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Quantification of δ-, γ- and α-Tocopherol in Tomatoes Using an Improved Liquid-Dispersive Solid-Phase Extraction Combined with Ultrahigh Pressure Liquid Chromatography

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Abstract Tomato (*Solanum lycopersicum* L.) consumption has been correlated with a lower incidence of cardiovascular diseases and cancer. This protective effect has been ascribed to different bioactive compounds present in this fruit. Therefore, to gain insights on the potential of *S. lycopersicum* L. as bioactive food, a fast and sensitive methodology, based on liquid–liquid extraction (LLE), dispersive solid phase extraction (dSPE) followed by ultrahigh pressure liquid chromatography (UHPLC-FLR) analysis, was developed and validated to quantify δ-, γ- and α-tocopherol in tomatoes. Upon the optimization of different parameters, a fast extraction and separation, and simultaneously, increased resolution and sensitivity was attained. The methodology was validated, retrieving better analytical performance than most methods reported so far. This included good linearity, (r^2 > 0.99) and precision (<6.4%), high recoveries (>79.5%) and improved limits of detection and quantification (LODs of 2.15, 5.52 and 1.67 ng/mL and LOQs of 7.18, 18.40 and 5.58 ng/mL, for δ- γ- and α-tocopherol, respectively). These limits are about 1000 times lower than those reported in literature. Furthermore, as far we are aware, this is the first time δ-tocopherol presence in tomato is fully characterized and quantified. The methodology was applied to different tomato varieties, ripening stages and fruit sections, revealing high levels of δ-tocopherol that increase along fruit ripening, while the α-tocopherol follows the inverse trend. Moreover, δ-tocopherol is almost fully concentrated in the seeds and skin of ripe tomato. Finally, ORAC and DPPH assays revealed that the selected tocopherols contribute to approximately half of tomato total antioxidant capacity.

Keywords LLE-dSPE · UHPLC-FLR · δ- γ- and α–tocopherols · Method validation · *Solanum lycopersicum* L

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Introduction

Originally from the Andean region, tomatoes (*Solanum lycopersicum* L.) came to Europe in the fifteenth century, being nowadays one of the most popular and extensively consumed vegetable crops worldwide (Capanoglu et al., 2008; Frusciante, et al. 2007). This fruit presents a high water content and up to 10% of dry matter and organic acids (mainly citric acid and malic acid) (Shi and Le Maguer 2000; Figueira et al., 2014). Nevertheless, the most interesting constituents of tomato are the bioactive compounds, as tocopherols, carotenes, lycopenes, ascorbic acid, chlorogenic and gallic acids (phenolic acids), and the flavonoids quercetin, kaempferol, rutin, myricetin and naringenin (Hallmann 2012; Georgé, et al. 2011). All these compounds have been widely associated with additional protection against different diseases, namely cancer and cardiovascular diseases (Sharoni, et al. 2012; Rao and Rao 2007; Giovannetti, et al. 2012). Tomato is therefore regarded as a functional food, being an important constituent of different diets across the planet, notably the Mediterranean diet. Tocopherols (α, β, γ and δ isoforms, differing in the number and position of alkyl groups) and tocotrienols (also α, β, γ and δ isoforms, differing from tocopherols in the unsaturated side chains) (Fig. 1) are important naturally occurring plant antioxidants (Chong-Han 2010). These compounds constitute the forms of vitamin E characterized in

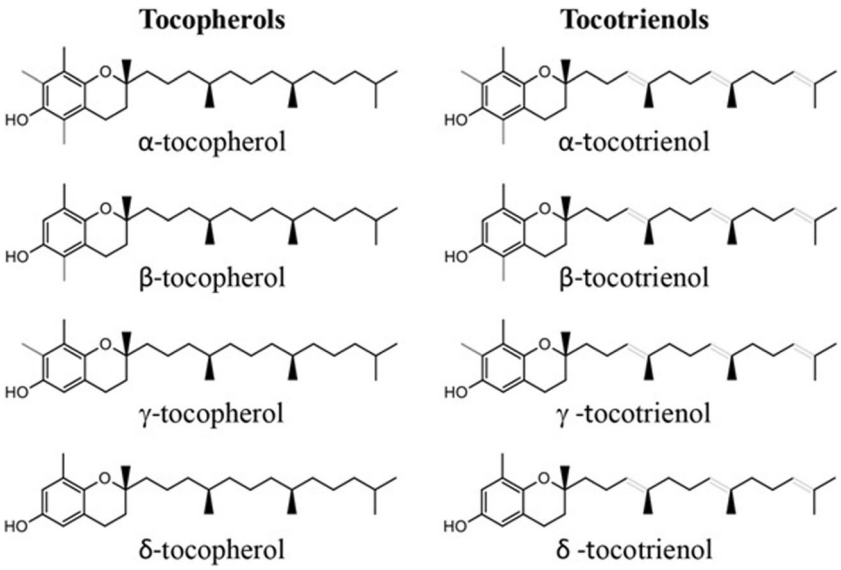

Fig. 1 Tocopherols and tocotrienols structures

1922 by Evans and Bishop (Zingg 2007; Azzi 2007) and are considered the most important lipid-soluble antioxidants in our organism (Sircelj and Batic 2007).

The α-tocopherol is the most bioactive of the tocopherol isoforms, being widely distributed in plant tissues, while δ-tocopherol is much less abundant and simultaneously the less bioactive isoform (Schneider 2005; Stocker and Keaney 2004). Nonetheless, γ- and δ-tocopherol have been suggested to have stronger anti-inflammatory activity than α-tocopherol (Wada 2012; Yang, et al. 2013) and have shown greater ability to reduce inflammation, cell proliferation and tumour burden (Wada 2012; Smolarek and Suh 2011). Considering specifically the fruit, vitamin E activity is usually assessed by the levels of α-tocopherol, which is reported to be mainly found in the seeds (Marsiv et al., 2010) and is comparable to β-carotene, another important dietary antioxidant (up to 1.8 mg/100 g FW) (Chun et al., 2006; Frusciante, et al. 2007; Gomez-Romero et al., 2007). The vitamin E activity of tocopherols, however, is not limited to their antioxidant capacity which lies in their ability to donate phenolic hydrogen (Kamal-Eldin and Appelqvist 1996; Yang, et al. 2013; Schneider 2005). Instead, they also include the regulation of the activity of important enzymes, as the inhibition of cyclooxygenase-2 and 5-lipoxygenase (involved in the synthesis of inflammatory mediators such as prostaglandin E2 and leukotriene B4) and SR-A and CD36 (inhibits the uptake of oxidized LDL into monocyte-derived macrophages) (Schneider 2005). Moreover, tocopherols have been associated to the inhibition of monocyte-endothelial cell adhesion and platelet adhesion and aggregation, as well as to the modulation of gene expression and cellular signalling (Borel et al., 2013; Brigelius-Flohé and Traber 1999; Schneider 2005; Salinthone et al., 2013). The evaluation of the total antioxidant capacity (TAC) of a certain bioactive compound can be obtained through different assays, being the oxygen radical absorbance capacity (ORAC) (Cao et al., 1993) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays (Xie and Schaich 2014; Tabart et al., 2009; Kedare and Singh 2011) often used.

In this work, we report a noteworthy improved, fast and reliable methodology based on LLE-dSPE technique followed by UHPLC-FLR analysis for quantification of δ- γ- and α-tocopherol in tomato fruits from *S. lycopersicum* L. species. An univariate experimental design, involving as independent variables, extraction solvent and clean-up sorbents, was performed and used to investigate the effects of different experimental parameters on the extraction performance. The analytical performance of the proposed LLS-dSPE/UHPLC-FLR was evaluated in terms of selectivity, linear dynamic range, LOD, LOQ, precision, accuracy and uncertainty. The antioxidant profiles of four *S. lycopersicum L.* varieties were evaluated by using DPPH and TBARS assays.

Materials and Methods

Reagents, Standards and Materials

The tocopherols (α- and γ-tocopherol, HPLC grade 96% and δ- tocopherol, 90%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (absolute PA, 99.5%) was acquired from Panreac (Valencia, Spain) and acetonitrile (ACN) and methanol (MeOH) (both HPLC grade, 99.99%) from Thermo Fisher Scientific (Leicestershire, UK). The clean-up salt multiwalled carbon nanotubes (MWCNTs), primary secondary amine (PSA), graphene oxide and PSA/C18/$MgSO_4$ (25/25/150 mg, DisQuE) were purchased from Waters (Milford, MA, USA).

Tomato Samples

Gordal tomato varieties (regional variety, 1500 g) at different ripening stages (full mature green -FMG, breaker and ripe) were collected from different plants of the same crop at different time points (during 90 days), while *campari*, *cherry* and *roma* samples (200 g) were imported from mainland and acquired in the local market. The samples were lyophilized (Christ Alpha 1–2 LD plus freeze dryer, Osterode am Harz, Germany), grounded to powder (IKA A11 basic analytical mill, Staufen, Germany) and immediately stored under nitrogen at −80 °C, in several aliquots, which were used only once to prevent sample degradation.

Optimization of Experimental Factors Affecting LLE-dSPE Performance

Different parameters affecting the efficiency of the extraction procedure were tested and optimized. This included the (*i*) extraction solvent (MeOH, ethanol (EtOH), ACN, ACN/MeOH 4:1 and MeOH/EtOH 4:1); and (*ii*) clean-up salts (PSA, graphene oxide, MWCNT and PSA/C18/MgSO4). The selection of the best conditions was based in the highest total peak areas for the target analytes and resolution

LLE-dSPE Procedure

Upon the tomato sample processing described above, sample aliquots of 0.50 g were diluted (1:10) with 5 mL of ACN/MeOH (4:1, *v*/*v*) and vortexed for 1 min to homogenize. Then, 1 mL of the extract was collected to Eppendorf ($n = 3$), mixed with 20 mg of PSA/C18/$MgSO_4$ (1:1:6; w, w, w) and submitted to centrifugation (5000×*g*, Espresso Personal microcentrifuge, Thermo Fisher Scientific (Leicestershire, UK) for 5 min. The supernatant was collected and evaporated (Heidolph Collegiate, Schwabach, Germany) to dryness and the residue reconstituted in 500 μL of initial mobile phase. After filtration over a PTFE syringe filter (0.20 μm; 13 mm, Millipore Corporation, Bedford, USA), the extract was collected in a 200-μL insert and placed into an LC amber glass vials for further UHPLC-FLR analysis.

UHPLC-FLR Analysis and Operating Conditions

Analysis of tocopherols was carried out on a Waters Ultra Pressure Liquid Chromatographic Acquity system (UPLC, Acquity H-Class) combined with a Waters Acquity quaternary solvent manager (QSM), an Acquity sample manager (SM), a column heater and a FLR detector. The whole configuration was driven by Empower software v2.0 from Waters (Milford, MA, USA). Optimum separation was achieved with a binary mobile phase composed by (a) ACN and (b) MeOH, with a constant flow rate of 500 μL min^{-1} and the following gradient conditions: 75% A until 1 min, increasing to 78% A (3 min), continuing up to 4 min, returning to 75% A (5 min), remaining until the end of the run. A re-equilibration time of 2 min regenerates the column to the initial conditions after each analysis was used. Overall, during the 8-min run, a maximum back pressure of 3.800 psi was reached, which is within the capabilities of the UHPLC. The samples were kept at 20 °C in the SM and 2 μL was injected in the thermostated (30 °C) Acquity UPLC BEH C18 analytical column (1.7 μm particle size, 2.1 mm × 50 mm, Waters, Milford, MA, USA). For quantification purposes, the FLR detection was conducted by using a channel with $\lambda_{Exc} = 296$ nm and $\lambda_{Em} = 330$ nm. The identification of tocopherols in real sample chromatograms was based on the comparison of retention time and spectral characteristics with standards and confirmed using the standard addition method. Quantification was also based on the standard addition method.

Method Validation

After the sample extraction optimization, the performance of the proposed LLE-dSPE/UHPLC-FLR approach was assessed by studying the selectivity, linearity, limits of detection (LODs) and quantification (LOQ), linear dynamic range (LDR), precision, accuracy and matrix effect. The selectivity of the method for tocopherols was assessed by the absence of interfering peaks in fluorescence spectra with $\lambda_{Exc} = 296$ nm and $\lambda_{Em} = 330$ nm. Linearity was evaluated using the external standard addition method, through analyte standard linear regression ($n = 3$). This involved eight different concentrations and the least-squares method to obtain the respective correlation coefficient (r^2). Sensitivity of the method was assessed through determination of the LOD (the lowest analyte concentration that produces a response detectable above the noise level of the system) and LOQ (the lowest level of analyte that can be accurately and precisely measured), obtained from the linear regression, with LOD defined as $a + 3S_{a/b}$ and LOQ as $a + 10S_{a/b}$, where "a" represents origin ordinate, "S_a" the origin ordinate variance and "b" the slope. Precision is a function of concentration, and it was calculated by dividing the standard deviation (SD) by the means of concentration to obtain the coefficient of variation, which when expressed on a percentage basis gives the relative standard deviations (RSDs). For method precision assessment, three concentrations, low level (LL), medium level (ML) and high level (HL) were evaluated four times ($n = 4$). Four trials were executed in the same day, resulting in intraday precision which retrieved the repeatability. The other four trials were executed in non-consecutive days, resulting in interday precision, retrieving the reproducibility. Accuracy was evaluated through a recovery study and expressed as recovery percentage (R%) according to the following formula: $\% R = 100 \times [(S_F - S)/\text{Std}]$, where "$S_F$" represents concentration of target analytes in

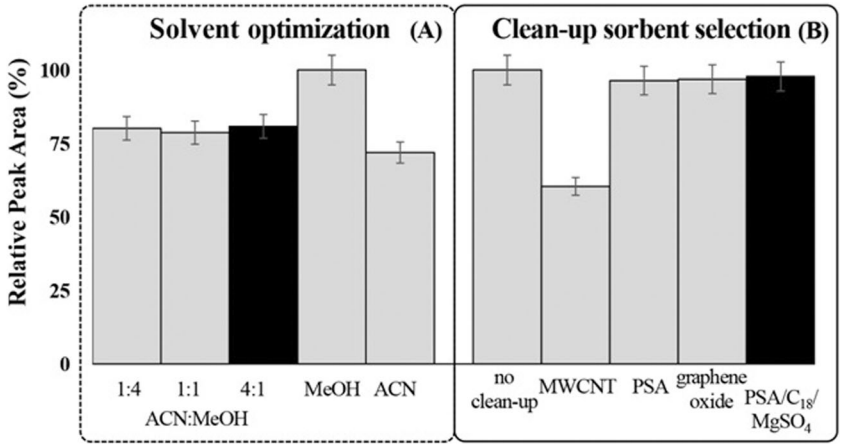

Fig. 2 Experimental optimization of the LL_{USAE} procedure: **a** solvent optimization using methanol (MeOH), acetonitrile (ACN) and three ACN/MeOH gradients (1:4, 4:1, and 1:1); **b** clean-up sorbent selection among multiwalled carbon nanotubes (MWCNT), PSA, graphene oxide and a PSA/C18/$MgSO_4$ mixture. Selection of the best conditions was based in the relative peak area and chromatographic conditions involved (as detailed in the text)

the fortified sample, "S" represents the concentration of target analytes in the sample and "Std" represents the concentration of target analytes added to the sample. Three different standard concentration levels corresponding to the LL, ML and HL were evaluated (n = 3) in S_F and Std. Matrix effect (ME) is the effect on an analytical method caused by all other components of the sample and was determined according to the formula: % ME = 100 × (m_{Sol}/m_{FS}), where "m_{Sol}" represents the slope of standard linear regression and "m_{FS}" the slope of fortified sample linear regression.

Total Antioxidant Capacity

Tomato TAC determination was performed using the ORAC and DPPH assays. The ORAC assay measures the oxidative degradation of a florescent probe, fluorescein, by a peroxyl radical ($ROO^{\bullet}$) generator, as the azo-initiator 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH). This degradation is obviously affected by the quenching ability of the sample extract being measured, allowing its TAC determination. The methodology here used was adapted from Bernaert et al. (2012). Briefly, 25 μL of the sample (diluted 1000 times) was added to 150 μL of fluorescein solution (40.0 nM), incubated at 37 °C for 30 min and added with 25 μL AAPH (153.0 mM). The values of fluorescence (λ_{Exc}. 485 nm and λ_{Em}. 520 nm) were subsequently determined every 90 s, for about 1 h through Victor3 Multilabel Plate Counter 1420 fluorescence reader (Perkin Elmer, Waltham, USA). Instead of the 25-μL sample, 25 μL of 10 mM phosphate buffer at pH 7.4 was used for the reaction control or different trolox solutions (ranging from 1 to 60 μM) to obtain the standard linear regression. The blank was prepared using only 200 μL of phosphate buffer. The results were expressed in mM Trolox/100 g FW.

The DPPH methodology relies in the scavenging ability of the antioxidants present in the matrix being assayed against the free-radical DPPH. This compound has deep violet colour (maximum absorption around 515 nm in alcoholic solution) that is lost upon its reduction (Xie and Schaich 2014; Tabart et al., 2009; Okoh et al., 2014; Kedare and Singh 2011). The DPPH assays here used were adapted from Xie and Schaich (2014) with minor differences. Briefly, 10 mg of DPPH was dissolved in 250 mL of MeOH and allowed to rest overnight (DPPH stock solution). Then, 500 μL of sample extracts (diluted ten times) was mixed in 1000 μL of DPPH stock solution and allowed to rest for 10 min in the dark. Finally, the absorbance was taken at 515 nm using a UV–Vis spectrophotometer (UV–Vis LAMBDA 25, Perkin Elmer, Waltham, USA). The blank assays were prepared using MeOH instead of the sample extract. The DPPH % inhibition was obtained using the formula $((A_{Ctr} - A_S)/A_{Ctr}) \times 100$, where A_{Ctr} is the absorbance of the control reaction and A_S is the absorbance of the sample extracts or standards used, as described by Okoh et al. (2014).

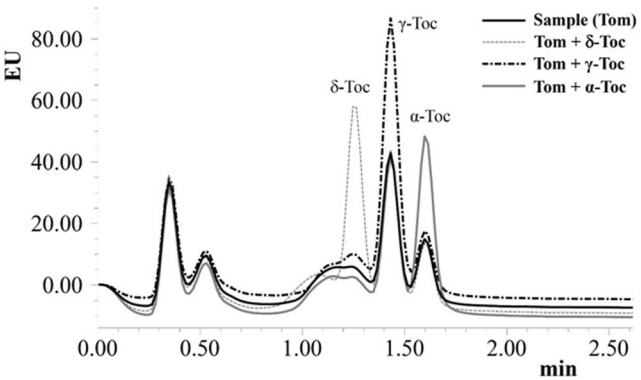

Fig. 3 Representative UHPLC-FLR chromatograms obtained at λ_{Exc} = 296 nm and λ_{Em} = 330 nm for *gordal* tomato sample (Tom) spiked with δ-, γ- and α-tocopherol standards (δ-, γ- and α-Toc, respectively)

Table 1 Validation parameters of LLE-dSPE/UHPLC-FLR for δ-, γ- and α-tocopherol determination

Tocopherols λ_{exc} = 296 nm λ_{em} = 330 nm	RT (min)	Linearity		Sensitivity		Spiking levels	Precision (%)		Recovery (%)	Matrix effect (%)
		LDR	r^2	LOD	LOQ		Intra-day (n = 4)	Inter-day (n = 9)		
δ-Tocopherol	1.25	0.01–4.0	0.9987	2.15	7.18	0.1 (LL)	3.0	6.4	105.3	96.8
						1.0 (ML)	2.1	5.8	105.3	
						4.0 (HL)	2.1	4.9	96.9	
						Average	2.4	5.7	102.5	
γ-Tocopherol	1.45	0.01–4.0	0.9974	5.52	18.40	0.1 (LL)	4.0	6.3	81.6	98.8
						1.0 (ML)	3.6	3.4	96.1	
						4.0 (HL)	1.9	2.6	94.1	
						Average	3.2	5.7	90.6	
α-Tocopherol	1.60	0.01–4.0	0.9998	1.67	5.58	0.1 (LL)	3.0	6.3	80.1	84.9
						1.0 (ML)	2.2	6.1	79.5	
						4.0 (HL)	1.9	5.8	85.2	
						Average	2.4	6.1	81.6	

RT retention time, *LDR* linear dynamic range (μg/mL), *LOD* limit of detection (ng/mL), *LOQ* limit of quantification (ng/mL), Spiking Levels (μg/mL): *LL* low level, *ML* medium level; HL high level

Results and Discussion

To implement a fast and sensitive method for the quantification of tocopherols, the LLE approach was developed, optimized and combined with a fast UHPLC-FLR analysis.

Optimization of the LLE Procedure

LLE optimization involved the selection of the best extraction solvent time and sample extract clean-up.

Extraction Solvent

To select the best extraction solvent, ACN, MeOH and different ratios between these two solvents (4:1; 1:1 and 1:4, *v*/*v*) were tested and compared. As shown in Fig. 2a, although the best results are obtained with MeOH, there is no significant difference for the other conditions assayed and so, ACN/MeOH (4:1; *v*/v) was selected to match the conditions used in the following chromatographic separation. In addition, MeOH extraction is very broad, extracting many interferents (Delgado-Zamarreño et al., 2016), while the selected ACN/MeOH mixture promotes protein precipitation (Polson et al., 2003), allowing obtaining of cleaner extracts (data not shown).

Sample Clean-Up

To simplify even more the extract composition before the chromatographic separation, discarding part of the interferents that could affect tocopherol analysis and quantification, different sorbents, namely, MWCNT, PSA, graphene oxide and PSA/C18/$MgSO_4$, were used. As shown in Fig. 2b, this procedure did not affect tocopherol extraction, with exception of MWCNT, which shows a very significant retention of the target analytes. Therefore, the selection of the best clean-up sorbent was made between PSA, graphene oxide and the PSA/C18/$MgSO_4$ mixture. It was selected the last option due to the cleaner extracts it produces (observed by the lower noise signals in the chromatographic separations, data not shown).

Method Validation

The optimized LLE-dSPE/UHPLC-FLR was validated for the determination of δ-, γ- and α-tocopherol using ripe tomato

Table 2 Comparison of the proposed extraction procedure with other published methods for the extraction of tocopherols in different samples

Sample	Extraction (method/solvents)	Analytical conditions (equipment/mobile phase)	Detection FLD (λExc/λEm) DAD (λ)[a]	TA (min)	LODs (δ-T/γ-T/α-T)[b] (ng/mL)	Recovery (δ-T/γ-T/α-T) (%)	Ref.
Tomatoes	LLE-dSPE	UHPLC: Acquity BEH C18 column/ACN/MeOH (75:25, *v*/v)	296/330	2	2.2/5.5/1.7	96.8/98.8/84.9	Method proposed
Mushrooms	LLE/MeOH and Hex	HPLC: Polyamide II column/Hex/ethyl acetate (70:30, *v*/v)	290/330	27	–/20/8	99/110/114	Barros et al., (2008)
Corn, walnut, grape seed, rice, virgin olive, sesame, peanut, sunflower oils	LLE/MeOH/Hex: tetrahydrofuran (80:10:10, *v*/v/v)	HPLC: Alltima RP C-18 column/ACN:MeOH (50: 50, *v*/v)	290/325	10	8/8/9	101/99/98	Bele et al., (2013)
Butter	LLE with H_2O and 2-propanol	HPLC: Phenomenex Luna PFP column/MeOH:H_2O, (93:7 *v*/v)	295/330	13	0.4/0.2/0.5	–	Górnaś, et al. (2014)
Several vegetables	LLE/acetone (0.025% BHT)	UHPLC: Kinetex PFP column/MeOH/H_2O (85:15, *v*/v) and MTBE/MeOH/H_2O (80:18:2, *v*/v/v)	295/330	24	100/100/500	88–100/85–98/99–108	Knecht et al., (2015)
Grass	LL-USAE/BHT/EtOH (10:1, *w*/*v*) and calcium carbonate/acetone (1:2, *w*/*v*)	HPLC: Zorbax RX-SIL column/Hex:2-propanol (99.3/0.7, *v*/v)	290/330	20	38/50/72	104–135	Valdivielso, et al. (2015)
Lipid emulsions	LLE/MeOH, and Hex (0.05% BHT)	HPLC: Pinnacle DB silica column/1,4-dioxane/Hex (2:98, *v*/v)	292/330	11	6/12/98	98/107/101	Xu et al., (2015)
Powdered milk	LLE/Hex and EtOH	HPLC: Tracer Spherisorb ODS2 C18 column/MeOH	292	7	21/33/33	101/98/98	Mendoza et al., (2003)
Oils of date fruits	Soxhlet extractor/petroleum ether as a solvent	UHPLC: Acquity BEH C18 column/ACN:F.A. (99.9/0.1, *v*/v)	291	10	–/–/83	–/–/97	Habib et al., (2013)
Table Olives	n-hexane/ethyl acetate SPE	HPLC-DAD: Merck Chromolith RP-18e column/acetonitrile/methanol (7/3) and i-propanol	252	6	[c]0.3/–/–	95.3–99.6/–/–	Sagratini, et al. (2012)
Several tropical fruits	LLE/H_2O and Hex/acetone (1:1 *v*/*v*)	HPLC: C18 Poroshell 120 column/ACN, MeOH and ethyl acetate	285	5	67/7/25	–/–/96	Stinco et al., (2014)
Several tropical fruits	Saponification/ether extraction	UPC^2: ACQUITY UPC^2 BEH column/CO_2 (99.999%) and methanol	293	2	60/–/–	95.4–101.4 /–/–	Gong et al., (2014)
Several nuts	QuEChERS/MeOH (PSA was selected as the clean-up)	HPLC: Zorbax Eclipse XDB-C18/MeOH/H_2O (98:2, *v*/v)	205	9	210/40/80	–/80/78	Delgado-Zamarreño et al., (2016)
Moringa oleifera Lam.	Saponification followed by LLE/ether	UPC^2: BEH 2-EP column/CO_2 and MeOH/isopropanol (1:1, *v*/v)	294	3	47/23/49	94/91/98	Qi, et al. (2016)
Seafood	*n*-hexane extraction	HPLC-DAD/FLR	(290/330) 210	8	50/–/–	–	Cruz, et al. (2012)
Human plasma	LLE/H_2O, EtOH and Hex	UHPLC: Acquity BEH C18 column/ACN and MeOH	295/330	5	–/10/50	–/97/99	Bell et al., (2014)
Human plasma	LLE/H_2O, EtOH and Hex	HPLC: Discovery HS C18 column/MeOH and EtOH	292	5	–/–/13	–/–/96	Kand'ár et al., (2013)
Human serum	LLE/EtOH and DCM/Hex (1:5, *v*/v)	HPLC: Spheri-5-ODS column/ACN/MeOH (85:15) and ACN/DCM/MeOH (70:20:10)	294	10 3	–/–/2154	–/–/95	Granado-Lorencio et al., (2010)

Table 2 (continued)

Sample	Extraction (method/solvents)	Analytical conditions (equipment/mobile phase)	Detection FLD (λExc/λEm) DAD (λ)[a]	TA (min)	LODs (δ-T/γ-T/α-T)[b] (ng/mL)	Recovery (δ-T/γ-T/α-T) (%)	Ref.
Human serum	LLE/H_2O/MeOH (1:2, vv), followed by Hex	UHPLC: HSS T3 column/ACN/MeOH (85:15) and ACN/DCM/MeOH (70:20:10) HPLC: Pursuit PFP/H_2O/MeOH/F.A. (97.9/2/0.1, v/v/v), MeOH/F.A. (99.9/0.1, v/v)	MS/MS (+)	28	−/−/430.7	−/−/101	Albahrani et al., (2016)

ACN acetonitrile, *BHT* butylhydroxytoluene, *DAD* diode array detector, *DCM* dichloromethane, *dSPE* dispersive solid phase extraction, *EtOH* ethanol, *FA.* formic acid, *FLD* fluorescence detector, *Hex* hexane, *LLE* liquid–liquid extraction, *LODs* limits of detection, *MeOH* methanol, *MTBE* methyl tert-butyl ether, *PSA* primary–secondary amine, *UPC*^{2} ultra-performance convergence chromatography, λ_{Exc} excitation wavelength, λ_{Em} emission wavelength in nm, *TA* time of analysis

[a] Wavelengths in nm

[b] δ-, γ- and α-tocopherol

[c] mg per kg

from the *gordal* variety. First, the method was applied to a mixture of tocopherol standards, yielding three distinct peaks with retention time of 1.25 min (δ-tocopherol), 1.45 min (γ-tocopherol) and 1.60 min (α-tocopherol). The selectivity of the method was therefore confirmed by the absence of any interferent in the chromatographic separation of the selected tocopherols using their specific excitation and emission wavelengths (Fig. 3).

Linearity was evaluated through external standard addition method, by applying the least-squares method elsewhere. A good correlation coefficient ($r^2 > 0.997$) was obtained in the LDR 0.01–4.0 μg/mL (Table 1). Regarding LODs and LOQs, determined from ordinary least squares regression data, the limits obtained (LODs of 2.15/5.52/1.67 ng/mL and LOQs of 7.18/18.40/5.58 ng/mL for δ-/γ-/α-tocopherol, respectively; Table 1) are substantially lower than those reported in literature for tomato extracts (1000 times lower) (Chun et al., 2006; Frusciante, et al. 2007) and serum (10 times lower) (Traber 2007; Charão, et al. 2012; Chauveau-Duriot et al., 2010), making LLE-dSPE/UHPLC-FLR a powerful strategy for tocopherol quantification. A further comparison of the analytical performance of selected methodologies to quantify tocopherols can be appreciated in Table 2.

For precision assessment, three concentrations were evaluated (LL, ML and HL, $n = 4$) and the RSD calculated. Intraday precision (repeatability) and interday precision (reproducibility) were also calculated using the same concentration levels (LL, ML and HL, $n = 9$). The results obtained (Table 1) range between 2.4 to 6.1%. As expected, repeatability is lower than reproducibility and both are far below the reference limit of 20% (Naidis and Turpeinen 2009; Shah, et al. 2000). In addition to the evaluation of the method accuracy, a recovery study was carried out by spiking a tomato sample at three concentration levels, with a known amount of each tocopherol (see Table 1). The average recoveries obtained, ranging from 81.6 to 02.5% with RSDs lower than 6.1% (Table 1) are within the tolerance range (80 to 120%) (Shah, et al. 2000) and in agreement with the matrix effect results. These ranged between 84.9 and 98.8, being therefore also within the tolerance range (80 to 120%) (Rodrigues et al. 2012; Shah, et al. 2000).

Determination of δ-, γ- and α-Tocopherol in Tomato by LLE-dSPE/UHPLC-FLR

Tocopherol composition in plant and fruits is affected by several abiotic and biotic factors, as temperature of the cultivation area, intercepted solar radiation to the plants, ripening stage and genotypic variety (reviewed in Monge-Rojas and Campos 2011). In sea buckthorn berries, for instance, the abundance of δ-tocopherol is greatly affected by the ripening stage of the

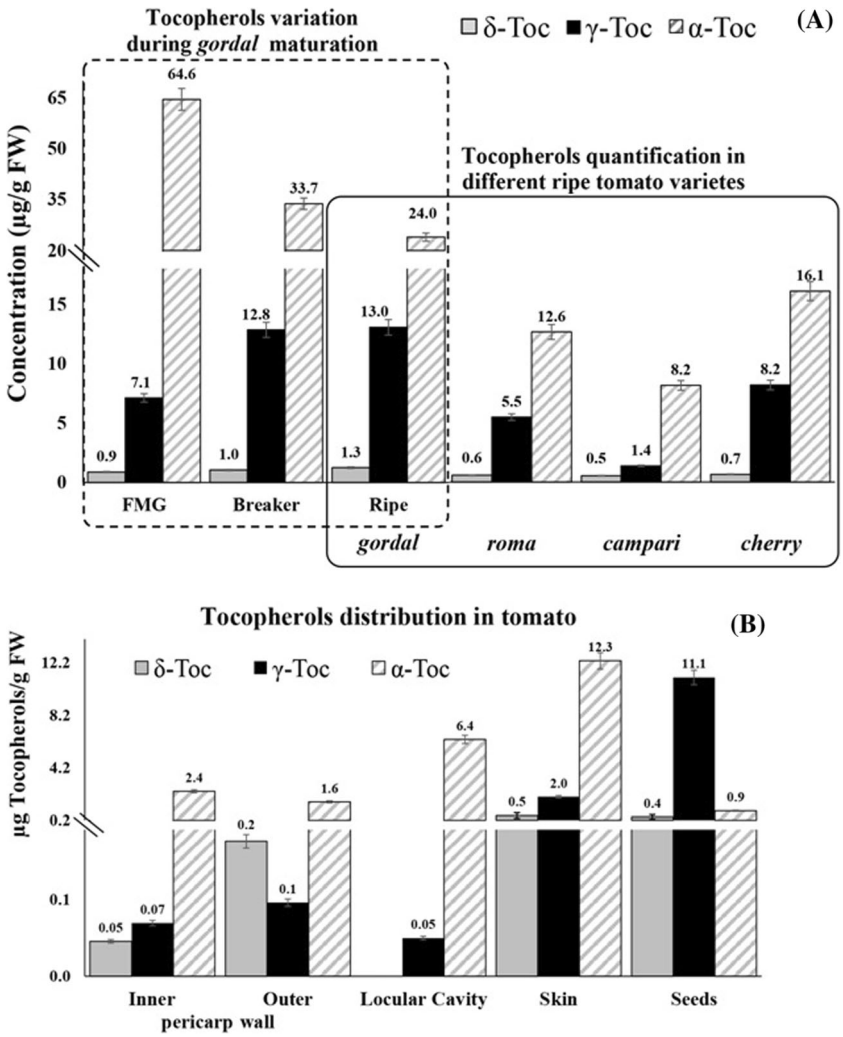

Fig. 4 Selected tocopherols concentration in different **a** tomato ripening stage (full mature green—FMG, breaker and ripe) and variety (*gordal*, *campari*, *cherry* and *grape*) and **b** fruit sections (inner and outer pericarps walls, locular cavity, skin and seeds)

fruit, as well as the cultivars and season harvesting (Andersson et al., 2008; Bal et al., 2011). Therefore, we use the methodology developed, LLE-dSPE/UHPLC-FLR, to assess δ-, γ- and α-tocopherol content in tomato samples from different ripening stages, varieties and fruit sections. The results obtained reveal that α-tocopherol is the most abundant of the selected tocopherols, followed by γ-tocopherol and finally δ-tocopherol with much lower levels than of the other tocopherols analysed. Furthermore, while α- and γ-tocopherol levels are affected by the ripening stage of the fruit, δ-tocopherols remains almost constant during this stage (Fig. 4a). Accordingly, the levels of α-tocopherol decrease by almost one third to 23.95 μg/g FW as the fruit ripening progresses from the full mature green (FMG) to breaker and finally ripe stage; the γ- isoform displays the opposite trend, raising its initial concentration from 7.1 up to 13.0 μg/g FW, and δ-tocopherols reveal a very narrow variation from 0.9 to 1.3 μg/g FW during tomato maturation (Fig. 4a, left dashed box). Following this, tocopherol levels were assessed in different tomato varieties, namely the regional *gordal* variety and the *campari*, *cherry* and *grape* varieties imported from mainland, in the ripe stage. As the results show, tocopherols levels present some variations among the four varieties analysed, but the three isoforms are significantly more abundant in the *gordal* variety (Fig. 4a, right box). This result agrees with previous reports showing evidences of the great influence of the tomato genetic diversity in its antioxidant potential and consequently in the relative composition of the antioxidant compounds (Aldrich, et al. 2010; Hanson, et al. 2004). Regardless of the ripening stage considered, the δ-tocopherol levels we found are particularly interesting because δ-tocopherol is rarely quantified in tomato and the amounts reported range from not detected (Vági, et al. 2007; Botinestean et al., 2013), to trace levels (Marsiv et al., 2010) and some mg/kg of industrial tomato dry weight (Kalogeropoulos et al., 2012). Even for most vegetables and other fruits, δ-tocopherol has been scarcely reported (Caretto et al., 2010; Piironen et al., 1986) and we were able to find this tocopherol described only in some legumes (Kalogeropoulos, et al. 2010), banana (Piironen et al., 1986; Caretto et al., 2010)

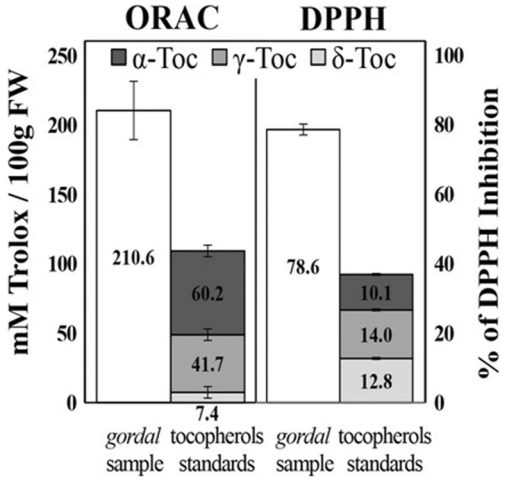

Fig. 5 Evaluation of tomato TAC and contribution of the selected tocopherols for this activity. TAC activity was assessed through the ORAC and DPPH assays as described in the experimental section (*Total antioxidant capacity* (*TAC*)) and using ripe *gordal* tomato samples. The relative contribution of δ-, γ- and α-tocopherol for TAC (δ-, γ- and α-Toc bars) was estimated using pure standards of the selected tocopherols in the same concentrations found in the ripe *gordal* tomato samples used

and a few other tropical fruits with a very limited production and consumption (Andersson et al., 2008; Konczak and Roulle 2011; Costa et al., 2010; Monge-Rojas and Campos 2011; Chun et al., 2006). Previously, it has been reported that the relative abundance of tocopherols can vary significantly in different tomato sections, with α-tocopherol mainly found in the outside (59%) and inside layers (39%) (Seybold et al., 2004). Here, we have performed a more detailed analysis of δ-, γ- and α-tocopherol isoform distribution in tomato, considering five different fruit sections, inner and outer pericarp walls, locular cavity, skin and seeds of the ripe *gordal* variety.

The results shown in Fig. 5b confirm the heterogeneous distribution of δ-, γ- and α-tocopherol in the fruit, with the α-tocopherol more abundant in the skin, followed by locular cavity and minor amounts in the pericarp walls and seeds. In turn, δ-tocopherol is almost exclusively found in the skin and seeds, in minor amounts in the outer pericarp wall and vestigial in the inner pericarp walls and not detected in the locular cavity. Finally, γ-tocopherol is almost totally concentrated in the seeds, in minor levels in the skin and vestigial in the remaining sections analysed.

Contribution of δ-, γ- and α-Tocopherols for the Total Antioxidant Capacity

The evaluation of tomato TAC and the respective contribution of α- and δ-tocopherols for this activity were performed through the ORAC and DPPH assays, using ripe *gordal* samples and the pure standards. As shown in Fig. 5, δ-, γ- and α-tocopherol antioxidant potential is quite significant, representing half of tomato TAC. This contribution could be even more relevant if synergetic effects with other tomato antioxidants, namely between α-tocopherol and β-carotene (Kotíková et al., 2011; Zanfini et al., 2010), could be assayed. Furthermore, if we take into account that these tocopherols are much more abundant in the tomato skin and seeds, as discussed in the previous section (Fig. 4b), then our results about the contribution of δ-, γ- and α-tocopherol for the tomato TAC agrees and support the observation that peeling and seeding tomatoes for cooking considerably affects their nutritional value (Vinha, et al. 2014).

Conclusions

This paper reports the successful development, validation and application of a fast, simple and reliable LLE-dSPE/UHPLC-FLR methodology for the characterization of δ-, γ- and α-tocopherol. Moreover, the methodology developed is precise, accurate and sensitive, retrieving LODs and LOQs about 1000 times lower than previously reported in literature and 10 times lower than the tocopherol levels found in serum. This anticipates the use of the developed LLE/UHPLC-FLR methodology as a powerful strategy for tocopherol quantification in other matrices beyond tomato extracts. It was also shown that δ-, γ- and α-tocopherols localize preferentially in tomato skin and seeds and have a very important contribution for tomato TAC. Therefore, at the one hand, this raises important nutritional concerns regarding the tomato peeling and seeding habits, particularly before its processing and cooking. On the other hand, tomato by-products contain high levels of tocopherols (Kalogeropoulos et al., 2012), having therefore great potential as ingredients in the food chain as shown very recently for the tomato seed oil (Shao, et al. 2015).

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Compliance with Ethical Standards

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Conflict of Interest José A. Figueira declares that he has no conflict of interest. Jorge A. M. Pereira declares that he has no conflict of interest. José S. Câmara declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants performed by any of the authors.

Informed Consent Not Applicable. This article does not contain any studies with human participants or animals performed by any of the authors.

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