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Full Length Research Paper

Extraction of pure lycopene from industrial tomato waste in water using the extractor Naviglio®

Daniele Naviglio[1*], Fabiana Pizzolongo[1], Lydia Ferrara[2], Biagio Naviglio[3], Alejandro Aragòn[4], and Antonello Santini[1]

[1]Dipartimento di Scienza degli Alimenti, Università di Napoli Federico II, Via Università 100, 80055 Portici (Napoli), Italy.
[2]Dipartimento di Chimica Farmaceutica e Tossicologia, Università di Napoli Federico II, Via Montesano 49, 80131 Napoli, Italy.
[3]Stazione Sperimentale per l'Industria delle Pelli e delle Materie Concianti, Via Poggioreale 39, 80143 Napoli, Italy.
[4]Facultad de Ciencias Agrarias y Forestales, Universidad de La Plata, Calle 60 y 119, 1900 - La Plata (Buenos Aires), Argentina.

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In this paper an innovative process for the extraction of pure lycopene from tomato-waste in water that uses the Extractor Naviglio® and water as solvent is presented. The use of water as extracting solvent considerably reduces the cost of the entire process if compared with the commonly used solvent-based procedure or with the newer supercritical extraction process of lycopene from tomato-waste. Exhausted tomato-waste treated with water can be then easily dried at room temperature and further used, e.g. in agriculture or as food ingredient in animal nutrition. Lycopene, not soluble in water, was recovered in a quasi-crystalline solid form and purified by SPE (Solid Phase Extraction) using a small amount of organic solvent. The all *trans* lycopene was obtained at a very high grade of purity, not less than 98% (w/w), with an average recovery from tomato waste of 14% (w/w). The availability of high purity *all trans* lycopene allowed us also to measure the molar absorption coefficient, unique for each molecule. An alternative procedure for the HPLC analysis, that uses a phenyl-hexyl silicone stationary phase as inverse phase and a linear gradient in water and acetonitrile, is also described.

Key words*:* Lycopene, Extractor Naviglio®, tomato-waste, HPLC-Diode array, solid-liquid extraction, chromatography, solid phase extraction.

INTRODUCTION

Lycopene, whose structural formula is reported in Figure 1, is a fat soluble carotenoid with 11 conjugated double bonds in the molecule, and it is a precursor of the β-carotene with a well known antioxidant activity, reported as at least twice that of the β-carotene (Sies and Stahl, 1998; Di Mascio et al., 2002). Lycopene can be easily degraded by atmospheric oxygen and by light and converted from the *all trans* to the *cis* forms, that show a decreased biologic activity (Lee and Chen, 2002 Wang and Chen, 2006.). For these reasons it must be stored at a very low temperature (≤-70°C) away from the light and from the atmospheric oxygen. In the last years the importance of lycopene rapidly increased due to its pharmacological and anti carcinogenic properties (Franceschi et al., 1994; Giovannucci, 1999; Livny et al., 2002) and antioxidant activity (Di Mascio et al., 2002; Heber and Lu, 2002; Sies and Stahl, 1998). Epidemiological studies indicate a protective effects of lycopene against some types of cancer, e.g., stomach and prostate cancer (Stacewicz – Sapuntzakis and Bowen, 2005; Stahl and Sies, 1996), so the demand for this molecule and for the carotenoids in general, is rapidly growing (Ulrich, 2000). In the literature, different synthetic pathways for this molecule have been reported, but in all cases the synthesis of lycopene seems to be a very expensive and economically not convenient procedure (Zhuo-cai et al., 2006). On the other hand, good amounts of lycopene are contained in many natural products, like tomato (*Lycopersicon esculentum* Mill.), water-melon, red pep-

*Corresponding author. E-mail: naviglio@unina.it. Tel./fax: +39 81 2539348.

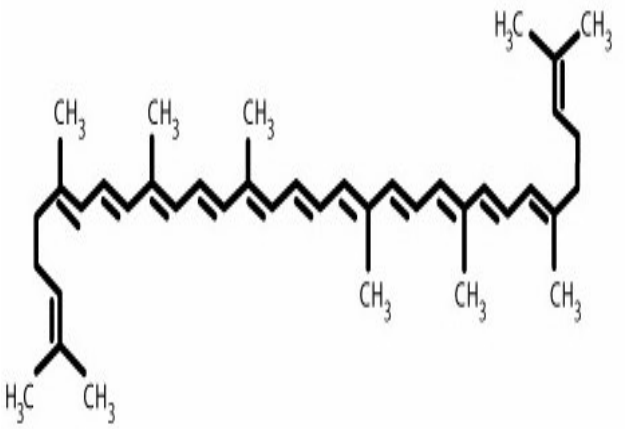

Figure 1. Structural formula of lycopene.

per, papaya etc. (Britton, 1991) This molecule is also responsible for the red intense colour of these vegetables, and a lycopene content in the range between 5.40-1500 mg/kg in tomato paste (wet weight) (Roldán-Gutiérrez and Luque de Castro, 2007) and between 20-30 mg/kg in tomato peels as raw material (Rozzi et al., 2002) were determined, respectively.

It is worth noting that conventional methods for the extraction of lycopene from natural vegetal origin sources use great quantities of organic solvents and the extraction procedure is usually followed by a complex purification step (Olives Barba et al., 2005 Sadler et al., 1990.). In the case of lycopene recovery from the tomato-waste, the extraction can also be conducted using carbon dioxide in supercritical state (Rozzi et al., 2002), and this method allows to recover a greater quantity of carotenoids even if partly mixed in an oleoresin containing around 6% (w/w) in lycopene (Vági et al., 2007). This method, unfortunately, is actually quite expensive, making the entire procedure economically not suitable for a large scale process. Biotechnological production of lycopene using micro-organisms (Sandmann, 1994), has been proposed too, but always at the bench scale and, in most cases, with a weak specific yield, even if recently a new approach that can be scaled-up to an industrial application has been proposed (Lopez-Nieto et al., 2004).

Since tomato by-products quantities deriving from industrial processes are growing annually, e.g. in Europe it is evaluated that about ten million tons of tomatoes are processed by the food industry, the deriving wastes can be quantified in approximately 0.1 million tons, and represent an interesting low cost source of lycopene. Italy is among the main producers of tomatoes in Europe and the by-products and the residues of the industrial processing of tomatoes that can be reincorporated in low quality tomato products or used as an ingredient in animal food, could be more efficiently used as a source of pure lycopene. Considering both the availability of the by-products from tomato industry, the relevant interest for this molecule and its biological activity, and starting from the consideration that better methods for the characterization and determination of lycopene are needed (Roldán-Gutiérrez and Luque de Castro, 2007), the main aim of this work is to propose a new method for the extraction and characterization of lycopene from tomato wastes. The extraction procedure was realized using the Naviglio Extractor® (Naviglio, 2003) that uses a new solid-liquid extraction technology (Naviglio, 2000), and tap water as extracting solvent followed by a simple and rapid purification step, that requires a minimum quantity of organic solvent.

Experimental

Reagents

Acetone, methanol, acetonitrile, chloroform, dichloromethane, n-hexane (all solvents were of analytical grade from Fluka, Bucks, Switzerland); de-ionized water of HPLC grade was produced by means of Milli-Q (Millipore); lycopene standard was purchased from Sigma Aldrich; Extractor Naviglio® *Mod. 500* cc (Nuova Estrazione S.a.s., Naples, Italy); Spectrophotometer UV 1601 (Shimadzu Corp., Tokyo, Japan); HPLC-Diode array, mod. 1100 (Agilent Technologies, Santa Clara, CA, USA); Cartriges octadecyl Solid Phase Extraction (SPE-C18) 10 g (Restek Corp., Bellefonte, PA, USA); filters 0.20 micron (Millipore, Bedford, MA, USA); Phenyl-hexyl silicone HPLC column 250x4.6 mm, 5 micron particle size (Phenomenex, Torrance, CA, USA).

Brief description of the Naviglio Extractor®

In this work the Naviglio Extractor® shown in Figure 2 and described elsewhere (Naviglio, 2003) was used. This device is a rapid and dynamic solid-liquid extractor that applies the principle that in a suitable solvent, generating a negative pressure gradient and letting it to go to equilibrium between outside and inside of a solid matrix, that contains compounds that can be extracted in the solvent followed by a rapid equilibrium condition restoring, a forced extraction of the not chemically bound compounds contained in the solid matrix is produced (Naviglio's Principle). The Naviglio Extractor® can operate at room temperature or at sub-ambient temperature, and it works applying a pressure increase on the surface of the liquid phase containing the solid material (matrix) to be extracted. The use of low or room temperature greatly reduces the thermal stress for any heat susceptible substances present in the matrix, e.g. lycopene that can be degraded when using high temperature for the extraction (Lee and Chen, 2002).

The device used consists of one extracting chamber equipped with a cylinder and a piston where, at the bottom, one porous set let the liquid phase and liquid soluble substances pass through, while the solid particles are blocked. The solid raw material is put in the chamber that is then filled with the solvent (organic, inorganic or a mixture of solvents). Pressure gradient is applied allowing the system to reach equilibrium (Static phase) at a pressure of about 8 atm. When the piston is moved from its equilibrium position, the dynamic phase starts; this step is performed for five times and for a brief period of time with aim of remixing the solutions and to allow the diffusion of the extracted compounds. The movement of the piston and hence the static and dynamic steps alternate till the

Figure 2. Naviglio Extractor®, Mod. 500 cc. (Depurex88, Padua, Italy).

extraction process was efficiently completed. One extraction cycle is formed by one static and one dynamic step; repeating more times these operations, complete exhausting of the solid matrix can be obtained.

When the maximum value set for the pressure is reached, the device stops for 2 min, allowing the outside and inside part of the solid matrix to equilibrate (Static phase) with the solvent pressure. Immediately after this step, the piston moves, the air quickly leaves the pneumatic chambers and the system causing a lowering of the pressure inside the extraction chamber. This induces the starting of the dynamic phase and at the beginning of this step the substances that are soluble in the used solvent and substances not chemically bonded to the matrix are extracted from the solid matrix and transferred to the solvent. Extractor Naviglio was recently employed in the innovative production of lemon liquor (Naviglio et al., 2007).

Chemical extraction of by-products of tomato

To compare the recovery of carotenoids in the starting material obtained using the Naviglio Extractor with those obtained with a conventional chemical solvent based extraction procedure, chemical extraction of lycopene from tomato by product was performed as reported by others (Rozzi et al., 2002). A 2 g sample of tomato by-products was placed in an extraction tube; 20 mL of chloroform were added and the tube was treated with ultra sounds for 30 min. The sample was then centrifuged for 15 min at 2000 rpm, and an aliquot was analysed by high-performance liquid chromatography (HPLC) to determine *all trans* lycopene. The extraction procedure was repeated to recover residual lycopene in the sample. Exhaustive extraction of tomato seeds and skins with additional volumes of chloroform did not result in additional recovery of lycopene.

Extraction by means of Extractor Naviglio®

Ten samples each constituted of 100 grams of tomato peels and by-products from different farms in the Naples area, were placed in a bag made of 50 µm filtering membrane and transferred into the chamber of the Extractor Naviglio mod. 500 cc. where 500 mL of tap water was added. Extraction parameters used are the following: the total extraction time was set to four hours for a total of 60 cycles. The dynamic phase used 5 cycles (Piston was idle for 12 s in the up and down positions) and for the static phase a 2 min time was used. After extraction the bag was removed and strongly pressed in order to completely recover the water (About 450 mL). The aqueous extract was loaded on octadecyl Solid Phase Extraction (SPE-C18) 10 g column vacuum packed; column was washed with methanol, that does not dissolve lycopene, in order to remove pigments and then the minimum quantity of acetone was used to recover lycopene. The acetone extract was analysed by spectrophotometric and HPLC analysis to confirm the presence of lycopene.

To verify if the use of tap water could affect the extraction, the same extraction procedure was repeated using deionised water (See Table 1).

Spectrophotometric analysis

Extracts were filtered through 0.20 micron membrane (Millipore, Bedford, MA, USA) and anhydrified on sodium sulphate anhydrous in order to eliminate water traces and rough impurities and analysed by spectrophotometer in the 350-550 nm wavelength range. The spectrophotometric analysis allowed to identify the *all trans* lycopene molecule, by comparing the spectra of absorbance and the absolute and relatives maxima peak of absorbance with spectra of absorbance and maxima of absorbance exhibited by lycopene standard and the relative values reported in literature.

HPLC analysis

In order to better verify the lycopene purity, filtered extracts were analysed by high-performance liquid chromatography (HPLC) system equipped with Diode array detector mod. 1100 (Agilent Technologies, Waldbronn, Germany). A phenyl-hexyl silicon column (250 x 4.6 mm I.D. 5 micron particle size) (Phenomenex, Torrance, CA, USA) was used for the separation. The flow rate was 1 ml/min and the gradient water-acetonitrile was: time 0-3 min, acetonitrile 0%; time, 20 min, acetonitrile 100%; hold for 5 min. Lycopene standard was purchased from Sigma-Aldrich (Milan, Italy).

Calibration curve

An amount of lycopene extract was dried under a nitrogen current. A stock solution of the extracted lycopene was prepared by dissolving 10.0 mg of lycopene in 100 ml of dichloromethane. From this standard solution by diluting 5 solutions containing from 1 to 5 ppm were prepared. The same procedure was used to prepare the stock solution and the diluted solutions from the purchased standard of lycopene. In this case 1.0 mg of lycopene was used. Correlation coefficient of calibration line was 0.998. In the same conditions a calibration line was prepared for the lycopene standard and in this case the correlation coefficient was 0.996 and angular coefficient was 5.8% lower than the previous one because its lower purity. The observed linear response for lycopene was comprised in

Table 1. Lycopene recovery from tomato by products using tap and demineralized water at different values of the extraction time.

Sample	Lycopene content (mg/Kg)				
	Demineralized water (4 h)	Tap water (4 h)	Tap water (2 h)	Tap water (6 h)	Tap water (8 h)
1	2.3	3.2	1.5	2.6	2.5
2	3.4	3.0	1.9	2.8	3.2
3	2.7	3.5	1.1	2.5	3.3
4	1.9	1.5	0.9	1.4	1.6
5	2.8	3.9	2.1	2.9	3.5
6	1.7	1.7	1.2	1.8	1.8
7	2.9	2.5	1.8	2.4	2.6
8	3.9	3.6	1.8	2.9	3.5
9	2.2	2.0	1.4	1.7	1.9
10	2.6	2.9	1.5	2.0	2.8

Table 2. Recovered lycopene (A) and lycopene standard (B) maxima wavelenght of absorbance in four different solvents and specific coefficient of absorption and molar extinction values.

Solvent	λ_1 (nm)	λ_2 (nm)	λ_3 (nm)	$E^{1\%}$ (L/g·cm)	ε (L/mol*cm)
n-Hexane	444.5±0.5	470.5±0.5	502.0±0.5	3105±30	166774±1610
Acetone	448.5±0.5	473.5±0.5	505.0±0.5	2855±30	153240±1610
Dichloromethane	456.0±0.5	482.0±0.5	515.0±0.5	2540±30	136308±1610
Chloroform	457.5±0.5	484.0±0.5	517.0±0.5	2850±30	152989±1610

(A)

Solvent	λ_1 (nm)	λ_2 (nm)	λ_3 (nm)	$E^{1\%}$ (L/g·cm)	ε (L/mol*cm)
n-Hexane	445.5±0.5	471.0±0.5	502.0±0.5	3140±50	168571±2684
Acetone	449.5±0.5	474.0±0.5	505.5±0.5	2803±50	150479±2684
Dichloromethane	455.0±0.5	482.0±0.5	516.0±0.5	2480±50	133139±2684
Chloroform	456.5±0.5	485.0±0.5	518.0±0.5	2810±50	150855±2684

(B)

the range 0-100 ppm.

Determination of molar absorption and specific extinction coefficient of lycopene in different solvents

Four solutions of lycopene extracted with the Extractor Naviglio were prepared in n-hexane, chloroform, dichloromethane and acetone, at a concentration of 1.00 mg/l starting from the previously prepared stock solution (100 ppm). Table 2 reports the maximum value for absorbance measured at a wavelength between 350-550 nm for each solution, the relative molar absorption coefficient and specific extinction coefficient.

RESULTS AND DISCUSSION

Use of the Extractor Naviglio® for the recovery of lycopene from tomato waste

The Naviglio Extractor® was used to recover a solid lycopene fraction in a para-crystalline form from tomato-waste in tap water, using the pressure and depressure effect that operate in the solid-liquid extractor device. In this case the extraction is allowed because of the effect of depression generated in the extractor and lycopene is

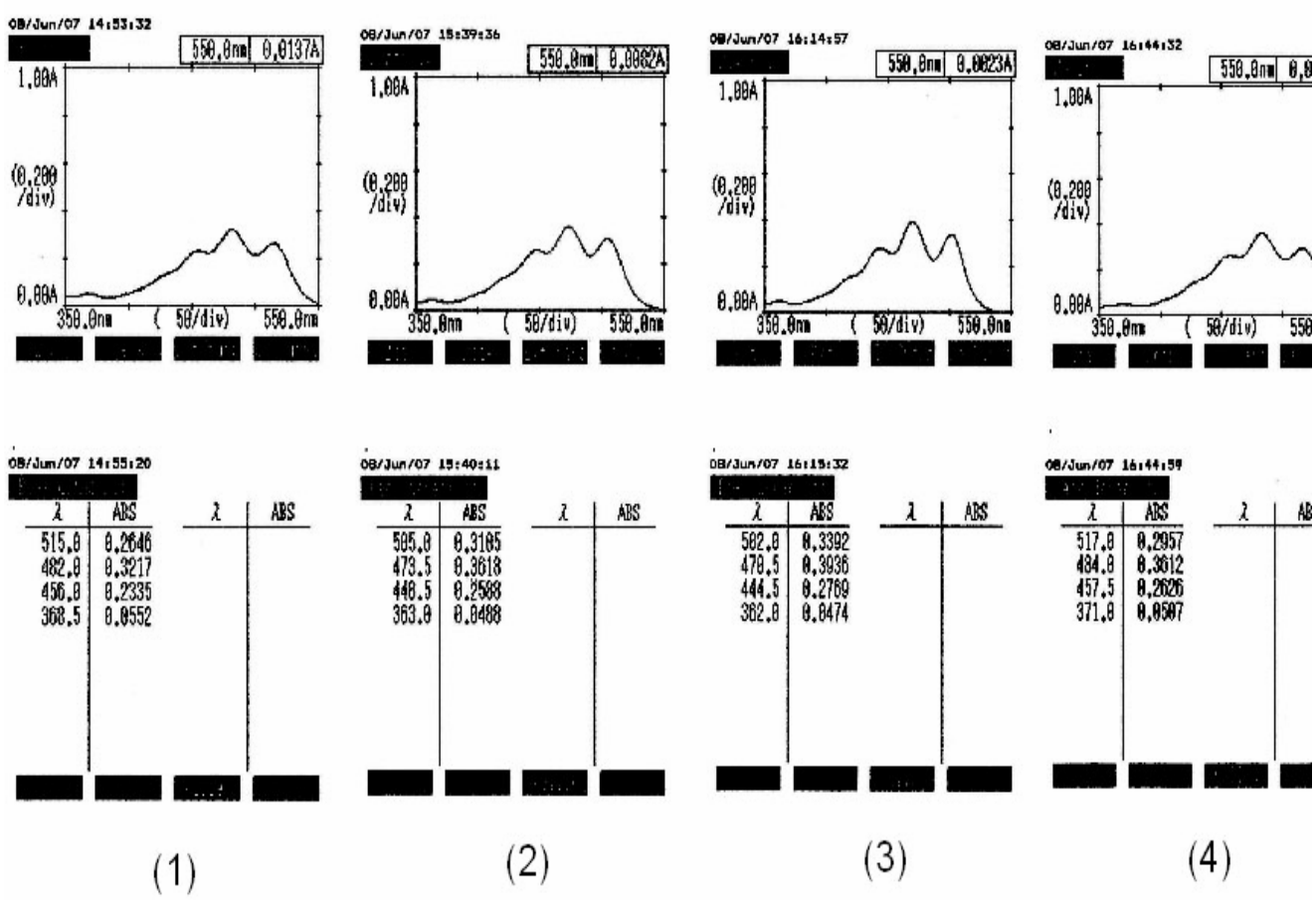

Figure 3. Visible spectra of licopene in dichloromethane (1), acetone (2), n-hexane (3) and chloroform (4).

found in heterogeneous phase. For this reason during the following purification on SPE-C18, *all-trans* lycopene present in the solid form, was separated on the filter column, and a little fraction of the other pigments present in the starting material were retained on the column head of the octadecylsilicone stationary phase. Pigments were removed by washing with methanol, solvent in which lycopene is totally not soluble, while washing with acetone allowed the lycopene, very soluble in this solvent, to be completely separated. Purity of extracted lycopene was checked by HPLC showing the presence of one major peak corresponding to the all-*trans* lycopene, while its quantification was possible through the calibration curve.

Table 1 reports the recovery obtained for the lycopene using deionized and tap water, respectively. As it can be observed, there is no significant difference between the two liquids, suggesting that it is preferable to use tap water to keep low the cost of the entire industrial process. Optimum extraction time was 4 h and a recovery between 1.5 and 3.9 mg/kg of lycopene was obtained; it can also be observed that continuing the extraction beyond this time period, recoveries did not increase and degradation events could occur, suggesting that 4 h is the optimum extraction time.

Qualitative and quantitative analysis of lycopene

The elution of lycopene from the C18-SPE can be made directly using acetone or washing the column with methanol first ad then eluting with acetone. In the first case, due to the contemporary presence of other carotenoids like β-carotene and lutein, lycopene at not elevated purity grade was obtained while washing the column with methanol first and allowed to obtain lycopene at a higher purity. At this point, it is worth noting that the washing of lycopene is made after the extractive procedure and the quantity of solvent employed is minimal; however the great quantity of by products are only touched with water and do not come in contact with solvent. Lycopene purity was investigated byspectrophotometric UV-Vis and HPLC analysis, allowing to observe the presence of one peak corresponding to the all *trans* pure lycopene. Figure 3 reports

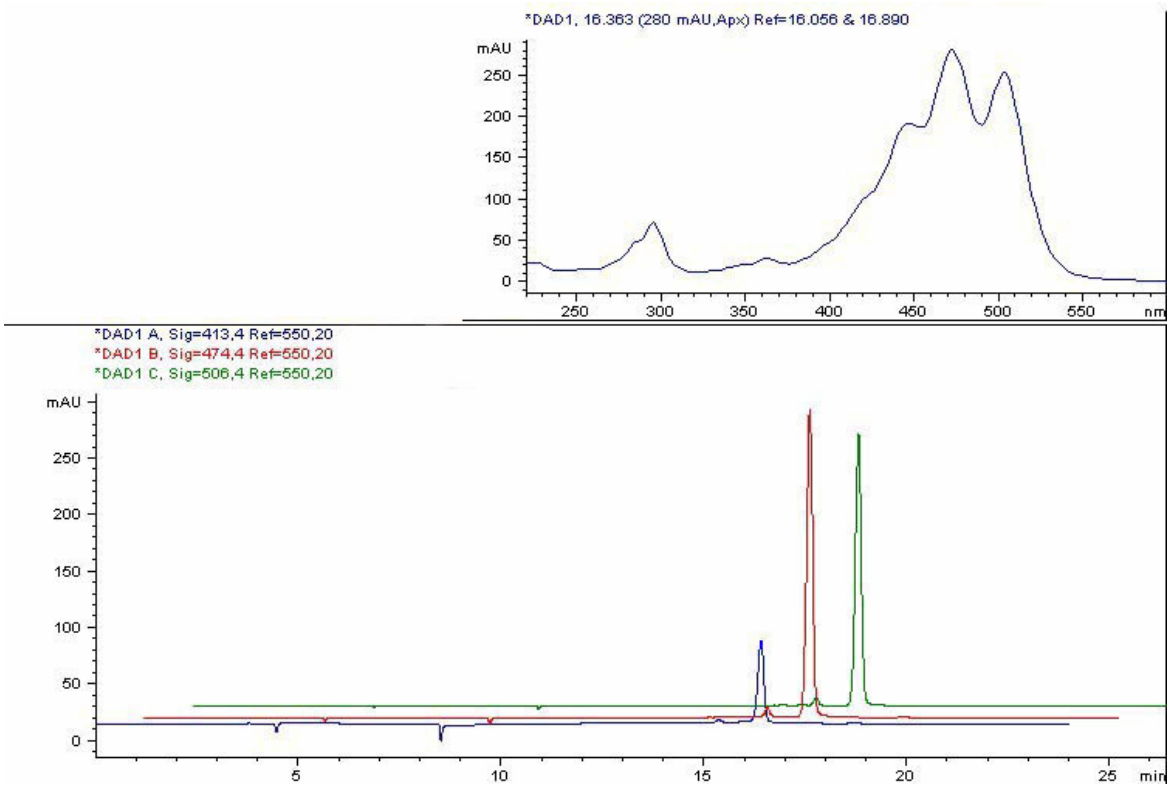

Figure 4. HPLC chromatograms of extracted lycopene at three different wavelengths, 413.4, 474.4, 506.4 (lower part), and DAD acquisition between 200 and 600 nm of the peak measured at a retention time of 16.363 min. (upper part).

visible spectra of lycopene in dichloromethane, acetone, n–hexane and chloroform respectively. The comparison of the maxima with those observed for the lycopene standard shows an excellent agreement (Tables 2a and 2b) and is consistent with data reported in the literature (Davis, 1949; Roldàn-Gutiérrez and Luque de Castro, 2007). Figure 4 reports HPLC results at three maxima wavelengths 413.4, 474.4 and 506.4 nm. One minor component, an impurity, was detected before the elution of lycopene peak and it was identified as beta-carotene. The impurity did not exceed the 2% of total signal intensity, allowing us to conclude that lycopene is at least of 98% purity (at λ = 474.4 nm). The DAD acquisition confirmed that no other contaminant was present in the range of 200-600 nm. Chromatographic conditions reported in this paper are different from the ones used in literature; in this case a phenyl-hexyl silicone as stationary phase and a simple water-acetonitrile gradient were used and for this reason the method here reported could be proposed as a valid alternative to the ones reported in literature (Brumann and Grimme, 1981; Olives Barba et al., 2005). This procedure allows us to reduce times of analysis in the case of a high purity lycopene containing solutions for which it is not necessary the use of complex gradients of solvent for the elution.

Chemical extraction on the ten samples of raw materials gave an average value of 20.3 mg/Kg. Table 1 reports recovery from tomato by products using tap water and demineralized water at different values of the extraction time. At the optimum extraction time (4 h) the recovery in tap water was comprised between 1.5 and 3.9 mg/Kg, corresponding to a percentage recovery of 7.5 and 19.5% (w/w), respectively, compared with chemical extraction. In demineralised water, the recovery ranged in the same conditions, from 1.7 and 3.9 mg/Kg, corresponding to a recovery percentage of 8.5 and 19.5% (w/w), respectively. The possibility of using tap water as extracting liquid notably reduces the costs of industrial processes. The efficiency of the proposed process is lower than the other procedures proposed in the literature, because of minor quantity of lycopene present in para-crystalline form in tomato waste. But when considering the cost of the tomato waste and the easy dumping of the exhausted material, it turns to be cost reducing since no organic solvent is used in the extractive phase, moreover a higher grade pure lycopene (>98% (w/w)) is achieved in the extraction using the Extractor Naviglio to be compared with the chemical extraction procedure that allowed a 20-30 mg/Kg recovery at a 10-20 % (w/w) purity. Finally, the exhausted material

can be dried at room temperature and the weight reduces about 95% (w/w). In this way it can be employed as cattle feed or as a manure in agriculture.

Determination of molar absorption coefficient and relative specific coefficient of absorption

The possibility of isolating pure lycopene from tomato wastes gave us the possibility to measure the molar absorption coefficient (ε) and the relative specific coefficient of absorption ($E^{1\%}$) in some of the most commonly used solvents, namely dichloromethane, acetone, n-hexane and chloroform. Table 2 reports the obtained values for ε and $E^{1\%}$. In the literature different values of ε and $E^{1\%}$ for lycopene (Davis, 1949; Davis et al., 2003; Roldàn-Gutiérrez and Luque de Castro, 2007) are reported, and the difficulty of obtaining accurate values principally derives from the lack of a technique to obtaining all-*trans* licopene at high purity. Moreover spectrum of absorbance and molar absorption coefficient, unique for each molecule, allows the unique identification of the lycopene and can be used to confirm the presence of all-*trans* lycopene. The high value of the measured molar absorption coefficient can be correlated to the high number of double bonds and the high conjugation of double bonds present in the lycopene molecule, that is one of the compounds with the highest molar absorption coefficient value.

Conclusions

In this work an extractive process is described that could be easily scaled-up to become an industrial application for the production of very high grade of purity (≥98%) all-*trans* lycopene, whose demand is constantly increasing. The average recovery of lycopene from tomato by-products obtained using Extractor Naviglio® was of 14% (w/w) in respect of chemical extraction at a purity of 98% at the optimum extraction time of 4 h. This result could be further improved using a different value for the pressure applied in the extraction process. The described procedure uses tap water to extract lycopene from tomato by-products and a new pressured extraction technology. Considering that the costs are lower if compared with the cost of a conventional solvent or supercritical fluid phase extraction procedure, and since no special by-product to be wasted are produced, the overall process appears to be economically convenient. The material obtained after the extraction of the lycopene e.g. the exhausted tomato-waste residues can be easily dried and used in agriculture or as feeding for cattle or animals after the recovery of a high economical value molecule like lycopene.

Compared to the use of water as extracting liquid, the existing procedures to extract lycopene from vegetal sources that use organic solvents present some disadvantages: (i) organic solvents are generally toxic, so they have to be completely removed from exhausted material making necessary to use a complex purification step before dumping it; (ii) exhausted matrixes must be dumped as a special residue after extraction and they cannot be re-used; (iii) organic solvents are not specific for lycopene, but at the same time other pigments or hydrophobic compounds (e.g. carotenes, xanthophylls, fat, etc.) present in the starting material are simultaneously extracted. Moreover, the purification of lycopene is obtained using HPLC on inverse phase, the most used technique, is particularly difficult and time consuming, since long time gradient and ternary or quaternary solvent gradients must be used (Brumann and Grimme, 1981) due to the co-presence of unwanted compounds in the organic extract. Finally, the high purity of the lycopene obtained with use of the extractor Naviglio makes the industrial process very appealing. The pure all-*trans* lycopene extracted makes it possible to use it, at a known dosage, as a drug and not only as a food integrator.

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