Abstract

FUNDAMENTAL AND APPLIED STUDIES ON CHROMATOGRAPHIC SEPARATION OF COLD DRUGS AND SKINCARE CREAMS AND EXTRACTION OF *SALVIA MILTIORRHIZA* USING SUBCRITICAL WATER

by

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Subcritical water chromatography (SBWC) and subcritical water extraction (SBWE) are two green techniques that use subcritical water as the sole solvent for separations, thus eliminating the use of toxic and expensive organic solvents. This dissertation research was mainly focused on the development of SBWC through both fundamental and applied studies. Fundamental studies include the solubility of parabens and stability of preservatives and stationary phases under subcritical water conditions. Solubility of parabens increased by 11 to 36 folds with temperature raise from 25 to 150 ^{o}C, but decreased at 200 ^{o}C due to degradation. A new approximation model developed in this work successfully estimated the solubility of parabens in subcritical water. The studies on the stability of preservatives in subcritical water revealed that the preservatives were stable up to 150 ^{o}C and there was approximately 10% degradation of preservatives at 200 ^{o}C. The stationary phase evaluation indicated that the Waters XBridge C18 and phenyl columns were stable for up to 30,000 column volume at 150 ^{o}C and the ZirChrom-DB-C18 column up to 14,250 column volume at 200 ^{o}C.

Applied studies of SBWC were focused on separation and analysis of pharmaceuticals from cold drugs and niacinamide, preservatives, and sunscreens present in skincare products. Our best SBWC quantification results achieved in this work are in the range of 97.4 to 103.4% recoveries and RSDs less than 1.9%. A large number of replicate chromatographic runs and the comparison with high performance liquid chromatography results indicate that our SBWC methods for niacinamide and preservatives are quite accurate and precise.

The Subcritical water extraction and traditional herbal decoction (THD) of *Salvia miltiorrhiza* were carried out and the herbal extracts were tested for cytotoxicity on *Caenorhabditis elegans*. In general, the concentration of anticancer agents obtained by SBWE increased by 4 to 18 folds when the temperature was raised from 75 to 150 ^{o}C. The concentration of tanshinones, important anticancer agents, obtained by SBWE at all four temperatures was higher than that of the THD. Similarly, the cytotoxicity tests revealed that the SBWE herbal extracts were more potent than the THD extracts.

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Dedicated to my Mother

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TABLE OF CONTENTS

LIST OF FIGURES……. XVIII

LIST OF TABLES…….……..... XXXIII

LIST OF ABBREVIATIONS…….. XXXIX

Chapter 1: Introduction...…….……..……. 1

1.1 Subcritical Water Chromatography…….. 1

1.2 Subcritical Water Extraction……...…….…… 2

1.3 Dissertation Research…….…….. 5

Chapter 2: Background.……...…..……..……. 9

2.1 Properties of Subcritical Water……...…….. 9

2.1.1 Polarity of Water……..……..... 10

2.1.2 Surface Tension of Water……..…….. 11

2.1.3 Viscosity of Water……..…..…… 11

2.1.4 Solute Mass Transfer…….....…….. 11

2.2 Solubility of Analytes in Subcritical Water……..…… 12

2.2.1 Solubility Determination…….…… 12

2.2.2 Solubility Modeling……..…….. 15

2.2.2.1 Empirical Model……..…… 15

2.2.2.2 Pure Component Property Model……..……... 16

2.2.2.3 Modified-UNIQUAC Functional-Group Activity Coefficient Model…… 17

2.2.2.4 Dielectric Constant Model……..……...…… 17

2.3 Stability of Analytes in Subcritical Water…….…….. 18

2.4 Stability of Stationary Phases under Subcritical Water Chromatographic Conditions…… 20

2.5 Subcritical Water Chromatography.....…. 23

2.5.1 Instrumentation…… 23

2.5.2 Separation of Analytes by Subcritical Water Chromatography…….. 26

2.5.3 Industrial Applications……..…….. 29

2.5.4 Advantages……. 30

2.5.4.1 Green Separation…… 30

2.5.4.2 Fast Separation and Analysis…….. 30

2.5.4.3 Column Efficiency…….. 32

2.5.4.4 Detectors Compatibility……... 34

2.6 Subcritical Water Extraction of Medicinal Herbs…….. 36

2.6.1 Medicinal Herbs in Cancer Treatment…….. 36

2.6.2 Techniques for Characterization of Medicinal Herbs…… 38

2.6.3 Herbal Medication Preparation Techniques…….. 39

2.6.4 Instrumentation……..…….……. 41

2.6.5 Applications of Subcritical Water Extraction on Medicinal Herbs…. 42

Chapter 3: Solubility of Parabens in Subcritical Water…….. 44

3.1 Introduction…….……. 44

3.2 Experimental…….…… 44

3.2.1 Reagents and Supplies……. 44

3.2.2 Preparation of Solutions…….. 46

3.2.2.1 Preparation of Internal Standard Solutions……. 46

3.2.2.2 Preparation of Calibrated Standard Solutions……. 46

3.2.3 Heating and Mixing of Paraben-Water Mixtures……. 46

3.2.4 HPLC Analysis…… 48

3.3 Results and Discussion……. 49

3.3.1 Temperature Effect on the Solubility of Parabens…….. 49

3.3.2 Estimating the Solubility of Parabens in Subcritical Water……..... 53

Chapter 4: Stability of Preservatives under Subcritical Water Conditions……. 56

4.1 Introduction…….. 56

4.2 Experimental……. 57

4.2.1 Reagents and Materials……..…….. 57

4.2.2 Preparation of Solutions…… 58

4.2.2.1 Preparation of Internal Standard Solutions…….. 58

4.2.2.2 Preparation of Calibrated Standard Solutions…….. 58

4.2.3 Heating of Water-Preservatives or Water-Single Paraben Mixtures…. 59

4.2.4 HPLC Analysis…….. 60

4.2.5 GC/MS Analysis…… 62

4.3 Results and Discussion…….…….... 62

4.3.1 Stability Studies of Preservatives under SBWC Conditions by Chromatographic Evaluation……. 62

4.3.2 Degradation Studies of Preservatives in Heated Water-Preservatives Mixtures……. 63

4.3.3 Evaluation of Paraben Degradation Products at 200 ^{o}C……. 64

4.3.4 Paraben Degradation in Subcritical Water…..... 66

Chapter 5: Long-Term Stability of Stationary Phases under Subcritical Water Chromatographic Conditions……. 70

5.1 Introduction…… 70

5.2 Experimental…… 71

5.2.1 Reagents and Supplies…… 71

5.2.2 Preparation of Solutions…….. 71

5.2.2.1 Preparation of Internal Standard Solutions…..……. 71

5.2.2.2 Preparation of Calibrated Standard Solutions…… 71

5.2.3 HPLC Analysis…… 72

5.3 Results and Discussion…….…… 72

5.3.1 Long-Term Stability of Waters XBridge Phenyl Column…….. 72

5.3.2 Long-Term Stability of Waters XBridge C18 Column…….. 77

5.3.3 Long-Term Stability of ZirChrom-DB-C18 column…….. 82

Chapter 6: Separation and Analysis of Cold Drugs and Skincare Creams…… 83

6.1 Introduction……. 83

6.2 Experimental……..…….. 85

6.2.1 Reagents and Materials…… 85

6.2.2 Preparation of Solutions…… 90

6.2.2.1 Preparation of Internal Standard Solutions……..… 90

6.2.2.2 Preparation of Calibrated Standard Solutions……. 91

6.2.2.3 Preparation of Sample Solutions……. 92

6.2.3 Instrumentation…… 93

6.3 Results and Discussion……. 94

6.3.1 Analysis of Pharmaceuticals Present in Cold Drugs…… 94

6.3.1.1 SBWC Separation and Analysis of Pharmaceuticals in P&G Vicks Formula…… 94

6.3.1.2 SBWC Separation and Analysis of Pharmaceuticals in Bayer Alka-Seltzer Plus……..... 96

6.3.1.3 SBWC Separation and Analysis of Pharmaceuticals in CVS Cold Relief……. 98

6.3.2 Analysis of Niacinamide Present in Skincare Creams…….. 100

6.3.3 Analysis of Preservatives Present in Skincare Creams……. 102

6.3.3.1 Separation and Analysis of Preservatives on ZirChrom-DB-C18 Column…… 102

6.3.3.1.1 Separation of Preservatives by High Temperature Liquid Chromatography…….…… 102

6.3.3.1.2 Separation of Preservatives by Subcritical Water Chromatography……..……. 105

6.3.3.2 SBWC Separation and Analysis of Preservatives on ZirChrom-PS Column……. 107

6.3.3.3 Separation and Analysis of Preservatives on Waters XBridge C18 Column…… 109

6.3.3.3.1 Separation of Preservatives by an Integrated SBWC/HTLC Method…….…….. 109

6.3.3.3.2 Separation of Preservatives by Subcritical Water Chromatography……..... 112

6.3.3.3.3 SBWC Separation of Preservatives Using Programmed Temperatures…… 114

6.3.3.3.4 Potential System Building-up Studies……. 115

6.3.3.4 SBWC Separation and Analysis of Preservatives on Waters XBridge Phenyl Column……. 116

6.3.3.4.1 SBWC Separation of Preservatives at Constant Flow Rate……. 116

6.3.3.4.2 Optimization of SBWC Methods with Programmed Flow Rates…….. 118

6.3.3.4.3 SBWC Separation of Preservatives Using Programmed Temperatures…….. 120

6.3.3.4.4 Potential System Building-up Studies…… 122

6.3.4 Analysis of Sunscreens Present in Skincare Creams…… 122

6.3.4.1 HTLC Separation and Analysis of Sunscreens on ZirChrom-DB-C18 Column…….…… 122

6.3.4.2 Separation and Analysis of Sunscreens on Waters XTerra MS C18 Column…….……. 124

6.3.4.2.1 Separation of Sunscreens by High Temperature Liquid Chromatography……. 124

6.3.4.2.2 Separation of Sunscreens by Subcritical Water Chromatography…….…… 128

6.3.4.3 Separation and Analysis of Sunscreens on the Waters XBridge C18 Column……. 129

6.3.4.3.1 Separation of Sunscreens by High Temperature Liquid Chromatography…… 129

6.3.4.3.2 Separation of Sunscreens by Integrated SBWC/HTLC… 132

6.3.4.3.3 Separation of Sunscreens by Subcritical Water Chromatography……. 134

Chapter 7: Developing Efficacious Herbal Medicines Through Subcritical Water Extraction…….. 136

7.1 Introduction……..…….. 136

7.2 Experimental…… 138

7.2.1 Reagents and Supplies…… 138

7.2.2 Preparation of Solutions……..…… 140

7.2.2.1 Preparation of Internal Standard Solutions……... 140

7.2.2.2 Preparation of Calibrated Standard Solutions…… 140

7.2.3 Subcritical Water Extraction of *Salvia miltiorrhiza*..... 140

7.2.4 Traditional Herbal Decoction of *Salvia miltiorrhiza*…….. 141

7.2.5 Sonication Extraction of *Salvia miltiorrhiza*…….. 142

7.2.6 Sample Treatment…… 142

7.2.6.1 Sample Treatment for GC/MS Analysis of Herbal Extracts….. 142

7.2.6.2 Sample Treatment for HPLC Analysis of Herbal Extracts……. 142

7.2.7 HPLC Analysis……. 143

7.2.8 GC/MS Analysis…….. 143

7.2.9 Sampling for the Cytotoxicity Studies on *Caenorhabditis elegans*…. 144

7.3 Results and Discussion……. 148

7.3.1 Subcritical Water Extraction of *Salvia miltiorrhiza*……. 148

7.3.1.1 Identification of Anticancer Analytes by GC/MS…….. 148

7.3.1.2 Quantification of Anticancer Agents by HPLC…….. 152

7.3.2 Reproduction Assay of *C. elegans*……... 156

7.3.2.1 Optimization of Reproduction Assay Using Diluted Herbal Extracts…….. 156

7.3.2.2 Evaluating the Cytotoxity of SBWE Herbal Extracts on *C.elegans* Using Reproduction Assay…….. 157

Chapter 8: Conclusions…….. 159

Chapter 9: Bibliography……. 164

APPENDIX: List of Published Papers……..……. 191

LIST OF FIGURES

Figure 2.1: Phase diagram of water. (Reproduced with permission from reference 1 © John Wiley & Sons, Ltd., 2011.)…….. 9

Figure 2.2: Control of solvent dielectric constant (a), surface tension (b), and viscosity (c) by changing temperature with pure liquid water at 100 bar compared to mixing water with methanol or acetonitrile at 25 °C. (Reproduced with permission from reference 3 © Elsevier, 1998.)…….. 10

Figure 2.3: Schematic drawing of the HPLC system and the specially designed heating oven for temperature-programmed applications. (1) Solvent reservoir; (2) pumps; (3) autosampler; (4) high-pressure mixing chamber; (5) preheating unit; (6) column heating unit; (7) cooling unit prior to detection; (8) UV detector; (9) back-pressure regulator. (Reproduced with permission from reference 170 © Elsevier, 2006.)… 24

Figure 2.4: Block diagram of a subcritical water chromatography system with the preheating coil placed before the injector. (Reproduced with permission from reference 1 © John Wiley & Sons, Ltd., 2011.)..... 25

Figure 2.5: Schematic diagram of an HTLC system: (1) solvent reservoir; (2) pumps; (3) injector; (4) T union; (5) stainless steel tubing; (6) heating bath; (7) heat exchanger; (8) low dead volume T union; (9) in-line filter; (10) analytical column; (11) cooling bath; (12) cooling tubing; (13) UV detector; (14) data system; (15) backpressure adjuster. (Reproduced with permission from reference 132 © American Chemical Society, 2000.)……..……. 26

Figure 2.6: Temperature effect on flow rate for the separation of caffeine derivatives (1, hypoxanthine; 2, theobromine; 3, theophilline; 4, caffeine; 5, β-hydroxy-ethyl-theophilline), column Zirchrom-DB-C18 4.6 mm i.d. 50 mm length, 254nm UV detection, 300 ng injected, (a) 25 °C, 1 mL/min, water–methanol 60:40 v/v, backpressure resulting from tubing and column 40 bars (b) 150 °C, 7 mL/min, water, 2 m × 0.127 mm preheating tube, backpressure resulting from tubing and column 300 bars. (Reproduced with permission from reference 22 © Elsevier, 2004.)……. 31

Figure 2.7: 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 ^{o}C, octanophenone, k = 3.87, ▽ = 80 ^{o}C decanophenone, k = 3.15, □ = 120 ^{o}C, decanophenone, k = 5.70, ○ = 150 ^{o}C, decanophenone, k = 1.65. (Reproduced with permission from reference 190 © Wiley-VCH, 2007.)…… 33

Figure 2.8: Comparison of the separation of purines and pyrimidines under conventional conditions and with high-temperature water. Column: Hypercarb 5, 100 × 4.6 mm ID; detection: UV at 254 nm. (a) Mobile phase: water + 0.1% formic acid/acetonitrile (85:15, v/v); flow rate: 0.8 mL/min; temperature: 50 °C. (b) Mobile phase: 100% water; flow rate: 2.0 mL/min; temperature: 190 °C. Analytes: 1, cytosine; 2, uracil; 3, thymine; 4, hypoxanthine; 5, guanine; 6, xanthine.

(Reproduced with permission from reference 17 © Wiley-VCH, 2007.)…….. 34

Figure 2.9: Block diagram of the subcritical water extraction system……..... 42

Figure 3.1: Structures of preservatives……..……..... 45

Figure 3.2: Schematic diagram of the heating/mixing system……..... 48

Figure 3.3: High performance liquid chromatography chromatograms obtained on the Alltech Adsorbosil C18 column. (a) 60-min heating solubility vessel at at 25 ^{o}C; (b) 100 ^{o}C; (c) 150 ^{o}C; (d) 200 ^{o}C. Peak identification: 1, 2-phenoxy ethanol; 2, methyl paraben; 3, degradation product……. 52

Figure 3.4: Temperature influence on paraben solubility in subcritical water: ●, methyl paraben; ■, ethyl paraben; ▲, butyl paraben. (Reproduced with Permission from Reference 234 © American Chemical Society, 2014.)…….. 55

Figure 4.1: Structure of benzyl alcohol…..……..... 58

Figure 4.2: Stainless steel vessel with an end cap……... 60

Figure 4.3: Block diagram of home-made subcritical water chromatography System……. 61

Figure 4.4: GC/MS chromatogram and mass spectra of a water-butyl paraben mixture obtained after heating at 200 ºC for 30 min. (a) Total ion chromatogram; (b) Mass spectrum of the phenol peak; (c) Mass spectrum of the *p*-hydroxybenzoic acid peak. Peak identification: 1, phenol; 2, *p*-hydroxybenzoic acid; 3, butyl paraben……... 66

Figure 4.5: Paraben degradation pathway…… 67

Figure 4.6: HPLC chromatograms obtained by the separation of heated water-butyl paraben mixtures over Alltech Adsorbosil C18 column at ambient temperature. (a) Water-butyl paraben mixture was prior heated at 200 ^{o}C for 60 min; (b) 90 min; (c) 120 min. Flowrate: 1.0 mL/min. UV detection: 256nm. Mobile Phase: A, deionized water; B, 100% methanol. Gradient: 0-7 min, 50-40% methanol; 7-9 min, 40-90% methanol; 9-13 min, 90%methanol; 13-14 min, 90-50% methanol; 14-16 min, 50%methanol. Peak identification: 1, *p*-hydroxybenzoic acid; 2, phenol; 3, butyl paraben…… 69

Figure 5.1: Long-term stability of XBridge phenyl column through the heating effect on theoretical plate number. Flow rate: 1 mL/min. (a) Temperature: 150 ºC for column volume of 0-9247; (b) Programmed temperature (100 ºC to 150 ºC at 15 ºC/min and then remained at 150 ºC for the remainder of each run) for column volume of 9247 to 30,000…… 73

Figure 5.2: Long-term stability of XBridge phenyl column through the heating effect on retention factor. Flow rate: 1 mL/min. (a) Temperature: 150 ºC for column volume of 0-9247; (b) Programmed temperature (100 ºC to 150 ºC at 15 ºC/min and then remained at 150 ºC for the remainder of each run) for column volume of 9247 to 30,000…….. 74

Figure 5.3: Subcritical water chromatography chromatograms of preservatives mixture obtained on the XBridge phenyl column using 1 mL/min at

different points of evaluation period. (a) At the beginning of the evaluation at 150 °C (after 1 hour exposure, 36 column volume); (b) After 62 hours exposure to 150 ºC (2240 column volume); (c) At the end of the evaluation at 150 ºC (256 hours or 9247 column volume); (d) After 273 hours exposure to the programmed temperature or a total of 529 hours exposure to both 150 ºC and the programmed temperature (a total of 19,108 column volume)…… 76

Figure 5.4: Long-term stability of the the XBridge C18 column through the heating effect on theoretical plate number of the XBridge C18 column. Flow rate: 1 mL/min. (a) Temperature: 150 ºC for column volume of 0-9428; (b) Programmed temperature (100 ºC to 150 ºC at 15 ºC/min and then remained at 150 ºC for the remainder of each run) for column volume of 9,428 to 23,912……. 78

Figure 5.5: Long-term stability of the the XBridge C18 column through the heating effect on retention factor of the XBridge C18 column. Flow rate: 1 mL/min. (a) Temperature: 150 ºC for column volume of 0-9428; (b) Programmed temperature (100 ºC to 150 ºC at 15 ºC/min and then remained at 150 ºC for the remainder of each run) for column volume of 9,428 to 23,912……. 79

Figure 5.6: Subcritical water chromatography chromatograms of preservatives obtained on the XBridge C18 column using 1 mL/min at different points of evaluation period. (a) At the beginning of the evaluation at 150 °C (after 1 hour exposure, 36 column volume); (b) After

141 hours exposure to 150 ºC (5,093 column volume); (c) At the end of the evaluation at 150 ºC (261 hours or 9428 column volume); (d) After 216 hours exposure to the programmed temperature or a total of 478 hours exposure to both 150 ºC and the programmed temperature (17,266 column volume)……..... 81

Figure 6.1. Structures of pharmaceuticals in cold drugs, niacin niacinamide, and sunscreens in skincare creams…… 90

Figure 6.2: Subcritical water chromatography chromatograms of pharmaceuticals obtained on the Alltech Adsorbosil C18 column with 1.0 mL/min using programmed temperatures. (a) Pharmaceutical standard mixture; (b) Vicks formula 44 custom care cough and cold sample. UV detection: 210 nm. Mobile phase: A, deionized water; B, 100 mM phosphoric acid. Gradient: 100% water for 1 min and then 100 mM phosphoric acid for rest of the run. Programmed temperatures: Initial temperature of 25 ^{o}C for 3 min and then increased at 15 ^{o}C/min to 150 ^{o}C and maintained at 150 ^{o}C for rest of the run. Peak identification: 1, dextromethorphan hydrobromide; 2, chlorpheniramine maleate; 3, phenylephrine hydrochloride; 4, acetaminophen; 5, matrix peak…… 96

Figure 6.3: Subcritical water chromatography chromatograms of pharmaceuticals obtained on the Alltech Adsorbosil C18 column at programmed temperatures with 1.0 mL/min. (a) Pharmaceutical standard mixture; (b) Alka-Seltzer plus cold and flu formula sample. UV detection:

210 nm. Mobile phase: A, deionized water; B, 100 mM phosphoric acid. Gradient: 100% water for 1 min and then 100 mM phosphoric acid for rest of the run. Programmed temperatures: Initial temperature of 25 ^{o}C for 3 min and then increased at 15 ^{o}C/min to 150 ^{o}C and maintained at 150 ^{o}C for rest of the run. Peak identification: 1, dextromethorphan hydrobromide; 2, chlorpheniramine maleate; 3, doxylamine succinate; 4, phenylephrine hydrochloride; 5, acetaminophen; 6, matrix peak…….. 97

Figure 6.4: Subcritical water chromatography chromatograms of pharmaceuticals obtained on the Alltech Adsorbosil C18 column at programmed temperatures with 1.0 mL/min using 100 mM phosphoric acid as mobile phase. (a) Pharmaceutical standard mixture; (b) CVS multi-symptom severe cold relief sample. Programmed temperatures: Initial temperature of 90 ^{o}C for 5.5 min and then increased at 15 ^{o}C/min to 150 ^{o}C and maintained at 150 ^{o}C for rest of the run. Peak identification: 1, dextromethorphan hydrobromide; 2, phenylephrine hydrochloride; 3, acetaminophen; 4, benzyl alcohol; 5, guaifenesin…….. 99

Figure 6.5: Subcritical water chromatography chromatograms of niacinamide obtained on the XBridge C18 column using pure water as the mobile phase at 60 ºC with 2 mL/min. (a) Standard mixture containing niacin and niacinamide; (b) Olay total effects, 7-in-1 anti-ageing UV moisturizer sample. UV detection:

245 nm. Peak identification: 1, niacin; 2, niacinamide; 3, 4-acetamidophenol; 4, matrix peak…… 101

Figure 6.6: High temperature liquid chromatography chromatograms obtained on ZirChrom-DB-C18 column at 150 ºC. (a) SC-EC1 skincare cream sample; (b) SC-EC3 skincare cream sample. Flow rate: 2.0 mL/min. UV detection: 256 nm. Mobile phase: A, deionized water; B, 100% methanol. Gradient: 0-2 min, 10-20% methanol; 2-6 min, 20-50% methanol; 6-10 min, 50% methanol; 10-10.5 min, 50-10% methanol. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)…….. 103

Figure 6.7: Subcritical water chromatography chromatograms obtained on ZirChrom-DB-C18 column at 200 ºC using 100% water as the mobile phase. (a) SC-EC1 skincare cream sample; (b) SC-EC2 skincare cream sample; (c) SC-EC3 skincare cream sample. UV detection: 256 nm. Programmed flow rates: 0-6.5 min, decreased from 1.0 mL/min to 0.75 mL/min; 6.5-27 min, 0.75 mL/min; 27-27.5 min, increased from 0.75mL/min to 1.0 mL/min. Peak identification: 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)…… 107

Figure 6.8: SBWC chromatograms obtained on ZirChrom-PS column at 180 ºC and 1.25 mL/min using 100% water as the mobile phase. (a) Preservatives

standard mixture; (b) SC-EC3 skincare cream sample. UV detection: 256 nm. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)…… 109

Figure 6.9: Integrated SBWC/HTLC chromatograms obtained on XBridge C18 column at 150 ºC and 1.0 mL/min (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. UV detection: 210 nm. Gradient: 0-7.9 min, 0% methanol; 7.9-8 min, 0-50% methanol; 8-11 min, 50% methanol; 11-11.1 min, 50-0% methanol; 11.1-15 min, 0% methanol. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)…… 111

Figure 6.10: SBWC chromatograms obtained using 100% water on XBridge C18 column at 150 ºC and 1.0 mL/min with the best chromatogram mode. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, while the other parabens were detected at 256 nm. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)……. 113

Figure 6.11: SBWC chromatograms obtained using 100% water on XBridge C18 column with programmed temperatures at 1.0 mL/min. (a)

Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, while the other parabens were detected at 256 nm. Programmed temperatures: initial temperature of 100 ^{o}C was increased to 150 ^{o}C at 15 ^{o}C/min and then maintained at 150 ^{o}C. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)…….. 115

Figure 6.12: SBWC chromatograms obtained using 100% water on XBridge phenyl column at 150 ºC and 1.0 mL/min. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, while the other parabens were detected at 256 nm. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)…… 118

Figure 6.13: SBWC chromatograms obtained using 100% water on XBridge phenyl column at 150 ºC with programmed flow rates. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, while the other parabens were detected at 256 nm.

Programmed flow rates: 0-6.5 min, decreased from 1.0 mL/min to 0.75 mL/min; 6.5-47 min, 0.75 mL/min; 47-50 min, increased from 0.75mL/min to 1.0 mL/min. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)……..... 119

Figure 6.14: SBWC chromatograms obtained using 100% water on XBridge phenyl column using programmed temperatures at 1.0 mL/min. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, while the other parabens were detected at 256 nm. Programmed temperatures: initial temperature of 100 ^{o}C, then increased to 150 ^{o}C at 15 ^{o}C/min and then maintained at 150 ^{o}C. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)…… 121

Figure 6.15: HTLC chromatograms obtained on the ZirChrom-DB-C18 column at 190 ^{o}C. (a) Sunscreen standard mixture; (b) SC-EC2 skincare cream sample. Gradient: 0-3 min, 10% methanol; 3-4 min, 10-40% methanol; 4-9 min, 40% methanol; 9-10 min, 40-70% methanol; 10-15 min, 70% methanol. Programmed flow rates: 0-3 min, 2.0 mL/min; 3-4 min, decreased from 2.0 mL/min to 1.5 mL/min; 4-9 min, 1.5 mL/min; 9-10

min, increased from 1.5 mL/min to 2.0 mL/min; 10-15 min, 2.0 mL/min. Peak identification: 1, ensulizole; 2, oxybenzone; 3, octocrylene; 4, octisalate; 5, avobenzone. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)…… 124

Figure 6.16: HTLC sunscreen chromatograms obtained on the XTerra MS C18 column at 200 ºC and 1.0 mL/min. (a) Sunscreen standard mixture; (b) SC-EC2 skincare cream sample. Isocratic elution using a mobile phase containing 2% of methanol in water. Peak identification: 1, ensulizole; 2, oxybenzone; 3, avobenzone; 4, octisalate; 5, octocrylene. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)…… 126

Figure 6.17: HTLC sunscreen chromatograms obtained on the XTerra MS C18 column at 200 ºC and 1.0 mL/min. (a) Sunscreen standard mixture; (b) SC-EC3 skincare cream sample. Isocratic elution using a mobile phase containing 2% of methanol in water. Peak identification: 1, oxybenzone; 2, avobenzone; 3, homosalate-cis; 4, octisalate; 5, homosalate-trans; 6, octocrylene. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)…… 127

Figure 6.18: SBWC chromatogram of SC-EC2 skincare cream sample obtained on the XTerra MS C18 column at 250 ^{o}C and 1.0 mL/min using 100% water as the mobile phase. Peak identification: 1, ensulizole; 2, oxybenzone; 3, avobenzone; 4, octisalate; 5, octocrylene. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)…….. 128

Figure 6.19: HTLC sunscreen chromatograms obtained on the XBridge C18 column

at 200 ºC and 0.75 mL/min. (a) Sunscreen standard mixture; (b) SC-EC2 skincare cream sample. Isocratic elution using a mobile phase containing 2% of methanol in water. Peak identification: 1, ensulizole; 2, oxybenzone; 3, avobenzone; 4, octisalate; 5, octocrylene. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)……. 130

Figure 6.20: HTLC sunscreen chromatograms obtained on the XBridge C18 column at 200 ºC and 0.75 mL/min. (a) Sunscreen standard mixture; (b) SC-EC4 skincare cream sample. Isocratic elution using a mobile phase containing 2% of methanol in water. Peak identification: 1, oxybenzone; 2, octisalate; 3, octinoxate; 4, octocrylene. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)…… 131

Figure 6.21: SBWC/HTLC chromatograms obtained on the XBridge C18 column at 200 ºC and 0.75 mL/min. (a) Sunscreen standard mixture; (b) SC-EC2 skincare cream sample. Gradient: 0-10 min, 0% methanol; 10-20 min, 0-4% methanol; 20-24 min, 4% methanol. Peak identification: 1, ensulizole; 2, oxybenzone; 3, avobenzone; 4, octisalate; 5, octocrylene. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)…… 133

Figure 6.22: SBWC chromatograms of sunscreens obtained on the XBridge C18 column at 230 ºC and 1.0 mL/min using 100% water as the mobile phase. (a) SC-EC3 skincare cream sample; (b) SC-EC4 skincare cream sample. Peak identification: 1, oxybenzone; 2, avobenzone; 3, homosalate-cis; 4, octisalate; 5, homosalate-trans; 6, octocrylene;

7, octinoxate. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)……... 135

Figure 7.1: Structure of anticancer agents found in *Salvia miltiorrhiza*…….... 139

Figure 7.2: Schematic diagram of the SBWE extraction system……... 141

Figure 7.3: The life cycle of *C. elegans*. Under favorable conditions animals pass through direct development to adulthood in as little as 3 to 4 days. In response to harsh environmental conditions, such as food shortage, crowding or high temperatures, animals can enter into an arrested dauer stage. (Reproduced with permission from reference 273 © BioMed Central Ltd, 2010.)…… 146

Figure 7.4: Reproduction assay of *C. elegans*. (‘∿‘ L1 larva, ‘⁓‘ adult worm, and ‘ᴑ‘ egg).....…… 147

Figure 7.5: GC/MS chromatogram and mass spectra of a *Salvia miltiorrhiza* herbal extract obtained by SBWE at 150 ºC for 30 min. (a) Total ion chromatogram; (b) Mass spectrum of the protocatechualdehyde peak; (c) Mass spectrum of the caffeic acid peak; (d) Mass spectrum of the ferulic acid peak; (e) Mass spectrum of the tanshinone IIA peak; (f) Mass spectrum of the tanshinone I peak. Peak identification: 1, protocatechualdehyde; 2, propyl paraben (internal standard); 3, caffeic acid; 4, ferulic acid; 5, tanshinone IIA; 6, tanshinone I…….. 151

Figure 7.6: HPLC chromatograms of *Salvia miltiorrhiza* herbal extract obtained at 125 ^{o}C evaluated on the Alltech Adsorbosil C18 column at ambient temperature. (a) Analyte standard solution; (b) Methylene chloride

phase; (c) Water phase. Flow rate: 1.0 mL/min. UV detection: 254 nm. Mobile phase: A, 100 mM phosphoric acid in water; B, 100% methanol. Gradient: 0-4 min, 2% methanol; 4-8 min, 2-10% methanol; 8-23 min, 10-30% methanol; 23-32 min, 30-60% methanol; 32-43 min, 60% methanol; 43-49 min, 60-70% methanol; 49-61 min, 70-80% methanol; 61-68 min, 80-2% methanol. Peak identification: 1, protocatechualdehyde; 2, caffeic acid; 3, ferulic acid; 4, propyl paraben; 5, tanshinone I; 6, tanshinone IIA…… 154

LIST OF TABLES

Table 2.1: Comparison of Dielectric Constant of Selected Common Organic Solvents with that of Subcritical or Supercritical Water at Various Temperature and Pressure Conditions (Reproduced With Permission From Reference 1 © John Wiley & Sons, Ltd., 2011.)…….. 11

Table 2.2: Solubility Enhancement of Various Analytes in Subcritical Water…..… 13

Table 2.3: Both Commercial and Lab-Packed Columns Tested in Subcritical Water Chromatography…….…….. 21

Table 2.4: Long-Term Stability Evaluation of Commercial and Lab-Packed Columns under Subcritical Water Chromatography Conditions…..…... 22

Table 2.5: Subcritical Water Chromatographic Separation of Various Analytes (Reproduced With Permission From Reference 1 © John Wiley & Sons, Ltd., 2011.)…… …. 27

Table 2.6: Subcritical Water Chromatographic Separation of Various Analytes and their Detection Through Flame Ionization Detector and Nuclear Magnetic Resonance Spectroscopy (Reproduced With Permission From Reference 1 © John Wiley & Sons, Ltd., 2011.)…….. 35

Table 2.7: Some of the Famous Chinese Medicinal Herbs Used in Fighting Cancer…… 38

Table 2.8: Summary of Experimental Conditions for Various Extraction Techniques……. 39

Table 2.9: Subcritical Water Extraction of Active Pharmaceutical Ingredients and

Essential Oils from Medicinal Herbs…….. 43

Table 3.1: HPLC Gradient Elution Conditions for Separation of Parabens…… 49

Table 3.2: Comparison of Paraben Solubility[a] at 25 ^{o}C Obtained by This Method and Reference Values (Reproduced with Permission from Reference 234 © American Chemical Society, 2014.)…….…….. 50

Table 3.3: Solubility of Parabens Found in Water-Paraben Mixtures after Heating at Each Temperature for 60 min…….…...…….… 50

Table 3.4: Comparison of Experimental Solubility of Parabens with Values Predicted by Equations 1-5 (Reproduced with Permission from Reference 234 © American Chemical Society, 2014.)…….. 55

Table 4.1: Comparison of Solute Peak Areas Obtained by SBWC and HPLC at Ambient Temperature on Three Columns Tested…… 63

Table 4.2: Percent Recovery of Preservatives Found in Water-Preservatives Mixtures after Heating at High Temperatures…….. 64

Table 4.3: Parabens Degraded and Their Degradants Produced after Heating Paraben-Water Mixtures at 200 ^{o}C for 30 min…….. 66

Table 6.1: Concentration of Pharmaceuticals Found in Vicks Formula 44 Custom Care Cough and Cold Sample Obtained by SBWC Using Alltech Adsorbosil C18 Column…… 95

Table 6.2: Concentration of Pharmaceuticals Found in Bayer Alka-Seltzer Plus Cold and Flu Formula Sample Obtained by SBWC Using Alltech Adsorbosil C18 Column…… 96

Table 6.3: Concentration of Pharmaceuticals Found in CVS Multi-Symptom

Severe Cold Relief Sample Obtained by SBWC Using Alltech Adsorbosil C18 Column……..…… 98

Table 6.4: Recovery of Niacinamide Present in SC-EC2 Skincare Cream Sample Achieved by Subcritical Water Chromatography Compared with the Concentration Obtained by HPLC at 25 °C Using 30% Methanol in the Mobile Phase.…… 100

Table 6.5: Comparison of Recoveries for Preservatives Present in SC-EC1 Skincare Cream Obtained by HPLC, HTLC, and SBWC Using ZirChrom-DB-C18 Column (Reproduced with Permission from Reference 20 © The Authors ICS, 2012.)…….……. 104

Table 6.6: Comparison of Recoveries for Preservatives Present in SC-EC2 Skincare Cream Obtained by HPLC, HTLC, and SBWC Using ZirChrom-DB-C18 Column (Reproduced with Permission from Reference 20 © The Authors ICS, 2012.)…….. 104

Table 6.7: Comparison of Recoveries for Preservatives Present in SC-EC3 Skincare Cream Obtained by HPLC, HTLC, and SBWC Using ZirChrom-DB-C18 Column (Reproduced with Permission from Reference 20 © The Authors ICS, 2012.)…….. 104

Table 6.8: Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by 24 Replicate HTLC Runs Using ZirChrom-DB-C18 Column at 150 ºC with 2.0 mL/min (Reproduced with Permission from Reference 20 © The Authors ICS, 2012.)…… 105

Table 6.9: Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by SBWC Using ZirChrom-PS Column at 180 ºC and 1.25 mL/min (Reproduced with Permission from Reference 20 © The Authors ICS, 2012.)…… 108

Table 6.10: Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by the Integrated SBWC/HTLC at 150 °C Using Waters XBridge C18 Column with Gradient Elution as Described in Figure 6.8 legend (Reproduced with Permission from Reference 20 © The Authors ICS, 2012.)……. 110

Table 6.11: Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by SBWC at 150 °C Using Waters XBridge C18 Column with 1.0 mL/min and the Best Chromatogram Mode (Reproduced with Permission from Reference 20 © The Authors ICS, 2012.)…… 112

Table 6.12: Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by SBWC at 150 ºC with the Best Chromatogram Mode Resulted from the Building-up Studies (Reproduced with Permission from Reference 20 © The Authors ICS, 2012.) ……..……. 116

Table 6.13: Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by SBWC at 150 °C Using Waters XBridge Phenyl Column and the Best Chromatogram Mode (Reproduced with Permission from Reference 20 © The Authors ICS, 2012.)…….. 117

Table 6.14: Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by Traditional HPLC with Ambient Methanol-Water Mixtures as the Eluent on Waters XBridge Phenyl Column (Reproduced with Permission from Reference 20 © The Authors ICS, 2012.)……. 120

Table 6.15: Concentration of Sunscreens Found in SC-EC2 Skincare Cream Sample Obtained by HTLC Using the ZirChrom-DB-C18 Column at 190 °C with Programmed Flow Rates as Described in Figure 6.14 Legend (Reproduced with Permission from Reference 19 © The Authors ICS, 2011.)……..……..….. 123

Table 6.16: Concentration of Sunscreens Found in SC-EC2 Skincare Cream Sample Obtained by HTLC with 2% Methanol in the Mobile Phase on the Waters XBridge C18 Column at 200 °C with 0.75 mL/min……..... 129

Table 6.17: Concentration of Sunscreens Found in SC-EC4 Skincare Cream Sample Obtained by HTLC with 2% Methanol in the Mobile Phase on the Waters XBridge C18 Column at 200 °C with 0.75 mL/min…….……. 132

Table 6.18: Concentration of Sunscreens Found in SC-EC2 Skincare Cream Sample Obtained by the Combined SBWC/HTLC Method Using the Waters XBridge C18 Column at 200 °C with 0.75 mL/min (Reproduced with Permission from Reference 19 © The Authors ICS, 2011.)……. 134

Table 7.1: Comparison of Anticancer Agent Concentrations Found in *Salvia miltiorrhiza* Obtained by Sonication, Traditional Herbal Decoction, and Subcritical Water Extraction…… 155

Table 7.2: Percent Reproduction Inhibition and Mortality of *C. elegans* After 30 Hour Exposure to the Diluted *Salvia miltiorrhiza* Herbal Extract Obtained by SBWE at 150 ^{o}C…….... 157

Table 7.3: Percent Reproduction Inhibition and Mortality of *C. elegans* After 30 Hour Exposure to the 10-time Diluted Traditional Herbal Decoction and Subcritical Water Extractions of *Salvia miltiorrhiza* at 75 to 150 ^{o}C……. 158

LIST OF ABBREVIATIONS

atm	Atmosphere
CO_2	Carbon dioxide
cm	Centimeter
cm^3/mol	Centimeter cube/mole
cm/s	Centimeter/second
cm^2/s	Centimeter square/second
cP	Centipoise
^oC	Degree Celsius
ε	Dielectric constant
Da	Damkohler number
dyn	Dyne
EI	Electron impact
eq	Equation
e.g.	Exempli gratia, for Example
FID	Flame ionization detector
FDA	Food and Drug Administration
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
g	Gram
g/mol	Gram/mole
HPLC	High-performance liquid chromatography

HTLC	High temperature liquid chromatography
H	Hours
H_2O_2	Hydrogen peroxide
OH	Hydroxyl functional group
IR	Infrared
i.d.	Internal diameter
J	Joule
K	Kelvin
L	Length
Ltd	Limited
u	Linear velocity
LC	Liquid chromatography
MS	Mass spectrometer
m/z	Mass-to-charge ratio
MPa	Megapascal
MΩ-cm	Megohm centimeter
M-UNIFAC	Modified-UNIQUAC functional-group activity coefficient
min	Minute
mL	Milliliter
mm	Millimeter
mM	Milli-molar
m	Meter
M	Molar concentration

mol	Mole
µL	Micro-liter
µm	Micro-meter
nm	Nanometer
NGM	Nematode growth media
n	Number of replicate experiments
NMR	Nuclear magnetic resonance
ppm	Parts-per-million
RSD	Relative standard deviation
H	Plate height
N	Plate number
PS-DVB	Polystyrene divinylbenzene
PGC	Porous Graphitic Carbon
P&G	Procter & Gamble
Ref	Reference
RPLC	Reversed-phase liquid chromatography
SD	Standard deviation
SBWC	Subcritical water chromatography
SBWE	Subcritical water extraction
SPF	Sun protection factor
SFE	Supercritical fluid extraction
T	Temperature in ^{o}C
TCM	Traditional Chinese medicine

THD	Traditional Herbal Decoction
vol%	Volume percentage
UFLC	Ultrafast liquid chromatography
UHPLC	Ultra high-pressure liquid chromatography
UV	Ultraviolet
UV-DAD	Ultraviolet-diode array detector
wt%	Weight percentage
%w/v	Weight/volume percentage solution

Chapter 1: Introduction

1.1 Subcritical Water Chromatography

One popular chromatographic technique used in analytical laboratories around the world is reversed-phase liquid chromatography (RPLC). The mobile phase used in RPLC analysis is a mixture of organic solvents and water (e.g., methanol-water or acetonitrile-water). These solvents are both toxic and flammable. Therefore, they are not safe for laboratory personnel and require waste disposal [1]. Recently developed analytical techniques to replace traditional RPLC are ultra high-pressure liquid chromatography (UHPLC), high temperature liquid chromatography (HTLC), and subcritical water chromatography (SBWC). Although the first two techniques, UHPLC and HTLC, help to minimize the usage of organic solvents, still small amounts of organic solvents are needed and their wastes require disposal. For subcritical water chromatography, no organic solvents are required since it uses only high temperature water as the mobile phase. So the development of green separation techniques like subcritical water chromatography is of great interest [1].

As defined in our previous review [1], subcritical water is the water heated and pressurized under any temperature and pressure conditions below its critical point of 374 ^{o}C and 218 atm [1]. Water is too polar at ambient conditions to serve as the sole mobile phase for liquid chromatographic separations. Fortunately, subcritical water may potentially replace organic solvents for RPLC since the dielectric constant, surface tension, and viscosity of water decrease with increasing temperature [1-7]. Therefore, subcritical water can mimic organic solvent programming (gradient elution) to achieve green reversed-phase chromatographic separations [1-2]. It is reported by our group that 2 to 3.5 ^{o}C rise in temperature is equivalent to 1%

methanol increase in methanol-water mixtures and 3 to 8 ^{o}C increase in temperature is equivalent to 1% acetonitrile rise in acetonitrile-water mixtures [8].

In 1981, Guillemin et al. first used hot water as the mobile phase for the separation of alcohols using liquid chromatography [9]. However, it was not until the late 90's that researchers started to explore subcritical water chromatography. This led to the green chromatographic separation of many classes of organic compounds, such as aldehydes [10], alkanols [10], alkylbenzenes [11], aminoacids [12], anilines [8], benzenes [13], carbohydrates [14], carboxylic acids [15], phenols[16], purines[17], and pyrimidines [17] etc. The main advantage of SBWC is the elimination of hazardous organic solvents used in traditional reversed phase liquid chromatography [1, 18-20]. As high temperatures are employed in SBWC separations, the water only mobile phase becomes less viscous with increase in temperature leading to a pressure drop across the column [1]. This decreased pressure helps in the usage of longer columns packed with smaller particle size materials for efficient separation [21, 22]. SBWC is compatible with both liquid phase and gas phase detectors due to water as the mobile phase. Some other benefits are the temperature dependent selectivity and resolution [1, 2, 4].

1.2 Subcritical Water Extraction

Similar to chromatography, organic solvents are used in most extraction techniques. Some of the existing extraction techniques include Soxhlet extraction, sonication, heating under reflux, pressurized liquid extraction, accelerated solvent extraction, microwave-assisted extraction, and supercritical fluid extraction [23-27]. Although carbon dioxide used in supercritical fluid extraction is nontoxic, it is nonpolar and not efficient for extraction of polar and moderately polar constituents from various sample matrices. Therefore, organic modifiers

have to be added to carbon dioxide to efficiently extract polar compounds [27]. In order to eliminate hazardous organic solvents from extractions, it is necessary to develop green extraction techniques such as subcritical water extraction (SBWE) where only subcritical water is used as the extraction fluid.

As disused earlier, with the increase of water temperature, there is an increase in diffusion rate of water with the decrease in dielectric constant, surface tension, and viscosity of water. These properties enhance the solvating power of water, which improves the solubility of organics in water at higher temperatures. The improved solubility makes water a better extraction fluid for polar, moderately polar, and some non polar analytes from different matrices [1, 28, 29, 30]. Miller at al. reported that the solubility of hydrophobic organic, chlorothalonil increased by about 130,000 folds and solubility of polycyclic aromatic, chrysene increased by about 120,000 folds with the increase of water temperature from 25 ^{o}C to the melting point of solutes [31, 29]. After the introduction of subcritical water extraction by the Hawthorne et al. in early 90's [32], this green extraction technique gained much attention and moved from bench scale analysis to pilot scale environmental cleanup and remediation [1, 33-40]. These subcritical water extractions have also been explored in determination of polycyclic aromatic hydrocarbons, pesticide residues in environmental solids [30, 32, 33], soil remediation [35], food analysis [41, 42], and herb analysis [43-46]. Later Yang et al. and Smith et al. developed both off-line and on-line SBWE-SBWC systems with a sorbent trap for the separation and analysis of several classes of analytes [47-50]. Yang et al. and Pross et al. compared SBWE with other extraction techniques such as Soxhlet and supercritical fluid extraction and achieved at least comparable or better results through SBWE [28, 33, 51].

For centuries, herbal medicine is a treatment of choice for various diseases in countries like China and India. The widespread knowledge on herbal medicine for its green nature and low side effects in disease remedy helped to promote the herbal medicine in the western world. Today cancer is a major life threatening disease around the world. There are millions of cancer patients in the US [52]. So researchers are also exploring the possibility of treating cancer using medicinal herbs. Some of the popular Chinese medicinal herbs and their bioactive compounds used in the cancer treatment are *Citrus aurantium*-limonene [53-58], *Artemisia Annua*-artemisinin [59-62], *Paeonia suffruticosa Andrews*-paeonol [63-66], and *Magnolia officinalis*-honokiol and magnolol [67-70].

There are various existing techniques for the preparation of herbal medicines such as maceration, vertical or turbo extraction, ultrasonic extraction, percolation, and counter current extraction. These preparation techniques also require organic solvents and in some cases benzene, a potential carcinogenic, was also used [71]. Just like the use of pesticides for crop fields left their traces in the fruits and vegetables, these techniques will leave organic solvent traces in the herbal extracts causing additional health problems for patients. There is a green extraction technique called traditional herbal decoction (THD), which is a traditional Chinese medicine (TCM) preparation method for over a thousand years. It is simply hot water extraction at 100 ^{o}C and open to the environment. As it is open to the environment there is a loss of volatile active constituents. The use of only one temperature setting in THD may not be sufficient for the extraction of moderately polar and non polar analytes. These less polar analytes normally requires high temperatures for efficient extraction [72]. Therefore the use of SBWE may help in the preparation of efficacious herbal medication, because SBWE is performed in a closed environment and the temperature can be optimized to achieve more

efficient extraction. Various anticancer constituents like anthraquinones, phenolics, and polyphenolics were already successfully extracted from medicinal herbs using SBWE [73-75, 43].

After years of subcritical water chromatography and subcritical water extraction research, these green separation technologies are mainly limited to the academic level. The main problem that keeps industry from adopting these green separation technologies is the concerns with the stability of analytes and stationary phases as well as analyte solubility in subcritical water. Therefore, fundamental studies such as organic solubility, analyte stability, and the life of chromatographic columns under SBWC conditions are needed. By conducting these fundamental studies will help to develop the green subcritical water separation technologies.

1.3 Dissertation Research

In this work, extensive research was conducted for the development of SBWC and SBWE green separation technologies through both fundamental and applied studies. Fundamental studies include solubility of analytes as well as stability of solutes and stationary phases under subcritical water conditions. Applied studies were focused on the SBWC separation and analysis of pharmaceuticals in cold drugs and niacinamide, preservatives, and sunscreens present in skincare creams. The last part of this dissertation research was focused on the development of an efficacious herbal medicine using subcritical water extraction.

Solubility of parabens in high-temperature water was performed using a solubility measuring system. This study is critically important in developing and evaluating feasible SBWC methods for parabens. Solubility experiments were performed on three parabens, methyl, ethyl, and butyl parabens in water at temperatures of 25 to 200 ^{o}C with a maximum

pressure of 35 atm to keep water in the liquid state. A model was developed to predict the solubility of parabens in subcritical water.

Stability studies on benzyl alcohol, methyl, ethyl, propyl, and butyl parabens were carried out using two different approaches. In the first approach, the stability of preservatives under SBWC conditions were investigated through the comparison of peak areas obtained by SBWC at higher temperatures with those obtained by conventional HPLC at ambient temperature. In the second approach, stability of preservatives were evaluated under much tougher conditions by heating the water-preservative mixtures in static steel vessels for 30 and 60 min at temperatures ranging from 25 to 200 ^{o}C.

The long-term stability of three stationary phases, Waters XBridge phenyl, Waters XBridge C18, and ZirChrom-DB-C18 columns under subcritical water chromatographic conditions were evaluated to determine the column life. These columns were selected due to their previous applications in the development of various SBWC methods for preservatives and sunscreens at much higher temperatures [19, 20]. Waters XBridge phenyl and Waters XBridge C18 columns were evaluated under SBWC conditions at 150 ^{o}C and ZirChrom-DB-C18 column at 200 ^{o}C.

Several green HTLC and SBWC methods were developed for pharmaceuticals in cold drugs and niacinamide, preservatives, and sunscreens present in skincare creams at temperatures ranging from 25 to 250 ^{o}C. These studies were designed to replace the existing HPLC methods currently used in industry with the green SBWC methods. The analytes investigated in this work are dextromethorphan hydrobromide, chlorpheniramine maleate, doxylamine succinate, phenylephrine hydrochloride, acetaminophen, and guaifenesin in cold drugs and niacin, niacinamide, benzyl alcohol, methyl paraben, ethyl paraben, propyl paraben, ensulizole,

octocrylene, octisalate, homosalate, octinoxate, and avobenzone present in skin care creams. These SBWC separations were performed using either a home-made SBWC system or a Shimadzu Nexera UFLC commercial system. The columns used for these studies are Alltech Adsorbosil C18 column, Hamilton PRP-1, Waters XBridge C18, Waters XBridge phenyl, Waters XTerra MS C18, ZirChrom-DiamondBond-C18, and ZirChrom-PS columns. Real life samples analyzed in this work include Vicks formula 44 custom care, cough and cold PM; Alka-seltzer plus, night, cold and flu formula; CVS multi-symptom severe cold relief, daytime, non-drowsy; Olay total effects, 7-in-1 anti-ageing daily moisturizer, fragrance free (coded as SC-EC1); Olay total effects, 7-in-1 anti-ageing UV moisturizer, plus SPF-15, fragrance free (coded as SC-EC2); Olay complete ageless skin-renewing UV lotion, SPF-20 (coded as SC-EC3); Olay Complete SPF 30 defense, daily UV moisturizer with vitamins E, B3 and pro-vitamin B5 (coded as SC-EC4).

Salvia miltiorrhiza, a medicinal herb, used for potential cancer treatment [76-78] was extracted using subcritical water extraction in order to develop an efficacious herbal medication preparation technique. Sonication and traditional hot water extraction of the herb were also conducted to evaluate the efficiency of SBWE extraction. Subcritical water extraction of *Salvia miltiorrhiza* was carried out by heating the water-herbal mixture in a vessel for 30 min at temperatures ranging from 75 to 150 ^{o}C. Five anticancer compounds present in the SBWE herbal extracts, protocatechualdehyde, caffeic acid, ferulic acid, tanshinone I, and tanshinone IIA were not only identified by HPLC and GC/MS but also quantified using HPLC. These SBWE herbal extracts were then tested for the cytotoxicity on *Caenorhabditis elegans* animal model using reproduction assay. The outcome of the cytotoxicity test can facilitate the

optimization of SBWE temperature in yielding the most efficacious herbal medicine that may potentially treat various cancers including breast, colon, lung, and liver cancers.

Chapter 2: Background

2.1 Properties of Subcritical Water

As shown in Figure 2.1, subcritical water normally refers to the liquid water heated from 100 to 374 ^{o}C and pressurized up to 218 atm. Water is highly polar at ambient temperature due to its ability to form intermolecular hydrogen bonding. Fortunately, by heating the water to higher temperatures, the hydrogen bonding will break down and leading to the decrease in polarity, surface tension, and viscosity of water as shown in Figure 2.2 [1].

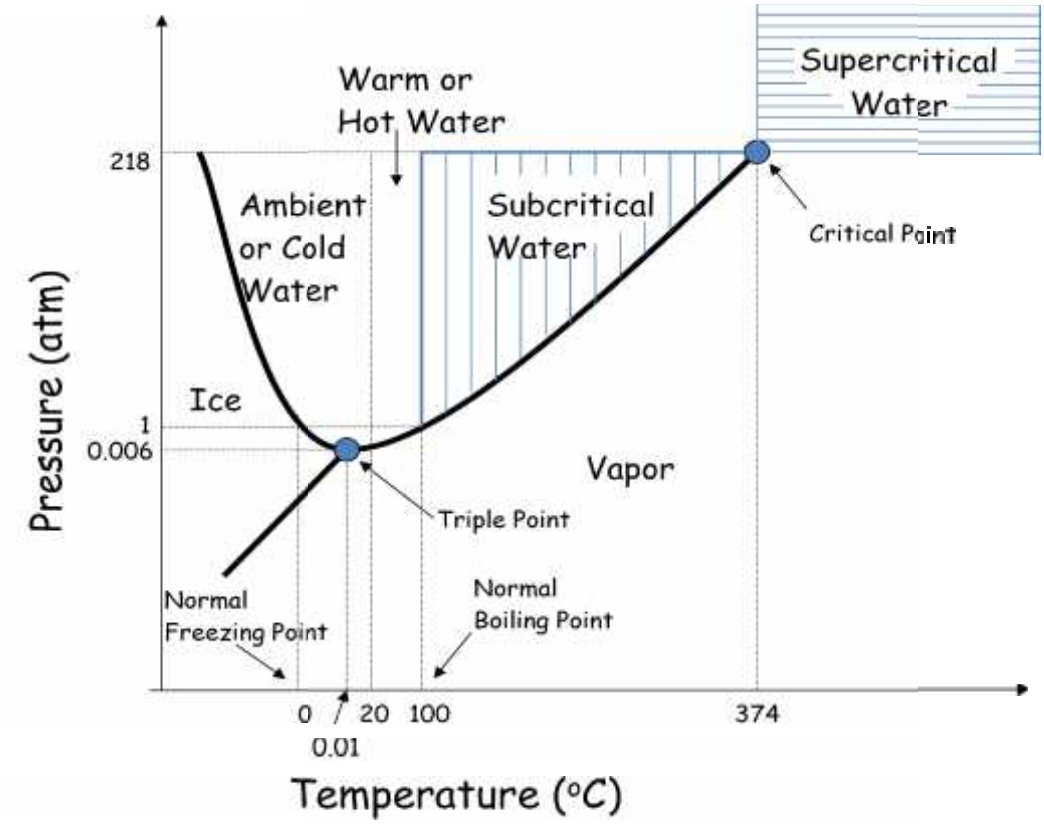
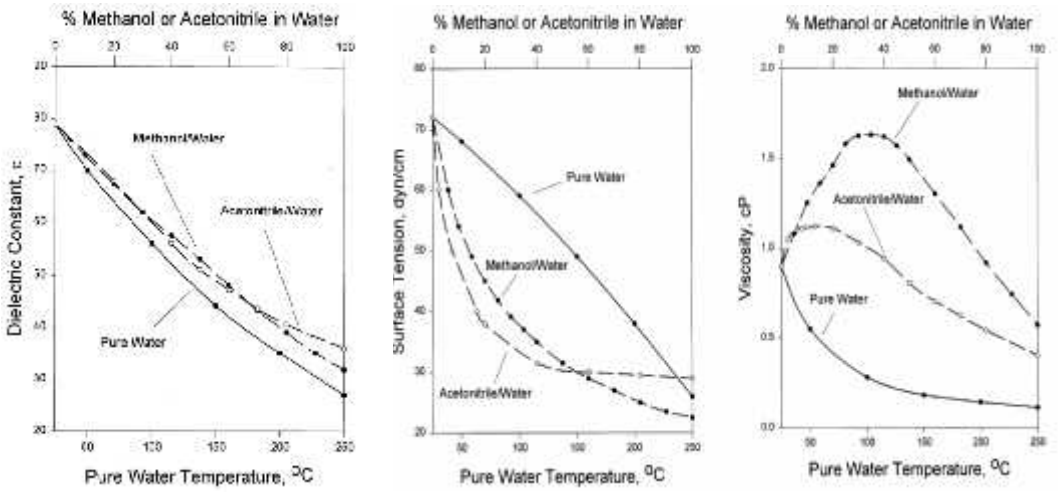


Figure 2.1. Phase diagram of water. (Reproduced with permission from reference 1 © John Wiley & Sons, Ltd., 2011.)



a b c

Figure 2.2. Control of solvent dielectric constant (a), surface tension (b), and viscosity (c) by changing temperature with pure liquid water at 100 bar compared to mixing water with methanol or acetonitrile at 25 °C. (Reproduced with permission from reference 3 © Elsevier, 1998.)

2.1.1 Polarity of Water

Polarity of water cannot be measured directly, but it can be measured indirectly through the dielectric constant of water. As shown in Figure 2.2a, the dielectric constant of water decreases from 79 to 27 by raising the water temperature from 25 to 250 ^{o}C at 100 bar pressure. This infers the decrease in polarity of water with increasing temperature. As shown in Figure 2.2a, the polarity of water also decreases with the addition of organic solvents such as methanol or acetonitrile to water at room temperature as displayed on the top x-axis. This means that the water at high temperatures can replace the RPLC gradient mixtures, such as methanol-water or acetonitrile-water. As shown in Table 2.1, by fine tuning temperature and pressure of water, the dielectric constant of water can be made equivalent to that of various organic solvents at room temperature and pressure [1].

Table 2.1. Comparison of Dielectric Constant of Selected Common Organic Solvents with that of Subcritical or Supercritical Water at Various Temperature and Pressure Conditions (Reproduced With Permission From Reference 1 © John Wiley & Sons, Ltd., 2011.)

Dielectric constant of common organic solvents at ambient temperature and pressure	Dielectric constant of water under specified temperature and pressure conditions [7].
1.9 (n-Hexane)	2.0 (275 °C, 200 bar)
7.6 (tetrahydrofuran)	7.5 (500 °C, 800 bar)
8.9 (methylene chloride)	8.5 (450 °C, 600 bar)
21 (acetone)	20 (300 °C, 100 bar)
25 (ethanol)	25 (275 °C, 300 bar)
33 (methanol)	35 (200 °C, 100 bar)
39 (acetonitrile)	39 (175 °C, 100 bar)

2.1.2 Surface Tension of Water

Surface tension of water also follows the similar trend of dielectric constant. As shown in Figure 2.2b, the surface tension of water declines from 72 dyn cm^{-1} to 26 dyn cm^{-1} at 100 bar pressure by heating the water from 25 to 250 ^{o}C. Again the same figure also shows the decrease in surface tension of water with the addition of organic solvents at room temperature [1].

2.1.3 Viscosity of Water

Similar to the dielectric constant and surface tension of water, viscosity of water also follows the same trend. As shown in Figure 2.2c, viscosity of water reduces from 0.89 cP to 0.11 cP by increasing water temperature from 25 to 250 ^{o}C at 100 bar pressure. Again the same figure also shows the viscosity of high-temperature water much lesser than the mixtures of organic solvent water at room temperature [1].

2.1.4 Solute Mass Transfer

The diffusion coefficient of solute over a chromatographic column can be known by the Wilke-Chang equation (eq 2.1) [79]. This equation clearly infers the increase in diffusion coefficient (mass transfer) of solute with the decrease in viscosity of mobile phase. As there is a

decrease in viscosity of water with increasing water temperature, the mass transfer of solute over the column increases with the increase in temperature of water due to the enhanced diffusion coefficient.

$$D_m = 7.4 \times 10^{-8} \frac{(\emptyset M)^{\frac{1}{2}} T}{\eta V_a^{0.6}} \quad (2.1)$$

Where η is the solvent viscosity (cP); D_m is the diffusion coefficient of solute in (cm^2/s); Φ is the association factor for the solvent that accounts for hydrogen bonding; M is the molecular weight (g/mol) of the solvent; T is the absolute temperature (K); and V_a is the solute molar volume (cm^3/mol).

2.2 Solubility of Analytes in Subcritical Water

2.2.1 Solubility Determination

Water solubility of a solid analyte in subcritical water is defined as the the amount of nondegraded or nontransformed dissolved analyte in liquid phase at that pericular temperature. But for the solubility determination of analytes at temperatures higher than melting point made the undissolved analytes also in liquid state, thereby the solubility of solid analytes at temperatures are only approximated values. As discussed in section 2.1, due to the weakend intermolecular hydrogen bonding and lower polarity enhances the solubility of moderately polar and nonpolar analytes in subcritical water. Solubility data of various analytes in subcritical water may help in the optimization of subcritical water chromatography methods [1]. Solubility of hydrophobic compounds like polycyclic aromatic hydrocarbons and nonpolar pesticides was improved by about four to five orders of magnitude with the increase in water temperature from ambient to 250 ^{o}C [29, 31, 80, 81]. There is a tremendous increase in the solubility of benzo[*a*]pyrene in subcritical water by about 2.5 million folds with temperature increase from

25 to 350 ^{o}C [80]. Solubility of liquid organics and organic acids also showed solubility enhancement in subcritical water [82-86].

In addition to the subcritical water chromatography studies, solubility results also help in the optimization of various green processes like subcritical water extractions [30, 87-89], environmental remediation [1, 33-40], micronization [90, 91], decomposition of non-biodegradable polymers [92], hydrothermal degradation of silk protein to amino acids [93], and a medium for chemistry reactions [94]. Table 2.2 summarizes the solubility enhancement of various moderately polar and non polar compounds like hydrophobic organics, fatty acids, and polycyclic aromatics in subcritical water.

Table 2.2. Solubility Enhancement of Various Analytes in Subcritical Water

Compound	X_2[a] at Low Temperature (^{o}C)	X_2[a] at High Temperature (^{o}C)	Enhancement in folds	Ref
Alkylbenzenes				
Toluene	1.05×10^{-4} (21)	2.46×10^{-3} (200)	23	83
Ethyl benzene	2.80×10^{-5} (25)	8.10×10^{-4} (200)	29	84
m-xylene	3.70×10^{-5} (25)	1.02×10^{-3} (200)	28	84
Benzene	4.20×10^{-4} (25)	4.60×10^{-3} (200)	11	84
p-Cymene	3.00×10^{-6} (25)	2.00×10^{-4} (200)	67	84
Octane	1.40×10^{-7} (25)	2.90×10^{-5} (200)	207	84
2,2,4- Trimethylpentane	4.40×10^{-7} (25)	6.10×10^{-5} (200)	139	84
Tetrachloroethylene	2.30×10^{-5} (25)	5.90×10^{-3} (200)	26	84
Tetraethyl Tin	3.40×10^{-9} (25)	8.80×10^{-7} (200)	259	84
1,2-Dichlorobenzene	1.70×10^{-5} (25)	5.70×10^{-4} (200)	34	84
Carbohydrates				
Glucose	4.45×10^{-2} (20)	2.32×10^{-1} (140)	5	95
Maltose.H_2O	2.44×10^{-2} (20)	1.47×10^{-1} (140)	6	95
Xylose	5.08×10^{-2} (20)	2.07×10^{-1} (140)	4	95
Cycloalkanes				
Adamantane	2.85×10^{-8} (25)	1.70×10^{-4} (150)	5965	96
Diamantane	0.91×10^{-9} (25)	3.77×10^{-5} (220)	41338	96
Fatty Acids				

Caprylic acid	7.13×10^{-2} (60)	9.79×10^{-1} (160)	14	97
apric acid	8.77×10^{-3} (60)	2.27×10^{-1} (160)	26	97
Lauric acid	9.65×10^{-4} (60)	5.91×10^{-2} (160)	61	97
Myristic acid	1.36×10^{-4} (65)	2.36×10^{-1} (230)	1735	97
Palmytic acid	4.02×10^{-4} (150)	5.53×10^{-2} (230)	138	97
Stearic acid	1.17×10^{-3} (180)	3.35×10^{-2} (230)	29	97
Flavor & Fragrants				
d-Limonene	1.00×10^{-6} (25)	5.70×10^{-5} (200)	57	98
Carvone	9.70×10^{-5} (25)	2.50×10^{-3} (200)	26	98
Eugenol	1.90×10^{-4} (25)	4.90×10^{-3} (200)	6	98
1,8-Cineole	3.30×10^{-4} (25)	5.30×10^{-4} (200)	2	98
Nerol	6.20×10^{-5} (25)	5.10×10^{-4} (150)	8	98
Catechin	1.32×10^{-4} (26)	3.52×10^{-2} (143)	267	99
Glucocorticoid Steroid				
Budesonide	2.24×10^{-6} (100)	9.84×10^{-5} (160)	44	100
Hydrophobic Organics				
Naphthalene	4.50×10^{-6} (25)	3.04×10^{-5} (65)	7	31
Benzo[a]pyrene	2.90×10^{-10} (25)	7.82×10^{-5} (250)	269651	31
Propazine	4.90×10^{-7} (25)	2.10×10^{-3} (200)	4282	31
Chlorothalonil	1.20×10^{-8} (25)	1.58×10^{-3} (200)	131937	31
Endosulfan II	1.20×10^{-8} (25)	1.99×10^{-4} (200)	16603	31
Organic Acids				
Benzoic acid	2.22 (25)	1.36×10^{2} (200)	61	85
Salicylic acid	4.69×10^{-1} (25)	1.02×10^{2} (150)	217	85
Gallic acid	1.24×10^{-3} (26)	2.33×10^{-1} (143)	188	99
Protocatechuic acid	3.55×10^{-3} (26)	1.26×10^{-1} (143)	36	99
Oxygenated Aromatics				
Xanthene	2.52×10^{-7} (40)	4.27×10^{-6} (90)	17	101
Anthrone	3.45×10^{-7} (40)	1.26×10^{-4} (150)	365	101
Xanthone	7.09×10^{-7} (40)	2.71×10^{-4} (160)	382	101

Thioxanthone	1.18×10^{-7} (40)	1.35×10^{-4} (180)	1144	101
9,10-Anthraquinone	7.25×10^{-8} (40)	2.96×10^{-5} (160)	408	101
9,10-Phenanthraquinone	5.50×10^{-7} (40)	1.83×10^{-3} (160)	3327	101
Polycyclic Aromatics				
Anthracene	8.10×10^{-9} (25)	2.10×10^{-4} (200)	25926	29
Pyrene	1.07×10^{-8} (25)	1.00×10^{-7} (100)	9	29
Chrysene	6.30×10^{-10} (25)	7.58×10^{-5} (225)	120317	29
Perylene	2.90×10^{-10} (25)	5.00×10^{-6} (200)	17241	29
Carbazole	1.10×10^{-7} (25)	1.90×10^{-3} (200)	17273	29
Phenanthrene	2.17×10^{-7} (40)	3.27×10^{-6} (90)	15	102
Phenanthridine	6.29×10^{-6} (40)	5.92×10^{-5} (90)	9	102
Acridine	9.10×10^{-6} (40)	6.09×10^{-5} (90)	7	102
Phenazine	7.17×10^{-6} (40)	8.27×10^{-4} (160)	115	102
Thianthrene	1.50×10^{-8} (40)	1.61×10^{-5} (150)	1073	102
Phenothiazine	2.92×10^{-7} (40)	4.31×10^{-4} (180)	1476	102
Phenoxathiin	2.78×10^{-7} (40)	7.51×10^{-7} (55)	3	102
Phenoxazine	1.94×10^{-6} (40)	2.23×10^{-4} (100)	115	102
Carbazole	2.72×10^{-7} (40)	1.68×10^{-4} (160)	618	102
Dibenzofuran	9.17×10^{-7} (40)	7.04×10^{-6} (80)	8	102
Dibenzothiophene	2.06×10^{-7} (40)	3.49×10^{-6} (90)	17	102
4,6-DMDBT	3.17×10^{-9} (40)	5.15×10^{-6} (150)	1625	102
Fluorene	3.82×10^{-7} (40)	1.58×10^{-5} (110)	41	103
Fluoranthene	3.99×10^{-8} (40)	$1.89\ x_2\ 10^{-6}$ (110)	47	103
1,2-benzanthracene	3.37×10^{-9} (40)	2.96×10^{-6} (150)	47	104
Terphenylene	1.82×10^{-9} (40)	2.83×10^{-5} (195)	15549	104
p-terphenyl	0.849×10^{-10} (60)	3.93×10^{-5} (210)	46290	104

[a] *Molefraction solubility of analytes.*

2.2.2 Solubility Modeling

Solubility modeling is necessary to guide the estimation of organics solubility in water at higher temperatures. Carr et al. [82] recently reviewed the solubility models developed over past ten years for the solubility of hydrophobic organic compounds in subcritical water. These solubility models are divided in to four types, empirical, pure component, modified-UNIQUAC functional-group activity coefficient (M-UNIFAC), and dielectric constant models.

2.2.2.1 Empirical Model

This model is developed by Miller et al. [29], where this model (eq 2.2) considered the Gibbs function, entropy, and enthalpy changes in estimating the solubility of analytes in water at higher temperatures.

$$\ln x_2(T) \approx \left(\frac{T_0}{T}\right) \ln x_2(T_0) \tag{2.2}$$

Where $X_2\,(T)$ is the mole fraction solubility of analyte at higher temperature and $X_2\,(T_0)$ is the mole fraction solubility of analyte at ambient temperature. Please note T is the absolute temperature. This is the Miller basic equation and without any modification it is not suitable to estimate the solubility of various kinds of analytes in subcritical water. In order to make this model applicable for the solubility of polycyclic aromatic hydrocarbons in water at higher temperatures [29], Miller et al. added a cubic part as shown in eq 2.3.

$$\ln x_2(T) = \left(\frac{T_0}{T}\right) \ln x_2(T_0) + 15\left(\frac{T}{T_0} - 1\right)^3 \tag{2.3}$$

Yang et al. also modified the empirical model to predict the solubility of alkylbenzenes (eq 2.4) and organic acids (eq 2.5) in subcritical water [84, 85].

$$\ln x_2(T) = \left(\frac{T_0}{T}\right) \ln x_2(T_0) + 2\left(\frac{T - T_0}{T_0} - 1\right)^3 \tag{2.4}$$

$$\ln x_2(T) = \left(1.85\frac{T_0}{T} - 1\right) \ln x_2(T_0) \tag{2.5}$$

2.2.2.2 Pure Component Property Model

Roth et al. developed a semi-empirical model [103] for the solubility estimation of polycyclic aromatic hydrocarbons in subcritical water. They considered the cohesive energy density and dielectric constant of a solution in developing the model as shown in eq 2.6.

$$\ln x_2 = \ln\left(\frac{f_2^{sc}}{f_2^{1}}\right) - U_2^{1}\left[Ac_1 + BP^{ii}{}_1 + C(\epsilon_{r1} - 1)\right] \tag{2.6}$$

Where f_2^{S0} is fugacity of pure component analyte; f_2^{10} is fugacity of pure component solvent; *A*, *B*, and *C* are coefficients of analytes at temperatures of 313 - 498 K and pressures within 0.1 – 40 MPa; *c* is the cohesive energy density (MPa); *p* is pressure (Mpa); ε_r is the relative permittivity of solvent. Roth et al. also used the semi-empirical model (eq 2.7) to estimate the solubility of adamantine [96], diamantine [96], phenanthrene [102], and solid three-ring aromatic heterocycles [102] in subcritical water.

$$\ln x_2 = a_1 + a_2\left(\frac{T_0}{T}\right) + a_3 \ln\left(\frac{T}{T_0}\right) \tag{2.7}$$

Where a_1, a_2, a_3 are coeeficients of analytes.

2.2.2.3 Modified-UNIQUAC Functional-Group Activity Coefficient Model

A modified UNIFAC model (eq 2.8) was developed by Fornari et al. by considering the interactions between analyte side groups and the solvent to calculate the activity coefficient of analytes in solution. Then by using the thermodynamic relation between melting properties of the solute and the solute/solvent ratio, activity coefficients will be converted to the mole fraction solubility of compounds for their prediction of solubility in high temperature water. This model successfully predicted the solubility of polycyclic aromatic hydrocarbons in water at higher temperatures [105]. This model was modified from the UNIFAC model developed by Fredenslund et al. to estimate active coefficients of liquid organics [106]. This model partially mimics the pure component model developed by Roth et al. was discussed earlier [103].

$$\ln x_2 = -\frac{\Delta H_{2}}{R}\left(\frac{T_{2}}{T} - 1\right) - \ln \gamma_2 \tag{2.8}$$

Where f_2^S is fugacity of pure analyte; f_2^0 is fugacity of analyte in hypothetical liquid state; *R* is gas constant; Tm_2, is analyte melting temperature; Hm_2, is enthalpy of fusion.

2.2.2.4 Dielectric Constant Model

Carr et al. developed this model by considering the dielectric constant of the solvent at higher temperatures. They used this model (eq 2.9) to estimate the solubility of budenoside in water at higher temperatures [100]. This dielectric constant model was based on mathematical relationships developed by the Akerlof [107] and a method developed by the Astin [108].

$$\ln x_2 = A\epsilon + B \tag{2.9}$$

Where *A* and *B* are the linear constants and ε is the dielectric constant of solvent.

2.3 Stability of Analytes in Subcritical Water

As discussed in Chapter 1, one of the main hindrances for the development and application of subcritical water chromatography and subcritical water extraction is the stability of analytes. As high temperatures are used in either chromatographic separation or extraction of analytes, stability of analytes is always a great concern. As discussed by the Carr et al. [109], reactions that take place during the retention time of the analyte on column determines the stability of analyte in SBWC. The undesirable reactions that take place during the retention time are hydrolysis, oxidation, isomerization, and epimerization in SBWC [110-113]. Keller and Giddings first demonstrated the occurrence of first order reactions during liquid chromatography leading to the degradation of analytes [114]. Horvath et al. observed much faster degradation of hydroxyquinones on silica columns than in liquid solutions. Based on these observations, he suggested that these on-column reactions can be decreased by adjusting the temperature, pH, column length, and flow velocity [115]. Based on reaction rate and on-column reaction time, Horvath et al. developed a Damkohler number (eq 2.10) to determine the on-column reactions [109, 115, 116]. If Da << 1, there is no on-column reaction and if Da > 50, there is a fast on-column reaction. In both cases only single peaks are expected, whereas for 0.1

< Da < 50, intermediate values means broad and irregular shape peaks are expected. So at higher temperatures, retention time and reaction rate of the analyte on that column determine the stability of analyte.

$$D = \frac{K\ (1+K')}{u_0} \quad (2.10)$$

Where K is the pseudo-first-order reaction rate (S^{-1}); L is the column length (cm); K' is the retention factor; u_0 is the interstitial linear velocity (cm/s). Reaction rate of water increases with increase in temperature [117]. The examples for the degradation of compounds in subcritical water that occurred due to the faster reaction rate are organochlorine compounds [118], oils [119], fatty acids [117], polycyclic aromatic hydrocarbons [120], pharmaceuticals like thiazides [121], aspirin [122], and thiamine [123]. Yang et al. also observed the degradation of terpenes [89]; salicylic acid [85]; phenanthrene [120]; benzoic acid and its derivatives [124], sunscreens and preservatives in subcritical water at 423-473 K [125]. Despite of disadvantages, some advantages with the increase in reaction rate of water are organic synthesis of aromatic acids [126], oxidation of organics [127], processing of triglyceride based oils and fats without catalysts [119], dehalogenation of aliphatic organochlorine compounds for environmental remediation [118], and pilot scale remediation of explosives and pesticides from contaminated soils [128, 129].

According to the van't Hoff equation, retention time of analytes on a column decreases with increase in temperature. Carr et al. reported that some of the pharmaceuticals are stable up to 190 ^{o}C in HTLC conditions [109]. As discussed in our previous review [1], small amounts of analytes degradations may not affect the quantitative analysis of analytes under subcritical water chromatographic conditions. As both standard and sample analyte mixtures are analyzed through SBWC at same time, they expose to the same temperatures and have the same amount

of degradations. Thus these minor degradations may not affect the quantification results of analytes through SBWC.

2.4 Stability of Stationary Phases under Subcritical Water Chromatography Conditions

Similar to the stability of analytes, stability of stationary phase under SBWC conditions also hinders the development and applications of SBWC. Both commercial and lab-packed columns have been used for the SBWC separations at higher temperatures [1]. These columns usually consist of silica, hybrid silica, carbon, polymer, and zirconia based materials [2, 4]. The manufacturer temperature limitations for these columns are 60 ^{o}C for silica based columns and 100-200 ^{o}C for other stationary phases [1].

Yang et al. evaluated several stationary phases under subcritical water chromatographic conditions [130, 131]. He and Yang evaluated the long-term stability of five commercial columns under SBWC conditions at temperatures ranging from 100-150 ^{o}C [130]. Of them three are silica based columns including Zorbax RX-C_8, Nucleosil C_{18}, and Hypersil BDS C_{18}. The fourth one is a Zirconia based column, ZirChrom-PS and the fifth one is a polymer based column, PRP-1. Silica based columns evaluated at 100 ^{o}C showed the Zorbax RX-C_8 column was stable for up to 250 hours, followed by the Nucleosil C_{18} column (245 hours) and least stable one is the Hypersil BDS C_{18} column, only stable for 42 hours at 100 ^{o}C. Whereas ZirChrom-PS was stable for up to 120 hours at 100 ^{o}C and PRP-1 to a total of 499 hours at 100 and 150 ^{o}C. So this indicates that the PRP-1 column is the most stable of all the columns tested.

Carr et al. developed zirconia based stationary phases and also tested their stability at temperatures up to 200 ^{o}C under high temperature liquid chromatographic conditions [109, 132-

135]. Through column bleeding studies, Tuetenberg et al. [136] tested the stability of silica, zirchrom, polymer, and titanium metal-oxide based columns at 200 ^{o}C under SBWC conditions and found that the silica based column showed highest column bleed and metal oxide columns showed the lowest column bleed demonstrating their better stability at higher temperatures than the other columns tested. Later in a separate stability study conducted by Tuetenberg obtained the similar results with silica and metal oxide based columns under SBWC conditions at higher temperatures [137]. The following Table 2.3 summarizes the stability testing of both commercial and Lab-packed columns in SBWC conditions and Table 2.4 summarizes the long-term stability evaluation of both commercial and Lab-packed columns under SBWC conditions.

Table 2.3. Both Commercial and Lab-Packed Columns Tested in Subcritical Water Chromatography

No	Base Material	Column Name	Temperature Tested (^{o}C)	Reference
1.	Carbon	Hypercarb	Up to 260	138-141
2.	Polymer	Nucleogel RP	160	142
		Oasis HLB	185	143
		Oasis (M81883D01)	up to 210	140
		Oasis (9M90762D01)	up to 208	140
		PRP-1	200-225	138, 10, 8, 130, 192
		PLRP-S	200-210	123, 144-148
3.	Lab-packed polymer	PRP-1	180	149
		Poly(divinylbenzene)	250	150
4.	Resin	CS11 G	180	14
5.	Silica	Chromatorex C18	140	8
		Discovery HS PEG	150	47
		Gemini C18	110	151
		Hypersil BDS C18	100-160	130, 140
		Hypersil ODS	140	152

		Nucleosil C18 AB	up to 200	130, 11
		Partisil ODS2	125	13
		Spherisorb ODS2	170	81, 146
		Zorbax RX-C8	100	152, 130, 8
6.	Lab-packed silica	C18	130	153
		Kovasil MS-H	300	154
		Lichrosorb RP-2	300	154
		ODS-BP	36	155
		Poly(acryloyl-L-Proline methyl ester)	up to 40	156
		Poly(N-isopropylacryl -amide- co-n-butylmethacrylate)	35-50	12, 157, 158
7.	Hybrid Silica	Acuty BEH C18	180-220	159-161
		Xbridge C18	200	162, 163, 121
		XBridge phenyl	200	164
		XTerra (C8)	160	165
		XTerra (C18)	160	165
		XTerra MS (C18)	110-130	164, 166
		XTerra RP (C18)	165	140
		XTerra Phenyl	130	16
8.	Zirconia	ZirChrom-C18	170	167
		Zirchrom carb	220	140
		Zirchrom-carb-TiO_2	185	137
		ZirChrom-DB-C18	150-200	22
		ZirChrom-PBD	175-200	168-170
		ZirChrom-PS	100-145	47, 130, 170
9.	Lab-packed zirconia	PBD coated zirconia	260	171
		ZirChrom-carb	370	172
		ZirChrom-PBD	300	172
		Zirchrom PS	120	132

Table 2.4. Long-Term Stability Evaluation of Commercial and Lab-Packed Columns under Subcritical Water Chromatography Conditions

Column	Base Material	Long-Term Stability	Reference

Hypercarb	Carbon	208 Hous (200 ^{o}C)	17
PRP-1	polymer	499 Hours (100 & 150 ^{o}C)	130
CS11 G	Resin	Several Days (180 ^{o}C)	14
Acuty BEH C18	Hybrid Silica	One Month (200 ^{o}C)	161
Hypersil BDS C18	Silica	42 Hours (100 ^{o}C)	130
Nucleosil C18 AB	Silica	245 Hours (100 ^{o}C)	130
Zorbax RX-C8	Silica	250 Hours (100 ^{o}C)	130
ZirChrom-PBD	Zirconia	50 Hours (185 ^{o}C)	137
Zirchrom-carb-TiO_2	Zirconia	50 Hours (185 ^{o}C)	137
Zirchrom-carb	Zirconia	50 Hours (185 ^{o}C)	137
ZirChrom-PS	Zirconia	120 Hours (100 ^{o}C)	130

2.5 Subcritical Water Chromatography

As discussed in Chapter 1, SBWC is a green separation technique using subcritical water as the only mobile phase, thus eliminating the use of toxic and expensive organic solvents. In literature, different terminologies have been used for subcritical water chromatography; they are hot water chromatography, super heated water chromatography, and pressurized hot water chromatography [4]. Subcritical water generally refers to the liquid water below critical temperature and pressure [1].

2.5.1 Instrumentation

The instrumentation of SBWC is similar to the regular HPLC system except the addition of an oven to provide the temperature and a cooling unit to cool the hot eluent to protect the detector incase of temperature sensitive detectors such as UV-vis is used. A back pressure regulator is also necessary to keep the hot water in liquid state at temperatures higher than 100

^{o}C. For example approximately 16 bar of pressure is sufficient to keep water at 200 ^{o}C in the liquid state [2]. In SBWC separations, thermal mismatch is a major problem. The mobile phase when entering the system is at ambient temperature. Therefore, the water temperature is lower at the head of the column but will be getting warmer towards the end of the column, causing thermal mismatch, leading to peak broadening and poor reproducibility. This problem can be minimized by adequately heating the mobile phase before entering the column. It is necessary to discuss the instrumentation developed by various chromatographers to resolve thermal mismatch in SBWC separations, because its helps in the better understanding of SBWC green separation technology [1].

Various approaches used by the chromatographers to minimize thermal mismatch in SBWC separations are discussed here. Teutenberg et al. developed a specially designed heating system as shown in Figure 2.3 [170]. Here the heating system used in subcritical water chromatography was designed based on the temperature measurements of the mobile phase flowing through the stainless steel capillary at both constant and gradient flow at higher temperatures. Although this system has the ability to quickly heat and cool the eluent up to 225 ^{o}C, but due to the presence of preheating and post-column cooling coils add the void volume and causes peak broadening.

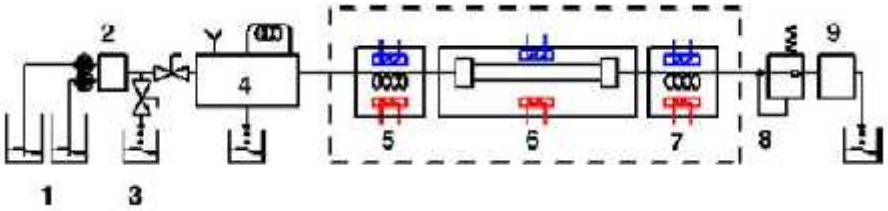


Figure 2.3. Schematic drawing of the HPLC system and the specially designed heating oven for temperature-programmed applications. (1) Solvent reservoir; (2) pumps; (3) autosampler; (4)

high-pressure mixing chamber; (5) preheating unit; (6) column heating unit; (7) cooling unit prior to detection; (8) UV detector; (9) back-pressure regulator. (Reproduced with permission from reference 170 © Elsevier, 2006.)

The second approach is by Yang et al. as shown in Figure 2.4, where they added the preheating coil before the injector to avoid the void space [18]. By this approach void space problem is resolved, but the injector at ambient temperature cools down the eluent and may lead to a minimal thermal mismatch. Yang et al. used this instrument setting for SBWC separation of niacinamide, preservatives, and sunscreens present in P&G skincare creams and did not encountered any major thermal mismatch problems [18-20].

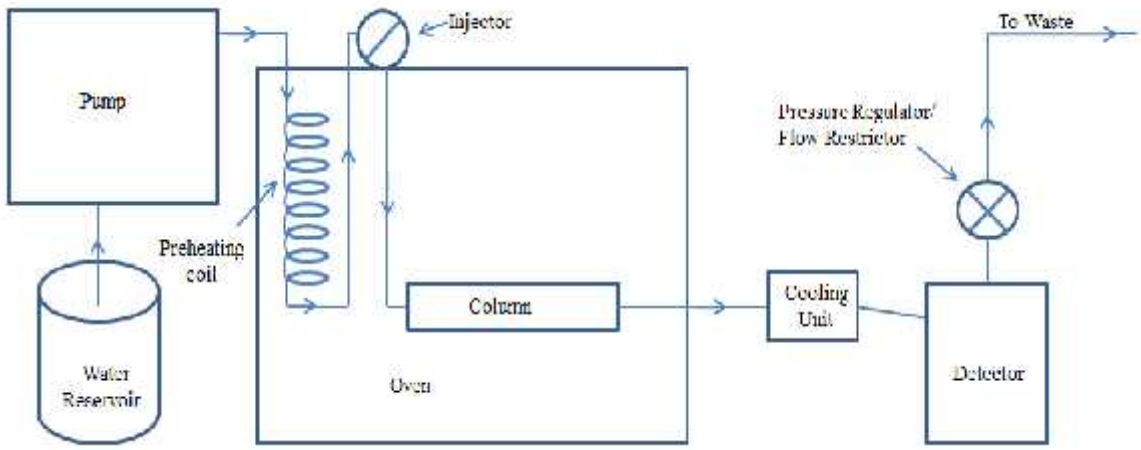


Figure 2.4. Block diagram of a subcritical water chromatography system with the preheating coil placed before the injector. (Reproduced with permission from reference 1 © John Wiley & Sons, Ltd., 2011.)

The third approach is by Carr et al. as shown in Figure 2.5. They added a preheating coil before the separation column similar to what Yang et al did but used a separate pump to introduce the sample into the separation column to avoid thermal mismatch [132]. Here the use

of multiple pumps increases the cost of SBWC instrumentation and the use of post-column cooling coil causes peak broadening.

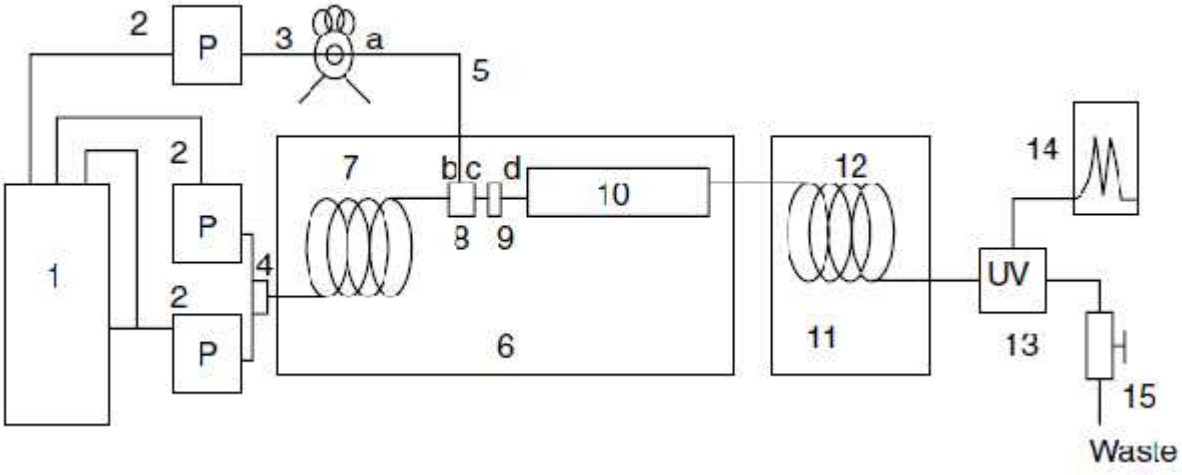


Figure 2.5. Schematic diagram of an HTLC system: (1) solvent reservoir; (2) pumps; (3) injector; (4) T union; (5) stainless steel tubing; (6) heating bath; (7) heat exchanger; (8) low dead volume T union; (9) in-line filter; (10) analytical column; (11) cooling bath; (12) cooling tubing; (13) UV detector; (14) data system; (15) backpressure adjuster. (Reproduced with permission from reference 132 © American Chemical Society, 2000.)

Presently, the commercial subcritical water chromatography system such as Schimadzu Nexera is available with pre-column heating (up to 160 ^{o}C) and post-column cooling. This commercial instrument was used by our group for successful SBWC separation of niacinamide and preservatives present in P&G skincare cream samples [18, 20].

2.5.2 Separation of Analytes by Subcritical Water Chromatography

As most of the SBWC work in this dissertation was performed using UV-visible as the detector, so the analytes separated through the subcritical water chromatography using UV-visible detector is summarized in Table 2.5. It is evident that most of the polar analytes was

separated through the SBWC at lower temperatures and moderately polar analytes at higher temperatures and some non polar analytes at much higher temperatures.

Table 2.5. Subcritical Water Chromatographic Separation of Various Analytes (Reproduced With Permission From Reference 1 © John Wiley & Sons, Ltd., 2011.)

No.	Analytes	Column Name	Temperature (^{o}C)	Reference
1.	Alkanols	Brownlee Spheri-5	Ambient	173
		Hybrid C18	190	161
		Spherosil XOA 600	150	9
		Silica column*	Ambient	174
2.	Alkylbenzenes	Hybrid C18	190	161
		Spherosil XOA 600	150	9
		Zirchrom-DB-C18	150	22
		ZirChrom-PBD	175	168
		Silica column*	Ambient	46
		Polymer GC column*	Ambient	175
		ZirChrom PBD, ZirChrom CARB capillary column	Up to 370	172
3.	Alkyl aryl ketones	PLRP-S	100–200	144
4	Aliphatic and aromatics	PLRP-S 100*	Up to 180	176
5.	PTH-aminoacids	Silica column*	Up to 50	12, 156, 157
6.	Aniline and its derivatives	Ethyl-bridged hybrid C18	200	163
		Nucleosil C18 AB	100–150	11
		PRP-1, Zorbax RX C18, Chromatorex C-18	Up to 200	8
		XBridge C18	Up to 200	162
		ZirChrom-PS	100	47
7.	Aryl amides	PLRP-S	Up to 180	146
8.	Barbiturates	PLRP-S	100–200	144, 145, 148
9.	Benzenes mixture	Polymer proline*	Up to 40	156

10.	BTEX	Nucleosil C18 AB	100–200	11
		Partisil ODS2	Up to 125	13
11.	Polyhydroxy-	Partisil ODS2	Up to 125	13
	-benzenes	PRP-1, Zorbax RX C18, Chromatorex C-18	Up to 140	8
12.	Diethyl phthalate	ZirChrom-PBD	175	168
13.	Ecdysteroids (plant extracts)	XTerra C8, C18	160	165
14.	Ginger extracts (plant extracts)	XTerra RP 18	50–130	177
15.	Kavalactones (plant extracts)	Zirconia PBD	Up to 160	178
16.	Flavones	Discovery HS PEG	100- 150	47
17.	Isoflavanoids	PLRP-S	170	179
18.	Hydrocarbons and	Brownlee Spheri-5	Ambient	173
	Its derivatives	Silica C18 column	170	81
		Partisil ODS2	Up to 125	13
		Polymer GC column*	Ambient	175
19.	Organic acids and bases	Silica column*	Up to 50	157
20.	Parabens	PLRP-S	210	145
		Spherisorb-ODS	170	146
		Discovery ZirChrom-C18	100–200	167
21.	Pharmaceutical drugs	Nucleogel RP (PS-DVB)	160	142
		PLRP-S	75–185	48
		PLRP-S, Novapak C18	50–200	122-123
		PLRP-S, Oasis 40, ZirChrom PDB Hypersil C18 BDS, ZirChrom CARB, Hypercarb BDS,	Up to 225	140

		XTerra RP18		
		XBridge C18	Up to 200	121
		XTerra C8, Oasis HLB	Up to 185	143
		ZirChrom-PS	130	170
22.	Phenol and its derivatives	Brownlee Spheri-5	Ambient	173
		Nucleosil C18 AB	100–150	11
		Partisil ODS2	Up to 113	13
		PLRP-S 100	Up to 180	176
		PLRP-S	Up to 180	145-146
		PRP-1	100–150	16
		PRP-1, Hypersil ODS, Zirchrom PBD, Zorbax RX-C8	Up to 160	152
		Spherisorb-ODS	120	146
		XTerra MS (C18), XTerra Phenyl, XBridge phenyl	Up to 200	164
		Zirchrom-PS	Up to 130	180, 47
		Zirchrom-PS*	120	132
23.	Polyethylene glycols (PEG 200)	PRP-1*	100-180	149
24.	Purines, pyrimidines	Hypercarb	100–200	17
25.	Sulfonamides	PLRP-S	70–200	181, 147
26.	Steroids	XTerra MS (C18)	130	166
		ZirChrom-PBD	160-200	169, 170
		Polymer column*	Up to 50	156, 158
22.	Triazine herbicides	Hypercarb	Up to 260	141
23.	Triazole fungicides	ZirChrom PBD	100–150	182

** Lab-packed column.*

2.5.3 Industrial Applications

Analytical companies are spending millions of dollars in purchasing organic solvents for RPLC separations and also for the disposal of organic waste [1]. The replacement of such

methods with SBWC will help in saving money for companies. It may be difficult to replace RPLC methods requiring greater amount of organic solvents, but traditional RPLC separations consuming smaller amounts of organic solvents easier to be replaced with SBWC methods. Yang et al. developed several SBWC methods for separation of niacinamide from skincare creams at up to 80 ^{o}C. These SBWC methods may replace the existing P&G RPLC methods that require organic solvents [18]. Development of such subcritical water chromatography methods at relatively low temperatures will promote the applications of SBWC separations in the industries.

Miller et al., Yarita et al., and Nakajima et al. applied SBWC-FID for quantitative determination of ethanol in various types of alcohol beverages [183-185]. Wilson et al. employed SBWC in the analysis of pharmaceuticals from urine samples [140].

2.5.4. Advantages

2.5.4.1 Green Separation

Complete elimination of organic solvents is achieved with the usage of subcritical water chromatography in analytical separations. Again water used in the mobile phase is economical, safe, non toxic and does not require waste disposal [1-2, 4].

2.5.4.2 Fast Separation and Analysis

Pressure drop across the column can be estimated by the Heinisch and Rocca derived equation [186].

$$\Delta P = N \frac{hv}{K_0} \eta D_m \qquad (2.11)$$

Where ΔP is the pressure drop across the column; K_0 is the specific column permeability; h and v are the reduced plate height and velocity parameters; N is the plate number; η is the solvent

viscosity (cP); *Dm* is the diffusion coefficient of the solute in (cm^2/s). This equation infers the increase of pressure drop (decrease in column back pressure) with the increase in diffusion coefficient. As discussed in section 2.1, diffusion coefficient increases with increasing temperature.

There are two advantages with the decrease of column back pressure. Firstly, the usage of higher flow rates in RPLC is normally restricted due to the associated column back pressure. Due to the decrease of column back pressure with temperature increase, now the usage of higher flow rates is possible. Figure 2.6 shows that caffeine derivatives were separated by 1 mL/min flow rate at 25 ^oC as well as by 7 mL/min flow rate at 150 ^oC [22]. One can clearly see that the retention time of the same mixture was shortened from 7 min to less than 1 min by increasing both temperature and flow rate as shown in Figure 2.6. There is about five folds decrease in back pressure with the increase of column temperature from 25 to 180 ^oC [187]. Again it was reported that the usage of higher flow rates in subcritical water chromatography decreased the retention of analytes, leading to the ultra fast liquid chromatography [21]. Edge et al developed a temperature model to predict the pressure and flow rates at higher column temperatures to develop fast separation methods for various organic compounds [159].

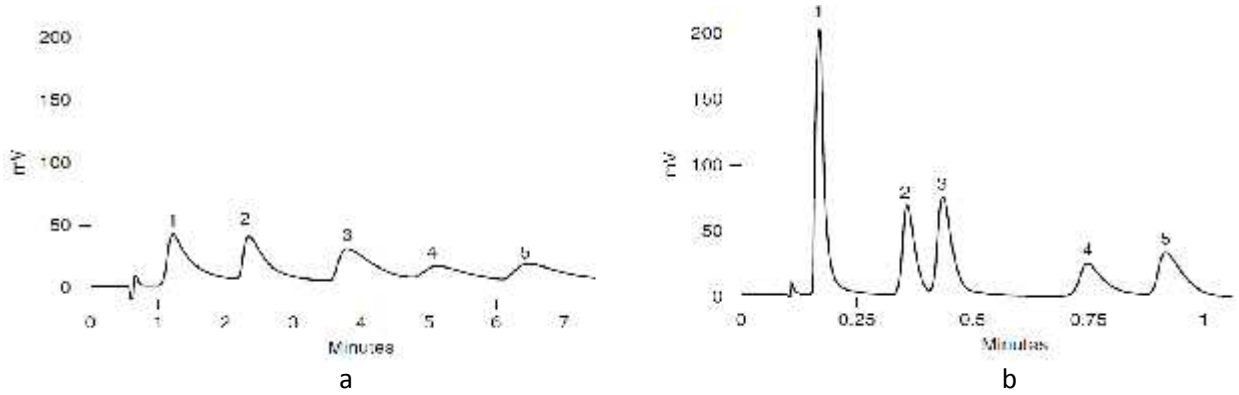


Figure 2.6. Temperature effect on flow rate for the separation of caffeine derivatives (1, hypoxanthine; 2, theobromine; 3, theophilline; 4, caffeine; 5, β-hydroxy-ethyl-theophilline), column Zirchrom-DB-C18 4.6 mm i.d. 50 mm length, 254nm UV detection, 300 ng injected, (a)

25 °C, 1 mL/min, water–methanol 60:40 v/v, backpressure resulting from tubing and column 40 bars (b) 150 °C, 7 mL/min, water, 2 m × 0.127 mm preheating tube, backpressure resulting from tubing and column 300 bars. (Reproduced with permission from reference 22 © Elsevier, 2004.)

Secondly, the usage of longer columns packed with smaller particle size is possible due to the decrease in back pressure with SBWC separations. Using long columns generally leads to higher column efficiency. Desmet et al. used standard column with 3 μm particle size for the separations of alkyl benzenes with in 400 bar pressure at 120 ^{o}C [188].

2.5.4.3 Column Efficiency

Yang developed a model (eq 2.12) from van Deemter equation to study the fundamental aspect of temperature effect on column efficiency [189]. According to this model, the column efficiency increases with increasing temperature in the lower temperature range and decreases with the rising temperature in the higher temperature range.

$$H = a + b(T^2 - 273T) + C/T \tag{2.12}$$

Where *H* is plate height; *a*, *b*, *c* are constants at fixed linear velocity; *T* is temperature.

A separate study conducted by Yang et al. on peak width and column efficiency infers that the maximum column efficiency can be obtained for a SBWC separation in the temperature range of 100 to 120 ^{o}C [152]. Later McNeff and Yan demonstrated the relation between column efficiency and linear velocity using the representative van Deemter plots [190], as shown in Figure 2.7. These plots demonstrate the use of higher temperatures and flow rates for better column efficiency. In general, increased column efficiency means the increase in plate number or narrow peaks. Figure 2.8, demonstrates the increased column efficiency for SBWC separation of purines and pyrimidines than the HPLC separation of analytes [17].

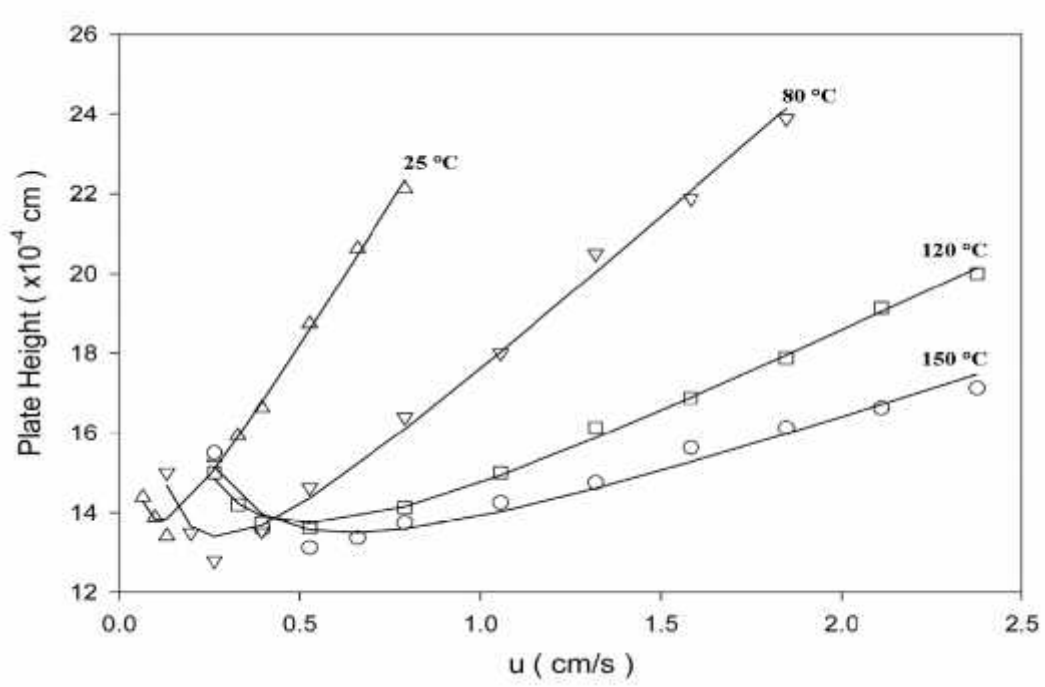


Figure 2.7. Plate height vs. linear velocity at various temperatures for moderately retained solutes. Experimental conditions: 3 µm ZirChrom-PS column (ZirChrom Separations), 5 cm x 4.6 mm id, 40% ACN/60% water, Δ = 25 °C, octanophenone, k = 3.87, ▽ = 80 °C decanophenone, k = 3.15, □ = 120 °C, decanophenone, k = 5.70, ○ = 150 °C, decanophenone, k = 1.65. (Reproduced with permission from reference 190 © Wiley-VCH, 2007.)

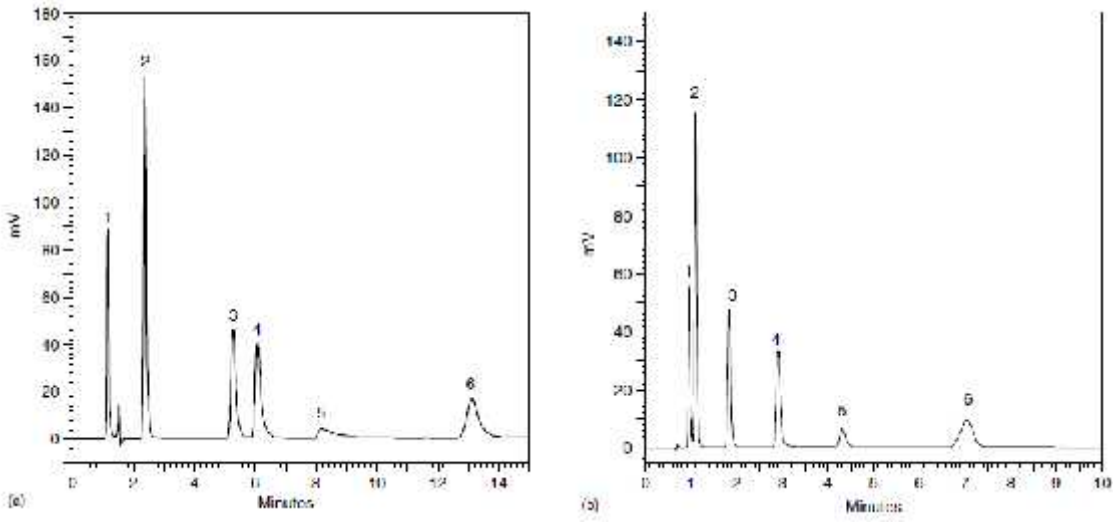


Figure 2.8. Comparison of the separation of purines and pyrimidines under conventional conditions and with high-temperature water. Column: Hypercarb 5, 100 × 4.6 mm ID; detection: UV at 254 nm. (a) Mobile phase: water + 0.1% formic acid/acetonitrile (85:15, v/v); flow rate: 0.8 mL/min; temperature: 50 °C. (b) Mobile phase: 100% water; flow rate: 2.0 mL/min; temperature: 190 °C. Analytes: 1, cytosine; 2, uracil; 3, thymine; 4, hypoxanthine; 5, guanine; 6, xanthine. (Reproduced with permission from reference 17 © Wiley-VCH, 2007.)

2.5.4.4 Detectors Compatibility

In RPLC separations, there is a restriction with compatible detectors due to the use of organic solvents as mobile phase. UV-visible and fluorescence spectroscopic detectors are common detection sources in RPLC separations [2, 191]. Universal detectors like NMR and FID are not compatible with RPLC, because organic solvent mobile phases have strong background. These universal detectors are compatible with subcritical water chromatography because water and deuterated water do not have any strong background with FID and NMR. The analytes separated by SBWC using FID and NMR universal detectors are summarized in the following Table 2.6.

Table 2.6. Subcritical Water Chromatographic Separation of Various Analytes and their Detection Through Flame Ionization Detector and Nuclear Magnetic Resonance Spectroscopy (Reproduced With Permission From Reference 1 © John Wiley & Sons, Ltd., 2011.)

Detection	Analyte	Column Name	Temperature (^{o}C)	Reference
FID	Aldehydes	PRP-1	175	138
	Alkanols	Brownlee Spheri-5	Ambient	173
		Develosil C30-UG-5	150	183
		Hypercarb	150	139
		PRP-1	100-225	184, 10, 138, 192, 193
		Zirchrom-PBD	120	139
		C18 column*	up to 130	153
		Lichrosorb RP-2*	up to 300	154
		Silica column*	Ambient	174
		Zirconia-PBD monolith*	up to 260	171
		Polymer monolith*	250	150
	Alkylbenzenes	Silica column*	Ambient	174
		ZirChrom PBD, ZirChrom CARB capillary	up to 370	172
	Aliphatic acids & alkylphosphoric acids	Kovasil MS-H*	up to 300	154
	Aminoacids	PRP-1	80–210	184, 15
		PRP-1 column*	up to 100	155
	Aminophenols	PRP-1 column*	100	155
	Aniline & its derivatives	PRP-1	150	192
		PRP-1 column*	Up to 100	155
		XBridge C18	Up to 200	162
	BTEX	PRP-1	200	192
	Carbohydrates	Hypercarb	100	15
		Kovasil MS-H*	Up to 300	154

		ODS-BP*	36	155
	Carboxylic acids	PRP-1	150-160	192, 15
	Free fatty acids	PRP-1	150	192
	Hydrocarbons & its derivatives	Brownlee Spheri-5	Ambient	173
	Organic acids & bases	PRP-1	Up to 150	155
	Phenols & its derivatives	Brownlee Spheri-5	Ambient	167
		PRP-1	100–210	169, 10
		Zirconia-PBD monolith*	Up to 220	171
NMR	Barbiturates	PLRP-S	200	148
	Ecdysteroids (plant extracts)	XTerra C8, C18	160	165
	Ginger extracts (plant extracts)	XTerra RP 18	50–130	177
	Kavalactones (plant extracts)	Zirconia PBD	Up to 160	178
	Pharmaceutical drugs	PLRP-S, Novapak C18	50–200	122, 123
		XTerra C8, Oasis HLB	Up to 185	143
	Sulfonamides	PLRP-S	160–200	147

** Lab-packed column.*

2.6 Subcritical Water Extraction of Medicinal Herbs

2.6.1 Medicinal Herbs in Cancer Treatment

In the US, medicinal herbs are classified under dietary supplements and botanicals. These plant products in pure and processed form are widely used in traditional medicine [40]. Due to the growing awareness of chemical synthetic products and their side effects, now the western

world is turning attention towards traditional medicine [194]. This fact is evident in last ten years by the tremendous growth in herbal supplement market to a billion dollar industry [195]. According to the Food and Drug Administration reports, some 29,000 different supplement products are available for sale in the United States [196]. These herbal medicines are now available as the synthetic medicine like tablets, capsules, and beverages [197].

Cancer is a life threatening disease, which may leads to death in many cases. It has been reported that about 13.7 million Americans with cancer are alive on January 1st 2012 and expecting to diagnose about 2 million new cases in a year [52]. Recently in both health promotion and adjuvant therapy, Chinese medicinal herbs are gaining attention in the west. It is common in Asian countries like India, China, and Japan to use medicinal herbs for chronic conditions and western synthetic medicine for acute and serious conditions [198]. Recently Wang et al. summarized the active ingredients isolated from Chinese medicinal herbs in fighting cancer, which are found to inhibit cancer proliferation by inducing apoptosis or by suppressing angiogenesis. These herbs are also found to retard metastasis, enhance chemotherapy, and showed anticancer effects in both in vitro and in vivo research [199]. Hence some of the famous Chinese medicinal herbs reported in the literature for fighting cancer are summarized in Table 2.7.

Table 2.7. Some of the Famous Chinese Medicinal Herbs Used in Fighting Cancer

Herb	Chinese Name	Active Ingredient	Analytical Technique	Cancer Literature	Clinical Trial	Reference
Citrus aurantium	Zhi Ke	Limonene	GCMS	Breast, colon, lung, prostrate	Phase l/ll conducted	53-58
Artemisia annua	Qing Hao	Artemisinin	GCMS	Breast, colon, leukemia	For malaria conducted	59-62
Paeonia suffruticosa	Mu Dan Pi	Paeonol	GCMS	Liver, colon kidney	Not conducted	63-66
Magnolia officinalis	Hou Po	Magnolol	HPLC	Lung, colon liver	For asthma conducted	67-70
Salvia miltiorrhiza	Dan Shen	Tanshinone IIA	LCMS	Breast, brain luekemia	For ischemic diseases conducted	76-78, 200-201

2.6.2 Techniques for Characterization of Medicinal Herbs

Selection of proper extraction technique is necessary for the analysis of various active constituents in medicinal herbs. Normally the choice of extraction technique is based on the type of analytes. There are many age-old extraction practices stated in the US pharmacopeia for medicinal herbs extractions including Soxhlet extraction, sonication, and heating under reflux [40]. Presently these extraction techniques are not in practice due to the consumption of excess organic solvents in the characterization of analytes. In the past decade, extraction techniques like microwave assisted extraction, supercritical fluid extraction (SFE), accelerated solvent extraction, pressurized liquid extraction, and subcritical water extraction [23, 24, 40, 202, 203] are developed to decrease the organic content in the extraction methods. Except in SBWE and SFE, organic solvents are still necessary in the remaining extraction methods. As discussed before, SFE needs additives like methanol to extract the polar analytes [203]. In case of subcritical water extraction, temperature will act as a tool to modify the polarity of water for the extraction of polar analytes at lower temperatures and non polar analytes at higher temperatures

becoming a green extraction technique and ideal for herbal medicine preparation [23, 24]. The experimental conditions for all the above mentioned extraction techniques are summarized in Table 2.8.

Table 2.8. Summary of Experimental Conditions for Various Extraction Techniques

Extraction	Common Solvent &	Extraction Temperature	Extraction Time	Solvent Consumption	Cost	Reference
Soxhlet	Methanol, ethanol, or alcohol-water mix	Depend on solvent	6-48h	200-600 mL	Cheap	24, 202, 203
Sonication	Methanol, ethanol, or alcohol-water mix	NA	1-12h	50-100 mL	Cheap	24, 203
Microwave assisted	Methanol, ethanol, or alcohol-water mix	80-150 ^{o}C	10-40 min	20-50 mL	Less expensive	24, 202-206
Supercritical fluid	Carbondioxide (CO_2) or CO_2 with modifier	40-100 ^{o}C	30-100 min	>10 mL	Expensive	24, 202-203, 207-208
Accelerated solvent	Methanol	80-200 ^{o}C	20-40 min	20- 40 mL	Expensive	24, 202-203
Pressurized liquid	Methanol,	80-200 ^{o}C	20-40 min	20- 30 mL	Expensive	23, 209-210
Subcritical water	Water	80-300 ^{o}C	40-60 min	40- 45 mL	Cheap	23, 211

2.6.3 Herbal Medication Preparation Techniques

Guidelines for various processes necessary in the quality control of medicinal herbs can be obtained from the United States Food and Drug Administration [40]. According to these guidelines, development of a proper efficient extraction technique is necessary to improve the efficacy of the medicinal herb preparation. The first step in the sample preparation is to obtain a

homogeneous herbal sample to improve the kinetics of extraction. Normally, the homogeneous herbal sample can be obtained by the basic operations such as pre-washing and then normal or freeze drying followed by grounding.

The oldest herbal medication preparation technique is traditional Chinese herbal decoction method. THD is not a good practice for medicinal herb preparation, because it is open to the environment and operates only at boiling temperature. Hence there is a loss of volatile active constituents and the temperature limitation on herbal extraction may not be sufficient for the extraction of different kinds of herbal analytes. Various organic solvent consuming extraction procedures have been reported in the literature for the preparation of herbal medications [71]. Vinatoru divided those extraction methods in to four types including distillation, solvent extractions, cold compression, and Non-conventional extractions [212].

Steam is used as the extraction fluid in distillation. Solvent extractions using different solvents are again divided in to four types including, percolation (organic solvents), maceration (organic solvents), infusion (steam), and enfleurage (hot fat). Compression energy is used as the extraction fluid in cold compression. Non-conventional extractions are again divided in to three types including supercritical fluid extraction (supercritical CO_2), vertical (turbo) extraction (electrical energy), and ultrasonic extraction (ultrasonic extraction). Thus these herbal preparation techniques still require steam or organic solvents for the herbal medication preparation [212]. Previously in some of the herbal medicine preparations, carcinogenic like benzene has been used [71]. As discussed in Chapter 1, the use of organic solvents in the herbal medication preparations may cause additional side effects to the patients. Unlike the synthetic medicines, these herbal medicines do not have strict FDA guidelines. So it is critically needed

to replace the organic solvent consuming herbal medication preparation techniques with green extraction techniques such as subcritical water extraction for the safety of patients.

Subcritical water extractions have been already widely used in extraction of many types of sample matrices. Before going to discuss in detail about the subcritical water extractions of medicinal herbs, it is worthwhile to know about SBWE of other sample matrices. Subcritical water extractions have been used in the extraction of various compounds from natural foods such as seeds, fruits, and leaves [41, 42]. For example, SBWE in the extraction of catechins and proanthocyanidins from dried grape seeds at higher temperatures showed comparable results with the organic solvent extraction using methanol [213]. SBWE has also been used to extract organic contaminants from foods [214, 215], such as carcinogenics like sulfonamides extraction from meat samples using SBWE [214]. It has also been applied to the extraction of various analytes from environmental samples, such as subcritical water extraction of polycyclic aromatic hydrocarbons [32], nitrogen-based pollutants [216], dioxins [217], brominated compounds [218], liquid organics [219], and surfactants [219] from the environmental solids and liquids.

2.6.4 Instrumentation

Subcritical water extraction system usually consists of an oven as shown in Figure 2.9 to heat the extraction vessel containing herbal mixture. A pump is used to let water in to the extraction system. A preheating coil is also used to heat the water before entering the extraction vessel to eliminate the thermal mismatch problem. A pressure restrictor is employed to keep the hot water in liquid state at higher temperatures. It is reported that there are many differences in the subcritical water extractions systems used by various researchers [40, 89]. For example, some SBWE systems require additional gas cylinders, pumps, and cooling coils [40, 89].

Commercial instrument like Dionex ASE 200 was also reported to use for the subcritical water extraction of active ingredients like ginsenosides from medicinal plants [220]. Kuosmanen et al. combined the SBWE with GC via microporous membrane liquid-liquid extraction trap for the extraction of polycyclic aromatic compounds from soil [221]. Yang and smith et al. combined SBWE with SBWC via sorbent trap for the extraction of pharmaceuticals [49, 50].

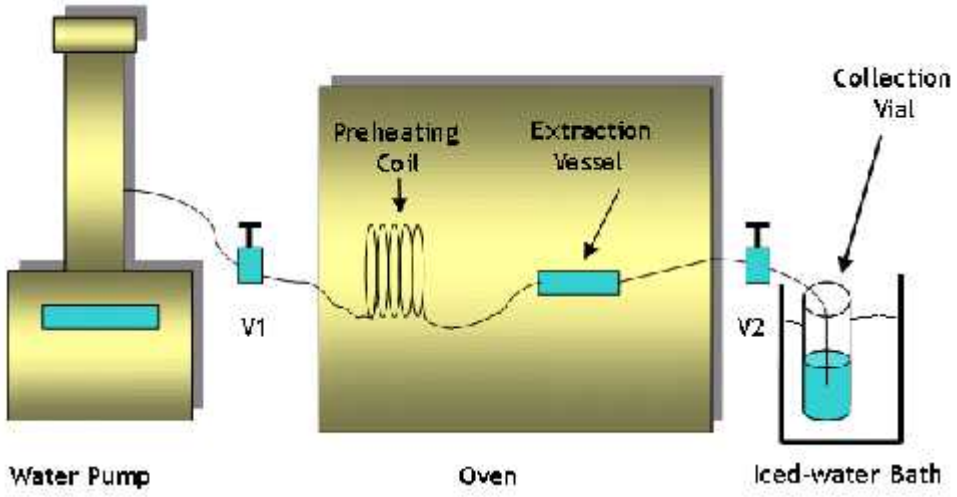


Figure 2.9. Block diagram of the subcritical water extraction system.

2.6.5 Applications of Subcritical Water Extraction on Medicinal Herbs

Subcritical water extraction is a reliable extraction technique when compared with the existing extraction techniques for medicinal herbs [72]. For example, in the extraction of APIs from medicinal herbs such as *Gastrodia elata* and *Stevia rebaudiana*, SBWE is more efficient than heating under reflux [26, 222]. In case of volatile essential oil extraction from *Cuminum cyminum*, SBWE produced similar yields when compared with the Soxhlet extraction and steam distillation [223]. As water is cheaper and economical, it saves large amounts of money in the extraction of medicinal herbs by SBWE. Table 2.9 summarizes the applications of SBWE in the

extraction of APIs and essential oil from medicinal herbs and also their comparisons with the existing organic solvent consuming methods.

Table 2.9. Subcritical Water Extraction of Active Pharmaceutical Ingredients and Essential Oils from Medicinal Herbs (Reproduced With Permission from Reference 72 © Elsevier, 2010.)

Herb	Analyte(s)	Temperature	Compared Method	Benefits	Reference
Stevia rebaudiana	Stevioside, rebaudioside A	100 °C	Reflux	Weight loss, cold & flu remedy	222
Gastrodia elata	Gastrodin, vinylalcohol	100 °C	Reflux	Blood deficiency, migrane relief	26
Salvia miltiorrhiza	Tanshinone I&IIA	95-140 °C	Soxhlet extraction	Anti-cancer, asthma, angina, glaucoma relief	224
Fructus amomi	Essential oil	150 °C	Recovery	Anti-Cancer, blood pressure, diuretic relief	225
Ziziphora taurica	Pulegone, terpinen-4-ol, trans-carveol verbenone	150 °C	Distillation, thermal desorption	Antibacterial, fever, cough migrane remedy	226
Glycyrrhiza glabra	glycyrrhizin	30-120 °C	No compared method	Anti-Cancer, antiperspirant, antitussive	227
Terminalia chebula Retz	Gallic acid, ellagic acid, corilagin	120-200 °C	Soxhlet, ultra sonic extraction	Anti-cancer, cardiovascular, hepatoprotective	43
Vaccaria segetalis Garcke	Saponins, cyclopeptides	160 °C	Ultrasonic extraction	Anti-Cancer, promotes lactation	228
Rosmarinus officinalis	Rosmarinic acid, carnosic acid	60-100 °C	No compared method	Promotes hair growth, mood reduces anxiety	229

Chapter 3: Solubility of Parabens in Subcritical Water

3.1 Introduction

As discussed in Chapter 1 and 2, subcritical water was already used as a green mobile phase in SBWC [1, 4] and a green extraction fluid in SBWE [37]. But these green separation technologies are still restricted to the academic level due to the lack of fundamental data such as solubility of analytes in subcritical water. The solubility data is critically important in the optimization of subcritical water chromatography and subcritical water extraction.

Recently our group successfully carried out subcritical water chromatographic separation of preservatives present in P&G skincare creams at temperatures ranging from 150 to 200 ^{o}C [20]. In order to further optimize the SBWC methods for preservatives, the solubility of methyl, ethyl, and butyl parabens in high temperature water was evaluated at temperatures ranging from 25 to 200 ^{o}C in this work. These solubility experiments were conducted using the home-made system. Then the solubility data obtained from this work was compared with the values predicated by the existing solubility models [29, 84, 85]. A new solubility model was developed in this work to predict the paraben solubility in water at higher temperatures, since the existing solubility models failed to predict them.

3.2 Experimental

3.2.1 Reagents and Supplies

Methyl paraben, ethyl paraben, propyl paraben, and 2-phenoxyethanol were obtained from Sigma Aldrich (St. Louis, MO, USA). Butyl paraben was purchased from SAFC (St. Louis, MO, USA). HPLC-grade methanol was received from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water (18 MΩ-cm) was obtained in our laboratory using a PURELAB Ultra

system from ELGA (Lowell, MA, USA). GD/X PVDF membrane filters (0.45 μm) were acquired from Whatman (Florham Park, NJ, USA). An Adsorbosil C18 column (4.6 x 150 mm, 5 μm) was purchased from Alltech Associates, Inc. (Deerfield, IL, USA). An empty stainless steel tube (15 x 1.00 cm I.D. with 1.27 cm O.D.) and endfittings were received from Chrom Tech, Inc. (Apple Valley, MN, USA). Magnetic stir bar (2.2 x 0.6 cm) was acquired from Bel-Art Products (Pequannock, NJ, USA). A ring magnet (1.9 cm I.D.) was obtained from AMF Magnetics (Botany, NSW, Australia).

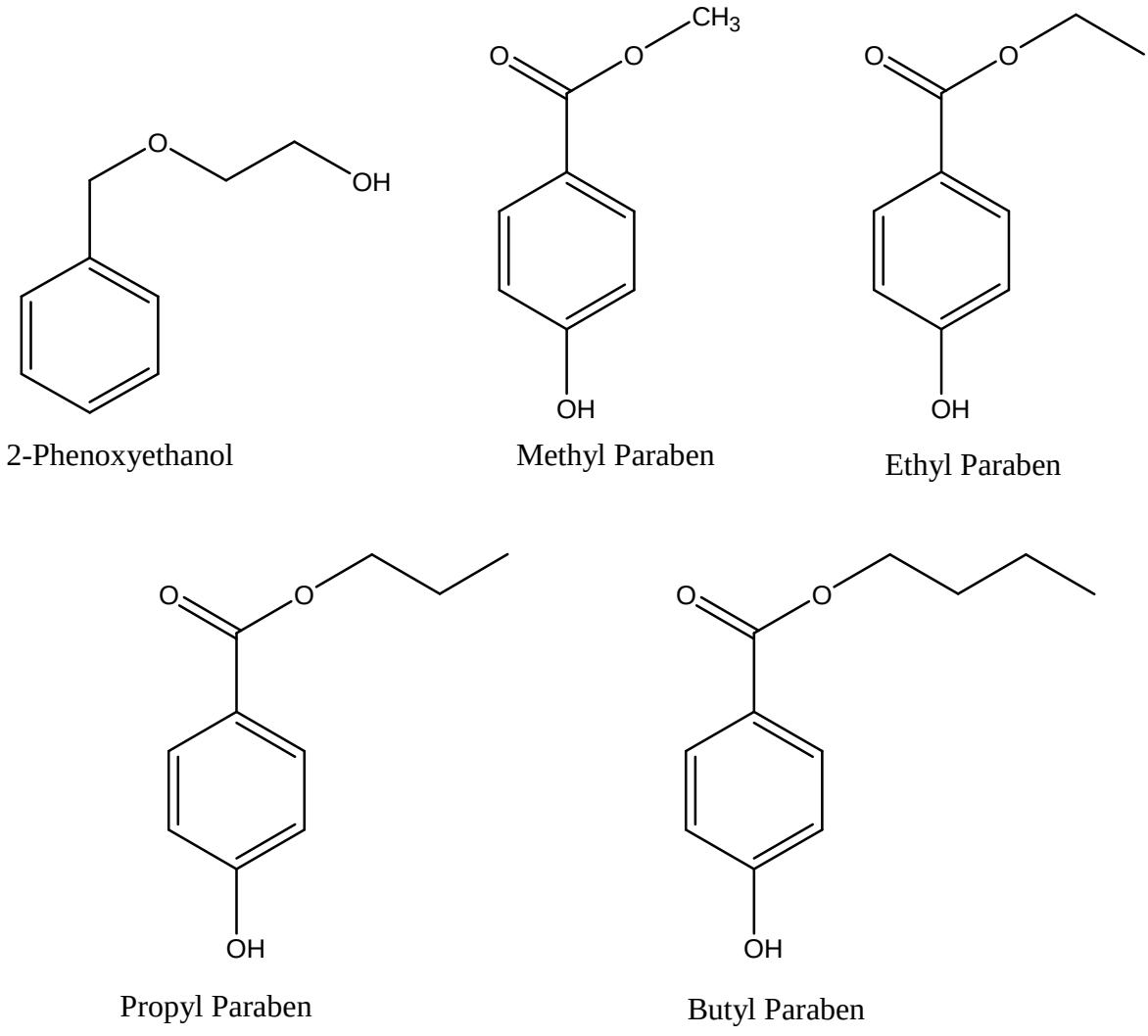


Figure 3.1. Structures of preservatives.

3.2.2 Preparation of Solutions

3.2.2.1 Preparation of Internal Standard Solutions

For paraben solubility studies at 25 to 150 ^{o}C, 2-phenoxyethanol was used as internal standard. This solution was prepared by adding approximately 0.2 g (accurately weighed) of 2-phenoxyethanol to a 100-mL volumetric flask and then diluted to the mark with methanol.

During the evaluation of solubility studies at 200 ^{o}C, parabens were degraded and the respective degradants were coeluted with 2-phenoxyethanol. Therefore propyl paraben was used as internal standard for the solubility studies at 200 ^{o}C. This solution was prepared by adding approximately 0.2 g (accurately weighed) of propyl paraben to a 100-mL volumetric flask and then diluted to the mark with methanol.

3.2.2.2 Preparation of Calibrated Standard Solutions

For each paraben, an individual stock solution was prepared by adding approximately 0.01 g (accurately weighed) of paraben to a 10-mL volumetric flask and then diluted to the mark with methanol. From this stock solution, calibrated standard solutions were prepared by adding an appropriate internal standard solution with methanol as the solvent.

3.2.3 Heating and Mixing of Paraben-Water Mixtures

Solubility of paraben in water was conducted using the solubility measuring system as shown in Figure 3.2. At first both endfittings of a stainless steel equilibration vessel were wrapped with Teflon tape for proper sealing. Then one end of the vessel was sealed with an endfitting. Approximately 1 g of paraben (methyl, ethyl, or butyl paraben) was added to the stainless steel vessel. Then a magnetic stir bar was placed inside the vessel. In order to mix the paraben-water mixtures during heating inside an equilibration vessel, a ring magnet was

attached to the outside of vessel. The other end of the stainless steel vessel was then sealed with another endfitting. The loaded vessel was then placed in a gas chromatograph oven (HP 5890 Series 2, Hewlett Packard, Avondale, PA, USA) as shown in Figure 3.2.

Both V_1 and V_2 are high-pressure valves made from stainless steel. An ISCO model 260 D syringe pump (Lincoln, NE, USA) was used to supply 18 MΩ water by opening V_1 and closing V_2 to fill the loaded vessel. Leak check was performed using the ISCO 260D syringe pump in the constant-pressure mode. After setting the desired temperature on the oven, it was turned on to heat the paraben-water mixture in the equilibration vessel for 60 min. Throughout the heating time, mixing handle attached to the ring magnet was used to mix the contents inside the equilibration vessel. A delay between the actual temperature of the equilibration vessel and oven temperature was determined. The delay was 12 min for 100 ^{o}C, 16 min for 150 ^{o}C, and 20 min for 200 ^{o}C. Therefore the counting of SBWE time started after the delay time was compensated. A pressure of 5 to 35 atm was applied to keep hot water in the liquid state for all experiments. After 60 min of heating, approximately 1.5 mL of saturated sample mixture was collected at 1.0 mL/min into a 10-mL volumetric flask by opening V_2. To this sample, 1.00 mL of internal standard was added and diluted to the mark with methanol. Then the sample was filtered through a Whatman GDX filter into a glass vial for chromatographic analysis. For each paraben, triplicate solubility experiments were conducted at all temperatures tested.

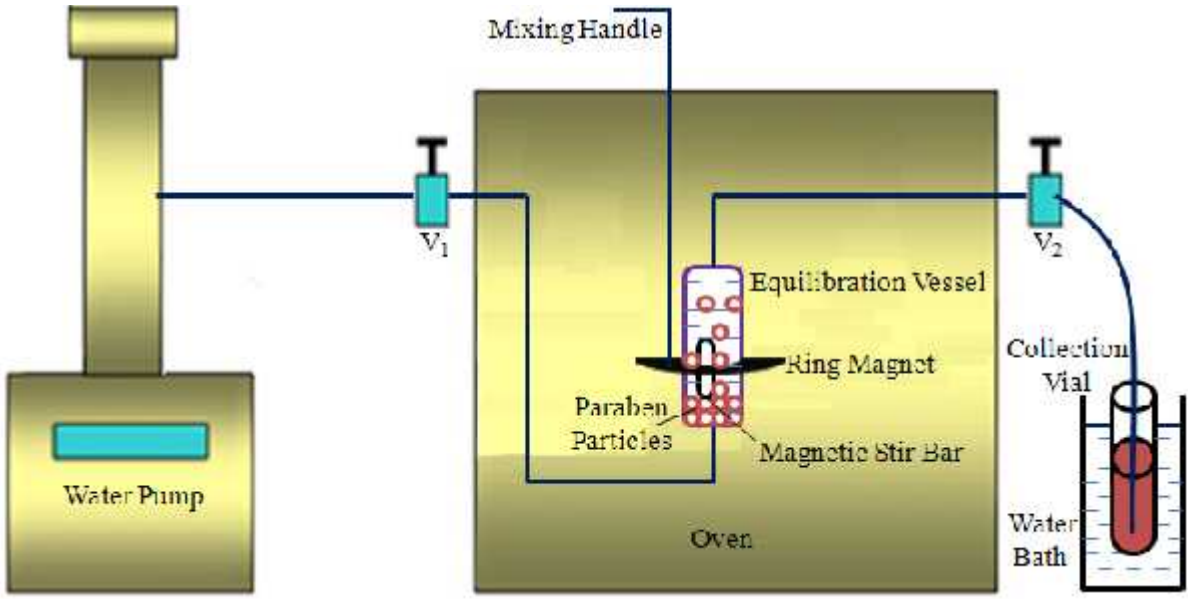


Figure 3.2. Schematic diagram of the heating/mixing system.

3.2.4 HPLC Analysis

Shimadzu Nexera UFLC with a UV-vis dual wavelength detection system (Shimadzu Corporation, Chiyoda-ku Tokyo, Japan) was utilized for the quantitative analysis of parabens in sample solutions. Paraben separations were carried out on the Adsorbosil C18 column using methanol-water gradient mixtures as the mobile phase at 1.0 mL/min. Analytes were detected at 256 nm wavelength. For each paraben (methyl, ethyl, or butyl paraben) a different gradient elution was developed for the paraben-water mixtures collected at 25 to 150 ^{o}C. Due to the severe degradation of parabens at 200 ^{o}C, a common gradient elution was developed for all the paraben sample solutions collected at 200 ^{o}C. These HPLC gradient conditions are summarized in Table 3.1.

Table 3.1. HPLC Gradient Elution Conditions for Separation of Parabens

Samples at 25, 100, 150 °C						Samples at 200 °C	
Methyl Paraben		Ethyl Paraben		Butyl Paraben		Methyl, Ethyl, Butyl Paraben	
Time	%Methanol	Time	%Methanol	Time	%Methanol	Time	%Methanol
0	40	0	60	0	70	0	50
9	50	5	67	7	80	8	50
10	50	6	60	8	70	9	80
11	40	10	60	10	70	13	80
12	40	-	-	-	-	14	50
-	-	-	-	-	-	16	50

3.3 Results and Discussion

3.3.1 Temperature Effect on the Solubility of Parabens

The solubility determination of all three parabens, methyl, ethyl, and butyl parabens in subcritical water was carried out at four different temperatures, 25 °C, 100 °C, 150 °C, and 200 °C. In order to demonstrate the reliability of results, the solubility values at 25 °C are compared with the reference values at same temperature as shown in Table 3.2 [230-233]. The experimental vales compare well with the reference values achieved by the other researchers. Table 3.3 shows the mole fraction solubility of all three parabens in subcritical water at all temperatures tested. This data infers the increase in solubility of parabens from 25 to 150 °C. Solubility of parabens increased by about 6 to 19 folds with the increase of water temperature from 25 to 100 °C and another 2 folds increase with the raise of water temperature from 100 to 150 °C. But with the further increase of water temperature from 150 to 200 °C caused decrease in solubility of parabens due to their severe degradation at 200 °C. It should be pointed out that the solubility value obtained at 200 °C does not represent the "real" solubility since the portion of solutes degraded is excluded in our solubility measurements. The evaluation of paraben degradation including the identification as well as quantification of paraben degradants at 200 °C will be discussed in Chapter 4. Figure 3.3, shows the increase in solubility of methyl paraben

in water by increasing water temperature from 25 to 150 ^{o}C and paraben degradation at 200 ^{o}C. As discussed in Chapters 1 and 2, degradation of analytes in water at higher temperatures is not unusual. Our group previously observed the degradation of phananthrene as well as benzoic acid and its derivatives in subcritical water [120, 124].

Table 3.2. Comparison of Paraben Solubility[a] at 25 ^{o}C Obtained by This Method and Reference Values (Reproduced with Permission from Reference 234 © American Chemical Society, 2014.)

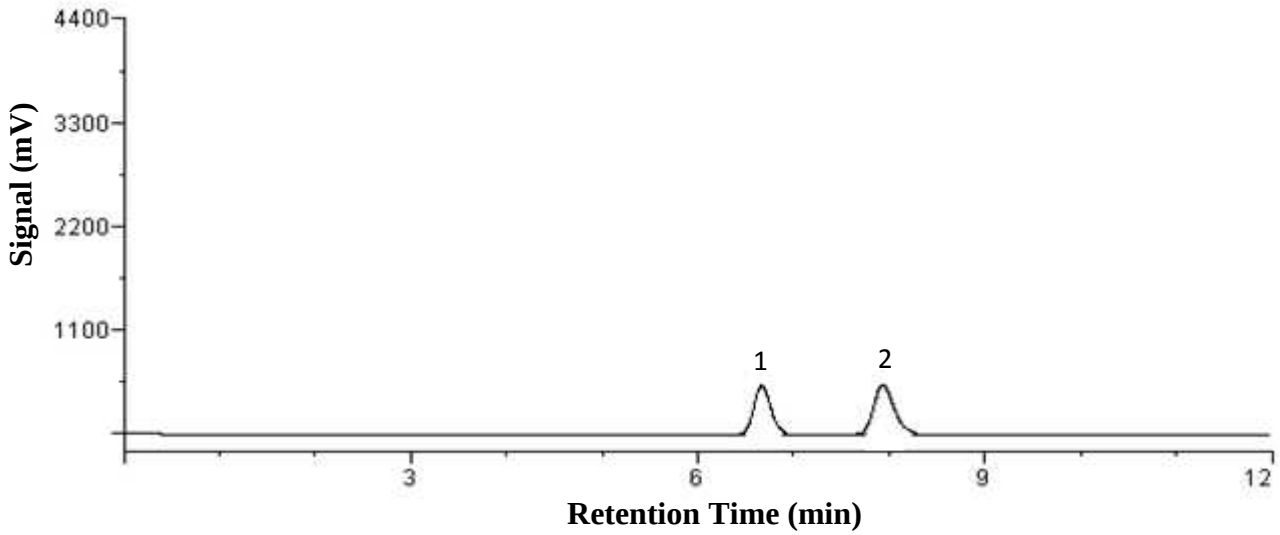
	Solubility, Mole Fraction x 10^3				
	This Work	Ref. 16	Ref. 17	Ref. 18	Ref. 19
Methyl Paraben	0.25	0.25	0.29	0.30	0.26
Ethyl Paraben	0.074	0.13	0.10	0.18	0.096
Butyl Paraben	0.018	0.015	0.019	0.019	0.023

[a]*All parabens are in solid phase.*

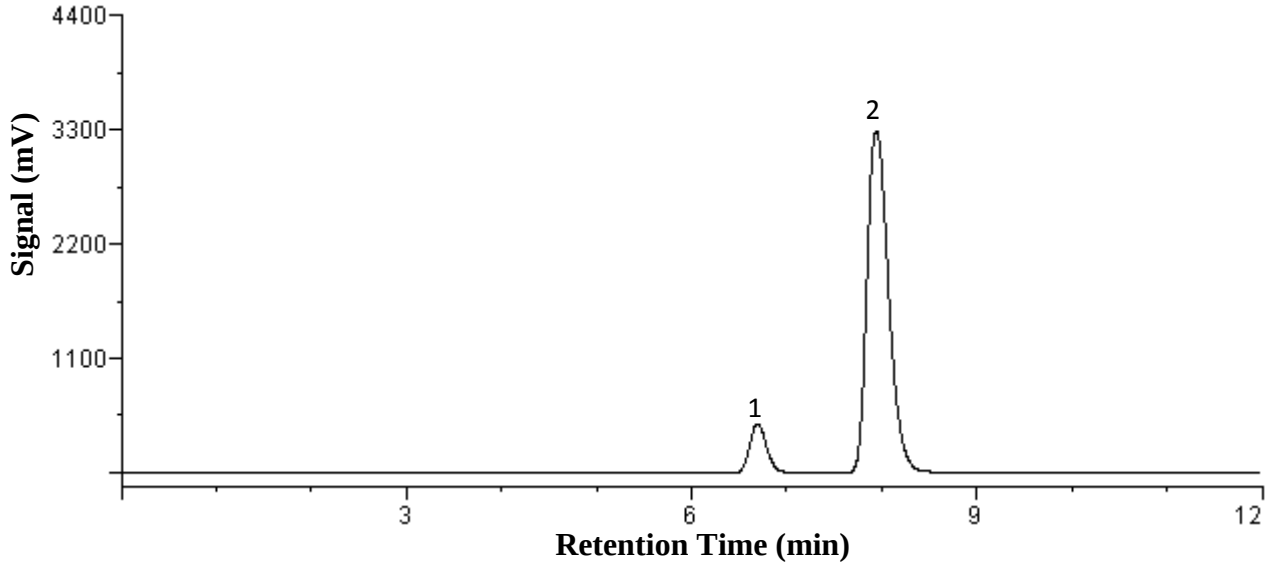
Table 3.3. Solubility of Parabens Found in Water-Paraben Mixtures after Heating at Each Temperature for 60 min

	Mole Fraction x 10^3 ±(SD)[a]			
Temperature (^{o}C)	25	100	150	200[b]
Pressure (atm)	5	15	25	35
Methyl Paraben	0.25 (0.01)[c]	1.4 (0.2)[c]	2.7 (0.4)[d]	1.5 (0.09)[d]
Ethyl Paraben	0.074 (0.007)[c]	0.74 (0.08)[c]	1.5 (0.2)[d]	0.91 (0.07)[d]
Butyl Paraben	0.018 (0.001)[c]	0.34 (0.01)[d]	0.65 (0.08)[d]	0.41 (0.05)[d]

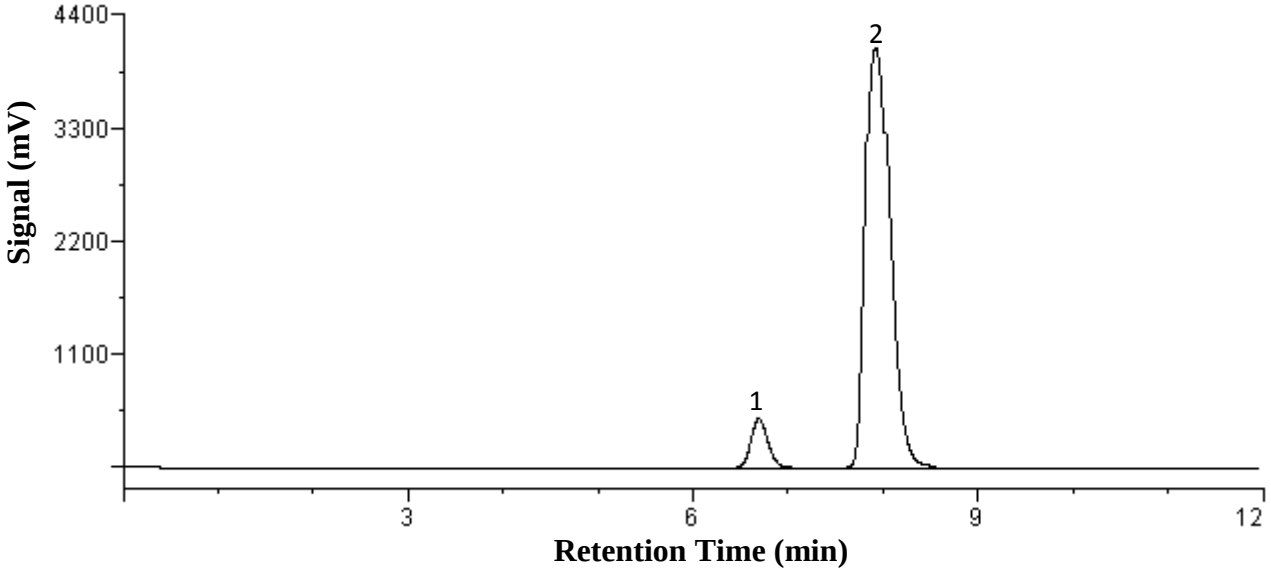
[a]*Based on triplicate measurements.*
[b]*Degradation of all three parabens at 200 ^{o}C.*
[c]*Parabens in the solid phase.*
[d]*Parabens in the liquid phase.*


4400
3300
2200
1100
Signal (mV)
1
2
3
6
9
12
Retention Time (min)

a


4400
3300
2200
1100
Signal (mV)
1
2
3
6
9
12
Retention Time (min)

b



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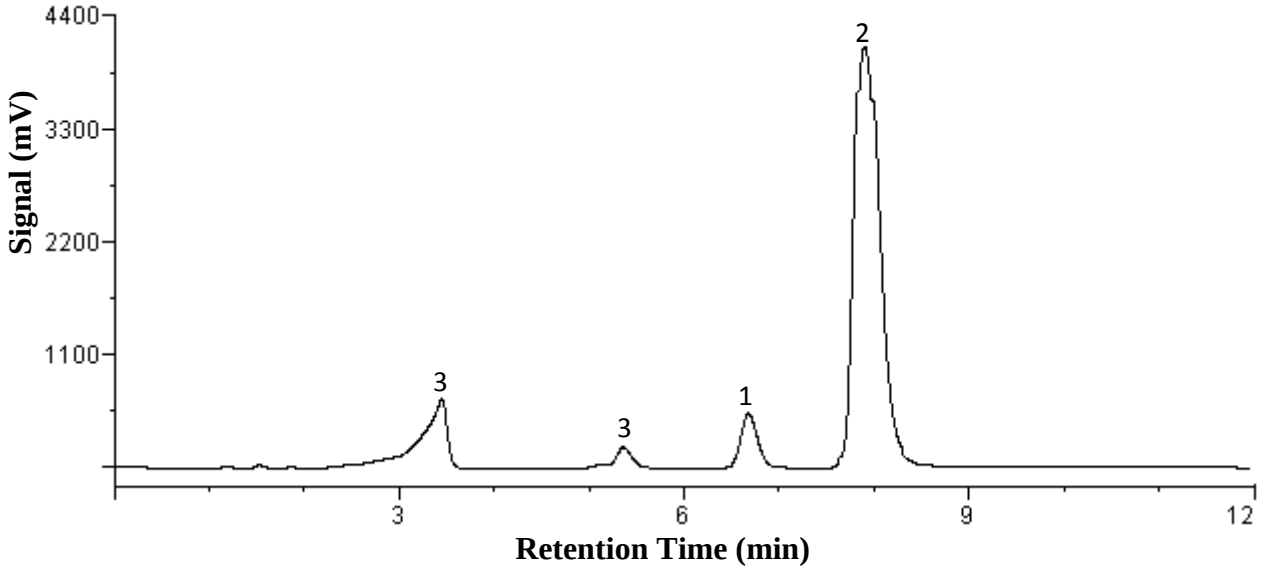


d

Figure 3.3. High performance liquid chromatography chromatograms obtained on the Alltech Adsorbosil C18 column. (a) 60-min heating solubility vessel at at 25 ^{o}C; (b) 100 ^{o}C; (c) 150 ^{o}C; (d) 200 ^{o}C. Peak identification: 1, 2-phenoxy ethanol; 2, methyl paraben; 3, degradation product.

3.3.2 Estimating the Solubility of Parabens in Subcritical Water

As parabens were degraded at 200 ^{o}C, the solubility values obtained at that specific temperature are false. Therefore those values were excluded from the development of a solubility model as shown in Table 3.4. We first tried the zeroth approximation model (eq 2.2) developed by Miller et al. [29] to estimate the solubility of parabens in subcritical water. This equation infers that the mole fraction solubility data at higher temperatures $x_2(T)$ can be predicted with the mole fraction solubility data at ambient temperature $x_2(T_o)$. Please note T in absolute temperature. This eq 2.2 only predicts the solubility of methyl paraben and fails to predict the solubility of ethyl and butyl parabens at higher temperatures as shown in Table 3.4.

$$\ln x_2(T) \approx \left(\frac{T_0}{T}\right) \ln x_2(T_0) \tag{2.2}$$

Another Miller approximation model (eq 2.3) [29] as discussed in the Chapter 2 is also used to predict the solubility of parabens in high-temperature water. The paraben solubility values predicted by this model are greater than the experimental solubility values at 150 ^{o}C as displayed in Table 3.4.

$$\ln x_2(T) = \left(\frac{T_0}{T}\right) \ln x_2(T_0) + 15\left(\frac{T}{T_0} - 1\right)^3 \tag{2.3}$$

Then we used an approximation model (eq 2.4), developed in our lab for the solubility of alkylbenzenes in subcritical water [84]. This model was also failed to predict the hot water solubility of parabens as shown in Table 3.4.

$$\ln x_2(T) = \left(\frac{T_0}{T}\right) \ln x_2(T_0) + 2\left(\frac{T - T_0}{T_0} - 1\right)^3 \tag{2.4}$$

Then we tried our recently developed approximation model (eq 2.5), for organic acids in high-temperature water [85]. Again this model fails to predict the paraben solubility values and

it also produced very high values when compared with the previous three models as shown in Table 3.4.

$$\ln x_2(T) = \left(1.85\frac{T_0}{T} - 1\right)\ln x_2(T_0) \tag{2.5}$$

As all of the above mentioned solubility models are failed to predict the solubility of parabens in subcritical water, therefore the development of new solubility model is necessary. For that temperature influence on paraben solubility was plotted as shown in Figure 3.4. It is clear from the figure, with the increase in carbon atom number in paraben alkyl group there is a decrease in linear correlation. Therefore a new approximation model is developed in this work including the carbon atom number to reduce the deviations caused by the molecular structure of solute as shown in eq 3.1. Please note C in this equation is the number of carbon atoms in the alkyl group of paraben. As shown in Table 3.4, this newly developed model was able to reasonably predict the paraben solubility values at all temperatures than the other previous developed solubility models.

$$\ln x_2(T) = \left(\frac{T_0}{T}\right)\ln x_2(T_0) + 0.5\,(C-1)\left(\frac{T}{T_0} - 1\right) \tag{3.1}$$

Table 3.4. Comparison of Experimental Solubility of Parabens with Values Predicted by Equations 1-5 (Reproduced with Permission from Reference 234 © American Chemical Society, 2014.)

		Mole Fraction x 10^3					
	Temperature (°C)	Experimental	Eq 3.1	Eq 2.5	Eq 2.4	Eq 2.3	Eq 2.2
Methyl Paraben	25	0.25	0.25	0.87	0.034	0.25	0.25
	100	1.4	1.3	19	0.57	1.7	1.3
	150	2.7	2.9	81	2.0	8.8	2.9
Ethyl Paraben	25	0.074	0.074	0.31	0.010	0.074	0.074
	100	0.74	0.57	11	0.22	0.64	0.50
	150	1.5	1.5	56	0.83	3.7	1.2
Butyl Paraben	25	0.018	0.018	0.093	0.0024	0.018	0.018
	100	0.34	0.24	5.4	0.070	0.21	0.16
	150	0.65	0.85	36	0.31	1.4	0.45

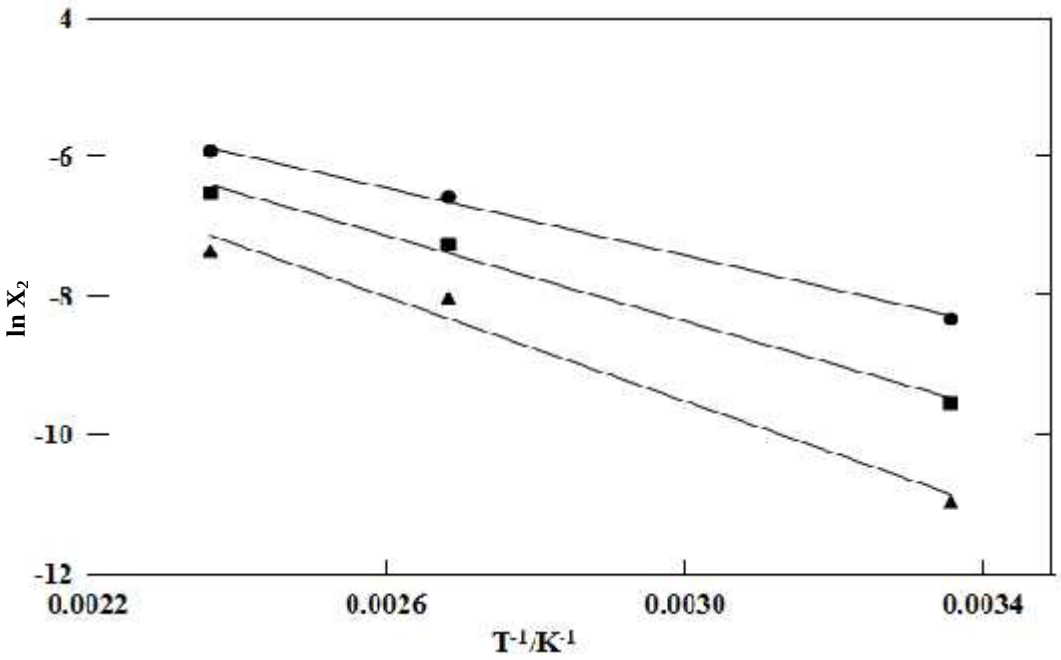


Figure 3.4. Temperature influence on paraben solubility in subcritical water: ●, methyl paraben; ■, ethyl paraben; ▲, butyl paraben. (Reproduced with permission from reference 234 © American Chemical Society, 2014.)

Chapter 4: Stability of Preservatives under Subcritical Water Conditions

4.1 Introduction

As discussed in Chapters 1 and 2, subcritical water chromatography and subcritical water extraction are gaining attention due to their economical, environmental, and human health benefits [1, 2, 4, 40]. The temperatures employed in SBWC separations depend on the polarity of analytes. In reversed-phase chromatographic separations, less polar solute elution requires less polar mobile phase. As discussed earlier, polarity of water can be decreased with the increase in temperature. Therefore higher temperatures are necessary for the elution of both moderately polar and nonpolar analytes using SBWC. The use of such higher temperatures in SBWC and SBWE may lead to the stability concerns of analytes. Therefore the stability study of analytes under SBWC conditions is critically needed.

Pharmaceuticals like thiazides [121], aspirin [122], and thiamine [123] showed degradation under SBWC conditions. Our group also observed the degradation of benzoic acid and its derivatives [124], phenanthrene [120], and terpene [89] in high-temperature water. Carr et al. reported that some of the pharmaceuticals are stable up to 190 ^{o}C under high temperature liquid chromatography conditions [109].

Our group recently carried out the subcritical water chromatographic separation of preservatives using high-temperature water as the only mobile phase [20]. In order to determine the reliability of these results, stability study of preservatives were carried out using two different approaches. In the first approach, stability of preservatives in subcritical water was tested by comparing the peak areas obtained by SBWC separations at elevated temperature with the peak areas achieved through HPLC separations at ambient temperature. We think that this is the best way to evaluate the stability of preservatives under subcritical water chromatography

conditions. In the second approach, stability of preservatives was tested under much tougher conditions by heating the water-preservatives mixtures at higher temperatures in a static steel vessel for 30 and 60 min. These two approaches were conducted at temperatures ranging from 100 to 200 ^{o}C. Degradation products of parabens were evaluated by heating water-single paraben mixture at 200 ^{o}C for 30 min, followed by the sample evaluation using GC/MS and HPLC analysis. The pathway of preservative degradation in high-temperature water was discussed.

4.2 Experimental

4.2.1 Reagents and Materials

Benzyl alcohol, methyl paraben, ethyl paraben, propyl paraben, and 2-phenoxyethanol were received from Aldrich (St. Louis, MO, USA). Butyl paraben was purchased from SAFC (St. Louis, MO, USA). GD/X PVDF membrane filters (0.45 μm) was obtained from Whatman (Florham Park, NJ, USA). HPLC-grade methanol was acquired from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water (18 MΩ-cm) was produced in our laboratory using a Sybron/Barnstead system (Sybron/Barnstead, Boston, MA, USA). ZirChrom-DiamondBond-C18 (4.6 x 100 mm, 3 μm) column was received from ZirChrom Separations, Inc. (Anoka, MN, USA). XBridge C18 (4.6 x 100 mm, 3.5 μm) and XBridge phenyl (4.6 x 100 mm, 3.5 μm) columns were purchased from Waters Corporation (Milford, MA, USA). Adsorbosil C18 (4.6 x 150 mm, 5 μm) was obtained from Alltech Associates, Inc. (Deerfield, IL, USA). Stainless steel vessels (7.07-mL, 9 cm x 1 cm ID) were received from Raleigh Valve and Fitting Company (Raleigh, NC, USA).

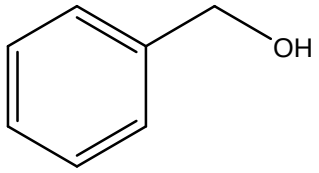


Figure 4.1. Structure of benzyl alcohol.

4.2.2 Preparation of Solutions

4.2.2.1 Preparation of Internal Standard Solutions

For the chromatographic evaluation of preservatives stability (first approach), butyl paraben was used as internal standard. This solution was prepared by adding 0.025 g (accurately weighed) of butyl paraben to a 50-mL volumetric flask and then diluted to the mark with methanol.

For the degradation studies of water-preservatives mixtures (second approach), 2-phenoxyethanol was used as internal standard. However for the degradation studies of water-single paraben mixtures at 200 ^{o}C, propyl paraben was used as internal standard due to the coelution of paraben degradants with 2-phenoxyethanol as mentioned in Chapter 3. 2-phenoxyethanol solution was prepared by adding 0.25 g (accurately weighed) of 2-phenoxyethanol to a 50-mL volumetric flask and then diluted to the mark with methanol. Propyl paraben solution was prepared by adding 0.15 g (accurately weighed) of propyl paraben to a 100-mL volumetric flask and then diluted to the mark with methanol.

4.2.2.2 Preparation of Calibrated Standard Solutions

For the chromatographic evaluation of preservatives stability, a stock standard solution was prepared by adding 0.075 g (accurately weighed) of benzyl alcohol and 0.025 g (accurately weighed) each of methyl, ethyl, and propyl paraben to a 50-mL volumetric flask and then

diluted to the mark with methanol. Then a calibrated standard solution was prepared by transferring 2.00 mL of each stock standard solution and butyl paraben internal standard solution to a 25-mL volumetric flask and then diluted to the mark with methanol.

For the degradation studies of water-preservatives mixtures, a stock standard solution was prepared by adding 0.015 g (accurately weighed) of benzyl alcohol and 0.01 g (accurately weighed) each of methyl, ethyl, propyl, and butyl paraben to a 10-mL volumetric flask. Then 5.00 mL of water was added to the volumetric flask and then diluted to the mark with methanol. From this stock solution, calibrated standard solutions were prepared by adding an appropriate internal standard solution with methanol as the solvent.

For the degradation studies of water-single paraben mixtures, a stock standard solution was prepared by adding 0.015 g (accurately weighed) of phenol and 0.01g (accurately weighed) each of *p*-hydroxybenzoic acid, benzoic acid, and paraben (methyl or ethyl or butyl paraben) to a 10-mL volumetric flask. Then 5.00 mL of water was added to the volumetric flask and then diluted to the mark with methanol. From this stock solution, calibrated standard solutions were prepared by adding an appropriate internal standard solution with methanol as the solvent.

4.2.3 Heating of Water-Preservatives or Water-Single Paraben Mixtures

Stainless steel vessel as shown in Figure 4.2 was cleaned with acetone before each use. At first, both ends having threaded portion of a vessel was wrapped with Teflon tape for proper sealing. Then one end of the vessel was sealed with an end cap. For degradation studies of water-preservative mixtures, to each vessel 0.015 g (accurately weighed) of benzyl alcohol and 0.01 g (accurately weighed) each of methyl, ethyl, propyl, and butyl paraben were added. Similarly for the degradation studies of water-single paraben mixtures, to each vessel 0.01 g (accurately weighed) of paraben (methyl or ethyl or butyl paraben) was added. Then 5.00 mL of

water was added to each vessel leaving a small amount of void space in the vessel for thermal expansion of mixture. The other end of the stainless steel vessel was then sealed with another end cap. Four replicate stability experiments were conducted at all temperatures.

The loaded vessels were then heated inside a Fisher Scientific Isotemp Oven (Pittsburg, PA, USA). Water-preservatives mixtures were heated at temperatures of 100 ^{o}C, 150 ^{o}C, and 200 ^{o}C for either 30 or 60 min. Similarly, water-single paraben mixtures were only heated at 200 ^{o}C for 30 min. After heating, these vessels were taken out from oven and allowed to cool for some time. Then the solution inside each vessel was transferred to a 10-mL volumetric flask. To each 10-mL flask, 1.00 mL of appropriate internal standard was added and then diluted to the mark with methanol. Then these sample solutions were filtered through the 0.45 μm Whatman GDX filter into a clean glass vial prior to the chromatographic analysis.

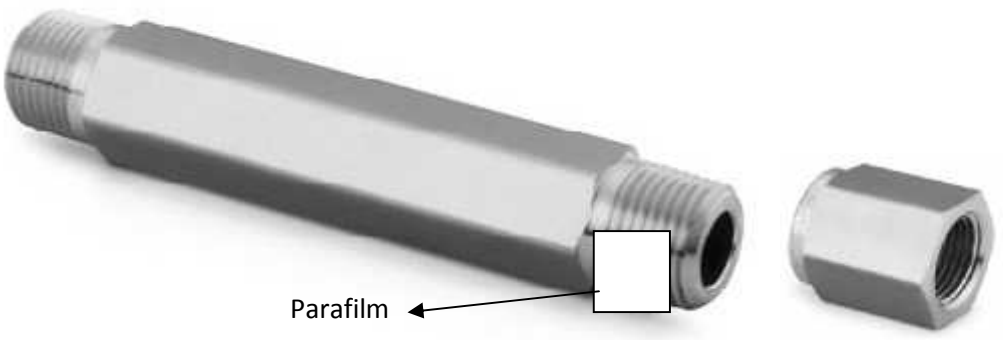


Figure 4.2. Stainless steel vessel with an end cap [235].

4.2.4 HPLC Analysis

Shimadzu Nexera UFLC with a UV-vis dual wavelength detection system (Shimadzu Corporation, Chiyoda-ku Tokyo, Japan) operated at wavelength of 256nm was employed for the evaluation of preservative stability in subcritical water. The separation of heated water-preservatives mixtures and water-single paraben mixtures were carried out on the Alltech Adsorbosil C18 column using HPLC at ambient temperature. The chromatographic evaluations

for the stability of preservatives under SBWC conditions were conducted on the Waters XBridge C18 and XBridge phenyl columns at 150 ^{o}C temperature. This system has a built in pre-column heating unit, column oven and a post-column cooling unit. The column oven can operate up to 160 ^{o}C temperature.

A home-made SBWC system as shown in Figure 4.3 was used for the chromatographic evaluation of preservatives stability on ZirChrom-DB-C18 column under SBWC conditions at 200 ^{o}C. Analytes were detected at 256nm. A Hitachi L-7100 HPLC pump (Hitachi, Ltd., Tokyo, Japan) is used to deliver the mobile phase. A Valco injector (Valco Instruments Company Inc., Houston, TX, USA) with a 10-µL loop is connected to the outlet of a preheating coil (HP 5890 Series 2, Hewlett Packard, Avondale, PA, USA). The hot eluent from column is cooled with an iced-water bath before entering a Hitachi L-7400 UV detector to protect the UV flow cell. In order to keep the mobile phase in liquid state, a back pressure regulator (Restek, Bellefonte, PA, USA) is connected to the outlet of the UV flow cell. A PC/Chrom (H&A Scientific, Greenville, NC, USA) interface is used to connect the UV detector with the computer. The software used for data acquisition and sample analysis is by the PC/Chrom.

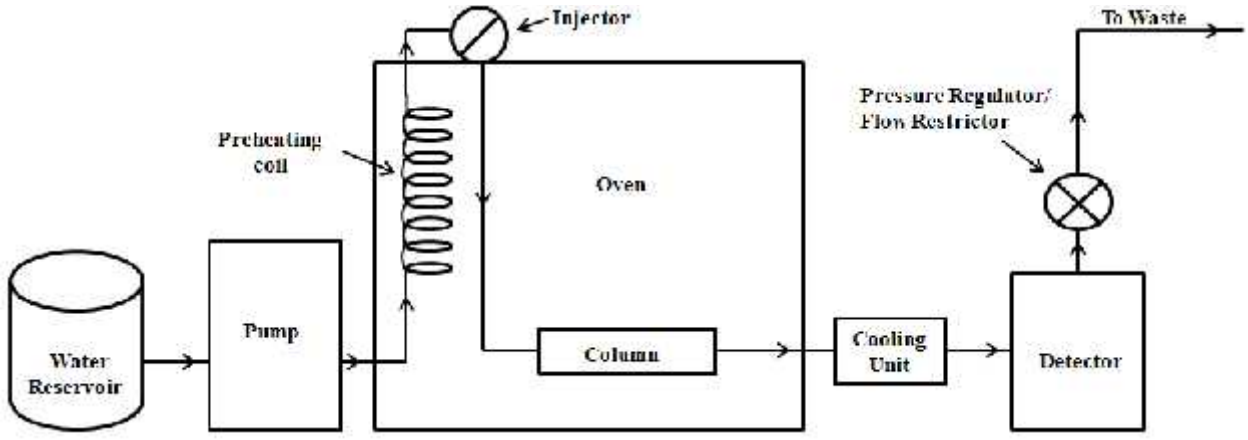


Figure 4.3. Block diagram of home-made subcritical water chromatography system.

4.2.5 GC/MS Analysis

Agilent Technologies 6890N Network GC System (Santa Clara, CA, USA) coupled with a JEOL Ltd. JMS-GCmate II MS System (Tokyo, Japan) is employed for the degradation studies of water-single paraben mixtures heated at 200 ^{o}C. The GC separations were carried out on an Agilent HP-5MS (5%-Phenyl)-methylpolysiloxane (30 m x 0.250 mm, 0.25 μm film thickness) capillary column with 1.0 mL/min flow of a helium carrier gas. The sample volume was 1 μL and injected using split mode by keeping the injector temperature at 250 ºC. The GC/MS interface and the MSD ion chamber were set at 250 ºC. The MS solvent delay time was 3 min. The GC oven temperature programming was as follows: The initial temperature was held at 30 ºC for 3.00 min. Then it was increased at 20 ºC/min to 250 ºC and maintained at 250 ºC for 5.00 min. TSSPro Version 3.0 (Shrader Analytical and Consulting Laboratories, Inc., Detroit, Michigan, USA) was used for data acquisition and analysis.

4.3 Results and Discussion

4.3.1 Stability Studies of Preservatives under SBWC Conditions by Chromatographic Evaluation

Chromatographic evaluation of preservatives stability under subcritical water chromatography conditions was carried out on the Waters XBridge phenyl and XBridge C18 columns at 150 ^{o}C and on the ZirChrom-DB-C18 column at 200 ^{o}C. The stability experiments were performed by comparing the preservatives peak areas achieved by SBWC at higher temperatures (150 ^{o}C & 200 ^{o}C) with those obtained by HPLC at 25 ^{o}C on the same column as shown in Table 4.1. The % differences in preservatives peak areas were calculated using the difference in preservative peaks area of SBWC and HPLC over a HPLC peak area. Based on

these %differences, preservatives are stable at 150 ^{o}C, as there is no significant difference between the peak areas obtained by SBWC at 150 ^{o}C and by HPLC at 25 ^{o}C. But there is a slight degradation of preservatives at 200 ^{o}C especially for the ethyl and propyl paraben, because of their peak areas obtained by SBWC are lower than the HPLC peak areas. As pointed in Chapter 2, slight degradation of analytes under SBWC conditions can be compromised by running both standard and sample solutions under SBWC conditions.

Table 4.1. Comparison of Solute Peak Areas Obtained by SBWC and HPLC at Ambient Temperature on Three Columns Tested

	%Difference in Peak Area (+SD)[a]		
	XBridge Phenyl[b]	XBridge C18[b]	ZirChrom-DB-C18[c]
Temperature	150 °C	150 °C	200 °C
Benzyl Alcohol	- 6 (1)	3 (1)	12 (3)
Methyl Paraben	- 4 (2)	4 (2)	11 (1)
Ethyl Paraben	8 (1)	- 5 (4)	- 9 (2)
Propyl Paraben	6 (1)	- 4 (3)	-11 (2)

[a] *Based on five replicates.*

[b] $\%Difference = \frac{SBWC\ peak\ area\ at\ 150\ ^{o}C - HPLC\ peak\ area}{HPLC\ peak\ area} \times 100$

[c] $\%Difference = \frac{SBWC\ peak\ area\ at\ 200\ ^{o}C - HPLC\ peak\ area}{HPLC\ peak\ area} \times 100$

4.3.2 Degradation Studies of Preservatives in Heated Water-Preservatives Mixtures

Stability of preservatives under much tougher conditions was evaluated by heating water-preservatives mixtures at temperatures ranging from 100 to 200 ^{o}C for 30 and 60 min as shown in Table 4.2. The % recoveries of preservatives in heated-water preservatives mixtures were calculated using the preservative mass recovered after heating over the mass added to the static vessel before heating. Based on these % recoveries, preservatives are stable at 100 ^{o}C and showed a slight degradation at 150 ^{o}C due to the lower percent recoveries especially for butyl

paraben. Similar to the chromatographic evaluation, preservatives in heated water-preservatives mixtures showed about 10 % degradation at 200 ^{o}C.

Table 4.2. Percent Recovery of Preservatives Found in Water-Preservatives Mixtures after Heating at High Temperatures

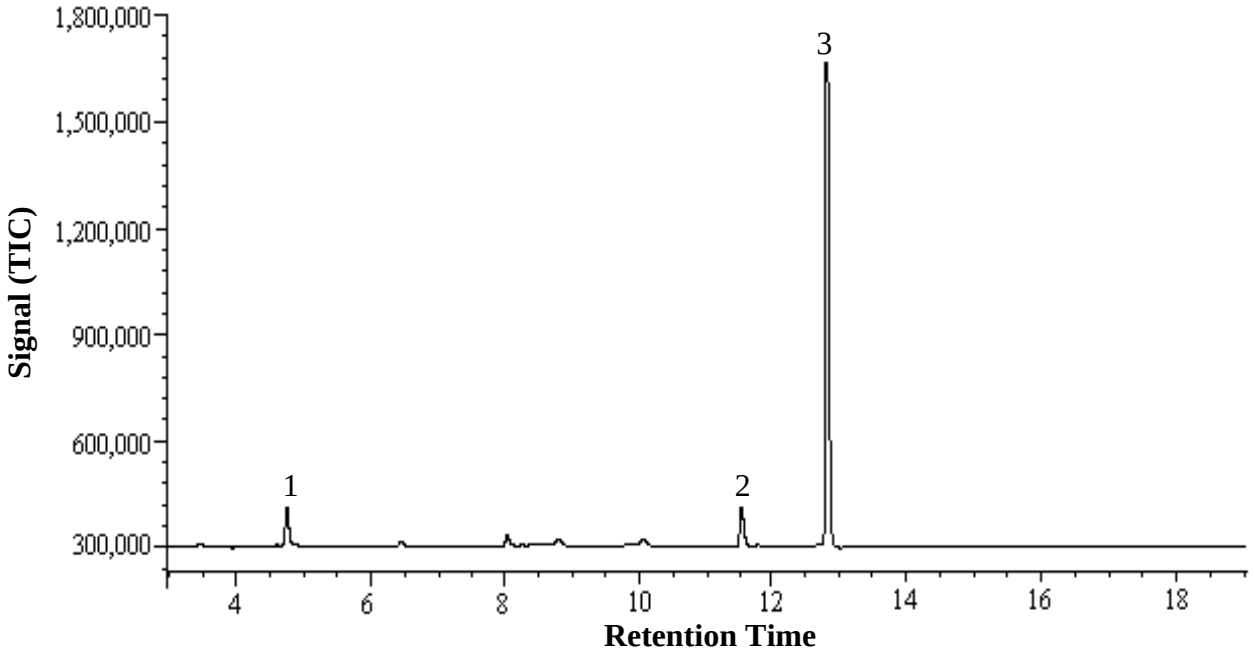
	%Recovery[a] (±SD)[b]					
	30 min			60 min		
	100 ^{o}C	150 ^{o}C	200 ^{o}C	100 ^{o}C	150 ^{o}C	200 ^{o}C
Benzyl Alcohol	99 (4)	101 (1)	91 (2)	102 (2)	101 (1)	95 (1)
Methyl Paraben	103 (2)	105 (1)	89 (2)	103 (1)	100 (1)	85 (1)
Ethyl Paraben	103 (2)	104 (1)	92 (3)	104 (1)	99 (1)	89 (2)
Propyl Paraben	103 (3)	102 (1)	88 (4)	104 (3)	97 (2)	90 (2)
Butyl Paraben	101 (3)	97 (2)	88 (4)	101 (2)	95 (3)	91 (2)

[a] $\%R = \frac{M_r \, a}{M_a \, b} \frac{he}{he} \times 100.$

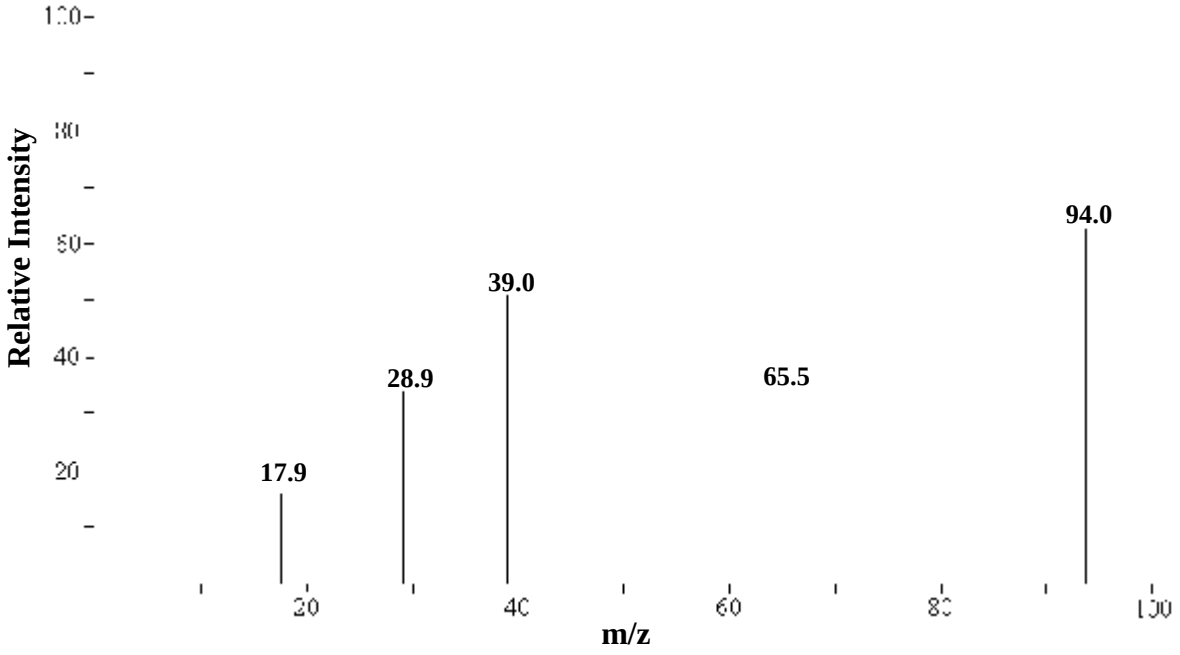
[b] *Based on four replicates.*

4.3.3 Evaluation of Paraben Degradation Products at 200 ^{o}C

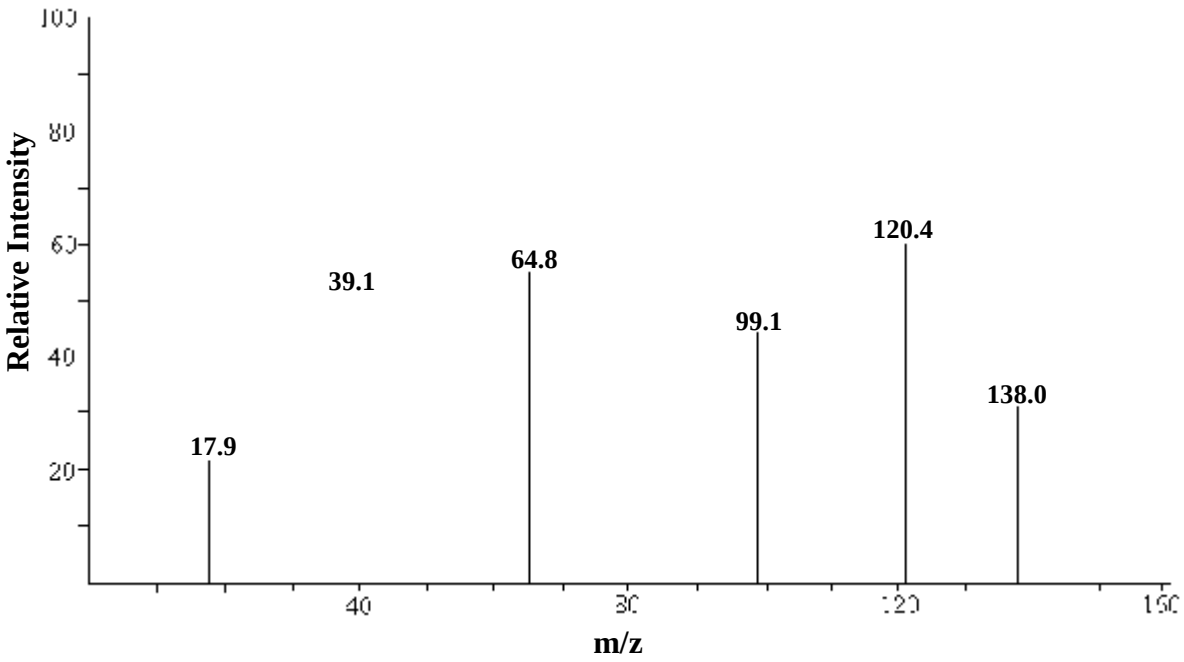
Degradation products of parabens were investigated by heating the water-single paraben mixtures at 200 ^{o}C for 30 min followed by the GC/MS analysis. Two degradation peaks were evident from Figure 4.4a obtained by the GC/MS separation of heated water-butyl paraben mixture. The two degradation peaks were confirmed by the MS spectra as phenol (4.3b) and *p*-hydroxybenzoic acid (4.3c). Then these two degradants were estimated by the HPLC separation on Alltech Adsorbosil C18 column at ambient temperature as shown in Table 4.3. These micromolar recoveries of degradants, infer that the formation two degradants (phenol and *p*-hydroxybenzoic acid) from methyl paraben was greater than the other parabens.


1,800,000
1,500,000
1,200,000
900,000
600,000
300,000
Signal (TIC)
1
2
3
4
6
8
10
12
14
16
18
Retention Time

a


Relative Intensity
94.0
39.0
28.9
65.5
17.9
20
40
60
80
100
m/z

b



c

Figure 4.4. GC/MS chromatogram and mass spectra of a water-butyl paraben mixture obtained after heating at 200 ºC for 30 min. (a) Total ion chromatogram; (b) Mass spectrum of the phenol peak; (c) Mass spectrum of the *p*-hydroxybenzoic acid peak. Peak identification: 1, phenol; 2, *p*-hydroxybenzoic acid; 3, butyl paraben.

Table 4.3. Parabens Degraded and Their Degradants Produced after Heating Paraben-Water Mixtures at 200 ^{o}C for 30 min

	µMoles (+SD)[a]		
	Paraben Degraded	Phenol Produced	*p*-Hydroxybenzoic acid Produced
Methyl Paraben	9 (1)	1.3 (0.1)	3.1 (0.4)
Ethyl Paraben	11 (2)	0.4 (0.1)	1.4 (0.1)
Butyl Paraben	19 (3)	0.4 (0.1)	1.0 (0.2)

[a] *Based on four replicates.*

4.3.4 Paraben Degradation in Subcritical Water

Based on the literature information, a paraben degradation pathway is developed for the prediction of paraben degradation products in the heated water-single paraben mixtures at 200 ^{o}C [236, 237]. According to our degradation pathway, shown in Figure 4.5, parabens in high-

temperature water first undergoes hydrolysis to form *p*-hydroxybenzoic acid and an alcohol. This *p*-hydroxybenzoic acid formed again undergoes decarboxylation on further heating to form phenol and carbon dioxide. Similar mechanism for the degradation of parabens using *Enterobacter cloacae* was developed by the Lepine et al. [238]. The feasibility of the developed degradation mechanism is evident from the separation of heated water-butyl paraben mixture on Alltech Adsorbosil C18 column as shown in Figure 4.6. One can clearly see the degradation of paraben to *p*-hydroxybenzoic acid and phenol at 200 ^{o}C and with further increase in heating time, decarboxylation step dominates leading to the raise of phenol formation.

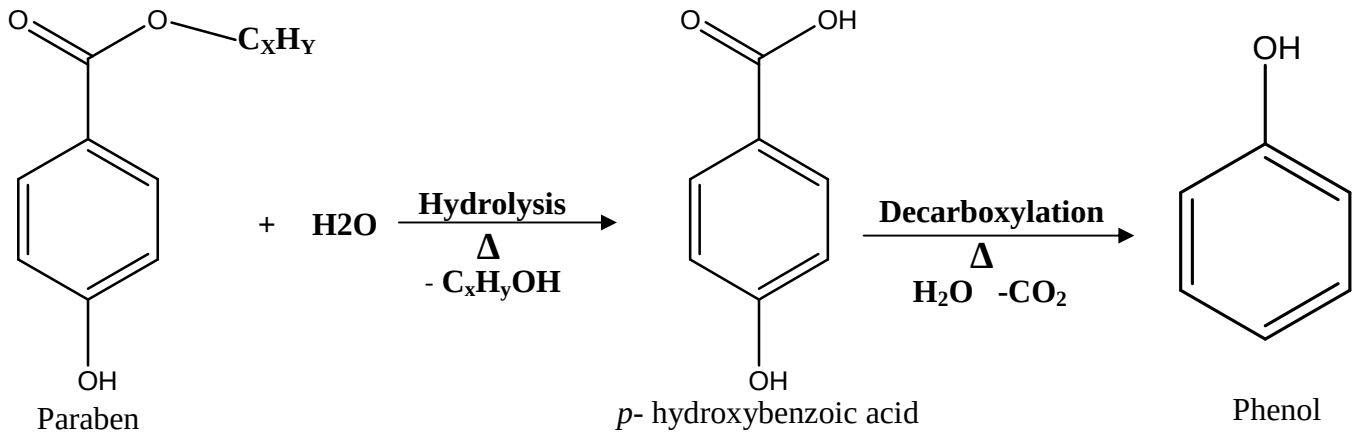
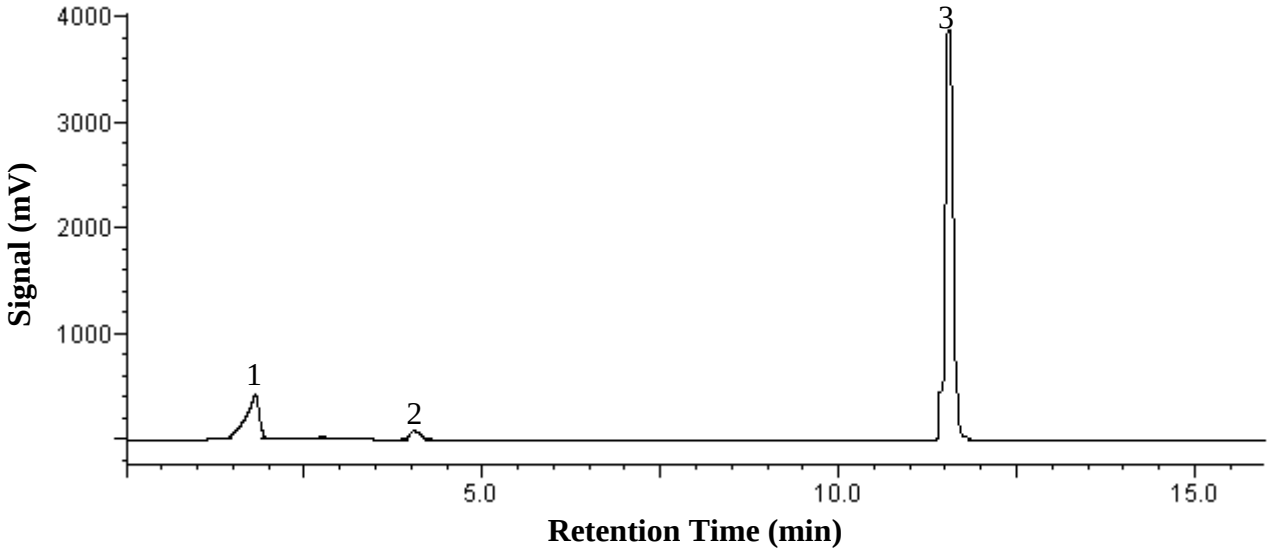
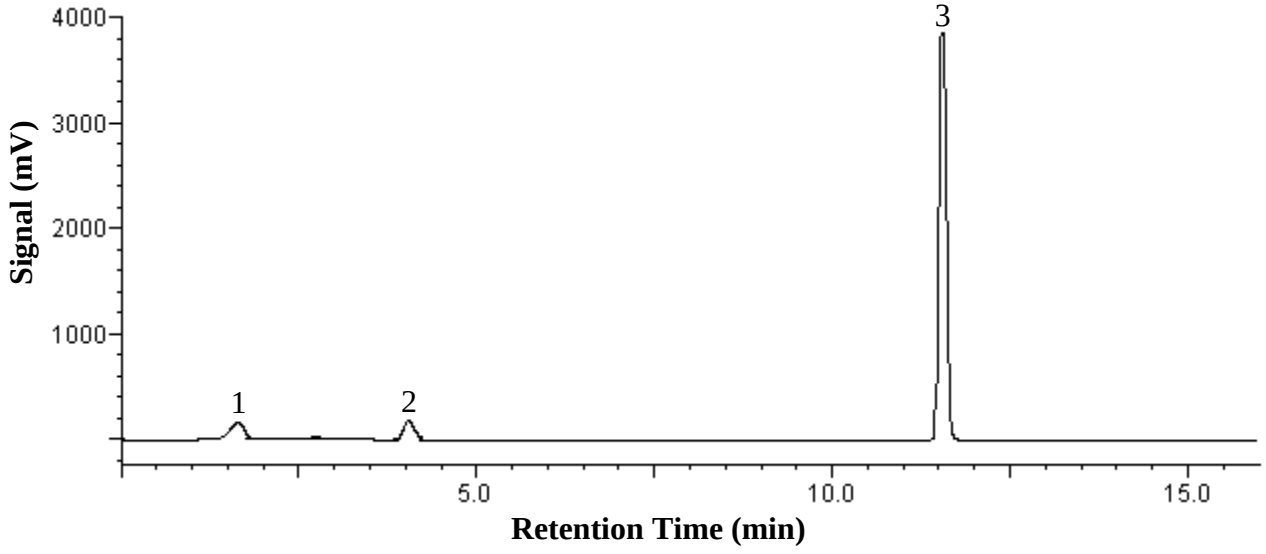


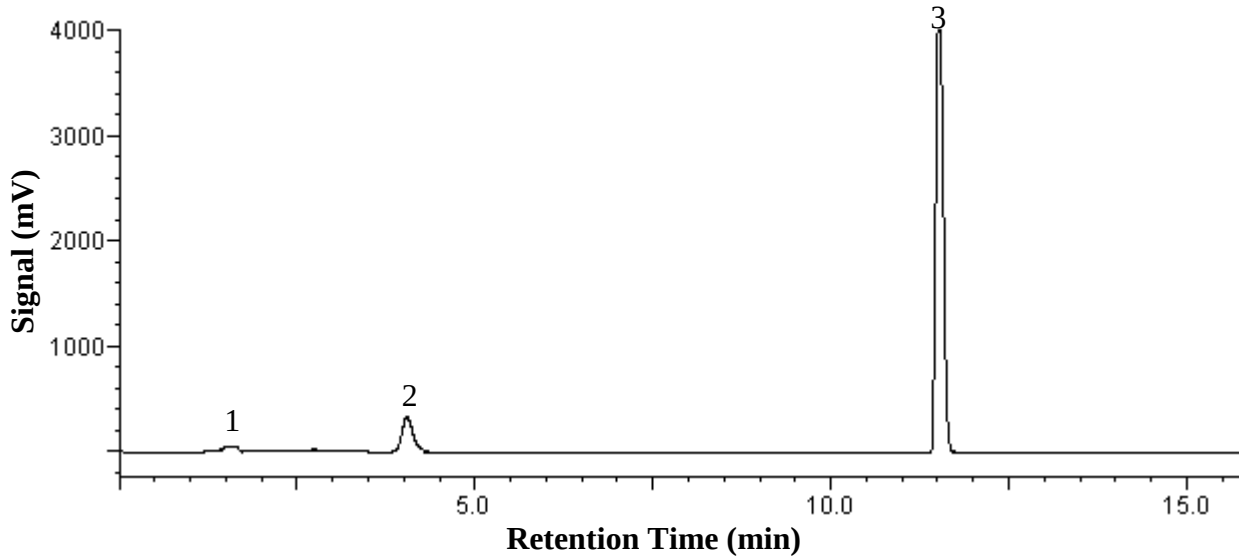
Figure 4.5. Paraben degradation pathway.



a) 60min



b) 90 min



c) 120 min

Figure 4.6. HPLC chromatograms obtained by the separation of heated water-butyl paraben mixtures over Alltech Adsorbosil C18 column at ambient temperature. (a) Water-butyl paraben mixture was prior heated at 200 ^{o}C for 60 min; (b) 90 min; (c) 120 min. Flowrate: 1.0 mL/min. UV detection: 256nm. Mobile Phase: A, deionized water; B, 100% methanol. Gradient: 0-7 min, 50-40% methanol; 7-9 min, 40-90% methanol; 9-13 min, 90%methanol; 13-14 min, 90-50% methanol; 14-16 min, 50%methanol. Peak identification: 1, *p*-hydroxybenzoic acid; 2, phenol; 3, butyl paraben.

Chapter 5: Long-Term Stability of Stationary Phases under Subcritical Water Chromatographic Conditions

5.1 Introduction

As discussed in Chapter 3, high temperatures used in the SBWC separations may lead to degradation of analytes and stationary phases [1-2]. While the stability of analytes was already addressed in Chapter 3, this chapter concentrates on the stability of columns under SBWC conditions.

Various commercial and lab packed columns made of different packing materials including Hypercarb-carbon [138]; Nucleogel RP-polymer [142, 149]; Chromatorex-silica; Acuity BEH C18-hybrid silica [8, 153]; ZirChrom-C18-zirconia [167, 171] were used in SBWC separations. Most of these columns have a temperature limit higher than ambient temperature. Thus these columns may facilitate the development of SBWC methods. In literature, sometimes these columns have been used at temperatures much higher than the temperature limit set by the manufacturers [1-2]. Therefore long-term stability study of these stationary phases under SBWC conditions is critically needed.

In this study, we selected three commercial columns used in our previous SBWC studies to evaluate their long-term stability. Among those three columns, two are hybrid silica based columns, Waters XBridge phenyl and Waters XBridge C18 and the third column is a zirconia based column, ZirChrom-DB-C18. The silica based columns were evaluated at 150 ^{o}C and the zirconia based column was evaluated at 200 ^{o}C under SBWC conditions.

5.2 Experimental

5.2.1 Reagents and Supplies

Benzyl alcohol, methyl paraben, ethyl paraben, propyl paraben, and 2-phenoxyethanol were obtained from Aldrich (St. Louis, MO, USA). Butyl paraben was received from SAFC (St. Louis, MO, USA). HPLC-grade methanol was received from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water (18 MΩ-cm) was produced in our laboratory using a Sybron/Barnstead system (Sybron/Barnstead, Boston, MA, USA). XBridge C18 (4.6 x 100 mm, 3.5 µm) and XBridge phenyl (4.6 x 100 mm, 3.5 µm) columns were received from Waters Corporation (Milford, MA, USA). ZirChrom-DB-C18 (4.6 x 100 mm, 3 µm) column was obtained from ZirChrom Separations, Inc. (Anoka, MN, USA).

5.2.2 Preparation of Solutions

5.2.2.1 Preparation of Internal Standard Solutions

Butyl paraben was used as the internal standard. This solution was prepared by adding 0.025 g (accurately weighed) of butyl paraben to a 50-mL volumetric flask and then diluted to the mark with methanol.

5.2.2.2 Preparation of Calibrated Standard Solutions

A stock standard solution was prepared by adding 0.075 g (accurately weighed) of benzyl alcohol and 0.025 g (accurately weighed) each of methyl, ethyl, and propyl paraben to a 50-mL volumetric flask and then diluted to the mark with methanol. Then a calibrated standard solution was prepared by transferring 2.00 mL of each stock standard solution and butyl paraben internal standard solution into a 25-mL volumetric flask and then diluted to the mark with methanol.

5.2.3 HPLC Analysis

Shimadzu Nexera UFLC with a UV-vis dual wavelength detection system (Shimadzu Corporation, Chiyoda-ku Tokyo, Japan) was employed for the stability evaluation of hybrid silica based columns at 150 ^{o}C under SBWC conditions. A home-made SBWC system was used for the stability determination of zirconia based stationary phases at 200 ^{o}C under SBWC conditions. In the previous chapters, specifications of these two instruments were already described in detail. UV detection wavelength for analytes is 256 nm.

5.3 Results and Discussion

5.3.1 Long-Term Stability of Waters XBridge Phenyl Column

At first the stability of XBridge phenyl column was evaluated at constant temperature of 150 ^{o}C using the Shimadzu Nexera UFLC system. Both plate number and retention factor of preservatives were decreased with the continuous stability evaluation at 150 ^{o}C as shown in Figure 5.1 & 5.2. After the XBridge phenyl column evaluated for 256 hours (9,247 column volume) at 150 ^{o}C, benzyl alcohol and methyl paraben were coeluted and plate number of parabens was also reduced to a much lower value. Therefore the stability evaluation was changed from constant temperature to the programmed temperature. In the programmed temperature evaluation, the initial temperature of 100 ^{o}C was increased to 150 ^{o}C at a rate of 15 ^{o}C/min and maintained at 150 ^{o}C for rest of the run. Surprisingly, column degradation was slowed down leading to the long-term stability evaluation of column extended for another 575 hours (20,753 column volume). Therefore the column life of XBridge phenyl column under SBWC conditions of combined isothermal and programmed temperatures is 831 hours (30,000 column volume).

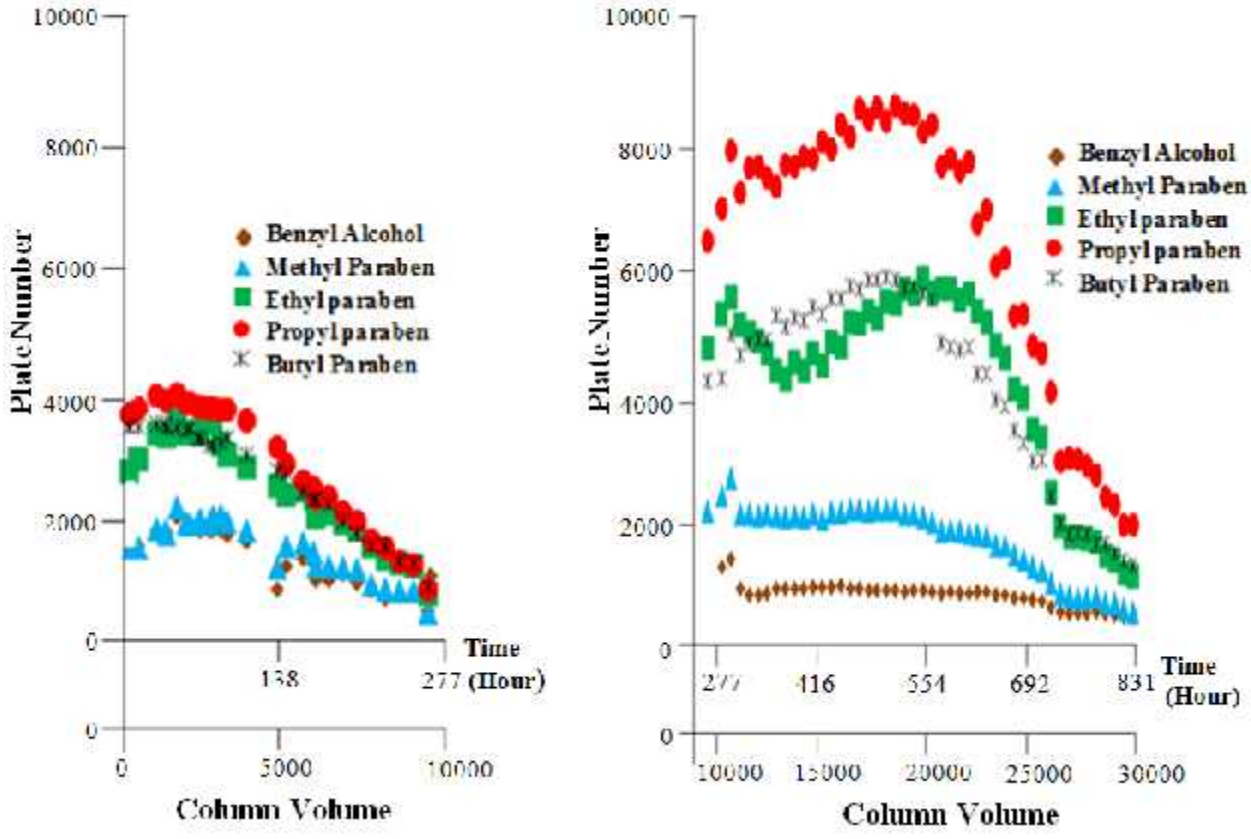


Figure 5.1. Long-term stability of XBridge phenyl column through the heating effect on theoretical plate number. Flow rate: 1 mL/min. (a) Temperature: 150 ºC for column volume of 0-9247; (b) Programmed temperature (100 ºC to 150 ºC at 15 ºC/min and then remained at 150 ºC for the remainder of each run) for column volume of 9247 to 30,000.

It is evident from Figure 5.1b that there is a greater increase in plate number with the change of column stability evaluation from constant temperature to programmed temperature. The raise of plate number can be understood by observing the temperature effect on column efficiency as explained by the Yang model [152, 189]. According to this model, the maximum column efficiency for a separation is in the temperature range of 100 to 120 ^{o}C. Due to the decrease of temperature in the column stability evaluation from 150 ^{o}C to 100 ^{o}C, increased the column efficiency means the decrease of peak width. It is also clear from Figure 5.2b that

retention factor also increased due to the decrease of temperature in the stability evaluation of column. As plate number is a ratio of retention time over peak width, both increase in retention time and decreased peak width should have dramatically increased the plate number of the preservative peaks.

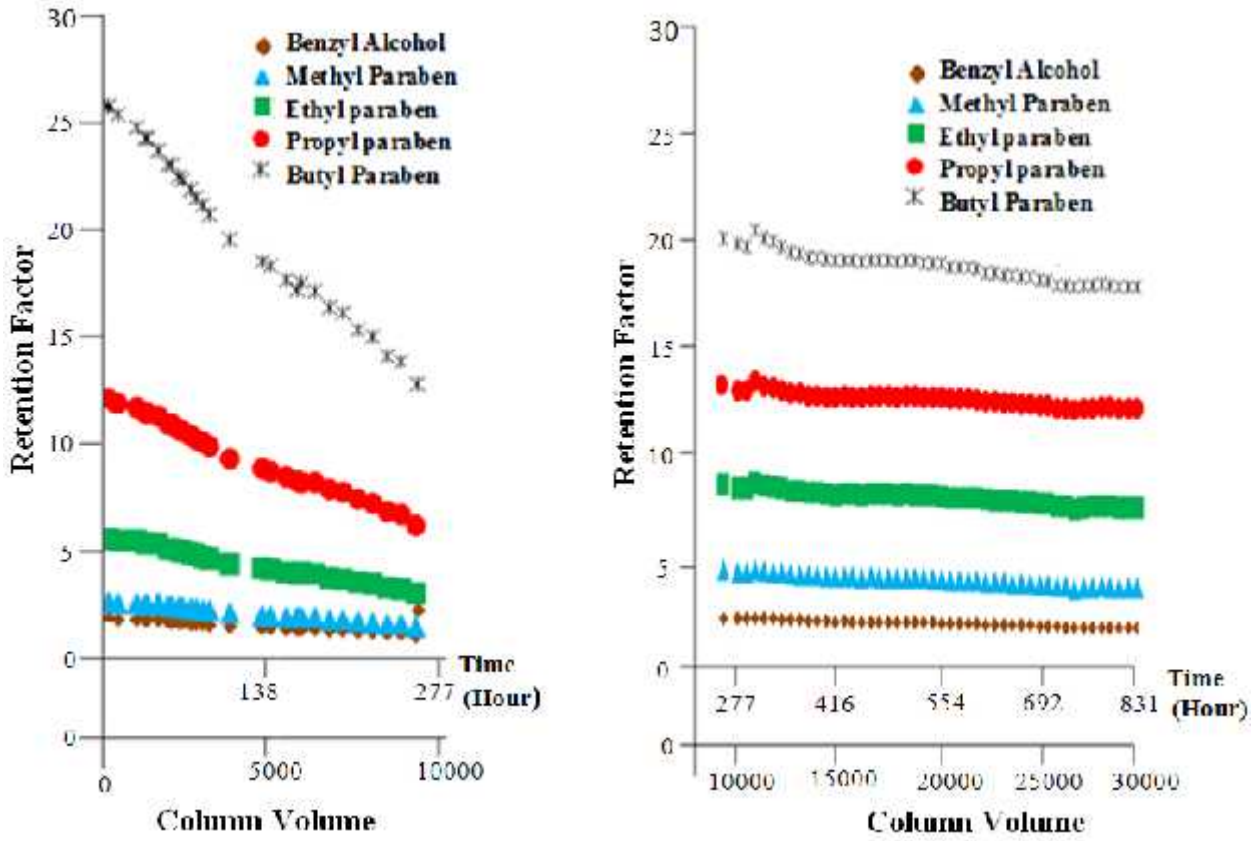
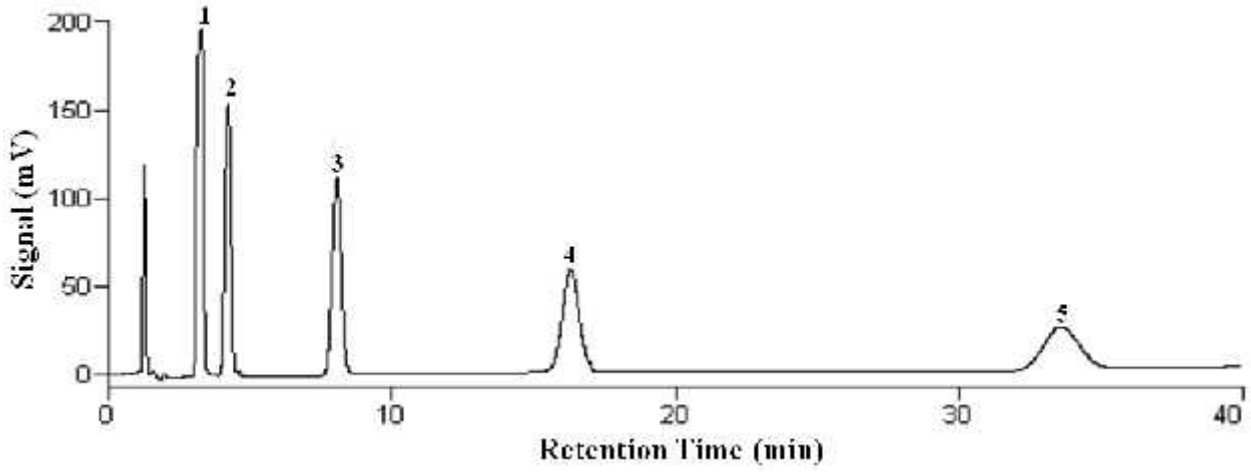


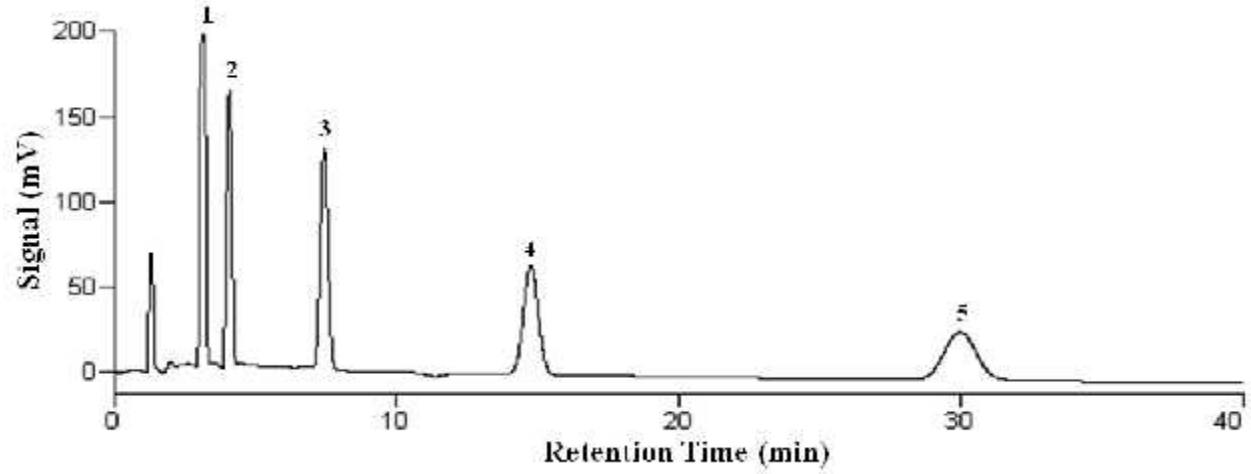
Figure 5.2. Long-term stability of XBridge phenyl column through the heating effect on retention factor. Flow rate: 1 mL/min. (a) Temperature: 150 ºC for column volume of 0-9247; (b) Programmed temperature (100 ºC to 150 ºC at 15 ºC/min and then remained at 150 ºC for the remainder of each run) for column volume of 9247 to 30,000.

Figure 5.3a-c shows the chromatograms of SBWC separation of preservative mixture obtained during the course of constant temperature stability evaluation of XBridge phenyl column at 150 ^{o}C. Figure 5.3c shows the coelution of methyl paraben with benzyl alcohol after

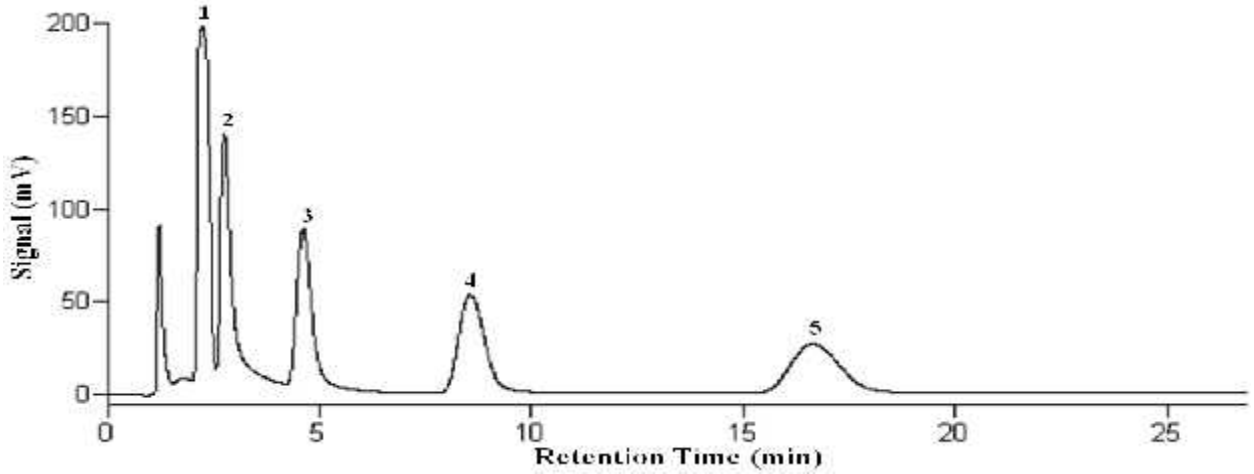
256 hours of column stability evaluation at 150 ^{o}C. These chromatograms (Figure 5.3a-c) show the good separation of preservative peaks until 256 hours of heating time. Figure 5.3d shows the chromatogram obtained in the middle of programmed temperature evaluation at 529 hours of heating time. This chromatogram also shows the enhanced column efficiency (narrow peaks) and increased retention with the programmed temperature.



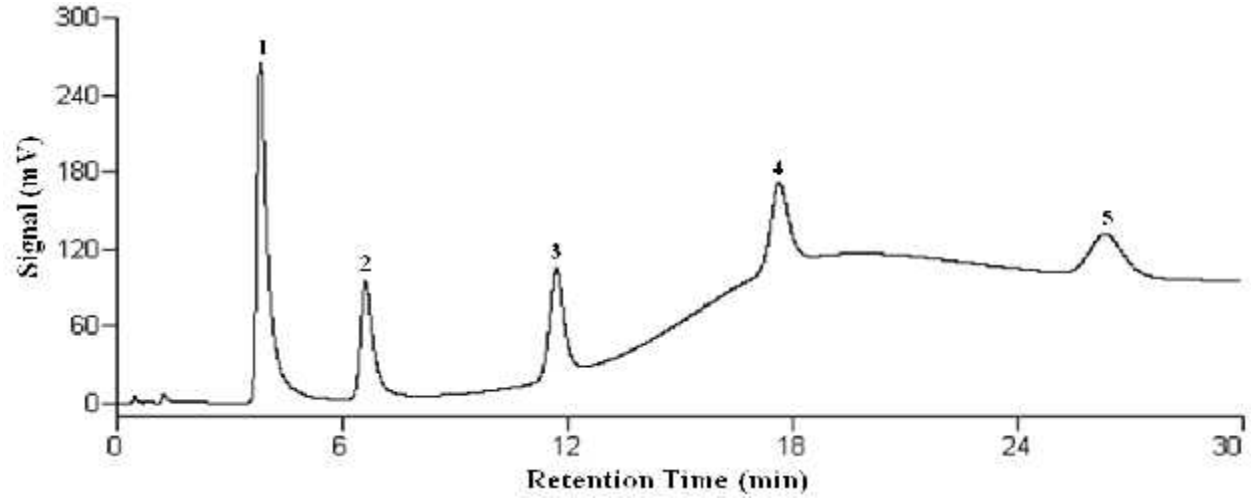
a



b



c



d

Figure 5.3. Subcritical water chromatography chromatograms of preservatives mixture obtained on the XBridge phenyl column using 1 mL/min at different points of evaluation period. (a) At the beginning of the evaluation at 150 °C (after 1 hour exposure, 36 column volume); (b) After 62 hours exposure to 150 ºC (2240 column volume); (c) At the end of the evaluation at 150 ºC (256 hours or 9247 column volume); (d) After 273 hours exposure to the programmed temperature or a total of 529 hours exposure (19,108 column volume) to both isothermal and the programmed temperature at 150 ºC.

5.3.2 Long-Term Stability of Waters XBridge C18 Column

The long-term stability evaluation of XBridge C18 column was also carried out using the commercial Shimadzu system at 150 ^{o}C similar to the XBridge phenyl column evaluation. These stability evaluations were also conducted at both isothermal and programmed temperatures. Both plate number and retention factor decrease with the continuous stability evaluation of column are visible from Figures 5.4 & 5.5. From Figure 5.6c, it can be inferred that the stability of XBridge C18 Column under constant temperature evaluation at 150 ^{o}C is 261 hours (9428 column volume) due to the coelution of methyl paraben with benzyl alcohol. Then with the application of programmed temperature evaluation, starting at 100 ^{o}C, XBridge C18 column lasted for another 401 hours (14,664 column volume). Thus the column life of XBridge C18 column under SBWC conditions of combined isothermal and programmed temperatures is 662 hours (23,912 column volume). Again there is a tremendous increase in plate number for the SBWC separations of preservatives with the change of stability evaluation from isothermal to the programmed temperature as shown in Figure 5.4b.

Figures 5.6a-c shows the chromatograms obtained at the beginning, middle, and end of the stability evaluation of XBridge C18 column under constant temperature heating at 150 ^{o}C. Figure 5.6d shows the chromatogram obtained after 477 hours of column heating time during the programmed temperature stability evaluation (initial temperature of 100 ^{o}C).

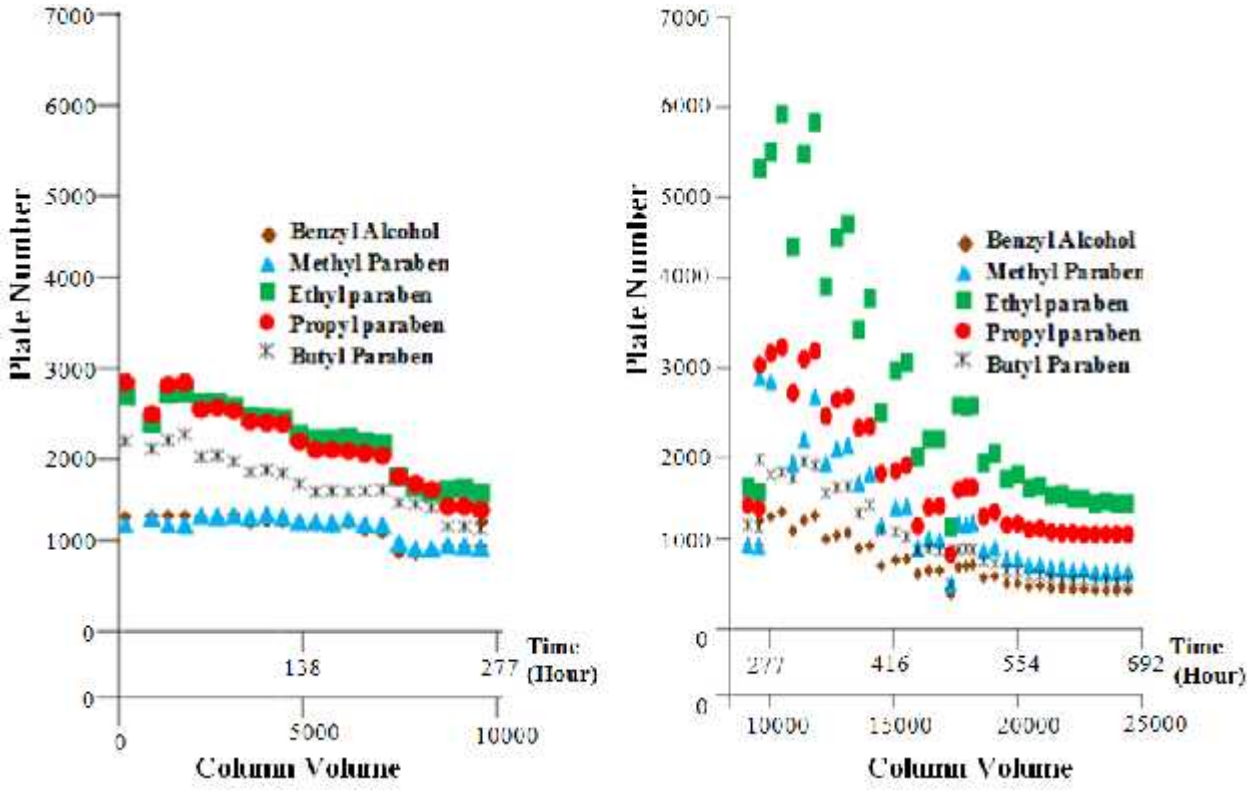


Figure 5.4. Long-term stability of the XBridge C18 column through the heating effect on theoretical plate number of the XBridge C18 column. Flow rate: 1 mL/min. (a) Temperature: 150 ºC for column volume of 0-9428; (b) Programmed temperature (100 ºC to 150 ºC at 15 ºC/min and then remained at 150 ºC for the remainder of each run) for column volume of 9,428 to 23,912.

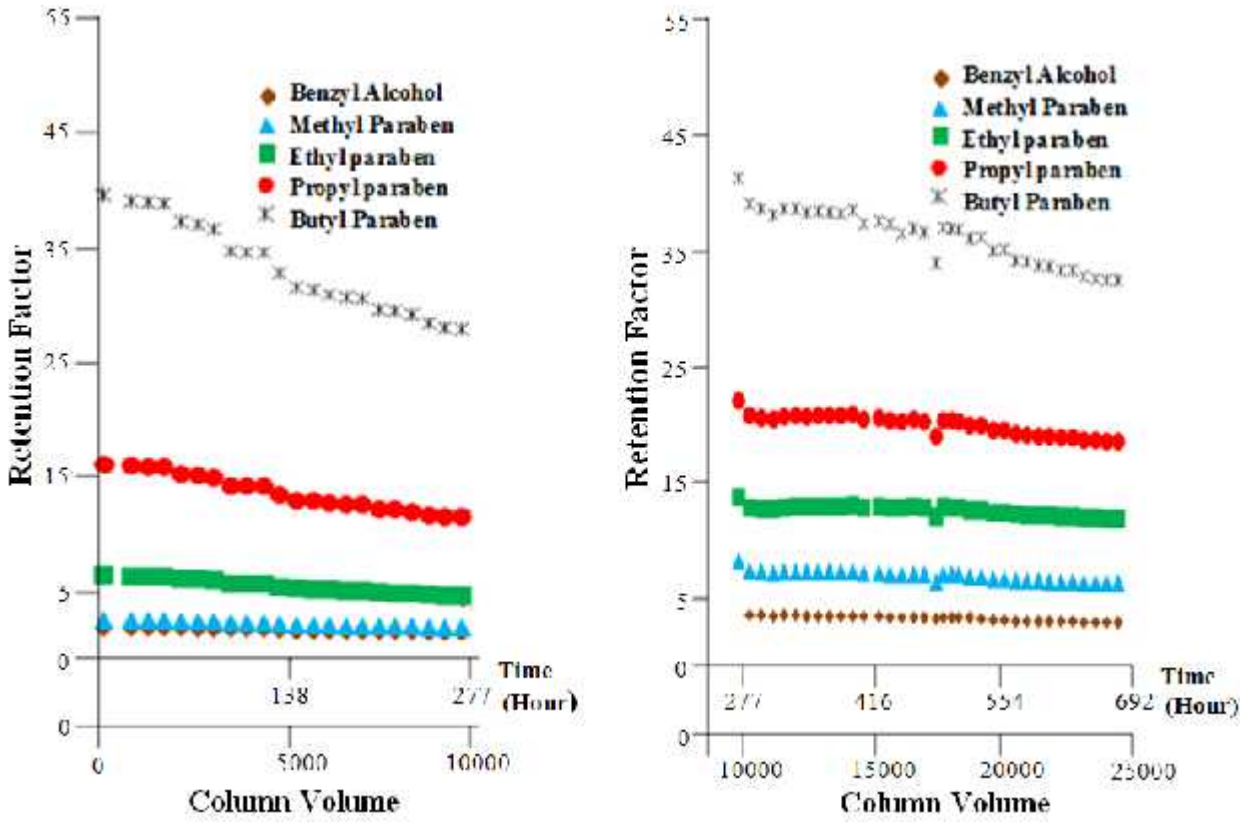
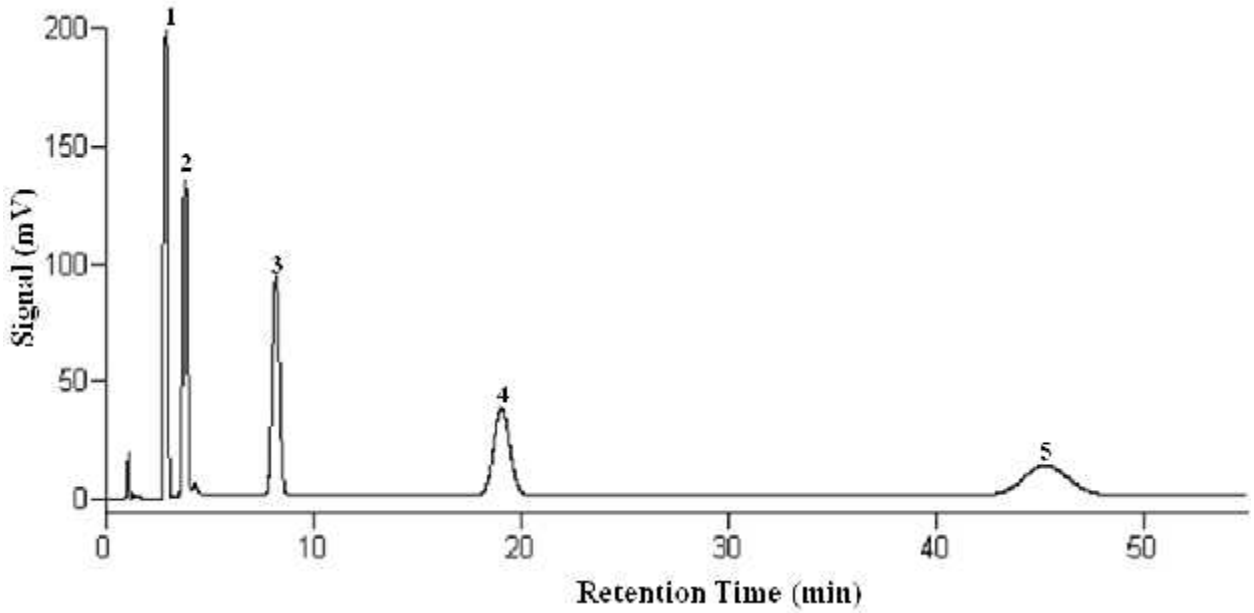
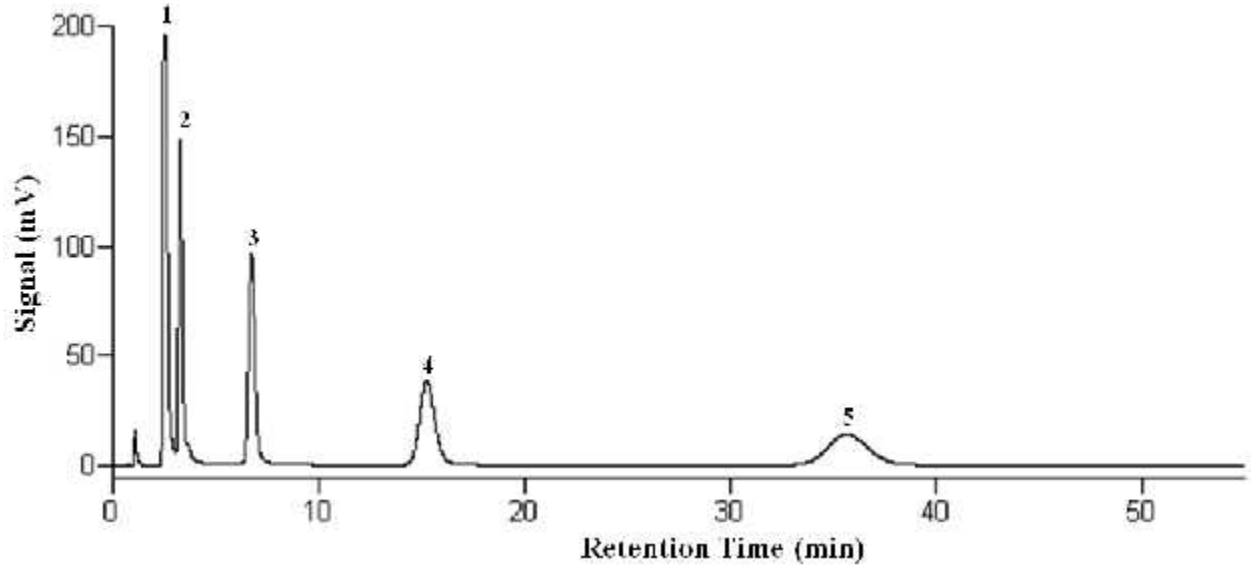


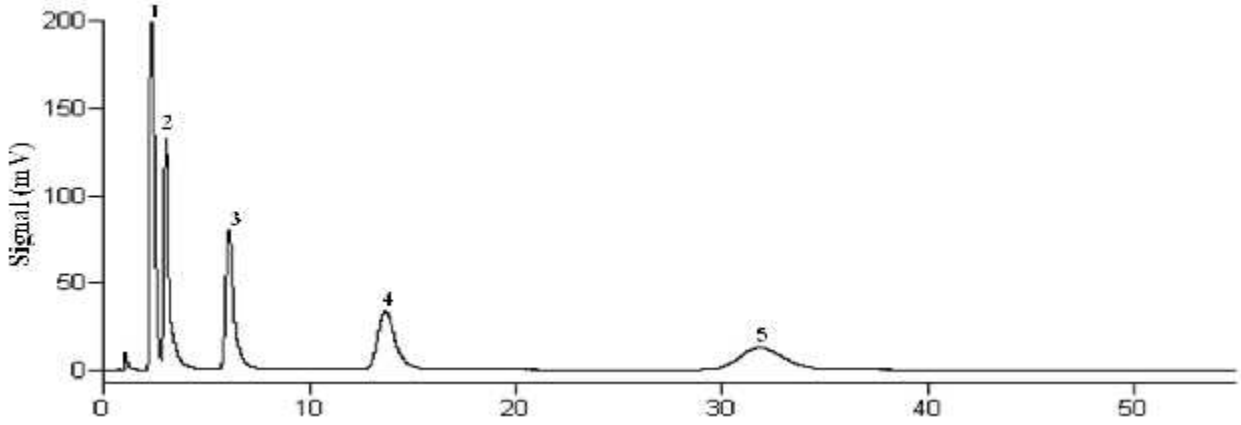
Figure 5.5. Long-term stability of the XBridge C18 column through the heating effect on retention factor of the XBridge C18 column. Flow rate: 1 mL/min. (a) Temperature: 150 ºC for column volume of 0-9428; (b) Programmed temperature (100 ºC to 150 ºC at 15 ºC/min and then remained at 150 ºC for the remainder of each run) for column volume of 9,428 to 23,912.


Signal (mV)
200
150
100
50
0
1
2
3
4
5
0
10
20
30
40
50
Retention Time (min)

a


Signal (mV)
200
150
100
50
0
1
2
3
4
5
0
10
20
30
40
50
Retention Time (min)

b



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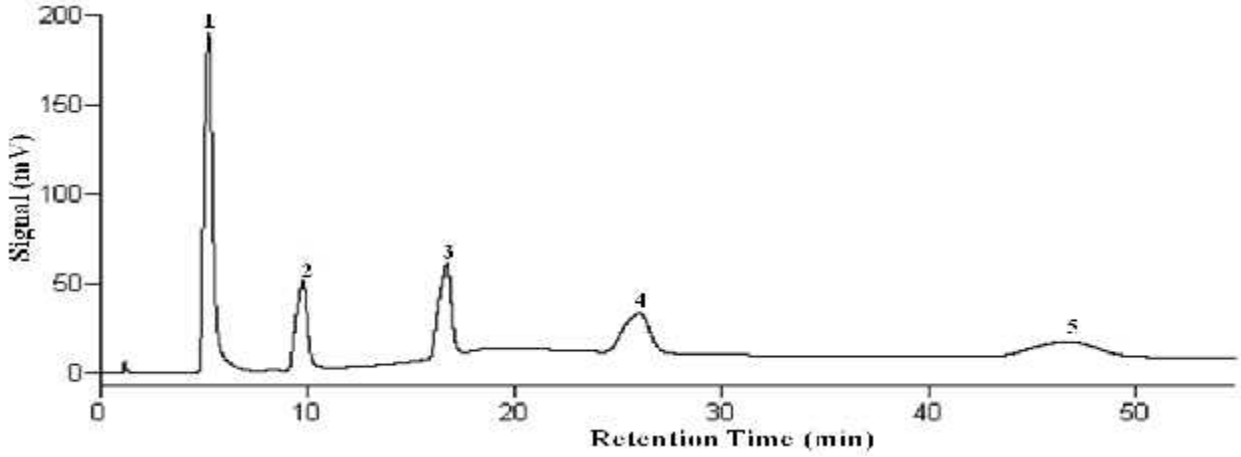


d

Figure 5.6. Subcritical water chromatography chromatograms of preservatives obtained on the XBridge C18 column using 1 mL/min at different points of evaluation period. (a) At the beginning of the evaluation at 150 °C (after 1 hour exposure, 36 column volume); (b) After 141 hours exposure to 150 ºC (5,093 column volume); (c) At the end of the evaluation at 150 ºC (261 hours or 9428 column volume); (d) After 216 hours exposure to the programmed temperature or a total of 478 hours exposure (17,266 column volume) to both isothermal and the programmed temperature at 150 ºC.

5.3.3 Long-Term Stability of ZirChrom-DB C18 Column

The long-term stability of ZirChrom-DB C18 column was conducted by Leena Gujjar from our group [239]. According to her data, the long-term stability of ZirChrom-DB-C18 column was conducted only using constant temperature evaluation at 200 ^{o}C. The column life of ZirChrom-DB C18 column under SBWC conditions at 200 ^{o}C was determined to be 263 hours or 14,250 column volume.

Chapter 6: Separation and Analysis of Cold Drugs and Skincare Creams

6.1 Introduction

Common cold is a disease of upper respiratory tract due to viral infections. Sneezing, sore throat, and cough are the common symptoms of cold. Common cold is not caused by a single microorganism infection, rather by the infection from a group of viruses belong to different families [240]. Medications belong to antipyretics, decongestant, anhistamine and antitussives are available in the market to treat several problems of common cold [240, 241].

Combinations of drugs are normally used in the treatment of cold. Antihistamines such as pyrilamine maleate and chlorpheniramine maleate are used in combination to treat allergic reactions associated with cold [242]. Pheniramine maleate (antihistamine) combined with pseudoephedrine hydrochloride (decongestant) is used in the treatment of cold, and also for sinusitis, bronchitis, and respiratory allergies [243]. Dextromethorphan hydrobromide (antitussive) combined with guaifenisin (expectorant) is used to treat the cough associated with cold [244]. These cold medications are available in the market as syrups, sachets, capsules and tablets [244, 245].

Skin is the outer layer of body that protects from environment and microorganisms. Often our skin is damaged due to UV radiation and pollution [246]. There are many skincare products available in the market that protects the skin from UV radiation by acting as UV filter [247]. In addition to the protection of skin from UV radiation, these skincare products also used in the treatment of other skin ailments like acne and ageing [248, 249].

Skincare products usually contain antioxidants, preservatives, and active ingredients that act as sun protection factors. Antioxidants like niacinamide (nicotinamide) is used in the skincare products to improve skin barrier, reduce skin pore size, reduce facial red blotchiness,

antiwrinkle, reduce hyperpigmentation, reduce yellowing and antiacne [249]. Preservatives such as benzyl alcohol and parabens (methyl, ethyl, propyl and butyl parabens) are widely used in these products due to their effectiveness against yeast, mold, and bacteria [250]. Active ingredients such as sunscreens are used in these products to protect the skin from deleterious sun light by acting as barriers [247, 251]. Popular sunscreens present in the skincare products are para-aminobenzoic acid esters, cinnamates, salicylates, benzophenones, dibenzoylmethanes, anthranilates, and benzylidene camphors etc. [248]. These skincare products are normally available as creams and lotions to protect the skin from sunburn and tanning [248]. The efficacy of these products is normally measured in terms of sun protection factors (SPF) [251].

It is necessary to accurately analyze the analytes present in various cold drugs and skincare products for quality control, product release, and other regulatory purposes. The separation and analysis of these compounds by HPLC involve hazardous solvents such as methanol [252-255]. As discussed in Chapter 1, the development of SBWC methods for the separation of analytes is necessary to completely eliminate the organic solvents from mobile phase.

In this work, several green HTLC and SBWC methods were developed for the analysis of pharmaceuticals (dextromethorphan hydrobromide; chlorpheniramine maleate; doxylamine succinate; phenylephrine hydrochloride; acetaminophen; guaifenesin) present in cold drugs, and niacinamide, preservatives (benzyl alcohol; methyl paraben; ethyl paraben; propyl paraben), and sunscreens (ensulizole; octocrylene; octisalate; homosalate; octinoxate; avobenzone) present in P&G skincare creams at temperatures ranging from 25-250 ^{o}C. This work is mainly aimed for the application of subcritical water chromatography in the industries. The stationary phases used in these separations were polymer based- (Hamilton PRP-1), silica based- (Alltech Adsorbosil

C18, Waters XBridge C18, Waters XBridge phenyl, and Waters XTerra MS C18), and zirconia based columns (ZirChrom-DiamondBond-C18, and ZirChrom-PS). Both commercial and home-made SBWC systems were employed in these separations. Cold drugs used for the pharmaceutical SBWC separations were Vicks formula 44 custom care, cough and cold PM; Alka-seltzer plus, night, cold and flu formula; CVS multi-symptom severe cold relief, daytime, non-drowsy. Commercial P&G skincare creams used for the separation of niacinamide, preservatives and sunscreens were Olay total effects, 7-in-1 anti-ageing daily moisturizer, fragrance free (coded as SC-EC1 in this dissertation); Olay total effects, 7-in-1 anti-ageing UV moisturizer, plus SPF-15, fragrance free (coded as SC-EC2); Olay complete ageless skin-renewing UV lotion, SPF-20 (coded as SC-EC3); Olay Complete SPF 30 defense, daily UV moisturizer with vitamins E, B3 and pro-vitamin B5 (coded as SC-EC4).

6.2 Experimental

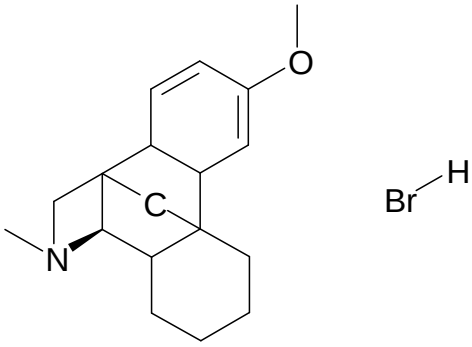
6.2.1 Reagents and Materials

Dextromethorphan hydrobromide, chlorpheniramine maleate, acetaminophen, doxylamine succinate, phenylephrine hydrochloride, guaifenesin, niacin, niacinamide, ammonium acetate, and 4-acetamidophenol were received from Sigma-Aldrich Chemical (Milwaukee, WI, USA). Benzyl alcohol, methyl paraben, ethyl paraben, propyl paraben, and calcium chloride were purchased from Aldrich (St. Louis, MO, USA). Butyl paraben was obtained from SAFC (St. Louis, MO, USA). Ensulizole, oxybenzone, octisalate, and octocrylene were acquired from Sigma-Aldrich Chemicals (Milwaukee, WI, USA). Avobenzone and homosalate were received from Procter and Gamble (P&G, Cincinnati, OH, USA). Octinoxate was obtained from the Science Lab.com, Inc. (Houston, TX, USA). HPLC-

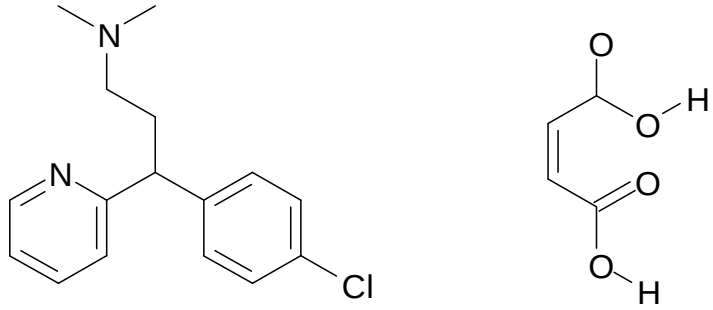
grade methanol, ortho phosphoric acid and formic acid (90%) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water (18 MΩ-cm) was obtained in our laboratory using a Sybron/Barnstead system (Sybron/Barnstead, Boston, MA, USA).

GD/X PVDF membrane filters (0.45 µm) were purchased from Whatman (Florham Park, NJ, USA). Copper tubing of 1/8-inch O.D. was obtained from Chromatography Research Supplies, Inc. (Louisville, Kentucky, USA). Glass vials were acquired from Supelco (Bellefonte, PA, USA). Real life samples of Vicks formula 44 custom care, cough and cold PM; Alka-seltzer plus, night, cold and flu formula; CVS multi-symptom severe cold relief, daytime, non-drowsy; Olay total effects, 7-in-1 anti-ageing daily moisturizer, fragrance free; Olay total effects, 7-in-1 anti-ageing UV moisturizer, plus SPF-15, fragrance free; Olay complete ageless skin-renewing UV lotion, SPF-20; Olay Complete SPF 30 defense, daily UV moisturizer with vitamins E, B3 and pro-vitamin B5 were purchased at a local store.

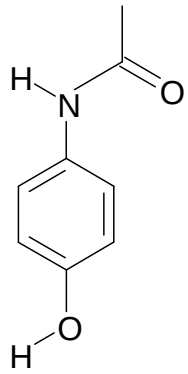
Hamilton PRP-1 column (150 x 4.1 mm, 3-µm) was purchased from Hamilton (Reno, NV, USA). Adsorbosil C18 (4.6 x 150 mm, 5 µm) was obtained from Alltech Associates, Inc. (Deerfield, IL, USA). XTerra MS C18 (2.1 x 100 mm I.D., 3.5 µm), XBridge C18 (4.6 x 100 mm, 3.5 µm) and XBridge phenyl (4.6 x 100 mm, 3.5 µm) columns were received from Waters Corporation (Milford, MA, USA). ZirChrom-DB-C18 (4.6 x 100 mm, 3 µm) and ZirChrom-PS (4.6 x 100 mm, 3 µm) columns were purchased from ZirChrom Separations, Inc. (Anoka, MN, USA).



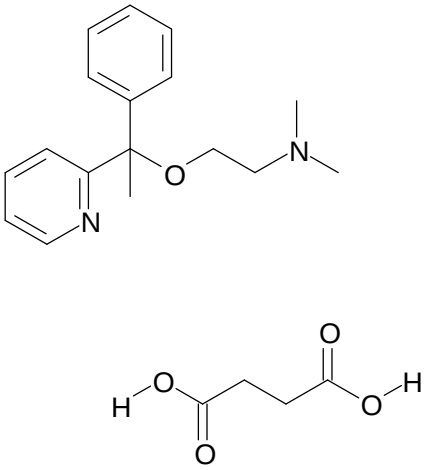
Dextromethorphan Hydrobromide



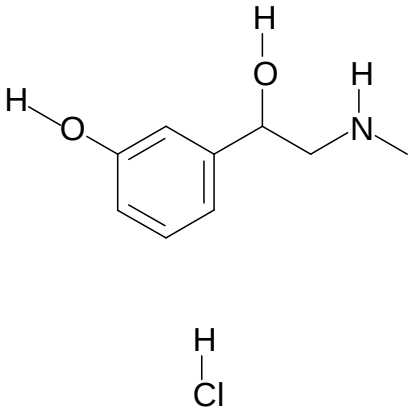
Chlorpheniramine Maleate



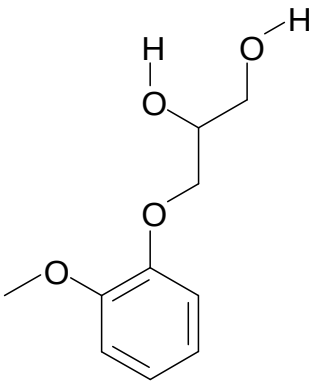
Acetaminophen



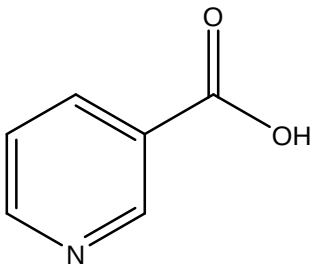
Doxylamine Succinate



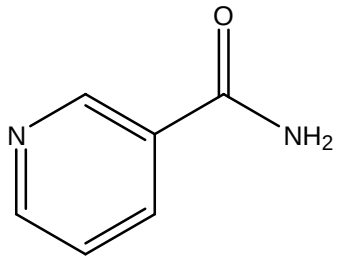
Phenylephrine Hydrochloride



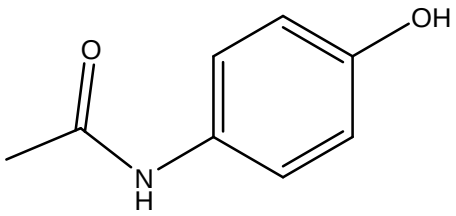
Guaifenesin



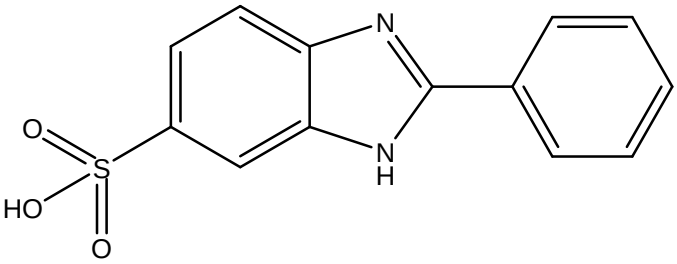
Niacin



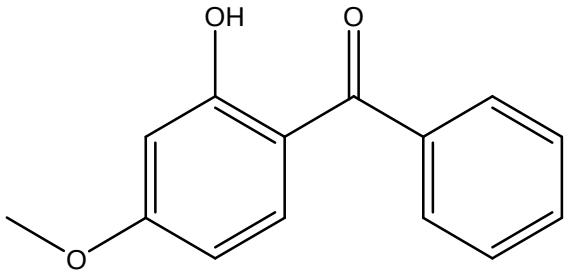
Niacinamide



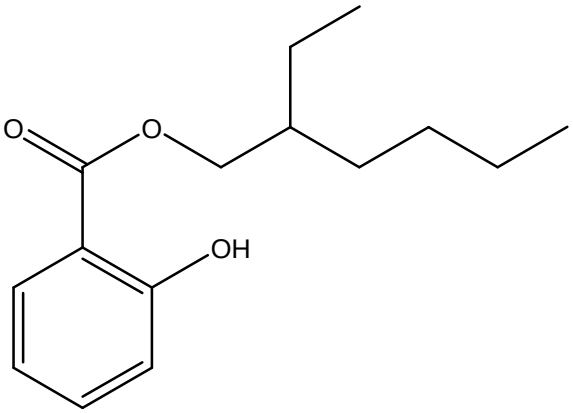
4-Acetamidophenol



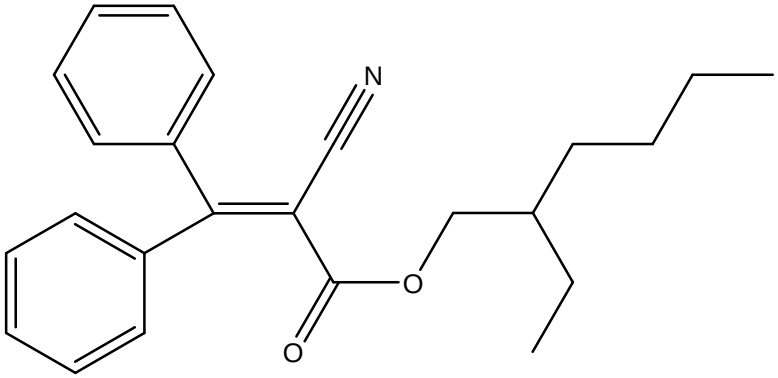
Ensulizole



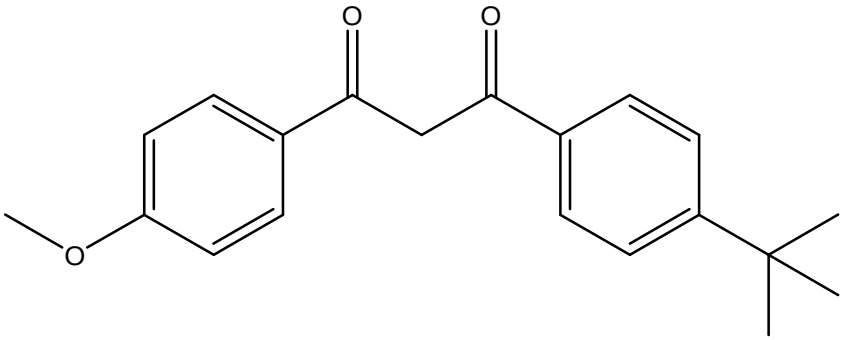
Oxybenzone



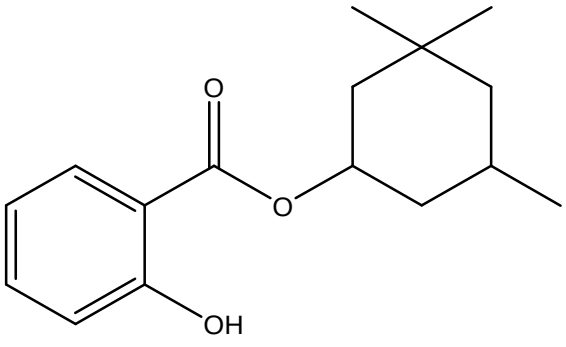
Octisalate



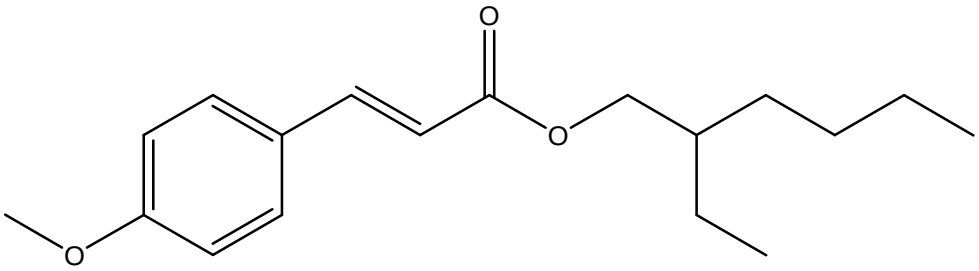
Octocrylene



Avobenzone



Homosalate



Octinoxate

Figure 6.1. Structures of pharmaceuticals in cold drugs, niacin niacinamide, and sunscreens in skincare creams.

6.2.2 Preparation of Solutions

6.2.2.1 Preparation of Internal Standard Solutions

For the analysis of pharmaceuticals in three real life cold drugs, different internal standards were used. Phenylephrine hydrochloride (Vicks formula 44 custom care), benzyl alcohol (CVS multi-symptom severe cold relief), and chlorpheniramine maleate (Alka-seltzer plus) were used as internal standards. Each internal standard solution was prepared by adding 0.05 g (accurately weighed) of appropriate internal standard to a 50-mL volumetric flask and then diluted to the mark with methanol.

For niacinamide analysis, 4-acetamidophenol was used as internal standard. This solution was prepared by adding 0.1 g (accurately weighed) of 4-acetamidophenol to a 100-mL volumetric flask and then diluted to the mark with methanol.

For preservatives analysis, butyl paraben was used as internal standard. This solution was prepared by adding 0.025 g (accurately weighed) of butyl paraben to a 50-mL volumetric flask and then diluted to the mark with methanol.

For sunscreens analysis, oxybenzone was used as the internal standard. This solution was prepared by adding 0.1 g (accurately weighed) of oxybenzone to a 100-mL volumetric flask and then diluted to the mark with acetonitrile.

6.2.2.2 Preparation of Calibrated Standard Solutions

For pharmaceuticals analysis, a stock standard solution was prepared by adding Q mg (accurately weighed) of each pharmaceutical compound (Q = Q mg of each pharmaceutical compound in cold drugs) to a 50-mL volumetric flask and then diluted to the mark with methanol. Then five series calibration standard solutions were prepared from this stock standard solution with an appropriate amount of internal standard solution using methanol as solvent.

For niacinamide analysis, a diluent was used as the solvent in calibrated standard solution preparation. This diluent was prepared by adding 200.00 mL of 50 mM formic acid to a 500-mL volumetric flask and then diluted to the mark with 50 mM ammonium acetate. Then a stock solution was prepared by adding 0.1 g (accurately weighed) of niacinamide and niacin to a 10-mL volumetric flask and diluted to the mark with diluent. The calibrated standard solution was prepared by adding 1.00 mL of each stock and 4-acetamidophenol internal standard solution to a 10-mL volumetric flask and then diluted to the mark with diluent.

For preservatives analysis, a stock standard solution was prepared by adding 0.075 g (accurately weighed) of benzyl alcohol and 0.025 g (accurately weighed) of each methyl, ethyl, and propyl paraben to a 50-mL volumetric flask and then diluted to the mark with methanol. Then, a calibrated standard solution was prepared by adding 2.00 mL of each stock and butyl paraben internal standard solution to a 25-mL volumetric flask and diluted to the mark with methanol.

For sunscreens analysis, a calibrated standard solution was prepared by adding Q mg (accurately weighed) of each sunscreen compound (Q = 10 x % each sunscreen compound in skincare product) to a 250-mL volumetric flask. Then, 20.00 mL of oxybenzone internal standard solution was added to the flask and then diluted to the mark with methanol.

6.2.2.3 Preparation of Sample Solutions

All the cold drugs and skincare cream samples were mixed well before sampling to ensure a homogeneous mixture.

For Vicks formula 44 custom care (syrup) analysis, a diluent was used as the solvent in sample preparation. This diluent was prepared by adding approximately 11 g of calcium chloride to a 50-mL volumetric flask and then diluted to the mark with deionized water. Then the Vicks pharmaceutical sample was prepared by adding 1.00 mL of each syrup and phenylephrine hydrochloride internal standard to a 25-mL volumetric flask and diluted to the mark with diluent. This solution was then vortexed to obtain a homogeneous sample mixture and filtered through a 0.45 µm Whatman GD/X filter into a 2-mL sample vial for chromatographic analysis.

For CVS multi-symptom severe cold relief (caplet) and Alka-seltzer plus (capsule) analysis, one caplet or capsule was added to the 50-mL volumetric flask and diluted to the mark with methanol. In case of caplet, it was brocken down in to pieces before adding it to the 50-mL volumetric flask for faster sample preparation. This mixture was then vortexed to obtain a homogeneous mixture. Then a sample solution was prepared by transferring 2.00 mL of homogeneous mixture and 1.00 mL of appropriate internal standard solution to a 25-mL volumetric flask and diluted to the mark with methanol. This solution was again vortexed and

filtered through a 0.45 µm Whatman GD/X filter into a 2-mL sample vial for chromatographic analysis.

For niacinamide analysis, 0.2 g (accurately weighed) of skincare cream was added to the 25-mL glass vial. Then 1.00 mL of 4-acetamidophenol internal standard solution and 5.00 mL of methanol were added to the glass vial. This mixture was then vortexed to obtain a homogeneous mixture. Then 5.00 mL of diluent was added to the glass vial and mixed thoroughly. This solution was then filtered through a 0.45 µm Whatman GD/X filter into a 2-mL sample vial for chromatographic analysis.

For preservatives analysis, a diluent was used as the solvent in sample preparation. This diluent was prepared by adding approximately 1.25 g of calcium chloride to a 50-mL volumetric flask and then diluted to the mark with deionized water. Preservative sample was then prepared by adding 0.3 g (accurately weighed) of skincare cream to each 25-mL glass vial. Then 2.00 mL of each butyl paraben internal standard and calcium chloride solution and 21.00 mL of methanol were added to the glass vial. This solution was then vortexed and filtered through a 0.45 µm Whatman GD/X filter into a 2-mL sample vial for chromatographic analysis.

For sunscreens analysis, 0.1 g (accurately weighed) of skincare cream was added to the 25-mL glass vial. Then 2.00 mL of oxybenzone internal standard and 23.00 mL of methanol were added to the glass vial. This solution was then vortexed and filtered through a 0.45 µm Whatman GD/X filter into a 2-mL sample vial for chromatographic analysis.

6.2.3 Instrumentation

As discussed in Chapters 3 & 4, Shimadzu Nexera UFLC with a UV-vis dual wavelength detection system (Shimadzu Corporation, Chiyoda-ku Tokyo, Japan) was used for the separation and analysis of analytes in cold drug and skincare cream samples. As discussed in

Chapter 4, a home-made SBWC system with UV detector was used for the separation and analysis of analytes in skincare cream samples. Pharmaceuticals were detected at 210 nm, niacinamide at 245 nm, preservatives at 210 or 256 nm, sunscreens at 300 nm using UV detector.

6.3 Results and Discussion

6.3.1 Analysis of Pharmaceuticals Present in Cold Drugs

The SBWC separations of pharmaceuticals in cold drugs were evaluated on Alltech Adsorbosil C18 column with 1.0 mL/min using the Schimadzu system. The pharmaceutical analytes were detected at 210 nm.

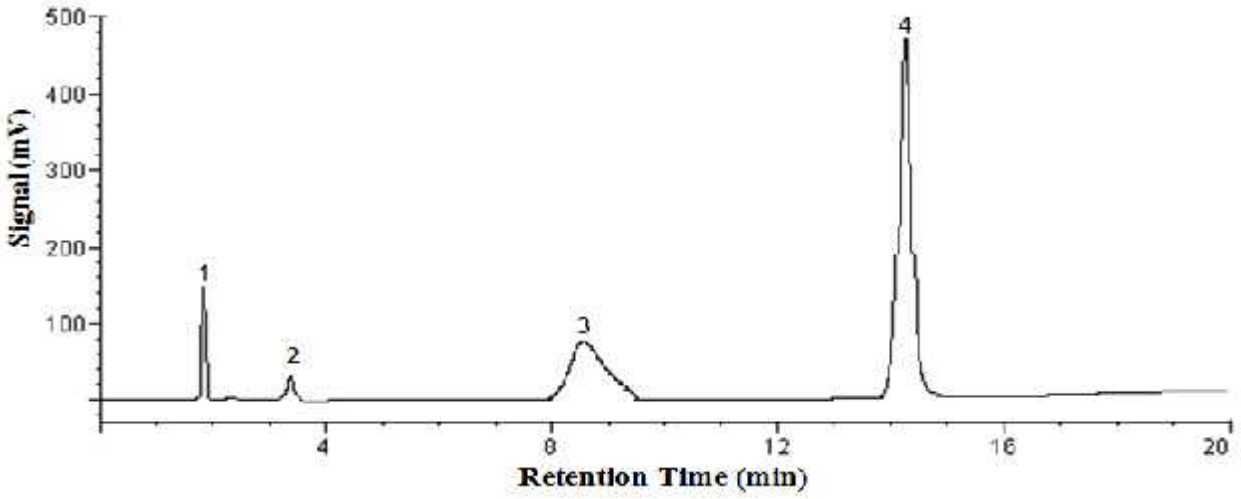
6.3.1.1 SBWC Separation and Analysis of Pharmaceuticals in P&G Vicks Formula

In order to completely eliminate the organic solvents from the mobile phase for the analysis of P&G cold drug, subcritical water chromatographic methods were developed. As the analytes of interest are ionizable compounds, therefore the pH of the mobile phase needs to be adjusted to achieve better resolution. Both pure water and 100 mM phosphoric acid mixture are used as the mobile phase. These SBWC methods were optimized and the best separation was achieved using the programmed temperatures and gradient elution. Figure 6.2 shows the separation of pharmaceuticals standard and P&G cold drug sample obtained by subcritical water chromatography. The separation conditions are given in Figure 6.2 legend. Table 6.1 shows the five replicate experiments of pharmaceuticals separation with %recoveries ranging from 94.1 to 105.0 and %RSDs lower than 3.6.

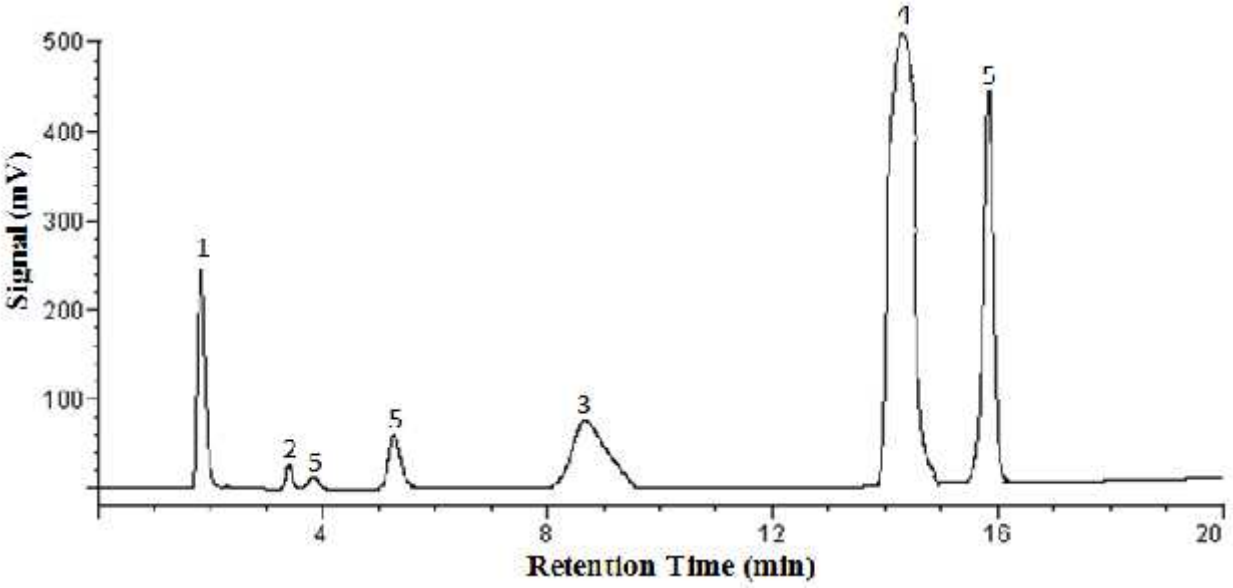
Table 6.1. Concentration of Pharmaceuticals Found in Vicks Formula 44 Custom Care Cough and Cold Sample Obtained by SBWC Using Alltech Adsorbosil C18 Column

Pharmaceuticals	P&G Stated Concentration (W)	Concentration Found by This Method (W)	%Recovery	%RSD[a]
Dextromethorphan Hydrobromide	30.0	28.8	96.0	3.6
Chlorpheniramine Maleate	4.0	4.2	105.0	1.8
Acetaminophen	650.0	611.5	94.1	2.1

[a]*Based on five replicates.*



a



b

Figure 6.2. Subcritical water chromatography chromatograms of pharmaceuticals obtained on the Alltech Adsorbosil C18 column with 1.0 mL/min using programmed temperatures. (a) Pharmaceutical standard mixture; (b) Vicks formula 44 custom care cough and cold sample. UV detection: 210 nm. Mobile phase: A, deionized water; B, 100 mM phosphoric acid. Gradient: 100% water for 1 min and then 100 mM phosphoric acid for rest of the run. Programmed temperatures: Initial temperature of 25 ^{o}C for 3 min and then increased at 15 ^{o}C/min to 150 ^{o}C and maintained at 150 ^{o}C for rest of the run. Peak identification: 1, dextromethorphan hydrobromide; 2, chlorpheniramine maleate; 3, phenylephrine hydrochloride; 4, acetaminophen; 5, matrix peak.

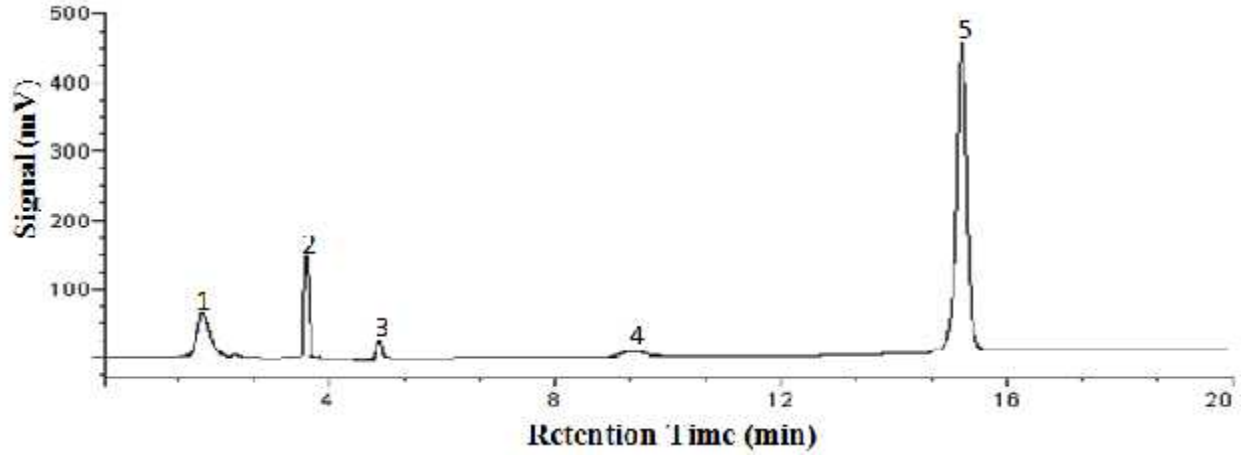
6.3.1.2 SBWC Separation and Analysis of Pharmaceuticals in Bayer Alka-Seltzer Plus

Similar to the SBWC separation of pharmaceuticals in P&G cold drug, further SBWC experiments were carried out for the separation of pharmaceuticals in Bayer cold drug. These separations were optimized and the best separation was achieved using the same programmed temperatures and gradient elution as the one used for P&G cold drug. Figure 6.3 shows the separation of pharmaceuticals standard and Alka-Seltzer cold drug sample obtained by subcritical water chromatography. The separation conditions are given in Figure 6.3 legend. Table 6.2 shows the quantification results of pharmaceuticals separation with %recoveries ranging from 92.8 to 98.7 and %RSDs less than 4.7.

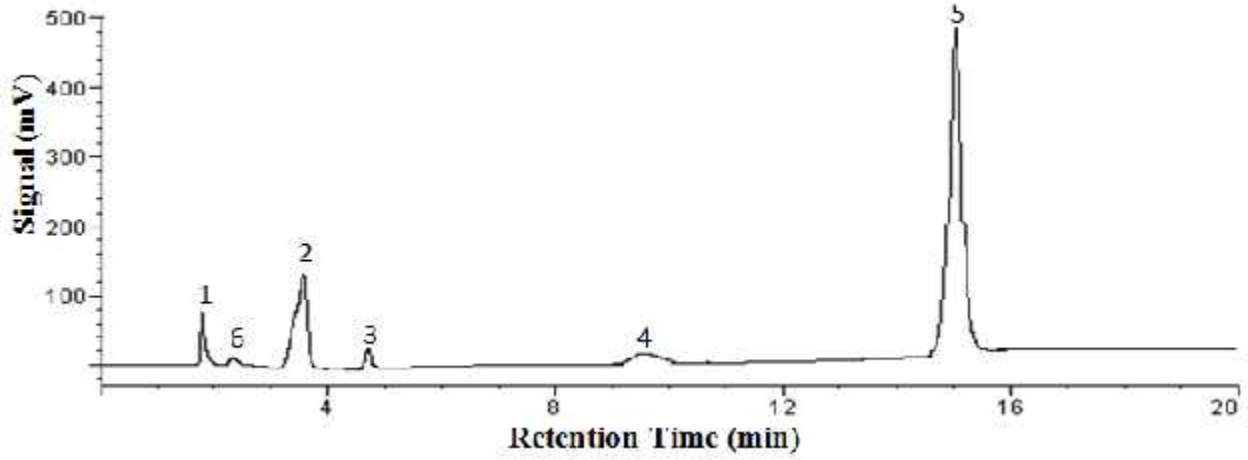
Table 6.2. Concentration of Pharmaceuticals Found in Bayer Alka-Seltzer Plus Cold and Flu Formula Sample Obtained by SBWC Using Alltech Adsorbosil C18 Column

Pharmaceuticals	Bayer Stated Concentration (W)	Concentration Found by This Method (W)	%Recovery	%RSD[a]
Dextromethorphan Hydrobromide	10.0	9.6	96.0	1.3
Doxylamine Succinate	6.2	5.8	92.8	4.7
Phenylephrine Hydrochloride	5.0	4.7	94.0	1.6
Acetaminophen	325.0	320.7	98.7	1.3

[a]*Based on five replicates.*



a



b

Figure 6.3. Subcritical water chromatography chromatograms of pharmaceuticals obtained on the Alltech Adsorbosil C18 column at programmed temperatures with 1.0 mL/min. (a) Pharmaceutical standard mixture; (b) Alka-Seltzer plus cold and flu formula sample. UV detection: 210 nm. Mobile phase: A, deionized water; B, 100 mM phosphoric acid. Gradient: 100% water for 1 min and then 100 mM phosphoric acid for rest of the run. Programmed temperatures: Initial temperature of 25 ^{o}C for 3 min and then increased at 15 ^{o}C/min to 150 ^{o}C and maintained at 150 ^{o}C for rest of the run. Peak identification: 1, dextromethorphan hydrobromide; 2, chlorpheniramine maleate; 3, doxylamine succinate; 4, phenylephrine hydrochloride; 5, acetaminophen; 6, matrix peak.

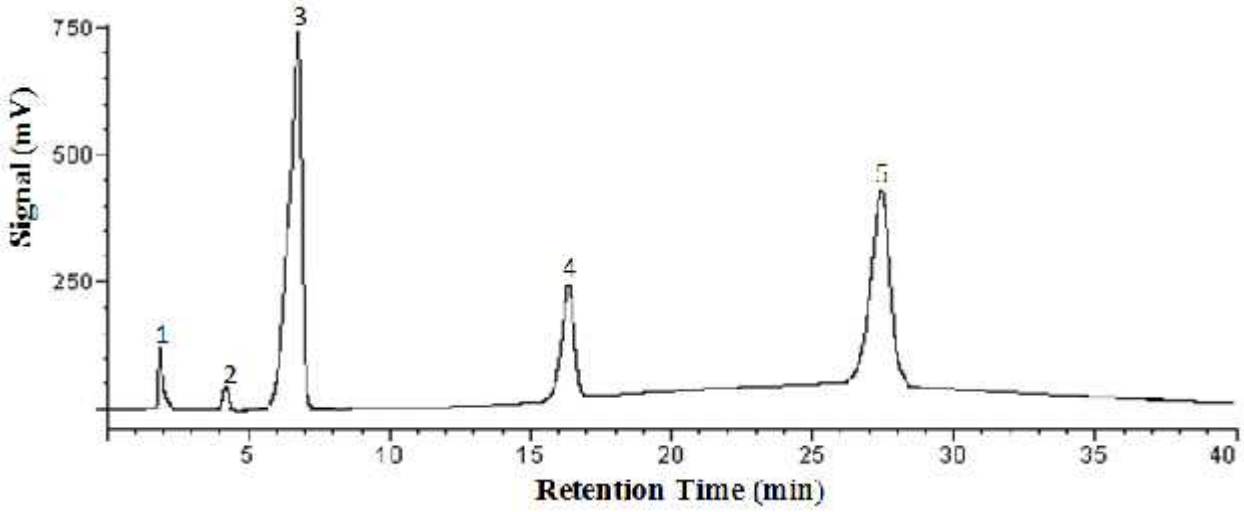
6.3.1.3 SBWC Separation and Analysis of Pharmaceuticals in CVS Cold Relief

The SBWC separation of pharmaceuticals in CVS cold drug was carried out using only 100 mM phosphoric acid as the mobile phase at higher temperatures. These SBWC separations were optimized and the best separation was achieved using programmed temperatures. Figure 6.4 shows the chromatograms of pharmaceuticals standard and CVS cold drug sample. Table 6.3 shows the five replicate experiments of pharmaceuticals with %recoveries ranging from 97.4 to 102.0 and %RSDs lower than 1.9. The pharmaceuticals in these SBWC separations have better resolutions, when compared with the SBWC results of Vicks and Alka-Seltzer cold drug analysis. The quantification results for the green separations of these three real life samples indicate a potential for applications of subcritical water chromatography in industry.

Table 6.3. Concentration of Pharmaceuticals Found in CVS Multi-Symptom Severe Cold Relief Sample Obtained by SBWC Using Alltech Adsorbosil C18 Column[a]

Pharmaceuticals	CVS Stated Concentration (W)	Concentration Found by This Method (W)	%Recovery	%RSD[a]
Dextromethorphan Hydrobromide	10.0	10.1	101.0	1.9
Phenylephrine Hydrochloride	5.0	5.1	102.0	1.1
Acetaminophen	325.0	319.1	98.2	1.1
Guaifenesin	200.0	194.8	97.4	1.9

[a]*Based on five replicates.*



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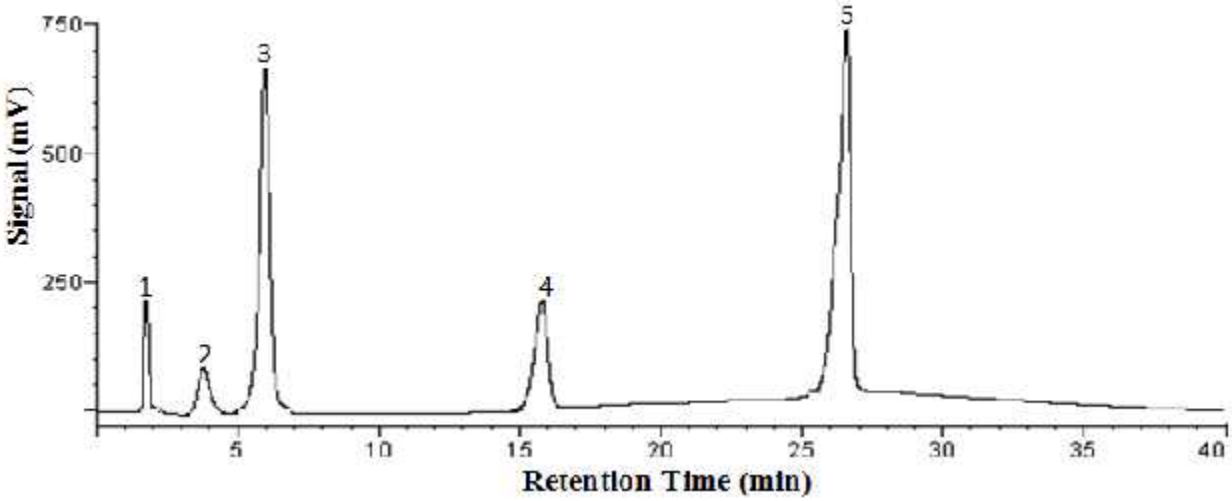


b

Figure 6.4. Subcritical water chromatography chromatograms of pharmaceuticals obtained on the Alltech Adsorbosil C18 column at programmed temperatures with 1.0 mL/min using 100 mM phosphoric acid as mobile phase. (a) Pharmaceutical standard mixture; (b) CVS multi-symptom severe cold relief sample. Programmed temperatures: Initial temperature of 90 ^{o}C for 5.5 min and then increased at 15 ^{o}C/min to 150 ^{o}C and maintained at 150 ^{o}C for rest of the run. Peak identification: 1, dextromethorphan hydrobromide; 2, phenylephrine hydrochloride; 3, acetaminophen; 4, benzyl alcohol; 5, guaifenesin.

6.3.2 Analysis of Niacinamide Present in Skincare Creams

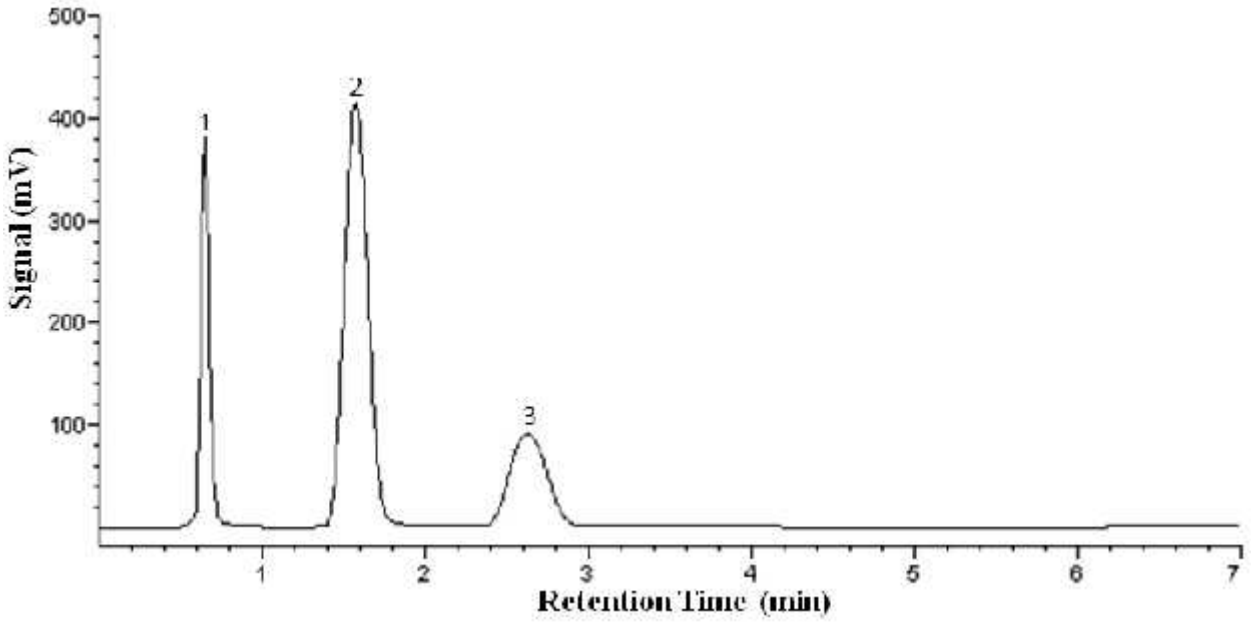
In addition to a large portion of this work [18] conducted by Zackary Strickland from our group, the SBWC separation of niacinamide and niacin was carried out on Waters XBridge C18 column at 60 ^{o}C. Although niacin is not present in skincare cream, it was added to the standard for the broader applications of this developed SBWC method. These separations were optimized and the best separation was achieved with 2.0 mL/min using Shimadzu system. Figure 6.5 shows the chromatograms of niacinamide standard and P&G SC-EC2 skincare cream sample. Table 6.4 shows the five replicate measurements of niacinamide with 100.5 %recovery and 1.3% RSD. These results demonstrate the accuracy and precision of the developed SBWC method for niacinamide in skincare creams.

Table 6.4. Recovery of Niacinamide Present in SC-EC2 Skincare Cream Sample Achieved by Subcritical Water Chromatography Compared with the Concentration Obtained by HPLC at 25 °C Using 30% Methanol in the Mobile Phase

	%Recovery[a]	%RSD[b]
Niacinamide	100.5	1.3

[a] $R\quad \% = \frac{W\quad h\varepsilon\%\ o}{W\quad h\varepsilon\%\ o} \times 100\%$

[b] *Based on five replicate sample preparations analyzed by both pure water chromatography and HPLC.*



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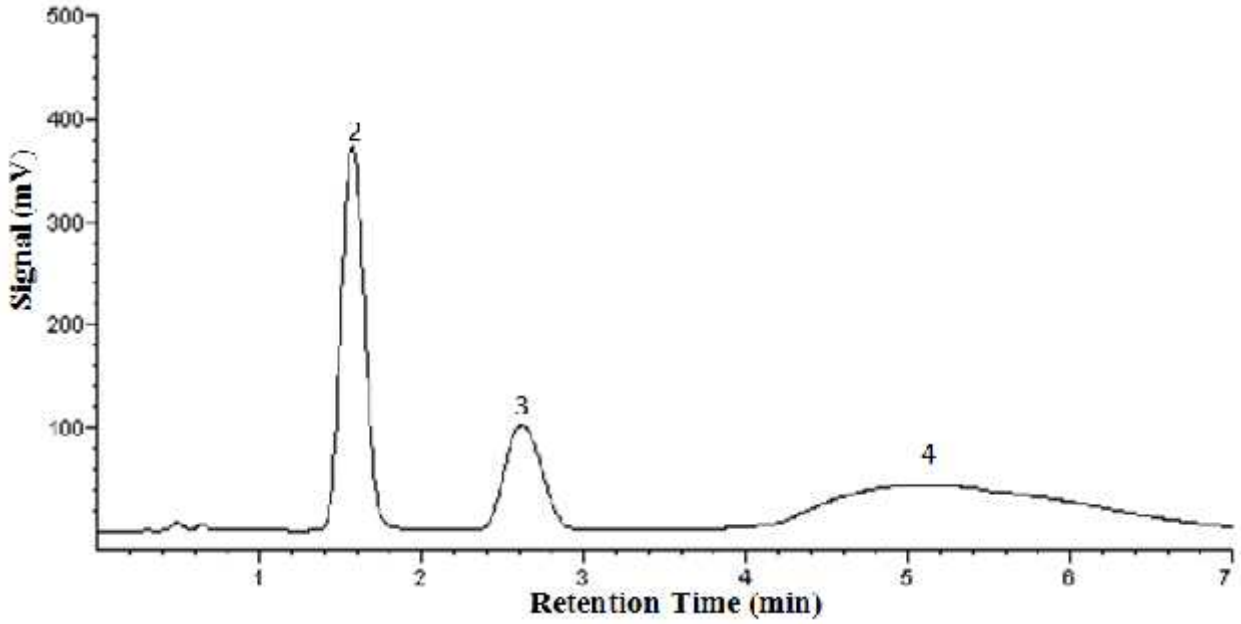


b

Figure 6.5. Subcritical water chromatography chromatograms of niacinamide obtained on the XBridge C18 column using pure water as the mobile phase at 60 ºC with 2 mL/min. (a) Standard mixture containing niacin and niacinamide; (b) Olay total effects, 7-in-1 anti-ageing UV moisturizer sample. UV detection: 245 nm. Peak identification: 1, niacin; 2, niacinamide; 3, 4-acetamidophenol; 4, matrix peak.

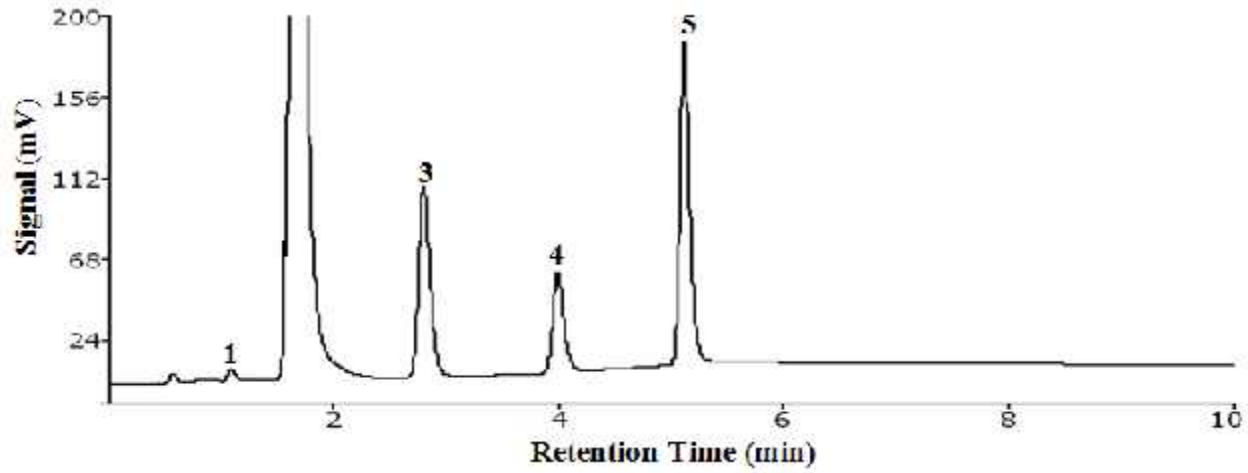
6.3.3 Analysis of Preservatives Present in Skincare Creams

6.3.3.1 Separation and Analysis of Preservatives on ZirChrom-DB-C18 Column

All the HPLC, HTLC and SBWC methods developed for the separation of preservatives on ZirChrom-DB-C18 column were carried out using the home-made system.

6.3.3.1.1 Separation of Preservatives by High Temperature Liquid Chromatography

High temperature liquid chromatography methods were developed for the separation of preservatives to decrease the organic content in the mobile phase. At first the HTLC separation of preservatives was conducted on ZirChrom-DB-C18 column at 90 ^{o}C using methanol-water mixture as the mobile phase. Although good HTLC separation of preservatives was achieved at 90 ^{o}C, but this method does not saved much of the organic content in the mobile phase when compared with the HPLC method for the separation of preservatives carried out in our lab at room temperature. Then further HTLC separations of preservatives were conducted at 150 ^{o}C. These separations were optimized using different flow rates and the best separation was achieved with 2 mL/min on ZirChrom-DB-C18 column. Figure 6.6 shows the HTLC separation of preservatives in SC-EC1 and SC-EC3 skincare cream sample at 150 ^{o}C. The separation conditions are stated in Figure 6.6 legend. When compared with the HPLC method, this HTLC method at 150 ^{o}C, saved about 75% of methanol in the mobile phase. Tables 6.5 to 6.7 shows the quantification results of preservatives present in three Olay skincare creams samples.



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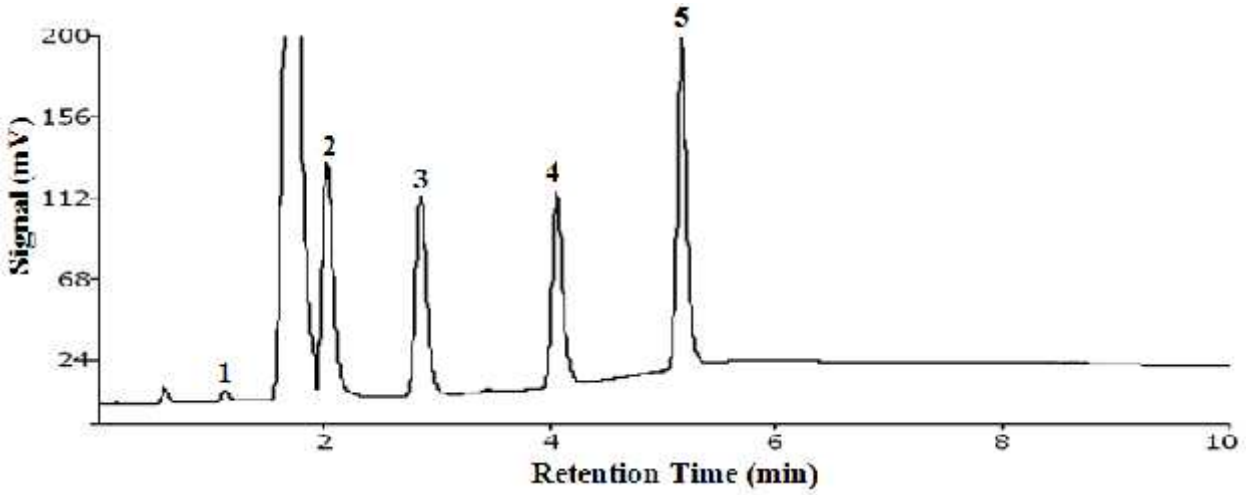


b

Figure 6.6. High temperature liquid chromatography chromatograms obtained on ZirChrom-DB-C18 column at 150 ºC. (a) SC-EC1 skincare cream sample; (b) SC-EC3 skincare cream sample. Flow rate: 2.0 mL/min. UV detection: 256 nm. Mobile phase: A, deionized water; B, 100% methanol. Gradient: 0-2 min, 10-20% methanol; 2-6 min, 20-50% methanol; 6-10 min, 50% methanol; 10-10.5 min, 50-10% methanol. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)

Table 6.5. Comparison of Recoveries for Preservatives Present in SC-EC1 Skincare Cream Obtained by HPLC, HTLC, and SBWC Using ZirChrom-DB-C18 Column (Reproduced With Permission from Reference 20 © The Authors ICS, 2012.)

	%Recovery (%RSD[a])		
	HPLC at 25 ºC	HTLC at 150 ºC	SBWC at 200 ºC
Methanol Saved	0%	75%	100%
Benzyl Alcohol	104.3 (3.2)	97.2 (4.0)	Co-eluted
Ethyl Paraben	105.5 (5.1)	106.2 (5.9)	101.8 (1.5)
Propyl Paraben	104.0 (4.8)	96.8 (5.2)	102.4 (1.9)

[a] *Based on five replicates.*

Table 6.6. Comparison of Recoveries for Preservatives Present in SC-EC2 Skincare Cream Obtained by HPLC, HTLC, and SBWC Using ZirChrom-DB-C18 Column (Reproduced With Permission from Reference 20 © The Authors ICS, 2012.)

	%Recovery (%RSD[a])		
	HPLC at 25 ºC	HTLC at 150 ºC	SBWC at 200 ºC
Methanol Saved	0%	75%	100%
Benzyl Alcohol	104.1 (3.9)	97.4 (2.9)	Co-eluted
Methyl Paraben	104.2 (4.1)	104.6 (1.9)	104.2 (3.7)
Ethyl Paraben	105.0 (4.4)	104.8 (2.1)	102.1 (3.2)
Propyl Paraben	106.0 (4.3)	97.6 (2.7)	100.9 (2.1)

[a] *Based on five replicates.*

Table 6.7. Comparison of Recoveries for Preservatives Present in SC-EC3 Skincare Cream Obtained by HPLC, HTLC, and SBWC Using ZirChrom-DB-C18 Column (Reproduced with permission from reference 20 © The Authors ICS, 2012.)

	%Recovery (%RSD[a])		
	HPLC at 25 ºC	HTLC at 150 ºC	SBWC at 200 ºC
Methanol Saved	0%	75%	100%
Benzyl Alcohol	103.2 (4.7)	99.4 (2.3)	Co-eluted
Methyl Paraben	102.2 (3.9)	103.9 (3.4)	98.8 (2.5)
Ethyl Paraben	105.0 (4.7)	102.3 (2.8)	103.0 (3.2)
Propyl Paraben	105.9 (4.9)	102.7 (2.6)	101.4 (2.3)

[a] *Based on five replicates.*

In order to determine the system building-up with real sample analysis by HTLC method developed at 150 ^{o}C, a total of 24 replicate injections of SC-EC3 sample preparation separations were conducted continuously. Table 6.8 shows the 24 replicate measurements with %recoveries ranging from 94.6 to 101.0 and %RSDs less than 2.2. These results infer that there is no system building-up with the real sample analysis by the HTLC method developed at 150 ^{o}C.

Table 6.8. Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by 24 Replicate HTLC Runs Using ZirChrom-DB-C18 Column at 150 ^{o}C with 2.0 mL/min (Reproduced With Permission from Reference 20 © The Authors ICS, 2012.)

	%Recovery	%RSD[a]
Benzyl Alcohol	94.6	2.2
Methyl Paraben	99.3	1.8
Ethyl Paraben	99.1	1.8
Propyl Paraben	101.0	1.2

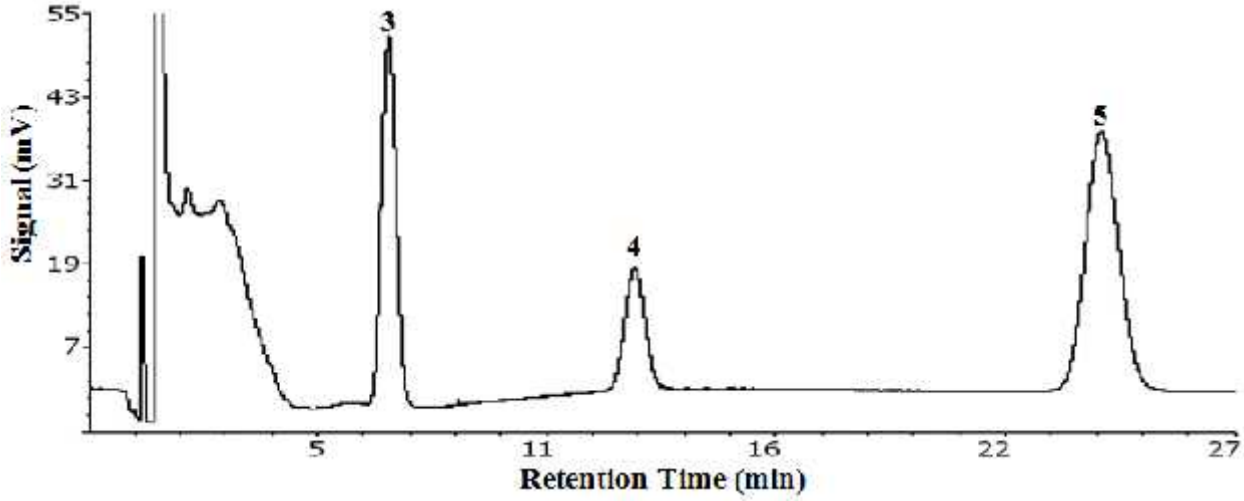
[a] *Based on 24 replicate injections of a single sample solution.*

6.3.3.1.2 Separation of Preservatives by Subcritical Water Chromatography

In order to completely eliminate the organic solvents from mobile phase, separation of preservatives were carried out using water as the mobile phase on ZirChrom-DB-C18 column at 200 ^{o}C. These SBWC separations were optimized at different flow rates and the best separation was achieved using the programmed flow rates as stated in Figure 6.7 legend. Figure 6.7 shows the SBWC separation of preservatives from three of the Olay skincare creams. Accuracy and precision of these SBWC methods can be known from the reasonable %recoveries and %RSDs given in Tables 6.5 to 6.7. The only limitation with these SBWC methods is the co-elution of benzyl alcohol with a matrix peak.

HPLC separations of preservatives present in P&G skincare creams were also conducted to further evaluate the HTLC and SBWC separations of preservatives mentioned above. These HPLC separations were also carried out on ZirChrom-DB-C18 column at 25 ^{o}C using methanol-

water mixture as mobile phase. Tables 6.5 to 6.7 also show the quantification results for the HPLC separation of preservatives on ZirChrom-DB-C18. These results infer that the developed HTLC methods at 150 ^{o}C and SBWC methods at 200 ^{o}C are as good as HPLC separations of preservatives at 25 ^{o}C.



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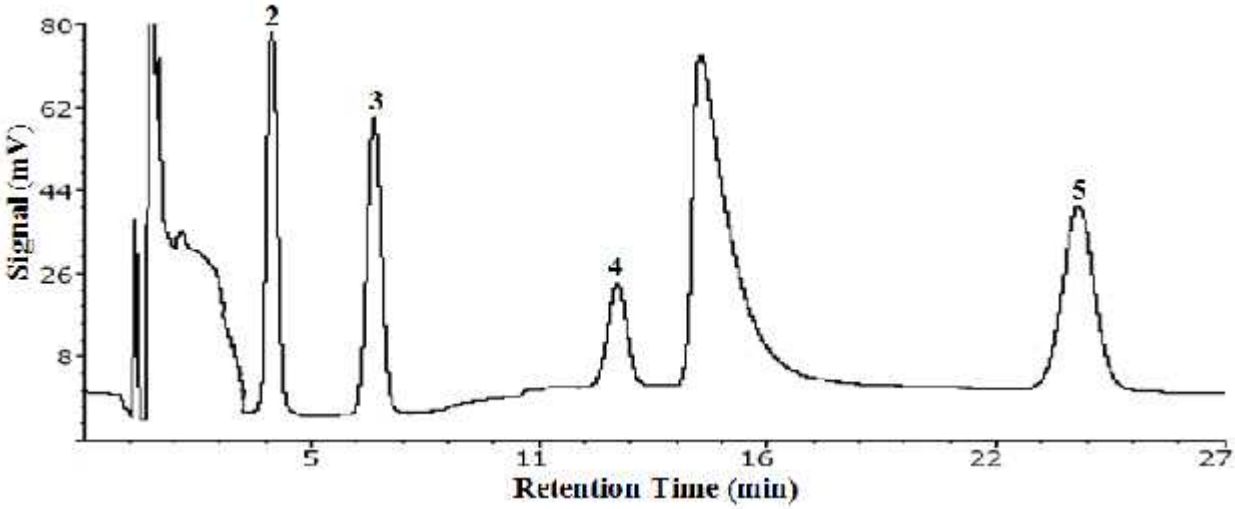

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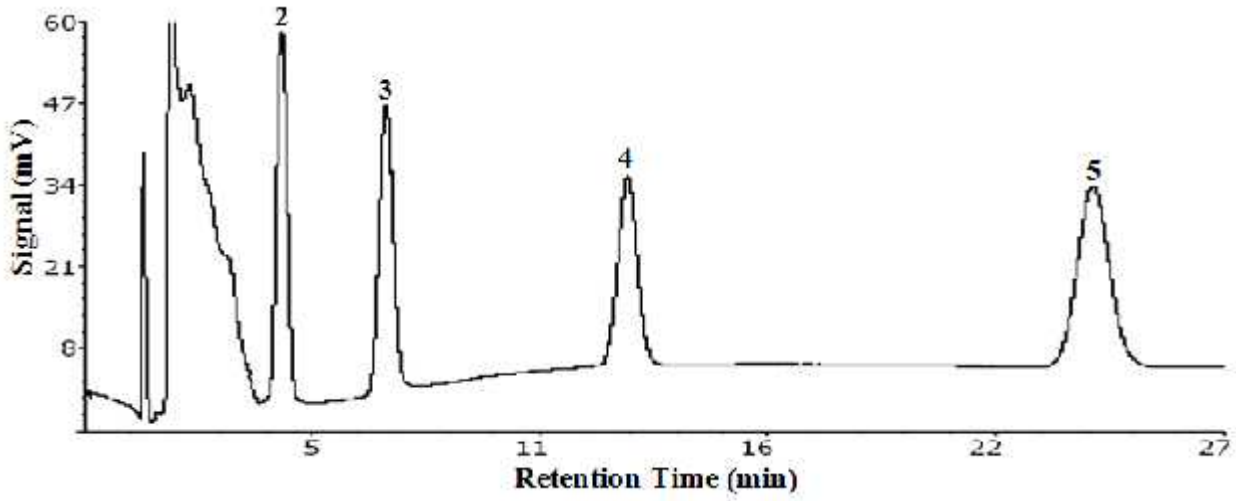


c

Figure 6.7. Subcritical water chromatography chromatograms obtained on ZirChrom-DB-C18 column at 200 ºC using 100% water as the mobile phase. (a) SC-EC1 skincare cream sample; (b) SC-EC2 skincare cream sample; (c) SC-EC3 skincare cream sample. UV detection: 256 nm. Programmed flow rates: 0-6.5 min, decreased from 1.0 mL/min to 0.75 mL/min; 6.5-27 min, 0.75 mL/min; 27-27.5 min, increased from 0.75mL/min to 1.0 mL/min. Peak identification: 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)

6.3.3.2 SBWC Separation and Analysis of Preservatives on ZirChrom-PS Column

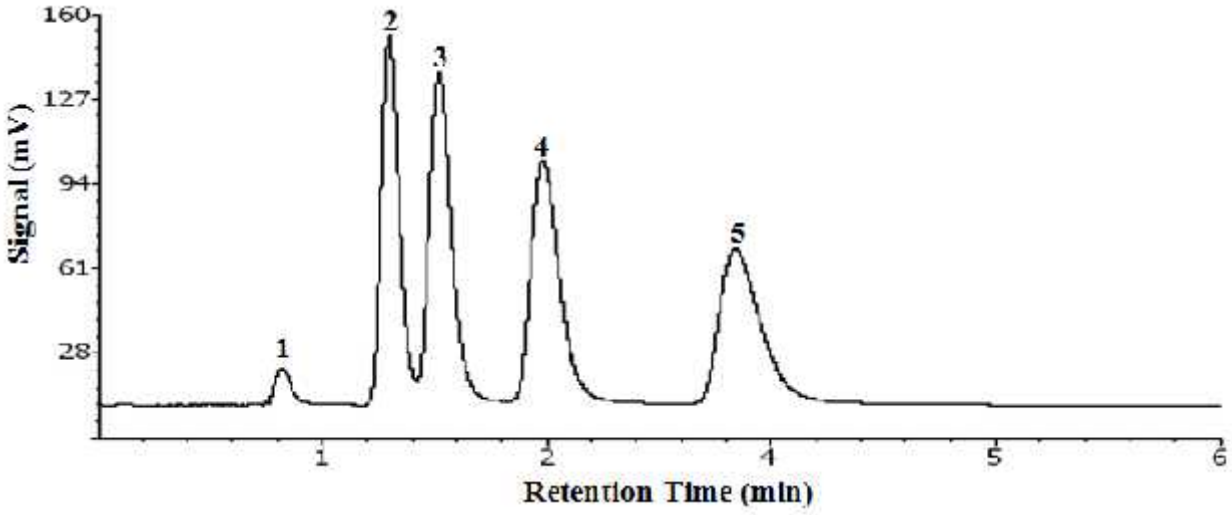
Similar to the SBWC studies on ZirChrom-DB-C18 column, SBWC separation of preservatives were carried out on the ZirChrom-PS column at 180 ^{o}C. These SBWC separations were conducted using only subcritical water as the mobile phase with a home-made system. Figure 6.8 shows the separation of preservatives standard and SC-EC3 skincare cream sample achieved by subcritical water chromatography. These SBWC separations were optimized and the best separation was achieved with 1.25 mL/min. Table 6.9 shows the five replicate measurements of preservatives separation with %recoveries ranging from 101.7 to 105.3 and

%RSDs less than 3.9. There is a significant decrease in retention time for the SBWC separation of preservatives on ZirChrom-PS column, when compared with the SBWC separation of preservatives on ZirChrom-DB-C18 column. Although parabens were well separated on the ZirChrom-PS column, benzyl alcohol was still co-elutied with a matrix peak as shown in Figure 6.8.

Table 6.9. Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by SBWC Using ZirChrom-PS Column at 180 ºC and 1.25 mL/min (Reproduced With Permission from Reference 20 © The Authors ICS, 2012.)

	%Recovery	%RSD[a]
Benzyl Alcohol	Co-eluted	
Methyl Paraben	104.5	3.8
Ethyl Paraben	101.7	1.3
Propyl Paraben	105.3	3.9

[a] *Based on five replicates.*



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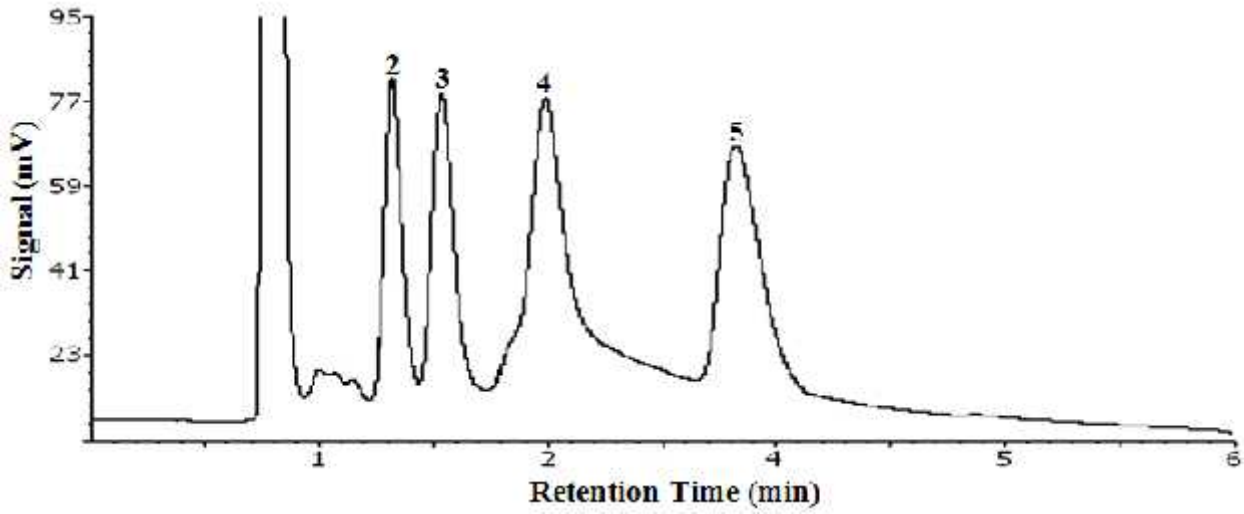


b

Figure 6.8. SBWC chromatograms obtained on ZirChrom-PS column at 180 ºC and 1.25 mL/min using 100% water as the mobile phase. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. UV detection: 256 nm. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)

6.3.3.3 Separation and Analysis of Preservatives on Waters XBridge C18 Column

All the HTLC and SBWC methods developed for the separation of preservatives were carried out using the commercial Shimadzu system on Waters XBridge C18 column. These separations were optimized at different flow rates and the best separation was achieved with 1.0 mL/min.

6.3.3.3.1 Separation of Preservatives by an Integrated SBWC/HTLC Method

An integrated SBWC/HTLC method was developed for the separation of preservatives on Waters XBridge C18 column at 150 ^{o}C. Subcritical water was the only mobile phase component for most of the run and a methanol-water mixture was used for the last 3 min of the run. Figure

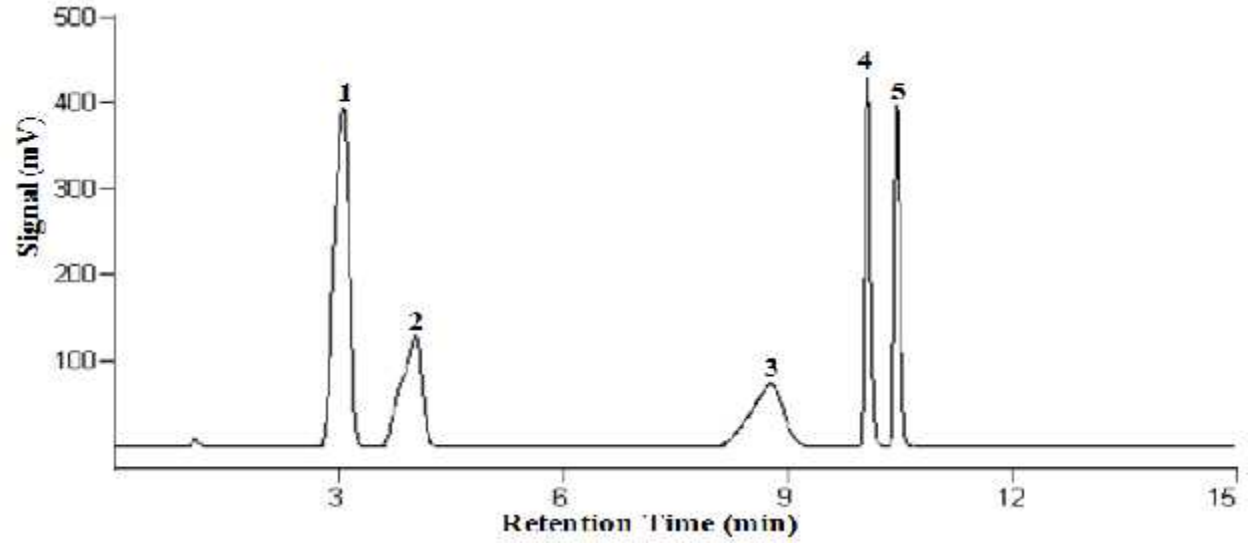
69 shows the separation of preservatives standard and SC-EC3 skincare cream sample achieved by the integrated SBWC/HTLC method. The separation conditions are stated in Figure 6.9 legend. This integrated SBWC/HTLC method differs from HTLC methods in terms of the methanol use in the mobile phase. As integrated SBWC/HTLC methods use methanol for only a short period of run, a large portion of the chromatography waste does not require waste disposal. Where as in HTLC methods, all chromatography waste requires waste disposal due to the use of methanol in the mobile phase throughout the run.

The main advantage with the preservatives separations on XBridge C18 column is the separation of benzyl alcohol from the matrix peak, when compared with the methods developed on zirconia columns. One more advantage is the increase of benzyl alcohol peak intensity by 40 folds with the use of UV detection at 210 nm as shown in Figure 6.9. The Quantification results obtained for the preservatives separations using 210 nm and 256 nm by the integrated SBWC/HTLC method are shown in Table 6.10. These results prove that the use of either 210nm or 256nm does not affect the quantification results of preservatives.

Table 6.10. Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by the Integrated SBWC/HTLC at 150 °C Using Waters XBridge C18 Column with Gradient Elution as Described in Figure 6.9 legend (Reproduced With Permission from Reference 20 © The Authors ICS, 2012.)

	%Recovery (%RSD[a])	
	Detection at 210 nm	Detection at 256 nm
Benzyl Alcohol	100.3 (1.1)	98.1 (0.8)
Methyl Paraben	102.9 (0.4)	104.4 (0.4)
Ethyl Paraben	101.8 (0.3)	103.7 (0.4)
Propyl Paraben	105.3 (1.3)	104.5 (1.3)

[a] *Based on five replicates.*



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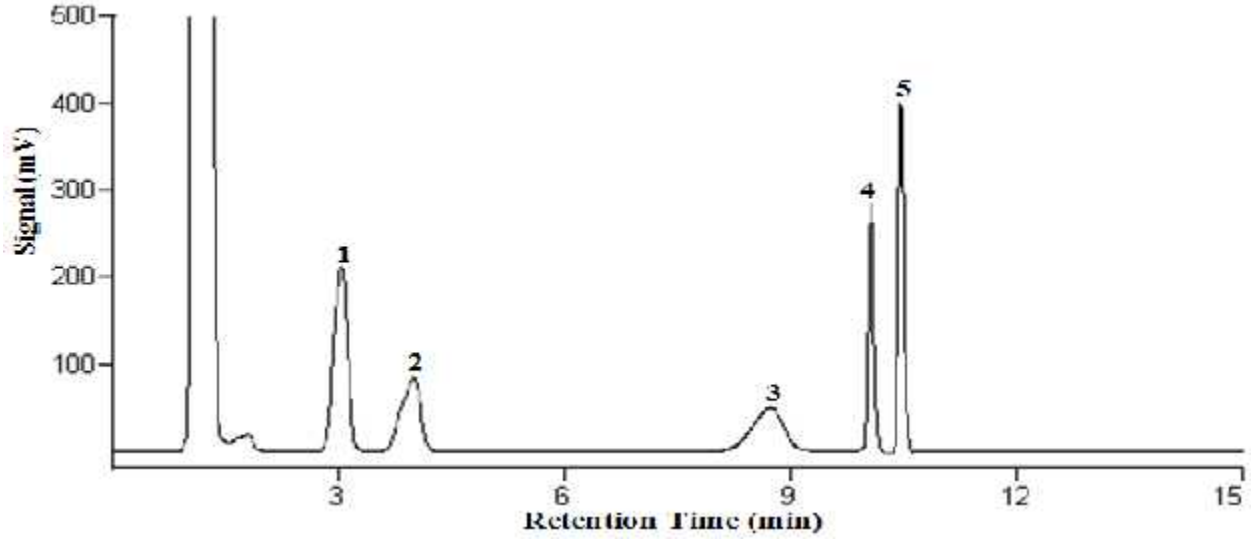

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Figure 6.9. Integrated SBWC/HTLC chromatograms obtained on XBridge C18 column at 150 ºC and 1.0 mL/min (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. UV detection: 210 nm. Gradient: 0-7.9 min, 0% methanol; 7.9-8 min, 0-50% methanol; 8-11 min, 50% methanol; 11-11.1 min, 50-0% methanol; 11.1-15 min, 0% methanol. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)

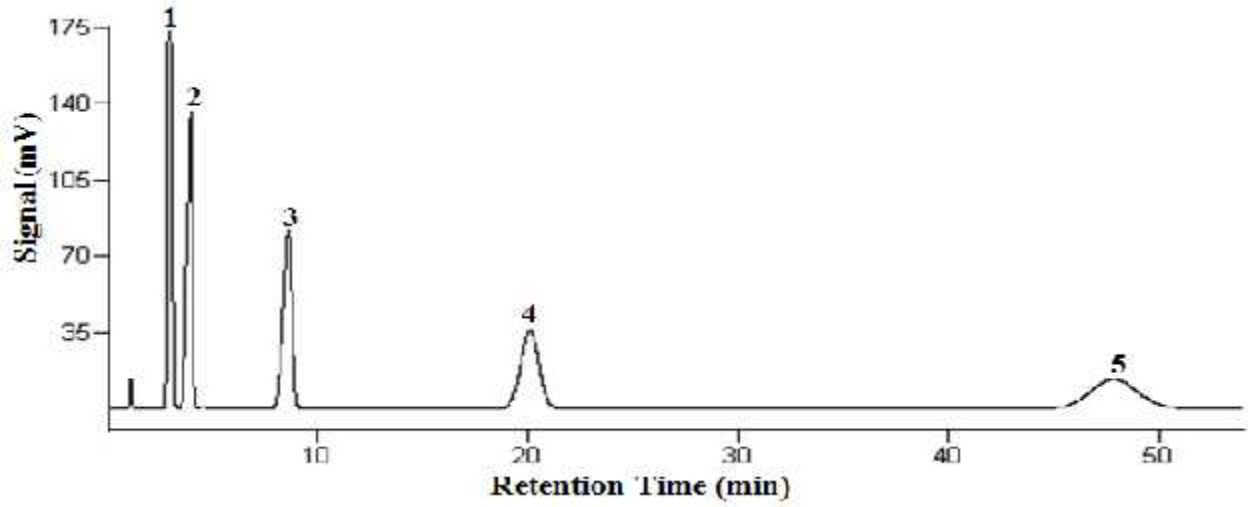
6.3.3.3.2 Separation of Preservatives by Subcritical Water Chromatography

Similar to the previous SBWC separation of preservatives on zirconia columns, the SBWC separations were carried out on the Waters XBridge C18 column at 150 ^{o}C. These separations were conducted using the best chromatogram mode. In the best chromatogram mode, first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm and the remaining paraben peaks at 256 nm. Figure 6.10 shows the separation of preservatives standard and SC-EC3 skincare cream sample by the subcritical water chromatography. Table 6.11 shows the five replicate measurements of preservatives separation with reasonable %recoveries ranging from 101.8 to 103.4 and %RSDs lower than 1.0.

Table 6.11. Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by SBWC at 150 °C Using Waters XBridge C18 Column with 1.0 mL/min and the Best Chromatogram Mode[a] (Reproduced With Permission from Reference 20 © The Authors ICS, 2012.)

	%Recovery	%RSD[b]
Benzyl Alcohol	101.8	0.6
Methyl Paraben	104.4	0.6
Ethyl Paraben	103.6	0.4
Propyl Paraben	103.4	1.0

[a] *Best chromatogram mode: Detection at 210 nm during the first 7 min; detection at 256 nm during the remainder of the chromatography run.*
[b] *Based on five replicates.*



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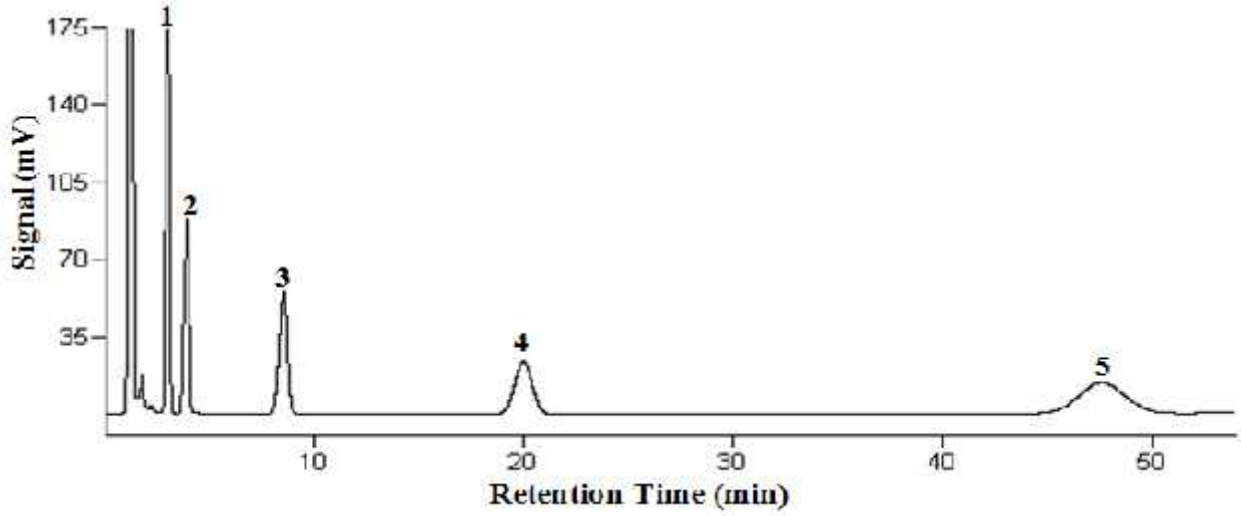
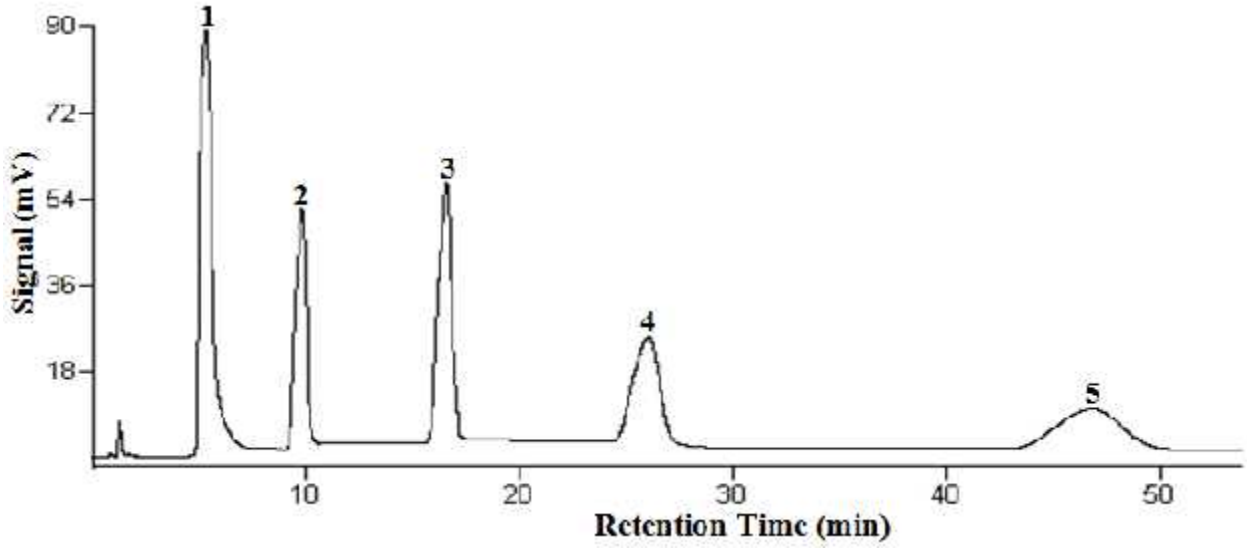


b

Figure 6.10. SBWC chromatograms obtained using 100% water on XBridge C18 column at 150 ºC and 1.0 mL/min with the best chromatogram mode. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, while the other parabens were detected at 256 nm. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)

6.3.3.3.3 SBWC Separation of Preservatives Using Programmed Temperatures

As discussed in Chapter 5, the long-term stability of the column was improved when a programmed temperatures was employed. In order to enhance the stability of the Waters XBridge C18 column, the SBWC separation of preservatives was conducted using a programmed temperatures. In the programmed temperatures evaluation, the initial temperature of 100 ^{o}C was increased to 150 ^{o}C at a rate of 15 ^{o}C/min and maintained at 150 ^{o}C for rest of the run. Figure 6.11 shows the reasonable SBWC chromatograms of preservatives standard and SC-EC3 skincare cream sample using programmed temperatures on Waters XBridge C18 column.



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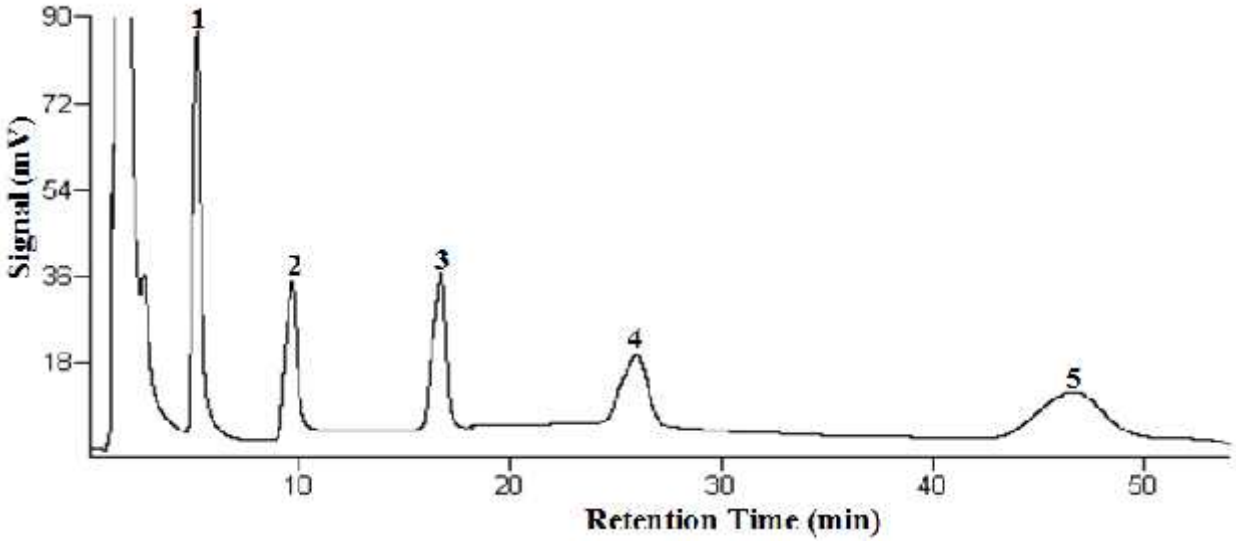


b

Figure 6.11. SBWC chromatograms obtained using 100% water on XBridge C18 column with programmed temperatures at 1.0 mL/min. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, while the other parabens were detected at 256 nm. Programmed temperatures: initial temperature of 100 ^{o}C was increased to 150 ^{o}C at 15 ^{o}C/min and then maintained at 150 ^{o}C. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)

6.3.3.3.4 Potential System Building-up Studies

In order to know whether subcritical water can adequately clean the sample matrix after each SBWC run, potential building-up studies were conducted with a large number of replicate injections of a single sample solution. These studies were conducted under subcritical water chromatographic conditions on Waters XBridge C18 column at 150 ^{o}C. Table 6.12 shows

quantification results of replicate injections of a single preservatives sample solution. The recoveries range from 101.0 to 106.3% and the relative standard deviations is less than 1.6%. These results indicate that there is no potential building-up with the continuous SBWC separations of preservatives on Waters XBridge C18 column at 150 ^{o}C.

Table 6.12. Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by SBWC at 150 ºC with the Best Chromatogram Mode Resulted from the Building-up Studies (Reproduced With Permission from Reference 20 © The Authors ICS, 2012.)

	%Recovery (%RSD[a])	
	Waters XBridge C18 Column[b]	Waters XBridge Phenyl Column[c]
Benzyl Alcohol	101.8 (1.2)	99.6 (1.8)
Methyl Paraben	106.3 (1.6)	105.0 (1.7)
Ethyl Paraben	101.0 (0.8)	99.9 (1.5)
Propyl Paraben	102.8 (0.8)	98.3 (1.6)

[a] *Based on 21 replicate injections of a single sample solution.*
[b] *Flow rate of 1.0 mL/min.*
[c] *Programmed flow rates as described in Figure 6.13 legend.*

6.3.3.4 SBWC Separation and Analysis of Preservatives on Waters XBridge Phenyl Column

All the SBWC separations of preservatives were conducted on XBridge Phenyl column using the commercial Shimadzu system.

6.3.3.4.1 SBWC Separation of Preservatives at Constant Flow Rate

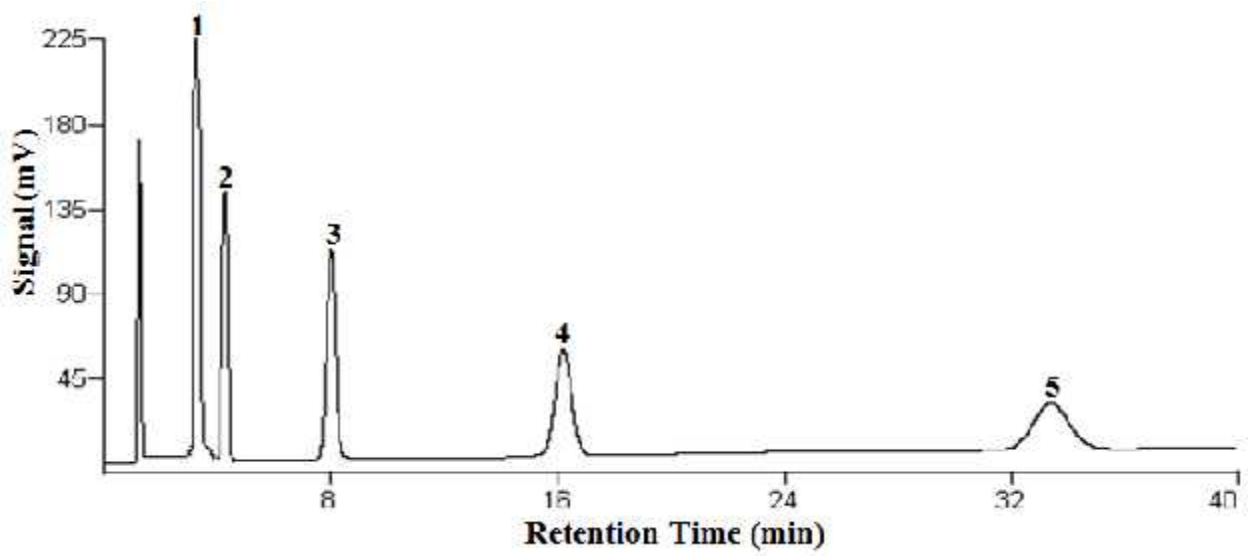
The separations of preservatives were carried out on Waters XBridge Phenyl column using subcritical water as the mobile phase at 150 ^{o}C. These separations were conducted using the best chromatogram mode with 1.0 mL/ min. In the best chromatogram mode, the first two peaks were detected at 210 nm, while the other parabens were detected at 256 nm. As methyl paraben has a similar molar absorptivity at both 210 and 256 nm, therefore methyl paraben was detected at 210 nm. Figure 6.12 shows the separation of preservatives standard and SC-EC3

skincare cream sample obtained by the subcritical water chromatography. Benzyl alcohol was again well separated from sample matrix peak in these SBWC separations. Table 6.13 shows the five replicate measurements of preservatives separations with reasonable %recoveries ranging from 102.7 to 107.3 and %RSDs lower than 1.5.

Table 6.13. Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by SBWC at 150 °C Using Waters XBridge Phenyl Column and the Best Chromatogram Mode[a] (Reproduced With Permission from Reference 20 © The Authors ICS, 2012.)

	%Recovery (%RSD[b])	
Flow Rate	1 mL/min	Programmed Flow[c]
Benzyl Alcohol	102.7 (1.1)	99.5 (2.5)
Methyl Paraben	103.6 (0.6)	102.5 (2.2)
Ethyl Paraben	105.1 (1.2)	101.2 (1.5)
Propyl Paraben	107.3 (1.5)	101.6 (2.1)

[a] *Best chromatogram mode: Detection at 210 nm during the first 7 min; detection at 256 nm during the remainder of the chromatography run.*
[b] *Based on five replicates.*
[c] *Programmed flow rates as described in Figure 6.13 legend.*



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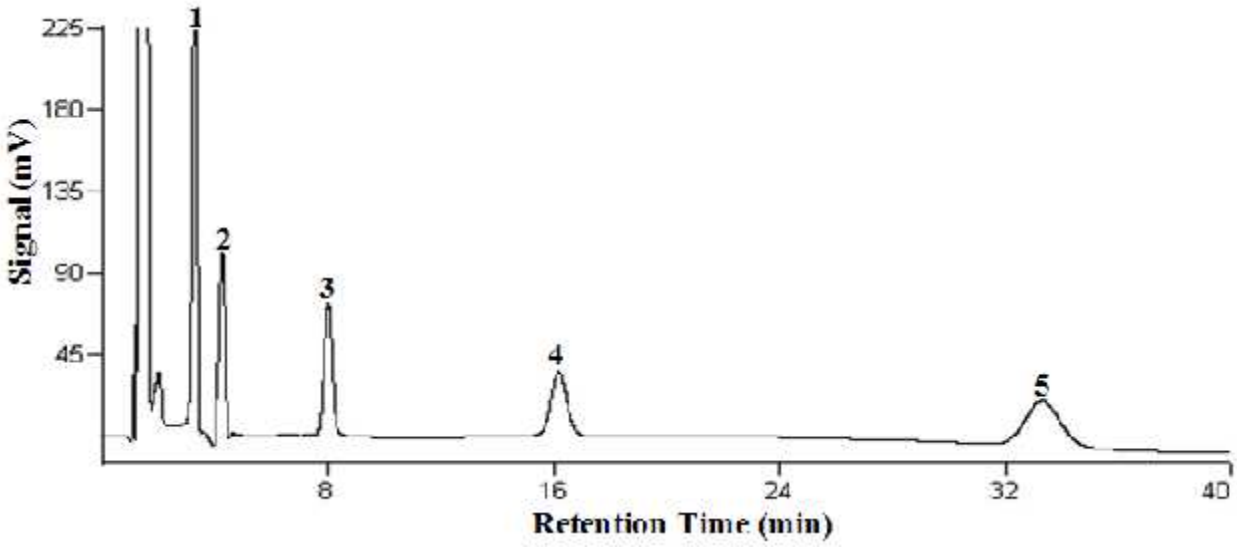
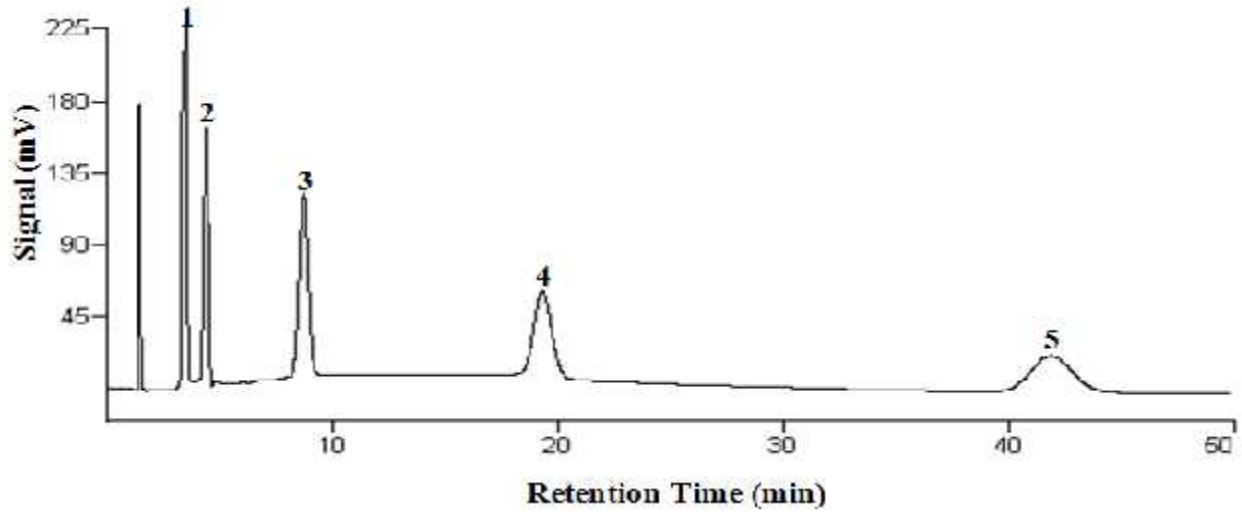


b

Figure 6.12. SBWC chromatograms obtained using 100% water on XBridge phenyl column at 150 ºC and 1.0 mL/min. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, while the other parabens were detected at 256 nm. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)

6.3.3.4.2 Optimization of SBWC Methods with Programmed Flow Rates

The SBWC methods developed for the separation of preservatives on Waters XBridge Phenyl column at 150 ^{o}C were also carried out using a programmed flow rates. The programmed flow rates conditions are given in Figure 6.13 legend. Figure 6.13 shows the separation of preservatives standard and SC-EC3 skincare cream sample achieved by the subcritical water chromatography using the programmed flow rates. Table 6.13 also shows the quantification results of preservatives separations by programmed flow rates with reasonable %recoveries ranging from of 99.5 to 102.5 and %RSDs less than 2.5.



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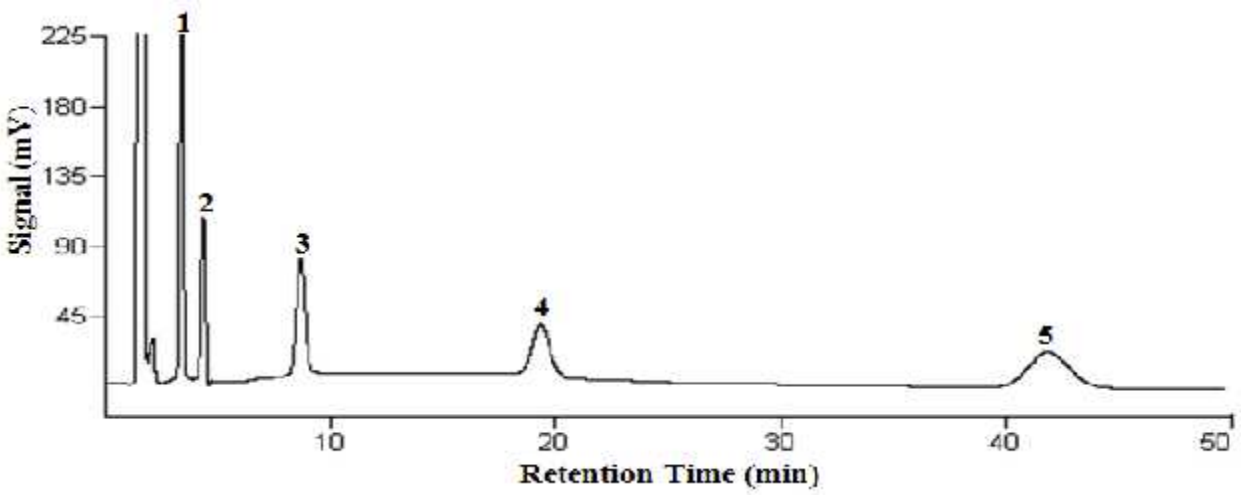


b

Figure 6.13. SBWC chromatograms obtained using 100% water on XBridge phenyl column at 150 ºC with programmed flow rates. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, while the other parabens were detected at 256 nm. Programmed flow rates: 0-6.5 min, decreased from 1.0 mL/min to 0.75 mL/min; 6.5-47 min, 0.75 mL/min; 47-50 min, increased from 0.75mL/min to 1.0 mL/min. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)

6.3.3.4.3 SBWC Separation of Preservatives Using Programmed Temperatures

Similar to the stability enhancement studies on the Waters XBridge C18 column, SBWC methods were developed for the separation of preservatives on Waters XBridge Phenyl column using the same programmed temperatures. Figure 6.14 shows reasonable SBWC separation of preservatives standard and SC-EC3 skincare cream sample using the programmed temperature elution given in Figure 6.14 legend.

To further validate the developed SBWC methods on Waters XBridge Phenyl column, HPLC methods were developed for the separation of preservatives on the same column using methanol-water mixture as mobile phase at ambient temperature. Table 6.14 shows the five replicate measurements of preservatives separations by HPLC with %recoveries ranging from 101.7 to 105.2 and %RSDs less than 1.2. When comparing the HPLC results with that of SBWC, both quantification results are at the same level, demonstrating the reliability of the developed SBWC method for the separation of preservatives on the Waters XBridge Phenyl column.

Table 6.14. Recovery of Preservatives Present in SC-EC3 Skincare Cream Obtained by Traditional HPLC with Ambient Methanol-Water Mixtures as the Eluent on Waters XBridge Phenyl Column (Reproduced With Permission from Reference 20 © The Authors ICS, 2012.)

	%Recovery	%RSD[a]
Benzyl Alcohol	101.7	0.6
Methyl Paraben	104.8	0.7
Ethyl Paraben	101.8	0.9
Propyl Paraben	105.2	1.2

[a] *Based on five replicates.*

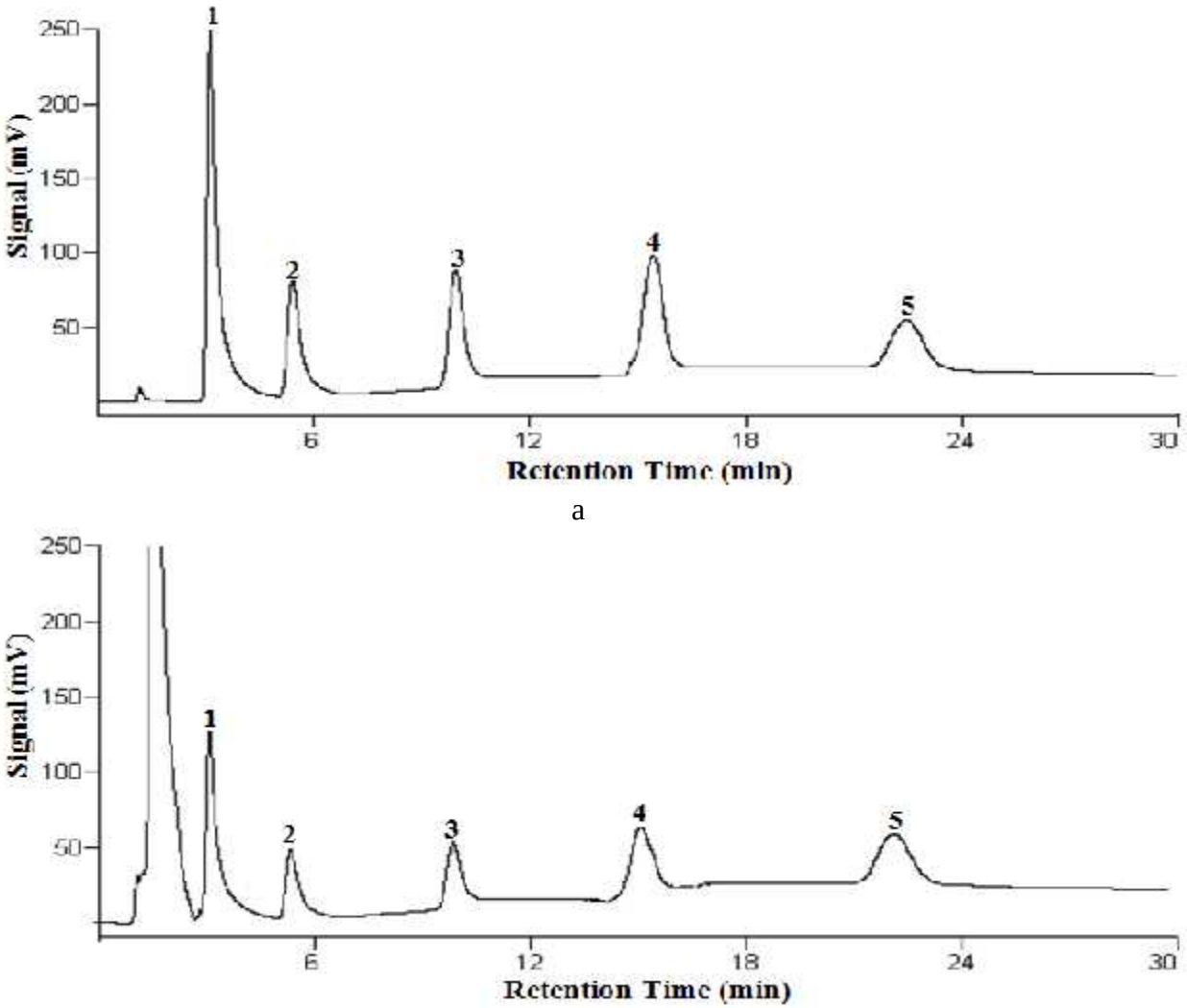


Figure 6.14. SBWC chromatograms obtained using 100% water on XBridge phenyl column using programmed temperatures at 1.0 mL/min. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, while the other parabens were detected at 256 nm. Programmed temperatures: initial temperature of 100 ^{o}C, then increased to 150 ^{o}C at 15 ^{o}C/min and then maintained at 150 ^{o}C. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben. (Reproduced with permission from reference 20 © The Authors ICS, 2012.)

6.3.3.4.4 Potential System Building-up Studies

Once again the potential building-up studies were conducted for the SBWC separations of preservatives on Waters XBridge Phenyl column at 150 ^{o}C with 1.0 mL/min. These separations were also conducted using a large number of replicate injections of a single preservatives sample solution. As shown in Table 6.12, the recovery ranges from 98.3 to 105.0% and RSDs lower than 1.8%. Again these results infer that there is no potential system building-up during the continuous SBWC separations of preservatives on the XBridge Phenyl column.

6.3.4 Analysis of Sunscreens Present in Skincare Creams

All the HTLC and SBWC separations of sunscreens were conducted using the home-made system. The sunscreen analytes were detected at 300 nm.

6.3.4.1 HTLC Separation and Analysis of Sunscreens on ZirChrom-DB-C18 Column

Separation of sunscreens was carried out on the ZirChrom-DB-C18 column using high-temperature liquid chromatography to decrease the methanol usage in the mobile phase. The HTLC separations were initially conducted at 90 ^{o}C and then at 150 ^{o}C. When comparing the methanol usage for the existing P&G HPLC method for sunscreens, the HTLC methods developed at 90 ^{o}C did not save any methanol in the mobile phase, where as the HTLC methods developed at 150 ^{o}C saved about 28% methanol.

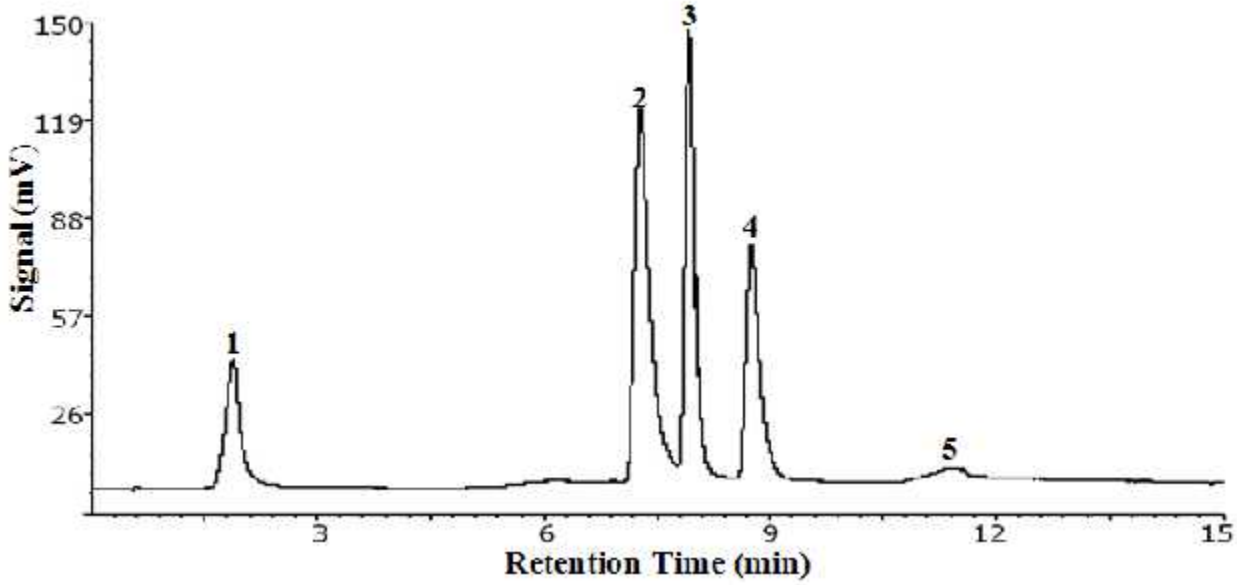
In order to further decrease the organic content in the mobile phase, HTLC separations of sunscreens were conducted at 190 ^{o}C on ZirChrom-DB-C18 column. The HTLC separations were optimized and the best separation was achieved using the gradient elution and programmed flow rates shown in Figure 6.15 legend. Figure 6.15 shows the separation of sunscreens standard and SC-EC2 skincare cream sample obtained by high-temperature liquid

chromatography. This HTLC method at 190 ^{o}C saved about 52% of methanol in the mobile phase. Table 6.15 shows the five replicate measurements of sunscreen separations by HTLC with reasonable %recoveries and RSDs. In this sunscreen separation, the precision of avobenzone is poor due to peak broadening.

Table 6.15. Concentration of Sunscreens Found in SC-EC2 Skincare Cream Sample Obtained by HTLC Using the ZirChrom-DB-C18 Column at 190 °C with Programmed Flow Rates as Described in Figure 6.15 Legend[a] (Reproduced With Permission from Reference 19 © The Authors ICS, 2011.)

	P&G Stated Concentration (%W)	Concentration Found By This Method (%W)	%Recovery	%RSD[b]
Ensulizole	1.00	1.06	105.5	3.7
Avobenzone	2.00	2.06	103.0	37.2
Octisalate	4.00	3.98	99.4	4.2
Octocrylene	1.00	1.02	101.8	5.2

[a] *Methanol saved: 52%.*
[b] *Based on five replicates.*



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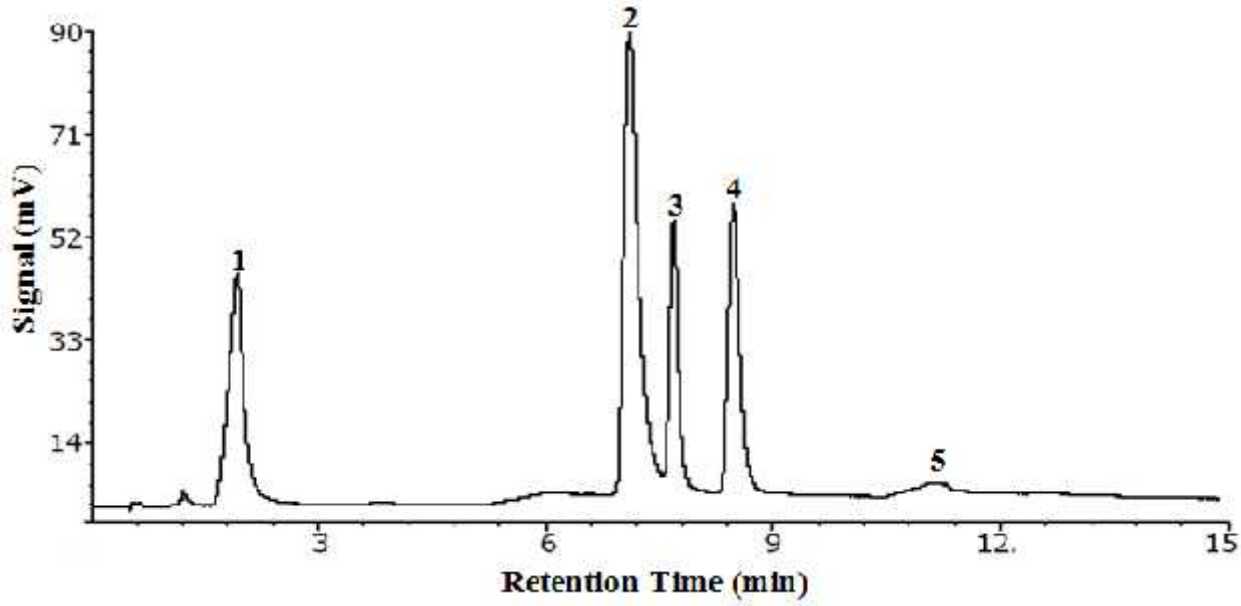


b

Figure 6.15. HTLC chromatograms obtained on the ZirChrom-DB-C18 column at 190 ºC. (a) Sunscreen standard mixture; (b) SC-EC2 skincare cream sample. Gradient: 0-3 min, 10% methanol; 3-4 min, 10-40% methanol; 4-9 min, 40% methanol; 9-10 min, 40-70% methanol; 10-15 min, 70% methanol. Programmed flow rates: 0-3 min, 2.0 mL/min; 3-4 min, decreased from 2.0 mL/min to 1.5 mL/min; 4-9 min, 1.5 mL/min; 9-10 min, increased from 1.5 mL/min to 2.0 mL/min; 10-15 min, 2.0 mL/min. Peak identification: 1, ensulizole; 2, oxybenzone; 3, octocrylene; 4, octisalate; 5, avobenzone. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)

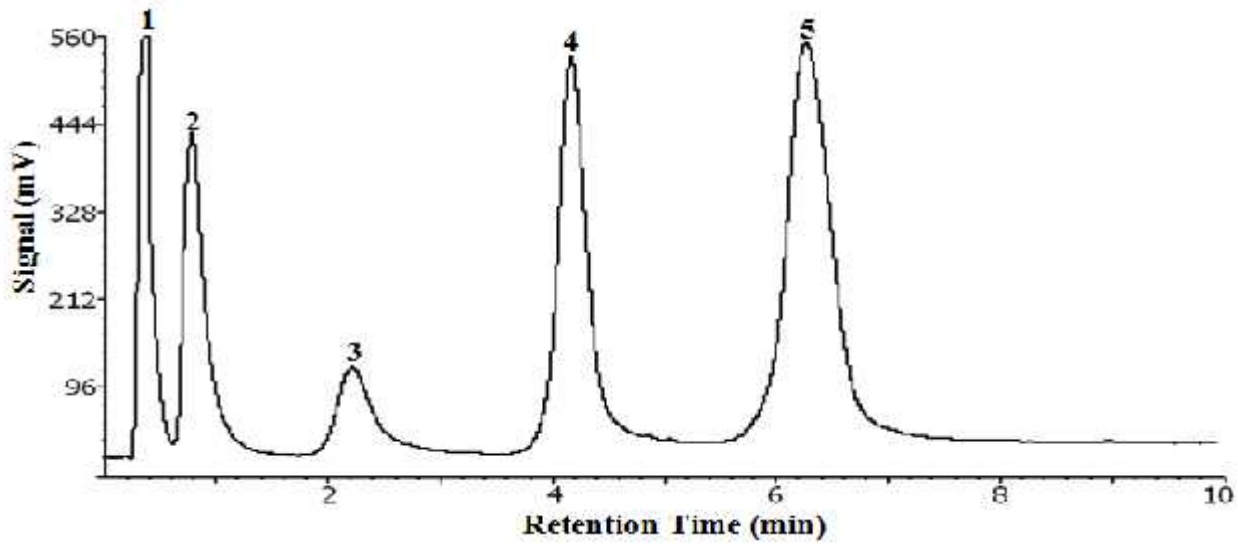
6.3.4.2 Separation and Analysis of Sunscreens on Waters XTerra MS C18 Column

6.3.4.2.1 Separation of Sunscreens by High Temperature Liquid Chromatography

As HTLC methods developed for the separation of sunscreens on the ZirChrom-DB-C18 column did not saved much of the methanol in the mobile phase. Therefore, further HTLC experiments were carried out on the Waters XTerra MS C18 column at 150 ^{o}C. Both SC-EC2

and SC-EC3 skincare cream samples containing sunscreens were separated by high temperature liquid chromatography at 150 ^{o}C. This HTLC method at 150 ^{o}C saved approximately 84% of methanol used in the mobile phase when comparing with the existing P&G HPLC method for sunscreens.

In order to further decrease the organic quantity in the mobile phases, HTLC separations of sunscreens were further explored at 200 ^{o}C. These HTLC separations were conducted using 2% methanol in water with 1.0 mL/min. Figure 6.16 shows the HTLC chromatograms of sunscreens standard and SC-EC2 skincare cream sample at 200 ^{o}C. Figure 6.17 demonstrates the HTLC separation of sunscreens standard and SC-EC3 skincare cream sample at 200 ^{o}C. It should be pointed out that over 99% of methanol was saved by employing these HTLC methods at 200 ^{o}C.



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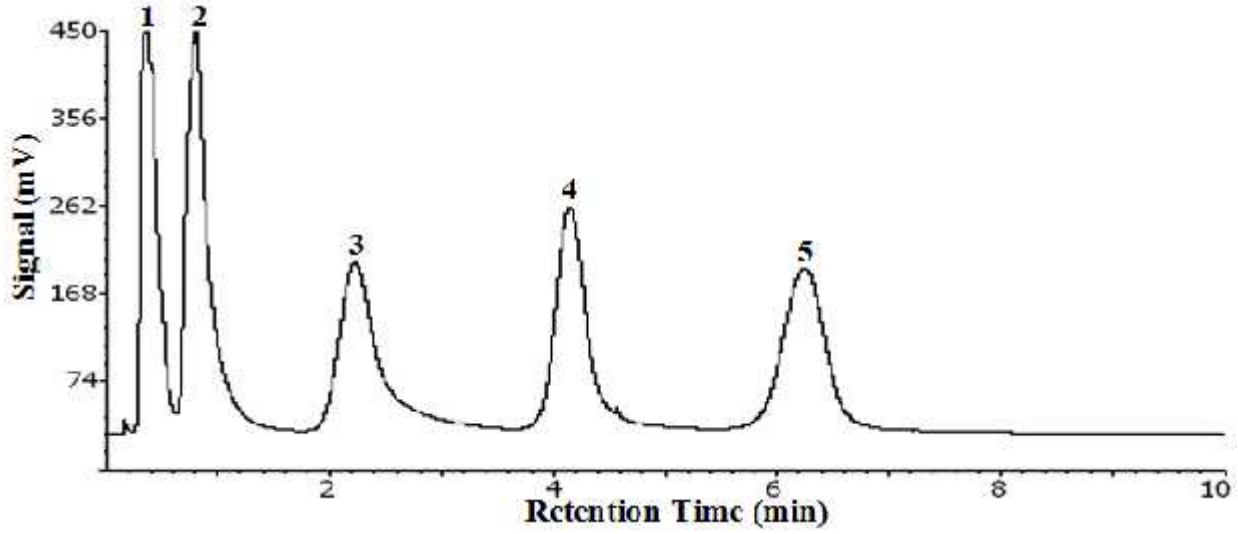
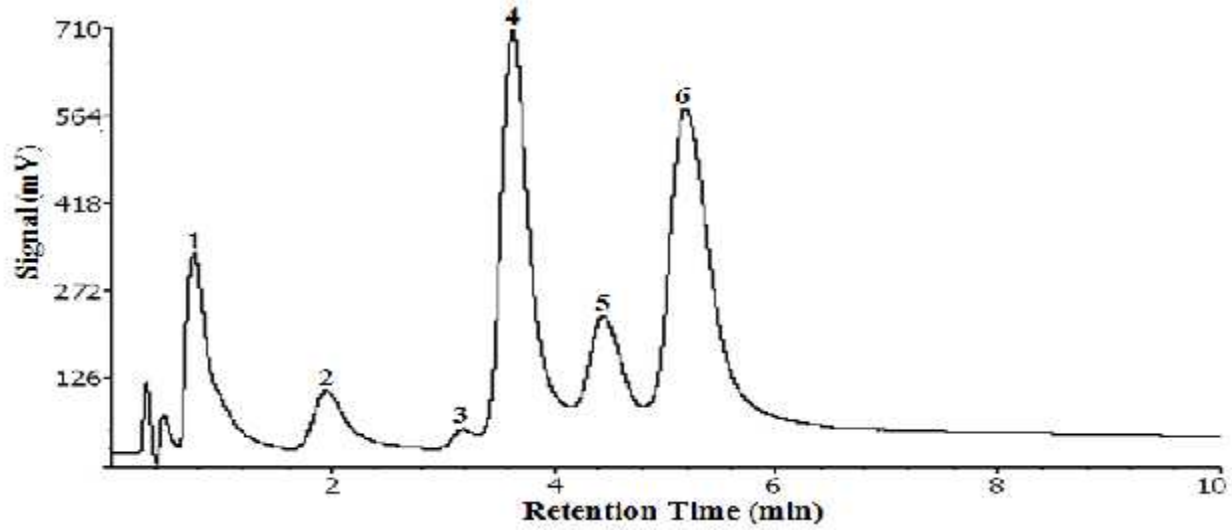


b

Figure 6.16. HTLC sunscreen chromatograms obtained on the XTerra MS C18 column at 200 ºC and 1.0 mL/min. (a) Sunscreen standard mixture; (b) SC-EC2 skincare cream sample. Isocratic elution using a mobile phase containing 2% of methanol in water. Peak identification: 1, ensulizole; 2, oxybenzone; 3, avobenzone; 4, octisalate; 5, octocrylene. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)



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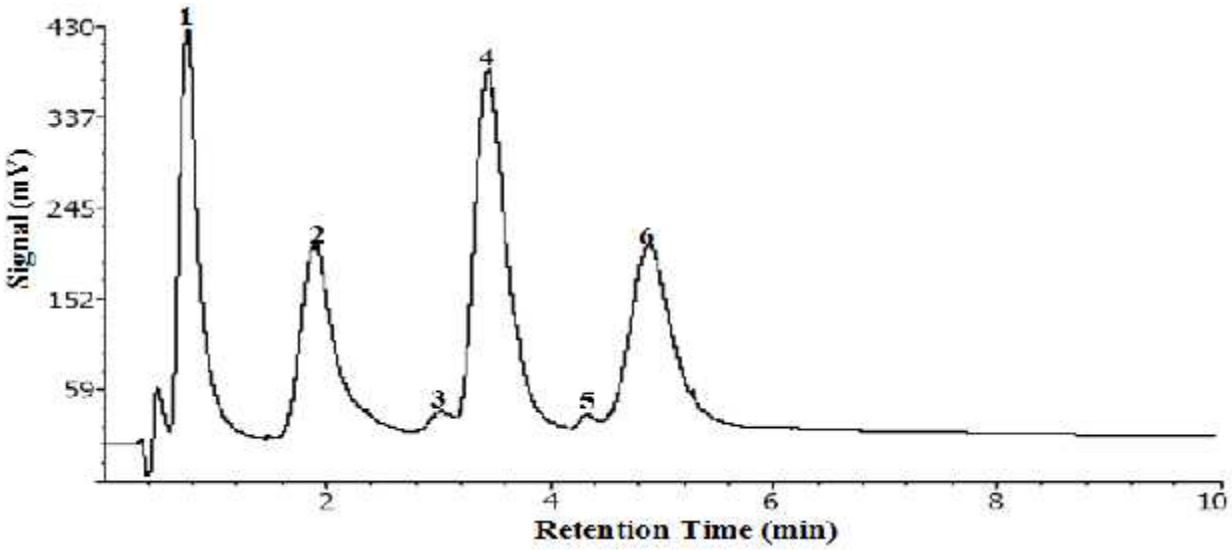


b

Figure 6.17. HTLC sunscreen chromatograms obtained on the XTerra MS C18 column at 200 ºC and 1.0 mL/min. (a) Sunscreen standard mixture; (b) SC-EC3 skincare cream sample. Isocratic elution using a mobile phase containing 2% of methanol in water. Peak identification: 1, oxybenzone; 2, avobenzone; 3, homosalate-cis; 4, octisalate; 5, homosalate-trans; 6, octocrylene. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)

6.3.4.2.2 Separation of Sunscreens by Subcritical Water Chromatography

In order to completely eliminate the organic solvents from the mobile phase, SBWC separation of sunscreens was carried out on Waters XTerra MS C18 column at 250 ^{o}C. Figure 6.18 shows the separation of SC-EC2 skincare cream sample containing sunscreens at 250 ^{o}C using only subcritical water as the mobile phase with 1.0 mL/min. However the elution strength of subcritical water at 250 ^{o}C is still too weak to efficiently separate sunscreens, when compared with the HTLC separation of sunscreens on the same column.

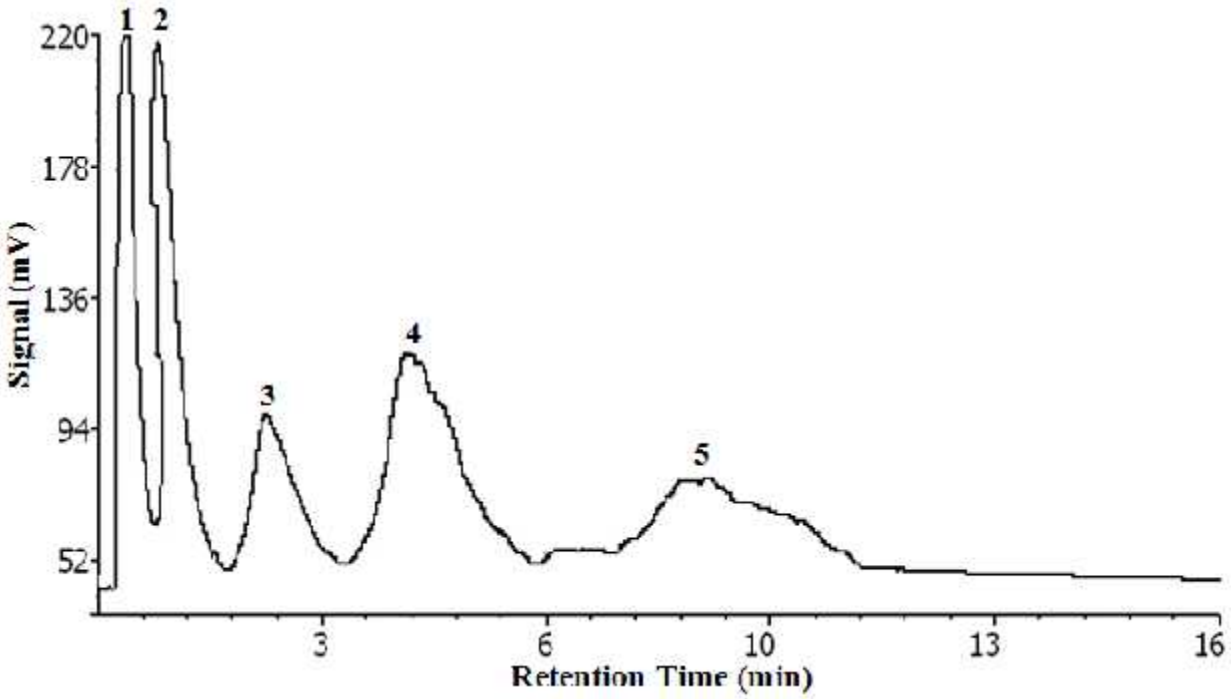


Figure 6.18. SBWC chromatogram of SC-EC2 skincare cream sample obtained on the XTerra MS C18 column at 250 ^{o}C and 1.0 mL/min using 100% water as the mobile phase. Peak identification: 1, ensulizole; 2, oxybenzone; 3, avobenzone; 4, octisalate; 5, octocrylene. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)

6.3.4.3 Separation and Analysis of Sunscreens on the Waters XBridge C18 Column

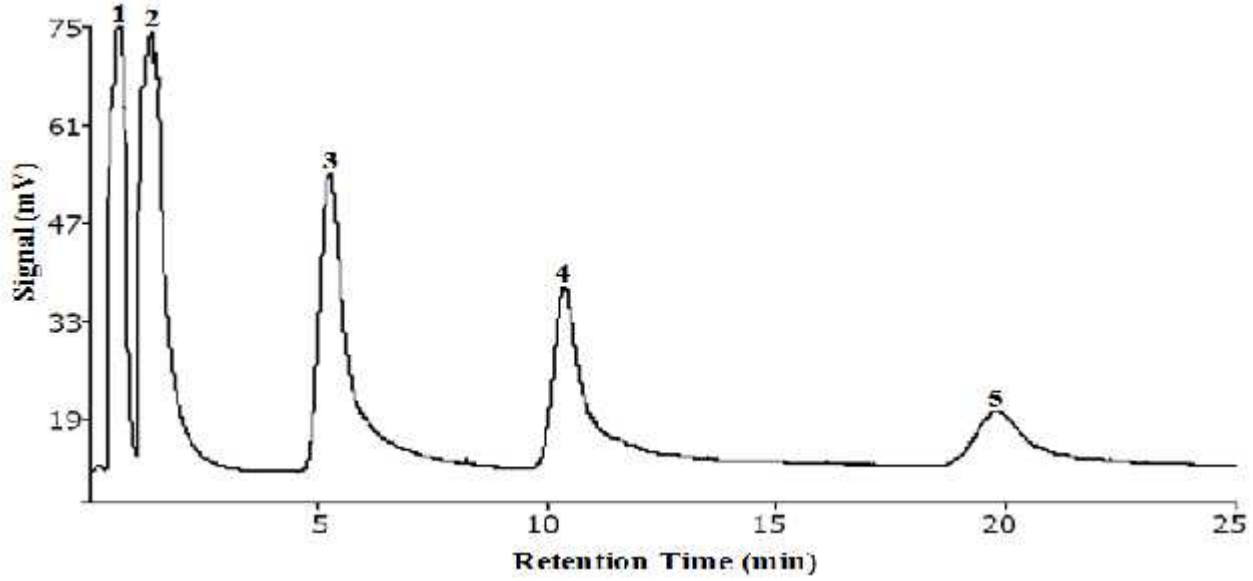
6.3.4.3.1 Separation of Sunscreens by High Temperature Liquid Chromatography

HTLC methods were developed for the separation of sunscreens on Waters XBridge C18 column at 200 ^{o}C to reduce the organic usage in the mobile phase. The HTLC separations were optimized and the best separation was achieved using 2% methanol in the mobile phase with 0.75 mL/min. Figure 6.19 shows the HTLC separation of sunscreens standard and SC-EC2 skincare cream, while Figure 6.20 demonstrates the HTLC separation of sunscreens standard and SC-EC4 skincare cream sample both at 200 ^{o}C. Table 6.16 shows the quantification results of SC-EC2 skincare cream containing sunscreens separations by HTLC with %recoveries ranging from 103.2 to 123.4. Table 6.17 shows the five replicate measurements of SC-EC4 skincare cream containing sunscreens separations by HTLC with %recoveries ranging from 81.4 to 98.0. Approximately 97% of methanol was saved by using these HTLC methods, when comparing with the existing P&G HPLC method for sunscreens.

Table 6.16. Concentration of Sunscreens Found in SC-EC2 Skincare Cream Sample Obtained by HTLC with 2% Methanol in the Mobile Phase on the Waters XBridge C18 Column at 200 °C with 0.75 mL/min[a]

	P&G Stated Concentration (%W)	Concentration Found By This Method (%W)	% Recovery	%RSD[b]
Ensulizole	1.00	1.03	103.2	0.9
Avobenzone	2.00	2.30	115.2	4.3
Octisalate	4.00	4.94	123.4	5.2
Octocrylene	1.00	1.17	116.8	12.8

[a] *Methanol saved: 97%.*
[b] *Based on five replicates.*



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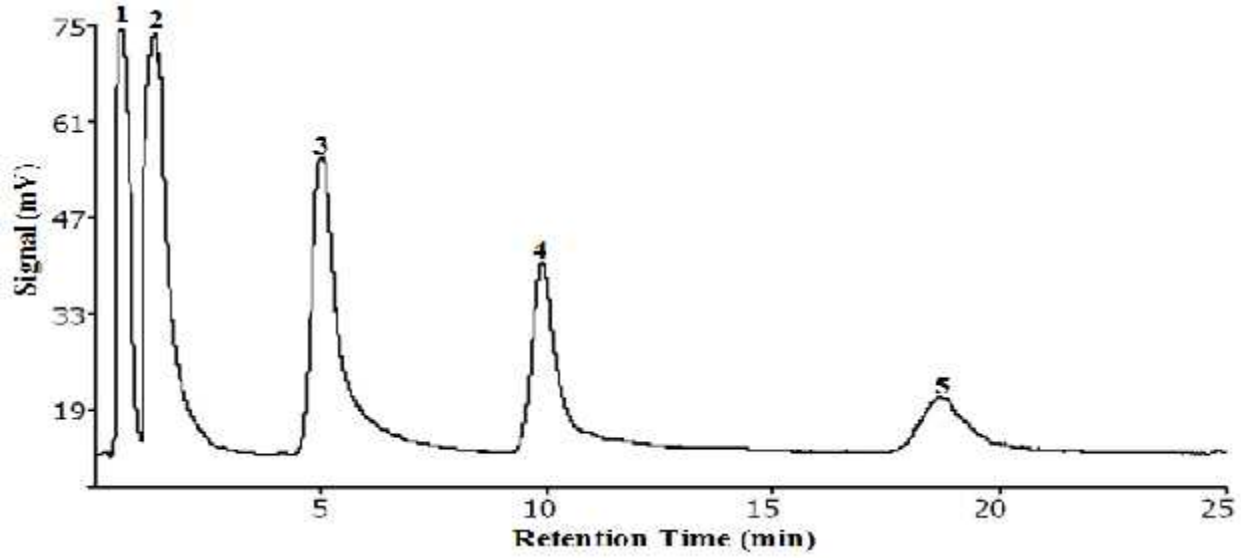
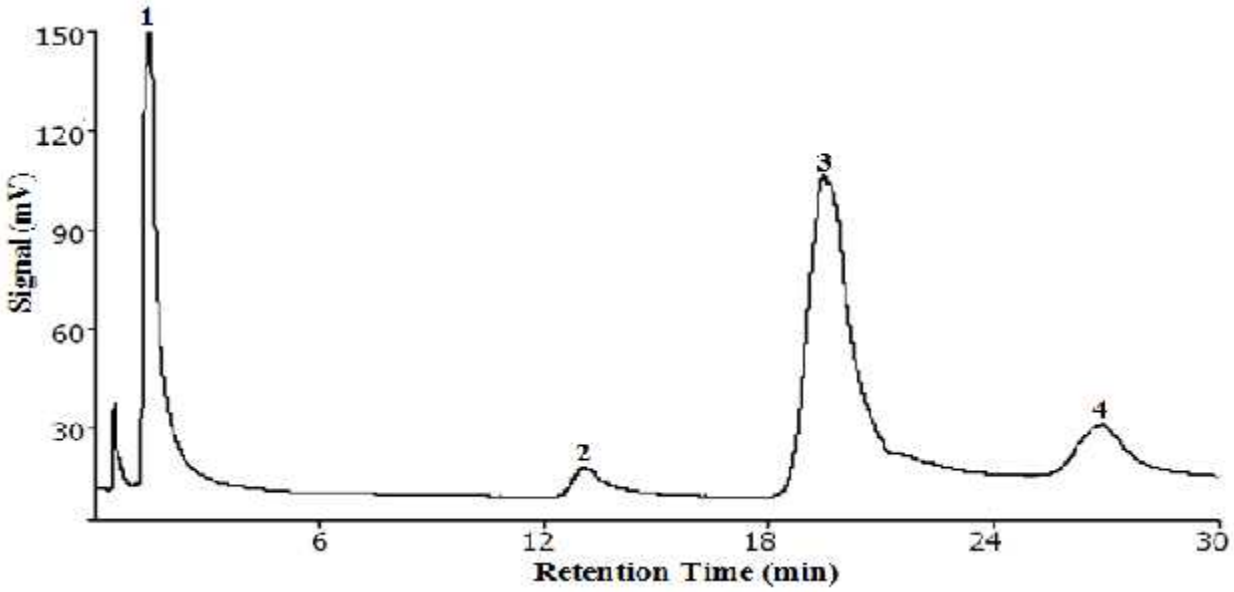


b

Figure 6.19. HTLC sunscreen chromatograms obtained on the XBridge C18 column at 200 ºC and 0.75 mL/min. (a) Sunscreen standard mixture; (b) SC-EC2 skincare cream sample. Isocratic elution using a mobile phase containing 2% of methanol in water. Peak identification: 1, ensulizole; 2, oxybenzone; 3, avobenzone; 4, octisalate; 5, octocrylene. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)



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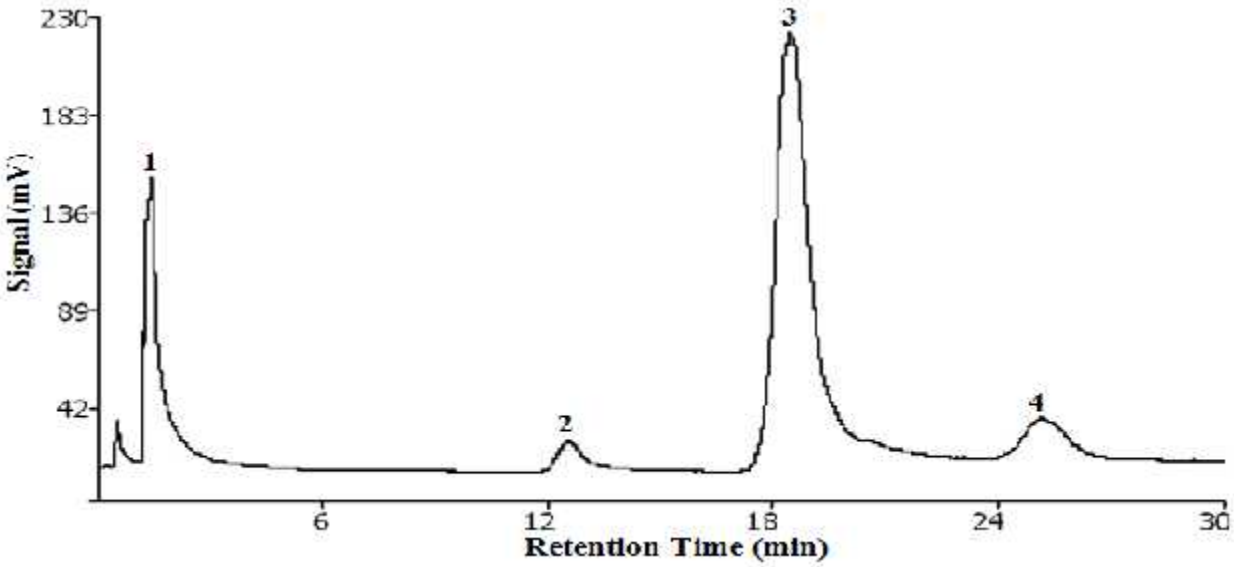


b

Figure 6.20. HTLC sunscreen chromatograms obtained on the XBridge C18 column at 200 ºC and 0.75 mL/min. (a) Sunscreen standard mixture; (b) SC-EC4 skincare cream sample. Isocratic elution using a mobile phase containing 2% of methanol in water. Peak identification: 1, oxybenzone; 2, octisalate; 3, octinoxate; 4, octocrylene. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)

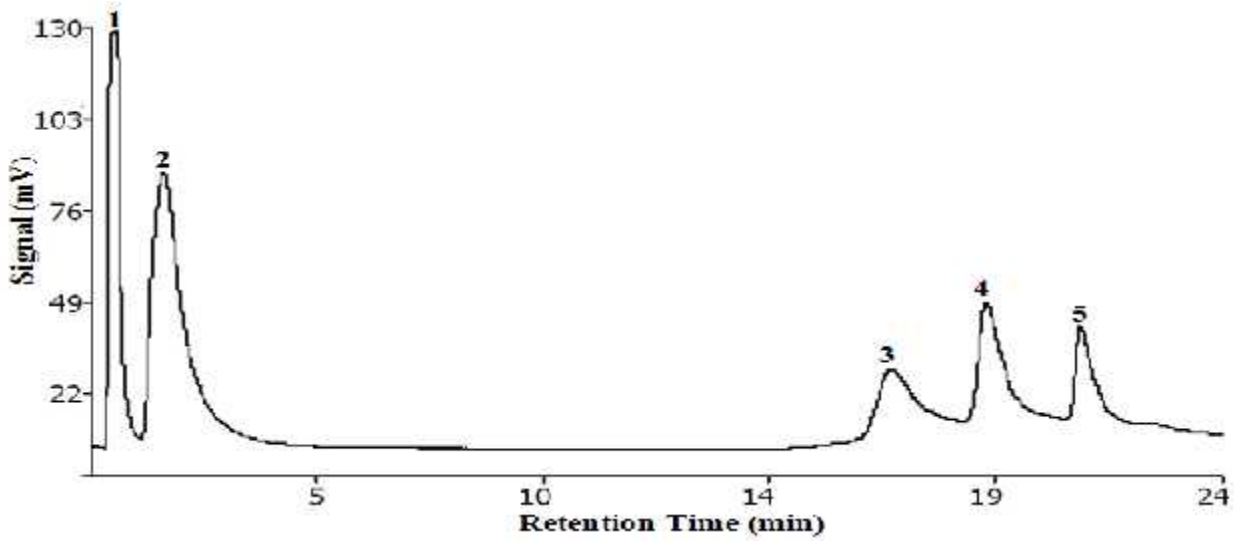
Table 6.17. Concentration of Sunscreens Found in SC-EC4 Skincare Cream Sample Obtained by HTLC with 2% Methanol in the Mobile Phase on the Waters XBridge C18 Column at 200 °C with 0.75 mL/min[a]

	P&G Stated Concentration (%W)	Concentration Found By This Method (%W)	% Recovery	%RSD[b]
Octisalate	2.50	2.32	92.6	8.7
Octinoxate	7.50	7.35	98.0	6.9
Octocrylene	2.50	2.04	81.4	8.5

[a] *Methanol saved: 97%.*
[b] *Based on five replicates.*

6.3.4.3.2 Separation of Sunscreens by Integrated SBWC/HTLC

Similar to the integrated SBWC/HTLC experiments for preservatives, the same approach was used for the separation of sunscreens in SC-EC2 skincare cream sample at 200 ^{o}C. Figure 6.21 shows the separation of sunscreens standard and SC-EC2 skincare cream sample using methanol-water gradient elution with 0.75 mL/min. Pure water was used as the mobile phase for the first 10 min, followed by the mobile phase containing 4% methanol for rest of the run. As pointed out earlier, the main advantage with these integrated methods is that only a very small portion of the chromatographic waste requires waste disposal. This integrated method enhanced the plate number especially for octocrylene as evidenced by its much narrower peak when compared with that obtained by the previous HTLC separations on the Waters XBridge C18 column. In addition, about 99% of methanol was saved by using this integrated SBWC/HTLC method. Table 6.18 shows the quantification results of SC-EC2 skincare cream containing sunscreens separations by integrated HTLC/SBWC method with %recoveries ranging from 90.3 to 113.2 and %RSDs less than 5.0.



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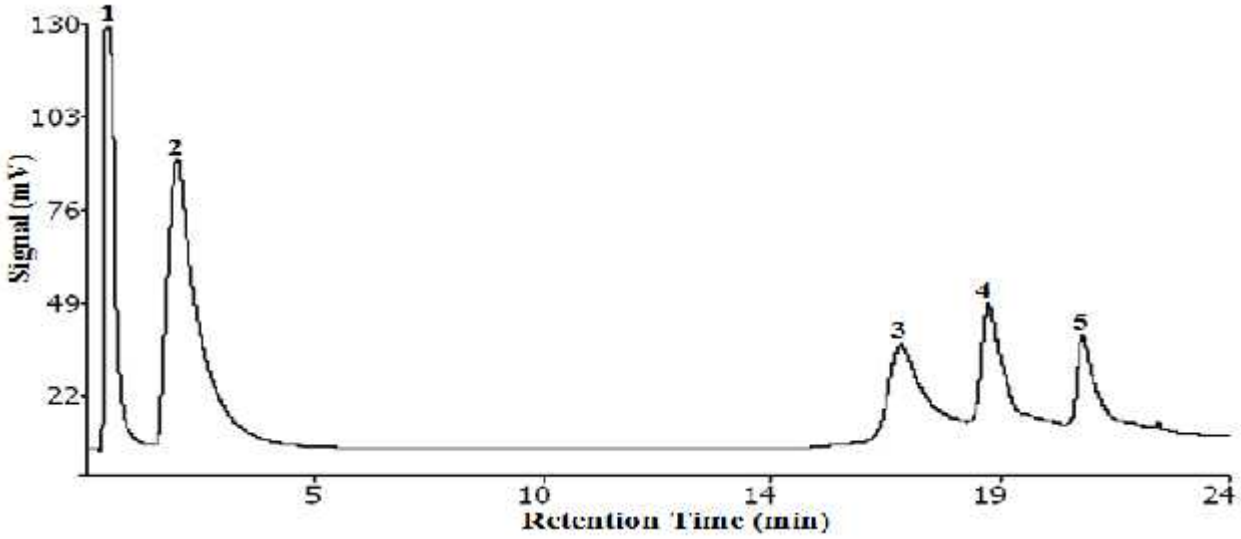


b

Figure 6.21. SBWC/HTLC chromatograms obtained on the XBridge C18 column at 200 ºC and 0.75 mL/min. (a) Sunscreen standard mixture; (b) SC-EC2 skincare cream sample. Gradient: 0-10 min, 0% methanol; 10-20 min, 0-4% methanol; 20-24 min, 4% methanol. Peak identification: 1, ensulizole; 2, oxybenzone; 3, avobenzone; 4, octisalate; 5, octocrylene. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)

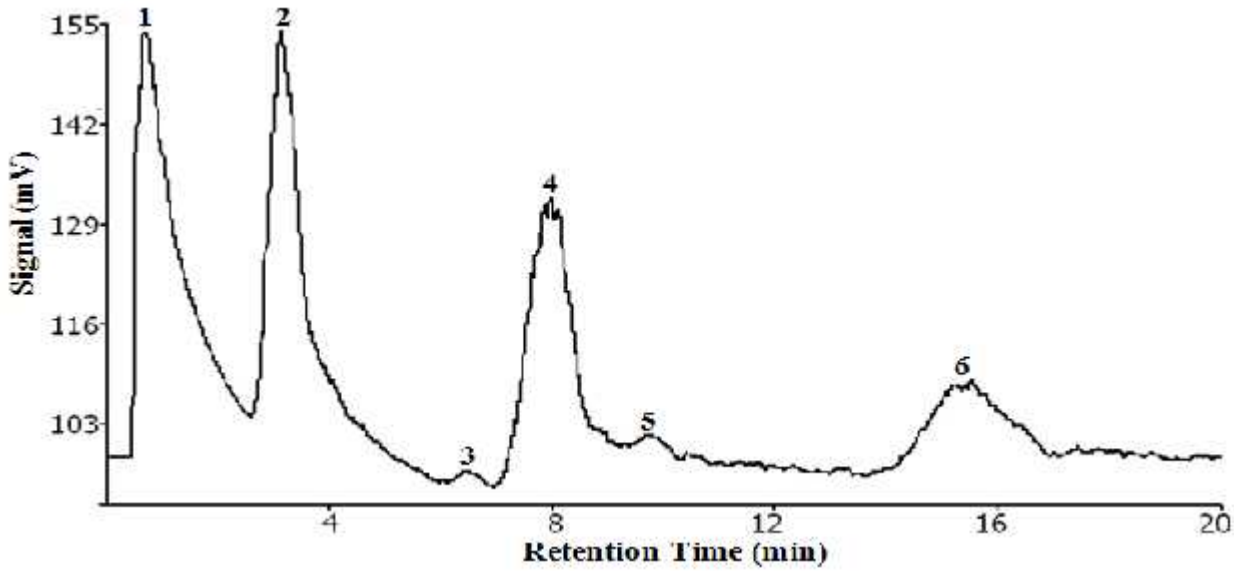
Table 6.18. Concentration of Sunscreens Found in SC-EC2 Skincare Cream Sample Obtained by the Combined SBWC/HTLC Method Using the Waters XBridge C18 Column at 200 °C with 0.75 mL/min[a] (Reproduced With Permission from Reference 19 © The Authors ICS, 2011.)

	P&G Stated Concentration (%W)	Concentration Found By This Method (%W)	% Recovery	%RSD[b]
Ensulizole	1.00	1.02	102.3	2.9
Avobenzone	2.00	2.26	113.2	2.8
Octisalate	4.00	4.00	100.1	3.2
Octocrylene	1.00	0.90	90.3	5.0

[a] *Methanol saved: 99%.*
[b] *Based on five replicates.*

6.3.4.3.3 Separation of Sunscreens by Subcritical Water Chromatography

In order to completely eliminate the organic solvents from mobile phase, further sunscreen separations were evaluated by SBWC on the Waters XBridge C18 Column at 230 ^{o}C. As shown in Figure 6.22, the SBWC separation of SC-EC2 and SC-EC4 skincare cream samples were carried out using subcritical water as the mobile phase with 1.0 mL/min. Again the weaker elution strength of water at 230 ^{o}C, resulted in poor efficiency and resolution as shown in Figure 6.22.



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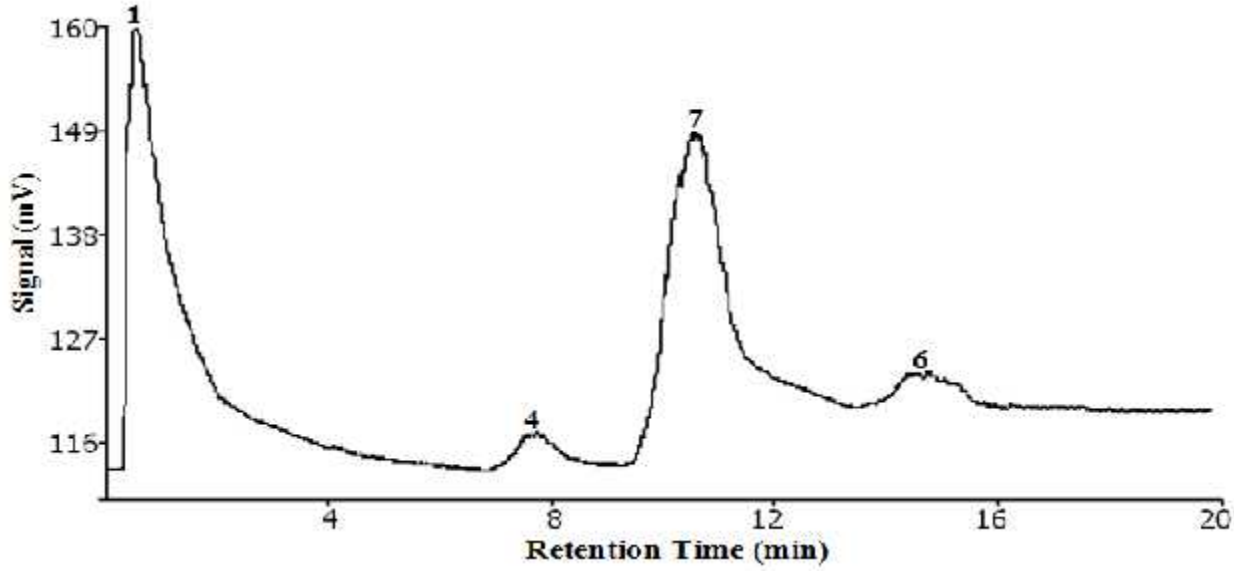

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Figure 6.22. SBWC chromatograms of sunscreens obtained on the XBridge C18 column at 230 ºC and 1.0 mL/min using 100% water as the mobile phase. (a) SC-EC3 skincare cream sample; (b) SC-EC4 skincare cream sample. Peak identification: 1, oxybenzone; 2, avobenzone; 3, homosalate-cis; 4, octisalate; 5, homosalate-trans; 6, octocrylene; 7, octinoxate. (Reproduced with permission from reference 19 © The Authors ICS, 2011.)

Chapter 7: Developing Efficacious Herbal Medicines Through Subcritical Water Extraction

7.1 Introduction

Salvia miltiorrhiza, also called as Danshen, is a traditional Chinese medicinal herb. It is used by the Chinese in the treatment of various diseases such as blood circulation, cardiovascular, and hepatic diseases [256-260]. Researchers isolated about 70 compounds from the extract of *Salvia miltiorrhiza*, and also structurally identified them [261]. Some of the structurally identified anticancer compounds present in Danshen include tanshinone I, tanshinone IIA, protocatechualdehyde, caffeic acid, and ferulic acid. These compounds already demonstrated their anti-proliferative effect on various cancer cells such as colon, leukemia, lung, and breast cancers [76-78, 262-265]. Presently, Danshen and Danshen containing medicinal preparations gain wide interest of many researchers [261].

In the literature, the extraction of *Salvia miltiorrhiza* has been carried out using microwave-assisted extraction [205], supercritical fluid extraction [266], pressurized liquid, extraction [224], and pressurized hot water extraction (ethanol-water as extraction solvent) [224]. As discussed in Chapter 2, these extraction techniques require organic solvents. Therefore, these organic solvent consuming extraction techniques are not good choices for the preparation of herbal medicine. Subcritical water extraction is the best choice, because it does not require organic solvents. SBWE has been already applied to extractions of food samples, environmental solids, and other samples [36, 40, 72, 214-218]. Subcritical water was also employed in the extractions of anticancer analytes from medicinal plants [43, 73].

Caenorhabditis elegans is a free living nematode that grows by consuming bacteria and slime molds. It belongs to the family of Rhabditidae with metazoan genome that was fully sequenced. The phenotypic effects of *C. elegans* are normally used for cytotoxicity testing. They include embryonic lethality, constitutive dauer arrest, sterility, egg laying defects, locomotory defects, and adult survival rate [267, 268]. Various fruit, aquatic, and drug extracts were evaluated for the cytotoxicity on *C. elegans* [268-271].

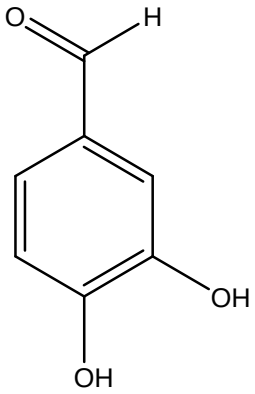
For the preparation of efficacious herbal medicine through subcritical water extraction of *Salvia miltiorrhiza*, the optimization of SBWE is necessary. The best way to optimize the medication preparation is to test the cytotoxicity of herbal extracts on animal model such as *C. elegans*. The outcome of the cytotoxicity test can be used to optimize SBWE temperature to yield the most potent herbal extract that may potentially treat cancer.

The main goal of this work is to prepare an efficacious herbal medicine for cancer using Subcritical water extraction. Therefore, subcritical water extraction of *Salvia miltiorrhiza* was carried out at four different temperatures (75, 100, 125, and 150 ^{o}C). For comparison and evaluation purposes, the traditional herbal decoction of *Salvia miltiorrhiza* using boiling water and sonication extraction using pure methanol were also conducted. Then these herbal extracts were characterized using GC/MS and HPLC to identify and quantify various anticancer agents. The cytotoxicity of SBWE and THD herbal extracts were tested on *C. elegans* using reproduction assay.

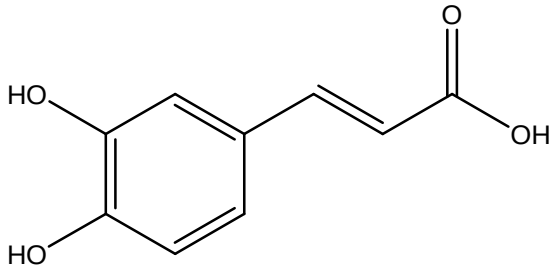
7.2 Experimental

7.2.1 Reagents and Supplies

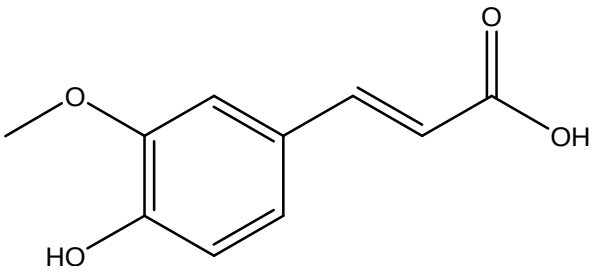
Tanshinone I and tanshinone IIA were obtained from LKT Laboratories, Inc. (St. Paul, MN, USA). Protocatechualdehyde, caffeic acid, ferulic acid, sodium chloride, sodium hydroxide, agar, cholesterol, calcium chloride, calcium chloride dehydrate, and sodium phosphate dibasic heptahydrate were purchased from Sigma Aldrich (St. Louis, MO, USA). Sand, peptone, tryptone, magnesium sulfate and magnesium sulfate heptahydrate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Potassium phosphate, dipotassium phosphate, yeast extract and HPLC-grade methanol were purchased from Alfa Aesar (Ward Hill, MA, USA). Methylene chloride was obtained from Acros Organics (Fair Lawn, NJ, USA). Top Job bleaching solution was purchased from the local store. Deionized water (18 MΩ-cm) was prepared in our laboratory using a Purelab Ultra system from ELGA (Lowell, MA, USA). GD/X PVDF membrane filters (0.45 μm) were acquired from Whatman (Florham Park, NJ, USA). Strata SPE silica-2 sample (3-mL) tubes were obtained from Phenomenex (Torrance, CA, USA). Petri dishes (6-cm) were obtained from BD Falcon (Franklin Lakes, NJ, USA). Alltech Adsorbosil C18 column (4.6 x 150 mm, 5 μm) was purchased from Alltech Associates, Inc. (Deerfield, IL, USA). An Empty stainless steel tube (5 x 1.00 cm I.D. with 1.27 cm O.D.) and endfittings were received from Chrom Tech, Inc. (Apple Valley, MN, USA). OP50 and *Caenorhabditis elegans* N2 Bristol Wild type worm were received from *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA).



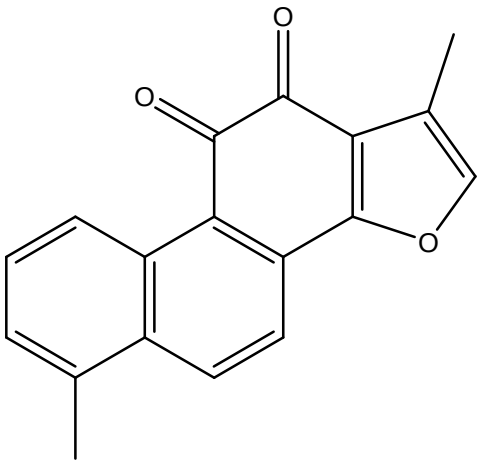
Protocatechualdehyde



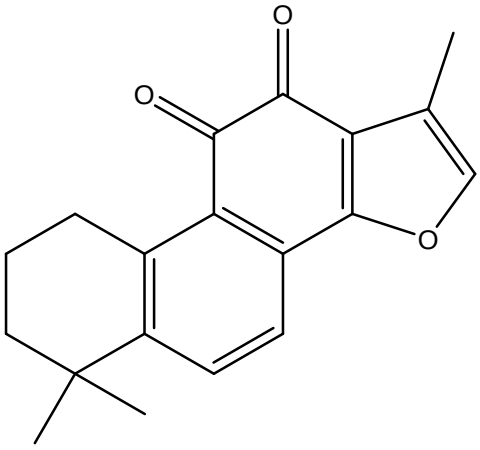
Caffeic Acid



Ferulic Acid



Tanshinone I



Tanshinone IIA

Figure 7.1. Structure of anticancer agents found in *Salvia miltiorrhiza*.

7.2.2 Preparation of Solutions

7.2.2.1 Preparation of Internal Standard Solutions

Propyl paraben was used as internal standard. This solution was prepared by adding 0.050 g (accurately weighed) of propyl paraben to a 50-mL volumetric flask and diluted to the mark with methanol.

7.2.2.2 Preparation of Calibrated Standard Solutions

A stock solution was prepared by adding 0.00020 g of each tanshinone I and tanshinone IIA to a 10-mL volumetric flask. Then 4.00 mL of dichloromethane was added into the volumetric flask. The volumetric flask was then vortexed to obtain a homogeneous mixture. Then 0.01 g (accurately weighed) of each protocatechualdehyde, caffeic acid, and ferulic acid were added to the same volumetric flask and diluted to the mark with methanol. Calibrated standard solutions were prepared using both stock and internal standard solutions.

7.2.3 Subcritical Water Extraction of *Salvia miltiorrhiza*

The extraction of *Salvia miltiorrhiza* was carried out using the subcritical water extraction system as shown in Figure 7.2. At first both endfittings of a stainless steel extraction vessel were wrapped with Teflon tape for proper sealing. One end of the vessel was sealed with an endfitting. Then 2.000 g of *Salvia miltiorrhiza* herbal powder was added to the stainless steel vessel. The void space of extraction vessel was completely filled with sand. The other end of the stainless steel vessel was then sealed with another endfitting. The loaded vessel was then placed in a gas chromatograph oven (HP 5890 Series 2, Hewlett Packard, Avondale, PA, USA) as shown in Figure 7.2.

Both V_1 and V_2 are high-pressure valves made from stainless steel. An ISCO model 260 D syringe pump (Lincoln, NE, USA) was used to supply 18 MΩ water by opening V_1 and

closing V_2 to fill the loaded vessel. Leak check was performed using the ISCO 260D syringe pump in the constant-pressure mode. After setting the desired temperature on the oven, it was turned on to heat the herbal-water mixture in the extraction vessel for 30 min. A delay between the actual temperature of the extraction vessel and oven temperature was determined. The delay was 10 min for 75 ^{o}C, 12 min for 100 ^{o}C, 14 min for 125 ^{o}C, and 16 min for 150 ^{o}C. Therefore the counting of SBWE time started after the delay time was compensated. A pressure of 8 to 25 atm was applied to keep hot water in the liquid state for all experiments. After 30 min of heating, approximately 10 mL of herbal extract was collected at 1.0 mL/min into a 25-mL glass vial by opening V_2. Triplicate SBWE experiments were conducted at all temperatures.

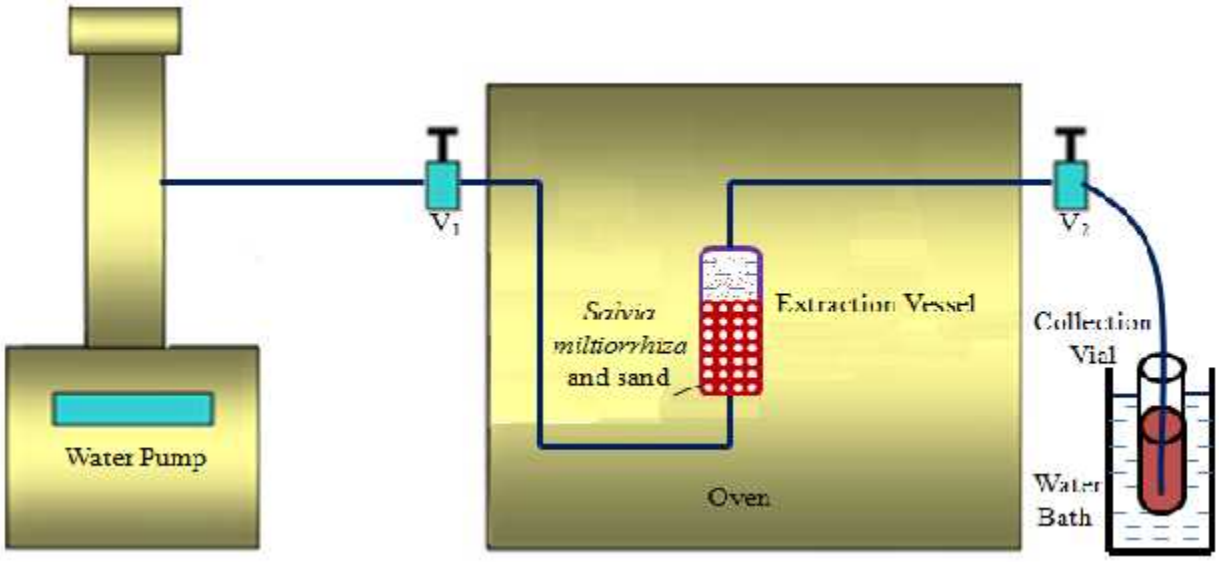


Figure 7.2. Schematic diagram of the SBWE extraction system.

7.2.4 Traditional Herbal Decoction of *Salvia miltiorrhiza*

Traditional herbal decoction of *Salvia miltiorrhiza* was carried out on a hot plate. At first, 2.000 g of *Salvia miltiorrhiza* herbal powder was added to the 50-mL glass beaker. Then 10.00 mL of deionized water was added to it. The glass beaker was then covered with a watch glass.

The beaker was placed on the hot plate and heated up to boiling. Then the temperature was adjusted to ensure the water keep boil for 30 min. Triplicate THD experiments were conducted.

7.2.5 Sonication Extraction of *Salvia miltiorrhiza*

Sonication of *Salvia miltiorrhiza* was carried out using a Sonicor SC-150T sonicator (Copiague, NY, USA). At first, 2.000 g of *Salvia miltiorrhiza* herbal powder was added to each 25-mL glass vial. Then 5.00 mL of methanol was added. These vials were then sealed with aluminum lined caps. The glass vials were then sonicated in the sonication bath for 8 hours. After 8 hours, these sonicated vials were taken out. The samples were then filtered through a Whatman GDX filter into a 25-mL glass vial for chromatographic analysis. Then 30 µL of propyl paraben internal standard was added to each vial. Triplicate sonication extractions were conducted.

7.2.6 Sample Treatment

7.2.6.1 Sample Treatment for GC/MS Analysis of Herbal Extracts

For characterization of SBWE herbal-water extracts on GC/MS, solid phase extraction (SPE) was carried out using a silica phase catridge and methanol as the elution fluid. At first, the silica catridge was cleaned with approximately 5 mL of methanol followed by 10 mL of water. Then the herbal extract was run through the silica catridge and eluted using 1.00 mL of methanol into a 2-mL glass vial. Then 30 µL of propyl paraben internal standard solution was added.

7.2.6.2 Sample Treatment for HPLC Analysis of Herbal Extracts

For HPLC analysis of SBWE and THD herbal-water extracts, liquid-liquid extraction was conducted for the quantification of tanshinones while methanol was added to the herbal-water extracts for quantification of protocatechualdehyde, caffeic acid, and ferulic acid.

For liquid-liquid extraction, first 1.00 mL of methylene chloride was added to each glass vial containing SBWE water-herbal extract. These vials were then sealed with aluminum lined caps. These vials were vortexed for mixing. After separation of the two phases, the methylene chloride phase was removed into the 5-mL glass vial. The liquid-liquid extraction was again repeated with another 1.00 mL of fresh methylene chloride. Again the methylene chloride phase was removed and combined with the first fraction of methylene chloride. Then 30.00 µL of propyl paraben internal standard was added to the methylene chloride phase.

To the left over herbal extract sample, 1.00 mL of methanol was added. Then 300 µL of propyl paraben internal standard was added and mixed well. This sample was then filtered through a Whatman GDX filter into a glass vial for chromatographic analysis.

7.2.7 HPLC Analysis

The Shimadzu Nexera UFLC system was employed for the evaluation of herbal extracts obtained by the SBWE, THD, and sonication of *Salvia miltiorrhiza*. The HPLC separations were carried out on the Alltech Adsorbosil C18 column using methanol-water mixture as the mobile phase with 1.0 mL/min at ambient temperature. The eluents were detected at 254 nm with the UV detector of the Shimadzu system.

7.2.8 GC/MS Analysis

Agilent Technologies 6890N Network GC System (Santa Clara, CA, USA) coupled with a JEOL Ltd. JMS-GCmate II MS System (Tokyo, Japan) was employed for the characterization of SBWE herbal extracts of *Salvia miltiorrhiza*. The GC separations were carried out on an Agilent HP-5MS (5%-Phenyl)-methylpolysiloxane (30 m x 0.250 mm, 0.25 µm film thickness) capillary column with 1.0 mL/min flow of a helium carrier gas. The sample volume was 1 µL and injected using split mode by keeping the injector temperature at 250 ºC. The GC/MS

interface and the MSD ion chamber were set at 250 ºC. The MS solvent delay time was 3 min. The GC oven temperature programming was as follows: The initial temperature was held at 30 ºC for 3.00 min. Then it was increased at 7.4 ºC/min to 250 ºC and maintained at 250 ºC for 16.00 min. TSSPro Version 3.0 (Shrader Analytical and Consulting Laboratories, Inc., Detroit, Michigan, USA) was used for data acquisition and analysis.

7.2.9 Sampling for the Cytotoxicity Studies on *Caenorhabditis elegans*

For the preparation of nematode growth media (NGM), appropriate quantities of sodium chloride, peptone, and agar were accurately weighed and added to each Erlenmeyer flask. A predetermined volume of water was added to each flask. Then 1.00 mL of herbal extract (SBWE or THD) was added to the labeled flasks and autoclaved. In case of control, there is no addition of the herbal extract. After sterilization, the flasks were placed on a hot plate located in a laminar fume hood. The hot plate was set at 55 ^{o}C. Appropriate quantities of cholesterol, calcium chloride, magnesium sulfate, and potassium phosphate were added to each flask and mixed well. Then these solutions were poured into the Petri dishes, called as nematode growth media plates.

A hermaphrodite, *C. elegans* N2 Bristol Wild type (worm), was used for the reproduction assay to determine the cytotoxicity of herbal extracts. Figure 7.3 shows the life cycle of *C. elegans*. These worms were first age-synchronized using a bleaching method described by Sulston and Hodgkin. This means that the worms were brought to the same age for reproduction assay [272]. For synchronization, these worms were transferred to the NGM plates seeded with bacteria (OP50). These bacteria will serve as food for the worms. These plates were then stored in an incubator at 20 ^{o}C for the growth of worms. Adult gravid worms were then washed off from the NGM plate using M9 buffer (appropriate quantities of sodium hydrogen phosphate,

potassium dihydrogen phosphate, sodium chloride and magnesium sulfate heptahydrate were added to the Erlenmeyer flask and made up to the volume with deionized water) into a Falcon tube. These tubes were centrifuged at 2000 rpm for 2 min to get a worm pellet. After the supernatant was discarded, fresh M9 buffer was again added. The worms were then centrifuged at 2000 rpm for 2 min and supernatant was discarded again. This step removes most bacteria and debris from worms. Then synchronization or bleaching solution was added to kill the worms, but not the eggs. This bleaching solution not only kills the worm, but it also breaks the worm to release eggs. These tubes were vortexed and again centrifuged using the same conditions. The supernatant was discarded and approximately 5 mL of M9 buffer was added and centrifuged. This centrifugation step was carried out two more time to completely remove the synchronization solution. After the third centrifugation, approximately 5 mL of M9 buffer was again added and these tubes containing only eggs were incubated at 20 ^{o}C on a shaker for about 14 to 19 hours. The eggs were hatched in that period and reached L1 stage as shown in Figure 7.3 which will remain struck at that stage due to absence of food. The dauer larva that struck at L1 stage due to absence of food can also be seen in Figure 7.3.

These worms at L1 stage were transferred to six treatment groups of NGM plates. They were control, traditional herbal decoction, and SBWE herbal extracts obtained at extraction temperatures of 75, 100, 125, and 150 ^{o}C. Approximately 15 worms in L1 stage were transferred to each NGM plate seeded with OP50 food. Five replicate studies were conducted. The plates were then sealed with parafilm and incubated for about 30 hours. This exposure time of worms to the herbal extract coincided with the worm development from L1 to L4 stages. After 30 hours, these worms were washed off from each plate using M9 buffer into an Eppendorf tube. The tubes were then centrifuged twice with M9 buffer to wash worms of herbal

extract. Then from each tube, about 4 worms were transferred to each plate already seeded with OP50 food. These plates were again incubated at 20 ^{o}C. These plates were continuously monitored for egg laying. When worms started laying eggs, time was noted and labeled the plates as day 1 plates. These plates were incubated for another 24 hours. Figure 7.4 shows the complete summary of the 3-day reproduction assay.

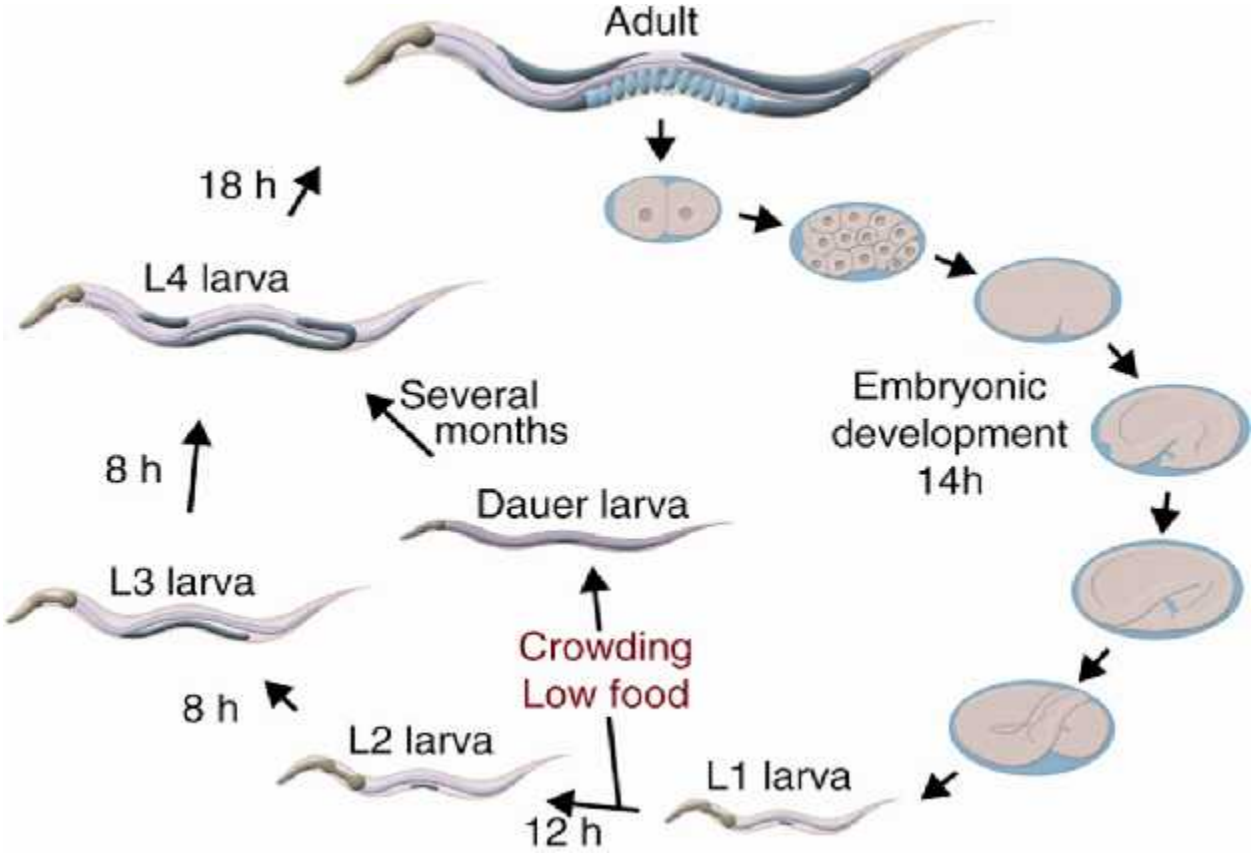


Figure 7.3. The life cycle of *C. elegans*. Under favorable conditions animals pass through direct development to adulthood in as little as 3 to 4 days. In response to harsh environmental conditions, such as food shortage, crowding or high temperatures, animals can enter into an arrested dauer stage. (Reproduced with permission from reference 273 © BioMed Central Ltd, 2010.)

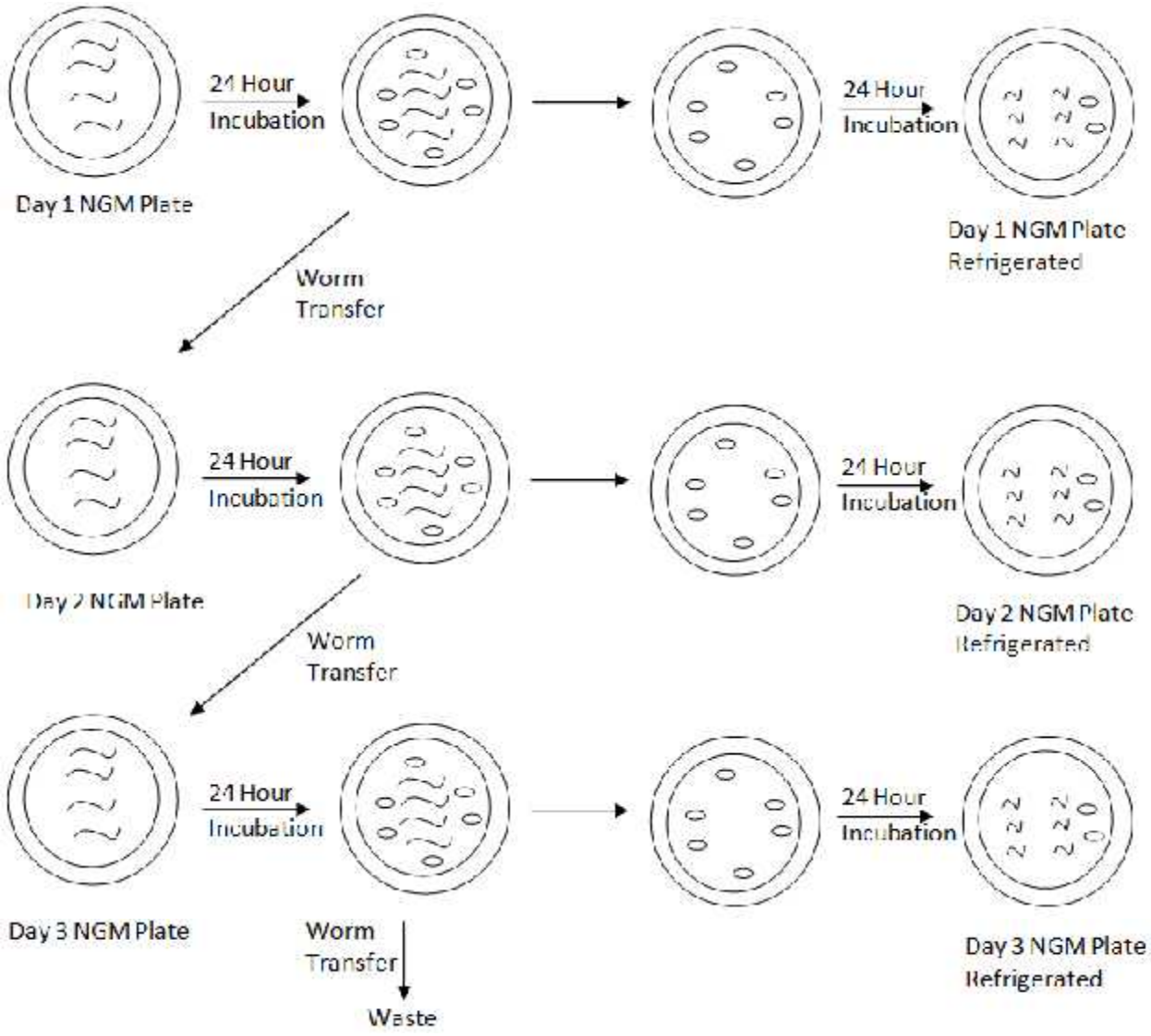


Figure 7.4. Reproduction assay of *C. elegans*. (‘ ∿ L1 larva, ‘ ⁓ adult worm, and ‘ ⊖gg)

As shown in Figure 7.4, after 24 hours of incubation, only the adult worms were transferred from day 1 plates to the fresh treatment free NGM plates seeded with OP50 food. Then these plates were labeled as day 2 plates. These plates were incubated along with the day 1 plates for 24 hours. As normal egg hatching time is around 10-12 hours, eggs that were laid at the last min will also hatch in day 1 plates. In day 2 plates, worms started laying eggs again. After 24 hours, day 1 plates were refrigerated. This will preserve the worms in their respective

development stages (adult, larva, or eggs) as well as their location on the plate. Due to refrigeration, all the worms, eggs, and larva will not move while counting under VWR VistaVision inverted microscope (Suwanee, GA, USA).

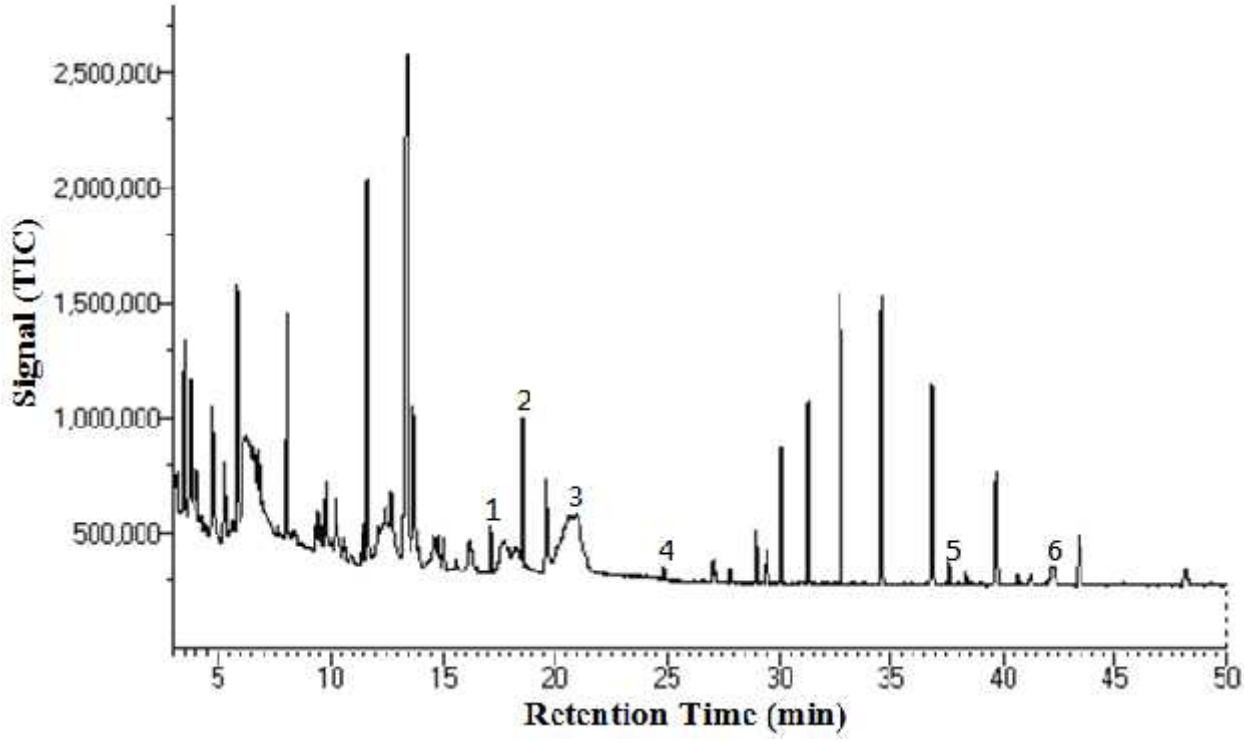
After 24 hours of transfer to the day 2 plates, worms were again transferred to the fresh treatment free NGM plates seeded with OP50 food. These plates were labeled as day 3 plates. Then day 2 and day 3 plates were incubated for 24 hours. After 24 hours, day 2 plates were refrigerated and worms were thrown away to waste from day 3 plates. Then day 3 plates were incubated for another 24 hours and refrigerated. After one to two days, all the day 1, day 2, and day 3 plates were taken out from the refrigerator and counted the fertilized eggs, unfertilized eggs, and larva for reproduction assay.

7.3 Results and Discussion

7.3.1 Subcritical Water Extraction of *Salvia miltiorrhiza*

7.3.1.1 Identification of Anticancer Analytes by GC/MS

The subcritical extraction of *Salvia miltiorrhiza* was carried out at four different temperatures of 75, 100, 125, and 150 ^{o}C. Then the obtained SBWE extracts were characterized using GC/MS. Various analytes in the herbal extracts were identified by GC/MS through matching of both GC retention times and mass spectra of standard analytes [261]. Among the identified analytes, five of them are anticancer agents including protocatechualdehyde, caffeic acid, ferulic acid, tanshinone I, and tanshinone IIA [76-78, 262-265]. Figure 7.5a, shows the elution of the five anticancer compounds with an internal standard. Figures 7.5b-f depict the mass spectra of those five solutes.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500,000
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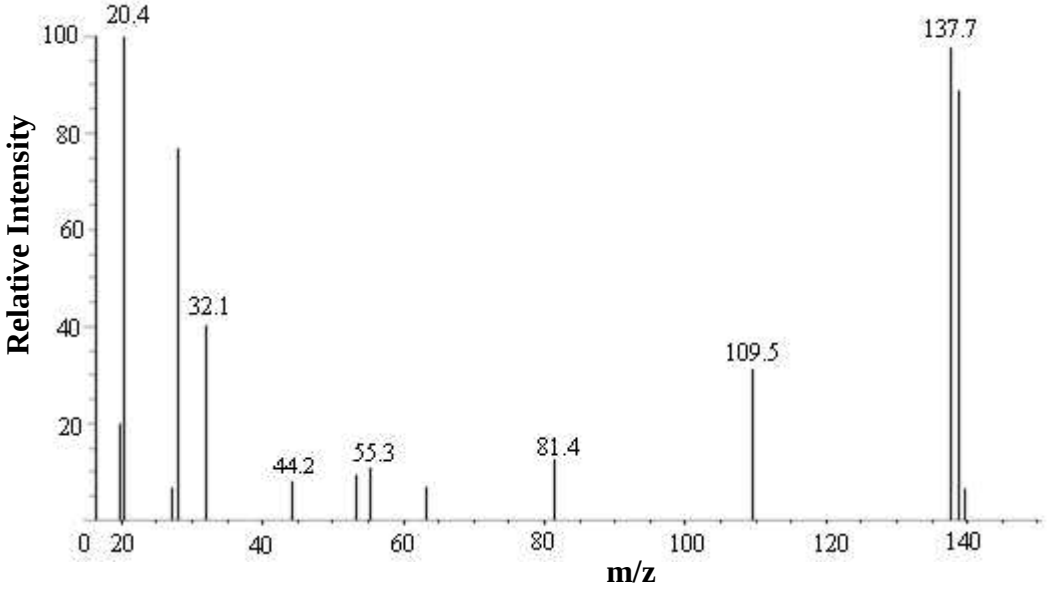
2
3
4
5
6
5
10
15
20
25
30
35
40
45
50
Retention Time (min)

a


Relative Intensity
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60
40
20
0
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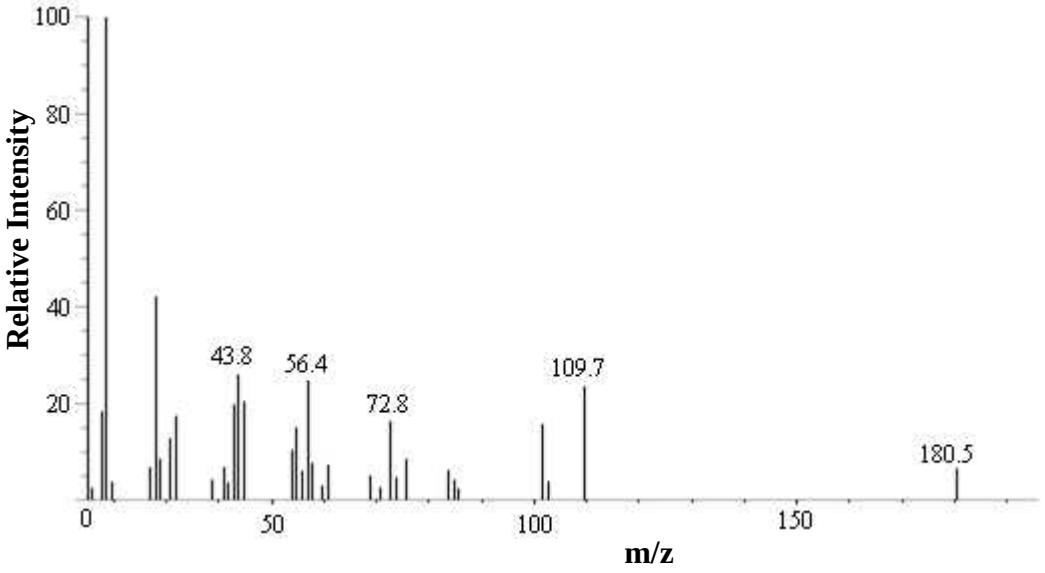
40
60
80
100
120
140
m/z

b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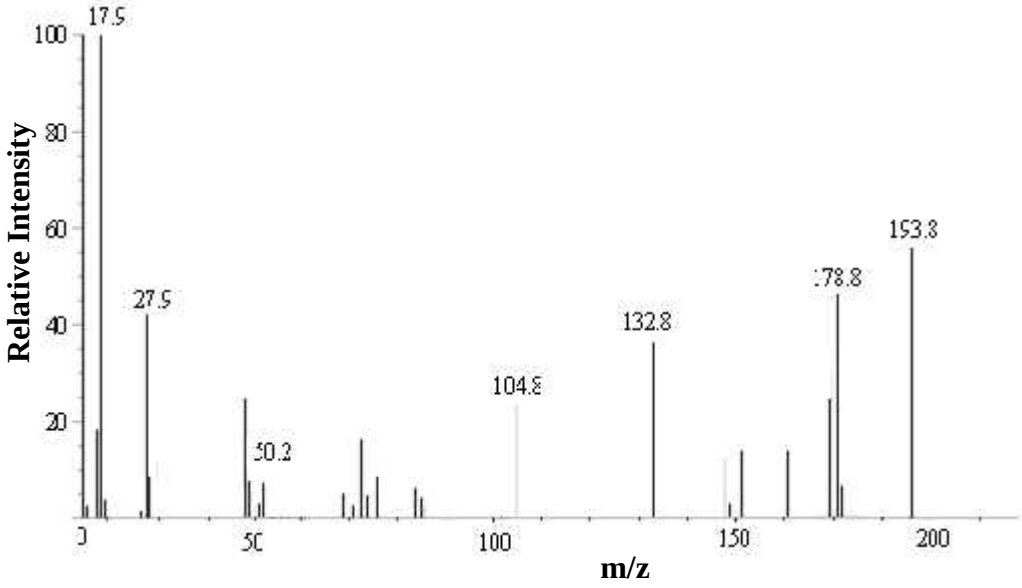
m/z

c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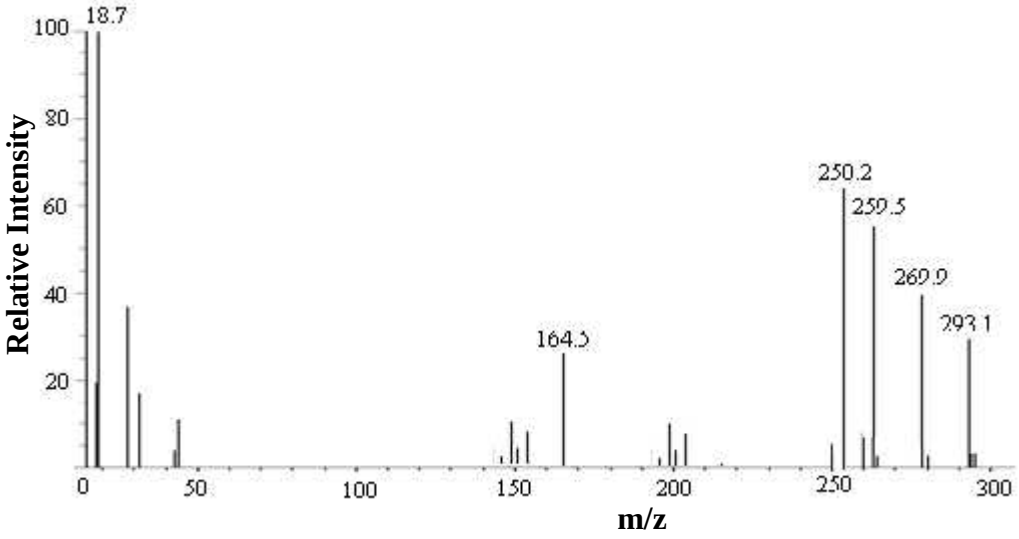
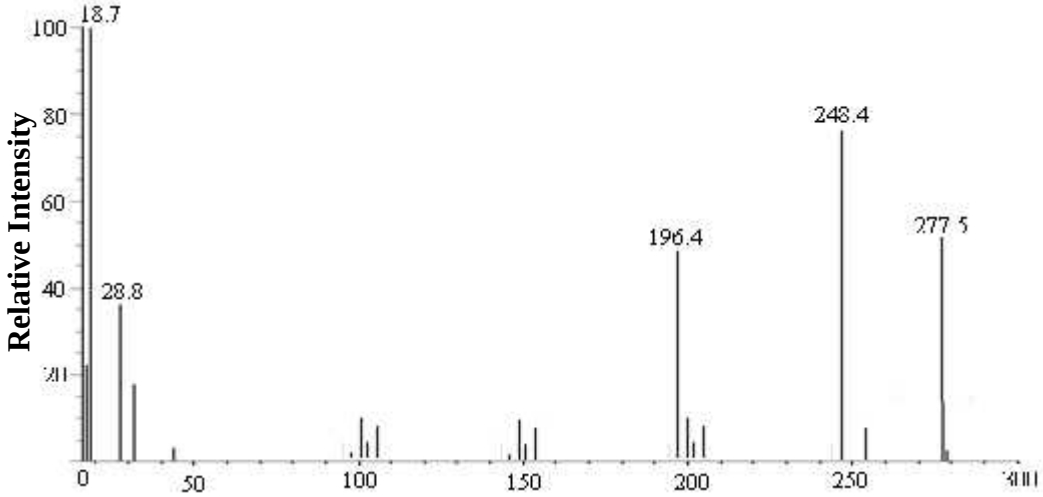
193.8
m/z

d



e

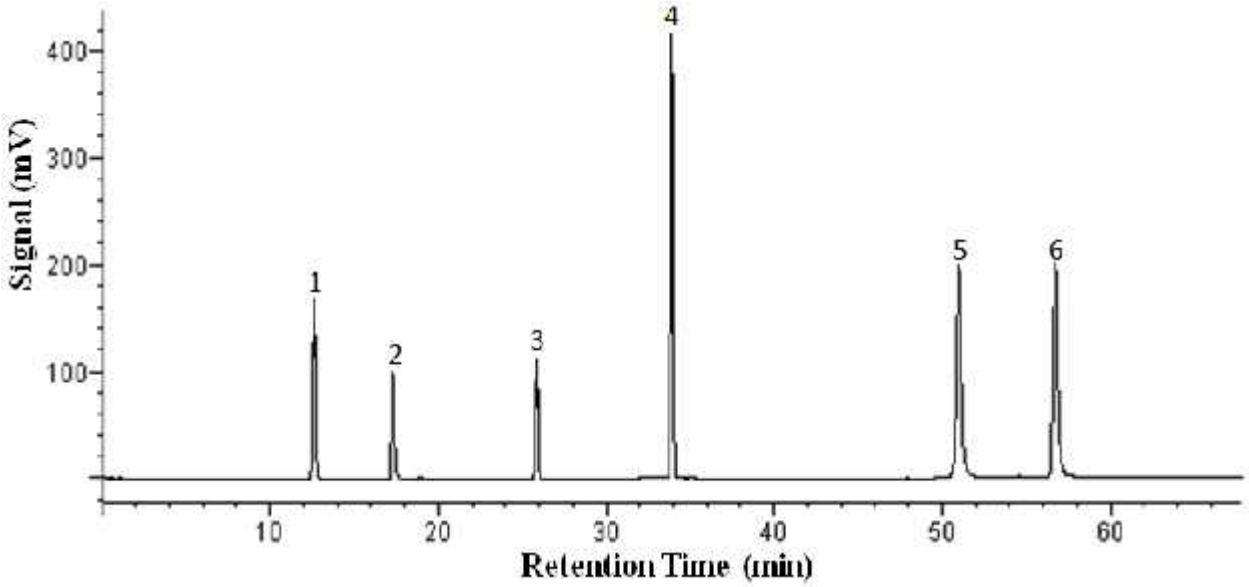


f

Figure 7.5. GC/MS chromatogram and mass spectra of a *Salvia miltiorrhiza* herbal extract obtained by SBWE at 150 ºC for 30 min. (a) Total ion chromatogram; (b) Mass spectrum of the protocatechualdehyde peak; (c) Mass spectrum of the caffeic acid peak; (d) Mass spectrum of the ferulic acid peak; (e) Mass spectrum of the tanshinone IIA peak; (f) Mass spectrum of the tanshinone I peak. Peak identification: 1, protocatechualdehyde; 2, propyl paraben (internal standard); 3, caffeic acid; 4, ferulic acid; 5, tanshinone IIA; 6, tanshinone I.

7.3.1.2 Quantification of Anticancer Agents by HPLC

The quantification of the five anticancer analytes (protocatechualdehyde, caffeic acid, ferulic acid, tanshinone I and tanshinone IIA) in *Salvia miltiorrhiza* extracts was carried out using HPLC at ambient temperature. HPLC separations were conducted on the Alltech Adsorbosil C18 column using methanol-water mixture as the mobile phase with 1.0 mL/min. The gradient conditions are given in Figure 7.6 legend. Figure 7.6 shows the HPLC separation of an analyte standard solution and methylene chloride phase after liquid-liquid extraction using methylene chloride and herbal extract obtained by SBWE at 125 ^{o}C. The last chromatogram in Figure 7.6 is from the water phase (methanol was added). Table 7.1 shows the quantification results of the five anticancer analytes present in the SBWE herbal extracts obtained at four different temperatures of 75, 100, 125, and 150 ^{o}C. The quantification results indicate that analyte concentrations increased by up to 4 folds with the increase of extraction temperature from 75 to 100 ^{o}C and up to 26 folds enhancement with further rise of extraction temperature from 100 to 125 ^{o}C, except for caffeic acid due to degradation. As discussed in Chapter 3, degradation of analytes in subcritical water at higher temperatures is not unusual [120, 124]. Then with further increase of temperature from 125 to 150 ^{o}C, the concentrations of ferulic acid and tanshinones were enhanced up to 4 folds, except for protocatechualdehyde and caffeic acid concentrations due to severe degradation at 150 ^{o}C. Please note that the extraction efficiency, measured by analyte concentration in herbal extracts, of tanshinones was improved with increasing temperature.


Signal (mV)
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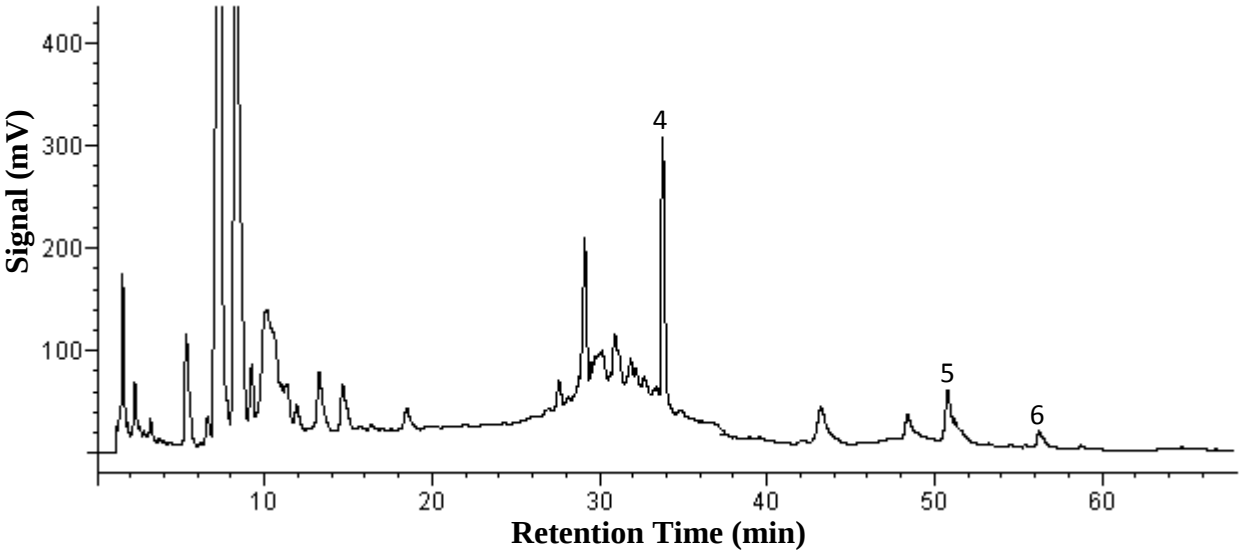
200
100
1
2
3
4
5
6
10
20
30
40
50
60
Retention Time (min)

a


Signal (mV)
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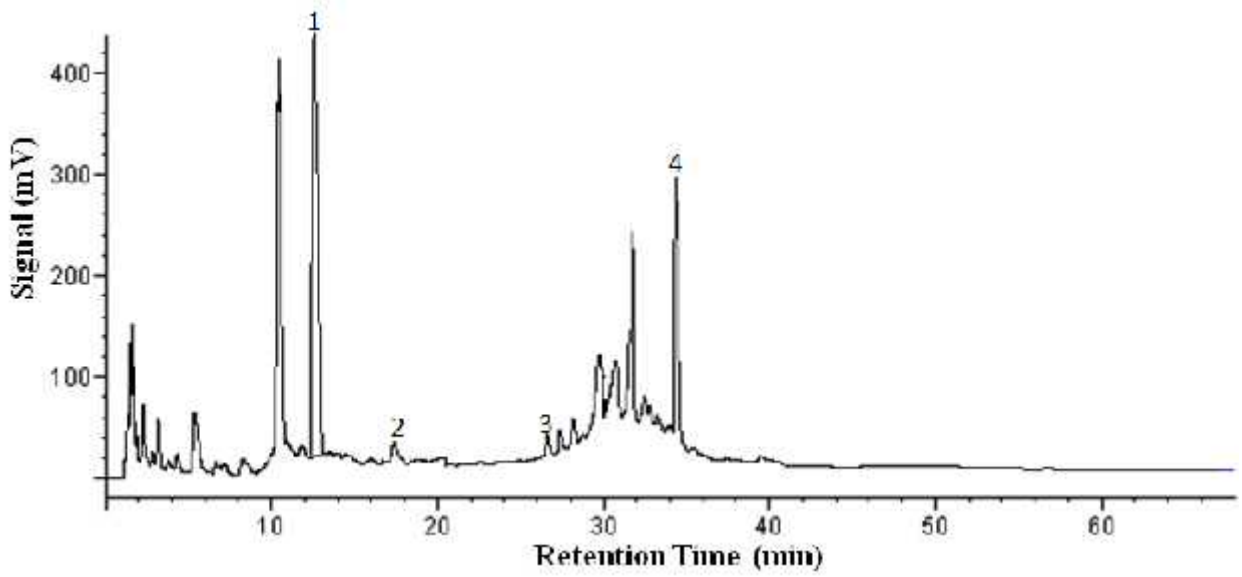
300
200
100
4
5
6
10
20
30
40
50
60
Retention Time (min)

b



c

Figure 7.6. HPLC chromatograms of *Salvia miltiorrhiza* herbal extract, obtained with 125 ^{o}C extraction temperature, evaluated on the Alltech Adsorbosil C18 column at ambient temperature. (a) Analyte standard solution; (b) Methylene chloride phase; (c) Water phase. Flow rate: 1.0 mL/min. UV detection: 254 nm. Mobile phase: A, 100 mM phosphoric acid in water; B, 100% methanol. Gradient: 0-4 min, 2% methanol; 4-8 min, 2-10% methanol; 8-23 min, 10-30% methanol; 23-32 min, 30-60% methanol; 32-43 min, 60% methanol; 43-49 min, 60-70% methanol; 49-61 min, 70-80% methanol; 61-68 min, 80-2% methanol. Peak identification: 1, protocatechualdehyde; 2, caffeic acid; 3, ferulic acid; 4, propyl paraben; 5, tanshinone I; 6, tanshinone IIA.

Table 7.1. Comparison of Anticancer Agent Concentrations Found in *Salvia miltiorrhiza* Obtained by Sonication, Traditional Herbal Decoction, and Subcritical Water Extraction

Analyte	Concentration in µg/g (%RSD)[a]					
	Sonication of Fresh Herb	Traditional Herbal Decoction	Subcritical Water Extraction			
			75 °C	100 °C	125 °C	150 °C
Protocatechualdehyde	12.8 (9.1)	112.3 (19.2)	55.6 (4.5)	196.3 (9.0)	5055.3 (4.8)	36.2 (10.4)
Caffeic Acid	47.0 (7.5)	525.3 (19.8)	217.0 (5.0)	351.4 (7.1)	92.9 (17.8)	1.1 (8.9)
Ferulic Acid	2.0 (5.9)	13.6 (16.6)	9.1 (13.0)	28.4 (13.6)	33.7 (10.3)	39.1 (20.8)
Tanshinone I	175.5 (34.4)	0.2 (13.5)	4.0 (2.2)	5.8 (7.3)	19.1 (13.0)	74.0 (2.5)
Tanshinone IIA	329.1 (36.3)	0.8 (17.5)	3.3 (1.3)	5.0 (13.1)	5.18 (16.4)	15.3 (13.0)

[a] *Triplicate measurements.*

The traditional herbal decoction of *Salvia miltiorrhiza* was carried out for the comparison of *Salvia miltiorrhiza* SBWE extraction data. Table 7.1 shows the concentrations of the five anticancer analytes from *Salvia miltiorrhiza* achieved by THD. As discussed earlier, tanshinones showed significant anticancer effect [76-78, 262]. What promising with SBWE of *Salvia miltiorrhiza* is tanshinone concentrations obtained by SBWE at all temperatures are higher than that achieved by THD extractions, the traditional herbal preparation technique used for thousands of years in eastern Asia. Especially tanshinone I concentration achieved by SBWE at 150 °C is 370 folds higher than the concentration obtained by THD extractions.

Similar to the THD extraction of *Salvia miltiorrhiza*, sonication extraction of *Salvia miltiorrhiza* was also conducted for the comparison purpose. Table 7.1 shows the concentrations of five anticancer compounds achieved by sonication of *Salvia miltiorrhiza*. The concentrations of protocatechualdehyde and ferulic acid obtained by SBWE at all temperatures are higher than the sonication. The caffeic acid concentration achieved by SBWE at most temperatures is higher than that of sonication. The tanshinones concentrations obtained by sonication are higher than the SBWE of *Salvia miltiorrhiza* at all temperatures.

7.3.2 Reproduction Assay of *C. elegans*

7.3.2.1 Optimization of Reproduction Assay Using Diluted Herbal Extracts

In order to optimize the dilution factor of the herbal extract to be used in the reproduction assay of *C. elegans*, *Salvia miltiorrhiza* herbal extract obtained by SBWE at 150 ^{o}C was evaluated. *C. elegans* consume the food through oral and skin, whereas chemicals only through skin absorption. If the herbal extract is too concentrated, it may not be absorbed through the skin. Therefore the optimization of the dilution factor for herbal extract is critically important in testing the cytotoxicity of *Salvia miltiorrhiza* herbal extract on *C. elegans*.

At first, the subcritical water extraction of *Salvia miltiorrhiza* was conducted at 150 ^{o}C. Then the herbal extract was diluted by a factor of 2, 10, and 50 with deionized water. The diluted herbal extracts were evaluated for the cytotoxicity on *C. elegans* using reproduction assay. Table 7.2 shows the reproduction inhibition and mortality of *C. elegans* after 30 hour exposure to the three different diluted SBWE herbal extracts obtained at 150 ^{o}C. The results indicate that the 2-time diluted herbal extract (high concentration) shows greater reproduction inhibition than the other diluted herbal extracts. Whereas the 10-time diluted herbal extract was associated with higher mortality rate than the other diluted herbal extracts. These results show that 23% of worms were found dead in the 10-time diluted treatment group. This concentration was also associated with a 21% decrease in reproductive capacity of the surviving worms. Therefore the 10-time dilution factor was chosen for the reproduction assay of *C. elegans* for the remainder of the cytotoxicity study. The higher mortality of *C. elegans* associated with 10-time diluted herbal extract (medium concentration) may be attributed to the higher uptake of 10-time diluted herbal extract than the 2-time diluted herbal extract through the skin of *C. elegans*.

Table 7.2. Percent Reproduction Inhibition and Mortality of *C. elegans* After 30 Hour Exposure to the Diluted *Salvia miltiorrhiza* Herbal Extract Obtained by SBWE at 150 ^{o}C

Treatment NGM Plates	Average Production[a] (SE)[b]	%Reproduction Inhibition[c]	% Mortality[d]
Control	134 (15)	0	0
02-time dilution	96 (18)	28	0
10-time dilution	105 (11)	21	23
50-time dilution	114 (7)	15	17

[a] *Total of eggs and larva average per worm over 3 days.*

[b] *Standard error obtained by five replicate measurements* $\left(S = \frac{S \quad di \quad a \ m}{\sqrt{N} \quad a \ m}\right)$.

[c] $\%R \quad ti\ hil = \frac{C \quad a \quad rej \quad -H \quad u\ e \quad a \quad re}{C \quad a \quad rej} \times 100.$

[d] $\%M = \frac{N \quad a \ w \quad ai}{T \quad n \quad a \ w} \times 100.$

7.3.2.2 Evaluating the Cytotoxity of SBWE Herbal Extracts on *C. elegans* Using Reproduction Assay

In order to optimize the preparation of efficacious herbal medication through subcritical water extraction, the cytotoxicity of SBWE herbal extracts obtained at four different temperatures (75, 100, 125, and 150 ^{o}C) was evaluated on *C. elegans*. All SBWE herbal extracts were diluted by 10-times with deionized water. Cytotoxicity testing was again evaluated through both reproduction inhibition and mortality of *C. elegans*. Table 7.3 shows the reproduction assay of *C. elegans* after 30 hour exposure to the 10-time diluted SBWE water extracts achieved at 75 to 150 ^{o}C. As shown in Table 7.3, the reproduction inhibition of the extracts increased with increasing temperature. The SBWE extraction temperature also has an effect on mortality. In general, the worm survival rate decreased with the increase in extraction temperature except at 150 ^{o}C. Again the main reason for lower mortality of *C. elegans* with the cytotoxicity evaluation of herbal extract achieved at 150 ^{o}C, may be attributed to the less intake of high concentrated herbal extract through the skin of *C. elegans*. One more reason for the

lower mortality of worms, may be due to the degradation of compounds associated with the mortality of worms such as protocatechualdehyde at 150 ^{o}C.

The reproduction assay of *C. elegans* was also carried out using the 10-time diluted THD extract of *Salvia miltiorrhiza* for comparison. As shown in Table 7.3, both reproduction inhibition and mortality achieved by SBWE extracts at all four temperatures are higher than those obtained by the traditional herbal decoction. The cytotoxicity results indicate that SBWE is a much more efficient extraction technique and it might be used to develop efficacious herbal medicine in the future.

Table 7.3 Percent Reproduction Inhibition and Mortality of *C. elegans* After 30 Hour Exposure to the 10-time Diluted Traditional Herbal Decoction and Subcritical Water Extractions of *Salvia miltiorrhiza* at 75 to 150 ^{o}C

Treatment NGM Plates	3Day Average Production[a] (SE)[b]	%Reproduction Inhibition[c]	%Mortality[d]
Control	111 (3)	0	0
THD	106 (3)	4	10
SBWE-75 ^{o}C	104 (2)	6	17
SBWE-100 ^{o}C	89 (6)	20	33
SBWE-125 ^{o}C	67 (9)	40	42
SBWE-150 ^{o}C	60 (12)	46	14

[a] *Total of eggs and larva average per worm over 3 days.*

[b] *Standard error obtained by five replicate measurements* $\left(S = \frac{S \quad d \quad a \quad m}{\sqrt{N} \quad a \quad m}\right)$.

[c] $\%R \quad ti\,hil = \frac{C \quad a \quad re \quad -N \quad e \quad a \quad re}{C \quad a \quad re} \times 100.$

[d] $\%M = \frac{N \quad a \quad w \quad d}{T \quad n \quad a \quad w} \times 100.$

Chapter 8: Conclusions

This chapter summarizes the results of both fundamental and applied studies of subcritical water chromatography and subcritical water extraction involved in this work. The fundamental studies include the solubility of parabens and stability of preservatives and stationary phases under subcritical water conditions. The applied studies of SBWC include the separation and analysis of pharmaceuticals in cold drugs and niacinamide, preservatives, and sunscreens in skincare creams. *Salvia miltiorrhiza*, a medicinal herb was extracted using subcritical water and the cytotoxicity of the herbal extracts was tested.

The solubility of all three parabens, methyl, ethyl, and butyl parabens in subcritical water increased by increasing the water temperature from 25 to 150 ^{o}C. The solubility enhancement was approximately 6 to 19 folds with the increase of temperature from 25 to 100 ^{o}C and about 2 folds with further increase from 100 to 150 ^{o}C. When the temperature was raised from 150 to 200 ^{o}C, the solubility of parabens in subcritical water decreased due to degradation. Since existing approximation solubility models failed to predict the solubility of parabens in high temperature water, a new model was developed in this work, and it can reasonably estimate the solubility of parabens in subcritical water.

The chromatographic evaluation revealed that there was no degradation of preservatives during SBWC runs at 150 ^{o}C when compared with HPLC runs at 25 ^{o}C. However there was a slight degradation of preservatives at 200 ^{o}C, especially for ethyl and propyl parabens. The evaluation of preservative stability under much tougher conditions (heated-water preservatives mixtures for up to 60 min) indicated that there was no degradation of preservatives at 100 ^{o}C and a slight degradation at 150 ^{o}C. However, the degradation of preservatives was approximately 10% at 200 ^{o}C. The major degradation products of parabens at higher

temperatures were identified as phenol and *p*-hydroxybenzoic acid using GC/MS as mentioned in Chapter 4. A paraben degradation mechanism developed in this study also shows the paraben degradation products as phenol and *p*-hydroxybenzoic acid.

As discussed in Chapter 2, the manufacturer temperature limit for silica based columns is 60 ^{o}C and zirconia based column is 200 ^{o}C [1, 133]. Fortunately, the stability evaluation of hybrid silica based columns at 150 ^{o}C under SBWC conditions revealed that the Waters XBridge phenyl column was stable for up to 831 hours (30,000 column volume) and the Waters XBridge C18 up to 662 hours (23,912 column volume). Similarly, the ZirChrom-DB-C18 column was stable for up to 263 hours (14,250 column volume) at 200 ^{o}C.

Pharmaceuticals present in cold drugs (Vicks formula 44 custom care, Alka-seltzer plus, and CVS multi-symptom severe cold relief) were successfully separated using SBWC on the Alltech Adsorbosil C18 column at temperatures ranging from 25 to 150 ^{o}C.

Niacinamide present in the Olay total effects UV moisturizer was well separated using SBWC on the XBridge C18 column at 60 ^{o}C. Preservatives present in the Olay daily moisturizer, Olay UV moisturizer, and Olay skin-renewing UV lotion were separated using both HTLC and SBWC methods on the Zir-Chrom-DB-C18 column at temperatures ranging from 150 to 200 ^{o}C. The HTLC methods at 150 ^{o}C on the Zir-Chrom-DB-C18 column saved about 75% of methanol in the mobile phase when compared with the HPLC methods at 25 ^{o}C. A much faster SBWC separation of preservatives in Olay skin-renewing UV lotion was also obtained on the ZirChrom-PS column at 180 ^{o}C. The disadvantage with the SBWC separations of preservatives on the Zir-Chrom-DB-C18 and ZirChrom-PS columns is the co-elution of benzyl alcohol with a matrix peak. Additional HTLC and SBWC separations of preservatives present in the Olay skin-renewing UV lotion were further evaluated on both Waters XBridge

C18 and Waters XBridge phenyl columns at 150 ^{o}C. There are several advantages with the separation of preservatives on XBridge columns. Firstly, benzyl alcohol was separated from the matrix peak. Secondly, the temperature required for the SBWC separations of preservatives on XBridge columns (100 to 150 ^{o}C) is much lower than the zirconia based columns (200 ^{o}C). Thirdly, the methanol saved with the HTLC methods on XBridge columns is about 90%.

Separation of sunscreens present in the Olay UV moisturizer was achieved using HTLC on the ZirChrom-DB-C18 column at 190 ^{o}C with programmed flow rates. Approximately 54% of methanol was saved by using this HTLC method. The HTLC separations of sunscreens on the XTerra MS C18 column were more promising than those achieved on the ZirChrom-DB-C18 column. Separation of sunscreens present in the Olay UV moisturizer and Olay skin-renewing UV lotion were obtained at both 150 and 200 ^{o}C using a weak gradient elution. The HTLC method at 150 ^{o}C saved up to 84% methanol while the HTLC method at 200 ^{o}C saved up to 99% methanol used in the mobile phase. At last, sunscreens present in the Olay UV moisturizer were separated using SBWC at 250 ^{o}C on the XTerra MS C18 column. Separations of sunscreens on the XBridge C18 column yielded the best results. Both HTLC separation using only 2% methanol and the integrated SBWC/HTLC separation at 200 ^{o}C resulted in narrower peaks compared with those obtained on the other two columns. Approximately 97% methanol was saved by using this HTLC method and approximately 99% methanol saved with the integrated SBWC/HTLC method. Again the SBWC separation of sunscreens was achieved on the XBridge C18 column at 230 ^{o}C.

A large number of replicate SBWC runs of a single preservative sample solution as well as a single niacinamide sample solution indicated no system building-up. Our best SBWC quantification results achieved in this work is in the range of 97.4 to 103.4% recoveries and

RSDs less than 1.9% for the separation of pharmaceuticals in cold drugs as well as niacinamide and preservatives in skincare creams. HPLC quantification results for the separations of niacinamide and preservatives indicate that some of the developed SBWC methods for these analytes are as good as the HPLC methods. Therefore, the results of SBWC methods demonstrate a potential for the application of subcritical water chromatography in industry.

The anticancer agent concentrations obtained by subcritical water extraction of *Salvia miltiorrhiza* increased by up to 4 folds with the raise of the extraction temperature from 75 to 100 ^{o}C. The analyte concentrations were further enhanced up to 26 folds with the increase from 100 to 125 ^{o}C, except for caffeic acid. When the temperature was raised from 125 to 150 ^{o}C, analyte concentrations were further increased by up to 4 folds except for caffeic acid and protocatechualdehyde. Both caffeic acid and protocatechualdehyde might be degraded at 125 ^{o}C or higher. When compared the tanshinone concentrations achieved by SBWE of *Salvia miltiorrhiza* with the THD, the SBWE extracts have higher tanshinone concentrations than the THD extracts.

The cytotoxicity evaluation of diluted (2, 10, and 50-time dilution) SBWE herbal extracts obtained at 150 ^{o}C on *C. elegans* indicated that the 10-time diluted herbal extract was more cytotoxic than the 2-time and 50-time diluted herbal extracts. The 10-time diluted herbal extract was associated with 23% mortality of worms as well as 21% reproduction inhibition on *C. elegans* in reference to the control. Further cytotoxicity evaluation of the 10-time diluted SBWE herbal extracts collected at four different temperatures (75, 100, 125, and 150 ^{o}C) on *C. elegans* revealed that the extraction temperature of 125 ^{o}C showed more cytotoxicity on *C. elegans*. The SBWE herbal extract obtained at 125 ^{o}C was associated with 42% mortality of worms and 40% reproduction inhibition on *C. elegans* in reference to the control. The cytotoxicity of SBWE

herbal extracts obtained at all temperatures from 75 to 150 ^{o}C was higher than the traditional herbal decoction extracts. Both SBWE and cytotoxicity results demonstrated that SBWE is a more efficient technique than the traditional herbal decoction to extract the anticancer agents from medicinal herbs and its extracts are also more potent than the THD extracts. These findings show a potential of employing the subcritical water extraction technique in developing more efficacious herbal medicine.

Chapter 9: Bibliography

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APPENDIX: LIST OF PUBLISHED PAPERS

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Industrial application of green chromatography—I. Separation and analysis of niacinamide in skincare creams using pure water as the mobile phase

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ABSTRACT

In this work, chromatographic separation of niacin and niacinamide using pure water as the sole component in the mobile phase has been investigated. The separation and analysis of niacinamide have been optimized using three columns at different temperatures and various flow rates. Our results clearly demonstrate that separation and analysis of niacinamide from skincare products can be achieved using pure water as the eluent at 60 °C on a Waters XTerra MS C18 column, a Waters XBridge C18 column, or at 80 °C on a Hamilton PRP-1 column. The separation efficiency, quantification quality, and analysis time of this new method are at least comparable with those of the traditional HPLC methods. Compared with traditional HPLC, the major advantage of this newly developed green chromatography technique is the elimination of organic solvents required in the HPLC mobile phase. In addition, the pure water chromatography separations described in this work can be directly applied in industrial plant settings without further modification of the existing HPLC equipment.

1. Introduction

Niacinamide is a chemical compound belonging to the vitamin B group that is commonly found in foods and used in cosmetic skincare products. Niacinamide is an amide derivative of niacin and is found in bound forms in nicotinamide adenine dinucleotide (NAD), its phosphorylated derivative NAD(P), and their reduced forms NAD(H) and NAD(PH), which are coenzymes important for cellular redox reactions [1,2]. These cofactors have many antioxidant properties and are involved in many enzymatic reactions in the skin [2]. Some of the cosmetic effects of niacinamide include improved skin barrier, reduced skin pore size, facial blotchiness, hyperpigmentation, and skin yellowing, antiwrinkle, and antiacne properties [2]. Niacinamide is also used as a bleaching agent in bleaching cosmetics and can control the transfer of melanin from melanocytes [3]. In addition, niacinamide is widely used as a color fixative in meats to maintain fresh color [4,5]. Its antioxidant properties help to reduce the speed of myoglobine oxidation and prolong the red fresh color [5].

It is important to be able to accurately analyze niacinamide levels in various products. Quantitative analysis of niacinamide in cosmetics is required for quality control, product release, and regulatory purposes. HPLC with UV or fluorometric detection has been widely used for the determination of niacinamide in pharmaceuticals, biological fluids, food, and cosmetics [1,3–6]. However, these traditional HPLC methods require organic solvents in the mobile phase that are hazardous and expensive.

With growing awareness about the environment and the increased initiative for the development of "green" technologies throughout the world, reversed-phase liquid chromatography using water as the sole component in the eluent has received increased attention. The ability to eliminate the enormous amounts of hazardous organic solvents that are consumed everyday worldwide and replace them with an efficient separation method that utilizes environmentally benign water offers many benefits both environmentally and economically.

Water is too polar to serve as a reversed-phase eluent at ambient temperature using normal RPLC stationary phases such as silica-based C18 and polymer PRP-1 columns. Therefore, there are two ways to achieve LC separation of organics using pure water as the sole mobile phase component. One way is to modify the stationary phase and the other is to heat the water mobile phase. Synovec

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and co-workers developed a special type of packing material by coating the stationary phase on non-porous glass beads or silica that allowed LC separation of organics using pure water at room temperature to occur [7–10].

Although ambient water is very polar, it can act like an organic solvent at elevated temperatures and has widely tunable properties such as dielectric constant, surface tension, viscosity, and dissociation constant that can be achieved by simply adjusting the temperature [11,12]. Pressure has little effect on the properties of the water but it is needed to keep the water in the liquid state at high temperatures [11–13].

Because of the unique properties of the high-temperature water, it has been used as the sole mobile phase component in reversed-phase liquid chromatography to achieve separation of many classes of organic compounds [11,13]. For over a decade, high-temperature (subcritical) water has been used to achieve chromatographic separation of polar, moderately polar, and even some non-polar solutes [11–22]. Most of the findings indicate that high-temperature water can be used to replace hazardous and expensive organic solvents required in the traditional RPLC to achieve comparable separation using water-only mobile phase [11,23–35].

However, much of the high-temperature water chromatography work was limited to academic studies. There are numerous industrial HPLC methods where a low percentage of organic solvents such as ~33% methanol are required in the mobile phase. Although one may argue that the methanol consumption is relatively low, the HPLC waste generated by such methods is threefold the volume of the methanol consumed and needs to be disposed of. For example, if an HPLC system is running continuously at 1 mL/min in an QC lab for a year, a total of 525 L of HPLC waste are generated for 175 L of methanol consumed using such an HPLC method. The industry has to pay not only for purchasing the 175 L of methanol but also for disposing of the 525 L of the methanol-containing waste. Fortunately, the organic solvents required in these HPLC methods can be replaced by heating pure water with only mild temperatures. Because the current commercial HPLC systems are equipped with column ovens capable of achieving 80 °C, the pure water chromatography separations may be directly applied in industrial plant settings without any further additions or modifications.

In this work the original Procter & Gamble (P&G) HPLC niacinamide method was identified as one of the existing industrial methods that may be replaced by a green method employing pure water chromatography. Therefore, the goal of this study was to develop a green chromatography method using pure water as the eluent for the analysis of niacinamide in skincare products. Waters XTerra MS C18 and XBridge C18 as well as Hamilton PRP-1 columns were used in this work. These columns were chosen because of their good stability at elevated temperatures [11]. Pure water chromatography separation has been optimized at different temperatures and flow rates. HPLC separation of niacinamide using methanol in the mobile phase was also performed for comparison purposes. Because niacin is often associated with niacinamide, we also included niacin in the last part of this study to broaden the application of this pure water chromatography method in other areas where the analysis of niacin is a must.

2. Experimental

2.1. Reagents

Niacin, niacinamide, ammonium acetate, and 4-acetamidophenol were purchased from Sigma–Aldrich Chemical (Milwaukee, WI, USA). HPLC-grade methanol and formic acid (90%) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water (18 MΩ cm) was prepared in our laboratory. Olay skincare creams were purchased at a local store. The niacinamide placebo sample was received from Procter and Gamble (Cincinnati, OH, USA).

2.2. Preparation of diluent and standard solutions

The diluent was prepared by combining 200-mL 50 mM formic acid with 300-mL 50 mM ammonium acetate and mixed well. Internal standard solution was prepared by adding 0.10 ± 0.01 g of 4-acetamidophenol to a 100 mL volumetric flask and then diluting to the mark with methanol. Stock solution was prepared by adding 0.1 ± 0.05 g of niacinamide to a 10-mL volumetric flask and diluting to the mark with diluent. The working standard solution was prepared by transferring 1.00 mL of stock solution and 1.00 mL of internal standard to a suitable container and adding 8.00 mL diluent.

2.3. Preparation of samples

Each skincare cream sample was mixed well before sampling to ensure a homogeneous mixture. 0.2 ± 0.05 g of sample were weighed directly into a tared 25-mL glass vial (Supelco, Park Bellefonte, PA, USA). 1.00 mL of internal standard solution and 5 mL of methanol were added to the vials. The mixture was vortexed to completely disperse the cream. Once dispersed, approximately 5 mL of diluent was added to each vial and shaken to mix thoroughly. The solution was then filtered through a 0.45 μm Whatman GD/X filter (VWR, West Chester, PA, USA) into a 2-mL sample vial for chromatographic analysis.

The placebo sample obtained from P&G was spiked with 1.00 mL of the stock solution for niacinamide recovery study. The spiked placebo samples were then treated in the same manner as other regular cream samples prior to chromatographic separation and analysis.

2.4. Instrumentation

A homemade chromatography system was mainly used in this study. A Hitachi L-7100 HPLC pump (Hitachi, Ltd., Tokyo, Japan) was used to deliver the mobile phase. A Valco injector (Valco Instruments Company Inc., Houston, TX, USA) with a 5-μL loop was connected to the outlet of the Hitachi pump using a stainless steel tubing that included a preheating coil. The tubing passed into a GC oven (HP 5890 Series 2, Hewlett Packard, Avondale, PA, USA) that was used to control the column temperature. The GC oven was allowed to heat up to the set temperature for the experiment and the first injection was made ~20 min after the set temperature was reached. The column was located inside the GC oven and connected to the outlet of the injector with a stainless steel tubing. A Hamilton PRP-1 column with 3-μm particles (150 mm × 4.1 mm, Reno, NV, USA) and a Waters XTerra MS C18 column (2.1 mm × 100 mm, 3.5 μm, Waters Corporation, Milford, MA, USA) were used in this work. The tubing then exited the GC oven and passed through an iced water bath before entering the Hitachi L-7400 UV detector set at 245 nm. After exiting the UV detector flow cell, the eluent passed through a back pressure regulator (Restek, Bellefonte, PA, USA) and then was collected in a waste container. Please note that the chromatography system used in this study was constructed to also allow high temperature applications. It is likely that both the backpressure regulator and the iced water bath could be eliminated for applications at lower temperatures such as the ones used in this work, provided that the UV detector precision is maintained. 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.

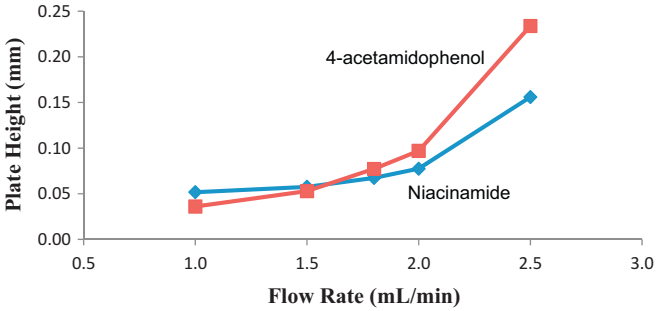

Fig. 1. Van Deemter plots obtained by pure water chromatography on the PRP-1 column at 80 °C.

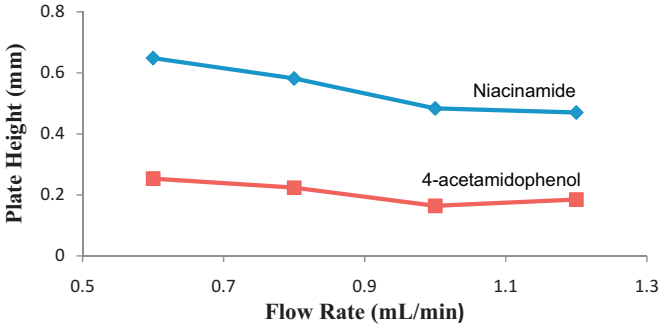

Fig. 3. Van Deemter plots obtained by pure water chromatography on the XTerra MS C18 column at 60 °C.

Additional experiments were performed using a Shimadzu Nexera UFLC system (Shimadzu Corporation, Tokyo, Japan). A Waters XBridge C18 column (4.6 mm × 100 mm, 3.5 μm, Waters Corporation) was used in this part of the work. Since niacin is normally associated with niacinamide, we also included niacin in this part of the study even though only trace amount of niacin is contained in the cream samples analyzed in this work.

3. Results and discussion

3.1. Hamilton PRP-1 column

3.1.1. Effects of temperature and flow rate on separation

The Hamilton PRP-1 column was tested for niacinamide separation using 100% water as the mobile phase. After initial evaluation of temperature effect on separation, the remainder of the pure water chromatography experiments on this column was carried out at 80 °C due to the efficient separation of niacinamide at this temperature. Please note that this temperature is within the manufacturer's temperature limit. Many reports show that PRP-1 columns are stable at much higher temperatures [11]. Another reason for the selection of 80 °C is that many commercially available HPLC systems are equipped with column ovens capable of operating at temperatures up to 80 °C. The HPLC experiments were performed at 25 °C.

Five different flow rates ranging from 1.0 to 2.5 mL/min were tested in optimizing the separation of niacinamide and 4-acetamidophenol. As shown in Fig. 1, the plate height for niacinamide stays relative flat in the range of 1–2 mL/min. However, the

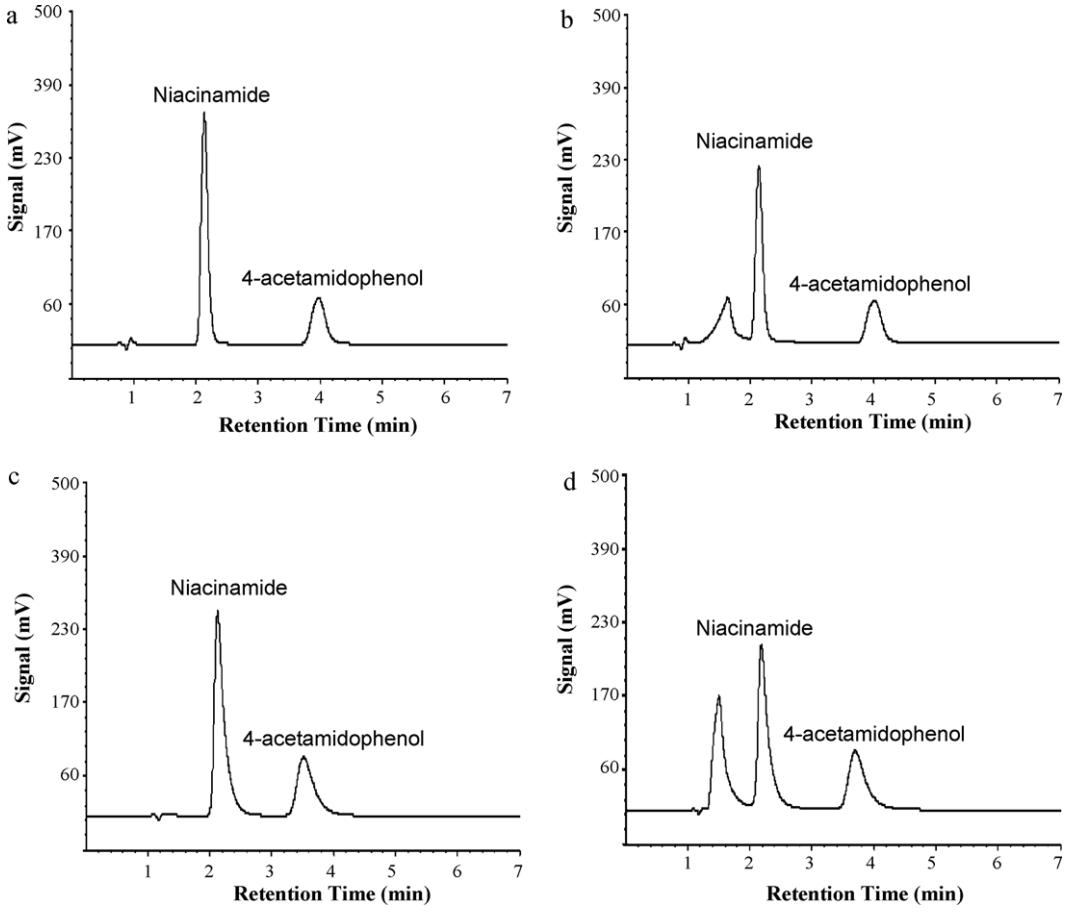

Fig. 2. Chromatograms of skincare cream samples on the PRP-1 column. (a) Pure water chromatography at 80 °C with 1.8 mL/min for SC-EC1; (b) pure water chromatography at 80 °C with 1.8 mL/min for SC-EC2; (c) HPLC at 25 °C with 1.2 mL/min for SC-EC1; and (d) HPLC at 25 °C with 1.2 mL/min for SC-EC2.

Table 1
Niacinamide recovery obtained by pure water chromatography compared with HPLC at 25 °C using 30% methanol in the mobile phase.

Samples	Column	Temperature (°C)	Flow rate (mL/min)	Niacinamide recovery (%)	%RSD
SC-EC1	PRP-1	80	1.8	101.0[a]	1.2[b]
SC-EC1	PRP-1	80	1.8	100.1[a]	1.3[c]
SC-EC2	PRP-1	80	1.8	100.2[a]	1.5[b]
SC-E	XTerra	60	1.0	100.4[d]	2.5[b]
SC-EC2	XBridge	60	2.0	100.5[a]	1.3[b]

[a] Recovery% = $\frac{\text{Weight\% obtained by this method}}{\text{Weight\% obtained by HPLC}} \times 100\%$.
[b] Based on five replicate sample preparations analyzed by both pure water chromatography and HPLC.
[c] Based on 21 replicate injections of one sample preparation.
[d] Recovery% = $\frac{\text{Mass of nicacinamide recovered by this method}}{\text{Mass of niacinamide added to the placebo}} \times 100\%$.

plate height increases significantly at 2.5 mL/min. Considering both separation efficiency and speed, 1.8 mL/min was chosen as the flow rate of pure water for the remainder of this study with the PRP-1 column.

3.1.2. Comparison of pure water chromatography and HPLC separations

HPLC separation of niacinamide was conducted at 25 °C using a mobile phase of 70% deionized water:30% methanol. For fair comparison purposes, similar retention times were obtained by both HPLC and pure water chromatography. Under the condition of similar retention times for each solute, the plate height obtained by HPLC is 0.154 and 0.180 mm for niacinamide and 4-acetamidophenol, respectively. These plate height values are about 2.3 times higher than that obtained by pure water chromatography with 1.8 mL/min as demonstrated in Fig. 1. In this case, pure water chromatography not only eliminates the use of methanol in the mobile phase but also yields better efficiency than HPLC with methanol involved in the mobile phase. The improvement in pure water chromatography separation efficiency can also be seen by comparing the chromatograms of both pure water chromatography and HPLC shown in Fig. 2.

3.1.3. Separation and analysis of niacinamide in skincare creams

Fig. 2a and b shows the chromatograms of two skincare cream samples, SC-EC1 and SC-EC2, obtained by pure water chromatography at 80 °C with 100% water as the mobile phase. The chromatograms of the same two cream samples obtained by HPLC

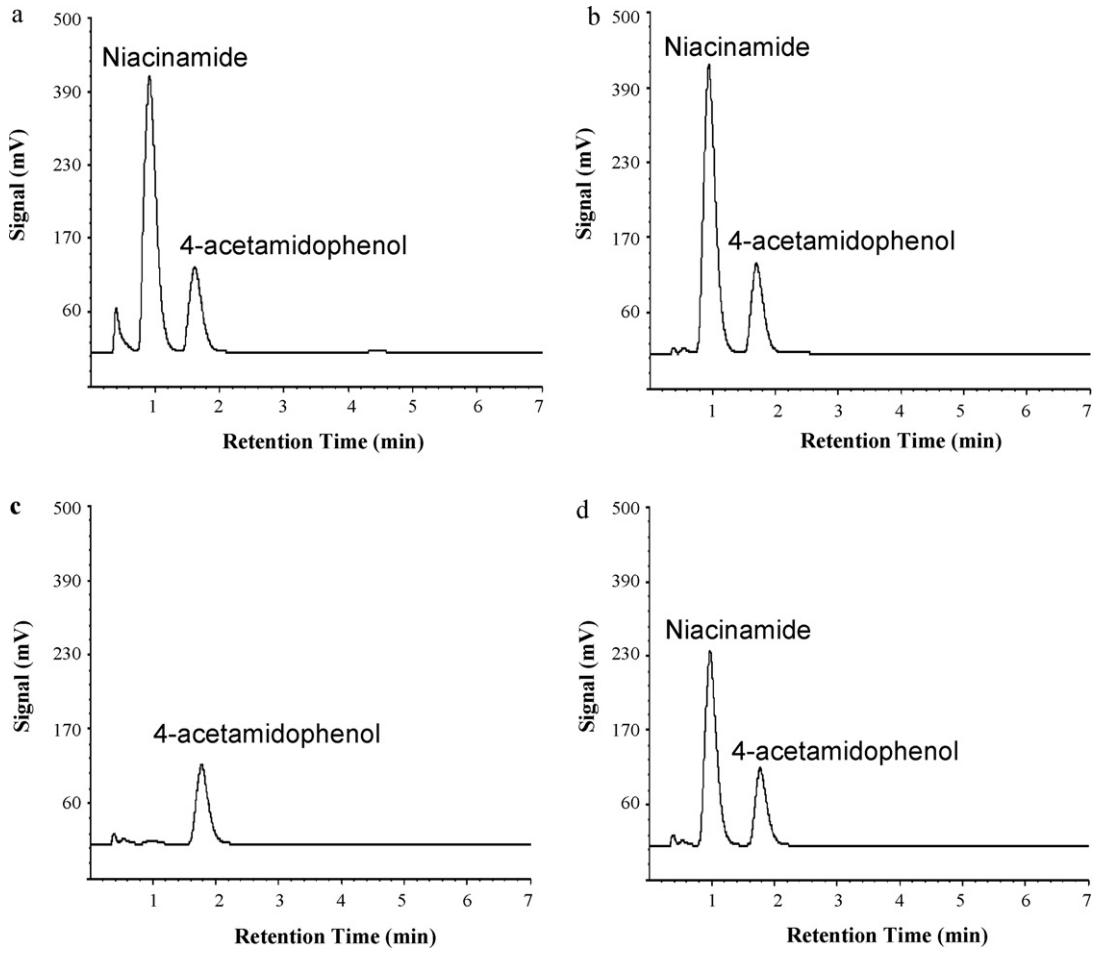

Fig. 4. Chromatograms on the XTerra MS C18 column using pure water as the mobile phase at 60 °C with 1 mL/min. (a) SC-EC1 cream sample; (b) SC-E cream sample; (c) SC-E placebo; and (d) SC-E placebo spiked with 9.6 mg of niacinamide.

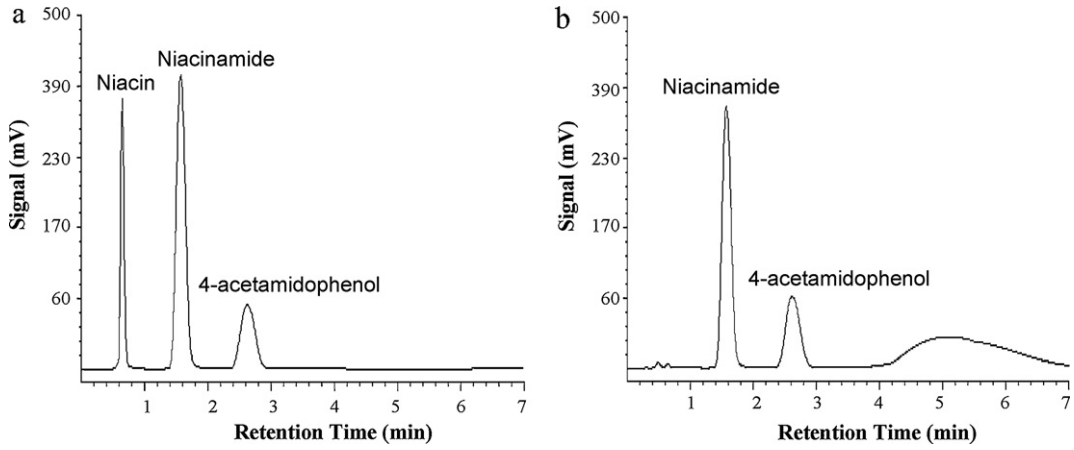

Fig. 5. Chromatograms on the XBridge C18 column using pure water as the mobile phase at 60 °C with 2 mL/min. (a) Standard mixture containing niacin and (b) SC-EC2 cream sample.

with 30% methanol in the mobile phase are given in Fig. 2c and d for comparison. The extra peak eluted before niacinamide as shown in Fig. 2b and d is from the sample matrix.

In order to evaluate the pure water chromatography method, the percent weight niacinamide determined by this green chromatography method was compared with that obtained by HPLC. As shown in Table 1, the recoveries for skin care creams SC-EC1 and SC-EC2 were 101.0% (RSD = 1.2%) and 100.2% (RSD = 1.5%), respectively. The percent recovery and RSD values were based on five replicate experiments. These recoveries are not significantly different from 100%, indicating that this new method provides the same results as the traditional HPLC method. In addition, the relative standard deviation obtained by pure water chromatography is below 2%, meaning that this green method is very reproducible.

3.1.4. Study of potential building-up problem

The reproducibility of this method was further evaluated by performing 21 replicate injections of one sample preparation using the SC-EC1 cream to check for building-up in the chromatography system resulting from numerous injections of real samples. As shown in Table 1, the niacinamide recovery is 100.1% with RSD of 1.3%, indicating that no sample building-up occurred. The excellent accuracy and precision determined from multiple injections of real samples suggests that pure water chromatography could be used in real industrial applications.

3.2. Waters XTerra MS C18 column

3.2.1. Effects of temperature and flow rate

Further investigation of separations using 100% water was conducted on the Waters XTerra MS C18 hybrid column. Temperatures of 60 and 80 °C were tested. Because the separation at 80 °C was not significantly better than that at 60 °C, the latter temperature was used for pure water chromatography separation on this Waters column. Another reason for choosing 60 °C is that it is within the manufacturer's recommended temperature limit for this column. It should be noted that other researchers found this column's actual temperature limit is much higher than the recommended limit of 60 °C [11].

Different flow rates ranging from 0.6 to 1.2 mL/min were evaluated to optimize the pure water chromatography separation. Fig. 3 shows that 1.0 mL/min flow rate yields the best separation. Therefore, this flow rate was used for the remaining experiments using this Waters XTerra column.

3.2.2. Separation and analysis of niacinamide in skincare creams

The optimized temperature (60 °C) and flow rate (1 mL/min) conditions for separations using pure water were applied to skincare cream and placebo samples. Fig. 4 shows the chromatograms of (a) SC-EC1 (skincare cream), (b) SC-E (skincare cream), and (c) SC-E placebo (skincare cream containing no niacinamide). As expected, no niacinamide peak appeared in the placebo sample (Fig. 4c). The niacinamide concentrations obtained by pure water chromatography was quantitative and the RSD is again below 2%.

3.2.3. Recovery study using a spiked placebo sample

To further test the reliability and validity of this green method, the placebo sample was spiked with 9.06 mg of niacinamide. The spiked placebo sample was treated in the same manner as a real skincare cream sample and analyzed using this new method. The chromatogram of the spiked placebo sample is shown in Fig. 4d. The percent recovery of niacinamide is 100.4% (RSD = 2.5%). The good recovery and the low %RSD value again demonstrate that this green separation method is accurate and precise.

3.3. Separation of niacin and niacinamide on the Waters XBridge C18 column

To further explore the industrial application of this green method, a commercial system, Shimadzu Nexera was employed in this part of the study. As mentioned earlier, niacin often co-exists in niacinamide-containing samples. To broaden the application of this green chromatography method, we also included niacin in this part of the study.

The conditions used here for separation using pure water were 60 °C and 2 mL/min. As shown in Fig. 5a, niacin was well separated from niacinamide using only pure water as the eluent. Fig. 5b shows the chromatogram of SC-EC2 cream sample. Only trace amount of niacin can be seen in the chromatogram. The last peak is from the sample matrix. The quantification shown in Table 1 reveals a niacinamide recovery of 100.5% with 1.3% RSD, again indicating the good accuracy and precision of this new method using a commercial system.

4. Conclusions

Our results clearly demonstrate that separation and analysis of niacin and niacinamide using only water as the eluent can be achieved at temperatures of 60–80 °C on either Waters XTerra MS C18 and XBridge C18 or Hamilton PRP-1 columns. The quantification quality and reproducibility of the organic-free methods

developed in this study are comparable with those of traditional HPLC methods. In addition, the separation efficiency obtained by the pure water chromatography methods is even higher than that of ambient HPLC methods with methanol required in the mobile phase.

Considering that most current commercial HPLC systems are equipped with column ovens capable of achieving 80 °C, the pure water chromatography separations described in this work can be directly applied in industrial plant settings without any modifications. There are numerous existing HPLC methods in industry where a fraction of organic solvents (e.g., 30% methanol) are required in the mobile phase. Although the methanol percentage is relatively low, the HPLC waste generated by such methods is threefold the volume of the methanol consumed. Fortunately, organic solvents are eliminated in pure water chromatography and a significant amount of money spent on HPLC solvents and waste disposal can be saved if a company is willing to adopt this green chromatography technique.

Acknowledgements

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Subcritical Water Chromatography – An Economical and Green Separation Technique

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1 Introduction 1
2 Terminology Clarification 1
3 Unique Characteristics of Subcritical Water 2
4 Beneficial Aspects of Subcritical Water Chromatography 3
5 Subcritical Water Chromatography Instrumentation Development 6
6 Analytes Separated by Subcritical Water Chromatography 7
7 Analyte Stability Under Subcritical Water Conditions 10
8 Columns Tested in Subcritical Water Chromatography and Their Temperature Tolerance 10
9 Long-Term Stability of Selected Stationary Phases Under High-Temperature Conditions 11
10 Analyte Detections in Subcritical Water Chromatography 13
11 Coupling of Subcritical Water Extraction with Subcritical Water Chromatography 14
12 Industrial Applications of Subcritical Water Chromatography 15
13 Studies on Subcritical Water Chromatography Efficiency and Retention 16
14 Conclusions 16
Abbreviations and Acronyms 17
Related Articles 17
References 17

Subcritical water chromatography (SBWC) refers to a new reversed-phase liquid chromatography (RPLC) technique where high-temperature water is used as the sole mobile phase component. The major advantage of SBWC is the elimination of toxic mobile phase organic solvents required in traditional RPLC. These organic solvents are not only expensive in terms of purchasing price but also costly in their waste disposal. Therefore, the SBWC technique offers both economical and environmental benefits. Additional advantages of SBWC are fast analysis time; temperature-dependent efficiency, selectivity, and resolution; temperature-programmed elution; and ability to accommodate both gas- and liquid-phase detectors. Most importantly, after years of academic studies, industry started paying attention to this economical and green separation technique. For example, Procter & Gamble has recently funded an SBWC project in developing green SBWC methods for the analysis of skincare products.

1 INTRODUCTION

Topics addressed in this review include terminology clarification; characteristics of subcritical water; solubility of organic compounds in subcritical water; beneficial aspects of SBWC; instrumentation development; analytes separated by SBWC; organic stability under subcritical water conditions; columns tested in SBWC and their temperature tolerance; long-term stability of stationary phases used in SBWC; analyte detection in SBWC; coupling of subcritical water extraction with SBWC; industrial applications; and studies on retention and efficiency in SBWC. Although the focus of this review is SBWC at temperatures higher than 100 °C, separation using pure water at lower temperatures is also included in this review.

2 TERMINOLOGY CLARIFICATION

Although theoretically speaking, water under any temperature and pressure conditions below its critical point of 374 °C and 218 atm can be called subcritical water, subcritical water normally refers to the liquid water that is heated between 100 and 374 °C and pressurized up to 218 atm as shown in Figure 1. However, different terminologies have been used interchangeably in the literature. They include superheated water, pressurized hot water, high-temperature water, hot water, and very hot water.[1] Liquid chromatographic separation using pure subcritical water as the mobile phase is termed as subcritical water chromatography (SBWC) in this review. Other terminologies used in the literature referring the same technique are superheated water chromatography, high-temperature water chromatography, pure water chromatography, pressurized hot water chromatography, or thermal aqueous liquid chromatography.

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3 UNIQUE CHARACTERISTICS OF SUBCRITICAL WATER

As we all know, water molecules form hydrogen bonding that makes water a very polar solvent at ambient conditions. However, the hydrogen bonding is weakened with increasing temperature. Thus, water becomes less polar at elevated temperatures.

The polarity of subcritical water can be described using the dielectric constant as a function of temperature.[2–4] As shown in Figure 2, the dielectric constant of water decreases from 79 at 25 °C to 27 at 250 °C and 100 bar.[4,5] It must be pointed out that the same decreasing trend of the dielectric constant is achieved by mixing methanol or acetonitrile with water under ambient conditions as demonstrated in Figure 2. Also as shown in Table 1, by manipulating the temperature and pressure of water, its dielectric constant can be tuned to have values equivalent to common organic solvents.

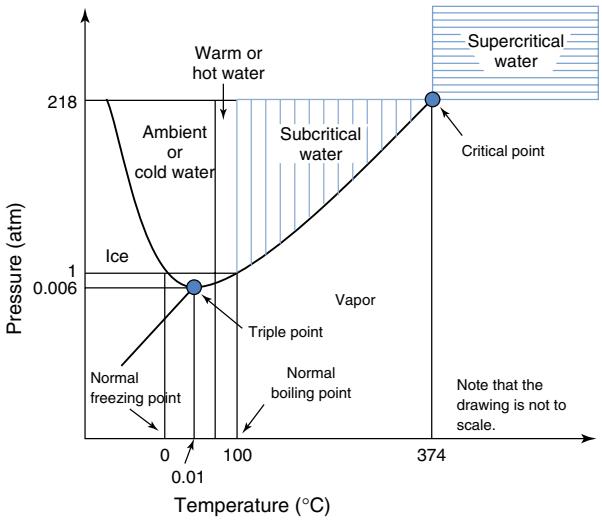

Figure 1 Phase diagram of water.

Table 1 Comparison of dielectric constant of selected common organic solvents with that of subcritical or supercritical water at various temperature and pressure conditions

Dielectric constant of common organic solvents at ambient temperature and pressure	Dielectric constant of water under specified temperature and pressure conditions[4]
1.9 (*n*-hexane)	2.0 (275 °C and 200 bar)
7.6 (tetrahydrofuran)	7.5 (500 °C and 800 bar)
8.9 (methylene chloride)	8.5 (450 °C and 600 bar)
21 (acetone)	20 (300 °C and 100 bar)
25 (ethanol)	25 (275 °C and 300 bar)
33 (methanol)	35 (200 °C and 100 bar)
39 (acetonitrile)	39 (175 °C and 100 bar)

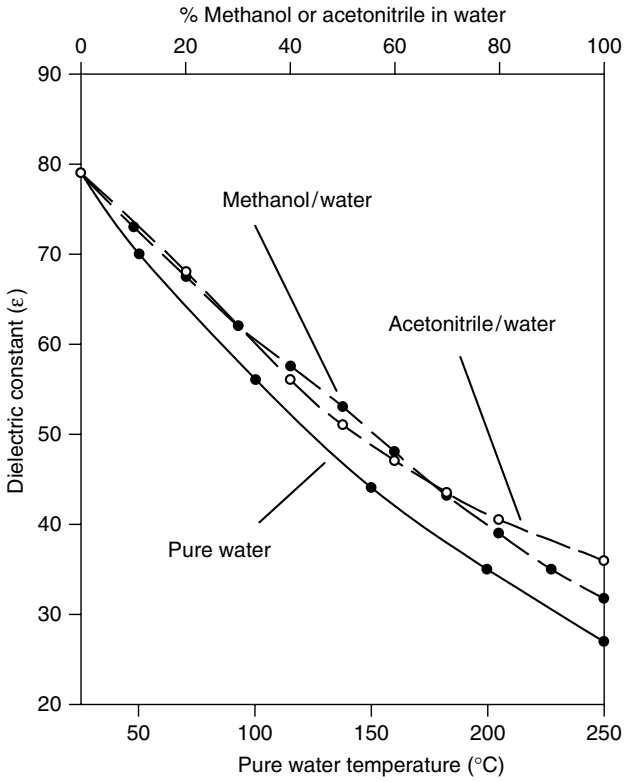

Figure 2 Control of solvent dielectric constant by changing temperature (at 100 bar) with pure water compared to mixing water with methanol or acetonitrile at 25 °C and ambient pressure. (Reproduced from Ref. 5 © Elsevier, 1998.)

Similarly, the surface tension of water also decreases with increasing temperature as depicted in Figure 3. For example, the surface tension is reduced from 72 dyn cm^{-1} at 25 °C to 26 dyn cm^{-1} at 250 °C. Again, the same decreasing pattern in surface tension is obtained by increasing the organic percentage in the RPLC mobile phase as shown in Figure 3.[5]

Viscosity follows the same decreasing trend as the dielectric constant and surface tension do with increasing temperature. As shown in Figure 4, the viscosity of water decreases from 0.89 cP at 25 °C to 0.11 cP at 250 °C.[4] Note that compared with RPLC using methanol–water or acetonitrile–water mixtures, the viscosity of pure water in the entire temperature range of 25–250 °C is lower than that of the both organic–water mixtures as depicted in Figure 4.

When pure water is used as the mobile phase to carry out liquid chromatographic separation at high temperatures, the backpressure of the SBWC system must be maintained at the pressure higher than the vapor pressure at a given temperature to keep the mobile phase in the liquid state. For example, the SBWC system pressure has to be higher than 16 bar if the SBWC separations are performed at 200 °C. The density of

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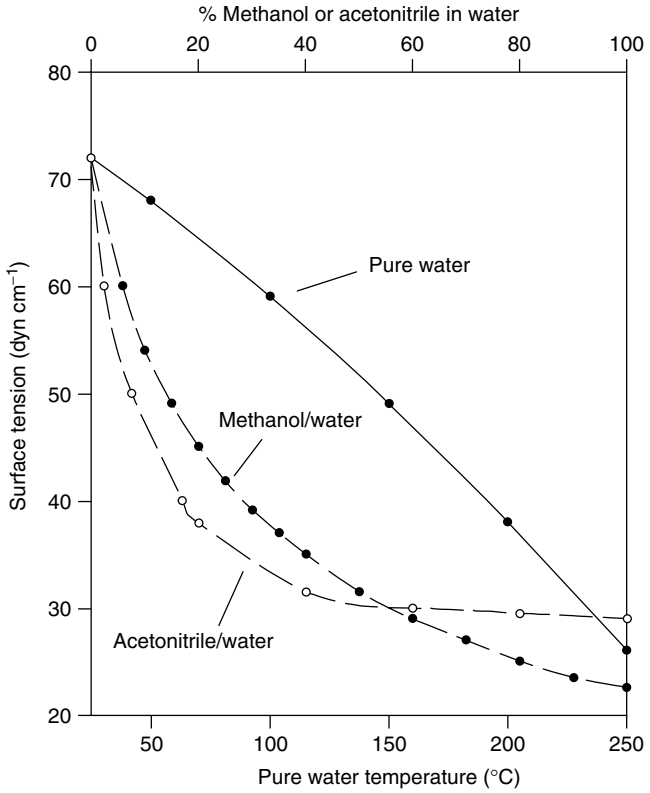

Figure 3 Control of surface tension by changing the temperature (at vapor pressure) with pure water compared to mixing water with methanol or acetonitrile at 25 °C and ambient pressure. (Reproduced from Ref. 5 © Elsevier, 1998.)

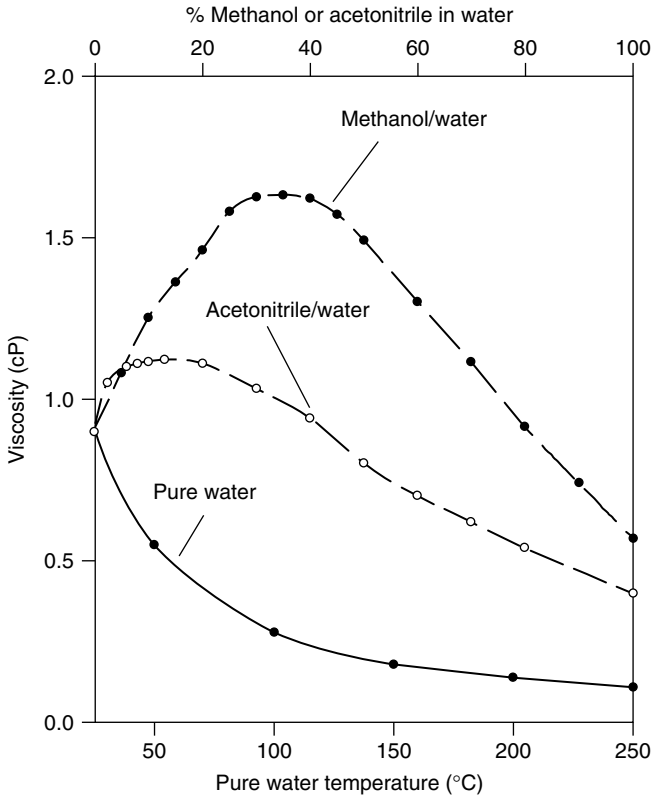

Figure 4 Control of viscosity by changing the temperature (at 100 bar) with pure water compared to mixing water with methanol or acetonitrile at 25 °C and ambient pressure. (Reproduced from Ref. 5 © Elsevier, 1998.)

water under the typical SBWC temperature and pressure conditions of 100–200 °C and 10–220 bar ranges from 0.86 to 0.97 g mL^{-1}.

Another characteristic property of subcritical water is its temperature-dependent dissociation constant. The ionization constant of water at temperatures of 200–300 °C is significantly greater than that of ambient water. Thus, subcritical water acts as a natural acid and base, but several orders of magnitude stronger than the ambient water. Owing to this property change with varying temperature, many acid- and base-catalyzed and other chemical reactions can be carried out under subcritical water conditions.[6–14]

Owing to the weakened hydrogen bond and decreased polarity of water at higher temperatures as discussed earlier, the solubility of organic compounds in subcritical water is dramatically enhanced. For example, the solubility of benzo[*a*]pyrene is increased 2.5 million folds by increasing the temperature from 25 to 350 °C.[15] Generally speaking, the solubility of polycyclic aromatic hydrocarbons and nonpolar pesticides is enhanced by four to five orders of magnitude when the temperature of water is raised from ambient to 250 °C or higher.[15–18] It is also very impressive for the solubility enhancement of liquid organics and organic acids in subcritical water with increasing temperature.[19–23]

4 BENEFICIAL ASPECTS OF SUBCRITICAL WATER CHROMATOGRAPHY

While RPLC is a widely employed separation and analysis technique, it requires a huge amount of hazardous mobile phase organic solvents that not only are expensive but also need to be disposed off after the consumption. Several liquid chromatographic separation techniques have been developed to minimize the use of organic solvents in the RPLC mobile phase. These techniques include ultrahigh high-pressure liquid chromatography (UHPLC) and high-temperature liquid chromatography (HTLC). While the quantity of organic solvents required in both UHPLC and HTLC techniques may be significantly reduced, the chromatography waste still contains organic solvents and has to be disposed of. Therefore, it is of great importance to develop a green liquid chromatography technique that eliminates the use of organic solvents in the mobile phase.

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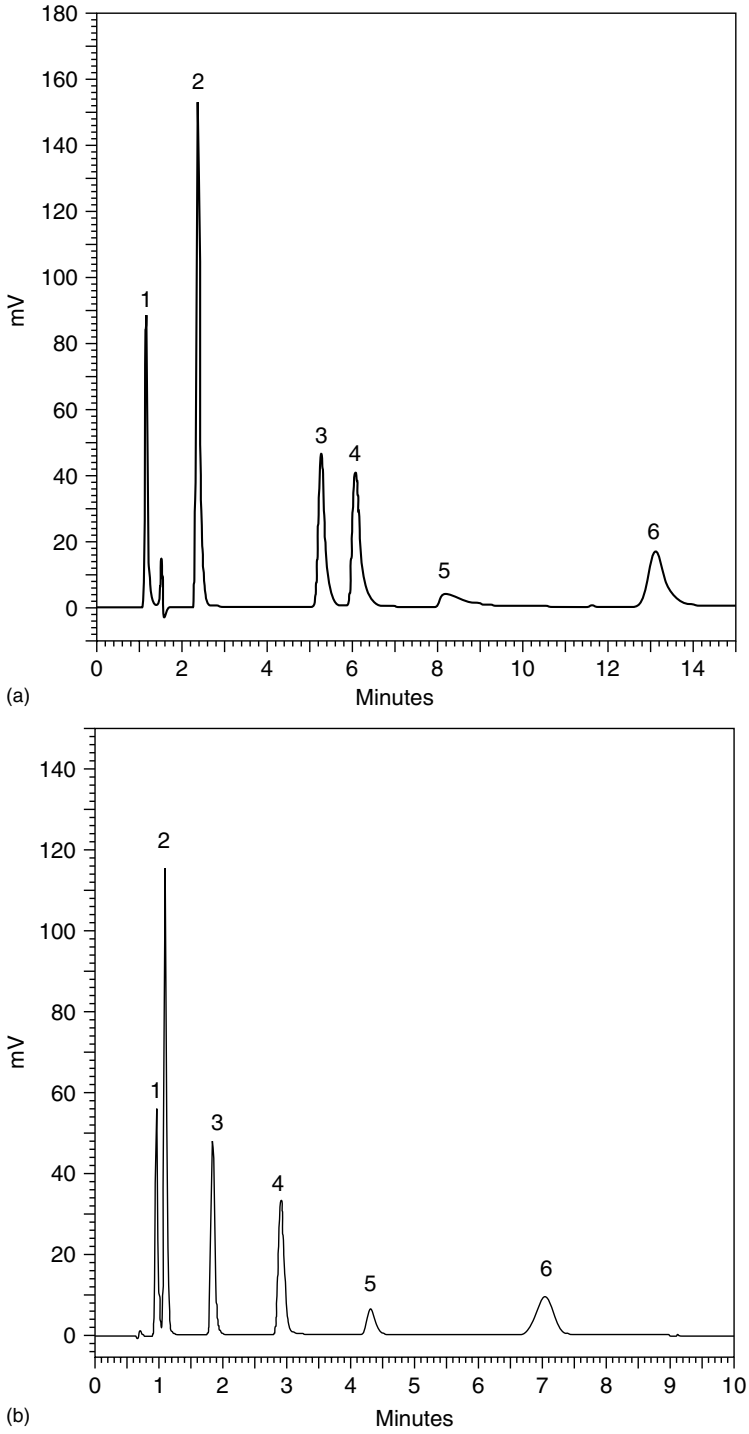

Figure 5 Comparison of the separation of purines and pyrimidines under conventional conditions and with high-temperature water. Column: Hypercarb 5, 100 × 4.6 mm ID; detection: UV at 254 nm. (a) Mobile phase: water + 0.1% formic acid/acetonitrile (85:15, v/v); flow rate: 0.8 mL min^{-1}; temperature: 50 °C. (b) Mobile phase: 100% water; flow rate: 2.0 mL min^{-1}; temperature: 190 °C. Analytes: 1, cytosine; 2, uracil; 3, thymine; 4, hypoxanthine; 5, guanine; and 6, xanthine. (Reproduced from Ref. 24 © Wiley-VCH, 2007.)

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Because increasing the water temperature from 25 to 250 °C causes similar changes in solvent polarity, surface tension, and viscosity as those achieved by conventional mixing of methanol or acetonitrile with ambient water in RPLC as shown in Figures 2–4, subcritical water has been used to mimic organic solvent–water mixtures as the mobile phase to achieve reversed-phase separation. Figure 5 shows an example of efficient reserved-phase separation of purines and pyrimidines achieved using SBWC.[24] The major advantage of SBWC is the obvious elimination of toxic organic solvents normally required in RPLC separation, making the SBWC technique more economical and the laboratory and environment greener.

As described by van't Hoff equation, solute retention can be shortened with increasing temperature. In addition, the reduced viscosity of the mobile phase at elevated temperatures results in much lower backpressure in an SBWC system. It was reported that the backpressure of an SBWC system was reduced by fivefold over a temperature range of 25–180 °C.[25] The combined effects of high-temperature and low system backpressure make it possible to use very high flow rates in SBWC to achieve fast separations even within seconds, a truly ultrafast LC separation technique.[26] The fast SBWC separation can also be achieved using ordinary HPLC pumps with a maximum pressure of 400 bar due to the much lowered backpressure at high temperatures. For example, 400 bar may tolerant only 2–3 mL min^{-1} of flow rates for a 25-cm column (4.6 mm I.D.) packed with 5-μm particles at ambient temperature, but the same chromatography system allows the use of much higher flow rates at 150–200 °C. Figure 6 shows an application of high flow rate in SBWC.[27]

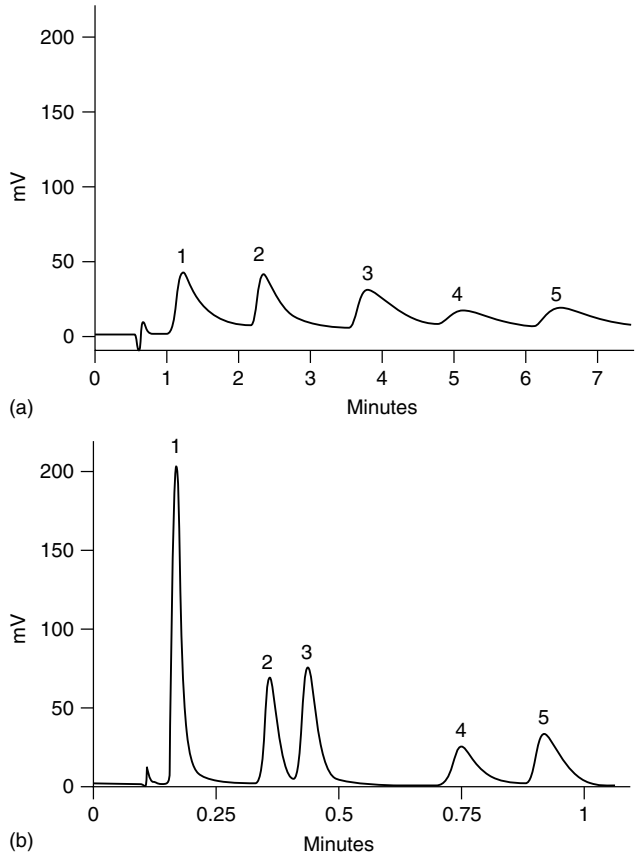

Figure 6 Chromatograms of caffeine derivatives (1, hypoxanthine; 2, theobromine; 3, theophylline; 4, caffeine; and 5, β-hydroxy-ethyl-theophylline) on a ZirChrom-DB-C18 column (50 × 4.6 mm I.D.) with UV detection at 254 nm. (a) Mobile phase: 60% water–40% methanol; 25 °C; 1 mL min^{-1}. (b) Mobile phase: 100% water; 150 °C; 7 mL min^{-1}. (Reproduced from Ref. 27 © Elsevier, 2004.)

We all agree that column efficiency is improved with deceasing particle size of the stationary phase. However, the backpressure of an LC system dramatically increases with decreasing particle size since the pressure is inversely proportional to the square of the particle diameter. Therefore, only short columns packed with 3-μm or smaller packing particles can be used at ambient temperature in HPLC systems equipped with ordinary pumps as these pumps can handle pressures only up to ~400 bar. Fortunately, the backpressure of SBWC systems is significantly reduced due to the much lowered viscosity of water at elevated temperatures as shown in Figure 4. Therefore, longer columns packed with smaller particles can be tolerated by the same ordinary pumps at higher temperatures. This further leads to enhanced SBWC separation efficiency resulted by the smaller packing particles and the increased length of the separation column.

Since mass transfer is enhanced at higher temperatures due to the reduced viscosity as shown in Figure 4, peaks become narrower. However, the retention time is also dramatically decreased with rising temperature. Since the plate number is determined by the ratio of retention and peak width, the temperature effect on column efficiency is dependent on the decreasing rate of the retention and peak width. Thus, column efficiency can be optimized by tuning the temperature in addition to the traditional optimization of the linear velocity of the eluent as done in conventional RPLC.[26,28]

Additional benefits of SBWC include temperature-dependent selectivity and resolution, temperature-programmed elution, and compatibility with both liquid- and gas-phase detectors.

Since 2002, Yang periodically reviewed SBWC in a section of his subcritical water and HTLC review articles.[25,26,28–30] Smith also addressed SBWC in a section of his review articles for related techniques.[31,32] In addition, other recent HTLC review articles[33–37] also contain partial reviews on SBWC. Su et al.[38] and Coym

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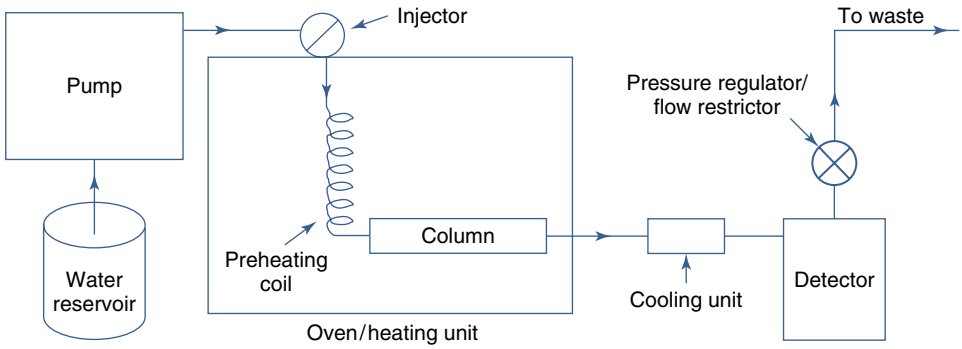

Figure 7 Block diagram of a subcritical water chromatography system with the preheating coil placed after the injector.

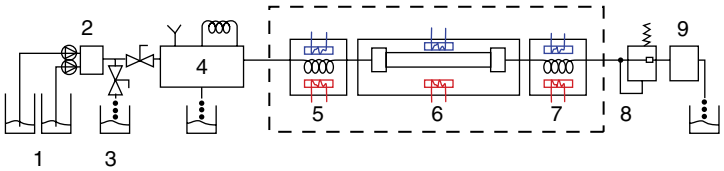

Figure 8 Schematic diagram of an HTLC system and the specially designed heating oven for temperature-programmed applications: 1, solvent reservoir; 2, pumps; 3, autosampler; 4, high-pressure mixing chamber; 5, preheating unit; 6, column heating unit; 7, cooling unit prior to detection; 8, UV detector; and 9, backpressure regulator. (Reproduced from Ref. 42 © Elsevier, 2006.)

and Dorsey[39] briefly reviewed SBWC in 2005. In early 2007, Yang published a thorough SBWC review article.[1] The SBWC technique was later reviewed by Smith[40] and by Hartonen and Riekkola[41] in 2008.

5 SUBCRITICAL WATER CHROMATOGRAPHY INSTRUMENTATION DEVELOPMENT

A homemade SBWC system as shown in Figure 7 can be easily realized by simply modifying an ordinary HPLC system. To eliminate or minimize thermal mismatch, a heating unit for preheating the eluent and the column is needed. This can be a built-in small column heater as provided by various HPLC manufacturers or can be an external oven, such as a used GC oven. If an UV detector is used for detection in SBWC, a cooling unit, either a built-in device or an external iced-water bath, must be employed for SBWC separations at temperatures higher than 80–100 °C, depending on the temperature tolerance of the UV flow cell.

A postcolumn backpressure regulator or a flow restrictor is needed to apply adequate pressure required to maintain water inside the separation column in the liquid state at elevated temperatures. In other words, the backpressure must be higher than the vapor pressure of the high-temperature water. For example, the backpressure has to be at least 16 bar for SBWC separations at 200 °C.

After these few additions are complete, a conventional HPLC system is converted into an SBWC system that is capable of carrying out subcritical water separations at temperatures as high as 200 °C if precolumn heating and postcolumn cooling are adequate enough.

Because the mobile phase reservoir is normally kept at room temperature while the analytical column is heated in SBWC, the low temperature of the incoming mobile phase does not match the high temperature of the separation column if the mobile phase is not adequately preheated. This thermal mismatch causes peak broadening and poor reproducibility. To minimize thermal mismatch, several approaches have been reported.[26] Most commonly, a preheating coil between the injector and the column is placed inside an oven where the separation column is located. In this case, the length of the preheating coil needs to be optimized to achieve the most efficient separation. Although this approach can reduce or even eliminate thermal mismatch, it causes additional peak broadening due to the added void volume of the preheating coil.

To decrease the void volume added by the preheating coil, a specially designed heating system was developed based on experimental measurements of eluent temperature inside a stainless steel capillary using very thin thermocouples as shown in Figure 8.[42] Although the thermal mismatch is eliminated, the extra tubing located inside the preheating unit still contributes to minor additional peak broadening.

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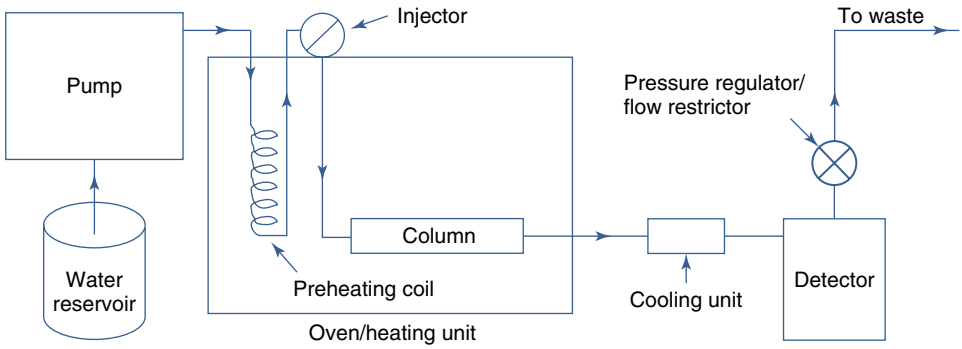

Figure 9 Block diagram of a subcritical water chromatography system with the preheating coil placed before the injector. (Reproduced from Ref. 44. © American Chemical Society, 2000.)

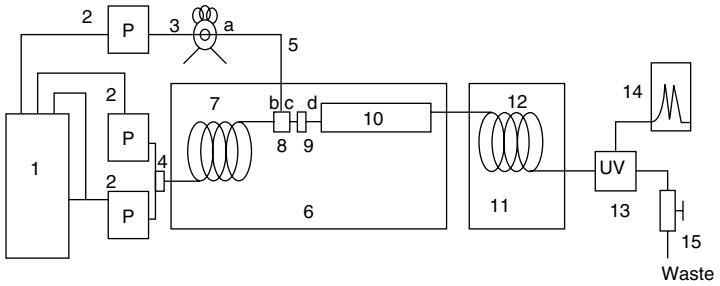

Figure 10 Schematic diagram of an HTLC system: 1, solvent reservoir; 2, pumps; 3, injector; 4, T union; 5, stainless steel tubing; 6, heating bath; 7, heat exchanger; 8, low dead volume T union; 9, in-line filter; 10, analytical column; 11, cooling bath; 12, cooling tubing; 13, UV detector; 14, data system; and 15, backpressure adjuster.

Another arrangement of the preheating coil as depicted in Figure 9 was developed to overcome the additional peak broadening problem caused by the first two approaches as described earlier.[43] In this arrangement, the preheating coil is still placed inside an oven, but between the pump and the injector instead of between the injector and the analytical column. The low-temperature mobile phase eluent coming from the pump is heated by the preheating coil located inside the oven and then the high-temperature mobile phase flows to the injector that is placed outside the oven. Since the analytes are injected into the column without passing through the preheating coil, additional peak broadening is eliminated. However, thermal mismatch may not be effectively minimized in this approach because the injector outside the oven acts as a cooling device that lowers the temperature of the preheated eluent. A combination of this and the first approach may be an economical solution to resolve the thermal mismatch problem than the other more expensive system as discussed below.

The experimental setup as demonstrated in Figure 10 can eliminate both thermal mismatch and additional peak broadening.[44] While the use of additional pumps connecting to the preheating coil eliminates thermal mismatch, the band broadening problem caused by the preheating coil is also eliminated in this approach because a separate pump enables the injected analytes to bypass the preheating coil.[44] The obvious drawback of this approach is that more pumps are required to deliver the mobile phase through different channels.

Also note that the postcolumn cooling loop may cause analyte precipitation and also lead to postcolumn peak broadening due to the additional void volume of the cooling loop. It is better to heat the UV flow cell at temperatures around 40–60 °C if all possible. In this case, the effluent temperature after postcolumn cooling does not need to be as cold as iced water, rather 40–60 °C may be low enough. This will reduce the risk for analyte precipitation and postcolumn band broadening. Some commercial HPLC-UV systems such as the Shimadzu Nexera UFLC system can accommodate the heating of the UV flow cell at temperatures around 50 ° C.

6 ANALYTES SEPARATED BY SUBCRITICAL WATER CHROMATOGRAPHY

As shown in Table 2, a number of classes of organic compounds have been separated using SBWC. These analytes range from water-soluble organics, polar and

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Table 2 Analytes separated by subcritical water chromatography

Analytes	Column name	Temperature	Detection	References
Aldehydes	PRP-1	175 °C	FID	45
Alkanols	Brownlee Spheri-5	Ambient	UV, FID	46
	Develosil C30-UG-5	35 °C	FID	47
	Hybrid C18	190 °C	UV	48
	Hypercarb	150 °C	FID	49
	PRP-1	100–225 °C	FID	45, 50–53
	Spherosil XOA 600	150 °C	UV, RI	54
	ZirChrom-PBD	120 °C	FID	49
	C18[a]	Up to 130 °C	FID	55
	Lichrosorb RP-2[a]	Up to 300 °C	FID	56
	Bonded nonporous silica[a]	Ambient	UV, FID	57
	PBD-coated zirconia monolith[a]	Up to 260 °C	FID	58
	Poly(divinylbenzene) monolith[a]	250 °C	FID	59
Alkylbenzenes	Hybrid C18	190 °C	UV	48
	Spherosil XOA 600	150 °C	UV, RI	54
	ZirChrom-DB-C18	150 °C	UV	27
	ZirChrom-PBD	175 °C	UV	60
	Bonded nonporous silica[a]	Ambient	UV, FID	57, 61
	Polymer-coated GC column[a]	Ambient	UV	62
	ZirChrom-PBD, ZirChrom-Carb capillary column[a]	Up to 370 °C	UV, FID	63
Aliphatic acids and alkylphosphoric acids	Kovasil MS-H[a]	Up to 300 °C	FID	56
Alkyl aryl ketones	PLRP-S	100–200 °C	UV	64
Aliphatic and aromatic test compounds	PLRP-S 100[a]	Up to 180 °C	UV	65
Amino acids	PRP-1	80–210 °C	FID	50, 66
	PRP-1[a]	Up to 100 °C	FID	67
PTH-aminoacids	Polymer-modified silica[a]	Up to 50 °C	UV	68–70
Aminophenols	PRP-1[a]	100 °C	FID	67
Aniline and its derivatives	Ethyl-bridged hybrid C18	200 °C	UV	71
	Nucleosil C18 AB	100–150 °C	UV	72
	PRP-1	150 °C	FID	52
	PRP-1[a]	Up to 100 °C	FID	67
	PRP-1, Zorbax RX C18, Chromatorex C-18	Up to 200 °C	UV	73
	XBridge C18	Up to 200 °C	FID, UV	74
	ZirChrom-PS	100 °C	UV	75
Aryl amides	PLRP-S	Up to 180 °C	UV	76
Barbiturates	PLRP-S	200 °C	UV/NMR	77
	PLRP-S	100–200 °C	UV	64, 78
Benzenes	Polymer of proline derivative[a]	Up to 40 °C	UV	70
	Nucleosil C18 AB	100–200 °C	UV	72
	Partisil ODS2	Up to 125 °C	UV	79
	PRP-1	200 °C	FID	52
Polyhydroxybenzenes	Partisil ODS2	Up to 125 °C	UV	79
	PRP-1, Zorbax RX C18, Chromatorex C-18	Up to 140 °C	UV	73
Bromobenzoic acids	Gemini C18	Up to 135 °C	ICP-MS	80
Carbohydrates	CS11G	Up to 150 °C	RI	81
	Hypercarb	100 °C	FID	66
	Kovasil MS-H[a]	Up to 300 °C	FID	56
	ODS-BP[a]	36 °C	FID	67

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Table 2 (*continued*)

Analytes	Column name	Temperature	Detection	References
Carboxylic acids	PRP-1	150–160 °C	FID	52, 66
Diethyl phthalate	ZirChrom-PBD	175 °C	UV	60
Ecdysteroids	XTerra C8, C18	160 °C	UV/IR/NMR/MS	82
Ginger extracts	XTerra RP 18	50–130 °C	UV/NMR (D_2O)	83
Kavalactones	ZirChrom-PBD	Up to 160 °C	UV/NMR	84
Flavones	Discovery HS PEG	100–150 °C	UV	75
Isoflavanoids	PLRP-S	170 °C	UV	85
Free fatty acids	PRP-1	150 °C	FID	52
Hydrocarbons and its derivatives	Brownlee Spheri-5	Ambient	UV, FID	46
	ODS-bonded silica	170 °C	UV	18
	Partisil ODS2	Up to 125 °C	UV	79
	Polymer-coated capillary[a]	Ambient	UV	62
Organic acids and bases	Polymer-modified silica[a]	Up to 50 °C	UV	69
	PRP-1	Up to 150 °C	FID	67
Parabens	PLRP-S	210 °C	UV	78
	Spherisorb-ODS	170 °C	UV	76
	ZirChrom-C18	100–200 °C	UV	86
Pharmaceuticals	Acquity C18	40–180 °C	MS/MS	87
	Acquity BEH C18	124–220 °C	MS/MS	88, 89
	Nucleogel RP (PS-DVB)	160 °C	UV	90
	PLRP-S	75–185 °C	UV	91
	PLRP-S, Novapak C18	50–200 °C	UV/FL/MS/NMR	92, 93
	PLRP-S, Oasis 40, ZirChrom-PDB, Hypersil C18 BDS, ZirChrom-Carb, Hypercarb BDS, XTerra RP18	Up to 225 °C	UV	94
	XBridge C18	Up to 200 °C	UV	95
	XTerra C8, Oasis HLB	Up to 185 °C	NMR/IR/UV/MS	96
	ZirChrom-PS	130 °C	UV	42
Phenols and its derivatives	Brownlee Spheri-5	Ambient	UV, FID	46
	Nucleosil C18 AB	100–150 °C	UV	72
	Partisil ODS2	Up to 113 °C	UV	79
	PLRP-S 100[a]	Up to 180 °C	UV	65
	PLRP-S	Up to 180 °C	UV	76, 78
	PRP-1	100–210 °C	FID	50, 51
	PRP-1	100–150 °C	UV	97
	PRP-1, Hypersil ODS, ZirChrom-PBD Zorbax RX-C8	Up to 160 °C	UV	98
	Spherisorb-ODS	120 °C	UV	76
	XTerra MS C18, XTerra Phenyl, XBridge phenyl	Up to 200 °C	UV	99
	ZirChrom-PS	Up to 130 °C	UV	75, 100
	ZirChrom-PS[a]	120 °C	UV	44
	PBD-coated zirconia monolith[a]	Up to 220 °C	FID	58
Polyethylene glycols (PEG 200)	PRP-1[a]	100–180 °C	UV	101
Purines, pyrimidines	Hypercarb	100–200 °C	UV, MS	24
Sulfonamides	PLRP-S	70–190 °C	UV	102
	PLRP-S	160–200 °C	UV, NMR, MS	103

(continued overleaf)

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Table 2 (*continued*)

Analytes	Column name	Temperature	Detection	References
Steroids	XTerra MS C18	130 °C	UV	104
	ZirChrom-PBD	160–200 °C	UV	42, 105
	Polymer-modified stationary phase[a]	Up to 50 °C	UV	70, 106
Triazine herbicides	Hypercarb	Up to 260 °C	UV	107
Triazole fungicides	ZirChrom-PBD	100–150 °C	UV	108

[a] Laboratory packed columns.

moderately polar solutes, to even nonpolar analytes. In general, all polar organic compounds can be separated using SBWC. Most moderately polar solutes are also doable with SBWC but will require higher temperatures than what needed for polar analytes. In addition, SBWC may be able to separate some nonpolar analytes if much higher temperatures and much less retentive stationary phases are employed. Therefore, the future of SBWC is for the analysis of polar and moderately polar solutes, not for nonpolar analytes.

7 ANALYTE STABILITY UNDER SUBCRITICAL WATER CONDITIONS

Since high temperature is employed in SBWC, the analyte stability at high temperatures needs to be addressed. Recent studies indicated that certain organic compounds including polycyclic aromatic hydrocarbons, pesticides, terpenes, fatty acids, and benzoic acid derivatives did experience some degree of degradation in subcritical water.[109–114] However, the temperature that caused such degradation is generally higher than what used in SBWC. Also, the heating time employed in these studies is longer than the analysis time of typical SBWC runs. According to van't Hoff equation, retention decreases with increasing temperature. In addition, since the high temperature employed in SBWC allows much higher flow rate, the SBWC retention is further reduced. Thus, SBWC separation shortens the analyte exposure time to high temperature, and, in turn, analyte degradation is minimized. Therefore, most of the analytes are stable at elevated temperatures on the timescale of the subcritical water chromatographic run. It has been reported that even some pharmaceuticals can withstand high temperatures on the timescale of fast LC separation.[115]

Recently, Yang et al.reported that degradation of some sunscreen compounds at temperatures of 150–200 °C was observed.[116] Huang et al.also noticed degradation of some thiazide compounds during SBWC separation.[95]

It should be noted that minor degradation of analytes should not significantly affect quantitative analysis of such analytes. This is because both standard and sample solutions are run under the same SBWC conditions and the same level of degradation should be expected for analytes in both calibration and unknown solutions.

8 COLUMNS TESTED IN SUBCRITICAL WATER CHROMATOGRAPHY AND THEIR TEMPERATURE TOLERANCE

As shown in Table 3, a large variety of commercially available columns ranging from microbore to standard ID of 4.6 mm have been tested in SBWC. These commercial columns are mainly packed with silica-based, hybrid silica-based, zirconia-based, or polymer packing materials. Among these stationary phases, most of the polymer and a large number of zirconia-based columns can tolerant high temperatures ranging from 100 to 200 °C. For example, the manufacturer's temperature limit for ZirChrom DiamondBond-C18 column is 200 °C. Some hybrid silica-based columns such as Waters XTerra and XBridge columns may stand temperatures higher than 100 °C although the manufacturer claimed temperature limit is much lower. The temperature tolerance for silica-based columns is the lowest among all columns studied in SBWC.

Besides the microbore and standard sized columns discussed above, capillary columns packed with various types of particle or monolith materials have also been reported.[56,58,59,63] Faster thermal equilibration can be achieved by the application of capillary columns in SBWC. Figure 11 shows chromatograms of alcohols and phenols on a capillary column packed with polybutadien-encapsulated zirconia particles.[58]

In addition to polymer, silica-, hybrid silica-, and zirconia-based columns, carbon and other metal oxide-based stationary phases were also tested for use in SBWC.[27,45,94,118]

Although the emphasis of this review is SBWC separation at high temperatures, it is worthwhile to mention pure water chromatographic separations at temperatures lower than 100 °C and even at ambient condition. A new type of HPLC packing material, the temperature-responsive stationary phase, has been

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Table 3 Commercial columns used in SBWC and their stability under SBWC conditions

Column name	Base material	Surface coating	Temperature	Stability	References
Hypercarb	Carbon	Carbon	Up to 260 °C	208 h up to 200 °C[a]	(24,45,49,94,107)
CS11 G	Cation exchange resin	Poly(styrene-*co*-divinylbenzene)	Up to 180 °C	Several days at 180 °C	(81)
Nucleogel RP	Polymer	Poly(styrene-divinylbenzene)	Up to 160 °C	Not reported	(90)
Oasis HLB	Polymer	Poly(styrene-divinylbenzene +nitro-vinylpyrrolidone)	185 °C	Not reported	(96)
Oasis (M81883D01)	Polymer	Poly(styrene-divinylbenzene)	Up to 210 °C	Not reported	(94)
Oasis (9M90762D01)	Polymer	Poly(styrene-divinylbenzene)	Up to 208 °C	Not reported	(94)
PRP-1	Polymer	Poly(styrene-divinylbenzene)	Up to 225 °C	499 h at 100–150 °C	(45,51,53,73,117)
PLRP-S	Polymer	Poly(styrene-divinylbenzene)	Up to 210 °C	Not reported	(64,76–78,92,94,103)
PLRP-S-100	Polymer	Poly(styrene-divinylbenzene)	Up to 180 °C	Not reported	(65)
Chromatorex C18	Super pure silica	C18	Up to 140 °C	Not reported	(73)
Discovery HS PEG	Silica	Polyethylene glycol	100 °C	Not reported	(75)
Gemini C18	Super pure silica	C18	Up to 135 °C	Not reported	(80)
Acuty BEH C18	1,2-bis(siloxy)ethane	C18	Up to 220 °C	Not reported	(88)
Hypersil BDS C18	Base deactivated silica	C18	Up to 160 °C	42 h at 100 °C	(94,117)
Hypersil ODS	Silica	C18	Up to 140 °C	Not reported	(98)
Nucleosil C18 AB	Silica (narrow-bore)	C18	Up to 200 °C	245 h at 100 °C	(72,117)
Partisil ODS2	Silica	C18	Up to 125 °C	Not reported	(79)
Spherisorb ODS2	Silica	C18	Up to 170 °C	Not reported	(18,76)
XBridge C18	1,2-bis(siloxy)ethane	C18	Up to 200 °C	One month at 200 °C	(48,71,74,95)
XBridge phenyl	1,2-bis(siloxy)ethane	Phenyl	Up to 200 °C	Not reported	(99)
XTerra C8	Methylethoxysilane	C8	160 °C	Not reported	(82)
XTerra C18	Methylethoxysilane	C18	160 °C	Not reported	(82)
XTerra MS C18	Methylethoxysilane	C18	110–130 °C	Not reported	(99,104)
XTerra RP C18	Methylethoxysilane	C18	165 °C	Not reported	(94)
XTerra Phenyl	Methylethoxysilane	Phenyl	Up to 200 °C	Not reported	(99)
ZirChrom-C18	Zirconia	C18	100–200 °C	Not reported	(86)
ZirChrom-Carb	Zirconia	Elemental carbon	188–220 °C	Not reported	(94)
ZirChrom-DB-C18	Zirconia	C18	150 °C	Not reported	(27)
ZirChrom-PBD	Zirconia	Polybutadiene	Up to 200 °C	50 h at 185 °C	(42,60,105)
ZirChrom-PS	Zirconia	Polystyrene	Up to 130 °C	120 h at 100 °C	(42,75,117)
Zorbax RX-C8	Silica	C8	Up to 100 °C	250 h at 100 °C	(98,117)
Zorbax RX-C18	Silica	C18	Up to 140 °C	Not reported	(73)

[a] Using water–organic solvent as the mobile phase.

developed by Kanazawa and coworkers.[68–70,106] By changing water temperature in the range of 5–50 °C or pH, the properties of this type of stationary phase can be altered from hydrophilic to hydrophobic and from charged to noncharged. Thus, solute retention can easily be controlled. Since the temperature used is relatively low, this type of packing material may be applicable to the separation of pharmaceuticals and biomolecules.[68–70,106]

To achieve LC separations using only ambient water in the mobile phase, Synovec and coworkers developed packing materials by coating the stationary phase on nonporous glass beads or silica.[57,61,62] Reasonable solute retention was achieved by decreasing the phase volume ratio of the stationary phase relative to the mobile phase volume.[62] A more polar stationary phase, Spheri-5 cyano propyl, was also tested to obtain chromatographic separation using ambient water as the eluent.[46]

9 LONG-TERM STABILITY OF SELECTED STATIONARY PHASES UNDER HIGH-TEMPERATURE CONDITIONS

Carr and coworkers have successfully developed several commercially available zirconia-based stationary phases

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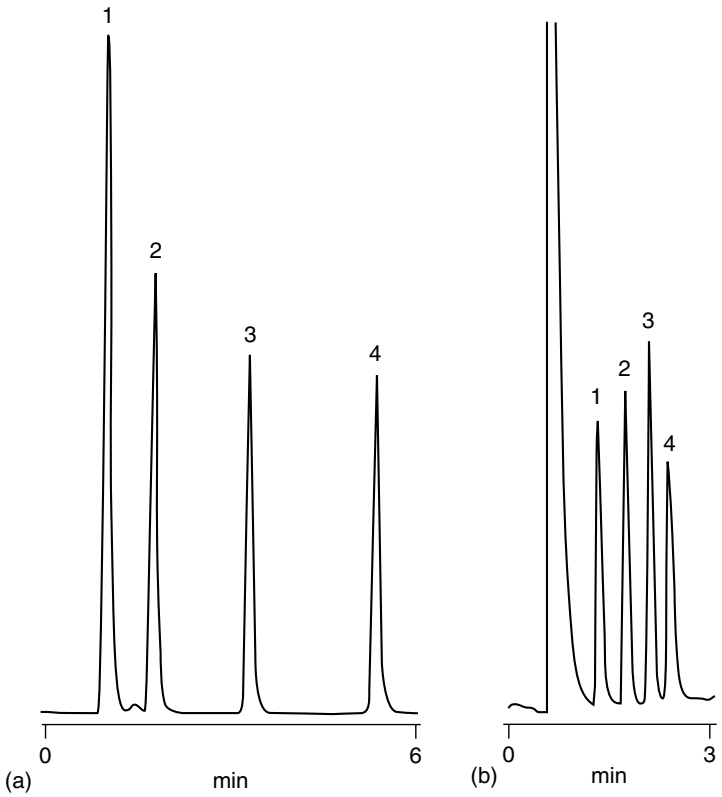

Figure 11 Chromatograms of (a) alcohols and (b) phenols. Conditions: (a) 25.2 cm × 250 μm I.D continuous-bed capillary column, 200 atm, 125–165 °C at 5 °C min^{-1}. Peak identifications: 1, methanol; 2, 2-propanol; 3, 3-methylbutanol; and 4, 1-heptanol. (b) 33 cm ×250 μm ID continuous-bed capillary column, 250 atm, 165–200 °C at 10 °C min^{-1}. Peak identifications: 1, 4-chloro-phenol; 2, 3-methyl-4-chlorophenol; 3, 2,4,6-trimeth-ylphenol; and 4, 2,4-dichlorophenol. (Reproduced from Ref. 58 © John Wiley & Sons, Inc., 2001.)

that are branded as ZirChrom columns. These researchers have published a number of papers reporting the long-term stability of ZirChrom columns at temperatures mostly ranging from 100 to 200 °C.[44,115,119–122] In these evaluation studies, methanol–water mixtures, acetonitrile–water mixtures, or pure water was used as the mobile phase. Their results demonstrate that the zirconia-based columns are more stable and last longer than most silica-based columns at high temperatures.[119–121]

He and Yang studied the long-term stability of five silica-based, zirconia-based, and polymer columns and their findings are rather encouraging than many chromatographers thought.[117] All five commercial columns tested were stable after exposure to subcritical water at 100 °C for at least 6000 column volumes based on retention factors. On the basis of the evaluation of column efficiency at 100 °C, Zorbax RX-C8, ZirChrom-PS, and PRP-1 columns were stable for several thousands of column volumes. However, the column efficiency obtained by Nucleosil C18 AB and Hypersil BDS C18 columns was decreased after heating for a prolonged period of time.[117] By comparing the performance of retention and efficiency of the three silica-based columns at 100 °C, the Zorbax RX-C8 column was the most stable one followed by the Nucleosil C18 AB column and the Hypersil BDS C18 column. The ZirChrom-PS column was more stable than the three silica-based columns and survived at 100 °C for at least 7600 column volumes.[117] Among all five columns, the polymer PRP-1 column turned out to be the most stable one. The column was found to be stable at 100 °C for over 11 000 column volumes. After the same PRP-1 column was exposed to 150 °C for another 9000 column volumes, the column was still stable based on the evaluation of both retention and efficiency.[117] The PRP-1 packing material was also reported to be resistant at temperatures up to 225 °C in a separate study reported by Ingelse et al.[45]

Yang and coworkers are currently studying the long-term performance of two hybrid silica-based columns and one zirconia-based column under SBWC conditions. So far, this study has revealed that both Waters XBridge C18 and XBridge Phenyl columns can be used in SBWC at temperatures ranging from 100 to 150 °C for ~20 000–30 000 column volumes. The evaluation of the stability of a ZirChrom-DiamondBond-C18 column at

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200 °C is also under way, and the column has been relatively stable for over 12 000 column volumes. Please note that the manufacturer's temperature limit for this ZirChrom-DiamondBond-C18 stationary phase is 200 °C.

A column bleeding study on the performance of several different types of packing materials was reported by Teutenberg et al.[118] Columns evaluated in this study are a silica-based Luna C18 column, a zirconia-based ZirChrom-Carb column, a Hypercarb Carbon column, a PLRP-S polymer column, and a TiO_2-based prototype Carb-TiO_2 column. This study concluded that the prototype Carb-TiO_2 column gave the least column bleeding, while the silica-based Luna column experienced the most serious column bleeding.[118] These authors later evaluated three reversed-phase and four normal-phase stationary phases at temperatures ranging from 120 to 185 °C.[123] After exposing to high temperatures for 50 h, metal oxide-based stationary phases, ZirChrom-Carb and Carb-TiO_2, were still stable based on retention factor, resolution, and peak asymmetry.[123]

10 ANALYTE DETECTIONS IN SUBCRITICAL WATER CHROMATOGRAPHY

Since an ordinary HPLC-UV system can be easily modified to accommodate SBWC separation, UV is the most popular detector used in SBWC. To keep water in the liquid state at higher temperatures, a flow restrictor or a backpressure regulator needs to be attached to the outlet of the UV detector. In addition, a cooling unit between the column and the UV flow cell is also necessary for performing SBWC separation at higher temperatures. If a cooling loop inside an iced-water bath is used, peak broadening may occur as a result of increased void volume caused by the cooling loop. The postcolumn cooling may also cause deposition of moderately polar and nonpolar solutes in the cooling tubing. However, if the SBWC temperature is lower than 100 °C and the UV flow cell can tolerant some temperature, iced-water cooling may not be necessary. Some commercial HPLC/UV flow cells can be heated up to 40–60 °C that is about the temperature of the water effluent coming out of the SBWC column before reaching the UV flow cell if the SBWC separation temperature is below 100 °C. In this case, the peak broadening caused by the extra cooling loop is eliminated.

While UV is the most popular detector used for SBWC, it cannot detect analytes without chromophores. A universal and sensitive GC detector such as the flame ionization detector (FID) is an ideal detector for SBWC. Unlike an SBWC-UV system, the SBWC-FID system requires no postcolumn cooling and eliminates the additional peak broadening originated by the cooling loop in an SBWC-UV system. FID has not been employed by traditional HPLC because the organic solvents used in HPLC mobile phase cause very strong FID background response. Fortunately, since water does not contain C–H bond, FID has no response to water. Thus, FID has been tested as a gas-phase detector in liquid chromatography with water as the sole eluent. Synovec and coworkers constructed a drop headspace interface to allow FID detection of alcohol and hydrocarbons in liquid chromatography using ambient water as the mobile phase.[46,57]

The first direct coupling of a nonmodified FID with SBWC was reported by Miller and Hawthorne.[50] A restrictor was placed between the separation column and FID to ensure that water stays in the liquid state at temperatures higher than 100 °C. The optimized position of the capillary restrictor is that the end of the capillary restrictor should be placed about 3 cm below the tip of the FID as shown in Figure 12.[50] This position was later confirmed by other researchers.[45,55,66,67] Separation using programmed temperature was also investigated in this work.[50] The problem with this direct coupling technique is the limited volume flow rate of the subcritical water eluent. The FID signal was found to be unstable at water flow rates faster than 0.2 mL min^{-1}.[50]

Yang and coworkers developed a split SBWC-FID system to solve the FID stability problem at higher flow rates.[66] The high-temperature water eluent was split using a T union before reaching the FID. This split system allows much higher volume flow rate of water while stable FID signal can still be maintained. This split technique was also evaluated later by other researchers.[47,53,124] Although this split approach works fine for analytes with adequate concentrations, it does not work for analytes with low concentrations because only a small fraction of solute reaches FID. Another

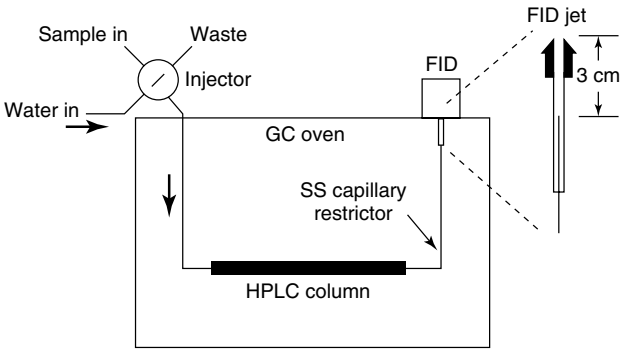

Figure 12 Schematic diagram of an SBWC-FID system. (Reproduced from Ref. 50 © American Chemical Society, 1997.)

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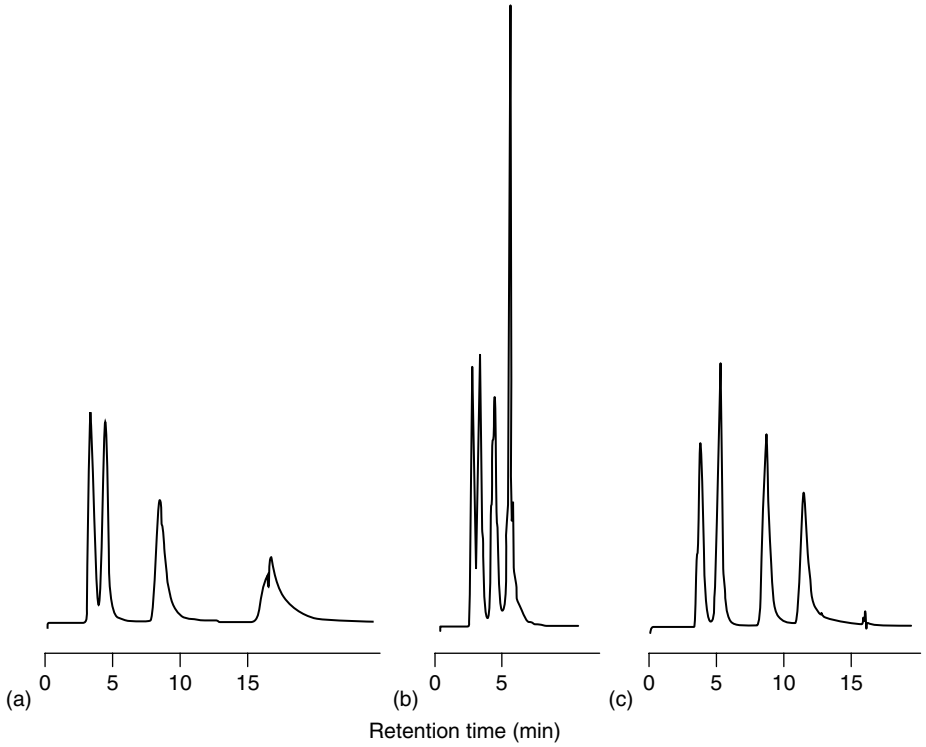

Figure 13 SBWC-FID chromatograms of amino acids on a microbore column packed with PRP-1 material (250 × 0.5 mm ID), 20 μL min^{-1}, (a) 50 °C, (b) 100 °C, (c) initial temperature of 50 °C (4 min), raised to 100 °C at the rate of 10 °C min^{-1}. Peak order: L-proline, L-leucine, D-phenylalanine, and L-tryptophan. (Reproduced with permission from Ref. 67. © Preston Publications, 2005.)

approach to ensure a stable FID signal is the use of microbore or capillary columns that require much smaller eluent volume flow rate to achieve optimized separation.[45,49,55,56,63,67] Figure 13 shows SBWC-FID chromatograms of amino acids on a microbore column packed with PRP-1 particles.[67] The microbore column SBWC-FID technique was applied to fast analysis of linear alcohols.[49] The separation and analysis time was dramatically shortened by using higher temperature and faster flow rate compared with those achieved at lower temperature and slower flow rate.[49] It is fair to say that FID is the second popular detector used in SBWC.

In addition to UV and FID detection, more expensive spectroscopic detection techniques such as NMR and MS were also tested for use in SBWC. Smith and coworkers reported the use of NMR and MS detection in SBWC.[77,83,92,93,103] Because proton NMR was used in this approach, high-temperature heavy water, deuterium oxide, was used as the mobile phase. ICP-MS[125] and a multiarrangement of IR-UV-NMR-MS[82,96] were also reported.

Other detectors employed in SBWC include charged aerosol detector (CAD),[118] refractive index (RI), and conductivity detectors.[81]

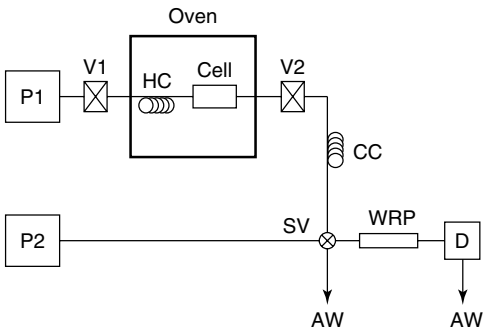

Figure 14 Schematic of subcritical water extraction–water only reversed-phase liquid chromatography (WRP-LC) instrument. P1 and P2, water pump for subcritical water extraction and WRP-LC systems, respectively; V1 and V2, needle valves; HC, heating coil; CC, cooling coil; Cell, subcritical water extraction cell; SV, switching valve; WRP, WRP-LC column; D, UV/VIS detector; and AW, aqueous waste. (Reproduced from Ref. 61 © Elsevier, 1998.)

11 COUPLING OF SUBCRITICAL WATER EXTRACTION WITH SUBCRITICAL WATER CHROMATOGRAPHY

As mentioned earlier, subcritical water extraction (SBWE) started gaining increased attention since mid-1990s.[1] There has been a great success for SBWE

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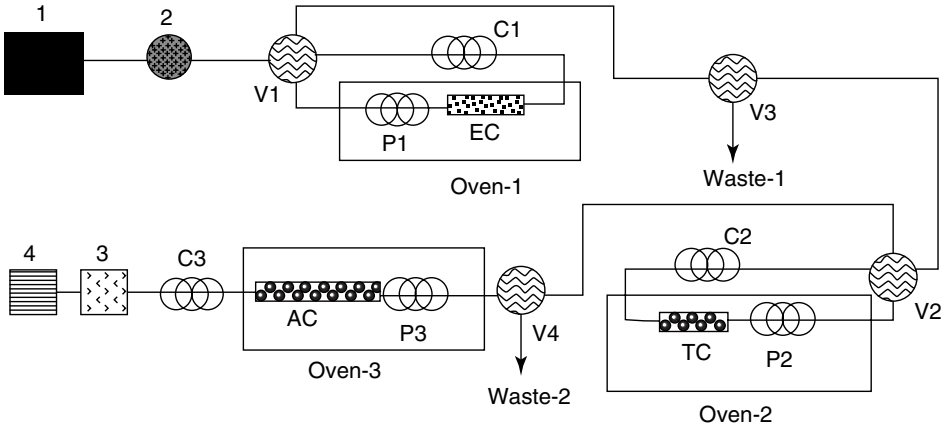

Figure 15 On-line coupled SBWE/SBWC system: 1, water pump; 2, injector; 3, UV detector; 4, backpressure regulator; EC, extraction cell; TC, trap column; AC, analytical column; V1–V4, switching valves; P1–P3, preheating coils; and C1–C3, cooling coils. (Reproduced from Ref. 107 © Elsevier, 2005.)

not only in bench scale analytical analysis but also in the area of pilot scale environmental cleanup and remediation.[111,126–139] Owing to the rapid development of SBWE, researchers investigated the possibilities of coupling SBWE with SBWC.[61,75,83,91,107] The biggest advantage of this SBWE/SBWC coupling technique is that it offers a potentially promising green sample preparation and chromatographic separation approach that completely eliminates the use of hazardous organic solvents involved in the entire extraction and chromatographic process.

Young et al. reported the coupling of SBWE with RPLC using ambient water as the mobile phase for extraction and analysis of benzene, ethylbenzene, and naphthalene.[61] Figure 14 shows the experimental setup for the coupling of SBWE with pure water chromatography. SBWE was performed at 200 °C while the pure water chromatography separation and analysis were carried out at room temperature. The coupling was achieved by taking 10 μL of SBWE extract using heart cuts and the 10 μL of collected SBWE extract was then injected into the pure water chromatography system as shown in Figure 14.[61]

In the late 1990s, Yang and coworkers developed both off-line and on-line SBWE/HPLC coupling systems.[140,141] On the basis of these SBWE/HPLC coupling arrangements, they further constructed a coupling system of SBWE/SBWC to achieve efficient extraction and chromatographic separation using only water as both extraction fluid and chromatography eluent.[75] In this system, the SBWE and SBWC steps are linked together using a sorbent trap. The trap in this SBWE/SBWC coupling system plays two roles. It collects the extracted analytes during the SBWE step and later acts as the injector during the SBWC step. The injection of the analytes collected in the trap into the SBWC column is achieved by applying thermal desorption to the trap.[75] Smith and coworkers reported on-line coupling systems for SBWE with SBWC.[83,91,107] The coupling of SBWE and SBWC in these works was also achieved by using a sorbent trap. Their on-line coupling of SBWE/SBWC/UV is shown in Figure 15.[107] Three sets of ovens, preheating coils, and cooling coils were used in this work for the three extraction, trapping/desorption, and chromatography units.[107]

12 INDUSTRIAL APPLICATIONS OF SUBCRITICAL WATER CHROMATOGRAPHY

As we all know, HPLC methods are used globally every day in many industries. The large amounts of organic solvents consumed by HPLC are classified as hazardous wastes that not only have a negative impact on the environment but also are very expensive for purchasing them and their waste disposal. Such costs can easily exceed $1 million per year for a large company. Therefore, the ultimate goal of SBWC development should be the industrial application of this green chromatographic separation technique.

However, much of the SBWC work reported so far was limited to academic studies. On the basis of what revealed by SBWC literature, it is highly possible for SBWC to replace the traditional RPLC that requires a relatively low concentration of methanol or acetonitrile in the mobile phase. There are numerous existing industrial RPLC methods where a low percentage of organic solvents such as ~ 33% methanol or acetonitrile are required in the mobile phase. Please note that although the methanol or acetonitrile consumption is relatively low, the HPLC waste generated by such methods is threefold the volume

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of the methanol or acetonitrile consumed and needs to be disposed of.

Yang and coworkers are currently working with their Procter & Gamble collaborators, Marple and Gamsky, on SBWC separation and analysis of niacinamide, preservatives, and sunscreens contained in skincare cream samples.[43,116] They were able to achieve separation and analysis of niacinamide in skincare formulations using pure water as the eluent at 60 °C on a Waters XTerra MS C18 column and a Waters XBridge C18 column or at 80 °C on a Hamilton PRP-1 column.[43] The separation efficiency, accuracy and precision of quantification, and analysis time of the green niacinamide method are at least comparable with those of the traditional HPLC method. In addition, the pure water chromatography method for niacinamide analysis can be directly applied in industrial plant settings without further modification of the existing HPLC system since the temperature required here is relatively low and many current commercial HPLC systems are equipped with column ovens capable of reaching 80 °C.[43]

Yang and coworkers also demonstrated successful SBWC separation and analysis of several preservatives in skincare products on Waters XBridge C18 and Phenyl columns at 150 °C and on a ZirChrom-DB-C18 column at 200 °C.[116] Again, the SBWC separation and quantification quality are as good as those obtained by room-temperature HPLC methods. For both niacinamide and preservative methods, organic solvents are eliminated from the mobile phase.[43,116] Although no quantification was performed, the work reported by Dugo et al.also shows the potential of SBWC separation and analysis of preservatives in cream samples.[86]

Sunscreens are more retentive than niacinamide and preservatives. Therefore, it is more challenging to achieve efficient separation of sunscreens using only aqueous mobile phase. However, our ongoing study shows that the percentage of organic solvents required in the mobile phase can be reduced to about 80% when the separation temperature is increased to ~200 ° C.

Although the method validation was not as thorough as at the industrial standard, the SBWC-FID methods reported by Miller and Hawthorne[50], Yarita et al.,[47] and Nakajima et al.[124] for the separation and analysis of alcohols may have a potential for application in the wine industry. In addition, Wilson's study on SBWC-UV analysis of model drugs contained in urine samples may also be appeal to pharmaceutical and medical industries.[94]

13 STUDIES ON SUBCRITICAL WATER CHROMATOGRAPHY EFFICIENCY AND RETENTION

Since temperature is the most important parameter in SBWC, Yang and coworkers investigated the temperature effect on peak width and column efficiency.[98] This study revealed that the number of theoretical plates can be optimized by varying the separation temperature at constant flow rate.[98] A model developed later by Yang was used to explain the effect of temperature on SBWC separation efficiency.[142]

Pawlowski and Poole studied the salvation characteristics of subcritical water.[65] The results of their study indicate that, for method development, the SBWC and RPLC processes are complementary, rather than redundant.[65] Edge et al.developed a model studying the relationship between temperature, flow rate, and pressure to facilitate rapid method.[87]

Yang et al.studied the retention behavior of polar, moderately polar, and nonpolar solutes on both normal- and reversed-phase columns in SBWC.[72] Coym and Dorsey studied retention thermodynamics using ambient water and high-temperature water as the mobile phase[60] while both kinetic and thermodynamic behaviors were studied by Guillarme et al.using methanol–water, acetonitrile–water, and pure water as the mobile phases.[27] The thermodynamic changes observed as the temperature is changed are similar to those seen when mobile phase composition, organic solvent and water, is changed at constant temperature.[60] This is similar to the observations reported by Yang and coworkers.[79,143] Allmon and Dorsey recently studied SBWC retention mechanism and the disruption of the hydrogen-bond network under subcritical water conditions.[100,144]

14 CONCLUSIONS

To date, researchers have studied many aspects of the SBWC technique. The areas studied include development of stable packing materials, instrumentation development, evaluation of commercially available columns, method development, and studies on retention mechanism and separation efficiency. SBWC is capable of separating most polar and moderately polar solutes. Even some nonpolar compounds can be separated using SBWC if high-temperature and less retentive columns are used. Over 30 classes of organics have been separated using SBWC. Most of these solutes are stable during the relatively short SBWC run. Polymer, several zirconia-based, and hybrid silica-based stationary phases are quite stable and robust under subcritical water conditions.

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The biggest advantage of SBWC is the elimination of hazardous solvents used in the mobile phase, thus saving money and making the environment greener. SBWC separation is also fast and may improve selectivity, resolution, and separation efficiency. Temperature-programmed SBWC elution can also be carried out. The use of FID provides a sensitive and universal detection technique for SBWC, making SBWC-FID a unique LC separation/detection technique. In addition, the coupling of SBWE with SBWC eliminates the use of organic solvents in both extraction and chromatographic separation processes.

Although some currently available stationary phases may be used for high-temperature applications, it is still important to further develop more thermally and chemically durable packing materials that can be safely used at temperatures up to 200 °C. Instrumentation companies will also focus more on the development of HTLC systems that integrates efficient precolumn heating and postcolumn cooling to minimize the extra void volume caused by these additions. The Shimadzu Nexera system with an CTO-30A column oven can handle SBWC separation at temperatures up to 150 °C. At last, it is time for industry to pay attention to this promising green SBWC technology and to develop SBWC methods to replace some of the existing RPLC methods currently used in industry. This will save a tremendous amount of money for companies that are willing to adopt SBWC.

ABBREVIATIONS AND ACRONYMS

CAD	Charged Aerosol Detector
FID	Flame Ionization Detector
HTLC	High-Temperature Liquid Chromatography
RI	Refractive Index
RPLC	Reversed-Phase Liquid Chromatography
SBWC	Subcritical Water Chromatography
SBWE	Subcritical Water Extraction
UHPLC	Ultrahigh High-Pressure Liquid Chromatography
WRP-LC	Water Only Reversed-Phase Liquid Chromatography

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Separation of sunscreens in skincare creams using greener high-temperature liquid chromatography and subcritical water chromatography

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Synopsis

In this study, high-temperature liquid chromatographic (HTLC) and subcritical water chromatographic (SBWC) separations of sunscreens contained in skincare creams were achieved at temperatures ranging from 90 to 250°C. The columns employed in this work include a ZirChrom-DiamondBond-C18, a XTerra MS C18 and a XBridge C18 column. The quantity of methanol consumed by the greener HTLC sunscreen methods developed in this project is significantly reduced although the HTLC separation at this stage is not as efficient as that achieved by traditional HPLC. SBWC separation of sunscreens was also achieved on the XTerra MS C18 and the XBridge C18 columns using pure water at 230–250°C. Methanol was eliminated in the SBWC methods developed in this study.

Introduction

Sunscreens are used to protect the skin by absorbing the ultraviolet (UV) light and act as UV filters [1–3]. These sunscreen compounds include para-aminobenzoic acid esters, cinnamates, salicylates, benzophenones, dibenzoylmethanes, anthranilates, benzylidene camphors and others. There are many skincare products available in the market as lotions and creams with various sun protection factors [4, 5]. Sunscreens are mostly effective in the prevention of skin ageing and skin cancer [6, 7].

Several extraction techniques are used to prepare lotion and cream samples for the analysis of sunscreens. These techniques include liquid–liquid extraction, solid-phase extraction, supercritical fluid extraction and microwave-assisted extraction [8–13]. In addition, direct dispersion is also used. The separation and analysis of sunscreens are then carried out mainly by liquid chromatography [12–18]. However, most of these liquid chromatography methods require a large amount of toxic and expensive organic solvents such as methanol in the HPLC mobile phase.

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Fortunately, the amount of organic solvents required in HPLC mobile phase can be significantly reduced by high-temperature liquid chromatography (HTLC) [19–24]. Even better, separation of certain classes of organics has been achieved by subcritical water chromatography (SBWC) where high-temperature water is used as the sole mobile-phase component [25–45]. Obviously, hazardous organic solvents such as methanol and acetonitrile are eliminated from the SBWC mobile phase.

Although a major concern with HTLC and SBWC is the stability of the stationary phase under high-temperature conditions, several hybrid silica-based and zirconia-based commercial columns are quite robust at high temperatures ranging from 100 to 200°C [19, 25]. These stable columns make it possible for HTLC and SBWC method development. As most of the HTLC and SBWC publications up to date are limited to academic studies, it is important to develop HTLC methods that can later be adopted by industry. Our recent work on SBWC separation and analysis of niacin and niacinamide and preservatives clearly demonstrates that it is indeed feasible for industry to replace certain existing HPLC methods with the green SBWC ones [43–45].

In this study, we examined the potential application of HTLC/SBWC methods for the separation of sunscreens in skincare creams. Separation columns used in this study include a ZirChrom-DiamondBond-C18, a Waters XTerra MS C18 and a Waters XBridge C18 column. These three commercial columns are reasonably stable at elevated temperatures [25, 43–45]. The sunscreen compounds investigated in this work include avobenzone, ensulizole, homosalate, octinoxate, octisalate and octocrylene. Oxybenzone was used as the internal standard. Separation and analysis of these sunscreens contained in three Olay skincare creams have been carried out by either HTLC or SBWC at temperatures ranging from 90 to 250°C.

Materials and methods

Reagents and materials

Ensulizole, oxybenzone, octisalate and octocrylene were purchased from Sigma-Aldrich Chemicals (Milwaukee, WI, U.S.A.). Avobenzone and homosalate were received from The Procter and Gamble

Company (P&G, Cincinnati, OH, U.S.A.). Octinoxate was obtained from the Science Lab.com, Inc. (Houston, TX, U.S.A.). HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Deionized water (18 MΩ-cm) was prepared in our laboratory. Olay skincare creams were obtained from a local store. Whatman GD/X filters of 0.45-μm and 5-mL syringes were purchased from VWR (West Chester, PA, U.S.A.). Copper tubing of 1/8-inch O.D. was received from Chromatography Research Supplies, Inc. (Louisville, KY, U.S.A.). Glass vials were obtained from Supelco (Bellefonte, PA, U.S.A.). Both XTerra MS C18 (2.1 × 100 mm I.D., 3.5 μm) and XBridge C18 (2.1 × 100 mm I.D., 3.5 μm) columns were purchased from Waters Corporation (Milford, MA, U.S.A.),

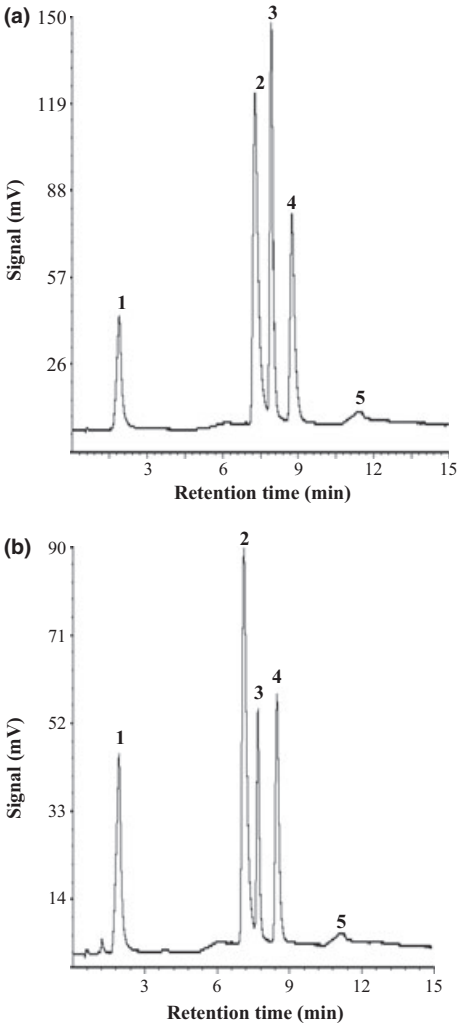

Figure 1 HTLC chromatograms obtained on the ZirChrom-DB-C18 column at 190°C. (a) Sunscreen standard mixture; (b) SC-EC2 skincare cream sample. Gradient: 0–3 min, 10% methanol; 3–4 min, 10–40% methanol; 4–9 min, 40% methanol; 9–10 min, 40–70% methanol; 10–15 min, 70% methanol. Programmed flow rates: 0–3 min, 2.0 mL min^{-1}; 3–4 min, decreased from 2.0 to 1.5 mL min^{-1}; 4–9 min, 1.5 mL min^{-1}; 9–10 min, increased from 1.5 to 2.0 mL min^{-1}; 10–15 min, 2.0 mL min^{-1}. Peak identification: 1, ensulizole; 2, oxybenzone; 3, octocrylene; 4, octisalate; 5, avobenzone. HTLC, high-temperature liquid chromatography.

Table I Concentration of sunscreens found in SC-EC2 skincare cream sample obtained by high-temperature liquid chromatography using the ZirChrom-DB-C18 column at 190°C with programmed flow rates as described in Fig. 1 legend

	P&G stated concentration (%W)	Concentration found by this method (%W)	%Recovery	%RSD[a]
Ensulizole	1.00	1.06	105.5	3.7
Avobenzone	2.00	2.06	103.0	37.2
Octisalate	4.00	3.98	99.4	4.2
Octocrylene	1.00	1.02	101.8	5.2

[a]Based on five replicate measurements.

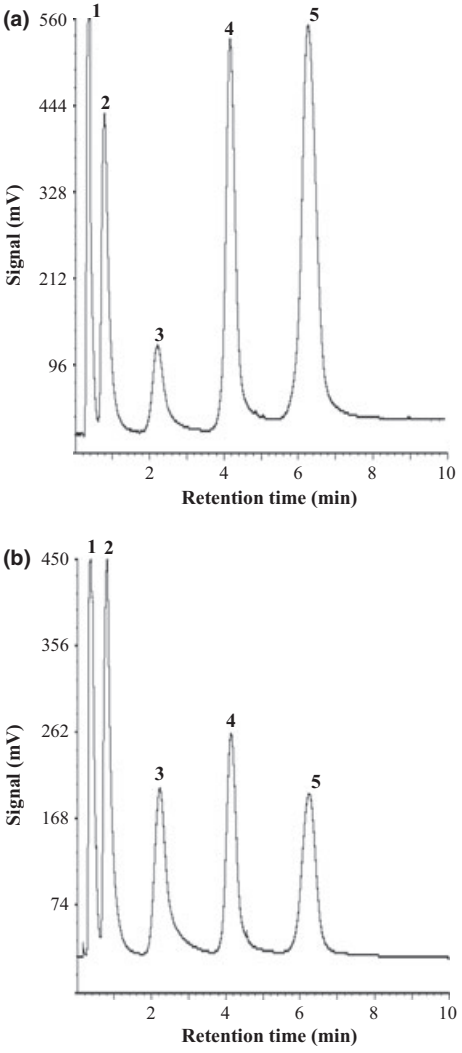

Figure 2 HTLC sunscreen chromatograms obtained on the XTerra MS C18 column at 200°C and 1.0 mL min^{-1}. (a) Sunscreen standard mixture; (b) SC-EC2 skincare cream sample. Isocratic elution using a mobile phase containing 2% of methanol in water. Peak identification: 1, ensulizole; 2, oxybenzone; 3, avobenzone; 4, octisalate; 5, octocrylene. HTLC, high-temperature liquid chromatography.

whereas the ZirChrom-DiamondBond-C18 (4.6 × 100 mm I.D., 3 μm) column was obtained from ZirChrom Separations, Inc., (Anoka, MN, U.S.A.). These columns were chosen owing to their good stability at high temperatures [25].

Preparation of standard solutions

Internal standard solution was prepared by adding 0.1 ± 0.01 g of oxybenzone into a 100-mL volumetric flask and then diluted to the mark with acetonitrile. Calibration mixture was prepared by weighing Q mg of each sunscreen compound (Q = 10 × % each sunscreen compound in skincare product) into a 250-mL volumetric flask. Then, 20 mL of internal standard was added to the flask, and the solution was diluted to the mark with methanol.

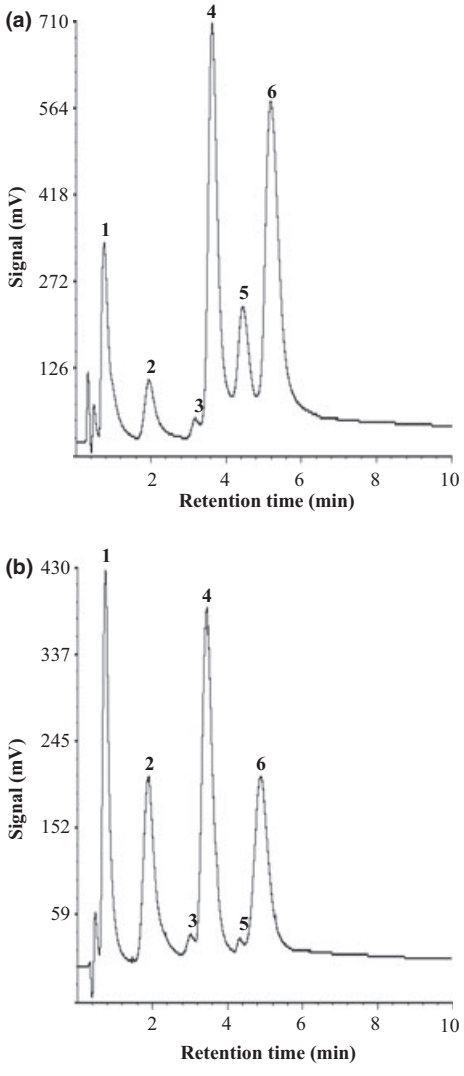

Figure 3 HTLC sunscreen chromatograms obtained on the XTerra MS C18 column at 200°C and 1.0 mL min^{-1}. (a) Sunscreen standard mixture; (b) SC-EC3 skincare cream sample. Isocratic elution using a mobile phase containing 2% of methanol in water. Peak identification: 1, oxybenzone; 2, avobenzone; 3, homosalate-cis; 4, octisalate; 5, homosalate-trans; 6, octocrylene. HTLC, high-temperature liquid chromatography.

Preparation of sample solutions

Each skincare cream sample was mixed well before weighing to ensure homogeneous sampling. Approximately 0.1 ± 0.02 g of Olay skincare cream sample was weighed directly into a 25-mL glass vial. Exactly 2.00 mL of internal standard solution and approximately 23 mL of methanol were added to the sample vial. The vial was capped and vortexed to completely disperse the cream. Once dispersed, the solution was filtered through a 0.45-μm Whatman GD/X filter into a 2-mL autosampler vial for chromatographic analysis.

Instrumentation

A home-made HTLC/SBWC chromatography system was used in this study. This system and its operating procedures were described in a previous work [43]. However, there was a minor modification for the HTLC/SBWC system used in this work. To reduce the temperature of the injection port and to ease the manual injection, copper tubing was wrapped around the portion of the pre-heating coil outside the oven. Please note that the pre-heating coil was connected between the pump and the injector. The pre-heating coil was heated inside the oven, but its exit end was connected to the injector located outside the oven. The injector was cooled by a copper tubing by continuously running room temperature water through it. The injection volume was 10 μL for all experiments.

Results and discussion

ZirChrom-DiamondBond-C18 column

Initial HTLC experiments were performed at 90 and 150°C. Although reasonable separation of sunscreens was obtained, only a small fraction of methanol was saved by these HTLC methods.

To further reduce the consumption of methanol in our HTLC methods, HTLC separation and analysis of the SC-EC2 skincare

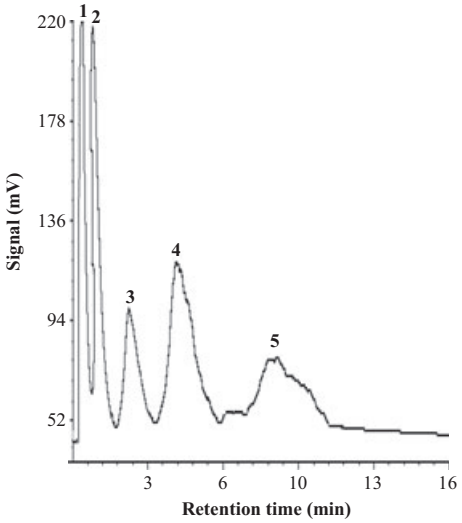

Figure 4 SBWC chromatogram of SC-EC2 skincare cream sample obtained on the XTerra MS C18 column at 250°C and 1.0 mL min^{-1} using 100% water as the mobile phase. Peak identification: 1, ensulizole; 2, oxybenzone; 3, avobenzone; 4, octisalate; 5, octocrylene. SBWC, subcritical water chromatography.

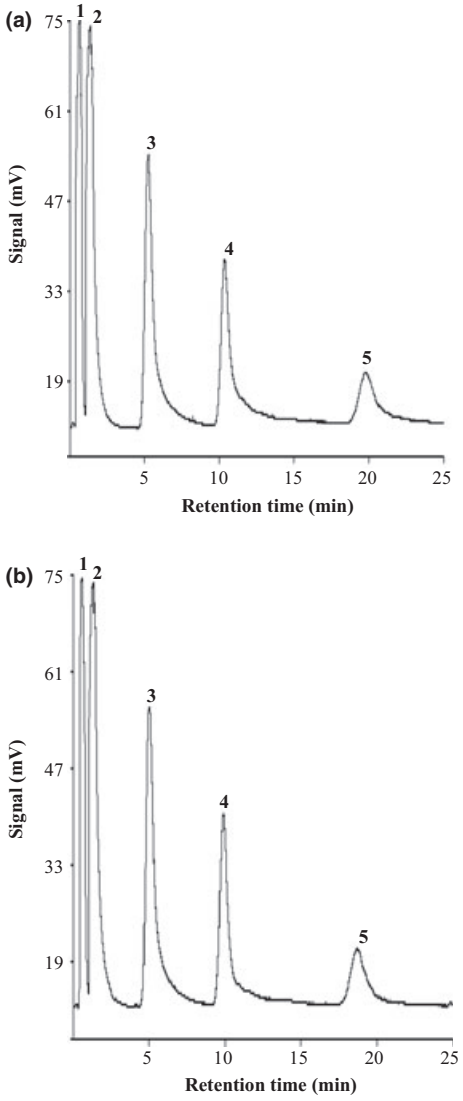

Figure 5 HTLC sunscreen chromatograms obtained on the XBridge C18 column at 200°C and 0.75 mL min^{-1}. (a) Sunscreen standard mixture; (b) SC-EC2 skincare cream sample. Isocratic elution using a mobile phase containing 2% of methanol in water. Peak identification: 1, ensulizole; 2, oxybenzone; 3, avobenzone; 4, octisalate; 5, octocrylene. HTLC, high-temperature liquid chromatography.

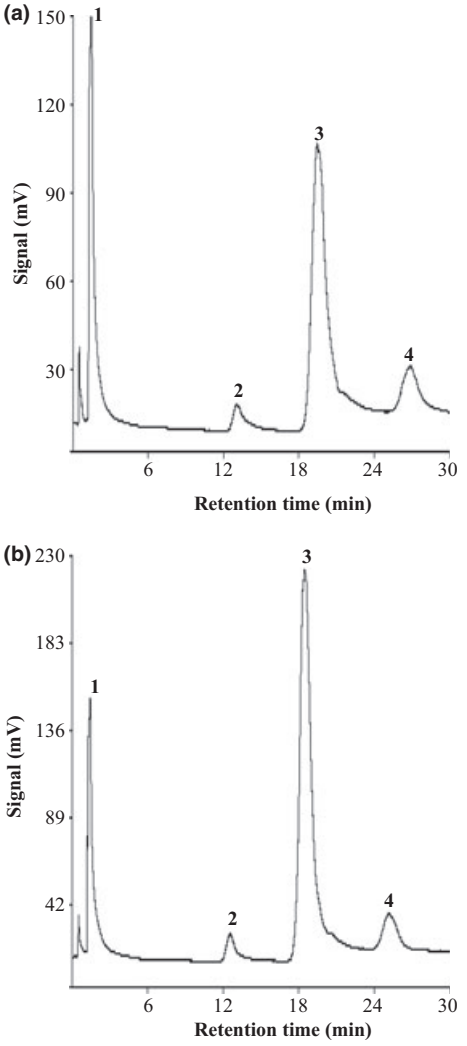

Figure 6 HTLC sunscreen chromatograms obtained on the XBridge C18 column at 200°C and 0.75 mL min^{-1}. (a) Sunscreen standard mixture; (b) SC-EC4 skincare cream sample. Isocratic elution using a mobile phase containing 2% of methanol in water. Peak identification: 1, oxybenzone; 2, octisalate; 3, octinoxate; 4, octocrylene. HTLC, high-temperature liquid chromatography.

cream sample were performed at 190°C. Figure 1 shows the separation of sunscreens contained in SC-EC2 skincare cream. The gradient elution and programmed flow rate conditions used for this separation are given in Fig. 1 legend. The lowest resolution in both chromatograms is 2 for oxybenzone and octocrylene. Quantification of sunscreens found in SC-EC2 cream was further carried out using this HTLC method, and the results are given in Table I. Although the recovery is reasonably good for all four sunscreens contained in the sample, the precision for avobenzone is poor because of peak broadening as shown in Fig. 1. The fraction of methanol saved in the HTLC mobile phase is still fairly small.

Waters XTerra MS C18 column

As the methanol percentage was not greatly reduced using the ZirChrom-DB-C18 column, we further evaluated a Waters XTerra MS C18 column in hoping to reduce the quantity of methanol required in the mobile phase. HTLC separation of sunscreens was carried out on the Waters XTerra MS C18 column at 150°C using different gradient elution for SC-EC2 and SC-EC3 skincare cream samples. Much greater fraction of methanol was saved using the 150°C HTLC methods when comparing with the methanol consumption required for HTLC sunscreen separation on the ZirChrom-DB-C18 column.

Further separation of sunscreens was performed on this XTerra column at 200°C using isocratic elution with a mobile phase

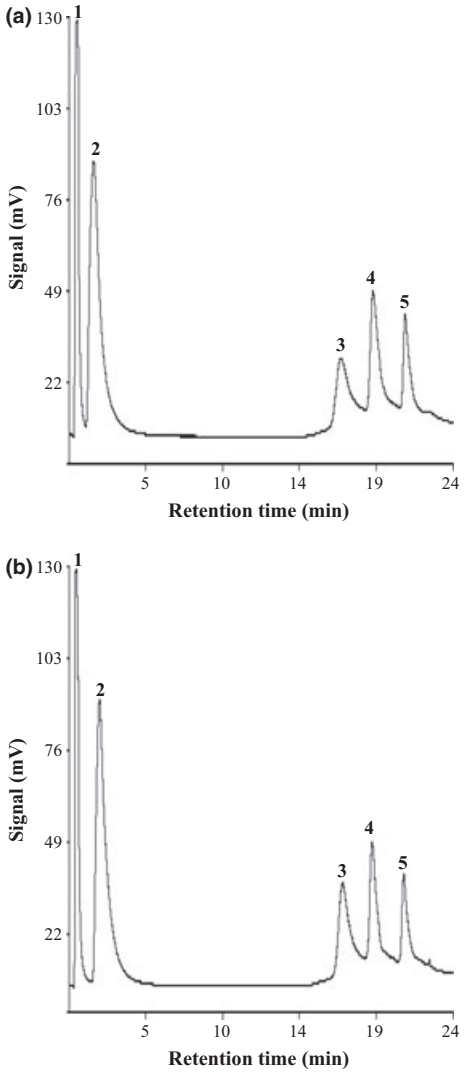

Figure 7 SBWC/HTLC chromatograms obtained on the XBridge C18 column at 200°C and 0.75 mL min^{-1}. (a) Sunscreen standard mixture; (b) SC-EC2 skincare cream sample. Gradient: 0–10 min, 0% methanol; 10–20 min, 0–4% methanol; 20–24 min, 4% methanol. Peak identification: 1, ensulizole; 2, oxybenzone; 3, avobenzone; 4, octisalate; 5, octocrylene. SBWC, subcritical water chromatography; HTLC, high-temperature liquid chromatography.

containing only 2% methanol in water. Flow rate of 1 mL min^{-1} was used in this experiment. Figure 2 shows the separation of sunscreens present in SC-EC2 skincare cream sample, whereas Fig. 3 demonstrates the separation of sunscreens present in SC-EC3 skincare cream. The resolution between the first two peaks is 1.9 although all other peaks are well separated with resolution >3 for chromatograms shown in Fig. 2. The resolution in Fig. 3 is getting worse with 1.2 for homosalate-cis and octisalate.

The last experiment performed on this XTerra column was the separation of sunscreens with pure water at 250°C. As no methanol was required in the mobile phase, this separation technique is termed SBWC. Figure 4 shows the chromatogram of SC-EC2 skincare cream sample obtained by SBWC at 250°C with 1 mL min^{-1}.

Table II Concentration of sunscreens found in SC-EC2 skincare cream sample obtained by the combined subcritical water chromatography/high-temperature liquid chromatography (HTLC) method using the Waters XBridge C18 column at 200°C with 0.75 mL min^{-1} and HTLC gradient conditions as described in Fig. 7 legend

	P&G stated concentration (%W)	Concentration found by this method (%W)	%Recovery	%RSD[a]
Ensulizole	1.00	1.02	102.3	2.9
Avobenzone	2.00	2.26	113.2	2.8
Octisalate	4.00	4.00	100.0	3.2
Octocrylene	1.00	0.90	90.3	5.0

[a]Based on five replicate measurements.

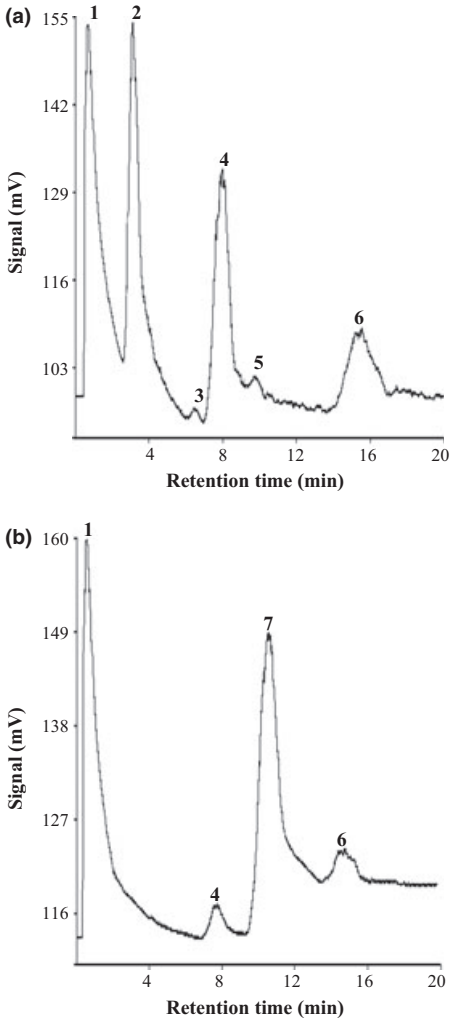

Figure 8 SBWC chromatograms of sunscreens obtained on the XBridge C18 column at 230°C and 1.0 mL min^{-1} using 100% water as the mobile phase. (a) SC-EC3 skincare cream sample; (b) SC-EC4 skincare cream sample. Peak identification: 1, oxybenzone; 2, avobenzone; 3, homosalate-cis; 4, octisalate; 5, homosalate-trans; 6, octocrylene; 7, octinoxate. SBWC, Subcritical water chromatography.

Because the elution strength of pure water is still weak even at this high temperature, the resolution became poorer compared with those achieved in HTLC. However, this result indicates that there is a potential to achieve reasonable separation of sunscreens using the green SBWC technique if a viable stationary phase is available.

Waters XBridge C18 column

As the results obtained on the Waters XTerra column look more promising, HTLC and SBWC separations of sunscreens were further evaluated using a Waters XBridge C18 column. We started the evaluation of this XBridge column by performing HTLC separation at 200°C using isocratic elution with 2% methanol in water at a flow rate of 0.75 mL min^{-1}. Figures 5 and 6 show the chromatograms of SC-EC2 and SC-EC4 skincare cream samples achieved on the XBridge column using this HTLC method. The resolution is 1.3 for ensulizole and oxybenzone but well exceeds 5 for all other pair of peaks in Fig. 5. All peaks in Fig. 6 are well separated with a resolution >2.4.

Although only 2% methanol was used for the isocratic elution in the aforementioned HTLC method, the HTLC waste also contains 2% of methanol and needs to be disposed of. To reduce the amount of methanol-containing HTLC waste, the following unconventional gradient elution was tested. In this new method, SBWC and HTLC were integrated into a unique separation approach. The separation was conducted again at 200°C. During the first 10 min, pure subcritical water was used as the mobile phase. After this initial stage of SBWC elution, a gradient elution using a few percentages of methanol in the mobile phase followed. The gradient detail is given in Fig. 7 legend. The advantage of using this combined SBWC/HTLC method is that the waste generated during the SBWC step (with no methanol in the mobile phase) does not require disposal. Figure 7 shows the SBWC/HTLC chromatogram of SC-EC2 cream sample obtained by this method. It should be pointed out that the octocrylene peak is much narrower than that achieved by the previous HTLC method using 2% methanol isocratic elution. Quantification results of SC-EC2 cream obtained by this combined SBWC/HTLC method are given in Table II. While reasonable precision was achieved, the recovery was poor for avobenzone and octocrylene mainly because of not so efficient separation for these solutes as shown in Fig. 7.

The ultimate goal of this work was to seek potential SBWC separation of sunscreens present in skincare products. Therefore, the last set of experiments in this work involved separation of sunscreens using pure water at 230°C and with a flow rate of 1.0 mL min^{-1}. This SBWC method was applied to SC-EC3 and SC-EC4 skincare cream samples. Figure 8 shows the chromatograms of the two Olay sunscreen creams. Although the separation here was not efficient enough for quantitative analysis, it does show a potential for the separation of hydrophobic sunscreens using pure water as the only mobile-phase component.

Conclusions

High-temperature liquid chromatography separation of sunscreens contained in SC-EC2 skincare cream was achieved on the ZirChrom-DB-C18 column at 190°C using programmed flow rates. While reasonable accuracy was achieved using this method, the reproducibility for avobenzone was poor.

Separation of sunscreens contained in SC-EC2 and SC-EC3 cream samples were also achieved on the XTerra MS C18 column at 150°C using a weak gradient elution. However, better separation was obtained at 200°C using isocratic elution with only 2% methanol in the mobile phase. Separation of sunscreens was also obtained using pure subcritical water at 250°C although the separation was not as efficient as the HTLC ones at 150 and 200°C. Please note that methanol was eliminated using this SBWC method at 250°C.

Both HTLC separation using only 2% methanol and the combined SBWC/HTLC separation on the XBridge C18 column at 200°C were reasonably successful. However, the accuracy for avobenzone and octocrylene obtained by the combined HTLC/SBWC method was poorer than that achieved by traditional HPLC. Reasonable separation of sunscreens was further achieved using pure water at 230°C. In this case, no methanol was required in the SBWC mobile phase.

It should be pointed out that although HTLC and SBWC methods developed in this study for separation of sunscreens can either greatly reduce or completely eliminate methanol consumption, the separation efficiency and resolution obtained by HTLC/SBWC are poorer than those achieved by traditional HPLC. Therefore, further instrumentation and method development is needed before industry can adopt the greener HTLC and SBWC technologies for the separation and analysis of sunscreen compounds in the future.

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Industrial application of green chromatography – II. Separation and analysis of preservatives in skincare products using subcritical water chromatography

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Synopsis

Several high-temperature liquid chromatography (HTLC) and subcritical water chromatography (SBWC) methods have been successfully developed in this study for separation and analysis of preservatives contained in Olay skincare creams. Efficient separation and quantitative analysis of preservatives have been achieved on four commercially available ZirChrom and Waters XBridge columns at temperatures ranging from 100 to 200°C. The quantification results obtained by both HTLC and SBWC methods developed for preservatives analysis are accurate and reproducible. A large number of replicate HTLC and SBWC runs also indicate no significant system building-up or interference for skincare cream analysis. Compared with traditional HPLC separation carried out at ambient temperature, the HTLC methods can save up to 90% methanol required in the HPLC mobile phase. However, the SBWC methods developed in this project completely eliminated the use of toxic organic solvents required in the HPLC mobile phase, thus saving a significant amount of money and making the environment greener. Although both homemade and commercial systems can accomplish SBWC separations, the SBWC methods using the commercial system for preservative analysis are recommended for industrial applications because they can be directly applied in industrial plant settings.

Résumé

Plusieurs méthodes de chromatographie liquide à haute température (HTLC) et de chromatographie à eau sous-critique (SBWC) ont été développées avec succès dans cette étude pour la séparation et l'analyse des conservateurs contenus dans les crèmes de soin OlayTM. Une séparation efficace et un dosage des agents conservateurs ont été réalisés sur quatre colonnes ZirChrom et WatersTM Xbridge, disponibles dans le commerce, à des températures allant de 100 à 200°C. Les résultats de quantification obtenus par ces méthodes à la fois HTLC et SBWC, développées pour l'analyse des agents conservateurs sont précis et reproductibles. Un grand nombre de répétitions d'analyses SBWC et HTLC indique également l'absence d'accumulations ou d'interférences dans l'analyse des crèmes de soin. Par rapport à la séparation traditionnelle CLHP réalisée à température ambiante, la méthode de chromatographie liquide à haute température permet d'économiser jusqu'à 90% du méthanol nécessaire dans la phase mobile CLHP. Toutefois, les méthodes de chromatographie à eau subcritique développées dans ce projet éliminent complètement la nécessité de l'utilisation de solvants organiques toxiques nécessaires dans la phase mobile CLHP, ce qui permet d'économiser une quantité importante d'argent et de rendre l'environnement plus vert. Bien que les deux systèmes "maison" et ceux disponibles dans le commerce peuvent réaliser des séparations SBWC, les méthodes SBWC utilisant les systèmes commerciaux pour l'analyse des conservateurs sont recommandés pour les applications industrielles, car ils peuvent être directement appliquées dans ces contextes.

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Introduction

Preservatives are added to products such as foods, pharmaceuticals, biological samples, paints and cosmetics to prevent microbial spoilage and therefore to prolong the shelf life of the product and to protect the consumer from a potential infection [1]. Some of the common preservatives used in cosmetic industry are benzoic acid, benzyl alcohol and parabens (hydroxybenzoates). Preservatives are frequently used in combination to achieve the required broad coverage [2].

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 methyl, ethyl, propyl and butyl paraben. They are inexpensive, colourless, odourless and effective against yeasts, moulds and bacteria [3,4]. There has been a controversy about the use of parabens in cosmetics over the years. The main concern is that parabens may be carcinogenic and have oestrogenic effects. However, research revealed that there has been no correlation between parabens and cancer [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 cosmetics at levels up to 0.8% for mixtures of parabens or up to 0.4% for a single paraben. Therefore, accurate analysis of preservative levels in various cosmetics products is important and required for quality control, product release and regulatory purposes. Currently, high-performance liquid chromatography (HPLC) has been widely used by the industry for the determination of preservatives in foods, cosmetics and pharmaceuticals [6–11]. However, the major drawback of the conventional HPLC is the large amount of organic solvents required in the mobile phase. These organic solvents such as methanol and acetonitrile are toxic, flammable, expensive and require waste disposal.

Fortunately, several greener chromatography techniques have been developed over the last decade. These techniques include high-temperature liquid chromatography (HTLC) and subcritical water chromatography (SBWC). HTLC refers to HPLC separations performed at elevated temperatures using organic solvent–water mixtures as the mobile phase. The consumption of organic solvents in HTLC mobile phase is significantly reduced with increasing temperature [12–16]. Subcritical water chromatographic separation, on the other hand, is performed at elevated temperatures using pure water as the sole mobile-phase component. Obviously, organic solvents are not required in the SBWC mobile phase and thus eliminated. Therefore, SBWC is truly a green chromatographic separation technique [17–42]. Unfortunately, the vast majority of the SBWC research up to date is still at the academic level, and SBWC quantitative analysis of real samples has rarely been investigated. This led to our recent effort in evaluating the potential of applying SBWC methods to industrial applications [36–42].

The goal of this study was to develop greener HTLC and SBWC methods for quantitative analysis of preservatives present in skincare cream samples. These new methods were evaluated against industrial standards for potential applications in industrial plant settings. To accomplish these objectives, both a homemade system and a Shimadzu Nexera UFLC system (Shimadzu Corporation, Tokyo, Japan) and more thermally stable stationary phases such as zirconia- and hybrid silica-based columns were used in this study. Separation and analysis of three Olay skincare cream samples were optimized at both isothermal and programmed temperatures ranging from 100 to 200°C in combination with either constant or programmed flow rates.

Materials and methods

Reagents and materials

Benzyl alcohol, methyl paraben, ethyl paraben, propyl paraben and calcium chloride were purchased from Aldrich (St. Louis, MO, U.S.A.). Butyl paraben was obtained from SAFC (St. Louis, MO, U.S.A.). Ortho phosphoric acid and HPLC-grade methanol were acquired from Fisher Scientific (Fair Lawn, NJ, U.S.A.). GD/X PVDF membrane filters (0.45 μm) were received from Whatman (Florham Park, NJ, U.S.A.). Olay total effects, 7-in-1 anti-ageing daily moisturizer, fragrance free (coded as SC-EC1 in this paper); Olay total effects, 7-in-1 anti-ageing UV moisturizer, plus SPF-15, fragrance free (coded as SC-EC2 in this paper) and Olay complete ageless skin-renewing UV lotion, SPF-20 (coded as SC-EC3 in this paper) were purchased at a local store. The deionized water (18 MΩ-cm) was prepared in our laboratory using a Sybron/Barnstead system (Sybron/Barnstead, Boston, MA, U.S.A.).

Separation columns

Owing to their better stability at high temperatures, four commercial columns were used in this study. ZirChrom-DiamondBond-C18 (4.6 × 100 mm, 3 μm) and ZirChrom-PS (4.6 × 100 mm, 3 μm) columns were purchased from ZirChrom Separations, Inc. (Anoka, MN, U.S.A.). Both XBridge C18 (4.6 × 100 mm, 3.5 μm) and XBridge phenyl (4.6 × 100 mm, 3.5 μm) columns were obtained from Waters Corporation (Milford, MA, U.S.A.).

Preparation of 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.

Preparation of standard solutions

A stock standard solution was prepared by adding 0.075 g of benzyl alcohol and 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. Then, a calibration standard solution was prepared by transferring 2.00 mL of the internal standard solution and 2.00 mL of the stock standard solution to a 25-mL volumetric flask and then diluting to the mark with methanol. The solution was mixed thoroughly.

Preparation of sample solutions

Preparation of calcium chloride solution. First, a calcium chloride solution was prepared by adding 1.25 g of calcium chloride to a 50-mL volumetric flask and then diluting to the mark with deionized water.

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 25-mL glass vial. Then 2.00 mL of the internal standard solution, 2.00 mL of calcium chloride solution and 21.00 mL of methanol were added to the vial. Sample in the vial was vortexed for 15 min or until the sample was completely dissolved. An aliquot of the sample solution was then filtered through a 0.45-μm Whatman GDX filter into a clean glass vial for chromatography analysis.

SBWC/HTLC system and its operation

A homemade system was used in the first part of this study for both HTLC and SBWC separations. Figure 1 shows the block diagram of the homemade SBWC/HTLC system. A Hitachi L-7100 HPLC pump (Hitachi, Ltd., Tokyo, Japan) is used to deliver the mobile phase. A Valco injector (Valco Instruments Company Inc., Houston, TX, U.S.A.) with a 10-μL loop is connected to the outlet of the Hitachi pump using 1/16″ stainless steel tubing. Part of this tubing acts as a pre-heating coil that is located inside a used GC oven (HP 5890 Series 2; Hewlett Packard, Avondale, PA, U.S.A.) as shown in Fig. 1.

The GC oven was allowed to heat up to the set temperature for a given experiment, and the first injection was made approximately 30 min after the set temperature was reached to ensure thermal equilibration. After leaving the oven, the effluent was cooled with an iced water bath before entering the Hitachi L-7400 UV detector

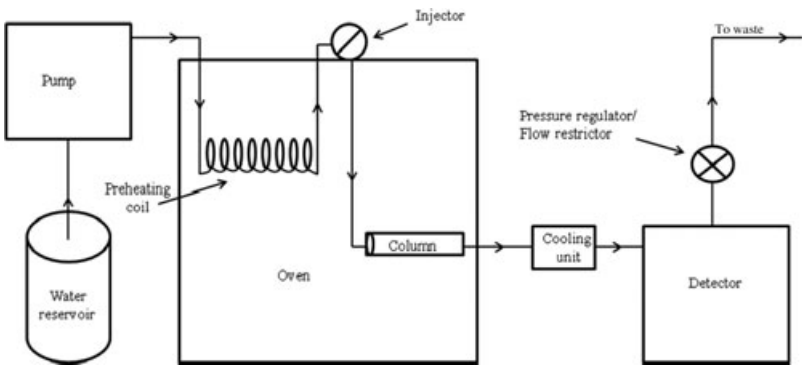

Figure 1 Block diagram of the homemade subcritical water chromatography/high-temperature liquid chromatography system.

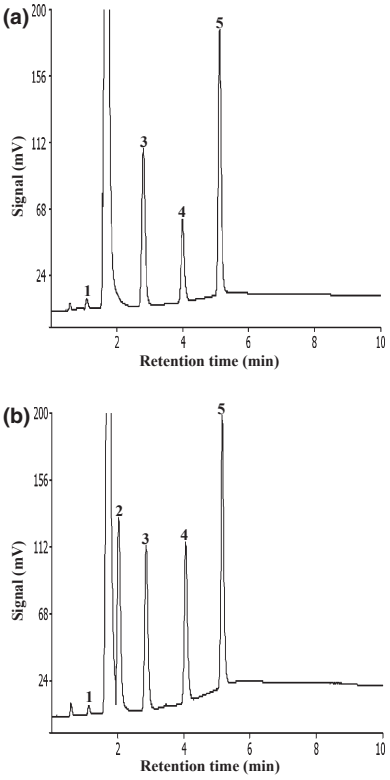

Figure 2 High-temperature liquid chromatography chromatograms obtained on ZirChrom-DB-C18 column at 150°C. (a) SC-EC1 skincare cream sample; (b) SC-EC3 skincare cream sample. Flow rate: 2.0 mL min^{-1}. UV detection: 256 nm. Mobile phase: A, deionized water; B, 100% methanol. Gradient: 0–2 min, 10–20% methanol; 2–6 min, 20–50% methanol; 6–10 min, 50% methanol; 10–10.5 min, 50–10% methanol. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben.

Table I Comparison of recoveries for preservatives present in SC-EC1 skincare cream obtained by HPLC, HTLC and SBWC using ZirChrom-DB-C18 column

	%Recovery (%RSD*)		
	HPLC at 25°C	**HTLC at 150°C**	**SBWC at 200°C**
Methanol saved	0%	75%	100%
Benzyl alcohol	104.3 (3.2)	97.2 (4.0)	Co-eluted
Ethyl paraben	105.5 (5.1)	106.2 (5.9)	101.8 (1.5)
Propyl paraben	104.0 (4.8)	96.8 (5.2)	102.4 (1.9)

HTLC, high-temperature liquid chromatography; SBWC, subcritical water chromatography.
*Based on five replicates.

Table II Comparison of recoveries for preservatives present in SC-EC2 skincare cream obtained by HPLC, HTLC and SBWC using ZirChrom-DB-C18 column

	%Recovery (%RSD*)		
	HPLC at 25°C	**HTLC at 150°C**	**SBWC at 200°C**
Methanol saved	0%	75%	100%
Benzyl alcohol	104.1 (3.9)	97.4 (2.9)	Co-eluted
Methyl paraben	104.2 (4.1)	104.6 (1.9)	104.2 (3.7)
Ethyl paraben	105.0 (4.4)	104.8 (2.1)	102.1 (3.2)
Propyl paraben	106.0 (4.3)	97.6 (2.7)	100.9 (2.1)

HTLC, high-temperature liquid chromatography; SBWC, subcritical water chromatography.
*Based on five replicates.

set at 256 nm. A back pressure regulator (Restek, Bellefonte, PA, U.S.A.) was connected to the outlet of the UV flow cell.

The UV detector was connected to a computer via an interface of PC/CHROM (H&A Scientific, Greenville, NC, U.S.A.). Data acquisition and analysis were made available by the PC/CHROM software.

Additional experiments were performed using a commercial system, Shimadzu Nexera UFLC (Shimadzu Corporation, Tokyo, Japan). This system has a built-in pre-heating unit, a column oven and a post-column cooling unit. It can be operated at temperatures up to 150°C without any modification. The effluent

Table III Comparison of recoveries for preservatives present in SC-EC3 skincare cream obtained by HPLC, HTLC and SBWC using ZirChrom-DB-C18 column

	%Recovery (%RSD*)		
	HPLC at 25°C	HTLC at 150°C	SBWC at 200°C
Methanol saved	0%	75%	100%
Benzyl alcohol	103.2 (4.7)	99.4 (2.3)	Co-eluted
Methyl paraben	102.2 (3.9)	103.9 (3.4)	98.8 (2.5)
Ethyl paraben	105.0 (4.7)	102.3 (2.8)	103.0 (3.2)
Propyl paraben	105.9 (4.9)	102.7 (2.6)	101.4 (2.3)

HTLC, high-temperature liquid chromatography; SBWC, subcritical water chromatography.
*Based on five replicates.

Table IV Recovery of preservatives present in SC-EC3 skincare cream obtained by 24 replicate high-temperature liquid chromatography runs using ZirChrom-DB-C18 column at 150°C with 2.0 mL min^{-1}

	%Recovery	%RSD*
Benzyl alcohol	94.6	2.2
Methyl paraben	99.3	1.8
Ethyl paraben	99.1	1.8
Propyl paraben	101.0	1.2

*Based on 24 replicate injections of a single sample solution.

after exiting the column was cooled using a built-in cooling unit, and UV flow cell was maintained at 50°C. UV detection at both 210 and 256 nm was achieved using the best chromatogram mode.

Results

ZirChrom-DiamondBond-C18 column

All experiments described in this section for separation of preservatives on the ZirChrom-DB-C18 column were performed using the homemade system.

HTLC separations of preservatives

Initially, separation of preservatives was carried out on the ZirChrom-DB-C18 column using methanol–water gradient elution at 90°C. Although good separations were achieved, this 90°C HTLC method still required a large portion of methanol in the mobile-phase mixture. To reduce the methanol consumption, further HTLC experiments were performed at 150°C with different flow rates. As 2 mL min^{-1} yielded good separation and short analysis time, this flow was used in this HTLC method. Figure 2 shows examples of chromatograms of Olay skincare cream samples obtained by this 150°C HTLC method. The separation conditions are given in Fig. 2 legend. Compared with HPLC method for preservatives carried out in our laboratory at East Carolina University, approximately 75% methanol was saved using this HTLC method.

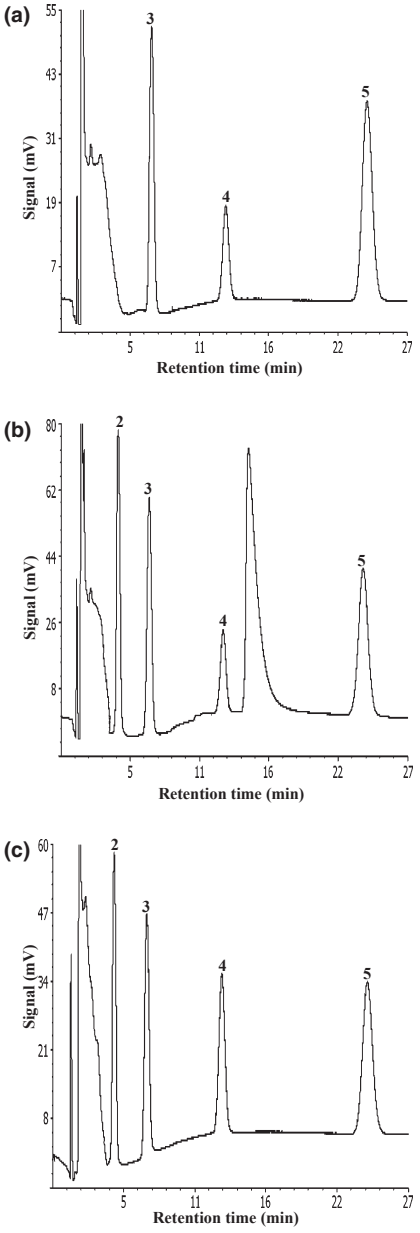

Figure 3 Subcritical water chromatography chromatograms obtained on ZirChrom-DB-C18 column at 200°C using 100% water as the mobile phase. (a) SC-EC1 skincare cream sample; (b) SC-EC2 skincare cream sample; (c) SC-EC3 skincare cream sample. UV detection: 256 nm. Programmed flow rate: 0–6.5 min, decreased from 1.0 to 0.75 mL min^{-1}; 6.5–27 min, 0.75 mL min^{-1}; 27–27.5 min, increased from 0.75 to 1.0 mL min^{-1}. Peak identification: 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben.

As efficient separation as shown in Fig. 2 was achieved, quantification of preservatives contained in three Olay skincare creams

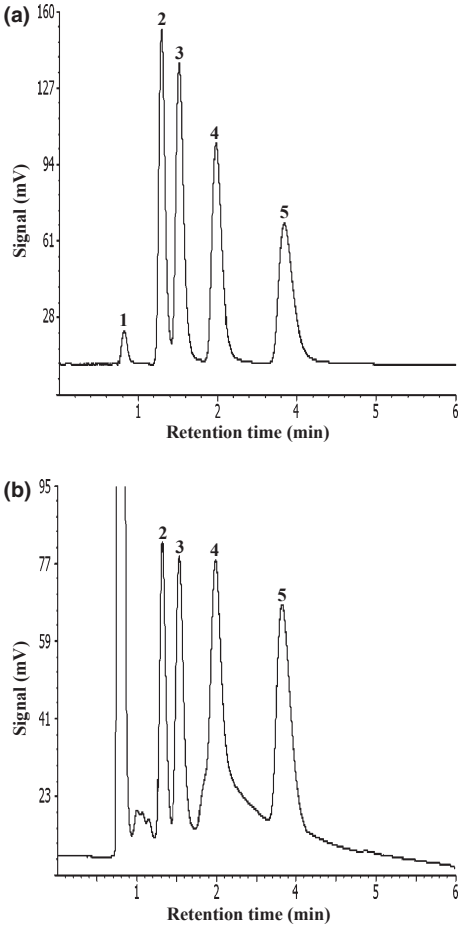

Figure 4 Subcritical water chromatography chromatograms obtained on ZirChrom-PS column at 180°C and 1.25 mL min^{-1} using 100% water as the mobile phase. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. UV detection: 256 nm. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben.

was also carried out using this 150°C HTLC method. Per cent recovery of preservatives was calculated based on P&G reference values. As shown in Tables I–III, the recoveries obtained by this HTLC method ranges from 97% to 106%.

To check if any building-up occurred for analysis of real cream samples, a total of 24 replicate injections of a single sample preparation were made continuously. As shown in Table IV, both good accuracy and precision were obtained for the 24 replicate measurements, indicating that no system building-up interfered the separation and analysis of preservatives in the skincare cream sample.

SBWC separations of preservatives

To eliminate the use of methanol in the mobile phase, the separation of preservatives was performed at 200°C using pure water as the mobile phase. The SBWC was optimized with various flow rates, and the best separation was achieved using a programmed flow as described in Fig. 3 legend. Figure 3 shows SBWC chromatograms of the three Olay skincare cream samples obtained at 200°C using a programmed flow.

Table V Recovery of preservatives present in SC-EC3 skincare cream obtained by subcritical water chromatography using ZirChrom-PS column at 180°C and 1.25 mL min^{-1}

	%Recovery	%RSD*
Benzyl alcohol	Co-eluted	
Methyl paraben	104.5	3.8
Ethyl paraben	101.7	1.3
Propyl paraben	105.3	3.9

*Based on five replicates.

As shown in Fig. 3, good separations of all parabens were achieved using pure water at 200°C. The only limitation of this method is that benzyl alcohol was co-eluted with a matrix peak. The recovery of parabens obtained by SBWC at 200°C is also

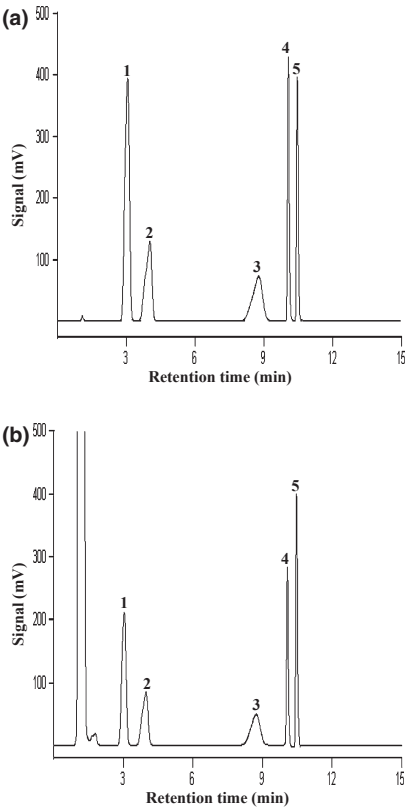

Figure 5 Integrated subcritical water chromatography/high-temperature liquid chromatography chromatograms obtained on XBridge C18 column at 150°C and 1.0 mL min^{-1} (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. UV detection: 210 nm. Gradient: 0–7.9 min, 0% methanol; 7.9–8 min, 0–50% methanol; 8–11 min, 50% methanol; 11–11.1 min, 50–0% methanol; 11.1–15 min, 0% methanol. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben.

Table VI Recovery of preservatives present in SC-EC3 skincare cream obtained by the integrated subcritical water chromatography/high-temperature liquid chromatography at 150°C using Waters XBridge C18 column with gradient elution as described in Fig. 5 legend

	%Recovery (%RSD*)	
	Detection at 210 nm	Detection at 256 nm
Benzyl alcohol	100.3 (1.1)	98.1 (0.8)
Methyl paraben	102.9 (0.4)	104.4 (0.4)
Ethyl paraben	101.8 (0.3)	103.7 (0.4)
Propyl paraben	105.3 (1.3)	104.5 (1.3)

*Based on five replicates.

reported in Tables I–III. One can easily see that the quantification of preservatives achieved by SBWC is accurate and precise.

To further evaluate the SBWC and HTLC methods developed so far, traditional HPLC separation was performed at 25°C using the same chromatography system. The results of HPLC separation and analysis are also included in Tables I–III. It is clear that the quantification quality achieved by HTLC at 150°C and by SBWC at 200°C is as good as that obtained by the traditional HPLC using the same chromatography system and the same separation column.

ZirChrom-PS column

Further SBWC experiments were performed using the homemade system on the ZirChrom-PS column at 180°C with pure water as the eluent. The flow rate evaluation revealed that 1.25 mL min^{-1} was the optimized flow. As shown in Fig. 4, the SBWC saparation time was significantly reduced by this ZirChrom-PS column when comparing with that obtained by SBWC separation on the ZirChrom-DB-C18 column. While the parabens were well separated, benzyl alcohol was still co-eluted with a matrix peak of SC-EC3 cream sample. Table V shows the recovery of parabens present in SC-EC3 skincare cream sample obtained by SBWC on the ZirChrom-PS column at 180°C.

Waters XBridge C18 column

All experiments reported in this section for the separation of preservatives on the Waters XBridge C18 column were performed using the Shimadzu Nexera UFLC system. The injection volume used was 10 μL, and the flow rate was 1 mL min^{-1}.

Integrated SBWC/HTLC separations of preservatives

A combined HTLC and SBWC separation approach was used here to separate preservatives on the XBridge C18 column at 150°C. The chromatography run was first started using pure water, then with a methanol–water mixture and finally using pure water again to finish the HTLC/SBWC separation run. The gradient conditions are detailed in Fig. 5 legend. As only 3 min of the total 15-min chromatography run time involved methanol in the mobile phase, only 20% of the chromatography waste contained methanol and required waste disposal. This is more advantageous than HTLC elution using a small percentage of methanol in the mobile phase throughout the entire run because all HTLC waste generated this way contains methanol and has to be disposed of.

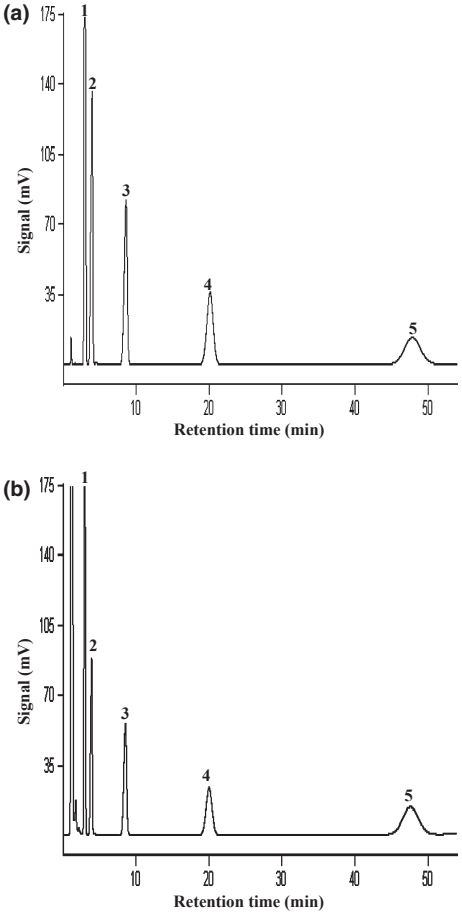

Figure 6 Subcritical water chromatography chromatograms obtained using 100% water on XBridge C18 column at 150°C and 1.0 mL min^{-1} with the best chromatogram mode. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, whereas the other parabens were detected at 256 nm. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben.

Compared with separations on the two ZirChrom columns, the major advantage of this XBridge C18 column is that the benzyl alcohol is well separated as depicted in Fig. 5. Because the molar absorptivity of benzyl alcohol is low at 256 nm, 210 nm was also used in this experiment to improve the absorbance of benzyl alcohol. The quantification results of preservatives obtained by detection at both 210 and 256 nm are given in Table VI for comparison purposes.

SBWC separations of preservatives at 150°C

To eliminate methanol used in the mobile phase, further experiments were performed at 150°C using pure water as the eluent. The 'best chromatogram mode' was used in this experiment, mean-

Table VII Recovery of preservatives present in SC-EC3 skincare cream obtained by subcritical water chromatography at 150°C using Waters XBridge C18 column with 1.0 mL min^{-1} and the best chromatogram mode*

	%Recovery	%RSD†
Benzyl alcohol	101.8	0.6
Methyl paraben	104.4	0.6
Ethyl paraben	103.6	0.4
Propyl paraben	103.4	1.0

*Best chromatogram mode: detection at 210 nm during the first 7 min; detection at 256 nm during the remainder of the chromatography run.
†Based on five replicates.

ing that the first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm to enhance the detection sensitivity, whereas the other parabens were detected at 256 nm. Figure 6 shows the chromatograms of SBWC separation of SC-EC3 skincare cream. It should be pointed out that the sensitivity of benzyl alcohol at 210 nm was enhanced approximately by 40-fold than that at 256 nm. The recoveries of preservatives contained in SC-EC3 skincare cream sample obtained by SBWC at 150°C are given in Table VII.

SBWC separations of preservatives at programmed temperature

Because the stability of the stationary phase of most reversed phase columns tend to decrease with increasing temperature, we also tested SBWC separation using programmed temperature started with lower temperature, 100°C, and then increased to 150°C in order to extend the life time of the column under subcritical water conditions. As shown in Fig. 7, reasonable separation of preservatives present in SC-EC3 skincare cream was achieved using this programmed temperature.

Study on potential SBWC system building-up

As the elution strength of pure subcritical water at 150°C is still relatively weak and it may not adequately clean the sample matrix from the separation column after each SBWC run, potential system building-up study was carried out using a large number of replicate injections of a single sample preparation under the same SBWC conditions. Based on the per cent recoveries of preservatives and relative standard deviations reported in Table VIII, no apparent system building-up occurred during the 21 replicate SBWC runs.

Waters XBridge phenyl column

All experiments described in this section for separation of preservatives on the Waters XBridge phenyl column were performed using the Shimadzu Nexera UFLC system. The injection volume was 10 μL for all runs.

SBWC separations of preservatives at 150°C

First, SBWC separation of preservatives was performed at isothermal condition of 150°C using constant flow rate of 1 mL min^{-1}. The best chromatogram mode was also used in this experiment. In this best chromatogram mode, 210 nm was used for the first 7 min to enhance the signal for benzyl alcohol. As methyl paraben

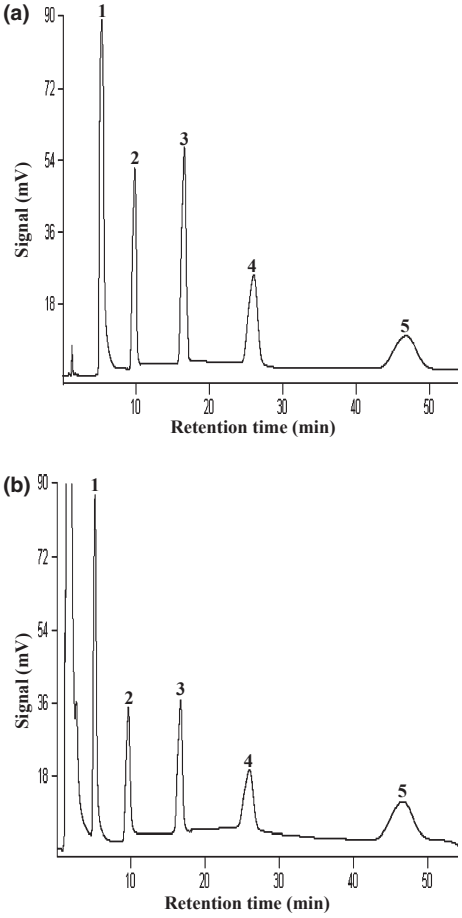

Figure 7 Subcritical water chromatography chromatograms obtained using 100% water on XBridge C18 column with programmed temperature at 1.0 mL min^{-1}. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, whereas the other parabens were detected at 256 nm. Programmed temperature: initial temperature of 100°C was increased to 150°C at 15°C min^{-1} and then maintained at 150°C. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben.

has similar molar absorptivity at both 210 and 256 nm, it was detected at 210 nm. After the first 7 min, 256 nm was used to detect the other parabens to achieve better sensitivity.

Figure 8 shows the chromatograms of the standard solution and SC-EC3 skincare cream obtained by SBWC on the Waters XBridge phenyl column at 150°C with 1 mL min^{-1} using the best chromatogram mode. Again, benzyl alcohol was well separated from the sample matrix. The quantitative data are shown in Table IX.

We also tried elution with programmed flow rates as described in Fig. 9 legend. Figure 9 shows the chromatograms of preservative standards and SC-EC-3 skincare cream sample obtained by SBWC at 150°C with programmed flow. Again, the best chromatogram mode was employed here. The recoveries of preservatives determined by this SBWC method are also given in Table IX.

Table VIII Recovery of preservatives present in SC-EC3 skincare cream obtained by subcritical water chromatography at 150°C with the best chromatogram mode resulted from the building-up studies

	%Recovery (%RSD*)	
	Waters XBridge C18 column†	Waters XBridge phenyl column‡
Benzyl alcohol	101.8 (1.2)	99.6 (1.8)
Methyl paraben	106.3 (1.6)	105.0 (1.7)
Ethyl paraben	101.0 (0.8)	99.9 (1.5)
Propyl paraben	102.8 (0.8)	98.3 (1.6)

*Based on 21 replicate injections of a single sample solution.
†Flow rate of 1.0 mL min^{-1}.
‡Programmed flow rate as described in Fig. 9 legend.

Once again, the potential building-up study was carried out using 21 replicate SBWC runs of a single SC-EC3 cream sample solution. The results shown in Table VIII demonstrate that building-up is not a concern.

SBWC separations of preservatives using programmed temperature
Additional experiments were performed using programmed temperature with initial temperature of 100°C in order to extend the life time of the column. Figure 10 shows the chromatograms of preservatives' standard solution and preservatives found in SC-EC3 skincare cream obtained by SBWC on the Waters XBridge phenyl column at 1 mL min^{-1} using programmed temperature. The initial temperature of 100°C was increased to 150°C at a rate of 15° C min^{-1}, and then the temperature was maintained at 150°C.

To further validate this particular SBWC method on the XBridge phenyl column, traditional HPLC separation using methanol–water gradient was carried out at 25°C. The quantitative results are given in Table X. Please note that the best chromatogram mode was also used in this HPLC experiment. As shown in Table X, the quantification quality obtained by this traditional HPLC method is at the same level as that obtained by our SBWC methods developed in this study, clearly showing that our SBWC methods are as good as the reliable HPLC methods.

Discussion and conclusions

The homemade chromatography system was used for the separation of preservatives on the two ZirChrom columns. Both HTLC and SBWC separations of preservatives were achieved on the ZirChrom-DiamondBond-C18 column at temperatures ranging from 150 to 200°C. While HTLC separation at 150°C still required methanol in the mobile phase, 75% methanol was saved when comparing with the methanol consumption in the traditional HPLC method for analysis of preservatives. The percentage of methanol saved can be further reduced with either increasing separation temperature or decreasing flow rate. Although separation of preservatives was also achieved by SBWC using pure water at 200°C, the benzyl alcohol peak was co-eluted with a sample matrix peak of the skincare creams.

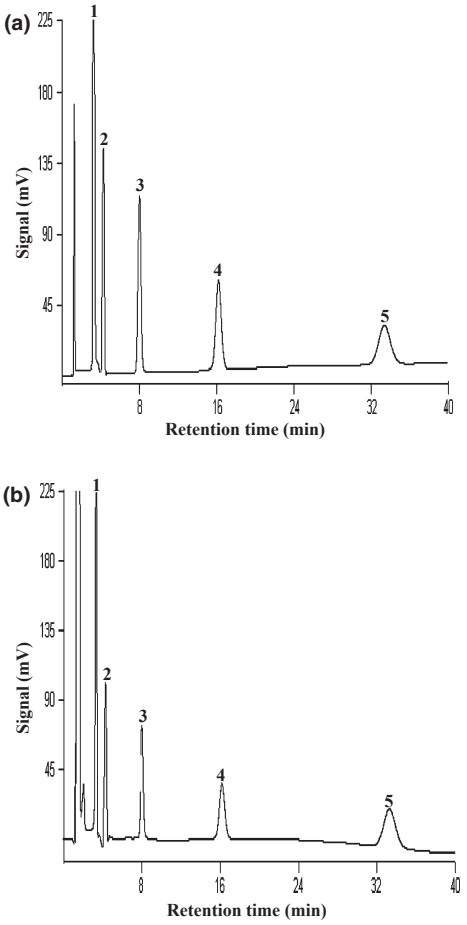

Figure 8 Subcritical water chromatography chromatograms obtained using 100% water on XBridge phenyl column at 150°C and 1.0 mL min^{-1}. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, whereas the other parabens were detected at 256 nm. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben.

Much faster SBWC separation was obtained using pure water at 180°C on the ZirChrom-PS column compared with the separation speed on the ZirChrom-DB-C18 column. Unfortunately, the benzyl alcohol peak was again co-eluted with the sample matrix.

The study was then switched to the Shimadzu Nexera UFLC system and using Waters XBridge columns. Good separations of preservatives were achieved on both XBridge C18 and XBridge phenyl columns using several HTLC and SBWC methods at temperatures lower than that required by the ZirChrom columns. These methods include elution with constant and programmed flow rates.

It should be pointed out that the XBridge columns used in the Shimadzu system offer several advantages compared with the ZirChrom columns used in the homemade system. First, the benzyl alcohol was well separated from the sample matrix using any HTLC and SBWC methods developed for both XBridge columns. Secondly, the temperature required (100–150°C) for efficient separation by the two XBridge columns is lower than what is

Table IX Recovery of preservatives present in SC-EC3 skincare cream obtained by subcritical water chromatography at 150°C using Waters XBridge phenyl column and the best chromatogram mode*

	%Recovery (%RSD†)	
Flow rate	**1 mL min^{-1}**	**Programmed Flow‡**
Benzyl alcohol	102.7 (1.1)	99.5 (2.5)
Methyl paraben	103.6 (0.6)	102.5 (2.2)
Ethyl paraben	105.1 (1.2)	101.2 (1.5)
Propyl paraben	107.3 (1.5)	101.6 (2.1)

*Best chromatogram mode: detection at 210 nm during the first 7 min; detection at 256 nm during the remainder of the chromatography run.
†Based on five replicates.
‡Programmed flow rate as described in Fig. 9 legend.

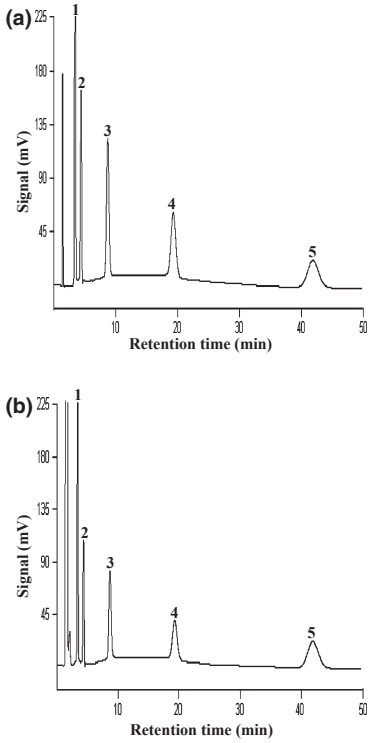

Figure 9 Subcritical water chromatography chromatograms obtained using 100% water on XBridge phenyl column at 150°C with programmed flow rates. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, whereas the other parabens were detected at 256 nm. Programmed flow rate: 0–6.5 min, decreased from 1.0 to 0.75 mL min^{-1}; 6.5–47 min, 0.75 mL min^{-1}; 47–50 min, increased from 0.75 to 1.0 mL min^{-1}. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben.

required (180–200°C) by the two ZirChrom columns. Thirdly, the XBridge HTLC methods saved more methanol (saved 90% methanol) than the ZirChrom HTLC methods (saved up to 75% methanol). Among the two XBridge columns, the phenyl column is less

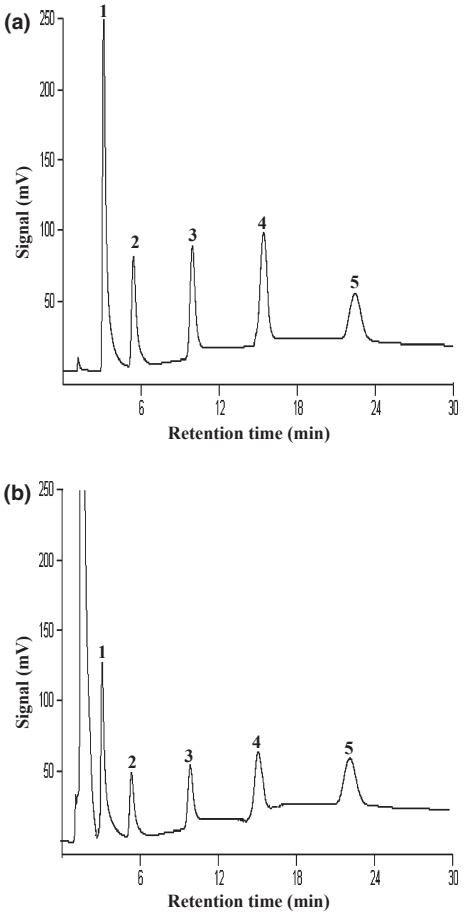

Figure 10 Subcritical water chromatography chromatograms obtained using 100% water on XBridge phenyl column using programmed temperature at 1.0 mL min^{-1}. (a) Preservatives standard mixture; (b) SC-EC3 skincare cream sample. Best chromatogram mode for UV detection: first two peaks (benzyl alcohol and methyl paraben) were detected at 210 nm, whereas the other parabens were detected at 256 nm. Programmed temperature: initial temperature of 100°C, then increased to 150°C at 15°C min^{-1} and then maintained at 150°C. Peak identification: 1, benzyl alcohol; 2, methyl paraben; 3, ethyl paraben; 4, propyl paraben; 5, butyl paraben.

Table X Recovery of preservatives present in SC-EC3 skincare cream obtained by traditional HPLC with ambient methanol–water mixtures as the eluent and Waters XBridge phenyl column

	%Recovery	%RSD*
Benzyl alcohol	101.7	0.6
Methyl paraben	104.8	0.7
Ethyl paraben	101.8	0.9
Propyl paraben	105.2	1.2

*Based on five replicates.

retentive than the C18 column and thus offers faster separation and analysis.

Quantitative analysis of preservatives was carried out using both homemade and commercial systems. Our results demonstrate that the preservative recoveries found by all HTLC and SBWC methods developed in this study are accurate and reproductive. A large number of replicate chromatographic runs also indicate that no system building-up occurred during the SBWC and HTLC runs. The separation and quantitative analysis results of SBWC and HTLC also compared very favourably with those of traditional HPLC, further confirming the reliability of the SBWC and HTLC methods.

As SBWC eliminates the use of methanol in the mobile phase, it is truly a green chromatographic separation technique. The evaluations of the SBWC methods developed using the Shimadzu commercial system indicate that the green SBWC technique may be applied to industrial plant settings for quantitative analyses. It should also be pointed out that there are numerous other existing industrial HPLC methods that may be replaced by the promising SBWC technique. Therefore, a significant amount of money spent on HPLC solvents and waste disposal can be saved for a company which is willing to adopt this green chromatography technique.

Acknowledgements

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Solubility of Parabens in Subcritical Water

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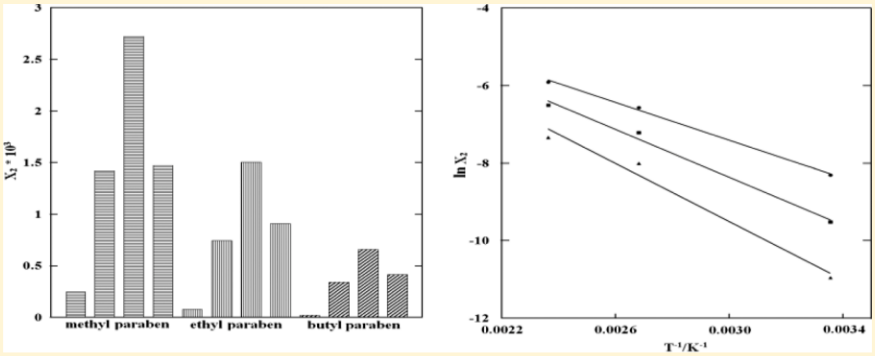

ABSTRACT: The solubility of methyl, ethyl, and butyl parabens (4-hydroxybenzoate) in water was determined at (298, 373, 423, and 473) K using a homemade heating/mixing system and by high performance liquid chromatography analysis. The solubility increased for all three parabens studied when water temperature was raised from 298 K to 423 K. However, the solubility decreased with a further temperature increase from 423 K to 473 K. This was due to the degradation of parabens at 473 K as revealed by a separate study. A new model was developed to guide the estimation of paraben solubility in subcritical water. The solubility values predicted using this new model compare reasonably well with our experimental values.

■ INTRODUCTION

Subcritical water has been used as a green separation fluid for both extraction[1−5] and chromatography.[6−9] Recently, chromatographic separations of preservatives, sunscreens, and niacinamide in skincare products using subcritical water as the mobile phase have been reported.[10−12] However, organic solubility in subcritical water is largely unknown. To promote and develop green subcritical water extraction and chromatography technologies, fundamental data such as the solubility of analytes in water at elevated temperatures are critically needed.

In this work, we studied the solubility of methyl, ethyl, and butyl parabens (4-hydroxybenzoate) in water at temperatures ranging from 298 K to 473 K. A homemade system was used to carry out the solubility experiments. The experimental solubility data of this work was then compared with values predicated using existing solubility models.[13−15] Unfortunately, none of these models[13−15] yields favorable predictions for solubility of all three paraben studied. Thus, we developed a new model that can reasonably predict the solubility of parabens in high-temperature water.

■ EXPERIMENTAL SECTION

Reagents and Materials. Methyl paraben, ethyl paraben, propyl paraben, and 2-phenoxyethanol were purchased from Sigma Aldrich (St. Louis, MO, USA). Butyl paraben was obtained from SAFC (St. Louis, MO, USA). High performance liquid chromatography (HPLC)-grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). The purity of the above-mentioned chemicals are summarized in Table 1.

Table 1. Purity and Sources of Materials

materials	mass fraction purity	analysis method	source
methyl 4-hydroxybenzoate	0.990	titration[a]	Sigma Aldrich
ethyl 4-hydroxybenzoate	0.998	GC[b]	Sigma Aldrich
propyl 4-hydroxybenzoate	0.999	GC[b]	Sigma Aldrich
butyl 4-hydroxybenzoate	0.998	GC[b]	SAFC
2-phenoxyethanol	0.999	GC[b]	Sigma Aldrich
methanol	0.998	GC[b]	Fisher Scientific

[a]Titration with sodium hydroxide. [b]Gas−liquid chromatography.

Deionized water (18 MΩ-cm) was prepared in our laboratory using a PURELAB Ultra system from ELGA (Lowell, MA, USA). GD/X PVDF membrane filters (0.45 μm) were received from Whatman (Florham Park, NJ, USA). An Adsorbosil C18 column (4.6 mm × 150 mm, 5 μm) was acquired from Alltech Associates, Inc. (Deerfield, IL, USA). An empty stainless steel tube (15 cm × 1.00 cm i.d. with 1.27 cm o.d.) and endfittings were purchased from Chrom Tech, Inc. (Apple Valley, MN, USA). Magnetic stir bar (2.2 cm × 0.6 cm) was obtained from Bel-Art Products (Pequannock, NJ, USA). A ring magnet (1.9

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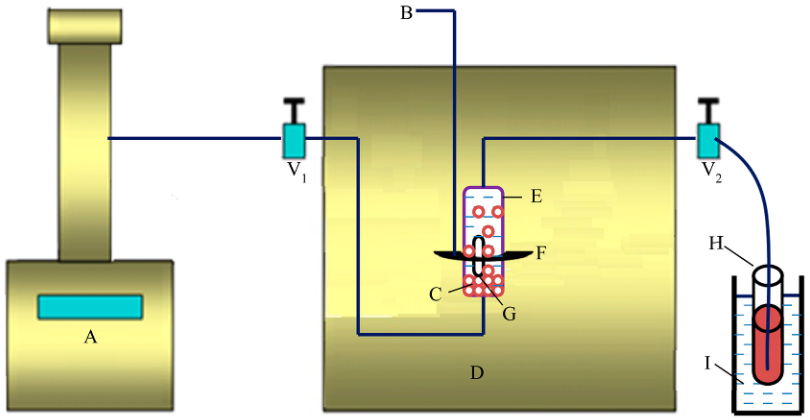

Figure 1. Schematic diagram of the heating/mixing system: A, water pump; B, mixing handle; C, paraben particles; D, oven; E, equilibration vessel; F, ring magnet; G, magnetic stir bar; H, collection vial; I, iced water bath; V_1 and V_2, high pressure valves.

Table 2. Gradient Conditions for HPLC Separation of Parabens

samples at (298, 373, 423) K						samples at 473 K	
methyl paraben		ethyl paraben		butyl paraben		methyl, ethyl, butyl paraben	
time/min	MeOH/%	time/min	MeOH/%	time/min	MeOH/%	time/min	MeOH/%
0	40	0	60	0	70	0	50
9	50	5	67	7	80	8	50
10	50	6	60	8	70	9	80
11	40	10	60	10	70	13	80
12	40					14	50
						16	50

cm i.d.) was acquired from AMF Magnetics (Botany, NSW, Australia).

Preparation of Solutions. An internal standard (2-phenoxyethanol) solution was prepared by adding approximately 0.2 g (accurately weighed) of 2-phenoxyethanol to a 100-mL volumetric flask. The mixture was then diluted to the mark with methanol. For solubility studies at 473 K, degradation of parabens was observed. Unfortunately, one of the degradants was coeluted with 2-phenoxyethanol, and thus propyl paraben was used as the internal standard. This propyl paraben solution was prepared by adding approximately 0.2 g (accurately weighed) of propyl paraben to a 100-mL volumetric flask, and then the mixture was diluted to the mark with methanol.

An individual stock solution was prepared for each of the parabens by adding approximately 0.01 g (accurately weighed) of a paraben to a 10-mL volumetric flask, and then the mixture was diluted to the mark with methanol. Calibration standard solutions were prepared from this stock solution and an appropriate internal standard solution using methanol as the solvent.

Heating and Mixing of Water−Paraben Mixtures. Solubility experiments were carried out using our solubility measuring system as shown in Figure 1. One end of a stainless steel vessel was sealed using an endfitting. Approximately 1 g of a paraben (methyl, ethyl, or butyl paraben) was added to the vessel. A magnetic stir bar was then placed inside the vessel. A ring magnet was attached to the outside of the vessel in order to mix the water−paraben mixture during heating. The open end of the vessel was sealed with another endfitting. Both endfittings were then tightened with wrenches. The loaded vessel was connected to an ISCO 260D syringe pump (Lincoln, NE, USA) and placed inside a gas chromatograph (GC) oven (HP 5890 Series 2, Hewlett-Packard, Avondale, PA, USA) as shown in Figure 1.

The loaded vessel was filled with deionized water through the pump with V_1 opened and V_2 closed. The oven was then turned on and set to a desired temperature. Please note that there is a delay time between the actual oven temperature and the displayed oven temperature. This delay time varies with the set temperature, ranging from 12 min for 373 K to 20 min for 473 K. Therefore, we started counting the heating time after the delay time. During the heating time, the mixture inside the equilibration vessel was mixed by moving the mixing handle as shown in Figure 1. A pressure of 0.5 MPa to 3.5 MPa was applied to experiments at 298 K to 473 K. After 60 min of heating and mixing, approximately 1.5 mL (accurately measured) of the saturated mixture was collected into a 10-mL volumetric flask by opening V_2. To this volumetric flask, 1.00 mL of internal standard solution was added and then diluted to the mark with methanol. This solution was filtered through a 0.45 μm Whatman GDX filter into a clean suitable glass vial prior to chromatographic analysis. Triplicate solubility experiments were carried out for each paraben at all temperatures tested.

HPLC Analysis. A Shimadzu Nexera UFLC system with a UV−vis detector (Shimadzu Corporation, Chiyoda-ku Tokyo, Japan) was employed for quantitative analysis of the parabens. Analyte detection was done at 256 nm. Separation of parabens was achieved on the Adsorbosil C18 column using water−methanol mixtures at 1 mL/min. For each paraben (methyl, ethyl, or butyl paraben) a different gradient elution was used for samples collected at temperatures lower than 473 K. But because of the degradation of parabens at 473 K, a separate

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gradient was developed for all paraben−water mixtures collected at 473 K. All four gradient conditions are summarized in Table 2.

RESULTS AND DISCUSSION

Effect of Temperature on the Solubility of Parabens. The solubility of all three parabens, methyl, ethyl, and butyl parabens, was measured at four different temperatures, (298, 373, 423, and 473) K. Table 3 shows paraben solubility measured at room temperature in comparison with literature values. As one can see, our experimental values compare reasonably well with that reported by other researchers.[16−19] The solubility in mole fraction obtained by our method at all four temperatures is given in Table 4. It is clear that the solubility of all three parabens increased when the temperature was raised from 298 K to 423 K. The solubility increase ranges from 6-fold to 19-fold with temperature increase from 298 K to 373 K. Another 2-fold increase was observed by further increasing the temperature from 373 K to 423 K as shown in Table 4. However, further increasing the temperature from 423 K to 473 K caused the solubility to decrease. Please note that this abnormal decrease in solubility with increasing temperature was caused by a severe degradation of parabens occurring at 473 K.[20] Our separate study on the stability of parabens in high-temperature water confirmed that a high percentage of methyl, ethyl, and butyl parabens decomposed in water at 473 K. It should be pointed out that the degradation of organics in high-temperature water is not unusual since it has been reported that polycyclic aromatic hydrocarbons as well as benzoic acid and its derivatives underwent degradation in subcritical water.[21,22]

Table 3. Comparison of Paraben Solubility[a] at 298 K Obtained by This Method and Reference Values

	solubility, mole fraction × 10^3				
	this work	ref 16	ref 17	ref 18	ref 19
methyl paraben	0.25	0.25	0.29	0.30	0.26
ethyl praben	0.074	0.13	0.10	0.18	0.096
butyl paraben	0.018	0.015	0.019	0.019	0.023

[a]All parabens are in the solid phase.

Predicting Solubility of Parabens in Subcritical Water. Because all three parabens significantly degrade at 473 K as discussed earlier, the "solubility" data obtained at 473 K are false, and hence excluded in developing a solubility model. We first used the zeroth approximation model (eq 1) developed by Miller et al.[13] to predict the paraben solubility. As shown in eq 1, the mole fraction solubility at higher temperatures $x_2(T)$ can be predicted with the knowledge of the mole fraction solubility at ambient temperature $x_2(T_o)$. The solubility values computed using eq 1 are given in Table 5. While eq 1 yields good predictions for methyl paraben solubility, the predicted values are lower than the experimental data for both ethyl and butyl parabens at higher temperatures.

$$\ln x_2(T) \approx \left(\frac{T_o}{T}\right) \ln x_2(T_o) \tag{1}$$

We then tried the first approximation model also developed by Miller et al. as described by eq 2.[13] As shown in Table 5, the values predicted by eq 2 are much higher than the experimental values at 423 K.

$$\ln x_2(T) = \left(\frac{T_o}{T}\right) \ln x_2(T_o) + 15\left(\frac{T}{T_o} - 1\right)^3 \tag{2}$$

Another approximation model (eq 3) was developed in our lab to predict the solubility of alkylbenzenes in subcritical water.[14] As shown in Table 5, this model also failed to predict paraben solubility in subcritical water.

$$\ln x_2(T) = \left(\frac{T_o}{T}\right) \ln x_2(T_o) + 2\left(\frac{T - T_o}{T_o} - 1\right)^3 \tag{3}$$

We also tried to predict the solubility of parabens using a model developed originally for the solubility of organic acids in high-temperature water.[15] The mole fraction solubility of parabens predicted using this model (eq 4) is also included in Table 5. One can clearly see that this model does not work at all for any of the parabens.

$$\ln x_2(T) = \left(1.85\frac{T_o}{T} - 1\right) \ln x_2(T_o) \tag{4}$$

It is obvious that a new model needs to be developed in order to predict the solubility of parabens in subcritical water. After careful examination of the temperature influence on paraben solubility as shown in Figure 2, it can be seen that the linear correlation decreases with increasing number of carbon atoms in the alkyl group of parabens. Therefore, we introduced eq 5 to compensate for the deviation possibly caused by the molecular structure of solutes. Please note that C in eq 5 is the number of carbon atom(s) in the alkyl group of the parabens. As shown in Table 5, this newly developed model can reasonably predict all three parabens solubility at temperatures up to 423 K.

$$\ln x_2(T) = \left(\frac{T_o}{T}\right) \ln x_2(T_o) + 0.5(C - 1)\left(\frac{T}{T_o} - 1\right) \tag{5}$$

Table 4. Solubility of Parabens Found in Water−Paraben Mixtures after Heating at Each Temperature for 60 min

	mole fraction × 10^3 (u)[a]			
temperature/K	298	373	423	473[b]
pressure/MPa	0.5	1.5	2.5	3.5
methyl paraben	0.25 (0.006)[c]	1.4 (0.1)[c]	2.7 (0.2)[d]	1.5 (0.05)[d]
ethyl paraben	0.074 (0.004)[c]	0.74 (0.05)[c]	1.5 (0.1)[d]	0.91 (0.04)[d]
butyl paraben	0.018 (0.0006)[c]	0.34 (0.006)[d]	0.65 (0.05)[d]	0.41 (0.03)[d]

[a]Standard uncertainties based on triplicate measurements. [b]Degradation of all three parabens occurred at 473 K. [c]Parabens in the solid phase. [d]Parabens in the liquid phase.

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Table 5. Comparison of Experimental Solubility of Parabens with Values Predicted by eqs 1 to 5

		mole fraction × 10^3					
temp/K		exptl	eq 5	eq 4	eq 3	eq 2	eq 1
methyl paraben	298	0.25	0.25	0.87	0.034	0.25	0.25
	373	1.4	1.3	19	0.57	1.7	1.3
	423	2.7	2.9	81	2.0	8.8	2.9
ethyl paraben	298	0.074	0.074	0.31	0.010	0.074	0.074
	373	0.74	0.57	11	0.22	0.64	0.50
	423	1.5	1.5	56	0.83	3.7	1.2
butyl paraben	298	0.018	0.018	0.093	0.0024	0.018	0.018
	373	0.34	0.24	5.4	0.070	0.21	0.16
	423	0.65	0.85	36	0.31	1.4	0.45

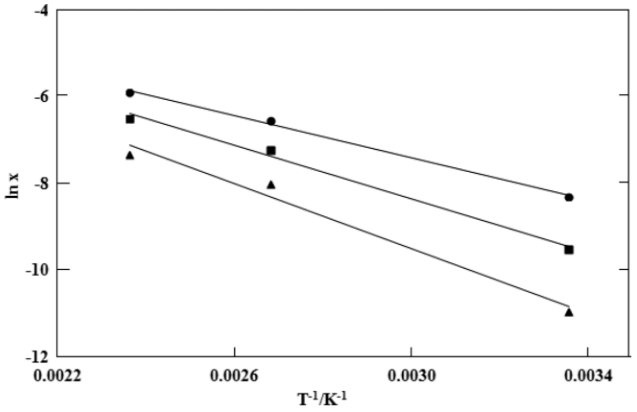

Figure 2. Temperature influence on paraben solubility in subcritical water: ●, methyl paraben; ■, ethyl paraben; ▲, butyl paraben.

■ CONCLUSIONS

The solubility of methyl, ethyl, and butyl parabens in water was enhanced with increasing temperature. When water temperature was raised from 298 K to 373 K, the solubility increase was significant, ranging from 6-fold to 19-fold. Further increasing the temperature from 373 K to 423 K yielded another 2-fold solubility increase. However, severe degradation of all three parabens studied occurred at 473 K. Because of the decomposition of solutes, the experimental solubility of parabens decreased when the temperature was raised from 423 K to 473 K. An approximation model developed in this work can better predict paraben solubility in high-temperature water than other models published earlier.

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