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Chromatographic And Spectroscopic Characterisation Of Lycopen Extracted From Fresh And Thermally Processed Tomato Fruit.

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ABSTRACT

The aim of this study was to extract, purify and analyse the extracted lycopene from different tomato samples. Samples used were fresh and thermally treated tomatoes from Bosnia and Herzegovina and imported from abroad (early June and late September), as well as canned tomatoes. Lycopene was extracted by solid-liquid extraction and purified by Column Chromatography. Extracts were analysed before and after recrystallization with Thin Layer Chromatography. Chemical characterization was done using melting point determination and Fourier transform infraredspectroscopy technique. The highest content of lycopene was found in fresh tomato grown in open garden in September 19,896 mg mL^{-1} and the lowest in all thermally processed tomatoes. The measured melting points were in good agreement with literature values (171.10 and 173.71°C) as well as the Rf values of samples.In conclusion, the degree of ripeness of the fruit leads to an increase in lycopene content, while thermal treatment results in lower lycopene content.

Keywords: tomato, lycopene, extraction, Chromatography, Fourier transform infrared spectroscopy

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INTRODUCTION

Recent studies suggest that the consumption of tomatoes and tomato-based food products reduce the risk of cancer (pharynx, oesophagus, stomach, colon, rectum, urinary bladder, prostate and breast) in humans[1-3]. This beneficial effect is attributed to carotenoids, which are one of the main phytochemical classes in this fruit[4] Lycopene is used for medical purposes as a nutritional supplement to prevent cancer and to protect the blood vessels[5]. Carotenoids include over 600 plant pigments that are fat-soluble and give colour to many plants we see in nature, and they are important antioxidant defence components from lipid peroxidation in living cells [6]. Lycopene synthesis takes place in chloroplasts under the influence of sunlight and in non-photosynthetic conditions, and in the tissue it accumulates in chloroplasts [7]. Lycopene is a carotenoid whose structure consists of a long chain of conjugated double bonds, with two open end rings. It has the longest structure of all arytenoids. Lycopene ($C_{40}H_{56}$, molecular weight 536.85) is an unsaturated hydrocarbon containing 13 carbon-carbon double bonds, 11 of which are conjugated and arranged in a linear array. These conjugated double bonds are responsible for the vibrant red colour of lycopene [8-9]. It is a lipophilic compound that is insoluble in water, but soluble in organic solvents [10].

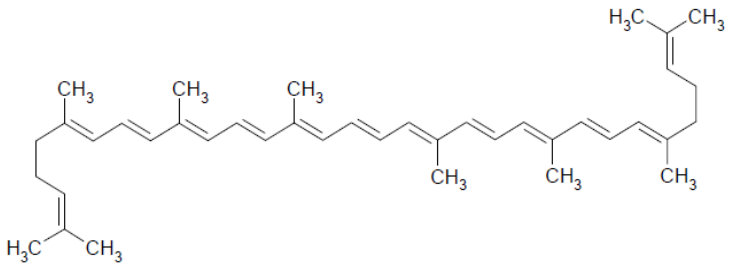

Figure 1: Chemical structure of lycopene

Tomato and tomato products have high concentrations of lycopene, but it is also present in watermelon, red grapes, apricots, peaches, goji berry, pink grapefruit and cranberries. Lycopene is the most common carotenoid in tomato (0.9 to 4.2 mg per 100 g); followed by β- carotene, γ-carotene and other carotenoids.It cannot be produced by human body so it must be obtained from food sources [11].

Lycopene degradation affects not only the quality of finished products, but also the health benefits of tomato-based food for human health. The main causes of lycopene degradation in tomatoes during processing are isomerisation and oxidation. Lycopene in fresh tomato fruit appears mostly in the all-trans configuration. The isomerisation converts all trans-isomers into cis-isomers due to additional energy input and results in an unstable, energy-rich form. The degree of lycopene isomerisation during processing affects the potential health benefits of tomato-based foods. Thermal treatment generally causes loss of lycopene in tomato-based foods. Heat causes the isomerisation of the all-trans form in the cis form. The content of the cis-isomer increases with the temperature and processing time. Generally, dehydrated and spiced tomatoes have poor lycopene stability, unless carefully treated and placed immediately in a hermetically sealed and inert atmosphere for storage. Frozen foods and heat sterilized foods show excellent lycopene stability during their normal temperature storage shelf life [12-14].

MATERIAL AND METHODS

Reagents

All reagents and solvents used were analytical-grade, methanol, and puriss. p.a., methylene chloride, puriss. p.a., sodium sulfate anhydrous, puriss. p.a., hexane, puriss. p.a., acetone, puriss. p.a., petroleum ether, puriss. p.a,ethanol absolute, puriss. p.a. (Sigma-Aldrich, St. Louis, USA), diethylether, p.a (Merck, Germany), aluminium oxydatum, (Carlo Erba, Italy).

Samples

Determination of lycopene content was made in eight samples shown in Table 1.

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Table 1: Investigated samples.

Samples	
LIK 1	Untreated tomato fruit from greenhouse, June (Jablanica, BiH)
LIK 2	Untreated tomato fruit from greenhouse, June, imported (Turkey)
LIK 3	Tomato fruit paste, factory processed, "Podravka - Passato"
LIK 4	Spelled tomato fruit in canned, factory processed, "Podravka - Pellato"
LIK 5	Tomato Fruit Pasta, Home Processing (Jablanica, BiH)
LIK 6	Peeled Tomato Fruit, Homemade Processing (Jablanica, BiH)
LIK 7	Untreated tomato fruit, grown in outdoor garden, September (Jablanica, BiH)
LIK 8	Lycopene extract, obtained by multiple extractions carried out using the same method, from the Faculty of Natural Sciences and Mathematics of the University of Sarajevo (students sample)

EXPERIMENTAL

Lycopene extraction and purification procedure

The extraction was carried out as follows:10 g of peeled and chopped fresh tomato sample, 25 mL of methanol and 50 mL of methylene chloride are placed in a flask and heated under reflux in the water bath for 5 minutes at 50-55ºC.After filtration, extraction of the precipitate was repeated with 50 mL of methylene chloride. The cooled mixture was again filtered and both of the filtrates are combined and three times shaken in a separating funnel with 50 mL of water. The collected organic layers were dried using 1-2 g of anhydrous sodium sulphate. After 5-10 minutes, the solution was filtered through a wrinkled filter paper and the volume was reduced in a rotary evaporator to approximately 1 mL.The pigment extract was washed with small amount of ether. The solution was evaporated and 0.5 mL of ether was added and warmed with absolute ethanol until blurry (turbidity). The solution washeated and cooledalternately to separate crystals. Lycopene and β-carotene are present (Figure 2.). Thecrystals are dried and tested.

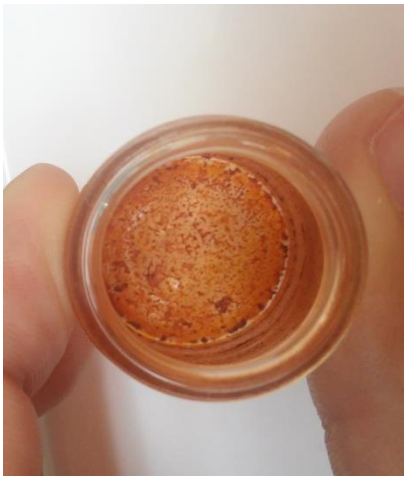

Figure 2: Lycopene crystals after the recrystallization process

Lycopene samples were confirmed by melting point, Thin layer chromatography (TLC) and Fourier transform infrared spectroscopy (FTIR).

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. This is a solid - liquid technique in which the stationary phase is a solid and mobile phase is a liquid. The principle of column chromatography is based on differential adsorption of substance by the adsorbent. The usual adsorbents employed in column chromatography are silica, alumina, calcium carbonate, calcium phosphate, magnesia, starch, etc., and the selection of solvent is based on the nature of both the solvent and the adsorbent. The rate at which the components of a mixture are separated depends on

the activity of the adsorbent and polarity of the solvent.Column chromatography is generally used as a purification technique: it isolates desired compounds from a mixture.

Alumina was used as a stationary phase, and a solvent mixture of methylene chloride and petroleum ether was used as a mobile phase in a 90:10 volume fraction. The aim was to purify lycopene by extracting β-carotene first from the column. The β-carotene yellow band began to move down the column along with the mobile phase (Figure 3). β-carotene fractions were caught, while lycopene in the form of red-orange strips remained at the beginning of the stationary phase in column. After extraction of β-carotene,the mobile phase was changed to extract lycopene from the column. In this case, as a mobile phase, methylene chloride and petrol ether were used in volume fractions 90:10. Lycopene wascollected as a red-orange fraction from the column (Figure 3). Chromatographic separation was successfully performed.

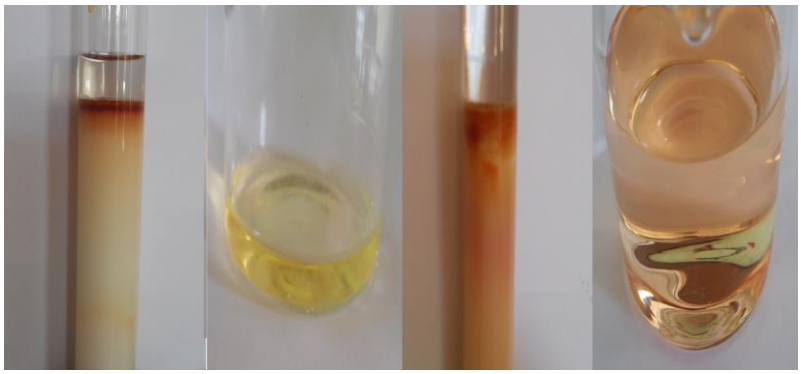

Figure 3: Separation of β-caroten (yellow fraction) and lycopene (red-orange fraction) by column chromatography

Melting point determination

Melting point determination was performed on capillary melting point apparatusKrüss, Germany.Capillary Melting Points, either in an oil bath or a melting-point apparatus, are most often used for the determination of the melting point of a solid. A few crystals of the compound are placed in a thin walled capillary tube 10-15 cm long, about 1 mm in the inside diameter, and closed at one end.The capillary with the substance was placed into the heated chamber, and the temperature was noted when the substance became completely transparent. This is considered to be the melting point[16].

Thin Layer Chromatography (TLC) characterization

The Thin Layer separation methods are very simple, fast and cheap, and allow relatively accurate determination of a number of organic and inorganic compounds. Low-temperature chromatography is a suitable method for the preliminary testing of samples for the presence of active and secondary substances, for pollutant testing and for the presence of decomposition products. The spots of coloured components divided along the panels are visible at daylight. Colourless spots are observed under ultraviolet light, UV lamp at wavelength of 254 or 360 nm. Spots can also be detected by means of certain reagents.

After lycopene extraction and evaporation on Rotavaporwith 10 µL of extract, each sample of lycopene extract (10 µL) was applied to a silica gel plate and developed with a mixture of hexane and acetone (70:30 volume fractions) as a mobile phase. The Rf value was calculated and the plate was observed under UV lamp. After recrystallization, TLC was again carried out in the same manner.

Fourier transform infrared spectroscopy (FTIR)

The identification of an unknown compound is based on the presence of a “fingerprint region” in the spectrum which is compared to a known molecule spectrum. Each organic compound has a characteristic spectrum in the fingerprint area which is not shown by any other substance. Typical IR spectrophotometers

record areas corresponding to vibrations of stretching and bending in the molecule. Radiation absorption is recorded and the IR spectrum of the sample is obtained.

The spectrum or fingerprints ofthe selected samples were obtained using FTIR spectroscopy. The samples of FTIR were prepared by using potassium bromide disks. The IR spectrum with KBr plates was obtained on a Perkin-Elmer FTIR spectrometer SPECTRUM BX.

RESULTS AND DISCUSSION

Structural characterization of the compounds present in the obtained extracts was performed by TLC and FTIR spectroscopy on samples prior to and after recrystallization. Double bonds within the lycopene structure are prone to isomerisation under the influence of heat and sunlight, resulting in transition from trans form to cis form. The trans form is thermodynamically more stable[18]. Trans-cis isomerisation facilitates absorption because cis isomers (5-cis, 9-cis, 13-cis, 15-cis) are much easier to absorb [11].

Lycopene bioavailability increases during processing because fine chopping or cooking makes it easier to absorb from the matrix contained within the tomato fruit. Bioavailability is higher from tomato fruit paste than from fresh fruits[19,11].

Yield of lycopene after recrystallization and purification of the crude extract

After crude extraction, the dried extract was reconstituted with 2 mL of methylenechloride, followed by recrystallization using the described method. The yield obtained was generated from the volume of the reconstituted extract. After the recrystallization of crude extracts, tomato fruits and tomato fruit products, the amount of crystals obtained for each sample was weighed to determine the yield by extraction. Table 2. shows the results obtained.

Table 2: Lycopene fraction yield after recrystallization

Samples	Yield (mg mL^{-1})
LIK 1	9.958
LIK 2	5.033
LIK 3	4.983
LIK 4	10.003
LIK 5	4.951
LIK 6	4.899
LIK 7	19.896
LIK 8	15.012

From Table 2. it is apparent that by thermal treatment the content of the lycopene fraction with the accompanying components decreases. It comes to small losses. It alsoindicatesthat the fruit that has ripened for a long time and harvested in September has a higher content than the fruit harvested in June.

After the recrystallization, the yield of each sample was calculated. For the samples 1 and 2, which matured in June in the greenhouse, the yield was 9.958 mg mL^{-1} for sample 1 and 5.033 mg mL^{-1} for sample 2. For sample 7, harvested in September, the scale was 19.896 mg mL^{-1}. These results point to the fact that, due to the effects of sunlight and light, the lycopene content of the fruit grows[20]. Plants that matured in June in the greenhouse did not complete the ripening and the lycopene was not completely biosynthesized. The fruit harvested in September has long been in the sun, outside the greenhouse, so lycopene was biosynthesized.

Thermally processed fruits in the industry as well as heat treatment in the household have lower yields, because heat processing slightly lowers lycopene content in the products. The yield of lycopene fraction was 4.983 mg mL^{-1} for samples 3, 4.951mg mL^{-1} for sample 5 and 4.899mg mL^{-1} for sample6, while yield of sample 4 was 10.003 mgmL^{-1}. Comparison of lycopene yield can be seen in the Figure 4.

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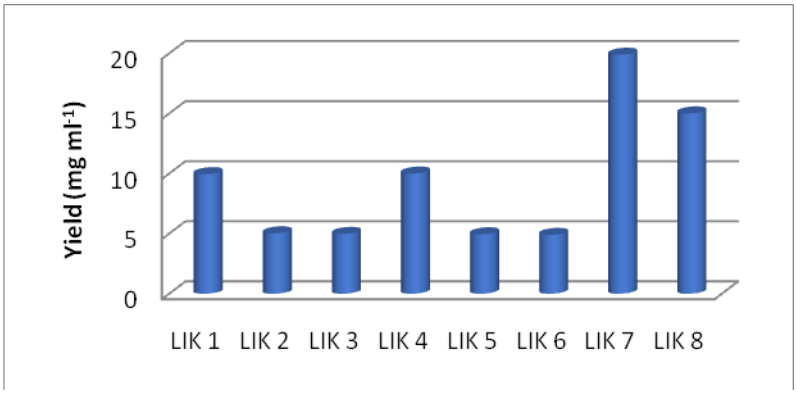

Figure 4: Lycopene fraction yield after recrystallization.

The results of thin layer chromatography (TLC)

The resulting chromatograms with the fraction samples before and after recrystallization were observed under a UV lamp. In both cases, there are many stains on the plates. β-carotene and lycopene, due to a different retention factor (Rf), travel at different speeds and are retained at different sites on the plate. The stains are not completely separated and clearly visible but there is already a complete trace of the molecules along the solvent pathway. This is due to the presence of different isoforms of β-carotene and lycopene, as well as other molecules within the extract such as lipids, amides, acids which are otherwise present within tomato fruit. The existence of these molecules has been proven when capturing the FTIR spectrum, which will be discussed further below.

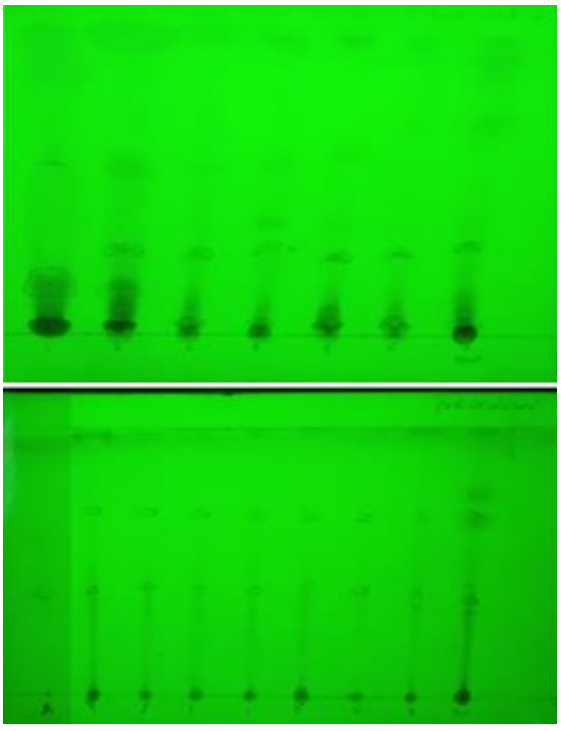

Figure 5: Obtained TLC chromatograms before and after recrystalization.

As a TLC solvent, a mixture of hexane and acetone was used in the volume ratio 70:30. From the ratio it is seen that more non-polar hexane is used. Since lycopene is less polar than β-carotene it remained halfway down. The β-carotene was present on the chromatographic plate due to the presence of a small amount of polar acetone, as seen from different Rf values. Recrystallized samples should be of greater purity, but according to Rf values it is seen that post-recrystallization has become more polarized by lycopene and has gone a long way compared to lycopene from un-recrystallized samples. During recrystallization, with the usage

of chemicals and heat,the isomerized lycopene adds itself various functional groups and its accompanying compoundspresent in the fruit. According to the literature, for this mixture of solvents, the Rf value of lycopene is about 0.34[21].

Lycopene stains were labelled on the chromatographic plate and Rf value was calculated for each sample. The obtained values are given in Table 3.

Table 3: Rf values of extracted lycopene before and after recrystallization

Samples	Rf values before recrystallization	Rf values after recrystallization
LIK 1	0.231	0.416
LIK 2	0.280	0.429
LIK 3	0.270	0.403
LIK 4	0.301	0.403
LIK 5	0.269	0.429
LIK 6	0.276	0.403
LIK 7	0.300	0.377
LIK 8	0.301	0.377

Results obtained by FTIR spectroscopy

In order to identify the chemical structure, i.e. the structural characterization of the isolated lycopene obtained by extraction, the FTIR spectroscopy method was used. Sampling spectrawas obtained after recrystallization and a sample spectrum with lycopene was separated by column chromatography, i.e. purified lycopene. Spectrum shows specific peaks and strips that are characteristic of lycopene as well as the strips of accompanying molecules within tomato fruits extracted together with lycopene, such as lipids, amides, acids, water etc. The specific peaks on the FTIR spectrum are determined using literature[22,23].

The spectral data showed the interpretation of the most significant group frequencies for the functional groups and structural components found in the present compound. The hydrocarbon and methyl group are molecular fragments that contribute their own set of characteristic of absorptions to the spectrum of the compound. In fact, the bonding between the functional group and the backbone is only the part of the overall picture used for spectral interpretation[24]. In Figure 6. FTIR spectrum of all samples with mutual overlapping can be seen.

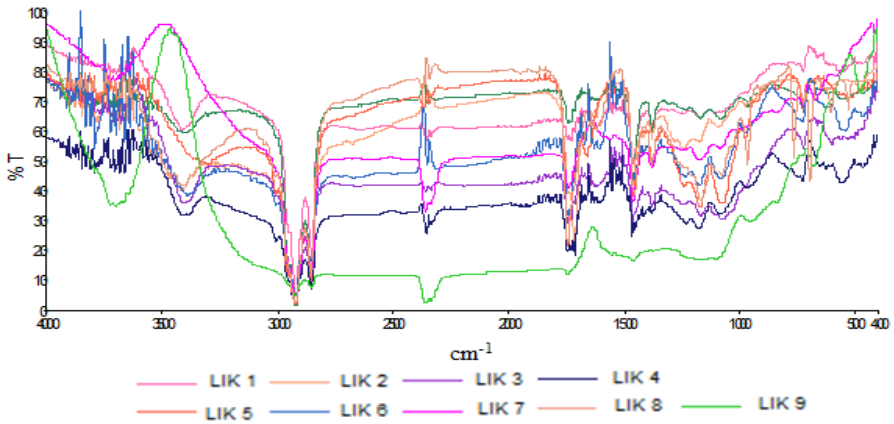

Figure 6: FTIR spectra of all samples (LIK 9 – sample obtained by column chromatography).

Figure 6. shows FTIR spectra with characteristic lycopene peaks of all samples corresponding to the wavelength range from 850 to 1200 cm^{-1}.It can be noted that in this area, the spectra of samples 3, 4, 5 and 6

with a greater deviation from the base line (3 and 4 are samples of industrial heat treated, and 5 and 6 are thermally treated in the household). Stronger and more pronounced peaks in the mentioned wave area confirm the validity of the results of this paper in relation to the literature data. After the thermal processing of tomato fruit, the content of cis-isomers that are less stable increased, produced higher energy, and therefore gave stronger and more noticeable peaks on these wavelengths.The wavelength of about 960 cm^{-1} shows trans C-H vibrations. For samples 3, 4, 5 and 6 on this wavelength wecan see a large deviation from the base line, which proves that the lower content of trans isomers in these samples aredue to the presence of cis isomers.

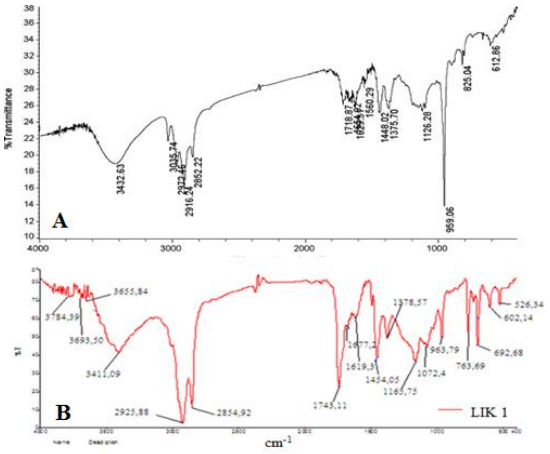

Figure 7: FTIR spectra. (A) [25],Spectrum of lycopene standard. (B) Spectrum of purified lycopene.

From Figure 7. we can distinguish the following points:3784.49 cm^{-1}, 3693.50 cm^{-1}, 3655.84 cm^{-1}, 3411.09 cm^{-1}, four points can be attributed to the presence of water that gives strong and broad strips, also the presence of water in the shape of peaks at1677.2 and 1619.3 cm^{-1}.At 2925.88 cm^{-1} and 2854.92 cm^{-1} there is evidence of the presence of lipids from tomato fruits, as well as at 1743.11 cm^{-1}.Within the region 2850-2960 cm^{-1} we find C-H symmetric and C-H asymmetric stretching.The bands between 1400 and 1477 cm^{-1} originate from C-H bending;specifically, in the picture the peak is at 1454.05 cm^{-1}.1100-1400 cm^{-1} shows the C-C and C-C-H stretching; in our spectra the strips are at 1378.57, 1165.75 and 1072.4 cm^{-1}.The peak at 1165.75 cm^{-1} can also be attributed to C-O stretching of an acid or lipid.Region 900 - 1200 cm^{-1} contains strips resulting from C-O-C vibrations; in our case the peaks are 963.79, 1072.4 and 1165.75 cm^{-1}.Spectral signal at 963.79 cm^{-1} can be attributed to trans C-H deformation vibrations of the lycopene molecule.All types of deformation within the lycopene molecule are below 1000 cm^{-1}[26,27].

Melting point determination

Determining the melting point of a compound is one way to test if the substance is pure. A pure substance generally has a melting range (the difference between the temperature where the sample starts to melt and the temperature where melting is complete)of one or two degrees. Impurities tend to depress and broaden the melting range so the purified sample should have a higher melting range than the original, impure sample.

The difference between the purely theoretical definition of the melting temperature and the results obtained in practice is now widely recognized. The measurement of the triple point is done in a highly complicated experiment. Determination of the melting point was done for all samples. Literature data[11]. indicate that the melting point of lycopene is 172-175 ºC. The obtained melting point values were between171.1 and 173.71°C for all samples.

CONCLUSIONS

Tomato is proved to be a good natural source of lycopene. The yield after recrystallization, calculated from the obtained crystals, proves that the lycopene content changes depending on the condition of extraction. For lycopene to be completely purified from extract, several consecutive recrystallizations and

column chromatography's are needed. Thermal treatment of samples causes a slight loss of lycopene in processed tomatoes, which corresponds to literary data. The degree of ripeness of tomato fruits had a significant effect on increasing the fat content in the lycopene fraction in the extract, and the autumn fruit has a higher lycopene content than the fruit harvested in early June.However, thermal processing makes the lycopene more available in processed tomato. In tomato fruit products, the cis isomer concentration increases due to thermal treatment. These isomers are easier to absorb.This nutrition information could be a guide to processors and consumers of tomato products.Consumption of naturally occurring carotenoid-rich fruits and vegetables, particularly processed tomato products containing lycopene, should be encouraged with positiveimplications in health and disease.Lycopene intake can be seen as a preventive measure and non-pharmacological therapy for different types of diseases, but the work of professionals in nutrition and health is required to increase its intake through food education and to propose daily intakes from results of scientific research.

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