Abstract

SEPARATION AND ANALYSIS OF PRESERVATIVES IN SKINCARE CREAMS BY HIGH TEMPERATURE LIQUID CHROMATOGRAPHY AND SUBCRITICAL WATER CHROMATOGRAPHY

By

Leena Gujjar

May 2011

Chair: Dr. Rickey P. Hicks

Major Department: Chemistry

Preservatives are chemicals with antimicrobial activity commonly added to foods, pharmaceuticals and cosmetics in order to prolong products' shelf life and to protect the consumer from potential infection. Parabens, the most widely used preservatives worldwide are a family of alkyl esters of *para*-hydroxybenzoic acid. The most widely marketed *para*-hydroxybenzoic acid esters are methyl, ethyl, propyl and butyl parabens. Their microbial activity increases as the alkyl chain increases. Parabens are reported to have weak estrogen-like properties. According to Cosmetic Ingredient Review (CIR), limit up to 0.4% (single paraben) or up to 0.8% (mixtures of parabens) can be added to the cosmetic products. Therefore, to monitor the levels of preservatives in cosmetic products is important.

High-performance liquid chromatography (HPLC) is the most commonly used separation and analysis technique for the determination of preservative in skincare creams. HPLC involves a consumption of large quantities of organic solvents in the mobile phase. These HPLC organic solvents are toxic, expensive for purchasing as well as their proper disposal.

At ambient temperature, water is too polar to serve as a sole chromatographic solvent. Fortunately, at elevated temperatures and under moderate pressures, the polarity of water significantly decreases and liquid water behaves more like an organic solvent. Thus, high-temperature water can mimic organic solvent-water mixtures used in HPLC to achieve liquid chromatographic separation.

The goal of this research is to develop high temperature liquid chromatography (HTLC) and subcritical water chromatography (SBWC) methods for the separation and analysis of preservatives in skincare creams to either reduce or completely eliminate the consumption of the harmful organic solvents used in traditional HPLC. A ZirChrom®-DiamondBond-C18 column was used in this study to carry out the separation of preservatives. Preservatives studied include benzyl alcohol, 2-phenoxyethanol, methyl, ethyl, propyl, and butyl paraben. Quantitative analysis of preservatives in three Olay® skincare creams was achieved by HTLC and SBWC. The recoveries obtained by HTLC and SBWC are efficient. The major advantage of HTLC and SBWC techniques is the reduction or elimination of organic solvents used in traditional HPLC.

SEPARATION AND ANALYSIS OF PRESERVATIVES IN SKINCARE CREAMS BY HIGH TEMPERATURE LIQUID CHROMATOGRAPHY AND SUBCRITICAL WATER CHROMATOGRAPHY

A Thesis

Presented To

The Faculty of the Department of Chemistry

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

Leena Gujjar

May 2011

SEPARATION AND ANALYSIS OF PRESERVATIVES IN SKINCARE CREAMS BY HIGH TEMPERATURE LIQUID CHROMATOGRAPHY AND SUBCRITICAL WATER CHROMATOGRAPHY

By

Leena Gujjar

APPROVED BY:

DIRECTOR OF THESIS: _____

Yu Yang, PhD

COMMITTEE MEMBER: _____

Shouquan Huo, PhD

COMMITTEE MEMBER: _____

Eli G. Hvastkovs, PhD

COMMITTEE MEMBER: _____

Tim Christensen, PhD

CHAIR OF THE
DEPARTMENT OF CHEMISTRY: _____

Rickey P. Hicks, PhD

DEAN OF THE
GRADUATE SCHOOL: _____

Paul J. Gemperline, PhD

DEDICATION

This thesis is dedicated to my mom, Suguna Gujjar, my dad Kashinath Gujjar and my husband, Sreenivas Punna.

Without their love, support, and patience I would not have been able to succeed in this endeavor.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude and appreciation to my advisor and guide, Dr. Yu Yang for his constant advice, support and guidance, but most importantly, for making time to have an open door and encouraging word, throughout my graduate endeavors. I thank him for giving me the opportunity to work under his guidance and hone my knowledge under his supervision. Thank you for everything.

This project was funded by The Procter & Gamble Company (P&G). I would like to extend my thanks to Dr. Ronita Marple and Dr. Chris Gamsky from P&G for their continuous support during this project.

I would like to express my thanks to Dr. Shouquan Huo, Dr. Eli G. Hvastkovs, and Dr. Tim Christensen for being on my thesis committee.

My thank also go to Phil Hodges, Kym Rispress, Jerry Mizell and Jay V. Wilkerson of Metrics, Inc. for their encouragement and support.

I appreciate the assistance given by the members of our research group: Edward Lindquist, Zack Strickland, Samar Hardous, Ann Marie Toebes, Marina Rybnikova, Bryan Pate, Aaryn D. Jones, Brahmam Kaplavavi and Amandeep Gujral.

I also thank Craig Hamilton of H&A Scientific, Inc. for providing the PC/Chrom interface and software.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

atm	Atmosphere
cP	Centipoise
cm	Centimeter
CIR	Cosmetic Ingredient Review
ºC	Degree Celsius
ε	Dielectric constant
dyn	Dyne
e.g.	exempli gratia, for Example
FIA	Flow injection analysis
FID	Flame ionization detector
FDA	Food and Drug Administration
FT-IR	Fourier transform-Infrared
g	Gram
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HTLC	High temperature liquid chromatography
Hrs	Hours
i.d.	Internal diameter
IR	Infrared
J	Joule
K	Kelvin

L	Length
u	Linear velocity
LC	Liquid chromatography
MS	Mass spectrometer
m	Meter
MΩ-cm	Megohm centimeter
min	Minute
mL	Milliliter
mm	Millimeter
mM	Milli-molar
mol	Mole
M	Molar concentration
µL	Micro-liter
µm	Micro-meter
ng	Nanogram
nm	Nanometer
n	Number of replicate experiments
NMR	Nuclear magnetic resonance
H	Plate height
N	Plate number
PGC	Porous Graphitic Carbon
PS-DVB	Polystyrene divinylbenzene
P&G	Procter & Gamble

RPLC	Reverse-phase liquid chromatography
% RSD	Percent relative standard deviation
SBWC	Subcritical water chromatography
SBWE	Subcritical water extraction
SPF	Sun protection factor
T	Temperature in Kelvin
TLC	Thin layer chromatography
UV	Ultraviolet
UV-DAD	Ultraviolet-diode array detector
UFLC	Ultrafast liquid chromatography
wt%	Weight percentage

CHAPTER 1: INTRODUCTION

1.1 Preservatives

A preservative is a naturally occurring or synthetic agent that is added to products such as food, wood, pharmaceuticals, biological samples, paints and cosmetics. The preservatives are added to products, first, to prevent microbial spoilage and therefore to prolong the shelf life of the product and second, to protect the consumer from a potential infection [1]. They are very minor ingredients in raw materials and formulations with concentrations of typically lower than 1%, yet they are essential in providing the requisite shelf life and use life in all aqueous systems that contain significant levels of hydrocarbons as a potential food source for a wide variety of microflora. They are frequently used in combination to achieve the required broad coverage; thus combining parabens with phenoxyethanol combats gram-negative and gram-positive bacteria, moulds, and yeast [2]. Some of the common preservatives used in cosmetic industry are sorbic acid, benzoic acid, benzyl alcohol, and hydroxybenzoates (parabens).

Parabens are a family of alkyl esters of *para*-hydroxybenzoic acid that differ at the para position of the benzene ring by various chemical substitutions. The most widely marketed parabens are methylparaben, ethylparaben, propylparaben, butylparaben and benzylparaben. They are popular because they are inexpensive, colorless, odorless, and non-toxic. They are effective over an extensive range of hydrogen ion concentration (pH) and have a wide spectrum of activity against yeasts, molds, and bacteria [3]. Parabens act as microbiostatic agents by increasing cell wall permeability and thereby disrupting membrane transport process. They also alter cellular respiration, electron transport, and oxidative enzyme systems of microbes [4, 6]. They were first

used as antimicrobial preservatives in pharmaceutical products in the mid 1920s. Parabens are prepared in the presence of a catalyst and are generally oil soluble. Their antimicrobial activity increases as the ester chain length increases. These compounds are stable in air as well as in acidic solutions, and conditions of sterilization.

There has been controversy about the use of parabens in cosmetics over the years. The main concern is that parabens are carcinogenic and have estrogenic effects. However, research revealed that no correlation between parabens and cancer has been found [5].

The Cosmetic Ingredient Review (CIR) Expert Panel [6] in 2008 reviewed the safety of parabens and concluded that parabens are used in over 22,000 cosmetics as preservatives and are safe for use in cosmetic levels up to 0.8% (mixtures of parabens) or up to 0.4% (single paraben). The industry estimates of the daily use of cosmetic products containing parabens are 17.76 g per adult and 0.378 g per infant.

The accurate analysis of preservative levels in various products is important. The quantitative analysis of preservatives in cosmetics is required for quality control, product release, and regulatory purposes. High-performance liquid chromatography (HPLC) with UV detection has been widely used for the determination of preservatives in foods, cosmetics, and pharmaceuticals [6-11]. As HPLC is the commonly used technique for the separation of preservatives, an obvious disadvantage of this technique is the use of large amounts of organic solvents in mobile phases.

1.2 The Need for Greener Chromatography Technique

In recent years there has been a considerable interest in the adoption of clean technology to reduce the usage of organic solvents or complete elimination of them in chromatographic separations as these organic solvents used in reversed-phase liquid chromatography (RPLC) are toxic, flammable and expensive in terms of both purchasing as well as their waste disposal costs. Fortunately, high temperature liquid chromatography or subcritical water chromatography can reduce or eliminate the use of organic solvents used in the mobile phase.

1.2.1 High Temperature Liquid Chromatography (HTLC)

High temperature liquid chromatography refers to any HPLC separations carried out at elevated temperatures in order to reduce the use of organic solvents in the mobile phase. Recently, temperature is considered as another important variable in traditional high-performance liquid chromatography method development. At elevated temperature, the viscosity of the mobile phase is decreased, thus increasing the analyte diffusivity [12]. The consequence of the reduced viscosity is a lowered backpressure over the column, which allows high speed of analysis and the possibility to use longer columns with smaller particle size [13]. The increased mass transfer usually results in improved efficiency [14]. According to Kondo and Yang [15], an increase of 3.5 °C in separation temperature is comparable to a 1% increase in methanol and a temperature increase of 5-8 °C can be compared to a 1% increase in acetonitrile in the mobile phase. Water at elevated temperatures can thus replace a large proportion of organic solvent in the mobile phase.

1.2.2 Subcritical Water Chromatography (SBWC)

Subcritical water refers to the water that is heated under pressure to below its critical point of 374 °C and 218 atm. Other terminologies used for water under subcritical conditions include superheated water, pressurized hot water and high temperature water. Chromatographic separation using subcritical water has been termed subcritical water chromatography, pressurized water chromatography or superheated water chromatography [16, 17].

Water at ambient temperature has very high polarity (dielectric constant, ε = ~78 at 25 °C) that precludes it from being an effective mobile phase in traditional reversed phase liquid chromatography, as it has minimal eluotropic strength [18]. Fortunately, the polarity of water significantly decreases at elevated temperatures and under moderate pressure that keeps water in the liquid state [19]. Thus high temperature water has similar properties such as polarity, surface tension and viscosity to typical methanol-water or acetonitrile-water mixtures used in RPLC [20, 21]. Thus by controlling the temperature, subcritical water has the potential to replace organic solvents in reverse-phase separations.

1.3 Goal of This Research

As mentioned earlier, large amounts of organic solvents are still required in existing HPLC methods for separation and analysis of preservatives. Therefore, the main objectives of this research are:

1. To investigate the potential of using high temperature liquid chromatography separation technique for the separation and analysis of preservatives in skincare creams in order to reduce the amount of organic solvent used in the mobile phase.
2. To investigate the potential of using subcritical water chromatography separation technique for the separation and analysis of preservatives in skincare creams in order to eliminate the consumption of organic solvent in the mobile phase.

To accomplish these objectives, a homemade system was constructed and used in this study. The detailed procedure and experimental conditions are described in Chapter 3. High temperature liquid chromatography separation and analysis were carried out on a polystyrene divinylbenzene (PS-DVB) PRP-1 column using gradient elution at temperature of 80 °C and flow rates of 1.2 – 1.4 mL/min. Then, HTLC separation and analysis were conducted on a zirconia-based ZirChrom®-DiamondBond-C18 column using gradient elution at temperatures ranging from 80 °C to 150 °C and flow rates of 1.4 – 3.5 mL/min. UV detection at 256 nm was used in this study. Quantification analyses were performed on three Olay® skincare cream samples. The potential building-up problem was also studied.

Subcritical water chromatography separation and analysis were carried out on the same ZirChrom®-DiamondBond-C18 column with pure water as the mobile phase at 200 °C with flow

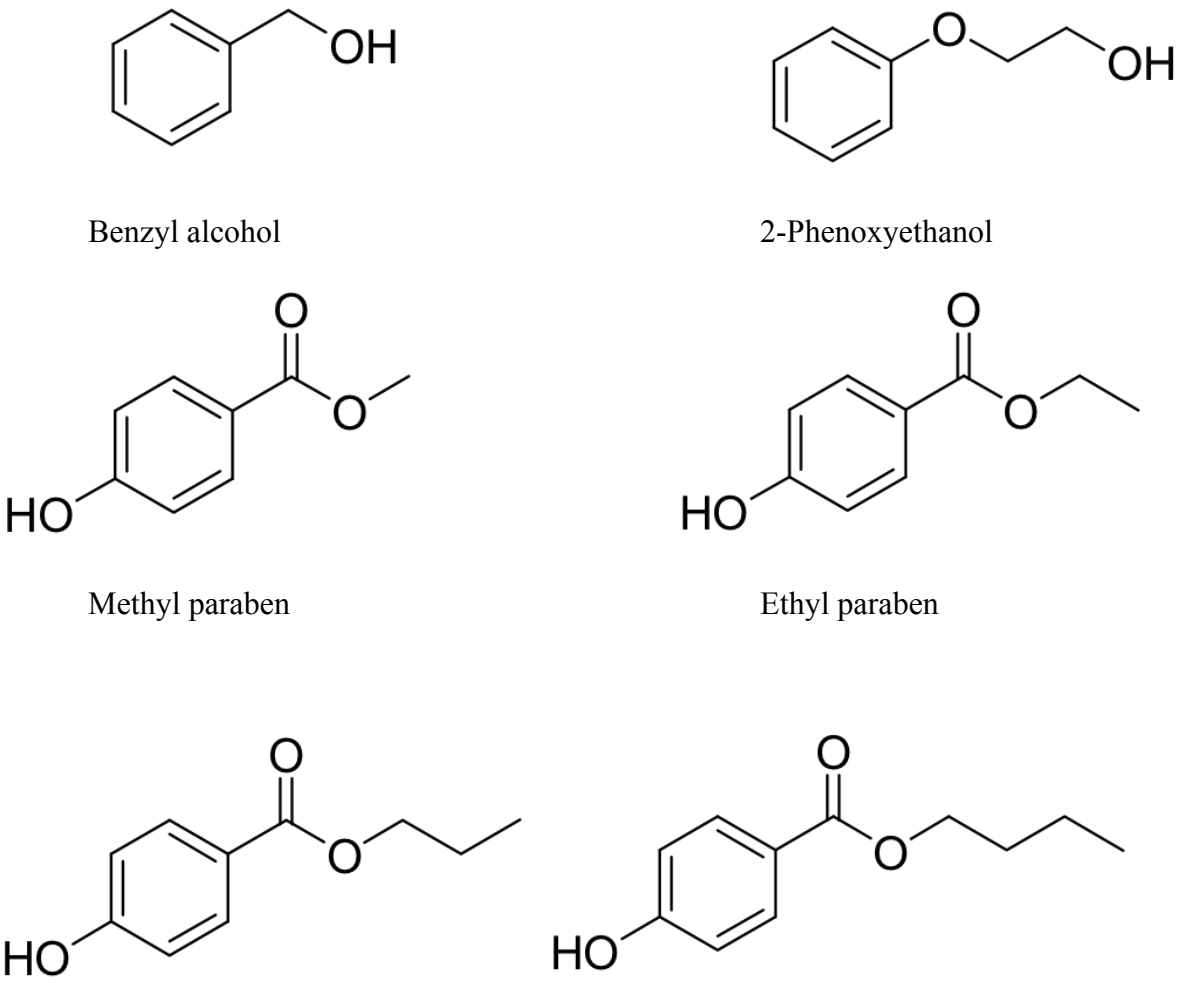
rates ranging from 1.25 – 2.5 mL/min. UV detection of 256 nm was used in this study. Quantification of an Olay® skincare cream sample was carried out. The long-term stability of the ZirChrom®-DB-C18 column was also studied at 200 °C using pure water as mobile phase with 1.5 mL/min flow rate.

CHAPTER 2: BACKGROUND

2.1 Preservatives

2.1.1 Preservative Structures

The structures of preservatives are shown in Figure 2.1.



Benzyl alcohol

2-Phenoxyethanol

Methyl paraben

Ethyl paraben

Propyl paraben

Butyl paraben

Figure 2.1. Structures of preservatives.

2.1.2 Preservative Properties

Parabens in pure form are generally small colorless crystals or white crystalline powders with practically no odor. They are soluble in alcohol, ether, glycerine, and propylene glycol and slightly soluble or almost insoluble in water. As the alkyl chain increases, water solubility decreases. Parabens are hygroscopic in nature and have a high oil/water partition coefficient. They are prepared by esterification of *p*-hydroxybenzoic acid with the corresponding alcohol in the presence of an acid catalyst. They are generally stable in air and are resistant to hydrolysis in water and in acidic solutions. As alkyl chain length of parabens increases, the resistance to hydrolysis increases and appreciable hydrolysis occurs at pH above 7. The other physical and chemical properties of preservatives are summarized in Tables 2.1 and 2.2.

Table 2.1. General Description and Physicochemical Properties of Parabens

Characteristics	Methyl Paraben	Ethyl Paraben	Propyl Paraben	Butyl Paraben
CAS no.	99-76-3	120-47-8	94-13-3	94-26-8
Chemical formula	$C_8H_8O_3$	$C_9H_{10}O_3$	$C_{10}H_{12}O_3$	$C_{11}H_{14}O_3$
Molecular weight	152.16	166.18	180.21	194.23
Melting point (°C) [5, 6]	131	116-118	96-98	68-69
Boiling point (°C) [5, 6]	270-280	297-298	-	-
Refractive index [5, 6]	1.525	1.505	1.505	-
pK_a [5, 6]	8.17	8.22	8.35	8.37
Solubility in water (g/100 mL at 25 °C) [22]	0.25	0.17	0.05	0.02

Table 2.2. General Description and Physicochemical Properties of Benzyl Alcohol & 2-Phenoxyethanol [23]

Characteristics	Benzyl Alcohol	2-Phenoxyethanol
CAS no.	100-51-6	122-96-6
Chemical formula	C_7H_8O	$C_8H_{10}O_2$
Molecular weight	108.14	138.16
Melting point (°C)	-15	11-13
Boiling point (°C)	205	247
Refractive index	-	-
pK_a	15.4	-
Solubility in water (g/100 mL)	4 (17 °C)	3 (20 °C)

2.1.3 Preservatives Applications

2.1.3.1 Uses in Food

According to the literature [4], preservatives have been added to food for more than 50 years to preserve the natural characteristics and appearance of food and to increase the shelf life of food for storage. Over the years, the use of parabens in food has steadily increased. Parabens are added in several foods including processed vegetables, baked goods, fats and oils, seasonings, sugar substitutes, coffee extracts, fruit juices, pickles, sauces, soft drinks and frozen dairy products. The major uses of parabens in the food industry are cakes, pastries, pie-crusts,

icings, toppings and fillings (0.03 – 0.06% of a 3:1 in ratio methyl and propyl Parabens); soft drinks (0.03 - 0.05% of a 2:1 ratio of methyl and propyl parabens); creams and pastes (0.1% of a combination of parabens); jams and preserves (0.07% of a 2:1 ratio of methyl and propyl parabens).

2.1.3.2 Uses in Cosmetics

Parabens are routinely used as preservatives in cosmetics, with propyl and methyl paraben being the most commonly used. These parabens are used in nearly all types of cosmetics, and as mentioned earlier in over 22,000 cosmetics. The popular use of paraben preservatives in cosmetics and toiletries arises from their low toxicity, broad spectrum of activity, inertness, worldwide regulatory acceptance, biodegradability and low cost. Parabens are used individually, or in combination in all cosmetic product formulation categories such as bath products (soaps & detergents), eye makeup products (eyebrow pencil, eyeliner, eye shadow, mascara, eye lotion, etc.), fragrance products (colognes, perfumes, sachets, fragrance powders, etc.), hair products (shampoos, conditioners, sprays, straighteners, dyes and colors, etc.), makeup products (blushers, face powders, foundations, lipsticks, etc.), nail care products (cuticle softeners, nail creams and lotions, nail polish and enamel, etc.), oral hygiene products (mouthwashes, dentifrices), personal cleanliness products (underarm deodorants, douches, etc.), shaving products (aftershave products, beard softeners, preshave lotion, shaving creams, etc.), and skin care products (cleansing creams, lotions, liquids, moisturizers, wrinkle removers, etc.) [4, 6].

2.1.3.3 Pharmaceutical Uses

Parabens were first used in drugs in the mid 1920s and since then they have been employed frequently in a wide variety of formulations as preservatives. Parabens are used in a variety of drug formulations including suppositories, anesthetics, eyewashes, pills, syrups, weight-gaining solutions, injectable solutions and contraceptives. It has been reported that the combinations of parabens are more active than the individual esters. The use of preservative concentration varies from product to product, but rarely exceeds 1% [4, 6].

2.1.4 Preservatives Analysis

The analytical determination of preservatives in food, cosmetics and pharmaceuticals is not only important for quality assurance purposes but also for consumer interest and protection. The most common analytical methods for the separation and determination of the preservatives has been reversed-phase HPLC [6-11, 24-26], although other analytical methods such as TLC [27], capillary electrophoresis [28, 29], micellar liquid chromatography [30], flow injection analysis (FIA) [31] and gas chromatography [32] have also been reported.

2.2 High Temperature Liquid Chromatography

Temperature affects significantly on liquid physico-chemical parameters, such as viscosity, surface tension, or dielectric constant. All of these parameters play a considerable role in LC. HPLC at high temperatures is found to be beneficial in terms of both speed and efficiency. The influence of temperature on LC separations is discussed below.

2.2.1 Influence of Temperature on Retention

The contribution of temperature to the retention is mainly given by the enthalpy term of the van't Hoff equation for the retention factor,

$$\ln k = -\Delta H/RT + \Delta S/R + \ln \beta \qquad (2\text{-}1)$$

Here, ΔH is the enthalpy change associated with the transfer of the solute between phases, ΔS is the corresponding entropy change, R is the molar gas constant (8.31441 J . K^{-1} mol^{-1}), T is the absolute temperature in Kelvin, k is the retention factor and β is the volume phase ratio of the stationary phase and mobile phase. For neutral compounds, in the limit of ΔH, ΔS and β being invariant with temperature in the above equation, a plot of ln k versus 1/T, also called van't Hoff plot, usually gives a straight line with a slope of $-(\Delta H/RT)$ and an intercept of $\Delta S/R + \ln \beta$. [33, 34].

Carr and co-workers [35] presented some data about the dependence of temperature on retention for a mixture of alkyl benzenes. Their data within a temperature range of 40 to 100 °C could be fitted to a straight line. Yarita *et al.* [36] also observed linear van't Hoff plots for selected phenolic compounds in a temperature range of 100 and 150 °C using subcritical water as the mobile phase. Also Shen *et al.* [37] observed linear van't Hoff plots for a mixture containing substituted anilines on a C18 hybrid stationary phase at temperatures ranging from 150 to 200 °C.

However, there are cases when the curves deviate from linearity were observed and attributed to the so-called "phase transition" phenomenon. The transition is a consequence of a conformational change of the stationary phase going from a solid-like (low temperature) to a

liquid-like (high temperature) state. As a result, the curve can be sometimes divided into two linear plots, which intersect at the transition temperature [38].

Guillarme *et al.* [39] observed a dependence of the solute behavior on the type of solvents. The van't Hoff plots were linear for water-methanol mixtures while curved in the case of water-acetonitrile when mixing organic polymer as the stationary phase. Yang reported that selective separation may be achieved by varying the separation temperature, as reduction in retention does not always follow the linear van't Hoff equation for each individual solute [40].

Chen and Horvath have shown that temperature increase of 4-5 °C has roughly the same effect on retention as a 1% increase in organic solvent concentration for neutral compounds, on silica-based columns [40, 41]. This correspondence was shown to be the same on zirconia-based columns [42]. Thus, by increasing the temperature under reversed phase conditions, it is possible to significantly reduce the content of organic solvent while keeping the same eluent strength.

Another consequence of increased temperature is the lowered viscosity of the mobile phase that results in much lower back pressure in an HTLC/SBWC system. Figure 2.2 from Anita and Horvath's study [43] shows a plot of retention vs. temperature T relative to that at 25 °C under conditions of fixed pressure drop, particle size, and plate number vs. column temperature. As can be seen, analysis time drops by more than a factor of 20 as the temperature is increased to 200 °C.

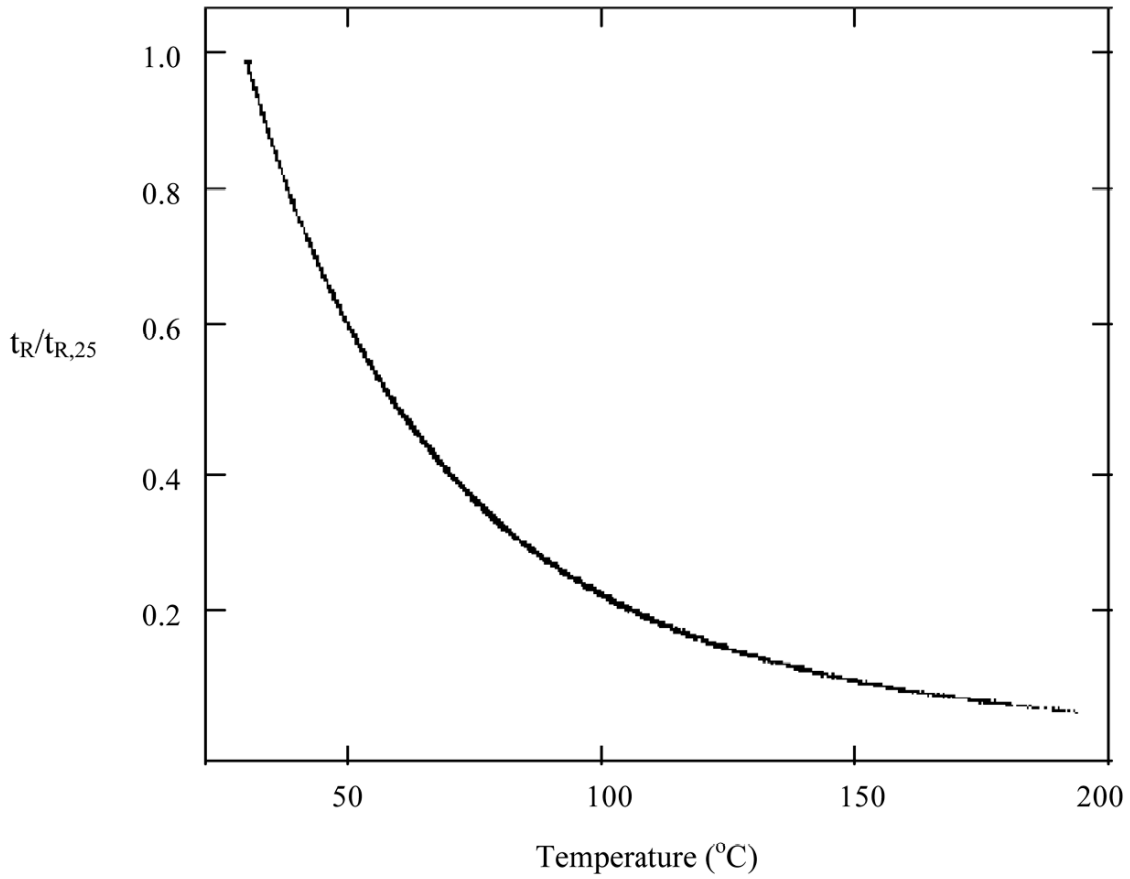


Figure 2.2. Reduction in analysis time due to increasing temperature. Adopted from Reference [43] with permission.

According to Yang's study [16], the back pressure of an HTLC system decreases over threefold by simply raising the temperature from 25 °C to 90 °C when a mixture of methanol-water (40:60) was used. Guillarme *et al.* [44] reported that the back pressure was reduced by fivefold over a temperature range of 25-180 °C in SBWC, where pure water was used as the mobile phase. The combined effect of high temperature and low back pressure make it possible for HTLC to achieve fast separations by the use very high flow rates.

2.2.2 Influence of Temperature on Efficiency

The column efficiency is commonly given by the plate number N, which is related to the plate height, H by

$$N = L/H \qquad (2\text{-}2)$$

where, L being the column length. H varies with the linear velocity of the mobile phase, u, and its variation may be expressed by the van Deemter equation.

$$H = A + B/u + Cu \qquad (2\text{-}3)$$

where, A is the eddy diffusion term reflecting band broadening due to the uniformity of the column packing, the B term accounts for longitudinal (axial) diffusion, while the C term represents the resistance to mass transfer in the stagnant mobile phase and stationary phase, and u is the linear velocity of the mobile phase [45]. Here both the coefficients B and C heavily depend on the solute retention factor and the C-term dominates the band broadening terms at higher retention factors. Thus the effect of temperature on solutes in terms of efficiency can be more pronounced for well-retained solutes that spend more time in the stationary phase than less retained solutes.

The influence of temperature on the efficiency under different flow rates is shown in Figure 2.3. It is clear that there is a dramatic effect of temperature on the mass transfer within the stationary phase zone. This improvement in efficiency is especially pronounced at higher linear velocities where the C-term is the dominant contribution to the plate height [46]. Elevated temperatures increase the interphase mass transfer which results in improved column efficiency and faster analysis at higher flow rates. Higher mobile phase linear velocities are also enabled by

the dramatic drop in eluent viscosity as the temperature increases [46]. Thus, it is beneficial to operate a liquid chromatography at both high temperatures and very high flow rates to improve efficiency and optimize resolution.

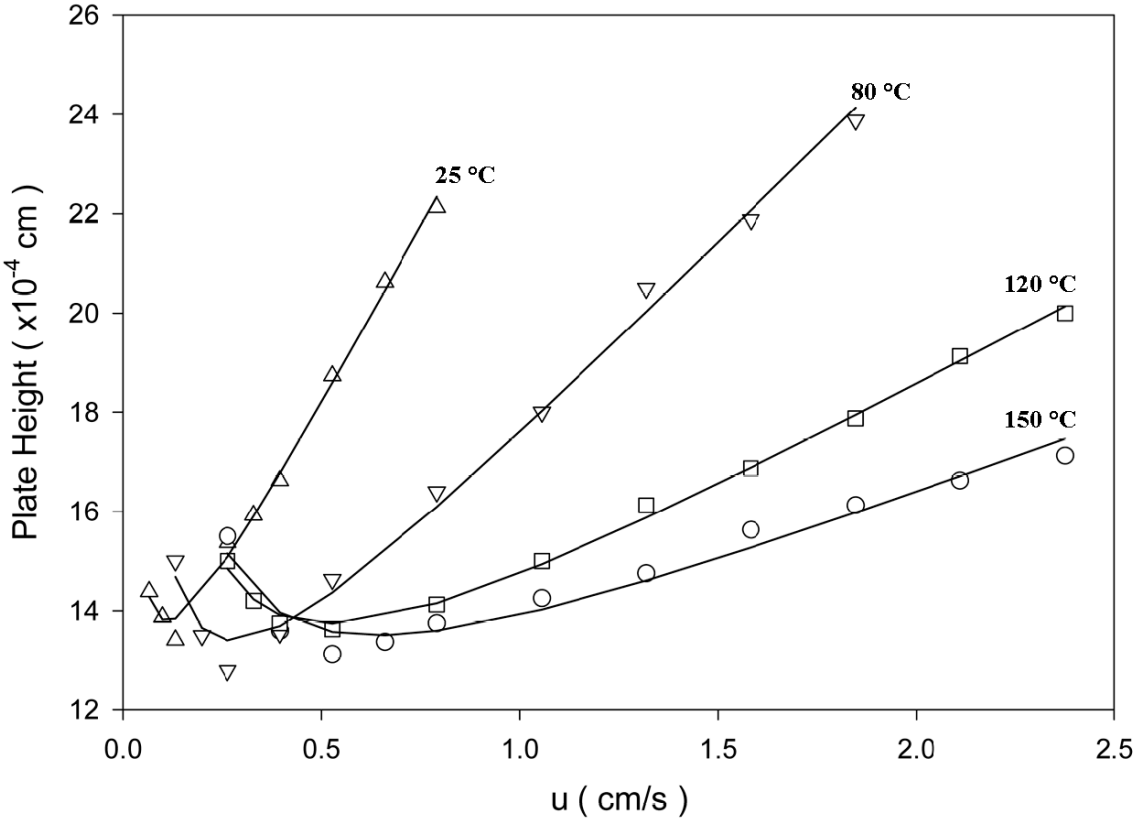


Figure 2.3. Plate height vs. linear velocity at various temperatures for moderately retained solutes. Experimental conditions: 3 µm ZirChrom-PS column (ZirChrom Separations), 5 cm x 4.6 mm id, 40% ACN/60% water, Δ = 25 °C, octanophenone, k = 3.87, ∇ = 80 °C decanophenone, k = 3.15, □ = 120 °C, decanophenone, k = 5.70, o = 150 °C, decanophenone, k = 1.65. Adopted from Reference [46] with permission.

Yang [40] developed a model to study the effect of temperature on column efficiency in high temperature LC. He concluded that the analysis temperature in RPLC could be optimized to achieve better efficiency under the conditions of constant linear velocity. At lower temperature, the mass transfer term in the van Deemter equation dominates the plate height, and at higher temperature the longitudinal diffusion term dominates the plate height. Consequently, under constant linear velocity conditions, the plate height decreases with increasing temperature at low temperature range and increases with increasing temperature in the higher temperature range. Therefore, a minimal plate height (a maximum in column efficiency) may occur for high temperature liquid chromatographic separations.

2.3 Subcritical Water Chromatography

2.3.1 Subcritical Water

Subcritical water is also termed as high-temperature water, superheated water, and pressurized hot water. Subcritical water refers to the water that is heated and pressurized at conditions below its critical point of 374 °C and 218 atm. Recent studies clearly demonstrate that subcritical water can be used as an alternative solvent to replace the organic solvents in extraction [20, 21, 47-55] and chromatographic process [15, 17, 40, 46, 49, 56-66]. Water compared to organic solvents, is environmentally benign, but at ambient temperature is too polar to serve as the sole eluent for reversed-phase separations. Fortunately, at elevated temperature and under moderate pressure, the physical properties of water are similar to organic solvent-water mixtures used in reversed-phase separations. The graphical representations of these properties are shown in Figures 2.4 – 2.6. At elevated temperature and pressure, these properties

can be widely adjusted. The decrease in dielectric constant and surface tension with increasing temperature has greatly enhanced the solvating power of water.

The dielectric constant of a solvent is a relative measure of its polarity. Therefore, dielectric constant of a solvent is higher if it has high polarity [67]. As shown in Figure 2.4, the dielectric constant of water at ambient temperature is approximately 78. As the temperature of water is increased from ambient to 250 °C, the dielectric constant of water gradually decreases to 27, which is similar to the dielectric constant of pure methanol ($\varepsilon = 33$) and pure acetonitrile ($\varepsilon = 36$) at ambient conditions [21]. Pressure changes (<400 bar) have no significant effect on the dielectric constant of subcritical water as long as water remains in the liquid state [68]. Therefore, the polarity of subcritical water is comparable to commonly used polar organic solvents. The same decreasing trend in the dielectric constant is normally achieved by increasing the organic portion in organic solvent-water mixtures in traditional reversed-phase liquid chromatography.

The two additional important physical parameters that change markedly as the temperature of water is increased are the reduced surface tension and viscosity. In order to increase a liquid's surface area, molecules in liquid would have to move from the interior to the surface, thus breaking some intermolecular forces, which requires energy. The surface tension is defined as the work done by an increase in the surface area of a liquid. In general, the stronger the forces are between the particles in a liquid, the greater is the surface tension. Water has a high surface tension because its molecules form multiple H (hydrogen) bonds [67, 69]. The viscosity is also dependent on intermolecular forces. It is the measurement of a liquid's

resistance to an applied force. In other words, it is the resistance to flow which results from intermolecular attractions. Thus, stronger the intermolecular forces, higher the viscosity [69].

Figures 2.5 and 2.6 shows the decrease in surface tension and viscosity of water with increasing temperature. The surface tension of water at ambient temperature is 72 dyn/cm. As shown in Figure 2.5, with increasing the temperature, the surface tension decreases to approximately 25 dyn/cm at 250 °C, which is similar to the surface tension of pure methanol and pure acetonitrile at ambient temperature [21].

As shown in Figure 2.6, the viscosity of water is approximately 0.82 cP at ambient temperature but decreases gradually with the increasing temperature and at 250 °C the viscosity of water is approximately 0.25 cP which is much lower than that of pure methanol and pure acetonitrile at room temperature [21].

Due to the decreased dielectric constant, surface tension and viscosity of water at high temperatures, subcritical water can mimic the traditional organic solvent-water mixtures to achieve reversed-phase LC separations

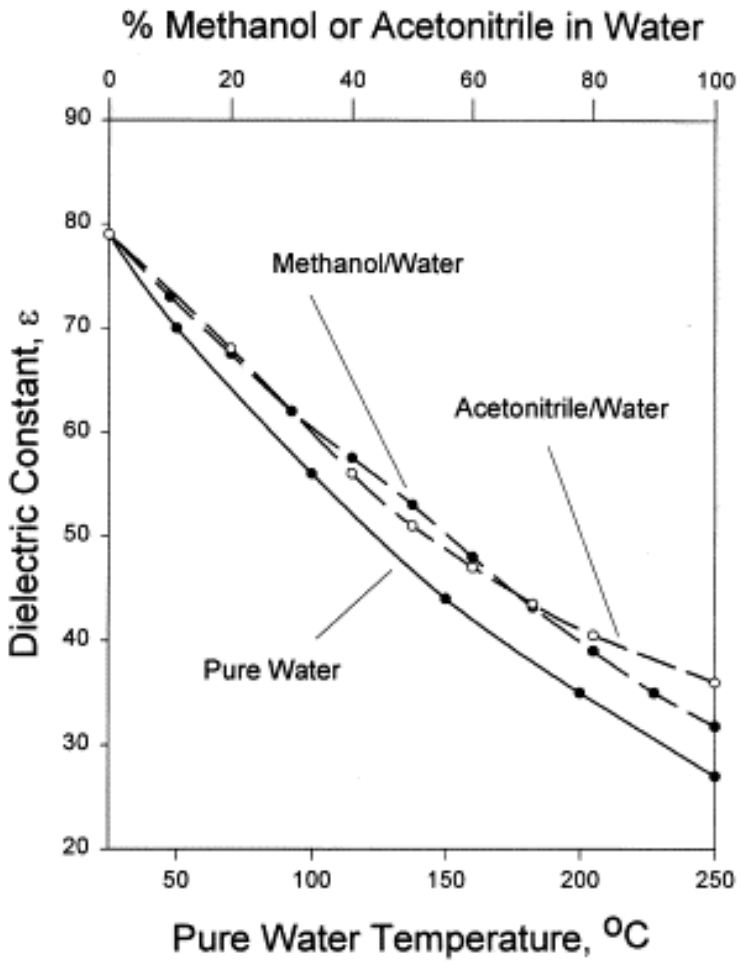


Figure 2.4. Control of solvent dielectric constant by changing temperature of pure water at 50 atm compared to mixing water with methanol or acetonitrile. Adopted from Reference [21] with permission.

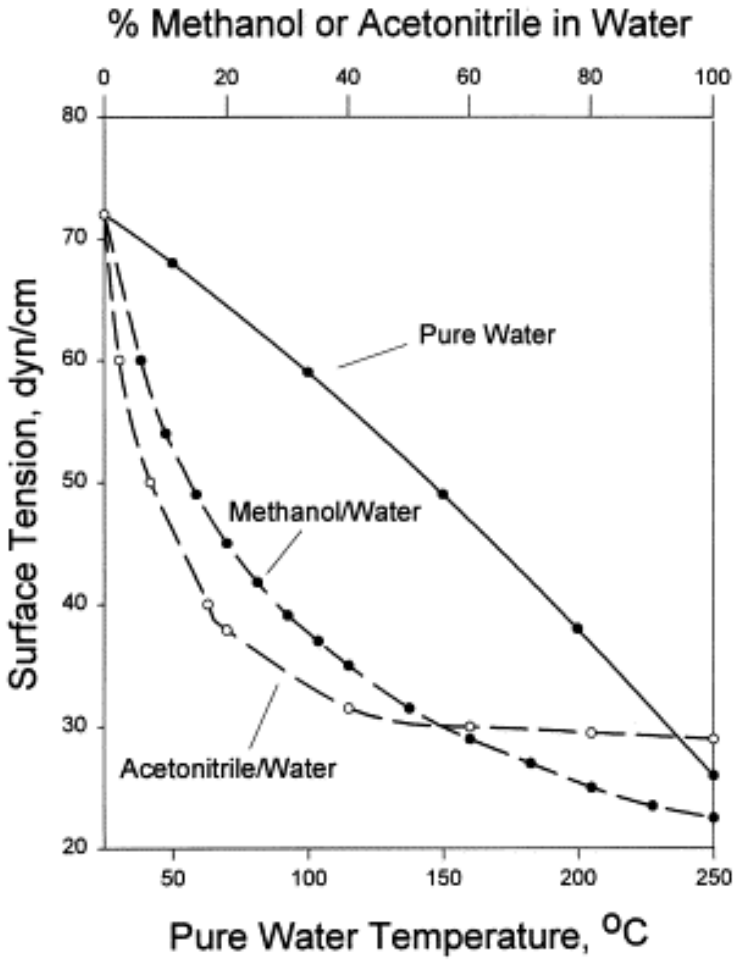


Figure 2.5. Control of solvent surface tension by changing temperature of pure water at 50 atm compared to mixing water with methanol or acetonitrile. Adopted from Reference [21] with permission.

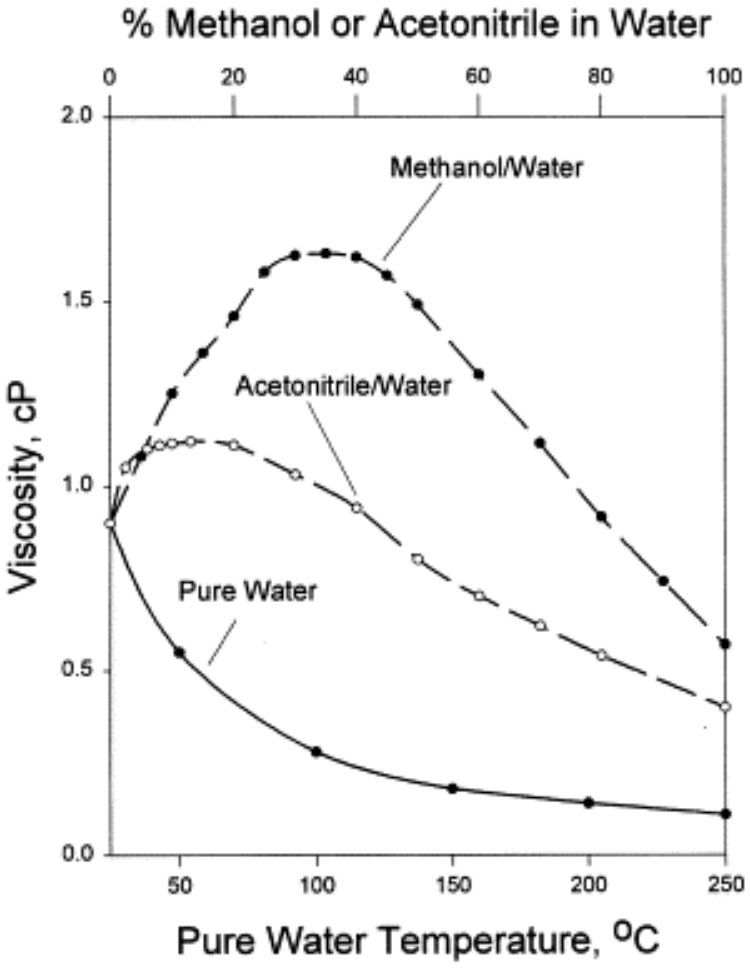


Figure 2.6. Control of solvent viscosity by changing temperature of pure water at 50 atm compared to mixing water with methanol or acetonitrile. Adopted from Reference [21] with permission.

2.3.2 Detection in Subcritical Water Chromatography

2.3.2.1 Spectroscopic Detectors

UV detection is the widely used detection technique in subcritical water chromatography. The reason for its popularity is that an SBWC-UV system can be easily setup by little modification of a regular HPLC system. A high temperature oven is needed to heat the column to the required temperature and a backpressure regulator that is attached to the outlet of the UV detector to keep the water in liquid state inside the separation column at elevated temperatures. Additional care need to be taken when using UV detector at high temperature is that the hot eluent before it reaches the UV detector flow cell need to be cooled to lower temperature. One of the advantages of using only water as the mobile phase is that water is transparent down to 190 nm and gives no background UV signal. This was explored by Yarita *et al*. [70] to detect polyethylene glycols at 190 nm. The detection wavelength in SBWC-UV is typically 254 nm [44, 49, 60, 71-78], but 220 nm [36], 265 nm [79] and 280 nm [80] have also been employed.

2.3.2.2 GC-based Detectors

The lack of a sensitive and universal detector such as the flame ionization detector (FID) used in gas chromatography is a well-known limitation of traditional RPLC. The presence of organic solvents in the traditional RPLC mobile phase has significant background signal and therefore FID could not be used. Fortunately, water has no response to FID, so it can be used as a gas-phase detector in LC with water as a sole eluent. The advantage of using FID when compared to UV detection with SBWC is that solutes with no chromophores can be detected, thus broadening the range of analytes.

Bruckner *et al*. used a drop headspace interface between the LC and the detector in which the analytes were blown from the droplets at the end of the column into the flame with a steam of helium. They were able to separate and detect volatile analytes (alcohols and hydrocarbons) using only water as the mobile phase with this drop headspace interface. The schematic representation of the drop headspace interface is shown in Figure 2.7 [81].

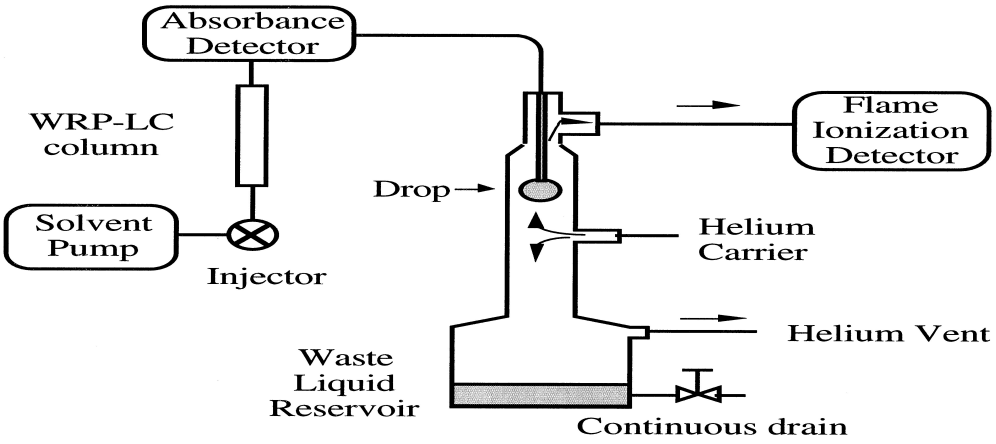


Figure 2.7. The Schematic diagram of an LC-UV-FID system using ambient water as the mobile phase and the drop headspace interface that transports volatile analytes from the LC eluent to the FID. Adopted from Reference [81] with permission.

Miller and Hawthorne reported the direct coupling of FID with SBWC for the separation of alcohols, hydroxy-substituted benzenes, and amino acids. They used a stainless steel restrictor between the column and the FID. As shown in Figure 2.8 the position of the restrictor is important and they discovered that it should be placed about 3 cm just below the tip of the FID jet in order to maintain stable FID signal. This position provided minimum noise and maximum sensitivity. This restrictor was also required to maintain the pressure in the column, so that the

water remained in the liquid state when temperatures greater than 100 °C were employed. The only limitation of this direct coupling is that a stable flame and FID signal could not be achieved when water flow rates greater than 200 µL/min were used [82].

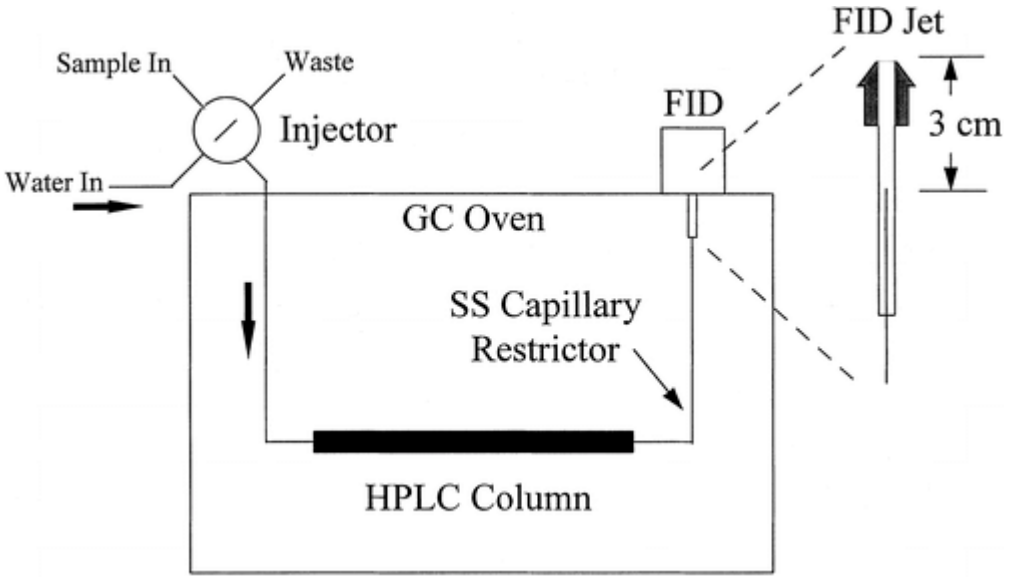


Figure 2.8. Schematic diagram of an SBWC-FID system. Adopted from Reference [82] with permission.

Ingelse *et al*. confirmed the position of the tip of the restrictor in FID jet as discovered by Miller and Hawthorne. In their study they found that an improvement in the stability of the FID signal was achieved when they used a wide bore FID jet with water flow rates of 100 µL/min or higher, however, there was a decrease in FID sensitivity. Therefore, standard FID jet was used at water flow rates of 50 µL/min or lower. They reported the detection of polar and moderately polar compounds such as alcohols and aldehydes by using water as a mobile phase at 175 ºC on

PRP-1 column by separately thermostating 50 μm capillary restrictor at 75 ºC which gave significantly improved signal stability [83].

Yang *et al*. reported the coupling of SBWC with FID in the split mode. A tee union was connected between the separation column and the FID system to split the water flow, since the FID signal was unstable at flow rates greater than 200 μL/min. The advantages of using this split mode is that a stable FID signal can be maintained at higher volume flow rates up to 1.24 mL/min. They reported the separation of several carbohydrates, carboxylic acids, and amino acids at different temperatures. A linear dynamic range of up to three orders of magnitude and the limit of detection of 38 to 111 ng were obtained for several analytes [59]. Yarita *et al.* [84], Fogwill *et al.* [85] and Yu *et al.* [86] also reported similar kind of split systems.

Kephart and Dasgupta reported coupling of SBWC-FID with no final restrictor. They have used a capillary column and directly linked the end of the column outlet to the FID. This configuration allowed the water to vaporize at some point along the column during a temperature ramp from 50 to 250 °C at 50 °C/min. They reported the separation of mixture of polar and non-polar benzene derivatives [86].

Yang *et al.* reported the use of micro-bore columns for SBWC separation with FID detection. The advantage of using micro-bore columns is that a very low volume flow rate of eluent was required because of the small internal diameter of the column. A stable FID was maintained due to the low flow rate of water. They reported the separation of carbohydrates, amino acids, and other organic acids and bases [56]. Many other researchers also reported the use of micro-bore and capillary columns [83, 87-89].

2.3.2.3 NMR, MS and IR Detection

LC coupled with NMR or MS is an important technique for the identification and structural elucidation of the analytes. In recent years, on-line HPLC-NMR spectroscopy has become practical for routine applications. Complications still arise from strong background signals in the NMR spectrum resulting from the proton containing solvents, which can overlap with resonance from the analyte. The purest HPLC grade mobile phase constituents can also contain impurities, which can contribute additional interfering signals. To solve this problem, NMR quality deuterated solvents such as deuterated acetonitrile (CD_3CN) can be used, but they are expensive.

The use of SBWC with NMR removes the need for the expensive deuterated organic solvents and the use of moderately expensive deuterium oxide as the eluent is possible.

Smith *et al.* have demonstrated the first coupling of SBWC with NMR. A schematic diagram of the instrument is shown in Figure 2.9. A 3-m length of 0.13 mm i.d. PEEK tubing was used to connect the outlet of the UV detector to the NMR detector. This tubing provided sufficient backpressure for the separation column and ensured that the eluent stayed in the liquid state. It also cooled the eluent to room temperature before it reached the NMR flow cell. Using this system Smith *et al.* were able to separate and identify salicylamide and barbiturates on polystyrene divinylbenzene (PS-DVB) column and UV wavelength of 254 nm [90].

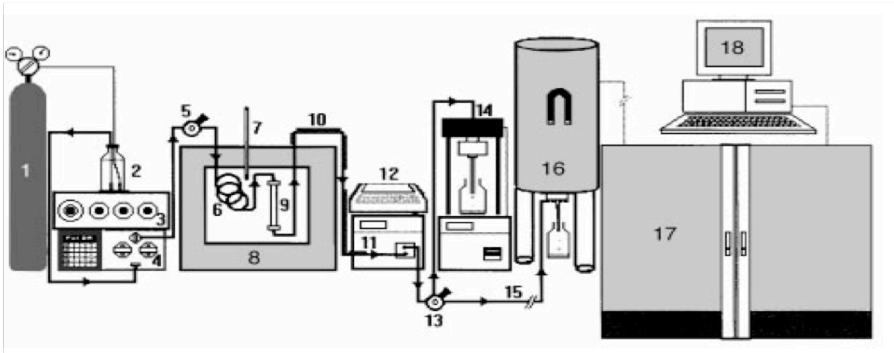


Figure 2.9. Schematic diagram of SBWC-NMR instrument. Part identification: 1) nitrogen cylinder; 2) water reservoir; 3) temperature programmer; 4) pump; 5) loop injector; 6) preheating coil; 7) thermometer; 8) column oven; 9) PLRP-S column; 10) cooling fins; 11) UV/VIS detector; 12) UV integrator; 13) switching valve; 14) back pressure controller and regulator; 15) 3 m PEEK tubing; 16-18) NMR magnet and data system. Adopted from Reference [90] with permission.

Saha *et al.* used a similar type of instrument. They reported the separation and identification of an extract of ginger using superheated deuterium oxide as the mobile phase. The separation was performed on XTerra C18 bonded column and eluent signals were recorded at 280 nm for UV detection [80].

Smith *et al.* [91] reported the coupling of NMR and MS with SBWC. A schematic diagram of the instrument is shown in Figure 2.10. A T-piece junction was placed in the tubing leading to the NMR detector before the magnet, which connects the SBWC, NMR and MS. The flow was split through a second 3-m length PEEK tubing before the NMR flow cell so that 95% of the sample was directed to the NMR flow cell and the remainder 5% to the MS. This type of set up allowed the same sample to be examined by both NMR and MS in parallel. Using this

system, Smith *et al.* were able to separate and identify a mixture of paracetamol, phenacetin, and caffeine. The C18 column and UV detection at 254 nm was used.

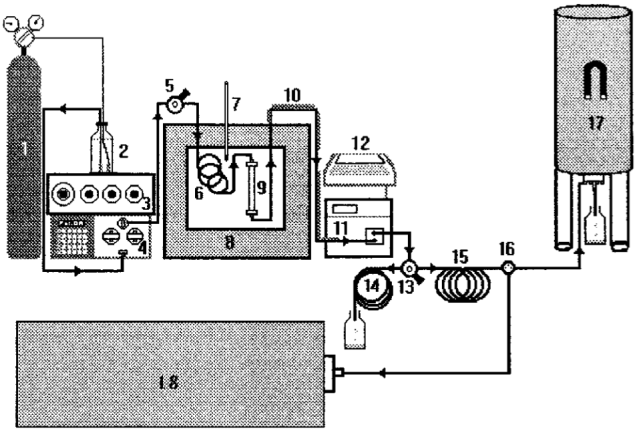


Figure 2.10. Schematic diagram of SBWC-NMR-MS instrument. Part identification: 1) nitrogen cylinder; 2) water reservoir; 3) temperature programmer; 4) pump; 5) loop injector; 6) preheating coil; 7) thermometer; 8) column oven; 9) HPLC column; 10) cooling fins; 11) UV/VIS detector; 12) UV integrator; 13) switching valve; 14 & 15) 3 m x 0.13 mm PEEK tubing; 16) three-way splitter; 17) NMR magnet and data system; 18) mass spectrometer and data system. Adopted from Reference [91] with permission.

In later studies Smith *et al.* used the same system to separate and identify a mixture of sulfacetamide, sulfadiazine, sulfamerazine and sulfamethazine. A polystyrene divinylbenzene (PS-DVB) column was used with a deuterium oxide phosphate buffered as mobile phase and a UV detection at 254 nm. During the separation, the weakly acidic protons of the methyl groups in sulfamerazine and sulfamethazine underwent an unexpected deuterium exchange, which was easily identified from the results of NMR and MS [92].

Smith *et al.* extended their SBWC-NMR-MS approach for the detection of water-soluble vitamins, which were well know for their instability towards light and heat. A PLRP-S polystyrene divinylbenzene (PS-DVB) column and C18 column were used with a deuterium oxide as mobile phase and a UV detection of 254 nm. Pyridoxine (vitamin B_6), Riboflavin (vitamin B_1) and Thiamine (Vitamin B_1) were successfully separated using superheated water as the mobile phase and were identified by UV and MS detectors. They used deuterium oxide as the eluent for the direct on-line NMR spectra [93].

Pereira *et al.* recently reported the separation of purines, pyrimidines and nucleic acids on porous graphitic carbon (PGC) column with MS [58].

Louden *et al.* have demonstrated the use of multiple detectors that could be extended to SBWC with LC-UV-IR-NMR-MS. A schematic diagram of the instrument is shown in Figure 2.11. The system was created by the addition of the oven to maintain the column at elevated temperatures and they used PEEK tubing to connect the column to the various detectors. This PEEK tubing acted as a backpressure regulator to maintain the D_2O in the liquid state. All of the instrumentation was located outside the magnetic field of the NMR spectrometer. They used a splitter as shown in Figure 2.11, so that the minor portion of the eluent from the column was

directed to a MS and the remainder of the flow was directed to FT-IR. The eluent from the FT-IR was directed to the UV-diode array detector and from UV-DAD detector finally to NMR flow probe. Using this system Louden *et al.* were able to detect and identify paracetamol, antipyrine, 4-aminoantipyrine, norantipyrine, caffeine, phenancetin, p-aminobenzoic acid, and propranolol. The columns used were XTerra and Oasis HLB based stationary phases at temperatures of 85 °C and 185 °C [94].

Louden *et al.* extended their studies using the same system to detect and identify ecdysteriods in a number of extracts of plants from the Silene family. A C8 XTerra and C18 XTerra columns at 160 °C with UV detection at 254 nm was used [95].

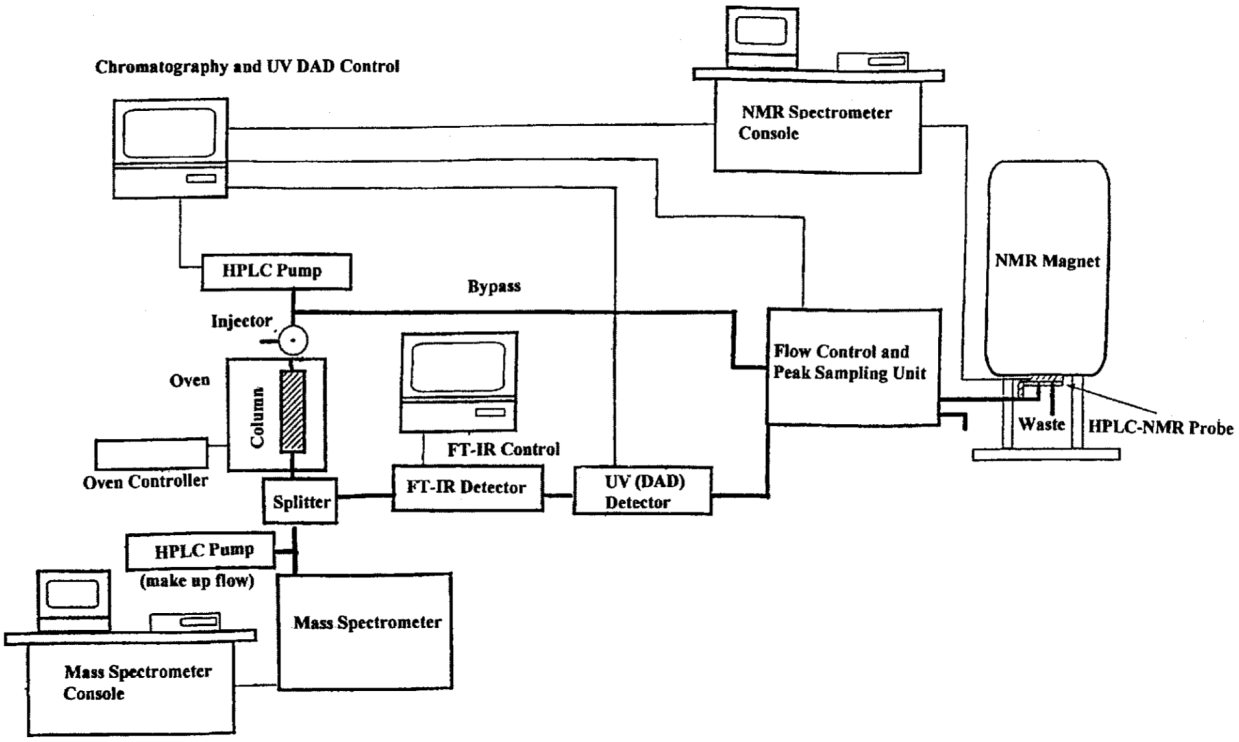


Figure 2.11. Schematic diagram of superheated water HPLC-MS-IR-UV-NMR instrument. Adopted from Reference [94] with permission.

2.4 Applications of HTLC and SBWC

Initially there were concerns about the stability of the analytes while working at high temperatures, which were employed in HTLC or SBWC. Fortunately, at elevated temperatures, the viscosity of the mobile phases is decreased significantly and the low backpressure allows HTLC or SBWC separations to be carried out with much higher flow rates. This shortens the separation time and thus shortens the analyte exposure time to high temperatures, which in turn reduces the degradation of the analyte.

Only few cases of degradation were reported by the researchers. Chienthavorn *et al.* reported that thiamine degraded at 160 °C into a number of breakdown products including 4-methyl-5-thiazole-ethanol [93]. Yang *et al.* studied the degradation of terpenes (α-pinene, limonene, camphor, citronellol, and carvacrol) and reported that with the increasing temperature the stability of terpenes decreased at 250 °C after 30 min [54]. Yang *et al.* also studied the degradation of polycyclic aromatic hydrocarbons such as phenanthrene. They reported that phenanthrene degraded and oxidized when heated at 150 °C for 30 min [96]. Lindquist and Yang recently studied the degradation of benzoic acid and their derivatives and reported that severe degradation occurred when they were heated in water at 200 °C for 30 min [97].

A wide range of analytes using different columns and conditions and with different methods of detection have been separated using HTLC or SBWC. They include polar analytes such as phenols, alcohols, etc. and non-polar analytes such as alkanes, alkyl benzenes, etc. as shown in Table 2.3. The HTLC/SBWC studies also extended to many pharmaceuticals such as anti-cancer drugs, paracetamol etc. as shown in Table 2.4 and to environmental and food industry (Table 2.5). While examining these wide ranges of analytes, the compatibility of different types of detectors and columns were tested with HTLC/SBWC.

Table 2.3. HTLC/SBWC Separations of Aliphatic, Aromatic and Polymeric compounds

Compound	Column	Temperature	Detector	Reference
n-alcohols	PRP-1	70-130 °C	FID	[85]
Metabolities [2-, 3- and 4-bromobenzoic acids (BBA's)]	C18 bonded	100-110 °C	MS	[98]
Acetone, p-cresol, ethylbenzene, & nitrobenzene	ZirChrom Carb	185 °C	UV, 254 nm	[99]
Parabens	Discovery ZR-Carbon C18	170 °C	UV, 254 nm	[100]
Alcohols, Phenols, & Carboxylic acids	Polymer RP-1 (PS-DVB)	160-210 °C	FID	[86]
Alcohols	Poly(divinylbenzene) monolithic capillary column (PS-DVB)	200 °C	FID	[101]
Aniline, N-methylaniline, 3-ethylaniline & N,N-dimethylaniline	XBridge C18	130-200 °C	UV, 220 nm	[102]
Anilines, Alcohols, Carboxylic acids	PRP-1	150 C	FID	[103]
Acetophenone, 4-aminoacetophenone, 4-hydroxyacetophenone, phenol, p-cresol, 4-ethylphenol, 4-propylphenol	XTerra phenyl, XBridge phenyl	60-200 °C	UV, 230 nm	[104]
Aromatic hydrocarbons	ODS silica	170 °C	UV	[105]
Alcohols, phenols	PBD-zirconia	125 - 200 °C	FID	[88]
Alcohols	Hypercarb, Zirchrom-PBD	150 °C	FID	[89]
Alkyl aryl ketones, Barbiturates	PLRP-S	100 - 150 °C	UV, 254 nm 220 nm	[76]
Anilines	XBridge C18	150 - 200 °C	UV, 220 nm FID	[37]
Phenols	PS-DVB	150 - 200 °C	UV, 220 nm FID	[106]
Phenols	PRP-1	100 - 150 °C	UV, 220 nm	[36]
Polyethylene glycols	PRP-1	100 -180 °C	UV, 190 nm	[70]

Table 2.4. HTLC/SBWC Separations of Pharmaceuticals

Compound	Column	Temperature	Detector	Reference
Niacin, Niacinamide	Waters Xterra MS C18 Waters XBridge C18 PRP-1	60 °C 80 °C	UV, 245 nm	[107]
Steroid (levonorgestrel)	Zirconia-based Zr-CarbonC18	150 °C	UV, 244 nm	[108]
Steroid (Estriol,1,4-Androstadiene-3,11, 17-trione, 19 nortestosterone,Testosterone, Progestrone)	XTerra MS C18	50-100 °C	UV, 230 nm	[109]
Sterols (cholesterol, lanosterol, stigmasterol, β-sitosterol, ergosterol)	Hypercarb, Pathfinder C18,ZirChrom-CARB	100-150 °C	UV, 254 nm	[110]
Octylphenol ethoxylates	Zorbax StableBond, Selerity Blaze C8, Zirchrom PBD, Hypercarb, Ultremex 3 Silica	65-100 °C	UV, 225 nm	[111]
Paracetamol (acetaminophen), antipyrine, 4-aminoantipyrine, norantipyrine, caffeine, phenacetin, *p*-aminobenzoic acid, propranolol, sulfacetamide, sulfanilamide.	XTerra C8 & Oasis HLB	>100 °C	UV, 188-1000 nm	[95]
Testosterone	Zirconia-PBD	160-200 °C	UV, 254 nm	[75]
Steroids (Estriol, Androstadiendione, Estrone, Dehydroepiandrosterone) Cytostatics & anti-antibiotic drugs)	ZirChrom-PS	185 °C	UV, 220 nm UV, 254 nm	[113]
Purines & pydimidines (cytosine, uracil, thymine, hypoxanthine, guanine, xanthine)	Porous grahitic carbon (PGC), hypercarb	190 °C	UV, 254 nm	[78]
Model drugs (antipyrine, aminohippuric acid, paracetamol, hydroxyantipyrine, aminoantipyrine, atenol, aminobenzoic acid, theophylline, phenacetin, and caffeine)	Acquity C18	40-180 °C	UV	[114]
Thiazide, Sulfonamide diuretics	XBridge C18	200 °C	UV, 271 nm	[115]
Antipyrine, aminohippuric acid, paracetamol, hydroxyantipyrine, aminoantipyrine, atenolol, aminobenzoic acid, theophylline, phenacetin, caffeine	Acquity BEH C18	60 - 180 °C	MS	[116]
AZD5438 (4-(1-isopropyl-2-methyl-1 H-imidazol-5-yl)-N-[4-(methylsulfonyl) phenyl]pyrimidin-2-amine)	Acquity BEH C18	60 - 150 °C	UV, MS	[117]

Anti-cancer drugs	Nucleogel RP (PS-DVB)	150 °C	UV, 215 nm	[73]
Salicylamide, bartitone, amylobarbitone, heptabarbitone	PLRS-S (PS-DVB)	200 °C	UV, 254 nm, NMR	[90]
Model drugs (analgesics and caffeine)	Novapak C18	80 to130 °C at 8 °C/min	UV, 254 nm, NMR, MS	[91]
Vitamins (pyridoxine, riboflavin, thiamine)	PLRP-S (PS-DVB)	200 °C	UV, 254 nm	[93]

Table 2.5. HTLC/SBWC Separations of Environmental and Food Samples

Samples	Column	Temperature	Detector	Reference
Kava lactones	PBD Zirconia	80-100-160 °C at 2 °C/min	UV, 254 nm NMR	[118]
Ginger extracts	Xterra RP 18	50 -130 °C at 4 °C /min.	UV, 280 nm, NMR	[80]
Ecdysteroids	C8 XTerra	160 °C	UV, 188-1000 nm, ,IR,NMR,MS	[94]
Triazole fungicides (hexaconazole, tebuconazole, propiconazole, difenoconazole)	ZirChrom-PBD	100-150 °C	UV, 195 nm	[112]

2.5 SBWC Coupled with Subcritical Water Extraction (SBWE)

Several researchers have reported the coupling of SBWE with SBWC where subcritical water was used for both extraction and chromatographic separations [48-49, 74, 80, 119-121]. This coupling technique offers a green analytical approach that completely eliminates the use of hazardous organic solvents involved in both extraction and chromatographic processes.

Young *et al.* reported the on-line coupling of SBWE with WRP-LC (water-only reversed phase liquid chromatography) for the analysis of hydrophobic analytes such as aromatic hydrocarbons [119]. The separations were carried out by using 100% water at ambient temperature with a very low retentive stationary phase. The UV detection at 200 nm was used.

Yang *et al.* reported the off-line coupling of SBWE with HPLC using a sorbent trap for the analysis of BTEX (benzene, toluene, ethylbenzene, and xylenes) and polycyclic aromatic hydrocarbons. The separations were carried out on an ODS column with UV detection at 254 nm [48].

Yang *et al.* later reported the on-line coupling of SBWE with HPLC via solid-phase trapping. Using this on-line coupling technique, several classes of compounds including caffeine, chlorinated phenols and anilines, nitrotoluenes and polychlorinated biphenyls. An ODS separation column was used with UV detection at 254 nm [120].

Yang *et al.* later demonstrated the possibility of off-line coupling of SBWE with SBWC via a sorbent trap and thermal desorption. Using this technique, anilines, and phenols from sand and flavones from orange peel were extracted and analyzed. A ZirChrom-PS (polystyrene-coated zirconia) and a discovery HS PEG (silica-based polyethylene glycol) separation columns were used with the UV detection at 254 nm [49].

Smith *et al.* reported on-line coupling of SBWE with SBWC [74, 121]. A sample of sand spiked with 6 compounds (paracetamol, salicylamide, caffeine, methyl paraben, phenacetin and ethyl paraben) was extracted at 120 °C and these extracted analytes were passed into the PS-DVB sorbent trap at ambient temperature. The trapped analytes were thermally released from the trap at 150 °C and passed to the cooler analytical column at 75 °C, where they were separated with a thermal gradient from 75 °C to 185 °C at a rate of 15 °C/min. UV detection at 254 nm was used [73]. In their latter attempt [121] SBWE coupled with SBWC were employed for extraction and analysis of triazine herbicides in spiked compost samples. A schematic diagram of the instrument is shown in Figure 2.12, where three sets of ovens, preheating coils, and cooling coils

were used for extraction, trapping and chromatography units. A Hypercarb PGC analytical column was used for the separations with UV detection at 222 nm.

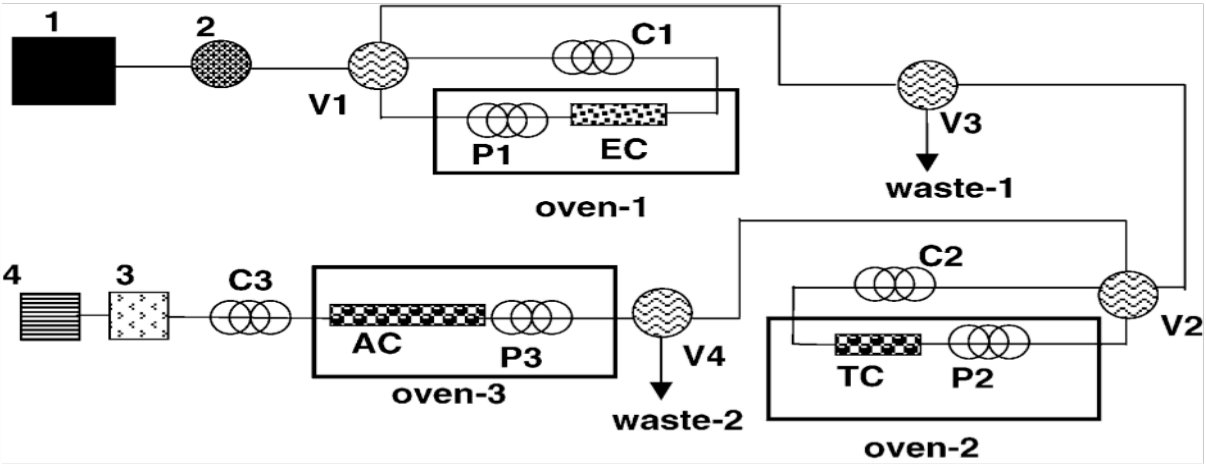


Figure 2.12. On-line coupled SBWE–SBWC system. 1, Water pump; 2, injector; 3, UV detector; 4, back-pressure regulator; EC, extraction cell; TC, X-Terra trap column; AC, PGC analytical column; V1–V4, switching valves; P1–P3, pre-heating coils; C1–C3, cooling coils [121].

2.6 Advantages and Limitations of HTLC and SBWC

2.6.1 Advantages

When compared to the traditional room temperature HPLC, the use of elevated temperature offers several advantages.

2.6.1.1 Lower Consumption or Elimination of Organic Solvents

Due to the decreased retention by increasing the temperature, the amount of organic solvent used in the mobile phase can be reduced significantly. As mentioned earlier, a temperature increase of 3.5 °C and 5-8 °C has similar effect on reducing retention as a 1% increase in methanol and acetonitrile, respectively [15]. Thus, water at elevated temperatures can replace a large proportion of organic solvent in the mobile phase. Even better, chromatographic separations using subcritical water eliminates the use of toxic organic solvents used in mobile phase and is considered as "green chromatography" [16, 17, 64].

2.6.1.2 Analysis Speed

The most obvious advantages are the gain in speed and reduced backpressure at increased temperature. In nearly all reversed phase separations, an increase in temperature will cause a decrease in retention. Additionally, decreased viscosity of the solvent at elevated temperature leads to lower backpressure. This allows the use of higher flow rates using standard equipment. Since high temperature leads to a flatter van Deemter curve, it enables the use of higher flow rates without hampering efficiency [45, 46].

2.6.1.3 Efficiency and Resolution

Due to decrease in viscosity at elevated temperature, the solute transfer from the mobile phase to the stationary phase is more efficient. This results in high efficiency even at high flow rates. The low backpressure allows the use of smaller particle sizes and or longer columns to increase the efficiency and resolution.

2.6.1.4 Selectivity

In traditional reversed-phase liquid chromatography, the stationary phase, mobile phase pH, organic modifier, and several other parameters determine the selectivity. Temperature can also play an important role in selectivity [122]. Since temperature is an instrumental setting, it is much easier to adjust during method development when compared to, e.g. a pH buffer or the stationary phase.

2.6.1.5 Improved Detectability

The number of available detectors is increased when subcritical water is used as an eluent. As mentioned earlier, UV detection could be done even at very short wavelength (190 nm) and therefore many species that do not absorb at longer wavelength can be easily detected [70]. Alternative detection techniques such as FID, NMR, MS also become an option in SBWC [80-95].

2.6.1.6 Temperature Programming

Since temperature of the column and mobile phase influences the retention, programmed temperature can be used to achieve more efficient separations [113, 123].

2.6.2 Limitations

The major drawback of HTLC/SBWC is the risk of stationary phase degradation, especially for silica-based columns. The development of a new generation of silica-based column, as well as non-silica based ones, has resulted in increased thermal stability. Several stationary phases can be used at elevated temperatures, such as columns based on polystyrene-divinylbenzene, graphitic carbon and ultra stable metal oxides such as zirconia [16, 17, 64].

The second concern with HTLC/SBWC is the lack of commercial equipment that can handle temperatures up to 200 °C. Currently there are only two commercially available column ovens that allow temperatures to be raised up to 200 °C. They are isothermal Metalox® 200 °C (ZirChrom Separation, Anoka, MN, USA) and the temperature programmable Polaratherm™ Series 9000 (Selerity, Salt Lake City, UT, USA). These two systems have an in-built mobile phase preheater and a mobile phase cooling unit. The Shimadzu Nexera UFLC system (Shimadzu Corporation, Tokyo, Japan) is one of the commercial instruments, which was used recently by Yang *et al.* [107] and is capable of heating up to 150 °C.

The limited availability of thermally and chemically stable stationary phase columns and the limited availability of commercial HTLC instruments capable of handling temperatures higher than 150 °C has restricted the adaption of HTLC/SBWC. Therefore, future improvement in these two areas is needed for the widespread adoption of HTLC/SBWC in industry.

CHAPTER 3: EXPERIMENTAL

3.1 Reagents and Materials

Benzyl alcohol, methyl paraben, ethyl paraben, 2-phenoxyethanol, propyl 4-hydroxybenzoate, and calcium chloride were purchased from Aldrich (St. Louis, MO, USA). Butyl-4-hydroxybenzoate was obtained from SAFC (St. Louis, MO, USA). Ortho phosphoric acid and HPLC-grade methanol was purchased from Fisher Scientific (New Jersey, USA). 0.45 μm GD/X PVDF membrane filter was received from Whatman (Florham Park, NJ, USA). Olay® total effects, 7-in-1 anti-aging UV moisturizer, plus SPF-15, fragrance free; Olay® complete ageless skin renewing UV lotion, SPF-20; and Olay® total effects, 7-in-1 anti-aging daily moisturizer, fragrance free were purchased from a local store. The deionized water (18 MΩ-cm) was prepared in the lab using a Sybron/Barnstead system (Sybron/Barnstead, Boston, MA, USA).

3.2 Separation Columns

Polystyrene divinylbenzene (PS-DVB) and zirconia-based columns were chosen due to their good thermal stability of stationary phases [16, 17]. A Hamilton PRP-1 column was purchased from Hamilton (Reno, Nevada, USA). The dimensions of the PRP-1 column are 150 x 4.1 mm i.d. and particle size is 5 μm. A ZirChrom®-DiamondBond-C18 column was purchased from ZirChrom (Anoka, MN, USA). The dimensions of this column are 100 x 4.6 mm i.d. and the particle size is 3 μm.

3.3 High Temperature Liquid Chromatography (HTLC) /Subcritical Water Chromatography System (SBWC)

A homemade system as shown in Figure 3.1 was used for HTLC/SBWC chromatographic separation. A Hitachi L-7100 model HPLC gradient pump (Hitachi, Ltd., Tokyo, Japan) was used to deliver the mobile phases at a flow rate of 1.0 – 3.5 mL/min. The outlet of the pump was connected to a Valco six-port injector (Valco Instruments Company Inc., Houston, TX, USA) with a 10-μL injection loop using stainless steel tubing that included a preheating coil to heat the mobile phase before entering into the column, in order to avoid band broadening related to thermal mismatch across the column. The tubing was passed into a gas chromatograph (GC) oven (Hewlett Packard 5890 Series II, Avondale, PA, USA). This GC oven was used to heat the analytical column during separations. The column was placed inside the GC oven and connected to the outlet of the injector with stainless steel tubing. The outlet of the column was connected to a Hitachi UV detector (model L7400). Detection at 256 nm was used for all experiments in this study. The outlet tubing of the column was placed in an iced water bath before reaching the UV flow cell to cool the eluent coming out of the column to ambient temperature to avoid any baseline noises. A backpressure regulator (Restek, Bellefonte, PA, USA) was placed right after the UV flow cell and before the tubing was placed in the waste container to maintain the mobile phase in liquid state at higher temperatures. The UV detector was connected to a computer via an interface of PC/Chrom (H&A Scientific, Greenville, NC, USA). Data acquisition and analysis were made available by the PC/Chrom software.

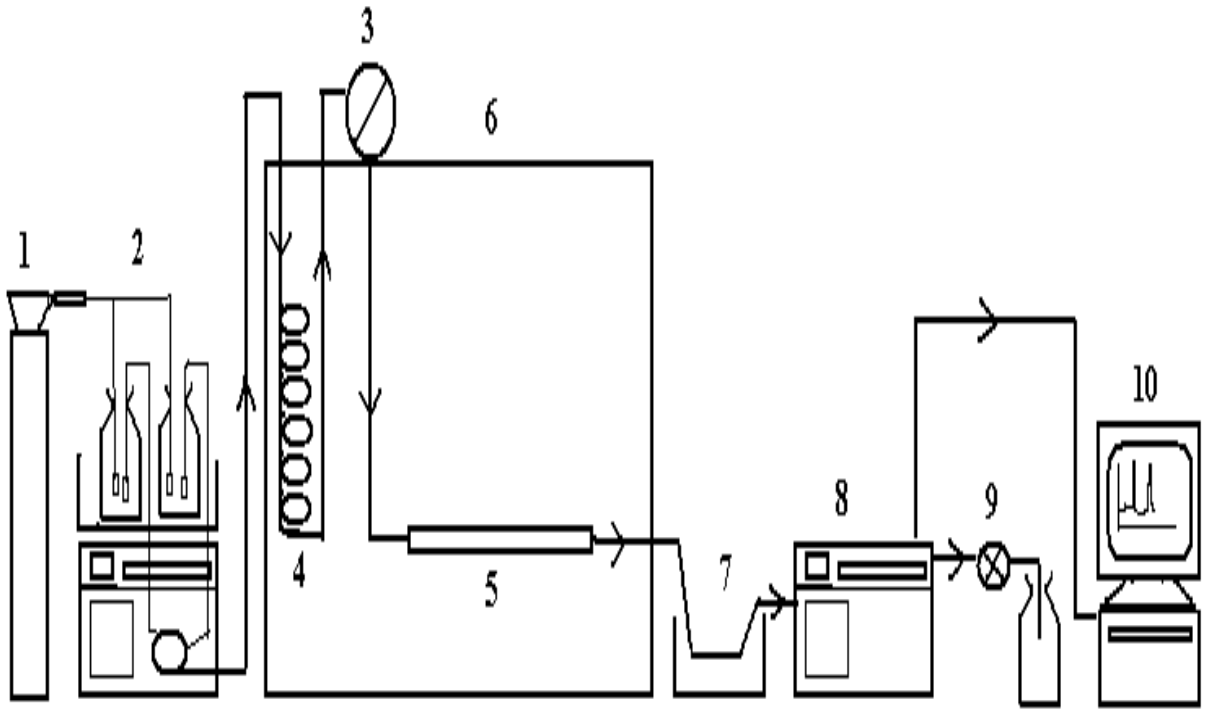


Figure 3.1. Schematic diagram of the HTLC/SBWC system: 1, helium for purging the mobile phase; 2, pump; 3, injection valve; 4, preheating coil; 5, column; 6, GC oven; 7, cooling bath; 8, UV detector; 9, back pressure regulator; 10, computerized data acquisition system.

3.4 Procedures

3.4.1 HTLC/SBWC Operation

Prior to each use the mobile phases were degassed with pure helium gas for 10 min. The purge valve of the pump was then opened to purge the mobile phases through the pump at 8.0 mL/min for 10 min to remove any residual air. After all the air bubbles were removed, the purge valve was closed and the flow rate was lowered to 1 mL/min. The flow was directed through the system. The oven and the UV detector were then turned on and set the appropriate temperature and wavelength respectively. At this point ice was added to the cooling bath and it was maintained throughout the run by adding ice every 15-20 min. It took approximately 30 min for the GC oven to reach the desired temperature. The pump was then programmed with desired gradient conditions shown in Tables 3.1-3.5. The uniform pressure of the HPLC pump confirmed the stabilized thermal equilibrium of the GC oven. The system was ready to perform a run when the output of the UV detector reached a steady state. First the output was adjusted to zero and then the injector port was set to load position. Then approximately 20 μL of analyte sample was loaded in to the injector loop. The injector was adjusted to the inject position while turning on the pump gradient and pressing F2 key on the computer keyboard to start collecting the data acquisition using PC/Chrom software. Once all the desired peaks were observed, the data acquisition was stopped. Above similar steps were followed for rest of the runs. After the completion of all the runs, the GC oven was turned off and then a (50:50) mixture of methanol and 18 MΩ-cm water was run through the system at 1 mL/min for approximately 20 min or until the oven reached to room temperature. Then the pump and UV detector were all turned off.

3.4.2 Preparation of Solutions

3.4.2.1 Preparation of Mobile Phases

Mobile phases for the HTLC separation of preservatives using Hamilton PRP-1 column at 80 ℃.

Mobile Phase A (0.1% phosphoric acid in water)

Mobile phase A was prepared by adding 1 mL of ortho phosphoric acid in 1000 mL of deionized water (18 MΩ-cm) in a suitable glass container and mixing well. The solution was degassed with helium prior to use.

Mobile Phase B (0.1% phosphoric acid in methanol)

Mobile phase B was prepared by adding 1 mL of ortho phosphoric acid to 1000 mL of HPLC grade methanol in a suitable glass container and mixing well. The solution was degassed with helium prior to use.

Mobile phases for the HTLC separation of preservatives using ZirChrom®-DiamondBond-C18 column at temperatures ranging from 80 – 150 ℃.

Mobile Phase A (40 mM phosphoric acid in water)

Mobile phase A was prepared by adding 2.5 mL of ortho phosphoric acid in 1000 mL of deionized water (18 MΩ-cm) in a suitable glass container and mixing well. The solution was degassed with helium prior to use.

Mobile Phase B

HPLC grade methanol was used as mobile phase B. The methanol was degassed with helium prior to use.

Mobile phase for the SBWC separation of preservatives using ZirChrom®-DiamondBond-C18 column at temperature 200 ℃.

Mobile Phase

The deionized water (18 MΩ-cm) was used as mobile phase. The water was degassed with helium prior to use.

3.4.2.2 Preparation of Calcium Chloride Solution

The calcium chloride solution was prepared by adding 0.625 g of calcium chloride to a 25-mL volumetric flask and then diluting to the mark with deionized water (18 MΩ-cm). The solution was mixed thoroughly.

3.4.2.3 Preparation of Standard Solutions

Preparation of the Internal Standard Solution

The internal standard solution was prepared by adding 0.025 g of butyl paraben to a 50-mL volumetric flask and then diluting to the mark with methanol. The solution was mixed thoroughly.

Preparation of Stock Standard Solution

The stock standard solution was prepared by adding 0.075 g of benzyl alcohol, 0.100 g of 2-phenoxyethanol, 0.025 g of each of methyl, ethyl and propyl paraben to a 50-mL volumetric flask and then diluting to the mark with methanol. The solution was mixed thoroughly.

Preparation of Calibration Standard Solution

A calibration standard solution was prepared by transferring 2 mL of the internal standard solution and 2 mL of stock standard solution to a 25-mL volumetric flask and then diluting to the mark with methanol. The solution was mixed thoroughly. A portion of calibration standard was then filtered into a clean suitable glass vial through a 0.45 μm whatman GDX.

3.4.2.4 Preparation of Samples

Each Olay® skincare cream sample was mixed well prior to sampling to ensure a homogeneous mixture. The samples were prepared by weighing 0.300 g of Olay® skin creams directly into a tared 25-mL glass vial. 2 mL of internal standard, 2 mL of calcium chloride solution and 11 mL of methanol were added to the glass vial. Samples were vortexed for 15 min or more until the sample was completely dissolved. An aliquot of the sample was then filtered through a 0.45 μm whatman GDX filter into a clean suitable glass vial.

3.5 Separation Conditions

The experimental parameters applied to the separation of the preservatives in skincare cream samples using HTLC/SBWC are summarized in Tables 3.1 - 3.5.

Table 3.1. Experimental Conditions Used for HTLC Separations at 80 °C on Hamilton PRP-1 Column

Injection Volume: 10 μL

Wavelength: 256 nm

Mobile Phase A: 0.1% phosphoric acid in water

Mobile Phase B: 0.1% phosphoric acid in methanol

Flow Rates: 1.2 – 1.4 mL/min

Run Time: 14 min

Gradient:

Time (min)	%A	%B
0	60	40
2	40	60
14	25	75

Table 3.2. Experimental Conditions Used for HTLC Separations at 80 °C on ZirChrom®-DiamondBond-C18 Column

Injection Volume: 10 μL

Wavelength: 256 nm

Mobile Phase A: 40 mM phosphoric acid in water

Mobile Phase B: 100% methanol

Flow Rates: 1.4 mL/min

Run Time: 8 min

Gradient:

Time (min)	%A	%B
0	60	40
2	40	60
8	25	75

Table 3.3. Experimental Conditions Used for HTLC Separations at 90 °C on ZirChrom®-DiamondBond-C18 Column

Injection Volume: 10 μL

Wavelength: 256 nm

Mobile Phase A: 40 mM phosphoric acid in water

Mobile Phase B: 100% methanol

Flow Rates: 2.0 – 3.0 mL/min

Run Time: 6 min

Gradient:

Time (min)	%A	%B
0	70	30
2	60	40
6	10	90

Table 3.4. Experimental Conditions Used for HTLC Separations at 150 °C on ZirChrom® -DiamondBond-C18 Column

Injection Volume: 10 μL

Wavelength: 256 nm

Mobile Phase A: 40 mM phosphoric acid in water

Mobile Phase B: 100% methanol

Flow Rates: 1.5 – 3.5 mL/min

Run Time: 10 min

Gradient:

Time (min)	%A	%B
0	90	10
2	80	20
6	50	50
10	50	50

Table 3.5. Experimental Conditions Used for SBWC Separations at 200 °C on ZirChrom®-DiamondBond-C18 Column

Injection Volume: 10 μL
Wavelength: 256 nm
Mobile Phase: 100% water
Flow Rates: 1.25 – 2.5 mL/min
Run Time: 30 min

CHAPTER 4: RESULTS AND DISCUSSION

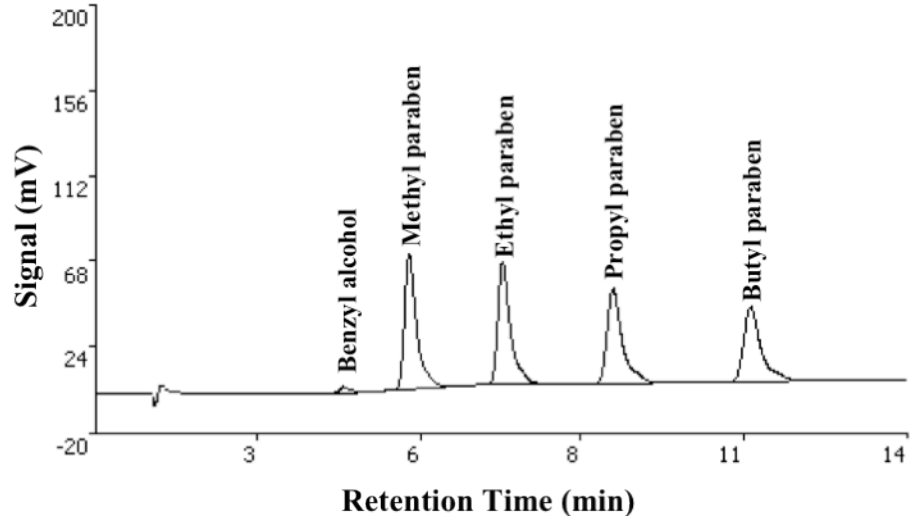
4.1 High Temperature Liquid Chromatography (HTLC) Separation at 80 °C

4.1.1 Hamilton PRP-1 Column

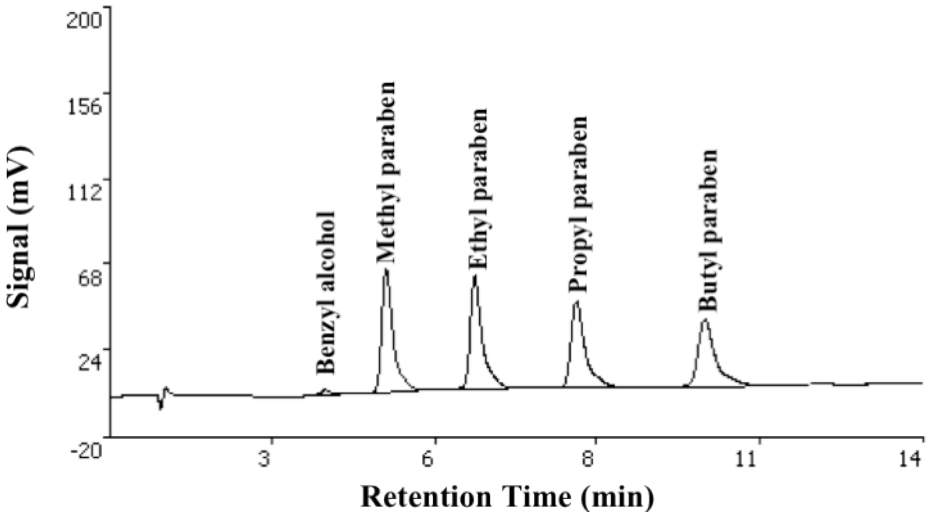
Evaluation of the separation of preservatives standard solution was initially performed at 80 °C using the Hamilton PRP-1 column with flow rates ranging from 1.2 to 1.4 mL/min. The optimized experimental conditions are shown in Table 3.1. The chromatograms obtained are shown in Figure 4.1. As you can see in Figure 4.1, all the preservatives were well separated from each other. The resolution was also found to be good. All preservatives were eluted well within 12 min. Table 4.1 shows the summarized values of the methanol volumes used in both traditional HPLC method and HTLC method at 80 °C**.** About 8 – 22% of methanol can be saved using this HTLC method. In order to save more methanol, our research was continued on another thermally stable zirconia-based column.

Table 4.1. Methanol Consumption (HPLC method vs. HTLC method at 80 °C) Using Hamilton PRP-1 Column

Average volume of methanol consumption, mL			**Methanol saved, %**	
HPLC method	HTLC at 80 °C			
10.0	1.2 mL/min	1.4 mL/min	1.2 mL/min	1.4 mL/min
	7.8	9.2	22	8.0



a)



b)

Figure 4.1. HTLC chromatograms of preservative standard at 80 °C using Hamilton PRP-1 column. a) 1.2 mL/min, b) 1.4 mL/min.

4.1.2. ZirChrom®-DiamondBond-C18 Column

Our research focus was shifted to using the ZirChrom®-DiamondBond-C18 column that yields better separation efficiency. Evaluation of the preservatives standard solution using this ZirChrom® column was initially performed at 80 °C. The optimized experimental conditions are shown in Table 3.2. The chromatogram obtained is shown in Figure 4.2. All preservative peaks were well separated within 7 min. As shown in Figure 4.2, the peaks have a narrow and symmetrical shape. Good resolution between peaks was observed. The values of methanol volumes used in traditional HPLC method and HTLC method at 80 °C are shown in Table 4.2. Approximately 54% of methanol can be saved by using HTLC method at 80 °C when compared to the P&G HPLC method at ambient temperature.

Table 4.2. Methanol Consumption (HPLC method vs. HTLC method at 80 °C) Using ZirChrom®-DiamondBond-C18 Column

Average volume of methanol consumption, mL		**Methanol saved, %**
HPLC method	HTLC method at 80 °C	54
10.0	4.6	

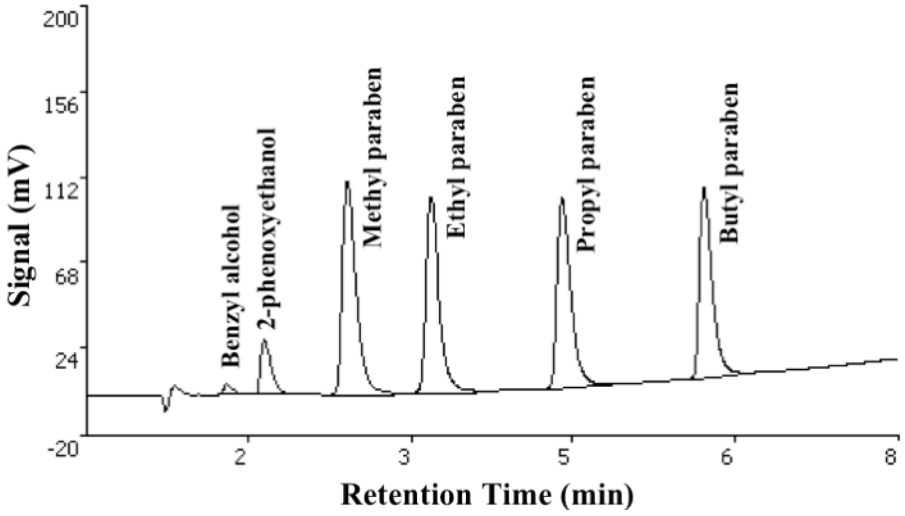


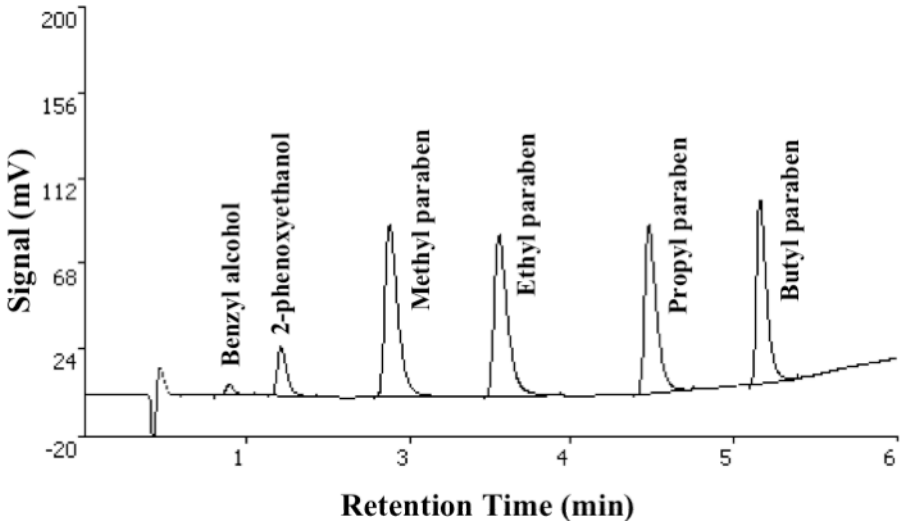
Figure 4.2. HTLC chromatogram of preservative standard at 80 °C using ZirChrom®-DB-C18 column at flow rate of 1.4 mL/min.

4.2 High Temperature Liquid Chromatography (HTLC) Separation at 90 °C

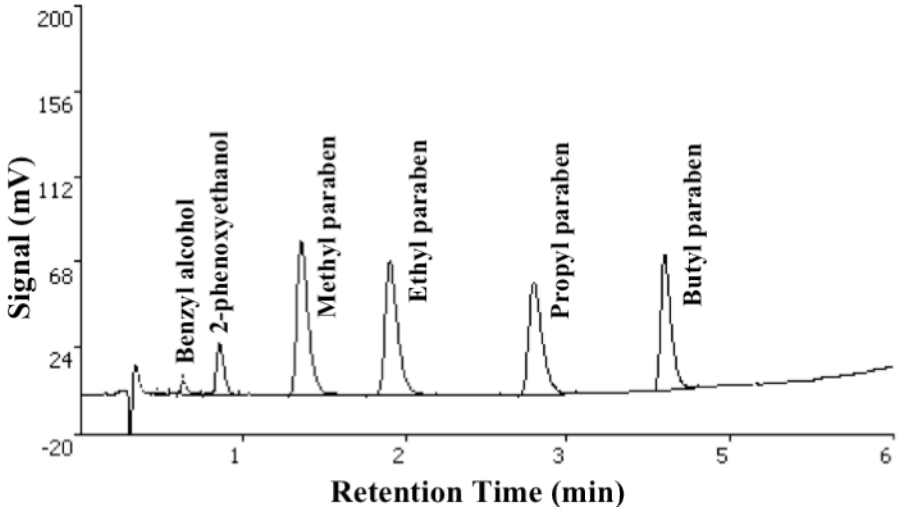
The separation of preservatives standard solution was then performed at 90 °C using the ZirChrom®-DiamondBond-C18 column with flow rates ranging from 2.0 to 3.0 mL/min. The optimized experimental conditions are shown in Table 3.3. The chromatograms obtained are shown in Figure 4.3. Good separation of preservative peaks was achieved in less than 6 min at both flow rates used. The peaks obtained at 2.0 mL/min flow rate are narrower than those obtained at 3.0 mL/min flow rate. Table 4.3 shows the methanol volumes used by both HPLC and HTLC methods. Approximately 26 - 34% of methanol can be saved by using HTLC method at 90 °C when compared to the P&G HPLC method at ambient temperature.

Table 4.3. Methanol Consumption (HPLC method vs. HTLC method at 90 °C) Using ZirChrom®-DiamondBond-C18 Column

Average volume of methanol consumption, mL			**Methanol saved, %**	
HPLC method	HTLC at 90 °C			
10.0	2.0 mL/min	3.0 mL/min	2.0 mL/min	3.0 mL/min
	6.6	7.4	34	26



a)



b)

Figure 4.3. HTLC chromatograms of preservative standard at 90 °C using ZirChrom®-DB-C18 column. a) 2.0 mL/min, b) 3.0 mL/min.

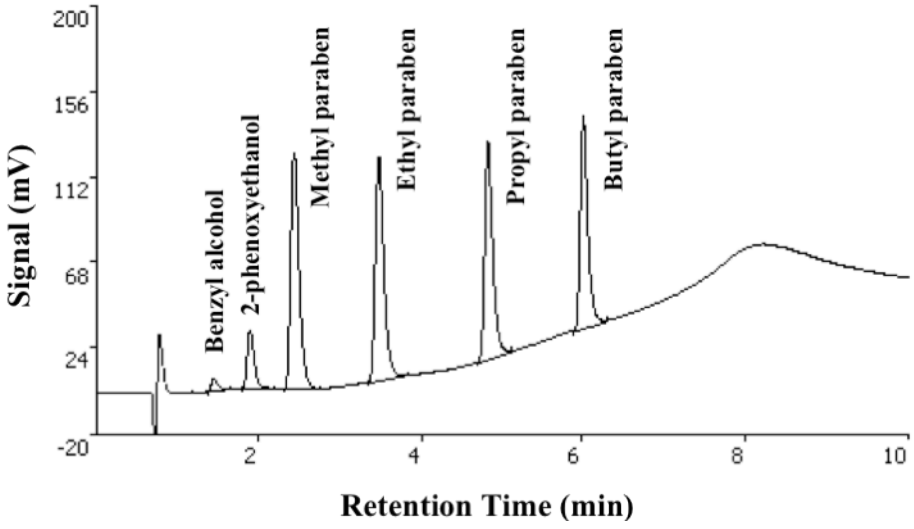
4.3 High Temperature Liquid Chromatography (HTLC) Separation at 150 °C

4.3.1 Separation and Analysis of Standard Preservative Solutions

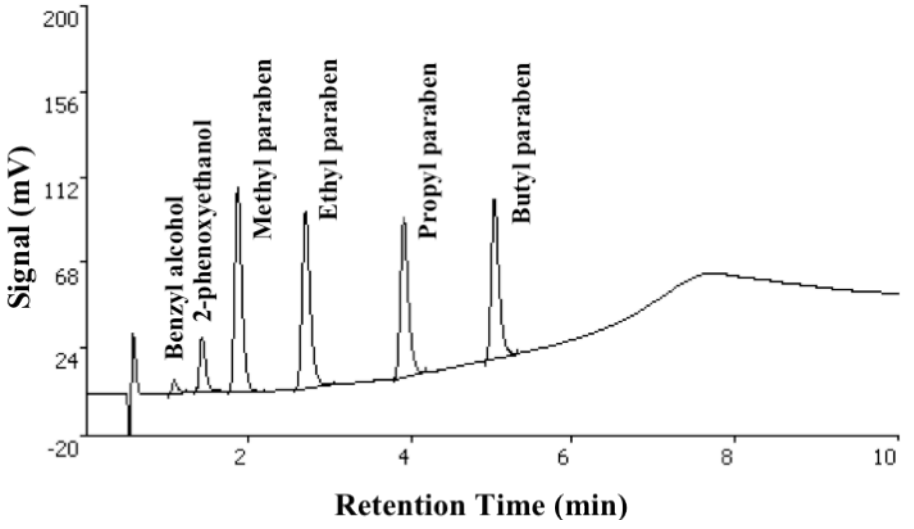
In order to further reduce the consumption of methanol, separation of preservatives standard solution was performed at 150 °C using optimized experimental conditions shown in Table 3.4. Four different flow rates were tested ranging from 1.5 to 3.5 mL/min in optimizing the separation of preservatives. Figure 4.4 shows the chromatograms while Figure 4.5 shows the van Deemter plot at these flow rates. As shown in Figure 4.5, the plate height of all the parabens stays relatively flat in the range of 1.5 – 2.0 mL/min. However, the plate height increases significantly at 3.0 mL/min. The methanol volumes used in both traditional HPLC and HTLC methods are given in Table 4.4. Considering the separation efficiency, speed and methanol consumption, flow rate of 2.0 mL/min was chosen for quantification of preservatives in Olay® skincare cream samples and potential building-up studies. Approximately 66% of methanol can be saved by using HTLC method at 150 °C with flow rate of 2.0 mL/min compared to the P&G HPLC method at ambient temperature.

Table 4.4. Methanol Consumption (HPLC method vs. HTLC method at 150 °C) Using ZirChrom®-DiamondBond-C18 Column

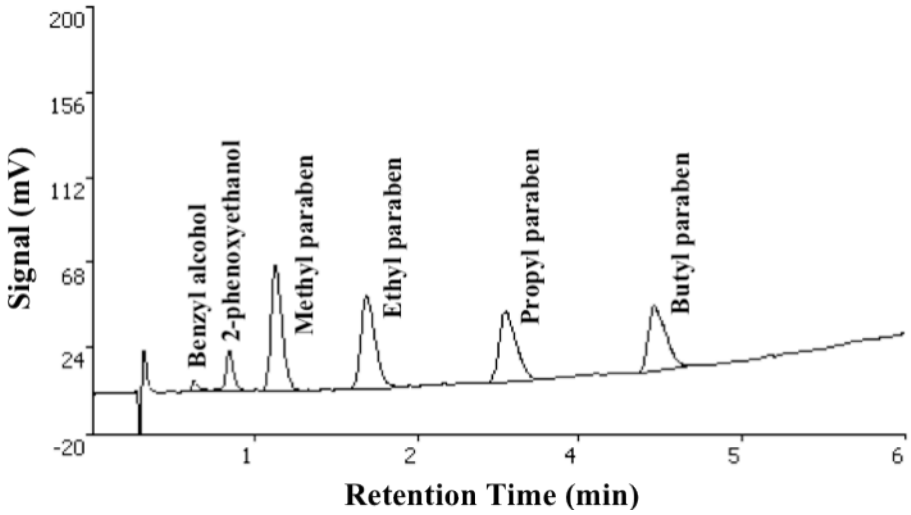
Average volume of methanol consumption, mL					**Methanol saved, %**			
HPLC method	HTLC method at 150 °C							
10.0	1.5 mL/min	2.0 mL/min	3.0 mL/min	3.5 mL/min	1.5 mL/min	2.0 mL/min	3.0 mL/min	3.5 mL/min
	2.6	3.4	5.1	6.0	74	66	49	40


Signal (mV)
200
156
112
68
24
-20
2
4
6
8
10
Retention Time (min)
Benzyl alcohol
2-phenoxyethanol
Methyl paraben
Ethyl paraben
Propyl paraben
Butyl paraben

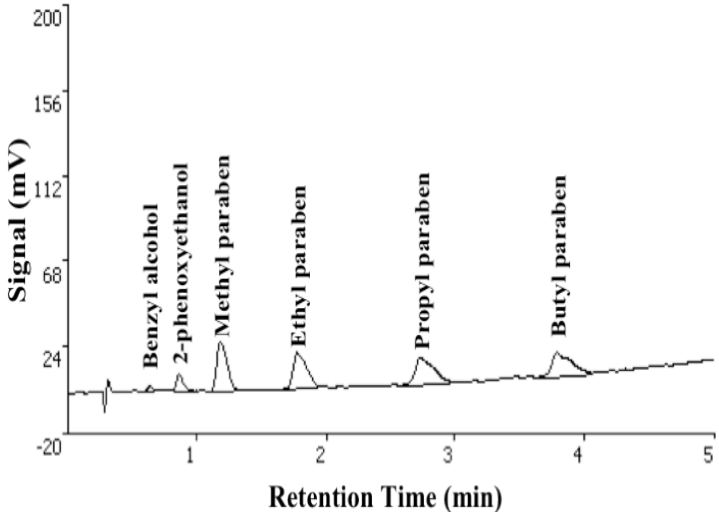
a)


Signal (mV)
200
156
112
68
24
-20
2
4
6
8
10
Retention Time (min)
Benzyl alcohol
2-phenoxyethanol
Methyl paraben
Ethyl paraben
Propyl paraben
Butyl paraben

b)



c)



d)

Figure 4.4. HTLC chromatograms of preservative standard at 150 °C using ZirChrom®-DB-C18 column. a) 1.5 mL/min, b) 2.0 mL/min, c) 3.0 mL/min, d) 3.5 mL/min.

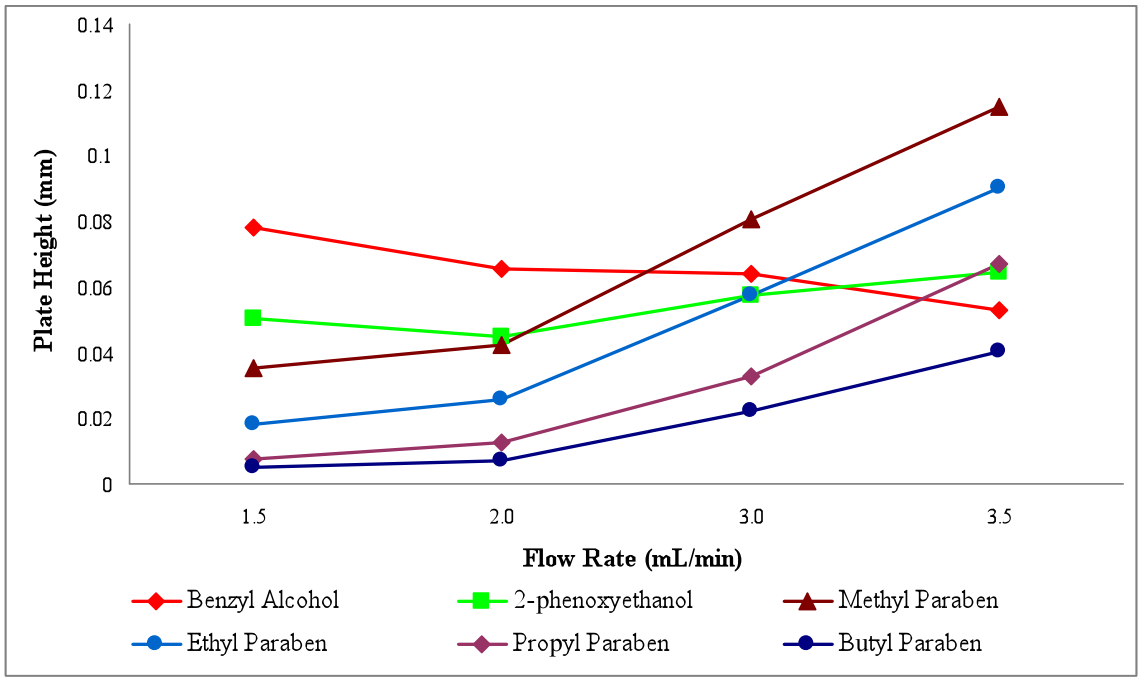
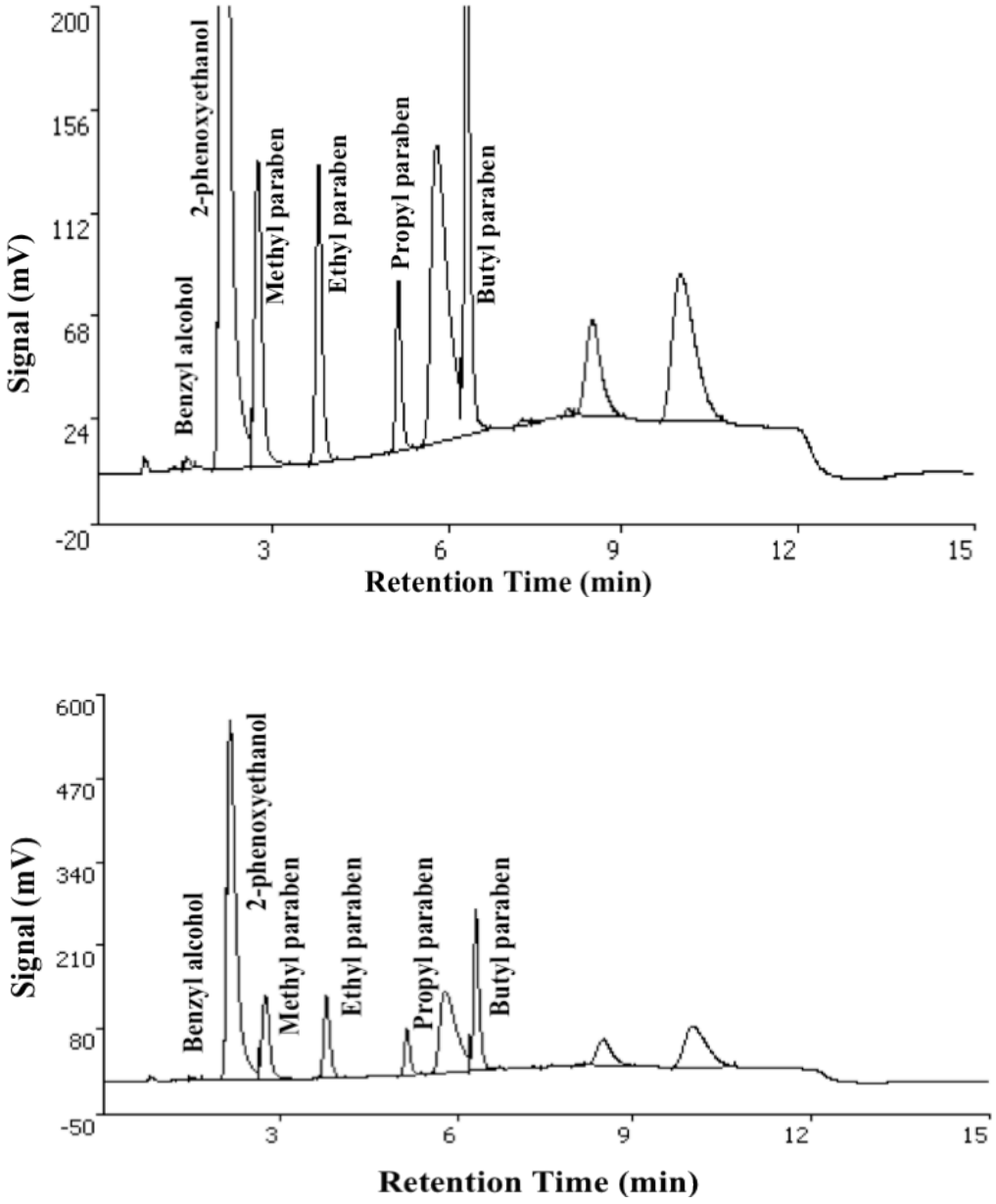


Figure 4.5. Van Deemter plots obtained by HTLC method on ZirChrom®-DiamondBond-C18 column at 150 °C.

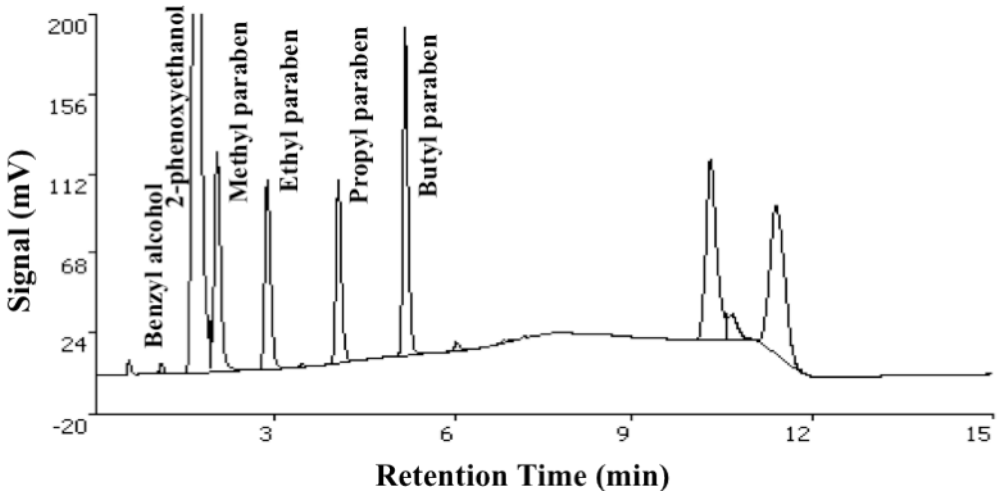
4.3.2 Separation and Analysis of Preservatives in Skincare Creams

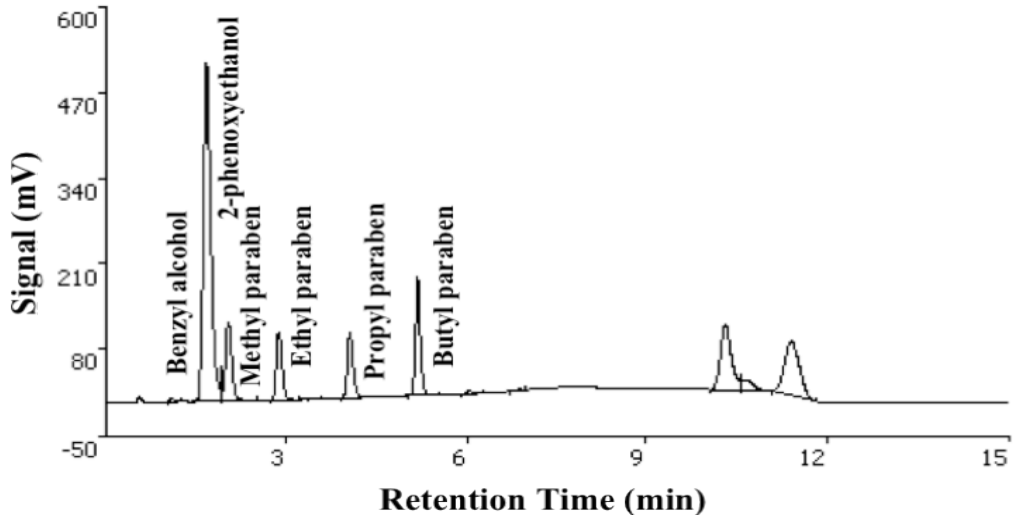
Quantification analysis of three Olay® skincare cream samples were performed using the optimized experimental conditions shown in Table 3.4 with a flow rate of 2.0 mL/min. Five independent sample preparations of each of the three Olay® samples were prepared as described in the procedure section of chapter 3. A single injection of each of the five sample preparations was injected along with a total of five injections of the preservative standard solutions. Figure 4.6 shows the chromatograms of the three Olay® skincare cream samples. The extra peaks eluted before and after butyl paraben as shown in Figure 4.6.a and extra peaks eluted after butyl paraben as shown in Figure 4.6.b are from the sample matrix. Butyl paraben was used as the internal standard. The % recoveries of samples were calculated based on P&G reference values. The formula is shown in the equation below. The results are shown in Tables 4.5 through 4.7.

$$\text{Recovery\%} = \frac{\text{wt\% obtained by HTLC method}}{\text{wt\% provided by P \& G}} \times 100 \qquad (4\text{-}1)$$

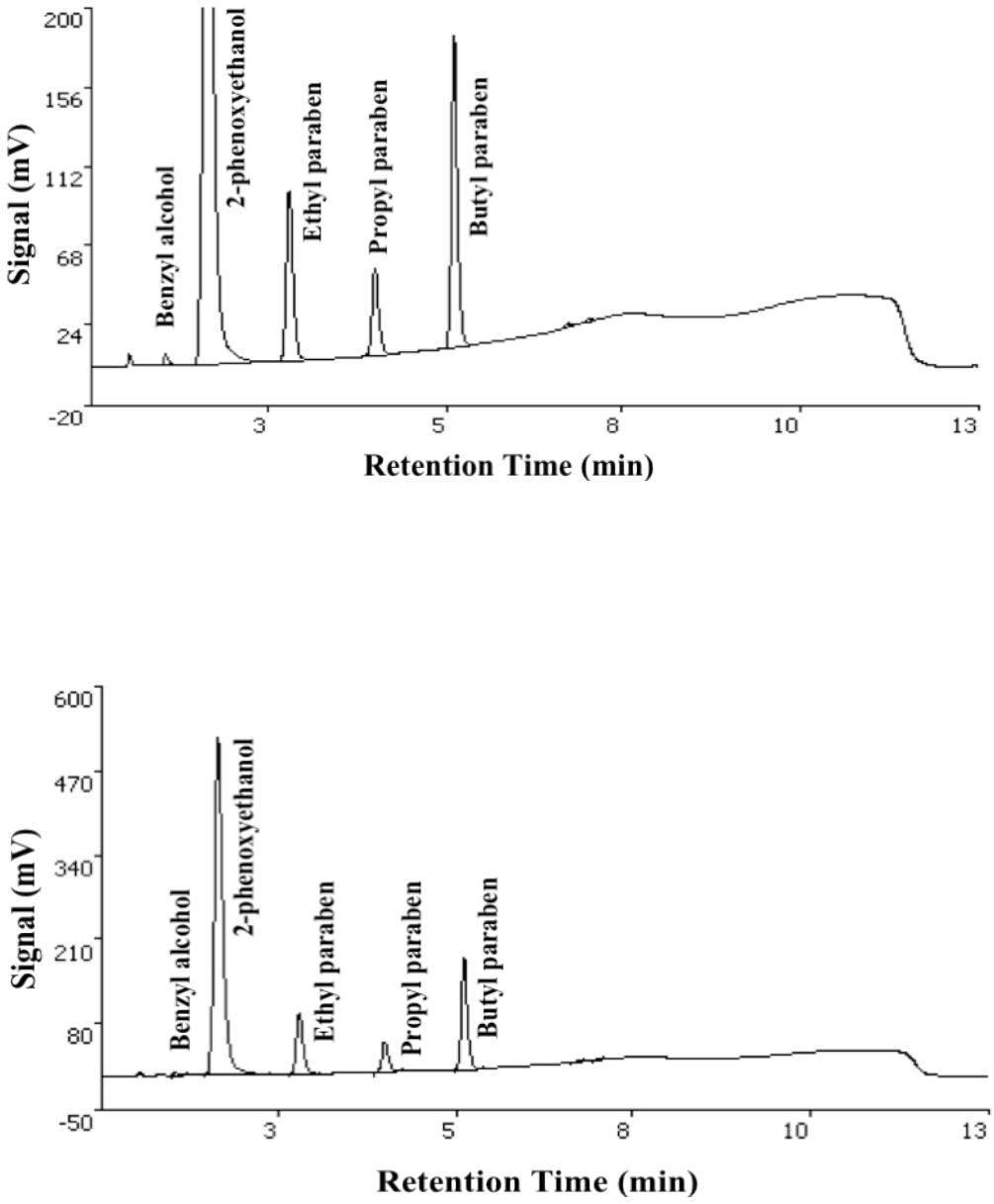

Signal (mV)
200
156
112
68
24
-20
3
6
9
12
15
Retention Time (min)
Benzyl alcohol
2-phenoxyethanol
Methyl paraben
Ethyl paraben
Propyl paraben
Butyl paraben
Signal (mV)
600
470
340
210
80
-50
3
6
9
12
15
Retention Time (min)
Benzyl alcohol
2-phenoxyethanol
Methyl paraben
Ethyl paraben
Propyl paraben
Butyl paraben

a)


Signal (mV)
200
156
112
68
24
-20
3
6
9
12
15
Retention Time (min)
Benzyl alcohol
2-phenoxyethanol
Methyl paraben
Ethyl paraben
Propyl paraben
Butyl paraben


Signal (mV)
600
470
340
210
80
-50
3
6
9
12
15
Retention Time (min)
Benzyl alcohol
2-phenoxyethanol
Methyl paraben
Ethyl paraben
Propyl paraben
Butyl paraben

b)



c)

Figure 4.6. HTLC chromatograms of skincare cream samples at 150 °C using ZirChrom®-DB-C18 column. a) Olay® SPF-15 (expanded and full scale), b) Olay® SPF-20 (expanded and full scale), c) Olay® 7-in-1 anti-aging moisturizer (expanded and full scale).

Table 4.5. Quantification Results of Olay® SPF-15 Skincare Cream Sample Obtained by HTLC at 150 °C

Preservatives	% RSD of Retention Time (n=5)	% RSD of Peak Area (n=5)	% Recovery (n=5)	%RSD (n=5)
Benzyl alcohol	0.8	3.5	78.6	3.2
Methyl paraben	0.8	6.1	99.5	4.8
Ethyl paraben	0.4	3.4	90.8	2.7
Propyl paraben	0.6	2.8	88.8	2.5

As shown in Table 4.5, the percent recoveries of all preservatives are between 79 – 100. The repeatability %RSD of all the recoveries was in the range of 2.5 – 4.8%, which demonstrates that this HTLC method was reproducible.

Table 4.6. Quantification Results of Olay® SPF-20 Skincare Cream Sample Obtained by HTLC at 150 °C

Preservatives	% RSD of Retention Time (n=5)	% RSD of Peak Area (n=5)	% Recovery (n=5)	%RSD (n=5)
Benzyl alcohol	2.8	6.9	94.3	7.2
Methyl paraben	3.6	8.2	114.5	4.8
Ethyl paraben	2.9	4.4	92.4	4.4
Propyl paraben	2.2	2.3	95.9	0.6

The recoveries of all preservatives in Olay® SPF-20 skincare cream sample as shown in Table 4.6 are close to 100% except for methyl paraben (114.5%). Co-elution was the cause for the high recovery of methyl paraben. The repeatability %RSD of all the recoveries was in the range of 0.6 – 7.2% which demonstrates that this HTLC method was reproducible.

Table 4.7. Quantification Results of Olay Total Effects 7-in-1 Anti-aging Daily Moisturizer Skincare Sample Obtained by HTLC at 150 °C

Preservatives	% RSD of Retention Time (n=5)	% RSD of Peak Area (n=5)	% Recovery (n=5)	%RSD (n=5)
Benzyl alcohol	1.0	4.9	95.0	2.6
Ethyl paraben	0.7	5.1	90.0	2.6
Propyl paraben	0.7	6.0	96.3	2.1

As shown in Table 4.7, the percent recoveries of all preservatives in Olay® total effects 7-in-1 anti-aging daily moisturizer are between 90 – 100. The repeatability %RSD of all the recoveries was in the range of 2.1 – 2.6%, which clearly demonstrates that this HTLC method was reproducible.

4.3.3. Study on Potential Building-up

Evaluation of a potential building-up caused by real sample analysis was performed with the experimental conditions shown in Table 3.4 at 2.0 mL/min. In this evaluation, 20 replicate injections of one Olay® skincare cream sample preparation was analyzed to check for any potential building-up in the system due to continuous injections of real samples. The results obtained are shown in Table 4.8.

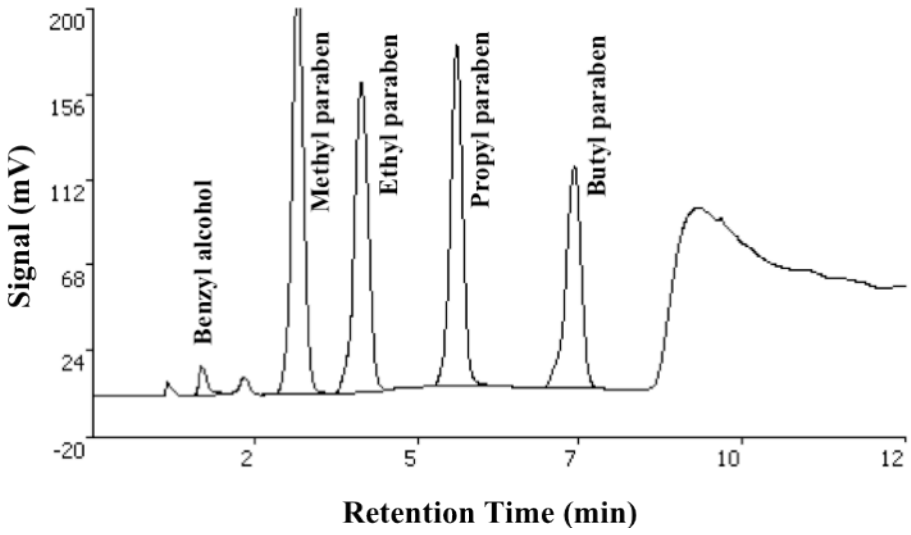
Table 4.8. Quantification Results of Olay SPF-20 Skincare Sample Obtained by HTLC at 150 °C with 20 Replicate Injections

Preservatives	% RSD of Retention Time (n=20)	% RSD of Peak Area (n=20)	% Recovery (n=20)	%RSD (n=20)
Benzyl alcohol	1.7	4.4	92.2	6.5
Methyl paraben	1.2	3.1	104.6	4.6
Ethyl paraben	1.0	3.3	94.6	5.2
Propyl paraben	0.9	3.7	96.9	4.6

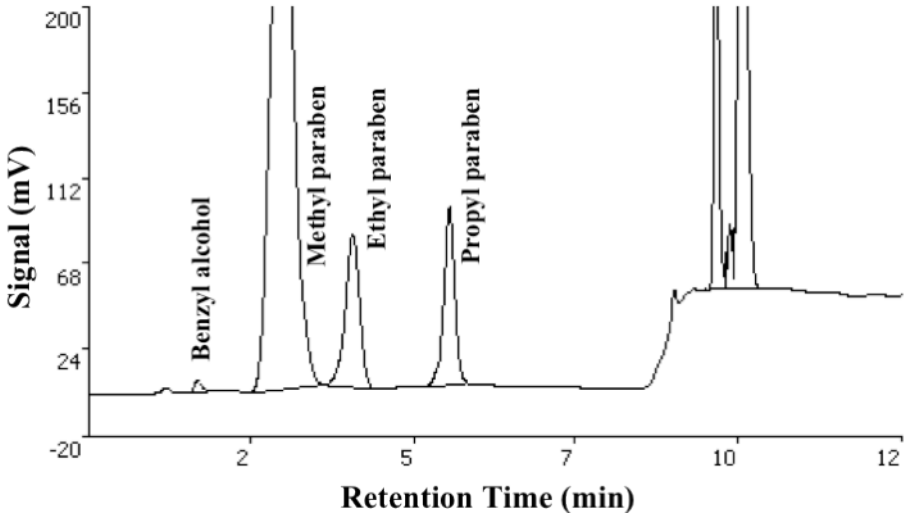
The recoveries of benzyl alcohol, methyl, ethyl, and propyl paraben from potential building-up study as shown in Table 4.8 are between 90-105 %. The repeatability of all recoveries was in the range of 4.6 – 6.5%. These recovery and %RSD results clearly indicate that there was no significant building-up of the sample occurred during HTLC analysis.

Based on the quantification analysis results and the potential building-up study results, separation and analysis of the preservatives in the skincare cream samples can be achieved by using HTLC separation at 150 ºC on ZirChrom®-DiamondBond-C18 column. This HTLC method not only reduces the analysis time but also approximately 66% of the methanol consumption can be saved when compared to the P&G HPLC method at ambient temperature.

Experiments were conducted to show that the Olay® skin care cream samples did not contain butyl paraben in them. In order to confirm its absence, we have prepared Olay® SPF-20 sample as described in the procedure section of Chapter 3 without adding internal standard (butyl paraben). One of the sample injections was made along with the preservatives standard using the optimized experimental conditions described in Table 3.4 at flow rate of 2.0 mL/min. Figure 4.11 shows the chromatograms of the standard and the sample which clearly confirms that the butyl paraben is not present in the samples and therefore used as the internal standard to quantify the other preservatives.



a)



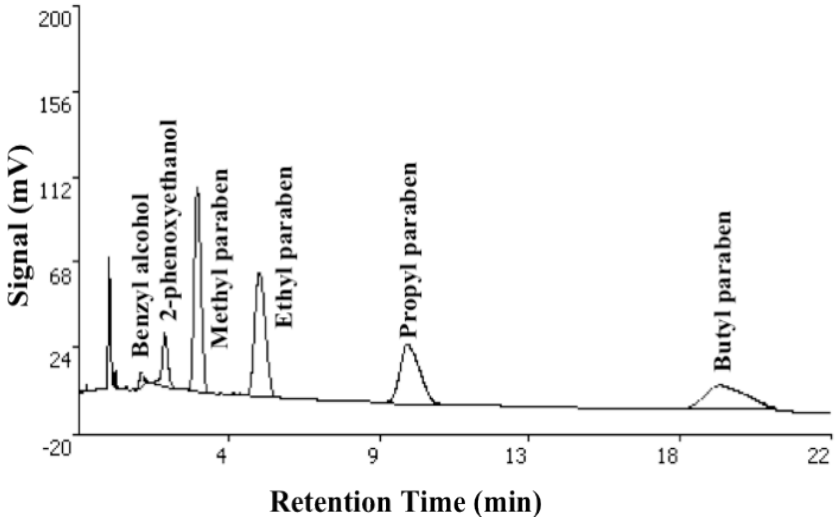
b)

Figure 4.7. HTLC chromatograms of Olay® samples without butyl paraben obtained at 150 °C. a) Preservatives standard, b) Olay® SPF-20 skin care cream sample.

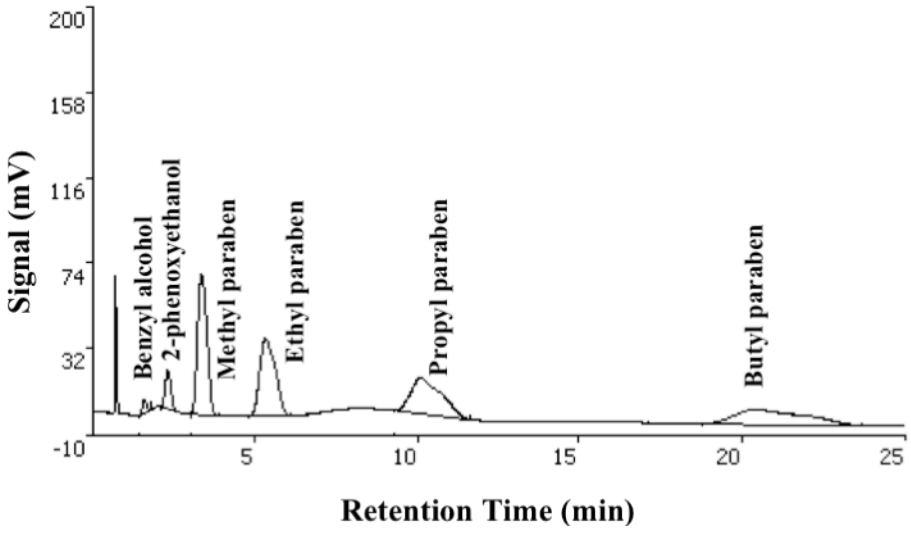
4.4 Subcritical Water Chromatography (SBWC) Separation at 200 °C

4.4.1 Separation and Analysis of Standard Preservative Solutions

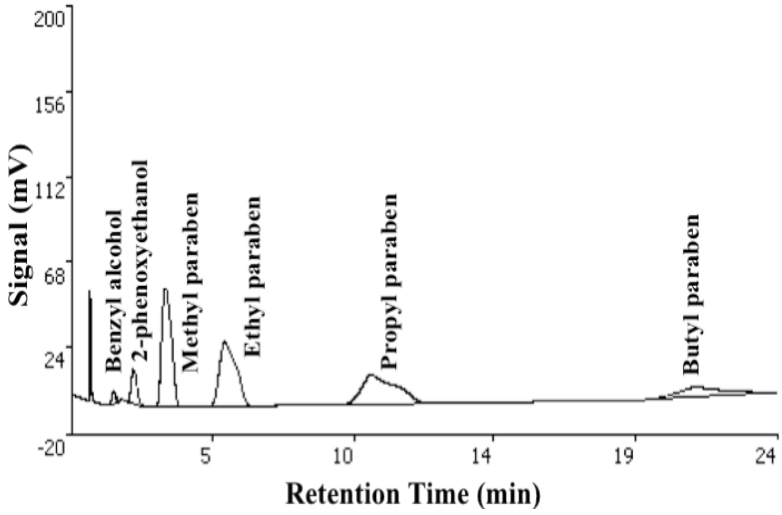
Our next research goal was to eliminate the consumption of methanol completely, so we have evaluated the separation of the preservatives using pure water at 200 °C with flow rates ranging from 1.25 to 2.5 mL/min on the ZirChrom®-DiamondBond-C18 column. Experimental conditions are shown in Table 3.5. Figure 4.8 depicts the chromatograms obtained from SBWC method. As shown in Figure 4.8, separation of preservatives achieved at 1.25 and 1.50 mL/min flow rates was good when compared to the other tested flow rates.



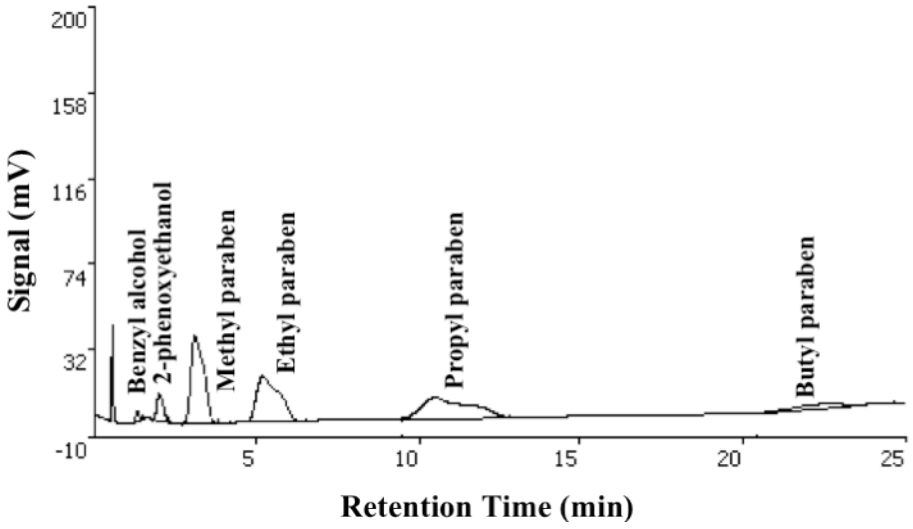
a)


200
158
116
74
32
-10
Signal (mV)
Benzyl alcohol
2-phenoxyethanol
Methyl paraben
Ethyl paraben
Propyl paraben
Butyl paraben
5
10
15
20
25
Retention Time (min)

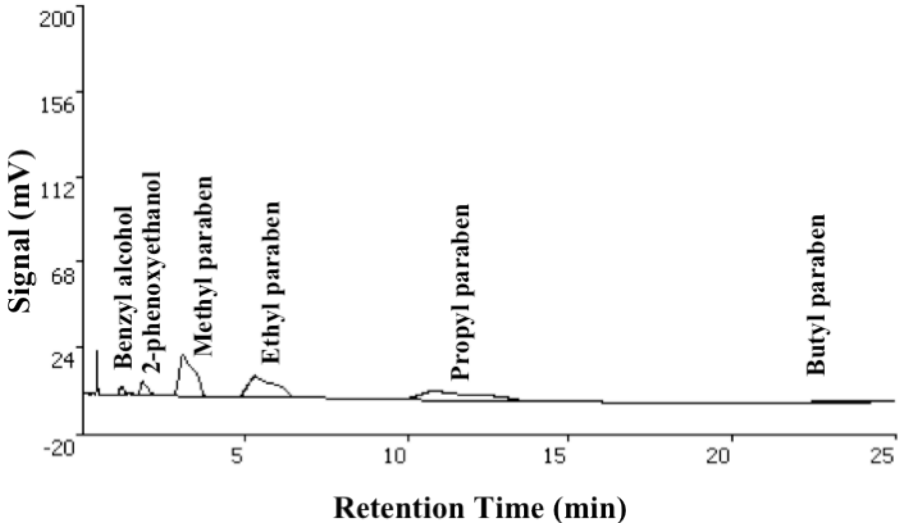
b)


200
156
112
68
24
-20
Signal (mV)
Benzyl alcohol
2-phenoxyethanol
Methyl paraben
Ethyl paraben
Propyl paraben
Butyl paraben
5
10
14
19
24
Retention Time (min)

c)



d)



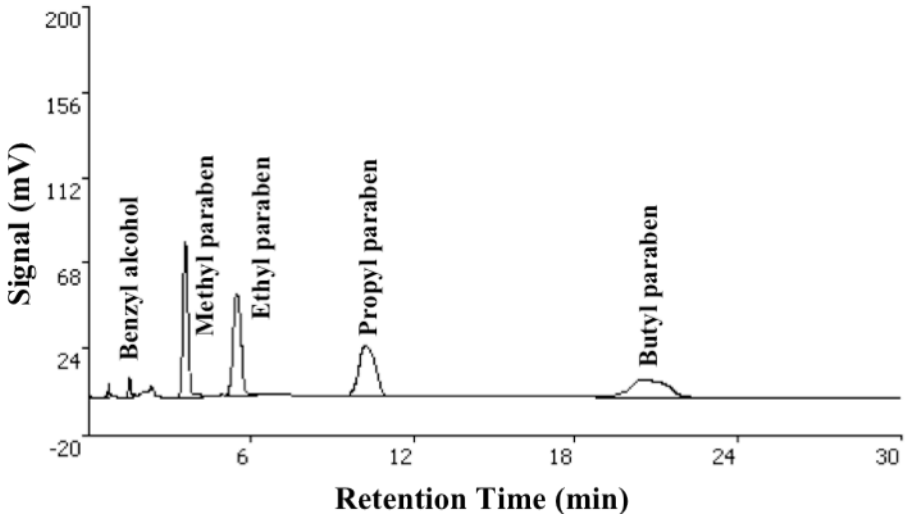
e)

Figure 4.8. SBWC Chromatograms of preservative standard at 200 °C using ZirChrom®-DB-C18 column. a) 1.25 mL/min, b) 1.50 mL/min, c) 1.75 mL/min, d) 2.0 mL/min, e) 2.50 mL/min.

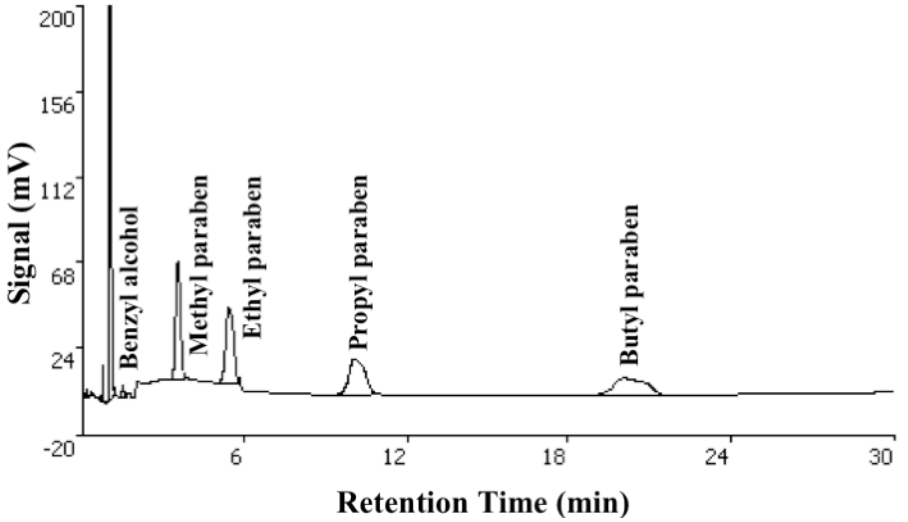
As shown in Figure 4.8, the retention of propyl and butyl parabens was increased with faster flow rates. This is an abnormal trend in column chromatography. Although the cause of this phenomenon is not clear at this time, the hydrophobic nature and poor solubility of these two parabens may contribute to the longer retention with increasing flow rate.

4.4.2. Separation and Analysis of Preservatives in Skincare Creams

Quantification analysis of Olay® SPF-20 skincare cream samples was performed using the experimental conditions shown in Table 3.5 with 1.5 mL/min flow rate. Five independent preparation of the Olay® SPF-20 sample were made as described in procedure section in chapter 3. A single injection of each of the five sample preparations was injected along with a total of five injections of the preservatives standard solution. Figure 4.9 shows the chromatogram of the Olay® SPF-20 skincare cream sample along with the preservatives standard solution. The percent recoveries and the percent RSD are shown in Table 4.9.



a)



b)

Figure 4.9. Chromatograms of SBWC at 200 °C using ZirChrom®-DB-C18 column with pure water as mobile phase, Flow rate: 1.5 mL/min. a) Preservatives standard, b) Olay® SPF-20 skin care cream sample.

Table 4.9. Quantification Results of Olay® SPF-20 Skincare Sample Obtained by SBWC at 200 °C

Preservatives	% RSD of Retention Time (n=5)	% RSD of Peak Area (n=5)	% Recovery (n=5)	%RSD (n=5)
Benzyl alcohol	0.7	4.2	98.5	3.8
Methyl paraben	0.4	6.4	108.6	2.7
Ethyl paraben	0.7	3.9	108.2	5.4
Propyl paraben	0.6	4.3	101.2	3.7

The recoveries of all preservatives in Olay® SPF-20 skincare cream sample as shown in Table 4.9 are between 98 – 110%. The repeatability of all recoveries was in the range of 2.7 – 5.4%, which demonstrates that this SBWC method was reproducible.

Based on the quantification analysis results, the separation and analysis of the preservatives in the skincare cream samples can be achieved by using SBWC separation at 200 ºC on ZirChrom®-DiamondBond-C18 column. This SBWC method completely eliminates the methanol consumption.

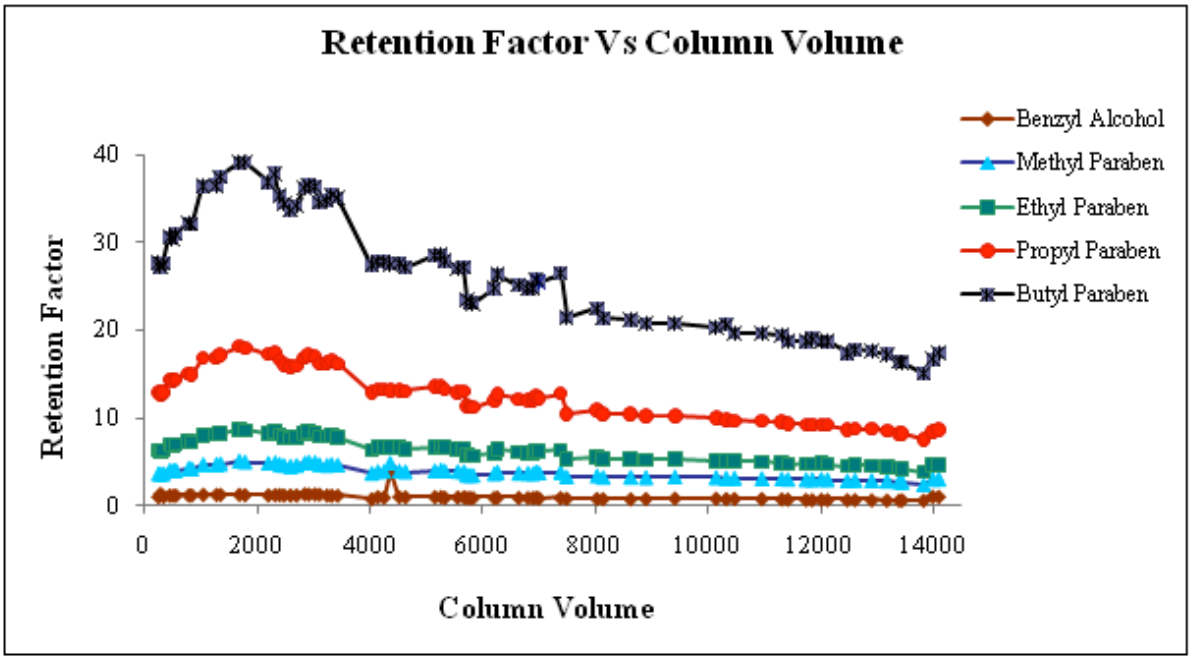
4.4.3 Long-term Stability of the ZirChrom®-DiamondBond-C18 column

The long-term thermal stability of ZirChrom®-DiamondBond-C18 column was evaluated under subcritical water conditions at 200 °C. The column was evaluated for 14,000 column volumes at elevated temperature of 200 °C. Retention factors ($k = (t_R-t_M)/t_M$, where k is the retention factor, t_R is the retention time of the analyte species, and t_M is the retention time of the unretained species.) and plate numbers ($N = 16(t_R/W_{1/2})^2$, where N is the plate number, t_R is the retention time of the analyte species, and $W_{1/2}$ is the peak width at the half peak height) were calculated for each peak at a given column volume to monitor any degradation of the stationary phase under subcritical water conditions.

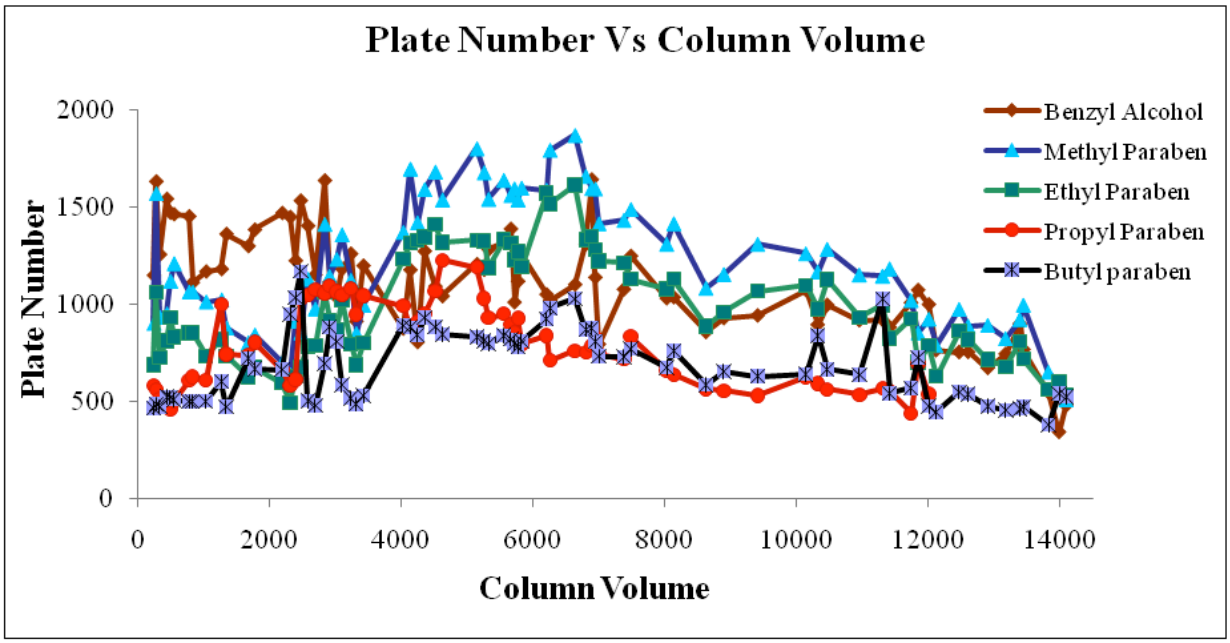
The 18 MΩ-cm water was used as the mobile phase for this evaluation. The mobile phase was degassed with helium before each use. Then the pump was turned on and the mobile phase was purged at 8.0 mL/min for 10 min by turning on the purge valve to remove any air residue in the mobile phase. After purging the mobile phase, the purge valve was turned off and the mobile phase at the flow rate of 1.5 mL/min was directed to the instrument. At this time oven and detector were turned on. The oven was set to 200 °C and the detector was set to 256 nm. Approximately one hour after the desired temperature was reached, the first injection of the day was made. The preservatives standard solution was used for this evaluation. The injection time was recorded and the data were collected. Then the same mixture was injected periodically at the same separation temperature to monitor the long-term change in retention time, plate number, and peak areas to evaluate the thermal stability of the column.

The column dimensions are 10 cm in length and 0.46 cm in inner diameter. Therefore, one column volume is equivalent to 1.66 mL ($V = \pi r^2 l = 3.14 \times (0.46/2)^2 \times 10 = 1.66$ cm = 1.66

mL). Thus at a flow rate of 1.5 mL/min, 1 min is equivalent to 0.90 column volumes (1.5 mL/min x 1 min/1.66 mL). As shown in Figure 4.10.a., the retention factors for the analytes initially had a slight increase from 0 to 3,000 column volumes. This may be due to the column which was flushed with methanol for 10 min after each analysis. A slight decreasing trend was observed from 3,000 to 7,000 column volumes. After 7,000 column volumes, retention factor for the analytes did not change very much after the column was exposed to subcritical water for more than 12,000 column volumes. As shown in Figure 4.10.b., the plate number gradually decreased with prolonged exposure to 200 ºC



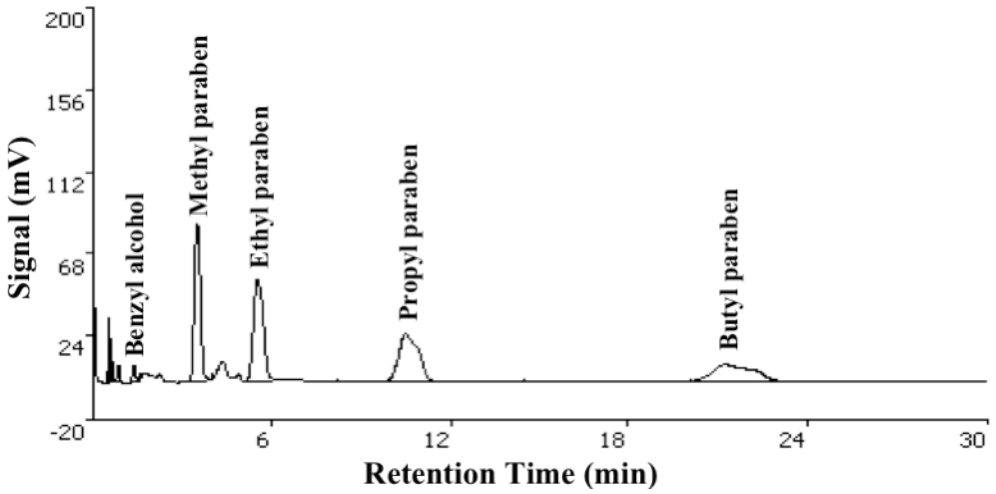
a)



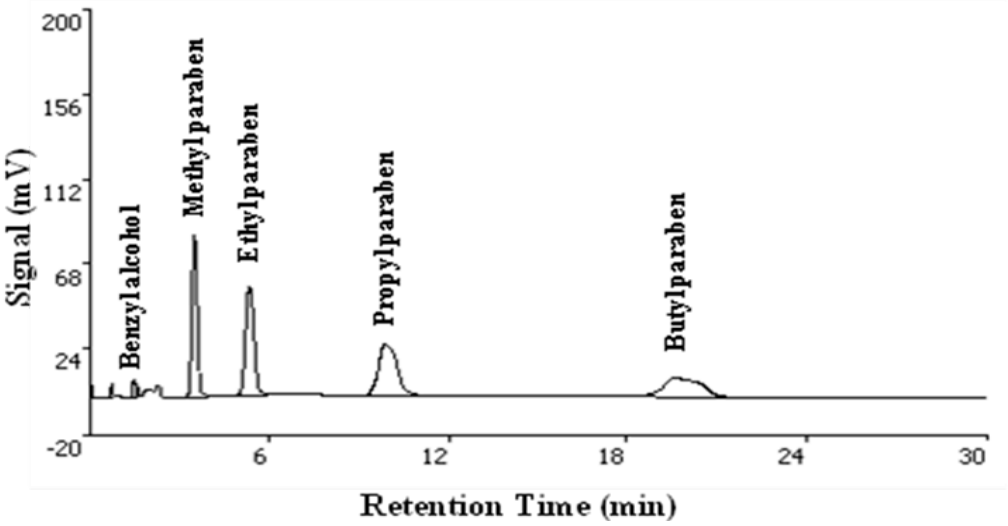
b)

Figure. 4.10.a) Retention factor vs. Column volume, b) Plate number vs. Column volume for SBWC separation at 200 ºC.

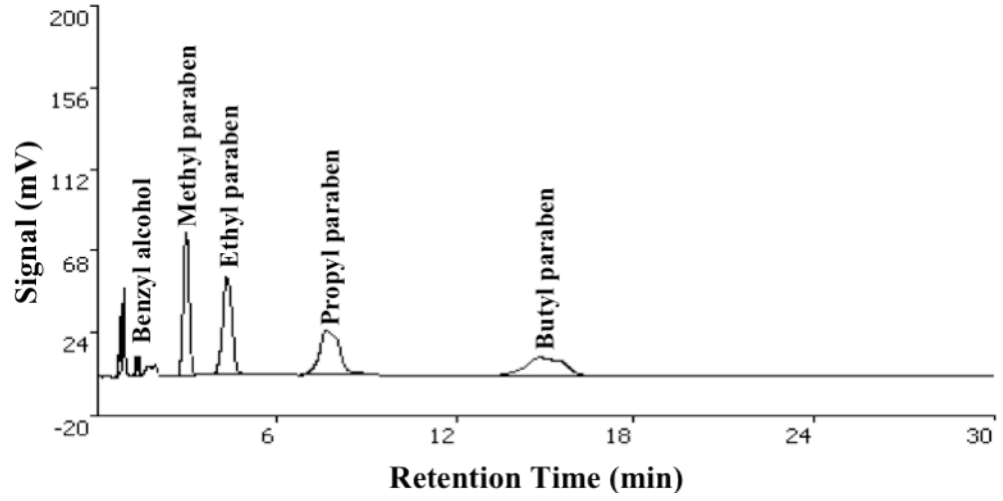
Figure 4.11 shows the chromatograms obtained at various points during the long-term stability evaluation at 200 ºC. As shown in Figure 4.11.c, the retention times of propyl paraben and butyl paraben shifted from 11 and 24 min to ~7 and 14 min respectively after the column was exposed to 200 ºC for 12,000 column volumes (224.0 hrs). The decrease in the retention times for propyl and butyl parabens after 12,000 column volumes indicated that the thermal stability became poorer due to prolonged heating under subcritical water condition.


Signal (mV)
200
156
112
68
24
-20
Benzyl alcohol
Methyl paraben
Ethyl paraben
Propyl paraben
Butyl paraben
6
12
18
24
30
Retention Time (min)

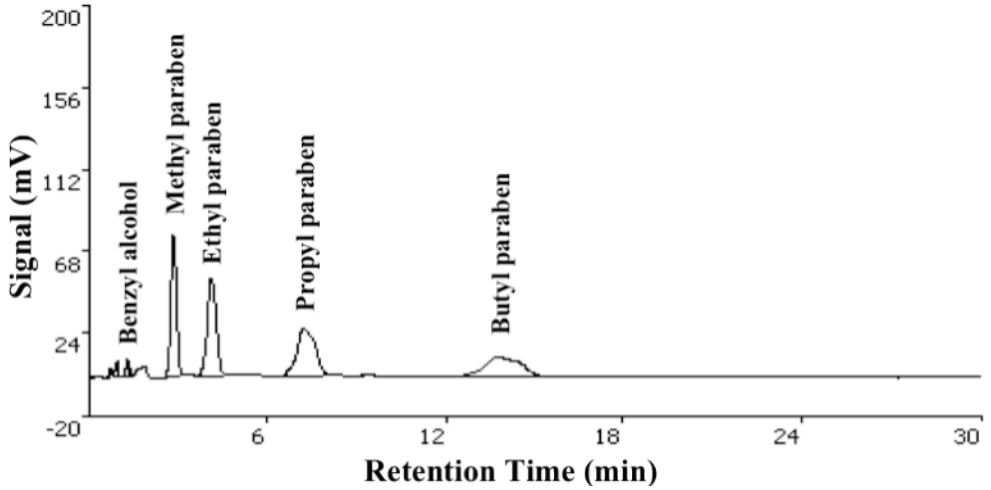
a)


Signal (mV)
200
156
112
68
24
-20
Benzylalcohol
Methylparaben
Ethylparaben
Propylparaben
Butylparaben
6
12
18
24
30
Retention Time (min)

b)



c)



d)

Figure 4.11. Chromatograms of preservatives standard obtained by SBWC at 200 ºC using 1.5 mL/min at different times during the long-term stability evaluation of the ZirChrom®-DB-C18 column. a) At the beginning of the evaluation (after 74.5 hrs., column volume = 4,140); b) After 124.0 hrs. exposure (column volume = 11,032) c) After 224.0 hrs. exposure (column volume = 12,015); d) At the end of the evaluation (263 hrs. or column volume = 14,100).

CHAPTER 5: CONCLUSIONS

In this study, HTLC and SBWC systems were constructed and used for the separation and analysis of preservatives in skincare creams. The percent recoveries obtained by HTLC at 150 ºC using ZirChrom®-DiamondBond-C18 column ranged between 79 – 115% with %RSD ranging typically from 3 – 7 for three Olay® skincare cream samples. This HTLC technique can significantly reduce the amount of methanol used in the mobile phase and achieve efficient separation of preservatives. The operation of this system is easy and the total analysis time is significantly reduced.

The percent recoveries obtained by SBWC method using pure water as the sole eluent for Olay® SPF-20 skincare cream sample are between 98-110 with %RSD of less than 5%. These SBWC results clearly demonstrate that the separation and analysis of preservatives in skincare creams using pure water as the sole eluent can be achieved at temperature 200 °C on the ZirChrom®-DiamondBond-C18 column. This SBWC technique completely eliminates the consumption of methanol, which is traditionally used in the mobile phases at ambient temperature.

The results obtained from the long-term stability study on ZirChrom®-DiamondBond-C18 column under subcritical water conditions at 200 ºC indicate that the column is relatively stable for up to 14,000 column volumes (or 263 hrs). A gradual decrease was noticed in the retention factor and plate number with prolonged exposure of the column to 200 ºC though there was slight increasing trend was initially observed.

Although a few columns are relatively stable at higher temperature and the commercial Shimadzu Nexera UFLC instrument is capable of handling high temperature up to 150 ºC, more stable stationary phases, commercial HTLC systems equipped with efficient heating up to 200 ºC and efficient cooling systems are needed for the adoption of HTLC/SBWC in industry.

REFERENCES

1. A. Varvaresou, S. Papageorgiou, E. Tsirivas, E. Protopapa, H. Kintziou, V. Kefala, C. Demetzos, *Int. J. Cosmet. Sci. 31* **(2009)** 163.
2. C. Edser, *Focus on Surfactants, 2006* **(2006)** 1.
3. A.L. Cashman, E.M. Warshaw, *Dermatitis 16* **(2005)** 57.
4. M.G. Soni, I.G. Carabin, G.A. Burdock, *Food and Chemical Toxicology 43* **(2005)** 985.
5. P.W. Harvey, P. Darbre, *J. Appl. Toxicol. 24* **(2004)** 167.
6. F.A. Andersen, *Int. J. Toxicol. 27* **(2008)** 1.
7. L. Gagliardi, A. Amato, A. Basili, G. Cavazzutti, E. Gattavecchia, D. Tonelli, *J. Chromatogr. 315* **(1984)** 465.
8. F.O. Serrano, I.S. Lopez, G.N. Revilla, *J. Liq. Chromatogr. 14* **(1991)** 709.
9. E. Sottofattori, M. Anzaldi, A. Balbi, G. Tonello, *J. Pharm. Biomed. Anal. 18* **(1998)** 213.
10. B. Saad, M.F. Bari, M.I. Saleh, K. Ahmad, M.K.M. Talib, *J. Chromatogr. A 1073* **(2005)** 393.
11. T. Wu, C. Wang, X. Wang, Q. Ma, *Int. J. Cosmet. Sci. 30* **(2008)** 367.
12. C.R. Wilke, P. Chang, *AM. Inst. Chem. Eng. J. 1* **(1955)** 264.
13. P. Sandra, G. Vanhoenacker, *J. Sep. Sci. 30* **(2007)** 241.
14. J.H. Knox, *J. Chromatogr. Sci. 15* **(1977)** 352.
15. T. Kondo and Y. Yang, *Anal. Chim. Acta 494* **(2003)** 157.
16. Y. Yang, *LC/GC, LC column Technology Supplement* **(2008)** 3.
17. R.M. Smith, *J. Chromatogr. A 1184* **(2008)** 441.
18. S.D. Allmon, J.G. Dorsey, *J. Chromatogr. A 1217* **(2010)** 5769.
19. Y. Yang, S. Bowdat, S.B. Hawthorne, D.J. Miller, *Anal. Chem. 67* **(1995)** 4571.
20. S.B. Hawthorne, Y. Yang, D.J. Miller, *Anal. Chem. 66* **(1994)** 2912.
21. Y. Yang, M. Belghazi, A. Lagadec, D.J. Miller, S.B. Hawthorne, *J. Chromatogr. 810* (**1998)** 149.
22. D.C. Steinberg, Preservatives for Cosmetics (2nd edition), Allured Publishing Corporation, 2006.

23. Materials Safety Data Sheet, http://www.wikipedia.org.

24. M.-R. Lee, C.-Y. Lin, Z.-G. Li, T.-F. Tsai, *J. Chromatogr. A 1120* **(2006)** 244.

25. T.A.M. Msagati, T. Barri, N. Larsson, J.A. Jonsson, *Int. J. Cosmet. Sci. 30* **(2008)** 297.

26. A. Zotou, I. Sakla, P.D. Tzanavaras, *J. Pharm. Biomed. Anal. 53* **(2010)** 785.

27. M. Thomassin, E. Cavalli, Y. Guillaume, C. Guinchard, *J. Pharm. Biomed. Anal. 15* **(1997)** 831.

28. J. Wang, D. Zhang, Q. Chu, J. Ye, *Chin. J. Chem. 28* **(2010)** 313.

29. H.-Y. Huang, Y.-C. Lai, C.-W Chiu, J.-M. Yeh, *J. Chromatogr. A 993* **(2003)** 153.

30. N. Memon, M. Iqbal Bhanger, M.Y. Khuhawer, *J. Sep. Sci. 28* **(2005)** 635.

31. J.F. Garcia-Jimenez, M.C. Valencia, L.F. Capitan-Vallvey, *Anal. Chim. Acta 594* **(2007)** 226.

32. L. Sanchez-Prado, J.P. Lamas, M. Lores, C. Garcia-Jares, M. Llompart, *Anal. Chem. 82* **(2010)** 9384.

33. L.A. Cole, J.G. Dorsey, *Anal. Chem. 62* **(1990)** 641.

34. A. Tchapla, S. Heron, H. Colin, G. Guiochon, *Anal. Chem. 60* **(1988)** 1443.

35. J. Li, P.W. Carr, *Anal. Chem. 69* **(1997)** 837.

36. T. Yarita, R. Nakajima, M. Shibukawa, *Anal. Sci. 19* **(2003)** 269.

37. S. Shen, H. Lee, J. McCaffrey, N. Yee, C. Senanayake, N. Grinberg, *J. Liq. Chromatogr. Related Technol. 29* **(2006)** 2823.

38. Y. Liu, N. Grinberg, K.C. Thompson, R.M.Wenslow, U.D. Neue, D.Morrison, T.H. Walter, J.E.O Gara, K.D. Wyndham, *Anal. Chim. Acta 554* **(2005)** 144.

39. D. Guillarme, S. Heinisch, J.L. Rocca, *J. Chromatogr. A 1052* **(2004)** 39.

40. Y. Yang, *Anal. Chim. Acta 558* **(2006)** 7.

41. M.H. Chen, C. Horvath, *J. Chromatogr. A 788* **(1997)** 51.

42. S. Heinisch J.L. Rocca, *J. Chromatogr. A 1216* **(2009)** 642.

43. F.D. Anita, C. Horvath, *J. Chromatogr. 435* **(1988)** 1.

44. D. Guillarme, S. Heinisch, J.L. Rocca, *J. Chromatogr. A 1052* **(2004)** 39.

45. C. Horvath, H. Lin, *J. Chromatogr. 149* **(1978)** 43.

46. C.V. McNeff, B. Yan, D.R. Stoll, R.A. Henry, *J. Sep. Sci. 30* **(2007)** 1672.

47. Y. Yang, *J. Sep. Sci. 30* **(2007)** 1131.

48. Y. Yang, B. Li, *Anal. Chem. 71* **(1999)** 1491.

49. L.J. Lamm, Y. Yang, *Anal. Chem. 75* **(2003)** 2237.

50. Y. Yang, S. Bowadt, S.B. Hawthorne, D. J. Miller, *Environ. Sci. Technol. 31* **(1997)** 430.

51. T.M. Pawlowski, C.F. Poole, *J. Agric. Food Chem. 46* **(1998)** 3124.

52. R.M. Smith, *J. Chromatogr. A 975* **(2002)** 31.

53. J. Kronholm, K. Hartonen, M.L. Riekkola, *Trends Anal. Chem. 26* **(2007)** 396.

54. Y. Yang, B. Kayan, N. Bozer, B. Pate, C. Baker, A.M. Gizir, *J. Chromatogr. A 1152* **(2007)** 262.

55. I.R. Meizoso, L. Jaime, S. Santoyo, F.J. Senoráns, A. Cifuentes, E. Ibáñez, *J. Pharm. Biomed. Anal. 51* **(2010)** 456.

56. Y. Yang, T.J. Kennedy, T. Kondo, *J. Chromatogr. Sci. 43* **(2005)** 518.

57. P. He, Y. Yang, *J. Chromatogr. A 989* **(2003)** 55.

58. Y. Yang, L. Lamm, P. He, T. Kondo, *J. Chromatogr. Sci. 40* **(2002)** 107.

59. Y. Yang, A.D. Jones, J.A. Mathis, M.A. Francis, *J. Chromatogr. A 942* **(2001)** 231.

60. Y. Yang, A.D. Jones, C.D. Eaton, *Anal. Chem. 71* **(1999)** 3808.

61. T.M. Pawlowski, C.F. Poole, *Anal. Commun. 36* **(1999)** 71.

62. R.M. Smith, *Anal. Bioanal. Chem. 385* **(2006)** 419.

63. J.W. Coym, J.G. Dorsey, *Anal. Lett. 37* **(2005)** 1013.

64. K. Hartonen, M. Riekkola, *Trends Anal. Chem. 27* **(2008)** 1.

65. G. Vanhoenacker, P. Sandra, *Anal. Bioanal. Chem. 390* **(2008)** 245.

66. H.A. Claessens, M.A.V. Straten, *J. Chromatogr. A 1060* **(2004)** 23.

67. P. Atkins, J. de Paula, Physical Chemistry (7th Edition), W. H. Freeman and Company, New York, 2006.

68. Y. Yang, D. J. Miller, S. B. Hawthorne, *J. Chem. Eng. Data 42* **(1997)** 908.

69. M.S. Silberberg, Chemistry – The Molecular Nature of Matter and Change (4th Edition), McGraw Hill Higher Education, New York, 2006.

70. T. Yarita, R. Nakajima, K. Shimada, S. Kinugasa, M. Shibukawa, *Anal. Sci. 21* **(2005)** 1001.

71. R.M. Smith, R. J. Burgess, *J. Chromatogr. A 785* **(1997)** 49.

72. O. Chienthavorn, R.M. Smith, *Chromatographia 50* **(1999)** 485.

73. T. Teutenberg, O. Lerch, H.J. Gotze, P. Zinn, *Anal. Chem. 73* **(2001)** 3896.

74. R. Tajuddin, R.M. Smith, *Analyst 127* **(2002)** 883.

75. S.M. Fields, C.Q. Ye, D.D. Zhang, B.R. Branch, X.J. Zhang, N. Okafo, *J. Chromatogr. A 913* **(2001)** 197.

76. M.M. Sanagi, H.H. See, *J. Liq. Chromatogr. 28* **(2005)** 3065.

77. B. Yan, J. Zhao, J.S. Brown, J. Blackwell, P.W. Carr, *Anal. Chem. 72* **(2000)** 1253.

78. L. Pereira, S. Aspey, H. Ritchie, *J. Sep. Sci. 30* **(2007)** 1115.

79. T. Kondo, Y. Yang, L. Lamm, *Anal. Chim. Acta 460* **(2002)** 185.

80. S. Saha, R.M. Smith, E. Lenz, I.D. Wilson, *J. Chromatogr. A 991* **(2003)** 143.

81. C.A. Bruckner, S.T. Ecker, R.E. Synovec, *Anal. Chem. 69* **(1997)** 3465.

82. D.J. Miller, S.B. Hawthorne, *Anal. Chem. 69* **(1997)** 623.

83. B.A. Ingelse, H.G. Janssen, C.A. Cramers, *J. High Resol. Chromatogr. 21* **(1998)** 613.

84. T. Yarita, R. Nakajima, S. Otsuka, T. Ihara, A. Takatsu, M. Shibukawa, *J. Chromatogr. A 976* **(2002)** 387.

85. M.O. Fogwill, K.B. Thurbide, *J. Chromatogr. A 1139* **(2007)** 199.

86. F. Yu, S.R. Juan, Y. Na, L.Y. De, H.T. Bao, *Chinese J. Anal. Chem. 35* **(2007)** 1335.

87. T.S. Kephart, P.K. Dasgupta, *Talanta 56* **(2002)** 977.

88. N. Wu, Q. Tang, J.A. Lippert, M.L. Lee, *J. Microcol. Sep. 13* **(2001)** 41.

89. D. Guillarme, S. Heinisch, J.Y. Gauvrit, P. Lanteri, J.L. Rocca, *J. Chromatogr. A 1078* **(2005)** 22.

90. R.M. Smith, O. Chienthavorn, I.D. Wilson, B. Wright, *Anal. Commun. 35* **(1998)** 261.

91. R.M. Smith, O. Chienthavorn, I.D. Wilson, B. Wright, S. D. Taylor, *Anal. Chem. 71* **(1999)** 4493.

92. R.M. Smith, O. Chienthavorn, S. Saha, I.D. Wilson, B. Wright, S.D. Taylor, *J. Chromatogr. A 886* **(2000)** 289.

93. O. Chienthavorn, R.M. Smith, S. Saha, I.D. Wilson, B. Wright, S.D. Taylor, E.M. Lenz, *J. Pharm. Biomed. Anal. 36* **(2004)** 477.

94. D. Louden, A. Handley, R. Lafont, S. Taylor, I. Sinclair, E. Lenz, T. Orton, I.D. Wilson, *Anal. Chem. 74* **(2002)** 288.

95. D. Louden, A. Handley, S. Taylor, I. Sinclair, E. Lenz, I.D. Wilson, *Analyst 126* **(2001)** 1625.

96. Y. Yang, F. Hildebrand, *Anal. Chim. Acta 555* **(2006)** 364.

97. E. Lindquist, Y. Yang, *J. Chromatogr. A 1218* **(2011)** 2146.

98. C.J. Smith, S. Shillingford, A.M. Edge, C. Bailey, I.D. Wilson, *Chromatographia 67* **(2008)** 67.

99. T. Teutenberg, J. Tuerk, M. Holzhauser, S. Giegold, *J. Sep. Sci. 30* **(2007)** 1101.

100. P. Dugo, K. Buonasera, M.L. Crupi, F. Cacciola, G. Dugo, L. Mondello, *J. Sep. Sci. 30* **(2007)** 1125.

101. T.J. Causon, R.A. Shellie, E.F. Hilder, *Analyst 134* **(2009)** 440.

102. Y. Wang, N. Grinberg, J. McCaffrey, D.L. Norwood, *J. Liq. Chromatogr. & Rel. Technol. 31* **(2008)** 2305.

103. M.O. Fogwill, K.B. Thurbide, *J. Chromatogr. A 1200* **(2008)** 49.

104. L.A. Khateeb, R.M. Smith, *J. Chromatogr. A 1201* **(2008)** 61.

105. N. Jones, A.A. Clifford, K.D. Bartle, P. Myers, *J. Sep. Sci. 33* **(2010)** 3107.

106. R.M. Smith, R.J. Burgess, O. Chienthavorn, J.R. Stuttard, *LC–GC North Am. 17* **(1999)** 938.

107. Y. Yang, Z. Strickland, B. Kapalavavi, R. Marple, C. Gamsky, *Talanta 84* **(2011)** 169.

108. R. Berta, M. Babják, M. Gazdag, *J. Pharm. Biomed. Anal. 54* **(2011)** 458.

109. L.A. Al-Khateeb, R.M. Smith, *Anal. Bioanal. Chem. 394* **(2009)** 1255.

110. L.A. Riddle, G. Guiochon, *J. Chromatogr. A 1137* **(2006)** 173.

111. G. Vanhoenacker, P. Sandra, *J. Chromatogr. A 1082* **(2005)** 193.

112. M.M. Sanagi, H.H. See, W.A.W. Ibrahim, A.A. Naim, *J. Chromatogr. A 1059* **(2004)** 95.

113. T. Teutenberg, H.J. Goetze, J. Tuerk, J. Ploeger, T.K. Kiffmeyer, K.G. Schmidt, W.gr. Kohorst, T. Rohe, H.D. Jansen, H.Weber, *J. Chromatogr. A 1114* **(2006)** 89.

114. A.M. Edge, S. Shillingford, C. Smith, R. Payne, I.D. Wilson, *J. Chromatogr. A 1132* **(2006)** 206.

115. G. Huang, R.M. Smith, H.M. Albishri, J.M. Lin, *Chromatographia 72* **(2010)** 1177.

116. A.M. Edge, I.D. Wilson, S. Shillingford, *Chromatographia 66* **(2007)** 831.

117. S. Shillingford, L. Bishop, C.J. Smith, R. Payne, I.D. Wilson, A.M. Edge, *Chromatographia 70* **(2009)** 37.

118. O. Chienthavorn, R.M. Smith, I.D. Wilson, B. Wright, E.M. Lenz, *Phytochem. Anal. 16* **(2005)** 271.

119. T.E. Young, S.T. Ecker, R.E. Synovec, N.T. Hawley, J.P. Lomber, C.M. Wai, *Talanta 45* **(1998)** 1189.

120. B. Li, Y. Yang, Y. Gan, C.D. Eaton, P. He, A.D. Jones, *J. Chromatogr. A 873* **(2000)** 175.

121. R. Tajuddin, R.M. Smith, *J. Chromatogr. A 1084* **(2005)** 194.

122. J. W. Dolan, L. R. Snyder, T. Blanc, L. Van Heukelem, *J. Chromatogr. A 897* **(2000)** 37.

123. T. Greibrokk, T. Andersen, *J. Sep. Sci. 24* **(2001)** 899.

APPENDIX A: NAME

Signed copyright form goes here