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ORIGINAL PAPER

Separation and Determination of Wax Content Using 100-Å Phenogel Column

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Abstract Rice bran wax (RBW) is a by product of rice bran oil refinery. Crude RBW from refineries in Thailand had only 20–40% of the wax ester. The major impurity was triglyceride (TG). Purification of RBW requires a rapid and reliable method of analysis. In this study, a modified size exclusion HPLC column (100-Å Phenogel) was reported. Degree swellings of the gel matrix were controlled by isooctane–toluene mobile phase ratio. With pure toluene as the mobile phase, the gel matrix is fully swollen. Wax and TG could not be separated. With 65:35 (v/v) of isooctane–toluene, wax and TG as well as other lipids were baseline separated. The resolution (Rs) between wax and TG was greater than 1.5. Acetic acid (0.1% or higher) in the mobile phase could suppress peak tailing and improved separation of the lipid containing active hydroxyl groups such as free fatty acid, diglyceride and monoglyceride without affecting retention times of the wax and the TG. Separation of lipids in crude RBW could be completed in a single run on the modified Phenogel column (100 Å) with the total analysis time less than 15 min. The relationship between the amount of wax in the sample and the peak area was linear with the R^2 greater than 0.98.

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Introduction

By strict chemical definition, wax is the fatty acid ester of a long chain alcohol. However, most commercial and natural waxes contain many other lipids, including hydrocarbons, free fatty acids and fatty alcohols. Natural waxes have great potential applications in cosmetics, food [1] and fruit coating [2]. Rice bran wax (RBW) is a co-product of rice bran oil refinery. It is separated in the crystallization/winterization step of the refining process. The crude RBW usually contain up to 60–80% oil and they are rich sources of long chain fatty alcohols, known as policosanol. RBW is reported to be one of the best sources of octacosanol and triacontanol. The benefits to health, such as blood lipid or cholesterol lowering, anti-inflammatory and athletic performance improving, have been studied and proven to be completely without side-effects [3–5].

Methods for determination of wax composition have been widely reported [1, 6–9]. The common methods were usually carried out by saponification followed by GC analysis of the cleavage products. Bonaduce and Colombini [7], working on beeswax, commented that beeswax was very resistant to saponification. It is a lengthy process (2–3 h) and gives poor recovery of the hydrolytic products. According to Nota et al. [10], the EEC Commission in 1993 introduced the wax ester parameter to distinguish pressure oils from solvent-extracted oils and the analysis of wax esters was carried out according to the method described in document IV of the modification of EEC Regulation 2568/91 (EEC, 1991). The method requires great amount of times and labor. Thus, a simpler and faster

procedure was proposed. The wax was separated from triglyceride by a silica gel column and quantified by GC. The column chromatography, which was time consuming was replaced by a faster and more reliable HPLC method [11]. In contrast, methods for determination of wax content in vegetable oil are few compared to those for wax composition.

Wax content in vegetable oil is generally determined by crystallization, filtration and weighing the solid residue. This method is more accurate for oils rich in crystallizable wax, such as crude sunflower oil or rice bran oil [1]. However, the method requires large amount of sample and very time consuming. A rapid turbidimetric method for determining wax content in sunflower seed oil was described by Morrison [12]. The oil was heated to 130 °C, filtered, and after cooling, an equal volume of acetone was added to precipitate the wax. The mixture was then reheated under tap water to dissolve waxes and cooled in an ice bath for 5 min. Turbidity was then measured. The method can determine wax down to the ppm level and requires no special equipment but heating the oil up to 130 °C and filtering at this temperature might not be suitable for routine analysis. A rapid method for the isolation and quantitative determination of fennel and caraway wax in seed oils was reported by Reiter et al. [13]. The wax in oil was separated from the TG by mean of silver nitrate solid-phase extraction. The method allows rapid sample preparation in parallel and therefore a high sample throughput. The wax obtained was then analyzed by high temperature gas chromatography. Most of the methods reported in the literature were good for determination of wax in oil. On the other hand, a method for determination of the amount of oil in the wax or the purity of the wax has not been reported. In this study, the lipid samples are separated into lipid classes with a Phenogel (100 Å) column and each lipid is quantitated from its peak area. Thus, the purity of the wax can be easily proved.

Experimental

Materials

Crude rice bran waxes were a gift of the Rice Bran Oil Refinery. Solvents were HPLC grade obtained from RCI Lab Scan Co. Ltd. (Bangkok, Thailand). Refined and dewaxed rice bran oil was obtained from a local supermarket.

Sample Preparation

Lipid standards (1,000 ppm each) and RBW samples were dissolved in toluene. The samples were kept at 60 °C in a heating box before analysis.

HPLC

The HPLC system consisted of a pump model 510 (Waters Associates, Milford, MA, USA), a Rheodyne 7125 valve injector, a 20-μl loop and a Sedex 55 Evaporative Light Scattering Detector (ELSD; Sedere, Alfortville, France). Detector temperature was set at 30 °C and N_2 gas was at 2 bar. Data were collected and processed by CSW32 HPLC software (DataApex Ltd, Prague, Czech Republic). Sample was analyzed on a 100-Å Phenogel column (300 mm × 7.8 mm ID, 5 μm) (Phenomenex, Torrance, CA) protected with a Bondapak C18 Guard Pak (Millipore Co., Milford, MA, USA). The column and injector were in an oven set at 65 °C. The mobile phase was at a flow rate of 1.0 ml/min, its composition is reported in the text.

Statistical Analysis

Repeatability was carried out by relative standard deviation (RSD) during the day from values of four replicates. The wax content was quantitated from the average peak areas. Statistical analysis was performed by Microsoft Excel Version 8.0.

Results and Discussion

Separation of Crude Rice Bran Wax

It was demonstrated that the 100-Å Phenogel column, which had been tailored to separate compounds according to their sizes showed a characteristic adsorption to the hydroxyl group [14]. Thus, components which had the same molecular size could be separated with respect to their polarities. Accordingly, lipids in the biodiesel reactor could then be easily separated into lipid classes. When the separation condition was applied to separate RBW from rice bran oil (0.25% acetic acid in toluene), it was speculated that RWB has a lower MW (730) than rice bran oil (MW 870) and should be eluted after the oil, but both RBW and oil were co-eluted (Fig. 1). The result suggested that resolution of the column was too low. Increasing the column length (by connecting another column) would be a simple solution for increasing the separation power of RBW and oil, but this would increase the analysis time and cost. Modification of the pore structure by controlling degree of swelling of the gel matrix would be a good alternative. According to the manufacturing catalog, toluene and THF are the two most powerful swelling solvents for the Phenogel. Hexane is the lowest but isooctane is not listed in the catalog. It is speculated that isooctane would be just as poor as a swelling solvent as hexane. Thus, a mixture of a different percentage of isooctane in toluene

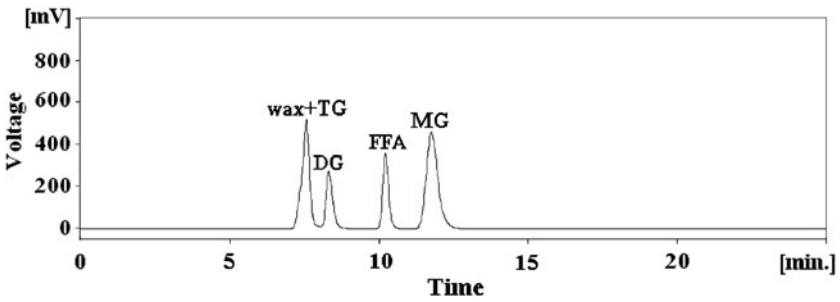

Fig. 1 Elution profile of standard lipids from a 100-Å Phenogel column eluted with 0.25% acetic acid in toluene

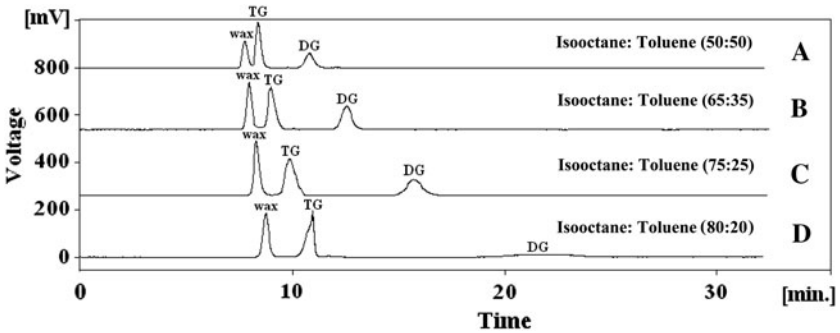

Fig. 2 Separation of wax from TG on a 100-Å Phenogel column eluted with toluene:isooctane at different ratios

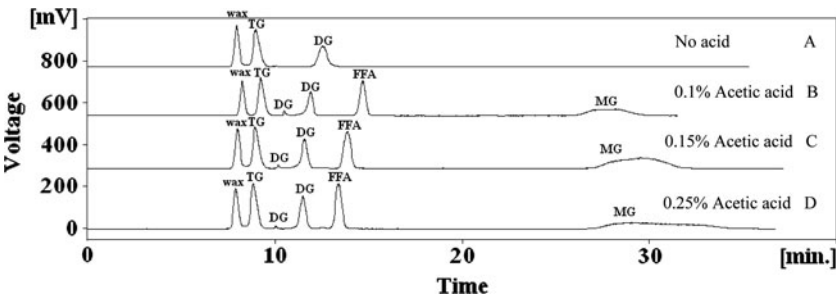

Fig. 3 Separation of standard lipids on a 100-Å Phenogel column eluted with isooctane:toluene (65:35 v/v) with different percentages of acetic acid

would control the degree of swelling of the gel matrix. Figure 2A–D is an overlay chromatogram of the standard lipids. The detection limit for each class of lipid is 0.10 μg [14] and the linearity (correlation coefficient) for all lipids is better than 0.99. Separation of the RBW and TG was increased as percent of isooctane in the mobile phase was increased or degree of swelling of the gel-matrix was decreased. For a 50:50 (v/v) of isooctane to toluene, partial separation of RBW and TG was observed (peak resolution; Rs = 1.3). Baseline separations of the RBW and TG were observed as the isooctane percentages were 65 and higher (Fig. 2B–D). The resolutions of RBW and TG were 1.9, 2.1 and 3.8 for 65:35, 75:25 and 80:20 (v/v) of isooctane to toluene, respectively. The wax was eluted out of the column faster than the TG. The Phenogel column is designed for use as size exclusion chromatography and the MW of the wax is lower than TG, hence it should be eluted out of the column after TG. Both RBW and oil do not contain a hydroxyl group which may interact with the gel matrix and elution order should follow the molecular size. Thus, molecular size of the long linear chain of RBW may be larger than the TG.

It was reported that molecules having a hydroxyl group were adsorbed and tailed badly in the absence of acetic acid [14]. A small amount of acid could shorten the retention times and suppress the tails of these peaks. Figure 3 shows the effect of acetic acid on the elution of molecules containing –OH groups. The mobile phase without acetic acid cannot elute monoglyceride (MG) and free fatty acid (FFA) out of the column (Fig. 3A). FFA was eluted out of the column at 13 min with 0.1% acetic acid in isooctane–toluene (65:35 v/v), while the MG peak was very broad (t_R about 28 min). The chromatogram in Fig. 3 simply suggests that MG cannot be quantified by this method. The problem of MG may be partly due to its low solubility in the mobile phase. Further incrementing the acetic acid, retention time of the FFA was shortened but retention times of the wax and the oil were unchanged,

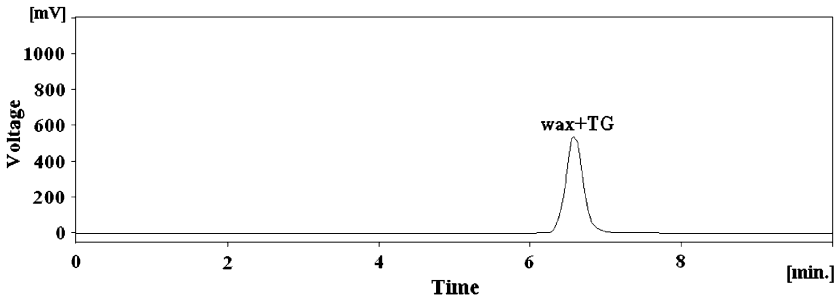

Fig. 4 Co-elution of wax and triglyceride on 50-Å Phenogel column eluted with 100% toluene

Table 1 The average area of wax from four replicates with SD and %RSD

Wax content (%)	Average	SD	%RSD
1	50.4	0.86	1.70
5	210.8	12.62	5.99
10	547.3	37.27	6.81
15	835.2	65.16	7.80
20	1243	32.1	2.58
25	1570	14.65	0.93

suggesting that there was no interaction between wax or oil and the gel matrix. Increasing column temperature from 65 to 75 °C and 85 °C shortened the retention time of MG while those of TG, wax, DG and FFA were almost not affected.

Although, optimization of RBW separation into lipid classes was done by mixing isooctane with toluene to control the degree of swelling of the gel matrix, the experiment was also performed on a column of the smallest pore size (50 Å), commercially available. By simple mathematics, the pore size of this column would be equivalent to the 50:50 (v/v) of isooctane to toluene. However, wax and TG were co-eluted when toluene was used as the mobile phase (Fig. 4). Thus, it may be concluded that the pore structure of the 50 Å column is not equivalent to the half swollen of the 100 Å column.

Analysis of RBW from Different Sources

Repeatability was carried out by relative standard deviation (RSD) during one day from values of four replicates. The percentages of RSD for each wax content (1–25%) are summarized in Table 1.

Variation of retention times during one day and within a week was within ±0.1 min and was highly reproducible. However, the peak area fluctuated slightly. It is very characteristic of ELSD, which is sensitive to the detector temperature and nebulizer gas flow rate [15, 16].

Table 1 shows that the peak area of wax is linearly related to the amount of wax in the oil ($R^2 = 0.9809$). The average areas of four injections for wax at 1, 5, 10, 15, 20 and 25% were 50.4 (1.70% RSD), 210.8 (5.99% RSD), 547.3 (6.81% RSD), 835.2 (7.80% RSD), 1243 (2.58% RSD) and 1570 (0.93% RSD), respectively. The standard deviation of the slope is 2.54. Thus, the precision of the determination for the wax content is acceptable. The HPLC–ELSD method in this study is less sensitive than the turbidimetric method reported by Morrison [12]. However, the method would be good for quantitative analysis of wax in the oil in the range of 1–50%. In addition, impurities other than TG, e.g. diglyceride (DG), free fatty acid (FFA) can be identified and quantitated.

Figure 5 is the overlay chromatograms of RBW from different refineries. It can be seen that all the crude waxes contain an appreciable amount of TG. In addition, soft wax (1) contains a large amount of DG. The presence of a large amount of DG implies the use of aged raw material (rice bran) where TG had been partially hydrolyzed by lipase to DG. On the other hand, soft wax (2) was obtained by gravitation filtration. Thus, the crude wax (2) contained very large amounts of TG (78%). The hard wax still retained up to 65% of TG.

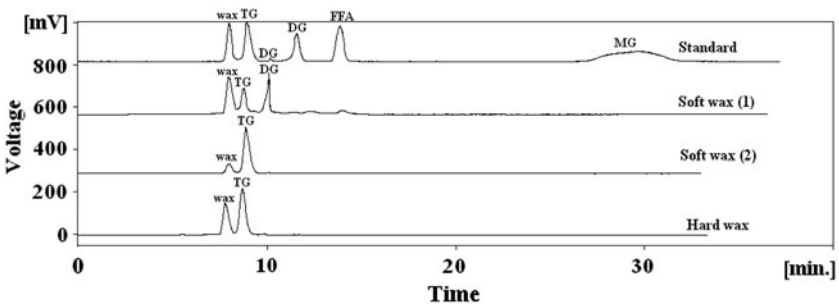

Fig. 5 Composition of crude waxes from different refineries/ processes

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