CHROMATOGRAPHY TODAY

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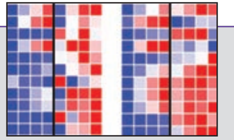

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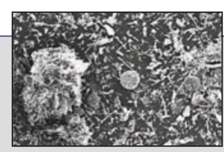

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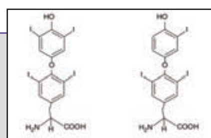

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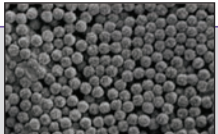

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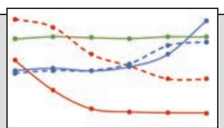

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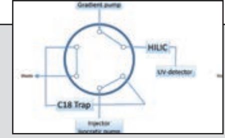

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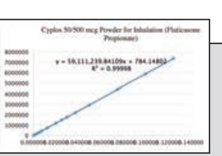

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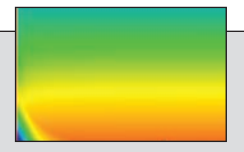

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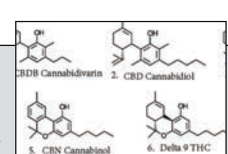

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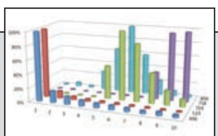


Foreword

Welcome to a brand-new year and decade at Chromatography Today and the first edition of 2020. Last year was another great year for Chromatography Today with our Buyers' Guide - now an annual feature, a growing readership and an increase in unsolicited article submission.

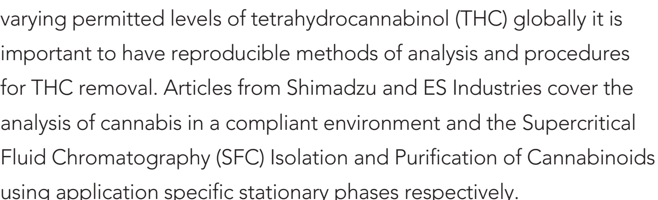
Trevor Hopkins - *Editor*

To keep the articles and features fresh and topical for 2020 we will retain the two general topics format throughout the year. The Fundamental Aspects of Chromatography and Modern Chromatography Applications are designed to provide you with both advances in the fundamental aspects, method development and new instrumentation in analytical separation science along with modern cutting edge and practical applications of analytical separation. The focus areas within these two themes for this edition are 'Sample Preparation' and 'The cannabis explosion - THC & CBD Analysis' respectively. These two themes will be separated by the Chromatography Today Knowledgebase which covers the use of mobile phase pH as a method development tool in liquid chromatography.

In the current issue for the Sample Preparation focus area, we present an article by Agilent explain the development and validation of a sample preparation method employing a novel synthetic SLE sorbent (96-well plate format) for the quantitative determination of an antiepileptic drug and metabolite in tissue samples. This article is followed by an article by Professor Giorgia Purcaro et. al. from the University of Liege which discusses the significantly improved kinetics of extraction when using vacuum assisted headspace solid-phase microextraction as a powerful tool for olive oil analysis.

This issue also has two off-topic articles which have been submitted by the authors - the first by Mattias Malm and Johan Kjellström of Ferring Pharmaceuticals, Denmark discusses the elimination of the sample solvent effect when analysing water based solutions of basic peptides by HILIC by using a column switching approach to enable the use of large injection volumes of aqueous samples. The second off-topic article is by Serkan Acar et. al. from Arven Pharmaceuticals, Turkey who provided a guide to the method development and validation of an improved method for dry powder inhalers.

The 'The cannabis explosion - THC & CBD Analysis' focus area covers the challenges of the analysis and isolation of cannabinoids in a rapidly changing arena. Interest in the therapeutic properties of compounds from cannabis such as cannabinoids has exploded in recent years. This has led to a significant increase in the number of products being released as nutraceuticals into a market which has fragmented regulations at best. With no defined testing guidelines agreed for potency or impurities and varying permitted levels of tetrahydrocannabinol (THC) globally it is important to have reproducible methods of analysis and procedures for THC removal. Articles from Shimadzu and ES Industries cover the analysis of cannabis in a compliant environment and the Supercritical Fluid Chromatography (SFC) Isolation and Purification of Cannabinoids using application specific stationary phases respectively.



The next issue of Chromatography Today will be focusing on Advances in Chiral Separations and Agricultural Applications.

Each edition of Chromatography Today is packed with valuable information, however it is impossible to include everything that is submitted. The new revised Chromatography Today website (www.chromatographytoday.com) is the repository where you will find the other submissions, the latest news from the world of separation science and access to all the historical articles. Perusing the trending news and article sections, it is surprising how much separation science plays a part in daily life without us ever appreciating it, or even realising it. There is a diverse range of areas highlighting just how important separation science is to society with a calendar of events and articles on food, biofuels, medicine, forensics, brewing and environmental clean-up. Please take a look and if you are not a current recipient of the Chromatography Today ebulletin - please sign up today.

As always, we welcome article submissions, topic ideas for inclusion in future issues and feedback from our readership. If you have any feedback on this edition of Chromatography Today, please contact us as we are always looking to improve the publication.

Next Issue -

Fundamental Aspects - *Advances in Chiral Separations*

Modern & Practical Applications - *Agricultural Analysis*

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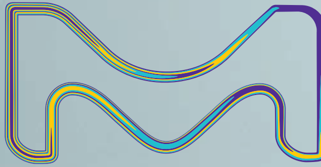

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Vacuum Assisted Headspace Solid-phase Microextraction: A Powerful Tool for Olive Oil Analysis

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The performance of vacuum-assisted headspace solid-phase microextraction (Vac-HS-SPME) was compared to regular headspace solid-phase microextraction (HS-SPME) for the analysis of extra-virgin olive oil. Vac-HS-SPME proved beneficial in particular for semi-volatile compounds, significantly improving the kinetics of extraction. Moreover, for viscous oil samples combining the effects of heating the sample and vacuum was proven beneficial since it reduced the viscosity of the sample, increased the diffusivity of compounds in the liquid phase and improved volatilisation of less volatile compounds.

1. Introduction

Volatile secondary metabolites are an important class of compounds in many fields of applications such as clinical [1-3], environmental [4-7, and food [4,8,9]. In the latter one, volatiles can provide highly informative hints on botanical and geographical origin and/or on the quality of food in terms of aroma profile, spoilage, or technological impact on the secondary metabolite profile. Among the many high-value food commodities for whom volatile metabolites play an essential role, extra virgin olive oil represents an urgent challenge. The goal is to support the official sensor evaluation with a more objective and robust method.

Headspace (HS) solid-phase microextraction (SPME) is most widely applied for volatile profiling and fingerprinting since it provides easy automation, solvent-free applications, and flexibility due to the different sorbents commercially available [10]. HS-SPME is a technique based on the equilibrium between three-phases, namely sample-headspace-fibre. The equilibrium can be reached in a few minutes or several hours, depending on the physicochemical properties of the analytes and the sample. Therefore, a compromise is needed between sensitivity and throughput.

An exciting possibility to minimise such a compromise is the use of reduced pressure conditions during sampling, a technique termed vacuum-assisted HS–SPME. The theory for water-based and solid samples was effectively clarified in a tutorial published by Psillakis *et al.* [11]. More recently, the theory was extended to oily samples [12]. From a thermodynamic viewpoint, the equilibrium concentration is not affected by reduced pressure conditions [11,13], while the kinetics is mostly dependent on medium, temperature, and pressure. This means that an increase in the mass transfer is recorded when increasing the temperature and/or decreasing the pressure [11,13].

The mass transfer in the HS towards the SPME fibre is not considered a limiting process [14,16], while the mass transfer from the liquid to the HS, although highly analyte-matrix dependent, is usually the limiting step [11,16]. This behaviour is explained by considering the concentration gradient located in the stagnant film layers at the liquid/HS interface, assuming that the bulk of the two phases is well mixed. Such a theory proved successful in describing the Vac-HS-SPME process in water-based samples [11,14]. Recently [12], it was clarified that the overall resistence to transfer from the liquid to the HS ($1/k_O$) is due to two diffusional resistances in series, namely the sum of the gas-phase resistance ($1/(K_{GL} k_G)$) and the liquid-phase resistance ($1/k_L$), in particular for viscous liquid as olive oil, according to the following equation:

$$\frac{1}{k_0} = \frac{1}{k_L} + \frac{1}{K_{GL} k_G}$$

where, k_G and k_L are the mass transfer coefficients for the gas and olive oil boundary layers and K_{GL} is the gas phase-olive oil partition coefficient representing the ratio of the equilibrium concentrations in the gas phase over that in the liquid sample. Moreover, the diffusivity should be taken into account in viscous liquid samples, leading to additional resistance in the liquid-film compared to an aqueous phase [17]. On the other hand, the diffusion coefficient in the gas-phase shows an inverse proportionality to the total pressure in the system, regardless of the model chosen for describing it [14]. Therefore, sampling by Vac-HS-SPME is beneficial for analytes where gas-phase resistance controls their volatilisation rate, improving their extraction kinetics, while for compounds where the limiting process is the liquid diffusion, the temperature will play a beneficial role.

This work aimed to investigate the Vac-HS-SPME sampling on the extra-virgin olive oil profile, comparing the extraction temperature and time profile under reduced and normal pressure conditions.

2. Materials and Methods

2.1. Chemicals and reagents

A mixture of normal alkanes (C_7-C_{30}) (Supelco, USA) dissolved in hexane (HPLC grade, MilliporeSigma®, USA) was used for calculating the linear retention index (LRI) for confirming peak identity. A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) d_f 50/30 µm 1 cm length fibre was used (offered by Millipore Sigma, Bellefonte, PA, USA).

Extra-virgin olive was purchased in a local supermarket (Gembloux, Belgium).

2.2. HS-SPME procedure for regular and reduced pressure conditions

The fibre was conditioned as suggested by the manufacturer before the first use. Blanks were run periodically to verify the absence of carryover.

1.5 g of oil sample was weighed into a 20 mL screw top vial (Restek, Bellefonte, USA). The sample was allowed to equilibrate with the headspace for 5 min at the temperature set for extraction (30°C and 43°C). Then, the SPME fibre was exposed to headspace and sampling was performed under agitation (250 rpm) at the selected sampling time (10, 20, 30, 40 min).

The fibre was desorbed at 250°C for 2 min (split 1:5) into the GC-MS inlet equipped with an SPME glass liner. All experiments were run in triplicate.

The difference between regular and Vac-HS-SPME is in the vial cap used. For the former, metallic caps with a central hole and a polytetrafluoroethylene (PTFE)/silicone septa (Restek, Bellefonte, USA) were used. For Vac-HS-SPME a custom-made closure design was implemented (provided by the Laboratory of Aquatic Chemistry, School of Environmental Engineering, Technical University of Crete [18]). The air inside the vial was evacuated for 1 min using a MD 4C diaphragm vacuum pump (7 mbar = 0.007 atm ultimate vacuum without gas ballast) (Vacuubrand GmbH & Co KZ, Wertheim, Germany). Then a 5 mL gastight syringe (SGE, Australia) was used to introduce 1.5 g of oil samples in the vial (Figure 1).

2.3. Central Composite Design

Central composite experimental design (CCD) was used to optimise extraction temperature and time, both for regular and Vac-HS-SPME. The extraction temperature was tested between 30°C and 55°C, and the exposition time from 10 to 30 minutes, based on conditions reported in previous works [19,20].

2.4. GC-MS analysis

An Agilent 7890B GC coupled to a 5977 MSD was used for all analyses. Carrier gas: helium at 1 mL/min flow rate. GC column: a 30 m x 0.25 mm i.d. x 0.5 µm df SLB-5ms capillary column [(silphenylene polymer, practically equivalent in polarity to poly(5%diphenyl/95% methylsiloxane)] kindly obtained from MilliporeSigma (Bellefonte, PA, USA). GC oven temperature program: 35°C (hold 2 min) to 250°C at 3°C/min and to 300°C at 25°C/min.

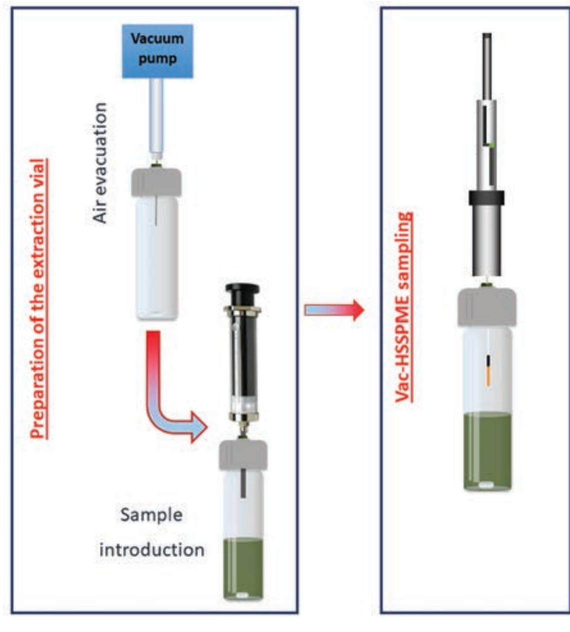


Figure 1: Schematic of the Vac-HSSPME

MS: in EI mode at 70 eV; source temperature: 230°C; quadrupole temperature: 150°C; full scan mode in the 35-500 m/z range.

2.5. Data elaboration and statistical analysis

Twelve compounds were selected over the entire chromatogram (Table 1). All the chemical-physical properties of the 12 compounds selected (reported in and obtained from the ChemSpider website (http://www.chemspider.com/).

All statistical analyses were performed using R v3.3.2 (R Foundation for Statistical Computing, Vienna, Austria) and Minitab 19 (https://www.minitab.com/en-us/) and https://software.broadinstitute.org/morpheus/.

Table 1: List of the 12 selected compounds together with their Chemical Abstracts Service (CAS) number, boiling point (B_p), Henry's constant (K_H, atm m^3 mol^{-1}), octanol-air partition constant (K_{oa}, atm m^3 mol^{-1}), octanol-water partition constant (K_{ow}, atm m^3 mol^{-1}), Vapour pressure (V_p, mmHg at 25°C), Molecular weight (M_W, g mol^{-1}) and Molecular volume (V_M, cm^3) , and linear retention index (IT) experimentally calculated and reported in the literature.

#	Compound Name	CAS	M_W (g mol^{-1})	V_M (cm^3)	B_P (°C)	Log K_H (atm m^3 mol^{-1})	log K_{oa} (atm m^3 mol^{-1})	log K_{ow} (atm m^3 mol^{-1})	V_p (mmHg at 25°C)	*m/z*	I^T_{ex}	I^T_{Lib}
v1	Acetic acid	64-19-7	60.1	56.2	117.1	-5.54	5.218	0.09	13.9	60	634	641
v2	Penten-3-one	1629-58-9	84.1	103.3	104.3	-3.71	3.748	0.90	31.1	55	691	683
v3	Hexanal	66-25-1	100.2	124.9	127.0	-3.45	3.84	1.80	10.9	56	801	801
v4	1-Hexanol	111-27-3	102.2	125.0	158.2	-4.76	5.185	1.82	0.9	56	872	867
v5	2(E)-Heptenal	18829-55-5	112.1	135.0	166.0	-3.81	4.341	2.07	1.8	83	960	956
v6	Benzaldehyde	100-52-7	106.1	101.1	178.7	-4.64	4.442	1.71	1.0	106	965	960
v7	Octanal	124-13-0	128.2	157.9	163.4	-3.20	4.457	2.78	2.1	84	1004	1006
v8	Hex-(3Z)-enyl acetate	3681-71-8	142.2	157.7	175.2	-3.35	4.195	2.61	1.4	67	1005	1008
v9	ß-Ocimene,(E)	13877-91-3	136.2	175.5	175.2	-0.62	3.398	4.80	1.6	93	1047	1046
v10	Nonanal	124.19-6	142.2	174.4	190.8	-3.10	4.793	3.27	0.5	57	1105	1107
v11	Methyl salicylate	119-36-8	152.1	125.8	222.0	-5.24	4.947	2.60	0.1	120	1197	1192
v12	α-Farnesene, (E,E)	502-61-4	204.3	251.5	279.6	-0.19	5.067	7.10	0.0	93	1506	1504

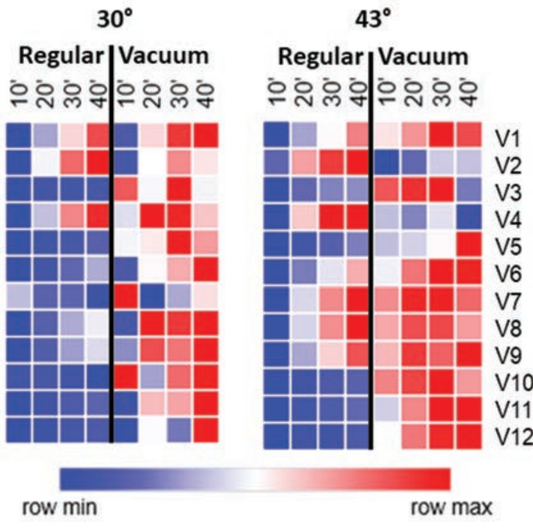


Figure 2: Heat-maps showing the extraction response obtained using regular and Vac-HS-SPME at 30 and 43 °C for different extraction time, namely 10, 20, 30 and 40 min. Compounds identification as reported in Table 1.

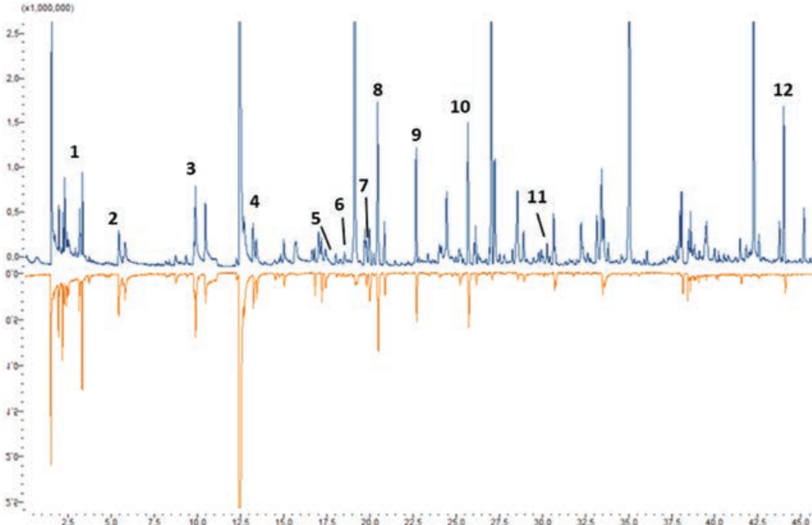


Figure 3: Total ion chromatogram obtained using regular (yellow, lower chromatogram) and Vac-HS-SPME (blue, upper chromatogram). Compound identification as for Table 1.

3. Results and Discussion

The heat maps reported in Figure 2 illustrate the overall change in the profile of the 12 targeted compounds when sampled using Regular and Vac-HS-SPME at both 30 and 43°C for different extraction times (10, 20, 30, and 40 min). The two heatmaps are normalised separately to emphasise the change in the response within a single temperature tested.

It can be observed as the Vac-HS-SPME sampling increased the general profile (colour turning toward red). However, an important distinction needs to be made between the compounds with the highest and lowest volatilities. For the former, generally, the same performance can be observed between regular and Vac-HS-SPME at 30 and 43°C, with even a slightly lower extraction yield at 43°C. The kinetics of these compounds is usually rapid; thus the effect of using reduced pressure conditions is limited or even non existent as these analytes have reached 'equilibrium'. The slight reduction of the signal at 43°C using Vac-HS-SPME indicates that the effect of temperature for the earlier eluted compounds is comparable to the gain in extraction yield obtained using vacuum. This behaviour can be related to both an acceleration of the extraction kinetic (not assessable in the range of extraction time tested) and competition effect. It is interesting to notice that for the rest of the compounds the effect of vacuum and temperature is instead synergic, significantly improving the extraction efficiencies at an earlier sampling time, in fact for the latest compounds (V6-V12) almost the same intensity of response is obtained at 43°C after 20 min using Vac-HS-SPME, meaning that the equilibrium is almost reached; while under regular conditions, the response is much lower compared to Vac-HS-SPME and in a clear ascending trend moving from 10 to 40 min.

Noteworthy is the improvement obtained for α-farnesene (V12), reported as an important marker for discriminating the geographical origin of extra-virgin olive oil [21,22]. An almost 10-fold increment was observed when Vac-HS-SPME sampling and higher temperatures are applied. Figure 3 shows the comparison of the chromatographic traces between regular- and Vac-HS-SPME when sampling at 43°C for 10 min.

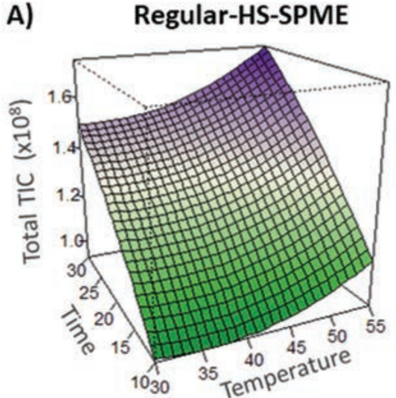
It is important to highlight that the effect of increasing the temperature in Vac-HS-SPME of water-based solutions was not always successful especially for absorbent type SPME fibres [11]. In fact, the increased humidity in the headspace increased the pressure in the vial, thus reducing its benefits. In edible oil samples, like olive oil, water is not present (or is in trace amounts); therefore, heating can be exploited with beneficial effect, although care must be paid to avoid artifact formation and thermal degradation products. Moreover, in high viscous samples like olive oil, the increase in temperature decreases the viscosity of the fluid. The high viscosity value of olive oil (49 mPa at 30°C, 60-times larger than water) increased the liquid-phase resistance, 'delaying' the analyte diffusion through the liquid boundary layer of the olive oil matrix. This phenomenom is of high importance when targeting analytes with a small affinity for the headspace, regardless of the pressure conditions used [16]. In fact, for these analytes, a multi-stage process occurs: analyte molecules are transferred from the liquid sample to the gas phase every time the headspace concentrations fall below equilibrium levels [14,16]. This 'replenishment' process depends on the resistance in the liquid phase (related to the viscosity). It was shown here, that heating the sample from 30°C to 43°C led to a 40% decrease in viscosity of the sample (*i.e.*, ~30 mPa s) improving the liquid-phase diffusivity and thus the overall extraction yield. Since Vac-HS-SPME sampling impact significantly on the kinetics of extraction, this process of 'replenishment' becomes even more limiting than in regular HS-SPME. However, the use of still milder temperature (43°C) in combination with reduced pressure provide a synergic improvement on the overall extraction yield..

Two-variable CCD: a study of the response surfaces

To better characterise the gain in performance obtained using Vac-HS-SPME over regular, a full factorial central composite design (CCD) was used to optimise at the same time and temperature ranges both the regular and Vac-HS-SPME. Based on previous findings that highlighted that time and temperature were the main significant variables [19,20], a two-variable (k=2; temperature and time) CCD was applied. The extraction temperature

was tested over a range of 30 (minimum temperature settable in the autosampler) and 55°C, and the extraction time was set between 10 and 30 min.

In fingerprinting and profiling studies, the goal is to maximise the overall response, maximising the number of peaks detected and their intensities. The response surface (Figure 4) was built considering the cumulative area intensity of all the peaks detected. It is evident from Figure 4 that the maximum is reached at a milder temperature and shorter time using Vac-HS-SPME, while using regular-HS-SPME, a maximum is not reached even after 55°C and 30 min of extraction. The total number of peaks detected increased from ~150 to ~180 using Vac-HS-SPME.



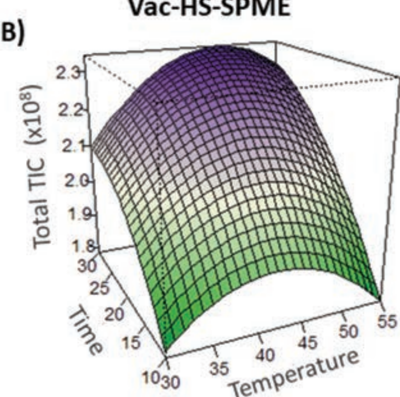


Figure 4: Surface responses are obtaining applying the two-variable inscribed rotatable CCD. A) Regular HS-SPME and B) Vac-HS-SPME. Temeprature range: 30-55°C; time range: 10-30 min.

Conclusions

The advantages of using Vac-HS-SPME compared to regular-HS-SPME were shown and discussed. Heating the sample (43°C compared to 30°C) further enhanced the benefit of vacuum. Moreover, for viscous liquid samples, the temperature remained an important parameter since it increased the diffusivity through the liquid phase facilitating mass transfer at the interface.

The use of Vac-HS-SPME for untargeted studies of olive oil can importantly increase the level of information obtainable and the effectiveness of cross-sample comparison applying pattern recognition algorithms, allowing more effective markers identification for quality and authenticity studies.

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Compliance with ethical standards

Note: The authors declare no competing financial interest.

Ethical Approval: The authors have declared that no ethical issues exist.

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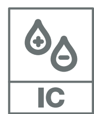

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Analysis of Fenfluramine and Norfenfluramine in Mouse Brain and Cerebellum by Liquid Chromatography Tandem Mass Spectrometry using a Novel Solid-Supported Liquid Extraction

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This study outlines the application of a new synthetic supported liquid extraction (SLE) sorbent for the quantitative determination of the antiepileptic drug fenfluramine (FNN) and metabolite, norfenfluramine (NFNN), in mouse brain by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Additionally, a comparison of the synthetic SLE sorbent with diatomaceous earth (DE) was conducted, wherein the synthetic SLE sorbent eliminated a greater content of phospholipid while demonstrating higher analyte recovery with improved reproducibility. The validated method supported a two-fold dynamic range with a limit of quantitation (LOQ) of 0.05 μg/g in mouse brain, demonstrated acceptable calibration curve linearity ($r^2 > 0.99$), intra- and inter-run precision (C.V. < 10%) and accuracy (100 ±10%), and matrix effect (100 ± 10%).

Introduction

LC-MS/MS has been widely adopted for the high throughput quantitative bioanalysis of small molecules, attributed mainly to the high selectivity, sensitivity, and sampling frequency of the approach. However, even with highly selective analyte monitoring, LC-MS/MS analysis can be deleteriously impacted by changes in ionisation efficiency due to coeluting matrix components such as salts, proteins, lipids (including phospholipids) and other various organic molecules. Such ionisation effects can influence the achievable limit of quantitation (LOQ), method reliability and reproducibility, chromatography and MS source contamination [1]. Therefore, sample preparation is required not only to extract target analytes from matrix, but also to remove unwanted components potentially impacting ionisation efficiency - the latter is often referred to as the matrix effect. The use of appropriate sample preparation techniques is dependent upon the complexity of the matrix, requirements for the detection of target analytes, and the selected instrument detection method. It is understood that sample preparation can be both time-consuming and costly, but these are the unavoidable factors in order to gain reliable quality analytical results, and to preserve high-value instruments from damage.

Sample preparation products for bioanalysis are often based on a 96-well plate format, which allows automated / semi-automated simultaneous sample processing, thereby supporting the preparation of a large number of samples aligned with high throughput LC-MS/MS analysis. The format also accommodates the relatively small sample sizes associated with biological matrices, typically within several hundreds of microliters. Protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE) are the techniques most commonly implemented in the preparation of biological samples for LC-MS/MS analysis, with pros and cons for each approach [2-4].

Although LLE represents a sample preparation process with advantages such as high recovery and extract cleanliness, its mainstream adoption into the modern bioanalytical lab is confounded by the disadvantages associated with automation (time consumption, labour-intensive processes, and the potential for emulsion formation). In contrast, supported liquid extraction (SLE) as a flow-through technique has been increasingly used as an alternative approach to LLE, overcoming many of the disadvantages associated with LLE. [5] The SLE substrate provides a chemically inert but highly hydrophilic surface upon which an aqueous sample adsorbs. When the aqueous sample is loaded onto SLE substrate, a thin layer of aqueous phase is generated and coated onto the SLE sorbent surface. This thin layer of aqueous phase on the sorbent significantly increases the contact surface area during extraction. Following a brief equilibration period, analytes are extracted with a water immiscible solvent either by gravity or through the application of positive or negative pressure while the aqueous phase is retained on the sorbent. The extraction mechanism and workflow process are outlined in Figure 1. Since insignificant mixing of aqueous and organic phases occurs with the SLE workflow, emulsions are eliminated and the intimate contact between phases allows very efficient analyte partitioning, often resulting in high analyte recovery. Due to the simplicity of the SLE workflow (load, soak and elute), labour and

time demands are significantly reduced. Lastly, SLE in the 96-well plate format is especially amenable to automation, increasing overall sample throughput.

Traditionally, the sorbent used for SLE is highly purified diatomaceous earth (DE). However, as a naturally occurring material, DE consists of irregular fossilised micro-organisms. Consequently, variance in particle-size distribution can generate issues with product manufacturing and batch-to-batch quality control, in turn leading to inconsistent product performance. Figure 2 shows the scanning electron microscope (SEM) image of natural DE sorbent and synthetic SLE sorbent particles, using the same SEM settings. As shown in the Figure 2A, there is noticeable amount of debris in the DE sorbent with particle size inconsistencies. However, for synthetic SLE sorbent (Figure 2B), the particle size is much more uniform, without obvious debris. Further, the inconsistency in DE sorbent particles results in the reduced and inconsistent aqueous phase holding capacity [6,7], leading to a higher risk of sample loss and matrix breakthrough during sample loading and elution. The use of the synthetic SLE substrate allows control of the particle size distribution, in turn leading to improved consistency of method performance.

Fenfluramine (FNN) is an anti-epileptic drug whose mechanism of action is poorly understood. In order to study the distribution of FNN and the accumulation of its major metabolite norfenfluramine (NFNN) in mouse cerebellum, it was necessary to develop a sensitive assay given the limitation in tissue mass (ca. 60 mg). Brain homogenate represents a matrix complexity greater than that of traditional plasma owing to significantly higher phospholipid content, and it was therefore necessary to deplete as many of these potential ion suppressors as possible in order to minimise accumulation within the LC/MS system. To this end, the novel synthetic SLE sorbent was evaluated in terms of the efficacy of phospholipid removal, recovery of FNN and NFNN, assay specificity and matrix effect, all benchmarked against traditional diatomaceous earth sorbent.

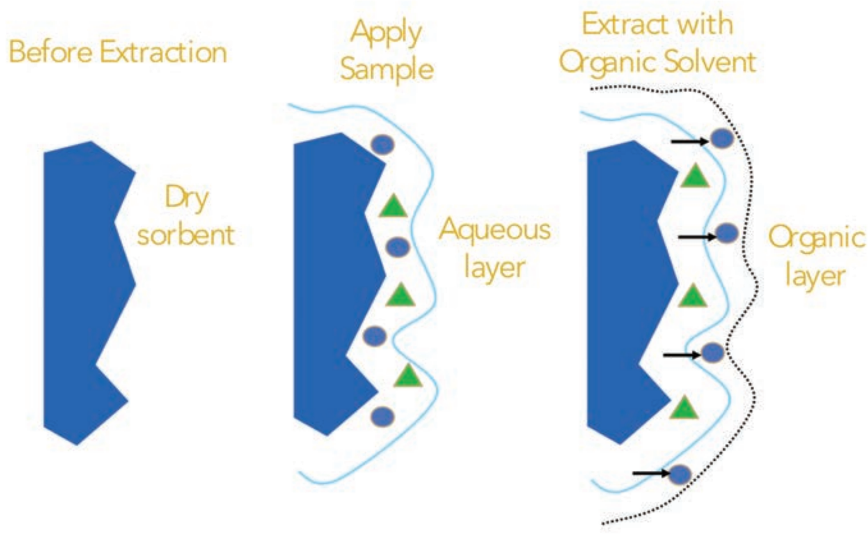


Figure 1: The process and analyte extraction mechanism of supported liquid extraction (SLE).

Experimental

Chemicals and Standards

High Performance Liquid Chromatography (HPLC) or Omnisolv grade solvents were sourced from Millipore Sigma, including methanol (MeOH), dichloromethane (DCM), methyl tert-butyl ether (MTBE), 1-chlorobutane, and hexane. Ethyl acetate (EtOAc) and chloroform Optima grade solvents were supplied by Fisher. Other chemicals were purchased from Sigma-Aldrich, including ammonium hydroxide (NH_4OH), concentrated hydrochloric acid (HCl), and ammonium bicarbonate (NH_4HCO_3).

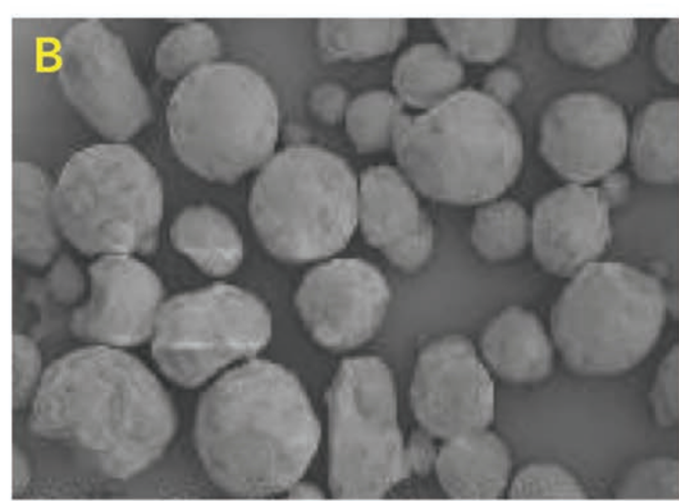


Figure 2: SEM images of DE sorbent (A) and synthetic SLE sorbent (B).

Table 1: MRM transitions for target analytes and phospholipids.

Target analytes	Q1/Q3
FNN	232.0/159.0
FNN-D5	237.0/159.0
NFNN	204.0/109.0
NFNN-D6	210.1/161.0
Phospholipid	**Q1/Q3**
Lysophosphatidylcholine (18:2)	520.3/184.1
Lysophosphatidylcholine (18:1)	522.4/184.1
Lysophosphatidylcholine (18:0)	524.4/184.1
Lysophosphatidylcholine (20:4)	544.3/184.1
Phosphatidylcholine (30:1)	704.5/184.1
Phosphatidylcholine (34:2)	758.6/184.1
Phosphatidylcholine (34:1)	760.6/184.1
Phosphatidylcholine (36:3)	784.6/184.1
Phosphatidylcholine (36:2)	786.6/184.1
Phosphatidylcholine (38:6)	806.6/184.1
Phosphatidylcholine (38:5)	808.6/184.1
Phosphatidylcholine (38:4)	810.6/184.1

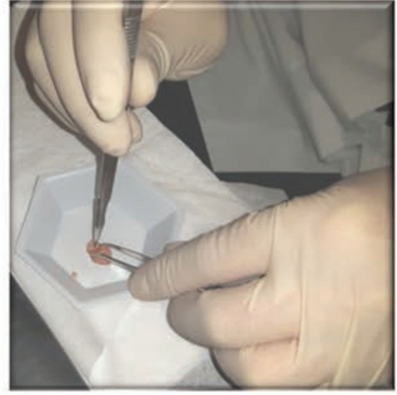
Reference standard and internal standard (IS) stock solutions were provided by Altasciences (100 µg/mL FNN and NFNN in MeOH, 100 µg/mL FNN-D_5, and NFNN-D_6 in MeOH).

LC-MS/MS Instrumentation

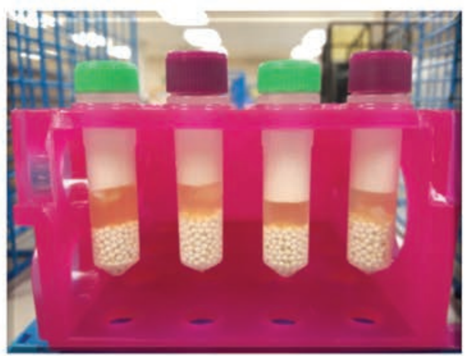
Chromatographic separations were conducted on a C18 column (2.1 x 100 mm, 2.0 µm) with 10 mM NH_4HCO_3, pH 10.0 (MP-A), and MeOH (MP-B), delivered using an UPLC system under the following gradient: MP-B was ramped from 70 to 90% over 1.75 min and held isocratic for 0.5 min after which the column was re-equilibrated at 70% MP-B for 0.75 min. Column flow rate was 0.70 mL/min at a column temperature of 60°C; the LOQ was achieved using an injection volume of 8 µL. A triple quadrupole mass spectrometer was operated under positive electrospray ionisation (ESI) conditions with detection in multiple-reaction monitoring (MRM) mode for the transitions outlined in Table 1.

Sample Preparation

Mouse whole brain (CD-1 strain) was purchased from BIOIVT. Cerebellum was harvested from whole brain by dissection (Figure 3A). Either dissected cerebellum or whole brain were treated with water (10 µL per mg of tissue), after which ceramic beads (Matrix D, MP Biomedicals™) were added (Figure 3B) and the sample was homogenised (Figure 3C). An aliquot of homogenate (10 µL) was fortified with internal standard spiking solution (25 µL) and 5% NH_4OH (165 µL), then vortexed (1 min) and centrifuged (2 min, 738 g). The entire sample homogenate was loaded onto SLE plates using synthetic SLE sorbent (Chem Elut S 96-well plate, 200 mg, Agilent Technologies) for SLE extraction followed with the procedure shown in Figure 4.
In order to prevent analyte loss during evaporation, a keeper consisting of HCl in MeOH was added in the collection plate prior to the elution step, which will create the analytes salt form. Consequently, this eliminated well to well variations previously noticed from the evaporation step.



(A)



(B)

(C)

Figure 3: Mouse brain pre-treatment, including A) dissection and weighing, B) Lysing buffer (water) and ceramic bead addition; C) homogenisation with MP Biomedicals FastPrep-96 homogeniser at 1,600 rpm for 40 seconds.

Results and Discussion

SLE Method Optimisation

The optimisation of SLE methodology involved the evaluation of analyte recovery, average reproducibility, and matrix effect as a function of elution solvent, solvent volume and sample soaking time. Elution solvents investigated included: MTBE, 1-Chlorobutane, DCM:EtOAc (1:1), MTBE:Chloroform (4:1) and MTBE:Hexane (4:1). A total volume of 1.2 mL was used for each elution solvent, applied in different aliquots: 3 x 400 µL, as two aliquots of 600 µL, 2 x 600 µL and 1 x 1200 µL. Results demonstrated in Figure 5 indicate that (a) the synthetic SLE sorbent provides higher analyte recovery, more consistent reproducibility, and reduced matrix effect when compared to DE, and (b) DCM/EtOAc (1:1) and EtOAc provide optimal recovery for each analyte, however elution with EtOAc exhibited higher matrix effect for NFNN. Consequently, DCM/EtOAc (1:1) was selected as the preferred elution solvent. Notably, when using the synthetic SLE sorbent, recovery was largely independent of the number of elution aliquots; 2 x 600 µL provided marginally improved recovery and was therefore selected as the final elution scheme. However, when using DE SLE, changes in recovery were more significant with different elution steps.

Equilibration time following sample loading on the SLE sorbent was investigated for up to 40 min. Results indicate no significant variation in recovery at different equilibrium time, and therefore 5 min was used to optimise sample throughput. Fact that recovery was not impacted with additional equilibration time ensures a level of robustness in the methodology, since analytes do not irreversibly bind to the sorbent substrate with extended soaking.

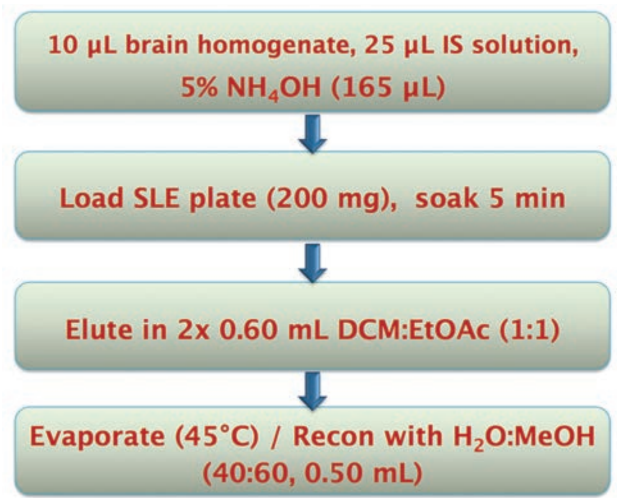
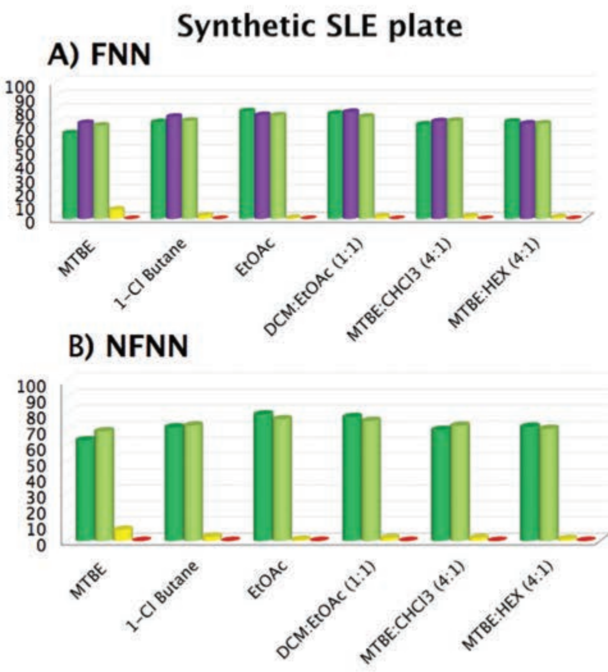


Figure 4: SLE procedure for mouse brain homogenate using synthetic SLE sorbent.

Phospholipid Depletion

Phospholipids (PPLs) have been identified as a major source of matrix effect in LC-MS/MS assays, leading to signal supression under ESI conditions. As PPLs elute over a wide range of retention times, their removal via sample preparation is critical to minimise the likelihood of coelution with analyte, or accumulation within the LC/MS system. Within many different classes of phospholipids in biological matrix, phosphatidylcholine (PC) and lysophosphatidylcholine (Lyso-PC) are the two most abundant classes. Therefore, eight PC and four Lyso-PC compounds, together with total PPLs (184 > 184), were monitored for depletion following SLE on both synthetic SLE sorbent and diatomaceous earth substrates. Results reported in Figure 6 demonstrate significant improvement for PPL depletion using the synthetic SLE sorbent, with > 50% of total PPL retained on the sorbent compared to diatomaceous earth.

With greater PPL depletion efficiency using the synthetic SLE sorbent, many benefits are conferred to the bio-analytical scientist, including (a) reduced likelihood for ionisation suppression, (b) the ability to develop faster chromatography without fear of PPL co-elution, and (c) increased assay robustness by elimination of PPL accumulation on-column and in the ion source, leading to extended column lifetime and reduced instrument downtime.



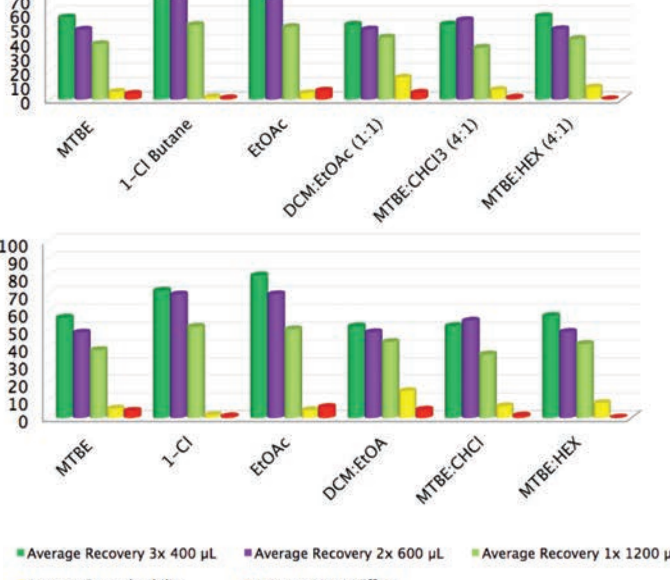


Figure 5: SLE elution solvent optimisation.

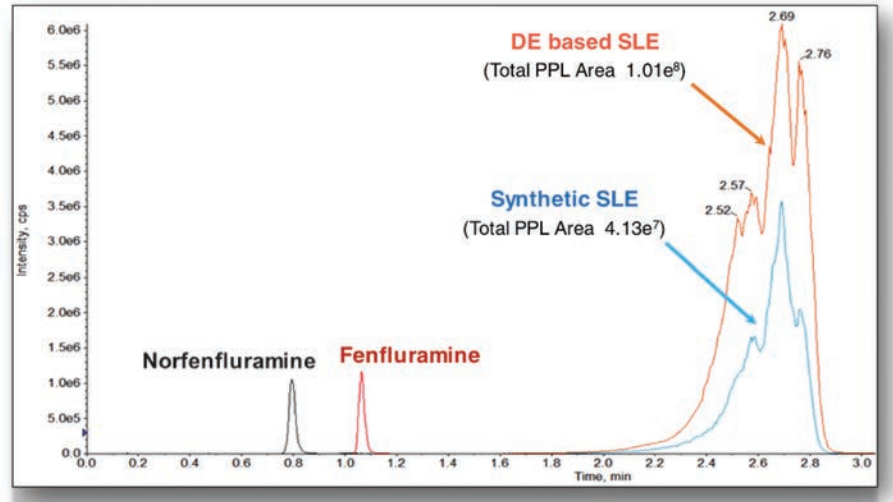
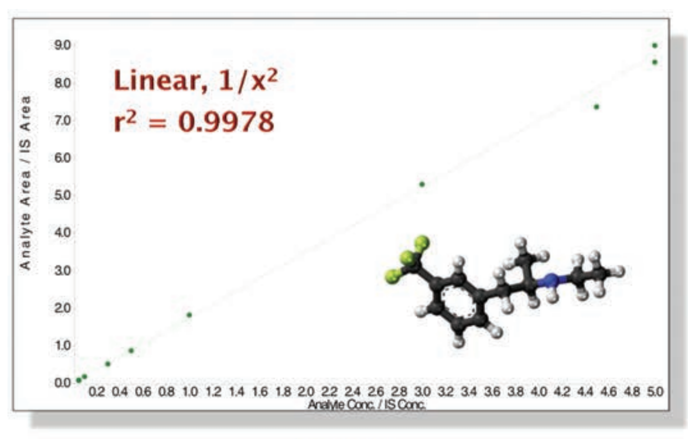


Figure 6: Phospholipid profile comparison for samples extracted using synthetic SLE sorbent (blue) vs. diatomaceous earth (red).

Method Validation

Following method optimisation, the synthetic SLE procedure was validated for the quantitative determination of FNN and NFNN in mouse brain from 0.05 – 5.0 μg/g (Figure 7), with subsequent cross validation in mouse cerebellum. Exemplary selectivity and sensitivity results are presented in Figure 8.

Method accuracy and precision derived from three separate batches using three different lots of synthetic SLE plates to prepare mouse brain extracts are summarised in Table 2. Four levels of QC samples were fortified into brain homogenate and extracted in replicates of six. Method accuracy and precision for QC samples prepared in mouse cerebellum whose concentrations were determined from a calibration curve prepared in whole brain are reported in Table 3. Matrix effect, as determined from eight lots of brain homogenate, met all acceptance criteria (Table 4).



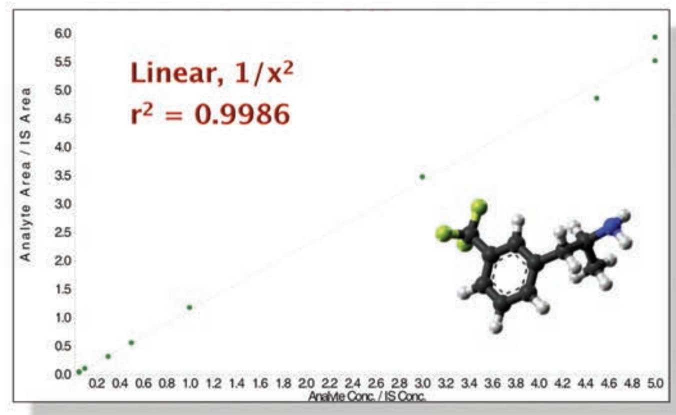


Figure 7: Method calibration curve linearity from 0.05 - 5.0 μg/g extracted from mouse brain. Responses of peak area ratio for analyte/IS were used for calibration curve plot.

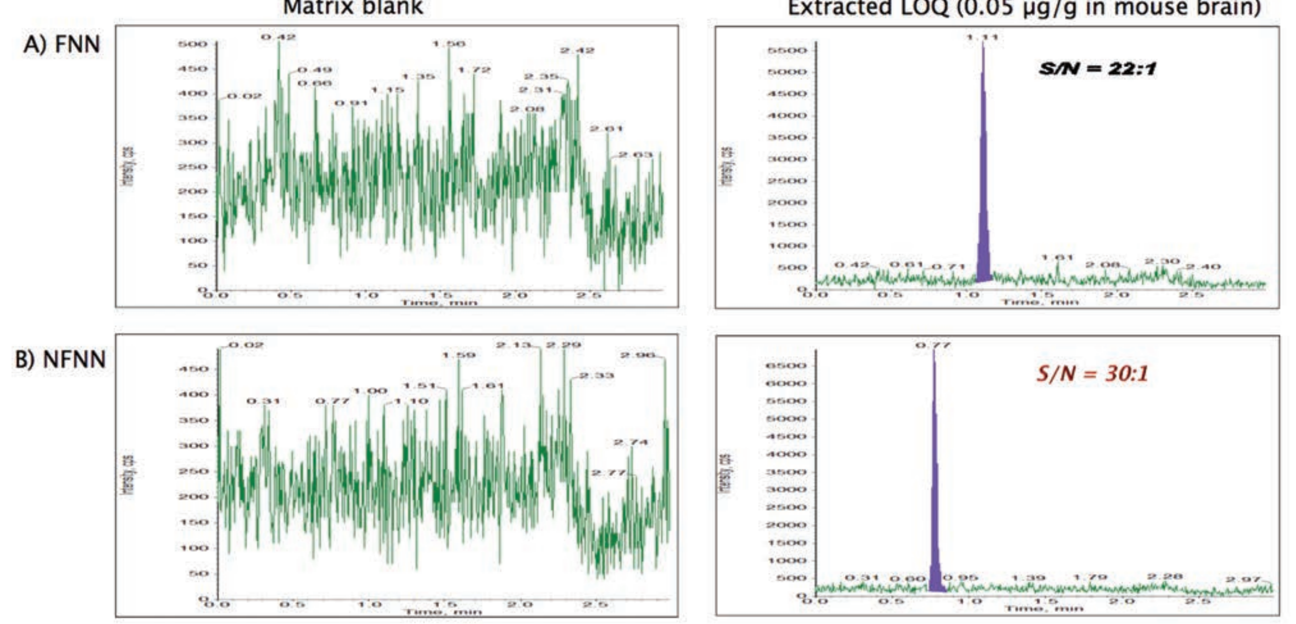


Figure 8: Method selectivity and sensitivity of FNN and NFNN extracted from mouse brain using validated method with synthetic SLE sorbent.

Table 2: Method accuracy and precision results for FNN and NFNN extracted from mouse brain for three extracted batches using three different lots of synthetic SLE plates. Six replicates were extracted for each QC concentration level.

Statistics	LLOQ QC 0.050 μg/g		Low QC 0.150 μg/g		Mid QC 2.50 μg/g		High QC 4.00 μg/g	
	FNN	NFNN	FNN	NFNN	FNN	NFNN	FNN	NFNN
Calculated concentration (μg/g)	0.053	0.053	0.148	0.153	2.500	2.498	3.825	3.832
SD	0.003	0.003	0.007	0.007	0.067	0.100	0.109	0.090
%CV	5.8	5.0	4.5	4.9	2.7	4.0	4.2	2.4
%Nominal	105.4	106.2	98.5	102.3	100.0	99.9	95.6	95.5

Table 3: Method accuracy and precision results for three batches extracted from mouse cerebellum. Six replicates were extracted for each QC concentration level. Quality control samples were spiked in cerebellum homogenate, and then quantified against a calibration curve derived from brain homogenate.

Statistics	Low QC 0.150 μg/g		High QC 4.00 μg/g	
	FNN	NFNN	FNN	NFNN
Calculated concentration (μg/g)	0.161	0.163	4.051	4.044
SD	0.005	0.004	0.207	0.203
CV [%]	3.0	2.2	5.1	5.0
Nominal [%]	107.0	108.4	101.3	101.1

Table 4: Matrix effect evaluation from eight lots of brain homogenate. Three replicates were extracted at each QC concentration level.

Statistics	Low QC 0.150 μg/g		High QC 4.00 μg/g	
	FNN	NFNN	FNN	NFNN
Calculated concentration (μg/g)	0.161	0.163	4.051	4.044
SD	0.005	0.004	0.207	0.203
CV [%]	3.0	2.2	5.1	5.0
Nominal [%]	107.0	108.4	101.3	101.1

Conclusions

A sample preparation method using a synthetic SLE sorbent (96-well plate format, 200 mg) was developed and validated for the quantitative determination of fenfluramine and norfenfluramine in mouse brain, and cross-validated in mouse cerebellum. The SLE method was optimised for elution solvent, elution volume, number of elution aliquots, and sample equilibrium time based on analyte recovery, method reproducibility and matrix effects. The developed SLE method using synthetic SLE sorbent was subsequently validated for method selectivity and sensitivity, calibration curve linearity, accuracy and precision, and matrix effect in multiple donor lots. All acceptance criteria were met for calibration curve linearity and intra- and inter-day accuracy and precision, the latter derived from three synthetic SLE sorbent lots. When compared to diatomaceous earth SLE, the synthetic SLE sorbent provided higher overall analyte recoveries, improved assay reproducibility and greater phospholipid depletion for brain and cerebellum extracts. The developed SLE assay in the 96-well plate format is amenable to fast and automated sample preparation in high throughput laboratories.

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Online Solid Phase Extraction and LC/MS Analysis of Thyroid Hormones in Human Serum

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Thyroid hormones play critical roles in the regulation of biological processes, such as growth, metabolism, protein synthesis, and brain development. Specifically, both 3,3',5,5'-tetraiodo-L-thyronine (thyroxine or T4) and 3,3',5-triiodo-L-thyronine (T3), are essential for development and maintenance of normal physiological functions [1]. For a clinical laboratory, measurements of total T4 and total T3, along with estimates of free T4 (FT4) and free T3 (FT3), are important for the diagnosis and monitoring of thyroid diseases. Most clinical laboratories measure thyroid hormones using immunoassays. The immunoassay-based methods offer a relatively rapid, high patient sample throughput that lends itself to automation, but are significantly compromised by problems with assay interference and are complicated by changes in protein levels that alter the free hormone availability [1,2]. These drawbacks lead to inaccuracies of immunoassays and can lead to false high or low results [3].

Liquid chromatography mass spectrometry (LC/MS) has been reported [1-3] to offer superior specificity and speed over the immunoassays for determination of thyroid hormones in biological matrices such as serum and tissues. Nevertheless, the reported sample preparation procedures, typically liquid-liquid extraction followed by solid phase extraction (SPE), involve multiple time-consuming steps, and are less compatible with automation [3,4]. The present work demonstrates successful on-line SPE with LC/MS for rapid determination of T4, T3, and 3,3',5'-triiodo-L-thyronine (rT3) from biological matrices. The method development process included the use of 2 on-line SPE cartridge chemistries: C8 and RP-Amide. The serum samples underwent protein precipitation procedure to release the protein-bound thyroid hormones. The capture of analytes on the on-line SPE cartridges was confirmed by washing the cartridges directly into analytical HPLC column using higher concentrations of organic solvent and tandem mass spectrometry detection.

Experimental:

Materials: SupelTMGenie C8 and RP-Amide (RPA) on-line cartridges (2 cm length x 4.0 mm i.d.), human serum (MilliporeSigma Cat. H-1388), protein crashing solvent: methanol with 1% (w/v) ammonium formate.

Chemical structures of the thyroid analytes

Thyroxine (T4), $C_{15}H_{11}I_4NO_4$, Monoisotopic mass: 776.686 Da

3, 3', 5-Triiodothyronine (T3), $C_{15}H_{12}I_3NO_4$, Monoisotopic mass: 650.790 Da

3, 3', 5'-Triiodothyronine (Reverse T3), $C_{15}H_{12}I_3NO_4$, Monoisotopic mass: 650.790 Da

Figure 1: The chemical structures of the thyroid analytes. Note T3 and rT3 are isobaric.

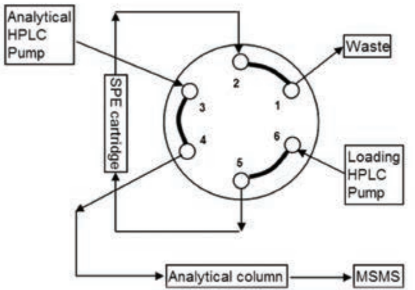
Sample processing procedure: the human serum spiked with analytes was protein precipitated by vortex mixing with the crashing solvent at a 1:3 ratio. Then the mixture was centrifuged at 10,000 x g for 3 min and the resulting supernatant was collected and directly injected for LC/MS analysis.

On-line SPE-LC/MS setup

As shown in Figure 2, the on-line SPE-LC/MS setup consists of a 6-port switching valve and two pumps; one for sample loading and washing, the other for sample elution. To minimise the potential peak broadening from the cartridges, the flow of sample loading/washing and the subsequent elution are in reversed directions.

On-line SPE-LC/MS:

Instrument:	Shimadzu LCMS-8030 with 2DLC setup
HPLC column:	Ascentis Express Biphenyl 10 cm x 2.1 mm (MilliporeSigma Cat# 64065-U)
Mobile phase:	(A) Water; (B) MeOH, each with 0.1% acetic acid
Isocratic:	70% B% for 10 min
Flow:	0.3 mL/min
Column temperature:	35°C
Sample loading/ washing:	0.3 mL/min for 2 min, then the valve switches to in-line with HPLC column, before sample loading the cartridge is equilibrated with the loading solvent for 2.5 min.
Sample loading solvent:	10% methanol
Injection Vol:	2 µL injection
Detection:	MS, ESI(+), MRM mode



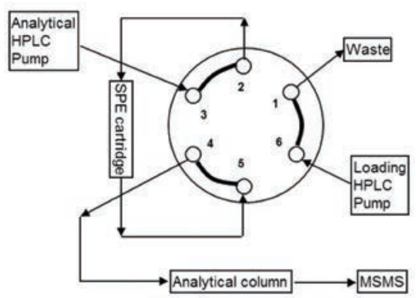
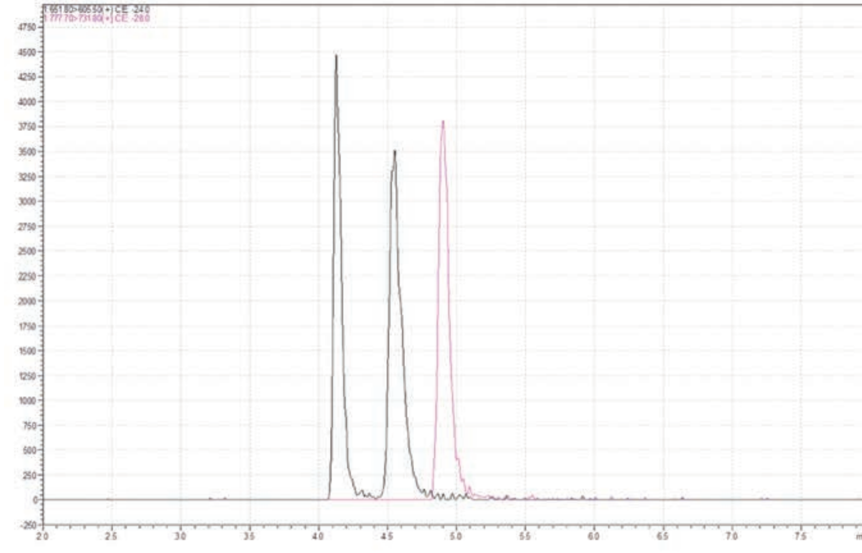


Figure 2: Configuration of the on-line SPE-LC/MS system.

Results and Discussion:

The conventional (off-line) sample preparation by SPE typically involves multiple labour-intensive and time-consuming steps, including: conditioning, sample loading, washing, elution, and finally evaporation and reconstitution of the sample in mobile phase. The on-line cartridges were developed to automate the sample preparation process, minimise hands on time and human error, and reduce overall sample processing time. The present work utilised the C8 and RPA on-line cartridges with LC/MS for the detection of thyroids from in human serum with C8 and RPA on-line cartridges, respectively. The human serum samples were simply protein precipitated with methanol containing ammonium formate and then directly injected for on-line SPE and LC/MS analysis. The sample loading/washing were carried out entirely by the instrument, without any hands-on effort. Additionally, the time-consuming solvent evaporation and reconstitution steps were eliminated. As can been seen from Figures 3 and 4, both C8 and RPA were capable of capturing a trace amount (100 ng/mL x 2 µL in this case) of thyroids from complicated human serum. All three thyroids are resolved from each other, with a peak width at half height <6s and tailing factor from 1.4-1.8. These indicate sharp and nice peak shapes with the on-line cartridges. The total run time is within 6 min.

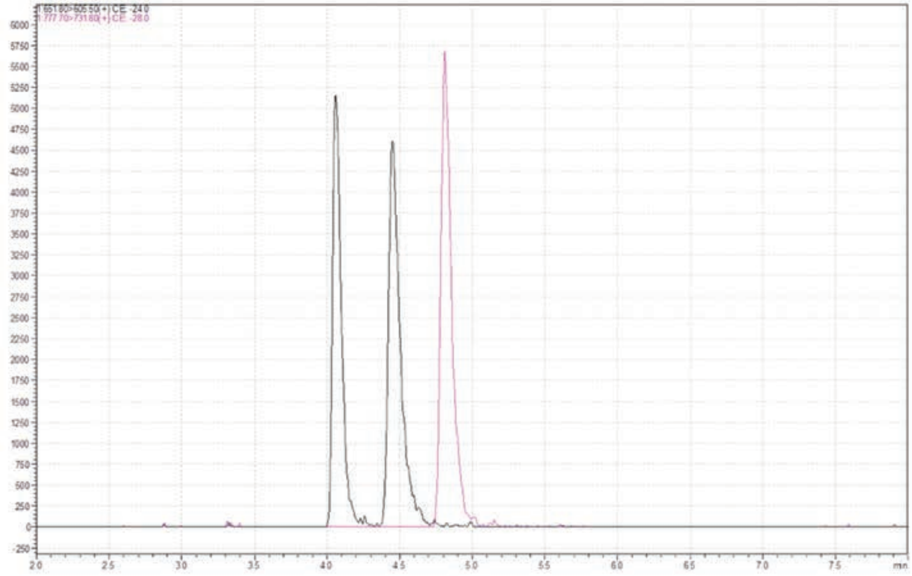
Table 1 and 2 shows the ruggedness of the on-line SPE-LC/MS with C8 and RPA cartridge, respectively, from 120 consecutive injections of the dirty human serum samples. As can be seen, the retention time of the thyroid analytes with C8 or RPA is very reproducible, with RSD's of 0.1%-0.2. The reproducibility (RSD%) of the peak area of the thyroid analytes with C8 and RPA cartridges is 6.2-7.0% and 5.1%-7.7%, respectively, which indicates great reproducibility.



Peak	Analyte	Peak width at 50% height (s)	Tailing factor
1	T3	3.7	1.6
2	rT3	5.2	1.5
3	T4	4.9	1.4

- Sample: 100 ng/mL spiked in human serum, 2 µL injection, the 120th injection.
- All peaks are narrow: <6s peak width at half height
- Peaks are all symmetric with a bit of tailing: tailing factors 1.4-1.6.
- Baseline is low and clean: no interference peaks

Figure 3: Representative LC/MS chromatogram of thyroids in human serum with C8 on-line cartridge.



Peak	Analyte	Peak width at 50% height (s)	Tailing factor
1	T3	4.4	1.5
2	rT3	5.6	1.8
3	T4	5.2	1.6

- Sample: 100 ng/mL spiked in human serum, 2 μL injection, the 100th injection.
- All peaks are narrow: <6s peak width at half height
- Peaks are all symmetric with a bit of tailing: tailing factors 1.5-1.8.
- Baseline is low and clean: no interference peaks

Figure 4: Representative LC/MS chromatogram of thyroids in human serum with RPA on-line cartridge.

Comparing the two types of on-line cartridges, RPA appears to deliver greater signals (peak height and area) for all three thyroid analytes compared to the C8 cartridge. The RPA phase has amide groups that are known to form hydrogen bonds and can provide better retention for analytes through hydrogen bonding interactions. Although the retention times and peak widths for thyroid hormones on the analytical column is very similar whether using the RPA or the C8 SPE cartridges, there appears to be a slight advantage of using RPA SPE with regards to the analyte's retention from the direct injection of protein precipitated serum samples.

Summary

An on-line SPE-LC/MS method has been developed for the rapid detection of thyroid hormones in human serum with minimal hands-on effort and time-consuming steps. Both C8 and RP-Amide on-line cartridges were shown to be capable of capturing trace amounts of thyroids from protein precipitated human serum samples. All three thyroid analytes, T3, rT3 and T4 were resolved on a Biphenyl column, with sharp and symmetrical peak shapes. In addition, reproducibility (RSD%) of the retention time of the thyroids from 120 consecutive injections is between 0.1% and 0.2%, with either C8 or RPA on-line cartridges, while the peak area reproducibility (RSD%) is between 5.1% and 7.7%. These RSD's indicate great ruggedness of the on-line SPE-LC/MS system.

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Table 1: Ruggedness of the system with C8 Cartridge.

Reproducibility of retention time and peak area

Analyte	MRM Quantifier	Retention time (min) (Avg. n = 120)	Retention time reproducibility (RSD%, n=120)	Peak area (Avg. n = 120)	Peak area reproducibility (RSD%, n = 120)
3,3',5-triiodo-L-thyronine (T3)	651.8 / 605.5	4.03	0.2	27046	5.1
3,3',5-triiodo-L-thyronine (rT3)	651.8 / 605.5	4.43	0.2	33723	6.2
3,3',5,5'-tetraiodo-L-thyronine (T4)	777.7 / 731.8	4.79	0.2	23766	7.7

Table 2: Ruggedness of the system with RPA Cartridge.

Reproducibility of retention time and peak area

Analyte	MRM Quantifier	Retention time (min) (Avg. n = 120)	Retention time reproducibility (RSD%, n=120)	Peak area (Avg. n = 120)	Peak area reproducibility (RSD%, n = 120)
3,3',5-triiodo-L-thyronine (T3)	651.8 / 605.5	4.13	0.1	17711	6.9
3,3',5-triiodo-L-thyronine (rT3)	651.8 / 605.5	4.53	0.2	22081	7.0
3,3',5,5'-tetraiodo-L-thyronine (T4)	777.7 / 731.8	4.89	0.1	22233	6.2

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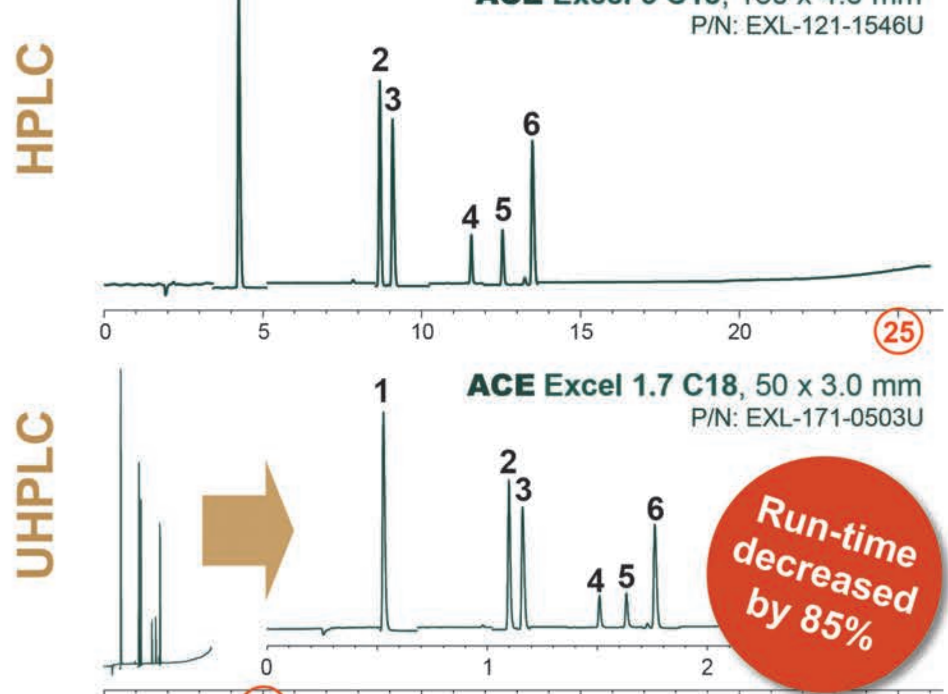

Mobile Phases: A = 0.05% TFA in H_2O; B = 0.05% TFA in MeCN
Temperature: 60 °C
Detection: UV, 220 nm
HPLC: VWR Hitachi Chromaster
UHPLC: VWR Hitachi ChromasterUltra Rs

Sample: 1. Gly-Tyr, 2. Tyr-Tyr-Tyr, 3. Val-Tyr-Val, 4. Oxytocin, 5. Angiotensin II, 6. Leu-enkephalin

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Uniform and Reliable Magnetic Beads for Protein Immunocapture Workflows

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Magnetic beads are ubiquitous in the field of genomics but are also a critical component of protein therapeutic analysis [1,2]. Typically utilised in the pharmacokinetic (PK) and pharmacodynamic (PD) laboratories, magnetic beads are used to extract and quantitate proteins from biologic matrices, which is predominantly considered a ligand binding assay (LBA) or immunocapture. The sensitivity, accuracy, and reliability of these quantitation methods are essential during safety and efficacy testing. Enzyme-linked immunosorbent assays (ELISA) are the most established LBA protocol, but magnetic beads are emerging as the tool of choice to streamline the immunocapture process. A common magnetic bead immunocapture approach starts with streptavidin-coated beads that are activated with a biotinylated anti-idiotypic reagent. This strategy allows specific binding to the protein of choice and takes steps toward platforming the protocol.

More modern strategies are geared toward combining LBA with LC-MS/MS which creates a more sensitive process with increased linear dynamic range (LDR) [3,4]. While magnetic beads are a mature technology, advances in the grafting process can lead to increased binding capacity and more dependable data. This article demonstrates a hybrid LBA/LC-MS/MS approach [5-7], utilising immunocapture of large molecule therapeutics with streptavidin-coated magnetic beads, followed by quantitation of signature peptides using LC-MS/MS. This strategy necessitates a consistent and reliable sample preparation procedure that can be platformed to different large molecule therapeutic modalities, which offers a considerable benefit over traditional methods [8,9].

Materials and Methods

Rituximab and insulin aspart (Novolog) were purchased from Myoderm® (Norristown, PA). Trypsin was purchased from Promega® Corporation (Madison, WI). SiLuMab and Dulbecco's Phosphate Buffered Saline (DPBS) were purchased from Sigma-Aldrich® (St Louis, MO). bioZen™ MagBeads, bioZen Peptide XB-C18 LC column, and bioZen Peptide PS-C18 LC column are from Phenomenex® (Torrance, CA). LC-MS/MS methods were performed on an Agilent® 1290 equipped with a SCIEX® 6500+ or a SCIEX X500B QTOF.

Magnetic Bead Activation – Representative Protocol (Figure 1)

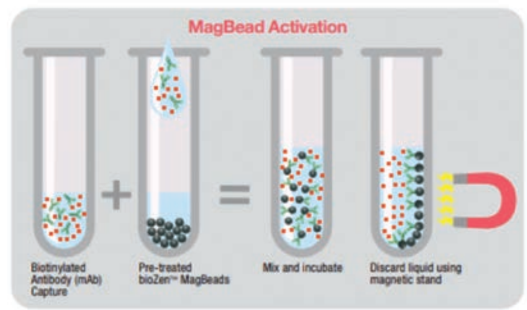
A 25 µL aliquot of bioZen MagBeads (20 mg/mL) was washed with 500 µL PBS buffer. Excess liquid was discarded using a magnetic stand (3x). The beads were reconstituted to original volume with PBS. 5 µg of anti-insulin and proinsulin antibodies were added and incubated at room temperature for 1 h with shaking speed of 1200 RPM using a deep well plate thermoshaker. The excess liquid was discarded using a magnetic stand. The beads were washed with 500 µL PBS buffer and the excess liquid was discarded using a magnetic stand (3x). The beads were reconstituted to original volume with PBS.

Immunocapture – Representative Protocol (Figure 1)

250 µL plasma samples were added to a 96-well plate. The activated beads were vortexed to mix thoroughly and 25 µL was added to each well containing plasma. The plate was covered and spun down at 800 RPM for 3 s before incubating at 1200 RPM for 2 h on a thermoshaker. The excess liquid was discarded using a magnetic stand.

Washing and Elution – Representative Protocol

200 µL of 0.5 % CHAPS in PBS buffer was added to the beads and the solution was mixed then centrifuged at 800 RPM for 3 s. The liquid was then discarded using a magnetic stand. The beads were washed with 200 µL PBS, mixed and shaken for 10 m at 1200 RPM using a deep well plate thermoshaker. The resultant mixture was centrifuged at 800 RPM for 3 s and the excess liquid was discarded using a magnetic stand for 2 m. 70 µL methanol/water/acetic acid (50:48:2) was added, mixed, and shaken at 1200 RPM for 10 min using a deep well plate thermoshaker. The mixture was centrifuged at 800 RPM for 3 s then placed on a magnetic stand for 10 m. The supernatant was transferred to a different 96-well plate and 45 µL water was added and



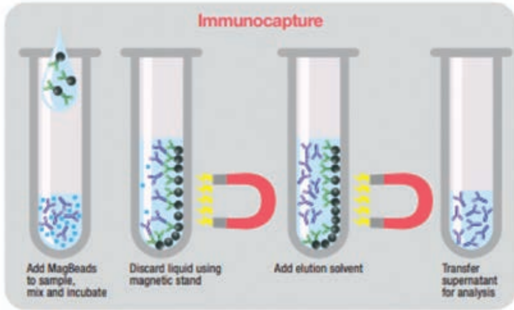


Figure 1: Sample Preparation Procecure Visual.

mixed. The plate was plated on a magnetic stand for 10 m then the supernatant was transferred to an injection plate.

Trypsin Digestion Protocol

For samples requiring a trypsin digestion, the supernatant of the wash/elution step was placed into a 96-well plate. The eluted samples were diluted with ammonium bicarbonate, ensuring pH >7.0. Samples were then heat denatured to 95°C. After cooling to <50°C, trypsin was added, and the mixture was shaken at 300 RPM for 1 h at 50°C. After centrifugation, the sample was used for LC-MS/MS analysis.

Results and Discussion

The uniformity of the bioZen MagBeads was assessed by scanning electron microscopy (SEM). The particle size of the streptavidin-coated beads was 1 µm and displayed excellent uniformity (Figure 2). Along with a patented streptavidin coating process, the consistent particle size lends to more efficient binding, thus lower background.

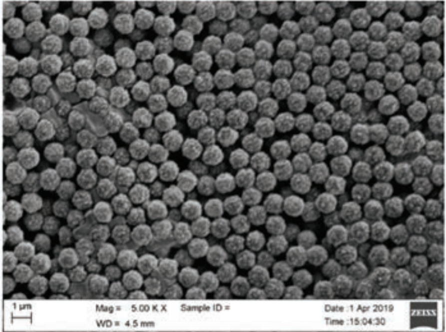


Figure 2: Uniform Particle Size of Magnetic Beads Determined by SEM.

To assess consistent immunocapture, two different lots of the activated magnetic beads were evaluated (Figure 3). With

Mag Bead	Correlation Coefficient
bioZen MagBeads Lot 1	0.9914
bioZen MagBeads Lot 2	0.9941

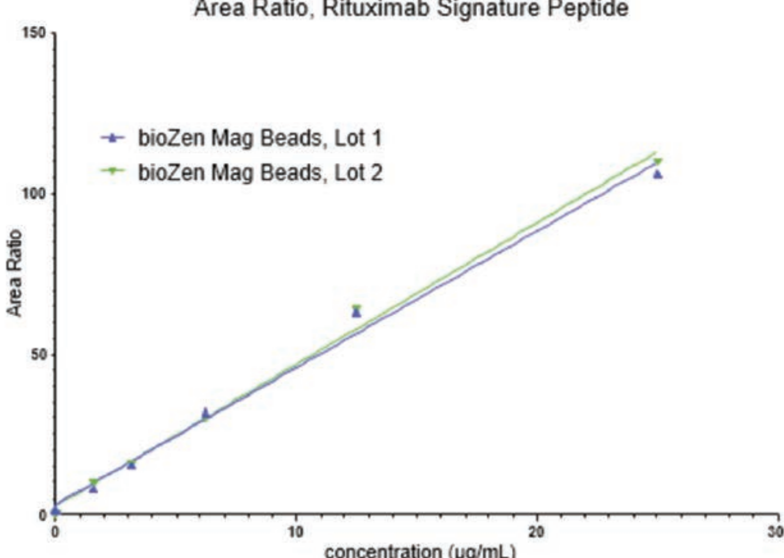


Figure 3: Correlation Coefficient Assessment of Multiple Bead Lots.

correlation coefficients greater than 0.99 for both lots, a robust LBA protocol was confirmed. This lot-to-lot reproducibility assessment is important to ensure consistency in biotinylated capture antibody, which could lead to potential variation in the linear dynamic range of the assay.

Insulin analogues are growing in the biotherapeutic industry [10] and thus, the bioanalytical immunocapture workflow is significant. The experiment commenced with a calibration curve to ensure good linearity with the external standard (Figure 4). A correlation coefficient of 0.99814 was determined for a LDR from 50-10,000 pg/mL. Proceeding to analyse insulin, we assessed samples at 50 pg/mL and 500 pg/mL to qualify the binding capacity of the magnetic beads (Figures 5 and 6, respectively).

Excellent recovery of each sample was observed even at 50 pg/mL, although some inherent background noise was also observed at this concentration.

Hybrid LBA/LC-MS/MS

Rituximab is a classic example of a commercial therapeutic monoclonal antibody in the biopharmaceutical industry. Utilising the magnetic bead LBA protocol in tandem with the trypsin digestion protocol described above, six rituximab signature peptides were analysed on a bioZen Peptide XB-C18 LC column (Figure 7). Good chromatographic separation of the peptides was observed, and each peptide demonstrated appropriate ionisation for the LC-MS/MS analysis. Notably, the integrity

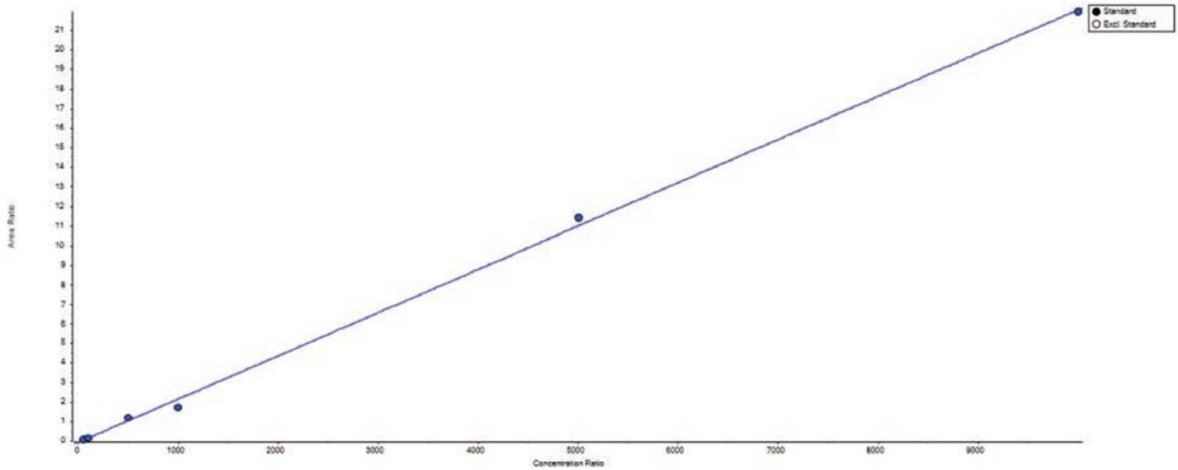


Figure 4: Calibration Curve from 50 pg/mL-10,000 pg/mL.

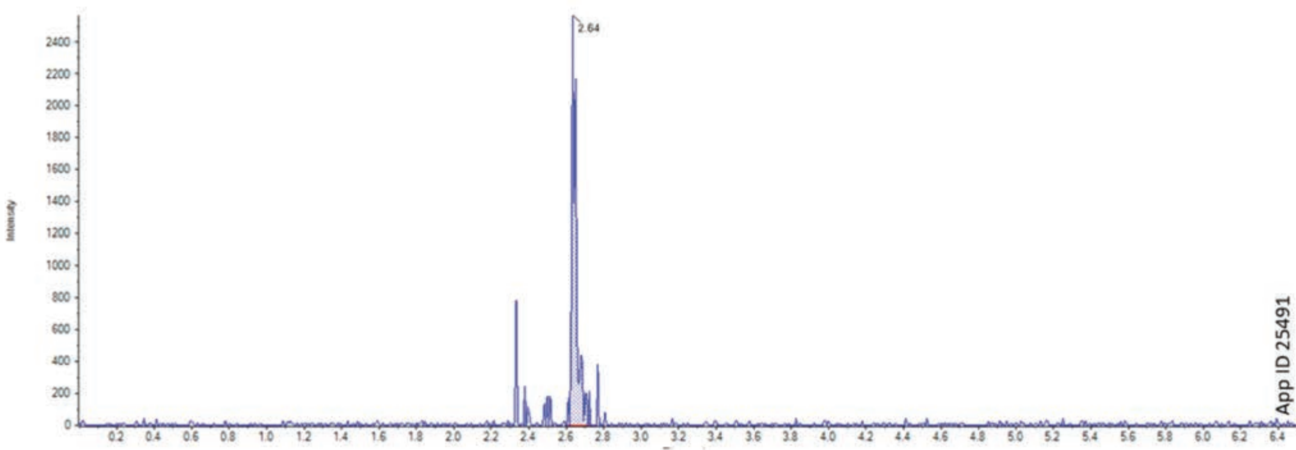


Figure 5: 50 pg/mL Extracted Insulin Aspart Standard Using bioZen MagBeads.

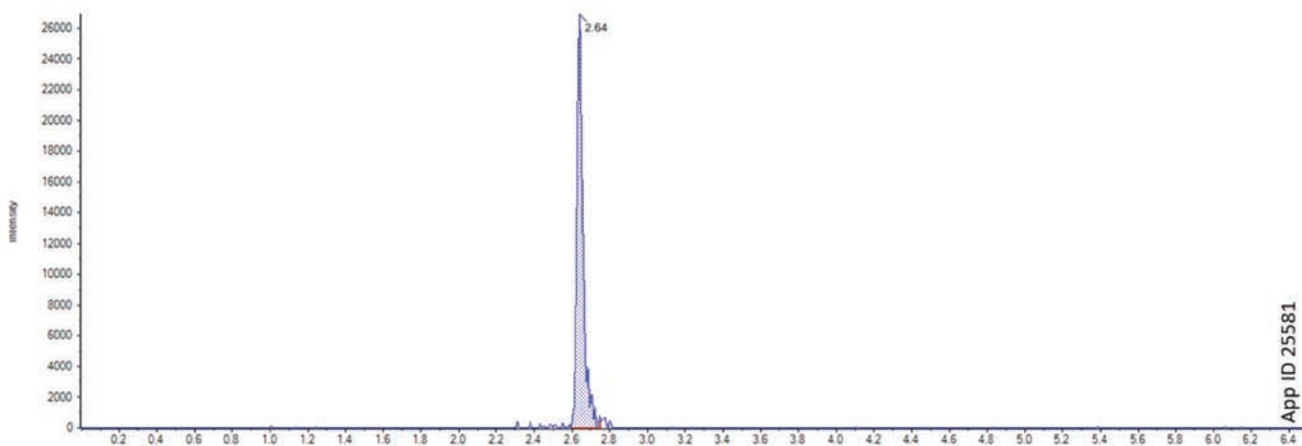


Figure 6: 500 pg/mL Extracted Insulin Aspart Standard Using bioZen MagBeads.

of the signature peptides is excellent which confirms a robust sample preparation protocol.

An important feature of this protocol is the similarity of the immunocapture procedure of rituximab compared to insulin. While the proteins are very different in structure, the immunocapture procedure had minimal modifications to achieve similar results. This fact suggests the broad applicability of the magnetic bead protocol and the ability to adopt it as a platform method.

Conclusion

As the complexity of therapeutic modalities increases, the bioanalytical field must adapt. Robust immunocapture and hybrid LBA/LC-MS/MS protocols using bioZen MagBeads are demonstrated. Because hybrid LBA/LC-MSMS utilises MS as the detection, specificity of the assay can be improved while sensitivity can be similar to traditional ELISA methods.

By utilising streptavidin-coated magnetic beads, the method can be platformed in such a way that only the capture antibody or biotinylated target can be modified. This concept is demonstrated by the workflow being used for both a monoclonal antibody, as well as for a peptide therapeutic. Finally, this workflow not only extends to different therapeutic modalities, but it also applies to other large molecule bioanalytical workflows including intact quantitation or biotransformation, which also could implement a similar sample preparation strategy.

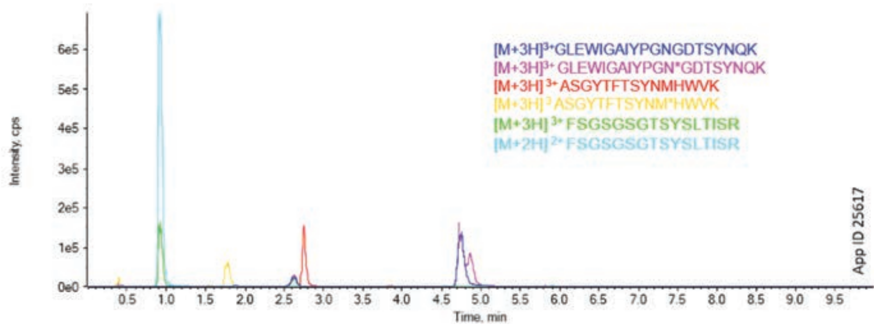


Figure 7: XIC of the Signature Peptides of Rituximab on a bioZen Peptide XB-C18 column.

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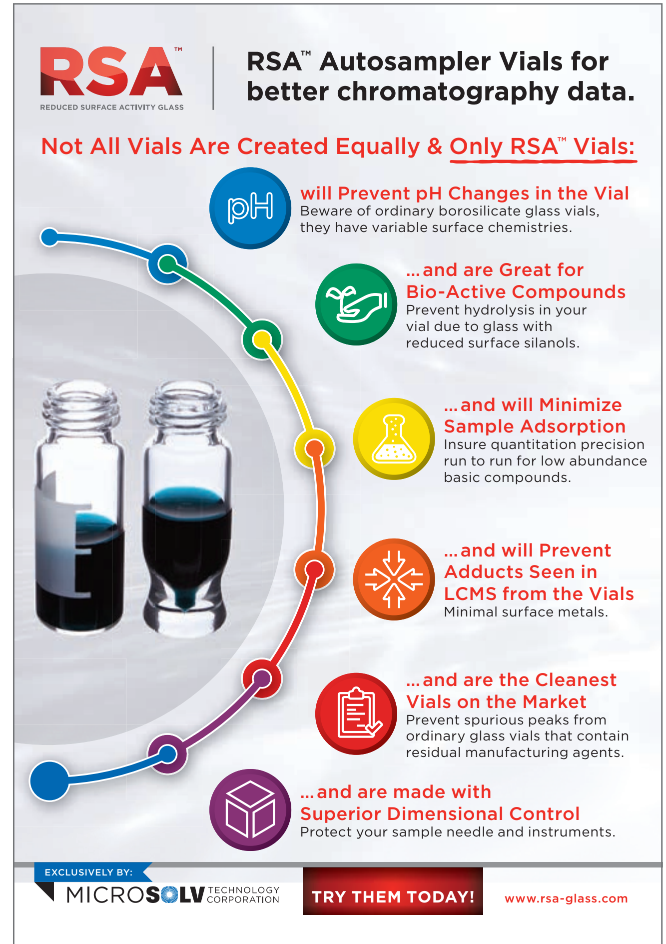

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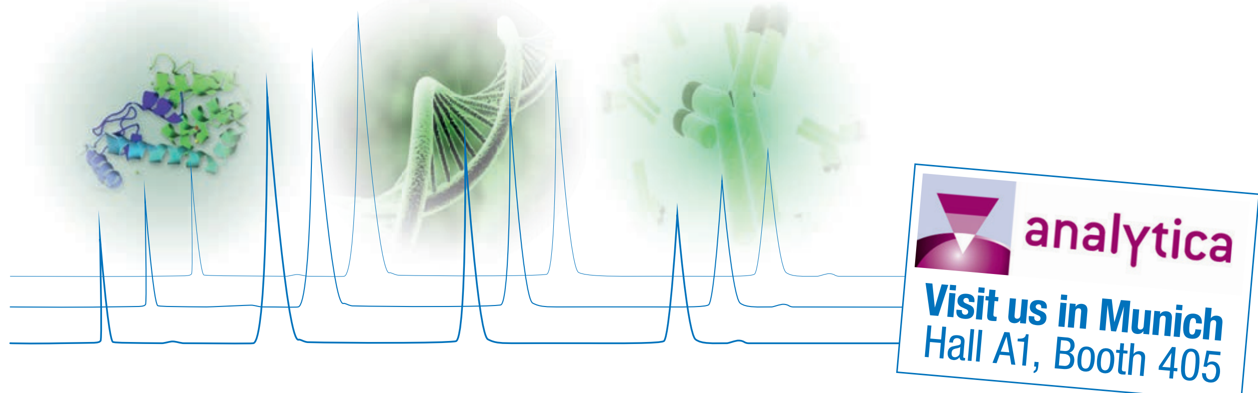

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The use of Mobile Phase pH as a Method Development Tool

In LC separations, the mobile phase pH determines the ionisation state of ionisable analytes. The mobile phase pH can therefore be varied and used as a powerful tool to control analyte retention, peak shape and selectivity. This short article explains how retention of acidic, basic and neutral analytes is affected by mobile phase pH, as well as the requirements for carrying out separations at high and low pH. It also discusses how the chromatographer can utilise pH during method development.

Introduction

The fundamental goal of any LC separation is to obtain suitable resolution of the key analytes of interest. The fundamental resolution equation states that the resolution between two analyte peaks is governed by the separation efficiency (N), analyte retention (k) and separation selectivity (α) [1]. Of these three parameters, α has the biggest impact on resolution and therefore, it pays to invest method development time in optimising the separation selectivity. Many analytical parameters can be used to affect the selectivity, in particular, optimisation of the column stationary phase, the organic modifier, percentage organic and temperature etc. For separations involving ionisable analytes, mobile phase pH can have a profound effect on analyte retention and selectivity. It is, therefore, an important parameter to investigate during method development.

Analyte retention at different mobile phase pH

To a large degree, analyte retention in reversed-phase is dictated by analyte hydrophobicity. For ionisable analytes, as the degree of ionisation increases, retention typically decreases (providing that no alternative modes of interaction such as ion exchange are present). Figure 1 summarises how the mobile phase pH affects the degree of ionisation for simple acidic and basic compounds. For basic analytes, at mobile phase pH's below their pK_a, the analyte will primarily be positively charged. At high pH (above their pK_a), they will be in their neutral form and will be better retained by reversed-phase. Conversely, acidic species show their strongest retention with a mobile phase below their pK_a and are more weakly retained at high pH, in their deprotonated form.

To demonstrate the effect of mobile phase pH on analyte retention, a set of basic, acidic and neutral analytes were chromatographed at different pH's (Figure 2) on a high pH stable reversed-phase C18 column. All other separation parameters such as gradient profile, buffer concentration and temperature were kept constant and changes in analyte retention are therefore determined by the mobile phase pH.

Toluene contains no ionisable functionality and is therefore neutral over the entire pH range. This means that mobile phase pH has no significant effect on the retention of toluene. At low pH, the acidic analytes (3,4-dichlorobenzoic acid and mefenamic acid) are present in their non-ionised, neutral form and therefore show their strongest retention. As the mobile phase pH is increased to the analytes pK_a and beyond, the degree of ionisation increases and a gradual decrease in retention is observed. In contrast, the basic analytes (nortriptyline and carvedilol) are positively charged at low pH and consequently show shorter retention. As the pH increases, the ionisation is suppressed and analyte retention increases. Protonated basic analytes may exhibit low retention and/or poor peak shape when analysed at low pH. Performing the analysis at high pH (neutral form), is an approach which can dramatically improve both peak shape and retention and also provide gains in sensitivity (Figure 3).

It is important to note that, for maximum column lifetime, many silica based reversed-phase columns should be used within a limited pH range of approximately 2-8 and are therefore not suited to high pH work. To perform a high pH separation, it is therefore essential to use a stationary phase that is compatible with high pH mobile phases. A number of columns that can

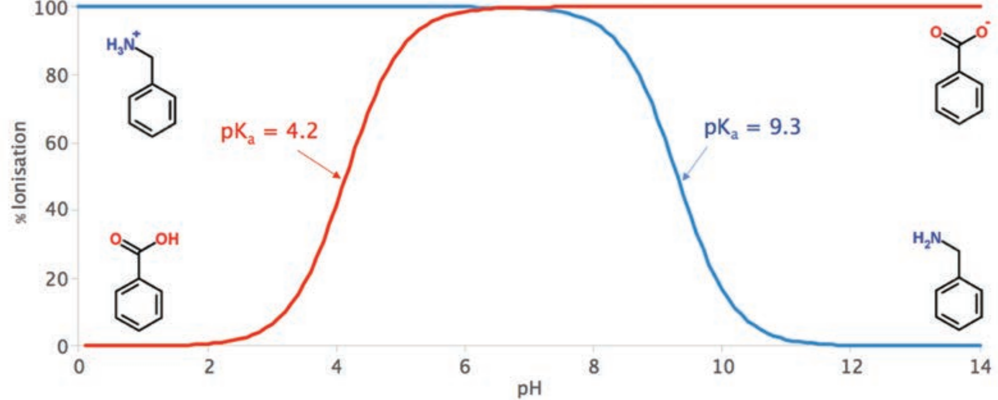
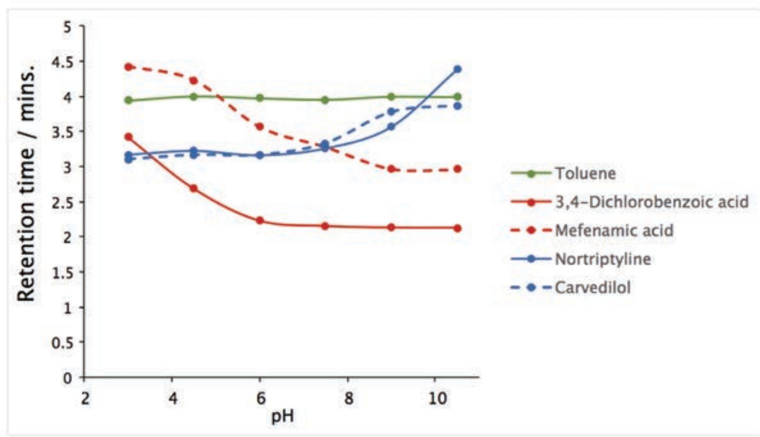


Figure 1: Percentage ionisation of acidic (red) and basic (blue) analytes at various pH values.



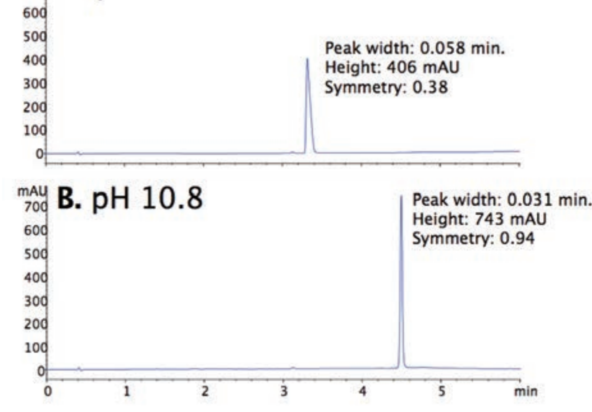


Figure 2: Effect of mobile phase pH on analyte retention.
Column: ACE Excel 3 SuperC18, 50 x 2.1 mm; Mobile phase: A: 20 mM ammonium formate pH 3.0, 4.5, 6.0, 7.5, 9.0 and 10.5 (aq), B: 20 mM ammonium formate pH 3.0, 4.5, 6.0, 7.5, 9.0 and 10.5 in MeCN/H_2O 9:1 v/v; Gradient: 3 to 100% B in 5 minutes; Flow Rate: 0.6 mL/min; Injection Volume: 1 µL; Temperature: 40°C; Detection: UV, 214 nm.

Figure 3: Chromatograms of carvedilol on an ACE Excel 3 SuperC18, 50 x 4.6 mm column with (A) low pH and (B) high pH mobile phases.

tolerate high pH are commercially available (such as those shown in Figures 2, 3 and 4) and are typically manufactured from a hybrid organo-silica material, polymer based, or utilise modified bonding technology.

Using pH to control analyte selectivity

From Figures 2 and 3, it is clear that when ionisable analytes are present in a sample, the selectivity between analytes can vary significantly with mobile phase pH. For such samples, it is highly recommended that mobile phase pH is explored during initial method development, to determine the most suitable option for the sample. When beginning any method development, it is useful to consider analyte structures and properties, if known, to anticipate any acidic/basic behaviour. If unknown, screening the sample on a generic gradient with low and high pH mobile phases can be a productive starting point. Figure 4 shows an example gradient separation of a set of acidic, basic and neutral analytes at low and high pH; analysed on a novel stationary phase with extended pH compatibility (pH 1.5-11.0). Neutral analytes, or analytes whose ionisation state remains unchanged within the pH range examined, show little change in retention with pH (e.g. peak #11). In contrast, all the acidic analytes show a sharp decrease in retention as mobile phase pH increases, whereas the opposite is observed for basic compounds. Clear differences in selectivity are observed: for example, peaks 5 and 12 show complete reversal in elution order. Mobile phase pH can also provide useful selectivity for samples containing just acidic or basic components: for example, the resolution between the bases carvedilol (9) and trimipramine (12) increases dramatically from low to high pH.

For method robustness, it is generally recommended to work at a mobile phase pH at least 2 pH units away from the analyte pK_a. Often this may not be possible, e.g. for complex samples or for multifunctional analytes containing moieties with overlapping pK_a values. In these situations, it is important to accurately control and document mobile phase preparation to ensure consistent pH between batches of mobile phase. Examining the effect of small changes in pH on the separation is also highly recommended to assess the method robustness.

Additional benefits of changing mobile phase pH

Careful selection of mobile phase pH can also provide several other benefits for the chromatographer. For LC/MS applications, pH can provide enhanced sensitivity in some cases. For example, using a high pH mobile phase to ionise acidic components may enhance detection in negative ion mode. For preparative applications and analyses involving one or more overloaded peaks (e.g. impurity testing), working at a pH where the analyte is neutral can be beneficial. Figure 5 summarises a loading study for amitriptyline at 2 different pH's. At low pH, amitriptyline is positively charged and shows peak tailing, whereas at high pH (non-ionised form), peak shape is vastly improved. As the sample load on column is increased, the peak width at low pH increases significantly. This could lead to loss of resolution or difficulty in observing/quantifying smaller impurity peaks. At high pH, however, the peak width is more constant as the mass of analyte on column is increased.

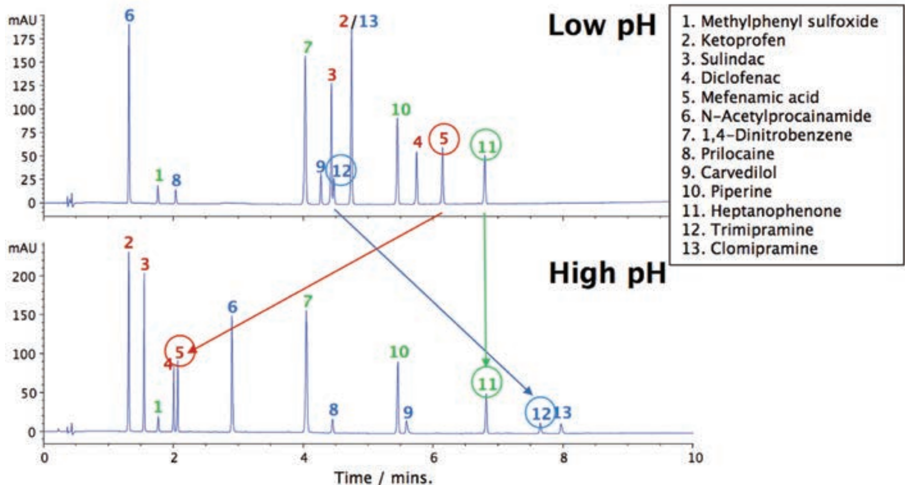
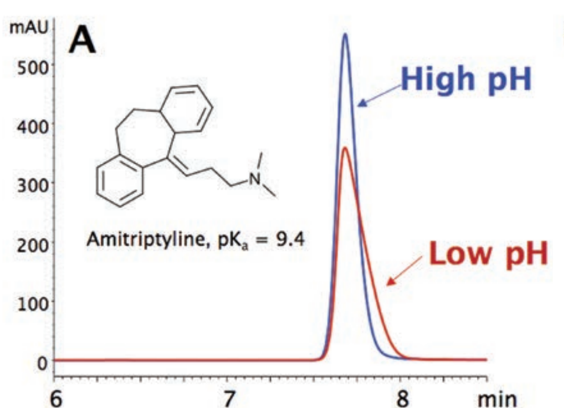


Figure 4: Separation of a range of acidic, basic and neutral analytes on a high pH compatible solid-core column with novel encapsulated bonding.
Column: ACE UltraCore 2.5 SuperPhenylHexyl, 100 x 3.0 mm; Mobile phase (low pH): A: 15 mM ammonium formate pH 3.0 (aq), B: 15 mM ammonium formate pH 3.0 in MeCN/H_2O 9:1 v/v; Mobile phase (high pH): A: 0.1% NH_3 (aq) B: 0.1% NH_3 in MeCN/H_2O 9:1 v/v; Gradient: 5 to 100% B in 10 minutes; Flow Rate: 1.2 mL/min; Injection Volume: 1 µL; Temperature: 40°C; Detection: UV, 260 nm



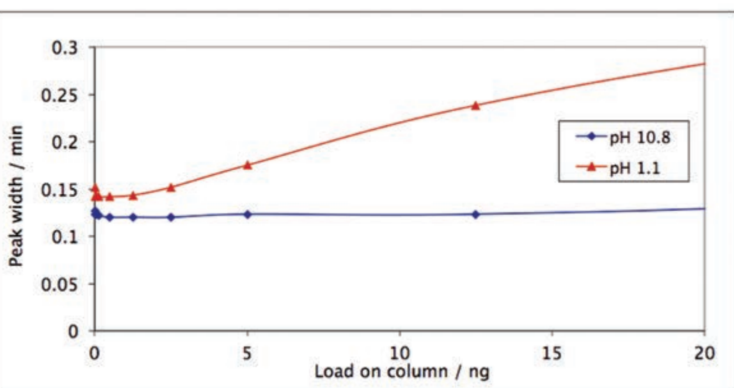


Figure 5: Loading study for amitriptyline at low and high pH. (A) Comparison of peak shape (normalised retention time) and (B) comparison of peak width as analyte load on column is increased. Note that mobile phase composition was set to obtain a retention factor (k) of approximately 5 at both pH values.
Column: ACE Excel 3 SuperC18, 150 x 4.6 mm; Mobile phase (low pH): 1% TFA in MeCN/H_2O 44:56 v/v; Mobile phase (high pH): 0.1% NH_3 in MeCN/H_2O 77:23 v/v; Flow Rate: 1.0 mL/min; Injection Volume: 5 µL; Temperature: 40°C; Detection: UV, 254 nm.

Conclusion

When working with ionisable analytes, the mobile phase pH can dramatically affect analyte retention behaviour. The mobile phase pH is therefore an important parameter to consider during method development. This article has shown how pH can be utilised during method development to alter selectivity and help optimise the separation. Appropriate pH selection also provides additional gains in retention, peak shape and sensitivity.

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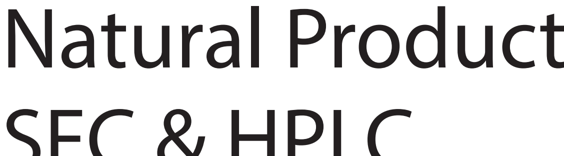

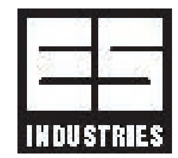

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Elimination of the Sample Solvent Effect when Analysing Water Solutions of Basic Peptides by HILIC

by Mattias Malm, Johan Kjellström, Global Pharmaceutical R&D, Ferring Pharmaceuticals A/S, Copenhagen, Denmark*

The Hydrophilic Interaction Chromatography (HILIC) separation mode offers many advantages for the analysis of basic peptides, such as good peak shape and separation efficiency, compared to, e.g., reversed-phase chromatography. However, the need to match the sample solvent with the highly organic mobile phase is limiting the use of HILIC. This article explores the suitability of a column-switch approach to enable the use of HILIC for large injection volumes of aqueous samples.

Introduction

The determination of potency and purity of the active pharmaceutical components is important in many stages of drug development, such as formulation development and stability studies for determination of shelf-life. Reversed-phase liquid chromatography (RPLC) is commonly used for this type of determinations. RPLC is a rugged and well-established technique for both small molecules and biomolecules. However, basic molecules, such as peptides containing the amino acids lysine or arginine, are difficult to analyse by RPLC, due to unwanted silanol interactions with the analyte, which can cause peak tailing and peak broadening [1].

An ion-pair reagent (such as TFA), or high amounts of salt in the mobile phase, can reduce such unwanted interactions, but can, on the other hand, cause problems if MS-detection is used. TFA causes ion suppression [2] and thus a decreased MS response, and non-volatile buffer salts are directly unsuitable to use in combination with MS.

Based on our experience, HILIC is an attractive technique for basic peptides since it provides good peak shape with MS-compatible mobile phases.

It is generally assumed that the retention in HILIC is mainly caused by partitioning of the analytes between the mobile phase and a water-enriched solvent layer close to the hydrophilic column surface [3,4]. This mechanism could explain the reduced secondary interactions in HILIC since the analytes are not interacting with the column material itself, or at least to a lesser extent, compared to RPLC.

What is limiting the use of HILIC is the need to match the sample solvent to the highly organic mobile phase. Since water is the strong solvent in HILIC, injection of aqueous solutions will lead to partial elution of the analytes at time of injection. This is called the sample solvent effect, which can lead to peak distortion and loss of retention and efficiency [5]. Thus, peptides formulated as aqueous solutions are not suitable for direct analysis by HILIC.

This article presents a fully automated way to eliminate the sample solvent effect also for large injection volumes of aqueous peptide samples in HILIC, by using a column-switch approach.

Experimental (HILIC method)

Acetonitrile (JT Baker, Ultra Gradient Grade) was used as HILIC mobile phase A. HILIC mobile phase B was prepared by adding 7.7 g ammonium acetate (Merck, p.a.) and 2000 µl glacial acetic acid (Merck, p.a.) to 1000 ml of water (measured pH 5.1). The trapping mobile phase was 5% acetonitrile in water.

[Lys^{8}]vasopressin and [Arg^{8}]vasopressin

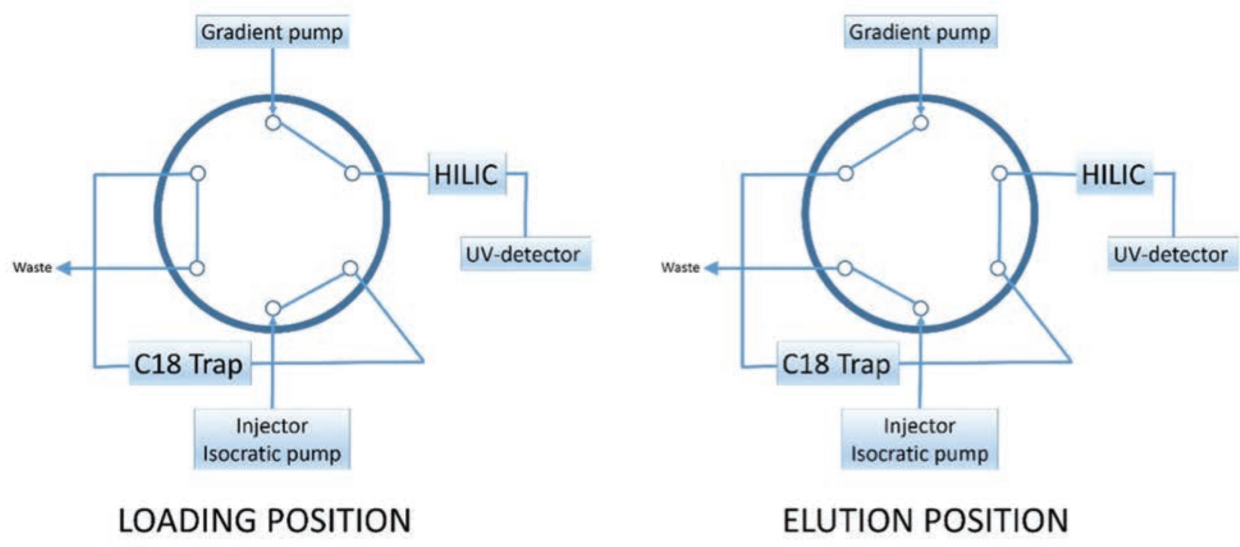

Figure 1. Configuration of the switching valve.

was purchased as lyophilised powder from Sigma-Aldrich and were dissolved in mobile phase B and mixed to contain 20 μg/ml of each component.

The experiment was performed on an Agilent 1260 HPLC with UV-detector, two pumps and one six port – two position switching valve, see Figure 1 for the instrument configuration. The trapping column was a 2.1x10 mm XTerra MS C_{18} precolumn (Waters). The HILIC column was an Accucore HILIC, 3.0 x 150 mm, 2.6 μm (Thermo Fischer Scientific). The column temperature was 60°C, and the detection wavelength was 277 nm. The injection volume was 100 μl.

The HILIC gradient is presented in Table 1. The flow of the trapping mobile phase was 1.0 ml/min.

Table 1: The HILIC gradient

HILIC gradient	%A	%B	Flow (ml/min)
0 min	85	15	0.8
1 min	85	15	0.8
20 min	62	38	0.8
21 min	85	15	0.8
30 min	85	15	0.8

For comparison the mixed vasopressin sample was also injected without using the column switching, i.e., the aqueous solution was injected directly on the HILIC column. All other method parameters remained the same.

Experimental (Reversed Phase method)

The same mobile phases, sample, and equipment were used in the reversed-phase experiment as in the HILIC experiment. The column switch was disconnected and the sample was injected directly on the column. The reversed-phase column was a Kinetex C_{18}, 3.0 x 150 mm, 2.6 μm (Phenomenex). All instrument settings were identical to the HILIC run, except the gradient and flow, see Table 2.

Table 2.

RP gradient	%A	%B	Flow (ml/min)
0 min	10	90	0.6
1 min	10	90	0.6
20 min	22	78	0.6
21 min	10	90	0.6
30 min	10	90	0.6

The reversed-phase method was not optimised for vasopressins and served only as a comparison to HILIC in terms of peak asymmetry and peak width with similar mobile phases.

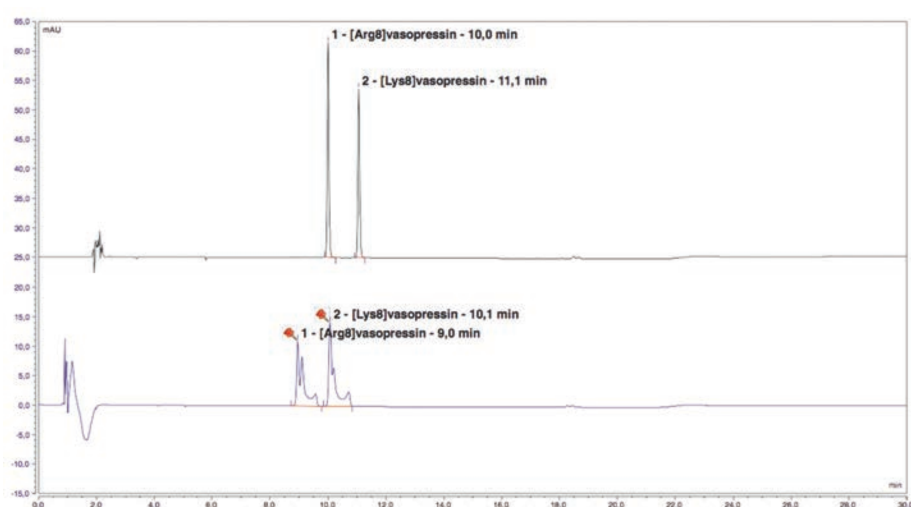

Figure 2: Overlay HILIC chromatograms of a) aqueous sample injected with the column switch approach (upper) and b) aqueous sample injected directly on the HILIC column (lower).

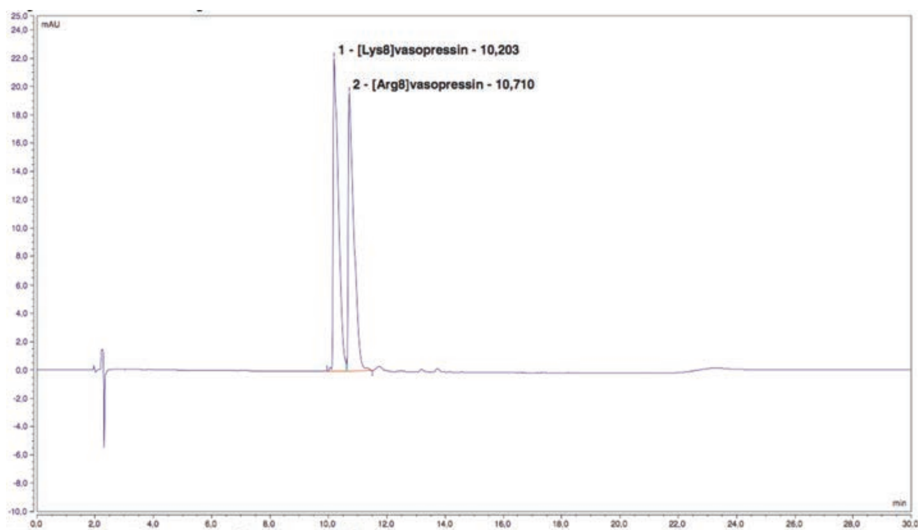

Figure 3: Reversed-phase chromatogram.

Results and Discussion

The HILIC and the reversed-phase chromatograms are presented in Figures 2 and 3, respectively, and the peak data comparison in Tables 3 and 4. The results show that HILIC is superior to reversed-phase chromatography for vasopressins in terms of peak width, asymmetry and resolution. In HILIC, the positively charged peptides elute as Gaussian peaks with an asymmetry factor of 1.0, whilst using MS-friendly mobile phases. In reversed-phase chromatography, the same peptides interact with the negatively charged residual silanol groups of the reversed phase bonded silica and show a high degree of tailing and peak broadening. Increased tailing and peak broadening decrease the peak capacity and the possibility to separate closely related impurities, which is observed by the decrease in resolution from 9.5 to 1.5 for HILIC and RPLC, respectively. It can be noted that the elution order of the two vasopressins is reversed in HILIC compared with reversed-phase, due to opposite column polarities.

Table 3. Peak characterisation data HILIC using column-switch.

Peak	Asymmetry factor (EP)	Resolution (EP)	Peak width (50% height)
[Arg^{8}]vasopressin	1.0	9.5	4 sec
[Lys^{8}]vasopressin	1.0	N/A	4 sec

Table 4. Peak characterisation data reversed-phase.

Peak	Asymmetry factor (EP)	Resolution (EP)	Peak width (50% height)
[Lys^8]vasopressin	3.3	1.5	11 sec
[Arg^8]vasopressin	3.4	N/A	13 sec

The chromatograms from injecting the aqueous sample in HILIC, with and without the column-switch installed, is compared in Figure 2. The results illustrate the problem of injecting an aqueous solution directly on to the HILIC column. With direct injection on to the column, the sample solvent effect causes severe peak splitting and peak broadening. However, when using the column switch approach, the sample solvent is removed before the HILIC column and thereby also the sample solvent effect.

The proposed setup with column switching and a trapping column works like an on-line solid-phase extraction method coupled with HILIC. Theoretically, there is no upper limit for the injection volume of aqueous sample since the sample is concentrated on the trap column and the sample solvent removed.

It should also be noted that the column-switching setup also de-salts the sample before being transferred to the HILIC column, since any buffer salts in the sample are not retained by the reversed-phase trap column and are sent to waste. Buffer salts are a potential problem in HILIC, since they are typically poorly soluble in high amounts of acetonitrile which could lead to precipitation and column blockage.

In summary, the presented technique provides a fully automated method to eliminate the sample solvent effect when analysing aqueous solutions by HILIC. A suggestion of a potential expansion of the principle can be to test different types of trap columns in combination with different mobile phases to explore selectivity and trapping.

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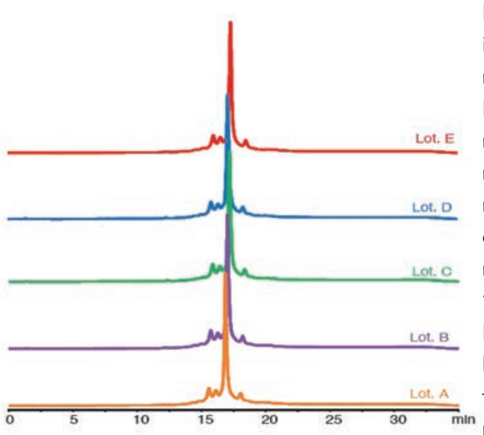

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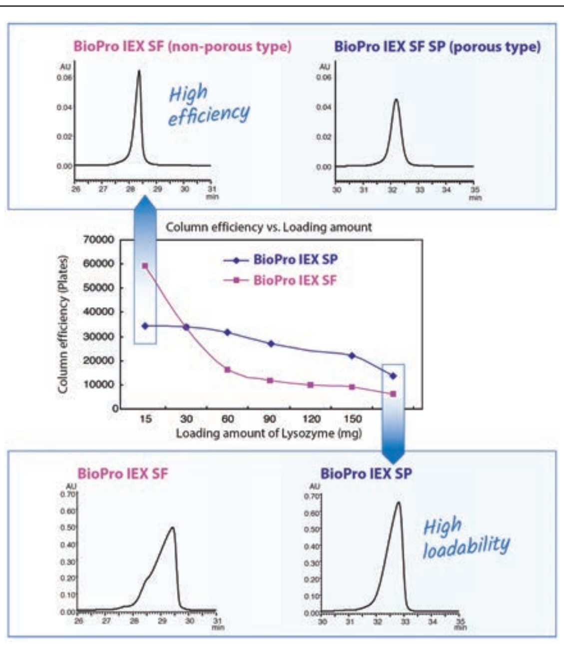

Method Development and Validation of Simultaneous Determination of Assay of Salmeterol Xinafoate and Fluticasone Propionate in Dry Powder Inhalers

by Serkan Acar[1], Devrim Çelik Sakızcı[1], Emine Yılmaz[1]*

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A new HPLC method has been developed as an alternative to existing pharmacopeia methods for the assay determination of Salmeterol and Fluticasone propionate Inhalation Powder. The chromatographic separation utilises an isocratic elution in which mobile phase consisting of a buffer potassium dihydrogen phosphate (pH 3.0) and acetonitrile at 1.5 mL min^{-1} flow rate, 40°C column temperature, 25°C tray temperature and gradient wavelength UV detection between 210-239 nm. A stainless steel column (15cm x 4.6mm, 5µm) packed with octadecylsilyl silica gel (Hypersil BDS) was employed.

Being validated in accordance with ICH guidelines, this method provides a safer and easier solution for assay determination of Salmeterol and Fluticasone propionate compared to pharmacopeia methods. The major benefits of the new method are; using a wavelength gradient for the quantitation of Salmeterol and Fluticasone propionate which give max. UV absorptions at different wavelengths, using a simple buffer solution that is prepared simply and which is non-toxic to the analyst.

1. Introduction

Being developed as a potent β2-adrenoceptor agonist and having the long acting bronchodilator profile, Salmeterol Xinofotate (SX) is used to open the airways in the lungs to make breathing easier in the treatment of asthma and chronic obstructive pulmonary disease known as COPD [1].

Largely used as an inhaled corticosteroid (ICS), Fluticasone propionate (FP) is commonly used in combination with Salmeterol Xinofoate [2], forming an inhalation product consisting of a long-acting beta2-adrenoceptor agonist (LABA) plus a corticosteroid [3]. It is proven that the twice daily therapy of combining Salmeterol and Fluticasone Propionate is more effective than the monotherapy of inhaled corticosteroids alone particularly in terms of enhancing lung function and reducing asthma symptoms. Moreover, as mentioned by McKeage and Keam, the combination of Salmeterol and Fluticasone Propionate ensures a powerful, strongly tolerated choice in the maintenance and treatment of asthma [3].

Cyplos 50/500 mcg Powder for Inhalation (Arven, Turkey) was developed as a fixed dose combination of Salmeterol and Fluticasone Propionate. For the assay determination of Salmeterol and Fluticasone Propionate Inhalation Powder, there are several assay methods provided by several authorities.

The US pharmacopeia (USP) published a pre-dispensed monograph for Fluticasone Propionate and Salmeterol inhalation powder. According to this monograph the assay method shows the separation of FP and SX with an octadecylsilyl bonded silica gel 5cm x 4.6mm, 3.5µm column with an isocratic elution of 0.01 M sodium dodecyl sulfate, methanol and acetonitrile at a flow rate of 2.0 mL/min. The column temperature is 40°C. Fluticasone Propionate UV is detected at 239 nm wavelength and Salmeterol detected using Fluorescence (FLR) detection with an excitation wavelength of 225 nm and a resulting emission of 305 nm [4].

The monograph for Fluticasone Propionate and Salmeterol Inhalation Powder published by British Pharmacopeia (BP) states that FP and SX are separated by a method using a flow rate of 1.5 mL/min, with a stainless steel column (20 cm x 4.6 mm) packed with 5 µm octadecylsilyl silica gel at 40°C. The method proposes a detection wavelength of 239 nm and a fluorescence detection with an excitation wavelength of 225 nm and an emission wavelength of 305 nm. The proposed mobile phase contains acetonitrile, methanol and a solution containing 0.2M ammonium acetate and 0.5% w/v tetrabutylammonium hydrogen sulphate in water [5].

The assay method provided by USP 41 employs Sodium Dodecyl Sulphate, a surfactant in the mobile phase preparation that is known to be flammable, harmful if swallowed, causes skin irritation and serious eye damage plus many more hazards [6]. Moreover, in the USP method and the method provided by the BP, FLR detector is used, which while being quite sensitive to any contamination caused by working conditions. Any contamination detected by

Compound	Chemical Name
Salmeterol EP Impurity A	(1RS)-1-[4-Hydroxy-3-(hydroxymethyl)phenyl]-2-[(4-phenylbutyl)amino]ethanol
Fluticasone Propionate EP Impurity C	6α,9-difluoro-17-[[(fluoromethyl)sulphanyl]carbonyl]-11β-hydroxy-16α- methyl-3-oxoandrosta-1,4-dien-17α-yl acetate
Fluticasone Propionate EP Impurity D	6α,9-difluoro-17-[(methylsulphanyl)carbonyl]-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17α-yl propanoate
Fluticasone Propionate EP Impurity G	6α,9-difluoro-17-[[(fluoromethyl)sulphanyl]carbonyl]-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17α-yl-6α,9-difluoro-11β,17-dihydroxy-16α-methyl-3- oxoandrosta-1,4-diene-17β-carboxylate

this detector is exhibited as an unknown peak in the chromatogram.

The new suggested method developed and proposed here utilises Potassium Dihydrogen Phosphate which is not classified as hazardous [7] instead of Sodium Dodecyl Sulphate. In addition, UV detection is used which is more common and shows fewer working condition contamination peaks compared to FLR detection. Furthermore, UV detection causes no selectivity difference in terms of the SX and FP peaks.

The purpose of this manuscript is to explain the assay method developed by Arven Pharmaceuticals for SX and FP fixed dose combination drug product, Cyplos 50/500 mcg Powder for Inhalation.

2. Materials and Methods

2.1. Reagents

SX, FP and their related impurities were purchased from a reputable API producer. Ortho-phosphoric Acid and Potasium Dihydrogen Phosphate Monohydrate were purchased from Merck Ltd. HPLC grade Acetonitrile was purchased from J.T.Baker. High purity deionised water was obtained from a Sartorius stedim, arium 611VF (Goettingen, Germany) purification system. Cyplos 50/500 mcg Salmeterol/Fluticasone Propionate Powder for Inhalation (Arven, Turkey) was used as the finished product. All impurities were European Pharmacopeia impurities as stated in Table 1.

2.2. Instrumentation

A Waters HPLC system consisting of inbuilt autosampler and quaternary gradient pump with an on-line degasser was used. The column compartment with temperature control and UV detector were engaged.

Empower software was used to obtain chromatographic data.

2.3. Chromatographic Conditions

Equipment: HPLC System

Column: Thermo BDS Hypersil C18
150 x 4.6 mm, 5 um

Flow Rate: 1.5 mL / min

Injection Volume: 40 µL

Column Temperature: 40°C

Tray Temperature: 25°C

Run Time: 10 mins

Wavelength: UV, 210 nm (Gradient Starting Wavelength)

Wavelength gradient:

Time (min)	Wavelength (nm)
0.00	210
5.50	210
5.60	239
9.00	239
9.10	210
10.00	210

A Thermo Fisher Hypersil BDS C18 (15 cm x 4.6 mm) 5 µm column was used as stationary phase and maintained at 40°C.

The Injection volume was 40 µL and the initial wavelength was 210 nm. In the analysis a wavelength gradient was applied representing the maximum wavelength response for SX and FP in Cyplos 50/500 mcg Salmeterol/Fluticasone Propionate Powder for Inhalation given above.

2.4. Preparation of Solutions

2.4.1. Mobile Phase

The mobile phase involved a fixed composition solvent A (pH 3.0 buffer) which was prepared by dissolving 1.0 g Potassium dihydrogen phosphate in a 1000 mL volumetric flask containing high purity deionised water and stirred at 1000 rpm for 20 minutes on a magnetic stirrer. The pH was adjusted to 3.00 ± 0.05 with 85% Ortho-phosphoric acid and stirred for a further 10 minutes.

Solvent A: Acetonitrile (520: 480 v / v) was prepared. Stirred for 15 minutes at 1000 rpm in a magnetic stirrer. The mixture was filtered through a 0.2 µm membrane filter and degassed for 2 minutes. The mobile phase was pumped through the column with a flow rate of 1.5 mL/min.

A Diluent mixture of acetonitrile : high purity deionised water (50:50, v/v) was also prepared for standard solution preparation.

2.4.2. Standard Solutions

Salmeterol Stock Standard Solution: ~14.53 mg Salmeterol Xinafoate standard (equivalent to 10.0 mg Salmeterol) was weighed and transferred into a 100 mL amber coloured volumetric flask and then diluted to volume with diluent stated above and dissolved by sonication in an ultrasonic bath for 15 minutes. The stock solution was then permitted to come to room temperature. ($C_{Salmeterol}$ = 0.1 mg/mL)

Fluticasone Propionate Stock Standard Solution: ~25.00 mg Fluticasone Propionate standard was weighed and transferred into a 100 mL amber coloured volumetric flask and then diluted to volume with aforementioned diluent. It was dissolved and dissolved by sonication in an ultrasonic bath for 15 minutes and allowed to come to room temperature. ($C_{Fluticasone\ propionate}$ = 0.25 mg/mL)

Standard Solution: 1.25 mL of the standard stock solution of Salmeterol and 5.0 mL of the standard stock solution of Fluticasone Propionate, transferred into a 50 mL amber coloured volumetric flask. The solution was made up to volume with dilution solution and mixed by shaking. The solution was filtered through 0.45 µm filter to a sample vial. ($C_{Salmeterol}$ = 0.0025 mg/mL, $C_{Fluticasone\ propionate}$ = 0.025 mg/mL)

2.4.3. Test solution from blister

Approximately 13.0 mg Cyplos 50/500 mcg Powder for Inhalation (containing 0.05 mg SX and 0.5 mg FP) was weighed in a 20 mL amber colour volumetric flask and made up to volume with dilution solution and dissolved by sonication in an ultrasonic bath for 15 minutes. The solution was then filtered through a 0.45 µm filter into a sample vial. ($C_{Salmeterol}$ = 0.0025 mg/ mL, $C_{Fluticasone\ propionate}$ = 0.025 mg/ mL)

2.5. System Suitability

The relative standard deviations for SX and FP peak areas of six replicate injections of standard solution should not be more than 2.0%.

2.6. Optimisation of the chromatographic conditions

Optimising the reverse phase HPLC parameters, several chromatographic conditions were tested in order to achieve a suitable peak resolution and peak shape for SX and FP.

2.6.1. Column Selection

Injections on to different columns types were conducted to achieve the best separation for the analyte peaks and other interfering blank and placebo peaks. The optimum peak shape, retention time, tailing factor, and column efficiency was achieved using a Hypersil BDS C18 column (15 cm x 4.6 mm, 5 µm).

2.6.2. Mobile Phase Composition

Different compositions of mobile phase were tested to obtain sufficient selectivity and retention time for the analyte peaks. With ammonium dihydrogen phosphate buffer, high sensitivity and selectivity were achieved when compared with other buffers. Based on peak shape, symmetry, retention time and peak tailing, pH 3.0 Potassium dihydrogen phosphate was selected as the buffer preparation to be used. Different gradient programs of pH 3.0 Potassium dihydrogen phosphate buffer and organic solvents were conducted and according to experiments with acetonitrile and methanol, higher retention time, higher column pressure and higher peak tailing were observed with methanol. Hence, acetonitrile was selected as the organic modifier. After many trials, based on the peak shape, peak symmetry, retention time and peak tailing a 1.5 mL/min flow rate was selected.

2.6.3. Detection Wavelength

SX, FP and their related substance peaks were scanned between 200 nm – 400 nm by photo-diode array detector. The maximum absorption of SX and FP was determined at 210 nm and 239 nm. Therefore, to obtain the maximum absorbance in one chromatogram a wavelength gradient was applied.

2.6.4. Buffer pH

Various trials on the pH of the Potassium dihydrogen phosphate buffer were made to achieve the optimum pH at which all API peaks are well separated. For the optimum peak shape and peak tailing, a buffer pH of 3.0 was selected.

The chromatographic conditions were optimised with a mobile phase of pH 3.0 Potassium dihydrogen phosphate buffer and acetonitrile mixture at gradient wavelength with a flow rate of 1.5 mL/min at 40°C column temperature and 40 µL injection volume. The typical HPLC chromatogram (Figure 1) shows a satisfactory separation of SX and FP.

2.7. Method Validation

The validation of the developed method was performed in accordance with the ICH Q2 (R1) guideline [8] for the following parameters: specificity, linearity, limit of quantitation (LOQ), accuracy, precision, robustness, and solution and mobile phase stability.

2.7.1. Specificity

Specificity is the ability of the substance to be analysed to be precisely determined in the presence of the matrix effect and additives ensuring the identity of the analyte(s) of interest.

Blank solution, placebo solution, standard solution and assay test solution were analysed and peaks from each of the solutions were determined for SX and FP. Moreover, the impurities in Table 1 were injected to the system in order to determine their relative retention times in the

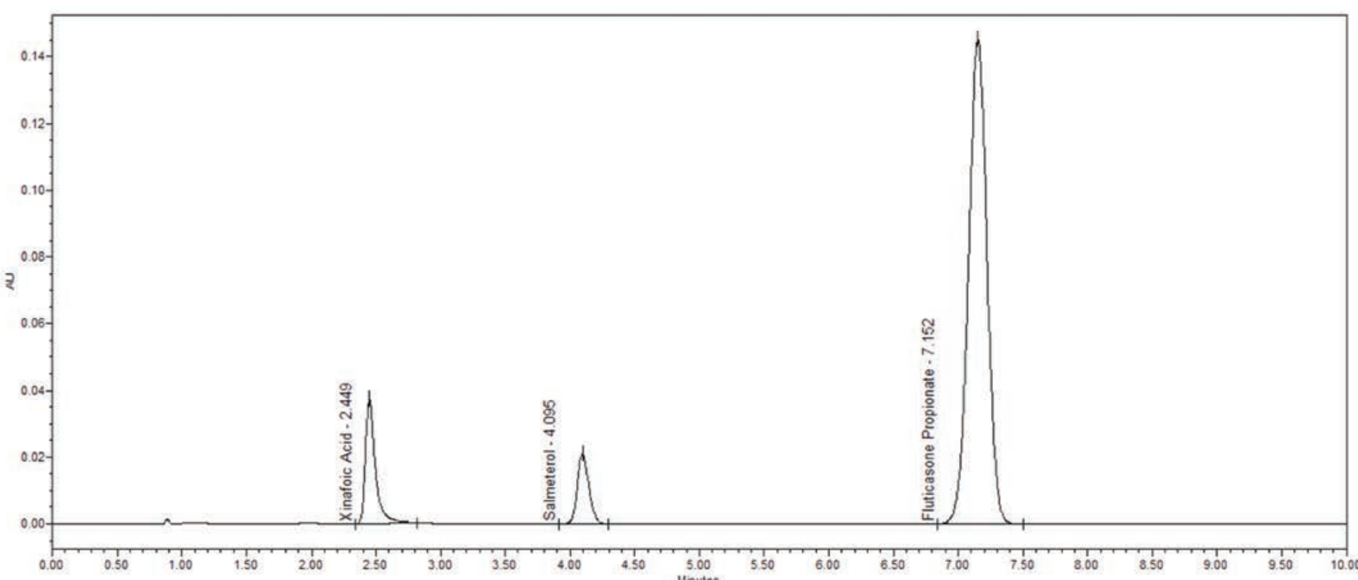


Figure 1: HPLC chromatogram showing the separation of SX and FP standard mixtures.

chromatogram hence preventing interfering peaks. With the given chromatographic conditions the PDA detector was used to detect the peak purity.

For a peak to be considered pure, purity angle must be less than purity threshold. SX and FP peaks in the chromatogram were found to be pure as it is seen in Table 2. All compounds were completely separated and no drift in analyte retention time was observed.

Table 2: Purity results for SX and FP.

	Salmeterol		Fluticasone Propionate	
Compounds	Peak Purity Angle	Peak Purity Angle	Peak Purity Angle	Peak Purity Angle
Test Solution	0.087	0.541	0.040	0.260
Standard solution	0.202	0.556	0.044	0.244

2.7.2. Linearity and range

Fourteen different concentrations of standard solutions were prepared to test the linearity range. The calibration curve was plotted as peak area versus concentration of the standard solutions.

The nominal concentration of test solutions for SX and FP are 0.0025 mg/mL and 0.025 mg/mL, respectively. Relative response factors were determined by preparing standard solutions at different concentration levels ranging from LOQ concentration to 0.0125 mg/mL for SX and to 0.125 mg/mL for FP.

The correlation coefficient (r) should not be lower than 0.998 to establish the criterion of linearity according to Kazakevich and LoBrutto [9]. As can be seen in the Graph 1, the correlation coefficients (r) were greater than 0.998. Therefore, it can be concluded that the analytical method was linear for this concentration range.

2.7.3. Limit of Quantification (LOQ)

LOQ values for SX and FP were determined based on signal-to-noise approach according to ICH guidelines. The results were tabulated in Table 3.

Table 3. Limit of quantification (LOQ) of SX and FP.

Impurities	LOQ (µg/mL)
Salmeterol Xinafoate	0.030
Fluticasone Propionate	0.067

2.7.4. Accuracy

The accuracy of the method was determined by recovery experiments. Recovery studies were carried out with three injections at three different concentrations. Three concentration levels of 80%, 100% and 120% of the specification level of SX and FP were prepared. Three samples were prepared for each level. The experimental results (shown in Table 4) reveal that recoveries were obtained between 80% - 120% for SX and FP.

Table 4: Average Accuracy (recovery, %) results for SX and FP.

Compounds	80 %	100 %	120 %
Salmeterol Xinafoate	99.5	100.2	99.2
Fluticasone Propionate	100.4	101.0	100.5

2.7.5. Precision

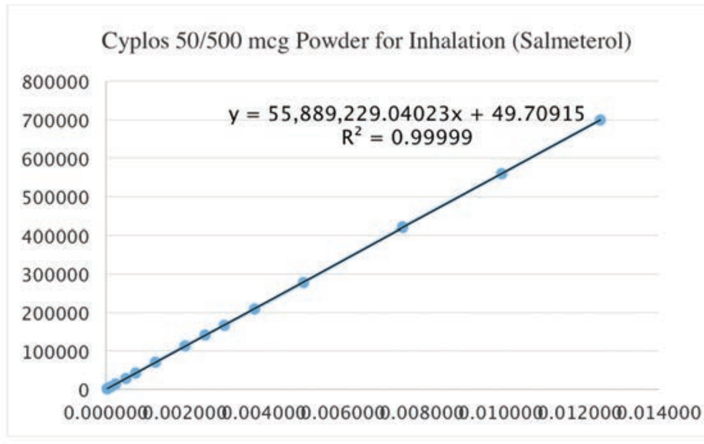
For the precision parameter of the method validation, system precision, repeatability and intermediate precision studies were performed. System precision studies were carried out by consecutively injecting the standard solutions for six times. Repeatability was studied by consecutively injection of six test solutions, which were prepared separately. Intermediate precision was carried out by injecting six injections of standard and sample solutions within-laboratory variations: different days, different analysts, and different equipment. The relative standard deviation and difference between two analysts were calculated. The lower RSD % values (<10.00) indicate good precision of the developed method shown in Table 5.

Table 5: Precision results for SX and FP.

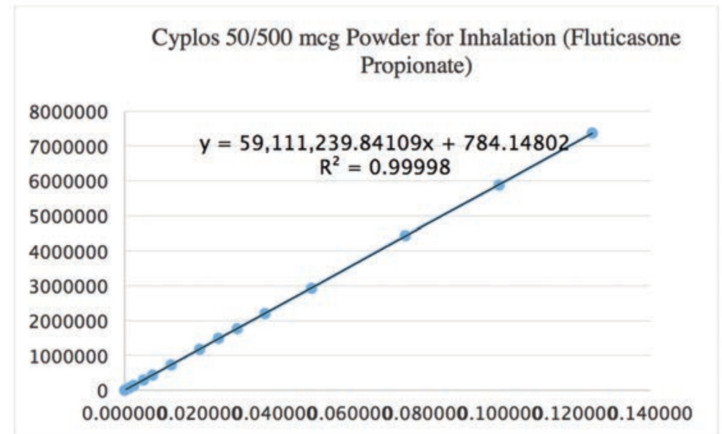
	Values%		
Compounds	System Precision	Repeatability	Intermediate Precision
Salmeterol Xinafoate	0.36	1.4	1.0
Fluticasone Propionate	0.18	1.8	1.2

2.7.6. Robustness

To demonstrate the robustness of the method, system suitability parameters were verified by making changes in chromatographic conditions such as change in column temperature ± 5°C, change in mobile phase ratio ± 10 (v/v), change in flow rate ± 0.1 mL/min., change in buffer pH ± 0.1. The retention time and the difference between the results at normal conditions and modified conditions were calculated. According to these modifications excluding the change in mobile phase ratio, it can be concluded that method was robust over an acceptable working range of its HPLC operational conditions.



Graph 1: Salmeterol Linearity Results of Assay Analytical Method Validation for Cyplos 50/500 mcg Powder For Inhalation.



Graph 2: Fluticasone Propionate Linearity Results of Assay Analytical Method Validation for Cyplos 50/500 mcg Powder For Inhalation.

2.7.7. Stability

The stability of mobile phase, standard and sample solutions were carried out by keeping the solutions for 28 days for mobile phase and 2 days for standard and sample solutions, and observing for changes in the area and the retention of the peaks. In addition, the standard and test solutions kept refrigerated and at room temperature (RT) were also monitored at the determined periods and the % change calculated and compared a chromatogram of freshly prepared solutions. Relative difference (%) of SX and FP was calculated based on the values of initial conditions and it should be smaller than 2.0%. The results show that the standard solution was stable for 30 hours at 25 °C, stable for 7 days at RT and 14 days when refrigerated. The test solution was found to be stable for 30 hours at 25°C for the assay, and mobile phase was stable for 28 days in ambient conditions.

3. Discussion & Conclusion

Replacing Sodium Dodecyl Sulphate with Potassium Dihydrogen Phosphate makes this method safer in terms of working conditions. In addition, employing UV detection provides simplicity when considering sample preparation compared to FLR detection which is more sensitive to any kind of contamination. Moreover, using the wavelength gradient, produced a maximum response at the wavelength maximum for SX and FP.

To conclude, the validation studies done in accordance with ICH guidelines prove that the suggested method is accurate, precise, robust, specific and selective.

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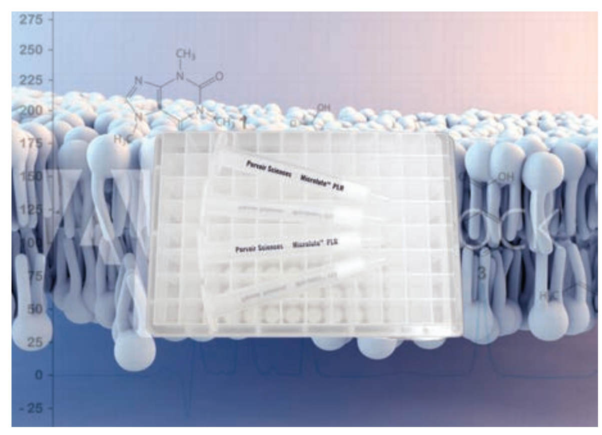

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Development of a Cannabinoid Analysis within a Regulated Environment

by Raymond Wong, Angela Jein
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Interest in the therapeutic properties of organic compounds from cannabis such as cannabinoids has exploded in recent years. This has led to a significant increase in the number of products hitting the market focussed on what the industry terms nutraceuticals. These nutraceuticals are food or fortified food products that are purported to supplement the diet but, also potentially assist in treating or preventing disease. Examples include cannabidiol (CBD) fortified oils which must adhere to the lower legal limits of tetrahydrocannabinol (THC) [1]. Since nutraceuticals are not as rigorously tested and regulated to the extent of pharmaceutical drugs, in recent times there has been a strong movement within the nutraceutical industry towards improving standards and regulation. On 1st November 2018, the United Kingdom legalised medicinal cannabis, allowing the pharmaceutical industry to provide medicinal cannabis extracts approved through clinical trials under pharmaceutical regulations. Unlike the common nutraceuticals these medicinal cannabis extracts can include THC. This article describes the development of a robust analytical method for the analysis of eleven primary cannabinoids within an FDA 21 CFR Part 11 ready chromatography data system (CDS), supporting laboratories seeking to follow the FDA fundamental elements of electronic data quality: ALCOA+.

Introduction

Cannabis contains a number of chemical alkaloids known as cannabinoids. Primary cannabinoids of interest to most laboratories are tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN). In extracts from the plant, THC and CBD exist as the native acid forms, tetrahydrocannabinolic acid (THCA) and cannabinolic acid (CBDA). These gradually decarboxylate to THC and CBD through exposure to heat and light [2].

Cannabis may be analysed for different purposes, the most common of which is the potency, characterised by the quantitation of THC, CBD and CBN. The Analytical Monograph Cannabis Flos (Version 7.1, November 28 2014) released by the Dutch Office for Medicinal Cannabis describes a methodology for analysis of cannabinoids for release testing of Cannabis Flos (flowers / granulated) [3]. Furthermore, based on this monograph method the typical solvents used to extract cannabinoids are typically 'strong' organic solvents due to their lipophilicity, however; early eluting compounds can suffer from poor peak asymmetry using this monograph method due to the strength of the extraction solvent. This can be solved by using the co-solvent injection mechanism previously published [4]. This article highlights the use of a high-resolution UHPLC method to determine the potency of cannabis extracts with the Shimadzu Cannabis Analyser for Potency within an FDA 21 CFR Part 11 ready CDS environment and the use of intelligent Peak Deconvolution Analysis (i-PDeA) for challenging separations.

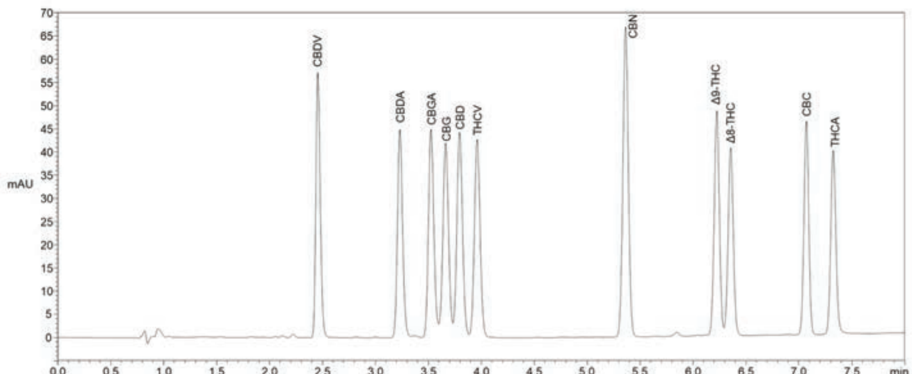

Figure 1: HPLC chromatographic analyses of 11 common cannabinoids. Chromatographic conditions are included in section Method Details.

Cannabinoid methods carried out on HPLC instruments analysed all 11 common cannabinoids in under 8 minutes with a low-pressure maximum of 193 bar / 2,800 psi as depicted in Figure 1. [5]

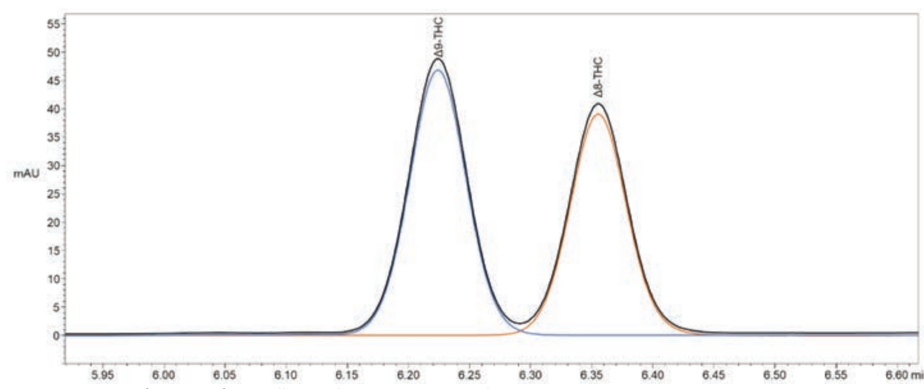

Figure 2: Quantification of partially co-eluting Δ9-THC and Δ8-THC using i-PDeA.

Fully resolving cannabinoids with similar structural properties can prove challenging, the result in Figure 1 demonstrated several components with a resolution factor of <1.5. It is possible to use an intelligent algorithm previously reported for such challenging separations, in order to successfully quantify these partially co-eluting compounds more readily. This is called the intelligent Peak Deconvolution Algorithm (i-PDeA) [6]. Using this technique, it has been reported to deconvolute even positional isomers of o-methyl acetophenone, m-methyl acetophenone and p-methyl acetophenone [7]. As depicted in Figure 2 the same technique can be used for the deconvolution of the Δ9-THC and Δ8-THC co-eluted peak.

It was the objective of this study to further improve the resolution, whilst maintaining faster analysis of these cannabinoids. This was paramount where baseline separation was sought in order to quantify each component successfully, such as within medicinal cannabis analysis.

Retention modelling

Although retention modelling has been successfully employed in optimising analytical separations of small molecules for over 30 years, it is still not universal. Published chromatographic methods using trial and error approaches continue to be prevalent. Retention modelling software packages provide a fast and efficient means to optimise analytical separations whilst selecting conditions that provide the most robust methods. This type of Quality by Design (QbD) approach has become popular within the pharmaceutical industry and the FDA has cited a risk-based approach to drug development as a desirable state for the near future [7]. These reasons lead to the method described in this article being optimised using ACD/LC Simulator, Advanced Chemistry Development, Inc, Toronto, Canada. In Figure 3, regions of colours depicting resolution >1.5 correspond to LC conditions fully [baseline] resolving all 11 cannabinoids. The higher the resolution (Rs) number the greater the resolution of the critical pair. With this information it is possible to choose the analytical conditions which provide the optimal separation within a desired run time. Furthermore, it is also possible to select a region which offers robustness by simulating potential variance in temperature or tG. In addition, other variables such as pH and ternary mobile phase compositions can also be investigated using this strategy.

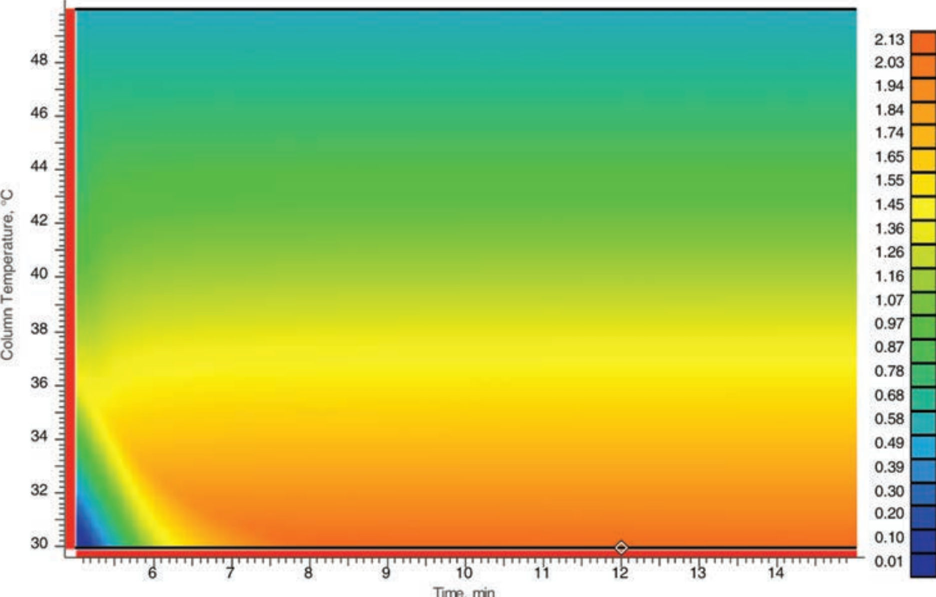

Figure 3: Simulated analytical conditions of 200 experiments.

Method Details

The Shimadzu Cannabis analyser equipped with a photodiode array detector was used for the analysis. Accurate and reproducible resolution for all 11 common cannabinoids were achieved using the NexLeaf CBX column over a 12-minute analytical gradient. The analytical conditions are shown in Table 1.

Table 1. LC Method Parameters.

LC System	Shimadzu Cannabis Analyser
Column	NexLeaf CBX uHPLC column, 2.7 µm, 150 x 4.6mm
Mobile phase A	Water + 0.1% formic acid
Mobile phase B	Methanol + 0.1% formic acid
Rinse solution	Methanol + 0.1% formic acid
Flow rate	1.5 mL/min
Gradient program	0 - 12 min, 70-95%B
Column temperature	30 °C
Injection volume	10 µL
Detector	3D-PDA
Co-injection	Water

The accompanying FDA 21 CFR Part 11 ready CDS used was the Shimadzu LabSolutions DB software. This analysis data system provides ER/ES compliance in regulated environments and included multi-data report functionality.

Materials

All solvents and diluents used were HPLC grade and pre-filtered via 0.45 µm filters from Romil Ltd. All diluents were isopropyl alcohol and methanol. Standards listed in Table 2 were obtained from Sigma-Aldrich® at a concentration of 1 mg/mL (in methanol). Formic acid (puriss p.a.) was purchased from Sigma-Aldrich®.

Table 2: Analysed compounds.

Abbreviation	Item Description
CBC	Cannabichromene
CBD	Cannabidol
CBDA	Cannabidiolic acid
CBDV	Cannabidivarin
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBN	Cannabinol
Δ8-THC	Δ8-Tetrahydrocannabinol
Δ9-THC	Δ9-Tetrahydrocannabinol
THCA	Δ9-Tetrahydrocannabinolic acid
THCV	Tetrahydrocannabivarin

Sample preparation

Varying sample matrix within the nutraceutical industry, has led to a plethora of dilution methods being reported. The most common on the market are oils containing CBD, these can be manufactured with varying types of oil such as hemp, olive or medium chain triglycerides (MCT) which are derived from coconuts. The next common sample type is vape/e-liquids, which have seen growth in general over the past few years, these have now been produced to include CBD within the e-liquids. Those companies that are producing these products from the raw materials, need to also test the flower and/or bud they are using.

Due to oil-based products being used in both nutraceuticals and pharmaceutical production we tested a single MCT based product multiple times. Previous

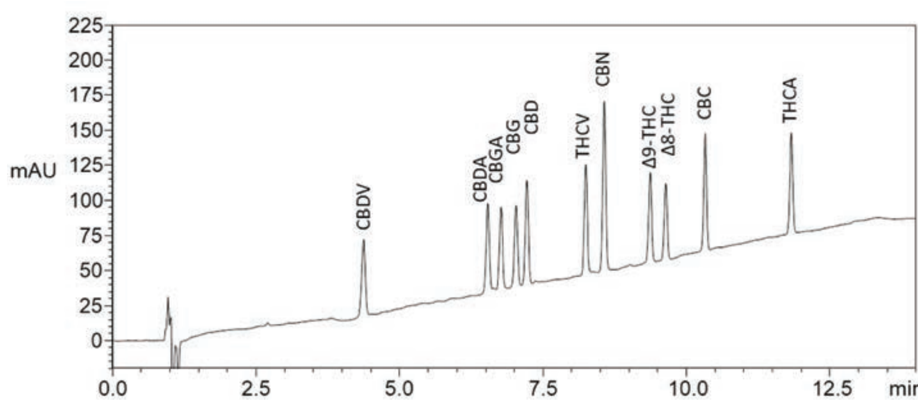

Figure 4: Resolution of 11 common cannabinoids, 10-ppm mix at 220 nm.

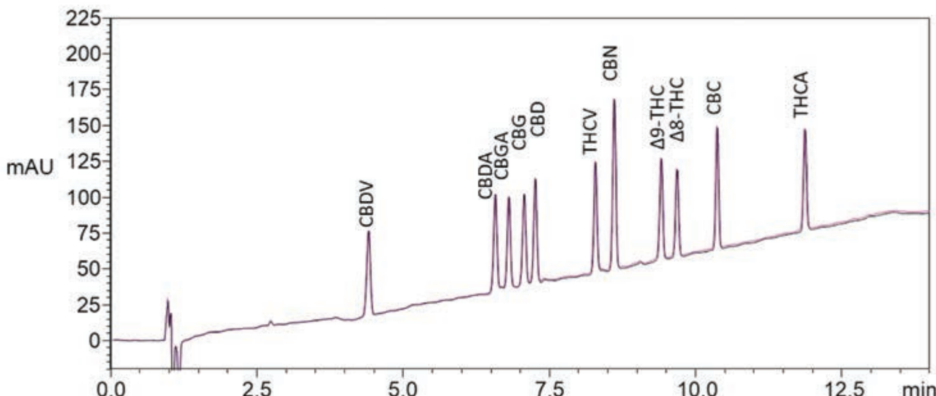

Figure 5: Six replicate injections of 10-ppm cannabinoid mixture, %RSD of 0.6%.

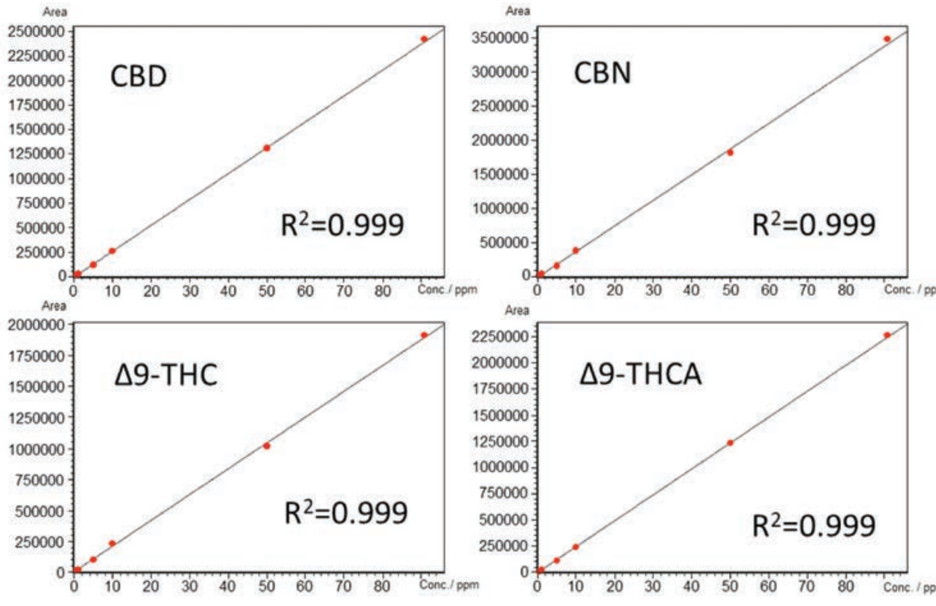

Figure 6: Linearity plots for CBD, CBN, Δ9-THC and Δ9-THCA, 0.5, 1, 5, 10, 50 and 90.9 ppm.

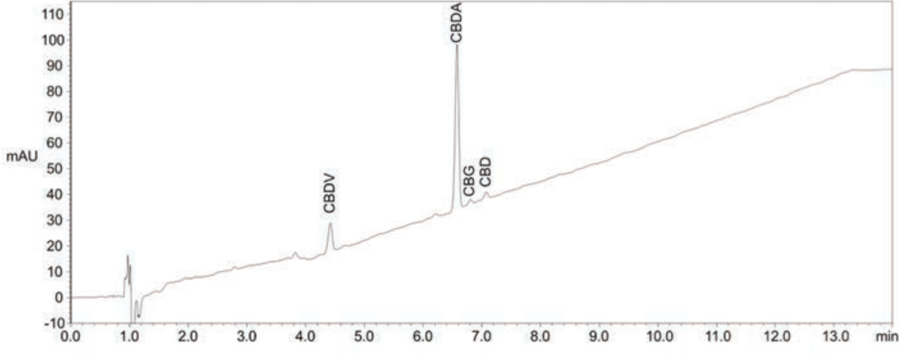

Figure 7: Overlay of 5 prepared CBD oil samples, CBD was found with a %RSD Rt 0.116%, Area 1.178%.

experience of the oil used had shown that only olive oil required any additional dilution procedure. We also tested an e-liquid sample at a higher concentration of CBD.

The sample was diluted in isopropyl alcohol (IPA) and methanol. The solution was filtered and further diluted with methanol to obtain a sample with a CBD concentration within the linear range.

Results and Discussion

As simulated by the retention modelling software; baseline resolution of all 11 cannabinoids was achieved in Figure 4 with good peak asymmetry for all cannabinoids including early eluting compounds, which can be affected by strong diluent sample injections. The maximum observed back pressure was registered at 486 bar / 7,050 psi (column dependent).

As shown in Figure 5, chromatographic repeatability was demonstrated via 6 replicate injections of a 10-ppm cannabinoid mixture with a %RSD of 0.6%.

A 6 level linearity plot was generated for each cannabinoid standard from 0.5 ppm through to 90.9 ppm. With a 10 µL injection this means the low standard equates to only 0.005 µg of cannabinoid on column can be routinely detected, see Figure 6.

Sample analysis

A CBD Oil sample was prepared five times, employing the methodology described in the sample preparation section, to ensure both the analytical method and sample preparation were robust. The chromatogram below demonstrates the robustness of the methods.

A vape sample was then prepared following the same procedure and tested using the same analytical method, the chromatogram can be seen in Figure 8.

Reporting

Compliant industries have seen a push towards validated computer systems over the last decade. This move towards automated processes within analytical laboratories has seen an increase in compliant laboratories dedicating time and resources to complex reports.

As part of this study we also worked with data to build a custom report based on two injections of the same sample at differing concentrations, the final report not only reports all relevant details, but amalgamates the two injections data. This data allows for

either an average result if both sets of data are within the calibration curve, or selection of data if only one is within the calibration curve. It is also possible to add a detection limit if no peak is detected or lower than the bottom standard used.

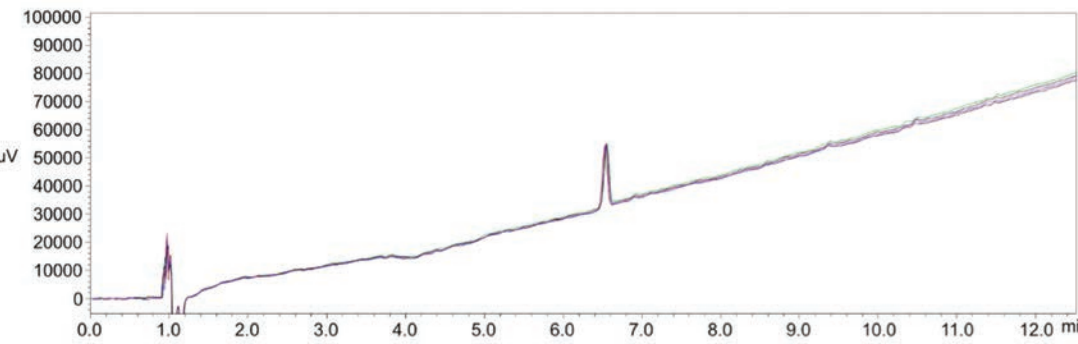

Figure 8: A single e-liquid vape sample

This type of report would usually involve an analyst manually creating a document from the various sample injections, here we can see that the report shows which results are outside the calibration data set, and the amalgamated results, including use of a limit value.

Conclusion

This study demonstrated the development of a new robust liquid chromatography method successfully resolving 11 common cannabinoids using 3D-photodiode array detection. Furthermore, the ability to use intelligent tools such as i-PDeA to help quantify challenging separations or co-eluting peaks caused by impurities or matrices from real samples, will improve laboratory productivity.

Shimadzu has the use of both Labsolutions DB and CS, which are fully compliant. The two options both enable full data integrity, including all data being encrypted to ensure security. The full compliance (if needed) has transparency, legitimacy and validity of the data generated within a regulated environment.

The additional option, Multidata Report, as shown in the reporting section, is another intelligent tool that can be used to customise the sample report generated to highlight results (pass/fail can be coloured or labelled), making report summaries easily understood.

Acknowledgements

Many thanks to Veronica Paget at ACD Labs for her continued support with the LC Simulator software. Thanks also Shimadzu Europe for supplying the necessary column for the assay. Finally, thanks to Shimadzu Scientific Inc. for the initial methodology which was used as the basis for this work.

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Cells highlighted are within calibration range

Sample Information

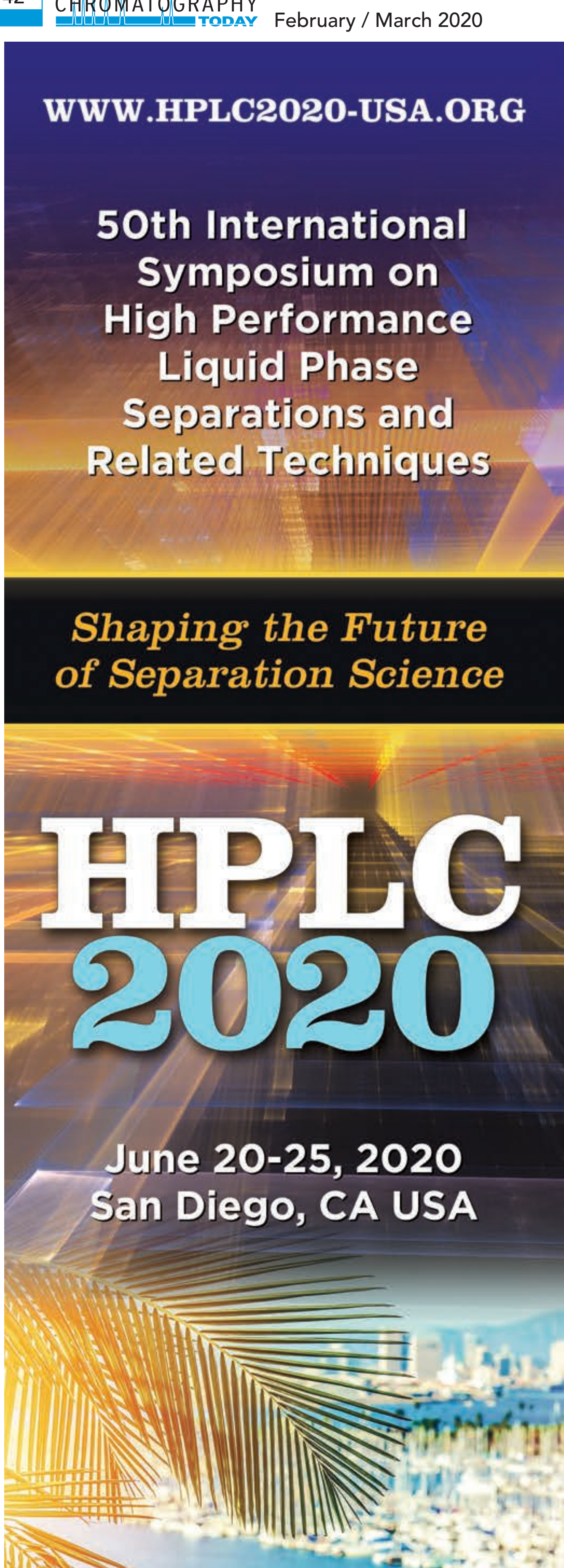
Sample Name	Sample ID	CBDV Area	CBDA Area	CBGA Area	CBG Area	CBD Area	THCV Area	CBN Area	d9-THC Area	d8-THC Area	CBC Area	THCA Area	Dil. Factor
Vape 1	A	118018	13719.76	1287	19548	595168	25601	527	1161	0	201	503	281
Vape 1	B	11333	1480.84	0	1773	57741	2421	0	0	0	0	0	2810
Vape 2	A	117797	13629.28	1542	19606	594045	25704	589	1178	0	197	207	281
Vape 2	B	11398	1394.68	0	1820	58032	2430	0	0	0	0	0	2810
Vape 3	A	115923	13488.08	1520	19286	584638	25221	574	1162	0	184	0	281
Vape 3	B	11348	1461.80073	0	1783	57741	2434	0	0	0	0	0	2810
Oil 1	A	21980	3953.64	361	10371	1039441	3607	27899	50609	2246	26192	4037	81
Oil 1	B	440	0	0	0	20877	0	579	989	0	482	671	4050
Oil 2	A	21505	3973.92	365	10145	1019966	3462	27534	49590	2116	25699	4550	81
Oil 2	B	384	0	0	0	19204	0	514	882	0	447	608	4050
Oil 3	A	21430	3964.64	354	9884	1013527	3399	27255	49182	2118	25356	4220	81
Oil 3	B	394	0	0		19166	0	511	905	0	450	616	4050

Table below generated from sample areas above, utilising data only within calibration range. If both dilutions are within range an average is represented. A limit of detection can be displayed if all data is lower than the calibration curve.

Sample Information

Sample Name	Sample ID	CBDV Conc. ppm	CBDA Conc. ppm	CBGA Conc. ppm	CBG Conc. ppm	CBD Conc. ppm	THCV Conc. ppm	CBN Conc. ppm	d9-THC Conc. ppm	d8-THC Conc. ppm	CBC Conc. ppm	THCA Conc. ppm
Vape 1		6444.79	825.58	LIMIT	1317.22	33573.65	1467.30	LIMIT	LIMIT	LIMIT	LIMIT	LIMIT
Vape 2		6433.15	821.03	LIMIT	1320.48	33728.38	1472.91	LIMIT	LIMIT	LIMIT	LIMIT	LIMIT
Vape 3		6334.49	813.93	LIMIT	1302.53	33573.65	1446.57	LIMIT	LIMIT	LIMIT	LIMIT	LIMIT
Oil 1		400.20	96.41	LIMIT	231.30	20136.77	77.19	339.68	871.59	126.39	491.59	95.52
Oil 2		392.99	96.70	LIMIT	227.65	18854.60	74.91	336.27	855.62	123.92	483.81	104.01
Oil 3		391.85	96.57	LIMIT	223.43	18825.48	73.92	333.67	849.22	123.96	478.39	98.55

Figure 9: Selected section of the generated report



44th ISCC and 17th GCxGC 2020: Italy May 24-29

Over the years, the International Symposium on Capillary Chromatography (ISCC) has established its reputation as a forum for microcolumn separation techniques. Since the first meeting in Hindelang in 1975, the most important developments in capillary gas chromatography, microcolumn liquid chromatography and electromigration techniques have been presented in this symposium series. The format and the atmosphere of the 44th meeting will be similar to the previous meetings.

This year the "Palazzo dei Congressi" in Riva del Garda, Italy, will accommodate the 44th ISCC and 17th GC×GC meeting from May 24-29, 2020. The six-day event will feature recent findings from leading academic and industrial experts in the form of lectures and posters. Apart from the most recent advances in the fields of pressure and electrodriven microcolumn separation techniques and comprehensive two dimensional gas chromatography (2D GC), this year particular emphasis will be directed to all Comprehensive Separation Technologies in combinations of capillary chromatography and 2D GC with various forms of MS from unit-mass to high resolution and from single to hybrid analysers. The conference offers sessions on capillary GC, microcolumn liquid chromatography (LC), electromigration methods and microfabricated analytical systems, which are expected to cover lab-on-a-chip, column technology, coupled and multidimensional techniques, comprehensive techniques, hyphenated techniques, sampling and sample preparation, trace analysis and automation. Application sessions include environmental applications, energy/petrochemical/industrial applications, biomedical/pharmaceutical applications, and the analysis of natural products, food, flavors, and fragrances.

Workshop seminars of instrument manufacturers and an extensive exhibition of instrumentation, accessories, and supplies will run in parallel to the scientific program.

At the meeting, the 2020 Marcel Golay award sponsored by PerkinElmer will be presented in recognition of outstanding contributions in the field of separation science. The Leslie Ettre award, sponsored by PerkinElmer, will be presented to a young scientist for research on capillary GC applied to environmental or food analyses. The Giorgio Nota award, sponsored by Waters, will be presented to a scientist in recognition of a lifetime of achievement in capillary LC. The John Phillips award, sponsored by LECO and Restek, will be awarded to individuals who have made outstanding contributions to the field of GC×GC analysis. The GC×GC Lifetime Achievement award, sponsored by LECO and Restek, honors an experienced GC×GC scientist who has made significant contributions to the field.

At the symposium, Chromaleont, ISCC & GCxGC, Fort Worth, Texas 2021, Division of Analytical Chemistry of the Italian Chemical Society (SCI) and the Interdivisional Group of Separation Science of the Italian Chemical Society, Elsevier, Secyta and Interdivisional Group on Food Chemistry, will promote scholarships for young researchers.

For more information please click here or visit www.chromaleont.it/iscc; e-mail: iscc@chromaleont.it

To encourage scientific exchange and friendship building, the scientific program will be enhanced with the well-known "Riva Social Program."

Considering the interest in comprehensive techniques, the 17th GC×GC Symposium will be organised during the same period to allow scientists to attend both meetings. The 17th GC×GC Symposium will start on 24 May 2020 with a courses presented by experts in the field covering the fundamental aspects of comprehensive techniques and a plenary session on 15 May 2020. For both meetings, abstracts for consideration as lecture or poster presentations can be submitted on-line at http://www.chromaleont.it/iscc. All abstracts will be reviewed on the basis of scientific merit, novelty and practical application.

Presenters at the meeting may also have their work published in a special volume of the Journal of Chromatography A.

The SFC Isolation and Purification of Cannabinoids using Application Specific Stationary Phases Under Optimised Conditions

by Matthew Przybyciel, PhD and David A. Kohler, ES Industries
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Cannabis sativa is comprised of hundreds of individual compounds that can be classified in many chemical families, such as terpenes, amino acids, fatty acids, hydrocarbons, flavonoids, sugars and cannabinoids [1,2]. Cannabinoids represents a class of chemicals that are classified as terpenophenolic compounds. There are about 70 terpenophenolic compounds in the cannabinoid class. These are only found in cannabis plants [3]. Of the 70 cannabinoids found in *Cannabis* there are several cannabinoids that are of human physiological and medicinal interest [4]. These include the psychoactive Δ-9-tetrahydrocannabinol (THC), non-psychoactive cannabidiol (CBD) and the non-psychoactive tetrahydrocannabivarin (THCV) (5-8). THC, THCV and CBD are neutral forms of cannabinoids, obtained after a non-enzymatic decarboxylation of delta 9-tetrahydrocannabinolic acid (THCA), tetrahydrocannabivarinic acid (THCVA) and cannabidiolic acid (CBDA). It is the focus of this manuscript to utilise SFC chromatographic stationary phases that have been specifically developed for the isolation and purification of THCA, CBDA, THC, CBD and THCV. These specific cannabinoids require the use of several different stationary phases for optimised separation and purification of them individually.

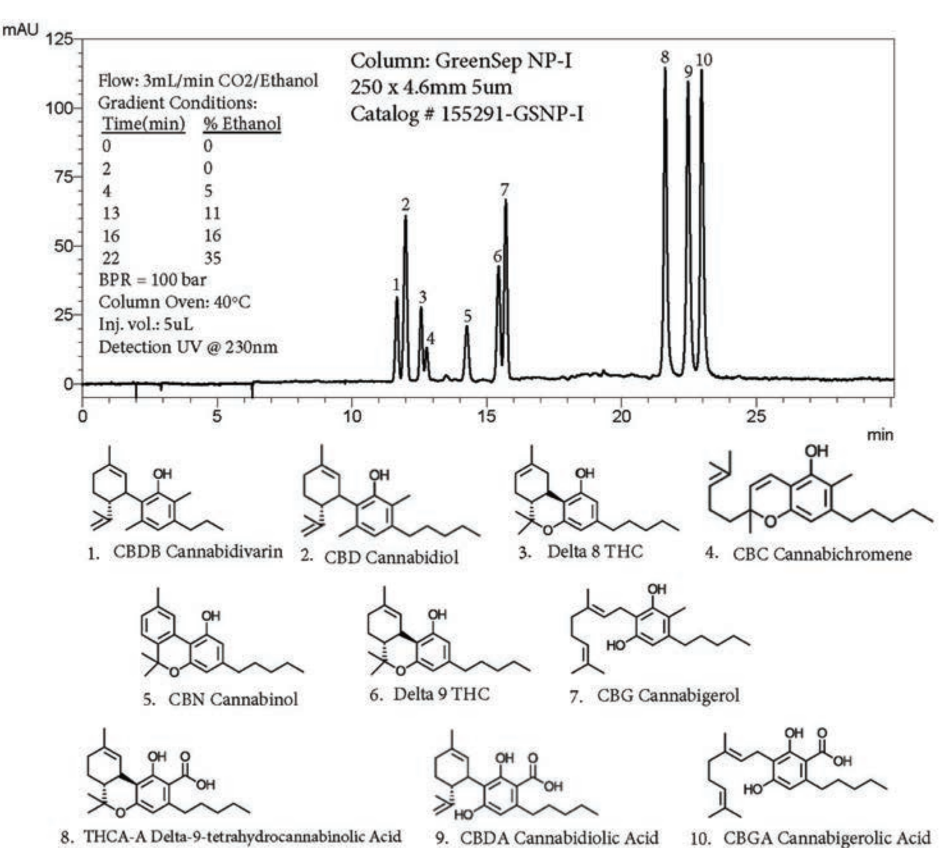

Figure 1: Separation of 10 cannabinoids chromatographed on GreenSep NP-I, a coated polysaccharide stationary phase.

Supercritical fluid chromatography (SFC) is a powerful chromatographic technique for the separation and isolation of complex mixtures from natural products. It has been useful in the area of preparative chromatography [9-11]. Virtually all current practitioners of SFC use carbon dioxide (CO_2) which offers several advantages when compared to preparative liquid chromatography [12]. The use of carbon dioxide (CO_2) as the primary component of the mobile phase is one of the key features that benefits preparative SFC chromatography since the CO_2 used for SFC is considered a 'Green' solvent. It is miscible with a wide range of organic solvents, nonflammable, has low UV absorbance at short wavelengths [13-15]. CO_2 SFC is particularly well suited in the area of preparative chromatography where it can be easily removed enabling the rapid recovery of isolated compounds. In addition, any residual amounts of CO_2 in isolated products are considered to be non-toxic [16]. Another advantage of SFC as a technique is that the diffusion coefficient of solutes in the SFC mobile phases have been shown to be 3-10 times higher than in normal liquids potentially allowing for very rapid separations. In addition the viscosity of SFC mobile phases are significantly lower than LC mobile phases hence producing a much lower pressure drop across the column

allowing the use of much higher mobile phase flow rates producing rapid preparative separations [17]. Given these attributes SFC chromatography is ideally suited to isolation and purification of cannabis extracts. In addition, super critical CO_2 extraction (SFE) of cannabis is routinely performed to produce a cannabis oil [18,19].

SCOPE

The preparative separation of cannabis mixtures to isolate specific components can be challenging. Traditional preparative liquid chromatography can be used to separate and isolate specific cannabis components. However, preparative liquid chromatography has several draw backs including the limits on flow rates and ultimately production throughput due to the relatively high viscosity of the mobile phase used. In addition, considerable amounts of ethanol and water are required for the liquid chromatographic separation of cannabis. In order to isolate the components, the ethanol/water mixture has to be removed or reduced in volume. This removal process is time consuming. The mixtures of CO_2/ethanol mobile phase are very low viscosity which can be used at very high flow rates to encourage higher production levels. In addition, CO_2 is rapidly released during component isolation and ethanol amounts are low and quickly removed.

One of the key factors for a successful SFC preparative separation and isolation of cannabinoids is stationary phase selection. There are a several of attributes that are necessary for the optimal stationary phase including:

1. The stationary phase should be designed to deliver the desired separation at the lowest level of organic modifier possible (in the case of Cannabis ethanol would be the organic modifier).

2. The stationary phase should be robust and easily scalable for preparative applications.

3. The stationary phase should not be expensive to manufacture.

Preliminary Investigations

Preliminary investigations for the SFC separations of cannabinoids employed modified polysaccharide phases coated phases for the SFC separations of natural products (NP) since they can be useful for the separation of structurally similar compounds. The GreenSep NP-I has been specifically optimised for the separation of 10 different cannabinoids. The chromatogram shown in Figure 1 is an example of the peak shape, performance and separation capacity obtainable with the GreenSep NP-I column with SFC for a high-resolution separation of a mixture of cannabinoids. Unfortunately, these polysaccharide phases whether coated or immobilised are expensive to manufacture, making these types of columns a major contributor to isolation costs.

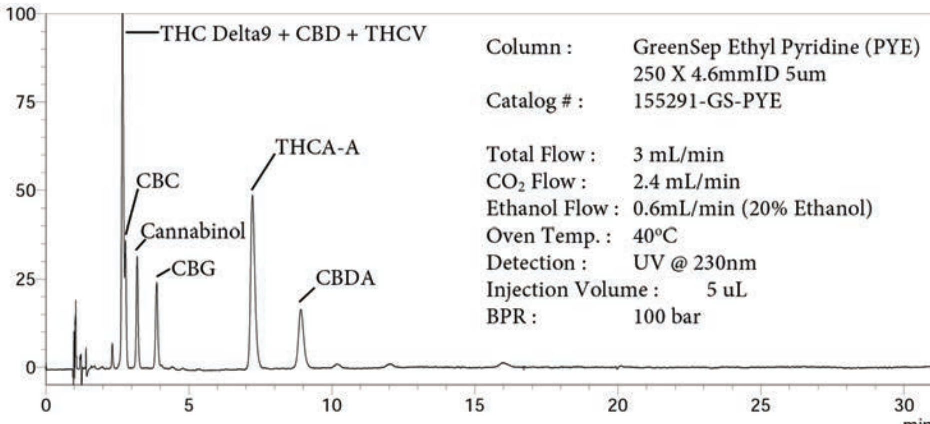

Figure 2: Cannabinoid mixture chromatographed on GreenSep Ethyl Pyridine.

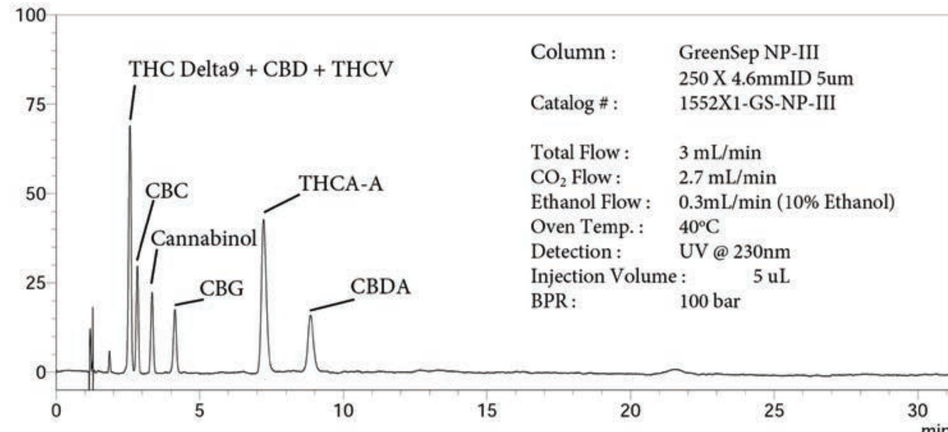

Figure 3: Cannabinoid mixture chromatographed on GreenSep NP-III using 10% Ethanol modifier.

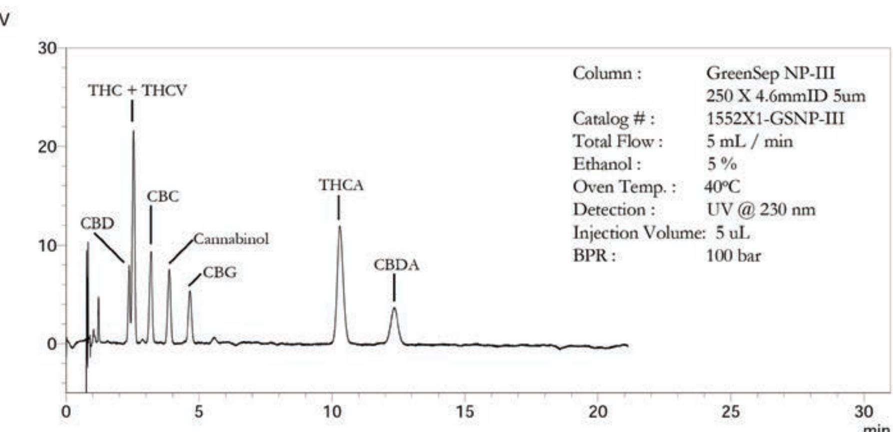

Figure 4: Cannabinoid mixture chromatographed on GreenSep NP-III using 5% Ethanol modifier.

Isolation of THCA and CBDA

Preparative SFC separations of cannabinoids have been performed using a column with 2-Ethyl pyridine bonded to silica as a stationary phase with ethanol used as co-solvent since it is less toxic compared to methanol or other organic solvents. This is of vital importance if the resulting isolate is for human consumption as no toxic solvent residues are present. A chromatogram showing the separation of mixture of cannabinoids is shown in Figure 2. CBDA and THCA are both well separated from the other cannabinoids, however, to elute these two components in less than 10 minutes 20% ethanol co-solvent is required.

GreenSep NP- III permits both CBDA and THCA to elute in less 10 minutes with only 10% ethanol (chromatogram shown in Figure 3), half as much when compared to 2-ethyl pyridine.

GreenSep NP-III can be used at higher total flow rates requiring only 5% ethanol to elute both THCA and CBDA in less than 13 minutes, while still maintaining good chromatographic resolution (chromatogram shown in Figure 4).

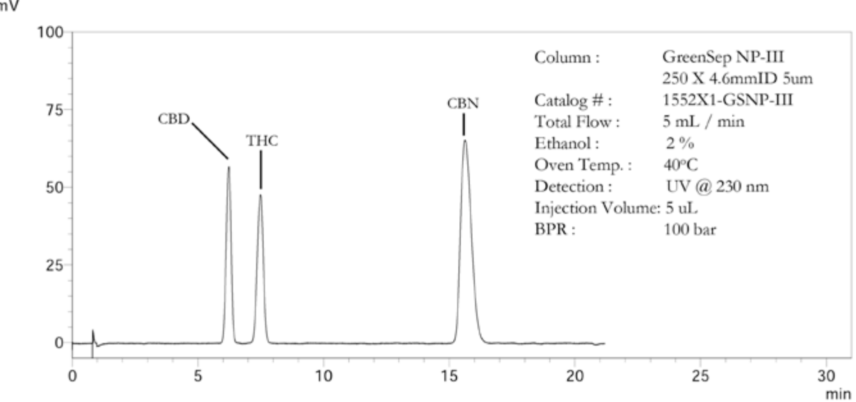

Figure 5: Separation of CBD, THC and CBN on GreenSep III using 2% ethanol.

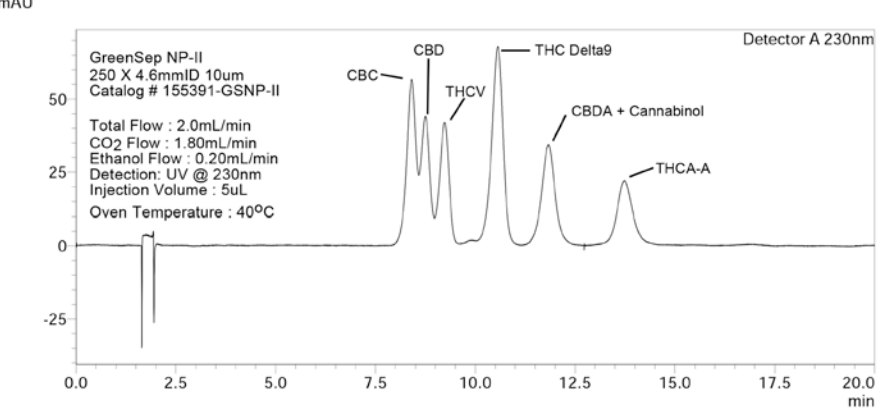

Figure 6: Cannabinoid mixture chromatographed on GreenSep NP-II using 10% ethanol.

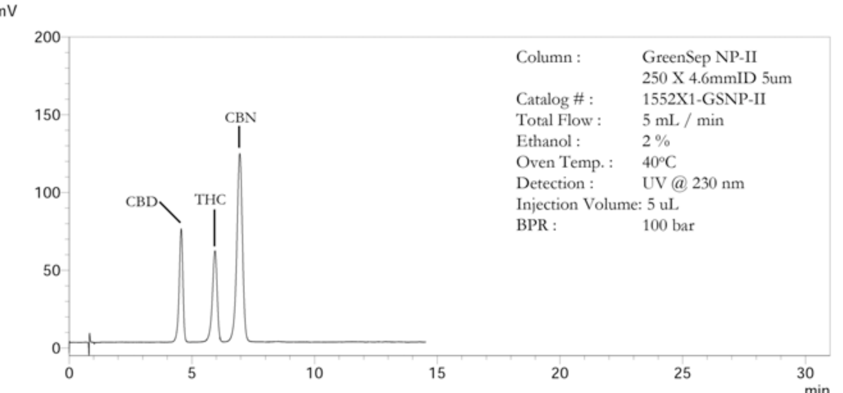

Figure 7: Separation of CBD, THC and CBN on GreenSep III using 2% ethanol.

Isolation of CBD and THC

During our investigation we discovered that GreenSep NP-III could be used for the SFC preparative separation of CBD and THC as shown in Figure 5 where both CBD and THC are eluted with only 2% ethanol.

The separation of CDB and THC on GreenSep NP-III provided the motivation to develop other new products specifically designed for optimised SFC preparative separation of cannabinoids. A chromatogram showing the separation of a cannabinoids mixture chromatographed on a GreenSep NP-II is shown in Figure 6 where THC can easily be removed from a cannabis extract and CBDA and THCA are still eluted in less than 15 minutes.

In some cases, it would be desirable to isolate full spectrum CBD without THC. Full spectrum CBD contains cannabinoids without THC and THCA. This full spectrum CBD may have additional therapeutic benefits when compared to pure CBD. Figure 7 shows the separation of CBD and THC. Based upon this chromatography THC and CBN can be removed from an extract to produce full spectrum CBD.

The separation of CBD from THC was further improved using another new stationary phase GreenSep NP-9. The chromatogram with enhanced separation between CBD and THC is shown in Figure 8. Using GreenSep NP-9 provides separation factor to effectively remove THC from a complex mixture of cannabinoids.

Isolation of CBD, THCV and THC

The cannabinoid THCV is another cannabinoid that has some medicinal interest. However it is difficult to separate THCV from THC and CBD by SFC. However, the SFC isolation of THCV from CBD and THC was achieved on GreenSep NP-12. A chromatogram of this separation is shown in Figure 9.

Conclusion

Several new stationary phases have been developed (GreenSep NP-III, GreenSep NP-II, GreenSep NP-9 and GreenSep NP-12) optimised for the preparative SFC separation and isolation of cannabinoids. GreenSep NP-III is optimised for the rapid separation of CDBA and THCA. GreenSep NP-II is useful for THC and THCA removal with a quick cycle time. GreenSep NP-9 is optimised to deliver the maximum separation alpha between CBD and THC and is best for the removal of THC. GreenSep NP-12 is designed to separate CBD, THCV and THC with maximum alpha value. The recommended use for each of these stationary phases are shown in Table 1. Loading studies are currently being conducted to define preparative loading and output for these cannabinoid isolates. These stationary phases separate the desired components and are designed to deliver the desired separation at the lowest level of liquid ethanol modifier possible. Ethanol minimisation is important since it is more expensive than CO_2 and more difficult to remove than CO_2. In addition, these stationary phases are robust, cost effective and designed for preparative SFC separations.

Table 1: Recommended Cannabis Component Isolation for the New GreenSep NP Stationary Phases.

GreenSep Column	Recommended Use
NP-II	THC and THCA removal with a quick cycle time
NP-III	Rapid isolation of CBDA and THCA
NP-9	Optimal THC removal to produce full spectrum CBD
NP-12	Optimal separation of THCV

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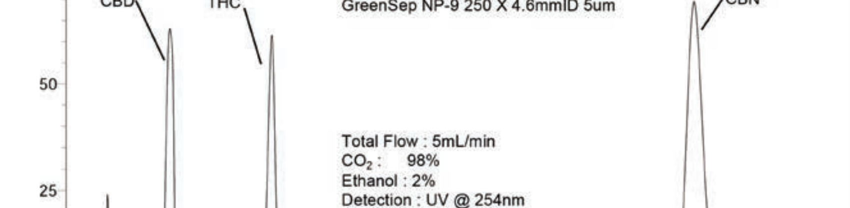

Figure 8: Separation of CBD, THC and CBN on GreenSep 9 using 2% ethanol.

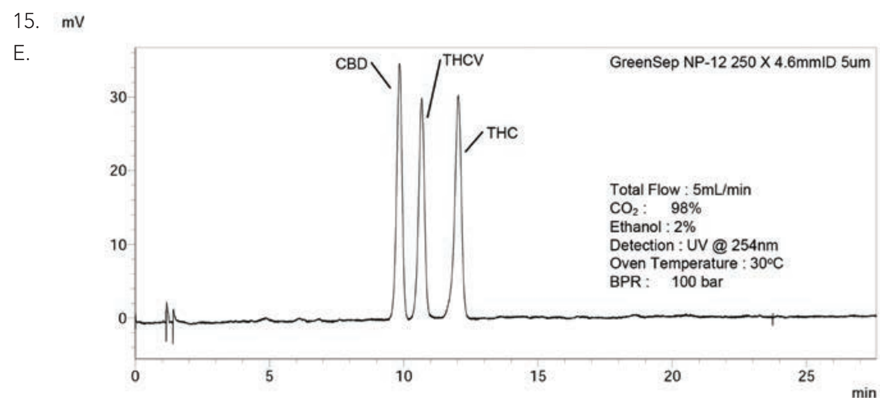

Figure 9: Separation of CBD, THCV and THC on GreenSep 12 using 2% ethanol.

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Chromatography Today Help Desk

Challenges with Sample Preparation

The introduction of liquid chromatography mass spectrometry (LC-MS) into the analytical laboratory has transformed the ability to identify and quantify compounds at low concentrations. Initially scientists had thought that the use of this technology, which allowed for much greater specificity, would eliminate the need for any sample preparation, and the concept of dilute and shoot was readily applied to a range of samples. It was very evident that this approach has limited applicability in disciplines which require quantitive analysis as the detected levels for the same concentration of sample could vary substantially depending on the nature of the matrix components. The explanation for the variability is due to the ionisation process, which can be greatly affected by co-eluting components, or indeed the analyte itself since mass spectrometers have a limited concentration range over which they give a linear response as a function of analyte concentration. The use of sample preparation can reduce or even eliminate co-eluting species derived from the matrix which will also reduce the suppression effects caused by matrix components.

However, even when some form of sample preparation is performed, the matrix can still affect the ionisation efficiency and the performance of an assay. So called 'matrix effects' [1-3] are well recognised for their potential to distort the analytical data, the use of appropriate sample preparation or chromatography, however where the sample matrix varies the analyst can never be truly confident, and in this scenario the use of isotopically labelled internal standards can provide greater levels of assurance to the assay. These matrix effects arise because of the complexity of the matrix, which for a biological fluid, can contain several tens of thousands of different compounds with a very wide range (>10^7) of concentrations [4]. Each of the endogenous compounds can, and does, vary from sample to sample [5]. Many of these compounds will interfere with the analyte ionisation process which results in them either;

- competing for the available charge in the ion source of the mass spectrometer [6]
- enhancement of the ionisation capabilities of other compounds [7]
- reduction in solvent evaporation [8]

There are also other processes, including space charge effects, micelle formation and gas phase interactions [9] that exist and can also cause variable responses from the mass spectrometer.

The variability in matrix composition potentially means that the degree of ionisation will vary from one sample to another with possible adverse effects on the analysis of target analytes. Therefore; it is critical that the compound is resolved from any endogenous materials that produce matrix effects in order to reduce or eliminate ion suppression within the mass spectrometer source. This can be achieved either through the initial sample preparation or by the final chromatographic separation to eliminate co-elution of the matrix component and the analyte. It should be noted that in biological samples which contain tens of thousands of matrix components this will be challenging to say the least.

An interesting observation is the variability of analyte response that can be observed with the same sample and the helpdesk will look at what can cause this issue. The introduction of Incurred Sample Reanalysis (ISR) [10] as part of the validation criteria in 2009 has resulted in this issue having much greater significance and as such is a necessary component of bioanalytical method validation. ISR is intended to verify the reliability of the reported subject sample analyte concentrations and is conducted by repeating the analysis of a subset of subject samples from a given study in separate runs on different days to critically support the precision and accuracy measurements established with spiked QCs; the original and repeat analysis is conducted using the same bioanalytical method procedures.

Repeating the analysis on the same sample can potentially highlight when there is an issue with the assay. There are a variety of reasons that could cause the assay not to give the same result, some pertaining to the sample stability and some relating to the performance of the assay. If the sample deteriorates over a period of time, then the assay performance should pick this up. This article will, however, focus on sample preparation issues that can affect the assay stability.

Sample Preparation

Within many bioanalytical laboratories, the typical workflow will be to perform some form of sample preparation followed by a LC-MS/MS based analysis. There are a range of different sample preparation techniques that can be employed including dilution, protein precipitation, liquid-liquid extraction, and solid phase extraction. Optimisation of each of these approaches can require some effort, making method development quite daunting. In general, the less selective the extraction technique the more economical will be the process and the quicker will be the sample preparation approach. However, the disadvantage is that there will be substantially more matrix components that reaches the chromatographic system and ultimately this will have a detrimental effect on the performance of the system.

Two common approaches of sample preparation that are often employed are protein precipitation and solid phase extraction. Protein precipitation has been successfully applied to the analysis of a wide range of compounds within a variety of biological matrices. It relies on altering the solubility of the protein by changing the configuration of the protein using a variety of chaotropic reagents, with the most common being acetonitrile and acids such as trichloroacetic acid (TCA). Different chaotropic reagents will preferentially affect different bonding mechanisms within the protein structure. Proteins commonly cause significant issues, either due to irreversible adsorption to active surface sites on the column, co-elution or causing MS ion suppression. The removal of these matrix components increases column lifetime and also significantly reduces ion suppression effects within the detector. However, this approach does not remove all of the matrix components, and one particular classification of compounds, phospholipids, which are present in high concentrations within a biological matrix can cause high levels of ion suppression.

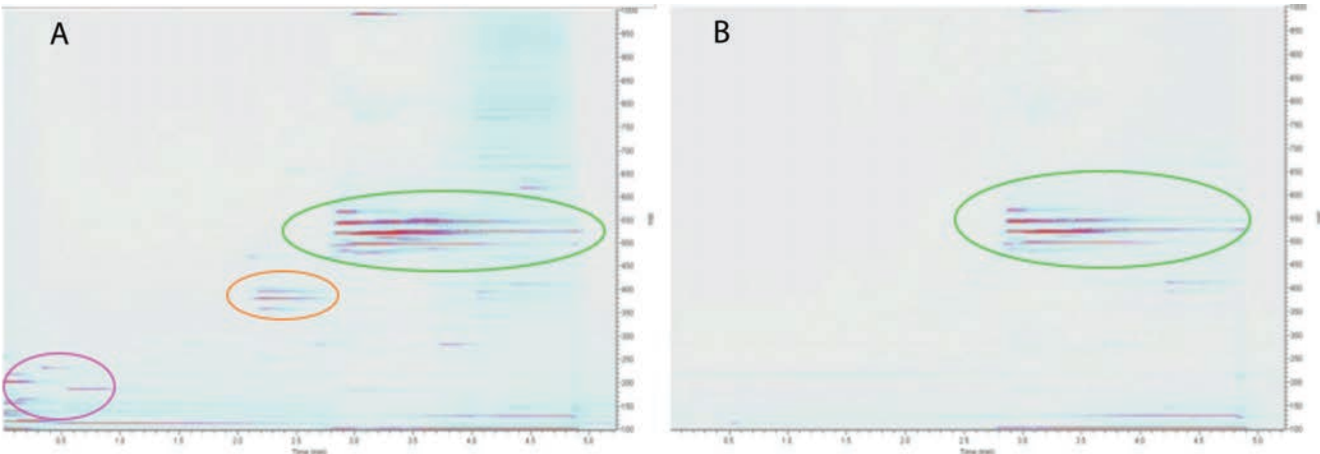

Figure 1.
Comparison of a full mass spectra over time between a plasma sample prepared using protein precipitation (A) and that prepared using solid phase extraction (B). Areas of detected ions are circled.

Figure 1 demonstrates the effect that different sample preparation techniques can have. This figure looks at the full scan spectra of a blank matrix extracted either using protein precipitation or by using solid phase extraction as a function of time with the intensity of a particular mass being highlighted by the intensity of the colour. The protein precipitation is performed using 3:1 acetonitrile to blank rat plasma (100 µL), whereas the solid phase extraction utilises a polymeric stationary phase, and washing with 30% methanol in water and eluting with 100% methanol. For the SPE method the 100 µL blank rat plasma was added to 900 µL of water prior to addition to the 1 mL 30 mg cartridge.

The chromatography was obtained on a C18 column. The mobile phases were 0.1% formic acid in water [A] and 0.1% formic acid in methanol [B]. A gradient program was used in the elution of the analytes from the column; 95% [A] and 5% [B] for 0.5 min, linear change to 5% [A] and 95% [B] over 3 min and hold for 1 min, then revert back to 95% [A] and 5% [B] and hold for 0.5 min. The flow rate was 0.6 mL/min, with the injection volume of 10 µL.

It can be seen that the protein precipitation results in a higher background level of ions, which would not be observed with many traditional assays that focus on a single parent daughter transition and do not look at a full scan spectra. In particular, there is a higher intensity of ions at longer elution times and also at the beginning of the chromatogram. Figure 2 highlights another issue with the protein precipitation approach in that it takes several aqueous blank injections before the matrix is removed. An interesting observation is that the mass spectra obtained with the first aqueous blank has higher molecular masses eluting when compared to the plasma extracted sample [11].

In the previous scenario the use of SPE would be beneficial to improving the robustness of the assay; however the use SPE does require a degree of dexterity to ensure that optimal performance is maintained. Figure 3 highlights one of the issues associated with SPE and one that can be quite common when dealing with multiple samples being processed simultaneously, either on a SPE manifold or using a 96 deep well (DW96) plate format. In both of these scenarios it is not uncommon to have different flow rates in different tubes/wells. There are a variety of reasons why this might exist; from poor manufacture of the SPE frits (pore structure variability etc.), to variations in the samples that are being tested, resulting in very different inter tube/well flow rates being experienced during the sample preparation step which can affect the recovery. Figure 3 shows an elution profile obtained from two

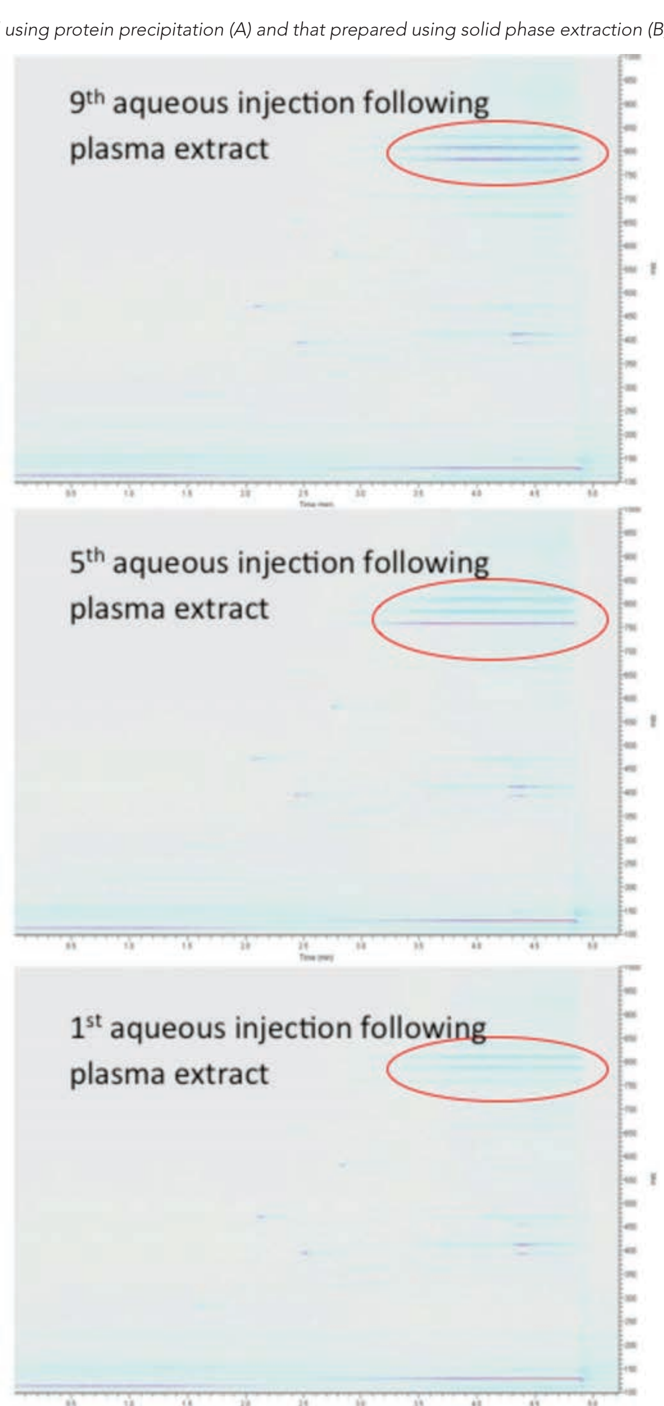

Figure 2
Comparison of full mass spectra over time for 1st, 5th and 9th aqueous injections subsequent to a protein precipitated sample.

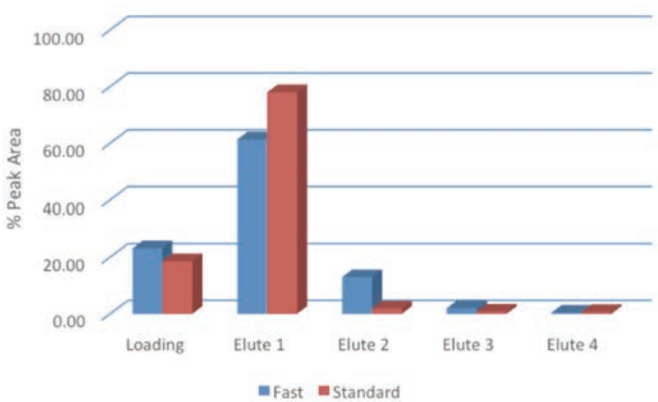

Figure 3
Effect of flow rate on the elution profile obtained for benzoic acid on a polymeric SPE media.

samples where the flow rate has been intentionally altered to simulate this effect. It can be seen from the experiments performed that with the higher flow rate the analyte results in a greater level of breakthrough for the loading stage and that the amount of analyte that is eluted in the initial 100% methanol step is reduced, both of which have an effect on the effective recovery of the analyte.

This phenomenon is caused by the difference in time taken for the pressure driven flow compared to the time required for diffusion into the pores. Diffusion into the pore structure is required to initially capture the analyte of interest since this is where the majority of the surface area resides, thus at higher flow rates the compound simply does not get time to diffuse into the pore structure, and so analyte breakthrough is higher. During the elution part of the process the eluent is required to diffuse into the process to allow the analyte molecule to elute from the SPE media. If sufficient time is not given for this process to occur then the analyte molecule remains within the pore structure during that elution step. Robust assay development will take this effect into consideration, however the use of generic methodologies means that this is not always considered.

Chromatography

A chromatography column is designed to be used for multiple samples, and it is generally assumed for sample analysis that the chromatographic performance does not vary outside of specified performance criteria during the assay. However, it is evident that when using biological extracts that changes to the column are occuring, since the back pressure and chromatographic performance can alter throughout a batch of samples. The changes in back pressure and chromatographic performance are indicators that the surface of the column is changing and that interstitial space and/or frit porosity is being affected by matrix component build-up. Figure 4 demonstrates the effect of running a series of peptides, GSTAENAEYLR (GST), GSHQISLDNPYDQQDFFPK (GSH) and RPAGSVQNPVYHNQPLNPAPSR (RPAG) over a 6 hour period and the chromatographic deterioration that is observed. The chromatography was performed using a binary gradient from 10 - 40% of 0.025% tri-fluoroacetic acid (TFA) aq. and acetonitrile with 0.025% TFA over 10 minutes on a C18 based column. It can be seen that the peak shape deteriorates for all three components (GSH, RPAG and GST) and that there is a shift in the peak retention for one of the compounds as the stationary phase is modified.

In itself the deterioration of the stationary phase due to build-up of

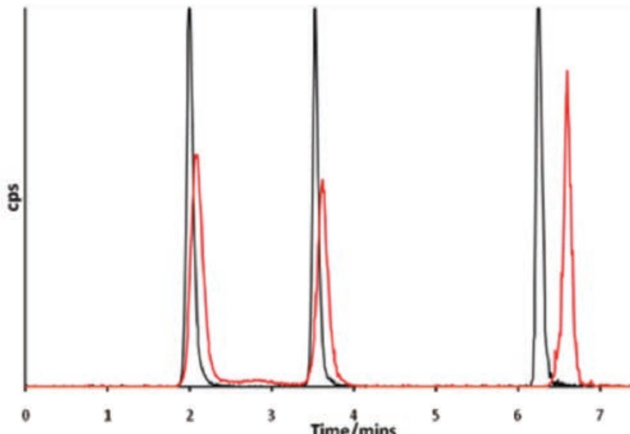

Figure 4
The effect on chromatographic performance of running a column for 6 hours for 3 peptides.

matrix components is detrimental, however at least in the previous example there is an obvious effect that can be seen, and so it would be possible to troubleshoot the assay with a degree of confidence in the data. A different scenario exists however when considering components that are being injected onto the chromatographic system and are not being detected, such as non-ionisable compounds, or compounds with low ionisation efficiencies under the source parameter settings. For most bioanalytical assays this is the majority of the extracted sample, with phospholipids being a good example of compounds that are not routinely detected but which can have a potential effect on the mass spectrometry. Since the elution of these components of the extracted sample are not monitored, the chromatography will not be optimised, which can result in matrix component not eluting during a single chromatographic run.

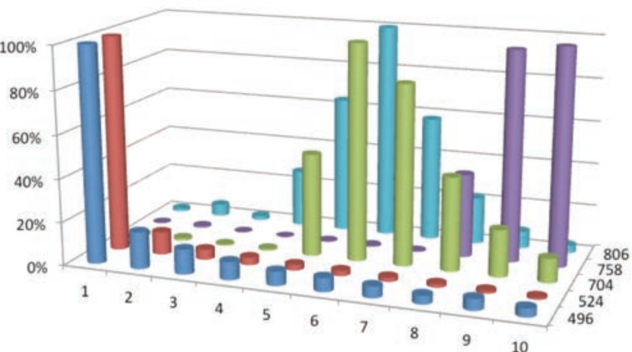

Figure 5
Normalised detector response for phospholipids in ten water injections subsequent to an injection from a protein precipitated extract. Phospholipid m/z transistion labeled.

Figure 5 demonstrates this effect for a protein precipitated sample. Five phospholipid components are monitored, 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine, 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine, glycerophosphocholine lipid, 1-hexadecanoyl-2-(9Z, 12Z–octadecadienoyl)-sn-glycero-3-phosphocholine and 1-(9Z, 12Z–octadecadienoyl)-2-(5Z, 8Z, 11Z, 14Z–eicosatetraenoyl)-sn-glycero-3-phosphocholines. All of these compounds have the same phosphocholine daughter group which has a characteristic mass of 184.3, with the parent masses being; 496.4, 524.4, 704.4, 758.4 and 806.4 respectively. The chromatography has been described earlier in association with the data obtained for Figure 1. It can be clearly seen that the

lower molecular mass phospholipids elute in a very small number of chromatographic cycles, however the heavier molecular mass phospholipids require a substantial number of cycles to elute from the column, indeed even after 10th injection cycles some of the phospholipids are still eluting from the C18 column. The consequence of this is that the amount of suppression will vary from one injection to the next and that the amount of suppression can depend on the nature of the previous sample. Selective removal of the phospholipids will alleviate this issue, which can occur with the appropriate choice of SPE.

Conclusion

The use of sample preparation to remove matrix components is something that separation scientists need to be aware of, however it is also important to be aware of the consequences that not performing adequate sample preparation can have on the overall performance characteristics of the assay. This has greater significance within the regulated environment with the introduction of ISR, which was introduced to ensure the robustness of an assay. It has been demonstrated that the use of simple, cost effective approaches such as protein precipitation can result in greater matrix components being present in the final sample which can have a detrimental effect on the assay performance, due to the common use of mass spectrometry within a bioanalytical laboratory. Where variability is seen in a bioanalytical assay then time should be spent investigating the effects that the matrix has on the system, and then looking to address these issues through improved chromatography or the application of more selective sample preparation techniques.

In order to reduce the deleterious effects of matrix components co-eluting, it is important to be aware of the effects that the matrix components can have, and one approach is to monitor the TIC to identify when co-eluting components are coming off the column. It will also aid in determining potential types of matrix components which will allow for more selective choice of sample preparation.

Phospholipids are present at high concentrations within a range of biological fluids and are renowned for causing ion suppression with a range of compounds. Monitoring these common transitions will allow the extraction process to be optimised to remove a large proportion of these components.

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PREP 2020	Baltimore, USA	31st May-3rd June	http://prepsymposium.org/
HPLC2020	San Diego, USA	20-25th June	www.hplc2020-usa.org/
41st BMSS Meeting	Sheffield, UK	8-10th September	www.bmss.org.uk/41st-bmss-annual-meeting/
ISC2020	Budapest, Hungary	21-25th September	www.isc2020.hu
Gulf Coast Conference	Galveston, USA	13-14th October	https://www.gulfcoastconference.com/
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