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DOKTORSKA DISERTACIJA

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THE ESTABLISHMENT OF AN ANALYTICAL PROCEDURE FOR ASSESSMENT OF THE GENUINENESS AND THE DEGREE OF PROCESSING OF PUMPKIN SEED OIL

DOCTORAL DISSERTATION

Ljubljana, 2012

POPRAVKI

Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa 29. seje Komisije za doktorski študij UL z dne 13.6.2012 je bilo potrjeno, da kandidat izpolnjuje pogoje za opravljanje doktorata znanosti na Interdisciplinarnem doktorskem študijskem programu **Bioznanosti**, znanstveno področje živilstvo. Za mentorja je bil imenovan **prof. dr. Peter Raspor** in za somentorja **prof. dr. Rajko Vidrih**.

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AI V prvi raziskavi smo uvajali nov vpogled v matrico bučnega olja na osnovi faz »drevesa odločanja« in pri sedmih bučnih oljih ugotavljali vsebnost *trans*-izomerov maščobnih kislin, maščobnokislinsko, sterolno ter tokoferolno sestavo. Na osnovi vrednosti *trans*-oleinske kisline in vsote *trans*-linolne in *trans*-linolenske kisline smo pokazali na potvorbo pri nekaterih vzorcih, ki se je potrdila s kislinami C 18:3, C 20:1 in C 22:0, razmerjem Δ-5/Δ-7 sterolov in analizo tokoferolov na osnovi deležev beta- in delta-tokoferola. Združeni rezultati so potrdili skladnost ugotovitev parcialnega preverjanja in pravilnost predlaganega drevesa odločanja. V nadaljevanju smo v istih vzorcih z uporabo HPLC ločili 29 TAG. Izbrali smo devet signifikantnih: LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL in SLS. Analiza PCA na osnovi diagrama teže glavnih komponent in razsevnega diagrama je potrdila rezultate prejšnjih parcialnih ugotovitev potvorb pri istih vzorcih in pokazala, da vzorci tvorijo pet različnih skupin. V zadnji raziskavi smo v olju iz praženih semen 'Slovenske golice' poročali o dveh novo odkritih izomerih vitamina E – alfa-tokomonoenol (17,6 ± 0,6 µg/g) in gama-tokomonoenol (118,7 ± 1,0 µg/g). Kvantificirali smo alfa-tokoferol (77,9 ± 1,9 µg/g), gama-tokoferol (586,0 ± 4,6 µg/g), beta-tokoferol (5,4 ± 0,0 µg/g) in delta-tokoferol (14,1 ± 0,3) µg/g. Koncentracija gama-tokotrienola je bila le 6,9 ± 0,2 µg/g. Koncentracija vitamina E je naraščala v seriji od nepraženih do praženih semen. Vsebnost gama-tokotrienola je bila nizka že na začetka procesa (1,6 ± 0,1 µg/g). V frakciji vitamina E sta dva kromatografska vrhova, ki sta najbolj izražena v praženih semenih (olju 10 µg/g), najmanj pa v nepraženih semenih. Ta vrhova, stereospecifični TAG in razmerje prostih in esterificiranih biofenolov so označevalci procesa praženja. Na osnovi eksperimentalno dobljenih podatkov smo predlagali tri sheme dreves odločanja. Z njimi na osnovi parcialnih določitev posameznega preskusa preverimo pristnost vzorca bučnega olja, njegovo kakovost in pa stopnjo predelave.

KEY WORDS DOCUMENTATION

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AA RASPOR, Peter (supervisor)/VIDRIH, Rajko (co-advisor)
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AB The research introduced a »Decision tree« approach in the matrix of pumpkin seed oil analyzing *trans*-fatty acids isomers, fatty acids, sterols and tocopherols composition in seven pumpkin seed oils. On the basis of *trans*-fatty acids certain samples were assigned as adulterated, this was confirmed with the aid of acids C 18:3, C 20:1 and C 22:0 content, the calculated Δ-5/Δ-7 sterols ratio and tocopherols analysis. The main principle of the platform for genuineness assessment lies in starting with rapid and less demanding analytical approaches and continuing with more complex ones. We successfully HPLC-determined 29 stereospecific TAGs in the samples. Nine significant were chosen: LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL and SLS. PCA plot of component weights revealed good distribution of the significant TAGs. PCA scatter plot confirmed the righteousness of the proposed analytical platform confirming the adulteration status of previously judged samples. The samples were furthermore clustered in five different groups. Two new isomers of vitamin E in roasted 'Slovenska golica' seed oil were reported: alpha-tocomonoenol (17.6 ± 0.6 µg/g) and gamma-tocomonoenol (118.7 ± 1.0 µg/g). Concentrations of alpha-tocopherol (77.9 ± 1.9 µg/g), gamma-tocopherol (586.0 ± 4.6 µg/g), beta-tocopherol (5.4 ± 0.0 µg/g) and delta-tocopherol (14.1 ± 0.3) µg/g) were determined. The gamma-tocotrienol concentration found was 6.9 ± 0.2 µg/g. It was low in the unroasted seeds as well (1.6 ± 0.1 µg/g). It was further shown that roasting process concentrates the vitamin E isomers. Detailed look at the vitamin E fraction revealed two additional chromatographic peaks. Their concentration increased with process and was found to be 10 µg/g in the final product. These compounds could serve as chemical markers for the establishment of the degree of processing of pumpkin seed oil. Three different “decision tree” schemes for establishing the genuineness, the quality and the degree of processing of the pumpkin seed oils are proposed.

KAZALO VSEBINE

KAZALO ZNANSTVENIH DEL

Butinar B., Bučar-Miklavčič M., Krumpak, A., Raspor P. 2009. Experiences in olive oil purity and quality assessment as a tool for pumpkin seed oil evaluation. What can consumers benefit? Acta Alimentaria, 38,2: 219-227

Butinar B., Bučar-Miklavčič M.,Valenčič V., Raspor P. 2010. Stereospecific analysis of triacylglycerols as a useful means to evaluate genuineness of pumpkin seed oils: lesson from virgin olive oil analyses. Journal of Agricultural and Food Chemistry, 58,9: 5227-5234

Butinar B., Bučar-Miklavčič M., Mariani C., Raspor P. 2011. New vitamin E isomers (gamma-tocomonoenol and alpha-tocomonoenol) in seeds, roasted seeds and roasted seed oil from the Slovenian pumpkin variety 'Slovenska golica'. Food Chemistry, 128, 2: 505-512

KAZALO PREGLEDNIC

KAZALO SLIK

KRATICE IN OKRAJŠAVE

AT	alfa-tokoferol: 2,5,7,8-tetrametil-2-(4,8,12-trimetiltridecil)kroman-6-ol
AT1	alfa-tokomonoenol: 2,5,7,8-tetrametil-2-(4,8,12-trimetiltridec-11-enil)kroman-6-ol
C 18:3 n-3	(9*Z*,12*Z*,15*Z*)-oktadeka-9,12,15-trienojska kislina (alfa-linolenska kislina)
C 20:0	eikozanojska kislina
C 20:1 n-9	(*Z*)-eikoz-11-enojska kislina
C 22:0	dokozanojska kislina
C 22:1 n-9	*(Z)*-dokoza-13-enojska kislina
EDOOSIZOP	Ekstra Deviško Oljčno Olje Slovenske Istre z Zaščiteno Označbo Porekla
FEMC	angl. Free and Esterified Minor Compounds (Proste in esterificirane minorne spojine)
GT	gama-tokoferol: 2,7,8-trimetil-2-(4,8,12-trimetiltridecil)kroman-6-ol
GT1	gama-tokomonoenol: 2,7,8-trimetil-2-(4,8,12-trimetiltridec-11-enil)kroman-6-ol
GT2	gama-tokodienol: 2,7,8-trimetil-2-(4,8,12-trimetiltrideka-7,11-dienil)kroman-6-ol
GT3	gama-tokotrienol: 2,7,8-trimetil-2-(4,8,12-trimetiltrideka-3,7,11-trienil)kroman-6-ol
LOQ	angl. Limit of Quantification (Meja določanja)
PCA	angl. Principal Component Analysis (Analiza glavnih komponent)
TAG	Triacilglicerol. Imena posameznih TAG so akronimi začetnih črk karboksilnih kislin, vezanih na glicerol, in sicer: P – palmitoil, S – stearoil, O – oleoil, L – linoleoil in Ln – linoleolenil. TAG so razvrščeni po naraščajočem redu eluiranja (Andrikopoulos in sod., 2004; Ollivier in sod., 2003)

LnLnLn	trilinoleolenil-glicerol
LLnLn	linoleoil-dilinoleolenil-glicerol
LLLn	dilinoleoil-linoleolenil-glicerol
OLnLn	oleoil-dilinoleolenil-glicerol
LLL	trilinoleoil-glicerol
OLLn	oleoil-linoleoil-linoleolenil-glicerol
PLLn	palmitoil-linoleoil-linoleolenil-glicerol
OLL	oleoil-dilinoleoil-glicerol
OOLn	dioleoil-linoleolenil-glicerol
PLL	palmitoil-dilinoleoil-glicerol
LnLS	linoleolenil-linoleoil-stearoil-glicerol
POLn	palmitoil-oleoil-linoleolenil-glicerol
LOO	linoleoil-dioleoil-glicerol
SLL	stearoil-dilinoleoil-glicerol
PLO	palmitoil-linoleoil-oleoil-glicerol
PLP	palmitoil-linoleoil-palmitoil-glicerol
OOO	trioleoil-glicerol
SOL	stearoil-oleoil-linoleoil-glicerol
POO	palmitoil-dioleoil-glicerol
SPL	stearoil-palmitoil-linoleoil-glicerol

	POP	palmitoil-oleoil-palmitoil-glicerol
	SOO	stearoil-dioleoil-glicerol
	SLS	stearoil-linoleoil-stearoil-glicerol
	POS	palmitoil-oleoil-stearoil-glicerol
	PPS	dipalmitoil-stearoil-glicerol
t-C 18:1	(*E*)-oktadec-9-enojska kislina (*trans*-oleinska (elaidinska) kislina)	
t-C 18:2	vsota izomerov (9*E*,12*E*)-oktadeka-9,12-dienojske kisline in (9*Z*,12*E*)-oktadeka-9,12-dienojske kisline in (9*E*,12*Z*)-oktadeka-9,12-dienojske kisline (*trans*-linolna kislina)	
t-C 18:3	vsota izomerov (9*E*,12*Z*,15*E*)-oktadeka-9,12,15-trienojske kisline in (9*Z*,12*Z*,15*E*)-oktadeka-9,12,15-trienojske kisline in (9*Z*,12*E*,15*Z*)-oktadeka-9,12,15-trienojske kisline in (9*E*,12*Z*,15*Z*)-oktadeka-9,12,15-trienojske kisline (*trans*-alfa-linolenska kislina; *trans*-linolenska kislina)	
TIC	angl. Total Ion Current (Signal skupnega ionskega toka)	
ZGO	Zaščitena Geografska Označba	
ZOP	Zaščitena Oznaka Porekla	

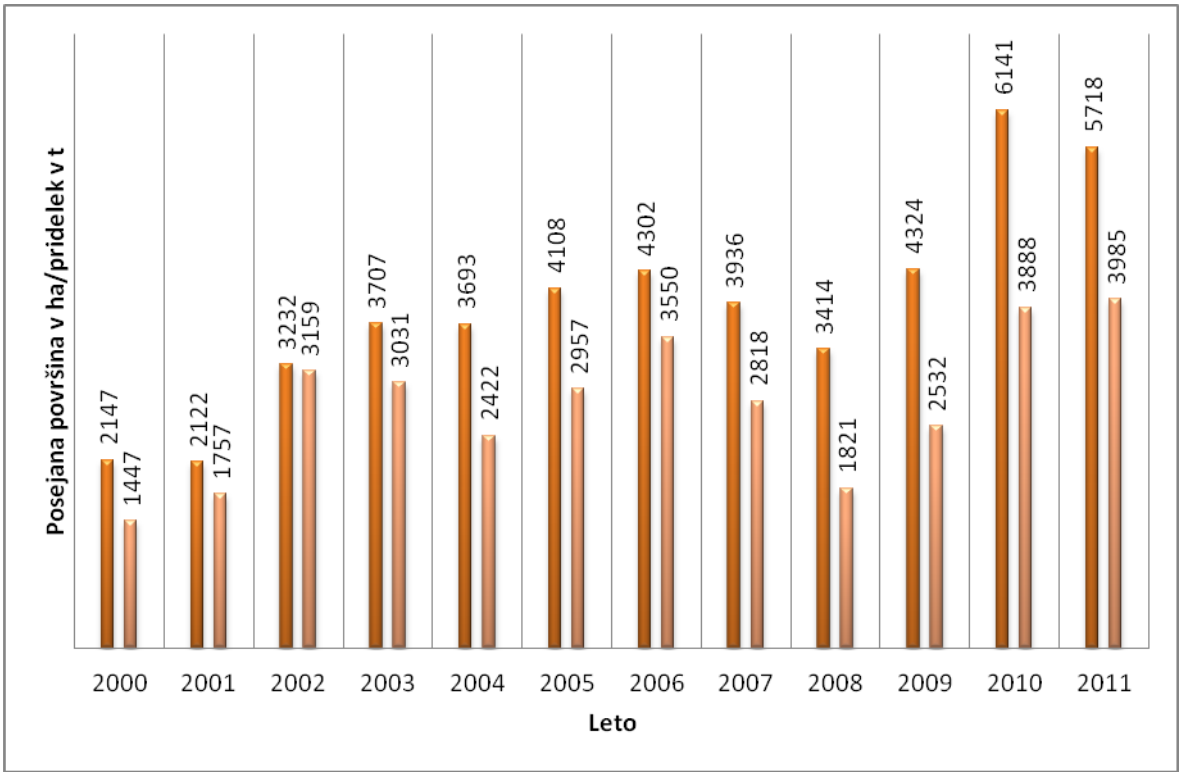
1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE

Leta 2005 je trg jedilnih olj v Sloveniji obsegal količino 17 milijonov litrov in je bil vreden 26 milijonov evrov (Škerlič in sod., 2006). Oljčno in bučno olje sta imeli posebni mesti v njem (ob njunih siceršnjih prehranskih, kulturnih in hedonističnih poudarkih): oljčno olje zaradi svojega tretjega cenovnega (prek 4 milijone evrov) in količinskega mesta (4,5 %), ki si ga je zaslužilo zaradi svoje relativno visoke cene v primerjavi z ostalimi semenskimi olji, bučno olje pa zaradi kar 43-odstotnega porasta cene ob 11-odstotnem količinskem porastu v obdobju 2004-2005.

Po podatkih družbe Food for Thought je bil zahodno evropski trg maščob leta 2009 vreden 24,2 milijardi evrov (2,2 % vrednosti vseh živil in pijač). Le-ta je bil v omenjenem pregledu razdeljen na sedem področij, vse od oljčnega olja prek margarine in margarini podobnih namazov do masla. Največji delež – kar 31 %, je pripadlo maslu, 29 % oljčnemu olju in kategoriji 'druga jedilna olja' 16 % te vrednosti. Po tej delitvi je bilo bučno olje vključeno v 16 % kategorije 'druga jedilna olja' (Oils and fats … , 2011).

Ti podatki so še toliko bolj zanimivi, če jih primerjamo s svetovno porabo jedilnih olj v letu 2011. Ta je ocenjena na več kot 141,4 milijona ton, od tega je palmovemu olju pripadlo 34 %, sojinemu pa kar 30 % te količine, medtem ko je bil delež oljčnega olja le 2 % (Oil crops yearbook … , 2011).

Pri bučnem olju so bolj podrobni podatki dostopni le za avstrijsko področje. Fruhwirth in Hermetter (2007) poročata, da so na avstrijsema Štajersema leta 2006 na 13000 ha gojili oljno bučo, ki je bila tretja najpomembnejša poljščina in so pridelali 11100 ton buč, iz katerih so v povprečju pridobili 500 do 600 kg semen/ha. Upoštevaje dejstvo, da je potrebnih 2,5 kg semen (oziroma 30 do 40 buč) za 1 L bučnega olja, je to najmanj 6000 ton semen ali 2,6 milijona litrov bučnega olja. Lelley in sod. (2010) so za leto 2006 prikazali rahlo drugačne podatke (18151 ha in 0,61 t semen/ha). Leta 2006 je bila vrednost avstrijskega bučnega olja 30 milijonov € (cca 1,5 % vrednosti vseh zahodno evropskih semenskih olj s trga). S hitrim računom lahko pokažemo, da je bil trg avstrijskega bučnega olja leta 2006 vreden približno toliko kolikor slovenski trg jedilnih olj. Slovenija je leta 2006 na 4302 ha pridelala 3550 t bučnih semen (825 kg semen/ha). Ta količina bi morala zadoščati za približno 1,4 milijona litrov bučnega olja. Leta 2010 je bila ta količina glede na obdobje 2000-2011 največja s kar 1,6 milijona litrov (hipotetična količina, izračunana na osnovi 2,5 kg semen za liter olja). Gibanje zasejane površine z bučami za olje in pridelek prikazuje Slika 1 (SURS, 2012). Historičen pogled na slovensko pridelavo oljnih buč in olja pokaže kako so leta 1916 na Štajerskem (O izdelovanju … , 1916) v opisanem poskusu sejanja buč in predelave semen v olje (preračunano) pridelali 470 kg semen golice/ha in stisnili 2,8 L olja/kg. Ocenjeni proizvodni stroški so bili 1 krona in 7 vinarjev z upoštevanjem prodaje bučne pogače (sicer pa 1 K 60 v).



Slika 1: Z bučami za olje (*Cucurbita pepo* L. var. *oleifera*) posejana površina v hektarjih (levi stolpci v seriji za posamezno leto) in pridelek semen v tonah (desni stolpci v seriji za posamezno leto) v Sloveniji v letih 2000-2011 (SURS, 2012). x-os: leto pridelka, y-os: površina (ha)/skupni pridelek (t)

Figure 1: Oil pumpkin (*Cucurbita pepo* L. var. *oleifera*) plantings in hectares (left columns in year series) and seeds yield in tons (right columns in year series) cultivated in Slovenia for years 2000-2011) (SURS, 2012). x-axis: year of cultivation, y-axis: cultivated area (ha)/yield (tons)

Vsi ti podatki kažejo na pomembnost ustreznih in točnih orodij, ki bi omogočila vpogled v kakovost in pristnost nekaterih bolj kakovostnih in dražjih olj, kamor spadata predvsem oljčno in bučno olje (Kreft in sod., 2009) ter seveda njuno ovrednotenje. Vsaj dva očitna razloga potrjujeta omenjeno trditev, eden je možna potvorba tako deviškega oljčnega olja kot tudi bučnega olja, drugi razlog pa je pravica potrošnika, da z nakupom pridobi kakovost in pristnost, za katero je plačal in pravico, da izbira med različnimi vrstami rastlinskih ali semenskih olj v luči dodane prehranske vrednosti. Sodobni potrošnik namreč povezuje prehransko vrednost in hedonističen užitek pri uživanju olja z vedenjem o zmanjšanem procesiranju olja kot tudi s posledično bolj bogato aromo (Matthaus, 2008).

Še posebej to velja za segmenta kakovosti in pristnosti v nutricionistični luči, v luči dodane prehranske vrednosti oz. zdravstvenih trditev in trditev v zvezi z zmanjšanjem tveganja za bolezni, ki so posledica uživanja živila oziroma njegove sestavine (Uredba Komisije … , 2012). Pri oljčnem olju so to enkrat nenasičene maščobne kisline - oleinska kislina in pa

minorne spojine, predvsem biofenoli in triterpenski dialkoholi ter posamezni triacilgliceroli - OOO, POO, SOL, OOL, LnPP, PLO, SLL, SOL in OLA in predvsem steroli (4-demetil steroli) kot inhibitorji tumorske rasti zaradi inducirane apoptoze in seveda skvalen, ki je inhibitor 3-hidroksi-3-metil-glutaril-reduktaze koencima A in domnevno tudi inhibitor farnezilacije onkoproteinov Ras (Garcia-Gonzalez in sod., 2008). Povzamemo lahko, da gre predvsem za varovalni učinek v primeru kardiovaskularnih in rakavih obolenj.

Podobno velja za bučna olja, saj se trditve, v zvezi z zmanjšanjem tveganja za bolezni, ki so posledica uživanja živila oziroma njegove sestavine nanašajo na maščobne kisline, fitosterole, minerale npr. selen (Kreft in sod., 2002) ter vitamine, biofenole, in pigmente (Fruhwirth in sod., 2003). Posebno poglavje gre seveda na račun zmanjševanja tegob povezanih z mehurjem in benigno hiperplazijo prostate (BHP) prek inhibicije testosteron 5-alfa-reduktaze (Lelley in sod., 2010), ko gre učinek pripisati predvsem kombinaciji fitosterolov, linolne kisline, vitamina E, luteina in lignanov, predvsem sekoizolaricirezinola (Fu in sod., 2006; Kuhnle in sod., 2008; Murkovic in sod., 2004; Sicilia in sod., 2003).

V tej luči se zdi dikcija v predlogu združenja GIZ Golica za priznanje zaščitene geografske označbe (ZGO) 'Štajersko prekmurskega bučnega olja' (Štajersko prekmursko … , 2005) pomanjkljiva; »Štajersko prekmursko bučno olje je jedilno nerafinirano rastlinsko olje, proizvedeno s stiskanjem praženih bučnih semen najboljše kvalitete, katera so pridobljena iz oljnih buč. Štajersko prekmursko bučno olje je temno zelene do rdeče barve, z značilnim aromatičnim vonjem in okusom. Ima ugodno maščobno kislinsko sestavo, saj vsebuje okrog 20 % nasičenih maščobnih kislin, okrog 35 % mononenasičenih maščobnih kislin in okrog 45 % polinenasičenih maščobnih kislin. Bučno olje je bogat vir tokoferolov, saj vsebuje okrog 50 mg vitamina E v 100 g olja. V bučnem olju so prisotni tudi drugi vitamini, mikroelementi, karotenoidi, redke aminokisline, naravno barvilo (klorofil).« Smotrno bi bilo tako dikcijo razširiti – vse v luči bolj zavezujoče in preverljive zaščite po eni strani in bolj verodostojnega obveščanja potrošnika po drugi.

Potrebno je upoštevati potrošnika in njegovo pravico po zahtevanju pristnosti in kakovosti, vključno s pravico vedeti, biti osveščen in s pravico do učenja oz. izobraževanja (Garcia-Gonzalez in Aparicio, 2010), tudi v smislu poudarjene butičnosti izdelkov različnih olj. Kakovost je namreč večplasten, velikokrat nejasen in neurejen koncept, ki je za povrhu še hierarhičen; le-ta prek varnosti živil združuje tako senzorično kakovost, prehransko kakovosti kot tudi emotivno kakovost glede na parcialne senzorične in kemijske preskuse.

Še posebej pereč je problem bučnega olja, saj je na tržišču mnoštvo različnih poimenovanj, vrst in definicij, ki potrošniku in posledično inšpekciji povzročajo znatne težave. Oznake kot rafinirano, nerafinirano, hladno stiskano in deviško, so prisotne v polju jedilnih olj, za njimi pa praviloma ne stoji ustrezno tehnološko, procesno oziroma prehransko ozadje (Matthaus, 2008).

Potvorbe kakovostnih semenskih ali rastlinskih olj s semenskimi olji nižje kakovosti lahko preprečimo s sledenjem dveh včasih vzporednih poti; prva je pot registriranja olj v skladu z evropsko zakonodajo (ustrezna etiketa s pripadajočimi podatki), druga pa je kemijska oziroma senzorična analiza, ki bi v naboru kompleksnih določitev morala potrditi deklarirano kategorijo olja. In zato je še posebej važno, da so kategorije olj točno definirane in sledljive: kot npr. način pridobivanja in priprave plodov oziroma semen, temperaturni režim njihove priprave, stiskanja in čiščenja.

Če se ozremo na prvo pot, ugotovimo, da se v Evropi lahko registrira posamezna imena in živila z vpisom v Nacionalne registre oziroma v register Evropske unije. Avstrija je za svoje bučno olje (olje iz semen buče *Cucurbita pepo* L.) to storila že leta 1992 kot »zaščitena geografska označba«, takrat na osnovi tedaj veljavne uredbe Evropskega Sveta, ki jo je leta 2006 zamenjala nova uredba (Council Regulation … , 2006), a ohranila ključni definiciji »Zaščitena označba porekla – ZOP« in »Zaščitena geografska označba – ZGO«. Ključna razlika med njima leži v povezavi med pridelavo in predelavo glede na definiran in zaščiten geografski prostor (Butinar in sod., 2010b).

V Sloveniji je že nekaj živil, ki jih pokriva ta uredba (oziroma so v postopku pridobitve), a v luči te raziskave sta zanimivi predvsem dve: 'Štajersko prekmursko bučno olje' (Bavec in sod., 2002; Fruhwirth in Hermetter 2007, 2008), ki je v fazi pridobivanja označbe ZGO (Publication … , 2009) in 'Ekstra deviško oljčno olje Slovenske Istre' s pridobljeno označbo ZOP (Council Regulation … , 2006; Objava … , 2006; Uredba Komisije … , 2007). V elaboratu za pridobitev označbe ZOP za »Ekstra deviško oljčno olje Slovenske Istre« so točno predpisani vsi parametri, ki vplivajo na zagotavljanje kakovosti in pristnosti tako označenega Ekstra deviškega oljčnega olja; med ostalim kultivar oljk, najdaljši dopusten čas med pobiranjem oljk in njihovo predelavo v olje, način hranjenja pobranih oljčnih plodov ter temperatura predelovalnega procesa. Te parametre je potrebno v praksi spremljati, dokumentirati in nadzorovati. To po eni strani počnejo oljarji sami in pa ustrezne nadzorne institucije, ki so imenovane in pooblaščene za nadzor.

V primeru slovenskega bučnega olja oziroma 'Štajersko prekmurskega bučnega olja' po nam dostopnih informacijah razmere niso tako urejene in tudi ni zelo podrobnih smernic in vodil, da bi lahko ovrednotili parametre kakovosti, predvsem pa parametre pristnosti bučnega olja – z izjemo vodil za senzorično ocenjevanje in nekaterih parametrov kakovosti (Štajersko prekmursko … , 2005). Še posebej so pomanjkljivi podatki o vrsti uporabljenih buč, o samem postopku praženja – o temperaturi in času, ki znatno vplivata na vsebnost pirazinov in zato posledično na senzorične značilnosti olja (Nikiforov in sod. 1996), o morebitnem hladnem stiskanju praženih oziroma nepraženih semen (delovni tlak je med 300 in 600 bar, zato bi bilo smiselno vpeljati termin – hlajenje pri stiskanju o čemer podrobneje govorimo v nadaljevanju, ko izpostavimo »pasivno« segrevanje zaradi visokega delovnega tlaka) (Bavec in sod., 2007; Pojbič, 2008; Vujasinović in sod., 2010). Sam proces praženja zelo vpliva na vsebnost pirazinov, ki so ključni za »praženo« aromo:

2-etil-3,5-dimetilpirazin; 2,3-dietil-5-metilpirazin in 3-etil-2,5-dimetilpirazin, furanov: 2-pentilfuran in tudi nenasičenih aldehidov: (*E*,*E*)-2,4-dekadienal in (*E*,*E*)-2,4-nonadienal.

1.1 PREDSTAVITEV PROBLEMATIKE

Vzporedna pot pri ugotavljanju morebitnih potvorb olja je pot kemijske oziroma senzorične analize. V primeru deviškega oljčnega olja so to v Evropski uniji edinstveno in izvrstno opravili z dvema uredbama Evropske komisije. Ti uredbi podajata med ostalim analitične postopke (Commission Regulation … , 1991) in pa »drevo odločanja« (decision tree) (Commission Regulation … , 2003), s pomočjo katerega lahko posamezno kategorijo olja razvrstimo v skladu z analiziranimi parametri oziroma preverimo ali je v skladu z deklarirano kategorijo. Commission Regulation … (1991) je krovna uredba, ki se s spreminjanjem tržnih in prehranskih zahtev, stanja na trgu in razvoja metod tudi sama spreminja. Spremembe so vsebovane v novih uredbah, a se nanjo nanašajo. Drevo odločanja se od leta 2003 ni spremenilo, spreminjajo pa se posamezne mejne vrednosti, ki so ključne za odločitev in uvrstitev v kategorijo.

To lahko storimo na osnovi nekaterih podatkov in mejnih vrednosti za posamezno analizo oziroma preskus. Ključnega pomena je dejstvo, da je skupina dobrih poznavalcev oljčnih kultivarjev in analitikov-raziskovalcev izdelala ustrezne analitične postopke in prišla do relevantnih vrednosti, le-te pridobila na osnovi velikega števila analiziranih in preverjenih vzorcev oljčnega olja. Na tak način je bila izgrajena baza podatkov in nabor mejnih vrednosti, ki pomagajo tako pri ugotavljanju parametrov kakovosti kot tudi parametrov pristnosti oljčnega olja. Glavna zasluga gre italijanskim, španskim in nemškim raziskovalcem, ki so delovali tudi v okrilju tedanjega Mednarodnega sveta za oljke (Amelio in sod., 1993; Cert in sod., 1994; Grob in sod., 1995; Homberg, 1974; Kalo in Kuuranne, 2001; Lanzon in sod. 1989, 1994). Na osnovi serije opravljenih analiz se lahko zanesljivo ugotovi skladnost posameznega vzorca z deklarirano kategorijo oziroma morebitno potvorbo – v skladu z vrednostmi in analiznimi postopki. V primeru ekstra deviškega oljčnega olja se najprej opravi serijo kakovostnih določitev (kislost, peroksidno število, preiskava v UV področju ter vsebnost alkilnih estrov) z vključeno senzorično analizo, nato sledi preverjanje pristnosti. V primeru ekstra deviškega oljčnega olja je potrebno opraviti sedem zahtevnih analiznih preskusov (določitev stigmastadienov, določitev *trans*-izomerov maščobnih kislin, določitev maščobnokislinske sestave, določitev razlike med praktično in teoretsko vsebnostjo triacilglicerolov z ekvivalentnim ogljikovim številom 42 (ΔECN42), vsebnost in sestavo sterolov, vsebnost triterpenskih dialkoholov eritrodiola in uvaola ter vsebnost voskov) preden se potrdi oziroma ovrže deklarirano kategorijo oljčnega olja. Mejne vrednosti in preskusne metode se za oljčno olje nenehno dopolnjujejo, zadnjič npr. z uredbo EU 61/2011 za vsebnost alkilnih estrov (Uredba Komisije … , 2011). Zanimiva je tudi historična primerjava različnih tržnih kategorij oljčnega olja na italijanskem tržišču v zadnjem stoletju (od leta 1890), iz katere je

razvidno, kako je kemijska okarakterizacija lahko v pomoč pri urejanju trga (Caponio in sod., 2012).

Preskusne metode za ugotavljanje pristnosti ekstra deviškega oljčnega olja in ustrezne meje so bile določene v skladu s spoznanji in značilnostmi tako ekstra deviškega oljčnega olja kakor tudi v skladu z značilnostmi posameznih kategorij oljčnega olja – to velja predvsem za razločevanje med ekstra deviškim oljčnim oljem in olji ostalih kategorij. Iz prej omenjenih določitev pristnosti je razvidno, da so določitve take, ki preverjajo maščobnokislinsko sestavo in triacilglicerolno sestavo olja (ki lahko pokažejo na prisotnost »tujih« maščobnih kislin in triacilglicerolov), prisotnost morebitnih *trans*-izomerov maščobnih kislin in stigmastadienov (reduciranih sterolov, ki pokažejo na postopek rafinacije oziroma toplotne obdelave olja, ki je v nasprotju s kategorijo ekstra deviškega oljčnega olja), po drugi strani pa preverjajo vsebnost in sestavo ne-triacilglicerolnih sestavin, ki so zelo karakteristične za posamezno vrsto oljčnega olja (in tudi za ostala semenska olja) – sestava in vsebnost sterolov ter vsebnost triterpenskih dialkoholov (uvaol in eritrodiol).

Sodobni raziskovalni pristopi se čedalje bolj poslužujejo novejših statističnih orodij, ki raziskujejo potvorbe bolj kakovostnih olj z jedilnimi olji (Hajimahmoodi in sod., 2005). Ključno je seveda, da mora statistično podprto raziskovanje in ugotavljanje razločevanja ostajati zvesto osnovni matrici – definiranemu olju in seveda zakonsko osnovanim zahtevam potrošnika.

Že v primeru ekstra deviškega oljčnega olja je tako, da je senzorična ocena, ki jo da ustrezno šolan, izkušen, pooblaščen in preverjan panel eden ključnih izključevalnih kakovostnih dejavnikov: v prihodnosti bi bilo le-to smiselno nadgraditi z instrumentalno kvantifikacijo ključnih »nosilnih« spojin, ki bi bile definirane tudi v luči posamezne geografske zaščite. Termin avtentičnost bi zatorej vključeval tudi instrumentalno podprto senzoriko (Aparicio in sod., 1997).

Na področju ugotavljanja potvorbe bučnih olj s cenejšimi semenskimi olji ni bilo opravljenih veliko raziskav, še posebej ne v Sloveniji. Nekaj poskusov je bilo na teritoriju Sovjetske zveze (Baratova in sod., 1982) in Jugoslavije (Marković in Bastić, 1976). Objavljene raziskave so se ukvarjale z določevanjem maščobnokislinske sestave bučnih olj in s povezavo med maščobnokislinsko sestavo in kultivarjem (Applequist in sod., 2006; Murkovic in sod., 1996a; Parry in sod., 2006; Stevenson in sod., 2007; Yoshida in sod., 2005). Nekaj podobnih raziskav je bilo opravljenih tudi v Sloveniji (Brodnjak-Vončina in sod., 2005; Erčulj Rogel, 2002; Vuga, 2002). Soroden pristop so uporabili pri določevanju karakterističnih triacilglicerolov – a predvsem v luči primerjave bučnih olj z nekaterimi manj pogostimi olji (Andrikopoulos in sod., 2004; Jakab in sod., 2002; Tuberoso in sod., 2007). Nekaj raziskav je poskusilo diskriminirati posamezna bučna olja glede na vrsto semen in njihov geografski izvor z uporabo spektroskopskih tehnik (Joebstl in sod., 2010; Saucedo-Hernandez in sod., 2011).

Po analogiji z oljčnim oljem lahko potrdimo, da sta vsebnost in sestava sterolov v bučnih oljih lahko zelo pomembna in karakteristična (Bastić in sod., 1977; Breinholder in sod., 2002; Fruhwirth in Hermetter, 2007; Garg in Nes 1985, 1986).

Z raziskovanjem sterolov v bučnih oljih so se ukvarjali tudi slovenski raziskovalci (Makovšek, 2003; Rodošek, 2009). Zanimivo je, da so prav v primeru razmerja med Δ-5 in Δ-7 steroli potekale raziskave, ki so skušale na osnovi njihovega razmerja ugotavljati potvorbe bučnega olja z drugimi semenskimi olji (Breinholder in sod., 2002; Mandl in sod., 1999; Wenzl in sod., 2002).

Precej pozornosti so namenili določevanju vsebnosti in vrste izomerov vitamina E (Gemrot in sod., 2006; Murkovic in sod., 1996b; Murkovic in Pfannhauser, 2000; Murkovic in sod., 2004; Stevenson in sod., 2007; Suturovic in Marjanovic, 1999; Yoshida in sod., 2006). Zanimivo je, da so se Murkovic in sod. (1996b, 2004) ukvarjali tudi z morebitnimi spremembami koncentracije vitamina E v odvisnosti od termične obdelave semen. Murkovic in Pfannhauser (2000) sta tudi določila vsebnost izomerov vitamina E – alfa-tokotrienola in gama-tokotrienola v semenih, ki naj bi v nekaterih primerih celo dosegla vsebnost svojih (nasičenih) tokoferolnih izomerov.

Pri zasnovi analitičnega postopka ugotavljanja pristnosti in stopnje predelave bučnega olja bomo obogatili nabor obstoječih analiznih postopkov za matrico bučno olje. Glede na rezultate raziskav pričakujemo, da bo doktorska študija pomagala poenotiti in predvsem poenostaviti uporabo in povezavo rezultatov različnih analiznih postopkov pri ugotavljanju pristnosti bučnega olja in tako omogočila temelje za izdelavo splošnega analitičnega pristopa, namenjenega ugotavljanja pristnosti in stopnje predelave bučnega olja. V tem pogledu je nujen korak izgradnje obširne baze podatkov posameznih bučnih olj – tako glede izvora bučnih semen (domača, uvožena) kot tudi glede na posamezne sorte ('Slovenska golica', 'Muškatna buča', 'Slovenska belica' 'Gleisdorfer Ölkurbis', 'Wies 371', 'Olinka'). Podatki o le-teh pa se omejijo zgolj na njihovo naštevanje in agronomske značilnosti (Erčulj Rogel, 2002; O izdelovanju …, 1916; Štajersko prekmursko … , 2005; Vuga, 2002) ter način predelave. O izdelovanju … (1916) je časopisni članek iz Časopisa Štajerc, ki razglablja o tem, kako modro bi bilo pridelovati buče oziroma bučna jedrca (semena) za predelavo v olje in pridobivanje oljne pogače, ki bi bila primerna za krmilo. Podrobneje ne omenja bučne vrste (sorte), a iz konteksta: »… Naglašamo pa, da imamo tukaj v mislih takozvane koščice brez luščin, katerih toraj ni treba luščiti.« je razvidno, da je šlo za golico – *Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb., ki naj bi se na Štajerskem pojavila okrog 1870-1880 in razmahnila v letih do začetka prve svetovne vojne (Teppner, 2004). Po podatkih iz Štajersko prekmursko … (2005), ki v prilogi prikaže faksimile dokumenta, ki priča o prvi stiskalnici oljčnega olja v Framu iz leta 1750 in podatkih iz Teppner (2004) je razvidno, da so se prve stiskalnice za olje na Štajerskem pojavile v letih 1738–1750. Baza v Sloveniji predelanih olj bi morala vsebovati vse signifikantne podatke – maščobnokislinsko sestavo, vsebnost *trans*-izomerov maščobnih

kislin, stereospecifično triacilglicerolno sestavo, vsebnost sterolov in razmerje med Δ-5 in Δ-7 steroli ter vsebnost in sestavo vseh izomerov vitamina E. Rezultati tega raziskovanja lahko pripomorejo k oblikovanju baze, pomembne za (re)definiranje pojmov pristnost oziroma avtentičnost v luči geografske zaščite bučnega olja, torej – ZGO ali ZOP. V kolikor je zaščita ravni ZGO lahko govorimo o pristnosti, ZOP pa seveda zahteva avtentičnost. Pristnost je namreč povezana z nepotvorjenostjo posameznega izdelka oz. živila – bučnega olja, medtem ko se za avtentičnost ob pristnosti nujno zahteva še preverljivo in izsledljivo povezavo na definiran in zaščiten geografski izvor (teritorij) surovine – bučnih semen.

Delno se povezava na geografski izvor – teritorij že dogaja v smislu dokumentiranih zapisov, a zaželena je nadgradnja s kemometrično obravnavo nekaterih dodatnih kemijskih določitev. Na kemometrijo bi bilo potrebno pogledati kot na umetnost ekstrahiranja kemijsko relevantnih informacij iz podatkov, dobljenih v kemijskem eksperimentu z uporabo statističnih in matematičnih orodij (Aparicio in Aparicio-Ruiz, 2002). Iz tako pridobljenih spoznanj se lahko ustvarijo orodja za klasifikacijo in kasnejšo diskriminacijo posameznih izdelkov – olj z ZOP oziroma ZGO (Aparicio in Garcia-Gonzalez, 2012; Aparicio in Morales, 1995; Garcia-Gonzalez in sod., 2009). Pri urejanju vseh dodatnih značilnosti izdelkov, ki jo zahteva vključitev teritorija v definicijo avtentičnosti, bi bilo v prihodnje ob nujnih, večinoma že definiranih kemijskih parametrih, o katerih delno govori tudi ta razprava, vključiti tudi izsledke, dobljene s pomočjo metabolomike (Garcia-Gonzalez in Aparicio, 2010; Ryan in Robards 2006a, 2006b).

Pomemben uporaben doprinos tega dela je, do bodo izsledki uporabni za razlikovanje med bučnim oljem iz praženih semen in hladno stiskanim bučnim oljem upoštevajoč temperaturni režim v samih postopkih, ki ni vedno natančno nadzorovan, saj definicija hladnega stiskanja ni enoznačno razumljena in sprejeta v praksi.

Codex Alimentarius (1981) je že v standardu za jedilne maščobe, ki jih ne pokrivajo drugi individualni standardi iz leta 1981, revizija 1987, dopolnilo 2009, omenil kategorijo hladno stiskane maščobe (masti, olja) – »Cold pressed fats and oils are edible vegetable fats and oils obtained, without altering the oil, by mechanical procedures, e.g. expelling or pressing, without the application of heat only. They may have been purified by washing with water, settling, filtering and centrifuging only.« Zanimivo je, da je ta definicija, ki eksplicitno omeni »brez uporabe toplote«, v veljavi vse od leta 1981! Druga zanimivost je, da Codex Alimentarius ne definira kategorije nerafiniranega olja ki je pri naših bučnih oljih često v uporabi (Štajersko prekmursko … , 2005). Zdi se, da bi bilo nujno začeti z ustreznim razumevanjem definicij, ki so že v veljavi (Matthaus in Spener, 2008).

'Codexov standard za imenovana rastlinska olja' iz leta 1999, revizije iz let 2001, 2003, 2009 in dopolnili 2005 in 2011 (Codex Alimentarius, 1999) se je v zadnjih letih zelo oplemenitil z novimi olji – brez oljčnega olja, ki ima svoj standard, jih je že 24. Od teh jih je kar osem takih, ki so povezane z maščobo palme (mdr.*Orbignya* spp. (babasu) in tudi

Elaeis guineensis), dodana so bila orehovo olje, olje pistacije, konopljino olje in lešnikovo olje (Codex Alimentarius, 2011). Zanimivo je, da so že leta 1974 na sedmem zasedanju Codex alimentarius v Londonu (Codex Alimentarius, 1974) razpravljali o morebitni vključitvi petih vrst olja v sekcijo imenovanih olj – 'Codex standard for named vegetable oils' in med njimi je bilo tudi bučno olje. Kot sledi iz dokumenta, je predsedujoči ugotovil, da je iz predstavljenih gradiv izšlo, da so bili pomanjkljivo predstavljeni (premalo podatkov) maslo illipe (*Shorea stenoptera*), karitejevo maslo (*Vitellaria paradoxa*) in bučno olje. Takrat sta v nadaljnjo obravnavo šli le olje grozdnih pešk in olje babassu. Prav tako so takrat ugotovili, da je bila proizvodnja bučnega olja v tedanji Jugoslaviji in Avstriji tako majhna, da nadaljnje aktivnosti ne bi bile smiselne. Ostaja pa zanimivost, da je po več kot tridesetih letih in avstrijski zaščiti navkljub področje bučnega olja v luči Codex Alimentarius nedefinirano in zdi se, da je tak status quo skoraj dobrodošel.

Že Bavec in sod. (2007) so za slovensko hladno stiskano bučno olje vpeljali svojo definicijo – to naj bi bilo mehansko stiskanje posušenih semen brez predhodnega mletja. Tudi Vujasinović in sod. (2010) v svojih raziskavah opisujejo hladno stiskano bučno olje in povzamejo definicijo iz standardov Codex Alimentarius, a termin »brez uporabe toplote« razumejo kot vsako stanje sistema, čigar temperatura ne preseže 50 ºC. Vedeti je potrebno, da se pri delovnem tlaku 300 do 600 barov (pa tudi pri nekaj nižjem tlaku, ki je potreben, da se olje iztisne s pomočjo vijačne preše) sam medij in olje izjemno segrejeta in so temperature, ki za deset ali več stopinj presežejo temperaturo prostora, običajne. Preveč ad literam sledenje dikciji »brez uporabe toplote« bi preprosto pomenilo «naravno« fizikalno segrevanje stisnjenega olja. V tej fazi je olje že podvrženo oksidacijskim in hidrolitskim procesom, ki znatno zmanjšajo njegovo kakovost in prehransko vrednost. Upoštevaje ta dejstva bi seveda morali razmišljati v smeri spremembe osnovne definicije hladnega stiskanja, ki bi morala vsebovati dodatek »brez uporabe toplote in tako, da zaradi segrevanja pri stiskanju temperatura ne preseže 27 ºC«. Temperatura 27 ºC je normalna priporočena temperatura pri predelavi oljčnega olja, saj jamči minimalen vpliv toplote na kakovost olja zaradi procesov stiskanja oziroma predelave (Objava … , 2006). Mislimo, da bi moral sam proces stiskanja - navkljub malce ohlapni definiciji – v luči dobre prehranske in živilske prakse potekati tako, da ne bi bila presežena arbitrarno določena temperatura. Vemo namreč, da sodobne stiskalnice omogočajo temperaturni nadzor medija. Torej – sploh ne bi smeli razpravljati o temperaturah 50 ºC ali 40 ºC (Vujasinović in sod., 2012), temveč kategorično sprejeti vse pod 30 ºC. Le-tako bo hladno stiskano bučno olje obdržalo svoj primat, ki ga ima zaradi svojih surovin, izpričanega prehransko zdravstvenega vpliva in zgodovinskega izročila.

Novejše raziskave v Srbiji in Makedoniji operirajo z dvema kategorijama bučnega olja, ki sta v skladu s standardi Codex Alimentarius. Prva je hladno stiskano bučno olje pridobljeno s kontinuiranim hladnim stiskanjem nepraženih in očiščenih ter posušenih bučnih semen s pomočjo vijačne preše, ki hkrati melje in stiska (ponovno – termin hladno avtorji razumejo kot ne več kot 50 ºC). Vujasinovic in sod. (2012) trdijo, da je tak način

stiskanja olja v Srbiji v uporabi od konca devetdesetih let prejšnjega stoletja. Morebitno motnost tako predelanega olja odstranijo s sedimentacijo oziroma s filtriranjem pri sobni temperaturi. Isti avtorji in Fruhwirth in Hermetter (2008) poročajo tudi o drugem stiskanju – ko iz bučne pogače po prvem stiskanju poskusijo pridobiti še nekaj olja. Eden glavnih dejavnikov za tako početje je relativno visoka cena bučnih semen. Menimo, da je tako početje contradictio in adiecto v odnosu na osnovne prehranske in zdravstvene trditve, ki dajejo izpričan pečat bučnemu olju (podobno kot pasivno segrevanje pri hladnem stiskanju). V takih primerih, ki so pogojeni z zaslužkom in podobno kot je bilo na začetku uveljavljanja (ekstra) deviškega oljčnega olja izrabljajo zaupanje manj zahtevne populacije potrošnikov s tem, da jim ponudijo (zanje) bolj sprejemljiv in manj moteč ter bolj blag okus olja z manj intenzivno aromo. Verjetno prihaja tudi do tretjega stiskanja, ko je že enkrat ali dvakrat stisnjena pogača v že toplejšem režimu ekstrahirana z nizkocenovnim jedilnim oljem. Tak primer smo opisali v svoji prvi publikaciji, ko je bil tak vzorec namerno vključen v nabor analiziranja (Butinar in sod., 2009).

Druga kategorija je deviško bučno olje, ki ga pridobijo tako, da iz posušenih zmletih in pri 100 do 130 °C do 60 minut praženih semen izotermno stisnejo olje pri tlaku, ki je v območju med 300 in 600 barov. Pri praženju koagulirajo proteini, obenem pa se izostri senzoričen profil olja ob hkratnem povečanju stabilnosti. V Sloveniji in v Avstriji med praženjem mletih bučnih semen zaradi lažjega, hitrejšega in boljšega izločanja olja le-tem dodajo tudi mešanico vode in natrijevega klorida (Murkovic in sod., 2004; Pojbič, 2008). Taka kemijska modifikacija izvorne bučne matrice pa je verjetno v nasprotju z osnovno definicijo, ki jo podaja Codex alimentarius (Codex Alimentarius, 1981). Raziskava makedonskih avtorjev (Srbinoska in sod., 2012) uvede oziroma redefinira kategorijo bučnega olja, ki je »ekstra deviško« bučno olje in prilije kanček terminološke zmede k vsemu povedanemu.

Glede na vse predstavljene terminološke zanke, vezane na definicijo hladno stiskanega olja in deviškega olja, na značilnosti zaščit ZGO in ZOP, bo tako zastavljena diskriminacija lahko služila kot osnova za bodočo izgradnjo podatkovne baze »domačih« in »tujih« bučnih kultivarjev in bo zatorej korak k redefiniranju ustrezne zaščite slovenskega bučnega olja (zaščita ZGO oziroma ZOP). Tako se bosta lahko tudi bolj sledljivo osmislila termina pristnost oziroma avtentičnost.

1.2 HIPOTEZE

Izhajajoč iz dosedanjih spoznanj na področju ugotavljanja pristnosti oljčnega olja in poznavanja značilnosti bučnega olja in njegove matrice trdimo, da bo:

- uporaba zasnovanega analitičnega postopka ugotavljanja pristnosti in stopnje predelave bučnega olja na osnovi izdelanega koncepta analiz določevanja pristnosti

oljčnega olja, ki temelji na »drevesu odločanja«, razločila med pristnimi in potvorjenimi bučnimi olji,

- s stereospecifično (HPLC) analizo triacilglicerolov bučnega olja možno razlikovati med pristnimi in potvorjenimi bučnimi olji,
- analiza eksperimentalno pridobljenih in objavljenih podatkov vsebnosti izomerov vitamina E v bučnem olju pomagala razlikovati med potvorjenimi in pristnimi bučnimi olji,
- analiza eksperimentalno pridobljenih in objavljenih podatkov vsebnosti nekaterih izomerov vitamina E (alfa-monotokoenol, gama-monotokoenol, gama-tokotrienol) v semenih in olju buče 'Slovenska golica' poglobila vedenje o razmerju med nasičenimi, enkrat nenasičenimi in večkrat nenasičenimi izomeri vitamina E v olju buče 'Slovenska golica',
- analiza izomerov vitamina E v olju iz praženih semen buče 'Slovenska golica' potencialni marker iz katerega bo možno sklepati na pris(o)tnost olja iz praženih semen.

2 ZNANSTVENA DELA

2.1 IZKUŠNJE PRI UGOTAVLJANJU PRISTNOSTI IN KAKOVOSTI OLJČNEGA OLJA SO ORODJE ZA OVREDNOTENJE OLJA IZ BUČNIH SEMEN. KAJ LAHKO PRIDOBIJO POTROŠNIKI?

Experiences in olive oil purity and quality assessment as a tool for pumpkin seed oil evaluation. What can consumers benefit?

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Oljčna in bučna olja imajo pomembno vlogo na trgu slovenskih jedilnih olj. To je razlog za potrebo po obstoju orodij za ugotavljanje njihove kakovosti in pristnosti (potvorbe). Eno od orodij je vpis imen in živil v Nacionalne registre in v Register Evropske unije. Drugo orodje je kemijska analiza, ki pomaga olja ovrednotiti in jih razvrstiti v ustrezne kategorije. V polju oljčnega olja je bilo to izvrstno storjeno z uredbami Evropske komisije. V pričujočem delu smo skušali preskusiti nekaj bučnih olj in ugotoviti njihovo vsebnost maščobnih kislin, vsebnost *trans*-maščobnih kislin, sterolno sestavo in vsebnost tokoferolov. Naš glavni namen je bil preveriti pristnost bučnih olj in doumeti stanje na področju bučnih olj. Analitični podatki so pokazali, da so bili nekateri vzorci potvorjeni s semenskimi olji. Kakšen nauk za potrošnike sledi iz ugotovitev? Iz predstavljene izkušnje sledi, da pot k boljši kakovosti olj ne sloni nujno na predstavljeni kemijski analitiki, temveč skuša slediti dobro utečenim pravilom za kakovost in pristnost. Potrošnik se mora izobraževati in se zavedati, da sta kakovost in pristnost analitično dokazljivi. Podatki iz analiz so pokazali, da nekateri vzorci bučnih olj niso mogli dokazati svoje pristnosti zaradi zelo verjetne potvorbe s cenejšimi semenskimi olji, ki je bila evidentno dokazana z analizo sterolne in tokoferolne sestave. Pri preverjanju pristnosti se namerno nismo dotaknili ne sorte ne teritorialnega izvora semen/olj ne pogostoma zavajajočega označevanja z uporabo dikcije 100-odstotnega bučnega olja. Razlog za tako stanje je verjetno precej ohlapna definicija Zaščitene geografske označbe, ki velja lokalno za 'Štajersko prekmursko bučno olje', a bolj verjetno gre glavni razlog iskati v pomanjkanju ustrezne kontrole in v pomanjkljivi zavesti potrošnika, da bi razumel in plačal za dokazljivo kakovost.

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EXPERIENCES IN OLIVE OIL PURITY AND QUALITY ASSESSMENT AS A TOOL FOR PUMPKIN SEED OIL EVALUATION. WHAT CAN CONSUMERS BENEFIT?

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Olive and pumpkin seed oils play a specific role in Slovenian edible oil market. That is why exact and accurate tools for assessing the oils' quality and purity (adulteration) are needed. One of the tools is registering certain names and foodstuffs in National registers and the Register of European Union. Another tool is the analysis which can help to assess the characteristics of the oil and to classify it in categories. In the field of olive oils, this has been done excellently with the European Commission regulations. In the present work we tried to test few pumpkin seed oils for fatty acids content, trans isomers of fatty acids, composition of sterols and tocopherols. The main goal was to check the purity of the oils and understand the present situation in the field. The analytical results show that some samples are adulterated with seed oils. What can consumers benefit? From the Slovenian olive oil experience it can be concluded that the path towards better quality oils does not strictly follow analytical methods but tries to track well-established rules and definitions of quality and purity. The consumers must learn and be aware that the quality and purity can be analytically proven.

Keywords: analytical assessment, olive oil, pumpkin seed oil, purity, quality

According to a recent survey, the edible oil (plant oil) market in 2005 in Slovenia was 17 million litres and was worth over 26 millions €. Olive and pumpkin seed oils took specific roles in it (besides their special nutritional, cultural and hedonic accents): olive oil being worth over 4 millions € at the 3rd place owing it to the relatively high price when compared to other seed oils and the pumpkin seed oil for its 43% price increase in period 2004–2005 (ŠKERLIČ et al., 2006). All these data show the importance of exact and accurate tools for assessing the oils' quality and especially purity and genuineness. Definitely, one of the reasons is possible adulteration due to relatively higher prices in

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the case of olive and pumpkin oils, and the other one is the consumer's right to have on his table the quality he chose to pay for and as well the right to choose between various types of vegetal oils in the light of the added nutritional values.

One of the tools is therefore registering certain names and foodstuffs in National registers and the Register of European Union. Austria did it in 1992 with the oil of pumpkin seeds (*Cucurbita pepo* L.), which has been approved by the Commission of the European Union as a "protected geographic name" (SIEGMUND & MURKOVIC, 2004) in accordance with the COUNCIL REGULATION (EEC, 1992) which was replaced by COUNCIL REGULATION (EC, 2006a), but left the two crucial definitions: "Protected Designation of Origin" (PDO) and "Protected Geographical Indication" (PGI) intact. The rough difference between the two lays in the sentence: "The production and/or processing and/or preparation of which (i.e. particular foodstuff) take place in the defined geographical area". In the case of PDO foodstuffs all logical operators must be "and" while in the case of PGI only one of the mentioned activities must be performed in the defined geographical area.

Slovenia has many foodstuffs which are covered by this regulation, but two of them are of our particular interest now: "Štajersko prekmursko bučno olje (pumpkin seed oil from Štajerska and Prekmurje region)" which carries a "Protected Geographical Indication", PGI, and "Ekstra deviško oljčno olje Slovenske Istre (Extra virgin olive oil from Slovene Istria)" with a "Protected Designation of Origin", PDO foodstuff registered in the Register of European Union (EC, 2007), based on an application for registration according to Council Regulation (EC, 2006a) published in the Official Journal of the European Union (EC, 2006b).

Other valuable tools are the chemical and sensory analyses which can help to assess the characteristics of the oil. In the field of olive oils, this has been done excellently and uniquely with the European Commission regulations. The regulations (EEC, 1991; EC, 2002a; EC, 2003) give inter alia the methods of analysis, the decision tree for establishing the conformity of the declared type of olive oil with the analysed parameters, the limit values needed to assess the characteristics of olive and olive- pomace oils. They include the sensory (organoleptic) assessment as an important quality parameter. The usual procedure is as follows: first the quality parameters of the declared category are checked, and then the purity criteria are challenged, but only if the sensorial (organoleptic) criteria are met for the declared category.

Seven different and rather tedious analytical procedures from the instrumental point of view must be performed before the sample can be classified as a sample of Extra virgin olive oil. All these procedures are a result of a long and cooperative work performed in many years between the experts from the olive field and analytical chemists who gathered their knowledge and experiences.

The present situation in Slovenian pumpkin seed oil production is not so strict and as far as we know there are no such regulations and procedures one can follow to establish/assess an oil's quality or purity parameters (KARAS, 2001). Furthermore, we think there are no guides considering the pumpkin seed composition (species and cultivars, temperature and duration of the roasting process, etc.) (DOLŠEK, 1997;

SIEGMUND & MURKOVIC, 2004) especially, there are various interpretations of "cold pressing" procedure and temperature ranges. On the contrary, with PDO Extra virgin olive oil from Slovene Istria where the various olive cultivars, maximum time between picking of the olives and pressing, temperature of the pressing process, etc. are strictly regulated and must be followed, registered and are controlled in accordance with EC (2006a) and Application for registration "Ekstra deviško oljčno olje Slovenske Istre" (BUČAR-MIKLAVČIČ et al., 2003). There are many brands, types, denominations on the Slovenian pumpkin seed oil market and the consumer hardly, if at all, recognizes what is exactly in the bottle. Another problem, which is not yet completely understood, is possible PAH content in the oils due to improper roasting processes demanding quality control procedures in the pumpkin seed oil production.

In the present work, we tried to test few pumpkin seed oils (purchased locally in the Prekmurje region) to determine purity indicators which, we thought, could give us a valuable answer. We followed the guidelines from the olive oil quality and purity assessment based on the unique specifics regarding pumpkin seed oil that must be followed and met if the product is indeed the PGI pumpkin seed oil from Sajerska and Prekmurje region. We did not check any quality parameters for the simple reason that we presumed the purity differences would be so huge that quality didn't really count. Sensory analysis is another step we did not take for its high specificity (SIEGMUND & MURKOVIC, 2004).

Purity parameters checked were trans isomers of fatty acids content, fatty acids content and composition of sterols. We determined tocopherols as well by HPLC.

1. Materials and methods

1.1. Materials

1.1.1. Pumpkin seed oil samples. We examined 7 pumpkin seed oil samples which were bought in the local stores and/or purchased locally at the pumpkin oil pressing facilities. Table 1 gives detailed information about the samples.

Table 1. Information about samples regarding their origin, declared type or data on the label or personal communication when purchasing the sample (PO facility – pumpkin oil roasting/pressing facility)

Sample	Origin	Declaration-label-explanation
1	Food store	Pumpkin oil from Prekmurje region
2	PO facility	Oil from dehulled seeds of *Cucurbita moschata*, D.
3	PO facility	Oil from dehulled seeds of *Cucurbita moschata*, D.
4	PO facility	Oil from dehulled seeds of *Cucurbita moschata*, D., second pressing
5	Food store	Pumpkin seed oil ("Kmečko bučno olje")
6	PO facility	Warm pressed pumpkin oil
7	PO facility	Cold pressed pumpkin oil

All samples were gathered and analysed between June and October 2006.

1.1.2. Chemicals. All chemicals used are crucial segment in the analytical methods which are standardized and validated and accredited in our laboratory.

1.2. Methods

1.2.1. Determination of trans isomers of fatty acids. Method used is the standardised method from the EEC (1991), Annexes XA & XB and accredited in our laboratory.

1.2.2. Determination of fatty acids composition. The method and the procedure are the same as the method for Determination of trans isomers of fatty acids.

1.2.3. Determination of sterols composition. Method used is the standardised method from the EEC (1991), Annex V and accredited in our laboratory. For the pumpkin seed oil a somehow more polar column must be used in order to separate some Δ-5 sterols from the Δ-7 ones, e.g. HP35.

1.2.4. Determination of tocopherols content. Method used is the standardised method (ISO, 1997) and accredited in our laboratory.

2. Results and discussion

As mentioned before, we followed the decision tree for olive oils from the EC (2003) Regulation because it gives the logical way of judging the oil's characteristics which are in the case of (extra) virgin olive oils and pumpkin seed oils quite similar. Both are or should be produced in a process which does not allow mixing with other (seed) oils, both should be in the form as they are thus not allowing refining and both should be cold pressed (the term "cold" is rather dubious in the pumpkin seed oil production). In the case of PDO 'Extra virgin olive oil from Slovene Istria' this temperature should not exceed 27 °C (EC, 2002b). The decision tree leads the analyst through analyses in the preset order from trans fatty isomers coming first and sterols last. The tocopherols determination is not included in the mentioned olive oil procedures, but we decided to determine its content in pumpkin seed oils because of its relatively easy determination and of the great importance of certain isomers in the case of adulteration with seed oils, especially rapeseed or soybean oils. The decision tree clearly states that when a criterion is not met, the analyses, which follow in the decision tree, are stopped because the fact itself clearly shows that the purity criterion is not met.

2.1. Content of trans isomers of fatty acids

The limits for the content of trans isomers of fatty acids in virgin and extra virgin olive oils are 0.05 wt% (of the total cis and trans isomers) for trans oleic acid and 0.05 wt% for the sum of trans linoleic and trans linolenic isomers. These numbers are derived experimentally and simply mean that if the values are greater than the above-mentioned ones then refining took place. This usually means in the olive oil field that the sample is a mixture that contains refined olive oil or even seed oil. In the case of pumpkin seed oil, the higher value will show the presence of other, usually cheaper seed oils, which all

underwent the refining process. The data for trans fatty acids content range from 0.014 to 0.022 wt% for trans oleic acid and from 0.037 to 0.443 wt% for the sum of trans linoleic and trans linolenic acids thus showing possible adulteration in samples 1, 4, 5 and 6.

2.2. Fatty acids composition

Literature data for pumpkin seed oils show rather big variations in the shares of certain fatty acids (WENLI et al., 2004; BRODNJAK-VONČINA et al., 2005; APPLEQUIST et al., 2006). Experiences in the oils purity assessment teaches us that the crucial factor in predicting the oil purity is not the amount of major fatty acids, but rather the definite presence of minor ones which are not specific to a certain type of oil, e.g. erucic acid (C 22:1) in the mixture can evidence the admixture of a rapeseed oil, linolenic acid (C 18:3) and gadoleinic acid (C 20:1) can evidence the presence of soybean and rapeseed oils, arachidic acid (C 20:0) evidence the presence of soybean, rapeseed and peanut oils and behenic acid (C 22:0) rapeseed and peanut oils (KOPRIVNJAK, 2006). The same can be stated for pumpkin seed oils, possibly by adopting and using certain chemometric methods (BRODNJAK-VONČINA et al., 2005). Our results state that 2 samples, 1 and 4, are probably adulterated, sample 1 has elevated amount of linolenic, gadoleinic and arachidic acids and sample 4 elevated amount of arachidic acid.

2.3. Composition of sterols

The most evident indication of the seed oils adulteration in pumpkin seed oils comes from the fact that pumpkin seed oils contain almost exclusively the Δ7-phytosterols (alpha-spinasterol, Δ-7,22,25 stigmastatrienol, Δ-7 stigmastenol, Δ-7,25 stigmastadienol (MANDL et al., 1999). This peculiarity is restricted to only a few plant families, e.g. Cucurbitaceae and Theaceae (BREINHÖLDER et al., 2002). It was shown that genuine pumpkin seed oils contain almost nil quantities of Δ-5 sterols and that only 1% by weight of admixture of corn oil enhances the β-sitosterol content of the adulterated sample by about 35% (WENZL et al., 2002). The problem arises when the ratio of Δ-5 to Δ-7 sterols, as a limit of genuineness, has to be set. This limit was tentatively set to 10% by BREINHÖLDER and co-workers (2002). The presence of brassicasterol is clear evidence of rapeseed oil admixture according to BREINHÖLDER and co-workers (2002) and MANDL and co-workers (1999). The results of our sterols determination in samples are presented in Table 2.

The picture can get somewhat clearer when we calculate the proposed Δ-5 to Δ-7 sterols ratio and plot it in a graph shown in Fig. 1.

Table 2. Sterols composition (wt%) in samples

Sterol\Sample No	1	2	3	4	5	6	7
Cholesterol	0.2	0.2	0.2	0.1	0.3	0.2	0.1
Brassicasterol	**2.4**	0.0	0.0	0.0	0.0	0.0	0.0
Campesterol	**16.9**	1.4	0.4	**4.9**	1.8	3.8	0.9
Stigmasterol	**6.3**	1.7	0.5	**4.7**	1.6	4.1	1.1
Δ-7Campesterol	1.3	0.0	0.0	0.0	0.0	0.0	0.0
24-Methyl-cholest-7-enol	1.4	3.9	3.1	2.8	1.9	2.2	1.9
Beta-sitosterol	**41.8**	3.0	0.8	**24.5**	**9.4**	**23.1**	**6.0**
Alpha-spinasterol	7.3	23.8	26.2	14.8	22.4	18.1	23.0
Δ-7,22,25 Stigmastatrienol	7.8	16.1	16.0	13.3	23.1	17.0	23.2
Δ-7 Stigmastenol	4.8	7.0	7.1	9.7	5.5	6.8	5.1
Δ-7.25 Stigmastadienol	5.2	22.4	21.4	11.0	18.3	14.2	21.1
Δ-7 Avenasterol	4.6	20.5	24.3	14.2	15.7	10.5	17.6

The bold numbers indicate the possible adulteration with seed oils (e.g. brassicasterol in sample No. 1)

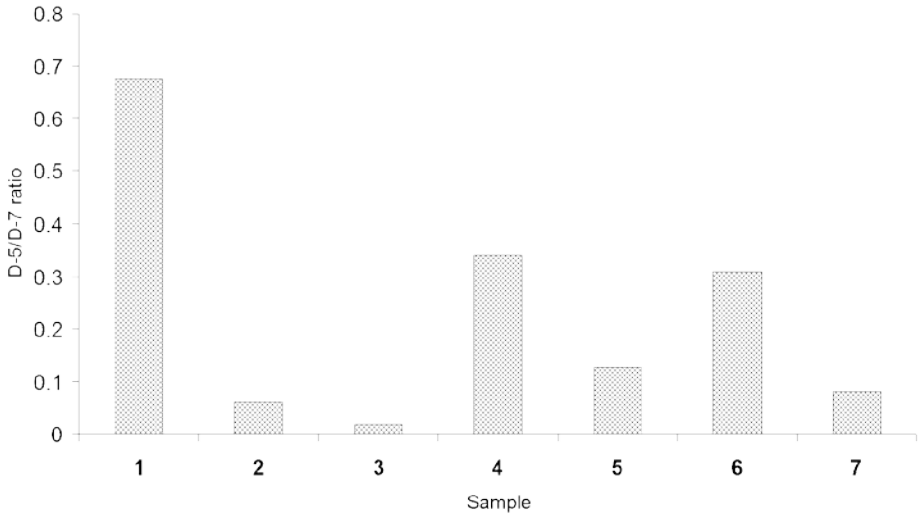

Fig. 1. The Δ-5 to Δ-7 sterols ratio in samples. Only samples No. 2, 3 and 7 can be judged genuine according to BREINHÖLDER and co-workers (2002)

2.4. Content of tocopherols

There are several publications dealing with the tocopherols content in pumpkin seeds (MURKOVIC et al., 1996; MURKOVIC & PFANNHAUSER, 2000) and in pumpkin seed oils (NEĐERAL NAKIĆ et al., 2006). The data show that the prevalent form is the γ-form with

more than 95%, the rest is almost entirely in α-form. The remaining 2 forms (β- and δ-) are practically nil.

Potential adulteration of pumpkin seed oil becomes evident in the case of admixtured seed oils with predominant or elevated tocopherols which are not in the γ-form. This is the case with soybean oil with δ-form and sunflower oil with α-form.

The graphical plot of α-, β- and δ-isomers content in the samples is shown in Fig. 2.

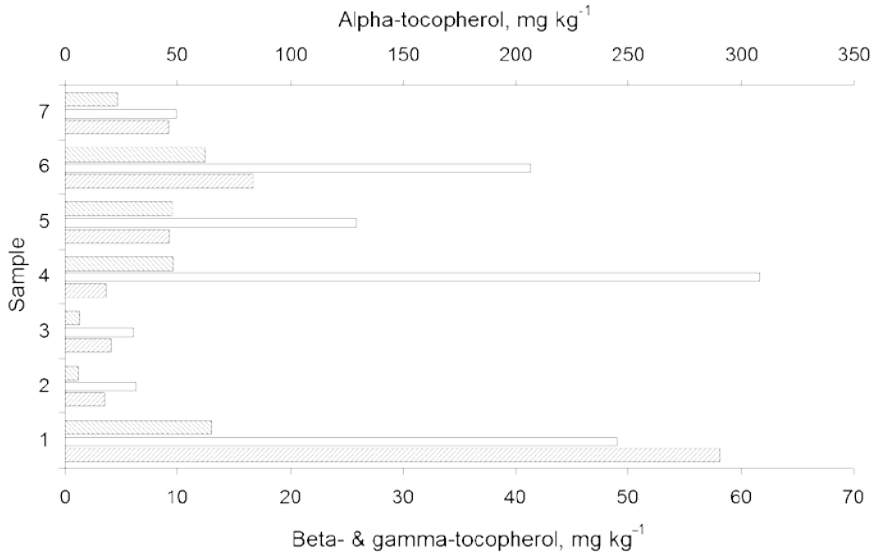

Fig. 2. The α-, β- and δ-tocopherol contents in samples in mg per kg oil showing the samples 1, 4, 5 and 6 being probably adulterated. □: Alpha-tocopherol; ▨: beta-tocopherol; ▨: delta-tocopherol

2.5. Summary

If we summarize all determinations' suppositions which indicate the various possible adulterations a very clear picture arises. It is presented in Table 3.

Table 3. Summarized partial decisions (Y: adulterated, N: genuine) for samples from different determinations (S: sample, D: type of determination T: trans fatty acids content, FA: fatty acids composition, ST: sterols composition, TOCO: content of tocopherol isomers)

S\D	T	FA	S	TOCO
1	Y	Y	Y	Y
2	N	N	N	N
3	N	N	N	N
4	Y	Y	Y	Y
5	Y	N	Y	Y
6	Y	N	Y	Y
7	N	N	N	N

The data from Table 3 show that according to partial determinations samples No. 2, 3 and 7 are probably genuine and samples No. 1, 4, 5 and 6 adulterated (not genuine). As expected, determination of sterols and tocopherols content gives the most profound evidence for adulteration. According to the presented data, fatty acids composition should serve only as an aid in assessing the pumpkin oil genuineness, together with data of trans isomers content.

3. Conclusion

The analytical results based mainly on trans fatty acids content, fatty acid composition, sterols composition and tocols content show that the purity of some samples was doubtful because of a very probable adulteration with cheaper seed oils which was clearly evidenced with sterols composition and tocols content. We deliberately did not enter into the cultivar- and territory-relevant issues and in labelling information being (mostly) misleading, thus making the consumer think that the oil he is buying is genuine and healthy because of the declared 100% pumpkin seed oil. One of the reasons is probably the relatively loose definition of the Protected geographical indications under which the Slovenian pumpkin oil falls, but the main reason could as well be the lack of quality field control or even some consumers not willing to understand and pay for the proven quality.

What can (other) consumers benefit? From the Slovenian olive oil experience it can be concluded that the path towards better quality oils does not strictly follow constant evolving and improving analytical methods, but instead tries to track well- established rules and precise definitions for the process of pumpkin seed oil production, (pumpkin seed oil) quality, purity and genuineness. The consumers (must) learn and are (be) aware that the quality and purity can be analytically proven and expect its evidence to be available and shown.

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References

APPLEQUIST, W.L., AVULA, B., SCHANEBERG, B.T., WANG, YAN-HONG & KHAN, I.A. (2006): Comparative fatty acid content of seeds of four *Cucurbita* species grown in a common (shared) garden. *J. Fd Comp. Anal., 19*, 606–611.

BREINHÖLDER, P., MOSCA, L. & LINDNER, W. (2002): Concept of sequential analysis of free and conjugated phytosterols in different plant matrices. *J. Chromat. B, 777*, 67–82.

BRODNJAK-VONČINA, D., CENCIČ KODBA, Z. & NOVIČ, M. (2005): Multivariate data analysis in classification of vegetable oils characterized by the content of fatty acids. *Chemometrics Intelligent Lab. Syst., 75*, 31–43.

BUČAR-MIKLAVČIČ, M., DUJC, V. & HLAJ, A. (2003): *Elaborat za postopek priznanja označbe geografskega porekla "Ekstra deviško oljčno olje Slovenske Istre"*. (Expert report for the procedure of recognition for the 'PDO' 'Extra virgin olive oil of Slovene Istria'.) DOSI, Koper, Slovenia, pp. 49–59.

DOLŠEK, K. (1997): *Bučno olje*. (Pumpkin seed oil.) Graduation Thesis, University of Ljubljana, Faculty for chemistry and chemical technology, Ljubljana, Slovenija, pp. 4–18.

EC (2002a): Commission Regulation (EC) No 796/2002 of 6 May 2002 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis and the additional notes in the Annex to Council Regulation (EEC) No 2658/87 on the tariff and statistical nomenclature and on the Common Customs Tariff.

EC (2002b): Commission Regulation (EC) No 1019/2002 of 13 June 2002 on marketing standards for olive oil.

EC (2003): Commission Regulation (EC) No. 1989/2003 of 6 November 2003 amending Regulation (EEC) No. 2568/91 on the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis.

EC (2006a): Council Regulation (EC) No 510/2006 of 20 March 2006 on the protection of geographical indications and designations of origin for agricultural products and foodstuffs.

EC (2006b): Publication of an application pursuant to Article 6(2) of Council Regulation (EC) No 510/2006 on the protection of geographical indications and designations of origin for agricultural products and foodstuffs (2006/C 127/08).

EC (2007): Commission Regulation (EC) No 148/2007 of 15 February 2007 registering certain names in the Register of protected designation of origin and protected geographical indications.

EEC (1991): Commission Regulation (EEC) No. 2568/91 of 11 July 1991 on the characteristics of olive oil and olive-residue oil on the relevant methods of analysis.

EEC (1992): Council Regulation No 2081/92 of 14 July 1992 on the protection of geographical indications and designations of origin for agricultural products and foodstuffs.

ISO (1997): *Animal and vegetable fats and oils – Determination of tocopherols and tocotrienols contents – Method using high-performance liquid chromatography*. International Organization for Standardization No. 9936, Geneva, Switzerland.

KARAS, R. (2001): Avstrijci so že zaščitili bučno olje. (Austrians have already registered their pumpkin seed oil.) *Vestnik, 53,* (49) 10.

KOPRIVNJAK, O. (2006): *Djevičansko maslinovo ulje od masline do stola*. (Virgin olive oil from the olive to the table.) MIH d.o.o., Poreč, Croatia, pp. 135–144.

MANDL, A., REICH, G. & LINDNER, W. (1999): Detection of adulteration of pumpkin seed oil by analysis of content and composition of specific Δ7-phytosterols. *Eur. Fd Res. Technol., 209*, 400–406.

MURKOVIC, M. & PFANNHAUSER, W. (2000): Stability of pumpkin seed oil. *Eur. J. Lipid Sci. Technol., 102*, 607–611.

MURKOVIC, M., HILLEBRAND, A., WINKLER, J. & PFANNHAUSER, W. (1996): Variability of vitamin E content in pumpkin seeds (*Cucurbita pepo* L.). *Z. Lebensm Unters Forsch., 202*, 275–278.

NEĐERAL NAKIĆ, S., RADE, D., ŠKEVIN, D., ŠTRUCELJ, D., MOKROVČAK, Ž. & BARTOLIĆ, M. (2006): Chemical characteristics of oils from naked and husk seeds of *Cucurbita pepo* L. *Eur. J. Lipid Sci. Technol., 108*, 936–943.

SIEGMUND, B. & MURKOVIC, M. (2004): Changes in chemical composition of pumpkin seeds during the roasting process for production of pumpkin seed oil (Part 2: volatile compounds). *Fd Chem., 84*, 367–374.

ŠKERLIČ, I., BUČAR-MIKLAVČIČ, M., BANDELJ MAVSAR, D., REŽEK-DONEV, N. & MARŠIČ, K. (2006): *Izdelava predhodne študije ekstra deviškega oljčnega olja z geografskim poreklom.* (Preliminary study for extra virgin olive oil with geographical origin.) University of Primorska, Science and Research Centre of Koper, Koper, Slovenia, pp. 4–8.

WENLI, Y., YAPING, Z., JINGJING, C. & BO, S. (2004): Comparison of two kinds of pumpkin seed oils obtained by supercritical CO_2 extraction. *Eur. J. Lipid Sci. Technol., 106*, 355–358.

WENZL, T., PRETTNER, E., SCHWEIGER, K. & WAGNER, F. (2002): An improved method to discover adulteration of Styrian pumpkin seed oil. *J. biochem. biophys. Methods., 53*, 93–202.

2.2 STEREOSPECIFIČNA ANALIZA TRIACILGLICEROLOV KOT UPORABNO ORODJE ZA OVREDNOTENJE PRISTNOSTI BUČNIH OLJ: LEKCIJA IZ ANALIZ DEVIŠKEGA OLJČNEGA OLJA

Stereospecific analysis of triacylglycerols as a useful means to evaluate genuineness of pumpkin seed oils: lesson from virgin olive oil analyses.
Bojan BUTINAR, Milena BUČAR-MIKLAVČIČ, Vasilij VALENČIČ in Peter RASPOR
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V Sloveniji proizvajajo dve vrhunski rastlinski olji z visoko dodano prehransko vrednostjo: "Ekstra deviško oljčno olje Slovenske Istre z zaščiteno označbo porekla" in "Štajersko prekmursko bučno olje". Njuno kakovost in pristnost je potrebno stalno nadzorovati, saj je verjetnost potvorbe precejšnja. Izkušnje iz preverjanja pristnosti oljčnega olja lahko pokažejo kako lahko izkustveno in podatkovno vodeni preskus, ki temelji na drevesu odločanja in zaobjame več kemijskih določitev ((*E*)-izomeri maščobnih kislin, določitev sterolne sestave in maščobnokislinske sestave) znatno pomaga pri ugotavljanju pristnosti slovenskih bučnih olj. V pričujoči raziskavi predstavljamo rezultate HPLC določitve triacilglicerolov na izboru različnih bučnih olj iz severovzhodne Slovenije, ki so temeljili na obdelavi devetih signifikantnih triacilglicerolv (LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL in SLS). Izvedene določitve so pokazale, da je stereospecifična analiza triacilglicerolov v navezi z ostalimi analitičnimi določitvami temelj zasnove postopka za presojo pristnosti slovenskih bučnih olj.

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Stereospecific Analysis of Triacylglycerols as a Useful Means To Evaluate Genuineness of Pumpkin Seed Oils: Lesson from Virgin Olive Oil Analyses

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In Slovenia two superb vegetable oils with high added nutritional value are produced: "Ekstra deviško oljčno olje Slovenske Istre (extra virgin olive oil from Slovene Istra)" and "Štajersko prekmursko bučno olje (pumpkin seed oil from Slovenia)". Their quality and genuineness must be monitored as adulteration can easily be undertaken. Olive oil genuineness determination experiences can show how analyses following an experience data-driven decision tree gathering several chemical determinations (fatty acids, *(E)*-isomers of fatty acids, sterol and tocopherol determinations) may be helpful in assessing the pumpkin seed oil from Slovenia genuineness. In the present work a set of HPLC triacylglycerol determinations was performed, based on the nine main triacylglycerols (LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL, and SLS) on a limited number of different pumpkin seed oils from northeastern Slovenia. The performed determinations showed that stereospecific analyses of triacylglycerols together with other chemical determinations can be useful in building a protocol for the evaluation of the genuineness of pumpkin seed oil from Slovenia.

KEYWORDS: Chemical assessment; *Cucurbita moschata* D.; *Cucurbita pepo* L.; genuineness; HPLC; principal component analysis; pumpkin seed oil; stereospecific triacylglycerol determination; virgin olive oil

INTRODUCTION

Pumpkin, the New World *Cucurbita* spp., has been known in Europe since the early 16th century (*1*) and soon became appreciated in the Štajerska and Prekmurje regions in northeastern Slovenia (mostly *Cucurbita pepo* L. and to a lesser extent *Cucurbita moschata* D.) as well as in southern Austria as a vegetable and as a source for excellent seed oil pressed from roasted seeds (*2*, *3*). On the other hand, olive has been known in Europe and in Slovenia or better in the part of Slovenia geographically known as the peninsula of Istra since ancient times.

In Slovenia two superb edible vegetable oils with high added nutritional and hedonistic value are produced, extra virgin olive oil from Slovene Istra (*4*) and pumpkin seed oil from the Štajerska and Prekmurje regions (*3*, *5*, *6*). Both oils contain certain pharmacodynamically active substances of non-triacylglycerol origin, mainly phytosterols (*3*, *7*). It was shown that unroasted pumpkin seeds contain dietary lignans, namely, secoisolariciresinol, isolariciresinol, and lariciresinol; however, secoisolariciresinol is destroyed after 20 min of roasting (*8*). Maybe with the pumpkin oils of a new era, the ones "cold" pressed from dry seeds without roasting or chemical treatments (*9*), lignan content may change. In the case of olive drupes dietary lignans are 1-acetoxypinoresinol, pinoresinol, and 1-hydroxypinoresinol (*10*), and some are retained in extra virgin olive oils (*11*). Both oils are known for their hedonistic, cultural, nutritional, and health-promoting aspects (*12*, *13*), which result in a relatively high price if compared to other seed oils. In 2005 the plant oil market in Slovenia was worth >26 million €, with olive oil taking third place with 4 million € and pumpkin seed oil with a 43% price increase for the period 2004−2005 (*14*). With respect to this it is logical to expect adulteration with cheaper seed oils. In the field of olive oil, adulteration can be revealed following the excellent and unique European Commission regulations (*15*, *16*). They give inter alia the methods of analysis, the decision tree for establishing the conformity of the declared type of olive oil with the analyzed parameters, and the limit values needed to assess the characteristics of olive and olive pomace oil.

Seven different and rather tedious analytical procedures from an instrumental point of view must be performed before the sample can be classified as a sample of extra virgin olive oil. All of these procedures are the result of long and cooperative work performed over many years by experts from the olive field and chemical analysts who gathered their knowledge and experiences (*14*).

The present situation in Slovene pumpkin seed oil production is not as strict, and to our best knowledge we are not aware of such

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regulations and procedures one can follow to establish/assess an oil's quality or purity parameters with an exemption of sensorial analyses and some quality determinations. Only genuine pumpkin seed oils can fully preserve the nutritive value the consumer has paid for, and the stereospecific analysis of triacylglycerols can add a value in assessing them. Other not so well-defined parameters are the roasting temperature and roasting time, which greatly influence the pressed roasted pumpkin seed oil aroma and therefore the quality (*17*).

Butinar and co-workers (*14*) tried to establish a procedure to assess a pumpkin seed oil's genuineness, performing a set of analyses described in the olive oil purity decision tree procedure (*15*). These were (*E*)-isomers of fatty acid content, fatty acid content, and composition and quantity of sterols. The tocopherols by normal phase HPLC were determined as well. It was shown that all determinations can lead to a very clear picture of the pumpkin seed's oil genuineness, especially if the final sterol composition is challenged (Δ-5 to Δ-7 sterol ratio). However, sterol composition is the major drawback in genuineness estimation due to complexity of the determination and because of the rather demanding procedure, which can cause problems even for trained sterol analysis personnel.

The efforts of the present work were aimed at another genuineness determination technique widely used in the virgin olive oil purity determination, which is basically a stereospecific analysis of triacylglycerols and is known as a ΔECN 42. It was implemented in 2006 and 2008 (*18*, *19*). The history of the ΔECN 42 determination in olive oils lies in the problem of the olive oils, which were in the past extensively adulterated with admixtures of other extraneous and cheaper seed oils. The method is based on the HPLC triacylglycerol determination. The chromatographic separation follows the equivalent carbon numbers (ECN) rule, where ECN is the number of carbon atoms in a triacylglycerol (not counting the glycerol carbons) minus twice the number of double bonds in a triacylglycerol. The "problematic" triacylglycerols are those with the ECN 42, especially LLL (trilinolein). Thus, the percentage of LLL in the examined oils was adopted as a criterion to detect seed oils in olive oils. Lately, LLL content was substituted by a new parameter, the difference between experimental and theoretical values of triacylglycerols with ECN equal to 42, thus giving more space to the genuine olive oils with higher (but still acceptable) content of linoleic acid (*20*). The theoretical value of ECN 42 is calculated from the fatty acid composition of the purified sample (purification removes possible oxidized products that could interfere with the lower ECN peaks) using a calculation procedure or a programmed macro (*19*). It was developed on the basis of fatty acid and triacylglycerol composition of pure olive oils with proven genuineness assuming a 1,3-random, 2-random distribution of fatty acids in the triacylglycerol, with restrictions for saturated fatty acids in the 2-position (*18*). The method uses a silica column to purify the sample, which is divided into two parts. One part is used to determine fatty acid composition and the other one to determine the practical triacylglycerol composition (HPLC). The liquid chromatographic separation is performed on an octadecylsilyl reverse phase column using almost equivoluminal mixtures of acetone and acetonitrile, thus allowing the optimal separation of all peaks of interest. The mobile phase composition is crucial. The detection is based on the refractive index change and monitored.

To follow the newer adulteration approaches using hazelnut oil, the official method was subjected to changes, introducing certain steps that should give more space to possible discovery of olive oils adulterated with oils with similar fatty acid composition. The improvements were the utilization of a silica SPE cartridge, substitution of the acetone/acetonitrile mobile phase with 100% propionitrile, and utilization of the column compartment heater/cooler, thus allowing the analysis to be performed in the stable temperature environment of 20 °C or less. Such a modification produces a much more stable baseline and allows more peaks to be separated and better peak resolution. The propionitrile mobile phase approach with a flow gradient was successful in chemometric characterization and differentiation of French virgin olive oils (*21*, *22*).

To our best knowledge there are not many such works performed; they were mainly focused on triacylglycerol determination in various seed oils with accent on particular chromatographic technique evaluation (*23*, *24*). With respect to this knowledge, the focus of the work was on triacylglycerol determination in the chosen pumpkin seed oil samples with known origin and previously determined status of adulteration/genuineness (fatty acids determination, (*E*)-isomers of fatty acids, Δ-5 to Δ-7 sterol ratio, tocopherols). Finally, their PCA elaborated stereospecific triacylglycerol composition data were questioned, in view of a possible complementary analytical tool to assess pumpkin seed oil genuineness. Consequently, we see the purpose of this research oriented to the development of a tool that will have discriminating capacity for closely related/adulterated pumpkin seed oils.

MATERIALS AND METHODS

Materials. *Pumpkin Seed Oil Samples.* Seven different pumpkin seed oil samples were examined. They were bought in local stores in the Prekmurje region or purchased locally at the pumpkin seed oil roasting/pressing facilities. All samples were gathered in June and July 2006 and analyzed between June and October 2006. After arrival at the laboratory, the samples were immediately divided into 50 mL portions and deep frozen at −30 °C until analysis. Just prior to analysis, they were simply room thawed and used according to the chosen procedure. **Table 1** gives detailed information about the samples. It should be emphasized that definition of the terms cold-pressed/warm-pressed oils is still very ambiguous.

Chemicals and Reference Material. All chemicals and materials needed to perform sample analyses were chosen and used in accordance with the laboratory's quality system accredited in accordance with ISO 17025.

TAG standards (LLL, LnLnLn, LLO, LLP, LOO, POL, OOO, POO) were purchased from Sigma-Aldrich Chemie Gmbh (Munich, Germany).

TAG CRM IRMM-801 standard (PPS, POP, PLP, POS, POO, PLS, PLO, SOO, SLS, SLO) was purchased from EC-JRC-IRMM (Geel, Belgium).

Olive oil reference samples with certified TAG composition were from International Olive Council (IOC, Madrid, Spain).

Various fat reference samples with certified TAG composition were from BIPEA (Gennevilliers, France).

Analytical Methods. The official method for the ΔECN 42 determination in olive oils (*19*) is COI/T.20/Doc. 20/Rev. 2 - 2008, Determination of the difference between actual and theoretical content of triacylglycerols with ECN 42.

The global method for the detection of extraneous oils in olive oils (*18*) is COI/T.20/Doc. No. 25 - 2006.

Analytical Method and HPLC Equipment Used. An SPE silica gel cartridge (1 g/6 mL) was washed with 6 mL of hexane, not allowing the cartridge to dry. Approximately 0.12 g of the oil dissolved in 0.5 mL of hexane was loaded into the cartridge; the solution was pulled through and then eluted with 10 mL of hexane/diethyl ether (87:13 v/v) under vacuum. The eluted solution was homogenized and divided into two equal parts. Both solutions were separately evaporated under reduced pressure, and the part for HPLC analysis was dissolved in 1 mL of acetone of such a purity that no extra spikes or interferences were seen when it was injected into the HPLC system. The other part was needed for GC fatty acid analysis after it had been dissolved in *n*-heptane, but this step was not performed because of the pumpkin seed oil specificity and because the fatty acid composition was established previously (*14*).

Ten microliters of acetone solution was injected into an Agilent HPLC 1100 HPLC apparatus equipped with a BinPump G1312A binary pump used together with a thermostabilized ALS G1329A autosampler, an ALS-Therm G1330B autosampler thermostat, a thermostabilized COLCOM

Table 1. Information about Samples Including the Code, Origin, Declared Type of Pumpkin Seed Oil (from the Label or from Personal Communication Gained When the Sample Was Obtained), and Previously Determined Adulteration Status[a]

sample	purchased in	label, explanation, origin	adulteration status
S1	food store A	pumpkin oil from Prekmurje region	A
S2	PO facility A	oil from dehulled seeds of *Cucurbita moschata* D., second pressing	G
S3	PO facility A	oil from dehulled seeds of *Cucurbita moschata* D., first pressing	G
S4	PO facility A	oil from dehulled seeds of *Cucurbita moschata* D., third pressing	A
S5	food store B	pumpkin seed oil ("Kmečko bučno olje")	A
S6	PO facility B	warm-pressed pumpkin seed oil	A
S7	PO facility B	cold-pressed pumpkin seed oil	G

[a] Food store A(B), two different local food stores; PO facility A(B), two different pumpkin oil roasting/pressing facilities; first pressing, the ground and roasted seeds were pressed in the freshly prepared unheated clean press; second pressing, the ground and roasted seeds were pressed in the heated press immediately following the first pressing; third pressing, the ground and roasted seeds and previously obtained seed cake (pumpkin seeds from first and second pressing) were mixed together in nonrevealed proportion and hot pressed (possibly with the aid of cheap seed oil to maximize the extracted oil quantity); warm-pressed pumpkin seed oil, ground and roasted pumpkin seeds were pressed in a heated medium press; cold-pressed pumpkin seed oil, according to the PO facility B owner ground pumpkin seeds were pressed in an unheated press without prior roasting; adulteration status, A (adulterated)/G (genuine), based on a set of chemical analyses described and performed in ref *14*.

G1316A column compartment, and a refractive index detector operating at 35 °C. A Phenomenex LiChrospher/Superspher RP18 80A (4 μm i.d., 250 × 4.0 mm) column was used and propionitrile as the mobile phase. To achieve better resolution, the column compartment temperature was kept at 15 °C. The chromatographic system was run with Agilent Chemstation v. 10.01 software. The software was used for the integration and elaboration of the chromatographic data as well.

Peak identification was performed on the basis of TAG standards, olive oil reference samples used in our accredited laboratory for performing the "Determination of the difference between actual and theoretical content of triacylglycerols with ECN 42" analysis and which are traceable to the International Olive Council (IOC, Madrid, Spain). IOC is (besides other important activities) an organizer of the Proficiency Test (PT) schemes from the olive oils area. Peak identification was performed as well on the basis of our laboratory's participation in PT schemes organized by BIPEA (Gennevilliers, France) for different fat and oil samples with certified triacylglycerol compositions and on the basis of our observations comparing certain pumpkin seed oil samples with the literature data dealing with triacylglycerol composition of pumpkin seed oils or with oils with similar fatty acid composition (*23*−*25*). The areas of integrated peaks were assigned as triacylglycerols and were evaluated with the assumption with identical reference factors (chromatographic peak vs peak area ratio) (*19*). All triacylglycerol areas were summed, and each triacylglycerol was expressed as a percent ratio versus the sum of all triacylglycerols (normalization method).

Each sample was determined in parallel, the results for each triacylglycerol were averaged, and the standard deviation was calculated.

Statistical Analysis. The analysis of chromatographic data was performed as one-way analysis of variance (ANOVA) for each triacylglycerol species in all seven samples using Statgraphics Plus 4.0 software. The differences for major triacylglycerols among samples (group means) were estimated using the Tukey honestly significant difference (Tukey HSD) test at $p < 0.05$. The principal component analysis (PCA) from the same software package was used as well.

Triacylglycerol Nomenclature. The triacylglycerols (TAG) are designated by letters corresponding to abbreviated names of fatty acid carbon chains that are linked to the glycerol. The fatty acids abbreviations together forming the TAG are as follows: P, palmitoyl; S, stearoyl; O, oleoyl; L, linoleoyl; and Ln, linoleolenyl (*21*).

RESULTS AND DISCUSSION

HPLC Approach. Using the official method (*19*) with propionitrile as mobile phase and column temperature control, we were able to separate 29 different triacylglycerols within 70 min as shown in **Figure 1**. Twenty-five of them were assigned, and the four unidentified triacylglycerols (marked NAS1, NAS2, NAS3, and NAS4) were almost evenly grouped across triacylglycerols with ECNs of 42 (NAS1), 48 (NAS2), and 50 (NAS3 and NAS4), and their sum ranged from 0.85 wt % (S2) to 1.10 wt % (S7). From these data it can be concluded their contribution to the overall triacylglycerol amount for the analyzed samples was not

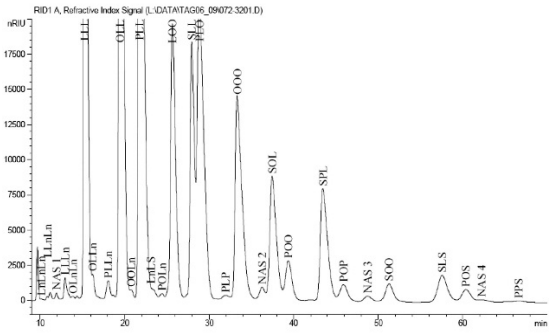


Figure 1. HPLC chromatogram of sample S2 using propionitrile as mobile phase. Chromatographic conditions: detection, RID (35 °C); flow, 0.638 mL/min; chromatographic column, Phenomenex LiChrospher/Superspher RP18 80A (4 μm i.d., 250 × 4.0 mm); column temperature, 15 °C; signal in nRIU, time in minutes. The assigned peaks are respective triacylglycerols.

relevant, because of their low amount and because of the rather small variations across the samples.

The triacylglycerol elution order was as follows: LnLnLn, LLnLn, NAS1, LLLn, OLnLn, LLL, OLLn, PLLn, OLL, OOLn, PLL, LnLS, POLn, LOO, SLL, PLO, PLP, OOO, NAS2, SOL, POO, SPL, POP, NAS3, SOO, SLS, POS, NAS4, PPS.

Elaboration of HPLC Data. From an examination of **Table 2** it can be concluded the number of 29 separated triacylglycerols is greater than previously reported (*23*, *24*). References *23* and *24* both report 12 triacylglycerols each. From the number of separated triacylglycerols it is obvious the separation using the thermostabilized column compartment and propionitrile can give much better results. A work dealing with the antioxidant compounds in commercial oilseeds for food use found 20 different triacylglycerol species in pumpkin seed oil (*26*). A comparable HPLC determination was used (without sample precleaning); however, the mobile phase used was acetone/acetonitrile in a volume ratio of 65:35. Unfortunately, the paper shows no chromatograms, so further comments would be purely speculation, although experience from our laboratory demonstrates the approach using propionitrile and temperature control gives more accurate and more repeatable data with greater baseline stability. They give data for a single pumpkin seed oil bought in a Polish market in the year 2004 or 2005 and analyzed in triplicate. Comparison of the six main triacylglycerols in their pumpkin seed oils with the S2, which our previous work showed to be genuine (four criteria of four) (*14*), thus being comparable, shows there exist similarities between our and their work as follows: PLL, 26.1 versus 16.95 wt %; LLL,

Table 2. Differences in Triacylglycerol Composition for Seven Pumpkin Seed Oils (S1−S7) in Weight Percent from Northeastern Slovenia Determined by HPLC Using Propionitrile as Mobile Phase[a]

TAG	S1	SD	S2	SD	S3	SD	S4	SD	S5	SD	S6	SD	S7	SD
LnLnLn	0.30	0.01	0.03	0.00	0.03	0.00	0.01	0.00	0.02	0.00	0.02	0.00	0.02	0.00
LLnLn	0.10	0.02	0.09	0.01	0.22	0.00	0.78	0.00	0.10	0.00	0.09	0.00	0.17	0.01
NAS1	0.05	0.00	0.09	0.02	0.18	0.00	0.06	0.00	0.11	0.01	0.13	0.00	0.22	0.01
LLLn	2.34	0.00	0.36	0.04	0.43	0.02	0.18	0.00	0.20	0.02	0.25	0.01	0.24	0.01
OLnLn	0.08	0.00	0.05	0.02	0.15	0.01	0.50	0.01	0.13	0.02	0.16	0.01	0.23	0.01
LLL	**16.09**	0.09	**16.95**	0.08	**17.36**	0.00	**26.17**	0.11	**14.51**	0.05	**16.47**	0.04	**11.11**	0.02
OLLn	2.41	0.01	0.36	0.07	0.38	0.01	0.17	0.02	0.35	0.02	0.39	0.01	0.31	0.01
PLLn	1.19	0.00	0.42	0.07	0.58	0.02	0.39	0.02	0.38	0.01	0.34	0.03	0.33	0.01
OLL	**19.97**	0.11	**16.26**	0.08	**16.64**	0.02	**23.31**	0.15	**20.17**	0.09	**21.69**	0.04	**18.26**	0.04
OOLn	1.83	0.01	0.15	0.02	0.23	0.00	0.43	0.04	0.23	0.02	0.26	0.02	0.26	0.01
PLL	**9.88**	0.04	**19.42**	0.07	**20.09**	0.01	**13.36**	0.08	**12.46**	0.07	**11.54**	0.02	**10.13**	0.01
LnLS	0.76	0.01	0.21	0.03	0.27	0.01	0.29	0.03	0.22	0.01	0.23	0.03	0.25	0.01
POLn	0.24	0.01	0.15	0.03	0.19	0.00	0.28	0.01	0.16	0.00	0.22	0.03	0.09	0.01
LOO	**13.27**	0.02	**7.17**	0.02	**6.41**	0.01	**7.36**	0.01	**14.40**	0.08	**13.83**	0.02	**15.97**	0.01
SLL	3.74	0.01	5.54	0.01	5.57	0.00	5.97	0.02	4.25	0.01	4.19	0.02	3.59	0.00
PLO	**7.58**	0.02	**10.53**	0.00	**10.70**	0.00	**6.81**	0.02	**9.75**	0.05	**9.00**	0.01	**10.64**	0.01
PLP	0.35	0.02	0.20	0.04	0.29	0.01	0.24	0.03	0.22	0.03	0.23	0.01	0.28	0.00
OOO	**8.37**	0.01	**7.37**	0.02	**6.78**	0.01	**3.90**	0.08	**8.23**	0.00	**7.68**	0.03	**10.59**	0.01
NAS2	0.36	0.02	0.40	0.02	0.44	0.01	0.47	0.03	0.35	0.01	0.34	0.00	0.30	0.01
SOL	3.26	0.00	4.37	0.01	4.09	0.02	3.06	0.02	4.55	0.00	3.98	0.01	4.70	0.00
POO	2.56	0.02	1.45	0.04	1.19	0.02	0.83	0.01	2.96	0.05	2.79	0.06	4.41	0.02
SPL	1.51	0.05	4.38	0.04	4.04	0.00	2.23	0.05	1.94	0.01	1.80	0.05	1.95	0.02
POP	0.83	0.01	0.83	0.13	0.89	0.02	0.94	0.04	0.73	0.00	0.88	0.03	1.10	0.04
NAS3	0.30	0.00	0.27	0.04	0.31	0.00	0.20	0.03	0.30	0.03	0.31	0.04	0.38	0.00
SOO	1.29	0.02	0.81	0.01	0.67	0.02	0.50	0.01	1.71	0.04	1.58	0.03	2.56	0.04
SLS	0.51	0.02	1.37	0.00	1.21	0.03	0.90	0.02	0.71	0.07	0.70	0.05	0.68	0.05
POS	0.42	0.00	0.63	0.01	0.52	0.01	0.31	0.03	0.57	0.03	0.52	0.04	0.84	0.02
NAS4	0.28	0.01	0.10	0.00	0.12	0.01	0.32	0.01	0.18	0.01	0.28	0.04	0.21	0.03
PPS	0.15	0.06	0.04	0.00	0.02	0.00	0.03	0.00	0.11	0.02	0.10	0.03	0.18	0.03

[a] SD columns represent the standard deviation in wt % from duplicate determinations. The rows in bold represent the first six triacylglycerols (PLL, LLL, OLL, PLO, OOO, and LOO), which account for >77% of the overall triacylglycerol amount in S2, which was previously found to be genuine (*14*).

9.2 versus 16.95 wt %; OLL, 17.2 versus 16.26 wt %; PLO, 5.1 versus 10.53 wt %; OOO, 5.4 versus 7.37 wt %; and LOO, 11.5 versus 7.17 wt% for their work and our results, respectively. Considering the well-known differences in fatty acid composition among different pumpkin varieties (*3*, *27*−*30*) and the not specified pumpkin variety in the oil analyzed by Tuberoso et al. (*26*), we can assume comparable agreement.

Our goal was to discriminate the analyzed pumpkin seed oils on the basis of their (assumed) adulteration or (assumed) genuineness.

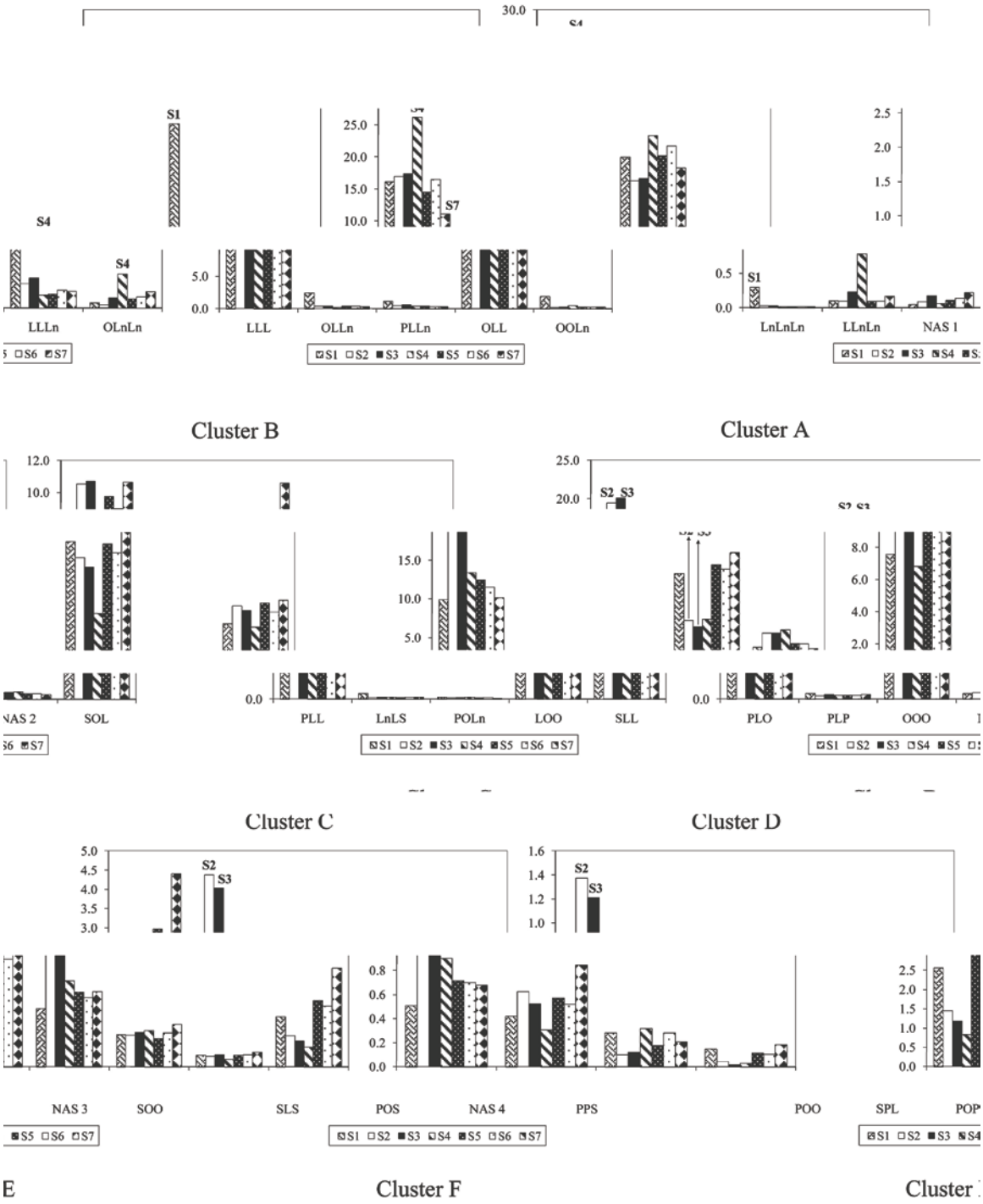
At first glance, the results from **Table 2** show some similarities, and two somehow distinct profiles in the composition pattern for samples S2 and S3 and for samples S5 and S6 can be spotted; however, this is not obvious for the rest of the samples, although PLL seems to dominate the discrimination among profiles. For that reason it is obvious that one should approach from a more focused triacylglycerol amount ratio perspective. Instead of finding clusters of samples with identical triacylglycerol patterns, one should determine which triacylglycerols can act as a discriminating tool for the samples. **Figure 2** thus gives the same data that were shown in **Table 2** but divided among six clusters with the following triacylglycerols in each: cluster A, LnLnLn, LLnLn, NAS1, LLLn, OLnLn; cluster B, LLL, OLLn, PLLn, OLL, OOLn; cluster C, PLL, LnLS, POLn, LOO, SLL; cluster D, PLO, PLP, OOO, NAS2, SOL; cluster E, POO, SPL, POP, NAS3, SOO; and cluster F, SLS, POS, NAS4, PPS. Analyzing **Figure 2** one could see which triacylglycerols might act as a discriminating tool for the samples. Cluster A reveals LLLn as dominating, clearly showing S1's accentuated amount of LLLn with >2 wt %. In cluster B LLL shows discrimination among samples and separates samples S4 and S7 from the rest. Cluster C gives two possibilities: PLL and LOO have samples S2 and S3 in a well-defined group in both cases. PLO and OOO are dominating triacylglycerols in cluster D, whereas cluster E shows discrimination in SPL for samples S2 and S3 again. To a lesser extent this stands for POO as well. Cluster F repeats the grouping mentioned before samples S2 and S3 with SLS.

Inspection of the data from the clusters thus identifies 9 possible triacylglycerols (LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL, and SLS) evenly distributed among all 29 triacylglycerols and covering the amount ratio from 0.18 to 26.17 wt %. These 9 triacylglycerols could act as a useful tool in discriminating the samples according to the triacylglycerol distribution.

A closer inspection of the data plotted in **Figure 3** reveals five different groups with samples S2 and S3 in the first group, samples S5 and S6 in the second one, and samples S1, S4, and S7 each defining its single group. Obviously these data per se cannot be too promising because the existing literature data are true only for defined nonadulterated samples, thus lacking the crucial set of parameters that could help one in assigning a pattern for sample recognition.

It has been shown so far that the different pumpkin seed oils can be discriminated according to some definite triacylglycerol species. However, this procedure could be satisfying only if a well-defined adulterated or genuine sample existed in the series. The analyzed triacylglycerols could then serve as a relative aid if compared to sets of known origin.

Principal Component Analysis (PCA). To find another and more revealing way of discriminating adulterated samples from genuine ones and vice versa, the PCA approach was challenged. PCA is a linear transformation of a set of original data to a set of uncorrelated components in such a way that only a few of the



amples of pumpkin seed oils (S1−S7) from northeastern Slovenia plotted in clusters
ıcylglycerols from each cluster and the *y*-axis triacylglycerol amount in wt %. Different
the text.

Figure 2. Triacylglycerol composition pattern distribution among seven s
A−F representing 29 determined triacylglycerols. The *x*-axis denotes tria
letters under the graphs represent different triacylglycerol clusters from

ır- set. The PCA approach and stepwise analysis of various triacyl- resulting variables account for the majority of the variability obse
fy glycerols finally elucidated seven variables: LLLn, LLL, sum of ved in the original data. Tsimidou et al. (*31*, *32*) tried to classi

The first group shown in the bottom of **Figure 4** is composed of

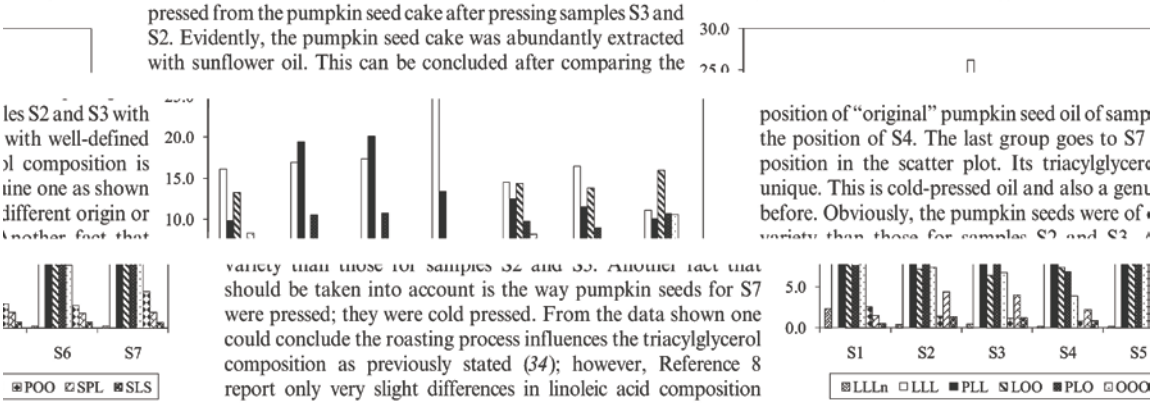
This fact could lead us to a conclusion that each sample has a

e much defined triacylglycerol composition. If we account for the samples S2 and S3, which were in fact pressed out from the sam
y fact that two of them are adulterated (S1 and S4) and for their pumpkin seeds in a differently driven press and shown previousl
5 relative positions (very well apart in the scatter plot), a conclusion to be genuine (*14*). The second group is composed of samples S
e can be drawn that either they were pressed from very different and S6, the first being bought in food store B and the second on
it pumpkin seeds or they were adulterated with very different seed warm pressed in PO facility B. For them as well it was shown tha
n oils or even both. Indeed, from the tocopherol analysis (S1 has they are adulterated: three criteria of four for each of ther

l; on the revealed the admixture of the seed oil. The only criterion that 58 mg/kg of δ-tocopherol and 245 mg/kg of α-tocopherc
lent (*14*) was not positive for the adulteration was fatty acid composition, other hand, S4 has 309 mg/kg of α-tocopherol) it is evic
wer oil. which reveals that the degree of adulteration was not very that S1 is adulterated with soy oil and S4 with sunflc
t isomer elevated and thus could not be proven in the fatty acid composi- Usually, in genuine pumpkin seed oils the predominan
rol (10– tion determination. The sample's vicinity in the scatter plot points is γ-tocopherol (> 500 mg/kg), followed by α-tocophe

he remain- 100 mg/kg) and δ-tocopherol (5–15 mg/kg). The Δ-5 to Δ-7 to possible admixture of the seed oil of the same type. I
:h (S1, S4, sterol ratio in S1 and S4 is much greater than 0.1, which again is a ing three groups are composed of a single sample eac
:atter plot. confirmation of the adulteration with seed oil(s) (*14*). S4 was and S7) and have very well-defined positions in the sc
pressed from the pumpkin seed cake after pressing samples S3 and
S2. Evidently, the pumpkin seed cake was abundantly extracted
with sunflower oil. This can be concluded after comparing the

les S2 and S3 with position of "original" pumpkin seed oil of samp
with well-defined the position of S4. The last group goes to S7
l composition is position in the scatter plot. Its triacylglycerc
ine one as shown unique. This is cold-pressed oil and also a genu
different origin or before. Obviously, the pumpkin seeds were of



variety than those for samples S2 and S3. Another fact that
should be taken into account is the way pumpkin seeds for S7
were pressed; they were cold pressed. From the data shown one
could conclude the roasting process influences the triacylglycerol
composition as previously stated (*34*); however, Reference 8
report only very slight differences in linoleic acid composition

54.6 vs 54.0%).

reconsideration and even before and after the roasting process (

sed pumpkin seed oils". These facts could lead us toward

es the temperature of the redefinition of the term "cold-pres

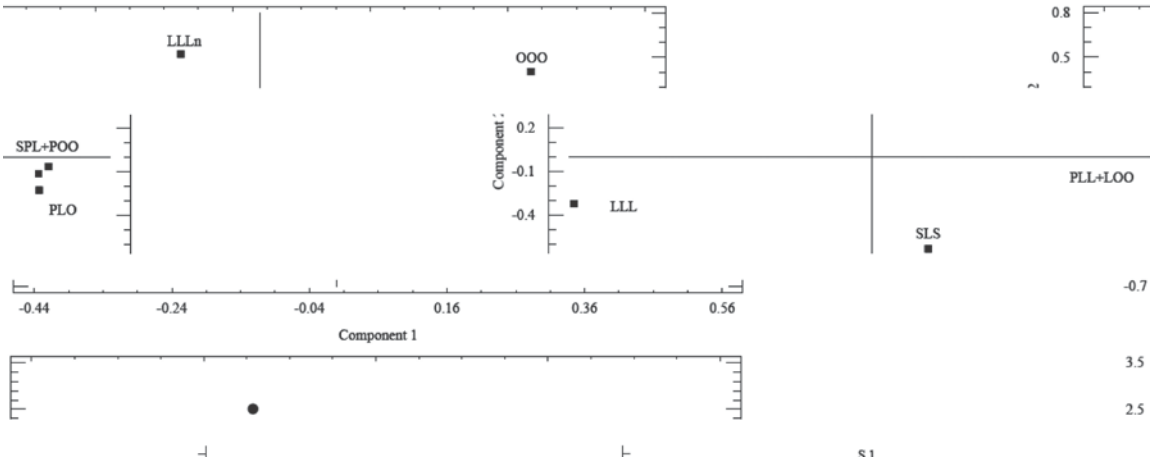
ed 40 °C but often reaches Usually, cold pressing simply denot

d (due to expansion of extra press medium, which should not exce

60 °C. Recently, this term was enlarge

Figure 3. Triacyglycerol composition pattern distribution among seven pumpkin seed oils (S1–S7) from northeastern Slovenia plot based on the selection of nine different triacylglycerols (LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL, and SLS). Legend denotes selected triacylglycerols. The *x*-axis denotes analyzed samples and the *y*-axis triacylglycerol amount in wt %.

virgin olive oil benefits) to cover seed oils as well (*35*), so the new



term "cold-pressed pumpkin seed oil" AND NOT "cold-pressed roasted pumpkin seed oil" should mean pressed ground seeds were not roasted before pressing (*9*), thus lacking their characteristic flavor.

It was demonstrated that guided chemical assessment can be very useful in checking pumpkin seed oil genuineness, especially if properly linked to stereospecific analysis of triacylglycerols. The set of analyzed pumpkin seed oils (adulterated and genuine) from northeastern Slovenia has well-defined intrinsic differences resulting from their different triacylglycerol compositions. In the future a more profound discrimination between various pumpkin seed oils from the market and/or from the field, based on the nine main triacylglycerols, LLLn, LLL, PLL, LOO, PLO, OOO, SPL, POO, and SLS needed for the PCA, can be undertaken. Stereospecific analysis of triacylglycerols per se gives a profound insight into a sample lipid composition and is less time-consuming if compared to sterol analysis; however, a larger number of samples with stated genuineness and/or adulteration state is needed to establish a comprehensive reference pool.

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LITERATURE CITED

(1) Paris, H. S.; Janick, J. Early evidence for the culinary use of squash flowers in Italy. *Chronica Hortic.* **2005**, *45* (2), 20–21.

(2) Bavec, F.; Gril, L.; Grobelnik-Mlakar, S.; Bavec, M. Production of pumpkin for oil. In *Trends in New Crops and New Uses*; Janick, J., Whipkey, A., Eds.; ASHS Press: Alexandria, VA, 2002; pp 187−190.

(3) Fruhwirth, G. O.; Hermetter, A. Seeds and oil of the Styrian oil pumpkin: components and biological activities. *Eur. J. Lipid Sci. Technol.* **2007**, *109* (11), 1128–1140.

(4) Bandelj, D.; Jakse, J.; Javornik, B. DNA fingerprinting of olive varieties by microsatellite markers. *Food Technol. Biotechnol.* **2002**, *40* (3), 185–190.

(5) Kreft, I.; Stibilj, V.; Trkov, Z. Iodine and selenium contents in pumpkin (*Cucurbita pepo* L.) oil and oil-cake. *Eur. Food Res. Technol.* **2002**, *215* (4), 279–281.

(6) Nakic, S. N.; Rade, D.; Skevin, D.; Strucelj, D.; Mokrovcak, Z.; Bartolic, M. Chemical characteristics of oils from naked and husk seeds of *Cucurbita pepo* L. *Eur. J. Lipid Sci. Technol.* **2006**, *108* (11), 936–943.

(7) Sivakumar, G.; Bati, C. B.; Perri, E.; Uccella, N. Gas chromatography screening of bioactive phytosterols from mono-cultivar olive oils. *Food Chem.* **2006**, *95* (3), 525–528.

(8) Murkovic, M.; Piironen, V.; Lampi, A. M.; Kraushofer, T.; Sontag, G. Changes in chemical composition of pumpkin seeds during the roasting process for production of pumpkin seed oil (part 1: non-volatile compounds). *Food Chem.* **2004**, *84* (3), 359–365.

(9) Bavec, F.; Grobelnik-Mlakar, S.; Rozman, C.; Bavec, M. Oil pumpkins: niche for organic producers. In *Issues in New Crops and New Uses*; Janick, J., Whipkey, A., Eds.; ASHS Press: Alexandria, VA, 2007; pp 185−189.

(10) Oliveras Lopez, M. J.; Innocenti, M.; Ieri, F.; Giaccherini, C.; Romani, A.; Mulinacci, N. HPLC/DAD/ESI/MS detection of lignans from Spanish and Italian *Olea europaea* L. fruits. *J. Food Compos. Anal.* **2008**, *21* (1), 62–70.

(11) Owen, R. W.; Mier, W.; Giacosa, A.; Hull, W. E.; Spiegelhalder, B.; Bartsch, H. Identification of lignans as major components in the phenolic fraction of olive oil. *Clin. Chem.* **2000**, *46* (7), 976–988.

(12) EC, Publication of an application pursuant to Article 6(2) of Council Regulation (EC) No. 510/2006 on the protection of geographical indications and designations of origin for agricultural products and foodstuffs. *Off. J. Eur. Union* **2009**, *C72* (52), 67−69.

(13) EU, Council regulation (EC) No. 510/2006 − Application for registration according to Article 5 and Article 17(2) − 'Ekstra deviško oljčno olje Slovenske Istre'. *Off. J. Eur. Union* **2006**, *C127*, 16−18.

(14) Butinar, B.; Bucar-Miklavcic, M.; Krumpak, A.; Raspor, P. Experiences in olive oil purity and quality assessment as a tool for pumpkin seed oil evaluation. What can consumers benefit? *Acta Aliment. Hung.* **2009**, *38* (2), 219–227.

(15) EC, Commission Regulation (EC) No. 1989/2003 of 6 November 2003 amending Regulation (EEC) No. 2568/91 on the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis. *Off. J. Eur. Union* **2003**, *L295*, 57−77.

(16) EEC, Commission Regulation (EEC) No. 2568/91 of 11 July 1991 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis. *Off. J. Eur. Union* **1991**, *L248*, 1−83.

(17) Siegmund, B.; Murkovic, M. Changes in chemical composition of pumpkin seeds during the roasting process for production of pumpkin seed oil (part 2: volatile compounds). *Food Chem.* **2004**, *84* (3), 367−374.

(18) IOC COI/T.20/Doc. No. 25 − 2006 Global method for the detection of extraneous oils in olive oils; http://www.internationaloliveoil.org/downloads/25.AVELLeng.pdf.

(19) IOC COI/T.20/Doc. 20/Rev. Two − 2008 Determination of the difference between actual and theoretical content of triacylglycerols with ECN 42.; http://www.internationaloliveoil.org/downloads/testing7.pdf.

(20) Moreda, W.; Perez-Camino, M. C.; Cert, A. Improved method for the determination of triacylglycerols in olive oils by high performance liquid chromatography. *Grasas Aceites* (*Sevilla*) **2003**, *54* (2), 175–179.

(21) Ollivier, D.; Artaud, J.; Pinatel, C.; Durbec, J. P.; Guerere, M. Triacylglycerol and fatty acid compositions of French virgin olive oils. Characterization by chemometrics. *J. Agric. Food Chem.* **2003**, *51* (19), 5723–5731.

(22) Ollivier, D.; Artaud, J.; Pinatel, C.; Durbec, J. P.; Guerere, M. Differentiation of French virgin olive oil RDOs by sensory characteristics, fatty acid and triacylglycerol compositions and chemometrics. *Food Chem.* **2006**, *97* (3), 382–393.

(23) Andrikopoulos, N. K.; Chiou, A.; Mylona, A. Triacylglycerol species of less common edible vegetable oils. *Food Rev. Int.* **2004**, *20* (4), 389–405.

(24) Jakab, A.; Heberger, K.; Forgacs, E. Comparative analysis of different plant oils by high-performance liquid chromatography−atmospheric pressure chemical ionization mass spectrometry. *J. Chromatogr., A* **2002**, *976* (1−2), 255–263.

(25) Fiebig, H. J. HPLC separation of triglycerides. *Fett. Wiss. Technol.* **1985**, *87* (2), 53–57.

(26) Tuberoso, C. I. G.; Kowalczyk, A.; Sarritzu, E.; Cabras, P. Determination of antioxidant compounds and antioxidant activity in commercial oilseeds for food use. *Food Chem.* **2007**, *103* (4), 1494–1501.

(27) Applequist, W. L.; Avula, B.; Schaneberg, B. T.; Wang, Y. H.; Khan, I. A. Comparative fatty acid content of seeds of four *Cucurbita* species grown in a common (shared) garden. *J. Food Compos. Anal.* **2006**, *19* (6−7), 606–611.

(28) Murkovic, M.; Hillebrand, A.; Winkler, J.; Leitner, E.; Pfannhauser, W. Variability of fatty acid content in pumpkin seeds (*Cucurbita pepo* L.). *Z. Lebensm. Unters. Forsch.* **1996**, *203* (3), 216–219.

(29) Stevenson, D. G.; Eller, F. J.; Wang, L. P.; Jane, J. L.; Wang, T.; Inglett, G. E. Oil and tocopherol content and composition of pumpkin seed oil in 12 cultivars. *J. Agric. Food Chem.* **2007**, *55* (10), 4005–4013.

(30) Zhong, H. Y.; Bedgood, D. R.; Bishop, A. G.; Prenzler, P. D.; Robards, K. Endogenous biophenol, fatty acid and volatile profiles of selected oils. *Food Chem.* **2007**, *100* (4), 1544–1551.

(31) Tsimidou, M.; Macrae, R.; Wilson, I. Authentication of virgin olive oils using principal component analysis of triglyceride and fatty-acid profiles. 1. Classification of Greek olive oils. *Food Chem.* **1987**, *25* (3), 227–239.

(32) Tsimidou, M.; Macrae, R.; Wilson, I. Authentication of virgin olive oils using principal component analysis of triglyceride and fatty-acid

profiles. 2. Detection of adulteration with other vegetable-oils. *Food Chem.* **1987**, *25* (4), 251–258.
(33) Aranda, F.; Gomez-Alonso, S.; del Alamo, R. M. R.; Salvador, M. D.; Fregapane, G. Triglyceride, total and 2-position fatty acid composition of Cornicabra virgin olive oil: comparison with other Spanish cultivars. *Food Chem.* **2004**, *86* (4), 485–492.
(34) Yoshida, H.; Tomiyama, Y.; Kita, S.; Mizushina, Y. Roasting effects on fatty acid distribution of triacylglycerols and phospholipids in the kernels of pumpkin (*Cucurbita* spp) seeds. *J. Sci. Food Agric.* **2005**, *85* (12), 2061–2066.
(35) Matthaus, B.; Spener, F. What we know and what we should know about virgin oils − general introduction. *Eur. J. Lipid Sci. Technol.* **2008**, *110* (7), 597–601.

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2.3 NOVA IZOMERA VITAMINA E (gama-TOKOMONOENOL in alfa-TOKOMONOENOL) V SEMENIH, PRAŽENIH SEMENIH IN OLJU IZ PRAŽENIH SEMEN SLOVENSKE BUČE *'SLOVENSKA GOLICA'*

New vitamin E isomers (gamma-tocomonoenol and alpha-tocomonoenol) in seeds, roasted seeds and roasted seed oil from the Slovenian pumpkin variety '*Slovenska golica*'
Bojan BUTINAR, Milena BUČAR-MIKLAVČIČ, Carlo MARIANI in Peter RASPOR
Food Chemistry, 2011, 128: 505-512

GC-MS analiza prostih in esterificiranih komponent bučnega olja iz slovenske buče 'Slovenska golica' (vzorec D) je razkrila prisotnost dveh do tedaj neobjavljenih izomerov vitamina E: alfa-tokomonoenola in gama-tokomonoenola. Oba sta bila okarakterizirana s pomočjo MS spektrov. Na osnovi podatkov GC-MS, referenčnega vzorca surovega palmovega olja ter tokoferolnih in tokotrienolnih standardov je bilo mogoče s pomočjo HPLC potrditi in koncentracijsko ovrednotiti vsebnost obeh novih oblik vitamina E v olju praženih semen 'Slovenske golice'. Vrednosti sta bili 17,6 ± 0,6 µg/g za alfa-tokomonoenol in 118,7 ± 1,0 µg/g za gama-tokomonoenol. Koncentraciji alfa-tokoferola in gama-tokoferola sta bili 77,9 ± 1,9 µg/g ter 586,0 ± 4,6 µg/g, vsaka. Preostala dva tokoferola – beta- in delta-izomer, sta obsegala vrednosti 5,4 ± 0,01 µg/g oziroma 14,1 ± 0,3 µg/g. Vse koncentracije tokoferolnih izomerov so bile v skladju s predhodno objavljenimi podatki. To pa ne drži za gama-tokotrienol, saj je bila njegova določena vsebnost 6,9 ± 0,2 µg/g, kar je znatno manj od podatkov, objavljenih v literaturi. To vrednost je potrdila tudi GC-MS analiza. To neskladje je bilo pojasnjeno z določitvijo gama-tokotrienola v nepraženih in praženih semenih sorte 'Slovenska golica'. Rezultati so pokazali, da je bila vsebnost gama-tokotrienola nizka že od vsega začetka in da se ni zmanjšala med procesom praženja, ravno nasprotno – med procesom je z 1,6 µg/g porastla na 2,2 µg/g. Podoben dvig se je zgodil tudi pri ostalih izomerih. HPLC spremljanje različnih spojin vitamina E med tehnološkim procesom od nepraženih bučnih semen do olja, je pokazalo porast dveh neidentificiranih spojin – prva eluira tik pred kromatografskim vrhom alfa-tokoferola, druga pa pred kromatografskim vrhom gama-tokoferola. Ti spojini utegneta služiti kot odlična kemijska markerja in kot uporabno orodje, namenjeno sledenju dogajanja v procesu stiskanja olja z namenom ugotavljanja stopnje predelave. In končno – služili bi lahko v postopku ugotavljanju pristnosti olja iz bučnih semen.

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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.internationalolive-oil.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.

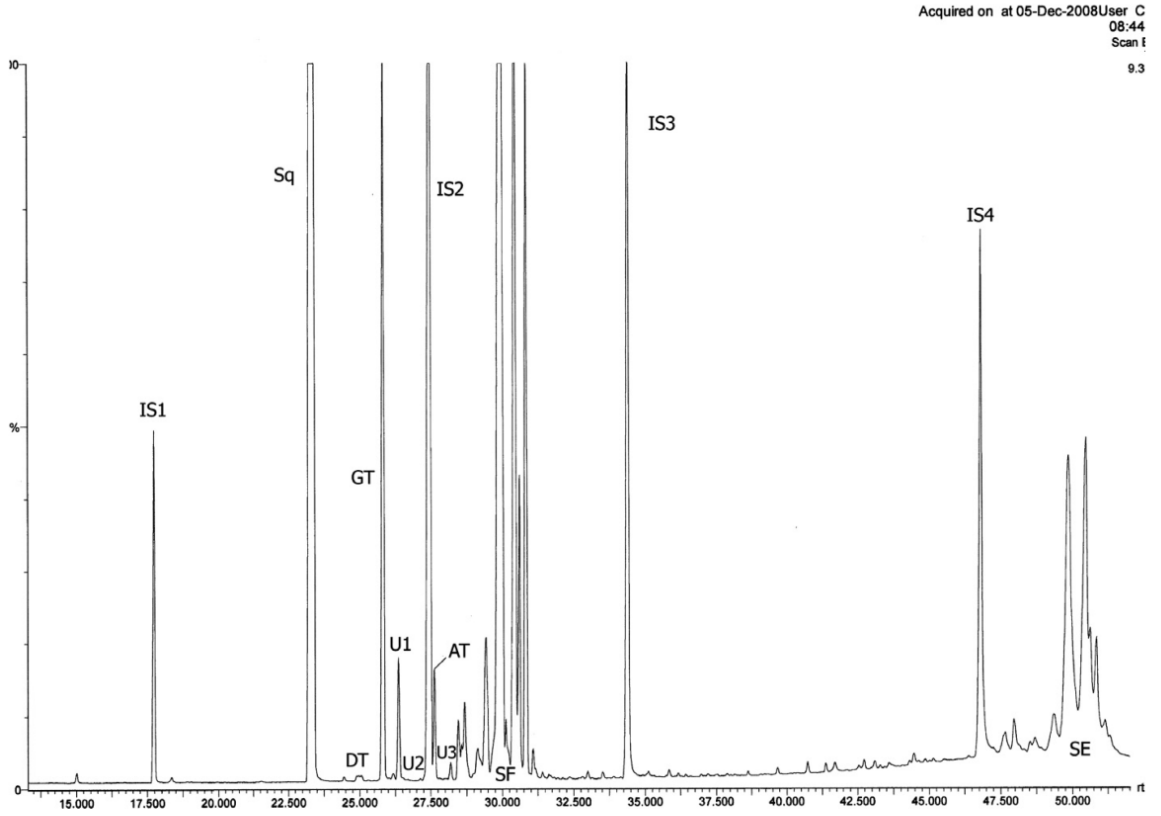


Fig. 1. Total Ion Current (TIC) chromatogram of free and esterified minor compounds (FEMC) of the GC-MS of the oil pressed from roasted ground pumpkin seeds (sample D). The peaks are assigned as follows: IS1 – internal standard 1, Sq – squalene, DT – delta-tocopherol, GT – gamma-tocopherol, U1 – unknown 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).

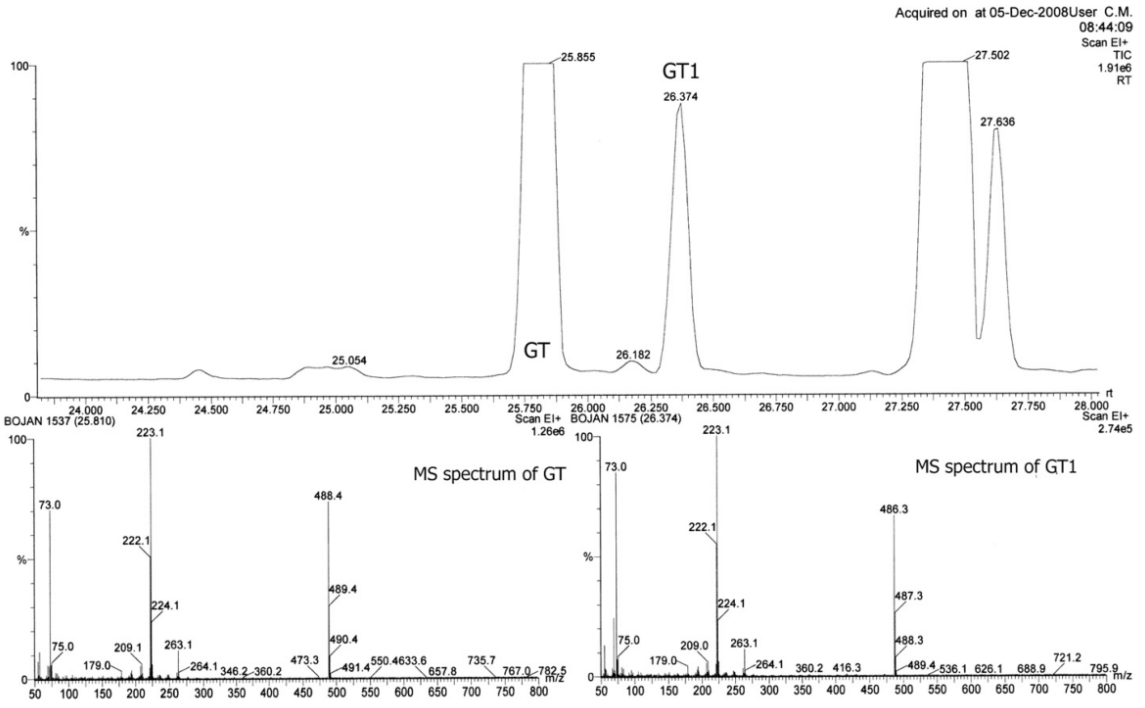


Fig. 2. TIC of the GC-MS of free and esterified minor compounds (FEMC) of the oil pressed from roasted ground pumpkin seeds (sample D, top) showing the gamma-tocopherol and gamma-tocomonoenol peaks with their respective MS spectra: gamma-tocopherol (bottom left) and gamma-tocomonoenol (bottom right).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

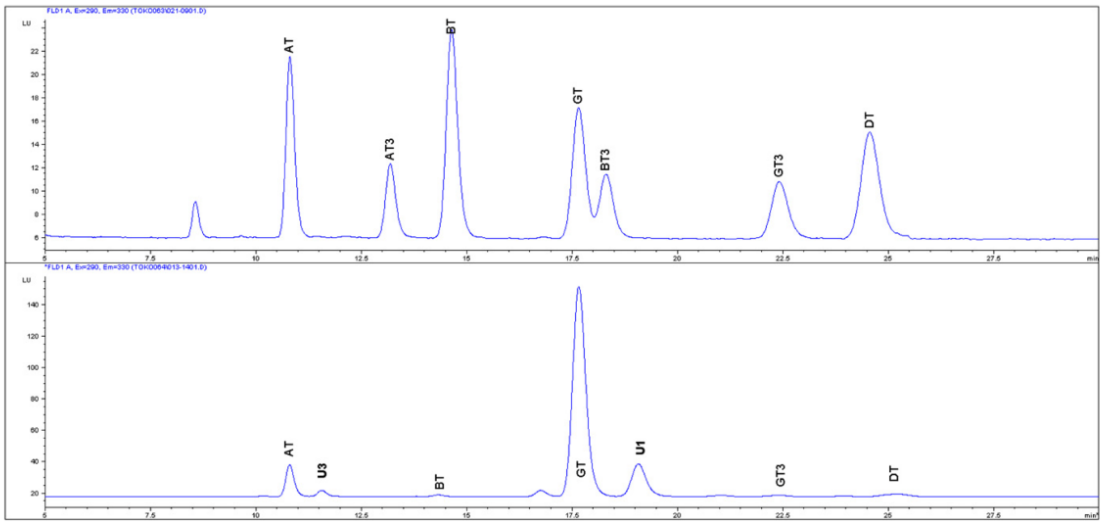


Fig. 3. HPLC chromatogram of tocopherol and tocotrienol standards (top), and HPLC chromatogram of vitamin E isomers in oil pressed from roasted ground pumpkin seeds (sample D, bottom). The peaks are assigned as follows: AT – alpha-tocopherol, AT3 – alpha-tocotrienol, BT – beta-tocopherol, GT – gamma-tocopherol, 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

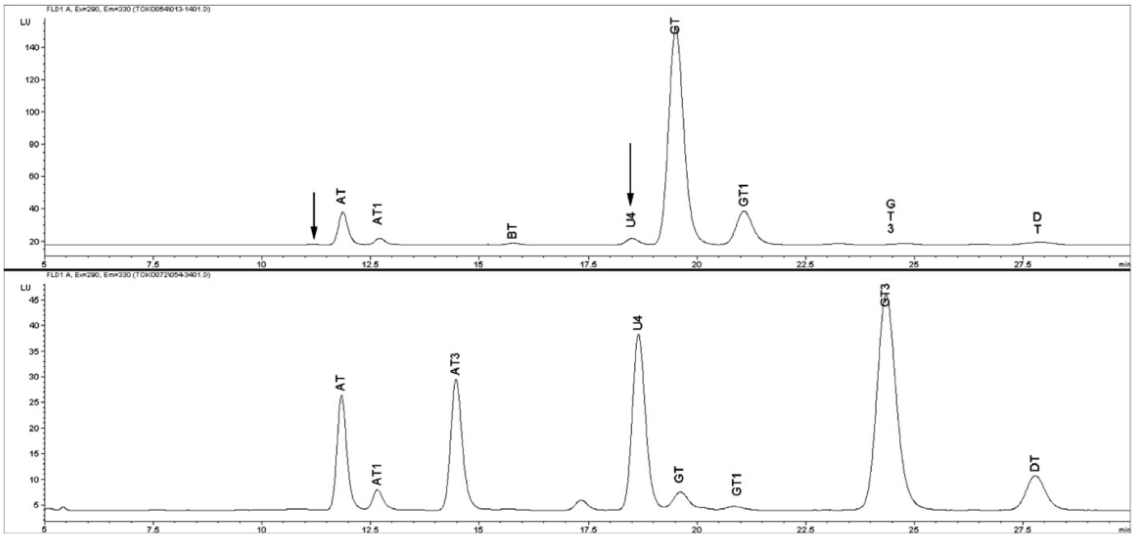


Fig. 4. HPLC chromatogram of vitamin E isomers in oil pressed from roasted ground pumpkin seeds in sample D (top) and HPLC chromatogram of vitamin E isomers in Crude Palm Oil (bottom). Chromatographic conditions were as in Fig. 3. AT1 – alpha-tocomonoenol, GT1 – gamma-tocomonoenol, U4 – the unknown peak 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.

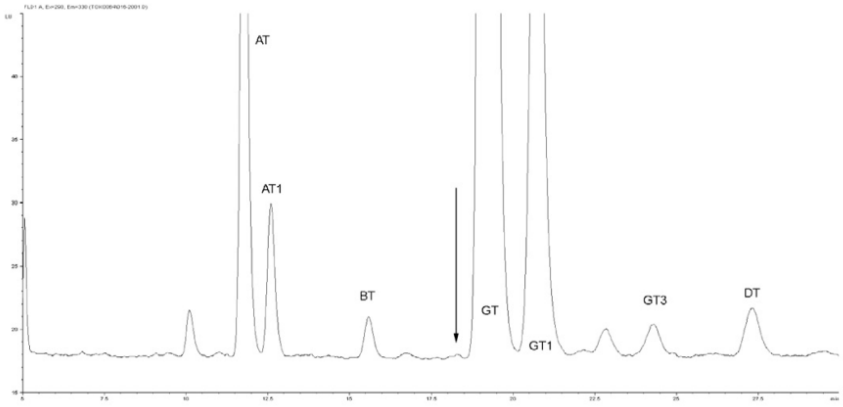
The concentration of gamma-tocotrienol determined in sample D is just 6.9 ± 0.2 μg/g, which is much less than reported by Murkovic et al. (2004) who state the concentration of tocotrienols is about one third of the corresponding tocopherols.

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

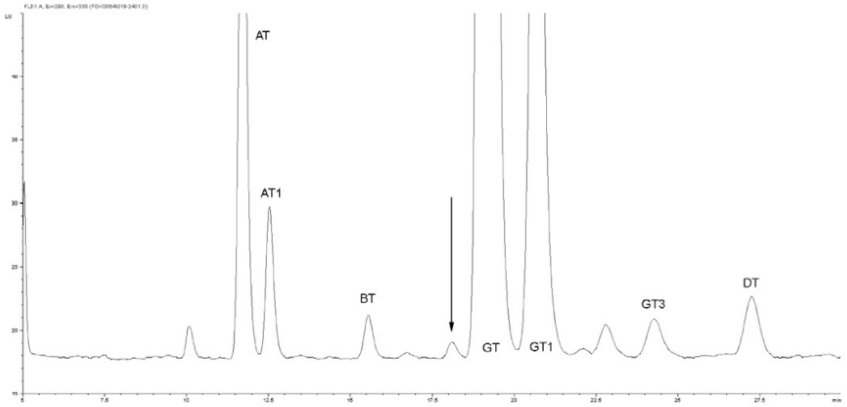
In order to explain the reason for the minor quantities of determined gamma-tocotrienol found in sample D compared to previously reported data, a vitamin E survey was undertaken. Attention was given to pumpkin seeds samples before they were processed as well (sodium chloride and water addition, roasting, 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).

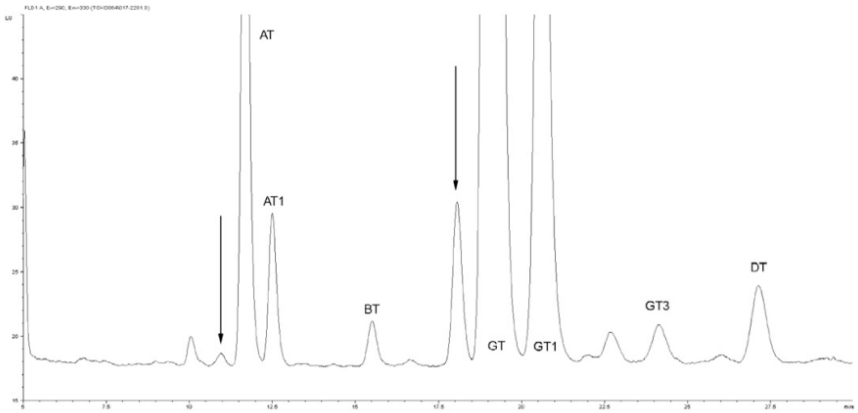
These data can be found in Table 1. A closer look at the results reveals significant changes in all vitamin E components from sample A to the sample C. The concentrations are reported on an 'as is' basis and increase from the initial state in the ground form (sample



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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References

Butinar, B., Bučar-Miklavčič, M., Krumpak, A., & Raspor, P. (2009). Experiences in olive oil purity and quality assessment as a tool for pumpkin seed oil evaluation. What can consumers benefit? *Acta Alimentaria, 38*(2), 219–227.

Fiorentino, A., Mastellone, C., D'Abrosca, B., Pacifico, S., Scognamiglio, M., Cefarelli, G., et al. (2009). [*delta*]-Tocomonoenol: A new vitamin E from kiwi (*Actinidia chinensis*) fruits. *Food Chemistry, 115*(1), 187–192.

Fruhwirth, G. O., Wenzl, T., El-Toukhy, R., Wagner, F. S., & Hermetter, A. (2003). Fluorescence screening of antioxidant capacity in a pumpkin seed oils and other natural oils. *European Journal of Lipid Science and Technology, 105*(6), 266–274.

Gemrot, F., Barouh, N., Vieu, J.-P., Pioch, D., & Montet, D. (2006). Effect of roasting on tocopherols of gourd seeds (*Cucurbita pepo*). *Grasas y Aceites, 57*(4), 409–414.

Gotoh, N., Watanabe, H., Oka, T., Mashimo, D., Noguchi, N., Hata, K., et al. (2009). Dietary marine-derived tocopherol has a higher biological availability in mice relative to alpha-tocopherol. *Lipids, 44*(2), 133–143.

ISO 5725-2, (1994). Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.

ISO/IEC 17025, (2005). General requirements for the competence of testing and calibration laboratories.

ISO 9936, (2006). Animal and vegetable fats and oils – Determination of tocopherol and tocotrienol contents by high-performance liquid chromatography.

ISO 3960, (2007). Animal and vegetable fats and oils – Determination of peroxide value – Iodometric (visual) endpoint determination.

ISO 660, (2009). Animal and vegetable fats and oils – Determination of acid value and acidity.

Mariani, C., & Bellan, G. (1996). Content of tocopherols, deidrotocopherols, tocodienols, tocotrienols in vegetable oils. *La Rivista Italiana Delle Sostanze Grasse, 73*(4), 533–543.

Mariani, C., & Bellan, G. (1997). Presence of tocopherol derivatives in vegetable oils. *Rivista Italiana Delle Sostanze Grasse, 74*(4), 545–552.

Mariani, C., & Fedeli, E. (1986). Detection of extraction oils in pressure ones. Note1. *La Rivista Italiana Delle Sostanze Grasse, 63*(1), 3–17.

Mariani, C., Fedeli, E., & Grob, K. (1991). Evaluation of free and esterified minor components in fatty materials. *La Rivista Italiana Delle Sostanze Grasse, 68*(5), 233–242.

Murkovic, M., Hillebrand, A., Winkler, J., & Pfannhauser, W. (1996). Variability of vitamin E content in pumpkin seeds (*Cucurbita pepo* L.). *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung, 202*(4), 275–278.

Murkovic, M., & Pfannhauser, W. (2000). Stability of pumpkin seed oil. *European Journal of Lipid Science and Technology, 102*(10), 607–611.

Murkovic, M., Piironen, V., Lampi, A. M., Kraushofer, T., & Sontag, G. (2004). Changes in chemical composition of pumpkin seeds during the roasting process for production of pumpkin seed oil (Part 1: Non-volatile compounds). *Food Chemistry, 84*(3), 359–365.

Nakic, S. N., Rade, D., Skevin, D., Strucelj, D., Mokrovcak, Z., & Bartolic, M. (2006). Chemical characteristics of oils from naked and husk seeds of *Cucurbita pepo* L. *European Journal of Lipid Science and Technology, 108*(11), 936–943.

Ng, M. H., Choo, Y. M., Ma, A. N., Cheng, H. C., & Hashim, M. A. (2004). Separation of vitamin E (tocopherol, tocotrienol, and tocomonoenol) in palm oil. *Lipids, 39*(10), 1031–1035.

Parry, J., Hao, Z. G., Luther, M., Su, L., Zhou, K. Q., & Yu, L. L. (2006). Characterization of cold-pressed onion, parsley, cardamom, mullein, roasted pumpkin, and milk thistle seed oils. *Journal of the American Oil Chemists Society, 83*(10), 847–854.

Puah, C. Wei., Choo, Y. May., Ma, A. Ngan., & Chuah, C. Hock. (2007). The Effect of Physical Refining on Palm Vitamin E (Tocopherol, Tocotrienol and Tocomonoenol). *American Journal of Applied Sciences, 4*(6), 4.

Ryan, E., Galvin, K., O'Connor, T. P., Maguire, A. R., & O'Brien, N. M. (2007). Phytosterol, squalene, tocopherol content and fatty acid profile of selected seeds, grains, and legumes. *Plant Foods for Human Nutrition, 62*(3), 85–91.

Sen, C. K., Khanna, S., & Roy, S. (2006). Tocotrienols: Vitamin E beyond tocopherols. *Life Sciences, 78*(18), 2088–2098.

Stevenson, D. G., Eller, F. J., Wang, L. P., Jane, J. L., Wang, T., & Inglett, G. E. (2007). Oil and tocopherol content and composition of pumpkin seed oil in 12 cultivars. *Journal of Agricultural and Food Chemistry, 55*(10), 4005–4013.

Yoshida, H., Tomiyama, Y., Hirakawa, Y., & Mizushina, Y. (2006). Microwave roasting effects on the oxidative stability of oils and molecular species of triacylglycerols in the kernels of pumpkin (*Cucurbita* spp.) seeds. *Journal of Food Composition and Analysis, 19*(4), 330–339.

Younis, Y. M. H., Ghirmay, S., & Al-Shihry, S. S. (2000). African *Cucurbita pepo* L.: Properties of seed and variability in fatty acid composition of seed oil. *Phytochemistry, 54*(1), 71–75.

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3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

Pri eksperimentalnem delu zasnove analitičnega postopka ugotavljanja pristnosti in stopnje predelave bučnega olja smo uvajali nov vpogled v zahtevno matrico bučnega olja na osnovi izključevalnih faz »drevesa odločanja«, poznanega iz ugotavljanja kakovosti in pristnosti oljčnih olj (Commission Regulation … , 2003). Za dokazovanje raziskovalne hipoteze smo večinoma uporabili že razvite, prenesene, validirane in akreditirane metode, ki jih uporabljamo v laboratoriju. V nekaterih primerih smo te metode tako spremenili, da smo se lahko prilagodili matrici bučnega olja (e.g. razmerje med Δ-5 in Δ-7 steroli). Pri raziskavah smo uporabili GC kromatografske metode, in sicer:

- za ugotavljanje maščobnokislinske sestave metode, objavljene v (Commission Regulation … 1991, 2003),
- za ugotavljanje vsebnosti *trans*-izomerov maščobnih kislin metode, objavljene v (Commission Regulation … 1991, 2003),
- za ugotavljanje sterolne sestave in razmerja med Δ-5 in Δ-7 steroli modificirane metode, objavljene v (Breinholder in sod., 2002;
  Commission Regulation … , 1991; Mandl in sod., 1999; Wenzl in sod., 2002).

ter HPLC kromatografske metode, in sicer:

- za ugotavljanje stereospecifične sestave triacilglicerolov modificirane metode, objavljene v (Commission Regulation … , 1991; IOC, 2006, 2010; Jakab in sod., 2002; Moreda in sod., 2003; Tuberoso in sod., 2007),
- za ugotavljanje sestave vitamina E v bučnem olju iz termično obdelanih in neobdelanih semen in v praženih semenih metode objavljene v (ISO 9936, 2006; Murkovic in sod., 2004),
- za ugotavljanje vsebnosti biofenolov v bučnem olju modificirano metodo, objavljeno v (IOC, 2009).

Postavljeno »drevo odločanja« podaja logičen redosled preskušanja, ugotavljanja in izključevanja na osnovi dejstev, ki so značilna za določeno kategorijo. Tako deviška oljčna olja kot bučna olja imajo skupno dejstvo, da ne smejo biti mešana z ostalimi (cenenimi) semenskimi olji, ne smejo biti rafinirana in v primeru hladno stisnjenega bučnega olja, morajo to trditev vzdržati. V primeru EDOOSI ZOP ne sme temperatura predelave preseči 27 °C (Objava … , 2006). Spomnimo se na v uvodu postavljeni trditvi, ko skladnost z zahtevami procesa dokazujemo tudi z dokumentiranimi zapisi – v tem primeru morajo biti vsi spremljevalni zapisi iz oljarne taki, da kažejo na temperaturo procesa, ki ne sme biti višja od omenjene. Podoben razmislek bi morda bil smiseln v primeru hladno stisnjenega

bučnega olja – seveda pa bi bilo potrebno najprej postaviti protokol, ki bi vzpostavil osnovne pojme režima hladnega stiskanja. Drevo odločanja vodi preskuševalca v vnaprej določenem vrstnem redu od začetne določitve vsebnosti *trans*-maščobnih kislin do končne določitve sterolne sestave. Vsebnost tokoferolnih izomerov v drevesu ni omenjena, v našo raziskavo pa smo jo vključili zaradi precejšnje raznolikosti tokoferolne sestave različnih olj, ki jih ponavadi uporabijo za potvorbe – e.g. sojino olje in olje oljne ogrščice. Drevo odločanja jasno pravi, da se s preverjanjem pristnosti preneha takoj, ko je eden od izključitvenih pogojev izpolnjen.

V prvi raziskavi smo na vzorcu sedmih različnih bučnih olj ugotavljali naslednje parametre pristnosti: vsebnost *trans*-izomerov maščobnih kislin, maščobnokislinsko sestavo, sterolno sestavo ter tokoferolno sestavo. Podatki o teh vzorcih so v Preglednici 1.

Preglednica 1: Podatki o vzorcih bučnih olj z oznako, izvorom in deklariranim tipom olja z deklaracijo na etiketi oziroma dobljeno informacijo v oljarni

Table 1: Pumpkin seed oils samples datasheet regarding their origin, declared type or data on the label or personal communication when purchasing the sample

Vzorec	Izvor	Tip olja – deklaracija - informacija
S1	Trgovina	Prekmursko bučno olje
S2	Oljarna	Olje iz muškatne buče (*Cucurbita moschata*, D.)
S3	Oljarna	Olje iz muškatne buče (*Cucurbita moschata*, D.)
S4	Oljarna	Olje iz muškatne buče (*Cucurbita moschata*, D.), drugo stiskanje
S5	Trgovina	Kmečko bučno olje
S6	Oljarna	Toplo stiskano bučno olje
S7	Oljarna	Hladno stiskano bučno olje

Največja dovoljena vsebnost *trans*-izomerov maščobnih olj v deviškem in ekstra deviškem oljčnem olju je 0,05 ut. % za *trans*-oleinsko kislino (*t*-C 18:1 (*E*)-oktadec-9-enojska kislina) ter 0,05 ut. % za vsoto izomerov *trans*-linolne (*t*-C 18:2 vsota izomerov (9*E*,12*E*)-oktadeka-9,12-dienojske kisline in (9*Z*,12*E*)-oktadeka-9,12-dienojske kisline in (9*E*,12*Z*)-oktadeka-9,12-dienojske kisline in in *trans*-linolenske kisline (*t*-C 18:3 vsota izomerov (9*E*,12*Z*,15*E*)-oktadeka-9,12,15-trienojske kisline in (9*Z*,12*Z*,15*E*)-oktadeka-9,12,15-trienojske kisline in (9*Z*,12*E*,15*Z*)-oktadeka-9,12,15-trienojske kisline in (9*E*,12*Z*,15*Z*)-oktadeka-9,12,15-trienojske kisline). Omenjene so tiste *trans*-maščobne kisline, ki nastanejo pri procesu rafinacije brez premestitvenih reakcij dvojne vezi, ki so domena adicijskih reakcij hidrogeniranja. Vrednosti so izkustvene in pomenijo, da je v primeru, ko so presežene, zelo velika verjetnost, da je v matrici prišlo do temperaturne obdelave, ki se dogaja pri rafinaciji olja. V polju oljčnega olja to pomeni, da je v mešanici bodisi rafinirano oljčno olje bodisi drugo rafinirano semensko olje. V naši raziskavi so se vrednosti za *trans*-oleinsko kislino gibale v razponu med 0,014 do 0,022 ut. % in za vsoto

trans-linolne in *trans*-linolenske med 0,037 do 0,443 ut. %, kar je pokazalo na verjetnost potvorbe pri vzorcih S1, S4, S5 in S6 (Butinar in sod., 2009).

Objave, ki podajajo maščobnokislinsko sestavo bučnih olj zgovorno pričajo, da je razpon le-teh precej širok (Applequist in sod., 2006; Brodnjak-Vončina in sod., 2005; Neđeral in sod., 2012; Vujasinović in sod., 2010; Yu in sod., 2004). Izkušnje preverjanja pristnosti na osnovi deležev posameznih maščobnih kislin pričajo, da ni izključevalni faktor delež tistih maščobnih kislin, ki so bolj zastopane v maščobni matrici, temveč ravno nasprotno – izključevalne so minorne maščobne kisline - e.g. *(Z)*-dokoz-13-enojska kislina (C 22:1 n-9), ki kaže na prisotnost olja oljne ogrščice, (9*Z*,12*Z*,15*Z*)-oktadeka-9,12,15-trienojska kislina (alfa-linolenska kislina, C 18:3 n-3) in (*Z*)-eikoz-11-enojska kislina (C 20:1 n-9), ki potrjujeta prisotnost sojinega olja ali olja oljne ogrščice, eikozanojska kislina (C 20:0) sojinega olja, olja iz kikirikija ali oljne ogrščice ter dokozanojska kislina (C 22:0), ki potrjuje prisotnost olja iz kikirikija ali oljne ogrščice (Christopoulou in sod., 2004; Koprivnjak, 2006). Podobno velja tudi za bučna olja v luči ugotovitev iz Brodnjak-Vončina in sod. (2005), upoštevaje kemometričen pristop. Naši rezultati so pokazali (Preglednica 2), da so trije vzorci: vzorec S1, vzorec S4 in vzorec S6 verjetno potvorjeni: vzorec S1 ima povišano vsebnost kislin C 18:3 n-3, C 20:1 n-9 in C 22:0, vzorca S4 in S6 pa imata povišano vsebnost kisline C 22:0 (Butinar in sod., 2009).

Preglednica 2: Masni deleži posameznih maščobnih kislin v vzorcih bučnih olj (%). MK – posamezna maščobna kislina, VZ. – oznaka vzorca bučnega olja, DA/NE – prisotnost vrha kisline C 22:1 v kromatografskem vrhu za skvalen

Table 2: Fatty acids wt. % composition in pumpkin seed oil samples. (MK – fatty acid determined, VZ. – pumpkin seed oil sample, DA/NE – presence of a C 22:1 peak in the squalene peak)

MK (%)\VZ.	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**
C 14:0	0,07	0,08	0,08	0,07	0,08	0,08	0,09
C 16:0	8,88	16,34	16,29	9,88	11,16	10,26	11,12
C 16:1	0,12	0,13	0,15	0,10	0,12	0,11	0,12
C 17:0	0,05	0,08	0,08	0,05	0,05	0,04	0,05
C 17:1	0,02	0,01	0,02	0,02	0,02	0,02	0,00
C 18:0	4,06	6,60	6,24	4,87	5,26	4,77	5,47
C 18:1	32,11	19,15	17,90	19,40	31,44	30,98	37,18
C 18:2	50,62	56,70	58,36	64,32	51,02	52,72	45,16
C 18:3	**2,76**	0,27	0,25	0,18	0,18	0,18	0,12
C 20:0	0,35	0,38	0,37	0,30	0,35	0,33	0,37
C 20:1	**0,43**	0,07	0,06	0,12	0,08	0,10	0,09
C 22:0	**0,40**	0,15	0,14	**0,51**	0,18	**0,29**	0,14
C 24:0	0,13	0,04	0,06	0,18	0,08	0,12	0,08
C 22:1	**DA**	NE	NE	**DA**	NE	NE	NE

Najbolj nazorna in prepričljiva indikacija prisotnosti semenskih olj pri potvorbi bučnih olj izvira iz dejstva, da bučna olja vsebujejo skoraj izključno Δ7-fitosterole alfa-spinasterol, Δ-7,22,25 stigmastatrienol, Δ-7 stigmastenol in Δ-7,25 stigmastadienol (Mandl in sod., 1999). Zanimivo je, da je to skoraj izključna značilnost družin *Cucurbitaceae* in *Theaceae* (Breinholder in sod., 2002). Pokazano je bilo, da je vsebnost drugih fito-Δ5-sterolov v bučnih oljih skoraj zanemarljiva. Že 1-odstotni masni dodatek koruznega olja k bučnemu olju poviša delež β-sitosterola za približno 35 % (Wenzl in sod., 2002). Pri postavitvi izključevalne meje pri razmerju Δ5-steroli/ Δ7-steroli so se Breinholder in sod. (2002) odločili za varnih 10 %. V primeru povišanega deleža brasikasterola je to evidenten dokaz za potvorbo z oljem oljne ogrščice (Breinholder in sod., 2002; Mandl in sod., 1999). Iz prikazanih podatkov sledi, da je indikacija potvorbe izrazita pri vzorcih S1, S4, S5 in S6.

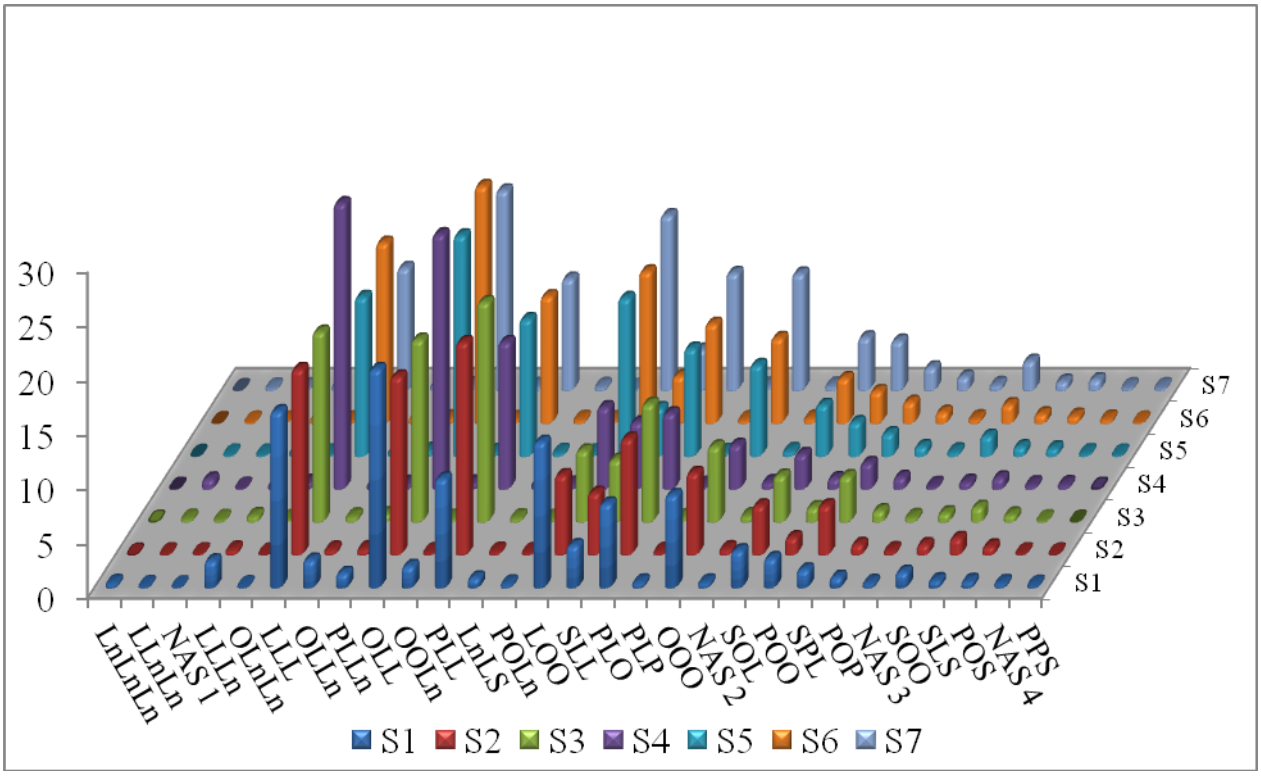
Veliko raziskav je določevalo vsebnost tokoferolov v bučnih semenih (Murkovic in sod., 1996b; Murkovic in Pfannhauser, 2000) in v bučnih oljih (Nakić in sod., 2006), če jih omenimo le nekaj, saj se s tem področjem podrobneje ukvarjamo v nadaljevanju raziskave. Rezultati določitve tokoferolov v vzorcih kažejo na prevladujočo obliko gama-izomera, a podobno kot pri deležu maščobnih kislin, so diskriminatorni deleži predvsem beta- in delta-oblika (potvorba s sojinim oljem) ter alfa-oblika (potvorba s sončničnim oljem) (Butinar in sod., 2009). Iz prikazanih rezultatov – deležev alfa-, beta- in delta-tokoferola v vzorcih je razvidno, da je zelo verjetna potvorba vzorcev S1, S4, S5 in S6. Še posebej je zanimiv vzorec št. 1 s skoraj 60 mg delta-tokoferola/kg (evidentna potvorba s sojinim oljem).

Združeni rezultati vseh parcialnih preverjanj v skladu z drevesom odločanja pokažejo na skladnost ugotovitev parcialnega preverjanja in pravilnost predlaganega postopnega ugotavljanja pristnosti. Izkaže se, da so vzorci S2, S3 in S7 zelo verjetno pristni in potrdijo predpostavko, da je pristop z določevanjem *trans*-izomerov maščobnih kislin, deleža Δ-5/Δ-7 sterolov in vsebnosti tokoferolov tak, da uspe diskriminirati pristna od potvorjenih olj. Princip platforme ugotavljanja pristnosti bo v bodoče lahko gradil od hitrejših, cenejših in analitično manj zahtevnih določitev do tistih bolj kompleksnih in zahtevnih (določevanje deleža Δ-5/Δ-7 sterolov). Rezultati potrdijo pričakovanje, da so vrednosti iz določitve maščobnokislinskega deleža lahko le dodatno pomagalo pri procesu ugotavljanja pristnosti.

Raziskavo smo razširili tudi na identifikacijo, speciacijo in statistično obdelavo stereospecifičnih triacilglicerolov v omenjenih vzorcih. Za te potrebe smo morali dopolniti in nadgraditi ustrezne analitične metode (Commission Regulation … , 1991; Fiebig, 1985; Jakab in sod., 2002; Tuberoso in sod., 2007) za kromatografsko določevanje triacilglicerolov v semenskih oljih, še posebej oljčnih (IOC, 2006, 2010; Moreda in sod., 2003; Ollivier in sod. 2003, 2006), kot je to opisano v nadaljevanju aktivnosti pri zasnovi analitičnega postopka ugotavljanja pristnosti bučnega olja (Butinar in sod., 2010a).

Z uporabo modificirane metode (IOC, 2010) in uporabo propionitrila kot mobilne faze in s temperaturno kontrolo kromatografske ločbe, nam je v vzorcih bučnih olj uspelo zadovoljivo ločiti 29 stereospecifičnih TAG v času 70 minut. Kromatogram ločbe TAG za vzorec S2 je prikazan v Butinar in sod. (2010a).

25 stereospecifičnih TAG nam je uspelo asignirati, preostali štirje (NAS 1 - 4) pa so bili enakomerno porazdeljeni med vse asignirane TAG. Njihov skupni delež je segal od 0,85 ut. % v vzorcu S2 do 1,10 ut. % v vzorcu S7, kar se nam je glede na preostale deleže zdelo sprejemljivo in nesignifikantno, še posebej, ker se njihovi deleži niso bistveno spreminjali med vzorci. Vrstni red eluiranja je bil naslednji (imena posameznih TAG so akronimi začetnih črk karboksilnih kislin, vezanih na glicerol, in sicer: P – palmitoil, S – stearoil, O – oleoil, L – linoleil in Ln – linolenil (Perona in Ruiz-Gutierrez, 2004)): LnLnLn, LLnLn, NAS1, LLLn, OLnLn, LLL, OLLn, PLLn, OLL, OOLn, PLL, LnLS, POLn, LOO, SLL, PLO, PLP, OOO, NAS2, SOL, POO, SPL, POP, NAS3, SOO, SLS, POS, NAS4 in PPS. Ločene TAG za vseh sedem vzorcev smo kvantificirali in dobili rezultate, ki so grafično zbrani na Sliki 2.



Slika 2: Grafično predstavljeni podatki za vsebnost TAG v vzorcih bučnih olj S1– S7. x-os: posamezen TAG, y-os: vzorec in z-os: masni delež

Figure 2: Graphical representation of TAG content in pumpkin seed oil samples S1–S7. x-axis: TAG species, y-axis: sample and z-axis: wt. %

Na osnovi dobljenih podatkov in po poglobljeni grafični analizi smo TAG vzorcev razvrstili v šest grozdov tako, da je vsak grozd predstavljal po pet TAG prikazanih po vrstnem redu eluiranja. Zadnji grozd je vseboval zadnje štiri TAG, in sicer SLS, POS, zadnji neidentificiran TAG (NAS4) ter PPS. Nadaljnja analiza v grozde razvrščenih podatkov je dala osnovo za določitev devetih signifikantnih TAG tako, da smo izbrali tiste, ki so obsegali najbolj diskriminantne vrednosti za vse analizirane vzorce. Izbrani triacilgliceroli so bili naslednji: LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL in SLS.

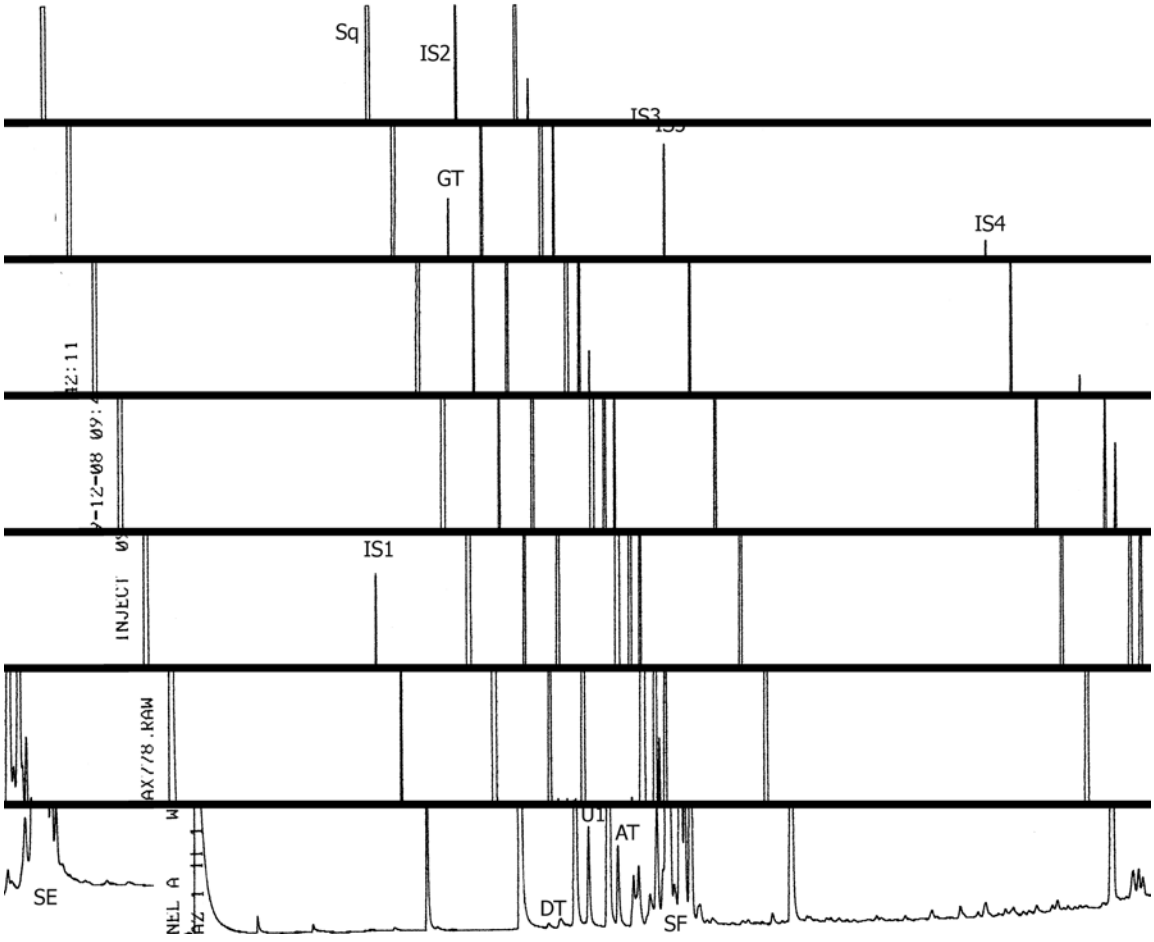
Podatki tako izbranih in predstavljenih TAG so bili osnova za nadgradnjo s pomočjo analize glavnih komponent, ki je prek diagrama teže glavnih komponent dala PCA razsevni diagram za TAG-e LLLn, LLL, PLL+LOO, PLO, OOO, POO+SPL in SLS za sedem vzorcev (S1-S7) bučnih olj iz SV Slovenije.

Diagram teže glavnih komponent kaže na zelo dobro porazdelitev izbranih signifikantnih TAG. PCA razsevni diagram potrdi tezo iz razprave v publikaciji (Butinar in sod., 2010a), da analizirani vzorci tvorijo 5 različnih skupin. In res, potrdi se tudi porazdelitev na pristne vzorce: vzorca S2 in S3, ki sta bila stisnjena iz istih bučnih semen, a v različnih procesnih režimih, sta si v diagramu blizu; podobno velja za potvorjena vzorca S5 in S6 z različnim izvorom – kriterij maščobnokislinske sestave je vzorec S6 uvrstil v razred potvorb, vzorca S5 pa ne (Butinar in sod., 2009). To dejstvo priča o ne »prehudi« potvorbi in da sta bila vzorca potvorjena z zelo podobnim ali istovrstnim semenskim oljem.

Preostali trije vzorci tvorijo zelo različne definirane skupine. Vzorca S1 in S4 sta potvorjena z različnima vrstama semenskega olja, o čemer priča tokoferolna sestava (Butinar in sod., 2010a) – vzorec S1 vsebuje 58 mg/kg delta-tokoferola in 245 mg/kg alfa-tokoferola, vzorec S4 pa kar 309 mg/kg alfa-tokoferola. Očitno je, da je prvi potvorjen s sojinim oljem, drugi pa s sončničnim oljem in da morata imeti različen položaj v razsevnem diagramu. Edinstven je tudi položaj vzorca S7, ki je dokazano pristen in deklarirano hladno stisnjen. Ponuja se sklep, da so vsi trije pristni vzorci iz različnih kultivarjev in/ali različnega izvora in da bo posledično zelo težko zaščititi in preverjati pristna olja z ZGO, če bodo geografsko in sortno zelo divergirala, kar se posledično lahko obrne proti zaščiti sami. Pa še ena ugotovitev se ponuja – primerjava položaja vzorcev S2, S3 in S7 kaže na drugo dejstvo, da utegne način procesiranja semen (Matthaus in Spener, 2008) (hladno stiskanje/praženje) – izstopajoč položaj vzorca S7 v razsevnem diagramu – vplivati na samo razliko sestave TAG, o čemer pričajo tudi japonski raziskovalci (Yoshida in sod., 2004; Yoshida in sod., 2005) in tudi rezultati novejših raziskav (Neđeral in sod., 2012). V nadaljevanju raziskovanja uporabe analitičnega postopka ugotavljanja pristnosti bučnega olja je bila izvedena raziskava v polju 15 vzorcev bučnih olj s slovenskega trga v letu 2008 (preverjali smo maščobnokislinsko sestavo, vsebnost *trans*-izomerov maščobnih kislin, tokoferolno sestavo, stereospecifične TAG in kakovostno določitev s preiskavo v UV), ki je potrdila vse navedene trditve pri ugotavljanju pristnosti in pravilnost izključevalnega pristopa (Butinar in sod., 2011b). Dejstvo, da je stereospecifična analiza

TAG močno orodje tako pri kemometrični obdelavi kot tudi pri ugotavljanju pristnosti in avtentifikaciji prehranskih maščob pa potrjujejo tudi nekatere novejše raziskave (Bosque-Sendra in sod., 2012).

Pri raziskovanju možnih analitičnih postopkov pri preverjanju pristnosti bučnega olja smo se še posebej usmerili v enega najbolj pogosto gojenih kultivarjev 'Slovensko golico' (Kljun, 2011; Rižnik, 2011; Štajersko prekmursko … , 2005) in v matrico bučnega olja, predelanega iz praženih semen 'Slovenske golice'. Tako smo želeli osvetliti segment prostih in esterificiranih minornih spojin (FEMC) iz oljne frakcije bučnega olja. Še posebej smo se osredotočili na segment različnih izomerov vitamina E, ki je viden v »nepotvorjeni« obliki v frakciji FEMC, o kateri poročajo Mariani in Bellan (1996), Mariani in Fedeli (1986) ter Mariani in sod. (1991). Najprej smo posneli FID kromatogram frakcije FEMC vzorca bučnega olja iz sorte 'Slovenska golica', ki ga prikazuje Slika 3, nato pa še signal skupnega ionskega toka GC-MS iste frakcije.

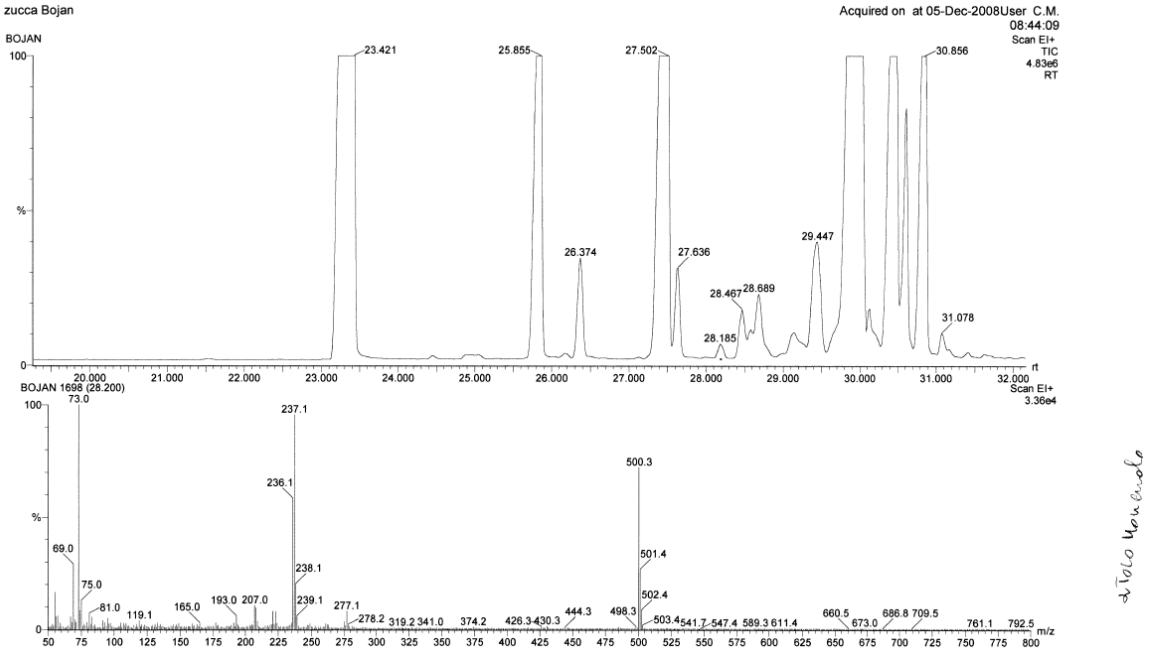


Slika 3: FID kromatogram prostih in esterificiranih minornih spojin (FEMC) olja iz praženih bučnih semen (vzorec D). Vrhovi predstavljajo: IS1 – interni standard 1, Sq – skvalen, DT – delta-tokoferol, GT – gama-tokoferol, U1 – neznani vrh 1, U2 – neznani vrh 2, IS2 – interni standard 2, AT – alfa-tokoferol, U3 – neznani vrh 3, SF – sterolna frakcija,

IS3 – interni standard 3, IS4 – interni standard 4, SE – sterolni estri. X-os: čas v minutah, y-os: signal FID

Figure 3: FID chromatogram of free and esterified minor compounds (FEMC) of the oil pressed from roasted ground pumpkin seeds (sample D). 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.

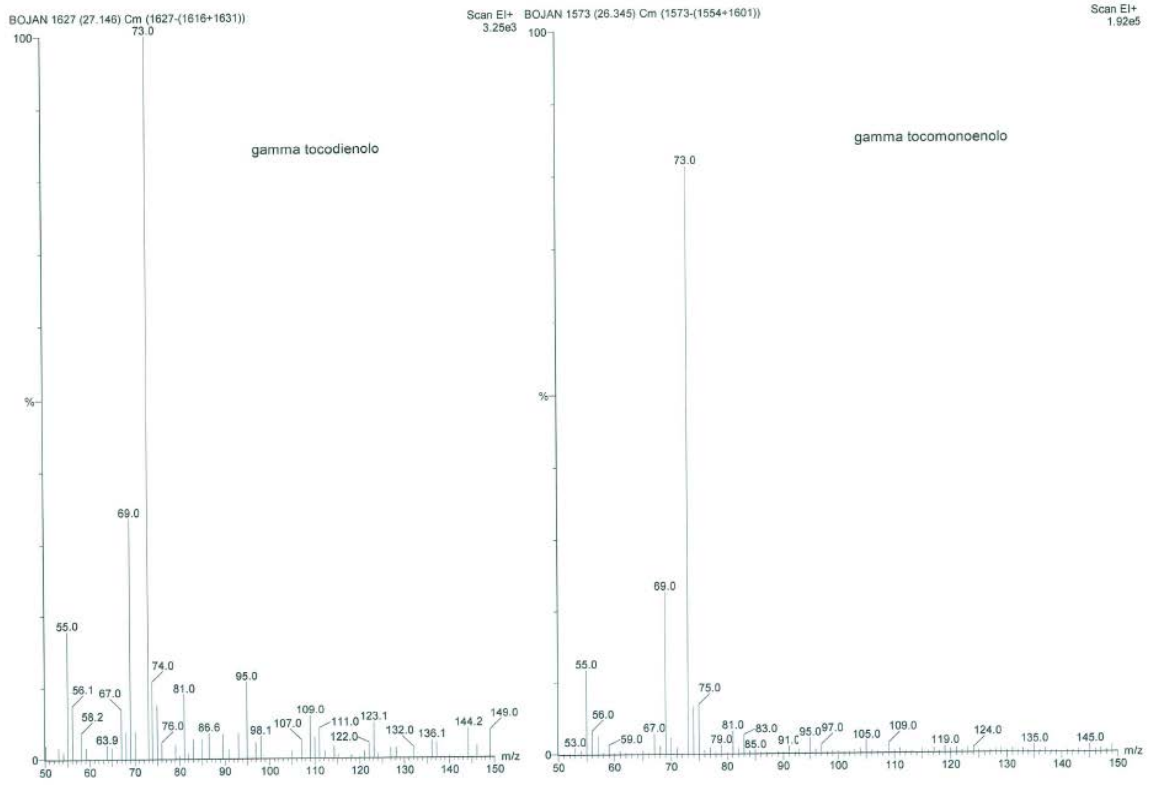
Iz signala skupnega ionskega toka se je izkazalo, da so v frakciji tri spojine, ki dotlej še niso bile dokazane in ni bilo objavljeno, da se pojavljajo v vzorcu bučnega olja iz praženih semen. Te tri spojine - trije neznani vrhovi so nenasičene oblike »glavnih« tokoferolnih izomerov. Vrh U1 je gama-tokomonoenol – GT1 (2,7,8-trimetil-2-(4,8,12-trimetiltridec-11-enil)kroman-6-ol), vrh U2 je gama-tokodienol – GT2 (2,7,8-trimetil-2-(4,8,12-trimetiltrideka-7,11-dienil)kroman-6-ol) in vrh U3, alfa-tokomonoenol – AT1 (2,5,7,8-tetrametil-2-(4,8,12-trimetiltridec-11-enil)kroman-6-ol) (Butinar in sod., 2011a; Ohnmacht in sod., 2008). Slika 4 prikazuje kromatogram in potrditveni MS spekter za AT1.



Slika 4: Signal skupnega ionskega toka izseka GC-MS kromatograma z izomeri vitamina E prostih in esterificiranih minornih spojin (FEMC) olja iz praženih bučnih semen (vzorec D) kaže izsek z izomeri vitamina E (zgoraj) in MS spekter za alfa-monotokoenol (spodaj). Vrh za AT1 je pri 28,185 minute.

Figure 4: 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 vitamin E isomers with alpha-tocomonoenol MS spectrum MS (bottom). AT1 peak is at 28,185 min.

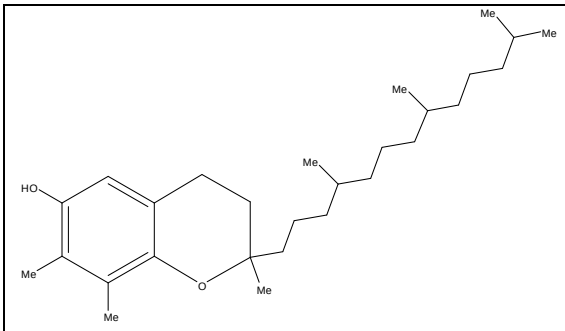
Z GC-MS je bil potrjen položaj Π vezi v AT1 in GT1 na ogljikovih atomih C11-C12 verige (Butinar in sod., 2011a; Dunlap in sod., 2002; Fiorentino in sod., 2009; Gotoh in sod., 2009; Puah in sod., 2007; Yamamoto in sod., 1999) – to prikazujeta MS spektra GT1 in GT2 s Slike 5, z razvidnim fragmentom m/z 69, ki potrjuje položaj Π vezi.



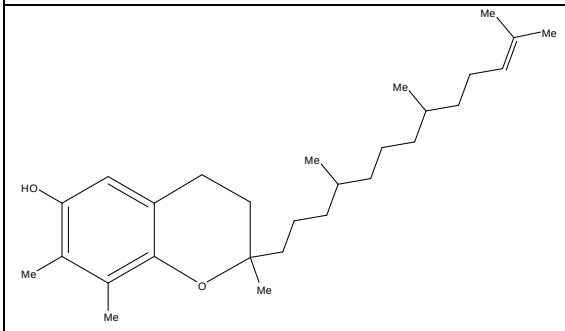
Slika 5: MS spektra za GT2 (levo) in GT1 (desno). Fragment m/z 69 pri obeh potrjuje položaj dvojne vezi na C11-12 verige

Figure 5: MS spectra of GT2 (left) and GT1 (right). Fragment with m/z of 69 proves the double bond position at C11-12 of the side chain

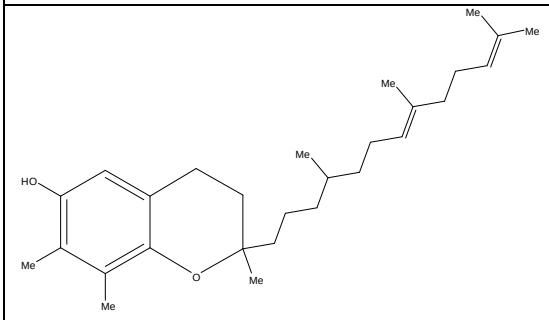
Skeletne formule in IUPAC poimenovanje gama-tokoferola, gama-tokomonoenola, gama-tokodienola in gama-tokotrienola so na Sliki 6, alfa-tokomonoenola pa na Sliki 7.



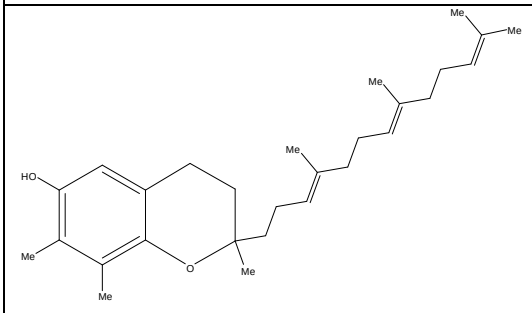
gama-tokoferol: 2,7,8-trimetil-2-(4,8,12-trimetiltridecil)kroman-6-ol



gama-tokomonoenol: 2,7,8-trimetil-2-(4,8,12-trimetiltridec-11-enil)kroman-6-ol



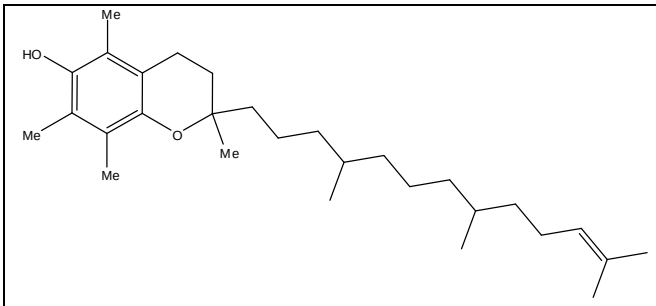
gama-tokodienol: 2,7,8-trimetil-2-(4,8,12-trimetiltrideka-7,11-dienil)kroman-6-ol



gama-tokotrienol: 2,7,8-trimetil-2-(4,8,12-trimetiltrideka-3,7,11-trienil)kroman-6-ol

Slika 6: Skeletne formule gama-tokoferola, gama-tokomonoenola, gama-tokodienola in gama-tokotrienola (Gotoh in sod., 2009; IUPAC-IUB, 1982; Ohnmacht in sod., 2008)

Figure 6: Sceletal formulas of gamma-tocopherol, gamma-tocomonoenol, gamma-tocodienol and gamma-tocotrienol (Gotoh in sod., 2009; IUPAC-IUB, 1982; Ohnmacht in sod., 2008)



alfa-tokomonoenol: 2,5,7,8-tetrametil-2-(4,8,12-trimetiltridec-11-enil)kroman-6-ol

Slika 7: Skeletna formula alfa-tokomonoenola (Dunlap in sod., 2002; Gotoh in sod., 2009; IUPAC-IUB, 1982; Ohnmacht in sod., 2008)

Figure 7: Sceletal formula of alfa-tocomonoenol) (Dunlap in sod., 2002; Gotoh in sod., 2009; IUPAC-IUB, 1982; Ohnmacht in sod., 2008)

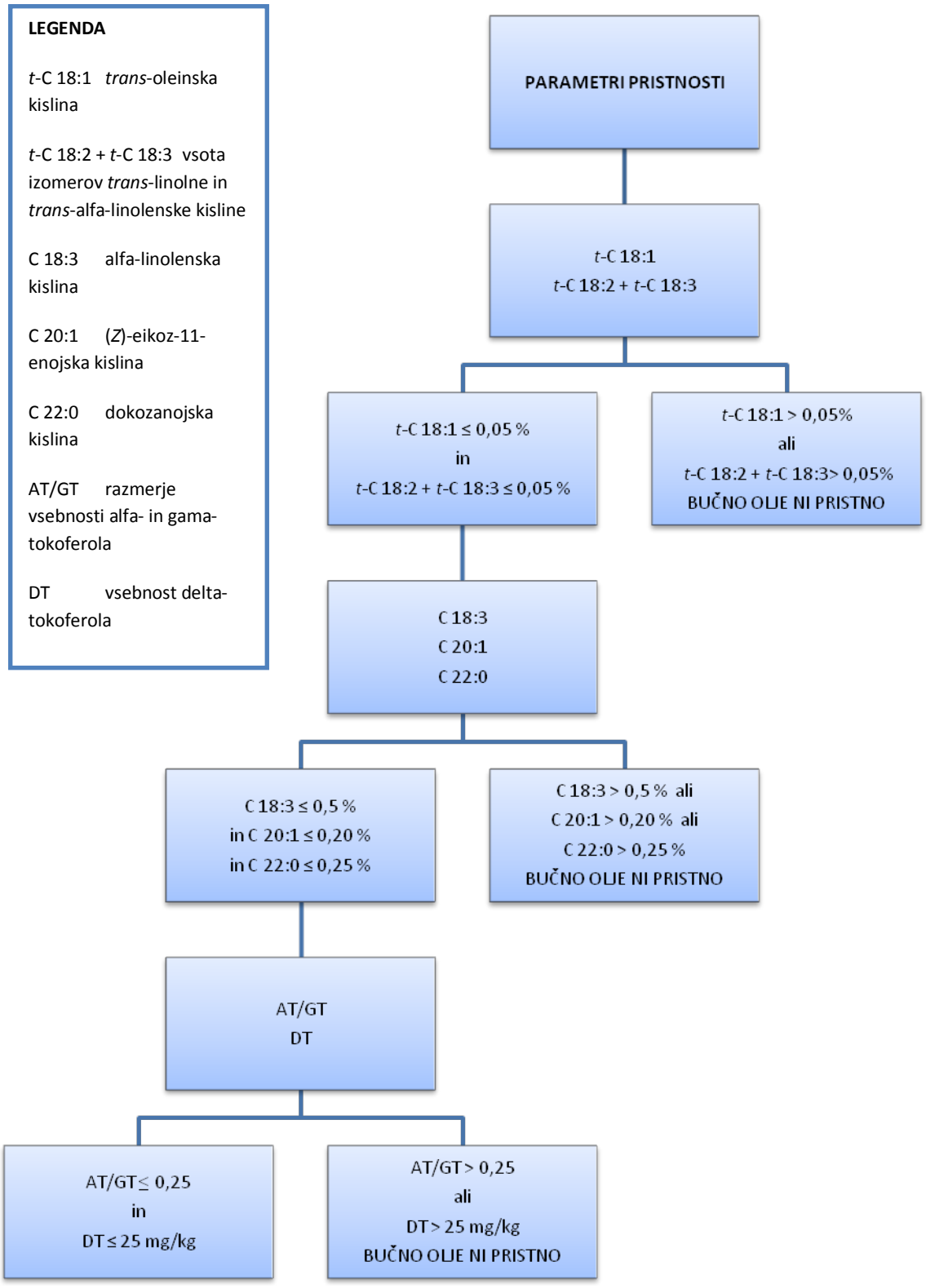
Iz pregleda nam dostopnih podatkih je razvidno, da smo prvi poročali o enkrat in dvakrat nenasičenih izomerih vitamina E v bučnem olju. Gemrot in sod. (2006) na objavljenem kromatogramu v svoji publikaciji prikažejo vrh gama-tokomonoenola, a ga ne asignirajo. Drugo zanimivo dejstvo pa je, da GC-MS v frakciji FEMC ni zaznal trikrat nenasičenega izomera gama-tokoferola - gama-tokotrienola kljub dejstvu, da je ta izomer večkrat omenjen v nekaterih publikacijah (Murkovic in sod. , 2004). Izsledke GC-MS smo s pomočjo tokoferolnih, tokomonoenolnih in tokotrienolnih standardov prenesli na HPLC in vse izomere vitamina E kvantificirali. V stolpcu D Preglednice 1 (Butinar in sod., 2011a) s podatki za olje, sta koncentraciji za alfa- in gama-tokoferol pričakovani in v sozvočju z ostalimi avtorji (Fruhwirth in sod., 2003; Murkovic in Pfannhauser, 2000; Neđeral in sod., 2012). Naša določitev poleg koncentracij za »nova« izomera AT1 in GT1 v nasprotju z nekaterimi avtorji (Fruhwirth in sod., 2003; Nakić in sod., 2006; Neđeral in sod., 2012) podaja tudi koncentracije za minorna beta-tokoferol in delta-tokoferol, kar se nam zdi še posebej pomembno v luči opisanega celovitega postopka ugotavljanja pristnosti bučnih olj. Določili smo tudi koncentracijo gama-tokotrienola, ki je bila 6,9 ± 0,2 µg/g, kar je znatno manj od podatkov, ki so jih objavili Murkovic in sod. (2004). Da bi razjasnili diskrepanco v objavljenih podatkih in da bi dokazali morebiten vpliv procesa praženja na domnevno zmanjšanje višje koncentracije GT3 v vzorcih nepraženih bučnih semen, smo določili vsebnost vitamina E v nepraženih zmletih semenih (vzorec A), v zmletih semenih z dodano

vodo in natrijevim kloridom (dejanski vzorec pripravljenih semen za praženje, vzorec B) ter v praženem vzorcu B (vzorec C) . Podatki so v stolpcih A, B in C v Preglednici 1 publikacije (Butinar in sod., 2011a). Rezultati HPLC določitve so pokazali dve zanimivosti. Prva zanimivost je dejstvo, da je vsebnost vseh določevanih izomerov narastla v seriji A do C, kar je v delnem soglasju z nekaterimi avtorji (Murkovic in sod., 2004; Yoshida in sod., 2006). Druga presenetljiva zanimivost pa je odkritje, da je vsebnost gama-tokotrienola že od vsega začetka procesa (vzorec A do vzorec C) nizka – v nepraženih semenih vzorca A samo 1,6 ± 0,1 µg/g, kar je na meji LOQ! Trdimo, da je v preiskovanem vzorcu koncentracija gama-tokotrienola prek celotnega procesa zelo nizka (v olju ne doseže 10 µg/g) in potrdimo, da proces praženja »koncentrira« izomere vitamina E. To potrjujejo tudi kasnejše raziskave drugih avtorjev (Neđeral in sod., 2012).

Na Sliki 4 v publikaciji (Butinar in sod., 2011a) sta s puščicami v območjih med 10–12 min in med 17–19 min označena dva kromatografska vrhova, ki sta najbolj izražena v vzorcu praženih semen (C) in najmanj v vzorcu nepraženih semen (A). Prvi, manjši, eluira pred alfa-tokoferolom, drugi – večji, pa pred gama-tokoferolom. Menimo, da sta dva vrhova označevalca procesa praženja – verjetno sta oksidacijska produkta alfa- in gama-tokoferola (Bayala in David, 2012; Butinar in sod., 2011b; Falk in Munne-Bosch, 2010; Sayago in sod., 2007), najdemo ju tudi v referenčnem vzorcu za kvalitativen prenos izomerov vitamina E z GC instrumentarija na HPLC v vzorcu surovega palmovega olja (Butinar in sod., 2011a). V primeru alfa-tokoferola gre verjetno za alfa-tokoferilkinon in/ali alfa-tokoferilkinon epoksid (Murkovic in sod., 1997; Rovellini in Cortesi, 2002). Vrh pred gama-tokoferolom je reda velikosti 10 µg/g in bi ga lahko po podrobnejši okarakterizaciji kvantitativno povezali s stopnjo praženja semen. Oba kromatografska vrhova bi v morebitnem nadaljevanju raziskave pokazala, da utegneta postati diskriminantni spojini za razlikovanje med hladno stiskanimi bučnimi olji in olji praženih bučnih semen in posredno tudi kot potrjevalna označevalca pri platformi pristnosti praženih bučnih olj. V navezi z izsledki stereospecifične analize triacilglicerolov hladno stiskanih bučnih olj (Butinar in sod., 2010a), ki so z analizo glavnih komponent pokazale na razliko med TAG hladno stiskanih in praženih olj in v navezi z izsledki nekaterih novejših publikacij s področja praženih in hladno stiskanih bučnih olj, ki so raziskovale biofenolno frakcijo bučnih olj in ugotovile znaten porast skupnih biofenolov kot tudi spremenjeno ravnovesje med enostavnimi in esterificiranimi biofenoli (Butinar in sod., 2011b; Neđeral in sod., 2012), trdimo, da bi ob ustrezni dodatni raziskavi ti relativno enostavno analizno določljivi spojini lahko bistveno obogatili, postavili in implementirali ne samo platformo preverjanja pristnosti, temveč tudi platformo preverjanja stopnje predelave bučnega olja.

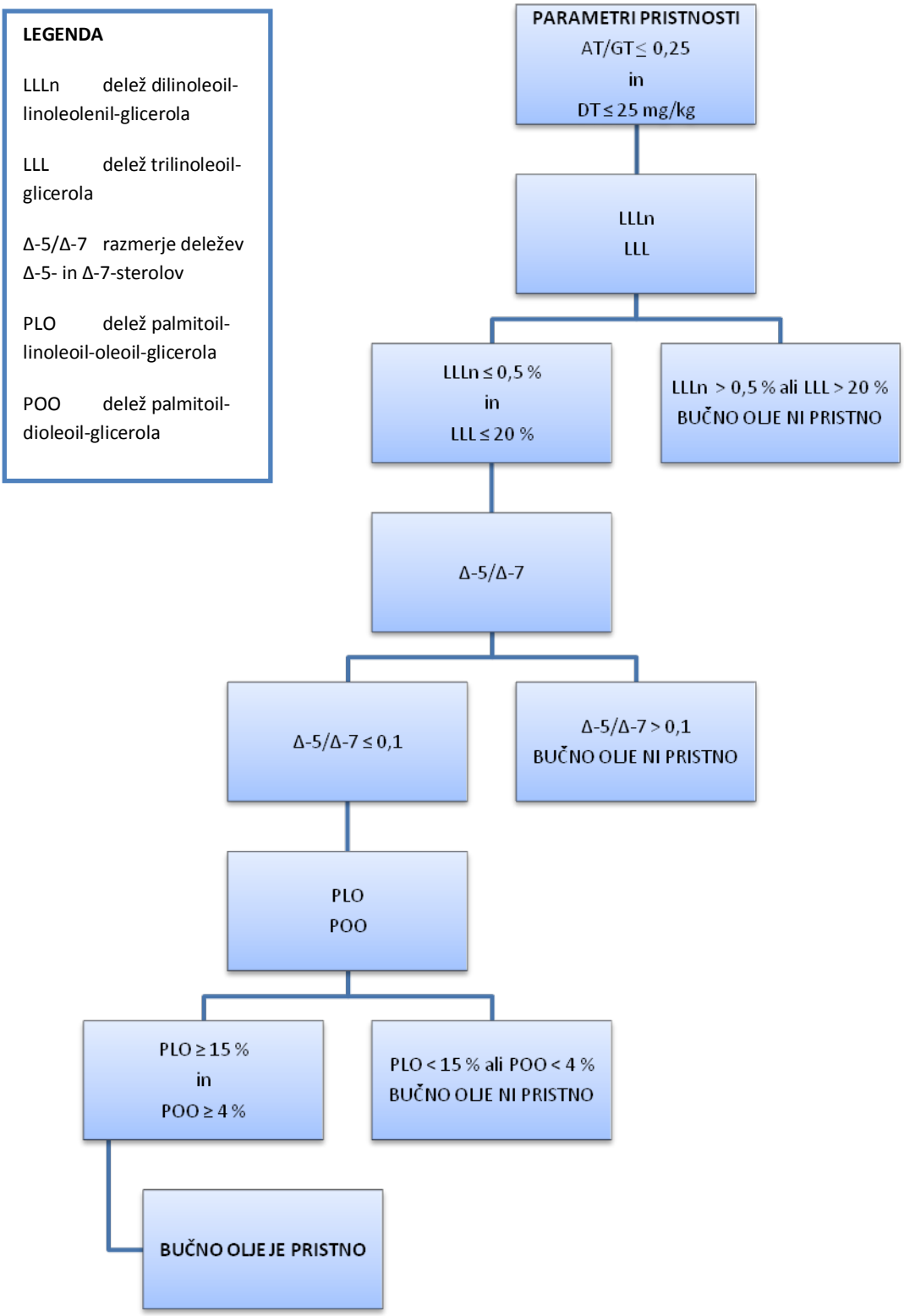
Na osnovi vseh ugotovljenih dejstev smo poskusili postaviti tri različna drevesa odločanja: za parametre pristnosti bučnega olja, za parametre kakovosti bučnega in za razlikovanje bučnega olja na osnovi stopnje predelave. Drevo odločanja za parametre pristnosti

upošteva že opisani analizni protokol, ki gre od bolj enostavnih in analitsko in časovno manj zahtevnih določitev do bolj zahtevnih kot je določitev sterolov. Presojanje se začne z ovrednotenjem vsebnosti izomera *trans*-oleinske kisline (*t*-C18:1) in vsote izomerov *trans*-linolne (*t*-C 18:2) in *trans*-linolenske (*t*-C 18:3) kisline. Nadaljuje se z ovrednotenjem vsebnosti alfa-linolenske kisline ter kislin C 20:1 in C 22:0. V kolikor je bilo bučnemu olju primešano semensko olje v večji količini, se to pokaže v preseženih vrednostih in olje je izločeno. S presojo lahko nadaljujemo, tako da preverimo še razmerje med alfa- in gama-tokoferolom (AT/GT) ter vsebnost delta-tokoferola (DT). S primerjavo relativnega deleža nekaterih TAG (par LLLn in LLL je marker za prisotnost semenskih olj, par LPO in POO pa je potrditveni par za pristnost bučnega olja) in z ugotavljanjem razmerja med Δ-5 in Δ-7 steroli (Δ-5/Δ-7) pa preverjanje zaključimo. Drevo odločanja za parametre pristnosti je v dveh delih prikazano na Sliki 8.



Slika 8: Drevo odločanja za presojanje pristnosti bučnega olja.
Figure 8: Decision tree for genuineness assessment of pumpkin seed oil.

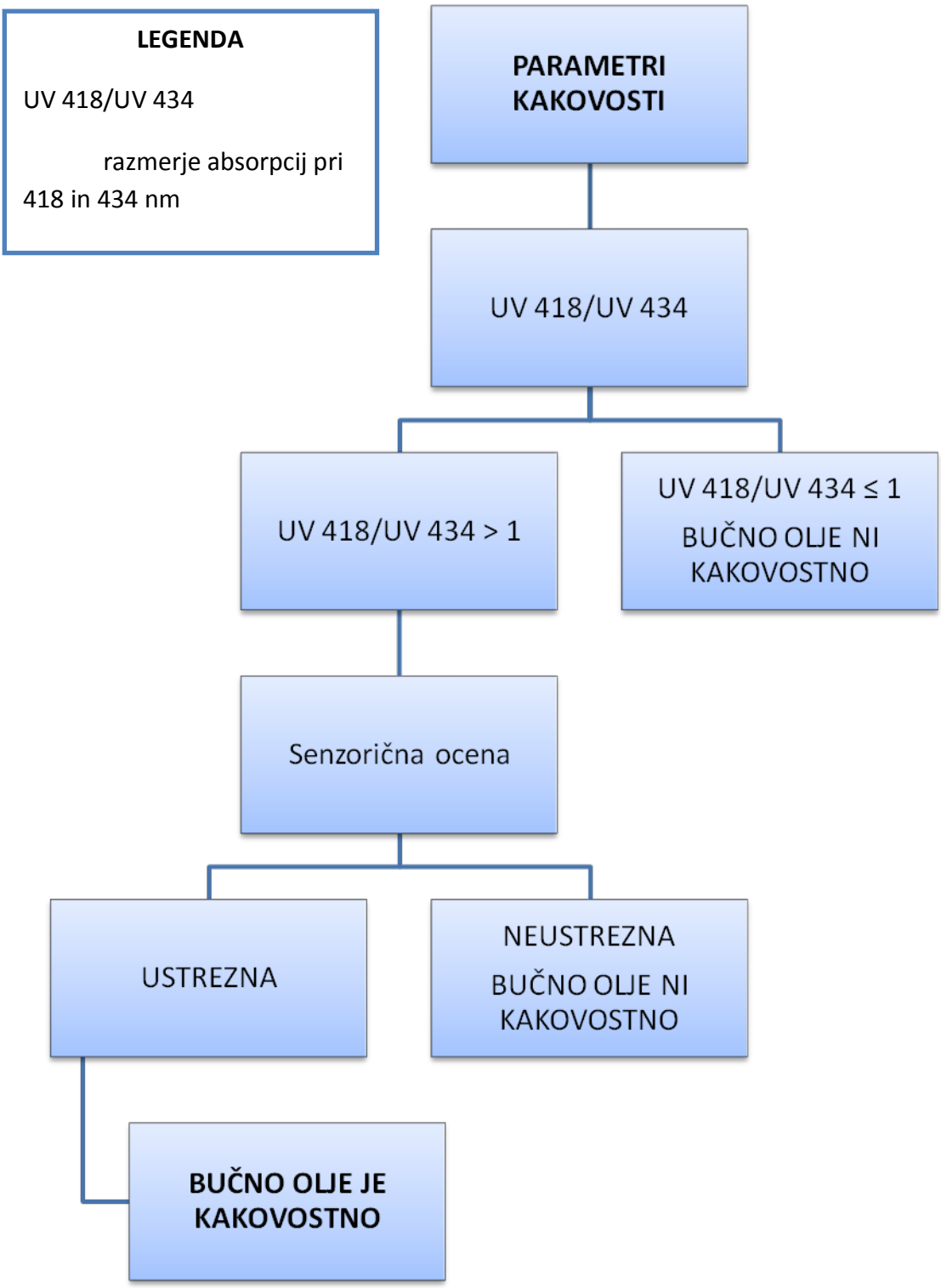
se nadaljuje



nadaljevanje Slike 8: Drevo odločanja za presojanje pristnosti bučnega olja
continuing of Figure 8: Decision tree for genuineness assessment of pumpkin seed oil

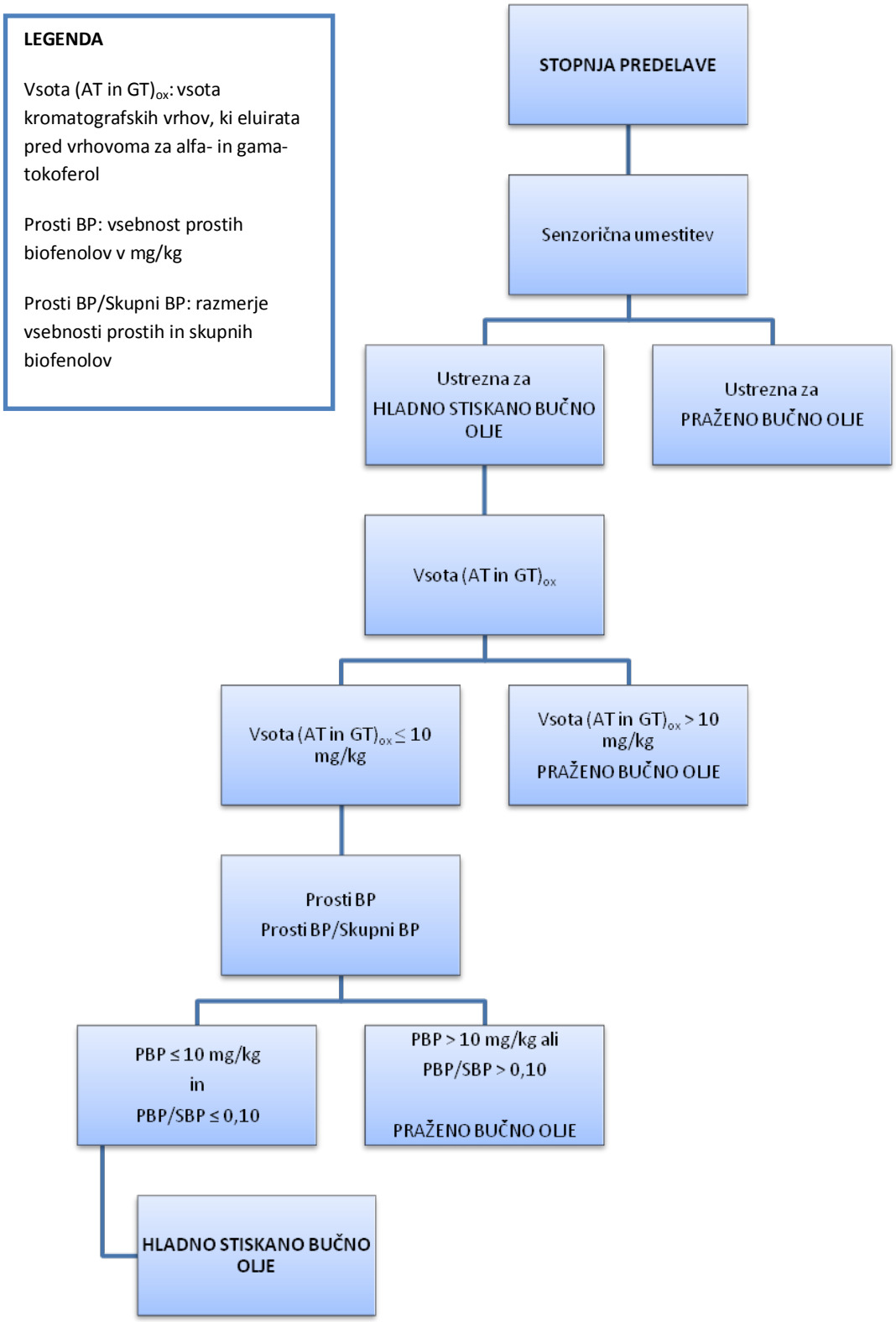
Ko je enkrat olje na osnovi parcialnih odločitev uvrščeno med pristna bučna olja, moramo na osnovi drevesa odločanja ugotoviti ali je tudi kakovostno. To lahko zaenkrat storimo na osnovi instrumentalne določitve s snemanjem UV/VIS spektra v območju med 250 in 600 nm in s primerjavo intenzivnosti absorpcijskih vrhov za klorofil pri 418 nm in za oksidirano obliko klorofila pri 434 nm (UV 418/UV 434). Pri kakovostnih in svežih oljih mora biti signal klorofila močnejši od signala njegove oksidirane oblike (Lankmayr in sod., 2004). Kakovostno umestitev nadaljujemo s senzoričnim ocenjevanjem, ki je po nam dostopnih podatkih zaenkrat definirano le v elaboratu za priznanje 'Štajersko prekmurskega bučnega olja' (Štajersko prekmursko … , 2005). Drevo odločanja za kakovostno preverjanje bučnega olja je prikazano na Slika 9. Ko nas zanima stopnja predelave oziroma ali je bilo olje stisnjeno iz praženih semen ali hladno stisnjeno, lahko uporabimo shemo s Slike 10. Mislimo, da je za smiselno uporabo drevesa odločanja za stopnjo predelave nujen ustrezno definiran protokol z vsemi deskriptorji za senzorični profil bučnega olja iz praženih semen kot tudi za senzoričen profil hladno stisnjenega bučnega olja. Ko s pomočjo senzoričnega profila olja potrdimo deklariran tip olja (praženo oziroma hladno stisnjeno), nadaljujemo s presojanjem na osnovi instrumentalnih določitev. Prva je valorizacija morebitnih kromatografskih vrhov, ki spremljata vrh alfa- oziroma gama-tokoferola. Če je njuna vsota večja od 10 mg/kg, obstaja resen dvom v hladno stisnjen tip olja; v nasprotnem primeru pa hladno stisnjen profil potrdimo s presojo količine prostih biofenolov (PBP) in količine esterificiranih oziroma skupnih (SBP) biofenolov in z razmerjem med prostimi in skupnimi biofenoli (PBP/SBP). V primeru relativno nizke vrednosti za proste biofenole in relativno nizkega deleža biofenolov v vzorcu pa lahko z gotovostjo zatrdimo, da je vzorec hladno stisnjen. V realnem preverjanju je seveda nesmiselno začeti s presojo pristnosti, saj je ta pristop bolj kompleksen, drag in zamuden. Začnemo s presojo kakovosti – saj je v primeru, ko olje ni kakovostno, skoraj nepotrebno preverjati še njegovo pristnost – seveda to velja predvsem za tržno – potrošniški aspekt.

Izbrani vrstni red preskušanja v vseh treh drevesih je postavljen na osnovi izkušenj in poznavanja preskuševalnih in analitskih dejstev kot tudi samega olja iz praženih bučnih semen in hladno stisnjenega bučnega olja. Vrstni red in število opravljenih preskusov nista strogo predpisana. Glede na potrebe vrstni red preskušanja lahko spremenimo in posamezne preskuse opustimo. Prav tako so mejne vrednosti, ki so postavljene v shemo, pridobljene na osnovi omejenega števila vzorcev (Butinar in sod. 2009, 2010a, 2011a, 2011b) in se lahko razlikujejo od dejanskega stanja v primeru izpričano pristnih ali avtentičnih vzorcev. S preskušanjem vseh parametrov v vzorcih različnih vrst bučnega olja je potrebno nadaljevati in (iz)graditi sortno in predelovalno specifične nabore podatkov. Le tako se bodo mejne vrednosti zbrane v drevesu odločanja lahko izboljšale in postale bolj točne in bolj diskriminatorne.



Slika 9: Drevo odločanja za presojanje kakovosti bučnega olja

Figure 9: Decision tree for quality assessment of pumpkin seed oil



Slika 10: Drevo odločanja za ugotavljanje stopnje predelave bučnega olja

Figure 10: Decision tree for assessment of the degree of processing of the pumpkin seed oil

3.2 SKLEPI

Naša raziskava je pokazala, da zasnova ugotavljanja pristnosti in stopnje predelave bučnega olja na osnovi sosledja analitičnih preverjanj in meritev predstavlja celovito platformo. Ta platforma analiz pristnosti bučnega olja temelji na »drevesu odločanja« in razloči med pristnimi in potvorjenimi bučnimi olji. Razločevanje sloni na postopnem preverjanju in potrjevanju parametrov pristnosti, ki so za bučno olje izključevalni: torej vsebnost *trans*-izomerov maščobnih kislin (previsok delež pokaže na prisotnost rafiniranega olj, ki je že sam po sebi izključujoč – vpeljani meji sta 0,05 % za *trans*-oleinsko kislino in 0,05 % za vsoto izomerov *trans*-linolne in *trans*-linolenske kisline), razmerje Δ-5/Δ-7 sterolov (z empirično mejo 0,1- ko je preseženo pokaže na prisotnost Δ-5 sterolov, ki so lastni drugim semenskim oljem in niso značilni za bučna olja, kjer prevladujejo Δ-7 fitosteroli alfa-spinasterol, Δ-7,22,25 stigmastatrienol, Δ-7 stigmastenol in Δ-7,25stigmastadienol), delež tokoferolnih izomerov (v pristnem bučnem olju prevladuje gama-tokoferol, vrednosti beta-tokoferola in delta-tokoferola morata biti relativno nizki in se gibljeta v dekadi okrog LOQ (2 – 20 µg/g), alfa-tokoferol pa obsega območje med 5 in 10 % celokupnih tokoferolov). Dodatni izključevalno komplementarni faktor je tudi delež nekaterih minornih kislin, kot so eikozanojska kislina (C 20:0), (*Z*)-eikoz-9-enojska kislina (C 20:1), dokozanojska kislina (C 22:0), *(Z)*-dokoz-13-enojska kislina (C 22:1) in (9*Z*,12*Z*,15*Z*)-oktadeka-9,12,15-trienojska kislina (alfa-linolenska kislina, C 18:3).

Na osnovi stereospecifične (HPLC) analize triacilglicerolov bučnega olja smo uspeli diskriminirati pristna bučna olja od potvorjenih. Z razvito metodologijo smo uspeli kromatografsko ločiti 29 različnih stereospecifičnih triacilglicerolov, od katerih smo jih 25 tudi asignirali. Še posebej smo se osredotočili na stereospecifično sestavo triacilglicerolov in z razvito metodologijo pokazali, da so triacilgliceroli LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL in SLS signifikantni. Izkazalo se je, da je stereospecifična analiza triacilglicerolov metoda, ki da globok vpogled v lipidno matrico bučnega olja, še posebej če jo primerjamo z bolj zamudno in kompleksno analizo sterolov, le obdelava večjega števila referenčnih vzorcev bi bila potrebna za vzpostavitev nosilnih parametrov. Prav tako smo z obdelavo kromatografskih podatkov v primeru signifikantnih triacilglicerolov LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL in SLS ob pomoči analize glavnih komponent pokazali na ključno vlogo le-teh pri razločevanju med pristnimi in potvorjenimi bučnimi olji tako v izolirani določitvi kot tudi v funkciji komplementarne analize z ostalimi kemijskimi določitvami.

Analiza večjega števila eksperimentalno pridobljenih in objavljenih podatkov vsebnosti izomerov vitamina E v semenskih oljih in v bučnem olju pomaga razlikovati med potvorjenimi in pristnimi bučnimi olji. Znano je, da je največ semenskih olj, ki so udeležena v potvorbah bučnega olja, iz najcenejšega kakovostnega ranga, kjer prednjačita sojino olje in sončnično olje in tudi olje oljne ogrščice. V primeru potvorbe s sojinim oljem se povišata koncentracija alfa-tokoferola (s približno sto na nekaj sto µg/g) in delta-

tokoferola (do nekaj deset µg/g), ob tem pa v manjši meri tudi koncentracija beta-tokoferola, od 10 do 15 µg/g.

HPLC analiza izomerov vitamina E in njihovega razmerja v olju iz praženih semen buče 'Slovenska golica' pokaže, da se v tokoferolnem kromatogramu skrivata dva kromatografska vrhova – dve neidentificirani spojini, ki sta v začetnem stanju – v nepraženih semenih neizraženi in se v procesu praženja semen in kasneje v samem olju tako izrazita, da ste kvantitativno določljivi. Spojini sta potencialni marker iz katerega bo možno sklepati na prisotnost olja iz praženih semen v oljih iz hladno stisnjenih semen. Še posebej uporabno je to dejstvo, če ga obravnavamo komplementarno v luči hkratne interpretacije analize glavnih komponent signifikantnih stereospecifičnih triacilglicerolov LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL in SLS, ki diskriminirajo med olji iz praženih semen in hladno stiskanimi olji.

Analiza eksperimentalno pridobljenih podatkov o vsebnosti nekaterih izomerov vitamina E (alfa-monotokoenol, gama-monotokoenol, gama-tokotrienol) v semenih in olju buče 'Slovenska golica' je poglobila vedenje o razmerju med nasičenimi, enkrat nenasičenimi in večkrat nenasičenimi izomeri vitamina E v olju iz praženih semen 'Slovenske golice'. Z GC-MS določitvijo nekaterih spojin v frakciji prostih in esterificiranih spojin v olju iz praženih semen 'Slovenske golice' smo uspeli poročati o dveh novih izomerih vitamina E v tem tipu olja – o alfa-tokomonoenolu (17,6 ± 0,6 µg/g) in gama-tokomonoenolu (118,7 ± 1,0 µg/g). V vzorcu olja smo potrdili koncentracije alfa-tokoferola (77,9 ± 1,9 µg/g), gama-tokoferola (586,0 ± 4,6 µg/g), beta-tokoferola (5,4 ± 0,0 µg/g) in delta-tokoferola (14,1 ± 0,3 µg/g). Koncentracija gama-tokotrienola je bila le 6,9 ± 0,2 µg/g in je bila v nasprotju s prejšnjimi publikacijami. Pokazali smo, da je bila leta nizka tudi v nepraženih bučnih semenih in da se je med procesom praženja celo rahlo zvišala: z 1,6 µg/g v nepraženih semenih na 2,2 µg/g v praženih semenih 'Slovenske golice'. Podobno se je zgodilo tudi z vrednostmi preostalih izomerov vitamina E.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

Pri eksperimentalnem delu zasnove analitičnega postopka ugotavljanja pristnosti in stopnje predelave bučnega olja smo uvajali nov vpogled v zahtevno matrico bučnega olja na osnovi principielnih faz »drevesa odločanja«, poznanega iz ugotavljanja kakovosti in pristnosti oljčnih olj. Pri raziskavah smo uporabili GC in HPLC kromatografske metode. »Drevo odločanja« predstavlja logičen redosled preskušanja, ugotavljanja in izključevanja na osnovi dejstev, ki so značilna za določeno kategorijo olja. Tako deviška oljčna olja kot tudi bučna olja imajo skupno iztočnico, da nikakor ne smejo biti mešana z ostalimi jedilnimi olji in ne smejo biti rafinirana. V primeru hladno stisnjenega bučnega olja mora biti ta trditev potrjena, seveda pa je potreben jasen protokol, ki bi vzpostavil osnovne pojme in procesne smernice režima hladnega stiskanja. »Drevo odločanja« vodi preskuševalca v vnaprej določenem vrstnem redu od začetne določitve vsebnosti *trans*-maščobnih kislin do končne določitve sterolne sestave. Vsebnost tokoferolnih izomerov v samem »Drevesu odločanja« pri deviških oljčnih oljih ni predvidena, v matrico ugotavljanja pristnosti bučnih olj smo jo uvrstili zato, ker je pestrost tokoferolne sestave jedilnih olj, ki se ponavadi uporabijo za potvorbe (sojino olje in olje oljne ogrščice) znatna. V prvi raziskavi smo na vzorcu sedmih različnih bučnih olj ugotavljali naslednje parametre pristnosti: vsebnost *trans*-izomerov maščobnih kislin, maščobnokislinsko sestavo, sterolno sestavo ter tokoferolno sestavo. Največja dovoljena vsebnost *trans*-izomerov maščobnih olj v deviškem in ekstra deviškem oljčnem olju je 0,05 ut. % za *trans*-izomer oleinske kisline in 0,05 ut. % za vsoto izomerov *trans*-linolne in *trans*-linolenske kisline. V naši raziskavi so se vrednosti za *trans*-oleinsko kislino gibale v razponu med 0,014 do 0,022 ut. % in za vsoto *trans*-linolne in *trans*-linolenske med 0,037 do 0,443 ut. %, kar je pokazalo na verjetnost potvorbe pri vzorcih S1, S4, S5 in S6. Objave, ki podajajo maščobnokislinsko sestavo bučnih olj zgovorno pričajo, da je razpon le-teh precej širok. Izkušnje preverjanja pristnosti na osnovi deležev posameznih maščobnih kislin pričajo, da ni izključevalni faktor delež tistih maščobnih kislin, ki so bolj zastopane v maščobni matrici, temveč ravno nasprotno – izključevalne so tim. minorne maščobne kisline - e.g. C 22:1, ki kaže na prisotnost olja oljne ogrščice, alfa-linolenska kislina, C 18:3 in C 20:1, ki potrjujeta prisotnost sojinega olja ali olja oljne ogrščice, C 20:0 sojinega olja, olja iz kikirikija ali oljne ogrščice ter C 22:0, ki potrjuje prisotnost olja iz kikirikija ali oljne ogrščice. Podobno velja tudi za bučna olja v luči ugotovitev iz kemometričnega pristopa. Naši rezultati so pokazali, da so trije vzorci: vzorec S1, vzorec S4 in vzorec S6 verjetno potvorjeni: vzorec S1 je imel povišano vsebnost kislin C 18:3, C 20:1 in C 22:0, vzorca S4 in S6 pa povišano vsebnost kisline C 22:0. Najbolj nazorna in prepričljiva indikacija prisotnosti semenskih olj pri potvorbi bučnih olj izvira iz dejstva, da bučna olja vsebujejo skoraj izključno Δ7-fitosterole. Iz analiziranih in izračunanih vrednosti razmerja Δ-5/Δ-7 sterolov je bilo pokazano, da je izrazita indikacija potvorbe pri vzorcih S1, S4, S5 in S6. Rezultati

določitve tokoferolov v vzorcih so pokazali na prevladujočo obliko gama-izomera, a podobno kot pri deležu maščobnih kislin, sta diskriminatorna deleža predvsem beta- in delta-oblika. Na njuni osnovi so bili izločeni vzorci S1, S4, S5 in S6. Združeni rezultati so pokazali na skladnost ugotovitev parcialnega preverjanja in pravilnost predlaganega postopnega ugotavljanja pristnosti. Izkazalo se je, da so vzorci S2, S3 in S7 zelo verjetno pristni in potrdili predpostavko, da je pristop z določevanjem *trans*-izomerov maščobnih kislin, deleža Δ-5/Δ-7 sterolov in vsebnosti tokoferolov tak, da uspe diskriminirati pristna od potvorjenih olj. Princip platforme ugotavljanja pristnosti bo v bodoče lahko gradil od hitrejših, cenejših in analitično manj zahtevnih določitev do tistih bolj kompleksnih in zahtevnih (določevanje deleža sterolov). Rezultati so dodatno potrdili pričakovanje, da so vrednosti iz določitve maščobnokislinskega deleža lahko le dodatno pomagalo pri procesu ugotavljanja pristnosti.

V nadaljevanju izgradnje platforme zasnove analitičnega postopka ugotavljanja pristnosti bučnega olja smo raziskavo razširili tudi na identifikacijo, speciacijo in statistično obdelavo stereospecifičnih triacilglicerolov v omenjenih vzorcih. Za te potrebe smo morali dopolniti in nadgraditi ustrezne analitične metode za kromatografsko določevanje triacilglicerolov v oljčnih oljih. Z uporabo modificirane metode in uporabo propionitrila kot mobilne faze in s temperaturno kontrolo kromatografske ločbe, nam je v vzorcih bučnih olj uspelo zadovoljivo ločiti 29 stereospecifičnih TAG v času 70 minut. Ločene TAG za vseh sedem vzorcev smo kvantificirali. Na osnovi dobljenih podatkov in po poglobljeni grafični analizi smo TAG vseh sedmih vzorcev razvrstili v šest grozdov in z nadaljnjo analizo v grozde razvrščenih podatkov izbrali devet signifikantnih TAG, in sicer: LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL in SLS. Diagram teže glavnih komponent je pokazal na zelo dobro porazdelitev izbranih signifikantnih TAG, PCA razsevni diagram pa je ne samo potrdil rezultate prejšnjih parcialnih ugotovitev potvorb pri vzorcih S1, S4, S5 in S6, temveč je nazorno pokazal, da analizirani vzorci tvorijo pet različnih skupin. Na osnovi prikazanih rezultatov sledi, da bo težko zaščititi in preverjati pristna olja z ZGO, če bodo geografsko in sortno zelo divergirala, kar se posledično lahko obrne proti zaščiti sami. Primerjava položaja vzorcev S2, S3 in S7 z izstopajočim položajem vzorca S7 v razsevnem diagramu je osvetlila drugo dejstvo, da utegne način procesiranja semen (hladno stiskanje/praženje) vplivati na samo razliko sestave TAG. V nadaljevanju raziskovanja uporabe analitičnega postopka ugotavljanja pristnosti bučnega olja, ki je bilo izvedeno v polju 15 vzorcev bučnih olj s slovenskega trga v letu 2008, so se potrdile vse navedene trditve in pravilnost zasnovanega analitičnega izključevalnega pristopa.

Pri raziskovanju možnih analitičnih postopkov pri preverjanju pristnosti bučnega olja smo se še posebej usmerili v enega najbolj pogosto gojenih kultivarjev 'Slovensko golico' in v matrico bučnega olja, predelanega iz praženih semen 'Slovenske golice'. Izkazalo se je, da so v frakciji FEMC tri spojine, o katerih še niso poročali. Te tri spojine so gama-tokomonoenol, gama-tokodienol in alfa-tokomonoenol. Koncentracije izomerov vitamina E v olju iz praženih semen 'Slovenske golice', ki smo jih kvantificirali, so bile naslednje:

alfa-tokomonoenol (17,6 ± 0,6 µg/g), gama-tokomonoenol (118,7 ± 1,0 µg/g), alfa-tokoferol (77,9 ± 1,9 µg/g), gama-tokoferol (586,0 ± 4,6 µg/g), beta-tokoferol (5,4 ± 0.0 µg/g) in delta-tokoferol (14,1 ± 0,3) µg/g. Določili smo tudi koncentracijo gama-tokotrienola, ki je bila 6,9 ± 0,2 µg/g, kar je znatno manj od vseh objavljenih podatkov. Da bi razjasnili diskrepanco v podatkih za GT3 in da bi dokazali morebiten vpliv procesa praženja na domnevno zmanjšanje višje koncentracije GT3 v vzorcih nepraženih bučnih semen, smo določili vsebnost vitamina E v nepraženih zmletih semenih, v zmletih semenih z dodano vodo in natrijevim kloridom ter v praženih zmletih semenih s pred praženjem dodanima vodo in natrijevim kloridom. Odkrili smo, da je vrednost vseh določevanih izomerov naraščala v seriji od nepraženih do praženih semen ter – presenetljivo - da je bila vsebnost GT3 že od začetka procesa nizka, v nepraženih semenih samo 1,6 ± 0,1 µg/g, kar je na meji LOQ! Potrdili smo, da je v preiskovanem vzorcu koncentracija GT3 prek celotnega procesa zelo nizka (v olju ne doseže 10 µg/g), in da proces praženja »koncentrira« izomere vitamina E. To koncentriranje so potrdile tudi kasnejše raziskave drugih avtorjev. Podroben pregled frakcije spojin vitamina E je pokazal na dva kromatografska vrhova, ki sta najbolj izražena v vzorcu praženih semen (oziroma v olju) in najmanj v vzorcu nepraženih semen. Menimo, da sta dva vrhova označevalca procesa praženja, verjetno sta oksidacijska produkta alfa- in gama-tokoferola. Vrh pred gama-tokoferolom je reda velikosti 10 µg/g in bi ga lahko po podrobnejši okarakterizaciji poskusili kvantitativno povezati s stopnjo praženja semen. Oba kromatografska vrhova bi v morebitnem nadaljevanju raziskave pokazala, da utegneta postati diskriminantni spojini za razlikovanje med hladno stiskanimi bučnimi olji in olji praženih bučnih semen in posredno tudi kot potrjevalna označevalca pri platformi pristnosti praženih bučnih olj. V navezi z izsledki stereospecifične analize triacilglicerolov hladno stiskanih bučnih olj, ki so z analizo glavnih komponent pokazali na razliko med TAG hladno stiskanih in praženih olj in v navezi z izsledki nekaterih novejših publikacij s področja praženih in hladno stiskanih bučnih olj, ki so raziskovale biofenolno frakcijo bučnih olj in ugotovile znaten porast skupnih biofenolov kot tudi spremenjeno ravnovesje med enostavnimi in esterificiranimi biofenoli, trdimo, da bi ob ustrezni dodatni raziskavi ti relativno enostavno analizno določljivi spojini lahko bistveno obogatili in implementirali ne samo platformo preverjanja pristnosti, temveč tudi platformo preverjanja stopnje predelave bučnega olja. Na koncu ponudimo tri sheme dreves odločanja z redosledom posameznih določitev in presoj, kjer na osnovi eksperimentalno dobljenih podatkov in na osnovi parcialnih določitev posameznega preskusa lahko preverimo pristnost vzorca bučnega olja, njegovo kakovost in pa stopnjo predelave.

4.2 SUMMARY

In the experimental part of the establishment of an analytical procedure for assessment of the genuineness and the degree of processing of pumpkin seed oil we introduced a new approach in the demanding matrix of pumpkin seed oil based on the principal phases of the »Decision tree« approach known from the quality and genuineness assessment of olive oils. The analytical techniques used were GC and HPLC. The »Decision tree« approach gives logical follow up of testing, assessing and exclusion steps based on data which are characteristic for the given oil category. The virgin olive oils and pumpkin seed oils must not be mixed with other edible oils and must not undergo refining process as well. In the assessing step of cold pressed pumpkin seed oils this »cold pressing« claim must be proved, logically based on a firm protocol, capable of setting basic terms and process indications of the "cold pressing" regime. »Decision tree« guides the assessing chemist from the *trans*-fatty acid determination to the final step of sterol composition determination. The tocopherols determination is not included in olive oil »Decision tree«, however we felt free to include it in pumpkin seed oils determination because of the very specific tocopherol composition edible oils used in adulteration (soya oil and rapeseed oil) have. The first research dealt with seven different pumpkin seed oils with following determinations: *trans*-fatty acids isomers content, fatty acids composition, sterols composition and tocopherols composition. The maximum allowed amount of *trans*-oleic acid in virgin olive oil is 0.05 %, as it is with the sum of *trans*-linoleic and *trans*-linolenic acids. The values determined in the samples ranged from 0.014 to 0.022 % for *trans*-oleic acid and from 0.037 to 0.443 % for the sum of *trans*-linoleic and *trans*-linolenic acids which was a clear indication for the admixture of edible oils in samples S1, S4, S5 and S6. Publications dealing with fatty acids composition of pumpkin seed oils reveal the rather broad distribution. Various experiences from fatty acids determination of pumpkin seed oils evidence the fact that the crucial factor in assessing the genuineness is not the major fatty acids content, but on contrary the content of minor ones. Acid C 22:1 points to the presence of the rapeseed oil, alpha-linolenic acid, C 18:3 and acid C 20:1 confirm the soya or rapeseed oils admixture, acid C 22:0 soya oil, peanut oil or rapeseed oil. The results of the fatty acids determination pointed at three samples – S1, S4 and S6 to be candidates for the adulteration. Sample S1 had higher amounts of acids C 18:3, C 20:1 and C 22:0 and samples S4 and S6 had higher amount of acid C 22:0. One of the most illustrative and confirmative indications of the seed oils presence in the pumpkin seed oils arises from the fact that pumpkin seed oils contain almost exclusively Δ7-phytosterols. From the data showing the sterols composition and from the calculated Δ-5/Δ-7 sterols ratio it was concluded again that samples S1, S4, S5 and S6 were very probably adulterated. Tocopherols analysis, following the "Decision tree" confirmed that the minor isomers – beta- and delta-tocopherols are the ones on which the discrimination between genuine and adulterated oils can be built. From tocopherols analysis it was shown and confirmed that samples S1, S4, S5 and S6 are adulterated. Pooled results were in agreement for the

conformity among partial assessment of the genuineness. It was shown that samples S2, S3 and S7 are genuine and that the stepwise approach gathering the *trans*-isomers of fatty acids data, Δ-5/Δ-7 sterols ratio and tocopherols concentration, was correct in being able to discriminate between adulterated and genuine oils. The main principle of the platform for genuineness assessment lies in the fact that more rapid and not too demanding analytical and cheaper approaches can be tried first and if the outcome is vague, it should be complemented with more complex and more time consuming (sterols determination) ones. The establishment of an analytical procedure for assessment of the genuineness of pumpkin seed oil was upgraded with the identification, speciation and statistical elaboration of the stereospecific TAGs data in the same seven samples from the beginning of the research. To fulfill this demand we had to implement and extend analytical method for chromatographic determination of TAGs in olive oils. We managed to successfully resolve 29 stereospecific TAGs with the use of propionitrile as a mobile phase and with the aid of temperature control of the chromatographic column in 70 minutes. Chromatographically separated TAGs for all seven samples were quantified and consequently statistically elaborated. TAGs were at first organized in six clusters and in consequence following the laborious elaboration analysis the nine most significant were chosen among them: LLLn, LLL, PLL, LOO, PLO, OOO, POO, SPL and SLS. PCA plot of component weights revealed excellent distribution of the chosen significant TAGs and the PCA scatter plot confirmed the righteousness of the previously proposed analytical platform discriminating samples S1, S4, S5 and S6 as the adulterated ones. Moreover – they illustrated the TAG richness of the samples, clustering them in 5 different groups. From the data shown it is evident that it will be a heavy task to protect and check/assess the genuine pumpkin seed oils with the PGI protection if they will be so geographically and cultivar diverse. This fact could – in the future – act as an obstacle for the efficient protection itself. The comparison of the relative positions of samples S2, S3 and S7 with the outstanding sample 7 (cold pressed oil) in the PCA scatter plot could testify for the different TAG composition of the roasted/cold pressed seeds. The continuing of the research of the establishment for an analytical procedure for assessment of the genuineness of pumpkin seed oil carried out in 2008 on a set of 15 samples of pumpkin seed oil confirmed all the conclusions set up in the establishment of the procedure proved to be correct. In the continuing research the cultivar 'Slovenska golica' and the matrix of roasted pumpkin seed oil from cultivar 'Slovenska golica' were investigated in greater extent, especially the FEMC fraction. It was shown that in the FEMC fraction there were three compounds not previously reported in pumpkin seed oils. These compounds were gamma-tocomonoenol, gamma-tocodienol and alpha-tocomonoenol. Concentrations of analysed isomers of vitamin E in the roasted 'Slovenska golica' seeds oil were as follows: alpha-tocomonoenol (17.6 ± 0.6 µg/g), gamma-tocomonoenol (118.7 ± 1.0 µg/g), alpha-tocopherol (77.9 ± 1.9 µg/g), gamma-tocopherol (586.0 ± 4.6 µg/g), beta-tocopherol (5.4 ± 0.0 µg/g) and delta-tocopherol (14.1 ± 0.3) µg/g. The gamma-tocotrienol concentration was determined as well and it accounted for 6.9 ± 0.2 µg/g which was

substantially less when compared to all previously reported data. To clarify this discrepancy and to prove the potential influence of the roasting process on the supposed decomposition of (high) concentration of GT3 in the unroasted seeds, the vitamin E isomers in unroasted seeds were analysed, as in ground seeds with added water and NaCl and in these ground seeds with added water and NaCl and roasted, as well. The analyses have shown – surprisingly – that the GT3 concentration was low from the very beginning – in the unroasted seeds as low as 1.6 ± 0.1 µg/g, which is comparable to the LOQ of the method. The fact that the GT3 concentration was low throughout the whole process (in oil not reaching 10 µg/g) was confirmed as well and the fact that the roasting process »concentrates« the vitamin E isomers. Detailed look at the vitamin E chromatographic fraction revealed the presence of two additional peaks. Their concentration increases with the process so that the final product – the oil – has their maximum amount; the one before gamma-tocopherol has the proposed concentration level of 10 µg/g. We think they are oxidation products of alpha- and gamma-tocopherol. These compounds could serve as excellent chemical markers and suitable tools for traceability purposes to indicate the roasting or non-roasting (cold pressing) history. Together with findings of the stereospecific analysis of TAGs of cold pressed pumpkin seed oils, which with the aid of PCA analysis discriminated between roasted and cold pressed oils, and together with some recent findings from the field of cold pressed and roasted seed pumpkin oils who dealt with biophenols fraction of the pumpkin seed oils and discovered big increase in the total biophenols amount and modified equilibrium between free and esterified biophenols, we can claim that these two compounds could substantially enrich and improve not only the assessing platform for the genuineness, but also the platform of the degree of processing of the pumpkin seed oil. At the end of the work three different “decision tree” schemes are proposed. On the basis of the follow up of proposed analytical determinations, established limits and assessments every particular “decision tree” serves as the aid for establishing the genuineness, the quality and the degree of processing of the pumpkin seed oil. This is acknowledged via analytical data gathered thru partial determinations.

5 VIRI

Amelio M., Amelotti G., Cozzoli O., Faraone V., Mariani C., Mattei A., Marzo G., Morchio G., Serani A., Spinetti M., Tiscornia E. 1993. Sottocommissione oli vegetali : determinazione degli acidi grassi trans insaturi negli oli non idrogenati mediante gascromatografia su colonna capillare. Risultati di una spermentazione interlaboratorio. Rivista Italiana delle Sostanze Grasse, 70, 11: 561-566

Andrikopoulos N.K., Chiou A., Mylona A. 2004. Triacylglycerol species of less common edible vegetable oils. Food Reviews International, 20, 4: 389-405

Aparicio R., Aparicio-Ruiz R. 2002. Chemometrics as an aid in olive oil authentication. V: Oils and fats authentication. Jee M. (ed.). Oxford, UK, Blackwell Publishing: 156-180

Aparicio R., Garcia-Gonzalez D.L. 2012. Chemical markers of sensory defects: the role of minor components in quality. V: 103rd AOCS annual meeting/Olive oil chemistry and sensory relationships short course. Long Beach, CA, 29. apr.–2. may 2012. Flynn D. (ed.). Urbana, IL, AOCS: 7 str. http://aocs.files.cms-plus.com/AnnualMeeting/Presentations/Aparicio-Chemical-Markers.pdf (6. avg. 2012)

Aparicio R., Morales M.T. 1995. Sensory wheels - a statistical technique for comparing QDA panels - application to virgin olive oil. Journal of the Science of Food and Agriculture, 67, 2: 247-257

Aparicio R., Morales M.T., Alonso V. 1997. Authentication of European virgin olive oils by their chemical compounds, sensory attributes, and consumers' attitudes. Journal of Agricultural and Food Chemistry, 45, 4: 1076-1083

Applequist W.L., Avula B., Schaneberg B.T., Wang Y.H., Khan I.A. 2006. Comparative fatty acid content of seeds of four *Cucurbita* species grown in a common (shared) garden. Journal of Food Composition and Analysis, 19, 6-7: 606-611

Baratova L.A., Bokova N.A., Yuskovich A.K., Samylina I A. 1982. Determination of the fatty-acid oil in pumpkin seeds by GLC method. Khimiya Prirodnykh Soedinenii, 2: 248-249

Bastić M., Bastić L., Jovanović J.A., Spiteller G. 1977. Sterols in pumpkin seed oil. Journal of the American Oil Chemists Society, 54, 11: 525-527

Bavec F., Gril L., Grobelnik-Mlakar S., Bavec M. 2002. Production of pumpkin for oil. V: Trends in new crops and new uses. Janick J., Whipkey A. (eds.). Alexandria, VA, ASHS Press: 187-190

Bavec F., Grobelnik-Mlakar S., Rozman C., Bavec M. 2007. Oil pumpkins: Niche for organic producers. V: Issues in new crops and new uses. Janick J., Whipkey A. (eds.). Alexandria, VA, ASHS Press: 185-189

Bayala I., David R. 2012. Extraction of α-tocopherolquinone from vegetable oil deodorizer distillate waste. European Journal of Lipid Science and Technology, DOI 10.1002/ejlt.201100380: 16 str. (v tisku)

Bosque-Sendra J.M., Cuadros-Rodríguez L., Ruiz-Samblás C., de la Mata A.P. 2012. Combining chromatography and chemometrics for the characterization and authentication of fats and oils from triacylglycerol compositional data: a review. Analytica Chimica Acta, 724: 1-11

Breinholder P., Mosca L., Lindner W. 2002. Concept of sequential analysis of free and conjugated phytosterols in different plant matrices. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 777, 1-2: 67-82

Brodnjak-Vončina D., Kodba Z.C., Novic M. 2005. Multivariate data analysis in classification of vegetable oils characterized by the content of fatty acids. Chemometrics and Intelligent Laboratory Systems, 75, 1: 31-43

Butinar B., Bučar-Miklavčič M., Krumpak A., Raspor P. 2009. Experiences in olive oil purity and quality assessment as a tool for pumpkin seed oil evaluation. What can consumers benefit? Acta Alimentaria, 38, 2: 219-227

Butinar B., Bučar-Miklavčič M., Valencič V., Raspor P. 2010a. Stereospecific analysis of triacylglycerols as a useful means to evaluate genuineness of pumpkin seed oils: lesson from virgin olive oil analyses. Journal of Agricultural and Food Chemistry, 58, 9: 5227-5234

Butinar B., Bučar-Miklavčič M., Raspor P. 2010b. Kemijsko voden pristop kot pripomoček pri ugotavljanju pristnosti bučnega olja : potrošniki in proizvajalci na isti strani? V: I. mednarodno znanstveni posvet/konferenca na temo Prehrana, gibanje in zdravje v interakciji. Rakičan, 20. 10. do 22.10. 2010. Katalinič D., Sekolovnik J., Jakob S. (ur.). Rakičan, RIS Dvorec: 31-36

Butinar B., Bučar-Miklavčič M., Mariani C., Raspor P. 2011a. New vitamin E isomers (gamma-tocomonoenol and alpha-tocomonoenol) in seeds, roasted seeds and roasted seed oil from the Slovenian pumpkin variety 'Slovenska golica'. Food Chemistry, 128, 2: 505-512

Butinar B., Bučar-Miklavčič M., Valenčič V., Rovellini P., Raspor P. 2011b. Ali so bučna olja na slovenskem trgu pristna? V: II. mednarodno znanstveni posvet/konferenca na

temo Prehrana, gibanje in zdravje. Rakičan, 19. okt. do 21. okt. 2012. Sekolovnik J., Katalinič D., Jakob S. (ur). Rakičan, RIS Dvorec: 35-40

Caponio F., Conte L., Summo C., Paradiso V.M., Pedone G., Gomes T. 2012. Evolution of regulations regarding the commercial classes of olive oils. Rivista Italiana Delle Sostanze Grasse, 89, 1: 29-39

Cert A., Lanzon A., Carelli A.A., Albi T., Amelotti G. 1994. Formation of stigmasta-3,5-diene in vegetable-oils. Food Chemistry, 49, 3: 287-293

Christopoulou E., Lazaraki M., Komaitis M., Kaselimis K. 2004. Effectiveness of determinations of fatty acids and triglycerides for the detection of adulteration of olive oils with vegetable oils. Food Chemistry, 84, 3: 463-474

Codex Alimentarius. 1974. Report of the seventh session of the Codex Committee on fats and oils. Rome, Codex Alimentarius Commission/Food and Agriculture Organization of the United Nations/World Health Organization/ Joint FAO/WHO Food Standards Programme: 28 str.

Codex Alimentarius. 1981. Codex-STAN 19-1981. Codex standard for edible fats and oils not covered by individual standards. Rome, Codex Alimentarius Commission/Food and Agriculture Organization of the United Nations/World Health Organization Joint FAO/WHO Food Standards Programme: 5 str.

Codex Alimentarius. 1999. Codex-STAN-210 1999 Codex standard for named vegetable oils. Rome, Codex Alimentarius Commission/Food and Agriculture Organization of the United Nations/World Health Organization/Joint FAO/WHO Food Standards Programme: 16 str.

Codex Alimentarius. 2011. Report of the twenty second session of the Codex Committee on fats and oils. Rome, Codex Alimentarius Commission Food and Agriculture Organization of the United Nations/World Health Organization Joint FAO/WHO Food Standards Programme: 48 str.

Commission Regulation (EEC) No 2568/91 of 11 July 1991 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis. 1991. Official Journal of the European Union, 34, L248: 1-83

Commission Regulation (EC) No 1989/2003 of 6 November 2003 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-pomace oil and on the relevant methods of analysis. 2003. Official Journal of the European Union, 46, L295: 57-77

Council Regulation (EC) No 510/2006 of 20 March 2006 on the protection of geographical indications and designations of origin for agricultural products and foodstuffs. 2006. Official Journal of the European Union, 49, L93: 12-25

Dunlap W.C., Fujisawa A., Yamamoto Y., Moylan T.J., Sidell B.D. 2002. Notothenioid fish, krill and phytoplankton from Antarctica contain a vitamin E constituent (alpha-tocomonoenol) functionally associated with cold-water adaptation. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 133, 3: 299-305

Erčulj Rogel K. 2002. Maščobnokislinska sestava in parametri kakovosti bučnega olja iz belic. Diplomsko delo. Ljubljana, Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za živilstvo: 69 str.

Falk J., Munne-Bosch S. 2010. Tocochromanol functions in plants: antioxidation and beyond. Journal of Experimental Botany, 61, 6: 1549-1566

Fiebig H.J. 1985. HPLC separation of triglycerides. Fette Seifen Anstrichmittel, 87, 2: 53-57

Fiorentino A., Mastellone C., D'Abrosca B., Pacifico S., Scognamiglio M., Cefarelli G., Caputo R., Monaco P. 2009. [delta]-Tocomonoenol: A new vitamin E from kiwi (*Actinidia chinensis*) fruits. Food Chemistry, 115, 1: 187-192

Fruhwirth G.O., Hermetter A. 2007. Seeds and oil of the Styrian oil pumpkin: Components and biological activities. European Journal of Lipid Science and Technology, 109, 11: 1128-1140

Fruhwirth G.O., Hermetter A. 2008. Production technology and characteristics of Styrian pumpkin seed oil. European Journal of Lipid Science and Technology, 110, 7: 637-644

Fruhwirth G.O., Wenzl T., El-Toukhy R., Wagner F. S., Hermetter A. 2003. Fluorescence screening of antioxidant capacity in a pumpkin seed oils and other natural oils. European Journal of Lipid Science and Technology, 105, 6: 266-274

Fu C., Shi H., Li Q.H. 2006. A review on pharmacological activities and utilization technologies of pumpkin. Plant Foods for Human Nutrition, 61, 2: 73-80

Garcia-Gonzalez D.L., Aparicio R. 2010. Research in olive oil: Challenges for the near future. Journal of Agricultural and Food Chemistry, 58, 24: 12569-12577

Garcia-Gonzalez D.L., Aparicio-Ruiz R., Aparicio R. 2008. Virgin olive oil: Chemical implications on quality and health. European Journal of Lipid Science and Technology, 110, 7: 602-607

Garcia-Gonzalez D.L., Luna G., Morales M.T., Aparicio R. 2009. Stepwise geographical traceability of virgin olive oils by chemical profiles using artificial neural network models. European Journal of Lipid Science and Technology, 111, 10: 1003-1013

Garg V.K., Nes W.R. 1985. Sterols of the family cucurbitaceae - an evolutionary recapitulation. Journal of the American Oil Chemists Society, 62, 4: 652-652

Garg V.K., Nes W.R. 1986. Occurrence of delta-5 sterols in plants producing predominantly delta-7 sterols - studies on the sterol compositions of 6 cucurbitaceae seeds. Phytochemistry, 25, 11: 2591-2597

Gemrot F., Barouh N., Vieu J.P., Pioch D., Montet D. 2006. Effect of roasting on tocopherols of gourd seeds (*Cucurbita pepo*). Grasas y Aceites, 57, 4: 409-414

Gotoh N., Watanabe H., Oka T., Mashimo D., Noguchi N., Hata K., Wada S. 2009. Dietary marine-derived tocopherol has a higher biological availability in mice relative to alpha-tocopherol. Lipids, 44, 2: 133-143

Grob K., Biedermann M., Bronz M., Mariani C. 1995. Composition of the sterol dehydration products in refined olive oil. Rivista Italiana delle Sostanze Grasse, 72, 2: 49-54

Hajimahmoodi M., Vander Heyden Y., Sadeghi N., Jannat B., Oveisi M.R., Shahbazian S. 2005. Gas-chromatographic fatty-acid fingerprints and partial least squares modeling as a basis for the simultaneous determination of edible oil mixtures. Talanta, 66, 5: 1108-1116

Homberg E. 1974. Alteration of sterols by industrial processing of fats and oils.1. Influence of refining conditions on sterol content and sterol composition. Fette Seifen Anstrichmittel, 76, 10: 433-435

IOC. 2006. COI/T.20/Doc. No 25 - 2006 Global method for the detection of extraneous oils in olive oils. Madrid, International Olive Council: 19 str. http://www.internationaloliveoil.org/documents/viewfile/3329-25-avelleng (7. avg. 2012)

IOC. 2009. COI/T.20/Doc. No 29. Determination of biophenols in olive oils by HPLC. Madrid, International Olive Council: 8 str. http://www.internationaloliveoil.org/documents/viewfile/4141-met29eng (7. avg. 2012)

IOC. 2010. COI/T.20/Doc. 20/Rev. 3 - 2010 Determination of the difference between actual and theoretical content of triacylglycerols with ECN 42. Madrid, International Olive Council: 23 str. http://www.internationaloliveoil.org/documents/viewfile/3884-testing7 (7. avg. 2012)

ISO 9936. 2006. Animal and vegetable fats and oils - Determination of tocopherol and tocotrienol contents by high-performance liquid chromatography: 17 str.

IUPAC-IUB. 1982. Nomenclature of tocopherols and related compounds - recommendations 1981. European Journal of Biochemistry, 123, 3: 473-475

Jakab A., Heberger K., Forgacs E. 2002. Comparative analysis of different plant oils by high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. Journal of Chromatography A, 976, 1-2: 255-263

Joebstl D., Bandoniene D., Meisel T., Chatzistathis S. 2010. Identification of the geographical origin of pumpkin seed oil by the use of rare earth elements and discriminant analysis. Food Chemistry, 123, 4: 1303-1309

Kalo P., Kuuranne T. 2001. Analysis of free and esterified sterols in fats and oils by flash chromatography, gas chromatography and electrospray tandem mass spectrometry. Journal of Chromatography A, 935, 1-2: 237-248

Kljun A. 2011. Pridelovanje oljnih buč (*Cucurbita pepo* L. var. *oleifera*) v Sloveniji. Diplomsko delo. Ljubljana, Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za agronomijo: 34 str.

Koprivnjak O. 2006. Djevičansko maslinovo ulje od masline do stola. Poreč, MIH,d.o.o.: 156 str.

Kreft I., Stibilj V., Trkov Z. 2002. Iodine and selenium contents in pumpkin (*Cucurbita pepo* L.) oil and oil-cake. European Food Research and Technology, 215, 4: 279-281

Kreft M., Zorec R., Janes D., Kreft, S. 2009. Histolocalisation of the oil and pigments in the pumpkin seed. Annals of Applied Biology, 154, 3: 413-418

Kuhnle G.G.C., Dell'Aquila C., Aspinall S.M., Runswick S.A., Mulligan A.A., Bingham S.A. 2008. Phytoestrogen content of beverages, nuts, seeds, and oils. Journal of Agricultural and Food Chemistry, 56, 16: 7311-7315

Lankmayr E., Mocak J., Serdt K., Balla B., Wenzl T., Bandoniene D., Gfrerer M., Wagner S. 2004. Chemometrical classification of pumpkin seed oils using UV-Vis, NIR and FTIR spectra. Journal of Biochemical and Biophysical Methods, 61, 1-2: 95-106

Lanzon A., Albi T., Cert A., Gracian J. 1994. The hydrocarbon fraction of virgin olive oil and changes resulting from refining. Journal of the American Oil Chemists Society, 71, 3: 285-291

Lanzon A., Cert A., Albi T. 1989. Detection of refined olive oil in virgin olive oil. Grasas y Aceites, 40, 6: 385-388

Lelley T., Loy B., Murkovic M. 2010. Hull-less oil seed pumpkin oil crops. V: Handbook of plant breeding. Vol. 4. Vollmann J., Rajcan I. (eds.). New York, Springer: 469-492

Makovšek K. 2003. Določanje sterolov v bučnih oljih s plinsko kromatografijo. Diplomsko delo. Maribor, Univerza v Mariboru, Fakulteta za kemijo in kemijsko tehnologijo: 50 str.

Mandl A., Reich G., Lindner W. 1999. Detection of adulteration of pumpkin seed oil by analysis of content and composition of specific Delta 7-phytosterols. European Food Research and Technology, 209, 6: 400-406

Mariani C., Bellan G. 1996. Content of tocopherols, deidrotocopherols, tocodienols, tocotrienols in vegetable oils. La Rivista Italiana Delle Sostanze Grasse, 73, 4: 533-543

Mariani C., Fedeli E. 1986. Detection of extraction oils in pressure ones. Note1. La Rivista Italiana Delle Sostanze Grasse, 63, 1: 3-17

Mariani C., Fedeli E., Grob K. 1991. Evaluation of free and esterified minor components in fatty materials. La Rivista Italiana Delle Sostanze Grasse, 68, 5: 233-242

Marković V.V., Bastić L.V. 1976. Characteristics of pumpkin seed oil. Journal of the American Oil Chemists Society, 53,1: 42-44

Matthaus B. 2008. Virgin oils - The return of a long known product. European Journal of Lipid Science and Technology, 110, 7: 595-596

Matthaus B., Spener F. 2008. What we know and what we should know about virgin oils a general introduction. European Journal of Lipid Science and Technology, 110, 7: 597-601

Moreda W., Perez-Camino M.C., Cert A. 2003. Improved method for the determination of triacylglycerols in olive oils by high performance liquid chromatography. Grasas y Aceites, 54. 2: 175-179

Murkovic M., Hillebrand A., Winkler J., Leitner E., Pfannhauser W. 1996a. Variability of fatty acid content in pumpkin seeds (*Cucurbita pepo* L.). Zeitschrift fur Lebensmittel-Untersuchung Und-Forschung, 203, 3: 216-219

Murkovic M., Hillebrand A., Winkler J., Pfannhauser W. 1996b. Variability of vitamin E content in pumpkin seeds (*Cucurbita pepo* L.). Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung, 202, 4: 275-278

Murkovic M., Pfannhauser W. 2000. Stability of pumpkin seed oil. European Journal of Lipid Science and Technology, 102, 10: 607-611

Murkovic M., Piironen V., Lampi A.M., Kraushofer T., Sontag G. 2004. Changes in chemical composition of pumpkin seeds during the roasting process for production of pumpkin seed oil (Part 1: non-volatile compounds). Food Chemistry, 84, 3: 359-365

Murkovic M., Wiltschko B., Pfannhauser W. 1997. Formation of alpha-tocopherolquinone and alpha-tocopherolquinone epoxides in plant oil. Fett-Lipid, 99, 5: 165-169

Nakić S.N., Rade D., Škevin D., Štrucelj D., Mokrovčak Z., Bartolić M. 2006. Chemical characteristics of oils from naked and husk seeds of *Cucurbita pepo* L. European Journal of Lipid Science and Technology, 108, 11: 936-943

Neđeral S., Škevin D., Kraljić K., Obranović M., Papeša S., Bataljaku A. 2012. Chemical composition and oxidative stability of roasted and cold pressed pumpkin seed oils. Journal of the American Oil Chemists' Society, DOI 10.1007/s11746-012-2076-0: 8 str. (v tisku)

Nikiforov A., Knapp M., Buchbauer G., Jirowetz L. 1996. Zur Bestimmung der dominierenden Geruchskomponenten (character impact compounds) von steirischem Kirbiskernöl. Ernährung/Nutrition, 20, 12: 643-644

Objava vloge na podlagi člena 6(2) Uredbe (ES) št. 510/2006 o zaščiti geografskih označb in označb porekla za kmetijske proizvode in živila (2006/C 127/08). 2006. Uradni list Evropske unije, 49, C127: 16-18

Ohnmacht S., West R., Sirnionescu R., Atkinson J. 2008. Assignment of the H-1 and C-13 NMR of tocotrienois. Magnetic Resonance in Chemistry, 46, 3: 287-294

Oil crops yearbook 2011. 2011. Ithaca, NY, United States Department of Agriculture, Economic Research Service: database http://usda.mannlib.cornell.edu/MannUsda/viewStaticPage.do?url=http://usda.mannlib.cornell.edu/usda/ers/89002/2011/index.html (9. avg. 2012)

Oils and fats markets in Western Europe - 2010. 2011. Geneva, Food for Thought: database.
http://www.fft.com (9. jul. 2012)

O izdelovanju olja in grozdnih peček in bučnih jederc. 1916. Štajerc, 17, 44: 4-5 http://www.dlib.si/?URN=URN:NBN:SI:doc-IOD82XE4 (6. avgust 2012)

Ollivier D., Artaud J., Pinatel C., Durbec J. P., Guerere M. 2003. Triacylglycerol and fatty acid compositions of French virgin olive oils. Characterization by chemometrics. Journal of Agricultural and Food Chemistry, 51, 19, 5723-5731

Ollivier D., Artaud J., Pinatel C., Durbec J.P., Guerere M. 2006. Differentiation of French virgin olive oil RDOs by sensory characteristics, fatty acid and triacylglycerol compositions and chemometrics. Food Chemistry, 97, 3: 382-393

Parry J., Hao Z.G., Luther M., Su L., Zhou K.Q., Yu L.L. 2006. Characterization of cold-pressed onion, parsley, cardamom, mullein, roasted pumpkin, and milk thistle seed oils. Journal of the American Oil Chemists Society, 83, 10: 847-854

Perona J.S., Ruiz-Gutierrez V. 2004. Analysis of neutral lipids: triacylglycerols. V: Handbook of food analysis. Nollet L.M.L. (ed.). New York, Marcel Dekker: 275-312

Pojbič M. 2008. Izboljšave v tehnološkem procesu izdelave bučnega olja. Diplomska naloga. Maribor, Živilska šola Maribor: 52 str.

Puah C. W., Choo Y.M., Ma A.N., Chuah C.H. 2007. The effect of physical refining on palm vitamin E (tocopherol, tocotrienol and tocomonoenol). American Journal of Applied Sciences, 4, 6: 374-377

Publication of an application pursuant to Article 6(2) of Council Regulation (EC) No 510/2006 on the protection of geographical indications and designations of origin for agricultural products and foodstuffs. 2009. Official Journal of the European Union, 52, C72: 67-69

Rižnik A. 2011. Proučitev postopkov pospešenega generacijskega cikla pri oljnih bučah (*Cucurbita pepo* L. subsp. *pepo* var. *stryriaca* Grebenšč.) s kulturo nezrelih embrijev *in vitro* in poskus medvrstnega križanja *C. pepo* in *C. maxima*. Diplomsko delo. Ljubljana, Univerza v Ljubljani, Biotehniška fakulteta, Študij biotehnologije: 53 str.

Rodošek M. 2009. Določanje posameznih in celokupnih fitosterolov v rastlinskih oljih s plinsko kromatografijo. Diplomsko delo. Maribor, Univerza v Mariboru, Fakulteta za naravoslovje in matematiko, Oddelek za kemijo: 78 str.

Rovellini P., Cortesi N. 2002. α-Tocopherol oxidation products. Rivista Italiana Delle Sostanze Grasse, 79, 10: 335-341

Ryan D., Robards K. 2006a. Analytical chemistry considerations in plant metabolomics. Separation and Purification Reviews, 35, 4: 319-356

Ryan D., Robards K. 2006b. Metabolomics: The greatest omics of them all? Analytical Chemistry, 78, 23: 7954-7958

Saucedo-Hernandez Y., Lerma-Garcia M.J., Herrero-Martinez J.M., Ramis-Ramos G., Jorge-Rodriguez E., Simo-Afonso E.F. 2011. Classification of pumpkin seed oils according to their species and genetic variety by attenuated total reflection Fourier-

transform infrared spectroscopy. Journal of Agricultural and Food Chemistry, 59, 8: 4125-4129

Sayago A., Marin M.I., Aparicio R., Morales M.T. 2007. Vitamin E and vegetable oils. Grasas Y Aceites, 58, 1: 74-86

Sicilia T., Niemeyer H.B., Honig D.M., Metzler M. 2003. Identification and stereochemical characterization of lignans in flaxseed and pumpkin seeds. Journal of Agricultural and Food Chemistry, 51, 5: 1181-1188

Srbinoska M., Hrabovski N., Rafajlovska V., Sinadinović-Fišer S. 2012. Characterization of the seed and seed extracts of the pumpkins *Cucurbita maxima* D. and *Cucurbita pepo* L. from Macedonia. Macedonian Journal of Chemistry and Chemical Engineering, 31, 1: 65-78

Stevenson D.G., Eller F.J., Wang L.P., Jane J.L., Wang T., Inglett G.E. 2007. Oil and tocopherol content and composition of pumpkin seed oil in 12 cultivars. Journal of Agricultural and Food Chemistry, 55, 10: 4005-4013

SURS. 2012. Pridelava poljščin (ha, t, t/ha) Slovenija, letno. Ljubljana, Statistični urad Republike Slovenije: 1 str. (baza podatkov) http://pxweb.stat.si/pxweb/Dialog/varval.asp?ma=1502402S&ti=&path=../Database/Okolje/15_kmetijstvo_ribistvo/04_rastlinska_pridelava/01_15024_pridelki_povrsina/&lang=2 (8. avg. 2012)

Šuturović Z.J., Marjanović N.J. 1999. Chronopotentiometric study of tocopherols in vegetable oils. Electroanalysis, 11, 3: 207-209

Škerlič I., Bučar-Miklavčič M., Bandelj Mavsar D., Režek-Donev N., Maršič K. Izdelava predhodne študije ekstra deviškega oljčnega olja z geografskim poreklom. 2006. Koper, Univerza na Primorskem, Znanstveno-raziskovalno središče Koper: 57 str.

Štajersko prekmursko bučno olje. 2005. Slovenska Bistrica, GIZ - Gospodarsko interesno združenje Golica: 43 str.

Teppner H. 2004. Notes on *Lagenaria* and *Cucurbita* (*Cucurbitaceae*) - Review and new contributions. Phyton-Annales Rei Botanicae, 44, 2: 245-308

Tuberoso C.I.G., Kowalczyk A., Sarritzu E., Cabras, P. 2007. Determination of antioxidant compounds and antioxidant activity in commercial oilseeds for food use. Food Chemistry, 103, 4: 1494-1501

Uredba Komisije (ES) št. 148/2007 z dne 15. februarja 2007 o vnosu nekaterih imen v Register zaščitenih označb porekla in zaščitenih geografskih označb. 2007. Uradni list Evropske unije, 50, L46: 14-17

Uredba Komisije (EU) št. 61/2011 z dne 24. januarja 2011 o spremembi Uredbe (EGS) št. 2568/91 o značilnostih oljčnega olja in olja iz oljčnih tropin ter o ustreznih analiznih metodah. 2011. Uradni list Evropske unije, 54, L23: 1-14

Uredba Komisije (EU) št. 432/2012 z dne 16. maja 2012 o seznamu dovoljenih zdravstvenih trditev na živilih, razen trditev, ki se nanašajo na zmanjšanje tveganja za nastanek bolezni ter na razvoj in zdravje otrok Besedilo velja za EGP. 2012. Uradni list Evropske unije, 55, L136: 1-40

Vuga J. 2002. Maščobnokislinska sestava in parametri kakovosti bučnega olja iz golic. Diplomsko delo. Ljubljana. Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za živilstvo: 60 str.

Vujasinović V., Djilas S., Dimić E., Romanić R., Takači A. 2010. Shelf life of cold-pressed pumpkin (*Cucurbita pepo* L.) seed oil obtained with a screw press. Journal of the American Oil Chemists Society, 87, 12: 1497-1505

Vujasinović V., Djilas S., Dimić E., Basić Z., Radočaj O. 2012. The effect of roasting on the chemical composition and oxidative stability of pumpkin oil. European Journal of Lipid Science and Technology, 114, 5: 568-574

Wenzl T., Prettner E., Schweiger K., Wagner F.S. 2002. An improved method to discover adulteration of Styrian pumpkin seed oil. Journal of Biochemical and Biophysical Methods, 53, 1-3: 193-202

Yamamoto Y., Maita N., Fujisawa A., Takashima J., Ishii Y., Dunlap W.C. 1999. A new vitamin E (alpha-tocomonoenol) from eggs of the Pacific salmon *Oncorhynchus keta*. Journal of Natural Products, 62, 12: 1685-1687

Yoshida H., Tomiyama Y., Hirakawa Y., Mizushina Y. 2004. Variations in the composition of acyl lipids and triacylglycerol molecular species of pumpkin seeds (*Cucurbita* spp.) following microwave treatment. European Journal of Lipid Science and Technology, 106, 2: 101-109

Yoshida H., Tomiyama Y., Hirakawa Y., Mizushina Y. 2006. Microwave roasting effects on the oxidative stability of oils and molecular species of triacylglycerols in the kernels of pumpkin (*Cucurbita* spp.) seeds. Journal of Food Composition and Analysis, 19, 4: 330-339

Yoshida H., Tomiyama Y., Kita S., Mizushina Y. 2005. Roasting effects on fatty acid distribution of triacylglycerols and phospholipids in the kernels of pumpkin (*Cucurbita* spp.) seeds. Journal of the Science of Food and Agriculture, 85, 12: 2061-2066

Yu W.L., Zhao Y.P., Chen J.J., Shu B. 2004. Comparison of two kinds of pumpkin seed oils obtained by supercritical CO_2 extraction. European Journal of Lipid Science and Technology, 106, 6: 355-358

ZAHVALA

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¡Oh!, qué llanura empinada con veinte soles arriba.
¡Qué ríos puestos de pie vislumbra su fantasía!
Pero sigue con sus flores,
mientras que de pie, en la brisa, la luz juega el ajedrez alto de la celosía.

To je Lorcova ***La monja gitana – Ciganska nuna*** iz *Ciganskega romansera*. In tako kot takrat, se tudi zdaj v hipu odločim – to! Takrat jo je prijatelj Jadran Sterle pomagal prevesti … to je njegov prevod, ki mi je veliko bolj všeč, ker je bolj za dušo – uradni champegnoise prevod (Aleš Berger, Tanji bo zaigralo srce) pa mi je preveč trd, čeprav razume bistvo – skoraj trdoerotično bistvo, ki naj si ga bralec sam poišče in primerja…

O privzdignjena ravnica,
dvajset sonc nad njo blešči se!
Dosti vzpetih rečnih tokov
zrejo njene fantazije!
A kar veze svoje rože.
Zunaj sapica se dvigne
in na okenskih rebračah
šah igrajo sončne lise.

In če kaj vem, vem zdaj: 2012 – 1981 = 31, po vseh enaintridesetih letih še bolj vem, da je potrebno slaviti učitelje!

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…my project
was to please: now I want
Spirits to enforce, art to enchant;
And my ending is despair

Hrpelje, poletje 2012