High Throughput Analysis of Caffeine and Other Food and Beverage Additives Present in Popular Soft Drinks using High Performance Reversed Phase HPLC

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Introduction

- Reversed phase liquid chromatography (RPC) is the analytical technique most widely used in the R&D and QC departments of the food and beverage industry.
- Many popular soft drinks have a high percentage of caffeine as a stimulant.
- The Food and Drug Administration does not regulate caffeine content of soft drinks, although the agency does require that the presence of caffeine is disclosed on labeling for energy drinks and cold coffee beverages.
- Popular soft drinks also contain a number of other chemicals as additives, such as: citric acid, acesulfame K, saccharin, DL-phenylalanine, sodium benzoate, potassium sorbate and aspartame.
- Saccharin is also used in artificial sweeteners such as Sweet'N Low
- In this era of high throughput analysis, the need to obtain shorter retention times while maintaining or improving resolution is very important for quality control analysis.
- The availability of a high efficiency column that can separate many ingredients varying in hydrophobicity is also useful for method development and formulation purposes.

Introduction Continued

- We used three different reversed phase columns to quantitate the caffeine and other additives found in soft drinks: one column packed with 2.3µm particles and two columns packed with very inert silicas.
- Eight chemicals covering a wide range of hydrophobicities which are used as additives in popular soft drinks were used in this study.
- We report the separation of caffeine and other chemicals used as additives in food and beverages found in popular soft drinks using a TSKgel ODS-140HTP column.
- A comparative analysis of soft drinks ingredients using two other reversed phase C18 columns, a TSKgel ODS-100V and a TSKgel ODS-100Z column, was included.
- Studies using a competitive column, forced degradation of caffeine, determination of limit of detection and of quantitation, calibration curve, etc. are also reported.
- High throughput separation of caffeine and other chemicals used in food and beverages as additives using a conventional HPLC with an upper pressure limit of 41.4MPa is valuable in method development for new formulations and for the identification and quantitation of ingredients.

Materials and Methods: Chromatographic Conditions

LC System:	HP-1100 HPLC with Chemstation (ver B.03.01)	
Columns:	TSKgel ODS-140HTP, 2.3µm, 2.1mm ID x 5cm TSKgel ODS-100V, 5µm, 4.6mm ID x 15cm TSKgel ODS-100Z, 5µm, 4.6mm ID x 15cm	
Mobile Phase:	Gradient –	A: 1% ACN in H_2O with 0.15% TFA B: 30% ACN in H_2O with 0.15% TFA
	Isocratic –	as noted
Temperature:	50°C, unless otherwise noted	
Injection volume:	10µL	
Detection:	UV@275nm except for aspartame @ 215nm	
Flow rate:	1.5mL/min (Gradient), 1.0mL/min (Isocratic) unless otherwise noted	

Material and Methods

- High purity Sigma-Aldrich brand chemical was used for the preparation of a standard solution of caffeine USP (1.427mg/mL). Working standards were prepared by dilution of the standard solution of caffeine USP in water and used to generate the calibration curve.
- High purity Sigma-Aldrich brand chemicals were also used for the preparation of stock standards of citric acid, acesulfame K, saccharin, DL-phenylalanine, sodium benzoate, potassium sorbate, and aspartame.
- Eleven commercially available soft drinks were used in the study: Red Bull®, Mountain Dew®, Diet Coke®, Pepsi®, Diet Pepsi®, Coca-Cola Classic®, Wawa MachW, Monster KHAOS Energy Juice®, NESTEA®, Seagram's® Ginger Ale, Sprite Zero®.
- Samples of each soft drink were prepared by 10 fold dilution in mobile phase A.
- All the standards and the samples were filtered thorough a 0.45µm membrane filter prior to injection.

Material and Methods Continued

- The limit of detection (LOD) is one of the limit tests defined as the lowest concentration of the analyte in a sample that can be detected, but not necessarily be quantitated, under the stated experimental conditions mentioned.
- LOD is determined by the following USP method:
    - The standard deviation of the response (mAU) of the baseline is calculated during a blank injection.
    - The standard deviation is multiplied by a factor of 2 to provide an estimate of the limit of detection (mAU).
    - The limit of detection is subsequently confirmed by the analysis of the sample near that calculated limit.
    - For the determination of the limit of quantitation (LOQ), the standard deviation of the blank injection is multiplied by a factor of 10.

Properties of TSK-GEL ODS-140HTP, ODS-100V and ODS-100Z Columns

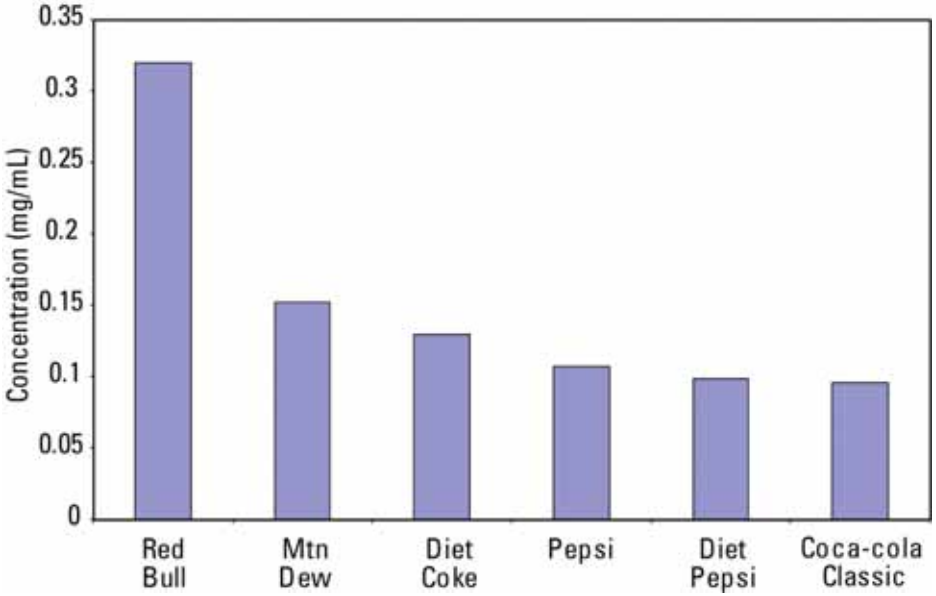
	TSKgel ODS-140HTP	TSKgel ODS-100V	TSKgel ODS-100Z
Carbon Content	8%	15%	20%
Endcapped	Yes[2]	Yes[1]	Yes[2]
Particle Size (µm)	2.3	3 and 5	5
Pore Size (Å)	140	100	100
Preferred Sample Type	Hydrophobic	Polar	Hydrophobic
Bonded Phase Structure	Polymeric	Monolayer	Monolayer
Specific Surface Area (m^2/g)	-	450	450
*Asymmetry Factor (10%)	0.90 - 1.3	0.90 - 1.15	0.90 - 1.15
*Theoretical Plates	>14,000	>14,000	>14,000

* Specifications for 4.6mm ID x 15cm columns packed with 5µm particles. Conditions: 70% methanol, 30% water; Flow rate: 1mL/min; Temp.: 40°C, N (Theoretical Plates) and As (Assymmetry Factor) are based on naphthalene peak. Typical pressure: 6MPa

[1] Prepared by an incomplete first reaction with a difunctional octadecylsilane reagent, which is followed by endcapping with a mixture of two difunctional dialkylsilane reagents.

[2] Prepared by bonding the surface with a difunctional octadecylsilane reagent, followed by repeated endcapping with monofunctional trimethylsilane reagent.

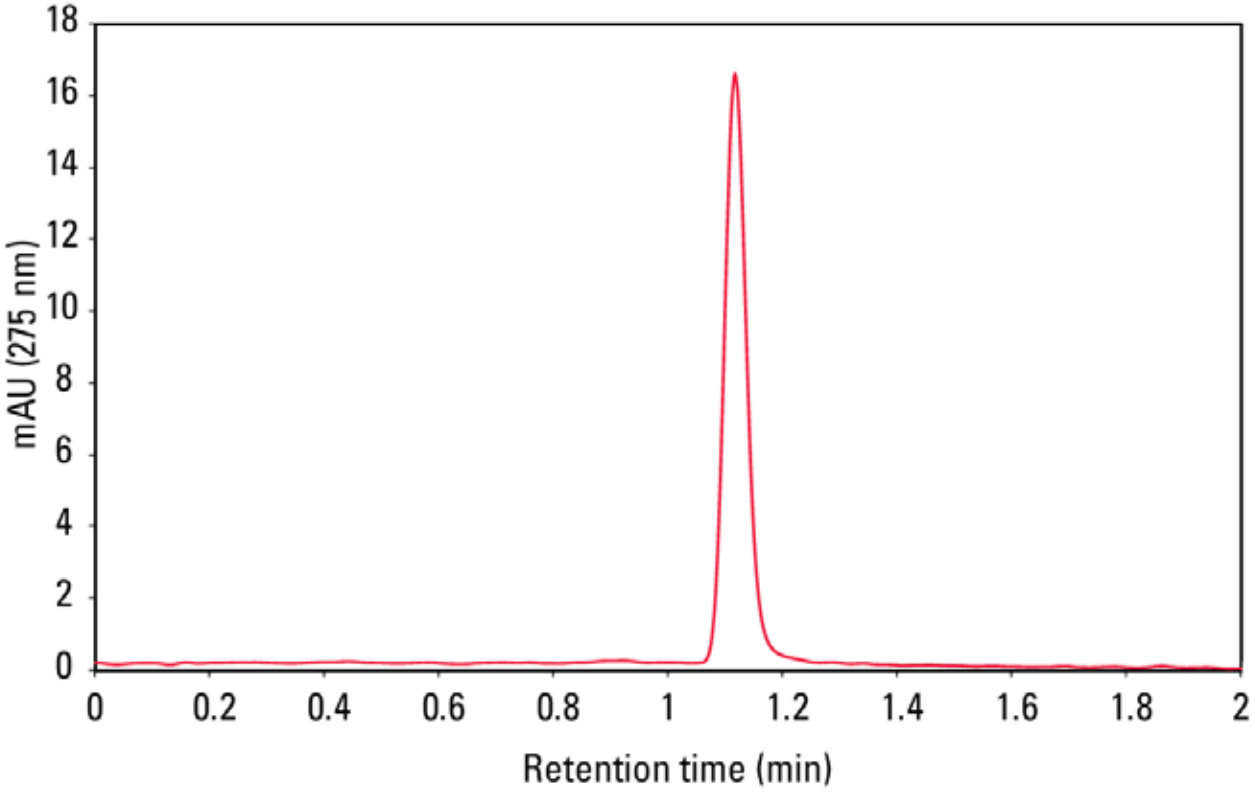
Figure 1: Soft Drinks Label Claims



Soft drink	Label claim
Red Bull	80 mg in 8.4 fl oz (250mL)
Mtn Dew	54 mg in 12 fl oz (355 mL)
Diet Coke	46 in 12 fl oz (355mL)
Pepsi	38 in 12 fl oz (355mL)
Diet Pepsi	35 in 12 fl oz (355mL)
Coca-cola Classic	34 in 12 fl oz (355mL)
Wawa MachW**	present but no label
Monster KHAOS energy juice**	present but no label
Nestea (Iced tea)	present but no label
Ginger Ale	labeled as "No Caffeine"
Sprite Zero	labeled as "No Caffeine"
**product of Canada	

- **Soft drinks Red Bull, Mountain Dew, Diet Coke, Pepsi, Diet Pepsi, Coca-Cola Classic, Wawa MachW, Monster KHAOS Energy Juice, NESTEA, Seagram's Ginger Ale, and Sprite Zero were analyzed for the presence of caffeine and other additives.**
- **Red Bull contains the maximum amount of caffeine compared to the other soft drinks studied in this report.**
- **Wawa MachW and Monster KHAOS energy juice (both products of Canada) did not have any label claim of the caffeine content although caffeine was present. NESTEA also contained caffeine, possibly from the tea extract, without any label claim on it.**
- **Seagram's Ginger Ale and Sprite Zero are caffeine free soft drinks and were used as negative controls.**

Figure 2: Analysis of Caffeine USP using a TSKgel ODS-140HTP, 2.3µm, 2.1mm ID x 5cm Column

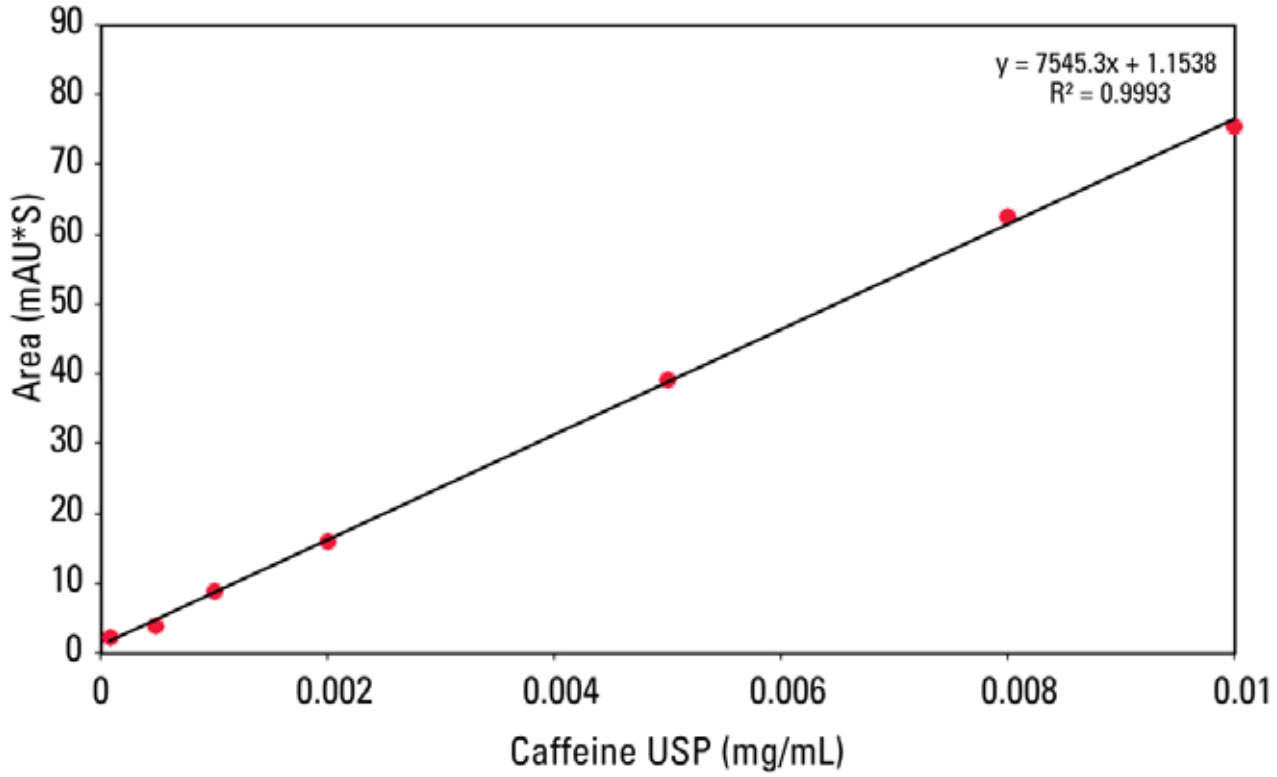


Caffeine USP standard eluted at 1.11 minute as a sharp peak with a USP tailing factor of 1.1 using a linear gradient in the method.

Calibration Curve and System Suitability Test – Caffeine USP

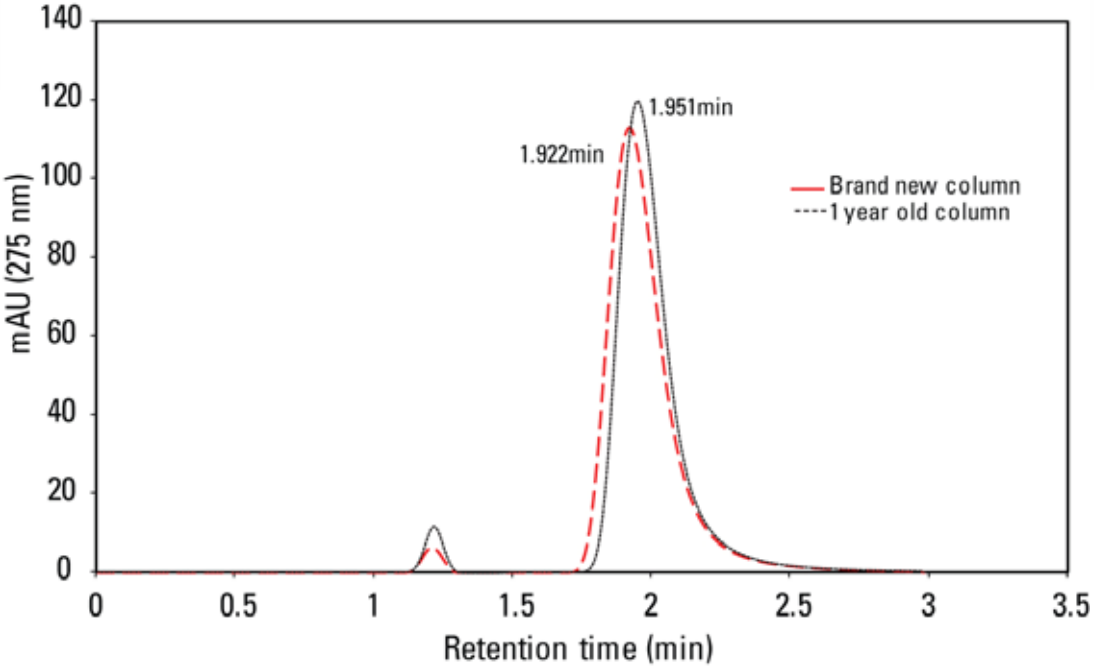
- A calibration curve was generated from a set of seven caffeine USP working standards bracketed between standard solutions 1 and 2.
- Standard solutions 1 and 2 were made separately from the solid powder of caffeine USP by weighing.
- Standard 1 was run at the beginning and end of the test to check the system suitability of the described method.
- The individual %RSD values for each set of 5 runs were calculated.
- The low %RSD values (~0.25 – 1.5) established the precision of the analysis method.

Figure 3: Calibration Curve of Caffeine USP using a TSKgel ODS-140HTP, 2.3µm, 2.1mm ID x 5 cm Column



The regression analysis of calibration curve yielded a very good correlation coefficient.

Figure 4: Isocratic Elution of Caffeine USP and Test of Column Durability using a TSKgel ODS-140HTP, 2.3µm, 2.1mm ID x 5cm Column



Isocratic conditions:

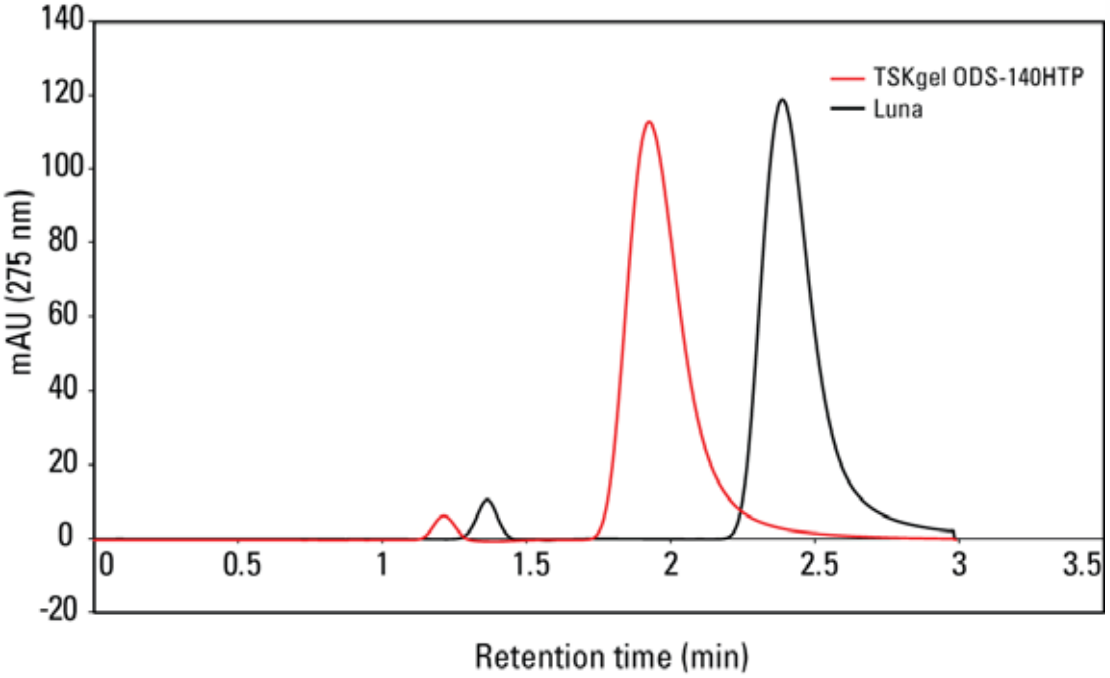
10% ACN in H_2O containing 0.15% TFA

Flow rate: **0.2mL/min**

Temperature: **40˚C**

- **Even under isocratic conditions, caffeine eluted within 2 minutes.**
- **No significant difference in elution profiles were noted between a brand new column and a column that was one year old. Caffeine eluted at 1.922 minutes from a brand new column, while a column used frequently for over a year yielded a retention time of 1.951 minutes.**
- **No significant change was observed in day to day reproducibility (data not shown).**

Figure 5: Analysis of Caffeine USP using a TSKgel ODS-140HTP, 2.3µm, 2.1mm ID x 5cm and a Competitive Column



TSKgel ODS-140HTP, 2.3µm, 2.1mm ID x 5cm

Luna C18(2)-HST, 2.5µm, 2.1mm ID x 5cm (Phenomenex)

Isocratic conditions:

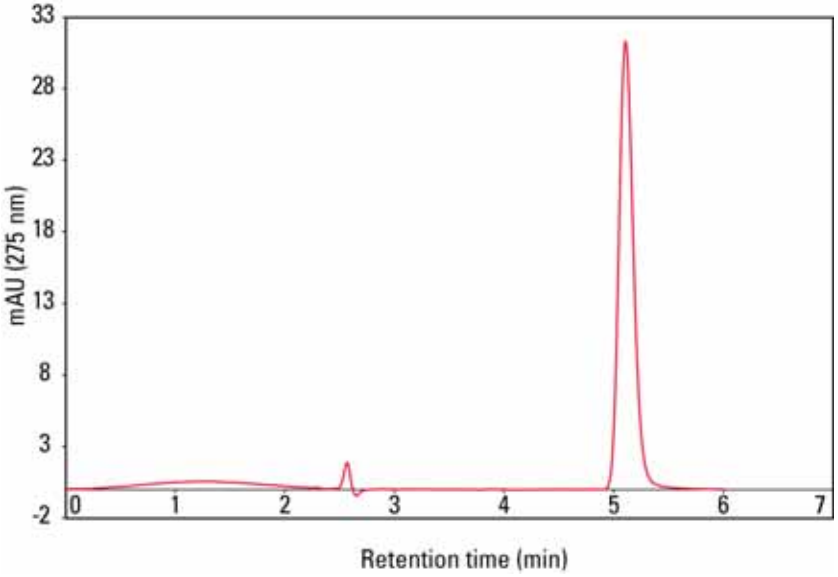
10% ACN in H_2O containing 0.15% TFA

Flow rate: 0.2mL/min

Temperature: 40˚C

The TSKgel ODS-140HTP column delivered a faster caffeine analysis (elution <2min) compared to a competitive column (elution >2.4 min).

Figure 6: Analysis of Caffeine USP using a TSKgel ODS-100V, 5µm, 4.6mm ID x 15cm Column



Isocratic conditions:

15% ACN in H_2O containing 0.15% TFA

Flow rate: 1.0mL/min

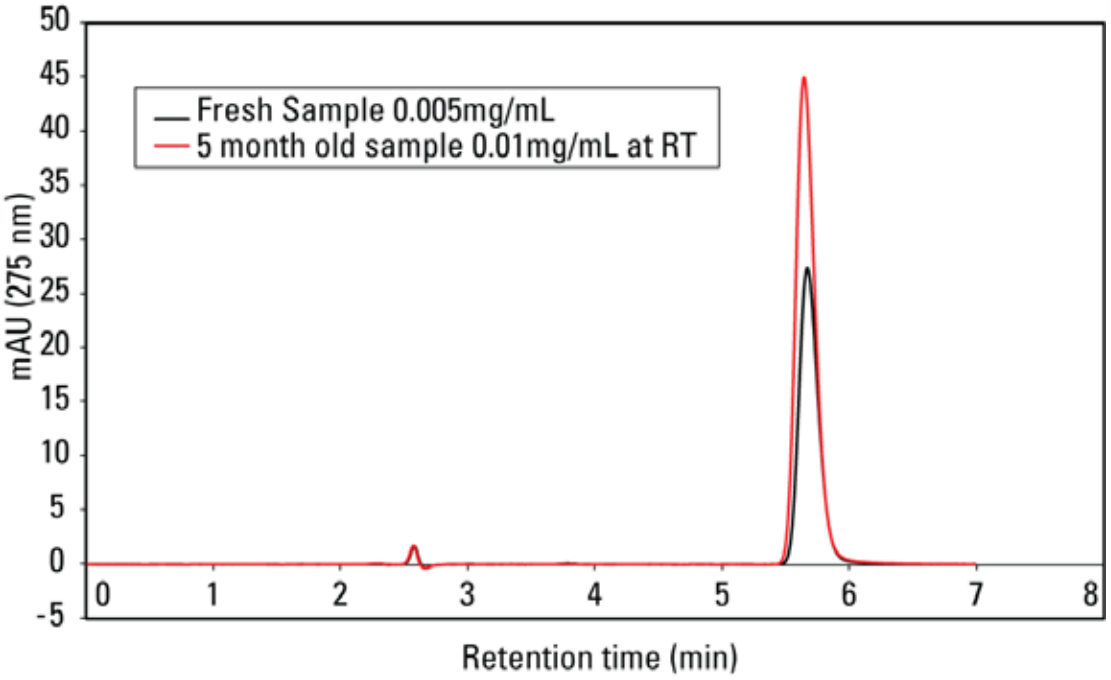
Temperature: 40°C

A TSKgel ODS-100V column was used successfully for the analysis of caffeine. %RSD values for different chromatographic parameters were very low (as shown in the table).

System Suitability Test: %RSD calculation in 5 consecutive injections in the analysis of caffeine using a TSKgel ODS-100V column

Caffeine	RT	Area (mAU*S)	Height (mAU)	Symmetry	Width (min)	Plates
	5.11	291.63	31.41	0.80	0.14	7306
	5.11	290.17	31.35	0.80	0.14	7296
	5.11	289.73	31.38	0.80	0.14	7300
	5.11	289.50	31.29	0.80	0.14	7292
	5.11	289.93	31.26	0.80	0.14	7291
Average	5.11	290.19	31.34	0.80	0.14	7297
Std Dev	0.00	0.84	0.06	0.00	0.00	6.16
%RSD	0.04	0.29	0.20	0.00	0.00	0.08

Figure 7: Stability Study of Caffeine USP using a TSKgel ODS-100V, 5µm, 4.6mm ID x 15cm Column



Isocratic conditions:

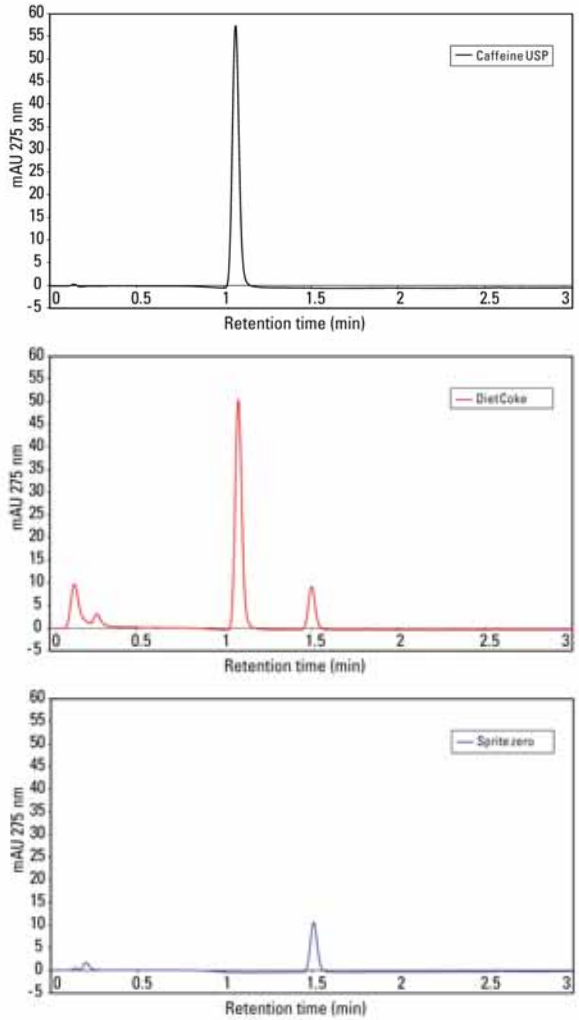
15% ACN in H_2O containing 0.15% TFA

Flow rate: **1.0mL/min**

Temperature: **40°C**

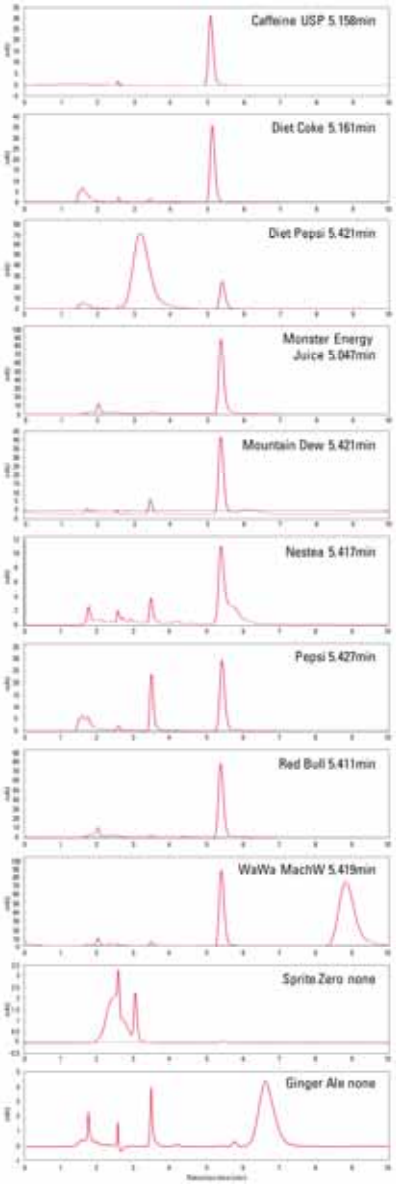
A TSKgel ODS-100V column produces sharp peaks for use in determining caffeine stability. No difference was found in the retention time of caffeine between an old sample and a fresh sample.

Figure 8: Comparative Separation Profile of Diet Coke and Sprite Zero using a TSKgel ODS-140HTP, 2.3µm, 2.1mm ID x 5cm Column



%RSD of caffeine peak of Diet Coke from 5 runs is 0.85, while it is 0.74 for Coca Cola Classic (data not shown). Sprite Zero did not exhibit a caffeine peak, confirming the label claim as a caffeine-free drink.

Figure 9: Analysis of Caffeine in Popular Soft Drinks using a TSK-gel ODS-100V, 5µm, 4.6mm ID x 15cm Column



Isocratic conditions:

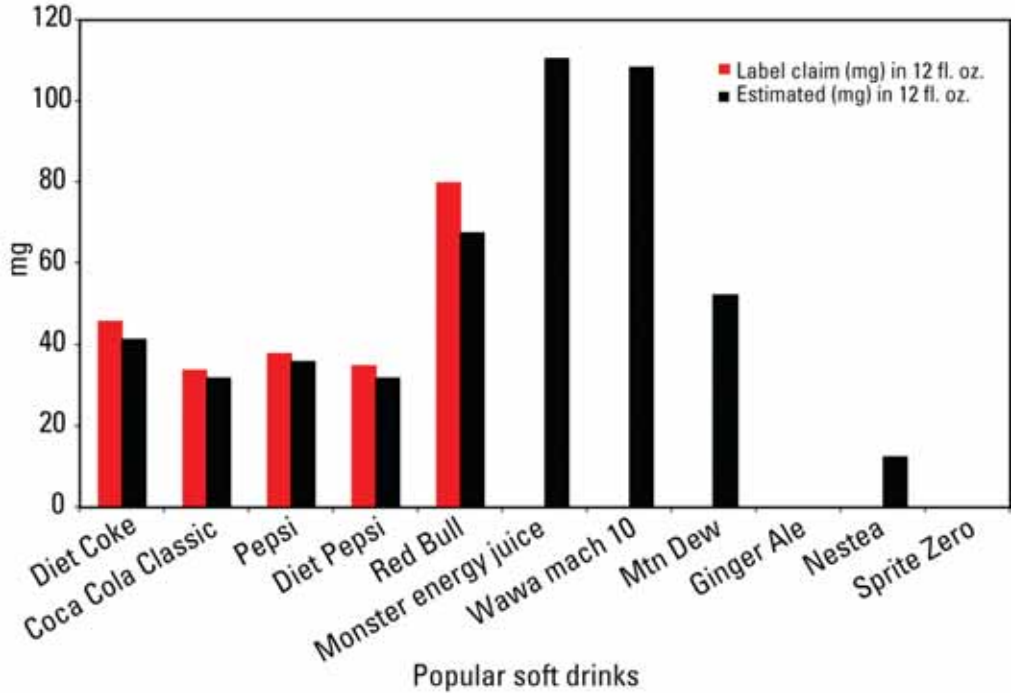
15% ACN in H_2O containing 0.15% TFA

Flow rate: 1.0mL/min

Temperature: 40˚C

Caffeine could be separated in several commercially available soft drinks on a TSKgel ODS-100V column. Pretreatment of the column was not required.

Figure 10: Estimation of Caffeine Content in Soft Drinks

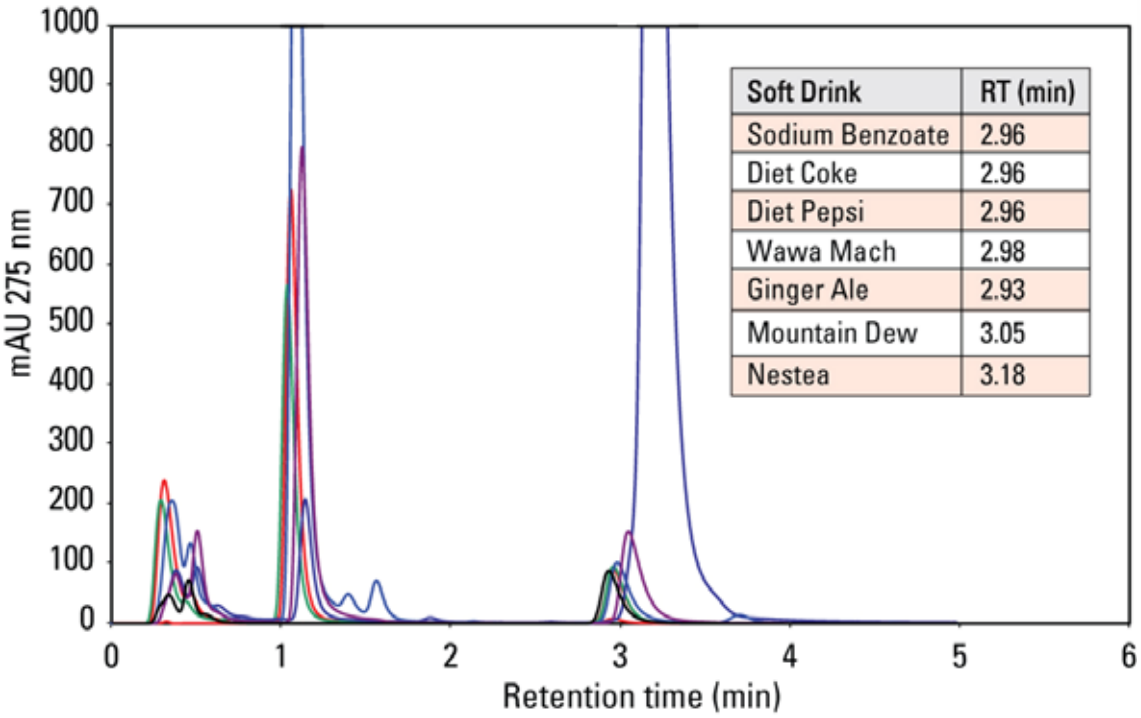


Soft drink	Label claim (mg) in 12 fl. oz.	Estimated (mg) in 12 fl. oz.
Diet Coke	46	41.3
Coca-cola Classic	34	31.7
Pepsi	38	35.7
Diet Pepsi	35	31.7
Red Bull*	80	67.5
Monster KHAOS energy juice	-	110.4
Wawa MachW	-	108.3
Mtn Dew	-	52.3
Ginger Ale	-	0
Nestea (Iced tea)	-	12.4
Sprite Zero	labeled as "No Caffeine"	0

*Red Bull label claim is based on 8 fl. oz. (250mL)

Caffeine content of the various soft drinks with/without label claims could be estimated using the calibration curve (figure 3).

Figure 11: Analysis of Sodium Benzoate in Soft Drinks using a TSKgel ODS-140HTP, 2.3µm, 2.1mm ID x 5cm Column



Soft Drink	RT (min)
Sodium Benzoate	2.96
Diet Coke	2.96
Diet Pepsi	2.96
Wawa Mach	2.98
Ginger Ale	2.93
Mountain Dew	3.05
Nestea	3.18

Isocratic conditions:

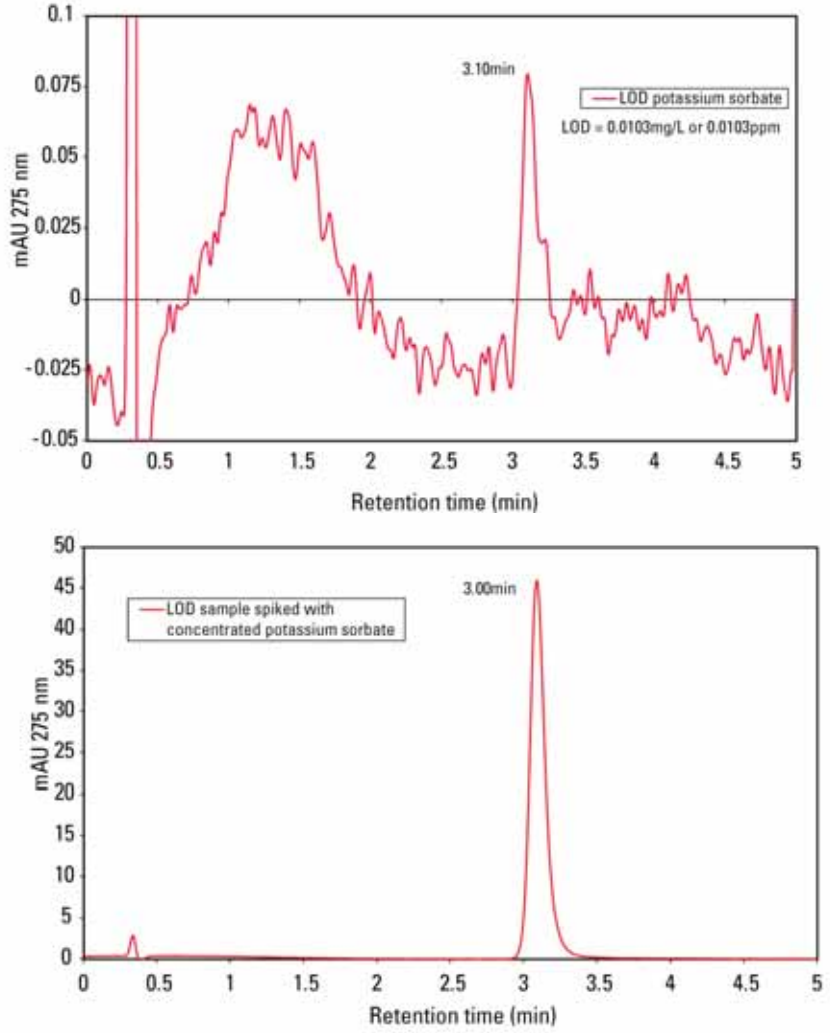
5% ACN in H_2O containing 0.15% TFA

Flow rate: 0.8mL/min

Temperature: 40˚C

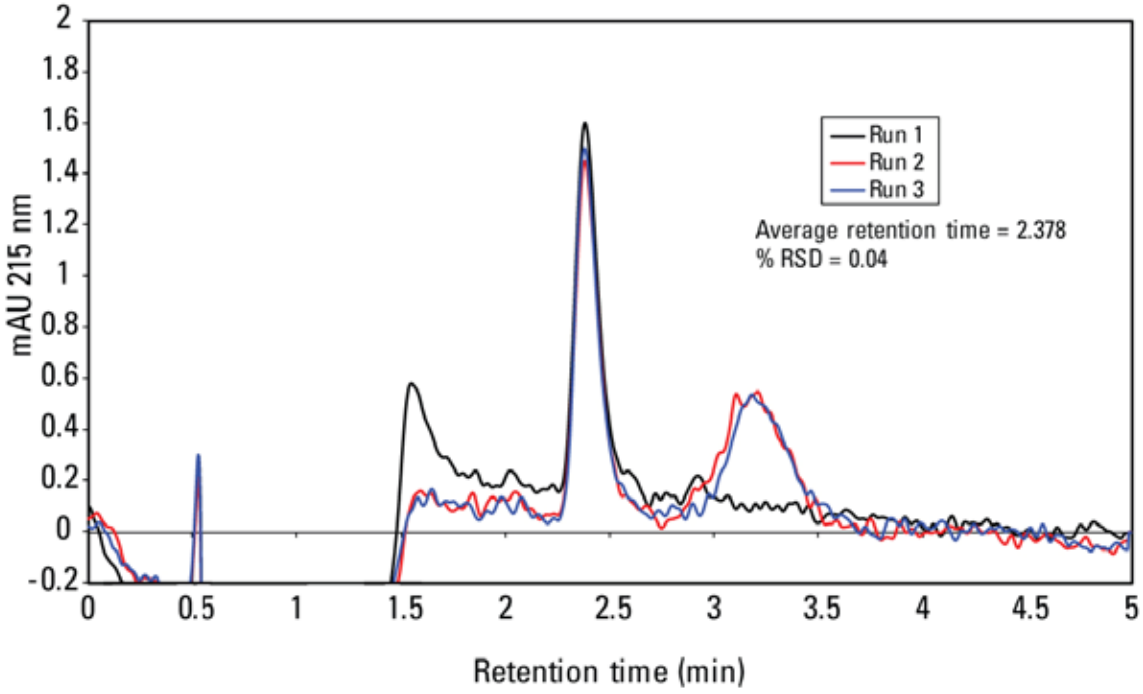
A TSKgel ODS-140HTP column was successfully used to analyze sodium benzoate in a number of soft drinks, in the presence of caffeine. The slight shift in the retention time of sodium benzoate is possibly due to the differences in composition of each individual soft drink. The identity of the individual peaks was established by spiking with sodium benzoate standard (data not shown).

Figure 12: Analysis and Determination of Limit of Detection of Potassium Sorbate using a TSKgel ODS-140HTP, 2.3µm, 2.1mm ID x 5cm Column



This study showed that a TSKgel ODS-140HTP column was used for limit of detection tests of potassium sorbate in soft drinks.

Figure 13: Determination of Limit of Detection of Aspartame using a TSKgel ODS-140HTP, 2.3µm, 2.1mm ID x 5cm Column



Isocratic conditions:

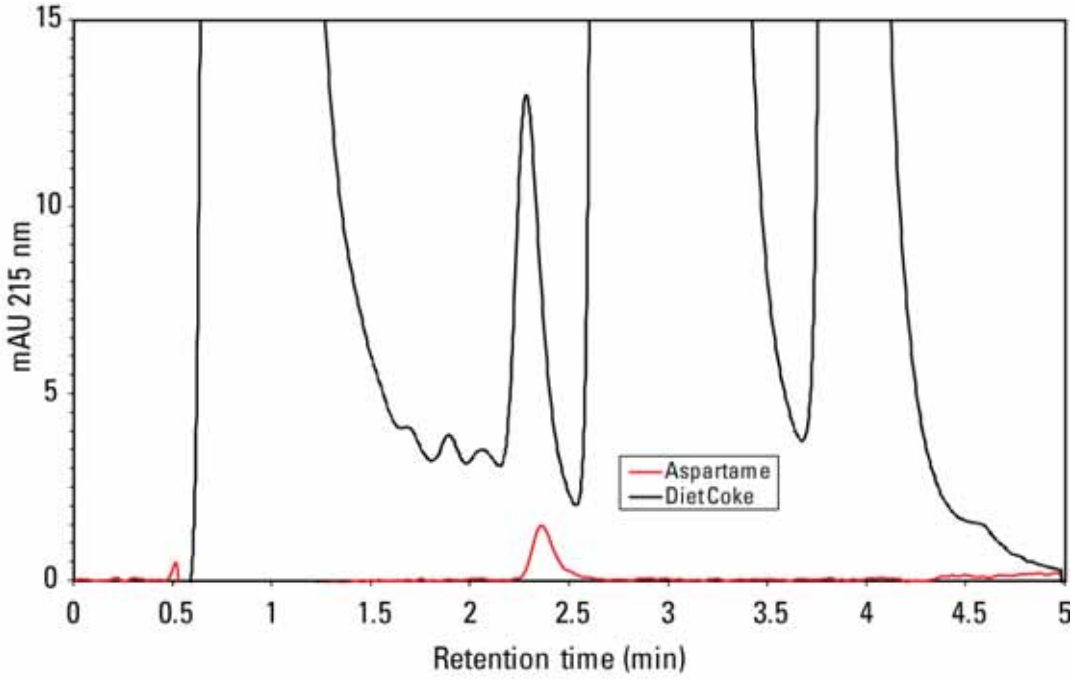
5% ACN in H_2O containing 0.15% TFA

Flow rate: 0.4mL/min

Temperature: 40˚C

The average retention time of aspartame was 2.378 minutes with a %RSD value of 0.04 over three consecutive injections. This low %RSD is compelling, given that the LOD concentration of aspartame is 0.001ppm. The LOQ is 0.01ppm.

Figure 14: Analysis of Aspartame in Diet Coke using a TSKgel ODS-140HTP, 2.3µm, 2.1mm ID x 5cm Column



Isocratic conditions:

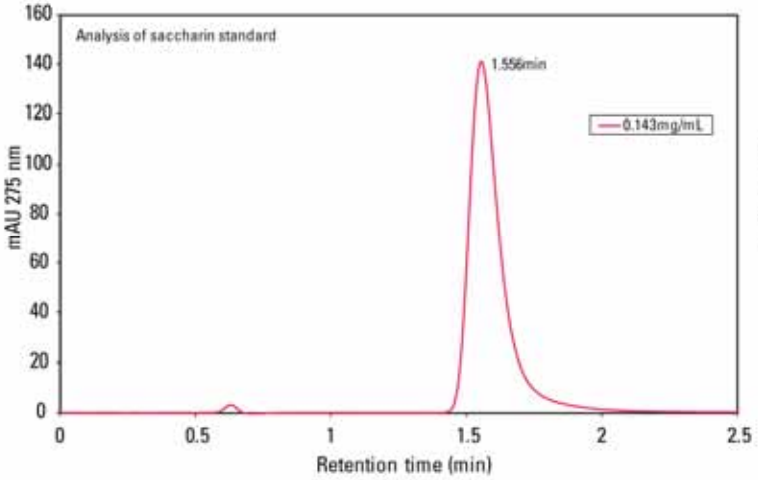
5% ACN in H_2O containing 0.15% TFA

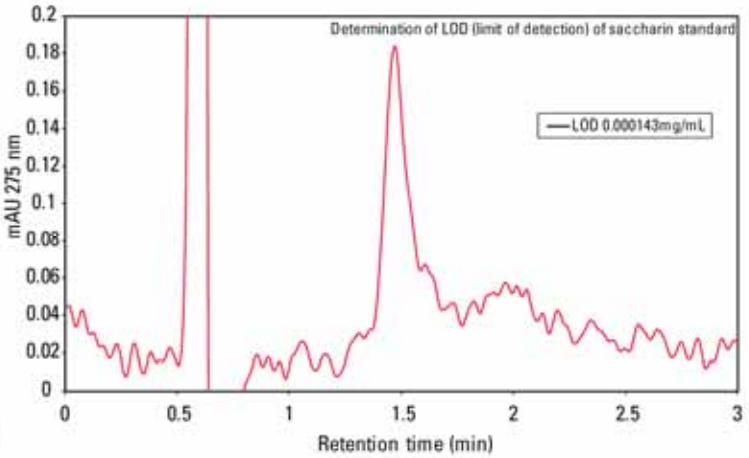
Flow rate: 0.4mL/min

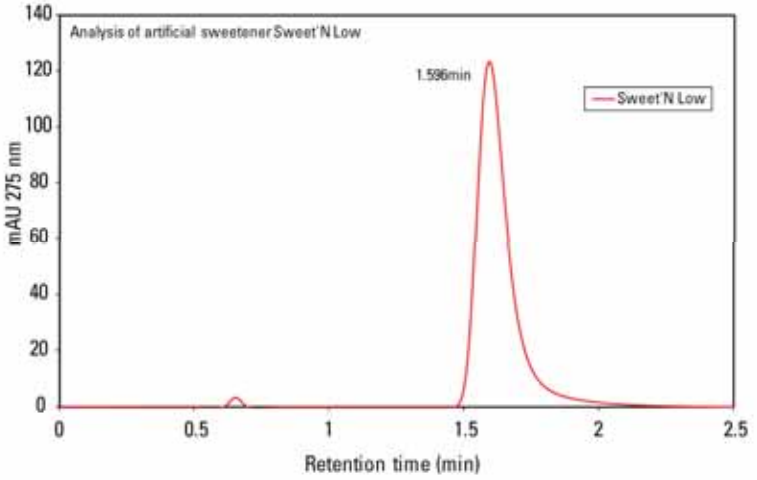
Temperature: 40˚C

Aspartame in Diet Coke could be quantitated using a TSKgel ODS-140HTP column.

Figure 15: Analysis of Saccharin in the Artificial Sweetener Sweet'N Low using a TSKgel ODS-140HTP, 2.3µm, 2.1mm ID x 5cm Column







Isocratic conditions:

15% ACN in H_2O containing 0.15% TFA

Flow rate: 1.0mL/min

Temperature: 40˚C

Linearity range – 1.43ng – 14.3µg

Limit of detection – 0.143mg/L or 0.143ppm

Limit of quantitation – 1.43ppm

Results and Discussion

- Analysis of caffeine and other additives from popular soft drinks such as Red Bull, Mountain Dew, Diet Coke, Pepsi, Diet Pepsi, Coca-Cola Classic, Wawa MachW, Monster KHAOS Energy Juice, NESTEA, Seagram's Ginger Ale, and Sprite Zero were carried out using reversed phase chromatography.
- Caffeine USP standard eluted from a TSKgel ODS-140HTP, 2.3μm, 2.1mm ID x 5cm column at 1.11 minutes as a sharp peak under linear gradient conditions (Figure 2).
- The low %RSD value establishes the precision of the analysis method.
- A calibration curve was generated using a TSKgel ODS-140HTP, 2.3μm, 2.1mm ID x 5cm column and the linear regression yielded a very good coefficient of 0.9993 (Figure 3).
- The caffeine USP standard could also be separated under isocratic conditions with an elution time of 1.9 minutes using the same TSKgel ODS-140HTP, 2.3μm, 2.1mm ID x 5cm column.
- The durability of the TSKgel ODS-140HTP 2.3μm, 2.1mm ID x 5cm column was established, showing no significant change in elution time after >1000 injections (Figure 4).
- A TSKgel ODS-140HTP, 2.3μm, 2.1mm ID x 5cm column delivered a faster analysis time for the separation of caffeine compared to a competitive column (Figure 5).
- Caffeine standards were also separated successfully using a TSKgel ODS-100V, 5μm, 4.6mm ID x 5cm column under isocratic conditions.

Results and Discussion Continued

- A system suitability test of the analysis yielded an average peak retention time of 5.11 minutes with an average symmetry of 0.80.
- The %RSD values calculated from the different chromatographic parameters, such as peak retention time, area, height, symmetry, plate count, resolution and selectivity were very low (Figure 6).
- It was shown that stability testing of caffeine could be performed using this column (Figure 7).
- The separation of caffeine from Diet Coke was achieved using a TSKgel ODS-140HTP, 2.3μm, 2.1mm ID x 5cm column. This same column was used to confirm that Sprite Zero is a caffeine-free soft drink (Figure 8).
- A TSKgel ODS-100V, 5μm, 4.6mm ID x 5cm column was used to analyze caffeine in commercially available popular soft drinks under isocratic chromatographic conditions without any pre-column treatment (Figure 9).
- Caffeine content of the various soft drinks with/without label claims could be estimated using the calibration curve in figure 3 (Figure 10).

Results and Discussion Continued

- Sodium benzoate, in addition to caffeine, could also be separated from soft drinks using a TSKgel ODS-140HTP, 2.3μm, 2.1mm ID x 5cm column (Figure 11).
- Potassium sorbate, another soft drink additive, was separated using a TSKgel ODS-140HTP, 2.3μm, 2.1mm ID x 5cm column, eluting at 3.10 minutes. The limit of detection (LOD) was 0.0103mg/L or 0.0103ppm with a signal to noise ratio of >2. (Figure 12).
- Aspartame was analyzed at 215nm using a using a TSKgel ODS-140HTP, 2.3μm, 2.1mm ID x 5cm column since aspartame does not absorb at 275nm. The average retention time was 2.378 minutes with %RSD value of 0.04 over three consecutive injections, even at an LOD concentration of 0.001ppm (Figure 13).
- This same column was used to separate aspartame in Diet Coke (Figure 14).
- Saccharin was separated from the artificial sweetener Sweet'N Low, using a TSKgel ODS-140HTP, 2.3μm, 2.1mmID x 5cm column. The saccharin standard peak eluted at 1.555 minutes, while the saccharin in Sweet'N Low eluted at 1.596 minutes. The calibration curve shows a linear range from 1.43ng – 14.3μg. The limit of detection of saccharin was found to be 0.143mg/L or 0.143ppm (Figure 15).