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# Development of a Cloud Point Extraction Technique Based on Lecithin for the Recovery of Carotenoids from Liquid Tomato Wastewater

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**Abstract:** The traditional extraction methods used to recover natural antioxidants from food industry wastes involve significant amounts of hazardous solvents. A viable alternative is the use of non-toxic surfactants to remove organic compounds from wastes at temperatures above the cloud point. Cloud point extraction has only recently begun to be used to recover high-value added compounds from food industry wastes. In the current work, a method for the isolation of high-value added components from liquid tomato wastewater using a cloud point extraction method was developed and optimized. Food-grade lecithin was examined for its potential to be used as a surfactant in the developed procedure. Moreover, parameters affecting the extraction (ionic strength, sample pH, temperature of extraction, and surfactant concentration) were examined and optimized. According to the results, the maximum recovery of carotenoids from the sample could be achieved with the developed procedure, by simply adjusting the pH to 3.5, adding 35.6% (*w/v*) sodium chloride, and setting the temperature at 45 °C. Moreover, the amount of lecithin used was examined. In order to extract the total amount of carotenoids from a sample, it was found that either three extractions with 1% lecithin are needed, or two extractions with 2% lecithin. In addition, the antioxidant activity of the extract was examined and it was found to scavenge 36.3% of DPPH free radicals. This percentage was 10% lower compared to the initial sample, which suggests that the extracted compounds retain their activity. Overall, the developed procedure can be used to recover carotenoids in a cost-efficient and easy way.

**Keywords:** antioxidants; carotenoids; lecithin; lycopene; surfactant; tomato industry waste



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## 1. Introduction

To date, more and more antioxidant compounds are being employed in the food industry, in an effort to protect the food products from oxidation, as well as bestow health benefits to the food products. Currently, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are commonly employed in food production. However, reports hint that these compounds may be carcinogenic [1]. Therefore, naturally-derived antioxidant compounds are being sought after to replace artificial antioxidants. Carotenoids are among the most significant dietary antioxidants [2,3]. Carotenoids are natural pigments that contribute significantly to the organic colors seen in foods, flowers, and plants. The main ones include lutein, zeaxanthin, lycopene, α-carotene, β-carotene, and cryptoxanthin [4,5]. In the food industry, lycopene is a coloring and antioxidant agent. Due to its high antioxidant activity, it is also utilized as a nutraceutical, lowering the risk of atherosclerosis and coronary heart disease [2,6]. Additionally, epidemiological research has linked lycopene

consumption to a decreased chance of developing some malignancies [7]. Consequently, lycopene extraction from tomato waste is a good substitute for valorizing this by-product.

The process of isolating carotenoids requires labor- and energy-intensive extraction techniques that, for example, use a lot of organic compounds ethanol or ethyl acetate, or require high pressure and temperature equipment in the case of supercritical fluid extraction [5,8,9]. Using surfactants, cloud point extraction (CPE) is a cheap and straightforward method for separating bioactive compounds from liquid media (micellar systems) [10]. Carabias-Martinez et al. [11] described the micellar system properties and CPE parameters that affect the extraction of compounds. The main parameters are the pH and the ionic strength of the sample, as well as the temperature of the sample and the amount of surfactant employed. Moreover, the superiority of CPE over commonly employed procedures employing organic solvents has been acknowledged [12,13]. This is due to the fact that many ionic surfactants are non-volatile, regarded as either moderately non-toxic or innocuous reagents, and are not related to toxicological or dermatological issues [14]. Among the various surfactants that can be used in CPE procedures, very few are naturally derived [15]. Lecithin is a naturally derived surfactant, commonly employed in the food industry. It is of low cost and it lacks toxicity. This is the reason why it has been labeled as "GRAS" (generally recognized as safe) by the U.S. Food and Drug Administration (FDA) [16]. As such, it holds great promise to be used in CPE procedures. So far, CPE has been used for the extraction of various compounds [15]. Among others, there is a limited number of reports regarding the use of CPE for carotenoids extraction [13,17]. For instance, Chatzilazarou et al. [17] reported the use of Genapol X-080 in a CPE procedure to recover carotenoids from red-fleshed orange juice, achieving 92.3% recovery. Similarly, Katsoyannos et al. [13] examined the use of Span 20, PEG 400, Tween 80 and 20 in a CPE procedure for the same reason, achieving a recovery of 72.4%. In regards to carotenoids, there are other types of extraction techniques that can also be used, such as atmospheric liquid extraction with maceration, ultrasound-assisted extraction, Soxhlet extraction, etc. [5]. However, these methods are accompanied by some drawbacks. First of all, the presence of water limits the extraction of carotenoids, since the latter are hydrophobic. As such, the drying of the sample is needed [5]. However, heat-based drying results in degradation of carotenoids. As such, samples need to be freeze dried, which increased the overall time and cost of the method. All the above are not applicable in the case of CPE, and as such, it is considered a better option [5].

Liquid tomato wastewater (LTW) is a waste product of the agri-food industry and a major polluter in many nations that process tomatoes. Approximately 100 million tons of fresh tomatoes are produced yearly, the second-most important vegetable crop, in 144 different nations [18]. Large amounts of by-products, such as peel, pulp, and seeds, which make up approximately 10–40% of all processed tomatoes, are produced by tomato products like ketchup and sauce [7]. As a result, enormous amounts of LTW are being produced. Given the high concentration of carotenoids in tomatoes, LTW is also a good source to recover carotenoids. However, up to date, reports regarding the extraction of carotenoids from LTW are scanty and sparse. As such, a hypothesis was made on whether CPE could be used to extract the carotenoids from LTW in an efficient and fast way. To examine this hypothesis, the main objective of this study was to develop a CPE-based procedure using lecithin. The next objective was to optimize the developed procedure, so as to recover the full amount of carotenoids contained. In this context, the procedure was optimized in terms of sample pH, ionic strength, and temperature, as well as surfactant concentration, so as to achieve maximum recovery of the compounds and render the method as efficient as possible.

## 2. Materials and Methods

### *2.1. Chemicals and LTW Sample*

Lycopene (analytical standard, $\geq$85%) and DPPH (1,1-diphenyl-2-picrylhydrazyl) were obtained from Sigma–Aldrich (Steinheim, Germany). Ethanol, methanol, and *n*-

hexane were purchased from Panreac (Barcelona, Spain). Sodium chloride, sodium hydroxide, citric acid, and soya lecithin (>97%) were obtained from Carlo Erba (Milano, Italy). The deionized water used in the experiments was produced using a deionizing column. Fresh LTW samples were delivered from a nearby tomato processing industry (Damavand S.A., Filia, Karditsa, Greece). Samples were stored at 4 °C until used.

### *2.2. Determination of Physicochemical Parameters*

The amount of water, fat, and total solids was determined as previously described [13]. In brief, centrifugation (20 min, 4500 rpm) was used to first separate the solids in the LTW. After that, the LTW sample was dried at 105 °C for 24 h to assess its water content. Utilizing a Soxhlet apparatus with *n*-hexane as the solvent for 4 h, the fat content was determined.

### *2.3. Total Carotenoid Content Determination*

The total carotenoid content was determined using a colorimetric assay developed by Biswas et al. [19] with some modifications. An aliquot of 100 μL of the sample was mixed with 900 μL of ethanol and the solution was vigorously shaken for 30 s. The absorbance was read at 471 nm, using a Shimadzu spectrophotometer (UV-1700, Shimadzu Europa GmbH, Duisburg, Germany). The total carotenoids were determined using a calibration curve ($R^2$ = 0.9981), using lycopene as the standard compound.

### *2.4. CPE Procedure*

The CPE method was a modification of previously published methods [17,20,21]. Citric acid and sodium hydroxide were used in the solids-free LTW sample to adjust the pH to 3.5. Next, sodium chloride was added (36.5% *w/v*) and the sample was vigorously stirred, using a magnetic stirrer. The temperature of the sample was raised to 45 °C and then an appropriate amount of lecithin solution was added so that the final concentration in the solution was 1% or 2%. After 20 min, the solution was centrifuged at 4500 rpm for 5 min. The lower aqueous phase was separated from the upper micellar (surfactant) phase, and used for further study.

### *2.5. Carotenoid Recovery by CPE*

The % carotenoid recovery (carotenoids recovered by the surfactant from LTW) was calculated using a carotenoid mass balance. The surfactant's recovery was approximated in accordance with earlier descriptions [17,20–22]:

$$\text{Recovery } (\%) = \frac{CsVs}{CoVo} \times 100 = \frac{CoVo - CwVw}{CoVo} \times 100 \quad (1)$$

where *C*o is the analyte (carotenoid) concentration in the volume *V*o (10 mL) of the initial sample, *C*w is the analyte concentration in the volume *V*w of the water phase, and *C*s is the carotenoid concentration in the volume *V*s' of the surfactant phase. Absorption (*A*) in carotenoids was used to calculate concentration (*C*). As a result, *C*s = *A*s, *C*o = *A*o, and *C*w = *A*w were assumed. Following phase separation, the following equations are used to calculate the concentration factor (*F*c) and analyte concentration (*A*s) in the surfactant phase:

$$Fc = \frac{As}{Ao} \text{ where, } As = \frac{AoVo - AwVw}{Vs} \quad (2)$$

### *2.6. Determination of the Antioxidant Activity*

The antioxidant activity of the carotenoids extracted in the surfactant phase as well as those remaining in the sample after CPE treatment was estimated by the DPPH method described by Tsaknis and Lalas [23] with some modifications. Briefly, a 1 mL 0.1 mM solution of methanolic DPPH was added to 4 mL of the sample solution. The mixture was thoroughly shaken before being let to stand at room temperature in the dark for 30 min and

the absorbance was measured at 517 nm. The formula used to determine the % scavenging was as follows:

$$\% \text{ Scavenging} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (3)$$

where $A_{\text{control}}$ and $A_{\text{sample}}$ are the absorbances.

### *2.7. Statistical Analysis*

All analyses were conducted three times. Results were expressed as mean values of the three replicates ± standard deviation. Statistically significant differences were examined with the Kruskal–Wallis test, after testing the data with the Kolmogorov–Smirnov test. Statistically significant differences were considered for $p < 0.05$.

## 3. Results and Discussion

### *3.1. Physicochemical Parameters of the LTW*

Some physicochemical parameters of the LTW samples were examined, before developing and optimizing the extraction procedure. Moreover, the total carotenoids contained in the sample were examined. Results are given in Table 1. It can be seen that carotenoids represent 0.034% *w/w* of the sample, highlighting that LTW is a valuable source of carotenoids that is usually discarded. According to Kotikova et al. [24] carotenoids contained in ripe red tomato fruits consist of lycopene (~ 90%), β-carotene (5–10%), and lutein (1–5%) while other carotenoids are present in trace amounts (<1%).

**Table 1.** Physicochemical composition of LTW used for the CPE experiments.

| | |
|---|---|
| Water content (%, *w/w*) | 88.7 ± 0.3 |
| Total Solids (%, *w/w*) | 10.2 ± 0.3 |
| Total Fat (%, *w/w*) | 1.0 ± 0.1 |
| Total Carotenoids (%, *w/w*) | 0.034 ± 0.002 |
| pH | 5.6 ± 0.3 |

Values are expressed as the mean values (± SD) of triplicate analyses.

### *3.2. Optimization of the Extraction Procedure*

To maximize the recovery of carotenoids from the LTW the main parameters that affect the extraction procedure were optimized. To this end, the effect of sample pH, the use of sodium chloride to tune the ionic strength of the solution, as well as the temperature of the extraction were firstly examined. Next, the use of lecithin at two different concentrations was also examined, in an attempt to maximize the recovery of the compounds. For the first three parameters examined, the concentration of lecithin was set at 1% (*w/v*).

#### 3.2.1. Effect of Ionic Strength

It is well known that the ionic strength of a solution plays a major role in the extraction of organic compounds. In general, the increase of the ionic strength of a solution results in increased extraction of the compounds, due to the salting out effect, causing a decrease in the solubility of organic compounds and as such facilitating the extraction process [25,26]. To this end, the addition of sodium chloride on the recovery of carotenoids was assessed. Results are presented in Figure 1. It can be seen that as the concentration of sodium chloride increases, an increase in the recovery of carotenoids is also recorded. Maximum recovery (25%) was achieved when 35.6% *w/v* sodium chloride was added. Aside from the salting out effect, it was reported that the addition of salt decreases the cloud point temperature and enhances phase separation, resulting in an overall enhanced extraction [27,28]. Thus, the enhanced extraction percentages achieved in our case cannot be solely attributed to the salting out effect, but to the combination of the aforementioned effects that may be applicable. The results of this optimization step were in accordance with previous reports [13,17].

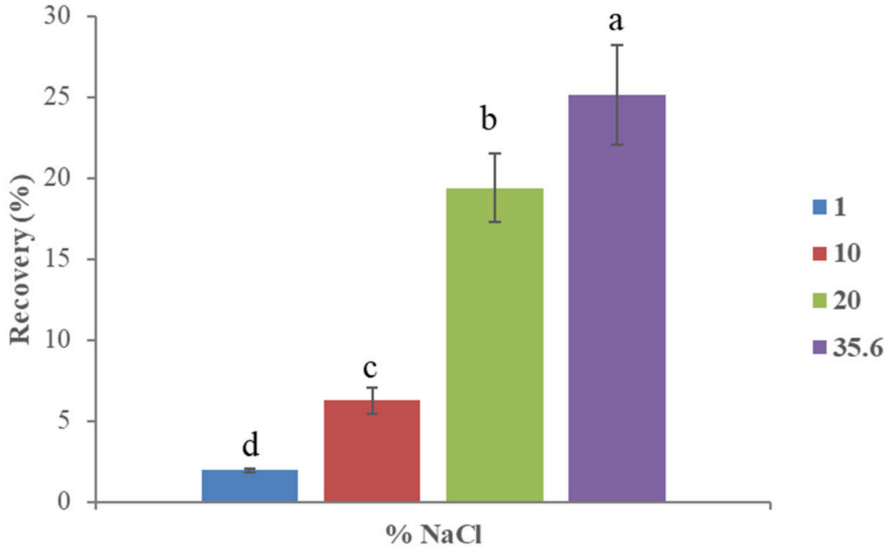


**Figure 1.** Effect of NaCl addition at various concentrations on the extraction recovery of carotenoids from LTW; standard deviation of three replicate analyses is presented with error bars; different letters (i.e., a–d) denote samples that differ significantly (statistical difference for $p < 0.05$).

### 3.2.2. Effect of Sample pH

As with most extraction procedures, the pH of the sample may have an impact on the extraction recovery. Therefore, we next examined the effect of different pH on the recovery of carotenoids. Results can be seen in Figure 2. It can be seen that pH has a big impact on the extraction of carotenoids. More specifically, maximum recovery was achieved when the pH was adjusted to 3.5. When the pH was adjusted to 2.5 a 4.4% decrease in the extraction recovery was observed (statistically significant for $p < 0.05$). Likewise, an increase of the pH value from 3.5 to 4.5 resulted in a 14% decrease in the recovery of carotenoids, whereas a further increase in the pH of LTW caused a further decrease in the recovery of carotenoids. Authors found that neutral and slightly alkaline pH values resulted in 26% reduced carotenoid content of fresh carrot juice, while maximum content was observed at pH ~3 [29]. The results are also in accordance with previous reports, mentioning that the optimum pH for the CPE of carotenoids is between 2.5–3.5 [13,17].

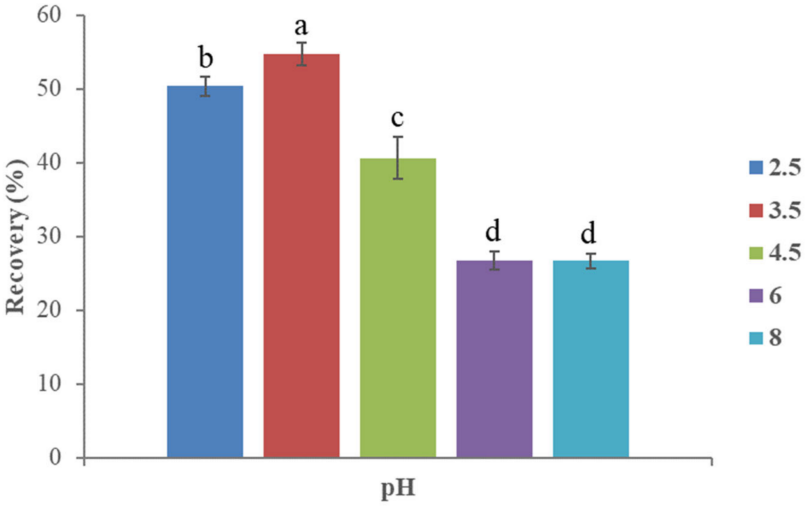


**Figure 2.** Effect of sample pH on the extraction recovery of carotenoids from LTW; standard deviation of three replicate analyses, is presented with error bars, different letters (i.e., a–d) denote samples that differ significantly (statistical difference for $p < 0.05$).

3.2.3. Effect of Temperature

Heating is necessary for the CPE to be carried out. However, excessive heating may damage the molecules to be extracted, and thus caution is needed [29,30]. In order to enhance the recovery of carotenoids, the temperature was studied in the range of 25–65 °C. Higher temperatures were not examined, since it has been reported that the thermal degradation of carotenoids starts from 70 °C onwards. Results can be seen in Figure 3. As can be seen, maximum recovery can be achieved at 45 °C. When higher than 45 °C temperatures were employed, the decreased recovery of carotenoids observed could be attributed to the partial loss of color of the carotenoids, due to reactions that can take place on the polyene chromophore moieties [31]. Similarly, lower temperatures resulted in lower recovery of carotenoids from LTW. Ideally, the extraction temperature should be 15–20 °C higher than the cloud point temperature, so that better separation of the surfactant-rich phase and the sample is achieved, supporting the overall enhanced recovery. In our case it was found that this optimum temperature was 45 °C, although a temperature of 25 °C could also be used without taking a big toll on the recovery of carotenoids since a ~6% decrease in the recovery was recorded. The results were in accordance with previous reports [13,17]. The addition of a high amount of salt in the sample, as mentioned above, resulted in a decrease in the cloud point. This is beneficial since less energy is consumed, rendering the extraction process more environmentally friendly, compared to the case where the temperature would be more elevated.

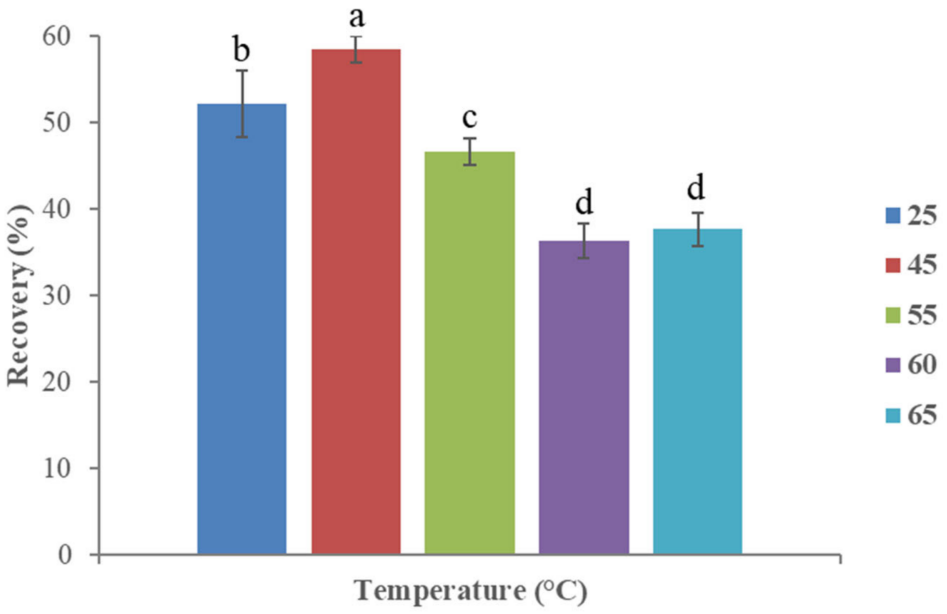


**Figure 3.** Effect of temperature on the extraction recovery of carotenoids from LTW; standard deviation of three replicate analyses, is presented with error bars; different letters (i.e., a–d) denote samples that differ significantly (statistical difference for $p < 0.05$).

3.2.4. Effect of Surfactant Concentration and Number of Extractions

The final parameter that we examined was the concentration of the surfactant and the number of extractions needed to achieve total extraction of carotenoids. Lecithin concentrations of 1% and 2% were examined. Increased concentrations were not deemed necessary, according to preliminary experiments, which is an asset for the developed procedure, needing only small amounts of surfactant. The effect of surfactant concentration (Cs) on $Vs/Vw$, $Fc$, and $As$ can be seen in Table 2. It can be seen that the use of the examined lecithin concentrations is advantageous since carotenoids recovery values were moderate, $Fc$ and $As$ were comparatively high, and $Vs/Vw$ ratio showed very low values. The recoveries of carotenoids on three consecutive extractions, using the two examined concentrations of lecithin, are presented in Figure 4. It can be seen that by carrying out

the extraction three times, all carotenoids from the sample can be extracted, using both lecithin concentrations. However, when 2% lecithin was used, a 98.5% extraction recovery could be achieved by carrying out the extraction two times. As such, it is recommended that the extraction can either be carried out three times with 1% lecithin or two times with 2% lecithin, so as to achieve comparable results.

**Table 2.** Consecutive CPE extraction steps for the recovery of carotenoids from LTW.

| CPE Step | % *Cs* | *Vs*/*Vw* | *Fc* | *As* |
|---|---|---|---|---|
| 1st | 1% | $0.021 \pm 0.001^{f}$ | $7.25 \pm 0.17^{a}$ | $5.21 \pm 0.23^{a}$ |
| | 2% | $0.055 \pm 0.001^{d}$ | $6.46 \pm 0.25^{b}$ | $4.63 \pm 0.26^{b}$ |
| 2nd | 1% | $0.036 \pm 0.002^{e}$ | $4.41 \pm 0.10^{c}$ | $1.65 \pm 0.06^{e}$ |
| | 2% | $0.069 \pm 0.002^{c}$ | $2.75 \pm 0.10^{d}$ | $2.47 \pm 0.05^{d}$ |
| 3rd | 1% | $0.039 \pm 0.001^{e}$ | $4.81 \pm 0.27^{c}$ | $1.58 \pm 0.06^{e}$ |
| | 2% | $0.041 \pm 0.001^{e}$ | $4.72 \pm 0.21^{c}$ | $2.71 \pm 0.11^{d}$ |
| Total | 3 × 1% | $0.096 \pm 0.006^{b}$ | $6.07 \pm 0.45^{b}$ | $4.36 \pm 0.31^{b}$ |
| | 3 × 2% | $0.165 \pm 0.009^{a}$ | $4.71 \pm 0.35^{c}$ | $3.42 \pm 0.21^{c}$ |

Values are expressed as the mean values (±SD) of triplicate determinations and means within each column with different superscript letters (i.e., a–f) are significantly ($p < 0.05$) different.

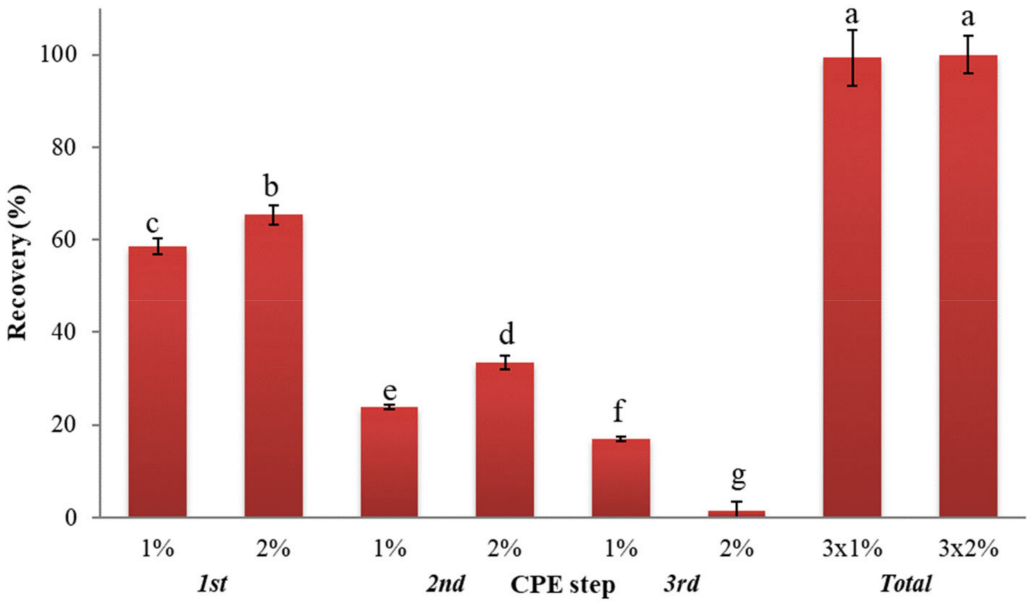


**Figure 4.** % Carotenoid recovery with lecithin on LTW; Standard deviation is presented with error bars; Different letters (i.e., a–g) denote samples that differ significantly (statistical difference for $p < 0.05$).

In the study of Chatzilazarou et al. [17] the use of Genapol X-080 in a CPE procedure to recover carotenoids from red-fleshed orange juice was reported. Under optimum conditions, a 5% surfactant concentration was needed, in order to achieve a 92.3% recovery rate of carotenoids. As such, it can be seen that the proposed method is beneficial, since half the amount of surfactant is needed to achieve a 100% recovery of carotenoids. Likewise, Katsoyannos et al. [13] examined the use of Span 20, PEG 400, and Tween 80 and 20. In a CPE procedure for carotenoids extraction, authors found that the use of 10% *v*/*v* surfactant resulted in recoveries for carotenoids ranging between 8.7% and 64.3%, with the highest recovery achieved by Tween 80. Next, they evaluated the amount of surfactant and found that 25% *v*/*v* of Tween 80 is needed in order to achieve 90.3% recovery. Based on these, it is obvious that the use of 1% lecithin is much more beneficial, compared to the use of increased amounts of other surfactants, while at the same time a better recovery is achieved.

### *3.3. Antioxidant Activity of the Recovered Carotenoids*

Extracting compounds from a sample would only be beneficial if the extracted compounds retain their properties. As such, it was necessary to evaluate whether CPE extraction of carotenoids from LTW, using lecithin, affected their antioxidant activity. For the assessment of the antioxidant activity, the DPPH assay was employed. The antioxidant activity of the initial LTW sample was first examined. Furthermore, a lycopene solution was prepared at a concentration of 10 ppm, so that it exhibited similar antioxidant activity to the initial LTW sample. After CPE extraction, both solutions were re-examined for their antioxidant activities. The results of the antioxidant activity of the samples are shown in Table 3. It can be seen that the antioxidant activity of the initial LTW sample was 47.4%. The extract obtained from the CPE was found to have a 36.3% antioxidant activity, which is nearly 10% lower. However, this was not the case when a lycopene solution was examined. Before the extraction, the lycopene solution exhibited a 47.2% antioxidant activity, while the extract exhibited a 46.9% antioxidant activity, which was not found to be statistically different from the initial solution ($p < 0.05$). According to that, it can be inferred that the CPE extraction does not affect the antioxidant activity of lycopene. The decreased antioxidant activity recorded in the case of LTW extract can be attributed to the presence of other compounds, such as polyphenols, that are present in the initial LTW sample and exhibit antioxidant activity, which, however, are poorly extracted during the CPE procedure. This can also be an asset for the developed procedure, since the extraction of other compounds may be minimized.

**Table 3.** Antioxidant activity of the initial LTW sample, a 10-ppm lycopene solution, and the surfactant phases after CPE of the two solutions.

| Phase | % Scavenging |
|---|---|
| Initial LTW sample | $47.4 \pm 3.1^{a}$ |
| Lycopene (10 ppm) | $47.2 \pm 2.8^{a}$ |
| CPE extract from LTW | $36.3 \pm 1.7^{b}$ |
| CPE extract from lycopene solution | $46.9 \pm 0.9^{a}$ |

Values are expressed as the mean values (±SD) of triplicate determinations and means with different superscript letters are significantly ($p < 0.05$) different.

## 4. Conclusions

Based on the results of our study, our hypothesis was validated. A CPE procedure was developed and optimized for the recovery of carotenoids from LTW samples, and used successfully. Lecithin was employed as a surfactant since it is a common food-grade surfactant, which is also widely used in pharmaceutical products, that has a low cost. Regarding our second objective, in order to maximize the recovery of carotenoids, an adjustment of pH at 3.5, the addition of 35.6% (*w/v*) of sodium chloride, and a temperature of 45 °C were needed: parameters that can easily be tuned without significantly increasing the cost of the procedure. More importantly, from the results, it was apparent that minimal surfactant concentrations (1–2%) and two or three extraction steps were sufficient to achieve the total recovery of carotenoids. The carotenoids extracted by the proposed method maintained high antioxidant activity (36.3%), according to the DPPH method. Given the low toxicity of the surfactant, the low consumption needed, and the easiness of the extraction procedure, the proposed procedure not only overcomes the hindrances of the commonly employed methods, but also achieved total extraction of carotenoids. Overall, the proposed procedure can be used for the easy and cost-efficient recovery of carotenoids from LTW and replace existing methods that are less environmentally friendly or are more laborious to carry out.

**Author Contributions:** Conceptualization, I.G., O.G., and S.I.L.; methodology, V.A. and T.C.; software, V.A.; validation, I.G. and V.A.; formal analysis, I.G., V.A., and T.C.; investigation, I.G. and V.A.; resources, S.I.L.; data curation, I.G. and V.A.; writing—original draft preparation, I.G., V.A., and T.C.;

writing—review and editing, I.G., V.A., T.C., O.G., G.D.N., and S.I.L.; visualization, V.A.; supervision, O.G., G.D.N., and S.I.L.; project administration, S.I.L. All authors have read and agreed to the published version of the manuscript.

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