Determination of fat-soluble vitamins in vegetable oils through microwave-assisted high-performance liquid chromatography

Silvia Carballo, Soledad Prats*, Salvador Maestre and José-Luis Todolí

Analytical Chemistry, Nutrition and Food Science Department.University of Alicante. (Spain).

Running title: Microwave assisted liquid chromatography for vegetable oil analysis

**Corresponding author: Soledad Prats. University of Alicante. Analytical Chemistry, Nutrition and Food Science Department. P.O. Box 99, 03080 Alicante. (Spain).Tel.: +34 965903777; Fax: +34 9653697. E-mail: maria.prats@ua.es*

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Abbreviations

High Temperature Liquid Chromatography (HTLC); microwave (MW); number of theoretical plates (N); photodiode arrayed detector (PDA); evaporative light scattering detector (PDA); fluorescence detector (FD)

Keywords

Microwaves; phylloquinone ;tocopherols; vegetable oils;

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Abstract

In this manuscript, a study of the effect of microwave radiation on the high-performance liquid chromatography separation of tocopherols and vitamin K1 was conducted. The novelty of the application was the use of a relatively low polarity mobile phase in which the dielectric heating effect was minimized to evaluate the non-thermal effect of the microwave radiation over the separation process. Results obtained show that microwave-assisted high-performance liquid chromatography had a shorter analysis time from 31.5 to 13.3 min when the lowest microwave power was used. Moreover, narrower peaks were obtained; hence the separation was more efficient maintaining or even increasing the resolution between the peaks. This result confirms the fact that the increase in mobile phase temperature is not the only variable for improving the separation process but also other non-thermal processes must intervene. Fluorescence detection demonstrated better signal-to-noise compared to photodiode arrayed detector mainly due to the independent effect of microwave pulses on the baseline noise, but photodiode array detector was finally chosen as it allowed a simultaneous detection of non-fluorescent compounds. Finally a determination of the content of the vitamin E homologues was carried out in different vegetable oils. Results were coherent with those found in the literature.

1 Introduction

Novel LC techniques are being explored to respond to the demands and needs of society [1]. In this context, techniques such as high-temperature liquid chromatography (HTLC) [2] and UHPLC have been developed. These new approaches lead to an improvement on HPLC separation capability due to the increase in analytes diffusivity leading to faster and more sensitive and efficient analysis. Thus, by setting the mobile phase temperature at 150°C, the analysis time was considerably shortened with respect to room-temperature HPLC in a separation of mono and disaccharides [3]. Moreover, analytical sensitivity was increased as the temperature went up [2]. Alternatively, UHPLC allows increasing efficiency, optimum mobile phase velocity and mass transfer through the use of stationary phase containing particles with diameters below 2 μm. In this case, the analysis time was shortened by a factor close to one order of magnitude [1]. However, with these techniques some constraints must be fulfilled. Thus, HTLC requires: (i) the use of thermally resistant stationary phases; (ii) mobile phase pre-heating to avoid temperature gradients inside the column; (iii) a mobile phase cooling system prior the detector; and, (iv) the use of specific instrumentation, *e.g.* pressure restrictors. UHPLC, in turn, requires specific columns and components compatible with pressures beyond 400 bars.

Alternatively, microwave (MW) radiation is widely used in laboratories for many purposes such as organic synthesis and sample treatment. However, this radiation is seldom used to assist chromatographic separations. MW radiation can interact with matter in three different ways: dipolar

polarization, ionic conduction and interfacial polarization [4–5]. Under the influence of a static electromagnetic field, molecules that present a dipole moment align themselves with the applied static field. When an oscillating field is used instead, such as MW radiation, the interaction of MWs with molecules results in changes of the dipoles orientations, and energy is dissipated as heat from internal resistance to these changes. For each material the conversion rate of electromagnetic energy into thermal one is reflected by the loss tangent (tanδ), which can be calculated as the ratio of loss factor (ϵ''), which accounts for the loss of energy through dissipative mechanisms and the relative permittivity (ϵ') that reflects the ability of a material to store electrical potential energy and re-orientate by means an electric field. For materials with dielectric constants in the same range, tanδ is a very useful tool for comparison purposes because the greater the loss tangent, the faster the increase of the temperature of the irradiated material [5].

From the instrumental needs viewpoint the use of MW in chromatographic separations can be assayed with no significant increase in costs due to the use of domestic MW oven operating at 2.45 GHz. However, method transfer from conventional HPLC implies the use of components made of a MW transparent material such as polyether ether ketone (PEEK).

Taking into account the interaction of MW radiation with matter several attempts have been published trying to use MW for improving the separation of compounds in chromatography [6–12]. In TLC [6] a perpendicular arrangement of the MW field relative to the TLC plate allowed obtaining a considerable improvement in the separation of dyes and pesticides compared with separation under usual conditions. A different application was the design of a MW GC oven in which only the column was heated because the column contains the only appropriate MW absorbing material. With this oven faster cooling can be achieved because less material is heated [7]. More specifically, in relation to LC, published studies involving application of MW radiation to the separation reported an increase in the chromatographic efficiency in terms of number of theoretical plates (*N*) when MW was used [9,10]. In both studies the possible contribution of temperature to the observed results was discarded by comparison of the results obtained when heating the column with a conventional oven. Galinada et al. conducted studies dealing with the influence of MW radiation on the mass transfer kinetics in reversed-phase HPLC [11]. In their work they concluded that an enhancement in the analyte intraparticle diffusion coefficient could lead to an increase in the analyte diffusivity into the particle

pores. Although the obtained results are particularly important none of those preliminary studies evaluated the influence of MW radiation on peaks resolution.

There is only one precedent in which MW radiation was applied to the chromatographic separation of five water-soluble vitamins [12]. According to this study a MW field induced a shortening in the analytes retention times with a concomitant 50% increase in sensitivity as compared to conventional HPLC. That study was performed in a discontinuous mode, so the MW oven was switched on 3 min after the sample injection and it was switched off once the last compound left the column. Therefore, it was necessary to equilibrate the column between injections during 25 min. Moreover, the aqueous mobile phase employed contained perchloric acid and, thus, ions were present. Under these circumstances, heating of mobile phase was favored due to dipolar polarization and ionic conduction processes [5] which favored the separation.

The purpose of the present work was, thus, to apply MW-assisted HPLC (MW-HPLC) with a mobile phase having low permittivity and loss tangent. In this case, the influence of the mobile phase heating on the separation was minimized. This allowed us to discern whether MW radiation favored the separation through non-thermal mechanisms. The method was validated for the separation of vitamin E homologues in vegetable oil samples.

2 Materials and methods

2.1 Reagents and samples

α-Tocopherol standard and butylated hydroxytoluene (BHT) were purchased from Sigma–Aldrich (Steinheim, Germany) and δ, γ-tocopherol and phylloquinone from Supelco (Bellefonte, PA, USA). Organic solvents as acetonitrile (ACN), tetrahydrofuran (THF) and propan-1-ol were also supplied by Sigma–Aldrich and were all of HPLC grade. Ultrapure water was obtained by a Milli-Q system (Millipore, Bedford, MA, USA).

2.2 Standard and sample preparation

Standards series of 1, 3, 7, 10, 40, 70, 100 mg L^{-1} for α-, γ-, δ-tocopherol and vitamin K1 were prepared in propan-1-ol or in acetonitrile containing 0.01% of BHT, kept in amber glass flasks and maintained at 4°C in the fridge until the analyses. Commercial samples of vegetable oils (corn, soybean, olive and sunflower) were obtained from a local supermarket. Commercial Soybean oil was a mixture of soybean oil with seed and fish oils and the sunflower oil was enriched in vitamin E. Samples were diluted 1:10 v/v with propan-1-ol and homogenized using a vortex stirrer. All solutions were filtered through a nylon syringe filter of 0.45 µm pore diameter before their chromatographic analysis.

2.3 Chromatographic separation

A HPLC pump Model PU-2089 Plus (Jasco International, Tokyo, Japan) with a 20 µL injection valve (Mod.7725i, Rheodyne, USA) was used. The mobile phase flow rate was adjusted, and the best results in terms of vitamin separation were obtained at 1 mL min^{-1}.The detectors employed for fat-soluble vitamins determination were: a Waters 996 photodiode arrayed detector (PDA) (Waters, Milford, MA, USA) operating at 270 nm for phylloquinone analysis and at 295 nm for tocopherols determination and also a Waters 474 scanning Fluorescence Detector (FD) (Waters, Milford, MA, USA) set at excitation and emission wavelength of 290 nm and 325 nm, respectively. Additionally an evaporative light scattering detector (ELSD) was also used (SoftA, Tokyo, Japan). For this detector the selected conditions were 50°C for the spray chamber and 65°C for the drift tube, being the argon gas pressure 4.5 bars for nebulization. Chromatograms were recorded and processed with the Millenium 32 Software (Waters, 1999).

Three mobile phases made up of different proportions of ACN, THF and ultrapure water were tested to achieve the best chromatographic performance in terms of resolution and retention time. Mobile phase polarity and permittivity were calculated taking into account solvents polarity indexes and their proportion in the mixture [13].

The HPLC column selected was a polymeric RP column, PRP-1, 5 µm, 100 x 4.6 mm (Hamilton, Reno, NV, USA) with a PEEK holder. A PRP-1 guard column from Hamilton was also used. This column was selected as it is packed with polystyrene divinylbenzene, a material that is highly inert and transparent to MW. For conventional heating of the column an HPLC column heater oven (Gecko 2000, Sainte Foy La Grande, France) was employed. Additionally, for studying the MW effect over the separation, the chromatographic column was placed inside a conventional LG MW oven (Mod. LG MH6340FS) in a hanging position as it was previously reported [12]. Three positions of the HPLC column inside of the MW oven were assayed, at 1.5, 4 and 6 cm from the top of the oven. The only difference from the former assembly was that a Pyrex vessel containing 1400 mL of tap water was placed inside the oven instead of several vessels. This water volume (heat well) absorbed the energy not transferred to the column and mobile phase hence avoiding magnetron damage. MW radiation frequency was 2.45GHz, and nominal power supplied by the MW oven ranged from 170 to 850 W. The actual power absorbed by the column was estimated by subtracting the energy absorbed by the heat well during a whole chromatogram run when the column was inside the oven, to the energy absorbed by the heat well when the column was not inside the oven. The absorbed energy was calculated by multiplying the water mass, the water specific heat and the temperature increment registered. Thus the actual MW power absorbed by the column was calculated dividing the energy absorbed by the irradiation time.

As regards mobile phase temperature measurements a digital thermometer TL-1 (ThermoProbe, USA) was placed just against the exit of the tubing evacuating the eluate of the column. This thermometer has an accuracy of ± 0.1°C, and 1 s response time.

2.4 Statistical analysis of data

The program SPSS 11.5 for windows (SPSS, Chicago, IL, USA) was used to analyze the results. Data were analyzed using one-way ANOVA and if significant differences were found comparisons among means were carried out using Tukey test ($P < 0.05$).

3 Results

3.1 Selection of the chromatographic conditions for fat soluble vitamin analysis

In this study low polarity organic mobile phases were investigated to minimize the impact of MWs on heating. The first mobile phase tested was that recommended by the column manufacturer, *i.e.*, a mixture of ACN, THF and ultrapure water at 70, 20 and 10% v/v/v contents. Optimization of the mixture composition was split in two parts because of the different MW-solvent interaction of organic solvents and water.

In a first stage the proportion of water in the mixture was maintained constant (i.e. 10%). The other two components of the mobile phase have similar tanδ values; hence changing the proportion of both solvents would not strongly influence the MW absorption and heating capability of the mobile phase. Nevertheless, THF is less polar than acetonitrile so when THF proportion was decreased from 20 to 5% retention times of analytes increased and the separation of tocopherol homologues was higher. 0% of THF was also assayed but the analytes did not elute from the column and therefore it was dismissed. Consequently, the first mobile phase chosen contained 85% ACN, 5% THF and 10% water.

As regards water, the third mixture component, it should be stressed that this component presents higher polarity and tanδ values than the other two components and so it could modify the MW adsorption and heating properties of the mobile phase. Therefore a reduction of the level of water in the mixture was investigated to minimize MW heating effect maintaining constant THF in 5. The limit in water reduction percentage was 5% because from that proportion tocopherols peaks coeluted. Finally, a compromise of conditions was obtained at a mixture of 87.5:5:7.5 ACN/THF/Water proportions.

Furthermore, the selection of the detector was done on the basis of the LODs obtained when compounds eluted at room temperature (22 ± 1°C; Table 1). As it can be seen the ELSD provided the highest limits of detection meanwhile UV and fluorescence showed similar values. Consequently, as fat soluble vitamins are present in low concentrations in food samples only PDA and FD were considered in the following assays.

3.2 Influence of some variables on the MW-assisted HPLC separation of fat soluble vitamins

Firstly, the influence of the position of the column inside of the oven was studied since it is known that MW radiation is not distributed homogeneously in the oven cavity [14]. The chromatographic column was placed centered inside the MW oven in a hanging position [12] and the influence in the separation process of the column position inside the oven was assayed at 1.5, 4 and 6 cm from the top of the oven. The best results in terms of retention factor and efficiency were obtained for the shorter distance (i.e. 1.5 cm). At 1.5 cm retention times of all compounds were significantly shorter (P <5) than the ones obtained at 4 and 6 cm (Fig 1S). So this position of the column in the oven was selected for the following experiences.

Secondly, another variable to take into account is the type of solvent used to dissolve the samples. To study the possible influence of sample solvent permittivity and tanδ values over the separation process two compatible solvents for fat soluble vitamins were assayed. Acetonitrile was chosen for having a low value of loss tangent (0.052) and, propan-1-ol for having a high loss tangent value of 0.757 [5]. A separation of tocopherols and phylloquinone assisted by MW radiation applying a nominal power of 340 W was conducted injecting 10 µL of a vitamins standard. The comparison of retention times, resolution and number of theoretical plates showed no significant differences among any of the samples diluted in acetonitrile or propan-1-ol for a $P < 0.05$. This result demonstrated that the possible MW radiation influence of type of sample solvent on the separation is negligible compared to other factors such as the mobile phase and stationary phase.

Afterwards, the effect of increasing nominal MW power on the chromatographic separation was studied. Unlike a previous study [12], the MW oven was switched on just after the sample injection. Once a chromatogram was obtained, the water inside the heat well was entirely replaced by a new volume of water at room temperature. The time required for replacing water and doing the next injection was just 2 min. Replacing water at room temperature at the beginning of each injection ensured that the heat well absorbed the same amount of energy in each injection. In this study, working with the conditions previously mention, nominal MW power of 170 and 340 W corresponded to an actual power of 5 ± 2, and 43 ± 2 W, respectively. Nominal MW powers above 43 W were assayed but they were discarded as overlapping of δ- and γ-tocopherol peaks occurred.

Fig.1 compares the chromatograms obtained for the separation of δ-, γ- and α-tocopherols and phylloquinone at an actual MW applied power of 5 W (a) and at room temperature (b).The variation of the pressure applied by the HPLC pump and mobile phase temperature at the exit of the column for 5 W are also shown. When the actual MW power was 5 W, it was verified that neither the temperature of the mobile phase nor the pressure changed with time. In fact, stationary separation conditions were reached after the first chromatographic run (15 min). This observation was in agreement with previously published work [9, 11]. In the present approach the total analysis time including vitamin K1 was shortened from 31.5 min with no MW radiation to 13.3 min when the lowest MW power was used. Furthermore, when the actual power was increased to 43 W the analysis time was only 11.6 min.

The reduction in analysis time evidences the potential of MW radiation to assist chromatographic separations. It is noteworthy that the increase in temperature of the mobile phase at the exit of the column was only 5 ± 1°C at 5 W and 7 ± 1°C at 43 W compared to HPLC at room temperature. At 5 W, the temperature at the exit of the column for mobile phases containing 5 and 10% of water increased by 4 to 6°C, respectively. The corresponding temperature increases at 43 W were only 6 and 8°C. As in previous studies, it was also verified that the temperature of eluate increased with increasing MW power [9–12]. This moderate increase in mobile phase temperature corroborates the influence of water proportion in mobile phase. In a previous work, Terol et al. [12] obtained a higher increased in the mobile phase temperature in a shorter space of time applying the lowest nominal MW power (160 W) to assist the separation of water soluble vitamins. In that application the mobile phase consisted on a mixture of 0.1 M perchloric acid in Milli-Q water (90%) and acetonitrile (10%).

3.3 Effect of power MW radiation on S/N

Even for the lowest MW power, peak height increased significantly by as much as 100% for α-tocopherol. This increase was more conspicuous as the MW power increased. Thus, using a nominal power of 43 W, improvements up to 170% were achieved for α-tocopherol and vitamin K1. Regarding the analytes retention on the column, it was found that those analytes with longer retention times undergone a slightly higher increase in peak height due to their increased exposure to MW radiation. This increase in analyte height was also observed by Galinada et al. [11], Stone et al. [10], and Terol et al. [12].

As regards the baseline noise, two different trends could be identified depending on the detection system employed using the MW-HPLC system of this work. Baseline noise remained nearly unchanged for fluorescence detection independently of the MW power applied. On the contrary, when the PDA detector was used, a periodic oscillation of the background appeared when MW was applied. This phenomenon was commented by Terol et al. and [12] attributed to the pulsing supply of MW energy. An oven pulse produced an increase in the background signal followed by a drop in this signal once the pulse ceased. It is important to note that in that work signal was specifically monitored at 210 nm. Interestingly in this work, it was verified that the magnitude of the oscillations depended strongly on the wavelength selected. Thus, for instance, at 295 nm the differences in background levels with and without MW radiation were minimal. Meanwhile, at shorter wavelengths a noisier chromatogram was found when applying MW radiation (Fig.2S). The specific behavior suggested that the origin of this fluctuating signal is the presence of a substance in the mobile phase with a high extinction coefficient at lower wavelengths. This could be due to the light absorption of impurities of the mobile phase components or maybe the column packing or hardware (i.e. frits or holder) material bleeding. The problem of HPLC column bleeding was studied in HTLC [15] and needs further consideration when MW-HPLC is employed as well. Notwithstanding, in this work no loss of separation efficacy of the column was observed after nearly 100 injections using MW-HPLC.

As a consequence of the increase on peak height and the stability of background, the calculated S/N ratio was higher for the irradiated than for the non-irradiated tocopherols. Thus, at 43 W S/N values were more than two times higher than that calculated without MW radiation, which was consistent with the increase in peak height. However, the S/N ratio for vitamin K1 at 254 nm (maximum absorbance) was found to be less favorable at 43 W due to an increase in background noise that cannot be compensated for by the increase on peak height (Fig.2). This is the reason why 270 nm was selected as suitable wavelength for phylloquinone detection in this application.

3.4 Comparison between MW-HPLC and conventional heating HPLC

For the purpose of this study, it is essential to identify the effects of dielectric heating on column performance. Temperature of the column effluent was measured when MW-HPLC was used to clarify whether improvements in separation efficiency and sensitivity rely entirely on heating of mobile phase (as it happens when HTLC is used) or in additional radiative mechanisms. Turner et al. proved that MW radiation modifies the sorption selectivity of the analytes in a stationary phase such as zeolites [8]. In this work, the eluent temperature at the exit of the column was 31 and 33°C when the actual MW power applied to the column was 5 and 43 W, respectively. To compare the results with conventional heating, the chromatographic column was inserted into an HPLC oven providing a temperature of the mobile phase at the exit of the column of approximately 33°C.

Taking conventional HPLC as reference, several differences were found when considering the results afforded by MW-HPLC. Firstly, in Fig.3a a representation of the logarithm of retention coefficient for all the analytes studied is shown. It can be observed that the decrease in retention time for α-tocopherol and phylloquinone at 33°C with respect to HPLC at room temperature was up to two times lower than in the case of MW-HPLC at 43 W. Moreover, the decrease of log *k*' with MW radiation for phylloquinone was more pronounced than for the three tocopherols as it eluted later, and it was longer under the influence of MWs. Secondly, the influence of MW on the separation of peaks was studied calculating the resolution values (Rs) using PDA. Rs at 33°C was virtually identical in comparison with HPLC at room temperature, in contrast, Rs improved by up to 25% for PDA at 43 W. Additionally, the column performance was verified calculating N. At 43 W, N was twice as high as it was obtained with conventional HPLC (Fig.3b), also evidenced by other authors [9–11]. Consequently, *N* enhancement is also in agreement with the increase in column performance when the MW power is increased.

3.5 Validation of the MW-assisted LC method

The MW-HPLC method for the determination of fat-soluble vitamins was validated through several parameters such as linearity, inter- and intra-day repeatability of retention time and peak area, LOD, and accuracy. Results for MW-HPLC at 43 W and UV-Vis detection are shown in Table 1. Good linearity of the calibration curves were obtained as the coefficients of regression (R^2) were close to 1. Intra-day and inter-day repeatability in terms of retention time and peak area were acceptable as RSDs were lower than 5% for all the analytes. Percentage of recovery ranged from 89 to 107% depending on the analyte considered, thus giving indication of a good accuracy [16]. Moreover, the LOD found for MW-HPLC (Table 1) was nearly half of the values obtained when HPLC at room temperature was used and slightly lower compared to conventional heating.

3.6 Analysis of fat-soluble vitamins in vegetable oil samples

Finally, the novel MW-HPLC technique was applied to the analysis of some commercially available vegetable oil samples. Concentrations of δ-, γ- and α-tocopherol obtained by conventional and MW HPLC at 43 W are summarized in Table 2. Soybean oil was the only sample that contained δ-tocopherol above limits of quantification. Measured concentrations of γ- and α-tocopherols for corn, olive and sunflower oils were in the same order as those obtained by other authors [17–19] who found δ-tocopherol concentrations ranging from 0.1 to 3 mg/100 g oil. Moreover γ-tocopherol for olive oil samples was only quantified when the MW-HPLC was used, as the concentration in these samples was under the LOD for the conventional HPLC method. Furthermore, in another study of composition of edible oils, concentrations of 66 and 2.5 mg/100 g of γ- and α-tocopherol were found, while undetectable amounts of δ-tocopherol in sunflower oil was reported [20]. Phylloquinone was not detected in oil samples as it is present in quantities lower than the limits of detection. A preconcentration treatment before the chromatographic analysis should be advisable but it was not the purpose of the present work. A one-way analysis of variance was applied to the mean scores of each tocopherol homologue. After the application of a Tukey's post hoc test, no significant differences between conventional HPLC and MW HPLC data were found except for α-tocopherol in corn oil and soybean oil for $P<0.05$.

4 Conclusions

The results of this work confirmed that MW radiation could successfully assist the elution of analytes more efficiently than conventional heating even if low MW power is applied. The overall significance in this study is that the increase in sensitivity was of the same order as that obtained with an ionic mobile phase [12], even though in this case the mobile phase had lower polarity and relaxation time. Although it is known that the efficiency in HPLC usually enhances with temperature, under the MW influence this effect is magnified and so "non-thermal" processes appear to have some effect [9–11]. Taking into account the Van Deemter equation, the MW radiation can have an effect over all coefficients of that equation. The band broadening due to Eddy diffusion (*A*-term) could be improved due to lateral mixing of molecules among different flow channels in the column due to higher molecular agitation induced by the perturbation in the molecular dipole moments under the effect of MWs [21]. Longitudinal diffusion coefficient (*B*-term) and resistant to mass transfer coefficient between the mobile and stationary phases (*C*-term) are temperature dependent and also directly or inversely dependent on the diffusion coefficient. Higher enhancement in diffusion of reactants compared to conventional heating has been documented in literature as a possible effect of MW radiation over the increase in reaction rates [4, 21]. The increase in the rate of adsorption desorption equilibria of the analytes in the mobile phase could also explain the reduction of retention time. Nonetheless, due to the complex interaction phenomena of MW radiation with material it is difficult to go further in the explanation of the exactly causes of the improvement obtained when MW assisted the separation.

Moreover, the use of mobile phases of low tanδ is also important as the chromatographic column works all the time at safe temperatures and so the durability of the column can be higher than using water mobile phases. Future enhancements in the implementation of MW-HPLC technique, for routine analysis could aim to the development of a MW suitable device for HPLC columns that allows working at low powers in a stationary MW mode, free of radiation pulses that perturb baseline noise, and preventing losses and dissipation of the supplied radiation, achieving a better control of system conditions.

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Fig.1 Chromatograms obtained for standard containing 3 mg L^{-1} of three tocopherol homologues and phylloquinone by MW-HPLC at 5 W (a) and by conventional HPLC (b). Changes on system pressure and temperature are presented in black dashed lines. Flow rate: 1 mL min-1 and a mobile phase which consisted in a mixture of 87.5:5:7.5 ACN/THF/Water. Detection: PDA

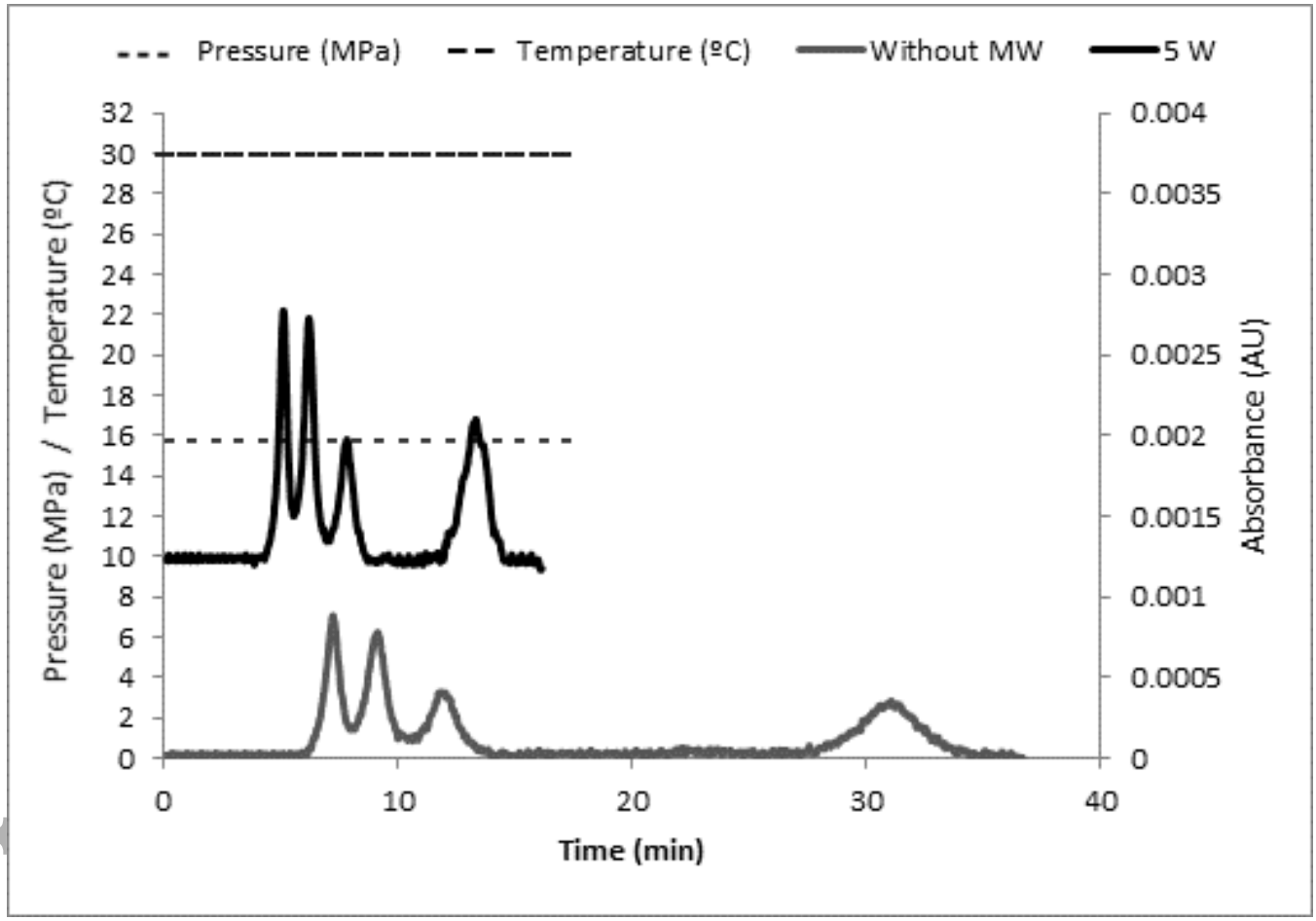


Fig.2 S/N for the three tocopherols homologues and phylloquinone calculated working under conventional HPLC (0 W), MW-HPLC at 5 and 43 W and, HPLC at 33°C. Flow rate: 1 mL min^{-1} and a mobile phase that consisted of a mixture of 87.5:5:7.5 ACN/THF/Water. Detection: PDA

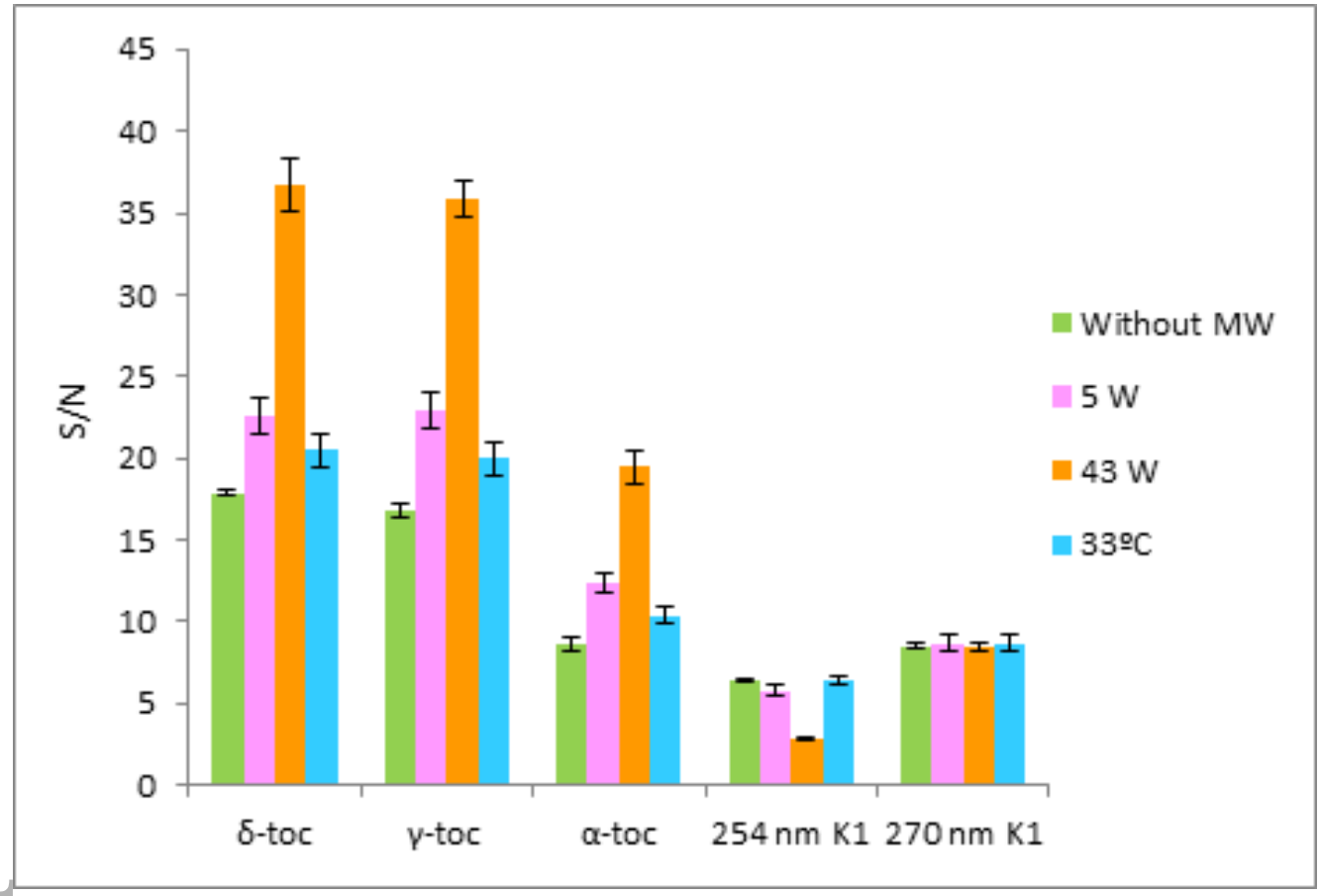
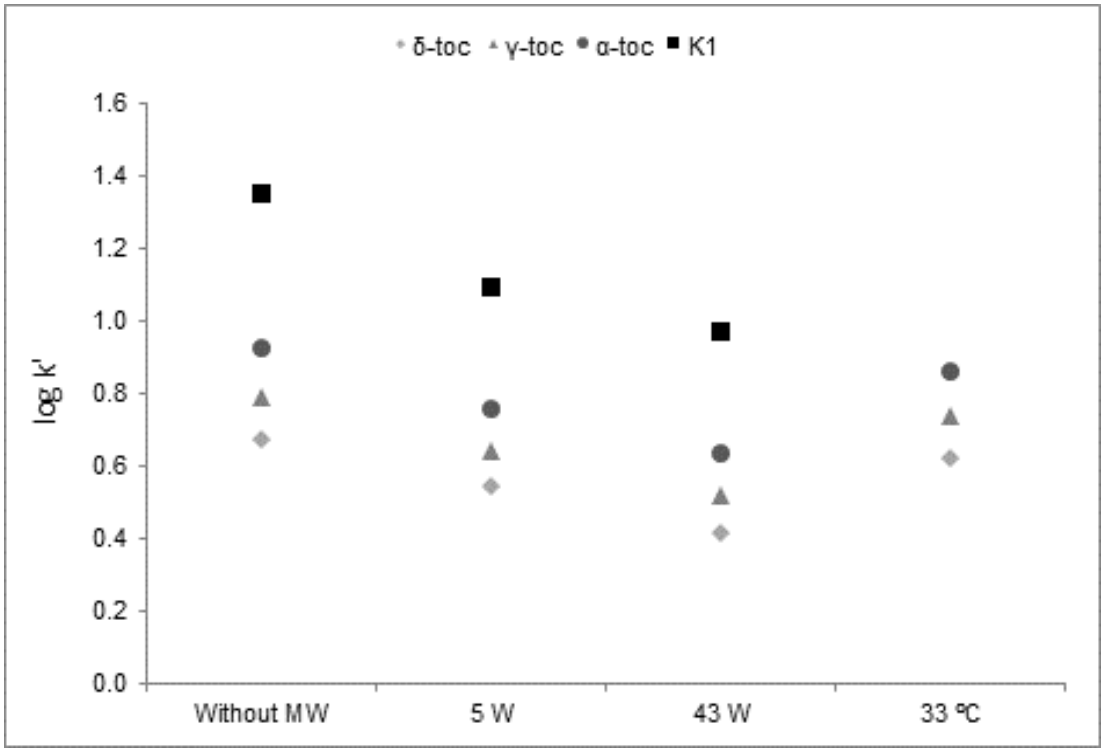


Fig 3 Plot of log *k'* (a) and *N* (b) as a function of MW-supplied power for the four analytes of interest.



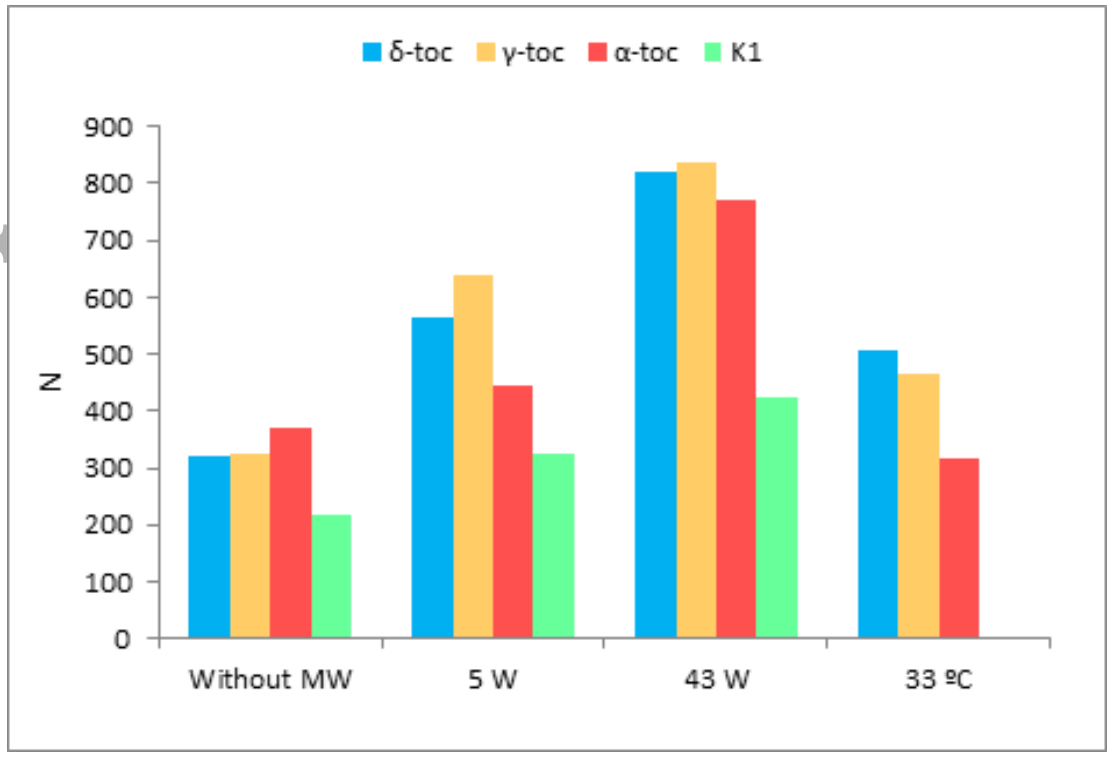


Table 1. Validation parameters for MW-HPLC technique at 43 W microwave supplied power.

		δ-tocopherol (295 nm)	*γ-tocopherol (295 nm)*	*α-tocopherol (295 nm)*	*Phylloquinone (270 nm)*
Concentration range (mg L^{-1})		1-100	1-100	1-100	1-100
Linearity					
Standard solutions		7	7	7	7
Regression equation		y = 9442.4x - 1367.1	y = 10993x - 1704	y = 7871.3x - 8459.8	y = 21162x - 22248
Regression coefficient (R^2)		0.9997	0.9997	0.9995	0.9996
Accuracy (% recovery)[a]					
20 mg L^{-1}		90 ± 4	89 ± 3	96 ± 6	107 ± 2
Repeatabilityintra-day[b]					
Retention time (% RSD)		3.62	3.47	3.19	3.37
Area (% RSD)		1.34	0.95	0.85	0.33
n		6	6	6	6
Repeatability inter-day[c]					
Retention time (% RSD)		2.70	3.06	3.26	4.57
Area (% RSD)		0.63	0.94	3.04	2.59
n		3	3	3	3
LOD (mg L^{-1})[d]					
0 W	PDA	0.96 ± 0.07	1.09 ± 0.06	2.0 ± 0.2	1.10 ± 0.08
	FD	0.66 ± 0.09	1.16 ± 0.11	4.2 ± 0.6	

	ELSD	5.3 ± 0.3	7.5 ± 0.8	21.3 ± 0.4	18.7 ± 0.3
5 W	PDA	0.55 ± 0.11	0.60 ± 0.13	1.07 ± 0.07	0.58 ± 0.08
	FD	0.32 ± 0.05	0.47 ± 0.09	2.3 ± 0.4	
43 W	PDA	0.47 ± 0.07	0.47 ± 0.09	1.01 ± 0.15	0.44 ± 0.07
	FD	0.34 ± 0.08	0.48 ± 0.10	2.7 ± 0.7	
33 ºC	PDA	0.68 ± 0.07	0.71 ± 0.03	1.35 ± 0.02	0.78 ± 0.03
	FD	0.61 ± 0.01	0.93 ± 0.03	4.32 ± 0.06	

(a) The confidence interval was calculated as $\pm\frac{ts}{\sqrt{n}}$ where *s* was standard deviation of three measurements (n=3), *t* was obtained using the 95 % confidence limit and *n* was the number of replicates.

(b) Repeatability intra-day was calculated as % RSD for six consecutive injections of a standardwithin a day.

(c) Repeatability inter-day was calculated as % RSD of a standard injectedby triplicateover the course of 4 days.

(d) LODs calculated according to LOD = $\frac{h\ noise}{(\frac{h\ peak}{C})}$ where *h noise* and *h peak* were noise and peak height, respectively, and *C* analyte standard concentration.

Table 2. Concentration (mg/100 g) of δ-, γ-, α-tocopherol in different commercial vegetable oil samples obtained by HPLC and MW-HPLC at 43 W with UV-Vis detection[1].

HPLC	Olive oil "for frying"		Corn oil		Extra virgin olive oil		Sunflower oil		Soybean oil mixture	
	HPLC	MW-HPLC	HPLC	MW-HPLC	HPLC	MW-HPLC	HPLC	MW-HPLC	HPLCs	MW-HPLC
δ-tocopherol	-	-	-	-	-	-	-	-	24.6±0.7[a]	24.7±1.0[a]
γ-tocopherol	< LOD	0.9 ±0.1	83.3±3.4[a]	86.0±3,9[a]	< LOD	0.6±0.1	4.8±0.1[a]	4.3±0.4[a]	59.9±0.1[a]	60.2±1.2[a]
α-tocopherol	13.5±0.6[a]	13.6±1.2[a]	24.1±1.6[a]	19.7±1.4[b]	13.6±0.3[a]	12.6±1.1[a]	67.1±0.8[a]	65.4±1.1[a]	14.8±1.0[a]	12.4±1.2[b]

[1] Results are given as mean values obtained by triplicate ± a 95 % confidence interval calculated as it is shown at the foot of the Table 1.

a,b for each sample values in the same row with different superscript letter are significantly different (P <0.05)