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Extraction of Lipophilic Antioxidants from Native Tomato Using Green Technologies

Running title: Use of Green Technologies to Obtain Tomato Antioxidants

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SUMMARY

Research background. Tomato (*Solanum lycopersicum* L.) fruit is highly consumed worldwide and contains high levels of carotenoids and tocopherols, two powerful antioxidants. Native tomato genotypes are rarely used in large-scale commercialisation and industries but serve as a reservoir to diversify the species gene pool and can be employed to obtain functional compounds. Extraction methodologies are

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currently experimenting changes towards cleaner methodologies that are more efficient and environmentally friendly, including avoiding toxic or polluting solvents.

Experimental approach. In this study, factorial and fractional factorial designs were used to evaluate the efficiency of digestive enzymes, sonication and green solvents to extract carotenoids and tocopherols from native tomatoes.

Results and conclusions. Digestive enzymes and sonication increased the carotenoid content and the antioxidant activity of the obtained extracts when applied individually. However, when these treatments were applied together and in combination with green solvents (ethyl lactate and isopropyl acetate), the obtained extracts had the highest carotenoid and tocopherol contents as well as the maximal antioxidant activity, compared with non-combined treatments. Moreover, a correlation analysis suggested that antioxidant activity resulted from synergistic effects between several antioxidants rather than individual compounds. The tomato extracts were obtained through a relatively rapid extraction method (2 h) and maintained their functional activity.

Novelty and scientific contribution. Tomato is one of the most studied fruits, and both the plant and its fruit have been the subject of numerous studies. Functional compound extraction, mainly through environmentally friendly methods, remains an attractive research field for the utilisation of native tomato fruit, enhancing its limited production and use or harnessing the large amount of industrial waste from commercial tomato processing. There are few reports where clean extraction methods are combined; even rarer are those where green solvents are also used. In this work, the combination of different clean extraction technologies improved the extraction of carotenoids and tocopherols and allowed to establish a more efficient process. We believe that these results will stimulate the use of clean technologies and make the native tomato more attractive to harvest for industrial processing or to extract carotenoids and tocopherols for supplements.

Key words: carotenoids, tocopherols, enzyme-facilitated extraction, sonication, green solvents, clean extraction

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a fruit native to South America (*1*). It is the second most important crop in the world (*2*) and a significant source of antioxidants in the human diet due to its high consumption (*3,4*). The main hydrophobic antioxidants in tomatoes are lycopene, β-carotene, and α-tocopherol, while vitamin C and polyphenolic compounds (such as quercetin, kaempferol, naringenin,

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and rutin) are the main hydrophilic functional compounds (*5,6*). It has been reported that native tomato genotypes (tomato landraces) have higher contents of functional compounds such as lycopene or polyphenols, as well as greater *in vitro* antioxidant capacity than commercial varieties (*7,8*). Hence, native tomatoes have the potential to be incorporated into genetic improvement programmes or to be used at the commercial and industrial levels.

Reactive oxygen species play a role in the development of several degenerative diseases, including cancer, diabetes, cardiovascular and inflammatory diseases, neuronal degeneration, and ageing (*9,10*). Some studies have linked tomato consumption to a lower incidence of these diseases (*11-13*); this association may be mediated by antioxidants (*14,15*).

Developing adequate and sustainable methodologies for extracting functional compounds from plant matrices is an area of great potential; ideally, these methodologies should be environmentally friendly and safe (*16*). The use of these compounds is of interest to industries such as food, cosmetics, and pharmaceuticals, among others. Because many functional compounds are prone to degradation when isolated from their original sources, the extraction methodology should be selected or developed to reduce the possible stages of degradation (*17*). Some simple non-conventional technologies like ultrasound and lytic enzymes enhance extraction efficiency compared to traditional methods (*18,19*). In addition, the use of green solvents, which are more friendly and safer than conventional solvents, is highly recommended (*20*).

Different experimental designs, applied to the extraction processes, provide a better understanding of the effect of parameters related to yield (time, temperature, solvent, *etc.*), making them powerful tools for researchers. In recent years, the use of incomplete designs such as a fractional factorial designs has increased because they provide valuable information with a relatively low resource investment (*21*). This study aimed to optimise the extraction of functional compounds from native tomato by using hydrolytic enzymes, sonication, and green solvents to achieve a higher yield in an environmentally friendly manner.

MATERIALS AND METHODS

Biological samples

Saladette commercial tomatoes at consumption maturity were acquired from a local market in Iztapalapa, Mexico City and used to perform experiments 1 and 2. Samples for experiment 3 were two native tomato genotypes (cherry type, code 209, ID LOR88, collected in Teotitlán de Flores Magón, Oaxaca [18°07'57"N 97°04'20"W], and deer-eye type, code 210, ID LOR118, collected in La Ceiba,

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Puebla [20°23'N, 97°52'W]) (*22*) collected and donated by Ph.D. Ricardo Lobato. For its study, the tomato plants were cultured in the Postgraduate College, in Montecillo, Texcoco, Mexico State (19°30'N, 98°53'W) in 2018. Both native genotypes used in this study are part of the Mexican Network of Plant Genetic Resources (Red Mexicana de Recursos Fitogenéticos).

Obtaining lipophilic tomato extracts

Three experiments, denoted as experiments 1, 2, and 3 were carried out for obtaining the extracts. In experiment 1, the application of digestive enzymes was tested by using a factorial design with two factors, the enzymatic cocktail and the reaction medium. Subsequently, experiments 2 and 3 comprised fractional factorial designs (2^{4-1}). In experiment 2, the reaction medium, the reaction time, the enzyme cocktail concentration, and the reaction temperature were studied. In experiment 3, the evaluated factors were the tomato genotype, the sonication application, the enzyme cocktail application, and the green solvent used.

Experiment 1: enzyme cocktail and reaction medium tests

The enzyme cocktails tested were NS-22002/CNN02196 (CNN) with glucanase, xylanase, hemicellulase; and cellulase activities; NS-50012/KTN02163 (KTN) with cellulase, glucanase, hemicellulase, pectinase, and xylanase activities; and Viscozyme L (VIS) with hemicellulase, glucanase, cellulase, and xylanase activities. These enzymes were obtained from *Humicola inolenses*, *Aspergillus aculeatus*, and *Aspergillus* sp., respectively and were acquired from Novozymes (Mexico City, Mexico). The enzymatic treatments were carried out in 0.2 M acetate buffers (pH=4, 5 or 6; sodium acetate-acetic acid, J.T. Baker, Xalostoc, Mexico) or distilled water to test the enzymatic reaction at unregulated pH. The added enzyme cocktail was 5 mL/100 g of fresh mass of tomato fruit (% *V/m).*

Briefly, 1 g of commercial saladette tomato pulp was frozen and pulverized in a mortar with liquid N_2. After that, it was mixed with 3 mL of one of the enzymatic reaction medium. Subsequently, the enzyme cocktail was added and the mixture was kept under constant stirring (300 rpm) in an incubator (Incubator Shaker II, model 136400, Boekel Industries Inc., Feasterville-Trevose, PA, USA) for 3 h at 40 °C in the dark. Afterwards, the tomato pulp was separated from the reaction medium by filtration. The residue was washed with 10 mL of distilled water and extracted with 10 mL of dichloromethane (DCM; J.T. Baker, Xalostoc, Mexico) by vortexing (Genie II SI-0236, Scientific industries, Bohemia, NY, USA) for 15 min at medium intensity. After this time, 4 mL of ethanol (J.T. Baker, Xalostoc, Mexico) and 6 mL of distilled water were added and the mixture was centrifuged at 4500x*g* for 10 min (Avanti-JCI, Beckman Coulter

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Inc., Brea, CA, USA). The aqueous and organic phases were collected separately; the organic phase was filtered through 0.45 µm nylon membranes (Merck Millipore Corporation, Burlington, MA, USA), and adjusted to 10 mL with DCM. The product was stored at -70 °C (Thermo Fisher Scientific, Ultra-low temperature freezer 88400V, Waltham, MA, USA) until analysis.

Experiment 2: enzymatic reaction conditions

Four factors were studied to evaluate the enzymatic reaction conditions, including reaction medium (-1: pH=5, 1: distilled water), reaction time (-1: 1 h, 1: 5 h), enzyme cocktail concentration (KTN, -1: 1 %, 1: 5 %), and temperature (-1: 50 °C, 1: 40 °C) (Table 1). The studied factor levels were selected by preliminary assays or following recommendations in the literature (*18,19,29,31,34*). The treatment arrangement was generated by the Statgraphics Centurion XVI software (*27*). Briefly, 1 g of commercial saladette tomato pulp was frozen with liquid N_2 and pulverised in a mortar. The powder was mixed with 3 mL of distilled water or 0.2 M acetate buffer (pH=5). The tested amount of enzyme cocktail (10 or 50 µL) was added and incubated with constant stirring for 1 or 5 h and at 40 or 50 °C, depending on the experimental design. Once the incubation was over, the extraction was carried out following the methodology applied in experiment 1.

Table 1

Experiment 3: non-conventional extraction methods

For the combination of non-conventional methods, two genotypes of native tomatoes (209 and 210) were studied. The other three factors studied were sonication (-1: no, 1: yes), KTN cocktail (-1: no, 1: yes), and green solvent (-1: isopropyl acetate, 1 ethyl lactate); the treatment arrangement was generated by the Statgraphics Centurion XVI software (*27*) (Table 2).

Table 2

The procedure was as follows: 0.5 g of tomato was frozen and pulverised in a mortar with liquid N_2. Subsequently, the tomato was mixed with 5 mL of 20 % *V*/*V* ethanol, vortexed for 5 min, and centrifuged (Avanti-JCI) at 4500x*g* for 10 min. The supernatant was separated and 2 mL of 0.2 M acetate buffer (pH=5) was added to the plant residue. According to the experimental design, 5 % *V/m* of the enzyme was added and incubated for 1 h at 50 °C with stirring (300 rpm). Subsequently, the samples were centrifuged at 4500x*g* and the plant material was separated by filtration, recovered and washed with 5 mL of distilled water. In cases where sonication was applied, the pellet was mixed with 5 mL of the solvent (isopropyl acetate or ethyl lactate; Sigma-Aldrich, St Louis, MO, USA) and sonicated for 10 min

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with an ultrasonic probe (Vibra-Cell, 130 W, 20 kHz, Sonics and Materials Inc., Newtown, CT, USA) in an ice bath. It was subsequently centrifuged at 4500x*g* for 10 min and the organic phase was collected for analysis. In the treatments where no sonication was applied, after enzymatic treatment and subsequent recovery of the plant material, 5 mL of solvent were added to the tomato, vortexed for 10 min, and centrifuged at 4500x*g*. The organic phase was collected and stored at -70 °C until analysis.

Determination of antioxidant activity

The method reported by Re *et al.* (*23*) was followed. A solution of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical (Sigma-Aldrich, St Louis, MO, USA) was prepared with 16.5 mg of potassium persulfate (J.T. Baker, Xalostoc, Mexico) and 96.2 mg of ABTS in 100 mL of distilled water. An aliquot from this solution was taken and diluted in ethanol 96 % *V*/*V* until it reached an absorbance of 0.7 at 734 nm. One hundred microlitres of the sample were mixed with 1000 µL of ABTS solution, incubated in the dark for 10 min, and the absorbance was read using a spectrophotometer (DU650 Spectrophotometer, Beckman Instruments Inc., Brea, CA, USA) at 734 nm. Quantification was performed by using a Trolox standard (Sigma-Aldrich, St Louis, MO, USA) curve and results are expressed as µmol Trolox equivalents per gram of fresh mass (µmol TE/g fm).

Determination of carotenoid content by spectrophotometry

The carotenoid content in tomato extracts was measured by spectrophotometric readings at an adequate dilution to obtain an absorbance between 0.25 and 0.85. Dilutions were made with DCM, which was used as the blank. The residue of other solvents did not affect the scan between 350 and 750 nm. The measurement was carried out at 482 nm by using a specific percentage absorption coefficient $E^{1\%}$=248.0 L/(g·cm), which is reported for lycopene in DCM (*24*). The results are expressed as µmol lycopene equivalents/g fm.

Determination of carotenoids by high-performance liquid chromatography (HPLC)

The technique reported by Fraser *et al.* (*25*) was followed with some modifications. The samples were injected into an Agilent Technologies 1260 (Agilent Technologies, Santa Clara, CA, USA) machine with a quaternary pump and autosampler. A Waters Xterra MS C18 column (5 µm, 4.6 × 250 mm; Waters Corporation, Milford, MA, USA) at 25 °C was used with acetonitrile:methanol:DCM (43:43:14 *V/V/V*; J.T. Baker, Xalostoc, Mexico) as the mobile phase in an isocratic run. The flow was 1 mL/min and the detector (diode-array detection [DAD]) was fixed at 455 nm. Quantification was performed by using standard

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curves of lycopene, β-carotene, and lutein (Sigma-Aldrich, St Louis, MO, USA). The results are expressed as µg carotenoid/g fm.

Determination of tocopherols by HPLC

The tocopherols content of the extracts was quantified according to the method of Méne-Saffrané *et al.* (*26*) with the following modifications. An HPLC system (Shimadzu Prominence 20, Shimadzu Corporation, Kyoto, Japan) equipped with a Shimadzu RF-20 fluorescence detector and a LiChrospher 100 Diol column (4.6 × 250 mm, 5 µm; Merck Millipore Corporation, Burlington, MA, USA) was used. The mobile phase was hexane:methyl *tert*-butyl ether, 90:10 *V*/*V* (J.T. Baker, Xalostoc, Mexico) in an isocratic run at 0.8 mL/min flow rate. The wavelengths used were 296 nm for excitation and 340 nm for emission. For the identification and quantification, standard curves of α-, β-, γ-, and δ-tocopherol (Sigma-Aldrich, St Louis, MO, USA) were used. The results are expressed as µg tocopherol/g fm.

Statistical analysis

The experimental designs and the data analysis were performed by using the Design of Experiment (creation of new design and analyse design, respectively) tool of Statgraphics Centurion XVI.II software (*27*). For fractional factorial designs (2^{4-1}), the software sets the level values (negative or positive) for the first three factors (A, B and C) and the fourth is selected by multiplication of the previous three, either -(A·B·C) or A·B·C (complement). The results were analysed by analysis of variance (ANOVA) followed by Tukey's test. Three replicates were made for each experiment. Significance was fixed at α=0.05. Pareto diagrams (Figs. S1 and S2) were also obtained with the analyse design tool.

RESULTS AND DISCUSSION

Effect of enzyme cocktails and reaction medium on extraction efficiency

Enzymatic treatment of different vegetal materials has been reported to be an effective way to improve extraction efficiency of various compounds of interest (*28,29*). We first evaluated whether the enzymatic treatment had significant effects on carotenoid content and antioxidant activity of the tomato extracts compared to a control sample without enzymatic treatment. At the same time, we determined which enzyme cocktail and reaction medium had the greatest benefits because enzymes are susceptible to modifications in their structure, activity, or affinity to their substrate depending on the environment in which they are immersed (*16,30*).

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Although the reported lytic activity of the different cocktails is similar, the specific composition of each one as well as the source of the obtained enzymes are different, so particularities such as optimal conditions for their activity and affinity with the substrates affected their performance in the tests. For this experiment, the reaction time and temperature were fixed at 3 h and 40 °C, respectively.

Fig. 1 shows the average values of carotenoid content and antioxidant activity in extracts obtained with the enzymatic treatments in the different reaction media tested. Both factors, reaction medium and enzyme cocktail, as well as their interaction significantly influenced ($p<0.001$) the carotenoid content (expressed as lycopene) and the antioxidant activity of the extracts. The KTN cocktail exhibits high performance in terms of carotenoid extraction as well as the maximum value of the antioxidant activity, with a significant difference compared to the control sample without enzyme. Concerning pH, when the reactions were carried out at pH=5, the average values of carotenoid content and antioxidant activity were the highest when using the KTN cocktail, a finding that is consistent with the previous reports for this cocktail (*31*). Recently, Ladole *et al.* (*19*) reported that pH=5 is optimal for tomato lycopene extraction using a pectinase-cellulase enzyme mixture. Interestingly, the KTN cocktail is the only cocktail for which pectinase activity has been reported (*31-33*), which might account for the results obtained. Enzymes can increase the yield of carotenoids in tomato extracts because these pigments are accumulated in specialised intracellular organelles and are not readily reached by solvents (*19*). In earlier reports, better yields of tomato carotenoids have been reported by using pectinases (alone or in combination with cellulase) for lycopene enzyme-assisted extraction from tomato fruit and waste compared with non-pectinase activity enzymatic treatments (*28,34*).

Fig. 1

Surprisingly, the treatment with the KTN cocktail using distilled water as the reaction medium had the second-highest values of carotenoid content and antioxidant activity. This result is interesting because it suggests that the external regulation of the pH of the enzymatic reaction might not be mandatory to obtain good results, perhaps due to the composition of tomato extracts. This would be an advantage for economical and simplicity reasons. Both factors, enzyme and reaction medium, as well as their interaction were significant. It was, therefore, necessary to study them in a more detailed experiment. Based on these results, the KTN enzyme cocktail and two media, the acetate buffer (pH=5) and distilled water were selected for use in experiment 2.

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Optimization of the enzymatic reaction conditions

Fractional factorial experiments have the advantage of allowing the incorporation of qualitative factors (*35*); the reaction medium corresponds to a qualitative factor while enzyme concentration, time of enzymatic reaction and temperature of reaction are quantitative factors. In addition to the pH of the medium, which was partially tested in the initial factorial experiment, the temperature, the enzyme concentration, and the reaction time are factors that can affect the effectiveness of the enzyme activity (*29,30*). The results of experiment 2 are presented in Fig. 2.

Fig. 2

Extracts obtained by treatments 1 (pH=5, time=1 h, enzyme %=1 and T=40° C), 4 (distilled water, time=1 h, enzyme %=5, T=40 °C), and 5 (pH=5, time=1 h, enzyme %=5, T=50 °C) showed the highest carotenoid content ((49.4±1.2), (53.5±0.8) and (55.2±1.4) μg lycopene eq./g fm, respectively, Fig. 2a); treatment 5 (pH=5, time=1 h, enzyme %=5, T=50 °C) had the highest antioxidant activity (1.1±0.5 μmol TE/g fm) followed by treatments 1 (pH=5, time=1 h, enzyme %=1 and T=40° C), 4 (distilled water, time=1 h, enzyme %=5, T=40 °C), and 6 (distilled water, time=5 h, enzyme %=1, T=50 °C) (Fig. 2b). For both the carotenoid content and the antioxidant activity of the extracts, the treatment time was the most influential factor, followed by the temperature, the pH of the medium, and finally the concentration of enzyme cocktail added (ignoring interactions among factors, α=0.05, Fig. S1). This last factor was not significant in the tested conditions.

A fractional factorial design methodology defines, in addition to the significance of the factors, the optimal levels for each factor to maximise the response variables. It is worth mentioning that even if some factors are not significant, as the enzyme concentration was, the method selects an optimal level. The conditions of treatment 5 maximised the values of both studied parameters: reaction medium pH=5, a reaction time of 1 h, a temperature of 50 °C, and an enzyme concentration of 5 % *V*/*m*. The optimal pH, temperature, and enzyme concentration are similar to those reported by Ladole *et al.* (*19*) (pH=5, T=50 °C, and enzyme %=3, respectively) while those authors used a shorter enzymatic treatment time (20 min). This difference could be because those authors used oven-dried material, which produces more uniform and smaller particles, while fresh frozen tomatoes were used in this study.

Optimisation of the extraction by combining clean technologies

From experiments 1 and 2, the conditions of enzymatic treatment to be applied in experiment 3 were selected. In this experiment, the enzymatic treatment and later probe sonication combined with green solvents were tested. Moreover, the sonication conditions were assayed previously (data not

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shown), with the sonication time being the only factor studied (5, 10, and 15 min). From these previous experiments, 10 min of sonication was selected to maximise the carotenoid content and antioxidant activity. Table 2 shows the treatment arrangement.

The experiment 3 results are shown in Fig. 3. The carotenoid content was similar in treatments 3 (genotype=209, sonication=yes, enzyme=yes, solvent=isopropyl acetate), 5 (genotype=210, sonication=yes, enzyme=no, solvent=isopropyl acetate) and 6 (genotype=210, sonication=no, enzyme=yes, solvent=isopropyl acetate) ((165.3±43.6), (149.5±3.7), (150.3±4.7) µg lycopene eq./g fm, respectively) and higher than those of the other treatments. However, the highest antioxidant activity was found in the extract obtained by treatment 3 (genotype=209, sonication=yes, enzyme=yes, solvent=isopropyl acetate) (1.4±0.1 µmol TE/g fm). Interestingly, treatment 4 (genotype=209, sonication=no, enzyme=no, solvent=isopropyl acetate) had the lowest antioxidant activity (0.2±0.1 µg TE/g fm). Unexpectedly, one of the lowest carotenoid values was found in treatment 1 (genotype=210, sonication=yes, enzyme=yes, solvent=ethyl lactate; 51.1±0.1 µg lycopene eq./g fm) despite the application of enzymatic treatment and sonication, as well as treatments 3 (genotype=209, sonication=yes, enzyme=yes, solvent=isopropyl acetate) and 4 (genotype=209, sonication=no, enzyme=no, solvent=isopropyl acetate) from the same tomato genotype exhibited the highest and lowest antioxidant activity. In addition, two treatments with the application of enzymes and sonication (treatments 1 [genotype=210, sonication=yes, enzyme=yes, solvent=ethyl lactate] and 3 [genotype=209, sonication=yes, enzyme=yes, solvent=isopropyl acetate]) had the highest and lowest carotenoid content. It is important to note that, while in treatments 1 and 3 different solvents were used, in treatments 3 and 4 was used the same solvent. These contradictory behaviours reflect the importance of studying the extraction conditions by using experimental designs and statistics because these factors can significantly modify the properties of the extracts obtained.

Fig. 3

Contrary to the results of experiment 2, the factors studied had a different influence on each parameter. While the solvent had the major impact on the carotenoid content (Fig. S2a), the application of digestive enzymes had the most significant influence on the antioxidant activity (Fig. S2b). Interestingly, in the case of the carotenoid content, neither the application of enzymes nor the genotype studied were significant factors, while for the antioxidant activity, the solvent was the factor with the most negligible significance (fractional factorial experiment analysis, α=0.05).

Although the factors had variable relevance according to their effect on the carotenoid content and the antioxidant activity, the method indicated the same optimal levels – treatment 3, namely genotype

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209, sonication, enzyme treatment, and isopropyl acetate – to maximise both responses. Sonication is used to damage tissue integrity by the cavitation phenomenon, in which gas bubbles are formed and grow until they violently collapse, causing implosions that can break cellular walls (*36*). This treatment has been applied successfully to extract lycopene from tomatoes (*37,38*). Regarding the solvent, isopropyl acetate and ethyl lactate are green solvents with good results based on previous publications (*28,39*). Besides providing better extraction in this work, isopropyl acetate has some advantages over ethyl lactate such as having a lower boiling point, limiting the temperature reached during the sonication process and making it easier to obtain a dried extract as well as protecting thermolabile compounds. The two tomato genotypes studied showed a similar carotenoid content because the genotype was not a significant factor for this parameter according to statistics (α=0.05, Fig. S2a); however, the antioxidant activity depended on this factor, with genotype 209 exhibiting the highest antioxidant activity.

Due to the reduction in the number of experimental runs in a fractional factorial design compared with complete factorial experiments, the information that can be obtained from them is limited. If there is an interest to know more precisely the effect of intermediate levels of one factor on the results or the effect of interactions among factors and levels, it would be necessary to study that factor in another type of experimental design, such as factorial designs or by using response surface designs (*35*). However, as only two levels were possible for each variable in this experiment, a fractional factorial design was suitable. Besides, Pareto diagrams (Figs. S1 and S2) are helpful to choose effectively between levels, as well as to decide whether it is necessary to carry out more experiments according to the significance of the factors.

Quantification of carotenoids and tocopherols in native tomato extracts by HPLC

As experiment 3 was the final experiment (experiments 1 and 2 were preliminary experiments), the extracts obtained in it were characterised in more depth to determine the most abundant carotenoids and tocopherols by HPLC. These results are presented in Table 3.

Table 3

Lycopene was the most abundant carotenoid (56.2-179.4 µg/g fm) followed by β-carotene (0.2-20.5 µg/g fm). These results are similar to previous reports of red tomato extracts (*39,40*). Significant differences between treatments were observed; the treatment with the highest carotenoid content was treatment 3 (genotype=209, sonication=yes, enzyme=yes, solvent=isopropyl acetate), which is in line with our spectrophotometric results presented in experiment 3 section determined spectrophotometrically. The lower contents, also in accordance with the spectrophotometric results, were

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presented in treatments 1 (genotype=210, sonication=yes, enzyme=yes, solvent=ethyl lactate) and 2 (genotype=210, sonication=no, enzyme=no, solvent=ethyl lactate). In this work, lutein was found only in trace amounts in the extracts, so its values are not presented.

In the literature, it is possible to find very wide ranges of lycopene and β-carotene contents in cherry tomatoes or native tomatoes. Recent works have reported lycopene contents in Mexican native 'cherry' tomatoes in a range of 44-82 µg/g fm (*7,41*). Vela-Hinojosa *et al.* (*41*) also reported a β-carotene content between 1.4 and 3.0 µg/g fm in cherry red native tomatoes. Kavitha *et al.* (*42*) analysed some cherry and Indian native tomatoes and reported lycopene contents between 20.0 and 151.0 µg/g fm and β-carotene contents between 10.0 and 90.0 µg/g fm (the latter only reported for cherry genotypes). However, Zanfini *et al.* (*43*) reported lycopene values as high as 42.2-60.9 mg/g fm for Italian native red genotypes, while the cherry Shiren genotype had 184.4 mg/g fm. In the same work, native genotypes had 8.4-12.4 mg/g fm of β-carotene and the cherry genotype had 64.8 mg/g fm of β-carotene; these values are markedly higher than those reported in the present work.

In the extracts obtained by applying non-conventional technologies, the four known isoforms of tocopherols were detected. The abundance of them was α>β>γ>δ (Table 3), a finding that is consistent with previous results (*39*). Similarly to the carotenoid content, treatment 3 (genotype=209, sonication=yes, enzyme=yes, solvent=isopropyl acetate) had the highest tocopherol content (α-tocopherol 4.24±0.45 µg/g fm and total tocopherols 6.5±0.3 µg/g fm). However, a significant difference was found only *versus* treatment 4 (genotype=209, sonication=no, enzyme=no, solvent=isopropyl acetate), which had the lowest tocopherol content (2.2±0.3 µg/g fm of α-tocopherol and 3.7±0.3 µg/g fm of total tocopherols). Extracts obtained by treatments 3 (genotype=209, sonication=yes, enzyme=yes, solvent=isopropyl acetate) and 4 (genotype=209, sonication=no, enzyme=no, solvent=isopropyl acetate) also had the highest and lowest antioxidant activity values, respectively (Fig. 3), suggesting a high contribution of tocopherols to antioxidant activity.

The extract with the highest carotenoid and tocopherol contents also had the highest antioxidant activity (genotype=209, sonication=yes, enzyme=yes, solvent=isopropyl acetate); however, the lowest antioxidant activity was found in the extract with the lowest tocopherol content and not with the lowest carotenoid content (genotype=209, sonication=no, enzyme=no, solvent=isopropyl acetate and genotype=210, sonication=yes, enzyme=yes, solvent=ethyl lactate, respectively), suggesting that tocopherols have a better correlation with antioxidant activity than carotenoids. To test this hypothesis, a correlation analysis between the functional compounds and the antioxidant activity of the extracts was carried out (Table 4). The analysis showed that both the carotenoid and tocopherol contents correlated

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positively with the antioxidant activity, with a better correlation for the tocopherol content (Pearson coefficients 0.66 *versus* 0.83). Interestingly, in these correlations, the lycopene content was correlated with the antioxidant activity (0.68) but the α-tocopherol content did not correlate with antioxidant activity (0.58), these two compounds are the main representatives of carotenoids and tocopherols, respectively. These results make it difficult to conclude about the contribution of each compound or group of compounds to the antioxidant activity, suggesting a synergistic effect among different components present in the extracts. As an example of how intricate these antioxidant interactions can be, two studies tested the synergistic effect among carotenoids (including lycopene) and α-tocopherol (*3,15*). While Kotíková *et al.* (*3*) reported a non-synergistic interaction among lycopene and α-tocopherol, Zanfini *et al.* (*15*) reported that the lycopene+α-tocopherol mixture had the maximum synergistic effect. These differences could be due to (among other factors) differences in tested concentrations. Interestingly, Zanfini *et al.* (*15*) also reported no synergistic effects in a lycopene+β-carotene+lutein+α-tocopherol mixture. Another example of positive interactions among carotenoids and tocochromanols was previously published by our group (*36*).

Table 4

CONCLUSIONS

There are very few reports in which simple and clean extraction methods, including green solvents, are combined to obtain antioxidants from tomato. The use of various experimental stages provided a more comprehensive study about the extraction process. Preliminary experiments – experiments 1 and 2 in this study – are always required to test the performance of enzyme cocktails and to establish optimal enzyme cocktail conditions to ensure the maximum yield of carotenoids and antioxidant activity, because each vegetal material has a distinct behaviour. Experiment 1 showed that the enzyme cocktail containing pectinase had the best performance regarding the carotenoid content and antioxidant activity. In experiment 2, the conditions for the enzyme cocktail reaction were optimised by using a fractional factorial design in order to save resources, increasing the efficiency of the extraction procedure. Experiment 3 was performed to test the effect of the combination of the enzyme cocktail and sonication. A significant increase of approximately 40 % to 100 % in carotenoids, 30 % to 100 % in tocopherols, and 20 % to 400 % in antioxidant activity was observed comparing to the extraction method without sonication and without enzymes. Interestingly, antioxidant activity had a significant positive correlation with lycopene and with total tocopherols. Taken together, these results suggest that combination of clean technologies to obtain antioxidant compounds increase the antioxidant activity of

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native tomato lipophilic extracts. Moreover, since the antioxidant activity of tomato extract is a result of the interaction among all its components, our results suggest that extraction methods that increase the extraction of all lipophilic antioxidants are preferable to more selective methods.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material concerning the Pareto diagrams of experiment 2 and experiment 3 is available at www.ftb.com.hr.

AUTHORS' CONTRIBUTION

D.R. Gómez-Linton contributed to the design, performed the experiments, collected and analysed the data, drafted the article, and approved the final version to be published. A. Navarro-Ocaña contributed to the design of the experiments, data analysis, critical revision of the article, and approved the final version to be published. S. Alavez contributed to the design of the experiments, data analysis, critical revision of the article, and approved the final version to be published. R. Lobato-Ortiz contributed to the collection, identification, and propagation of the native tomato genotypes; provided critical revision of the article; and approved the final version to be published. A. Román-Guerrero helped with the design of the experiments and obtaining tomato extracts, provided critical revision of the article, and approved the final version to be published. A. Mendoza-Espinoza helped with the HPLC determinations and data analysis, and approved the final version of the article to be published. J.M. Villa-Hernández contributed to drafting the article, provided critical revision of the article, and approved the final version to be published. L.J.

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Pérez-Flores contributed to the experimental design, provided the material used, helped with the data analysis, provided critical revision of the article, and approved the final version to be published.

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Table 1. Experimental design for experiment 2, enzymatic reaction conditions

Treatment	Reaction medium (-1=pH, 1=DW)	Time/h (-1=1, 1=5)	*V*/% (-1= 1, 1=5)	*Temperature/°C (-1=50, 1=40)
1	-1	-1	-1	1
2	1	1	1	-1
3	-1	1	-1	-1
4	1	-1	1	1
5	-1	-1	1	-1
6	1	1	-1	1
7	-1	1	1	1
8	1	-1	-1	-1

*Complement: -(A·B·C). DW=distilled water

Table 2. Experimental design for experiment 3, combination of clean extraction methods

Treatment	Genotype -1=209, 1=210	Sonication -1=no, 1=yes	Enzyme -1=no, 1=yes	*Solvent -1=IA, 1=EL
1	1	1	1	1
2	1	-1	-1	1
3	-1	1	1	-1
4	-1	-1	-1	-1
5	1	1	-1	-1
6	1	-1	1	-1
7	-1	1	-1	1
8	-1	-1	1	1

*Complement: A·B·C. ET=ethyl lactate; IA=isopropyl acetate

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Table 3. Carotenoid and tocopherol contents in native tomato extracts

Treatment	*w*/(μg/g)					
	Lycopene	β-Carotene	α-toc	β-toc	γ-toc	δ-toc
1	$(56.24±5.74)^{e}$	$(1.63±0.24)^{c}$	$(3.24±0.64)^{ab}$	$(1.03±0.12)^{c}$	$(0.27±0.02)^{c}$	$(0.18±0.03)^{b}$
2	$(61.31±3.67)^{e}$	$(0.20±0.10)^{d}$	$(3.88±0.93)^{ab}$	$(0.99±0.14)^{c}$	$(0.45±0.05)^{b}$	$(0.41±0.08)^{a}$
3	$(179.41±15.32)^{a}$	$(20.51±3.93)^{a}$	$(4.24±0.45)^{a}$	$(1.56±0.09)^{a}$	$(0.43±0.09)^{b}$	$(0.29±0.04)^{b}$
4	$(102.54±7.51)^{c}$	$(10.38±3.87)^{b}$	$(2.23±0.33)^{b}$	$(1.10±0.10)^{bc}$	$(0.37±0.06)^{b}$	$(0.04±0.01)^{c}$
5	$(147.64±11.38)^{b}$	$(14.32±5.78)^{ab}$	$(3.15±0.70)^{ab}$	$(1.36±0.21)^{ab}$	$(0.77±0.27)^{a}$	$(0.42±0.06)^{a}$
6	$(114.08±8.23)^{c}$	$(1.78±0.13)^{c}$	$(2.69±0.54)^{ab}$	$(1.17±0.18)^{bc}$	$(0.97±0.21)^{a}$	$(0.34±0.05)^{b}$
7	$(145.71±14.76)^{b}$	$(3.39±1.77)^{c}$	$(4.15±0.61)^{a}$	$(1.13±0.19)^{bc}$	$(0.44±0.04)^{b}$	$(0.27±0.03)^{b}$
8	$(82.56±10.64)^{d}$	$(1.96±0.62)^{c}$	$(3.20±0.38)^{ab}$	$(1.24±0.19)^{bc}$	$(0.42±0.13)^{b}$	$(0.33±0.08)^{b}$

Mean values and SD. toc=tocopherol. Different letters indicate significant differences according to the Tukey test, α=0.05.

Table 4. Correlations between carotenoids, tocopherols and antioxidant activity

	Carotenoids	Tocopherols	α-tocopherol	Lycopene	Antioxidant activity
Carotenoids		0.478/0.116	0.260/0.267	0.996/0.001*	0.664/0.036*
Tocopherols	0.478/0.116		0.880/0.002*	0.494/0.107	0.828/0.005*
α-tocopherol	0.260/0.267	0.880/0.002*		0.273/0.257	0.582/0.065
Lycopene	0.996/0.001*	0.494/0.107	0.273/0.257		0.683/0.031*
Antioxidant activity	0.664/0.036*	0.828/0.005*	0.582/0.065	0.683/0.031*	

Correlation coefficients/p values. α=0.05

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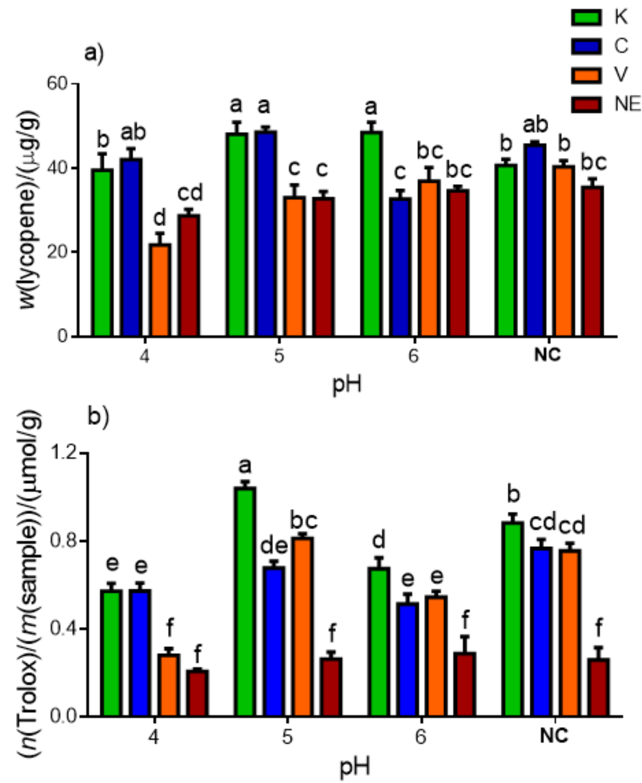

Fig. 1. Carotenoid contents (a) and Antioxidant activities (b) (mean and SD) in commercial tomato extracts applying different enzyme cocktails in different reaction media. Different letters indicate significant difference according to the Tukey test (α=0.05)

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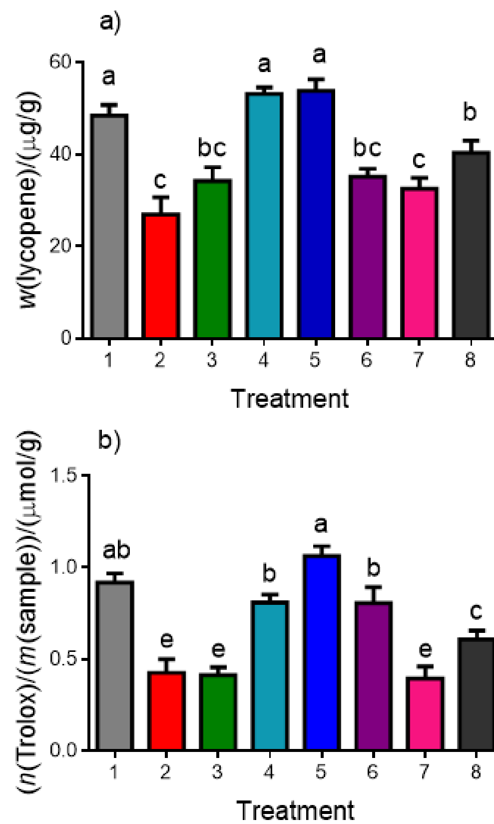

Fig. 2. Carotenoid contents (a) and Antioxidant activities (b) of extracts obtained using the KTN cocktail under different enzymatic reaction conditions. Different letters indicate significant difference according to the Tukey test (α = 0.05)

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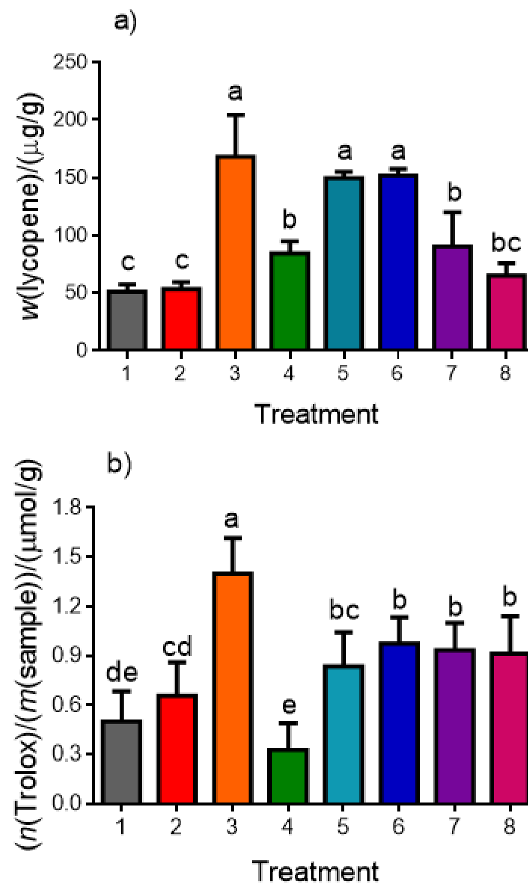

Fig. 3. Carotenoid contents (a) and Antioxidant activities (b) of extracts obtained from native tomatoes by application of digestive enzymes (KTN) and sonication. Different letters indicate significant difference according to the Tukey test (α=0.05)

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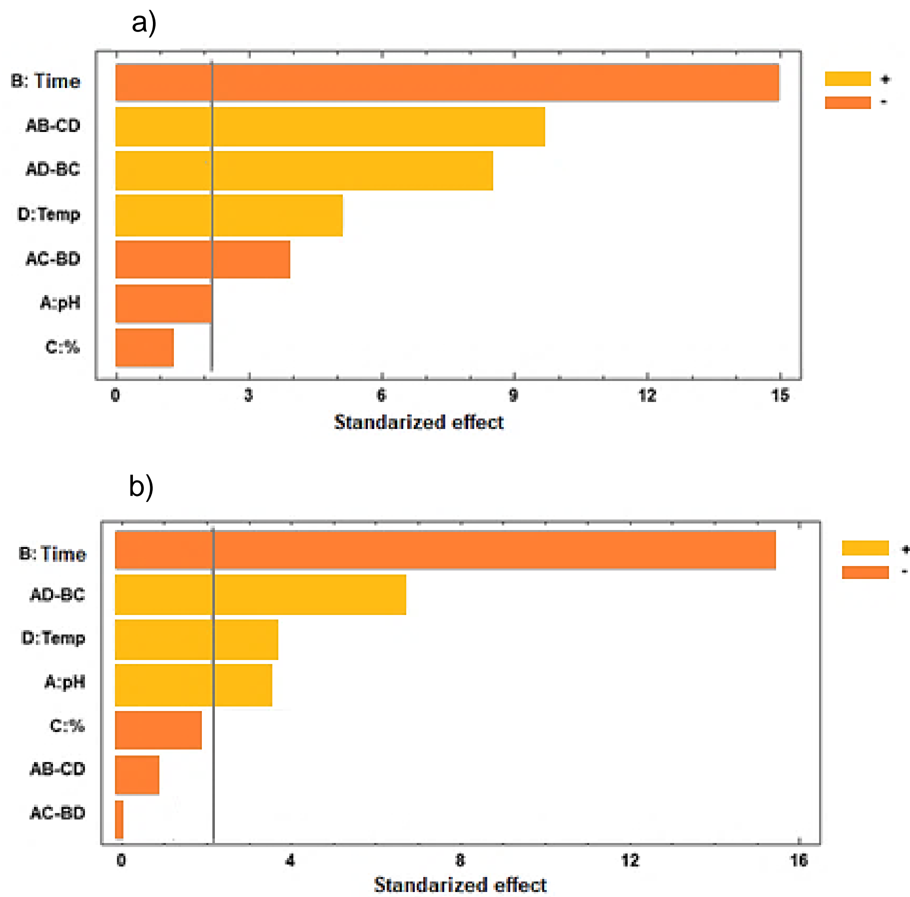

Fig. S1. Pareto diagrams of (a) carotenoids analysis and (b) antioxidant activity analysis corresponding to experiment 2

a)

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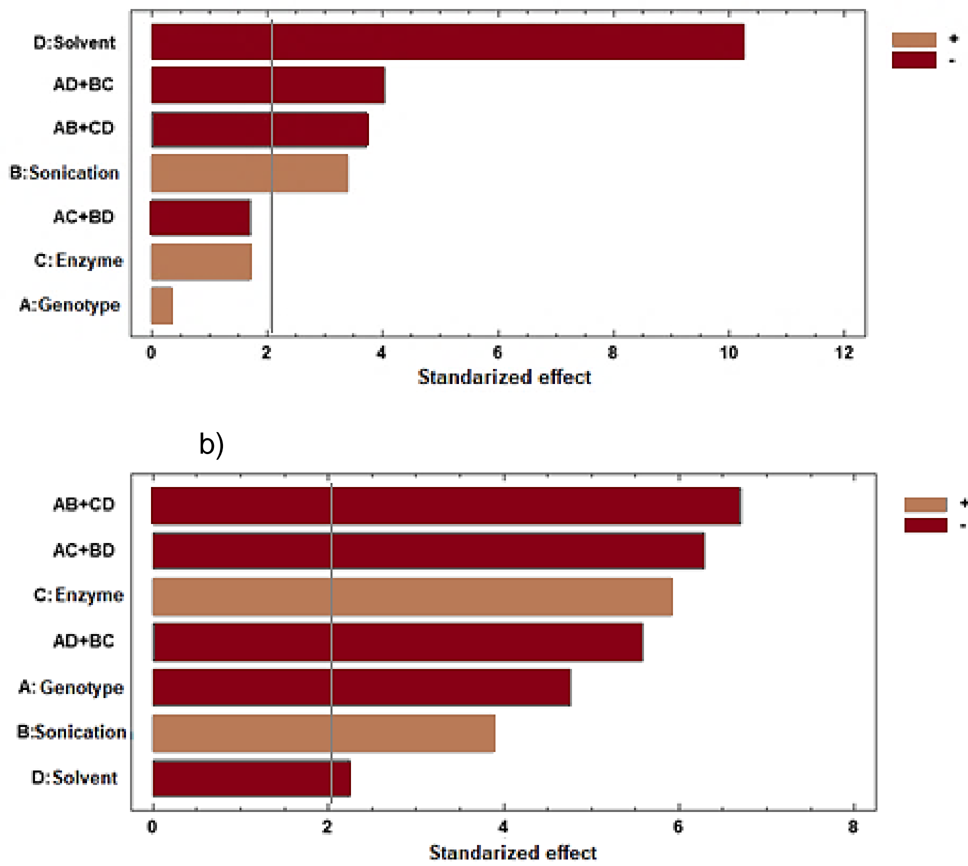

Fig. S2. Pareto diagrams of carotenoids analysis (a) and antioxidant activity analysis (b) corresponding to experiment 3