 3-glucoside (CGE).

The total antioxidant capacity was determined using the DPPH (free radical scavenging activity) and FRAP (ferric reducing antioxidant power) assays. Trolox, a water-soluble vitamin E analogue, was used as a reference in both assays and the antioxidant capacity was expressed as equivalents of Trolox (TE).

DPPH assay was based on the method of Brand-Williams et al. (1995) modified as follows: 2250 µL of a DPPH methanolic solution (6×10^{-5} M) was mixed with 250 µL of sample solution (diluted with distilled water 1:3, v/v); absorbance was measured at 515 nm after 15 min of reaction in a cap-sealed cuvette kept in the dark at room temperature.

FRAP assay was performed according to Benzie and Strain (1996) with some modifications. The FRAP reagent was prepared by mixing 2.5 mL of a TPTZ solution (10 mmol/L) in hydrochloric acid (40 mmol/L), 2.5 mL of a $FeCl_3$ water solution (20 mmol/L) and 25 mL of an acetate buffer (0.3 mol/L, pH 3.6). In the assay, 2250 µL of FRAP reagent and 250 µL of sample solution (diluted with distilled water 1:3, v/v) were mixed in a cuvette and absorbance was measured at 593 nm after 4 min of reaction.

Experimental design

According to Kalcheva-Karadzhova et al. (2014), a simplex-centroid design for a mixture with three components was applied (Fig. 1). Enzyme preparations (single or mix) were used as 1.2% (v/v) solution and the incubation time was 120 min.

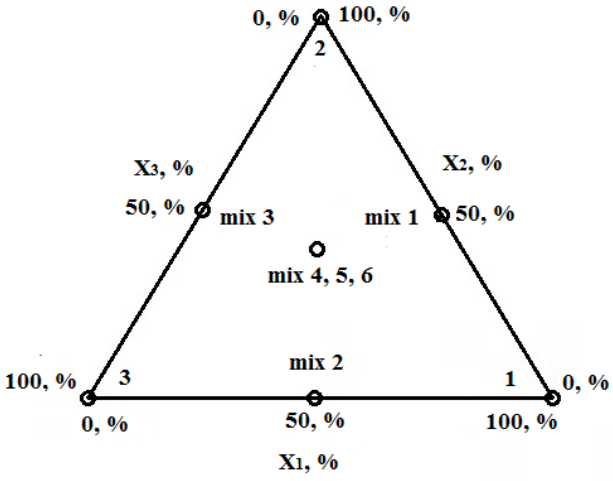

Fig. 1. Ternary diagram for the simplex-centroid design: 1 – 100% Pectinex Ultra Color (X_1), 2 – 100% Celluclast BG (X_2), 3 – 100% Xylanase AN (X_3), mix 1 – X_1:X_2 = 1:1; mix 2 – X_1:X_3 = 1:1; mix 3 – X_2:X_3 = 1:1; mix 4, 5, 6 – X_1:X_2:X_3 = 1:1:1

An optimal central composite design (OCCD) of type $2^n + 2n + n_0$ was applied. The influence of the independent variables was determined by means of the response surface methodology (Kalcheva-Karadzhova et al., 2016). Table 1 shows the levels of the two independent variables – enzyme dose (0.02–0.18%) and reaction time (30–210 min). The enzyme used was a 1:1 mixture of the cellulolytic and hemicellulolytic preparations. The experimental data were fitted to a second-degree regression equation:

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} b_{ij} x_i x_j \qquad (1)$$

where:

y – the dependent variable (response),
b_0 – the model intercept,
b_i, b_{ii}, b_{ij} – the linear, quadratic, and interaction regression coefficients, respectively,
x_i, x_j – the independent variables,
n – equal to the number of the tested factors ($n = 2$ in this study).

Table 1. Values of the independent variables at the corresponding levels

Factor	Minima	Center point	Maxima	Axial point
Enzyme dose X_1, %E/S*	0.02	0.1	0.18	$-\alpha = -1$
				$+\alpha = +1$
Time X_2, min	30	120	210	$-\alpha + = -1$
				$+\alpha = +1$

* Grams of enzyme mixture per 100 g of substrate (suspension).

Statistical analysis

The results reported in the present study are the mean values of at least three analytical determinations, and the coefficients of variation expressed as the percentage ratios between the standard deviations and the mean values were found to be <5% in all cases. The means were compared using one-way ANOVA and Tukey's test at a 95% confidence level.

RESULTS AND DISCUSSION

Selection of the mixture of enzyme preparations

Significant increases in the recovery rates of total polyphenols and total anthocyanins were observed due to the enzymatic treatments (Table 2, Fig. 2a and 2b).

The binary combination containing cellulolytic and hemicellulolytic preparations (X_2:X_3 = 1:1) resulted in the highest yield of total polyphenols, reaching a 45% higher value as compared to the control sample (without enzymatic treatment). This value (30.9 g GAE/kg saffron tepals) is higher than that (3.4 g GAE/kg) reported for methanolic extract (Goli et al., 2012), but lower than that (112.2 g GAE/kg) obtained for 60% ethanolic extract (Lakka et al., 2019). However, possible differences in the polyphenolic content of the raw materials should be taken into account.

The yield of total anthocyanins was 38% higher due to the enzymatic treatment. This value (2.0 g CGE/kg saffron tepals) is lower than that (6.7 g CGE/kg) reported by Lotfi et al. (2015), which could be attributed to the different raw materials and enzyme preparations used.

Consistent with Østby et al. (2020), these results confirm the co-operativity between cellulases and hemicellulases in the enzymatic hydrolysis of lignocellulosic cell wall material.

Interestingly, similar effects with respect to the total polyphenols and total anthocyanins were observed for the pectinolytic and cellulolytic preparations mixture (X_1:X_2 = 1:1), which is probably due to the secondary xylanase activity of commercial pectinase (Kalcheva-Karadzhova et al., 2014).

The two assays used represent different mechanisms for evaluating antioxidant capacity. While the DPPH assay measures the ability of plant extracts to scavenge free radicals, the FRAP assay quantifies the total concentration of redox-active compounds (Magalhães et al., 2008). In general, the changes in the total antioxidant capacity (Table 2, Fig. 2c and 2d) correspond to the results obtained for the total polyphenols.

Optimisation of the process parameters

Significant variations in the yields of total polyphenols and total anthocyanins were observed in response to the different enzymatic treatments (Table 3).

Table 2. Treatment variants and results[1] for the simplex-centroid design

Variant	Extract yield %	TPP[2] g GAE/kg	TMA[3] g CGE/kg	DPPH[4] µmol TE/kg	FRAP[4] µmol TE/kg
Control (no enzyme)	58.96	21.32 ±1.07a	1.45 ±0.07ab	35.89 ±1.80a	63.15 ±3.16ab
1	60.40	22.16 ±1.11ad	1.51 ±0.08a	41.83 ±2.09bc	62.05 ±3.10a
2	62.40	22.96 ±1.15ad	1.34 ±0.07b	40.40 ±2.02b	61.62 ±3.08a
3	69.52	26.83 ±1.34b	1.47 ±0.07ab	45.06 ±2.26c	73.72 ±3.69b
Mix 1	79.63	30.23 ±1.51ce	2.00 ±0.10c	51.06 ±2.56d	91.00 ±4.55c
Mix 2	61.78	24.20 ±1.21d	1.51 ±0.08a	40.26 ±2.02b	69.93 ±3.50b
Mix 3	79.56	30.90 ±1.55c	2.00 ±0.10c	53.45 ±2.66d	91.41 ±4.57c
Mix 4, 5, 6	77.73	28.30 ±1.42be	1.86 ±0.09c	43.33 ±2.17bc	89.30 ±4.47c

[1] Means ±standard deviation (n = 3). Different letters within a column indicate significant differences (Tukey's test, $P < 0.05$).
[2] Results are expressed as grams of gallic acid equivalents (GAE) per 1 kg saffron tepals (dry weight basis, dwb).
[3] Results are expressed as grams of cyaniding 3-glucoside equivalents (CGE) per 1 kg saffron tepals (dwb).
[4] Results are expressed as micromoles of Trolox equivalents (TE) per 1 kg saffron tepals (dwb).

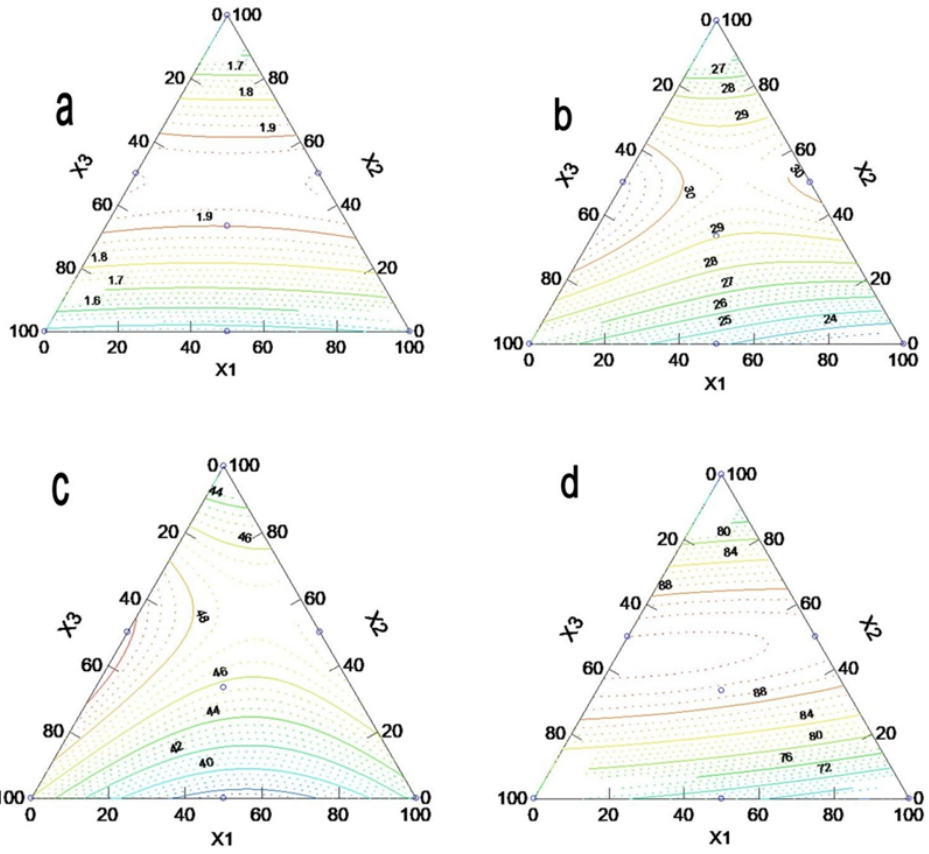

Fig. 2. Simplex contour plots for: a – TMA, b – TPP, c – DPPH, d – FRAP

Table 3. Experimental design matrix and results[1] for the optimal central composite design

No	Coded values		Enzyme dose %E/S[2]	Time min	TMA[3] g CGE/kg	TPP[4] g GAE/kg	DPPH[5] µmol TE/kg	FRAP[5] µmol TE/kg	Extract yield %
			X_1	X_2	Y_1	Y_2	Y_3	Y_4	Y_5
1	–	–	0.02	30	0.22 ± 0.01a	7.30 ± 0.37a	19.10 ± 0.96a	31.04 ± 1.55a	46.03
2	+	–	0.18	30	0.19 ± 0.01b	7.98 ± 0.40a	16.56 ± 0.83b	35.78 ± 1.79bf	47.67
3	–	+	0.02	210	0.23 ± 0.01a	8.52 ± 0.43a	22.13 ± 1.11c	38.41 ± 1.92cf	51.87
4	+	+	0.18	210	0.43 ± 0.02ci	24.96 ± 1.25b	25.20 ± 1.26de	45.22 ± 2.26d	59.64
5	–	0	0.02	120	0.31 ± 0.02d	20.28 ± 1.01c	22.18 ± 1.11c	34.67 ± 1.73b	49.79
6	+	0	0.18	120	0.38 ± 0.02eh	22.86 ± 1.14de	22.91 ± 1.15c	36.59 ± 1.83bg	55.49
7	0	–	0.1	30	0.34 ± 0.02f	21.48 ± 1.07cd	25.38 ± 1.27de	38.41 ± 1.92cfg	50.67
8	0	+	0.1	210	0.40 ± 0.02e	24.46 ± 1.22b	26.67 ± 1.33d	39.62 ± 1.98ce	56.29
9	0	0	0.1	120	0.47 ± 0.02g	22.40 ± 1.12de	26.72 ± 1.34d	41.90 ± 2.10eh	53.91
10	0	0	0.1	120	0.45 ± 0.02gi	23.90 ± 1.20be	26.55 ± 1.33d	43.70 ± 2.19dh	54.23
11	0	0	0.1	120	0.37 ± 0.02h	21.35 ± 1.07cd	23.79 ± 1.19ce	34.13 ± 1.71b	57.39

[1] Means ±standard deviations (n = 3). Different letters within a column indicate significant differences (Tukey's test, $P < 0.05$).
[2] Grams of enzyme mixture per 100 g substrate (suspension).
[3] Results are expressed as grams of cyaniding 3-glucoside equivalents (CGE) per 1 kg saffron tepals (dry weight basis, dwb).
[4] Results are expressed as grams of gallic acid equivalents (GAE) per 1 kg saffron tepals (dwb).
[5] Results are expressed as micromoles of Trolox equivalents (TE) per 1 kg saffron tepals (dwb).

In accordance with Lotfi et al. (2015), with an increase in the enzyme dose, the recovery rate of total anthocyanins increased until the middle of the experiment and afterwards started to decline. The negative effects of the higher enzyme dose indicate that the preparations might possess secondary enzyme activities that catalyse the degradation of anthocyanins. Indeed, the presence of some glycosidases would cause deglycosylation of the anthocyanidin glycosides, which in turn results in unstable aglycons (Wrolstad et al., 1994).

The positive effects of incubation time on the total polyphenol yield imply that extensive enzymatic cell wall digestion promotes releasing of insoluble-bound phenolic compounds (Wang et al., 2017).

The experimental data in Table 3 were used to determine the coefficients of four second-order polynomial equations as follows:

$$Y_1 = 0.117 + 2.93X_1 + 0.00225X_2 - 16.9X_1^2 + 0.00799X_1X_2 - 0.0000103X_2^2, \text{ g CGE/kg}, \quad R^2 = 0.98 \quad (2)$$

$$Y_2 = 3.88 + 176.1X_1 + 0.133X_2 - 1003.5X_1^2 + 0.547X_1X_2 - 0.00062X_2^2, \text{ g GAE/kg}, \quad R^2 = 0.85 \quad (3)$$

$$Y_3 = 16.5 + 126.6X_1 + 0.0411X_2 - 736.6X_1^2 + 0.195X_1X_2 - 0.000152X_2^2, \text{ g TE/kg}, \quad R^2 = 0.93 \quad (4)$$

$$Y_4 = 26.4 + 197.4X_1 + 0.0434X_2 - 861.5X_1^2 - 0.0226X_1X_2 - 0.0000233X_2^2, \text{ g TE/kg}, \quad R^2 = 0.94 \quad (5)$$

where:

Y_1, Y_2, Y_3, Y_4 – the predicted responses for TMA, TPP, DPPH and FRAP, respectively,

X_1 – the enzyme dose,

X_2 – the incubation time.

All of the R^2 (coefficient of determination) values were greater than 0.80, implying that the models accurately represent the experimental data (Iglesias-Carres et al., 2018).

Both the incubation time and enzyme dose produced positive linear and negative quadratic effects on total

anthocyanins and total polyphenols. This means that the yields of total anthocyanins (Fig. 3a) and total polyphenols (Fig. 3b) increase when the incubation time or enzyme dose increases up to a certain point, after which they begin to decrease. Positive linear and negative quadratic effects of incubation time were also reported for total polyphenols and total anthocyanins in extracts from rose petals (Kalcheva-Karadzhova et al., 2016).

Positive linear effects of incubation time and negative quadratic effects of enzyme dose were obtained for the total antioxidant capacity values (Fig. 3c–3d), suggesting similar changes to those observed for the total polyphenols.

A graphical optimisation of the extraction conditions was carried out in order to maximise the yields of total polyphenols and total anthocyanins. Figure 4 shows the overlapping region, defining the intervals of variation of the enzyme mixture dose (0.12–0.15%) and treatment time (145–185 min) that satisfy the optimisation criterion.

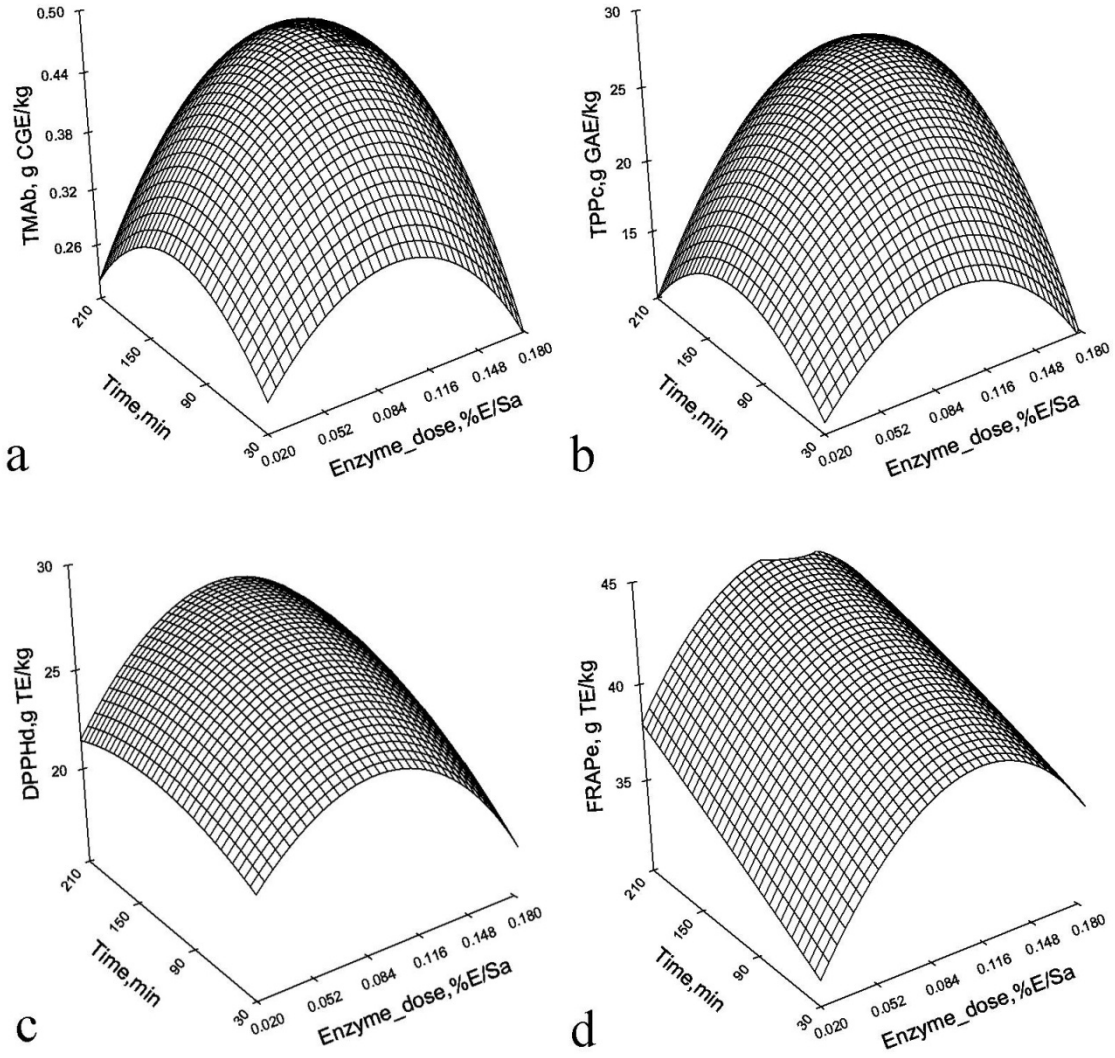

Fig. 3. Response surfaces showing the effects of enzyme mixture dose, %E/Sa – grams of enzyme mixture per 100 g substrate, and incubation time, min, on: a – TMA, b – TPP, c – DPPH, d – FRAP

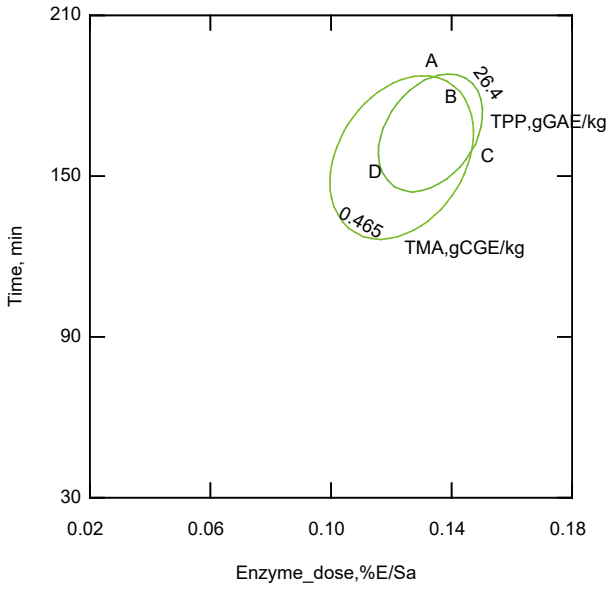

Fig. 4. Graphical optimisation of the extraction conditions – enzyme mixture dose, %E/Sa – grams of enzyme mixture per 100 g substrate, and incubation time, min

CONCLUSION

The results obtained demonstrate that the enzyme-assisted extraction enhances the recovery of polyphenolic antioxidants from saffron tepals, especially using the binary enzyme combination comprising cellulolytic and hemicellulolytic preparations (1:1). The intervals of variation of the enzyme mixture dose (0.12–0.15%) and incubation time (145–185 min) define the optimal region for obtaining extracts with high yields of both total polyphenols and total anthocyanins. This new process can be considered to be a green technology, offering an environmentally friendly alternative to conventional organic solvent extraction.

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