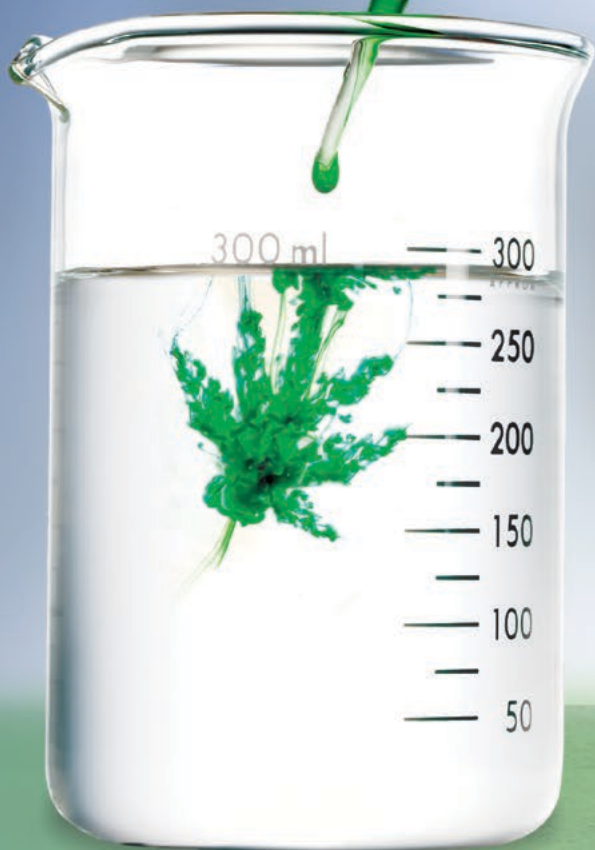


Analytix Reporter



Cannabinoid Analysis of Hemp: Developing an Efficient HPLC Method Workflow

New Carotenoid Standards

Chiral Separation of Pesticide Diniconazole by SFC

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

Efficient Analysis of Kava Extract

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Pattern and ADCC Activity of a Therapeutic Antibody

Lopinavir Assay Following European
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Cannabinoid Analysis of Hemp: Developing an Efficient HPLC Method Workflow

Edited article, refer to [SigmaAldrich.com/Analytix \(Issue 8\)](https://www.sigmaaldrich.com/Analytix) for full version

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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.

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 THCA, 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

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

Analyte*	w/w % (Dry Weight)	
	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

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.

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.

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5. AOAC SMPR 2017.001. Standard Method Performance Requirements (SMPRs) for Quantitation of Cannabinoids in Cannabis Concentrates
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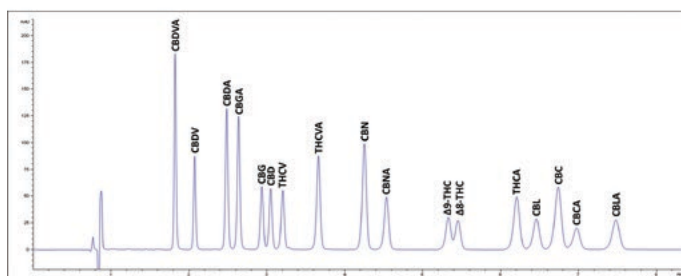


Figure 3. Separation of Cannabinoids in the Peak Identification Mix Using an Isocratic Method on a 2.7 μm Ascentis[®] Express C8 Column.

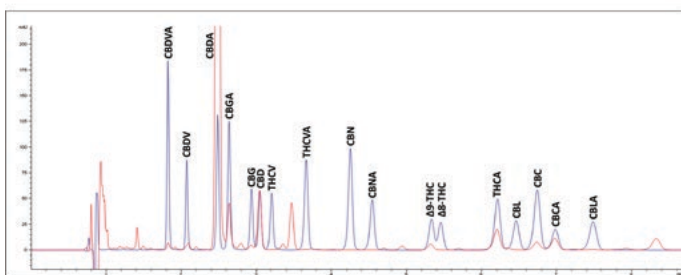


Figure 4. Overlay of Hemp Sample and Peak Identification Mix, Isocratic Method on Ascentis Express C8 2.7 μm Column

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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
Cannabicyclic 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
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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

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Ascentis® Express C8, 5 mm x 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
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Millex® Samplicity® Hydrophilic PTFE Filters, 0.20 µm, Pk.96	SAMPLG001
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New Carotenoid Standards

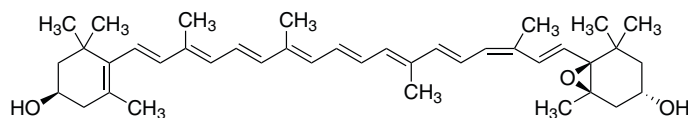
Matthias Nold, Product Manager Reference Materials, Analytix@milliporesigma.com

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.

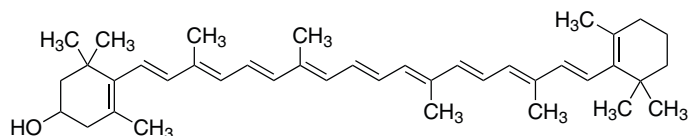
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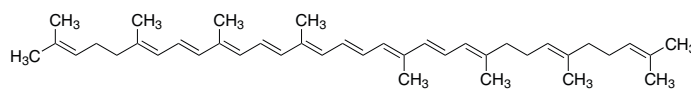
Description	Package Size	Cat. No.
9-cis-Antheraxanthin	1 mg	47999
(+/-)-beta-Cryptoxanthin	1 mg	51772
all-trans-Neurosporene	1 mg	59739



47999 9-cis-Antheraxanthin



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

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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).

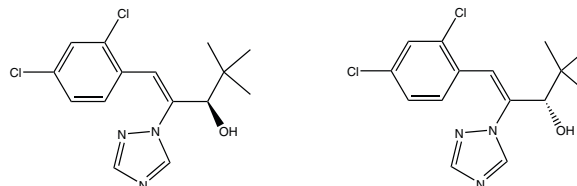


Figure 1. Chemical structure of R- and S- Diniconazole, respectively.

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 (CO₂) which offers several advantages, particularly when compared to liquid chromatography.⁶ The use of CO₂ as the primary component of the mobile phase is one of the key features that benefits the preparative SFC chromatographer, and, since the CO₂ 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 CO₂. 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 CO ₂
flow rate:	2 mL/min.
pressure:	140 bar
column temp.:	25 °C
detector:	UV, 254 nm
injection:	5 μ L
sample:	diniconazole 200 μ g/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**).
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.

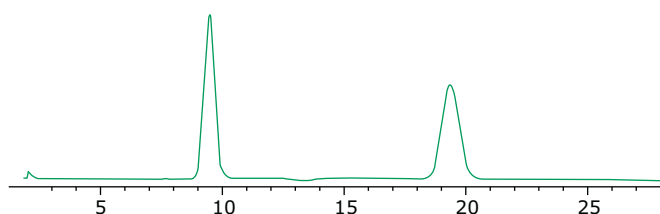


Figure 2. Enantiomeric separation of diniconazole on ChromegaChiral™ CCS (15 cm x 4.6 mm), Conditions see Table 2.

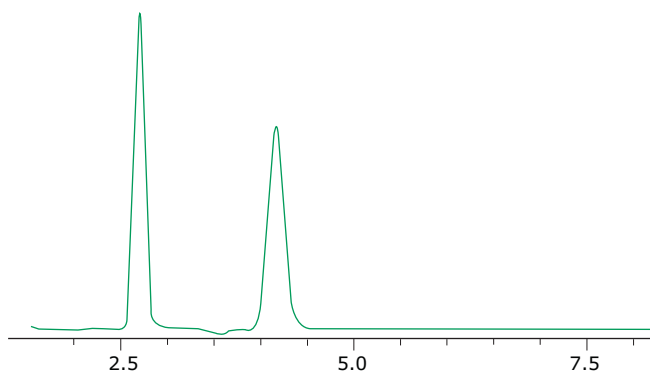


Figure 3. Enantiomeric separation of diniconazole on ChromegaChiral™ CCS. Conditions different from Table 2: 15% IPA in CO₂.

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.

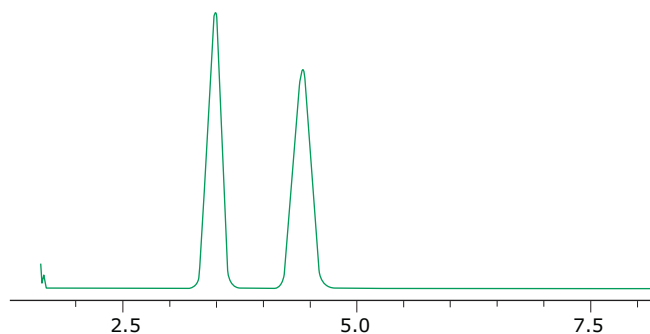


Figure 4: Enantiomeric separation of diniconazole on ChromegaChiral™ CCS (15 cm x 4.6 mm) . Conditions different from Table 2: 5% Methanol in CO₂.

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.

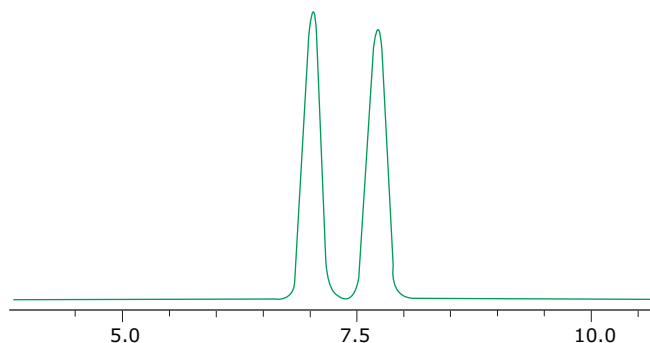


Figure 5: Enantiomeric separation of diniconazole on ChromegaChiral™ CCO-F4. Conditions different from Table 2: 5% Methanol in CO₂.

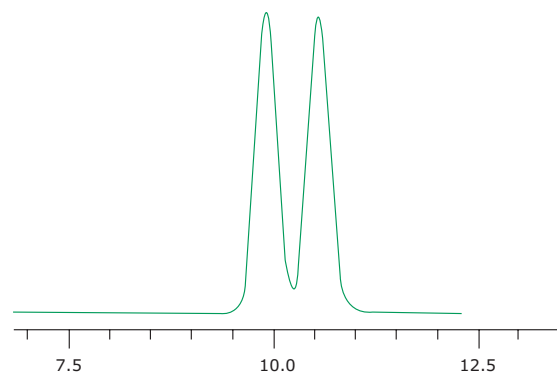


Figure 6: Enantiomeric separation of diniconazole on ChromegaChiral™ CC4. Conditions different from Table 2: 5% Methanol in CO₂.

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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Featured Products

Description	Cat. No.
ChromegaChiral™ Chiral CC3; 25 cm x 4.6 mm., 5.0 µm	ES5689
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

Related Products

Description	Cat. No.
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Methanol gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur	1.06007

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

Katherine K. Stenerson, Analytical Sciences Liaison, Analytix@milliporesigma.com

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-d₆ 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 ₂ HPO 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:	SUPELCO WAX® 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

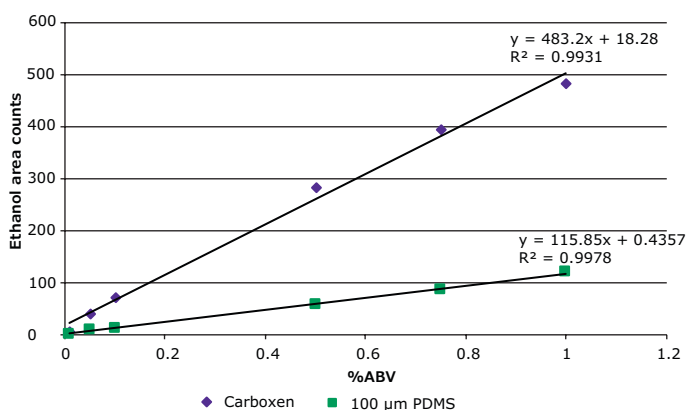


Figure 1. Comparison of Alcohol Response (GC-FID) from Water obtained by HS-SPME; 100 µm PDMS and Carboxen®/PDMS Fibers.

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.

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

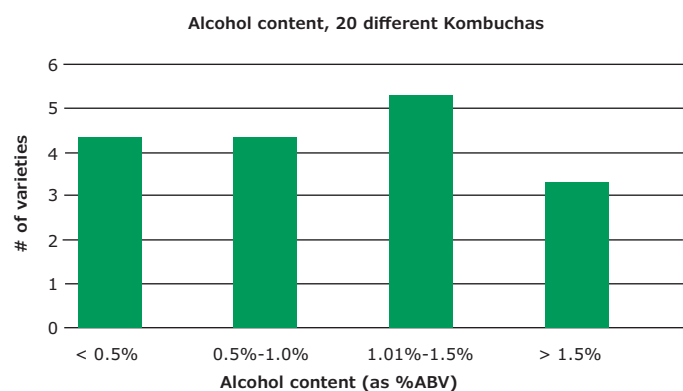


Figure 2. Alcohol (Ethanol) Content, as % ABV, Measured on 20 Kombucha Tea Samples using HS-SPME.

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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SPME fiber holder for autosampler	57347-U
Inlet Liner, Direct (SPME) Type, 0.75 mm I.D. for Agilent, Pk.1	2637501
Headspace vial with screw top, clear, 10 mL, Pk.100	SU860099
Magnetic screw cap for headspace vial w/1.5 mm PTFE silicone septa, Pk.100	SU860103
Ethanol, 200 proof anhydrous, >99.5% purity*	459836
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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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@milliporesigma.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 preparations¹, 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 flavokavains (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 countries⁴, 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 flavokavains. 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.

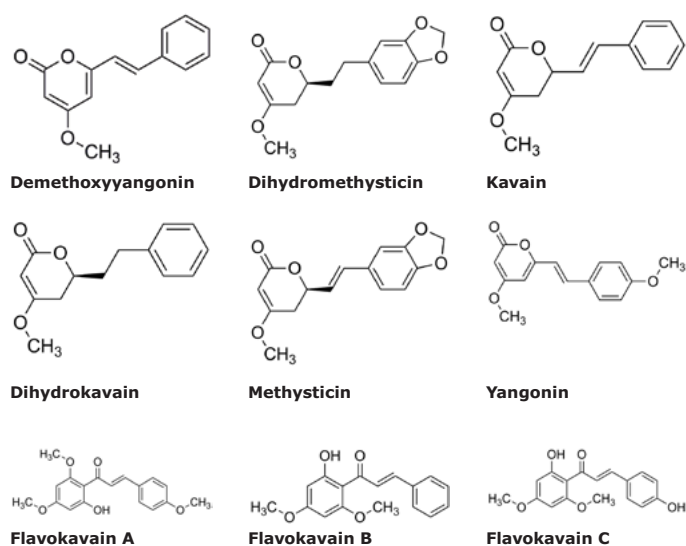


Figure 1. Chemical Structures of Kava Ingredients

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 ₃ PO 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					

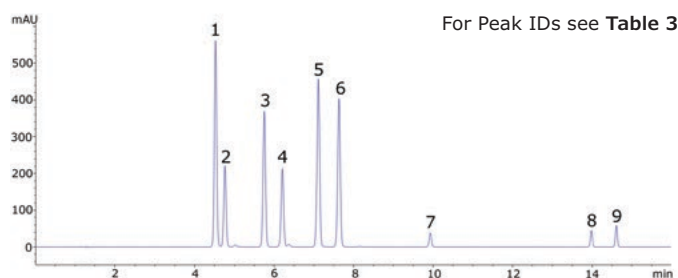


Figure 2. Chromatogram of the Kava CRM Mix Cat.No. K-007

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**).

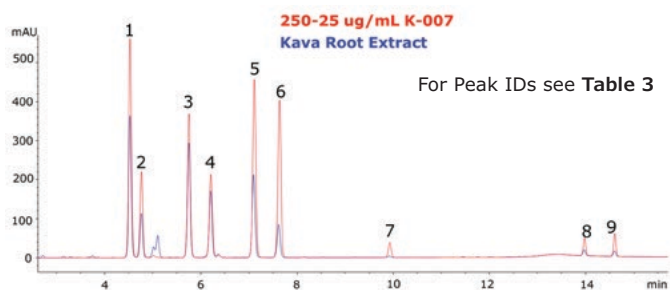


Figure 3. Chromatogram of the Kava CRM Mix Cat.No. K-007 (red) and Kava root extract (blue)

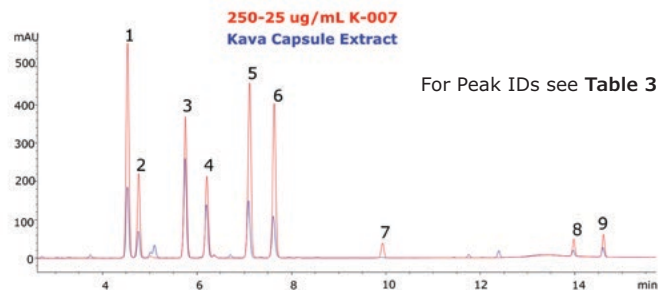


Figure 4. Chromatogram of the Kava CRM Mix Cat.No. K-007 (red) and Kava capsule extract (blue)

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	
		#	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.

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Featured Products

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Ascentis® Express Guard Cartridge Holder	53500-U
Kava Mix, Cerilliant® Certified Reference Material	K-007
Milllex® 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
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Methanol for UHPLC	900688
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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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To see our complete offer on phytochemical reference materials visit us at [SigmaAldrich.com/Phytochemicals](https://www.sigmaaldrich.com/Phytochemicals)

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

Cara Tomasek, Leader, Product Management, Tosoh Biosciences, North America
 Cory E. Muraco, Product Manager Liquid Chromatography Technologies, Analytix@milliporesigma.com

Introduction

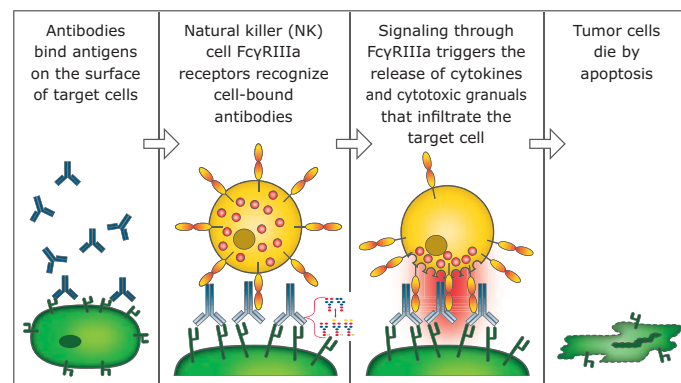
Antibody-dependent cell-mediated cytotoxicity (ADCC) is an important mechanism of action (MOA) of monoclonal antibodies used in cancer treatment. Selecting suitable cell lines and optimizing culture conditions towards the expression of antibody candidates with desired ADCC activity is an essential part 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).



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Figure 1. Antibody-dependent cell-mediated cytotoxicity

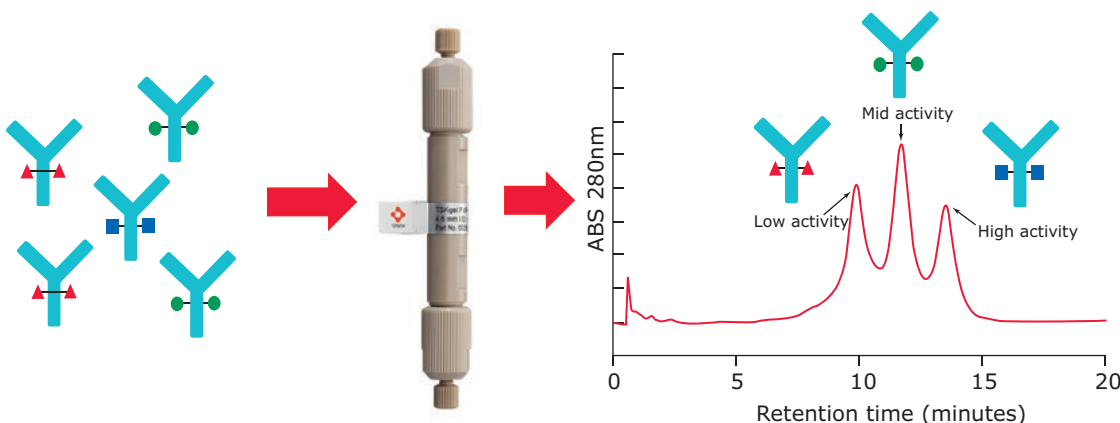


Figure 2. FcγRIIIa affinity chromatography

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 IgG₁ 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.

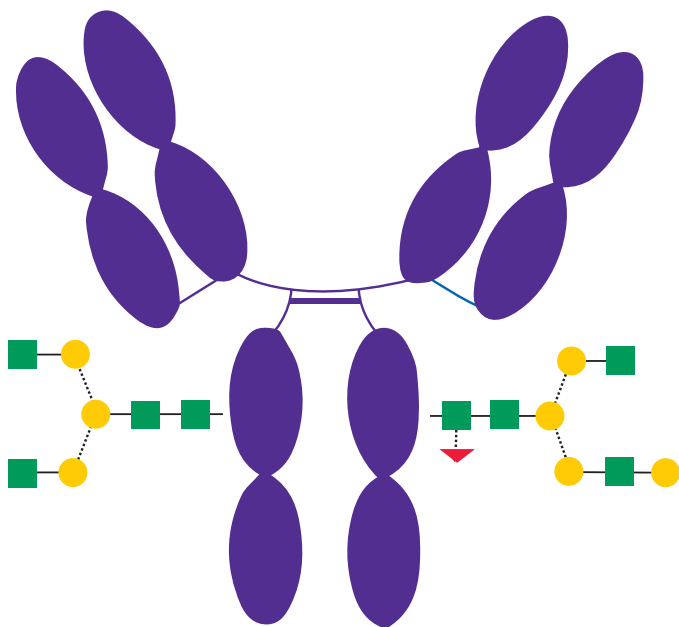
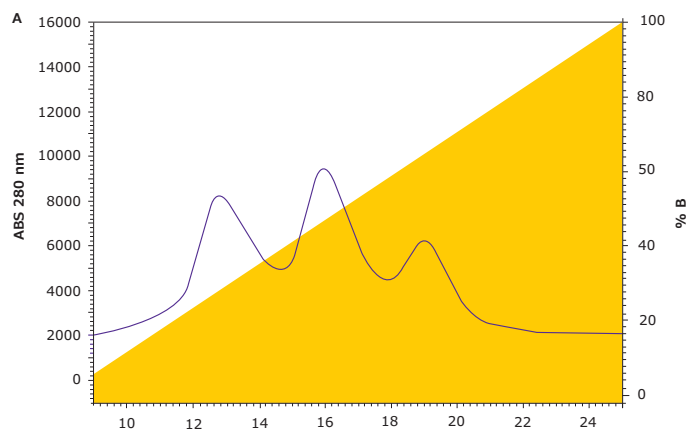


Figure 3. Schematic Diagram of Rituximab

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 Conditions³

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® 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 FcγR 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.

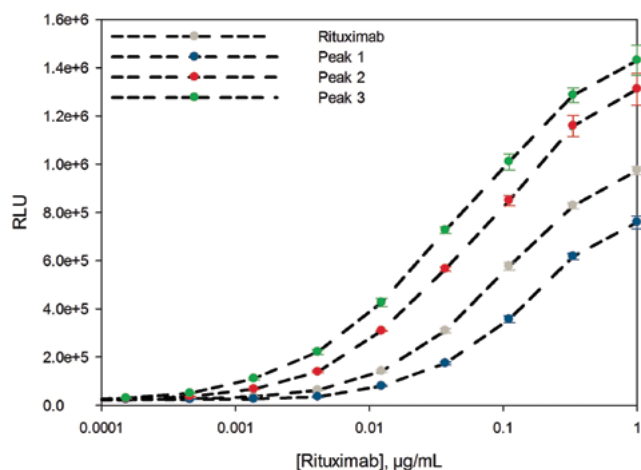


Figure 5. ADCC Reporter Bioassay Response to Rituximab

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

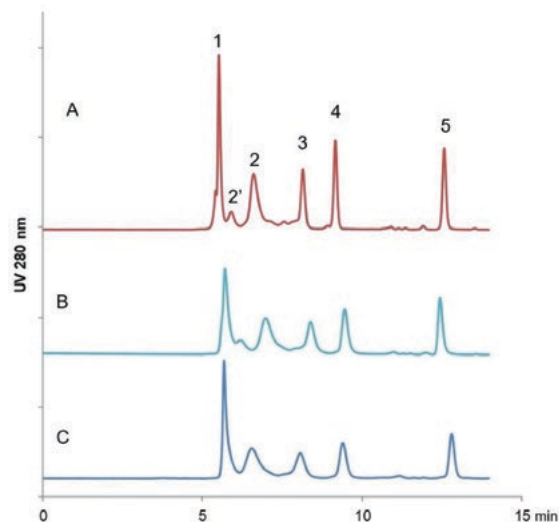


Figure 6. HILIC Analysis of Oligosaccharides of the three FcR Affinity Fractions (Peak 1 blue, Peak 2 red, Peak 3 green) compared with a 2-AB Labeled Biantennary Glycan Library (grey).⁴

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

References

1. D. Reusch and M. L. Tejada, *Glycobiology* 2015, 25 (12): 1325-1324; doi:10.1093/glycob/cwv065
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. Kioishi 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

TSKgel and Tosoh Bioscience are registered trademarks of Tosoh Corporation.

Rituximab was kindly provided by Rentschler Biopharma.

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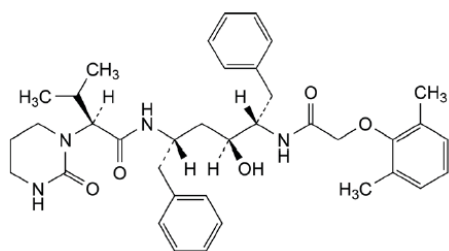
Lopinavir Assay Following European Pharmacopoeia 10 Guidelines Using an Ascentis® Express C18 Column and UV Detection

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

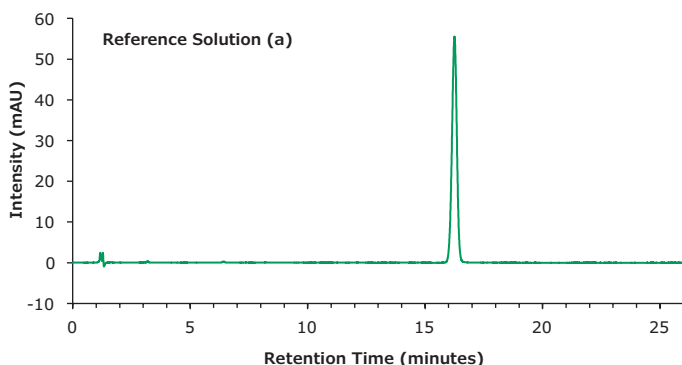
Introduction

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 μ m 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

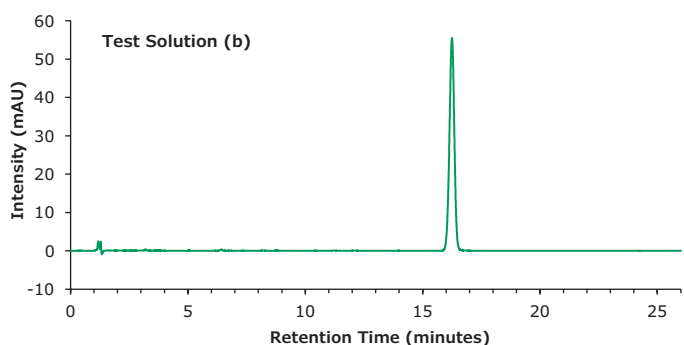


Experimental

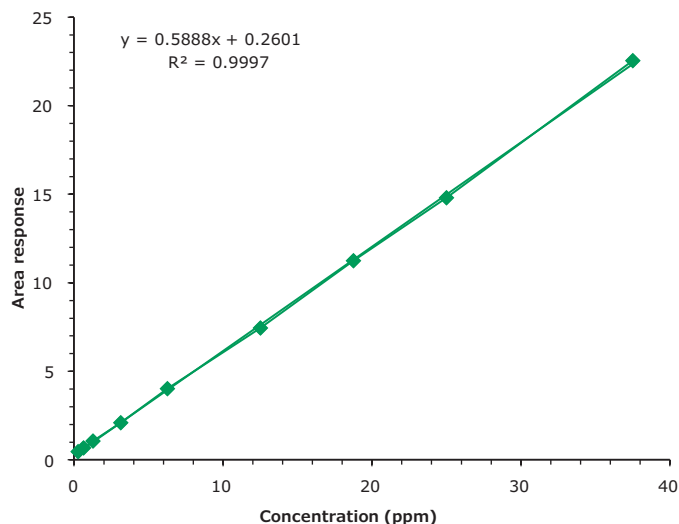
Conditions	
column:	Ascentis® Express C18, 15 cm x 4.6 mm, 2.7 μ m (53829-U)
mobile phase:	acetonitrile/phosphate buffer solution 45/55 (v/v)
buffer preparation:	dissolve 0.9 g of dipotassium hydrogen phosphate and 2.7 g of potassium dihydrogen phosphate in 900 mL of water and mix well. Adjust to pH 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 °C
detector:	UV, 215 nm UV = 215 nm (micro flow cell; 1.4 μ L/7 mm)
injection:	12 μ L
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 Pharmacopoeia 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 Pharmacopoeia (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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CLINICAL & FORENSIC

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@milliporesigma.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* transcribed (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:

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



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

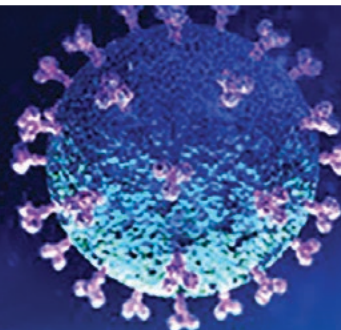
Please find more information at SigmaAldrich.com/EURM019

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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@milliporesigma.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) guidelines¹ and USP 665 guidelines for polymeric components and systems² (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

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

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.

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 protocol¹, 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-d₁₄ as LLE extraction standard. The sample was fortified with phenanthrene-d₁₀ as internal standard prior to analysis. The sample and the standard mix ran in one sequence.

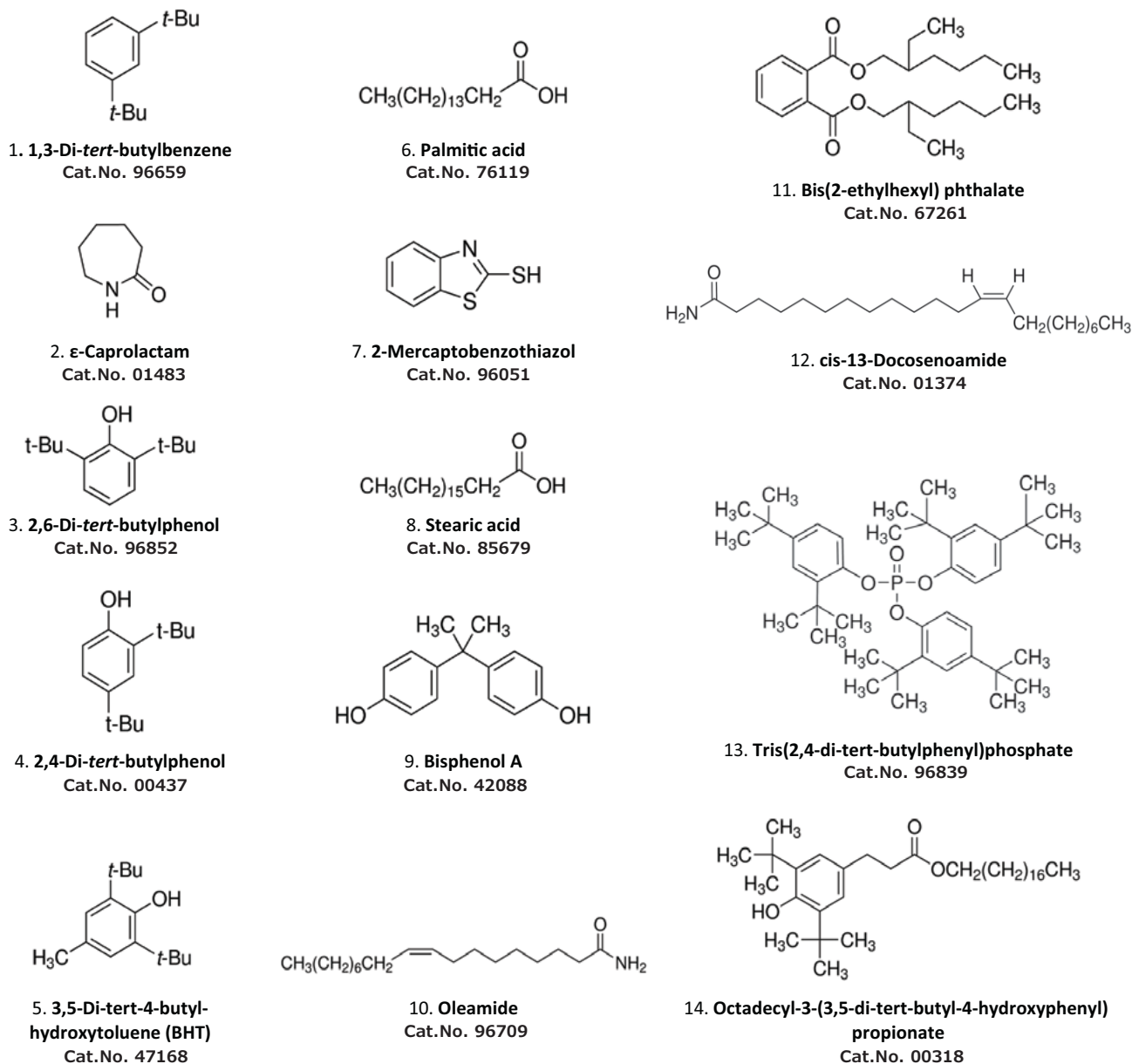


Figure 1. Components of the Extractables and Leachables Screening Standard for GC Cat. No. **01829**; in Elution Order of Below Method. Concentration is 50 mg/L in *tert-Butyl* methyl ether for each component. For all the analytes, individual neat reference materials are also available under the catalog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

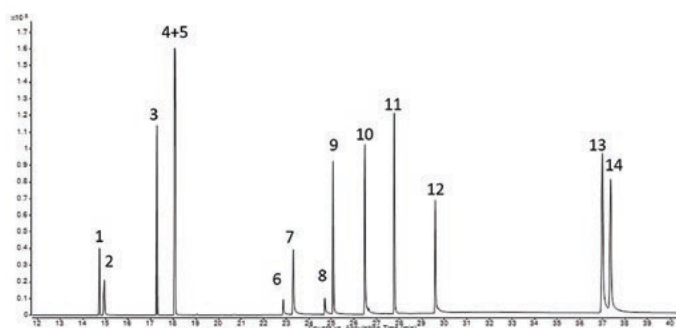


Figure 2. Extractables and Leachables Screening Standard for GC, 50 mg/L in *tert*-Butyl methyl ether (Peak IDs - Table 2).

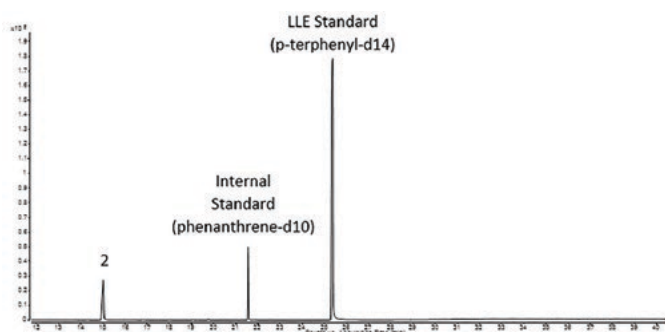


Figure 3. Representative Sample of a Single-use Equipment Extraction (Peak IDs - Table 2).

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- <i>tert</i> -butylbenzene	1014-60-4
2	ϵ -Caprolactam	105-60-2
3	2,6-Di- <i>tert</i> -butylphenol	128-39-2
4	2,4-Di- <i>tert</i> -butylphenol	96-76-4
5	3,5-Di- <i>tert</i> -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	<i>cis</i> -13-Docosenoamide	112-84-5
13	Tris(2,4-di- <i>tert</i> -butylphenyl)phosphate	95906-11-9
14	Octadecyl-3-(3,5-di- <i>tert</i> -butyl-4-hydroxyphenyl) propionate	2082-79-3

References

- Ding et al. Standardized Extractables Testing Protocol for Single-Use Systems in Biomanufacturing, Pharmaceutical Engineering, (2014).
- <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 μ m	28471-U
Dichloromethane, for gas chromatography ECD and FID SupraSolv®	1.06054
<i>p</i> -Terphenyl- d_{14} , analytical standard, 100mg	442734
Phenanthrene- d_{10} , analytical standard, 100mg	442753

Related Products

Description	Cat. No.
Internal Standard Solutions	
<i>p</i> -Terphenyl- d_{14} solution, certified reference material, 2000 μ g/mL in dichloromethane	48418
Phenanthrene- d_{10} solution, certified reference material, 2000 μ g/mL in methanol	48094
Single Component Reference Materials	
1,3-Di- <i>tert</i> -butylbenzene, reference material, 100 mg	96659
2,4-Di- <i>tert</i> -butylphenol, reference material, 100 mg	00437
2,6-Di- <i>tert</i> -butylphenol, reference material, 100 mg	96852
2-Mercaptobenzothiazol, reference material, 100 mg	96051
3,5-Di- <i>tert</i> -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
<i>cis</i> -13-Docosenoamide, reference material, 100 mg	01374
Octadecyl-3-(3,5-di- <i>tert</i> -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- <i>tert</i> -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](https://www.sigmaaldrich.com/Extractablesandleachables)

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

Titripur® volumetric solutions certified according to ISO/IEC 17025

The Titripur® volumetric solutions are now certified according to DIN EN ISO/IEC 17025. The Quality Control Laboratory at Merck KGaA, Darmstadt, Germany is now accredited by DAkkS (Deutsche Akkreditierungsstelle - German Accreditation Service) 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® certified 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 pH buffers. 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 certified according to ISO/IEC 17025.




 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).



Figure 1. DAkkS certificate for the QC calibration lab at Merck KGaA Darmstadt, Germany. [SigmaAldrich.com/ISO17025](https://www.sigmaaldrich.com/ISO17025)



Certificate of Analysis

1.09141.1000 Sodium hydroxide solution $c(\text{NaOH}) = 0.1 \text{ mol/l}$ (0.1 N) Titripur®
 Reag. Ph Eur
 Batch HC02423141

	Spec. Values		Batch Values	
Form	liquid		liquid	
Amount-of-substance concentration	0.0995 - 0.1005	mol/l	0.1000	mol/l
Measurement uncertainty	+/- 0.0003	mol/l	+/- 0.0003	mol/l
Traceability	NIST SRM		84L	

Accreditation: This volumetric solution is certified according to DIN EN ISO/IEC 17025. Merck KGaA, Darmstadt, Germany is accredited as calibration laboratory D-K-15185-01-00 for amount-of-substance concentrations in volumetric solutions according to DIN EN ISO/IEC 17025 by DAkkS (Deutsche Akkreditierungsstelle). The accreditation certificate can be found at www.sigmaaldrich.com/ISO17025.

The concentration is determined by volumetric titration and refers to 20°C.

The certified value is traceable to a primary standard 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). This volumetric standard is certified by the accredited calibration laboratory of Merck KGaA, Darmstadt, Germany according to DIN EN ISO 17025. The uncertainty is expressed as expanded measurement uncertainty with a coverage factor $k=2$ covering a confidence level of 95%. All measurements are carried out in the calibration laboratory D-K-15185-01-00 of Merck KGaA, Darmstadt, Germany.

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) 07.05.2020
 Minimum shelf life (DD.MM.YYYY) 31.05.2023

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 Certified According to ISO/ICE 17025.

Traceability & Uncertainty

With the ISO/IEC 17025 accreditation as a calibration laboratory, the traceability of certified values 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 certified according to ISO/IEC 17025 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

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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 ($\sim 10\%$) or adding sodium azide ($\sim 0.05\%$) 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 personally 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 ($\sim 90\%$) 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 studies¹ 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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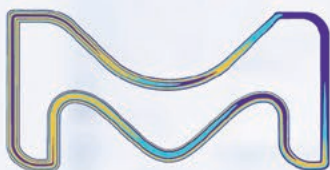
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