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Determination of Ascorbic Acid and Carotenoids in Food Commodities by Liquid Chromatography with Mass Spectrometry Detection

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Two methods, one to determine ascorbic acid and one to determine lycopene and β-carotene, in vegetables and fruits by liquid chromatography coupled with mass spectrometry (LC-MS) have been established. The chromatographic separation of the studied compounds and their MS parameters were optimized to improve selectivity and sensitivity. In both methods, separation was carried out with two coupled columns, first a C_{18} and then a dC_{18}, using as mobile phase 70% methanol (0.005% acetic acid) and 30% acetic acid 0.05% for ascorbic acid determination and a mixture of methanol, tetrahydrofuran, and acetonitrile (60:30:10 v/v/v) for carotenoid analysis in isocratic mode. The molecular ion was selected for the quantification in selective ion monitoring (SIM) mode. Ascorbic acid was detected with electrospray ionization probe (ESI) in negative mode, while chemical ionization atmospheric pressure (APCI) in positive mode was used for the target carotenoids. The methodology for ascorbic acid analysis is based on an extraction with polytron using methanol and a mixture of methaphosphoric acid and acetic acid. Extraction of the carotenoids was carried out with tetrahydrofuran/methanol (1:1) (v/v). The proposed methods were applied, after their corresponding validations, to the analysis of four varieties of tomatoes, tomato in tin enriched and dried tomato, and to the analysis of mango and kiwi fruits, to compare the content in these compounds. Moreover, the influence of the process of freezing and the effect that the manipulation/preservation has in the content of ascorbic acid in tomato have also been studied.

KEYWORDS: Ascorbic acid; carotenoids; liquid chromatography; mass spectrometry; vegetables; fruits

INTRODUCTION

Ascorbic acid (vitamin C) and carotenoids are among the most important compounds present in fruits and vegetables postulated to have a beneficial role in health-promoting effects (*1*−*6*). Both of them act as antioxidants against a variety of diseases. Ascorbic acid, a water-soluble compound, is an important micronutrient that, besides its antioxidant effect, is valuable for its vitamin action playing many physiological roles (*7*). During recent years, the interest in ascorbic acid has also been increased because of its involvement in the liberation of nitric oxide under various conditions (*8*). It can be synthesized by plants and by many mammals, but not by man, and it is present in small quantities in food and is indispensable for life, health, and physical and daily activity. The best way of deriving benefit of ascorbic acid is eating fresh fruits and vegetables or with a minimum of processing (cooking) (*9*). However, losses of ascorbic acid are produced when the samples are frozen or refrigerated (*10*, *11*).

Carotenoids are vegetal natural pigments responsible for diverse biological functions and for the coloration of many fruits and vegetables. They are fat-soluble compounds with excellent properties for the human health. In research, both the lycopene (ψ,ψ -carotene) and β-carotene (β,β -carotene) are responsible for the anticarcinogenic effects of fresh fruits and vegetables (*10*). Both of them present nine conjugated double bonds capable of quenching reactive oxygen species such as singlet oxygen and other free radicals. Lycopene and β-carotene are structural isomers **(Figure 1)**. Lycopene is naturally present in trans configuration in raw tomato (*11*), and it accumulates inside the chloroplast during the ripeness of the fruits. It is converted to its cis form during processing and storage of foods. β-Carotene is a precursor of vitamin A, therefore making fruits and vegetables essential contributors to vitamin A status in humans.

Liquid chromatography (LC) coupled with UV−vis detection seems to be the preferred method for analysis of ascorbic acid, lycopene, and β-carotene. The overwhelming majority of these methods involve the photodiode array detector, which allows examining all wavelengths at the chromatographic peak to confirm the presence of a previously identified compound by retention time. However, food samples require the application of more reliable and selective methods for definitive peak identification that only can be achieved by mass spectrometry (MS). This technique presents a rich amount of qualitative information from which compound identity may be inferred with

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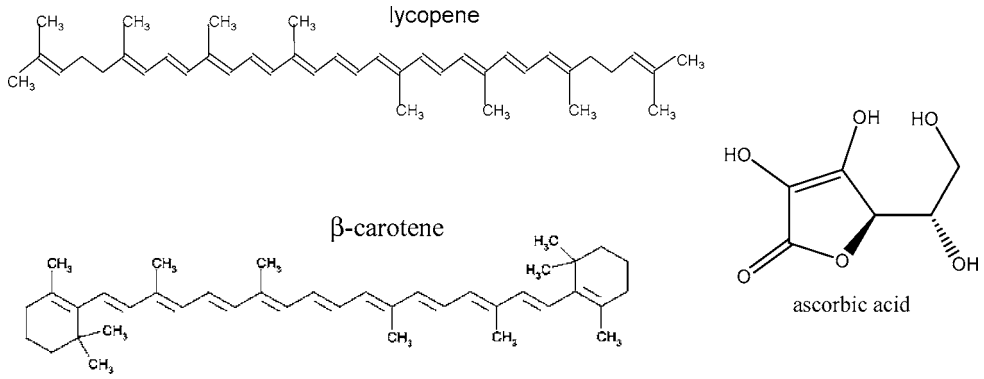

Figure 1. Chemical structure of the target analytes.

a high degree of certainty. In addition, MS has the advantage of providing information for confirmation purposes. Once the identity is established, the quantitative information obtained can be correctly interpreted without producing false positive results. No references have been found which use LC interfaced with MS detection for the quantitative determination of the target analytes in such matrixes although this is not the case for biological samples (*12*−*15*) or recently for fortified infant formulas (*16*). LC-MS has been used in several studies only to confirm the identity of carotenoids from food samples (*17*−*20*).

Despite that the same analytical technique is used for determining the target analytes, methods for their extraction are based on the use of solvents of different properties. Aqueous solvents, such as metaphosphoric acid and acetic acid mixtures, are used for the extraction of ascorbic acid (*9*). Mixtures of organic solvents with different polarity, such as acetone, methanol, *n*-hexane, tetrahydrofuran, and so forth, are used for the extraction of carotenoids (*21*−*24*). In addition, for the latter compounds, methods based on matrix solid-phase dispersion (MSPD) are also found (*25, 26*).

In this paper, we describe the development and validation of the analytical methodologies, on the basis of LC-MS, for determining ascorbic acid on one hand and lycopene and β-carotene on the other hand in food commodities. Finally, the methodologies have been applied to the analysis of ascorbic acid, lycopene, and β-carotene in four varieties of tomato widely cultivated in agricultural areas on the south of Spain (Almería) and in kiwi and mango fruits. Also, the target compounds were determined in processed/manipulated foods (dried tomato and tin tomato). Until now, analytical methods have not been published for determining the target compounds in the matrixes of the study by LC-MS. This allows the identification, confirmation, and quantification of the analytes in one single injection. Both LC-MS methods described in this work are simple (no cleanup steps) and rapid (analysis time lower than 5, 16, and 24 min for ascorbic acid, lycopene, and β-carotene, respectively). Moreover, the proposed methods include internal quality controls to ensure the reliability of the analytical measures.

MATERIAL AND METHODS

Reagents. L(+)- Ascorbic acid (vitamin C) was purchased from Panreac (Barcelona, Spain), meta-phosphoric acid (33.5−36.5%) was purchased from Fluka (Buchs, Switzerland), and β-carotene and lycopene (ψ,ψ-carotene) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Methanol for HPLC was obtained from Merck (Darmstadt, Germany). Other reagents such as petroleum ether, tetrahydrofuran, magnesium carbonate, and sodium chloride were obtained from Panreac. Doubly distilled water was purified using a Milli-Q system (Millipore, Bedford, MA). A stock solution (340 mg/L) of ascorbic acid was prepared in 50 mL of methanol. β-carotene and lycopene (248 and 40 mg/L, respectively) were prepared in *n*-hexane; first, lycopene was dissolved in 1 mL of tetrahydrofuran and then was diluted with *n*-hexane. Working solutions of lower concentrations were prepared by appropriate dilution with methanol. They were kept in polypropylene bottles at −24 °C. All operations were performed in subdued light and with dark volumetric flasks.

Instrumentation. HPLC system was an Alliance 2695 equipped with autosampler, degasser, and heater column purchased by Water (Milford, Massachusetts). The mass spectrometer system was a ZQ 2000 single quadrupole purchased from Water-Micromass (Manchaster, U.K.). Data were collected by MassLynx 4.0 software in a personal computer. The compounds were separated by two coupled columns, first a Symmetry C_{18} 75 × 4.6 mm i.d. 3.5 μm and then an Atlantis dC_{18} 150 × 2.0 mm i.d. 5 μm, both from Water. A mobile phase composition of 70% methanol (0.005% acetic acid) and 30% acetic acid 0.05% in isocratic mode was used for ascorbic acid determination, while for carotenoid analysis a mixture of methanol, tetrahydrofuran, and acetonitrile (60:30:10 v/v/v) was used. Both columns were set to 30 °C during all runnings, and the injection volume was 10 μL for ascorbic acid and 100 μL for carotenoids.

Ascorbic acid was detected with electrospray ionization probe (ESI) in negative mode, while chemical ionization atmospheric pressure (APCI) in positive mode was used for the target carotenoids. The temperature source was set at 130 °C, the flows for desolvation and cone gas were 400 L/h and 60 L/h, respectively, and the capillary was set to 3.5 kV. Selective ion monitoring (SIM) was acquired by previous infusion of each standard in full scan mode at a concentration of 15 mg/L.

Samples. The food commodities analyzed were tomato, kiwi, and mango, which were frozen in our laboratory. Four cultivars of tomatoes (raf, cherry, rambo, and daniela) were analyzed. The contents of the target compounds in dried tomato and tomato in tin enriched were quantified too.

Extraction Procedures. The extraction procedure for ascorbic acid was slightly modified in relation to the one described in ref *27*. Briefly, 2 g of vegetable or fruit sample was mixed with 5 mL of methanol and 25 mL of a mixture of meta-phosphoric acid 3% and acetic acid 8%. The mixture was homogenized with a polytron mixer (model PT2100, Kinematica AG, Littan/Luzern, Switzerland) at 19 000 rpm for 1 min and then was filtered through a funnel büchner, and 10 mL of this extract was made up to 25 mL with acetic acid 0.1%. This diluted extract was used for quantification by the standard additions method because the matrix contained the target analyte. For it, 1 mL, after addition of standard, was made up to 2 mL with mobile phase and was injected into LC-MS (10 μL).

The extraction method used for carotenoid analysis was similar to that described elsewhere (*28*). Briefly, it consists of mixing 1 g of the vegetal in a polytron with 0.2 g of $MgCO_3$ and 50 mL of a mixture of tetrahydrofuran/methanol (1:1) (v/v). The solvent was removed by filtration and the filter cake was re-extracted again. Then, 50 mL of aqueous NaCl solution (10%) was added and a liquid−liquid extraction was carried out with 50 mL of petroleum ether (twice). The organic phases were joined, and the solvent was removed under vacuum in a

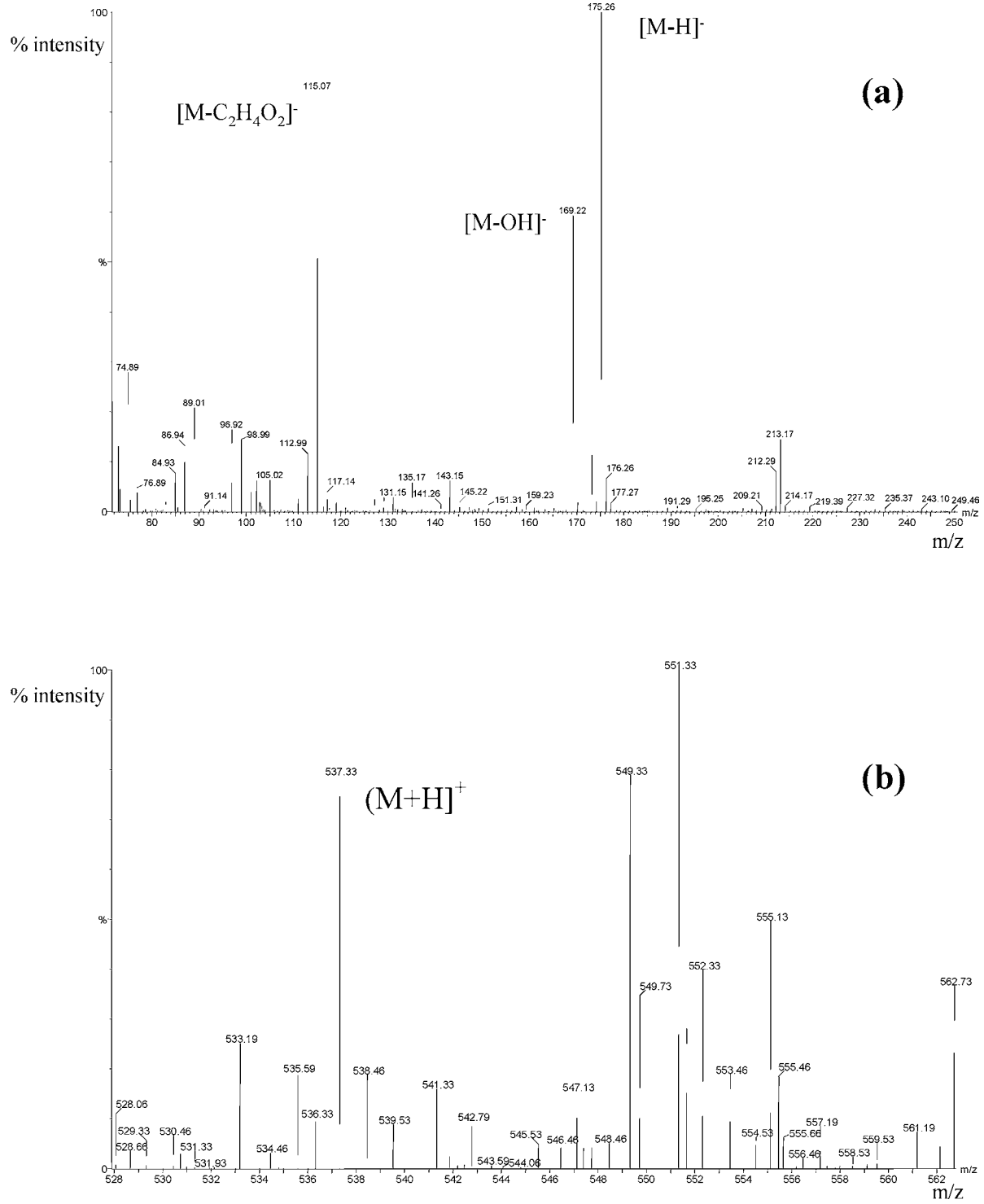

Figure 2. Mass spectrum of (a) ascorbic acid and (b) β-carotene.

rotatory evaporator until almost dry and then just to the point of dryness with a slight N_2 stream. The residue was redissolved in 10 mL of initial mobile phase. This solution was used for quantification by the standard additions method, as in the previous determination, and was injected into LC-MS (100 μL).

RESULTS AND DISCUSSION

Optimization of the Instrumental Conditions. To optimize the MS parameters, individual standards of 15 mg/L of the target compounds were injected by infusion in full scan in positive and negative modes. For ascorbic acid, ESI negative ion mode provided the best response where the unprotonated molecular ion $[M-H]^-$ at *m/z* 175 is monitored at a cone voltage of 20 V. Even different fragment ions were found as confirmation ions when the voltage was increased **(Figure 2a**). On the other hand, the more typical adduct in LC-MS in positive mode ($[M + Na]^+$) was not found in the characterization experiments by infusion. Ascorbic acid is a relative soft molecule with low weight (MW = 176.13), and its molecular ion $[M + H]^+$, as well as its fragment ion at *m/z* 114.8, was selected for monitoring this compound. For carotenoids analysis, APCI was used because a better response was found when this ionization mode was applied. Only APCI in positive ion mode allowed monitoring both carotenoids. **Figure 2b** shows the mass spectrum for

Table 1. MS Conditions and Validation Parameters of the LC-MS Method

compound	ionization method	ion (*m*/*z*)	cone voltage (V)	RTW (min)	recovery (%)	RSD (%)	LOD (μg/L)	LOQ (μg/L)
ascorbic acid	ESI	175	20	3.90–4.10	85	8.7	10	50
β-carotene	APCI	537	30	23.40–23.70	90	10.3	25	100
lycopene	APCI	537	30	15.70–15.90	93	9.4	25	100

β-carotene, and the majority of the ion is at *m*/*z* 537 corresponding to $(M + H)^+$. Lycopene and β-carotene are structural isomers and showed similar mass spectra.

Chromatographic conditions were varied to improve the retention time of the compounds in the column (**Figures 3** and **4**). Two columns were chosen in the two methods because when one single column was used the retention time was short, and at these times there were more matrix interferences. In addition, it was shown that the presence of acetic acid in the mobile phase for ascorbic acid determination improved its interaction with the analytical column. The percentage of acetic acid was optimized with 0.05% being sufficient to find the ascorbic acid protonated. Other parameters optimized were the flow rate of mobile phase and the injection volume, the best conditions being 0.3 mL/min and 10 μL, respectively, for ascorbic acid analysis. For carotenoid determination, the flow and injection volume used were 0.4 mL/min and 100 μL, respectively, which are higher than those in the previous case because of the ionization mode used.

Method Validation. To check the feasibility of the LC-MS methods for the analysis of ascorbic acid, lycopene, and β-carotene, in food commodities they were validated in tomato matrix.

Table 1 summarizes the main LC-MS and validation parameters determined for the compounds in this study. The identification of the target compounds was carried out by searching the appropriate retention time windows (RTWs). The RTW is defined as the average of the retention times plus or minus 3 times the standard deviation of retention time when 10 samples were analyzed. For ascorbic acid, the confirmation of the previously identified compound was done by the ion ratio obtained from the ratio of the intensity of two ions for the same compound. However, for β-carotene and lycopene, it was not possible to find fragment ions, and the confirmation was carried out monitoring the protonated molecular ion $[M + H]^+$.

The linearity of the method was studied by the method of standard additions of the compounds at different concentration levels in the range 0.1−1 mg/L. In this calibration method, a sample extract is used for preparing the calibration curve. It was used because neither blank matrix samples for the target analytes can be found nor matrix matched calibrations, that is, calibrations that have a similar composition to that of tomato or fruit samples, can be prepared. So, linear calibration graphs were constructed by least-squares regression of concentration versus peak area of the calibration standards. Good linearity was found in the concentration range tested, with determination coefficients (r^2) higher than 0.98 in all cases.

Detection (LODs) limits and quantification (LOQs) limits of target analytes were determined as the lowest concentration levels that yielded a signal-to-noise (S/N) ratio of 3 and 10 (when the quantification ion was monitored) ($n = 5$). They are shown in **Table 1** with values of 10 and 25 μg/L for LOD and 50 and 100 μg/L for LOQ for ascorbic acid and carotenoids, respectively.

The recovery rate of each compound was evaluated to assess the extraction efficiency of the proposed methods. For that, different aliquots of a tomato sample ($n = 5$) were spiked independently with the target analytes at a concentration level of 0.5 mg/L and were extracted with the corresponding methods (S1); also, different aliquots ($n = 10$) of the same tomato sample without fortifying were extracted with the corresponding methods, and half ($n = 5$) were spiked at a concentration level of 0.5 mg/L (S2) and the other half were not spiked (S0). Recoveries (R) were calculated as follows: $R = (S1 - S0)/S2 \times 100$. Satisfactory results were found with recoveries between 85 and 93% (**Table 1**). Settling down as a criterion for validation recoveries of the compounds ranged between 70 and 110%, and all the compounds gave acceptable recoveries within the mentioned validation interval. The precision (repeatability, $n = 5$) of the overall methods was also evaluated at the same concentration levels and was expressed as relative standard deviation (RSD). **Table 1** shows the results, with RSD values lower than 11% for all compounds.

Table 2. Contents (mg/Kg) of Ascorbic Acid, Lycopene, and β-Carotene Found in Different Food Commodities

sample	ascorbic acid	lycopene	β-carotene
raf tomato	180	37	15
cherry tomato	157	8	3
rambo tomato	97	14	7
daniela tomato	126	19	11
ketchup	67	11	5
tomato in tin enriched	238	18	6
kiwi	307	<0.1	<0.2
mango	216	<0.1	2

Application to the Analysis of Samples. Once the analytical methods were validated, they were used for monitoring the target compounds in food commodities. Our laboratory is certified by ISO 9001:2000, and so internal quality control criteria must be routinely applied to check if the system is under control. The set of samples analyzed each day was processed together with (i) analysis of an extract of sample, analysis of an extract of sample spiked after the extraction, and analysis of an extract of sample spiked before the extraction to assess the extraction efficiency; real samples were analyzed if recoveries were between 70% and 120%; (ii) calibration curves prepared daily to check both sensitivity and linearity in the working range of concentrations ($r^2 > 0.9$ are requested); and (iii) 10% of duplicated and blind samples.

The proposed methods have been applied to the analysis of the target compounds in four varieties of tomatoes to compare their contents of these important compounds. The analyzed samples were chopped and stored in individual polyethylene bags at −24 °C in our laboratory for 1 month. The ascorbic acid analysis and the preparation of the standards were realized in a short period of time because the ascorbic acid is easily oxidized in solution, especially when it is exposed to the heat. It was found that in all the varieties of tomato, the raf variety showed the higher amount (**Table 2**). The influence of the process of freezing was evaluated for the cases of daniela and rambo tomatoes. When the samples are chopped, extracted, and analyzed the same day, the content in ascorbic acid is higher than in the case when frozen vegetables samples are used. 231 and 228 mg/Kg were found in daniela and rambo tomatoes, respectively, when vegetables were analyzed the same day that they were bought. Thus, in the process of freezing, losses of approximately 50% are produced.

β-Carotene and lycopene were also found in the four varieties of tomatoes. The results indicated that the raf variety also has the highest content in both vegetable pigments (**Table 2**). In general, according to the bibliography, tomatoes have a higher

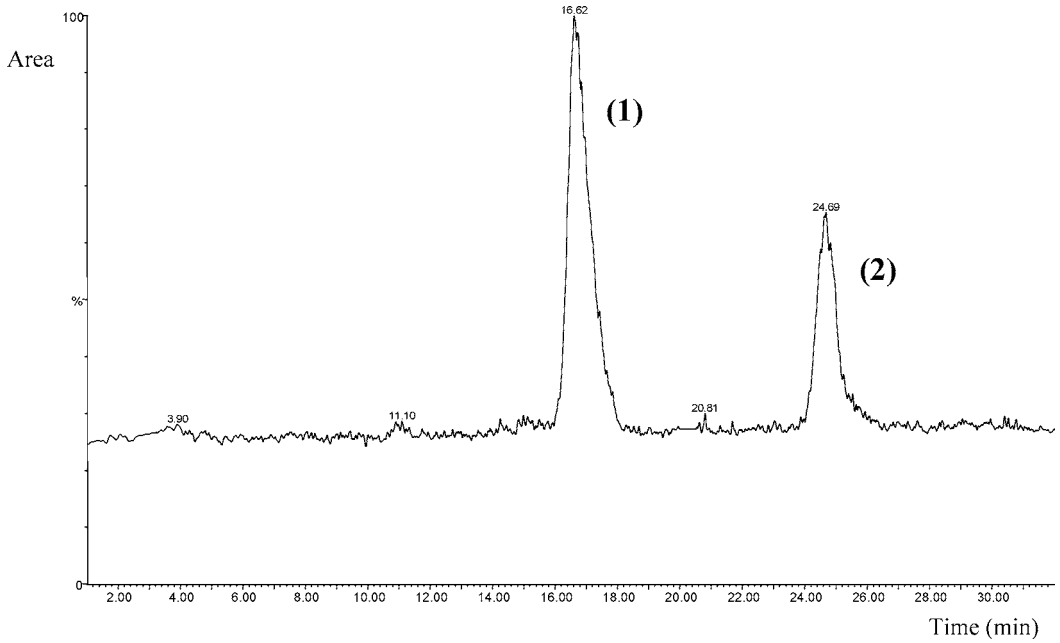

Figure 3. Chromatogram of lycopene (1) and β-carotene (2) in a tomato sample, obtained by two coupled C_{18} columns at 30 °C, and using as mobile phase a mixture of methanol, tetrahydrofuran, and acetonitrile (60:30:10 v/v/v) in isocratic mode.

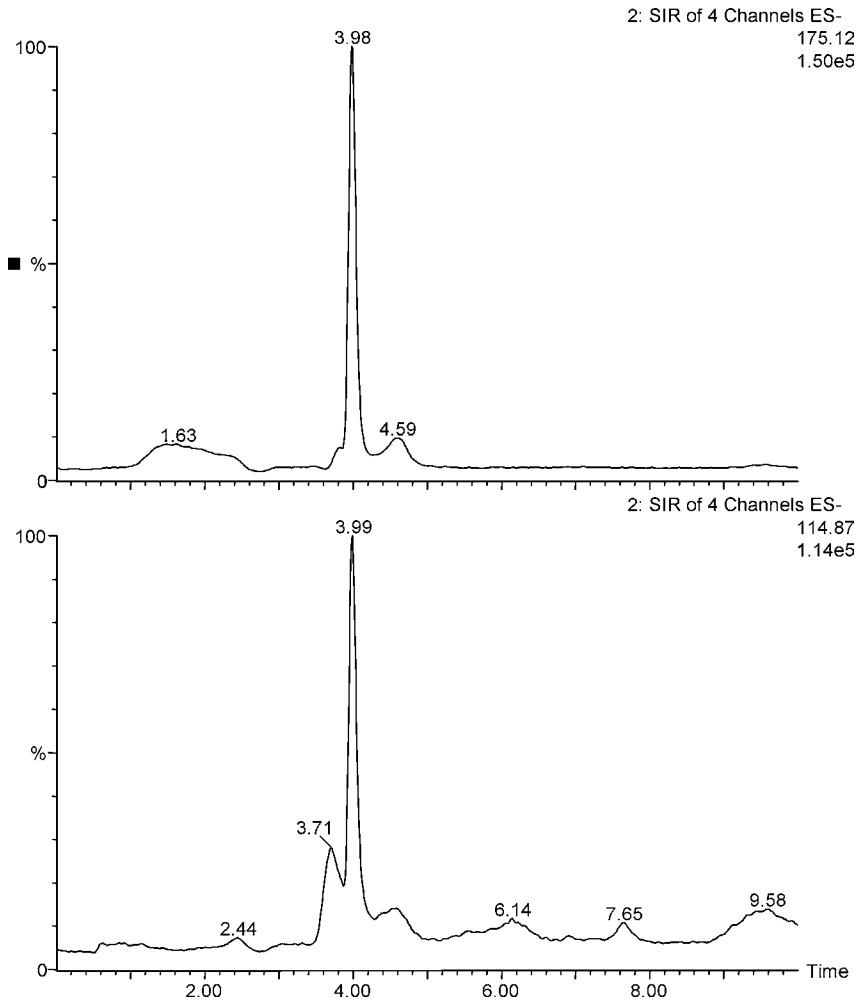

Figure 4. Chromatogram of ascorbic acid in a tomato sample, obtained by two coupled C_{18} columns at 30 °C, and using as mobile phase a mixture of 70% methanol (0.005% acetic acid) and 30% acetic acid 0.05% in isocratic mode.

amount of lycopene than β-carotene, mainly because β-carotene is more efficiently converted to vitamin A than other carotenoids.

On the other hand, analyses on the contents of the target analytes in processed food samples (dried tomato and tinned tomato enriched) were also carried out. A lower amount of

ascorbic acid in dried tomato than in tinned tomato was found because of the accused manipulation of the sample. In addition, the ascorbic acid is a water-soluble vitamin, so usually it is eliminated in the water and it is easily oxidized in solution. The tomato in tin had a high content in acid because it was an entire tomato enriched with the juice of a tomato. Finally, the methods were applied to the analysis of ascorbic acid, lycopene, and β-carotene in kiwi and mango fruits, with kiwi being the commodity with the highest content of ascorbic acid and the lowest content of lycopene of all the analyzed commodities. Mango also showed a low content in lycopene.

Conclusion. To minimize time, labor, and cost, a rapid method for the determination of ascorbic acid in food commodities was optimized using reversed phase liquid chromatography with mass spectrometry detection. There were differences in the amount of ascorbic acid in the different varieties of tomatoes studied, with the raf tomato being the richest in ascorbic acid, although the highest ascorbic acid content was found in kiwi. Losses of ascorbic acid are produced when the samples are frozen, as well as in manipulated foods because of, for example, the oxidation process. Processed tomatoes still contained high amounts of the investigated parameters, which is one reason for recommending the intake of these products. On the other hand, a method for the determination of two carotenoids is also proposed. As in the previous case, there were differences in the amount of these compounds in different tomatoes studied. The raf variety also showed the highest concentrations of lycopene and β-carotene. Kiwi and mango showed low contents of both carotenoids.

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