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Fatty Acid and Sterol Profile of Nutmeg (*Myristica fragrans*) and Star Anise (*Illicium verum*) Extracted by Three Different Methods †

Marko Obranović [1], Joanna Bryś [2], Maja Repajić [1], Sandra Balbino [1], Dubravka Škevin [1], Andrzej Bryś [3], Petra Tonković [1], Ana Marija Medved [1], Verica Dragović Uzelac [1] and Klara Kraljić [1]

[1] Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia; email1@gmail.com (M.O.); email2@gmail.com (M.R.); email3@gmail.com (S.B.); email4@gmail.com (D.S.); email5@gmail.com (P.T.); email6@gmail.com (A.M.M.); email7@gmail.com (V.D.U.); email8@gmail.com (K.K.)

[2] Institute of Food Science, Warsaw University of Life Sciences, Poland; email1@gmail.com

[3] Institute of Mechanical Engineering, Warsaw University of Life Sciences, Poland; email1@gmail.com

* Correspondence: e-mail@e-mail.com; Tel.: +xx-xxxx-xxx-xxxx

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Abstract: Nutmeg (*Myristica fragrans*) and star anise (*Illicium verum*) are world popular spices originally from South East part of Asia. Apart as food condiment they have been used extensively in traditional medicine and lately as a subject of research in the field of pharmacology and medical sciences. Most of the research were done on the subject of essential oils, especially for the star anise, while the data on seed oil properties is much scarcer. The main problems in oil extraction of nutmeg is hardness of the nut while for star anise seed is relatively low oil yield (around 10 %). This presents significant problem for screw press production and demands different methods of extraction for better oil yield and quality. Aim of this research was to compare three different methods of oil extraction with *n*-hexane Agitation Assisted Extraction (AAE), the Soxhlet Extraction (SE) and Accelerated Solvent Extraction (ASE) at 25 and 100 °C and to compare the processes on the basis of invested time, oil yield, fatty acid and sterol profile of the oils. The determination of fatty acid composition was carried out by GC-FID analysis of fatty acid methyl esters while sterols composition was determined with GC-MS. The highest yield of oil was obtained using the Soxhlet method while the best results combining time and yield were obtained with the ASE method at 100°C. The main fatty acid in star anise seed oil was lauric (average 62.30%) with significant differences between extraction methods ($p < 0.05$) while myristic (average 75.69%) was the most abundant in all samples of nutmeg oil. The main sterol in all of the samples was β-sitosterol.

Keywords: nutmeg; star anise; fatty acids; sterols; accelerated solvent extraction

1. Introduction

Nutmeg (*Myristica fragrans*) and star anise (*Illicium verum*) are native herbs from South East Asia which were traditionally used as spices or as a part of traditional medicine and as cosmetic ingredient. Main focus of previous scientific research done in this field was conducted on essential/volatile oils while there is little data regarding fixed oil properties. Nutmeg belongs to the family Myristicaceae and is a medium sized, evergreen aromatic tree. Its seed (nutmeg) and its fleshy aril (mace) are used as spices. Guatemala, Indonesia, India, Nepal, Sri Lanka, Lao and Bhutan produce more than 97 % of global nutmeg ("FAO," 2020). The Nutmeg tree is indigenous to Banda islands in the Moluccas in east Indonesia. Nutmeg butter, a fat derived from the seed is used in perfumery, tobacco and toothpaste. Medicinally, it is used to support digestion and to treat rheumatism. *Myristica fragrans*

seed is also used for diarrhoea, mouth sore and insomnia (Nagja et al., 2016). Nutmeg contains an essential oil, a fixed oil, proteins, fats, starch, and mucilage. Yield of the essential oil is 5–15 % and it was the main focus of published scientific research. The intoxicating effects of the seeds have been assigned to the presence of myristin and elemicin in volatile oil while the myristin is responsible for most of its pharmacological effects (Kuete, 2017). Seeds also contains 25–50 % fixed oils depending on the source (Abdurrasheed and Janardanan, 2009; Kuete, 2017; Niyas et al., 2003) with dominant myristic, stearic, palmitic, oleic, linoleic and lauric acids (Kuete, 2017; Manasa et al., 2020; Niyas et al., 2003). Significantly less research has been done on sterol composition of nutmeg oil with dominant representatives in β-sitosterol and campesterol (Al-Khatib et al., 1987; Hou et al., 2012). Star anise is an aromatic evergreen tree with red flowers and star fruits. It belongs to the family Schisandraceae and grows mainly in southern China and Vietnam. Its dried fruit is used as a spice and is very important in Chinese medicine where it is used to relieve vomiting, stomach pain, insomnia, skin inflammation, and rheumatic pain. It possesses antimicrobial, antiviral, and antioxidant properties and the fruits are also referred in Ayurveda, traditional Indian system of medicine, in variety of health problems. (Patra et al., 2020). The shikimic acid, which is used as a primary ingredient for antiviral drug Tamiflu (oseltamivir phosphate) is extracted from star anise. Oseltamivir is considered the only drug available which may reduce the severity of bird flu (Dinesha et al., 2014; Patra et al., 2020; Wang et al., 2011). Other components of star anis which were focus of scientific research are ssential oils, prenylated C_6–C_3 compounds, lignans, sesquiterpenes and flavonoids (Wang et al., 2011). Accelerated solvent extraction at elevated pressure (ASE) is a newer and more advanced extraction technique that has recently been increasingly used due to its many advantages. It is an automated method that represents an alternative to Soxhlet extraction with shorter extraction time and less solvent usage. This extraction method involves the use of elevated pressure which keeps the solvent in a liquid state during extraction at higher temperatures and thus prevents it from evaporating. The application of elevated temperature increases the kinetic energy of molecules in the system leading to an increase in the rate of chemical reactions, higher solubility and higher diffusion rate of solutes in the solvent (Jentzer et al., 2015). Furthermore, the ASE principle makes it possible to carry out extraction in several cycles, the purpose of which is to introduce a fresh solvent and thus maintain a favourable extraction balance (Sarker, 2012). The extraction is performed on a specially designed ASE device which contributes to the automation and ease of use of this extraction method. The aims of this present paper were (1) to give more detail information about fatty acid and sterol composition of nutmeg and star anise seed oils (2) to try to give alternative methods for oil extraction taking in account hardness of nutmeg and low oil yield in star anise and (3) to give some basis for further research of this seeds using ASE.

2. Statistical Analysis

The analysis of the studied samples was performed in triplicate. The results were presented as the mean ± standard error (SE). The statistical analysis was carried out with ExcelStat 2020. One-way analysis of variance (ANOVA) was used to compare results between the studied seed oils. Difference between samples were examined using a Tukey`s test and were considered significant at $p < 0.05$.

3. Methods

3.1. Materials and Reagents

Nutmeg (*Myristica fragrans*) and star anise (*Illicium verum*) were obtained from the local supplier of spices in Zagreb, Croatia. Origin of nutmeg was Thailand and star anise from Vietnam. All chemicals and solvents were of analytical grade and used without further purification.

3.2. Oil Extraction

Soxhlet extraction—oil yield: Oil and moisture contents of the seeds were determined by using ISO methods 659 and 665 (ISO 659:2010, 2010; ISO 665:2004, 2004). The data obtained were used to calculate oil yield, defined as the percentage of oil extracted by each process on a total oil extractable

basis. **Agitation Assisted Extraction:** Seeds were crushed and ground in Waring Blender WSG60E grinder into a fine powder. Extraction was performed by *n*-hexane. Sample mass of 4 g was extracted with 40 mL of *n*-hexane at room temperature using the table shaker. After 20 min, the sample was centrifuged for 10 min at 5000 rpm. Supernatant was decanted and the pellet was returned to the flask and extracted two more times with 40 mL of hexane for 20 min. Extracts were combined and evaporated to dryness at 60°C on a rotary evaporator and afterwards purged under the stream of nitrogen to remove any residual solvent. **Accelerated solvent extraction:** Accelerated solvent extraction (ASE) was applied for oil extraction from nutmeg and star anise seeds. The procedure was conducted on Dionex™ ASE™ 350 Accelerated Solvent Extractor (Thermo Fisher Scientific Inc., Sunnyvale, CA, USA) using *n*-hexane as the extraction solvent. A mixture of sample (8 g) and diatomaceous earth (0.5 g) was placed into 34 mL stainless steel cells fitted with 2 cellulose filters at the bottom of the cells. Extraction conditions were set according to the method described by (Lohani and Fallahi, 2015), slightly modified: temperature at 25 and 100°C, static extraction time 10 min and 6 extraction cycles, constant pressure of 10.34 MPa, 30 s of purge with nitrogen and 50% of flushing. Obtained extracts were collected in 250 mL glass vessel with Teflon septa, evaporated at 60°C under vacuum and afterwards purged under the stream of nitrogen to remove any residual solvent.

3.3. Fatty Acid and Sterol Composition

Fatty acid composition was determined by using gas chromatography. Fatty acid methyl esters (1 μL), prepared by ISO method 12966-2 (ISO, 2017) were injected into a GC equipped with an FID detector. Fatty acid methyl esters were separated on a TRACE TR-FAME capillary column (30m x 0.22mm × 0.25 μm) using a stationary phase of 70% cyanopropyl polysilphenylene-siloxane (Thermo Scientific, Waltham, MA, USA). Helium was used as the carrier gas at a 0.7 mL/min flow rate. The temperature of the injector was set at 250 °C and of the detector at 280 °C. The temperature of the oven was programmed to increase 4 °C/min from an initial value of 120 to 160 °C, and then at 10 °C/min to 190 °C, where it was held for 10 min. The split ratio was 75:1. Fatty acid methyl ester peaks were identified by comparing their retention times with those of FAME standards (C8–C22)**.** Sterol composition was determined by using standard ISO method (ISO, 2014). Nonpolar extracts were spiked with α-cholestanol as an internal standard and saponified by potassium hydroxide. Unsaponified fractions were eluted by diethyl ether on aluminum oxide filled glass column and then converted to trimethylsilyl derivates. Individual components were separated and determined on the ATI Unicam 610 (Boston, MA) gas chromatograph equipped with a flame ionization detector. Gas chromatography column Agilent DB-17 (30 m × 0.32 mm, film thickness 0.25 μm; Santa Clara, CA) was heated from of 180 to 270°C at the rate of 6°C/min and then kept at 270°C for 30 min. Helium flow rate was set to 1.5 mL/min, split ratio of 13.3:1 was used and 1 μL of sample was injected. Injector was kept at 290°C and detector at 250°C.

4. Results and Discussion

4.1. Oil Extraction Yield

Oils used in this study were extracted from seeds using *n*-hexane and three different methods. Every method has its advantages and disadvantages where SE is done in longer time period (8 h), ASE at significantly shorter time under elevated pressure and with much less solvent usage while AAE avoids any application of higher temperature during the extraction in time more similar to ASE. Average results for oil yield of nutmeg and star anise and average yield of oil depending on the extraction method are presented on Figure 1. Average yield of oil in nutmeg is 24.46% with highest value obtained by AAE at 26.90% and lowest with ASE25 at 22.10%. These values are lower or at the lower limits of previously published papers at 25–50 % (Abdurrasheed and Janardanan, 2009; Kuete, 2017; Niyas et al., 2003). Unfortunately all of the citied papers are review papers which refer to much older research from 1992 (Abdurrasheed and Janardanan, 2009; Kuete, 2017) and book published in 1981 (Niyas et al., 2003). Original research published in 1987 gives similar result of 30% of oil (Al-Khatib et al., 1987). There is a need for much more detailed and contemporary research in this

scientific field. Published research in English on oil quantity from star anise is even scarcer with most of the work published in Chinese with short abstracts in English through China National Knowledge Infrastructure (CNKI). Average oil yield for star anise was 10.53% with lowest values at 9.55% with AAE and highest at 11.95 with ASE100. In their research of CO_2 oil extraction optimisation from star anise Li at el. (Li et al., 2010) published a span between 6.80 and 23.72% of oil depending on extraction conditions.

The highest yield in oil extraction was obtained with ASE100 and lowest with ASE25 (Figure 1). It is evident that the influence of elevated pressure alone (ASE25) does not result in satisfactory utilization and that the combination of elevated temperatures and pressures is key to more efficient ASE. Following all the above, for future research on the extraction of oil from spice seeds with ASE, it is important to further optimize the extraction conditions using temperatures >100 °C, but also other solvents to increase efficiency or recovery.

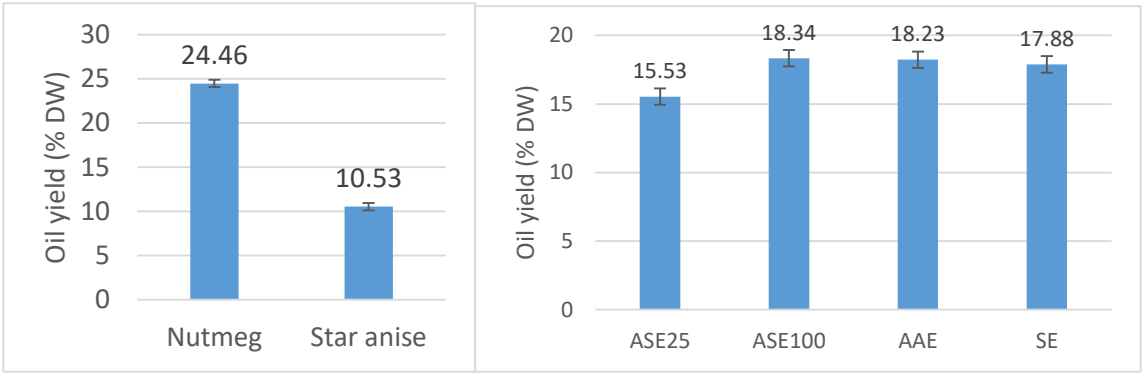

Figure 1. Oil yield in samples and average yield obtained by different extraction methods ($p < 0.05$). Results are shown as mean ± standard error.

4.2. Fatty Acid Composition

Fatty acid composition of analysed samples are presented in Table 1. Eight different fatty acids have been identified in nutmeg oil. Dominant fatty acid in all nutmeg oil samples was myristic C14:0 with average of 75.69% which is similar to other research with 79.20% (Al-Khatib et al., 1987). Together with oleic (C18:1n9 – 13.00%) and palmitic (C16:0 – 7.89%) it covers more than 96 % of total fatty acids. Kuete (Kuete, 2017) in its chapter on "Chemistry of *Myristica fragrans*" cites myristic, stearic, palmitic, oleic, linoleic, and lauric acids as the main components. Contrary to his findings presented results had significantly lower lauric acid quantities. Abdurasheed and Janardanan (Abdurrasheed and Janardanan, 2009) in their research also had higher values of lauric acid (8.00%) but much lower myristic fatty acid (55.10%). The fatty acid composition of seeds depends on its genetic characteristics, but the latitude and climatic conditions of cultivation also have a strong influence on the biosynthesis of fatty acids (Linder, 2000). Dominant fatty acid in star anise oil was lauric with average of 62.30%. Other significant components include linoleic (13.33%), oleic (12.57%) and palmitic (8.43%). Together they include more than 96% of all fatty acids. Contrary to results published by Li at el. (Li et al., 2010) of 71% of unsaturated fatty acids our results were average 72.57% saturated fatty acids which could be explained by Methods of extraction had significant effect only on lauric acid and few smaller components $p \leq 0.05$ (Table 2).

Table 1. Influence of seed type and extraction method on composition and content (%) of fatty acids in nutmeg and star anise seed oils.

	SFA	**MUFA**	**PUFA**	**C12:0**	**C14:0**	**C14:1**	**C16:0**	**C16:1**	**C17:0**	**C18:0**	**C18:1n9**	**C18:2n6**	**C18:3n6**	**C20:0**
Type of seed	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p < 0.01$ *	$p = 0.09$	$p < 0.01$ *	$p < 0.01$ *	$p = 0.13$	$p < 0.01$ *	$p < 0.01$ *	$p = 0.32$
Nutmeg	85.59 ± 0.42 [a]	13.12 ± 0.24 [a]	1.29 ± 0.19 [a]	0.00 ± 0.41 [a]	75.69 ± 0.19 [a]	0.00 ± 0.04[a]	7.89 ± 0.12 [a]	0.12 ± 0.11 [a]	0.84 ± 0.02 [a]	1.00 ± 0.04 [a]	13.00 ± 0.12 [a]	1.15 ± 0.19 [a]	0.15 ± 0.00 [a]	0.17 ± 0.02 [a]
Star anise	72.57 ± 0.42 [b]	14.05 ± 0.24 [b]	13.38 ± 0.19 [b]	62.30 ± 0.41 [b]	0.06 ± 0.19 [b]	1.06 ± 0.04 [b]	8.43 ± 0.12 [b]	0.42 ± 0.11 [a]	0.02 ± 0.02 [b]	1.62 ± 0.04 [b]	12.57 ± 0.12 [a]	13.33 ± 0.19 [b]	0.04 ± 0.00 [b]	0.14 ± 0.02 [a]
Extraction method	$p = 0.28$	$p = 0.18$	$p = 0.08$	$p < 0.01$ *	$p = 0.05$	$p < 0.01$ *	p = 0.31	$p < 0.01$ *	$p = 0.27$	$p = 0.25$	$p = 0.51$	$p = 0.08$	$p < 0.01$ *	$p = 0.08$
ASE25	79.27 ± 0.59 [a]	13.61 ± 0.34 [a]	7.12 ± 0.26 [a]	31.95 ± 0.58 [a]	37.47 ± 0.27 [a]	0.43 ± 0.54 [a]	8.02 ± 0.17 [a]	0.10 ± 0.16 [a]	0.39 ± 0.02 [a]	1.28 ± 0.05 [a]	13.08 ± 0.28 [a]	6.97 ± 0.27 [a]	0.15 ± 0.01 [a]	0.18 ± 0.03 [a]
ASE100	79.81 ± 0.59 [a]	13.37 ± 0.34 [a]	6.82 ± 0.26 [a]	32.24 ± 0.58 [b]	37.71 ± 0.27 [a]	0.63 ± 0.54 [b]	8.01 ± 0.17 [a]	0.14 ± 0.16 [b]	0.45 ± 0.02 [a]	1.32 ± 0.05 [a]	12.60 ± 0.28 [a]	6.72 ± 0.27 [a]	0.10 ± 0.01 [b]	0.09 ± 0.03 [a]
AAE	79.12 ± 0.59 [a]	13.13 ± 0.34 [a]	7.75 ± 0.26 [a]	30.43 ± 0.58 [c]	38.63 ± 0.27 [a]	0.46 ± 0.54 [c]	8.19 ± 0.17 [a]	0.10 ± 0.16 [c]	0.44 ± 0.02 [a]	1.25 ± 0.05 [a]	12.57 ± 0.28 [a]	7.68 ± 0.27 [a]	0.07 ± 0.01 [c]	0.17 ± 0.03 [a]
SE	78.12 ± 0.59 [a]	14.23 ± 0.34 [a]	7.66 ± 0.26 [a]	29.99 ± 0.58 [d]	37.69 ± 0.27 [a]	0.62 ± 0.54 [d]	8.43 ± 0.17 [a]	0.73 ± 0.16 [d]	0.43 ± 0.02 [a]	1.39 ± 0.05 [a]	12.88 ± 0.28 [a]	7.59 ± 0.27 [a]	0.07 ± 0.01 [d]	0.19 ± 0.03 [a]

ASE = Accelerated Solvent Extraction (at 25 and 100 °C), AAE = Agitation Assisted Extraction, SE = Soxhlet Extraction; Results are shown as mean ± standard error.

* Statistically significant variation at $p \leq 0.05$. The values within the column marked with different letters differ statistically at $p \leq 0.05$.

4.3 Terol Composition

Results for sterol composition are presented in Table 2. Dominant sterol in all samples was β-sitosterol (average nutmeg 214.8 and star anise 256.9 mg/100 g). Other significant sterols in nutmeg oil were campesterol and Δ5-avenasterol while in star anise oil were campesterol and stigmasterol. Higher values for total sterols were in nutmeg oil and oils extracted with ASE25 and ASE100. Moreau et al. (Moreau et al., 2003) used ASE at 40 and 100 ° C to extract the sterol fraction from maize and oats and found that applying a higher temperature increased its concentration in the extract.

Table 2. Influence of seed type and extraction method on composition and content (mg/100g) of sterols in nutmeg and star anise seed oils.

	Campe-Sterol	Campe-Stanol	stigma-Sterol	β-Sitosterol	Sito-Stanol	Δ5-Avena-Sterol	Δ5.24-Stigmasta-Dienol	Δ7-Stigmasterol	Citro-Stadienol	Δ7-Avena-Sterol	Total Sterols
Type of seed	$p <$ 0.01*	$p <$ 0.01*	$p =$ 0.85	$p < 0.01$ *	$p =$ 0.34	$p <$ 0.01 *	$p < 0.01$ *	$p = 0.05$ *	$p < 0.01$ *	$p =$ 0.03 *	$p <$ 0.01 *
Nutmeg	131.5 ^{a} ± 12.8	0.0 ^{b} ± 0.6	15.3 ^{a} ± 0.8	214.8 ^{b} ± 8.2	0.1 ^{a} ± 0.0	32.9 ^{a} ± 1.1	0.0 ^{b} ± 0.2	0.0 ^{b} ± 0.8	4.7 ^{a} ± 1.0	0.0 ^{b} ± 0.3	399.2 ^{a} ± 14.6
Star anise	47.1 ^{b} ± 12.8	4.1 ^{a} ± 0.6	15.5 ^{a} ± 0.8	256.9 ^{a} ± 8.2	0.0 ^{a} ± 0.0	0.8 ^{b} ± 1.1	5.5 ^{a} ± 0.2	2.6 ^{a} ± 0.8	0.0 ^{b} ± 1.0	1.1 ^{a} ± 0.3	333.6 ^{b} ± 14.6
Extraction method	$p =$ 0.02 *	$p = 0.03$ *	$p =$ 0.05	$p = 0.14$	$p =$ 0.02 *	$p =$ 0.43	$p = 0.22$	$p = 0.08$	$p = 0.05$	$p =$ 0.05	$p =$ 0.05 *
ASE25	135.8 ^{a} ± 18.1	2.1 ^{ab} ± 0.9	16.1 ^{a} ± 1.1	250.5 ^{ab} ± 11.6	0.1 ^{a} ± 0.1	18.1 ^{a} ± 1.5	2.7 ^{a} ± 0.2	0.0 ^{a} ± 1.2	5.0 ^{a} ± 1.4	0.0 ^{a} ± 0.4	430.4 ^{a} ± 20.6
ASE100	102.0 ^{ab} ± 18.1	0.0 ^{b} ± 0.9	17.2 ^{a} ± 1.1	259.5 ^{a} ± 11.6	0.0 ^{b} ± 0.1	16.7 ^{a} ± 1.5	3.0 ^{a} ± 0.2	0.6 ^{a} ± 1.2	4.3 ^{a} ± 1.4	0.4 ^{a} ± 0.4	403.6 ^{ab} ± 20.6
AAE	63.7 ^{ab} ± 18.1	2.0 ^{ab} ± 0.9	15.0 ^{a} ± 1.1	231.0 ^{ab} ± 11.6	0.0 ^{b} ± 0.1	14.2 ^{a} ± 1.5	3.1 ^{a} ± 0.2	4.7 ^{a} ± 1.2	0.0 ^{a} ± 1.4	1.8 ^{a} ± 0.4	335.3 ^{bc} ± 20.6
SE	55.8 ^{b} ± 18.1	4.0 ^{a} ± 0.9	13.3 ^{a} ± 1.1	202.4 ^{b} ± 11.6	0.0 ^{b} ± 0.1	18.5 ^{a} ± 1.5	2.2 ^{a} ± 0.2	0.0 ^{a} ± 1.2	0.0 ^{a} ± 1.4	0.0 ^{a} ± 0.4	296.3 ^{c} ± 20.6

* Statistically significant variable at $p \leq 0.05$. Results are expressed as mean ± SE. Values in the same column with different letters are statistically different at $p \leq 0.05$.

5. Conclusions

Nutmeg and star anise seed oils present interesting source of myristic and lauric fatty acids while the application of ASE in its extraction is far more suitable with using at higher temperatures. Apart from higher oil yield it enhances sterol extraction of around 50%.

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