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IDENTIFICATION OF PHYTOSTEROLS AND TOCOPHEROLS IN TRAU AND DAU DAU SEED KERNELS AS THEIR TRIMETHYLSILYL DERIVATIVES USING GAS CHROMATOGRAPHY/MASS SPECTROMETRY

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ABSTRACT

Trau and Dau dau are two of input raw materials for biodiesel production. The quality of biodiesel could be reduced with presence of phytosterols in the raw materials. Most plants contain tocopherols which are helpful in medicine. However, the information of the phytosterol and tocopherol compositions of Trau and Dau dau seeds is limited. This study presented the identification of the main phytosterols and tocopherols in Trau and Dau dau seed kernels as their trimethylsilyl derivatives using gas chromatography/mass spectrometry. As the results, γ-tocopherol was the principal tocopherol in these seed kernels, and β-tocopherol was practically undetected in Trau seeds. This showed both kinds of seeds in this study are valuable materials for γ-tocopherol production. The results also indicated that β-sitosterol was the most abundant phytosterol in Trau seeds, whereas, stigmasterol and β-sitosterol were equivalent in the seeds of Dau dau.

1. INTRODUCTION

Many researchers have seriously concerned biodiesel production because biodiesel, one kind of biofuels, could replace fossil fuels that has been rapidly exhausted. According to (Thanh et al., 2012), many methods have been studied to produce biodiesel, e.g., mechanical stirring, transesterification using various catalysts, ultrasonic irradiation, supercritical alcohol and co-solvent. The oilseeds/oils that support biodiesel production could be divided into two principal groups: non-edible oils and edible oils (Kumar et al., 2013; Silitonga et al., 2013). As the results of (Bondioli et al., 2008; Moreau et al., 2008; Songtawee et al., 2014), that derivatives of phytosterols form the precipitate in biodiesel leads to the negative effects on engine. In other words, the presence of phytosterols in the raw materials and also in the final biodiesel could reduce the quality of biodiesel. Hence, identification and quantification of phytosterols are necessary in biodiesel production.

In recent years, there are a large amount of plant materials that could provide for biodiesel production. Among them, Trau, as known as *Vernicia montana* Lour. and Dau dau, as known as *Pongamia pinnata* seeds are the non-edible

materials used for biodiesel production due to their high oil content. Trau belongs to the family Euphorbiaceae with 40-60% oil in seed kernels, whereas Dau dau belongs to the family Fabaceae with 18-27% oil in seeds/kernels (Axtell and Fairman, 1992; Bala et al., 2011). There are a lot of scientists studied the utilization of these raw materials for biodiesel production (Chen et al., 2010; Mukta et al., 2009; Naik et al., 2008; Sharma et al., 2010). However, to our knowledge, the information of phytosterols and tocopherols in both seeds is limited.

For medical purposes, phytosterols have positive effects, e.g., anti-cholesterol, anti-cancer as well as additives in cosmetics due to their similarity with cholesterol (Fernandes and Cabral, 2007; Kritchevsky and Chen, 2005). In addition, a lot of plants contain tocopherols and tocotrienols, known as two major components of vitamin E. It plays an important role in the human body as an antioxidant, anti-inflammatory, anticancer and antiplatelet. Tocopherols and tocotrienols are classified into four kinds of isomers, namely α-, β-, γ-, δ-tocopherol and α-, β-, γ-, δ-tocotrienol (Guralp, 2014; Thomas, 2006).

In this study, phytosterols and tocopherols in Trau and Dau dau seed kernels cultivated in Vietnam and India respectively, were identified using GC/MS technique and their components were evaluated. The results were compared with sunflower oil (Schwartz et al., 2008) in order to estimate phytosterols and tocopherols in these materials and demonstrate their ability for medicinal purposes and enhancement of the quality of biodiesel.

2. MATERIALS AND METHODS

2.1 Seed materials

Trau seeds used for experiments were collected in Vietnam and Dau dau seeds were taken from India. The seed kernels were powdered and homogenized before extracted by methanol.

2.2 Reagents and standards

Organic solvents, methanol, ethyl acetate were GC or HPLC analytical grade and pyridine was a reagent grade, and they were purchased from Wako Chemical Industry (Tokyo, Japan). The purified water was produced in Osaka Prefecture University (Sakai, Japan). Standards of stigmasterol, campesterol and β-sitosterol standards were supplied by Tama Biochemical Company (Japan). A reagent for silylation , *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA)) and the mixtures of n-alkane C_{16}-C_{44} , and the standards of (±) α-tocopherol, *rac*-β-tocopherol, (+) γ-tocopherol, (+) δ-tocopherol were purchased from Sigma-Aldrich/Supelco (Japan). Standard solutions were prepared using methanol individually and kept at 4 ˚C for experiments.

2.3 Extraction of samples and trimethylsilyl (TMS) derivatization

Each sample (5.00 g) was extracted with methanol (50 mL) three times in ultrasonic bath for 30 minutes/time. The combined methanol extracts were filtered and evaporated by the vacuum rotary evaporator. The crude methanol extract was continuously extracted with ethyl acetate (25 mL) and water (10 mL) three times in the separation funnel.

A 100μL of ethyl acetate and water extracts of each sample were dried under the gentle pure nitrogen gas stream. Afterward, pyridine (200 μL) and BSTFA (200 μL) were added to form their TMS derivatives at 60˚C for 1 hour. The mixtures were diluted with 2 mL of ethyl acetate before injected into GC/MS system. The standards were performed under the above conditions in order to produce TMS derivatives.

2.4 GC/MS analysis

Standards and samples after the silylation procedure were analyzed on GC/MS apparatus,

consisting of Agilent 6890 gas chromatograph with mass selective detector 5973 and autosampler HP 7683. The DB-5ms capillary column (30 m×0.25 mm I.D., film thickness of 0.25μm, Agilent, Palo Alto, CA, USA) was used to separate analytes. The temperatures of injector, mass source and mass quadrupoles were held at 270, 230 and 150˚C, respectively. The electron impact ionization was at 70eV with mass scan range was 50-700 Da. The oven temperature program begun at 65˚C for 2 minutes, afterward the temperature was increased to 310˚C with the rate of 5˚C min^{-1} and maintained isothermally at 310˚C for 10 minutes. The splitless mode (1μL of injection volume) with splitless time 2 minutes and helium gas as the carrier gas at a constant flow rate of 1.3 mL min^{-1} were applied. The data were acquired and processed by HP-Chemstation software.

The mixtures of n-alkane C_{16}-C_{44} were separated in the same GC/MS conditions with TMS derivatives after dissolved in n-hexane. Based on the retention time of n-alkane and analytes, the retention indexes, known as Kovats indexes, were determined. Data were processed using Microsoft Excel 2013.

3. RESULTS AND DISCUSSION

3.1 Standard analysis

The information of TMS derivatives of phytosterols and tocopherols analyzed by GC/MS are in Table 1. Identification was based on the mass spectra of analytes and authentic compounds. However, in some cases, it is difficult to distinguish the components whose mass spectra are similar, e.g., β-tocopherol and γ-tocopherol (Table 1). Moreover, the peaks of β-tocopherol and γ-tocopherol partly overlapped during eluting on the column. One of the solutions for this problem is retention indexes. The comparison of the retention indexes between our results with the previous studies about phytosterols and tocopherols was performed and given Table 1. The retention indexes of some phytosterols and tocopherols in this study were higher than the data provided by (Isidorov and Szczepaniak, 2009). This difference could be due to the replacement of phenyl groups on the stationary phase of DB-5ms capillary column by methyl groups present in TMS derivative samples during a long time. This problem was also confirmed by (Isidorov and Szczepaniak, 2009).

Table 1. The information of phytosterols and tocopherols in standards and samples

Compounds	Molecular fomula	Molecular mass	Molecular mass-TMS	Kovats index			Major fragment ions (m/z (%))
				Sample	Standard	Ref.*	
(+) δ-Tocopherol	$C_{27}H_{46}O_2$	402	474	2915[a] 2916[b]	2916	2916	474(100), 475(37), 209(30), 208(26)
rac-β-Tocopherol	$C_{28}H_{48}O_2$	416	488	3005[a,b]	3006	3010	488(100), 223(41), 489(37), 222(36), 73(29)
(+) γ-Tocopherol	$C_{28}H_{48}O_2$	416	488	3014 [a,b]	3014	3000	488(100), 223(70), 489(40), 222(29), 73(28)
(±) α-Tocopherol	$C_{29}H_{50}O_2$	430	502	3157[a] 3158[b]	3158	3147	502(100), 237(53), 503(45), 236(25), 73(19)
Campesterol	$C_{28}H_{48}O$	400	472	-	3272	3251	129(100), 343(89), 382(75), 73(35), 472(35), 367(35), 75(30), 95(30), 145(28), 107(27), 121(26), 119(24), 81(24), 344(24), 383(23), 105(22), 342(22), 159(20)

Compounds	Molecular fomula	Molecular mass	Molecular mass-TMS	Kovats index			Major fragment ions (m/z (%))
				Sample	Standard	Ref.*	
Stigmasterol	$C_{29}H_{48}O$	412	484	3303[a] 3304[b]	3303	3286	83(100), 129(88), 255(72), 394(66), 484(66), 69(42), 73(40), 55(39), 81(39), 159(35), 75(34), 133(33), 355(31), 145(31), 119(28), 351(28), 486(27), 105(27), 95(25), 147(24), 379(24), 93(23), 107(23), 97(22), 213(21), 395(21), 91(21), 131(20), 139(19), 131(20)
β-Sitosterol	$C_{29}H_{50}O$	414	486	3361[a] 3362[b]	3361	3342	129(100), 357(94), 396(83), 486(41), 381(36), 73(34), 145(28), 75(28), 95(28), 121(26), 358(26), 397(26), 107(26), 119(26), 356(23), 81(23), 159(22), 105(21)

*[a] Trau , [b] Dau dau , * referenced from (Isidorov and Szczepaniak, 2009)*

3.2 Phytosterols in samples

The components of phytosterols in Trau and Dau dau seed kernels are displayed in Table 2. There are few researchers who have reported the phytosterol and tocopherol contents in these samples. According to (Shameel et al., 1996), two sterols and three sterol derivatives from Dau dau seeds were isolated, yet their amounts were not determined. Similarly, in the information of Trau, the fruits contain phytosterols, but identification and quantification were also not reported (Oyen, 2007). In our study, stigmasterol and β-sitosterol were identified as two major phytosterols in Trau and Dau dau seeds. Most phytosterol presented in Trau seeds was β-sitosterol, whereas, β-sitosterol and stigmasterol occupied the similar amount in Dau dau seeds (Table 2). Moreover, comparison with the content of phytosterols in sunflower oil (Schwartz et al., 2008), β-sitosterol and stigmasterol in both seed kernels are significant and should be quantified exactly in order to estimate their potential in medicinal purposes.

Table 2. Percentage of peak area of the main phytosterols in Trau and Dau dau seed kernels

No.	Compound	Trau	Dau dau	Sunflower oil* (% in total phytosterols)
1	Stigmasterol	5.3 ± 0.1	50.1 ± 1.1	6.2
2	β-Sitosterol	94.7 ± 0.1	49.9 ± 1.1	45.7

** referenced from (Schwartz et al., 2008)*

3.3 Tocopherols in samples

Identification and estimation of tocopherols in Trau and Dau dau seed kernels is given in Fig.1 and Table 3. To our knowledge, no information of tocopherols in Trau and Dau dau seed had been reported. Hence, this study revealed valuable results for future researchers. Four kinds of tocopherols, consisting of α-, β-, γ-, δ-tocopherol, were identified and estimated in Dau dau seed kernels (Table 3).

Table 3. Percentage of peak area of the main tocopherols in Trau and Dau dau seed kernels

No.	Compound	Trau	Dau dau	Sunflower oil* (% in total tocopherols)
1	δ-tocopherol	0.3 ± 0.0	8.9 ± 0.2	0.4
2	β-tocopherol	ND	6.1 ± 0.3	3.8
3	γ-tocopherol	96.8 ± 0.1	70.3 ± 0.6	2.2
4	α-tocopherol	2.8 ± 0.1	14.6 ± 1.0	93.6

ND means that not dectected, * referenced from (Schwartz et al., 2008)

Similarly, the presence of α-, γ-, δ-tocopherol was confirmed in Trau. seeds, yet β-tocopherol was practically undetected in our experiments (Fig.1). Most tocopherol present in the seeds of Trau and Dau dau was γ-tocopherol. Although δ-tocopherol was also detected in Trau seeds, its content was trace level (Fig 1 and Table 3). Tocotrienols, another group of vitamin E, were found in Dau dau, but their ratio of contents was insignificant compared with tocopherols. Otherwise, in Trau seeds, the presence of tocotrienols are not determined. In the review article of (Jiang et al., 2001), they showed that γ-tocopherol should be evaluated its important play in medicine similar to α-tocopherol because in the recent years, there are many studies indicate its significant distribution for human health. If α-tocopherol is inhibitor of new free radicals, γ-tocopherol is known as trap and/or neutral compound for existent free radicals. And in the US diet, γ-tocopherol presents as the main form of vitamin E beside α-tocopherol. The content of γ-tocopherol in Trau and Dau dau seeds is higher than that in sunflower oil (Table 3). This comparison demonstrates both samples have great potential for γ-tocopherol production and the precise quantification of their content in these seeds is necessary to assess their roles in medicinal purposes.

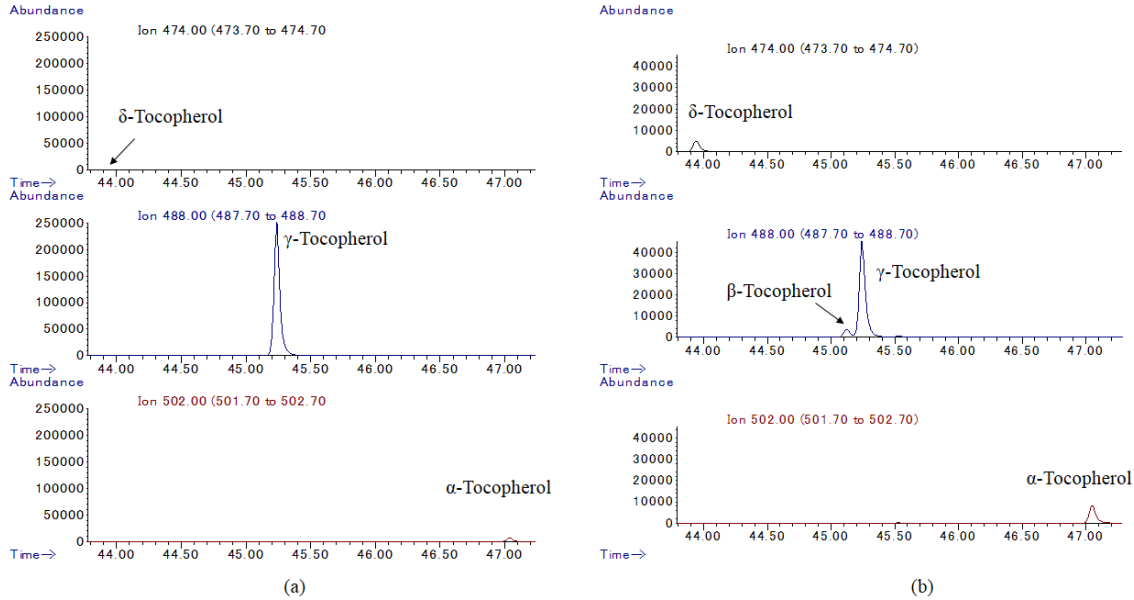

Fig. 1. Chromatogram of tocopherols in Trau (a) and Dau dau (b) seed kernels

4. CONCLUSIONS

The main phytosterols and tocopherols were identified and their ratio of content was estimated in the seeds of Trau and Dau dau. β-sitosterol and stigmasterol were the major phytosterols and γ-tocopherol was the principal tocopherol in both materials. Quantification of these components is necessary for assessment of their exact potential in medical purposes as well as enhancement of biodiesel.

REFERENCES

Axtell, B.L., & Fairman, R. (1992). Minor oil crops. Part I-Edible oils. Part II-Non-edible oils. Part III-Essential oils. Switzerland: Springer Nature Switzerland AG.

Bala, M., Nag, T.N., Kumar, S., Vyas, M., Kumar, A., & Bhogal, N.S.(2011). Proximate Composition and Fatty Acid Profile of Pongamia pinnata, a Potential Biodiesel Crop. *J Am Oil Chem Soc, 88, 559-562*.

Bondioli, P., Cortesi, N., & Mariani, C. (2008). Identification and quantification of steryl glucosides in biodiesel. European Journal of Lipid Science and Technology 110, 120-126.

Chen, Y.-H., Chen, J.-H., Chang, C.-Y., & Chang, C.-C. (2010). Biodiesel production from tung (Vernicia montana) oil and its blending properties in different fatty acid compositions. *Bioresource Technology, 101*, 9521-9526.

Fernandes, P., & Cabral, J.M.S. (2007). Phytosterols: Applications and recovery methods. *Bioresource Technology, 98*, 2335-2350.

Guralp, O. (2014). Effects of vitamin E on bone remodeling in perimenopausal women: Mini review. *Maturitas 79*, 476-480.

Isidorov, V.A., & Szczepaniak, L. (2009). Gas chromatographic retention indices of biologically and environmentally important organic compounds on capillary columns with low-polar stationary phases. *Journal of Chromatography A, 1216*, 8998-9007.

Jiang, Q., Christen, S., Shigenaga, M.K., & Ames, B.N. (2001). γ-Tocopherol, the major form of vitamin E in the US diet, deserves more attention. *The American Journal of Clinical Nutrition, 74*, 714-722.

Kritchevsky, D., & Chen, S.C. (2005). Phytosterols—health benefits and potential concerns: a review. *Nutrition Research, 25*, 413-428.

Kumar, N., Varun. & Chauhan, S.R. (2013). Performance and emission characteristics of biodiesel from different origins: A review. *Renewable and Sustainable Energy Reviews 21*, 633-658.

Moreau, R., Scott, K., & Haas, M. (2008). The Identification and Quantification of Steryl Glucosides in Precipitates from Commercial Biodiesel. *J Am Oil Chem Soc 85*, 761-770.

Mukta, N., Murthy, I.Y.L.N., & Sripal, P. (2009). Variability assessment in Pongamia pinnata (L.) Pierre germplasm for biodiesel traits. *Industrial Crops and Products 29*, 536-540.

Naik, M., Meher, L.C., Naik, S.N., & Das, L.M. (2008). Production of biodiesel from high free fatty acid Karanja (Pongamia pinnata) oil. *Biomass and Bioenergy 32*, 354-357.

Oyen, L.P.A. (2007). Vernicia montana Lour, in: van der Vossen, H.A.M., Mkamilo, G.S. (Eds.), Record from PROTA4U. PROTA (Plant Resources of Tropical Africa / Ressources végétales de l'Afrique tropicale), Wageningen, Netherlands.

Schwartz, H., Ollilainen, V., Piironen, V., & Lampi, A.-M. (2008). Tocopherol, tocotrienol and plant sterol contents of vegetable oils and industrial fats. *Journal of*

Food Composition and Analysis, 21, 152-161.

Shameel, S., Usmanghani, K., Ali, M.S., & Ahmad, V.U. (1996). Chemical constituents from the seeds of Pongamia pinnata (L.) Pierre. *Pakistan journal of pharmaceutical sciences 9, 11-20.*

Sharma, Y.C., Singh, B., & Korstad, J. (2010). High Yield and Conversion of Biodiesel from a Nonedible Feedstock (Pongamia pinnata). *Journal of Agricultural and Food Chemistry 58, 242-247.*

Silitonga, A.S., Masjuki, H.H., Mahlia, T.M.I., Ong, H.C., Chong, W.T., & Boosroh, M.H.(2013). Overview properties of biodiesel diesel blends from edible and non-edible feedstock. *Renewable and Sustainable Energy Reviews, 22*, 346-360.

Songtawee, S., Ratanawilai, S., & Tongurai, C. (2014). Effect of Sterol Glucosides in Biodiesel Production.

Thanh, L.T., Okitsu, K., Boi, L.V., & Maeda, Y. (2012). Catalytic Technologies for Biodiesel Fuel Production and Utilization of Glycerol: A Review. Catalysts.

Thomas, D.R. (2006). Vitamins in aging, health, and longevity. Clinical interventions in aging 1, 81.