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New vitamin E isomers (gamma-tocomonoenol and alpha-tocomonoenol) in seeds, roasted seeds and roasted seed oil from the Slovenian pumpkin variety '*Slovenska golica*'

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ABSTRACT

The Štajerska region in north-eastern Slovenia and the Styria region in southern Austria have a long tradition of growing pumpkins (*Cucurbita pepo* L.) as an oil crop. GC-MS determination of the free and esterified minor compounds in oil of roasted pumpkin seeds from the Slovenian *C. pepo* L. variety '*Slovenska golica*' revealed the presence of two previously unreported compounds: alpha-tocomonoenol and gamma-tocomonoenol. Using the GC-MS data, reference samples (Crude Palm Oil) and tocopherol and tocotrienol standards it was possible to assign and quantify alpha-tocomonoenol (17.6 ± 0.6 μg/g) and gamma-tocomonoenol (118.7 ± 1.0 μg/g) compounds in roasted '*S. golica*' seed oil using HPLC. The concentrations of alpha-tocopherol and gamma-tocopherol were 77.9 ± 1.9 μg/g and 586.0 ± 4.6 μg/g, respectively. Surprisingly the gamma-tocotrienol concentration found was only 6.9 ± 0.2 μg/g. Analysis of the seeds from which the oil was pressed showed the initial gamma-tocotrienol amount was even lower (1.6 ± 0.1 and 2.2 ± 0.1 μg/g in the ground and roasted seeds, respectively) than in the roasted seed oil.

1. Introduction

The Štajerska region in north-eastern Slovenia and the adjacent Styria region in southern Austria have a long tradition of growing pumpkins (*Cucurbita pepo* subsp. *pepo* var. Styriaca (in Slovenia called the '*Slovenska golica*'), *Cucurbitaceae*) as an oil crop. The significant characteristic of the Styrian oil-pumpkins is its thin seed coat without any sclerifications, thus allowing the protochlorophyll to be visible. The seed oil is used for salad dressings but also has uses in pharmacology and alternative medicine, especially when produced organically. The oil content of pumpkin seeds varies from 40% to 50% depending on the genotype. The oil pressed from roasted seeds has a unique chemical composition characterised by fatty acids (triacylglycerols), vitamins (vitamin E), minerals, phytosterols, pigments (dichroism), pyrazine derivatives (aroma) and phenolics.

Vitamin E comprises 4 tocopherols (alpha-, beta-, gamma- and delta-tocopherol) with a saturated side chain and 4 tocotrienols (alpha-, beta-, gamma- and delta-tocotrienol) with an unsaturated side chain with three double bonds (Gemrot, Barouh, Vieu, Pioch, & Montet, 2006). Recent publications have elucidated monounsaturated tocol (tocomonoenols) isomers coming from the plant world, namely: alpha-tocomonoenol in palm oil (2,5,7,8-tetramethyl-2-(4,8,12-trimethyltrideca-11-enyl)chroman-6-ol) (Mariani & Bellan, 1996; Ng, Choo, Ma, Cheng, & Hashim, 2004; Puah et al., 2007) and delta-tocomonoenol in kiwi (*Actinidia chinensis*) fruit (2,8-dimethyl-2-(4,8,12-trimethyltrideca-11-enyl)chroman-6-ol) (Fiorentino et al., 2009). Mariani and Bellan (1996) found traces of diunsaturated tocols, e.g. alpha-tocodienol: 2,5,7,8-tetramethyl-2-(4,8,12-trimethyltrideca-7,11-dienyl)chroman-6-ol in palm oil as well.

Tocopherols are molecules exerting antioxidant activity and their primary task is to prevent the damage caused by free radicals to tissues (Gemrot et al., 2006). However, gamma-tocopherol, which is the prevalent form of vitamin E in pumpkin seed oil, may be a more potent cancer chemo-preventive than alpha-tocopherol. It has been shown (Sen, Khanna, & Roy, 2006) that the biological and antioxidative properties of tocopherols can often diverge. The tocotrienols are more effective antioxidants because they are unsaturated. The novel tocomonoenols which were

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detected in human plasma and their relative availabilities have still to be investigated (Gotoh et al., 2009).

Several authors dealt with vitamin E determination in pumpkin seeds and/or seed oils; the most often used technique was normal phase HPLC (Butinar, Bučar-Miklavčič, Krumpak, & Raspor, 2009; Fruhwirth, Wenzl, El-Toukhy, Wagner, & Hermetter, 2003; Gemrot et al., 2006; Murkovic, Hillebrand, Winkler, & Pfannhauser, 1996; Murkovic & Pfannhauser, 2000; Murkovic, Piironen, Lampi, Kraushofer, & Sontag, 2004; Nakic et al., 2006; Stevenson et al., 2007; Yoshida, Tomiyama, Hirakawa, & Mizushina, 2006; Younis, Ghirmay, & Al-Shihry, 2000), followed by RP HPLC (Parry et al., 2006; Ryan, Galvin, O'Connor, Maguire, & O'Brien, 2007) and to the best of our knowledge one chronopotentiometric approach. RP HPLC and chronopotentiometric determinations are unable to distinguish between beta- and gamma-tocopherol which can be crucial if cultivar or adulteration is the issue. Murkovic et al. (1996, 2000, 2004) examined the variability of vitamin E content in pumpkin seeds and pumpkin seed oils and followed the changes in the vitamin E content during thermal treatment when roasting the seeds for production of pumpkin seed oil. They reported significant levels of tocotrienols vs. their corresponding tocopherols (alpha- and gamma-isomers). To the best of our knowledge there are no data on the GC approach for determining pumpkin seed or seed oil vitamin E. Mariani, Fedeli, and Grob (1991) published a method which allowed GC determination of sterols, alcohols and triterpenic alcohols in their original forms without a saponification step, together with squalene and tocols. They determined various tocopherols (including beta- and gamma-tocotrienol) in many different seed oils. Mariani and Bellan (1996) widened their research to cover tocomonoenols and tocodienols with the aid of a GC-MS separation of their trimethylsilyl (TMS) derivatives, especially in palm and grape seed oils, including their tocopherol esters (Mariani & Bellan, 1997). Fiorentino et al. (2009) determined delta-tocomonoenol via GC-MS in hexane/ethyl acetate extracts of the freeze-dried peel and pulp of kiwi fruits.

Regarding the current state of knowledge about the pumpkin seed oil matrix and the complexity of the production process, the aim of this study was to determine all the vitamin E isomers present in pumpkin seed oil, throughout the production chain. This was achieved by the MS aided GC approach, by transferring the GC-MS qualitative results to HPLC determination on the basis of a Crude Palm Oil reference standard and by quantifying the vitamers using HPLC, seeking for a process marker relevant for oil genuineness and oil processing history traceability.

2. Materials and methods

2.1. Chemicals

All chemicals needed to perform sample analyses were chosen and used in accordance with the laboratories' quality systems accredited in accordance with ISO/IEC 17025 (2005) standard (Laboratory for olive oil testing, Science and Research Centre of Koper, University of Primorska and Stazione Sperimentale per le Industrie degli Oli e dei Grassi). Tocopherol isomers were purchased as follows: alpha-tocopherol – Fluka (Sigma–Aldrich, St. Louis, USA), beta-tocopherol – Matreya, LLC (Pleasant Gap, USA), gamma-tocopherol and delta-tocopherol – Supelco (Sigma–Aldrich, St. Louis, USA). Tocotrienol isomers were purchased from Davos Life Science Pte Ltd, Singapore – (http://www.davoslife.com).

Crude Palm Oil (CPO) used as a reference material was a gift from 'Stazione sperimentale per le industrie degli oli e dei grassi', Milano, Italy.

2.2. Samples

2.2.1. Pumpkin seeds from different processing stages (grinding, water and sodium chloride addition, roasting) and roasted pumpkin seed oil

- Ground pumpkin seeds (sample A).
- Pumpkin seeds (*C. pepo* subsp. *pepo* var. *Styriaca* –'*S. golica*' were cultivated near Bogojina, Slovenia (detailed info known to authors) in the summer/autumn of 2008. They were ground at a roasting and pressing plant.
- Ground pumpkin seeds with added water and sodium chloride (sample B).
- Ground pumpkin seeds with added water and sodium chloride and roasted (sample C).
- Oil pressed from roasted ground pumpkin seeds (sample D).

2.3. Roasting and pressing plant process

50 kg of dried seeds were processed on October 30th, 2008 at the roasting and pressing plant located in north east of Slovenija (exact location known to authors). The seeds were ground and water and NaCl were added and mixed thoroughly to a final NaCl concentration of 1 wt.% in the whole mass. Malaxation time was 10 min and after that the mixture was roasted at 110–120 °C for 20–25 min – the time needed for complete evaporation of the water. The heating process was based on a wood burning stove.

2.4. Vitamin E extraction from pumpkin seeds (samples A–C)

The procedure used was the one described in Murkovic et al. (2004). In short: to 1 g of ground pumpkin seeds 5 g of anhydrous sodium sulphate were added to remove the water and subsequently vitamin E was extracted with *n*-hexane in an ultrasonic bath for 30 min. To prevent oxidation, 8 mg of butylated hydroxytoluene were added. At the end of extraction the extract was filtered and its volume was reduced on a rotary evaporator and brought to 10 mL with *n*-hexane.

2.5. GC vitamin E analysis

The sample of roasted '*S. golica*' pumpkin seed oil (sample D) was prepared for analysis according to procedures previously described (Mariani & Bellan, 1996; Mariani & Fedeli, 1986; Mariani et al., 1991) for the determination of free and esterified minor compounds (FEMC) in plant oils. In short: to 200 mg of fatty substance 4 different internal standards in solution were added (IS1: 1-eicosanol (C21–OH) for determination and quantification of aliphatic alcohols; IS2: alpha-cholestanol for determination and quantification of sterols and tocols; IS3: heptadecanol-stearate for determination of waxes and IS4: cholesteryl-heptadecanoate for the determination of sterol esters; the solvent was evaporated and the sample was silylated. The non-polar fraction was separated with the aid of 1% ethyl ether in hexane. The elute was evaporated on a rotary evaporator, re-dissolved in 5 mL heptane and injected into the GC-MS system. The GC-MS system consisted of a Trace (Thermo) GC instrument and a Thermo Voyager mass spectrometer; the chromatographic column was DB5 with a film thickness of 0.1 μm, 20 m long and with a diameter of 0.32 mm. The oven was set to 80 °C for 1 min and then heated to 200 °C at a rate of 20 °C/min and then to 340 °C at a rate of 5 °C/min. The interface temperature was set to 260 °C and the ion source to 220 °C operating at 70 eV. The carrier gas was He at 0.709 bars.

2.6. HPLC vitamin E analysis

The ISO 9936 (2006) standard method was used. This method for determination of tocopherols is accredited in accordance with ISO standard (ISO/IEC 17025, 2005). The content of vitamin E in pumpkin oil and in pumpkin seed extracts was determined by high-performance liquid chromatography (HPLC) on an Agilent 1100 Series HPLC system equipped with a BinPump G1312A binary pump, a thermostatted ALS G1329A auto sampler, an ALSTherm G1330B auto sampler thermostat, a COLCOM G1316A thermostatted column compartment, an FLD G1321A fluorescence detector operating at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. The analytical column used was a Phenomenex (Torrance, USA) Luna (250 × 4.60 mm), packed with 5 μm silica, S/N 00G-4274-E0. The mobile phase used was heptane-THF (1000 + 40 v/v) at a constant flow rate of 1.0 mL/min. The injection volume was in the range from 5–100 μL in order for all the vitamin E isomers to elute in the calibration range. The retention times of alpha-tocopherol and gamma-tocopherol were in the range between 11.5 and 12.0 and 19.0 and 20.0 min, respectively. The elution order was as follows: alpha-tocopherol, alpha-tocotrienol, beta-tocopherol, gamma-tocopherol, beta-tocotrienol, gamma-tocotrienol, delta-tocopherol and delta-tocotrienol. All 8 tocopherol and tocotrienol isomers were resolved to baseline with the exception of the gamma-tocopherol – beta-tocotrienol pair which had a calculated resolution of 0.90. For each of the four tocopherol isomers a five level calibration graph was constructed covering the concentration range from 2 to 1000 μg for each tocopherol isomer per g of oil and from 0.2 to 200 μg per g of seeds, with a linearity correlation coefficient r^2 greater than 0.9998 for all tocopherol isomers. The concentrations of tocotrienol isomers, gamma-tocomonoenol and alpha-tocomonoenol were calculated on the basis of the response factors of their corresponding tocopherol isomers. The exact concentration of each tocopherol stock standard which served as a basis for five working calibration standards was calculated from the measured absorbance of the standard and the known absorbance coefficients of the isomers as fully described in the ISO method (ISO 9936, 2006). The calibration graphs were used to calculate the tocopherol concentrations. For each tocopherol and tocotrienol isomer the LOQ was determined, as well as the expanded measurement uncertainty, U, with a coverage factor of 2 based on the calibration data and the laboratory's international proficiency testing schemes results.

The precision of the data were calculated from the laboratory control chart data for alpha-tocopherol gathered over a 21 month time period with total of 41 single determinations clustered in 12 different time and operator-independent sections, which served for calculation of the repeatability limit (r) and reproducibility limit (R) according to ISO 5725-2 (1994) standard, Annex B. The calculated values for r and R were 13.0 and 23.7 μg/g, respectively and were within the limits specified by the standard method (ISO 9936, 2006).

The accuracy of the method was evaluated on the basis of the laboratory's participation (several samples per year) in international proficiency testing schemes (http://www.internationaloliveoil.org; http://www.bipea.org) on various seed oil samples with the overall (sum) and individual tocopherol data with z values ranging from −0.68 to 0.33.

2.7. Chemical parameters of processed oil

- Acidity in the processed oil (ISO 660, 2009): 0.61 wt.%.
- Peroxide value (ISO 3960, 2007): <LOQ (0.04 mmol/kg).
- PAH's, FAME, trans-FAME, free and esterified biophenols and sterols were also determined (data not shown).

2.8. Statistical analysis

The software for the calculations used was that included in MS Office Excel 2003 package and the Statistics Calculator StatPac version 3.0 (StatPac Inc., Bloomington, USA). The analyses were carried out in duplicate except where stated differently.

3. Results and discussion

3.1. GC of FEMC of the oil pressed from roasted ground pumpkin seeds (sample D)

On Fig. 1 two distinct groups of peaks can be seen – the first one lying between the squalene peak and Internal Standard 3 peak (comprising tocols and free sterols), and the second one after Internal Standard 4 and comprising sterol esters. Of main interest are the peaks of the first group eluting after squalene. The Total Ion Current (TIC) signal reveals (in ascending Rt order) delta-tocopherol, gamma-tocopherol, an unknown peak eluting after gamma-tocopherol (U1), another unknown and small peak eluting after U1 (U2), Internal Standard 2, alpha-tocopherol and a third unknown peak (U3).

The U2 and U3 peaks are minor if compared to the U1 peak. The TIC GC-MS section from 24 to 28 min from Fig. 1 is shown in Fig. 2. The MS spectrum of the peak at 25.855 min is shown in the lower left part of Fig. 2 and confirms that the compound is gamma-tocopherol with an M^+ of 488 as described in detail by Mariani and Bellan (1996). The lower right part of Fig. 2 shows the MS spectrum of the first unknown peak U1 at 26.374 min. Comparison of these two spectra shows they differ only in the molecular ion – the unknown peak (U1) being reduced by 2 mass units (486). This means the peak U1 is gamma-tocomonoenol, i.e. gamma-tocopherol with one double bond. Gamma-tocomonoenol was found in traces in sesame and corn oil as well (Mariani and Bellan, 1996). The second unknown peak (U2) from the TIC section of Fig. 1 is a small peak just prior to Internal Standard peak 2 with an Rt of 27.131 min. Its MS spectrum when compared to the spectra of gamma-tocopherol and gamma-tocomonoenol reveals that the only difference is its M + value which is reduced by 2 MU compared to gamma-tocomonoenol (data not shown). These data confirm that the second unknown peak (U2) is gamma-tocodienol, i.e. gamma-tocopherol with 2 double bonds.

The third unknown peak (U3) from the TIC section of Fig. 1 elutes after alpha-tocopherol and has its Rt at 28.185 min. Its MS spectrum is similar to that of alpha-tocopherol (data not shown). Its molecular ion is 500 instead of 502 as is the case with alpha-tocopherol. This evidence leads us to conclude that the third unknown peak (U3) should have one unsaturated bond in the side chain of alpha-tocopherol. We can conclude the structure should be assigned to that of alpha-tocomonoenol.

Ng et al. (2004) and Puah et al. (2007) working on Crude Palm Oil (CPO) tocols using RP C30 HPLC with GC-MS and NMR techniques showed that the structure of alpha-tocomonoenol has a double bond at the C11-C12 position. Fiorentino et al. (2009) assigned the same C11-C12 double bond position to delta-tocomonoenol from kiwi fruit when investigating the delta-tocomonoenol structure with the aid of GC-MS and NMR showing that the allylic fragment with m/z of 69 was the 3-methylbut-2-en-1-ylium cation. Considering all these facts and comparing our recorded MS spectra of alpha-tocomonoenol, gamma-tocomonoenol and gamma-tocodienol, especially the region from 50 to 150 MU with a fragment of m/z 69, we reasoned that the first double bond position in gamma-tocomonoenol, gamma-tocodienol and alpha-tocomonoenol is at C11-C12 as well.

If we look at the two main tocopherols in the GC-MS of the FEMC of roasted pumpkin seed oil on Fig. 1 (sample D), i.e.

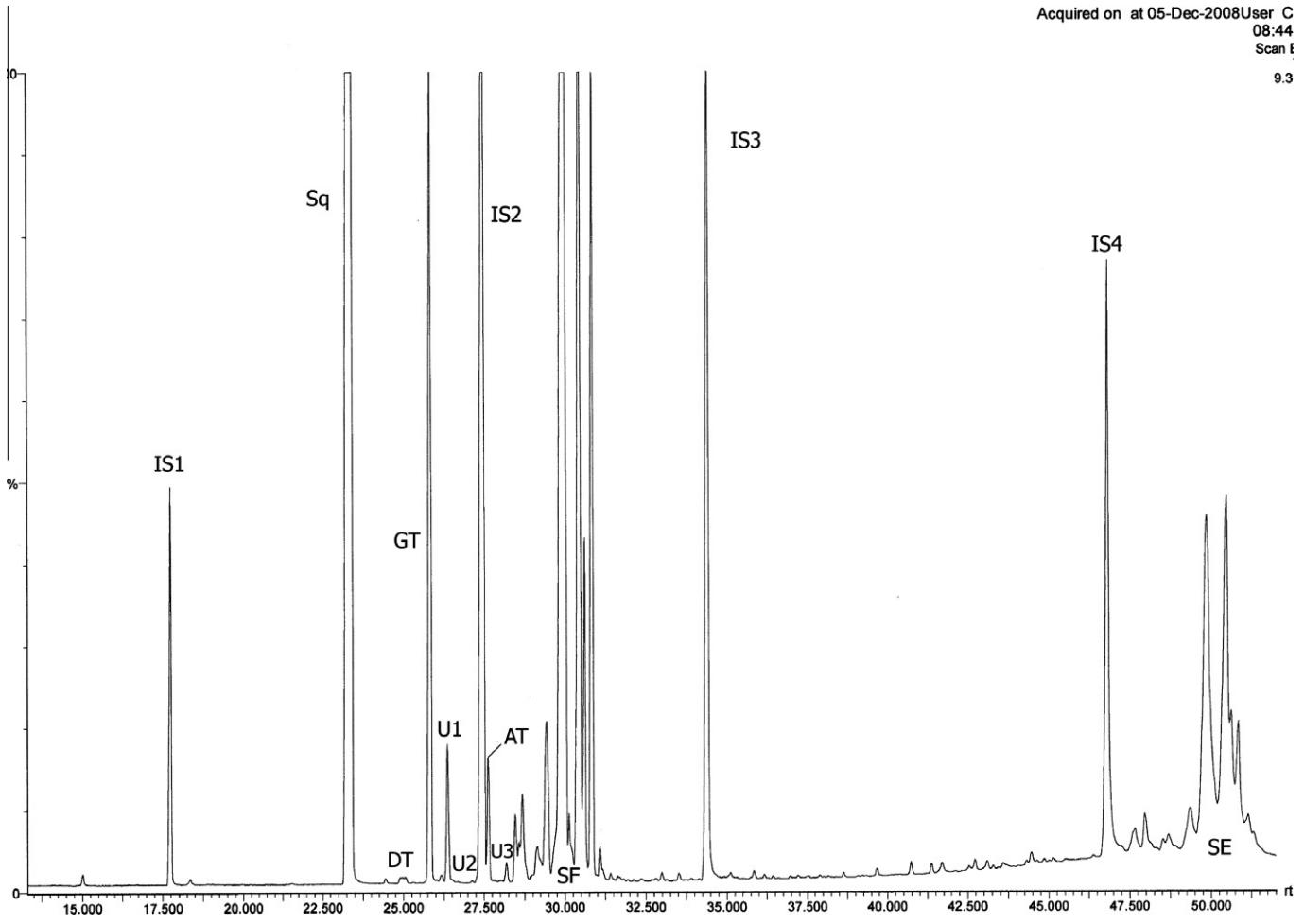


Fig. 1. Total Ion Current (TIC) chromatogram of free and esterified minor compounds (FEMC) of the GC-MS of the oil pressed from roasted ground pumpkin seeds (sample D). The peaks are assigned as follows: IS1 – internal standard 1, Sq – squalene, DT – delta-tocopherol, GT – gamma-tocopherol, U1 – unknown peak 1, U2 – unknown peak 2, IS2 – internal standard 2, AT – alpha-tocopherol, U3 – unknown peak 3, SF – sterols fraction, IS3 – internal standard 3, IS4 – internal standard 4, SE – sterol esters. X-axis: time in minutes; Y-axis: TIC signal.

gamma-tocopherol and alpha-tocopherol and at their monounsaturated forms gamma-tocomonoenol and alpha-tocomonoenol, we can confirm what has been stated in Mariani and Bellan (1996) that all the major tocopherols in seed oils also have their "dehydro" forms present.

3.2. HPLC of the oil pressed from roasted ground pumpkin seeds (sample D) and pumpkin seeds from different process stages (samples A–C)

3.2.1. Oil pressed from roasted ground pumpkin seeds (sample D)

Comparison of the HPLC chromatogram of tocopherol and tocotrienol standards with the HPLC chromatogram of vitamin E isomers of sample D shown in Fig. 3 reveals that the roasted '*S. golica*' pumpkin seed oil has a substantial amount of gamma-tocopherol, followed by alpha-tocopherol. The amounts of delta-tocopherol, beta-tocopherol and gamma-tocotrienol are minor. In addition, two extra peaks which were not so far identified as tocopherols or tocotrienols, can be observed; the first elutes soon after the alpha-tocopherol peak (U3) and the second one soon after the gamma-tocopherol peak (U1).

With the aid of the reference Crude Palm Oil sample (used as a reference sample for vitamin E tocopherols, tocomonoenols and tocotrienols) the peaks U3 and U1 were assigned as alpha-tocomonoenol (AT1) and gamma-tocomonoenol (GT1), respectively.

Fig 4 confirms the presence of a minor quantity of gamma-tocotrienol in sample D. One can see another peak, assigned U4, which is probably a degradation product of one of the isomers.

The D^{HPLC} data column in Table 1 shows all the tocols, tocomonoenols and tocotrienols in the roasted '*S. golica*' pumpkin seed oil determined via HPLC analysis. The concentrations of gamma-tocopherol and alpha-tocopherol are 586.0 ± 4.6 and 77.9 ± 1.9 μg/g, respectively. Fruhwirth et al. (2003), Murkovic et al. (2000) and Nakic et al. (2006) who analysed the same hull-less cultivar pumpkin oils, reported alpha-tocopherol concentrations in the range from 7 to 201 μg/g and gamma-tocopherol in the range from 441 to 860 μg/g. The beta-tocopherol and delta-tocopherol concentrations found in sample D are 5.4 ± 0.0 and 14.1 ± 0.3 μg/g. As mentioned in the introduction there are few reported determinations of these low abundance tocols. Some authors did not detect any of them (Fruhwirth et al., 2003), others reported 4 μg/g of delta-tocopherol in industrially pressed oil from hull-less seeds, but did not mention beta-tocopherol (Nakic et al., 2006). Examination of the adulteration status of some Slovenian pumpkin seed oils revealed beta-tocopherol levels between the LOQ (2 μg/g) and 5 μg/g, and delta-tocopherol levels between 4 and 9 μg/g in 3 genuine samples (Butinar et al., 2009).

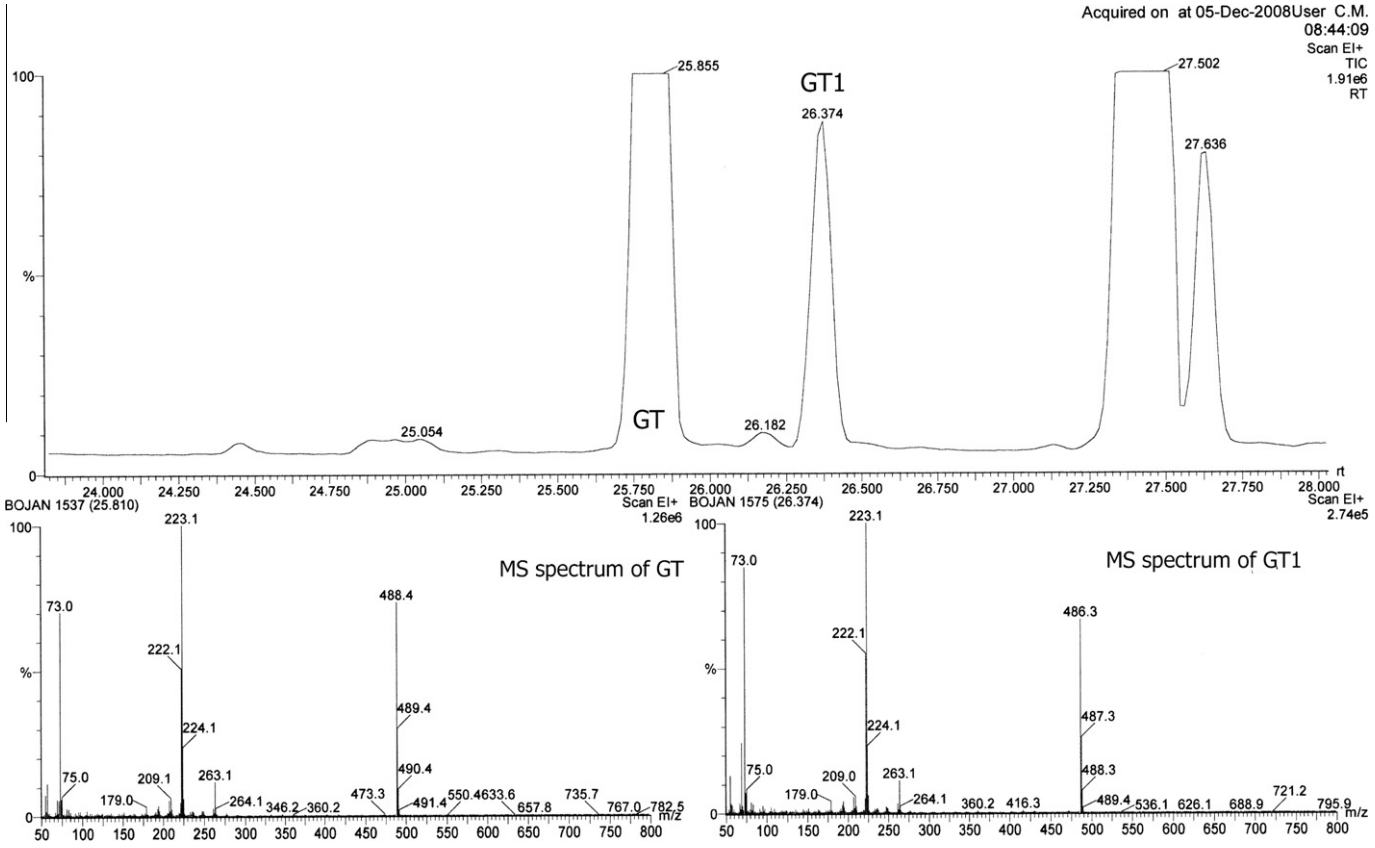


Fig. 2. TIC of the GC-MS of free and esterified minor compounds (FEMC) of the oil pressed from roasted ground pumpkin seeds (sample D, top) showing the gamma-tocopherol and gamma-tocomonoenol peaks with their respective MS spectra: gamma-tocopherol (bottom left) and gamma-tocomonoenol (bottom right). Chromatographic conditions were described in the Section 2. The peaks are assigned as follows: GT – gamma-tocopherol, GT1 – gamma-tocomonoenol (U1).

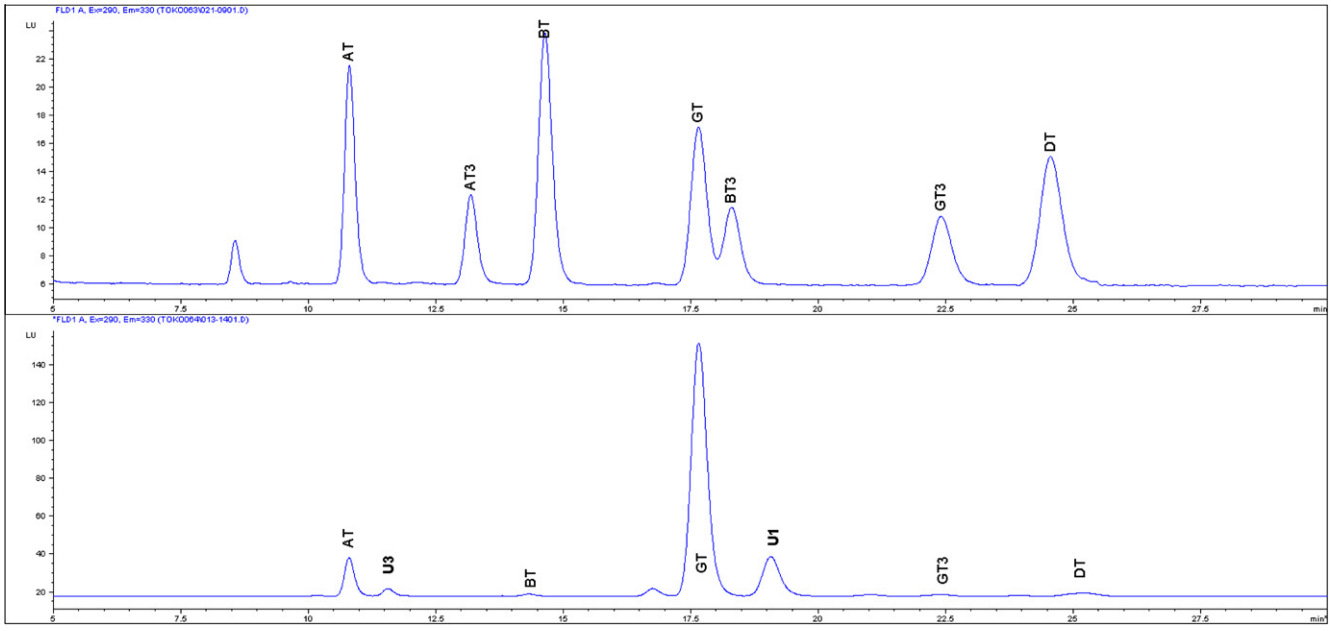


Fig. 3. HPLC chromatogram of tocopherol and tocotrienol standards (top), and HPLC chromatogram of vitamin E isomers in oil pressed from roasted ground pumpkin seeds (sample D, bottom). The peaks are assigned as follows: AT – alpha-tocopherol, AT3 – alpha-tocotrienol, BT – beta-tocopherol, GT – gamma-tocopherol, BT3 – beta-tocotrienol, GT3 – gamma-tocotrienol, DT – delta-tocopherol. Delta-tocotrienol elutes at 35 min (not shown). Chromatographic conditions were described in the Section 2. X-axis: time in mins. Y-axis: FLD signal. U3 – the unknown peak eluting after alpha-tocopherol, U1 – the unknown peak eluting after gamma-tocopherol.

To the best of our knowledge there are no data available on alpha-tocomonoenol and gamma-tocomonoenol in pumpkin seeds or (roasted) pumpkin seed oils, though according to Gemrot et al. (2006) and Murkovic et al. (1996, 2000, 2004), who used a normal phase separation technique with a dioxane/hexane mobile phase, one should expect such data. Indeed, according to contemporary

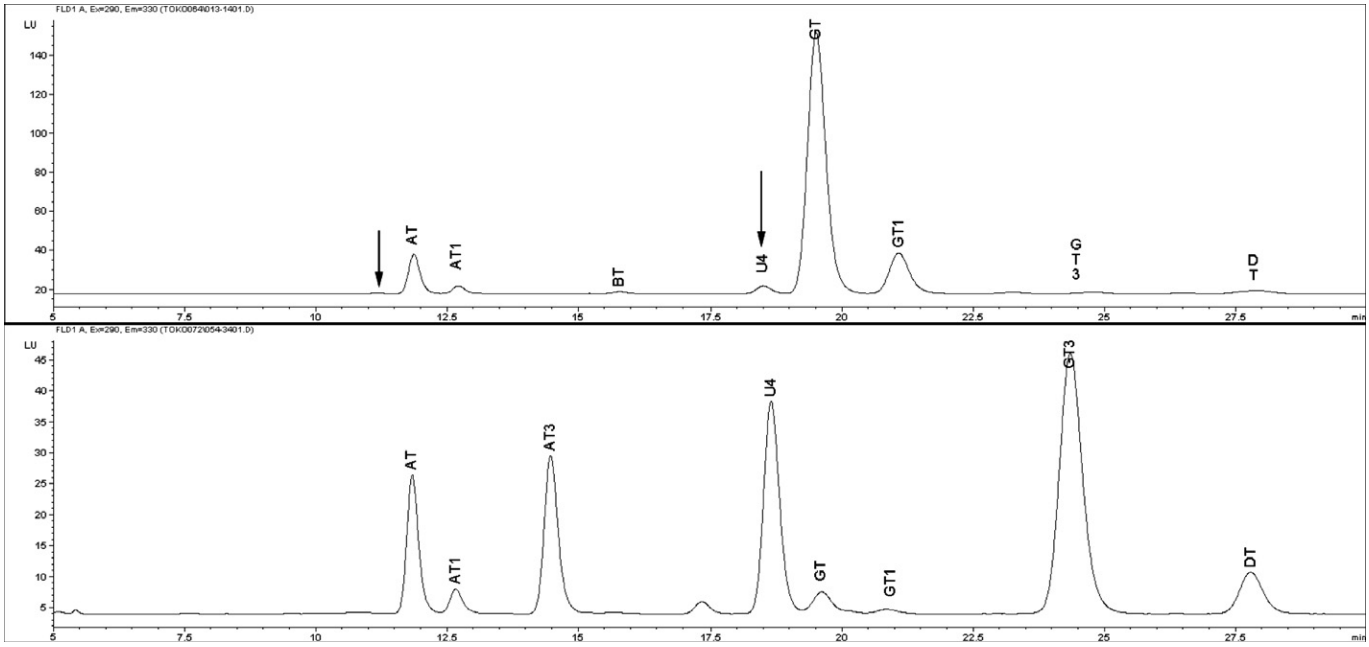


Fig. 4. HPLC chromatogram of vitamin E isomers in oil pressed from roasted ground pumpkin seeds in sample D (top) and HPLC chromatogram of vitamin E isomers in Crude Palm Oil (bottom). Chromatographic conditions were as in Fig. 3. AT1 – alpha-tocomonoenol, GT1 – gamma-tocomonoenol, U4 – the unknown peak eluting before GT. Delta-tocotrienol elutes at 35 min (not shown). The arrows denote the peaks which were increased or generated during roasting process (see text for details).

Table 1
Concentrations of vitamin E isomers in samples A–D in μg/g determined via HPLC. Concentrations and absolute errors (AE) or standard deviations (SD) were based on two determinations (samples A–C for AE) or on three determinations (sample D, SD) in μg/g. <LOQ – less than Limit of Quantification (2 μg/g). Results are significantly influenced by the process ($p \leqslant 0.05$).

	A^{HPLC} (μg/g)	AE (μg/g)	B^{HPLC} (μg/g)	AE (μg/g)	C^{HPLC} (μg/g)	AE (μg/g)	D^{HPLC} (μg/g)	SD (μg/g)
Alpha-tocopherol	18.3	0.2	16.1	0.0	20.0	0.2	77.9	1.9
Alpha-tocomonoenol[a]	5.1	0.2	4.4	0.1	5.5	0.2	17.6	0.6
Beta-tocopherol	<LOQ		<LOQ		<LOQ		5.4	0.0
Delta-tocopherol	2.2	0.1	2.4	0.1	3.6	0.1	14.1	0.3
Gamma-tocopherol	156.8	0.3	163.6	0.2	191.7	0.4	586.0	4.6
Gamma-tocomonoenol	32.[b]	0.2	33.9[b]	0.1	39.5[b]	0.2	118.7[b]	1.0
Gamma-tocotrienol[c]	1.6	0.1	1.9	0.1	2.2	0.1	6.9	0.2

[a] Expressed as alpha-tocopherol.
[b,c] Expressed as gamma-tocopherol.

analytical experience such a system should separate all the major tocopherols, tocotrienols and tocomonoenols in pumpkin seeds and their oils, e.g. alpha-tocopherol, alpha-tocomonoenol, gamma-tocopherol, gamma-tocomonoenol and gamma-tocotrienol. The system used in Nakic et al. (2006) with 0.7% of propan-2-ol which on a 15 cm NP column could very probably not resolve gamma-tocopherol from gamma-tocomonoenol. Based on analytical experience in HPLC with tocopherols, the HPLC chromatograms of pumpkin seed extracts published in Murkovic et al. (1996) may in fact show gamma-tocomonoenol under peak No. 5 (their Fig. 1). Probably the same situation is evident in Gemrot et al. (2006). Fig. 2 of their publication shows an unassigned peak which could be attributed to gamma-tocomonoenol, if we consider the findings of this present work.

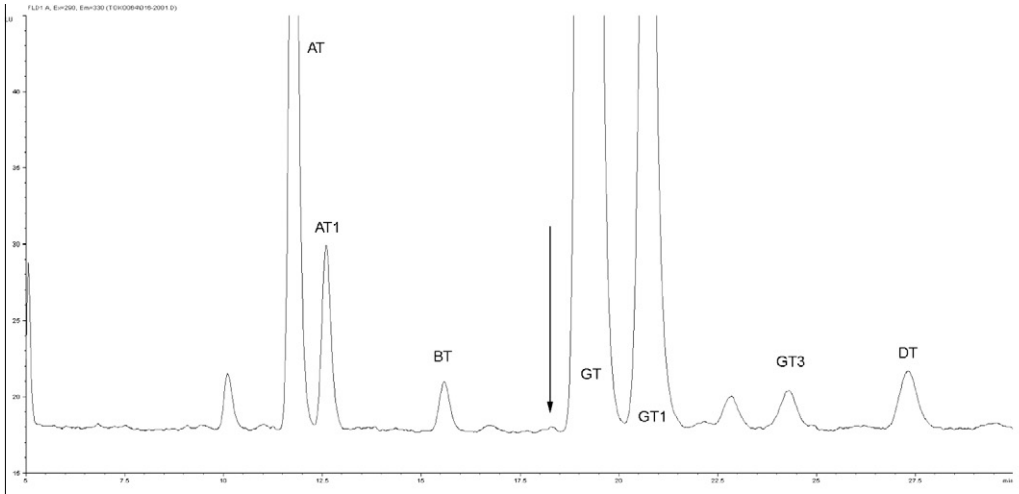
The concentration of gamma-tocotrienol determined in sample D is just 6.9 ± 0.2 μg/g, which is much less than reported by Murkovic et al. (2004) who state the concentration of tocotrienols is about one third of the corresponding tocopherols.

3.3. Pumpkin seeds from different process stages (samples A–C)

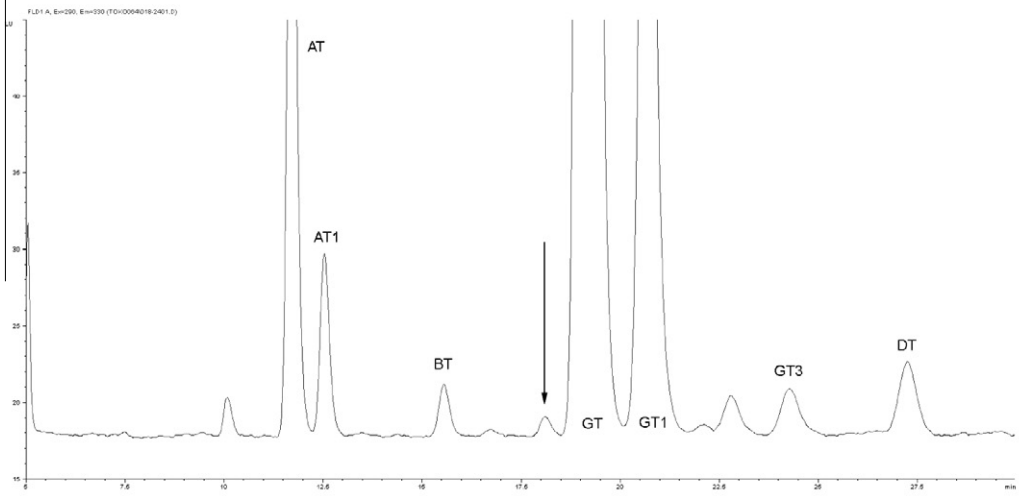
In order to explain the reason for the minor quantities of determined gamma-tocotrienol found in sample D compared to previously reported data, a vitamin E survey was undertaken. Attention was given to pumpkin seeds samples before they were processed as well (sodium chloride and water addition, roasting, pressing). The goal was to monitor the possible gamma-tocotrienol decomposition or chemical changes occurring during processing in order to clarify the difference in the concentrations of gamma-tocotrienol. In fact, all the previously mentioned authors who reported elevated tocotrienols quantities also surveyed the vitamin E isomers in unprocessed seeds. Surprisingly, Murkovic et al. (2004) found the concentrations of sterols and vitamin E increased during the roasting process. Yoshida et al. (2006) showed that microwave-assisted pumpkin seed roasting did not significantly influence the content of tocopherols. In contrast, Gemrot et al. (2006) showed a substantial loss of tocopherols (25–41%, depending on their antioxidant capacity) after 5 min roasting at 140 °C.

The vitamin E content of the pumpkin seed samples used for monitoring actually reflected the production process and all eventual oxidative changes (due to ground seeds contacting air during malaxation and the influence of temperature during roasting).

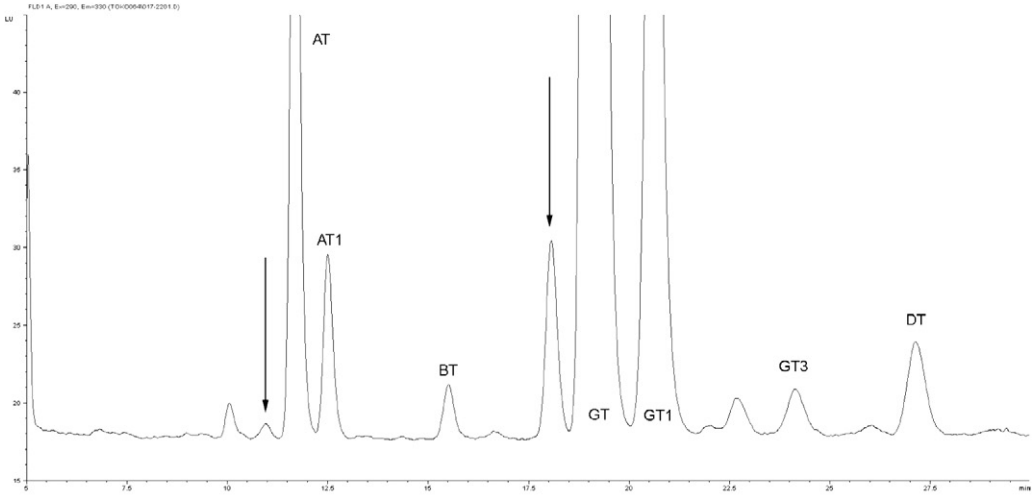
These data can be found in Table 1. A closer look at the results reveals significant changes in all vitamin E components from sample A to the sample C. The concentrations are reported on an 'as is' basis and increase from the initial state in the ground form (sample



Sample A – ground ‘Slovenska golica’ pumpkin seeds



Sample B – ‘Slovenska golica’ pumpkin seeds with water/NaCl



Sample C – roasted ‘Slovenska golica’ pumpkin seeds

Fig. 5. HPLC chromatogram of vitamin E isomers in pumpkin seeds from different process stages (samples A–C). Chromatographic conditions were as in Fig. 3. AT – alpha-tocopherol, AT1 – alpha-tocomonoenol, BT – beta-tocopherol, GT – gamma-tocopherol, GT1 – gamma-tocomonoenol, GT3 – gamma-tocotrienol, DT – delta-tocopherol. The unassigned peak between GT1 and GT3 should probably be attributed to GT2. The arrows denote the peaks which were increased or generated during the roasting process (see text for details). X-axis: time in mins, Y-axis: FLD signal.

A) to the dry, roasted and pre-pressing state (sample C). The increase is substantial and ranges from 7.8% for alpha-tocomonoenol to 63.6% for delta-tocopherol. The increase in gamma-tocopherol amounts to 22.3%.

A detailed look at the data confirms that the gamma-tocotrienol concentration is very low compared to the data published by Murkovic et al. (2004), and surprisingly, it increases during the process. The data presented in Table 1 show that all vitamin E isomers determined are present from the very beginning of the process (sample A) to the end of roasting (sample C) and are present in the final product, the oil (sample D) as well, which is a clear finding of this work.

A detailed look at the time ranges from 10–12 min and from 17–19 min in the HPLC chromatograms of samples A–C as shown on Fig. 5 and of sample D as shown on Fig. 4 reveals two additional peaks. The first (U4) precedes the gamma-tocopherol peak and the second one (not labelled) precedes the alpha-tocopherol peak in samples C (Fig. 5) and D (Fig. 4). They are denoted with arrows. Their concentration increases with the process from sample A to the sample C (D). These two compounds could serve as roasting process markers. Their presence could discriminate between oils pressed from roasted seeds from the cold pressed oils.

4. Conclusion

GC-MS determination of free and esterified minor compounds of roasted pumpkin seed oil from the Slovenian pumpkin variety '*S. golica*' (sample D) revealed the presence of two previously unreported compounds in this type of roasted pumpkin seed oil, namely alpha-tocomonoenol and gamma-tocomonoenol. They were chemically assigned from the MS spectra.

Using the GC-MS data, the reference sample of Crude Palm Oil and tocopherol and tocotrienol standards, it was possible to identify alpha-tocomonoenol and gamma-tocomonoenol in roasted '*S. golica*' seed oil. They were quantified using HPLC and the amounts found were 17.6 ± 0.6 μg/g and 118.7 ± 1.0 μg/g, respectively. The alpha-tocopherol and gamma-tocopherol concentrations were 77.9 ± 1.9 μg/g and 586.0 ± 4.6 μg/g, respectively. The concentrations of the other two tocopherol isomers beta- and delta-tocopherol were in agreement with previously reported data cited in the introduction section of this publication (5.4 ± 0.0 μg/g and 14.1 ± 0.3 μg/g).

The concentration of gamma-tocotrienol determined was as low as 6.9 ± 0.2 μg/g, which is much less than the data of other researchers. GC-MS determination confirmed these data as well.

This disagreement was clarified by analysing the vitamin E concentrations in unroasted and roasted pumpkin seeds of the '*S. golica*' cultivar. The results showed that the initial gamma-tocotrienol concentration was low and did not diminish during the roasting process; on contrary, its concentration rose from 1.6 to 2.2 μg/g. Such an increase in concentration was found in other vitamin E compounds as well.

HPLC monitoring of the various vitamin E compounds during the production steps from unroasted pumpkin seeds to oil showed an increase of two unidentified compounds: one eluting prior to the alpha-tocopherol peak and the other just prior to the gamma-tocopherol peak. These compounds could serve as excellent chemical markers and suitable tools for traceability purposes to indicate the roasting or non-roasting processing history. Finally, they could also be used for assessment of the genuineness of pumpkin seed oil.

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