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## **Enzyme-aided extraction of lycopene from high-pigment tomato cultivars by supercritical carbon dioxide**

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### **Abstract**

This work reports a novel enzyme-assisted process for lycopene concentration into a freeze-dried tomato matrix and describes the results of laboratory scale lycopene supercritical CO<sub>2</sub> (SC-CO<sub>2</sub>) extractions carried out with untreated (control) and enzyme-digested matrices. The combined use of food-grade commercial plant cell-wall glycosidases (Celluclast/Novozyme plus Viscozyme) allows to increase lycopene (~153%) and lipid (~137%) concentration in the matrix and rises substrate load onto the extraction vessel (~46%) compared to the control. The addition of an oleaginous co-matrix (hazelnut seeds) to the tomato matrix (1:1 by weight) increases CO<sub>2</sub> diffusion through the highly dense enzyme-treated matrix bed and provides lipids that are co-extracted increasing lycopene yield. Under the same operative conditions (50 MPa, 86 °C, 4 mL min<sup>-1</sup> SC-CO<sub>2</sub> flow) extraction yield from control and Celluclast/Novozyme + Viscozyme-treated tomato matrix/co-matrix mixtures was similar, exceeding 75% after 4.5 h of extraction. However, the total extracted lycopene was ~3 times higher in enzyme-treated matrix than control.

### **Keywords**

Carotenoids, Cell-wall hydrolases, Enzymatic digestion, Freeze-dried tomato matrix, Green chemistry, *Lycopersicon esculentum* (Mill.), Oleoresin

### **1. Introduction**

Lycopene, the red pigment synthesised and stored in tomato berry chromoplasts, is one of the over 750 carotenoids found in nature (Britton, 2004). It is used in food, pharmaceutical and cosmetic industry as natural dye, antioxidant, cancer-preventing and anti-ageing product. A number of *in vitro*, *in vivo* and *ex vivo* studies have demonstrated that lycopene supplementation is inversely associated to cardiovascular diseases, cancer risk and diabetes, through its powerful antioxidant activity alongside other, not yet fully understood, mechanisms of action (Kong et al., 2010, Palozza

et al., 2012). Lycopene extraction with supercritical carbon dioxide (SC-CO<sub>2</sub>) from red-ripe tomato berries is an excellent technique suitable to replace the use of harmful organic solvents and satisfy the increasing demand for biological solvent-free lycopene (Konar, Haspolat, Poyrazoğlu, Demir, & Artık, 2012). The preparation of a freeze-dried tomato matrix is a key step to maximize the efficiency and yield of lycopene extraction by SC-CO<sub>2</sub> (Lenucci et al., 2010). In the tomato matrix, consisting of dehydrated clumps of berry mesocarp parenchyma cells, lycopene is present as red crystals within the chromoplasts. Lycopene crystals are enclosed into newly synthesised membranes originating by infoldings of the inner membrane plastid envelope (Simkin, Zhu, Kuntz, & Sandmann, 2007). Thus, to solubilise and extract lycopene, the supercritical fluid must reach these organelles penetrating through the pecto-cellulosic primary cell-wall and the membrane bilayers of cell which represent potential obstacles to fluid free diffusion.

Enzymes have been extensively used to improve the yield and quality of several plant natural products, including flavouring, colorants and bioactives [see Sowbhagya and Chitra (2010) and Puri, Sharma, and Barrow (2012) for excellent reviews]. Pre-treatments with cell wall hydrolases have proved effective in enhancing the extraction rate and quality of oily products from a variety of oleaginous seeds and fruits by mechanical or organic solvent based methods (Domínguez, Núñez, & Lema, 1994). Since the primary cell wall of dicotyledonous plants comprises mainly of cellulose, hemicelluloses and pectins, most of the works in this field utilise cocktails of cellulase, xyloglucanase and pectinase to hydrolyse and degrade the polysaccharide network surrounding the cell, improving, in this way, the release of intracellular contents by hydro-distillation or conventional solvent extraction (CSE). Following this approach, enzymatic mixtures have been explored as a mean to enhance the extraction of capsaicinoids and carotenoids from chilli pepper (Santamaría et al., 2000),  $\alpha$ - and  $\beta$ -carotene from orange peel, sweet potatoes and carrots (Çinar, 2005), lycopene from tomato tissues (Choudhari and Ananthanarayan, 2007, Zuorro et al., 2011, Papaioannou and Karabelas, 2012) lutein from marigold flowers (Barzana et al., 2002), and flavonoids from *Ginkgo biloba* (Chen, Xing, Huang, & Xu, 2011) or pigeonpea (Fu et al., 2008) leaves. However, as far as we know, the combined use of cell-wall hydrolytic enzymes and supercritical fluids to extract plant metabolites has been only marginally explored. Recently, a similar approach has been assessed for the enhancement of supercritical fluid extraction of grape seed oil obtaining a considerable increase (+44%) in oil yield compared to untreated seeds (Passos, Silva, Da Silva, Coimbra, & Silva, 2009).

The present work reports a novel enzyme assisted process for the deconstruction of primary cell-wall of tomato parenchyma cells aimed at the preparation of a matrix suitable for an improved lycopene extraction by SC-CO<sub>2</sub>. In addition, we describe the results of laboratory scale SC-CO<sub>2</sub> extraction tests carried out with control and treated tomato matrices, pure or blended with different co-matrices, in order to optimise lycopene extraction yield.

This work is the first comprehensive report on the possible use of hydrolytic enzyme pretreatments for SC-CO<sub>2</sub> extraction of carotenoids from plants. It also suggests some technological solutions to overcome the problems related to the excessive packing density of the enzyme digested tomato matrices.

## 2. Materials and methods

### 2.1. Chemicals

Celluclast 1.5 L (Cellulase from *Trichoderma reesei*, declared activity 700 EGU g<sup>-1</sup>), Novozyme 188 (Cellobiase from *Aspergillus niger*, declared activity  $\geq 250$  U g<sup>-1</sup>), Viscozyme L (multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase,  $\beta$ -glucanase, hemicellulase, xylanase and pectinase from *Aspergillus* sp., declared activity  $\geq 100$  FBG g<sup>-1</sup>) and Flavourzyme 500 L (Protease from *Aspergillus oryzae*, declared activity  $\geq 500$  U g<sup>-1</sup>) were from Novozymes A/S (Bagsvaerd, Denmark).

(all-*E*)-lycopene standard was purchased from CaroteNature (Ostermundigen, Switzerland), the other lycopene (*Z*)-isomers were prepared by using iodine or heat isomerisation and fractionation using preparative C<sub>30</sub>-HPLC as described by Fröhlich, Conrad, Schmid, Breithaupt, and Böhm (2007), and showed a purity of 97–99%. All the HPLC grade solvents were purchased from Sigma–Aldrich (Milan, Italy). High purity carbon dioxide (99.995%) for supercritical fluid extraction was purchased from Mocavero Ossigeno (Lecce, Italy).

## **2.2. Plant material**

All experiments were carried out using a tomato purée prepared from red-ripe tomatoes of the high-pigment cultivar HLY 18 (COIS '94 Srl, Belpasso, Italy) as previously described (Lenucci et al., 2010). This variety, obtained by conventional breeding techniques, is characterised by a high lycopene content.

Briefly, tomatoes were subjected to extensive washing under running water, blanched in water at 70 °C for 5 min, crushed and sieved by a Reber 9004 N tomato squeezer (Reber, Luzzara, Italy) in order to obtain a tomato purée made up of cell clumps of similar size to the sieve used (1 mm), separated from skins, seeds and vascular tissues. Subsequently, the tomato purée was packed into 1 L screw top glass jar, pasteurised at 121 °C, and stored in the dark at room temperature until use.

## **2.3. Tomato purée digestion and matrix preparation**

For studies aimed to assess the effect of enzyme concentration and digestion time on freeze-dried matrix weight and lycopene titer, experiments were carried out in small volumes. Triplicate aliquots (50 mL) of tomato purée were digested with 0–2% v/v of: Celluclast/Novozyme (3:1 by vol.), Viscozyme or Flavourzyme, for 0–48 h, at 50 °C, under constant stirring. Enzyme-treated tomato purées were centrifuged at 27,000g for 10 min by using a J2-21 Beckmann centrifuge (Beckman Coulter, Fullerton, CA, US) to remove water soluble substances. The pellets were dehydrated to constant weight by a Christ ALPHA 2-4 LSC freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Freeze-dried tomato matrices were ground in a laboratory ultra centrifugal mill (ZM200, Retsch GmbH, Haan, Germany) through 35 mesh (500 µm) sieve, vacuum-packaged in food grade oxygen impermeable plastic bags and stored in a freezer at –20 °C. The procedure was scaled-up to obtain an amount of lyophilised tomato matrix sufficient to perform pilot-scale SC-CO<sub>2</sub> extractions of lycopene. In this case the freeze-dried matrices were prepared by digesting, for 24 h, at 50 °C, under constant stirring, 1L of tomato purée with 0.25% v/v of: (1) Celluclast/Novozyme (3:1 by vol.); (2) Viscozyme; (3) Flavourzyme; (4) Celluclast/Novozyme (3:1 by vol.) + Viscozyme; (5) Celluclast/Novozyme (3:1 by vol.) + Flavourzyme; (6) Celluclast/Novozyme + Viscozyme + Flavourzyme. A control matrix was prepared from undigested tomato purée.

## **2.4. Residual moisture determination**

Residual moisture in freeze-dried tomato matrices was measured gravimetrically, on 1.0 g aliquots (three independent replicates), after further drying at 105 °C to constant weight in a Büchi TO-50 infrared drier (Büchi Labortechnik AG, Postfach, Switzerland).

## **2.5. Determination of lycopene, total lipids and total proteins in the freeze-dried matrices**

Triplicate aliquots of each freeze-dried tomato matrix (50 mg) were used for the extraction of lycopene by the method of Perkins-Veazie, Collins, Pair, and Roberts (2001). All extractions were carried out in the dark to prevent lycopene degradation and/or isomerisation.

Total lycopene content was determined by HPLC as previously described (Tlili et al., 2011). Briefly, carotenoids were extracted with 0.05% (w/v) BHT in acetone and 95% ethanol (1:1, v/v), separated by partition into hexane and directly assayed by a Dionex HPLC (Dionex s.r.l., Milan, Italy) with an AD 25 UV–Vis detector. The separation was performed at 31 °C on an Acclaim HPLC column C<sub>18</sub> (5 µm, 250 × 4.6 mm) by using a linear gradient of acetonitrile (A), hexane (B) and

methanol (C) as follow: from 70% A, 7% B, 23% C to 70% A, 4% B, 26% C within 35 min, with a flow rate of 1.5 mL min<sup>-1</sup>. Peaks were detected at 503 nm.

Lycopene isomers were analysed, in the same above mentioned extracts, using a Merck-Hitachi (Darmstadt, Germany) HPLC system (pump L-7100, degasser L-7614, autosampler L-7200, diode array detector L-7450, interface L-7000) and a Jetstream plus column oven (JASCO, Gross-Umstadt, Germany). An analytical polymeric C<sub>30</sub>-column [YMC Carotenoid S, 5 µm, 250 × 4.6 mm (YMC Europe, Dinslaken, Germany)], preceded by a C<sub>18</sub> ProntoSil 120-5-C<sub>18</sub> H (5 µm, 10 × 4.0 mm) column (Bischoff, Leonberg, Germany), was used. As mobile phase (1.3 mL min<sup>-1</sup>) the following gradient of methanol (A) and methyl *tert*-butyl ether (B) was used: 0 min, 90:10 A:B; 40 min, 50:50 A:B; 60 min, 50:50 A:B; 70 min, 90:10 A:B; 75 min, 90:10 A:B. Column temperature was 23 ± 1 °C and detection wavelength 450 nm.

Total lipids were gravimetrically determined using the Folch method (Folch, Lees, & Sloane-Stanley, 1957) starting from triplicate 500 mg aliquots of each freeze-dried matrix.

Proteins in the freeze-dried matrices treated with Flavourzyme were determined by the Kjeldahl method, using 6.25 as conversion factor (AOAC, 1994). Three determinations were performed for each sample.

### **2.6. Scanning electron microscopy of tomato matrices**

Microstructure observation of control and enzyme digested tomato matrices were carried out by scanning electron microscopy (SEM) using a ZEISS EVO HD 15 (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) operated under high-vacuum at an accelerating voltage of 3 kV.

### **2.7. Packing density**

The packing density of tomato matrices in the extractor was determined gravimetrically weighing the material loaded (at a compressive pressure of 2.0 MPa) in a 25 mL extraction vessel. The measure was repeated three times.

### **2.8. Extraction of lycopene from freeze-dried matrices by supercritical CO<sub>2</sub>**

SC-CO<sub>2</sub> extraction was carried out using a laboratory apparatus (Spe-ed SFE system, Applied Separations, Allentown, PA, USA) fitted with a 25 mL stainless-steel extraction vessel ( $\phi = 1$  cm;  $h = 25$  cm). For each extraction, the fed material (from 19 to 29 g in keeping with the packing density) was packed into the vessel and extracted statically (no fluid flow) for 15 min and subsequently dynamically, for 60 min, or up to 270 min in the experiments aimed to study the time course of SC-CO<sub>2</sub> lycopene extraction yield. The carbon dioxide flow rate was kept constant at 4 mL min<sup>-1</sup>. The other operative parameters were 50 MPa and 86 °C for pressure and temperature, respectively. The extracted oils were stored, under enriched CO<sub>2</sub> atmosphere and protected from light, at -20 °C until further analyses. Each extraction was repeated at least three times starting from the same batch of freeze-dried tomato matrix. In some experiments the freeze-dried matrices were blended with an inert co-matrix (PSE-matrix, Applied Separation, Lehigh, PA – USA) in a ratio of 4:1 by weight or with an oleaginous co-matrix consisting of roughly crushed hazelnuts, a seed particularly rich in oil (~75% w/w).

Triplicate aliquots (30 mg) from each oleoresin were dissolved in 5 mL of hexane and assayed for lycopene or lycopene isomer distribution by HPLC, as described above.

### **2.9. Statistical analysis**

Results are presented as the mean value ± standard deviation of three independent experiments ( $n = 3$  for each trial). Statistical analysis was based on a one-way ANOVA test. Holm–Sidak Test method was applied to establish significant differences between means ( $p < 0.05$ ). All statistical comparisons were performed using SigmaStat version 11.0 software (Systat Software Inc., Chicago, IL).

### 3. Results and discussion

#### 3.1. *Tomato purée digestion and matrix preparation*

In a previous study (Lenucci et al., 2010) we reported the use of red-ripe berries of a hybrid selection of high-lycopene tomatoes (cultivar HLY 18) to prepare a freeze-dried matrix suitable for lycopene SC-CO<sub>2</sub> extraction which had a lycopene titer of ~10 mg g<sup>-1</sup> freeze-dried matter (f-dm). SC-CO<sub>2</sub> extraction of this matrix gave an amount of total extracted lycopene (~7.1 mg g<sup>-1</sup> f-dm) much higher compared with the use of the matrix from the ordinary tomato cultivar Incas (~4.0 mg g<sup>-1</sup> f-dm). We also highlighted the variability in the glycosyl residue composition of cell wall polysaccharides of high-pigment tomato cultivars (Lenucci, Leucci, Piro, & Dalessandro, 2008) and assayed their enzymatic hydrolysis by a commercial glycosidase mixture (Driselase) for bioethanol production. We found that the polysaccharide typology and amount of red-ripe HLY 18 tomato cell-walls overlap with that of ordinary red-ripe tomato cultivars (Lenucci, Durante, Montefusco, Dalessandro, & Piro, 2013). The data showed, beside cellulose, the presence of large amounts of pectic polysaccharides mainly constituted of homogalacturonan and rhamnogalacturonan I substituted with arabinosyl-, galactosyl- and arabino-galactosyl-containing side chains, and of hemicelluloses including xyloglucans, xylans and glucomannans (Lenucci et al., 2010).

As more than 80% of the weight of the freeze-dried tomato matrix is due to cell wall polysaccharides (Lenucci et al., 2013), while the small amount of structural proteins contribute to the maintaining of cell wall architecture (Lamport, Tan, Kieliszewski, 2011). The enzymatic hydrolysis of such polymers by mixtures of specific cell-wall glycosidases and/or proteases could contribute to a further concentration of lycopene within the matrix and, at least in theory, facilitate its extraction by SC-CO<sub>2</sub>.

In this work two different commercial glycosidase mixtures (Celluclast/Novozyme and Viscozyme) and a fungal protease-peptidase complex (Flavourzyme), commonly used in food industry, were employed, individually and in combination, in the preparation of freeze-dried tomato matrices to be tested for SC-CO<sub>2</sub> extraction of lycopene.

Preliminary experiments demonstrated that all three enzymatic commercial preparations were active at the acidic media (pH 5.0) of the tomato purée with no need of pH buffering. Both glycosidases mixtures determined, in fact, a significant increase in lycopene titer of the freeze-dried matrix with respect to the control, but no statistically significant differences were found among buffered and not buffered samples neither for Celluclast/Novozyme ( $p = 0.723$ ) nor for Viscozyme ( $p = 0.775$ ) digestions (Fig. S1). Besides, Flavourzyme digested samples, while not presenting any significant difference in lycopene titer compared to control, showed a reduction of over 50% of the total amount of protein regardless of buffering (data not shown).

#### 3.2. *Effect of enzyme concentration on matrix weight and lycopene titer*

The effect of hydrolytic enzyme concentration on the weight and lycopene titer of the freeze-dried matrices prepared from tomato purée of the cv. HLY 18 is reported in Fig. 1A. Both glycosidase treatments caused a significant decrease in the weight of the obtained freeze-dried matrices ( $p < 0.01$ ). Celluclast/Novozyme was more effective than Viscozyme in catalysing cell wall polysaccharide degradation. A concentration of 0.25% by vol. of Celluclast/Novozyme determined a weight reduction of the freeze dried matrix of ~51% with respect to the undigested tomato purée (control). A higher concentration of the mixture did not further improve weight loss. When increasing concentrations of Viscozyme were used, an almost linear weight loss was observed up to 2% by vol.; in this case, a 35% weight loss was reached at the maximum enzyme concentration tested (2% by vol.). Similar concentrations of commercially available cell-wall degrading enzymes were found to enhance the recovery of carotenoids from different plant organs extracted by CSE (Barzana et al., 2002, Choudhari and Ananthanarayan, 2007). Pectinase was more effective than cellulase for lycopene extraction from whole tomatoes, tomato peel and laboratory pulper tomato wastes, but not

from industrial tomato wastes, on account of the differences in the chemical composition of these fractions (Choudhari & Ananthanarayan, 2007).

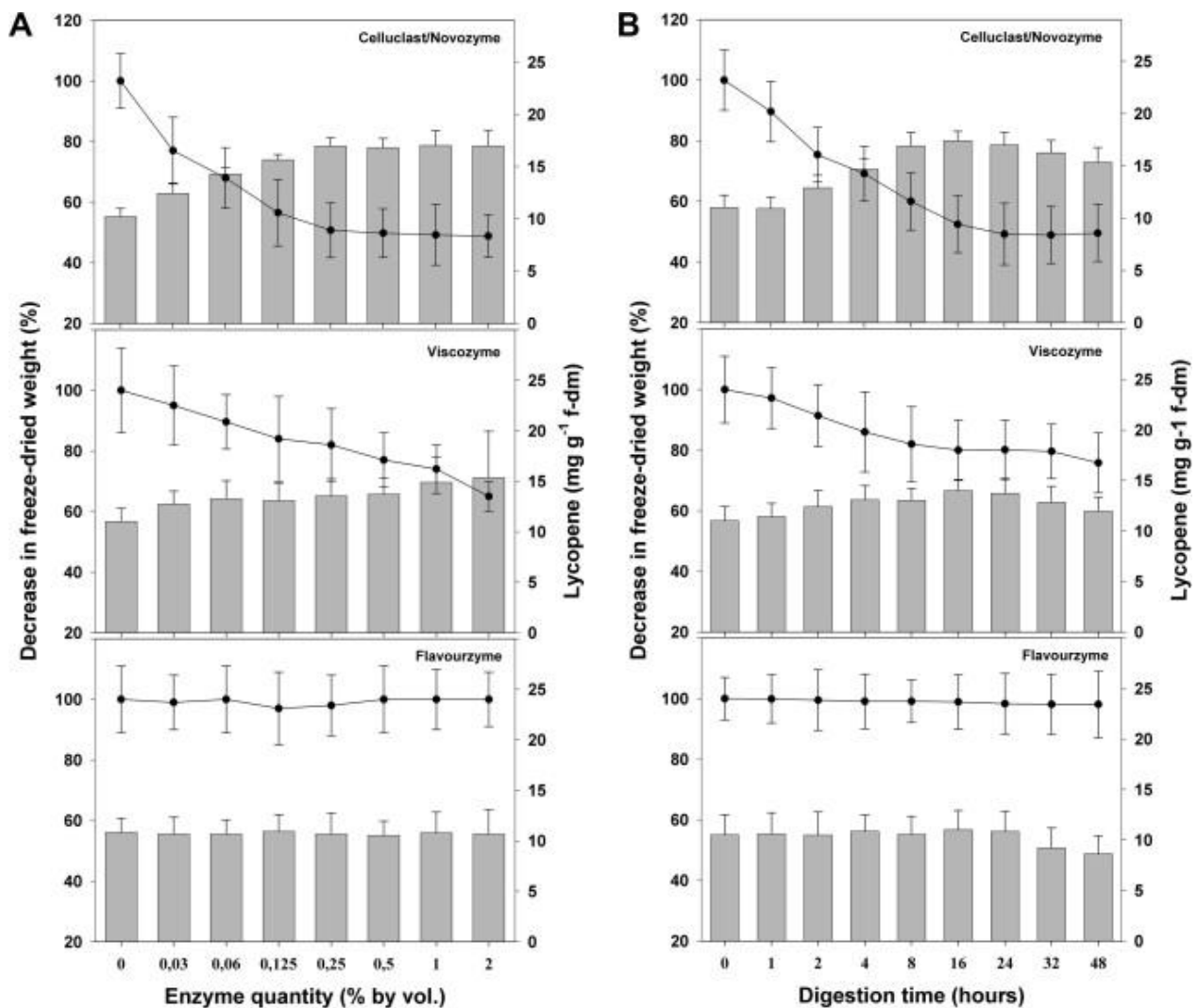


Fig. 1. Effect of enzyme concentration (A) and digestion time (B) on the freeze-dried weight and lycopene titer of tomato matrices prepared by digesting tomato purée (cv. HLY 18) with Celluclast/Novozyme (3:1 by vol), Viscozyme or Flavourzyme, at 50 °C, under continuous stirring. In the enzyme concentration studies the incubation time was 24 h; during the time course enzyme concentration was 0.25% by vol. Data are means  $\pm$  standard deviation of three independent replicates ( $n = 3$ ).

In line with the weight reduction of the freeze-dried tomato matrix, a significant increase in lycopene titer was observed. Lycopene strongly concentrated in the Celluclast/Novozyme (0.25% by vol.) treated freeze-dried matrix passing from  $\sim 10.2 \text{ mg g}^{-1} \text{ f-dm}$  of the control matrix to  $\sim 17.0 \text{ mg g}^{-1} \text{ f-dm}$ . The increase in lycopene titer of Viscozyme digested freeze-dried tomato matrix was much smaller (+39%) even when 2% by vol. of the enzyme was used, probably due to the greater specificity of Viscozyme for pectins. All tested concentrations of Flavourzyme did not affect either weight or lycopene titer of the freeze-dried tomato matrix, although a  $\sim 40\%$  protein reduction was evidenced in 0.25% by vol. Flavourzyme treated freeze-dried matrix with respect to the control. In this regard, it is important to emphasise that most of soluble proteins were previously removed by the tomato purée centrifugation step during matrix preparation (see Section 2.3 of Materials and methods for details).

### **3.2.1. Effect of enzyme digestion time on matrix weight and lycopene titer**

At the enzyme concentration of 0.25% by vol. (a good compromise between the desired reduction of enzyme consumption and the need of performing the hydrolysis), an incubation time of 16 h was sufficient for both Celluclast/Novozyme and Viscozyme to halve the weight of the lyophilised matrix and concentrate lycopene into the freeze-dried tomato matrix (Fig. 1B); while no differences were observed after Flavourzyme digestion. For all treatments, an increase of the incubation time did not result in further reductions of the freeze-dried matrix weight, but it caused a slight decrease in lycopene titer, possibly due to oxidation. It is well known that lycopene stability is strongly affected by physical and chemical factors including heat, light, oxygen and extreme pH exposure (Nguyen & Schwartz, 1999). Studying thermal stability of standard lycopene dissolved in HPLC-grade hexane, Lee and Chen (2002) found a significant decrease of total lycopene after heating at 50 °C for 9 h, time after which degradation reaction dominated over isomerisation. The higher stability of lycopene found in our study is possibly due to the protective effect of other substances within the matrix. Actually, increasing piece of evidence indicates that matrix system plays a fundamental role in lycopene resistance or susceptibility to degradation during food processing and storage (Xianquan, Shi, Kakuda, Yueming, 2005).

### **3.2.2. Effect of enzyme blends on the concentration of lycopene and total lipids in the matrices**

Fig. 2 shows the titer of lycopene (A) and total lipids (B) in the freeze-dried matrices resulting from digestion of tomato purée using the three commercial enzyme preparations, individually or in combination, at the concentration of 0.25% by vol., for 16 h, at 50 °C. With the exception of treatment with Flavourzyme, the use of all other enzymes, or enzyme blends, led to a significant increase of lycopene and total lipid concentrations in the freeze-dried matrices with respect to the control. This was mainly due to the partial removal of cell-wall polymers and also, at least partially, to a more efficient precipitation of lycopene from the 27,000 g supernatant obtained after the tomato purée centrifugation (step essential to remove water and soluble solutes from the fresh matrix) possibly caused by changes in the viscosity of the media (Supplementary Fig. S2). The freeze-dried matrix obtained from Celluclast/Novozyme + Viscozyme hydrolysate showed the highest concentration of lycopene (~27.6 mg g<sup>-1</sup> f-dm) and total lipids (118.0 mg g<sup>-1</sup> f-dm), respectively 2.4- and 2.5-fold the quantities measured in the control (~10.9 mg g<sup>-1</sup> f-dm and ~50.0 mg g<sup>-1</sup> f-dm, respectively), consistently with the fact that pectinase improves cell-wall lysis. No statistically significant increase in lycopene and total lipid concentration in the freeze-dried matrix was obtained by adding Flavourzyme to the Celluclast/Novozyme + Viscozyme mixture. Furthermore, the addition of Flavourzyme to Celluclast/Novozyme did not significantly affect neither lycopene content with respect to Celluclast/Novozyme digested matrix (~19.5 mg g<sup>-1</sup> f-dm vs. 16.5 mg g<sup>-1</sup> f-dm) nor lipid concentration (82.6 mg g<sup>-1</sup> f-dm vs. 73.2 mg g<sup>-1</sup> f-dm). A 8- to 10-fold increase in lycopene concentration was recently obtained incubating homogenised tomato peels, for 4 h at 50 °C, with 0.3% or 3% cellulase (Cellulyve 50LC) by Cuccolini, Aldini, Visai, Daglia, and Ferrari (2013), while the addition of pectinase (Peclyve) did not result in further increase of lycopene concentration. The authors reported an additional improvement in lycopene concentration (30-fold with respect to the untreated peels) by a second enzymatic treatment using a protease cocktail (Prolyve 1000), demonstrating the strong action of enzymatic treatments to concentrate lycopene within the matrix. Enzymatic treatment has been reported to enhance carotenoid content in dehydrated marigold flower meal by Delgado-Vargas and Paredes-López (1997).

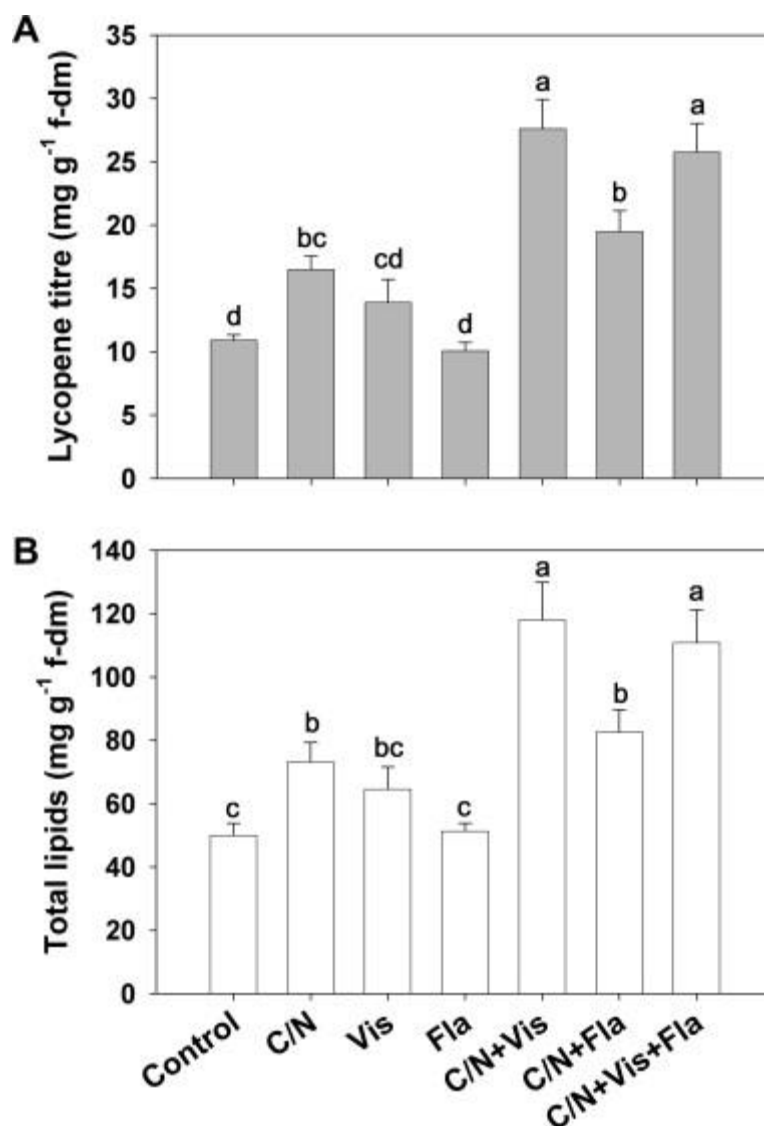


Fig. 2. Lycopene (A) and total lipids (B) titers in freeze-dried matrices resulting from the hydrolysis of tomato purée (cv. HLY 18) using the three commercial enzyme preparation individually or in combination. Each enzyme was added at the concentration of 0.25% by vol. Digestions were carried out at 50 °C, for 16 h, under continuous stirring. Data, expressed as mean  $\pm$  standard deviation, are representative of three independent replicates ( $n = 3$ ). Data were submitted to one-way analysis of variance (ANOVA), bars marked with different letters indicate significant differences among treatments (Holm–Sidak post-hoc test,  $p < 0.05$ ).

Lipids concentration linearly correlated with that of lycopene in the freeze-dried matrices ( $r^2 = 0.995$ ;  $p < 0.001$ ) according to the function:  $\text{mg lipids g}^{-1} \text{ f-dm} = 4.37 \text{ mg lycopene g}^{-1} \text{ f-dm}$ . Lipid increase is a positive feature of the digested matrices since it is known that lipids contribute to enhance lycopene extraction efficiency by increasing the solubility of the analyte in the supercritical fluid and/or the fluid flow rate of carotenoids through the matrix and pipelines (Durante et al., 2014, Lenucci et al., 2010).

### 3.3. Scanning electron microscopy of freeze-dried tomato matrices

Scanning electron micrographs of milled (35 mesh) freeze-dried tomato matrices (Fig. 3) showed that all samples suffered extensive damage at the cellular level. Control and Flavourzyme treated matrices (Fig. 3A and D, respectively) exhibited a similar microstructure characterised by large but porous and flaky particles. Celluclast/Novozyme and Viscozyme treatments (Fig. 3B and C, respectively), and even more digestion with multiple enzyme mixtures (Fig. 3E and F), determined significant structural



changes in the matrix which appeared more compact than control and constituted of smaller and more regular particles.

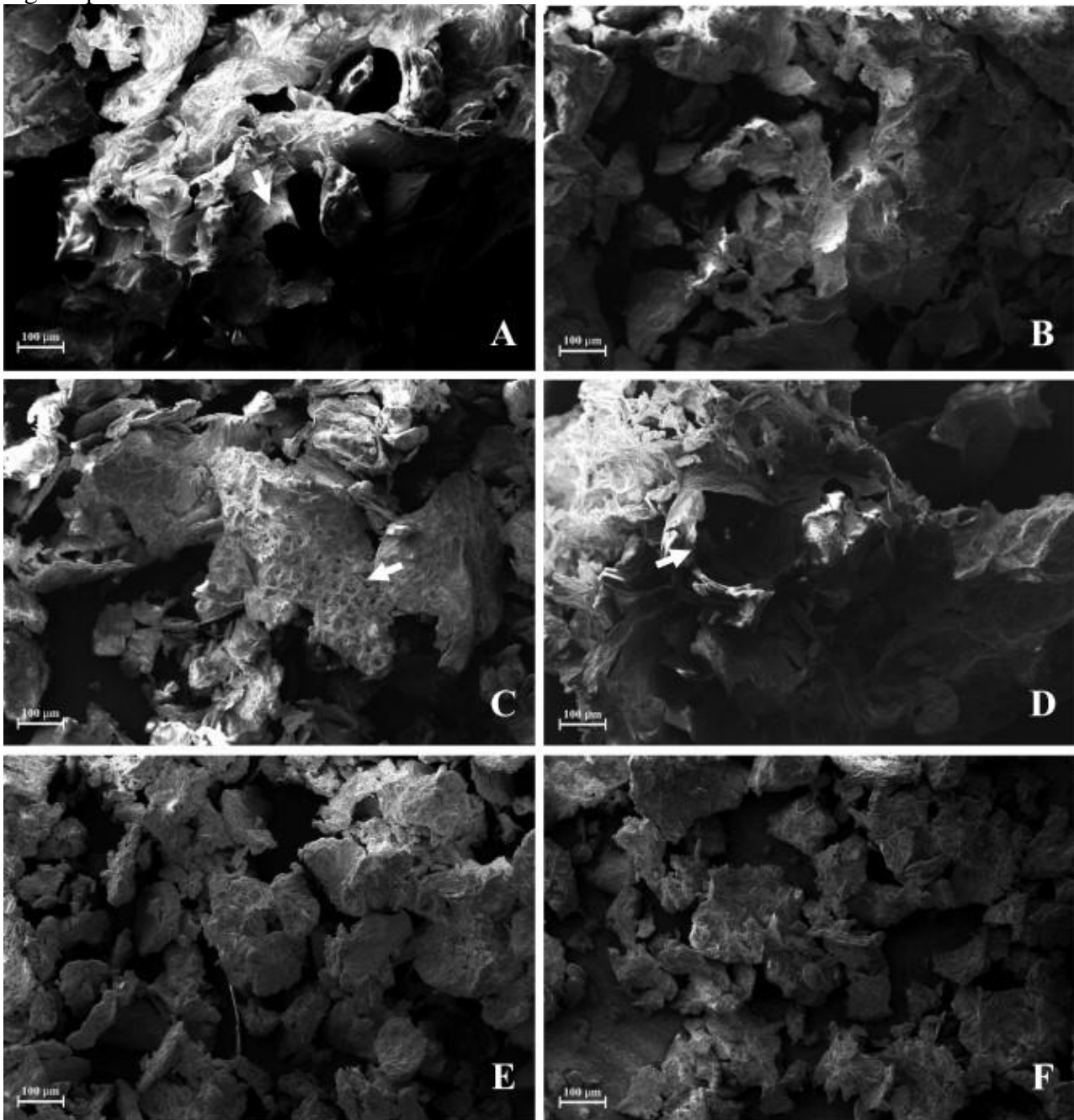


Fig. 3. Scanning electron micrographs of (A) control freeze-dried tomato matrix and (B) Celluclast/Novozyme, (C) Viscozyme, (D) Flavourzyme, (E) Celluclast/Novozyme + Viscozyme, (F) Celluclast/Novozyme + Viscozyme + Flavourzyme freeze-dried tomato matrices. Bars indicates 100 µm.

### 3.4. Matrix packing density and SC-CO<sub>2</sub> extraction

In SC-CO<sub>2</sub> extraction, matrices are packed into cylindrical steel vessels where they are permeated by the supercritical fluid. The packing density is a very important parameter because it strongly influences the extraction yield. Excessive density prevents the supercritical fluid to permeate uniformly the matrix and determines the formation of preferential paths (McHugh & Krukoniš, 1986). This leads to a reduction in the extraction yield. If, on the contrary, the packing density is low, the amount of matrix loaded into the vessel decreases, with a consequent reduction of the potentially extractable lycopene. The packing density is a function of both the compressive force imposed during vessel loading, and the micro-structural characteristics of the freeze-dried matrix (grain size, average

weight of the granules, etc.). At a defined compression (2 MPa), the use of hydrolytic enzymes resulted in a significant increase ( $p < 0.001$ ) in the packing density of the matrices (Table 1) from a density of  $\sim 0.8 \text{ g cm}^{-3}$  of the control to values higher than  $1.0 \text{ g cm}^{-3}$  with the exception of Flavourzyme digested matrix whose packing density was not statistically different from the control ( $p = 0.167$ ). This, jointly with the highest titer of lycopene of the hydrolysed matrices, resulted in a substantial rise of lycopene packed into the vessel per volume unit (Table 1) from  $\sim 8.3 \text{ mg cm}^{-3}$  in the control matrix to  $\sim 30 \text{ mg cm}^{-3}$  in the matrices hydrolysed with Celluclast/Novozyme + Viscozyme and Celluclast/Novozyme + Viscozyme + Flavourzyme.

*Table 1. Effect of enzyme digestion on packing density of freeze-dried matrices and lycopene concentration into the SC-CO<sub>2</sub> extraction vessels. Digestion were carried out as reported in Fig. 2; freeze-dried matrices were loaded with a packing pressure of 2 MPa. Data, expressed as mean  $\pm$  standard deviation, are representative of three independent replicates (n = 3). Different letters indicate significant differences among treatments (Holm–Sidak post-hoc test,  $p < 0.05$ ).*

Treatment	Matrix packing density (mg f-dm cm <sup>-3</sup> )	Lycopene concentration (mg cm <sup>-3</sup> )
Control	0.76 $\pm$ 0.07 <sup>c</sup>	8.3 $\pm$ 0.8 <sup>d</sup>
Celluclast/Novozyme	1.10 $\pm$ 0.16 <sup>a</sup>	18.1 $\pm$ 1.3 <sup>bc</sup>
Viscozyme	1.16 $\pm$ 0.09 <sup>a</sup>	16.1 $\pm$ 1.5 <sup>c</sup>
Flavourzyme	0.86 $\pm$ 0.05 <sup>bc</sup>	8.7 $\pm$ 0.6 <sup>d</sup>
Celluclast/Novozyme + Viscozyme	1.11 $\pm$ 0.05 <sup>a</sup>	30.6 $\pm$ 2.1 <sup>a</sup>
Celluclast/Novozyme + Flavourzyme	1.09 $\pm$ 0.07 <sup>ab</sup>	21.2 $\pm$ 1.5 <sup>b</sup>
Celluclast/Novozyme + Viscozyme + Flavourzyme	1.17 $\pm$ 0.03 <sup>a</sup>	30.1 $\pm$ 2.3 <sup>a</sup>

#### **3.4.1. Effect of the addition of an inert co-matrix on SC-CO<sub>2</sub> extraction**

SC-CO<sub>2</sub> extraction of tomato matrices leads to an oil-solid bi-phasic extract (oleoresin) containing lycopene dissolved or dispersed as crystal-like structures (Lenucci et al., 2010, Longo et al., 2012). Fig. 4A shows the amount of oleoresin, expressed in mg g<sup>-1</sup> f-dm, extracted from each matrix as such or mixed with a solid inert co-matrix (PSE-matrix) in a ratio of 4:1 w/w. In the absence of co-matrix the hydrolysis with Celluclast/Novozyme, Viscozyme and Flavourzyme did not cause any significant increase ( $p > 0.05$ ) in the amount of oleoresin extracted compared with the control ( $\sim 53 \text{ mg g}^{-1} \text{ f-dm}$ ). The use of the enzymes in combination, however, determined a highly significant increase of the extracted oleoresin ( $p < 0.01$ ) up to a maximum of  $\sim 107 \text{ mg g}^{-1} \text{ f-dm}$  from the Celluclast/Novozyme + Viscozyme digested matrix. In general, the addition of an inert co-matrix did not significantly increase the amount of oleoresin extracted, except for the Celluclast/Novozyme + Viscozyme and Celluclast/Novozyme + Viscozyme + Flavourzyme hydrolysed matrices from which over  $124 \text{ mg g}^{-1} \text{ f-dm}$  of oleoresin were obtained. These variations were significant for  $p \leq 0.05$ . The increase is likely due to the combined effect of (i) the deconstruction of cellular and sub-cellular organisation of tomato tissues which enhances the broken/intact cell ratio, thus facilitating oleoresin extraction (Sovovà, 2005), and (ii) the increased matrix packing density that augments lipid load onto the extraction vessel.

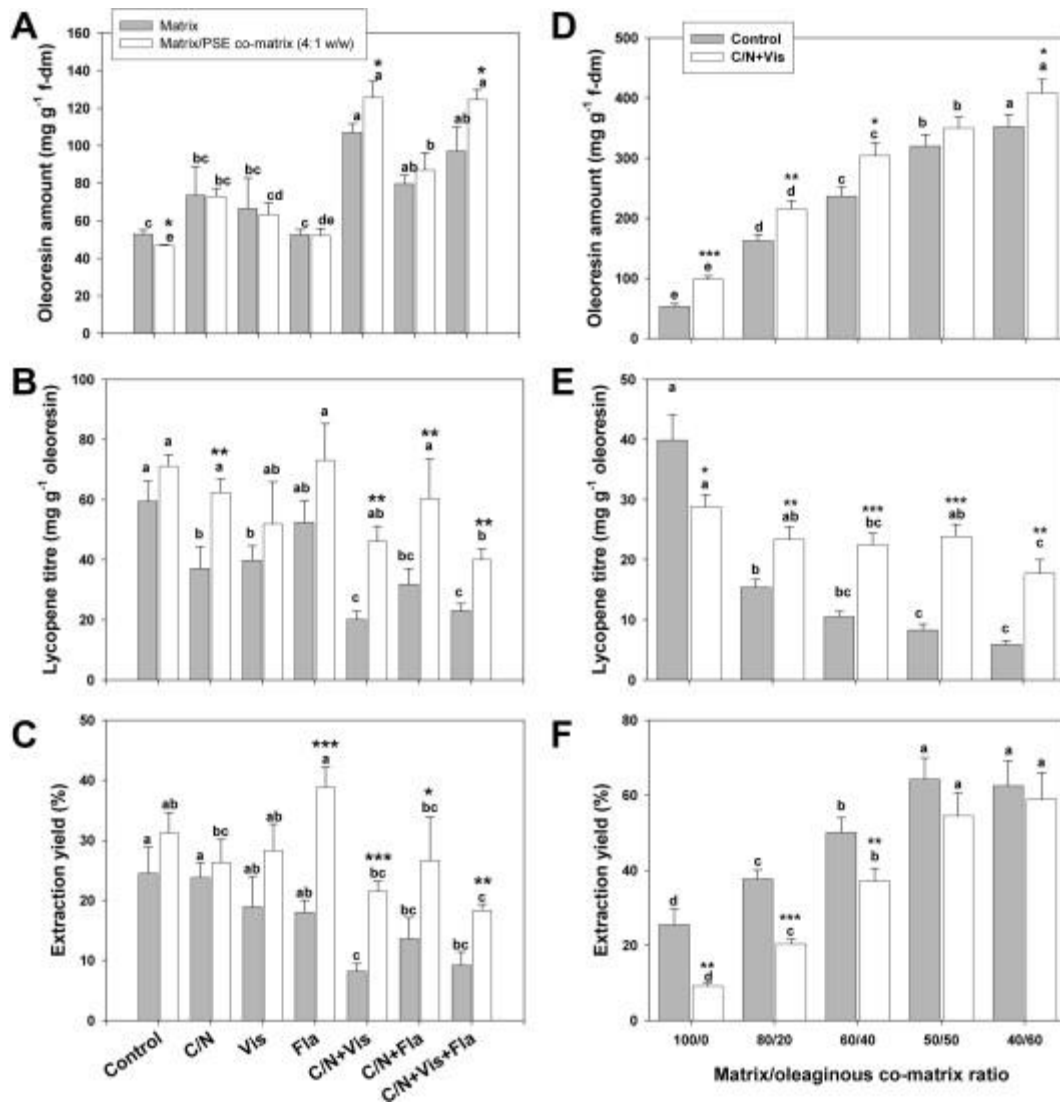


Fig. 4. Amount (A) and lycopene titer (B) of the oleoresins extracted by SC-CO<sub>2</sub> from control and enzyme digested freeze-dried tomato matrices pure or blended (4:1 w/w) with an inert inorganic co-matrix (PSE co-matrix); lycopene extraction yield (C). Effect of the addition of increasing amounts of oleaginous co-matrix (roughly ground hazelnut seeds) on the weight (D) and lycopene titer (E) of the oleoresins extracted by SC-CO<sub>2</sub> from control and Celluclast/Novozyme + Viscozyme digested tomato matrices and on lycopene extraction yield (F). Extraction operative parameters were: pressure = 50 MPa, temperature = 86 °C, SC-CO<sub>2</sub> flow = 4 mL min<sup>-1</sup>, extraction time 75 min. Data, expressed as mean ± standard deviation, are representative of three independent replicates (n = 3). Bars of the same series marked with different letters indicate significant differences among treatments (Holm–Sidak post-hoc test, p < 0.05). Asterisks describe values levels of statistical significance within each group (\*p < 0.05, \*\*p < 0.01; \*\*\*p < 0.001).

In absence of the solid inert co-matrix, all enzyme treatments, except Flavorzyme, led to a significant reduction ( $p < 0.001$ ) in oleoresin lycopene titer with respect to the control (Fig. 4B), while co-matrix addition caused a statistically significant ( $p < 0.01$ ) increase in this parameter in all samples where Celluclast/Novozyme was used, compared to the pure corresponding matrix. It is known that matrix microstructure can affect inner diffusion coefficients which, in turn, influence extraction rates (Araus, Uquiche, & Del Valle, 2009). This likely differentially affects the extraction of molecules poorly soluble in CO<sub>2</sub>, such as lycopene, compared to lipids or other fat-soluble vitamins with higher solubility in the supercritical fluid (Shi et al., 2009).

Lycopene extraction yields, calculated as the percentage of the total extracted lycopene with respect to the total amount of lycopene loaded into the extractor vessel, are reported in Fig. 4C. After 1 h of extraction, control, Celluclast/Novozyme, Viscozyme and Flavourzyme digested matrices gave no

statistically different ( $p > 0.05$ ) lycopene yields within the range 18–24%. The other enzymatic treatments (Celluclast/Novozyme + Viscozyme, Celluclast/Novozyme + Flavourzyme and Celluclast/Novozyme + Viscozyme + Flavourzyme) lead to a significant reduction (44–67%;  $p < 0.05$ ) in lycopene extraction yields with respect to the control. In general, the inert co-matrix addition caused a differential increase of lycopene yield that only in some samples was statistically significant (Fig. 4C). Among them, Flavourzyme digested matrix gave the best result with a lycopene extraction yield of almost 40%. Flavourzyme treatment may have been responsible for desorption/dissolution phenomena favouring SC-CO<sub>2</sub> lycopene extraction by weakening or disrupting lycopene-protein complexes (Shi & Le Maguer, 2000). The positive effect of inert co-matrix addition on lycopene extraction yields is likely due to a reduction in the packing density of the matrix in the extraction vessel. Although higher packing density increases the amount of total extractable lycopene in the vessel, it could negatively affect internal (solid phase) mass transfer mechanisms (Del Valle, Jiménez, Napolitano, Zetzl, & Brunner, 2003), lead to fluid channeling effects, prevent the eluting solvent from performing homogeneous extraction and, ultimately, result in a lower overall lycopene yield. Regardless of the enzymatic treatment, in the absence of co-matrix, SC-CO<sub>2</sub> did not efficiently permeate the tomato matrix using as a preferential path the interface between matrix and extraction vessel inner surface (Supplementary Fig. S3).

#### **3.4.2. Effect of the addition of an oleaginous co-matrix on SC-CO<sub>2</sub> extraction**

It is well known that the addition of an oleaginous co-matrix (hazelnuts) to the tomato matrix results in a substantial increase in SC-CO<sub>2</sub> lycopene recovery and that the hazelnut/tomato matrix ratio is fundamental to maximize the yield of recovered lycopene and to obtain a standardised product (Ciurlia et al., 2009, Lenucci et al., 2010). This was confirmed by Machmudah et al. (2012) which improved SC-CO<sub>2</sub> extraction of lycopene from tomato peels by blending the matrix with tomato seeds in a ratio of 37/63 by weight. Therefore we performed SC-CO<sub>2</sub> extraction tests on control (undigested) and Celluclast/Novozyme + Viscozyme digested matrices mixed with increasing amounts (from 0% to 60% by weight) of roughly ground hazelnut seeds as oleaginous co-matrix (Fig. 4D–F; Supplementary Fig. S3).

Obviously the amount of extracted oleoresin from both control and enzyme digested matrices increased proportionally to the percentage of oleaginous co-matrix added (Fig. 4D). Co-matrix addition determined a sharp decrease in lycopene titer of the oleoresin extracted from the control matrix due to a dilution effect (Fig. 4E), but, simultaneously, an increase in lycopene extraction yield which reached values slightly lower than 65% when a ratio matrix/co-matrix of 50/50 or 40/60 by weight was used (Fig. 4F), confirming our previous results (Lenucci et al., 2010). Glycosidase digested matrix mixed with the oleaginous co-matrix gave oleoresins with a lycopene titer significantly higher than those extracted from the respective control matrix/co-matrix mixture, reaching a maximum value ( $\sim 24 \text{ mg g}^{-1}$ ) at the matrix/oleaginous co-matrix ratio of 50/50 (Fig. 4E). In this case the dilution effect is fully compensated, and even exceeded, by the greater amount of extracted lycopene as confirmed by the increase in the extraction yield which, for matrix/oleaginous co-matrix ratios higher than 50/50 by weight, was not significantly different from the control and reached values close to 60% (Fig 4F).

The time course of SC-CO<sub>2</sub> lycopene extraction yield from control and Celluclast/Novozyme + Viscozyme digested tomato matrices blended (1:1 w/w) with the oleaginous co-matrix is reported in Fig. 5A. From the curves it is evident that, prolonging the extraction time, the differences in lycopene yields are reduced and become almost negligible after 3 h extraction.

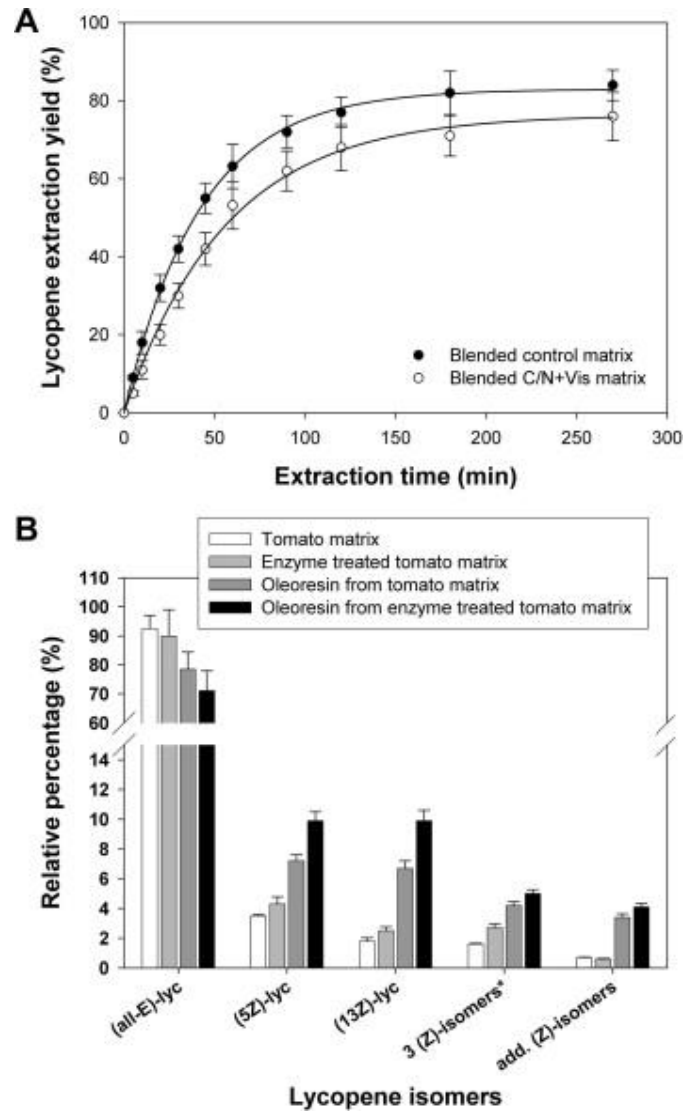


Fig. 5. (A) Time course of SC-CO<sub>2</sub> lycopene extraction yield from control and Celluclast/Novozyme + Viscozyme digested tomato matrices blended (1:1, w/w) with roughly ground hazelnut seeds (oleaginous co-matrix). Extraction operative parameters were as described in Fig. 4. (B) Distribution of lycopene isomers in control and Celluclast/Novozyme + Viscozyme digested tomato matrices and in the corresponding SC-CO<sub>2</sub> extracted oleoresins. Data, expressed as mean  $\pm$  standard deviation, are representative of three independent replicates (n = 3).

### 3.5. Lycopene isomerisation during processing

The effect of matrix processing and extraction on the isomer distribution of lycopene is reported in Fig. 5B. In both control and digested matrices (*all-E*)-lycopene was the most represented isomer (~90%) indicating that enzymatic treatment followed by matrix freeze-drying did not substantially affect lycopene isomerisation. In contrast, a shift in favour of the (*Z*)-isomers was observed by comparing the isomer distribution in the matrices with that of the oleoresins, regardless of the enzymatic treatment. Temperature and time dependent isomerisation of (*all-E*)-lycopene to (*Z*)-isoforms was reported in different oleoresins (Hackett et al., 2004, Longo et al., 2012) showing that oleoresin storage conditions are important to preserve the product characteristics.

## 4. Conclusion

The exploitation of enzymes in industry for extracting phytochemicals is a promising field of investigation. Different enzyme-assisted extraction techniques, often combined with CSE have been explored to improve the extraction yield of lycopene from tomato tissues or tomato industrial wastes. Most of the authors demonstrated that treatment with cell wall degrading enzymes improves solvent

penetration and lycopene dissolution as a result of cell separation and loss of cell integrity resulting in a remarkable increase in lycopene extraction yield. In this study we evaluated the effect of enzymatic pre-treatments of the tomato purée on the microstructure of the resulting freeze-dried tomato matrices and on lycopene extraction by SC-CO<sub>2</sub>.

We demonstrated that the use of plant cell-wall glycosidases allows to increase considerably the concentration of lycopene and lipids in the tomato matrix and simultaneously increases substrate load onto the extraction vessel compared to the control matrix. The more dense microstructure of the digested matrix and the smaller particle size cause the sample to compact and restrict CO<sub>2</sub> diffusion through the sample leading to a channeling effect and reducing lycopene extraction yield, nevertheless, the addition of an oleaginous (roughly grounded hazelnut seeds) co-matrix to the freeze-dried tomato matrix (i) facilitates CO<sub>2</sub> diffusion through the matrix, (ii) allows the co-extraction of lipids that increases both lycopene solubility in SC-CO<sub>2</sub> and its fluid flow rate through the tomato matrix and pipeline. SC-CO<sub>2</sub> extraction yield from enzyme-treated and untreated tomato matrix, successively blended with the oleaginous co-matrix (ratio 1:1, w/w) was very much similar, whereas the total amount of extracted lycopene was approximately three times higher in enzyme-treated tomato matrix than control. Thus, the economic convenience of the use of enzymatic mixtures in preparing a tomato matrix suitable for SC-CO<sub>2</sub> lycopene extraction has to be evaluated mainly on the base of the enzyme cost. These findings encourage further studies in order to scale up the process for possible industrial production of high quality lycopene from tomato matrices to be possibly used for functional food or cosmeceutical formulations.

## Notes

The authors disclose any actual or potential competing interests.

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