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U-HPLC-MS/MS To Quantify Liposoluble Antioxidants in Red-Ripe Tomatoes, Grown under Different Salt Stress Levels

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ABSTRACT: The growing interest of consumers in healthy food challenges growers to continuously improve the nutritional quality of their crops. In this research, the potential of a more saline growth environment for improved antioxidant concentration in tomato fruit was studied, and an U-HPLC-MS/MS method for the determination of lycopene, β-carotene, and α-tocopherol was optimized. Analytes were thereby separated on a 1.9 μm Hypersil GOLD C_{18} column and quantified on a TSQ Vantage triple-quadropole mass spectrometer. The method displayed a short analysis time (6 min), a high specificity, and an excellent repeatability ($\leq$6.39%). Furthermore, it was demonstrated that the electrical conductivity level of the applied nutrient solution did not unambiguously influence antioxidant concentration in tomato fruits. Future research should focus on moderate salt stress only and should aim at reducing natural variation by more closely controlling the growth environment and a more objective determination of the ripening degree.

KEYWORDS: *tomato, antioxidants, salt stress, ultrahigh-performance liquid chromatography, tandem mass spectrometry, liquid−liquid extraction, electrical conductivity*

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most popular and extensively consumed vegetable crops worldwide and is consequently fulfilling a key role in the human diet. As a major component of daily meals in many countries, tomato constitutes an important source of health-promoting compounds including antioxidants.[1] In tomato fruit, especially carotenoids, phenolics, tocopherols, and ascorbate are compounds with important antioxidative properties. Among the carotenoids, mainly lycopene and β-carotene are of interest. Lycopene is the most abundant carotenoid in red-ripe tomato fruits (about 80−90%) and exerts the highest antioxidant activity among all dietary antioxidants, whereas β-carotene ranks second (about 7−10%) and is of interest due to its provitamin A activity.[2,3] Among the tocopherols (collectively known as vitamin E), α-tocopherol is the most abundant form and exhibits the highest biological activity.[4] As a consequence, the intake of α-tocopherol, through primarily plant products, determines whether or not vitamin E deficiency, with related cardiovascular and degenerative diseases, occurs.[5]

Improvement of tomato fruit nutritional quality is a challenge for greenhouse tomato growers who want to meet the ever-increasing demand of consumers in a highly competitive fresh market.[1,6] The antioxidant concentration in a certain fruit or vegetable is, however, not fixed and is strongly influenced by differences in tomato variety and ripening stage, as well as by agronomical, geographical, and environmental factors.[3,7] Tomato fruit nutritional quality can be optimized by either genetic modification (by conventional breeding or more advanced molecular techniques) or application of suitable cultural practices. These approaches, however, require the use of analytical procedures to assay tomato fruit antioxidant concentrations and select the optimal cultural practices and/or most suitable varieties with respect to nutritional quality.

Therefore, a first objective of this research was to optimize a liquid−liquid partition based extraction procedure and an ultrahigh-performance liquid chromatography (U-HPLC) atmospheric pressure chemical ionization (APCI) tandem mass spectrometry (MS/MS) analysis procedure by which lycopene, β-carotene, and α-tocopherol, the main fat-soluble antioxidants in tomato, could be quantified. For chromatographic separation, U-HPLC was preferred due to the ability to speed the analysis and increase the sample throughput and resolving power of the chromatographic separation process. This type of chromatography is characterized by the use of columns packed with porous sub-2 μm particles, which results in an increased interaction between stationary phase and analytes with an improved chromatographic separation as outcome.[8] In earlier research the chromatographic separation process of carotenoids and tocopherols was exclusively based on classical HPLC, which generally resulted in extensive analysis times (>30 min).[9−13] For carotenoid quantification most authors use UV−vis detection,[14] particularly diode array detectors (DAD), because rapid and acceptable results are provided.[15] UV−vis identification of a compound is based on the absorption spectrum and the previously determined retention time of the authentic marker.[16,17] Although when a complex food sample has to be analyzed, a more reliable and selective method such as (tandem) mass spectrometry is desirable. Using tandem mass spectrometry, a specific

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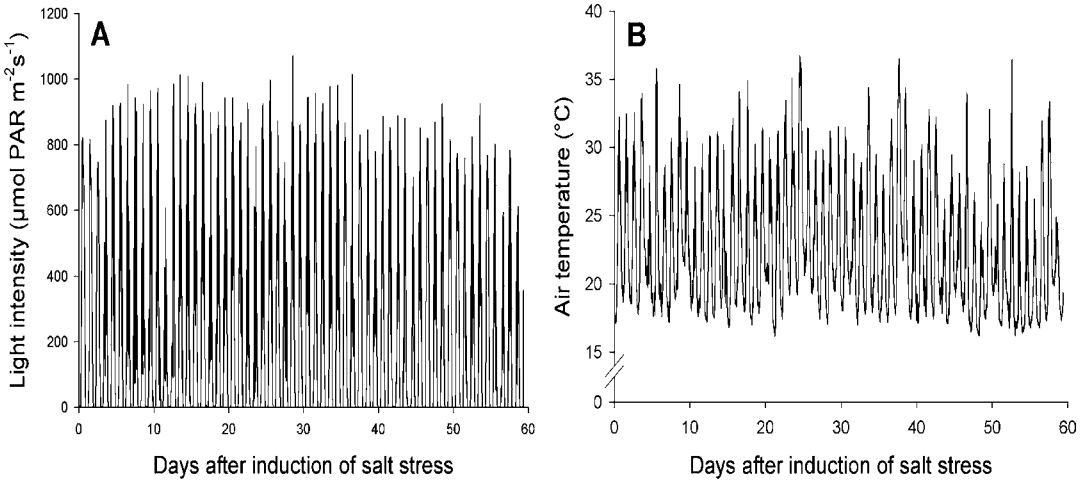

Figure 1. Light intensity (A) and air temperature (B), measured during the entire experiment.

antioxidant can be identified with higher certainty because information about both molecular mass and the presence of functional groups is used.[15,18]

Second, the optimized procedures were applied on hydroponically grown greenhouse tomatoes, subjected to nutrient solutions with various electrical conductivity (EC) levels. After all, it is postulated that increment of the EC level of the nutrient solution may be beneficial to crops by improving the organoleptic quality[19] and antioxidant content.[20,21] Initially, this improvement was exclusively attributed to the concentration effect of compounds in the fruits, which results from the osmotic stress and related impaired water uptake into the fruits. However, physiological responses may be activated as well. More specifically, moderate salinity may improve nutritional quality due to an up-regulation of gene encoding enzymes, involved in the key steps of antioxidant biosynthesis, whereas under higher salinity inhibition may occur, resulting in a reduced antioxidant content.[6] For example, De Pascale et al. observed a gradual increase in total carotenoids (on fresh and dry weight bases) until the nutrient solution reached an EC level of 4.4 dS m^{-1} and a decrease at higher salinity levels.[22] However, the literature is not unambiguous; other authors did not observe any influence of osmotic stress on antioxidant levels.[23,24] After all, many factors such as the growing conditions, selected cultivar, genotype-dependent changes in plant architecture, and specific composition of the nutrient solution, determine the final response in terms of nutritional quality toward the induced salt stress.[6]

Therefore, the dual objective of this research may contribute to a further elucidation of the correlation between osmotic stress and antioxidant concentration in tomato fruit and may be helpful in the determination of an optimum EC value, applicable in the production of greenhouse tomatoes.

MATERIALS AND METHODS

Plant Material and Experimental Setup. Tomato plants (*S. lycopersicum* L. cv. Doloress) were grown in a 360 m^2 compartment of a Venlo-type glasshouse at the Provincial Research and Advisory Centre for Agriculture and Horticulture (POVLT, Beitem, Belgium). Four-week-old tomato seedlings were transplanted on January 9, 2009, in 15 L rock wool slabs (Grodan Expert, Hedehusene, Denmark) whereby a final plant density of 2.3 plants m^{-2} was pursued. Plants were grown under natural light conditions (Figure 1A), and temperature (Figure 1B) was regulated with heating set points of 15 °C at night and 17 °C during the day. Irrigation of the plants was based upon the sum of radiation, whereby 150 mL of nutrient solution was applied each time a value of 150 J cm^{-2} was reached.

For the experimental setup, four treatments, characterized by a specific EC level of the nutrient solution, were considered. Manipulation of the concentration of macronutrients (N, P, S, K, Ca, and Mg), whereby relative concentration ratios were kept constant, resulted in average EC levels of 2.4, 4.0, 5.3, and 8.0 dS m^{-1} for the different treatments, respectively. The nutrient solution with an EC level of on average 2.4 dS m^{-1} consisted of (in mg L^{-1}) 225 N, 38 P, 102 S, 300 K, 171 Ca, 43 Mg, 0.84 Fe, 0.55 Mn, 0.33 Zn, 0.05 Cu, 0.32 B, and 0.05 Mo, which is a common practice in commercial tomato production. The average pH values of the various nutrient solutions were similar and varied between 5.2 and 5.6. From July 13 until September 10, 2009, each treatment was applied to 12 plants. Harvest time of the trusses was based on visual color assessment, whereby trusses were harvested when fruits were deep red. Individual fruits were immediately weighed and stored at −20 °C. To eliminate border effects, tomato fruits from the outer plants (four for each treatment) were discarded. Therefore, the effect of increased salinity on nutritional quality and fresh yield was assessed on eight plants per treatment.

Chemicals and Reagents. *all-trans*-β-Carotene, α-tocopherol, and the internal standards β-apo-8′-carotenal and α-tocopherol acetate were all purchased from Sigma-Aldrich Co. (St. Louis, MO), whereas *all-trans*-lycopene was obtained from LGC Standards (Wesel, Germany).

Acetonitrile, chloroform, isopropanol, methanol, and triethylamine (TEA) were purchased from Fisher Scientific U.K. (Loughborough, U.K.) and ethanol and sodium chloride from VWR International (Merck, Darmstadt, Germany); magnesium carbonate was from Sigma-Aldrich Co. and butylated hydroxytoluene (BHT) from SAFC Supply Solutions (St. Louis, MO). Water, used for sample preparation, was obtained by a purified water system (VWR International, Merck).

Preparation of Stock Solutions. Preparation of stock solutions was adapted from the method of Karppi et al.[25] In brief, stock solutions of lycopene (1 mg mL^{-1}), β-carotene (1 mg mL^{-1}), and β-apo-8′-carotenal (1 mg mL^{-1}) were prepared in a solution containing acetonitrile/methanol/chloroform (18:7.5:74.5, v/v/v) and 0.01% (w/v) BHT, whereas stock solutions of α-tocopherol acetate (1 mg mL^{-1}) and α-tocopherol (1 mg mL^{-1}) were prepared in ethanol + 0.01% (w/v) BHT solution. To prevent any form of degradation, solutions were stored in amber glass bottles at −20 °C.

Extraction of Lycopene, β-Carotene, and α-Tocopherol from Tomato Fruit. Extraction of the various, liposoluble antioxidants from a single, fresh tomato fruit was modified after the procedure of Lin and Chen.[12]

Because variability in antioxidant concentration within a tomato fruit may be expected, homogenization of the tomato fruit was required for representative sampling. This was realized by cutting and

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mixing with an ultraturrax. Subsequently, 10 g of the homogenate was weighed in a plastic tube of 50 mL to which 200 mg of magnesium carbonate and 35 mL of ethanol/hexane (4:3, v/v) were added. After 15 min of rotation (42 rpm), tomato residue and extract were separated by filtration through a Whatman no. 1 filter paper (GE Healthcare, Munich, Germany). The filtrate was collected and poured in a separating funnel. The obtained residue was re-extracted with a second volume of 35 mL of ethanol/hexane (4:3, v/v) and again rotated for 15 min. After rotation, a new filtration step was executed, after which the residue was washed with 30 mL of pure hexane. The obtained filtrate was collected and poured into the previously mentioned separation funnel. To improve the partitioning of ethanol and hexane, 150 mL of distilled water and 100 mL of a 10% (w/v) sodium chloride solution were added to the pooled filtrates. After partitioning, the supernatant was collected, whereas the lower phase was once again extracted with 20 mL of hexane. Both supernatants were combined, and a subvolume of 100 μL was transferred to a HPLC vial and diluted with acetonitrile/methanol (50:50, v/v) + 0.01% (w/v) BHT solution until a final volume of 1 mL before analysis.

Reversed Phase Ultrahigh-Performance Liquid Chromatography. Chromatographic separation of lycopene, β-carotene, and α-tocopherol was conducted by an Accella U-HPLC system (Thermo Finnigan, San Jose, CA), equipped with a reversed-phase C_{18} Hypersil GOLD column (100 × 2.1 mm, 1.9 μm, Thermo Fisher, San Jose, CA). Two solvent systems were compared in terms of separation efficiency of the aimed antioxidants and were selected on the basis of the paper of Rodríguez-Bernaldo de Quirós and Costa.[26] A first mobile phase consisted of a mixture of acetonitrile, methanol, and chloroform (50:35:15, v/v/v) containing 0.01% BHT and 0.05% TEA, whereby an isocratic elution program was implemented. A second mobile phase consisted of acetonitrile, methanol, and isopropanol, and various gradient programs were tested. The flow of the mobile phase and the column oven temperature for both solvent systems were 300 μL min^{-1} and 30 °C, respectively.

Triple-Stage Quadrupole Tandem Mass Spectrometry. Mass spectrometry was performed using a TSQ Vantage triple-quadrupole mass spectrometer (Thermo Finnigan), equipped with an APCI interface.[27] APCI was operated in the positive ion mode, and the following instrumental parameter values were determined as optimal: a nitrogen sheath and auxiliary gas pressure of 70 and 5 units, respectively; a vaporizer temperature of 275 °C; a corona discharge current of 4 μA; a capillary temperature of 250 °C; and an argon collision cell gas pressure of 1.5 mTorr. Remaining parameter values, to be optimized for each analyte separately, and m/z ratios for precursor and product ions are given in Table 1.

Quality Assurance. Prior to the sample analysis, a standard mixture of the targeted compounds was injected to check the operational conditions of the U-HPLC-MS/MS device. To every sample was added the internal standard β-apo-8′-carotenal at a concentration of 50 μg g^{-1}, prior to extraction. Identification of the antioxidants was based on their retention time relative to the internal standard and m/z ratios of selected product ions. After identification, the concentrations of analytes were calculated by fitting their area ratios in a seven-point calibration curve, established by green tomato samples spiked with the internal standard (50 μg g^{-1}) and the three antioxidants in the ranges of 25.00−750.00 μg g^{-1} for lycopene, 5.00−100.00 μg g^{-1} for β-carotene, and 5.00−150.00 μg g^{-1} for α-tocopherol. Area ratios were determined by integration of the area of an analyte under the specific selected reaction monitoring (SRM) chromatograms in reference to the integrated area of the internal standard.

Statistical Analysis. All data were statistically analyzed by the S-Plus software package S-Plus 8.0 (TIBCO Software Inc., Palo Alto, CA). Data sets were first tested for normality and equality of variance. On the basis of the obtained results, it could be decided whether a parametric Kruskall−Wallis test or a nonparametric ANOVA F test (Tukey method) was performed. The different salinity treatments were compared with each other for the antioxidant content of their tomato fruits, both for each harvest date separately as for the entire treatment period.

Table 1. Precursor and Product Ion m/z Values and Instrumental Parameter Values, Optimized for Antioxidants and Internal Standards

	m/z				
analyte	precursor ion	product ion	collision energy (eV)	S-lens voltage (V)	retention time (min)
β-apo-8′-carotenal	417	91	61	92	1.73
		105	46	92	1.73
		119	36	92	1.73
		157	39	92	1.73
α-tocopherol acetate	473	137	51	82	2.79
		165	34	82	2.79
		207	20	82	2.79
		431	17	82	2.79
α-tocopherol	430	121	55	106	2.54
		136	36	106	2.54
		164	28	106	2.54
		165	32	106	2.54
lycopene	537	105	56	106	2.93
		119	36	106	2.93
		145	36	106	2.93
		157	46	106	2.93
β-carotene	537	105	57	122	4.53
		119	47	121	4.53
		121	43	122	4.53
		157	42	121	4.53

RESULTS AND DISCUSSION

U-HPLC Optimization. The first solvent system, consisting of acetonitrile, methanol, chloroform, BHT, and TEA, proved not to be suitable because separation of lycopene and β-carotene was not possible, with the application of neither an isocratic nor a gradient elution (data not presented). In addition, when the column was flushed with methanol after usage of this solvent system, signals corresponding with BHT were observed in the mass spectrum. As a result, the strong adsorption of BHT to the stationary phase of the selected column would head up for an undesirable extensive cleaning after each run.

Therefore, a second solvent system without any antioxidative compounds such as BHT and TEA and consisting of acetonitrile, methanol, and isopropanol was tested. Analytes could be separated by using a gradient elution program starting with a mixture of 50% methanol and 50% acetonitrile for 1.50 min. Next, in 1.50 min the amount of acetonitrile was decreased to 44%, whereas the amount of isopropanol was increased to 6%. Then, in 0.10 min isopropanol was further increased to 15% and acetonitrile was further decreased to 35%. This mobile phase composition was kept for 1.90 min and was then switched back to initial conditions in 0.10 min and was kept there for 0.90 min. Under these conditions antioxidants and internal standards were successfully separated within only 6 min (Figure 2A), resulting in a significant shortening of the total analysis time in comparison with earlier research.[9−13] Because of the short analysis time, it could be expected that potential

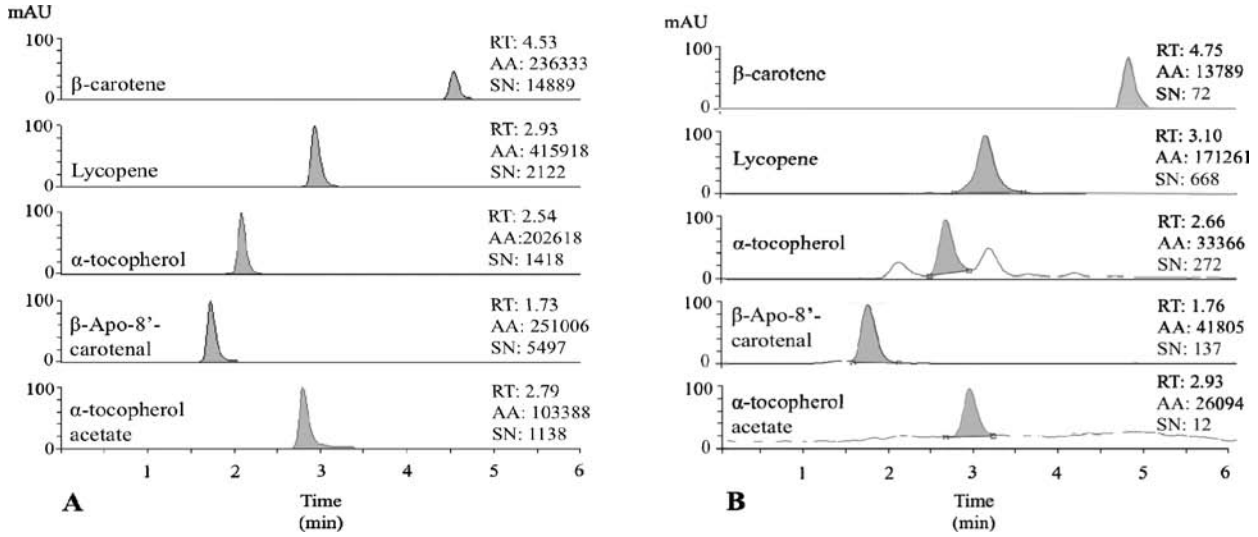

Figure 2. U-HPLC chromatogram for (A) 1 ng standard mixture of β-carotene, lycopene, α-tocopherol, β-Apo-8'-carotenal and α-tocopherol acetate and (B) antioxidants in a tomato sample, enriched with the internal standards.

degradation of the antioxidants will not take place and that the usage of antioxidants such as BHT and TEA in the mobile phase is not necessary.

APCI-MS/MS Optimization. Before the start of the optimization process, for every antioxidant standard and internal standard (precursor ions), four daughter ions (product ions) with the highest signal intensities and signal-to-noise ratios, as previously reported in Table 1, were selected. Subsequently, optimal instrumental parameter values, as earlier described, were determined by evaluating signal intensity and signal-to-noise ratio of chromatographic peaks of selected daughter ions.

Identification of the antioxidants found in the tomato fruit matrix was therefore based on both retention time and the m/z ratio of daughter ions. As such, the application of U-HPLC-APCI-MS/MS with SRM allowed the detection of lycopene, β-carotene, α-tocopherol, β-apo-8′-carotenal, and α-tocopherol acetate without significant interferences from the tomato fruit matrix (Figure 2B).

Performance Characteristics of the Chemical–Analytical Procedures. *Linearity.* Linearity was evaluated on the basis of calibration curves, set up in a solution of acetonitrile/methanol (50:50) + 0.01% BHT (eluent) and in green tomato homogenate (matrix), which was subsequently extracted using the optimized extraction procedure. Green tomato is a suitable biological matrix for the evaluation of this performance characteristic because minimal interferences from endogenous antioxidants and a strong similarity with deep red tomato matrix can be expected. These curves were used to determine the correlation between the concentration of antioxidant standards and peak area ratios, calculated as the ratio of the peak area of each antioxidant standard and the internal standard (either β-apo-8′-carotenal or α-tocopherol acetate). Standard concentrations of antioxidants in eluent or obtained green tomato extract ranged from 0.50 to 15.00 ng μL^{-1} for lycopene, from 0.10 to 2.00 ng μL^{-1} for β-carotene, and from 0.10 to 3.00 ng μL^{-1} for α-tocopherol. These concentration ranges were selected in such a way that the endogenous antioxidant concentrations in tomato extract were expected to be found within these ranges. The concentration of internal standards was always 1 ng μL^{-1}. Regression coefficients (R^2) for the calibration curves in eluent and matrix were in the ranges of 0.9910–0.9993 and 0.9900–0.9951, respectively. The optimized analytical procedure could consequently be determined as properly linear in the considered concentration ranges.

Matrix Effects. Calibration curves, set up in eluent and in the extract of a green tomato, were compared for the evaluation of matrix effects. Concentration ranges were identical for both matrices and were similar to the ones mentioned for linearity evaluation. Regression coefficients for the calibration curves in the extract were in the range of 0.9901–0.9998 (R^2 for the curves in eluent were already described in the previous section). Because calibration curves did not perfectly converge, matrix effects could be assumed. Internal standards β-apo-8′-carotenal and α-tocopherol acetate were used to counteract these effects. For β-carotene both internal standards had a similar and good rectifying action toward the matrix effects. However, for lycopene and α-tocopherol differences between the internal standards were noticeable. For both antioxidants it could be observed that upon application of β-apo-8′-carotenal, calibration curves in extract and eluent converged quite well. Although when α-tocopherol acetate was used, calibration curves did not converge well. Therefore, it could be stated that α-tocopherol acetate suffered more from ionization suppression by matrix compounds than the considered antioxidants lycopene and α-tocopherol. Consequently, because β-apo-8′-carotenal showed a better overall rectifying action toward matrix effects, this internal standard was selected as the only internal standard for the quantification of the selected antioxidants in tomato fruit.

Repeatability. Repeatability was evaluated by calculating the precision. For this, five tomato fruits from the same truss were considered, whereby each fruit was extracted three times and subsequently analyzed. Quantification of the antioxidant concentrations was based on curves, correlating antioxidant concentration and peak area ratio (with β-apo-8′-carotenal as internal standard). For each fruit the coefficient of variance was calculated and the overall precision was determined as the average of these coefficients. The average coefficients of variance for lycopene, β-carotene, and α-tocopherol were, respectively, 6.4, 2.8, and 4.9%. The low values meet the requirements (<10%) of the Commission Decision 2002/657[28]

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Table 2. Average Concentration Levels of Lycopene, β-Carotene, and α-Tocopherol for Each Treatment with Indication of the Variation by Means of the Standard Error (n = 30 on a Fresh Weight (FW) Basis; n = 10 on a Dry Weight (DW) Basis)[a]

	lycopene (mg kg^{-1})		β-carotene (mg kg^{-1})		α-tocopherol (mg kg^{-1})	
	FW	DW	FW	DW	FW	DW
2.4 dS m^{-1}	46.57 (±2.90)	972.40 (±114.20)	1.24 (±0.02)	23.80 (±0.97)	2.60 (±0.06)	48.71 (±2.13)
4.0 dS m^{-1}	51.19 (±3.71)	895.57 (±91.46)	1.23 (±0.02)	22.02 (±0.66)	2.63 (±0.08)	45.71 (±1.65)
5.3 dS m^{-1}	48.69 (±3.85)	875.84 (±150.55)	1.22 (±0.02)	21.86 (±0.98)	2.78 (±0.09)	49.05 (±2.78)
8.0 dS m^{-1}	52.98 (±4.90)	795.03 (±116.64)	1.23 (±0.03)	19.37* (±1.25)	2.68 (±0.09)	41.68 (±0.97)

[a]Significant differences ($p \leq 0.05$) in average concentration of an antioxidant of any treatment toward the salinity treatment with an average EC level of 2.4 dS m^{-1} are indicated with an asterisk (*).

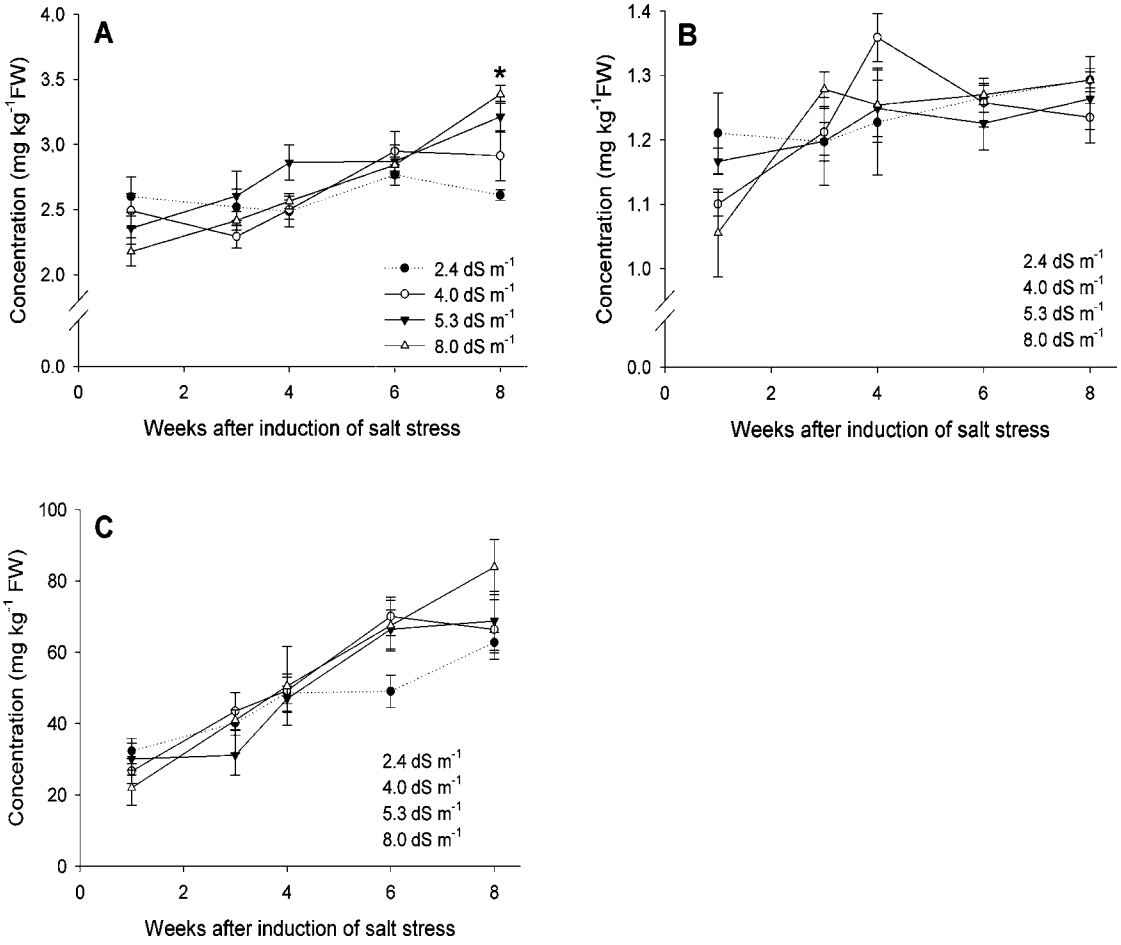

Figure 3. Average concentration levels on a fresh weight basis for α-tocopherol (A), β-carotene (B), and lycopene (C) for each harvest period and each salt stress level. The variation in average concentration is indicated by means of the standard error (n = 6). Significant differences ($p \leq 0.05$) in antioxidant concentration of any treatment toward the salinity treatment with an average EC level of 2.4 dS m^{-1} are indicated with an asterisk (*)

and indicate that the optimized extraction and analysis procedures are applicable to quantify lycopene, β-carotene, and α-tocopherol concentrations in tomato fruit with high precision.

When antioxidants were quantified by means of α-tocopherol acetate as internal standard, repeatability did not score as high as when β-apo-8′-carotenal was used. This statement confirmed the conclusion made earlier, that β-apo-8′-carotenal is the most suitable internal standard for the selected antioxidants.

Recovery. The recovery of each antioxidant during extraction was determined according to the ratio of the amount added to that measured experimentally after extraction. Because a green tomato was used as matrix, it was desirable to determine possible trace amounts of antioxidants, which could be utilized for a more correct calculation of the recovery. To this end, besides two spiking levels for each antioxidant (n = 3), also some blank samples (n = 3) were considered. The amounts spiked aimed at final concentrations of 0.50 and 1.50 ng μL^{-1} for lycopene and 0.10 and 0.50 ng μL^{-1} for both β-carotene and α-tocopherol. Quantification was based on curves, correlating concentration, and peak area ratio (with β-apo-8′-carotenal as internal standard). The average recoveries for lycopene, β-carotene, and α-tocopherol were found to be 86, 73, and 92%, respectively.

Correlation between Salt Stress and Antioxidant Concentration in Tomato Fruit. To evaluate the effect of salinity (osmotic stress) on antioxidant content, 30 fruits of each treatment were quantified for their lycopene, β-carotene,

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and α-tocopherol concentrations. To this end, five harvest periods (the first, third, fourth, sixth, and eighth week after induction of salt stress) were considered, at which for each treatment six ripe fruits (from three different trusses, from three different plants) were harvested and separately analyzed for the aimed antioxidants. Concentration levels on a fresh weight (FW) basis, calculated as the average of the entire treatment period, are presented in Table 2. When treatments were compared with each other, significant differences ($p \leq 0.05$) in terms of concentration could not be detected for any of the antioxidants. However, because not all harvested fruits were exposed to an EC treatment for their entire growing period, treatments were also compared for each harvest period separately (Figure 3). Only for α-tocopherol in the latest harvest period (eighth week) could a significant difference between the standard EC level of 2.4 dS m^{-1} (2.61 mg kg^{-1} FW) and the treatment of 8.0 dS m^{-1} (3.39 mg kg^{-1} FW) be detected.

Expression of the antioxidant concentration on a dry weight (DW) basis is a specific indicator for physiological responses, in particular, altered antioxidant synthesis. Therefore, the dry matter content of 40 tomato fruits (10 for each treatment) was determined, and the average antioxidant concentration on dry weight basis was calculated for each treatment (Table 2). A statistical analysis was performed, which indicated a significant difference ($p \leq 0.05$) in β-carotene concentration between the treatments with average EC levels of 2.4 (23.80 mg kg^{-1} DW) and 8.0 dS m^{-1} (19.37 mg kg^{-1} DW). As a result, an unambiguous effect of induced salt stress on antioxidant concentration, expressed on either a fresh or dry weight basis, could not be identified.

For the explanation of these results, the specific growth conditions, in particular, light intensity and air temperature (Figure 1), were considered. It is stated that light intensities of 650 W m^{-2} or higher exert an inhibiting effect on the accumulation of lycopene in the tomato fruit.[24] Because during the time course of our experiment natural light intensities (data presented only for photosynthetic active radiation in Figure 1A) frequently crossed this threshold, synthesis-stimulating effects from increased salt stress on antioxidant concentration were possibly counteracted by the light-inhibiting activity. In addition, lycopene synthesis is highest when temperatures range between 12 and 21 °C,[6] whereas at temperatures above 32 °C the lycopene synthesis is completely inhibited.[24] Figure 4 presents the evolution of maximum and daily average temperature as a function of time and indicates a decreasing trend, in particular, during the second half of the experiment. Because temperatures, measured during the experiment, were frequently above the optimal range, this decreasing trend was expected to result in a higher lycopene synthesis as the experiment progressed, which is evidenced by Figure 3. Because of this, a possible, relatively small effect of increased salinity on lycopene concentration was probably masked by the large effect of an altering temperature on lycopene synthesis. This statement could also be extended to β-carotene and α-tocopherol (Figure 3), although the effect of temperature on the synthesis of these antioxidants has not yet been properly assessed.[24]

Changing the EC level of the nutrient solution undoubtedly influences the plant's water availability and hence the fresh weight of tomato fruits.[29] For each treatment, the average marketable fresh yield per shoot was determined for the entire treatment period (Figure 5), whereby fruits with blossom end

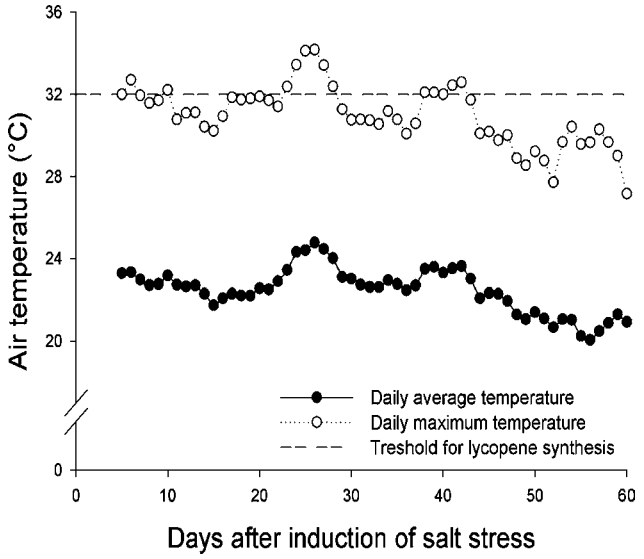

Figure 4. Daily average temperatures and daily maximum temperatures, calculated as the average of the daily average or maximum temperature of the considered day and the daily average or maximum temperatures of the previous four days.

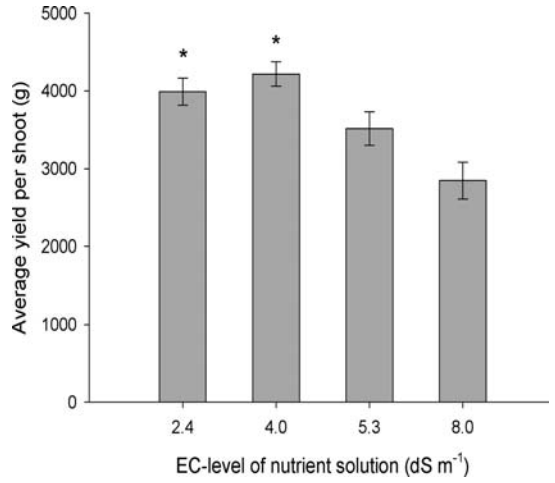

Figure 5. Average marketable fresh yield of tomatoes per shoot for each treatment with indication of the variation by means of the standard error ($n = 8$). Significant differences ($p \leq 0.05$) in average yield per shoot of any treatment toward the salinity treatment with an average EC level of 8.0 dS m^{-1} are indicated with an asterisk (*).

rot were excluded. In comparison with the salinity levels of 2.4 and 4.0 dS m^{-1}, the yield per shoot significantly ($p \leq 0.05$) decreased under the severest salinity treatment (8.0 dS m^{-1}) from about 3990 and 4218 g FW $shoot^{-1}$ to only 2846 g FW $shoot^{-1}$, which was mainly due to a higher incidence of blossom end rot. Although not statistically significant, application of the moderate saline nutrient solution of on average 4 dS m^{-1} resulted in a higher fresh yield per shoot (+5.7%) compared to the standard EC level. This increase could be mainly assigned to a significantly ($p \leq 0.05$) higher number of harvested fruits (data not presented). The hypothesis is postulated that increased salinity stimulates lycopene synthesis, which results in a shorter development period to achieve the deep red fruit stage. Because in our experiment the harvest time was based on fruit color, which was visually assessed, a reduced fruit development period could be expected. As a consequence, in

the same period of time a higher production in terms of number of fruits and trusses and consequently fresh yield could be realized under the applied moderate salinity. However, the duration of the salinity treatment was rather short, so a long-term (whole season) evaluation may be desirable to confirm these results.

In conclusion, numerous studies focus their activity on bioactive compound concentration and antioxidant activity in foods, so the development of reliable, selective, and fast methods for the analysis of the compounds of interest is of great importance. By completing the first objective of our research, an U-HPLC-MS/MS method was optimized, which is described by the targeted performance characteristics. The selectivity and reliability allowed a reproducible identification and quantification of the relative lowly abundant fat-soluble antioxidants lycopene, β-carotene, and α-tocopherol in the matrix of a tomato fruit. In addition, the short analysis time is both time-saving and solvent-reducing, of which the latter entirely fits within the concept of green chemistry.

The application of the different salt stress levels on tomato plants did not allow us to unambiguously identify the effect that moderate or more severe salt stress exerted on antioxidant concentration in tomato fruits. A reduction of the present variation in antioxidant concentration could possibly result in a better understanding of the salinity effects. A more controlled experiment, mainly in terms of ripening degree at harvest, is consequently desirable. It was also found that severe salinity led to unacceptable losses in fresh yield. Therefore, when sufficient evidence is available to assume there exists a significantly positive correlation between increased salinity and tomato fruit antioxidant concentration, it should be advised that only moderate salt stress is commercially applied to improve nutritional quality.

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