GREEN APPROACHES INVOLVING LIQUID PHASE MICROEXTRACTION, MONOLITHIC COLUMN AND CAPACITIVELY COUPLED CONTACTLESS CONDUCTIVITY DETECTION IN FLOW TECHNIQUES

by

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Specially dedicated to:

My Dad,

My Mum,

Brothers & sisters,

My Wife & sons,

My relatives and friends

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LIST OF ABBREVIATIONS

AP	Acceptor phase
pK_a	Acid dissociation constant
α	Alpha
β	Beta
BEH	Bridge ethane hybrid
C^4D	Capacitively coupled contactless conductivity detection
CE	Capillary electrophoresis
CS	Carrier stream
cm	Centimeter
C	Column
L	Column length
C	Concentration
C_{AP}	Concentration of analyte in the acceptor phase
C_a	Concentration of analyte in the extractor phase
C_{DP}	Concentration of analyte in the donor phase
CNLSD	Condensation nucleation light scattering detection
CPO	Crude palm oil
δ	Delta
D	Detector
DLLME	Dispersive liquid-liquid microextraction
DP	Donor phase
ECD	Electrochemical detection
EF	Enrichment factor
ELSD	Evaporative light scattering detection
ER	Extraction recovery

FA	Fatty acid
fg	Femtogram
FID	Flame ionization detection
FIA	Flow injection analysis
Fl	Fluorescence detection
FFA	Free fatty acid
γ	Gama
GC	Gas chromatography
g	Gram
HETP	Height equivalent to a theoretical plate
Hz	Hertz
HPLC	High performance liquid chromatography
HF	Hollow fiber
h	Hour
IκB	Inhibitor factor kappa-B
C_d	Initial concentration of analyte in the sample solution before extraction
I	Injector
I.D	Internal diameter
IPA	Isopropyl alcohol
Kg	Kilogram
KHz	Kilohertz
LLSD	Laser light scattering detection
LOD	Limit of detection
LOQ	Limit of quantitation
LA	Linoleic acid
LC	Liquid chromatography
LLE	Liquid-liquid extraction

LPME	Liquid phase microextraction
LPME-BE	Liquid phase microextraction-back extraction
L	Liter
Log *p*	Log octanol-water partitioning coefficient
MPOB	Malaysian Palm Oil Board
MS	Mass spectrometry
MΩ	Mega ohms
µg	Microgram
µL	Microliter
µm	Micrometer
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Millimolar
mmol	Millimole
min	Minute, Minutes
M	Molar
MALS	Multi-angle light scattering detection
MCFS	Multi-commutation flow system
MA	Myristic acid
ng	Nanogram
2-NPOE	2-nitrophenyl octyl ether
ND	Not detected
NF-κB	Nuclear factor kappa-B
N	Number of theoretical plates
OA	Oleic acid
OD	Outer diameter
PA	Palmitic acid

PO	Palm olein
W	Peak width
%	Percentage
PPARs	Peroxisome proliferator-activated receptors
PC	Personal computer
psi	Per square inch
pg	Picogram
P	Pump
rpm	Rate per minute
RI	Refractive index detection
r^2	Regression coefficient
rcf	Relative centrifugal force
RSD	Relative standard deviation
t_r	Retention time
RP	Reverse phase
RNA	Ribonucleic acid
ROSI	Rosiglitazone
s	Second
SIA	Sequential injection analysis
S/N	Signal-to-noise ratio
SDME	Single-drop microextraction
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SD	Standard deviation
SA	Stearic acid
SBSE	Stir-bar-sorptive extraction
SCPO	Stored crude palm oil
SRBD	Stored refined bleached deodorized palm oil

SLE	Supported liquid-liquid extraction
SLM	Supported liquid membrane
°C	Temperature in degree Celsius
IUPAC	The International Union of Pure and Applied Chemistry
UHPLC	Ultra high pressure liquid chromatography
UV	Ultraviolet detection
UV-vis	Ultraviolet-visible detection
V	Voltage
v	Volume
V_{AP}	Volume of acceptor phase
V_{DP}	Volume of donor phase
W	Waste
w	Weight

PENDEKATAN HIJAU MELIBATKAN PENGEKSTRAKAN MIKRO FASA CECAIR, TURUS MONOLITIK DAN PENGESAN KEKONDUKSIAN TANPA SENTUH KUPEL KAPASITIF DALAM KAEDAH ALIRAN

ABSTRAK

Tesis ini berkaitan dengan perkembangan kaedah analisis baru di dalam kaedah aliran (kromatografi cecair prestasi tinggi (HPLC) dan analisis suntikan aliran). Tujuan penting kaedah ini adalah penggunaan pendekatan hijau (pengekstrakan mikro tanpa pelarut, turus monolitik, pengesan kekonduksian tanpa sentuh kupel kapasitif (C^4D), dan automasi untuk mencapai objektif masing-masing.

Teknik pengekstrakan mikro fasa cecair gentian berongga telah digunakan sebagai penyediaan sampel bagi pengekstrakan surihan rosiglitazon (ubat anti-diabetis) di dalam cecair biologi. Kaedah pengekstrakan mikro, bersama dengan HPLC, telah dioptimum dan ditentusahkan dengan jayanya. Keadaan optimum adalah; pelarut pengekstrakan, diheksil eter; pH fasa penderma, 9.5; fasa penerima, 0.1M HCl; halaju pengacauan, 600 rpm; masa pengekstrakan, 30 min dan tanpa penambahan garam. Faktor pemerkayaan 280 telah dicapai.

Betulin dan asid betulinik di dalam ekstrak hasil semulajadi telah dipisahkan dengan jayanya dengan menggunakan turus monolitik. Kaedah HPLC telah dioptimumkan menggunakan model plat dengan kecekapan optimum turus telah dipilih. Di bawah keadaan optimum (fasa gerak 95:5 % (v/v: asetonitril:air); kadar aliran, 1.0 mL min^{-1}; suhu ambien), kedua-dua sebatian telah dipisahkan dalam masa kurang daripada 5 min. Kelinearan yang baik telah diperoleh bagi kedua-dua analit dalam

julat kepekatan 1 - 200 mg L^{-1} dengan pekali kolerasi > 0.999. Kaedah dibangunkan telah digunakan untuk menganalisis betulin dan asid betulinik di dalam ekstrak tumbuhan. Kaedah cadangan adalah jelas lebih baik daripada kaedah-kaedah yang telah dilaporkan menggunakan turus C_{18}.

Suatu kaedah HPLC fasa terbalik dengan C^4D telah diperkembangkan untuk pemisahan dan penentuan serentak lima asid lemak (FAs) rantai panjang tanpa diterbitkan (asid miristik, palmitik, stearik, oleik, dan linoleik). Mod elusi isokratik menggunakan metanol/1 mM natrium asetat (78:22, v/v) sebagai fasa gerak dan kadar alir yang digunakan ialah 0.6 mL min^{-1}. Keluk penentukuran bagi lima FAs adalah > 0.999 di julat 5 – 200 μg mL^{-1} bagi asid stearik, dan 2 – 200 μg mL^{-1} bagi FAs yang lain. Persetujuan yang baik telah didapati dengan kaedah kromotrografi gas (GC) apabila digunakan untuk analisis minyak-minyak labu, kacang soya, dedak padi dan olein sawit. Kaedah cadangan mempamerkan kebaikan ketara berbanding kaedah GC piawai, terutamanya dari segi kemudahannya, masa pemisahan pantas dan kepekaan.

Satu kaedah analisis suntikan alir (FIA) tunggal yang membabitkan turus prapemekatan (dipadat dengan zarah C_{18}) dan C^4D telah diperkembangkan bagi penentuan asid lemak bebas (FFA) di dalam minyak sayuran. Aliran pembawa ialah metanol/1.5 mM natrium asetat (pH 8) 80:20 (v/v) dan dioperasikan pada kadar alir 1.0 mL min^{-1}. Keluk penentukuran adalah baik ($r^2 = 0.9995$) di dalam julat 1 – 200 mg L^{-1} FFA (sebagai asid palmitik). Kadar pensampelan 40 – 60 jam^{-1} telah tercapai. Persetujuan yang baik telah didapati antara kaedah titrimetri tanpa akueus dan kaedah cadangan apabila digunakan untuk penentuan FFA di dalam minyak kelap sawit (mentah, olein, dan ditapis, diluntur dan dinyah bau) dan minyak sayuran lain (kacang soya, dedak padi, walnut, jagung and zaiton). Kaedah cadangan adalah

lebih baik daripada kaedah resmi, terutamanya dari segi kemudahannya, kadar pensampelan lebih tinggi, ekonomi pelarut dan sampel.

GREEN APPROACHES INVOLVING LIQUID PHASE MICROEXTRACTION, MONOLITHIC COLUMN AND CAPACITIVELY COUPLED CONTACTLESS CONDUCTIVITY DETECTION IN FLOW TECHNIQUES

ABSTRACT

This thesis deals with the developments of new analytical methods in flow techniques (high performance liquid chromatography (HPLC) and flow injection analysis). An important goal of these methods is the use of green approaches (solventless microextraction, monolithic column, capacitively coupled contactless conductivity detector (C^4D), and automation) to achieve the respective objectives.

A hollow fiber liquid phase microextraction technique was used as sample preparation for the extraction of trace amounts of rosiglitazone (anti-diabetic drug) in biological fluids. The microextraction method, in tandem with HPLC, was successfully optimized and validated. The optimum conditions were: extraction solvent, dihexyl ether; donor phase pH, 9.5; acceptor phase, 0.1M HCl; stirring speed, 600 rpm; extraction time, 30 min and without addition of salt. Enrichment factor of 280 was achieved.

Betulin and betulinic acid in natural product extracts were successfully separated using a monolithic column. The HPLC method was optimized using plate model where the optimum efficiency of the column was selected. Under the optimized conditions (mobile phase 95:5 % (v/v: acetonitrile:water); flow rate, 1.0 mL min^{-1}; ambient temperature) the two compounds were separated in less than 5 min. Good linearities were obtained for both analytes over the concentration range 1 - 200 mg

L^{-1} with correlation coefficients > 0.999. The method was used to analyze betulin and betulinic acids in plant extracts. The proposed method was clearly superior over the other reported methods using conventional C_{18} columns.

A reversed-phase HPLC method with C^4D was developed for the separation and simultaneous determination of five underivatized long chain fatty acids (FAs), namely myristic, palmitic, stearic, oleic, and linoleic acids. An isocratic elution mode using methanol/1 mM sodium acetate (78:22, v/v) as mobile phase with a flow rate of 0.6 mL min^{-1} was used. Calibration curves of the five FAs were well correlated ($r^2 > 0.999$) within the range of 5 – 200 μg mL^{-1} for stearic acid, and 2 – 200 μg mL^{-1} for the other FAs. Good agreement was found with the standard gas chromatographic (GC) method when applied to the analysis of pumpkin, soybean, rice bran and palm olein oils. The proposed method offers distinct advantages over the official GC method, especially in terms of simplicity, faster separation times and sensitivity.

A single line flow injection analysis (FIA) method that incorporated a preconcentrator column (packed with C_{18} particles) and C^4D was developed for the determination of free fatty acids (FFA) in vegetable oils. The carrier stream was methanol/1.5 mM sodium acetate (pH 8) 80:20 (v/v) and operated at a flow rate of 1.0 mL min^{-1}. Calibration curve was well correlated ($r^2 = 0.9995$) within the range of 1 – 200 mg L^{-1} FFA (expressed as palmitic acid). Sampling rate of 40 – 60 h^{-1} was achieved. Good agreement was found between the standard non-aqueous titrimetry method and the proposed method when applied to the determination of FFA in palm (crude, olein, and refined, bleached and deodorised) and other vegetable (soybean, rice bran, walnut, corn and olive) oils. The proposed method is superior over the

official method, especially in terms of simplicity, higher sampling rate, economy of solvents and sample.

CHAPTER ONE

INTRODUCTION

1.1 Background

Analytical laboratories can be considered as small-scale factory where the incoming raw materials (a designated problem) need to go through the production line (analytical process) to produce a product (answers and solutions) (Fig. 1.1).

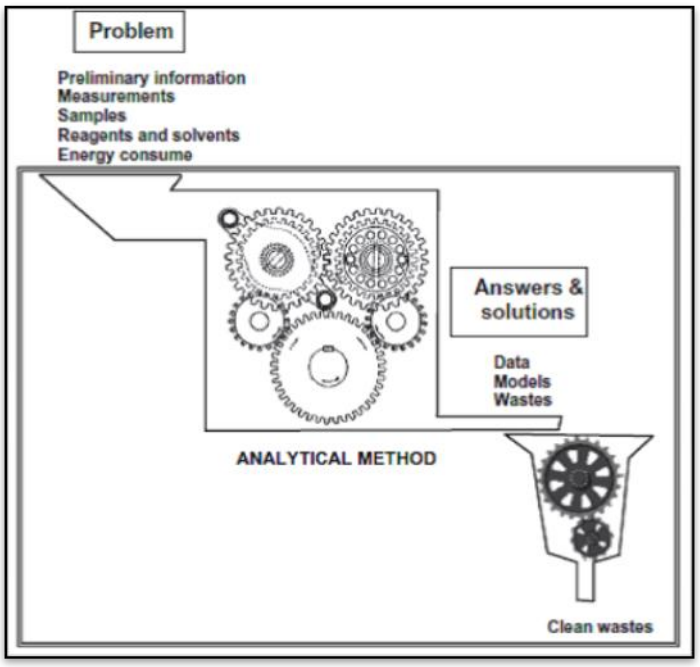

Fig 1.1 Input and output of analytical method (Garrigues et al., 2010).

The modern quality control and monitoring laboratories have to deal with a huge number of samples routinely. These analytical laboratories can produce wastes similar to those of the fine chemicals industry. The E-factor (environmental factor, which is defined as the ratio of the amount of by-products (waste) produced per unit

of the desired products) is considered high, especially, in the pharmaceutical production as shown in Table 1.1 (Sheldon, 1994). Thus, the quest towards green chemistry for these laboratories is highly justified.

Table 1.1: E-factor across the chemical industries (Sheldon, 1994).

Industry sector	Annual production (ton)	E-factor	Waste produced (ton)
Oil Refining	10^6-10^8	Ca. 0.1	10^5-10^7
Bulk Chemicals	10^4-10^6	<1-5	10^4-5×10^6
Fine Chemicals	10^2-10^4	5-50	5×10^2-5×10^5
Pharmaceuticals	10-10^3	25-100	2.5×10^2-10^5

1.2 Green analytical chemistry

Analytical chemistry is generally related to green chemistry in two ways. Firstly, analytical chemistry is frequently used as a confirmation tool of the green approaches in the production of chemicals. Secondly, analytical methods require solvents, reagents, energy, etc., and wastes are generated as by-products.

Anastas and Warner suggested twelve principles of green chemistry (see Appendix A) (Anastas and Warner, 1998). Green chemistry was stated as "the use of chemical techniques and methodologies that reduce or eliminate the use or generation of feedstocks, products, by-products, solvents, reagents, etc. that are hazardous to human health or the environment" (Anastas, 1999). Among the twelve principles, six are directly related to analytical chemistry, which are:

(i) Prevention. The prevention of generation of waste is better than the treating or cleaning up of the waste after it has been created.

(ii) Safer solvents and auxiliaries. The use of auxiliary substances (e.g., solvents, separation agents) should be reduced and avoided where possibly can.

(iii) Design for energy efficiency. Minimization the use of energy in the chemical processes should consider the environmental and economic impacts (e.g., conducting the derivatization procedure at ambient temperature and pressure if that is possible).

(iv) Reduce derivatives. Minimize or avoid unnecessary derivatizations as possibly can, since such steps require additional reagents that will generate wastes.

(v) Real-time analysis for pollution prevention. Analytical methods need to be improved so that the analysis can be conducted in real times. This prevents the generation of wastes.

(vi) Safer chemicals for accident prevention. Inert chemicals and reagents should be chosen so that they pose minimum potential for chemical accidents, including releases, explosions, and fires.

The term “green analytical chemistry” has been proposed by Namieśnik where several features were discussed (Namieśnik, 1999; 2001). In recent years, a steady growth in this topic as reflected by the number of publications was observed (Fig. 1.2).

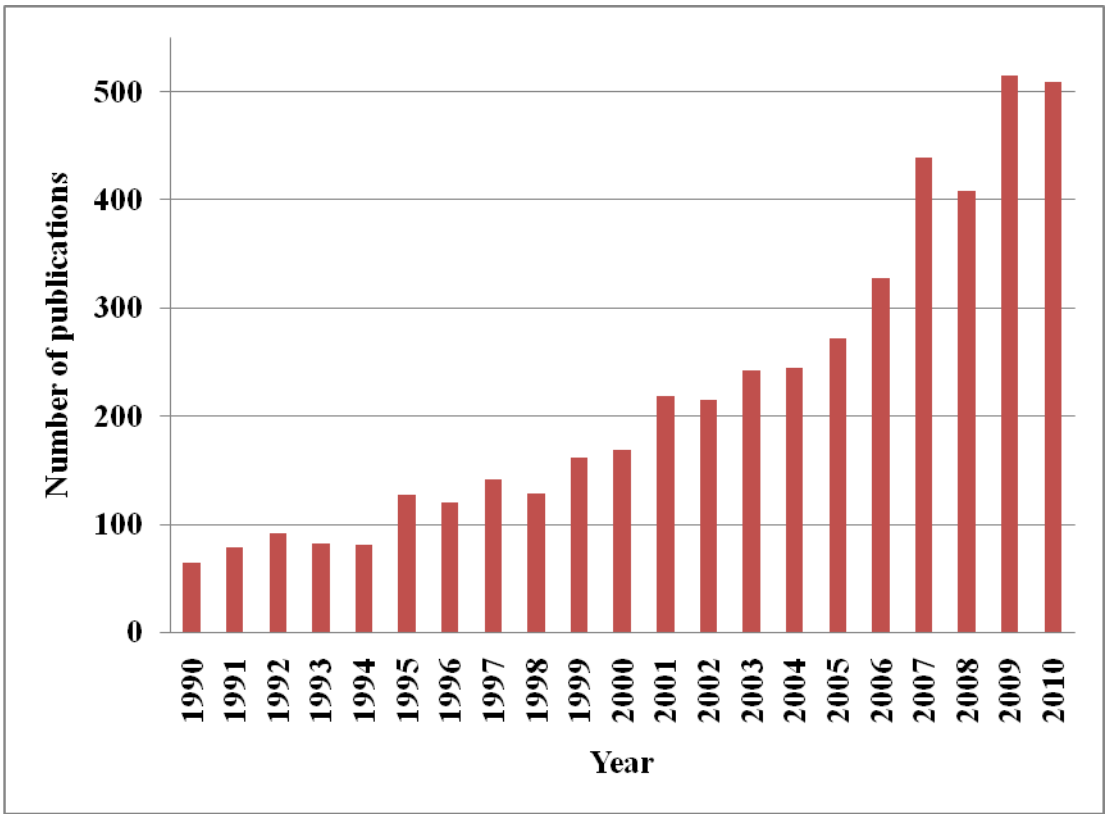

Fig 1.2 Publications on green analytical methods (obtained from Web of Science) by typing the key words "green analytical methods"

Replacing wet chemistry is a common trend in green analytical chemistry. The main target of developing a new analytical method is to increase the reliability of analysis, to produce more precise data, to save time, and can reduce the production of waste. Moreover, the use of instrumental methods will decrease the amount of sample and solvent required. The use of micro-scale sample preparations, new approaches in separations and detections are some common strategies to meet the objective.

Instrumental methods also lead to optimum use of energy, especially when the method is highly automated. The combination of several sample treatment methods

together with innovative separation methods and/or new methods of detection (e.g., photochemical and electrochemical methods) provides efficient use of energy.

In some cases, there is a choice of direct techniques of analysis using different detection methods (e.g., evaporative light scattering detection for non chromophoric analytes instead of derivatization for ultraviolet detection) or solventless processes of analysis (e.g., microextraction methods for samples with complex matrices). Finding alternative solvents is also an important strategy to produce greener methods. The main target of this process is not only replacing the non-green solvents, but also introducing additional advantages such as improving the selectivity, sensitivity, and reliability of the analysis, as well as reducing the analysis time. Furusawa reported the classifications of some common solvents used in analysis (Table 1.2) (Furusawa, 2004). The use of alternative solvents such as supercritical fluids and ionic liquids are also attractive to replace some of these solvents (e.g., chloroform).

Table 1.2: Classification of some solvents used in analytical chemistry (Furusawa, 2004).

Solvent	Poison class[a]	Harmful class[b]
Acetone	5	H
Acetonitrile	2	T
Chloroform	1	T
Dichloromethane	4	H
Diethyl ether	4	H
Ethanol	–	–
Ethyl acetate	4	T
n-heptane	5	–
n-hexane	4	H
Methanol	3	T

[a]Toxicity classification, 1 = very strong toxin (carcinogenic, mutagenic, and teratogenic), 2 = very strong toxin, 3 = strong toxin, 4 = solvent is considered harmful, 5 = solvent with a low hazard potential (negligible hazard),– = no toxicity classification; [b]Harmful classification, T = toxic, H = harmful,– = not harmful.

1.3 Steps in chemical analysis

The chemical analysis of any sample involves several major steps as shown in Fig. 1.3.

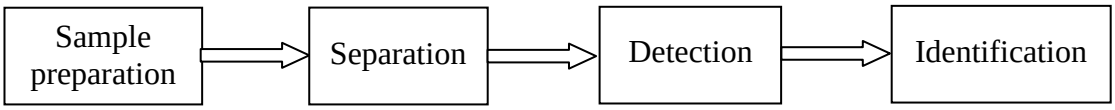

Fig. 1.3 Major steps in chemical analysis

The analysis usually starts with sample treatment and preparation for further separation. The separated components are detected and its identity established (Koel and Kaljurand, 2006).

There are many ways to prepare or treat the sample. The same also applies to the separation process. Unfortunately, there is no universal method for sample

pretreatment or separation because of the huge variation of samples. Furthermore, samples are complex, and always contain many unwanted components (matrix effect) that can pose as a source of interference. Therefore, analytical laboratories are expected to provide solutions to overcome these problems.

Separation and detection of an analyte are other areas where green chemistry can be adopted. The development of new columns such as monolithic and smaller particles columns is another approach towards green chemistry. The introduction of new detectors such as the evaporative light scattering, electrochemical and capacitively coupled contactless conductivity detections which are sensitive, require small amounts of sample without any pre-derivatization process helps to fulfill the requirements of green analytical method and reduce the waste. In short, green analysis demands innovative approaches to be adopted for the major steps in the analysis, and some of these strategies are summarized in Fig. 1.4.

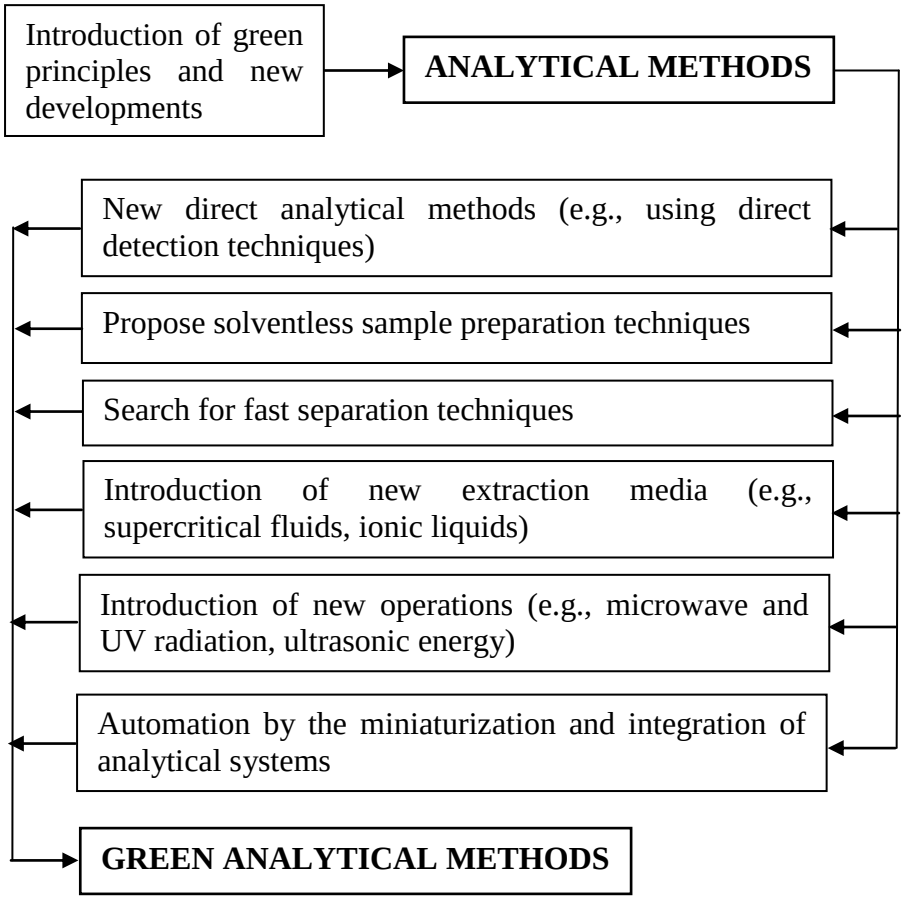

Fig. 1.4 Major strategies to achieve green analytical methods.

1.3.1 Innovative sample preparation methods

Classical wet methods for the preparation of samples are time consuming, use large amounts of solvents, generate wastes, and slow down the entire analytical process. Many new techniques have been developed to replace the existing wet methods. Miniaturization has been a key factor in the design of new sample preparation techniques. Some examples of novel developments in the field of miniaturized sample preparation for chemical analysis are listed below (Ramos et al., 2005; Tzschucke et al., 2002).

(i) Sorption microextraction

These microextraction techniques involve the use of solids (e.g., C_{18}, silica) to perform the task, and several varieties are available, e.g., solid-phase extraction (SPE), solid-phase microextraction (SPME), in-tube solid-phase microextraction (in-tube SPME), and stir-bar-sorptive extraction (SBSE).

(ii) Solvent microextraction

These microextraction techniques involve the use of liquids placed in special formats to carry out the extraction process. Several forms of the technique have been proposed, e.g., single-drop microextraction (SDME), hollow fiber liquid-phase microextraction (HF-LPME), and dispersive liquid-liquid microextraction (DLLME).

SPE was the first method introduced, and it represents a new paradigm shift in sample preparation method. The on-line and automated SPE coupled with liquid chromatography (SPE-HPLC) is now available.

SPME was the first successful microextraction technique developed by Arthur and Pawliszyn in 1990 (Arthur and Pawliszyn, 1990). SPME comprises of a small polymer-coated fiber which can be used to extract analytes from solution or headspace region (Fig. 1.5). The extract is thermally desorbed in the injector of a gas chromatography (GC) or stripped at the high performance liquid chromatography (HPLC) injector using special interface, for further separation of the analytes. SPME is used to a lesser extent in HPLC due to the need of extraction solvent to strip the analytes from the fiber which can slow down the process compared to thermal

desorption. To overcome some of these problems, an in-tube SPME technique was developed using short piece of GC column which was internally coated with a suitable stationary phase (Eisert and Pawliszyn, 1997). The analytes were sorbed at the stationary phase by repeatedly aspirating and dispensing of the sample liquid. The sorbed analytes are transferred by the HPLC solvent to the column for analysis (Eisert and Pawliszyn, 1997).

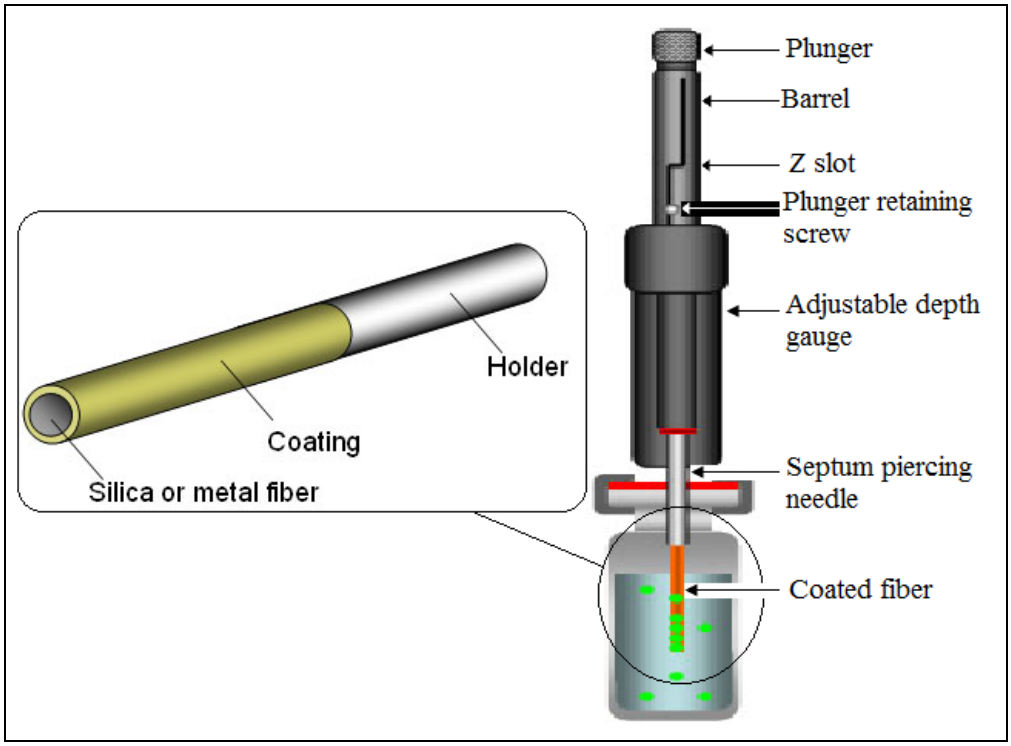

Fig. 1.5 Schematic of the SPME apparatus (Agilent technologies, 2010)

SBSE consists of a glass magnetic stir bar that was coated with a polymeric sorbent (Fig. 1.6) (Baltussen et al., 1999). The surface area of the coated bar is 50 - 250 times higher than the surface area of the coated fiber in the SPME. Therefore, higher extraction efficiency can be achieved compared to the SPE or SPME techniques (David et al., 2003). The stir bar is placed in the sample solution, stirred

for predetermined time (usually a few minutes), removed, dried to remove water, and placed in a thermal desorption unit to desorb the analytes for further analysis using the GC or alternatively stripped in small amount of solvent to be injected into a HPLC unit. This technique can be used in the analysis of a wide range of analytes. Moreover, it is easy to be automated, easy to handle, miniaturized and is solvent-free.

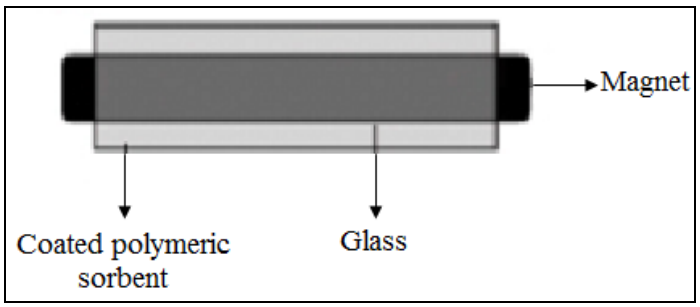

Fig. 1.6 Schematic of the SBSE bar

In the late 1990s, the SDME was proposed to replace the liquid-liquid extraction (LLE) technique (Jeannot and Cantwell, 1996; Liu and Dasgupta, 1996). It is considered as the first liquid microextraction technique that attempted to emulate the SPME technique. An immiscible single drop (1–10 μL) of organic solvent (acceptor phase) was suspended at the end of a micro-syringe needle in an aqueous solution (donor phase) with continuous stirring (Figure 1.7). Once equilibrium was achieved between the two phases, the hanging drop is withdrawn into the syringe barrel and injected directly into a GC, HPLC, or capillary electrophoresis (CE) unit. The extraction of analytes from the matrix depends on the diffusion and partition process of compounds of interest between the organic solvent and the aqueous phase. Therefore, the whole process is based on equilibrium principles rather than exhaustive extraction. Parameters (e.g., stirring rate, increasing the extraction

temperature) can accelerate the diffusion process, thereby, increasing the extraction efficiency. Other parameters such as volume of the solvent drop, type of solvent, pH, and salting-out effect also affect the extraction efficiency.

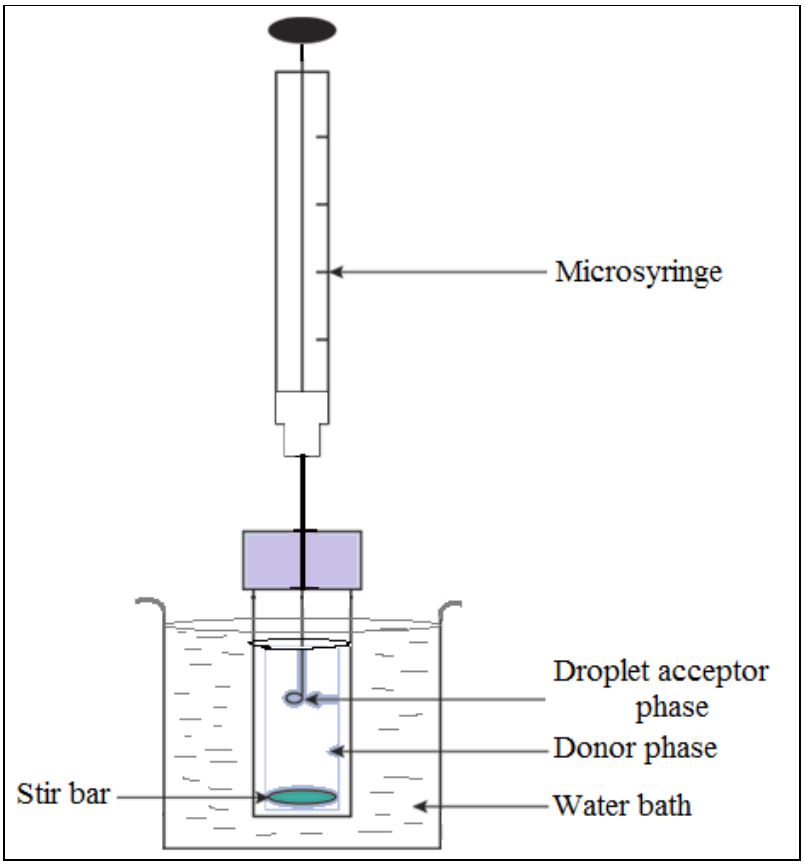

Fig. 1.7 Schematic of the SDME apparatus.

Solvent microextraction techniques usually use a very small amount of organic solvent (few μL) compared to the donor aqueous phase. The high volume ratio of the donor aqueous phase to the acceptor organic phase provides a high enrichment.

Although the SDME is inexpensive, simple, and efficient, the stability of the hanging drop has always been a problem. High stirring speed and the slight solubility of the acceptor organic liquid in the aqueous solvent may cause the drop to be

dislodged from the syringe needle. Moreover, the analysis of biological samples such as plasma can emulsify considerable amounts of organic solvents which may also affect the stability of the drop during the extraction process. However, the selection of a sharp needle syringe, suitable organic solvent, and the use of a small volume of organic solvent (1–2 μL) can minimize this difficulty (Pena-Pereira et al., 2010).

The extraction of analytes using SDME in complicated matrices may not be successful due to the presence of particles or bubbles in the sample which may affect the extraction efficiency. These particles can also affect the drop stability, or may be potentially detrimental to the functioning of the analytical instrument. This problem can be overcome by compromising the extraction parameters (e.g., short equilibrium time, use low stirring speed, etc.) during the method optimization. In other words, generally SDME is a very good sample preparation technique, but it is not suitable as a clean-up technique. Furthermore, the process is considered slow (usually more than 30 min).

The HF-LPME technique overcomes the problem of drop stability by protecting the drop (or small volume of organic solvent) within the lumen of a porous hydrophobic polypropylene hollow fiber from mechanical disturbance (Pedersen-Bjergaard and Rasmussen, 1999). More details about the HF-LPME technique will be discussed in chapter 2 (section 2.1).

The DLLME technique involves a small volume of extraction solvent (density higher than water and 1–3% of the total volume of the sample) and a disperser

solvent which is miscible with the extraction solvent and the aqueous sample. The extraction and disperser solvent are mixed together and is rapidly injected into the aqueous sample to form a cloudy mixture (Fig. 1.8). The sample is centrifuged and the sediment phase is then collected for analysis or further processing (Majors, 2008; Razaee et al., 2006). The extraction efficiency is quite high due to the high surface area of the extraction solvent droplets. Moreover, the extraction equilibrium is extremely rapid (few minutes) (Pena-Pereira et al., 2010).

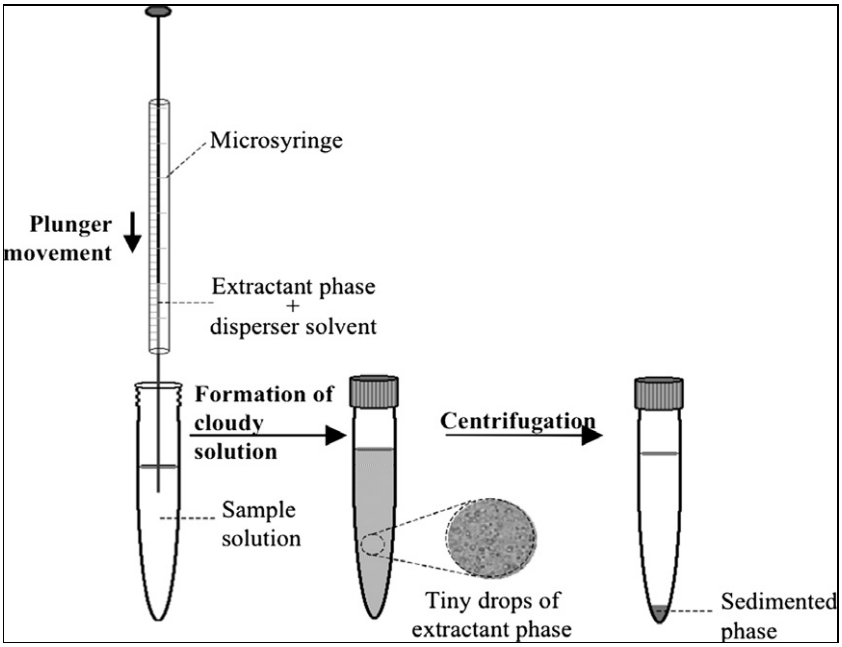

Fig. 1.8 Schematic of dispersive liquid-liquid phase microextraction (DLLME) (Pena-Pereira et al., 2010)

1.3.2 New approaches in separation

Green separation techniques are becoming increasingly important as solvents costs continue to increase and laboratories are moving towards minimization of solvent consumption and even the possible complete elimination of wastes.

Generally, conventional liquid chromatography (LC) need long run times. The separations are effected on large internal diameter particles columns (>3.0 mm) which can create a large amount of waste and consume considerable amount of energy. Recently, a wide range of developments were carried out on LC separations to overcome these problems. The key developments are to improve column efficiencies and at the same time provide lower analysis costs. Therefore, a trend towards faster separation using smaller amounts of solvent but giving better separation efficiencies and faster analysis times were the main interests for researchers (Cheng et al., 2001).

To achieve this target smaller particle columns (< 2 μm) and faster flow rates (up to 10 mL min^{-1}) have been used. Furthermore, elevating the column temperature which lowers the viscosity of mobile phase and thus increasing the mass transfer due to the increase in the diffusivity of the analytes, has also been investigated (Neue and Mazzeo, 2001). However, to achieve these goals using the conventional particle size column and pressures is difficult due to the loss of resolution and efficiency. Therefore, developing new columns that can provide improved resolution, high efficiency and tolerate high pressure have been attempted. Monolithic and sub-2 μm LC columns have been introduced. These columns have accelerated the separations down to a few minutes or even seconds.

1.3.2 (a) Sub-2 μm particle size column

Based on the van Deemter equation, a significant gain in efficiency as the particle size is decreased to less than 2.5 μm can be anticipated. Furthermore, the efficiency is not affected as the flow rate or linear velocity is increased (Fig. 1.9). The use of smaller particle (sub-2 μm) column will speed up the separation process and increase the peak capacity (number of peaks separated per unit time). The development of sub-2 μm particles is a big challenge, and this area has attracted lots of attention (Jerkovitch et al., 2003; Unger et al., 2000; Wu et al., 2001). Non-porous 1.5 μm particles column was introduced. However, although this column offered high efficiency, but poor loading capacity and retention were observed due to its small surface area. Silica based particles have been proposed, it offers good mechanical strength, but has many limitations (e.g., tailing of basic analytes and limited pH range). Therefore, polymeric particles were proposed which overcome the pH limitations, but unfortunately suffers from low efficiency and limited capacity.

In 1999, hybrid particles (1.7 μm) were for the first time introduced (Cheng et al., 2000; Neue et al., 1999). These particles were synthesized using the classical sol-gel technique with addition of methyl groups. The columns are mechanically strong, highly efficient, and are able to operate over wide pH range. This column was used for the separation of some benzodiazepines, herbicides, and various pharmaceutical compounds (Lippert et al., 1999). Later, further improvement was carried out by bridging the methyl groups in the silica matrix to produce a second generation particles, known as the bridged ethane hybrid (BEH) technology which provided better mechanical stability (Mazzeo et al., 2005).

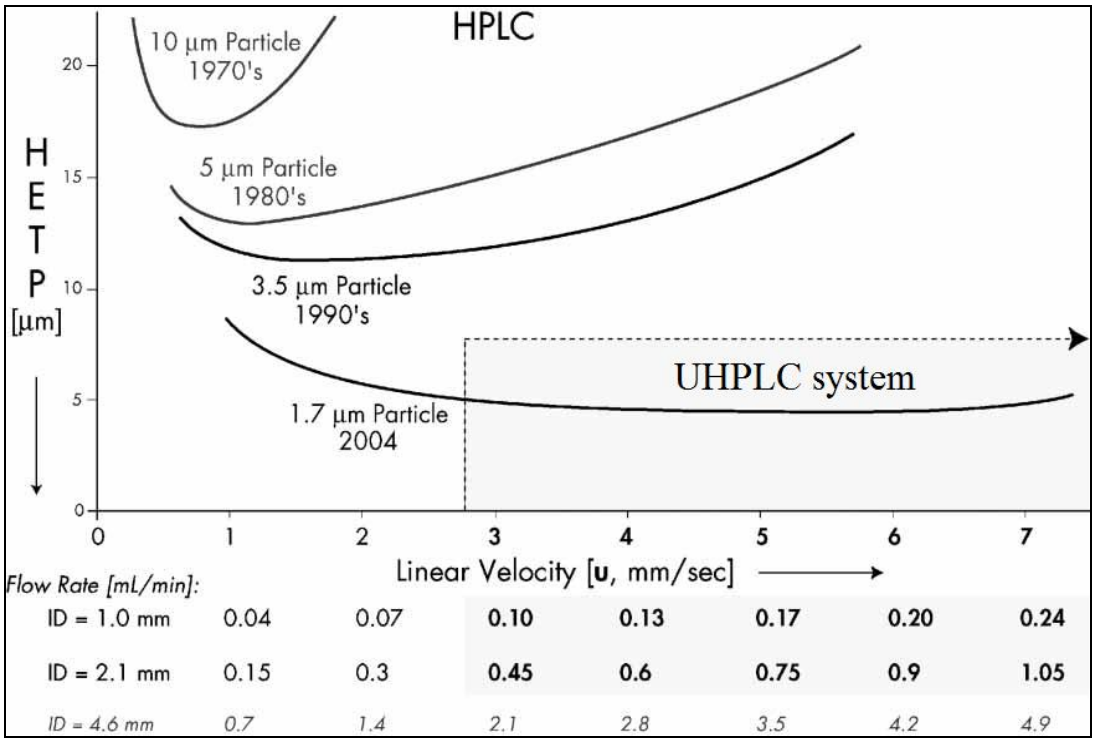

Fig. 1.9 van Deemter plots for different particle sizes (Swartz, 2005)

Further improvements in column efficiency cannot be realized by using 1.7 µm particles sizes, mainly due to the high back pressures (Fig. 1.9). Therefore, further improvement can be realized using instrument technology to afford faster speed in analysis, superior resolution and sensitivity (Tolley et al., 2001; Wu et al., 2001).

In 2001, Wu et al. illustrated the design of injection valves and the employ of carbon dioxide to improve the slurry packing process on the capillary (Wu et al., 2001). In the same year, Jorgenson et al. also modified a commercial HPLC pump to be operated at 17,500 psi to analyze proteins using a 22 cm long capillaries packed with 1.5 µm C_{18}-modified particles (Tolley et al., 2001).

The previous reports show that, to take full advantage of the small particles technology, greater pressure range than the normal HPLC is required. Furthermore,

sample introduction is also critical. Therefore, a modified injection valve is also needed to protect the column from pressure fluctuations and must be relatively pulse-free. The delay volume of the device should also be as minimum possible to avoid band spreading. A fast injection cycle time, low volume injections with nominal carryover, and high sensitivity detector are also required to increase the sensitivity.

In early 2004, a first commercial system known as the ultra high pressure liquid chromatography (UHPLC) to fulfill these requirements was described for the separation of pharmaceuticals, small organic molecules, proteins, and peptides (Swartz, 2005). The UHPLC takes advantage of the chromatographic principles to run separations using shorter column, but with superior resolution and sensitivity. The UHPLC significantly reduce the solvent consumption and waste generation without sacrificing the quality of the separation. The field has since witnessed significant growth in the number of publications that use UHPLC using sub-2 μm particle size column since 2004 (Fig. 1.10). It is safe to predict that the future LC techniques will be predominantly based on UHPLC.

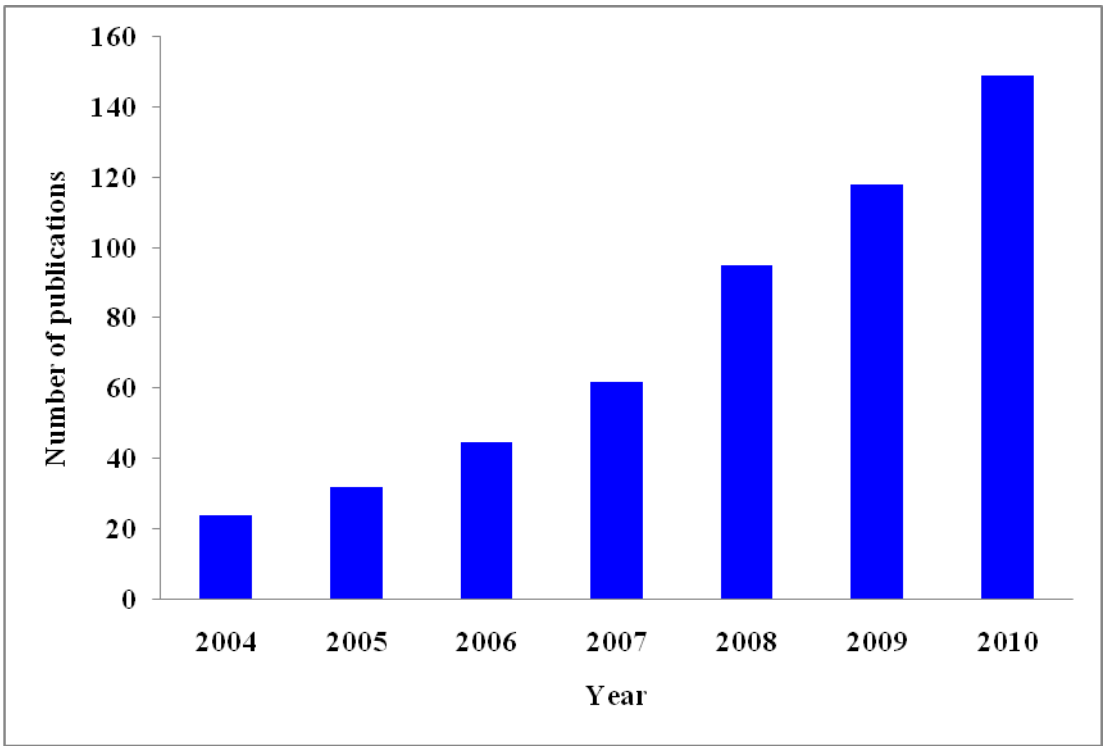

Fig. 1.10 Publications on UHPLC (obtained from the Web of Science) by typing the key words “ultra pressure liquid chromatography”

1.3.2 (b) Monolithic column

Monoliths are rod structures with porous channels rather than beads of the conventional HPLC, and it is characterized by mesopores and macropores structures (Fig. 1.11). This column has gained considerable attention due to its high permeability (low pressure, due to its high bed porosity and no frits is used), good separation efficiency, easy to fabricate and is highly reproducible. The unique structure of monolith columns gives rise to several physico-mechanical characteristics that allow it to perform competitively or even better than the traditionally packed columns.

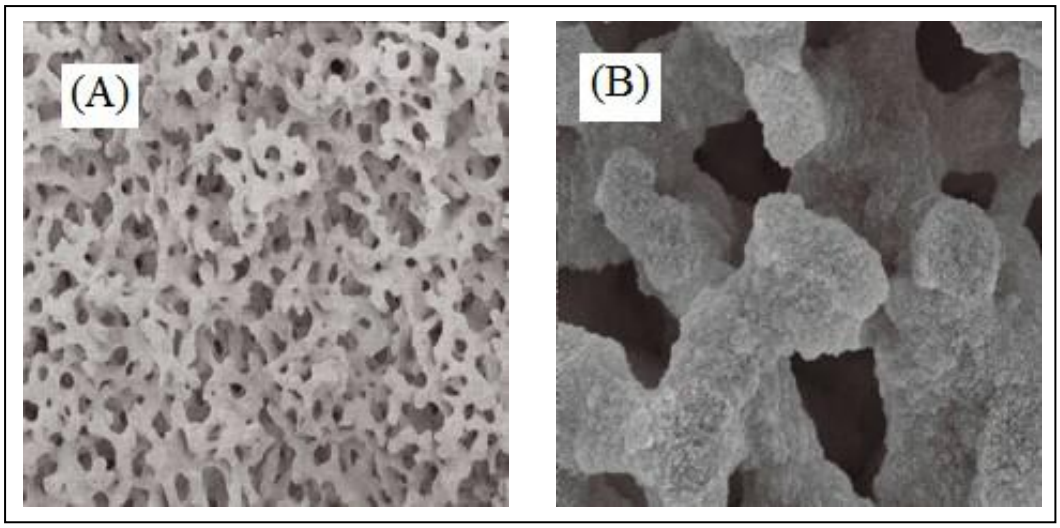

Fig. 1.11 Scanning electron microscope images for (A) macropores, (B) mesopores structures in monolith column (Merck KGaA).

The unique structure of monoliths column helps to explain the differences in characteristics compared to traditionally packed column such as, the absence of interstitial voids, the very short diffusion distances and multiple pathways are available for solute dispersion (Svec, 2003). Furthermore, the pore connectivity value of traditionally packed particles column is about 1.5, while monolith has values ranging from 6 to 10. This means that, the analyte in the traditionally packed column may diffuse in the same pore (in and out), or enter through one pore and exit through another pore. By contrast, the analyte in a monolith column is able to enter one channel and exit through any of six or more other different locations (Svec, 2003). Due to the small-size skeletons of monolith and its wide number of channels and outcroppings, higher efficiency and faster analysis time can be achieved.

Unlike in traditionally packed particles column, monoliths are mainly used for the separation of large molecules (i.e., proteins, DNA and RNA). As was previously mentioned, the better efficiency and higher resolution are easily achieved as the

particle sizes are decreased, which caused higher backpressures. Furthermore, the separation of biomolecules using smaller particle sizes (sub-2 μm) will increase the backpressures because of the large molecule size. By contrast, monoliths have lower backpressure and larger channel size, therefore, the separation of small molecules is generally less efficient (Svec, 2003).

Polymeric monoliths were firstly synthesized in the 1960s. However, the first successful fabricated column was introduced in the late 1980s for protein separations (Tennikova et. al., 1990). Thus, commercial polymeric monolith columns have become widely used after that, and were mainly used for the analysis of large biomolecules (Svec and Krenkova, 2008). In 1993, Tanaka et al. proposed a silica-based monolith (Tanaka et al., 1993) which was later (in 2001) commercialized by Merck KGaA (Darmstadt, Germany). Tanaka and his research group have conducted further developments on silica-based monolith later by immobilizing different functional groups. Apart from reversed-phase ligands, modified monoliths with ion exchange, hydrophilic, chiral, and mixed modes interactions have also been developed (Núñez et al., 2008).

Recently, the synthesis of second generation polymeric monolith materials to provide high performance for the separation of small molecules has been reported (Urban (a) et al., 2010). These materials contained large throughpores with very large surface areas in the mesopores that allow the efficient separation of small molecules (Urban (a) et al., 2010). The new monoliths was synthesized as poly(styrene-co-vinylbenzyl chloride-co-divinylbenzene) which was prepared and subsequently

modified using hypercrosslinking reaction to produce monolith containing an array of small pores (Urban (b) et al., 2010). These monolithic columns exhibit a large surface area (up to 500 m^2 g^{-1}) which is larger than the non-modified precursor columns (Urban (b) et al., 2010). The mesopores in the hypercrosslinked monolithic columns allow good separation of small molecules. The same research group was able to increase the surface area of polymeric monolithic column by the addition of carbon nanotubes which are chemically cut (oxidative cutting) into short lengths and implanted into the structure of the monolith (Chambers et al., 2011). The carbon nanotubes increase the hydrophobicity, and the large surface area have led to improvements in the separation especially for small molecules (Chambers et al., 2011).

1.3.3 New approaches in detection

In the 1940s, the qualitative or quantitative analysis in HPLC was carried out by collecting the fractions and conducting the analysis off-line either using gravimetric or wet chemical techniques. The first online detections for LC were the refractive index (RI), and conductivity detectors (James et al., 1951; Tiselius and Claesson, 1942). Although these detectors possess considerable advantages over the off-line detection but they were not particularly sensitive. Therefore, the need for more sensitive detectors led to the adaption of GC detectors for use in HPLC (Dolan and Seiber, 1977; Haati and Nikkari, 1963; Julin et al., 1975; Scott and Lawrence, 1970). However, the removal of the mobile phase was challenging for these detectors to have any practicality. In 1966, Horvath and Lipsky introduced the first ultraviolet

(UV) detector (Horvath and Lipsky, 1966), and in 1968, Kirkland improved the UV detector to achieve better sensitivity (Kirkland, 1968). Further improvements such as variable wavelength and diode array UV detectors had been introduced. Later, many different types of detectors (i.e., fluorescence (Fl), electrochemical (ECD), and more recently the evaporative light scattering (ELSD), and capacitively coupled contactless conductivity (C^4D) detectors) have been introduced. Table 1.3 summarizes the properties of these common LC detectors.

Table 1.3: Properties of common HPLC detectors (Scott, 2003)

Property	**UV-vis**	**Fl**	**RI**	**ELSD**	**ECD**	**C^4D**
Range of applications	Sel[a] & Uni[b] ($\lambda < 210$)	Very Sel	Uni	Uni	Very Sel	Sel
LOQ	ng	pg	μg	High ng	fg-ng	High ng

[a]Sel: Selective
[b]Uni: Universal

The selection of a suitable detector can lead to green analytical method. Therefore, many characteristics need to be considered in choosing a detector (Parriott, 1993; Scott, 1998; Katz et al., 1998; Blau and Halket, 1993), some of which are:

(i) High sensitivity and reproducibility.

(ii) Response to the analytes of interest.

(iii) Wide linear range.

(iv) Response should not be affected by changes in temperature, mobile phase and flow rate.

(v) Should not involve in extra peak broadening.

(vi) Non-destructive.

(vii) Reliable and convenient to use.

(viii) Provide both qualitative and quantitative information.

(ix) Fast response.

(x) Easy to handle.

(xi) Inexpensive.

(xii) Easy to service and inexpensive maintenance.

However, it is almost impossible to find one detector that can fit all of these characteristics. Therefore, and to fit with green analytical chemistry, multi-detectors have been designed. Recently, there has been an increased interest on the flow-cell development to provide less peak broadening and faster detector responses to achieve the requirements of the UHPLC system (Grumbach et al., 2009; Swartz, 2005).

Generally, there is no green or non-green detector, but the selection of suitable detector for the analytes of interest can be considered as green analytical approach. In the next sections, the ECD which is considered as the most common green detector (Koel and Kaljurand, 2006), and some of the recently developed detectors which have the elements of green methods will be highlighted.

1.3.3 (a) Electrochemical detectors (ECD)

ECD is one of the most sensitive and selective HPLC detectors available for analytes that can be oxidized or reduced (Ackworth, 1997; Kissinger and Heineman, 1984). The mobile phase in ECD should have electrical conductivity properties.