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Cannabinoid Analysis of Hemp: Developing an Efficient HPLC Method Workflow

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Aman Bhattacharjee
Head, Reference Materials.

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FOOD & BEVERAGE

Cannabinoid Analysis of Hemp: Developing an Efficient HPLC Method Workflow

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Abstract

Several different HPLC method options are explored for the efficient and time effective analysis of cannabinoids in a sample of hemp flower. The differences between gradient and isocratic analyses are examined, and the two methods compared in determining the weight percentage of cannabinoids.

Introduction

Consumption of both marijuana and hemp products for medical use stems from the proposed benefits of cannabinoids and other natural compounds present in the plants. In the case of marijuana, the need for cannabinoid testing, often referred to as "potency testing", is driven by the need to assure product quality and safety. Cannabinoid testing of hemp and hemp-derived products is also on the rise. Hemp, classified as a strain of *Cannabis sativa* with low THC content, was made legal on a federal level in the U.S. with the passing of the Agriculture Improvement Act of 2018, also known as the "2018 Farm Bill".[1] This resulted in the establishment of the U.S. Domestic Hemp Production Program by the United States Department of Agriculture (USDA). Guidelines issued under this program as the "interim final rule" (IFR) designate that for a product to be classified as hemp, its THC content must be < 0.3%.[2] This has subsequently driven not only quality testing of hemp for cannabinoid content, but also the need for accurate THC measurement to ensure that the product meets legal requirements.

The two major cannabinoids of interest in both marijuana and hemp are Tetrahydrocannabinol (THC) and Cannabidiol (CBD), which are commonly reported on product information labels. However, there are many more cannabinoids present, and some states are increasing their requirements for the number that must be reported. Thereby, testing must be able to distinguish and provide accurate results for multiple cannabinoids.

In the case of marijuana and hemp flower, a common approach to cannabinoid testing incorporates a simple liquid extraction with methanol or ethanol, followed by HPLC-UV analysis. UV detector is preferred as it is easier and less expensive to operate than a mass spectral (MS) detector; however, it requires peaks to be separated chromatographically for proper identification and accurate quantitation.

Many HPLC methods for cannabinoid analysis utilize a gradient elution. But this often adds to the overall run time as an equilibration to initial conditions is required for every sample. Using an isocratic method eliminates the need for repeat equilibration, which may in turn allow more samples to be run per unit time. However, the tradeoff can be a loss in resolution during some portions of the run. In this work, we demonstrate both gradient and isocratic conditions capable of separating 17 cannabinoids on Ascentis® Express HPLC columns. For each set of conditions, the overall elution time was maintained at <8 minutes. We then applied these methods to the analysis of dried hemp flower. The cannabinoids targeted for testing consisted of those listed by the AOAC in standard method performance requirements (SMPRs) for dried plant material, chocolates, and concentrates, plus three additional cannabinoids of interest.[3,4,5]

Experimental

The sample preparation method used was taken from a first action AOAC method for the quantitation of cannabinoids in cannabis.[6] A 5 g sample of hemp flower of unknown origin, donated by an external source, was homogenized to a particle size of <1 mm using a Cryo-Cup™ grinder. In order to homogenize the sample without degradation of the cannabinoids, use of a technique in which the sample is cooled is preferred. The low temperature prevents analyte degradation and produces uniform particle sizes.

A 500 mg sample of homogenized hemp flower was weighed into a 50 mL centrifuge tube. A 20 mL aliquot of ethanol was added, and the tube was vortexed briefly to mix. The tube was then shaken at 250 rpm for 30 min and centrifuged at 4000 rpm for 5 min to pelletize the plant material. The supernatant was carefully decanted into an amber 50 mL volumetric flask and set aside. A second extraction was performed from the plant material in the same 50 mL centrifuge tube using an additional 20 mL aliquot of ethanol. The resulting supernatant was then combined with the first and brought to a final volume of 50 mL with ethanol. To allow quantitation of high and low levels of cannabinoids in the hemp sample, the extract was diluted 1:10 and 1:100 with methanol. The diluted extract was filtered directly into a glass autosampler vial using a Mini-Uniprep™ G2 filter vial with a 0.20 µm PTFE membrane.

A peak identification standard at 50 µg/mL (CBLA at 25 µg/mL) containing 17 cannabinoids was prepared from certified reference materials of each cannabinoid. A separate mix containing the cannabinoids that were subsequently quantitated in the sample was prepared at 100 µg/mL and then diluted down to produce six calibration standards ranging from 0.25 to 100 µg/mL. HPLC analysis was performed on the same set of hemp flower extracts using two different sets of conditions (**Table 1**), that are described in more detail in the following sections. The instrumentation used for the analyses was a modern UHPLC, with a low-pressure mixing system and a 1 µL UV flow cell.

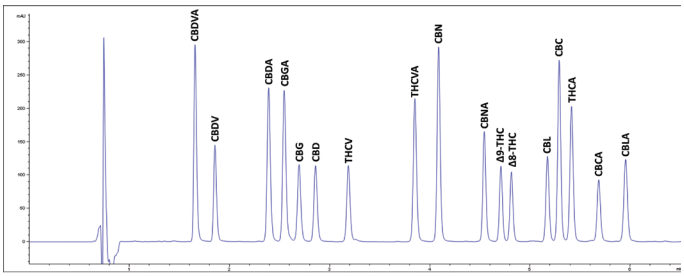

Figure 1. Separation of Cannabinoids in the Peak Identification Mix Using a Gradient on a 2 µm Ascentis® Express C18 Column

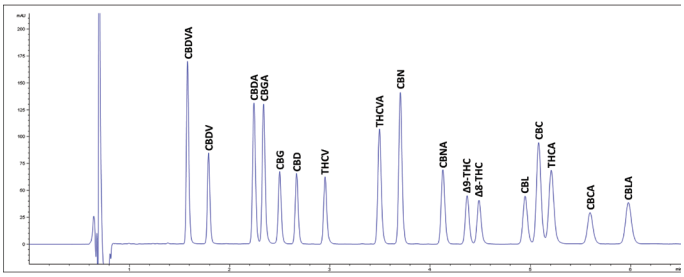

Figure 2. Separation of Cannabinoids in the Peak Identification Mix Using a Gradient on a 2.7 µm Ascentis® Express C18 Column

Results & Discussion

HPLC method optimization. Several factors considered when developing the HPLC methods for this workflow included: separation efficiency, speed, mobile phase composition, flow rate, and isocratic vs. gradient conditions.

The columns chosen for this work were from the Ascentis® Express line. This family of columns contains particles with a solid core/porous shell architecture (also referred to as superficially porous), which provides higher separation efficiency for a given particle size, compared to conventional fully porous particles.

Gradient methods. Gradient methods were developed on C18 columns with two different particle sizes: 2 µm and 2.7 µm. The gradient conditions and injection size were optimized for each column, as noted in **Table 1**. Formic acid and ammonium formate were added to the mobile phase to improve peak shape and resolution. Chromatograms showing the separation of the 17 cannabinoids in the peak identification mix on the two columns are shown in **Figures 1** and **2**. Separation is achieved in a little over 6 minutes. As expected the resolution is better on the 2 µm column. Another advantage of the 2 µm C18 method is the lower flow rate (0.4 mL/min vs. 0.8 mL/min), which uses less mobile phase per run and will ultimately result in cost savings both in solvent usage and waste disposal. However, with the smaller 2 µm particles there is higher backpressure, which requires the use of a UHPLC system.

Isocratic method. Isocratic HPLC methods are simpler than gradient methods and can be faster since no pre-run equilibration is required between samples. An isocratic method was developed for the 17 cannabinoids on an Ascentis® Express C8 column (**Table 1**). The organic and aqueous constituents of the mobile phase were of the same composition as those used for the gradient methods, with the ratio optimized for the

Table 1. HPLC Method Conditions

Column	Ascentis® Express C18 15 cm x 2.1 mm, 2.0 µm (50814-U)	Ascentis® Express C18 15 cm x 3 mm, 2.7 µm (53816-U)	Ascentis® Express C8 15 cm x 3 mm, 2.7 µm (53853-U)
mobile phase:	[A] 5 mM ammonium formate + 0.1% formic acid in water [B] 0.1% formic acid in acetonitrile		
mobile phase conditions:	75% to 90% B in 2 min; held at 90% B for 5 min	75% to 85% B in 2 min; held at 85% B for 5 min	Isocratic: 27:73, A:B
flow rate:	0.4 mL/min	0.8 mL/min	0.7 mL /min
pressure:	530 bar	250 bar	220 bar
column temp.:	25 °C	25 °C	30 °C
detector:	UV, 228 nm	UV, 228 nm	UV, 228 nm
injection:	3 µL	5 µL	5 µL

application. The resulting separation (**Figure 3**) yielded broader peaks as compared to the gradient methods, especially for Δ^8- and Δ^9-THC, and the run time was 2 minutes longer. This was expected since the gradient method utilizes an increased solvent strength to speed up elution of more retained analytes. In optimizing the isocratic method, a C8 phase was used to help counter this to some degree, as retention would have been even longer using C18 under the same conditions. The C8 phase also showed different selectivity towards some of the acidic cannabinoids compared to the C18 phase. Specifically, the retention of THCVA, CBNA, THCA, CBCA, and CBLA shifted relative to their neutral counterparts, resulting in a different elution order for two of these cannabinoids. Note: After the last peak, an additional 6 minutes (8 min + 6 min) proved sufficient for washing the column. The method uses a high ACN composition, which also helped reduce the wash time. However, to prevent any further build-up on the column, it's good practice to perform a high organic wash after each sample batch.

Analysis of hemp flower for cannabinoids. Analysis of the hemp sample was performed using both the 2.7 µm C8 and 2 µm C18 stationary phases. The hemp extract prepared as previously described was first screened by comparison to the 17-component peak ID mix. The six most prevalent cannabinoids were then quantitatively analyzed on both columns, yielding similar results (**Table 2**). This dual approach confirmed a high abundance of total CBD, which is characteristic of hemp. Total THC and CBD contents were calculated as a sum of their neutral and acidic forms after applying a conversion factor of 0.877 to account for the mass difference from decarboxylation. This dual column approach confirmed a high abundance of total CBD, which is characteristic of hemp. However, the total THC content exceeded the 0.3% limit to qualify as hemp, as per the USDA Interim Final Rule.

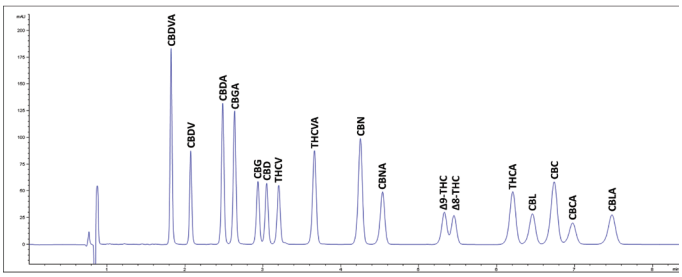

Figure 3. Separation of Cannabinoids in the Peak Identification Mix Using an Isocratic Method on a 2.7 µm Ascentis® Express C8 Column.

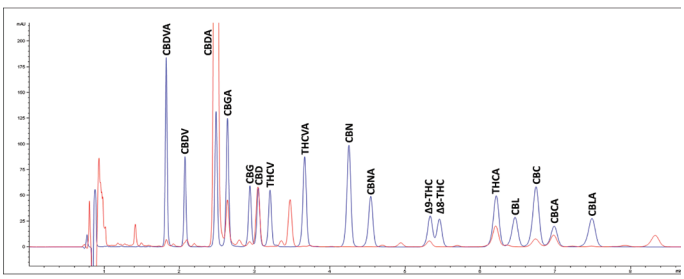

Figure 4. Overlay of Hemp Sample and Peak Identification Mix, Isocratic Method on Ascentis Express C8 2.7 µm Column

Table 2. Results of Cannabinoid Analysis of Hemp Sample on C8 and C18 Columns

	w/w % (Dry Weight)	
Analyte*	**C8, 2.7 µm**	**C18, 2 µm**
CBDA	18.20	18.10
CBG	0.13	0.10
CBD	1.40	1.40
CBN	ND	ND
Δ^9-THC	0.22	0.24
THCA	0.46	0.50
Total THC	0.62	0.68
Total CBD	17.36	17.26

*For analyte abbreviations, see featured products table on the next page

Conclusion

A complete HPLC workflow for the analysis of 17 cannabinoids was developed using both gradient and isocratic HPLC methods. The total THC content of a hemp sample was evaluated by two different HPLC methods, and was found to significantly exceed the 0.3% total THC limit necessary to be classified as hemp by the USDA Interim Final Rule. The results confirmed the high total CBD content characteristic of hemp, with the majority detected in the acidic form (CBDA). Results from both methods were in agreement and demonstrated a high degree of selectivity despite the abundance of matrix components. The workflow can easily be adopted to quantitate cannabinoids in plant material and concentrates with concentrations spanning 0.05 – 100% by weight. The short run times and low solvent consumption of both methods also makes them cost-effective for high-throughput potency testing.

References

1. Hemp: USDA.gov/topics/hemp, accessed Apr. 6th,2020.
2. Establishment of a Domestic Hemp Production Program. Fed. Regist., 2019, 84 (211), 58522-58564.
3. AOAC SMPR 2017.002. Standard Method Performance Requirements (SMPRs) for Quantitation of Cannabinoids in Dried Plant Materials
4. AOAC SMPR 2017.019. Standard Method Performance Requirements (SMPRs) for Quantitation of Cannabinoids in Edible Chocolate
5. AOAC SMPR 2017.001. Standard Method Performance Requirements (SMPRs) for Quantitation of Cannabinoids in Cannabis Concentrates
6. 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. J. AOAC 2019, 6, 1822-1833.

Featured Products

Description	Cat. No.
HPLC Columns	
Ascentis® Express C18, 15 cm x 2.1 mm, 2 µm	**50814-U**
Ascentis® Express C18, 15 cm x 3.0 mm, 2.7 µm	**53816-U**
Ascentis® Express C8, 15 cm x 3.0 mm, 2.7 µm	**53853-U**
Solvents & Reagents	
Ultrapure water from Milli-Q® System e.g. IQ 7005 or bottled water	**Milli-Q® or 1.01262**
Acetonitrile Solution, contains 0.1 % (v/v) formic acid, for UHPLC, for MS	**900686**
Water Solution, contains 0.1 % (v/v) formic acid, for UHPLC, for MS	**900687**
Ammonium Formate, eluent additive for LC-MS, LiChropur®, ≥99.0%	**70221**
Formic Acid, for LC-MS LiChropur®	**5.33002**
Ethyl Alcohol, HPLC/spectrophotometric grade*	**459828**
Methanol, UHPLC, for mass spectrometry	**900688**
Certified Reference Materials (Packs of 1 mL)	
Cannabidivarinic acid (CBDVA), 1 mg/mL in acetonitrile	**C-152**
Cannabidiolic acid (CBDA), 1 mg/mL in acetonitrile	**C-144**
Cannabigerolic acid (CBGA), 1 mg/mL in acetonitrile	**C-142**
Tetrahydrocannabivarinic acid (THCVA), 1 mg/mL in acetonitrile	**T-111**
Δ^9-tetrahydrocannabinolic acid (THCA), 1 mg/mL in acetonitrile	**T-093**
Cannabinolic acid (CBNA), 1 mg/mL in acetonitrile	**C-153**
Cannabichromenic acid (CBCA), 1 mg/mL in acetonitrile	**C-150**
Cannabicyclolic acid (CBLA), 0.5 mg/mL in acetonitrile	**C-171**
Cannabidivarin (CBDV), 1 mg/mL in methanol	**C-140**
Cannabigerol (CBG), 1 mg/mL in methanol	**C-141**
Cannabidiol (CBD), 1 mg/mL in methanol	**C-045**
Tetrahydrocannabivarin (THCV), 1 mg/mL in methanol	**T-094**
Cannabinol (CBN), 1 mg/mL in methanol	**C-046**
Δ^9-tetrahydrocannabinol (Δ^9-THC), 1 mg/mL in methanol	**T-005**
Δ^8-tetrahydrocannabinol (Δ^8-THC), 1 mg/mL in methanol	**T-032**
Cannabichromene (CBC), 1 mg/mL in methanol	**C-143**
Cannabicyclol (CBL), 1 mg/mL in acetonitrile	**C-154**

Description	Cat. No.
Sample Preparation Hardware, Tubes and Flasks	
Corning® 50 mL centrifuge tubes, polypropylene, conical bottom w/ CentriStar cap	**CLS430828**
BRAND® BLAUBRAND® Volumetric Flask, Glass Stopper, Amber Glass, 50 mL	**Z327050**
BRAND® BLAUBRAND® Volumetric Flask, Glass Stopper, Amber Glass, 20 mL	**Z327026**
BenchMixer™ Shaker/Vortexer, 115V, US 2-pin plug	**Z742705**
BenchMixer™ Shaker/Vortexer, 230V, Schuko plug	**Z742706**
BenchMixer™ Shaker/Vortexer, 230V, UK Plug	**Z742707**
Corning® LSE™ Compact Centrifuge, 120 V	**CLS6755**
Corning® LSE™ Compact Centrifuge, 230 V, EU plug	**CLS6759**
Corning® LSE™ Compact Centrifuge, 230 V, UK plug	**CLS6758**
Corning® LSE Rotor fixed angle rotor for 6 x 50 mL tubes	**CLS480136**
Whatman® Mini-UniPrep G2 Filter Vial Starter Kit, Pk.100	**WHAGN203 APEORGSP**

*Not Available in all countries. Please visit SigmaAldrich.com to check for alternatives.

Related Products

Description	Cat. No.
Ascentis® Express Guard Cartridge Holder, Pk.1	**53500-U**
Ascentis® Express C18, 5 mm × 2.1 mm, 2 µm Guard Cartridge, Pk.3	**50822-U**
Ascentis® Express C18, 5 mm × 3 mm, 2.7 µm Guard Cartridge, Pk.3	**53504-U**
Ascentis® Express C8, 5 mm × 3 mm, 2.7 µm Guard Cartridge, Pk.3	**53511-U**
BRAND Seripettor Bottle-top Dispenser	**Z627577**
Certified Vial Kit - Amber	**29653-U**
Millex® Syringe Filter, Hydrophilic PTFE Filters, 0.20 ®µm	**SLLG033**
Samplicity® G2 Filtration System	**SAMP2SYSB**
Millex® Samplicity® Hydrophilic PTFE Filters, 0.20 µm, Pk.96	**SAMPLG001**
Millipore® Chemical Duty Pump, 115 V/60 Hz	**WP6111560**
Millipore® Chemical Duty Pump, 220 V/50 Hz	**WP6122050**
Corning Glass Media Storage Bottles (1L)	**CLS13991L**

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FOOD & BEVERAGE

New Carotenoid Standards

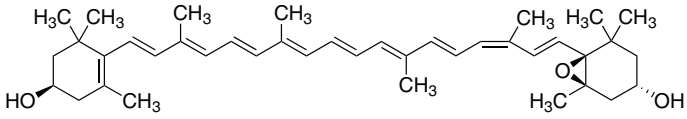
Matthias Nold, Product Manager Reference Materials, Analytix@merckgroup.com

Carotenoids, belonging to the class of isoprenoids, are very abundant in nature. Due to their chemical structure with a long chain of conjugated double bonds, they show antioxidant properties, and play a major role in the photosynthesis process. They are often used as food additives to add color or flavor.

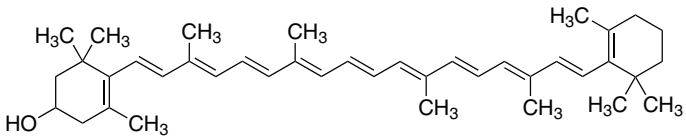
Our portfolio of analytical standards comprises of more than 25 highly purified carotenoid standards. The most recent additions are listed below.

New Carotenoid Standards

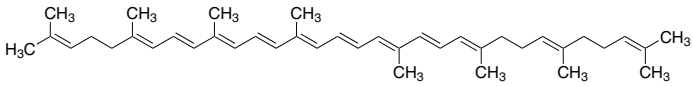
Description	Package Size	Cat. No.
9-cis-Antheraxanthin	1 mg	**47999**
(+/-)-beta-Cryptoxanthin	1 mg	**51772**
all-trans-Neurosporene	1 mg	**59739**



47999 9-cis-Antheraxanthin



51772 (+/-)-beta-Cryptoxanthin



59739 all-trans-Neurosporene

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FOOD & BEVERAGE

Chiral Separation of the Pesticide Diniconazole by Supercritical Fluid Chromatography (SFC)

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Cory E. Muraco, Global Franchise Product Manager, Liquid Chromatography Technologies, Analytix@merckgroup.com

Abstract

Diniconazole is a broad-spectrum triazole fungicide used to prevent plant diseases such as powdery mildew, bunt, rust, smut, and septoria leaf spot. Diniconazole is a chiral chemical with one chiral center and is sold commercially as a mixture of R- and S-enantiomers. It has been reported that R-(-)-diniconazole has higher bactericidal activity, while S-(+)-diniconazole shows higher plant growth regulator activity. It is important to know if environmental factors have altered the ratio of R- and S-diniconazole. Chiral Supercritical Fluid Chromatography (SFC) and mass spectrometry detection have been used as a tool to study the different ratios of diniconazole in various plant materials. For that study a ChromegaChiral™ CCA column was used providing baseline resolution of the two diniconazole enantiomers, however, they are closely separated. In this study 14 different chiral stationary phases were evaluated for the chiral SFC separation. From this evaluation we discovered that a better separation can be achieved by using a newly developed ChromegaChiral™ stationary phase for a high resolution separation of diniconazole enantiomers.

Introduction

A variety of diseases can attack agricultural crops for many reasons including changes in climatic conditions. These include many fungal diseases such as smut and rust, that can significantly affect crop yields and agricultural output unless they are controlled or eliminated. The application of chemical pesticides can effectively eliminate plant pests, however, with increasing doses and accumulated application, they may potentially impact human health.[1] Additionally, about 30% of the commonly used chemical pesticides are chiral, however most of these are sold and used as racemic mixtures.[2] A racemic mixture, or racemate, is one that has equal amounts of left- and right-handed enantiomers of a chiral molecule. Enantiomers of the same chemical have identical physicochemical properties, but may exhibit differences in biological activity, pharmacokinetics, pharmacodynamics, and toxicity.[3] One widely used pesticide used to control plant fungal diseases is diniconazole which is sold and used as a racemic mixture (**Figure 1**).

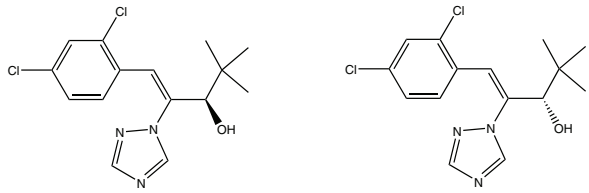

Figure 1. Chemical structure of R- and S- Diniconazole, respectively.

Diniconazole is a broad-spectrum triazole fungicide used to prevent diseases in fruits, vegetables, wheat and tea plants. Its mode of action includes the suppression of 14-α-demethylation in the biosynthesis of ergosterol causing a deficiency of ergosterol which prevents the occurrence of rust and smut disease.[4] Diniconazole is a chiral chemical with one chiral center and is sold commercially as a mixture of R- and S- enantiomers. It has been reported that R-(-)-diniconazole has higher bactericidal activity, while S-(+)-diniconazole shows higher plant growth regulator activity. In many cases it is important to know if environmental factors have altered the ratio of R- and S-diniconazole residues when applied on agricultural crops.[5]

Supercritical fluid chromatography (SFC) is a powerful chromatographic technique for the separation, isolation, and analysis of complex mixtures from many different samples. Many chemicals can potentially be used as a supercritical mobile phase for SFC; however, virtually all current practitioners of SFC use carbon dioxide (CO_2) which offers several advantages, particularly when compared to liquid chromatography.[6] The use of CO_2 as the primary component of the mobile phase is one of the key features that benefits the preparative SFC chromatographer, and, since the CO_2 used for SFC is recovered from the atmosphere, it is considered a “Green” solvent. Carbon dioxide is miscible with a wide range of organic solvents, is nonflammable, and has low UV absorbance at low wavelengths.[7-9] Other advantages of SFC as a technique are the diffusion coefficients of solutes in the SFC mobile phases that have been shown to be 3-10 times higher than in normal liquids potentially allowing for very rapid separations, and the viscosity of SFC mobile phases being significantly lower than LC mobile phases and producing a much lower pressure drop across the column thus allowing the use of much higher mobile phase flow rates.[10]

Building A Better Method

In a recent manuscript, SFC Chiral chromatography coupled with quadrupole-time-flight mass spectrometry had been used as a tool to determine the enantiomeric residue of diniconazole in tea, grape, and apples.[11] The authors of this manuscript developed a methodology to determine if environmental and processing factors have altered the ratio of R- and S-diniconazole residues on the selected agricultural products. Of particular interest to them was the alteration of residual diniconazole on black tea during processing. The increased activity of the enzyme polyphenol oxidase leads to browning and ultimately the production of black tea from green tea leaves. In this recent manuscript, the authors found changes in the enantiomeric ratios of diniconazole as a result of black tea processing. A significant part of the methodology utilized by the authors, was the SFC separation of enantiomeric diniconazole residues using a chiral column. The chiral column selected by the authors was a ChromegaChiral™ CCA (tris-(3,5-di-methylphenyl) carbamoyl amylose produced by ES Industries. ChromegaChiral™ CCA produces near baseline resolution of the two enantiomers in about 10.5 minutes with some peak tailing using 5% isopropanol (IPA) in CO_2. The ChromegaChiral™ CCA chiral column enabled the authors to complete their study; however, we wanted to investigate if other chiral columns could be utilized to improve the resolution of R,S-diniconazole with a significantly faster analysis time. It is the goal of this current study to investigate and identify other chiral columns to improve the separation and analysis time for the determination of R,S-diniconazole.

Chiral Column Selection

For this study, we selected ten different ChromegaChiral™ stationary phases for evaluation (**Table 1**). These have been found to have high rates of applicability to many chiral separations and have been successfully applied to a wide variety of chiral applications. Initially, all tested stationary phases were evaluated at conditions listed in **Table 2**. In addition to the stationary phases evaluated, **Table 1** also includes the chemical structure, USP resolution for the two enantiomers, and retention time (RT) of the first eluting enantiomer.

Table 1. ChromegaChiral™ Columns Employed in Study (25 cm x 4.6 mm if not indicated otherwise)

Stationary Phase Type	Chemical Structure	Resolution	RT (min)
ChromegaChiral™ CCA*	Tris-(3,5-dimethylphenyl) carbamoyl amylose	1.23	10.85
ChromegaChiral™ CCO	Tris-(3,5-dimethylphenyl) carbamoyl cellulose	No resolution	10.54
ChromegaChiral™ CCO-F4	Tris-(4-fluoro 3-methylphenyl) carbamoyl cellulose	No resolution	12.18
ChromegaChiral™ CC4	Tris-(4-chloro 3-methylphenyl) carbamoyl cellulose	No resolution	14.94
ChromegaChiral™ CCS*	Tris-((S)-α-methylbenzyl) carbamoyl amylose	9.44	8.86
ChromegaChiral™ CC3	Tris-(5-chloro 2-methylphenyl) carbamoyl amylose	No resolution	7.35
ChromegaChiral™ CCJ	Tris-(4-methylbenzoate) Cellulose	No resolution	9.56
ChromegaChiral™ CCU	Bis-(3-chloro 4-methylphenyl) & (methylbenzyl) carbamoyl amylose	2.94	19.33
ChromegaChiral™ CCA-F4	Tris-(4-fluoro 3-methylphenyl) carbamoyl amylose	1.00	8.22
ChromegaChiral™ CCC	Bis-(3,5-dichlorophenyl) & (3-chloro 4 methylphenyl) carbamoyl cellulose	0.68	21.42

*15 cm x 4.6 mm

Table 2. Chromatographic Conditions Overview

columns:	25 cm x 4.6 mm (15 cm x 4.6 mm for CCA & CCS)
mobile phase:	5% IPA co-solvent in CO2
flow rate:	2 mL/min.
pressure:	140 bar
column temp.:	25 °C
detector:	UV, 254 nm
injection:	5 µL
sample:	diconazole 200µg/mL in acetonitrile

There are several points that are notable in **Table 1**:

1. ChromegaChiral™ CCS provided the highest resolution for diniconazole enantiomers (**Figure 2**).

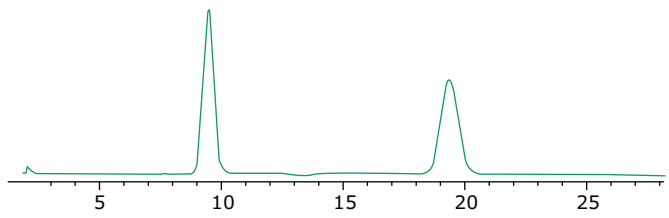

Figure 2. Enantiomeric separation of diniconazole on ChromegaChiral™ CCS (15 cm x 4.6 mm), Conditions see Table 2.

2. Most of the amylose based stationary phases resolved the diniconazole enantiomers except for ChromegaChiral™ CC3.
3. All the cellulose based phases did not adequately separate the diniconazole enantiomers.

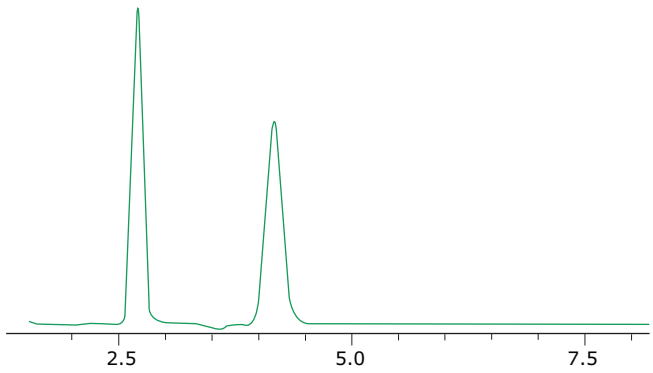

Figure 3. Enantiomeric separation of diniconazole on ChromegaChiral™ CCS. Conditions different from Table 2: 15% IPA in CO_2.

The resolution of the diniconazole enantiomers on ChromegaChiral™ CCS is clearly superior to any of the stationary phases evaluated using a co-solvent of 5% IPA and, based on this the percentage of IPA co-solvent was risen in order to try to reduce the analysis time. We found acceptable conditions with 15% IPA (**Figure 3**). At 15% IPA, the resolution (5.44) of the diniconazole enantiomers on ChromegaChiral™ CCS is excellent and the separation completed in less than 4.50 minutes. The resolution and rapid analysis at 15% IPA on ChromegaChiral™ CCS are striking when compared to the resolution (1.23) on ChromegaChiral™ CCA which has an analysis time of 10.85 minutes.

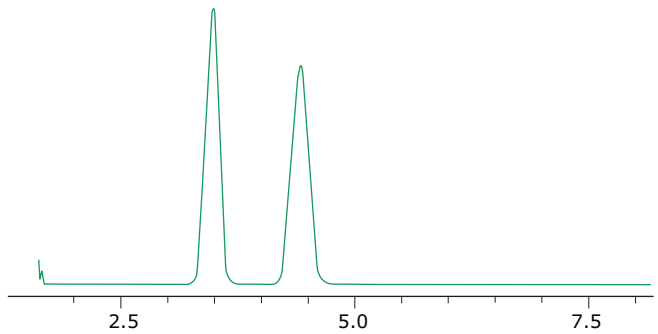

Figure 4: Enantiomeric separation of diniconazole on ChromegaChiral™ CCS (15 cm x 4.6 mm) . Conditions different from Table 2: 5% Methanol in CO_2.

Co-Solvent Selection

The selection of the optimal co-solvent is critical for SFC chiral separations, and the initial selection of IPA as a co-solvent was based on previous work.[11] In order to further optimize the enantiomeric separation of diniconazole, tests were carried out with other commonly used co-solvents for SFC, chiefly ethanol and methanol. In the case of ethanol vs. IPA co-solvent, no substantial differences were observed. However, for methanol vs. IPA, several differences were observed. An enantiomeric separation of diniconazole with an analysis time of less than 4.5 minutes and a resolution of 2.84 could be obtained with only 5% methanol on ChromegaChiral™ CCS (**Figure 4**). However, on ChromegaChiral™ CCA the enantiomeric separation of diniconazole was a complete co-elution with 5% methanol. Alternatively, an enantiomeric separation of diniconazole was achieved using 5% methanol on both ChromegaChiral™ CCO-F4 (**Figure 5**) and ChromegaChiral™ CC4 (**Figure 6**) which are both based on cellulose. It is also noted that the separation is faster on ChromegaChiral™ CCO-F4, the fluorinated version of ChromegaChiral™ CC4 (chlorinated). General observation was that the fluorinated ChromegaChiral™ stationary phase required less co-solvent than the similar chlorinated phase to obtain the same separation.

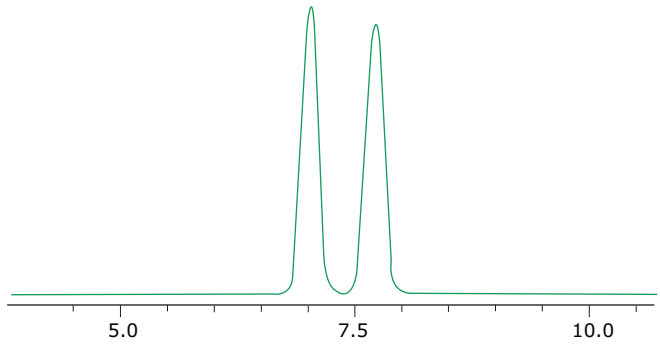

Figure 5: Enantiomeric separation of diniconazole on ChromegaChiral™ CCO-F4. Conditions different from Table 2: 5% Methanol in CO_2.

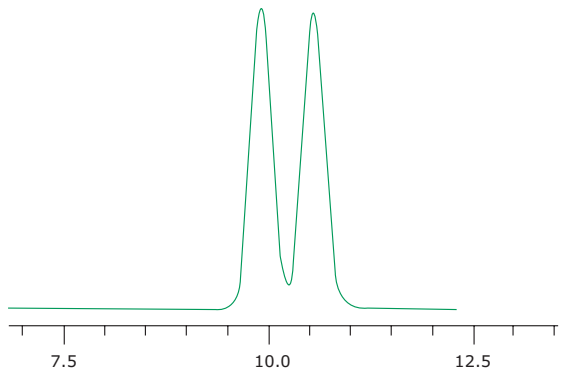

Figure 6: Enantiomeric separation of diniconazole on ChromegaChiral™ CC4. Conditions different from Table 2: 5% Methanol in CO_2.

Conclusion

We have investigated ten different chiral stationary phases in order to optimize the enantiomeric separation of diniconazole by SFC. The ChromegaChiral™ CCS provided the highest resolution separation and, with adjustments to co-solvent strength, we were able to obtain a high-resolution separation in less than 4.5 minutes, which is a substantial improvement over the previously reported enantiomeric separation of diniconazole on ChromegaChiral™ CCA.[11] In addition, the use of methanol co-solvent improved the enantiomeric separation of diniconazole on several chiral stationary phases tested. When developing chiral separations for either analytical or preparative applications, it is important to test several different chiral stationary phases to obtain the optimal separation conditions for a given enantiomeric separation.

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ChromegaChiral™ Chiral CC4; 25 cm x 4.6 mm, 5.0 µm	**ES5722**
ChromegaChiral™ Chiral CCA; 15 cm x 4.6 mm, 5.0 µm	**ES5409**
ChromegaChiral™ Chiral CCA-F4; 25 cm x 4.6 mm, 5.0 µm	**ES5437**
ChromegaChiral™ Chiral CCC; 25 cm x 4.6 mm., 5.0 µm	**ES5459**
ChromegaChiral™ Chiral CCJ; 25 cm x 4.6 mm, 5.0 µm	**ES5492**
ChromegaChiral™ Chiral CCO F4; 25 cm x 4.6 mm, 5.0 µm	**ES5591**
ChromegaChiral™ Chiral CCO; 25 cm x 4.6 mm, 5.0 µm	**ES5525**
ChromegaChiral™ Chiral CCS; 15 cm x 4.6 mm, 5.0 µm	**ES5620**
2-Propanol, for HPLC, 99.9%	**34863**
Methanol, for HPLC, ≥99.9%	**34860**
Diniconazole, PESTANAL®, analytical standard, 250 mg	**46049**

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Methanol gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur	**1.06007**

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FOOD & BEVERAGE

Validation of a Simple Method for the Alcohol Content in Kombucha Tea by Headspace SPME and GC-MS

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Abstract

An assortment of different Kombucha teas was analyzed for ethanol content using an SPME method developed and validated specifically for this application. It was found that many had ethanol levels >0.5%, which is the limit for a beverage to be sold as non-alcoholic in the United States.

Introduction

Kombucha is a fermented tea beverage. It is produced by the addition of a mixture of yeast and acetic acid bacteria, sometimes referred to as "tea fungus", to a solution of sugar and tea. After fermentation, the result is an effervescent solution containing phenolics, water soluble vitamins, organic acids, as well as some alcohol (ethanol). The health benefits often associated with Kombucha stem from the antioxidant activity of many of these compounds.[1,2]

In order for Kombucha to be sold as a non-alcoholic beverage in the United States, the alcohol content must be <0.5% by volume.[3] Headspace gas chromatography (HS-GC) is a technique that is readily amenable to the analysis of ethanol in a variety of aqueous-based matrices. In the case of Kombucha tea, which can contain a variety of sweeteners and flavoring ingredients, headspace can be used to isolate the volatile alcohol from other constituents in the sample, thus, protecting the GC system. Conventional headspace analysis often requires instrumentation additional to the GC. Headspace solid phase microextraction (HS-SPME) is an alternative approach to the analysis of alcohol content in beverages such as Kombucha. This technique can be performed manually or by automation, does not require the use of a separate concentrator or headspace analyzer external to the GC, and is typically faster and less expensive to perform than other more traditional approaches.

In this work, we have developed an HS-SPME method for the determination of alcohol content in Kombucha. GC-MS was used to allow for accurate and confirmative determination. The HS-SPME method developed is quick, simple, accurate, highly sensitive, and easy to automate.

Experimental

The final, optimized HS-SPME/GC-MS method is described in **Table 1**. Alcohol calibration standards were prepared in deionized water at concentrations of 0.10, 0.40, 0.80, 1.00, 1.50, and 2.00 percent alcohol by volume (% ABV) by direct dilution of aliquots of 200 proof ethanol in 25 mL of water. An internal standard/sample diluent solution was prepared at a concentration of 0.08% ABV by direct dilution of neat ethanol-d_6 in a 0.05 M sodium phosphate buffer solution (pH=7) containing 25% sodium chloride. This solution was then used in the dilution of samples prior to SPME. The internal standard/diluent solution was prepared daily and chilled prior to use. All samples and calibration standards were diluted 10:1 prior to SPME by addition of 400 µL of each to 3.6 mL of the chilled internal standard/sample diluent solution.

All Kombucha samples tested were purchased at local grocery stores and kept under refrigeration until analyzed. A ginger flavored Kombucha found to have very low alcohol content was used for the preparation of spikes in the method validation process.

Table 1. Optimized HS-SPME / GC-MS Method

sample/matrix:	400 µL tea sample + 3.6 mL salt/buffer soln. (0.05 M Na_2HPO_4 at pH 7 w/25% NaCl) containing ethanol-d_6 (I.S.) at 0.08% ABV in 10 mL headspace vial
SPME fiber:	polydimethylsiloxane (PDMS), 100 µm film, 23 ga (**57341-U**)
incubation:	7 min, 40 °C, agitation at 250 rpm
extraction:	2 min, headspace, 40 °C, agitation at 250 rpm
desorption process:	3 min, 250 °C, split 10:1
fiber post-bake:	5 min, 260 °C
column:	SUPELCOWAX® 10, 30 m x 0.25 mm I.D., 0.50 µm (**24284**)
oven:	40 °C (5 min), 8 °C/min to 70 °C, 20 °C/min to 250 °C (5 min)
carrier gas:	helium, 1 mL/min, constant flow
detector:	MSD, full scan, m/z=25-300 (m/z=45 used for quant of ethanol, m/z=49 for ISTD)
MSD interface:	250 °C
injection:	SPME, 10:1 split
liner:	0.75 mm I.D. SPME

Results and Discussion

Optimization of the HS-SPME Procedure

SPME is a very sensitive technique and is normally applied to test for analytes at very low levels. The targeted analytical range for this method was 0.10 to 2.00% ABV, which is considered to be too concentrated for SPME. Thus the goal was to develop an SPME method that could be used with high accuracy and reproducibility over this entire range. In order to prevent overloading the fiber and detector, sample dilution and the use of a 10:1 split during desorption of the SPME fiber were necessary. Initially, a Carboxen®/PDMS fiber was chosen for method optimization. However, a linear response could not be obtained over the entire analytical range. **Figure 1** shows a comparison of absolute response by GC-FID using the Carboxen®/PDMS fiber and a 100 µm PDMS fiber over a range of 0.10 to 1.00% ABV. Linearity within this range was slightly better using the PDMS fiber, as evidenced by the higher correlation coefficient (r^2) value. Also, the response curve obtained using the Carboxen®/PDMS fiber started to show some leveling off between 0.80 and 1.00% ABV, and this was expected to become more pronounced at higher concentrations. Since sufficient sensitivity was obtained using the PDMS fiber, it was chosen for further work. After fiber choice, additional parameters such as extraction temperature, time, sample additives, and sample dilution were evaluated during method development. All were optimized to minimize variability in alcohol response from both water and Kombucha tea samples. (See expanded version of this article at SigmaAldrich.com for more details.) With the final method, ethanol response was unaffected by sample matrix. This allowed quantitation of samples to be done against a calibration curve made with ethanol in water.

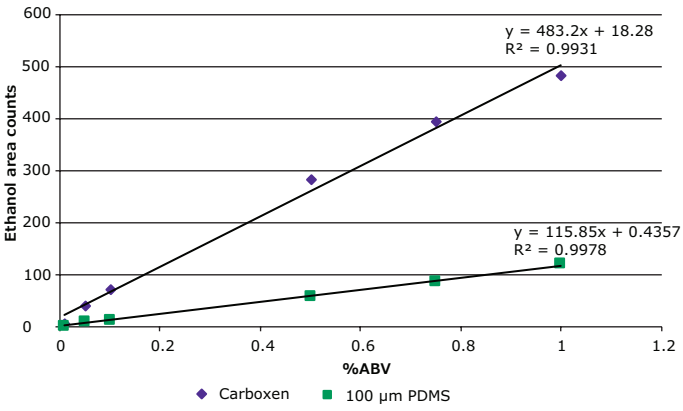

Figure 1. Comparison of Alcohol Response (GC-FID) from Water obtained by HS-SPME; 100 µm PDMS and Carboxen®/PDMS Fibers.

Method Validation

Accuracy of the HS-SPME GC/MS method was evaluated using ginger flavored Kombucha tea replicates, spiked at concentrations from 0.10 to 2.00% ABV. For the determination of method detection and quantitation limits, replicates spiked at 0.1% ABV were used. Method accuracy, repeatability, detection, and quantitation limits are summarized in **Table 2**. For the Kombucha spikes, good linearity was obtained, with a linear correlation coefficient of 0.999 from 0.10 to 2.00% ABV. Excellent accuracies of 98-100% ABV were obtained over the analytical range, with method repeatability of <4% RSD. Using the 0.1% ABV Kombucha spikes (n=8), the limit of detection (LOD) and limit of quantitation (LOQ) for the method were calculated as 3x and 10x the standard deviation (0.003) respectively. These calculated values were verified experimentally with the analysis of Kombucha samples spiked at 0.010 and 0.030% ABV. For n=5 spikes at each concentration, accuracy at the LOQ was 95% with a repeatability value of 6% RSD. Accuracy at the LOD was 83% with repeatability of 21% RSD. These low accuracy and poor repeatability values at the LOD can be attributed to the poor response of ethanol at this level, as the signal-to-noise ratio was approximately 2x lower than that obtained at the LOQ, and 4.5x times lower than the 0.1% spiking level. In case of a necessity to quantitate at 0.01% ABV, simple modifications to the method, such as using a splitless SPME injection, could be used to increase the ethanol response.

Analysis of Certified Reference Materials

The method was further validated using certified reference materials of low alcohol beer and pre-prepared solutions of alcohol in water. The results are summarized in **Table 3**. Each was analyzed multiple times over different days, and in some cases by different analysts on different instruments. The daily accuracies ranged from 96-101% for the beer samples and 94-103% for the alcohol in water solutions with reproducibility of <6% RSD for all sample sets.

Table 2. HS-SPME Method Accuracy and Repeatability from 0.10 % to 2.00% ABV; Spiked Kombucha Tea

Spiking Level (% ABV)	Amt. Measured in Unspiked Kombucha (% ABV)	Avg. Measured (% ABV)	Avg. Measured Less Unspiked (% ABV)	Repeatability % RSD (n=5)	% Accuracy	LOD (% ABV)	LOQ (% ABV)
0.10 (used for LOD & LOQ)	0.011	0.11	0.098	3*	98	0.01	0.03
0.50	0.011	0.51	0.50	2	100		
1.00	0.011	1.00	0.99	1	99		
2.00	0.026	1.99	1.96	1	98		

*n=8

Table 3. Method Accuracy, Determined using Certified Reference Materials

Sample	Certified conc. (% ABV)	# of Replicates	# Instruments/ Analysts	Range (% ABV)	Avg. Amt. Measured (% ABV)	Avg. % Accuracy	Reproducibility % RSD
Low alcohol beer	0.51	10	2/2	0.48-0.51	0.50	98	2
Alcohol in water, 80 mg/dL	0.10	4	2/1	0.095-0.11	0.099	98	5
Alcohol in water, 200 mg/dL	0.25	3	2/2	0.24-0.26	0.25	99	3
Alcohol in water, 400 mg/dL	0.51	7	2/2	0.49-0.52	0.51	100	3

Analysis of Kombucha tea samples

A total of twenty different Kombucha tea samples from ten different producers (including one homemade sample) were tested using the optimized HS-SPME/GC-MS procedure. All were stored under refrigeration after purchase, and testing was performed on freshly opened bottles before the "best by" date indicated on the label. The results are summarized in **Figure 2**. All samples in this set were analyzed in replicates of 2 or 3. To determine method reproducibility over multiple days, a subset of eight samples was analyzed in triplicate on two separate days. The samples in this subset varied in alcohol content from 0.39 to 1.66% ABV. Measurement variability was

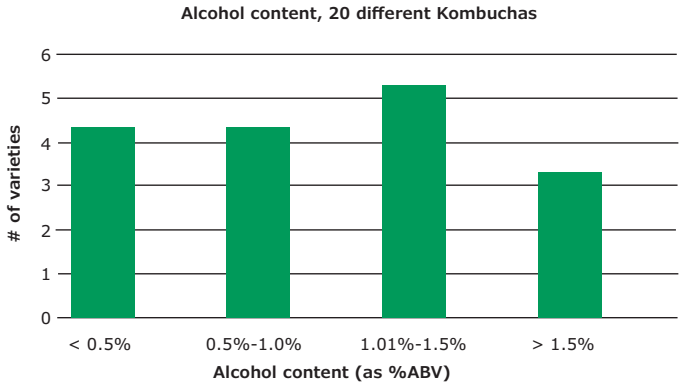

Figure 2. Alcohol (Ethanol) Content, as % ABV, Measured on 20 Kombucha Tea Samples using HS-SPME.

determined as the % RSD in the average value for the 6 replicates of each sample. For all samples, % RSD was < 3%, indicating good repeatability of the method over separate sample batches analyzed on separate days (with all other parameters such as instrument and analyst being the same).

Conclusions

An HS-SPME/GC-MS method was developed which can be used to accurately and precisely measure alcohol content in Kombucha tea samples. The optimized method allowed for accurate determination in the range of 0.1 to 2.0% ABV; however, the limit of quantitation indicates that accurate measurement is possible as low as 0.03% ABV. Method repeatability, as demonstrated with analysis of eight different Kombucha varieties, was demonstrated as <4% RSD for replicate measurements made over two days. The dilution approach used in the procedure minimizes matrix effects, thus making it possible to use this HS-SPME method for other low alcohol matrices such as non-alcoholic beer and wine. Applying the HS-SPME/GC-MS method to twenty different varieties of commercially available Kombucha tea samples from nine producers, it was found that most had alcohol levels above 0.5% ABV. This indicates that either the current methodology used for alcohol measurement of these products is not accurate, and/or fermentation is continuing after bottling, despite refrigeration, and resulting in elevation of alcohol level.

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Ethanol-d_6, >99.5% atom %D	**186414**
Sodium phosphate, monobasic, for HPLC, 99-101%	**52074**
Sodium phosphate, dibasic, for HPLC, ACS grade*	**SX0723**
Sodium chloride, EMSURE®	**106404**

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FOOD & BEVERAGE

Efficient Analysis of Kava Extract

New phytochemical certified reference material mixes for ginger and kava

Adam J. Kuszak; Stephen A. Wise - Office of Dietary Supplements, National Institutes of Health, U.S. Department of Health and Human Services, Bethesda, Maryland, USA

Chris Leija, Senior Scientist, Reference Materials & Workflows; Uma Sreenivasan, Head of Reference Materials & Workflows R&D; Matthias Nold, Product Manager Reference Materials; Analytix@merckgroup.com

Introduction

Plant-based products are widely used for herbal medicinal drugs, dietary supplements, or cosmetic applications. The complexity and the natural variations in the chemical composition of plants pose big challenges to quality control and authenticity confirmation of botanical products. To verify the plant species and detect potential adulteration with different plant parts, different plant species, or the addition of synthetic components, chromatographic fingerprint analysis of the most common chemical markers is an efficient method. We have a comprehensive portfolio of more than 1500 phytochemical neat standards of the most important marker compounds for a large variety of plants.

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Quantitative and qualitative analysis of botanical materials, usually requires testing of multiple key components. Analysis requires time-consuming preparation of multi-component solutions from the neat materials. The stability of the solutions must be established, possibly requiring frequent preparations. To offer an effective alternative, we supply a series of plant-specific Certified Reference Material (CRM) mixes.

These Supelco® branded products, produced at the Cerilliant® site in Round Rock Texas under ISO/IEC 17025 and ISO 17034 accreditation, offer a cost-efficient and precise tool for calibration and verification of multiple key components for a series of popular medicinal plants and dietary supplements. The CRMs are supplied with a comprehensive certificate of analysis, including traceable values and expanded uncertainties for all the analytes.

A new kava mix has recently been added to this portfolio (composition shown in table 1). This new product was developed in collaboration with the U.S. National Institutes of Health (NIH) Office of Dietary Supplements (ODS) along with other products in development (e.g. a CRM mix for gingerols and shaogols from ginger). Responding to the increased biomedical interest in kava's health effects and safety, as well as an expressed need to more rigorously characterize food and dietary supplement preparations[1], the ODS Analytical Methods and Reference Materials Program contracted with Cerilliant to develop a CRM calibration solution of major kava constituents to aid industry, academic, and regulatory scientists and analysts.

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Table 1. Composition of the new Kava CRM Mix **K-007** (1 mL) in Acetonitrile

Components	Concentration (µg/mL)
Yangonin	250
Desmethoxyyangonin	250
Dihydrokavain	250
D,L-Kavain	250
Methysticin	250
Dihydromethysticin	250
Flavokavain A	25
Flavokavain B	25
Flavokavain C	25

Background

The kava plant, also called kava kava (*Piper methysticum* G. Forst.), has its origins in the islands

of the South Pacific and is increasingly popular as an herbal medicine and dietary supplement in other parts of the world. Traditionally consumed as a ceremonial beverage prepared from the rhizomes, kava also has a long history of use in traditional medicines for its relaxing effects.[2] Kava contains numerous kavalactones and flavokawains (also referred to as flavokavains) which may have unique and/or synergistic bioactivities, and different kava cultivars can contain varying proportions of these constituents. Current biomedical research is investigating the safety and efficacy of kava constituents for treating anxiety disorders and their potential in preventing or modulating cancer progression.[3] The early 2000's saw a number of cases of hepatotoxicity associated with kava consumption, causing public health concerns and leading to import restrictions in several countries[4], though many regulatory controls have recently been eased. Some research suggests kava's potential for hepatotoxicity may be related to the types of solvents used in commercial extracts or due to herb-drug interactions with specific flavokawains. However, an understanding of the safety of kava's constituents continues to be investigated.

Sample Preparation and Analytical Method

Analysis of samples of ground kava root (noble cultivar) as well as capsules containing Kava Kava extract was performed. For the quantitative and qualitative evaluation, the samples were compared with the new Kava CRM Mix. **Figure 1** shows the chemical structure of the kavalactones and flavokavains included in the CRM Mix cat.no. **K-007**. Using the Kava CRM solution, a 5-point calibration curve was constructed spanning 6.25 – 250 µg/mL for the kavalactones and 0.625 – 25 µg/mL for the flavokavains.

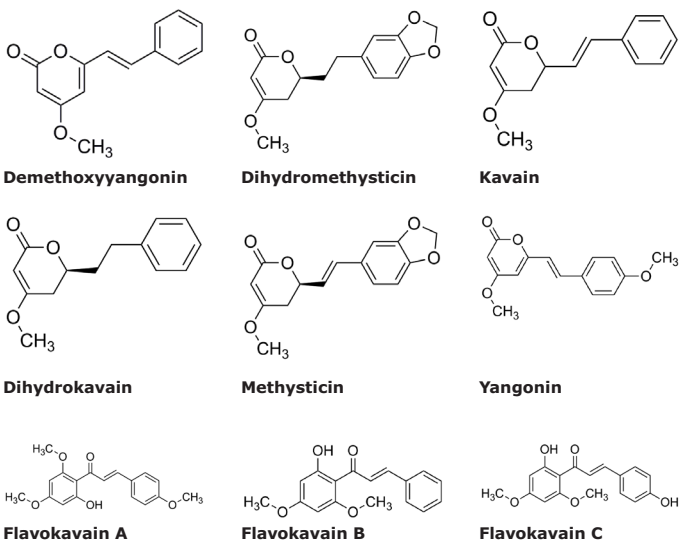

Figure 1. Chemical Structures of Kava Ingredients

For the analysis, 750 mg of kava root or kava capsules respectively was suspended in 50 mL extraction solvent consisting of a 70:30 mixture of methanol and water and sonicated for 60 min. After centrifugation at 4000 rpm for 5 minutes, the soluble fraction was passed through a 0.2 µm PTFE syringe filter and injected into the HPLC (conditions **Table 2**.).

Table 2. Chromatographic Conditions

column:	Ascentis® Express C18, 15 cm x 3.0 mm, 2.7 µm (**53816-U**)
mobile phases:	[A] 0.1% H_3PO_4; [B] isopropyl alcohol; [C] acetonitrile
gradient:	Time (min) 0 6 16 18 18.1 A 70 58 15 15 70 B 20 20 10 10 20 C 10 22 75 75 10
flow rate:	0.5 mL
pressure:	460 bar
column temperature:	30 °C
detection:	UV, 220 nm
injection volume:	1 µL
sample:	kava root or capsules, 750 mg in 50 mL 70:30 MeOH:Water

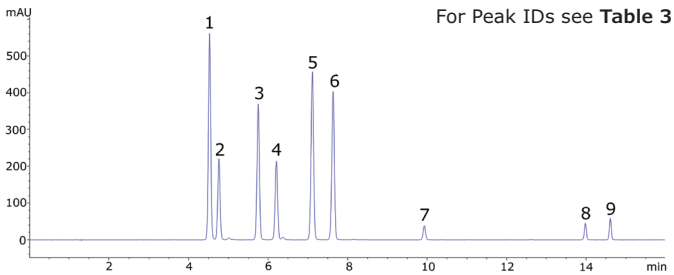

Figure 2. Chromatogram of the Kava CRM Mix Cat.No. **K-007**

Results

The Ascentis® Express C18 column and Kava CRM solution were used to successfully separate and quantitate kavalactones and flavokavains in two types of Kava products. Single component solutions of each analyte were prepared for peak identification and determination of elution order. The analysed kava root extract, as well as the kava capsules showed a typical distribution of kavalactones (**Figure 3** and **4**).

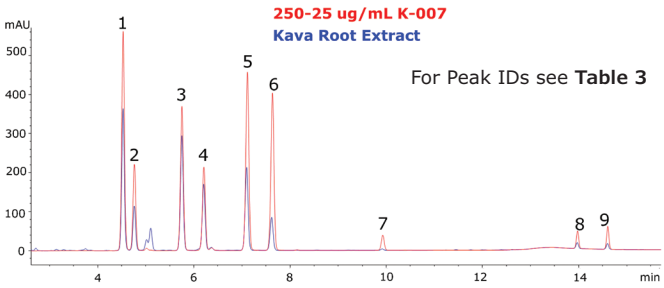

Figure 3. Chromatogram of the Kava CRM Mix Cat.No. **K-007**(red) and Kava root extract (blue)

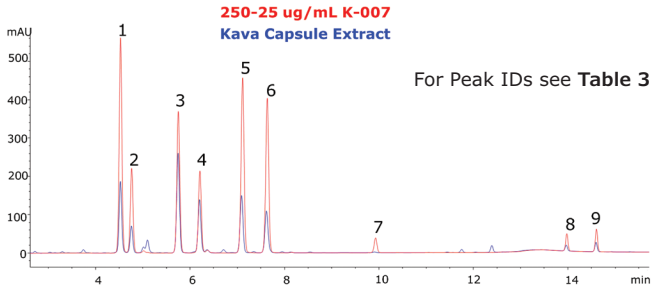

Figure 4. Chromatogram of the Kava CRM Mix Cat.No. **K-007**(red) and Kava capsule extract (blue)

The calibration curve demonstrated excellent linearity across the analyte ranges (R^2 > 0.999). Quantitation of each analyte revealed a low abundance of flavokavains relative to kavalactones in both samples. The total sum (% weight basis) of kavalactones and flavokavains in the root and capsule samples was found to be 5.68% and 17.90%, respectively (**Table 3**).

Table 3. Composition (% weight) of Kavalactones and Flavokavains in Kava Samples.

Peak #	Analyte	% Weight #	% Weight Capsules
1	Methysticin	1.03	2.18
2	Dihydromethysticin	0.82	2.09
3	Kavain	1.29	4.60
4	Dihydrokavain	1.31	4.40
5	Yangonin	0.75	2.14
6	Demethoxyyangonin	0.35	1.93
7	Flavokavain C	0.02	0.04
8	Flavokavain A	0.06	0.23
9	Flavokavain B	0.04	0.28
	Total	**5.68**	**17.90**

Conclusion

The results of this analysis demonstrate that the Kava CRM solution can easily be adopted for use in HPLC-UV analysis of kavalactones and flavokavains in kava products. The use of kava CRMs in a solution format provides the convenience and efficiency of material usage, consistency, accuracy, and metrological traceability. The new Kava CRM mix is a very efficient tool to analyse the chemical compositions of kava products to confirm their potency and authenticity.

References

1. Standard Method Performance Requirements (SMPRs®) 2018.005: Determination of Kavalactones and/or Flavokavains from Kava (Piper methysticum). Journal of AOAC INTERNATIONAL, Volume 101, Issue 4, 1 July 2018, Pages 1256–1260, https://doi.org/10.5740/jaoacint.SMPR2018.005
2. Singh, Y.N. and M. Blumenthal. 1997. Kava - An Overview. HerbalGram 39:3356.
3. PubMed ID: 30396607; PubMed ID: 29641222; PubMed ID: 31172600
4. HerbalGram. 2002; 55:26-32 American Botanical Council.

Featured Products

Description	Cat.No.
Ascentis® Express C18, 15 cm x 3.0 mm I.D., 2.7 µm	**53816-U**
Ascentis® Express C18, 2.7 µm Guard Cartridge	**53504-U**
Ascentis® Express Guard Cartridge Holder	**53500-U**
Kava Mix, Cerilliant® Certified Reference Material	**K-007**
Millex® Syringe Filters, Hydrophilic PTFE 0.20 µm	**SLLG033**
Solvents & Reagents	
Ultrapure water from Milli-Q® system IQ 7005 or bottled water	**Milli-Q or 101262**
Phosphoric Acid, for HPLC, LiChropur®, 85%	**49685**
Acetonitrile for UHPLC	**900667**
Methanol for UHPLC	**900688**
Isopropanol, HPLC Plus	**650447**

Related Products

Description	Cat.No.
Powdered kava extract, United States Pharmacopeia (USP) Reference Standard	**1355709**
DL-Kavain, primary reference standard	**05790585**
Methysticin, phyproof® Reference Substance	**PHL89250**
Dihydromethysticin, phyproof® Reference Substance	**PHL89186**
(±)-Kavain, phyproof® Reference Substance	**PHL89239**
Dihydrokavain, phyproof® Reference Substance	**PHL89185**
Yangonin, phyproof® Reference Substance	**PHL89293**
Desmethoxyyangonin, phyproof® Reference Substance	**PHL89184**
Flavokawain A, phyproof® Reference Substance	**PHL83762**
Flavokawain B, phyproof® Reference Substance	**PHL83763**
Flavokawain C, phyproof® Reference Substance	**PHL83854**

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Correlation of FcR Affinity Chromatography with Glycan Pattern and ADCC Activity of a Therapeutic Antibody

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Cory E. Muraco, Product Manager Liquid Chromatography Technologies, Analytix@merckgroup.com

Introduction

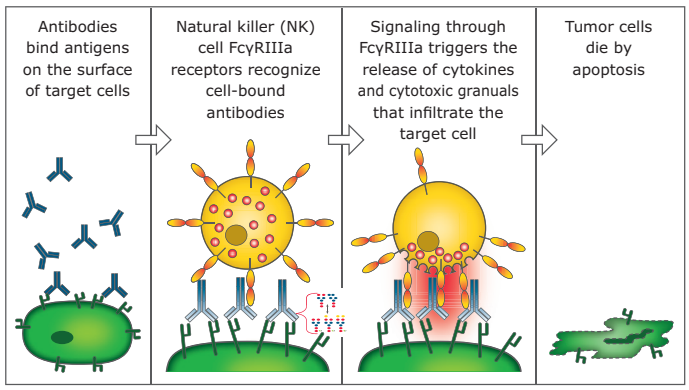
Antibody-dependent cell-mediated cytotoxicity (ADCC) is an important mechanism of action (MOA) of monoclonal antibodies used in cancer treatment. Selecting suitable cell lines and optimizing culture conditions towards the expression of antibody candidates with desired ADCC activity is an essential part of the R&D process. A fast and straightforward approach to easily access ADCC activity would facilitate screening of many clones or monitoring the effect of upstream process variations. Other stages of R&D and production could benefit from fast ADCC assessment as well: comparing biosimilar and originator, detecting lot-to-lot variations, monitoring product stability, to name but a few.

Fc Receptor and ADCC Activity

ADCC starts with the binding of the Fab region of an antibody to a target cell, *e.g.,* a cancer cell. Binding of the Fc domain of that antibody to Fcγ receptors on the outer membrane of natural killer (NK) cells triggers degranulation into a lytic synapse and finally the apoptosis of the cancer cell (**Figure 1**). The glycan microheterogeneity of the Fc domain, on the galactose and core-fucose levels, influences the binding of the Fc domain to Fcγ receptors.[1]

Current ADCC activity tests are either cell-based bioassays or surface plasmon resonance (SPR) measurements using immobilized Fcγ receptors. A new approach combines the specificity of the FcγIIIa receptor (FcγRIIIa) with the easy handling of an HPLC method.

For Fc receptor affinity chromatography, a recombinant FcγIIIa receptor ligand is immobilized on a stationary phase. Glycoforms of an antibody sample can be partly separated based on the strength of their binding to the FcR ligand. Resulting peaks can be assigned to low, medium, and high ADCC activity (**Figure 2**).



Original image by Satchmo2000, distributed under a CC-BY 3.0 license.

Figure 1. Antibody-dependent cell-mediated cytotoxicity

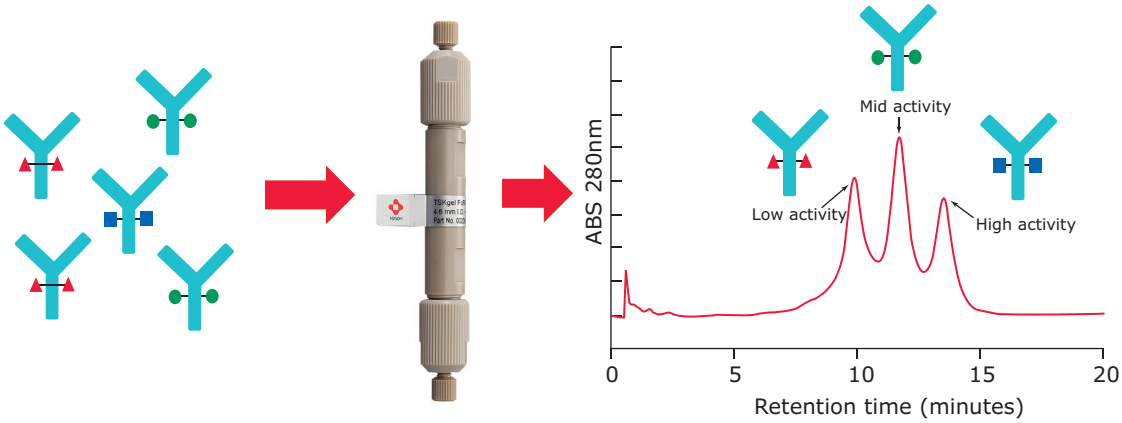


Figure 2. FcγRIIIa affinity chromatography

Taking the well-known therapeutic antibody rituximab as an example, this application note demonstrates that the pattern of FcγRIIIa affinity chromatography shows a good correlation with the results obtained by an ADCC reporter assay. Fractions collected from HPLC peaks with different receptor affinity also show different glycosylation patterns at the Fc domain.

Rituximab (**Figure 3**) is a recombinant chimeric human/mouse monoclonal IgG_1 antibody approved in 1997 and used to treat certain autoimmune diseases and types of cancer. Besides other effects of rituximab, its Fc portion mediates antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).[2] N-glycans bound to the Fc domain of rituximab contain mainly G0F and G1F structures.

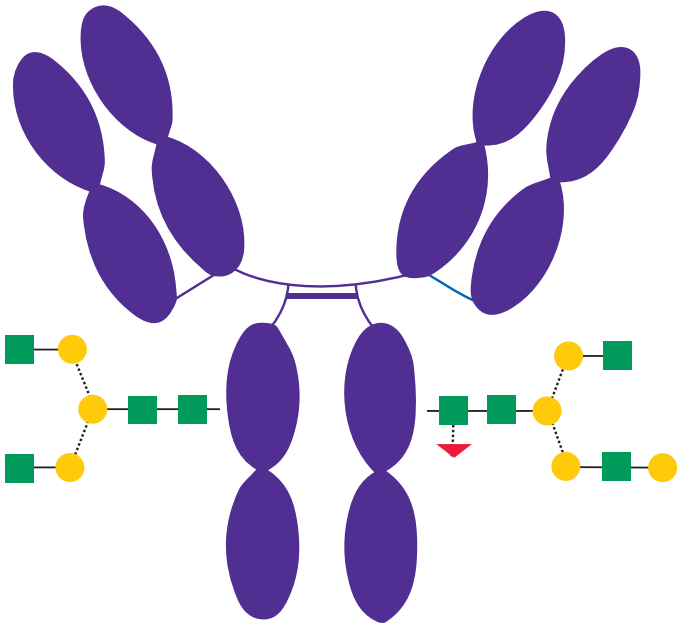
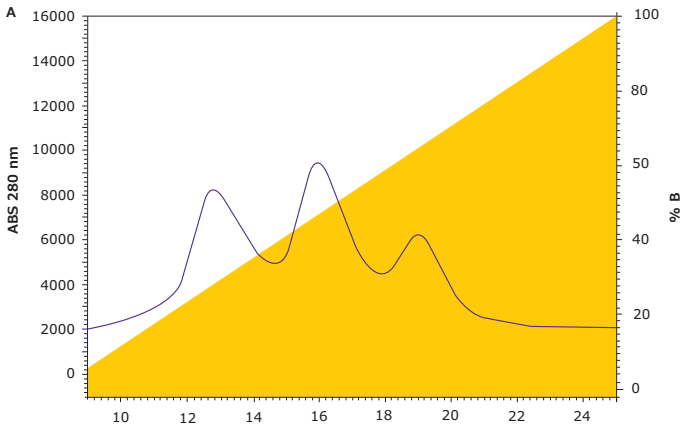


Figure 3. Schematic Diagram of Rituximab

FcR Affinity Chromatography of Rituximab

In FcγRIIIa affinity chromatography, purified antibody or cell culture supernatant is injected under conditions that promote binding of monoclonal antibodies (mAbs) to the FcγRIIIa ligand. Elution of bound mAb variants is performed by lowering the pH of the mobile phase in order to disrupt the target/ligand interactions. The higher the affinity of a mAb variant to the receptor, the higher the retention time of the respective peak.

FcR affinity chromatography analysis of rituximab on the new TSKgel® FcR-IIIA-NPR column results in three peaks representing variants with low, medium, and high FcγRIIIa affinity (**Figure 4**). Subsequent characterization of the three peaks was carried out using a semi-preparative prototype FcR-IIIA column to collect fractions. For each peak, ADCC activity was analyzed with a standard ADCC reporter bioassay kit (Promega). Glycan analysis was also carried out on each fraction using HILIC-UHPLC.



Chromatographic Conditions[3]	
column:	TSKgel® FcR-IIIA-NPR, 7.5 cm x 4.6 mm I.D., 5 µm, PEEK (**823513**)
mobile phase:	[A] 50 mM Citrate, pH 6.5 ; [B] 50mM Citrate, pH 4.5
flow rate:	1 mL/min
column temp.:	25 °C
detector:	UV, 260 nm
injection:	30 µL
sample:	Rituximab, 1 µg/µL, Rituximab kindly provided by Rentschler Biopharma

Figure 4. FcγR Affinity Analysis of Rituximab on TSKgel®I FCR-IIIA-NPR

ADCC Bioassay of Rituximab and FcR Affinity Fractions

The Fc effector reporter bioassay uses the FcγR and Nuclear Factor of Activated T-cells (NFAT)-mediated activation of luciferase activity in effector cells to determine ADCC efficacy and potency of antibodies. **Figure 5** shows the ADCC reporter bioassay response to rituximab and to the three fractions collected from FcR affinity chromatography (low, medium, high FcγR affinity). Rituximab is shown as the grey points; fraction 1, representing low FcγR affinity, is shown in blue; fraction 2, representing medium FcγR affinity, is shown in red; and fraction 3, representing high FcγR affinity, is shown in green.[3]

Table 1 shows the half maximal effective concentration (EC50) values obtained by the reporter bioassay test. The lower the EC50 value, the higher the ADCC potency. As expected, peak 3 (high FcRγ affinity, green) shows the highest ADCC potency and efficacy in the bioassay. Peak 2 shows the intermediate and peak 1 shows the lowest ADCC efficacy and potency. ADCC efficacy and potency of the original rituximab lies between the low and medium affinity fractions.

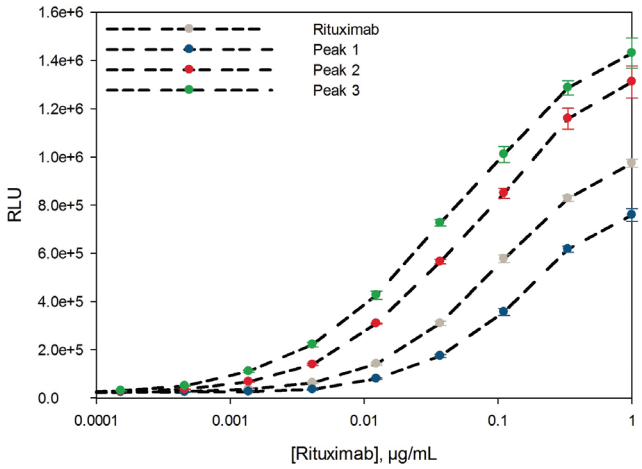


Figure 5. ADCC Reporter Bioassay Response to Rituximab

Table 1. The Dose-Response Curve of ADCC Data was fitted with a 4-Parameter Model using a Sigma Plot

EC50 values obtained by the reporter bioassay test	
Antibody	EC50 (µg/mL)
Rituximab	0.098
Peak 1	0.153
Peak 2	0.072
Peak 3	0.049

Glycan Analysis of FcR Affinity Fractions

For each peak in **Figure 4**, cleaved and 2-aminobenzamide-labeled (2AB-labeled) N-glycans were characterized by HILIC-UHPLC. **Figure 6** shows the glycan pattern of the FcR affinity fractions compared to a glycan library. The antibody glycoforms collected in peak 3 (highest affinity) show mainly galactose containing N-glycans (G1F and G2F). Peak 2 glycoforms contain more G0F glycans than peak 3 and glycoforms collected in peak 1 (lowest affinity) show predominantly fucosylated glycans without galactose units (G0F).

Conclusions

The ADCC activity bioassay results show that high retention on the TSKgel® FcR-IIIA-NPR column corresponds to a high ADCC activity. The HILIC-UHPLC glycosylation pattern analysis of the FcR affinity fractions also matches the common understanding that terminal galactose units of Fc-glycans typically enhance affinity to FcγRIIIa and ADCC activity while core fucose

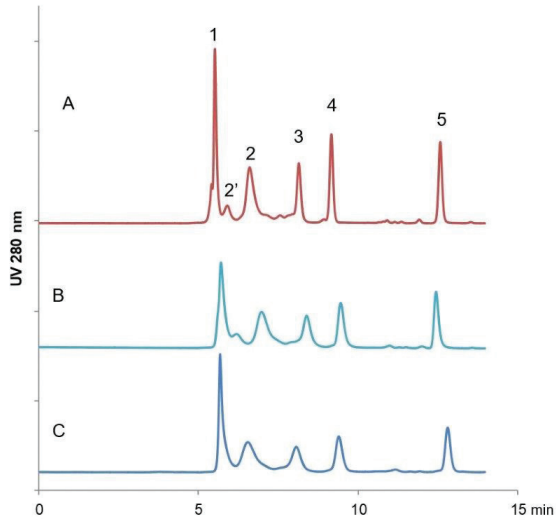


Figure 6. HILIC Analysis of Oligosaccharides of the three FcR Affinity Fractions (Peak 1 blue, Peak 2 red, Peak 3 green) compared with a 2-AB Labeled Biantennary Glycan Library (grey).[4]

units decrease ADCC activity of antibodies. These results confirm that FcγRIIIa affinity chromatography allows fast assessment of biologic activity and glycoform pattern of antibodies.

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2. G.J. Weiner; Semin Hematol. 2010; 47 (2): 115-123; doi:10.1053/j. seminhematol.2010.01.011
3. Master Thesis Leila Ghaleh, TU Darmstadt
4. M. Kioshi et. al. Nature Scientific Reports | (2018) 8:3955 | DOI:10.1038/s41598-018-22199-8

Featured Products

Description	Cat. No.
TSKgel® FcR-IIIA-NPR HPLC Column, 7.5 cm x 4.6 mm I.D., 5 µm, PEEK	**823513**
Sodium citrate tribasic dihydrate, > 98%	**C7254**
PNGase Fast, recombinant, expressed in E. coli	**EMS0001**
GlycoProfile™ 2-AB Labeling Kit	**PP0520**

Rituximab was kindly provided by Rentschler Biopharma.

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PHARMA & BIOPHARMA

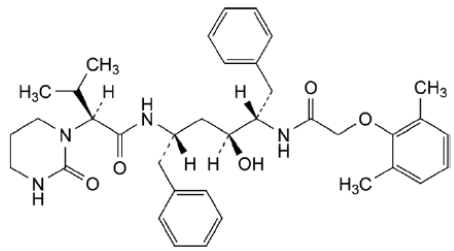
Lopinavir Assay Following European Pharmacopoeia 10 Guidelines Using an Ascentis® Express C18 Column and UV Detection

Anita Piper, Scientist Instrumental Analytics R&D, Analytix@merckgroup.com

Introduction

This paper illustrates the possible setting-up of an assay method for Lopinavir testing following the current European Pharmacopoeia guidelines (10.2). The monograph assay method calls for a column with L = 0.25 m, I.D. = 4.6 mm end-capped octadecylsilyl silica gel for chromatography with 4 µm particle size. No particular HPLC column is referenced in the EP knowledge database for the assay method, and the method is of isocratic nature.

This provides an opportunity to replace the monograph column geometry/particle size with a shorter and faster alternative column (up to 70% reduction in length) packed with smaller particles (up to 50% reduction). This can save valuable time and ensure improved separation efficiency, which typically translates into better method performance and sensitivity. In this study, the limit of detection (LOD) is better than 1 ppm using HPLC-UV detection.



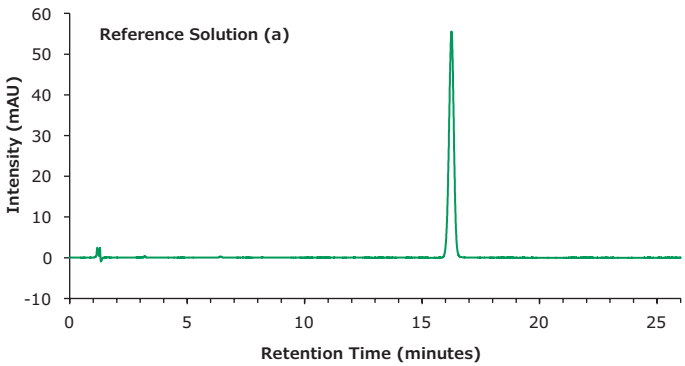
Lopinavir

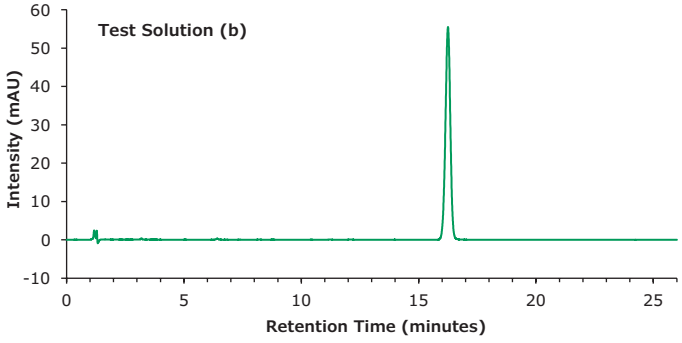
Experimental

Conditions	
column:	Ascentis® Express C18, 15 cm x 4.6 mm, 2.7 um (**53829-U**)
mobile phase:	acetonitrile/phosphate buffer solution 45/55 (v/v)
buffer preparation:	dissolve 0.9 g of dipotassium hydrogen phosphate and 2.7 g of potassium dihydrogen phosphate in 900 mL of water and mix well. Adjust to pH 6.0 with phosphoric acid, dilute to 1000 mL with water and filter.
flow rate:	1.0 mL/min
pressure:	2219 psi (153 bar)
column temp.:	50 ºC
detector:	UV, 215 nm UV = 215 nm (micro flow cell; 1.4 µL/7 mm)
injection:	12 µL
samples:	
solvent mixture:	acetonitrile/water 50/50 (v/v)
test solution (a):	dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 100 mL with the solvent mixture.
test solution (b):	dilute 5.0 mL of the test solution (a) to 100 mL with the solvent mixture.
reference solution (a):	dissolve 50.0 mg of Lopinavir CRS in the solvent mixture and dilute to 100 mL with the solvent mixture. Dilute 5 mL of this solution to 100 mL with the solvent mixture.

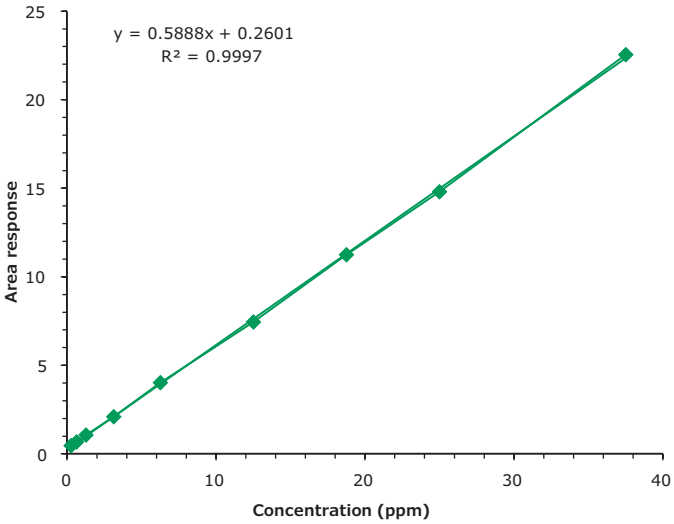
Concentration

No.	Compound	Retention Time (min)	Tailing Factor
1	t_0 void volume	1.1	
2	Lopinavir CRS	16.2	0.97





Chromatographic Data



1. Specificity: Inject Reference Solution (a) and Determine the Retention Time and the Content of Desired Analyte				
		Retention Time (min)	Area (%)	Tailing Factor
1	Lopinavir CRS	16.3	96.3	0.95

2. Standard Repeatability (25 ppm)	
Measurement	**Area (mAU*min)**
STD 1	14.79
STD 2	14.82
STD 3	14.86
STD 4	14.90
STD 5	14.93
Mean	**14.86**
Standard Deviation	**0.06**
(%) RSD	**0.4**

3. LOD & LOQ	
Conc. (ppm)	**Mean Area, n=5 (mAU*min)**
0.25	0.46
0.63	0.67
1.25	1.06
3.13	2.10
6.25	4.02
12.50	7.44
18.75	11.24
25.00	14.86
37.50	22.54
STEYEX	**0.1348**
Slope	**0.5888**
LOD (ppm)	**0.8**
LOQ (ppm)	**2.3**

Conclusion

In this study in reference to European Pharmacopeia 10 Guidelines, a shorter and faster Fused-Core® (Superficially Porous Particle, SPP) column was evaluated for an assay method for Lopinavir, achieving a limit of detection (LOD) of better than 1 ppm using HPLC-UV detection.

Featured Products

Description	Cat. No.
Ascentis® Express C18, 15 cm x 4.6 mm, 2.7 µm	**53829-U**
Acetonitrile gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur	**1.00030**
Water for chromatography (LC-MS grade) LiChrosolv® or tap fresh from an appropriate Milli-Q® water purification system	**1.15333**
Millex® syringe filter Durapore® (PVDF), non-sterile, 0.45 µm pore size, 13 mm diameter	**SLHVX13**
Ortho-phosphoric acid, EMSURE® ACS,ISO, Reag. Ph Eur	**1.00573**
Potassium di-hydrogen phosphate, EMSURE® ISO	**1.04873**
Di-Potassium hydrogen phosphate, anhydrous for analysis EMSURE®	**1.05104**
Reference Materials	
Lopinavir, European Pharmacopoeia (EP) Reference Standard	**Y0001498**
Lopinavir for system suitability, European Pharmacopoeia (EP) Reference Standard	**Y0001505**
Lopinavir for peak identification, European Pharmacopoeia (EP) Reference Standard	**Y0001506**

Related Products

Description	Cat. No.
Lopinavir, United States Pharmacopeia (USP) Reference Standard, 350 mg	**1370101**
Di-Potassium hydrogen phosphate, anhydrous for HPLC, LiChropur®	**5.43839**

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… that thin-layer chromatography allows the quantitative determination of Dexamethasone and Dexamethasone sodium hydrogen sulfate in blood and in pharmaceutical preparations. (M. Amin, Fresenius Z Anal Chem (1988) 329: 778- 780).Find our TLC products at

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New Positive Quality Control Reference Material EURM-019 for Testing of Coronavirus SARS-CoV-2

JRC issues a synthetic single stranded RNA (ssRNA) for real-time PCR analysis

Matthias Nold, Product Manager Reference Materials, Analytix@merckgroup.com

The global outbreak of the respiratory disease COVID-19, caused by the coronavirus SARS-CoV-2, also referred to as 2019-nCoV has had a massive impact on people's lives in all parts of the world. The WHO declared the outbreak a Public Health Emergency of international concern.

To ensure efficient and reliable analysis of the virus, the Joint Research Centre (JRC), a Directorate-General of the European Commission, has issued a single stranded RNA (ssRNA) reference material intended for use as a positive quality control sample in testing for the presence of the coronavirus SARS-CoV-2.

It can be used to verify the correctness of the transcription and amplification steps for the SARS-CoV-2 real-time RT-PCR assays.

EURM-019 is a solution containing a stabilized *in vitro* transcripted (IVT) synthetic single-stranded RNA (ssRNA) in buffer. It does not contain any viable virus.

This universal synthetic ssRNA of 880 nt contains the target regions that can be amplified by the following RT-PCR assays:

a. N1, N2, and N3 gene, developed by the Centers for Disease Control and Prevention (USA)
b. E gene, and the RdRP gene developed by the Charité (DE)
c. N gene, developed by the Japanese National Institute of Infectious Diseases (JP)
d. N gene, developed by the Ministry of Heath of Thailand (TH)
e. S gene, developed by the Joint Research Centre of the European Commission (EU).

European Commission

The JRC's mission is to promote a common and reliable European measurement system in support of EU policies. This involves the development and manufacturing of certified reference materials (pure and matrix materials) for various applications including environmental analysis, food analysis, clinical chemistry, physical properties or industrial applications.

We are an authorized distributor of JRC reference materials.

Featured Product

Description	Cat. No.
Single stranded RNA (ssRNA) fragments of SARS-CoV-2	**EURM019**

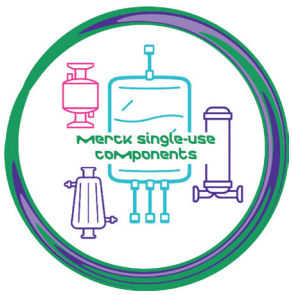
Please find more information at **SigmaAldrich.com/EURM019**

For our complete offering of reference materials, visit us at **SigmaAldrich.com/standards**

Extractables Studies of Single-Use Equipment

Immediate identification and quantification of unknown extractables with GC/MS due to a certified reference material mix for Extractables and Leachables

Maria Viehoff, Project Manager for Extractables and Leachables Studies
Sebastian Siefert, Scientist Gas Chromatography-Mass Spectrometry
Saskia Haehn, Manager Extractables and Leachables Laboratory
Matthias Nold, Product Manager Reference Materials, Analytix@merckgroup.com



The manufacture of active pharmaceutical ingredients, drug substances, or drug products generally involves several production steps, during which the process stream may inadvertently pass along components of the production unit consisting of polymeric materials. Caused by this direct contact, an accumulation of so-called leachables in the process stream could occur, potentially compromising the safety and quality of the desired product.

One such example of components made up of polymeric materials within the production process are single-use systems (SUS). Manufacturers of single-use equipment are interested in studies on their materials to provide an overview of possible leachables in the end users' products. Extractables data are generated in a laboratory under a variety of possible production conditions, thereby providing valuable information on what could potentially occur during the process.

According to BPOG (BioPhorum Operations Group) guidelines[1] and USP 665 guidelines for polymeric components and systems[2] (draft version), investigations regarding extractables should be performed using various solvents and incubation times and analyzed using a variety of analytical methods applied to the extracts. One of these analytical techniques is gas chromatography-mass spectrometry (GC-MS), which is used for the determination of semi-volatile extractables such as additives, impurities, polymer components, degradation products, or extraction solvent-material interaction products. If the exact composition of the polymeric material used to make the single-use equipment is unknown, a non-targeted analysis is crucial. To accelerate the identification, we have launched a certified reference material (CRM) mixture of the most common extractables detected via GC/MS (**Figure 1**) as well as neat reference materials of the components. Using these materials, not only can these 14 components be identified, but also their quantification with traceability to NIST SRM is possible. In the following, an application is described for the Extractables and Leachables Screening Standard for GC mix, for the identification and quantification of the main extractables present within an extractable study of a filter.

Leachables: Chemical compounds that migrate into a drug formulation from any product contact material (e.g., single-use systems) as a result of direct contact under typical process or storage conditions; Leachables may affect the toxicity or efficiency of the drug product

Extractables: Chemical compounds that are extracted from any product contact material usually under extreme conditions (harsh solvents, exaggerated time and temperature); an Extractables profile represents a worst-case Leachables profile

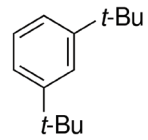
Single-use Systems (SUS): Usually polymeric, disposable equipment for bioprocessing used for the manufacturing of pharmaceuticals

Advantages: Flexibility, no need for cleaning validation, low investment, no cross contamination

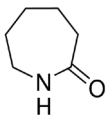
Examples of SUS: Bioreactors, disposable filters or tubing

GC Method for Extractables Testing

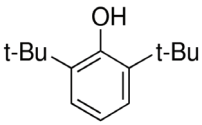
The applied instrument parameters for an extractable study on single-use equipment are summarized in **Table 1**. According to the BPOG protocol[1], the separation was performed on a Supelco® SLB®-5ms Capillary Column. A representative sample taken after seven days of extraction at 40 °C under orbital rotation with WFI (water for injection) was prepared and further processed by liquid-liquid extraction (LLE) using dichloromethane as extraction solvent and p-terphenyl-d_{14} as LLE extraction standard. The sample was fortified with phenanthrene-d_{10} as internal standard prior to analysis. The sample and the standard mix ran in one sequence.



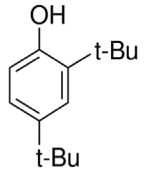
1. **1,3-Di-*tert*-butylbenzene**
Cat.No. 96659



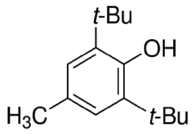
2. **ε-Caprolactam**
Cat.No. 01483



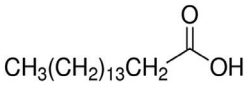
3. **2,6-Di-*tert*-butylphenol**
Cat.No. 96852



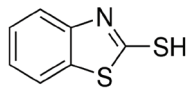
4. **2,4-Di-*tert*-butylphenol**
Cat.No. 00437



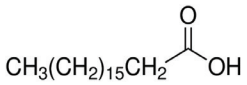
5. **3,5-Di-tert-4-butyl-hydroxytoluene (BHT)**
Cat.No. 47168



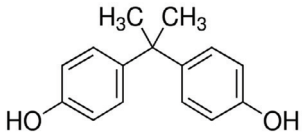
6. **Palmitic acid**
Cat.No. 76119



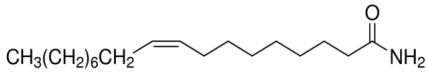
7. **2-Mercaptobenzothiazol**
Cat.No. 96051



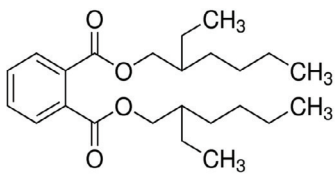
8. **Stearic acid**
Cat.No. 85679



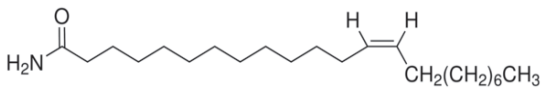
9. **Bisphenol A**
Cat.No. 42088



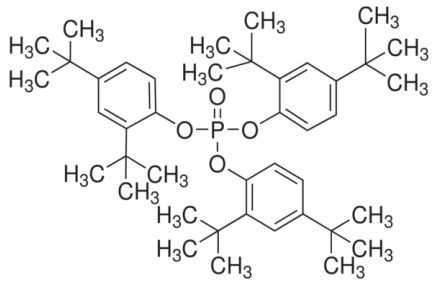
10. **Oleamide**
Cat.No. 96709



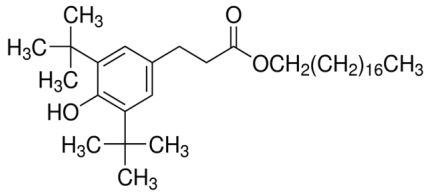
11. **Bis(2-ethylhexyl) phthalate**
Cat.No. 67261



12. **cis-13-Docosenoamide**
Cat.No. 01374



13. **Tris(2,4-di-tert-butylphenyl)phosphate**
Cat.No. 96839



14. **Octadecyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate**
Cat.No. 00318

Figure 1. Components of the Extractables and Leachables Screening Standard for GC Cat. No. **01829**; in Elution Order of Below Method. Concentration is 50 mg/L in *tert-Butyl* methyl ether for each component. For all the analytes, individual neat reference materials are also available under the catalog numbers listed.

Table 1. Experimental Conditions

instrument:	Agilent® HP7890, QToF 7200
column:	SLB®-5ms, 30 m x 0.25 mm, df=0.25 µm (**28471-U**)
oven:	50 °C (3 min), 10 °C/min to 320 °C (15 min)
inj. temp.:	280 °C
carrier Gas:	helium, 1.2 mL/min
detector:	MSD, full scan, m/z 25-800 Transfer line 250 °C, EI, Ion Source 230 °C, Electron Energy 70 eV
injection:	1 µL, pulsed splitless 350 kPa, 0.5 min, then 1:25
liner:	4 mm I.D. Inlet liner, split, focus, tapered, glass wool, deactivated
sample:	1. Extractables and Leachables Screening Standard for GC (**01829**), 50 mg/L in *tert*-butyl methyl ether 2. Product of a 1 day filter extraction with water for injection (WFI)

Results & Discussion

The chromatogram of the Extractables and Leachables Screening Standard for GC is shown in **Figure 2**. All 14 reference compounds were detected and almost completely separated. Due to mass spectrometric detection, coeluting components could be identified by their extracted ion chromatogram. By matching retention time and m/z ratio, ε-caprolactam was identified as the main extractable during the extraction of the single-use filter (**Figure 3**). A quantitative analysis against the caprolactam peak within the Extractables and Leachables Screening Standard for GC could be performed based on the extracted ion chromatograms.

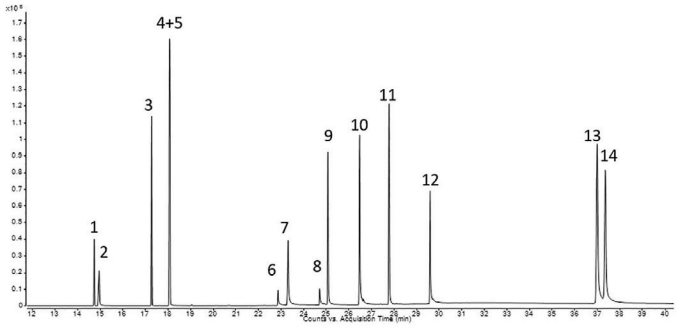


Figure 2. Extractables and Leachables Screening Standard for GC, 50 mg/L in *tert-Butyl* methyl ether (Peak IDs - **Table 2**).

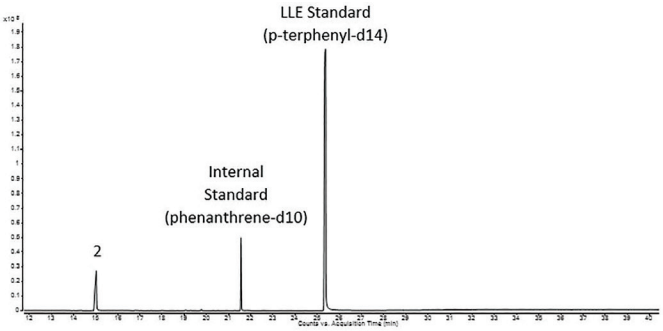


Figure 3. Representative Sample of a Single-use Equipment Extraction (Peak IDs - **Table 2**).

Conclusion

The example shown demonstrates the applicability and value of the Extractables and Leachables Screening Standard for GC for the reliable identification and quantification of the most common extractables resulting from single-use equipment.

Table 2. Peak Identifications

Peak#	Name	CAS#
1	1,3-Di-*tert*-butylbenzene	**1014-60-4**
2	ε-Caprolactam	**105-60-2**
3	2,6-Di-*tert*-butylphenol	**128-39-2**
4	2,4-Di-*tert*-butylphenol	**96-76-4**
5	3,5-Di-*tert*-4-butylhydroxytoluene (BHT)	**128-37-0**
6	Palmitic acid	**57-10-3**
7	2-Mercapto-benzothiazol	**149-30-4**
8	Stearic acid	**57-11-4**
9	Bisphenol A	**80-05-7**
10	Oleamide	**301-02-0**
11	Bis(2-ethylhexyl) phthalate	**117-81-7**
12	*cis*-13-Docosenoamide	**112-84-5**
13	Tris(2,4-di-*tert*-butylphenyl)phosphate	**95906-11-9**
14	Octadecyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) propionate	**2082-79-3**

References

1. Ding et al. Standardized Extractables Testing Protocol for Single-Use Systems in Biomanufacturing, Pharmaceutical Engineering, (2014).
2. <665> Plastic Materials, Components and Systems Used in the Manufacturing of Pharmaceutical Drug Products and Biopharmaceutical Drug Substances and Products, third draft, published on March 1, 2019 by the USP.

Featured Products

Description	Cat. No.
Extractables and Leachables Screening Standard for GC, 1 mL or 5 mL	**01829**
SLB®-5ms, 30 m x 0.25 mm, df = 0.25 µm	**28471-U**
Dichloromethane, for gas chromatography ECD and FID SupraSolv®	**1.06054**
p-Terphenyl-d_{14}, analytical standard, 100mg	**442734**
Phenanthrene-d_{10}, analytical standard, 100mg	**442753**

Related Products

Description	Cat. No.
Internal Standard Solutions	
p-Terphenyl-d_{14} solution, certified reference material, 2000 µg/mL in dichloromethane	**48418**
Phenanthrene-d_{10} solution, certified reference material, 2000 µg/mL in methanol	**48094**
Single Component Reference Materials	
1,3-Di-*tert*-butylbenzene, reference material, 100 mg	**96659**
2,4-Di-*tert*-butylphenol, reference material, 100 mg	**00437**
2,6-Di-*tert*-butylphenol, reference material, 100 mg	**96852**
2-Mercaptobenzothiazol, reference material, 100 mg	**96051**
3,5-Di-*tert*-4-butyl-hydroxytoluene (BHT), reference material, 500 mg	**47168**
Bis(2-ethylhexyl) phthalate, certified reference material, TraceCERT®, 100 mg	**67261**
Bisphenol A, certified reference material, TraceCERT®, 100 mg	**42088**
cis-13-Docosenoamide, reference material, 100 mg	**01374**
Octadecyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) propionate, reference material, 100 mg	**00318**
Oleamide, reference material, 100 mg	**96709**
Palmitic acid, analytical standard, 5 g	**76119**
Stearic acid, analytical standard, 500 mg or 5 g	**85679**
Tris(2,4-di-*tert*-butylphenyl)phosphate, reference material, 100 mg	**96839**
ε-Caprolactam, reference material, 100 mg	**01483**

To see our complete portfolio of reference materials for extractable and leachable testing, visit us at **SigmaAldrich.com/Extractablesandleachables**

Volumetric Solutions Analyzed by an ISO/IEC 17025 Accredited Laboratory

The Highest Standard for Realibility Ensuring Accurate and Reliable Titration Results

Bettina Straub-Jubb, Product Manager Titration, Analytix@merckgroup.com

Titripur Volumetric solutions analyzed by our DIN EN ISO/IEC 17025 accredited calibration laboratory

The Quality Control Laboratory at Merck KGaA, Darmstadt, Germany is now accredited by DAkkS (Deutsche Akkreditierungsstelle - German Accreditation Body) as calibration laboratory D-K-15185-01-00 for the “amount-of-substance concentrations in volumetric solutions” according to DIN EN ISO/IEC 17025 (**Figure 1**). This accreditation as a calibration laboratory is one of the highest standards for laboratories.

The Titripur® volumetric solutions in conjunction with Certipur® volumetric standard CRMs (Certified Reference Materials) ensure consistently good and reliable titration results. Laboratories working in regulated environments or having their own ISO/IEC 17025 accreditation are better prepared for their audits. The ISO/IEC 17025 is an internationally recognized accreditation for laboratories made possible through the ILAC (International Laboratory Accreditation Organization) and is accepted worldwide. Every country has its own accreditation bodies who are members of the ILAC.

What is the purpose of our accreditation?

As early as 1994, Merck KGaA Darmstadt, Germany received its first ISO/IEC 17025 accreditation, for the QC laboratory measuring pH values for buffer solutions. Over the past 25 years, the company has extended its scopes of accreditation for analytical products, procedures, and laboratories to better support customers in their daily analytical processes. Therefore, we are accredited as a reference material producer according to ISO 17034 for our CRMs and now additionally, our Titripur® Volumetric Solutions are analyzed by our ISO/IEC 17025 accredited QC laboratory.

ILAC is the international organization for accreditation bodies operating in accordance with ISO/IEC 17011 and involved in the accreditation of conformity assessment bodies including calibration laboratories (according ISO/IEC 17025) and testing laboratories (according ISO/IEC 17025).

DAkkS
Deutsche Akkreditierungsstelle

Deutsche Akkreditierungsstelle GmbH

Entrusted according to Section 8 subsection 1 AkkStelleG in connection with Section 1 subsection 1 AkkStelleGBV
Signatory to the Multilateral Agreements of EA, ILAC and IAF for Mutual Recognition

Accreditation

The Deutsche Akkreditierungsstelle GmbH attests that the calibration laboratory

Merck KGaA
Kalibrierlaboratorium für chemische Messgrößen
Frankfurter Straße 250, 64293 Darmstadt

is competent under the terms of DIN EN ISO/IEC 17025:2018 to carry out calibrations in the following fields:

Chemical and medical quantities
Chemical analysis and reference materials
- **pH value**
- **Electrolytic conductivity**
- **Mass fraction of elements in standard solutions**
- **Amount of substance concentration of elements in standard solutions**
- **Mass fraction of titrimetric standards**
- **Mass fraction in water and titrimetric standards**
- **Amount-of-substance concentration in volumetric solutions**

The accreditation certificate shall only apply in connection with the notice of accreditation of 18.11.2019 with the accreditation number D-K-15185-01. It comprises the cover sheet, the reverse side of the cover sheet and the following annex with a total of 3 pages.

Registration number of the certificate: **D-K-15185-01-00**

Braunschweig, 18.11.2019 — Dr. Heike Manke, Head of Division — Translation issued: 18.11.2019 — Head of Division

The certificate together with its annex reflects the status at the time of the date of issue. The current status of the scope of accreditation can be found in the database of accredited bodies of Deutsche Akkreditierungsstelle GmbH.
https://www.dakks.de/en/content/accredited-bodies-dakks

This document is a translation. The definitive version is the original German accreditation certificate.
See notes overleaf.

Figure 1. DAkkS certificate for the QC calibration lab at Merck KGaA Darmstadt, Germany. **SigmaAldrich.com/ISO17025**

Certificate of Analysis

1.09141.1000 Sodium hydroxide solution c(NaOH) = 0.1 mol/l (0.1 N) Titripur® Reag. Ph Eur,Reag. USP

Batch HC14487741

	Spec. Values		Batch Values	
Form	liquid		liquid	
Amount-of-substance concentration	0.0995 - 0.1005	mol/l	0.0999	mol/l
Measurement uncertainty	+/- 0.0003	mol/l	+/- 0.0003	mol/l
Traceability	NIST SRM		84L	

Accreditation: This volumetric solution is analyzed by our calibration laboratory D-K-15185-01-00 which is accredited according to DIN EN ISO/IEC 17025 for analysis of amount-of-substance concentrations in volumetric solutions by DAkkS (Deutsche Akkreditierungsstelle - German National Accreditation Body). The accreditation certificate can be found at www.sigmaaldrich.com/ISO17025.

The concentration is determined by volumetric titration and refers to 20°C.
The amount-of-substance concentration of this volumetric solution is traceable to a primary standard reference material (SRM) from the National Institute of Standards and Technology, Gaithersburg, USA (NIST SRM 84 potassium hydrogen phthalate) by means of volumetric standard potassium hydrogen phthalate (article number 1.02400), certified reference material according to ISO 17034, analyzed by our accredited calibration laboratory of Merck KGaA, Darmstadt, Germany according to DIN EN ISO/IEC 17025. The uncertainty is expressed as expanded measurement uncertainty with a coverage factor k=2 covering a confidence level of 95%.

Note: The titer is a correction factor to correct for variations of the volumetric solution, the titration equipment, the temperature and other laboratory conditions. For correct titration results it is recommended to determine a titer with the laboratory specific equipment and under laboratory specific conditions directly after opening a new bottle and at regular time intervals.

Date of release (DD.MM.YYYY) 22.01.2021
Minimum shelf life (DD.MM.YYYY) 31.01.2024

Ayfer Yildirim
Responsible laboratory manager quality control

This document has been produced electronically and is valid without a signature.

Figure 2. Example Certificate of Analysis for Titripur® Volumetric Solutions analysed by our ISO/IEC 17025 accredited laboratory.

Traceability & Uncertainty

With the DIN EN ISO/IEC 17025 accrediation of laboratories as calibration lab, the traceability of the analyzed value of a product to a SI unit is completely ensured. For the Titripur® volumetric solution, we trace back to primary NIST (National Institute of Standards and Technology) standards. All sources of uncertainty, such as repeatability or reproducibility, are documented and included in the expanded measurement uncertainty which can be found in the Certificate of Analysis for each product (**Figure 2**) as well as the specification range. (See the annex at **SigmaAldrich.com/ISO17025**.)

The benefits of using volumetric solutions analyzed by our ISO/IEC 17025 accredited lab and volumetric standards, Certified Reference Materials (CRMs) :

- Confidence in analytical results
- Documentation available for audits
- Safety for the quality management system
- Consistent quality of the reagents & standards
- Increased confidence in testing, calibration data, and in the personnel performing work
- Cost and time savings - no multiple testing
- Trust in the quality of products being tested and evaluated with this volumetric solution & standards

For our complete titration portfolio visit us at **SigmaAldrich.com/Titration**

HPLC Tips & Tricks

Proper Storage of HPLC/UHPLC Columns

Dr. Egidijus Machtejevas, Lead Expert, Chromatography Product & Portfolio Management, Analytix@merckgroup.com

The concept may seem simple. Once the last chromatogram of the day or the project is finished, we disconnect the column and put it into the drawer. However, what exactly should be done with the column before storing it? Does the procedure vary depending on the planned storage time? There is actually quite a lot to: planned storage time, column modification (stationary phase), buffer concentration, pH, etc. In all the column storage scenarios, special care must be taken if buffers, which provide a microbe friendly environment, are used. In such cases, fresh buffers are to be prepared daily and filtered using 0.45 or 0.22 µm membrane filters. Also adding a small amount of organic solvent (~ 10%) or adding sodium azide (~ 0.05%) in the storage solvent - if buffers are used for storing e.g. needed for some HILIC columns - can be sufficient to prevent microbial growth. The easiest and safest way to store the column, however, is by using the same solvent in which it was delivered to you.

This applies in particular to the polymer-based stationary phases. Depending on their material properties, these might not be compatible with some organic solvents.

For silica-based normal phase columns, it is typically recommended that heptane or isopropanol are used. I personnaly have also had good experience with dioxane, as it nicely removes residual water, but this cannot be generalized. Some stationary phases such as aminopropyl- or diol-modified stationary phases might be effectively stored in 2-propanol, which is in fact, compatible with both Reversed Phase and Normal Phase modes. Size exclusion columns should be stored in a solvent compatible with the swelling properties of the packing.

Column storage may be short, middle, and long term.

For **short term storage**, i.e., overnight, either the mobile phase used in the last analysis can remain in the column, or it is possible that the mobile phase passes at a very low flow rate (especially if the buffer concentration in the mobile phase is high, >50 mM). In these cases, column conditioning can potentially be skipped before continuing the analysis the next day. This option is particularly recommended for normal phase separations, where change in mobile phase composition can result in lengthy re-equilibration. However, if the buffer concentration in the mobile phase is very high (>0.5 M), then the lifetime of the pump parts (e.g. injector & switching valves) could depend on the length of time they are in contact with high concentration buffer. The same is true for the column if the pH is close to the limit of the column (for most silica-based columns - pH 2 to pH 7). Some salts, such as chloride salts used in ion chromatography in particular, are very corrosive to stainless steel and might attack the column wall as well as the inlet-outlet frits. In such cases, column (and all system) should be flushed with a less harsh mobile phase. In this case, I would recommend rinsing the column with a water-rich mobile phase (~90%) with about 10 column volumes (the approximate column volumes for some popular dimension are listed in **Table 1**).

Table 1. Approximate Column Volumes for some Popular Column Dimensions and Their Multiples

Length (mm)	ID (mm)	Approximate Column Volume (mL)	10 Column Volumes (mL)	15 Column Volumes (mL)
250	4.6	4.15	41.5	62.3
250	2.0	0.79	7.9	11.8
150	4.6	2.49	24.9	37.4
150	2.0	0.47	4.7	7.1
100	4.6	1.66	16.6	24.9
100	2.0	0.31	3.1	4.7
50	4.6	0.83	8.3	12.5
50	2.0	0.16	1.6	2.4
25	4.6	0.42	4.2	6.2
25	2.0	0.08	0.8	1.2

Note: The volume of the recommended mobile phase as indicated in the table must actually pass the column. Be aware that, if you exchange the solvent bottle and remove tubing from one solvent and place it into another container, you have to consider the volume of the tubing (~2-3 mL), degasser (older degassers could be up to 15 mL, newer ~ 4 mL), pump (~1 mL), and injector until the new solvent reaches the column. Depending on the flow rate, additional time has to be added for the rinsing procedure.

If you disconnect a column from the instrument, end plugs should be tightly fitted to prevent solvent evaporation, otherwise a drying of the stationary phase could happen. The worst-case scenario is an improperly washed column previously used with a high salt concentration and allowed to dry over time, resulting in the formation of salt crystals. The column most likely will be irreversibly damaged. However, it might be permissible for some columns to be stored dry, others should not. Please check the manufacturer's column care guidelines. Standard HPLC columns should only be stored at room temperature and never in a freezer (exceptions are protein modified affinity

or active enzyme reactor columns). This paragraph's recommendations are also valid for mid- and long-term column storage.

Medium interval storage, i.e., 2 days or over the weekend. Columns should be flushed. Flush intensity or volume depends on the buffer concentration used during analysis. It is generally advisable to first flush buffering agents off the column with about 10 column volumes of mobile phase with 10% organic solvent in the water. In this case, washing will be effective, and we would also avoid buffer precipitation and possible column dewetting problems. When the buffer is washed out, pump 100% organic for 15 column volumes. The column could then be left connected to the instrument or disconnected and closed with end plugs. Please consider short term column storage advice too, such as referencing column documentation for recommended storage solvent.

Storing a HILIC column in an acetonitrile–water mixture may take a long time to re-equilibrate if a low ionic strength buffer such as 5 mM ammonium acetate is used for the analytical method. Therefore, for HILIC columns, it is recommended that they are stored in solvents containing 80–90% acetonitrile and buffers containing 5–10 mM ammonium acetate or ammonium formate. But for some HILIC phases this may differ, please check the column product information.

Ion-exchange and mixed-mode phases containing carboxylic acid functional groups (for example, weak cation-exchange phases) cannot be stored in solutions containing alcohols, because of a possible slow esterification and the resulting change in selectivity/capacity.

For long term storage (>2-3 days), silica based columns, after proper washing with a minimum of 15 column volumes (**Table 1**) using ~ 10% organic solvent in water, should then be flushed with an organic-rich mobile phase for a minimum of 10 column volumes and should then be stored in an aprotic solvent. If water is also present, it should not be in higher concentrations (less than 50%). The best storing solvent recommended in the literature is acetonitrile or methanol (some exceptions exist, such as columns with amide modification, which should be stored in acetonitrile only). Some studies[1] also indicate that at RP conditions, rates of erosion and corrosion of the stainless steel components of the HPLC using pure acetonitrile or methanol were higher compared to when they were mixed with water. Therefore, 90% acetonitrile or methanol are perfect long-term storage agents for most reverse-phase columns. However, my personal favorite storage solution is a mixture of isopropanol and water (80/20), because of isopropanol's higher vapor pressure and the reduced chance for column dry-out, even if end fittings are not completely sealed. Isopropanol is also a stronger eluent, therefore, after storing in isopropanol, we can be sure that even more impurities will be removed than with acetonitrile or methanol gradients. Last but not least, isopropanol is also less toxic. It is also important to note that all mobile phases used for flushing, washing, or column storing must be of the same quality grade as the ones used for the analysis. Columns should be stored at room temperature (exceptions include affinity columns, as mentioned before) in their original box, with a copy of the certificate of analysis (CoA)/Column Report, and possibly with the column log book to show previous uses and to help the user evaluate the column prior to future use.

How long can columns be stored? This depends on many factors. Some columns do not change even after 5 or 10 years of storage. If you decide to use a column after such a long period of time, assume that the column most likely has dried out, and needs to be rewetted by first flushing with 100% acetonitrile (RP-phases), and then equilibrated in mobile phase for about 1 hour before making any selectivity measurements. Additionally, consider running a column test mix and compare the data to the CoA or previous column tests.

Correct column storage is essential for proper chromatography and a prolonged column life. In addition, always follow the manufacturer's guidelines for column operation details!

Reference

1. R.A. Mowery, Jr., J. Chromatogr. of Science, Volume 23, Issue 1, January 1985, Pages 22-29, https://doi.org/10.1093/chromsci/23.1.22

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