CHAPTER I

INTRODUCTION

1.1 Glucosamine and its properties [1]

Glucosamine is an amino monosaccharide found in chitin, glycoproteins and in glycosaminoglycans (GAGSs) (formerly know as mucopolysaccharide), such as hyaluronic acid and heparan sulphate. It is the basic building block of the amino sugars and hence is an important constituent of the cell wall and interstitial proteins.

The chemical structure of glucosamine is shown in Figure 1.1. Glucosamine (2-amino-2-deoxyalpha-D-glucose) is one of the two hexosamine sugars (six carbon amino sugars) common in human cells. Structurally, glucosamine is modified glucose, with an $–NH_3$ group replacing the –OH group found on carbon two (C-2). G6-P is an amino monosaccharide (amino sugar) produced in the body by the combination of glucosamine with fructose, through the enzymatic action of glucosamine synthetase.

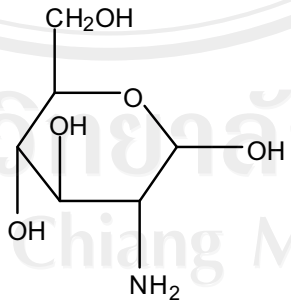

Figure 1.1 Chemical structure of glucosamine [1]

Glucosamine has two natural stereoisomers (α andβ) (Figure 1.2), and the interconversion of these two in aqueous solution is not preventable.

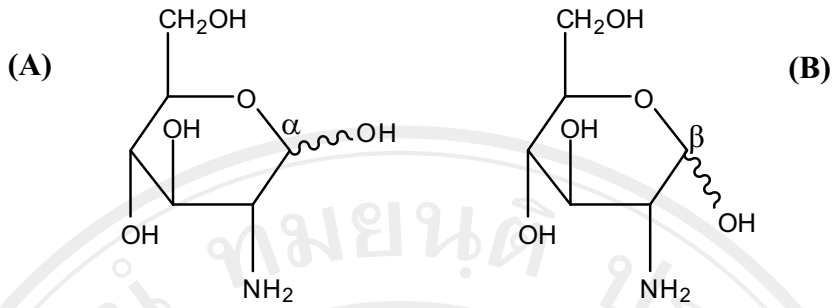

Figure 1.2 (A) Alpha-D- glucosamine (B) Beta-D- glucosamine

Glucosamine is available commercially as a nutritional supplement in three forms: glucosamine HCl, glucosamine sulphate and *N*- acetyl-glucosamine. All three forms are water soluble, the salt acting as a delivery vehicle. At neutral and physiological pH, the amino group in glucosamine is protonated, resulting in a positive charge. Salt forms of glucosamine contain negative anions to neutralize the charge.

In the case of glucosamine hydrochloride, the anion is chloride, and in glucosamine sulphate the anion is sulphate. *N*- acetyl-glucosamine is a delivery form of glucosamine in which the amino group is acetylated, thus neutralizing its charge. To date, most of the clinical studies examining the effect of glucosamine on osteoarthritis (OA) have been performed with either the sulphate or the chloride salts of glucosamine.

The glucosamine used in supplements is typically derived from marine exoskeletons. Synthetic glucosamine ia also available.

1.1.1 Pharmacokinetics [1]

About 90% of glucosamine administered orally as a glucosamine salt gets absorbed from the small intestine and from there it is transported, via the portal circulation, to the liver. It appears that a significant fraction of the ingested

glucosamine is catabolized by first-pass metabolism in the liver. Healthy men have serum glucosamine concentrations of 0.04 mmol/L when they are not consuming supplemental glucosamine. Ingestion of recommended oral doses of glucosamine in humans achieves serum levels of approximately 0.06 mmol/L. It is not presently known how much of an ingested dose is taken up in the joints in humans.

Most relevant clinical trials have used patented crystalline glucosamine sulphate in a soluble powder form, where 1500 mg is taken once a day. This is a prescription drug in most European and non European countries. However, the Dietary Supplement Health and Education Act of 1994 favored the appearance of several undocumented glucosamine salts (e.g. hydrochloride), derivatives (e.g., *N*-acetyl-glucosamine) on the dietary supplement market in the USA and other countries, and various other dosage forms and regimens.

1.1.2 Health effects [2]

Oral glucosamine is commonly used for the treatment of osteoarthritis. Since glucosamine is a precursor for glycosaminoglycans, and glycosaminoglycans are a major component of joint cartilage, supplemental glucosamine may help to rebuild cartilage and treat arthritis. Its use as a therapy for osteoarthritis appears safe, but there is conflicting evidence as to its effectiveness. A randomized, double-blind, placebo-controlled trials have found glucosamine sulfate is no better than placebo in reducing the symptoms or progression of hip osteoarthritis.

1.1.3 Use [2]

A typical dosage of glucosamine salt is 1,500 mg per day. Glucosamine contains an amino group that is positively charged at physiological pH. The anion included in the salt may vary. The amount of glucosamine present in 1500 mg of

glucosamine salt will depend on which anion is present and whether additional salts are included in the manufacturer's calculation. Glucosamine is often sold in combination with other supplements such as chondroitin sulfate and methylsulfonylmethane.

Glucosamine is a popular alternative medicine used by consumers for the treatment of osteoarthritis. Glucosamine is also extensively used in veterinary medicine as an unregulated but widely accepted supplement.

1.2 The method of glucosamine

A variety of analytical methods are available for determination of glucosamine such as Tin Layer Chromatography (TLC) [3], Spectrophotometry [4,5], High Performance Liquid Chromatography (HPLC)[6-17], Gas chromatography (GC) [18]. For more details of these methods, see Table 1.1

Table 1.1 Some analytical methods reported for the determination of glucosamine

Technique	Method	Linear Range	Detection Limit	Sample	Analyte	Ref.
Tin Layer Chromatography	High Performance Tin Layer Chromatography (HPTLC), detection with ninhydrin chromagenic reagent solution and scaning wavelength 580 nm	1.0-4.0 µg	1.0 µg	Dietary supplement	Glucosamine	[3]
Spectrophotometry	The analytes form complex with *o*-hydroxydroquinonephthaiein and palladium(II) and UV spectrophotometer detection at 630 nm	0.02-0.18 mg/L	-	Dietary supplement	- Glucosamine - Amino sugars	[4]
	The analytes form complex with phenylisothiocyanate, UV spectrophotometer detection at 240 nm	5.0-25.0 mg/L	-	Dietary supplement	Glucosamine sulfate	[5]
HPLC Spectrofluorimetry	Precolumn derivatization with Fmoc-Cl and HPLC with fluorescence detection: λ_{ex} = 263 nm λ_{em} = 315 nm	0.1-10.0 mg/L	15 µg/L	Human plasma	Glucosamine sulfate	[6]

Table 1.1 (Continued)

Technique	Method	Linear Range	Detection Limit	Sample	Analyte	Ref.
HPLC Spectrofluorimetry	Derivatization with 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate and HPLC fluorescence detection: λ_{ex} = 250 nm λ_{em} = 395 nm	0.1-13 μM	49-780 fmol	- Soya protein - Chitin - Soils	- Amino acid - Amino sugar	[7]
HPLC Spectrophotometry	Pre-column derivatization with phenylisothiocyanate and HPLC with UV detection at 254 nm.	6.65-16.63 mg/L	-	- Raw materials - Dog plasma	Glucosamine hydrochloride	[8]
	Pre-column derivatization with N-(9-fluorenylmethoxy-carbonylony succinimide (FMOC-Su) and HPLC with diode array detector at 265 nm	-	-	Dietary supplement	Glucosamine sulfate/ Hydrochloride	[9]
	Reversed phase ion-paring HPLC with refractive index (RI) detector at 40 ^{o}C	0.05-0.20%	-	Nutritional supplements	Glucosamine hydrochloride	[10]

Table 1.1 (Continued)

Technique	Method	Linear Range	Detection Limit	Sample	Analyte	Ref.
HPLC Spectrophotometry	Pre-column derivatization with FMOC-Su and analyzed by HPLC with UV detection.	2.0-150 mg/L	1 mg/L	- Raw materials - Dietary supplement	Glucosamine sulfate/ hydrochloride	[11]
	HPLC-RI detection and separation by using aminophase column	20-1000 mg/L	-	- Raw materials - Dosage forms	- Glucosamine sulfate - Chitosan	[12]
	Pre-column derivatization with 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) and HPLC with UV detection at 254 nm GC-MS detection	100-500 mg/L	-	Chitin	Glucosamine	[13]
	HPLC- UV detection 195 nm and separation by using amino column	1.88-5.62 mg/L	0.037 mg/L	Pharmaceutical	Glucosamine	[14]
HPLC Mass spectrometry	HPLC-tandem mass spectrometric method	10-1000 µg/L	10 µg/L	Human plasma	Glucosamine	[15]

Table 1.1 (Continued)

Technique	Method	Linear Range	Detection Limit	Sample	Analyte	Ref.
HPLC Electrochemistry	HPLC-exchang chromatography with a pulsed amperometric detector	-	-	Lipid	- Glucosamine - Glucosamine-4- phosphate	[16]
	HPLC- PED-2 cell, gold working electrode and Ag/AgCl electrode standard	0.25-40 μM	1-5 pmol	- Plasma - Serum	- Galactosamine - Glucosamine - Galactose - Glucose - Mannose	[17]
Gas Chromatography	Derivatization with hydroxylamine hydrochloride and 4-(dimethylamino) pyridine and GC-MS detection	20-640 mg/L	-	Soils	- Glucosamine - Mannosamine - Galactosamine	[18]

1.3 Food Preservatives [19]

Preservatives added to inhibit or kill microorganisms may be classified on various bases, such as their chemical composition, mode of action, specificity, effectiveness, and legality. Some, e.g., sugar, are effective because of their physical action, others, e.g., sodium benzoate, because of their chemical action, and others e.g., sodium chloride, because of a combination of these effects. Some preservatives are incorporated into foods and usually are antiseptic rather than germicidal, while others are used only to treat outer surfaces and may kill organisms as well as inhibit them. Some are employed to treat wrappers or containers for foods, while others are used as gases or vapors above the food. Some have been incorporated in ice used to chill foods, such as fish. Preservatives may be fairly specific against microorganisms, e.g., they may be effective against molds or yeast and less against bacteria, or vice versa, and may act against definite groups or species of bacteria or other organisms.

1.3.1 Benzoate [19-22]

Benzoate, the sodium salt of benzoic acid (Figure 1.3) has been used extensively as an antimicrobial agent in foods. It has been incorporated into jams jellies, margarine, carbonated beverages, fruit salads, pickles, relishes, fruit juice, etc. Sodium benzoate is relatively ineffective at pH values near neutrality, and the effectiveness increases with the increase in acidity, an indication that the undissociated acid is the effective agent. The pH at which sodium benzoate is most effective (2.5 to 4.0) is in itself enough to inhibit the growth of most bacteria; but some (not all) yeasts and molds are inhibited at pH levels that would otherwise permit their growth.

Figure 1.3 (A) benzoic acid (B) sodium benzoate

Benzoic acid occurs naturally in free and combined forms in cranberries, prunes, greengage, apples, cinnamon, and ripe cloves. Gum benzoin contains as much as 20% benzoic acid. Benzoic acid is synthesized in a variety of ways. The manufacturing processes include the air oxidation of toluene, the hydrolysis of benzotrichloride and the decarboxylation of phthalic anhydride.

Physical properties of benzoic acid are listed in Table 1.2.

Table 1.2 Physical properties of benzoic acid [19]

Chemical names	Benzenecarboxylic acid, phenylformic acid, dracyclic acid
Molecular formula	$C_7H_6O_2$
Molecular weight	122.12 g/mol
Appearance	Colorless or white needles or leaflets
Melting point	122.4°C (begins to sublime at ~ 100°C)
Boiling point	1.266 – 1.321 g/cm^3 at 20°/4°C
Acidity (pK_a)	4.21
Specific gravity	2.9 at 20°C, 12.0 at 60°C, 68.0 at 95°C

A broader spectrum of microbiocidal activity in foods is often achieved by using a combination of benzoic acid and sorbic acid as food preservatives.

For example, combinations of these two acids inhibit several bacterial strains better than either sorbic acid or benzoic acid alone.

Benzoates do not appear to be accumulated in the body. They are absorbed from the intestine and detoxified and excreted as hippuric acid via the formation of benzoyl CoA intermediate.

The FAO established the levels of benzoate causing no toxicological effect at 1% level in the diet, or equivalent to 500 mg/kg body weight. The acceptable daily intake for total benzoates in the human diet is established at 0-5 mg/kg bodyweight.

1.3.2 Sorbates [19-20]

Sorbic acid (Figure 1.4), is used as a direct antimicrobial additive in foods and as a spray, dip, or coating on packing material.[1]

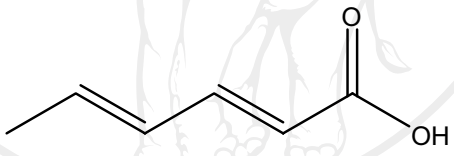

Figure 1.4 Sorbic acid

Sorbic acid and its salts are effective antimicrobial agents against many yeasts and molds, as well as bacteria. As yeast inhibitors, the compounds are useful in fermented vegetable products, fruit juice, wines, dried fruits, meat, and fish products. Specific products protected from yeasts by sorbates include carbonated beverages, salad dressing, tomato products, syrups, jams, candy, and chocolate syrup. Physical properties of sorbic acid are summarized in Table 1.3.

Table1.3 Physical properties of sorbic acid [19]

Chemical names	(2-Butenylidene) acetic acid, crotylidene acetic acid, hexadienic acid, 2,4-hexadienoic, 1,3-pentadiene-1-carboxylic acid, 2-propenylacrylic acid
Molecular formula	$CH_3CH=CH-CH=CH-COOH$ ($C_6H_8O_2$)
Molecular weight	112.14 g/mol
Appearance	Colorless needles, or white crystalline powder, odorless with slightly acidic taste
Melting point	134.5°C
Boiling point	228°C (decomposes)
Solubility, (g/100ml)	0.16 (water, 20°C)
	14.8 (ethanol, 25°C)
Acidity (pK_a)	4.76 at 25 °C

Sorbic acid and its salts are known to inhibit yeast and molds but are less effective against bacteria. They are most effective at low pH values with a maximal level of use at about pH 6.5. These compounds are more effective than sodium benzoate at pH values above 4.0. Under normal metabolic conditions. Sorbates are completely oxidized to carbon dioxide and water in the same way as other fatty acids, releasing 6.6 kcal/g energy. As a result of the extensive favorable toxicological and physicological aspects of sorbic acid, the FAO has allowed for its highest acceptable daily intake of all feed preservation at 25 mg/kg body weight.

1.3.3 Salicylic acid [23]

Salicylic acid (Figure 1.5) is a beta hydroxy acid. This colorless crystalline organic acid is widely used in organic synthesis and functions as a plant hormone. It is derived from the metabolism of salicin. In addition to being a compound that is chemically similar to but not identical to the active component of aspirin (*acetylsalicylic acid*), it is probably best known for its use in anti-acne treatments. The salts and esters of salicylic acid are known as salicylates. Salicylic acid is the key additive in many skin-care products for the treatment of acne, callouses and corns, keratosis pilaris and warts. It treats acne by causing skin cells to slough off more readily, preventing pores from clogging up. This effect on skin cells also makes salicyclic acid an active ingredient in several shampoos meant to treat dandruff.

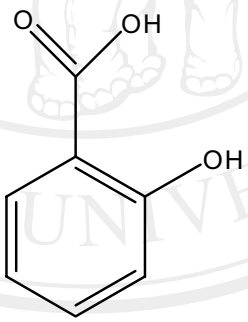

Figure 1.5 Salicylic acid

Although toxic in large quantities, salicylic acid is used as a food preservative. For some people with salicylate sensitivity even these small doses can be harmful. Physical properties of salicylic acid are summarized in Table 1.4

Table 1.4 Physical properties of salicylic acid [23]

Molecular formula	$C_6H_4(OH)COOH$
Molecular weight	138.12 g/mol
Appearance	Colorless needles, or white crystalline powder
Melting point	159 °C
Boiling point	211 °C (20 mmHg)
Solubility, (g/100ml)	0.2 g/100 mL H_2O (20 °C)
Acidity (pK_a)	2.97 at 25 ^oC

1.4 The method of detecting food preservatives

There are several analytical approaches reported for the determination salicylic, benzoic and sorbic acids. The most commonly used analytical procedures are High Performance Liquid Chromatography (HPLC) [24-34], Capillary Electrophoresis (CE) [35-38], and Gas Chromatography (GC) [39-42]. Other methods include Biosensors [43], Spetrophotometry [44], Potentiometry [45], and Flow Injection chromatography (FIA) [46]. Details of these analytical methods are summarized in Table 1.5.

Table 1.5 Some analytical methods reported for the determination of food preservatives.

Technique	Method	Linear Range	Detection Limit	Sample	Analyte	Ref.
HPLC Spectrophotometry	Extraction with steam distillation, ethanol, methanol and via Amberlite XAD2 and HPLC with diode array detection at 235 nm	BA: 5-500 mg/L SOA: 1-500 mg/L	BA: 25 mg/kg SOA: 6.25 mg/kg	Quince jam	BA, SOA	[24]
	Solid phase extraction(SPE) using Bond-Elute SI cartridges and HPLC – UV detection at 235 nm	DHA, BA: 1-20 mg/L 0.5-10 mg/L for SOA 1.5-30 mg/L for SA	DHA: 2.5 ng BA: 4.0 ng SOA: 2.0 g SA: 5.5 ng	Cosmetic	DHA, BA, SOA, SA	[25]
	Extraction with organic solvent and HPLC-UV detection at 200-400 nm	0.2-10 mg/L of BA and SOA	BA: 0.06-0.1 mg/L SOA: 0.05-0.2 mg/L	- Soft drink - Fruit juice - Margarine - Yoghurt - Cheese	BA, SOA	[26]

Table 1.5 (Continued)

Technique	Method	Linear Range	Detection Limit	Sample	Analyte	Ref.
HPLC Spectrophotometry	Extraction with methanol in a sonicator and HPLC – UV detection at 254 nm	BA: 5.0-120 mg/L SOA: 1.-75 mg/L MP: 3.0-100 mg/L PP: 1.0-75 mg/L	BA: 0.5 mg/L l SOA: 0.1 mg/L MP: 0.3 mg/L PP: 0.1 mg/L	Foodstuffs	BA, SOA, MP, PP	[27]
	Solid phase extraction (SPE) by sep-pak C18 column and HPLC-UV-VIS detection at 235 nm	BA, SOA: 0-100 mg/L	BA: 0.2 mg/L SOA: 0.1 mg/L	Wine	BA, SOA	[28]
	Micellar Electrokinetic Capillary Chromatography (MEKC) UV detection at 230 nm	BA: 10-100 mg/L SOA: 10-100 mg/L	-	Beverages, Foods	BA, SOA	[29]
	Micellar Electrokinetic Capillary Chromatography (MEKC) UV detection at 254 nm	Aspartame : 0-200 mg/L Acesulfame, BA, SOA : 0-100 mg/L Saccharin, caffeine : 0-50 mg/L	-	Low-jule soft drink, Other foods	Aspartame Acesulfame, Caffeine, BA,SOA	[30]

Table 1.5 (Continued)

Technique	Method	Linear Range	Detection Limit	Sample	Analyte	Ref.
HPLC Spectrophotometry	The samples were diluted in water and HPLC – UV detection at 254 nm, mobile phase methanol:water(10:90)	BA: 5-145 mg/L SOA: 0.2-4 mg/L	BA: 0.05 mg/L SOA: 0.004 mg/L	Soft drinks	BA, SOA	[31]
	The sample extraction by methanol and HPLC-UV detection at 235 nm, mobile phase acetate buffer : methanol (65:35)	BA: 5-500 mg/L SOA: 1-500 mg/L	BA: 2.0 mg/L SOA: 0.5 mg/L	Food stuffs	BA,SOA	[32]
	The preparation sample was diluted with 0.1M HCl or mobile phase and HPLC – UV detection at 220 and 228 nm Chemometric methods in spectrophome-tric analysis, PLS-1 and PCR	CP: 2.4-5.6 mg/L B1, B2, B3, B6 : 1.4-3.4 mg/L SOA: 6-14 mg/L	CP: 0.91 mg/L B1, B3: 0.06 mg/L B2: 0.07 mg/L B6: 0.08 mg/L SOA: 0.02 mg/L	Pharmaceu-tical	CP, B1, B2, B3, B6, SOA	[33]
	The samples were prepared by International Dairy Federation (IDF) standard method and HPLC-UV detection at 227 nm	BA, SOA : 0-300 mg/l	BA : 5.0 mg/kg SOA : 3.0 mg/kg	Yogurt	BA, SOA	[34]

Table 1.5 (Continued)

Technique	Method	Linear Range	Detection Limit	Sample	Analyte	Ref.
Capillary Electrophoresis	The sample was directly diluted with double-distilled water and determine by capillary electrophoresis (CZE)	Quinic acid: 5.41-288.67 mg/L Anisic acid: 3.19-106.54 mg/L SA: 1.12-112.13 mg/L BA: 2.93-73.27 mg/L SOA: 3.17-96.68 mg/L	Quinic acid:1.80 mg/L Anisic acid:1.21 mg/L SA: 0.44 mg/L BA: 2.19 mg/L SOA: 2.07 mg/L	Vinegar Soy sauce	Quinic acid, Anisic acid SA, BA, SOA	[35]
	Thermo-optical absorbance detection at 248 nm compare to HPLC with UV absorbance and samples were diluted with buffer	-	BA: 0.27 mg/L DHA: 0.25 mg/L SA: 0.93 mg/L	Food	BA, DHA, SOA	[36]
	Electro kinetic flow analysis-ion pair solid phase extraction capillary zone electrophoresis and detection at 214 nm	BA: 0.06-20 mg/L SOA: 0.03-20 mg/L	BA: 0.02 mg/L SOA: 0.01 mg/L	Food	BA,SOA	[37]

Table 1.5 (Continued)

Technique	Method	Linear Range	Detection Limit	Sample	Analyte	Ref.
Capillary Electrophoresis	Capillary electrophoresis with inspection of ionic mobilities and direct UV detection at 200 and 254 nm for BA and SOA, respectively	BA: 4-45 mg/L SOA: 2-20 mg/L	BA: 0.9 mg/L SOA: 0.3 mg/L	Beverage	BA, SOA	[38]
Gas Chromatography	Solid phase extraction element combined with thermal desorption-gas chromatography (TD-GC)	BA, SOA: 2-1000 mg/L MP: 0.2-300 mg/L EP, PP: 0.02-300 mg/L	BA: 0.08 mg/L SOA: 0.2 mg/L M-PHBA, E-PHBA, P-PHBA: 0.002-0.1 mg/L	Soft drink, Yogurts, Sauces	BA, SOA, M-PHBA, E-PHBA, P-PHBA	[39]
	The on-line pyrolytic methylation by tetra-methylammonium hydroxide (TMAH) and direct-injection in GC	1-1000 mg/L	0.1 mg/L	Soft drink	BA	[40]
	Flow injection on-line SPE for the preconcentration and isolation of preserva-tives and detection with GC-FID	BA: 0.2-25 mg/L SOA: 0.3-25 mg/L MP: 0.5-25 mg/L EP, PP: 0.4-25 mg/L	BA: 0.10 mg/L SOA: 0.07 mg/L M-PHBA: 0.20 mg/L E-PHBA, P-PHBA: 0.15 mg/L	Food	BA, SOA, M-PHBA, E-PHBA, P-PHBA	[41]

Table 1.5 (Continued)

Technique	Method	Linear Range	Detection Limit	Sample	Analyte	Ref.
Gas Chromatography	Headspace solid-phase microextraction (HS-SPME) with GC-FID	SOA, BA: 0.1-20 mg/L	SOA: 5.83 mg/L BA: 11.4 mg/L	Beverages	BA,SOA	[42]
Biosensor	Immobilization of tyrosinase (Tyro) by calcium carbonate nano-materials(nano $CaCO_3$) and amperometric detection	5.6×10^{-7} M	8.0×10^{-8} M	Food, Soft drink	BA	[43]
Spectrophoto-metry	Chemometrics were applied to quantitatively resolve the overlapped UV Spectra and multivariate calibration and artificial neural networks	BA, MP, PP: 0.5 mg/L SOA: 0.25-10.0 mg/L	BA: 0.22 mg/L MP: 0.19 mg/L PP: 0.17 mg/L SOA: 0.08 mg/L	BA, MP, PP, SOA	Foodstuffs	[44]
Potentiometry	Potentiometric sorbate ion sensor immobilized in a graphite matrix Pt | Hg | $Hg_2(SOB)_2$ | Graphi-te	$5.0\times10^{-7} - 1.0\times10^{-2}$ M	4.3×10^{-7} M	SOA	Food	[45]

Table 1.5 (Continued)

Technique	Method	Linear Range	Detection Limit	Sample	Analyte	Ref.
Flow Injection Analysis	Spectrotometric based on second-order data provided by diode array detection system with an imposed double pH gradient, analyzed by both parallel factor analysis (PARAFAC) and multivariate curve resolution-alternating least-squares (MCR-ALP)	-	-	BA, SOA	Orange juice	[46]

1.5 Sequential Injection Chromatography [47]

1.5.1 Principle of SIC

Sequential Injection Chromatography (SIC) is a relatively new technique and it poses as a hybrid technique of sequential injection analysis (SIA) and liquid chromatography. SIC was introduced by D. Satinsky in 2003 and one of the major advantages, against conventional SIA, is that it allows simultaneous efficient separation and quantitation of more than two compounds. The SIC consisted of syringe pump, holding coil, multi position valve, column, detector and recorder, as shown in Figure 1.6.

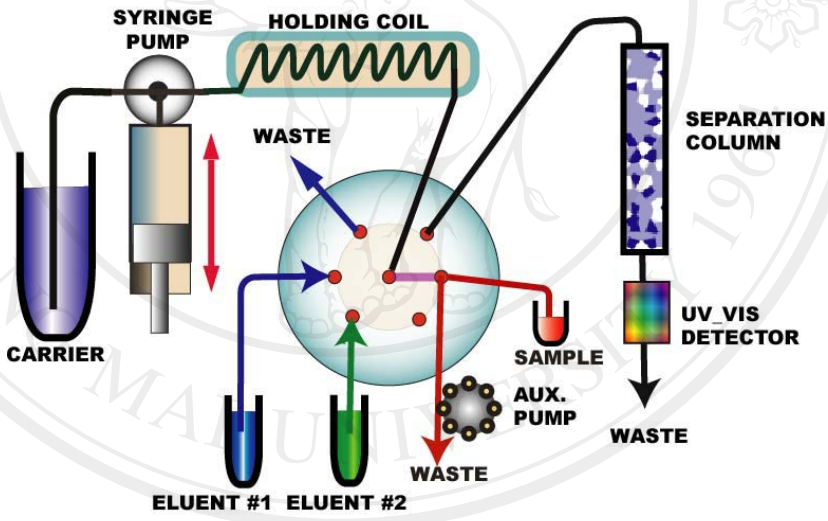

Figure 1.6 A SIC system [47]

Operationally, the well-defined sample zone was injected into the system and it is led towards the column for separation. Then mobile phase, acting as carrier, is employed to elute each compound from the monolithic column at relatively high flow rate. The height or area of the detected peaks is proportional to analyte concentration. Basically all detectors equipped with a flow-through cell can be used with the SIC setup, covering a wide range of detection modes (UV-Vis, fluorescence, etc).

1.5.2 Monolithic column [48]

The research in the field of liquid chromatography columns has tremendously accelerated during last years. Innumerable types of chromatography columns have been developed to solve particular problems of separation requirements. One important direction of this research area is the development of monolithic columns with high porosity sorbent (it permits high flow rate of mobile phase at low back pressures without losing efficiency) as a new separation tool used both in HPLC and other flow analytical methods. This feature can be utilized for integrating these columns into a SIA manifold (low pressure flow method with limited range of pump back-pressure to about 2.5 MPa) for extending the possibilities of this technique. Two bands of monolithic columns suitable for SIC are nowadays available commercially-Merck® Chromolith™ and Phenomenex® Onyx™ (in usable lengths of columns-25 mm or 50 mm are with silica based ODS-C18 sorbent only). The monolithic columns consist of a single piece of high-purity polymeric silica gel rod with a bimodal pore structure (macropores and mesopore-a porosity exceeding 80% in Figure 1.7).

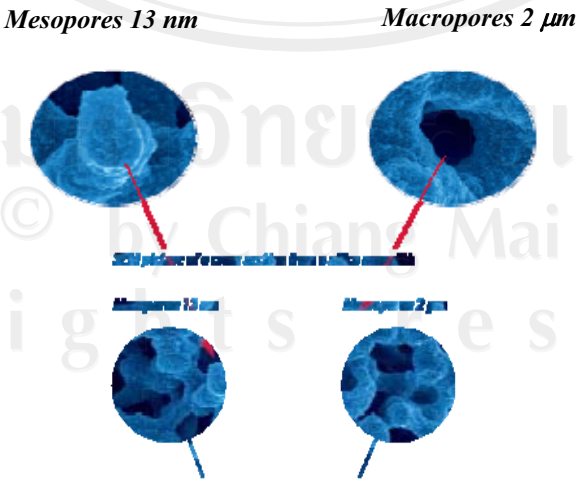

Figure 1.7 SEM picture of a cross section from a silica monolith

Macropores (average size 2 μm) dramatically reduce the column back-pressure and allow the use of higher flow rates. The mesopores (average size 13nm) form the fine porous structure and create the large uniform active surface enabling high performance chromatographic separation. A monolithic rod demonstrates very high mechanical stability and long operative lifetimes, in most cases far exceeding the lifetime of a particulate column. Monolithic columns also exhibit similar chromatographic properties with respect to retention and selectivity as particle columns of the same specific surface area and pore diameter.

1.5.3 Advantages of SIC [47]

The Sequential Injection Chromatography (SIC) configuration combines the advantages of SIA and liquid chromatography:

· Fast analysis - high sample throughput

· Automation in sample pretreatment

· Operational simplicity

· Low reagent and mobile phase consumption

· Robustness

· Reliability

· Lower instrumentation cost compared to HPLC

· Portability of the instrument

1.5.4 Applications of SIC [49]

Early work of SIC analysis were focused on the analysis of relatively simple multi-component samples such as pharmaceuticals. Solutions, drops, and syrups, etc. without interfering incompatibilities and containing 2-5 compounds of interest can be determined directly only by diluting the sample by a mobile phase. Other samples

(topical creams, tablets, capsules, etc.) have to be pre-treated (for example by extraction into organic solvent). Length of column was chosen depending on chromatographic features of all substances in the sample. Mobile phase was usually methanol or acetonitrile based and the amount used was only the volume needed to elute all substances from the column. Volume of syringe pump used in SIC system was 5.0 or 10.0 mL. A smaller syringe was more preferable due to the possibility of achieving a higher working pressure in the system. Flow rate of mobile phase was optimized according to the length of column and peak shapes (usually less than 1.5 mL min^{-1}). Detection by DAD UV-VIS detector (S 2000, Ocean Optics Inc., USA) was used, coupled with a Z flow cell with 10 mm active optical length. Two or three wavelengths were used simultaneously if it was necessary to increase the detection sensitivity. Volume of sample used for one analysis was usually 10-20 μL depending on the column length. The whole system was controlled by commercial FIAlab® software with predefined sequence. The SIC served as a good automatic analyzer for fast chromatographic determination of simple mixtures with the possibility of easy handling and pre-treatment of sample. Table 1.6 shows a summary of the relevant applications of SIC in pharmaceutical analysis.

Table1.6 Applications of SIC

hMatrix	Analytes	Column (mm)	Flow rate ($mLmin^{-1}$)	Mobile phase	Detection UV(nm)	Pre-treatment of sample	Ref.
Pharmaceutical syrups and drops	Ambroxol, Methylparaben, Benzoic acid	50+10	0.48	Acetonitrile:tetrahydrofuran: water (10:10:90,v/v/v) pH 3.75 adjusted with triethy-lamine and acetic acid	245	Extraction by mobile phase	[49]
Pharmaceutical drops	Naphazolinenitras Methylparaben	25+5	0.9	Methanol:water(40:65,v/v),pH 5.2 adjusted with triethylamine 0.8 ml $mL^{-1}$and acetic acid	220; 256	Dilution of drops	[50]
Pharmaceutical drops	Triamcinoloneace -tonid, Salicylic acid	50+5	0.9	Acetonitrile:water(35:65,v/v), pH 3.2 adjusted with acetic acid	240	Dilution of drops	[51]
Pharmaceutical drops	Betamethasone, Chloramphenicol	25+5	0.48	Acetonitrile:water(30:80,v/v)	241; 271	Extraction by methanol with 1% of H_3PO_4	[52]

Table 1.6 (Continued)

Matrix	Analytes	Column (mm)	Flow rate ($mLmin^{-1}$)	Mobile phase	Detection UV(nm)	Pre-treatment of sample	Ref.
Topical cream	Triamcinoloneacetonid, Methylparaben, Propylparaben	25+10	0.6	Acetonitrile:methanol:water (35:5:65,v/v/v), pH 2.5 adjusted with 0.05% of nonylamine and H_3PO_4	243	Extraction by methanol	[53]
Topical cream	Salicylic acid, Methylsalicylate	50	0.6	Acetonitrile:water(35:60,v/v), pH 2.5 adjusted with 98% H_3PO_4	240	Extraction by methanol	[54]
Topical cream	Diclofenacnatrium, Methylparaben, Propylparaben	25	0.48;0.9;1.2	Acetonitrile:water(40:70,v/v), pH 2.5 adjusted with 0.05% triethylamine and H_3PO_4	275	Extraction by methanol	[55]
Pharmaceutical capsules	Ambroxolhydrochloride, Doxycycline	25	0.48	Acetonitrile:water(20:90,v/v), pH 2.5 adjusted with 98% H_3PO_4	213	Extraction by methanol with 1% of H_3PO_4	[56]

Table 1.6 (Continued)

Matrix	Analytes	Column (mm)	Flow rate ($mLmin^{-1}$)	Mobile phase	Detection UV(nm)	Pre-treatment of sample	Ref.
Pharmaceutical tablets	Paracetamol, Caffeine, Acetylosalicylic acid	25	0.6	Acetonitrile:water(10:90,v/v, pH 4.05 adjusted with 98% H_3PO_4	210	Extraction by methanol and mobile phase	[57]
Topical cream	Lidocaine, Prilocaine	25	0.6	Acetonitrile : water (40:80, v/v), pH7.1 adjusted with 0.01% triethylamine and H_3PO_4	212	Automated system for the release testing of semisolid dosage forms	[58]
Spray, Foam, Foam Mechanical	Fenoxycarb, Permathrin	10	0.6; 1.2	Acetonitrile : water (60:40,v/v)	225	Dilution with mobile phase	[59]
Pharmaceutical tablets	Thiamine, Pyridoxine, Cyanocobalamin	25+5	0.5	Ammonium acetate pH 7 : ammonium acetate - methanol pH 7 (20:80, v/v)	280, 325,360	Dilution with Milli-Q water	[60]

Table 1.6 (Continued)

Matrix	Analytes	Column (mm)	Flow rate ($mLmin^{-1}$)	Mobile phase	Detection UV(nm)	Pre-treatment of sample	Ref.
Green alga Tetraselmis gracilis	Aspartic acid, Glutamic acid, Asparagine, Serine, Glutamine, Glycine, Threonine, Citruline, Arginine, Alanine, Tyrosine, Phenylalanine, Ornithine, Lysine	25	0.6	Methanol : phosphate buffer (pH 7.2)(20:80, 35:65, 50:50, 65:35)	340, 450	Extraction by water	[61]

1.6 The Aims of This Research

The aims of this research work can be summarized as follows:

1. To design, setup and investigate a low-cost SIC system
2. To optimize the condition for separation GLcN 1 and GLcN 2.
3. To optimize the condition for separation SA, BA and SOA.
4. To apply the developed methods for the determination of GLcN in dietary supplement samples
5. To apply the developed methods for the determination of SA, BA and SOA in food and beverage samples