

High Throughput Separation of Xanthines by Rapid Resolution HPLC

Application Note

Biochemistry, Food and Beverage, Pharmaceutical

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from 8 minutes to 1.5 minutes without a major loss in resolution. A simple isocratic HPLC method was used and described to analyze theobromine, theophylline and caffeine in liquid refreshments (tea, chocolate syrup, and cocoa).

Abstract

Xanthines were found to be optimally separated by reversed-phase HPLC on a C18 column. By a reduction in column length and particle size, the separation time for a mixture of the xanthines investigated was reduced

Introduction

Xanthines are a group of alkaloids with the most common xanthine being caffeine, found in foods such as coffee beans, tea, kola nuts, and in small amounts in cacao beans. The chemical structures of these xanthines and some of their metabolites are depicted in Figure 1.

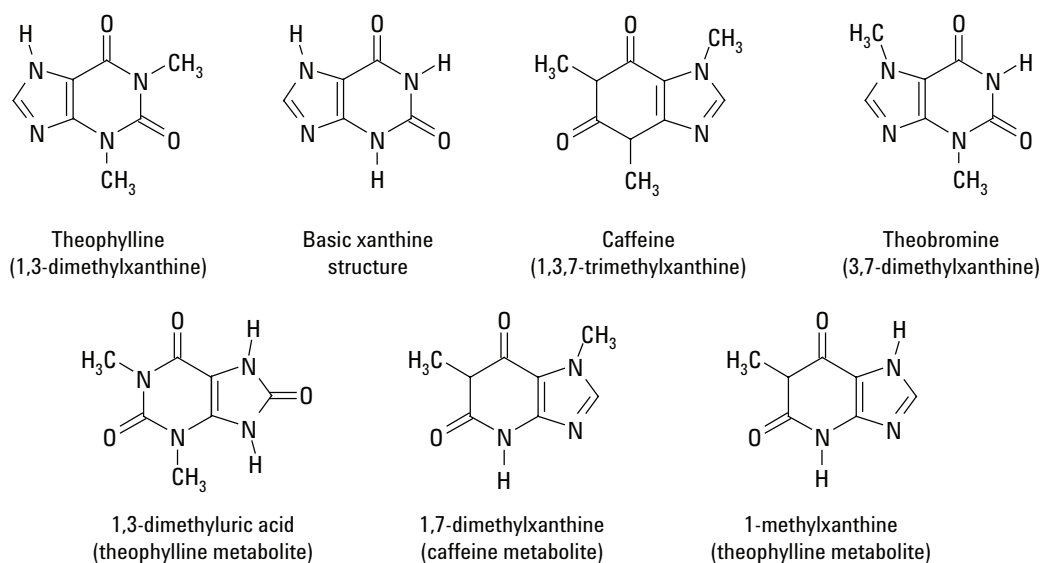


Figure 1. Structures of selected xanthines and metabolites used in this study.



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Xanthines are most often separated by reversed-phase HPLC (RP-HPLC) on a C18 column [1–3]. Although ion pair chromatography has been used for xanthine separation [2], RP-HPLC with buffered water and acetonitrile requires a much simpler mobile phase system. This application note will show how different stationary phases may impart different selectivities for xanthine separations and will also investigate the effect of particle size and column length on the separation speed. Finally, it will show an application of a method for the analysis of caffeine and theobromine in chocolate-based drinks.

Selection of the Stationary Phase for the Separation of Xanthines

HPLC allows for the resolution of peaks of interest in the shortest possible time. Selection of the appropriate stationary phase is an important step in analytical method

development. Initially, several different stationary phases were tried in order to choose an appropriate one for these investigations. Figure 2 shows the separation of the xanthine components in the test mixture using ZORBAX StableBond phases [cyano (CN), phenyl, C18] and a polar embedded stationary phase (ZORBAX Bonus RP).

Although the particle size was different for the Bonus column, our objective here was to choose the phase with the best selectivity and the shortest retention time. Under the conditions employed, the CN column gave least retention (by virtue of its shorter alkyl chain length), but the column failed to resolve two of the test xanthines. The SB-C18 column gave the best overall separation in the shortest time and thus became the stationary phase of choice.

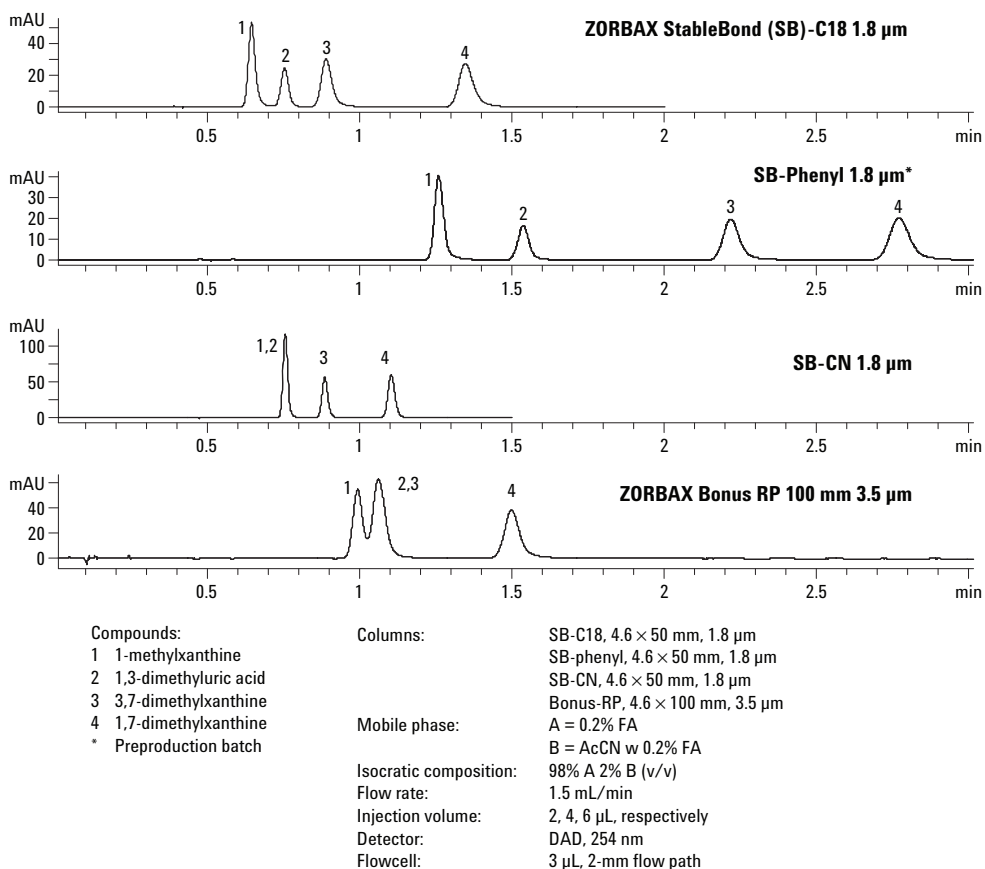


Figure 2. ZORBAX stationary phase selectivity comparisons for xanthines.

The chromatographic conditions chosen for subsequent experiments appear below:

LC Conditions

Column: ZORBAX SB-C18 (various lengths and particle diameters shown on chromatograms),
 Mobile phases: A= 0.2% Formic acid (FA)
 B=Acetonitrile with 0.2% FA
 Isocratic composition: 98% A 2% B (v/v)
 Flow rate: 1.5 mL/min; Injection volumes are shown on chromatograms
 Detection: DAD, 254 nm
 Flowcell: 3 μ L, 2-mm flow path

The Effect of Particle Size and Column Length on the Separation of Xanthines

Recent trends in HPLC have pointed to the use of shorter columns with smaller particles. The end result is a faster separation with the same or similar resolution. Figure 3 depicts the isocratic separation of the xanthine test mixture on three different columns (250 mm, 100 mm, and 50 mm) packed with three different particle sizes of ZORBAX StableBond C18 (5 μ m, 3.5 μ m, 1.8 μ m, respectively). As the column length decreases, one would expect to see shorter retention times, proportional to the decrease in length. Indeed Figure 3 clearly shows a decrease in overall separation time from 8 minutes to 1.5 minutes.

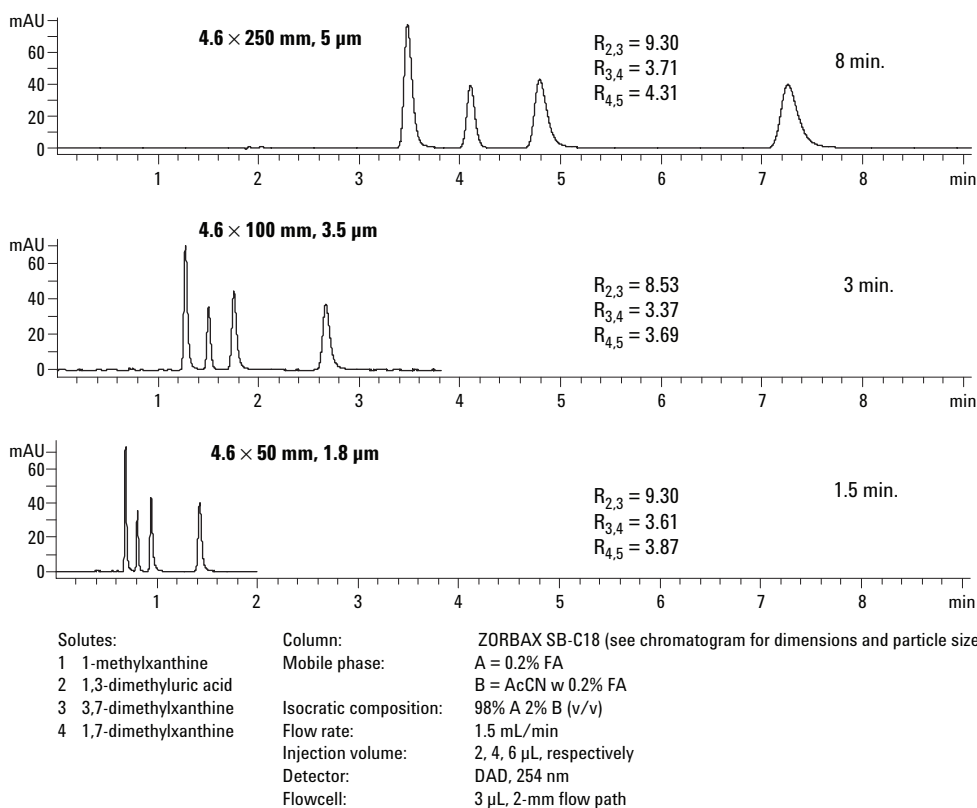


Figure 3. Column scalability: change in column configuration to increase speed while maintaining resolution.

On the other hand, one would also expect to see a reduction in column efficiency. However, by reducing the particle size, the overall efficiency and resolution is nearly the same. The calculated resolution for all pairs of xanthenes is shown on Figure 3. This is the rapid resolution concept where a combination of shorter columns and smaller particles led to equivalent separations at greatly reduced separation time. Since the flow rate is the same, in this case 1.5-mL/min, the solvent use is decreased proportional to column length resulting in an overall cost reduction. Another advantage when converting to shorter columns is that the peaks are narrower. Thus, if the same sample mass is injected the resulting increase in peak height provides greater sensitivity. In Figure 3, the sample volume was reduced proportional to column length to keep peaks nearly the same peak height.

Of course, as one decreases the particle size of a column, the column backpressure increases with the inverse square of the particle diameter. Thus, if the same column length was used, the pressure at the same flow rate (or more correctly the linear velocity), the pressure would go up by a factor of 2 for a 3.5- μm particle versus a 5.0- μm particle and a factor of almost 8 for a 1.8- μm particle. However, with the increase in plate count for the smaller

particles, columns can be shortened and the actual pressure increase is more nominal as can be seen in Table 1. Agilent's engineered particle size distribution helps to keep the pressure lower than what one would anticipate for a 1.8- μm column.

Table 1. Pressure as a Function of Particle Diameter and Column Length*

Particle diameter, μm	Column length, mm	Pressure, bar	Pressure increase (relative to 5.0)
5	250	181	1.0
3.5	100	155	0.86
1.8	50	264	1.46

* Conditions of Figure 3

In order to demonstrate that a change in the particle size of the column packing has a minimal effect on selectivity, the isocratic separation of the xanthine test mix as a function of particle size at constant column length was investigated. Figure 4 shows a minimal variation in retention but a significant decrease in peak width in going from the 5- μm column to the 1.8- μm column. In other words, the column showed more efficiency and subsequent better resolution for the 1.8- μm column but the selectivity was mostly unaffected. The HPLC conditions are shown on the chromatogram in Figure 4.

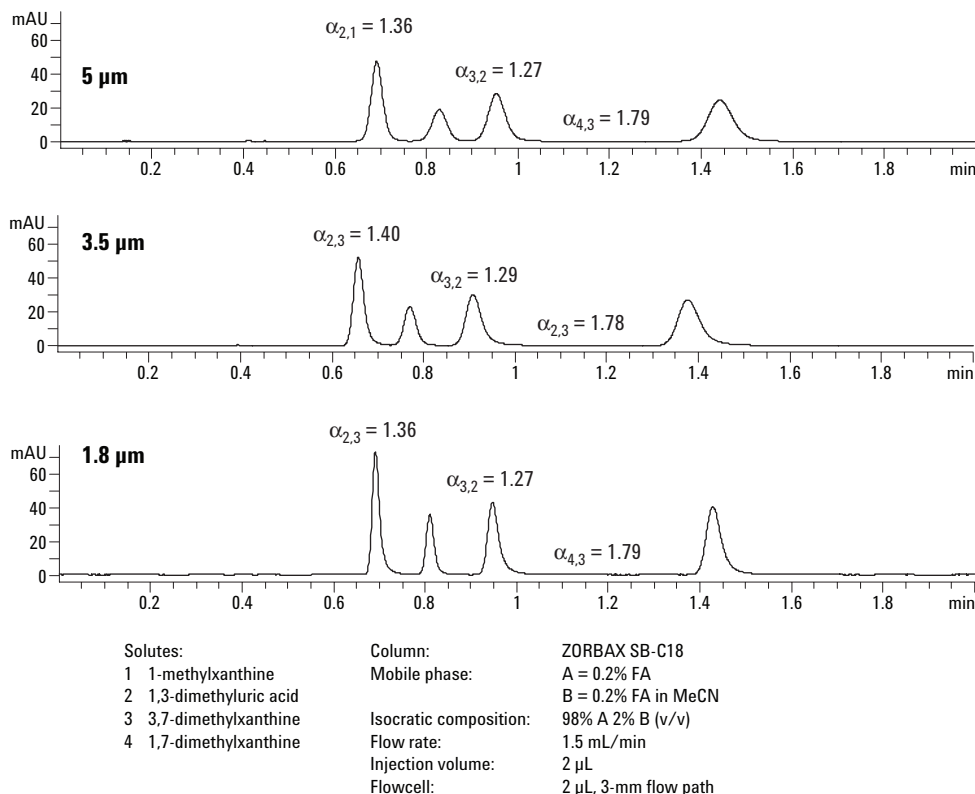


Figure 4. Column selectivity as a function of particle size.

Analysis of Xanthines in Liquid Refreshments

The three most common xanthines are caffeine, theophylline, and theobromine. We developed a simple isocratic method to analyze for them in chocolate drink and tea. Using the same chromatographic conditions described earlier; an excellent separation of a standard xanthine mixture was achieved. See Figure 5.

Next, three different liquid refreshments-hot cocoa, chocolate syrup, and black tea (bag) were prepared using directions on the container, but using sonication for mixing. After preparation, all solutions were centrifuged and then the aqueous portion was filtered through a 0.45-micron filter to remove any particulates that may foul the HPLC column. In particular, the hot cocoa gave a distinctive fat layer, but only the aqueous layer was sampled for analysis. For each sample, a 3- μ L injection of the aqueous extract was made. See results in Figure 6.

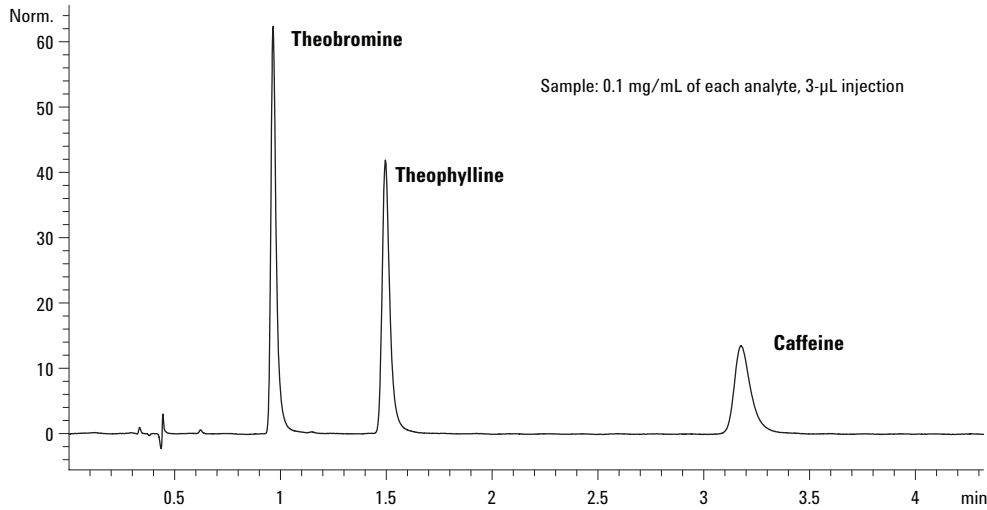


Figure 5. Separation of xanthine standards.

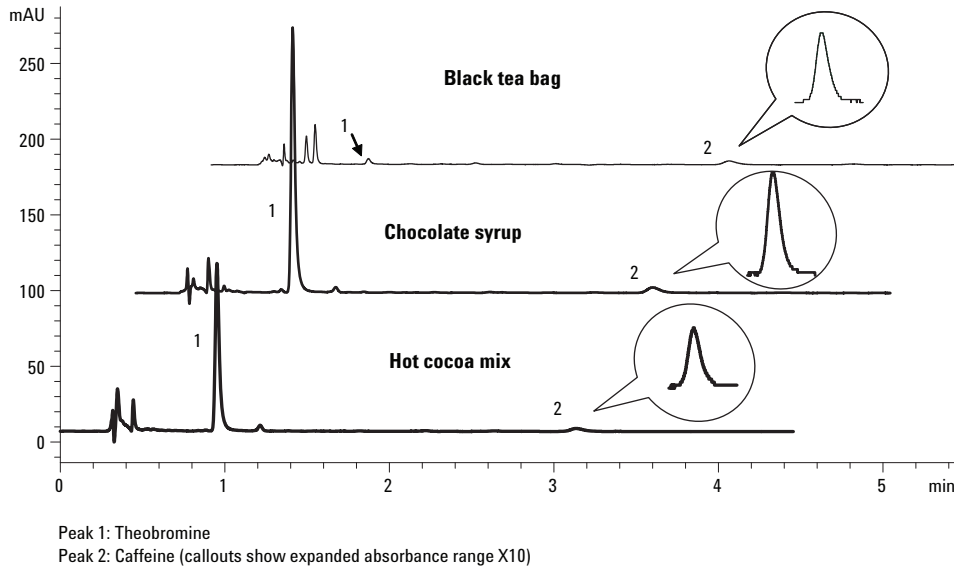


Figure 6. Analysis of liquid refreshments for xanthines.

From the raw areas we were able to do a semi-quantitative analysis (single-point calibration) of the three xanthines in the drinks. As can be seen from Table 2, in the chocolate drinks, relatively large levels of theobromine were observed but smaller amounts of caffeine while for the tea sample, caffeine was in an excess. The results of Table 2 were based on a weight/weight basis and not on total milligrams in the drink solution itself. These results are within the concentrations expected based on the manufacturer's approximations. No theophylline was observed in any of the drinks.

Table 2. Determination of Xanthines in Liquid Refreshments

Beverage	Theobromine (%, wt/wt)	Caffeine (%, wt/wt)
Hot chocolate	0.15	0.011
Chocolate syrup	0.13	0.011
Tea	0.056	0.17

Conclusions

Xanthines were found to be optimally separated by reversed-phase HPLC on a C18 column. By a reduction in column length and particle size, the separation time for a mixture of the xanthines investigated was reduced from 8 minutes to 1.5 minutes without a major loss in resolution. A simple isocratic HPLC method was used to analyze theobromine, theophylline and caffeine in liquid refreshments (tea, chocolate syrup, and cocoa).

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