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Tips & Tricks for Thin-Layer Chromatography

Not with standing recent changes in certain state laws in the US and federal law in Canada, federal law in the US remains unchanged and the cultivation, possession, and/or sale of marijuana and related cannabis products continues to be illegal under such law. In addition, the distribution of marijuana to minors under the age of (i) 21 years remains illegal under state law in the US and (ii) 18 or 19 years (depending on the state) remains illegal under state law in Canada.

Supelco®
Analytical Products

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Analytix Reporter

Dear Reader,

The first records of the medicinal use of cannabis by ancestral cultures date back to the 500s BC. The Greek, Roman, and Asian civilizations used it for the therapeutic treatment of various diseases like arthritis, inflammation, and pain among others. After being considered an illicit drug for decades, cannabis has now regained prominence in the medical field. Several scientific studies have proved its beneficial effects in the treatment of various pathologies. This has made the countries to start reviewing the legal status of the plant. Paraguay, Canada, and the United States have led efforts to legalize cannabis in recent years. They are now being followed by countries from Europe, Latin America, Asia, and Oceania.

Currently, the laws regulating the manufacturing practices and quality standards of cannabis source materials are few or nonexistent. Besides, they vary greatly between countries or even between different states, in the case of the USA.

Cannabis quality control is mandatory to alleviate patient concerns about the efficacy and safety of cannabis-derived therapies, as well as other legal derivatives. With the rise of cannabis and hemp legislation, the industry has seen an increased demand for accurate development and validation of data, in addition to accurate testing methods.

The potency testing, the assessment of the content of the active cannabinoids in a cannabis/hemp plant materials, is in this context a key requirement. In this edition of the Analytix Reporter we display a workflow for this analysis using monolithic silica columns, that demonstrate due to their bi-modal pore structure, high efficiency paired with exceptional low back pressure and matrix robustness. This in particular is handy when dealing with natural plant sample extracts while still requiring reliability, speed and robustness for efficient and high throughput analytical methods. Please have a read and see how this can benefit your current workflow.

For more applications and solutions for “Cannabis Testing” see our special edition of the Analytix Reporter on **SigmaAldrich.com/analytix** and/or visit us on the bellow mentioned cannabis dedicated website.

Stay beyond the leaf.

Sincerely yours,

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Testing Segment Marketing Manager

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CANNABIS

Workflow for Cannabinoids Analysis in Cannabis using a High Resolution Monolithic Silica HPLC Column Providing Low Backpressure and Extended Column Lifetime

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Introduction

The legal use of recreational and medical cannabis is expanding globally along with hemp-based products (based on cannabidiol), for health and wellness. Hemp is defined legally in various geographies as cannabis varieties with limits on total tetrahydrocannabinol (THC) content. To ensure consumer safety, cannabis and hemp products need to be tested to determine accurate potency of the active cannabinoid constituents. Cannabis products in the market range from plants to distillates, and edibles to cosmetics. This broad variety of matrices underscores the need for robust columns and high throughput analytical methods.

This work provides a complete HPLC-DAD (high performance liquid chromatography-diode array detection) workflow for cannabinoids analysis using robust Chromolith® HighResolution (HR) HPLC columns which are based on monolithic silica. Chromolith® HPLC columns enable fast and cost-efficient separations due to low column backpressure and the very high robustness of the column. The low backpressure allows fast separation at high flow rates with the same mobile phase consumption per sample compared to slower low flow-rate methods. The workflow offers the following:

- Detailed hemp bud sample preparation for HPLC-UV analysis.
- Fast and cost-efficient use of low back pressure Chromolith® HPLC columns to determine potency of a hemp bud sample.
- Demonstration of robustness of the Chromolith® column.
- Separation of 14 cannabinoids within 10 minutes.
- Calibration curve preparation using Certified Reference Materials (CRMs).

Chemical Structures of 14 Cannabinoids

There are more than 100 distinct cannabinoids that have been isolated from cannabis. Delta-9-Tetrahydrocannabinol (Δ^9-THC) is the primary psychoactive compound and cannabidiol (CBD) is another major non-psychoactive constituent in cannabis. Structures of Δ^9-THC, CBD, and some other cannabinoids analyzed by the method are shown in **Figure 1**.

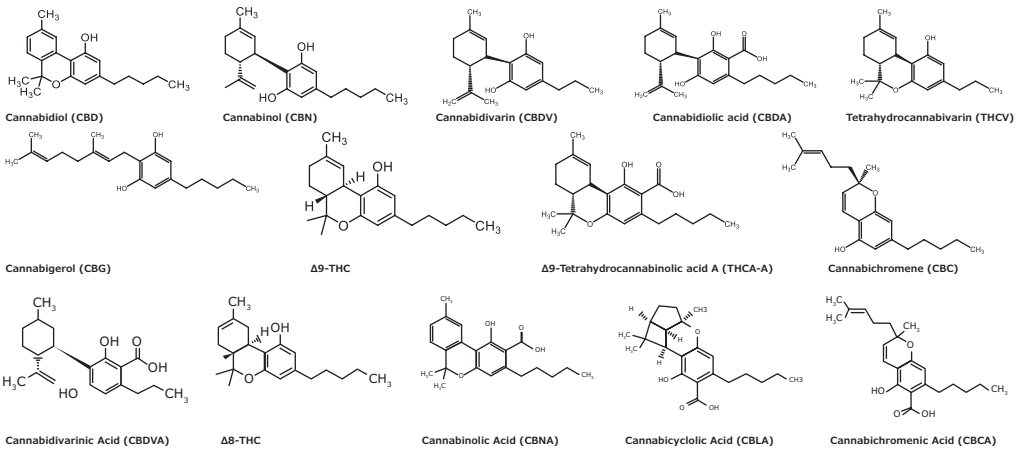

Figure 1. Chemical structures of fourteen cannabinoids included in this study.

Experimental

In this work, hemp bud samples were analyzed to determine their potency. Sample preparation involved ethanol extraction of cannabinoids from plant material. The extract was then analyzed applying an HPLC-UV method and using a Chromolith® HR RP-18e monolithic silica HPLC column. Quantitation was performed utilizing a 6-point calibration curve obtained from HPLC-UV analysis of standard solutions prepared from CRMs. Peaks were identified using the retention times from a chromatogram of a 14 cannabinoids mix. Cannabinoid peaks were also verified by comparing UV absorption spectra of both samples and standards. Furthermore, robustness of the monolithic silica based Chromolith® was demonstrated via retention time stability and separation efficiency after 1400 injections.

Preparation of Mobile Phases

For mobile phase A, 0.1% H_3PO_4 (aq.) was prepared by adding 1 mL H_3PO_4 to 1000 mL of water. Pure methanol was used as mobile phase B.

Preparation of Standard Solutions

Standard solutions containing six major analytes were prepared using Supelco® CRMs as shown in the **Table 1**.

Table 1. Preparation of standard solutions for 6 cannabinoids determined

Step	Instructions				
1	To a 1.5 mL autosampler vial, add the following volumes of CRM solutions to prepare the stock solution:				
	#	**Cannabinoid**	**Cat. No.**	**Conc. (mg/mL)**	**Volume (µL)**
	1	CBD	**C-045**	1.0	100
	2	CBG	**C-141**	1.0	100
	3	CBDA	**C-144**	1.0	100
	4	CBN	**C-046**	1.0	100
	5	Δ⁹-THC	**T-005**	1.0	100
	6	THCA	**T-093**	1.0	100
2	Add 400 µL of methanol and mix well. Final concentration is 100 µg/mL for all cannabinoids.				
3	Prepare the solution for 6-point calibration curve following the dilution scheme below using methanol as a diluent:				
	Conc. (µg/mL)		**Solution ID**	**Dilution Factor**	**Source Solution**
	100		Stock	1	Stock
	25		A	4	Stock
	5		B	5	A
	1		C	5	B
	0.5		D	2	C
	0.25		E	2	D

Preparation of Peak Identification Solution

A peak identification solution containing 14 cannabinoids was prepared using CRMs, as shown in **Table 2**.

Table 2. Preparation for peak identification solution for 14 compounds

Step	Instructions				
1	To a 1.5 mL autosampler vial, add the following volumes of CRM solutions:				
	#	**Cannabinoid**	**Cat. No.**	**Conc. (mg/mL)**	**Volume (µL)**
	1	8-mix (neutrals)	**C-219**	0.5	100
	2	CBDVA	**C-152**	1.0	50
	3	CBDA	**C-144**	1.0	50
	4	CBNA	**C-153**	1.0	50
	5	THCA	**T-093**	1.0	50
	6	CBLA	**C-171**	0.5	50
	7	CBCA	**C-150**	1.0	50
2	Add 600 µL of methanol and mix well. Final concentration is 25 µg/mL for CBLA and 50 µg/mL for all other cannabinoids.				

Preparation of Hemp Bud Extract

Cannabinoids were extracted from hemp buds using ethanol extraction as explained below:

1. Homogenize 1 g hemp bud (particle size <1 mm). (Low temperature homogenization such as frozen ball-milling is the preferred method of homogenization without sample degradation.[1])
2. Transfer the homogenized sample to a 50 mL polypropylene centrifuge tube.
3. Dispense 20 mL ethanol and vortex for 30 s.
4. Incubate sample on horizontal shaker for 30 min.
5. Centrifuge sample at 4000 rpm for 5 min to pellet plant material.
6. Transfer the supernatant into amber 100 mL volumetric flask and keep the pellet for second extraction.
7. Perform second extraction with 20 mL ethanol and add the supernatant to amber 100 mL volumetric flask containing contents of the first extraction.
8. Fill flask to 100 mL mark with ethanol and mix well.
9. Perform 1:10 and 1:100 dilution of sample with ethanol.
10. Filter samples into HPLC vials with 0.2 µm PTFE membrane. Here, syringeless filter-vials were used.

Subsequent analysis was performed applying a 2 mm I.D. Chromolith® HR RP-18e HPLC column using conditions described in **Table 3**.

Table 3. Chromatographic conditions for determination of 14 cannabinoids by HPLC-UV.

HPLC Parameters	
Instrument:	Agilent 1290 Infinity II LC System; Quaternary Pump; 0.12 mm ID tubing; 10 mm Max-light Cartridge Cell 1.0 µL
Column:	Chromolith® HighResolution RP-18e 50-2mm (**1.52321**)
Mobile phase:	[A] Water with 0.1% H_3PO_4; [B] methanol
Gradient:	Time (min) / %A / %B: 0 / 38 / 62; 1 / 38 / 62; 8 / 10 / 90; 10 / 10 / 90; 10.1 / 38 / 62
Flow:	0.5 mL/min
Max Pressure:	150 bar
Column temp.:	40 °C
Detection:	DAD, UV 228 nm
Injection:	1.0 µL

Results and Discussion

Hemp bud sample was homogenized at low temperature to prevent analyte degradation using cryo-cup grinder followed by double extraction with ethanol. Resulting solution was diluted, filtered, and subjected to HPLC-DAD analysis. Calibration curves were obtained by analyzing solutions prepared from CRMs. Cannabinoids in hemp bud extract were identified based on retention time match with standards and cross verified with UV absorption spectra.

System Suitability: Peak Identification Solutions

CRMs as 1.0 mg/mL or 0.5 mg/mL solutions in methanol or acetonitrile, were used to prepare calibration and peak identification solutions. Separation of 14 cannabinoids was demonstrated with good resolution and analyte signal reproducibility (**Table 4**). Separation of 14 cannabinoids was achieved in less than 10 minutes (**Figure 2**).

Table 4. Peak resolution and system reproducibility.

Analyte	Resolution	%RSD of area count (n=5)	Analyte	Resolution	%RSD of area count (n=5)
CBDV	-	0.20	Δ⁹-THC	4.5	0.18
CBDVA	6.3	0.17	Δ⁸-THC	2.1	0.23
THCV	7.7	0.18	CBC	6.9	0.16
CBD	1.7	0.19	CBNA	2.8	0.20
CBG	2.1	0.19	THCA	4.6	0.18
CBDA	2.7	0.17	CBLA	1.8	0.17
CBN	6.9	0.18	CBCA	2.3	0.19

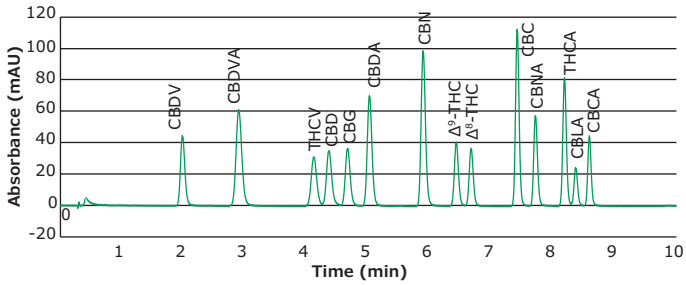

Figure 2. Chromatogram of 14 cannabinoids mixture obtained with a Chromolith® HR RP-18e 50×2 mm column at 228 nm.

Quantitation

Calibration curves were obtained for six major cannabinoids (CBD, CBG, CBDA, CBN, Δ⁹-THC, and THCA); see chromatogram in **Figure 3**. Linearity of R^2>0.995 was obtained for all analytes within the range of 0.25-100 µg/mL (**Figure 4**).

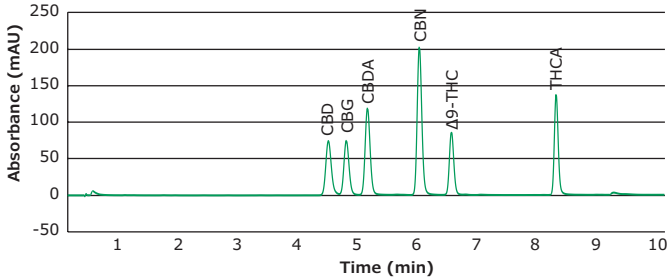

Figure 3. Chromatogram of calibration mixture at 100 µg/mL obtained with a Chromolith® HR RP-18e 50×2 mm column at 228 nm.

Results showed that the "as is" hemp bud sample contained 7.37% (wt/wt) total CBD and 0.25% (wt/wt) total THC (**Table 5**). Stable retention time for cannabinoids was observed during 1400 injections, demonstrating the robustness of the column towards a complex matrix like hemp bud extract.

Table 5. Potency of hemp bud sample (Total CBD = CBD + 0.877xCBDA, and Total THC = Δ⁹-THC + 0.877xTHCA).

Analyte	Conc*. (% by weight)
CBD	0.37
CBG	0.00
CBDA	7.37
Δ⁹-THC	0.04
CBN	0.05
THCA	0.21
Total CBD	7.74
Total THC	0.25

*Note: The stated cannabinoid concentrations were calculated on "as is" basis and were not adjusted to dry weight. For many countries "official" potency testing the THC content needs to be referred to a dry weight sample base.[2] For example: the USDA definition is that samples should be dried to a consistent weight (typically 5-12% moisture content). Alternatively, Karl Fischer titration can be applied to determine moisture content.

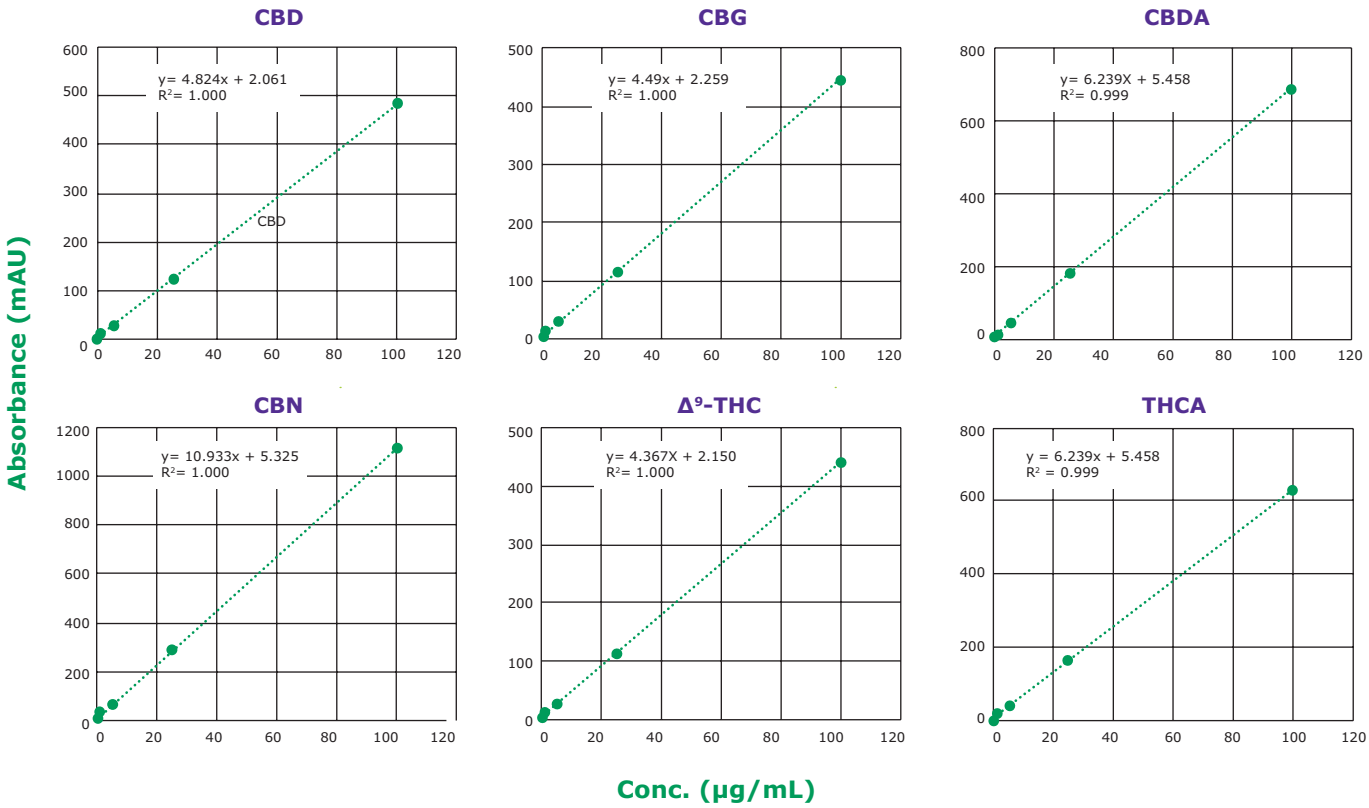

Figure 4. Calibration curves of six cannabinoid analytes obtained with a Chromolith® HR RP-18e 50×2mm column at 228 nm. Calibration curve ranges from 0.25 to 100 µg/mL. Linearity: R^2 >0.995 for all six analytes.

In HPLC-UV analysis, identity of analytes depends on retention times and can be compromised by co-eluting peaks. Therefore, it is necessary to ensure that no co-elution of matrix compounds with the peak of interest is taking place. Here, we checked for the effects of matrix impurities by comparing the UV absorption spectra of the analytes identified in the sample with those of the standards. As can be seen in **Figure 6**, most analytes in the hemp bud extract display absorption profiles similar to those of the standard. Among them, the spectra of CBN seems to contain an impurity which is visible as an extra peak in the chromatogram in **Figure 5** as well. This additional verification with UV absorption spectra further ensures the identity of detected analytes.

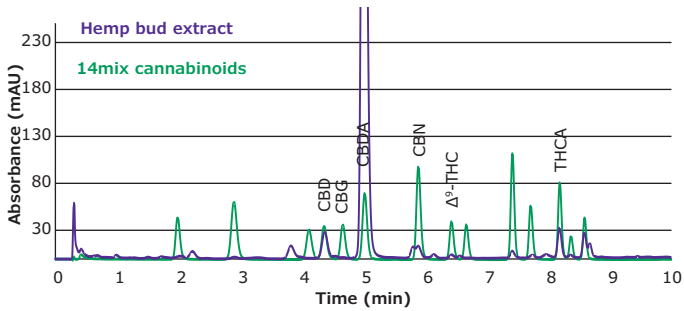

Figure 5. Overlay of chromatograms of peak identification solution and hemp bud extract obtained with a Chromolith® HR RP-18e 50 x 2 mm I.D. column at 228 nm.

Robustness of Chromolith® Columns

The separation of matrix-rich samples, such as herbs, food, or biological samples tends to reduce the lifetime of particulate columns if insufficient sample preparation/cleanup is performed before HPLC separation. Chromolith® monolithic silica HPLC columns due to their bimodal pore structure allow the separation of matrix-rich samples with extended column lifetime, with no or very reduced sample preparation required. Extended column lifetime and reduced sample preparation significantly reduces the overall cost of operation. **Figure 7** shows the stability of the retention factors for the analysis of cannabinoids in a hemp extract sample with a Chromolith® HR RP-18e 100×2 mm column over 1400 injections. Results demonstrate the robustness of monolithic silica based Chromolith® columns.

In between cannabinoid sample analyses, the separation efficiency of the Chromolith® column was tracked with a performance test using anthracene. Separation efficiency was tracked by calculating plate count (N). Results were compared with those obtained for two other HPLC columns with small particle size: Ascentis® Express 2 µm (superficially porous particles, SPP) and Purospher® STAR 2 µm (fully porous particles, FPP) (**Table 6**). In addition, retention

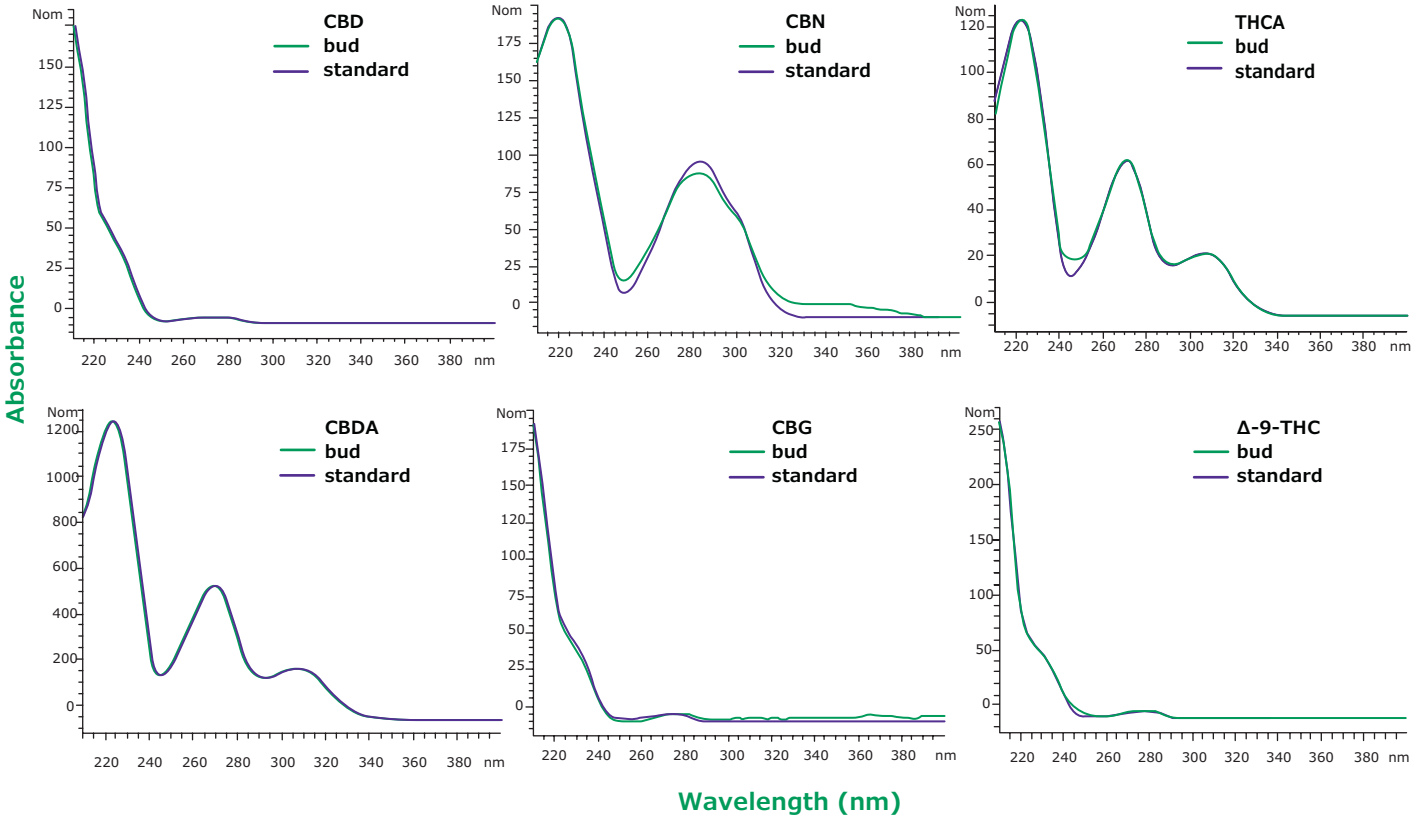

Figure 6. Overlay of UV spectra from hemp bud sample (green) and standard solution at same retention time (purple) for six analytes. Slight differences in CBN spectra between sample and standard might be due to the overlap with another peak as can be seen in **Figure 5**.

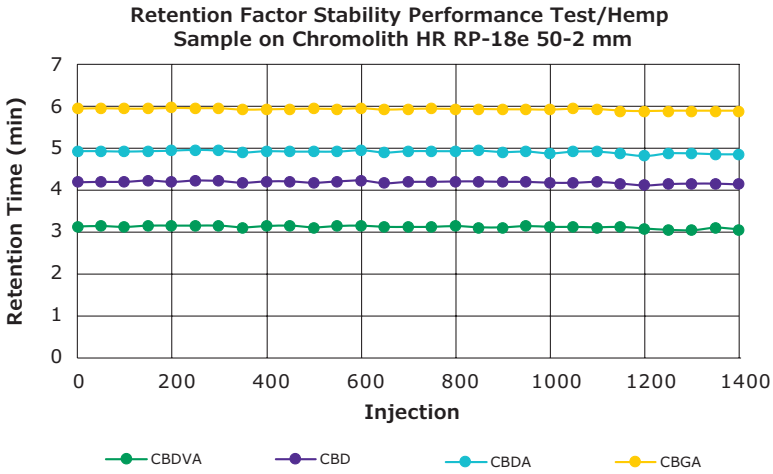

Figure 7. Retention factor stability performance test/hemp sample on a Chromolith® HighResolution RP-18e 100×2 mm I.D. (Column temp.: 25 °C, mobile phase A: 0.1% H_3PO_4, mobile phase B: methanol, flow rate: 0.38 mL/min, injection volume: 0.2 µL, gradient: 72% B for 0.1 min, 72-90% B in 7 min, hold at 90% B for 3 min).

Table 6. Separation efficiency for anthracene and retention time of CBD using three different HPLC column types

Column	Separation Efficiency Anthracene			Retention Time CBD		
	Initial (N/m)	After 1400 injections (N/m)	Relative Change	Initial (min)	After 1400 injections (min)	Relative Change
Chromolith® HR RP-18e	165,990	161,500	-2.7%	4.19	4.14	-1.2%
Purospher® STAR RP-18e 2 µm (FPP)	188,450	140,770	-25.3%	6.42	6.34	-1.2%
Ascentis® Express C18 2 µm (SPP)	241,690	214,310	-11.3%	5.28	5.14	-2.7%

time stability was compared using CBD. For the CBD retention time comparison, the cannabinoid separation was optimized for the Chromolith® column and was then transferred to Purospher® STAR and Ascentis® Express columns in order to create comparable stability data. No significant change in the CBD retention time was observed after 1400 injections. The efficiency for the monolithic silica based Chromolith® column only slightly decreased (2.7%), while for the fully porous and superficially porous, a reduction of 25.3% and 11.3%, respectively, was determined. These results again show the robustness of both bimodal pore structure and rigid monolithic silica skeleton over an extended analysis period.

Conclusion

This work demonstrates an HPLC-DAD workflow, using a monolithic silica based Chromolith® HR RP-18e HPLC column, for the determination of cannabinoids in hemp bud samples. Sample homogenization, use of accurate CRMs, separation of 14 cannabinoids with good selectivity, and robustness of Chromolith® columns are important elements of the workflow. Hemp bud samples were homogenized at low temperature to prevent analyte degradation using a cryo-cup grinder, followed by double extraction with ethanol. The resulting solution was diluted, filtered, and subjected to HPLC-DAD analysis. Calibration curves were obtained by analyzing solutions prepared from CRMs. Cannabinoids in hemp bud extract were identified based on retention time match with standards and cross verified with UV absorption spectra. Results showed that the hemp bud samples contained 7.37% (wt/wt) total CBD and 0.25% (wt/wt) total THC on as is basis without determining dry weight data. The robustness of Chromolith® columns was also demonstrated by the analysis of hemp bud and stable retention factors for cannabinoids over 1400 injections, proving once more the suitability of these columns for matrix-rich samples.

References

1. Vaclavik L, Benes F, Fenclova M, Hricko J, Krmela A, Svobodova V, Hajslova J, Mastovska K. Quantitation of cannabinoids in Cannabis dried plant materials, concentrates, and oils using liquid chromatography–diode array detection technique with optional mass spectrometric detection: Single-laboratory validation study, first action 2018.11. Journal of AOAC International. 2019;102(6):1822–1833. https://doi.org/10.1093/jaoac/102.6.1822
2. AOAC SMPR 2019.003. Standard Method Performance Requirements (SMPRs) for quantitation of cannabinoids in Plant Materials of Hemp (Low THC Varieties Cannabis sp.). https://www.aoac.org/wp-content/uploads/2019/10/SMPR-2019_003.pdf

Featured Products

Description	Cat. No.
HPLC Columns	
Chromolith® HighResolution RP-18e 50-2 mm HPLC Column	**1.52321**
Chromolith® HighResolution RP-18e 100-2 mm HPLC Column	**1.52322**
Sample Preparation, Solvents & Reagents	
Ultrapure Water from Milli-Q® IQ 7005 water purification system or bottled water	**ZIQ7005T0C or 1.01262**
Phosphoric acid, suitable for HPLC, LiChropur™, 85%	**49685**
Methanol, suitable for HPLC, ≥99.9%	**34860**
Ethanol 200 proof, HPLC/spectrophotometric grade	**459828**
Filter Vial, PTFE 0.2 µm, Whatman® Mini-UniPrep® G2 standard septum	**WHAGN203NPEORG**
Certified Reference Materials and System Suitability Reagents (Cerilliant®)	
Cannabichromene (CBC), 1.0 mg/mL in methanol	**C-143**
Cannabichromenic Acid (CBCA), 1.0 mg/mL in acetonitrile	**C-150**
Cannabicyclolic Acid (CBLA), 0.5 mg/mL in acetonitrile	**C-171**
Cannabidiol (CBD), 1.0 mg/mL in methanol	**C-045**
Cannabidiolic Acid (CBDA), 1.0 mg/mL in acetonitrile	**C-144**
Cannabidivarin (CBDV), 1.0 mg/mL in methanol	**C-140**
Cannabidivaric Acid (CBDVA), 1.0 mg/mL in acetonitrile	**C-152**
Cannabigerol (CBG), 1.0 mg/mL in methanol	**C-141**
Cannabinol (CBN), 1.0 mg/mL in methanol	**C-046**
Cannabinolic Acid (CBNA), 1.0 mg/mL in acetonitrile	**C-153**
Δ^9-Tetrahydrocannabinol (Δ9-THC), 1.0 mg/mL in methanol	**T-005**
Δ^8-Tetrahydrocannabinol (Δ8-THC), 1.0 mg/mL in methanol	**T-032**
Tetrahydrocannabinolic Acid (THCA), 1.0 mg/mL in acetonitrile	**T-093**
Tetrahydrocannabivarin (THCV), 1.0 mg/mL in methanol	**T-094**
8 Cannabinoids Mix (Neutrals)	**C-219**
Accessories	
Clear Glass Vial, 2 mL, pk of 100, Natural PTFE/silicone septa	**29652-U**
Amber Glass Vial, 2 mL, pk of 100, Natural PTFE/silicone septa	**29653-U**

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CLINICAL & FORENSIC

Steroid Hormone Analysis in Serum using Supel™ Swift HLB DPX Tips

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Introduction

Routine hormone analysis is necessary for establishing and monitoring patient diseases. For example, the continuous monitoring of cortisol levels can help diagnose a patient with Cushing disease (high cortisol) or Addison disease (low cortisol).[2,3] A robust method for steroid hormone determination in serum is imperative in diagnostics and treatment. The Supel™ Swift HLB DPX Tips (3 mg bed, Hamilton®) allow for reduced sample volume, sample evaporation mitigation, and offer a fully automated approach. In this method, a total of 9 steroids (cortisone, cortisol, 11-deoxycortisol, androstenedione, testosterone, dehydroepiandrosterone, 5α-dihydrotestosterone, 17α-hydroxyprogesterone, and progesterone) were analyzed as a panel to provide a variety of testing applications and disease diagnostics.

The Supel™ Swift HLB sorbent provides good selectivity and sensitivity for steroids in a neutral solution[1], allowing for dilution with water prior to injection. The sorbent has significant versatility in analyte binding due to the co-polymer phase containing both hydrophilic and lipophilic functional groups.[1]

This validated method used 100 µL of serum and the final volume available for injection was approximately 100 µL, allowing for a 1:1 concentration factor without solvent evaporation. Recoveries for the 9 analytes range from 65-86% **(Table 7).** The LOQs for all analytes fall below clinically relevant values, and linear dynamic ranges were between 0.025 ng/mL and 250 ng/mL. The automated extraction method allows up to 96 samples to be processed simultaneously in approximately 20 minutes prior to LC-MS/MS analysis.

Figure 1. Supel™ Swift DPX HLB 3 mg (bed) Tips. The tips are being actively picked up by the automated liquid handler.

Experimental

Methods

A Hamilton® Microlab NIMBUS96 was utilized to automate sample preparation using the Supel™ Swift DPX HLB Tips (**Figure 1**). The analysis was performed on an Agilent 1290 LC system coupled with a SCIEX Triple Quad™ 6500+ tandem mass spectrometer. The LC column used was an Ascentis® Express C18 (2.7 µm particle size, L × I.D. 10 cm × 3 mm) joined with an Ascentis® Express C18, 2.7 Micron Guard Cartridge in an Ascentis® Express Guard Cartridge Holder (Millipore Sigma, Burlington, MA); this combination allowed for optimal separation of all steroids. An injection volume of 15 µL was found to be optimal to meet required cutoffs. The LC conditions are shown in **Table 1.** Ammonium fluoride additives are common in steroid analysis[4] but were found to decrease retention time stability and were therefore omitted. The mass spectrometer source parameters are available in **Table 2** with the transitions monitored in **Table 3.**

Sample Preparation

Serum was aliquoted (100 µL) into a 2 mL V-bottom polypropylene well plate. The internal standard mixture (200 ng/mL for all internal standards except for DHT and progesterone which were 500 ng/mL) was added (10 µL) and allowed to incubate for 1 hour at ambient temperature. The well plate was then loaded onto the NIMBUS96 system for the rest of the automated protocol. The automated liquid handler (ALH) picked up a set of standard transfer tips, added 200 µL of aqueous 0.4% formic acid to the sample and mixed thoroughly. This solution was then incubated for 15 minutes prior to sample extraction. While the protein dissociation step occured, the ALH picked up a second set of transfer tips for aliquoting the wash solvents into appropriate well plates (**Figure 4**). After that the ALH picked up the Supel™ Swift HLB DPX Tips and conditioned the HLB sorbent by aspirating and dispensing 300 µL of 20% methanol from a buffer

reservoir two times. Once the protein dissociation timer was complete, the ALH moved to the sample well plate and aspirated and dispensed the sample five times to bind analytes to the HLB sorbent. The ALH moved to the first wash location (300 µL of 100% water) and aspirated/dispensed three times, and sequentially moved to the second wash location (300 µL of 20% methanol) and aspirated/dispensed three times. The ALH ejected the Supel™ Swift HLB DPX Tips back into the original deck position and picked up the transfer tips to aliquot the elution solvent into the appropriate well plate. This was done to avoid solvent evaporation of the low elution volume while the previous steps of the method were completed. Finally, the ALH picked up the Supel™ Swift HLB DPX Tips again and moved to the elution location (75 µL 50/50 MeOH/ACN), aspirated/dispensed three times. The tips were ejected, and standard tips were picked up to dilute the eluent with 25 µL of water. The final sample plate was then sealed and vortexed briefly for 5-10 seconds before submitting for analysis by LC-MS/MS injection. **(Figures 2, 3,** & **4)**

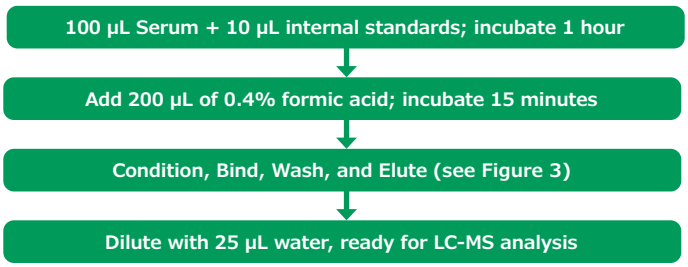


Figure 2. Sample preparation method.

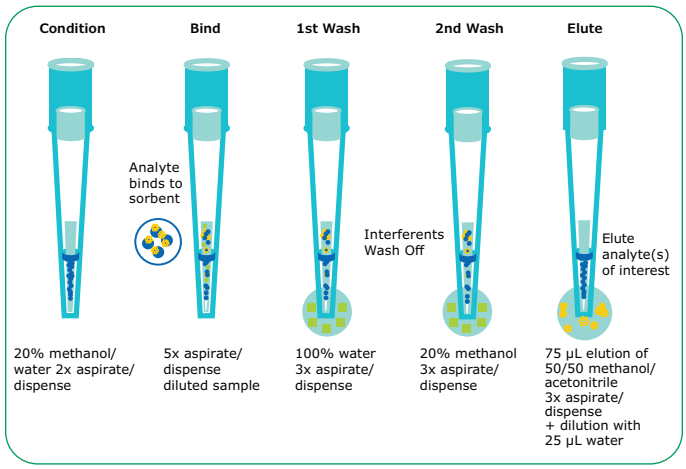
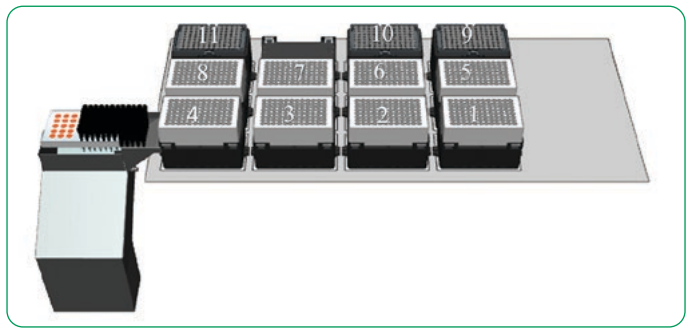


Figure 3. Schematic of the automated bind/wash/elute steps.



#	Usage/Step Performed
1	**Sample plate** Preloaded manually with 100 µL serum + ALH adds 200 µL 0.4% formic acid (#5) in water, timer to wait 15 mins
2	**Wash Plate #1** + 300 µL water (from 7)
3	**Wash Plate #2** + 300 µL 20% methanol in water (from 6)
4	**Elution Plate** + 75 µL 50/50 methanol/acetonitrile (7) for elution + 25 µL water post-elution (6)
5	**0.4% Formic Acid in Water** • Used for protein dissociation of sample (1)
6	**20% Methanol in Water** • Used for loading wash plate #2 (3) and • Used for conditioning of tips
7	**Water** • Used for loading wash plate #1 (2)
8	**50/50 Acetonitrile and Methanol** • Used for elution in (4)
9	**Standard Transfer Tips (300 µL)** • One set used for the formic acid addition
10	**Supel™ Swift HLB DPX Tips, 3 mg (bed)**
11	**Standard Transfer Tips (300 µL)** • Used for the remaining transfer steps (adding elution solvent to the well plate, etc.)

Figure 4. Representative Hamilton® Microlab NIMBUS96 deck overview with designation of lab equipment and/or role in the method

Ionization Effects and Recoveries

Ionization effects and recovery studies were performed as outlined by Scientific Working Group for Forensic Toxicology, SWGTOX.[5]

Briefly, recoveries were evaluated by preparing two sets of serum samples; the first that were spiked with internal standards prior to extraction, and the second set were serum samples that were spiked with internal standards after extraction.

Ion suppression/enhancement was evaluated by preparing two sets of samples. The first set being internal standards prepared in final solution composition (3 equivalents of 50/50 methanol/acetonitrile to 1 equivalent of water) and the second set being the internal standards spiked into the post-extracted solution. All internal standard concentration were as described earlier (200 ng/mL, except for DHT and progesterone which were 500 ng/mL).

Results & Discussion

Method Development

When analyzing endogenous compounds in complex biological matrices, optimal compound separation is imperative. While the panel here consisted of 9 compounds, there are dozens of known endogenous compounds to monitor to ensure analysis was selective and accurate. In the initial method development, a 50 mm column was evaluated, however, isobars were nearly impossible to separate. For example, DHEA and testosterone are isobars (both with a molecular weight of 288.42 g/mol). To achieve baseline separation, a 100 mm column was necessary. A second set of isobars includes three isomers; 11-deoxycortisol, 17-deoxycortisol and 21-deoxycortisol. These three co-eluted and required isocratic separation. Without incorporation of an isocratic plateau (0.5 - 3.7 min) and using a longer column (100 mm), separation of the isobars proved to be unachievable. 17-Deoxycortisol and 21-dexoycortisol were not evaluated further in the analysis. Refer to **Figure 5** for chromatographic separation achieved by this method (LC method in **Table 1).**

Table 1. Instrument Parameters and LC Method

Instrument:	Agilent 1290 and AB SCIEX Triple Quad™ 6500+
Column:	Ascentis® Express C18, 10 cm x 3 mm I.D., 2.7 µm, with attached Ascentis® Express C18 guard cartridge, 5 mm x 3 mm I.D., 2.7 µm
Mobile Phase A:	10 mM ammonium formate + 0.05% formic acid in water, pH 3.
Mobile Phase B:	50:50 methanol:acetonitrile
Gradient/Flow rate:	

Time (min)	%A	%B	Flow Rate (µL/min)
0	50	50	500
0.5	45	55	400*
3.7	45	55	500
3.8	20	80	500
6.0	10	90	500
9.5	0	100	500
12.0	0	100	500
12.1	50	50	500
17.0	50	50	500

Column Temp:	40°C
Detector:	MS/MS (see settings **Table 2 & 3**)
Injection Volume:	15 µL

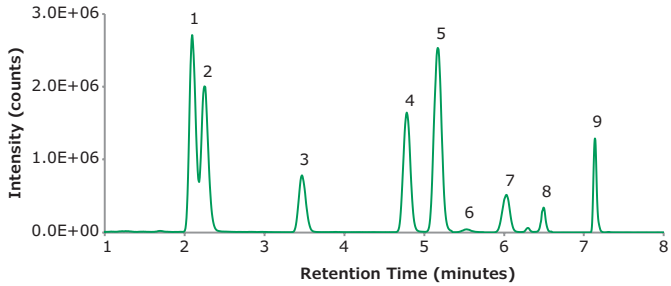
* Slower flow rate allowed for the separation of 11-deoxycortisol from 17-deoxycortisol

Table 2. MS Parameters

Source/Gas Parameters	
Curtain Gas (CUR):	35
Collision Gas:	8
IonSpray Voltage (IS):	4500
Temperature (TEM):	600° C
Ion Source Gas 1 (GS1):	60
Ion Source Gas 2 (GS2):	60

Method Repeatabilty

A three-day precision and accuracy study was performed for 8 analytes utilizing external quality control serum from UTAK and NIST-971a (UTAK Laboratories, Inc., Valencia, CA, USA and NIST, Gaithersburg, MD, USA). Neither source offered verified values for DHT, therefore it was omitted. Ultimately, the inter-day precision of the 8 analytes (excluding DHT) varied from 0.30% to 12%. Intra-day precision ranged from 1.9% to 8.5%. Samples were performed in triplicates over three days.



1. cortisone (50 ng/mL)
2. cortisol (50 ng/mL)
3. 11-deoxycortisol (5 ng/mL)
4. testosterone (5 ng/mL)
5. androstenedione (5 ng/mL)
6. DHEA (5 ng/mL)
7. 17α-hydroxyprogesterone (5 ng/mL)
8. DHT (5 ng/mL)
9. progesterone (5 ng/mL)

Figure 5. Representative TIC of a spiked blank serum with 9 steroids.

Using the data summarized in **Table 4, Figures 6,** and **7** were created to compare theoretical UTAK versus measured values. A correlation graph comparing the UTAK provided values to the InTip™ dispersive SPE is shown in **Figure 6**. Overall, a slightly higher average steroid concentration was found using Supel™ Swift HLB DPX compared to the UTAK provided values with a slope of 1.19 with excellent linearity represented by R^2 = 0.9974, when considering all controls sampled. Another representation of this data is presented in **Figure 7** using the Bland-Altman analysis. The near zero bias (-3.02) and the evenly scattered error (positively and negatively) demonstrates that the two methods are interchangeable.

Like with the UTAK data, using the data summarized in **Table 5**, **Figures 8,** and **9** were created. A correlation graph comparing the NIST values to the InTip™ dispersive solid phase extraction (dSPE) is shown in **Figure 8**. Overall, a slightly higher average steroid concentration was shown using Supel™ Swift HLB DPX Tips compared to the NIST provided values with a slope of 1.11 with excellent linearity represented by R^2 = 0.9922 with a near zero y-intercept. Another representation of this data is presented in **Figure 9** using the Bland-Altman analysis. The near zero bias (-0.09) and the evenly scattered error (positively and negatively) demonstrates once again that the two methods are interchangeable.

Great sensitivity and chromatographic separations allowed for levels of detection in the sub-nanogram per milliliter range. Limits of quantification (LOQ)

Table 3. Transitions monitored by MS/MS

#	Analyte	RT (min)		Q1	Q3	Dwell Time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
1	Cortisone	2.14	Quant	361.2	163.1	80	140	10	33	18
			Qual	361.2	90.9	80	140	10	87	12
	Cortisone-$^{13}C_3$	2.14	IS	364.1	165.9	80	140	10	38	11
2	Cortisol	2.30	Quant	363.2	121.1	80	105	10	24	10
			Qual	363.2	91.1	80	105	10	72	10
	Cortisol-$^{13}C_3$	2.30	IS	366.1	124.2	80	105	10	31	10
3	11-Deoxycortisol	3.56	Quant	347.2	109.1	80	156	10	31	10
			Qual	347.2	97.1	80	156	10	29	8
	11-Deoxycortisol-D_5	3.53	IS	352.2	100.0	80	156	10	31	10
4	Androstenedione	5.25	Quant	287.1	97.1	120	80	10	30	6
			Qual	287.1	109.1	120	80	10	30	10
	Androstene-3,17-dione-2,3,4-$^{13}C_3$	5.25	IS	290.2	100.1	120	171	10	27	14
5	Testosterone	4.87	Quant	289.2	97.3	120	100	10	31	10
			Qual	289.2	108.8	120	100	10	31	12
	Testosterone-2,3,4-$^{13}C_3$	4.86	IS	292.2	99.9	120	196	10	29	12
6	Dehydroepiandrosterone (DHEA)	5.53	Quant	289.1	253.3	400	60	10	18	12
			Qual	289.1	213.2	400	60	10	18	12
	Dehydroepiandrosterone-D_5 (DHEA-D_5)	5.53	IS	294.2	276.3	400	151	10	13	8
7	17α-Hydroxyprogesterone	6.10	Quant	331.4	109.2	200	100	10	40	10
	(OH-progesterone)		Qual	331.4	97.1	200	100	10	40	8
	OH-progesterone-2,3,4-$^{13}C_3$	6.09	IS	334.2	100.1	200	231	10	29	12
8	5α-Dihydrotestosterone (DHT)	6.55	Quant	291.2	255.2	180	110	10	24	10
			Qual	291.2	77.1	180	158	10	80	10
	5α-Dihydrotestosterone-D_3 (16,16,17-D_3)	6.52	IS	294.2	258.2	180	156	10	23	14
9	Progesterone	7.18	Quant	315.1	96.9	120	160	10	15	10
			Qual	315.1	109.0	120	160	10	15	14
	Progesterone-D_9	7.14	IS	324.2	99.9	120	201	10	29	10

Table 4. Real patient sample validation with UTAK Quality Controls. Concentration Range correspond to values from standards. Average Concentrations correspond to the experimental values obtained with Supel™ Swift HLB DPX Tips. %CVs are representative of 9 total replicates.

UTAK Quality Control Samples		Low (ng/mL)	High (ng/mL)
Progesterone	Concentration Range	7.5-12	20-24
	Average Concentration (%CV)	6.55 (6.5%)	17.9 (6.4%)
OH-Progesterone	Concentration Range	3-4.49	8-9.2
	Average Concentration (%CV)	3.9 (3.3%)	9.37 (5.5%)
Testosterone	Concentration Range	3-3.99	8-8.37
	Average Concentration (%CV)	3.49 (5.2%)	9.23 (7.3%)
Androstenedione	Concentration Range	1.5-1.74	4-4.38
	Average Concentration (%CV)	1.42 (0.4%)	4.03 (4.7%)
Cortisone	Concentration Range	15	40
	Average Concentration (%CV)	17.6 (3.4%)	49.4 (6.8%)
Cortisol	Concentration Range	60-65	180-200
	Average Concentration (%CV)	66.3 (3.9%)	226 (6.4%)
11-Deoxycortisol	Concentration Range	1-1.06	3-3.61
	Average Concentration (%CV)	1.05 (6.1%)	3.17 (2.3%)
DHEA	Concentration Range	3-3.8	8-11
	Average Concentration (%CV)	3.55 (9.5%)	9.67 (0.39%)

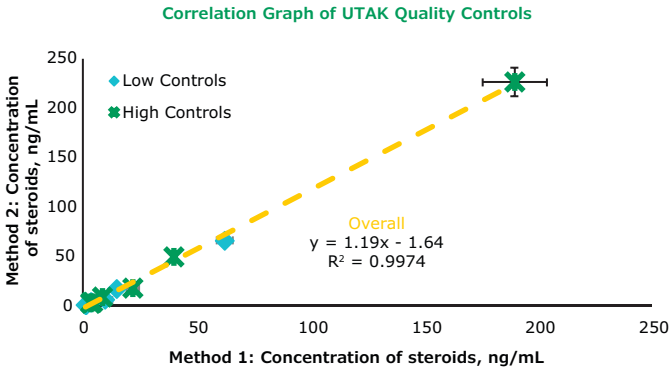


Figure 6. Correlation Graph of the Total Steroid Concentration comparing the two different approaches for the eight hormones (progesterone, OH-progesterone, testosterone, androstenedione, cortisone, cortisol, 11-deoxycortisol, DHEA). Method 1 corresponds to the UTAK provided values and Method 2 corresponds to using Supel™ Swift HLB DPX Tips. Low Controls: $y = 1.07x - 0.44$, $R^2 = 0.9953$, High Controls: $y = 1.20x - 1.70$, $R^2 = 0.9985$.

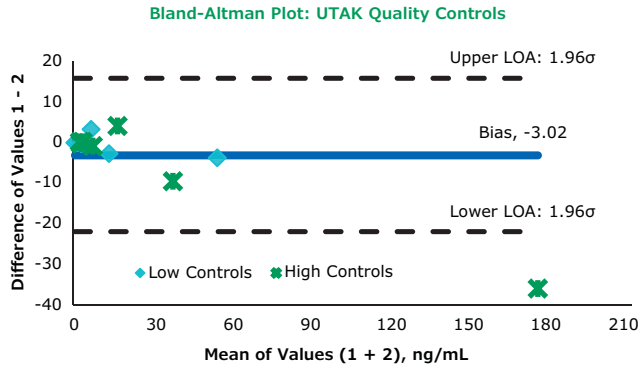


Figure 7. Total Steroid Concentration across eight different hormones simultaneously determined. Method 1 corresponds to the UTAK provided values and Method 2 corresponds to using Supel™ Swift HLB DPX Tips. The dash lines represent a 95% confidence interval.

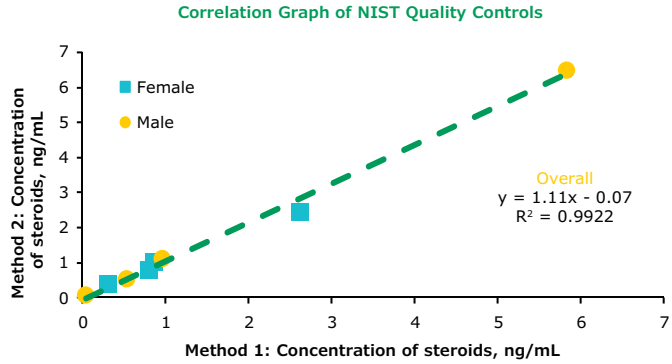


Figure 8. Correlation graph of the Total Steroids Concentration comparing the two different values for the four hormones (progesterone, OH-progesterone, testosterone, and androstenedione). Method 1 corresponds to the NIST values and Method 2 corresponds to using Supel™ Swift HLB DPX Tips. Female Controls: $y = 0.88x + 0.11$, $R^2 = 0.9934$, Male Controls: $y = 1.13x - 0.04$, $R^2 = 1.00$.

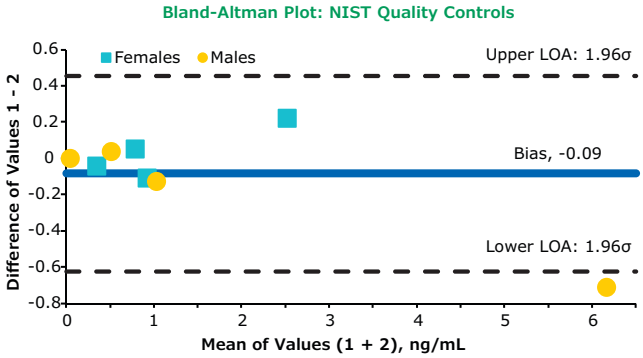


Figure 9. Total Steroid Concentration across four different hormones simultaneously determined. Method 1 corresponds to the NIST values and Method 2 corresponds to using Supel™ Swift HLB DPX Tips. The dash lines represent a 95% confidence interval.

Table 5. Real patient sample validation with NIST Quality Controls. Percent coefficients of variation (%CV) are representative of 9 total replicates.

NIST Quality Control Samples		Female (ng/mL)	Male (ng/mL)
Progesterone[a]	Concentration	2.63	0.0421
	Average Concentration (%CV)	2.41 (8.3%)	0.0416 (6.8%)
OH-Progesterone[b]	Concentration	0.8725	0.9635
	Average Concentration (%CV)	0.981 (3.4%)	1.09 (2.8%)
Testosterone[a]	Concentration	0.3231	5.808
	Average Concentration (%CV)	0.366 (5.3%)	6.52 (4.9%)
Androstenedione[b]	Concentration	0.8103	0.5359
	Average Concentration (%CV)	0.759 (0.2%)	0.498 (3%)

[a]The testosterone and progesterone levels were certified values via isotopic dilution(ID)-LC-MS/MS (NIST SRM971a).

[b] The "non-certified" values, OH-progesterone and androstenedione, were certified by Center of Desease Control (CDC) via ID-LC-MS/MS as well, however they were not certified by multiple sources outside the CDC.

range below the lowest calibrator level at 0.025 ng/mL for all analytes except cortisone and cortisol, which had a lowest calibrator of 0.25 ng/mL. The LOQ was calculated based on a signal-to-noise above 10 and the limit of detection (LOD) was based on a signal-to-noise above 3. In all cases, the reproducibility at the lowest calibrator is well within the Bioanalytical Validation Guidelines (BAVG)[6] of 15% which corresponds with the non-lowest accepted calibrator **(Table 6)**. According to the BAVG, the accepted reproducibility criteria for the lowest calibrator is 20%.

Using the SWGTOX guidelines and a total of eight replicates for each analyte, the recoveries and the matrix effects for each analyte were determined **(Table 7).** Using **Eq 1**, the recovery of the method was determined in the range of 65-86% with an average of 71% recovery. The influence of matrix was determined using **Eq 2** with an average of 33% ionization suppression.

Table 6. The signal-to-noise (S/N) and reproducibility for each analyte at the lowest calibrator tested. Percent coefficients of variation (% CV) are represented from triplicate calibration curves over three days.

Analyte	Level 1 Concentration (ng/mL)	S/N	% CV
Cortisone	0.25	229	10%
Cortisol	0.25	98.4	4%
11-Deoxycortisol	0.025	18.9	6%
Testosterone	0.025	80.8	7%
Androstenedione	0.025	34.8	3%
DHEA	0.025	23.6	8%
OH-Progesterone	0.025	31.2	14%
DHT	0.025	11.6	8%
Progesterone	0.025	19.4	5%

Eq 1.

$$Recovery = \frac{Spiked\ sample\ prior\ to\ extraction}{Spiked\ sample\ post\text{-}extraction} \times 100\%$$

Eq 2.

$$Ionization\ Effects = \frac{Spiked\ sample\ post\text{-}extraction}{Neat\ sample\ in\ solution} \times 100\%$$

Table 7. Recovery and matrix effects. Values found using SWGTOX guidelines.

Analyte	Average Recovery (n=9)	% RSD	Matrix Effects (n=8)*
Cortisone	72%	10%	29%
Cortisol	70%	10%	30%
11-Deoxycortisol	69%	9%	40%
Androstenedione	67%	9%	34%
Testosterone	69%	10%	30%
DHEA	86%	10%	12%
OH-Progesterone	67%	9%	41%
DHT	70%	12%	38%
Progesterone	65%	8%	46%

*Matrix effects are defined as: (+) positive indicates ion suppression, and a (-) negative indicates ion enhancement.

Conclusion

The use of Supel™ Swift HLB DPX Tips for the analysis of various steroids in blood serum was shown to be reproducible across two different standards (UTAK and NIST) and offers an alternative that is faster and programmable for clinical testing laboratories. This method provides the necessary sensitivity relevant to clinical values while also enabling the ability for high throughput sample processing for fast turnaround times. The accurate and sensitive method described here can be a valuable tool for quantification of steroids in serum.

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6. Food and Drug Administration. (2018) Bioanalytical Method Validation Guidance for Industry. Washington, DC: Center for Drug Evaluation and Research

Featured Products

Description	Cat. No.
Sample Preparartion & HPLC	
Supel™ Swift HLB DPX Tip Hamilton®, weight 3 mg (bed), volume 300 µL, box of 96 tips	**53001-U**
Ascentis® Express C18, 10 cm x 3 mm I.D., 2.7 µm	**53814-U**
Ascentis® Express Guard Cartridge Holder	**53500-U**
Ascentis® Express C18, Guard Cartridge, 5 x 3 mm I.D., 2.7 µm, Pk. 3	**53504-U**
Solvents & Reagents	
Formic acid 98-100%, LC-MS LiChropur™	**5.33002**
Methanol, for UHPLC-MS LiChrosolv®	**1.03726**
Acetonitrile, hypergrade for LC-MS LiChrosolv®	**1.00029**
Water, for chromatography (LC-MS Grade) LiChrosolv®	**1.15333**
Certified Reference Materials - Cerilliant® & Sample Matrix	
Androstenedione, 1.0 mg/mL in acetonitrile, 1 mL	**A-075**
Androstene-3,17-dione-2,3,4-$^{13}C_3$, 100 µg/mL in acetonitrile, 1 mL	**A-084**
Cortisone, 100 µg/mL in methanol, 1 mL	**C-130**
Cortisone-$^{13}C_3$, 100 µg/mL in methanol, 1 mL	**C-160**
Cortisol, 1.0 mg/mL in methanol, 1 mL	**C-106**
Cortisol-$^{13}C_3$, 100 µg/mL in methanol, 1 mL	**C-216**
11-Deoxycortisol, 1.0 mg/mL in methanol, 1 mL	**D-061**
11-Deoxycortisol-D_5 (2,2,4,6,6-D_5), 100 µg/mL in methanol, 1 mL	**D-078**
Dehydroepiandrosterone (DHEA), 1.0 mg/mL in methanol, 1 mL	**D-063**
Dehydroepiandrosterone-D_5 (DHEA-D_5) (2,2,3,4,4-D_5) solution 100 µg/mL in methanol, 1 mL	**D-064**
5α-Dihydrotestosterone (DHT), 1.0 mg/mL in methanol, 1 mL	**D-073**
5α-Dihydrotestosterone-D_3 (16,16,17-D_3), 100 µg/mL in methanol, 1 mL	**D-077**
17α-Hydroxyprogesterone, 1.0 mg/mL in methanol, 1 mL	**H-085**
17α-Hydroxyprogesterone-2,3,4-$^{13}C_3$, 100 µg/mL in methanol, 1 mL	**H-100**
Progesterone, 1.0 mg/mL in methanol, 1 mL	**P-069**
Progesterone-D_9, 100 µg/mL in methanol, 1 mL	**P-070**
Testosterone, 1.0 mg/mL in acetonitrile, 1 mL	**T-037**
Testosterone-2,3,4-$^{13}C_3$, 100 µg/mL in acetonitrile, 1 mL	**T-070**
DDC Mass Spect Gold® Serum	**MSG3000**
Suggested Consumables	
Zone-Free™ Sealing Films	**Z721646**
SealPlate Film	**Z369659**
Nunc™ 96 DeepWell™ plate, non-treated, U-bottom natural polypropylene wells, 2 mL	**Z717266**
Corning® 96 well Polypropylene Deep Well Plate, V-bottom clear, 2 mL, non-sterile	**CLS3960**
BRAND® 96-well deep well plate Stackable volume 2.2 mL, polypropylene, non-sterile	**BR701354**
Axygen® Deep well plate size 96 wells, size 2.0 mL	**AXYPDW20C**

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Nitrosamine Testing using LC-MS Methods from the USP General Chapter <1469>

Tim Mueller, Site Management-Analytical; Patrik Appelblad, Senior Principal Scientist; Analytix@milliporesigma.com

Introduction

Nitrosamines are unwanted side products present in many substances and are suspected to have toxic and carcinogenic properties. In pharmaceutical raw materials and finished drug products, nitrosamines may also be formed as side-products from synthesis, during storage, or from packaging, etc. A demand for nitrosamine analysis has rapidly increased worldwide. The list of concerned products manufactured from drug substances using specific synthetic routes has grown after extensive synthetic route assessments.

The United States Pharmacopeia (USP) published in December 2021 new procedures in response to the unexpected detection of nitrosamines, such as N-nitrosodimethylamine (NDMA), **Figure 1**, in certain active pharmaceutical ingredients (APIs) and corresponding final formulations.[1] The new USP chapter <1469> provides recommendations regarding the creation of controls for nitrosamine levels to ensure their elimination or reduction, and analytical method performance characteristics for procedures to monitor nitrosamine levels using both GC-MS (procedures 2 and 4) and LC-MS (procedures 1 and 3).

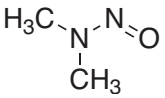

Figure 1. Chemical Structure of N-Nitrosodimethylamine (NDMA).

This paper focuses on the LC-MS based test procedures (procedure 1 and 3) for quantitative analysis of known nitrosamine impurities in pharmaceutical raw materials and finished products using liquid chromatography and mass spectrometric detection. Even though both methods were evaluated, final run conditions and data from the procedure 3 will be presented. System suitability criteria for the procedure 1 could not be met with the instrumentation available, but will be described.

Procedure 1 designates the use of a high-resolution mass spectrometer (HRMS), and can be used for the quantitation of NDMA, NDEA (nitrosodiethylamine), NDBA (nitrosodibutylamine), NDIPA (N-nitrosodiisopropylamine), NEIPA (N-nitrosoethylisopropylamine), NMBA (N-nitrosomethylaminobutyric acid), and NMPA (N-nitrosomethylphenylamine) in selected sartans (valsartan, irbesartan, and losartan potassium). Procedure 3 uses MS/MS and can be used for the quantitation of NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA in selected sartans (valsartan, losartan potassium, olmesartan medoxomil, candesartan cilexetil, and telmisartan).

Experimental

Sample and Standard Preparation

The used standard and sample solutions were prepared as follows:

Internal Standard Solution: 10 µg/mL each of NDMA-d6 and NMBA-d3, as well as 1 µg/mL each of NDEA-d10 and NDBA-d18 were prepared in water

Nitrosamine standards stock solution mixture: A mixture containing 200 ng/mL each of NDMA, NEIPA, NDIPA, NDBA and NMBA was prepared by mixing appropriate volumes of the respective USP Reference Standards and diluted with water.

NDEA standard stock solution: A solution of 132 ng/mL of NDEA was prepared by diluting USP N-Nitrosodiethylamine RS with water.

Standard solutions: Depending on the targeted nitrosamine concentration in the sample, a set of 5 consecutive linearity solutions were prepared as described in **Table 1** from the nitrosamine standards stock solution mixture and NDEA standard stock solution by mixing specified volumes of each solution as indicated.

The samples were prepared as follows:

1. 80 mg of the drug substance transferred into a 2 mL lidded centrifuge tube.
2. Addition of 1188 µL diluent (1% formic acid in water) and 12 µL of the Internal standard solution.
3. Vortexing at 2500 rpm for 20 min (except for losartan potassium, which should be vortexed NMT 5 min).
4. Centrifuging at about 10,000 rpm for 10 min.
5. Filtering into a vial using a PTFE filter with 0.45-µm pore size.

Valsartan Samples

The valsartan sample solution was prepared following the sample preparation protocol. “Valsartan” in **Table 8** means drug product (tablet/tablet powder).

Table 1. Preparation of the nitrosamine standard solutions (dilution protocol) for procedure 3

Linearity Solution #	Concentration Level	Concentration of NDMA, NMBA, NDBA, NEIPA, NDIPA/NDEA (ng/mL)	Content of NDMA, NMBA, NDBA, NEIPA, NDIPA/NDEA (ppb)	Nitrosamine Standard Stock Solution Mixture (µL)	NDEA Standard Stock Solution (µL)	Water (µL)	Internal Standard (µL)	Total Volume (µL)
1	L1	1.33/0.66	19.95/10	8	6	1174	12	1200
2	L2	2/0.88	30/13.5	12	8	1168	12	1200
3	L3	5/3.3	75/49.5	30	30	1128	12	1200
4	L4	7.5/4.95	112.5/74.25	45	45	1098	12	1200
5	L5	10/6.6	150/99	60	60	1068	12	1200
6	L6	15/9.9	225/148.5	90	90	1008	12	1200
7	L7	30/19.8	450/297	180	180	828	12	1200
8	L8	60/39.6	900/594	360	360	468	12	1200
9	L9	90/59.4	1350/891	540	540	108	12	1200

Losartan Samples

The losartan sample solution was prepared following the sample preparation protocol. "Losartan" in **Table 10** corresponds to losartan tablets or ground losartan tablets

LC-MS Conditions (Procedure 3)

Separations were conducted on an Agilent 1290 Infinity II HPLC system (Agilent, Waldbronn, Germany), equipped with a 6495C triple quadrupole MS detector having an APCI Source. Chromatographic separations were performed in gradient mode on an Ascentis® Express C18 (USP L1 Packing) 150×3.0 mm I.D., 2.7 µm column (see **Table 2**).

Data Handling

Data acquisition and processing were performed using Masshunter software version 10.0.

Table 2. Experimental conditions for procedure 3

HPLC Parameters			
Column:	Ascentis® Express C18, 150×3.0 mm I.D., 2.7 µm (**53816-U**)		
Mobile Phase:	[A] 0.1% formic acid in H_2O; [B] 0.1% formic acid in methanol		
Gradient:	**Time (min)**	**% A**	**% B**
	0.0	97	3
	1.5	97	3
	4.0	50	50
	7.0	25	75
	8.1	15	85
	9.2	5	95
	12.0	5	95
	12.1	97	3
	15.0	97	3
Flow Rate:	0.5 mL/min		
Column Temperature:	60 °C		
Autosampler Temperature:	18 °C		
Detection:	MRM, APCI (+), see **Table 3** & **4**		
Injection:	20 µL		
Samples:	Standards & valsartan/losartan solutions as indicated		

Table 3. MS instrument parameters used for quantitative purposes for procedure 3

MS Detector Parameters	
Instrument:	Agilent 6495C Triple quadrupole MS
Source:	APCI
Detection:	Instrumental parameters as shown in this table. Further detailed detector settings can be found in the procedure 3, USP chapter<1469>
Source Parameter	
Gas Temperature:	290 °C
Drying Gas Flow:	11 L/min
Nebulizer Pressure:	25 psi
Vaporizer Temperature:	350 °C
Capillary Voltage:	3000 V
Corona Current:	4 µA
Fragmentor:	166 V
Ion polarity:	positive

Table 4. MRM Transitions for nitrosamine impurities -procedure 3

Nitrosamine	Polarity	MRM Transitions (m/z) MRM-1 (Quantitation)	MRM-2
NDMA	Positive	75.0 → 43.0	75.0 → 44.1
NDMA-D_6	Positive	81.2 → 46.0	81.2 → 64.1
NDEA	Positive	103.1 → 75.1	103.1 → 47.1
NDEA-D_{10}	Positive	113.2 → 34.2	113.2 → 49.1
NMBA	Positive	147.1 → 44.1	147.1 → 117.1
NMBA-D_3	Positive	150.1 → 47.1	150.1 → 120.2
NDBA	Positive	159.2 → 41.1	159.2 → 29.1
NDBA-D_{18}	Positive	177.3 → 66.2	177.3 → 46.2
NEIPA*	Positive	117.1 → 75.1	117.1 → 47.2
NDIPA*	Positive	131.2 → 89.1	131.1 → 47.1

*NDEA-D_{10} was used as internal standard for NEIPA and NDIPA

Results and Discussion

Evaluation of Procedure 1

The USP procedure 1 method describes the use of liquid chromatography and high-resolution mass spectrometric detection (LC-HRMS), but the given experimental conditions[1] appear not generic enough to allow implementation and validation on any given HRMS platform. Our laboratory experienced sensitivity issues with an ultra-modern HRMS detector (Agilent 6546 Q-TOF), and to verify the results, different HPLC columns, solvents, and reagents were tested. Chromatographic system suitability criteria could be met, but it was not possible to meet system suitability for the overall identification and sensitivity.

Comparable signal intensities were attained at 1 µg/mL, 100 ng/mL, and 50 ng/mL for NDEA, NEIPA, NDIPA, NDBA and NMPA (ESI positive mode) with a Supelco® L43 column (Ascentis® Express F5), see **Figure 2** and two other manufacturers L43 columns (not shown). NDMA was not detected with ANY column at all concentration levels, and NMBA was not detected with any column in ESI(-) mode. The comparison of different L43 columns (pentafluorophenyl groups chemically bonded to silica particles by a propyl spacer) showed similar overall behaviour with procedure 1 using the LC-HRMS instrument, but it was not possible to meet the system suitability for identification and sensitivity. More recently, there has been further clarification posted on the USP Pharmacopeial Forum (USP-PF) regarding procedure 1. It mentions that analyses were performed and validated with an Orbitrap Fusion Lumos Tribrid brand of mass spectrometer. Since this type of instrumentation was not available in our laboratory, further validation of procedure 1 was not pursued.

Evaluation of Procedure 3

This method describes the use of liquid chromatography and tandem-mass spectrometric detection (LC-MS/MS), for the quantitation of NDMA, NDEA, NDIPA, NEIPA, NMBA, and NDBA in selected sartans (valsartan, losartan potassium, olmesartan medoxomil, candesartan cilexetil, and telmisartan). The procedure listed two system suitability criteria: 1. Correlation coefficient: NLT 0.99 and 2. y-Intercept: Not more than (NMT) 25% of the response of the medium concentration solution used in standard curve generation. Going forward, analytical data will be presented from the work establishing a validated analytical procedure 3 using a 150 x 3.0 mm Ascentis® Express C18 column (USP L1 packing) with 2.7 µm particles. An example of a nitrosamine impurity standard on this column is shown in **Figure 3**.

The method linearity was determined over nine calibration levels after optimizing the instrumental set-up. Triplicate injections were made of each linearity solution. The USP Chapter <1469> has defined two system suitability requirements for procedure 3. The correlation coefficient should not be less than (NLT) 0.99 and the y-Intercept for each calibration graph

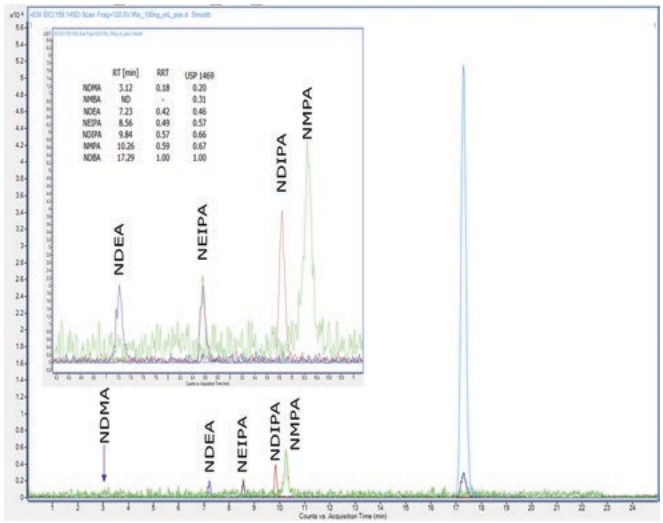

Figure 2. Chromatogram of a 100 ng/mL nitrosamine mixture analysed with a Supelco L43 column (Ascentis® Express F5), ESI (+) mode.

should not be more than (NMT) 25% of the response of the medium concentration solution used in standard curve generation. As shown in **Table 5** both of these requirements were met.

Table 5. The method system suitability requirements, procedure 3

Analyte	Correlation coefficient	y-Intercept (max. y-Intercept)
NDMA	0.9960	0.001851(< 0.031125)
NMBA	0.9932	0.000409 (< 0.022675)
NDEA	0.9986	0.000167 (< 0.018575)
NEIPA	0.9952	0.001380 (< 0.0939)
NDIPA	0.9959	0.000144 (< 0.032175)
NDBA	0.9936	0.019615 (< 0.2201)

The method precision (**Table 6**) and accuracy (**Table 7**) were determined using data from ten injections of calibration levels 1, 5 and 9 (L1, L5 and L9). Accuracies of the level 1, 5 and 9 solutions were calculated using the 9-point calibration curves described in **Table 1**.

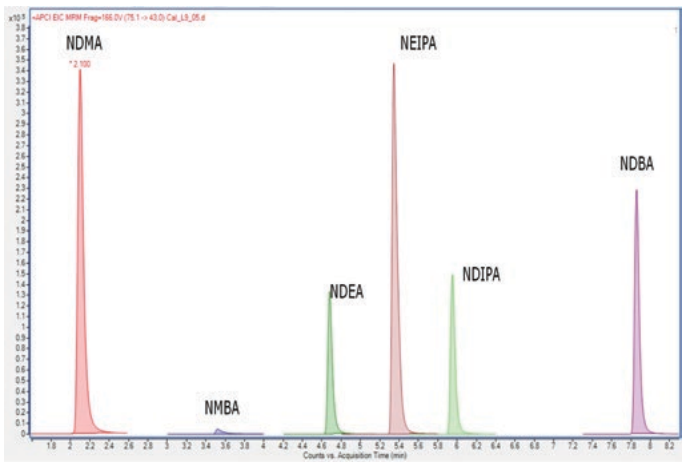

Figure 3. MRM Chromatogram (no scaling) of a 90 ng/mL nitrosamine standard solution using an Ascentis® Express C18 column for procedure 3.

Table 6. Procedure 3 method precision determined from calibration level 1, 5 and 9 (n=10 at each level)

Analyte	Concentration (ng/mL)	Average Response	Relative Standard Deviation (%)
NDMA	1.33	21687	1.7
	10.00	168086	2.3
	90.00	1513884	0.7
NMBA	1.33	398	5.6
	10.00	3093	7.6
	90.00	27009	2.7
NDEA	0.66	4443	2.5
	6.60	46576	2.4
	59.40	455404	1.1
NEIPA	1.33	18033	1.6
	10.00	148983	2.9
	90.00	1395374	0.9
NDIPA	1.33	6470	1.6
	10.00	54903	3.8
	90.00	523857	0.8
NDBA	1.33	12255	4.0
	10.00	92155	6.2
	90.00	827071	3.3

Table 7. Procedure 3 method accuracy determined for level 1, 5 and 9 using the calibration curve of each corresponding analyte (n=10 at each level)

Analyte	Concentration (ng/mL)	Average Accuracy (%)	Relative Standard Deviation (%)
NDMA	1.33	100.85	0.6
	10.00	97.90	1.1
	90.00	100.03	0.5
NMBA	1.33	109.93	7.0
	10.00	95.65	5.3
	90.00	98.66	3.7
NDEA	0.66	106.56	2.4
	6.60	96.04	1.0
	59.40	103.51	0.9
NEIPA	1.33	105.93	1.4
	10.00	97.86	2.4
	90.00	100.38	1.1
NDIPA	1.33	106.34	1.4
	10.00	96.80	3.6
	90.00	100.55	1.0
NDBA	1.33	95.64	2.2
	10.00	100.86	3.7
	90.00	98.44	2.2

The methods limit of detection (LOD) and limit of quantification (LOQ) were determined by spiking 3.3 ng/mL (2.2 ng/mL for NDEA) into a valsartan/losartan sample solution and using the signal-to-noise (S/N) ratio the for calculation. The limit of detection is defined as a signal-to-noise S/N ratio of 3, whilst the limit of quantification is defined as a S/N ratio of 10. The S/N ratio was calculated by the instrument software, and where the S/N ratio for each peak is established automatically using the peak height and a defined region of noise. Resulting limits for the measured samples are shown in **Table 8-11**.

Table 8. The method limit of detection (LOD), and limit of quantification (LOQ), in valsartan sample solution

NDMA		NBMA		NDEA		NEIPA		NDIPA		NDBA	
LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)
12	41	103	343	19	63	19	63	7	22	63	211

Table 9. The method limit of detection (LOD), and limit of quantification (LOQ), for analyte content in valsartan

NDMA		NBMA		NDEA		NEIPA		NDIPA		NDBA	
LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)
0.18	0.62	1.55	5.15	0.28	0.94	0.28	0.95	0.10	0.33	0.95	3.16

Table 10. The method limit of detection (LOD), and limit of quantification (LOQ), in losartan sample solution

NDMA		NBMA		NDEA		NEIPA		NDIPA		NDBA	
LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)	LOD (ng/L)	LOQ (ng/L)
12	41	96	320	25	85	13	43	8	27	474	1580

Table 11. The method limit of detection (LOD), and limit of quantification (LOQ), for analyte content in losartan potassium

NDMA		NBMA		NDEA		NEIPA		NDIPA		NDBA	
LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)
0.18	0.62	1.44	4.80	0.38	1.27	0.19	0.64	0.12	0.41	7.11	23.70

The method specificity was determined by monitoring the analytes retention time, and their relative retention to the retention of NDBA for a series of injections of nitrosamine standard solutions (n=40).

Table 12. Procedure 3 - method specificity

Analyte	Average Retention time (min)	Relative Retention time (RRT)	Relative Retention time (RRT) per USP 1469
NDMA	2.100	0.27	0.20
NMBA	3.528	0.45	0.31
NDEA	4.681	0.60	0.46
NEIPA	5.345	0.68	0.57
NDIPA	5.951	0.76	0.66
NDBA	7.859	1.00	1.00

The analyte recovery was determined in one valsartan batch and in one losartan potassium batch (**Table 13**). The drug substance batches were spiked with all analytes over three concentration levels as triplicates during the sample preparation procedure. The prepared sample solutions were measured and evaluated against an external calibration curve to calculate the individual analyte concentration. The ratio of internal standard signal versus analyte signal was determined in the sample solution and in the solutions of the (external) calibration row, i.e., signal NDMA-D_6 / signal NDMA. Then the signal ratios were used to calculate the concentration in the sample solution against the calibration solutions.

Table 13. Procedure 3 method - analyte recovery

Analyte	Spiked concen-tration* (ng/mL)	NDMA (%)	NMBA (%)	NDEA (%)	NDEIPA (%)	NDIPA (%)
Valsartan	3.3 / 2.2	96.70	105.00	106.83	93.52	96.84
	16.6 / 11	94.72	95.50	102.50	80.52	82.79
	33.3 / 22.2	99.27	99.32	108.09	84.06	85.54
Losartan potassium	3.3 / 2.2	100.50	97.58	82.45	87.67	94.31
	16.6 / 11	103.25	97.19	109.20	92.29	96.04
	33.3 / 22.2	105.94	95.30	115.22	97.54	103.74

* for all analytes / NDEA

During determination of the analyte recovery a systematic issue with the determination of the spike recovery for NDBA was observed (data not shown), as the found concentrations of this analyte were always too high (recoveries > 130% and thus excluded).

The analysis of possible reasons for this issue showed that coelution with one or several unknown substances occurs during the elution of NDBA.

Conclusion

This paper shows intriguing findings from work with USP Chapter <1469>, Procedure 1, and a successful implementation of Procedure 3 meeting all system suitability requirements.

References

1. United States Pharmacopeia. 2022. General Chapter, <1469> Nitrosamine Impurities. USP-NF. Rockville, MD: United States Pharmacopeia. DOI: https://doi.org/10.31003/USPNF_M15715_02_01

Product List (USP Chapter <1469>, Procedure 1)

Description	Cat. No.
HPLC	
Ascentis® Express F5 column 100×4.6 mm I.D., 2.7 µm	**53590-U**
Methanol hypergrade for LC-MS LiChrosolv®	**1.06035**
Water for chromatography (LC-MS Grade) LiChrosolv®	**1.15333**
Formic acid for LC-MS LiChropur™	**00940**
Reference Materials and Accessories	
N-Nitrosodimethylamine (NDMA) USP Reference Standard	**1466674**
N-Nitrosodiisopropylamine (NDIPA) USP Reference Standard	**1466663**
N-Nitrosoethylisopropylamine (NEIPA) USP Reference Standard	**1466685**
N-Nitrosomethylaminobutyric Acid (NMBA) USP Reference Standard	**1466696**
Nitrosodibutylamine (NDBA) USP Reference Standard	**1466641**
Nitrosodiethylamine (NDEA) USP Reference Standard	**1466652**
N-Nitrosomethylphenylamine (NMPA)	**PHR3611**
Millex® Syringe Filter LG 0.20 µm hydrophilic PTFE, 13 mm	**SLLGX13**

Product List (USP Chapter <1469>, Procedure 3)

Description	Cat. No.
HPLC	
Ascentis® Express C18 column 150×3.0 mm I.D., 2.7 µm	**53816-U**
Methanol hypergrade for LC-MS LiChrosolv®	**1.06035**
Water for chromatography (LC-MS Grade) LiChrosolv®	**1.15333**
Formic acid for LC-MS LiChropur™	**00940**
Reference Materials and Accessories	
N-Nitrosodimethylamine (NDMA) USP Reference Standard	**1466674**
N-Nitrosodiisopropylamine (NDIPA) USP Reference Standard	**1466663**
N-Nitrosoethylisopropylamine (NEIPA) USP Reference Standard	**1466685**
N-Nitrosomethylaminobutyric Acid (NMBA) USP Reference Standard	**1466696**
Nitrosodibutylamine (NDBA) USP Reference Standard	**1466641**
Nitrosodiethylamine (NDEA) USP Reference Standard	**1466652**
Nitrosodimethylamine-d6 (NDMA-d6)	**591068**
Millex® Syringe Filter LCR 0.45µm hydrophilic PTFE, 13 mm	**SLCRX13**

For a determaniation of N-nitrosamine in valsatran by GC-MS/MS see the article in Analytix Reporter issue 11 at **SigmaAldrich.com/analytix**

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PHARMA & BIOPHARMA

Analysis of Nucleotide Activated Sugars by LC-MS/MS

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Introduction

Nucleotide activated sugars are highly energetic forms of monosaccharides that act as key metabolites in glycosylation reactions, during which the glycosyl group from the activated sugars is transferred to an acceptor molecule, e.g. a protein.

In mammals, the most common nucleotide for sugar activation is uridine diphosphate (UDP), which is found in combination with Glucose (Glc), Galactose (Gal), N-Acetylglucosamine (GlcNAc), N-Acetylgalactosamine (GalNAc), Glucuronic acid (GlcA) and Xylose (Xyl). Furthermore, Guanosine diphosphate (GDP) linked to Mannose (Man) and Fucose (Fuc) as well as Cytidine monophosphate (CMP) linked to sialic acid (Neu5Ac) are used for glycan assembly. Plants and bacteria utilize an even larger variety of nucleotides and sugars.

Understanding the nucleotide sugar metabolism is of interest in different scientific areas, e.g. for the production of glycosylated therapeutic proteins in cell culture, where a sufficient glycosylation needs to be ensured in order to obtain a product of high quality and efficacy.[1] For this purpose, a sensitive, quantification method is needed that is capable of simultaneously analyzing a set of polar analytes with similar structures and physicochemical properties. In this application note, an LC method using a Supel™ Carbon LC column in combination with selective MS/MS detection is described.

Experimental

The used conditions for the analysis of 11 nucleotide activated sugars in a standard solution with a concentration of 80 µM each are described in **Table 1** & **2**.

Results

The 5 cm x 3 mm I.D. Supel™ Carbon LC column provided, under reversed phase conditions, good retention of the 11 activated nucleotide sugar compounds in the applied standard solution (**Figure 1** & **Table 3**).

Table 1. HPLC Conditions for analysis of 11 nucleotide activated sugars

Column:	Supel™ Carbon LC, 50 x 3 mm I.D., 2.7 µm (**59991-U**)
Mobile phase:	[A] Water + 0.5 % diethylamine, pH 10 (adjusted with acetic acid); [B] acetonitrile
Gradient:	**Time (min) / A% / B%** 0 / 100 / 0 2.0 / 100 / 0 12.5 / 90 / 10 21.0 / 25 / 75 26.0 / 25 / 75 26.5 / 100 / 0 33.0 / 100 / 0
Flow rate:	0.4 mL/min
Column temp.:	60 °C
Detector:	MS/MS, negative mode (Instrument: Agilent 6495 Triple Quadrupole); for used transitions see **Table 2**
Injection:	10 µL
Needle Wash Solution	Water + 5% isopropanol
Sample(s):	10 mM individual stock solutions of the activated sugar standards were prepared in water and mixed to yield a concentration of 80 µM each.

Table 2. MS/MS Transitions nucleotide activated sugars

Analyte	Q1 mass (m/z)	Q3 mass (m/z)
ADP-Glucose	588.1	346.0
ADP-Ribose	558.1	346.1
CMP-Neu5Ac	613.2	322.0
GDP-Fucose	588.1	442.1
GDP-Mannose	604.0	424.0
UDP-Galactose	565.0	323.0
UDP-GalNAc/UDP-GlcNAc	606.1	385.1
UDP-Glucose	565.0	323.1
UDP-Glucuronic acid	579.0	403.1
UDP-Xylose	535.0	323.1
TDP-Glucose	563.1	321.1

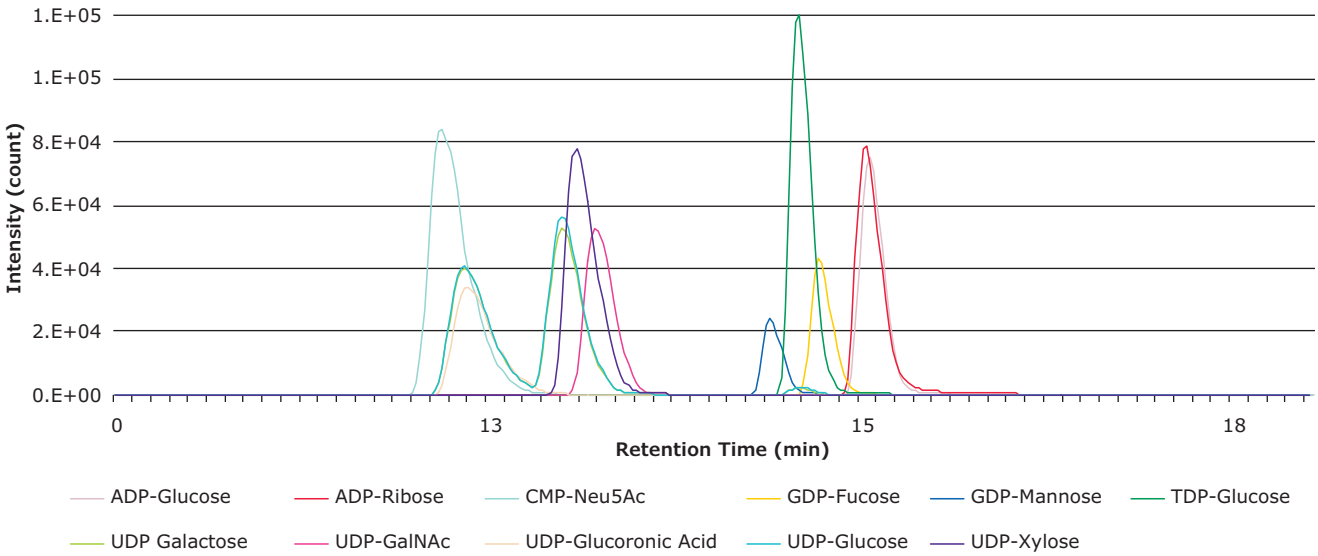

Figure 1. LC-MS/MS Analysis of nucleotide activated sugars on Supel™ Carbon LC.

Table 3. Peak identification and retention times of nucleotide activated sugars on Supel™ Carbon LC

Peak no.	Compound	Retention time (min)
1	CMP-Neu5Ac	12.75
2	UDP-Glucose	12.89
3	UDP-Glucuronic acid	12.91
4	UDP-Galactose	13.41
5	UDP-Xylose	13.49
6	UDP-GalNAc	13.62
7	GDP-Mannose	14.55
8	TDP-Glucose	14.65
9	GDP-Fucose	14.81
10	ADP-Ribose	15.05
11	ADP-Glucose	15.11

Conclusion

This application demonstrated the use and suitability of the Supel™ Carbon LC column for the analysis of 11 nucleotide activated sugars. The group of highly polar compounds with similar structures and physicochemical properties was sufficiently retained and separated on the Carbon LC column to be selectively detected with the described MS/MS method. Its ability to retain polar compounds makes the Supel™ Carbon LC column a viable choice for the analysis and quantitation of nucleotide activated sugars under reversed phase (RP) Conditions.

Reference

1. Bertozzi CR, Freeze HH, Varki A, et al. Glycans in Biotechnology and the Pharmaceutical Industry. In: Varki A, Cummings RD, Esko JD, et al., editors. Essentials of Glycobiology. 2nd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009. Chapter 51.

Featured Products

Description	Cat. No.
HPLC Column	
Supel™ Carbon LC, 5 cm x 3.0 mm I.D., 2.7 µm	**59991-U**
Solvents and Reagents	
Acetonitrile, hypergrade for LC-MS LiChrosolv®	**1.00029**
Water for UHPLC-MS LiChrosolv®	**1.03728**
Acetic acid, glacial, glacial, ≥99.99% trace metals basis	**338826**
Diethylamine, ≥99.5%	**471216**
Substances used as Standards	
Adenosine-5′-diphosphoglucose disodium salt, ≥93%	**A0627**
Adenosine-5′-diphosphoribose sodium salt, ≥93%	**A0752**
Cytidine-5′-monophospho-N-acetylneuraminic acid sodium salt, ≥85% (HPLC)	**C8271**
Guanosine 5′-diphospho-β-L-fucose sodium salt, ≥85%	**G4401**
Guanosine 5′-diphospho-D-mannose sodium salt from Saccharomyces cerevisiae, Type I, ≥97% (HPLC)	**G5131**
Uridine 5′-diphosphogalactose disodium salt, ≥97.0%	**U4500**
Uridine 5′-diphospho-N-acetylgalactosamine disodium salt, ≥97%	**U5252**
Uridine 5′-diphospho-N-acetylglucosamine sodium salt, ≥98%	**U4375**
UDP-α-D-Glucose, Disodium Salt, ≥80% (HPLC)	**670120**
Uridine 5′-diphosphoglucuronic acid ammonium salt, 98-100%	**U5625**

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Determination of 2-Chloroethanol as Marker for Fumigant Ethylene Oxide in Sesame Seeds by HS-SPME-GC-MS

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Abstract

The main goal of this work was to develop and optimize an HS-SPME-GC-MS sampling method to measure 2-chloroethanol as a marker of ethylene oxide in sesame seed samples. The developed method was then applied to quantify the marker in a variety of sesame seeds of different origins. It was found that 2-chloroethanol was present in two of the samples with concentrations in the range of 84-151 ng/g, values above the permitted concentration levels established by the Europe Union regulation (EC) No 396/2005 in oil seeds.

Introduction

Ethylene oxide (EO) gas is used as a fumigant for the control of insects and microorganisms in food commodities.[1] It reacts with natural chlorides present in the food matrix to form 2-chloroethanol, a known carcinogen, that may persist in the food product for long periods of time, even throughout food processing.[2,3] Due to the demonstrated harmful health effects of these compounds, the employment of EO as a fumigant for food commodities is being progressively regulated or banned in several countries.[4] Currently, Europe controls the use of this fumigant in food by regulation (EC) 396/2005, which defines its concentration as a sum of EO and 2-chloroethanol, with a permissible concentration of 0.05 mg/kg in nuts, oil fruits, and oil seeds.[5] The aim of this study was to develop a high-throughput HS-SPME-GC-MS method to detect and quantify 2-chloroethanol as a marker for EO fumigation in sesame seeds.

Four commercially available SPME fibers were used to determine the selectivity of the SPME fiber coating for the headspace extraction of sesame seeds followed by GC-MS analysis. Carboxen-PDMS coating on a nitinol core was more effective in the extraction and desorption of 2-chloroethanol, and the optimized HS-SPME-GC-MS method demonstrated overall good linearity, reproducibility, and sensitivity. This shows that the method exhibits great potential as a quality control methodology for the fast screening of 2-chloroethanol. Additionally, this method was successfully applied for analyzing 2-chloroethanol in 3 sesame samples of different origins.

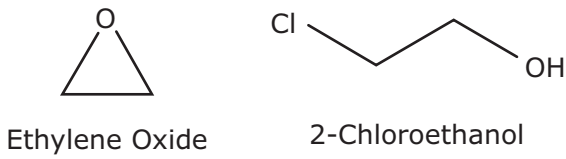

Figure 1. Structure of ethylene oxide and its marker 2-chloroethanol.

Experimental Conditions

The HS-SPME method optimization was achieved using sesame seed samples obtained from a local market with an undetectable GC-MS level of 2-chloroethanol. During the method development, fiber selectivity, extraction time (1, 2, 5, 10, 15, 20 min), and temperature (30, 40, 50, and 60 °C) parameters were studied. For this purpose, 1 g of 2-chloroethanol-free sesame seeds were spiked with 1 µL of a 20 ng/g solution of 2-chloroethanol prepared in methanol. The HS-SPME-GC-MS method is summarized in **Table 1** and **Table 2**.

Table 1. HS-SPME Sampling Conditions

Autosampler:	Gerstel MPS II with cooled tray holder
Sample/ matrix:	1 g of 2-chloroethanol-free sesame seeds in 10 mL headspace vial
SPME Fibers:	PDMS on Fused silica core, 100 µm, 23 ga, 1 cm
	DVB/PDMS on Nitinol core, 65 µm, 23 ga, 1 cm
	CAR/PDMS on Nitinol Core, 75 µm, 23 ga, 1 cm
	DVB/CAR/PDMS on StableFlex core, 50/30 µm, 23 ga, 1 cm
Incubation:	2 min at 40 °C with agitation
Extraction:	Headspace; 10 min, 40 °C, 250 rpm
Desorption:	3 min at 300 °C for CAR/PDMS Fiber
	3 min at 270 °C for DVB/CAR-PDMS, PDMS, and DVB/PDMS Fibers
Fiber post conditioning:	2 min at 300 °C for CAR/PDMS Fiber
	2 min at 270 °C for DVB/CAR-PDMS Fiber
	2 min at 250 °C for PDMS and DVB/PDMS Fibers

Table 2. GC/MS Conditions

GC/MS:	Agilent 7890B/5977A
Column:	SUPELCOWAX® 10, 60 m x 0.25 mm, df 0.25 µm
Oven:	40 °C (0.5 min) to 200 ºC at 10 °C/min (0.5 min)
Inj. temp.:	300 °C CAR-PDMS Fiber
	270 °C for DVB/CAR-PDMS, PDMS, and DVB/PDMS Fibers
Carrier gas:	Helium at 1 mL/min constant flow rate
Detector:	MSD Quadrupole, full scan, m/z=40-200
	SIM Mode, 2-Cloroethanol (m/z 80)
MS Parameters:	Source at 230 °C; Quad at 150°C; Interface temperature at 220 °C; Electron energy at 70 eV
Injection:	SPME, splitless for 3 min then vent at 20 mL/min
Liner:	0.75 mm ID SPME Liner

Results and Discussion

HS-SPME Method Optimization Procedure

Coating selectivity

Initial tests were conducted using 4 different fibers including PDMS, DVB/PDMS, CAR/PDMS, and DVB/CAR/PDMS to evaluate the performance and effectiveness of each fiber coating material for the headspace extraction of 2-chloroethanol in sesame seed samples. The extraction conditions were as follows: equilibrium time of 2 min, extraction time of 10 min, and extraction temperature of 40 °C. Further sample preparation conditions are mentioned in the experimental section. Additionally, a 3-min desorption time was adopted since preliminary tests proved that it allowed a complete desorption of analytes from the fiber. The results of comparing different fibers are shown in **Figure 2**, which depicts 2-chloroethanol chromatogram peak response. CAR/PDMS fiber renders the highest peak response, which indicates that this fiber is the most efficient chemistry for the extraction of 2-chloroethanol in terms of sensitivity and selectivity. The presence of micropores in the coating structure of the CAR/PDMS fiber allows the retention and release of small analytes (i.e., 2-chloroethanol) more efficiently. Thus, CAR/PDMS was used for further HS-SPME method optimization.

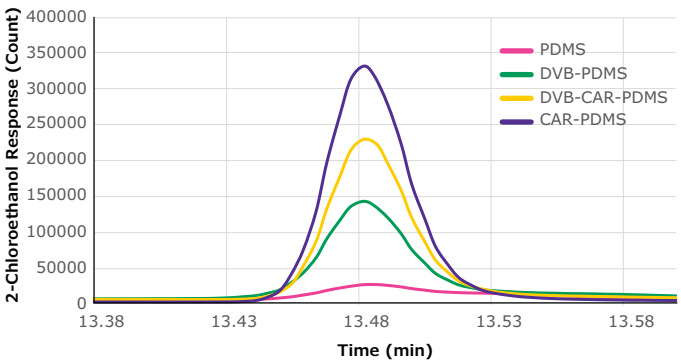

Figure 2. Evaluation of four SPME coating chemistries on the extraction of 2-chloroethanol in sesame seeds.

Effect of extraction temperature and extraction time

The SPME extraction time profile was obtained by repeated measures (n=3) of matrix-matched standard samples at increasing extraction time (up to 20 min), following the sample preparation described previously. The results plotted in **Figure 3** pointed out a change in the sorption dynamic after 10 minutes of fiber extraction, as evidenced by the decrease in the curve slope. The extraction temperature of 40 °C allowed the highest analytical response. Additionally, the minimum equilibrium time was found to be 2 min (data not shown).

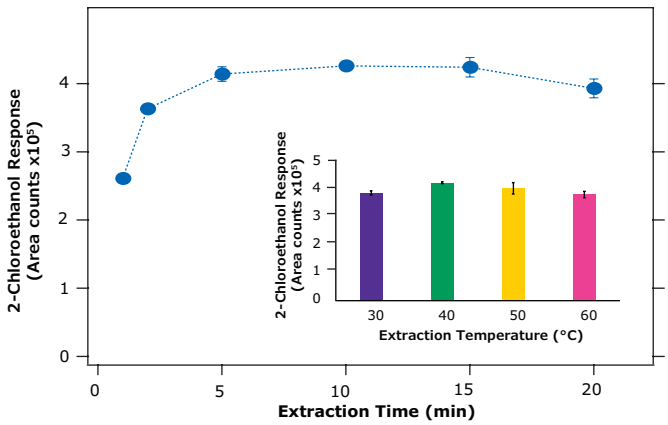

Figure 3. Time and temperature extraction profiles for 2-chloroethanol obtained via HS-SPME-GC-MS with CAR-PDMS. Mean values and standard deviation of 2-chloroethanol peak area (n=3). Sample: 1 g sesame seeds spiked with 20 ng/g of 2-chloroethanol.

Method Validation

The method was validated in terms of linearity, limits of detection (LOD), limits of quantification (LOQ), accuracy, and repeatability. The results of the study are summarized in **Table 3**. Linearity was assessed through the construction of a multipoint calibration curve, at seven different concentration levels from 5 ng/g-150 ng/g. The calibration curve range was selected based on the permitted concentration level established by the European regulation (50 ng/g) for ethylene oxide and 2-chloroethanol in nuts, oil fruits, and oil seeds.[5] The calibration curve was prepared by adding proper volumes of 2-chloroethanol standard into SPME vials containing 1.0 g of 2-chloroethanol-free sesame seeds. **Figure 4** shows the calibration curve obtained in which good linearity across the calibration range was obtained with a correlation coefficient value (R^2) of 0.9997.

Excellent accuracy of 99% was obtained over the analytical range with method repeatability of 1% RSD. This was achieved by analyzing 7 replicates of SPME extractions of sesame seed samples spiked at 5 ng/g. For the calculation of LODs and LOQs, seven spiked samples (5 ng/g) were tested. LOD and LOQ were determined by calculating the uncertainty of the calibration curve in the range of the LOQ.[6] LOQ was measured as 10 times the standard deviation used for LOD. LOD of 2.0 ng/g and LOQ of 6.8 ng/g for 2-chloroethanol were achieved using the CAR/PDMS chemistry.

Table 3. HS-SPME method accuracy and repeatability in spiked white sesame seeds

Analyte	Spike level (ng/g)	Avg. amount Measured (ng/g)	Repeatability % RSD (n=7)	Accuracy (%)	LOD (ng/g)	LOQ (ng/g)
2-Chloroethanol	5	4.98 ± 0.06	1%	99%	2.0	6.8

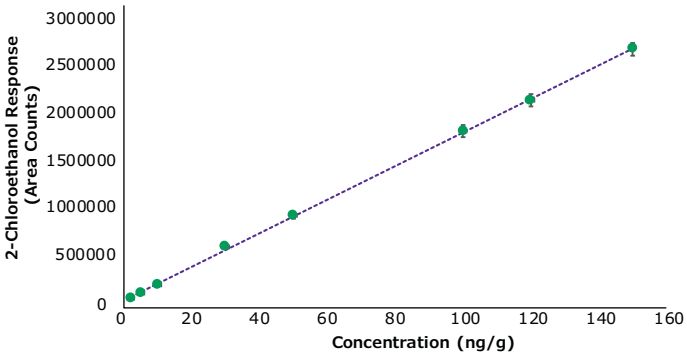

Figure 4. Calibration curve of absolute responses of 2-chloroethanol. Triplicates were carried out for each point of the curve.

Quantification of 2-chloroethanol in real sesame seed samples

White raw, roasted white, and black sesame seeds from different origins were acquired from local markets and analyzed as per the given HS-SPME method. 2-Chloroethanol concentrations in the tested samples are reported in **Table 4**. 2-Chloroethanol was not detected in white raw sesame seed samples. However, it was detected in roasted black and white sesame seeds samples with concentrations of 84 and 151 ng/g, respectively. These concentrations are above the permissible values established by European regulation (50 ng/g) in sesame seeds. A possible source of contamination during the processing of sesame seed samples with ethylene oxide or 2-chloroethanol can occur when combustible gases containing ethylene oxide contaminate the sample during the drying or roasting processes.[6] Thus, the roasting process could be the source of contamination of the analyzed samples in which high content of 2-chloroethanol was observed.

Table 4. Concentration of 2-chloroethanol measured in un-spiked sesame seed samples using HS-SPME

Sample	Measured Concentration (ng/g Sample)	%RSD (n=5)
White raw sesame seeds	Not Detected	---
Roasted black sesame seeds	84 ± 3	3%
Roasted white sesame seeds*	151 ± 2	1%

* 0.5 g of sample was analyzed

Conclusions

This study developed and optimized an HS-SPME-GC-MS method using a Carboxen®-PDMS fiber on a nitinol core for the analysis of 2-chloroethanol as a marker of ethylene oxide in sesame seeds. The SPME method yielded good sensitivity, accuracy, and reproducibility when applied to the analysis of sesame seed samples. The Carboxen®-PDMS chemistry allows the efficient retention and release of small analytes such 2-chloroethanol due to the presence of micropores in the fiber structure.

This study demonstrated that HS-SPME-GC-MS employing a CAR/PDMS fiber can be used as a quality control methodology for fast screening of 2-chloroethanol as a marker of ethylene oxide in food commodities.

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Description	Cat. No.
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Divinylbenzene-polydimethylsiloxane (DVB-PDMS), 65 µm, Nitinol, needle size 23 ga, for use with manual holder/autosampler	**57923-U**
Carboxen-Polydimethylsiloxane (CAR-PDMS), 75 µm, Nitinol, needle size 23 ga, for use with manual holder/autosampler	**57907-U**
Divinylbenzene-Carboxen-Polydimethylsiloxane (DVB-CAR-PDMS), 50/30 µm, Stableflex, needle size 23 ga, for use with manual holder/autosampler	**57298-U**
SPME fiber holder for CTC, Combipal, Gerstel MPS2	**57347-U**
SPME fiber holder for manual sampling	**57330-U**
0.75 mm Inlet Liner, Direct (SPME) Type, Straight Design	**2637505**
Molded Thermogreen® LB-2 Septa, with injection hole	**28338-U**
Headspace vial, screw top, 10 mL rounded bottom	**SU860099**
Magnetic Screw Cap for Headspace Vials, 18 mm thread	**SU860101**
Analytical Standards	
2-chloroehanol solution, certified reference material, 2000 µg/mL in methanol, 1 mL	**CRM48085**

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FOOD & BEVERAGE

Determination of Ascorbic Acid and Dehydroascorbic Acid in Different Food Products and Supplements - A Simple HPTLC Based Approach

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Introduction

Vitamin C or ascorbic acid is a vitamin found naturally in many fruits and some vegetables or is added to certain processed foods or dietary supplements. It is water-soluble and has antioxidative capacities by degrading to dehydroascorbic acid. The human organism cannot produce ascorbic acid and it must be ingested through food or supplements. It has essential functions in human body and maintains numerous vital processes. When vitamin C is deficient (scurvy), symptoms can occur such as fatigue, tiredness, and inflammations. The recommended daily dose of ascorbic acid is about 100 mg per day and can easily be reached with a healthy, balanced diet. Typically, ascorbic acid is quantified by iodometric titration according USP method.[1] An additional substance identification is required and performed by e.g. infrared analysis.[1]

In the following application, we show an easy and fast screening approach for the simultaneous analysis of ascorbic acid and dehydroascorbic acid by High Performance Thin Layer Chromatography (HPTLC). Thin layer chromatography (TLC) and HPTLC are convenient, fast, and efficient separation techniques that enable the development of analytical methods without the need for extensive sample preparation or high investments.[2] When combined with MS, a subsequent substance identification is possible. Low cost and short analysis time per sample are given by the parallel analysis of many samples on one plate. Furthermore, the high matrix tolerance of TLC offers additional opportunities to existing routine methods. The high viscosity and high sugar content of many ascorbic acid products (e.g. fruit juice) make them very complex and matrix-rich samples to analyze.

Experimental

Five different commercially available ascorbic acid containing products, like juice concentrate, fruit gums, vitamin C effervescent tablet, multi vitamin effervescent tablet, and a tablet with cranberry extract were analyzed using conditions in **Table 1**.

Table 1. Experimental conditions for determination of ascorbic acid and dehydroascorbic acid

HPTLC Conditions	
Plate:	HPTLC Silica gel 60 F_{254}s, 20x10cm (**1.15696**)
Application volume:	0.8 – 4.0 µL, bandwise application, 6 mm bands
Detection:	366 nm
Chamber:	20x10 Chamber without filter paper
Mobile Phase:	Acetone/toluene/formic acid 60:30:10 (v/v/v)
Migration distance:	6 cm
hR_f:	Ascorbic acid = 45; Dehydroascorbic acid = 58
Drying:	50 °C
Samples	
Standard preparation:	Ascorbic acid: about 10 mg of ascorbic acid was weighed in a 10.00 mL volumetric flask and filled up with a solution of ethanol/water 1:1 (v/v) (solvent mixture)
	Dehydroascorbic acid: about 10 mg of dehydroascorbic acid was weighed in a 10.00 mL volumetric flask and filled up with a solution of ethanol/water 1:1 (v/v) (solvent mixture)
Sample:	Juice concentrate: 1.0 mL of the juice was transferred to a volumetric flask and filled up with the solvent mixture
	Fruit gums: the fruit gums were stored in a freezer for several hours. Afterwards they were grounded with a mixer (blender) for a few seconds. About 2.0 g of the chunks were weighed into a flask, filled up with 10.0 mL solvent mixture and centrifuged. The supernatant was used for the analysis
	Vitamin C effervescent tablet: the tablet was pulverized with a mortar. About 250 mg was weighed into a flask and filled up with 10.0 mL solvent mixture
	Multi vitamin effervescent tablet: the tablet was pulverized with a mortar. About 250 mg was weighed in a flask and filled up with 10.0 mL solvent mixture
	Tablet with cranberry extract: the tablet was pulverized with a mortar. About 100 mg was weighed in a flask and filled up with 10.0 mL solvent mixture
MS Measurement:	The samples extracted with the Plate Express and measured with the single-quadrupole mass spectrometer expression CMS from Advion
	MS Mode: ESI Mode-, Spectrum 1, ascorbic acid, Measured molecule mass 175.0 (M-H). Spectrum 2, dehydroascorbic acid, measured molecule mass 173.1 (M-H)
Extraction solvent:	Acetonitrile/water 95:5 (v/v) + 0.1% formic acid

Due to its oxidative capacity, ascorbic acid gets rapidly decomposed and dehydroascorbic acid is formed. A reliable quantification of ascorbic acid can be challenging and requires a gentle but rapid quantification of the samples. In practice, ascorbic acid might be quantified together with a low amount of its dehydration product dehydroascorbic acid. (**Figure 1**). To simulate this effect, in this experiment, standards of ascorbic acid were over-spotted with dihydroxyascorbic acid.

Calibration curves of ascorbic acid and dehydroascorbic acid were established based on 3 different applied standard volumes (**Table 2**). After separation, a fast and simple substance confirmation by MS was performed.[3]

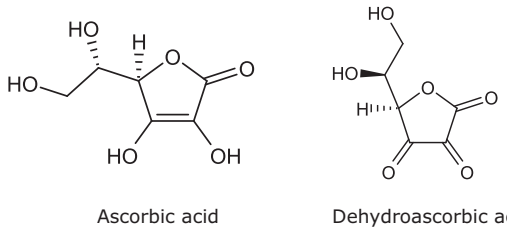

Figure 1. Chemical structure of ascorbic acid and dehydroascorbic acid.

Table 2. Calibration solutions applied

Spots	Application volume (µL)	Description
1, 9, 17	1.5	Ascorbic acid standard solution, applied first to standard tracks
2, 10, 18	3.0	
3, 11, 19	5.0	
1, 9, 17	0.3	Dehydroxyascorbic acid standard solution, applied over ascorbic acid spots.
2, 10, 18	1.0	
3, 11, 19	2.0	

The samples and standards were applied bandwise (6.0 mm). At first, the concentration series of the ascorbic acid standards were applied and afterwards over-spotted with the dehydroascorbic acid standards. Due to expected lower concentration of dehydroascorbic acid, the sample volume was lower than for ascorbic acid.

The plate was developed with the mobile phase and afterwards dried at 50°C until completely dry. To quantify, the plate was heated at 110 °C for 10 minutes. Examination of the plate was done at 366 nm.

Results and Discussion

At 366 nm illumination, ascorbic acid appears at hRf 45 and dehydroascorbic acid at hR_F 58 (**Figure 2**). MS measurement of the spots (before heating) were carried out to confirm substance identification.[3]

The calibration solution profiles (**Table 2**) at 366 nm were used for establishing the calibration curves and quantification (**Table 3**, **Figure 3** & **4**) related to amount applied to the plates.

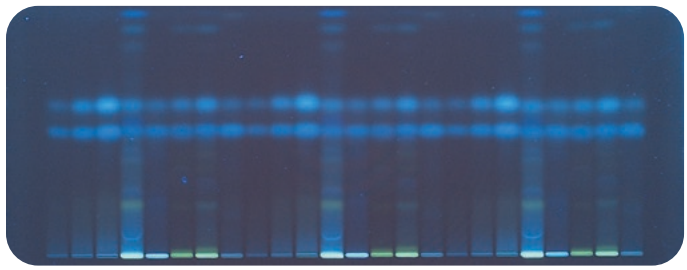

Figure 2. Visualization of the plate under UV 366 nm. Ascorbic acid appears at hR_F 45 and dehydroascorbic acid at hR_F 58.

Table 3. Results of 3 calibration solutions for ascorbic acid and dehydroascorbic acid

Compound	Amount (µg)	Mean Area AU
Ascorbic acid	1.54	0.00647
	3.08	0.01144
	5.15	0.01867
Dehydroascorbic acid	0.30	0.00461
	1.00	0.01286
	1.99	0.02822

The calibration data was used to quantify the vitamin C content of the five applied samples. In all 5 samples ascorbic acid and also dehydroascorbic acid could be determined. The results are displayed in **Table 4** a & b.

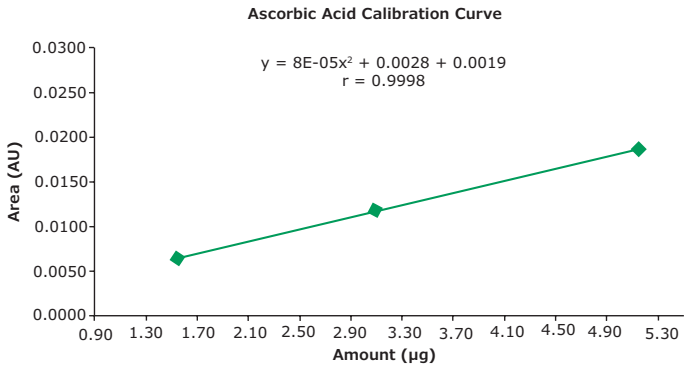

Figure 3. Calibration plot with corresponding calibration function of ascorbic acid.

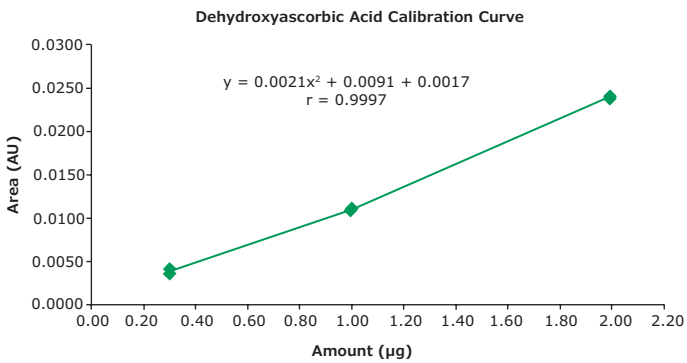

Figure 4. Calibration plot with corresponding calibration function of dehydroxyascorbic acid.

Table 4a. Quantitative Results of measured samples

Samples	Application position	Conc. Sample (mg/mL)	Application-volume (µL)	Substance	Mean Area (AU)	Mean Amount (µg)	RSD %	Vitamin Content in the Sample Solution
Juice concentrate	4, 12, 20	100.0	4.0	Ascorbic acid	0.01046	2,790	2.01	0.70 mg/mL
				Dehydroascorbic acid	0.01113	0.862	3.38	0.22 mg/mL
Fruit gums	5, 13, 21	200.0	3.0	Ascorbic acid	0.01076	2,881	2.08	0.96 mg/mL
				Dehydroascorbic acid	0.00749	0.562	3.74	0.19 mg/mL
Vitamin C effervescent tablet	6, 14, 22	25.0	2.5	Ascorbic acid	0.01026	2,731	3.63	1.09 mg/mL
				Dehydroascorbic acid	0.01014	0.783	4.78	0.31 mg/mL
Multi Vitamin effervescent tablet	7, 15, 23	24.9	3.5	Ascorbic acid	0.01254	3,412	2.25	0.97 mg/mL
				Dehydroascorbic acid	0.01604	1,225	1.87	0.35 mg/mL
Tablet with Cranberry extract	8, 16, 24	9.9	5.0	Ascorbic acid	0.01368	3,744	0.90	0.75 mg/mL
				Dehydroascorbic acid	0.00676	0.498	0.95	0.10 mg/mL

Table 4b. Calculated recovery rates (expected values are data listed on the packages of the tested products)

Samples	Conc. Sample (mg/mL)	Substance	Content Vitamin in the Sample Solution (mmol/L)	Combined content (mmol/L)	Expected content (mmol/L)	Recovery Rate %
Juice concentrate	100.0	Ascorbic acid	3,960	5,197	3,407	153
		Dehydroascorbic acid	1,238			
Fruit gums	200.0	Ascorbic acid	5,452	6,527	17,016	38
		Dehydroascorbic acid	1,075			
Vitamin C effervescent tablet	25.0	Ascorbic acid	6,202	8,001	5,683	141
		Dehydroascorbic acid	1,799			
Multi Vitamin effervescent tablet	24.9	Ascorbic acid	5,535	7,545	4,480	168
		Dehydroascorbic acid	2,010			
Tablet with Cranberry extract	9.9	Ascorbic acid	4,252	4,823	4,480	108
		Dehydroascorbic acid	0.571			

Conclusion

The developed application procedure provides a simple screening of ascorbic acid and dehydroascorbic acid for different kind of samples and matrices by HPTLC. This easy and straightforward approach represents an alternative method for a reliable screening of vitamin C in food & beverage samples.

It provides a fast, cheap, and simple semi-quantification of ascorbic acid and dehydroxyascorbic acid, and also demonstrates the main advantages of the TLC approach, such as quick sample preparation, high matrix tolerance, and high-throughput.

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3. Unpublished results. Contact the author to get more details.

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ENVIRONMENTAL

Efficient PAH Analysis (EU 15+1) using an Ascentis® Express PAH HPLC Column

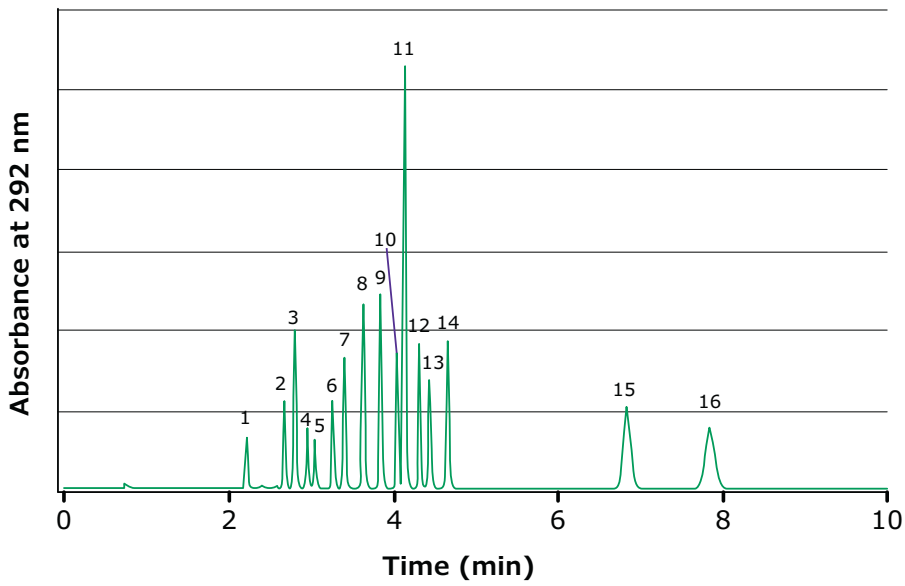
Petra Lewits, Global Product Manager HPLC Columns; Analytix@milliporesigma.com

In 2005, the European Commission (EU) recommended the monitoring of 15 priority PAHs along with an additional PAH highlighted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) which are considered toxic in food products.[1] The list contains eight of the EPA's priority PAHs along with eight other compounds that are known carcinogens.

In this application, we demonstrate a PAH analysis method for the separation of EU 15 + 1 PAH compounds on a 50 x 4.6 mm, 2.7 µm Ascentis® Express PAH column. The analysis was completed under the given conditions (**Table 1**) in less than ten minutes with an excellent resolution between the critical pair, peaks 4 and 5 (chrysene and 5-methylchrysene), that only differ by the presence of a methyl group (**Figure 1**).

Table 1. Experimental Conditions

Column:	Ascentis® Express 90 Å PAH, 2.7 µm, 5 cm x 4.6 mm I.D. (**53539-U**)
Mobile phase:	[A] Water; [B] acetonitrile
Gradient:	**Time** / **%B**: 0.0 / 50; 4.0 / 100; 15.0 / 100; 15.1 / 50
Flow rate:	1.8 mL/min
Column temp.:	30 °C
Detection:	UV @ 292 nm; data rate: 100 Hz
Injection volume:	10 µL



Peak Identities

1. Benzo[c]fluorene
2. Cyclopenta[cd]pyrene
3. Benzo[a]anthracene
4. Chrysene
5. 5-Methylchrysene
6. Benzo[j]fluoranthene
7. Benzo[b]fluoranthene
8. Benzo[k]fluoranthene
9. Benzo[a]pyrene
10. Dibenzo[a,l]pyrene
11. Dibenz[a,h]anthracene
12. Benzo[ghi]perylene
13. Indeno[1,2,3-cd]pyrene
14. Dibenzo[a,e]pyrene
15. Dibenzo[a,i]pyrene
16. Dibenzo[a,h]pyrene

Figure 1. Separation of EU 15 + 1 polycyclic aromatic hydrocarbons using an Ascentis® Express PAH HPLC Column.

Conclusion

The new Ascentis® Express PAH HPLC column allows a highly efficient separation of 16 PAHs in 10 minutes. The Fused-Core® technology of Ascentis® Express PAH HPLC columns enables fast, efficient, and rugged separations, which are paramount to environmental and food analysis of polycyclic aromatic hydrocarbons (PAHs).

References

1. European Union, Commission Recommendation 2005/108/EC, Off. J.Eur.Comm. L34 (2005) 43. http://data.europa.eu/eli/reco/2005/108/oj

Featured & Related Products

Description	Cat. No.
Ascentis® Express PAH, 2.7 µm, 5 cm x 4.6 mm, 90 Å	**53539-U**
Acetonitrile LiChrosolv®	**1.00030**
Water LiChrosolv®	**1.15333**
Reference Materials	
Benzo[a]anthracene certified reference material, TraceCERT®	**75451**
Benz[a]anthracene analytical standard	**48563**
Benz[a]anthracene solution certified reference material, 1000 µg/mL in methanol	**40070-U**
Chrysene analytical standard	**35754**
Chrysene certified reference material, TraceCERT®	**94035**

Description	Cat. No.
Benzo[j]fluoranthene	**BCR139**
Benzo[b]fluoranthene analytical standard	**48490**
Benzo[b]fluoranthene, certified reference material, TraceCERT®	**30958**
Benzo[k]fluoranthene, certified reference material, TraceCERT®	**03323**
Benzo[k]fluoranthene certified reference material, 1000 µg/mL in acetone	**40073**
Benzo[a]pyrene, certified reference material, TraceCERT®	**51968**
Benzo[a]pyrene, analytical standard	**48564**
Dibenzo[a,l]pyrene, certified reference material	**BCR096**
Dibenz[a,h]anthracene, analytical standard	**48574**
Dibenz[a,h]anthracene, certified reference material, TraceCERT®	**91861**
Benzo[ghi]perylene, certified reference material, TraceCERT®	**55488**
Benzo[ghi]perylene analytical standard	**48491**
Indeno[1,2,3-cd]pyrene, certified reference material, TraceCERT®	**94377**
Indeno[1,2,3-cd]pyrene, analytical standard	**48499**
Dibenzo[a,e]pyrene, certified reference material	**BCR133**
Dibenzo[a,h]pyrene, certified reference material	**BCR159**

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Universal HPTLC Mix (UHM) for simplified System Suitability Tests

A novel concept for HPTLC suitability test

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Abstract

We recently launched the HPTLC calibration mix (cat. no. **91816**) for use as a universal system suitability test (SST) solution, developed in collaboration with CAMAG, a leading manufacturer of HPTLC instrumentation.

Introduction

In HPTLC, the SST often qualifies only a limited region of the chromatogram (e.g., specific R_F values or narrow R_F ranges). If no deviation from the acceptance criteria is observed, the entire chromatographic system is typically considered compliant. However, in practice, the chromatographic quality of the other regions remains unknown. Additionally, HPTLC methods using developing solvents of different polarities resulting in different selectivities may require different sets of substances for different SST. Cost and stability are the other criteria to consider when selecting reference substances for a system suitability test. To offer convenience and reliability, a Universal HPTLC Mix (UHM) for use in SST was developed, that is applicable for use with a wide variety of solvents.[1]

The idea for a universal system suitability test (SST) for HPTLC originated from the company Anchrom (India). Dr. Manjusha Phanse started the evaluation of this concept. Thinking about the practical aspects of qualifying an HPTLC analysis and the needs of clients for routine analysis, the laboratory teams of CAMAG and Anchrom worked together to create a new SST concept for HPTLC. This project was later supported by Sigma-Aldrich Chemie GmbH (subsidiary of Merck KGaA, Darmstadt, Germany). The outcome was a joint publication in the Journal of Chromatography A[1] and the launch of the HPTLC calibration mix, a ready-to-use analytical standard solution, suitable for the CAMAG SST concept.

This mix is applicable for SST in a wide range of chromatographic systems, with different polarities and selectivities. The replacement of conventional substances for SST by the UHM will help laboratories to save time and money required for laborious in-house investigations of specific reference substances for each method to be qualified. Different fields of application can benefit from the UHM concept, such as herbal drugs, forensics, pharmaceuticals, cosmetics, etc.

Definition of Mix Composition

In the first step of the investigation, suitable substances for the UHM were selected. An initial list of 56 candidates was determined using the following criteria:

1. low hazard (not harmful and non-toxic substances)
2. detectability at UV 254 and 366 nm prior to derivatization
3. high stability in solution

The chromatographic behavior of those 56 compounds was evaluated with 20 developing solvents (8 are shown in **Table 1**), covering a wide range of polarities and selectivities.

Table 1. Examplary listing of developing solvents with their polarities and selectivity groups according to Snyder

N°	Developing solvent	Polarity index	Selectivity groups
A	Ethyl acetate, formic acid, acetic acid, water 100:11:11:26 (V/V/V/V)	5.63	VI, IV, VIII
B	Ethyl acetate, formic acid, water 15:1:1 (V/V/V)	4.76	VI, VIII
C	Dichloromethane, methanol, water 14:6:1 (V/V/V)	4.01	V, I, VIII
D	Toluene, acetic acid 4:1 (V/V)	3.12	VII, IV
E	Toluene, ethyl acetate 3:1 (V/V)	2.90	VII, VI
F	Toluene, ethyl acetate 9:1 (V/V)	2.60	VII, VI
G	Toluene, methanol, diethylamine 8:1:1 (V/V/V)	2.58	VII, I
H	Cyclohexane, ethyl acetate 5:3 (V/V)	1.73	VI

Chromatographic Conditions:

Plate: HPTLC plates silica gel 60 F_{254}, 20×10 cm (**1.05642**).

Standard solutions: In the development phase, 2.0 µL of individual compound solutions were applied as bands with the Automatic TLC Sampler (ATS 4), band length 8.0 mm, distance from left edge 20.0 mm, distance from lower edge 8.0 mm. For the HPTLC calibration

mix, an application volume of 2.0 µL is recommended for best results.

Chromatography: Plates were developed to 70 mm (from the lower edge) in the ADC 2 with chamber saturation (20 min, with saturation pad) and after activation at 33% relative humidity for 10 min using a saturated aqueous solution of magnesium chloride. 20 different developing solvents (eight of them are listed in **Table 1**) were investigated, followed by drying for 5 min.

Documentation: Images of the plates were captured with the TLC Visualizer 2 at UV 254 nm and 366 nm.

Densitometry: Absorbance measurement at 254 nm and fluorescence measurement at 366 nm with TLC Scanner 4 and visionCATS, slit dimension 5.00 mm x 0.20 mm, scanning speed 20 mm/s. For the fluorescence measurement, a mercury lamp and a cut-off filter 400 nm were used.

The objective was to find the ideal set of substances that provides an even distribution of zones throughout the entire chromatogram for a maximum number of different developing solvents. Additionally, each developing solvent should achieve a baseline separation for at least 3–4 substances. The finally chosen substances and their chromatograms with eight different developing solvents are shown in **Figure 1**.

To evaluate, whether the proposed UHM responds to variations in the chromatographic conditions, three experiments were performed:

In the first, plates were conditioned to different relative humidities (from 0% to 90%) prior to development. As shown in **Figure 2**, the UHM is sensitive to variations in relative humidity, particularly to the higher ones. The differences were more pronounced for developing solvents containing no water.

In the second experiment, the individual proportion of the solvents in developing solvents B and F (**Table 1**) was changed (±10%), and the effect on the chromatography was evaluated. A difference of up to 0.06 R_F units could be observed from the mean R_F values of the control track.

In the third experiment, different levels of chamber saturation were tested: unsaturated, partially saturated (20 min, no saturation pad), and saturated (20 min, with saturation pad). R_F values increased with partial saturation, but then decreased with full saturation (**Figure 3**), proving that the SST with the UHM may indicate chamber saturation problems.

The UHM performance was evaluated in intra- and inter-laboratory tests based on the ΔR_F in developing solvents B, F and G. For the intra-laboratory test, the confidence interval ΔR_F was 0.03, while for the inter-laboratory test, this value was 0.04.

Throughout the development of the final composition, we supported CAMAG with the individual components that were considered and at a later stage with several prototypes of the mix. The subsequent optimisation lead to the final composition (**Table 2**).

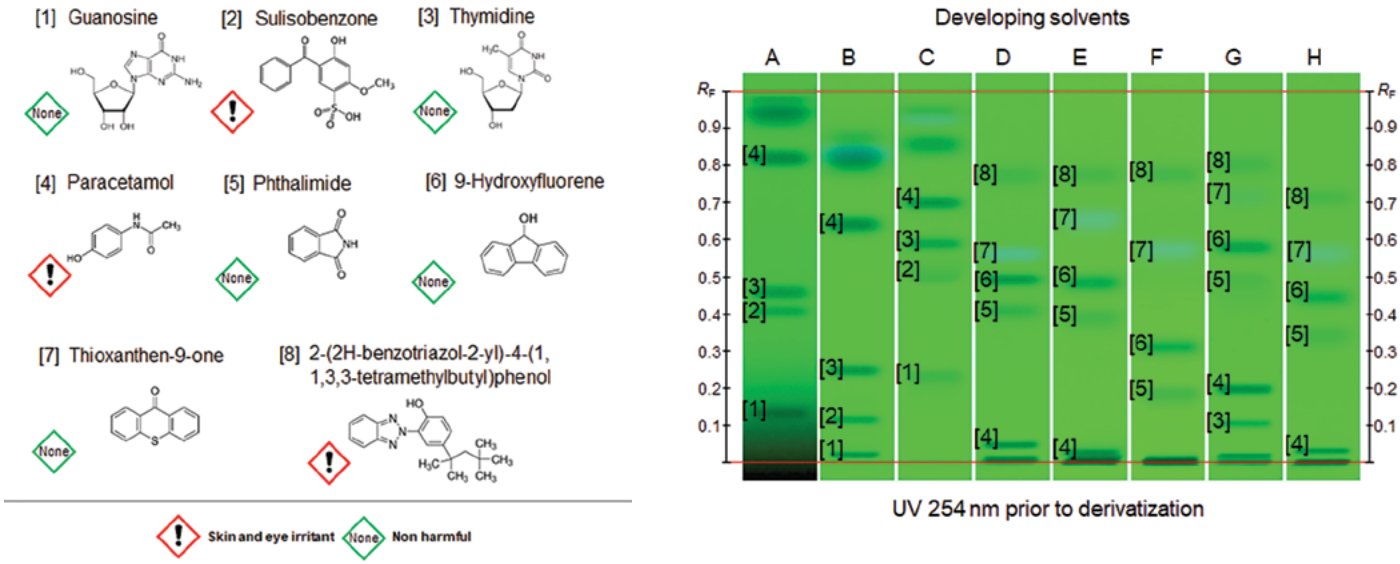

Figure 1. Substances selected for UHM and the HPTLC chromatograms of the UHM with eight different developing solvents (**Table 1**). Bands: 1. Guanosine, 2. Sulisobenzone, 3. Thymidine, 4. Paracetamol, 5. Phthalimide, 6. 9-Fluorenol (9-Hydroxyfluorene), 7. Thioxanthone, 8. Octrizole (2-(2H-Benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol).

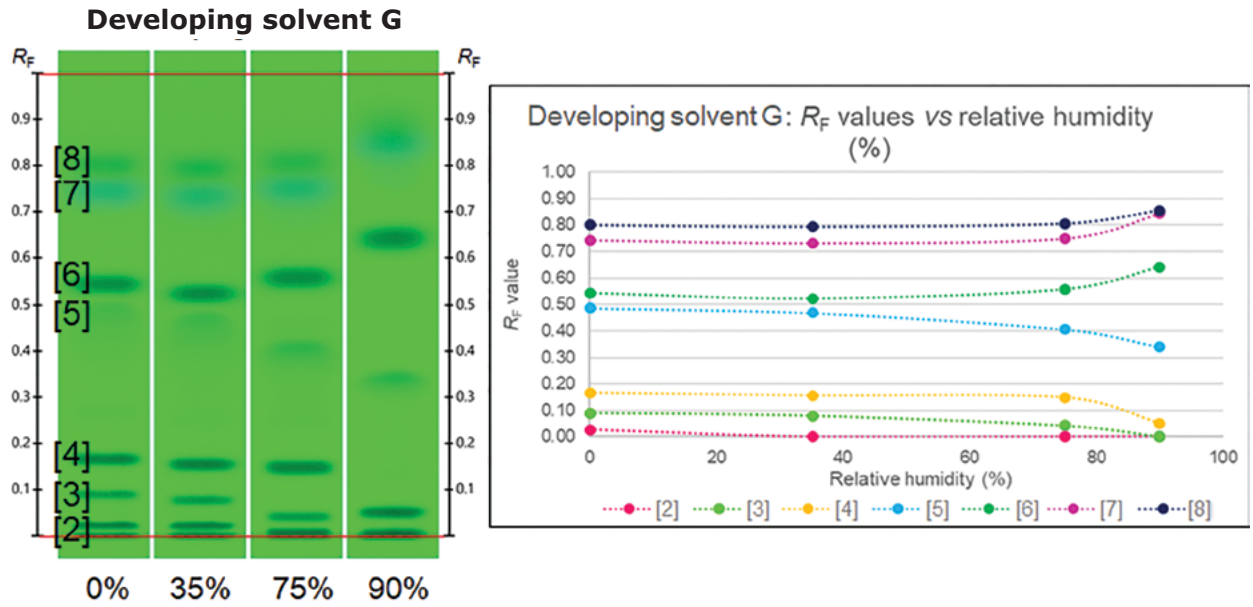

Figure 2. UHM evaluated with developing solvent G (see **Table 1**) and conditioned with different relative humidities prior to development. (Bands: 1. Guanosine, 2. Sulisobenzone, 3. Thymidine, 4. Paracetamol, 5. Phthalimide, 6. 9-Fluorenol, 7. Thioxanthone, 8. Octrizole)

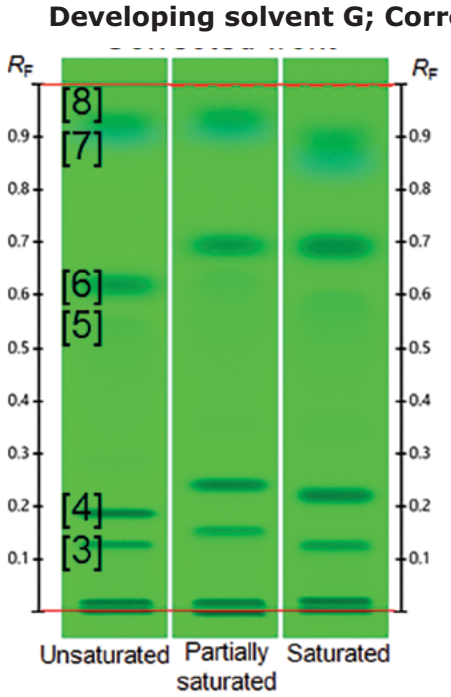

Figure 3. UHM evaluated with developing solvent G (**Table 1**) developed with different levels of chamber saturation (Bands: 1. Guanosine, 2. Sulisobenzone, 3. Thymidine, 4. Paracetamol, 5. Phthalimide, 6. 9-Fluorenol, 7. Thioxanthone, 8. Octrizole).

Table 2. Final UHM components in methanol

Compound	Concentration
9-Fluorenol	1000 mg/L
Guanosine	500 mg/L
Octrizole	1000 mg/L
Paracetamol	1000 mg/L
Phthalimide	2000 mg/L
Sulisobenzone	1000 mg/L
Thioxanthone	10 mg/L
Thymidine	1000 mg/L

The ready-to-use standard mix is available as cat. no. **91816** (**SigmaAldrich.com/uhm**). This product is manufactured under ISO 9001 management system as an analytical standard quality grade and is provided in a 1 mL amber glass ampoule. Stability checks were preformed to ensure that the mix is fit for purpose for the entire duration of the shelf life.

Conclusion

The newly developed universal HPTLC mix (UHM) enables HPTLC users to efficiently and reliably perform their system suitability testing (SST).

Reference:

1. Do TKT, Schmid M, Phanse M, Charegaonkar A, Sprecher H, Obkircher M, Reich E. 2021. Development of the first universal mixture for use in system suitability tests for High-Performance Thin Layer Chromatography. J Chromatogr A. 1638(461830):461830. doi:10.1016/j.chroma.2020.461830.

Featured Products

Description	Cat. No.
HPTLC calibration mix, 8 compounds in methanol, 1mL	**91816**
HPTLC Silica gel 60 F_{254}, 20 cm × 10 cm, glass support, Pk.50	**1.05642**

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Tips & Tricks for Thin-Layer Chromatography

Monika Bäumle, Global Product Manager TLC; Ilona Matus, Analytical Sciences Liaison; Analytix@milliporesigma.com

Thin-layer chromatography (TLC) is a fast, easy-to-use, and highly versatile separation technique for qualitative and quantitative analysis. It is ideal for rapid identification, screening, and reaction monitoring. Its high matrix tolerance and the possibility to separate many samples in parallel makes TLC highly time- & cost-efficient.

The TLC Working Principle

The general thin-layer chromatography process is simple, however it includes a range of steps and some precautions need to be considered (**Figure 1**). One example is the gas phase in the development chamber, which influences the TLC process. Therefore, it is critical to maintain controlled gas and humidity conditions to obtain reproducible performance and accurate TLC results. In the following we address some of the important aspects, which should be considered at different steps.

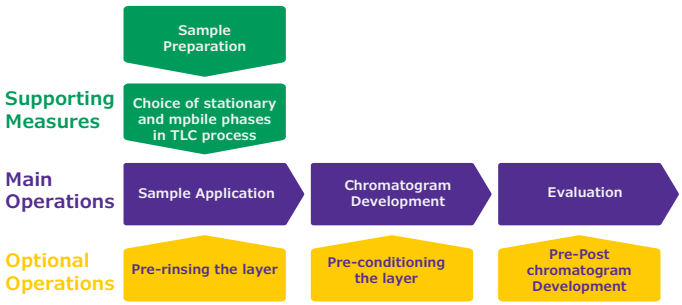

Figure 1. TLC process

Storage and Handling of TLC Plates

TLC layers are highly active materials and can adsorb moisture and contaminants originating from the lab environment. Therefore, a storage of plates in a clean and dry environment is recommended (e.g. in a desiccator). If possible, wrap the plates in aluminum foil and keep them away from chemical fumes and vapor.

Pre-Rinsing (Washing) and Activation of the Layer

Contaminants and moisture from the environment can alter the plate's performance, especially when not stored properly after opening a package. In addition, plates may have impurities from binders, packaging or previous handling. These contaminants can/should be removed by pre-rinsing the layer. This can be performed by either dipping them in a solvent (once or twice, 1-7 minutes) or by a blank run of the TLC plate e.g. with methanol. Mind the chromatographic direction, because impurities will concentrate at the top edge of the plate.

To remove bound water at the polar functional groups of the phase, it is advised to heat the plate for 20 – 30 min at 120 °C (in a clean oven) for a proper plate activation.

Sample Preparation

Thin-Layer Chromatography plates are single-use devices, hence they do not bear the risk for cross-contamination into future analysis. Because of this, sample preparation can typically be simplified and is therefore less time-consuming compared to HPLC. A sample crushing/homogenization and extraction with an appropriate diluent is recommended (e.g. for solid samples), eventually followed by filtration and concentration steps.

Choice of Stationary Phase in TLC

The choice of the TLC stationary phase is crucial as it determines the selectivity and separation performance. There is a broad range of options available – based on silica, aluminum oxide or cellulose, modified or non-modified. The selection should be in relation to the properties of your sample and your application goals. TLC can be run in two modes, normal and reversed phase mode. In normal phase (NP) mode, the mobile phase is less polar than the stationary phase on the TLC plate, whereas in reversed phase (RP), the mobile phase is more polar than the stationary phase. More than 80% of all TLC separations are done on silica gel as stationary phase, either bare or modified with e.g. C18.

Choice of Mobile Phase in TLC (Solvent System)

The choice of the mobile phase is another critical factor for an efficient separation result. The solvent dissolves the sample components on the sorbent layer and moves them across the plate. Ideal mobile phases transport all components from the baseline having final R_F (retention factor) values between 0.15 up to 0.85 (ideally 0.2 – 0.6). The retention factor is defined as

the distance traveled by the substance divided by the distance traveled by the solvent. Usually, these are also described as hR_F, which is defined as 100x R_F. When a method is developed from scratch, typically a mixture of a polar and non-polar solvent is used as a starting point. To increase R_F values in NP-TLC, an increase in the polarity of the mobile phase is needed. In case, a reduction of R_F is required, a decrease in polarity needs to be done. A very common mobile phase system in NP-TLC contains hexane and 10 – 50% ethyl acetate (EtOAc). Other prominent solvent systems are based on methanol and dichloromethane, toluene or acetone. In RP-TLC, the solvent systems are commonly mixtures based on water, methanol, acetonitrile, or aqueous buffers.

The employed solvents and solvent blends should have an adequate purity and stability, low viscosity, low vapor pressure, and low toxicity, if possible.

The addition of certain modifiers (basic or acidic) might improve separation results.

A detailed systematic approach to find the ideal solvent systems can be followed based on the published data.[1]

Sample Application

Samples can be applied as spots or bands by contact or spraying. Sample application by spraying in a band enables improved separation result and is the preferred method when having larger sample volume. There is a special equipment available to spray sample solutions onto a plate. This method avoids direct contact with the TLC layer and is typically used for band application.

The polarity of the sample diluent is a factor to be considered. In NP-TLC, non-polar diluents are used, like n-hexane, which ensure that the substances remain at the application point. However, with more polar diluents (e.g. toluene, dichloromethane, methanol), the sample substances are transported toward the edge of the "wet zone" (spot at the starting line), and typically form a circular chromatographic spot at the starting area (see top of **Figure 2**). Like in chromatographic development, peaks with an almost Gaussian distribution are achieved, which is widening with increasing polarity of the diluent (bottom **Figure 2**).

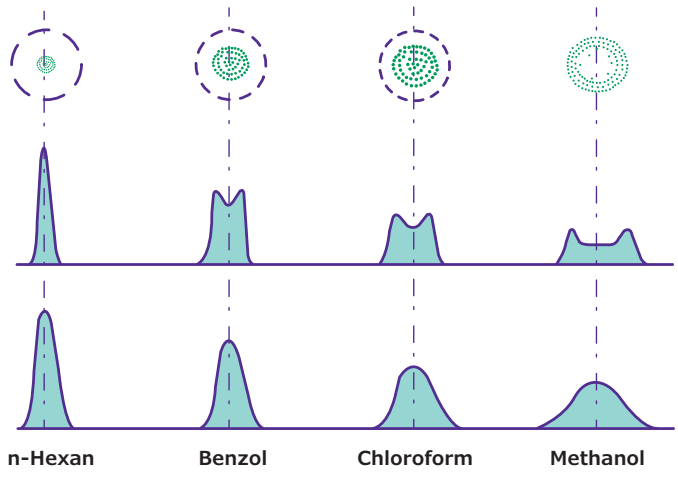

Figure 2. Substance distribution in a sample spot after application on a TLC plate as a function of the solvent

Based on the number of samples being analyzed, select a suitable plate size or cut a larger plate to the needed dimensions. Mark the application zone with a pencil by drawing lines across the plate. Take care not to damage/scratch the layer surface as this can influence the flow behavior and lead to errors. Be careful not to apply the sample too close to the bottom edge of the plate (8 mm from lower edge), as this can cause the starting point to spread/leach into the mobile phase. The sample volume to apply depends on the goal of the analysis and the concentration of the sample solutions. Typically a sample volume of 0.5-2.0 µL is recommended for identity tests, and a maximum of 10 µL for purity testing. The higher the sample volume, the more volatile and non-polar (for NP) the diluent should be and the slower the application should be carried out.

Drying Plates Prior to Development

After the sample is applied on the plate, the sample diluent must be completely removed by careful drying the plates prior to development. Avoid contamination with fumes during this step and chose an appropriate temperature in order not to cause sample diffusion or loss. The temperature depends on the kind of substances, their stability and boiling points.

Pre-Conditioning the Layer/ Humidity Control

Unless special precautions are taken during sample application, humidity in the laboratory can diminish the activity of the TLC layer within minutes, as an equilibrium is quickly etablished between the lab atmosphere and the sorbent. Pre-conditioning the TLC plate before development helps to avoid its deterioration. Therefore, condition your plates (after sample application) for 45 min over a saturated salt solution in a closed chamber. Depending on the relative humidity desired, several saturated salt solutions are typically recommended, for example a relative humidity of 33%, can be achieved by using a saturated $MgCl_2$ solution. Ensure, that there is still undissolved salt in the solution to ensure saturation. After conditioning it is important to develop the plate immediately to prevent recurrence of alterations.

Chromatogram/Plate Development

The development of the TLC plates can be done via various techniques, e.g. one-dimensional, two-dimensional or via forced flow processes. One-dimensional can be single or multiple development, and is run vertically, horizontally, or circular. In most cases,

the separation is done vertically in a development chamber.

First add the freshly prepared mobile phase in the development chamber to a level of max. 0.5 cm (immersion line). Before starting the development process, an equilibrium between the liquid mobile phase and the gas phase is required to achieve a chamber saturation. To facilitate that faster, add a saturating pad or place a filter paper inside the chamber (chamber wall) and equilibrate for 20 min (keep the chamber closed). After chamber saturation, place the plate quickly inside the chamber. As soon as the mobile phase has travelled two-thirds of the plate dimension (max. up to 1 cm from the top), remove the plate and mark the solvent front. After development, the plate should be properly dried to remove residual solvents before visualization.

Derivatization

After separation, typically a derivatization is done to enable visualization of analytes and enhancement of detection, if the sample components are colorless, or do not fluoresce. The ideally suited derivatization/ detection reagent is dependent on the target analytes and desired detection method. It may be applied before development (pre-chromatographic derivatization), with the solvent system (in-situ derivatization), or after development (post-chromatographic derivatization).

For post-chromatographic derivatization or visualization of the analytes, the reagent is applied to the plate by spraying or dipping. The advantages of spraying are its high flexibility and low reagent amount required, however, manual spraying often does not provide sufficient reproducibility.

Derivatization by dipping allows for more homogeneous and reproducible derivatization but requires more reagent. Besides that, background coloration may occur. Make sure to carefully wipe the back site of the plate after derivatization and before read out.

Visualization/Readout

Performing TLC in a proper way will provide you reproducible and precise results both for qualitative and quantitative analysis.

We will cover more Tips and Tricks for the visualization and documentation process in a separate article.

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Reference:

1. Snyder LR. Classification off the solvent properties of common liquids. Journal of chromatographic science. 1978;16(6):223–234. http://dx.doi.org/10.1093/chromsci/16.6.223

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