Chromatographic Problems Related to Mobile Phase Degassing and Fluorescence Detection

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Abstract

Two problems are encountered in degassing hexane-based mobile phases. First, when fluorescence is used for the detection of vitamins A and E, the peak responses observed for consecutive injections of the same sample decrease over time due to quenching by oxygen dissolved in the mobile phase.
Second, a rapid increase in retention time is observed with consecutive injections due to the preferential evaporation of the modifier, isopropanol (0.5%) in hexane. The rapid evaporation is due to the formation of an azeotrope between isopropanol and hexane with a boiling point of 63°C. A discussion of possible solutions to the two problems is given.

Introduction

Experiment I

High-performance liquid chromatography (HPLC) has been used for the determination of natural levels of vitamins A, E, and β-carotene in fat-containing products such as milk, milk powders, and cream after extraction using a modified Rose-Gottlieb procedure (1). All three vitamins were eluted within 15 min using fluorescence detection for vitamins A and E and ultraviolet–visible (UV–vis) detection for β-carotene; a silica column and a hexane-based mobile phase in isocratic mode were used.

It is common practice in our laboratory to analyze up to 40 samples by HPLC overnight (10–12 h). The mobile phase is thoroughly degassed with helium before use and a very low flow of helium is maintained in a blanket above the mobile phase. However, consecutive injections of the same extract for over 7 h revealed that the peak responses for vitamins A and E decreased with time. Several possibilities existed: gradual oxidation of vitamins A and E, photo-decomposition, drifts in detector response with time, or quenching of the fluorescence signal with time. These possibilities were explored in detail, and remedies to the problem were suggested.

Experiment II

A simplified saponification procedure (2) has also been used for the determination of vitamins A, E, and β-carotene in some dairy products. The vitamins were separated using a silica column and a mobile phase containing 0.5% isopropanol in hexane, isocratically. Vitamins A and E were detected using a programmable wavelength fluorescence detector. The wavelengths were programmed to detect the retinyl esters during the first 2–3 min, to detect the tocopherols for the next 10 min, and then to change the wavelengths back to the original to detect retinol. The total analysis time was about 25 min. When a high helium flow was maintained to prevent quenching of the fluorescence signal, we found that the retention time for consecutive separations dramatically increased. A lengthy equilibration of the column with the mobile phase did not stabilize the retention times. When the helium flow was shut down, the retention times were more stable but increased to a certain extent. This observation suggested the possibility of evaporation of the mobile phase. The fact that the retention time was increasing suggested a faster evaporation of the modifier (isopropanol) than hexane. Possible reasons for this problem and solutions were discussed.

Experimental

Chromatography was performed using Waters Associates chromatographic equipment (Milford, MA) consisting of a model 6000 A pump, a model 710B WISP autoinjector, a model 470 scanning fluorescence detector, a model 490 E programmable multi-wavelength UV–vis detector, the program Maxima 820 (version 3.10) data processing package, and a system interface module. A Hitachi F1000 fluorescence detector (Hitachi, Tokyo, Japan) was also used for some experiments. An Alltech econosphere silica column (15 × 4.6 cm, 3-µm film thickness) was used for all separations. Amber screw-cap vials with lids and Teflon septa (Alltech Associates, Deerfield, IL) were used to hold the samples for HPLC analysis.

Chemicals

HPLC-grade hexane was used. Diisopropyl ether (DIPE), isopropanol (IPA), 3-*t*-butyl hydroxy-anisole (BHA), and acetic acid were all analytical reagent grade. Retinyl acetate and α-tocopherol were USP reference standards (USP, Rockville, MD). Retinyl palmitate (R-3375), α-tocopherol acetate (T-3376), and β-carotene (C-9750) were purchased from Sigma Chemical (St Louis, MO). Retinol (38280) was from Serva (Heidelberg, Germany).

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Mobile phases

The HPLC mobile phase consisted of either 3% DIPE and 0.04% acetic acid (Experiment I) or 0.5% IPA and 0.01% acetic acid in hexane (Experiment II). Approximately 0.02 mg/L α-tocopherol was added to both mobile phases to obtain consistent retention times and to saturate the active sites of the column with α-tocopherol (1). The mobile phase was vacuum-filtered through a 0.45-µm filter, degassed, and stored in an amber reservoir.

Sample and standard solution preparation

Samples and standard solutions were prepared under subdued incandescent light to prevent photo-decomposition of vitamins. A combined standard solution containing 2.5 mg/L retinyl palmitate, 50 mg/L α-tocopherol, 0.5 mg/L β-carotene, and 200 mg BHT (as an antioxidant) was used for Experiment I. In Experiment II, the same combined standard was used, except retinol (2 mg/L) was added. Fat was extracted from samples such as milk, milk powder, and cream using a modified Rose-Gottlieb procedure (1), or samples underwent a saponification procedure (2) prior to HPLC separations.

HPLC procedure

Samples and standards were injected using the WISP autoinjector. The mobile phase flowrate was 1.5 mL/min, and the separations were carried out at ambient temperature. Vitamin A was detected using fluorescence (330 nm excitation and 470 nm emission) and UV (313 nm) detection. Vitamin E was detected using fluorescence at 295 nm excitation and 330 nm emission. The Waters 470 detector was programmed to detect vitamins A and E in the same chromatogram. β-Carotene was simultaneously detected at 450 nm using the Waters 490E detector, which was serially connected to the 470 detector. The Hitachi F1000 fluorescence detector was connected serially between the Waters 470 and 490E detectors to detect vitamin E in Experiment I.

Experiment IA: Reduction in fluorescence signal following normal degassing procedure

In the normal degassing procedure, the mobile phase was sparged with helium for about 10 min, and the stainless steel frit at the end of the helium line was then withdrawn to a position above the mobile phase to maintain a helium layer above the solvent. The helium outlet of the mobile phase reservoir was connected to a tube, the end of which was submerged in a water reservoir so that the speed of the gas flow could be observed. The helium flow was adjusted until there was a bubble approximately every 10 s. (It was later observed, however, that after a few hours, the helium flow rate gradually decreased and often ceased, possibly because of a fault in the adjustment valve.)

The normal degassing procedure was used while the mobile phase was continuously running through the column, and consecutive injections of 100-µL volumes of the combined standard solution were made at fixed time intervals up to about 9.3 h. The peak area value found on the first injection for each component was set to 100, and the values found on successive injections were ratioed to the corresponding peak area value of the first injection. This procedure was followed so that the response of all the mixture components could be compared on the same graph.

Experiment IB: Effect of helium sparging on the quantitation of vitamin A

Helium sparging was carried out for 10 min prior to commencement of HPLC in the usual manner. Following the initial sparge at approximately 5 L/min, the frit was left in the mobile phase rather than raised above it, and the helium flow rate was adjusted so that there were several (3–4) bubbles per second or approximately 100 mL/min. This was in excess of the flow rate used in normal practice (1 bubble every 10 s). Care was taken to adjust the helium flow so that this bubbling rate was maintained for the duration of the experiment.

Three consecutive 100-µL injections of the combined standard solution were made, and the responses were measured. The pump and the helium flow were then stopped, the mobile phase reservoir lid was opened, and the contents were left exposed to air for 20 min. Then the pump was restarted, and the mobile phase was passed through the column for another 20 min to allow the lines and the column to be flushed with the "air-exposed" mobile phase. Three further consecutive 100-µL injections of the combined standard solution were made, and the responses were measured. The response of vitamin A was measured with both UV and fluorescence detection.

Experiment IC: Effect of alternating helium and air sparges

Successive 100-µL volumes of combined standard were injected (22 injections in total) and the responses were measured using the fluorescence and UV detectors following the procedure described below.

The mobile phase was exposed to air overnight and was not degassed with helium prior to commencement of the HPLC run. Helium sparging commenced after 80 min using a rigorous gas flow (approximately 5 L/min). At 220 min, the helium flow was stopped, and air was blown through the mobile phase for 10 s to displace helium in the mobile phase. At 320 min, helium sparging was recommenced. The peak responses obtained for each injection (run time, approximately 19 min) were processed in the same way as in Experiment IA.

Experiment II: Effect of IPA–hexane mobile phase on retention time

Samples that were prepared using the saponification procedure and standard solutions containing retinyl palmitate, retinol, α-tocopherol, and β-carotene were injected consecutively onto an HPLC column and eluted with the mobile phase containing IPA prepared as described earlier.

To prevent rapid changes in the retention time, the helium was sparged through a flask containing hexane–IPA (78:22) before passing it through the mobile phase.

Results and Discussion

Experiment IA

Photo-decomposition of vitamins was unlikely because the samples were contained in brown glass vials, and the HPLC tubing was either covered with stainless steel jackets or made of stainless steel.

Figure 1 shows the change in peak responses obtained with the normal setup used for degassing the mobile phase, in which the helium flow rate was found to gradually decrease with time. The fluorescence response for vitamins A and E gradually decreased with time, whereas the UV response for vitamins A and β-carotene remained constant. The absorbance of vitamin E was too low to allow quantitative measurements with the UV detector. The fact that the vitamin E fluorescence signal decreased with time for both fluorescence detectors and that the UV signal for vitamin A remained constant suggests that this decrease was related to the mode of detection (fluorescence) rather than the detector itself.

Experiment IB

The fluorescence response was significantly reduced by approximately 50% when helium sparging was stopped. The UV signal was unaffected.

Experiment IC

The results of Experiment IC are shown in Figure 2. There was a marked increase in the fluorescence response following the commencement of helium sparging for both vitamins A and E. When a brief air sparge was given, there was a rapid decrease in the fluorescence responses. When helium sparging recommenced, the fluorescence response increased to its previous level. The time that elapsed between commencement of helium sparging and "full" fluorescence response was of the order of 40 min. The first change in fluorescence response was observed 10 min after sparging commenced, which was consistent with the lag time between the mobile phase reservoir and the detector.

The restoration of the fluorescence response for both vitamins A and E with adequate helium sparging eliminated the possibility of vitamin oxidation. The reversible nature of the fluorescence further confirmed that the problem was associated with the mobile phase degassing. The most likely cause for the reduction in the fluorescence signal was quenching of the fluorescence signal by oxygen.

Maintenance of a helium flow rate of at least 100 mL/min is adequate to prevent the problem with fluorescence quenching. Even with vigorous sparging at a flow rate of 5 L/min, the time required to totally expel oxygen was at least 30 min with 1.5 L of mobile phase in the reservoir. Depending on the seal of the mobile phase reservoir, it should be possible to reduce the helium flow rate to cut back on the flow rate maintained thereafter.

Experiment II

Consecutive injection of the saponified sample extract or the combined standard solution showed an increase in the retention times for all peaks (vitamins A, E, and β-carotene). For example, the retention time of retinol increased about 1.5 min per run, and the run time was 30 min. This suggested a reduction in the percentage of IPA in the mobile phase with time. In addition, a drastic reduction in the volume of the mobile phase (from 2 to 1.5 L) suggested rapid evaporation. Considering the above facts and IPA's considerably higher

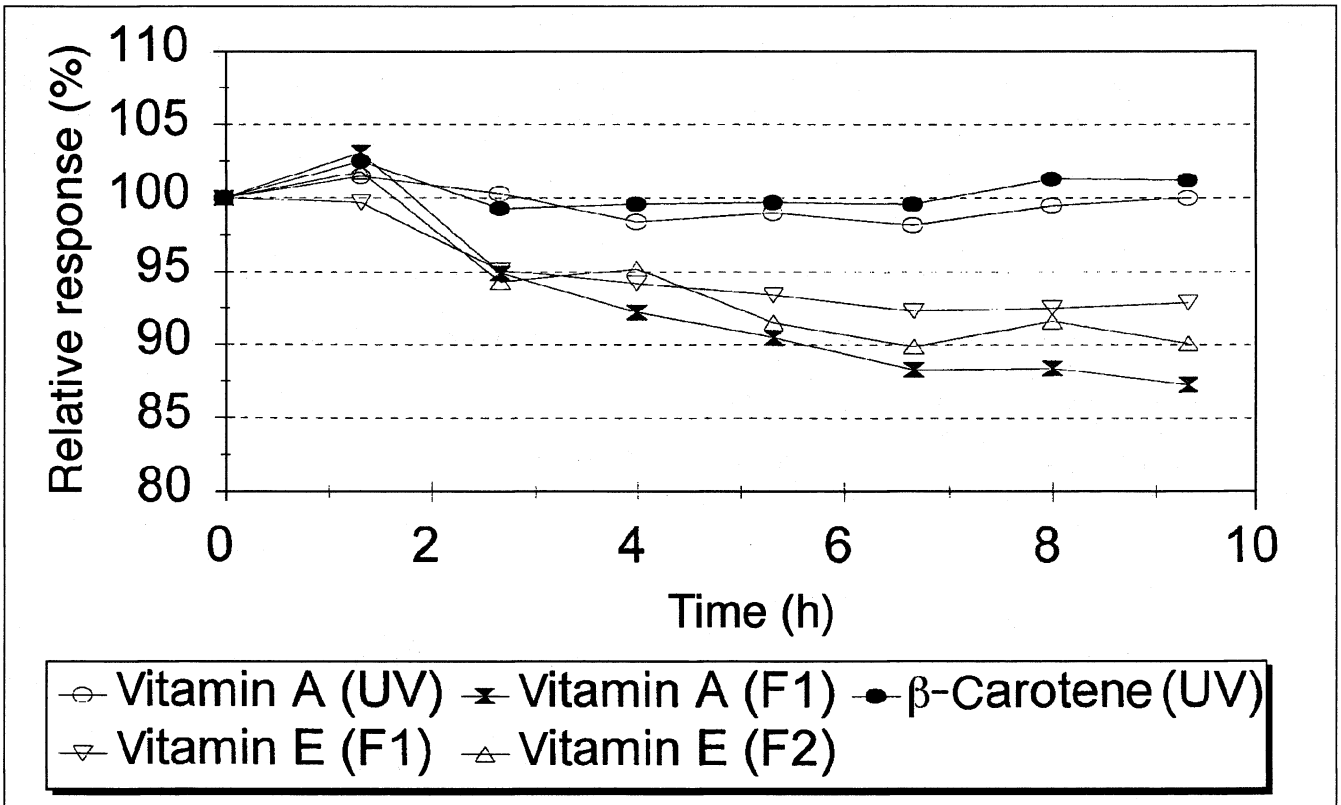

Figure 1. Variation of peak responses for vitamins A, E, and β-carotene with time when consecutive 100-µL injections of a standard solution containing the three vitamins were made. The chromatographic conditions used are in Experiment IA. Vitamin E was detected using a Waters 470 (F1) and a Hitachi F1000 (F2). Vitamin A was detected using a Waters 490 (UV) and a Waters 470 (F1). β-carotene was detected using a Waters 490 (UV).

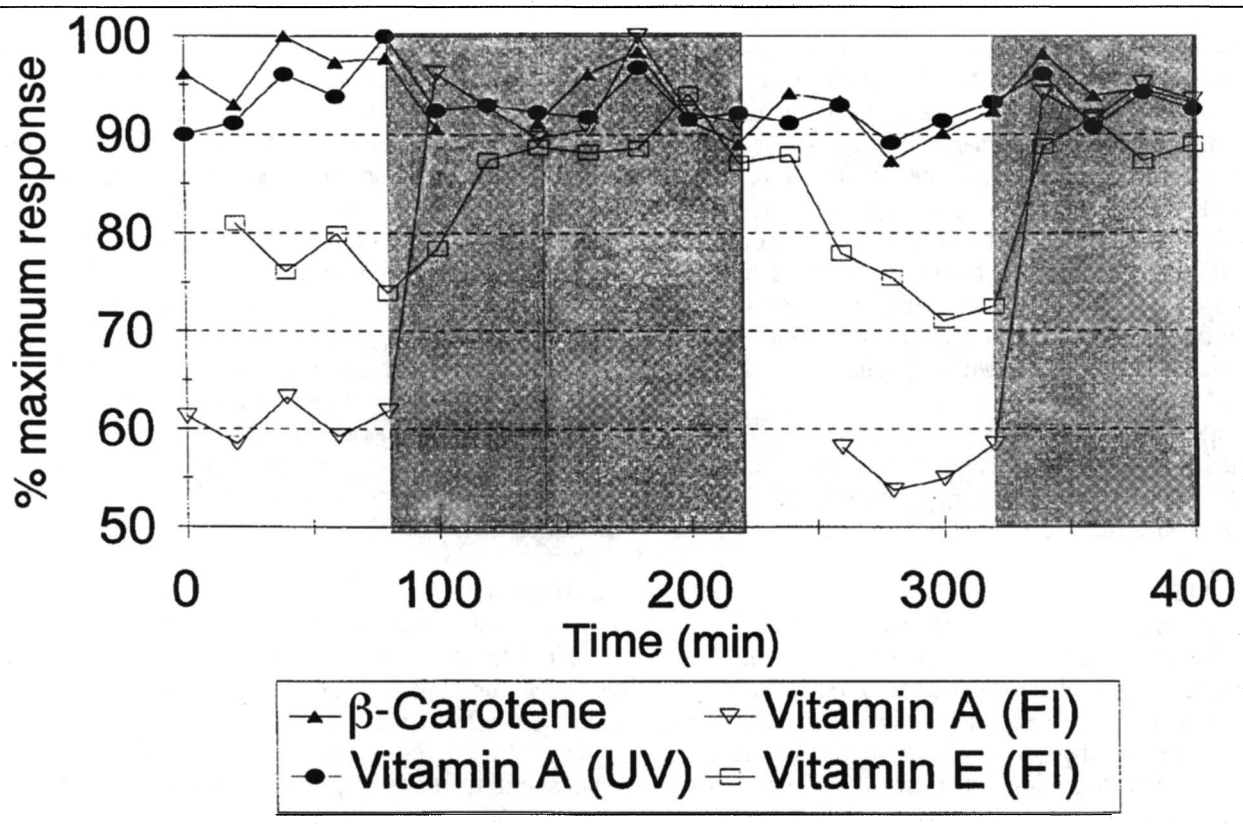

Figure 2. Effect of alternating between helium sparging and air sparging of the mobile phase on the peak responses of vitamins A, E, and β-carotene. The shaded area indicates when helium sparging was on, and the non-shaded area indicates when air sparging was on.

boiling point (82°C) than hexane's (69°C), the formation of an azeotrope between hexane and IPA was suspected. Hexane forms an azeotrope with IPA; the composition is 78% hexane and 22% IPA, and the boiling point is 63°C (3). Without azeotrope formation, hexane should be evaporated in preference to IPA, causing a decrease in retention time. Therefore, azeotrope formation explains the reduction in the solvent strength of the mobile phase due to preferential evaporation of IPA. All the above effects were reduced when the helium flow was shut down.

When helium was passed through a flask containing hexane–IPA before it reached the mobile phase, the helium was saturated with the azeotropic mixture of hexane and IPA; therefore, the IPA was no longer selectively removed from the mobile phase. With the introduction of the flask containing the azeotropic mixture, the retention times were stabilized but slowly decreased due to the slow introduction of IPA from the saturated helium flow to the mobile phase. However, this stability of retention time was sufficient for the work involving the switching of the detection wavelengths with time during each chromatographic run.

Conclusion

The effect of degassing the mobile phase on peak responses obtained with fluorescence detection is very important not only in the vitamin A and vitamin E analyses but also with any analysis involving fluorescence detection. It is recommended that the minimum helium flow rate required for the maintenance of a constant fluorescence signal be established for each analyte as well as each degassing system and that a flow rate above that level be maintained each time an analysis is carried out.

The increase in retention time with time when IPA is used as a modifier in hexane-based mobile phases is not entirely avoidable. However, as described here, a reduction in the effect can be achieved by modifying the HPLC system.

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