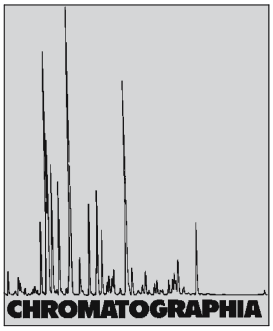
RP-LC for Determination of Plastochromanol, Tocotrienols and Tocopherols in Plant Oils



2007, *66*, 909–913

Jolanta Gruszka, Jerzy Kruk✉

Department of Plant Physiology and Biochemistry, Faculty of Biotechnology, Jagiellonian University, ul. Gronostajowa 7, 30-387, Kraków, Poland; E-Mail: jkruk@mol.uj.edu.pl

Received: 24 March 2007 / Revised: 3 August 2007 / Accepted: 28 August 2007
Online publication: 26 September 2007

Abstract

An isocratic high performance liquid chromatographic method, with the application of C_{18} and C_{30} reverse-phase column and fluorescence detection, is described for the analysis of plastochromanol, tocotrienols and tocopherols in plant seed oils. The solvent systems have been optimized to obtain high resolution for all tocochromanols and relatively short analysis time. The use of reverse-phase columns for plastochromanol analysis, previously not reported, enables very sensitive and selective detection of plastochromanol which under the described separation conditions did not interfere with tocochromanols or any other compounds. The sample extraction method is fast, simple and highly efficient. The obtained results show that plastochromanol was present in most of the investigated seed oils. Its level was the highest in flax (17–30 mg/100 g oil), rape (8.5–9), camelina (4.3), peanut (1.95), corn (1.69) and grape (1.31) seed oils. Its level in the other investigated oils was below 1 mg/100 g oil, and only in sesame and coconut oils it was not detected. Tocotrienols were found in most of the oils but their content was usually very low (<<1 mg/100 g oil) with the exception of grape, milk thistle and corn oils where it reached >1 mg/100 g oil. Tocopherol content and isomer composition was within the earlier reported literature values for the investigated oils.

Keywords

Column liquid chromatography
C_{30} silica phase
Plastochromanol
Tocotrienol
Tocopherol

Introduction

Tocopherols are lipophilic antioxidants synthesized exclusively by photosynthetic organisms, found mainly in leaves and seeds [1]. Their antioxidant function is mainly attributed to inhibition of lipids peroxidation and scavenging of reactive oxygen species [1–3]. The literature data on tocopherol content and isomer composition were mainly devoted to seed oils because of the nutritional importance of vitamin E. The reported tocopherol content in seed oils reach up to 2 mg g^{-1} oil or even higher [4], while in leaves it is usually considerably lower (10–50 μg g^{-1} fresh weight) with few exceptions where the content between 300 and 500 μg g^{-1} fresh weight in oil palm was reported [5] and a few tropical plants [6] or nearly 1 mg g^{-1} fresh weight in *Eucalyptus gunni* [7].

The occurrence of tocotrienols in plants is limited to nonphotosynthetic organs (seeds) [8] of only certain plant species, mainly monocotyledons. The richest source of tocotrienols are palm and rice bran oils [9, 10], but these compounds are also found in wheat germ oil [9–11], coconut [9, 10], seeds of amaranth [12], orobanche [13], lychee [14] and others [15–17], as well as in latex of rubber trees [18]. The recent interest in tocotrienols results from their broad therapeutic action in human organism, which apart from their strong antioxidant properties manifests in anticancer and neuroprotective action, as well as protection against atherosclerosis. Their function in cell signalling was also suggested [10].

Plastochromanol (PC) is a naturally occurring γ-tocotrienol (γ-Tt) homologue with a longer side chain of eight isoprenoid units, which is probably formed by plants from the reduced plastoquinone by tocopherol cyclase [19], the key enzyme in tocopherol biosynthesis. PC is found in the highest amounts in flax and rape seed oils [11, 20–22], as well as in minor amounts in seed oils of camelina [23], hemp [24], many Vietnamese plants [14], salvia [15], black pine [16] and others

DOI: 10.1365/s10337-007-0416-2
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[17]. In contrast to tocotrienols, PC was found also as minor component in leaves of rubber trees [25] and others [26]. Its function in seeds and leaves seems to be similar to that of tocopherols, i.e. antioxidant action but the literature data on that subject is very scarce [27]. It could also have a similar action as tocotrienols on human health, but this issue requires further study.

For the analysis of tocochromanol content in plant oils, usually a normal-phase HPLC with fluorescence detection is used that offers high sensitivity and good separation of all tocopherol and tocotrienol isomers [11]. PC is also separated by this method but it runs between tocotrienols and when the PC content is low, its peak can be difficult to identify and properly quantified.

In this report, we applied for the first time reverse-phase HPLC with fluorescence detection for the analysis of the PC content in plant seed oils, which gives superior separation of PC from other tocochromanols due to its considerably higher hydrophobicity as compared to tocopherols or tocotrienols. Using this method, there was no interference of other tochromanols and fluorescent impurities with PC, which allows for its very sensitive detection. Reverse-phase HPLC method also enables tocopherol and tocotrienol separation and quantitative estimation, although β- and γ-tocotrienols can be separated only using a C_{30} reverse-phase column which is known for its perfect separation of other compounds like carotenoids and protochlorophyllides [28, 29].

Experimental

Material

Plant seed oils (mostly cold-pressed) were obtained from the local market and used soon after opening. Seed oils from the following plants were analyzed: flax (*Linum usitatissimum*), olive (*Olea europea*), rape (*Brassica napus*), poppy (*Papaver somniferum*), hemp (*Cannabis sativa*), safflower (*Carthamus tinctorius*), hazelnut (*Corylus avellana*), walnut (*Juglans regia*), peanut (*Arachis hypogaea*), sesame (*Sesamum indicum*), evening primrose (*Oenothera biennis*), camelina (*Camelina sativa*), rice (*Oryza sativa*), pumpkin (*Cucurbita pepo*), grape (*Vitis vinifera*), milk thistle (*Silybum marianum*), corn (*Zea mays*) almonds (*Amygdalus communis*), sunflower (*Helianthus annuus*), coconut (*Cocos nucifera*) and castor (*Ricinus communis*).

Standards

HPLC-grade tocopherol isomers of the highest available purity (α-99.9%, β-99.5%, γ-99.5%, δ-99.9%) were purchased from Merck (No. 15496), while tocotrienol isomers were obtained from Calbiochem (No. 613432) with the purity of all isomers specified as $\geq$99.5%. PC was synthesized from natural plastoquinone (isolated from maple leaves [30]), according to the method described in Ref. [25] and purified by preparative HPLC on a C_{30} column in methanol. Standard stock solutions of tocopherols were prepared in absolute ethanol and their concentrations were determined spectrophotometrically, using the following molar extinction coefficients in ethanol: ε_{292} = 3260 for α-Toc, ε_{296} = 3720 for β-Toc, ε_{298} = 3810 for γ-Toc and ε_{298} = 3510 for δ-Toc [31]. The same ε values were taken for the corresponding tocotrienols and the coefficient of γ-Toc was taken for PC.

Sample Extraction

For tocopherol and tocotrienol analysis, 5–20 mg of an oil was precisely weighed in an 2-mL Eppendorf tube, then 1 mL of acetonitrile–methanol–water mixture (72:8:1, *v*/*v*) was added and the tube was shaken vigorously on a laboratory vortex for 15 min at 60 °C in the dark with 5 min preincubation. Afterwards, the extract was shortly centrifuged on a benchtop centrifuge (10,000*g* × 5 min) and the supernatant was analyzed by HPLC. After removal of the supernatant with a syringe, the extraction and centrifugation was repeated, followed by HPLC analysis and the obtained results were combined. This method gave 94–100% of extraction yield of the investigated tocochromanols.

For plastochromanol analysis, 2–6 mg of an oil was extracted using methanol–hexane mixture (85:15, *v*/*v*) under the conditions described above. The extraction yield of PC was in the range of 97–100%.

HPLC Measurements

The HPLC measurements were performed using Jasco PU-980 pump, UV-Vis detector UV-970, Shimadzu RF10-AXL fluorescence detector (excitation-emission detection at 290–330 nm), Borwin v. 1.50 software, Teknokroma (Barcelona, Spain) C_{18} reverse-phase column (Nucleosil 100, 250 × 4 mm, 5 µm) thermostated at 24 °C, and Rheodyne injector with 100 µL loop. For method optimization, different binary (acetonitrile–methanol) and ternary (acetonitrile–methanol–water) isocratic mobile phases were applied for tocochromanol separation, and methanol–hexane solvent systems for PC separation. For oil analysis, acetonitrile–methanol–water (72:8:1, *v*/*v*) was used at a flow rate of 2 mL min^{-1} for tocopherol and tocotrienol separation, while methanol–hexane (85:15, *v*/*v*) at a flow rate of 1.5 mL min^{-1} was used for PC analysis. Addtionally, for the separation of β- and γ-Tt, YMC (Europe GmbH) a C_{30} reverse phase column (250 × 4.6 mm, 3 µm) was applied using acetonitrile–methanol–water (72:8:1) at a flow rate of 1 mL min^{-1}.

Calculations of Tocochromanol Content

Tocochromanols were identified by comparison of the retention time of peaks in oil extracts with the peaks of standards. The concentration of tocochromanols in the HPLC-analyzed oil extract was calculated by comparison of the peak areas from the fluorescence detector with the corresponding peaks of standard solutions. The fluorescence intensity of peaks of the corresponding tocopherols and tocotrienols at the same concentration in a given solvent system should be the same, as the fluorescence is emitted only from the chromanol ring and it should not be influenced by the isoprenoid side chain. Therefore, the same is true for PC and γ-Toc or γ-Tt. Since the tocotrienol standards gave lower peak intensities than the corresponding tocopherols, due to the lower purity of tocotrienols than those of tocopherols, the appropriate corrections were taken into calculation. PC standard gave nearly the same response as γ-Toc, indicating its high purity.

Results and Discussion

Method Optimization and Validation

For quantitative calculations, it was important to use tocopherol standards of the highest purity because the impurities may overestimate the true standards concentration by contributing to the absorption in the 290–300 nm region, which is used for the spectrophotometric determination of the tocopherol concentration. Since the tocotrienol standards gave lower peak intensities in the fluorescence chromatograms than the corresponding tocopherols, the appropriate corrections were taken into calculation.

In finding the optimal solvent system for tocopherol and tocotrienol separation, using a C_{18} reverse-phase column, we tested different binary (acetonitrile–methanol) and ternary (acetonitrile–methanol–water) solvent systems. In pure methanol, tocotrienol and tocopherol peaks were narrow but the separation between tocopherols and tocotrienols was poor. On the other hand, in pure acetonitrile the separation was better but the peaks were broad and overlaid. When testing different binary acetonitrile–methanol mixtures we found that the small methanol content (5–10%) in acetonitrile resulted in considerable peak sharpness improvement and preserving good separation between tocopherols and tocotrienols. For further tests of ternary solvent mixtures (acetonitrile–methanol–water), 10% of methanol in acetonitrile was used. In order to improve the separation of individual tocotrienols and tocopherols, different water proportions were tested in acetonitrile–methanol–water mixtures. As can be seen in Fig. 1, the separation between individual tocopherols and tocotrienols increased with the increase of water content in the ternary solvent mixture but the retention time, especially of tocopherols increased considerably with the higher water content. For further applications, we found that the water content at proportions of 72:8:1 (*v*/*v*) in acetonitrile–methanol–water mixture was optimal for individual tocopherol and tocotrienol separation and preserving short analysis time.

In the case of PC separation in hexane–methanol binary mixtures, it can be seen that with the lowering of hexane content in methanol, the retention time of PC increased considerably (Fig. 1). Since there was no peak interference with that of PC in the investigated oil samples at 15% of hexane in methanol, we used this solvent composition throughout the further study.

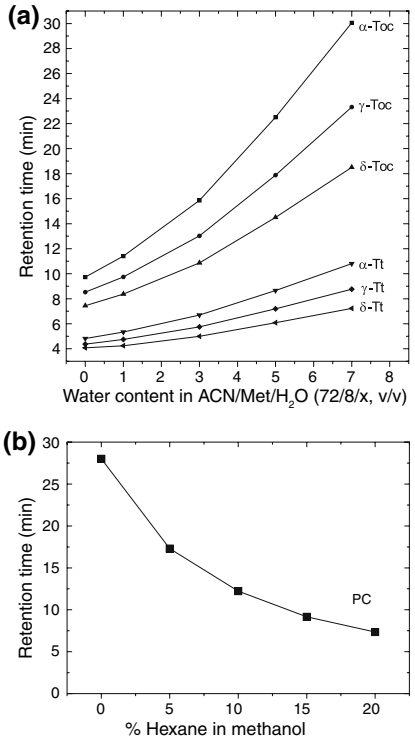

Fig. 1. **a** Dependence of retention time of tocopherols and tocotrienols on water content in acetonitrile–methanol–water (72:8:x, *v*/*v*) mixtures. **b** Dependence of retention time of PC on hexane content in methanol

Since the application of C_{18} reverse-phase phase did not enable separation of β and γ tocotrienol isomers, we additionally used a C_{30} reverse-phase for the purpose. The separation conditions using a C_{30} column were also found optimal using a acetonitrile–methanol–water mixture proportion of 72:8:1 (*v*/*v*). The increase of the water content in this ternary mixture increased the retention time of both tocotrienols but the separation was not further improved.

Relationships between the response of the fluorescence detector and prenyllipids concentration were linear over the concentration ranges of 5 nM–20 μM (3.7 ng mL^{-1}–15 μg mL^{-1}) for PC in methanol-hexane (85:15, *v*/*v*) and 2 nM–20 μM (0.85 ng mL^{-1}–8.5 μg mL^{-1}) for tocopherols and tocotrienols in acetonitrile–methanol–water (72:8:1, *v*/*v*) with regression coefficients (*r*) higher than 0.9999 for both PC and tocochromanols, indicating significant linearity of the calibration plots obtained from the method.

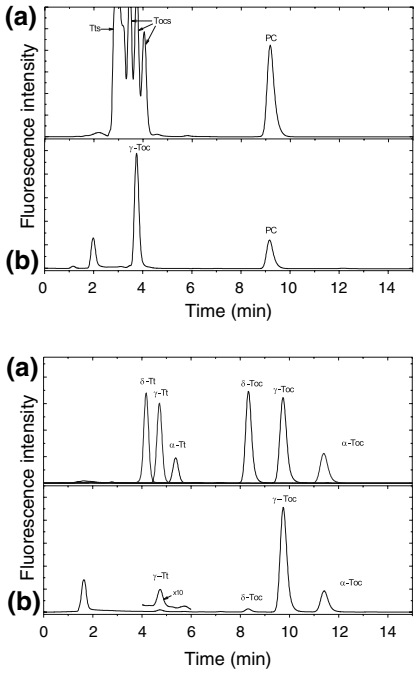

Fig. 2. Top Chromatograms of PC, tocotrienol and tocopherol standards (**a**) and lineseed oil "Eurolen" (**b**) using C_{18} reverse-phase column in methanol–hexane (85:15, *v*/*v*) using fluorescence detector (290 nm excitation–330 nm emission); flow 1.5 mL min^{-1}. Bottom Chromatogram of tocotrienol and tocopherol standards (**a**) and rapeseed oil "Kuchcik" (**b**) using C_{18} reverse-phase column in acetonitrile–methanol–water (72:8:1, *v*/*v*) and fluorescence detector (290 nm excitation–330 nm emission); flow 2 mL min^{-1}

Application

The application of a reverse-phase column gives fine separation of PC from tocopherols and tocotrienols (Fig. 2). The obtained data on PC content in the investigated oils (Table 1) show that flax and rape seed oils are the richest source of PC, as reported earlier in the literature. The content of PC in the four investigated flax oils amounted to 17–30 mg/100 g oil which is above the reported data (4–17 mg/100 g oil [11, 20, 21]). It is known that the tocochromanol content may vary considerably within the same type of oil depending on the cultivars and oil processing [32]. Most of the oils used in the present analysis were cold-pressed which should preserve the natural, high content of tocochromanols. The PC content of rape seed oils obtained in our study (Table 1) is at the higher limit of

Table 1. Content of tocopherols, tocotrienols and plastochromanol in plant seed oils

Oil	Toc (mg/100 g oil)			Tt (mg/100 g oil)			PC (mg/100 g oil)
	α	γ (+β)	δ	α	γ (+β)	δ	
[a]Flax 'Linolia'	0.25	21.35	0.33	tr.	tr.	–	21.88
[a]Flax "Vigean'	0.38	23.24	0.34	tr.	0.13	–	17.01
[a]Flax 'Eurolen'	1.04	40.51	0.85	0.03	0.06	–	30.15
[a]Flax 'Oleofarm'	1.58	26.98	0.38	–	0.02	–	17.92
[a]Olive 'Due Angeli'	4.22	1.04	0.03	0.07	tr.	–	0.46
Rape 'Kuchcik'	14.19	22.45	0.52	–	0.22	–	9.08
Rape "Primus'	17.63	27.24	0.63	–	0.05	–	8.57
[a]Poppy 'Vigean'	1.99	15.51	0.23	–	0.16	–	0.11
[a]Hemp 'Vigean'	5.27	30.46	1.07	–	0.11	–	0.16
[a]Safflower 'Vigean'	43.75	1.33	0.33	–	0.15(γ)	–	0.12
[a]Hazelnut 'Vigean'	22.22	6.88	0.41	–	0.03(γ)	0.02	0.01
[a]Walnut 'Vigean'	7.53	23.64	2.64	–	0.09	–	0.04
[a]Peanut 'Oleofarm'	10.92	11.08	0.88	–	0.01(γ)	0.03	1.95
[a]Sesame 'Oleofarm'	–	14.03	0.30	–	tr.	–	–
[a]Evening primrose 'Oleofarm'	7.48	27.39	0.52	–	0.01(γ)	–	0.15
[a]Camelina 'Vigean'	1.82	44.75	0.98	0.30	0.08	–	4.30
Rice bran 'Rizi'	4.93	4.74	0.30	7.76	9.99	–	0.11
[a]Pumpkin 'Vigean'	–	19.78	0.80	–	–	–	0.05
Grape	28.18	17.30	0.88	1.58	1.21(γ)	–	1.31
[a]Milk thistle 'Vigean'	19.33	5.21	0.61	0.87	0.37	0.10	0.24
[a]Corn	17.10	85.09	6.55	1.14	1.17(γ)	0.12	1.69
[a]Roasted almonds 'Bio Planete'	46.12	4.41	0.66	0.46	0.11	–	0.18
Sunflower 'Dolores'	59.37	7.95	1.33	0.04	0.07	–	0.25
Coconut	0.36	0.21	0.03	0.34	0.17	–	–
Castor	1.30	34.46	28.50	–	0.05	0.02	0.11

The value are means from three replicates. The standard error was ≤5 and ≤20% for the tocochromanol content above and below 1 mg/100 g oil, respectively
"–" not detected, tr. traces (< 0.01 mg/100 g oil), (γ) - only γ-Tt present as revealed by C_{30} column analysis
[a] Cold pressed oils

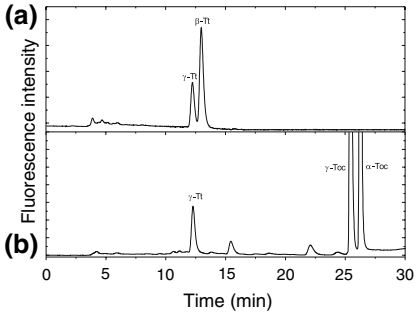

Fig. 3. Chromatograms of β- and γ-tocotrienol standards (**a**) and grape seed oil (**b**) using C_{30} reverse-phase column in acetonitrile–methanol–water (72:8:1, *v*/*v*) and fluorescence detector (290 nm excitation–330 nm emission); flow 1 mL min^{-1}

the previously reported values (2.8–8.2 mg/100 g oil [11, 20, 22]). The third PC most abundant oil was camelina seed oil with the content of 4.3 mg/100 g oil, which is higher than earlier reported (1.5 mg/100 g oil [23]). In contrast to the present results (Table 1), the reported earlier [24] PC content of hemp seed was higher. The other oils with a PC content above 1 mg/100 g oil are peanut (8% of total tocochromanol content), grape (2.6%) and corn (1.5%) seed oils that was not found before. Most of the other investigated oils also contain PC but the content is considerably lower (Table 1).

The applied system using a C_{18} column allowed for the simultaneous separation of tocopherol and tocotrienol isomers (Fig. 2). In this system PC was eluted at 85 min as a broad band. Although this system does not allow for separation of γ and β isomers, it was achieved using a C_{30} column (Fig. 3). The obtained data (Table 1) show that rice bran oil, which is known for its high tocotrienol content [9, 10], is the richest source of tocotrienols among the investigated oils. Nearly all the other investigated oils contained low but detectable amounts of tocotrienol isomers among which grape, corn and milk thistle seed oils contained the highest amount of tocotrienols. The available reports on the tocotrienols content in these plant oils are only for grape seed oil [33] the composition of which was found to be very variable regarding both tocopherols and tocotrienols. In some cases, the tocotrienol content exceeded even the tocopherol content and reached up to 35 and 78 mg/100 g oil for α-Tt and γ-Tt, respectively [33]. The investigated oils, analyzed for the composition of γ and β isomers using a C_{30} column, showed only the presence of γ-Tt (Table 1, Fig. 3).

The composition of tocopherol isomers of the investigated oils show (Table 1) that in most oils the γ isomer is dominating with the exception of olive, safflower, hazelnut, grape, milk thistle, roasted almond and sunflower seed oils. In the case of peanut oil, the α and γ-tocopherols were found in equal amounts. Castor oil is the only oil with the high content of δ-Toc, which was already reported in the literature [9]. The chromatographic system (C_{18} column) used for the determination of tocopherol isomers composition did not enable the separation of β and γ-tocopherols but it is known that β-Toc is a minor component of most oils [32].

The obtained data in our study on tocopherol contents of different plant oils are within relatively broad values reported in the literature [11, 20–24, 32–34]. However, the relative content of PC with respect to all tocochromanols in these oils is often clearly higher, as for flax (62–100% in our study vs. 6–48% in the literature data [11, 20, 21]), rape (19–24.4% vs. 6–11% [11, 20, 22]) and camelina (9% vs. 2% [23]). One of the possible reasons

of this discrepancy is prabably an overestimation of the tocopherol content values reported in the literature due to use of impure tocopherol standards.

The presented HPLC method for the tocochromanol analysis using an isocratic solvent system and reverse-phase columns shows that it can be successfully applied and is especially useful for the PC analysis where it is well resolved from other tocochromanols.

Ackowledgments

This work was supported by the grant N302 049 32 obtained from the Polish Ministry of Science and Education.

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