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Determination of lycopene in food by on-line SFE coupled to HPLC using a single monolithic column for trapping and separation

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Abstract

A method that would eliminate the degradation of lycopene during analysis was developed. Supercritical fluid extraction (SFE) with carbon dioxide as the extraction medium was connected on-line to high performance liquid chromatography (HPLC) where a single monolithic column was used for trapping and the subsequent separation of analytes. The method was linear over the studied range (0.1–2.5 μg), and it was repeatable (R.S.D. 3.9%), sensitive (LOD = 0.5 ng) and fast (35 min). Lycopene was determined in tomatoes, fruit and several food products. Because of the on-line construction, lycopene was not in contact with air or light during the whole procedure and the amount analysed should therefore correspond to the real amount in the sample.

Keywords: Lycopene; Food; Supercritical fluid extraction; Liquid chromatography; On-line coupled SFE–LC

1. Introduction

Lycopene is a carotenoid pigment, well known in red colour of tomatoes. The singlet oxygen-quenching constant of lycopene is double than that of β-carotene and as much as 10 times than that of α-tocopherol [1]. The ability of lycopene to trap peroxyl radicals is thus significant. This antioxidant property can be exploited in the protection against epithelial cancer [2,3,4,5] and vascular disease [6,7,8]. Consumption of food containing lycopene is thus recommended for health reasons. Knowledge of the content of lycopene in food and food products then becomes important.

Solid-liquid extraction (SLE) has been applied to the isolation of lycopene from solid matrices [9–12]. Replacement of toxic organic solvents, employed in SLE with carbon dioxide in supercritical state provides a more environmentally friendly and faster extraction procedure. Supercritical fluid extraction (SFE) offers other advantages as well associated with the high diffusivity and low viscosity of supercritical media [13]. SFE is also easy to connect on-line with chromatographic techniques because CO_2 is a gas at ambient conditions.

SFE has been used for the extraction of carotenoids, and especially of β-carotene [14], however employing of SFE for isolation of lycopene is a matter of recent years. The key parameter in the supercritical fluid extraction of lycopene has been the extraction temperature. In the work by Baysal et al., the best parameters for the isolation of lycopene (54% recovery) from tomato paste waste were 55 °C and 300 bar, with addition of 5% ethanol [15]. Cadoni et al. [16] investigated the removal of lycopene from ripe tomatoes and achieved 87% extraction yield at 80 °C and 275 bar. Cadoni et al. assumed that extraction recovery would increase with temperature, but this was not tested as lycopene degradation was expected to occur at elevated temperatures. In the study of the SFE of dried tomato skin at higher temperatures, Ollanketo et al. [17] observed degradation of lycopene above 120 °C. Parameters

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for the quantitative extraction were 110 °C and 405 bar. Recently, SFE was tested as a procedure for obtaining lycopene from processed tomato products [18], where the best extraction recovery (61%) was obtained at 86 °C and 34 MPa. The extraction yield has also been investigated as a function of the particle size of crushed tomatoes [19]. The extraction yield of tomato was poorer for small particles (0.080 mm) than for particles of 0.345 mm and the result was an inhomogeneous extraction caused by the channeling effect in a fixed bed.

HPLC is the most widely used method for the analysis of fruit and vegetable extracts because it is benign for thermally unstable carotenoids. Analysis of total lycopene can be performed with the use of C18 as the stationary phase [16]. However, when the separation of *cis*- and *trans*-isomers is required, C30 stationary phase has usually been employed [17,20–22]. Detection has frequently been done with the UV–vis detector but also electrochemical detection (ED) as well as mass spectrometry (MS) with atmospheric pressure chemical ionisation (APCI) have been employed [15,17,18, 20–26]. The use of other analytical techniques such as supercritical fluid chromatography (SFC) with UV–vis detection [27] and laser optothermal window (LOW) [28] has been reported as well. Traditional spectrophotometry [29,30] can be employed for the estimation of lycopene content in fruit extract.

A number of attempts have been made to combine SFE with LC and several interfaces have been developed [31]. Several types of interfaces have been developed for the on-line coupling of SFE with LC and of these, trapping onto a solid phase adsorbent is the most common approach. Solid phase trapping requires a separate trap column, because the high backpressure caused by the packed column prevents direct trapping to a conventional HPLC column. The high backpressure means that the fluid cannot be efficiently decompressed and thus it will retain (partially) its solvation properties, and efficient trapping will not be achieved, especially if modifiers are added in the fluid.

Coupling of extraction and analysis offers several advantages, and many of the problems associated with the traditional approaches can be avoided. The analysis is typically faster, less solvent is needed, and the cost of analysis decreases. As well, the reliability and repeatability of the analysis are improved since the analysis and sample clean up take place in a closed, usually automated system, and the risks of sample loss and contamination decrease. Furthermore, the negative effects of light, atmospheric oxygen and moisture are eliminated, which can be crucial for labile analytes.

In this study, a simplified coupling for on-line SFE–LC was developed. The constructed interface included a single monolithic column for both trapping and separation. The instrumentation was applied to the determination of lycopene in food with UV–vis detection. Differing from earlier work, lycopene was not exposed to atmospheric air or light during the analytical procedure, and unwanted and unexpected degradation was thereby eliminated.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (Labscan Ltd., Dublin, Ireland) and methyl-*tert*-butyl ether (Rathburn Chemicals, Walkerburn, Scotland) were used as mobile phase for liquid chromatography. SFE/SFC grade carbon dioxide was purchased from Messer (Vantaa, Finland). Methanol, a modifier for carbon dioxide, was from J.T. Baker, Deventer, Holland. *trans*-Lycopene standard (extract from tomato, 90–95% purity) was purchased from Sigma–Aldrich (Helsinki, Finland) and cholesterol (purity ≥95%) from Merck KGaA (Darmstadt, Germany). Sea sand (Riedel-de-Haën GmbH, Seelze, Germany) and Hydromatrix (Varian Inc., Harbor City, CA, USA) were used in sample preparation. Real samples bought in a local grocery shop were as follows: tomato (Spain), ruby grapefruit (USA), guava (not specified), pomelo red grapefruit (Israel), watermelon (Spain), papaya (not specified), dates (Tunis), tomato ketchup (Heinz, The Netherlands), tomato paste (Rainbow, Italy), pasta sauce (Raguletto, UK), and rosehip paste (Nestle, Finland).

2.2. Standard solutions

The *trans*-lycopene standard solution was prepared by diluting the commercial standard with methyl-*tert*-butyl ether to concentration of 100 μg ml^{-1}; it was stored in a brown-glass vial in a freezer. The same procedure was applied to the preparation and storage of cholesterol (employed as internal standard) standard solution with the difference that the concentration was 200 μg ml^{-1}.

2.3. SFE–HPLC

The on-line system was constructed from an SFE (Suprex Prep Master with Accutrap, Pittsburgh, PA, USA) and an HPLC (Agilent Technologies) with HP 1050 degasser and pump and HP 1100 DAD UV–vis detector (Espoo, Finland). For SFE, extraction cartridges of inner volume 0.8 ml were used for sample extraction. In HPLC, an isocratic mixture of 90% acetonitrile and 10% methyl-*tert*-butyl ether was employed as the mobile phase with a flow rate of 1 ml min^{-1}. The interface (Fig. 1) coupling SFE to HPLC consisted of a monolithic column Chromolith, RP-18e, 100–4.6 mm (Merck KGaA, Darmstadt, Germany), and one electrically controlled six-port valve (Vici AG, Schenkton, Switzerland). The CO_2 outlet from the SFE device was connected to the monolithic column, which was placed in a thermostated chamber (part of the SFE). The monolithic column was employed both for trapping and for analyte separation. Mere switching of the interface valve provided two modes: extraction mode and analysis mode. Moreover, in the analysis mode, the standard sample could be analysed by employing the injection valve (Vici AG, Schenkton, Switzerland) with a sampling

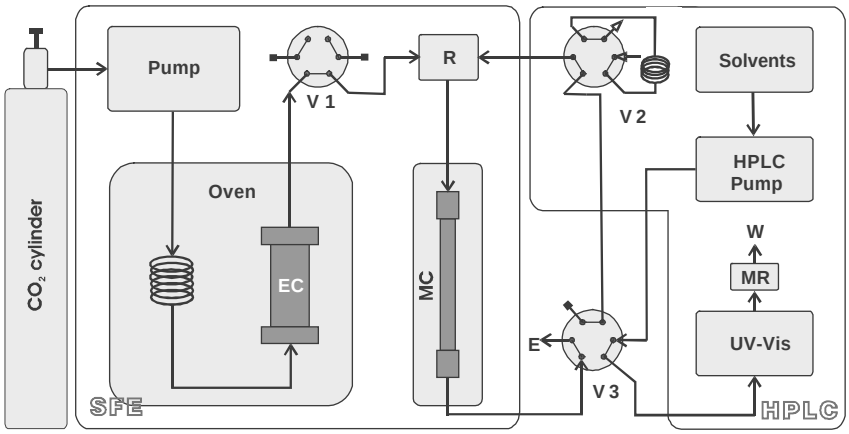

Fig. 1. SFE–HPLC apparatus displayed in extraction mode. EC: extraction column, R: restrictor, MC: monolithic column placed in thermostated chamber, V1: static/dynamic SFE valve, V2: HPLC injection valve with sampling loop, V3: interface valve switching between extraction and analysis mode, E: exhaust, MR: mobile phase restrictor, W: waste.

loop (50 μl) that was part of the HPLC segment. A pressure restrictor was attached to the output of the UV–vis detector to increase the pressure inside the detector cell. This improved separation performance as will be discussed below.

2.4. Sample preparation procedure

Sample preparation varied slightly for the different samples. Raw tomato and date were washed with distilled water, dried with a clean napkin, peeled, and any remaining pulp was carefully scraped from the skin, which was analysed. Grapefruit were washed, dried and peeled, and several cell-like pieces from the pulp were taken for analysis. Washed and dried guava, papaya and watermelon were cut in half and the pulp was sampled. Other food products were homogenised by stirring, and a part was sampled. Subsequently, the sample was weighed and transferred into a porcelain mortar where it was ground with sea sand (∼500 mg) and Hydromatrix (∼80 mg). After that, the contents of the mortar were quantitatively transferred to the extraction cartridge. In the cartridge, the homogenised sample was placed between two layers of Hydromatrix (∼40 mg) and capped with two round pieces of filter paper. Internal standard and modifier were added before the cartridge was tightly closed. Finally, the cartridge was attached to the extractor and the extraction was started.

3. Results and discussion

In the development of the SFE–LC method, the LC separation was optimised first separately, after which extraction and trapping were optimised with the whole on-line coupled system. The applicability of the total method to the quantitative analysis was evaluated by determining linearity, repeatability and limits of detection.

3.1. LC separation and analyte trapping onto the monolithic column

In the study of the trapping and the following LC separation, the separation conditions were optimised first. Isocratic conditions with fully organic solvent composition provided sufficient separation. Lycopene could then be separated from β-carotene, which absorbs at the same wavelengths as lycopene. The *cis*- and *trans*-lycopene were partly separated from each other. The flow rate of the eluent was not critical, and a flow rate of 1 ml min^{-1} was chosen to give an analysis time of 10 min.

Lycopene occurs in nature primarily in *trans* form, which possesses more bioactivity than the *cis* form. The isomerisation of *trans*-lycopene to *cis*-lycopene may take place during handling of the sample, e.g. during drying due to exposure of the sample to air and light [16]. More intense exposure causes degradation of lycopene, observable as the loss of red colour of a sample or an extract [13]. In our experiments, a minor amount of *cis*-lycopene was found in most of the samples (Fig. 2). The constancy of the *trans*/*cis* ratio under different extraction conditions (time, temperature) indicated that the *cis*-lycopene is formed during grinding of the sample.

Direct trapping onto a conventional packed column is not possible for several reasons. (1) The packing materials generally used in HPLC do not tolerate complete drying, as will occur in direct trapping. (2) The high flow-rate of CO_2 upset the stability of the packing of the stationary phase bed, causing problems in the separation. (3) The CO_2 cannot be decompressed and thus it will possess (part of) its solvation capabilities due to the backpressure created by the column. This, in turn, will cause serious band-broadening of the analyte bands during the trapping.

In general, monolithic columns are characterized by low backpressure, which allows high flow rates, and they also tolerate drying of the stationary phase. The monolithic column used in our experiments was run under a flow rate of gaseous

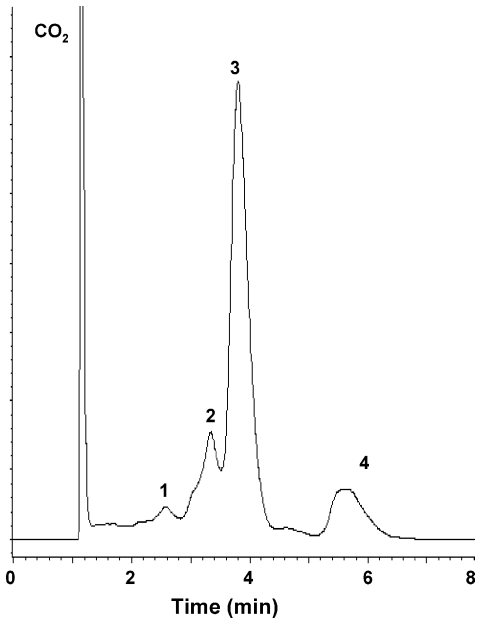

Fig. 2. Chromatogram of tomato extract. Peak eluting at 1.2 min comes from residual CO_2 present inside the column after extraction: (1) β-carotene; (2) *cis*-lycopene; (3) *trans*-lycopene; (4) cholesterol (internal standard).

CO_2 of about 500 cm^3 min^{-1}, varying with the extraction parameters. Continuous changes between extraction and analysis caused successive wetting and drying of the monolithic stationary phase, but this did not have any negative affect on the chromatographic profile of the standard sample of *trans*-lycopene during the time scale of the experiments. The same monolithic column was employed in all experiments.

The trapping efficiency of the monolithic column in the on-line system was studied with and without modifier. Even with longest extraction time (80 min) and with use of methanol as modifier, no significant band-broadening relative to direct injection of the standard was observed. Lycopene, as a highly nonpolar compound, was efficiently trapped at the beginning of the column.

In a test for possible analyte breakthrough, 10 μl of methanolic standard solution of *trans*-lycopene (0.5 μg) was injected to the top of the monolithic rod. The column was reattached to the system and a blank extraction was performed. The amount of analyte corresponded to the dosed amount, confirming that no breakthrough of *trans*-lycopene had occurred.

Gaseous CO_2, remaining in the column after the extraction, is partly dissolved in the mobile phase and as it changes the pH of the mobile phase and thus, chromatographic behaviour of certain compounds could be changed. This had no effect to lycopene, as it is a non-polar and non-dissociable compound.

The on-line arrangement was improved by attaching an additional pressure restrictor to the outlet of the UV–vis detector, which increased backpressure inside the UV–vis to 15 atm (backpressure of the whole system increased from 30 to 45 atm). This caused compression of the residual CO_2 band during elution and possible interference with analyte peaks was thereby minimised. Moreover this resulted also in much better reproducibility in retention times of the analyte peak in the extract: *trans*-lycopene exhibited just 1.1% relative standard deviation within seven runs.

3.2. Stability of lycopene during analysis

The sample preparation procedure was developed so as to minimise the contact time of the crushed sample with air and light and thereby avert the degradation of lycopene [17]. Most of previously published procedures employed drying of sample before extraction that could result in lycopene oxidation [16–19,22,37]. There are some papers describing removing of moisture in methanolic solution in the presence of $CaCO_3$ [24] or ethylenediamine tetraacetic acid with *tert*-butyl-4-methoxyphenol [32]. In our procedure, the water content of the sample was fixed with Hydromatrix during grinding, and immediately afterwards the sample was analysed. Another advantage of the procedure was associated with the instrumental arrangement. Lycopene extracted from a sample was immediately trapped onto the monolithic column and the HPLC analysis was performed immediately after the extraction step. During the whole procedure, the analyte was kept under an environment of carbon dioxide inside the column and no degradation due to contact with air or light could occur. This is in contrast to off-line methods where the extract is exposed to ambient conditions.

3.3. SFE extraction

The preliminary conditions for the extraction, i.e. extraction temperature, pressure and modifier, were chosen on the basis of our previous off-line study [17]. Here, we found that the most important parameters were the extraction temperature and the modifier, while the pressure did not have a significant effect on the recovery. The use of modifier improved the recovery of the extraction, and quantitative recovery was obtained faster. In an on-line system, however, modifier can affect the efficiency of trapping, and a study was made to determine whether use of modifier was advisable in the present system. The effect of temperature and the duration of the static and dynamic periods of extraction were also studied.

3.3.1. Effect of modifier

On the basis of our previous study, we chose methanol as modifier for the static period of extraction [17]. The enhancement of relative extraction recovery achieved with use of methanol is assumed to be due to two things. The first of these is the increase in solubility of a carotenoid in the presence of entrainer [33]. The *trans*-lycopene is less soluble in supercritical carbon dioxide than are the other major carotenoids [34] and a modifier is needed. The second cause is that *trans*-lycopene is contained inside chromoplasts [35], a plastid present in cell cytoplasm. The presence of modifier in CO_2 accelerates the rupturing of cell and chromoplast

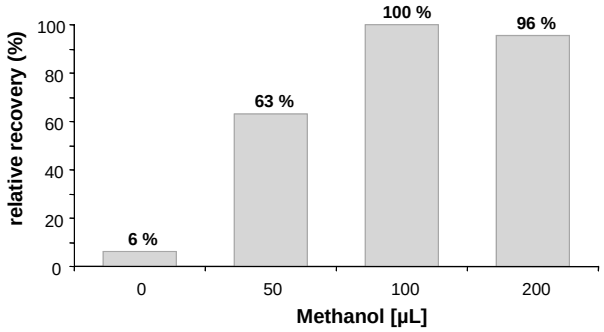

Fig. 3. Relative extraction recovery of lycopene in tomato skin obtained with different amounts of modifier added to the extraction chamber. Amount of sample extracted was 5 mg. Extraction parameters were: p = 400 atm, T = 90 °C, static time = 10 min, dynamic time = 5 min.

walls through elevated osmotic pressure, and *trans*-lycopene is thereby exposed to the extraction medium much faster than if is without use of the entrainer. Methanol was more suitable modifier than acetone, which possesses a similar modification effect, because it has weaker elution strength [17]. This minimised the possible elution during trapping and band-broadening of analytes at the trapping/analytical column. Study was made of the optimum amount of methanol for the extraction of lycopene and best results were obtained with 100 μl (Fig. 3).

3.3.2. Effect of temperature

Extraction temperature has a critical effect on the extraction yield of lycopene. While elevated temperature increases solubility, it may also cause degradation of lycopene, which is thermally unstable. Experiments carried out to determine the optimum extraction temperature were performed at 400 atm, with liquid CO_2 flow rate of 1.5 ml min^{-1}. Methanol (100 μl) was added to the extraction cell as a modifier, and the static and dynamic extraction times were 10 and 60 min, respectively. Extraction temperatures of 40–120 °C were tested. The maximum extraction yield was found at 80–100 °C. At higher temperatures the extraction yield decreased, indicating degradation of *trans*-lycopene. An extraction temperature of 90 °C was chosen as optimal.

3.3.3. Optimisation of extraction time

Shortening of extraction time while maintaining maximum extraction recovery is important from the cost point of view. The time required for dynamic extraction was studied (5 mg of tomato, 400 atm, 90 °C, 100 μl of methanol) with a 10 min static period and a dynamic extraction period ranging from 5 to 80 min. Relative extraction recovery was found to be the same within this time interval. The rapidity of the extraction process was attributed to the small amount of sample as well as static period of the extraction and in the presence of methanol with a dynamic period of 10 min. The whole process of lycopene determination (Table 1) takes 35 min.

Table 1
Duration of individual steps and total time for the determination of lycopene

Step	Time (min)	Conditions
Sampling and sample preparation	10	–
Static extraction	10	90 °C, 400 atm with 100 μl methanol
Dynamic extraction	5	90 °C, 400 atm at 1.5 ml min^{-1}
Analysis	10	90% acetonitrile and 10% methyl-*tert*-butyl ether, 1 ml/min
Total time	35	–

3.4. Quantitative analysis

To test the suitability of the method for quantitative analysis, we determined linearity, repeatability and limit of detection for the samples. The repeatability of retention times and peak areas was calculated using the analyses of tomato skin samples.

Only a small amount of sample (5–61 mg) was used for the SFE–LC determination. Since small sample size is not always desirable for solid samples, we took care to homogenise the sample well and weigh it carefully. When analytes are present in a sample in low concentration, the limits of detection can be enhanced by increasing the size of the sample. A sample of only 5 mg was used for the analysis of tomato, while in the analysis of dates, the amount of sample was increased to 61 mg. It should be noted that, for lycopene analysis, homogenisation and the subsequent analysis should be carried out without delay to avoid degradation of the analyte. Otherwise the homogenised sample should be kept under inert gas atmosphere.

Calibration was performed with the interface valve switched to analysis mode. Injections of 0.1, 0.25, 0.5, 1.0 and 2.5 μg of standard mixed solution of *trans*-lycopene (40 μg ml^{-1}) and cholesterol (200 μg ml^{-1}) were made to establish the calibration curve. The coefficient of regression (R^2) of the curve was 0.9988 for *trans*-lycopene and 0.9958 for cholesterol.

The limit of detection (LOD) was determined only for the HPLC part, since the LOD for an on-line system is determined by the amount of sample extracted. The LOD of *trans*-lycopene was 0.5 ng.

Sand spiked with *trans*-lycopene and cholesterol was extracted at 40 °C and 400 atm to test apparatus performance and reproducibility. Some degradation of *trans*-lycopene occurred even at this low temperature, probably caused by direct interaction of *trans*-lycopene with residual air inside the cartridge. This did not occur during extraction of tomato skin because the chromoplast in which lycopene resides [25] protects it at the beginning of the extraction. Cholesterol, used as internal standard, was released from the cartridge quantitatively, and the R.S.D. was 3.7% in three runs. The repeatability of the method was therefore tested using tomato skin as the

sample. With 5 mg of tomato skin ($n = 3$), the analyses were accomplished with 3.9% variability, A typical chromatogram is shown in Fig. 2.

The optimum extraction parameters (Table 1) were determined by extraction of 5 mg of tomato skin and correspond to 100% relative extraction recovery. Determination of the real extraction recovery of lycopene from solid matrices is not possible because extraction of spiked material does not simulate the real matrix sufficiently well and some degradation of lycopene was observed even during the extraction of spiked sea sand.

3.5. Determination of lycopene in vegetables and fruits

Several types of food available in Finland were analysed for lycopene (Table 2). The contents of *trans*-lycopene found in the samples were in agreement with previously published data [12,16,18,28–30,36–38]. The lycopene content in fruit depends on the growing area and the crop season [29], and varies over a wide range. Raw tomato has been found to contain 8.8–420 $\mu g\,g^{-1}$ and watermelon 23–72 $\mu g\,g^{-1}$ of lycopene [39]. With such wide ranges, a satisfactory comparison with other methods is difficult.

To demonstrate the ability of our method to determine *trans*-lycopene in small samples, we analysed two different parts of skin of the same tomato: the most reddish bottom part and the top part where red color was least. Analysis confirmed visual observation: the top greenish part of the tomato contained less *trans*-lycopene.

Tomato skin and tomato food products contained higher concentration of lycopene than other samples analysed. Guava was expected to contain *trans*-lycopene, but none was found, perhaps because the fruit obtained from a local grocery shop was unripe. Analysis of dates, which are a more complex matrix containing oils, was accomplished without any interference in the chromatogram, confirming the selectivity of both extraction (oils are not quantitatively extracted at higher temperatures) and analysis (selective wavelenght).

Table 2
Concentration of lycopene determined in food samples

Sample and extracted amount	*Trans*-lycopene (μg/g)
Tomato skin (bottom) (4.8 mg)	281.0
Tomato skin (top) (4.8 mg)	187.7
Dried tomato skin (bottom) (4.7 mg)	283.2
Tomato paste (6.8 mg)	320.4
Tomato ketchup (8.3)	25.4
Pasta sauce (10.7)	166.4
Ruby grapefruit (21.3)	53.7
Pomelo red grapefruit (51.5)	4.3
Rosehip paste (20.5)	2.9
Water melon (15.2)	30.8
Papaya (12.7)	16.1
Date (61.1)	0.2
Guava (35.3)	0.0

The amount of sample extracted is given in parentheses.

4. Conclusions

The on-line coupled SFE–LC system developed for the determination of lycopene is of simple construction and is easy to operate. It is also possible to automate the whole procedure. The total time required for determination of lycopene in samples was very short, and sensitivity was good compared with that of traditional methods, and the amount of sample required is small. The total efficiency of the method was good, as were the linearity and sensitivity. In addition, if the concentration of the analytes is very low, it is easy to increase the sensitivity by increasing the amount of sample. The main advantage of the system is the reliability: the whole analysis takes place in a closed system, so that degradation of lycopene due to atmospheric oxygen and UV light is avoided. The system can easily be adapted for the determination of other antioxidants.

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