Technical Review

High–Speed Analyses using Rapid Resolution Liquid Chromatography on ZORBAX column packed 1.8 μm Particles

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Abstract

The potential of high–speed analyses by Rapid Resolution Liquid Chromatography (RRLC) and RRLC/mass spectrometry (MS) on 1.8 μm porous particles packed into short columns operated at high flow rate was investigated and compared to the performance of 5 μm porous particles packed into conventional columns. Using similar chemistries, the ease of conversion from conventional HPLC to RRLC method was demonstrated. In order to display the practicality of RRLC separations, the isocratic analysis of pesticides and the gradient analysis of catechins in Japanese Green Tea were selected. In isocratic analysis, compared to the conventional method, the time could be reduced by a factor of 31 at the highest flow rate. In gradient elution, the fastest separation was achieved by RRLC on 50 mm×4.6 mm i.d. or ×2.1 mm i.d. RRLC column packed 1.8 μm particles. The analysis time was reduced by a factor of 15, compared to the conventional method.

Keywords: Rapid Resolution Liquid Chromatography, High throughput, Fast LC, sub–micron, 1.8 μm, pesticide, cathechin

1. Introduction

Over the years, separation resolution and reduction of analysis time has continually improved in High Performance Liquid Chromatography (HPLC). For further improvement, column efficiency must be increased. The relationship among separation efficiency, the mobile phase linear velocity and particle size was investigated in detail in the early 1970 s [1]. This and other systematic investigations have led to high–throughput and high–resolution HPLC that we know today. The major advantages of high–throughput HPLC are the increase in throughput and the reduction in the analysis cost. The shortening in analysis time is due to the use of a shorter column length. However, a shorter column may lead to a loss of theoretical plates, hence a decrease in chromatographic resolution that may be required for a complex mixture of compounds. To offset the potential loss of resolution, the use of smaller size particles has resulted in more efficient columns. Further, ultra high–pressure liquid chromatography using a capillary column packed 1–1.5 μm non–porous silica particles was introduced in 1997 [2]. Since then, HPLC using smaller particles has become more popular.

In this paper, the technique of the use of small particles (sub–2 micron) packed into short columns run at a high flow rate has been termed Rapid Resolution Liquid Chromatography (RRLC). Recently, RRLC analysis has become a routine method in the pharmaceutical industry. However, such a fast analysis technique is not only for the pharmaceutical field. In this study, we apply to the fast RRLC technique to a more general field in the analysis of chemical compounds. Today, the multi–residue screening method of pesticides established by the Japanese Food Hygiene Law method [3, 4] use a 250 mm×4.6 mm i.d. column packed with 5 μm octadecyl (C 18) silica gel used in the isocratic elution mode. These columns typically produce an analysis time of 30 min. However, with new

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RRLC technology, analysis time can be significantly reduced without losing chromatographic resolution. In addition, gradient elution RRLC has been applied for the analysis of nutritional components (catechins) in green tea using the same approach.

In this study, we had two goals: 1) to demonstrate the ease of conversion of a traditional HPLC method to a RRLC method and 2) to investigate the potential of RRLC and RRLC/MS on 1.8 μm porous particles packed into short columns operating at a high flow rate compared to the performance of 5 μm porous particles packed conventional columns.

2. Experimental

2.1. Apparatus

All analyses were performed on an Agilent 1200 Series Rapid Resolution LC system (1200 RRLC system; Agilent Technologies, Hachioji–shi 1–9, Tokyo, Japan) equipped with on–line degasser, high–pressure binary pump, autosampler with temperature–controlled sample compartment, thermostated column compartment, and photodiode array detector (DAD). For data processing and acquisition, Chemstation 01.03 (Agilent Technologies) was used. The LC/MS system included the 1200 RRLC / 1100 MSD – liquid chromatograph / mass spectrometer, and the LC/MS system was operated in an external injection mode. In general, conventional DAD analysis with the 1200 RRLC system was performed with a normal instrument plumbing configuration, which has an internal system volume of approximately 600–800 μL. Components installed included internal tubing connections with 0.17 mm i.d. capillary tubing, a 400 μL static mixer, damper (pressure dependent volume ranging from 80 to 280 μL), and 10 mm path length DAD flow cell (13 μL). For RRLC analysis using 2.1 mm i.d. column, studies were performed on a 1200 system with lowest delay volume configuration that had an internal volume of approximately 120 μL. Components installed included internal tubing connections with 0.12 mm i.d. capillary tube and a 6 mm path length DAD flow cell (5 or 1.7 μL) and without the static mixer and damper.

2.2. Reagents

HPLC grade acetonitrile (MeCN) and methanol (MeOH), pesticides standards (Diflubenzuron, Tebufenozide, Hexaflumuron, Teflubenzuron, Lufebenzuron, Flufenoxuron, and Chlorluazuron), catechin standards (Gallic acid, Gallocatechin, Epigallocatechin, Catechin, Caffeine, Epicatechin, Epigallocatechin gallate, Gallocatechin gallate, Epicatechin gallate, and Catechin gallate), alklyphenons, (acetanilide, acetophenon, propiophenon, butyrophenon, benzophenon, valerophenon, hexanophenon, heptanophenon, and octanophenon) were purchased from Wako Pure Chemical Industries, Co., Ltd (Osaka, Japan). All other reagents used were of the highest grade commercially available. Japanese Green Tea was also purchased commercially at a local supermarket.

ZORBAX columns with 600 bar upper pressure limit were purchased from Agilent Technologies (Santa Clara, CA, USA).

HPLC grade water was prepared by Milli–Q system (Millipore, Yokohama, Japan).

2.3. Chromatographic conditions

2.3.1. RRLC/DAD separation using Isocratic elution

Pesticides standards were dissolved using acetone or MeOH and diluted with MeOH. For Japanese Food Hygiene Law method, the separation of phenyl urea pesticides was performed on ZORBAX Eclipse XDB–C18 (250 mm×4.6 mm i.d., 5 μm) analytical column using the DAD detector set at a wavelength of 250 nm. The flow rate was 0.7 mL/min with the column compartment temperature set at 40℃ and an injection volume of 20 μL. Solvent A and solvent B were water and MeCN, respectively. The isocratic elution condition was A/B (3/7).

For RRLC/DAD method, the pesticide separation was performed on a ZORBAX Eclipse XDB–C18 Rapid Resolution HT (RRHT) (50 mm×4.6 mm i.d., 1.8 μm) analytical column with the same detection conditions. The flow rate was 0.7 mL/min with a column compartment temperature was set at 40℃ and an injection volume of 4 μL was used. The mobile phase was the same as above.

2.3.2. RRLC/MS approach using Isocratic elution

For RRLC/MS method, the pesticide separation was performed ZORBAX Eclipse XDB–C18 RRHT (50 mm×2.1 mm i.d., 1.8 μm) analytical column. The flow rate was 1.3 mL/min with a column compartment temperature of 40℃ and an injection volume of 0.1 μL. Solvent A and solvent B were 5 mM ammonium formate in water and MeCN, respectively. The isocratic elution condition was A/B (3/7). The LC/MS ionization was performed by Electro–Spray Ionization (ESI). Typical operating conditions of the ESI interface in negative mode were as follows: vaporized temperature, 350℃; nebulizer gas, nitrogen, at a of pressure of 50 psi; drying gas, also nitrogen, at a flow rate 10 L/min and capillary voltage, –3500 V. ESI in negative mode produces the $[M–H]^-$ molecular adduct ion. Quantitative analyses was performed by selected ion monitoring (SIM) mode. The monitoring m/z were as follows: 1) Diflubenzuron (m/z=309); 2) Tebufenozide (m/z=351); 3) Hexaflumuron (m/z=459); 4) Teflubenzuron (m/z=379); 5) Lufebenzuron (m/z=509); 6) Flufenoxuron (m/z=487); and 7) Chlorluazuron (m/z =540).

2.3.3. RRLC/DAD separation using Gradient elution

Catechin standards for identification were dissolved in and diluted with HPLC grade water. The Japanese green tea sample was diluted four fold using HPLC grade water.

The separation of the Japanese green tea sample was performed on three different ZORBAX SB–C18 (150 mm×4.6 mm i.d., 5 μm), (75 mm×4.6 mm i.d., 3.5 μm) and SB–C18 RRHT (50

mm×4.6 mm i.d., 1.8 μm) analytical columns and with a DAD wavelength setting of 210 nm. The flow–rate was 1.0 or 5.0 mL/min with a column compartment temperature of 40℃ and an injection volume of 5 μL. Solvent A and solvent B were 0.1 % phosphoric acid in water and MeCN, respectively. For the 150 mm×4.6 mm i.d. column, the gradient elution condition was: initial concentration of 10 % B with programming to 15 % B over 7.5 min, and to 27 % B over 7.5 min. Then, the column was equilibrated under the initial condition for 10 min prior to next injection. For 75 mm×4.6 mm i.d. size column, the gradient condition was 10–15 %B in 3.75 min, 15–27 %B in 3.75 min, and 5 min re–equilibration time at the initial condition. For 50 mm×4.6 mm i.d. size column, the condition was 10–15 %B in 2.5 min, 15–27 %B in 2.5 min, and 3 min re–equilibration time. For the high–throughput analysis at 5 mL/min flow rate using 50 mm×4.6 mm i.d. column, the condition was 10–15 %B in 0.5 min, 15–27 %B in 0.5 min, and 0.6 min re–equilibration time. For 50 mm×2.1 mm i.d. column at 0.21 or 1.05 mL/min, the gradient segments were the same conditions as that for the 50 mm×4.6 mm i.d. column.

2.3.4. RRLC/MS approach using Gradient elution

For RRLC/MS method, the catechins separation was performed on a ZORBAX SB–C18 RRHT (50 mm×2.1 mm i.d., 1.8 μm) analytical column. The flow–rate was 1.05 mL/min with a column compartment temperature of 40℃ and an injection volume of 0.5 μL. Solvent A and solvent B were 0.2 % formic in both water and MeCN, respectively.

The ESI conditions were as follows: vaporized temperature, 350℃; nebulizer gas, nitrogen, at a of pressure of 50 psi; drying gas, also nitrogen, at a flow rate 10 L/min and capillary voltage, –3500 V of negative and 3500 V of positive. ESI of catechins in negative mode produces the $[M–H]^-$ molecular adduct ion in the Caffeine in positive mode produces the $[M+H]^+$ molecular adduct ion. The monitoring m/z were as follows: 1) Gallic acid, m/z=169; 2) Gallocatechin and 3) Epigallocatechin, m/z=305; 5) Caffeine m/z=195; 5) Catechin and 6) Epicatechin, m/z=289; 7) Epigallocatechin gallate and 8) Gallocatechin gallate, m/z=457; 9) Epicatechin gallate and 10) Catechin gallate, m/z=441.

2.3.5. Analytical precision under the fastest RRLC condition

Alkylphenone standards were dissolved using acetone or MeOH and diluted with MeOH.

The analytical precision of the alkylphenones was tested on ZORBAX SB–C18 RRHT (50 mm×2.1 mm i.d., 1.8 μm) analytical column and on the DAD wavelength setting of 245 nm. The flow rate was 1.3 mL/min with a column compartment temperature of 60℃ and an injection volume of 1 μL. Solvent A and solvent B were 0.1 % triflouroacetic acid (TFA) in water and 0.1 % TFA in MeCN, respectively. The gradient elution condition was as follows: initial concentration 35 %B with programming to 95 %B over 0.7 min and an isocratic hold for 0.2 min. The column was then equilibrated at the initial condition for 0.4 min prior to next injection.

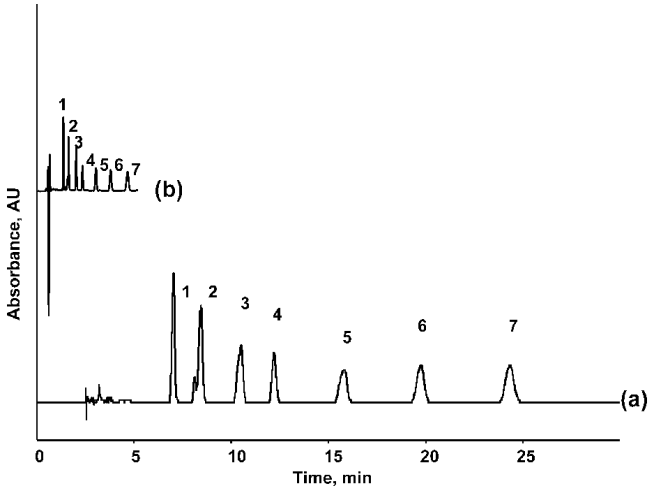

Figure 1. The simultaneous separation of the phenyl urea pesticides. Shown are separations of phenyl urea pesticides standard on (a)ZORBAX XDB–C18 (250 mm×4.6 mm i.d., 5 μm) and (b) ZORBAX Eclipse XDB–C18 RRHT (50 mm×4.6 mm i.d., 1.8 μm) analytical column. The Peaks, in order of elution, are 1= Diflubenzuron, 2=Tebufenozide, 3=Hexaflumuron, 4=Teflubenzuron, 5= Lufebenzuron, 6=Flufenoxuron, and 7=Chlorluazuron. Detail conditions are shown in text.

3. Results and discussion

When converting a conventional HPLC method to a fast or ultrafast HPLC method, the following parameters may require optimization: analysis time, gradient time and slope, flow rate and injection volume. The detail method transfer consideration was described in Ref [5, 6].

3.1. RRLC Separation

3.1.1. RRLC/DAD separation using Isocratic elution

These pesticides are widely used in agriculture for a large number of crops. The detection of their residues in food has caused a great deal public concern. The multi–residue screening method of these phenyl urea pesticides has been established by Japanese Food Hygiene Law method [3, 4]. In this conventional method, an isocratic separation has been developed using a 250 mm×4.6 mm i.d. column packed 5 μm octadecyl silica gel and the MeCN/H_2O (7/3) solution as a mobile phase. These phenyl urea pesticides have the positive charge in acidic aqueous solution because they have amine groups in the each compound. Therefore, these compounds could interact with the surface silanols on silica gel and cause the peak tailing. The ZORBAX Eclipse XDB–C18 column (250 mm×4.6 mm i.d., 5 μm), thoroughly endcapped to cover active silanols, was used for the separation of these pesticides. Fig.1 (a) shows the

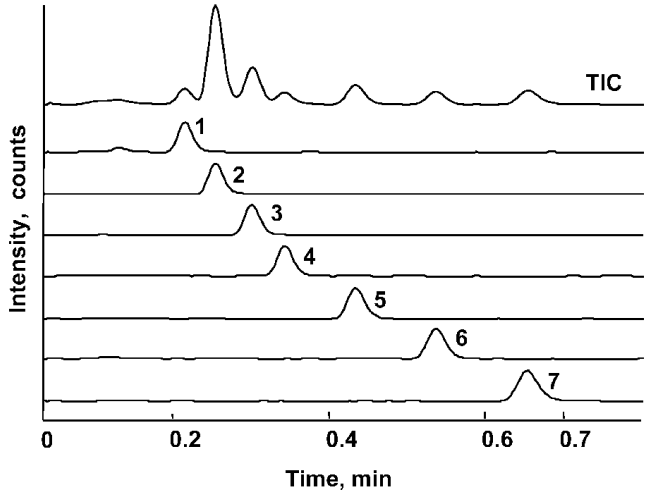

Figure 2. The ultrafast RRLC/MS of the phenyl urea pesticide standard.
The phenyl urea pesticides standard was separated using ZORBAX Eclipse XDB–C18 RRHT(50 mm×4.6 mm i. d., 1.8 μm) analytical column at 1.3 mL/min. The top chromatogram is the negative total ion chromatogram (TIC). The other chromatograms are obtained by the negative SIM mode. The peaks, in order of elution, are 1= Diflubenzuron (m/z=309), 2=Tebufenozide (m/z= 351), 3=Hexaflumuron (m/z=459), 4=Teflubenzuron (m /z=379), 5=Lufebenzuron (m/z=509), 6=Flufenoxuron (m/z=487), and 7=Chlorluazuron (m/z=540). Detail conditions are shown in text.

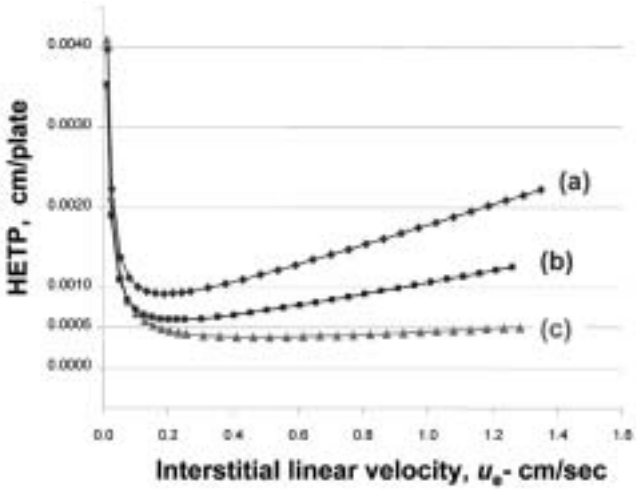

Figure 3. HETP versus linear velocity for small particle spherical bonded silica column [5].
Shown is a series of van Deemter curves for (a)5, (b)3.5 and (c)1.8 μm bonded spherical bonded silica column. The column (a), (b) and (c) is ZORBAX XDB–C18 (30 mm×4.6 mm i.d., 5 μm), ZORBAX XDB–C18 (30 mm ×4.6 mm i.d., 3.5 μm) and ZORBAX XDB–C18 RRHT (30 mm×4.6 mm i.d., 1.8 μm) analytical column, respectively. The solute is octanophenone and the mobile phase is 85:15 MeCN/water. The flow rate range is 0.05 mL (1.8 mm/sec) – 5.0 mL/min (18 mm/sec); temperature: 20℃.

25 min chromatogram of the seven standard phenyl urea pesticides.

Fig.1 (b) shows the 5 min chromatogram for the separation of the seven standard phenyl urea pesticide using the 50 mm×4.6 mm i.d. ZORBAX Eclipse XDB–C18 RRHT column packed with 1.8 μm particle size octadecyl silica gel. This column and demonstrates the same chemistry as the ZORBAX 3.5 μm and 5 μm bonded silica particles. As a result, both columns packed with 5 and 1.8 μm particles provided identical selectivity. RRLC method in isocratic elution could easily replace the conventional regulated method.

3.1.2. RRLC/MS approach using Isocratic elution

The RRLC/MS separation of same pesticide mixture was attempted and the chromatogram of standard sample is shown in Fig.3. In this case, a narrow bore ZORBAX Eclipse XDB–C18 RRHT (50 mm×2.1 mm i.d., 1.8 μm) column was used. In this experiment, the flow rate in this experiment was reduced to 1.3 mL/ min (23.4 mm/sec) for a resultant column backpressure of 550 bar. The linear velocity (23.4 mm/sec) on 2.1 mm i.d. column would correspond to the 8 mL/min on the 4.6 mm i.d. column. The same seven phenyl urea pesticides could be successfully separated using this narrow bore column at this high flow rate. In this case, the total analysis time by RRLC/MS method was only 0.7 min on the ZORBAX Eclipse XDB–C18 RRHT (50 mm×2.1 mm i.d, 1.8 μm) column without a loss in resolution. Furthermore, this analysis time by RRLC/MS method is 31 times faster than that by the method of Japanese Food Hygiene Law method. RRLC coupled with MS showed excellent experimental results in term of resolution, selectivity and sensitivity.

In a series of isocratic experiments, it is concluded that the RRLC method could achieve a faster separation using the shorter column packed with the 1.8 μm particles at a high flow rate without loss in resolution.

The reason that the rapid, high–resolution separation is maintained at a high flow rate is evident from van Deemter analysis. The separation efficiency H (height equivalent to theoretical plate, HETP) in micrometers as a function of mobile phase velocity is described by the van Deemter equation, shown simplistically in Equation 1.

$$H=A+B/\mu+C\mu \qquad (1)$$

where A, B, and C are constants and μ is the mobile phase linear velocity (proportional to flow rate), measured in centimeters per second. The A term is a measure of the packing efficiency and is a function of packing efficiency and particle size. The B term is a function of longitudinal diffusion, or diffusion in the mobile phase, and the C term is a function of the mass transfer between the stationary and mobile phase as well as within the mobile phase.

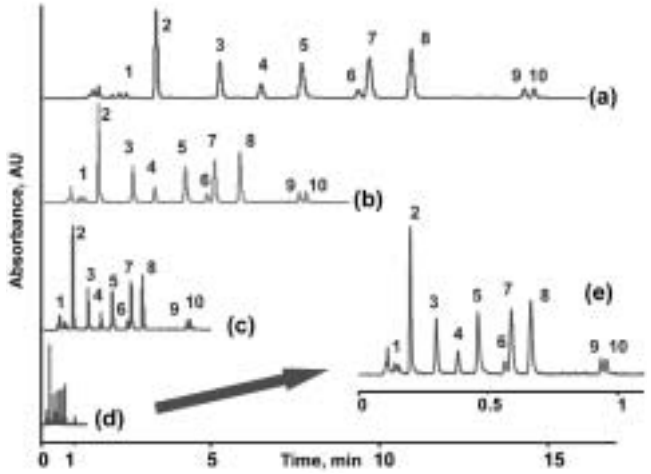

Figure 4. The separation of catechins in the Japanese Green tea on the three columns.
The chatechins in the Japanese Green tea were performed using (a) ZORBAX SB–C18 (150 mm×4.6 mm i.d., 5 μm), (b) ZORBAX SB–C18 (75 mm×4.6 mm i. d., 3,5 μm), and (c) ZORBAX SB–C18 RRHT (50 mm ×4.6 mm i.d., 1.8 μm) column at 1.0 mL/min. The chromatogram (d) are obtained on ZORBAX SB–C18 (50 mm×4.6 mm i.d., 1.8 μm) column at 5.0 mL/min. The chromatogram (e) is enlarged in a separation window. The Peaks, in order of elution, are 1=Gallic acid, 2= Gallocatechin, 3=Epigallocatechin, 4=Catechin, 5=Caffeine, 6=Epicatechin, 7=Epigallocatechin gallate, 8= Gallocatechin gallate, 9=Epicatechin gallate, and 10= Catechin gallate.

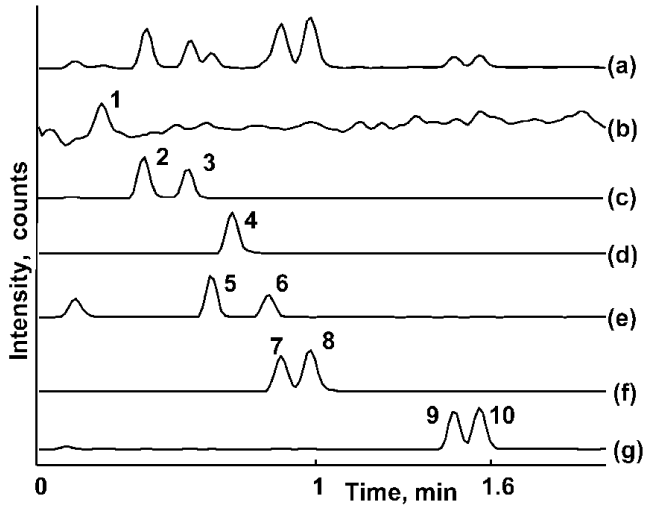

Figure 5. The ultrafast RRLC/MS of catechins in the Japanese Green tea.
The catechins in the Japanese Green tea were separated on ZORBAX SB–C18 RRHT (50 mm×2.1 mm i.d., 1.8 μm) column at 1.05 mL/min. The chromatogram (a) is the negative TIC. The chromatogram (b), (c), (d), (e), (f) and (g) is obtained by the SIM mode, (b) m/z=169, (c) m/z=305, (d) m/z=195, (e) m/z=289, (f) m/z=457 and (g) m/z=441. The peaks, in order of elution, are 1= Gallic acid, 2=Gallocatechin, 3=Epigallocatechin, 4= Catechin, 5=Caffeine, 6=Epicatechin, 7=Epigallocatechin gallate, 8=Gallocatechin gallate, 9=Epicatechin gallate, and 10=Catechin gallate.

Within the C term, there is also a proportional dependency of the particle diameter squared. Another fallout of the decrease in particle size is that the van Deemter curves tend to flatten out at higher linear velocities and the minimum shifts toward the right. Fig. 3 shows a series of van Deemter curves for 5, 3.5 and 1.8 μm bonded spherical silica columns. Researchers can easily see that the column packed with 1.8 μm particles gives a flatter curve at high linear velocity than the 5 μm column [5]. Thus, these column can be run at faster flow rates (linear velocities) and the chromatographic peaks maintain their efficiency yet the separation time decreases proportional to the increase in flow rate.

3.1.3. RRLC/DAD separation using Gradient elution

A comparative study of the chromatography of catechins in Japanese green tea on three different columns packed with 5, 3.5, and 1.8 μm reversed phase C18 bonded material is depicted in Fig. 4. The theoretical plate number among the three columns is almost same. The results for the ZORBAX SB–C18 (150 mm × 4.6 mm i. d.) column packed with 5 μm particles (Fig. 4(a)) are typical for this standard column that is routinely used in many HPLC laboratories. On this conventional column, the catechins could be completely separated as shown in Fig. 4(a). This gradient method required a total of 15 min analysis time. Fig. 4(b) shows the chromatogram obtained using a ZORBAX SB–C18 (75 mm × 4.6 mm i. d.) column packed with 3.5 μm particles. If the internal column diameter among three columns is the same, the gradient time is proportional to the column. In this case, as the column length is halved, each gradient time should be half (i.e. 1st segment, B 10 % (5 min) to B 10 %(2.5 min) and 2nd segment, B 27%(15 min) to B 27 %(7.5 min)). Even switching to a 75 mm column reduced the total time to just over 7.5 min (Fig. 4(b)). Fig. 4(c) shows the chromatogram separated using a ZORBAX SB–C18 RRHT (50 mm × 4.6 mm i.d.) column packed with 1.8 μm particles. The same separation could be achieved on the column that was only 50 mm in length but in a third of the time – less than 5 min. The flow rate among Figs. 4(a)–(c) was 1 mL/min (3.6 mm/sec). Using the shorter column length can save the analysis time and solvent.

Next, an ultra fast separation at a high flow rate (5 mL/min = 18 mm/sec) was attempted. As mentioned above and indicated in Fig.3, the column packed with 1.8 μm particles has a flatter curve at high linear velocity, so peaks maintain their efficiency yet the separation time decreases proportional to the increase in flow rate. The result of this approach is shown in Fig. 4(d). The total analysis time was less than 1 min. A nearly five fold faster separation speed could be achieved and the resolution of peaks maintained compara-

Table 1. Solvent consumption and column pressure drop

Column	Solvent A, B, consumption [mL]	Analysis time [min]	Column drop pressure [bar]
SB 5 μm 150 mm×4.6 mm i.d. 40℃, 1.0 mL/min	A;21.5 B;3.5 Total;25.0	15	56 bar
SB 3.5 μm 75 mm×4.6 mm i.d. 40℃, 1.0 mL/min	A;10.7 B;1.8 Total;12.5	7.5	72 bar
SB 1.8 μm 50 mm×4.6 mm i.d. 40℃, 1.0 mL/min	A;6.9 B;1.1 Total;8.0	5	95 bar
SB 1.8 μm 50 mm×4.6 mm i.d. 40℃, 5.0 mL/min	A;6.9 B;1.1 Total;8.0	1	500 bar
SB 1.8 μm 50 mm×2.1 mm i.d. 40℃, 0.21 mL/min	A;4.5 B;0.9 Total;5.1	5	88 bar
SB 1.8 μm 50 mm×2.1 mm i.d. 40℃, 1.05 mL/min	A;4.5 B;0.9 Total;5.1	1	485 bar

ble to the separation at 1 mL/min. The ultimate separation speed was obtained using the shortest column packed with the small particles (1.8 μm) at the highest flow rate without a loss in resolution.

With respect to gradient separation, system volume (also called the gradient delay volume or the dwell volume) must be noted. The gradient delay volume is the volume from the point of mixing of the binary eluents to the column entrance. The lower flow rates used with smaller–diameter columns will increase the gradient delay time because the time needed to traverse the gradient delay volume is long. When the gradient delay time is large, the gradient time should be changed in order to maintain resolution. Further, gradient delay volume will also affect regeneration time. Larger delay volume needs a much longer regeneration time. Therefore, the HPLC apparatus with the minimum gradient delay volume is required for low flow rate separations [7].

The solvent consumption for the chromatograms shown in Fig. 4 was calculated, and the results are summarized in Table 1. Furthermore, the pressure drops under the experimental conditions were also listed in Table 1. The shorter the column, the proportionally lower is solvent consumption when columns are run at the same flow rate. For the inner diameter of 4.6 mm i.d., the shortest analysis time (below 1 min) was obtained with ZORBAX SB–C18 RRHT (50 mm×4.6 mm i.d., 1.8 μm) column at flow–rate 5.0 mL/min (18 mm/sec) with total solvent consumption 8.0 mL. The smaller is inner diameter of column, the lower is the solvent consumption as well. For the inner diameter of 2.1 mm i.d., the shortest analysis time (below 1 min) was performed with ZORBAX SB –C18 RRTH (50 mm×2.1 mm i.d., 1.8 μm) column at flow rate 1.05 mL/min (18 mm/sec) with total solvent consumption 5.1 mL. The column pressure (485 bar) was not as high as observed with ZORBAX SB–C18 (50 mm×2.1 mm i.d., 1.8 μm) at highest flow rate 5.0 mL/min (18 mm/sec). It is always best to run the HPLC column at the lowest pressure drop as possible. Column pressure can be lowered by the use of shorter columns, lower flow rates, low viscosity mobile phase and higher temperatures.

3.1.4. RRLC/MS approach for Gradient elution

In the above experiments using DAD, orthophosphoric acid was used as modifier because of its low absorbance in the UV region of the spectrum, as low as 190 nm. However, this acid is unsuitable for use in MS. A volatile mobile phase must be used for MS. Formic acid is frequently used as a modifier for LC/MS work. Although the separation selectivity may be altered by changing the modifier, there are no clear method conversion protocols with a change in the modifier, and thus the suitable separation conditions must be determined by trial and error. After the optimum of gradient elution profile and mobile phase were determined, the catechins could be completely separated on a ZORBAX SB–C18 RRHT (50 mm × 2.1 mm i.d.) column packed with 1.8 μm particles. The MS chromatogram is shown in Fig. 5. The total analysis time was 1.6 min at 1.05 mL/min (18 mm/sec). This LC/MS separation was longer than the LC/DAD separation due to the change in selectivity caused by the modifier. Using the MS selectivity and MS parameter adjustment, the resolution of separation could be improved. In this MS experiment, the sampling rate was adjusted to 0.08 min (corresponding to peak half width). The sampling rate is also important in MS detection since sometimes a fast acquisition rate can cause a cross talk phenomena.

3.1.5 Analysis precision under fastest RRLC condition

The repeatability of the gradient analytical run was tested for retention time, peak height and area using the mixture of nine alkylphenones. The narrow bore ZORBAX SB–C18 RRHT (50 mm ×2.1 mm i.d., 1.8 μm) analytical column was used at 1.3 mL/min (23.4 mm/sec). This linear velocity (23.4 mm/sec) at 2.1 mm i.d. was corresponding to the 8 mL/min at 4.6 mm i.d.. At this flow rate, the column compartment temperature was adjusted to 60℃ and, therefore, the pressure drop was only 500 bar. The overlay result of six replicate injections of alkylphenones is shown in Fig. 6. The deviation between individual chromatograms was quite small. In the Table 2, the %RSD of retention time, peak height and area are listed. The mean retention time precision is 0.2 % RSD. The mean peak height and area precision are 0.61 % and 0.47 % RSD, respectively. At this high flow rate and operating pressure, the reproducibility was quite satisfactory for all test compounds.

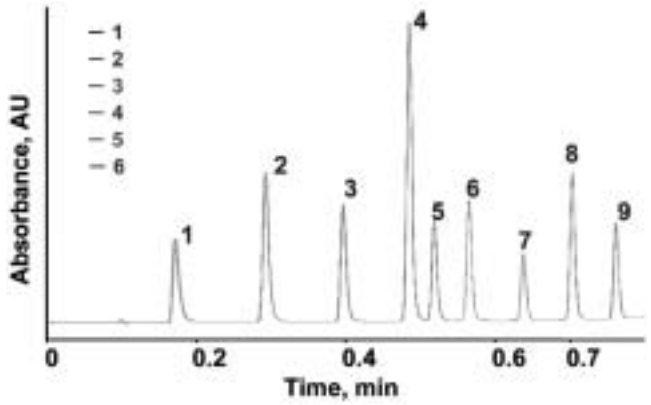

Figure 6. Repetitive ultrafirst separations of alkylphenones (six replicate injections).
The chromatogram of the mixture of 9 kinds alkylphenones was obtained on ZORBAX SB–C18 RRHT(50 mm×4.6 mm i.d., 1.8 μm) analytical column. The Peaks, in order of elution, are 1=acetanilide, 2=acetophenone, 3=propiophenone, 4=butyrophenone, 5=benzophenone, 6=valerophenone, 7=hexanophenone, 8=heptanophenone, and 9=octanophenone. See text for other chromatographic conditions.

Table 2. Relative Standard Deviation (RSD)s (n=6) of the retention times, peak heights and peak areas.

No. Alkylphenons	Retention time %STD	Height %STD	Area %STD
1 Acetanilide	0.64	0.45	0.69
2 Acetophenone	0.40	0.88	0.74
3 Propiophenone	0.13	0.26	0.27
4 Butyrophenone	0.16	0.79	0.35
5 Benzophenone	0.16	0.60	0.38
6 Valerophenone	0.09	0.59	0.73
7 Hexanophenone	0.08	0.57	0.36
8 Heptanophenone	0.07	0.83	0.23
9 Octanophenone	0.08	0.53	0.46
Average	0.20	0.61	0.47

4. Conclusions

In this study, we applied the fast RRLC technique to the analysis of general chemical compounds. Most applications of these small particle columns have been in the area of pharmaceutical analysis. Using a 1200 RRLC system equipped with higher acquisition rate of detector, low dead volume system configuration and combined with a high–pressure HPLC system with 600 bar pressure capability, the ultrafast RRLC analysis could be done with satisfactory analytical precision.

RRLC separations could be easily achieved from many traditional HPLC methods. In an isocratic example, one cycle analysis time by traditional HPLC/DAD method that required 25 min could be shortened to a RRLC/MS method to 0.8 min, a factor of 31. The same method transfer approach was carried out in a gradient separation. One cycle analysis time by conventional HPLC/DAD method of 15 min was shortened to a RRLC/DAD method of 1.0 min, a factor of 15. From the point of view of increasing sample throughput, lowering solvent consumption and in time savings, it was concluded that the fast RRLC technique is useful for not only pharmaceuticals but also for chemical compounds currently analyzed by conventional HPLC on 250 mm×4.6 mm i.d. columns.

In the future, the fast RRLC technique will be used more widely in other HPLC fields.

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