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Analytix Reporter

Allergenic Fragrance Testing - New Certified Reference Materials and GC-FID/GC-MS Application

HPTLC Application & Standards for Passiflora Incarnata

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Ultrasensitive Determination of Silicate in Water by Rapid Tests

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Dear Reader

One of the key steps in most analytical workflows is the sample preparation conducted before the analytical measurement. Sample preparation either cleans up the sample to ensure that sample matrix components that interfere with the measurement or contaminate the analytical system are removed or reduced, or enriches the analyte concentration to achieve required detection limits with the given analytical system used.

We have a long tradition in developing and providing innovative sample preparation tools and products for various fields of applications. Besides Solid Phase Extraction (SPE) and QuEChERS (see articles in Analytix Reporter Issues 1 and 2), one of the key technologies is Solid Phase Microextraction (SPME), which was invented by Professor Janusz Pawliszyn at the University of Waterloo, Canada, and with whom we have enjoyed collaborative work for over two decades. This year Prof. Pawliszyn was awarded the 2018 ACS Award in Chromatography. Read more on this award and Prof. Pawlizyn's views in the dedicated article at the end of this edition. Also in this edition you will find two articles on his invention, SPME, one demonstrating the use of SPME for natural product characterization and one on an improved determination of Volatile Organic Compounds (VOCs) in water by GC-MS, that became established in the ISO Standard 17943.

In this edition we also feature new reference and certified reference materials for allergens in fragrances (acc. to IFRA*), essential oils, terpenoids, passion flower ingredients (including a very interesting HPTLC application), milk proteins, as well as standards for neonicotinoid pesticides recently restricted for use in Europe. We also cover the determination of bispecific monoclonal antibodies by SEC-MS and ultrasensitive silicate determination in process and boiler water using rapid photometric tests.

We hope that one or more topics are of interest for you and will benefit your work. To learn more on our sample preparation tools and solutions mentioned above, please visit us at SigmaAldrich.com/sampleprep.

Happy Analysis!

*IFRA – International Fragrance Association

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Allergenic Fragrance Testing - New Certified Reference Materials and GC-FID/GC-MS Application

Calibration mixes for the new IFRA method now available

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Last year, the International Fragrance Association (IFRA) published a new method for the testing of 57 potentially allergenic fragrance compounds in response to an opinion by the European Scientific Committee on Consumer Safety (SCCS), which also proposed to correspondingly amend EC regulation No. 1223/2009.

This new analytical method has been developed by the IFRA Analytical Working Group to identify and to quantify the 57 fragrance substances and their relevant isomers at a concentration higher than 0.002% (20 mg/kg) in ready to inject fragrance materials and oils using Gas Chromatography with FID (GC-FID) or with Mass Spectrometry (GC-MS) detection. This new method will allow the screening of complex fragrance mixtures for the presence of these chemically defined allergens.

In this article we will discuss the introduction of new certified reference materials and their analysis by GC using an ionic liquid column as an alternative with unique selectivity and stability as compared to traditional wax/PEG phases.

New Certified Reference Material (CRM) Mixtures

We are proud to introduce two ready-to-use certified reference material mixtures suitable for use as calibrants for the above mentioned new IFRA method. The individual substances of these two mixes consist of a range of compound classes (predominantly of alcohol and carbonyl components), which were all individually thoroughly characterized and their content determined by quantitative nuclear magnetic resonance (qNMR) spectroscopy. qNMR is a relative primary method, as the signal intensity is proportional to the number of protons contributing to the resonance. It is therefore possible to directly compare the signal intensities of an analyte via an internal reference standard to a NIST SRM being a different compound. The individual qNMR certifications were all performed following the well-established qualification workflow developed in Buchs, Switzerland, according to the ISO/IEC 17025 accreditation.

This workflow typically starts with a series of preliminary investigations such as hygroscopy and volatility of the substances. Further determinations ensure the compatibility of each analyte with the internal standard used for the qNMR measurements.

Since reliable weighing values are mandatory, having a direct influence on the result, they are performed in a metrological way. Using a micro-balance in a specific, dedicated setup is the key to success because less sensitive equipment will lead to higher uncertainty contributions deriving from the weighing procedure. This is of the utmost importance for these components as they often have relatively low boiling points. In the qNMR measurements, a value for the mass fractions (g/g) was obtained and direct traceability to the SI unit was achieved through the use of acknowledged NIST primary reference materials. In addition, early insight into the homogeneity and stability of the components could be gained. If signals of interest of different isomers could not be completely separated in NMR, the ratio was determined by Gas Chromatography (GC) measurement under ISO/IEC 17025 accreditation.

The raw materials were then gravimetrically dissolved and diluted in methyl tert-butyl ether following the ISO 17034 accreditation workflow. Amber glass ampoules were then filled with the resulting bulk solutions and the process subsequently controlled by GC-MS homogeneity testing.

The final ampoules were thoroughly investigated to guarantee stability throughout the entire shelf life of the product at the storage temperature, as well as during transport to the customer at a potentially higher temperature. In order to be fully compliant with ISO 17034, Long-Term Stability studies (LTS) as well as Accelerated Stability Tests (AST) were performed by GC-MS.

The results from stability testing, from the conducted homogeneity experiments and from the qNMR measurements themselves all contribute to the overall uncertainty budget that is included individually for each component in the certificate. In addition to the certified

(continued on next page)

values of the concentrations and their respective overall expanded uncertainties in g/kg and g/L (through a certified density measurement at 20 °C), the certificate contains the lot number, an expiry date, intended use, accreditation stamps and signatures of the producing and releasing chemists.

To guarantee a suitable solution for the customer, the mixtures were intensively tested in a series of interlaboratory comparison studies before the launch.

Table 1 shows product number and composition of the two mixes. These products and example certificates can be found on our website at **SigmaAldrich.com/fragrancestandards.**

Table 1. Fragrance Allergen Mixes A1 and A2 with Composition. The nominal concentration for most components is 2000 mg/kg (exceptions are marked with *). The lot specific certified values are given in the certificate provided with the products. For both mixes, 2 mL and 5 mL packages are available (amber glass ampoules).

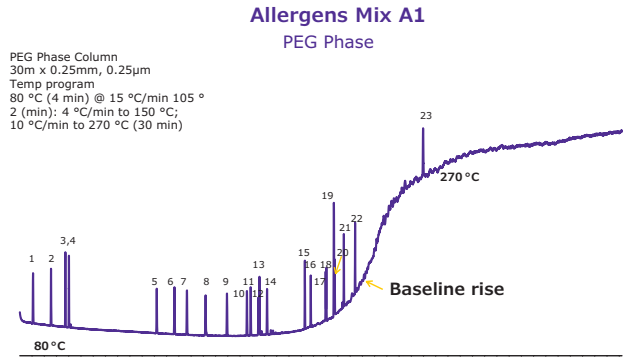
Fragrance Allergen Mix A1	Fragrance Allergen Mix A2
Cat. No.: 89131 Qty.: 1 mL, 5 mL	**Cat. No.: 16558 Qty.: 1 mL, 5 mL**
Solvent: Methyl-tert-butyl ether (MTBE)	Solvent: Methyl-tert-butyl ether (MTBE)
α-Amylcinnamyl alcohol	3-Propylidene phthalide
trans-Anethole	α-Acetyl Cedrene
Anise alcohol	Isoeugenyl Acetate
Benzyl alcohol	α-Amylcinnamaldehyde
β-Caryophyllene	Amyl salicylate
Cinnamyl alcohol	Benzaldehyde
Citronellol	Benzyl benzoate
Ebanol 1	Benzyl cinnamate
Ebanol 2	Benzyl salicylate
Eugenol	Butylphenyl methylpropional
trans,trans-Farnesol	Camphor
Geraniol	Carvone
Isoeugenol (E+Z)	Cinnamaldehyde
R(+)-Limonene	Neral
Linalool	Geranial
Menthol	Coumarin
α-Pinene	β-Damascenone (Rose Ketone-4)
β-Pinene	α-Damascone
α-Santalol	ß-Damascone "E"
β-Santalol	δ-Damascone (Rose Ketone-3)
Sclareol	Dimethylbenzylcarbinyl acetate (DMBCA)
α-Terpinene	Eugenyl acetate
Terpineol, mainly α	Galaxolide 1
Trimethyl-benzenepropanol	Galaxolide 2
	Geranyl acetate
	Hexadecanolactone / Dihydroambrettolide
	α-Hexylcinnamaldehyde
	Hydroxycitronellal
	Hydroxyisohexyl 3-cyclohexene carboxaldehyde - major
	Hydroxyisohexyl 3-cyclohexene carboxaldehyde - minor*
	α-Isomethylionone
	Linalyl acetate
	Methyl salicylate
	Methyl-2-octynoate
	Salicylaldehyde
	Terpinolene
	α-Tetramethylacetyloctahydronaphthalene (ISO E® α)*
	β-Tetramethylacetyloctahydronaphthalene (ISO E® β)
	γ-Tetramethylacetyloctahydronaphthalene (ISO E® γ)*
	Vanillin

* Compounds deviate from the nominal concentration of 2000 mg/kg

GC-FID and GC-MS Application

In the following, the separation of the allergens of both mixes using an ionic liquid phase column is demonstrated. The chromatograms shown below have been generated using a polar phase SLB®-IL60i, which provides wax/PEG like polarity with unique selectivity. Unlike the wax/PEG column (**Figure 1**) there is minimal baseline rise when this polar column is used (**Figures 2 & 3**). The new IFRA methodology[1] discusses FID (higher concentrated samples) and MS use; therefore, the figures below show the bleed behavior of the SLB-IL60i for GC-FID (**Figure 2**) and GC-MS (**Figure 3**).

Figure 1. GC-MS Chromatogram of Mix A1 on a typical PEG phase column



Experimental

Two fragrance allergen samples Mix A1 (**Figure 2**) & Mix A2 (**Figure 3**) were dissolved in MTBE (100 µL/mL) and analyzed by GC-FID or GC-MS using SLB®-IL60i ionic liquid column applying the conditions in **Tables 2 and 3**.

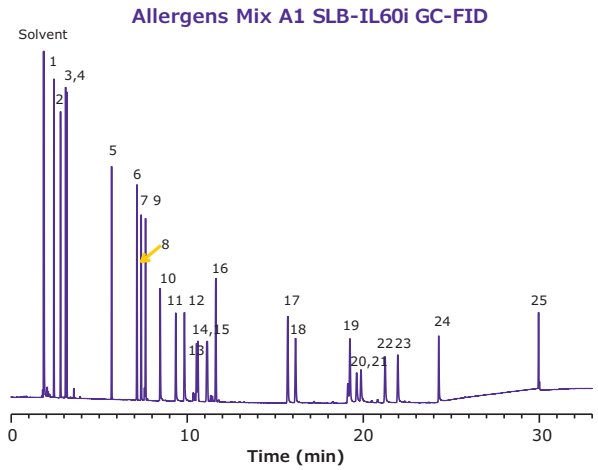
Table 2. Conditions GC-FID method Mix A1 (peak identification was done via separate GC-MS run)

Column:	SLB®-IL60i 30 m x 0.25 mm x 0.2 µm (29832-U)
Inlet:	250 °C
Oven:	70 °C @ 10 °C/min. to 135 °C (2 min.); 3 °C/min. to 170 °C (1 min.); 10 °C/min.to 270 °C (3 min.)
Flow:	31.457 cm/s Helium
Detector:	FID: 310 °C
Liner:	Cup liner
Split:	100:1
Sample:	1 µL (100 µL Mix A1 cat. no. 89131 in 1 mL of MTBE)

Table 3. Conditions GC-MS method Mix A2

Column:	SLB®-IL60i, 30 m x 0.25 mm x 0.2 µm (29832-U)
Inlet:	250 °C
Oven:	70 °C @ 10 °C/min. to 135 °C (2 min.); 3 °C/min. to 170 °C (1 min.); 10 °C/min. to 270 (30 min.)
Flow:	1.06 mL/min. (38 cm/s) Helium
Detector:	GC-MS, Ion Source: 250 °C, Interface Temp.: 200 °C
Liner:	Splitless with wool (Shimadzu 221-48876-03)
Split:	100:1
Sample:	1 µL (100 µL Mix A2 cat.no 16558 in 1 mL MTBE)

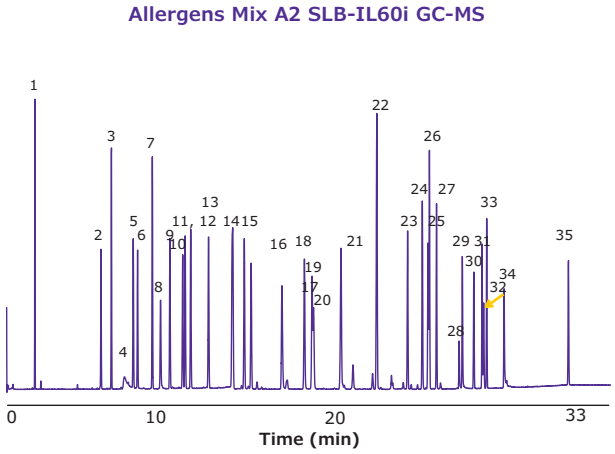
Figure 2. GC-FID Separation of Mix A1 on a SLB®-IL60i Column



Peak IDs

1. α-Pinene
2. β-Pinene
3. Limonene
4. α-Terpinene
5. Linalool
6. β-Caryophyllene
7. Menthol
8. γ-Terpineol
9. α-Terpineol
10. Citronellol
11. Geraniol
12. Benzyl alcohol
13. Ebanol 1
14. Ebanol 2
15. Anise alcohol
16. trans-Anethole
17. Trimethylbenzenepropanol
18. Eugenol
19. Santalol isomer
20. Cinnamyl alcohol
21. trans,trans-Farnesol
22. Santalol isomer
23. Isoeugenol (E+Z)
24. α-Amylcinnamyl alcohol
25. Sclareol

Figure 3. GC-MS of Fragrance Allergen Mix A2 on a SLB®-IL60i Column



Peak IDs

1. Terpinolene
2. Benzaldehyde
3. Linalyl acetate
4. Salicylaldehyde
5. Camphor
6. Methyl-2-octynoate
7. Geranyl acetate
8. Methyl salicylate
9. Neral
10. Carvone
11. Geranial
12. Dimethylbenzylcarbinyl acetate (DMBCA)
13. Damascone Isomer
14. β-Damascenone (Rose Ketone-4)/Damascone Isomer
15. α-Isomethylionone
16. Damascone Isomer
17. Hydroxycitronellal
18. Butylphenyl methylpropional
19. Amyl Salicylate
20. Cinnamaldehyde
21. Tetramethyl acetyl octahydronaphthalene (ISO E® Super) - all Isomers
22. Galaxolide 1 & 2
23. α-Amylcinnamaldehyde
24. α-Acetyl cedrene
25. Eugenyl acetate
26. Hexadecanolactone / Dihydroambrettolide
27. α-Hexylcinnamaldehyde
28. Vanillin
29. Hydroxyisohexyl 3-cyclohexene carboxaldehyde - major & minor
30. 3-Propylidene phthalide
31. Isoeugenol acetate
32. Coumarin
33. Benzyl benzoate
34. Benzyl salicylate
35. Benzyl cinnamate

Results and Discussion

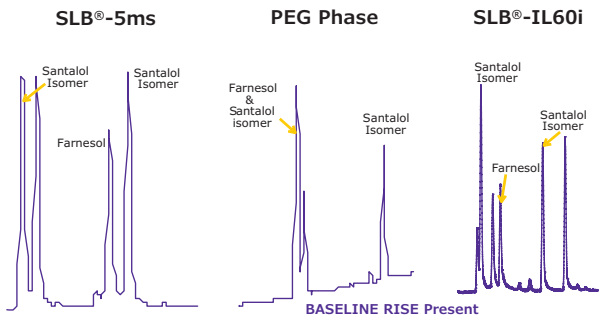
Figures 2 & 3 show the unique separation of the two Fragrance Allergen Mixes on a SLB®-IL60i. Both chromatograms show minimal baseline rise and unique selectivity. **Figure 4** shows the separation of farnesol and santalol isomers in Fragrance Allergen Mix A1 on the SLB®-IL60i compared to other commonly used columns (methods not shown here). The ionic liquid phase provides the best selectivity to separate these compounds.

Summary

With the two new ready-to-use Certified Reference Materials, fragrance manufacturers and testing labs are being provided with reliable, high quality standard calibration solutions suitable for the new IFRA method. This is a major advancement in improving the safety of personal care products and allowing manufacturers to retain their customer's trust.

For the GC analysis the SLB®-IL60i ionic liquid phase column is an alternative to the traditional capillary columns used for fragrance analysis, providing unique separation patterns and often fewer co-elutions (will be demonstrated in a later article). It shows a significantly reduced column bleed compared to traditional polar columns like PEG/wax phases.

Figure 4. Comparison of Santalol & Farnesol Separation on 3 column phases



Reference

1. THE INTERNATIONAL FRAGRANCE ASSOCIATION (IFRA) Analytical Method to Quantify 57 Suspected Allergens (and Isomers) in Ready to Inject Fragrance Materials by Gas Chromatography and Mass Spectroscopy, November 15th, 2016

Featured Products

Description	Qty.	Cat. No.
Fragrance Allergen Standard A1	2 mL/5 mL	**89131**
Fragrance Allergen Standard A2	2 mL/5 mL	**16558**
SLB®-IL60i, 30 m x 0.25 mm I.D., 0.20 µm	1 ea	**29832-U**

COSMETICS & PERSONAL CARE

New Essential Oil Reference Materials

Matthias Nold, Global Product Manager Reference Materials, **matthias.nold@sial.com**

Essential oils are volatile, hydrophobic liquids obtained from plant material by steam distillation or other extraction techniques. They have been used for centuries for personal care, as pharmaceutical products, or for aromatherapy. The world-wide demand for essential oils as natural fragrances is rapidly increasing, making them an attractive target for adulterations. Thus, cheaper essential oils or even synthetic compounds are added to a material to maximize profits. The detection of such adulterations can be quite challenging. Gas chromatography is most commonly used, but HPTLC is also a very efficient method for quick detection of compounds that are not supposed to be present in the product or to verify the product identity by fingerprint. For this purpose, we exclusively offer a range of essential oil reference materials from HWI pharma services. The products are graded as secondary reference materials, traceable to HWI primary reference materials quantified by qNMR (quantitative NMR). For each product, a mass percentage value is provided for at least one of the major components, while several other key components are qualitatively verified. The leaflet provided with the product also contains a GC method with chromatogram and peak assignment. For new lots, an HPTLC fingerprint of the material is also added.

Recently we added 10 new products to this portfolio, shown in the table below. For the entire product range, please visit **SigmaAldrich.com/essentialoils.**

Table 1. New essential oil reference materials

Cat. No.	Description	Quantitative Marker	Qualitative Marker	Package Size
05941501	Bergamot oil	Limonene	α-Pinene, β-Pinene, Linalool, Limonene, Geranyl acetate	1 mL
06031501	Cinnamon oil	Eugenol	Linalool, 1,8-Cineole, Eugenol	1 mL
06001501	Dwarf pine oil	α-Pinene	α-Pinene, β-Pinene, (+)-3-δ-Carene, Myrcene, Limonene	1 mL
05971501	Frankincense oil	α-Pinene, (+)-3-δ-Carene	α-Pinene, (+)-3-δ-Carene, β-Pinene, Limonene, γ-Terpinene, Sabinene hydrate, Terpinen-4-ol	1 mL
05981501	Lemon oil	Limonene	Limonene, β-Pinene, γ-Terpinene	1 mL
06011501	Mint oil	(-)-Menthol, (-)-Menthone	(-)-Menthol, (-)-Menthone, Limonene, Menthyl acetate, Isopulegol	1 mL
05991501	Pine needle oil	α-Pinene	α-Pinene, β-Pinene, (+)-3-δ-Carene, Myrcene, Limonene	1 mL
06021501	Sage oil	α-Thujone	α-Thujone, α-Pinene, D/L-Camphor, Bornyl acetate	1 mL
05961501	Spruce needle oil	(-)-Bornyl acetate	(-)-Bornyl acetate, α-Pinene, Camphene, (+)-3-δ-Carene, Limonene	1 mL
05951501	Ylang-ylang oil	Geranyl acetate	Geranyl acetate, Benzyl acetate, Linalool	1 mL

NUTRITIONAL SUPPLEMENTS

HPTLC Application for *Passiflora incarnata*

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In previous articles we presented HPTLC (High-Performance Thin-Layer Chromatography) applications for a series of medicinal plants, such as Ginkgo (Analytix 5/2016), Hypericum (Analytix 1/2017) or Ginseng (Analytix Reporter Issue 2/2018). With this article, we continue this series with an application note for passion flower, to further demonstrate the effectiveness of HPTLC for fingerprints of botanicals. Our comprehensive offering of analytical reagents and standards includes all consumables (TLC/HPTLC plates, solvents, analytical standards and extract reference materials) used for this application.

Passiflora incarnata (passion flower) has been familiar to native Latin Americans for many centuries. The Spanish physician Monardes discovered it in 1569 in Peru. In Europe, passion flower was long used as an ornamental plant. In the second half of the last century, passion flower entered the field of homeopathy. In low potencies it is used as a sedative, and also has a cardiotonic and nerve calming effect. Passion flower is primarily found in the tropical rainforest, and for production of the herbal drug, it is mainly grown in India, US (Florida), Italy and Spain.

TLC fingerprint analysis is stipulated for identification of herbal drugs in most pharmacopoeias. In the passion flower herb, several glycosylated flavonoids are present. After derivatization with Natural Product reagent, they can sensitively be detected via the HPTLC fingerprint analysis shown in this article.

Results & Discussion

With the demonstrated method according to the HPTLC Association[1], a rapid identification of *Passiflora incarnata* by HPTLC fingerprint analysis is possible. Reference

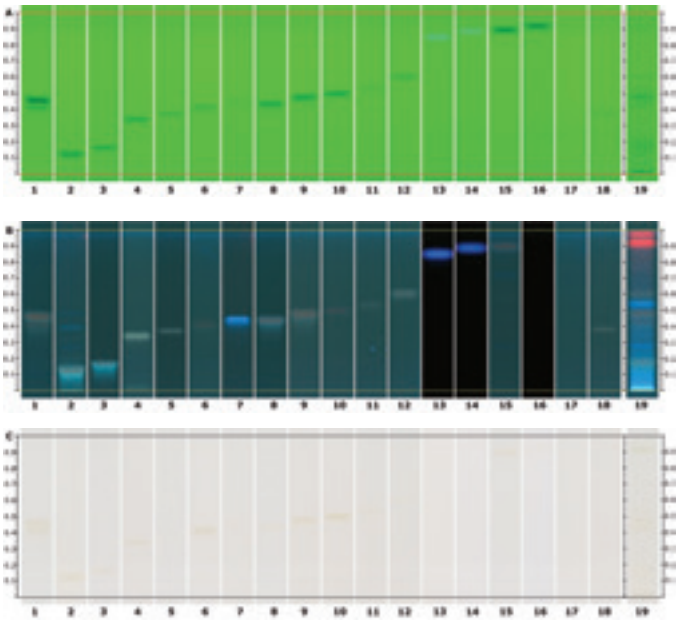


Figure 1: Chromatograms under UV 254 nm **(A)**, UV 366 nm **(B)** and white light **(C)** prior to derivatization, and under UV 366 nm after NP/PEG derivatization **(D)**, and white light after NP/PEG derivatization **(E)**.

Tracks: 1: SST isoorientin, isovitexin (with increasing R_F); **2:** vicenin-2 (03980585, lot HWI01702); **3:** schaftoside (42925, lot BCBP3755V); **4:** vitexin-2''-o-rhamnoside (00660585, lot HWI01189-1); **5:** hesperidin (50162, lot BCBT7241); **6:** isoorientin (78109, lot BCBR4394V); **7:** chlorogenic acid (00500590, lot HWI01268-1); **8:** swertisin; **9:** isovitexin (67135, lot BCBN8928V); track **10:** hyperoside (00180585, lot HWI00286-2); **11:** orientin (03810585, lot HWI01050-1); **12:** vitexin (49513, lot BCBR8611V); **13:** scopoletin (38332, lot BCBS2552V); **14:** umbelliferone (54826, lot BCBR1694V); **15:** apigenin (01760595, lot HWI00086-1); **16:** chrysin (95082, lot BCBP9460V); **17:** oleanolic acid (42515, lot BCBT0832); **18:** α-terpineol (04899, lot BCBS7535V); **19:** *Passiflora incarnata* Extract Reference Material (05085001, lot HWI01280).

(continued on next page)

substances are applied as a system suitability test to qualify the results (R_F values have to be in the specified tolerance window of ΔR_F 0.025). Furthermore, reference substances of glycosylated flavonoids present in *Passiflora incarnata* are applied.

System suitability test

(under UV 254 nm, ΔR_F 0.025; min_{height} 0.010 AU)

- Isoorientin (0.15 mg/mL): a quenching zone at R_F ~ 0.041
- Isovitexin (0.15 mg/mL): a quenching zone at R_F ~ 0.046

For our tests, a reference extract from HWI (track 19) was used. Zones present in the extract corresponded in color and position to those of the standards vicenin-2, schaftoside, isoorientin, isovitexin, orientin, and vitexin. The obtained HPTLC fingerprint was similar to the Passiflora herb chemotype isovitexin[1].

HPTLC fingerprint analysis allows a rapid identification of many samples in parallel without a time-consuming and cost-intensive sample preparation. Evaluation by visual comparison is simple and convenient. HPTLC can be considered the method of choice for identity testing of plant materials.

Reference

1. www.hptlc-association.org: Passion flower herb and passion flower dry extract identification (version 2018-05-07)

Chromatography according to USP <203>

Stationary phase	HPTLC Si 60 F_{254}, 20 x 10 cm
Sample application	Bandwise application with ATS 4, 15 tracks, band length 8 mm, track distance 11.4 mm, distance from left edge 20 mm, distance from lower edge 8 mm, application volume 4 µL for test solutions and standards
Developing solvents	Ethyl acetate, formic acid, water, ethyl methyl ketone, (50:10:10:30; *v/v/v/v*)
Development	In the ADC 2 with chamber saturation (with filter paper) for 20 min and after conditioning at 33% relative humidity for 10 min using a saturated solution of magnesium chloride ($MgCl_2$).
Developing distance	70 mm (from the lower edge)
Plate drying	Drying 5 min in the ADC 2
Derivatization	NP reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate; PEG reagent: 50 mg/mL of polyethylene glycol 400 in dichloromethane
For spraying, before the derivatization: mix NP reagent and PEG reagent (1:1, *v/v*)	Heat at 100 °C for 3 min, let the plate cool down, derivatize (spray with nozzle: green, spraying level: 3; spraying volume: 3 mL) with the mixture, dry the plate for 2 min in cold air flow and take picture

Featured Products

Description	Package Size	Cat. No.
Analytical Standards		
Chlorogenic acid*	25 mg	**00500590**
Vitexin 2-O-rhamnoside*	10 mg	**00660585**
Apigenin	10 mg	**01760595**
Chrysin	50 mg	**95082**
Hesperidin	10 mg	**50162**
Hyperoside*	25 mg	**00180585**
Isoorientin	10 mg	**78109**
Isoorientin*	10 mg	**03820585**
Isovitexin	5 mg	**67135**
Isovitexin*	10 mg	**01120590**
Oleanolic Acid	10 mg	**42515**
Oleanolic Acid*	10 mg	**03920590**
Orientin*	10 mg	**03810585**
Schaftoside	5 mg	**42925**
Scopoletin	10 mg	**38332**
α-Terpineol	250 mg	**04899**
α-Terpineol*	100 mg	**03420590**
Umbelliferone	50 mg	**54826**
Vicenin-2*	10 mg	**03980585**
Vitexin	10 mg	**49513**
Vitexin*	10 mg	**00840595**
Extract Reference Material		
Passiflora incarnata extract Quantified Components: Isovitexin Qualitatively Confirmed Components: Vitexin, Orientin, Homoorientin, Isovitexin	500 mg	**05085001**
TLC Plates		
HPTLC glass plate silica gel 60 F_{254} 20×10 cm	50 Plates	**1.05642**

*HWI reference standard

Find all available analytical standards for phytochemicals listed on **SigmaAldrich.com/medicinalplants**

An overview of all plant extract reference materials can be found at **SigmaAldrich.com/plantextracts**

Learn more about the features of High Performance Thin Layer Chromatography plates at **SigmaAldrich.com/tlc**

FOOD & BEVERAGE

Headspace SPME-GC/MS Analysis of Terpenes in Hops and Cannabis

Katherine K. Stenerson, Principal Scientist, **katherine.stenerson@sial.com**

In this application, headspace-SPME combined with GC/MS was used to analyze some of the terpenes present in both common hops and cannabis.

Terpenes are small molecules synthesized by some plants. The name terpene is derived from turpentine, which contains high concentrations of these compounds. Terpene molecules are constructed from the joining of isoprene units in a head-to-tail configuration (**Figure 1**). Classification is then done according to the number of these isoprene units in the structure (**Table 1**). The configurations of terpenes can be cyclic or open, and can include double bonds, and hydroxyl, carbonyl or other functional groups. If the terpene contains elements other than C and H, it is referred to as a terpenoid.[1]

Figure 1. Isoprene Unit

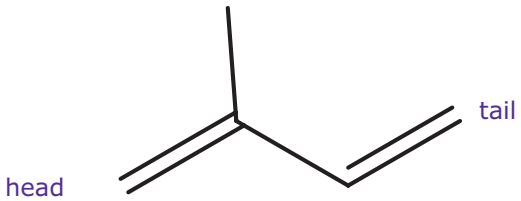


Table 1. Classification of Terpenes

Classification	Number of Isoprene Units
Monoterpene	2
Sesquiterpene	3
Diterpene	4
Triterpene	6
Tetraterpene	8

Terpenes are present in essential oils derived from plants and often impart characteristic aromas to the plant or its oil. For example, d-Limonene, which is found in lemon, orange, caraway and other plant oils, has a lemon-like odor. Essential oils, with their component terpenes and terpenoids, have been applied in therapeutic use known as aromatherapy to aid in the relief of conditions such as anxiety, depression, and insomnia.[2] This has led to the use of plants which contain these compounds in preparations such as oils, teas, and tonics.

Using Terpene Profile for Plant Identification

The *cannabis sativa* (cannabis or marijuana) plant contains over 100 different terpenes and terpenoids, including mono, sesqui, di, and tri, as well as other miscellaneous compounds of terpenoid orgin.[3] Although the terpene profile does not necessarily indicate geographic origin of a cannabis sample, it can be used in forensic applications to determine the common source of different samples.[4] In addition, different cannabis strains have been developed which have distinct aromas and flavors; a result of the differing amounts of specific terpenes present.[5] *Humulus lupulus* (common hops) and cannabis are both members of the family Cannabaceae.[6] Consequently, there are similarities in the terpenes each contains. Terpenes give both plant commodities characteristic organoleptic properties and, in the case of cannabis, produce characteristic aromas when the buds are heated or vaporized.[7]

Experimental

Dried cannabis sample was obtained courtesy of Dr. Hari H. Singh, Program Director at the Chemistry & Physiological Systems Research Branch of the United States National Institute on Drug Abuse at the National Institute of Health. The extract strain of the sample was not known. Hop flowers of an unknown variety were purchased from an on-line source. Pelletized of Cascade and US Golding hop varieties were purchased at a local home-brew supply shop. Chromatographic separation was performed on an Equity®-1 capillary GC column, and identification was done using retention indices and spectral library match. Final analytical conditions appear in the figures.

SPME Method Optimization

The SPME method was developed using a sample of dried hops flowers (0.2 g in 10 mL vial). The initial SPME parameters were based on previously published work.[8] The GC/MS results of this analysis are shown in **Figure 2**. This initial set of parameters used the 100 µm PDMS fiber, a 1 g sample size, and 60 minute equilibration at room temperature prior to extraction. The sample size was then scaled down to 0.2 g, and the equilibration temperature increased to 40 °C. This increased temperature allowed the equilibration time to be decreased from 60 to 30 minutes without a loss in sensitivity (**Figures 3 and 4**). The initial extraction

(continued on next page)

Figure 2. Headspace SPME-GC/MS Analysis of Dried Hops Flowers (100 µm PDMS Fiber, 1 g Sample)

Sample/matrix:	1 g ground hop flowers
SPME fiber:	100 µm PDMS (57341-U)
Sample equilibration:	60 min, room temperature
Extraction:	20 min, headspace, 40 °C
Desorption process:	3 min, 270 °C
Fiber post bake:	3 min, 270 °C
Column:	Equity®-1, 60 m x 0.25 mm I.D., 0.25 µm (28047-U)
Oven:	60 °C (2 min), 5 °C/min to 275 °C (5 min)
Inj. temp.:	270 °C
Detector:	MSD
MSD interface:	300 °C
Scan range:	full scan, m/z 50-500
Carrier gas:	helium, 1 mL/min constant flow
Liner:	0.75 mm ID SPME

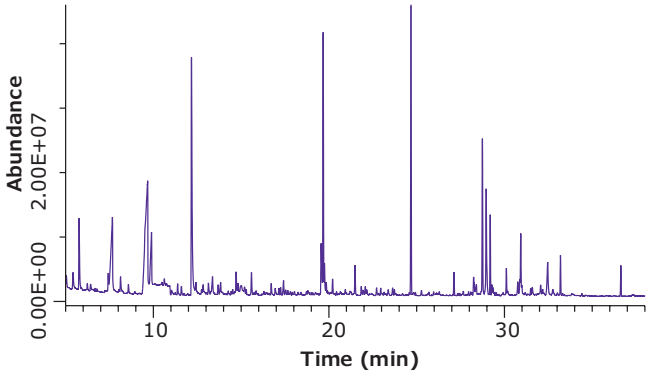
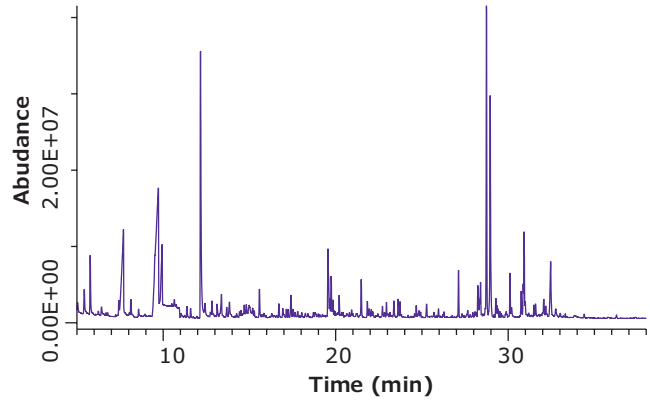


Figure 3. Headspace SPME-GC/MS Analysis of Dried Hops Flowers (100 µm PDMS Fiber, 0.2 g Sample)

Conditions same as Figure 2 except:
sample/matrix: 0.2 g ground hop flowers



time used was 20 min, and a shorter extraction time of 10 minutes was evaluated. However a loss in sensitivity was noted, thus extraction time was maintained at 20 minutes. The DVB/CAR/PDMS fiber was then evaluated (**Figure 5**). As expected, this fiber extracted more of the lighter compounds, which by MS spectral match, were identified as short chain alcohols and acids.

Figure 4. Headspace SPME-GC/MS Analysis of Dried Hops Flowers, Increased Sample Equilibration Temperature (100 µm PDMS Fiber, 0.2 g Sample)

Conditions same as Figure 2 except:	
sample/matrix:	0.2 g ground hop flowers
sample equilibration:	30 min, 40 °C

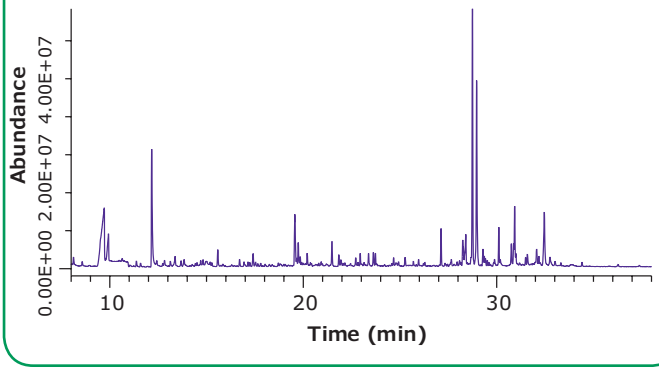
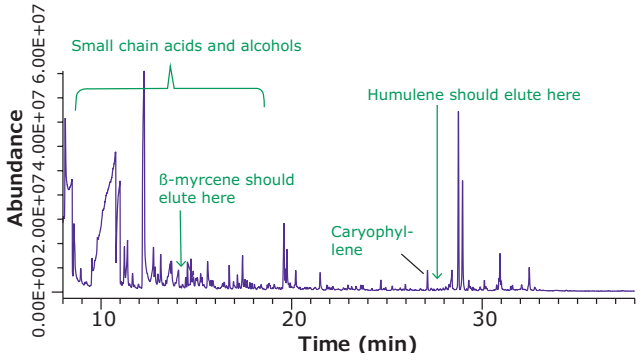


Figure 5. Headspace SPME-GC/MS Analysis of Dried Hops Flowers, Increased Sample Equilibration Temperature (DVB/CAR/PDMS Fiber, 0.2 g Sample)

Conditions same as Figure 2 except:	
sample/matrix:	0.2 g ground hop flowers
SPME fiber:	50/30 µm DVB/CAR/PDMS (57298-U)
sample equilibration:	30 min, 40 °C



Identification of Terpenes Using GC/MS

Using the DVB/CAR/PDMS fiber, samples of hops and cannabis were analyzed using the optimized SPME method. Peak identifications were assigned using MS spectral matching against reference spectra in the Wiley and NIST libraries. Confirmatory identification was done based on retention index. Retention indices were calculated for the compounds identified in each sample using an *n*-alkane standard analyzed under the same GC conditions. This data was compared with published values (**Tables 2 and 3**), and final identifications were assigned, as shown in **Figures 6 and 7**.

Terpenes in Hops Samples

For the dried hop flower sample (**Figure 5**), the terpene profile should have shown a predominance of β-myrcene, humulene, and caryophyllene, which are typical aroma compounds in hops and hop oil.[9] While caryophyllene was identified, both β-myrcene and humulene were not present at levels high enough to be detected by a library search. This may be due to the condition of the

Table 2. Terpenes in Hops Pellets Identified by MS Spectral Library Match and Retention Index

Peak No.	RT (min)	Name	RI (calculated)	RI (literature)	Reference
1	8.58	Hexanal	—	780	11
2	12.84	α-Pinene	939	942	11
3	13.28	Camphene	953	954	11
4	13.71	6-Methyl-5-hepten-2-one	966	968	11
5	14.1	β-Pinene	979	981	11
6	14.41	β-Myrcene	988	986	11
7	15.32	Cymene	1018	1020	11
8	15.65	d-Limonene	1030	1030	11
9	15.98	β-Ocimene	1041	1038	11
10	16.72	*cis*-Linalool oxide	1066	1068	11
11	17.49	Linalool	1089	1092	11
12	21.86	Geraniol	1239	1243	11
13	25.28	Geranyl acetate	1363	1364	11
14	25.85	α-Ylangene	1384	1373	8
15	25.97	α-Copaene	1388	1398	11
16	27.22	Caryophyllene	1437	1428	11
17	27.4	*trans*-α-Bergamotene + unknown	1445	1443	12
18	17.63	*trans*-β-Farnesene	1454	1450	8
19	28.11	Humulene	1473	1465	11
20	28.41	γ-Muurolene	1484	1475	11
21	28.45	γ-Selinene	1486	1472	12
22	28.68	Geranyl isobutyrate	1495	1493	11
23	28.79	β-Selinene	1499	1487	8
24	28.94	α-Muurolene	1505	1500	11
25	28.97	α-Selinene	1507	1501	12
26	29.31	γ-Cadinene	1521	1518	11
27	29.37	Calamenene	1524	1518	11
28	29.45	Δ-Cadinene	1527	1524	11
29	30.93	Caryophyllene oxide	1590	1584	8
30	31.5	Humulene oxide	1614	1599	12

Figure 6. Headspace SPME-GC/MS Analysis of Hops Pellets Using Final Optimized Method

The peak elution order is listed in Table 2.	
Conditions same as Figure 2 except:	
sample/matrix:	0.5 g ground hop flowers (hops pellets)
SPME fiber:	50/30 µm DVB/CAR/PDMS (57298-U)
sample equilibration:	30 min, 40 °C

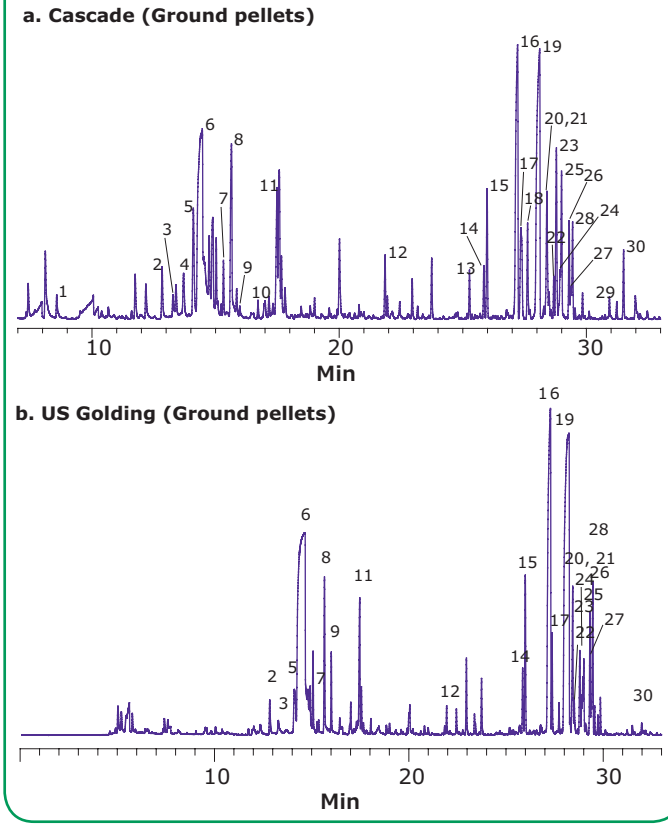
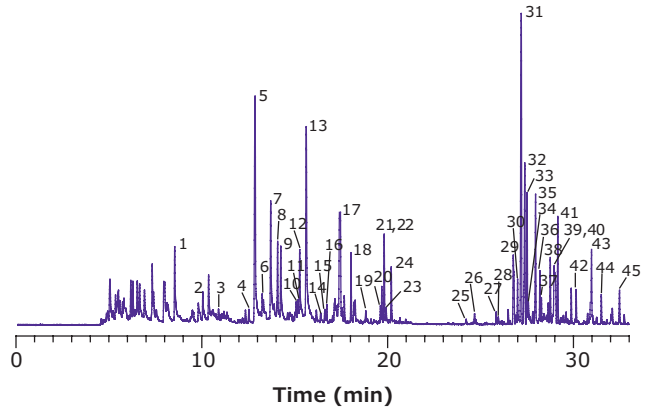


Figure 7. Headspace SPME-GC/MS Analysis of Dried Cannabis Using Final Optimized Method

The peak elution order is listed in Table 3.	
Same as Figure 2 except:	
sample/matrix:	0.5 g dried, ground cannabis
SPME fiber:	50/30 µm DVB/CAR/PDMS (57298-U)
sample equilibration:	30 min, 40 °C



sample or the actual variety of hops analyzed since terpene profiles are known to vary between different hop varieties[10]. The variety of the hop flowers analyzed is unknown, as the identity was not indicated on the packaging. For comparison, samples of two different varieties of pelletized hops were analyzed after grinding. These samples appeared green in color, and had a much more characteristic hops-like odor than the dried flowers. Analysis of these samples showed a characteristic terpene profile, with high levels of β-myrcene, caryophyllene, and humulene present in both (**Figure 6**). The SPME method was able to detect differences in the terpene profiles between the two hops varieties. For example, farnesene (peak 18) was identified in the Cascade hops, but was too low to be confirmed in the US Goldings sample. The level of farnesene in Cascade hops is expected to be 3-7% of total oils, while in US Goldings the level should be <1%.[13]

Terpenes in Cannabis Sample

The terpenes identified in the cannabis sample (**Figure 7**) are indicated in **Table 3**. The profile was similar to those found previously in the analysis of dried cannabis.[4,8] Peaks 1-27 in **Figure 7** (with the exception of peak 7) were monoterpenes and monoterpenoids. The later eluting peaks consisted of sequiterpenes and caryophyllene oxide,

(continued on next page)

which is a sequiterpenoid. The most abundant terpene was caryophyllene. The predominance of this compound could be due to the specific strain of cannabis tested, and/or the nature of the sample tested, which was dried. Previous studies have shown the level of this compound to increase significantly relative to other terepenes and terpenoids with drying.[4] Consequently, the levels of the more volatile monoterpenes and terpenoids would be expected to be less, and this was observed to some degree. Among the monoterpenes and terpenoids the most abundant were α-pinene and d-Limonene.

Table 3. Terpenes in Dried Cannabis Identified by MS Spectral Library Match and Retention Index

Peak No.	RT (min)	Name	RI (calculated)	RI (literature)	Reference
1	8.57	Hexanal	—	—	—
2	10.05	Hexene-1-ol	—	—	—
3	10.89	2-Heptanone	—	—	—
4	12.56	α-Thujene	928	938	11
5	12.86	α-Pinene + unknown	939	942	11
6	13.27	Camphene	953	954	11
7	13.69	6-Methyl-5-hepten-2-one	966	968	11
8	14.09	β-Pinene	979	981	11
9	14.27	β-Myrcene	984	986	11
10	15.09	δ-3-Carene	1010	1015	12
11	15.2	α-Terpinene	1014	1012	12
12	15.29	Cymene	1018	1020	11
13	15.6	d-Limonene	1028	1030	11
14	16.42	γ-Terpinene	1056	1057	11
15	16.6	*trans*-Sabinene hydrate	1062	1078	11
16	16.72	*cis*-Linalool oxide	1066	1068	11
17	17.43	Linalool	1087	1092	11
18	18.04	d-Fenchyl alcohol	1107	1110	11
19	18.82	*trans*-Pinocarveol	1135	1134	12
20	19.59	Borneol L	1161	1164	11
21	19.81	1,8-Methandien-4-ol	1168	1173	8
22	19.81	*p*-Cymen-8-ol	1168	1172	12
23	19.92	Terpinene-4-ol	1172	1185	11
24	20.22	α-Terpineol	1181	1185	11
25	24.2	Piperitenone	1322	1320	12
26	24.76	Piperitenone oxide	1344	1352	12
27	25.85	α-Ylangene	1384	1373	8
28	25.97	α-Copaene	1388	1398	11
29	26.76	γ-Caryophyllene	1419	1403	12
30	27.01	α-Santalene	1429	1428	12
31	27.16	Caryophyllene	1435	1428	11
32	27.36	*trans*-α-Bergamotene + unknown	1443	1443	12
33	27.49	α-Guaiene	1448	1441	8
34	27.56	*trans*-β-Farnesene	1451	1446	12
35	27.98	Humulene	1467	1465	11
36	28.17	Alloaromadendrene	1475	1478	11
37	28.25	α-Curcumene	1478	1479	12
38	28.75	β-Selinene	1497	1487	8
39	28.97	α-Selinene	1507	1497	8
40	28.97	β-Bisobolene	1507	1506	8
41	29.13	α-Bulnesene	1514	1513	12
42	30.12	Selina-3,7(11)-diene	1556	1542	12
43	30.94	Caryophyllene oxide	1590	1595	12
44	31.5	Humulene oxide	1614	1599	12
45	32.48	Caryophylla-3, 8(13)-dien-5-ol A	1658	1656	12

Conclusion

A simple headspace SPME-GC/MS method was used in the analysis of the terpene/terpenoid profiles of both hops and cannabis. The method was able to detect the characteristic terpenes and terpenoids of both, and to distinguish between different hops varieties.

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Description	Cat. No.
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Equity®-1, 60 m × 0.25 mm I.D., 0.25 µm	**28047**
SPME Fibers and Accessories	
SPME fiber assembly Divinylbenzene/Carboxen®/Polydimethylsiloxane (DVB/CAR/PDMS), d_f 50/30 µm, needle size 23 ga, StableFlex™, for use with autosampler, pk of 3	**57298-U**
SPME fiber assembly Polydimethylsiloxane (PDMS), d_f 100 µm (nonbonded phase), needle size 23 ga, for use with autosampler, pk of 3	**57341-U**
SPME fiber holder for CTC autosampler	**57347-U**
SPME fiber holder for manual sampling	**57330-U**
Accessories	
Inlet Liner, Direct (SPME) Type, straight design, 0.75 mm I.D. for Agilent® GC	**2637501**
Molded Thermogreen® LB-2 Septa, with injection hole, 11 mm, pk of 50	**28336-U**
Headspace Vial, screw top, rounded bottom, 10 mL, clear glass, pk of 100	**SU860099**
Magnetic Screw Cap for Headspace Vials, PTFE/silicone septum, pk of 100	**SU860103**

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As described in the previous application article (pages 9-12), terpenoids are an important group of natural products in many different fields. They play an important role in spices, aromatherapy, perfumes, phytochemicals and are responsible for the intense scent of cannabis. In addition, some of the medicinal or physiological effects found in cannabis are attributed to specific terpenoids. Since terpenes testing and profiling can be used to better understand the characteristics but even more to identify and classify different strains reliable reference materials are needed.

For this purpose, we developed two terpene mixes specifically required for cannabis testing (CRM40755 and CRM40937) and established more than 60 single component CRM solutions for terpenoids. The concentrations of these mixtures and solutions are typically 2000 µg/mL in methanol.

Find these listed below. We also offer nearly 250 neat standards of isoprenoids, which can be found at **SigmaAldrich.com/terpenoids.**

Please also find an overview of applications and products suitable for cannabis testing at **SigmaAldrich.com/cannabis.**

Table 1: Components of Cannabis Terpene Mix A CRM40755

#	Compound	Concentration	CAS Number
	Internal Standard	2000 µg/mL	N/A
2	α-Pinene	2000 µg/mL	80-56-8
3	Camphene	2000 µg/mL	79-92-5
4	β-Pinene	2000 µg/mL	127-91-3
5	3-Carene	2000 µg/mL	13466-78-9
6	α-Terpinene	2000 µg/mL	99-86-5
7	(R)-(+)-Limonene	2000 µg/mL	5989-27-5
8	γ-Terpinene	2000 µg/mL	99-85-4
9	L-(-)-Fenchone	2000 µg/mL	7787-20-4
10	Fenchol	2000 µg/mL	2217-02-9
11	(1R) (+) Camphor	2000 µg/mL	464-49-3
12	Isoborneol	2000 µg/mL	124-76-5
13	Menthol	2000 µg/mL	89-78-1
14	Citronellol	2000 µg/mL	106-22-9
15	(+)-Pulegone	2000 µg/mL	89-82-7
16	Geranyl acetate	2000 µg/mL	105-87-3
17	α Cedrene	2000 µg/mL	469-61-4
18	α Humulene	2000 µg/mL	6753-98-6
19	Nerolidol*	2000 µg/mL	7212-44-4
20	(+)-Cedrol	2000 µg/mL	77-53-2
21	(-)-α-Bisabolol	2000 µg/mL	23089-26-1

Table 2: Components of Cannabis Terpene Mix B CRM40937

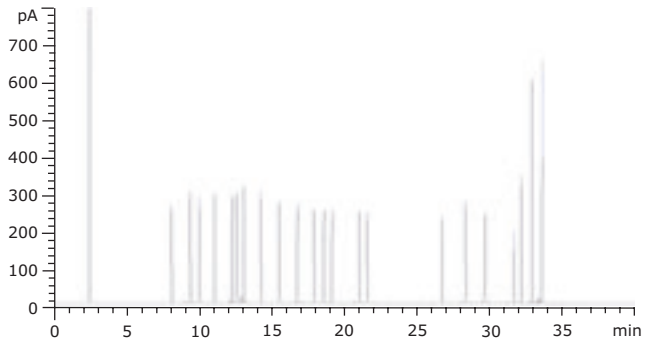
#	Compound	Concentration	CAS Number
	Internal Standard	2000 µg/mL	N/A
2	β-Pinene	2000 µg/mL	18172-67-3
3	(1S)-(+)-3-Carene	2000 µg/mL	498-15-7
4	p-Cymene	2000 µg/mL	99-87-6
5	Limonene	2000 µg/mL	138-86-3
6	Terpinolene	2000 µg/mL	586-62-9
7	Linalool	2000 µg/mL	78-70-6
8	(1S)-(-)-Camphor	2000 µg/mL	464-48-2
9	(+)-Borneol	2000 µg/mL	464-43-7
10	(-)-α-Terpineol	2000 µg/mL	10482-56-1
11	Geraniol	2000 µg/mL	106-24-1
12	β-Caryophyllene	2000 µg/mL	87-44-5
13	cis-Nerolidol	2000 µg/mL	3790-78-1
14	β-Eudesmol	2000 µg/mL	473-15-4
15	Phytol**	2000 µg/mL	7541-49-3

* Area sum of isomers
**Certified as sum of isomers

(continued on next page)

Cannabis Terpene Mix A - CRM40755

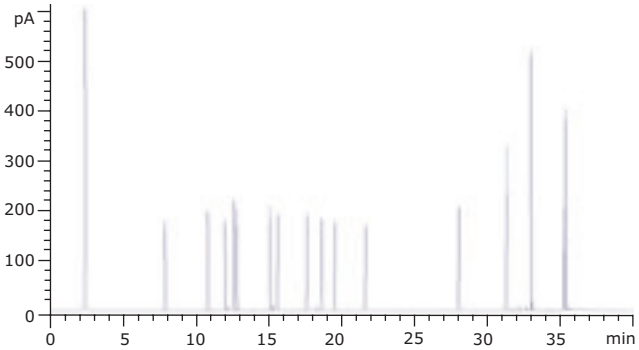
Column:	SLB®-5ms 30 m x 0.25 mm x 0.25 µm 60 °C for 3.5 min, 3.5 °C/min to 155 °C, 30 °C/min to 300 °C, hold 5 min
Detector:	FID, 310 °C
Injection Volume:	1 µL



Peak IDs: see listing in table 1

Cannabis Terpene Mix B - CRM40937

Column:	SLB®-5ms; 30 m x 0.25 mm x 0.25 µm df 60 °C for 3.5 min, 3.5 °C/min to 155 °C, 30 °C/min to 300 °C, hold 5 min
Detector:	FID,310 °C
Injection Volume:	1 µL



Peak IDs: see listing in table 2

Table 3: Single Component Solutions

Solution of	Qty.	Cat. No.
(-)-Bisabolol	1 mL	**CRM40763**
(+)-Borneol	1 mL	**CRM40901**
(-)-Borneol	1 mL	**CRM40456**
Camphene	1 mL	**CRM40378**
(±)-Camphor	1 mL	**CRM40393**
(1R)-(+)-Camphor	1 mL	**CRM40374**
(+)-3-δ-Carene	1 mL	**CRM40416**
β-Caryophyllene	1 mL	**CRM40483**
(-)-Caryophyllenne oxide	1 mL	**CRM40928**
Cedrene	1 mL	**CRM40482**
Cedrol	1 mL	**CRM40903**
Cetone	1 mL	**CRM40383**
Citronellol	1 mL	**CRM40469**
p-Cymene	1 mL	**CRM40419**
Dihydrocarveol	1 mL	**CRM40914**
Eucalyptol	1 mL	**CRM40684**
Farnesol	1 mL	**CRM40689**
Fenchol	1 mL	**CRM40746**
(+)-Fenchone	1 mL	**CRM40747**
L-(-)-Fenchone	1 mL	**CRM40762**
Geraniol	1 mL	**CRM40749**
Geranyl acetate	1 mL	**CRM40764**
Guaiacol	1 mL	**CRM40484**
(-)-Guaiol	1 mL	**CRM40917**
Humulene	1 mL	**CRM40921**
Hydroxycitronellal	1 mL	**CRM40908**
Isoborneol	1 mL	**CRM40471**
Isolongifolene	1 mL	**CRM40391**
Isopulegol	1 mL	**CRM40757**

Solution of	Qty.	Cat. No.
Lilial	1 mL	**CRM40184**
(R)-(+)-Limonene	1 mL	**CRM40422**
Linalool	1 mL	**CRM40437**
Longifolene	1 mL	**CRM40481**
Lyral	1 mL	**CRM40911**
(+)-Menthol	1 mL	**CRM40474**
β-Myrcene	1 mL	**CRM42262**
Nerol	1 mL	**CRM40744**
cis-Nerolidal	1 mL	**CRM40743**
trans-Nerolidal	1 mL	**CRM40742**
Nerolidol	1 mL	**CRM40906**
cis/trans-Ocimene	1 mL	**CRM40748**
Phytol	1 mL	**CRM40375**
(-)-β-Pinene	1 mL	**CRM40417**
(+/-)-α-Pinene	1 mL	**CRM40339**
(+/-)-β-Pinene	1 mL	**CRM40433**
Safrole	1 mL	**CRM40478**
α-Terpinene	1 mL	**CRM40443**
γ-Terpinene	1 mL	**CRM40431**
(-)-α-Terpineo	1 mL	**CRM40907**
α-Terpineol	1 mL	**CRM40428**
Terpinolene	1 mL	**CRM40929**
α & β-Thujone	1 mL	**CRM40909**
Thymol	1 mL	**CRM40188**
Valencene	1 mL	**CRM40934**
Verbenon	1 mL	**CRM40189**

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New Certified Reference Materials for α-Lactalbumin, β-Lactoglobulin A & B, and Lactoferrin

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Abstract:

We have developed Certified Reference Materials (CRMs) of fully intact milk proteins that allow accurate testing of the most critical proteins in infant formula; help labs to develop and test infant formula products with high accuracy, while ensuring legal and regulatory compliance; and support food and beverage manufacturers implement and routinely perform quality assurance and quality control programs with accuracy and confidence.

Introduction

Mother's milk is the best source of nutrition for nearly all infants. Beyond somatic growth, breast milk as a biologic fluid has a variety of other benefits, including modulation of postnatal intestinal function, immune ontogeny, and brain development. Although breastfeeding is highly recommended, it may not always be possible, suitable or solely adequate. In China for example, less than 30% of infants younger than 6 months are exclusively breastfed.[1]

The 2008 milk scandal was a widespread food safety incident in China. The scandal involved milk and infant formula along with other food materials and components being adulterated with melamine to give the appearance of higher protein content. Of an estimated 300,000 victims in China, six babies died and an estimated 54,000 babies were hospitalized. In a separate incident four years prior, watered-down milk had resulted in 12 infant deaths from malnutrition.

The World Health Organization referred to these incidents as one of the largest food safety events ever encountered. After this series of scandals involving contaminated milk and added pressure from the One Child Policy, Chinese parents lost confidence in domestically manufactured infant formula. Beginning January 1, 2018, the infant formula registration requirements in the new Food Safety Law came fully into force. Therefore, all infant formula products must obtain formula registration with CFDA before they can be sold in China.[2]

Since 2008, regulation of the global infant formula industry has undergone constant reform and great progress has been made in infant formula testing methods. Industry methods have been moving from immunoassay testing to LC-MS/MS for greater accuracy and sensitivity.

To help provide children around the world with safe and nutrient-rich infant formula, as well as support new regulations, we have developed Certified Reference Materials (CRMs) of fully intact milk proteins. These CRMs allow accurate testing of the most critical proteins in infant formula through development and routine calibration of LC-MS/MS methods to ensure quality of infant formula products with high accuracy and confidence, while ensuring legal and regulatory compliance.

Infant Formula

Infant formula is an industrially produced milk substitute designed for infant consumption that attempts to mimic the nutritional composition of breast milk as closely as possible, and is usually based on cow's milk or soy milk. Human and bovine milk differ substantially in the ratio of whey to casein protein, with whey protein content in human milk being significantly higher. Because of the differences in the protein profiles of human milk and infant formula, amino acid profiles also differ. An important goal in the improvement of infant formulas is to more closely match the total protein content and the protein profile of human milk.

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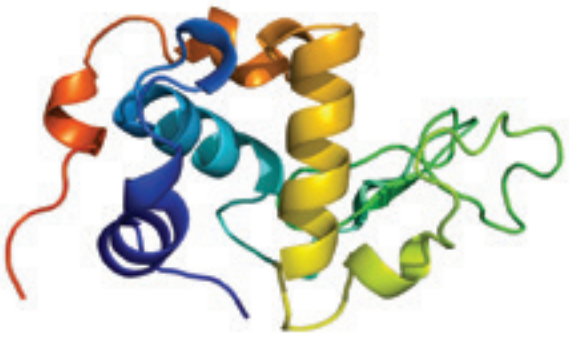
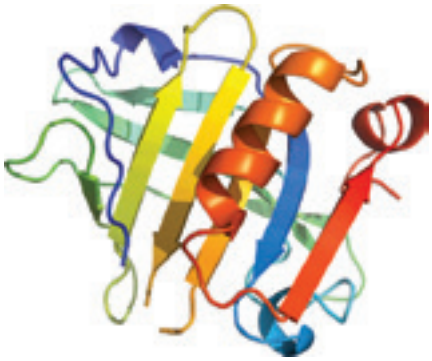
Milk whey protein represents a rich mixture of proteins with wide ranging nutritional, biological and food functional attributes. The main constituents are α-lactalbumin, β-lactoglobulin, and lactoferrin, which account for approximately 70–80% of total whey protein. These three proteins are of high nutritional value, which have made them ingredients of choice in the formulation of modern foods and beverages. They have physiological activity through moderating gut microflora, mineral absorption, and immune function.



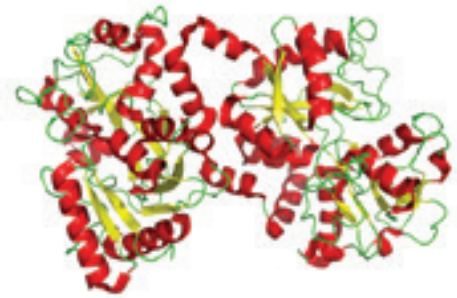
α-Lactalbumin

α-Lactalbumin is an important whey protein that regulates the production of lactose in milk and is present in the milk of almost all mammalian species.



β-Lactoglobulin A & B

β-Lactoglobulin A & B is the major whey protein of cow and sheep's milk (~3 g/l), and is also present in many other mammalian species with an interesting exception being humans. β-Lactoglobulin can cause severe allergic effects.



Lactoferrin

Lactoferrin, also known as lactotransferrin, is a glycoprotein widely represented in various secretory fluids including milk. Lactoferrin is part of the immune system and has antimicrobial activity.

Although current infant formulas closely mimic the ratio of total whey to casein in human milk, the concentration of α-lactalbumin, the dominant protein in human milk, is relatively low in formula, whereas β-lactoglobulin, a protein not found in human milk which can cause severe allergic effects, is the most dominant whey protein in formula. Lactoferrin is a multifunctional and biologically active iron-binding glycoprotein existing in both human and bovine milk. Its physiological functions act as an antimicrobial/antiviral, immunomodulatory agent, and antioxidant. Oral administration of lactoferrin can benefit both infants and adults.[3]

The new full-length protein CRMs suitable as starting material for use in calibrators or controls for a variety of LC-MS/MS testing applications including allergen testing, infant formula testing, dietary, or nutritional testing applications.

Product Features

- Certified Reference Material – stability, homogeneity, uncertainty, and traceability defined
- Product manufactured according to ISO 17025 & ISO Guide 34
- Stability predetermined and supported with testing data
- Comprehensive Certificate of Analysis

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Featured Products

Description	Qty.	Cat. No.
α-Lactalbumin, (from bovine milk), neat, Certified Reference Material	100 mg	**L-045-100MG**
β-Lactoglobulin A & B (from bovine milk), neat, Certified Reference Material	100 mg	**L-046-100MG**
Lactoferrin (from bovine milk), neat, Certified Reference Material	50 mg	**L-047-50MG**

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ENVIRONMENTAL

Improved Determination of Volatile Organic Compounds in Water by SPME and GC/MS: ISO Standard 17943

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The analysis of water for volatile organic compounds is important due to their toxicity. The current methods for this determination lack sensitivity, selectivity or capability for automation. This paper presents the new ISO 17943 Standard using Solid Phase Microextraction (SPME) and GC/MS. The sample preparation by SPME enables lower limits of detection and easy automation of the entire method. GC/MS provides the required sensitivity and selectivity. This ISO standard was validated by an interlaboratory trial, whose results confirm the outstanding performance of this method.

Introduction

Volatile Organic Compounds (VOCs) can occur from natural sources such as plant scents. However, a large amount of VOCs do have an anthropogenic origin, because they are released from products in daily use or emitted during the manufacturing of such products, as well as from polymers, adhesives, paints, petroleum products or pharmaceuticals. Typical applications for VOCs are use as additives for gasoline or as solvents and hydraulic fluids or for dry-cleaning. As many VOCs are toxic or are known or suspected human carcinogens, contamination of water resources is a serious human health concern worldwide.

Because of this, many international regulations have been established to limit and control the amount of VOCs in drinking water, groundwater or surface water. Examples of such regulations are the Safe Drinking Water Act (SDWA)[1] in the USA, and a corresponding law in Canada that established national standards for drinking water including VOC listings that are based on health considerations. Another example is the European Council Directive 98/83/EC on the quality of water intended for human consumption that regulates the values for individual volatile organic substances[2]. In the EU Water Framework Directive (WFD) in article 16 of the Directive 200/60/EC[3] a "strategy against pollution of water" is described.

Figure 1. SPME Manual Holder

According to Directive 2008/105/EC (EQS Directive)[4] Environmental Quality Standards (EQS) values for single VOCs should be in the range of 0.4 to 20 µg/L. In annex V of WFD (standards for monitoring of quality elements) the use of ISO and CEN standards for the analysis of water is required, if available.

The existing ISO and CEN standards for the determination of VOCs in water are not state-of-the-art methods anymore. ISO 10301[5] uses Liquid/Liquid Extraction (LLE) in combination with Gas Chromatography (GC) and detection using Flame Ionization Detection (FID) or Electron Capture Detection (ECD). ISO 11423[6] employs headspace (HS) sampling in combination with GC/FID or GC/ECD. For certain relevant VOCs, the required limits of detection cannot be achieved using these ISO standards because the detectors are not sensitive or selective enough. ISO 15680[7] exhibits an alternative by using purge-and-trap enrichment and Gas Chromatography-Mass Spectrometry (GC-MS) analysis leading to better selectivity and limits of detection. The downside of purge-and-trap is the susceptibility of the trap to become contaminated and that automation is rather challenging to achieve[8].

Improved Method for Determination of VOCs in Water by HS-SPME and GC/MS: ISO Standard 17943

Solid Phase Microextraction (SPME) in combination with GC-MS is an attractive alternative for the determination of VOCs in water. SPME was developed by Janusz Pawliszyn in 1990[9] (**Figure 1**). Since then SPME has gained broader acceptance in environmental, pharmaceutical and food analysis as demonstrated by the growing number of publications on SPME developments and applications. The prevalence of this technique was additionally increased by the automation of SPME using regular GC autosamplers beginning in 1993. The use of SPME for the extraction of VOCs from water is described in several publications[10-12]. In these publications, headspace SPME (HS-SPME) was proven to be a reliable and beneficial alternative to classical methods for VOC determination in water. Furthermore, SPME has been successfully used in many other official methods[13-15].

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Due to this, the new ISO standard 17943 was developed for VOCs in water. The scope of the standard is the determination of more than 60 VOCs from very different classes such as halogenated hydrocarbons, gasoline additives (like BTEX, MTBE and ETBE), volatile aromatic compounds and highly odorous substances like geosmin and 2-methylisoborneol in drinking water, groundwater, surface water and treated wastewater by HS-SPME and GC-MS. Of course the limit of detection depends on the matrix, on the specific compound and on the applied mass spectrometer, but for most compounds in ISO 17943, it is equal to or better than 0.01 µg/L. Additional validation data derived from standardization work show applicability of the method within a concentration range from 0.01 µg/L to 100 µg/L for individual substances.

Global Interlaboratory Trial for Validation of New ISO Standard 17943

As part of the development of this new ISO standard, an international interlaboratory trial was conducted to validate the new method[16]. Each of the labs had to determine the concentration of 61 compounds in the two water samples (one surface water, one wastewater). The surface water sample was taken from an urban and industrialized area (the Ruhr River in Muelheim, Germany). The municipal wastewater sample was taken from a plant effluent. Both samples had been pre-treated to stabilize them and had been spiked with concentrations unknown to the participating labs in the range of 0.02 – 0.80 µg/L (~ 50 % < 0.10 g/L) for the surface water and 0.05 – 3.0 µg/L (~ 50 % < 0.50 g/L) for the wastewater. The labs in the interlaboratory trial had to conduct four independent replicate analyses from each of the two samples, strictly following the procedure as prescribed in the draft standard method. All laboratories were provided with a set of calibration solutions placed in three ampoules each containing certified reference substances of the 61 VOCs dissolved in methanol. These stock solutions contained the individual substances in concentrations of 100 µg/mL each and were intended to be used for preparation of the corresponding aqueous multi-component reference solutions used for calibrating the total procedure. The results had to be delivered within 30 days after receipt of the samples.

The Supelco® Application Lab was one participant in the interlaboratory trial. The two water samples were analyzed according to the drafted ISO Standard 17943 (**Table 1 & 2**, **Figure 2**) using toluene-d_8, benzene-d_6 and fluorobenzene as internal standards. For the GC analysis a VOCOL capillary GC column was used, which is an intermediate polarity column that is designed for analysis of VOCs and provides great retention and resolution of highly volatile compounds. For HS-SPME a DVB/CAR/PDMS fiber was used which was also used by the majority of the interlaboratory trial participants. A smaller share of the labs used a CAR/PDMS fiber. According to ISO Standard 17943 both the Carboxen/PDMS (85 µm) and the DVB/Carboxen/PDMS (50/30 µm) fiber can be used.

Table 1. Conditions for HS-SPME Extraction

Sample volume:	10 mL
HS-Vial:	20 mL, addition of 3 g salt
SPME fiber:	DVB/CAR/PDMS, 24 gauge
Incubation time:	10 min @ 40 °C
Extraction time:	10 min @ 40 °C
Autosampler:	CTC CombiPAL™ (agitated by circular motion of the vial, velocity: 250 rpm)

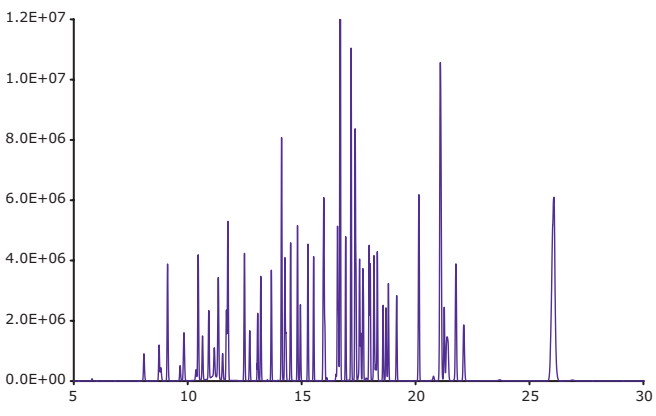
Table 2. Conditions for GC/MS Analysis

GC:	Agilent® GC/MS
Column:	VOCOL®, 60 m x 0,25 mm I.D., 1.5 µm
Carrier gas:	He, 1 mL/min
Injection/Liner:	Splitless, SPME liner w/ 0,75 mm ID
Desorption/ Injector:	10 min @ 270 °C
Oven program:	35 °C, 1 min; 10 °C/min to 150 °C; 20 °C/min to 250, 20 min
Sample:	61 VOCs, 1 ppm, in water plus three internal standards

Figure 2. Chromatogram of 61 VOCs in water after HS-SPME using a VOCOL® GC column on an Agilent® GC/MS

Compound Name	RT (min)
Vinyl chloride	5.8
1,1-Dichloroethene	8.077
Methylenechloride	8.743
MTBE	8.819
trans-1,2-Dichloroethylene	9.113
1,1-Dichloroethane	9.667
ETBE	9.834
2,2-Dichloropropane	10.365
cis-1,2-Dichloroethylene	10.46
Trichloromethane	10.649
Bromochloromethane	10.927
1,1,1-Trichloroethane	11.166
TAME	11.339
1,1-Dichloro-1-propene	11.344
Carbon tetrachloride	11.533
1,2-Dichloroethane	11.7
Benzene	11.761
Trichloroethylene	12.491
1,2-Dichloropropane	12.722
Bromodichloromethane	13.073
Dibromomethane	13.21
cis-1,3-Dichloro-1-propene	13.671
Toluene	14.119
trans-1,3-Dichloro-1-propene	14.267
2-Ethyl-4-methyl-1,3-dioxolane	14.311
1,1,2-Trichloroethane	14.52
1,3-Dichloropropane	14.817
Tetrachloroethylene	14.946
Dibromochloromethane	15.277
1,2-Dibromoethane	15.527
Ethylbenzene	15.945

Compound Name	RT (min)
Chlorobenzene	15.975
1,1,1-2-tetrachloroethane	15.983
p-Xylene	16.573
o-Xylene	16.573
Styrene	16.619
2-Ethyl-5,5-dimethyl-1,3-dioxane	16.695
Cumene	16.933
Bromoform	17.162
1,1,2,2,-Tetrachloroethane	17.175
1,2,3-Trichloropropane	17.346
Propylbenzene	17.386
Pseudocumene	17.544
Bromobenzene	17.596
2-Chlorotoluene	17.688
4-Chlorotoluene	17.688
tert-Butylbenzene	17.966
Mesitylene	18.015
sec-Butylbenzene	18.173
p-Cymene	18.32
1,3-Dichlorobenzene	18.577
1,4-Dichlorobenzene	18.698
Butylbenzene	18.807
1,2-Dichlorobenzene	19.17
DBCP	20.145
2-Methylisoborneol	21.087
1,2,4-Trichlorobenzene	21.257
Hexachlorobutadiene	21.386
Naphthalene	21.773
1,3,5-Trichlorobenzene	22.113
Geosmin	26.074



Evaluation of the Interlaboratory Trial

More than 40 labs from all over the world registered for this interlaboratory trial. Out of these a total of 27 labs reported results to be included in the evaluation process according ISO 5725-2[17]. Nine laboratories did not submit any results. Six labs had to be excluded from the valuation due to significant deviation from the prescribed procedure. Some single results had to be excluded due to outliers.

All 61 parameters had been analyzed by ten labs and nearly all parameters had been analyzed by nine labs. Expressed in a different way, this resulted in the fact that nearly each of the 61 VOCs had been analyzed by more than 20 labs, which provides a valid base for statistical evaluation. The data was analyzed for the overall mean of results (without outliers), the recovery rate (from assigned value), the reproducibility (variation between different labs) and the repeatability (variation within a lab).

One example of such an evaluation is shown in **Figure 3** for 2-chlorotoluene. For this compound, results from 24 labs could be evaluated. The overall mean value (green line) is very close to the assigned value (purple line). The majority of the 24 labs, even those labs that were new to SPME, achieved results very close to the assigned value. The recovery rate for more than 90% of the compounds was between 84 and 116 % (surface water) and 81 and 118 % (wastewater). The reproducibility (variation between laboratories), for more than 90% of the compounds, was less than 31% (surface water) and less than 35% (wastewater), while the repeatability (variation within a lab) for more than 90% of the compounds was less than 10% (surface water) and less than 8% (wastewater).

Summary

The outstanding results in the interlaboratory trial underscore the high performance, reliability and reproducibility of HS-SPME in combination with GC/MS for the determination of VOCs in water. The new ISO 17943 is an improvement on existing official methods for this determination in terms of sensitivity and selectivity. In addition, the capability for full automation of SPME is beneficial for running this analysis 24/7.

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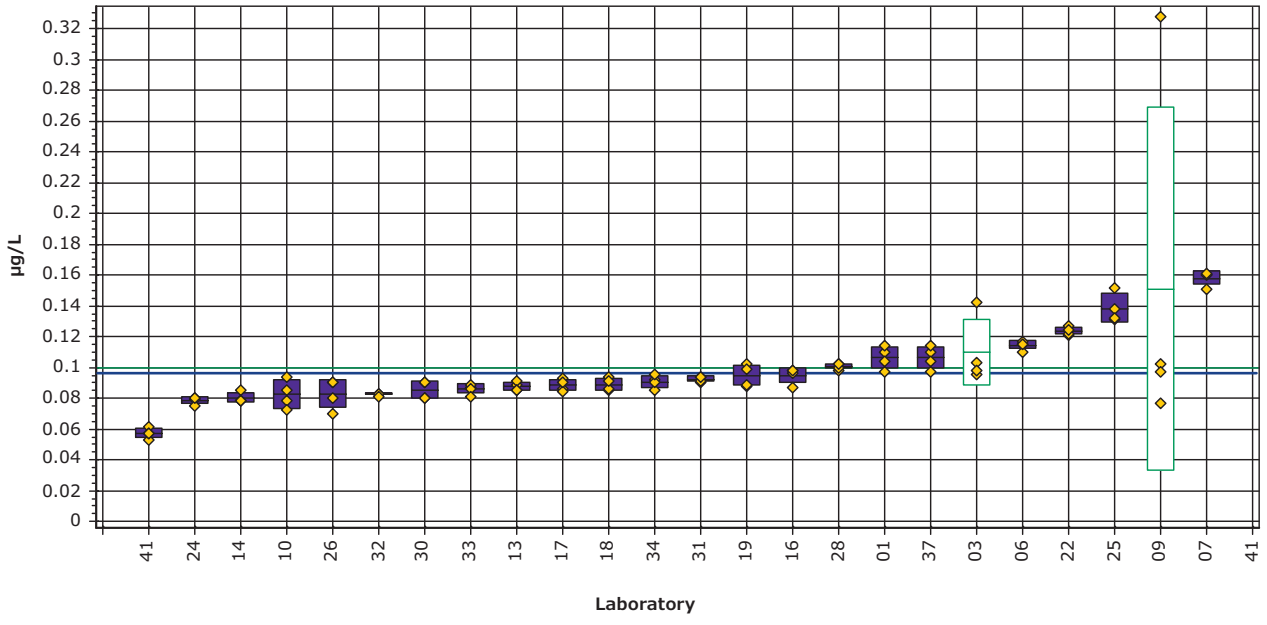
Figure 3. Graphical presentation of the example of 2-chlorotoluene which shows the results of the interlaboratory trial for the validation of ISO 17943. The purple horizontal line is the assigned value; the green horizontal line is overall mean.



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Description	Qty.	Cat.No.
SPME Fiber Divinylbenzene/Carboxen®/ Polydimethylsiloxane (DVB/CAR/PDMS), 24 ga, for use with autosampler	3	**57329-U**
SPME Fiber Holder for use with CTC CombiPAL™, Gerstel® MPS2 and Thermo® TriPlus Autosamplers	1	**57347-U**
VOCOL® Capillary GC Column, 60 m × 0.25 mm, df 1.50 µm	1	**24154**

Description	Qty.	Cat.No.
Reference Standards		
ISO 17943 57 Component VOC Mix, 200 µg/mL each component in methanol, 1 mL, Certified Reference Material	1 mL	**44926-U**
ISO 17943 Odor Compounds Mix, 200 µg/mL each component in methanol, 1 mL, Certified Reference Material	1 mL	**44923-U**
1,3,5-Trichlorobenzene Certified Reference Material, TraceCERT®	100 mg	**3824**
Vinyl chloride solution 200 µg/mL in methanol, analytical standard	1 mL	**48625**
Accessories		
Headspace vial, screw top, rounded bottom (vial only) volume 20 mL, amber glass vial, Pk. 100	100	**SU860098**
Magnetic Screw Cap for Headspace Vials, 18 mm thread PTFE/silicone septum, septum thickness 1.3 mm, Pk. 100	100	**SU860101**

Related Products

Description	Qty.	Cat.No.
SPME Fiber Carboxen®/Polydimethylsiloxane (CAR/PDMS) 85 µm, 24 ga, StableFlex™ fiber, for use with autosampler	3	**57335-U**
SPME fiber Divinylbenzene/Carboxen®/ Polydimethylsiloxane (DVB/CAR/PDMS), 23 ga, StableFlex™, for use with autosampler	3	**57298-U**
SPME fiber Carboxen®/Polydimethylsiloxane (CAR/PDMS) df 85 µm, needle size 23 ga, StableFlex™, for use with autosampler	3	**57295-U**

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CHEMICAL INDUSTRY

Ultrasensitive Determination of Silicate in Process and Boiler Water Using Rapid Photometric Tests

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Economic efficiency is becoming an ever more important aspect of everyday life, with the efficiency of industrial plants and equipment constituting one of the basic preconditions for sustainable economic operations. One avoidable problem that can result in losses of efficiency is posed by the undesirable build-up of deposits, i.e., scale – in pipes, boilers, and turbines.

A principal cause of scale in such equipment is silicate. Especially at high pressures – as in high-pressure turbines – silicate is deposited on the internal surfaces. This problem occurs mainly as a result of silicate dissolved in the steam.[1]

The expansion of the steam results in a reduction of the solubility capacity of silicate, which in turns leads to the formation of solid silicon dioxide on the surrounding surfaces, for example the turbine blades, reducing the efficiency of the plant.[2]

One measure that can help to minimize the need for time-consuming cleaning operations that interrupt the operation of the machine is to regularly inspect the boiler and boiler feed water for their silicate concentrations.

The guidance values depend on a variety of operating conditions (e.g., steam capacity, heating-surface load, and working pressure) of the boiler. In high-pressure turbines, even the slightest concentration of silicate in the steam can lead to deposits. To avoid such deposits, in most cases it is recommended to prevent steam silicate concentrations from exceeding 20 µg/L SiO_2.[3,4] Depending on the operating conditions, the limit for silicate may even be as low as 10 µg/L SiO_2 or less.[2]

Analytical methods

The determination of concentrations of silicate in such a low range requires an extremely sensitive detection method. Graphite furnace atomic absorption spectrometry (GF-AAS) is frequently the method of choice here, capable of detecting concentrations of silicate down into the lower ppb range. Besides the element-analytical methods, classic photometry has also proven to be a reliable method. This method is based on the reaction of silicate ions in acidic solution with molybdate ions to produce yellow silicomolybdic acid. The addition of a suitable reduction agent then produces deep blue silicomolybdenum blue, which is subsequently determined photometrically.[5]

Silicate test kit

The molybdenum blue method is also the principle used in the photometric silicate test (Cat. No. 101813) of our Spectroquant® test kit series.

The advantage of this test is that it is quick and easy to use without major instrument investment needed. All necessary reagents are supplied in the test kit in a ready-to-use format. Compared with classic photometry, the use of the corresponding Spectroquant® photometers enables the time-consuming calibration procedure to be dispensed with, since the method is already pre-programmed into the devices. Using the 100-mm cell, the Prove 600 spectrophotometer is capable of measuring silicate concentrations as low as 0.25 µg/L SiO_2, thus ensuring the detection of extremely low amounts of dissolved silicate. The overall measuring range of the test kit is 0.25–500.0 µg/L SiO_2.

Figure 1. Spectroquant® Prove 600 photometer

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Performance of the measurement with Spectroquant® Silicate Test

The silicate content of process-water samples lies within the lower part of the measuring range of the test kit. During the course of the experiments it was found that the precision in the lower part of the measuring range can be enhanced when reagents Si-1 and Si-2 are added with a pipette instead of dropwise.

The procedure for the tests was correspondingly adapted. Moreover, the procedure described in the instruction sheet enclosed with the product has been changed from dropping to pipetting to ensure the highest possible accuracy. Care was also taken to ensure that no glass equipment was used during the entire procedure. In the event of any turbidity of the sample solution, this must be filtered beforehand.

The silicate test starts by pipetting 20 mL of sample solution into a plastic test vessel, after which 200 µL of reagent Si-1 is added. The solution is mixed and then left to stand for 5 minutes. After the standing time, 200 µL reagent Si-2 is added and the solution mixed, then 1.00 mL of reagent Si-3 is added. The solution is mixed once again, left to react for 5 minutes, and then measured in the photometer against a reagent blank prepared with Ultrapure water in an analogous manner.

A detailed description of the procedure is described in the application "Ultrasensitive determination of silicate in process and boiler water". The application can be found online on the product page for Spectroquant® Silicate Test 101813.

Standard addition with Spectroquant® Silicate Test

In an experiment to gain an expressive statement on the suitability of the Spectroquant® Silicate Test for the determination of the silicate content in process water, the standard addition method was applied to five samples. Each sample was spiked with three different concentrations of silicate. In order to determine the recovered silicate concentration, the silicate concentration of the sample, also gained using the Silicate Test, was subtracted from the measured result of the spiked sample. For evaluation, the deviation of the recovered concentration from the target value (spiked concentration) was calculated. The results are shown in **Table 1**.

Table 1. Recovered content of silicate

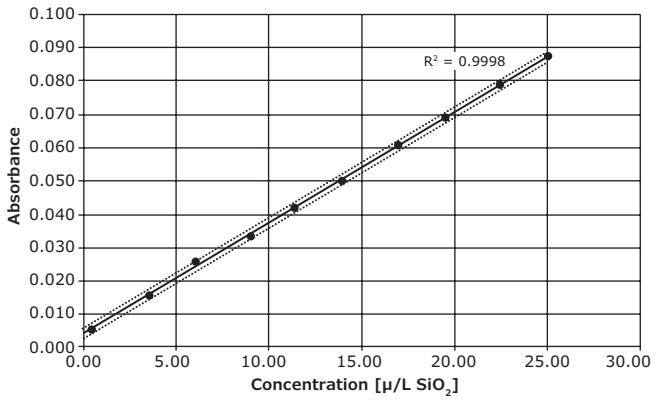
Sample	Addition [µg/L SiO_2]	Recovered concentration [µg/L SiO_2]	Deviation [µg/L SiO_2]
Ultrapure water	1.00	0.86	0.14
	5.00	6.25	1.25
	10.00	10.60	0.60
Steam water from power plant	1.00	1.83	0.83
	5.00	6.14	1.14
	10.00	11.01	1.01
Boiler water from power plant	1.00	1.24	0.24
	5.00	6.09	1.09
	10.00	10.20	0.20
DI water	1.00	1.97	0.97
	5.00	5.74	0.74
	10.00	11.31	1.31
Double-distilled water	1.00	1.75	0.75
	5.00	7.40	2.40
	10.00	11.53	1.53

The recovered silicate spikes all lie within the 95% confidence value of 3.33 µg/L SiO_2. The value of the deviation from the respective spike lies between 0.14 and 2.40 µg/L SiO_2, averaging out at 0.93 µg/L SiO_2.

The accuracy of the pre-programmed method is sufficient for many users. Users for whom the error rate of the pre-programmed method is still too large can, however, enhance the accuracy of the method by plotting their own custom calibration curve, thus eliminating batch-specific fluctuations and the user's own systematic errors.

A calibration curve was plotted for the Spectroquant® Silicate Test for the measurement range 0.50–25.00 µg/L SiO_2, see **Figure 2**.

Figure 2. Calibration curve of Spectroquant® Silicate Test 101813, measuring range 0.50–25.00 µg/L SiO_2



In the case of the silicate test, the custom calibration curve was able to improve the performance characteristics obtained acc. to ISO 8466-1, respectively DIN 38402 A51. A comparison of the performance characteristics of the pre-programmed method vs. the custom calibration is presented in **Table 2**.

Table 2. Comparison of performance characteristics

	Pre-programmed method 0.25 – 250.00 µg/L SiO_2	Custom calibration 0.50 – 25.00 µg/L SiO_2
Method standard deviation [µg/L]	± 0.790	± 0.185
Method coefficient of variation [%]	± 0.62	± 1.44
Confidence interval (P=95 %) [µg/L]	± 3.33	± 0.45

At a value of 1.44%, the method coefficient of variation is 2.5 times higher than that of the pre-programmed method. This can be attributed to the fact that deviations have, in relative terms, a stronger effect in the lower measurement range as a result of the custom calibration. Seen in absolute terms, the custom calibration procedure can, however, result in considerably lower method errors, as shown by the values of the standard deviation for the procedure and the confidence interval. The method standard deviation and the confidence interval for P=95 % of the custom calibration are 76% (standard deviation) and 86% (confidence interval) lower than those for the pre-programmed method.

When the standard additions are evaluated using the user-specific calibration function, the deviations can be reduced down to the expected values. On average, the value of the deviation was now 0.29 µg/L, indicating a reduction from the original value of 0.94 µg/L by almost 70%. The measurement values are presented in **Table 3**.

Table 3. Recovered contents of silicate, evaluated against the custom calibration

Sample	Addition [µg/L SiO_2]	Recovered concentration [µg/L SiO_2]	Deviation [µg/L SiO_2]
Ultrapure water	1.00	0.70	0.30
	5.00	4.83	0.17
	10.00	10.28	0.28
Steam water from power plant	1.00	1.15	0.15
	5.00	5.42	0.42
	10.00	10.13	0.13
Boiler water from power plant	1.00	1.00	0.00
	5.00	5.12	0.12
	10.00	9.54	0.46
DI water	1.00	1.15	0.15
	5.00	4.97	0.03
	10.00	10.28	0.28
Double-distilled water	1.00	0.85	0.15
	5.00	6.45	1.45
	10.00	10.57	0.57

Method comparison of GF-AAS and Spectroquant® Silicate Test

Besides the standard addition experiments, a reference analysis was also performed: the silicate content of the five water samples was quantified by a GF-AAS method. The Limit Of Quantitation (LOQ) of the GF-AAS method was determined using the ten-fold standard deviation of the blank, yielding a value of 1.93 µg/L SiO_2. **Table 4** compares the results from the GF-AAS method with those of the photometric determination method, calculated using the pre-programmed method and the custom calibration.

Table 4. Comparison of the results of the Spectroquant® Silicate Test 101813 and the GF-AAS reference analysis

Sample	GF-AAS	Concentration [µg/L SiO_2] Spectroquant® Silicate Test	
		Pre-programmed method	Custom calibration
Ultrapure water	< 1.93	< 0.25	< 0.25
Steam water from power plant	2.25	3.26	2.18
Boiler water from power plant	3.66	4.85	3.94
DI water	< 1.93	0.29	< 0.25
Double-distilled water	< 1.93	0.77	< 0.25

For three of the samples, the silicate concentration measured by the GF-AAS method lay below the LOQ of 1.93 µg/L SiO_2, a finding that could also be confirmed by the measurement with the Spectroquant® test kit.

The power plant samples lay above the LOQ and the results of the GF-AAS method are comparable with those of the Spectroquant® test kit. The deviations are all within the 95% confidence interval of the pre-programmed method and that of the custom calibration (see **Table 2**). As was also the case for the standard addition, it is apparent that a custom calibration can serve to reduce the error even further.

Summary

The above results show that the Spectroquant® Silicate Test is capable of yielding quantitative values for silicate concentrations in process water and deionized water in the lower ppb range. Users for whom the accuracy of the test for the determination of silicate is sufficient can use the pre-programmed method to determine the silicate content of their samples swiftly and effortlessly.

In the event that greater accuracy of the method is required, the creation of the user's own custom calibration curve is recommended, which was shown to be capable of reducing the mean deviation of the spike amounts that were added by almost 70%.

Chemicals and reagents used

All measurements were carried out as per the application on a Prove 600 spectrophotometer. The reference method that was used was a graphite furnace atomic absorption spectroscopy method on the SpectrAA 280Z instrument supplied by Agilent.

References

1. Bahadori, A.; Vuthaluru, H.B. APPL THERM ENG **2010**, 30, 250-253.
2. The International Association for the Properties of Water and Steam, Technical Guidance Document: *Steam Purity for Turbine Operation*, **2013**.
3. Zhou, S.; Turnbull, *A. Steam Turbine Operating Conditions, Chemistry of Condensates, and Environment Assisted Cracking – A Critical Review*, NPL Report MATC (A) 95, **2002**.
4. Mustafa, A.I. IJCT **2007**, 14, 195-199.
5. Koch,O.G, Koch-Dedic G.A., *Handbuch der Spurenanalyse 2*, Springer-Verlag, **1974**.
6. Package leaflet for the Spectroquant® Silicate (Silicic Acid) Test, Cat. No. 101813, April 2016.

Featured Products

Description	Qty.	Cat.No.
Spectroquant® Silicate (Silicic Acid) Test, 0.25 - 500.0 µg/L SiO_2	100 tests	**101813**
Silicon standard solution, 1000 mg/L Si, Certipur®	100 mL, 500 mL	**112310**
Water Ultrapure	500 mL, 1 L	**101262**

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PHARMA & BIOPHARMA

Analysis of a Bispecific Monoclonal Antibody Using Size Exclusion Chromatography and Mass Spectrometry

Stacy Shollenberger, Product Manager II, HPLC Columns Tosoh Bioscience LLC

Cory E. Muraco, Senior R&D Scientist, Technology and Workflow R&D, **cory.muraco@sial.com**

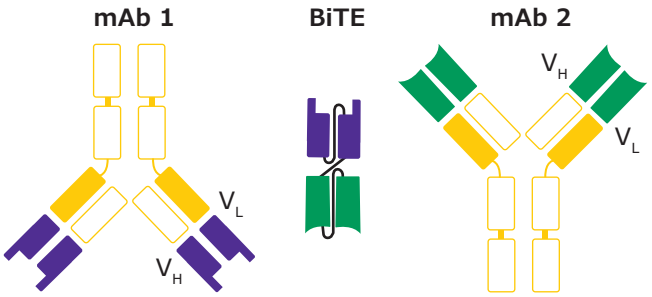
Introduction

More potent formats of monoclonal antibodies (mAbs), such as bispecific antibodies (bsAbs), are on the rise in the area of biotherapeutics. bsAbs recognize two different epitopes. This dual specificity increases the potency of these molecules compared to mAbs and expands the range of possible applications. bsAbs can be used to redirect T cells to tumor cells, block two different signaling pathways simultaneously, dually target different disease mediators, and deliver payloads to targeted sites. At this time, more than 50 bsAb products are currently undergoing clinical evaluation.[1]

Characterization of bsAbs is essential to ensuring product safety and efficacy. Size Exclusion Chromatography (SEC) coupled with Mass Spectrometry (MS) is increasingly being used to identify the accurate molecular mass of biomolecules, including bsAbs. SEC-MS, however, requires the use of mobile phases that do not contain high concentrations of non-volatile salts and the use of columns that do not exhibit column bleed, both of which will interfere with the MS signal response.

In this application, a Bispecific T cell Engager (BiTE®) consisting of two single-chain variable fragments (scFvs) recombinantly linked by a nonimmunogenic five-amino-acid chain (**Figure 1**) was analyzed by SEC-MS using a TSKgel® UP-SW3000, 2 µm column.

Figure 1. Formation of Bispecific T Cell Engager (BiTE)



Experimental HPLC Conditions

Column:	TSKgel® UP-SW3000, 30 cm x 4.6 mm, 2 µm
HPLC Instrument:	Nexera® XR UHPLC system
MS Instrument:	Q Exactive™ Plus*
Mobile phase:	20 mmol/L ammonium acetate, 10 mmol/L ammonium bicarbonate; pH 7.2
Gradient:	Isocratic
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	30 °C
Injection vol.:	5.0 µL
Samples:	BiTE, 0.3 mg/mL (Creative Biolabs) parent mAb shown, 0.5 mg/mL (Creative Biolabs)
Ionization mode:	Electrospray ionization, positive mode
MS mode:	Scanning, m/z 800-6000

*SEC-MS analysis was performed by the Wistar Proteomics and Metabolomics Facility (*Philadelphia, PA*)

Results and Discussion

The ~55 kDa BiTE and ~150 kDa parent mAbs were injected separately onto a TSKgel UP-SW3000 column coupled to a Q Exactive Plus mass spectrometer for molar mass determination. **Figure 2** shows the (a) total ion chromatogram, (b) mass spectrum, and (c) deconvoluted mass spectrum of the BiTE. A main peak can be seen at m/z 54,143; adjacent peaks at m/z 54,181, 54,219 and 54,086 correspond to different salt adducts.

Figure 3 shows the (a) total ion chromatogram, (b) mass spectrum, and (c) deconvoluted mass spectrum of one of the parent mAbs. A main peak can be seen at m/z 149,264; adjacent peaks at m/z 149,426, and 149,592 correspond to different glycoforms. Similar results (not shown) were obtained for the other parent mAb.

These results demonstrate accurate molar mass determination for the BiTE and both parent mAbs utilizing a 20 mmol/L ammonium acetate, 10 mmol/L ammonium bicarbonate (pH 7.2) mobile phase with SEC-MS compatibility.

Prior to analysis, a blank injection was run in order to assess column bleed. **Figure 4a** shows the total ion chromatogram of a blank injection that was run on a new TSKgel UP-SW3000 column. MS data indicates that there is no column bleed from the TSKgel UP-SW3000 column prior to sample injection. Additionally, a blank injection was run between each of the sample injections in order to monitor sample carryover. **Figure 4b** shows the total ion chromatogram of a blank injection run between the BiTE and parent mAb. No evidence of carryover can be seen in the run after sample injection. The lack of column bleed and carryover indicate that the TSKgel UP-SW3000 column is suitable for use with MS.

Figure 2. SEC-MS analysis of the BiTE. Accurate molar mass of the BiTE was calculated to be 54.1 kDa via SEC-MS .

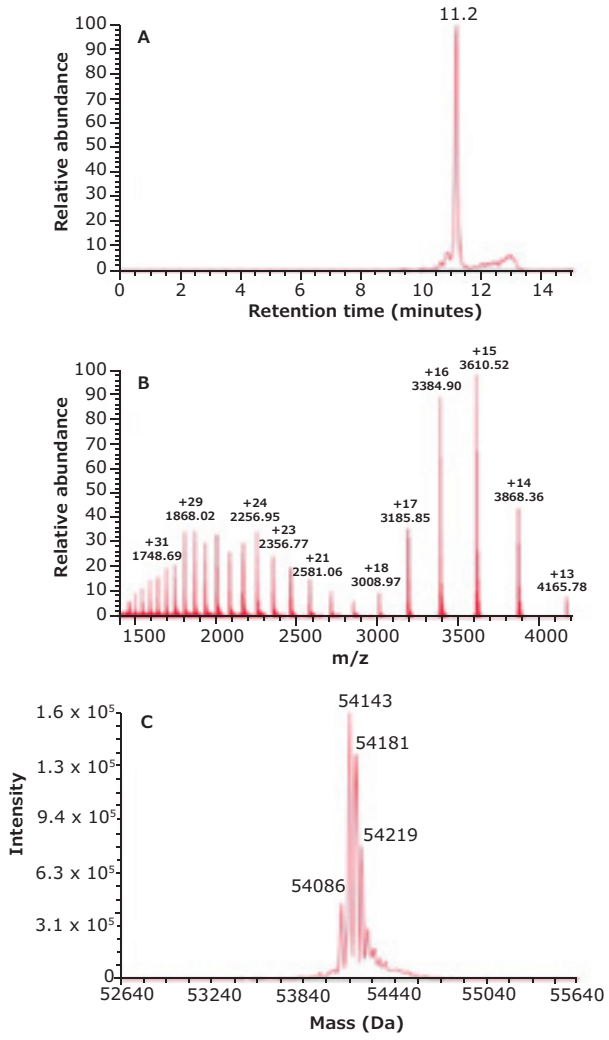
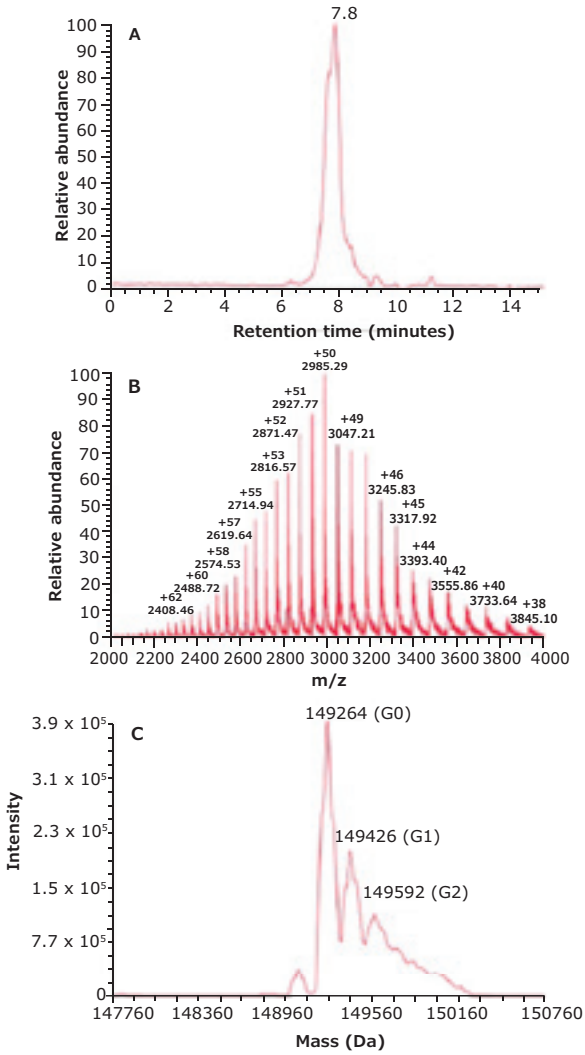


Figure 3. SEC-MS Analysis of Parent mAb. Accurate molar mass of the parent mAb was calculated to be 149.3 kDa via SEC-MS .



Conclusion

The TSKgel UP-SW3000, 2 µm SEC column can be used as a platform method for bispecific antibody accurate mass determination using SEC-MS. A MS compatible mobile phase under non-denaturing conditions was successfully used with the TSKgel UP-SW3000 column. No signs of column bleed or sample carryover, which may interfere with MS signal response, were noted with the TSKgel UP-SW3000 column.

Reference

1. Trivedi, A.; Stienen, S.; Zhu, M.; Li, H.; Yuraszeck, T.; Gibbs, J.; Heath, T.; Loberg, R.; Kasichayanula, S. *Clin. Transl. Sci.*, 10, 147-162 (2017).

Featured Products

Description	Cat.No.
TSKgel® UP SW3000, 2 µm, 4.6 mm ID × 30 cm	**80023448**
Related products	
Ammonium acetate for LC-MS LiChropur®	**5.33004**
Ammonium hydrogen carbonate for LC-MS LiChropur®	**5.33005**
Water for chromatography (LC-MS Grade) LiChrosolv®	**1.15333**

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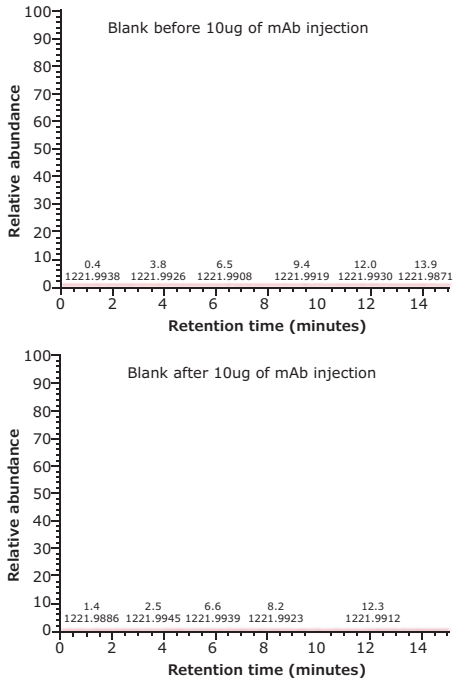
Figure 4. Column Bleed and Carryover Analysis. No column bleed or carryover was observed via MS total ion chromatogram.



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Description	Pack size	Cat. No.
Silver nitrate solution 0.1 mol/L for 3S adapter technology	1 L	**1.50689.1000**
Perchloric acid solution 0.1 mol/L in glacial acetic acid for 3S adapter technology	1 L	**1.50695.1000**
Hydrochloric acid solution 1 mol/L for 3S adapter technology	1 L	**1.50696.1000**
Hydrochloric acid solution 0.1 mol/L for 3S adapter technology	1 L	**1.50677.1000**
Iodine solution 0.05 mol/L for 3S adapter technology	1 L	**1.50690.1000**
Sodium thiosulfate solution 0.1 mol/L for 3S adapter technology	1 L	**1.50691.1000**
Sulfuric acid solution 0.25 mol/L for 3S adapter technology	1 L	**1.50692.1000**
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IN ESSENCE

Banned and Other Neonicotinoids from Plant Material

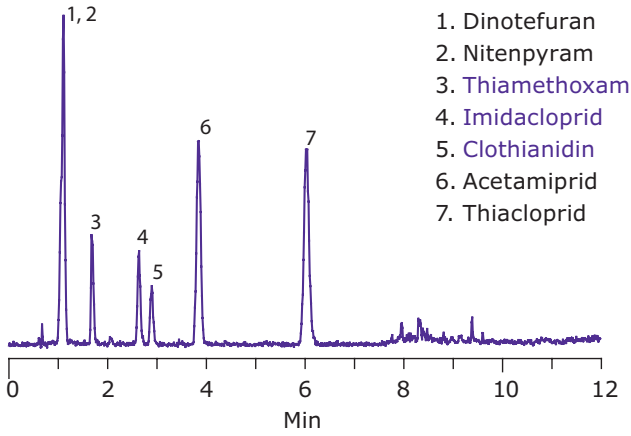
QuEChERs Sample cleanup and LC-MS method using a Fused-Core® particle column complemented by a comprehensive line of reference materials

On Friday, April 27, 2018, representatives of the Member States of the European Union decided to ban the use of three products from the neonicotinoid family of insecticides as they have been deemed dangerous to bees when used on all outdoor crops. Proposed by the European Commission, the text concerns three neonicotinoids (clothianidin, imidacloprid, and thiamethoxam), whose use it had already partially restricted since December 2013. This time, the moratorium was not only expanded, but it was also generalized to include all field crops, leaving only greenhouse use permitted.

The application below demonstrates the extraction and clean up of these banned compounds and others of the neonicotinoid class from dandelion blossoms using the QuEChERS methodology.

For the QuEChERS approach, Supel™ QuE tubes were used for extraction (Acetate) and clean up (PSA/C18) prior to analysis of seven neonicotinoid pesticides. For the LC-MS analysis (**Figure 1**), an Ascentis® Express C18 Fused-Core® particle column was chosen to achieve high efficiency at relatively low back pressure compared to sub-2 micron UHPLC columns.

Figure 1. Separation of Neonicotinoids on Ascentis® Express C18



Conditions

sample/matrix:	3 g pulverized dandelion blossoms, homogenized in 10 mL water
extraction process:	add 25 mL of acetonitrile; add contents of Supel™ QuE acetate extraction tube (55234-U); shake immediately for 1 min; centrifuge at 3400 rpm for 5 min
cleanup process:	Transfer 1 mL of the acetonitrile layer to Supel™ QuE PSA/C18 cleanup tube (55288-U); shake 1 min.; centrifuge at 3500 rpm for 3 min.; (draw off 700 µL of supernatant, evaporate to dryness at 50 °C under nitrogen; reconstitute in 200 µL of 50:50 0.1% formic acid:0.1% formic acid in methanol)
column:	Ascentis® Express C18, 10 cm x 3.0 mm I.D., 2.7 µm particles (53814-U)
column temp.:	25 °C
mobile phase:	[A] 0.1% formic acid in water; [B] 0.1% formic acid in methanol
gradient:	30% B from 0 to 5 min; to 100% B in 0.2 min; held at 100% B for 5.3 min; to 30% B in 0.5 min; held at 30% B for 5 min
flow rate:	500 µL/min
pressure:	3800 psi (262 bar)
injection:	3 µL
detector:	MS, ESI(+), MRM, m/z 203.2/129.2, 271.2/225.0, 292.1/211.0, 256.0/175.2, 250.0/132.0, 223.2/126.0, 253.0/125.8

(continued on next page)

Featured Products

Sample Preparation & HPLC

Description	Qty.	Cat. No.
Supel™ QuE Acetate extraction tube, AOAC 2007.01	50	**55234-U**
Supel™ QuE PSA/C18 cleanup tube, 2 mL, AOAC 2007.01	100	**55288-U**
Supel™ QuE Empty 50 mL centrifuge tube with lid	50	**52248-U**
Ascentis® Express C18, 10 cm x 3.0 mm I.D., 2.7 µm particles	1	**53814-U**
Acetonitrile hypergrade for LC-MS LiChrosolv®	1 L, 2.5 L	**1.00029**
Methanol hypergrade for LC-MS LiChrosolv®.	1 L, 2.5 L	**1.06035**
Water for chromatography (LC-MS Grade) LiChrosolv®	1, 2.5 & 4 L	**1.15333**
Formic acid 98% - 100% for LC-MS LiChropur®	50 mL	**5.33002**

Standards

Description	Qty.	Cat. No.
Clothianidin		
Clothianidin PESTANAL®, analytical standard	100 mg	**33589**
d_3-Clothianidin PESTANAL®	50 mg	**56816**
Imidacloprid		
Imidacloprid PESTANAL®, analytical standard	100 mg	**37894**
Imidacloprid certified reference material, TraceCERT®	50 mg	**68694**
Imidacloprid solution, 100mg/mL, PESTANAL®, analytical standard	2 mL	**46341**
d_4-Imidacloprid PESTANAL®, analytical standard	10 mg	**34170**
Imidacloprid-olefin PESTANAL®, analytical standard	10 mg	**34534**
Desnitro-imidacloprid hydrochloride PESTANAL®, analytical standard	25 mg	**37052**
Thiamethoxam		
Thiamethoxam PESTANAL®, analytical standard	100 mg	**37924**
d_3-Thiamethoxam PESTANAL®, analytical standard	25 mg	**38176**
N-Desmethylthiamethoxam	50 mg	**73348**
Acetamiprid		
Acetamiprid PESTANAL®, analytical standard	100 mg	**33674**
d_3-Acetamiprid, analytical standard	50 mg	**39246**
Acetamiprid-N-desmethyl PESTANAL®,analytical standard	10 mg	**32979**
Thiacloprid		
Thiacloprid PESTANAL®, analytical standard	100 mg	**37905**
Thiacloprid certified reference material, TraceCERT®	50 mg	**14783**
Thiacloprid-amide PESTANAL®, analytical standard	100 mg	**33897**
Thiacloprid-(thiazolidin ring-d_4), analytical standard	10 mg	**30673**
Others		
Nitenpyram PESTANAL®, analytical standard	100 mg	**46077**
Dinotefuran PESTANAL®, analytical standard	50 mg	**32499**
Metabolites		
6-Hydroxypyridine-3-carboxylic acid, analytical standard	100 mg	**19386**
6-Chloropyridine-3-carboxylic acid, analytical standard	100 mg	**68678**
2-Imidazolidone PESTANAL®, analytical standard	250 mg	**31534**

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IN ESSENCE

The 2018 ACS Award in Chromatography Sponsored by MilliporeSigma

The American Chemical Society (ACS) recognized pioneers in the chromatography field by establishing the Award in Chromatography and Electrophoresis in 1959. Supelco has sponsored this prestigious award since 1970 when the award became dedicated solely to chromatography. Our uninterrupted sponsorship continues now through MilliporeSigma.[1]

To receive this recognition, the winner must have made an outstanding contribution to the field of chromatography with particular consideration given to development of new methods. The 2018 winner is Dr. Janusz Pawliszyn, University Professor and Canada Research Chair, Department of Chemistry, at the University of Waterloo.[2] Dr. Pawliszyn was cited for the invention, development, and commercialization of universal, ultraviolet, and fluorescence modes of whole-column imaging detection technology.[3] We posed some questions to Dr. Pawliszyn and wanted to share his insightful answers with our readers.

1. MilliporeSigma: Our Company's mission statement "solving the toughest problems in the industry by collaborating with the global scientific community" seems to mirror your career path. Can you give us some highlights on your experience collaborating with your industry partners?

- **Janusz Pawliszyn (JP):** I am an inventor. I strive to come up with new concepts - paradigm shifts that no one else has thought of. These concepts may actually go against the common understanding. I realize the technology, but I need partners to bring it to users, to make it into something that can be adopted into routine methods. My industry partners are necessary to bring these concepts into reality. The whole column imaging detection technology was commercialized in combination with capillary isoelectric focusing initially by Convergent Bioscience, which was acquired by Protein Simple. As another example, I started working with Supelco in the early 1990's to develop and commercialize SPME (solid phase microextraction).[4] That fruitful collaboration continues today. However important these partnerships are, there is a challenge keeping the technology in focus as companies are bought and sold, strategies evolve, and people move on or change position. I have a role to keep the technology fresh and interesting and demonstrate why the innovation is important to the scientific community.

Dr. Pawliszyn (center) receiving his award from Dr. Paul Ross (right), Director R&D and Technology, Analytical Separations, MilliporeSigma. On the left is ACS President Dr. Peter Dorhout.

2. MilliporeSigma: You are well known as the inventor of the SPME technique, which is such a breakthrough technology. Can you tell us what the inspiration was that caused you to go down the research path that led to SPME?

- **JP:** Although I enjoy my life very much, I don't need to drive a Ferrari to be happy! I prefer to minimize my footprint I leave on this earth. So I was always interested in the development of green technologies. I saw the irony that many environmental methods used a lot of organic solvents and toxic, hazardous reagents. I wanted to replace these with more green methods. In the late 1980's I was working with SFE and microfluidics devices that used optical fibers as a component of the detection system. I thought, why don't I use the optical fibers to introduce the sample to the analytical system via laser desorption, volatilizing the sample directly into the injector of the GC? This was the beginning. From then, my group experimented with different fiber clads (coatings) to optimize the adsorption-desorption process based on the analyte and matrix. The original fiber holders used parts of syringes. The first application we used to demonstrate the potential of SPME was the analysis of BTEX compounds in water using a bare fiber without any cladding.

*The life science business of Merck KGaA, Darmstadt, Germany operates as MilliporeSigma in the U.S. and Canada.

(continued on next page)

3. MilliporeSigma: SPME is beginning to find application in life sciences, like blood and tissue sampling in clinical and medical labs. Can you talk a bit about where you see this application going, why the medical and scientific community should get excited about it?

- **JP:** We have been working on SPME for a long time in environmental and food matrices, which are very complex and are similar in many ways to clinical and medical samples. We always were successful in showing that indeed you get a good representation of the sample onto the fiber; our fundamental studies confirmed that. Now you ask: Why would any medical researcher want to use it? Well, talk to surgeons, they'll tell you why. The small fiber doesn't damage the tissue like a biopsy does, so it is obviously a huge advantage. Not only surgeons, but scientists working with muscle and brain disorders, are all very interested in SPME for non-destructive tissue sampling. This is where SPME is differentiated from sampling techniques that can only handle fluids, which SPME also does well. Looking into metabolomics applications, with SPME not only can you pick out the target analyte, but you can also pick out the full representative metabolite coverage for the given tissue. Besides tissues and fluid sampling, applications of SPME include breath analysis to detect cancer biomarkers. So you see, SPME is unique in being able to perform non-invasive microsampling of all types of biological samples – tissue, fluids, and breath. There is no question of what the technology can do. The question is how to put it into practice. The more people try the technology, the more they become excited by it. We have all the ammunition, in terms of fundamental studies and actual usage, to say this really works, there is so much evidence for it.[5]

4. MilliporeSigma: I like the quote below your email signature "Life is a laboratory. Experiment." Can you give us some insight into what that means to you? How do you experiment in your daily life, at home or at work?

- **JP:** Some people misunderstand this quote. I promote the science to understand what is happening around us. What I mean is that life is an experiment – by someone or something – so investigate it, ask questions, seek answers. That is what is so exciting to me about the biological applications of SPME. It helps us understand how life works.

5. MilliporeSigma: For the ACS award, you were actually cited for the development of whole column imaging detection (WCID). What is the connection between WCID and SPME?

- **JP:** This device of whole column detection goes along with the goal of "on site" analysis or at least rapid analysis and screening. It is a combination of optical imaging technology and column separations. When I began using optical fibers for SPME, I was also interested in imaging technology. Imaging technology was also available for the communications industry, and you could buy components like LEDs, laser diodes, charge-coupled devices (CCD), quite cheaply, and they could be interfaced to a computer to read an image. Obviously you cannot look into the small 100 micron capillary by eye, but if you design the optics which can introduce the light in the center and have a CCD below, you can actually detect what is inside the capillary using spectroscopic means. One separation technology in particular, isoelectric focusing, was amenable to this approach because in focusing you have a stationary system without any electroosmotic flow (EOF). However controlling EOF is not that simple. So what I came up with is the concept of a stationary system using special deactivation of the capillary wall using a methylcellulose coating, which produces a layer that has no EOF. Then we used this method of focusing which is just mixing the protein samples with the carrier ampholytes to produce a pH gradient. Proteins are separated according to their isoelectric point (pI). When the proteins are stationary in the capillary, we can then take a picture of the assembly of separated proteins. Not only can we see the final separation, but we can also monitor the kinetics of the separation process so we can optimize the time of the separation, and perform other kinetic studies like protein-protein interactions and protein binding. And we don't need to mobilize the proteins into the detector; we can detect the separated proteins directly inside the capillary. So we have eliminated the mobilization step and subsequent band dispersion giving much higher resolution compared to mobilization. Detection is immediate, so it is much faster. You just need to wait until the proteins are focused. Since the capillary is very short, they focus very fast. And you can miniaturize the set-up because we have very short capillaries.[6] It is not surprising that the device has become a platinum standard instrumentation in product development and QC in the biotech industry.

6. MilliporeSigma: Looking ahead 25 years, what are your predictions on the breakthroughs that the 2043 ACS Award in Chromatography recipient may be cited for? It's interesting to think that person is just starting their career as we write this!

- **JP:** They will be cited for new green technologies! This has two parts. First, it involves eliminating the use of toxic substances in the labs, reduced or eliminated use of solvents and reagents. Second, they will be cited for the design of instrumentation that can be used at the point of need, to give accurate, rapid answers, possibly in place of sending the sample back to a central laboratory, or to rely on that laboratory only for confirmation.

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*The life science business of Merck KGaA, Darmstadt, Germany operates as MilliporeSigma in the U.S. and Canada.

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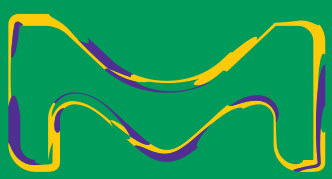

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