Separation of Vitamin E and γ-Oryzanols from Rice Bran by Normal-Phase Chromatography

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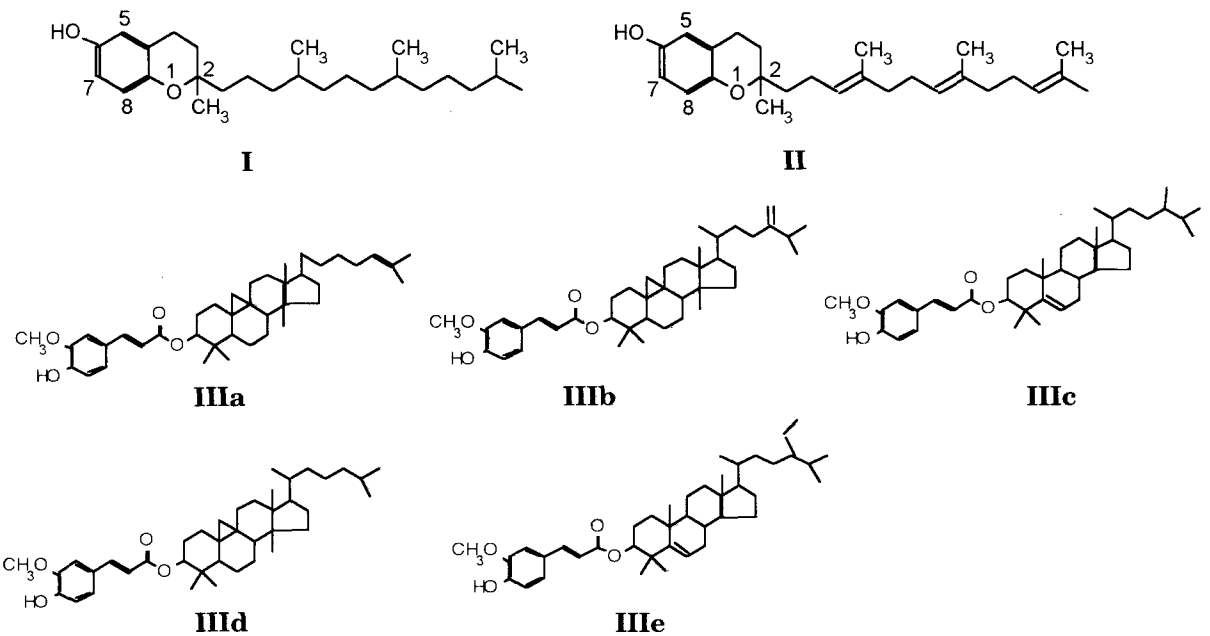
Five commercial silicas [Nova-Pak, μPorasil (Waters, Milford, MA), Kromasil (Eka Nobel, Bohus, Sweden) Spherisorb (Sigma, St. Louis, MO) and Select-KP (Biotage, Charlottesville, VA)] were tested for their efficiency in separating vitamin E and γ-oryzanol components from saponified rice bran extracts, with the perspective of future large-scale purification of the various compounds. The effects of mobile phase composition, eluent flow rate, column temperature and the physical properties of the silica on the selectivity and analysis time were studied. All silicas except Select-KP gave baseline separation of the vitamin E components with isooctane/ethyl acetate (97.5:2.5) at room temperature. However, only the 4-μm Nova-Pak spherical silica gave adequate resolution of the oryzanols into two distinct fractions with an analysis time of *circa* 20 min. Other analytical conditions, i.e., the content of the polar modifier in the mobile phase, temperature and flow rate, can be used to optimize the separation once the silica had been chosen. Speed of separation is enhanced by increasing the polarity of the eluent, temperature and flow rate, while the selectivity is reduced only slightly. The Nova-Pak silica appears resistant to deactivation from both residual water in the mobile phase and polar components left over after sample clean-up and is suitable for preparative separation of the target compounds.

KEY WORDS: Normal-phase chromatography, oryzanol, rice bran, vitamin E.

The extraction of vitamin E from natural sources (e.g., rice bran) and the chromatographic resolution of various constituents of this family of compounds have lately regained potential importance because of their proven or alleged health benefits and antioxidant activity (1–4). The term "vitamin E" covers a family of related compounds that possess as a common feature a hydroxychromane ring and a terpenoid side chain located at position 2 of the ring (Scheme 1). They are divided into two main groups: the tocopherols with a saturated isoprenic side chain (Structure **I** in Scheme 1) and the tocotrienols with three double bonds on the isoprenic chain (Structure **II**). Within these two groups, distinction is made between α-(5,7,8-trimethyltocol), β-(5,8-dimethyltocol), γ-(7,8-dimethyltocol) and δ-(8)methyltocol) tocopherols and tocotrienols according to the number and position of the methyl substituents of the chromane ring (5,6). A family of ferulic acid esters of unsaturated triterpenoid alcohols was identified in crude rice bran oil (7) and termed γ-oryzanols (**IIIa** = cycloartenyl ferulate, **IIIb** = 2,4 methylene cycloartenyl ferulate, **IIIc** = campestryl ferulate, **IIId** = cycloartanyl ferulate, **IIIe** = β-sitosteryl ferulate) by the authors (Structure **III**, Scheme 1). The oryzanols are antioxidants and are believed to possess curative functions for various human diseases (8–10).

Rice bran oil is a good source of most of the vitamin E and oryzanol components, and although their concentrations may vary substantially according to the origin of the rice bran, combined they form 2–5% of the oil (11). Various extraction techniques have been described in the literature, all in conjuction with sample preparation for high-performance liquid chromatography (HPLC) rather than as preparative processes. The quantitative and selective extraction of those compounds from the complex matrix of the bran is tedious (12), and care must be taken to avoid their oxidation during extraction.



SCHEME 1

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In most cases, the oil is first extracted with an organic solvent (i.e., *n*-hexane, ethanol, isopropanol or diethyl ether), the bran is removed, and waxes and triglycerides are removed from the micella by precipitation at subzero temperatures, molecular distillation or, most commonly, by saponification with KOH. The processes of extraction and saponification can also be accomplished simultaneously, in the presence of the bran (13–15). The target compounds are then further selectively recovered from the unsaponifiable fraction by liquid/liquid extraction (16–18) or adsorption on normal- or reverse-phase silica (6).

To our knowledge, no systematic comparison has been attempted regarding the merits of the various extraction techniques. It has been shown though (6), in the study of a cereal sample, that the recovery of α-tocopherol and α-tocotrienol by Soxhlet extraction was higher (99%) than their yield in a Soxhlet-extracted and saponified oil (83%). The loss in saponification is probably related to degradation in alkaline media and possibly to trapping of the target compounds in the precipitates of the fatty acid salts generated during saponification (19). Bradcock *et al.* (20) found a 50% loss of vitamin E during saponification under inert gas without an antioxidant, e.g., pyrogallol or ascorbic acid, which would be expected to protect the tocopherols from oxidation. Even if sample processing for most efficient extraction of vitamin E is still an open problem, because of fouling of the adsorbents by the saponifiables present in great excess in the extracts, the general consensus is that saponification is necessary (13,21,22) to achieve a satisfactory recovery of vitamin E in a liquid phase with properties permitting their quantitation, isolation and fractionation by liquid chromatography (LC).

Use of both normal-phase (6,13,15,23) and reverse-phase chromatography (3,7,24,25) for the analytical resolution of vitamin E has been reported. Those systems, either in isocratic or gradient mode, allow baseline resolution of the eight vitamin E compounds. Recently, five fractions of the γ-oryzanols (Scheme 1, Structures **IIIa–IIIe**) were isolated from vitamin E in a saponified rice bran sample in a C_{18} reverse-phase column with a mixture of acetonitrile/methanol/isopropanol/water as the mobile phase (7). To our knowledge, with a normal-phase system, separation of the five oryzanols has not been reported. Even though the advantages of reverse-over normal-phase systems for separating complex mixtures are undisputable, and quick column equilibration and better reproducibility are usually obtained, it has been proven, however, that normal-phase chromatography on silica presents better selectivity for the fat-soluble vitamins (2,16). In addition, the nonpolar mobile phase in normal-phase chromatography tends to be a better solvent for the lipid-rich vitamin extracts, therefore reducing the danger of poisoning the chromatographic packing. Deactivation of the silica surface may be a problem in normal-phase chromatography, from adsorption of water or other polar components, e.g., the residual fatty acids in the unsaponified vitamin E-rich extracts (26–28). Shin and Godber (15) recommended use of small amounts of acetic acid and dimethoxypropane in the mobile phase to prevent column deactivation.

Silicas for HPLC are commercially available under various trade names. Their physical properties and chromatographic behavior are related to their mode of preparation and can differ widely due to the surface pH, surface impurities, specific surface area, and their pore and particle size (29). For a given system (i.e., solute/mobile phase composition), its chromatographic behavior depends on the specific surface area, the column packing density and porosity according to a general equation (30):

$$k\text{-}\dot{\text{o}}\text{-} = K\ s\ (P_o/\varepsilon) \qquad [1]$$

where $k\text{-}\dot{\text{o}}\text{-}$ is the capacity factor (dimensionless retention time), defined as:

$$k\text{-}\dot{\text{o}}\text{-} = (t-t_0)/t_0 = Fa \qquad [2]$$

with t being the retention time, t_0 the retention time of the solvent, F the phase ratio (volume of sorbent/total volume of liquid), a the slope of the Langmuir isotherm at infinite dilution, K an adsorption coefficient reflecting primarily the factors related to the surface chemistry of a particular silica, P_d the packing density of the column, ε the total column porosity and s the specific surface area of the silica. Although a linear relationship is generally expected between the capacity factor and the specific surface area, provided that the column packing density, pore volume and the adsorption coefficient remain constant (30), exceptions must be expected (31) because it is difficult to obtain silica of widely different surface areas that would at the same time have identical surface chemistries (or K). Selectivity of a column is expected, in the first approximation, to be independent of surface area, although again exceptions are to be expected. For example, Scott and Kucera (31) found that the column selectivity for a pair of components increased when the specific surface area of the adsorbent decreased, which was attributed again to the difference in surface chemistries of the tested silicas. It was concluded that the selectivity depended exclusively on the relative competition for the silanol groups at the silica surface with the polar part of the mobile phase. It was demonstrated that, when ethyl acetate was used as an organic modifier, a layer of polar solvent was formed at the surface of the silica. The retention (i.e., $k\text{-}\dot{\text{o}}\text{-}$ values) and selectivity of a column for a given pair of eluting components depended on the polarity difference between the solutes and the polar organic modifier (32,33). Longer retention time is then expected when the polarity of the solute equals or exceeds the polarity of the organic modifier.

In view of the potential isolation and purification of these components by preparative LC, it was considered necessary to evaluate first various typical commercially available silicas for their relative advantages. The criteria were their selectivity, speed of analysis, long-term stability, ease of regeneration and saturation capacity. Commercial analytical columns, 15 cm in length and either 0.46 or 0.39 cm i.d., were used.

MATERIALS AND METHODS

All solvents were HPLC-grade from Mallinckrodt (Paris, KY) and were used as received. 2,2-Dimethoxypropane, L-ascorbic acid and citric acid were purchased from Aldrich (Milwaukee, WI), Mallinckrodt and EM Science (Gibbstown, NJ), respectively. The standard solutions of α- (95% purity), γ- and δ-tocopherol (95%) were purchased from Sigma (St. Louis, MO). A solution of γ-oryzanols, prepared by sequential solvent purification as described previously (34), was supplied to us by Godber and Shin

(Department of Food Science, LSU Agricultural Center, Baton Rouge, LA).

Crude rice bran oil was prepared by following the technique described previously (13–15) with slight modifications, which consisted primarily in the use of an ultrasonic bath at 80°C for better dispersion of the phases and faster mass transfer and the use of ascorbic acid and citric acid as antioxidant and metal-chelating agents, respectively. A 50-g sample of a raw Louisiana rice bran was weighed in a 1000-mL beaker and 5 g ascorbic acid, 150 mg citric acid and 500 mL ethanol were added. The mixture was then sonicated at 80°C for 15 min. Without removing the bran, 15 mL of 80% aqueous solution of KOH were added, and the mixture was saponified at 80°C for another 15 min. Then, after the reaction had been stopped (ice bath), 100 mL water and 100 mL hexane were added, and the mixture was again sonicated for 10 min. During the whole procedure, the reaction vessel was kept blanketed with nitrogen to minimize oxidation of the target compounds. The reaction mixture was allowed to settle at room temperature for 20 min and was then filtered through a Whatman cellulose filter disk in a closed stainless-steel filtration funnel pressurized with nitrogen. The filtrate was transferred in a separatory funnel, and the hexane layer was collected and washed three times with 100 mL deionized water. The resulting hexane solution was finally filtered through a layer of anhydrous Na_2SO_4, and the volume was reduced to 50 mL in a glass rotary vacuum evaporator and used without further treatment for the HPLC analysis.

The HPLC apparatus consisted of a Model 610 pump, a Model 600 controller, both from Waters (Milford, MA), and a Spectra Physics (San Jose, CA) 200 ultraviolet (UV) detector, set at 295 nm for the analytical injections and at 360 nm for the overloaded injections. A six-port air pressure-activated Dionex sliding valve with 15-, 20-, 40- and 50-μL fixed loops was used for injections. The sample to be injected was kept in a glass pressurized container, shielded from light. The repeated loop filling–injection cycle was executed with a series of 24 V solenoids, which were controlled with a Gould programmable controller for unattended operation. The data were collected with a PC-based A/D data conversion/collection system. In some cases, for identification purposes, a 470 fluorescence detector (Waters) with excitation at 290 nm and emission at 330 nm was coupled to the UV detector.

The UV spectra were recorded by using the stop-flow method with the Spectra 200 UV detector. The pump was stopped at the apex of the eluting peak, and the spectrum was scanned between 190–360 nm with a step wavelength of 2 nm. The signal was collected with the PC-based A/D data collection system.

The five following silica stationary phases were studied: Nova-Pak and μPorasil (Millipore Waters), Kromasil (Eka Nobel, Bohus, Sweden), Spherisorb (Sigma) and Select-KP (Biotage, Charlottesville, VA). The physical properties of the adsorbents, as provided by the different suppliers, are listed in Table 1. The five silicas are referred in the text according to their numbers in Table 1. The column efficiency was determined at ambient temperature with an analytical injection (15 μL) of a solution (0.195 g/L) of δ-tocopherol at a flow rate of 1.6 mL/min, with an isooctane/ethyl acetate (97.5:2.5) mobile phase. The column plate number (efficiency) N was calculated as:

$$N = 16\ (t/W_h)^2 \qquad [3]$$

where W_h is the baseline peak width.

Often in analytical LC, the useful capacity of a column is estimated as the sample load that gives a 10% reduction in k-ϕ- (relative to k-ϕ- at infinite dilution) or a 10% decrease in column efficiency (31). For preparative chromatography, where the columns do get purposely overloaded, a so-called "touching band" (35) approach can be used but, preferably, the capacity is estimated from the adsorption isotherm determined from analytical and overloaded injections (36,37). In this work, the saturation capacity of one of the packings, the Waters' Nova-Pak silica, was determined from analytical and overloaded chromatographs of γ-tocopherol.

The long-term stability and selectivity of the columns for the various vitamin E isomers and γ-oryzanols were studied by repeated injections of the saponified rice bran samples. After 100 injections, the columns were regenerated by flushing with a solution (10 column volumes) consisting of 95:2.5:2.5 (vol/vol/vol) hexane/acetic acid/2,2-dimethoxypropane (15), followed by equilibration with the mobile phase prior to further injections.

RESULTS AND DISCUSSION

Physical properties of the various silicas are given in Table 1. All data are from the respective manufacturers, except for the column efficiencies, which were measured here as described in the previous section. The pore size of a silica and its specific surface area appear unrelated, as do the column efficiency and the specific surface area, judging from the three silicas (**I**, **III** and **IV**) of comparable particle size. As expected, the efficiency is much higher for the 4- and 5-micron silicas (**I** and **III**), although the

TABLE 1

Physical Properties of the Five Silicas (suppliers' data except efficiency, N)[a]

Silica (column number)	Dimension (mm)	Pore size (Å)	Surface area (m^2/g)	Particle size (μm)	Shape (S, I)	Efficiency (N)
Nova-Pak (**I**)	3.9 × 150	60	120	4	S	4068
μPorasil (**II**)	3.9 × 150	125	330	10	I	1985
Kromasil (**III**)	4.6 × 150	60	550	5	S	4163
Spherisorb (**IV**)	4.6 × 150	80	220	5	S	2617
Select-KP (**V**)	4.6 × 150	60	475	32	S	<1200

[a]S = spherical, I = irregular. Nova-Pak, μPorasil (Millipore Waters, Milford, MA), Kromasil (Eka Nobel, Bohus, Sweden), Spherisorb (Sigma, St. Louis, MO) and Select-KP (Biotage, Charlottesville, VA).

efficiency of silica **IV** is lower than expected, in that regard.

The identification in a typical chromatogram (UV, 295 nm) of the α-, γ-, δ-tocopherols and the γ-oryzanols in the rice bran extract (Fig. 1) was performed by adding their standard solutions as internal markers. Because of the lack of tocotrienol standards, their identification was done by matching the chromatographs with those of Shin and Godber (15), which were collected at identical chromatographic conditions (column, mobile phase, temperature). Component 5 and the two minor components eluting at 27 and 30 min remained unidentified, but because the former did not give any fluorescence signal, it is probably not a part of the vitamin E complex. The triglycerides, left-over from saponification, are not retained in normal-phase chromatography (2), and they probably co-elute (peak 1) with toluene that had been added as a t_0 marker. The five oxidation products of α-tocopherol that were identified and isolated (38) previously are all less retained in the normal-phase system than α-tocopherol itself. Peak 2 is possibly one of the oxidation products of the vitamin E components.

The tocopherols and tocotrienols identified in this sample elute in the order α-tocopherol (α-T) < α-tocotrienol (α-T3) < γ-T < γ-T3 < δ-T < δ-T3, identical to previous studies (4,16,18,24). The retention time and relative selectivity for the vitamin E components in normal-phase chromatography are presumably attributed to the steric hindrance of the phenolic group by the methyl substituent of the chromane rings, which leads to different degrees of interactions between the molecules and the silanol groups of the silica (23). The slow eluting major peaks 10 and 11 were identified as γ-oryzanols. Furthermore, their UV spectra (Fig. 2) with three major maxima at 231, 291 and 315 nm, characteristic of their ferulic acid moieties, are similar to those reported previously (39). Considering that a total of five isomers of γ-oryzanols (Structure 1, Structure **IIIa–e**) were identified recently in a rice bran extract by reverse-phase chromatography, it appears that normal-phase does not give their full resolution. Because the retention time in reverse-phase chromatography depends on the solubility of the component in the polar mobile phase, the elution order (on reverse-phase) of the oryzanols **IIIa** < **IIIb** < **IIIc** < **IIId, IIIe** (7) indicates, in a first approximation, their order of decreasing polarity. The cycloartenyl ferulate (**IIIa**) and (**IIIb**) with a terminal double bond susceptible to polarization are likely the most polar. On a normal phase column, where the elution order is usually reversed, it is likely that the less polar isomers **III(c–e)** colute in peak 10, and the more polar structures **III(a–b)** in peak 11.

The comparison of the various silicas at identical analytical conditions is given in Figure 3. Here, for more reliable identification of the peaks, small amounts of α-T (peak 3), γ-T (peak 6) and δ-T (peak 8) standards were added to the sample. Except for column **V**, whose efficiency is too small, baseline separation of the tocopherols and tocotrienols was obtained with all columns. Surprisingly, despite the large difference in their specific surface areas, the retention times and the selectivity for vitamin E are quite similar on columns **I** and **II**, although the higher packing density of the 4-μm silica may have offset the effect of lower surface area. On column **II**, the oryzanols do not elute as distinct peaks. This may be in agreement with previous studies (31), which demonstrated the advantage of a low-surface area adsorbent for the resolution of mixtures of components with a wide range of polarities.

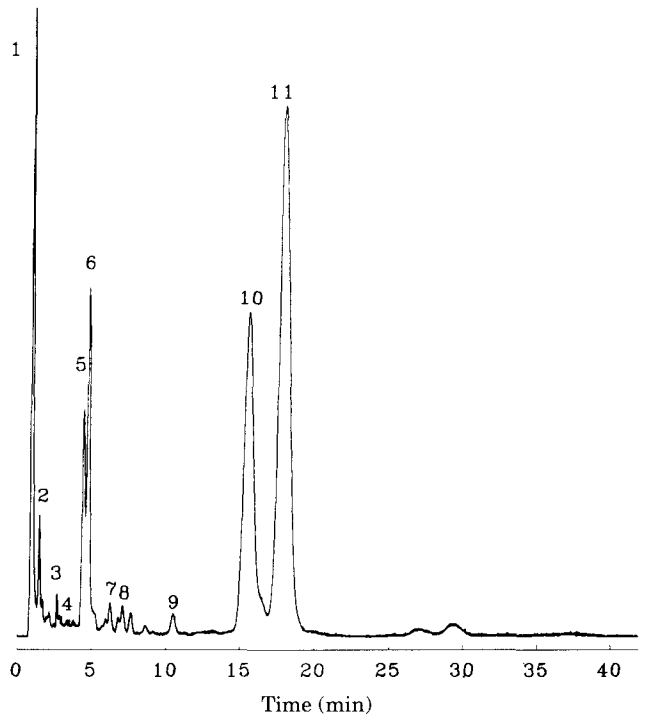

FIG. 1. High-performance liquid chromatography chromatogram of saponified rice bran oil. Conditions: Nova-Pak (Millipore Waters, Millford, MA) silica column (15 × 0.39 cm) at room temperature; injection: 15 μL of the extract; mobile phase: 2.5% ethyl acetate in isooctane at 1.6 mL/min; detection: ultraviolet (295 nm). Components: 1 = triglycerides + toluene (internal marker), 2 = oxidation products, 3 = α-tocopherol, 4 = α-tocotrienol, 5 = not identified, 6 = γ-tocopherol, 7 = γ-tocotrienol, 8 = δ-tocopherol, 9 = δ-tocotrienol, 10, 11 = γ-oryzanols.

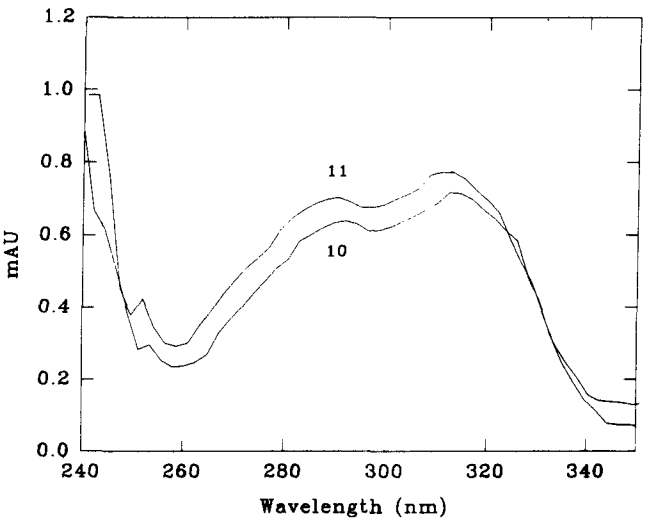

FIG. 2. Ultraviolet absorption spectra of the γ-oryzanols obtained from stop-flow scanning of peaks 10 and 11 (Fig. 1).

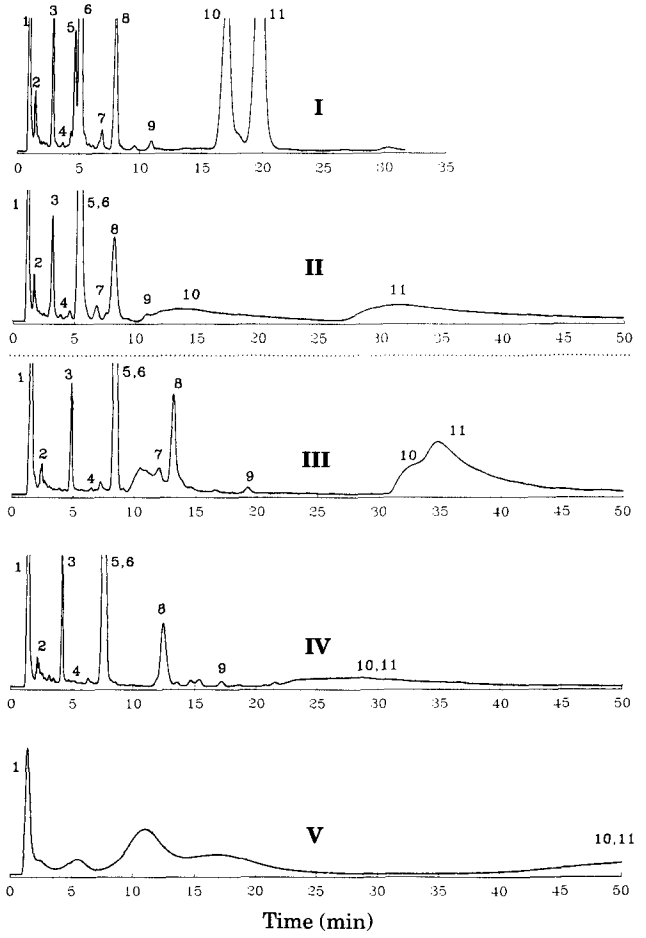

FIG. 3. High-performance liquid chromatography chromatograms of saponified rice bran oil on the five silica columns tested: I = Nova-Pak, II = μPorasil, III = Kromasil, IV = Spherisorb, V = Select-KP (see Fig. 1 for company sources). Same conditions as in Figure 1, except that 20 μL of each sample was injected.

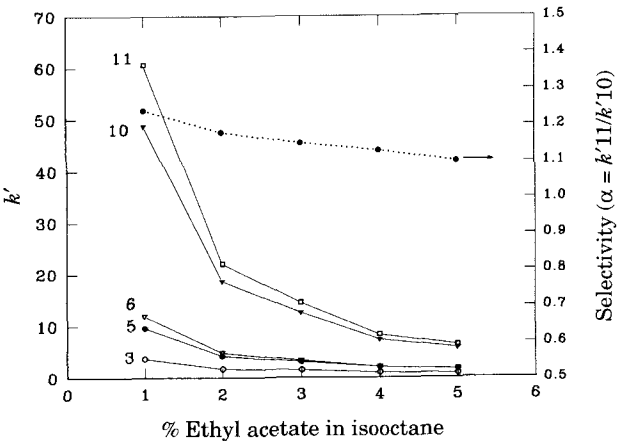

FIG. 4. Variations of the capacity factor and selectivity for the two γ-oryzanol fractions (peaks 10 and 11) with the composition of the mobile phase on the Nova-Pak column (see Fig. 1 for company source). The numbers refer to the peaks as numbered in Figure 1. All other analytical conditions were as in Figure 1.

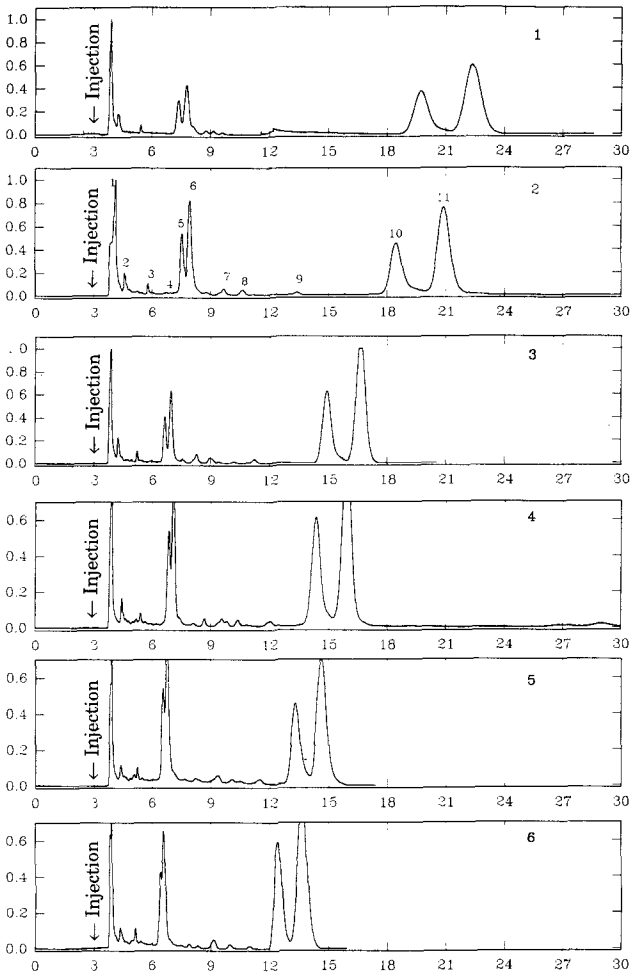

FIG. 5. The temperature effects on the retention time and selectivity of the Nova-Pak column (see Fig. 1 for company source). Same analytical conditions as in Figure 1. Temperature (°C): 1 = 5; 2 = 25; 3 = 45; 4 = 55; 4 = 65; 6 = 75.

Apparently, the lower the surface area of the silica, the wider the polarity range of components that can be chromatographed in isocratic mode. None of the columns, with the exception of Nova-Pak (I), resolves the oryzanols as distinct fractions, although it is possible that other analytical conditions (mobile phase) could be found to improve the separation on columns II, III and IV.

As has been often reported in the literature, the capacity factor on Nova-Pak (I) decreases with increasing content of the organic modifier in the mobile phase (Fig. 4). The selectivity for the γ-oryzanol pair decreased from $\alpha = 1.25$ to 1.1 when the percentage of ethyl acetate increased from 1 to 5%. Two to three percent of the organic modifier appears a good compromise between resolution and speed of analysis. Consistent with the exothermic adsorption concept, all retention times and k-◌- decreased with increasing temperature (Figs. 5 and 6). The slopes dk-◌-$/dT$ in Figure 6 clearly increase with k-◌-. In contrast, the selectivity between the two oryzanol fractions (10 and 11) remains nearly constant between 5 and 75°C. In view of the positive effects of temperature on the speed of analysis

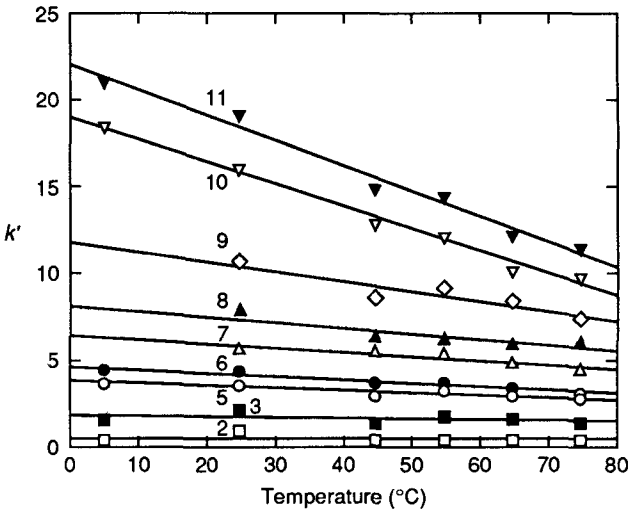

FIG. 6. Temperature effects on the capacity factors of the various components of saponified rice bran oil. Same analytical conditions and peak numbers as in Figure 1.

and column efficiency, a column temperature of 55°C appears a good compromise, taking into account the possibility of thermal degradation of the target compounds, and worsening of the separation of the 5:6 pair.

Three-hundred successive injections (Fig. 7) of an identical saponified oil sample do not indicate any systematic shortening of the retention times on the Nova-Pak silica, perhaps with the exception of the 5:6 pair. Although in retrospect it does not appear to have been necessary, the column was regenerated after every 100 injections as recommended by Shin and Godber (15), who observed much more severe changes after only 70 injections or so on a Supelco silica. To test independently whether any deactivation might come from the traces of water in the isooctane-ethylacetate mobil phase, the column was submitted to continuous solvent flow for 62 h, with a few injections only every 8 to 10 h, without any regeneration. No systematic changes in column behavior were observed.

The capacity factor of all components decreased in a nearly linear manner with the increase in flow rate (Fig. 8). As with the effect of temperature, the slope is higher for the more retained components (k-◇), perhaps because of the slower intraparticle mass transfer of the more polar compounds. The saturation capacity of Nova-Pak silica was determined from analytical and overloaded injections of δ-tocopherol (Fig. 9). The retention times of the two

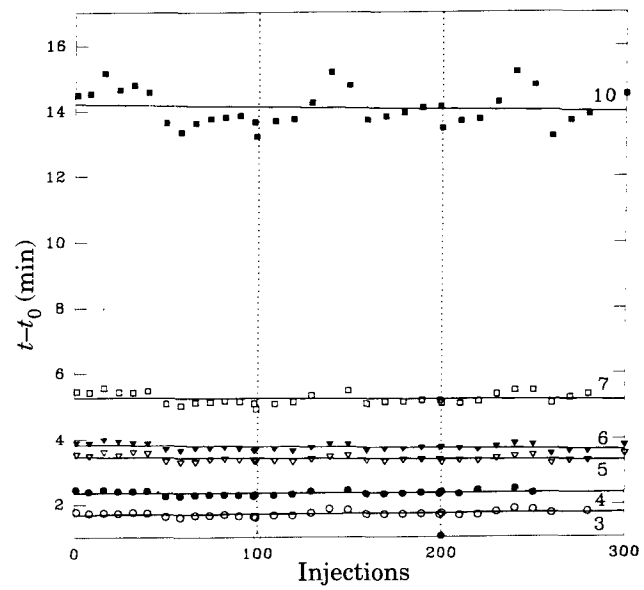

FIG. 7. Long-term stability of the Nova-Pak column (see Fig. 1 for source) for separation of the saponified rice bran oil components. Same peak numbers and analytical conditions as in Figure 1, except that a flow rate of 1.8 mL/min was used here. After each 100 injections, column regeneration was done as described in text. Only every seventh chromatogram was recorded to save PC memory storage space.

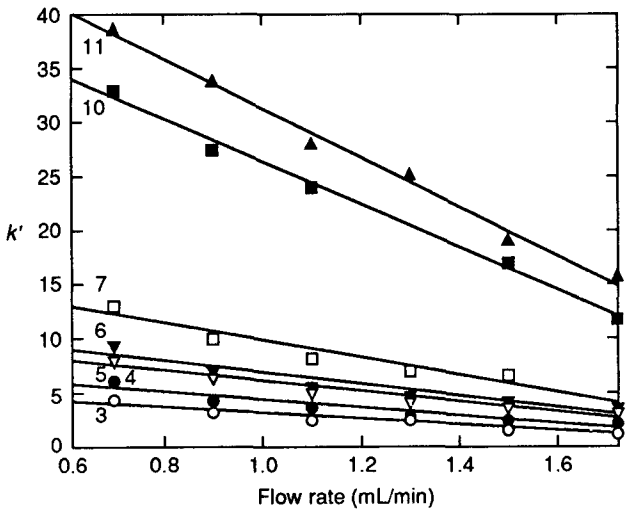

FIG. 8. The capacity factor of the Nova-Pak (see Fig. 1 for source) for the various components as a function of the flow rate. The same peak nomenclature and analytical conditions as in Figure 1.

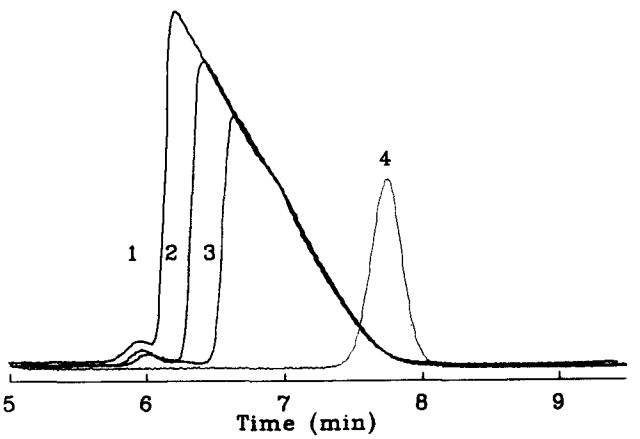

FIG. 9. Superimposed chromatograms of δ-tocopherol at overloaded (1,2,3) and analytical conditions (4, detector at a higher sensitivity). Nova-Pak silica column (see Fig. 1 for source) (15 × 0.39 cm), mobile phase 2.5% ethyl acetate in isooctane at 1.6 mL/min. Chromatograms 1, 2 and 3 correspond, respectively, to injections of 50, 40 and 20 μL of a 59.84 mM/L solution, chromatogram 4 to an injection of 15 μL of a 0.48 mM/L solution of δ-tocopherol in the mobile phase. The ultraviolet detection was set at 360 and 295 nm for the overloaded and the analytical injections, respectively.

overloaded profiles 1 and 2 (the loading factor of profile 3 was somewhat less than 1% and was not used for the calculation) were measured at half height of the peak front as 6.1 and 6.3 min, respectively, and the retention time at infinite dilution was 7.8 min. The total porosity of the column (1.424 mL) was determined with toluene as a t_0 marker. The Langmuir parameters a and b were 29.9 and 58,730 mL/M, respectively, giving a column capacity of 0.51 mM/mL. Even accounting for the difference in molecular size, this is substantially lower than the 12.5 mM/mL determined for acetophenone (38) in the system hexane/ethyl acetate (97.5:2.5) but on a different silica than used here.

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