METHOD DEVELOPMENT FOR THE ANALYSIS OF BETA-CAROTENE AND ASTAXANTHIN BY CAPILLARY ELECTROPHORESIS

KHIN THIDA NYUNT

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Khin Thida Nyunt

Miss Khin Thida Nyunt
Candidate

Leena S.

Assoc. Prof. Leena Suntornsuk,
Ph.D. (Pharmaceutical chemistry)
Major-Advisor

Nongluck R.

Assist. Prof. Nongluck Ruangwises,
Ph.D. (Pharmaceutical chemistry)
Co-Advisor

B. Mahaisavariya

Prof. Banchong Mahaisavariya,
M.D.
Dean
Faculty of Graduate Studies

Ampol Mitrevej

Prof. Ampol Mitrevej,
Ph.D. (Pharmaceutics)
Chair
Master of Pharmacy
Program in Pharmaceutical chemistry
Faculty of Pharmacy

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on

October 4, 2007

Miss Khin Thida Nyunt
Candidate

Assoc. Prof. Leena Suntornsuk,
Ph.D. (Pharmaceutical chemistry)
Major-Advisor

Assoc. Prof. Worapot Suntornsuk,
Ph.D. (Food Science)
Chair

Assist. Prof. Nongluck Ruangwises,
Ph.D. (Pharmaceutical chemistry)
Co-advisor

Prof. Banchong Mahaisavariya,
M.D.
Dean
Faculty of Graduate Studies
Mahidol University

Prof. Ampol Mitrevej,
Ph.D. (Pharmaceutics)
Dean
Faculty of Pharmacy
Mahidol University

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Khin Thida Nyunt

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KHIN THIDA NYUNT 4837395 PYPE/M

M.Sc. in Pharm. (PHARMACEUTICAL CHEMISTRY)

THESIS ADVISORS: LEENA SUNTORNSUK, Ph.D., NONGLUCK RUANGWISES, Ph.D.

ABSTRACT

Oil-in-water (o/w) and water-in-oil (w/o) MEEKC were investigated for the separation of beta-cartoene and astaxanthin. Due to the instability of the carotenoids in acid pH (2.5), o/w MEEKC at acid pH was not suitable. O/W microemulsion with basic pH (9.2) gave poor separation and sensitivity for both carotenoids because of highly hydrophobic nature and solubility problems. The second approach, w/o MEEKC offered complete resolution of both carotenoids due to its unique separation mechanism. Development of w/o MEEKC optimum condition was investigated by varying injection time, oil and surfactant types, surfactant and water compositions, additional oils and capillary length. The w/o microemulsion buffer containing 9% (w/w) SDS, 80% (w/w) 1- butanol, 11% (w/w) 70 mM sodium acetate (pH 8), using temperature of 25°C, the separating voltage of -30 kV and the total capillary length of 32 cm (effective length 23.5 cm) was optimized. Detection was by a diode array detector at 475 nm with 40 nm bandwidth. Both carotenoids could be resolved within 9 min with a resolution of 4.9. Method linearity was good with r^2 of 0.997 for β-carotene and r^2 of 0.996 for astaxanthin over the concentration of 20-120 μg/ml. The method precision was excellent with % RSD of 3.1 % for migration time, 3.8 % for peak area of β -carotene and 1.1% for migration time, 3.4 % peak area for astaxanthin. Limits of detections were 3.5 and 4 μg/ml (%RSD= 5.3%) and limit of quantitations were 11.5 and 14 μg/ml (%RSD = 5.8 %) for beta-carotene and astaxanthin, respectively.

KEY WORDS: MICROEMULSION ELECTROKINETIC CHROMATOGRAPHY (MEEKC)/ BETA-CAROTENE/ ASTAXANTHIN

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
APCI	Atmospheric pressure chemical ionization
BGE	Background electrolyte
CE	Capillary electrophoresis
CEC	Capillary electrochromatogrphy
CGE	Capillary gel electrophoresis
CIEF	Capillary isoelectric focusing
CTAB	Cetyltrimethylammonium bromide
CZE	Capillary zone electrophoresis
DAD	Photodiode array detector
DCM	Dichloromethane
ED	Electrochemical detection
EKC	Electrokinetic chromatography
EOF	Electroosmotic flow
ESI	Electrospray ionization
GC	Gas chromatography
HPCE	High performance capillary electrophoresis
HPLC	High performance liquid chromatography
ISP	Ion spray tandem
ITP	Isotachophoresis
LDL	Low density lipoprotein
LOD	Limit of detection
MEEKC	Microemulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
MEOH	Methanol
MS	Mass spectrophotometry

LIST OF ABBREVIATIONS (Continued)

MTBE	Methyl tetrabutyl ether
NACE	Non-aqueous capillary electrophoresis
NM	Normal mode
NMR	Nuclear magnetic resonance
RM	Reverse mode
RMEEKC	Reverse microemulsion electrokinetic chromatography
ROS	Reactive oxygen species
RP	Reverse phase
SDS	Sodium dodecyl sulphate
TEPA	Tetraethylammonium perchlorate
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TLS	Thermal lens spectrophotometry
VIS-UV	Visble-Ultraviolet
σ	Standard deviation of peak width
E	Electric field
F_e	Electric force (Frictional force)
i.d.	Inner diameter
k	Capacity factor
kV	kilovolt
l	Effective capillary length
L	Total length of capillary tube
μ_a	Apparent mobility of the analyte
μA	Microampere
μ_e	Electrophoretic mobility
η	Viscosity of BGE

LIST OF ABBREVIATIONS (Continued)

N	Number of theoretical plates
o.d.	External diameter
o/w	Oil-in-water microemulsion system
q	Charge of the molecule
r	Ion radius
t	Migration time of analyte
t_0	Migration time of netral solute (EOF marker)
t_{ME}	Migration time of microemulsion droplet
t_R	Migration time of analyte
t_{EOF}	Migration time of EOF
ν_{ep}	Velocity of anlayte
V	Voltage
w/o	Water-in-oil microemulsion system
$w_{1/2}$	Temporal peak width at ½ height
wb	Baseline peak width

CHAPTER I
INTRODUCTION

Carotenoids containing polyisoprenoid structures are generally found in plants, algae, photosynthetic bacteria, non-photosynthetic bacteria, yeasts and molds. Nowadays, the major interest of carotenoids is not only due to their provitamin A activity but also to their antioxidant activity by scavenging oxygen radicals and reducing oxidative stress in the animals and human beings. Many studies show strong correlations between carotenoids intake and a reduced risk of some diseases such as cardiovascular diseases [1, 2], cancer [3-5], eye degeneration [6-8], bone calcification [9], modulator of immunological system [10-12] and neuronal damage [13].

Among the carotenoids, astaxanthin belonging to the xanthophyll class is gaining popularity because of its higher antioxidant power than all the other carotenoids [14] and antioxidants such as vitamin E [15]. Therefore, it is industrially produced by synthetically and from the natural sources. Synthetic astaxanthin dominates the world market but recent interest in natural sources has increased substantially because synthetic astaxanthin contains large amount of undesirable compounds (e.g. meso form) during the processing and its biological activity is less than that of natural astaxanthin. Common sources of natural astaxanthin are the green microalgae *Haematococcus pluvialis*, the red yeast *Phaffia rhodozyma* and crustacean byproducts [16, 17]. Disadvantage of astaxanthin from natural sources is that these sources produce not only astaxanthin but also other carotenoids. Therefore, it is also important to establish an improved method for the separation and determination of carotenoids.

Several methods have been developed for the determination of carotenoids, including thin layer chromatography (TLC), column chromatography, derivative spectrophotometry, Raman sepctrophotometry and high performance liquid chromatography (HPLC). However, using column chromatography and TLC methods is time consuming, needs large amounts of samples and poor reproducibility.

Determination of carotenoids by derivative spectrophotometry is less precise than other separation techniques. The disadvantage of Raman spectrophotometry is that it is not routine instrument. Although HPLC is most commonly used for the determination of carotenoids, it can be expensive due to its column and high amount of organic solvent consumption. Capillary electrophoresis has been recently used for the determination of carotenoids, especially capillary electrochromatography [18]. Capillary electrophoresis methods represent a category of the most rapidly developing analytical techniques at the present time because of its higher resolving power, simplicity, low cost and short analysis time in comparison to other techniques. However, their potential for the separation of carotenoids has not yet been fully explored.

Among CE methods, microemulsion electrokinetic chromatography (MEEKC) is a relatively new method and gives highly efficient and relatively rapid separation for both ionic and neutral compounds with a wide range of hydrophobicity. In MEEKC, both oil-in-water (o/w) and water-in-oil (w/o) microemulsion systems are employed as the separating media and the analytes are generally separated by depending on their partitioning between the microemulsion droplets and continuous phase. The separation mechanism is similar to micellar electrokinetic chromatography (MEKC), but it offers advantages over MEKC because microemulsion has better solubilizing power of highly hydrophobic compounds than micelle and it can perform custom tuning of elution of window [19, 20]. Therefore, it is recognized as a useful alternative to MEKC.

In MEEKC, o/w microemulsion systems (o/w MEEKC) are most commonly used because it is less complex than w/o microemulsion system (w/o MEEKC) and a standard set of o/w microemulsion system (e.g. 3% (w/w) sodium dodecyl sulphate, 0.81% (w/w) n-octane, 6.6% (w/w) 1-butanol and 89.59% (w/w) 10 mM borate buffer pH 9.2) can separate various analytes. On the other hand, although w/o MEEKC is not common like o/w MEEKC, it offers great potential to acquire the separation of a range of anlaytes, especially those analytes with similar partition coefficient (log *P*) values which can not be resolved in o/w MEEKC or MEKC and the analytes with strongly solubility problem with aqueous phase. A typical w/o microemulsion system consists of 10% SDS, 78% 1-butanol, 2% n-octane and 10% aqueous buffer [21, 22].

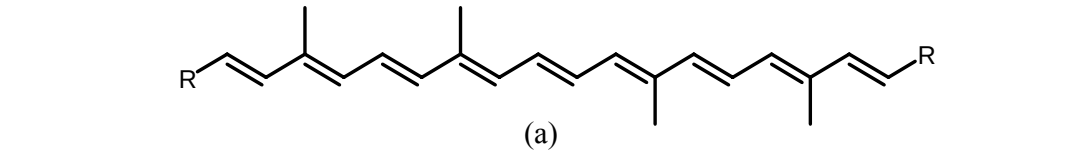
There are no reports on the separation of the carotenoids by using MEEKC techniques and this study was firstly developed. In this study, the development of both o/w MEEKC and w/o MEEKC was investigated for the separation of β-carotene and astaxanthin due to their highly non-polar property and highly poor solubility in water. And, the optimum method was validated.

CHAPTER II
LITERATURE REVIEW

1. Carotenoids

The term "carotenoids" refers to a family of more than 600 different plant pigments which are responsible for many colours (red, orange and yellow etc.) of plant leaves, fruits and flowers, as well as the colours of some birds, insects, fish and crustaceans. Some familiar examples of carotenoid coloration are the oranges of carrots and citrus fruits, the reds of peppers and tomatoes, and the pinks of flamingoes and salmon [23]. They are generally found in plants, algae, bacteria, yeasts and molds. Although human and animals are incapable to synthesize carotenoids in the body, they can uptake carotenoids from their diet.

Carotenoids are classified according to their chemical structures. The major carotenoids are derived from a 40-carbon polyene chain, which is the backbone of the molecule. The central portion of the molecule contains four isoprene units and this chain may be terminated by acyclic or cyclic ring (R) (Figure 1). Hydrocarbon carotenoids (i.e., carotenoids made up of only carbon and hydrogen) are collectively called carotenes (e.g. α-carotene, β-carotene) and those containing oxygen such as hydroxy, keto and epoxy are termed xanthophylls (e.g. astaxanthin, zeaxanthin) (Figure 1**)**. The polyene system gives carotenoids their distinctive molecular structures, chemical properties and light-absorption characteristics. Each double bond from the polyene chain may exist in two configurations; geometric isomers *cis* (*Z*) or *trans* (*E*). *Cis*- isomers are thermodymanically less stable than *trans* isomers. This is because in the *cis* form, the branching methyl (CH_3) groups and nearby hydrogen atoms on the linear portion of the molecule form greater steric hindrance. Therefore, most carotenoids found in nature are predominantly all *trans* isomers and only a few exhibit *cis*-*trans* configuration [24, 25]. In addition to geometric isomers, stereoisomers are found and about one half of natural carotenoids usually one to six chiral centers.



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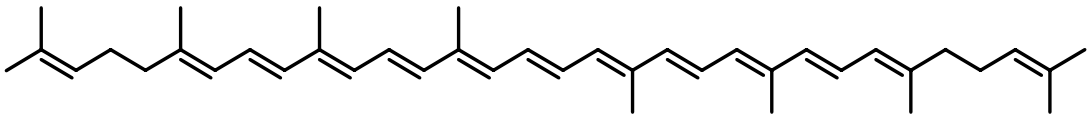

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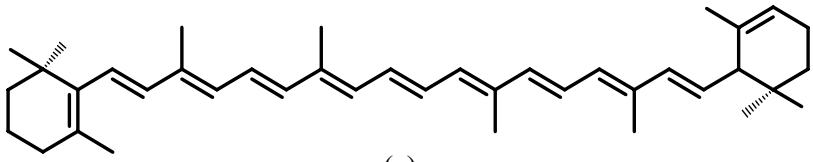

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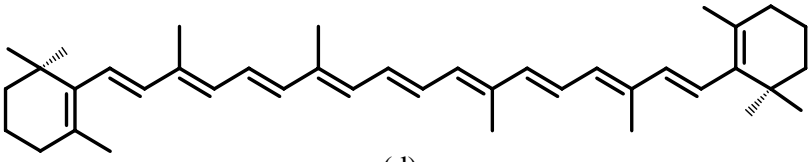

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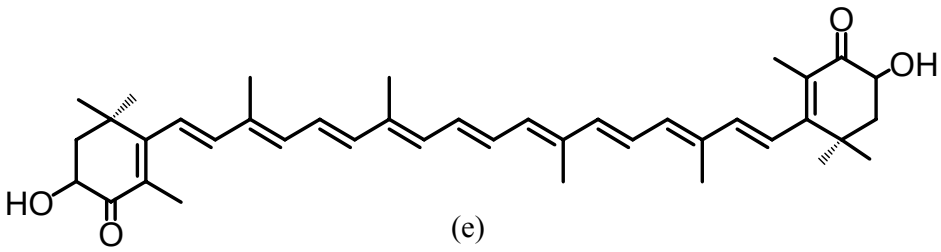

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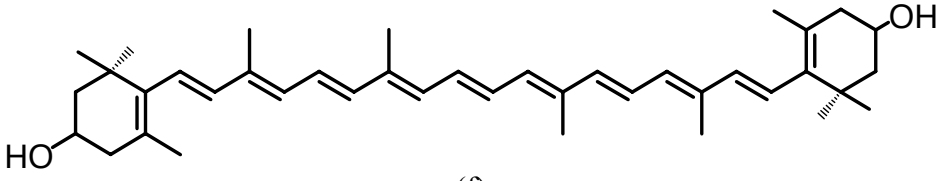

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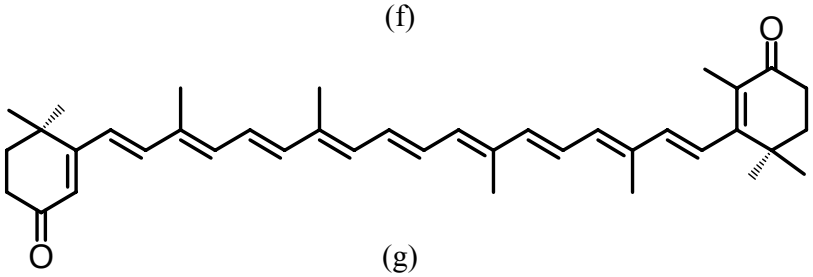

(g)

Figure 1 The skeleton of carotenoids (a) and structures of selected carotenoids (b) lycophene, (c) α-carotene (d) β-carotene (e) astaxanthin (f) zeaxanthin (g) canthaxanthin

1.1 Biological effects and health benefits of carotenoids

In human beings, carotenoids can serve several important functions. One of the most important physiological functions of carotenoids in human nutrition is to act as vitamin A precursors. Pro-vitamin A carotenoids support the maintenance of healthy epithelial cell differentiation, normal reproductive performance, and visual functions [6, 7]. Both pro-vitamin A carotenoids (e.g. α-carotene, β-carotene and cryptoxanthins) and non pro-vitamin A carotenoids (e.g. lutein, astaxanthin, zeaxanthin and lycopene) also play an important role in human health as biological antioxidants protecting cells and tissues from the oxidative damaging effects of free radicals and singlet oxygen. In mammalian and human cells, carotenoids protect from oxidative damage by two general mechanisms:

(1) quenching of singlet oxygen and dissipating the energy as heat [26-28]

(2) scavenging of radicals to prevent or terminate chain reactions [29, 30].

This capacity depends on the numbers of conjugated double bonds in the carotenoid molecule. In vivo, the carotenoid molecules are commonly located in various biological membranes because of their high lipophilicity. The localization and orientation of a carotenoid in membrane depends on its chemical structure and strongly influences on the reactions of the molecule with different radicals. Carotenes such as β-carotene and lycophene locate entirely within the hydrocarbon inner part of membrane. Thus, they are able to react efficiently only with the radicals generated from the inner hydrophobic part of the membrane. However, the presence of polar substituents in the carotenoids such as astaxanthin and lutein provides a profound effect because hydroxyl groups on the rings react with hydrophilic polar groups of phospholipids and the polyene chain is the interior of the membrane. This orientation enables the molecule to bind to the phospholipids membrane in a quite stable way, positioning itself crossway inside the phospholipids membrane and helps to react efficiently with radicals generated from the at/near the surface and interior part of the membrane [31]. Therefore, some of the carotenoids can be more effective than others as membrane based protective antioxidant.

Based on epidemiological studies, many studies show strong correlations between carotenoids intake and numerous health benefits such as

1. prevention of cardiovascular diseases by reduction in low density lipoprotein

(LDL) oxidation and oxidative stress at locations of plaque formations [1, 2]

2. protecting against developing certain types of cancers (e.g., lung, liver, , uterine,
3. skin, cervix, gastrointestinal tract) by inhibition cell proliferations, cell transformations and modulatation of the expression of gene determinants [3-5]
4. prevention of eye degeneration [6-8]
5. bone calcification [9]
6. function as booster and modulator of immunological system[10-12]
7. neurodegenerative diseases [13]

1.2 Astaxanthin

Astaxanthin (3, 3′- dihydroxy-β, β-carotene - 4, 4′- dione) is one of major pigments in the carotenoid family belonging to the xanthophyll class like lutein and zeaxanthin. It is highly liposoluble although its side rings have some polar substitute groups. It is not a vitamin A precursor. Its main role is to provide the desirable reddish-orange colour for some birds, crustaceans and salmons. Since these animals cannot synthesize astaxanthin, it must be added in their feed for them. The use of astaxanthin as a pigment for aquaculture and poultry industries is important not only from the standpoint of pigmentation and consumer appeal but also as an essential nutritional component for adequate growth and reproduction. In addition to its effect on colour, other several biological functions of astaxanthin have attracted more and more interest because of its health benefits to human beings.

. 1.2.1 Different forms of astaxanthin

Astaxanthin has several double bonds in the linear portion of the molecule and two asymmetric carbons at the 3 and 3′ position. This chemical features result in the existence of several different forms. They can be classified according to stereoisomers, two enantiomers (3R, 3′R and 3S, 3′S) and meso forms (3R, 3′S and 3S, 3R′) and several geometric isomers, *trans* (*E*) and *cis* (*Z*) forms [32-35]. The geometric isomers have been observed at positions 9, 13 and 15, singly or in combination such as all-*trans*, (9-*cis*), (13-*cis*), (15-*cis*), (9-*cis*, 13-*cis*), (9-*cis*, 15-*cis*),

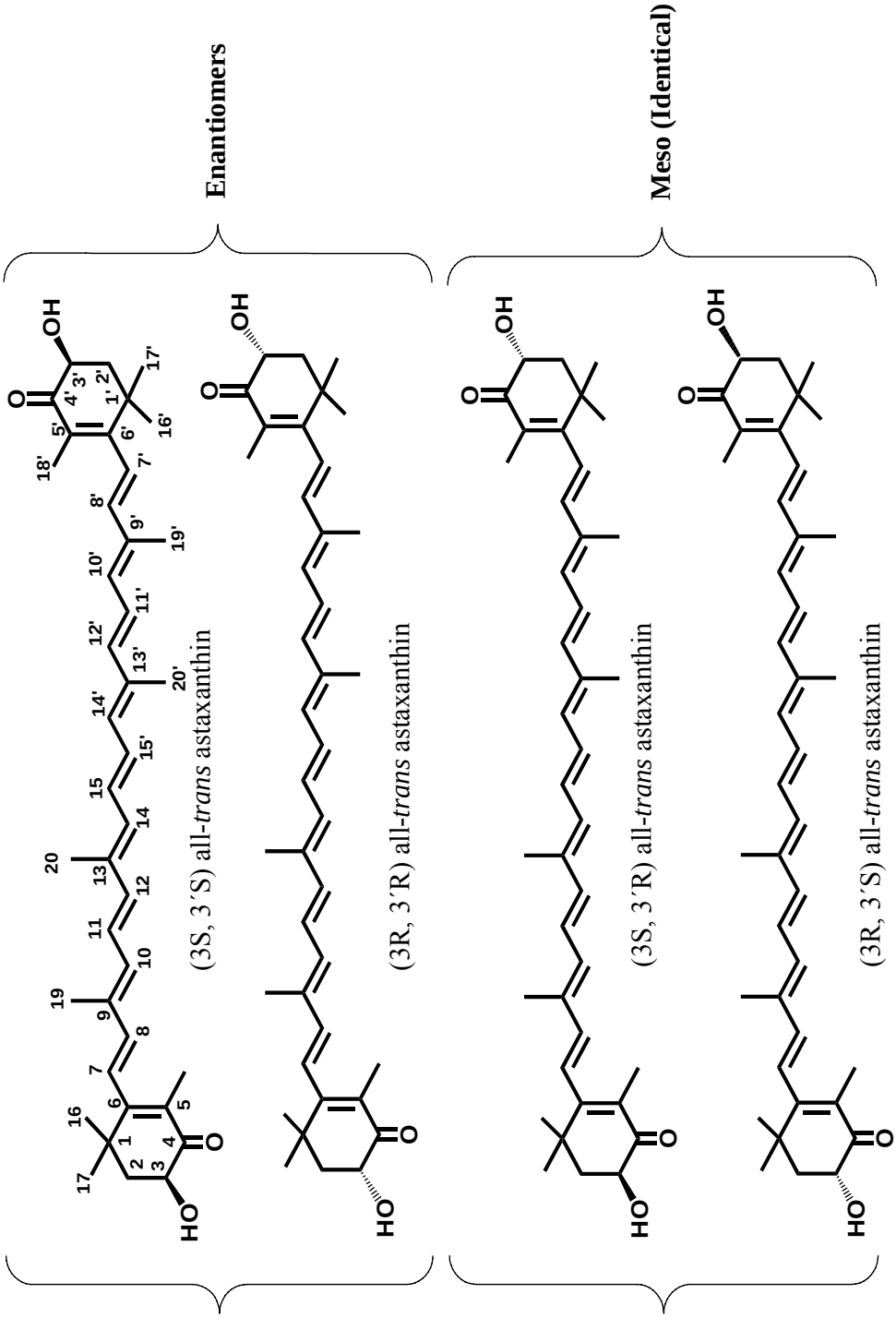

Figure 2 The stereoisomers of all-*trans* astaxanthins; the skeleton, xanthophyll; $C_{40}H_{52}O_4$, MW. 596.847, (Ref. 26)

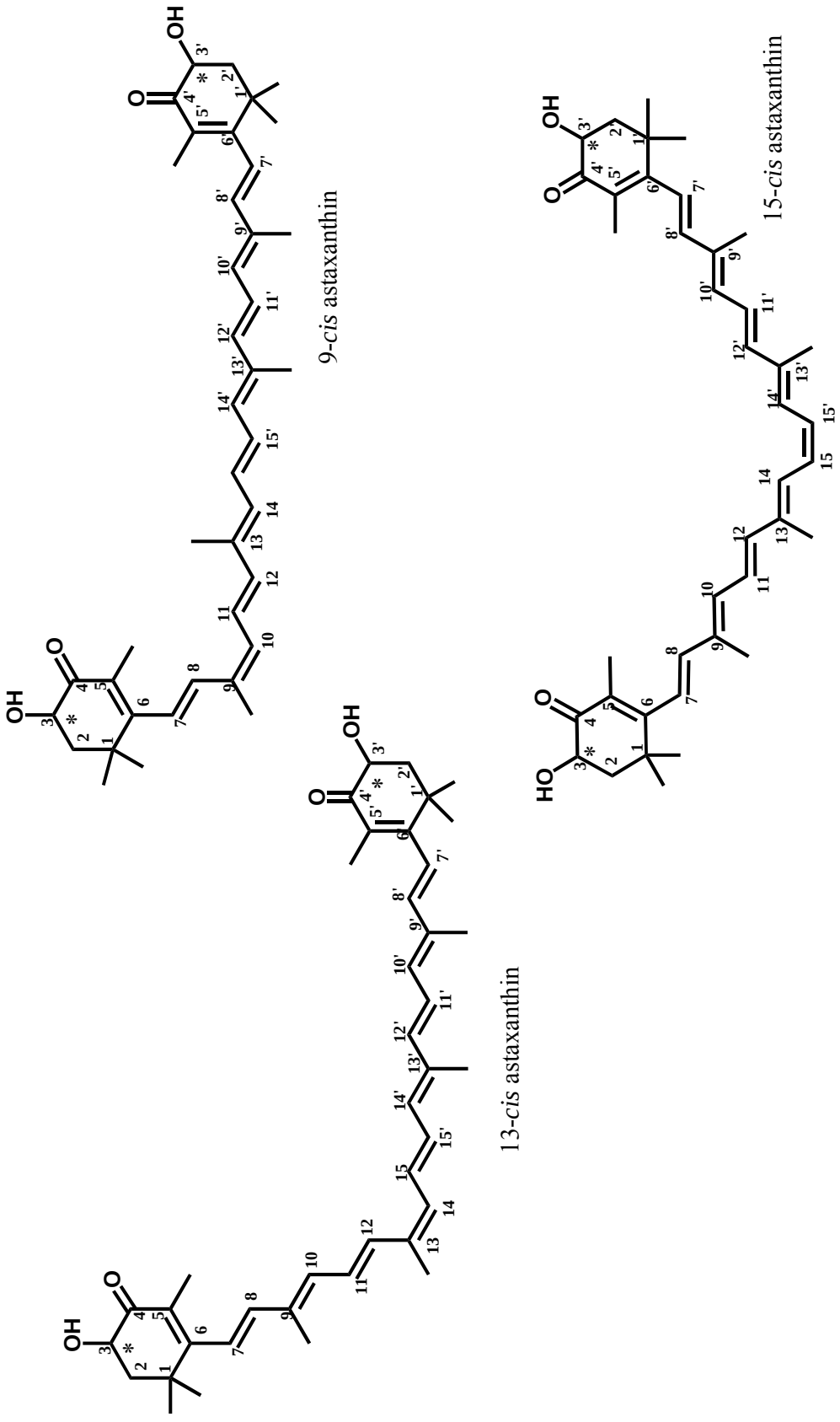

Figure 3 The structures of 9-cis-, 13-cis-, and 15-cis-astaxanthin. The chiral centers are designated by an asterisk (*), (Ref. 26).

(13-*cis*, 15-*cis*), (9-*cis*, 13-*cis*, 15-*cis*). Among all the isomers, the most abundant found in nature is the 3S, 3´S and all-*trans* isomers [33, 34, 36]. Synthetic astaxanthin consists of a racemic mixture of the two enantiomers (3R, 3´R and 3S, 3´S) and a meso form (3R, 3´S or 3S, 3´R) in the ratio of 1:1:2 [34]. Therefore, the meso form is abundant in synthetic astaxanthin. The structures of all-*trans* and *cis* isomers of astaxanthin are shown in Figure 2 and 3 respectively. In nature, based on their origins, all stereoisomers and geometric isomers of astaxanthin may be found in (1) free form (2) association with other compounds such as esterification with one or both hydroxyl groups with different fatty acids such as palmitic, oleic, stearic or linoleic acid [37, 38] or (3) chemically complex form with proteins (carotenoproteins) or lipoprotein (carotenolipoproteins) [33, 38]. For example, synthetic astaxanthin is not esterified, while that found in algae is always esterified [38].

1.2.2 Sources of astaxanthin

Most of the industrially used astaxanthin is synthesized chemically. The industrial producers of synthetic astaxanthin are Hoffmann-La Roche AG and BASF AG. Despite the availability of chemically synthetic astaxanthin, it may contain undesirable compounds such as unnatural configuration during the processing and the biological activity of synthesized astaxanthin is less than that of natural astaxanthin. Thus, the biological sources such as crustacean, crustacean extracts, algae and yeast become the important role to replace the synthetic astaxanthin. However, crustacean and crustacean extracts has very low concentration of astaxanthin and it can not compete economically with synthetic astaxanthin. The green microalga *Haematococcus pluvialis* and the heterobasidiomycetous yeast, *Xanthophyllomyces dendrorhous*, formerly known as *Phaffia rhodozyma*, are the only microbial sources with commercial potential for the production of astaxanthin [16, 17]. The *H. pluvialis* can produce high concentration of astaxanthin (0.2 to 2% on a dry weight basis) but industrial production is limited because autotrophic cultivation are manipulated in open freshwater ponds and the processes for disruption of the cell wall to liberate the carotenoids are required.

Although the concentration of the astaxanthin in *P. rhodozyma* is lower than that in green alga *H. pluvialis*, the yeast has the advantage of producing large amount

of astaxanthin through rapid self-propagation. More recently, other algae species such as *Chlorella vulgaris* and *Chlorococcum sp* was proposed as sources of astaxanthin. The former can produce the same amount as synthetic astaxanthin for pigmentation purposes [40] and the latter seems to be a promising source of astaxanthin as well as other carotenoids such as canthaxanthin and adonixanthin [40]. *Chlorella zofingiensis* was also investigated as a astaxanthin producer, however, the yield was relatively lower than *H. pluvialis* [41]. Some species of bacteria, *Brevibaterium Mycobacterium lacticola* [17, 42], marine bacteria *Agrobacterium aurantiacum* and *Alcaligenes sp* strain PC-1 also accumulated astaxanthin in considerable amounts [43]. A new aerobic gram-negative bacterium *Paracoccus carotinifaciens sp nov* were discovered to produce astaxanthin [44]. In addition to algae, yeast and bacteria, some of the fungi species, themselves, can produce astaxanthin [45]. Due to their low ability of astaxanthin production, they are more useful as a stimulator to increase the growth and to improve the production of astaxanthin for other microorganisms such as *P. rhodozyma* [46].

1.2.3 Astaxanthin and human health

Among the xanthophylls, astaxanthin molecule contains the longest polyene chain (with 13 double bonds compared to the 11 bonds of β-carotene) along with both hydroxyl and carbonyl groups at each end. This configuration provides significantly more antioxidant capacity than other carotenoids (10 times stronger than that of beta-carotene) [14] and antioxidants such as vitamin E (up to 100 to 500 times stronger than vitamin E) [15]. The astaxanthin molecule binds to the cell membrane like a bridge because its polar end groups span the cell membrane, thus, increasing its rigidity and mechanical strength and entraps free radicals by adding them to its long, double-bonded chain rather than donating an electron [31]. In fact, apart from inactivating free radicals, astaxanthin neutralizes singlet and triplet oxygen and inhibits all reactive oxygen species (ROS) [27]. Unlike other carotenoids, astaxanthin has many different mechanisms of antioxidant action at the same time. Due to its potent antioxidant activity, it may be beneficial in cardiovascular diseases [47, 48], immune response [12, 49, 50], anti-inflammatory [51, 52], skin health [53, 54] and anticancer [3, 55, 56]. Unlike β-carotene, astaxanthin is capable of crossing the

blood-brain barrier and can extend its antioxidant benefits beyond that barrier. Thus, it is very effective for the direct treatment of ocular diseases and an excellent candidate for testing neurodegenerative diseases such as Alzheimer's disease [57]. It encourages the release of vitamin A from the liver when needed and enhances the antioxidant actions of vitamin C [58] and vitamin E [59].

1.3 β-carotene

β-carotene is a part of the family of carotene class, which gives yellow and orange colour to fruits and vegetables. It is one of the most abundant found in the diet and is used as the colouring agents for food such as margarine. It is also one of the most widely studied carotenoid.

1.3.1 Different forms of β-carotene

β-carotene is a highly liposoluble hydrocarbon compound, which has a β-ionone structure as the terminal ring system at each side of the polyene chain. Unlike astaxanthin, since β-carotene has no asymmetric centers, stereoisomers can not be found. Most commonly found β-carotene naturally or synthetically are geometric isomers such as *all-trans*, 9-*cis,* 13-*cis* and 15-*cis* (Figure 4). Synthetic β-carotene is mainly consists of only one molecule, all-*trans* β-carotene. Natural β-carotene found in food and biological sources is made of two molecules, all-*trans* β-carotene and 9-*cis* β-carotene. However, the ratio of *trans* and *cis* β-carotene depends on the sources and can not be fixed. 13-*cis* and *15-cis* β-carotene can be found in a very small amount naturally and none synthetically.

1.3.2 Sources of β-carotene

The richest dietary sources of β-carotene are yellow, orange, and green leafy fruits and vegetables (such as carrots, spinach, lettuce, tomatoes, sweet potatoes, broccoli, and winter squash). In general, the greater the intensity of the color of the fruit or vegetable, the more β -carotene it contains**.** It can always be found along with α-carotene, lycopene, lutein, zeaxanthin and β-cryptoxanthin in food. Although β-carotene can be extracted from the various kinds of food and plant,

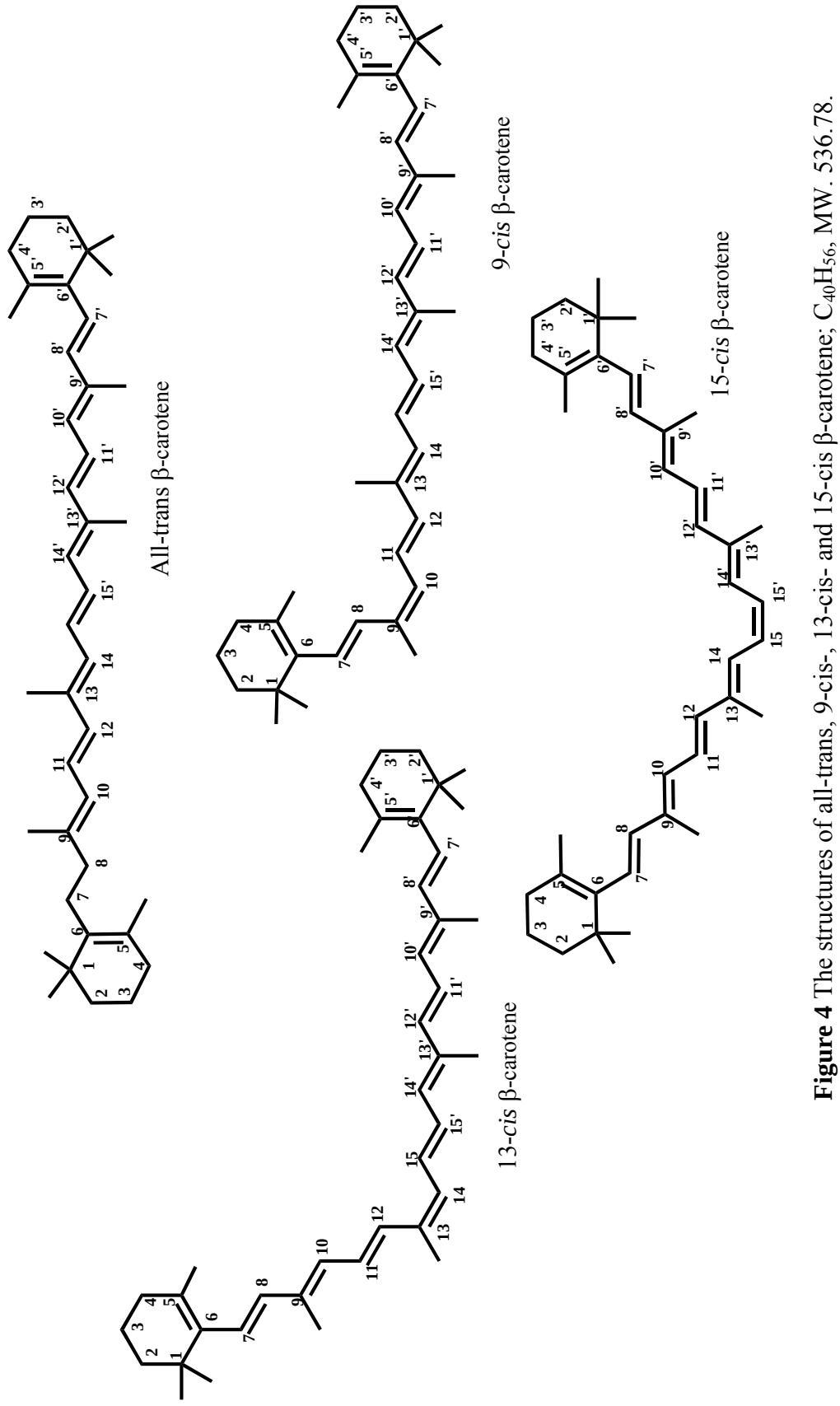

Figure 4 The structures of all-trans, 9-cis-, 13-cis- and 15-cis β-carotene; $C_{40}H_{56}$, MW. 536.78.

every extraction methods can not be justified for the commercial due to some levels of other carotenoids. Hence, dietary sources do not develop for the industrially production of β-carotene. There are two industrially predominant sources of production for β-carotene, synthetic manufacture (the main source for the market) and natural production. Several synthetic pathways have been developed for the industrial production of all-*trans* β-carotene such as BASF and Roche synthetic pathways [60]. As biological sources, microalgae such as *Dunaliella* and *Haematococcus* [16, 61], yeasts such as *Phaffia*, *Rhodotorula* and *Sporobolomyces* [62-64], fungi like *Blakeslea trispora* [65] and bacteria such as *Mycobaceterium sp, Brevibacterium sp, Flavobacterium sp* and *Micrococcus sp* [66-69] were used for the production of β-carotene. Among these, *Dunaliella* and *Blakeslea trispora* are two predominant commercially production sources. The quantity of β-carotene in naturally occurring strains of *P. rhodozyma and R. glutinis* is significantly low in comparison to other pigments. However, the hyperproduction of β-carotene from these yeasts could be performed by mutation programs [63]. Similarly, although the former carotenogenic bacterial sources produce a minor amount of β-carotene, their mutants could produce β-carotene in industrial amount by using recombinant DNA technology [69]. The differences between synthetic all-*trans* β-carotene and β-carotene from natural sources are significant according to the research evidences. This is because the 9-*cis* isomer which is lack in synthetic β-carotene is far more potent than the all-*trans* isomer as an antioxidant. For example, studies in both animals [70] and humans [71], the natural form showed antioxidant activity, but the synthetic form did not show the activity. Therefore, synthetic β-carotene in the supplement is used only as a vitamin A precursor.

1.3.3 β-carotene and human health

Carotenoids containing at least one unsubstituted β-ionone ring and a polyene chain are potential precursors of vitamin A. Thus, β-carotene is defined as a provitamin A. With the aid of dioxygenase enzymes, the human body can split one molecule of β-carotene into two vitamin A molecules which has many vital functions in the human body (1) the growth and repair to body tissues, (2) the formation of

bones and teeth (3) the resistance of the body infection and (4) the development of healthy eye tissues. β-carotene is a better source of vitamin A than vitamin A supplements because it is only converted to vitamin A based on the body requirement. Excess β-carotene is stored in the body and unlike vitamin A, it is not toxic when taken in amounts in excess of body needs. Like astaxanthin, it belongs to the polyene class of compounds, which accounts for its high reactivity as a free-radical inhibitor; hence, act as antioxidant [70-72]. In addition, it was investigated that β-carotene at high concentration (10 μM) can act as pro-oxidant [73]. β-carotene also improves immune function [10, 12, 74], increases lung function [75], reduces DNA damage [76], protects form the sun [77] and prevents the risks of some types of cancer [20, 78-80]. However, for people who drink and smoke excessively, β-carotene may increase their risk of lung cancer [81]. β-carotene rich diets also prevent cardiovascular diseases [2, 82].

2. Analytical techniques for determination of carotenoids

Classical-column chromatography and thin-layer chromatography (TLC) were originally used for the determination of carotenoids [83-85]. However, these methods are time consuming and require large amounts of samples. In addition, their separation efficiency and reproducibility are poor with low recoveries of the analytes. Therefore, TLC is mainly used for preliminary examinations to give an indication of the number and variety of carotenoids present and to help in the selection of a suitable separation and purification procedure for a given mixture

Classical spectrophotometric method can be used for the determination of total amounts of carotenoids. Information obtained by this technique is generally not sufficient for the quantitative analysis of the individual carotenoid because of the problem of spectra overlapping resulted from the chemical analogues that cause false results. Therefore, derivative spectrophotometry method has been investigated for the determination of carotenoid pigments. Third-derivative spectrophotometry method was developed to determine and quantify β-carotene and anthocyanoside [86]. Astaxanthin and β-carotene from *P. rhodozyma* was simultaneously determined by first-derivative ratio spectrophotometry [87].

Among the high performance separation methods, gas chromatography (GC) is not normally used because of low volatility and thermolability of carotenoids. Therefore, high performance liquid chromatography (HPLC) is commonly used for the determination of carotenoids. There have been several reports for the determination of different carotenoids by HPLC with C_{18} or C_{30} reverse phase (RP) column operated with an isocratic or a gradient elution using the mixtures of different organic solvents as mobile phase and different detectors such as VIS-UV, DAD, MS, NMR, TLS and ED. Selected HPLC methods for analysis of different carotenoids from different samples were shown in Table A1 (See Appendix) [88, 89, 90-100]. Capillary zone electrophoresis (CZE) is inapplicable because of the absence of charge on the carotenoid molecules. However, capillary electrochromatography (CEC) is a very promising method for carotenoid analysis. Highly hydrophobic carotenes, beta-carotene and lycophene and xanthopyll, lutein in vegetables was effectively separated in a short analysis time by CEC with a Hypersil ODS packed column [18].

Comparison to other methods, Raman spectroscopy exhibits not only good detection limits, but also allows non-invasive analysis. This method was used for detection of carotenoids in human skin [101], in the human eye [102], and in liver corpus luteum cells [103].

3. Capillary electrophoresis

Capillary electrophoresis (CE) has been developed into an extremely powerful analytical technique in recent years [104-107]. Along with advances in instrumentation and separation methodologies, a wide range of applications has been developed in many areas including chemical, biotechnical, pharmaceutical, and environmental analysis.

3.1 Theory and principle of capillary electrophoresis

Electrophoresis is a separation method based on the differential movement of ions by attraction or repulsion in an applied electric field. In high-performance capillary electrophoresis (HPCE), the components of typical instrument include a capillary, power supply, electrodes, two buffer vials and a detector (Figure 5).

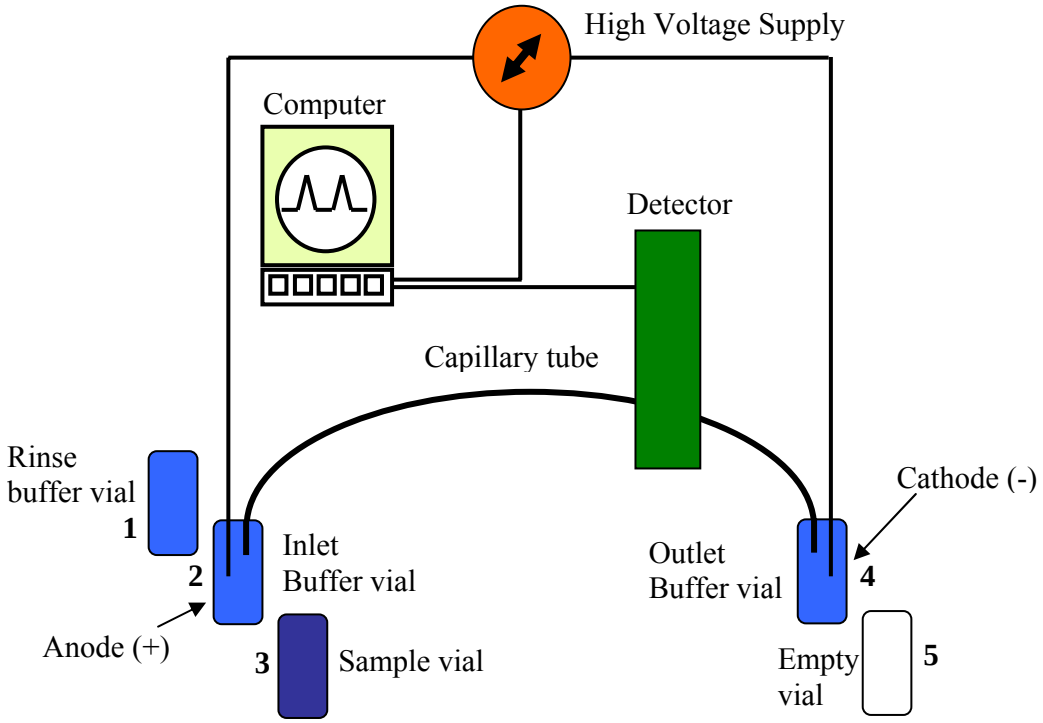

Figure 5 Schematic of basic capillary electrophoresis components.

Separation is commonly performed in narrow-bore fused silica capillaries, typically of < 100 μm inner diameter (i.d.), 300-400 μm external diameter (o.d.), ranging in length from 32 to 75 cm and externally coated with polyimide to get flexibility to the normally brittle glass. The polyimide coating, which is also used for GC column, is easily burned away to create an optically transparent, on-column detection window. The high voltage power supply provides up to 30kV with current level of 200 to 300 μA in either normal or reversed polarity mode. Under normal polarity, the anode is located at the sample injection end and the cathode is located towards the capillary outlet (near the detector). The potential is established via two platinum electrodes. Injection of sample into the capillary can be performed via three ways: (1) applying pressure (2) applying vacuum (or siphoning) or (3) applying voltage. The most commonly used detectors are VU/Vis and fluorescence detectors which are commercially available. The capillary ends are immersed into the buffer vials and the capillary tube is filled with buffer from the buffer vial at the capillary inlet before sample injection. And then, a specific quantity of sample is introduced into the capillary from the inlet by temporarily changing of the buffer vial with a

sample vial. After injecting the sample, the electric filed is applied and a bulk flow (electro-osmotic flow) will be generated from anode to cathode under normal polarity conditions. Separation is carried out by both electrophoretic mobility of the analytes being separated and electroosmotic flow (EOF) [104-107].

The electrophoretic mobility (μ_{ep}) is the migration of the analyte with a constant velocity under the influence of electric field. This mobility mainly depends on the electric force (F_e) which enhances the migrating of the analytes toward the opposite charged electrode and frictional force (F_f) which makes the movement of the analytes to be slow down. Both of the these two forces are in turn dependent on the charge of the molecule (q) , the ion radius (r) and the viscosity of the background electrolyte solution (η). Therefore, the electrophoretic mobility can also be described as noted in equation (1) by the application of stoke equation;

$$\mu_{ep} = \frac{q}{6\,\pi\eta r} \qquad (1)$$

According to the equation 1, a small molecule with greater charge will migrate more rapidly than a large molecule with lesser charge [104-107]. When the two former forces are equal at one time, the analyte migrates with constant velocity (ν_{ep}) which is proportional to the electrophoretic mobility and applied electric field ((E =V/L where V is the voltage applied over the total length of the column L).

$$\nu_{ep} = \mu_{ep}E \qquad (2)$$

Thus, the greater the electric field, the faster the anlaytes will migrate through the column. However, the magnitude of the electric field that can be applied through the capillary is limited by the amount of Joule heat generated by ions in the background electrolyte (BGE). All ions present in the BGE migrate in a manner governed by the equation 2. This migration generates heat (Joule heat) due to the frictional force of their movement in solution. This joule heat can cause not only the temperature increases but also create non-uniform temperature gradient in the buffer solution which produces convective, parabolic and/or non-uniform flow. Since these effects severely degrade analytical performance, analyses are typically conducted at minimal Joule heat ($\leq$ 1.5 W/m) [104].

One of the most important characteristics of CE is the electroosmotic flow (EOF). EOF is the bulk flow of electrolyte solution through the capillary. The capillary surface wall is composed of acidic silanol groups (Si-OH) which can partly dissociate to the silanol anions ($Si\text{-}O^-$) above pH 2 and give negative charge to the capillary surface [104-107]. The pH of the BGE solution can determine the extent of ionization and overall charge on the capillary wall. The negatively charged capillary wall attracts to cations from the BGE to maintain charge balance and form an immobilize cation layer along the wall by electrostatic forces. Since this layer does not electrically neutralize all the negatively charged silanol groups, the remaining cations in BGE accumulate at the wall and form another loose (diffuse) cation layer which is adjacent to the immobile layer. Hence, three layers, negatively charged silica, the immobile layer and the diffuse layer of cations become develop between the fused-silica tube wall and the buffer. Once the voltage is applied, the diffuse layer of solvatized cations migrates toward the cathode by drawing the bulk BGE solution with them. This result bulk flow of BGE solution from anode to cathode is called the EOF (Figure 6). Unlike pressure-induced or laminar flows that found in HPLC and GC, EOF in the capillary provides very flat flow profile (like plug flow) because the driving force of the flow originates from the capillary wall and no pressure drop within the capillary (Figure 7). Due to it flat flow, the flow velocity near the capillary wall is the same as that in the centre of the capillary. As a result, radical diffusion of the analytes between the capillary wall and centre can not be occurred and higher resolution with less band broadening of the analyte peaks is obtained.

The velocity of electroosmotic flow depends on the dielectric constant of the buffer (ε), the buffer viscosity (η), the electric field (E) and the zeta potential (ζ) as noted in equation (3) [126-129].

$$\nu_{eo} = \frac{\varepsilon\zeta}{4\pi\eta} \qquad (3)$$

Successful separations are usually achieved by manipulation of EOF. The key factors that influence EOF are applied voltage, buffer pH, temperature, buffer concentration or ionic strength, and buffer composition (including the presence or absence of organic modifiers) [104-107]. The applied voltage, temperature and buffer

pH have the linear relationship with the velocity of EOF. Among these, the pH of the buffer significantly affects on EOF. EOF at high pH is much stronger than at low pH because silanol groups on the capillary wall are more ionized and increase the zeta potential. The buffer concentration or ionic strength alters the zeta potential through the capillary according to double layer theory.

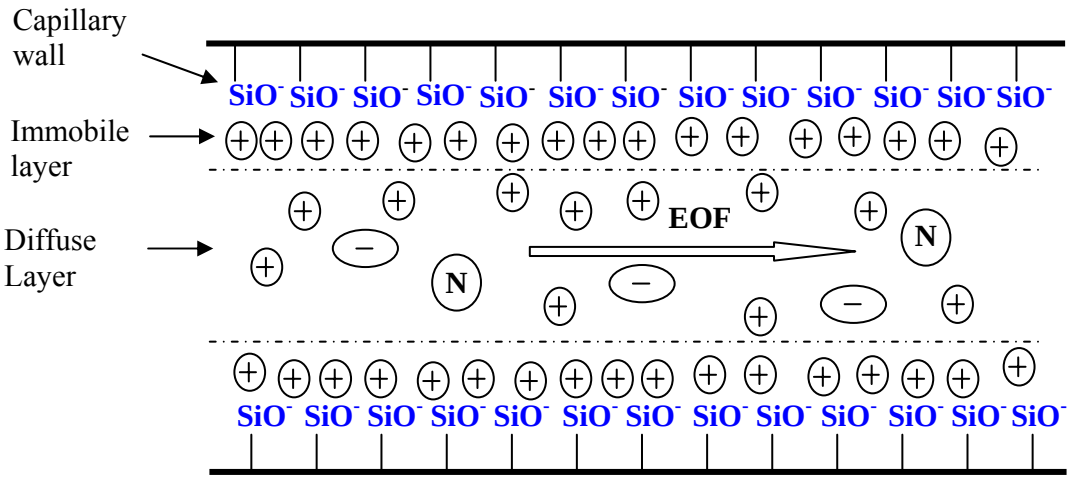

Figure 6 Electrical double layer at the capillary wall and creation of electro-osmotic flow. (N= Neutral analytes).

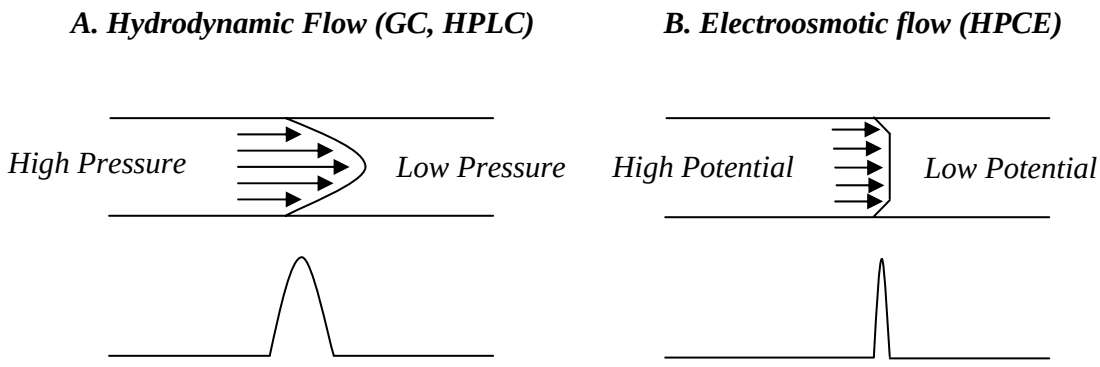

Figure 7 Comparison of (A) hydrodynamic flow profile and (B) Electroosmotic flow profile.

Increased ionic strength forms compact double layer at the capillary wall which cause rapid potential decay, decrease zeta potential and EOF. In general, addition of organic modifiers decreases the EOF because of their high viscosity and decreasing

the dissociation of the surface silanol group. The direction of EOF can be suppressed or reversed by either addition of cationic surfactants or capillary wall coating such as dynamic wall coating and permanent wall coating.

Without EOF, anions and cations move to the oppositely charged electrodes and neutral analytes can not move at all. Therefore, it is not possible to separate them simultaneously without EOF under the electric field in a single run. Generally, the strength of EOF is greater than the electrophoretic mobility of each analytes and carries them in the same direction (towards the detector), even anions migrate towards the cathode. In normal polarity mode, cations always migrate faster than anion and neutral analytes because their movement is enhanced by the EOF. After the cations, neutral analytes, which have no charge and no eletrophoretic mobility, are eluted with EOF. Finally, the anions are eluted since their electrophoretic mobility is opposite to EOF direction. The observed mobility of analyte in the presence of the EOF is called the apparent mobility ($\mu_a = \mu_e + \mu_{EOF}$). It can be calculated by the following equation;

$$\mu_a = \frac{l}{tE} = \frac{l\,L}{t\,V} \qquad (4)$$

where V is the applied voltage, l is the effective capillary length, L is total capillary length, t is the migration time of the analyte and E is the electric field. The separation efficiency and resolution are related to the direction and flow of EOF. It is expressed in number of theoretical plates N and can be experimentally determined by using:

$$N = 5.54\,\left(\frac{t}{w_{1/2}}\right)^2 \qquad (5)$$

where t is the migration time of the analyte, $w_{1/2}$ is temporal peak width at ½ height. In practice, the measured efficiency is usually lower than the calculated efficiency because theoretical zcalculation only accounts for zone broadening due to longitudinal diffusion. Zone broading (dispersion) can be defined as the baseline peak width , w_b;

$$w_b = 4\sigma \qquad (6)$$

where σ is standard deviation fo peak width (in time, length, volume).

3.2 Non-electrokinetic chromatographic separation modes

3.2.1 Capillary zone electrophoresis

Capillary zone electrophoresis, also known as free-solution CE (FSCE), is the simplest mode of CE and especially used for the separation of ionic species. The separation mechanism is based on differences in the charge-to-mass ratio of the analytes. In this mode, uniformity of the buffer solution and stable field strength throughout the length of the capillary are essential for the separation system. Disadvantage of this mode is that it can not separate neutral analytes.

3.2.2 Isotachophoresis (ITP)

Isotachophoresis is different from other CE modes because discontinuous buffer is used instead of uniform buffer. The samples are separated into zones between fast leading electrolyte solution and slow terminating electrolyte solution based on their individual migration rate. Disadvantage of this mode is that both cations and anions can not be separated at the same time. It is principally used as transient ITP for the on-line sample preconcentration.

3.2.3 Capillary isoelectric focusing (CIEF)

Capillary isoelectric focusing is mainly used for the separation of amphoteric molecules such as proteins based on their isoelectric points (Ip). The separation is occurred due to creating pH gradient in the capillary tube (in the absence of EOF). The solutes become net charge zero at its Ip and stop migration. As a result, the solutes focus into a tight zone and migrate towards the detector by either application of pressure or chemical means.

3.2.4 Capillary gel electrophoresis (CGE)

Capillary gel electrophoresis uses polymer network (e.g. polyacrylamide) as a sieving medium to perform size separation. When the molecules pass through the sieve medium based on their size, the migration of larger molecules is more delay than the small molecules because of their greater difficulty fitting with the sieve pores. This technique can resolve the analytes with the same charge to massratios, which are difficult to separate by other modes, according to their different

size. It is commonly employed in SDS-gel molecular weight analysis of proteins and the sizing applications of DNA sequencing and genotyping.

3.2.5 Non-aqueous capillary electrophoresis (NACE)

Non-aqueous capillary electrophoresis is somewhat similar to CZE except using non-aqueous (organic) solvents instead of aqueous media. The choice of organic solvents is the vital role in the selectivity because their physicochemical properties such as viscosity and dielectric constants are significantly affect on both ion mobility and the intensity of EOF. The use of a non-aqueous medium is beneficial for the separation of water-insoluble compounds.

3.2.6 Capillary electrochromatography (CEC)

Capillary electrochromatography is a separation method in combination of CZE and HPLC because the capillary tube is packed with the stationary phase materials which are the same as in HPLC and the separation is performed with electric field like CZE. The separation is based on both electrophoretic and chromatographic differences. The advantage of this technique is that backpressure of the column is very low in comparison to HPLC and it is possible to use small-diameter packing, which gives very high efficiencies. It is a technique with tremendous potential especially in the pharmaceutical and biomedical fields.

3.3 Electrokinetic chromatography (EKC) separation modes

The separation mechanism of electrokinetic chromatography is combination of electrophoresis and chromatographic separation. This mode is also similar to HPLC since it involves pseudostationary phase (PSP) used as secondary chromatographic separation. The main difference between EKC and HPLC is the stationary phases that used as the chromatographic selectors. The chromatographic selectors in HPLC are fixed and chemically bound to the column whereas those in EKC are moving and not stationary. Hence, the fixed chromatographic selectors are called true stationary phase and in EKC, those are called pseudostationary phase.

A variety of materials that can be used as PSP are anionic, nonionic, zwitterionic, and cationic surfactants[108-110], macrocyclic and macro-molecular

phases [111, 112], micelle polymers [113], polymer surfactants[114], vesicles [115], resorcarenes [116], dendrimers [117], polymer ions [118], ionic liquid [119] and microemulsions [19-22, 120-137].

Generally, EKC has two modes called normal migrating EKC (NM-EKC) and reversed migrating (RM-EKC) depending on the migration behavior of the pseudo-stataionary phase. Generally, NM-EKC is most commonly used and in which, EOF moves faster than PSP whereas in RM-EKC, the latter is faster than the former. EKC depends on the greater or lesser affinity of different solutes to the PSP which influences separation selectivities. Hence, the two modes can be assumed the analogous of normal-phase HPLC and reverse-phase HPLC respectively. Most commonly used EKC modes are micellar electrokinetic chromatography (MEKC) and microemulsion electrokinetic chromatograpy (MEEKC). These methods can be used for the separation of not only neutral solutes but also charged species.

3.3.1 Micellar electrokinetic chromatography (MECK or MECC)

In MEKC, micelles are used as a pseudostatiaonary phase. Therefore, the surfactant with a concentration higher than the critical micelle concentration (CMC) (individual surfactants aggregate to form micelle) is added to the background electrolyte (BGE) to achieve the separation. The analytes are separated based on their different partitioning between the micelle phase and aqueous background electrolyte phase. Although MEKC is especially used for the separation of hydrophobic neutral analytes which are very difficult to separate in capillary zone electrophoresis, charged species can also be separated successfully. Therefore, MEKC becomes great utility and valuable in the separation of very hydrophobic pharmaceuticals from their very polar metabolites**.**

While micelles are simple to create and small in size (3 to 5 nm), vesicles are formed from either double–tailed surfactants or non-stoichiometric combinations of appositely charged single-tailed surfactants. The resulting structure can be either unilamellar or multilamellar in nature, surrounding one or more internal cavities of solvent (Figure 8). Vesicles are also very attractive pseudostataionary phases because they offer larger elution ranges than micelles and form bilayer structures which function as a tool to study biological membrane process.

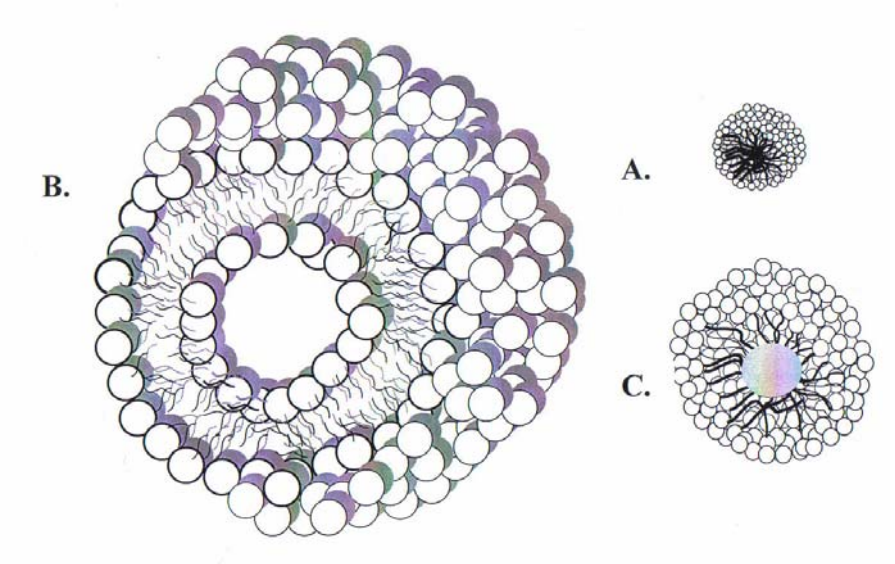

Figure 8 Representative cross-sections comparing various surfactant aggregates (A) Micelle (B) Vesicle (C) Microemulsion.

3.3.2 Microemulsion electrokinetic chromatography (MEEKC)

Microemulsion electrokinetic chromatography (MEEKC) is very similar to MEKC except microemulsion is used as a pseudostationary phase (PSP). Analytes are separated based on both their affinity to microemulsion droplets and electrophoretic mobilities. This method gives high efficient separations for not only neutral analytes but also charged species covering a wide range of water solubilities. Microemulsions were originally used in a variety of non-analytical application. Watarai first accessed them as PSP in microemulsion electrokinetic chromatography (MEEKC) [121]. Recently, microemulsion electrokinetic chromatography has been profitably applied to the separation of pharmaceuticals [22, 122], biomolecules [123], vitamins [124], environmental analysis [125], steroids [126], natural products [127], bioanalysis [128], chiral separations [129] and also partition coefficient determinations [130].

Microemulsion (it is called microemulsion although the droplet size is in a range 10- 100 nm is thermodynamically stable, isotropic, optically transparent solution and its lifespan is microsecond [131]. Although microemulsion droplet shapes are diverse, sphere shape is most common. The range of the shape can be known by measuring the polydispersity of microemulsion [132]. Lower

polydispersity indicates that the droplets are highly spherical and highly organized microemulsion is obtained. In contrast to micelle and vesicles, microemulsions are not only surfactant-based. Microemulsions are macroscopically homogenous mixture of surfactant, oil, water and often co-surfactant. Since there is high surface tension between oil and water, they are not feasible to mix wholly. Therefore, surfactant (amphiphile) is added to reduce the surface tension which allows formation of a stable microemulsion. Nevertheless, mixing of the specific ratio of surfactant, oil and water concentrations are required to obtain the stable emulsion. A co-surfactant such as short chain alcohol is often added to get better stability of microemulsion because it can prevent charge repulsion of surfactants which leads to less efficient packing and also decrease interfacial tension between oil and water. If the relative amount of surfactant, oil, co-surfactant and water are appropriate, the microemulsion system is greatly stable since its surface tension is at a significant lower energy and remains intact indefinitely. If the microemulsion system is unstable, it will separate into two liquid layers, oil and water, in a short period.

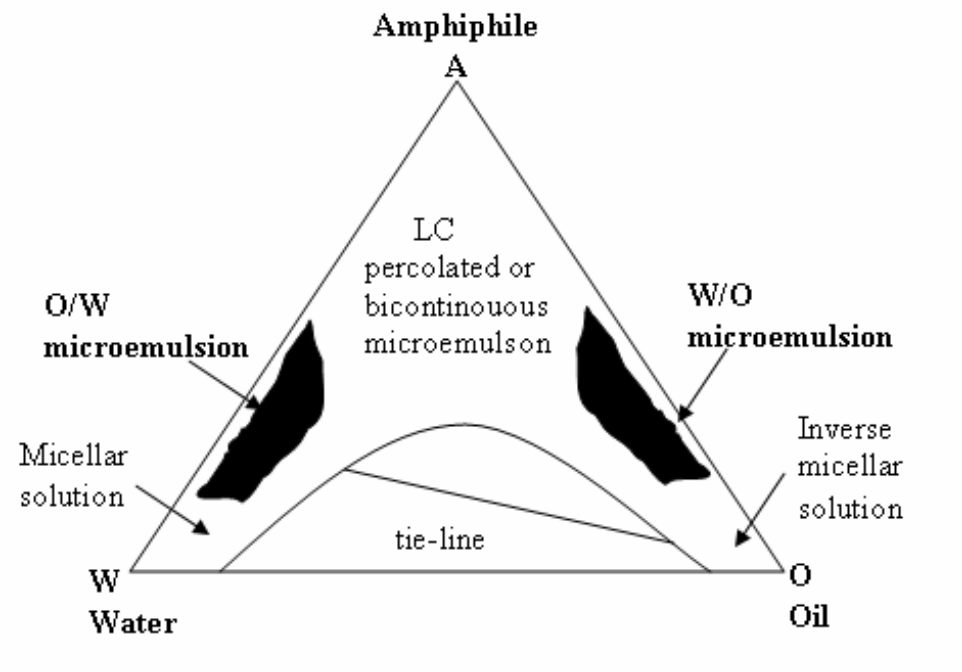

Figure 9 Phase diagram of water-surfactant-oil system (Ref. 133).

According to phase diagram of an oil-surfactant-water system (Figure 10), two regions of microemulsion can be seen, oil-in-water (o/w) and water-in-oil (w/o) [133]. Typically, oil-in-water microemulsions are most commonly used in microemulsion electrokinetic chromatography because they have suitable physical properties

3.3.2.1 Oil-in-water microemulsion electrokinetic chromatography (O/W MEEKC)

The microemulsion buffers used in o/w MEEKC are composed of minute water-immiscible oil droplets suspended in an aqueous buffer. Figure 10 shows the diagram of oil-in-water (o/w) microemulsion droplet composed of sodium dodecyl sulphate (SDS, surfactant), octane (oil droplet), short chain alcohol (butan-1-ol; co-surfactant) and the sodium ions in the aqueous phase surrounding the droplet. The non-polar part (alkyl chain) of surfactant is oriented into the oil core and its polar head (negatively charged sulphate groups) remain in the aqueous microemulsion phase. Thus, oil droplets acquire negative charges and it will migrate towards the anode due to its electrophoretic mobility which directs to the opposition of EOF. Like MEKC, the separation of the analytes depends on their partition coefficient between the oil droplets and aqueous phase of microemulsion.

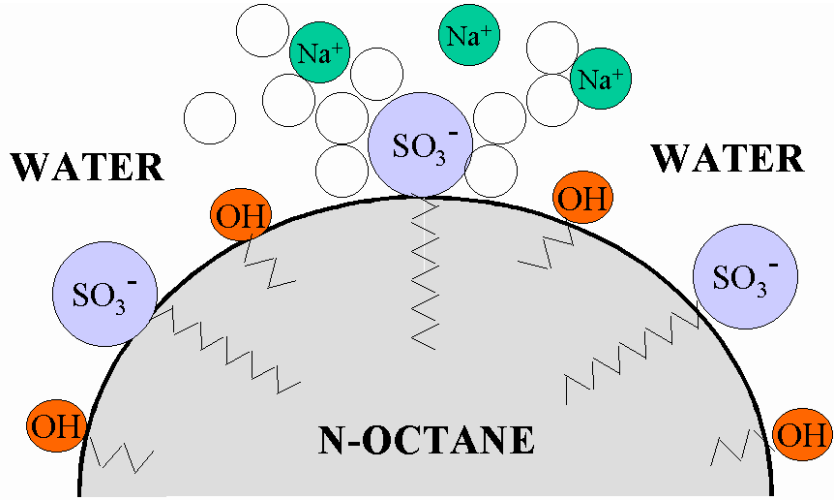

Figure 10 Schematic of oil-in-water (o/w) microemulsion droplets (Ref. 20)

The borate or phosphate buffers at high pH are generally used for aqueous phase. Due to high pH background electrolytes, EOF move from anode to cathode through the detector when the voltage is applied. In addition, at high pH, EOF is stronger than the electrophoretic mobility of oil droplets. Eventually, EOF sweeps the oil droplets to the cathode (detector end) although negatively charged oil droplets attempt to migrate towards the anodes. As a result, two phases with different migration rates are established and separation is accomplished by the different migration. The longer the analytes spend in the oil droplets, the more time it will take to reach the detector. The above separation process is illustrated in Figure 11 [20].

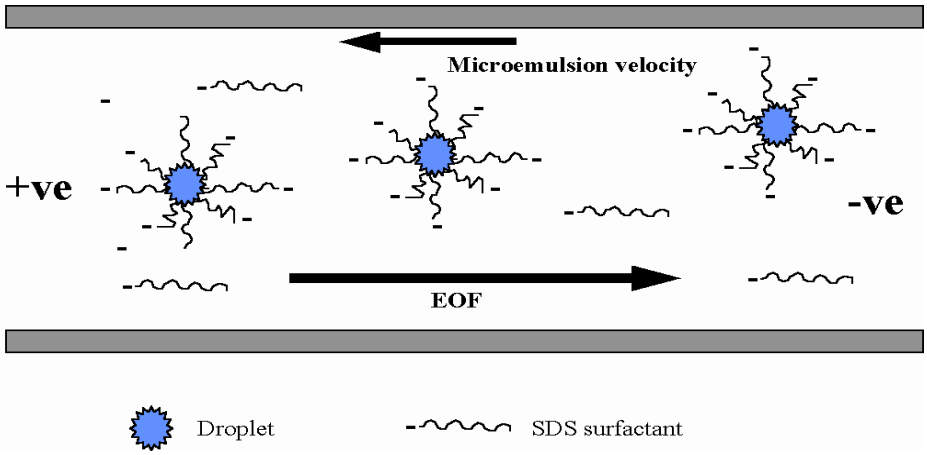

Figure 11 Schematic of MEEKC separation process (Ref. 20).

Extremely water-soluble neutral analytes such as methanol and dimethyl sulphoxide are entirely unretained in oil droplets and, hence, will largely prefer to the aqueous phase rather than partitioning into the oil droplets. Thus, these unretained analytes will migrate towards the cathode with EOF. The migration time of these analytes can be defined as t_{EOF} i.e. the time taken to reach detector from the injection end. Conversely, highly water-insoluble analytes such as dodecylbenzene will be strongly held in the oil droplets with high retention factor and can be used as t_{ME} marker. Other analytes with moderate polarity will be equilibrium partitioning between the oil droplets and aqueous phase. Their migration time of these analytes (t_R) may be between t_{EOF} and t_{ME} and can be described as the following equation.

$$t_R = \left(\frac{1 + k}{1 + \frac{t_{EOF}}{t_{ME}} k} \right) t_0 \qquad (7)$$

where k is the retention factor of the neutral analyte which is proportional to its equilibrium distribution constant between the two phases and can be calculated as the following equation.

$$k = \frac{\frac{t_R}{t_{EOF}} - 1}{1 - \frac{t_R}{t_{ME}}} \qquad (8)$$

However, for the practical purposes, k is calculated as follow;

$$k = \frac{t_R}{t_{EOF}} - 1 \qquad (9)$$

If the analytes can be ionized in the microemulsion background electrolyte, ionic interaction is formed between the charged species and charged oil droplets. If the analytes are anions, they can develop charge repulsion with the anionic oil droplets, which leads to low affinity for the pseudostationary phase and migrate towards the detector by their electrophoretic mobility. For the cationic solutes, ion-pair interaction with the negatively charged oil droplets can be formed and absolutely penetrate into microemulsion droplets. Thus, they will migrate to the detector with both their electrophoretic mobility and chromatographic manner.

Although a microemulsion system is more complicated than micellar solution, it has advantages than the latter. Micelle is composed of only surfactant monomer and their surface is rigid while in microemulsion, the presence of additional oil core provides the droplets larger and less rigid than micelles (Figure 9). Thus, analytes are more straightforwardly diffuse through the surface and enter into the oil droplets. Consequently, MEEKC can be utilized for very highly hydrophobic solutes which offer problems such as low solubility and precipitation during separation in MEKC. Due to its high solubilizing power, MEEKC can be used for the separation of analytes with a wide range of solubility. Another advantage is that the separation window is significantly larger than that of MEKC due to greater mobility of the droplets in comparison to micelle and it can be modified by either altering the surfactant

concentration or combining ionic surfactant with neutral surfactant. For example, increasing the surfactant concentration triggers greater opposition to the EOF because of increasing charge density on the droplets and result in larger elution window of the system. The last advantage is that MEEKC gives better separation efficiencies than MEKC.

A variety of different water-immiscible solvents used as the oil core of microemulsion are short chain hydrocarbon such as hexane [134], heptane [123, 126] or octane [122, 124, 125, 128]. The disadvantage in using these oils is that they require high concentration of surfactant to reduce the interfacial tension sufficiently to allow the droplet formation. Since the surfactant monomers are ionized, increasing their concentration results increase the current and joule heat of the system. Therefore, lower operation voltages are required. The lower the voltage offers the longer the analysis time and the lower the separation efficiency. "Low-interfacial tension" oil such as dibutyl ether, butyl acetate, disopropyl ether and 1-octanone were investigated instead of these "high-interfacial tension" oils [134,135]. Using low-interfacial oil requires low surfactant concentration to form a stable microemulsion. This allows using higher applied voltages for operation which results in much more rapid analysis times and higher overall efficiencies. In fact, the oil phase is advantageous only for the readily solubilizing or partitioning of the hydrophobic solutes. Changing the nature of oil phase does not notably affect on the separation selectivity [136].

In comparison to changing oil phase, changing the co-surfactants displays largely effect on the separation selectivity. Most commonly used co-surfactants are short chain and linear alcohol because they can readily penetrate into the surfactant monolayer, prevent electrostatic repulsion and promote aggregate formation. A co-surfactant, 1-butanol, is generally used in MEEKC [122-136]. However, 1-propanol, methanol, tetrahydrofuran and 2-ethoxyethanol have also been used [134, 135]. In addition to the selectivity, the migration times can be altered with varying co-surfactant concentration because it affects the solution viscosity which in turns affects the EOF rate.

The surfactant displays an important role in not only the stability of microemulsion but also significant effect on the selectivity. Changing type and

concentration of surfactants can vary the charge density and size of the droplets, the velocity and direction of EOF and the ability of ion-pair interaction with charged species [136]. The surfactants that used in MEEKC are anionic, cationic and non-ionic surfactants. Among these, anionic surfactant, sodium dodecyl sulphate (SDS), is most commonly used because of its high water solubility and lipid solubilizing power. Sodium cholate, anionic bile salt surfactant has also been used to generate negatively charged droplets, which gives different selectivity compared to SDS buffer. Cationic species can form ion-pair interaction with the anionic oil droplets. To eliminate this effect, cationic surfactants such as cetyltrimethylammonium bromide (CTAB) is used. CTAB not only produces positively charged droplets but also coats negative capillary wall and form positively charged surfactant bilayer surface which reverses the EOF direction. In this case, a negative polarity voltage (injection end is at the cathode and detector end is at the anode) has to be used. Microemulsion can be prepared by using non-ionic surfactants (e.g. Brij 35). This microemulsion is not possible to use in MEEKC because the oil droplets have not charge and they migrate with EOF velocity which leads to fail the separation. Especially, they are not suitable for the separation of neutral analytes. They are only used by mixing with charged surfactants in different composition for manipulation of elution window.

Other important variables to be consider in MEEKC include type, concentration and pH of the BGE, the use of buffer additives such as organic solvents, cyclodextrin, ion-pair reagents, temperature, choice of sample diluents and microemulsion preparation procedure. Although a microemulsion system is more complicated than micellar solution, it has advantages than the latter. Micelle is composed of only surfactant monomer and their surface is rigid while in microemulsion, the presence of additional oil core provides the droplets larger and less rigid than micelles (Figure 9). Thus, analytes are more straightforwardly diffuse through the surface and enter into the oil droplets. Consequently, MEEKC can be utilized for very highly hydrophobic solutes which offer problems such as low solubility and precipitation during separation in MEKC. Due to its high solubilizing power, MEEKC can be used for the separation of analytes with a wide range of solubility. Another advantage is that the separation window is significantly larger

than that of MEKC due to greater mobility of the droplets in comparison to micelle and it can be modified by either altering the surfactant concentration or combining ionic surfactant with neutral surfactant. For example, increasing the surfactant concentration triggers greater opposition to the EOF because of increasing charge density on the droplets and result in larger elution window of the system. The last advantage is that MEEKC gives better separation efficiencies than MEKC.

3.3.2.2 Water-in-oil or reversed microemulsion electrokinetic chromatography (RMEEKC or W/O MEEKC)

Water-in-oil MEEKC has not been developed like oil-in-water MEEKC. This technique uses water-in-oil microemulsion as BGE electrolytes. The difference between the two microemulsion systems is their continuous phase. In w/o microemulsion, the continuous phase is aqueous whereas for w/o microemulsion, it is oil phase. W/O microemulsion is normally the specific combination of two or more components within the oil. These components are water, surfactant and possibly a co-surfactant or additional oil phase. The diagram of w/o microemulsion droplets is shown in Figure 13. A central core of water is surrounded by surfactant and co-surfactant molecules which forms a droplet in the oil. The amount of water that can be solubilized in the system is strongly dependent on the surfactant/co-surfactant ratio [21, 22]. If the co-surfactant concentration is too high, the microemulsion will divide into two phase. If surfactant concentration is excessively involved, a lyotropic liquid crystalline phase is formed which differs from microstructure.

As the oil phase, long chain alkane or water-insoluble alcohols are generally used to prepare w/o microemulsion. However, in w/o MEEKC, short chain alcohols such as 1-butanol and 1-pentanol are commonly used because of their water mixable properties, viscosity and electrical conductivity [21, 22, 137]. Due to its high content of oil, the electrical current can not conduct as high as o/w microemulsion which has the same conductivity as water although buffer is added to the microemulsion to generate current. The buffers that used in o/w MEEKC such as borate, phsphoate and ammonium acetate buffer can not be employed in w/o MEEKC because they can not generate adequate current to separate the analyte. Therefore organic buffer such as sodium acetate, tetraethylammonium perchlorate (TEPA) and Tris buffer are used [21,

22, 137]. These buffers can generate sufficient current of nearly 10 μA. In comparison to o/w microemulsion system, it is difficult to adjust pH of w/o microemulsion because of very low content of water. pH is an important parameter for the level of EOF. Nevertheless, in this system, effective pH (pH*) is used and lower level of EOF is generated than o/w microemulsion due to the high viscosity.

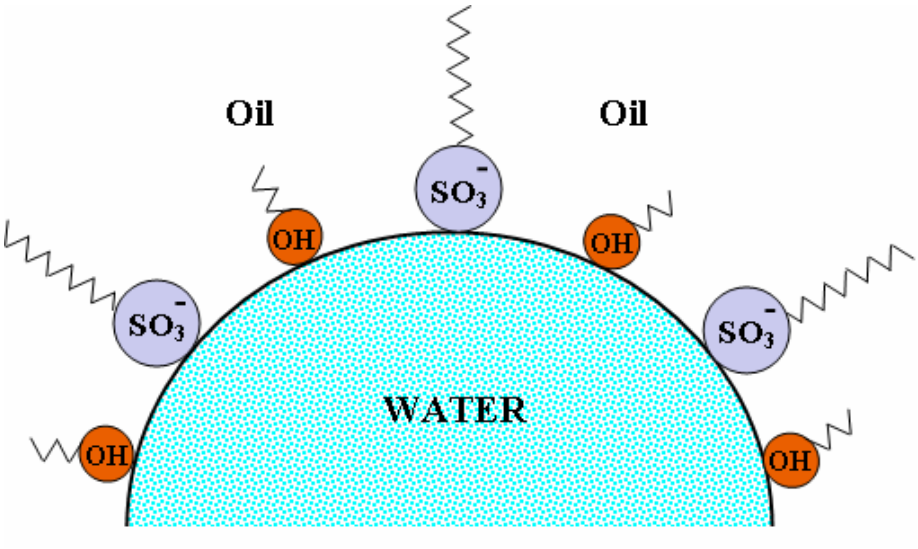

Figure 12 Schematic of water-in-oil (w/o) microemulsion droplets.

Although the o/w microemulsion system is easier to manipulate and most commonly used for the separation of analytes than w/o microemulsion system, the latter has some unique advantages. For example, due to the presence of high concentration of oil, very high hydrophobic compounds (steroids, complex aromatics) and pharmaceutical formulations (e.g. cream) can be analyzed without sample extraction and preparation process, which are necessary for o/w MEEKC [21, 22, 137]. Another remarkable aspect is that the separation mechanism for the neutral analytes is not solely dependent on their hydrophobicity of the analytes like o/w MEEKC. For the acidic and basic drug, the separation is based on their free mobility the analyte, partition into the water core and ion-pair interaction with the droplets [22]. Therefore, it has unique separation mechanism. Most commonly used w/o microemulsions are 1-butanol-SDS-water and 1-pentanol-SDS-water. In addition, four-component microemulsion systems are also used by mixing water, surfactant, co-surfactant and additional oil. Figure 13 displays the partial ternary phase diagram of

the SDS, butanol/ocatane, water outlining the L1 (w/o) and L2 (o/w) phases where the composition of the microemulsion is determined [21].

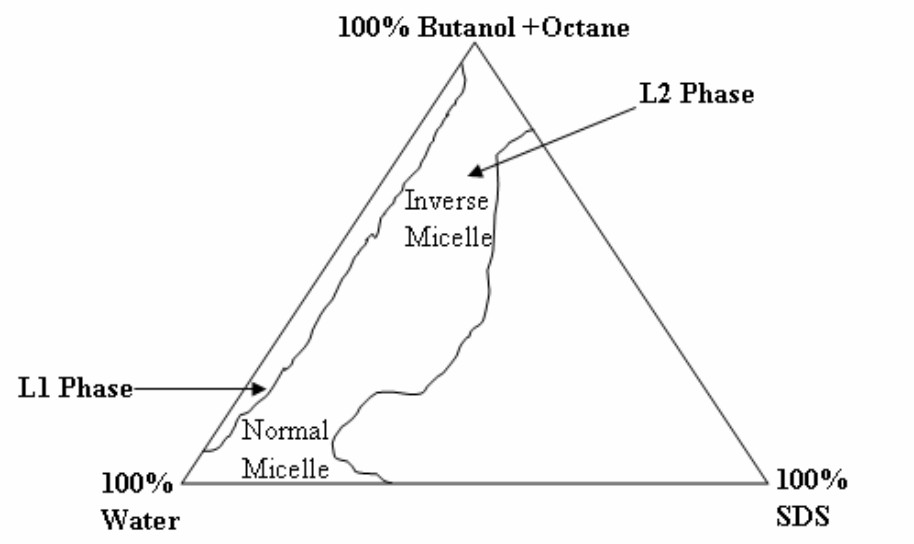

Figure 13 A partial ternary phase diagram of the SDS, Butanol/Octane, water outlining the L1 and L2 phases.

CHAPTER III
MATERIALS AND METHODS

1. Chemicals and reagents

Name	Grade	Source/Supplier
1-Butanol	99%	Sigma (St Louis, MO, USA)
1-Pentanol	98%	Fluka (Buchs, Switzerland)
2-Propanol	99.7%	Labscan Asia (Bangkok, Thailand)
Acetic acid	96%	Riedel-de Haën(Sydney, Australia)
Acetone	99.5%	Labscan Asia (Bangkok, Thailand)
Astaxanthin	98%	Sigma (St Louis, MO, USA)
Beta-carotene	97%	Sigma (St Louis MO, USA)
Buffer pH 10	AR	Ajaxchemical (Sydney, Austria)
Buffer pH 4	AR	Ajaxchemical (Sydney, Austria)
Buffer pH 7	AR	Ajaxchemical (Sydney, Austria)
Cetyltrimethyl ammonium bromide	99%	Sigma (Steinheim, Germany)
Chloroform	99.5%	Labscan Asia (Bangkok, Thaniland)
Dichloromethane	≥99.5%	Merck (Darmstadt, Germany)
Ethyl acetate	99.8%	Labscan Asia (Bangkok, Thailand)
Hydrochloric acid	AR	BDH (Poole, England)
N, N dimethyl formamide	99.8%	Carlo-Erba (Bacelona, Spain)
n-Heptane	> 99%	Fluka (Buchs, Switzerland)
n-Hexane	99.9%	J.T Baker (Philipsburg, USA)
n-Octane	95%	Labscan Asia (Bangkok, Thailand)
n-Octanol	99%	Sigma (St Louis, MO, USA)

1. Chemicals and reagents (continued)

Name	Grade	Source/Supplier
Petroleum ether	HPLC	Labscan Asia (Bangkok, Thailand)
Phosphoric acid	90%	May&Baker (Dagennam,England)
Sodium acetate	99-105%	Riedel-de Häen (Seelze, Germany)
Sodium dihydrogen phophate	≥99.8	Merck (Dmastadt, Germany)
Sodium cholate	98%	Sigma (St Louis, MO, USA)
Sodium dodecyl sulphate	≥ 98%	Fluka (Buchs, Switzerland)
Tetrahydrofuran stabilized with BHT	≥99.8	Merck (Dmastadt, Germany)

2. Instrument

Name	Source/Supplier
Capillary electrophoresis Instrument (^{3D}CE)	Hewlett-Packard (Waldbronn, Germany)
Capillary tube 50 μm (i.d.), 375 (o.d.), effective length 23.5 cm and 32 cm	Polymicro Technologies, Phoenix (U.S.A)
UV-VIS Spectrophotometry	PerkinElmer (Lamda 35) (USA)
pH meter	Consort model C830 (Turnhout, Belgium)
Ultrasonic bath (D-7700)	Elma (Singen, Germany)
Analytical balance (AE 160)	Sartorious (Goettingen, Germany)
13 mm syringe filter, 0.2μm Nylon	Vertical (Bangkok, Thailand)

3. Methods

3.1 Instrumentation

3.1.1 Spectrophotometry

The absorbances of both carotenoids were measured by PerkinElmer

(Lamda 35) spectrophotometer, with a fixed slit width of 2 nm, in a 1-cm quartz cuvette to evaluate the stability of β-carotene and astaxanthin in dissolution media and BGE. The UV spectra of the carotenoids were scanned from 250 to 650 nm and the maximum absorbancies were determined. The maximum absorbance of β-carotene and astaxanthin THF stabilized with BHT was directly measured at 458 nm and 482 nm, 455 nm and 484 nm in w/o microemulsion and 455 nm and 481 nm in o/w microemulsions, respectively. The sepctrophotometeric method was measured against THF stabilized with BHT and the respective microemulsions as the blanks.

3.1.2 Capillary electrophoresis

The CE system was a Hewlett-Packard instrument (^{3D}CE) system (model G1600A) controlled by PC through Agilent Chemstation Plus software version A.08 (G1601A) (Figure14). The software was designed to run on a compatible computer HP Pentium 4 (500 MHZ, RAM 256 MB, hardisk 20 GB) under Microsoft® Window NT 4.0. An instrument was defined as a single time base, but could collect data from diode array detector at various wavelengths simultaneously.

Figure 14 Instrumentation of capillary electrophoresis

The detector measured in the range of 190-600 nm (wavelength accuracy ± 2 nm) was a diode array detector, which consisted of a deuterium lamp and detected by continuous emission. The regulation of high voltage was varied in the range of 0-30

kV (current 0-300 μA, power 0-6 W). The temperature control system for the capillary tube could be varied from 5 to 60°C. The injection system could be done by applying pressure (hydrostatic injection) and voltage (electromigration injection). The capillary tubes used were with a total letngth of 40.5 cm (32 cm of effective length) and 32 cm (23.5 of effective length) of 50 μm inner diameter (i.d.). During the separation, the voltage was set at -30 kV for w/o MEEKC and +20kV for o/w MEEKC . The capillary tube was thermostated at 25°C in all experiments.

3.2 General procedure

All the separations were performed in the uncoated fused-silica capillary tubes with inner diameter. 50 μm and external diameter 375 μm. The temperature was kept at 25°C for all experiments.

For w/o MEEKC, the effective length of capillary tube was varied 32 cm (40.5 cm of total length) and 23.5 cm (32 cm of total length) and the voltage was set for -30 kV (The polarity was reversed which means that the anode was at the detector).

For o/w MEEKC, only the capillary tube with 23.5 cm of effective length (32 cm of total length) was used and the voltage of +20 kV was applied.

For the capillary conditions for o/w MEEKC and w/o MEEKC are described in Table 1 and 2, respectively.

All the samples were kept at ambient temperature in the autosampler and introduced by successively injection of 50 mbar per 2 s for sample and 50 mbar per 2 s for BGE unless otherwise stated. Detection wavelengths of 450 nm, 475 nm and 484 nm with 40 nm bandwidth were set for all experiments and 475 nm with 40 nm bandwidth was selected for the determination of carotenoids. Both the new BGE vials and sample vials were changed after 3 injections.

3.3 Preparation of stock and working standard solutions

Stock standard solutions were prepared in tetrahydrofuran (THF) stabilized with butylated hydroxyl toluene (BHT) at a concentration of 1,000 μg/ml for β-carotene and astaxanthin separately. From these stock solutions, 100 μg/ml of each

carotenoid solution was prepared by diluting with THF for optimization and the concentrations in a range of 20-120 μg/ml for method validation of w/o microemulsion. All the solutions were protected from the light and stored at -20°C and filtered with 0.2 μm membrane before injection.

Table 1 The capillary tube condition procedure for o/w MEEKC

Step	Duration	Solvents
New Capillary tube		
1) Rinse	10 min	1N NaOH
2) Rinse	10 min	0.1N NaOH
3) Rinse	10 min	Microemulsion
Between run		
1) Rinse	2 min	Microemulsion
Storage		
1) Rinse	10 min	1 N NaOH
2) Rinse	10 min	0.1N NaOH
3) Rinse	10 min	Deionized water
4) Rinse	5 min	air

Table 2 The capillary tube condition procedure for w/o MEEKC

Step	Duration	Solvents
New Capillary tube		
1) Rinse	10 min	1N NaOH
2) Rinse	10 min	0.1N NaOH
3) Rinse	10 min	Deionized water
4) Rinse	10 min	Microemulsion
Daily condition before use		
1) Rinse	5 min	1 N NaOH
2) Rinse	5 min	0.1 N NaOH
3) Rinse	5 min	Deionized water
4) Rinse	5 min	Microemulsion
Between run		
1) Rinse	3 min	0.1N NaOH
2) Rinse	3 min	Microemulsion
Storage		
1) Rinse	5 min	1N NaOH
2) Rinse	10 min	0.1N NaOH
3) Rinse	10 min	Deionized Water

3.4 Preparation of microemulsions

3.4.1. Preparation of o/w microemulsions

For the microemulsion with acid pH (ME-1), sodium dodecyl sulphate and 1-butanol was mixed in the volumetric flask and then, n-octane, 2-propanol and 25 mM phosphate buffer adjusted to 2.5 with 0.5 M phosphoric acid were added. After that, the mixture was sonicated for 30 min to obtain clear and highly stable microemulsion. Once sonicated, the microemulsions were left at room temperature for 1 h. Similarly, basic microemulsions (ME-2 and ME-3) were also prepared by using the same composition as the acid pH microemulsion, except borate buffer pH 9.2 adjusted with 1 M hydrochloric acid instead of phosphate buffer at pH 2.5. The o/w microemulsion systems are shown in Table 3. All the microemulsions were filtered with 0.2 μm membrane filter before use.

3.4.2 Preparation of w/o microemulsions

For each w/o microemulsion preparation (ME-4 to ME-15), an appropriate amount of sodium dodecyl sulphate (SDS) was weighed in the conical flasks to obtain the final concentrations from 9% to 4% (w/w) respectively. Then, 78% (w/w) of oil (1-butanol or 1-pentanol) and 2% (w/w) additional oil phase (n-octane, n-octanol, n-heptane, THF or ethyl acetate) were added orderly into the SDS and mixed thoroughly. For microemulsion without additional oil, 80% (w/w) oil was used instead of 78% (w/w). After mixing SDS, oil and additional oil well, the required amounts (from 11% to 16% w/w) of 70 mM sodium acetate buffer (pH 8.0), which has been pH adjusted to 8.0 with 1 M and 0.1 M acetic acid were added to the resulting mixture. The microemulsions were sonicated for 30 min to get the optically transparent solutions. Once the microemulsions were sonicated, they were filtered with Whattman no. 1 and left at room temperature for 30 min. Then, each microemulsion was transferred to volumentric flasks and closed tightly to prevent evaporation. All the solutions were filtered with 0.2 μm membrane filter before use. The compositions of w/o microemulsions used are shown in Table 4.

Table 3 O/W microemulsion compositions expressed in % w/w

ME	SDS	Octane	BuOH	2-PrOH	Aqueous buffer
1.	5.2%	0.81%	6.6%	20 %	67.39 % (25 mM phosphate buffer pH 2.5)
2	5.2%	0.81%	6.6%	20 %	67.39% (10 mM borate buffer pH 9.2)
3.	3%	0.81%	6.6%	15%	74.59% (5 mM borate buffer pH 9.2)

Table 4 W/O microemulsion compositions expressed in % w/w

ME	Surfactant	Oil	Additional oil	Aqueous buffer pH 8.0
4.	9% SDS	78% 1-butanol	2% n-octane	11% (70 mM NaOAc)
5.	10% SDS	78% 1-pentanol	2% n-heptane	10% (70 mM NaOAc)
6.	10% SC	78% 1-butanol	2% n-octane	12% (70 mM NaOAc)
7.	10% CTAB	78% 1-butanol	2% n-octane	10% (70 mM NaOAc)
8.	8% SDS	78% 1-butanol	2% n-octane	12% (70 mM NaOAc)
9.	6%SDS	78% 1-butanol	2% n-octane	14% (70 mM NaOAc)
10.	4% SDS	78% 1-butanol	2% n-octane	16% (70 mM NaOAc)
11.	9%SDS	80% 1-butanol	-	11% (70 mM NaOAc)
12.	9% SDS	78% 1-butanol	2% n-heptane	11% (70 mM NaOAc)
13.	9% SDS	78% 1-butanol	2% n-octanol	11% (70 mM NaOAc)
14.	9% SDS	78% 1-butanol	2% ethyl acetate	11% (70 mM NaOAc)
15.	9% SDS	78% 1-butanol	2% THF	11% (70 mM NaOAc)

3.5 Choice of dissolution media and sample diluent

Due to extremely hydrophobic nature of β-carotene and astaxanthin, there was encountered when dissolving the standards. Both o/w and w/o microemulsion was initially assessed for dissolution media. Due to low solubility in the microemulsions, some of organic solvents, petroleum ether, hexane, ethyl acetate, N. N dimethyl formamide, dimethyl sulfoxide, acetone, dichloromethane, chlororform and tetrahydrofuran. For the solubility tests, 1 mg of standard β-carotene and astaxanthin were dissolved in 10 ml of each organic solvent in each test tube separately and check

the solubility. For the sample diluent, both o/w and w/o microemulsion systems and THF stabilized with BHT were investigated.

3.6 Stability tests

The stability of β-carotene and astaxanthin in dissolution media was determined by spectrophotometer [138]. The stability of both carotenoids in THF stabilized with BHT was performed for working condition i.e. the samples were kept at 25°C and dim light. In this experiment, 3.9 μg/ml of β-carotene and 4.8 μg/ml of astaxanthin standard solutions were prepared separately and stored at 25°C and dim light. Their absorbances were measured for 24 h.

For the stability in BGE, the maximum absorbances of 3.9 μg/ml β-carotene and 4.8 μg/ml of astaxanthin solutions obtained by diluting separately with either o/w microemulsions with acid pH or basic pH were measured in 1 h. Similarly, each carotenoid solution was diluted with w/o microemulsion to get 4 μg/ml and their absorbance was measured for 1 h.

The stability of each microemulsion system was visually investigated by observing their turbidity or precipitation. Twenty five milliliters of each microemulsion were kept in a tightly closed volumetric flask to prevent evaporation. Then, the precipitation of microemulsions was observed for 2 months.

4. Oil-in-water microemulsion electrokinetic chromatography (O/W MEEKC)

O/W MEEKC is popularly used for the separation of neutral and highly hydrophobic compounds. In the present work, acid pH microemulsion (ME-1) could not be used for the separation of both carotenoids due to instability of β-carotene in this system. Therefore, basic pH two microemulsion systems, ME-2 and ME-3 were assessed. And, its separation efficiency was compared with that of w/o MEEKC.

5. Water-in oil microemulsion electrokinetic chromatography (w/o MEEKC)

W/O MEEKC was less developed in comparison with o/w MEEKC. But, it has advantages for the separation of analytes which has solubility problem with aqueous phases. Therefore, w/o microemulsion, ME-4 system was initially adopted

and used for the separation of both carotenoids. Its separation efficiency and resolution were compared with that of o/w MEEKC.

Optimization and validation of w/o MEEKC was performed because it gave better separation efficiency and better peak shape than o/w MEEKC. Optimization experiments for the separation of β-carotene and astaxanthin was investigated by assessing the effect of injection time, type of oils, type of surfactants, the effect of surfactants and aqueous buffer composition, type of additional oils and the effect of capillary length. The migration time (t_m), resolution (R_s) and sensitivity from each condition were compared.

5.1 Effect of injection time

The volume introduced into the capillary is an important factor in capillary electrophoresis. If the injection volume is too large, the separation efficiency can be decreased. Sample overloading causes poor peak shape, low separation efficiency and poor precision. Therefore, the injection of the carotenoids by 50 mbar per 1s, 2s and 3s were initially investigated by using butanol microemulsion system (ME-4).

5.2 Effect of oil types

In w/o microemulsion system, oil is one of the most important factors because its physicochemical properties especially viscosity, dielectric constant and conductivity significantly affect the separation of analytes.

1-butanol and 1-pentanol, which are most commonly used as oils and the separation efficiency of the microemulsions containing these oils, ME-4 and ME-5, were investigated.. ME-4 system was composed of 78% (w/w) 1-butanol, 9% (w/w) SDS, 2% (w/w) n-octane and 11% (w/w) 70 mM sodium acetate (pH 8.0). ME-5 contained 78% (w/w) 1-pentanol, 10% (w/w) SDS, 2% (w/w) n-heptane and 10 % (w/w) sodium acetate (pH 8.0).

5.3 Effect of surfactants

The types of surfactants in microemulsion give a marked effect on resolution, selectivity and migration time because it influences the droplet size, level and direction of EOF and ion pairing with the solutes in the system. The most frequently

used anionic surfactants, sodium dodecyl sulphate (ME-4)and sodium cholate (ME-6), and cationic surfactant, cetyltrimethyl ammonium bromide (ME-7) were assessed.

5.4 Effect of surfactant concentration

Changing of surfactant concentration affects the physical properties of microemulsion such as particle size, surface tension and conductivity, which subsequently influence on the retention and resolution. There is relationship between SDS concentration and the amount of water. When the SDS concentration is reduced, it must be compensated by an increase of water amount. In this experiment, surfactant concentrations were reduced from 9% to 4% (w/w) and aqueous buffer was increased from 11% to 16% (w/w) (ME1, ME-8, ME-9 and ME-10) .

5.5 Effect of additional oils

Additional oils are mainly used to decrease the water content in the microemulsion system and to improve the stability of microemulsion. In addition, different additional oils can give a marked effect on selectivity and migration time [22, 23]. Therefore, microemulsions with different additional oils and without additions oil (ME-11) were investigated. In this experiment, high surface tension oils (n-octane (ME-4), n-heptane (ME-12) and n-octanol (ME-13)) and low surface tension oils (ethyl acetate (ME-14) and tetrahydrofuran (ME-15)) were used as additional oil.

5.6 Effect of capillary lengths

Capillary dimension, especially effective length affects separation efficiency and migration time. The effective lengths of 32 cm and 23.5 cm were investigated for the separation of the carotenoids.

6. Method Validation

Under the optimized w/o MEEKC conditions, the developed method was used to validate for the determination of β-carotene and astaxanthin. Linearity, precision, limit of detection (LOD) and limit of quantitation (LOQ) of the method were evaluated.

6.1 Linearity

Linearity for standard β-carotene and astaxanthin were established in a range of 20-120 μg/ml. Each concentration was injected in triplicate. The calibration curves were plotted between peak area and peak height against the concentrations. The regression equation and regression coefficient (r^2) were calculated by using Microsoft EXCEL®

6.2 Precision

Precision of the optimized method was determined by assessing injection precision, intra-day precision and inter-day precision. The percent relative standard deviations (%RSDs) of migration time, peak area, and peak height were calculated.

6.2.1 Injection precision

Injection precision was determined at the middle point of the calibration curve (75 μg/ml) of each carotenoid by nine replicate injections.

6.2.2 Intraday precision

Intraday precision was determined by triplicate injections of standard solutions at 50 μg/ml, 75μg/ml and 100 μg/ml within one day.

6.2.3 Inter-day precision

Inter-day precision was determined on six different days at three different concentrations of 50 μg/ml, 75μg/ml and 100 μg/ml for both carotenoids. Each solution was injected in triplicate.

All of the precisions were determined from the percent relative standard deviations (%RSD), which was determined from the following formula.

$$\% \text{ RSD} = \frac{\text{SD} \times 100}{\overline{X}}$$

Where; SD = the standard deviation from the mean value

$\overline{X}$ = the mean value

6.3 Limit of detection (LOD) and Limit of quantification (LOD)

The detection limit (LOD) is the lowest amount of analytes in a sample that can be detected, typically acceptable signal to noise ratio of ≥ 3. The limit of quantification is a characteristic of quantitative assays. It is the lowest amount of analyte in the sample that can be determined with acceptable precision and accuracy with a signal to noise ratio should be ≥ 10.

In this experiment, LOD and LOQ were determined at 450 nm for β-carotene and 475 nm for astaxanthin with 40 nm bandwidth. And, the reference channel was set 380 nm with 80 nm bandwidth to obtain smooth baseline.

CHAPTER IV
RESULT AND DISCUSSION

Microemulsion electrokinetic chromatographic method (MEEKC) was demonstrated for the separation of carotenoids, β-carotene and astaxanthin. Before the optimization of MEEKC conditions, dissolution media for β-carotene and astaxanthin were chosen and the stability of the carotenoids in dissolution media, sample solvent and microemulsions was initially examined by spectrophotometry. In addition, the stability of microemulsions was also investigated. The separation of carotenoids was performed by both w/o and o/w microemulsions and their separation efficiencies were compared. In this present work, optimization and method validation was studied for w/o MEKKC because β-carotene gave the solubility problem in o/w microemulsion and w/o MEEKC offered better separation efficiency than o/w MEEKC. The optimization of w/o MEEKC was performed by varying injection time, oil and surfactant types, surfactant concentrations, types of additional oil, and capillary length. The optimum conditions were validated in term of linearity, precision, limit of detection (LOD) and limit of quantitation (LOQ) by using the standard mixture of β-carotene and astaxanthin for the method development.

1. Choice of dissolution media

The proper dissolution media for the analytes is very important in analytical practices. The samples should be freely soluble in the solvent and background electrolyte (BGE) is usually used as dissolution media. If the samples, especially highly hydrophobic compounds, do not dissolve in the BGE, organic solvents are used to dissolve them. Due to their lower conductivity than BGE, the analytes become stack and increase on-column concentration, which leads to the problems such as precipitation and/or peak tailing. Moreover, for MEEKC, improper dissolution media can cause microemulsion disintegration.

Both β-carotene and astaxanthin do not dissolve in both oil-in-water (o/w) and

water-in-oil (w/o) microemulsions. Due to the extremely hydrophobic nature of the carotenoids, appropriate solvent for the carotenoid should be searched for MEEKC. The solvents that are generally used in the separation of carotenoids with HPLC are acetone, tetrahydrofuran, chloroform, dichloromethane, petroleum ether, hexane, ethyl acetate, dimethyl sulfoxide, N, N-dimethylformamide and various mixtures of them. The carotenoids were extremely low soluble in methanol and acetonitrile, which are most commonly used as dissolution media in MEEKC [138]. The solubility of β-carotene and astaxanthin were investigated.

Results showed that acetone, petroleum ether, hexane, ethyl acetate and N, N dimethylformamide gave unsatisfactory solubilities for either one or both carotenoids (Table 5). However, tetrahydrofuran (THF), dichloromethane and chloroform could dissolve both carotenoids. In comparison to these three solvents, all could mix with w/o microemulsions whereas dichloromethane and chloroform could not mix with o/w microemulsions. However, THF could mix with both w/o and o/w microemulsion system. Therefore, THF was chosen as a solvent for the carotenoids since it could mix well with both o/w and w/o microemulsions. However, this solvent sometimes might be contaminated with traces of hydrogen peroxide which can oxidize carotenoids to their decomposition. Hence, THF stabilized with antioxidant such as butylated hydroxyl toluene (BHT) or hydroquinone was more suitable than the one with HPLC grade**.**

In MEEKC, the sample solutions are generally diluted with the microemulsion to prevent the disruption of microemulsion environment inside the capillary near the sample injection plug. Therefore, o/w and w/o microemulsions were firstly investigated as sample diluents for the carotenoid solutions. β-carotene and astaxanthin solutions were diluted with both w/o and o/w microemulsions separately at the concentrations of 10 μg/ml to 100 μg/ml. In this experiment, astaxanthin could mix well with both w/o and o/w microemulsions at all concentrations. However, β-carotene solution precipitate immediately at the concentrations above 10 μg/ml in o/w microemulsion. The precipitation of β-carotene is due to the large amount of water, which led to the formation of β-carotene microcrystals [139]. In w/o microemulsion, β-carotene solutions could mix well at the concentration up to 50 μg/ml without

precipitation until 30 min. Therefore, the microemulsions could not be used as sample diluents for β-carotene because of precipitation problem. To avoid this problem, direct injection of the carotenoid solution diluted with THF was investigated. To test the precipitation of β-carotene in the capillary tube, when a few drops of the standard solution of β-carotene (300 μg/ml) were added to the 1 ml of w/o and o/w microemulsions in each test tube before injections, there was no precipitation for several hours. Therefore, THF was used as both dissolution media and sample diluent and the mixture of β-carotene and astaxanthin which were dissolved in THF were injected directly.

Table 5 The solubility of β-carotene and astaxanthin in organic solvents

Solvent	β-carotene	Astaxanthin
Acetone	Partly soluble	Partly soluble
Petroleum ether	Soluble	Insoluble
Hexane	Soluble	Insoluble
Ethyl acetate	Partly soluble	Soluble
N, N dimethyl formamide	Soluble	Partly soluble
Dimethyl sulfoxide	Partly soluble	Soluble
Dichloromethane	Soluble	Soluble
Chloroform	Soluble	Soluble
Tetrahydrofuran	Soluble	Soluble

Partly soluble = Some carotenoid powder was left undissolve; Soluble = All carotenoid powder was dissolved; Insoluble = All carotenoid powder was not dissolved.

2. Stability tests

The stability of analytes in dissolution media and BGE is an important factor in CE separation because it can affect the precision of the method. Additionally, degradation or instability of analytes influences the method accuracy. Therefore, the stability of both carotenoids in THF stabilized with BHT, o/w microemulsions with acidic and basic pH and w/o microemulsion was evaluated by using UV-Visible spectrophotometer. The stability of BGE (microemulsions) was also investigated**.**

2.1 Stability in dissolution media and sample diluent

The stability of β-carotene and astaxanthin prepared in THF stabilized with BHT was determined for 24 h in working condition i.e. the carotenoid solutions were kept 25 °C and dim light. All the solutions were diluted to 50 ml with THF from the stock solutions to obtain suitable concentrations, which can be measured by spectrophotometry. The maximum absorbances were measured at 458 nm for β-carotene and 482 nm for astaxanthin against THF as the blank. Figure 15 shows that both carotenoids were stable in working condition for 24 h because the absorbance did not decrease and no wavelength shift was found, which means that there was no degradation and transformation from *trans* to *cis* isomers significantly [138].

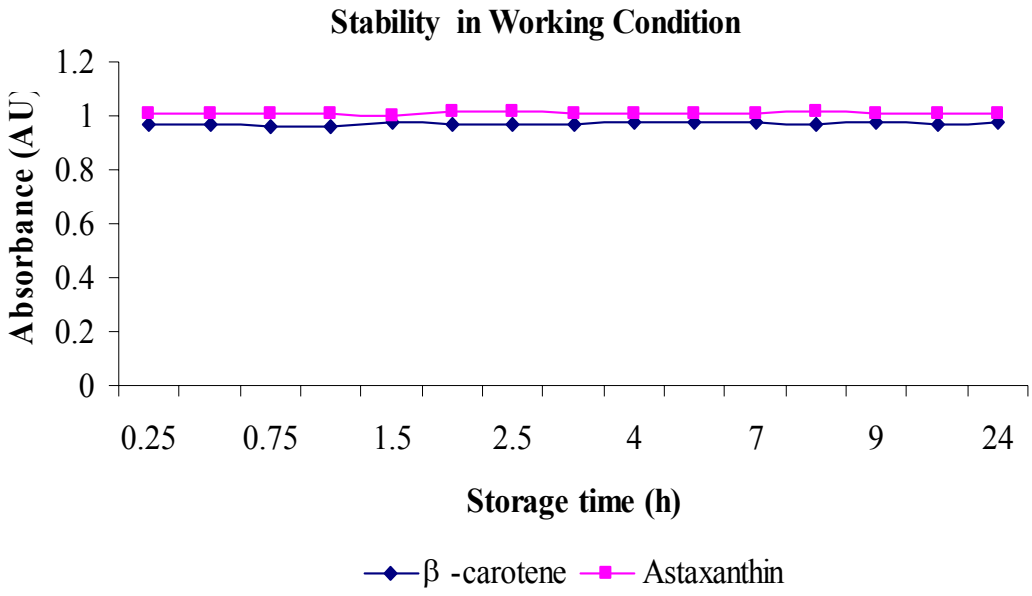

Figure 15 Stability for β-carotene and astaxanthin in THF stabilized with BHT in working condition (The carotenoid solutions were kept 25°C and dim light). The absorbance was measured at 458 nm for β-carotene (3.9 μg/ml) and 482 nm for astaxanthin (4.8 μg/ml) in 24 h.

2.2 Stability in BGE (Microemulsions)

2.2.1 Stability in o/w microemulsion

The stability of carotenoids was investigated in acid pH and basic pH o/w microemulsions. Acid pH microemulsion (ME-1) was composed of 0.81% (w/w)

n-octane, 5.2% (w/w) sodium dodecyl sulphate, 6.6% (w/w) 1-butanol, 20% (w/w) 2-propanol and 67.39% (w/w) 25 mM phosphate buffer pH 2.5. For basic microemulsion (ME-2), all the compositions are the same as the acidic microemulsion system except using borate buffer at pH 9.2 instead of phosphate buffer at pH 2.5. All the solutions were diluted to 50 ml with each microemulsion to obtain suitable concentrations which can be measured by spectrophotometry. Maximum absorbance was examined at the wavelength of 455 nm for β-carotene and 481 nm for astaxanthin in 1 h against their respective o/w microemulsions.

In o/w microemulsion systems, the acidic microemulsion systems were more commonly used than basic microemulsions for the separation of highly hydrophobic compounds to obtain a shorter migration time. However, the microemulsions with acid pH could not be possible to use for the separation of the carotenoids because β-carotene was unstable in acid pH. Figure 16 illustrate that only astaxanthin was very stable in both acid and basic pH. β-carotene was more stable in basic pH than acid pH. Hence, the basic pH microemulsion was used for the determination of carotenoids. According to the stability of astaxanthin in acidic pH, it was found that the microemulsion with acid pH may be used for the separation of carotenoids, especially for xanthophyll group.

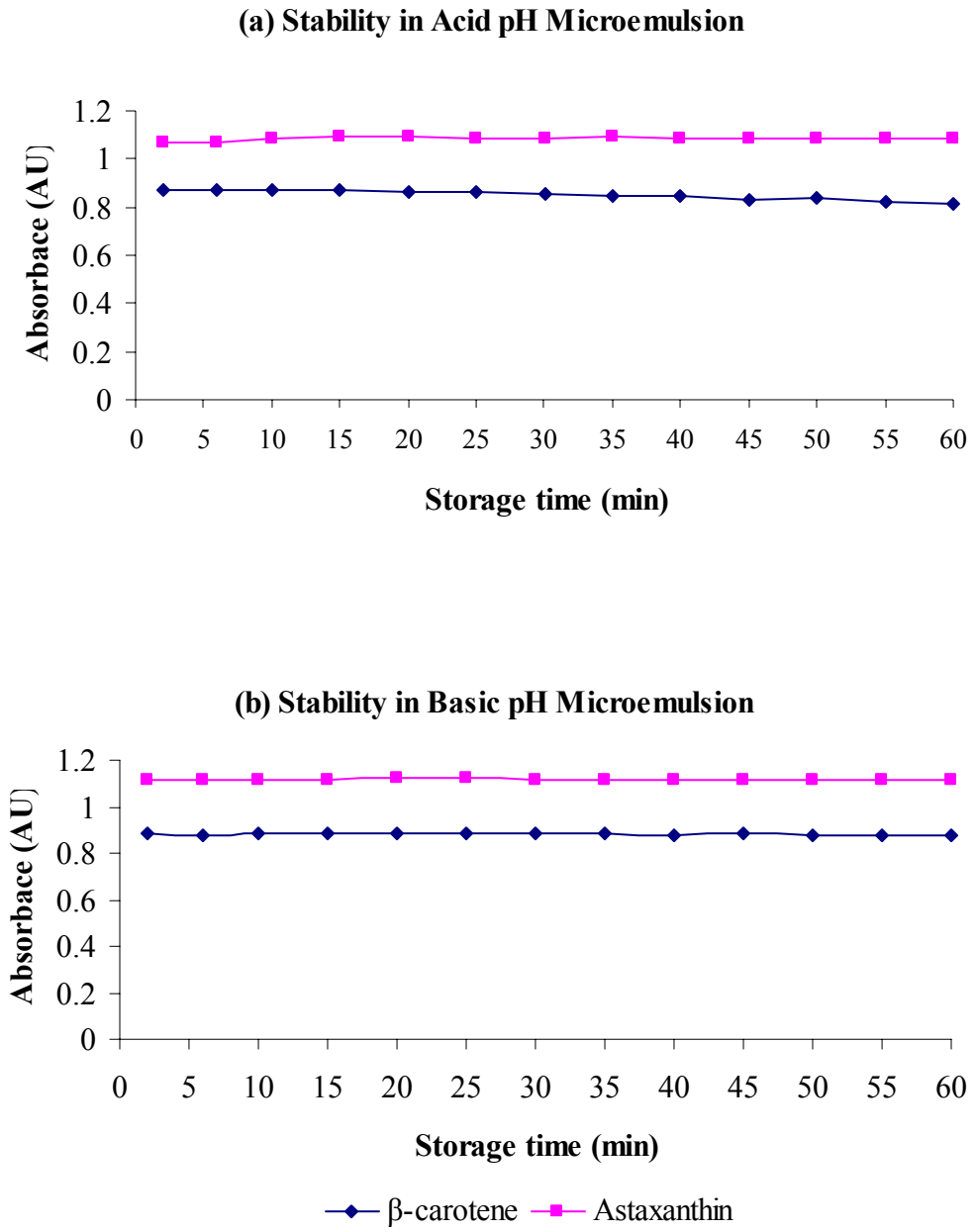

Figure 16 Stability of β-carotene and astaxanthin in (a) acid pH (ME -1) (b) basic pH (ME -2) o/w microemulsion system. Absorbance was measured at 455 nm for β-carotene (3.9 μg/ml) and 481 nm (4.8 μg/ml) for astaxanthin for 1h.

2.2.2 Stability in w/o microemulsion

The stability of β-carotene and astaxanthin in w/o microemulsion was assessed by using microemulsion with or without additional oil. Microemulsions, ME-

4 and ME-11 were applied for this experiment. All the carotenoid solutions were diluted to 50 ml with each w/o microemulsion to obtain suitable concentrations, which can be measured by spectrophotometry.

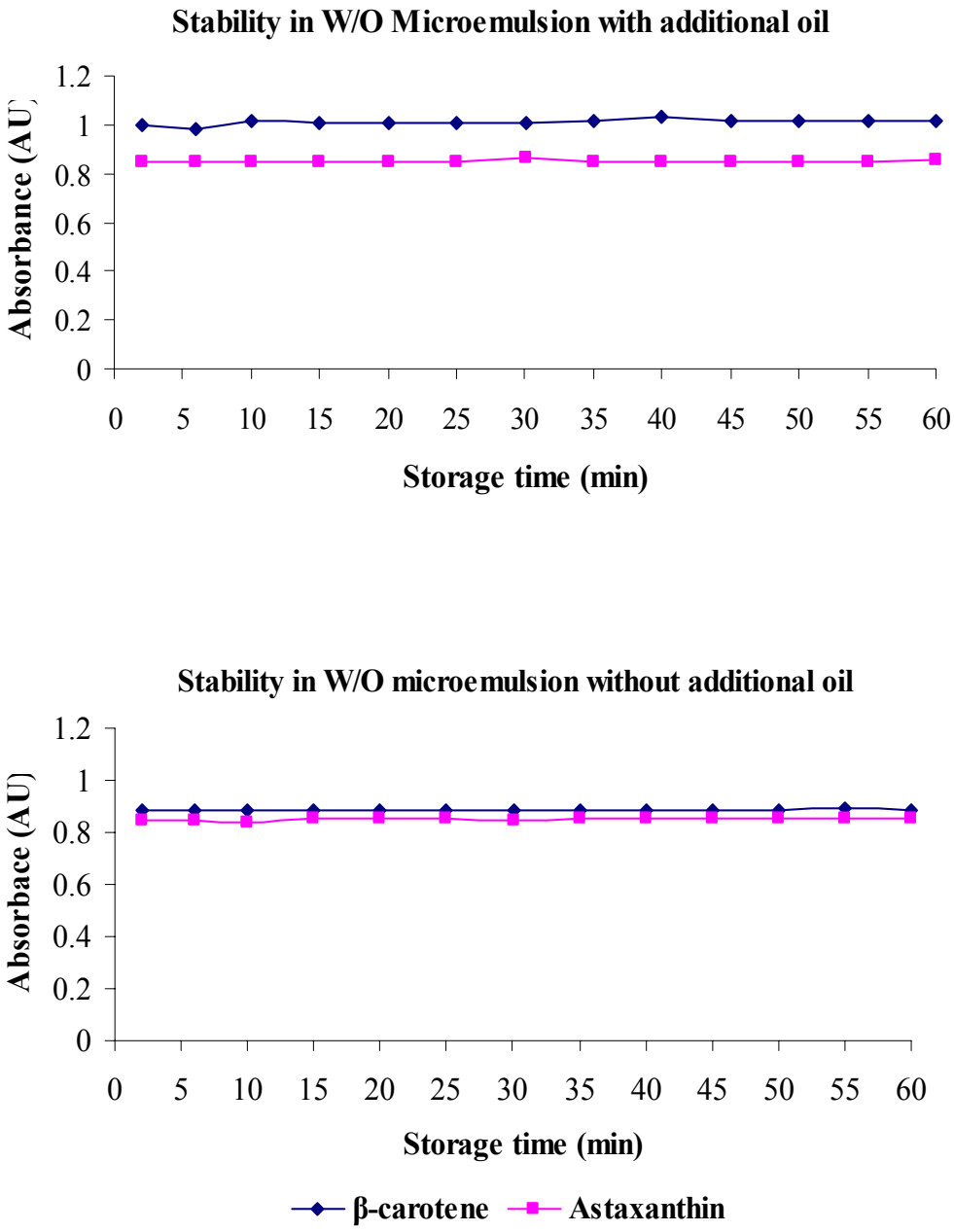

Figure 17 Stability of β-carotene and astaxanthin in w/o microemulsion system with (ME-4) or without additional oil (ME-11). Absorbance was measured at 455 nm for β-carotene (4 μg/ml) and 484 nm (4 μg/ml) for astaxanthin for 1h.

The maximum absorbance was measured at 455 nm for β-carotene and 484 nm for astaxanthin in 1 h against the w/o microemulsion as the blank. Figure 17 show that both β-carotene and astaxanthin were stable in w/o microemulsions with or without additional oil**.**

Results from Figure 16 and 17 revealed that both β-carotene and astaxanthin were stable in o/w microemulsion with basic pH and w/o microemulsion. Therefore, the separation of both carotenoids was carried out by using these o/w and w/o microemulsion systems.

2.3 Microemulsion stability

For the stability of BGE, all o/w and w/o microemulsions used in this experiment were determined for two months by checking the precipitation visually.

Many reports has described o/w microemulsions are stable at least 2 to 3 months. In this experiment, o/w microemulsions, ME-1 and ME-2 were studied and it was found that there was not any precipitation and it was stable up to two months.

Table 6 The stability of microemulsions

Microemulsion	Stability
ME-1	Stable for 8 weeks
ME-2	Stable for 8 weeks
ME-4	Stable for 8 weeks
ME-5	Stable for 8 weeks
ME-6	Stable for 8 weeks
ME-7	Stable for 8 weeks
ME-8	Stable for 8 weeks
ME-11	Stable for 8 weeks
ME-12	Stable for 8 weeks
ME-13	Stable for 8 weeks
ME-14	Stable for 3 days
ME-15	Stable for 6 weeks

The stability of different w/o microemulsion systems by addition of homologues series of alkanes used as additional oils has been investigated in [22, 23]. It was suggested that microemulsions containing additional oils are more stable than one without additional oil because the additional oil can further reduce the surface tension by 5% which lead to form more stable microemulsion. Additionally, among the microemulsions, the stability of the n-octane-1-butanol-SDS-water microemulsions are greater because the number of carbons atoms in the surfactant is equal to the number of carbon atoms in the co-surfactant and oil. In these reports, the additional oils used were high interfacial tension oils.

In the present work, the stability of microemulsions with or without additional oil were also observed by using not only high interfacial tension oils (n-octane, n-octanol and n-heptane) but also low interfacial tension oils (ethyl acetate, THF) as additional oils. The compositions of these microemulsions were described in Table 4 (Chapter III).

All the microemulsions investigated (ME-4, ME-11 to 15) were thermodynamically stable except the one with ethyl acetate system (ME-14). Ethyl acetate formulation initially gave an optically transparent solution, which means surfactant, oil, additional oil and aqueous buffer could readily mix. After three days, it started to precipitate and became turbid. Therefore, this microemulsion system was not true microemulsion system. In contrast, microemulsions with octane (ME-4), heptane (ME-12), octanol (ME-13) and without additional oil (ME-11) showed no precipitation up to 2 months. However, the precipitation was found in microemulsion prepared with THF used as additional oil (ME-15) after 6 weeks. Microemulsions containing low surface tension oils used as additional oils were less stable than the ones involving high surface tension oils.

The microemulsions containing sodium cholate (ME-6) and CTAB (ME-7) were used instead of sodium dodecyl sulphate (SDS). They could form stable microemulsions successfully for 8 weeks.

For the microemulsions with different surfactant concentrations and water compositions, ME-8 system was investigated and any precipitation was not found up to two months. It can be concluded that both o/w and w/o microemulsion systems were thermodynamically stable except ME-14.

3. Oil-in-water microemulsion electrokinetic chromatography (O/W MEEKC)

O/W MEEKC has been widely developed for the separation of neutral, highly hydrophobic compounds due to high solubilizing properties of microemulsion droplets. For the separation of highly hydrophobic compounds, acidic pH microemulsion buffer was generally employed to suppress the EOF and offer faster separation of the analytes. However, in the present work, acid pH microemulsion was not suitable for the separation of β-carotene and astaxanthin because of the instability of β-carotene in acid pH. Therefore, only microemulsion with basic pH could be investigated. The structures of β-carotene and astaxanthin are shown in Figure 1 (Chapter II).

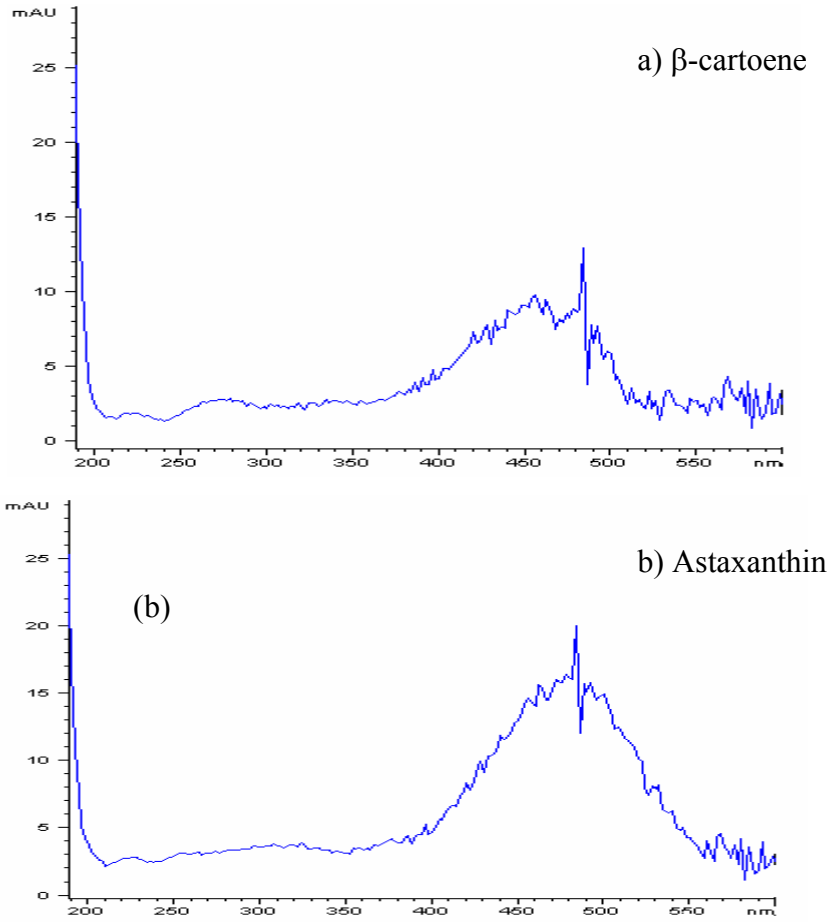

Figure 18 The UV-visible spectra of (a) 100 μg/ml of β-carotene and (b) 100 μg/ml astaxanthin in o/w microemulsion (ME-3) from the diode array detector of the capillary electrophoresis instrument.

The initial condition used for the separation of β-carotene and astaxanthin was microemulsion (ME-2) containing 5.2 % (w/w) SDS, 0.81% (w/w) n-octane, 6.6% (w/w) 1-butanol, 20% (w/w) 2- propanol and 67.39% (w/w) 10 mM borate buffer pH 9.2, a capillary tube with the total length of 32 cm with the effective length of 23.5 cm, 50 μm I.D., a voltage of 20 kV and temperature of 25°C. In this case, high voltage (30 kV) could not be used because the capillary tube was short which generates high current than longer tube and this high current causes baseline jump at the starting point. Although detection wavelengths were set at 450 nm, 475 nm and 484 nm with 40 nm bandwidth, 475 nm with 40 nm bandwidth was selected because of good absorbance of both carotenoids (Figure 18).

Under o/w microemulsion system (ME-2), both the carotenoids did not appear until 40 min. This is because the microemulsion droplets are moving against the EOF and hence migrate slowly towards the detector in basic condition. This means that more hydrophobic compounds which favor to inclusion of oil droplets will result in longer separation time. Therefore, the microemulsion composition was changed to ME-3 by decreasing surfactant concentration to 3% (w/w), 2-propanol to 15% (w/w) and the strength of borate buffer to 5 mM to obtain shorter migration time.

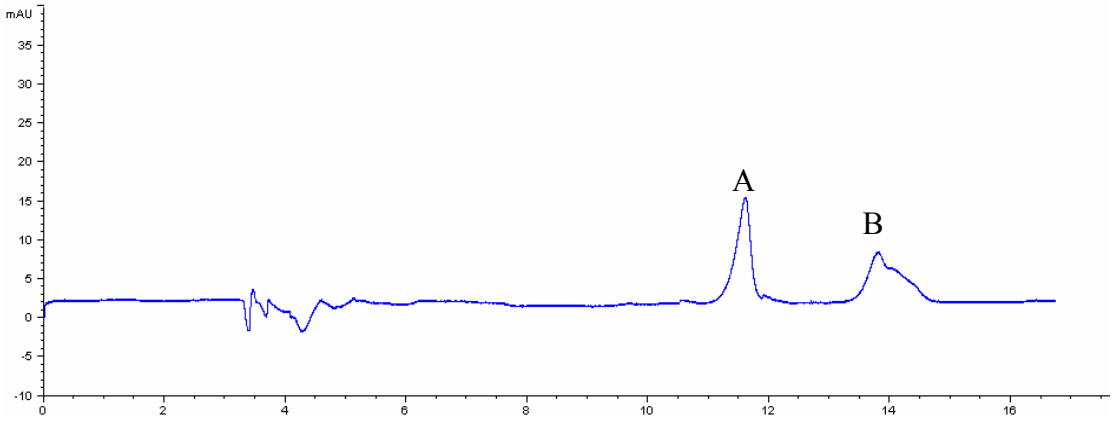

Figure 19 The separation of standard mixture of β-carotene (B) and astaxanthin (A) by o/w MEEKC. Separation conditions: O/W microemulsion (ME-3); Applied voltage 20 kV; Temperature 25°C; Capillary tube 23.5 cm (effective length) × 50μm (I.D); 475 nm with 40 nm bandwidth.

According to Figure 19, the carotenoids could be separated within 15 min and the order of elution was dependent on their partition coefficient because more polar carotenoid, astaxanthin, was eluted first. However, the separation efficiency of o/w MEEKC was not good because β-carotene peak shape was poor. In addition, the sensitivity of both carotenoids was low in o/w microemulsion. Due to poor separation efficiency and peak shape, w/o MEEKC system was alternatively investigated for the separation of the carotenoids.

4. Water-in-oil or reversed microemulsion electrokinetic chromatography (W/O MEEKC or RMEEKC)

In comparison to o/w MEKC, w/o MEEKC is less commonly used. However, in this experiment, o/w MEEKC gave poor separation efficiency, poor peak shape of both carotenoids and also offered low sensitivity. Therefore, the second approach w/o MEEKC was evaluated for the separation of β-carotene and astaxanthin.

The initial condition, using ME-4 system composed of 2% (w/w) n-octane, 9% (w/w) SDS, 78 % (w/w) 1-butanol, 11% (w/w) 70 mM sodium acetate buffer pH 8, a capillary tube with the total length of 40.5 cm with the effective length of 32 cm, 50 μm I.D., a voltage of -30 kV and temperature at 25°C was adopted for the separation of both carotenoids. All the detection wavelengths were the same as o/w MEEKC and 475 nm with 40 nm bandwidth was also selected to determine the carotenoids because of good absorbance at this wavelength (Figure 20).

In this experiment, negative polarity was used to facilitate the migration of the negative microemulsion droplets through the high viscosity medium toward the detector at anode. If the positive polarity is used, the analytes may not be eluted or their migration times may be very long. This is because when high pH is used, w/o microemulsion system can generates less EOF in high viscosity medium to sweep negative microemulsion droplets attempting to migrate towards the anode (opposite end of detector) and the analytes. Consequently, the electrophoretic mobility of the droplets is greater than EOF and the analytes effort to migrate against the microemulsion droplets and move slowly towards the detector. This result in lengthen the migration time of analytes or not eluted. So, the positive polarity may not be possible to use in w/o MEEKC.

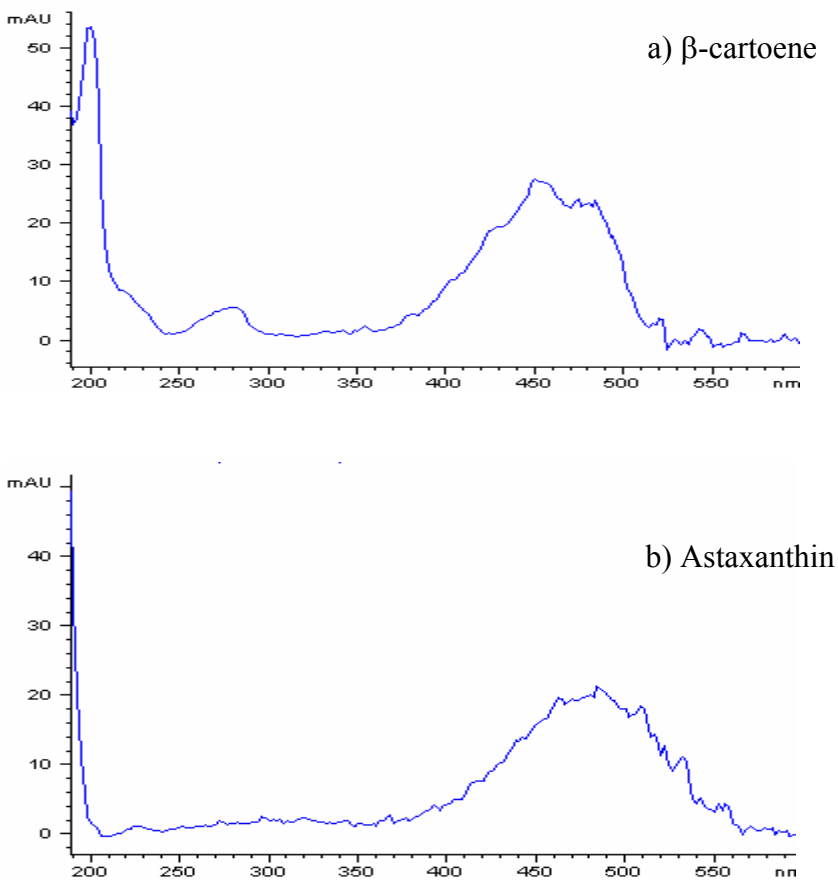

Figure 20 The UV-visible spectra of 100 μg/ml β-carotene (a) and 100 μg/ml of astaxanthin (b) in w/o microemulsion (ME-4) from the diode array detector of the capillary

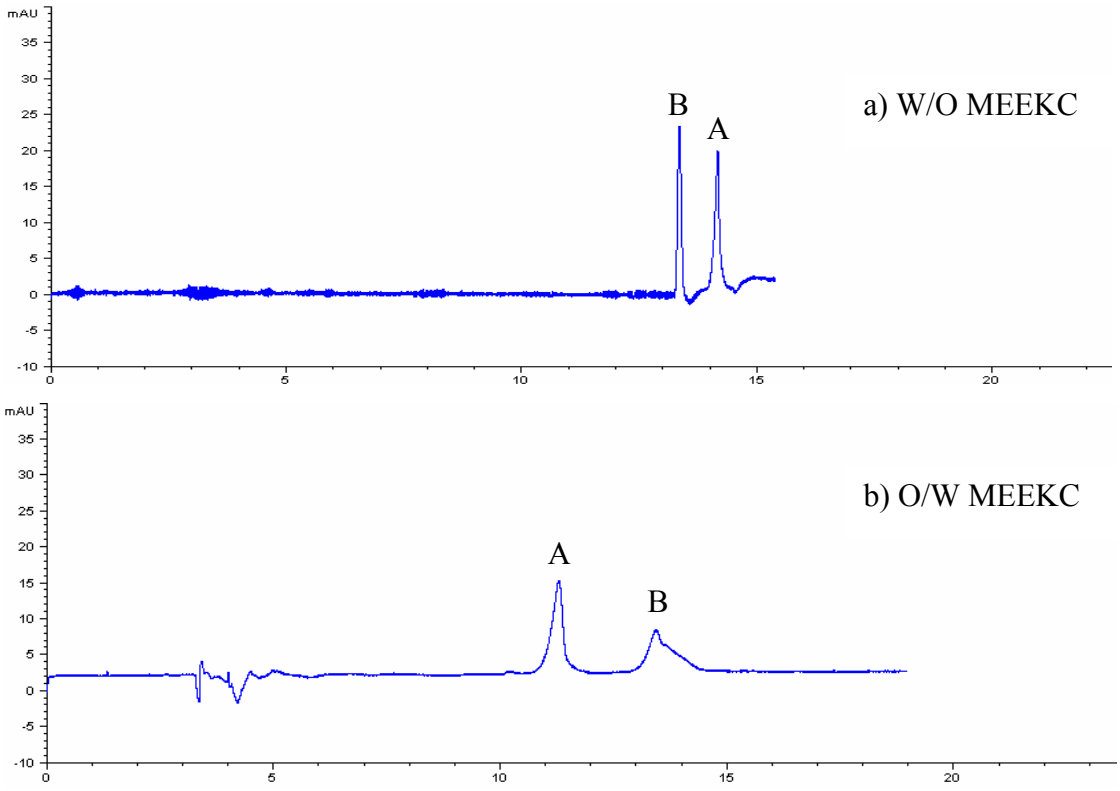

Figure 21 Comparison of separation of standard mixture of β-carotene (B) and astaxanthin (A) by w/o MEEKC and o/w MEEKC. Separation conditions: (a) ME-4, Applied voltage -30 kV, Capillary tube 32 cm (effective length) × 50 μm (I.D) for w/o MEEKC; (b) ME-3; Applied voltage +20 kV; Capillary tube 23.5 cm (effective length) × 50 μm (I.D) for o/w MEEKC; Temperature 25°C; Hydrodynamic injection at 50 mbar per 2 s; 475 nm with 40 nm bandwidth.

According to Figure 21 and Table 7, w/o microemulsion system (ME-4) offered better peak shape, resolution, number of theoretical plate, tailing factor and sensitivity than o/w microemulsion system. The order of elution was reversed of w/o MEEKC and the more hydrophobic compound β-carotene was eluted first. Results show that w/o MEEKC has unique separation mechanism for highly hydrophobic compounds which are difficult to separate by o/w MEEKC. Therefore, ME-4 system was used to carry out optimization

Table 7 Results of migration time, peak area, peak height, peak tailing, number of theoretical plate (N) and resolutions (R_s) of o/w MEEKC and w/o MEEKC for β-carotene (B) and astaxanthin (A)

	O/W MEEKC (ME-3)		W/O MEEKC (ME-4)	
	B	A	B	A
Migration time (min)	11.28	13.43	13.34	14.15
Peak area (mAU*S)	238.33	210.13	134.20	150.23
Peak Height (mAU)	12.85	5.76	23.86	19.48
TF	0.774	1.478	1.1	0.9
N	10843	3561	133863	120854
R_s	3.2		5.3	

4.1. Opimization

In the optimization, effects of injection time, type of oils, type of additional oils, type of surfactants, concentration of surfactants, and capillary lengths were investigated. Migration times (t_m), resolutions (R_s) and sensitivities for each condition were compared.

4.1.1 Effect of injection times

Suitable injection times were evaluated by using 1-butanol microemulsion system (ME-1). Injection times of 1-3 s at 50 mbar were assessed. Figure 22 shows that injection time of 3 s worsened astaxanthin peak shape due to sample overloading and the tailing factors for β-carotene is 1.1 and astaxanthin is 1.2. However, injection time of 1 s and 2 s gave good peak shapes with tailing factor of 1.1 for β-carotene and 0.8 and 0.9 for astaxanthin, respectively. In this case, injection by 50 mbar per 2 s was chosen for further optimization because of the acceptable peak shape and it gave better sensitivity than injection by 50 mbar per 1 s to determine the carotenoids. All the analytical data of different injection times were shown in Table 8.

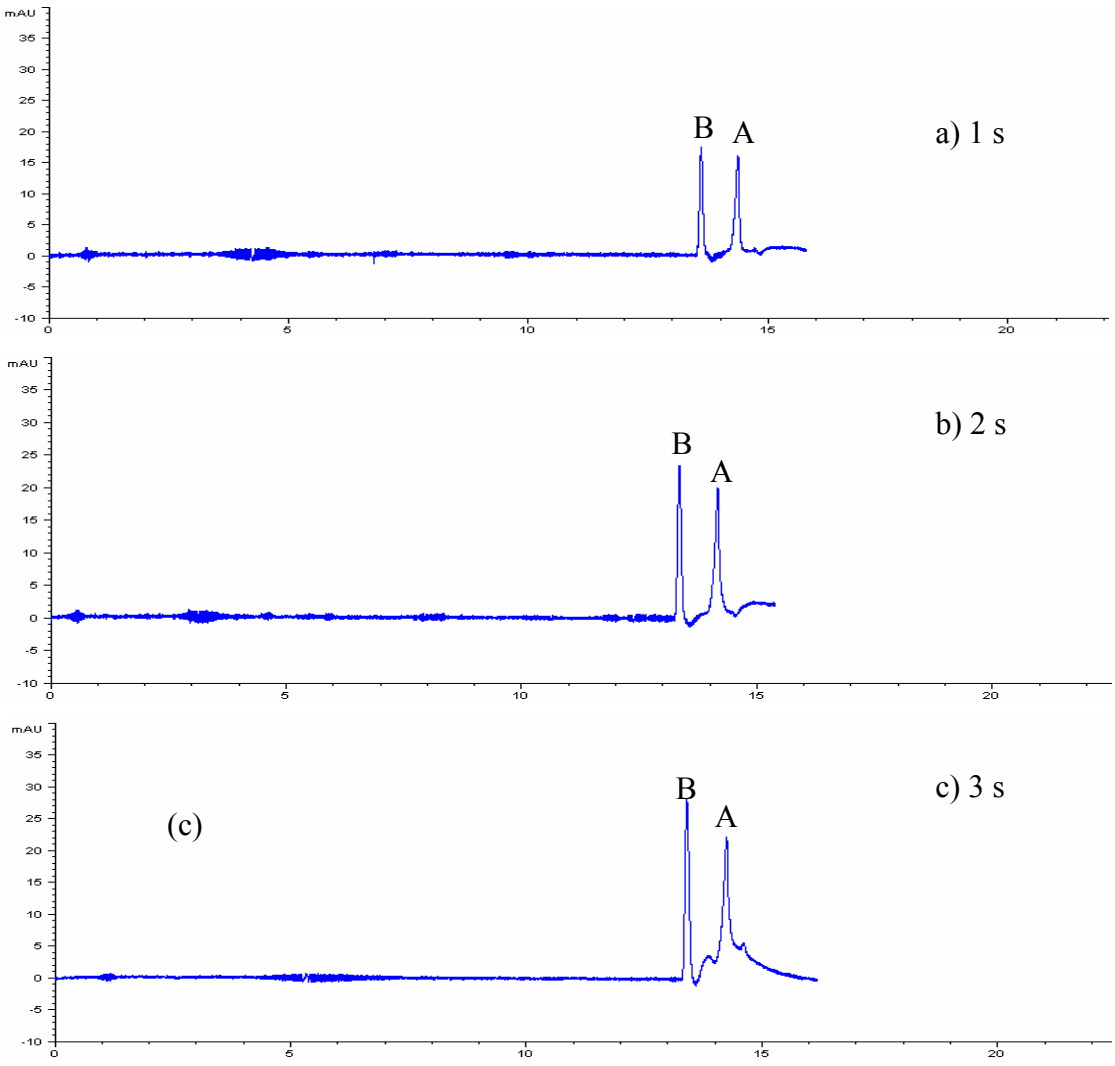

Figure 22 Influences of injection times for the separation of standard mixture of β-carotene (B) and astaxanthin (A) by w/o MEEKC. Separation condition: ME-4; Applied voltage: -30 kV, temperature 25°C, capillary: 32 cm (effective length) × 50 μm (I.D), Hydrodynamic injection: 50 mbar per (a) 1 s (b) 2 s (c) 3 s, 475 nm.

Table 8 Results of migration times, peak areas, peak heights, tailing factors (TF), number of theoretical plates (N) and resolutions (R_s) of different injection times for β-carotene (B) and astaxanthin (A)

	50 mbar per 1s		50 mbar per 2s		50 mbar per 3s	
	B	A	B	A	B	A
Migration time (min)	13.591	14.357	13.34	14.15	13.482	14.328
Peak area (mAU*S)	84.54	91.94	134.20	150.23	169.22	98.24
Peak Height (mAU)	17.24	15.40	23.86	19.48	27.79	17.34
TF	1.1	0.8	1.1	0.9	1.1	1.2
N	177955	149147	133863	120854	124321	107090
R_s	5.5		5.3		4.8	

4.2 Effect of type of oils

W/O microemulsions generate low separation current making the application of high voltage possible. However, high buffer concentrations are needed in w/o MEEKC to generate sufficient operation current to achieve stable and efficient resolutions. On the other hand, to generate adequate current, the oil phase involved is an important factor in w/o MEEKC because it occupies the highest amount and main background solvent in microemulsion buffer. Therefore, types of oil are potential and their physico-chemical properties such as viscosity, dielectric constant and conductivity significantly affect the separation of the analytes.

In this experiment, 1-butanol and 1-pentanol were used as oil phase and their physicochemical properties are described in Table A2 (See appendix). Both oils can form microemulsions well. However, successful separation of both carotenoids could be achieved in 1-butanol microemulsion (ME-4), but not in 1-pentanol microemulsion (ME-5) (Figure 23). It is because 1-butanol has a lower viscosity (3 cP at 25 °C) and more mixable with buffer (ε = 17.51) which generates adequate current (11.4 μA) to

separate the analytes in comparison with 1-pentanol (4.06 cP at 25 °C, ε =14.2). Ions can not migrate fast in higher viscosity solutions and therefore lesser current is generated than lower viscosity solutions. Therefore, ME-5 system could not give baseline separation of the carotenoids due to generating insufficient current (3.6 μA).

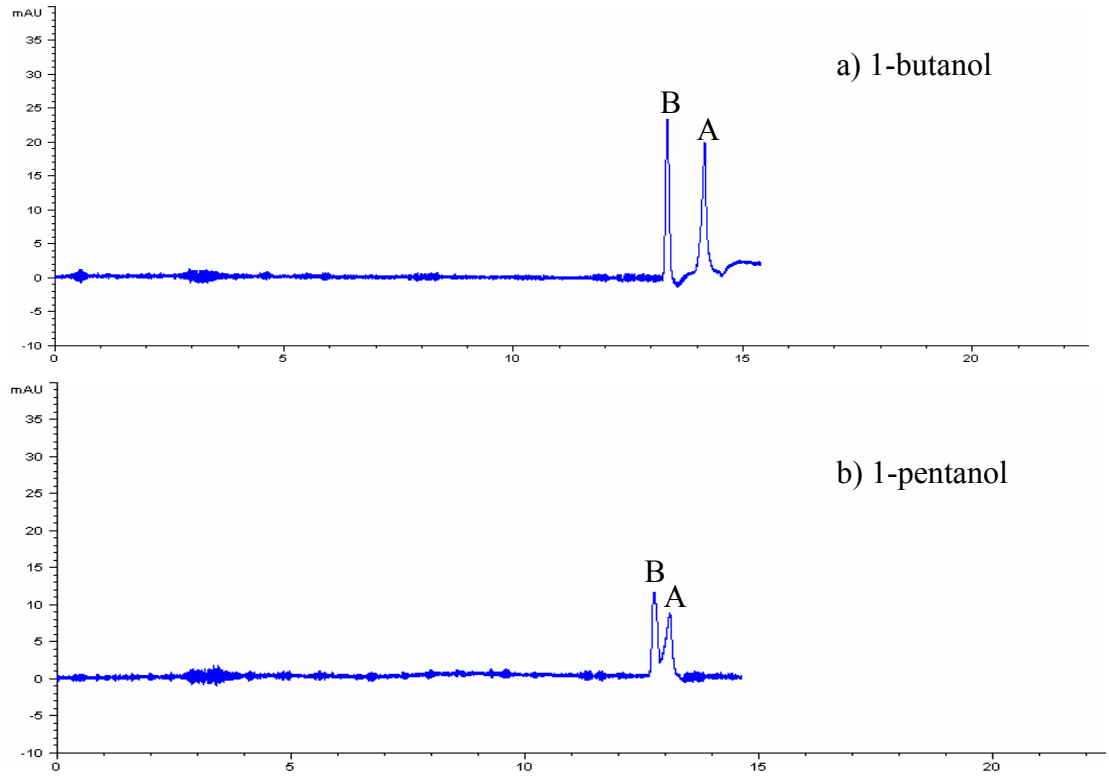

Figure 23 Effect of different of oils on the separation of standard mixture of β-carotene (B) and astaxanthin (A) by w/o MEEKC. Separation conditions: (a) ME-4 (b) ME-5; Applied voltage -30 kV; Temperature 25°C; Capillary tube 32 cm (effective length) × 50 μm (I.D); Hydrodynamic injection (50 mbar per 2 s); 475 nm with 40 nm bandwidth**.**

Table 9 show sthat results of migration times, peak areas, peak heights, tailing factors (TF), number of theoretical plate (N) and resolutions (R_s) of 1-butanol microemulsion (ME-1) and 1-pentanol microemulsion (ME-2). From these results, 1-butanol microemulsion (ME-4) gave good resolution and peak shapes; hence this system was used to continue further optimization.

Table 9 Results of migration times, peak areas, peak heights, tailing factors (TF), number of theoretical plates (N) and resolutions (R_s) of microemulsions with different oil type (ME-4) and (ME-5) for β-carotene (B) and astaxanthin (A)

	ME-4		ME-5	
	B	A	B	A
Migration time (min)	13.34	14.15	12.77	13.10
Peak area (mAU*S)	134.20	150.23	101.64	109.30
Peak Height (mAU)	23.86	19.48	12.04	9.17
TF	1.1	0.9	1.3	1.2
N	133863	120854	50796	31638
R_s	5.3		1.3	

4.3 Effect of type surfactants

The choice of surfactants gives a significant effect on the separation in both w/o and o/w MEEKC because it can change charge and size of the droplets, level and direction of the EOF, and ion pair interaction with the analytes.

Anionic surfactants, sodium dodecyl sulphate (SDS) and sodium cholate (SC), and cationic surfactant, cetyltrimethylammonium bromide (CTAB), are often employed in MEEKC. Microemulsions consisting of these surfactants were investigated for the separation of the carotenoids. The microemulsions were prepared the same as 1-butanol microemulsion except by using 10% sodium cholate (ME-6) or 10% (w/w) CTAB (ME-7) instead of 9% (w/w) SDS (ME-4). CTAB could be used to form microemulsion well whereas SC could not give clear microemulsion. Therefore, the composition of 70 mM sodium acetate buffer was increased to 12% in the microemulsion containing SC. Their separation efficiencies were compared with that of microemulsion containing SDS.

Using SC instead of SDS lengthened migration time of carotenoids to 80 min and both carotenoids may be co-eluted together (Figure 24b). Although SC is anionic

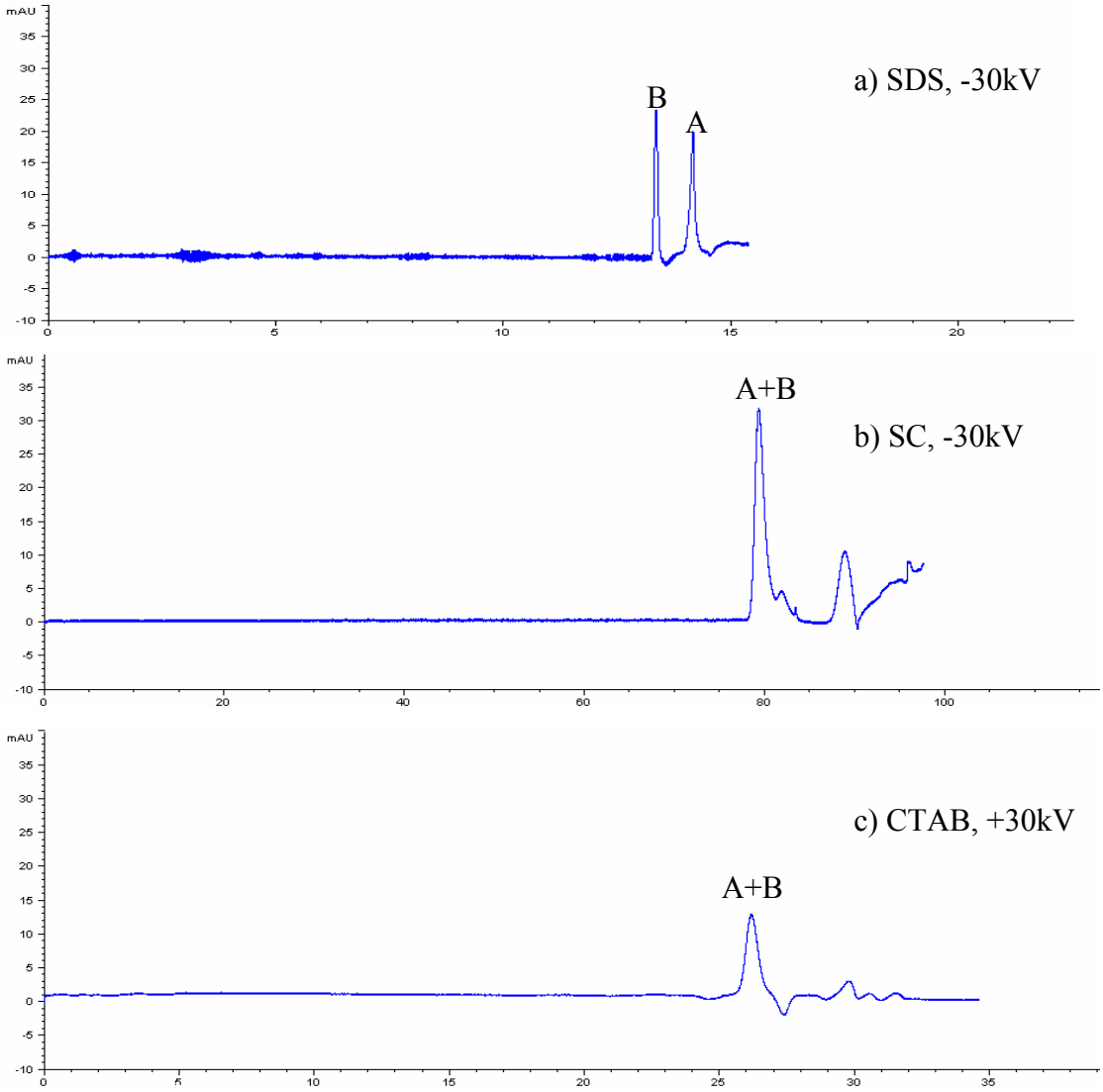

Figure 24 Effect of surfactant types on the separation of standard mixture of β-carotene (B) and astaxanthin (A) by w/o MEEKC. Separation conditions: (a) ME-4, Applied (b) ME- 6, (C) ME-7; Applied voltage -30 kV for (a) and (b) and +30 kV for (c); Temperature 25°C, Capillary tube 32 cm (effective length) × 50 μm (I.D), Hydrodynamic injection (50 mbar per 2 s), 475 nm with 40 nm bandwidth.

surfactant like SDS, the migration times of carotenoids were very long either due to lower charge to size ratio (q/r) of the microemulsion will result in a slower migration of the droplets. In addition, microemulsion containing SC gave insufficient current (5.7 μA) than SDS microemulsion.

For CTAB using negative polarity, both carotenoids did not appear until 100 min. This was due to the migration of the positive microemulsion droplets with their electrophoretic mobility toward the anode (opposite to the detector). Therefore, CTAB using positive voltage was re-investigated. Under this condition, the carotenoids were co-eluted within 27 min with low sensitivity (Figure 24c). Both carotenoids were co-eluted together because there may be some interaction between positive charge of microemulsion droplets and negative electron cloud of both carotenoids. Therefore, the microemulsion with SDS was determined to be the optimal surfactant and used to carry out optimization since it gave the shortest migration times and the best resolution among the surfactants.

4.4 Effect of surfactant concentrations

The effect of surfactant concentrations on the separation of the carotenoids was investigated. In this experiment, standard microemulsion system containing 10% (w/w) SDS, 10% (w/w) aqueous buffer [21, 22] composition did not give clear microemulsion. Therefore, ME-4 system was initially used as standard composition in this present work. Although microemulsion containing of 9% (w/w) SDS and 11% (w/w) aqueous buffer [ME-4] gave good peak shape and resolution, the migration time was long 13.34 and 14.15 min. Therefore, surfactant concentration was decreased to reduce migration time. Previous reports [21, 22] investigated that the surfactant SDS concentration gave a great effect on retention and resolution of the test mixtures in w/o and the concentration of surfactants show strong relationship with water content. If the surfactant concentration is decreased, it must be compensated by increasing the water content to obtain a stable microemulsion.

In this experiment, the surfactant (SDS) concentration was decreased from 9% (w/w) to 4% (w/w) and reducing SDS amounts was compensated by increasing their respective aqueous buffer content (ME-8, 9, and 10). According to Figure 25 and 26, decreasing the surfactant concentrations reduced the migration times and resolution. This was due to the increase of aqueous composition, which leads to the decrease of BGE viscosity. Consequently, the analytes migrated faster which caused reduction of migration times and resolution. The currents generated at decreasing SDS concentrations which were balanced with increasing water content were nearly the

same at 8.3, 8.9, 8.8, and 8 μA for 4, 6, 8 and 9 % (w/w) respectively. Therefore, the migration time and separation efficiency of the carotenoids was only dependent on not only surfactant concentration but also the viscosity of the BGE.

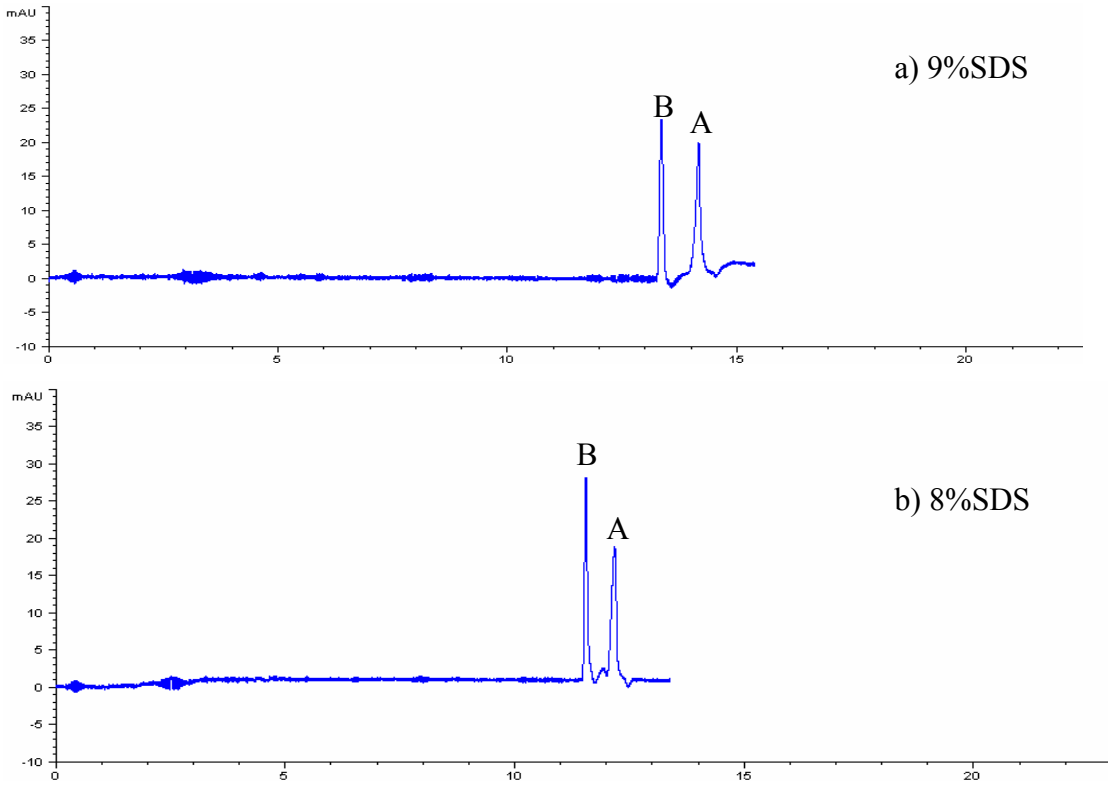

Figure 25 Comparison of the separation of standard mixture of β-carotene (B) and astaxanthin (A) by (a) ME4 (b) ME-8. Separation condition: (a) ME-4 (b) ME-8; Applied voltage -30 kV; Temperature 25°C; Capillary tube 32 cm (effective length) × 50 μm (I.D); Hydrodynamic injection (50 mbar per 2 s); 475 nm with 40 nm bandwidth.

According to this experiment, when the SDS was reduced to 4% (w/w) to 6% (w/w), migration times and resolutions were decreased with β-carotene peak split. Microemulsion containing 8%S DS decreased migration time and the resolution was drop to 4.1. Although the resolution was acceptable; however, the peak shape of astaxanthin was worse than that obtained from ME-4 (Figure 25). Therefore, ME-4 system was used for further optimization.

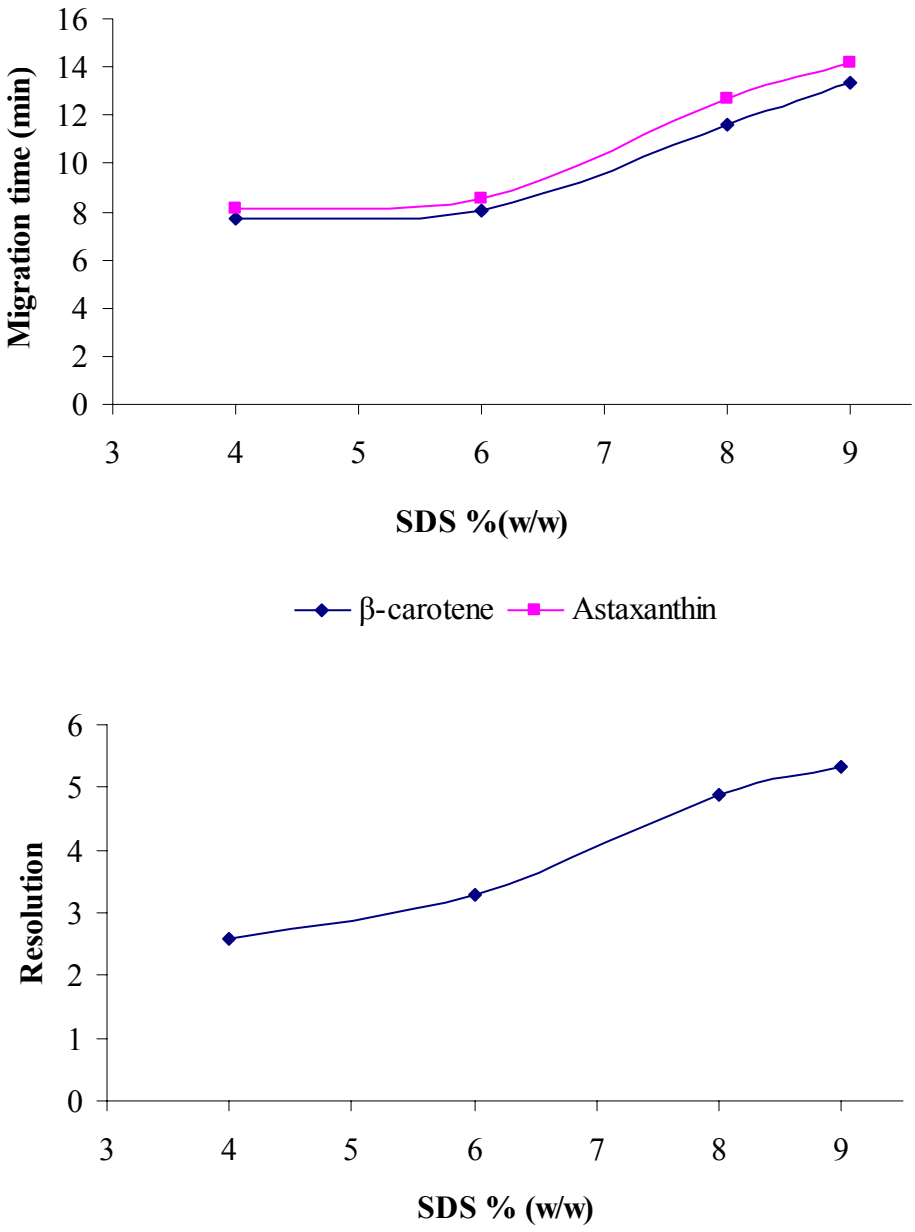

Figure 26 Effect of microemulsions with different surfactant and aqueous ratio (ME-4, ME-8, ME-9 and ME-10) on the separation of β-carotene and astaxanthin. Applied voltage: -30 kV, temperature 25°C, capillary: 32 cm (effective length) × 50 μm (I.D), Hydrodynamic injection (50 mbar per 2 s), 475 nm with 40 nm bandwidth.

4.5 Effect of additional oils

Although additional oil is mainly added for the stability of microemulsion, it can influence the selectivity, migration time and resolution [22, 23]. In this

experiment, high-interfacial oils (n-octane, n-heptane , n-octanol) and low-interfacial oil (tetrahydrofuran and ethyl acetate) are used as an additional oil and microemulsion with (ME-4 and ME-12 to 15) or without additional oil (ME-11) were investigated for carotenoid separation. All the microemulsion systems were prepared the same composition as ME-4 microemulsions except n-heptane (ME-12), n-octanol (ME-13), ethyl acetate (ME-14) and THF (ME-15) were used instead of n-octane (ME-4). The compositions of these microemulsions were shown in Table 4 (Chapter III). All microemulsions could be successfully prepared expect ethyl acetate microemulsion. Therefore, all the microemulsions except ethyl acetate microemulsion were evaluated for the separation of carotenoids.

According to Figure 27, all the microemulsions could separate the carotenoids and their migration times were not significantly different. However, among the microemulsions, n-heptane gave the best resolution value of 6.1. Although the microemulsions containing n-octane, n-octanol and THF offered good separation efficiency of 5.3, 4.7 and 4.1 respectively, the sensitivity was not as good as the one with n-heptane. n-Heptane microemulsion could not be used for the separation of the carotenoids because successive injection often caused baseline problems and migration time shift. Other microemulsions also found baseline problems and shifting the migration time, especially in THF microemulsion. Baseline problems and migration time shift may be due to capillary wall deterioration which leads to the variation of the EOF. Therefore, microemulsion without additional oil was re-investigated. This system also gave fewer baseline problems than others. Therefore, microemulsion without additional oil was used to carry out the optimization.

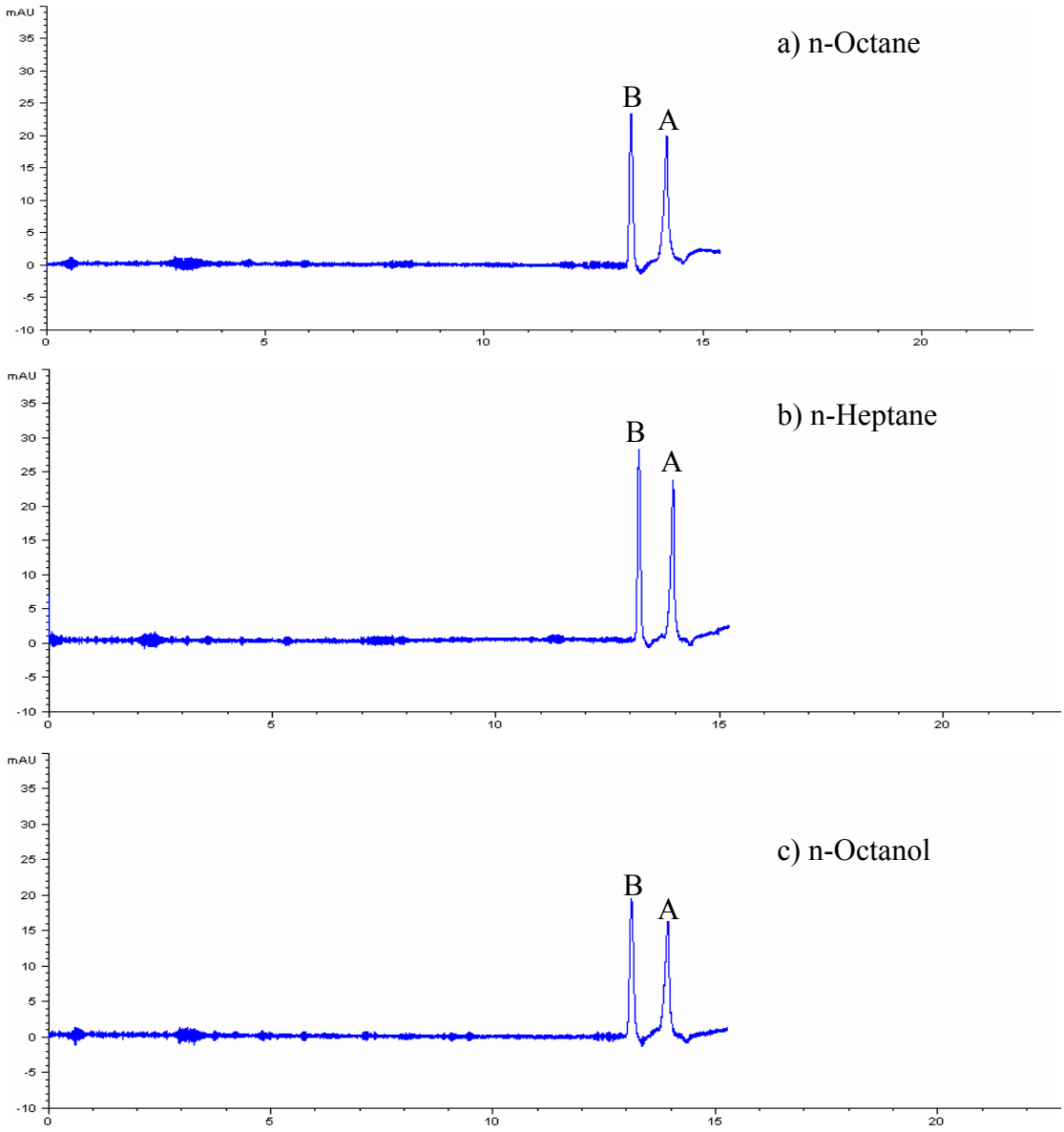

Figure 27 Effect of different additional oils on the separation of standard mixture of β-carotene (B) and astaxanthin (A) by w/o MEEKC. Separation conditions: (a) ME-4, (b) ME-12 (c) ME-13 (d) ME-14 (e) ME-11; Applied voltage -30 kV; Temperature 25°C; Capillary tube 32 cm (effective length) × 50μm (I.D), Hydrodynamic injection (50 mbar per 2 s), 475 nm with 40 nm bandwidth.

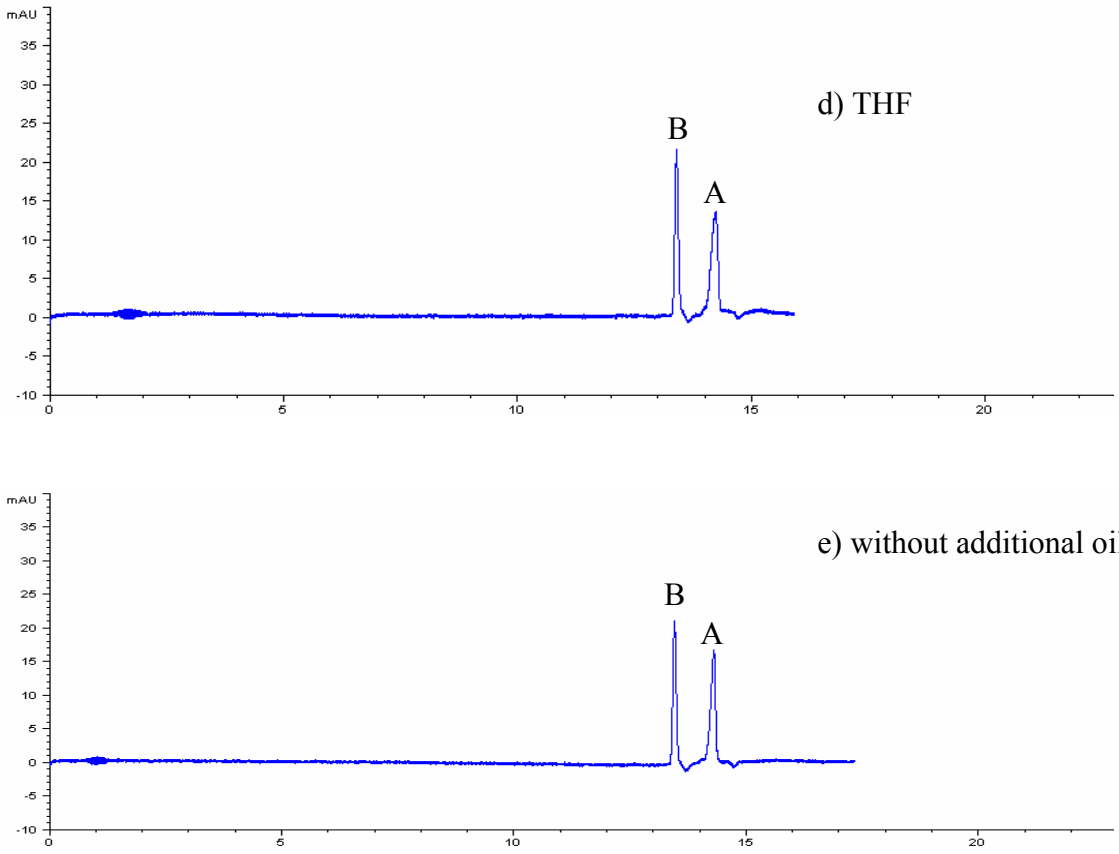

Figure 27 (Continued) Effect of different additional oils on the separation of standard mixture of astaxanthin and β-carotene by w/o MEEKC. Separation conditions: (a) ME-4, (b) ME-12 (c) ME-13 (d) ME-15 (e) ME-11; Applied voltage -30 kV; Temperature 25°C; Capillary tube 32 cm (effective length) × 50μm (I.D); Hydrodynamic injection (50 mbar per 2 s); 475 nm with 40 nm bandwidth. A = Astaxanthin, B= β-carotene.

Table 10 Results of migration times, peak areas, peak heights, tailing factors (TF), number of theoretical plates (N) and resolutions (R_s) of microemulsions with different additional oil (ME-4 and ME-11 to 15) for β-carotene and astaxanthin

Additional oil	β-carotene					Astaxanthin					R_s
	t_m (min)	Peak Area (mAU*S)	Peak Height (mAU)	N	TF	t_m	Peak Area (mAU*S)	Peak Height (mAU)	N	TF	
Octane (ME-4)	13.34	134.21	23.86	133,863	1.1	14.15	150.23	19.48	120,854	0.97	5.3
Heptane (ME-12)	13.18	129.48	27.98	191,872	1.1	13.95	134.14	23.23	183,371	0.83	6.1
Octanol (ME-13)	13.10	124.04	19.72	105,445	1.1	13.92	117.19	15.97	92,941	0.90	4.7
THF (ME-15)	13.38	120.31	21.60	145,858	1.1	14.22	119.96	12.56	45,180	0.77	4.1
without additional oil (ME-11)	13.44	114.39	21.55	156,565	1.1	14.30	116.67	16.35	96,563	0.73	5.3

4.6 Effect of capillary length

Capillary dimension, especially effective length, affect separation efficiency and migration time. In this study, the effective length was decreased from 32 cm to 23.5 cm to reduce the migration time. When the length was decreased, the migration time was reduced half and separation efficiency also decreased; however; it was not significant (Figure 28 and Table 11). In addition decreasing migration time, the peak shape became sharp. Therefore, 23.5 cm of capillary tube of effective length was used for further experiments.

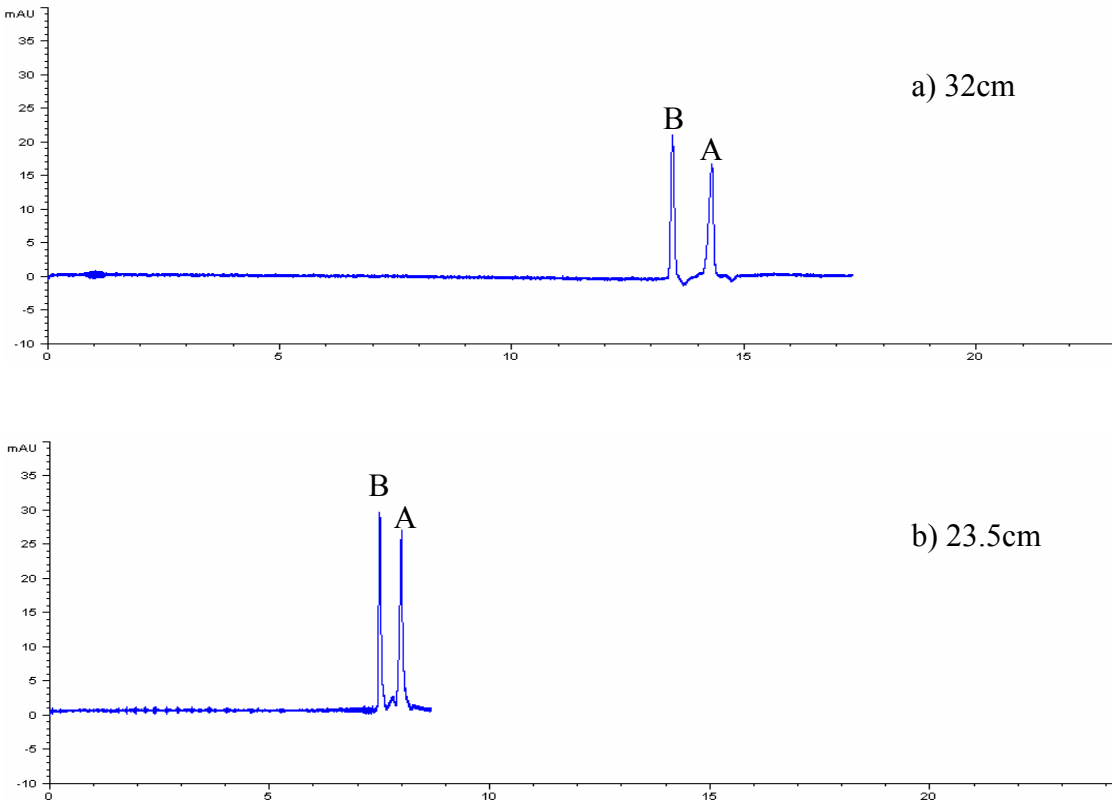

Figure 28 Effect of capillary length (a) 32 cm and (b) 23.5 cm for the separation of standard mixture of β-carotene (B) and astaxanthin (A) by w/o MEEKC. Separation conditions: ME-11; Applied voltage -30 kV; Temperature 25°C; Capillary tube 23.5 cm (effective length) × 50 μm (I.D); Hydrodynamic injection (50 mbar per 2 s); 475 nm with 40 nm bandwidth.

Table 11 Results of migration time, peak area, peak height, peak tailing, number of theoretical plate (N) and resolutions (Rs) of different effective capillary length for the β-carotene (B) and astaxanthin (A)

	32 cm		23.5 cm	
	B	A	B	A
Migration time (min)	13.34	14.15	7.50	8.00
Peak area (mAU*S)	134.21	150.23	108.71	126.94
Peak Height (mAU)	23.86	19.48	28.85	25.89
Peak tailing	1.1	0.9	1.5	1.1
N	133863	120854	102868	85880
Resolution (Rs)	5.3		4.9	

W/O microemulsion composed of 9% SDS, 80% 1-butanol and 11% sodium acetate, a capillary tube with the total length of 32 cm with effective length of 23.5 cm, 50 μm I.D, a voltage of -30 kV and temperature at 25°C was used as the optimized condition for the separation of carotenoids. Under this optimized condition, both carotenoids were separated within 9 min (Figure 28) with the acceptable peak tailing factor 1.5 for β-carotene and 1.1 for astaxanthin and gave good resolution of 4.9. Other analytical parameters, peak area, peak height and number of theoretical plates are shown in Table 11.

5. Method validation

The optimized condition for the separation of standard mixture of β-carotene and astaxanthin were validated by the assessing linearity, precision, limit of detection (LOD) and limit of quantitation (LOQ).

5.1 Linearity

The linearity of the method was performed by triplicate injection of standard mixture of β-carotene and astaxanthin at five concentrations in the range of 20 -120

Table 12 Calibration curve parameters and statistics of β-carotene

Curve	Slope	y-Intercept	Correlation coefficient (r^2)
Peak Area			
1	1.0207	2.1989	0.9980
2	1.0122	2.3858	0.9967
3	1.0143	2.1987	0.9965
Average	1.0157	2.2611	0.9971
%RSD	0.44	4.77	
Peak Height			
1	0.2478	1.2146	0.9718
2	0.2531	0.4302	0.9871
3	0.2318	0.9582	0.9304
Average	0.2442	0.8677	0.9631
%RSD	4.54	46.09	

Table 13 Calibration curve parameters and statistics of Astaxanthin

Curve	Slope	y-Intercept	Correlation coefficient (r^2)
Peak Area			
1	1.1657	11.203	0.9975
2	1.1589	12.703	0.9954
3	1.1598	13.691	0.9941
Average	1.1615	12.592	0.9960
%RSD	0.3180	9.99	
Peak Height			
1	0.1888	3.2704	0.9580
2	0.1989	1.3597	0.9850
3	0.1917	3.3953	0.9548
Average	0.1950	2.6750	0.9660
%RSD	2.85	42.64	

Three calibration curves from different days were established for each carotenoid. Calibration curve parameters and statistics for β-carotene and astaxanthin are in Table 12 and 13 and all the detailed data are shown in Table A3 and A4 (See appendix). The calibration data calculated from peak area and peak height with the signal, which provided the best correlation, was selected for establishing the calibration curve.

From Tables 12 and 13, it is evident that the calibration curve calculated from peak area provided the best correlation with the smallest %RSDs. Under the optimized condition, high linear relationship of peak area and concentration of each carotenoid was obtained more than 0.99. However, correlation coefficients obtained from the peak height was less than 0.97 and unacceptable % RSDs. Therefore, peak areas were employed to establish the calibration curve for both carotenoids.

5.2 Precision

Method reproducibility was determined by measuring repeatability of injection and intermediate precision (intra-day and inter-day precision) of the migration time, peak area and peak height.

5.2.1 Injection precision

Injection precision was firstly investigated at the middle point of the calibration curve (75 μg/ml) by nine replicate injections. The % RSDs of migration time, peak area and peak height were determined. The % RSDs calculated from migration times, peak areas and peak heights are shown in Table 13. Although % RSDs of migration time and peak area were less than 3.3, for both carotenoids. Those of peak height was very high, 12% for β-carotene and 10% for astaxanthin, respectively.

Table 14 Analytical data of injection precision β-carotene and astaxanthin

Injection no.	β-carotene			Astaxanthin		
	t_m (min)	Peak area (mAU*S)	Peak height (mAU)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)
1	8.35	87.45	21.46	8.91	107.38	23.69
2	8.27	92.09	20.88	8.86	106.37	19.73
3	8.43	89.15	20.54	9.02	107.55	22.64
4	8.19	84.47	19.43	8.75	105.76	19.43
5	8.38	85.60	18.69	8.95	108.50	25.30
6	8.18	82.39	19.18	8.74	105.90	22.31
7	8.16	86.56	15.99	8.71	108.08	19.64
8	8.81	84.91	16.16	9.42	104.95	20.75
9	8.83	85.09	15.38	9.47	107.73	19.15
Average	8.40	86.412	18.64	8.98	106.91	21.41
SD	0.25	2.8591	2.27	0.28	1.21	2.18
%RSD	3.03	3.3086	12.20	3.12	1.13	10.20

5.2.2 Intra-day precision

Intra-day precision was examined at three different concentrations (50 μg/ml, 75 μg/ml and 100 μg/ml) of standard mixture of β-carotene and astaxanthin by three replicate injections. The % RSDs of migration time, peak area and peak height of both carotenoids were determined and the results are shown in table 14 and 15. For intra-day, the % RSDs of all β-carotene were within 2.5%, 3.8%, and 7% for migration time, peak area and peak height respectively (Table 14). For astaxanthin, % RSDs for migration time and peak area were generally less than 2.7 %, however; those of peak height were from 4.8 to 8.1% (Table 15). The results show that the precisions of peak heights for both carotenoids were not good.

Table 15 Analytical data of intra-day precision of β-carotene

Injection no.	Concentration (μg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)
1	50.40	8.07	55.03	14.55
2		8.13	55.95	13.87
3		8.26	54.03	13.94
Average		8.16	55.01	14.12
SD		0.10	0.96	0.37
%RSD		1.25	1.74	2.63
1	76.61	7.65	75.51	20.97
2		7.99	74.14	21.59
3		7.99	79.72	21.45
Average		7.88	76.46	21.33
SD		0.20	2.91	0.32
%RSD		2.51	3.80	1.51
1	100.80	7.54	99.46	27.17
2		7.78	101.96	25.30
3		7.75	99.25	23.64
Average		7.69	100.22	25.37
SD		0.13	1.51	1.77
%RSD		1.73	1.50	6.96

Table 16 Analytical data of intra-day precision of astaxanthin

Injection no.	Concentration (μg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)
1	51.20	8.62	74.77	14.76
2		8.67	77.80	15.49
3		8.82	75.45	16.25
Average		8.70	76.00	15.50
SD		0.11	1.60	0.74
%RSD		1.21	2.10	4.80
1	77.83	8.15	105.13	21.07
2		8.55	105.29	18.85
3		8.53	108.97	22.11
Average		8.41	106.47	20.67
SD		0.22	2.18	1.67
%RSD		2.66	2.05	8.06
1	102.40	8.03	125.41	30.03
2		8.29	125.35	26.39
3		8.26	126.01	27.27
Average		8.20	125.59	27.90
SD		0.14	0.37	1.90
%RSD		1.74	0.29	6.81

5.2.3 Inter-day precision

Inter-day precision was determined by evaluating three different concentrations (50 μg/ml, 75 μg/ml and 100 μg/ml) on six different days. Each concentration was injected by three times. The %RSDs of migration time, peak area and peak height were shown in table 17 and 18. For inter-day precision, the % RSDs of all β-carotene concentrations were within 3%, 5%, 11.6% for migration time, peak area and peak height respectively (Table 17). For astaxanthin, % RSDs of migration time and peak area were in the range of 0.7 to 3.4% and those of peak height was 5 to 14.9% (Table 18).

Table 17 Analytical data of inter-day precision of β-carotene

Day	Concentration (μg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)
1	50.40	7.68	52.22	15.32
2		8.16	55.01	14.12
3		7.99	52.74	13.37
4		7.70	51.75	13.28
5		8.11	56.52	14.31
6		8.27	58.63	14.31
Average		7.98	54.48	14.12
SD		0.24	2.73	0.75
%RSD		3.07	5.01	5.29
1	76.61	8.04	79.15	17.42
2		7.88	76.46	21.33
3		8.34	77.85	18.02
4		7.84	77.16	17.48
5		7.87	85.73	19.73
6		7.82	82.39	22.90
Average		7.97	79.79	19.48
SD		0.2	3.58	2.26
%RSD		2.53	4.49	11.62
1	100.80	7.90	103.72	28.19
2		7.69	100.22	25.37
3		8.03	102.30	25.66
4		7.81	102.69	20.78
5		7.57	107.81	28.11
6		7.81	105.12	28.86
Average		7.80	103.64	26.16
SD		0.16	2.61	3.00
%RSD		2.06	2.52	11.47

Table 18 Analytical data of inter-day precision of astaxanthin

Day	Concentration (μg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)
1	51.20	8.18	72.87	15.18
2		8.70	76.00	15.50
3		8.56	75.43	10.95
4		8.21	77.38	15.45
5		8.68	76.47	11.91
6		8.89	77.35	12.61
Average		8.54	75.92	13.60
SD		0.29	1.68	2.02
%RSD		3.35	2.21	14.85
1	77.83	8.51	103.45	18.11
2		8.41	105.63	20.73
3		8.81	105.73	18.52
4		8.58	104.94	19.12
5		8.40	104.18	20.45
6		8.59	104.95	19.36
Average		8.55	104.82	19.38
SD		0.15	0.87	1.04
%RSD		1.80	0.83	5.36
1	102.40	8.41	127.09	22.50
2		8.19	125.59	27.90
3		8.61	127.47	20.90
4		8.57	128.20	23.05
5		8.08	126.19	27.97
6		8.32	126.74	26.06
Average		8.36	126.88	24.73
SD		0.21	0.93	2.99
%RSD		2.51	0.73	12.09

In conclusion, % RSDs of injection precisions, intra-day precisions and inter-day precisions of migration times and peak areas of both carotenoids were ≤ 5. However, those of peak height were increased up to 14.9. Their peak area and migration time gave better % RSD than peak height.

5.3 Determination of LOD and LOQ

LOD and LOQ of each carotenoids were performed by diluting 100 μg/ml of each standard solution to obtain the concentration which can give signal to noise ratio of 3 for LOD and of 10 for LOQ. All peaks were detected at the wavelength which gave the maximum absorbance. Therefore, β-carotene was detected at 450 nm with 40 nm bandwidth and astaxanthin was at 475 nm with 40 nm bandwidth by opening the reference channel which can reduce baseline line noise. All the detailed LOD and LOQ data for β-carotene and astaxanthin are described in Table A5-A8 (See appendix).

The LODs based on the S/N = 3 were 3.5 and 4 μg/ml with peak height % RSDs of 5.3 and the LOQs based on S/N = 10 were 11.5 and 14 μg/ml with % RSDs of 5.3 for β-carotene and astaxanthin, respectively.

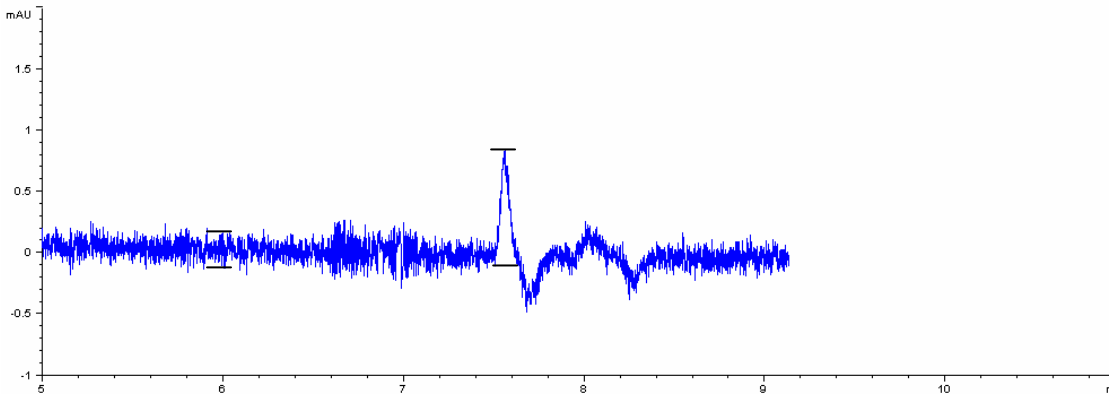

Figure 29 Limit of detection of β-carotene; BGE conditions: ME-11; Applied voltage -30kV; Temperature 25°C; Capillary tube 23.5 cm (effective length) × 50 μm (I.D); Hydrodynamic injection (50 mbar per 2 s); 450 nm with 40 nm bandwidth (reference channel 380 nm , 80 nm bandwidth).

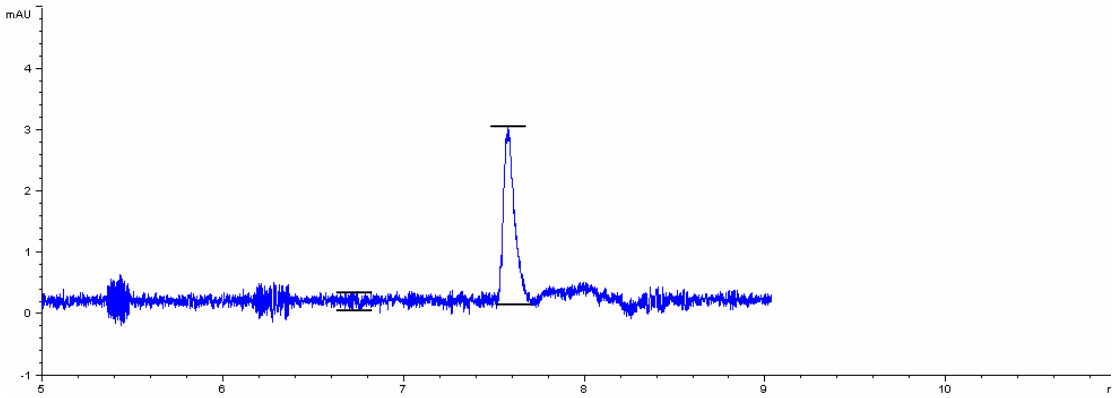

Figure 31 Limit of quantitation of β-carotene; BGE conditions: ME-11; Applied voltage -30kV; Temperature 25°C; Capillary tube 23.5 cm (effective length) × 50 µm (I.D); Hydrodynamic injection (50 mbar per 2 s); 450 nm with 40 nm bandwidth (reference channel 380nm , 80 nm bandwidth).

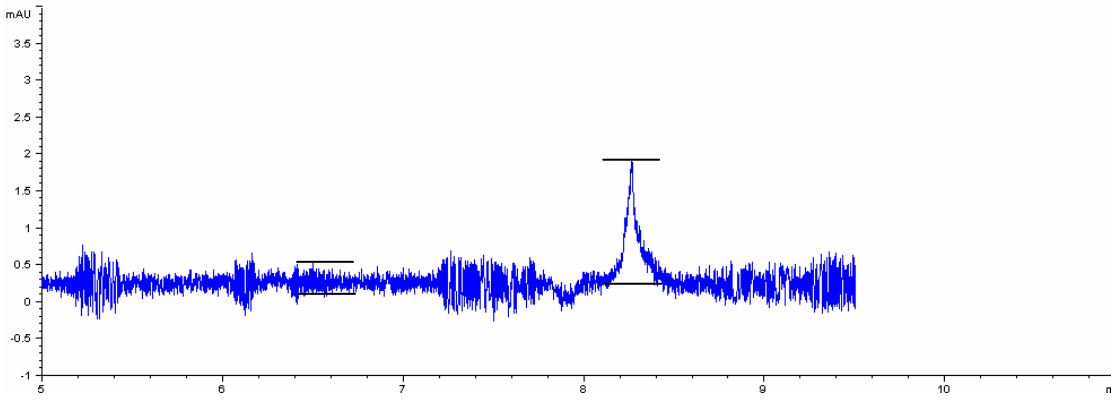

Figure 32 Limit of detection of astaxanthin; BGE conditions: 1-Butanol microemulsion composed of 9% SDS, 80% 1-butanol and 11% 70mM sodium acetate; Applied voltage: -30kV, temperature 25°C, capillary: 23.5 cm (effective length) × 50 µm (I.D), 475 nm with 40 nm bandwidth (reference channel 380nm , 80 nm bandwidth).

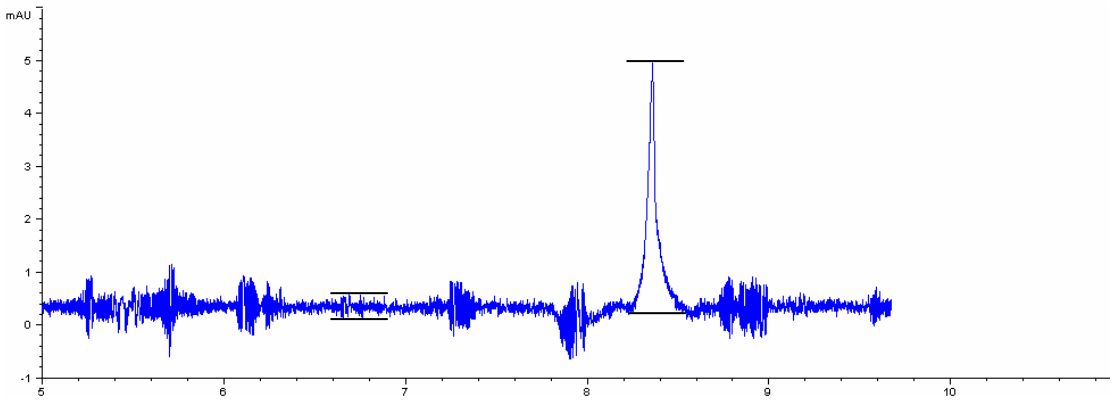

Figure 33 Limit of quantitation of astaxanthin; BGE conditions: 1-Butanol microemulsion composed of 9% SDS, 80% 1-butanol and 11% 70mM sodium acetate; Applied voltage: -30kV, temperature 25°C, capillary: 23.5 cm (effective length) × 50 μm (I.D), 475 nm with 40 nm bandwide (reference channel 380nm, 80 nm bandwidth).

CHAPTER V
CONCLUSION

The current study demonstrated for the development of w/o MEEKC method for the separation of carotenoids (e.g. β-carotene and astaxanthin). Development of w/o MEEKC condition was investigated by varying injection time, oil and surfactant types, surfactant and water compositions, additional oils and capillary length. The w/o microemulsion buffer containing 9% (w/w) SDS, 80% (w/w) 1- butanol, 11% 70 mM sodium acetate pH 8, using temperature of 25°C, voltage of -30 kV and the total capillary length of 32 cm (effective length 23.5 cm) was optimized. Both the carotenoids were detected at 475 nm with bandwidth of 40 nm. Baseline separation of the carotenoids with the R_s of 4.9 was achieved within 9 min.

The optimized method was validated by assessing its linearity, precision, LOD and LOQ as described in chapter IV. The linearity of the method was evaluated by injecting of five concentrations of working standard solutions in the range of (20-120 μg/ml). The linearity was expressed as linearity equation and correlation coefficient (r^2) which was calculated from the peak areas. Summary of all the linearity data are in Table 19.

Table 19 Summary of linearity data of carotenoids calculated from peak area

Carotenoid	Linearity equation	Correlation	% RSD of slope	%RSD intercept
β -carotene	Y=1.0157x+2.2611	0.9971	0.44	4.77
Astaxanthin	Y=1.1615x+12.592	0.9960	0.32	9.99

Method precision was determined by assessing the injection precision, intra-day precision and inter-day precision peak area, peak height and migration time of β-carotene and astaxanthin. Injection precision was determined by nine replicate

injections of the concentration at the middle point of calibration curves and % RSDs of migration time and peak area were calculated. %RSDs of injection precisions were within 3.3 for both carotenoids. For intra-day precision, three replicate injections of the standard solutions at three different concentrations were performed. For inter-day variability, the triplicate injections of three points of the calibration curve on six different days were determined. The data of method precisions were summarized in Table 20.

Table 20 Summary of precision data of β-carotene and astaxanthin

	%RSD (β-carotene)		% RSD (Astaxanthin)	
	t_m	Peak area	t_m	Peak Area
Injection precision	3.0	3.3	3.1	1.1
Intra-day precison	2.5	3.8	2.7	2.1
Inter-day precision	3.1	5	3.4	2.2

The LOD based on the S/N = 3 were 3.5 and 4 μg/ml and the LOQ based on the S/N =10 were 11.5 and 14 μg/ml with % RSD of 5.3 and 5.8% for β-carotene and astaxanthin respectively.

The % RSDs are acceptable in CE analysis although they are higher than those obtained from HPLC technique. The analysis was achieved in 9 min, which allowed the analysis of five samples per h. Comparison to HPLC, the developed w/o MEEKC technique was less expensive, rapid and generated minimal organic waste. However, the disadvantages of this method are w/o microemulsions are volatile and baseline problems were often found. These factors can affect the method precision. Although there are some disadvantages, this method can be used as an alternative for the analysis of carotenoids from different natural sources.

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APPENDIX

Table A1 Selected HPLC methods for the determination of carotenoids from different samples

Ref.	Carotenoid	Sample	Column	Mobile phase	Inj. Vol.	Detection	Significant result
88.	β-carotene *All-trans* astaxanthin 13-*cis* astaxanthin Canthaxanthin Echinenone 3-hydroxyehineneone HDC	*Phaffia rhodozyma*	Merck Lichrosorb Si 60	100% Hexane (eluent A) Hexane: Ethyl acetate 50:50 v/v (eluent B)	-	DAD	All the carotenoids were resolved in 10 min.
89.	β-carotene *All-trans* astaxanthin *cis* astaxanthin 3-hydroxyehineneone	*Phaffia rhodozyma*	(250 × 40) mm Beckman Ultrasphere silica 5μm with guard column (45 × 4.6) mm Ultrasphere silica 5μm	Petroleum ether:Ethyl acetate 1:1 v/v	-	UV-VIS 474 nm	All the carotenoids were resolved in 8 min.
90.	Astaxanthin Zeaxanthin Vitamin A Vitamin E	*Phaffia rhodozyma*	(250 × 40) mm stainless steel packed with molecular imprinted microspheres (MIMS) 8-20 μm	MeOH:DCM 8:2 v/v (eluent A) MeOH:H_2O 5:5 v/v (eluent B)	-	DAD 291nm	The method was successfully developed to separate astaxanthin in the saponified samples of algae and yeast. The analytes were separated in 20 min.

Table A1 (continued) Selected HPLC methods for the determination of carotenoids from different samples

Ref.	Carotenoid	Sample	Column	Mobile phase	Inj. Vol.	Detection	Significant result
91.	32 carotenoids	*Chlorella pyrenoisosa*	YMC C30 (250 × 4.6) mm, 5μm	MeOH: ACN: H_2O 84:14:2 v/v/v (eluent A), DCM (100%) (eluent B)	20μL	DAD (450nm)	All carotenoids could be resolved within 49 min.
92.	Astaxanthin β-carotene Chlorophyll Echinenone Canthaxanthin	*Haematococcus pluvialis*	Hypercil C_{18} 250 × 4 mm, 5 μm	H_2O (eluent A) MeOH (eluent B) Acetone (eluent C)	-	UV-VIS 447nm & 476 nm	A new low toxicity HPLC method was investigated for the separation of chlorophylls and carotenoids in a short-period of time, using low volumes of solvents and with an economical price.
93.	Astaxanthin β-carotene Canthaxanthin β-cryptoxanthin Lutein Zeaxanthin	*Spirulina platensis* microalgae	Tow ODS C18 Hypersil columns (200× 2.1)mm, 5μm & (100 × 2.1)mm, 5μm connected in series	ACN: MeOH (0.1M ammonium acetate): CH_2Cl_2 71: 22:7 v/v/v	1 to 5μL	Turbo ISP-MS	Detection limit by using ISP-MS were estimated in 0.1 to 1 ng. Better sensitivity under positive-ion than negative-ion conditions was investigated.

Table A1 (continued) Selected HPLC methods for the determination of carotenoids from different samples

Ref.	Carotenoid	Sample	Column	Mobile phase	Inj. Vol.	Detection	Significant result
94.	Astaxanthin All *trans* β-carotene 9-*cis* β-carotene 13-*cis* β-carotene Lutein Zeaxanthin Canthaxanthin β-cryptoxanthin Echinenone All *trans* lycophene *Cis*-lycophene α-carotene α-carotene	Human plasma	(150×4.6) mm, RP C_{18}, 3-μm Nucleosil column coupled with a (250 × 4.6) mm RP C_{18}, 5-μm and (20 × 4.6) mm C_{18}, 5-μm	ACN: MeOH (50mM ammonium acetate): H_2O: CH_2Cl_2	80 μl	UV-VIS (450 nm)	In addition to 13 carotenoids, unknown compounds or their metabolites were separated in the same run. All the carotenoids were resolved in 47 min.
95.	*trans*-β-carotene α-carotene Lycophene Cryptoxanthin	Blood plasma	4.6 × 250 mm Vydac 218TP54 column, 5μm, 30 nm pore size	10% THF in MeOH	10 μL	UV-VIS 450 nm & TLS	The advantages of TLS compared to UV–Vis detection included higher sensitivity and about a 100-times lower LOD for carotenoids in plasma.

Table A1 (continued) Selected HPLC methods for the determination of carotenoids from different samples

Ref.	Carotenoid	Sample	Column	Mobile phase	Inj. Vol.	Detection	Significant result
96.	Astaxanthin β-carotene Zeaxanthin Canthaxanthin Echinenone Lycophene	Vegetable juice	(250 × 4.6) mm column packed with Prontosil silica gel (3 μm, 200A°) modified with triacontyltrichlo-rosilane (C_{30})	MeOH:MTBE	-	UV-APCI-MS	Good separation, short analysis time and sharp peaks were obtained. Using APCI-MS detection, additional modification of chromatographic eluent is not necessary, which gives a robust method.
97.	Astaxanthin β-carotene Lutein All-*trans* retinol β-cryptoxanthin Canthaxanthin	Fish egg	C18 column (150 × 2.1) mm 4μm and a Phenomenex C18 guard column (40 × 2) mm, 4μm	MeOH : MTBE: H_2O 90:5:5 v/v/v (eluent A) MeOH: MTBE: H_2O (67:30:3) (eluent B) MeOH : MTBE: H_2O 57:40:3 v/v/v (eluent C)	-	ESI-MS	A method was developed for the chemical extraction and isolation, LC separation and ESI (+)–MS determination of carotenoids and all-*trans*-retinol in the eggs of Chinook salmon.

Table A1 (continued) Selected HPLC methods for the determination of carotenoids from different samples

Ref.	Carotenoid	Sample	Column	Mobile phase	Inj. Vol.	Detection	Significant result
98.	β-carotene 13-cis-β-carotene 9-cis--β-carotene Lycophene α-tocopherol γ-, δ-,α-tocotrienol Chlorophyll	Vegetable oil	4.6 × 250 mm Polymeric YMC^{TM} C30 column, 5µm coupled with C_{18} guard 4.6 × 50 mm and precolumn filter	MeOH: MTBE: Ammonium acetate : H_2O 88:5:5:2 v/v/v/v (eluent A) MEOH:MTBE: Ammonium acetate 20:78:2 v/v/v (eluent B)	25 µL	ED	Rapid and sensitive analyses of oil quality and adulteration could be achieved with this methodology.
99.	*All-trans*-β-carotene 9, 13-cis-β-carotene 9-cis-β-carotene *All-trans* zeaxanthin *All-trans* lutein *All-trans* canthaxanthin *All-trans* lycophene Chlorophyll a Chlorophyll b	Standard mixture and spinach extract	Self packed C30 capillary 15 cm×250µm, 3 µm and a pore width of 200 A°	Acetone:water 80:20 v/v	100 nl	NMR	The identification of UV light and air-sensitive carotenoids in the low ng range was accomplished with 1H NMR spectroscopy. Comparison with HPLC columns, Use of self-packed separation capillaries reducedretention times without a loss of selectivity.

Table A1 (continued) Selected HPLC methods for the determination of carotenoids from different samples

Ref.	Carotenoid	Sample	Column	Mobile phase	Inj. Vol.	Detection	Significant result
100.	*trans*-β-carotene α-carotene β-cryptoxanthin Alloxanthin Diadinoxanthin Diatoxanthin Fucoxanthin Lycophene Lutein Peridinine Sudan I Zeaxanthin 9′-hexano-yloxy-fucoxanthin Chlorophyll C_2 Chlorophyll C_3	Mixture of standards	C18 column, 33× 4.6 mm, 3 μm	MeOH: 1M ammonium acetate 80:20 v/v (eluent A) MeOH:Acetone 90:10 v/v (eluent B)	20 μL	UV-VIS 488 nm & TLS	For gradient chromatographic conditions, the LODs for the TLS detector were enhanced by as much as three times in comparison with UV-Vis detection. For the isocratic, part of the chromatogram, up to a tenfold improvement of LODs was achieved with TLS detection

Table A2 Physicochemical properties of surfactants and organic solvents used in microemulsions

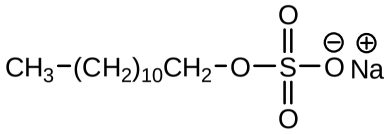
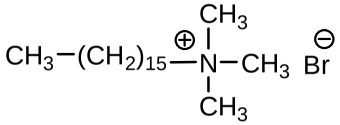
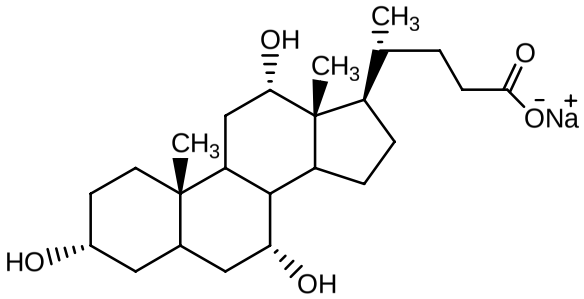
Chemical Name	Structure	MW (g)	Melting point (°C)	Boiling Point (°C)	Viscosity at 25°C (cP)	Dielectric constant (ε)
Sodium dodecyl sulphate (SDS)		288.38	204-207	-	-	
Cetyltrimethyl ammonium bromide (CTAB)		364.45	248-251	-	-	
Sodium cholate (SC)		430.55		-	-	
1-butanol	$CH_3-(CH_2)_2-CH_2-OH$	74.12	−90	116-118	3	17.51

Table A2 (continued) Physicochemical properties of surfactants and organic solvents used in microemulsions

Chemical Name	Structure	MW	Melting point (°C)	Boiling Point (°C)	Viscosity at 25°C (cP)	Dielectric constant (ε)
1-pentanol	$CH_3-(CH_2)_3-CH_2-OH$	88.15	−78	136-138	4.0608	14.2
n-hexane	$CH_3-(CH_2)_4-CH_3$	86.18	−95	69	0.294	2.02
n-heptane	$CH_3-(CH_2)_5-CH_3$	100.20	−91	98	0.386	1.9
n-octane	$CH_3-(CH_2)_6-CH_3$	114.23	−57	125-127	(20°C)0.542	2.02
n-octanol	$CH_3-(CH_2)_6-CH_2-OH$	130.23	−15	196	-	3.4
Ethyl acetate	$CH_3-COO-C_2H_5$	88.11	−84	76.5-77.5	0.426	6.02
Dichloromethane	CH_2Cl_2	84.93	−97	39.8-40	0.423	9.08
Chloroform	$CHCl_3$	119.38	60.5-61.5	−63	0.542	4.8

Table A2 (continued) Physicochemical properties of surfactants and organic solvents used in microemulsions

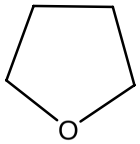
Chemical Name	Structure	MW (g)	Melting point (°C)	Boiling Point (°C)	Viscosity at 25°C (cP)	Dielectric constant (ε)
N, N dimethyl-formamide		73.09	-61	153	0.8	36.71
Tetrahydrofuran		72.11	−108	65-67	0.480	7.52

Table A3 Calibration data of β-carotene for three different days

Day	Standard no.	Concentration (μg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)
1.	1	20.16	7.79	24.59	6.26
	2	50.40	7.68	52.22	15.32
	3	76.61	8.04	79.14	17.42
	4	100.80	8.01	103.72	28.19
	5	120.96	7.73	127.88	32.24
	Slope			1.0207	0.2478
	y-intercept			2.1989	1.2146
	r^2			0.9980	0.9718
2.	1	20.16	7.79	24.59	6.26
	2	50.40	7.99	52.74	13.37
	3	76.61	8.34	77.85	18.02
	4	100.80	8.03	102.30	25.66
	5	120.96	7.73	127.88	32.24
	Slope			1.0122	0.2531
	y-intercept			2.3858	0.4302
	r^2			0.9967	0.9871
3.	1	20.16	7.79	24.59	6.26
	2	50.40	7.99	52.74	13.37
	3	76.61	8.34	77.85	18.02
	4	100.80	8.03	102.30	25.66
	5	120.96	7.73	127.88	32.24
	Slope			1.0122	0.2531
	y-intercept			2.3858	0.4302
	r^2			0.9967	0.9871

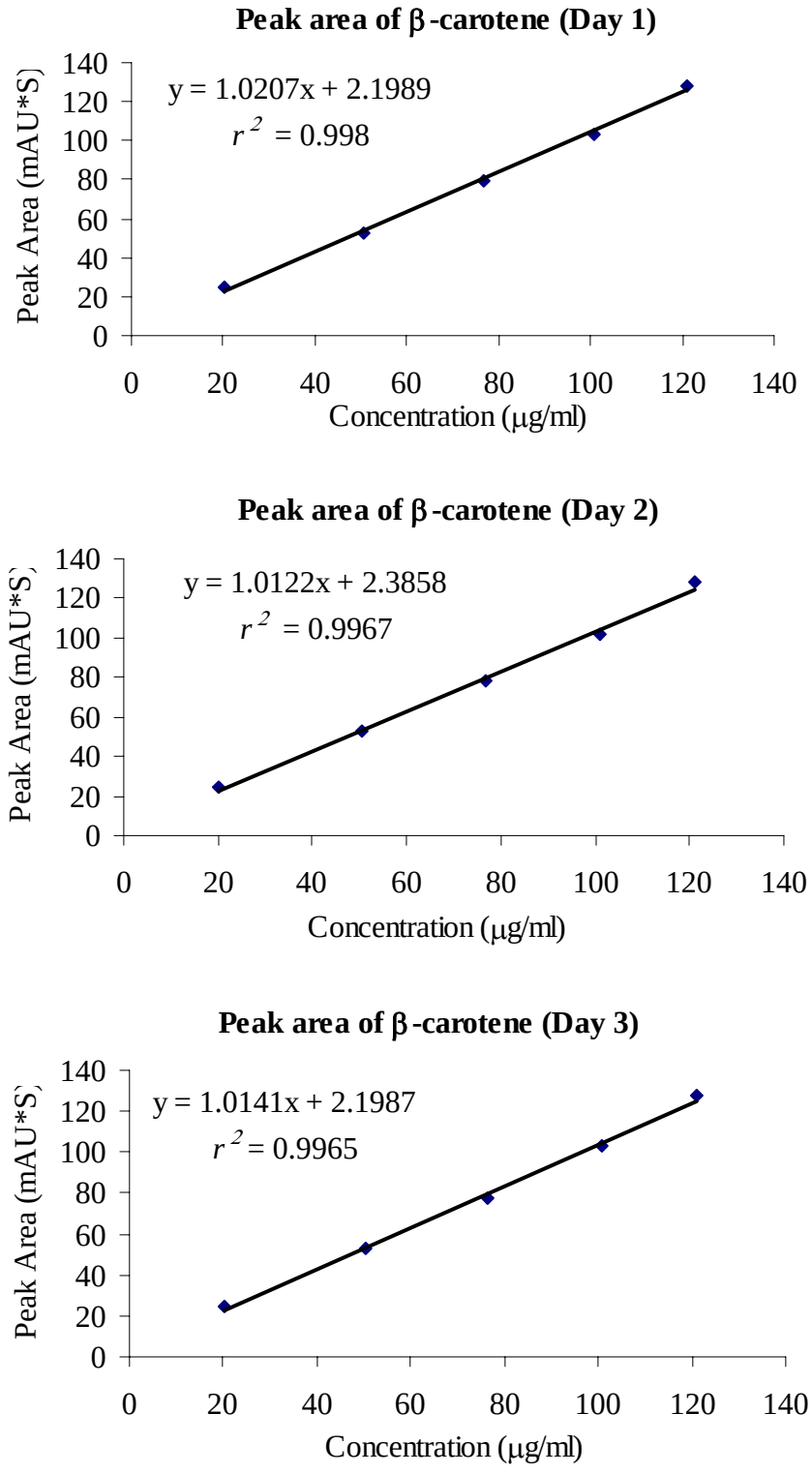


Figure A1 Calibration curves of β-carotene calculated from peak area for three different days

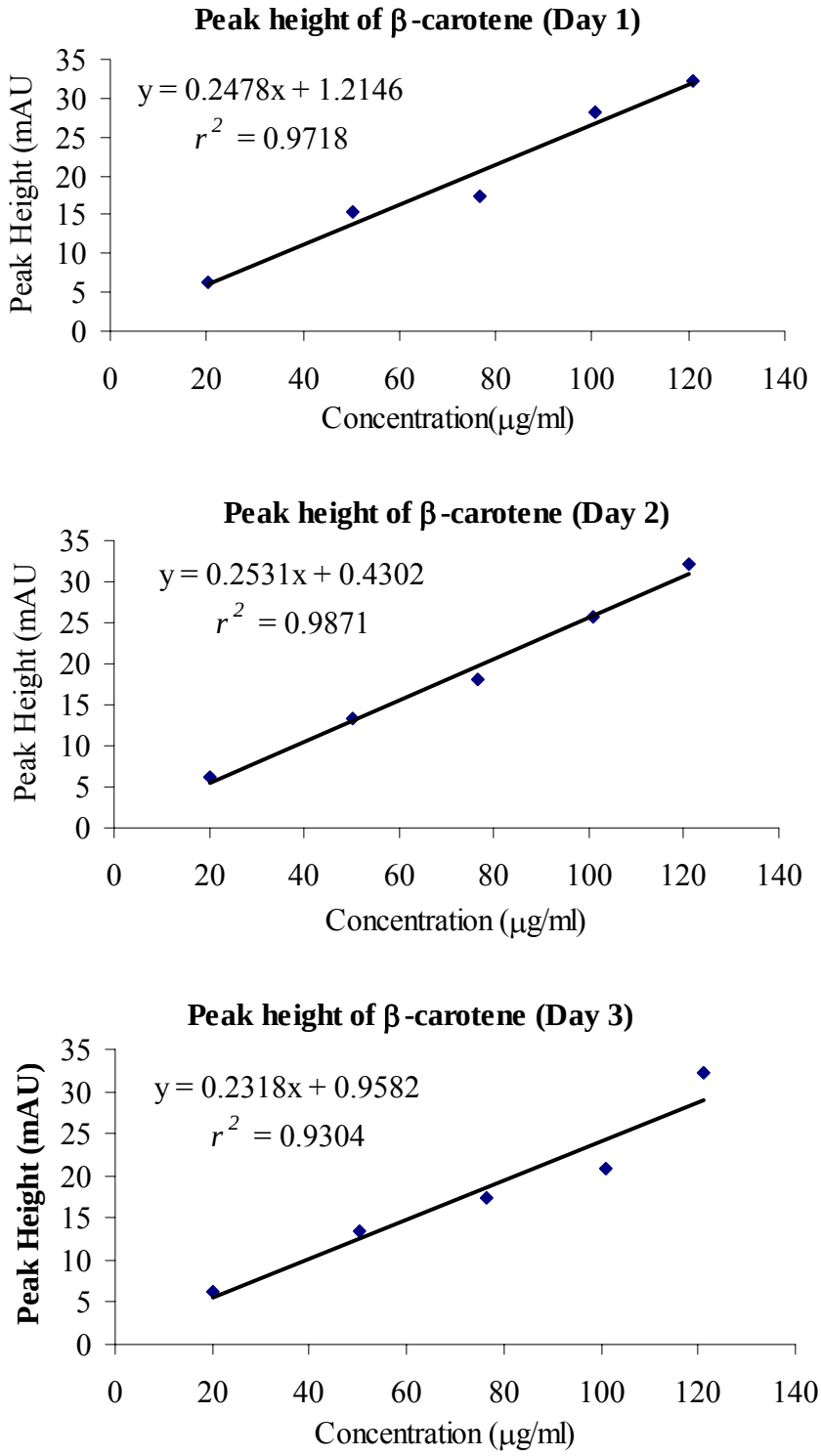


Figure A2 Calibration curves of β-carotene calculated from peak height for three different days

Table A4 Calibration data of astaxanthin for different days

Day	Standard no.	Concentration (μg/ml)	t_m (min)	Peak area (mAU*S)	Peak height (mAU)
1.	1	20.48	8.34	33.71	5.32
	2	51.20	8.18	72.87	15.47
	3	77.82	8.50	103.45	18.11
	4	102.40	8.41	127.09	22.50
	5	122.88	8.25	155.77	25.71
	Slope				
	y-intercept				
	r^2				
2.	1	20.48	8.34	33.71	5.32
	2	51.20	8.56	75.43	10.88
	3	77.82	8.93	105.47	18.52
	4	102.40	8.61	127.47	20.90
	5	122.88	8.25	155.77	25.71
	Slope				
	y-intercept				
	r^2				
3.	1	20.48	8.34	33.71	5.32
	2	51.20	8.21	77.38	15.45
	3	77.82	8.36	107.07	19.28
	4	102.40	8.17	129.20	23.05
	5	122.88	8.25	155.77	25.71
	Slope				
	y-intercept				
	r^2				

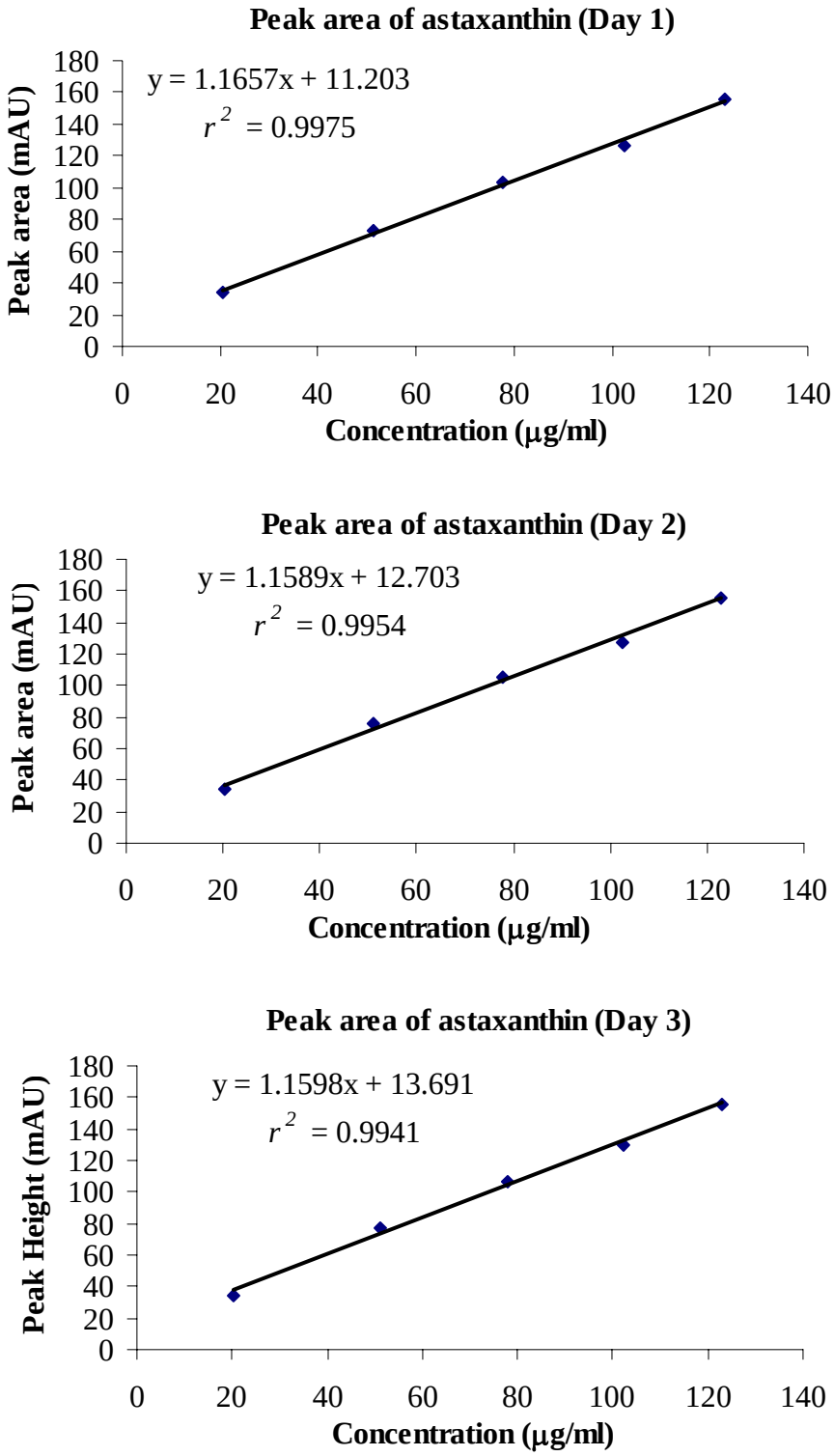


Figure A3 Calibration curves of astaxanthin calculated from peak area for three different days

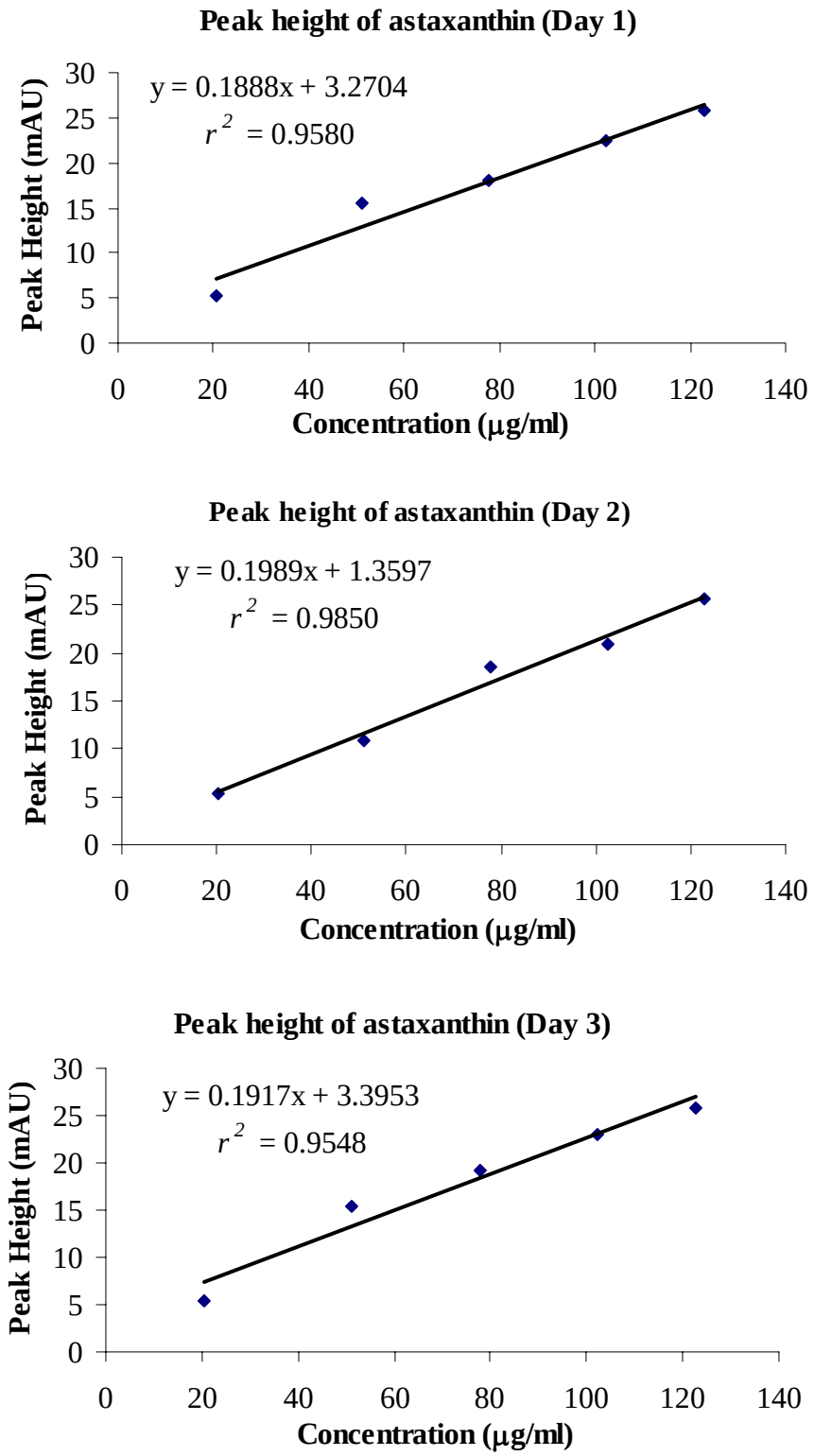


Figure A4 Calibration curves of astaxanthin calculated from peak height for three different days

Table A5 Precision of LOD of β-carotene

Limit of detection (LOD) of β-carotene			
Injection no.	t_m (min)	Peak area (mAU*S)	Peak height (mAU)
1	7.46	3.14	0.94
2	7.56	3.20	0.94
3	7.78	3.08	0.85
Average	7.60	3.14	0.91
SD	0.16	0.06	0.05
RSD	2.16	1.93	5.32

Table A6 Precision of LOQ of β-carotene

Limit of detection (LOQ) of β-carotene			
Injection no.	t_m (min)	Peak area	Peak height
1	7.45	3.64	1.15
2	7.56	3.72	1.15
3	7.51	3.97	1.24
Average	7.53	3.85	1.19
SD	0.03	0.17	0.06
RSD	0.39	4.48	5.30

Table A7 Precision of LOD of astaxanthin

Limit of detection (LOD) of astaxanthin			
Injection no.	t_m (min)	Peak area (mAU*S)	Peak height (mAU)
1	8.47	8.03	1.89
2	8.28	10.20	2.01
3	8.26	12.14	1.79
Average	8.34	10.12	1.90
SD	0.12	2.06	0.11
RSD	1.38	20.32	5.77

Table A8 Precision of LOQ of astaxanthin

Limit of detection (LOQ) of astaxanthin			
Injection no.	t_m (min)	Peak area	Peak height
1	8.562	18.64792	4.85780
2	8.356	21.16758	4.81374
3	8.324	23.44043	4.88330
Average	8.41	21.09	4.85
SD	0.13	2.40	0.04
RSD	1.54	11.37	0.73

BIOGRAPHY

NAM E	Miss Khin Thida Nyunt
DATE OF BIRTH	1 June 1975
PLACE OF BIRTH	Mandalay, Myanmar
INSTITUTIONS ATTENDED	University of Pharmacy, Yangon, 1997-2002 Bachelor of Pharmacy Mahidol University, 2005-2007: Master of Science in Pharmacy (Pharmaceutical chemistry)
GRADURATION GRANT	University of Pharmacy (Yangon, Myanmar) and China Medical Board (CMB)
POSITION & OFFICE	Demonstrator (2001-present) Department of Pharmaceutical chemistry University of Pharmacy, Yangon Myanmar Email: linlinjean@gmail.com