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Determination of sugars in atmospheric aerosols by hydrophilic interaction liquid chromatography-mass spectrometry

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Tiivistelmä/Referat – Abstract

Sugar and Sugar alcohol are indicative compounds in the environmental aerosol which make them really important. The concentration of sugar and sugar alcohol reveal biogenic and anthropogenic information such as climate, air quality, wood consumption, the activity of plantation and pollution.

The conventional analysis methods of sugar and sugar alcohol are reverse phase High Performance Liquid Chromatography–Mass Spectrometry (HPLC-MS/MS), and Gas Chromatography-Mass Spectrometry (GC-MS/MS). However, both of them have some limitations due to the sugar and sugar alcohol aerosol sample which are not easy to analyze. For reverse phase HPLC-MS/MS, the separation of analytes is not satisfied. For the GC-MS/MS, the derivatization process requires extra work and the derivatization compound is not stable. Besides, the matrix effect from the aerosol sample is a significant challenge which needs to be solved. Hence, the hydrophilic interaction chromatography (HILIC) and the Solid Phase Extraction (SPE) are introduced. The retention factors of HILIC column are the hydrophilic partition, the hydrogen bonding, and the electrostatic interactions. Polar stationary phase is used in HILIC mode, and the highly organic solvent is employed in mobile phase. Hence, a stagnant aqueous-rich layer is generated in HILIC mode, which can separate sugars and sugar alcohol efficiently. Furthermore, the interference and the matrix effect are solved by SPE.

The development and the optimization of SPE-HILIC-MS/MS method for sugars were done in the experimental part. Eventually, the real environmental aerosol was analyzed by the optimized parameters and methods. The sugars and sugar alcohols were analyzed successfully from atmospheric aerosol samples.

Avainsanat – Nyckelord – Keywords
Hydrophilic interaction liquid chromatography, high-performance liquid chromatography, solid phase extraction, sugar, sugar alcohol, aerosol

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Abbreviations:

OC = organic carbon

WSOC= water-soluble organic carbon

CCN= cloud condensation nuclei

HPLC = high-performance liquid chromatography

UV-Vis = ultraviolet –visible wavelength detector

MS= mass spectrometer

m/z= mass to charge ratio

EI= electron impact ionization

CI= chemical ionization

ESI= electrospray ionization

MS/MS= tandem mass spectrometry

CID= collision-induced dissociation

API= atmospheric pressure ionization

RI= refractive index

ELSD= evaporative light scattering detection

ANP= aqueous normal phase partition

HILIC= hydrophilic interaction liquid chromatography

SEC= size exclusionchromatography

IEx= ion-exchange

LExC= ligand exchange chromatography

PEG= poly(ethylene glycol)

POE= poly(oxyethylene)

β –CD=β-cyclodextrin

PC= phosphorylcholine

SPE= Solid phase extraction

LLE= Liquid-Liquid Extraction

MQ= milli-Q water

FA= formic acid

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1. Introduction

The environmental aerosol is important due to its consequences to a lot of aspects. For instance, weather, plantation, and pollution, etc. The environmental aerosol can be liquid droplets or suspension which is composed of fine solid particles. Environmental aerosol can be biogenic and anthropogenic. Hence, the scientist can get a lot of information from monitoring the composition of the environmental aerosol. The organic carbon (OC) ambient aerosol is an important cluster of the aerosol. Besides, the water-soluble organic carbon (WSOC) takes part in 11-95 % of the OC content in ambient aerosols.[2-3] The WSOC has been an important topic for several years. Some WSOC compounds are defined, which are correlated to the cloud condensation nuclei (CCN)[4-6]. Some of WSOC compounds are correlated to the wood consumption and some of them are produced by vegetation. Some of WSOC compounds are related to the variation of temperature and weather because WSOC compounds have diverse pathway and can also be transported over long distances. Therefore WSOC have some influence on global air quality, climate, and biogeochemical distribution of nutrients, such as cloud condensation nuclei. In recent studies, biomass burning and wood consumption are related to the CCN. The burning of cellulose produces smoke particles which are nearly 100 % water-soluble. According to research, WSOCs compounds might contribute toward the CCN activity of aerosols.[4] In addition, the WSOCs have high solubility in the water. Therefore, the concentrations of the WSOC compounds within the smoke aerosols may play a significant role in the aqueous-phase chemistry and in the nucleation of cloud droplets by smoke.[7] Hence, an increased knowledge and understanding of the WOSC cluster becomes an essential topic. [3, 8-14]

2. Sugar and sugar alcohol

Sugars (Fructose, glucose, sucrose, trehalose) and sugar alcohols (Arabitol, mannitol, inositol, and erythritol) are defined as part of nature background aerosol. Those sugars and sugar alcohols are derived from the airborne microorganism and other biogenic material. In general, the source of those sugars might be suspended biogenic which are formed by nature background material such as vegetation, plant detritus, airborne microorganism, bacteria, virus, algae, spores of lichens and fungi.[2, 15]

2.1. The diel patterns of sugars and sugar alcohols

Diel patterns of sugars and sugar alcohols are changing dramatically especially for the sugar alcohols. The maximal concentration of mannitol and arabitol are observed during the day-time, which indicate the higher release of fungal fragments during the daytime. On the contrary, the concentration of sugars and sugar alcohol are relatively lower during the night-time, which means the fungal fragments are less active during the same period.[16]

2.2. The seasonal variation of the sugar

Generally, during summer season, some saccharide compounds are more dominant due to the higher activity of the vegetation. It is known that solar radiation and biogenic volatile organic compounds reach their maximum activity, during summer time. Moreover, the emission of primary biological particles such as plant pollen and fungal spores may also increase the biological activity. During the winter, the sugar alcohols are correlated to the fungi, which are fungal marker compounds.[17-18] The monosaccharides compounds such as glucose and fructose, which are well defined, originate from the plant pollen that might be rich in glucose and fructose.[19]Hence, the concentration of sugar and sugar alcohol can be biomarkers of the seasonal variation.

3. The important sugars and sugar alcohols markers

As the discussion above, sugars and sugar alcohols can be good markers to gain more environmental information.[15, 19-21] Hence, I will list and discuss some important sugars and sugar alcohols marker below. Fructose and glucose dominate numerous primary biogenic sources, such as plant pollen, fruits, and plant detritus. Sucrose plays a key role in plant flowering processes, and sucrose is the major component of airborne pollen grains.[17, 19, 22] Levoglucosan is an anhydrous derivative of glucose which is formed through pyrolysis of cellulose at temperatures above 300°C. During the wood consumption, it is emitted in huge amounts and the emission of levoglucosan is more stable. Besides, levoglucosan is specific to cellulose-containing substances and serve as an excellent molecular marker indicator of wood consumption and biomass burning.[20] Therefore levoglucosan can be used to monitor tropical environments that are affected by deforestation[21], also it is an important marker in urban and rural environments.[9]Trehalose is well-defined and well-known. Trehalose is constituents of bacteria, fungi, lichen, and lower plants. In addition, for invertebrates, trehalose functions as carbohydrates and cell protectants against stressful conditions.[7, 21]Trehalose also acts as a fungal metabolite. Furthermore, the increasing of the trehalose in aerosol particles is frequently accompanied

by the soil particles resuspension and unpaved road dust.[20] Arabitol and mannitol are substances which storage in fungal spores commonly. Besides, the great fraction of coarse organic carbon is constituted by fungal spores in the atmospheric aerosol. Hence, monitoring the fungal spores is important. Arabitol and mannitol work as a good tracer for the fungal spores.[2, 23]

4. High performance liquid chromatography

High-performance liquid chromatography (HPLC) is an analytical technique widely used for separation, quantification, and identification of liquid samples. Typically, the separation of the HPLC system is operated by having a high-pressure pump that leads the liquid solvent (which contains mobile phase and liquid sample) into the HPLC system. The liquid sample mixture is introduced into the HPLC column from vials by the auto-sampler or injector. The main separation mechanism is caused by the interactions between the separation materials or packing materials of the column (stationary phase) and the compounds to be analysed. Eventually, the components will separate by the column and travel to the detector which analyzes the eluting components. The detector is connected to the computer which records all the electrical signals and shows the chromatogram, then quantification and identification can be done by those chromatograms. Different types of detectors can combine with HPLC. Generally, ultraviolet-visible wavelength detector (UV-Vis), mass spectrometer, electrochemical detector, and refractive index detector are combined with HPLC. The detection can be specific or universal which depends on the operation mode.[24]

4.1. Normal-phase and reserve-phase high performance liquid chromatography

Generally, HPLC performs the separation in different modes such as electrical charge, polar and molecular size. The utilization of polarity is most common, which involve different phases; normal phase, reverse phase. The separation mechanism depends on the mobile and stationary phases. In normal-phase HPLC, the mobile phase is non-polar and the stationary phase is polar. Silica is widely used as the stationary phase on the normal-phase HPLC due to its availability and low cost, and it can be modified by polar functional groups for separation purposes, e.g. cyano, amino, and the diol functional groups. The mobile phase of the normal phase is the solvent with low to medium polarities such as hexane and methanol. Since the stationary phase is polar, the non-polar and least polar compounds elute out first and the more polar compounds, which are retained to the polar stationary phase, elute out later. Reversed-phase chromatography (RPC) has a hydrophobic stationary phase. The stationary phase is made from alkyl chains covalently bonded to the stationary phase particles.

Generally, RPC is used for less polar or hydrophobic compounds. The hydrophobic stationary phase of RPC is a polar (aqueous) mobile phase. Therefore, in the polar mobile phase, the hydrophobic molecules incline to adsorb to the hydrophobic stationary phase, and hydrophilic molecules are eluted out the column first.

5. Mass spectrometry

Generally, a mass spectrometer (MS) is a powerful analytical instrument which is widely used in analytical chemistry by measuring the mass to charge ratio (m/z) of ions produced from analyte molecules, thus they can be quantified and identified. The instrumentation of mass spectrometry is composed of three major compartments; (1) ion source such as electron impact ionization (EI), chemical ionization (CI) and electrospray ionization (ESI) to ionize and fragment molecules in the sample, (2) mass analyzer such as ion trap, time-of-flight and quadrupole, which separate ions for example by magnetic and/or electric fields according to their m/z ratios. (3) Ion detector such as the electron multiplier which receives and amplifies the ion current from the analyzer in order to record the signals and improve the sensitivity. Then all the signals are sent to the data processor unit which generates the mass spectrum from the detected ions.

5.1. Tandem mass spectrometry (MS/MS)

Furthermore, in order to obtain more structural information of compounds, more than one stage of mass analysis can be applied, this technique is called tandem mass spectrometry (MS/MS). Generally, in MS/MS, the components of the sample are firstly ionized and fragmented by the ionization technique, leading to a mixture of ions to be separated in the first analyzer. Secondly, after the first round of mass analysis, all or just selected ions are collided with inert gas. This collision-induced dissociation (CID) fragments the ions further to product ions that are then analyzed in the second mass analyzer. Therefore, more selective, informative and reliable analysis can be obtained, for example to define molecular structure and for quantitative analysis. By tandem mass spectrometry, only fragments of interest can be selected and analyzed.[25-26]

5.2. Electrospray ionization and factors affecting ionization efficiency

Generally, electrospray ionization (ESI) method is widely used in the HPLC-MS system, due to the good compatibility. In this research, the ESI method is utilized together with the ion-trap mass analyzer. ESI is a soft atmospheric pressure ionization (API) technique which is commonly used for

biological molecules. The principle of ESI is that the liquid sample is introduced to the high voltage stainless steel capillary, and the liquid sample is converted to a fine aerosol of very small droplets. In addition, the nebulizing gas also decreases the size of the droplets. When the charge density of the droplet surface reaches the Rayleigh limit, the Coulombic explosion takes place, which cause splitting into smaller droplets. Evaporation of the solvent from the charged droplets continues (assisted with hot drying gas flow) until finally charged gas phase ions are obtained. ESI can be run in both positive and negative mode. Usually, in the positive ion mode, the molecular ion is protonated which presented as $[M+H]^+$ ion. On the contrary, deprotonating happens in the negative ion mode, and the negative ion is presented as $[M-H]^-$ ion commonly. Additionally, several parameters will affect the ionization efficiency of the electrospray ionization, such as flow rate of the sample solution, solvent of the sample solution, voltage of the ionization chamber, temperature and drying gas flow rate, nebulizing gas flow rate and pressure, distance between the electrospray emitter to the sampling inlet and inlet temperature. There are some important things which need to be noticed. First, in order to evaporate the solvent before solvent arriving at the analyzer inlet, the solvent used in the sample should be volatile. Moreover, the low surface tension of the solvent will be able to form fine spray sufficiently. "High surface tension solvent has difficulties forming stable spray and may lead to the decrease in the ionization efficiency." Besides, the solvent with the high ionic strength causes ion suppression effects due to the fact that too many ions in the solvent compete with the analyte ions which results in lower sensitivity, thus the ionic strength of the solvent should not be too high. The solvent should be adjusted to the appropriate pH according to the desired polarity of ionic and pH of the sample. Acetonitrile, ethanol, methanol, acetic acid, and acetone are used commonly as the solvent, due to those solvents possessing the benefits described above. In addition, the other important parameter is the flow rate of the sample solution. The increasing of the flow rate generates bigger droplets thus decreasing ionization efficiency while the decrease in flow rate gives higher peak intensity. The voltage of the ionization chamber is required to be sufficiently high, thus "solvent with high surface tension is utilized for the formation of stable electrospray". Moreover, nebulizing gas and the flow rate of drying gas should be optimized depending on the solvent used and instrumental settings. For instance, if the solvent flow rate is high, the gas flow rate and the gas temperature needs to be accordingly high too. Similarly, in order to improve the evaporation of solvent in larger droplets, the inlet temperature to the mass spectrometer usually requires to be high when the flow rate of solvent is high. Then the distance of the capillary tip and mass analyzer inlet also affects the efficiency of ionization, and the distance should be adjusted according to the instrument. Typically, the distance is between 2 to 10 mm, for example, the larger gap is used for the higher solution flow rate. Furthermore,

the droplet evaporation is not so efficient when the gap is smaller, which also decreases ionization efficiency.[27]

5.3. Ion trap mass analyzer in MS/MS

The ion trap mass spectrometer, MS/MS is done by first ionizing the molecules in the sample. Then the parent ion of interest is selected while all other ions are ejected from the trap. Generally, the collision-induced dissociation (CID) process with the buffer gas originates selected parent ions which are fragmented by applying radiofrequency voltage. Eventually, these productions are recorded by scanning the radiofrequency voltage for the second mass analysis scan.[27]

5.4. Advantages of MS/MS

Generally, biological and environmental aerosol samples are a great challenge to a single MS detection due to the analytes in the presence of very complex sample matrices. Hence, the analysis for environmental aerosol sample has a lot of problems. When using only a single MS in the analysis, the sample matrices result in suppression effects. In addition, the complex mass spectra obtained from a single MS is difficult to be interpreted. However, these problems can be solved and analysis improved by using MS/MS. Therefore, MS/MS provides more structural information of the certain ions of the analyte of interest, which makes the identification of biological and environmental samples possible. Typically, MS/MS are also used when there is the presence of impurities which co-elute at the same time with the analyte, when the compound structures are unknown and when the compounds in the sample share similar structures and similar mass. Besides, fragmentation mechanisms can also be determined by the MS/MS. MS/MS is high-sensitivity and high-selectivity analysis. Possibly, MS/MS can study ion-molecule reactions with the use of reactant gas, kinetic methods, thermochemical data determination and isotope analysis. MS/MS can be used for the complex sample matrices analysis and offers lower background noise which improves the signal-to-noise ratio of the detection, resulting in lower detection limits.[28]

6. Comparison of Gas Chromatography–Mass Spectrometry and High Performance Liquid Chromatography–Mass Spectrometry

The identification of sugars and sugar alcohols in the environmental aerosol sample has been achieved by using gas chromatography coupled with mass spectrometry (GC-MS) and with silylation derivatization. The processes of derivatization, which includes addition of the extra chemical group to the molecule, thus makes the GC-MS approach more complicated and difficult.[29-30] Besides, the silylated derivatives should be analyzed as soon as possible, because of its susceptibility to hydrolysis. On the other hand, the sample pretreatment of LC-MS is simplified greatly compared with the GC-MS. Furthermore, with LC-MS some factors can be optimized easily, such as the evaporation of the solvent, different concentration of the buffer, extraction processes and the reconstitution with the different solvent. Therefore, it turns out that the LC-MS possesses the higher feasibility than the GC-based processes.[31]The derivatization of the hydroxyl groups is a based method required for the GC-based methods. The silylation is the most-used technique which uses bis(trimethylsilyl)trifluoroacetamide(BSTFA) for the derivatization. However, this method forms both aldehyde and –OH group, both are unwanted silylation derivatives. The derivatization step includes adding the extra chemical, which involves extra sample handling and chemical manipulation. Hence, the experiment is prone to contamination and becomes more labor-intensive. Interestingly, derivatization is not required in liquid chromatography- mass spectrometer (LC-MS) and MS-MS coupled with electrospray ionization (ESI). Considering the information mentioned above, the GC-MS methods are still time-consuming, as the result LC-MS will be important for analyzing a large amount of environmental aerosol samples and become more wide-spread use. Besides, the GC-MS analysis are mainly used to detect non-polar compounds with the organic-solvent extractable portion. However, those non-polar compounds only take part in 10-30% of organic carbon, due to that the water-soluble polar organic compounds dominate the fraction of the organic carbon. Moreover, because of the complexity of the environmental aerosol sample, it requires diverse analytical techniques for separation, quantification, and determination.[32]

The LC-MS is used to quantify biological samples commonly. Typically, the concentration of biological sugar is higher compared with the atmospheric sugar, thus is relatively easy to detect biological samples. Besides, the atmospheric sample is complex sample matrices. The composition of the atmospheric aerosol sample is complicated. Lacking the chromatographic separation technique is prone to have interference, which is a tremendous obstacle for sugar analysis. Therefore, although

the HPLC method is really powerful and flexible, there are still some challenges. The suitable column for separation and proper detection methods need to be considered. For instance, the sugar compounds lack the fluorophoric and chromophoric moieties required for fluorescence and UV detection because refractive index (RI) and evaporative light scattering detection (ELSD) are less sensitive by HPLC detection methods. Additionally, the limit of detection for many compounds in environmental aerosols by fluorescence and UV are relatively high; therefore, compared with HPLC, the LOD is not low enough.[33]The other method is injecting the sample extracts into the LC-MS system directly without LC column. Although direct injecting of sample works, the coexistence of sample matrix components can cause negatively affect, which affects the sensitivity of the detection.[34-35]Therefore, the selection of chromatographic and mass spectrometric system is important. There are two criteria for carbohydrate analysis; first, the sample preparation part; second, to detect the sugars which are in the complicated matrix; mostly because sugars and sugar alcohol are at lower concentrations than other compounds present in complex aerosol samples.

Although the difficulty for analyzing the sugars by GC-MS still exist, the sugar had been analyzed successfully. Table 1 shows the result of the water-soluble hydroxylated organic compounds in German and Finnish environmental aerosol by GC-MS method.[36]

Table 1. Sugars and sugar alcohols concentration range in PM_{10} (ng m^{-3}) German and Finnish environmental aerosol. Reprinted with permission from Elsevier.[36]

Name	Formula	Melpitz **(ng m^{-3})**	Hyytiala **(ng m^{-3})**
Arabitol	$C_5H_{12}O_5$	4.2–35	1.4–241
Mannitol	$C_6H_{14}O_6$	1.6–23	0.5–88
Inositol (isomers)	$C_6H_{12}0_6$	1.0–6.9	0.07–3.5
Glucose	$C_6H_{12}O_6$	28–180	1.3–41
Sucrose	$C_{12}H_{22}O_{11}$	16–213	0.3–10
unidentified sugars		4.3–62	0.1–11

6.1. The analysis of carbohydrates by high-performance liquid chromatography

High-performance liquid chromatography (HPLC) plays a key role in the analysis of carbohydrates. There are various separation modes such as size exclusion chromatography, partition chromatography, and ion exchange chromatography. Among them, aqueous normal phase partition (ANP) chromatography are an aqueous mobile phase and polar stationary phase and ANP has been used in

various fields ANP is commonly used for the separation of the sugars group. Recently, there is one novel separation mode which is “hydrophilic interaction chromatography (HILIC)”. Because of more common use of the HILIC mode, the separation of the reducing sugar has been improved and developed by many novel stationary phases of HILIC mode. [37]

The progress of the HPLC promotes the advanced knowledge of carbohydrate chemistry. Although there are a large number of studies about this topic that are public, the methods and stationary phase for separation of the multiple sugars group simultaneously still are a difficult challenge. One of the reason is that there are so many carbohydrates. Monosaccharides include alditols, aldoses, aldonic acids, ketoses, aldaric acids, uronic acids, the number of carbon atoms defines these subclasses. Another reason is sugar derivatives, such as amino sugars and deoxy sugars, oligosaccharides. Moreover, sugar derivatives differ in linkage and composition, which are composed by the various combination of the monosaccharides. Therefore, the separation technique of sugars has been limited for a long time, until the utilization of the HILIC mode was introduced in the HPLC.[38]

Several separation modes have been applied to HPLC of saccharides already, such as size exclusion (SEC), normal phase partition (NP) and ion-exchange (IEx). Since 1984, the anion-exchange mode has been applied to HPLC, in IEx mode carbohydrate-borate complexes are often used.[39] Despite the success in the separation of sugar groups, the borate complex anion-exchange still has disadvantages regarding the broadening effect of the complex peaks and its long analysis time. In recent studies, direct anion exchange chromatography using alkaline as mobile phase and couple with the pulsed ampere metric detection has been used over borate-complex technique frequently..[40]

Although the difficulty for analyzing the sugars by HPLC-MS still exist, the sugar had been analyzed successfully. Figure1 shows the chromatogram of Sugars by reversed-phase HPLC-MS, Table 2 shows the concentrations of the water-soluble hydroxylated organic aerosol compounds in Hong Kong University of Science and Technology by HPLC-MS method.[41]

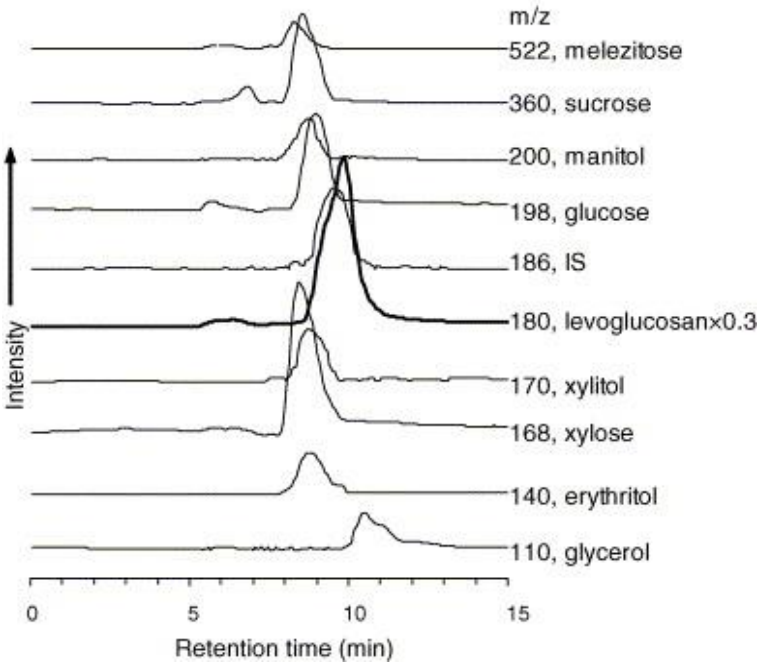

Figure 1. Chromatogram of Sugars by reversed-phase HPLC-MS.[41] Reprinted with permission from Elsevier. Copyright © 2003 Elsevier Science Ltd. All rights reserved.

Table 2. Sugars and sugar alcohols concentration range in $PM_{2.5}$ (ng m^{-3}) Hong Kong University of Science and Technology environmental aerosol.[41] Reprinted with permission from Elsevier. Copyright © 2003 Elsevier Science Ltd. All rights reserved.

Compound	Air concentrations (ng/m3)	
	Mean	Range
Glycerol	10.39	0.7–61.2
Erythritol	5.35	1.0–28.2
Xylitol	6.10	0.9–21.4
Mannitol	3.62	ND[a]–16.6
Xylose	71.97	0.7–474.8
Glucose	17.96	3.2–84.4
Levoglucosana	87.68	4.1–269.8
Sucrose	17.25	ND–202.5
Melezitose	6.22	ND–62.3
Sum	226.5	39.0–1309.6

[a]: not detected

6.1.1. Normal-phase partitionmode

Normal-phase partition mode (NP) is composed of nonpolar mobile phase with a polar stationary phase. NP has opposite characteristic to the reverse phase. In general, hexane is widely used as mobile phase in the NP mode. Nevertheless, because the solubility of reducing sugars are quite low in nonpolar mobile phase such as hexane, the aqueous alcohol and aqueous acetonitrile are commonly used instead of the hexane. In NP mode there are various chemically bonded silica stationary phases, such as amido-bonded silica, diol-bonded silica, amino-bonded silica and ion exchange resin. Because of the versatility of the stationary phase and the less broadening effect compared with the IEx mode, the NP mode offers the better separation of the multiple sugars. Recently, NP is defined as a type of "hydrophilic interaction chromatography "(HILIC)".[42]

6.1.2. Size exclusion mode

In size exclusion (SEC) mode, sulfonated polystyrene resin act as the stationary phase, and it has been used to separate sugars group since the early days of HPLC.[43] In SEC mode, salt aqueous solution or water work as the mobile phase. However, separation of the multiple monosaccharides in SEC mode is not so effective. Recently, the ligand exchange chromatography allows to separate multiple monosaccharides. Some studies show that various stationary phases have different counter-ions for ligand exchange chromatography (LExC) and have successfully separated fructose, glucose, mannose.[44]

7. The traditional stationary phase for the separation of sugars by high-performance liquid chromatography

7.1. Ion exchange resins

Ion exchange resins have been applied on the analysis of the sugars foryears.[45]The ion exchange resins work as the stationary phase, and the aqueous ethanol as a mobile phase.[46-47]However, the common drawback of the ion exchange resins is that the resins are easy to deform (shrinking and

swelling) under the high flow rates and high pressures of the HPLC. Hence, rigid sulfonated polystyrene resins were introduced because of the stable polymeric stationary phases, even under high pressure and flow rate condition this stationary phase scarcely swells or shrinks. In addition, due to the existence of the macro-pores structure, the ion-exchange resins can reach equilibrium rapidly. This stationary phase has been applied to the separation of sugars.[48]In this ion-exchanged mode, the hydrophilicity of the functional group and hydration water contents decides the retention time of reducing sugars, not by the ion exchange interaction. Typically, the ion-exchanged mode is operated under the high column temperature because of the anomeric separation at lower column temperature. The retention of anomers varies depending on the ligand exchange interaction between the hydroxyl group and the counter-ions in saccharide molecules.[49] The heavy metal ion-loaded sulfonated polystyrene resins are also developed in order to improve the separation ability in LEx mode. Also, the retention time order of the reducing sugars is different according to the heavy metal.[50-51]

7.2. Chemically-bonded silica phases modified with polar functional groups

Aminopropyl-bonded (amino-bonded) silica has been used widely in the aqueous normal-phase (ANP) mode for separation of sugars since the 1970s.[37] Additionally, aminopropyl-bonded is still used for this purpose. The amino-bonded silica affects the retention of saccharides. The retention of sugars is governed by the polar functional group and other important factors affect the retention such as the degree of hydration formed by the polar functional group. Compared with the ion-exchange modes, the chromatogram of the amino-bonded silica modes have shaper peaks and require less analysis time. Due to the alkalinity of the amino-group, anomers cannot be separated even in the room temperature. Nevertheless, the anomeric sugar can be separated at a column temperature of 0°C, in this condition it can prevent tautomerism.[52-53]

However, the amino-based silica has one important issue which is the formation of the Glycosylamines. Generally, it is formed by a reaction between the aldehyde (ketone) group in sugars and the amino group on the stationary phase. The Glycosylamine can cause the low recoveries and the column deterioration, the glycosyl amine problem can be solved by adding phosphate buffer (pH6) to adjust the mobile phase. The addition of buffer will modulate electrostatic of sugars which might be essential for the separation of the ionic saccharides. According to the studies, some acidic saccharides have been analyzed successfully by using the buffer containing aqueous acetonitrile.
A polymeric amino-bonded phase is commercially available (Shodex® Asahipak NH2P-50, Showa Denko). The aminopropyl group of the polymeric amino-bonded phase is bonded to a polyvinyl

alcohol resin. The elution order of sugars is different between the polymeric amino-bonded resin and the ion-exchange resin. Due to the polar functional group of the polymeric amino-bonded resin and the ion-exchange resin, it has different affinities for sugars. However, the retention order of sugars in the amino polymeric matrix stationary phase is almost as same as the amino-bonded silica.[54-55]Amide-bonded silica is used to separate peptides and sugars in HILC mode widely.[56] And amide-bonded silica is also commercial available as TSKgel® Amide-80 (amido-bonded silica, Tosoh) and as Partisil PAC (amido/cyano-bonded silica, Whatman). A great benefit of the amide-bonded silica is its high chemical stability. The limit of the operating temperature is up to 80°C, whereas the amino-bonded silica should operate below 50°C.Although the high chemical stability, the separation of the anomeric still causes peak broadening on amide-bonded silica. Hence, the temperature of the column should be higher than 50°C. The separation is also affected by the type of salt and the pH in the mobile phase. Therefore, the additional modifiers in the mobile phase play a big rule in these separation modes. Comparing the amide-bonded and the amino-bonded silica, the affinity between the sugars and polar functional groups are slightly different. Nevertheless, in both modes the retention order of sugars are similar.

Diol-bonded silica is the other traditional functional group-modified silica phase for separate reducing sugars. In diol-bonded silica mode, the retention properties of sugars are similar to the amino- and the amine- bonded silica. However, the resolution of sugar is lower than the amino- and the amine-bonded silica. Because there is no amino group in diol-bonded silica mode, the glycosylamines cannot form on diol-bonded silica. However, due to the anomeric separation happening at the low column temperature condition it can cause the peak broadening effect. Although adding amine to the mobile phase or heating the diol-bonded silica can improve anomeric separation, adding amine in the mobile phase will cause the dissolution of silica matrix which might shorten the column lifetime.[57]

8. Recent novel polar phases for separate sugar

HILIC separation mode becomes more popular for analysis of hydrophilic compounds (water-soluble) in ANP mode and it promotes the development of the many new stationary phases for carbohydrates separation.

8.1. Non-charged (neutral) stationary phases

The following are three types of novel stationary phases used for the analysis of sugars. First, polar compounds can be separated by Poly(ethylene glycol) (PEG)-bonded stationary phase in HILIC mode. The hydrophobic interaction is caused by the lower polarity of PEG. Second, poly(oxyethylene) (POE)

is a chemically-bonded stationary phase and it can work on three different modes 1) a reversed-phase mode for nonpolar analytes 2) an anion exchange mode which bases on anion-dipole interaction from the eluent cation fixed to POE-chains and 3) a HILIC mode for separate polar organic analytes. Third, polyol-type stationary phase such as sugar alcohol bonded phase has been invented by polymerization with sorbitol methacrylate.[58]Additionally, poly(succinimide) and its derivative stationary phase have also been studied and those HILIC stationary phases are modified to make them into the commercial product. Those stationary phases are used for the highly polar compound such as proteins, peptides, nucleic acid constituents, and carbohydrates. Although those stationary phases can retain the reducing sugars, the anomers are separated either due to the slow mutarotation in the mobile phase. However, adding 0.1% organic amine to increase the pH of the mobile phase which can solve the mutarotation problem.[59]

"Click chemistry", one novel synthesizing stationary phase which attracted much attention recently. In this new approach, azido-activated silica gel is covalently bonded to the alkyne-modifies saccharides in the presence of copper as a catalyst. By "click chemistry" a β-cyclodextrin (β -CD)-bonded phase was synthesized, and this stationary phase has separated polar compounds successfully such as glycopeptides, amino acids, oligonucleotides, saccharides and sugar alcohols. Various stationary can be produced by different highly polar groups via "click chemistry". [60]

8.2. Charged (ionic) stationary phases

Charged (ionic) stationary phase is not commonly used due to the electrostatic interaction interference with the separation of reducing sugars in both ANP mode and HILIC mode. The electrostatic interaction can cause low stability and low resolution. Although the anionic stationary phase poly(2-sulfoethyl aspartamide) separates polar compounds in HILIC mode successfully, this stationary phase mode is not so effective for separation of reducing sugars.[61]

8.2.1. Zwitterionic stationary phases

The materials of the zwitterionic separation surface are characterized by carrying both positive and negative charges. Compared with normal ion exchange stationary phase, in zwitterionic stationary phase the electrostatic interaction between charged analytes and the oppositely charged groups on the stationary phase are weaker. Different zwitterionic materials can be obtained by both dynamic coating and covalent bonding procedures, which lead to different zwitterionic functionalities. However, only the covalently bonded zwitterionic surface is stable enough to endure eluents with high organic

solvent mixtures in HILIC mode. Sulfobetaine-bonded zwitterionic phase possesses both positive charged quaternary ammonium and the negatively charged sulfonic group have been used widely. In addition, by utilizing the hydration water on the surface of the stationary phase, the water-soluble compounds can also be separated in HILIC mode. According to studies, the sulfobetaine-bonded zwitterionic phase can separate inorganic ions, organic molecules, sugars, and proteins. Applications of the sulfobetaine-bonded zwitterionic phase are really variable.[62] The spatial arrangement of the sulfonic group at the distal end of the zwitterionic moiety form a slightly negative surface charge. Besides, due to the highly hydrophilic surface layer, zwitterionic phases are suitable for HILIC mode.

The phosphorylcholine (PC) type is the other type of the zwitterionic phase. Compared with the sulfobetaine-bonded type, phosphorylcholine type also has a positively charged quaternary ammonium group and negatively charge phosphoric group. However, PC type possesses the opposite charge arrangement. PC type has been used to study drug permeability through phospholipids membranes as a result of the mimicry of nature biological membrane. Furthermore, in recent studies, PC mode also can apply to separation of peptides, sugars and sugar alcohols in HILIC mode. The phosphorylcholine stationary phase is commercially available as ZIC®-cHILIC (SeQuant), which was used in this experiment.[63]

ZIC®-cHILIC (SeQuant) HILIC column was used in this experiment, which was designed as excellent compatibility with HPLC and LC-MS of hydrophilic polar compounds. Generally, Sugars and sugar alcohols are difficult to separate by the HPLC columns operating in reversed-phase. However, due to the phosphoryl choline functional group in zwitterionic stationary phase, SeQuant ® ZIC®-cHILIC column can solve these problems, which provides the better selectivity and the easier method development.

Because the special zwitterionic stationary phase features of SeQuant® ZIC®-cHILIC column, the column can separate negatively charged polar compounds such as nucleotides and organic acids. The SeQuant® ZIC®-cHILIC column can also separate positively charged hydrophilic molecules, such as aminoglycosides and cations. SeQuant® ZIC®-cHILIC column is also very suitable for neutral

polar and zwitterionic hydrophilic substances. Applications and compatibility of this column are very versatile. The structure of the SeQuant ® ZIC®-cHILIC column is illustrated in Figure 2.[1]

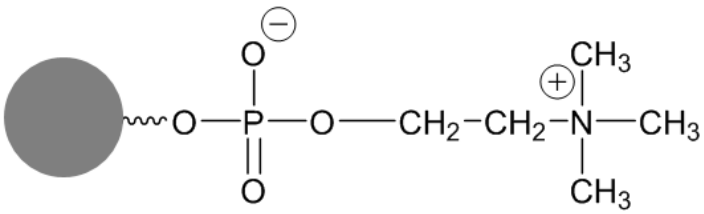

Figure 2. The functional group of the SeQuant ® ZIC®-cHILICcolumn[1]

In ZIC®-cHILIC column, phosphoric group has negative charge and the quaternary ammonium group possesses positive charge. Nevertheless, due to the nearly 1:1 charge balance of the zwitterionic stationary phase, the SeQuant ® ZIC®-cHILIC column has weak electrostatic interaction (repulsion and attraction). The weak electrostatic interaction enables optimization and separation tuning at low concentration of buffer in the mobile phase without interfering. Especially, the mass spectrometry (MS) has barely no interference.

SeQuant ® ZIC®-cHILIC column has good LC-MS compatibility. This column also has extraordinary low column bleed in LC-MS mood. Column bleed is one of the common problems in many HILIC columns; especially, several commercial "zwitterionic" HILIC columns have column bleed problems. The column bleed can cause signal suppression, increasing background signal and interference. Column bleed even can damage the MS instrument. Figure 3 shows, compared with other column, that SeQuant ® ZIC®-cHILIC column possesses the lower column bleed effect. All columns are 100 * 2.1 mm, operate at 50 °C with a flow rate 0.1 mL/Min, and the mass spectrometry intensity data is acquired from ESI+ mode with single quadruple, MS measured as total ion current for 20-2000 m/z. Average of 3 measurements each for 6 minutes. Eluent is 80 : 20 acetonitrile/ 25 mM aqueous ammonium acetate pH 6.8. All baselines of columns are stabilized before measurement.[1]

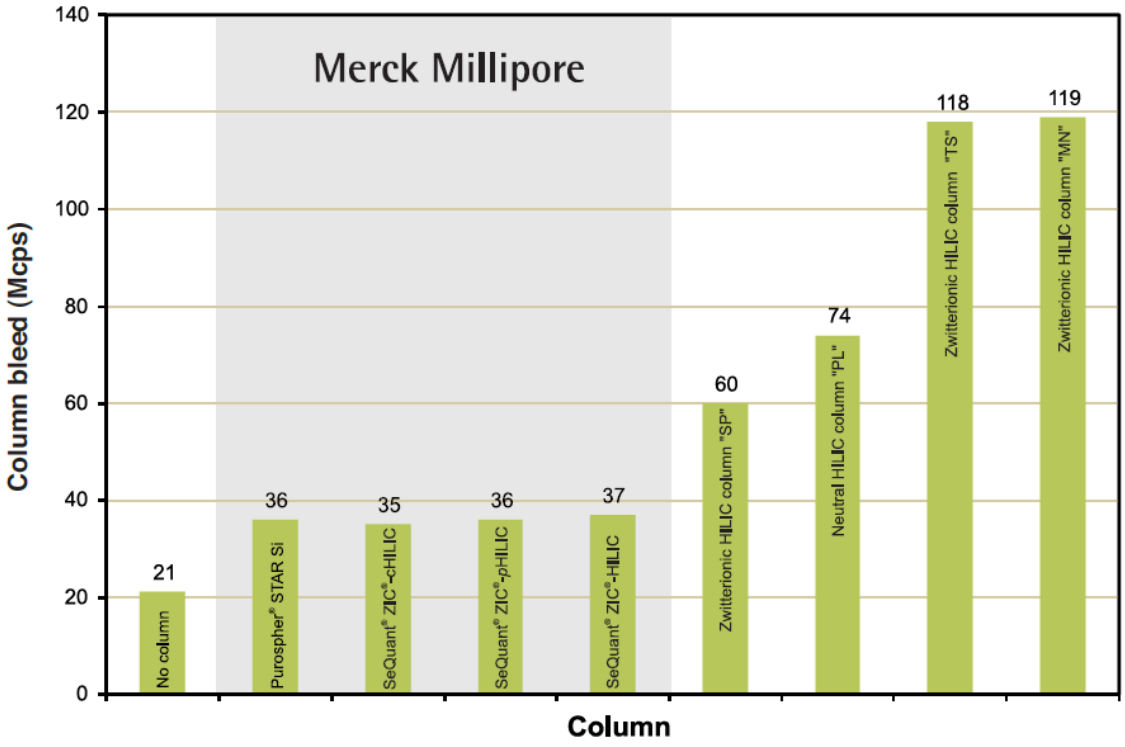

Figure 3. Column bleed effect of each different column[1].

SeQuant ® ZIC®-cHILIC column also shows high stability. Figure 4 shows the high reproducibility and stability of the SeQuant ® ZIC®-cHILIC column, which can withstand more than 1600 injections during 270 hours of continuous using and signals of the retention barely change. Retention factors on SeQuant® ZIC®-cHILIC (Ord. No. 1.50661.0001) 150 x 4.6 mm. Both operate at 23 °C with a flow rate of 0.5 mL/min. The mobile phase is 80: 20 acetonitrile/25 mM aqueous ammonium acetate pH 6.8. Injection of 10 µL toluene which is the void marker, uracil, and cytosine in eluent every 12 minutes. Measure by UV detector the absorbance at 254 nm.[1]

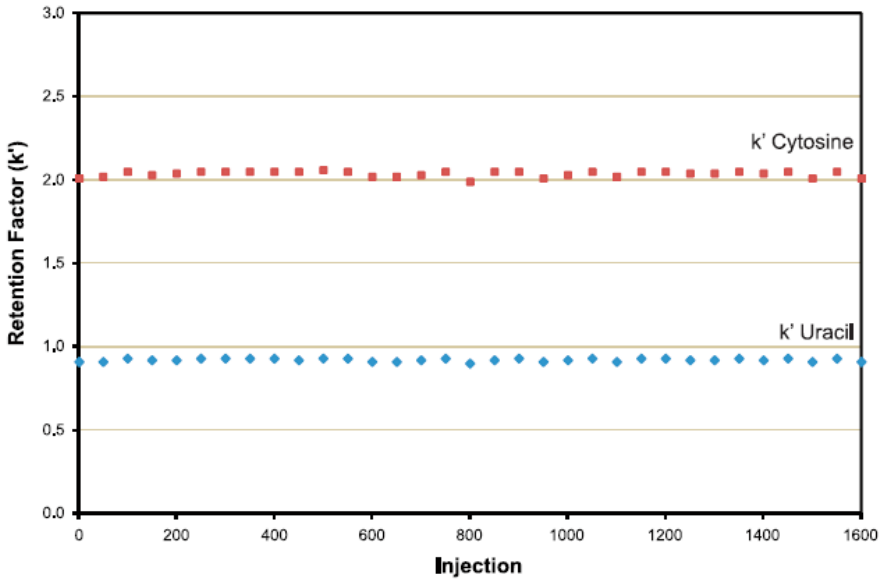

Figure 4. High reproducibility and stability of the SeQuant ® ZIC®-cHILIC column. SeQuant ® ZIC®-cHILIC can withstand more than 1600 injections during 270 hours of continuous using and signals of the retention barely change [1].

9. Hydrophilic Interaction Liquid Chromatography mode

Hydrophilic Interaction Liquid Chromatography (HILIC) is the novel and successful approach for the retention and separation of polar compounds. During the last decade, HILIC has increased in popularity. HILIC is widely used in the development of polar drugs within the pharmaceutical industry and the growing field of environmental chemistry which primarily involves the analysis of polar molecules. HILIC has been described as "reversed" reversed phase", due to that, in HILIC the stationary phase is polar and the aqueous mobile phase acts as the stronger solvent. Compared with the HILIC, the conventional reversed phase chromatography has the opposite property of the stationary phase and the mobile phase. In addition, there are many different stationary phases that can be used in HILIC mode. Besides, different stationary phase in HILIC mode possess different characteristics. Therefore, the application of the HILIC mode can be really versatile. However, owing to the complexity of the mechanism behind the HILIC mode, the strong knowledge of chromatogram and comprehensive understanding of the analyst are required.[64]

The origin of the HILIC mode can be traced back to 1951 when Gregor described a water-enriched layer on an ion-exchange resin surface.[65] Later on, monosaccharide was analyzed on an ion-exchange column by Samuelson and Sjöström. Then, in 1954, "a stagnant water layer could be responsible for the uptake of analytes" was assumed by Rückert and Samuelson. Then in 1975, the analysis of sugars

was accomplished successfully by the amino column.[66] Nowadays, the dominant mechanism of HILIC had been postulated that the existence of a semi-immobilized water-enriched layer on the polar stationary phase, combined with a partitioning equilibrium of analytes into the mobile phase. However, it was not until 1990 that Alpert introduced the acronym of HILIC, and the Hemström and Irgumhad written a comprehensive review on HILIC, which provide an excellent background to the technique.[67]

At the beginning, HILIC did not become widely recognized as a distinct chromatographic mode, it was only until the 2000's that was 'rediscovered' by the scientific community.[68]Accompanied by the rising popularity of HILIC, the versatile and particular HILIC stationary phases had been designed, which can offer higher retention and different selectivity for polar compounds. However, silica materials are still the most popular phases which include unmodified bare and/or hybrid silica materials. The statistics of the reported HILIC application are illustrated in Figure 5.

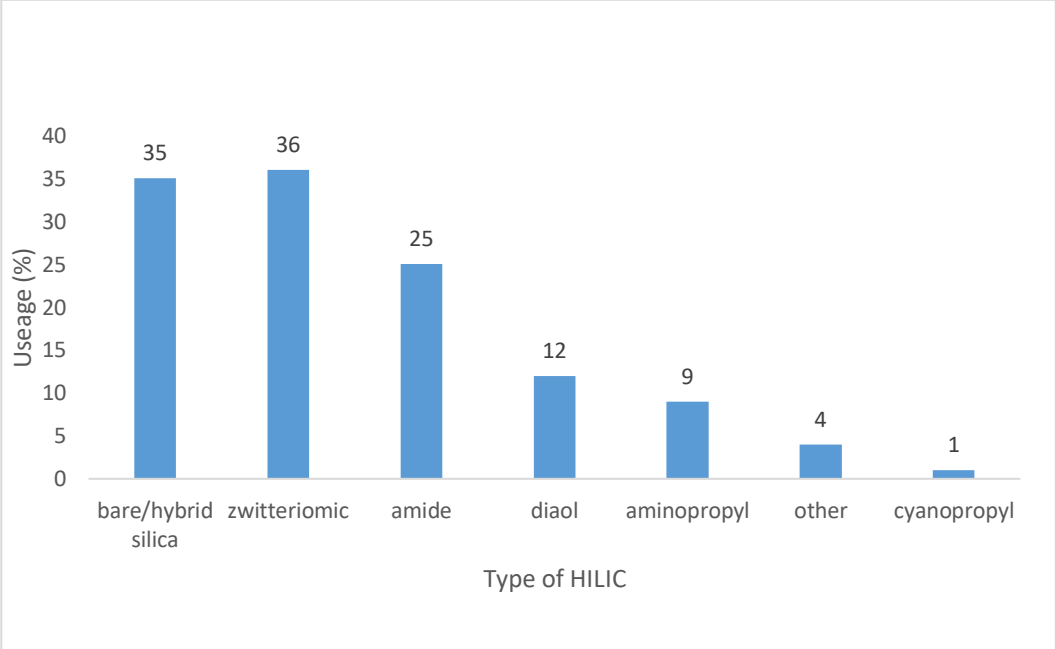

Figure 5. The statistics of the reported HILIC application[64]

The second most employed type of HILIC material is shown in Figure 5, which is the zwitterionic phases such as the sulfobetaine phase. Sulfobetaine-bonded zwitterionic phase possesses both positive charged quaternary ammonium and negatively charged sulfonic group in a 1:1 ratio so that the net surface charge is zero. Although the net surface charge is zero, meticulous electrostatic interaction are still generated by the negative charge of the sulfonic acid at the distal end of the phase with charged analytes. Therefore, a new type of zwitterionic phase phosphorylcholine (PC) has been

discovered by Irgum and his group. PC zwitterionic phase has a positively charged ammonium group at its distal end. Due to the special property of zwitterionic phases, it has successfully been used for the wide areas of analysis. Especially, the zwitter-ionic phase which is really compatible with sugars analysis.[69]

9.1. Retention mechanism of Hydrophilic Interaction Liquid Chromatography

The main difference between Hydrophilic Interaction Liquid Chromatography (HILIC) and reverse-phase chromatography (RPC) is the stationary phase. Compared with HILIC mode, Normal phase (NP) mode does not use water as one of the mobile phase components. HILIC mode uses a polar stationary phase, and the mobile phase employed in HILIC is highly organic solvent in nature such as acetonitrile also containing a small percentage of aqueous solvent/buffer or other polar solvents. "A stagnant aqueous-rich layer" adsorbed to the polar surface of the stationary phase which is formed by the water in the mobile phase. Polar analytes are retained through the partition into this stagnant aqueous rich layer. Basically, the mechanism of HILIC is composed by three main factors.[64, 70]

1. Hydrophilic partition: hydrophilic partitioning of the analyte between the mobile phase and the bulk of the aqueous-rich layer.
2. Hydrogen bonding: hydrogen bonding between the stationary phase and the polar functional group of analyte.
3. Electrostatic interactions: electrostatic interactions between ionized functional group of analyte and the stationary phase.

In addition, under the appropriate experimental conditions, van der Waals interactions can be present. The van der Waals interaction is between the non-polar part of the analyte and the hydrophobic portions of the bonded ligand of the stationary phase.

Due to HILIC has the opposite property compared with reverse-phase chromatography (RPC) and the high organic content mobile phase of HILIC, some advantages are obvious. The advantages of HILIC are shown below.

1. HILIC mode can separate hydrophilic compounds that are difficult to retain in RPLC without using an ion pair agent. Additionally, the elution order is mainly based on analyte hydrophilicity. Therefore, HILIC selectivity is complementary to RPLC selectivity.
2. In the ESI MS detection mode, signal/noise ratio (S/N) is strongly improved. In HILIC mode, mobile phases have high organic content that the ionization of highly organic mobile phase can be more efficient. Hence, HILIC leads to lower detection limits.
3. HILIC has low back pressures. Owing to the low viscosity of the organic-rich mobile phase, the pressure of HILIC mode is usually lower than RPLC mode. Generally, the pressure of HILIC is 2−3 times less than in RPLC. Because of the lower back pressure, the high flow rates for fast analysis can be fulfilled.
4. HILIC has good compatibility with SPE extracts. After SPE procedure, if analytes are 100% aprotic organic solvent then the 100% aprotic organic solvent extraction can be directly injected to HILIC system, without dry down and reconstitute in the mobile phase.[71]

9.2. Limitations of Hydrophilic Interaction Liquid Chromatography

1. Peak distortion is one of most common problems in HILIC mode, which is caused by the mismatch of mobile phase and sample solvent. When an aqueous sample solvent has high elution strength which it interferes with the partitioning between the stationary phase and analytes.
2. HILIC has relatively longer column equilibration times. Mechanisms of HILIC mode are kinetically slower than the common mechanisms in RPLC, which lead to the longer column equilibrium times.
3. The accurate pH of the mobile phase cannot be measured when using high proportions of the organic solvent. For instance, when the mobile phase is acetonitrile/ammonium formate 85/15 (v/v) pH 7 and the aqueous buffer pH 3. In fact, the pH to the column and analyst is about 5.2.

9.3. The stationary phase for Hydrophilic Interaction Liquid Chromatography mode

First of all, it’s critical to understand the compounds and the physiochemical properties of the molecules in order to reveal the possible retention mechanisms. Even for choosing the organic mobile phase, the organic solvent content, the buffer concentration and the pH value of the mobile phase are dependent on understanding the compounds and the physicochemical properties of analytes.

Therefore, in order to select a suitable HILIC column, understanding the physiochemical properties of the analyte is essential.

The partition coefficient is especially important, due to the fact that hydrophilic partition is the main mechanism of the HILIC. The partition coefficient (log P) is used for the neutral compound, or where the compound only exists in a single form. Log P is calculated from the concentration ratio of a compound in a mixture of two immiscible phases at equilibrium, being water and octanol the most commonly used. The calculation of the partition coefficient is shown in Equation (1).Log P shows the degree of the hydrophilicity of a compound. When log P <0 it indicates a relatively hydrophilic compound, and vice versa, when log P > 0 it indicates a more hydrophobic compound. Usually, HILIC mode is recommended for compounds with negative log P values due to the hydrophilic partition.

Equation (1) $$\text{Log } P_{oct/water} = \log \frac{[Aanalyte]_{octanol}}{[Aanalyte]_{water}}$$

However, the ionizable solutes may exist as several different species in each phase at any given pH, thus the log P is not appropriate anymore. In this case, we need to derive log D, which is the distribution coefficient for ionizable compounds. Log D is a pH-dependent measurement of differential solubility of all species in the octanol/waster system. Log D is the ratio of the sum concentration of all the forms of the analyte in each water phase and octanol phase. The calculation is illustrated in Equation (2). Log D provides an appropriate indication state of a compound in solution at a specific pH value and the hydrophobic character in the buffer system. Therefore, the appropriate stationary phase of HILIC mode can be chosen by understanding the knowledge with log P, log D and pKa of analytes comprehensively.[64]

Equation (2) $$\text{Log } D_{oct/water} = \log \frac{[ionizedAanalyte]_{octanol} + [un-ionizedAanalyte]_{octanol}}{[ionizedAanalyte]_{water} + [un-ionizedAanalyte]_{water}}$$

The four main criteria for picking up the appropriate stationary phase are illustrated below.

1. The degree of hydrophilicity.
2. Selectivity for conformational isomers and positional isomers.
3. Selectivity for hydrophilic-hydrophobic groups.
4. Electrostatic interactions.

9.4. The mobile phase for Hydrophilic Interaction Liquid Chromatography mode

Generally, the mobile phase in HILIC mode is highly organic (organic 60-70%; at least 3% water is required). The major factor for controlling the separation selectivity is the organic modifier/aqueous ratio. According to studies, an increase in the percentage of organic solvent will cause an increase in retention. Concerning the type of the organic solvent, although acetonitrile is the most dominant solvent used in HILIC mode, there still have several polar water-miscible organic modifiers that can be used, such as tetrahydrofuran (THF), acetone, isopropanol (IPA), ethanol (EtOH), methanol (MeOH) and water. Usually, the eluotropic strength of HILIC solvent is opposite to the RPLC mode. Nevertheless, it is recommended to use aprotic solvents in HILIC mode. Aprotic solvent cannot exchange a proton due to their hydrogen bonding interaction with the water molecule. Besides, the competition between the solvate surface and the surface of the HILIC stationary phase is important. The competition will decrease the strength of the partitioning and become less retentive. Additionally, when the gradient elution is introduced, it is really crucial to have a sufficient long post gradient re-equilibration stage. The re-equilibration stage can let the volume of the water layer to re-establish its initial conditions. Approximately 20 column volumes for the post gradient re-equilibration is recommended. Adding the buffer in both mobile phases is recommended in the gradient system. This way it allows constant buffer strength to be maintained during the running.[64, 71-72]

Furthermore, adding buffer to the mobile phase can reduce peak tailing and/or retention of charged analytes. According to studies, the absence of buffer salt in mobile phase results in peak boarding and extremely long retention times on cyclodextrin, amide, cyano and amino-based columns. Another thing that should be noticed is that the common buffers used in RPLC should be avoided in HILIC, owing to the poor solubility in highly organic mobile phase.

Electrostatic interaction also has an important contribution to the retention of HILIC, due to some polar compounds that can be charged at the mobile phase. Hence, the buffer plays a key role in this case. The existence of the buffer in the mobile phase can reduce electrostatic interaction between analytes and the stationary phase (both repulsive and attractive). As the salt concentration increases, the retention of positively charge solute on positively charge stationary phase will increase too. The increased population of solvated salt ions in the mobile phase led to the increase of hydrogen-bonding interaction (between the stationary phase and analytes). This phenomenon is called salting-out effect. Besides, the concentration of the buffers is important. In HILIC mode it is recommended that the concentration of the buffer should be maximum of 20 mM but not above when added to acetonitrile

and/or other organic mobile phases, in order to avoid solution crashing out. Besides, using a lower concentration buffer the suppression effect with electrospray source in MS can be reduced.

The effect of different type of mobile phase buffer on the retention of acid and basic mode compounds have been studied. Generally, ammonium formate and ammonium acetate are the most commonly used buffers. Ammonium formate has longer retention times for acidic compounds, which leads to weaker eluting strength in ion exchange. The acetate ion has the opposite property. The acetate ion has a great neutralizing effect of the electrostatic attraction between the negatively charged acid and the surface of the positively charged stationary phase. However, the retention mechanism of basic analytes on the amino column between ammonium formate and ammonium acetate does not have a great difference. Therefore, no numerous differences were observed between formate and acetate salt for neutral and zwitterionic phases.[64, 73]

Owing to the specific separation mechanism of HILIC column, choosing and understanding mobile phase become really important. Key points for HILIC mode mobile phase selection are shown below.

1. The common mobile phase can be acetonitrile or other polar, water-miscible organic modifiers. Aprotic solvents have longer retention than protic solvents. Besides, in HILIC the eluotropic strength is opposite to RPLC.
2. The mobile phase should be high organic content. Generally, the percentage of organic content is between 60 to 90%; the minimum percentage of water required to ensure a stagnant water layer that can be sufficiently formed on the stationary phase is 3%.
3. An increase in the organic solvent will lead to an increase in retention.
4. Ammonium acetate and ammonium formate are widely used buffer salts which can avoid peak tailing and control the retention times of charged analytes.
5. Although highersalt concentrations will increase the retention of charged solutes, buffer salt concentrations should not be above 20 mM due to the suppression effect to MS. Furthermore, the excess buffer salts cannot solve in the high level of organic solvent.
6. When running gradient elution, both mobile and stationary phase should have buffer salt.
7. When running gradient elution, do not run mobile phase from 100%organic to 100% aqueous.
8. The pH values of the mobile phase can change the state of the stationary phase which can affect HILIC retention of ionizable compounds.

9.5. Hydrophilic Interaction Liquid Chromatography method development

It is necessary to have the logical manner to conduct the HILIC method development. The systematic approach to method development is inevitable. Three main points mentioned below play the key role in the HILIC method development. Which type of the stationary phase in HILIC mode is most suitable?

1. Which type of the mobile phase is the most appropriate and which is the best composition of the mobile phase.
2. Which kind of the influent will be influenced by the pH value and the buffer during the analysis.
3. How the flow rate and the column temperature influence the HILIC analysis.

10. Solid phase extraction

Solid phase extraction (SPE) is a powerful sample preparation technique. In SPE, the separation of analytes from a mixture occurs by selective partitioning of the compounds between a liquid phase (solvent) and a solid phase (sorbent). Because of its practicability and convenience, SPE has become the powerful tool for rapid and selective sample preparation prior to analytical chromatography. With SPE, many problems associated with Liquid/Liquid Extraction (LLE) can be solved, such as matrix effect, incomplete phase separation, disposal of large quantities of organic solvents, less-than-quantitative, and fragile specialty glassware. Compared with LLE, SPE is more efficient and rapid. The yield quantitative extraction of SPE is easy to perform and can also be automated. The main aim of the SPE is to have better chromatographic separation and analytical results, longer column lifetime, less instrument downtime, and higher detection sensitivity for the sample. Generally, SPE is widely used to prepare liquid samples and extract nonvolatile or semi-volatile analytes. In addition, SPE makes the sample more compatible for separation and detection; especially, in the HPLC system and MS analysis. Usually, the sample matrix in the bio-sample or environmental aerosol sample can cause the ion suppression, such as human plasma or aerosol filter. However, due to the high selectivity of SPE which can remove undesired interferences, the signal to noise is decreased, and the ion suppression is improved.[74-85] The advantages of solid phase extraction are shown below.

1. Less clean-up time compares with LLE.
2. Simplify the lab works such as faster processing and less manipulation.
3. Reduce the total costs of the material.
4. Decrease organic solvent usage and lower the disposal.
5. Reduced the risk of exposure under the toxic organic solution compare with LLE.
6. Avoid the formation of the emulsion from the liquid phase compare with LLE.
7. It is possible to conduct the great number of the batch at the same time (high throughput capacity), and it can be automatic.
8. After SPE, analytes are compatible with the derivatization processes.
9. Possibility for solvent exchange and desalting.
10. Good platform for sample conservation from the sample site to the lab.
11. According to the matrix and compounds of interest to design more specific extraction mode, which is more flexible.

10.1. Reversed phase solid phase extraction

Commonly, the SPE can be classified into three different types. The first category is called reversed-phase SPE. The principle behind the reversed phase SPE is the same as RPLC. The separation mechanism involves a polar or slightly polar sample matrix (usually aqueous mobile phase) and a nonpolar stationary phase. The analyte of interest is typically non- to mid-polar compounds. The common absorbents of reverse phase SPE, such as LC-18, LC-8, ENVI-18, LC-4, and LC-Ph are widely used. Besides, the synthesis of reversed-phase SPE material is illustrated in Figure 6. The raw hydrophilic silanol groups on the surface of the silica packing (typically 40μm particle size, 60Å pore size) have been chemically modified with aryl functional groups or hydrophobic alkyl by reaction with the corresponding silanes.

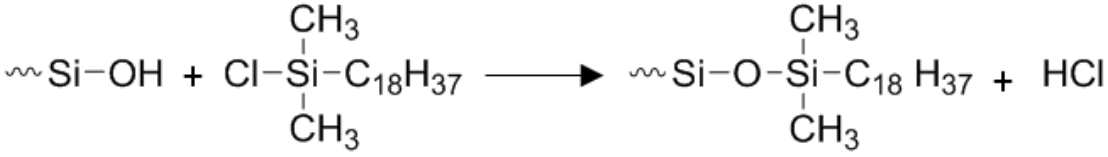

Figure 6. The modification process of the raw hydrophilic silanol groups on the surface of the silica packing.[74]

The retention factor of analytes in reversed phase SPE mode (typically organic analytes from polar solvents such as water) is caused by primarily attractive force between functional groups on the silica surface and the carbon-hydrogen bonds in analytes. This nonpolar to nonpolar attractive force is called van der Walls forces or dispersion force. Normally, a nonpolar solvent is used to elute sorbents in order to disrupt the binding forces between analytes and adsorbent. Generally, monomerically bonded silicas are LC-18 and LC-8. The polymeric adsorbent, such as ENVI-18, and ENVI-8, is more resistant to extreme pH conditions. The polymeric adsorbent has a more complete coverage of the silica surface and higher carbon loading thus is more suitable for trapping organic compounds from acidified aqueous, such as environmental samples.[86-87]

10.2. Normal phase solid phase extraction

Typically, a normal phase SPE mode involves a polar analyte and a mid-to nonpolar matrix, (e.g. Hexane, acetone, and chlorinated solvents), and a polar stationary phase. Polar adsorption media (LC-Si, LC-Alumina, ENVI-Florisil, and LC-Florisil) and polar-functionalized bonded silica (LC-CN, LC-Diol, and LC-NH_2) are applied in the normal phase mode. The main mechanism of retention is the interaction between polar functional groups of the analyte and polar groups on the sorbent surface. For instance, hydrogen bonding, dipole-dipole interactions, dipole-induced dipole interactions and pi-pi interactions. Generally, the eluting solvent is more polar than the original matrix of the sample in order to disrupt the binding mechanism thus the bonded compounds of interest can be eluted.[74, 88]

10.3. Ion exchange solid phase extraction

Ion exchange SPE mode is used for charged compounds. The solution usually is aqueous but sometimes can be organic. LC-SCX or LC-WCX bonded silica cartridges are used for cationic compounds, and the LC-SAX or LCNH2 bonded silica cartridges are used for anionic compounds. The primary retention force is between the electrostatic attraction of the charged functional group on the compound and the charged group bonded to the silica surface. The pH value plays a key role in ion exchange SPE mode. In order to retain charged compounds from an aqueous solution, functional groups on the bonded silica and the compounds of the interest need to be charged. Furthermore, in some cases, other species of the same charged compound in the matrix might interfere with the adsorption of the interest compound; therefore, the functional group of the compound or the functional group on the sorbent surface can be neutralized by tuning pH value. Because, when one of

these functional group is neutralized then electrostatic force, binding is disrupted, thus the compound of the interest can be eluted. Moreover, the solution contains anionic species that displaces the adsorbed compound, or the high ionic strength solution, both are used to elute the compound..[74, 89]

10.4. The step of the solid phase extraction

First step is the washing of impurities and pre-conditioning the SPE phase. Pre-conditioning the SPE material before using is important. The chemical and physical properties of interest compounds and the matrix should be well understood. Then, the proper SPE mode is chosen accordingly. Secondly, the SPE tube is conditioned by rinsing the tube up to one tube-full of solvent before extracting the sample. Generally, for the reversed phase, a water-miscible organic solvent, such as methanol, is used for conditioning. Later on, water or the aqueous buffer is used to condition the cartridge. The purpose of using methanol is that methanol penetrates bonded alkyl phases and wets the surface of the sorbent, allowing water to wet the silica surface more efficiently. Besides, in order to remove any impurities on the SPE tube, some pre-conditioning solvents are used before the methanol wash. Typically, normal phase SPE silica and polar adsorption media are conditioned in the organic solvent in which the sample exists. For ion exchange SPE, the sample is in the polar solvent and water-miscible organic solution with the proper pH, salt concentration, and solvent content is used to condition the cartridge. In addition, if the sample is in the nonpolar solvent, the cartridge is conditioned with the sample solvent.[74]

Then is the selective extraction step. In this step, an SPE sorbent which can bind selected components of the sample, either the sample impurities or the compounds of interest is selected. When the sample passes through the SPE cartridge, the selected components are retained. The extracted impurities are discarded, and the adsorbed compounds of interest are collected through elution. Then is the selective washing step. After loading sample, the impurities and the compounds of interest are retained on the SPE adsorbent. The impurities can be eluted using the wash solution which is able to selectively elute impurities but not the compounds of interest. Furthermore, the selective elution can also be used. In this mode, the solvent is appropriate to elute the adsorbed compounds of interest and keep the impurities bound to the adsorbent.

11. Experiment part

This chapter shows the chemicals (Table 3), equipment and devices (Table 4) that were used. In addition; the sampling detail and the sample preparation procedure are described at this chapter.

11.1. Materials and reagents

Table 3. Materials and reagents

Chemical	Manufacturer
Ammonium Acetate	Sigma aldrich
Acetonitrile	Sigma aldrich
Arabitol	Sigma aldrich
Fructose	Merck
Formic Acid	Sigma aldrich
Glucose	Merck
Inositol	Sigma aldrich
Levoglucosan	Merck
Methanol	Sigma aldrich
Mannitol	Sigma aldrich
Sucrose	Merck
Trehalose	Merck

11.2. Instruments and tools

Table4. Instruments and tools

Equipment or device	Model and Manufacturer
HPLC	Agilent 1260 Infinity
Ion Trap Mass Spectrometer	Bruker Esquire 3000 Plus
ULTRA HPLC In-Line Filter	KrudKatcher, 0.5µm Depth Filter x 0.004
HILIC column SeQuant® ZIC®-cHILIC	3µm, 100Å 100 x 2.1 mm
SPE cartridge	Strata™-X 33 µm, part number 8B-S100-EBJ
Balance	Sartorius BP analytical balances Model BP301S
Syringe filter forfiltratethesamle	VWR International 0.2 µm, 13 mm PTFE No. 514-0068
Glassware and measuring instrument	Test tubes, volumetric flasks, beakers, glass syringes, pipettes, HPLC vials and caps
Ultrasonic Cleaner	Branson 5510R-MT

11.3. Standard Solutions

The standard solution of each sugar and sugar alcohol was made by dissolving the solid compounds in deionized water containing 0.1% formic acid. The weighing procedure was operated by the balance (Sartorius BP analytical balances Model BP301S). The stock solutions were made from the standard solutions, and the concentration of the stock solution was 1250 mg/L. The stock solutions were also used for SPE/HPLC-MS method development and calibration. The concentrations of standard solutions were 100 mg/L, 10 mg/L, 5 mg/L, 3 mg/L, 1 mg/L and 0.5 mg/L. Each of the standard solution was prepared by pipetting an appropriate amount of stock solutions and diluted it with deionized water containing 0.1% formic acid.

11.4. Site and sampling condition

The environmental aerosol sample was extracted by the pump. The volume of samples was 360 m^3. The sample was extracted from 7:00 to 19:00 hours. The location of this sampling site was at the Kumpula campus, Helsinki, GustafHällströminkatu 2, longitude and latitude: 60.204953, 24.963738). The sampling procedure began from the 3rd to the 29th of May, 2015, except on the 9th and the 21st of May, 2015. Additionally, the blank filters from May/2015 were sealed in the plastic bag dailyandstoredin the freezer at +5°C before analysis.

11.5. Instrumentation

The Agilent 1260 Infinity HPLC was composed of an auto-sampler with the capacity for 100 vials, a gradient pump system, a UV detector, and an ion trap mass spectrometer. However, during this experiment, the UV detector was not used due to the usage of the ion trap tandem mass spectrometer as a detector (Bruker Esquire 3000 Plus Ion Trap Mass Spectrometer). The separation of the analytes was achieved by the HILIC column SeQuant® ZIC®-cHILIC (3µm, 100Å 100 x 2.1 mm). An auto-injector syringe pump (SP100I, World Precision Instruments) was used in order to optimize the ionization of the ion trap. The flow rate of the auto-injector syringe pump as 200 mL/min. The ultrasonication machine was used for the extraction and degassing. The extraction time was 20 min which was also used for homogenizing the mobile phase. Besides, for the degassing procedure, the mobile phase was under ultrasonication machine for 10 min.

11.6. Sample preparation

Prior to the experiment, all tools and glassware were washed in the following process to reduce the risk of contamination. The tools were cleaned with water and methanol under sonication for 10 min and dried in the oven which at 50 °C. After cleaning procedure, filters containing analytes were cut. All the filter samples were transferred using a tweezer to avoid contamination. Firstly, the filter samples were placed on the Teflon mat and cut with the mold and hammer. After cutting filter samples, the samples were transferred to test tubes. The filter samples were sonicated by sonicated for 20 minutes at room temperature with 2 mL deionized water with 0.1% of formic acid. The sonication process was repeated using new 2 mL deionized water with 0.1% of formic acid. The sample solutions were vortexed to obtain homogeneous mixtures. Then, the sample solutions were passed through the syringe filter (VWR International 0.2 µm, 13 mm PTFE No. 514-0068) into the test tubes. Later on, the SPE processes, the cartridge (Strata™-X 33 µm Polymeric Reversed Phase, 100 mg / 3 mL, and part number 8B-S100-EBJ) was preconditioned. Each cartridge was rinsed with 1 mL methanol, and this process was repeated twice. Next, the cartridge was rinsed with 1mL of deionized water and the process was repeated in triplicate. Then, the sample solution was loaded into the cartridge, and the sample was eluted only by gravity. After elution, the first fraction was collected into the test tube. Then, the second fraction eluted by 3 mL of methanol was collected. Later on, nitrogen gas was used to dry the eluted solution. Then, the dried samples were redissolved with 400 µL of deionized water with 0.1% formic acid, and samples were sonicated by sonication machine for 20 minutes at room temperature, and sample solutions were transferred to different HPLC vials. In addition, a blank filter sample was pretreated by the same procedure. The blank filter sample was operated with the normal filter sample simultaneously for blank correction. All prepared samples were stored in the 4 °C fridge. The samples were required to be analyzed within 72 hours. The sample preparation procedure is shown as Figure 7.

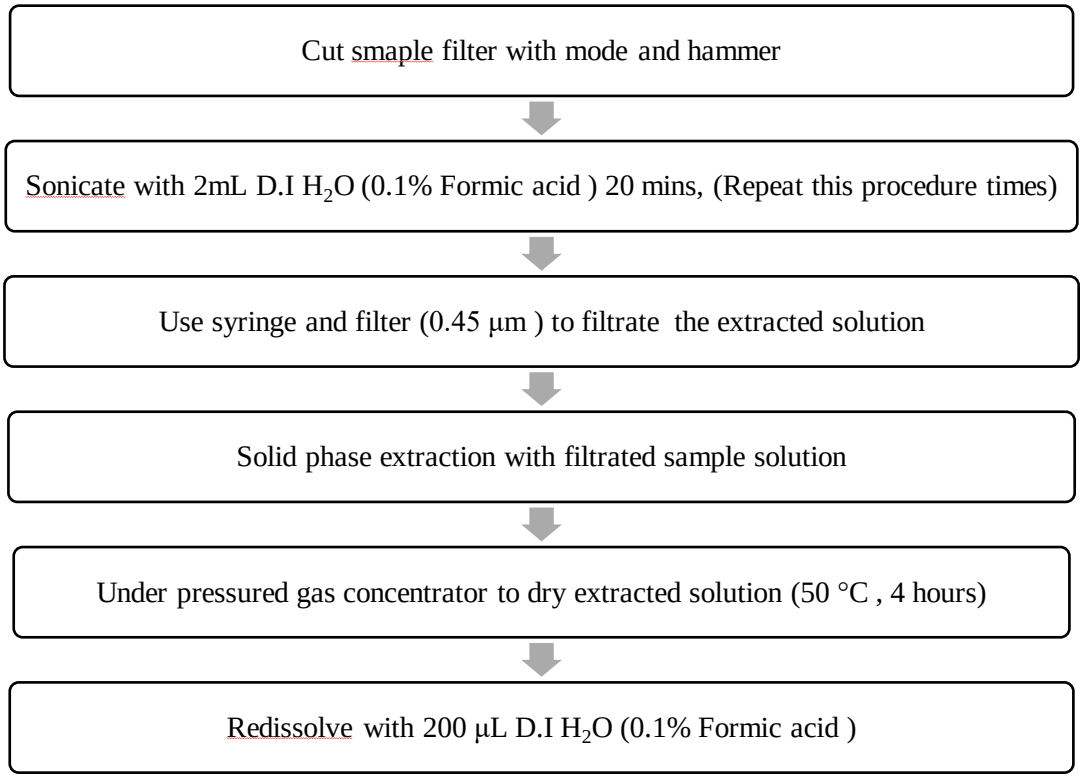

Figure7. Sample preparation procedure.

11.7. Chromatographic analysis

The experiment was operated with an Agilent 1260 Infinity HPLC coupled with Bruker Esquire 3000 Plus Ion Trap Mass Spectrometer (Bruker Corporation, Germany). Ionization of electrospray was in the positive mode. The HILIC column was SeQuant® ZIC®-cHILIC (3μm, 100Å 100 x 2.1 mm Merck KGaA, Germany). The column temperature was retained at room temperature, and the injection volume was 5 μL. Eluent A was 20 mM ammonium acetate, and the eluent B was acetonitrile (HPLC grade).The optimized gradient flow rate program is referred to Table 5. The gradient parameter is illustrated in Table 6.

Table 5. The gradient parameters of the HPLC pump.

Flow rate	0.45 mL/min
Stop time	12 min
Solvent A	H_2O
Solvent B	Acetonitrile
Time (min)	%B
0 min	80%
1.5 min	50%
3 min	20%
6 min	50%
9 min	80%

Table 6. The optimized parameters of ion trap mass spectrometer.

Optimized parameters	
Injectionrate	350 µL/h
Capillary	-4496.88 V
Cap exit	93 V
Skimmer	29.5 V
Oct 1DC	10 V
Oct 2DC	1.45 V
Trap drive	32 m/z
Oct RF	125.9 V
Lens 1	-3.9 V
Lens 2	-51.81 V

12. Results

This chapter shows the optimized results. In addition, the results of daytime sample filter from May/2015 3rd to 29th are illustrated in the chapter.

12.1. The mass-to-charge ratio of analytes and optimization of the ion source

The m/z of analysts were found by injecting the standard solution to the ion trap tandem mass spectrometer. The flow rate of the auto-injector syringe (auto-injector syringe pump SP100I, World Precision Instruments) was 350 µL/h, and the concentration of the standard solution was 5 mg/L (standard dissolve in deionized water with 0.1% of formic acid). The most optimal results were obtained using the positive ESI mode. On the other hand, when the negative ESI mode was applied, most of the analytes could not be detected. In addition, the intensity of the signal was really weak, and only a few sugars were detected. However, in the positive ESI mode, the intensity of the signal was much stronger. All the analytes could be detected in the positive ESI mode. In addition, the analyte inclined to bind with sodium. Therefore, the analyte became analyte-Na^+. Although some of the analytes still can be detected as the protonated products (analyte-H^+), the sensitivity of sodium products (Sugar-Na^+,) were higher than protonated products due to sodium ions from the test tubes. Besides, the optimization of parameters for the ionization part was also conducted at this session. The optimization parameters are referred to the Table5 and Table 6. The detection m/z and retention time of analytes are shown as Table7.

Table 7. Retention time and m/z of analytes.

Molecular	Molarmass	Detectionmass	Retentiontime
Fructose	180.16	203	2.5 min
Glucose	180.16	203	3.1 min
Sucrose	342.30	365	3.6 min
Arabitol	152.15	175	2.3 min
Inositol	180.16	203	3.8 min
Mannitol	182.17	205	3.1 min
Levoglucosan	162.14	185	1.2 min

12.2. Optimized the Flow rate

The mixture of sugars and sugar alcohol could not be separated by HPLC isocratic mode. Hence in here, the gradient system was used. The optimized flow rate is referred to Table 5. The mobile phases A and B were 20 mM ammonium acetate and HPLC grade acetonitrile, respectively. The standard injection volume was 5µL. The flow rate from bi-pump was 0.45 mL/min, and the condition time for HILIC column was 12 minutes. The maximum pressure of HILIC column was 400 bar.

12.3. Optimized the concentration of adduct compound for analysis

Due to the special property of Hydrophilic Interaction Liquid Chromatography (HILIC) column, the column needed to be conducted in the proper condition time. Therefore, the stagnant aqueous rich layer was formed after proper condition time in each injection, and the polar analytes can retain in this stagnant aqueous layer by the hydrophilic partition, hydrogen bonding and electrostatic interactions. In this session, parameters of the optimization procedures are shown below: 20 mM ammonium acetate worked as mobile phase A and acetonitrile worked as mobile phase B. The concentration of standard was 0.125 mg/L. The standard injection volume was 3µL. The flow rate was 0.45 mL/min. The condition time of HILIC column were11, 12, 13, 14 and 15 minutes. When the condition time was 12 min, the relative signal intensity and the peak shape was optimized. The result is illustrated in Table 8 and the Figure 8. Hence, in the end, the 12 min was chosen as the optimized condition time for HILIC column.

Table 8. Relative signal intensity by different mobile phase buffer.

	15 mM		20 mM		25 mM	
Analytes	Area	Ratio (%)	Area	Ratio (%)	Area	Ratio (%)
Fructose	8811984	100.43	8773679	100	6515690	74.26
Glucose	2530972	90.94	2782856	100	2339111	84.05
Sucrose	5292323	84.14	6289463	100	3479567	55.32
Arabitol	6955438	92.01	7559063	100	5868877	77.64
Inositol	1229994	91.13	1349638	100	1051258	77.89
Mannitol	9345649	56.25	16613383	100	6471734	38.95
Levoglucosan	1750701	52.62	3326575	100	1680487	50.51
Trehalose	73306	35.36	207261	100	103175	49.78

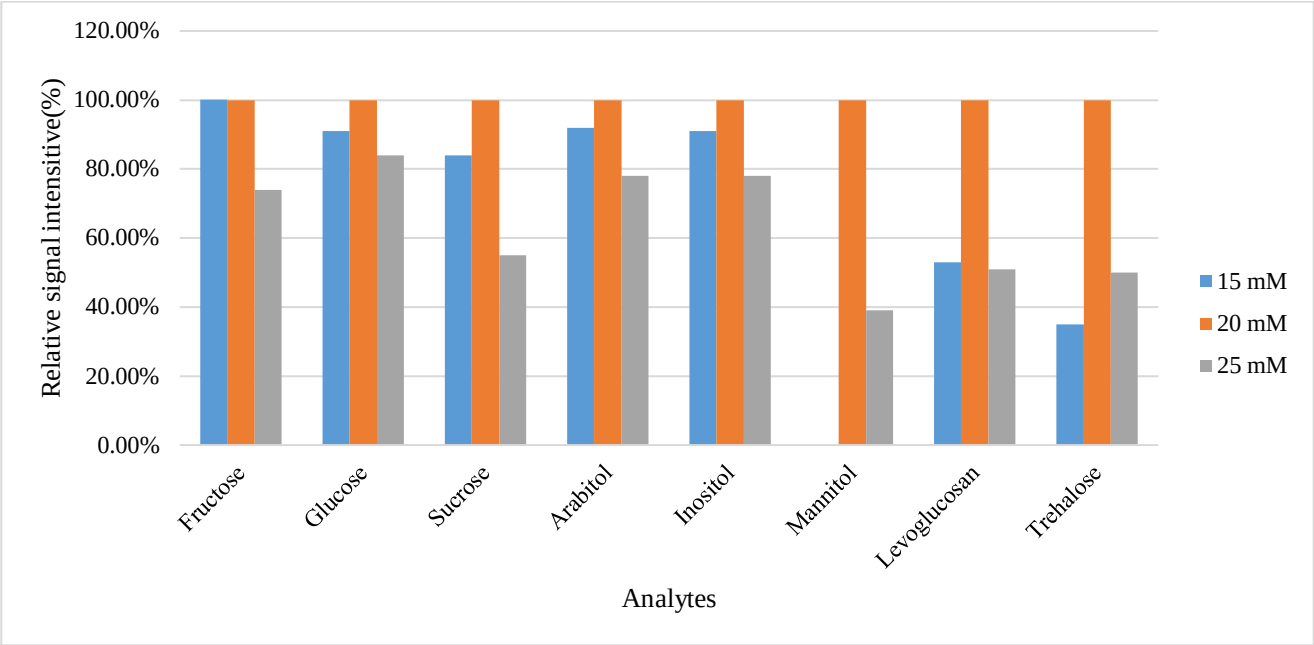

Figure 8. Relative signal intensity by different mobile phase.

12.4. Optimization of the condition time for the HILIC column

Due to the special property of Hydrophilic Interaction Liquid Chromatography (HILIC) column, the column needed to be conducted in the proper condition time. Therefore, the stagnant aqueous rich layer was formed after each injection with proper condition time, and the polar analytes could be retained in this stagnant aqueous layer. The main retaining mechanisms were generated by the hydrophilic partition, hydrogen bonding and electrostatic interactions. In this session, parameters of the optimization procedures are shown below: 20 mM ammonium acetate worked as mobile phase A and acetonitrile worked as mobile phase B. The concentration of standard was 0.125 mg/L. The standard injection volume was 3μL. The flow rate was 0.45 mL/min. The condition times of HILIC column were11, 12, 13, 14, and 15 minutes. Although inositol had relative good result in 14 minutes and 15minutes, the comprehensive analytes results were not optimized. When the condition time was 12 min, the relative signal intensity and the peak shape was optimized. The result is shown in Table 9 and Figure 9. Hence, 12 minutes was chosen as the optimized condition time for HILIC column.

Table 9. Condition time of the HILIC column.

	11 min		12 min		13 min		14 min		15 min	
Analytes	Area	Ratio (%)	Area	Ratio (%)	Area	Ratio (%)	Area	Ratio (%)	Area	Ratio (%)
Fructose	1168532	92.72	1260220	100	1334299	105.87	1319990	104.74	1264768	100.36
Glucose	347540	93.47	371809	100	304118	81.79	304969	82.02	264854	71.23
Sucrose	1010049	100.60	1003948	100	1053862	104.97	868318	86.49	946130	94.24
Arabitol	1339513	104.81	1278014	100	1391076	108.84	1276438	99.87	1322915	103.51
Inositol	95391	64.93	146910	100	119078	81.05	163765	111.47	193638	131.80
Mannitol	1518719	89.34	1699782	100	1541284	90.67	1462798	86.05	1326079	78.01
Levog	279977	76.97	363725	100	309420	85.06	334328	91.91	342202	94.08
Trehalose	51489	87.65	58741.1	100	49625	84.48	36226	61.67	45263	77.05

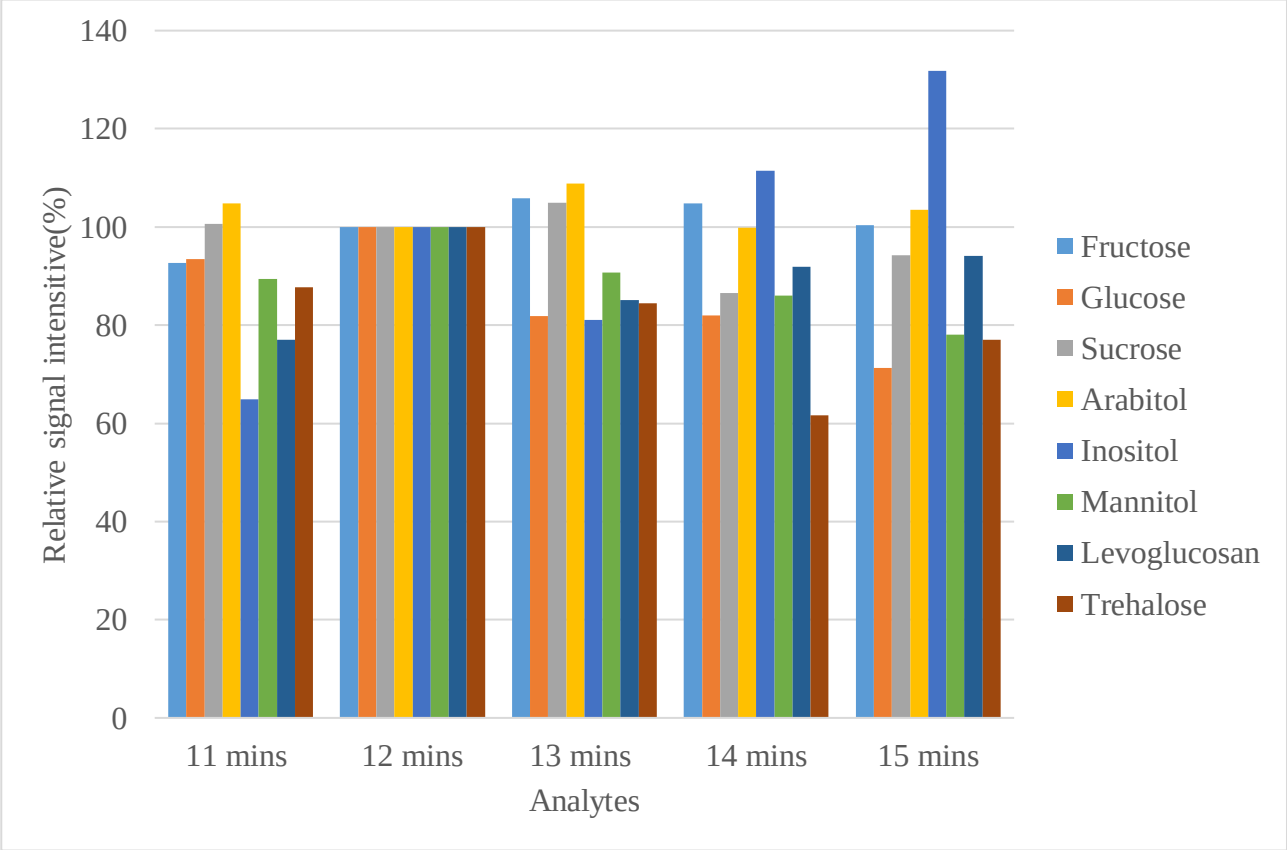

Figure 9. Relative signal intensity by different condition time, 12 min has optimized condition time for HILIC column.

12.5. Optimization of the injection volume for the high-performance liquid chromatography

The injection volume was another parameter needed to be optimized. 3 µL, 4 µL, and 5 µL injection volume of the standard analytes were tested. In this session, parameters of the optimization procedures are shown below: 20 mM ammonium acetate worked as mobile phase A and acetonitrile worked as mobile phase B. The concentration of standard was 0.125 mg/L. The flow rate was 0.45 mL/min. The condition time of HILIC column was 12 minutes. The most optimized injection volume is shown in Table 10 and Figure 10. The optimized result was 5 µL. Compared with 3 µL and 5 µL, the relative signal intensity of glucose was 1.83 times higher. When the injection volume was 5 µL, the signal of each analyte still had a good shape and there was no tailing or over injection. Hence, the 5µL was optimized injection volume.

Table 10. Relative signal intensity by injection volume.

	3 µL		4 µL		5 µL	
Analytes	Area	Ratio (%)	Area	Ratio (%)	Area	Ratio (%)
Fructose	6553698	74.42	7549540	85.73	8806485	100
Glucose	2486511	54.77	4134552	91.06	4540223	100
Sucrose	3099337	90.68	3388565	99.14	3417919	100
Arabitol	5587355	95.02	5779648	98.29	5880277	100
Inositol	1309965	76.91	1471494	86.39	1703268	100
Mannitol	5650061	78.63	7327648	101.98	7185390	100
Levoglucosan	1750701	64.54	2379099	87.71	2712428	100
Trehalose	85885	69.22	136488	110.00	124077	100

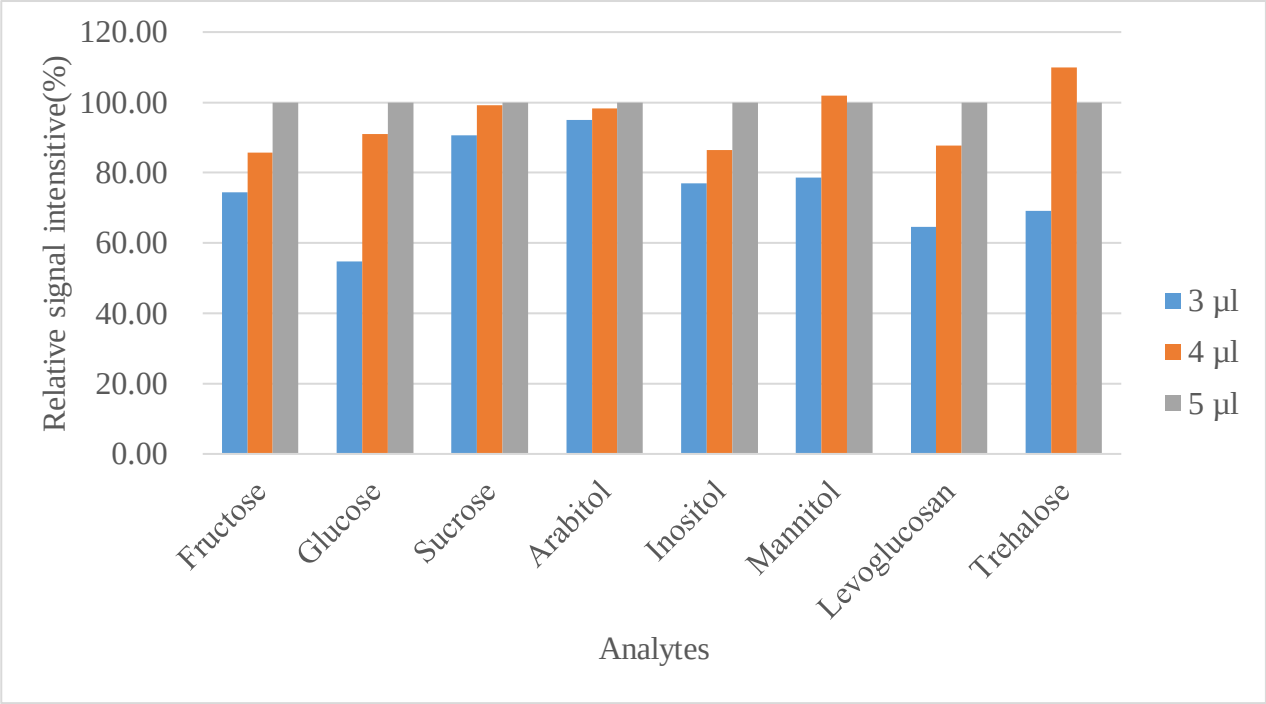

Figure 10. Relative signal intensity by injection volume. 5 µL has optimized signal intensity.

12.6. Run the standard in the optimized condition

All the optimized conditions are shown below. 20 mM Ammonium acetate worked as mobile phase A and HPLC grade acetonitrile work as mobile phase B. The standard injection volume was 5 µL, and the flow rate from bi-pump was 0.45 mL/min. The conditioning time for HILIC column was 12 minutes. In addition, the parameters of the eluting gradient are referred to Table 5. The ionization parameters are referred to Table 6. In Figure 11, there was a doublet peak (peak6), which was the signal of the glucose. The possible reason for this doublet peak was the decay of the glucose or contamination in the original standard chemical. Furthermore, in Figure 11 there was a small peak (peak 1), which was the signal of trehalose. Although the signal intensity of trehalose was increasing as the concentration of trehalose was increased, the peak shape was not qualified and the signal intensity did not reach the limit of quantitation. The resulting peak of each analyte is shown in Figure 12.

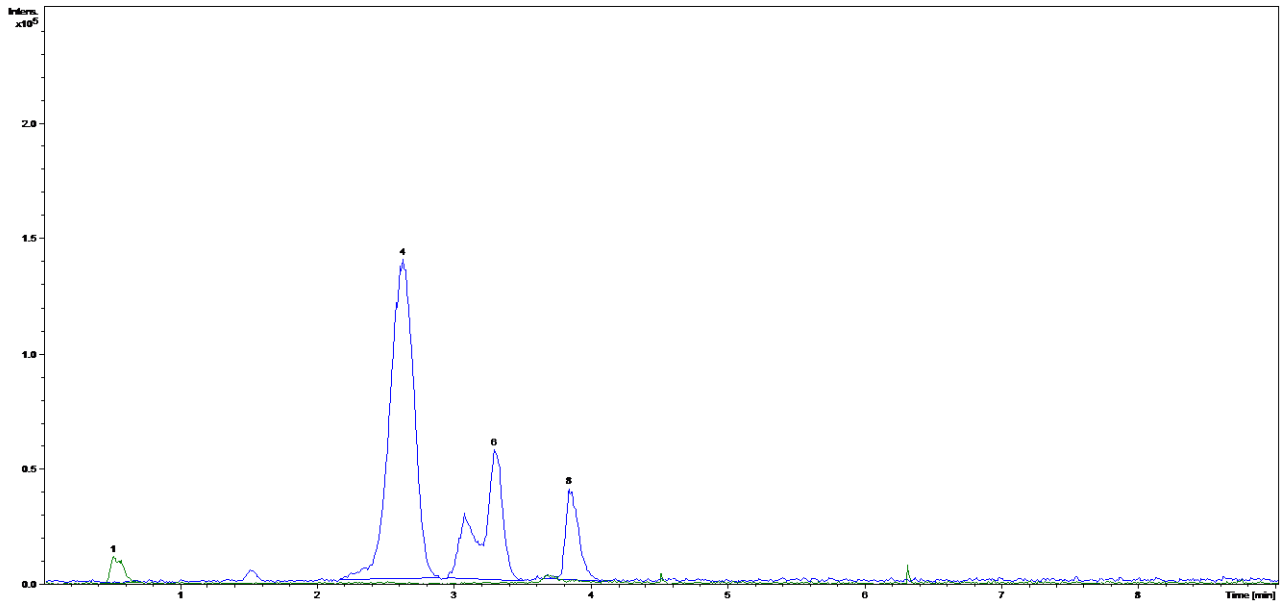

Figure 11. Chromatogram of 5 mg/L standard analytes injection.1.trehalose, 4.fructose, 6. Glucose, 8.inositol.(The range of chromatogram retention time: 9 min, signal intensity: 250000.)

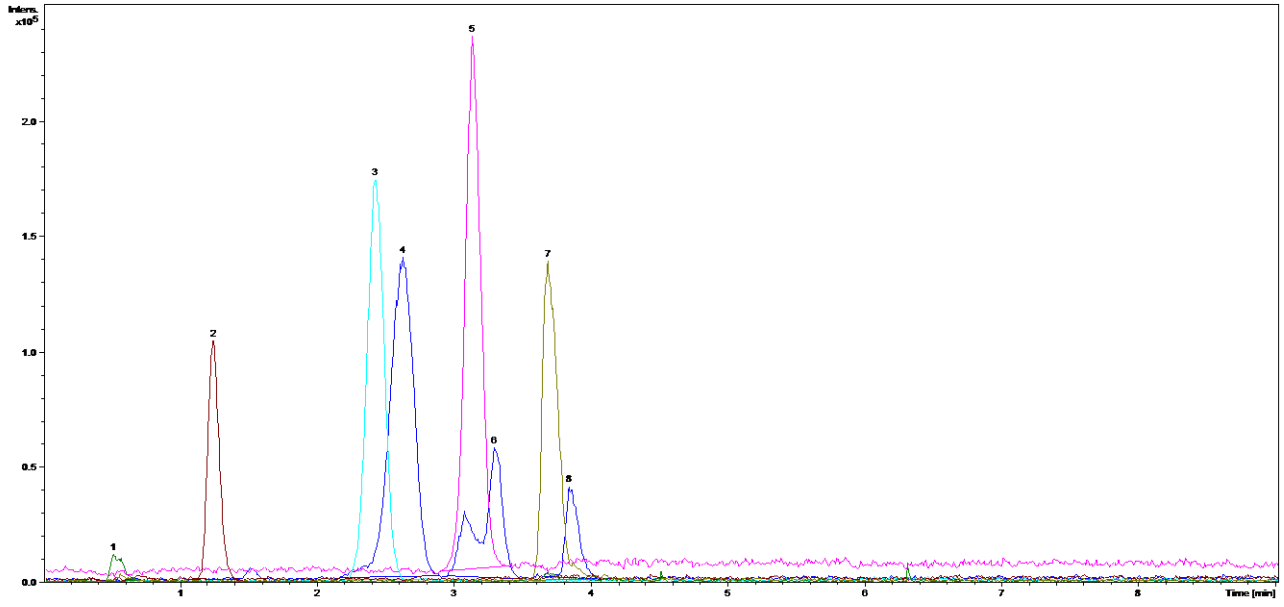

Figure 12. Chromatogram of 5 mg/L standard analytes injection.1.trehalose, 2.levoglucosan, 3.arabitol, 5.mannitol, 6.glucose, 7.sucrose, 8.inositol. (The range of chromatogram retention time: 9 min, signal intensity: 250000.)

Table 11 shows the calibration curves, R square and the limit of quantification for each analyte. However, the signals of the trehalose did not reach the limit of quantification. In addition, when the pre-filter was cascaded with the HPLC system the signal of trehalose could not be seen.

Table11. Calibration curves, R square and the limit of quantification for each analyte.

Sugar	Calibrationcurve	R square	LOQ (mg/L)
Fructose	y = 134708x + 650495	0.881	0.25-25
Glucose	y = 347724x + 488305	0.978	0.25-10
Sucrose	y = 4E+06x + 236081	0.997	0.1-1
Arabitol	y = 532144x + 278687	0.984	0.1-5
Inositol	y = 271178x + 384255	0.97	0.25-10
Mannitol	y = 192753x + 65304	0.99	0.1-5
Levoglucosan	y = 348327x + 108106	0.989	0.1-5
Trehalose	-	-	-

12.7. Optimized the extraction solution, and remove the interference

In order to remove the grease and the interference on the filter sample, the extraction solution required optimization. Four different groups of extraction solution were examined. The first solution was milli-Q water (1 mL). The second solution was a mixture of milli-Q water (500 μL) and toluene (500 μL). The third solution was deionized water with 0.1% formic acid (FA) (1 mL). The fourth solution was a mixture of deionized water with 0.1% formic acid (500 μL) and toluene (500 μL). Table 12 and Figure 13 show the optimization results. Not all the analytes were detected from the old filter sample (from 2003), and the strong interference effect existed. Fructose, glucose, inositol, and arabitol could still be optimized by this method. Hence, the third extraction solution deionized water with 0.1% of formic acid (1 mL) which had the optimized result. Figure 14 showed the interference of the mannitol. Furthermore, the overloading happened commonly for the mannitol. Figure 15 and Figure 16 show the interference of the levoglucosan and trehalose due to the strongest interference signal from 0 min to 2 min which were the same retention as levoglucosan and trehalose. As a result, the levoglucosan and trehalose could not be detected. Therefore, the dichloromethane was introduced in order to remove the medium polar interference. 200 μL dichloromethane and 400μLmilli-Q water (MQ) were added in the extracted solution. Nevertheless, dichloromethane was not effective in removing interferences. Hence, only deionized water with 0.1% of formic acid (1 mL) was chosen as the

extraction solution. Figure17 shows the chromatogram of the analysis by the deionized water with 0.1% of formic acid (1 mL).

Table 12. Relative signal intensity by different extraction solution.

	Mili-Q water (MQ)		MQ+Toluene		MQ+FA		MQ+Toluene+FA	
Analytes	Area	Ratio (%)	Area	Ratio (%)	Area	Ratio (%)	Area	Ratio (%)
Fructose	58813	45.92	55803	43.57	128071	100	58231	45.47
Glucose	250690	78.68	245349	77.00	318634	100	193194	60.63
Inositol	42455	94.13	53504	118.62	45104	100	45928	101.83
Arabitol	57608	73.60	17867	22.83	78277	100	42009	53.67

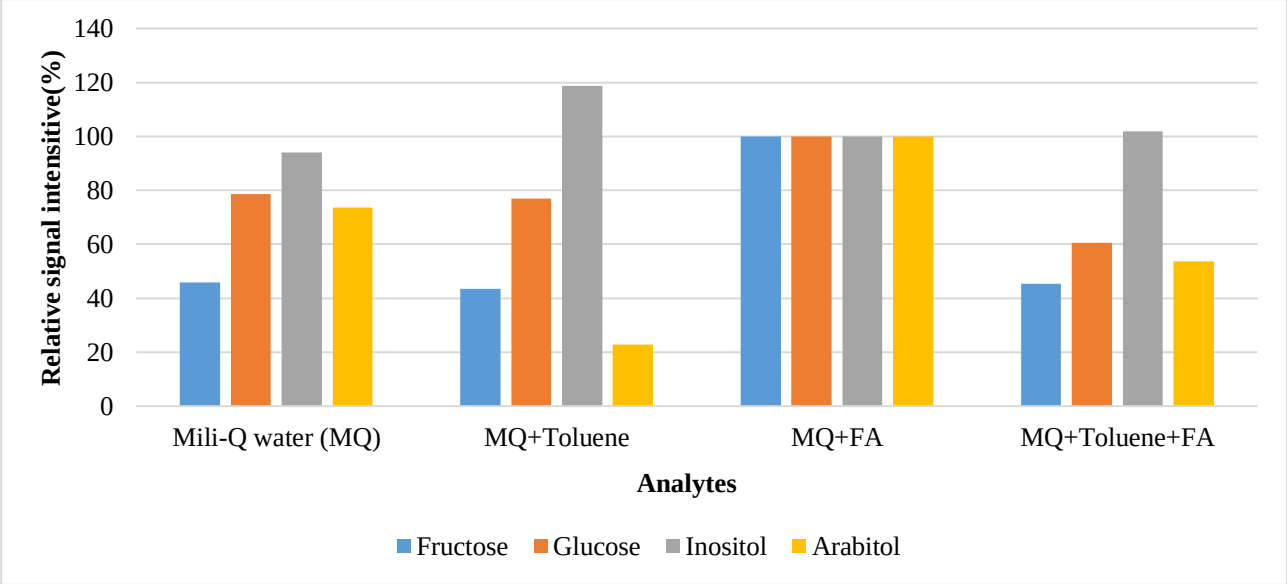

Figure 13. Relative signal intensity by different extraction solution.

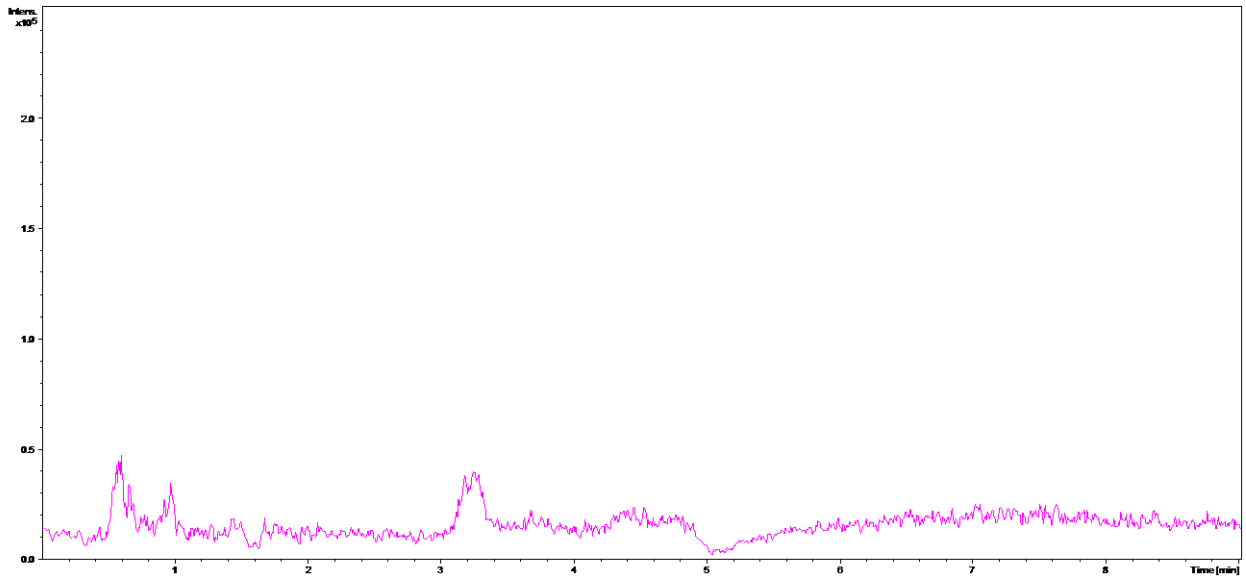

Figure 14. Chromatogram of mannitol, extraction from deionized water with 0.1% formic acid (1 mL), filter from 2003. (The range of chromatogram retention time: 9 min, signal intensity: 250000).

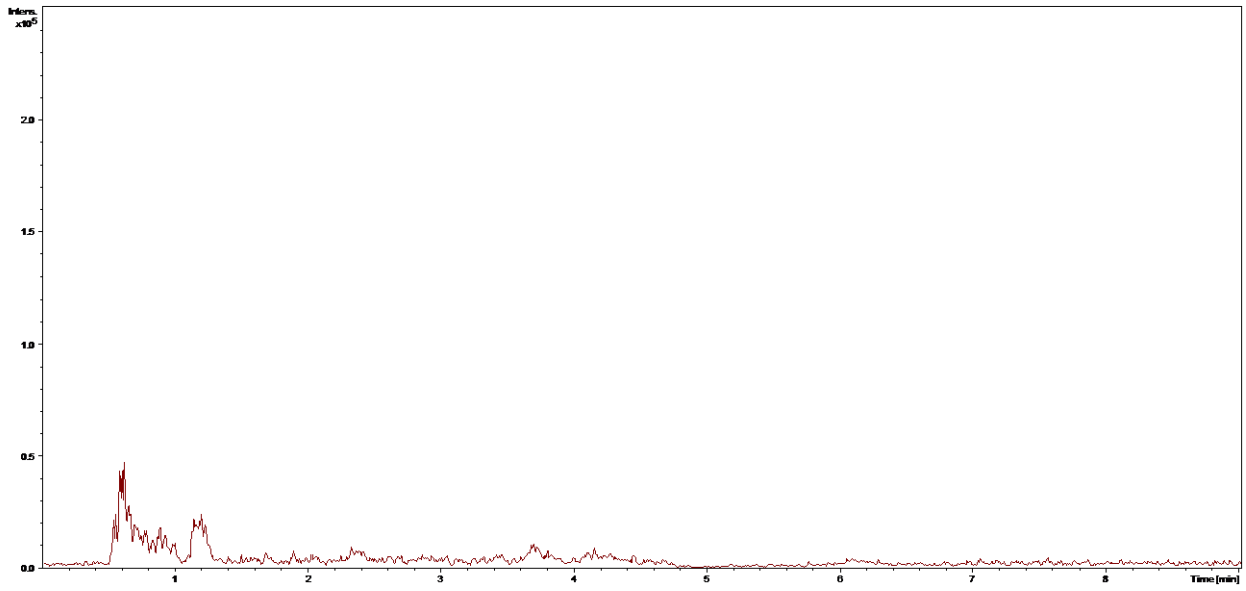

Figure 15. Chromatogram of levoglucosan, extraction from deionized water with 0.1% formic acid (1 mL), filter from 2003, (The range of chromatogram retention time: 9 min, signal intensity: 250000).

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Figure 16. Chromatogram of trehalose,extraction from deionized water with 0.1% formic acid (1 mL), filter from 2003, (The range of chromatogram retention time: 9 min, signal intensity: 250000).

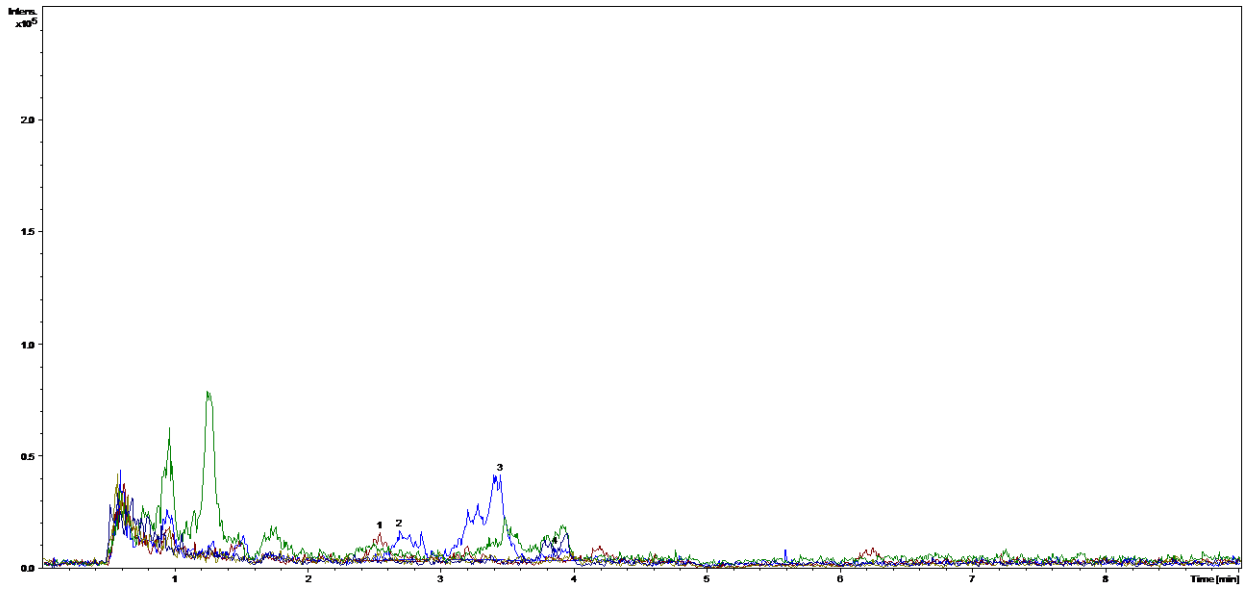

Figure 17. Extraction from deionized water with 0.1% formic acid (1 mL), filter from 2003, 1. arabitol, 2.fructose, 3.glucose, 4.Inositol. (The range of chromatogram retention time: 9 min, signal intensity: 250000).

12.8. Comparison of the filter samples from different years

In this session, two different batches of sample filters were examined. The first batch was collected in 2003, and another was collected in 2016. Table 13 and Figure 18 show the result. The filter from

2016 had the relatively optimized result. Due to the fact that analyte concentrations in the aerosol particles at different times are different. Figure 19 and Figure 20 show the chromatogram of the sample filter from 2003 and the sample filter from 2016. The signals of analyte form 2003 were difficult to detect. Although fructose and sucrose could be detected, the signals of other analytes did not reach the limit of detection, and the strong interference signals existed. However, the signals of analytes from filter 2016were obvious. Fructose, glucose, arabitol, and sucrose were easy to detect in filter 2016, especially the arabitol signal intensity of the new filter was a factor of 3.84 higher than the old filter. Although inositol and levoglucosan could be detected in filter 2016, interference still affected the signals. As a result, the aerosol sample collected from 2016 was chosen for the later experiment.

Table 13. Relative signal intensity by sample from different years.

year	2003		2016	
Analytes	Area	Ratio (%)	Area	Ratio (%)
Fructose	128071	37.48	341697	100
Glucose	318634	79.76	399515	100
Sucrose	45104	47.91	94137	100
Arabitol	78277	26.01	300922	100
Inositol	None	None	987117	100
Mannitol	None	None	None	None
Levoglucosan	None	None	None	None
Trehalose	None	None	None	None

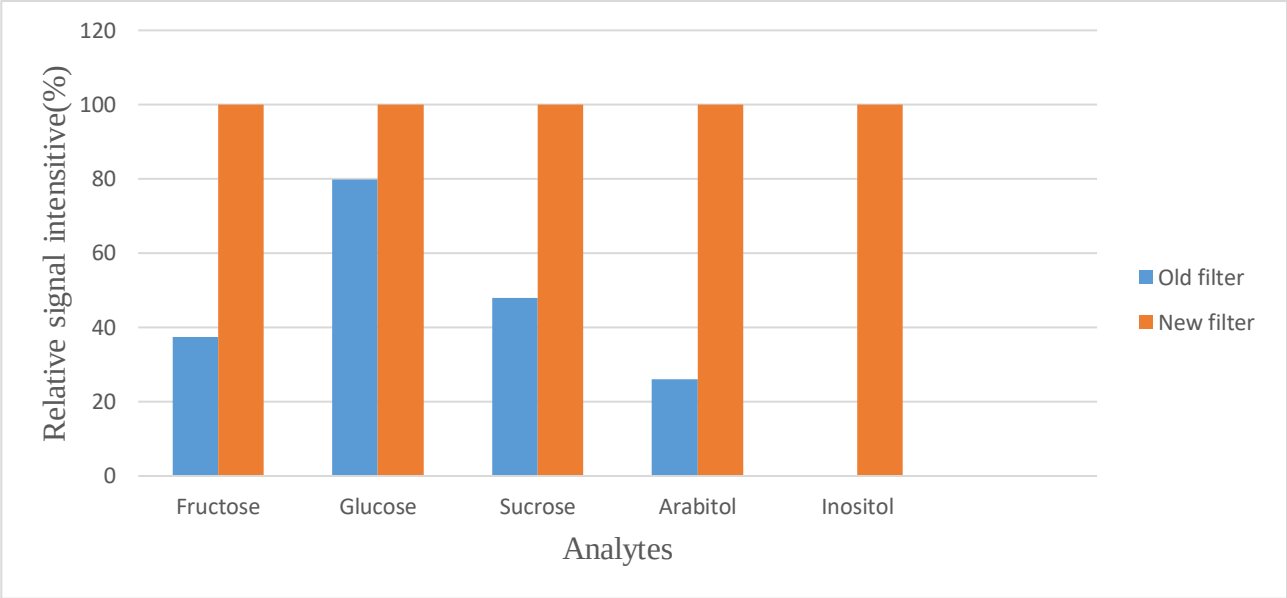

Figure 18. Relative signal intensity by sample from different year.

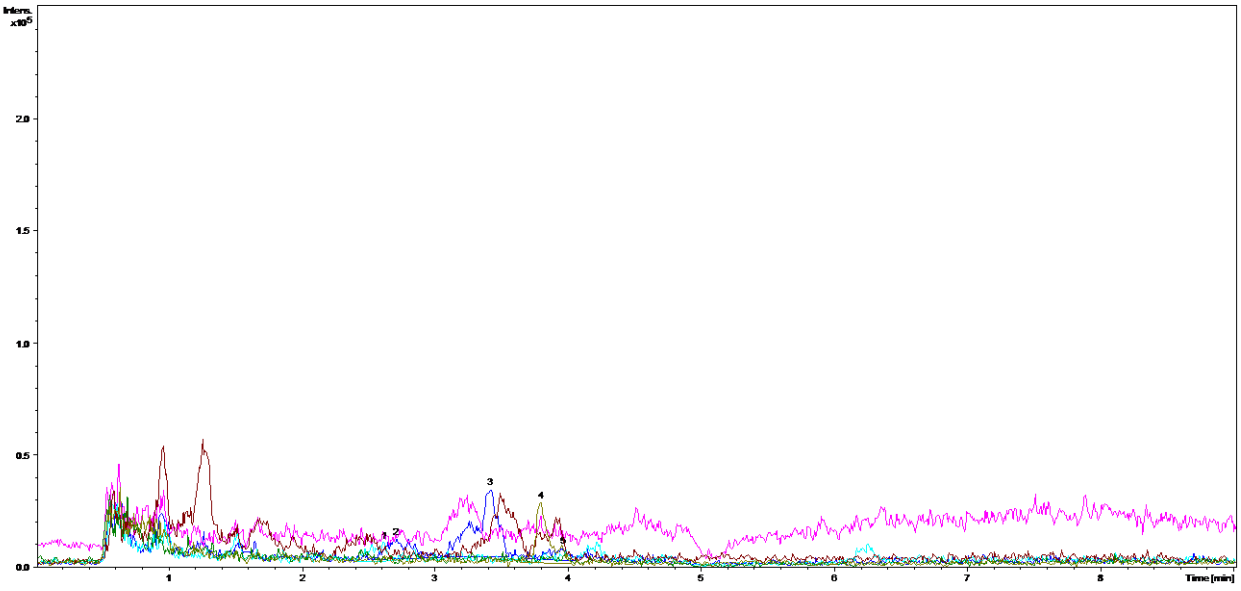

Figure 19. Filter from 2003. 1. arabitol, 2.fructose, 3.glucose, 4.Inositol. (The range of chromatogram retention time: 9 min, signal intensity: 250000).

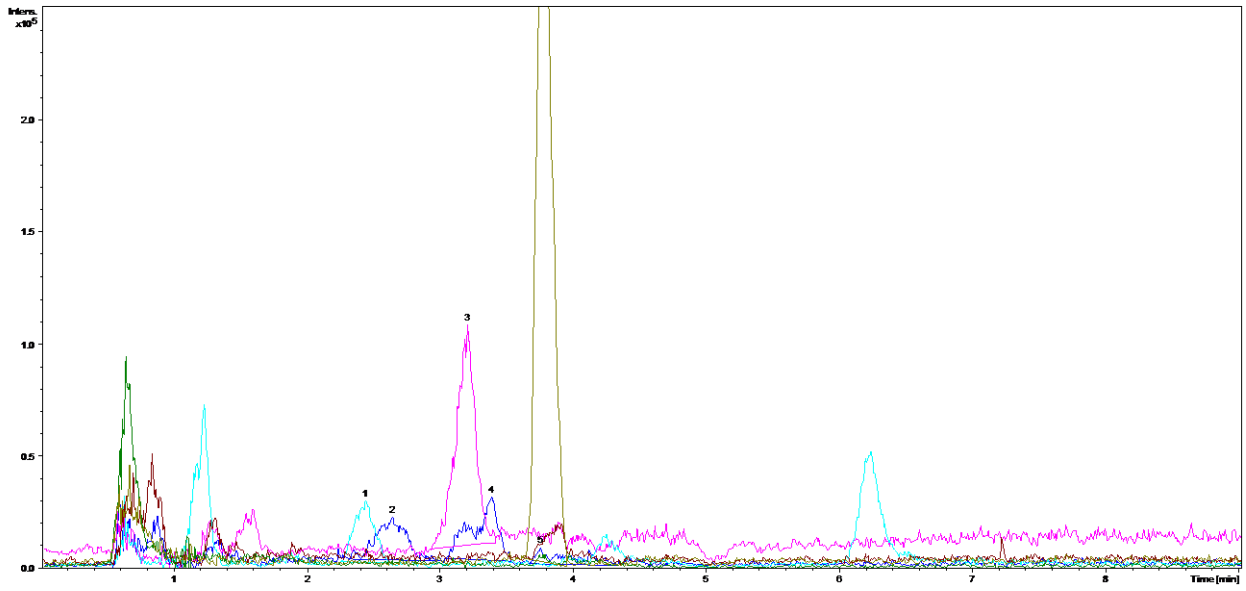

Figure 20. Filter from 2016. 1. arabitol, 2.fructose, 3. mannitol, 4. glucose, and the highest peak is sucrose. (The range of chromatogram retention time: 9 min, signal intensity: 250000).

12.9. Compare the daytime and nighttime sample filter

In order to find the differences between the daytime sample and the nighttime sample, the daytime filter collected on the 11th of May, 2016, and the nighttime filter collected on the 21st of June, 2016 which were chosen for the optimization. Table 14 and Figure 21 show the relative signal intensity of analytes from the daytime filter and nighttime filter. In this session, the daytime filter had the relatively good result. The daytime filter signal of fructose was a factor of 4 higher than the nighttime filter. The signal of sucrose from the daytime filter was 8.8 times higher than the nighttime filter. Thus, the daytime sample filter was chosen for the later experiment. The other analytes cannot see in the chromatogram due to the fact that there were no sugars on the filter and/or the interference effect.

Table 14. Relative signal intensity by diel filter.

	Day		Night	
Analytes	Area	Ratio (%)	Area	Ratio (%)
Arabitol	306042	100.00	191988	62.73
Fructose	264661	100.00	66116	24.98
Glucose	395420	100.00	139703	35.33
Sucrose	2597776	100.00	293667	11.30

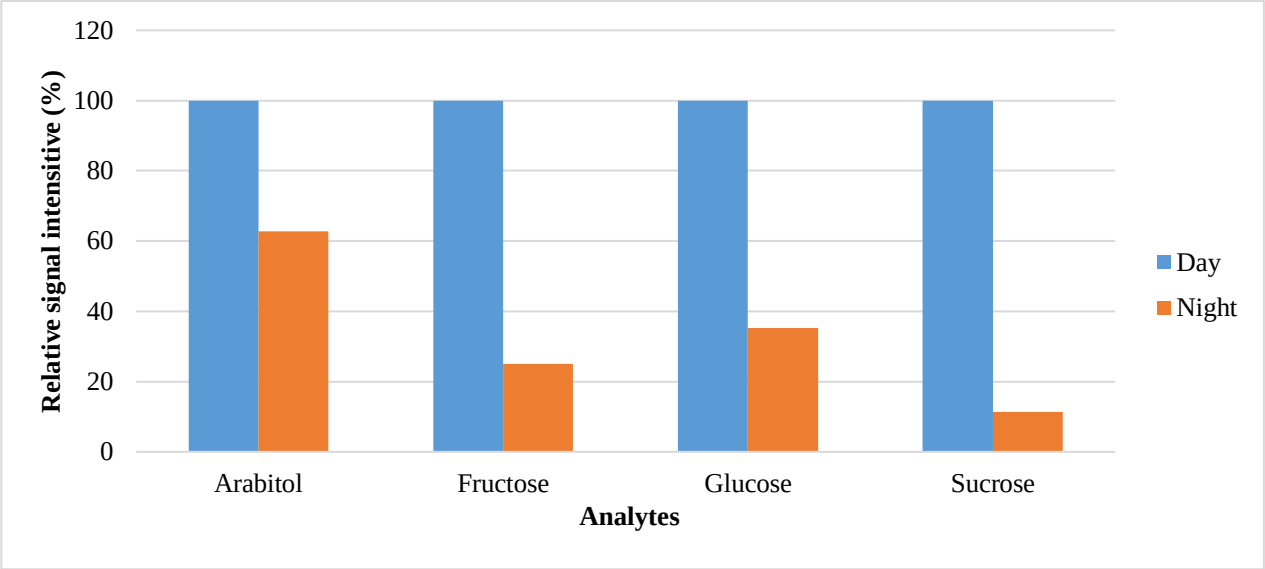

Figure 21. Relative signal intensity by diel filter.

12.10. Compare the daytime sample filter with and without solid phase extraction

Although the signals of analytes from filter 2016 were better, the matrix effect still existed. The nonpolar interference affected the analytes strongly, especially the analytes retention time between 0 to 3 minutes. In order to remove the interference, the solid phase extraction (SPE) was introduced. In this session, the sample filter from the 18th and 19th of June, 2016 was chosen for the experiment. The result is shown in Table 15 and Figure 22. The chromatograms with SPE without SPE are shown in Figure 23 and Figure 24. The sample filter with SPE procedure had optimized result. Without SPE, levoglucosan and trehalose could not be detected due to the interference effect. The retention time of the levoglucosan and trehalose overlapped with the interference signal. Besides, without SPE the signal of sucrose did not reach the limit of quantification, and the signal of arabitol was really small. When the SPE was introduced, comprehensive signal of analytes were improved significantly. The signal of arabitol with SPE was a factor of 8 higher than the signal of arabitol without SPE. Due to the matrix effect was solved, the average of the signal with SPE was a factor of 2.43 times bigger than the signal without SPE. Therefore, the SPE procedure was chosen for the later experiments.

Table 15. Relative signal intensity by SPE and without SPE.

	Without SPE		With SPE	
Analytes	Area	Ratio (%)	Area	Ratio (%)
Fructose	341697	49.97	683817	100
Glucose	399515	57.80	691161	100
Inositol	94137	43.02	218833	100
Arabitol	300922	13.72	2193106	100
Mannitol	987117	45.01	2193106	100
Levoglucosan	None	None	207229	100
Trehalose	None	None	None	None
Sucrose	None	None	1573173	100

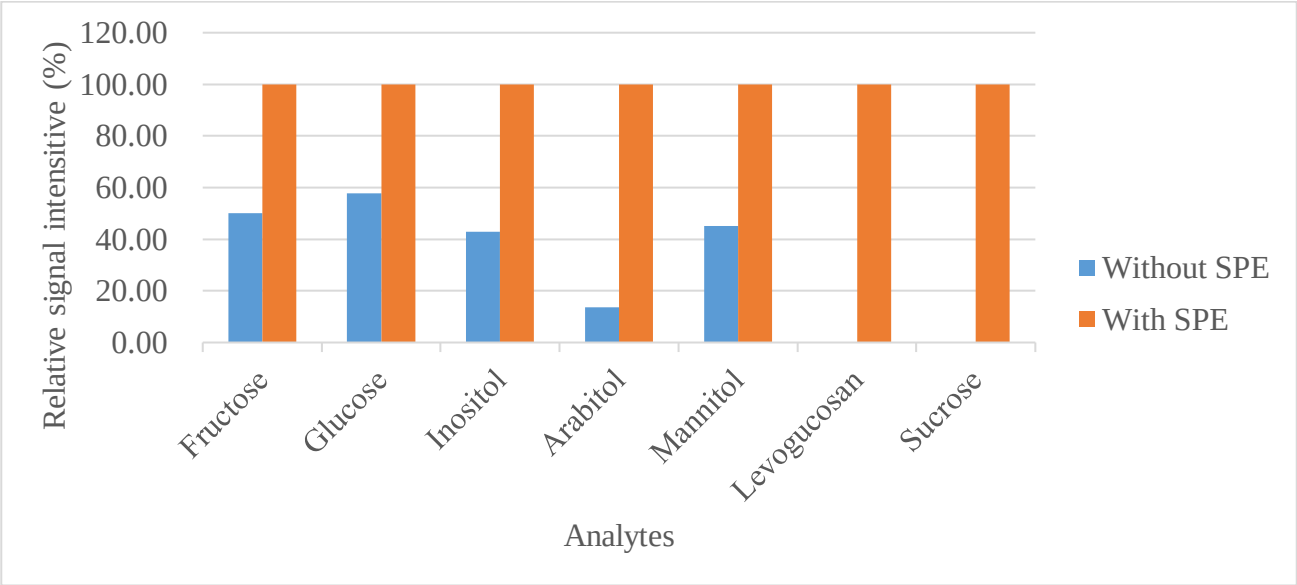

Figure 22. Relative signal intensity by SPE and without SPE.

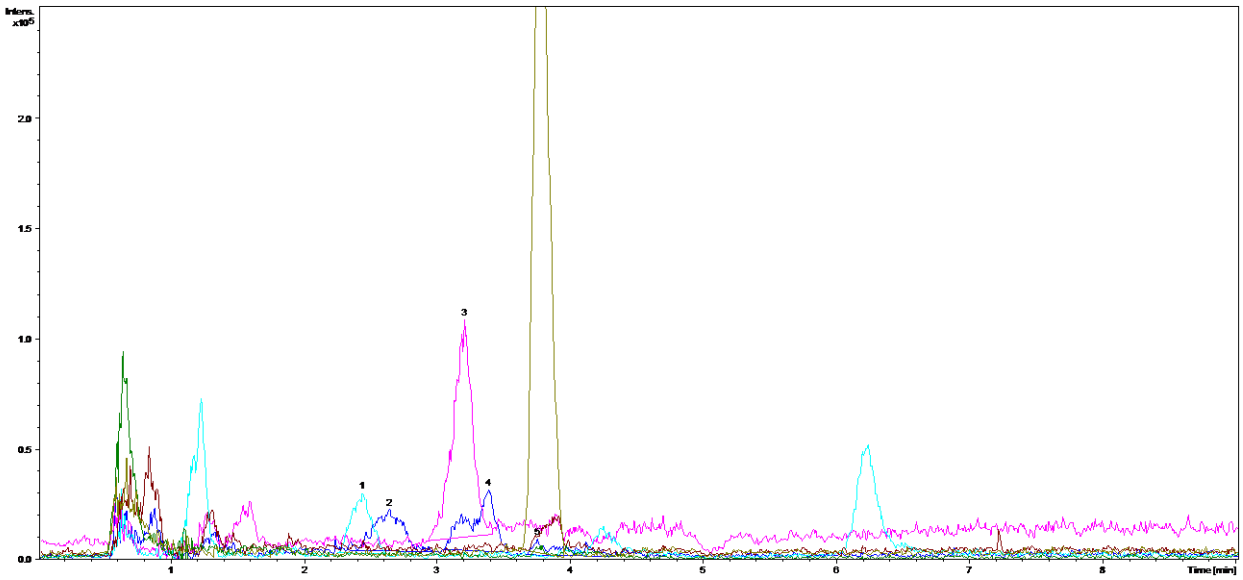

Figure 23. Filter from 18th/June/2016. 1.arabitol, 2.fructose, 3.mannitol, 4.glucose, and the highest peak is sucrose (The range of chromatogram retention time: 9 min, signal intensity: 250000).

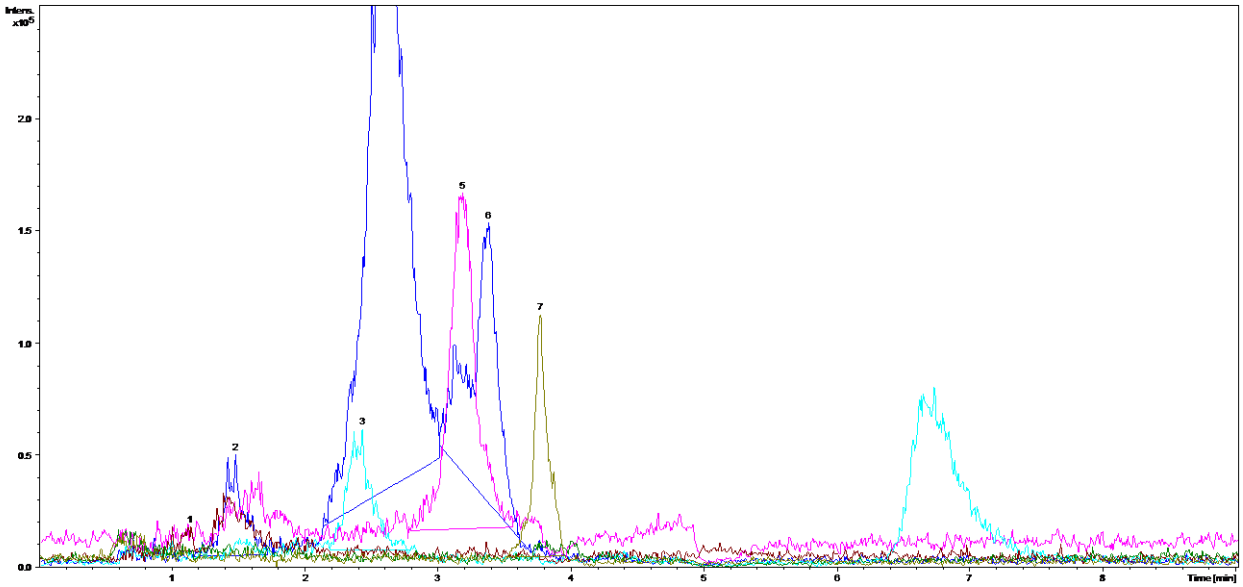

Figure 24. Filter from 19th/June/2016. 1.levoglucosan, 2.fructose, 3.arabitol, 5.mannitol, 6.inositol, 7.Sucrose, and the highest peak is glucose(The range of chromatogram retention time: 9 min, signal intensity: 250000).

12.11. Optimization eluent volume of the SPE

The fraction of the SPE was important. In order to make sure that all analytes were extracted by eluting from 4 mL extracted solution directly, 4 different fractions were introduced. The first fraction

was 4 mL direct elute which from the extracted solution. The second fraction was 1 mL deionized water. The third fraction was another 1 mL of deionized water. The fourth fraction was 3 mL methanol. Table 16 shows the result. In the first fraction, almost all the analytes were eluted out from the SPE cartridge. However, there still were some analytes in this fraction such as 23 % of sucrose and 5 % of arabitol in the SPE cartridge. In the fraction 3 showed that there were no analytes in this fraction. Infraction 4, although there were some signals, none of them were analytes, due to the fact the retention time did not match with the sugars and sugar alcohol. Most of the signal in the fraction 4 were interference because deionized water eluted out the relative polar analytes, and the non-polar interferences were eluted out by methanol. Hence, the SPE procedure was optimized by the original extracted solution with 1 mL of deionized water.

Table 16. Signal intensity of analytes by the fraction of SPE.

Analytes	4 mL direct elute	1 mL deionized water	1 mL deionized water	3 mL methanol
Fructose	81454	none	none	none
Glucose	337242	none	none	none
Sucrose	835895	195594 (23%)	none	none
Arabitol	219166	11627 (4%)	none	none
Inositol	347793	none	none	none
Mannitol	577063	none	none	none
Levoglucosan	none	none	none	none
Trehalose	none	none	none	none

12.12. Optimization of redissolving solution volume

The volume of redissolving solution needed to be optimized, as the solubility of the analytes affected the signal. Besides, by using redissolving procedure, the filter sample was easier for quantitation. Hence, the volume of redissolving solution needed to be optimized. Firstly, after SPE extracted solution was dried under the pressured gas concentrator (50 °C, 4 hours). Then, the dried analyte was redissolved in deionized water. Table 17 and Figure 25 show the results. 200 μL redissolving solution had the relative optimized result. Although there were no significant differences between 100 μL and

200 µL redissolving solutions, 200 µL was chosen as the optimized condition because 200 µL was relatively easier to conduct.

Table 17. Relative signal intensity by different redissolving solution volumes.

	200 µl		100 µl	
Analytes	Area	Ratio (%)	Area	Ratio (%)
Fructose	186754	100	218687	117.10
Glucose	559967	100	404486	72.23
Inositol	534108	100	439444	82.28
Arabitol	648002	100	596851	92.11

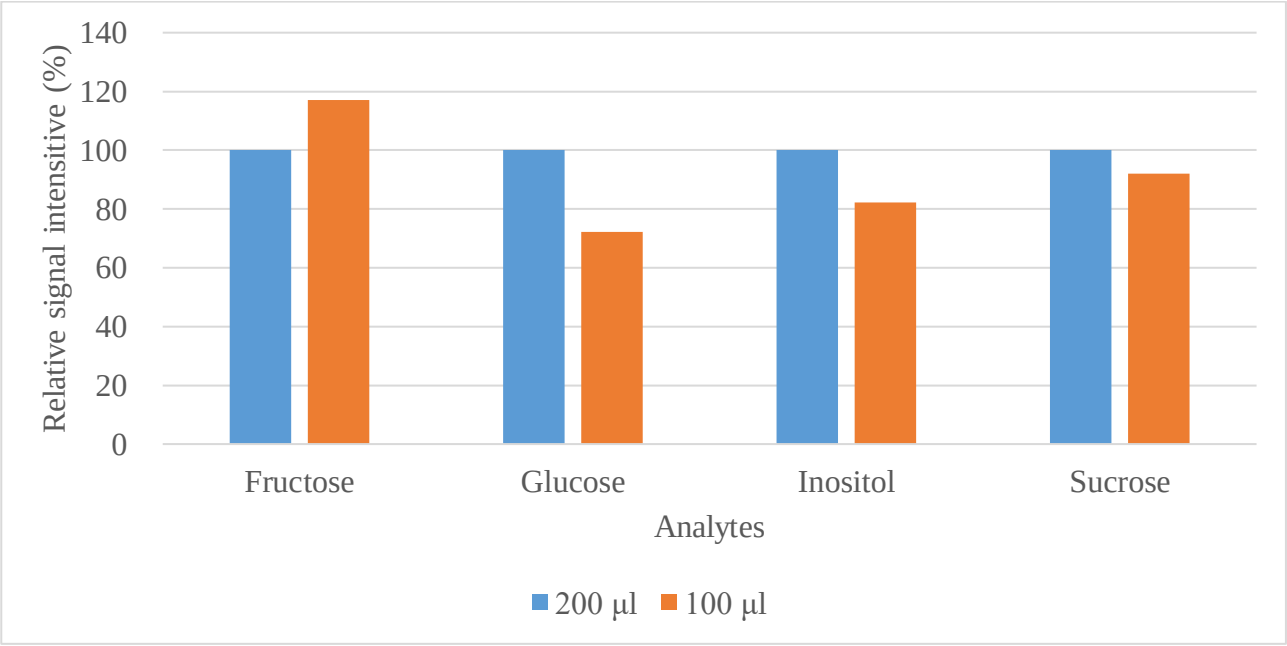

Figure 25. Relative signal intensity by different redissolving solution volumes.

12.13. Recovery of sample

In order to verify the methodology, the recovery testing was introduced. The blank sample filter from the 3rd of May, 2015 was chosen as the blank sample, and the 100 mg/L 10 µL standard mixture was added into the blank sample filter. Then, the optimized method was applied as discussed above. Optimized parameters: 20 mM Ammonium acetate worked as mobile phase A and HPLC grade

acetonitrile worked as mobile phase B. The standard injection volume was 5 µL and the concentration of standard was 5 mg/L. The flow rate from bi-pump was 0.45 mL/min, and the condition time for HILIC column was 12 minutes. Daytime filter sample was chosen. SPE procedure conducted by the original extracted solution plus 1 mL of deionized water and redissolving solution was 200 µL. Table 18 shows the recovery of each analyte. The sucrose had the better recovery which reached 67.97 % and levoglucosan had the leastrecovery which only reached 29.09 %. Although the optimization procedures had done before, the recovery of each analyte was not satisfied. Hence, the recovery needed to be considered for the real sample analysis and deducted the contamination from the blank filter sample. The chromatogram of blank filter and spike filter are shown in Figure 26 and Figure 27.

Table 18. Revovery of the analytes.

Analyst	Signal intensity of spike filter	Signal intensity of standard 5 mg/L	Recovery(%)
Fructose	3827690	7460006	51.31
Glucose	3431006	7071391	48.52
Sucrose	4160122	6120765	67.97
Arabitol	5374991	12872804	41.75
Inositol	2859172	6048722	47.27
Mannitol	8504148	17308599	49.13
Levoglucosan	1398984	4809032	29.09

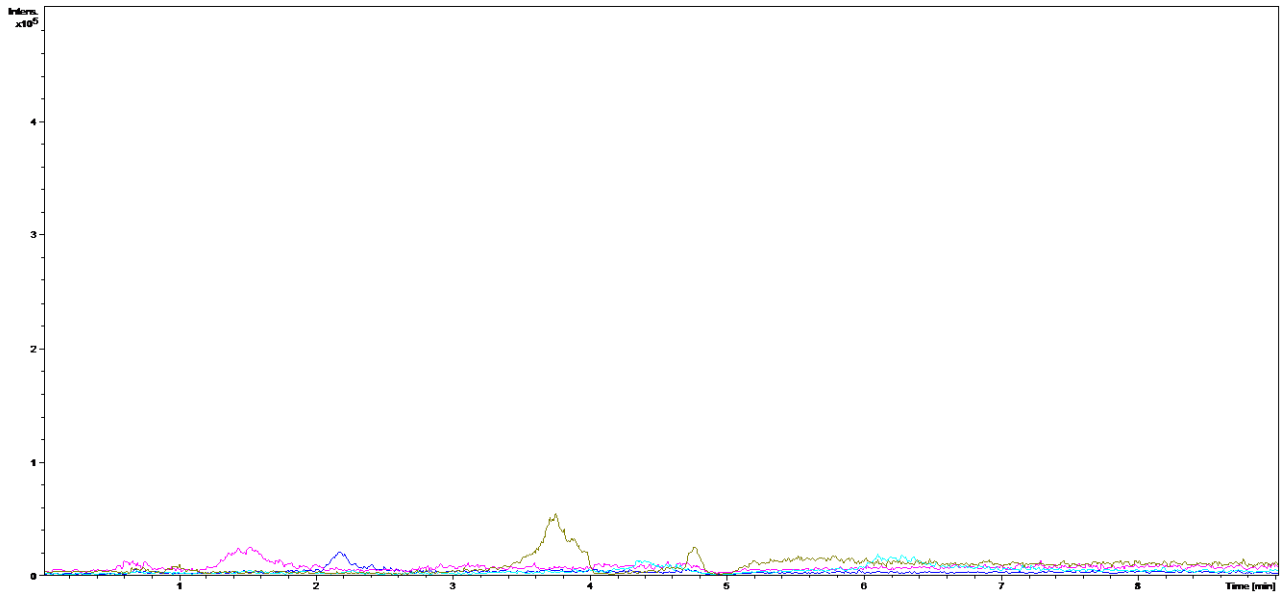

Figure 26. 15th/May/2016 Blank filter. (The range of chromatogram retention time: 9 min, signal intensity: 500000.)

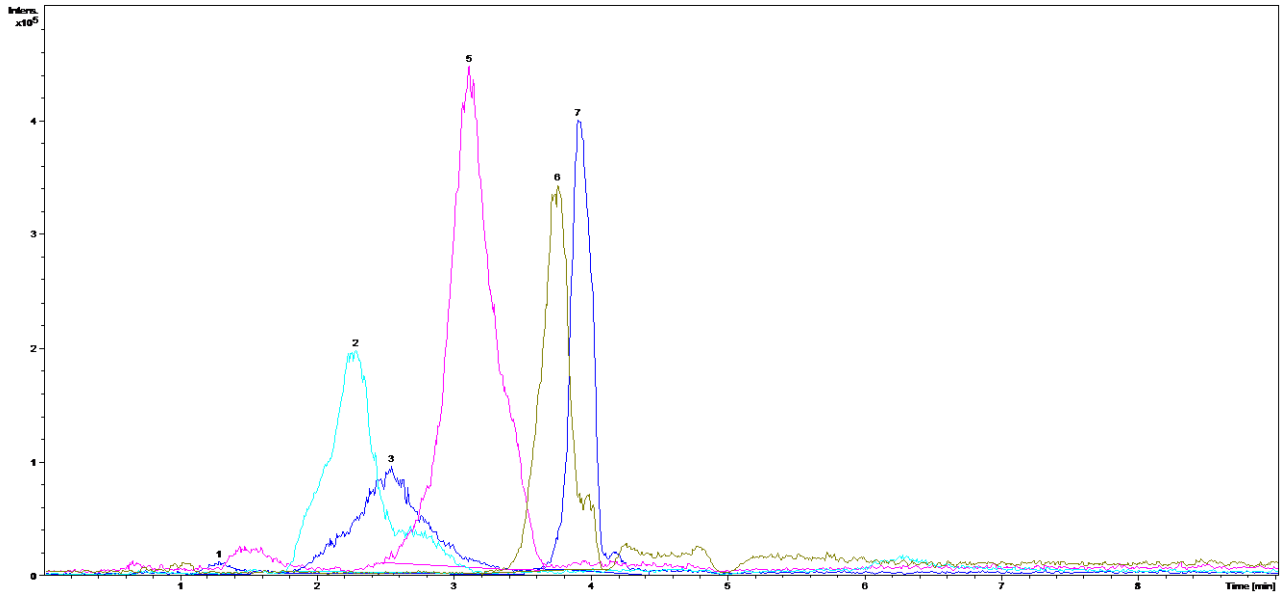

Figure 27. 15th/May/2016 Spike filter.1. fructose, 2. arabitol, 3. glucose, 5. mannitol, 6. sucrose, 7. inositol. (The range of chromatogram retention time: 9 min, signal intensity: 500000.)

12.14. Analyses of real samples

In the end, the daytime sample filter from May/2015 3rd to 29thwere chosen for the experiment. During the detection, the blank filter from May/2015 was inserted, in order to make sure there was no contamination during the procedure. The analytical procedure was followed by the optimized condition which discussed above already. Optimized parameters: 20 mM Ammonium acetate worked as mobile phase A and HPLC grade acetonitrile worked as mobile phase B. The standard injection volume was 5 µL and the flow rate from bi-pump was 0.45 mL/min. The conditioning time for HILIC column was 12 minutes. Daytime filter sample was chosen, SPE procedure conducted by the original extracted solution plus 1 mL of deionized water, and redissolving solution was 200 µL deionized water. Table 19 showed the result of each analyte at the different date.

Table 19. The concentration of analytes from sample filters May/2015.

Date	Average temperature (°C)	Fructose (µg m^{-3})	Glucose (µg m^{-3})	Inositol (µg m^{-3})	Mannitol (µg m^{-3})	Arabitol (µg m^{-3})	Sucrose (µg m^{-3})
3rd	10.50	3.83	63.32	11.21	12.86	3.28	19.01
4th	10.50	4.92	7.66	ND	0.55	ND	3.97
5th	9.50	4.65	18.60	2.46	8.48	4.10	21.20

6th	12.50	6.56	50.47	3.83	13.68	4.38	141.96
7th	12.50	6.29	46.64	3.15	16.82	4.24	116.52
8th	9.00	8.75	148.25	4.38	25.44	6.70	143.06
10th	13.00	ND[a]	83.84	ND	7.93	2.87	110.78
11th	11.50	ND	ND	ND	6.56	2.05	132.25
12th	13.00	ND	214.58	5.20	0.55	1.37	ND
13th	9.00	5.74	41.17	3.56	9.44	2.46	129.52
14th	13.00	7.52	133.07	2.32	15.32	4.65	146.47
15th	8.50	11.90	165.90	8.07	15.18	3.97	39.80
16th	11.50	9.16	221.42	3.56	31.18	6.70	92.18
17th	12.00	8.62	239.34	4.65	29.54	7.39	167.95
18th	10.00	8.48	204.74	5.33	35.70	7.39	153.59
19th	15.00	7.52	196.26	5.20	27.63	5.20	126.37
20th	14.50	9.44	199.68	4.79	21.47	4.51	116.39
22nd	16.50	6.02	113.92	3.42	9.16	2.60	103.26
23rd	12.50	4.65	75.22	3.15	7.11	2.32	54.16
24th	13.00	4.10	104.76	2.60	9.57	2.05	91.63
25th	13.00	5.47	198.58	2.87	3.69	1.91	170.00
26th	17.00	ND	31.32	24.48	4.24	1.64	138.27
27th	17.00	ND	20.24	14.50	3.15	1.78	110.51
28th	17.00	7.80	222.79	2.32	7.11	2.05	247.13
29th	14.00	8.62	188.46	2.19	4.38	1.64	239.75

[a]: not detected

13. Discussion

Table 19 shows the result of the analytes from May, 2015 filters. Fructose, glucose, mannitol, and arabitol from the 14th to 20th had relatively high concentrations. However, glucose and sucrose did not follow the same pattern; the concentrations of glucose and sucrose fluctuated over time. Interestingly, although fructose, glucose, mannitol, and arabitol had the relatively high concentrations from May/2015 14th to 20th, their concentrations were independent from the temperature. Furthermore, during the detection of the analysis, the pressure of HILIC column was not stable, possibly due to particles and impurities present in the analytes even after the sample preparation step using filters and SPE. In addition, the column exhibited high pressure and was required to be cleaned constantly. This could lead to sensitive changing and fluctuations of measured concentrations. In addition, the impurities might damage the HILIC column. Hence the sensitivity was not so stable, and the retention

time was not very repeatable. Although the methodology was established and optimized, there are still opportunities for further improvements for some factors, such as the addition of the internal standard to the whole system. The internal standard addition may increase the reliability of the results despite the fluctuation of the sensitivity and the high pressure, and the results can be more quantitative. Additionally, in order to remove the interferences and particles from the samples, centrifugation can be introduced after extraction from SPE. In addition, the recovery of the extraction process is not efficient. The average recovery of analyte was only 47.81%. Different extraction processed can still be introduced and discussed.

14. Conclusion

Sugar and Sugar alcohol are indicative compounds in the environmental aerosol. The concentrations of sugar and sugar alcohol reveal biogenic and anthropogenic information. Levoglucosan is referred to wood consumption. Arabitol and mannitol work as a good tracer for the fungal spores. Fructose, glucose, and sucrose are correlated with the activity of the vegetation. Therefore, analysis of sugar and sugar alcohol in the environmental aerosol are really important.

According to research, sugars and sugar alcohols were analyzed successfully from atmospheric aerosol samples by HPLC-MS/MS and GC-MS/MS. However, both of them have some limitations which contributed to the fact that sugar and sugar alcohol aerosol sample are not easy to analyze. For RPLC-MS/MS, sugars and sugar alcohols are not well dissolved in the highly organic content mobile phase and the separation of analytes is not satisfied. For the GC-MS/MS, the derivatization process requires extra work and the derivatization compound is not stable. Hence, the HILIC mode is introduced. HILIC is the novel and successful approach for the retention and separation of polar compounds. The retention mechanisms of HILIC column are the hydrophilic partition, the hydrogen bonding, and the electrostatic interactions. Polar stationary phase is used in HILIC mode, and the highly organic solvent is employed in mobile phase. Therefore, a stagnant aqueous-rich layer is generated in HILIC mode. “A stagnant aqueous-rich layer” is formed by the water in the mobile phase and it adsorb to the polar surface of the stationary phase. Polar analytes are retained through the partition into this stagnant aqueous rich layer. Hence HILIC can separate sugars and sugar alcohol efficiently. Besides, because of the complexity of the environmental aerosol sample, the SPE is introduced. The interference and the matrix effect are solved by SPE.

In this study, the environmental aerosol sample was examined by SPE-HILIC -MS/MS method. The method was optimized and 8 sugars were separated. Compared with the RP-HPLC column, HILIC column had better separation results, chromatograms referred to Figure 1 and Figure 12. During

analysis of environmental aerosol samples, 6 sugars have been detected successfully. Compared to other studies of sugars by GC-MS/MS and HPLC-MS/MS, the concentration of analytes in this experiment relatively higher (referred to Table 1, Table 2, and Table 19). Especially glucose and sucrose and their maximum concentrations are 239.34 and 247.13 $\mu g\ m^{-3}$, respectively.

In spite the methodology was established and optimized, the average recovery of analyte in SPE process was only 47.81%. Hence the extraction processed can still be improved and discussed. Although the SPE were applied, particles and impurities still presented in the environmental aerosol sample. The impurities might be stuck in the HILIC column; therefore, the pressure of HILIC column was not so stable and the retention time of analyte shifted during the experiment. Moreover, the high pressure HILIC column was required to be cleaned constantly, which caused sensitive fluctuations in measured concentrations. Hence, there are still opportunities for further improvements in this experiment, such as adding the internal standard to the whole system. Despite the fluctuation of the sensitivity and the high pressure existed, adding internal standard can increase the reliability of the results and the results can be more accurate.

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