Reversed-Phase Chromatographic Determination of Caffeine, Saccharin and Aspartame in Dietary Beverages

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Master of Science Thesis in Chemical Studies

Prince of Songkla University

1999

Thesis Title Reversed-Phase Chromatographic Determination of Caffeine, Saccharin and Aspartame in Dietary Beverages

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Academic Year 1999

Abstract

The use of reversed-phase chromatographic technique for direct determination of caffeine, saccharin and aspartame in dietary beverages was studied. The effects on retention time, separation and the determination of additives in dietary beverages such as mobile phase concentration, types and concentration of modifiers, pH and mobile phase velocity were investigated. The optimum mobile phase in the determination of additives in dietary beverages for saccharin, caffeine and aspartame was 12% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate buffer adjusted to pH 3.0 with NaOH, and the active compounds were determined using absorbance detection at wavelength of 214 nm using a C_{18} column. An acetonitrile-triethanolammonium phosphate buffer was used as mobile phase in order to provide optical transparency at short wavelength. Detection at 214 nm provided increased response to both saccharin and aspartame. By this technique, after degassing and diluting, the dietary beverages were injected into

the chromatographic system directly without solvent extraction. Saccharin, caffeine and aspartame were eluted at 4.2 min, 7.7 min and 9.2 min, respectively with flow rate of 1.0 mL / min. The total run time for analysis per one injection was approximately 10 minutes. Minimum detectable concentrations were 0.02 μg / mL, 0.02 μg / mL and 0.20 μg / mL for saccharin, caffeine and aspartame, respectively.

ชื่อวิทยานิพนธ์ การหาปริมาณคาเฟอีน ซัคคาริน และแอสปาแทม ในเครื่องดื่ม โดยวิธีรีเวอร์สเฟส โครมาโทกราฟี

ผู้เขียน นายทรงธรรม แก้วประถม

สาขาวิชา เคมีศึกษา

ปีการศึกษา 2542

บทคัดย่อ

ศึกษาการวิเคราะห์ปริมาณคาเฟอีน ซัคคาริน และแอสปาแทมในเครื่องดื่มน้ำอัดลม โดยวิธีรีเวอร์สเฟสโครมาโทกราฟี และศึกษาถึงปัจจัยต่าง ๆ ที่มีอิทธิพลต่อเวลาการแยกของสาร การแยกและการหาปริมาณของสารปรุงแต่ง ได้แก่ ความเข้มข้นของตัวเคลื่อนที่ ชนิดและความเข้มข้นของสารปรุงแต่ง ความเป็นกรดและอัตราความเร็วของตัวเคลื่อนที่ พบว่า สภาวะที่เหมาะสมที่สุดในการวิเคราะห์ปริมาณสารปรุงแต่งในเครื่องดื่มน้ำอัดลม สำหรับซัคคาริน คาเฟอีน และแอสปาแทม คือ 12% โดยปริมาตร acetonitrile ใน 0.1% โดยปริมาตร triethanolammonium phosphate buffer pH 3.0 ที่ความยาวคลื่น 214 นาโนเมตร โดยใช้คอลัมน์ C_{18} การวิเคราะห์ปริมาณสารปรุงแต่งในเครื่องดื่มน้ำอัดลมด้วยเทคนิคนี้ สามารถทำได้โดยไม่ต้องผ่านการสกัดหรือแยกสารตัวใดออกจากตัวอย่างก่อนการวิเคราะห์ หลังจากเจือจางตัวอย่าง ไล่ก๊าซและฉีดสู่ระบบ ซัคคาริน คาเฟอีน และแอสปาแทมถูกแยกออกจากคอลัมน์ที่เวลา 4.2 นาที 7.7 นาที และ 9.2 นาที ตามลำดับ เมื่ออัตราเร็วการเคลื่อนที่ของตัวเคลื่อนที่คือ 1.0 มิลลิลิตร/นาที การวิเคราะห์ปริมาณสารตัวอย่างในแต่ละครั้ง จะใช้เวลาประมาณ 10 นาที และความเข้มข้นต่ำสุดที่สามารถตรวจวัดได้ด้วยวิธีนี้ของ ซัคคาริน คาเฟอีน และแอสปาแทมคือ 0.02 ไมโครกรัม/มิลลิลิตร, 0.02 ไมโครกรัม/มิลลิลิตร และ 0.20 ไมโครกรัม/มิลลิลิตร ตามลำดับ

Acknowledgement

I wish to express my appreciation to a number of people for their help in this thesis. The most sincere thank is to my advisor, Assistant Professor Dr. Manop Arunyanart and co-advisor, Assistant Professor Panit Sherdshoopongs. This thesis will not be successful without the kindness, guidance and assistance from them.

Sincere thank is extended to Dr. Naiyana Srichai and Mr. Ekkarin Songtong for commenting and correcting the language of this thesis.

I would like to thank the Department of Chemistry, Faculty of Science for various chemicals and equipments, especially HPLC and the Graduate School, Prince of Songkla University Scholarship Award for their financial support enrollment.

A note of appreciation is also acknowledged to the Director of Demonstration School, Faculty of Education, Prince of Songkla University, Pattani Campus, for the opportunity to undergo further studies.

Special appreciation is granted to both of my dear parents and Mrs. Suchada Kaewprathom for their love, encouragement and understanding throughout the entire study.

Finally, I would like to extend my grateful acknowledgement to the members of the Examining Committee for approving this thesis.

Songtham Kaewprathom

Contents

Page

List of Tables

List of Figures

Page

Page

CHAPTER 1

INTRODUCTION

Additives such as caffeine, saccharin and aspartame in dietary beverages are important for different purposes. For example, caffeine is used as a central nervous system stimulant, saccharin and aspartame are used as artificial sweeteners or sugar substitutes. However, overdosage of these additives can give toxic response. For example, caffeine causes tachycardia, gastric symptoms, headaches, palpitations, vomiting, panic attacks and anxiety. Saccharin is a potential chemical carcinogen for cancer in the lower urinary tract and inalignant tumors. Aspartame causes hives, periorbital oedema and erythema nausea, vomiting and abdominal pain and adverse effects on the central nervous system such as headaches, mood changes, insomnia and seizures *(Guiso, et al., 1988 : 485-493).* Therefore, the quantitative analysis of these additives in dietary beverages needs to be studied.

The literature review of caffeine, saccharin and aspartame and chromatographic techniques used in this study were presented.

Caffeine

Caffeine is an alkaloid, a group of compounds obtained from plants, and is an additive in soft drink. The molecule consists of nitrogen-containing rings found in coffee, tea, chocolate and other natural foods. Caffeine structural formula is shown in Figure 1.

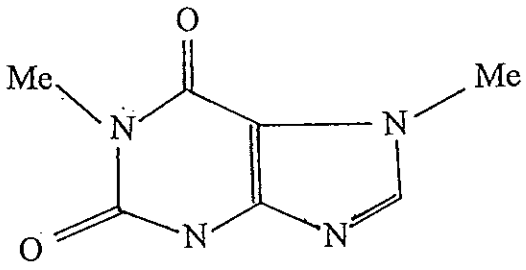

Figure 1. Structural Formula of Caffeine

Chemical name is 3, 7 - dihydro - 1, 3, 7 - trimethyl - 1H - purine - 2, 6 - dione and trademark is Theine, Methyl theobromine.. Some chemical and physical properties are as followed : It is fleecy white solid or long silky crystals. It is odorless with distinctive bitter taste. It will be hexagonal prisms by sublimation at 178°C.

Chemical Formula	: $C_8H_{10}O_2N_4.\ H_2O.$
Solubility	: One gram dissolve in 46 mL water, 5.5 mL water at 80°C, 1.5 mL boiling water, 66 mL alcohol at 60°C and 530 mL ether.
Melting Point	: 235.8°C

In general, alkaloids tend to have identifiable physiological effects on human body, although these effects vary greatly from compound to compound *(Anon, 1997 : 52 - 53).* Caffeine has been a part of the human diet for many centuries and is one of the most widely used as central nervous system stimulants in the world. Caffeine does not show adverse effects. In addition to its stimulation of the central nervous system, it is believed to be an adenosine blockade *(Rall, 1982 : 594).* Caffeine may in some people, causes tachycardia and gastric symptoms *(Darragh, et al., 1979 : 1196).* Caffeine has also been implicated as a cause of "food allergy", a non-specific term for a disorder which includes symptoms such as headache, palpitations, vomiting, panic attacks and anxiety *(Finn and Cohen, 1978 : 426).* Caffeine interferes with normal sleep patterns and may enhance the absorption of certain drugs. It affects drug-metabolizing enzymes by acting as an inducer of the microsomal system *(Mitoma, et al., 1968 : 145).*

It is well known that tea and coffee contains caffeine. However, it is less known that a number of other beverages, including carbonated soft drinks contain caffeine, although caffeine levels in certain foods, beverages and drugs have documented *(Lecos, 1984 : 2184)*. Lacking of information on addition of caffeine in beverages may produce developmental toxicity and may be a teratogen *(Collins, et al., 1983 : 763)*. For this reason, it has been suggested that pregnant women should keep their caffeine consumption relatively low. In addition, certain investigations and clinical conditions require patients to refrain from caffeine intake. Therefore, it is important to investigate the presence and amount of caffeine in popular beverages.

Saccharin

Saccharin is a well-recognized artificial sweetener which is used in numerous foods and beverages. The principal exposure of humans to saccharin is through diet soft drinks *(IARC Working Group, 1980 : 111)*. Figure 2 shows the structural formula of saccharin.

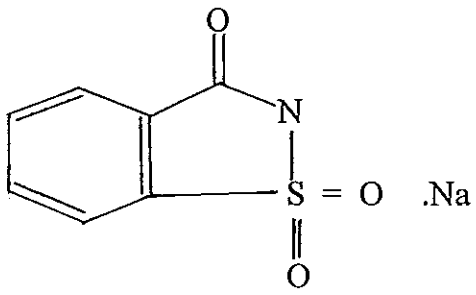

Figure 2. Structural Formula of Saccharin

The chemical name is 1, 2 - benzisothiazol - 3 (- 2H) - one 1, 1 - dioxide sodium dihydrate and trademark is 0 - benzoic acid sulfamide sodium saltdihydrate , Sodium benzosulfamide, Soluble gluside, Soluble saccharin. Some chemical and physical properties are as followed : It is white crystal or a crystaline powder, efflorescent in dry air, used in medicine and as non-nutritive food sweetener. In dilute aqueous solution, it is 500 times sweeter than sugar, and in aqueous solutions are neutral or alkaline to litmus, but not alkaline to phenolpthalein.

Chemical Formula	: $C_7 H_4NNaO_3 S.2H_2O$
Molecular Weight	: 205.19
Dissociation Constant	: pKa = 1.8 at 25°C.
Solubility	: One gram dissolves in 1.2 mL water and 50 mL alcohol.
Melting Point	: 228.8 - 229.7°C.

Saccharin came into use as a sweetening agent for canned fruits and vegetables, and in fact was also reported to inhibit spoilage that had been experience with sugar-sweetened products. Saccharin in small quantities (0.3 gram or less), added to food, does not show any deleterious or poisonous action *(Bernard , 1985 : 535 – 542)*, and is not injurious to the health of normal adults *(Ricciardi, et al.,1987 : 14-17)*. Saccharin is stable at physiological pH and temperature, well absorbed in the body and is not metabolized in the species in which it has been studied, including the rat, man and rhesus monkey. It does not accumulate inbody tissues and is excreted unchanged in the urine and faeces.

Saccharin is not electrophilic and does not bind covalently to DNA in rat liver or bladder, nor does it produce other cellular effects considered to be part of the process of carcinogenesis. Saccharin possesses surffactant properties and these may be related to its biological effects. *(Golberg, et al., 1985 : 543-546)*.

Aspartame

Aspartame is a dipeptide artificial sweetener, which is approximately 180 times sweeter than sucrose. It has the structural formula as shown in Figure 3.

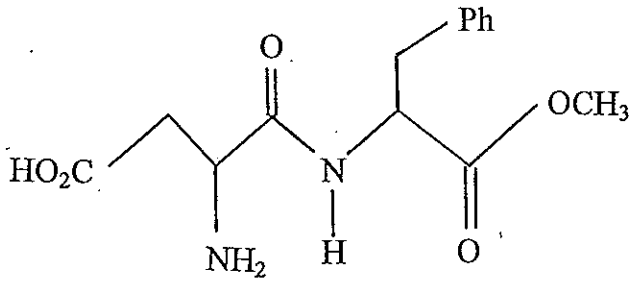

Figure 3. Structural Formula of Aspartame

The chemical name is N - L - α - aspartyl - L - phenylalanine - 1 - methyl ester and the trademarks are Nutrasweet, Sanecta and Tri - sweet. Some chemical and physical properties are as followed :

Chemical Formula	: $C_{14}H_{18}N_2O_5$
Molecular Weight	: 294.31
Dissociation Constant	: pKa 5.2 at 25°C.
Solubility	: One gram dissolves in about 11.52 mL water, at 25°C, 6.88 mL metanol, 384.61 mL ethanol, 2500 mL chloroform.
Melting Point	: 246 - 248°C.

Aspartame is used as a table-sugar substitute, as an additive in dry powder mixes, and as a sweetener in carbonated beverages *(Woodward, et al., 1979 : 1011-1019).* Aspartame ingestion can be associated with allergic reactions. These reactions have included hives, periorbita oedema and erythema, nausea, vomiting and abdominal pain, reportedly occurring within minutes to hours. However, it does not cause activation of human cutaneous mast cells *(Szucs, et al., 1986 : 171-174).* Further, there have been several recent anecdotal reports of adverse effects on the human central nervous system, associated with aspartame consumption, such as headaches *(Johns, 1986 : 456),* mood changes *(Drake, 1986 : 631),* insomnia and seizures *(Guiso, et al., 1988 : 485-493).*

Reversed Phase Liquid Chromatography

Reversed phase liquid chromatography (RPLC) employing hydrocarbonaceous bonded stationary phase has become one of the most widely used modes of liquid chromatography because of the impressive selectivity available. The lack of excessively strong solvent-surface interactions facilitates the use of a wider range of mobile phase, which allows for greater flexibility in the control of selectivity as well as more options in the choice of a suitable solvent for a sample. Separation in RPLC is achieved by means of differences in the interactions of the solutes with both the mobile and stationary phases. The mobile phase must be chosen to ensure solubility of the sample solutes. The use of a stationary phase that interacts strongly with solute relative to solute-mobile phase interactions will result in very long retention time (t_R), a situation which is not analytically useful. Retention in RPLC is dominated by the solute-mobile phase interactions, with solute-stationary phase interactions *(Dorsey, et al., 1983 : 924-928).* Thus, the key to selective separations is the ability to control solute-mobile phase interactions by varying the composition of the optimum mobile phase.

Reversed-phase (C_{18}) HPLC for identification and quantitation of caffeine was used by *De Andrage, et al. (De Andrage, et al., 1995 : 379-381).* Methanol-water (40 : 60, v/v) was used as mobile phase at 0.7 mL / min and absorbance was detected at 273 nm. The detection limit was 1 ng / mL and the

precision for ten different injections was under 3.2%. Caffeine levels found in Brazilian cola beverages (2-41 mg / 350 mL), tea (2-40 mg / 150 mL) and coffee (0.2 - 109 mg / 150 mL) were lower than those reported in the literature for the corresponding products available in the U.S.A. or Europe. The method proposed was suitable for quantifying of caffeine in beverages and did not require pretreatment nor derivatization of the samples.

A collaborative study for the determination of sodium saccharin, sodium benzoate and caffeine in 3 types of soda beverage, cola, grape and lemonlime was reported using reverse phase high pressure liquid chromatography with a C_{18} column and acetic acid mobile phase by *Woodward, et al. (Woodward, et al., 1979 : 1011-1019).* Recoveries were 98.6, 98.0 and 99.1% for sodium saccharin ; 100.6, 102.6 and 100.6% for sodium benzoate and 100.8, 101.4 and 101.1% for caffeine, respectively. This method thus has been adopted as official analysis.

Reverse phase high pressure liquid chromatographic method for the simultaneous separation and determination of saccharin, sodium benzoate and caffeine in soft drinks, fruit juices, fruit cocktails, fruit punches, coffee and artificial sweetener concentrates was reported by *Smyly, et al. (Smyly, et al., 1976 : 14-19).* Decarbonated soft drinks, fruit punches, and artificial sweetener concentrates were injected directly into the detecting instrument. But fruit juices and coffee solutions required filtration through a 0.45 micrometer membrane filter prior to injection. Samples were eluted with 5% glacial acetic

acid, and were quantitated with an UV detector. The results of saccharin, sodium benzoate and caffeine determinations in 34 soft drinks (representing 11 manufacturers and 20 flavors); 8 fruit juices, cocktails, and punches; 7 coffees; and 6 artificial sweetener concentrates were reported. Average recoveries of saccharin, sodium benzoate and caffeine from soft drinks were 99.0, 99.3 and 100.2%, respectively.

Aspartame was found to be about 160 times sweeter than 4% sucrose in aqueous solutions and compared favorable to sucrose in sensory attributes in the study by *Cloninger and Baldwin (Cloninger and Baldwin, 1974 : 347-349)*. This study was conducted to establish equivalents to various concentrations of sucrose, to evaluate the effects of selected ingredients on sweetness of the aspartame as compared to sucrose, and to monitor effects on pH. As an example of a typical application, the aspartame was evaluated in a noncarbonated orange-flavored beverage. Since sugar affects texture and body of food products as well as sweetness, the aspartame was evaluated in combination with gelatin, gum arabic (GA), methocal (MC), and carboxy-methylcellulose (CMC).

Furda, et al. (Furda, et al., 1975 : 340-343) reported that samples of L-aspartyl-L-phenylalanine methyl ester (APM) and APM hydrochloride were stored in aqueous solutions and their degradation products were identified by gas chromatography and mass spectrometry as their trimethylsilyl derivatives. Although both compounds furnished the same decomposition pattern, their

degree of decomposition differed considerably. It was believed that the low pH of the APM hydrochloride solution was the primary reason for its instability.

According to the literature review above, reversed phase chromatography was thus selected as a tool for separation and determination of the three additives in dietary beverages in this study.

The aim of this study was to use reversed phase liquid chromatography for the qualitative and quantitative analysis of saccharin, caffeine and aspartame in dietary beverages. In this study the optimum condition for separation such as capacity factor, selectivity, pH and concentration of mobile phase and the enhancement of efficiency in this chromatographic technique for the determination of these additives in dietary beverages were also investigated.

CHAPTER 2

EXPERIMENTS

APPARATUS

1. The high performance liquid chromatographic (HPLC) system consisted of :

- High pressure liquid chromatographic pump (JASCO, model 880 - PU; Japan)
- UV / VIS detector (JASCO, model 875 - UV; Japan)
- Sample injector with a 20 μL loop (Rheodyne, model 7125, USA)
- μBondapak C_{18} Column (Water Associates, USA, 300 x 3.9 mm.i.d. particle size is 10 μm)
- Chart recorder (WPA, model CG 95 UK)

2. pH meter (Corning Benchtop Meter, model 255)
3. Diode Array Spectrophotometer. (Hewlett Packard 8452 A)
4. Sonicator transsonic digitals (Elma D - 78224 singen / Htw, Germany)
5. 25 mm nylon membrane filter pore size 0.45 μm, (Pyrex, USA)
6. Suction flask (Pyrex, USA)

7. Volumetric flask
8. Cylinder
9. Beaker
10. Pipette

REAGENTS

1. Standard Chemicals
   - Caffeine (Sigma Chemical Company, USA)
   - Sodium saccharin (Fluka Chemica, Switzerland)
   - Aspartame (Fluka Biochemica, Switzerland)
2. General Solvents and Chemicals
   - Acetonitrile (J.B. Baker, USA)
   - Triethanolamine (Sigma Chemical Company, USA)
   - Orthophosphoric acid 85% (Deventer, Holland)
   - Sodium chloride (J.T. Baker, USA)
   - Sodium hydroxide (Merck, Germany)
   - Ethanol (Riedel - de Haen, Germany)
3. Samples
   - Beverages (Diet Coke, Pepsi Max, Coke, Pepsi, Sprite, Fanta, Red Fanta, Green Fanta, 7up and Green Spot)

All chemicals were analytical reagent grade. One bottle of each beverage brands was randomly bought from the supermarket in Haad Yai Town. Diet Coke, Pepsi Max, Coke, Pepsi, Sprite, Fanta, Red Fanta, Green Fanta, 7up and Green Spot were the selected brands. Solvent was routinely filtered through 25 mm nylon membrane filter pore size 0.45 μm prior to use.

METHODS

1. Preparation of Stock Standard Solutions

Each standard chemicals was accurately weighed to 200 mg in separate 100 mL beaker. Caffeine and saccharin were dissolved in water while aspartame was dissolved in 80 mL ethanol - water (1 : 1). Each standard was diluted in separate 100 mL volumetric flask with water. The concentration of each stock solution was 2 mg / mL.

2. Preparation of Working Standard Solutions

1. Stock solutions of caffeine and saccharin were pipetted for 10 mL in a 100 mL volumetric flask and diluted to volume with water to form the first mixed standard solution (200 μg / mL).

2. First mixed standard solution was pipetted for 10 mL and mixed with aspartame stock solution 10 mL in a 100 mL volumetric flask. Diluted to volume with water, final mixed standard was obtained (20, 20 and 200 μg / mL of caffeine, saccharin and aspartame, respectively).

3. Mixed standard was diluted over the range of 2.0 - 20.0 μg / mL both of caffeine and saccharin and 20.0 - 150.0 μg / mL of aspartame.

3. Buffer Solution

Ten millilitres of triethanolamine was diluted with water to 90 mL and 8 mL of phosphoric acid (85%) was mixed cautiously, cooled to room temperature, and brought to final volume of 1L with water. The solution was ready to be diluted with distilled water to prepare mobile phase solutions.

4. Degassing System

Degassing is an important step to eliminate dissolved gases in the mobile phase and to reduce the possibility of bubbles forming in the pump or detector during the separation. Elimination of oxygen in the mobile phase is also important to prevent reaction with the mobile and / or stationary phase within the analysis. In this work, the degassing systems of mobile phase were performed by sucking through 25 mm nylon membrane filter pore size 0.45 μm.The degassed mobile phases were kept ready to use in the mobile phase reservoir.

5. Optimum Wavelength Determination

The optimum wavelength of each standard was obtained with Diode Array Spectrophotometer (HP 8452A) which was scanned wavelength from 190 to 350 nm.

6. Equilibration System and Chromatographic Condition

The high performance liquid chromatographic system including pump and UV - detector was turned on for an hour before injection to stabilize the light source and equilibrate the reversed phase system. Equilibration was confirmed by constant retention time of all solutes. The operating temperature was ambient temperature and the flow rate was set at 1.0 mL / min. Detector was set at 214 nm and 0.08 absorbance unit of full scale (AUFS). Chromatograms were recorded with a chart recorder at sensitivity of 0.5 mv/cm and chart speed at 10 mm / min.

7. Preparation and Selection of Mobile Phase

In order to select the optimum mobile phase on k' or t_R, the studies of various effects in reversed phase systems, i.e., mobile phase concentration, pH, salt, organic modifiers and linear velocity were performed as follows :

7.1 Effects of Mobile Phase Concentration

1. The various concentrations of acetonitrile were prepared as 10, 15, 20 and 30% in distilled water.

2. The mobile phase was degassed before it was used as described previously.

3. Equal volumes of 20 µL of standard solution were injected for all of these mobile phases on µBondapak C_{18} column.

4. The k' of each standard was calculated from t_R.

5. The k' was plotted against acetonitrile concentration.

7.2 Effects of Sodium Chloride

1. The appropriate amounts of solid NaCl in 12% v/v acetonitrile solutions to make the concentrations of NaCl at 0.00, 0.05, 0.10, 0.15 and 0.20 M were dissolved respectively.

2. This mobile phase was used to analyse standard.

3. The k' of each standard was calculated.

4. The k' versus concentration of NaCl was plotted.

7.3 Effects of pH

1. The desired pH of phosphate buffer was prepared by diluting 10 mL buffer solution to 800 mL with distilled water, 150 mL of acetonitrile was added, and pH was adjusted with 1.0 M NaOH to 3.5, 4.5, 5.5 and 6.5 monitored with a pH meter. The final volume was brought to 1L with water.

2. Mobile phase solutions were passed through 25 mm nylon membrane filter pore size 0.45 µm.

3. Equal volumes of 20 µL of standard solution were injected for all these mobile phases on µBondapak C_{18} column.

4. The k' of each standard was calculated.

5. The k' versus pH was plotted.

7.4 Effects of Triethanolamine

1. The various concentrations of triethanolamine in phosphate buffer mobile phase consisting of 15% v/v acetonitrile to 0.05%, 0.1%, 0.2% and 0.3% were prepared.

2. The mobile phase was degassed and the standard solution was injected.

3. The k' of each standard was calculated.

4. The k' versus triethanolamine concentration was plotted.

7.5 Effects of Linear Velocity

1. The mobile phase consisting of 120 mL acetonitrile and 10.0 mL triethanolammonium phosphate buffer solution with pH adjusted to 3.0 and diluted to 1.0 L with distilled water was prepared (12% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate buffer).

2. The flow rate was adjusted at 0.50, 0.70, 1.00, 1.20 and 1.50 mL / min.

3. The t_R and peak width at half height of caffeine were measured.

4. The HETP and the mobile phase velocity were calculated.

5. The Van Deemter relationship was plotted.

7.6 Selection of the Optimum Mobile Phase

1. The mobile phases consisted of 12% v/v and 15% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate buffer were prepared. pH 3 and 4.3 were adjusted, respectively.

2. These mobile phases were used to study the separation of caffeine, saccharin and aspartame.

7.7 Reproducibility and Linearity of the Developed Method

1. For reproducibility study a mixed standard solution of 10 μg / mL caffeine, saccharin and 100 μg / mL aspartame was injected for 10 times and the standard deviation of resulting peak height was determined.

2. In linearity study various standard concentrations were prepared by using mixed intermediate standard solution which composed of aspartame 10 times higher than those of caffeine and saccharin. These mixed standard solutions contained caffeine and saccharin at the level of 2, 5, 10 and 20 μg / mL of caffeine, respectively. Each standard solution was injected for 3 times and peak height versus standard concentration was plotted.

7.8. Limits of Detection

The ability to quantify a trace element or molecule in chemical and biological matrixes using specific analytical methods is often viewed in terms of the limit of detection. This limit of detection is a number, expressed in units of concentration (or amount), that describes the lowest concentration level

(or amount) of the element that an analyst can determine to be statistically different from an analytical blank.

The minimum amounts of caffeine, saccharin and aspartame in aqueous solution were measured manually from chromatograms. The signal individual additive peak of chromatogram was two times higher than the noise or baseline.

8. Application of Developed HPLC Conditition for Dietary Beverages

1. The sample was degassed by using sonication.
2. Degassed sample was diluted 20 time of actual sample with water to form 5% v/v in aqueous solution.
3. Each sample solution was injected for 3 times.
4. Peak heights of sample solutions were compared with the standard linear calibration curve.
5. The result was calculated using the equation

$$M\ \mu g / mL = 20\ C\ \mu g / mL$$

where M is concentration of dietary beverage. C is concentration of diluted sample solution that can be measured from linear calibration curve.

CHAPTER 3

RESULTS AND DISCUSSIONS

1. Optimum Wavelength Determination

The optimum wavelengths of caffeine, saccharin and aspartame were determined by Diode Array Spectrophotometer in the range of 190 to 350 nm. The lists of absorbance data were shown in Table 1 and UV - spectra of caffeine, saccharin and aspartame were presented in Figures 4, 5 and 6, respectively.

The maximum absorbances of caffeine, saccharin and aspartame were 0.8341 AU, 1.5762 AU and 1.5053 AU at wavelenghs 202 nm, 202 nm, and 190 nm, respectively for concentration of 200 μg / mL. However, the optimum wavelength of caffeine, saccharin and aspartam was not selected at these maximum wavelengths but the wavelength at 214 nm with the medium absorbance of 0.2516 AU, 0.5224 AU and 0.8742 AU, respectively was selected. This is due to the fact that when using the UV photometric detector, it was not necessary to work at the maximum absorption . It was important to use the wavelength peak with the highest sensitivity. They were free from interferences and error from the shift of the absorption maximum of the peak.

Table 1. Absorbances of caffeine, saccharin and aspartame in aqueous solution (200 μg / mL) from Diode Array spectrophotometer.

wavelength (λ) nm	absorbance		
	caffeine	saccharin	aspartame
190	0.3998	0.6099	1.5053
200	0.7433	1.4920	0.5401
210	0.7078	1.0389	0.3364
214	0.5224	0.8742	0.2516
220	0.2935	0.7042	0.1088
230	0.1592	0.4416	0.0059
240	0.0962	0.0955	-0.0011
250	0.1013	0.0484	0.0014
260	0.2073	0.0631	0.0040
270	0.3167	0.0694	0.0017
280	0.2830	0.0564	0.0015
290	0.1049	0.0283	0.0031
300	0.0082	0.0008	0.0041
310	-0.0018	0.0016	0.0049
320	-0.0033	-0.0007	0.0054
330	-0.0034	-0.0012	0.0058
340	-0.0035	-0.0013	0.0062
350	-0.0034	-0.0014	0.0065

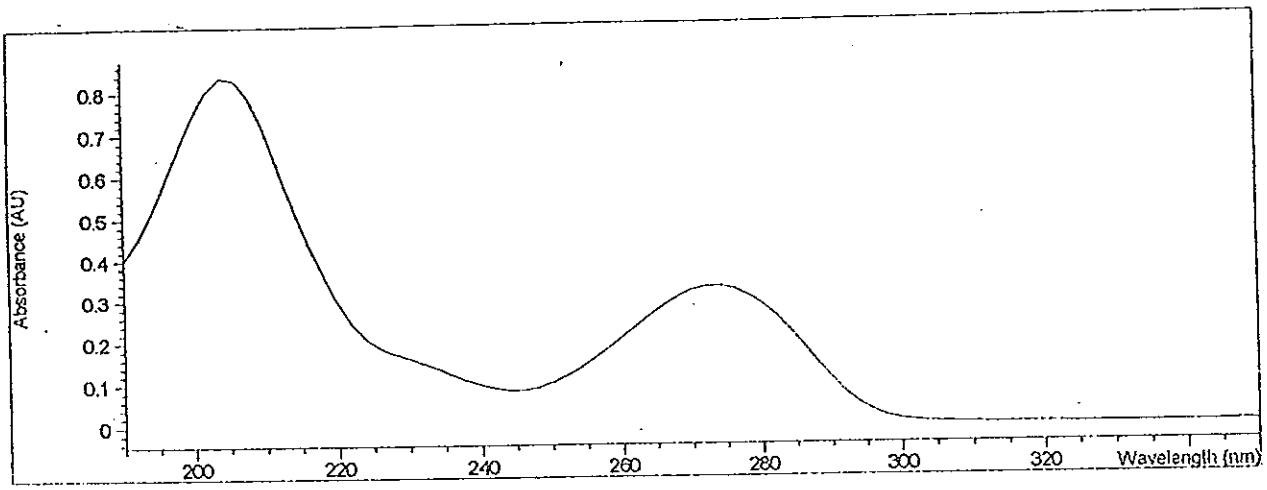

Figure 4. UV spectrophotometric absorption spectra of caffeine in aqueous solution at concentration of 200 μg / mL. The maximum absorbance was 0.834 AU at wavelength of 202 nm.

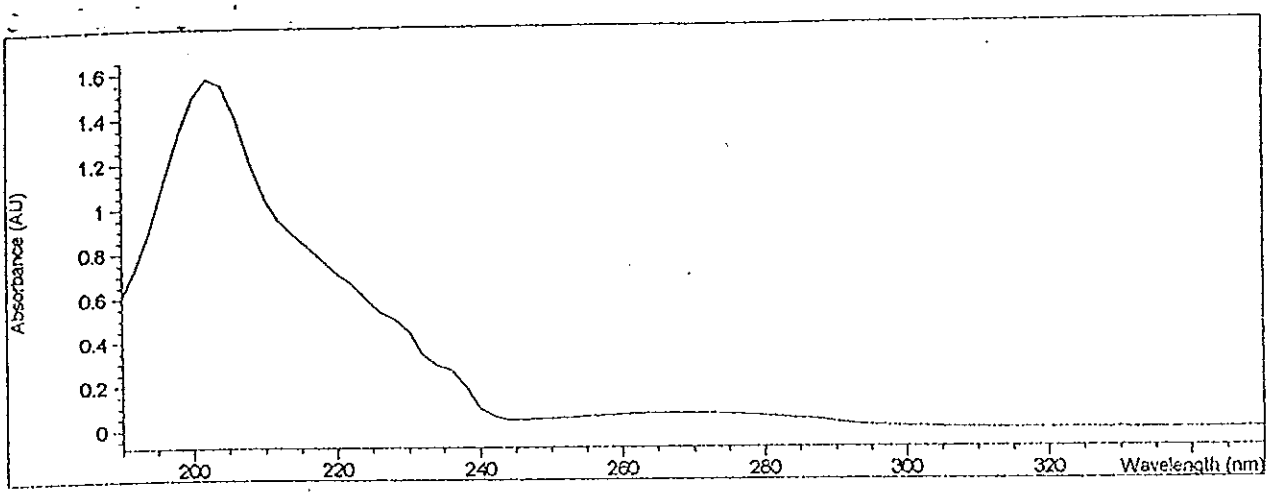

Figure 5. UV spectrophotometric absorption spectra of saccharin in aqueous solution at concentration of 200 μg / mL.

The maximum absorbance was 1.576 AU at wavelength of 202 nm.

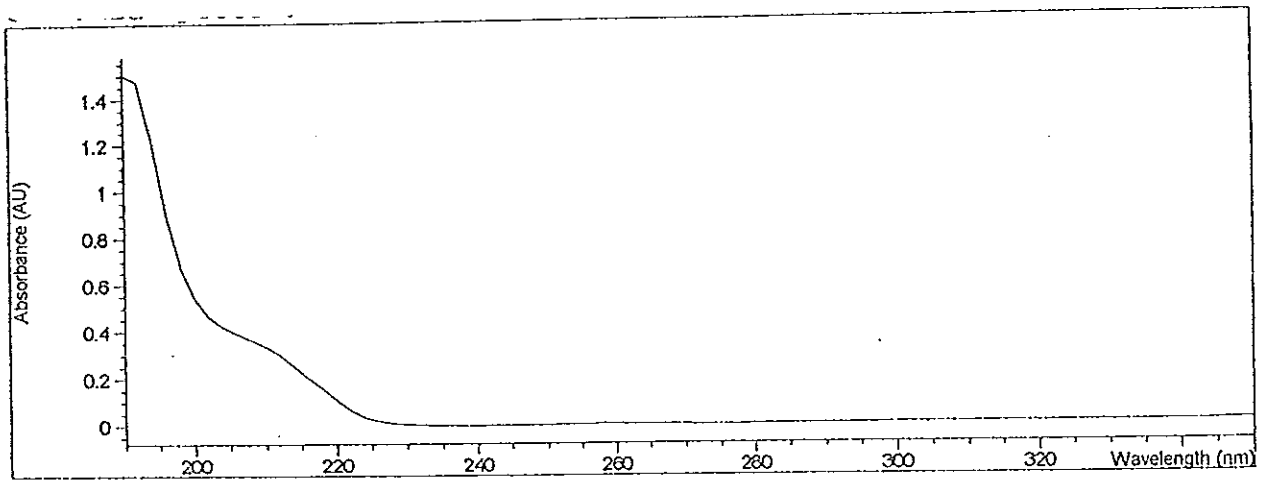

Figure 6. UV spectrophotometric absorption spectra of aspartame in aqueous solution at concentration of 200 µg / mL. The maximum absorbance was 1.505 AU at wavelength of 190 nm.

2. Preparation and Selection of Mibile Phase

2.1 Effects of Mobile Phase Concentration

Retention behaviors of saccharin, caffeine, and aspartame were tabulated in Table 2. The capacity factors of all solutes were plotted as a function of acetonitrile concentration and were shown in Figure 7. The capacity factors of both caffeine and aspartame decreased exponentialy with the increasing acetonitrile concentration. The capacity factor of saccharin was constant and the elution time was lower than that of unretained component. Saccharin is a polar compound and has less hydrophobic interaction. Moreover, molecule is the smallest when compared with caffeine and aspartame. Then, saccharin was eluted quickly on C_{18} column because of the dipole interactions with mobile phase. However, the capacity factor of caffeine was lower than aspartame at any acetonitrile concentration. Caffeine was more polar, bicyclic molecule than aspartame which is a long chain molecule. Therefore caffeine was more soluble in mobile phase than aspartame. This is why aspartame was retained in the column for a longer period of time.

Retention behavior in liquid chromatography is influenced by a wide variety of physical and chemical properties of stationary phase, mobile phase and the solute. The organic modifier in water was used as a mobile phase in reversed phase chromatography with certain C_{18} column. The retention behavior of solute depended on solvent strength. Solvent strength usually was adjusted by varying the composition of the solvent mixture, and the capacity

factor changes with changing solvent composition *(Snyder and Kirkland, 1979 : 285).*

Chromatographic work was studied by using a mobile phase of acetonitrile in water. Four different concentrations of acetonitrile in water were used to measure the retention behavior of saccharin, caffeine and aspartame. Saccharin, caffeine and aspartame were eluted firstly, secondly and lately, respectively. The mobile phase consisting of 15% v/v acetonitrile in aqueous solution was selected to study next conditions.

Table 2. The capacity factors of saccharin, caffeine and aspartame with various acetonitrile concentrations in the mobile phases.

acetonitrile concentration (% v/v)	capacity factor (k')		
	saccharin	caffeine	aspartame
10	- 0.55	2.57	3.86
15	- 0.57	1.00	1.86
20	- 0.57	0.45	0.97
30	- 0.57	0.11	0.38

HPLC conditions : μBondapak C_{18} column, 300 x 3.9 mm i.d.; flow rate, 1.0 mL / min; UV-detector, 214 nm.; sensitivity, 0.08 AUFS; chart speed, 10 mm / min.

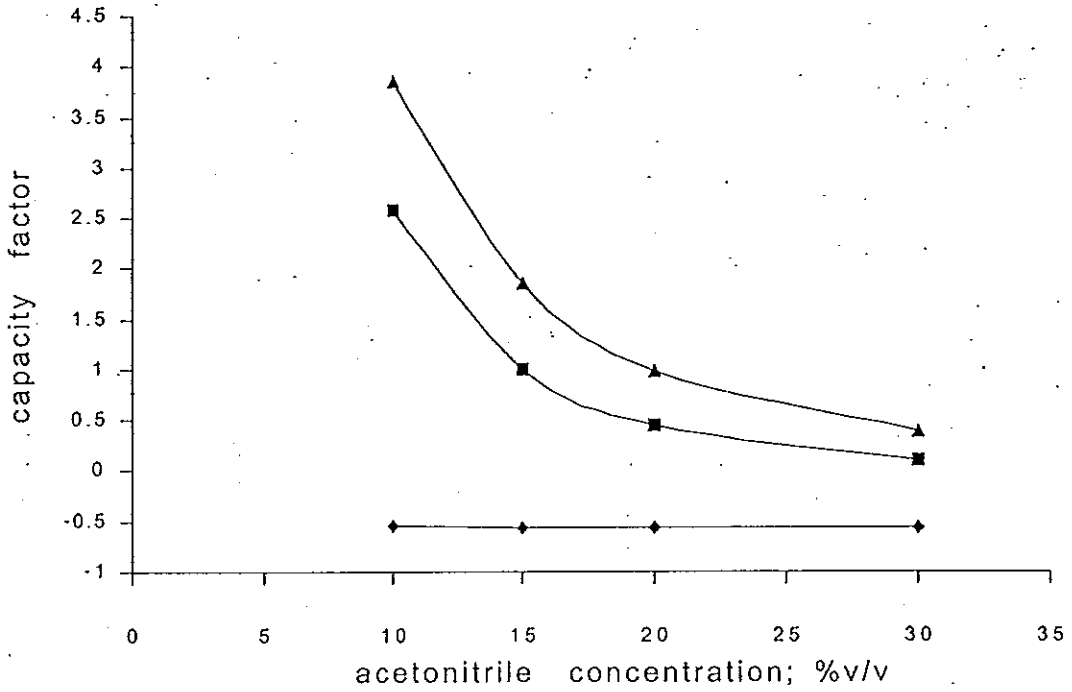

Figure 7. Dependence of k' on various acetonitrile concentrations of saccharin (◆), caffeine (■) and aspartame (▲).

HPLC conditions : μBondapak C_{18} column, 300 x 3.9 mm i.d.; flow rate, 1.0 mL / min; UV-detector, 214 nm; sensitivity, 0.08 AUFS; chart speed, 10 mm / min.

2.2 Effects of Sodium Chloride

The effect of salts added to the aqueous mobile phase in reversed phase liquid chromatography could vary solute retention or selectivity. Capacity factor of the solute would increase when neutral salt such as sodium chloride was added to the aqueous mobile phase. Addition of salt decreased the solubility of the solute in aqueous mobile phase, thus increasing the retention time of the solute. The effect of sodium chloride on capacity factor of three additives were shown in Table 3 and the chromatographic behavior of saccharin, caffeine and aspartame plotted between capacity factor versus sodium chloride concentration were shown in Figure 8. It can be seen that the elution behavior of these additives were relatively increased in capacity factor with increase in sodium chloride concentration, indicating that the addition of salt decreased the solubility of the solutes in the mobile phase (salting - out effect).

Table 3. Effects of sodium chloride in 12% v/v acetonitrile mobile phase on capacity factors of saccharin, caffeine and aspartame.

[NaCl] in 12% v/v acetonitrile; M	capacity factor (k')		
	saccharin	caffeine	aspartame
0.00	- 0.47	2.38	3.32
0.05	0.30	2.42	3.38
0.10	0.50	2.55	3.60
0.15	0.52	2.65	3.70
0.20	0.75	2.85	4.00

HPLC conditions : μBondapak C_{18} column; 300 x 3.9 mm. i.d.; flow rate, 1.0 mL / min; UV - detector, 214 nm; sensitivity, 0.08 AUFS; chart speed, 10 mm/min.

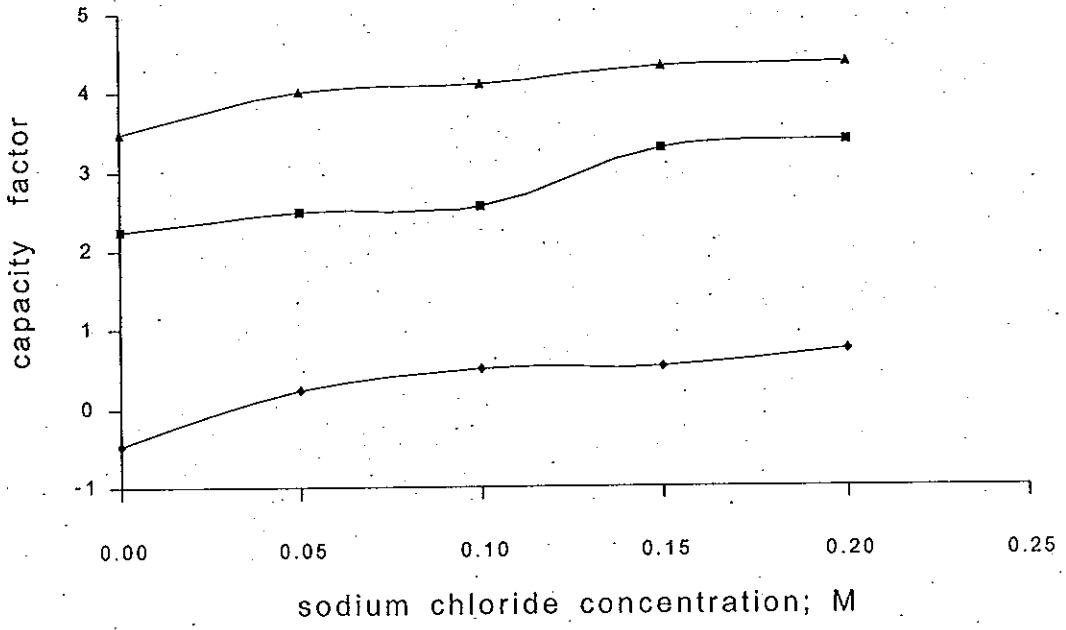

Figure 8. Influence of sodium chloride concentrations on capacity factors of saccharin (◆), caffeine (■) and aspartame (▲) in 12% v/v acetonitrile mobile phase; HPLC conditions : μBondapak C_{18} column, 300 x 3.9 mm. i.d.; flow rate, 1.0 mL / Min; UV - detector, 214 nm; sensitivity, 0.08 AUFS; chart speed, 10 mm/min.

2.3 Effects of pH

The pH dependence on retention times were examined with phosphate buffer mobile phase consisting of 15% v/v acetonitrile in pH range of 3.0 to 6.5. The results indicated the effect of various pHs on capacity factor of three additives were shown in Table 4. The values of capacity factor at various pH mobile phase were plotted in Figure 9. The capacity factors of saccharin, caffeine and aspartame hardly altered significantly but the capacity factor of aspartame decreased with increasing the pH. These effects were related to the ionization of the additives. Caffeine showed little change in retention with variation in pH because it did not contain ionizable group. Ionization changes ratio of ionized and non-ionized forms of solutes and this affects the partition of them in the hydrocarbon stationary phase, C_{18} bonded silica column which leads to changing of separation selectivity *(Snyder and Kirkland, 1979 : 286 - 288).* Saccharin was constant in capacity factor within the range of pH under study. That was higher than its pKa (1.8), therefore, the ratio of two species was negligible altered. Aspartame has pKa at 5.2, therefore, non-ionized forms of this additive at pH lower than its pKa was partitioned into hydrocarbon stationary phase and retained at stationary phase.

In conclusion pH 3.0 was selected for further studies since it provided good selectivity for the three additives (k' of saccharin 0.16, caffeine 0.90, aspartame 1.90).

Table 4. Influence of pH of mobile phase on the capacity factors of saccharin, caffeine and aspartame in 15% v/v acetonitrile in phosphate buffer.

pH	capacity factor (k')		
	saccharin	caffeine	aspartame
3.0	0.16	0.90	1.90
3.5	0.12	0.97	1.81
4.0	0.06	0.94	1.56
5.5	0.06	1.09	1.69
6.5	-0.10	1.00	1.50

HPLC conditions : μBondapak C_{18} column, 300 x 3.0 mm. i.d.; flow rate, 1.0 mL/min; UV-detector, 214 nm; sensitivity, 0.08 AUFS; chart speed, 10 mm/min.

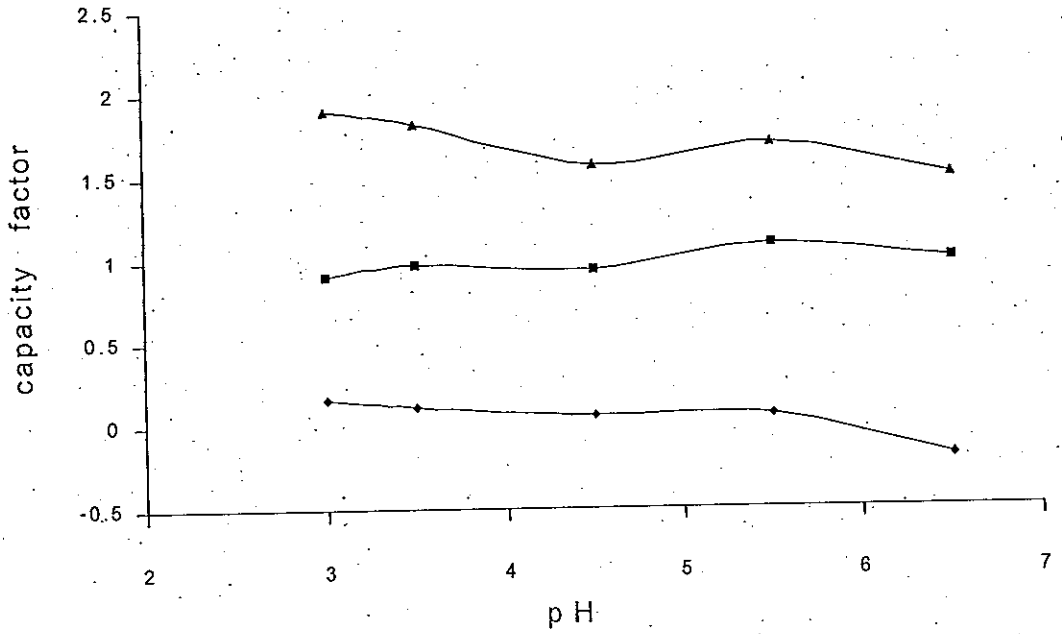

Figure 9. Capacity factors of saccharin (◆), caffeine (■) and aspartame (▲) versus pH of 15% v/v acetonitrile in phosphate buffer.HPLC conditions : μBondapak C_{18} column, 300 x 3.0 mm.i.d.; flow rate, 1.0 mL/min; UV-detector, 214 nm; sensitivity, 0.08 AUFS; chart speed, 10 mm/min.

2.4 Effects of Triethanolamine

In order to increase the retention time of saccharin, triethanolamine was added in 15% v/v acetonitrile in phosphate buffer to modify stationary phase.Retention behaviors of saccharin increased when triethanolamine were added (see Figure 10). The capacity factors of saccharin, caffeine and aspartame increased with triethanolamine concentration in mobile phase (15% v/v acetonitrile in phosphate buffer pH 3.0). The capacity factors were shown in Table 5.

The mobile phase consisting of 10 - 30 % v/v acetonitrile mobile phase gave small capacity factor values for all three additives, especially saccharin. This mobile phase strongly disolved with molecule of saccharin with dipole interaction. Therefore, saccharin was eluted immediately. Reversed phase chromatography could change retention behavior by adding a modifier such as triethanolamine. This polar modifier also suppressed adsorption of the additives onto the C_{18} surface. Triethanolamine concentration at 0.1% v/v in 15% v/v acetonitrile- phosphate buffer pH 3.0 was the best condition selected for further studies. It retained saccharin in column for a reasonable elution time (k' at 0.25) and provided a compromise separation with the other two additives at k' 1.56 and 2.19 for caffeine and aspartame, accordingly.

Table 5. Influence of triethanolamine concentrations on the values of capacity factors of saccharin, caffeine and aspartame of 15% v/v acetonitrile in phosphate buffer mobile phase pH 3.0.

triethanolamine concentration (% v/v)	capacity factor (k')		
	saccharin	caffeine	aspartame
0.05	0.12	1.25	2.00
0.10	0.25	1.56	2.19
0.20	0.31	1.50	2.38
0.30	0.38	1.31	2.06

HPLC conditions : μBondapak C_{18} column 300 x 3.9 mm.i.d.; flow rate, 1.0 mL/min; UV-detector, 214 nm; sensitivity, 0.08 AUFS; chart speed, 10 mm/min.

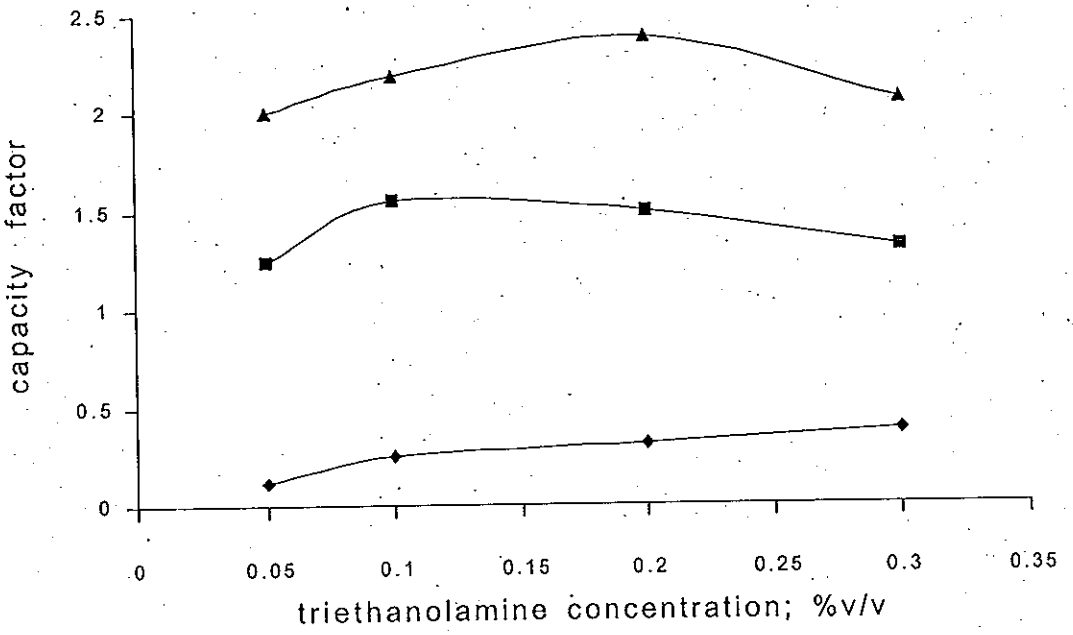

Figure 10. Influence of triethanolamine concentrations in phosphate buffer mobile phase pH 3.0 consisting of 15% v/v acetonitrile on capacity factors of saccharin (◆), caffeine (■) and aspartame (▲); HPLC conditions : μBondapak C_{18} column, 300 x 3.9 mm.i.d; flow rate, 1.0 mL/min; UV-detector, 214 nm; sensitivity, 0.08 AUFS; chart speed, 10 mm/min.

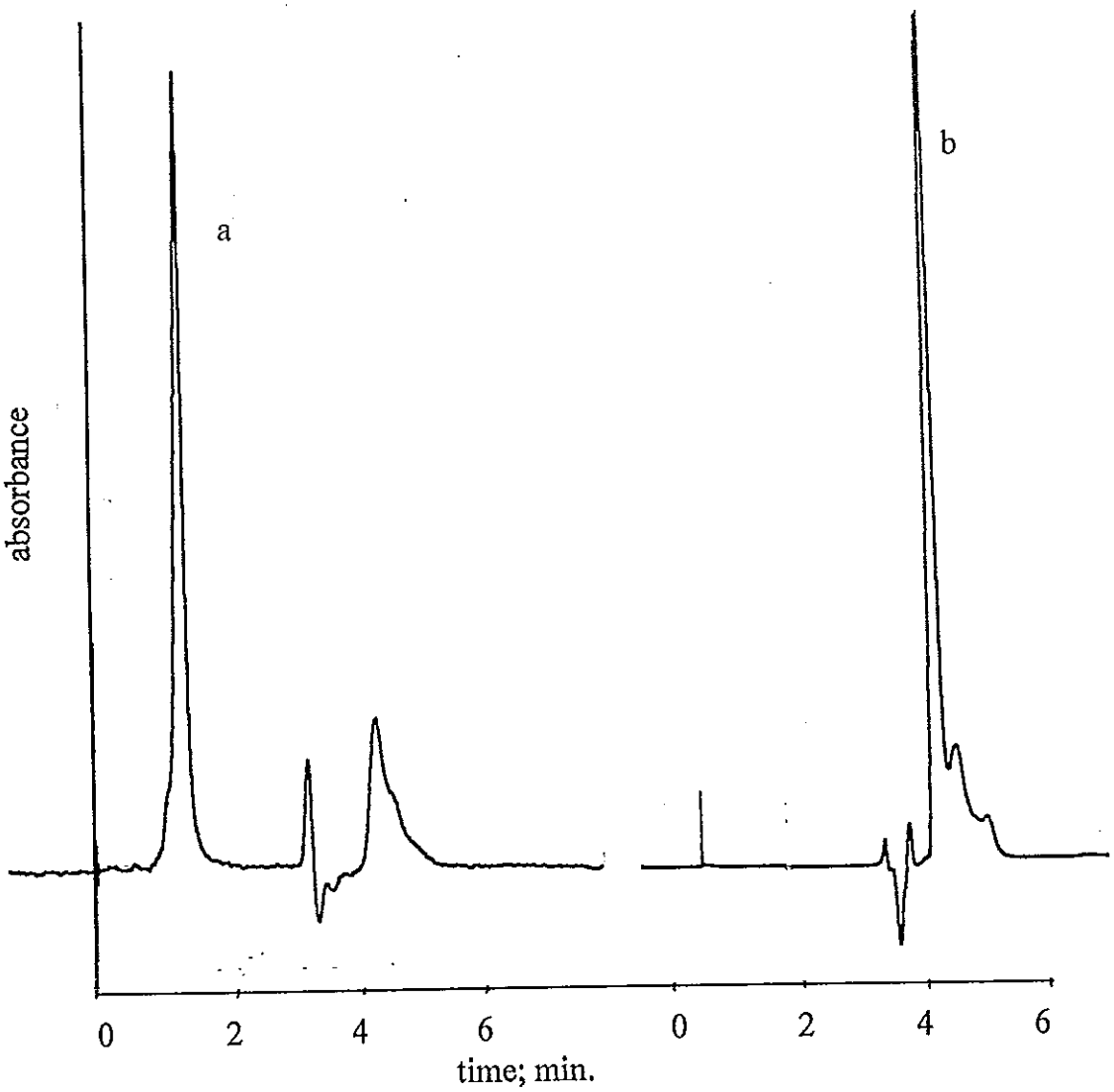

Figure 11. Chromatograms of saccharin on μBondapak C_{18} column, 300 x 3.9 mm.i.d.; using 15% v/v acetonitrile in phosphate buffer pH 3.0 mobile phase (a) and the modification with 0.1% v/v triethanolammonium (b). HPLC conditions : flow rate, 1.0 mL/min; UV-detector, 214 nm; sensitivity, 0.08 AUFS; chart speed 10 mm/min.

2.5 Linear Velocity

The column efficiency is normally expressed in terms of the *theoretical plate number (N)* of the column and can be calculated by using the equation :

$$N = 5.54 \ (t_R / W_{1/2})^2$$

where t_R is the retention time of the solute and $W_{1/2}$ is the peak width at the half height. The plate count N is approximately constant for different bands in the chromatogram, for a given set of operating conditions (a particular column and mobile phase, with fixed mobile phase velocity) N is proportional to column length (L), so that an increase in L results in an increase in N and better separation. This proportion of N and L can be expressed as :

$$N = L / H$$

where H is the so - called *height equivalent of a theoretical plate* (plate height) or HETP value. H measures the efficiency of a given column (operated under a specific set of operating conditions) per unit length of column. Small H or large N values indicate more efficient columns favored by slow mobile phase velocity (v) with long columns packed with small particles.

Column efficiency depends on H value on mobile phase velocity. The relationship between H and v for column efficiency is described in the Van Deemter equation as followed :

$$H = A + B/v + Cv$$

where A, B, and C are constants for a given column and v the linear velocity. Column efficiency also depends on some extent on the solutes, mobile phase and separation temperature. A which describes Eddy diffusion, is determined primarily by packing structure of the column bed and particle diameter of the packing material. B is a measurement of longtitudinal or axial diffusion and is proportional to the diffusion rate of the solutes in the mobile phase. In RPLC, the solutes diffusion rates are very low and this term is only significant at very low flow rates. C is concerned with mass transfer, both in mobile phase and stationary phase *(Snyder and Kirkland, 1979 : 27 - 31).*

In RPLC, the typical Van Deemter plots shows a decrease in efficiency with increasing linear velocity above the velocity optimum. In this study caffeine was selected for demonstration. The agreement of experimental results were shown in Table 6 and Van Deemter plot were given in Figure 12.

The high values of plate count at the flow rate of 0.5 mL/min retained caffeine for a long time. However, the flow rate at 1.0 mL/min was selected. because it was good separation and shorter analysis times. The flow rate at more than 1.00 mL/min was not good separation because the peaks of all additives were closed to one another.

Table 6. Influence of the linear velocity on the column efficiency of caffeine.

flow rate (mL / min)	t_o (sec)	linear velocity[a] (cm / sec)	plate counts (N)	HETP
0.50	330	0.09	3235	0.0093
0.75	219	0.14	2243	0.0134
1.00	160	0.19	1989	0.0151
1.20	134	0.22	1539	0.0195
1.50	105	0.29	1247	0.0241

[a]Linear velocity is calculated from L / t_o.

L is referred to the length of column (30 cm).

t_o is referred to the retention time of an unretained components.

HPLC conditions : μBondapak C_{18} column, 300 x 3.9 mm.i.d.; mobile phase, 15% v/v acetonitrile in triethanolammonium phosphate buffer pH 3.0; flow rate, 1.0 mL/min; UV-detector, 214 nm; sensitivity, 0.08 AUFS; chart speed 10 mm/min.

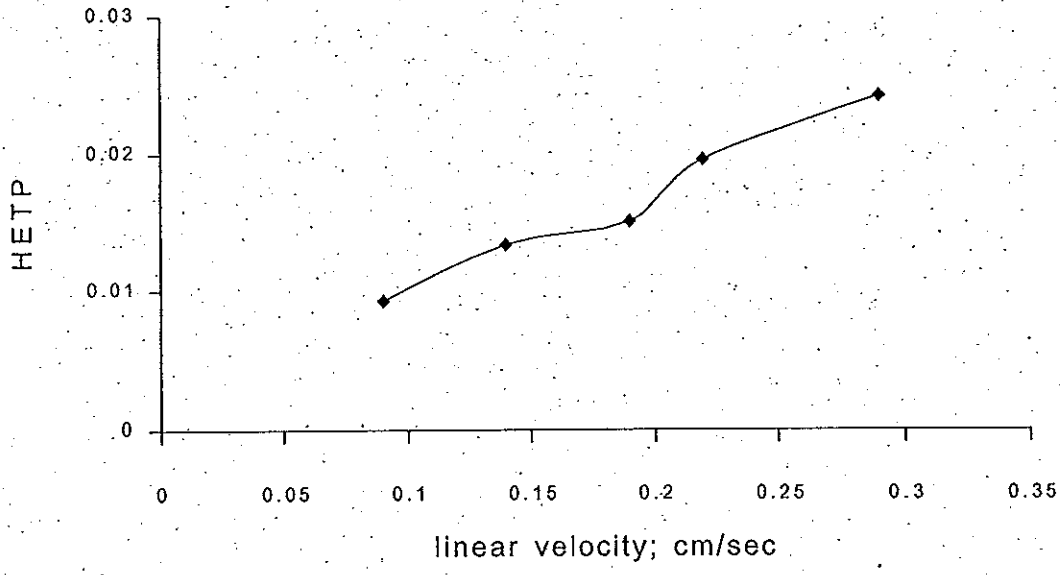

Figure 12. Van Deemter plot of caffeine in the mobile phase consisting of 15% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate buffer pH 3.0. HPLC conditions : μBondapak C_{18} column, 300 x 3.9 mm.i.d.; flow rate, 1.0 mL/min; UV-detector, 214 nm; sensitivity, 0.08 AUFS; chart speed 10 mm/min.

2.6 Selection of the Optimum Mobile Phase

The optimum mobile phase was confirmed by comparison of two values of pH at 3.0 and 4.3 , and two values of acetonitrile concentration at 12% v/v and 15% v/v in 1.0% v/v triethanolammonium phosphate buffer. Table 7 showed the variation of the retention time and selectivity. The retention time of all additives decreased with increasing acetonitrile concentration and increasing pH. The chromatogram of standard solution consisting of 10.0 μg / mL saccharin , 10.0 μg/mL caffeine and 100.0 μg/mL aspartame was shown in Figure 13. The mobile phase consisting of 15% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate buffer pH 3.0 gave the retention time of saccharin (3.8 min) near the unretained solute retention time (t_o = 2.9 min) (Table 7 and Figure 14). The peaks of caffeine and aspartame were closed to one another (α_2 at 1.88) with mobile phase consisting of 12% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate buffer pH 4.3 (Table 7 and Figure 15). The mobile phase consisting of 12% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate buffer pH 3.0 provided similar selectivity to the observed condition at 15% v/v acetonitrile in 0.1% triethanolammonium phosphate buffer pH 4.3 (α_1 at 3.69 , α_2 at 1.31 and α_1 at 4.33 , α_2 at 1.35, respectively.) However , the retention time of saccharin under the condition at 12% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate buffer pH 3.0 (4.2 min) was further from t_o than the condition at 15% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate buffer pH 4.3 (3.5 min).

Mobile phase containing 12% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate buffer pH 3.0 also gave clear resolution between the peaks of caffeine and aspartame (no peaks overlapping) as shown chromatograms in Figure 13 and thus could be concluded that the optimum mobile phase was 12% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate buffer pH 3.0.

Table 7. Variation of the retention time and selectivity of saccharin, caffeine and aspartame as a function of percentage of acetonitrile concentration at different pH of triethanolammonium phosphate buffer mobile phase.

pH	acetonitrile concentration (%)	t_o (min)	retention time (min)			selectivity (α)[a]	
			saccharin	caffeine	aspartame	1	2
3.0	12	2.9	4.2	7.7	9.2	3.69	1.31
3.0	15	2.9	3.8	5.9	7.3	3.33	1.47
4.3	12	2.9	3.9	7.8	8.7	4.90	1.18
4.3	15	2.9	3.5	5.5	6.4	4.33	1.35

[a]Selectivity 1 = $(t_2 - t_o) / (t_1 - t_o)$

Selectivity 2 = $(t_3 - t_o) / (t_2 - t_o)$

t_1, t_2 and t_3 was referred to the retention time of saccharin, caffeine and aspartame, respectively.

HPLC conditions : μBondapak C_{18} column, 300 x 3.9 mm.i.d.; flow rate, 1.0 mL/min; UV-detector, 214 nm; sensitivity, 0.08 AUFS; chart speed, 10 mm/min.

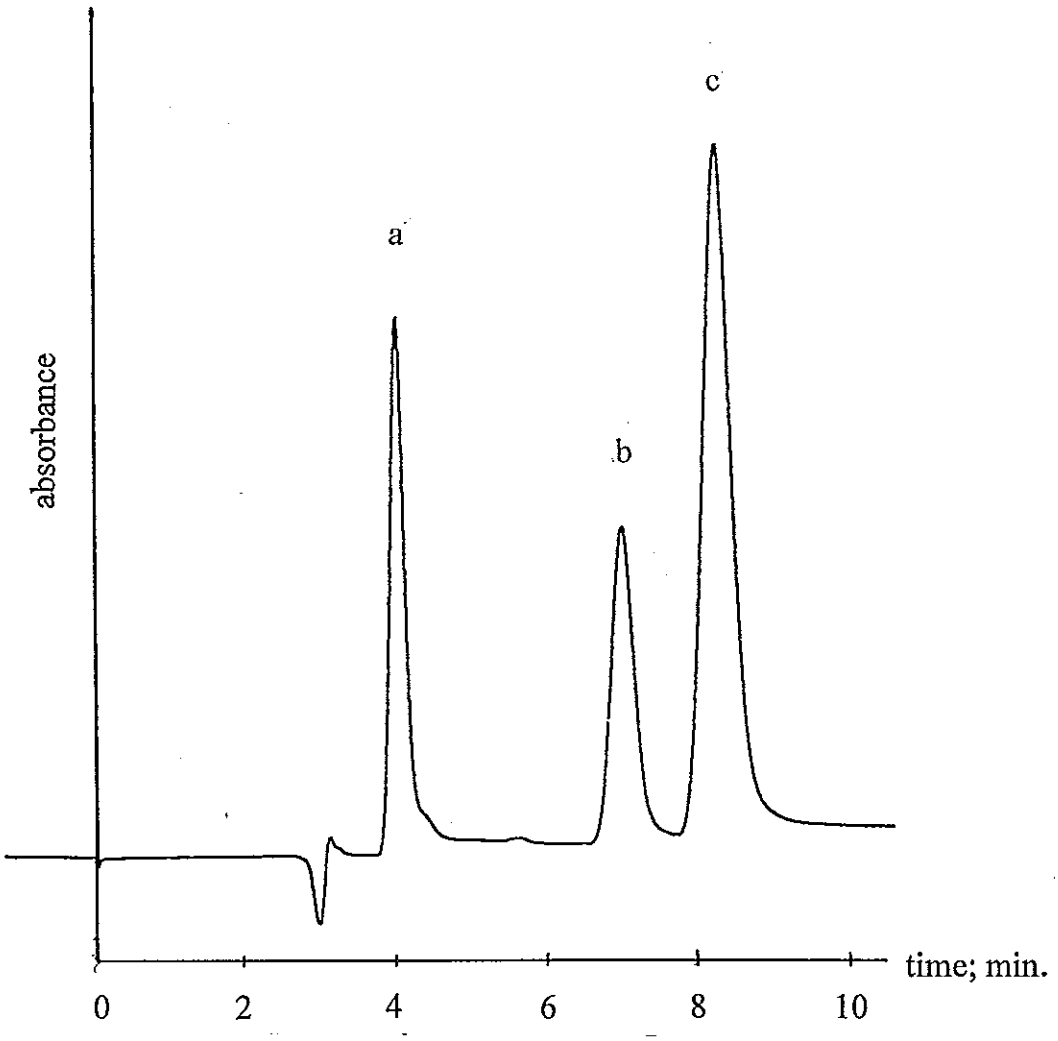

Figure 13. Chromatograms of standard solutions consisting of 10 μg / mL saccharin (a), 10 μg / mL caffeine (b) and 100 μg / mL aspartame (c). HPLC conditions : μBondapak C_{18} column, 300 x 3.9 mm.i.d.; 12% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate mobile phase buffer pH 3.0; flow rate, 1.0 mL/min; UV-detector, 214 nm; sensitivity, 0.08 AUFS.

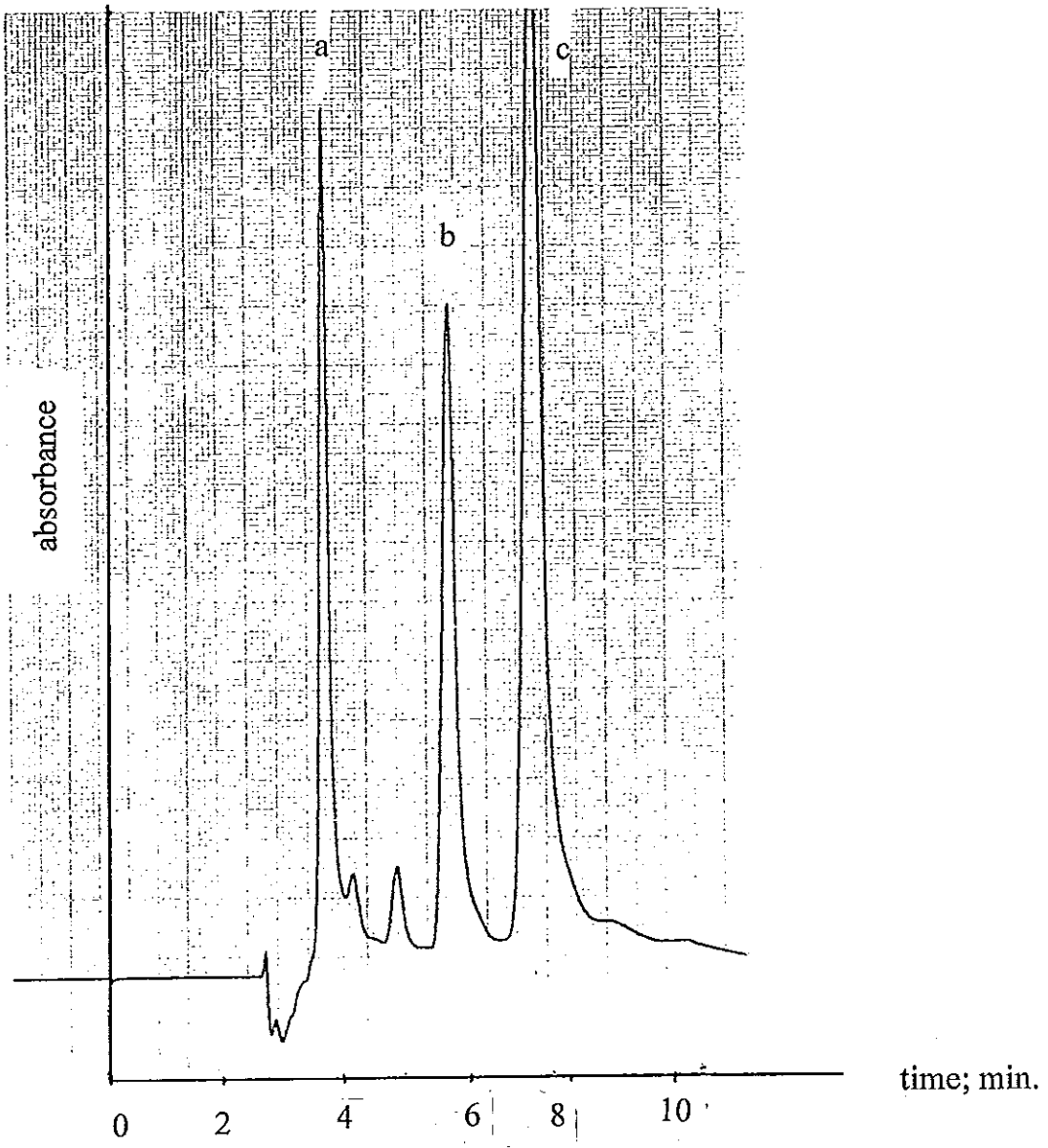

Figure 14. Chromatograms of standard solution consisting of 10 μg / mL saccharin (a), 10 μg / mL caffeine (b) and 100 μg / mL aspartame (c). HPLC conditions : μBondapak C_{18} column, 300 x 3.9 mm.i.d.;15% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate mobile phase buffer pH 3.0; flow rate, 1.0 mL/min.;UV-detector, 214 nm; sensitivity, 0.08 AUFS.

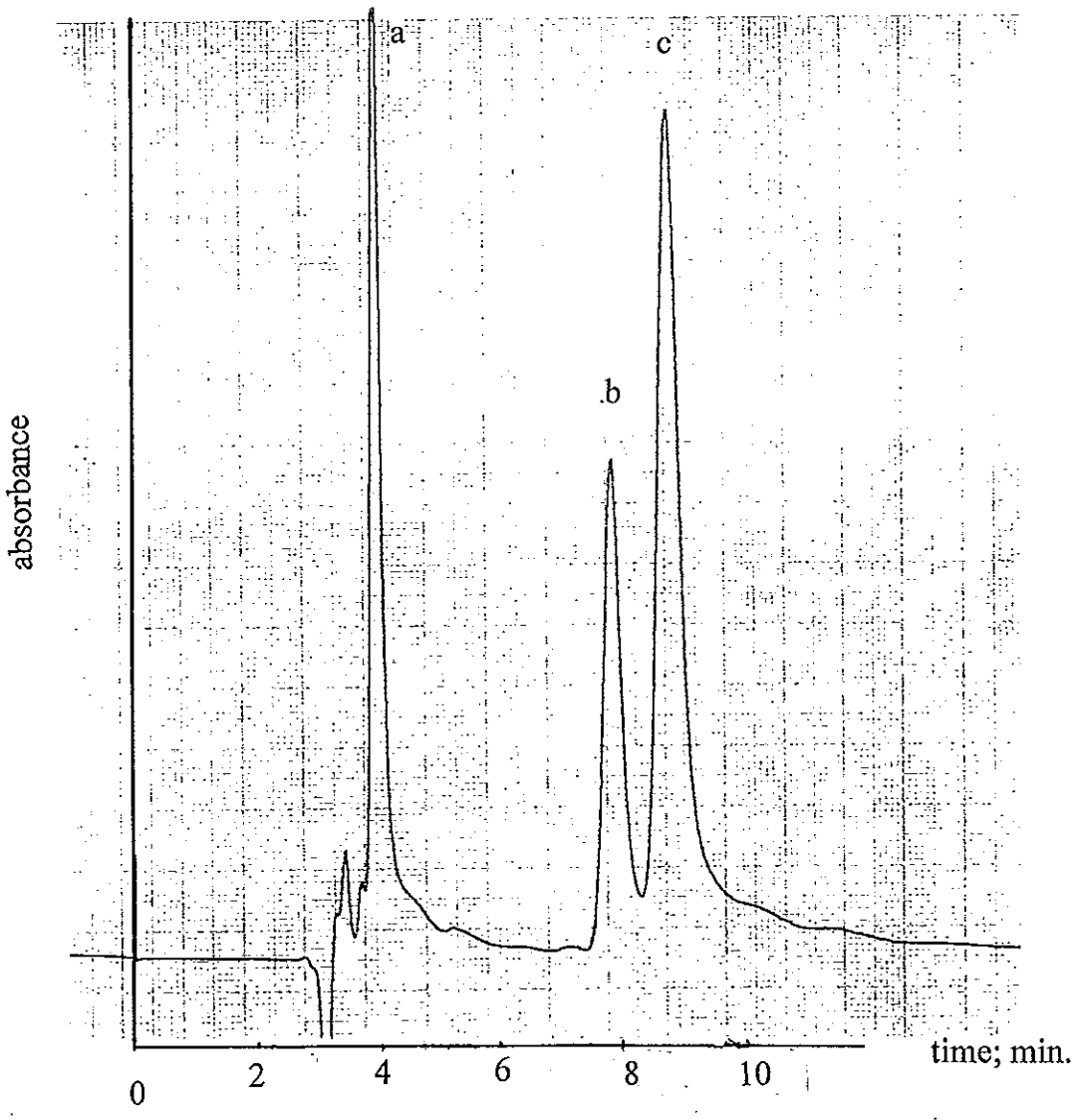

Figure 15. Chromatograms of standard solution consisting of 10 μg / mL saccharin (a), 10 μg / mL caffeine (b) and100 μg / mL aspartame (c). HPLC condition : μBondapak C_{18} column 300 x 3.9 mm.i.d.; 12% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate mobile phase buffer pH 4.3; flow rate, 1.0 mL/min; UV-detector, 214 nm; sensitivity, 0.08 AUFS.

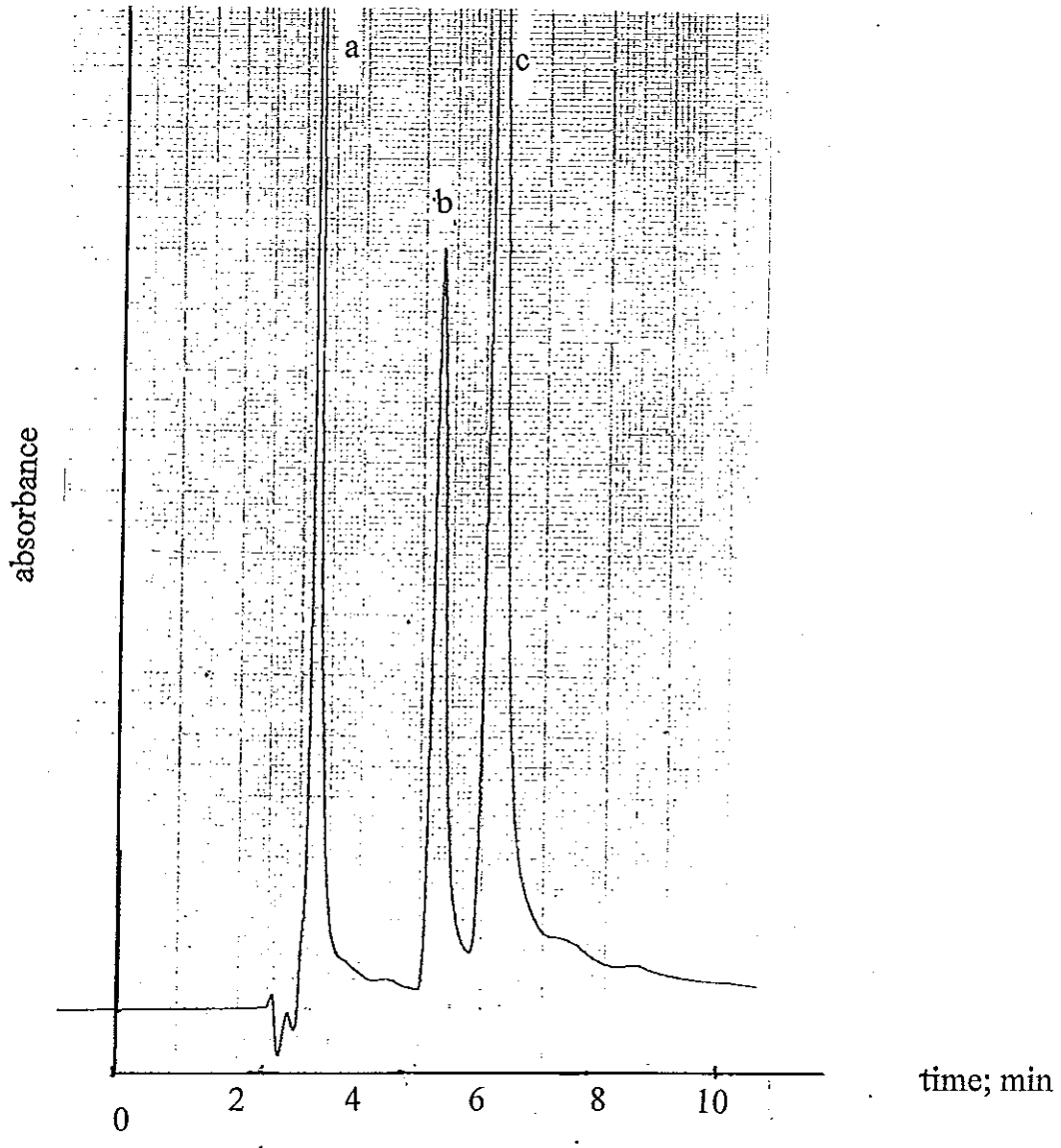

Figure 16. Chromatograms of standard solution consisting of 10 µg / mL saccharin (a), 10 µg / mL caffeine (b) and 100 µg / mL aspartame (c). HPLC conditions : µBondapak C_{18} column, 300 x 3.9 mm.i.d.; 15% acetonitrile in 0.1 % v/v triethanolammonium phosphate mobile phase buffer pH 4.3; flow rate, 1.0 mL/min; UV-detector, 214 nm; sensitivity, 0.08 AUFS.

2.7 Limits of Detection

The minimum amount of a species that can be reliable seen on the chromatogram is the detection limit. Usually detection limits are measured with an individual species in a standard solution. In a real sample, the detection limit for these species would usually be higher due to baseline disturbance or interference from the matrixes or other species. In this experiment, the detection limits of the standard solutions of saccharin, caffeine and aspartame were 0.02, 0.02 and 0.20 μg / mL, respectively. These detection limits were measured manually from chromatograms that gave individual peak where signal was higher than two times of the baseline noises.

3. Application of Developed HPLC Condition for Dietary Beverages

Saccharin, caffeine and aspartame in dietary beverages were successfully separated using the mobile phase consisting of 12% v/v acetonitrile in 0.1% v/v triethanolamonium phosphate buffer adjusted to pH 3.0 with NaOH. The sample was degassed, diluted in water and injected onto a C_{18} column. The retention times of saccharin, caffeine and aspartame were 4.2, 7.7 and 9.2 minutes, respectively. The chromatogram of standard solution containing three additives was shown in Figure 13.

The precision was confirmed by determinating the relative standard deviation of the resulting peak heights. The relative standard deviations were 2.24%, 2.21% and 0.46% for 10 μg/mL saccharin, 10 μg/mL caffeine and 100 μg/mL aspartame, respectively.

The calibration curves of saccharin, caffeine and aspartame were constructed by plotting the peak heights of each additive versus known concentrations of particular standard additive by using the data from Table 8 - Table 10. Typical calibration curves were shown in Figure 17 - Figure 19.

The calibration curves of all additives were linear over most of the concentration range studies, and therefore, accurate results could be obtained using the linear portion of the calibration curve. A computer software was used to calculate the regression line. These allowed the subsequent calculation of the concentrations in unkown samples, which would be more accurate and precise than direct reading from a graph. However, the concentration of an unknown sample must fall in the linear portion of the curve. In this procedure, dietary beverages were diluted with water (5 : 100 v/v), so the concentrations of additives calculating from linear regression were multiplied by dilution factor of 20. The concentrations of additives in dietary beverages and others were summarized in Table 11. Chromatogram of dietary beverage (Diet Coke) sample solution (dilute 5 : 100) was shown in Figure 20. Equations of the calibration curve of the studied additives were as follows :

The calibration curve of saccharin was $y = -0.144 + 0.679x$ with regression coefficient; $r = 1.000$

The calibration curve of caffeine was $y = -0.0492 + 0.422x$ with regression coefficient; $r = 1.000$

The calibration curve of aspartame was $y = -0.375 + 0.0981x$ with regression coefficient; $r = 0.999$

where y was the peak height; cm

x was the concentration; μg / mL

The response linearity of method was established by injecting three replications of the standard solutions. The range of relative standard deviations were 1.13 to 2.27 percent for saccharin, 0.00 to 2.21 percent for caffeine and 0.46 to 1.72 percent for aspartame at the range of concentration 2.0 - 20.0 μg/mL, 2.0 - 20.0 μg/mL and 20.0 - 150.0 μg/mL, respectively. Correlation coefficients of better than 0.999 were found for all standard curves.

Table 8. Standard concentrations of saccharin in aqueous solutions.

saccharin concentration (μg / mL)	peak height (cm)	RSD(%)[a]
2	1.27	2.27
5	3.22	0.89
10	6.60	2.24
20	13.47	1.13

Table 9. Standard concentrations of caffeine in aqueous solutions.

caffeine concentration (μg / mL)	peak height (cm)	RSD (%)[a]
2	0.80	0.00
5	2.02	1.40
10	4.24	2.21
20	8.38	0.91

Table 10. Standard concentrations of aspartame in aqueous solutions.

aspartame concentration (μg / mL)	peak height (cm)	RSD (%)[a]
20	1.73	1.34
50	4.43	1.72
100	9.27	0.46
150	14.47	1.06

[a] n = 3

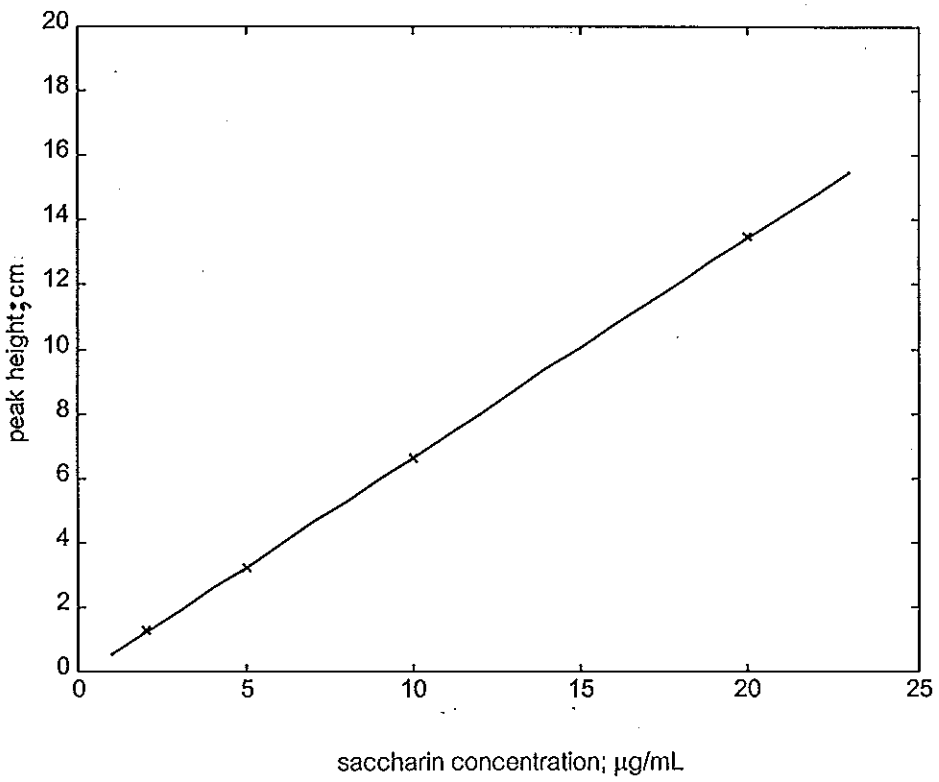

Figure 17. Calibration curve for the determination of saccharin in aqueous solution. Regression analysis by the least-squares method yielded a slope of 0.679 and an intercept of -0.144 ($r = 1.000$). HPLC conditions : µBondapak C_{18} column, 300 x 3.9 mm.i.d.; 12% v/v acetonitrile in triethanolammonium phosphate buffer pH 3.0; flow rate, 1.0 mL/min; UV-detector, 214 nm; sensitivity, 0.08 AUFS; chart speed, 10 mm/min.

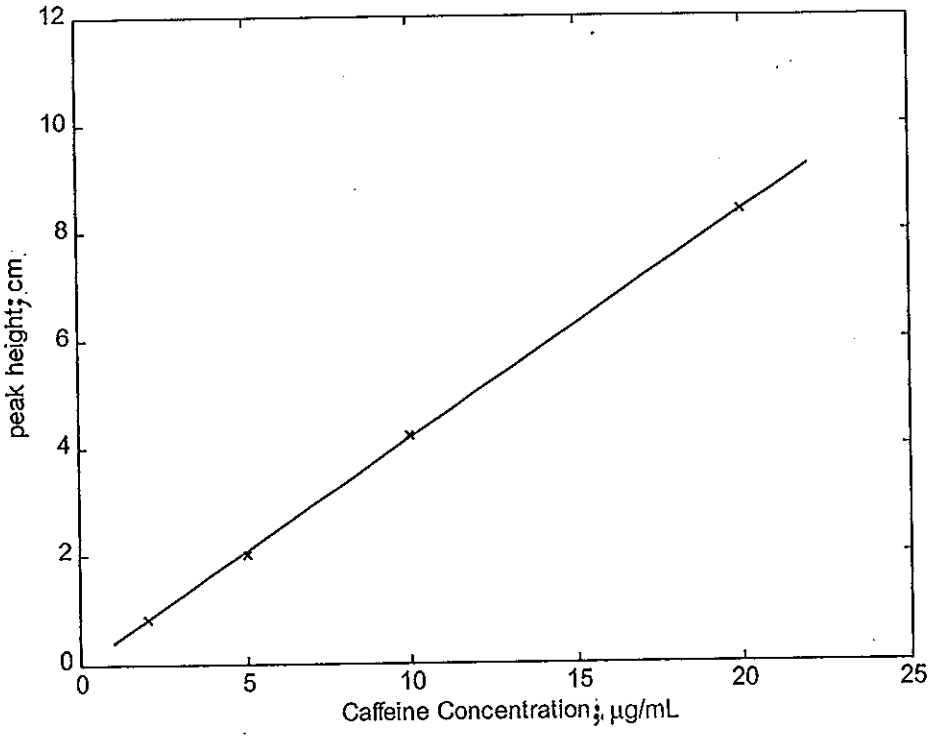

Figure 18. Calibration curve for the determination of caffeine in aqueous solution. Regression analysis by the least-squares method yielded a slope of 0.422 and an intercept of -0.0492 (r = 1.000). (HPLC conditions was as in Figure 17).

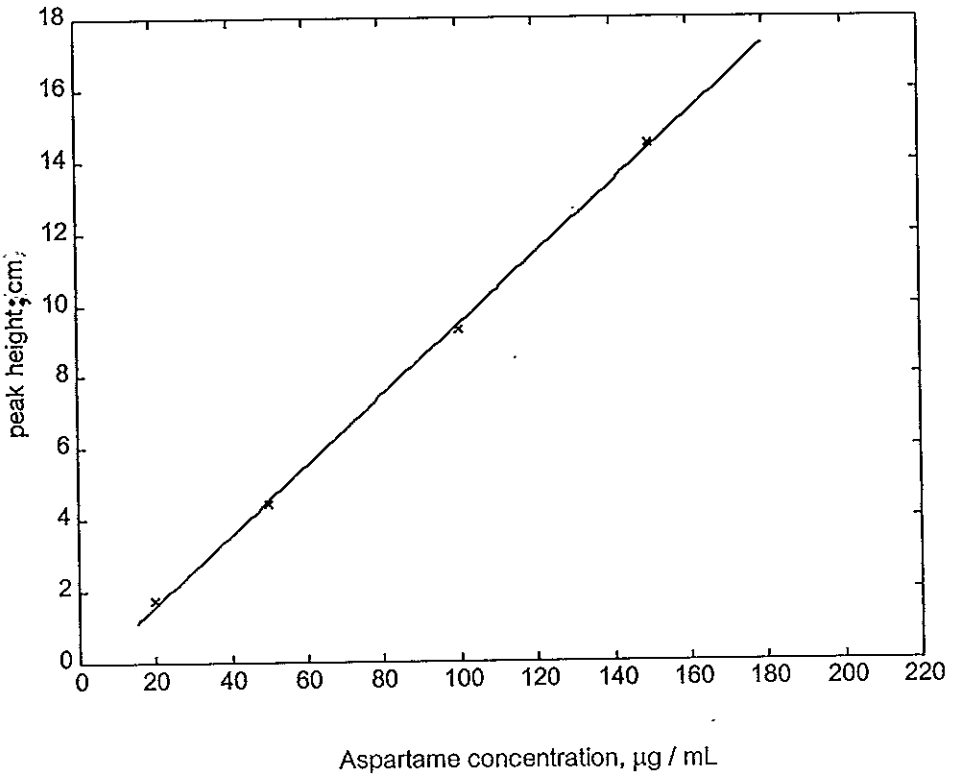

Figure 19. Calibration curve for the determination of aspartame in aqueous solution. Regression analysis by the least-squares method yielded a slope of 0.098 and an intercept of -0.375 ($r = 0.999$) (HPLC conditions was as in Figure 17).

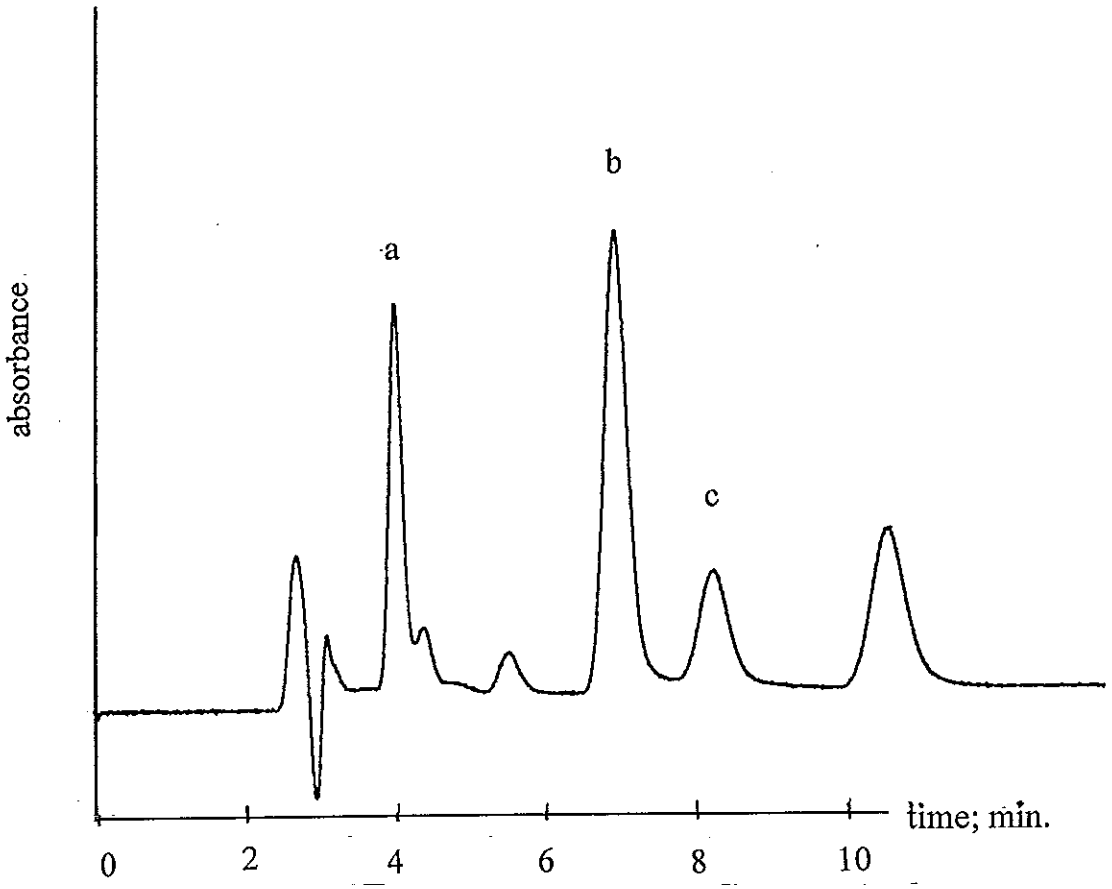

Figure 20. Chromatograms of dietary beverages (Diet Coke) sample solutions (dilute 5 : 100) consisted of saccharin (a), caffeine (b) and aspartame (c). HPLC conditions : μBondapak C_{18} column, 300 x 3.9 mm.i.d.; 12% v/v acetonitrile in 0.1 % v/v triethanolammonium phosphate buffer pH 3.0; flow rate, 1.0 mL/min; UV-detector, 214 nm; sensitivity, 0.08 AUFS.

Table 11. Concentrations of saccharin, caffeine and aspartame in dietary beverages.

sample	concentration (μg / mL)					
	saccharin	RSD[a] (%)	caffeine	RSD[a] (%)	aspartame	RSD[a] (%)
DIET COKE	81.41	2.20	134.09	1.04	192.62	0.50
PEPSI MAX	18.97	0.00	111.34	0.00	498.37	1.40
COKE	-	-	167.26	0.83	-	-
PEPSI	-	-	173.90	0.79	-	-
SPRITE	-	-	-	-	-	-
FANTA	-	-	-	-	-	-
RED FANTA	-	-	-	-	-	-
GREEN FANTA	-	-	-	-	-	-
7 UP	-	-	-	-	-	-
GREEN SPOT	-	-	-	-	-	-

(-) means undetectable

[a]n = 3

CHAPTER 4

CONCLUSION

According to the study of various effects on the elution behavior of the caffeine, saccharin and aspartame investigated by RPLC techniques, it is shown that :

1. The relationship between the capacity factors of caffeine and aspatame with acetonitrile concentrations in the mobile phase was an exponential curve. That was in agreement with the increase in concentration of mobile phase and resulted in a decrease in the capacity factor.

2. The addition of sodium chloride to the 12% v/v acetonitrile in aqueous mobile phase appeared to have a little change in retention time.

3. The elution behavior of the caffeine and saccharin did not significantly change their retention time with increase in pH.

4. Triethanolamine of 0.1% v/v in phosphate buffer containing 12% v/v acetonitrile at pH 3.0 had an effect on capacity factor of saccharin by increasing the retention time.

It could be concluded that the optimum of mobile phase condition for the determination of caffeine, saccharin and aspartame standard solutions was the 12% v/v acetonitrile in 0.1% v/v triethanolammonium phosphate buffer, which was adjusted to pH 3.0 with sodium hydroxide. This mobile phase provided optical transparency at short wavelengths. Detection at 214 nm provided increasing response to both saccharin and aspartame.

This developed method was similar to the report of *Tyler (Tyler, 1984 : 745-747)* that was the determination of sodium saccharin, caffeine, aspartame, and sodium benzoate in dietary beverages. The sample was degassed, diluted in water, and injected onto a C_{18} column. The mobile phase consisted of 15% v/v acetonitrile in triethylammonium phosphate buffer adjusted to pH 4.3 with NaOH, but this method used a smaller volume of acetonitrile (only 12% v/v).

This method could apply to dietary beverages containing caffeine, saccharin and aspartame. Moreover, there were many advantages in this technique including rapid method, using a small volume of sample, high precision, low detection limits and selectivity.

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