Effect of Mobile Phase Composition on the Separation of Neutral Lipids, γ-Oryzanol and its Saponified Compounds on a 100-Å Phenogel Column

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Abstract.

Monitoring of bioactive compounds in rice bran oil is generally required more than one approach. This study attempted to simultaneously analyze the bioactive compounds (γ-oryzanol (OZ), free sterols (both phytosterols and triterpene alcohols, FS), ferulic acid (FA), free fatty acid (FFA) and acylglycerols (triglycerides (TG), diglycerides (DG), monoglycerides (MG)) in RBO-based mixture using a size-exclusion HPLC column (100-Å Phenogel) with a modified mobile phase. These compounds cannot be separated on a 100-Å Phenogel column using 0.1% acetic acid in ethyl acetate as a mobile phase. To improve their separation, isooctane was added to the mobile phase to decrease the degree of swelling of the column gel matrix and reduced the elution strength of the mobile phase. The retentions of TG, FS and DG were less affected by the isooctane content. Whereas, it had a moderate effect on the elution of FFA and MG and strong effect on OZ and FA. The separation of these compounds was obviously improved with increasing the isooctane content up to 70%. However, peaks of the polar compounds were broadened, especially for the most polar, FA. The separation on the size-exclusion column did not follow the expected elution order (molecular sieving) and showed interactions between the polar groups of the compounds to the column gel matrix. Ethyl acetate/isooctane (50:50, v/v) with 0.1% acetic acid as a mobile phase shows a promising condition for simultaneous separation of neutral lipids, OZ and its saponified compounds with a total analysis time of 15 min.

Keywords: Elution Order, Ferulic Acid, Interaction, Phytosterols, Size Exclusion HPLC

1. Introduction

Preparation of rice bioactive compounds such as γ-oryzanol (OZ), ferulic acids (FA), free phytosterols and triterpene alcohols (FS) received much attention due to their various applications in foods, medical and cosmetics (Verma & Srivastav, 2020). Over the last few years, many studies have focused on methods for preparation of these compounds from low-cost by-products of rice bran oil (RBO) refining (Truong et al., 2017, Sombutsuwan et al., 2018). Generally, different of these compounds required different approaches for determination. For example, a reversed phase C18 column with a diode array detector (PDA) was used for analysis of FA (Bento-Silva et al., 2018) and FS (Pokkanta et al., 2019). Thin layer chromatography equipped with flame ionization detector (TLC-FID) and reverse-phase high performance chromatography (RP-HPLC) were used for analysis of lipid composition and OZ, respectively (Yoshie et al., 2009). TLC and high temperature gas chromatography (GC) were adopted in phytosterols, free fatty acids (FFA) and acylglycerols analysis (Ju & Zullaikah, 2013).

High-performance size-exclusion chromatography (HPSEC) with a modified mobile phase has been reported as alternate method for analysis of lipid classes in samples using just only a single 100-Å Phenogel column. It was successfully applied for monitoring the reaction components in biodiesel (Kittirattanapiboon & Krisnangkura, 2008) and refined vegetable oils (Kittiratanapiboon et al., 2009). In addition, resolutions of solute compounds and the separation power of a single 100-Å Phenogel column were substantially improved when non-polar solvent, isooctane were incorporated into the common mobile phase, toluene, and/ or tetrahydrofuran with additional a small amount of acetic acid. These were demonstrated for the separations of TG, DG, MG, FFA and wax ester in rice bran wax (Aryusuk et al., 2011), acyglycerols, FFA and OZ in rice bran acid oil (Sombutsuwan et al., 2018) and fatty acid methyl ester and steryl glucoside in biodiesel (Cheewaphan et al., 2019).

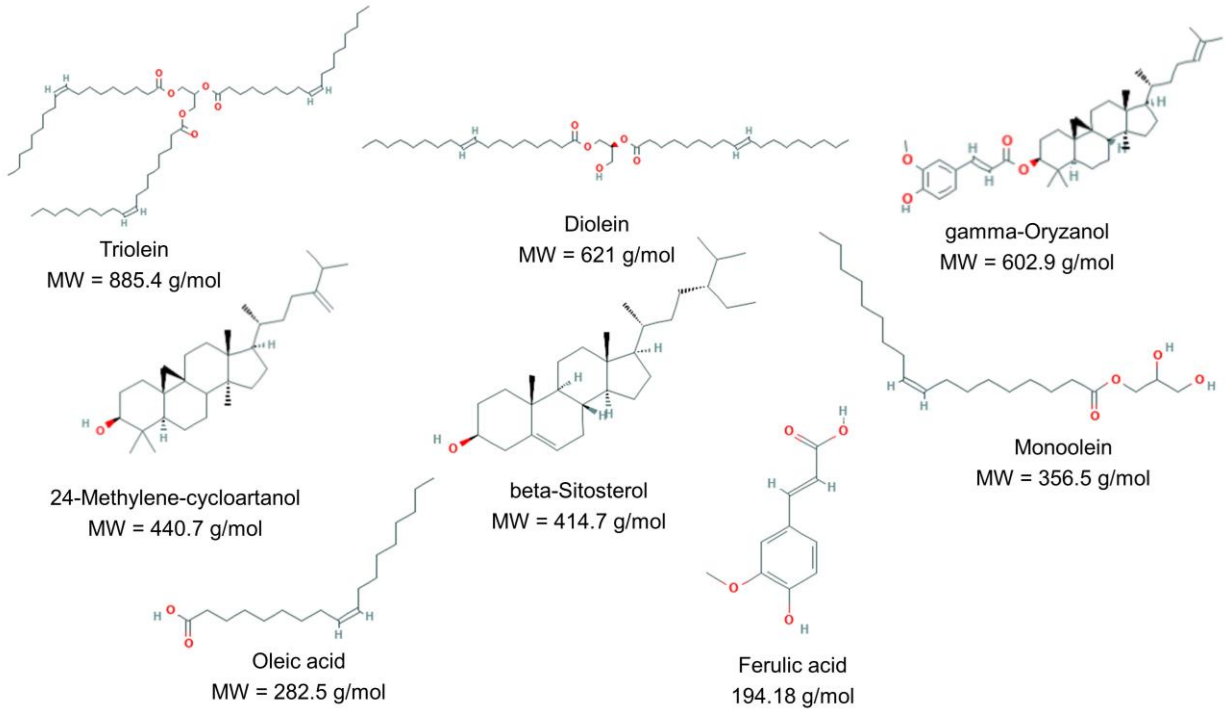
It was discovered that the separation of solutes on a 100-Å Phenogel column with a modified mobile phase was not only based on the molecular sieving, but also with their interactions between the polar groups of solutes and the gel matrix of the stationary phase (Kittirattanapiboon & Krisnangkura, 2008, Chumsantea et al., 2017). In addition, interaction of the polar groups was increased, and better separation was obtained when a silica guard column was connected to the separation column (Krisnangkura & Simamaharnnop, 1992, Chumsantea et al., 2014). Although, several mobile phase mixtures have been developed for the Phenogel column, separation of a mixture of compounds with a wide range in polarity such as acylglycerols, OZ, FA, FS has not been observed.

Thus, the objective of this study was to develop mobile phase composition for simultaneous determination of rice bioactive compounds (OZ, FA, FS) and neutral lipids (acylglycerols and FFA) on a 100-Å Phenogel column. We report their separations behavior caused by mobile phase composition composed of different ratios of ethyl acetate/isooctane/acetic acid. In addition, effect of silica guard column on the separation was also investigated.

2. Methods

The lipid standards glyceryl trioleate (triolein, TG), glycerol-1,3-dioleate (diolein, DG), glyceryl monooleate (monoolein, MG), oleic acid (FFA), ferulic acid (FA), β-sitosterol and 24-methylene cycloartenol were purchased from Sigma-Aldrich (St. Louis, MO, USA). γ-Oryzanol (OZ), 98.1% purity was obtained from Oryza Oil & Fat Chemical Co., Ltd. (Ichinomiya, Japan). β-sitosterol and 24-methylene cycloartenol, two mains components found in γ-oryzanol are representative of free sterol and triterpenoid (FS). Ethyl acetate, 2,2,4-trimethylpentane (isooctane) and acetic acid were of HPLC grade from RCI Lab Scan Co. Ltd. (Bangkok, Thailand).

Figure 1: Structures and molecular weight (MW) of compounds used in this study.



Source: (PubChem, 2021)

HPLC separations were performed on a 100-Å Phenogel column (300 mm × 7.8 mm ID, 5 µm, Phenomenex, Torrance, CA) equipped with a Phenogel guard column (50 mm × 7.8 mm ID, 5 µm) or a silica guard cartridge (20 mm × 3.9 mm ID, 4 µm, Water, USA). The HPLC system consisted of a pump model 515 (Waters Associates, Milford, MA, USA), a Rheodyne 7125 valve injector, a 20-µl loop and a Sedex 80 evaporative light scattering detector (ELSD; Sedere, Alfortville, France). Injector and column oven was set at 30 °C. The detector temperature and pressure of nitrogen gas were set at 30°C and 2 bars, respectively. Mobile phases were mixtures of ethyl acetate: isooctane: acetic acid and their ratios are reported in the text. To switch the swelling of gel matrix by changing the mobile phases, it is important to equilibrate column at least 5 days (store in the selected mobile phase). Flow rate of the mobile

phase was set at 1.0 ml/min. CSW32 HPLC software (DataApex, Prague, Czech Republic) was used for data analysis.

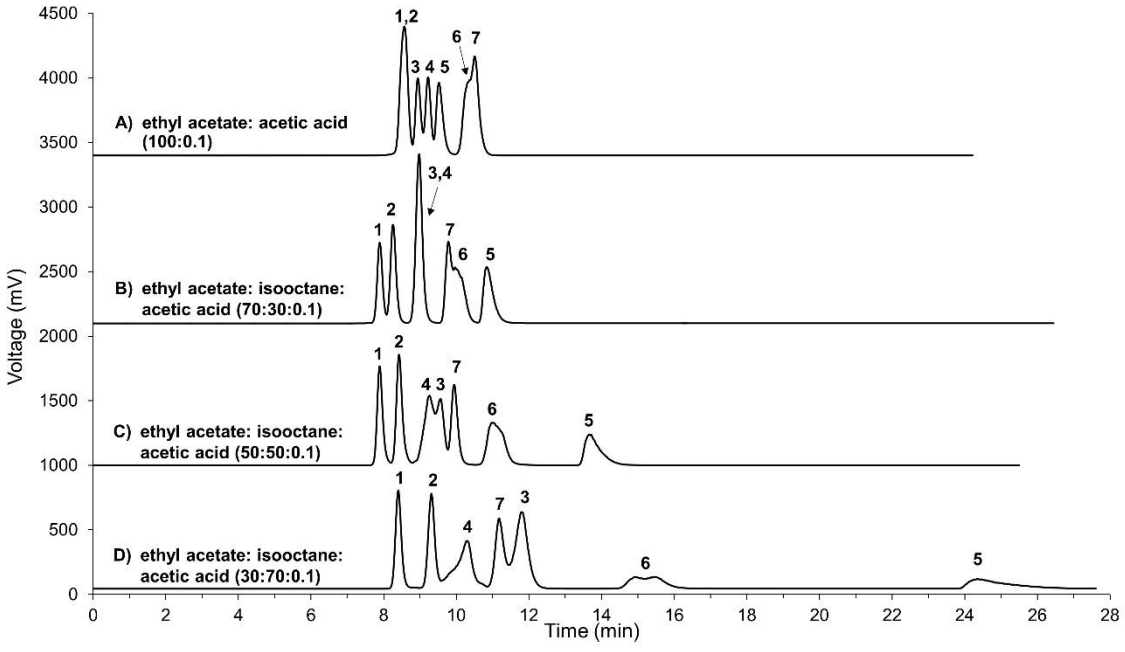
3. Results and Discussion

3.1 Effect of mobile phase composition on the elution of lipids

Ethyl acetate is one of a good swelling solvent for the Phenogel column (gel matrix is made from polystyrene divinylbenzene copolymer) as recommended by manufacturing catalog and it able to dissolve standard mixture containing compounds with a wide range in polarity as shown in Fig. 1. Acetic acid was suggested to be used as a solvent modifier to decrease retention, provide better separation, and peak shape of compounds containing hydroxyl or carboxylic groups (Kittirattanapiboon & Krisnangkura, 2008). Therefore, ethyl acetate and acetic acid were selected as a mobile phase to separate lipid compounds in this study.

By using ethyl acetate/ acetic acid (100:0.1) as a mobile phase (Fig. 2A), the analytes were mostly separated according to their MW on the 100-Å Phenogel column except OZ and FS.

Figure 2: HPSEC chromatograms of standard mixtures with ELSD detection. Column, 100-Å Phenogel column with a Phenogel guard; mobile phase, mixtures of ethyl acetate: isooctane: acetic acid with different volume ratios (A-D). Peak number, 1 triolein, 2 diolein, 3 monoolein, 4 oleic acid, 5 ferulic acid, 6 γ-oryzanol, 7 β-sitosterol and 24-methylene cycloartenol.



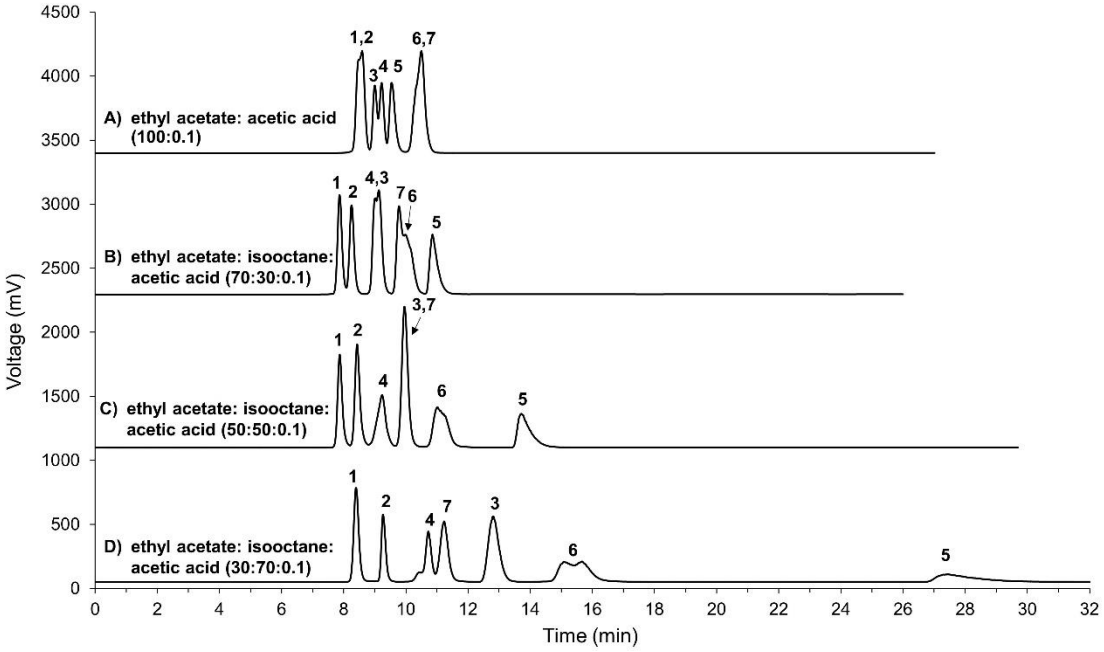
The MW and structure of FS, β-sitosterol and 24-methylene cycloartenol are very similar (Fig.1) thus, these compounds were co-eluted and depicted as peak No. 7. The OZ was more retained than the DG under this condition even though their MWs are very similar. OZ and FS were the most retained compounds. The molecular shapes of these compounds are more compact compared to other molecules and they contain hydroxyl and aromatic functional groups in their molecules that can be adsorbed by the column.

The separation in size exclusion chromatography is based on the molecular dimensions (size and shape). Therefore, molecular compounds having the same MW may not be of the same size (Meyers, 2002). Although the MW of TG and DG are different, they were not separated on the chromatographic condition (Fig. 2A). On the other hand, TG and DG was baseline separation when toluene were used as a mobile phase (Kittirattanapiboon & Krisnangkura, 2008). Thus, type of solvent used as mobile phase influences the elution strength of the mobile phase and the swollen of the gel matrix. To increase the separation power, isooctane was used to control the swelling degree of the gel matrix. The more addition of isooctane into the mobile phase, the more decreasing of the mobile phase polarity and the less swelling of the gel matrix (Aryusuk et al., 2011). Ethyl acetate in the mobile phase was incorporated with isooctane at various amounts ranging from 30 to 70% (v/v), the results are shown in Fig. 2B-2D. The separation of TG and DG was obviously improved with increasing isooctane content. However, retention times of the polar compounds having hydroxyl, carboxylic and aromatic functional groups were prolonged, especially for the FA. Resolutions of all compounds seemed to increase with increasing percentage of isooctane in the mobile phase up to 70%. However, elution time of OZ and FA were greatly increased, and their peaks were broadened (Fig. 2D). Mobile phase with 0.1% acetic acid in ethyl acetate/isooctane (50:50, v/v) was able to separate the OZ and its saponified compounds, FA, and FS from the neutral lipids with acceptable time and peak shape (Fig. 2C).

3.2 Effect of silica guard column on the separation of lipids

To improve the resolution between FFA and MG separated on a 100-Å Phenogel column using 0.1% acetic acid in ethyl acetate/isooctane (50:50, v/v) as a mobile phase, a small silica guard was connected to the separation column to retard the polar solutes as previously recommended (Krisnangkura & Simamaharnnop, 1992, Chumsantea et al., 2014). The separation profile of the compounds of the same chromatographic condition using high percentage of ethyl acetate as mobile phase with and without the silica guard (Fig. 2A-2B vs Fig. 3A-3B) were similar.

Figure 3: HPSEC chromatograms of standard mixtures with ELSD detection. Column: 100-Å Phenogel column with a silica guard; mobile phase mixtures of ethyl acetate: isooctane: acetic acid in different volume ratios. Peak numbers were the same as described Fig. 2.



This imply that interaction between the polar compounds and the silica guard was minimal for high percentage of ethyl acetate in the mobile phase. However, it was indeed seen to show stronger interaction when the mobile phases of low polarity (50-70% isooctane in the mobile phase) were used. Different degrees of retardation with the silica guard were observed (Fig. 3C-3D). Thus, relative retention times of the compounds to the TG were plotted to evaluate the effect of isooctane and the silica guard on their retentions (Fig.4).

The change in mobile phase composition had a small effect on the retention of TG, FS and DG, moderate effect on the FFA and MG but showed the strong effect on the OZ and FA. In addition, retentions of compounds containing polar groups separated on the column connected to silica guard were even more pronounced, when high percentage of isooctane was used in the mobile phase. For instance, MG was more retained than FFA when the silica guard was connected to the column, while retention of the FS was relatively unchanged. Consequently, MG and FS were co-eluted, even resolution of FFA and MG was greatly improved (Table 1).

Figure 4: Relative retention time of the compounds to the TG eluted with different percentages of ethyl acetate in isooctane separated on (A) 100-Å Phenogel column with Phenogel guard (B) 100-Å Phenogel column with silica guard.

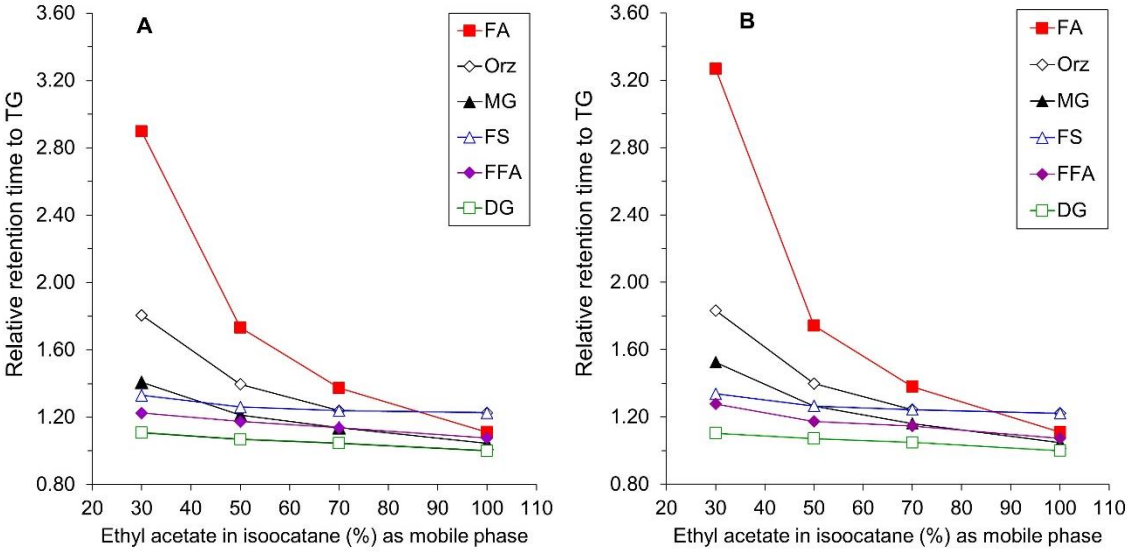


Table 1: Resolution factor of standard mixtures separated on a 100-Å Phenogel column connected with different guard eluted with ethyl acetate/isooctane/ acetic acid (50:50:0.1, v/v/v).

Guard column	Resolution factor					
	TG-DG	DG-FFA	FFA-MG	MG-FS	FS-OZ	OZ-FA
Phenogel guard	1.76	2.01	0.66	1.00	1.76	3.19
Silica guard	1.86	1.88	1.56	0.00	1.72	3.35

Thus, connection of a silica guard to the separation column seems likely not a good choice of selected for compounds of different polarity. Finally, monitoring of compounds in the sample containing neutral lipids, OZ and its saponified compounds could be performed by connecting a single 100-Å Phenogel column with a Phenogel guard and eluted with 0.1% acetic acid in ethyl acetate/isooctane (50:50, v/v). The analysis time was within 15 min at a mobile phase flow rate of 1.0 mL/min.

4. Conclusion

The separation mechanisms of compounds on the Phenogel column are quite complex. It is mostly based on the molecular dimensions (size and shape) of compounds when they are eluted with good swelling solvent or strong elution power mobile phase. However, interactions between the polar group (hydroxyl, carboxylic acid, or aromatic functional groups) of compounds and the stationary phase are also play a role in separation performances. The separation behavior is more interaction-dependent when low polarity and poor swelling solvent is used. Thus, the separation of compounds on the Phenogel column is considered as a mixed- mode mechanism due to several factors including molecular dimensions and functional group of solutes, degree swelling of the gel matrix as well as the polarity of mobile phase contribute simultaneously to the overall retention. The use of mid-polar solvent, ethyl acetate/isooctane (50:50, v/v) with 0.1% acetic acid without pre-column is useful for rapid monitoring the sample containing neutral lipids, OZ and its saponified compounds. Additional validation will be performed for further quantitative analysis.

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