APPLICATION NOTE
#Appn_SS011-0

SiliCycle

SiliaSep™

Separation of Caffeine and Other Natural Stimulants Using Reversed-Phase C18 SiliaSep Flash Cartridges

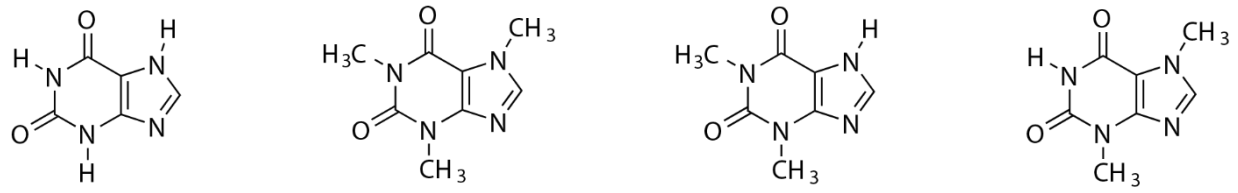
Xanthine derivatives are found in many food products because of their natural occurrence in tea leaves, cocoa beans, and coffee beans, to name a few. Caffeine, theobromine, and theophylline are methylated xanthines with similar chemical compositions and chemistries, making them a challenge to separate by chromatography. This Application Note aims to separate these compounds by using reversed-phase C18 SiliaSep flash cartridges.

LEARN MORE about SiliaSep in our brochure "*Solutions For Purification & Chromatography*".

Methyl xanthines are known for their physiological effects, the most notorious being the stimulant effect of caffeine. To a lesser extent, theophylline and theobromine are also well-known methyl xanthines with stimulant effects. Other alkylated xanthines (*either natural or synthetic*) are being explored for their health-related applications.

Xanthines are weak bases composed of an imidazole ring coupled with a pyrimidine ring. Caffeine is methylated on the 1, 3 and 7 positions, theophylline on the 1 and 3 positions and theobromine on the 3 and 7 positions. The similar chemical structures make these compounds challenging to separate by chromatography. The structures of the four natural stimulants are shown in Figure 1.

Figure 1: Compounds in the sample mixture



Xanthine Caffeine Theophylline Theobromine

Comparison Between SiliCycle's Silia*Sep* PREMIUM and Irregular Cartridges

Reversed-phase chromatography was used to separate a mixture of these compounds on C18 Silia*Sep* flash cartridges. SiliCycle's R&D team compared irregular and spherical silica gel. The sorbents' characteristics are presented in Table 1. Tests were performed with 40 g cartridges and then scaled up with 120 g cartridges.

Table 1: Comparison between irregular and spherical flash cartridges

Comparison of Silica Gels			
	Particle Shape	Particle Size (*µm*)	Pore Diameter (*Å*)
Silia*Sep* **PREMIUM**	Spherical	25	90
Silia*Sep*	Irregular	40 - 63	60

When using spherical rather than irregular sorbent, it is possible to decrease the particle size without affecting the back pressure, thus increasing resolution, separation efficiency, and loading capacity.

The sample was introduced by liquid loading. Larger loadings were not possible because of the poor solubility of the analytes in DMSO. Other injection solvents, namely water, acetic acid, acetonitrile, methanol, and DMF, were tested, but none offered enough solubility to increase the sample load.

Acetic acid and trifluoroacetic acid were both tested as acidic additives to the mobile phase. Acetic acid offered the best separation at a pH of approximately 4.5. Different gradients of acidified water and methanol were tested, and the best conditions are shown in Table 2 for the 40 g and the 120 g cartridges.

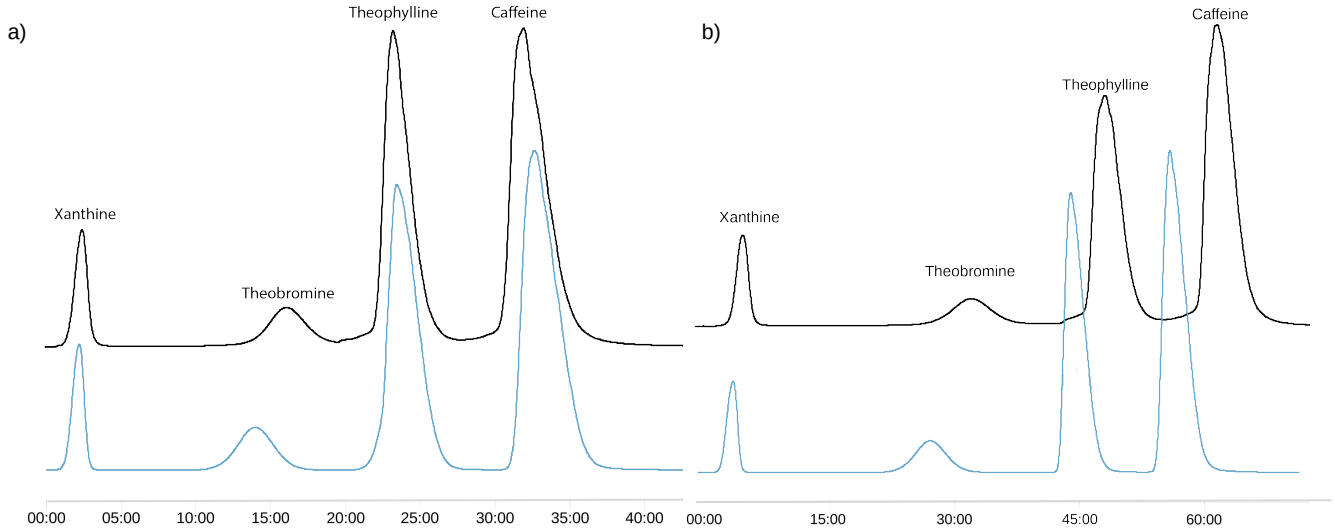
The method needed optimization while scaling up. Namely, a step was added to the gradient to offer similar separation efficiency with the larger cartridge.

Table 2: Chromatographic conditions

Chromatographic Conditions	
Parameter	**Value**
CARTRIDGE	#1: Silia*Sep* PREMIUM Flash Cartridge (*spherical silica*), C18, Monomeric, 25 µm, 90 Å
	#1: Silia*Sep* Flash Cartridge (*irregular silica*), C18, Monomeric, 40 - 63 µm, 60 Å
PART NUMBER	#1: FLH-03295D-A-ISO40 (*40 g*)
	FLH-03295D-A-ISO120 (*120 g*)
	#2: FLH-R33230B-ISO40 (*40 g*)
	FLH-R33230B-ISO120 (*120 g*)
GRADIENT	Mobile phase A: water/methanol (*95/5*) + 0.0125 % acetic acid
	Mobile phase B: water/methanol (*5/95*) + 0.0125 % acetic acid
	40 g cartridges 1. 0 % B (*22 CV*) 2. 5 % B to 20 % B (*24 CV*) 120 g cartridges 1. 0 % B (17 *CV*) 2. 5 % B to 20 % B (*12 CV*) 3. 20 % B (*2 CV*)
TEMPERATURE	25°C
FLOW RATE	50 mL/min for 40 g cartridges
	80 mL/min for 120 g cartridges
DETECTOR	UV at 270 nm
INJECTION	Liquid load of a solution of mixture in DMSO (*0.1 % sample load*)

Abbreviation used: CV = Column Volume

Figure 2: Separation of xanthine derivatives with Silia*Sep* C18 flash cartridges a) 40 g cartridges, irregular (*blue*) and PREMIUM (*black*); b) 120 g cartridges, irregular (*blue*) and PREMIUM (*black*)



The chromatograms comparing spherical and irregular sorbents are presented in Figure 2 for the 40 g and the 120 g cartridges. For the larger scale, the retention times were longer with the spherical sorbent, creating more distance between the peaks. Overall, both the spherical and irregular sorbents separated the compounds efficiently.

Comparison of Different Brands of Spherical Silica Gel

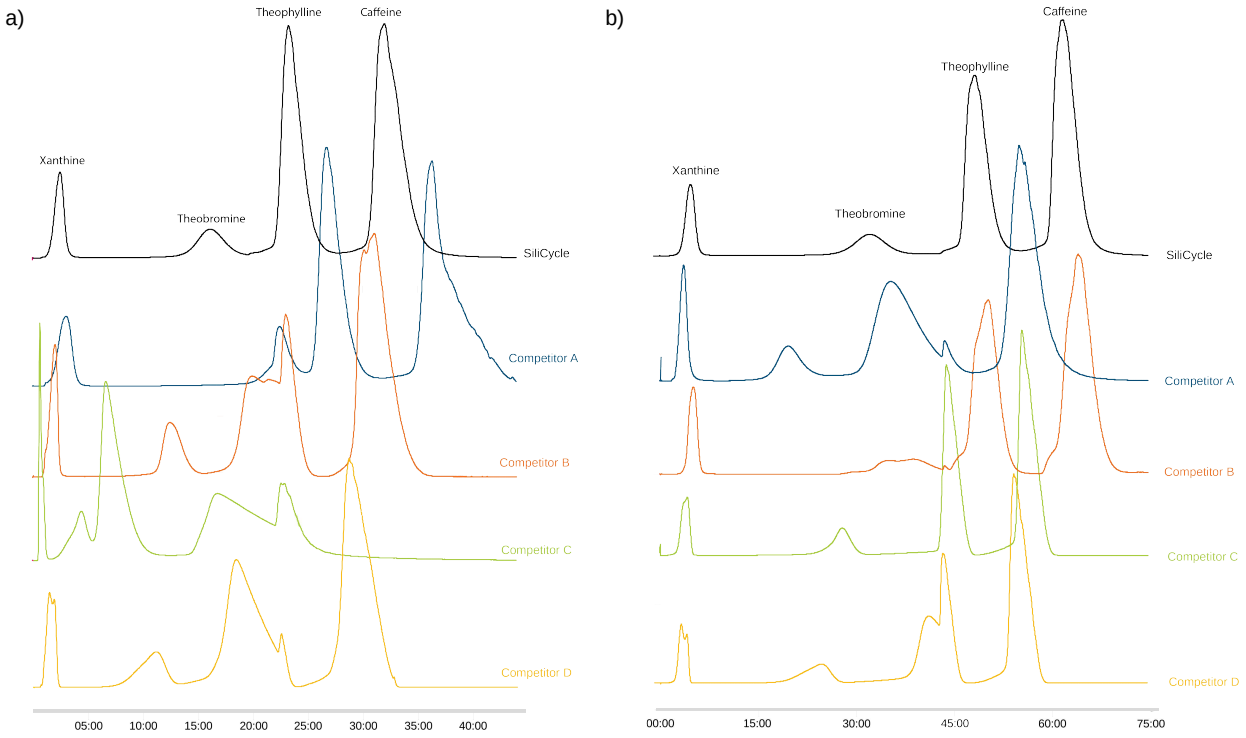
Spherical gel was chosen as the basis to compare the cartridges from different manufacturers. The sorbents characteristics for all cartridges tested can be found in Table 3. The chromatographic conditions were optimized for SiliCycle's cartridges and transposed directly on other cartridges without further optimization.

Table 3: Comparison of silica gels

Comparison of Silica Gels		
	Particle Size (*µm*)	Pore Diameter (*Å*)
Silia*Sep* PREMIUM	25	90
Competitor A	20 - 35	100
Competitor B	30	100
Competitor C	30	100
Competitor D	20 - 40	100

Injecting the compounds separately enabled the identification of the peaks as follows: first xanthine, the second and third peaks are theobromine and theophylline respectively, while caffeine is last. Caffeine is the least polar of the methylxanthine with the largest number of methyl chains on the backbone, hence its exit from the column last was expected. The chromatograms are shown in Figure 3 for the separation of the mixture using the different manufacturers' spherical flash cartridges.

Figure 3: Separation of xanthine derivatives with C18 spherical flash cartridges: a) 40 g cartridges; b) 120 g cartridges.



Using the 40 g cartridges, competitors B, C, and D produced shorter retention times, bringing the peaks closer together with lower resolution. Competitors B and D also showed peak broadening and splitting of the third peak (*i.e., theophylline*), while this effect is observed on the fourth (*caffeine*) peak on competitor C's chromatogram. Competitor A had sharper peaks than the other competitors, but with significant tailing on the last peak, making the analysis time increase. SiliCycle's Silia*Sep* PREMIUM resulted in the best separation with all peaks being sharper.

It must be noted that the run conditions were optimized on SiliCycle's Silia*Sep* PREMIUM cartridges and not individually on each of the competitors' cartridges. The results obtained using this protocol could be different with other conditions. With this in mind, conditions should not be replicated when changing supplier without taking the time to re-optimize the protocol.

All competitors showed improved resolution on the larger 120 g cartridges with sharper and more spaced-out peaks. The analysis time increased for the larger scale.

Conclusion

In conclusion, spherical C18 silica gel was used to successfully separate caffeine and other naturally occurring xanthine derivatives from tea leaves, a challenging mixture with similar chemical compositions. SiliCycle's cartridges were compared with its competitors on the 40 g and 120 g scales. The conditions were optimized on SiliCycle's cartridges and directly transposed on the other cartridges. Silia*Sep* PREMIUM resulted in the best resolution for both the 40 g and the 120 g cartridges.

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