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Improvement of Carrot Accelerated Solvent Extraction Efficacy Using Experimental Design and Chemometric Techniques

Vesna Tumbas Šaponjac [1], Strahinja Kovačević [1], Vanja Šeregelj [1], Olja Šovljanski [1], Anamarija Mandić [2], Gordana Ćetković [1], Jelena Vulić [1], Sanja Podunavac-Kuzmanović [1] and Jasna Čanadanović-Brunet [1,*]

[1] Faculty of Technology Novi Sad, University of Novi Sad, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia; vesnat@uns.ac.rs (V.T.Š.); strahko@uns.ac.rs (S.K.); vanjaseregelj@tf.uns.ac.rs (V.Š.); oljasovljanski@uns.ac.rs (O.Š.); cetkovic@tf.uns.ac.rs (G.Ć.); jvulic@uns.ac.rs (J.V.); sanya.podunavac@uns.ac.rs (S.P.-K.)

[2] Institute of Food Technology, University of Novi Sad, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia; anamarija.mandic@fins.uns.ac.rs

* Correspondence: jasnab@uns.ac.rs

Abstract: Human studies have demonstrated the multiple health benefits of fruits and vegetables. Due to its high fiber, mineral and antioxidant content, carrot is an ideal source for the development of nutraceuticals or functional ingredients. Current research assesses accelerated solvent extraction (ASE) traits which affect the antioxidant qualities of carrot extract using response surface methodology (RSM), hierarchical cluster analysis (HCA), and the sum of ranking differences (SRD). A mixture of organic solvents, acetone, and ethanol with or without the addition of 20% water was applied. The total carotenoid and polyphenol contents in extracts, as well as their scavenging activity and reducing power, were used as responses for the optimization of ASE extraction. RSM optimization, in the case of 20% water involvement, included 49% of acetone and 31% of ethanol (*Opt1*), while in the case of pure organic solvents, pure ethanol was the best choice (*Opt2*). The results of HCA clearly pointed out significant differences between the properties of extracts with or without water. SRD analysis confirmed ethanol to be optimal as well. RSM, HCA, and SRD analysis confirmed the same conclusion—water in the solvent mixture can significantly affect the extraction efficacy, and the optimal solvent for extracting antioxidants from carrot by ASE is pure ethanol.

Keywords: extraction; carotenoids; polyphenols; antioxidant activity; response surface methodology; hierarchical cluster analysis; sum of ranking differences

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1. Introduction

Epidemiological studies have suggested that a diet rich in bioactive compounds such as phenolics and carotenoids may have protective effects against various degenerative diseases, including cancers and cardiovascular diseases [1,2]. Most preventive effects of these bioactives are associated with their antioxidant activity and protection of the cell biomolecules against oxidative damage caused by various free radicals [3,4]. Fruits and vegetables are an important part of human diet and valuable sources of bioactive health-promoting substances [5,6]. Among them, carrot (*Daucus carota* L.) belongs to horticultural crops of importance due to its nutritional value and high content of bioactive compounds [6]. Carrot is rich in bioactive antioxidants of a lipophilic (carotenoids) and hydrophilic (phenolic compounds) nature [6,7]. These antioxidants have a special significance nowadays because of the potential for their application in the development of new functional and nutraceutical products [8]. The recommended level of β-carotene in a dietary allowance for a typical adult is 4.8 mg. Therefore, it is worthwhile to search for an appropriate means to extract β-carotene from plant sources [9].

The use of bioactive compounds in different sectors, such as the pharmaceutical, food, and chemical industries, has prompted a continuing search for the most appropriate and

efficient method to extract these active components from plant materials [10]. Conventional extraction methods of these compounds using solid/liquid extraction techniques at atmospheric pressures require large quantities of solvents with low selectivity and/or low extraction yields and long extraction times, and can result in extract exposition to excessive heat, light, and oxygen [11,12].

In recent years, accelerated solvent extraction (ASE) has been developed for extracting phytochemicals from various fruit and vegetable samples. ASE is a fully automated technique that uses common solvents or solvent mixtures at elevated temperatures and pressure to rapidly extract target compounds from solid and semisolid samples. High temperature leads to better solubility of the target compound, faster diffusion rates, lower solvent viscosities, and weakening of the solute–matrix interactions [13]. High pressure is capable of ensuring that the extraction cell is filled quickly and helps to force liquid into the sample pores and to keep the solvent liquid at operating temperatures. ASE method has become popular due to its feasibility, speed, and ability to process samples automatically using different solvents, pressures, and temperatures under nitrogen. Rapid extraction in an oxygen- and light-free environment minimizes phytochemical degradation [11,12].

Response surface methodology (RSM) has been a very popular technique for the optimization of solid–liquid extraction in recent years [14]. During solid–liquid extraction, several factors such as the solvent composition, temperature, time, pressure, particle size, and solvent to plant material ratio can influence the extraction process of bioactive compounds from plant materials [11]. While many factors and interactions may affect a desired process, RSM is an effective statistical technique to find a combination of factor levels that produce the optimal conditions [14,15]. This approach allows the prediction of the influence of the factors on the chosen response using a mathematical model [13]. The main aim of the application of chemometric methods (hierarchical cluster analysis (HCA) and sum of ranking differences approach (SRD) lies in the fact that these methods can reveal some potentially hidden features of the raw experimental data and present it in a clear way (numerically or graphically). In the present study, the main purpose of HCA and SRD analysis is to classify the conducted experiments, rank and group similar experiments, and gain an overview of the optimum experimental conditions that will result in extracts with desirable properties.

In this view, the aim of this study was to optimize the solvent composition for ASE extraction of carrot, planning the experiments with a simplex lattice experimental design. Two statistical methods were used for optimization: response surface methodology (RSM) and sum of ranking differences (SRD) analysis. The optimization was performed in terms of maximizing total carotenoid and polyphenol contents, as well as the radical scavenging activity and reducing power of the obtained carrot extracts.

2. Materials and Methods

2.1. Plant Material

Fresh carrots (*Daucus carota* L.) were purchased from a local supermarket. After washing, the fresh carrots were chopped in a kitchen blender (B 800 E, Gorenje, Slovenia), freeze-dried at −40 °C (Martin Crist Alpha 2–4, Osterode, Germany), ground, and stored at −20 °C until use.

2.2. Accelerated Solvent Extraction Procedure

A Dionex ASE 350 (Thermo Scientific, Waltham, MA, USA) system was used for the extraction of dried carrot samples using solvent mixtures guided by the simplex lattice experimental design. For this purpose, a stainless-steel Dionex cell was filled with diatomaceous earth (to reduce the volume of the extraction solvent). In our preliminary studies, the optimum diatomaceous earth:carrot sample ratio, temperature and extraction time were found to be 4:1, 80 °C and 5 min, respectively (data not shown), and they were applied as such in this study. To prevent the collection of suspended particles in the extract, a cellulose filter was placed at the bottom of the cell. Finally, the cell was placed in the cell

tray and used to extract the carrot sample. In all experimental runs, extraction pressure was constant at 10.34 MPa. The glass vials were used to collect the extracts, which were stored at −20 °C in darkness until further use.

2.3. Total Carotenoid Contents (TCar)

The content of carotenoids in carrot extracts was analyzed spectrophotometrically using the method of Nagata and Yamashita [16], using extracting solvent as the blank. The content of total carotenoids was calculated using the equation: TCar (mg β-carotene/100 mL) = 0.216A663 − 1.22A645 − 0.304A505 + 0.452A453, where A663, A645, A505, and A452 represent the absorbances measured at 663, 645, 505, and 453 nm, respectively. The total carotenoid content was expressed as mg of β-carotene equivalents per 100 g of dried carrot.

2.4. Total Polyphenol Content (TPh)

The total polyphenol contents in carrot extracts were determined spectrophotometrically by means of Folin–Ciocalteau method adapted to the microscale [17]. The results were expressed as gallic acid equivalents (GAE) per 100 g dried carrot.

2.5. Radical Scavenging Activity (SA) by DPPH• Assay

The free radical scavenging effect of carrot extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was measured spectrophotometrically in a 96-well micro-plate reader, according to Girones-Vilaplana et al. [18]. SA values were calculated using the following equation: SA (%) = [(AC − AS)/AC] × 100, where AC is the absorbance of control, and AS is the absorbance in the presence of extracts. Results were also expressed as μmol Trolox equivalents (TE) per 100 g of dried carrot.

2.6. Reducing Power (RP)

Reducing power was determined using the method of Oyaizu [19] adapted for a 96-well microplate. The calibration curve was made with Trolox, and results were expressed as μmol of Trolox equivalents (TE) per 100 g of dried carrot.

2.7. Experimental Design and Response Surface Methodology (RSM)

The composition of extracting solvent mixture was defined by adopting the simplex lattice experimental design for two variables at five levels. Independent variables were the volume fractions of acetone and ethanol (experiment 9–16), or their 80% water solutions (experiment 1–8), in extracting solvent, where their values were 0, 0.25, 0.5, 0.75, and 1. The complete design is presented in Table 1. Two augmented 2-component simplex lattice designs, with three replicated mixtures each, were applied. For the first experiment, the components of the extracting solvent were ethanol and acetone in up to an 80% water solution (levels 0.0–0.8, a in Table 1), while for the second experiment, ethanol and acetone were mixed without water (levels 0.0–1.0, b in Table 1). The response variables in both trials were total carotenoid and polyphenol contents, radical scavenging activity, and reducing power.

RSM analyses were executed using Design-expert 7 software (Stat-Ease, Inc., Minneapolis, MN, USA). Significant differences were calculated by means of ANOVA ($p < 0.05$) using the Origin 7.0 SRO software package (OriginLab Corporation, Northampton, MA, USA, 1991–2002).

Table 1. The calculated physicochemical properties solvent mixtures used in the SRD analysis of extraction experiments.

Experiment	Solvent Volume Fraction (*v*/*v*)			ε_m	RP_m	ρ_m (g/dm^3)	P'_m
	Ethanol	Acetone	Water				
(a) Organic solvent and water mixtures							
1	0.8	0	0.2	82.77	0.72	772.91	5.96
2	0	0.8	0.2	24.66	0.48	769.01	6.12
3	0	0.8	0.2	24.66	0.48	769.01	6.12
4	0.2	0.6	0.2	39.19	0.54	769.99	6.08
5	0.4	0.4	0.2	53.72	0.60	770.96	6.04
6	0.6	0.2	0.2	68.24	0.66	771.94	6.00
7	0.8	0	0.2	82.77	0.72	772.91	5.96
8	0.4	0.4	0.2	53.72	0.60	770.96	6.04
Opt1	0.31	0.49	0.2	47.18	0.58	770.53	6.06
Experiment	**Solvent Volume Fraction (*v*/*v*)**			ε_m	RP_m	ρ_m (g/dm^3)	P'_m
	Ethanol	**Acetone**	**Water**				
(b) Organic solvent mixtures							
9	1	0	0	88.21	0.65	725.63	5.20
10	0	1	0	15.57	0.36	720.76	5.40
11	0	1	0	15.57	0.36	720.76	5.40
12	0.25	0.75	0	33.73	0.43	721.98	5.35
13	0.5	0.5	0	51.89	0.50	723.20	5.30
14	0.75	0.25	0	70.05	0.58	724.42	5.25
15	1	0	0	88.21	0.65	725.63	5.20
16	0.5	0.5	0	51.89	0.50	723.20	5.30
Opt2	1	0	0	88.21	0.654	725.634	5.20

The calculation of dielectric constant of the solvent mixture (εm), mixture relative polarity (RPm), mixture density (ρm), and mixture polarity index ($P'm$), of the solvent mixtures is presented in Supplementary data.

2.8. Hierarchical Cluster Analysis (HCA) and Sum of Ranking Differences (SRD)

Hierarchical cluster analysis [20] is a simple chemometric method for clustering similar objects in groups on the basis of the analyzed variables. In the present study, the experiments were classified on the basis of the calculated dielectric constant of the solvent mixture (εm), mixture relative polarity (RPm), mixture density (ρm), mixture polarity index (P′m), total carotenoid content (TCar, mg/100 g), total polyphenol content (TPh, mg/100 g), the scavenging activity (SA, µmol TE/100 g), and reducing power (RP, µmol TE/100 g) of the carrot extracts. The clustering was carried out based on the non-standardized Euclidean distances as a distance metric and Ward's method as an amalgamation (joining) rule by using the Statistica 10 software.

The SRD analysis is a non-parametric chemometric approach introduced by Heberger and Kollar-Hunek [21,22]. It has become a very popular chemometric tool applicable in the ranking of mathematical models [23], food samples [24], stationary phases in chromatographic analysis [25], lipophilicity assessment [26]. In order to carry out SRD analysis, it is necessary to organize the data into matrix form, so the objects to be ranked (samples, models, experiments) are organized into columns and their features into rows. The ranking is performed in comparison with an established reference ranking or so-called "golden standard". The evaluation of SRD results depends on the established reference ranking, so the objects placed close to it could be considered favorable or not. In the present study, the SRD analysis of 16 extraction experiments, including optimum experiments defined by the RSM approach (optimum solvent, *Opt1*, and optimum ternary solvent mixture composition, *Opt2*) was carried out on the basis of the εm, RPm, ρm, P′m, TCar, TPh, SA, and RP. The reference ranking was based on average row values for εm, RPm, ρm, P′m, and maximum row values for TCar, TPh, SA, and RP. In order to scale the data in the range 0.01–0.99, the min-max normalization method was applied [27]. The normalized data and

the input matrix used in the SRD analysis are presented in the Supplementary Materials (Tables S1 and S2).

3. Results and Discussion

3.1. RSM Analysis of the Extraction Experiments

In the process of scaling-up, the careful optimization of the isolation of bioactive compounds from plant material is required, in order to save valuable material, time, and effort. For the conventional optimization of extraction, trial and error or the single factor variable method are commonly used to satisfy several performance requirements. In this study, the optimization of extraction solvent composition was carried out.

Usually, the selection of extraction solvent is performed depending on the polarity of the target compounds, but in some cases, solvent mixtures of polar and nonpolar solvents provide higher recoveries. [28]. Many organic solvents were used for the isolation of carotenoids, sensitive natural colorants. Hue et al. [29] isolated and purified the yellow fraction of sweet potatoes by extracting it with acetone, methanol, tetrahydrofuran, and petroleum ether:tetrahydrofuran mixture (4:1), out of which acetone was the most effective at removing a mixture of β-carotene and lutein. Three organic solvents, ethyl acetate, petroleum ether, and hexane/acetone (1:1 *v*/*v*), were used for the extraction of β-carotene from orange peel and carrot waste [30]. The highest yield was achieved with petroleum ether, whereas the other two solvents were nearly as good. Šeregelj et al. [31] used four organic solvents, ethanol, acetone, ethyl acetate, and hexane, for the extraction of carotenoids from carrots. The highest yields were achieved with hexane at a solid to solvent ratio 1:10 *w/v* and with acetone at a solid to solvent ratio 1:5 *w*/*v*. Badr [32] used a mixture of isoproanol:petrol ether, while Ting and Hendrickson [33] used acetone followed by hexane for the extraction of carotenoids from oranges. Weissenberg et al. [34] reported that diethyl ether and methanol were used sequentially as extraction solvents for carotenoid extraction from sour orange. In the study of El-Sharnouby et al. [35], acetone, petroleum ether, hexane, 90% ethanol, ethyl acetate, and methylen chloride were used for the extraction of carotenoids from sour orange and grape, where ethyl acetate was the most efficient extracting solvent. Cerón-García et al. [36] reported that the most suitable options for carotenoid recovery are the use of hexane, acetone, and ethanol, since they are already employed in the food industry. Based on "green" principles, ethanol and acetone are the preferred solvents, compared to hexane, diethyl ether, dichloromethane, and chloroform, which are commonly used for the extraction of carotenoids [37,38]. Our preliminary tests showed that for ASE as an extraction technique, acetone and ethanol were the best extracting solvents for carotenoids from carrot, employing previously optimized parameters (data not shown). Moreover, there are also polar carotenoids that these solvents are not capable of recovering, making it necessary to use a mixture of solvents with different polarity, including water. According to Zaghdoudi et al. [39], ASE at 40 °C, using methanol/tetrahydrofuran (2:8, *v*/*v*), 103 bar pressure, and 5 min of extraction time, were defined as the optimum conditions to extract the carotenoids from lyophilized apricot (Prunus armeniaca), peach (Prunus persica L.), and Tunisian Kaki (Diospyros kaki) fruits, with an average extraction yield of 87%. The maximum extraction efficiency of lutein and β-carotene from the orange carrot was achieved using a mixture of acetone/ethanol/hexane (1:2:3, *v*/*v*/*v*), compared to acetone/hexane (3:5, *v*/*v*), ethanol/hexane (4:3, *v*/*v*), and acetone/ethanol/hexane (3:2:1, *v*/*v*/*v*) [40]. Jaime et al. [41] found that the ASE extraction yield of carotenoids was higher with the polar organic solvent ethanol, when compared to hexane.

The mixture of pure organic solvents, acetone, and ethanol, with or without the addition of 20% water, was applied at pre-designed volume fractions following a simplex lattice experimental design. Total carotenoid and polyphenol contents in extracts, as well as their scavenging activity and reducing power, presented in Table 2, were used as responses for the optimization of ASE extraction.

Table 2. Total carotenoid (TCar) and polyphenol (TPh) contents in carrot extracts and their scavenging activity (SA) and reducing power (RP).

Experiment	TCar (mg/100 g)	TPh (mg/100 g)	SA (μmol TE/100 g)	RP (μmol TE/100 g)
1	3 ± 0^{a}	$131 \pm 5^{f,g,h}$	980 ± 37^{e}	205 ± 8^{i}
2	17 ± 1^{d}	$123 \pm 5^{f,g}$	710 ± 30^{c}	149 ± 6^{f}
3	$13 \pm 1^{c,d}$	117 ± 5^{f}	514 ± 21^{a}	$170 \pm 7^{f,g}$
4	8 ± 0^{b}	$127 \pm 6^{f,h}$	$575 \pm 23^{a,b}$	$183 \pm 7^{g,h}$
5	5 ± 0^{a}	$133 \pm 6^{g,h}$	671 ± 29^{b}	$189 \pm 6^{g,h}$
6	3 ± 0^{a}	142 ± 7^{h}	$783 \pm 34^{c,d}$	$185 \pm 8^{g,h}$
7	3 ± 0^{a}	121 ± 6^{f}	$943 \pm 46^{d,e}$	$190 \pm 9^{h,i}$
8	5 ± 0^{a}	141 ± 6^{h}	$776 \pm 30^{c,d}$	176 ± 7^{g}
9	21 ± 1^{e}	$115 \pm 5^{e,f}$	1920 ± 86^{g}	171 ± 6^{g}
10	14 ± 1^{d}	56 ± 2^{a}	720 ± 29^{c}	$55 \pm 2^{a,b}$
11	12 ± 1^{c}	77 ± 3^{b}	1330 ± 55^{f}	67 ± 2^{b}
12	$23 \pm 1^{e,f}$	59 ± 2^{a}	1020 ± 42^{e}	108 ± 4^{c}
13	23 ± 1^{e}	$83 \pm 3^{b,c,d}$	824 ± 37^{d}	41 ± 1^{a}
14	27 ± 2^{f}	$94 \pm 4^{c,d}$	888 ± 37^{d}	$155 \pm 6^{e,f}$
15	22 ± 1^{e}	$101 \pm 5^{d,e}$	1960 ± 79^{g}	142 ± 7^{e}
16	25 ± 1^{f}	$96 \pm 4^{c,d}$	$759 \pm 26^{b,c,d}$	126 ± 4^{d}

Results are expressed as mean values of three independent experiments ± SD; means with different letters in columns are significantly different ($p < 0.05$).

In this study, as expected, taking into account the polarity of carotenoids, the addition of water impaired carotenoid isolation from carrot, resulting in lower carotenoid contents in experiments 1–8 (2.797–16.902 mg/100 g), i.e., extracts with addition of 20% water, than in experiments 9–16 (11.574–26.649 mg/100 g), i.e., extracts with pure organic solvents. In the study of Saha et al. [40], the ethanol:hexane:acetone (2:3:1 *v*/*v*/*v*) solvent system recorded the maximum extraction of lutein and β-carotene from orange carrot, which ranged between 14.11 and 21.38 mg/100 g and 78.06 and 114.82 mg/100 g, respectively, using ASE, optimizing solvent mixture composition, extraction temperature (40, 50, and 60 °C), and static time (5, 10, and 15 min). Behsnilian and Mayer-Miebach [42] reported 156.3 mg/100 g of carotenoids (all-trans-β-carotene, cis-β-carotene, all-trans-lycopene, cis-lycopene, α-carotene and lutein) being extracted with acetone from frozen samples of Nutri Red carrot. When the freeze-dried carrots were extracted with pure ethanol, the total carotenoid contents were found to be 85 mg/100 g [43]. Šeregelj et al. [44], by optimizing extraction time (20–60 min), temperature (40–120 °C), and extraction cycles number (one to three) using pure ethanol as a solvent, found that the longest extraction (60 min) at medium temperature (80 °C) and in three cycles ensures the maximum yield of carotenoids (30.57 mg/100 g). It has been shown that carotenoid contents in carrot roots are modulated depending on growing locations [45] and water supply [46,47]. Contrary to carotenoids, the extraction of polyphenols was more efficient when water was included as a solvent (116.903–141.740 mg/100 g) compared to pure organic solvents mixtures (55.856–114.565 mg/100 g). Slightly higher values of total polyphenols extracted from freeze-dried carrots by ethanol (152 mg/100 g) were reported by Lee et al. [43], while Han et al. [48] extracted 23.31–23.53 mg/100 g soluble phenolics from wounded carrots (cut into slices, pies, and shreds). The scavenging activities of carrot ASE extracts were higher without water addition (720.328–1956.400 μmol TE/100 g), while for reducing power, organic solvents with water were favorable extracting solutions (148.698–205.297 μmol TE/100 g). Surjadinata and Cisneros-Zevallos [49] reported obtaining 150–235 μg Trolox/g carrot fresh weight using the DPPH assay when the samples were extracted with methanol using an Ultra-Turrax homogenizer. The same type of extraction using different parts of carrots (whole, shreds or inner tissue) yielded antioxidant activity in the range 232.2–436.1 μg/ 100 g fresh weight [50].

Employing response surface analysis/mixture design, the dependence of TCar and TPh on solvent composition, in the case of organic solvents with and without water addition, is presented in Figure 1a,b, respectively.

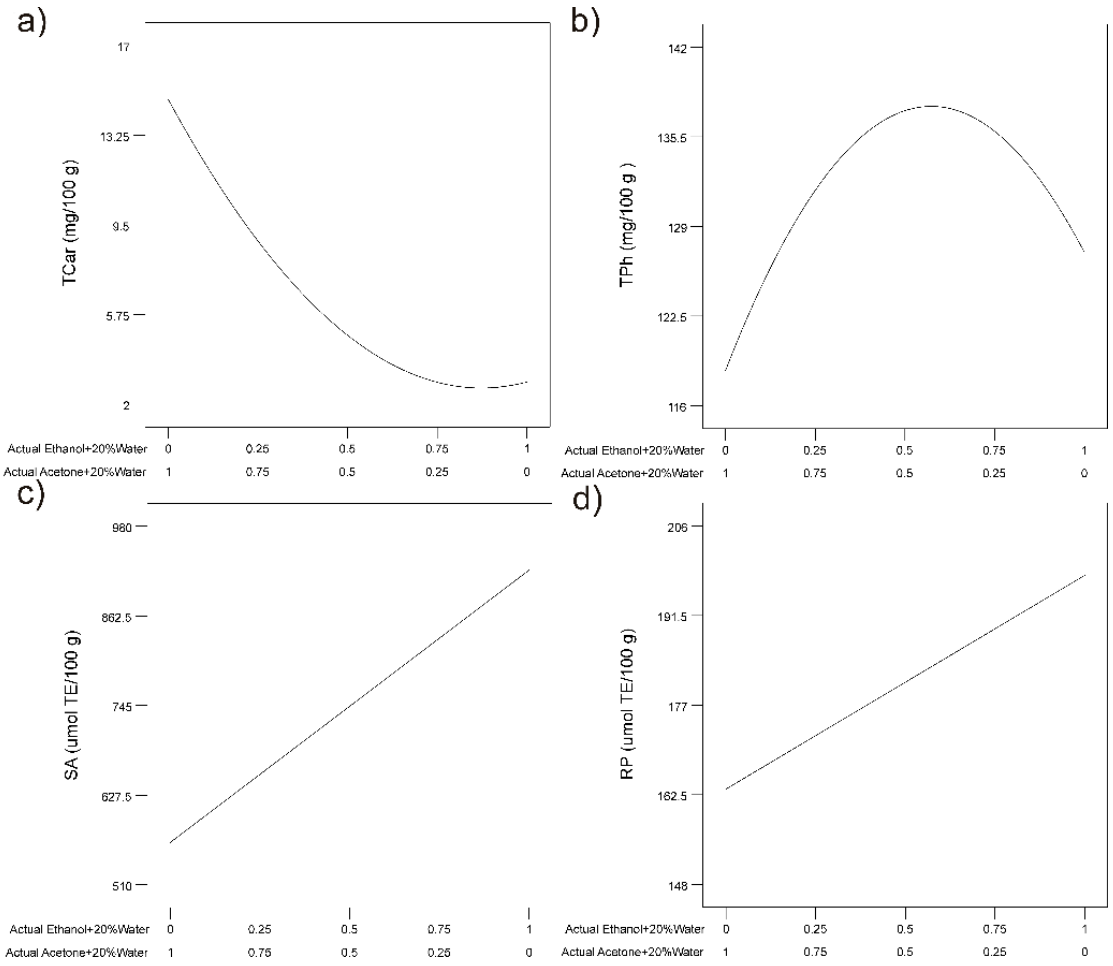

Figure 1. Total carotenoid (**a**) and polyphenol contents (**b**), scavenging activity (**c**), and reducing power (**d**) in ASE extracts obtained with acetone/ethanol mixtures with 20% of water.

For the isolation of carotenoids, from Figures 1a and 2a, it can be concluded that addition of water favors acetone as the organic solvent in the extracting mixture, while the sole organic solvents mixture favors an increase in the ethanol volume fraction, but only up to a point, after which the trend becomes inverted. On the other hand, polyphenols prefer equal amounts of ethanol and acetone in a mixture with water (Figures 1b and 2b), while scavenging activity and reducing power are enhanced by excluding acetone from the mixture, with or without water. The obtained results were used for single- and multi-response optimization, with the intention being to define the optimal solvent for each parameter (TCar, TPh, SA, RP) based on the individual as well as mutual response.

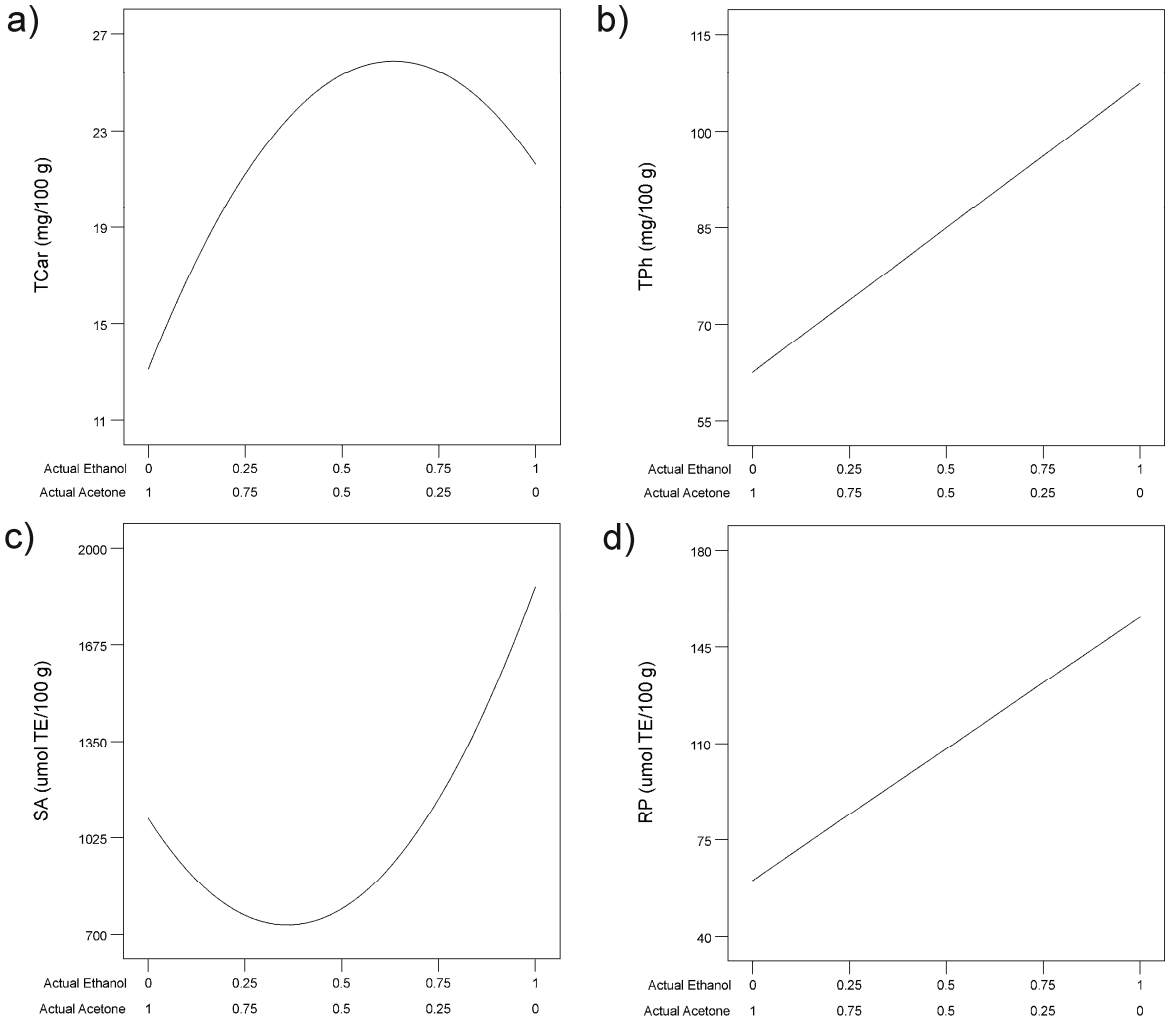

Figure 2. Total carotenoid (**a**) and polyphenol contents (**b**), scavenging activity (**c**), and reducing power (**d**) in ASE extracts obtained with acetone/ethanol mixtures.

The results of the optimization (Table 3) show that the mixture containing 63.4% ethanol and 36.6% acetone could isolate the highest amount of carotenoids (25.8774 mg/100 g), while the mixture with 34% acetone, 46% ethanol, and 20% water potentially enables the highest yield of polyphenols (137.708 mg/100 g). For the highest scavenging activity (1870.27 µmol TE/100 g) and reducing power (197.982 µmol TE/100 g), the involvement of acetone was excluded, i.e., pure ethanol and 80% ethanol were the most desirable, respectively. Multi-response optimization, in the case of 20% water involvement, included 49% acetone and 31% ethanol. In the case of pure organic solvents, pure ethanol was the best choice for overall optimization and a simplified procedure for achieving the highest levels of TPh, SA, and RP. Furthermore, ethanol as an optimal solvent can be suitable for carotenoid recovery as well, due to the ratio of ~64% ethanol and ~36% acetone corresponding to the highest value.

Table 3. Single and multi-response optimization of extraction solvent mixtures.

	TCar [a]	TPh [b]	SA [c]	RP [c]	Acetone	Ethanol	Water
Ethanol + Acetone + 20% Water							
Single response-$TCar_{max}$	14.750	–	–	–	0.8	0	0.2
Single response-TPh_{max}	–	137.708	–	–	0.34	0.46	0.2
Single response-SA_{max}	–	–	922.416	–	–	0.8	0.2
Single response-RP_{max}	–	–	–	197.982	–	0.8	0.2
Multi response	6.407	135.695	703.899	176.811	0.49	0.31	0.2
Ethanol + Acetone							
Single response-$TCar_{max}$	25.8774	–	–	–	0.366	0.634	–
Single response-TPh_{max}	–	107.394	–	–	0	1	–
Single response-SA_{max}	–	–	1870.27	–	0	1	–
Single response-RP_{max}	–	–	–	155.744	0	1	–
Multi response	21.6177	107.394	1870.394	155.744	0	1	–

TCar, total carotenoid content; TPh, total polyphenol content; SA, scavenging activity; RP, reducing power. [a] Expressed as mg β-carotene equivalents per 100 g carrot DW. [b] Expressed as mg gallic acid equivalents per 100 g carrot DW. [c] Expressed as µmol Trolox equivalents per 100 g carrot DW.

Various studies have been carried out to optimize the extraction of carotenoids and polyphenols. For example, De Andrade Lima et al. [51] optimized the extraction of carotenoids from carrot peel by supercritical CO_2, utilizing ethanol as a co-solvent. The optimal conditions for carotenoid recovery (86.1%) were found at 59.0 °C, 349 bar, and 15.5% ethanol, while model fitting confirmed the fast extraction trend and desorbing nature of carotenoids from the sample matrix. ASE at 7000 bar exhibited higher (64%) extraction yields from tomato waste for 10 min, compared to the conventional solvent extraction process of 30 min [52]. From the cryogenic ground biomass of the microalga *Neochloris oleoabundans*, the determined ASE conditions for extraction of carotenoids were: 100 °C temperature, extraction time of 20 min, extraction pressure of 103 bar, and 100% ethanol as the extraction solvent [53]. Saha et al. [40] utilized the Hildebrand solubility parameter (δ) to predict the extraction yield of carotenoids in ASE. The authors declared that maximum extraction efficiency of lutein and β-carotene from the orange carrot was achieved using a mixture of acetone/ethanol/hexane (1:2:3, $v/v/v$: δ value of 9.56 cal1/2 cm3/2), compared to acetone/hexane (3:5, v/v), ethanol/hexane (4:3, v/v), and acetone/ethanol/hexane (3:2:1, $v/v/v$). Optimal ASE extraction conditions for the isolation of polyphenols from olive leaves were achieved after two cycles, at 80 °C and 5 min [54]. Feuereisen et al. [55] optimized ASE extraction of phenolic compounds from Brazilian pepper (*Schinus terebinthifolius* Raddi) by RSM and found high yields of phenolic compounds from the exocarp/drupes at 100/75 °C, 10/10 min, 54.5/54.2% ethanol, and 5/0.03% acetic acid. In another study, del Pilar Garcia-Mendoza et al. [56] found that the highest antioxidant activity and concentration of phenolics in juçara (*Euterpe edulis* Mart.) residues' ASE extracts were obtained with the acidified mixture of ethanol + water at 80 °C.

Regression models for investigated response, total carotenoid and polyphenol contents, SA, and RP were significant for both organic solvent mixtures with 20% water ($p < 0.05$), as determined by analysis of variance (Tables 4 and 5). Models had no significant lack of fit ($p > 0.05$). Moreover, experimental data fitted well with the models, as presented by correlation coefficients, which are especially high for the total carotenoid contents (0.9540 and 0.9223).

Table 4. Analysis of variance (ANOVA) of the modelled responses for ethanol + acetone + 20% water.

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-Value	*p*-Value
		Total Carotenoids			
Model	182.23	2	91.12	51.81	0.0005
Residual	8.79	5	1.76		
Lack of fit	0.46	2	0.23	0.083	0.9228
Pure error	8.33	3	2.78		
Total	191.02	7			
		$R^2 = 0.9540$			
		Total Polyphenols			
Model	420.07	2	210.04	6.34	0.0426
Residual	165.74	5	33.15		
Lack of fit	66.45	2	33.23	1.00	0.4636
Pure error	99.28	3	33.09		
Total	1529.87	7			
		$R^2 = 0.7171$			
		Scavenging Activity			
Model	1.433×10^5	1	1.433×10^5	20.22	0.0041
Residual	42,513.88	6	7085.65		
Lack of fit	17,103.52	3	5701.17	0.67	0.6236
Pure error	25,410.36	3	8470.12		
Total	1.858×10^5	7			
		$R^2 = 0.7712$			
		Reducing Power			
Model	1345.02	1	1345.02	13.42	0.0105
Residual	601.35	6	100.22		
Lack of fit	175.36	3	58.45	0.41	0.7574
Pure error	425.99	3	142.00		
Total	1946.37	7			
		$R^2 = 0.6910$			

3.2. HCA of the Extraction Experiments

The dendrogram of the clustering of the analyzed experiments is presented in Figure 3. It is obvious that there is a clear division of the experiments into two main clusters, so the experiments in which water was used as a solvent (1–8, *Opt1*) are strictly separated from those in which water was not applied as a solvent or in combination with other solvents (9–16, *Opt2*). In addition, inside each main cluster, certain sub-clusters are observable: experiments *Opt2*, 15, and 9 belong to the same sub-cluster; in these experiments, pure ethanol was applied as a solvent, and the extraction resulted in the extracts with similar properties. Moreover, the experiments *Opt1*, 8, and 5 are placed in the same sub-cluster; in these experiments, the same or similar solvent mixture composition was used. The same can be noticed in the sub-cluster which contains experiments 1 and 7.

Generally, the results of the HCA clearly point out that there is significant statistical difference between the properties of the extracts obtained from the experiments in which water was used in the solvent mixture. This means that the use of water in the solvent mixture should be carefully considered if an extract with certain properties is to be obtained.

Table 5. Analysis of variance (ANOVA) of the modelled responses for ethanol + acetone.

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-Value	*p*-Value
		Total Carotenoids			
Model	181.48	2	90.74	29.69	0.0017
Residual	15.28	5	3.06		
Lack of fit	9.11	2	4.56	2.21	0.2566
Pure error	6.17	3	2.06		
Total	196.77	7			
		R^2 = 0.9223			
		Total Polyphenols			
Model	2258.84	1	2258.84	19.16	0.0047
Residual	707.35	6	117.89		
Lack of fit	312.30	3	104.10	0.79	0.5743
Pure error	395.05	3	131.68		
Total	2966.19	7			
		R^2 = 0.7615			
		Scavenging Activity			
Model	1.453×10^6	2	7.266×10^5	10.68	0.0157
Residual	3.403×10^5	5	68,066.93		
Lack of fit	1.517×10^5	2	75,858.05	1.21	0.4126
Pure error	1.886×10^5	3	62,872.85		
Total	1.794×10^6	7			
		R^2 = 0.8102			
		Reducing Power			
Model	10,233.03	1	10,233.03	9.46	0.0218
Residual	6489.54	6	1081.59		
Lack of fit	2340.70	3	780.23	0.56	0.6751
Pure error	4148.84	3	1382.95		
Total	16,722.57	7			
		R^2 = 0.6119			

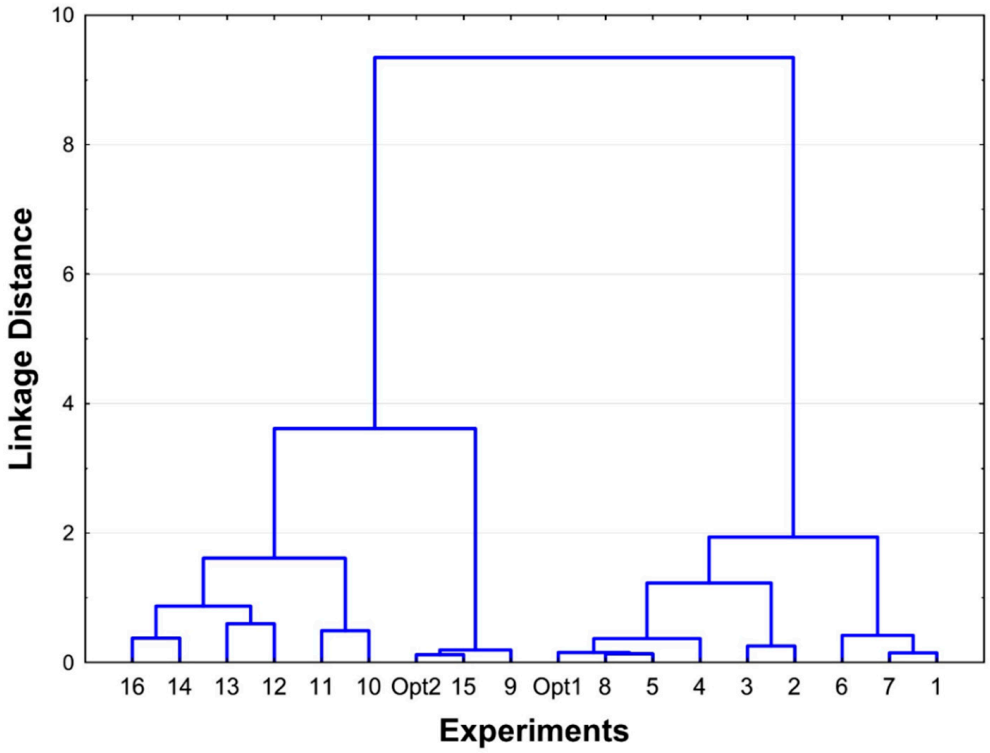

Figure 3. The dendrogram of the clustering of extraction experiments.

3.3. SRD Analysis of the Extraction Experiments

The results of SRD analysis of the extraction experiments 1–16 based on the normalized input data and the data predicted by the RSM approach (*Opt1* and *Opt2*) are presented in Figure 4. The ranking of the experiments 1–16, *Opt1*, and *Opt2* was related to the reference ranking calculated on the basis of the row average values for the ε_m, RP_m, ρ_m, and P'_m parameters and maximum row values for the TCar, TPh, SA, and RP parameters (the higher TCar, TPh, SA, and RP values, the better). The row average is usually considered the best choice for reference ranking (consensus ranking), meaning that errors cancel each other out [57,58]. According to the maximum likelihood principle, the most probable ranking will be ensured by using the average as a reference [58].

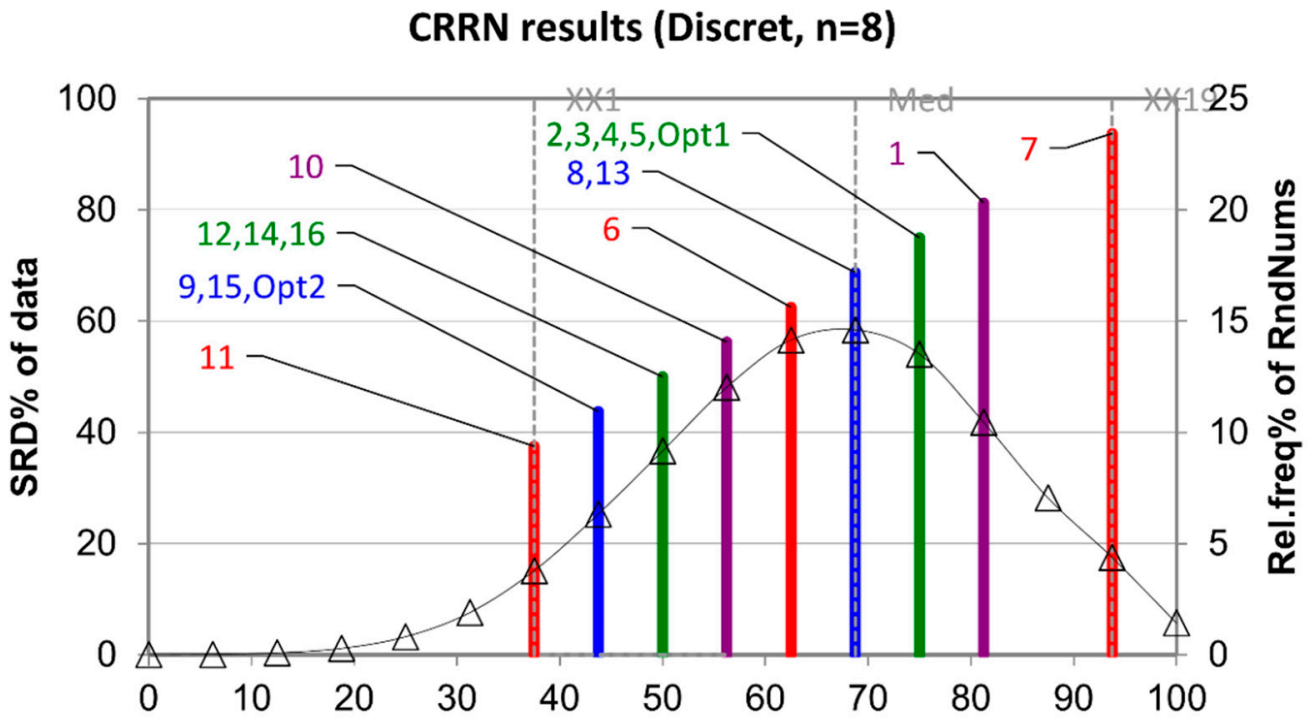

Figure 4. The graphical representation of the results of SRD analysis of the extraction experiments 1–16 and optimal solvent (*Opt1*) and optimal ternary solvent mixture composition (*Opt2*). The statistical characteristics of the theoretical distribution functions are first icosaile (5%), XX1 = 12; first quartile, Q1 = 18; median, Med = 22; last quartile, Q3 = 24; last icosaile (95%), XX19 = 30.

Figure 4 shows the distribution of the experiments 1–16, *Opt1*, and *Opt2* data in relation to the reference ranking. As it can be seen from the SRD graph, the experiments with water present in the solvent mixture are placed farther from the reference ranking. This is in agreement with the results obtained by HCA, despite the fact that these two methods are theoretically and computationally different. The established reference ranking can be used as a discriminating factor for the conducted experiments, dividing them in a previously described way. The conducted SRD analysis points out that the SRD values for each experiment can be considered a (dis)similarity measure, since similar experiments in the space of the analyzed variables have similar or the same rank number (9, 15, and *Opt2*; 12, 14, and 16; 8 and 13; 2, 3, 4, 5, and *Opt1*).

If the position of every experiment in the SRD graph is analyzed, it can be seen that the experiments described by low ρ_m and P'_m of the solvents will probably result in extracts with high TCar and SA, and low TPh and RP. Based on the results of the SRD analysis, it can be concluded that the experiment defined as *Opt2* in RSM analysis is confirmed to be optimal considering its place in the SRD graph, which is very close to the reference rank,

together with the experiments 9 and 15. In these three experiments, with pure ethanol as a solvent, the extracts with significant TCar, TPh, SA, and RP were obtained.

4. Conclusions

In order to gain an overview of the optimum experimental conditions and to classify the conducted experiments and rank and group similar experiments for the accelerated solvent extraction of carrot antioxidants, response surface methodology and chemometric methods (hierarchical cluster analysis and sum of ranking differences approach) were applied. The mixture of pure organic solvents, acetone, and ethanol with or without the addition of 20% water was applied following the simplex-lattice experimental design. Total carotenoid (TCar) and polyphenol contents (TPh) in extracts, as well as their scavenging activity (SA) and reducing power (RP), were used as responses for the optimization of ASE extraction. Multi-response optimization, in the case of the involvement of 20% water, included 49% acetone and 31% ethanol (*Opt1*), while in the case of pure organic solvents, pure ethanol was the best choice for overall optimization (*Opt2*). The results of the HCA clearly indicate that there is significant statistical difference between the properties of the extracts obtained from the experiments in which water was used in the solvent mixture. This means that the use of water in the solvent mixture should be carefully considered if an extract with certain properties should be obtained. The conducted SRD analysis ranked the extraction experiments based on their extraction efficacy, solvent polarity used in the experiments, and the solvent composition in the mixture. Based on the results of the SRD analysis, it can be concluded that the experiment defined as *Opt2* in RSM analysis is confirmed to be optimal considering its place in the SRD graph, which is very close to the reference rank. In the experiments with pure ethanol as a solvent, the extracts with significant TCar, TPh, SA, and RP were obtained. The conducted RSM, HCA, and SRD analysis confirmed the same conclusion—water in the solvent mixture can significantly affect the extraction efficacy. Finally, all the optimization analysis agreed that the optimal solvent for extracting antioxidants from carrot by ASE is pure ethanol.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr9091652/s1, 1. Calculation of dielectric constant (εm) of solvent mixtures, 2. Calculation of relative polarity (RPm) of solvent mixtures, 3. Calculation of density (ρm) of solvent mixtures, 4. Calculation of polarity index (P′) of solvent mixtures, Table S1. The normalized data used in the SRD analysis of extraction experiments, Table S2. The input matrix used in the SRD modeling.

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