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FOOD & BEVERAGE

Beverage Testing with the MQuant® StripScan Mobile App

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Abstract

pH and nitrate content are two of the key parameters used to measure the quality and safety of food and beverages (F&B). Generally, pH measurements are carried out using pH strips, pH meters, or electrodes, whereas spectrophotometers or electrodes are used for measuring nitrate content. This article examines the use and efficiency of the MQuant® StripScan mobile app to measure the pH of orange juice samples and nitrate concentration of mineral water samples. The results indicate towards the possible use of the MQuant® StripScan mobile app as a viable alternative to more sophisticated and instrument-based methods for pH and nitrate measurements in food and beverage analysis.

Introduction

Ensuring delivery of quality and safe food and beverage products is critical to the global F&B industry. pH plays an important role in preserving the color, flavor, texture, taste, and nutritional overall value of the product and significantly impacts the quality of final food and beverage product delivered. Moreover, maintaining the proper pH is a food safety issue.[1] For instance, keeping the pH of canned or preserved food at 4.6 helps prevent the growth of toxic bacteria, such as *Clostridium botulinum*.[2] Additionally, most microorganisms including bacteria, yeasts, and molds cannot survive or grow under very low or high pH values.

In the food industry, monitoring the pH of raw materials is essential to prevent their deterioration, which in turn, can affect the shelf-life of the final product. For example, in juices and brews, measuring the pH of water before addition in food processing guarantees a good quality and safe end-product.[3]

This is particularly important when the water source is a municipal water system, where quality can vary considerably over time.

The traditional method for pH measurement involves the use of a pH meter, which uses the difference in electrical potential between a pH electrode and a reference electrode to generate a reading. The logarithmic nature of pH scale makes even a small change very significant, with even a change of just 0.3 units denoting doubling of acid concentration.[4] Additionally, pH measurements by electrodes are greatly influenced by factors like temperature, electrode stability (drift and hysteresis), the quality of the response slope/calibration curve, as well as the accuracy of the instrument.[5]

A novel method for measuring pH involves the use of the MQuant® StripScan mobile app in combination with test strips. This method provides pH measurements in a few seconds, and also offers additional benefits of automatic data storage, generation of graphs for further documentation, and an automatic data transfer to desktop devices.[6]

Nitrate ($NO3^-$) is another compound that has a significant influence on the quality of F&B products. Although it occurs naturally as part of earth's nitrogen cycle, various human activities contribute to its presence through agricultural operations (via excessive use of inorganic fertilizer), sanitation, diffusion from industrial processes, and disposal of solid waste.[7]

Consumption of food or beverages with high levels of nitrates can have adverse health effects. For example, it can generate carcinogenic nitrosamines upon reaction with amines or amides. Under some conditions, nitrates can also produce nitrite ($NO2^-$) through bacterial reduction in the stomach, causing a rare blood disorder called methaemoglobinaemia, a serious condition resulting from impaired oxygen transport by red blood cells.[8] For this reason, regulatory agencies have set safety limits for nitrate in food and beverages, as well as in water supplies. For example, the current acceptable daily intake (ADI) for nitrate set forth by the European Food Safety Authority (EFSA) is 3.7 milligrams per kilogram of body weight per day (mg/kg bw/day). The U.S. Environmental Protection Agency (EPA) has implemented a maximum contaminant level (MCL) for nitrate in water at 10 mg/L (as nitrogen; equivalent to 45 mg/L of nitrate).[9] The Food and Drug Administration's (FDA) allowable nitrate

level in bottled water is the same as EPA, while in food, the level should not exceed 500 part per million (ppm) in the finished product.[10]

Different methods available for measuring nitrate content include spectrophotometric, chemiluminescence, electrochemical detection, chromatographic, capillary electrophoretic, and spectrofluorimetric methods.[11] Although these techniques offer high sensitivity and selectivity, they also involve laborious chemical work, and require specialized and expensive instrumentation. To address these issues, rapid detection techniques based on test strips are gaining popularity. Combined with the MQuant® StripScan smartphone app, test strips are fast, affordable, non-hazardous for most measurements, and do not require the handling of liquid chemical waste.

In this article, we describe the use of MQuant® StripScan mobile app and test strips for measuring pH and nitrate content of food and beverage products.

Methods and Materials

To measure pH and nitrate in F&B samples, MQuant® test strips (Merck KGaA, Darmstadt, Germany) were used; specifically pH test strips with the range 0-14 and nitrate test strips with the range 0-500 mg/L. The test strips were used according to manufacturer instructions, and readout was performed using the MQuant® StripScan app in conjunction with the appropriate reference cards (Merck KGaA, Darmstadt, Germany):

1. Dip the test strip in the sample and select the parameter to measure (pH or nitrate) in the app.
2. As the app displays a countdown, remove excess sample from the strip and place the strip on the reference card.
3. Position the reference card within the mobile phone's camera view. Align the marks on the screen with the reference card. An image is acquired automatically, and the result is displayed immediately.

A. pH of orange juice

Four orange juice samples were analyzed using the MQuant® pH-indicator strips (pH 0-14) and the corresponding pH reference card. Five individual measurements were made for each sample.

The pH measurements using the test strips were compared with the measurements made using a pH meter.

B. Nitrate in mineral water

Three samples of mineral water were tested using MQuant® nitrate test strips (0-500 mg/L) and the corresponding reference card. Five measurements were made for each sample.

The nitrate measurements were compared with two different reference methods:

1. photometric determination using a Spectroquant® photometer
2. reflectometric determination using the Reflectoquant® system (reflectometer)

Results and Discussion

A variety of modern analytical techniques are being used to support the quality control of food and beverages. These include mobile photometry (e.g. Spectroquant® Move 100) or reflectometry (Reflectoquant® RQflex 20), bench top spectrophotometry (e.g. Spectroquant® Prove series), chromatography, mass spectrometry, NMR, X-ray analysis, and atomic spectroscopy.[5, 6] Although these techniques offer high sensitivity and selectivity, most of them also involve laborious chemical work and expensive investment in instruments.

Rapid detection techniques based on test strips are gaining popularity. A visual observation of color or fluorescence forms the basis of the test strip detection method. Typically, the test substance reacts with chemicals on the reaction pad(s) of the test strip and results in a color change, which is then compared

with a color reference for validation.[12] One drawback of this technique, however, is that a semiquantitative readout is not very accurate, and is prone to individual variations and documentation errors. These aspects are addressed by a new app reader for test strips, the MQuant® StripScan app.[6]

A. pH measurement of orange juice samples

Amongst others, pH measurement is relevant in the food and beverage industry, for instance in quality monitoring of fruit juices. Juices are prone to spoilage due to their possible contact with air and microorganisms in the environment during handling. This is a concern because spoiled fruit juice products can lead to various foodborne illnesses. Despite this risk, microorganisms are not usually present in significant amounts because the low pH of these products is not conducive to their growth. Therefore, monitoring the pH of juice products is critical for both their shelf life and safety.

Four orange juice samples were analyzed using the MQuant® pH-indicator strips (pH 0-14), the MQuant® StripScan app, and the corresponding reference card. The pH measurements were compared with the measurements using a pH meter. The results are shown in **Table 1**.

Table 1. Comparison of pH values obtained by MQuant® StripScan and a pH meter

Orange juice sample number	pH	
	MQuant® StripScan	pH meter
1	4	3.95
2	4	3.85
3	4	3.83
4	4	3.87

The results in **Table 1** are averages of five measurements. The MQuant® StripScan app yields results in increments of 0.5 pH units, while the pH meter provides an accuracy of two decimal places. The experimental data indicates that results obtained by the app correspond with the values measured with the pH meter, showing that MQuant® test strips along with the StripScan app are an adequate alternative to measure pH, if the accuracy provided by the app is sufficient for the use case.

B. Nitrate in mineral water

Monitoring of nitrate is important because of its potentially adverse health effects when consumed in excess. An example is the preparation of infant formula, prepared usually with mineral water. In a hypothetical scenario, where a formula is made from water containing 50 mg of nitrate per liter (50 mg/L), that would average about 8.3–8.5 mg of nitrate per kilogram of body weight per day, which is more than double the current acceptable daily intake (ADI) of 3.7 mg/kg bw/day set forth by the EFSA. Water with high nitrate levels used in making infant formula has a serious impact on the daily exposure levels among the formula-fed infants.

Two mineral water samples were measured for their nitrate content. The rapid MQuant® StripScan method was compared to measurements using reflectometry with the Reflectoquant® system, and photometric measurements with the Spectroquant® system. The results are shown in **Table 2**.

Table 2: Nitrate concentration of mineral water samples measured using MQuant® StripScan app, Reflectoquant® system and Spectroquant® measurements

Sample	NO_3^- concentration [mg/L]		
	*MQuant® StripScan	*Reflectoquant®	Spectroquant®
Water 1	0	<3	2.7
Water 2	10	12	12.0
Citrus flavored water	10	10	>25.0

*MQuant® StripScan app and Reflectoquant® instrument results are based on the average of 5 measurements

As expected, the photometric approach provided the most accurate results out of the three methods that were compared in this experiment.

As the nitrate content in water sample 1 was very low, it was below the detection limit of the reflectometric and app readers. Accordingly, the Reflectoquant® system correctly determined NO_3^- content to be below the detection range of 3 mg/L. The MQuant® StripScan method also correctly determined the concentration to be below the detection limit of 5 mg/L.

The nitrate content of water sample 2 was concurrently determined by reflectometry and photometry to be 12 mg/L. The MQuant® StripScan app determination of 10 mg/L was also in accordance with these reference values, as the incremental values determined by the app are 0 - 5 - 10 - 15 - 20 - 25 - 35 - 50 - 75 - 100 - 250 - 500 mg/L. Thus, the increment value determined by the app is the closest match with the results of the other two methods.

The nitrate concentration of citrus flavored water was also measured. In this case, the Spectroquant® photometric measurements did not give an accurate result because the high sugar content of the sample interfered with the measurement. The results obtained by reflectometry and the MQuant® StripScan app correspond with each other, suggesting that similar method precision is observed here.

To summarize, the measurements obtained with the MQuant® test strips and StripScan app were in range with the reference methods. The accuracy of measurements made was lower due to the system's semiquantitative nature. For accurate values at very low concentrations, the photometric method is best suited. At the same time, the MQuant® test strips and StripScan app are well-suited as a rapid alternative if only binary answers are required (i.e. whether a concentration is above or below

a threshold) and to determine the general concentration range of nitrate content in a sample.

Test strip-based methods show advantages with samples containing additives that may interfere with photometric measurements. Here, measurements with test strips yielded adequate results in direct measurements of the sample without the necessity of sample preparation.

Conclusion

The pH of orange juice samples and nitrate concentration of mineral water samples were measured using the MQuant® StripScan mobile app and MQuant® test strips. In all samples, the data obtained with the MQuant® StripScan mobile app was in range in comparison with the results obtained by the reference methods. This allows for the conclusion that this smartphone-based analytical tool presents a viable alternative to more sophisticated, instrument-based methods, such as pH meters for measuring pH, and spectrophotometers for nitrate measurement.

A general advantage of using test strips over pH electrodes or wet chemical methods is their ease of use, speed, low cost as well as the fact that the strips can be discarded with regular waste after use. This can streamline measurement processes drastically, as no cleaning of equipment and disposal of hazardous liquid waste is necessary, saving time and money.

By combining test strips with the readout by the MQuant® StripScan mobile app, accuracy and reproducibility of the test strip readout are improved without the need to purchase a dedicated readout instrument. As an added value, digital data acquisition and traceability are provided by the app solution, together with an easy way to graph, share, and export data for better documentation. This makes this method suitable for on-site and in-process testing which does not require highly accurate results, and for routine use in laboratories or production sites where pH and chemical screenings are routinely executed.

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Description	Cat. No.
MQuant® pH-indicator strips pH 0-14 Universal indicator, Pk.100	**1.09535**
MQuant® StripScan Reference Card for analyzing MQuant® pH indicator strips pH 0-14 (Cat. No. **1.09535**)	**1.03736**
MQuant® Nitrate Test, colorimetric with test strips, Pk. 25 or 100	**1.10020**
MQuant® StripScan Reference Card for analyzing MQuant® Nitrate Test (Cat. No. **1.10020**)	**1.03733**

MQuant® StripScan App can be downloaded via the Apple Appstore and Google Play.

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Related Products

Description	Cat. No.
Reflectoquant® Reflectometer RQflex® 20	**1.17246**
Nitrate Test 5-225 mg/L (NO_3^-), for use with RQflex®20, 50 Tests	**1.16971**
Spectroquant® Prove 100 Photometer, suitable for UV/Vis spectroscopy	**1.73016**
Spectroquant® Prove 300 Photometer, suitable for UV/Vis spectroscopy	**1.73017**
Spectroquant® Prove 600 Photometer, suitable for UV/Vis spectroscopy	**1.73018**
Nitrate Test photometric, 0.3-30.0 mg/L (NO3-N), Spectroquant®, 100 Tests	**1.01842**

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FOOD & BEVERAGE

Sensitive Determination of Iron in Drinking Water, Mineral Water, Groundwater, and Spring Water Using Rapid Photometric Tests

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The quality of drinking water is regulated by a variety of guidelines, such as the EU Council Directive 98/83[1,2] and WHO guideline.[3] The key principles used to define these limits consider both health hazards and sensory and technical reasons. Iron, for example, does not exhibit a risk for health in concentrations usually found in drinking water.[2,3] However, increased concentrations of iron result in the formation of iron hydroxide products, which can form deposits in water pipe systems and a brown discoloration of the water.[4]

To ensure the supply of clear and colorless water, country-specific limits have been set for drinking water. The limit for iron set by the EU directive is 0.2 mg/L Fe,[2] while the U.S. EPA specifies 0.3 mg/l Fe.[5] To prevent the formation of iron deposits in water pipe systems, a limit of 0.02 mg/L should not be exceeded.[6] To ensure that the specified limits are met, drinking water is, in many cases, subjected to a treatment step in which the iron is precipitated. This method virtually eliminates any iron content, reducing the iron concentration to the lower ppb range.[6]

Analytical methods

Highly sensitive analytical methods for trace level quantification include flame atomic absorption spectroscopy (flame AAS, F-AAS) and optical emission spectrometry with inductively coupled plasma (ICP-OES). Depending on the dosage volume, the measuring range of the F-AAS method according to DIN EN ISO 38406-32 is 0.002–0.020 mg/L Fe. The limit of quantification (LOQ) for the ICP-OES method according to DIN EN ISO 11885 is 0.002 mg/L Fe.[7,8] In our lab an LOQ of 0.0007 mg/L Fe is achieved by ICP-MS according to the ICH Q2 standard.

Analysis of iron using analytical test kits (rapid photometric methods)

A practical alternative for swift, sensitive results without investment in expensive instruments are rapid photometric methods. Test kits are generally characterized by their ease of use and speed of the procedure. The choice of the method depends on the application, the measuring range, and the required accuracy. In the case of iron, two sensitive photometric methods can be chosen.

The determination of iron using the 1,10-phenanthroline method according to APHA 3500-Fe B and DIN 38406-1 enables photometric measurement down to a level of 0.01 mg/L, which is entirely sufficient for many samples.[9]

If lower LOQs are required, the triazine method can be chosen. In this method, all iron ions are reduced to iron (II) ions. These react in a thioglycolate-buffered medium containing a triazine derivative to form a red-violet complex, which is subsequently determined photometrically.[10] Using a 100 mm cell and the Prove 600 UV-VIS spectrometer, LOQs for iron as low as 0.0025 mg/L can be achieved. Due to iron removal treatment and the naturally low iron content of most drinking water, preference should be given to the more sensitive triazine method. The Spectroquant® Iron Test (Cat. No. **114761**) has an overall measuring range of 0.0025-5.00 mg/L Fe. In the Spectroquant® photometers, the methods are pre-programmed, so no time-consuming calibration curve must be created.

Sample preparation and performance of the measurement with Spectroquant® Iron Test

Samples must first be acidified with nitric acid to stabilize the iron, while carbonic acid-containing samples must also be degassed in an ultrasonic bath. A detailed description of the measurement procedure is given in the application "Sensitive Measurement of Iron in Water".[11]

Method comparison of ICP-MS vs. Spectroquant® Iron Test

The iron content of five different mineral waters was determined by Spectroquant® test kit and ICP-MS. All samples were below the LOQ of the respective method (0.0007 mg/L for ICP-MS, 0.0025 mg/L for Spectroquant® test kit.

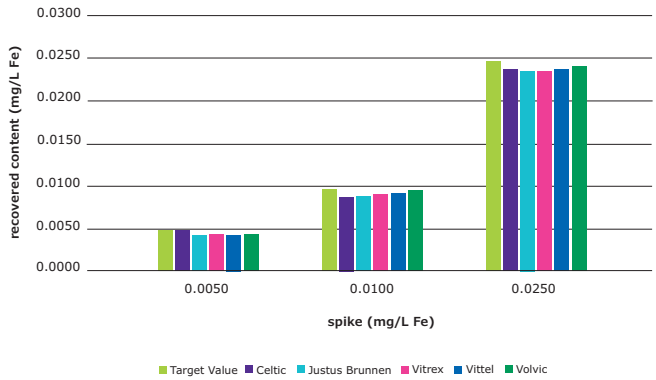
The five samples were spiked with iron at three different concentration levels by standard addition, and the respective recovery rates were determined by the photometric method. The results are shown in **Table 1** and **Figure 1**.

The added concentrations of iron were accurately recovered. The recovery rates in the spiked samples ranged between 89% and 99% over all experiments, with an average recovery rate of 95%.

Table 1. Iron Content Recovered After Standard Addition

Mineral water	Addition [mg/L Fe]	Recovered concentration [mg/L Fe]	Recovery rate
Celtic natural	0.0050	0.0050	99%
	0.0100	0.0089	89%
	0.0250	0.0239	96%
Justus Brunnen medium	0.0050	0.0046	91%
	0.0100	0.0091	91%
	0.0250	0.0239	96%
Vitrex natural	0.0050	0.0048	95%
	0.0100	0.0093	93%
	0.0250	0.0238	95%
Vittel natural	0.0050	0.0046	91%
	0.0100	0.0095	95%
	0.0250	0.0241	97%
Volvic natural	0.0050	0.0047	93%
	0.0100	0.0098	98%
	0.0250	0.0244	98%

Figure 1: Results of the standard addition



An even higher accuracy can be achieved by a custom calibration curve. **Table 2** shows the performance characteristics of the pre-programmed method for Cat. No. **114761** determined according to DIN 38402 A51 and ISO 8466-1 compared with a manually made calibration curve for the measurement range 0.0005 – 0.0100 mg/l Fe using the photometric test kit. The calibration curve is shown in **Figure 2**.

At 4.35%, the coefficient of variation of the custom calibration curve is 3.3 times higher than that of the pre-programmed method. This is due to the fact that at these lower concentrations, the deviations have a stronger relative effect in the custom calibration. Seen in absolute terms, the custom calibration procedure provides considerably lower method errors, as shown by the values of the method standard deviation and the method confidence interval for P=95%, which are 13 to 14 times lower than those of the pre-programmed method.

In the case of the standard additions, the use of such a custom calibration resulted in a further enhancement of the recovery rate, which now achieved a mean value of 101%. The individual values are between 95% and 106% (see **Table 3**).

Figure 2: Calibration curve for the measuring range 0.0005–0.0100 mg/L Fe

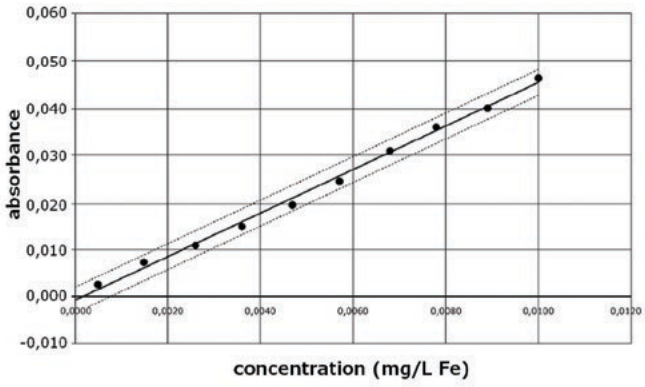


Table 2: Comparison of Performance Characteristics

	Pre-programmed method 0.0025 – 0.5000 mg/L Fe	Custom calibration 0.0005 – 0.0100 mg/L Fe
Method standard deviation [mg/L]	± 0.00328	± 0.00023
Method coefficient variation [%]	± 1.31	± 4.35
Confidence interval (P=95 %) [mg/L]	± 0.0079	± 0.0006

Table 3: Iron Content Recovered After Standard Addition With Custom Calibration

Mineral water	Addition [mg/L Fe]	Recovered concentration [mg/L Fe]	Recovery rate
Celtic natural	0.0050	0.0053	106%
	0.0100	0.0095	95%
	0.0250	0.0255	102%
Justus Brunnen medium	0.0050	0.0049	97%
	0.0100	0.0097	97%
	0.0250	0.0255	102%
Vitrex natural	0.0050	0.0051	102%
	0.0100	0.0099	99%
	0.0250	0.0254	102%
Vittel natural	0.0050	0.0049	97%
	0.0100	0.0102	102%
	0.0250	0.0257	103%
Volvic natural	0.0050	0.0050	99%
	0.0100	0.0105	105%
	0.0250	0.0261	104%

Since mineral waters have only low iron content, the experiments were also carried out using samples of groundwater and spring water, whose iron concentrations are naturally higher due to the lack of any water treatment. The measurement was carried out using the pre-programmed method. Here again the measurement results were verified by reference analysis using the ICP-MS method. **Table 4** shows a comparison of the results obtained with the two methods.

Table 4: Iron Content of Groundwater and Spring Water – Comparison of Icp-Ms And Spectroquant® Iron Test **114761**

	Concentration [mg/L Fe]	
Groundwater and spring water	ICP-MS	Spectroquant® Iron Test 114761
Spring water Bad König	0.0047	0.0041
Spring water Höchst Himmelsleiter	0.0043	0.0051
Spring water Breitenbrunn	0.0022	< 0.0025
Spring water Vielbrunn	0.0017	< 0.0025
Spring water Rai-Breitenbach	0.0059	0.0051
Groundwater Bensheim	2.70	2.71

The results yielded by the Spectroquant® Iron Test are in agreement with those obtained using the ICP-MS method. Due to the very high iron content of the Bensheim groundwater sample of 2.7 mg/L Fe, in deviation from the defined procedure, a 10 mm cell was used. The recovery rate here was 100%. These results show that even very high concentrations of iron can be precisely determined by means of the iron test.

In the case of the low iron concentrated spring water samples, the measurement results differed only by a maximum value of 0.0008 mg/L. Even those iron concentrations that are below the LOQ of the photometric method were confirmed by the ICP-MS measurements.

Summary

The Spectroquant® Iron Test offers a good alternative to ICP or AAS when it comes to determining the iron content in drinking water, mineral water, groundwater, and spring water. The method yields results comparable to those obtained by the ICP-MS method and is easy to perform. For all laboratories for which the purchase of an ICP-OES or ICP-MS system is inexpedient for economic reasons, the Spectroquant® Iron Test Cat. No. **114761** offers a swift, sensitive, and precise alternative for the determination of the iron content of drinking water, mineral water, groundwater, and spring water.

Chemicals, samples, and instruments used:

All measurements were conducted using a Prove 600 photospectrometer. The reference system was a Thermo Fisher Scientific HR-ICP mass spectrometer (method on the Element 2 device).

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Description	Cat. No.
Spectroquant® Prove 600 UV/VIS spectrophotometer 1,8 nm spectral bandwidth	**173018**
Spectroquant® Iron Test 0.0025-5.00 mg/L Fe	**114761**
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Nitric acid 65% for analysis EMSURE® ISO	**100456**
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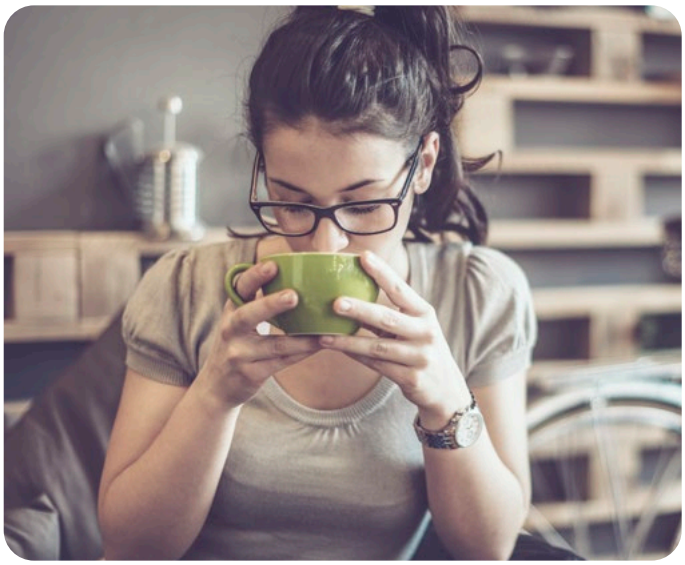
10. Package leaflet Spectroquant® Iron Test, Cat. No. **114761**, June 2016.
11. https://www.SigmaAldrich.com/catalog/product/mm/**114761**

FOOD & BEVERAGE

Water Determination in Instant Coffee

By Karl Fischer Titration

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



Foodstuffs include a very diverse group of products. Depending on whether carbohydrate-rich, fatty or protein-rich substances are under investigation, different working techniques are preferable. Complex matrices that dissolve slowly in the Karl Fischer solvent, or instances where the water can only be slowly extracted, necessitate the use of a solubilizer. In addition, titration under heating or the use of a homogenizer to accelerate water release are expedient. Coffee represents such a complex matrix.

Coffee is more than just a drink

In the 17th century, coffee was a luxury food and only affordable for the wealthy. However, since the 19th century it has become a mass produced product and a common daily drink for everyone. Additionally, it is now considered an important trading product and also developing into a life style product. Of primary concern to the consumer is that the coffee tastes good. To ensure this, one quality parameter is the water content. The determination of the water in coffee beans, roasted coffee and instant coffee is of interest as it has an influence on the roasting process of the beans and the quality, taste and shelf life of the coffee powder.

Karl Fischer Titration of Instant Coffee

Instant coffee contains very firmly bonded water. Its extraction by methanol is very slow and sluggish. For volumetric Karl Fischer Titration, the presence of formamide and salicylic acid accelerates the release of water. The salicylic acid has a buffering function to keep the pH in the right range. Additionally, titration under warm conditions, as well as the use of a homogenizer, are favorable. Alternatively, the Karl Fischer oven technique can be used in combination with Coulometry. For the release of water, a temperature of approx. 105 °C is suitable. A direct coulometric titration is not recommended.

Volumetric Karl Fischer Titration procedure

The titration medium is first placed into the cell and titrated dry. As titration medium, 40 mL of Aquastar® CombiMethanol or two component Aquastar® Solvent is filled into the titration cell and 20 mL formamide and 12 g salicylic acid are added. As titrant, Aquastar® CombiTitrant 5 or the two component Aquastar® Titrant 5, if the Aquastar® Solvent is used, can be selected. Then about 0.3 to 0.5 g of the instant coffee sample is added with a weighing boat and the titration is started. The exact sample weight is determined by weighing the boat before and after the sample addition. For a complete dissolution of the sample, a stirring time of three minutes is recommended. To accelerate the water release, the titration medium can be heated up to 50 °C, using a double wall titration cell connected to a water bath. If the coffee particles are too large, they may need to be crushed before they are added to the titration cell.

It is recommended to do a regular titer determination (e.g. with Aquastar® Water Standard 1%). It is important that the titer determination is done with the actual titration medium mixture (CombiMethanol or CombiSolvent) containing the formamide and salicylic acid.

Titration instrument parameters:

- Extraction time (stirring time): 180 sec.
- Default titration setting:
  I(pol) = 20 - 50 µA, U(EP) = 100 - 250 mV
  Stop criterion: drift < 20 µL/min

For a reagents list see the Ordering Information.

Karl Fischer Oven Method Procedure combined with Coulometry

A direct coulometric Karl Fischer Titration for instant coffee is not recommended, due to the low water content, the accuracy typically is not sufficient.

With the Karl Fischer Oven technique, the water can be extracted/released from the instant coffee sample and be determined in the coulometric titration cell.
The Karl Fischer reagent Aquastar® CombiCoulomat frit is suitable for both, the cathode and anode compartment of the titration cell with diaphragm, simplifying the handling/method by needing only one reagent. It is recommended to place about 10 mL of the solution into the cathode and 150 mL into the anode cell. Then the coulometer is started and the solvent is titrated dry. After the pre-titration and stabilization of drift, the series of measurements can be started by determining the blank value for the sample vials. Then 0.1 g sample is weighed into a sample vial, which is immediately tightly capped. The vial is either manually or automatically placed into the KF oven and heated to the chosen temperature program. The water thereby released is transferred to the titration cell by means of a gas stream (dry air or nitrogen) and coulometrically analyzed.

An oven standard (e.g. Aquastar® Oven Standard 1%) is recommended to be ran before the first sample determination, and for longer sample series in between, and at the end of your sample determinations to check/verify the performance of the Karl Fischer oven and titration system throughout the measurements.

The Titration Parameters:

Oven settings:

- Temperature: 105 °C Extraction time: 600 sec
- Default coulometer settings for cell with diaphragm:
  End point indication, e.g.:
  I(pol) = 5 - 10 µA, U(EP) = 50 - 100 mV
  Stop criterion: drift < 20 µg/min

Ordering Information

Description	Cat No
Volumetric Titration	
Aquastar® - CombiTitrant 5, one component reagent, 1 mL = approx. 5 mg water	**188005**
Aquastar® – Titrant 5, two component reagents, 1 mL = approx. 5 mg water	**188010**
Aquastar® - CombiMethanol, one component solvent, max. 0.01% water	**188009**
Aquastar® – Solvent, two component solvent	**188015**
Formamide	**109684**
Salicylic acid	**100635**
Aquastar® Water Standards 1% in ampoules	**188052**
Oven Method with Coulometric Titration	
Aquastar® - CombiCoulomat frit, Coulometric Karl Fischer reagent for cells with diaphragm	**109255**
Aquastar® – Oven Standard 1%, solid standard for the Karl Fischer oven method	**188054**

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FOOD & BEVERAGE

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

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

Abstract

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

Introduction

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

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

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

Experimental

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

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

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

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

Results and Discussion

Optimization of the HS-SPME Procedure

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

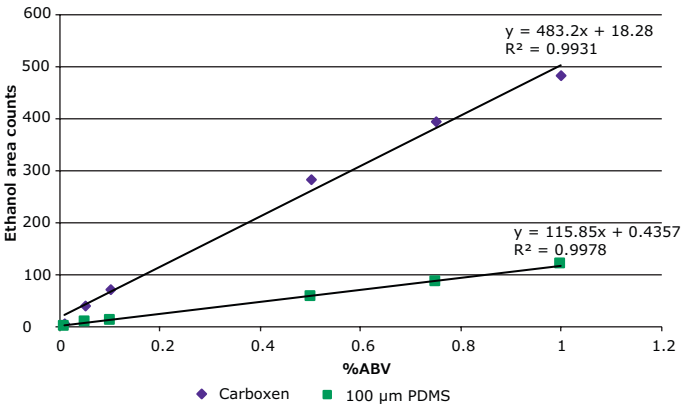

Figure 1. Comparison of Alcohol Response (GC-FID) from Water obtained by HS-SPME; 100 µm PDMS and Carboxen®/PDMS Fibers.

Method Validation

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

Analysis of Certified Reference Materials

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

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

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

*n=8

Table 3. Method Accuracy, Determined using Certified Reference Materials

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

Analysis of Kombucha tea samples

A total of twenty different Kombucha tea samples from ten different producers (including one homemade sample) were tested using the optimized HS-SPME/GC-MS procedure. All were stored under refrigeration after purchase, and testing was performed on freshly opened bottles before the "best by" date indicated on the label. The results are summarized in **Figure 2**. All samples in this set were analyzed in replicates of 2 or 3. To determine method reproducibility over multiple days, a subset of eight samples was analyzed in triplicate on two separate days. The samples in this subset varied in alcohol content from 0.39 to 1.66% ABV. Measurement variability was determined as the % RSD in the average value for the 6 replicates of each sample. For all samples, % RSD was < 3%, indicating good repeatability of the method over separate sample batches analyzed on separate days (with all other parameters such as instrument and analyst being the same).

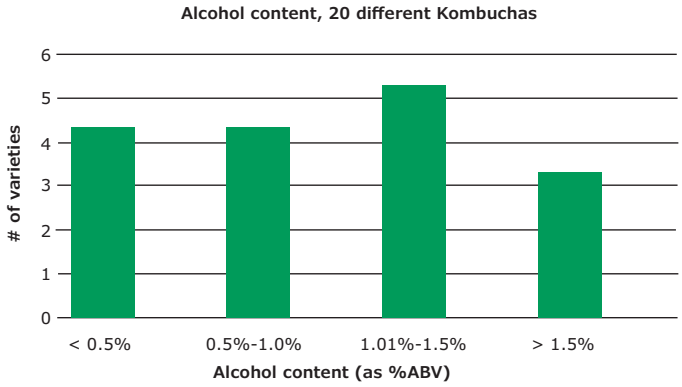

Figure 2. Alcohol (Ethanol) Content, as % ABV, Measured on 20 Kombucha Tea Samples using HS-SPME.

Conclusions

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

References

1. Sun, T., Li, J., Chen, C. (2015) J. Food and Drug Analysis 23, 709-718.
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3. Alcohol and Tobacco Tax Trade Bureau, "General Kombucha Information", ttb.gov/kombucha, accessed 07/08/2020.

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Featured Products

Description	Cat. No.
SUPELCOWAX®10, 30 m x 0.25 mm I.D., 0.50 µm	**24284**
SPME fiber assembly, 100 µm PDMS for autosampler, 23 ga, Pk.3	**57341-U**
SPME fiber holder for autosampler	**57347-U**
Inlet Liner, Direct (SPME) Type, 0.75 mm I.D. for Agilent, Pk.1	**2637501**
Headspace vial with screw top, clear, 10 mL, Pk.100	**SU860099**
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Ethanol-d_6, >99.5% atom %D	**186414**
Sodium phosphate, monobasic, for HPLC, 99-101%	**52074**
Sodium phosphate, dibasic, for HPLC, ACS grade*	**SX0723**
Sodium chloride, EMSURE®	**106404**

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

FOOD & BEVERAGE

Determination of Total Glucose and Xylose in Instant Coffee by Reverse Phase HPLC-UV

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Abstract

This is a method for the determination of total glucose and xylose in coffee samples by reverse phase HPLC-UV. The limit of detection for glucose and xylose is 53.2 and 33.8 ppm respectively for freeze dried coffee.

Introduction

Coffee is an indispensable beverage for many people. The adulteration of coffee with coffee husks, cereal grains and soy beans to raise the profit margin is well known. Typical markers for such adulteration include glucose and xylose. Instant coffee is considered to be adulterated if it contains more than 2.46% total glucose and 0.45% total xylose.[1,2]

As sugars lack UV chromophores, their determination is typically accomplished by HPLC-RID (Refractive Index Detector)[3] or by anion exchange chromatography with a pulsed amperometric detector (HPAEC-PAD)[1,4]. The RID is less sensitive compared to the UV detector and often requires a longer time to stabilize. It is therefore not the detector of choice for many HPLC users. The HPAEC-PAD is a more expensive setup with a limited set of applications and separation columns. It is therefore not a common instrument.

Although there are established methods for sugar determination in coffee e.g. AOAC Method 995.13 and ISO Method 11292:1995, they all require the HPAEC-PAD instrument.

Here, we demonstrate the determination of total glucose and xylose using a procedure to release the sugars from the coffee followed by an SPE cleanup. The released sugars are next derivatized with a UV tag, 1-phenyl-3-methyl-5-pyrazolone (PMP).[5,6,7] A final clean up by liquid-liquid extraction with dichloromethane is done before HPLC injection. The standard addition technique was chosen as it corrects for varying levels of matrix interferences with different coffee samples.

Instruments & Samples

The analysis was performed on a Thermo Dionex UltiMate 3000 UHPLC. An ultrasonic bath was used to dissolve the coffee samples. For sample digestion and derivatization, a water bath and vortex mixer were used. The Visiprep™ vacuum manifold with a vacuum pump was employed for the SPE cleanup. A bench and a mini centrifuge were used to spin down the samples.

Freeze dried coffee and milk coffee mixture samples were purchased from a local grocery store. Freeze dried coffee refers to pure and instant soluble coffee granules or powder whereas milk coffee mixture would have sugar, milk, emulsifier, flavoring agents etc. compounded with instant coffee powder.

Method

Glucose and xylose standard solutions

Prepare 1 L of 1 M hydrochloric acid. Weigh 100 mg of glucose and 100 mg of xylose into a 10 mL volumetric flask. Add 6 mL of hot 1 M hydrochloric acid (~80 °C) and swirl gently. Sonicate for 10 minutes to dissolve completely before topping up to the mark with 1 M hydrochloric acid. Mix well before use.

Coffee sample solutions and reagent blank

Weigh 1.5 g of the freeze dried coffee sample into a 10 mL volumetric flask. For milk coffee mixture sample, use 0.5 g. Add 6 mL of hot 1 M hydrochloric acid (~80 °C) to both. Swirl gently and sonicate for 10 minutes. Ensure all solids are dissolved (milk solids will remain insoluble) before topping up to mark with 1 M HCl.

Spike in glucose and xylose at 400, 800 and 1600 ppm for freeze dried coffee samples as in **Table 1**. Do a

reagent blank using water in place of sample. For milk coffee samples, use 500 µl of sample solution instead.

Table 1. Glucose and Xylose Spiking for Freeze Dried Coffee Sample

Identity	Freeze dried coffee, µl	Glucose, µl	Xylose, µl	1M HCl, µl	Dilution factor	Glucose ppm	Xylose ppm
A	2000	0	0	3000	Nil	0	0
B	2000	200	200	2600	25.00	400	400
C	2000	400	400	2200	12.50	800	800
D	2000	800	800	1400	6.25	1600	1600

Acid digestion

Incubate all spiked solutions at 80 ±2 °C for 3 hours. Then cool to room temperature, spin down contents and filter through a Millex PTFE hydrophilic 0.45 µm filter into a new tube.

SPE cleanup

This SPE cleanup is necessary to remove oils, fats and other organics present in the coffee samples. Set up the SPE cartridges (LiChrolut® RP-18 200 mg/3 mL PP SPE tubes) on the Visiprep™ SPE vacuum manifold system. Connect this to the vacuum pump. Condition the SPE cartridges first with 2 x 3 mL methanol followed by 2 x 3 mL 1 M HCl. Next, place a 15 mL centrifuge tube as a receiver for each of the SPE cartridges. Transfer 1 ml of the filtrate from the acid digestion step into the SPE cartridge. Control flowrate for a dropwise elution.

Derivatization: Tagging the sugars with PMP (UV label)

Prepare 10 mL of 0.5 M PMP in methanol and 10 mL of 1.2 M sodium hydroxide. Pipette 200 µL from the SPE cleanup step into a 2 mL microcentrifuge tube. Add 200 µL of 1.2 M sodium hydroxide and vortex for 30 seconds. Pipette 100 µL into a 5 mL microcentrifuge tube. Add 100 µL of 0.5 M PMP and vortex for 1 minute. Spin down and incubate at 70 ±2 °C for 100 minutes in a water bath. Cool to room temperature for the next step.

Cleanup of sample for HPLC

Prepare 10 mL of 0.2 M hydrochloric acid. Add 100 µL of 0.2 M hydrochloric acid to the tagged sample. Vortex for 30 seconds and spin down contents. Add 1800 µL of water and 1500 µL of dichloromethane to it. Vortex for 1 minute and centrifuge for 2 minutes at 7000 RCF. Draw off the top aqueous layer into another 5 mL tube. Discard the dichloromethane. Repeat extraction of the aqueous layer with 1500 µL of dichloromethane twice more. Filter the aqueous layer through a 0.22 µm 13 mm Millex PTFE hydrophilic filter into a 2 mL HPLC vial. Seal vials and proceed to HPLC injection. Chromatographic conditions are in **Table 2**.

Table 2. Chromatographic Conditions

column:	Purospher® STAR RP-18e, 15 cm x 3 mm, 3 µm (**1.50750**) with guard cartridge, 4-4 mm (**1.50270**) and pre-column holder (**1.16217**)
mobile phase:	[A] 200 mM ammonium acetate (pH 6.8 ± 0.05); [B] acetonitrile; (78% A / 22% B; isocratic elution). All filtered through hydrophilic PTFE, 0.2 µm
flow rate:	0.4 mL/min
column temp:	35 °C
detector:	UV, 245 nm
pressure:	~200 bar
injection volume:	20 µL

Results and Discussion

Both glucose and xylose peaks were symmetrical and eluted at ~9.8 and ~11.5 minutes respectively (**Figure 1**). The freeze dried coffee has a more complex HPLC profile compared to the coffee mixture sample. See **Figure 2** for spiked freeze dried coffee sample.

The freeze dried coffee samples 1 and 2 had a total xylose content >0.42% w/w (**Table 3**). The coffee mixture samples 1 and 2 had a high glucose content

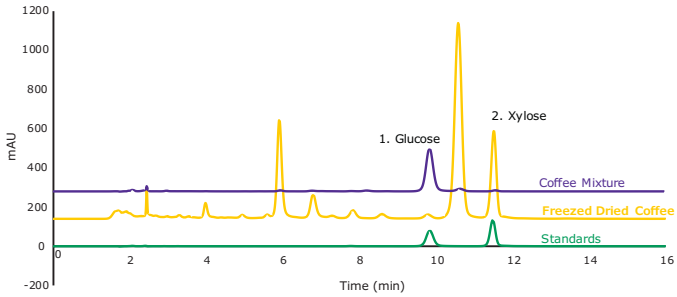


Figure 1. Coffee Mixture and Freeze Dried Coffee with Glucose and Xylose Standards

Sample	Peak	Compound	Retention Time (min)	Resolution	Peak Symmetry
Freeze dried coffee	1	Glucose	9.79	5.75	0.97
	2	Xylose	11.52	-	0.93
Coffee mixture	1	Glucose	9.83	5.72	1.03
	2	Xylose	11.55	-	0.93

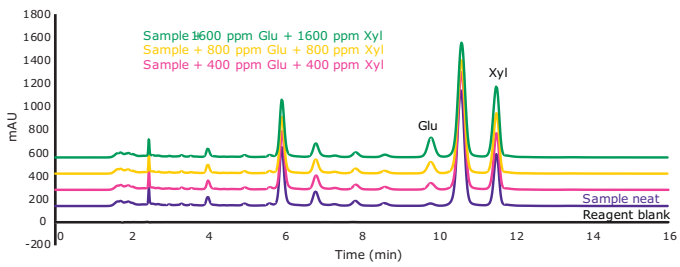
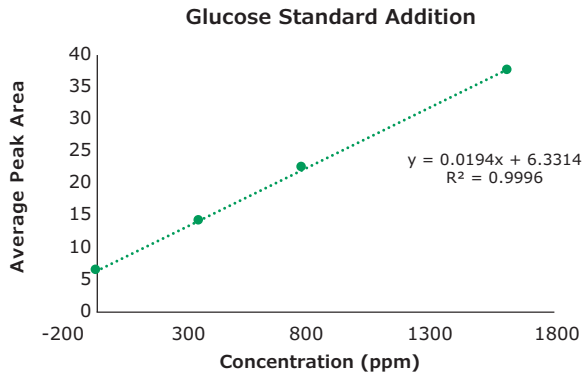
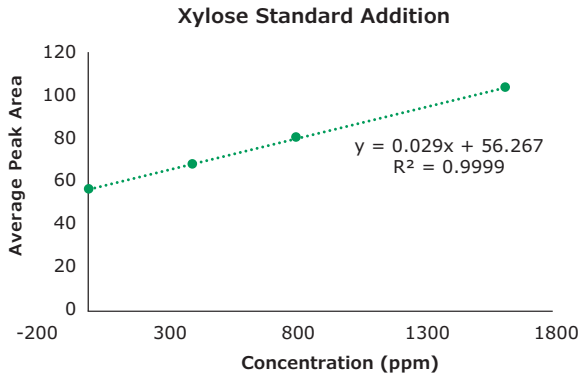


Figure 2. Freeze Dried Coffee Sample Spiked with Glucose and Xylose Standards





Linearity - Glucose	
Concentration (ppm)	**Mean Area**
0	6.1962
404.08	14.1098
808.16	22.4116
1616.32	37.5630
Slope	**0.0194**
STEYX	**0.3132**
LOD	**53.2 ppm**
LOQ	**161.2 ppm**

Linearity - Xylose	
Concentration (ppm)	**Mean Area**
0	56.0414
403.64	68.0976
807.28	80.0099
1614.56	102.9951
Slope	**0.0290**
STEYX	**0.2978**
LOD	**33.8 ppm**
LOQ	**102.5 ppm**

Figure 3. Standard Addition Calibration Plots and Data for Freeze Dried Coffee

>40% w/w as both have sugar and glucose syrup listed as ingredients.

Table 3. Results for Coffee Samples

Sample	Glucose (% w/w)	Xylose (% w/w)
Freeze dried coffee 1	0.35	4.10
Freeze dried coffee 2	0.55	3.23
Coffee mixture 1	43.31	0.68
Coffee mixture 2	46.59	0.13

CONCLUSION

We can determine total glucose and xylose in coffee by Reversed Phase HPLC-UV. This is a sensitive isocratic separation that can be completed by fifteen minutes with the Purospher® STAR RP-18e fully porous particle column. The method can be modified using Fused-Core® or Chromolith® columns for even faster separation while still applicable to conventional HPLC and to UHPLC instruments.

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Featured Materials

Description	Cat. No.
HPLC Columns	
Purospher® STAR RP-18e, 3 µm, 150-3 mm, Hibar® RT	**1.50750**
Purospher® STAR RP-18e 4-4 mm Guard Cartridge	**1.50270**
Pre-column holder for LiChroCART® cartridges 4-4 for capillary connection	**1.16217**
Sample Prep, Reagents and Accessories	
LiChrolut® RP-18, 200 mg, 3 mL SPE PP Tube, Pk.50	**1.02014**
Visiprep™ SPE Vacuum Manifold	**57030-U**
Millipore® Chemical Duty Pump, 220 V/50 Hz	**WP6122050**
Acetonitrile isocratic grade for liquid chromatography LiChrosolv®	**1.14291**
Ammonium Acetate for analysis EMSURE® ACS, Reag. Ph Eur	**1.01116**
Dichloromethane for liquid chromatography LiChrosolv®	**1.06044**
Hydrochloric Acid Fuming 37%,for analysis EMSURE® ACS, ISO, Reag. Ph Eur	**1.00317**
Methanol for analysis EMSURE® ACS, ISO, Reag. Ph Eur	**1.06009**
Methanol for liquid chromatography LiChrosolv®	**1.06018**
3-Methyl-1-Phenyl-2-Pyrazoline-5-one (PMP), 99%	**M70800**
Sodium Hydroxide pellets for analysis EMSURE®	**1.06498**
Omnipore® 0.2 µm 47mm Membrane Filters	**JGWP04700**
Millex® - LCR 0.22 µm 13 mm filter unit, Hydrophilic PTFE	**SLCR013NL**
Millex® - LCR 0.45 µm 33 mm filter unit, Hydrophilic PTFE	**SLCR033NB**
HPF Millex® - LCR 0.45 µm 33 mm filter unit	**SLLGM25NS**
Reference Materials	
D-(+)-Glucose, Pharmaceutical Secondary Standard	**PHR1000**
D-(+)-Xylose, Pharmaceutical Secondary Standard	**PHR2102**

FOOD & BEVERAGE

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

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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

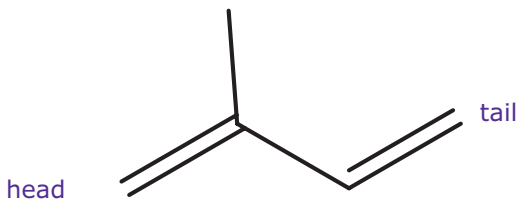


Table 1. Classification of Terpenes

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

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

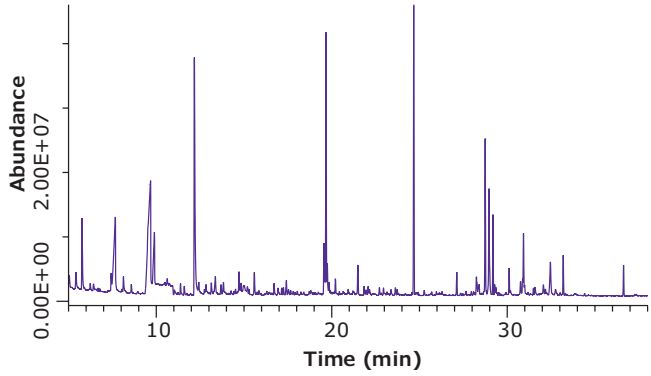
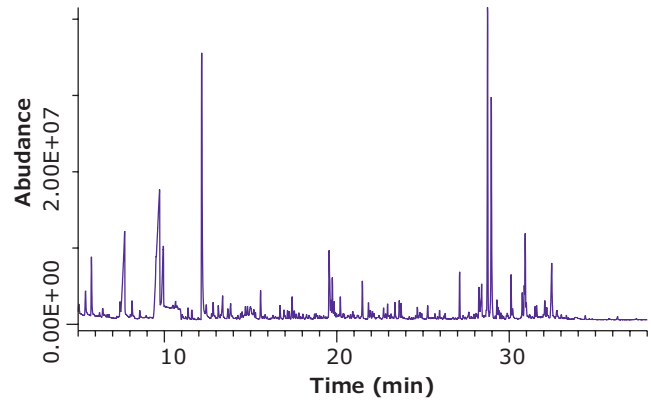


Figure 3. Headspace SPME-GC/MS Analysis of Dried Hops Flowers (100 µm PDMS Fiber, 0.2 g Sample)

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

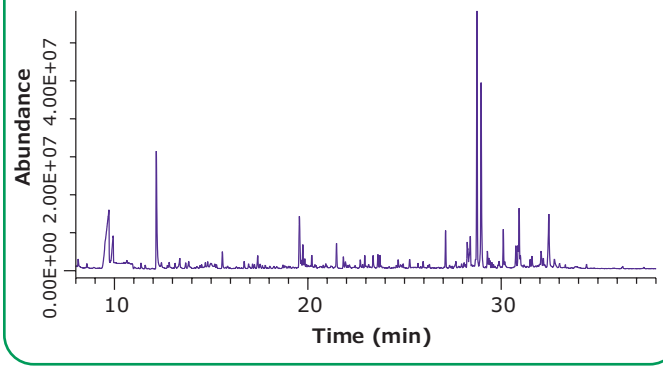
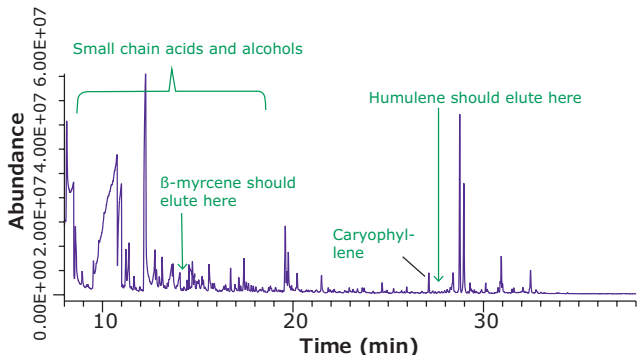


Figure 5. Headspace SPME-GC/MS Analysis of Dried Hops Flowers, Increased Sample Equilibration Temperature (DVB/CAR/PDMS Fiber, 0.2 g Sample)

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

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,

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

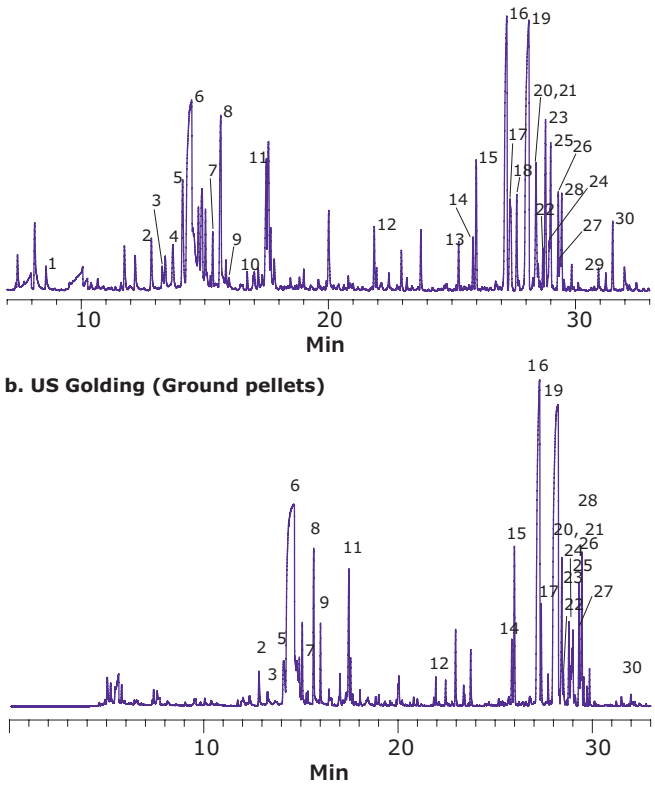
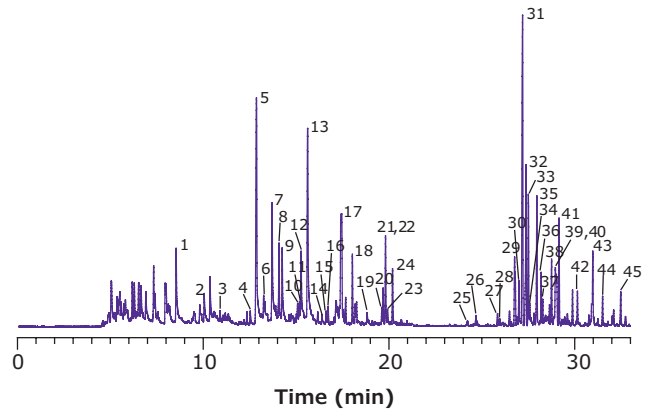


Figure 7. Headspace SPME-GC/MS Analysis of Dried Cannabis Using Final Optimized Method

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



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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Featured Products

Description	Cat. No.
Capillary GC column	
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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FOOD & BEVERAGE

Analysis of Active Cannabis Compounds in Edible Food Products: Gummy Bears and Brownies

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Margaret Wesley, 2016 R&D Summer Intern from Pennsylvania State University, State College, PA

Introduction

Potency testing in marijuana-infused edibles is a problematic task that analytical labs are facing due to the complexity of the involved matrices. Among the most popular matrices are gummy bear candies and brownies. According to one laboratory site, the concentration of active ingredients in the edibles can range from a few parts per million to 3.5 parts per thousand.[1] In this application, a procedure was developed to extract active cannabinoid compounds from gummy bears and brownies. The procedure included a simple and fast extraction of the active compounds from the studied foods, and analysis by HPLC-UV using a biphenyl stationary phase chemistry.

Experimental

Cerilliant® cannabinoid standards, available as 1 mg/mL solutions in either methanol or acetonitrile, were used for this experiment. The concentration of cannabinoids allowed for the spiking of both gummy extract and brownies at about 40 ppm with all compounds. The following compounds were included in this study: cannabidivarinic acid (CBDVA), cannabidivarin (CBDV), cannabigerolic acid (CBGA), cannabigerol (CBG), cannabidiolic acid (CBDA), cannabidiol (CBD), tetrahydrocannabivarin (THCV), cannabinol (CBN), (-)-Δ^9-Tetrahydrocannabinol (Δ^9-THC), (-)-Δ^8- Tetrahydrocannabinol (Δ^8-THC), and (-)-Δ^9-Tetrahydrocannabinolic acid A (THCAA). This list of 11 different cannabinoids includes several acidic forms; thus HPLC analysis was used in order to quantitate these in their native forms.

The HPLC column used was Ascentis® Express Biphenyl, 2.7 µm particle size, which gave the best separation of all 11 compounds in under 13 minutes. The use of this column with Fused-Core® particle architecture resulted in low back pressure, thus a standard pressure HPLC system could be used during this experiment.

Sample Preparation

One gummy bear candy, non-spiked, (2.3 g) was dissolved in 20 mL of warm water. This solution was then spiked with cannabinoids and extracted using a QuEChERS procedure. The average spiking level in each gummy bear was 45 ppm for each compound. Bears of four different colors were tested – orange, yellow, red, and green. After spiking, the water/candy solution was

transferred to a 50 mL plastic QuEChERS extraction tube (55248-U). Acetonitrile (10 mL) was added, and the tube was shaken for one minute by hand. Supel™ QuE non-buffered salts (55295-U) were added, and the samples were shaken for 5 minutes on an automated QuEChERS shaker. Post-shaking, the samples were centrifuged for 5 minutes at 5000 rpm. The top layer was collected and injected directly into the HPLC.

For brownies, a 2.5 g sample of a non-spiked brownie with frosting was added to the QuEChERS extraction tube. This sample was spiked with cannabinoids and allowed to sit for 30 minutes prior to extraction. The average spiking level for the brownies was 40 ppm. The QuEChERS extraction was performed as previously described for gummy bears. Post-extraction, the top acetonitrile layer was collected into a vial and kept under refrigeration for a minimum of 3 hours to remove fats prior to HPLC analysis.

A calibration curve was constructed in acetonitrile bracketing the expected concentration of 10 µg/mL in the final extracts. The following calibration points were included: 2 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL and 25 µg/mL.

Results and Discussion

For the gummy bear samples, it was found that neither the red, yellow, nor green color interfered with detection of cannabinoids at 220 nm. The red color was partially extracted into acetonitrile, while the green and yellow colors stayed in the aqueous layer upon extraction. However, the orange color from the gummy bear, when extracted into acetonitrile, was found to have an interfering peak that co-eluted with CBDVA. Thus, for the orange gummy bear, quantitation of CBDVA was done at 280 nm, where CBDVA has significant absorbance free of interference. Quantitation was done at 220 nm for the rest of compounds in this study (**Figure 1**).

While no cleanup was required for gummy bear samples post-extraction, the co-extractives in the brownie were found to decrease the recoveries of the analytes if the brownie extract was injected into HPLC without further processing. The brownie extract was cleaned by refrigeration to remove the co-extracted fats.

The ruggedness of the method for brownies was tested by injecting the brownie extract (**Figure 2**) multiple times followed by the injection of the 10 µg/mL standard. After 7 injections of the brownie extract, it was found that the peak retention times were not affected, indicating that the column was being thoroughly cleaned between injections. The peak areas for the standards showed a slight decrease of 4 %.

Excellent recovery values of above 90 % for gummies and above 80 % for brownies were achieved with good accuracies (**Table 1**).

Table 1. Recoveries From Spiked Gummy Bears and Brownies

Peak No.	Compound	Yellow Gummy	Orange Gummy	Red Gummy	Average Gummy and RSD	Average Brownie and RSD
1	CBDVA	90 %	92 %*	92 %	91 % (2 %)	91 % (1 %)
2	CBDV	93 %	100 %	100 %	98 % (3 %)	93 % (5 %)
3	THCV	87 %	93 %	90 %	90 % (3 %)	87 % (1 %)
4	CBDA	94 %	90 %	95 %	94 % (3 %)	95 % (1 %)
5	CBGA	87 %	91 %	89 %	91 % (4%)	90 % (2 %)
6	CBD	95 %	100 %	98 %	97 % (3 %)	89 % (5 %)
7	CBG	93 %	99 %	98 %	96 % (4 %)	91 % (5 %)
8	CBN	88 %	95 %	97 %	95 % (6 %)	84 % (4 %)
9	Delta-9-THC	93 %	99 %	100 %	97 % (3 %)	82 % (4 %)
10	Delta-8-THC	91 %	97 %	98 %	95 % (3 %)	80 % (4 %)
11	THCA-A	89 %	89 %	89 %	92 % (7 %)	91 % (2 %)

*The orange gummy was done at 280 nm due to the interfering background peak quantitation.
Note: THCA is the abbreviation used by AOAC

Conclusion

A method was developed for analysis of active cannabinoid compounds in both brownies and gummy bears. The extraction procedure involved a salting out step into acetonitrile and did not require intensive cleanup. The separation of eleven compounds was achieved on a biphenyl stationary HPLC phase and was completed in 13 minutes. The active compound CRMs are available from Cerilliant® through SigmaAldrich.com.

Reference

1. Analytical 360, Test Results, Sour Gummy Bears. http://analytical360.com/m/archived/216628, (accessed July 2016).

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Figure 1. HPLC Chromatogram of Orange Gummy Bear Extract at (a) 220 nm and (b) 280 nm **Figure 2**. HPLC of a Brownie Extract at 220 nm. The peak elution order is listed in **Table 1**.

column:	Ascentis® Express Biphenyl, 10 cm × 2.1 mm I. D., 2.7 µm particles (64065-U)
mobile phase:	(A) 0.1 % TFA in water; (B) 0.1 % TFA in acetonitrile
gradient:	at 47 % B, to 50 % B in 13 minutes, to 100 % B in 0.1 min, 100 % B for 3 minutes, to 47 % B in 0.1 min, at 47 % B for 2.5 minutes
flow rate:	0.70 mL/min
column temp.:	35 °C
detector:	UV, 220 nm and 280 nm
injection:	5 µL
pressure:	340 bar
instrument:	Agilent® 1200, with UV detector

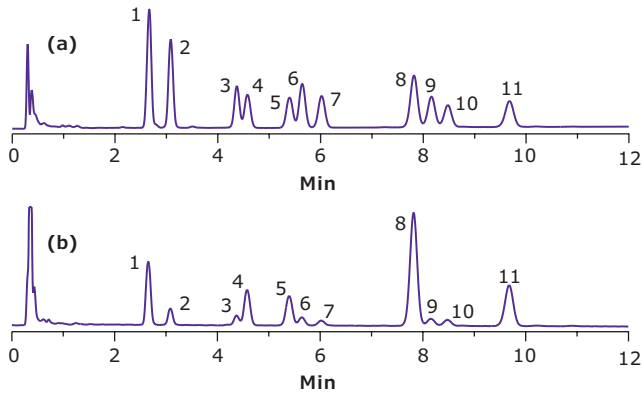
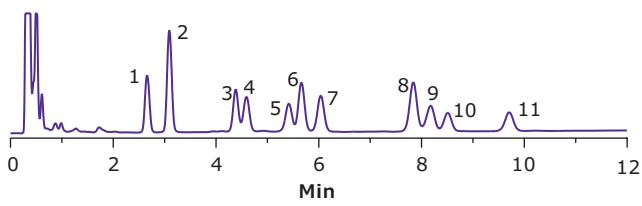


Figure 2. HPLC of a Brownie Extract at 220 nm
The peak elution order is listed in **Table 1**.

Conditions same as **Figure 1**.



Featured Products

Description	Cat. No.
Supel™ QuE QuEChERS Products	
Non-buffered Extraction Tube 2, 12 mL, pk of 50	**55295-U**
Empty Centrifuge Tube, 50 mL, pk of 50	**55248-U**
Ascentis® Express Biphenyl HPLC Column	
10 cm × 2.1 mm I.D., 2.7 µm particle size	**64065-U**
Cerilliant® Certified Reference Materials	
Cannabidivarinic acid (CBDVA), 1 mg/mL in acetonitrile, CRM	**C-152**
Cannabidivarin (CBDV), 1 mg/mL in methanol	**C-140**
Cannabigerolic acid (CBGA), 1mg/mL in acetonitrile	**C-142**
Cannabigerol (CBG), 1 mg/mL in methanol	**C-141**
Cannabidiolic acid (CBDA), 1 mg/mL in acetonitrile	**C-144**
Cannabidiol (CBD), 1 mg/mL in methanol	**C-045**
Tetrahydrocannabivarin (THCV), 1 mg/mL in methanol	**T-094**
Cannabinol (CBN), 1 mg/mL in methanol	**C-046**
(-)-Δ^9-Tetrahydrocannabinol (Δ^9-THC), 1 mg/mL in methanol	**T-005**
(-)-Δ^8-Tetrahydrocannabinol (Δ^8-THC), 1 mg/mL in methanol	**T-032**
(-)-Δ^9-Tetrahydrocannabinolic acid A (THCA-A), 1 mg/mL in acetonitrile	**T-093**
Accessories	
QuEChERS Shaker and Rack Starter Kit, USA compatible plug	**55278-U**
QuEChERS Shaker and Rack Starter Kit, EU Schuko plug	**55438-U**
Certified Vial Kit, Low Adsorption (LA), 2 mL, pk of 100	**29653-U**

FOOD & BEVERAGE

Analysis of Pesticide Residues in Pistachios

Using QuEChERS Extraction and Cleanup with Supel™ QuE Z-Sep+

Kathy Stenerson, Principle R&D Scientist, Megan Wesley, 2016 R&D Summer Intern, Analytix@merckgroup.com

Introduction

Pistachios are popular and enjoyed for both taste and health benefits such as decreased cholesterol, weight management, protection against diabetes and hypertension, and improved digestion.[1] These nuts are grown in the United States (specifically, California), Italy, and countries in Central Asia like Iran, Turkey, Afghanistan and Syria. Pesticide tolerances set by the US EPA for pistachios range from 0.01 - 0.7 µg/g before harvest to 3 - 200 µg/g after harvest, depending on the pesticide.[2] Testing for pesticide residues then requires a method which will allow for low level and accurate determination. The "quick, easy, cheap, effective, rugged and safe" (QuEChERS) approach has been used to analyze multiple pesticide residues found in pistachios.[3] Pistachios contain approximately 45% fat, which can result in a significant amount of co-extracted matrix in the acetonitrile extract generated using the QuEChERS procedure. The use of a cleanup sorbent which can reduce this fat is essential to prevent fouling of LC-MS/MS and GC-MS/MS systems, and minimize ion suppression, thus allowing low level detection. In this application, Supel™ QuE Z-Sep+ sorbent was used as part of the QuEChERS method in the analysis of pesticide residues in pistachios. Z-Sep+ is a zirconia and C18 functionalized silica sorbent which acts to retain fatty constituents through both Lewis acid/base and hydrophobic interactions. The selectivity of the zirconia present in Z-Sep+ offers retention of a wider range of fats than C18 alone. In this application, QuEChERS extraction and cleanup using Z-Sep+ sorbent were used before the LC-MS/MS and GC-MS/MS analysis of pesticide residues in pistachios. The targeted analyte list included pesticides relevant to pistachios.[4,5]

Experimental

Pistachios were purchased from a local grocery store. They were frozen with liquid nitrogen (shells on), ground, and spiked at 10 ng/g with the pesticides listed in **Tables 2** and **4**, and allowed to equilibrate for 1 hour. Samples were then subjected to QuEChERS extraction and cleanup with Z-Sep+ following the procedure in **Figure 1**. A 100 µL aliquot of the final extract was diluted to 1 mL with 5 mM ammonium formate/0.1% formic acid in water, and analyzed by LC-MS/MS using the conditions shown in **Table 1**. The remaining acetonitrile extract was analyzed directly by GC-MS/MS using the conditions shown in **Table 3**. Spiked samples were quantitated against 5-point matrix-matched calibration curves prepared in unspiked pistachio matrix blanks (after cleanup). No internal standard was used.

Figure 1. QuEChERS Extraction and Cleanup Procedure Used for Pistachios.

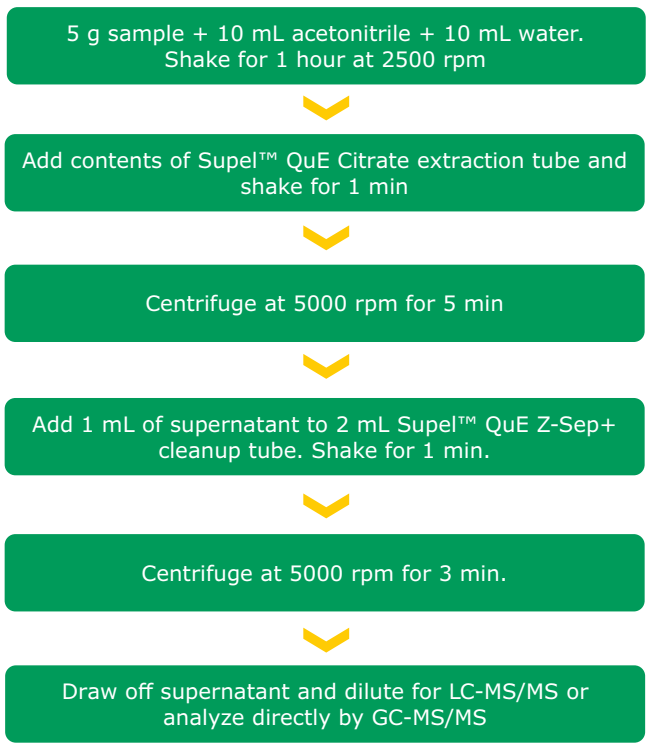


Table 1. LC-MS/MS Analysis Conditions

column:	Ascentis® Express RP-Amide, 10 cm × 2.1 mm I.D., 2 µm (51576-U)
mobile phase:	[A] 5 mM ammonium formate, 0.1% formic acid in water; [B] 5 mM ammonium formate, 0.1% formic acid in 95:5 acetonitrile:water
gradient:	5% B held for 1 min; 5 to 100% B in 12 min; held at 100% B for 1.5 min; 100 to 5% B in 0.5 min; held at 5% B for 1.5 min
flow rate:	0.4 mL/min
column temp.:	30 °C
detector:	MS, ESI (+), MRM (see Table 2)
injection:	5 µL

Table 2. MRMs Used for Quantitation, LC-MS/MS

Compound	CAS No.	MRM	Frag (V)	CE
Aclonifen	74070-46-5	265/182.1	115	28
Aldicarb	116-06-3	208.1/89.1	70	12
Aldicarb-sulfone	1646-88-4	223.1/86.1	80	8
Bifenazate	149877-41-8	301.1/170.1	95	16
Butocarboximsulfoxide	34681-24-8	207.1/132	65	0
Carbendazim	10605-21-7	192.1/160.1	105	16
Carbofuran	1563-66-2	222.1/165.1	80	20
Chlorantraniliprole	500008-45-7	483.9/452.9	105	16
Etrimfos	38260-54-7	293.1/125	120	28
Flufenoxuron	101463-69-8	489.1/158	100	20
Isoxathion	18854-01-8	314.1/105	135	12
Malathion	121-75-5	331/126.9	80	5
Methabenzthiazuron	18691-97-9	222.1/165.1	90	12
Methomyl	16752-77-5	163.1/106	50	4
Neburon	555-37-3	275.07/57.1	100	20
Omethoate	1113-02-6	214/109	80	24
Pyraflufen-ethyl	129630-19-9	413/339	120	25
Quinalphos	13593-03-8	299/163	90	20
Rotenone	83-79-4	395/213.1	145	20
Spinetoram	187166-40-1	748.5/142.2	206	32
Spiromesifen	283594-90-1	388/273	110	10
Thiacloprid	111988-49-9	253/126	100	16
Thiophanate-methyl	23564-05-8	343/151	90	20
Triazophos	24017-47-8	314.1/162.1	110	16
Trichlorfon	52-68-6	256.9/109	80	12

Table 3. GC-MS/MS Analysis Conditions

column:	SLB®-5ms, 20 m × 0.18 mm I.D., 0.18 µm (**28564-U**)
oven:	50 °C (2 min), 15 °C/min to 320 °C (5 min)
inj. temp.:	250 °C
carrier gas:	helium, 1.2 mL/min constant flow
detector:	MSD, scan and MRM (see Table 4)
MSD interface:	325 °C
injection:	1 µL, splitless (0.75 min)
liner:	4 mm I.D. FocusLiner™ with taper

Table 4. MRMs Used for Quantitation; GC-MS/MS

Compound	CAS #	MRM	CE
Chlorpyrifos-methyl	5598-13-0	286/93	20
Tolclofos-methyl	57018-04-9	265/250	15
Fenthion	55-38-9	278/169	15
MGK-264	18691-97-9	164/98	10
Endosulfan sulfate	1031-07-8	274/239	15
Etoxazole	153233-91-1	141/63	30

Results and Discussion

Background

Initially, cleanup using Z-Sep+ sorbent was compared to PSA/C18, a common QuEChERS cleanup sorbent for fat-rich samples. A visual comparison of the QuEChERS extracts (in acetonitrile) is shown in **Figure 2**. Both cleanups removed some green color, resulting in similar light yellow extracts. GC-MS-scan comparisons (**Figure 3**) show lower background after Z-Sep+ cleanup compared to PSA/C18. The predominant peaks present in the uncleaned extract are fatty acids and monoglycerides. While PSA/C18 only reduced the levels of these compounds, almost none were detected after Z-Sep+ cleanup.

Pesticide Recovery

Table 5 shows the average %Recovery and %RSD for n=3 replicates of spiked pistachio samples. The majority of the pesticides were analyzed by LC-MS/MS; and those without sufficient response were analyzed by GC-MS/MS. Out of the 30 pesticides analyzed, 22 had recoveries within the generally accepted range of 70-120 %. Reproducibility was good, with RSD values < 20% for all 30 pesticides, and < 10% for many. Two pesticides, etoxazole and trichlorfon, had recoveries < 50%. Trichlorfon was most likely retained by the Z-Sep+ sorbent during the cleanup step. This could be due to the Lewis base character of the phosphate group present in its structure. Etoxazole, on the other hand, does not contain a phosphate group. It is a very lipophilic pesticide, indicated by its log P value of 5.6. Extraction efficiency of this compound from the fatty pistachio matrix was probably very poor using acetonitrile. Spinetoram, with a log P of 6.3, also showed lower recovery (56%) than a majority of the pesticides studied. This trend of decreased recovery for high log P pesticides has been observed by others for high fat matrices.[6] Recovery of both of these compounds may be increased by addition of a less polar solvent such as ethyl acetate for the extraction; however, an increase in the level of co-extracted background can be expected.

Figure 2. Comparison of Pistachio Extracts; Before and After Cleanup.

No cleanup | PSA/C18 | Z-Sep +

Figure 3. GC-MS-Scan Comparison of Pistachio Extracts With (a) No Cleanup, (b) PSA/C18 Cleanup, and (c) Z-Sep+ Cleanup; All the Same Y-scale

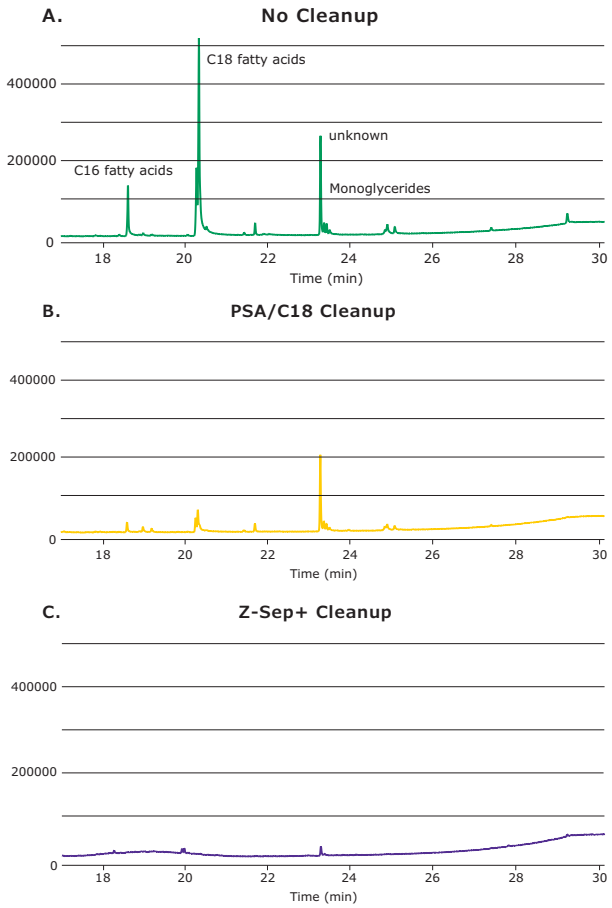


Table 5. Pesticide Recoveries from Pistachios Using Z-Sep+ Cleanup, Spike Level of 10 ng/g

Pesticide	Avg. % Recovery (n=3)	% RSD	Analysis
Aldicarb	102%	3%	LC-MS/MS
Aldicarb-sulfone	108%	1%	LC-MS/MS
Bifenazate	88%	4%	LC-MS/MS
Butocarboximsulfoxide	83%	5%	LC-MS/MS
Carbendazim	71%	4%	LC-MS/MS
Carbofuran	104%	4%	LC-MS/MS
Chlorantraniliprole	90%	5%	LC-MS/MS
Chlorpyrifos-methyl	66%	10%	GC-MS/MS
Endosulfan sulfate	58%	6%	GC-MS/MS
Etoxazole	45%	9%	GC-MS/MS
Etrimfos	90%	7%	LC-MS/MS
Fenthion	72%	9%	GC-MS/MS
Flufenoxuron	62%	15%	LC-MS/MS
Isoxathion	92%	3%	LC-MS/MS
Malathion	102%	4%	LC-MS/MS
Methabenzthiazuron	84%	3%	LC-MS/MS
Methomyl	106%	5%	LC-MS/MS
MGK-264 (avg. 2 isomers)	57%	17%	GC-MS/MS
Neburon	92%	7%	LC-MS/MS
Omethoate	66%	2%	LC-MS/MS
Pyraflufen-ethyl	97%	18%	LC-MS/MS
Quinalphos	104%	7%	LC-MS/MS
Rotenone	100%	3%	LC-MS/MS
Spinetoram	56%	10%	LC-MS/MS
Spiromesifen	83%	4%	LC-MS/MS
Thiacloprid	100%	2%	LC-MS/MS
Thiophanate-methyl	100%	3%	LC-MS/MS
Tolclofos-methyl	71%	10%	GC-MS/MS
Triazophos (avg. 2 isomers)	89%	3%	LC-MS/MS
Trichlorfon	14%	13%	LC-MS/MS

Conclusions

Pistachios, which contain 45% fat, present a challenging matrix when doing pesticide residue analysis. If using QuEChERS extraction, some fat will be co-extracted with the analytes of interest. Thus, the cleanup step must be able to reduce this background. In this application, the use of Supel™ QuE Z-Sep+ was demonstrated for the effective cleanup of these

extracts prior to LC-MS/MS and GC-MS/MS analysis. Fatty acid and monoglyceride background were significantly reduced using Z-Sep+, and compared to PSA/C18 cleanup, the resulting extract had lower background; as evidenced by GC-MS-scan data. Pesticide recovery was within the acceptable range of 70-120% for 22 out of 30 targeted pesticides, with excellent reproducibility demonstrated for spiked replicates.

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Supel™ QuE Citrate Extraction Tube, 12 mL, pk of 50	**55227-U**
Supel™ QuE Z-Sep+, 2 mL, pk of 100	**55414-U**
Supel™ QuE Z-Sep+, 15 mL, pk of 50	**55486-U**
Capillary GC Column	
SLB®-5ms, 20 m × 0.18 mm I.D., 0.18 µm	**28564-U**
HPLC Column	
Ascentis® Express RP-Amide, 10 cm × 2.1 mm I.D., 2 µm	**53913-U**
Accessories	
QuEChERS Shaker and Rack Starter Kit, USA compatible plug	**55278-U**
QuEChERS Shaker and Rack Starter Kit, EU Schuko plug	**55438-U**

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Thermo-O-Ring™ Inlet Liner O-Ring, pk of 10	**21003-U**
Gold-Plated Inlet Seal (Straight Design), pk of 2	**23318-U**
Capillary Column Nut for Agilent®MS, pk of 5	**28034-U**
Vials	
Certified Vial Kit, Low Adsorption (LA), 2 mL, amber, w/slit caps pk of 100	**29654-U**
Certified Vial Kit, Low Adsorption (LA), 2 mL, clear with marking spot, PTFE-silicone septa w/slit, pk of 100	**29652-U**

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Analysis of Fipronil and Fipronil Sulfone in Eggs, Chicken Meat and Mayonnaise

Katherine Stenerson, Principal R&D Scientist, Analytix@merckgroup.com

Fipronil is a broad spectrum insecticide that is in the European Union (EU)[1] not permitted to be use with food producing animals. Fipronil sulfone is formed by degradation of fipronil, and is actually more toxic to birds and other organisms than the parent compound.[2] The maximum residue limit designated by the EU for fipronil in eggs 5 ng/g (.005 mg/Kg), reported as a sum of the parent compound and the sulfone degradant.

In this application, QuEChERS extraction and cleanup (see **Figure 1**) followed by GC/MS/MS analysis (conditions listed in **Table 1**) were used for spiked samples that were quantitated against a matrix-matched calibration curve. No internal standard was used, thus recoveries reported are absolute.

Supel™ Que Z-Sep+, used for extract cleanup, was found to significantly reduce levels of co-extracted fatty compounds, including cholesterol. **Figure 2** shows the reduced background after cleanup with Z-Sep+. The fatty acids, eluting in the same retention range as the fipronil and fipronil sulfone, were removed by the Z-Sep+ cleanup, resulting in a clean signal for both compounds at 5 ng/mL in the final extract (**Figure 3**). Recovery and reproducibility of the method was good (**Table 2**). The method was also applied to chicken meat and mayonnaise. The ruggedness test of the GC method was done by repeated injections (>70) of egg sample extracts, resulting in only a small change in signal throughout the run, with a variation of 12%.

Figure 1. Sample Preparation, Procedure, QuEChERS Extraction and Cleanup with Z-Sep+

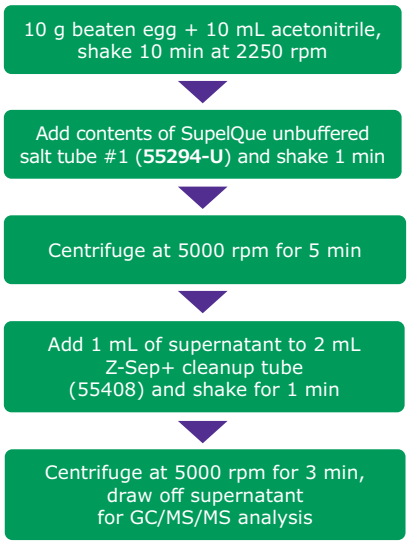

Table 1. GC-MS/MS Conditions

column:	SLB®-PAHms, 30 m x 0.25 mm I.D., 0.25 µm (**28340-U**)
oven:	50 °C (2 min), 15 °C/min to 340 °C (10 min)
inj. temp:	250 °C
carrier gas:	helium, 1.2 mL/min, constant
detector:	MRM, Fipronil: 254.9/228, 350.8/254.8, 366.8/212.8 Fipronil sulfone: 382.8/254.9, 384.8/256.8, 254.9/227.9
injection:	1 µL, pulsed splitless (50 psi until 0.75 min, splitter on at 0.75 min)
liner:	4 mm I.D. FocusLiner™ with taper

Figure 2. GC-MS Scan Analysis of Quechers Extract of Egg

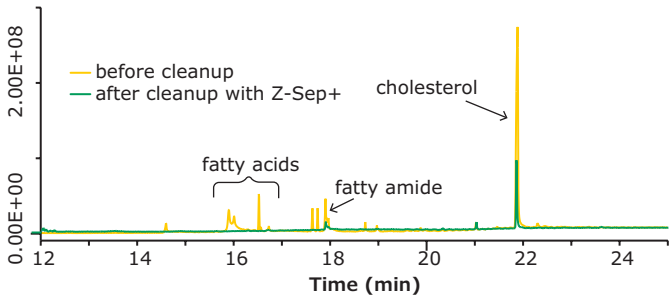

Figure 3. GC-MS/MS analysis of fipronil and fipronil sulfone in eggs at 5 ng/g

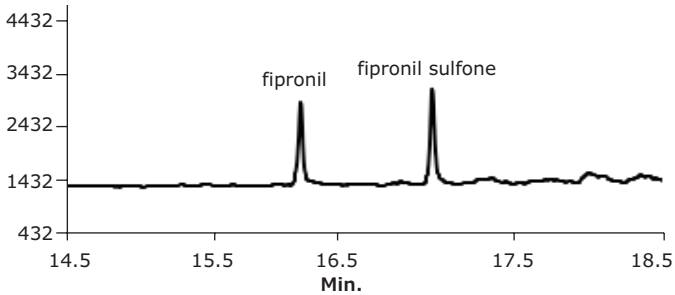

Table 2. Percent Recovery and Reproducibility (%Rsd); Spiking Level of 5 ng/g

n=3	Eggs	Chicken Meat	Mayonnaise
Fipronil	91 (0.5)	103 (3)	85 (3)
Fipronil Sulfone	91 (1.8)	116 (2)	87 (4)

For the full application data contact us or visit **SigmaAldrich.com/fipronil**

References

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2 . Madsen, J.E.; Sandstrom, M.W.; Zaugg, Steven D., Methods of Analysis of the U.S. Geological Survey Nation Water Quality Laboratory-A Method Supplement for the Determination of Fipronil and Degradates in Water by Gas Chromatography/Mass Spectrometry; Open-file report 02-462; U.S.G.S.: Denver, CO, 2003.

Featured Products

Description	Cat. No.
SLB®-PAH ms, 30 m x 0.25 mm I.D., 0.25 µm	**28340-U**
Supel™ Que nonbuffered extraction tube #1	**55294-U**
Supel™ QuE Z-Sep + Tube, 2 mL	**55408-U**
Fipronil, Pestanal® analytical standard	**56451-100MG**
Fipronil Sulfone, Pestanal® analytical standard	**32333-50MG**

FOOD & BEVERAGE

Analysis of Bisphenol A in Food by Solid Phase Microextraction Using an Overcoated Fiber

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Introduction

Bisphenol A (BPA) is commonly used for food packaging applications such as polycarbonate bottles and the linings of metal cans used for soups, juices, etc. It is a suspected endocrine disruptor, and therefore, low level, long term exposure as a result of migration into food from packaging materials is a concern. The use of BPA in food contact applications is regulated by the Food and Drug Administration (FDA), and in 2013 was prohibited for use in the packaging materials of infant formula.[1] For other food contact applications, margins of safety were published by FDA as "NOAEL", which stands for "no observed adverse effect level". This NOAEL was set at 5 mg/kg body weight per day, which is well above the estimated dietary intake.[2] Similarly, tolerable daily intake or "TDI" was set by the European Union (EU) at 4 µg/kg body weight per day.[3] While exposure to BPA through diet is thought to be low, testing continues in order to assess its migration into food from can and lid linings, plastic containers, etc. In the case of the EU, the specific migration limit (SML) for BPA from packaging into food has been amended in Sept 2018 to 0.05 mg/kg (formerly 0.6 mg/kg) food. In case of contact materials for food products with intended use for infants or young children, no BPA migration from coatings or varnishes is permitted at all.[4]

Extraction methods for determination of BPA in food include both solvent extraction (SE) and solid phase extraction (SPE), with the latter more commonly used with liquid samples and the former for solid samples. Analysis can be done by either LC or GC, and both have been used throughout the literature. Solid phase microextraction (SPME) has been used for the determination of BPA in water, but has not been widely used for this application in food matrices due to sensitivity and fiber ruggedness issues associated with exposure to matrix components such as fats and proteins.[5,6]

The purpose of this application was to revisit the use of SPME to develop a quick, easy, and sensitive method for analysis of BPA in a variety of food products. The issues mentioned previously related to food matrices and SPME were addressed through the use of an overcoated (OC) divinylbenzene (DVB) fiber. The overcoating, which consists of polydimethylsiloxane (PDMS), protects the DVB layer from contamination and increases the physical robustness of the fiber.

In addition, material adhering to the overcoating is more easily removed during the wash step typically performed for such samples. SPME extraction using the OC-DVB fiber was followed by GC-MS/MS analysis for optimum sensitivity. The steps taken in method development and optimization as well as durability comparison are outlined here. For more detailed information, please refer to the online version of this article under **SigmaAldrich.com/Analytix** (Issue 4).

Experimental

The final, optimized SPME method using the OC fiber is described in **Table 1**. After extraction, the fiber was desorbed in the inlet of a 7890/7000C GC-MS/MS system, and analysis proceeded following the conditions

Table 1. Optimized SPME Procedure for Extraction of BPA from Food Samples

sample/matrix:	10 mL vial containing 0.5 g of sample and 6.5 mL of water at pH 4 containing 25 % sodium chloride
SPME fiber:	Overcoated PDMS-DVB, 23 gauge
incubation:	10 min, 50 °C, 400 rpm
extraction:	immersion, 50 min, 50 °C, 250 rpm, vial penetration 34 mm
wash:	0.5 min, 250 rpm, vial penetration 34 mm
desorption:	3 min, 260 °C
post bake:	6 min, 270 °C

Table 2. GC-MS/MS Conditions

column:	SLB®-PAHms, 30 m x 0.25 mm I.D., 0.25 µm (**28340-U**)
oven:	100 °C (3 min), 15 °C/min to 300 °C (10 min)
inj.temp.:	260 °C
carrier gas:	helium, 1 mL/min constant flow
detector:	MRM: BPA: 213/119, 213/91, 119/91
	BPA-d16: 224/125, 224/97, 125/97
MSD interface:	325 °C
liner:	0.75 mm I.D. SPME

listed in **Table 2**. The samples analyzed (canned pumpkin, pureed carrot baby food, cream of chicken soup and canned energy drink) were obtained from a local grocery store and refrigerated prior to testing. They were prepared for SPME by weighing 0.5 g into a 10 mL autosampler vial. Spiked samples for determination of accuracy and repeatability were spiked at 10 ng/g by direct addition of 5 µL of a 1 µg/mL solution of BPA in methanol to the 0.5 g sample. Samples were then allowed to equilibrate for 30-60 minutes. 6.5 mL of SPME diluent (LC-MS/MS grade water containing 25 % NaCl by weight, and adjusted to pH=4 with H_3PO_4) was added to each, followed by 7 µL of a 1 µg/mL methanolic solution of BPA-d16 internal standard. To decrease BPA background from the laboratory, all measuring glassware and pipettes used were glass, and were triple rinsed with methanol prior to use. The salt used to make the SPME diluent was treated in a muffle furnace and stored in a glass jar.

Samples (spiked and unspiked) were quantitated against matrix-matched calibration curves prepared as described previously and extracted following the method in **Table 1**.

Results and Discussion

Method Optimization

A primary goal of method development was to determine a single set of SPME parameters that could be used with multiple sample types. In the following paragraphs, optimization of critical parameters is outlined (more details in the online version under **SigmaAldrich.com/Analytix**).

Salt, pH and dilution. Addition of salt and lowering of pH increased response significantly of both BPA and BPA-d16 (not shown). The samples to be analyzed were mostly very viscous, and required dilution prior to SPME; thus, a water diluent at pH 4 containing 25 % salt was chosen. After experimentation with different sample sizes/dilutions, 0.5 g diluted to 7 mL was found to work adequately for all the matrices evaluated, which included canned pumpkin, pureed carrots, condensed cream of chicken soup, and a fruit flavored energy drink (latter one probably could had been analyzed undiluted).

Post-extraction wash. Since the method was to be used with food samples, incorporation of a post-extraction wash step was critical in removing residual matrix on the surface of the fiber prior to desorption in the GC inlet. Past work found this step to be more effective with the overcoated rather than the standard DVB fiber.[7] To maximize washing, a 30-second time was chosen for the method. Since good response was still obtained, the loss in response compared to no/shorter wash time was not expected to severely impact the sensitivity of the method.

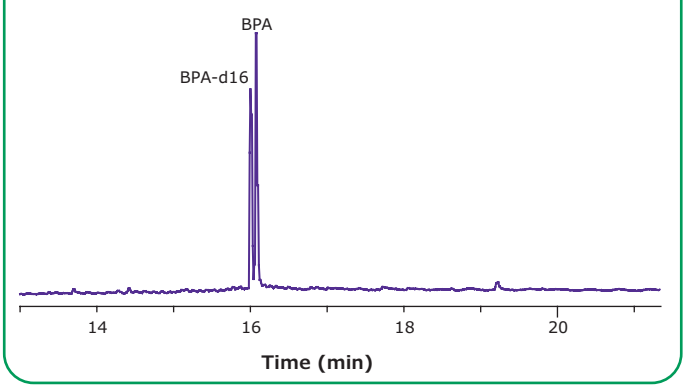
Extraction & equilibration conditions. Extraction times from 10 to 60 minutes were studied using 25 % saltwater at pH 4, spiked at 0.1 ng/mL (10 min equilibration @ 400 rpm agitation). Response steadily increased from 10 to 50 minutes and then leveled off from 50 to 60. Thus, 50 minutes was chosen as the extraction time.

Since temperature can influence the kinetics of the extraction, especially when working in heavy matrix like pureed carrot baby food, the effect of extraction temperature was studied at 30 °C, 40 °C, and 50 °C. Absolute response of BPA increased with temperature; thus 50 °C was chosen as the temperature for extraction and equilibration.

BPA background. BPA is a common laboratory contaminant, thus a major challenge in its analysis by any approach is managing background. SPME requires minimal sample preparation steps and materials, reducing sources of contamination compared to liquid-liquid extraction or SPE. Some steps taken to reduce background for the SPME method included methanol rinsing of pipettes and glassware, muffle furnace treatment of the sodium chloride, and use of LC-MS/MS water in a glass bottle for the diluent solution. Some background was generated upon injection in the GC, and increasing the septum purge setting from 3 to 6 mL/min reduced this. Some BPA background was still present from the SPME process; however, low level detection from samples was still possible.

Calibration. Since SPME is an equilibrium extraction technique, quantitation must be done against standards extracted using the same method as the samples. In the case of BPA, extraction efficiency varied by matrix, thus matrix calibration had to be used for accurate quantitation. An unspiked sample for each was included as a "0" concentration point.

Figure 1. GC-MS Analysis (full scan) of BPA and BPA-d16, Underivatized, on the SLB-PAHms 10 ppm Standard in Methanol



GC-MS/MS Analysis. A common approach to the GC analysis of BPA is derivatization using silylation or acetylation. This improves peak shape and response, allowing for better quantitation.[5,6] For this method, using an SLB®-PAHms column, as seen in **Figure 1**, derivatization was not necessary to obtain sufficient chromatographic performance and response.

SPME Method Performance

Analysis of spiked and unspiked samples. For all four matrices studied, both BPA and the internal standard, BPA-d16, could be detected free of interferences. An example is shown for the heaviest matrix, canned pumpkin, in **Figure 2**. As seen in **Figure 3**, the SPME method showed good linearity from the different matrices. The units are reported as ng/mL, which reflects the concentration from 0.5 g of sample diluted to a final volume of 7 mL prior to analysis, and translates to 7 to 140 ng/g BPA in the original sample. Accuracy and reproducibility of the method from these same matrices was determined by analysis of samples spiked at 10 ppb. The results of these evaluations are summarized in **Table 3**. Accuracy was >80% for all four matrices, with reproducibility as percent relative standard deviation (% RSD) or relative percent difference (%RPD) of <15 %. Since matrix-matched calibration curves were used to quantitate spiked samples, the level of BPA present in each sample prior to spiking could be determined with the "0" concentration or unspiked analysis using a standard addition approach. These values are reported in **Table 4**. The BPA detected in the carrot/baby food in the glass jar was probably a result of leaching from the lined cap, and the level detected is in the range found by others in the analysis of baby food in glass jars with metal lids.[8] In the canned samples, the highest level of BPA was detected in the cream of chicken soup. However, this level was still lower than past BPA levels determined by others in canned chicken soup.[9] It should also be noted that the soup was analyzed directly without water dilution. Normal preparation for consumption requires a 1:1 dilution with water, which would essentially cut the BPA level by 50 %.

As mentioned previously, when doing immersion SPME into heavy matrices, a post extraction wash step is essential in removing residual matrix prior to the desorption step in the GC inlet. A comparison of fiber durability and method ruggedness between the OC and standard, non-overcoated versions of the PDMS/DVB fiber was conducted by subjecting both to multiple extractions of canned pumpkin samples. Separate samples of pumpkin were weighed out and spiked at 10 ppb with BPA-d16. These were then run in a continuous sequence with 1 ng/mL BPA/BPA-d16 spiked water samples run every 6th extraction. The results

Figure 2. SPME-GC-MS/MS Analysis of BPA Spiked at 10 ppb in Canned Pumpkin

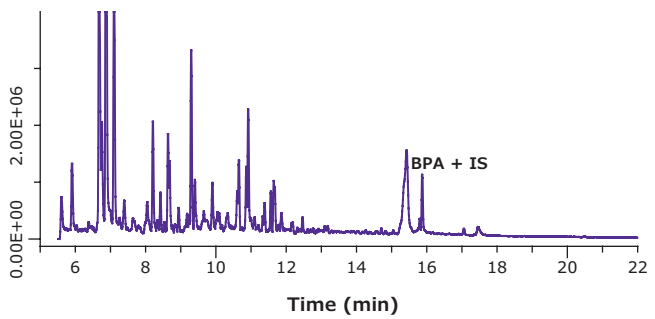


Figure 3. BPA / SPME Method Linearity from Different Matrices

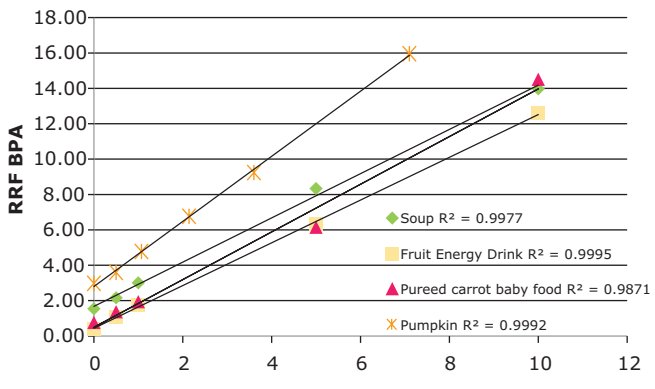


Table 3. Accuracy and Reproducibility for SPME Method Applied to Spiked Samples

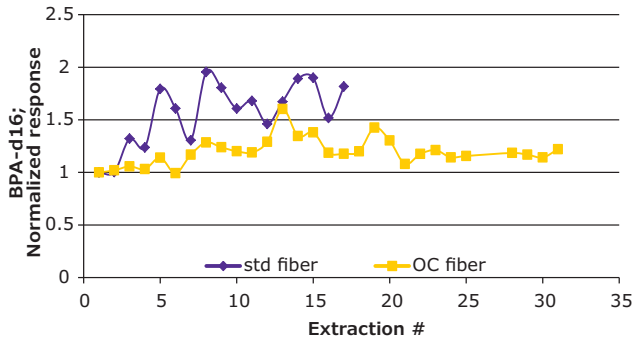
Sample	Spike Level	Avg. Amount Measured	Accuracy	RSD
fruit flavored energy drink	10 ng/mL	11.5 ng/mL	115 %	1 % (*n*=3)
baby food, carrots	10 ng/g	11.7 ng/g	117 %	2 %*
cream of chicken soup (condensed)	10 ng/g	8.2 ng/g	82 %	9 %*
pumpkin	10 ng/g	11.0 ng/g	110 %	13 % (*n*=6)

*%RPD, 2 replicates

Table 4. Level of BPA in Unspiked Samples; Calculated Using Standard Addition

Sample	Container Type	BPA Level - measured in unspiked sample
fruit flavored energy drink	can	0.8 ng/mL
baby food, carrots	glass jar with metal lid	0.65 ng/g
cream of chicken soup (condensed)	can	12.7 ng/g
pumpkin	can	1.6 ng/g

Figure 4. Response Trend of BPA-d16 from Spiked Pumpkin Samples Over Repeated Extractions. Response is Normalized to the First Sample.



are shown in **Figure 4**. For purposes of studying the trend in response, area counts were normalized to the first sample extraction. In 25 extractions of pumpkin using the OC fiber, response did not show a significant decline. The condition of the fiber after the testing sequence was fairly good, with some discoloration but no evidence of physical damage. By comparison, the standard fiber run was stopped after 18 extractions, as the coating had stripped completely off the fiber core. The response trend was erratic, as seen in **Figure 4**. The findings of the durability testing indicate that either the overcoating is protecting the phase from damage as a result of exposure to the pumpkin matrix, and/or the post-extraction wash step is more effective for the OC fiber at removing residual matrix. This then helps to prolong fiber life.

Summary and Conclusions

An immersion SPME-GC-MS/MS method using an overcoated PDMS/DVB fiber was developed for the low level analysis of BPA from various food products. Method linearity from different matrices - a fruit flavored beverage, canned pumpkin, pureed carrot baby food, and cream of chicken soup - was in the range of 0.9871 (carrots) to 0.9995 (beverage). Method accuracy and reproducibility at a 10 ppb spiking level was between 80-110%, with RSD/RPD values of <15%. Durability testing showed the OC fiber to be more physically robust, with more consistent response compared to a standard fiber; the SPME method had only a few steps and was easy to automate. In addition, it was highly sensitive, and when combined with GC-MS/MS, provided the selectivity necessary to be used with different matrices.

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Featured Products

Description	Cat. No.
SPME OC Fiber Assembly (PDMS/DVB), Pk.3	**57439-U**
SPME fiber holder for use with CTC autosampler	**57347-U**
SLB®-PAHms capillary GC column, 30 m x 0.25 mm I.D., 0.25 µm	**28340-U**
Bisphenol A, certified reference material, TraceCERT®, 100 mg	**42088**
Bisphenol A-d16, analytical standard, 50 mg	**442876**

Related Products

Description	Cat. No.
Sodium chloride, ACS reagent grade	**746398**
Clear vial, screw top, 10 mL, for CTC autosampler, Pk.100	**SU860099**
Magnetic screw cap, with 1.3 mm septa, for autosampler vial, Pk.100	**SU860101**
0.75 mm I.D. direct (SPME) liner for Agilent®	**2637501**
Molded Thermogreen™ LB-2 septa with injection hole, 11 mm, Pk.50	**28336-U**

To read more about overcoated SPME fibers, visit us at **SigmaAldrich.com/spme-ocf**

For our food safety tools, see **SigmaAldrich.com/food-and-beverage**

FOOD & BEVERAGE

New CRM Solutions for Paralytic Shellfish Toxins

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

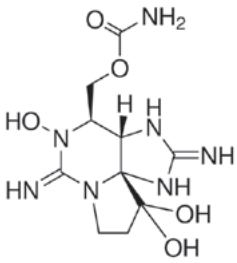
Paralytic Shellfish Toxins (PSTs) are a group of highly toxic, naturally occurring alkaloids produced by certain species of marine algae. During harmful algae blooms, these toxins can accumulate in bivalve mollusks and cause severe poisoning after consumption. The occurrence of PSTs in seafood, therefore, needs to be monitored. We are introducing a series of native and isotope labeled Certified Reference Material (CRM) solutions for paralytic shellfish toxins, suitable for calibration of LC-MS / LC-IDMS (isotope dilution MS) food testing methods.

The CRM solutions are manufactured in-house under our ISO/IEC 17025 and ISO 17034 accreditation, and feature:

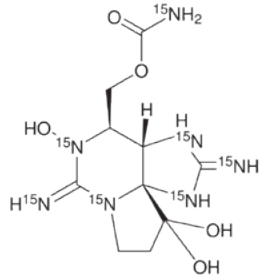
- Certification by qNMR or IDMS with traceability to SI unit via NIST or NRC primary standards
- Solutions produced gravimetrically
- Homogeneity, accelerated and long-term stability tested by LC-MS
- A comprehensive certificate including the overall uncertainty

For more information, and an up-to-date list of marine toxin CRMs, please visit:

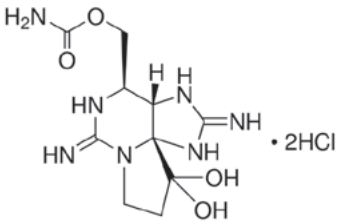
SigmaAldrich.com/marinetoxins



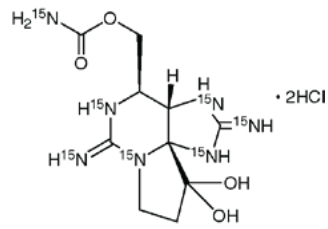
41619
Neosaxitoxin solution
20 µg/g in 3 mmol/L HCl



41206
Neosaxitoxin $^{15}N_7$ solution
10 µg/g in 3 mmol/L HCl



93665
Saxitoxin dihydrochloride solution
20 µg/g in 3 mmol/L HCl



30929
Saxitoxin $^{15}N_7$ dihydrochloride solution
10 µg/g in 3 mmol/L HCl

As a first set of products, we are introducing the PSTs Neosaxitoxin and Saxitoxin dihydrochloride as CRM solutions both in native and ^{15}N labeled form.

Description	Qty.	Cat. No.
Neosaxitoxin, 20 µg/g in hydrochloric acid, certified reference material, TraceCERT®	0.5 mL	**41619**
Neosaxitoxin-$^{15}N_7$, 10 µg/g in hydrochloric acid, certified reference material, TraceCERT®	0.5 mL	**41206**
Saxitoxin dihydrochloride, 20 µg/g in hydrochloric acid, certified reference material, TraceCERT®	0.5 mL	**93665**
Saxitoxin-$^{15}N_7$ dihydrochloride, 10 µg/g in hydrochloric acid, certified reference material, TraceCERT®	0.5 mL	**30929**

Surface Enhanced Raman Spectroscopy (SERS) – A new solution for food quality and safety analysis

Yanqi Qu, Siyue Gao, Lili He, Department of Food Science, University of Massachusetts, Amherst, MA

Winner our 2018 Lifesciences Award in Food & Beverage Safety 2018 - See full article online at SigmaAldrich.com/Analytix (Issue5)

Surface-enhanced Raman spectroscopy (SERS) is an emerging technology in environmental, agricultural, food, and medical applications.[1–7] In **Figure 1** (a), Raman spectroscopy provides a signature profile of an analyte according to its chemical structure, and with the attachment of nanometallic structures, Raman scattering can be dramatically enhanced. Compared to the standard analytical methods (e.g., HPLC, GC-MS, etc.), SERS showed advantages in simpler sample preparation, faster detection, easier operation, less instrumental complexity, and relative less expensive cost. Additionally, a handled or portable Raman instrument allows SERS to be an on-site solution for the field test.

SERS substrates

Colloidal nanoparticles

Colloidal silver and gold nanoparticles are commercially available and can also be easily fabricated in a lab. Sample preparation can be facilely done by mix a few drops of sample with colloidal nanoparticles and air-dried on solid surfaces for measurement. The biggest concern towards the colloidal nanoparticles is the "coffee ring" that can cause a huge variation.

Solid based substrates

As shown in **Figure 1** (c), nanoparticles can be driven by a "mediating solvent" to self-assembly a monolayer mirror substrate, which features a better signal consistency and an improved quantitative ability. It can be either fabricated with the targets in the solvent or preformed as a dried SERS active platform for sample immersing. In **Figure 1** (b), nanoparticles can also be deposited to targets concentrated on a Nitrocellulose Millipore® membrane. This providing a fast detection of low concentrations of pathogens and contaminants without pre-enrichment.

Application of SERS in food analysis

Pesticides detection

SERS is known for its sensitive and fast detection of many kinds of pesticides in different food or agricultural matricees, with a limit of detection lower than the regulation requirement.[10] In my project, the mirror substrate can detect fonofos pesticide in beverages at a low concentration (i.e. 0.5 ppm) with good recoveries (i.e. 99-106 %), which illustrated the reliability of mirror and SERS in pesticides detection.[8] SERS is also suitable to monitor the distribution and penetration of pesticides on plants (**Figure 1** (d)), also the efficacy of pesticides removal from fruits.[12]

Colorants and adulterants analysis[5]

A SERS database was developed including a wide variety of artificial and natural food coloring agents currently approved or banned in the United States. All colorants showed discriminative SERS signals and can be differentiated or quantified in commercial products.

Wine analysis

By forming the mirror substrate with the red wine extracts in the "mediating solvent", signature peaks in the spectra were successfully matched with condensed tannin, resveratrol, anthocyanins, gallic acid, and catechin, and the unique chemical information creates a specific bar code that could be beneficial for red wine quality assessment and authentication.

Bacteria detection[9,13]

Using the Millipore® filter membrane substrate, we developed a rapid bacteria screening method using SERS to successfully detect Escherichia coli, Salmonella enterica, and Listeria monocytogenes in 80 min in pond water and vegetable rinse water with reliable quantification (i.e. R^2 = 92 %) to a concentration as low as 10 CFU/mL.

References

Please see the online version of the article

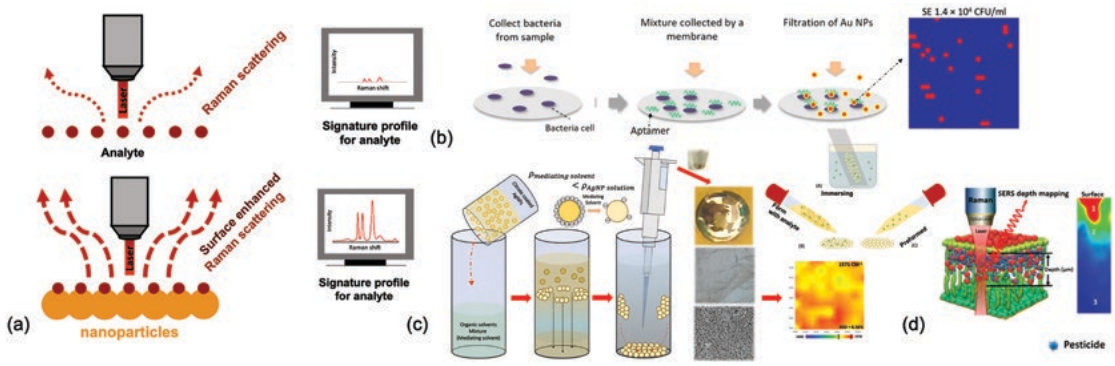


Figure 1. (a) Mechanism of SERS (b) Millipore® membrane substrate for bacterial detection (c) Mirror substrate (d) Monitoring of pesticide penetration using SERS mapping technique.

New Reference Materials for Elemental Analysis and Isotope Ratio Testing in Food

Expanded food matrix material offering

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

Everyone is concerned that the food we eat and the beverages we drink are safe and correctly labeled. Food scandals, which have included the detection of instances of food fraud and adulteration, frequently remind us that the quality of food is not to be taken for granted and should be monitored constantly by the food industry and regulators.

Enforcement of food regulations requires reliable and efficient testing methods. To ensure that these methods lead to traceable and comparable test results, well characterized reference materials need to be available.

As part of our comprehensive offering of reference materials for food and beverage testing, we provide a range of more than 150 food matrix materials intended for method development, method validation, or routine analysis. On our website **SigmaAldrich.com/foodmatrix** these products can be browsed by analyte type or matrix, making it easy to locate products for a certain area of interest.

This portfolio is now complemented with a new range of twelve products certified for element content of Carbon, Hydrogen, Nitrogen and/or Sulfur. Some of the products are certified for an isotope ratio of ^{13}C, ^{15}N and/or ^{34}S (see table for details).

Stable isotope analysis is often applied to detect adulteration of food, since the isotope ratio can be different depending on the source of a material.

The products are Certified Reference Materials (CRMs) manufactured under ISO/IEC 17025 and ISO 17034 double accreditation. The certified values for Carbon, Hydrogen, Nitrogen and Sulfur were determined by an elemental analyser calibrated to suitable materials from the National Institute of Standards and Technology (NIST), Maryland, USA. The isotope values are traceable to primary isotopic Certified Reference Materials (CRM) issued by IAEA Vienna.

Matrix	Parameters	Package Size	Cat. No.
Alfalfa	Carbon, Hydrogen, Nitrogen and Sulphur	30 g	**EMB2273**
Coconut Shell	Carbon, Hydrogen and Nitrogen	30 g	**EMB2168**
Olive Oil	$\delta\ ^{13}C$	5 g	**EMB2172**
Olive Stone	Carbon, Hydrogen and Nitrogen	30 g	**EMB2170**
Pasta	Nitrogen	100 g	**EMB2149**
Pasta	Nitrogen	10 g	**EMB2140**
Protein	Nitrogen	30 g	**EMB2154**
Rice Flour	Carbon, Hydrogen, Nitrogen and Sulphur	30 g	**EMB2278**
Sorghum Flour	Carbon, Nitrogen and Sulphur	30 g	**EMB2158**
Sorghum Flour	Carbon, Nitrogen and Sulphur, $\delta\ ^{13}C$, $\delta\ ^{15}N$, $\delta\ ^{34}S$	5 g	**EMB2159**
Wheat Flour	Carbon, Hydrogen and Nitrogen	30 g	**EMB2156**
Wheat Flour	Carbon, Nitrogen and Sulphur, $\delta\ ^{13}C$, $\delta\ ^{15}N$, $\delta\ ^{34}S$	5 g	**EMB2157**

For our complete range of reference materials for F&B testing, please see our new webpage below.

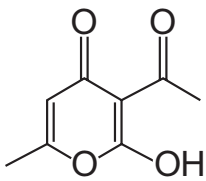
FOOD & BEVERAGE

Determination of Dehydroacetic Acid in Bread Using HPLC with UV Detection Following the Chinese National Standard (GB) Method

Dean Duan, Application Scientist, Analytix@merckgroup.com

Introduction

Dehydroacetic acid is an organic compound which has several industrial applications. It can be used as a plasticizer in synthetic resins; as a fungicide or bactericide; but also as a food preservative (European food additive number E265).

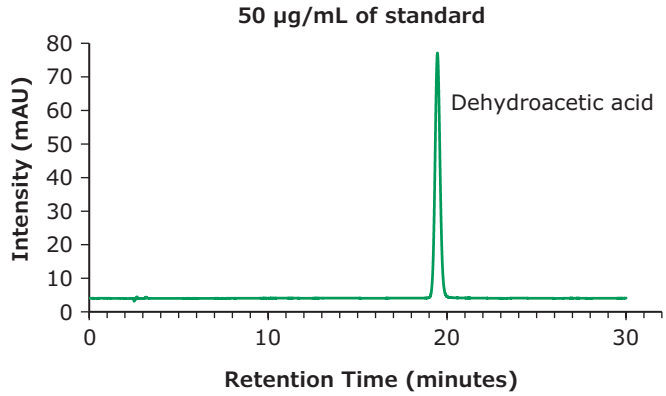


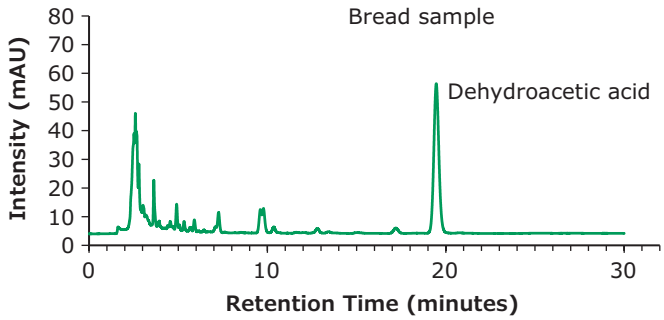
Dehydroacetic acid

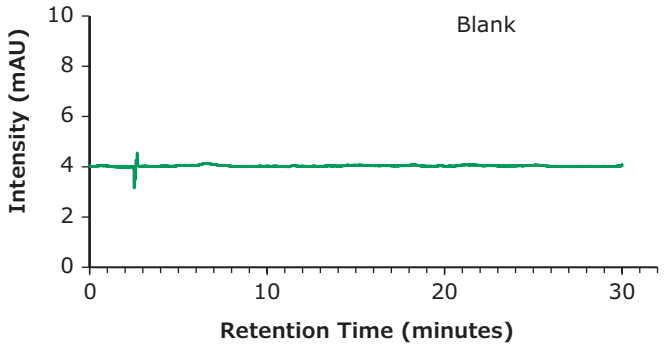
This application focuses on testing dehydroacetic acid in bread using a Discovery® HS C18 HPLC column, following the current Chinese national standard method (GB 5009.121-2016).

Obtained results show satisfactory chromatographic resolution of dehydroacetic acid from the bread sample matrix, and the method linearity, Limit of Detection (LOD) and Limit of Quantitation (LOQ) meet set testing requirements.

Experimental Conditions	
column:	Discovery® HS C18, 25 cm x 4.6 mm I.D., 5 µm (**568523-U**)
mobile phase:	[A] 20 mM ammonium acetate, pH 3.5 with acetic acid; [B] methanol; (70:30, A:B)
flow rate:	1.0 mL/min
column temp:	30 °C
detector:	UV, 293 nm
injection:	10 µL
sample:	standard stock solution: Weigh accurately 0.1 g of dehydroacetic acid into a 100 mL volumetric flask, add into 10 mL of 20 g/L of sodium hydroxide solution, add water to volume to obtain a 1.0 mg/mL of stock solution. Standard solution: Dilute the stock solution with water to obtain a 50 µg/mL of solution.
	sample preparation: Homogenize bread sample, weigh accurately 2–5 g of homogenized sample to a 25 mL centrifuge tube. Add into 10 mL of water, 5 mL of 120 g/L of $ZnSO_4$ aqueous solution, adjust pH value to pH 7.0 with 20 g/L of NaOH aqueous solution. Add water to volume, shake well. sonicate for 10 min. Transfer the supernatant over the bread crumb suspension to separating funnel, add into 10 mL of hexane for protein and fat removal, shake for 1 minute, stand for separating, remove the hexane layer, repeat the procedure twice, pool the aqueous layer to centrifuge, centrifuge at 4000 r/min for 10 minutes. Filter the supernatant with 0.45 µm filter membrane for HPLC analysis.







Chromatographic Data

	Compound	RT (min)	Resolution	Plates (USP)	Tailing Factor
1	Dehydroacetic acid	19.5	--	22832	1.0

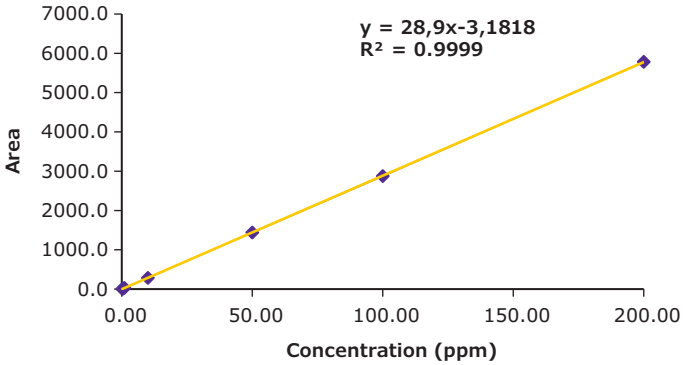
Specificity and Repeatability

1. Specificity: Inject Standard Solution and Determine the Retention Time and Monitor the Peak Purity

No.	Compound	RT (min)	Plates (USP)	Tailing Factor	Peak Purity
1	Dehydroacetic acid	19.5	22832	1.0	1.0000

2. Standard Repeatability (Dehydroacetic acid, 10 ppm)

Measurements	Mean Area
STD 1	283.7
STD 2	284.8
STD 3	284.2
STD 4	284.8
STD 5	283.2
Mean	284.1
Standard Deviation	0.7
RSD (%)	0.2



3. Linearity

Concentration (µg/mL)	Mean Area
0.20	5.7
0.50	14.8
1.00	28.8
10.0	284
50.0	1439
100.0	2875
200.0	5783

4. LOD & LOQ

Concentration (µg/mL)	Mean Area
0.20	5.73
0.50	14.79
1.00	28.76
STEYEX	0.35
Slope	28.70
LOD (ppm)	0.04
LOQ (ppm)	0.12

Featured Products

Description	Cat. No.
Discovery® HS C18 HPLC Column, 25 cm × 4.6 mm I.D., 5 µm particle size	**568523-U**
Syringe Filter Millex-HV Durapore® (PVDF), hydrophilic, non-sterile 0.45 µm pore size, 13 mm diameter, PK.100	**SLHVX13NK**
Dehydroacetic acid Pharmaceutical Secondary Standard; Certified Reference Material, 1 g	**PHR1582**
Solvents & Reagents	
n-Hexane for liquid chromatography LiChrosolv®	**1.04391**
Methanol gradient grade for liquid chromatography LiChrosolv®	**1.06007**
Water for chromatography (LC-MS Grade) LiChrosolv®	**1.15333**
Ammonium acetate for analysis EMSURE® ACS,Reag. Ph Eur	**1.01116**
Formic acid 98-100% for HPLC LiChropur®	**5.43804**
Sodium hydroxide pellets EMPLURA®	**1.06462**
Zinc sulfate heptahydrate for analysis EMSURE®ACS,ISO,Reag. Ph Eur	**1.08883**

For more infomation on food analysis, visit us at

SigmaAldrich.com/FoodTesting

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Subscribe to get regularly informed about news in our Reference Material portfolio for Food and Beverage testing including new product launches and useful applications.

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New Fapas® Food Matrix Reference Materials

Expanding our comprehensive offering of matrix reference materials for F&B testing

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

Matrix reference materials are an important tool for an analytical laboratory in order to develop, validate, or verify the results of analytical methods. While neat (or solution) reference materials are usually used for calibration or identification purposes of specific analytes, matrix materials take into account matrix effects and can serve to account for bias during sample workup and preparation. Matrix Materials are characterised in their composition of specified major, minor, or trace chemical constituents. The material can be naturally contaminated, or the samples can be fortified by spiking the analytes of interest to a blank matrix.

The closer the nature of the chosen matrix reference material is to the tested samples, the better it can help to validate the results of a method.

Manufacturing of Food Matrix Reference Materials is a very laborious and time consuming process. Most of the Food Matrix Materials currently available on the market are manufactured either by metrological institutes (like the National Institute of Standards and Technology (NIST) or the European Joint Research Center (JRC)) or by Capitalise Proficiency Testing (CPT) providers with access to a robust set of analytical data from accredited labs.

Our offering of close to 200 food matrix materials including products from NIST and JRC is now complemented by 41 new reference materials (RMs) manufactured by Fapas®, a provider of proficiency testing schemes for food analysis. Fapas® is the proficiency testing branch of FERA, a center of excellence for interdisciplinary investigation and problem solving across plant and bee health, crop protection, sustainable agriculture, food and feed quality, and chemical safety in the environment, based in York (UK).

These Reference Materials (RM) are derived from materials used for proficiency testing schemes and undergo formal testing for both short-term and long-term stability. The products are delivered with an associated datasheet, which lists the reference values and their expanded uncertainty U. The value of U is not a performance limit but is the uncertainty relating to the reference value. RMs therefore have a greater degree of trust in their values than, for example, quality control materials and can be used for method calibration purposes. Fapas® RMs are manufactured in accordance to the principles of ISO 17034, but they are not certified reference materials (CRMs).

fapas®
celebrating 30 years

As shown in the product table below, the product range includes a variety of analytes and matrix types.

Matrix Type	Analyte Types	Description	Cat. No.
Animal feed	Biotoxins	Aflatoxins in animal feed	**FAP80738**
		Fusarium toxins in animal feed	**FAP83946**
		OA in animal feed	**FAP83947**
Beverages	Biotoxins	OTA in coffee (processed)	**FAP84207**
	Carbohydrates and Sweeteners	Soft drinks ingredients	**FAP80679**
	Organic Pollutants	Acrylamide in coffee	**FAP80591**
	Trace Elements	Metals in wine	**FAP80561**
		Metals in soft drink	**FAP80562**
Cereals	Biotoxins	Aflatoxins in maize	**FAP80868**
		Fumonisins in cereals	**FAP80926**
		Fusarium toxins in cereals	**FAP80916**
		Multi-Mycotoxins in cereals	**FAP82171**
		OA in cereals	**FAP80836**
	Organic Pollutants	Acrylamide in potato products	**FAP80659**
	Trace Elements	Metals in infant cereal	**FAP80551**
		Metals in rice	**FAP80469**
		Metals in wheat	**FAP80467**
		Nutritional elements in breakfast cereal	**FAP88984**
Dairy products	Ash, Carbohydrates, Moisture, Nitrogen, Total Fat	Proximates in condensed milk	**FAP85259**
	Biotoxins	Aflatoxin M1 in milk powder	**FAP80733**
	Organic Pollutants	Melamine in milk powder	**FAP80673**
		Pesticides and PCBs in infant formula	**FAP88987**
		Pesticides and PCBs in milk powder	**FAP89036**
	Trace Elements	Metals in milk powder	**FAP80527**
		Nutritional elements in infant formula	**FAP88659**
		Nutritional elements in milk powder	**FAP88819**
Fish and seafood	Allergens	Histamine in fish	**FAP79864**
	Nitrogen	Total Volatile Basic Nitrogen in fish	**FAP89089**
	Trace Elements	Metals in seafood	**FAP80466**
Fruits and vegetables	Biotoxins	Patulin in fruit	**FAP84209**
	Trace Elements	Metals in fruit products	**FAP80553**
		Metals in vegetable puree	**FAP80554**
Meat	Ash, Moisture, Nitrogen, Total Fat, Trace Elements	Nutritional in canned meat product	**FAP85276**
		Nutritional and Hydroxyproline in meat	**FAP84231**
Oils and fats	Organic Pollutants	PAH in oils and fats	**FAP79868**
		Pesticides and PCBs in fat	**FAP89005**
		Pesticides in oil	**FAP89004**
Processed food	Allergens	Nut allergen in biscuit	**FAP79867**
	Allergens, Protein	Nut allergen in choc, quant	**FAP79859**
Spices	Biotoxins	Mycotoxins in spices	**FAP80775**
	Trace Elements	Metals in spices	**FAP79875**

The complete range of food matrix materials can be browsed by matrix type and analyte type on **SigmaAldrich.com/foodmatrix.**

Our full portfolio of reference materials can be found at **SigmaAldrich.com/Standards**

Certified Reference Materials of Brevetoxins

New product additions to our marine toxins certified reference materials range

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

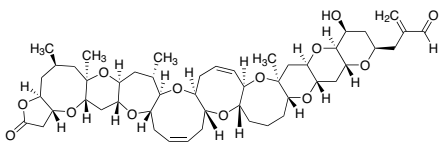
The pleasure of eating a good meal of fresh seafood can sometimes be abruptly dampened, if the food is contaminated by algal toxins accumulated through the food chain. Toxic algae can exponentially grow in unpredictably occurring algae blooms. Also, due to climate change such algae blooms can spread to new areas where they have been unknown before.

There is a big variety of naturally occurring marine toxins with very diverse chemical structures produced by various species of algae or phytoplankton. Some examples of marine toxin classes include amnesic shellfish toxins (domoic acid), diarrhetic shellfish toxins (okadaic acid and dinophysistoxins), and paralytic shellfish toxins (e.g. saxitoxin or neosaxitoxin).

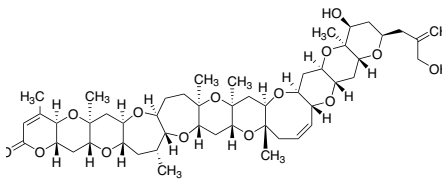
Brevetoxins (BTX) are neurotoxins produced by the dinoflagellate Karenia brevis and are responsible for neurotoxic shellfish poisoning (NSP). Acute symptoms of NSP include nausea, vomiting, diarrhoea, parasthesia, cramps, bronchoconstriction, paralysis, seizures, and coma. Brevetoxins have complex cyclic polyether structures as shown in **Figure 1**.

Although neurotoxic shellfish poisoning (NSP) predominantly occurs in the Gulf of Mexico and the east coast of the US, it can also be found in other regions, such as New Zealand. Particularly notable was the NSP outbreak observed in the New Zealand Hauraki Gulf region in 1993. In these regions, the brevetoxins in shellfish are regulated. The US FDA and New Zealand sets the action level at 0.8 mg BTX-2 equivalents per kg shellfish (MPI BMS RCS 2018, ref US FDA). In Australia, the maximum level for BTX-group toxins is 20 MUs/100 g, but the BTX analogue is not specified (FSANZ, 2010). In the EU, brevetoxins are currently not regulated but the EFSA published a scientific opinion assessing the risks to human health related to the consumption of brevetoxin-(BTX) group toxins in shellfish and fish.[1]

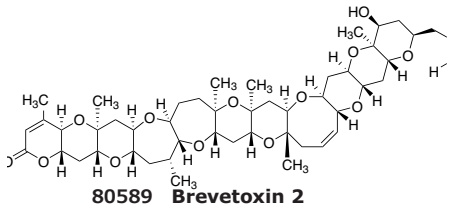
While traditional methods such as the mouse bioassay or ELISA are still being used for detection of marine toxins, the use of LC-MS is gaining importance.[2] Therefore, the availability of well characterized, reliable reference materials is critical. One of the main challenges hereby is the limited availability of such materials. The toxins often need to be isolated from the producing algae, which is a very laborious process that typically yields only a few mg of purified material.



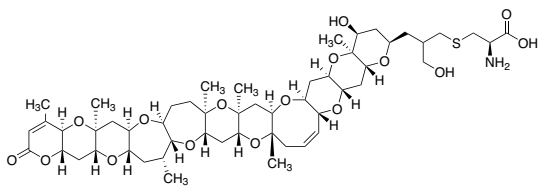
41014 Brevetoxin 1



07576 Brevetoxin 3



80589 Brevetoxin 2



78688 S-Desoxybrevetoxin B2

Figure 1. Chemical Structures of the Brevetoxins

In our ISO/IEC 17025 and ISO 17034 double accredited laboratory, we use a combination of quantitative NMR (qNMR) and Isotope Dilution MS (IDMS) enabling the manufacturing of Certified Reference Materials (CRMs) with very low quantities of starting materials.[3] A considerable number of marine toxin CRM solutions have been launched over the past years using this method. Recently, four new brevetoxins CRM solutions have been added to this range.

*Trace*CERT® Marine Toxin CRM Solutions for Brevetoxins

Description	Qty.	Cat No
Brevetoxin 1, 20 µg/g in acetonitrile	0.5 mL	**41014**
Brevetoxin 2, 20 µg/g in acetonitrile	0.5 mL	**80589**
Brevetoxin 3, 20 µg/g in acetonitrile	0.5 mL	**07576**
S-Desoxybrevetoxin B2, 20 µg/g in methanol	0.5 mL	**78688**

For more information and an up-to-date list of marine toxin CRMs please visit:
SigmaAldrich.com/marinetoxins

References:

1. EFSA Journal 2010; 8(7):1677
2. McNabb, Paul S.; Selwood, Andrew I.; et al. Journal of AOAC International, Volume: 95, Issue: 4, Pages: 1097-1105
3. Analytix Reporter, Issue 5, 2019

FOOD & BEVERAGE

Determination of Water in Sunflower Oil by Karl Fischer Titration

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

Sunflower oil is the most used cooking oil worldwide. It finds its use in food, as a frying oil, or in cosmetic products as a natural emollient. Additionally, it is used in the production of biodiesel and in some pharmaceutical and technical applications. Every year sunflower seeds produce more than 16 million tons of oil. Sunflower oil contains polyunsaturated fatty acids, such as linoleic acid, monounsaturated acids such as oleic acid, saturated fatty acids, and a high amount of vitamin E antioxidant.

The water content in sunflower oil influences the quality and shelf life of the oil and can chemically react with the oil's components, e.g. it can break up the ester bonds and form free fatty acids.

The amount of water in sunflower oil is typically 0.2 % or less, therefore the coulometric method is recommended and described in different norms e.g. DIN EN ISO 8534 - Animal and vegetable fats and oils -- Determination of water content -- Karl Fischer method (pyridine free).

Application Details

Special information concerning the sample

Due to the inadequate solubility of this sample in methanolic Karl Fischer reagent the addition of solubilizers (e.g. chloroform, decanol) is necessary. Because of the low water content of sunflower oil, coulometric Karl Fischer titration is the most appropriate method.

Titration method - Coulometry without diaphragm

Conditions	
Reagents	
Working Medium:	80 mL Anolyte Aquastar® (188079)
Solubilizer:	20 mL Decanol (**8.03463**)
Instrument parameters	**For end point indication (general recommendation)**
I(pol):	5 - 10 µA
U(EP):	50 - 100 mV
Stop Criterion:	drift < 10 µg/min
Sample size	
By weight:	~1 g (weight needs to be accurately determined)

Procedure

The Karl-Fischer reagent and solubilizer is placed in the titration cell without a diaphragm. The coulometer is started, and the solvent mixture is titrated dry. After pre-titration and stabilization of the drift, the sample (1 mL) is injected into the titration cell with a syringe (exact sample weight determination by weighing of syringe before and after injection) and the water content is determined. We recommend doing an instrument check before the sample titration and after a few sample titrations with a water standard 0.1 %.

Alternatively, we also have procedures available to determine water content in sunflower oils with the volumetric method and a low concentrated titrant. Find out more on our webpage at **SigmaAldrich.com/titration**

Featured Products

Description	Cat. No.
Anolyte for coulometric Karl Fischer Titration without diaphragm Aquastar®	**1.88079**
1-Decanol for synthesis	**8.03463**
Water standard 0.1% Standard for Karl Fischer Titration 1 g ≙ 1 mg H_2O Aquastar®	**1.88051**
Water Standard 0.01 % Standard for Karl Fischer Titration 1 g ≙ 0.1 mg H_2O Aquastar®	**1.88050**

FOOD & BEVERAGES

Effective Detection of Food Allergens

Purified Food Proteins from Indoor Biotechnologies. Now available on SigmaAldrich.com

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

Food allergies are an increasing global concern. In industrialized countries, up to 10% of infants suffer from food allergies, with an increasing prevalence noted in the last decades. Developing countries have also experienced an increase in occurrence.[1]

Food allergy symptoms can range from mild (itchiness, diarrhea) to potentially life-threatening (anaphylaxis). Allergic reactions typically involve an IgE-triggered immune response of the body caused by the presence of allergen proteins. To protect consumers, there is a need for more comprehensive food allergen labeling beyond precautionary labeling. Additionally, significant progress is being made with regards to food allergy immunotherapies and diagnostics.

Quantitative results of analytical testing of food allergens can vary depending on the method. The various analytical techniques used to detect food allergens include PCR, immunoassays, and LC-MS. The use of allergen proteins reference materials will provide more consistent, traceable, and comparable results.

We are proud to introduce a new comprehensive range of purified food allergen protein standards, manufactured by Indoor Biotechnologies. These proteins are purified by affinity chromatography and/or HPLC. The protein identity and amino acid composition is verified by Indoor Biotechnologies by using in-house mass spectrometry and amino acid analysis. In addition, their immune reactivity is validated by ELISA and IgE antibody binding. Allergens are manufactured under ISO 9001:2015 certified Quality Management System, consistently providing high-quality allergen proteins with limited lot to lot variability.

Allergen proteins are either isolated from a natural source (product codes NA) or expressed as a recombinant protein in *E. coli* or *P. pastoris* (RE, RP or RPI). Some proteins are also available in biotinylated form (BI) or Lo Tox™ proteins (LTN or LTR) which have very low endotoxin levels (<0.03 EU/µg protein). Lo Tox™ proteins are ideal for human and murine cellular studies using T-cells, APC's or dendritic cells.

Indoor Biotechnologies, headquartered in Charlottesville, VA, USA, is a leading manufacturer and supplier of highly purified allergen molecules and immunoassays for research, diagnostics, and pharmaceutical product development. With more than 25 years of experience, Indoor Biotechnologies is internationally recognized for its research on protein structure, function, and immune recognition.

To view our entire list of products, please visit us at **SigmaAldrich.com/foodallergens**

Table 1: List of Food Allergen Proteins

Product Description	Source	Scientific Name	Expression	Protein Family	Cat. No.
Peanut/Legume Allergens					
Gly m 4.0101	Soy	*Glycine max*	*P. pastoris*	PR-10	**RP-GM4-1**
Gly m 5			Natural	β-conglycinin	**NA-GM5-1**
Gly m 6				Glycinin	**NA-GM6-1**
Ara h 1	Blanched peanut	*Arachis hypogaea*	Natural	7S globulin	**NA-AH1-1**
Ara h 1 (LoTox™)			Natural	7S globulin	**LTN-AH1-1**
Biotinylated natural Ara h 1			Natural	7S globulin	**BI-NAH1-1**

Table 1. (cont.) List of Food Allergen Proteins

Product Description	Source	Scientific Name	Expression	Protein Family	Cat. No.
Ara h 2	Peanut flour,light roast	*Arachis hypogaea*	Natural	2S albumin	**NA-AH2-1**
Ara h 2 (LoTox™)			Natural	2S albumin	**LTN-AH2-1**
Biotinylated natural Ara h 2			Natural	2S albumin	**BI-NAH2-1**
Ara h 2.0201	Peanut		P. pastoris	2S albumin	**RP-AH2-1**
Ara h 3	Blanched peanut		Natural	11S globulin	**NA-AH3-1**
Ara h 3 (LoTox™)			Natural	11S globulin	**LTN-AH3-1**
Biotinylated natural Ara h 3			Natural	11S globulin	**BI-NAH3-1**
Ara h 6	Peanut flour,light roast		Natural	2S albumin	**NA-AH6-1**
Ara h 6 (LoTox™)			Natural	2S albumin	**LTN-AH6-1**
Biotinylated natural Ara h 6			Natural	2S albumin	**BI-NAH6-1**
Ara h 8.0101	Peanut		*E. coli*	PR-10	**RE-AH8-1**
Biotinylated recomb. Ara h 8			*E. coli*	PR-10	**BI-RAH8-1**
Ara h 9.0101			P. pastoris	nsLTP	**RP-AH9-1**
Peanut Protein (LoTox™)	Peanut flour		Natural	Multiple	**LTN-AHRE-1**
Tree Nut Allergens					
Cor a 1.0401	Hazelnut	*Corylus avellana*	P. pastoris	PR-10	**RP-CA1-1**
Cor a 8.0101			P. pastoris	nsLTP	**RP-CA8-1**
Cor a 9			Natural	11S globulin	**NA-CA9-1**
Cor a 14.0101			P. pastoris	2S albumin	**RP-CA14-1**
Ana o 3.0101	Cashew	*Anacardium occidentale*	P. pastoris	2S albumin	**RP-AO3-1**
Jug r 1.0101	Walnut	*Juglans regia*	P. pastoris	2S albumin	**RP-JR1-1**
Jug r 3.0101			P. pastoris	nsLTP	**RPI-JR3-1**
Jug r 5.0101			E. coli	PR-10	**RE-JR5-1**
Pru du 6	Almond	*Prunus dulci*	Natural	11S globulin	**NA-PD6-1**
Egg Allergens					
Gal d 1 (LoTox™)	Chicken egg	*Gallus domesticus*	Natural	Ovomucoid	**LTN-GD1-1**
Gal d 2 (LoTox™)				Ovalbumin	**LTN-GD2-1**
Gal d 3				Ovotransferrin	**NA-GD3-1**
Gal d 4 (LoTox™)				Lysozyme	**LTN-GD4-1**
Seafood Allergens					
Shrimp Tropomyosin	Carolina Shrimp		Natural	Tropomyosin	**NA-STM-1**
Pen a 1.0101	Brown shrimp	*Penaeus aztecus*	P. pastoris	Tropomyosin	**RPI-PA1-1**
Cyp c 1.0101	Carp	*Cyprinus carpio*	E. coli	Parvalbumin	**RE-CC1-1**
Milk Allergens					
Bos d 4	Cow's milk	*Bos domesticus*	Natural	a-lactalbumin	**NA-BD4-1**
Bos d 5				β-lactoglobulin	**NA-BD5-1**
Bos d 6				Serum albumin	**NA-BD6-1**
Bos d 8				Casein	**NA-BD8-1**
Bos d 11				β-casein	**NA-BD11-1**
Vegetable and Fruit Allergens					
Api g 1.0101	Celery	*Apium graveolens*	P. pastoris	PR-10	**RP-AG1-1**
Pru p 3.0102	Peach	*Prunus persica*		nsLTP	**RP-PP3-1**
Mal d 1.0108	Apple	*Malus domestica*		PR-10	**RPI-MD1-1**
Cereal and Seed Allergens					
Tri a 14	Wheat	*Triticum aestivum*	E. coli	nsLTP	**RE-TA14-1**
Sin a 1.0101	Mustard	*Sinapis alba*	P. pastoris	2S albumin	**RP-SA1-1**
Ses i 1.0101	Sesame	*Sesamum indicum*	P. pastoris	2S albumin	**RP-SI1-1**
Other Allergens					
Alpha-Gal	Red meat (cow)	*Bos domesticus*	Natural	Bovine Thyroglobulin	**AGAL-1**
Can s 3.0101	Cannabis - Indian Hemp	*Cannabis sativa*	E. coli	nsLTP	**RE-CS3-1**

References

1. 1. W. Loh, M. Tang; International Journal of Environmental Research and Public Health; v.15(9); 2018

FOOD & BEVERAGES

Multiclass Pesticide Analysis of Soy Milk Using a Matrix-Compatible SPME Fiber

Emanuela Gionfriddoa,b Dominika Gruszeckaa Xiujuan Lia,c Janusz Pawliszyna
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[b]Department of Chemistry and Biochemistry, The University of Toledo, Toledo OH, 43606, USA
[c]Key Laboratory of Environment Correlative Dietology (Ministry of Education), College of Food Science & Technology, Huazhong Agricultural University, Wuhan, China, Analytix@merckgroup.com

Abstract

A matrix-compatible direct-immersion solid-phase microextraction (SPME) fiber, named PDMS/DVB/PDMS or SPME-OC Fiber, was used for the determination of pesticides in soy milk via direct immersion. Combined with gas chromatography-mass spectrometry, it eliminated the need for extensive sample pre-treatment procedures. To extend the lifetime of the SPME device, rapid pre- and post-desorption cleaning steps were implemented. This method allowed to achieve limits of quantitation (1–2.5 µg/kg) for the targeted analytes that were below the Maximum Residue Levels mandated for soy-based products.[1,2]

Introduction

Soy-based products are a category of nutraceuticals extensively used worldwide for their health benefits and also as a more sustainable alternative to dairy products. Raw soy grains are the starting material for all soy-based products and are often exposed to agrochemicals from agricultural and post-harvesting practices. It is important to monitor the level of pesticide residues in soy derivatives to ensure their compliance with tolerance limits set by various regulatory agencies across the world. Soy milk, being a stable emulsion of oils, water, and proteins, is a challenging sample to treat for the extraction of pesticides residues at ultra-trace levels. To propose an automated and sensitive method, solid phase microextraction (SPME) was considered as an extraction technique in this work. This is because of SPME's ability to provide an automated analytical workflow and pre-concentration to achieve limits of quantitation for the targeted pesticides at low part-per-billion levels.[3] Moreover, the use of a matrix-compatible SPME fiber enabled direct immersion extraction from soymilk, improving the recovery of pesticides with good water solubility.

Experimental

Table 1 describes the final optimized method. Calibration was performed via matrix-matched calibration, spiking the analytes of interest and three deuterated internal standards: diazinon D_{10}, malathion D_6, and thiabendazole D_4. The soy milk samples were purchased at local grocery stores and were refrigerated until analyzed.

Table 1. Optimized DI-SPME-GC-MS method

Sample/matrix	4.5 g of soy milk + 45 µL of I.S. mix at 25 mg/kg + 4.5 mL of an acetone:water solution (3:7 v:v)
SPME fiber	SPME-OC Fiber (**57439-U**)
Incubation:	1 min, 35 °C, 500 rpm
Extraction:	40 min, 35 °C, 500 rpm
Post-extraction rinsing	10 s, 500 rpm, in acetone:water (1:9, v:v)
Desorption:	15 min at 270 °C
Post-desorption washing:	1 min, 500 rpm, acetone: water solution (1:1 v:v)
Column:	5% Phenyl MS capillary column (30 m × 0.25 mm x 0.25 µm)
Oven:	80 °C (2 min), 6 °C/min to 280 °C (4 min)
Carrier gas:	helium, 1.5 mL/min, constant flow
Detector:	MSD, full scan, m/z= 35-450
MSD transfer line	250 °C
Injection:	splitless
Liner:	0.75 mm I.D., SPME

Result and Discussion

Optimization of the DI-SPME Procedure

The SPME procedure necessitated the optimization of fiber washing after the extraction (rinsing) and desorption (washing), in order to prolong its lifetime. And previous studies demonstrated that this optimization needed to be performed based on the type of food matrix analyzed and the targeted analytes.[4-7] Several rinsing and washing solutions were tested (**Table 2**).

Table 2. Fiber rinsing and washing methods tested in this work, after extraction of pure soy milk, unless noted.

Post-extraction-rinsing	Post-desorption-washing
30 sec in ultra-pure	2 min MeOH: H_2O (1:1 v/v)
1 sec in acetone	2.5 min in acetone
1 sec in H_2O: acetone (1:9 v/v) (static)	30 s in acetone
10 sec in H_2O: acetone (9:1 v/v)	1 min in H_2O: acetone (1:1 v/v)
10 sec in H_2O: acetone (9:1 v/v)*	1 min in H_2O: acetone (1:1 v/v)

*After extraction of soy milk diluted 1:1 (w/w) with ultra pure H_2O, further used for analysis

The best cleaning method involved a rinsing step in water:acetone (9:1 v/v) for 10 s and 1 min washing in water:acetone (1:1 v/v), in combination with a 1:1 dilution of the soy milk sample with ultra pure water prior to SPME. This method allowed for 120 consecutive extractions with an average signal variation of +/- 25% and % RSD of less than 15%. Furthermore, the matrix modifiers were optimized for enhanced extraction of hydrophobic analytes. Salting out effects were investigated by varying the ionic strength of the solution, by adding sodium chloride within a range from 5 to 20% to the soy milk/water mixture (1:1, w:w). However, due to no significant improvement noticed in the recovery of the analytes, the addition of salt was discarded for further optimization. An alternative strategy to improve recovery is the addition of organic modifiers. For aqueous samples, optimal recoveries are obtained keeping the content of the organic solvent below 1%. But for complex samples containing matrix constituents that can bind the analytes, the addition of organic modifiers is useful to shift the binding equilibrium toward the free, unbound form thus improving recovery by SPME. In this work, four organic solvents were considered, namely, acetonitrile, acetone, methanol, and ethanol. Each solvent was added at concentrations of 10%, 20%, 30%, and 50% (v:v) to the samples. Solvent concentrations above 50% induced congealing of the soy milk, thus were not further tested. The results showed that the addition of a solution containing 30:70 acetone:water (v:v) to the soy milk sample (dilution ratio 1:1) allowed the best recovery of the targeted analytes. Further, other parameters were finely tuned to optimize both the extraction and desorption process (**Table 1**).

Method validation

A matrix-matched calibration approach was used by spiking pesticide-free soymilk samples with all analytes in a concentration range of 1-1000 µg/kg; with the exception of phosalone which was spiked at 2.5-1000 µg/kg.

Calculations were performed using linear regression for each of the targeted analytes, except phosalone, which required a 1/x2 weight. The accuracy and precision of the method were assessed at three concentration levels of 15, 75, and 200 µg/kg in quadruplicate measurements over three days. Limits of quantitation (LOQs) were determined at the lowest concentration level with an RSD of below 20%, and accuracy within 30% of the nominal concentration. LOQs ranged between 1 and 2.5 µg/kg. The LOQs achieved by this method allowed the detection of the targeted pesticides below the recommended limits set for soy products by the European Commission[6] and Office of the Federal Register[7] for the USA Market. A summary of the figures of merit for this work is provided in **Table 3**.

Table 3. Figures of merit of the DI-SPME-GC-MS method, reproduced from ref. 3 with permission from Elsevier, Elsevier Copyright 2020.

Analytes	Linearity (µg/kg)	LOQ	r^2	Concentration Level (µg/kg)	Day 1 Accuracy (%)	Day 1 %RSD (n=4)	Day 2 Accuracy (%)	Day 2 %RSD (n=4)	Day 3 Accuracy (%)	Day 3 %RSD (n=4)
Trifuralin	1-1000	1	0.9998	15	88	2	80	2	74	8
				75	94	4	91	2	58	4
				200	103	5	92	5	77	12
Dimethoate	1-1000	1	0.9958	15	94	14	102	6	128	1
				75	72	6	89	15	120	11
				200	96	3	114	16	118	23
Diazinon	1-1000	1	0.9996	15	99	6	107	8	102	9
				75	102	1	102	1	119	3
				200	109	2	102	2	130	3
Malathion	1-1000	1	0.9988	15	111	6	117	6	119	5
				75	78	1	85	2	100	4
				200	81	2	82	2	106	3
Chlorpyrifos	1-1000	1	0.9956	15	87	2	96	6	85	11
				75	94	3	94	4	93	5
				200	102	2	91	6	99	5
Thiabendazole	1-1000	1	0.9972	15	106	13	81	4	82	11
				75	98	16	90	16	121	12
				200	81	16	119	15	124	3
Phosalone	2.5-1000	2.5	0.9851	15	118	18	116	12	123	13
				75	116	4	121	4	122	11
				200	123	2	116	8	124	8
Cyhalothrin	1-1000	1	0.9989	15	111	4	101	13	87	11
				75	99	14	74	7	80	21
				200	80	4	77	5	71	9
Cyfluthrin	1-1000	1	0.9947	15	114	2	89	15	89	11
				75	97	4	77	8	89	19
				200	88	5	94	10	104	20
Esfenvalerate	1-1000	1	0.9971	15	90	14	105	16	97	13
				75	78	6	74	9	99	10
				200	79	3	124	10	112	11

Analysis of Real Samples

The validated method was further used for the analysis of different brands of soy milk samples obtained from local grocery stores. The results in **Table 4**, show the occurrence of several targeted pesticides, up to 118.9 µg/kg, in two different commercial brands of soy milk.

Table 4. Quantitative analysis of commercial soy milk samples, reproduced from ref. 3 with permission from Elsevier, Elsevier Copyright 2020.

Compound	Brand #1 Concentration detected (µg/kg)	Brand #2 Concentration detected (µg/kg)
Trifluralin	N.D.	N.D.
Dimethoate	118.90	6.50
Diazinon	N.D.	N.D.
Malathion	27.40	28.20
Chlorpyrifos	7.40	7.70
Thiabendazole	N.D.	N.D.
Phosalone	40.10	33.60
Cyhalothrin	N.D.	N.D.
Cyfluthrin	20.50	N.D.
Esfenvalerate	N.D.	N.D.

N.D.=not detected

Conclusions

A new method for the analysis of pesticides in soy milk was optimized and validated using a matrix-compatible SPME fiber. This DI-SPME-GC-MS method was able to quantitatively monitor the presence of pesticides with LOQs of 1-2.5 µg/kg, with a completely automated workflow including rinsing and washing of the SPME fiber. The excellent robustness of the SPME matrix compatible fiber enabled its use of up to 120 extraction/desorption cycles.

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Description	Cat.No.
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SPME-OC Fiber Assembly, Polydimethylsiloxane/Divinylbenzene (PDMS/DVB)	**57439-U**
Smart SPME-Overcoated (OC) Fiber Assembly, Polydimethylsiloxane/Divinylbenzene (PDMS/DVB)	**548651-U**
Inlet Liner, Direct (SPME) Type, Straight Design (unpacked), Pk.1 (Pk.5 2637505, Pk.252637525)	**2637501**
Reference Materials	
Diazinon-(diethyl-d10), PESTANAL®, analytical standard, 5 mg	**74332**
Malathion diacid-(dimethyl-d6) PESTANAL®, analytical standard, 10 mg	**34541**

Related Products

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GC	
SLB®-5ms, 30 m × 0.25 mm, df= 0.25 µm	**28471-U**
Solvents	
Acetone, for gas chromatography MS SupraSolv®	**1.00658**
Water for gas chromatography SupraSolv® (or high purity from Milli-Q® system)	**1.02699**
Reference Materials	
Trifluralin, PESTANAL®, analytical standard, 250 mg	**32061**
Dimethoate, reference material, 100 mg	**52994**
Diazinon, certified reference material, TraceCERT®, 50 mg	**68486**
Malathion, certified reference material, TraceCERT®, 50 mg	**91481**
Chlorpyrifos, certified reference material, TraceCERT®, 100 mg	**94114**
Thiabendazole, certified reference material, TraceCERT®, 50 mg	**67554**
Phosalone, reference material, 100 mg	**44988**
λ-Cyhalothrin, certified reference material, TraceCERT®, 50 mg	**72765**
β-Cyfluthrin, certified reference material, TraceCERT®, 50 mg	**93223**
Esfenvalerate, reference material, 100 mg	**67115**

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FOOD & BEVERAGE

Simplified LC-MS/MS Method for Glyphosate, AMPA, and Glufosinate in Oat-Based Cereals

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Abstract

A simplified LC-MS/MS method for the determination of glyphosate, (aminomethyl)phosphonic acid (AMPA), and glufosinate in cereals is described. The method enables the analysis of glyphosate and its metabolites without sample derivatization. The samples are prepared utilizing an extraction method based upon the Quick Polar Pesticides (QuPPe) Method and separated by high-performance liquid chromatography with MS detection. A carbon-based chromatography column allowed retention of the analytes while a basic ammonium carbonate buffer and acetonitrile:water mobile phase system ensured proper ionization under negative ESI conditions. The use of a sensitive Sciex 6500 MS instrument enabled low detection limits of 10 ppb in oat-based samples. In multiple analyzed cereals levels of glyphosate, AMPA and glufosinate were found to be above the detection limits of the method.

Introduction

Glyphosate is one of the most used herbicides in the world with more than 0.64 million tons of glyphosate applied to fields per year.[1] This chemical's usage increased after the introduction of genetically modified, glyphosate tolerant crops such as corn, soybeans, and cotton. In the USA, US Environmental Protection Agency (EPA) regulation document Code of Federal Regulations (CFR)-title 40-volume 26 sets the tolerance levels for the occurrence of glyphosate in food commodities and produce.[2] The EPA tolerance for glyphosate residues in cereal grains (also called crop group 15) are set at 30 ppm; this limit excludes rice, soy, and corn. In rice, the tolerance is 0.1 ppm whereas, in sweet corn it is 3.5 ppm.[2] For glufosinate, an herbicide that is often included with glyphosate in analytical methods, the tolerance values are 0.4 ppm for cereal and 1.0 ppm for rice. These tolerance values include metabolites and degradants. Therefore, a glyphosate metabolite, AMPA, was also included into this study (**Figure 1**). For comparison, in the European Union (EU), maximum residue levels (MRL) in oats are 20 mg/kg for glyphosate and 0.03 mg/kg for glufosinate (lower limit of analytical detection). For rice, the MRLs for glyphosate and glufosinate are 0.1 mg/kg (lower limit of analytical detection) and 0.9 mg/kg, respectively.[3]

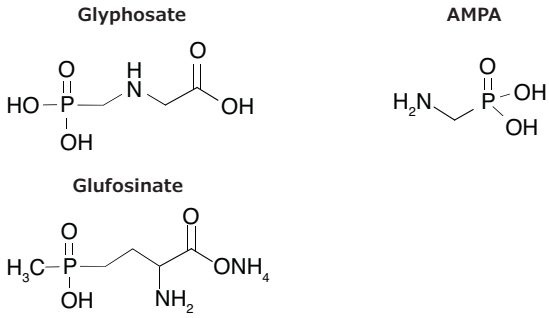


Figure 1. Structures of Glyphosate, AMPA and Glufosinate

In this application, the presence of glyphosate in cereal grains, and oat, in particular, used in the production of breakfast cereals, was explored. Various methods for glyphosate analysis were developed over the last 30 years. Some HPLC methods required derivatization of analytes with o-phthalaldehyde prior to fluorescence detection.[4] A method with glyphosate derivatization using fluorenylmethyloxycarbonyl chloride (FMOC) and fluorescence detection has also been proposed and widely used.[5] Recently, with the advent of modern, sensitive, and rugged LC-MS/MS instruments, it has become possible to analyze glyphosate and its metabolites without derivatization, as illustrated in this work with direct analysis of glyphosate by MS/MS. Multiple columns were previously used for mass spectrometry-based glyphosate analysis including ion-exchange, hydrophilic interaction liquid chromatography (HILIC), or carbon HPLC columns.[6] Some of the HILIC-based and ion-exchange methods used ESI(+) for detection of glyphosate and analogues in acidic mobile phases.[6,7] The HILIC-based methods present a challenge of solvating these very polar analytes in the non-polar diluent. Ion-exchange methods utilized negative ionization mode for detection and citric acid or citric salts in the mobile phase.[5] These additives are not volatile and therefore not fully compatible with mass spectrometric detection. We have shown previously that detection of glyphosate in ESI(-) was possible using carbonate buffer and an anion exchange column.[8] In this work, we used a volatile

bicarbonate buffer mobile phase and a Supel™ Carbon LC column. This column possesses a unique mixed-mode retention mechanism that allows better retention of polar analytes without the need for HILIC conditions.

Experimental

Glyphosate, AMPA and ammonium glufosinate were of analytical standard grade. Isotopically labelled internal standards were used including glyphosate-2-^{13}C,^{15}N, AMPA-^{13}C,^{15}N, and glufosinate-D_3. Solutions of internal standards and non-isotopically labelled standards were prepared in water at 1 mg/mL and used for spiking the grain matrices.

Organic oatmeal was selected as the test matrix and used during method development. Multiple samples of organic oatmeal from local stores were screened for the presence of glyphosate using the method described below (**Figure 2**). All oatmeals had some level of glyphosate and other compounds present. For the method development study, a cereal sample was selected that had the lowest overall background for all three analytes, containing only glyphosate at 24 ppb. This specific matrix was spiked to contain 80 ppb and 800 ppb of all 3 analytes. The spiked samples were used to evaluate the recovery of analytes during method development and for validation.

Additionally, multiple samples of oat cereals were purchased in the local grocery store and evaluated for glyphosate contamination using the developed method.

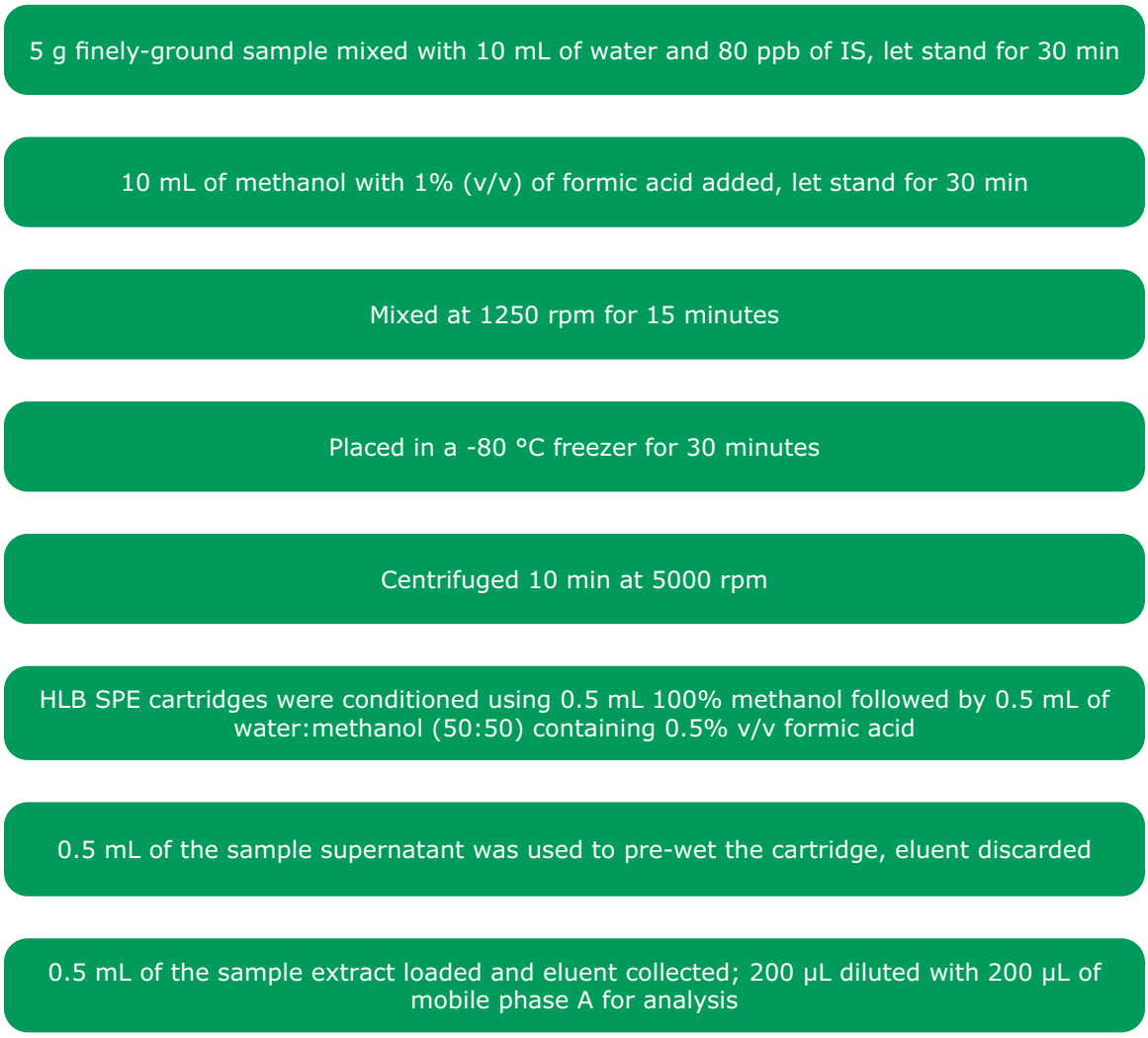


Figure 2. Sample preparation method

Sample preparation

The extraction method was based on the Quick Polar Pesticides (QuPPe) methodology developed in the European Union (EU) for fruits and vegetables, and used water:methanol (50:50) containing 0.5% formic acid (V/V) as the final extraction solvent.[6]

Solid phase extraction (SPE) cleanup using Supel™ Swift HLB cartridges was applied to the extract, similarly to a method reported by Chamkasem and Harmon.[9] Hydrophilic-lipophilic balance (HLB) SPE can be applied to a broad range of analytes using reversed phase methodology. The SPE cleanup method used is based on chemical "filtration" (interference removal). In this approach, the sample extract is simply passed though the HLB cartridge and the eluate collected for analysis. The HLB will retain impurities that are more hydrophobic in character than the target compounds, while the polar analytes will pass through.

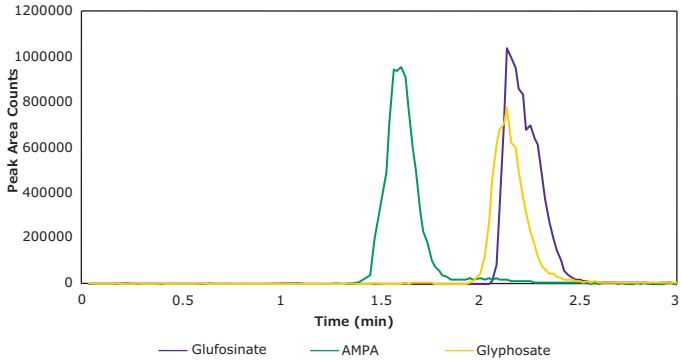
LC-MS/MS method

The analysis was completed using a Supel™ Carbon LC column, which provided good retention for these polar analytes. The aqueous mobile phase [A] used was an ammonium carbonate buffer, pH 9. This ensured proper ionization of the phosphate or phosphonate moiety in the analytes (**Figure 1**) monitored under ESI(-) conditions. In addition, ammonium carbonate buffer is volatile and is fully compatible with LC/MS instrumentation. The organic mobile phase [B] contained acetonitrile:water (95:5). The method operated under both a mobile phase and a flow rate gradient. **Table 1** lists the MS analyte parameters. **Figure 3** presents a chromatogram of a standard injection and LC instrument parameters.

Solvent-based external calibration curves used internal standards and were prepared in 75:25 water:methanol with 0.25% (v/v) formic acid. The concentration range of calibration curves was from 3 ng/mL to 200 ng/mL and the linearity was better than R^2 of 0.99 for each compound. A representative calibration curve for glyphosate is shown in **Figure 4**.

Table 1. MS parameters for analytes

Compound		Q1	Q3	DP	EP	CX
Glyphosate	Quant	168	63	-30	-5.9	-26
	Qual	168	124	-30	-5.5	-16
Glyphosate-2-^{13}C,^{15}N	Quant	167	63	-30	-6.5	-28
AMPA	Quant	110	63	-15	-10.0	-28
	Qual	110	80	-15	-10.0	-36
AMPA-^{13}C,^{15}N	Quant	112	63	-15	-6.5	-24
Glufosinate	Quant	180	63	-50	-6.0	-56
	Qual	180	95	-50	-10.0	-24
Glufosinate-D_3	Quant	183	63	-50	-5.0	-70



LC Conditions				
Instrument:	Agilent 1290 HPLC with AB Sciex Triple Quad 6500+			
Columns:	Supel™ Carbon LC, 10 cm x 2.1 mm I.D., 2.7 µm (**59986-U**)			
Mobile phase:	[A] 20 mM ammonium carbonate pH9; [B] acetonitrile:water (95:5)			
Gradient:	**Time (min)**	**A (%)**	**B (%)**	**Flow rate (mL/min)**
	0.0	100	0	0.2
	3.0	100	0	0.2
	3.1	0	100	0.5
	5.0	0	100	0.5
	5.1	100	0	0.2
	8.0	100	0	0.2
Column Temp.:	30 °C			
Detection:	ESI (-) MS/MS (See Table 1)			
Injection:	20 µL			
MS Parameters				
Voltage:	-4500 V			
Curtain gas:	30			
Source temp:	600 °C			
Gas 1 / Gas 2:	50 /70			

Figure 3. LC/MS Analysis of Glyphosate, AMPA, and Glufosinate using a Supel™ Carbon LC column. Quantitative transitions are shown for each analyte. 100 ng/mL calibration standard is shown.

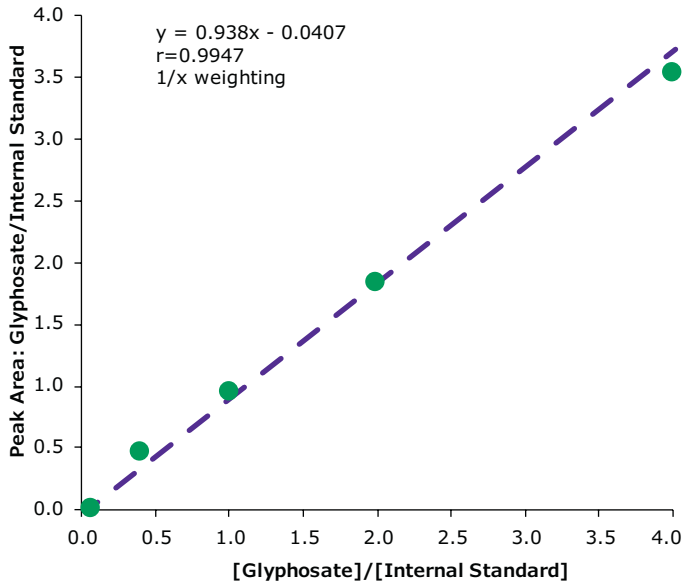


Figure 4. Representative calibration curve for glyphosate. Calibrators for glyphosate range from 3 to 200 ng/mL with an internal standard of 50 ng/mL.

Results

Method development

Sample preparation used fast solvent extraction. SPE was the first choice for sample cleanup as the sample can be simply passed through the cartridge. As all analytes in this method are polar, they were not retained on the Supel™ Swift HLB material and were expected to achieve good recoveries.

The chosen chromatographic method allowed the injection of the prepared extract after a simple dilution step. Elution off the HPLC column was performed isocratically using aqueous carbonate buffer as the mobile phase. A column wash step followed using acetonitrile. Multiple injections (n>100) of extracted samples did not result in significant retention time change. For example, the retention time variability across 5 days of injections and two different mobile phase preparations had an RSD of 3.2%, indicating the ruggedness of this LC method.

Organic oatmeal samples were screened for glyphosate and related compounds. These results are shown in **Table 2**. All compounds were found to be present in all samples indicating the good sensitivity of the proposed method. The sample with the lowest overall concentration for incurred analytes, Sample N, was chosen to be used for method validation.

Table 2. Background analysis results for organic oat cereals

Cereal	Glyphosate (ppb)	AMPA (ppb)	Glufosinate (ppb)
Sample S	8.0	38	44
Sample Z	4.8	198	39
Sample N	24.6	<LOQ	<LOQ
Water control	ND	ND	ND

Six replicates of Sample N were spiked with 24 ppb to determine the method's limit of quantitation (LOQ) for all three analytes. The recoveries were found to be within 80-120% with an RSD of below 15%. LOQ was calculated using a signal-to-noise ratio of 10:1. It was as follows for each analyte:

- Glyphosate 6 ppb
- AMPA 11 ppb
- Glufosinate 8 ppb

Sample N was spiked with the three analytes at both 80 ppb and 800 ppb levels. Glyphosate and AMPA chromatograms for the 800 ppb spiked sample are shown in **Figure 5**. The quantitation results are shown in **Table 3**. All three analytes were detected and quantified at both spiking levels. Accuracy of the method was measured as the percent recovery of the known spiked amounts. For 80 ppb and 800 ppb spikes, the recovery values for glyphosate were 124% and 96%, for glufosinate, they were 132%

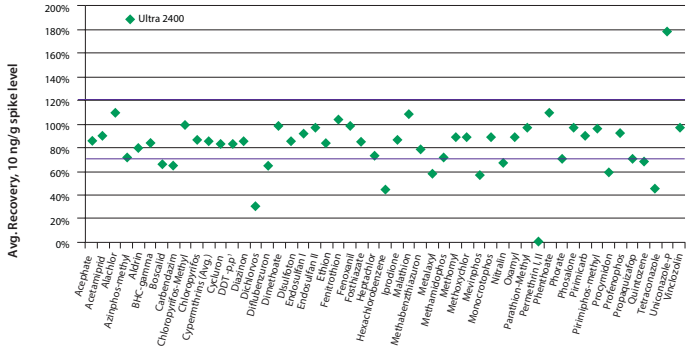


Figure 5. Glyphosate (green trace) and AMPA (purple trace) spiked into oatmeal at 800 ppb.

Table 3. Method validation results after spiking 80 ppb and 800 ppb into cereal/grains labelled "organic"

Spiking level (n=5)	Compound	Day 1 % recovery (%RSD)	Day 2 % recovery (%RSD)
80 ppb	Glyphosate	124 (6)	134 (7)
	AMPA	132 (6)	106 (13)
	Glufosinate	80 (18)	109 (6)
800 ppb	Glyphosate	96 (3)	96 (4)
	AMPA	104 (10)	91 (7)
	Glufosinate	89 (4)	111 (5)

and 104%, and for AMPA the recoveries were at 80% and 88% respectively on day one. Similar recoveries were achieved on the second day of testing when the samples were extracted and analyzed again. Reproducibility of the method was excellent with below 10% RSD for 800 ppb spiked samples and below 20% RSD for 80 ppb spiked samples.

Identification and quantitation of glyphosate in cereals

The results of glyphosate analysis in cereals using the proposed method are presented in **Table 4**. These samples were purchased in the grocery store, sample N was labelled "organic". Internal standards were used as described in the experimental section. The samples were found to contain 25-260 ppb of glyphosate. AMPA levels in oat-containing samples varied from non-detected to 40 ppb. Glufosinate levels were found to be below LOQ.

Table 4. Results of analysis for oat-containing cereals

Samples n=3	Glyphosate (ppb)	% RSD	AMPA (ppb)	%RSD	Glufosinate (ppb)	% RSD
Sample N	24.6	5.0	<LOQ	n/a	<LOQ	n/a
Sample C	178.0	3.0	12	1.4	<LOQ	n/a
Sample R	259.0	9.7	41	9.9	<LOQ	n/a

Conclusion

The developed method for glyphosate and related compounds uses LC-MS/MS with a Supel™ Carbon LC column that is stable under basic pH conditions. This column provided sufficient retention for the polar analytes in the presence of methanol as extraction solvent. The mobile phase used 20 mM ammonium carbonate buffer, fully compatible with mass spectrometry, and allows for efficient ionization. For the oat cereal samples, the extraction was based on the QuPPe method using a mixture of methanol and water followed by a cleanup using the Supel™ Swift HLB SPE. The use of stable isotope labelled internal standards resulted in good accuracy for glyphosate and related compounds. Further, it allowed the use of solvent-based calibration curves. The limit of quantitation (LOQ) for glyphosate for this method using triple-quad MS detection was determined at 6 ppb. The analyzed samples of oat-containing cereals were found to contain 25-260 ppb of glyphosate.

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Featured Products

Description	Cat. No.
HPLC & SPE	
Supel™ Carbon LC, 10 cm x 2.1 mm, 2.7 µm	**59986-U**
Supel™ Swift HLB SPE Tubes, 30 mg/1 mL, Pk. 108	**57493-U**
Reference Materials	
Aminomethylphosphonic acid (AMPA), analytical standard PESTANAL®, 50 mg	**05164**
Glyphosate, analytical standard PESTANAL®, 250 mg	**45521**
Glufosinate-ammonium, analytical standard PESTANAL®, 100 mg	**45520**
Glyphosate	**89432**
Glufosinate-ammonium	**49677**
Glyphosate-2-^{13}C,^{15}N, analytical standard PESTANAL®, 5 mg	**90479**
Solvents, Reagents & Accessories	
Acetonitrile hypergrade for LC-MS LiChrosolv®	**1.00029**
Methanol UHPLC, suitable for mass spectrometry (MS)	**900688**
Ammonium hydrogen carbonate, for LC-MS LiChropur™, 50 g	**5.33005**
Ammonium hydroxide, OmniTrace® Ultra	**AX1308-7***
Formic acid, puriss. p.a., ACS reagent, reag. Ph. Eur., ≥98%	**33015**
Brand® Centrifuge tubes, PP, w/caps, 50 mL, Pk. 300	**BR114820**
Pyrex® disposable culture tubes, rimless, 10 mL, Pk. 1000	**CLS9944513**

* US only product, see below for alternatives

Related Products

Description	Cat. No.
Methanol for UHPLC-MS LiChrosolv®	**1.03726**
Acetonitrile for UHPLC-MS LiChrosolv®	**1.03725**
Ammonia solution 25%, for HPLC LiChropur™	**5.43830**
Formic acid for LC-MS LiChropur™, 97.5-98.5% (T)	**00940**
Reference Materials	
Glyphosate, certified reference material, TraceCERT®, 100 mg	**89432**
Glyphosate-2-13C, PESTANAL®, analytical standard, 5 mg	**90007**
Glufosinate-ammonium, certified reference material, TraceCERT®, 100 mg	**49677**

Read more about the Supel™ Carbon LC column at **SigmaAldrich.com/CarbonLC**

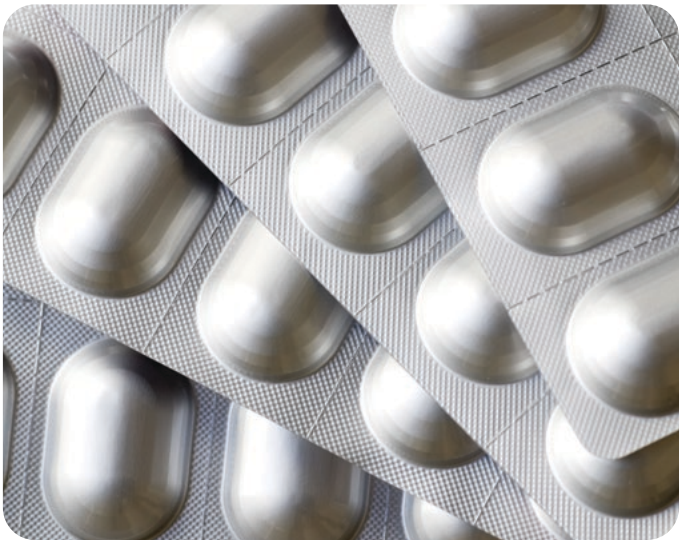
Find further information on Supel™ Swift HLB SPE at **SigmaAldrich.com/SupelSwiftHLB**

PACKAGING

Reference Materials for Extractables and Leachables Testing

New certified reference materials solution mixes

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



Extractables and leachables (E&L) are chemical compounds with the potential to migrate into pharmaceutical or clinical products from packaging materials, tubing, or medical devices. This can lead to patient exposure to these compounds.

Extensive E&L studies to identify compounds that might leach into the product are obligatory for pharmaceutical products and medical devices.

Since it is never entirely predictable which chemicals could migrate, it is crucial that no potential extractables and leachables are overlooked in the analysis. Depending on the nature of the packaging material, the product, and the applied conditions, new unexpected or unknown compounds may be found. There is, therefore, no finite list of analytes for which products should be tested. However, there are certain monomers or additives that are more commonly detected in studies examining extractables and leachables.

In Issue 6 of Analytix Reporter we presented a new series of neat reference materials for some of the most frequently found extractables and leachables. To facilitate your identification and quantification of these extractables and leachables, we developed two certified calibration mixes for extractables and leachables. One mix is designed for LC (21 components) and another one for GC detection (14 components). These two products are Certified Reference Materials (CRM) produced under ISO/IEC 17025 and ISO 17034 double accreditation:

- Certification of each individual component by qNMR (following ISO/IEC 17025 accreditation)
- Mixes produced following the ISO 17034 workflow
- Tested for homogeneity and long-term stability using GC-MS
- Traceability to NIST SRMs
- Supplied with a comprehensive certificate including the overall uncertainty

The components were chosen to reflect a broad spectrum of typical extractables and leachables compound classes, taking into account the toxicity and also how frequently they are typically found in E&L tests.

LC Mix - 21 Components

Catalog Number:	**95636**
Product Name:	Extractables and Leachables Screening Standard for LC
Type:	Certified Reference Material, TraceCERT®
Concentrations:	50 µg/mL per component in acetonitrile
Package Size:	1 mL

Compound	CAS
Irganox 1010 (Ir 1010)	**6683-19-8**
Irganox 1076 (Ir1076)	**2082-79-3**
Dometrizol (Dome) / Tinuvin P/2-(2H-Benzotriazol-2-yl)-p-cresol	**2440-22-4**
ε-Caprolactam (CAP)	**105-60-2**
Dibenzylamine (DBA)	**103-49-1**
Benzoic acid (BA)	**65-85-0**
2-Mercaptobenzothiazole (2-MBT)	**149-30-4**
Bisphenol A (BPA)	**80-05-7**
2-Ethylhexanoic acid (EHA)	**149-57-5**
Bis(4-chlorophenyl)sulfone (CPS)	**80-07-9**
2,6-Di-tert-butyl-4-hydroxymethyl-phenol (DBOHP)	**88-26-6**
Butylhydroxytoluene (BHT)	**128-37-0**
1,3-Di-tert-butyl-benzene (DBB)	**1014-60-4**
Oleamide (Ole)	**301-02-0**
Bis(2-ethylhexyl) phthalate (DEHP)	**117-81-7**
Stearic acid (SA)	**57-11-4**
Erucamide (Eruca)	**112-84-5**
Irganox 3114 (Ir3114)	**27676-62-6**
Irgafos 168-oxide	**95906-11-9**
2,4-di-tert-Butylphenol	**96-76-4**
Palmitic acid	**57-10-3**

GC Mix - 14 Components

Catalog Number:	01829
Product Name:	Extractables and Leachables Screening Standard for GC
Type:	TraceCERT® Certified Reference Material
Concentrations:	50 µg/mL per component in TBME
Package Size:	1 mL

Compound	CAS
Irganox 1076 (Ir1076)	**2082-79-3**
ε-Caprolactam (CAP)	**105-60-2**
2-Mercaptobenzothiazole (2-MBT)	**149-30-4**
Bisphenol A (BPA)	**80-05-7**
Butylhydroxytoluene (BHT)	**128-37-0**
1,3-Di-tert-butyl-benzene (DBB)	**1014-60-4**
Oleamide (Ole)	**301-02-0**
Bis(2-ethylhexyl) phthalate (DEHP)	**117-81-7**
Stearic acid (SA)	**57-11-4**
Erucamide (Eruca)	**112-84-5**
Irgafos 168-oxide	**95906-11-9**
2,4-di-tert-Butylphenol	**96-76-4**
2,6-di-tert-Butylphenol	**128-39-2**
Palmitic acid	**57-10-3**

Featured Products

Description	Cat.No.
Extractables and Leachables Screening Standard for LC, Certified Reference Material *Trace*CERT®, 1 mL	**95636**
Extractables and Leachables Screening Standard for GC, Certified Reference Material *Trace*CERT®, 1 mL	**01829**

In addition to certified reference materials (CRM) mixes for LC and GC we have also developed individual compounds found in extractables and leachables studies. Check our website regularly for the most recent product additions.

SigmaAldrich.com/extractablesandleachables

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...that our proficiency testing portfolio for environmental & pharmaceutical testing is now supported by a new portal to enter your data and download reporting packets?

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PACKAGING

The Effect of Container Materials on Production of Light Induced Off-Flavors in Milk - A Study using SPME-GC-MS

Robert E. Shirey, Principal R&D Scientist, Analytix@merckgroup.com

Introduction

Light induced off-flavors (LIOFs) in milk became an issue when dairies began to package milk in high density polyethylene (HDPE) plastic jugs instead of glass bottles. There are several types of LIOFs with the most common coming from oxidation of lipids and degradation of sulfur containing amino acids. Light induced lipid oxidation occurs when free radicals react with the unsaturated fatty acids in milk. The free radical reaction cleaves the double bond and forms hydroperoxides that degrade predominately to aldehydes and to a lesser degree, ketones and alcohols. The most common light activated analytes in this class are hexanal and pentanal, primarily induced from linoleic acid.[1]

The mechanism for the breakdown of sulfur containing amino acids in whey protein is not fully understood. The most common breakdown products in this class are dimethyl sulfide (DMS), methanethiol (MT) and dimethyl disulfide (DMDS). Due to the high volatility of DMS and MT, this study focused primarily on DMDS.

It is well documented that UV rays do not easily penetrate glass, but have been known to penetrate various types of plastic materials. In the US milk is predominately sold in HDPE jugs. Some of these jugs contain white or colored pigments to increase the effectiveness of the plastic to serve as a barrier to UV light. The goal of this study was to evaluate various types of plastics to determine which type provides the best barrier for preserving the integrity of the milk.

Several analytical methods have been used for the analysis of LIOFs in milk. In this study we chose solid-phase microextraction (SPME) to analyze the various milk samples, because of its automation capabilities. Furthermore this technique is sensitive, easy to automate, and is accurate with good precision.

Materials & Methods

Milk containing 2% fat was purchased from a local dairy farm and was stored in ½ gallon glass jugs with a wall thickness of approximately 5 mm. The plastic sealing cap was immediately covered with aluminum foil upon purchase and the milk was stored at 4 °C in the dark.

Different types of plastic containers were obtained from various sources throughout the lab. Each of the plastic containers contained a symbol indicating the type of plastic. Effort was taken to find containers with similar surface areas and volumes. The wall thickness of each container was measured with calipers. The containers were filled to 93% ±1% of the internal volume. The purpose was to keep the void volume of the containers consistent since the shape of the containers varied. The caps and container necks were wrapped with aluminum foil to prevent UV permeation through the cap. The container materials and dimensions are shown in **Table 1**.

Table 1. Container Materials and Dimensions Used in Milk Light Exposure Study

Container Material	Wall Thickness (mm)	Base shape	Total surface area (mm^2)	Volume of milk in container (mL)	Internal volume of container (mL)	Percent of fluid volume
PETE[1]	0.60	Circular	10241	55	59	93%
HDPE[2]	0.80	Circular	9864	65	71	92%
PP[3]	1.32	Circular	9694	50	54	93%
White HDPE[4]	1.50	Rectangular	10400	67	72	93%
Glass bottle	2.00	Circular	11327	75	80	94%

[1]PETE - polyethylene terephthalate ether

[2]HDPE – high density polyethylene

[3]PP-Polypropylene

[4]White HDPE – HDPE impregnated with white opaque pigment

A 500 mL volumetric flask was filled with cold milk and spiked with an internal standard, hexanal-d_{12}, at 5 µg/L. The milk was immediately dispensed into containers at the volume levels listed in **Table 1** and into two glass vials sealed and placed in the refrigerator at 4 °C. Caps were covered with aluminum foil to reduce UV permeation. The containers were placed in a foil-lined tray about 10 cm beneath Sylvania Octron 32 W fluorescent lights, which were used as a UV light source. The exposure time was 2 hours.

After the milk was exposed, the containers were placed in the refrigerator at 4 °C for 1 h to cool the milk and prevent rancidity. During the time the milk samples were being cooled, ten empty 10 mL vials were placed in a Peltier-cooled vial tray holder set at 4 °C on a Gerstel MPS II multi-purpose sampler. The sampler was also equipped with a needle conditioner to clean the fiber, and an agitator for sample mixing.

Five mL of milk was transferred in duplicate into ten cooled vials. The two vials containing the spiked fresh milk in the refrigerator were added to the tray. A Supelco® CAR/PDMS fiber on a Nitinol core was used to extract the samples. The extraction conditions used in the study are shown in **Table 2**.

Table 2. SPME Sampling Conditions

auto sampler:	Gerstel MPS II
sample:	5 mL cooled milk
fiber:	Carboxen®/PDMS (CAR/PDMS) on Nitinol core (**57907-U**)
incubation:	50 °C for 1 min with agitation at 255 rpm
extraction:	headspace, 15 min, 50 °C, with agitation at 250 rpm
desorption:	3 min, 300 °C
post desorption:	2 min, 280 °C, in needle cleaner

The samples were analyzed with an Agilent 7890B GC connected to a 5977 A MSD. The conditions used to analyze the desorbed analytes are shown in Table 3.

Table 3. GC/MS Analysis Conditions

GC:	Agilent® 7890
column:	VOCOL®, 30 m x 0.25 mm I.D., 1.5 µm df (**24205-U**)
oven program:	45 °C (2 min) to 100 °C at 8 °C/min to 140 °C at 12 °C/min to 180 °C at 16 °C min (0.2 min)
carrier gas:	helium at 1 mL/min constant flow rate
inlet:	300 °C with 0.75 mm ID liner (**2637501**)
injection port:	splitless for 0.75 min then vent at 20 mL/min
transfer line:	250 °C
detector:	MSD quadrupole, m/z 40-150
quantitation ions:	pentanal-44; hexanal-56; dimethyl disulfide-94; hexanal-d_{12} -64

Results

The CAR/PDMS fiber on the Nitinol core is an excellent choice for this application due to the small micropores of CAR/PDMS. These pores are ideal for extracting small and midsized analytes. The Nitinol core is very durable and inert. The coating process is produced with state-of-the-art coating equipment that assures good reproducibility by constant monitoring of the coating thickness.

The addition of sodium chloride does increase recovery of these analytes in water, but not in milk containing fat. The responses in milk samples were higher with better precision without added salt; therefore, salt was not added to the samples. Various extraction times were evaluated, but it was determined that 15 minutes enabled samples to be quantified below µg/L concentration levels. The other SPME parameters were optimized to provide good extraction and desorption efficiencies without compromising sample integrity.

A calibration curve was generated by spiking seven fresh milk samples with a standard of the LIOFs analytes from 1-10 µg/L sample concentration and with hexanal-d_{12} at 5 µg/L. Another vial of fresh milk was only spiked with hexanal-d_{12} at 5 µg/L. The samples were extracted and analyzed according to the methods

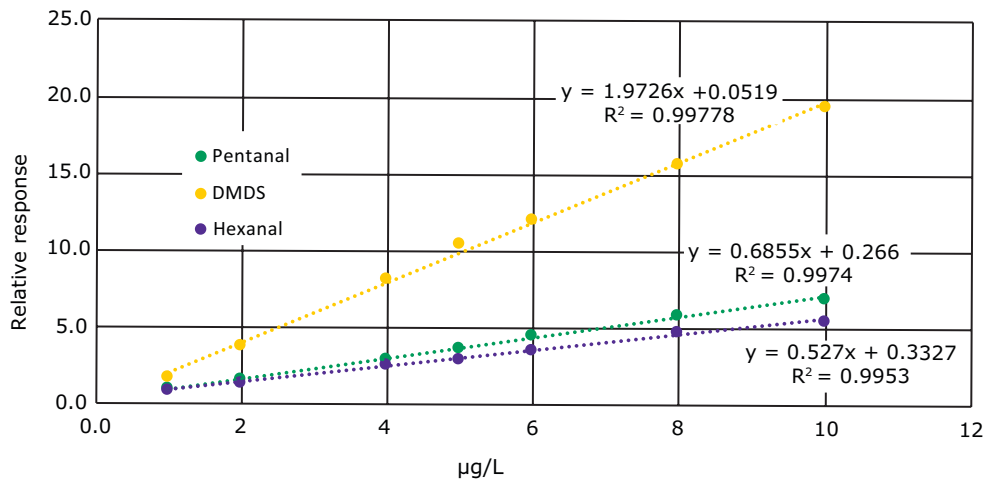


Figure 1. Calibration Curve of Relative Responses of LIOFs with Background Subtraction

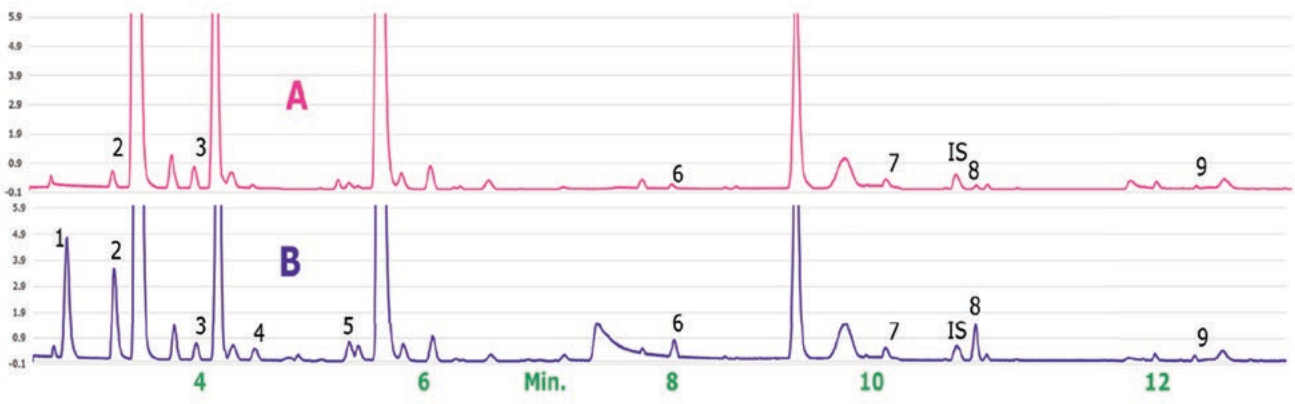


Figure 2. Chromatograms of Milk Spiked with IS not Exposed to Light (A); Milk Spiked with IS Exposed to Light Stored in a Polypropylene Container (B)

Peak IDs: 1. Pentane, 2. Isopropanol, 3. Dimethyl sulfide, 4. n-Hexane, 5. 2-Butanol, 6. Pentanal, 7. Dimethyl disulfide, IS. Hexanal-d_{12}, 8. Hexanal, 9. Heptanal. (See **Table 3** for Run Conditions)

listed in **Tables 2** and **3**. The relative responses of each analyte were calculated and the relative responses from the sample not spiked with the LIOF standard were subtracted from the seven LIOF spiked samples.

Relative responses of the three analytes over the 1-10 µg/L spiking range had regression coefficient values > 0.99, and low Y-intercept values (**Figure 1**). These results were obtained in full scan mode, so greater sensitivity could be obtained using SIM mode if needed.

Chromatograms of milk not exposed to light spiked with the IS (A) and milk exposed to light in a polypropylene container (B) are shown in **Figure 2**.

The comparison of the chromatograms shows that light exposure in the PP container increased the response of many of the analytes. Both chromatograms are at the same scale, and the response of the internal standard (hexanal-d_{12}) is similar in both plots. Even though this study focused on three analytes, other analytes are generated from the light exposure or some other mechanism. Two small volatile analytes, pentane and isopropanol, have much larger responses on the light exposed samples. Note that the samples were run in duplicate (data not shown here) and the responses of duplicate samples were similar.

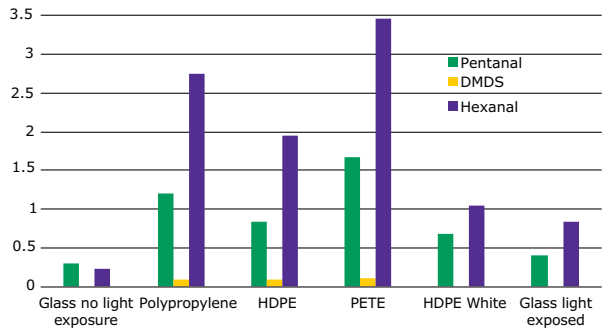


Figure 3. Concentration in µg/L of LIOFs in Milk with Background Subtraction

To calculate the concentration level of the selected LIOFs obtained from milk exposed in the various containers, the average relative responses from duplicate runs were calculated. The average relative responses are shown in **Table 4**.

Table 4. Relative Responses of LIOFs in Milk after Exposure to Light in Various Containers

	No Light	PP	HDPE	PETE	HDPE White	Glass
Pentanal	0.206	0.826	0.572	1.142	0.470	0.282
DMDS	0.000	0.172	0.170	0.196	0.000	0.000
Hexanal	0.122	1.454	1.027	1.826	0.551	0.438

The average relative responses for each analyte obtained from the no light exposed milk samples were subtracted from the average relative responses obtained from the various containers. The background subtracted relative responses were divided by the slope of the line as listed in **Figure 1**. **Figure 3** shows the calculated results.

The results show that two of the plastics, PETE and PP, were the least efficient barrier to UV light. The PETE container had the thinnest wall of the containers, which may have contributed to the barrier properties. PP had the thickest wall of any plastic but the formation of LIOFs was quite high. The addition of white pigment to the HPDE plastic made it a much better barrier to UV light. Its properties were similar to glass. The thickness of the glass does affect the barrier properties as we demonstrated in an additional study.

Conclusions

The type of material used to store milk can be critical in the prevention of lipid oxidation. This study shows that glass is still the best barrier to UV light, but HDPE impregnated with a pigment is a good option. In this case white pigmentation helped to reduce LIOF formation, but studies have shown that yellow or pink pigments may be even better.

The CAR/PDMS fiber on the Nitinol core was able to retain the small flavoring analytes. The micropores retain and release these analytes efficiently. In addition, the Nitinol core is highly inert and extremely durable. This fiber is a viable alternative to this coating on a fused silica core.

Reference:

1. Marsili, R. T., Journal of Chromatogrphic Science 37 (1999) 17–23

Featured Products

Description	Cat. No.
SPME Fiber Assembly Carboxen®/PDMS (CAR/PDMS) on Nitinol Core (NIT), Pk.3	**57907-U**
VOCOL®, 30 m x 0.25 mm I.D., 1.5 µm df	**24205-U**
Inlet Liner, Direct (SPME) Type, Straight Design (unpacked), for Agilent®, Pk.1	**2637501**
Reference Materials	
Dimethyl disulfide (DMDS), analytical standard, neat, 1 mL	**68986**
Hexanal, analytical standard, neat, 1 mL	**18109**
Hexanal-d_{12}, ≥98 atom % D, ≥96% (CP), neat, 250 mg	**732338**
Valeraldehyde (Pentanal), analytical standard, neat, 1 mL	**42272**

To read more about the new Nitinol-core SPME Fibers or to request an evaluation fiber, visit

SigmaAldrich.com/NITSPME

For more information on SPME in general and to download the "*SPME for GC - Setting Started with Solid Phase Microextraction*" brochure, visit

SigmaAldrich.com/spme

PLANTS, HERBS & SPICES

Analysis of Pesticides in Turmeric Powder by LC-MS/MS and GC-MS/MS

After Cleanup with a Novel Dual-Layer SPE Cartridge

Katherine K. Stenerson, Principal R&D Scientist, Analytix@merckgroup.com

Introduction

Turmeric is a plant indigenous to south Asia, with a majority of its production coming from India. The rhizome of the plant is used to produce powdered turmeric, which is used in foods, cosmetics, and some medicines. It is also an essential constituent of curry, which is a mixture of spices used extensively in Indian cooking. Turmeric has also been used in traditional medicines for thousands of years, and recently has garnered attention for studies showing its potential antioxidant, anti-inflammatory, antimutagenic, antimicrobial and anticancer properties.[1]

Pesticide residue testing of turmeric and other spices is required by many countries. For example, Canada has set maximum residue limits for 42 different pesticides in turmeric root.[2] The US EPA has set tolerance limits for a variety of pesticides in root and tuberous vegetables, of which turmeric is included.[3]

Turmeric contains more than 100 different components, with two of the main constituents being curcumin and volatile oils. Curcumin gives turmeric its distinctive yellow/orange color, while the volatile oils consist primarily of terpenes. Turmeric also contains some fats; specifically, sterols and fatty acids.[4] This complex composition makes extracts produced from turmeric a challenge in the chromatographic analysis of pesticides, as residual pigments and oils can contaminate both GC-MS and LC-MS systems.

When dealing with very high background samples such as turmeric, standard QuEChERS cleanup may not offer enough capacity. For better cleanup, solid phase extraction (SPE), including dual-layer cartridges, can be used. These cartridges often contain graphitized carbon black (GCB) in the top bed and primary-secondary amine (PSA) in the bottom bed. PSA retains acidic interferences such as fatty acids. GCB removes planar molecules such as pigments and sterols. Common GCBs, however, will retain all molecules with planar structures, including some pesticide analytes such as hexachlorobenzene. To increase recoveries of these pesticides, toluene is normally added to the elution solvent. However, there are issues associated with the use of toluene. It can affect the ability of the PSA to retain fatty acids, and its presence in the final extract is problematic for HPLC analysis.[5]

In this application, a different dual-layer SPE cartridge was used in the cleanup of extracts of turmeric powder prior to pesticide analysis by GC-MS/MS and LC-MS/MS. This cartridge, the Supelclean™ Ultra 2400, was designed for the cleanup of acetonitrile extracts made from difficult matrices such as dry commodities (spices, tea, etc.) prior to pesticide residue analysis. The top bed consists of a mixture of PSA, C18 and a graphitized, spherical carbon known as Graphsphere™ 2031. This carbon was engineered to remove sufficient pigmentation while allowing for better recoveries of planar compounds, without the need for toluene in the elution solvent. The bottom layer of the cartridge contains Z-Sep, a zirconia-coated silica. Z-Sep removes oily residues and provides additional retention of some pigments. The combination of these sorbents in an SPE format offers more capacity than QuEChERS cleanup, and compared to traditional GCB/PSA dual layer cartridges, does not require the use of toluene in the elution solvent to recover planar pesticides.

Experimental

Turmeric powder was obtained from a local grocery store. Samples were spiked at 100 ng/g with the pesticides listed in **Tables 1** and **2**. Sample extracts were prepared and cleaned following the procedures in **Figure 1**. A set of 3 spiked samples and 1 unspiked (blank) were prepared and analyzed for each set of pesticides. Analysis was done by GC-MS/MS and LC-MS/MS using the conditions listed in **Tables 3** and **4** (with MS/MS transitions shown in **Tables 1** and **2**). Quantitation was performed against multi-point calibration curves prepared in unspiked turmeric extract (after cleanup). Recoveries were calculated as the average of the three spiked replicates, less anything found in the unspiked extract. No internal standards were used, thus the values reported represent absolute recoveries.

Results and Discussion

Background

Prior to cleanup, the extract appeared orange-brown in color with a yellow oily residue (**Figure 2**). After cleanup for both LC and GC, the extracts appeared substantially lighter and clearer. **Figures 3** and **4** show

(continued on next page)

Table 1. Pesticides Studied in Turmeric Powder by GC-MS/MS Analysis

	MRM 1	CE	MRM 2	CE
Alachlor	188/160	10	188/130	40
Aldrin	263/193	35	263/191	35
γ-BHC	183/147	15	181/145	5
Azinphos-methyl	160/77	15	132/77	15
Chloropyrifos	314/286	5	314/258	15
Chloropyrifos-methyl	286/93	20	288/93	20
Cypermthrins	165/91	10	163/91	10
4,4'-DDT	235/199	15	235/165	25
Diazinon	199/135	15	137/84	10
Dichlorvos	185/93	25	145/109	25
Dimethoate	125/79	20	93/63	10
Disulfoton	88/60	5	88/59	15
Endosulfan β	241/206	15	241/170	30
Endosulfan-α	241/206	15	241/170	30
Ethion	231/129	20	121/65	10
Fenitrothion	277/125	20	277/109	20
Heptachlor	274/239	15	272/237	15
Hexachlorobenzene	284/249	20	284/214	35
Iprodione I	314/56	35	187/124	25
Iprodione II	316/56	35	187/124	25
Malathion	173/99	15	158/125	5
Metalaxyl	234/174	10	234/146	20
Methoxychlor	227/169	30	227/141	30
Mevinphos	192/127	25	192/109	25
Parathion-methyl	233/109	10	124/47	10
Permethrins	183/168	10	183/165	10
Phenthoate	274/125	15	274/121	10
Phorate	260/75	5	231/129	25
Phosalone	182/102	15	182/75	30
Pirimiphos-methyl	290/151	20	290/125	25
Profenophos	339/269	15	339/188	15
Quintozene	295/237	20	237/143	30
Vinclozolin	212/145	30	187/124	20

Table 2. Pesticides Studied in Turmeric Powder by LC-MS/MS Analysis

	MRM	Frag (V)	CE (V)	Cell Acc (V)
Acephate	184/143	70	0	5
Acetamiprid	223.1/126	80	27	2
Boscalid (Nicobifen)	343/307.1	145	16	6
Carbendazim (Azole)	192.1/160.1	105	16	2
Chlorbufam	224/172.02	120	5	3
Cycluron	199.2/72	120	20	2
Diflubenzuron	311/158	80	8	2
Fenoxanil	329.08/189	80	30	3
Fosthiazate	284/61	90	60	2
Methabenzthiazuron	222.1/165.1	90	12	2
Methamidophos	142/125	85	10	2
Methomyl	163.1/106	50	4	2
Monocrotophos (Azodrin)	224.1/193	65	0	5
Nitralin	346.11/304	100	10	3
Oxamyl	237.1/72	60	12	2
Pirimicarb	239.15/72.1	100	20	2
Procymidon	301/284*	70	8	2
Propaquizafop	444.12/100.1	125	16	2
Tetraconazole	372/159	130	36	2
Uniconazole-P	292.1/125	135	40	2

a comparison between extracts at the same level of dilution with and without cleanup. The LC extract (in 80% aqueous) was almost devoid of color, with very little cloudiness. The extract for GC analysis was a pale yellow color, with substantially less oily residue. Full scan GC-MS analyses of GC extracts are shown in

Figure 1. Extraction and Cleanup Procedure Used for Turmeric Powder, GC and LC

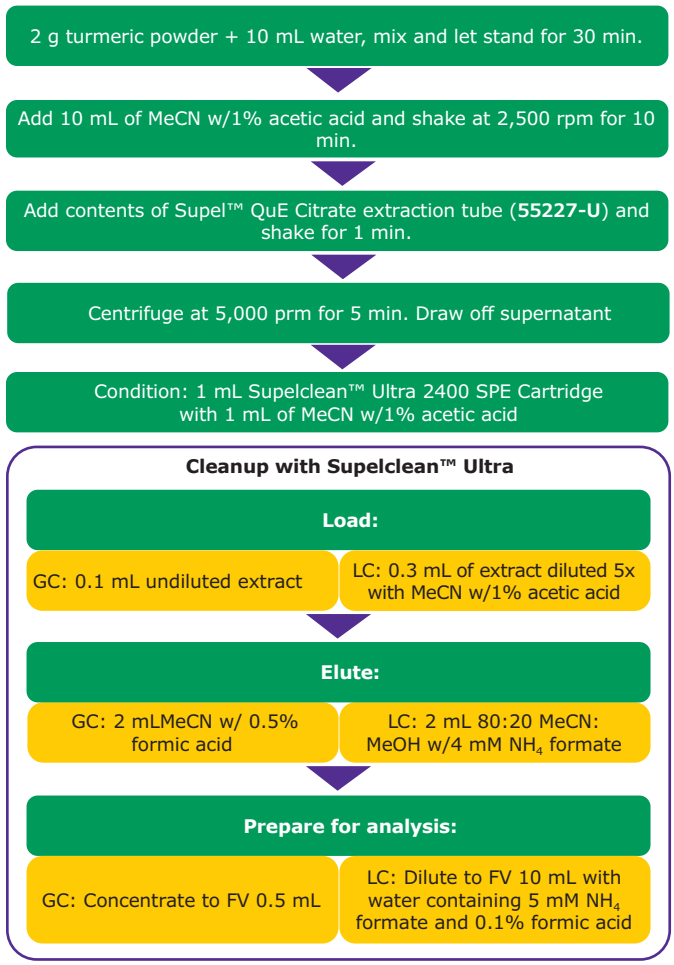


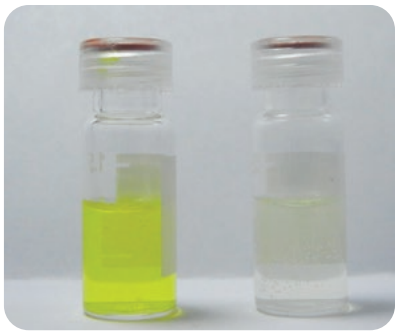
Table 3. GC-MS/MS Run Conditions for the Analysis of Pesticides in Turmeric

column:	SLB®-5ms, 30 m × 0.25 mm ID, 0.25 µm (**28471-U**)
oven:	50°C (2 min), 8°C/min to 320°C (5 min)
inj. temp.:	250°C
carrier gas:	helium, 1.4 mL/min, constant
detector:	MRM (see **table 1**)
MSD interface:	320°C
injection :	1µL, splitless (splitter open at 0.75 min)
liner:	4 mm I.D., split/splitless type, single taper wool packed FocusLiner™ design (**2879901-U**)

Table 4. LC-MS/MS Run Conditions for the Analysis of Pesticides in Turmeric

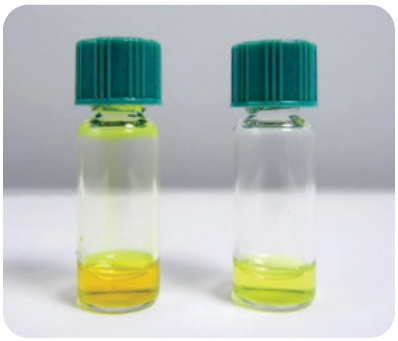
column:	Ascentis® Express C18, 10 cm × 2.1 mm ID, 2 µm (**50813-U**)
mobile phase:	[A] 5 mM ammonium formate, 0.1% formic acid in water; [B] 5 mM ammonium formate, 0.1% formic acid in methanol
gradient:	95% A, 5% B held for 1 min; to 50% A in 3 min; to 100% B in 8 min; held for 1 min;, to 95% A in 1.5 min; held at 95% A for 1.5 min
flow rate:	0.4 mL/min
detector:	MRM (see **table 2**)
injection:	5 µL

Figure 2. Undiluted Acetonitrile Extract of Turmeric Powder Before Cleanup



Without Cleanup After Cleanup

Figure 3. Turmeric Extracts at the Same Dilution (167X total); Without Cleanup, and After Cleanup for LC/MS/MS Analysis



Without Cleanup After Cleanup

Figure 4. Turmeric Extracts at the Same Dilution (5x); Without Cleanup, and After Cleanup for GC/MS/MS Analysis

Figure 5 as total ion chromatograms (TICs). The peak pattern is similar between the two, with the main peaks consisting primarily of terpenes. These compounds are easily volatilized in the GC inlet, and do not pose issues with system contamination, however they can interfere with mass spectral detection, requiring the use of MS/MS for selectivity. The overall amplitude of the peaks was less after cleanup, as is shown by a 21% reduction in the peak area sums for each in **Figure 5**.

Pesticide Recovery and Reproducibility

The average recoveries obtained from spiked turmeric samples (n=3) are presented in **Table 5**. Of the 51 pesticides spiked, all except hexachlorobenzene had recovery of greater than 70%. Hexachlorobenzene, a pesticide with a planar structure, was recovered at 67% after cleanup. It should be noted that this was without using toluene in the elution solvent, as is necessary to obtain good recoveries from dual-layer cartridges containing graphitized carbon black.[5] Although not shown here, higher recovery of hexachlorobenzene has been obtained by loading more turmeric extract (300 µL) on the Supelclean™ Ultra 2400 cartridge. This indicates that the presence of more matrix displaced the hexachlorobenzene, thus reducing its retention on the carbon. However the higher sample loading produced an extract with more color, a sign that the cleanup capacity of the cartridge had been reached or exceeded for this matrix.

Reproducibility, calculated as %RSD for the sets of spiked replicates, was less than 20% for 44 of the 51 pesticides. As is indicated in **Figure 6**, many compounds had RSD values of less than 10%. Pesticides with RSD values greater than 20% were attributed to those showing low response in the MS/MS method.

Table 5. Pesticide Recoveries and % RSD Values (***n***=3) for Spiked Replicates; Turmeric Spiked at 100 ng/g

Pesticide	Avg. Recovery	RSD	Analysis
Alachlor	99%	23%	GC-MS/MS
Aldrin	85%	10%	GC-MS/MS
Azinphos-methyl	89%	11%	GC-MS/MS
γ-BHC	83%	8%	GC-MS/MS
Chloropyrifos	96%	12%	GC-MS/MS
Chloropyrifos-Methyl	113%	6%	GC-MS/MS
Cypermthrin (isomer 1)	99%	15%	GC-MS/MS
4,4'-DDT	95%	8%	GC-MS/MS
Diazinon	92%	14%	GC-MS/MS
Dichlorvos	78%	31%	GC-MS/MS
DIsulfoton	86%	7%	GC-MS/MS
Endosulfan β	86%	35%	GC-MS/MS
Endosulfan-α	92%	23%	GC-MS/MS
Ethion	97%	7%	GC-MS/MS
Fenitrothion	63%	5%	GC-MS/MS
Heptachlor	81%	7%	GC-MS/MS
Hexachlorobenzene	67%	9%	GC-MS/MS
Iprodione (isomer 1)	103%	5%	GC-MS/MS
Malathion	90%	10%	GC-MS/MS
Metalaxyl	86%	21%	GC-MS/MS
Methoxychlor	78%	12%	GC-MS/MS
Mevinphos	73%	7%	GC-MS/MS
Parathion-Methyl	88%	8%	GC-MS/MS
Permethrin (isomer 1)	104%	24%	GC-MS/MS
Phenthoate	89%	7%	GC-MS/MS
Phorate	82%	10%	GC-MS/MS
Phosalone	90%	7%	GC-MS/MS
Pirimiphos-methyl	74%	3%	GC-MS/MS
Profenophos	88%	7%	GC-MS/MS
Quintozene	75%	8%	GC-MS/MS
Vinclozolin	90%	6%	GC-MS/MS
Acephate	89%	6%	LC-MS/MS
Acetamiprid	102%	4%	LC-MS/MS
Boscalid (Nicobifen)	86%	7%	LC-MS/MS
Carbendazim (Azole)	106%	7%	LC-MS/MS
Chlorbufam	92%	18%	LC-MS/MS
Cycluron	103%	5%	LC-MS/MS
Diflubenzuron	101%	5%	LC-MS/MS
Fenoxanil	91%	10%	LC-MS/MS
Fosthiazate	95%	4%	LC-MS/MS
Methabenzthiazuron	96%	4%	LC-MS/MS
Methamidophos	85%	5%	LC-MS/MS
Methomyl	106%	6%	LC-MS/MS
Monocrotophos (Azodrin)	97%	3%	LC-MS/MS
Nitralin	124%	55%	LC-MS/MS
Oxamyl	104%	3%	LC-MS/MS
Pirimicarb	97%	3%	LC-MS/MS
Procymidon	91%	13%	LC-MS/MS
Propaquizafop	97%	1%	LC-MS/MS
Tetraconazole	98%	2%	LC-MS/MS
Uniconazole-P	103%	19%	LC-MS/MS

Figure 5. GC-MS Scan Analyses of Turmeric Extracts Before and After Cleanup with Supelclean™ Ultra 2400 cartirgde Shown with same Y-scale. Sum of area counts for all peaks is indicated with each.

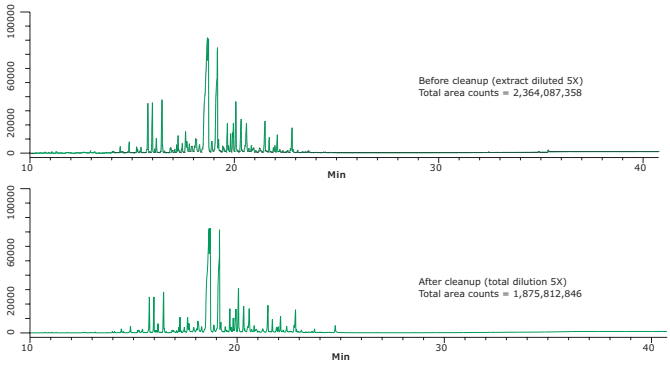
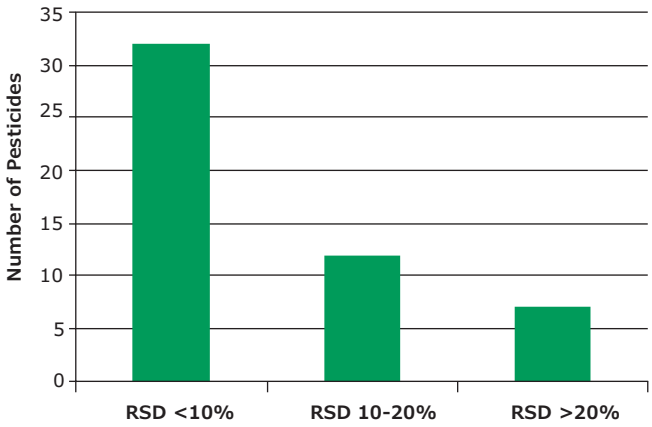


Figure 6. Number of Pesticides with Average Recoveries Within Indicated Percent Relative Standard Deviation (%RSD) Ranges After Cleanup with Supelclean™ Ultra 2400

(Recoveries from turmeric powder spiked at 100 ng/g.)



Conclusion

A new cleanup method has been developed using the Supelclean™ Ultra 2400 dual-layer SPE cartridge. The selection of sorbents in this cartridge allows for cleanup of acetonitrile extracts of very difficult samples such as spices and other dry commodities. The Graphsphere™ 2031 carbon used in the upper layer removes/reduces pigmentation while still allowing for recovery of planar pesticides without the use of toluene in the elution solvent. Z-Sep sorbent in the bottom layer of the cartridge removes oils and some pigments, as was indicated in the cleanup of turmeric extracts for both GC and HPLC analysis. Suitable recoveries for a wide range of pesticides of different polarities and classes were obtained from turmeric extract, and minimal background interference was noted. In this work, a 1 mL Supelclean™ Ultra 2400 cartridge was used. A larger 3 mL version of the cartridge is also available which can accommodate a higher sample loading.

Acknowledgements

The author would like to thank Richard Schriner and Bruce Morris of R.J. Hill Laboratories for their helpful discussions and input.

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5. Shimelis, O.; Yang, Y.; Stenerson, K.; Kaneko, T.; Ye, M. Evaluation of Solid-Phase Extraction Dual-layer Carbon/Primary Secondary Amine for Clean-up of Fatty Acid Matrix Components From Food Extracts in Multiresidue Pesticide Analysis. *J. Chrom. A*, 2007, 1165, 18-25.

Featured Products

Description	Cat. No.
Supelclean™ Ultra 2400 SPE Cartridges	
1 mL, pk of 108	**52779-U**
3 mL, pk of 54	**54281-U**
Supel™ QuE QuEChERS Products	
Citrate Extraction Tube, 12 mL, pk of 50	**55227-U**
Empty Centrifuge Tube, 50 mL, pk of 50	**55248-U**
Columns	
SLB®-5ms Capillary GC Column, 30 m × 0.25 mm I.D., 0.25 µm	**28471-U**
Ascentis® Express C18 HPLC Column, 10 cm × 2.1 mm I.D., 2 µm particle size	**50813-U**
Accessories	
QuEChERS Shaker and Rack Starter Kit, USA compatible plug, AC input 115 V	**55278-U**
QuEChERS Shaker and Rack Starter Kit, Schuko plug, AC input 230 V	**55438-U**
Visiprep™ DL 12-port Solid Phase Extraction Manifold	**57044**
Disposable valve liners, PTFE, 100 ea.	**57059**

Related Products

Description	Cat. No.
Solvents and Reagents	
Acetonitrile hypergrade for LC-MS LiChrosolv®	**1.00029**
Acetic acid 100% for LC-MS LiChropur®	**5.33001**
Formic acid 98% - 100% for LC-MS LiChropur®	**5.33002**
Ammonium formate for mass spectrometry, ≥99.0%	**70221**
Acetonitrile for GC-MS SupraSolv®	**1.00665**
Accessories	
Certified Vial Kit, Low Adsorption (LA), 2 mL, pk of 100	**29653-U**
Inlet Liner, Split/Splitless Type, Single Taper FocusLiner™ Design (wool packed)	**2879901-U**

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PLANTS, HERBS & SPICES

Analysis of Pesticide Residues in Ginger Powder Using QuEChERS Extraction and Cleanup with a Novel Dual Layer SPE Cartridge

Katherine K. Stenerson, Principal R&D Scientist, Analytix@merckgroup.com

Introduction

Ginger comes from the rhizome of the plant *Zingiber officinale*. It has been used for thousands of years as both a spice for cooking and a treatment for ailments such as nausea, gastrointestinal (GI) irritation, inflammations and cold & flu symptoms. In addition, recent studies indicate that it may be useful in pain reduction and supporting cardiovascular health.[1] Ginger is native to southeast Asia, however it is also grown in various countries in the western hemisphere. The world production of ginger was approximately 2.1 million metric tons in 2013, with about half coming from China and India.[2] After harvesting, ginger root is first washed and then boiled in a process known as "killing", which stops enzymatic activity. It is then dried and subjected to further processing such as grinding for powdered ginger, or solvent extraction and distillation for production of ginger oil and oleoresin.[3] Many pesticides used on the plant can be carried through these processes and end up in the final product. Since dried ginger is used for both cooking and as a dietary supplement, there is a risk for exposure to pesticides as a result of more frequent consumption. Thus, pesticide residue analysis of ginger is required by many countries. For example, Canada has maximum residue limits for a variety of pesticides in ginger root ranging from 0.01 to 0.15 ppm.[4]

The odor and pungency of ginger is due to the presence of terpenes, gingerols and shogaols.[5] These compounds contribute to the highly complex matrix of ginger, which subsequently presents a special challenge in low level analyses of contaminants. The analysis of pesticide residues in ginger can be achieved using the "Quick, Easy, Cheap, Effective, Rugged and Safe" (QuEChERS) approach for extraction. Following extraction, a cleanup step is essential to produce a sample that is amenable to both LC and GC chromatographic analysis. A common approach, included in the QuEChERS methodology is to use dispersive solid phase extraction (dSPE) with loose sorbents. However, dry commodities such as ginger powder often produce too much background for effective cleanup using dSPE. A traditional solid phase extraction (SPE) cartridge has more cleanup capacity than dSPE, and is recommended for these types of samples. In this application, a new dual-layer SPE cartridge, the Supelclean™ Ultra 2400, was used in the cleanup of QuEChERS extracts of ginger powder prior to analysis of pesticide residues by both LC-MS/MS and GC-MS/MS. This cartridge contains a blend of primary-secondary amine (PSA), C18, and graphitized carbon in the top layer and Z-Sep sorbent in the bottom layer. The cartridge contains sorbents traditionally used for cleanup (PSA and C18) as well as two novel materials; Graphsphere™ 2031 and Z-Sep. Graphsphere™ 2031 is a specially engineered carbon with a lower surface area than standard graphitized carbon black (GCB). The lower surface area provides a balance between removal of pigments with planar structures such as chlorophyll, and weaker retention of planar pesticides. The Z-Sep in the bottom layer is zirconia coated silica which can retain carotenoids as well as some fatty constituents. Together these two layers of sorbents provide more rigorous cleanup than dSPE. In addition, compared to current dual layer SPE cartridges containing GCB and PSA or aminopropyl silica, the Ultra 2400 is much smaller, but still provides sufficient sample cleanup with much less solvent consumption. The use of Graphsphere™ 2031 carbon in the Ultra 2400 offers an advantage over traditional GCB containing cartridges in the form of improved recoveries of planar pesticides without the use of toluene in the elution solvent.

In this study, ginger powder was spiked at 10 ng/g with a variety of pesticides, and extracted using a standard QuEChERS approach. The extract was then cleaned for LC-MS/MS and GC-MS/MS analyses using the 3 mL Supelclean™ Ultra 2400 SPE cartridge.

Experimental

Dry ginger powder was obtained from a local grocery store, and spiked at 10 ng/g with the pesticides listed in **Tables 1 and 2**. Samples were extracted as described in **Figure 1** and subjected to separate cleanups for LC and GC as described in **Figure 2**. Spiked replicates of ginger extract (n=3) and blanks were subjected to cleanup using the described procedures. Samples were analyzed by external standard analysis against matrix-matched calibration curves using the HPLC and GC conditions shown in **Tables 3 and 4**. The MRMs used for quantitation are indicated in **Tables 1 and 2**.

Figure 1. QuEChERS extraction procedure used for ginger powder

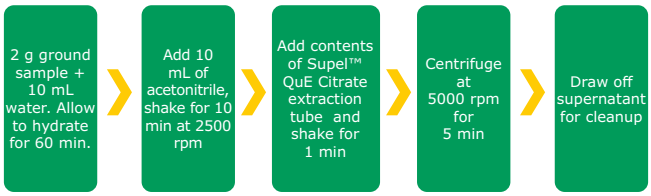


Figure 2. Cleanups used for QuEChERS extracts of ginger powder using 3 mL Supelclean™ Ultra 2400 cartridge

Table 1. Pesticides analyzed in dry ginger powder by LC-MS/MS

Compound	MRM	Frag (V)	CE (V)	Cell Acc (V)
Acephate	184/143	70	0	5
Acetamiprid	223.1/126	80	27	2
Boscalid	343/307.1	145	16	6
Carbendazim	192.1/160.1	105	16	2
Chlorbufam	224/172.02	120	5	3
Cycluron	199.2/72	120	20	2
Diflubenzuron	311/158	80	8	2
Fenoxanil	329.08/189	80	30	3
Fosthiazate	284/61	90	60	2
Methabenzthiazuron	222.1/165.1	90	12	2
Methamidophos	142/125	85	10	2
Methomyl	163.1/106	50	4	2
Monocrotophos	224.1/193	65	0	5
Nitralin	346.11/304	100	10	3
Oxamyl	237.1/72	60	12	2
Pirimicarb	239.15/72.1	100	20	2
Procymidon	301/284*	70	8	2
Propaquizafop	444.12/100.1	125	16	2
Tetraconazole	372/159	130	36	2
Uniconazole-P	292.1/125	135	40	2

*ammonium adduct

Table 2. Pesticides analyzed in dry ginger powder by GC-MS/MS

Compound	MRM	CE
Alachlor	188/160	10
Aldrin	263/193	35
Azinphos-methyl	160/132	0
γ-BHC	217/181	5
Chloropyrifos	197/169	15
Chloropyrifos-Methyl	125/47	15
Cypermethrins	163/127	5
4,4'-DDT	237/165	20
Diazinon	137/84	10
Dichlorvos	185/93	10
Dimethoate	87/46	15
Disulfoton	88/59	15
Endosulfan β	207/172	15
Endosulfan-α	195/160	5
Ethion	153/97	10
Fenitrothion	277/260	10
Heptachlor	274/239	15
Hexachlorobenzene	284/249	20
Iprodione I	187/124	25
Iprodione II	244/187	5
Malathion	173/99	15
Metalaxyl	220/192	5
Methoxychlor	227/169	30
Mevinphos	127/95	15
Parathion-Methyl	125/47	10
Permethrins	183/168	10
Phenthoate	274/125	15
Phorate	121/47	30
Phosalone	182/111	15
Pirimiphos-methyl	290/125	20
Profenophos	208/63	30
Quintozene	295/237	20
Vinclozolin	187/124	20

Table 3. LC-MS/MS analysis conditions

column:	Ascentis® Express C18, 10 cm × 2.1 mm I.D., 2 µm
mobile phase:	[A] 5 mM ammonium formate, 0.1% formic acid in water; [B] 5 mM ammonium formate, 0.1% formic acid in methanol
gradient:	95% A, 5% B held for 1 min; to 50% B in 3 min; to 100% B in 8 min; held at 100% B for 1 min, to 95% A, 5% B in 0.5 min, held 1.5 min.
flow rate:	0.4 mL/min
column temp.:	30 °C
detector:	MS, ESI(+), MRM (see **Table 1**)
injection:	5 µL

Table 4. GC-MS/MS analysis conditions

column:	SLB®-5ms, 20 m × 0.18 mm I.D., 0.18 µm
oven:	45 °C (3 min), 8 °C/min to 325 °C (2 min)
inj. temp:	250 °C
carrier gas:	helium, 1.2 mL/min, constant flow
detector:	MS, MRM (see **Table 2**)
MSD interface:	325 °C
injection:	1 µL pulsed splitless (40 until 0.5 min, splitter on at 0.5 min)
liner:	4 mm I.D. FocusLiner™ with taper

Table 5. Average recoveries and %RSD values for pesticides from ginger powder; QuEChERS extraction followed by cleanup with Supelclean™ Ultra 2400 SPE

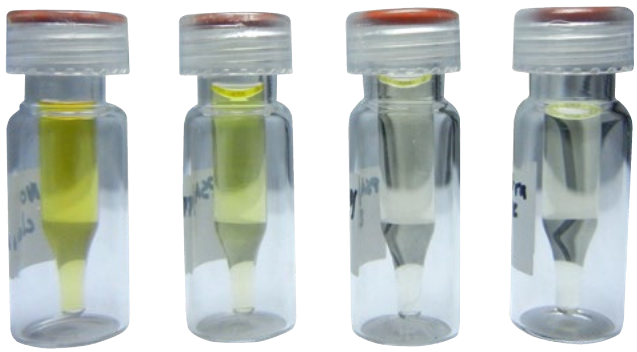
Compound	Avg. % Recovery (*n*=3)	RSD	Analysis
Acephate	86%	3%	LC
Acetamiprid	90%	2%	LC
Alachlor	110%	4%	GC
Aldrin	73%	2%	GC
Azinphos-methyl	79%	7%	GC
BHC- γ	84%	1%	GC
Boscalid	66%	7%	LC
Carbendazim	65%	2%	LC
Chlorpyrifos	100%	2%	GC
Chlorpyrifos-methyl	87%	3%	GC
Cycluron	85%	2%	LC
Cypermethrins (avg. isomers I-IV)	82%	8%	GC
DDT-p,p'	84%	6%	GC
Diazinon	86%	4%	GC
Dichlorvos	31%	72%	GC
Diflubenzuron	66%	2%	LC
Dimethoate	99%	2%	GC
Disulfoton	85%	5%	GC
Endosulfan I	92%	7%	GC
Endosulfan II	97%	23%	GC
Ethion	84%	14%	GC
Fenitrothion	102%	5%	GC
Fenoxanil	99%	60%	LC
Fosthiazate	85%	5%	LC
Heptachlor	73%	7%	GC
Hexachlorobenzene	45%	21%	GC
Iprodione	87%	4%	GC
Malathion	109%	4%	GC
Metalaxyl	79%	14%	GC
Methabenzthiazuron	58%	3%	LC
Methamidophos	72%	0%	LC
Methomyl	89%	5%	LC
Methoxychlor	90%	2%	GC
Mevinphos	57%	26%	GC
Monocrotophos	89%	4%	LC
Nitralin	67%	27%	LC
Oxamyl	89%	4%	LC
Parathion-methyl	97%	2%	GC
Permethrin I, II	not quantitated	–	GC
Phenthoate	110%	5%	GC
Phorate	70%	12%	GC
Phosalone	97%	5%	GC
Pirimicarb	90%	2%	LC
Pirimiphos-methyl	96%	3%	GC
Procymidon	59%	1%	LC
Profenofos	93%	3%	GC
Propaquizafop	70%	11%	LC
Quintozene	68%	8%	GC
Tetraconazole	46%	8%	LC
Uniconazole-P	179%	83%	LC
Vinclozolin	97%	4%	GC
Average	**82%**	**9%**	**–**

Results & Discussion

Background

After cleanup, the extract prepared for GC analysis showed substantially less color (**Figure 3**). For comparison, an aliquot of extract was also cleaned by dSPE using PSA/C18/GCB and PSA/C18. The PSA/C18/GCB and Ultra 2400 cleaned extracts were similar in color, while the extract cleaned using PSA/C18 was darker yellow, indicating the need for carbon to reduce pigmentation. GC-MS scan comparisons of the Ultra 2400, PSA/C18/GCB cleaned and uncleaned extracts are shown in **Figure 4**. Peak patterns are similar between the three extracts, however the overall amplitude of the peaks was reduced after cleanup. Most of the background peaks eluting prior to 15 minutes were removed by the Ultra 2400 cleanup. The reduction in background, measured as total peak areas, was 11% using PSA/C18/GCB and 21% using Ultra 2400.

Figure 3. QuEChERS extracts of ginger powder after cleanup by dSPE and Supelclean™ Ultra 2400 SPE



Pesticide Recovery

The pesticide recoveries and reproducibilities obtained after cleanup with Ultra 2400 are presented in **Table 5** and **Figure 5**. The average recovery obtained was 82%, with an average RSD of 9%. Of the 51 pesticides, 38 were within the 70-120% recovery range considered acceptable. Recovery of permethrin and uniconazole-P were affected by matrix interference. Procymidon was a very poor responding analyte by LC-MS/MS, and was difficult to detect at 10 ng/g. It is suspected that

recoveries of boscalid, carbendazim, diflubenzuron, nitralin and tetraconazole were reduced by the amount of sorbent to which the extract was exposed during cleanup. In past work done by the authors with cleanup of turmeric extract using the 3 mL Ultra 2400 SPE cartridge, these same pesticides had recoveries of >70%. Turmeric is much more oily and pigmented than ginger, and thus there was more matrix available to bind with active sites on the sorbents in the SPE cartridge. In the case of ginger, the relatively lower amounts of oil and pigment could have resulted in increased binding of these pesticides with the sorbents. A smaller 1 mL Ultra cartridge containing less sorbent weight could be used to increase recoveries.

Dichlorvos exhibited very low and variable recovery, and this same behavior has been observed by others using zirconia-based sorbents for cleanup.[6] This is most likely due to Lewis acid/base interaction between the Z-Sep and the phosphate group present in the structure of dichlorvos. Other pesticides containing phosphate groups: fosthiazate, methamidophos, mevinphos and monocrotophos, had better recoveries. All were recovered at >70%, except mevinphos, which was recovered at 57%. Both mevinphos and dichlorvos were analyzed by GC-MS/MS while the other phosphate containing pesticides were analyzed by LC-MS/MS. The difference in recoveries could be due to the variation in elution protocols used during the cleanups. Elution from the Ultra 2400 cartridge for GC analysis used acetonitrile containing formic acid, while LC used methanol:acetonitrile containing ammonium formate. Formic acid is a Lewis base, and is added to prevent interaction between the Z-Sep and weaker Lewis bases such as some acidic compounds. However, this was not entirely effective in the case of the phosphate groups in dichlorvos and mevinphos. The ammonium formate in the LC elution solvent acts as a Lewis base, and also acts to disrupt any weak cation exchange interactions that may be occurring between basic compounds and silanol groups present in the silica of the Z-Sep.[7] The addition of methanol to the elution solvent may also be contributing to increased recovery of the phosphate containing pesticides. It has been shown that methanol addition increases pesticide recovery when using Z-Sep sorbent; possibly by disrupting Lewis acid-base and/or electrostatic interactions.[8]

Figure 4. GC-MS scan analyses of ginger powder extracts

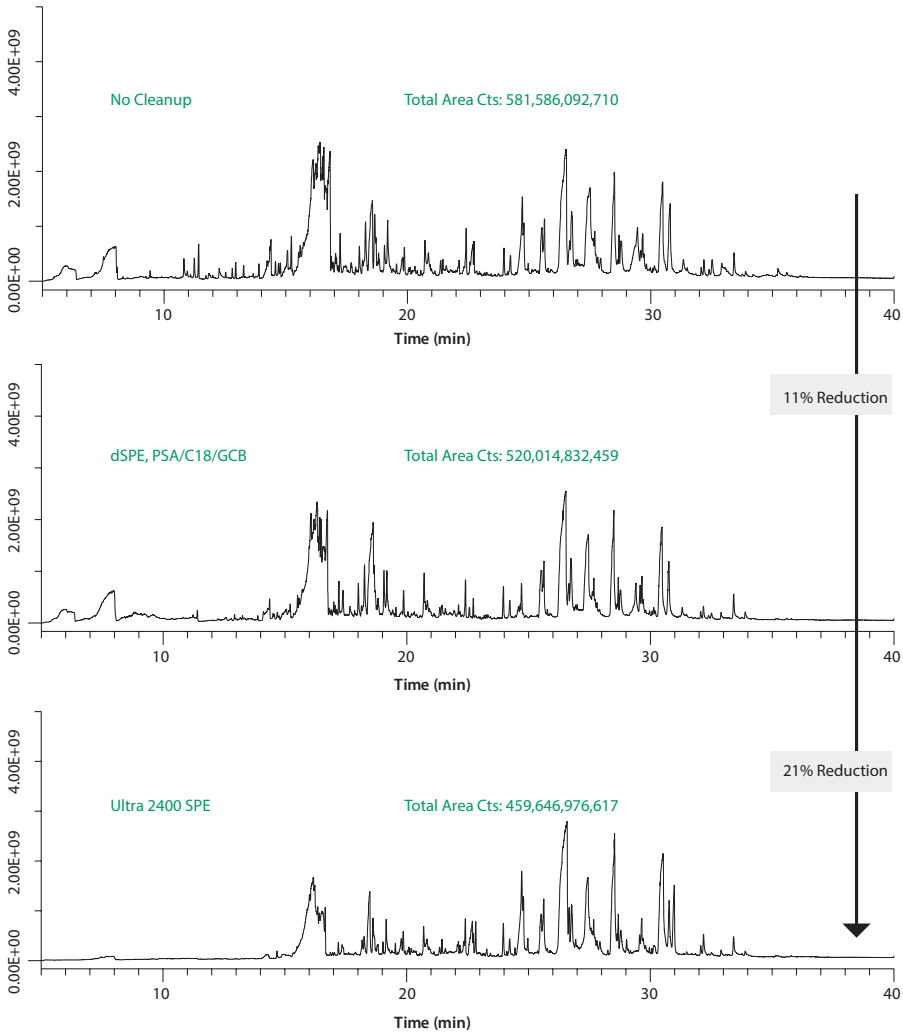
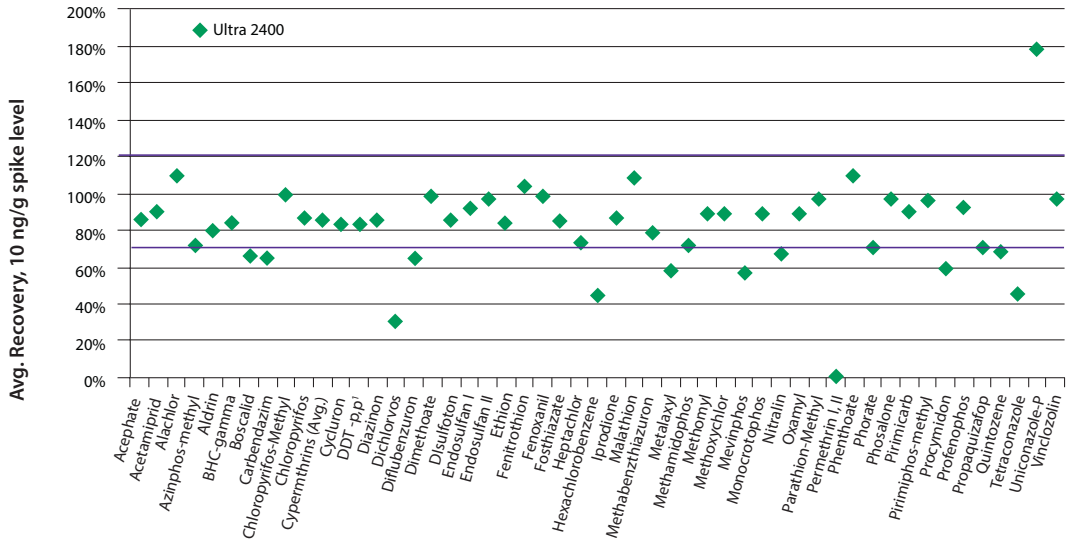


Figure 5. Average recoveries of pesticides from ginger powder spiked at 10 ng/g after cleanup with 3 mL Supelclean™ Ultra 2400 SPE



Recovery of the planar pesticide hexachlorobenzene (HCB) is traditionally problematic after cleanup using GCB. This compound is retained by conventional GCBs during cleanup. If using SPE cleanup, toluene in the elution solvent can increase recovery of this compound by displacing it from the carbon. However, toluene is then in the final extract, making it incompatible with HPLC analysis. Recovery of HCB after cleanup with the 3 mL Ultra 2400 cartridge was 45%. This is in the same range as recoveries reported by others for cleanup of acetonitrile extracts of botanicals using 6 mL dual layer SPE cartridges in combination with toluene-containing elution solvent.[9,10] To determine if recovery of HCB could be improved, the ginger extract was also cleaned using a smaller 1 mL Ultra 2400 cartridge. The elution protocol used was similar to that used for the 3 mL, with smaller solvent volumes. Recovery increased to 63%, indicating that, similar to other pesticides with recoveries <70%, the smaller 1 mL cartridge may provide a better balance between matrix and sorbent active sites for the cleanup of powdered ginger extract.

Conclusions

QuEChERS extraction of powdered ginger produces an extract containing enough background to require cleanup prior to chromatographic analysis. The use of Ultra 2400 SPE reduced this background, as evidenced by visual appearance of the extract and GC-MS-scan data. The greater capacity of the SPE cleanup reduced background more than dSPE using PSA/C18/GCB. Recoveries for 51 target pesticides, spiked at 10 ng/g, were in the range of 70-120% for 75% of the analytes. Recoveries of some of pesticides outside of this range could be improved through adjustments to the cleanup method such as use of a smaller, 1 mL Ultra SPE cartridge, and/or modifications to the elution solvent used. Compared to cleanup using larger 6 mL dual layer SPE cartridges containing GCB, the method using the 3 mL Ultra 2400 requires significantly less solvent: 11 mL vs. 20-30 mL. Recovery of the planar pesticide hexachlorobenzene was achieved without toluene in the elution solvent. Using the 3 mL cartridge, recovery from ginger was 45%; and this could be increased with use of the smaller 1 mL cartridge.

In summary, Supelclean™ Ultra SPE offers a cost effective alternative to the use of conventional 6 mL dual layer SPE containing GCB. The cartridge is available in 1 and 3 mL sizes, offering a choice to accommodate differing matrices and analyte lists.

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9. Chen, Y.; Lopez, S.; Hayward, D.G.; Park, H.Y.; Wong. J. W.; Kim, S.S.; Wan, J.; Reddy, R.M.; Quinn, D.J.; Steiniger, D. Determinatinon of multiresidue pesticides in botanical dietary supplements using gas-chromatography-triple-quadrupole mass spectrometry (GC-MS/MS). *J. Agric. Food Chem*. 2016, *64*, 6125-6132.
10. Hayward, D.G.; Wong, J.W.; Park, H.Y., Determinations for

PLANTS, HERBS & SPICES

What Makes Wasabi so Hot?

New reference materials for Glucosinolates from PhytoLab now available

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

PhytoLab is one of the leading manufacturers of phytochemicals internationally. We are proud to be able to offer their comprehensive portfolio of more than 1,300 extensively characterized and documented herbal reference substances of all classes of natural compounds. In Issue 5 of the Analytix Reporter we presented the compound class of pyrrolizidine alkaloids as an example of the extensive range covered by the PhytoLab portfolio. This time we will focus on a class of natural compounds that are responsible for the burning sensation we feel when eating mustard and horseradish: glucosinolates.

Glucosinolates are secondary plant metabolites that occur in a wide variety of plants mainly from the families of the Brassicaceae (e.g., horseradish (*Armoracia rusticana*), black mustard (*Brassica nigra*), wasabi (*Eutrema japonicum*), broccoli (*Brassica oleracea var. italica*), maca (*Lepidium meyenii*)), the Capparaceae (e.g., capers (*Capparis spinosa*)) and the Caricaceae (e.g., papaya (*Carica papaya*), but also from the Euphorbiaceae and Tropaeolaceae (e.g., garden nasturtium (*Tropaeolum majus*)). Besides being responsible for the pungent and bitter taste of these plants, the glucosinolates and their hydrolysis products also protect plants against herbivores and have been shown to have antimicrobial, antiviral, antifungal and anticarcinogenic properties. Due to their antimicrobial properties, herbal medicinal products containing nasturtium herb and horseradish root are used in the treatment of sinusitis, bronchitis and urinary tract infections.

All glucosinolates have a central carbon atom in common, which is bound via a sulfur atom to a glucose, and via a nitrogen atom to a sulfate group. Furthermore, a substance-specific side chain (its structure depending on the amino acid applied in the biosynthesis) is bound to the central carbon atom. As the sulfate group is negatively charged, glucosinolates are most often isolated as their potassium salts.

Upon contact with the enzyme myrosinase and water (myrosinase is kept in a separate compartment in the cell, but can be released, for example, during cutting or chewing), the glucose moiety is cleaved. The remaining molecule can then undergo various spontaneous reactions, usually resulting in the corresponding isothiocyanate. Depending on the reaction conditions, thiocyanates, nitriles or oxyzolidine-2-thiones can also be formed.

For a reliable quantitative analysis of glucosinolates, well characterized reference substances are essential. Currently we offer seventeen glucosinolates, all of which come with a comprehensive certificate of analysis. Due to the negative charge of the molecule, the counter ion has to be taken into account. For all glucosinolates characterized as primary reference substances, potassium was determined quantitatively and considered as an impurity in the calculation of the absolute content, which therefore refers to the pure glucosinolate only.

Available Glucosinolate Reference Materials

Description	Package Size	Cat. No.
Epiprogoitrin	10 mg	**PHL89657**
Glucobarbarin	10 mg	**PHL89684**
Glucoberteroin	5 mg	**PHL83241**
Glucobrassicanapin	10 mg	**PHL83242**
Glucobrassicin	10 mg	**PHL80593**
Glucocheirolin	10 mg	**PHL89685**
Glucoerucin	10 mg	**PHL89686**
Glucoiberin	10 mg	**PHL89687**
Gluconapin	10 mg	**PHL89688**
Gluconasturtiin	10 mg	**PHL89689**
Glucoraphanin	10 mg	**PHL89215**
Glucoraphenin	10 mg	**PHL89690**
Glucosibarin	10 mg	**PHL89691**
Glucotropaeolin	10 mg	**PHL89216**
Progoitrin	10 mg	**PHL89765**
Sinalbin	10 mg	**PHL89793**
Sinigrin	25 mg	**PHL89279**

Find all the products on our website at
SigmaAldrich.com/glucosinolates

For our complete offering on phytochemical reference materials visit us at
SigmaAldrich.com/phytochemicals

Vanilla - Natural or Out of the Reaction Flask?

HPLC Fingerprint Method and Reference Materials Help to Distinguish Natural from Synthetic or Adulterated Vanilla

Anita Piper, Scientist Instrumental Analysis R&D
Stephan Altmaier, Principal Scientist Advanced Analytical R&D
Matthias Nold, Product Manager Reference Materials, Analytix@merckgroup.com

Introduction

Vanilla is one of the most popular flavors in food and beverage products. The demand far exceeds the global supply of naturally grown vanilla; therefore, in addition to natural vanilla, artificial vanilla flavors are used in the food industry. Natural vanilla is commonly substituted for synthetically produced vanillin or by other compounds with a similar flavor such as ethyl vanillin.

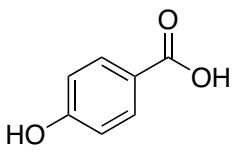
And because of the large price difference between natural and synthetic vanilla, this is a very attractive target for food criminals and frauds.

Analysis of the chromatographic fingerprint of a vanilla flavor represents an efficient method to detect these types of adulteration and mislabeling.[1] Characteristic markers for natural vanilla are vanillic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde and vanillin (**Figure 1**).

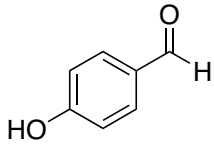
For artificially produced vanilla, cheap chemicals such as guaiacol or eugenol are typically used as starting materials. The presence of traces of these compounds are indicators of synthetically produced vanilla. Ethyl vanillin or coumarin are also often added to enhance the flavor (**Figure 2**).

We recently launched a set of two reference materials for natural and synthetic vanilla extracts for the testing of vanilla authenticity by chromatographical fingerprint. These two reference extracts are also available individually (**Table 1**).

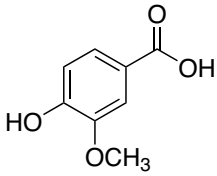
Figure 1. Compounds Found in Natural Vanilla



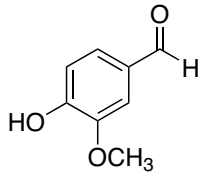
4-Hydroxybenzoic acid
(Cat.No. **92596**)



4-Hydroxybenzaldehyde
(Cat.No. **91554**)

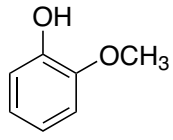


Vanillic acid
(Cat.No. **68654**)

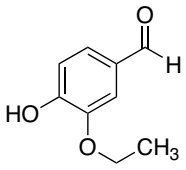


Vanillin
(Cat.No. **30304**)

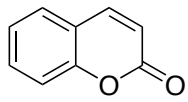
Figure 2. Markers for Artificial Vanilla



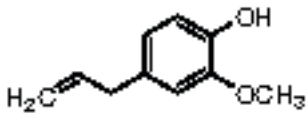
Guaiacol
(Cat.No. **PHR1136**)



Ethyl vanillin
(Cat.No. **75042**)



Coumarin
(Cat.No. **72609**)



Eugenol
(Cat.No. **79891**)

Table 1. Vanilla Extract Reference Materials (Natural and Synthetic)

Description	Quantified Components	Qualitatively Confirmed Components	Package Size	Cat. No.
Vanilla extract, natural	Vanillin, Vanillic acid	Vanillin, Vanillic acid, Ethyl Vanillin (absence)	1 mL	**06261501**
Vanilla extract, synthetic	Vanillin, Vanillic acid	Vanillin, Vanillic acid, Ethyl Vanillin	1 mL	**06271501**
Vanilla extract set, natural and synthetic	Vanillin, Vanillic acid	Vanillin, Vanillic acid, Ethyl Vanillin (absence / presence)	2 x 1 mL	**06281501**

The products are developed and manufactured by HWI pharma services GmbH in Rülzheim, Germany, and are qualified as secondary standards, traceable to HWI primary reference standards quantified by qNMR. These products add to a range of plant extract reference materials (**SigmaAldrich.com/plantextracts**) designed for rapid identification and quantification of typical constituents of plants used as food additives or as herbal medicinal products.

HPLC fingerprint method

In the following we present an HPLC method to detect natural and synthetic vanilla markers using a Chromolith® Performance RP-18 endcapped 100x2 mm column (**Table 2**). Results for both the synthetic and the natural vanilla reference material extract are shown. In addition, samples of food and beverage products containing vanilla flavor, such as bourbon vanilla, ice cream and Rooibos tea, were also tested (see **Table 2**. for sample preparation details)

For all the standards, extensive studies were made to determine LOD, LOQ, linearity, repeatability and standard deviation. The complete dataset incl. the data for Rooibos tea and validation data for the method can be viewed online in the full version of this article at **SigmaAldrich.com/Analytix**, Issue 7.

Table 2. Experimental Conditions & Sample Preparation

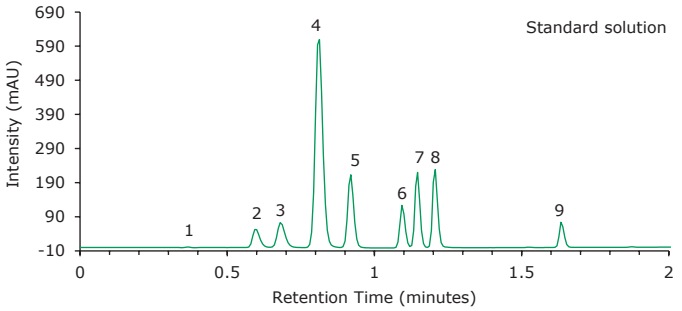
column:	Chromolith® Performance RP-18 endcapped 100x2 mm (**1.52006**)
mobile phases:	[A] 0.05% TFA in water, [B] acetonitrile
injection volume:	0.5 µL
gradient:	**Time** / **%A** / **%B** 0 / 90 / 10 0.5 / 65 / 35 1.3 / 25 / 75 1.5 / 0 / 100 2 / 0 / 100
flow rate:	0.8 mL/min
pressure:	75-118 bar (1088-1711 psi)
column temp.:	40 °C
detector:	Dionex Ultimate 3000 VWD-3400 @ UV = 280 nm (micro flow cell; 1.4 µL/7 mm)
Standard & Sample Preparation:	
standard solution :	the standards 4-hydroxybenzoic acid (c = 0.1 mg/mL), vanillic acid (0.1 mg/mL), 4-hydroxybenzaldehyde (0.2 mg/mL), vanillin (0.1 mg/mL), guaiacol (0.2 mg/mL), ethyl vanillin (0.1 mg/mL), coumarin (0.1 mg/mL), and eugenol (0.2 mg/mL) were dissolved in mobile phases A/B 90/10 (v/v).
matrix standard solution natural:	transfer approximately 100 mg of vanilla extract natural into a 5 mL volumetric flask, dissolve in mobile phases A/B 95/5 (v/v) and fill up to mark with mobile phases A/B 95/5 (v/v).
matrix standard solution synthetic:	transfer approximately 25 mg of vanilla extract synthetic into a 25 mL volumetric flask, dissolve in mobile phases A/B 95/5 (v/v) and fill up to mark with mobile phases A/B 95/5 (v/v)
sample solution Bourbon vanilla:	one piece of Bourbon vanilla was cut into small pieces and placed in a 50 mL volumetric flask. The flask was filled to the mark with ethanol and after ultrasonic extraction at room temperature for 30 min the sample mixture was filtered through a 0.45 µm membrane filter.
sample solution ice cream:	500 mg vanilla ice cream was placed in a 50 mL volumetric flask and filled up with ethanol to the mark. After ultrasonic extraction at room temperature for 30 min the sample mixture was filtered through a 0.45 µm syringe filter.
sample solution Rooibos tea:	one pad of vanilla flavored Rooibos tea was placed in a 50 mL volumetric flask and filled up with ethanol to the mark. After ultrasonic extraction at room temperature for 30 min the sample mixture was filtered through a 0.45 µm membrane filter.

Results & Discussion

The prepared standard solution was used for method development and validation. The chromatogram and retention data is shown in **Figure 3**. Vanillin is the main component of the natural vanilla extract reference material (cat. no. 06261501), in addition traces of 4-hydroxybenzoic acid, vanillic acid and 4-hydroxybenzaldehyde could be detected. No ethyl vanillin, guaiacol, coumarin or eugenol were present (**Figure 4**). For comparison, the chromatogram of a commercial Bourbon vanilla sample (**Figure 5**) is very similar to the natural vanilla extract reference material.

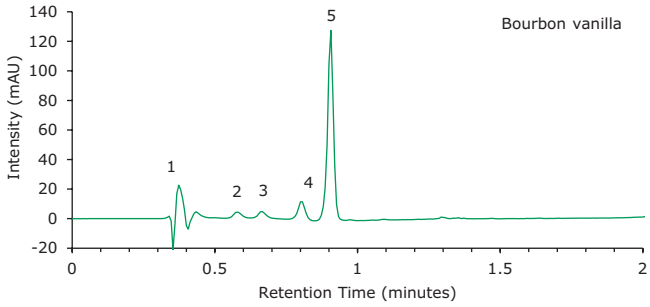
In contrast to natural vanilla, the synthetic vanilla extract reference material (cat.no. 06271501) shows, besides vanillin as the major peak, ethyl vanillin, coumarin and traces of eugenol (**Figure 6**). In the ice cream sample, guaiacol is the major peak (**Figure 7**). In addition, traces of ethyl vanillin, coumarin and eugenol were detected, indicating the synthetic nature of the material.

Figure 3. HPLC-UV Analysis of the Standard Solution



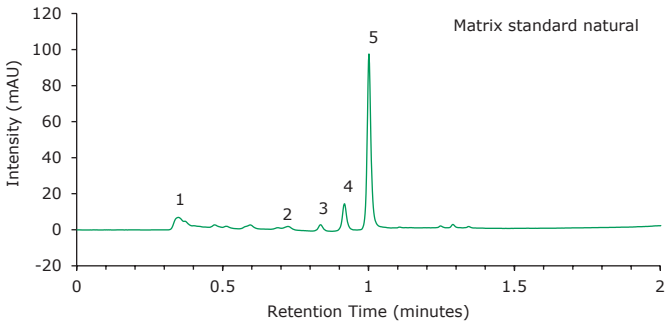
No.	Compound	Retention Time (min)	RRT	Area (mAU* min)	Tailing Factor
1	t_0 void volume	0.37			
2	4-Hydroxybenzoic acid	0.59	0.64	1.537	1.5
3	Vanillic acid	0.68	0.74	2.256	1.28
4	4-Hydroxybenzaldehyde	0.81	0.88	18.34	1.03
5	Vanillin	0.92	0.00	5.06	1.05
6	Guaiacol	1.09	1.18	2.391	1.16
7	Ethyl vanillin	1.15	1.25	4.243	1.02
8	Coumarin	1.21	1.32	4.384	1.05
9	Eugenol	1.63	1.77	1.416	1.32

Figure 5. HPLC-UV Analysis of a Commercial Bourbon Vanilla Sample



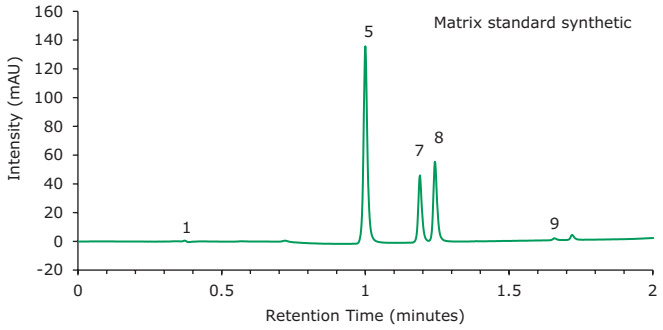
No.	Compound	Retention Time (min)	RRT	Area (mAU* min)	Tailing Factor
1	t_0 void volume	0.37			
2	4-Hydroxybenzoic acid	0.58	0.64	0.127	1.05
3	Vanillic acid	0.67	0.74	0.153	1.15
4	4-Hydroxybenzaldehyde	0.80	0.87	0.340	1.08
5	Vanillin	0.91	0.00	3.094	0.89
6	Guaiacol				
7	Ethyl vanillin				
8	Coumarin				
9	Eugenol				

Figure 4.HPLC-UV Analysis of the Matrix Standard Solution Natural (Vanilla Extract, Natural, Cat.No. **06261501**)



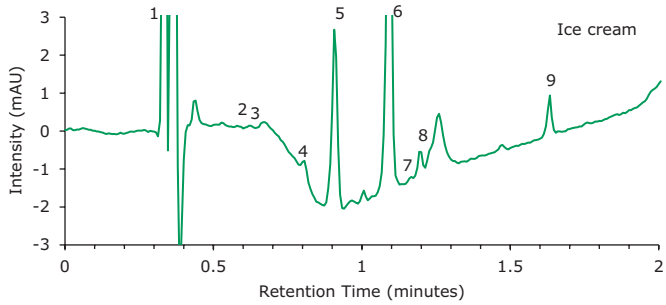
No.	Compound	Retention Time (min)	RRT	Area (mAU* min)	Tailing Factor
1	t_0 void volume	0.35			
2	4-Hydroxybenzoic acid	0.72	0.72	0.0317	1.13
3	Vanillic acid	0.84	0.84	0.0621	1.12
4	4-Hydroxybenzaldehyde	0.92	0.92	0.2579	1.03
5	Vanillin	1.00	0.00	1.5233	1.16
6	Guaiacol				
7	Ethyl vanillin				
8	Coumarin				
9	Eugenol				

Figure 6. HPLC-UV Analysis of the Matrix Standard Solution Synthetic (Vanilla Extract, Synthetic, Cat.No. **06271501**)



No.	Compound	Retention Time (min)	RRT	Area (mAU* min)	Tailing Factor
1	t_0 void volume	0.37			
2	4-Hydroxybenzoic acid				
3	Vanillic acid				
4	4-Hydroxybenzaldehyde				
5	Vanillin	1.00	0.00	2.2404	1.16
6	Guaiacol				
7	Ethyl vanillin	1.19	1.19	0.7156	1.22
8	Coumarin	1.24	1.24	0.9187	1.33
9	Eugenol	1.66	1.66	0.0216	1.27

Figure 7. HPLC-UV Analysis of an Ice Cream Sample



No.	Compound	Retention Time (min)	RRT	Area (mAU* min)	Tailing Factor
1	t_0 void volume	0.37			
2	4-Hydroxybenzoic acid	0.58	0.64	0.001	1.29
3	Vanillic acid	0.67	0.74	0.004	1.22
4	4-Hydroxybenzaldehyde	0.81	0.89	0.008	1.06
5	Vanillin	0.91	0.00	0.099	0.96
6	Guaiacol	1.09	1.20	0.244	0.85
7	Ethyl vanillin	1.14	1.25	0.001	
8	Coumarin	1.19	1.31	0.009	1.25
9	Eugenol	1.63	1.79	0.008	0.88

Conclusion

The examples shown demonstrate the applicability and value of matrix reference materials to help detect food adulterations and mislabeling.

Reference:

1. Cicchetti, Chaintreau J. Sep. Sci. 2009, 32, 3043 – 3052.

Featured Products

Description	Cat. No.
Vanilla extract reference materials (natural and synthetic)	
Vanilla extract, natural, secondary reference standard, 1 mL	**06261501**
Vanilla extract, synthetic, secondary reference standard, 1 mL	**06271501**
Vanilla extract set, natural and synthetic secondary reference standard, 2x1 mL	**06281501**
Reference Materials for ingredients of natural vanilla and markers for synthetic vanilla	
Coumarin, Certified Reference Material*, 100 mg	**72609**
Ethyl vanillin, Certified Reference Material*, 100 mg	**75042**
Eugenol, Certified Reference Material*, 100 mg	**79891**
Guaiacol, Certified Reference Material*, 1.5 g	**PHR1136**
4-Hydroxybenzaldehyde, Analytical Standard, 250 mg	**91554**
4-Hydroxybenzoic acid, Certified Reference Material*, 50 mg	**92596**
Vanillic acid, Certified Reference Material*, 50 mg	**68654**
Vanillin, Certified Reference Material*, 50 mg	**30304**
Sample Prep, HPLC Column, Solvents & Reagents	
Chromolith® Performance RP-18 endcapped 100-2 (100x2 mm)	**1.52006**
Millex® syringe filter units, disposable, Durapore® PVDF, pore size 0.45 µm, non-sterile, Pk.1000	**SLHVX13NK**
Ethanol gradient grade for liquid chromatography LiChrosolv®	**1.11727**
Acetonitrile gradient grade for liquid chromatography LiChrosolv®	**1.00030**
Water for chromatography (LC-MS grade) LiChrosolv®	**1.15333**
Trifluoracetic acid for spectroscopy Uvasol®	**1.08262**

* *Trace*CERT®

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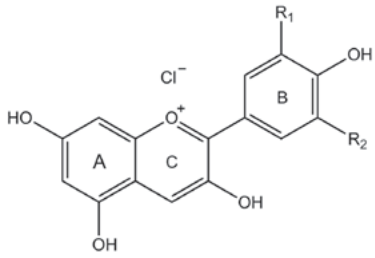
Reference Materials for Accurate Quantification of Anthocyanins & Anthocyanidins

New products from PhytoLab now available

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

Figure 1. Chemical Structure of Anthocyanidins



Anthocyanidin	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3
Pelargonidin	H	H
Peonidin	OCH_3	H
Petunidin	OH	OCH_3

In a series of articles, we have highlighted specific product groups of phytochemical standards from PhytoLab. These products represent the extensive portfolio of more than 1400 extensively documented herbal reference substances of all classes of natural compounds. The initial article in Analytix Reporter, Issue 5 dealt with Pyrrolizidine alkaloids and Issue 6 highlighted Glucosinolates. We will now focus on the product group of Anthocyanins & Anthocyanidins.

Anthocyanins are water-soluble secondary plant metabolites that can occur in all parts of higher plants, including leaves, stems, roots, flowers and fruits.

They are responsible for making bright-colored flowers and fruits attractive to pollinators or animals. Anthocyanins also act as a “sunscreen” and protect cells from damage due to exposure to UV-light. They may also act as antioxidants in the cell vacuoles.

Plants rich in anthocyanins include blueberries, cranberries, raspberries, blackberries, strawberries, cherries and grapes, among many other species.

Anthocyanins belong to the class of natural compounds known as flavonoids. Their 15-carbon skeleton consists of two phenyl rings and one heterocyclic ring containing a positively charged oxygen atom. In nature, usually carboxylate anions of water-soluble acids would act as counter ions, while the pure compounds are most frequently isolated as chloride salts. The most common anthocyanins exhibit hydroxyl functions in positions 3, 5, 7 and 4’. The anthocyanidins are the aglycones of the anthocyanins, which most often bear a sugar moiety bound to position 3. Structural variation is usually achieved by the substitution pattern in the B-ring and differences in the glycosidic profile as shown in **Figure 1**.

In the European Pharmacopoeia, a specification for total content of anthocyanins, calculated as cyanidin 3-glucoside, is given in the monographs for fresh bilberry fruit and fresh bilberry fruit dry extract, refined and standardized. The latter monograph also specifies a maximum limit for anthocyanidins, calculated as cyanidin, describes a certain chromatographic profile of 15 anthocyanins and 5 anthocyanidins to confirm identity. A minimum content of procyanidins, expressed as cyanidin, is given in the monograph on hawthorn berries.

In the United States Pharmacopoeia, the dietary supplements monographs on powdered bilberry extract and European elder berry extract specify a minimum content of anthocyanins, calculated as cyanidin 3-glucoside, and a maximum limit for anthocyanidins, calculated as cyanidin. Requirements on chromatographic profiles, including peak intensities of various anthocyanins, are given.

For a reliable quantitative analysis of anthocyanins & anthocyanidins, well characterized reference substances are essential.

Due to the positive charge of the molecule, the counter ion has to be taken into account. For all anthocyanins and anthocyanidins characterized as primary reference substances, chloride was determined quantitatively

and considered as an impurity in the calculation of the absolute content, which therefore refers to the pure anthocyanin or anthocyanidin only. Another very useful feature of the phyproof® standards is that the exact weight of each package is printed on the label of the product vial, which offers the convenience of dissolving the analyte directly in the vial.

Available Anthocyanins & Anthocyanidins Reference Materials

Description	Package Size	Cat. No.
Cyanidin chloride	20 mg	**PHL80022**
Cyanidin 3-arabinoside	10 mg	**PHL89614**
Cyanidin 3,5-diglucoside	10 mg	**PHL89615**
Cyanidin 3-galactoside	10 mg	**PHL89463**
Cyanidin 3-glucoside	10 mg	**PHL89616**
Cyanidin-3-(6''-malonylglucosid)	5 mg	**PHL85728***
Cyanidin 3-rutinoside	10 mg	**PHL80577**
Cyanidin 3-sambubioside	5 mg	**PHL89617**
Cyanidin 3-sophoroside	5 mg	**PHL80579**
Delphinidin chloride	10 mg	**PHL89625**
Delphinidin 3,5-diglucoside	5 mg	**PHL89626**
Delphinidin 3-galactoside	5 mg	**PHL89506**
Delphinidin 3-glucoside	10 mg	**PHL89627**
Delphinidin 3-rutinoside	10 mg	**PHL80735**
Delphinidin 3-sambubioside	5 mg	**PHL82249**
Malvidin chloride	10 mg	**PHL80083**

Description	Package Size	Cat. No.
Malvidin 3,5-diglucoside	10 mg	**PHL89727**
Malvidin 3-galactoside	10 mg	**PHL80600**
Malvidin 3-glucoside	10 mg	**PHL89728**
Pelargonidin chloride	10 mg	**PHL80084**
Pelargonidin 3,5-diglucoside	10 mg	**PHL80334**
Pelargonidin 3-glucoside	10 mg	**PHL89753**
Peonidin chloride	5 mg	**PHL80085**
Peonidin 3,5-diglucoside	10 mg	**PHL80335**
Peonidin 3-glucoside	5 mg	**PHL89754**
Petunidin chloride	5 mg	**PHL80225***
Petunidin 3-glucoside	5 mg	**PHL89755**

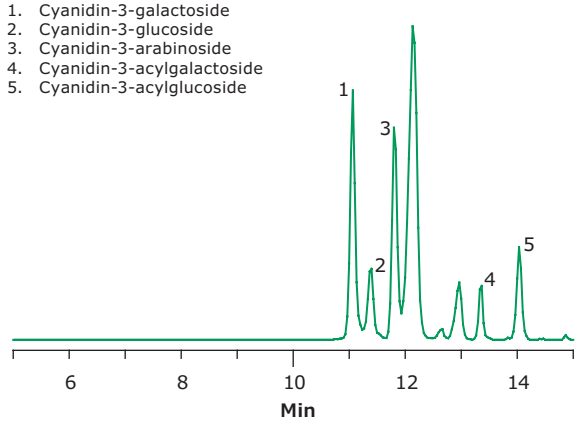
* = coming soon

All the phytochemical standards from PhytoLab can be found at **SigmaAldrich.com/phytolab**

Discover our complete range of phytochemical standards at **SigmaAldrich.com/medicinalplants**

LC/MS (TOF) Analysis of Cyanidin Glycosides from High Bush Blueberries

1. Cyanidin-3-galactoside
2. Cyanidin-3-glucoside
3. Cyanidin-3-arabinoside
4. Cyanidin-3-acylgalactoside
5. Cyanidin-3-acylglucoside



Analytical Conditions	
column:	Ascentis® Express C18, 10 cm x 2.1 mm I.D., 2.7 µm particles (**53823-U**)
mobile phase:	[A] 0.1% (v/v) formic acid in water; [B] 0.1% (v/v) formic acid in 75:25 (v/v) acetonitrile:water
gradient:	2% B for 2 min, to 100% B in 38 min
flow rate:	0.2 mL/min
column temp.:	35 °C
injection:	1 µL
detector:	ESI(+) TOF, extracted ions m/z 449.1100, 419.0979, 491.1213
sample preparation:	berries (1.0 g) were added to 1.0 mL of 1% (v/v) formic acid in methanol. Samples were crushed in the solvent mixture and extracted (refrigerated) for 2 hours. A portion of the extracted sample was removed, centrifuged and the supernatant was collected for HPLC analysis.

To see more applications for Anthocyanins, visit us at **SigmaAldrich.com** search for **"Anthocyanins"** and see the **"Site Content"**

Supelco®
Analytical Products

New Carotenoid Standards

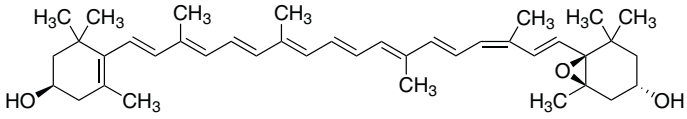
Matthias Nold, Product Manager Reference Materials, Analytix@merckgroup.com

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

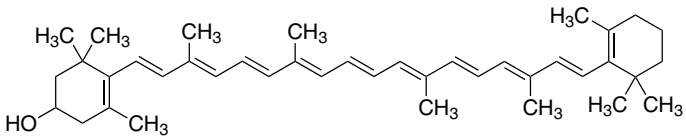
Our portfolio of analytical standards comprises of more than 25 highly purified carotenoid standards. The most recent additions are listed below.

New Carotenoid Standards

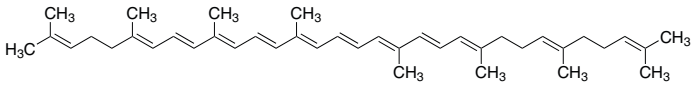
Description	Package Size	Cat. No.
9-cis-Antheraxanthin	1 mg	**47999**
(+/-)-beta-Cryptoxanthin	1 mg	**51772**
all-trans-Neurosporene	1 mg	**59739**



47999 9-cis-Antheraxanthin



51772 (+/-)-beta-Cryptoxanthin



59739 all-trans-Neurosporene

To see our complete portfolio of carotenoid standards, visit us at **SigmaAldrich.com/carotenoids**

New Furocoumarin and other Phytochemical Standards

Addition of new analytical standards of furocoumarins and other phytochemical standards to the portfolio of reference materials of plant constituents

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

We offer a comprehensive range of more than 2000 reference materials of plant constituents used in the quality control of herbal medicinal products and dietary supplements. And the portfolio keeps getting updated with new products. The following table shows the list of most recent product additions.

In the list you will find Cnidicin and Cnidilin, two newly added furocoumarin standards. Furocoumarins are a class of organic compounds that undergo activation by UV light and can form potentially harmful intermediates.[1] As a result, these compounds are regulated in cosmetic products.[2] In Analytix Reporter issue 11 we presented a new certified reference material (CRM) mix with 16 compounds (cat. no. **93102**) to test for furocoumarins. Now we extend our range with furocoumarin neat standards, complementing the furocoumarin portfolio beyond components of the mix. In the following list you will also find new product additions from other phytochemical substance classes.

Compound	Qty.	Cat. No.
Cnidicin	5 mg	**50014**
Cnidilin	5 mg	**44139**
Coniferyl alcohol	10 mg	**41402**
2,3-Dihydroxybenzoic acid	100 mg	**41398**
7-Ethoxycoumarin	10 mg	**41577**
Flavanone	100 mg	**41226**
Flavone	100 mg	**40862**
Gardenin A	5 mg	**49849**
5-Geranyloxy-7-methoxycoumarin	5 mg	**52006**
4-Hydroxycoumarin	100 mg	**40863**
7-Hydroxyflavone	50 mg	**41934**
2-Hydroxy-1,4-naphthoquinone	100 mg	**40911**
Meranzin	5 mg	**42460**
Meranzin hydrate	5 mg	**42230**
Phloroglucinol dihydrate	100 mg	**40846**
Tetra-O-methylscutellarein	5 mg	**43075**

Our entire offering of phytochemical reference materials, including standards and CRMs in neat and solution form, and the reference materials of plant extracts can be found on our website **SigmaAldrich.com/Medicinalplants**

References

1. Melough MM, Chun OK. Dietary furocoumarins and skin cancer: A review of current biological evidence. Food Chem Toxicol. 2018 Dec;122:163-171. doi: 10.1016/j.fct.2018.10.027

2. Regulation (EC) No 1223/2009 http://data.europa.eu/eli/reg/2009/1223/oj

50 years of TLC-MS

Thin-Layer Chromatography coupled to Mass Spectrometry and new perspectives by complementary use to HPLC as demonstrated in testing of honey

Michael Schulz, Head of Instrumental Analytics R&D | Michaela Oberle, Scientist, Instrumental Analytics R&D
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Introduction

Thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) are known to be convenient, fast and efficient separation techniques enabling analytical methods without the need for complicated sample preparation or high investments. Low cost and short analysis time per sample is given by parallel analysis of many samples on one plate. The high matrix tolerance of TLC offers additional opportunities to existing routine methods, such as cross-checking of HPLC results or complementary method development.

Various different detection approaches such as analyte visualization by application of derivatization reagents or coupling to other methods like UV detection can be used in combination with TLC. In 1969, Prof. R.E. Kaiser has reported the coupling of TLC with mass spectrometry (MS) for the first time.[1] TLC spots were heated and desorbed into a gas stream in front of the inlet of a mass spectrometer. Numerous publications have demonstrated convincing results and contribute strongly to the progress of TLC, today and in the future.[2]

High-performance liquid chromatography (HPLC) is an established analytical technique for quick and highly efficient analyses of a broad range of complex samples. In contrast to TLC, HPLC can suffer from matrix rich samples causing problems such as increased backpressure or column clogging by accumulation of matrix compounds at the column inlet. In addition, the detection of ghost peaks is possible during repeated sample injections under unsuitable gradient conditions.

The joint use of TLC and HPLC is an option to combine the best out of two chromatographic worlds: High matrix tolerance of TLC makes sample preparation facile or even obsolete and HPLC provides excellent peak capacity for the efficient separation of overlapping TLC bands and increases sensitivity, compared to TLC-MS, by band focusing. Combining 2 different phase selectivities can make the TLC-HPLC-MS hyphenation a true 2D-LC method.

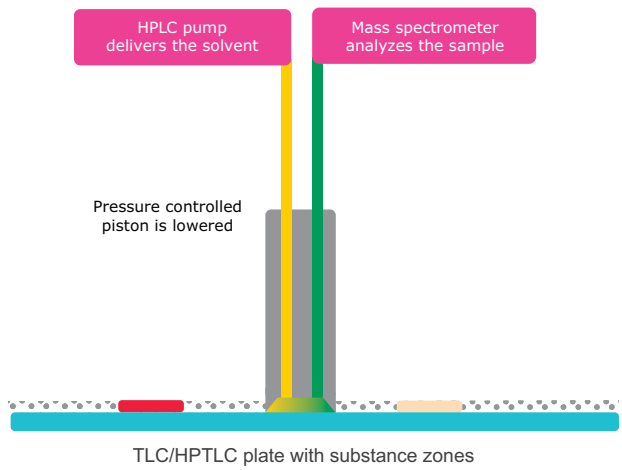
In this article we describe the coupling of thin layer chromatography to mass spectrometry (TLC-MS) and the combination of TLC-MS with high performance liquid chromatography (TLC-HPLC-MS) using as an example the detection of neonicotinoid pesticides in honey.

TLC-MS coupling techniques

The techniques for coupling TLC directly with mass spectrometry can be divided into elution- and desorption-based techniques.[2]

The elution-based approach utilizes a TLC-MS interface that enables the dissolution of the analyte from the silica plate by a solvent and transfer to the mass spectrometer in the liquid phase (see **Figure 1**).

Figure 1. Schematic working principle of elution-based TLC-MS.



Desorption-based techniques make use of vaporization of the analyte from the TLC surface and transfer to the MS in the gas phase. Vaporization techniques include, gas beam, ion bombardment and MALDI (matrix assisted laser desorption/ionisation) or DART (direct analysis in real time).

Both approaches work offline, and both are performed after a TLC separation is finished and the plate is dried. The sample transfer to the MS is fast and typically takes less than a minute.

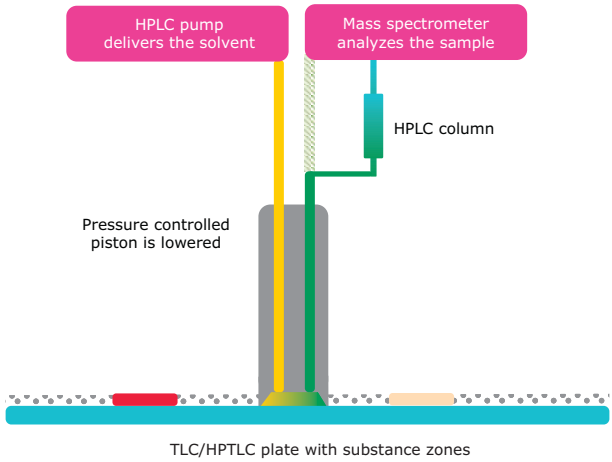
Features and benefits of TLC-MS

- Sample preparation mainly takes place on the TLC plate
- Direct MS analysis of spots or bands of interest – rapid results
- Chromatography is performed separately from MS infusion - high flexibility in choosing mobile phases
- MS-grade plates allow for high resolution separations combined with high sensitivity and reliability in MS detection

Combining TLC-MS and HPLC-MS

A flexible instrument setup allows for direct elution-based TLC-MS and TLC-HPLC-MS measurements (see **Figure 2**). A schematic overview over the entire workflows is displayed in **Figure 3**. A spot can be eluted from the plate and transferred to a HPLC column for detailed analysis. Here the TLC can act either as sample preparation or as the first dimension of 2D-LC.

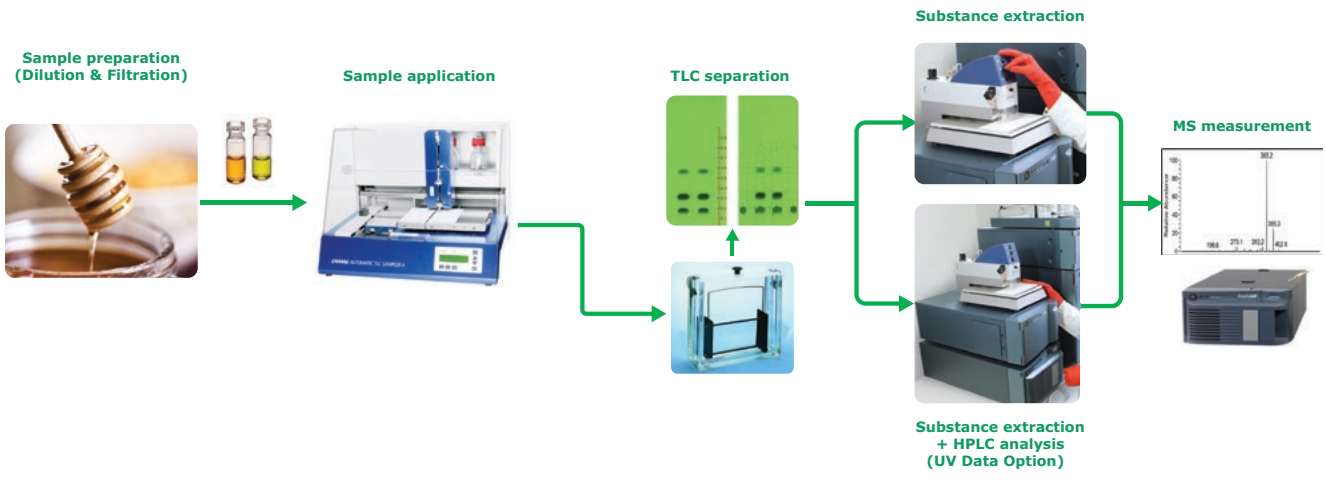
Figure 2. Schematic setup of TLC-HPLC-MS.



Features and benefits of TLC-HPLC-MS

- High matrix tolerance of TLC allows for analyses without complex sample preparation
- Screening and method development capabilities by parallel sample application on one TLC plate and by the option to apply a high variety of staining reagents for visual spot determination during the method development
- Bands overlapping (not resolved compounds) on the TLC plate can be separated by the high separation power of HPLC
- Increased sensitivity by TLC-HPLC-MS compared to TLC-MS

Figure 3. Overview of TLC-MS and TLC-HPLC-MS workflows including instruments and consumables.



Neonicotinoids in Honey

The highly effective group of neonicotinoid pesticides is under discussion regarding negative effects on bee health. (EU) No. 485/2013 prohibits the use and sale of seeds treated with plant protection products containing the neonicotionoids clothianidin, imidacloprid and thiamethoxam. In April 2018 the EU banned these compounds on all outdoor uses (EU) 2018/783-785. European Union maximum residue levels (MRLs) of neonicotionoids authorized in food and feed products are 50 ng/g for acetamiprid, imidacloprid and thiacloprid and 10 ng/g for clothianidin and thiamethoxam.[3]

Honey is a product of natural origin and it is one of the most frequently tested food products. Because of its high viscosity and high sugar content, honey represents a very complex matrix.

Experimental

All TLC analyses were performed utilizing HPTLC Silica gel 60 F_{254} MS-grade plates.

Neonicotinoid standard solutions (NSS) 1 and 2 were prepared by dissolving 0.2 mg/mL and 1 ng/mL, respectively, of each of the seven pesticides nitenpyram, dinotefuran, thiamethoxam, clothianidin, imidacloprid, acetamiprid and thiacloprid in acetone.

Sample preparation was done by diluting 1 g honey in 10 mL water/acetone 1/1 (v/v). The samples were applied bandwise (2.5 mm band width) using a CAMAG ATS4.

The thin layer chromatogram development was performed in two steps, using acetonitrile and acetonitrile/methanol 3/1 (v/v) as mobile phases. The development time was 1 and 3 minutes. **Table 2** displays an overview over all applied tracks and obtained hR_f values for the analytes.

Table 1. HPLC conditions

HPLC Column	Purospher® STAR RP-18 endcapped (2µm) Hibar® HR 100-2.1 (Cat.No. 1.50648)
Mobile phases	A) Water w/ 0.1 % formic acid
	B) Acetonitrile w/ 0.1% formic acid.
Gradient	100 to 90 % A in 3 min,
	90 to 70 % A in 2 min,
	70 to 60 % A in 7 min,
	60 to 100 % A in 0.4 min,
	100 % A for 3.2 min
Flow Rate	0.25 mL/min
Column Temp	Room temperature
MS mode	ESI (+)
TLC spot elution	100 % water, flow rate 0.25 mL/min

TLC-MS and TLC-HPLC-MS experiments were performed by an elution-based approach, using the CAMAG TLC-MS Interface 2 combined with a Waters Acquity® UPLC H-Class Bio System with an ACQUITY® QDa detector.

Results and Discussion

Analysis of neonicotinoids in honey

In total, 33 tracks of five different samples were applied onto the TLC plate:

a) NSS 1 with a pesticide concentration of 0.2 mg/mL of each neonicotinoid

b) NSS 2 with a pesticide concentration of 1 ng/mL of each neonicotinoid

c) honey sample spiked with 1 mg/g of each neonicotinoid

d) honey sample spiked with 10 ng/g of each neonicotinoid

e) unspiked honey sample

Table 2 displays an overview over all applied tracks and obtained hR_f values for the analytes.

Figure 4A shows the developed TLC plate under irradiation with UV light (254 nm). The neonicotinoids in spiked honey samples are visible at hR_f = 70 (nitenpyram) and hR_f = 93 (dinotefuran, thiamethoxam, clothianidin, imidacloprid, acetamiprid, thiacloprid). In order to visualize the high matrix load, the plate was stained with anisaldehyde sulfuric acid reagent (**Figure 4B**). The long, dark smearing zone can be attributed to the high sugar content of the sample. In addition, ninhydrin staining was applied in order to visualize compounds bearing aminofunctions (**Figure 4C**).

After TLC development, elution-based TLC-MS was used to elute the zone at hR_f = 70 and identify it as nitenpyram by subsequent single-quad MS detection. (**Figure 6A**).

The zone at hR_f = 93, resulting from the TLC separation of spiked honey samples and consisting out of six analytes, was eluted from the plate onto the HPLC column. Chromatograms were obtained using UV detection for the two spiked and one unspiked honey sample (**Figure 5** and **Table 3**). In addition, MS detection was utilized to identify the six neonicotinoids (see spectra in **Figure 6 B–G**).

The TLC-HPLC-MS setup was capable of detecting all neonicotinoids in the honey sample spiked at a level of 10 ng/g. As reproducibly (multiple TLC tracks) demonstrated by means of MS, the unspiked honey contained acetamiprid and thiacloprid at levels below the EU limits (MRLs) of 50 ng/g.

For precise quantification by this approach further studies are needed.

Table 2. TLC data: Track numbers with applied samples and volumes and obtained hR_f values.

Track	Substance	Application volume [µL]	hR_f
1, 12, 23	Neonicotinoid standard solution 1 – each 0.2 mg/mL	0.5 µL	70: Nitenpyram
3, 14, 25	Neonicotinoid standard solution 2 – each 1 ng/mL	0.8 µL	
4, 15, 26		1.0 µL	
5, 16 ,27		1.2 µL	
2, 13, 24	Honey sample – spiked with 1 mg/g of each neonicotinoid	1.0 µL	93: Dinotefuran, Thiamethoxam, Clothianidin, Imidacloprid, Acetamiprid, Thiacloprid
9, 20, 31	Honey sample – spiked with 10 ng/g of each neonicotinoid	1.0 µL	
10, 21, 32			
11, 22, 33			
6, 17, 28	Honey sample – without spiking	1.0 µL	
7, 18, 29			
8, 19, 30			

Figure 4. A) Visualization of the neonicotinoids under UV light (254 nm). B) Visualization of matrix compounds after staining with anisaldehyde sulfuric acid (white light). C) Visualization of amino group containing matrix compounds by ninhydrin staining (white light).

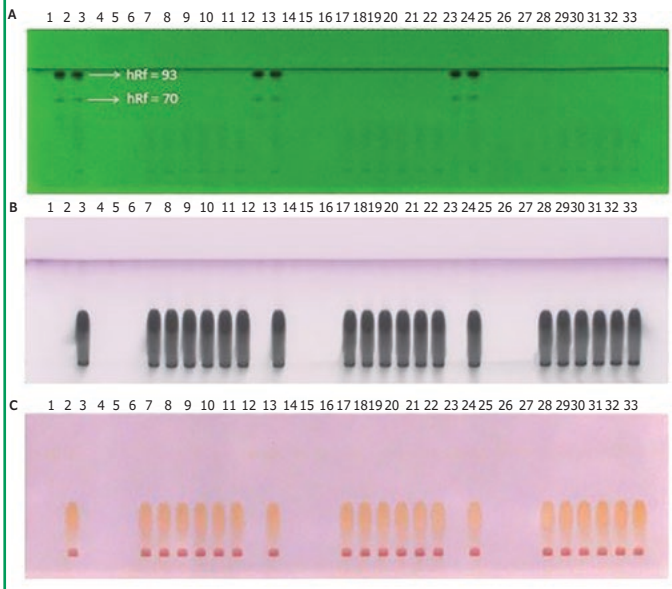


Table 3. HPLC retention times of neonicotionoids in spiked honey samples after spot elution and HPLC analysis of the TLC band at hR_f = 93.

Peak	Substance	Retention time [min]
1	Dinotefuran	6.6
2	Thiamethoxam	7.6
3	Clothianidin	8.1
4	Imadacloprid	8.4
5	Acetamiprid	8.8
6	Thiacloprid	10.1

Figure 5. HPLC chromatograms of spiked and unspiked honey samples after spot elution and HPLC analysis of the TLC band at hR_f = 93. A: Honey sample spiked with 1 mg/g. B: Honey sample spiked with 10 ng/g, C: unspiked honey sample. Peak IDs:1: Dinotefuran, 2: thiamethoxam, 3: clothianidin, 4: imidacloprid, 5: acetamiprid, 6: thiacloprid.

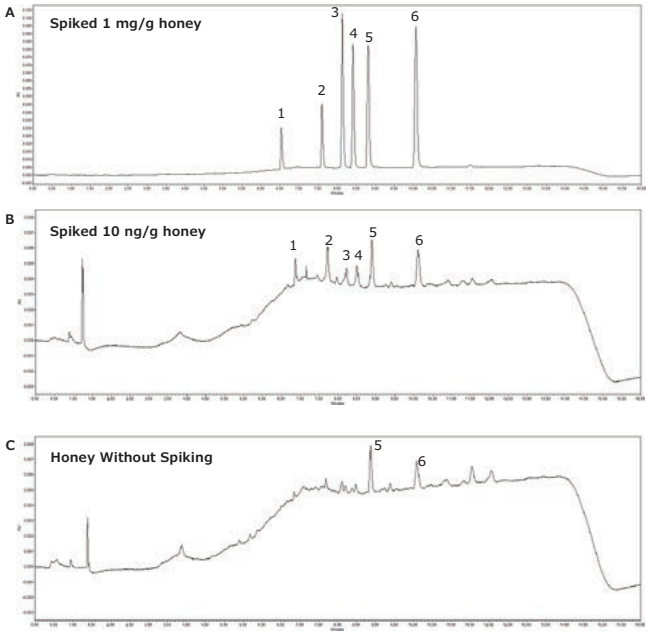
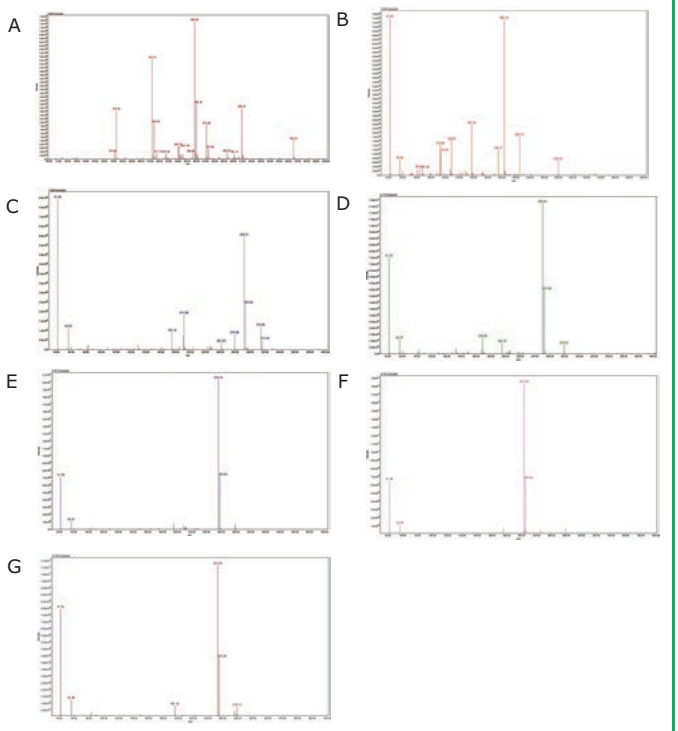


Figure 6. Analysis of honey spiked with pesticides. Mass spectra of seven neonicotinoids obtained by analysis of the TLC band at hR_f = 70 using TLC-MS (A: nitenpyram) and by analysis of the TLC band at hR_f = 93 using TLC-HPLC-MS (B: dinotefuran; C: thiamethoxam; D: clothianidin; E: imidacloprid; F: acetamiprid; G: thiacloprid).



Conclusion

An example for the analysis of different analytes in a complex and challenging food matrix was described by using TLC-MS and TLC-HPLC-MS as attractive and flexible methods. Target analytes can easily be separated and detected without time-consuming and labor-intensive sample preparation.

The flexible instrument setup enables the combination of elution-based TLC-MS and TLC-HPLC-MS measurements as complementary chromatographic methods in one setup. The applicability of this combination was demonstrated by means of the analysis of 7 neonicotinoid pesticides. Spiked and unspiked honey samples were analyzed. In the unspiked honey sample acetamiprid and thiacloprid were found at levels below the EU limit (MRLs) of 50 ng/g.

Screening and method development capabilities were shown by the application of 33 tracks (21 honey samples and 11 standard solutions). The high matrix load of the honey samples was visualized by staining with anisaldehyde sulfuric acid and the opportunity to obtain additional selective information was demonstrated by ninhydrin staining for amino group containing compounds.

The versatile TLC-HPLC-MS setup with its TLC strengths of high matrix tolerance, high sample capacity and derivatization flexibility in combination with the high separation power of HPLC enables new approaches especially for the analysis of matrix rich and complex samples.

Featured Products

Description	Cat. No.
HPTLC Silica gel 60 F_{254} MS-grade, 20 x 10 cm	**1.00934**
Purospher® STAR RP-18 encapped (2 µm) Hibar® HR 100-2.1, 100 x 2.1 mm	**1.50648**
Millex® Syringe Filter, Fluoropore™ PTFE, Hydrophobic, Non-sterile, 0.45 µm pore size, 25 mm diameter	**SLFH025**
Solvents & Reagents	
Methanol gradient grade for liquid chromatography LiChrosolv®	**1.06007**
Acetonitril gradient grade for liquid chromatography LiChrosolv®	**1.00030**
Water for chromatography (LC-MS Grade) LiChrosolv®	**1.15333**
Acetonitrile hypergrade for LC-MS LiChrosolv®	**1.00029**
Formic acid 98 % - 100 % for LC-MS LiChropur®	**5.33002**
Reference Materials	
Nitenpyram PESTANAL®, 100 mg	**46077**
Dinotefuran PESTANAL®, 100 mg	**32499**
Thiamethoxam PESTANAL®, 100 mg	**37924**
Clothianidin PESTANAL®, 100 mg	**33589**
Imadacloprid PESTANAL®, 100 mg	**37894**
Acetamiprid PESTANAL®, 100 mg	**33674**
Thiacloprid PESTANAL®, 100 mg	**37905**

Related Products

Description	Cat. No.
HPTLC Silica gel 60 F_{254} MS-grade, 20 x 10 cm glass plates	**1.00934**
HPTLC RP-18 F_{254}s MS-grade, 20 x 10 cm glass plates	**1.15161**
HPTLC Silica gel 60 F_{254} MS-grade for MALDI, 5 x 7.5 cm aluminum foils	**1.51160**
TLC Silica gel 60 F_{254} MS-grade, 20 x 10 cm glass plates	**1.00933**
TLC Silica gel 60 F_{254} MS-grade, 5 x 7.5 cm aluminum foils	**1.51022**
TLC RP-18 F_{254}s MS-grade, 5 x 7.5 cm aluminum foils	**1.51015**

To find more information on TLC and HPLC visit
SigmaAldrich.com/TLC
SigmaAldrich.com/HPLC

For a comprehensive overview on our standards visit
SigmaAldrich.com/standards

References:

1. Chem. Br. 5 (1969) 54. R. Kaiser
2. Trends Anal. Chem. Vol. 29 (2010), Issue 10, 1157-1171. G. Morlock, W.Schwack
3. Science Vol. 358 (2017), Issue 6359, 109-111. E.A.D. Mitchell, B. Mulhauser, M. Mulot, A. Mutabazi, G. Glauser, A. Aebi

Separation of Steviol Glycosides by HPTLC

Introducing a new Stevia Extract Reference Material

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HPTLC (High-Performance Thin-Layer Chromatography) is a fast and efficient tool to create molecular fingerprints of complex chemical mixtures. Therefore it is particularly well suited for the analysis of plants and plant derived products. In a series of articles in Analytix and Analytix Reporter journals,[1-4] we showed several examples of HPTLC of plants used as dietary supplements or as herbal medicinal products such as Ginkgo, Ginseng or St. John's Wort.[5] That series is continued here with a fingerprint method for *Stevia rebaudiana* leaves, including the analysis of our new stevia extract reference material.

Extracts from the leaves of the *Stevia rebaudiana* plant have a long tradition of being used as a sweetener. Native tribes of Latin America have known and consumed it for centuries because of the sweet taste. Stevia extracts have approximately 300 times more intense sweetness than sucrose while only having a negligible effect on blood glucose. Therefore, in recent years, the plant has increasingly been used in other parts of the world as an alternative to artificial sweeteners.

Stevia is FDA approved as a dietary supplement and rebaudioside A is considered to be "Generally Recognized As Safe (GRAS)". The European Community has allowed the use of steviol glycosides as food additives since December 2011.

The WHO defined the acceptable daily intake of steviol glycosides at 4 mg per kg body weight.[6]

We recently launched a new extract reference material, developed and manufactured by HWI pharma services GmbH in Rülzheim, Germany:

Description	Quantified Components	Qualitatively Confirmed Components	Package Size	Cat.No.
Stevia extract	Stevioside	Rebaudiosides A, B, C and D, Dulcoside A, Rubusoside, Steviolbioside, Stevioside	500 mg	**06295001**

This new product complements our range of plant extract reference materials designed for use as a rapid identification and quantification method for typical constituents of plants used as food additives or as herbal medicinal products (see the complete offer at **SigmaAldrich.com/plantextracts**).

The Stevia extract reference material is provided with comprehensive documentation including a quantitative value for the major component Stevioside as well as qualitative conformation of various other constituents (Rebaudiosides A, B, C and D, Dulcoside A, Rubusoside, Steviolbioside, Stevioside). In addition to an HPLC method including a chromatogram with assigned peak identities, the documentation also contains an HPTLC method according to [5].

For the analysis, Supelco® HPTLC plates and reagents have been used. The analytical standards of the pure steviol glycosides are listed below. Please find a comprehensive listing of our entire phytochemical standards range at **SigmaAldrich.com/medicinalplants**.

HPTLC method

The scope of the method is the identification of a *Stevia rebaudiana* leaf dry extract reference material based on HPTLC fingerprints of steviol glycosides obtained with the HPTLC method by Wald and Morlock 2017[5] by comparison with the fingerprint of Stevia leaf. Additionally, chemical reference substances were used for identification of the zones of the chromatogram.

Instrumentation

Automatic TLC Sampler (ATS 4), Automatic Developing Chamber (ADC 2), Chromatogram Immersion Device 3, TLC Plate Heater 3, TLC Visualizer, *visionCATS* (the software offers a Method Library that includes an SOP for each method, an instrument method, and a comparison file with reference images).

Samples

Extract: 50 mg were suspended in 50 mL of methanol and sonicated for 10 min. The suspension was centrifuged, and the supernatant used.

Leaf: 0.5 g of powdered leaf was suspended in 30 mL of water and boiled for 10 min. The solution was filtered into a 50 mL volumetric flask and the volume was made up with water.

Standards

Standard solutions were prepared in a concentration of 0.3 mg/mL in methanol. (Note: This is 9.09–fold more concentrated than in [5])

Chromatography according to USP <203>

Stationary phase	HPTLC Si 60 F_{254}, 20 x 10 cm (**1.05642**)
Sample application	Application with ATS 4, 10 tracks, band length 8 mm, track distance 11.4 mm, distance from left edge 20 mm, distance from lower edge 8 mm, application volume 2 µL for test solutions and standards
Developing solvent	Ethyl acetate, methanol and formic acid 93:40:1 (*v/v/v*)
Development	In the ADC 2 without chamber saturation and after conditioning at 33% relative humidity for 10 min using a saturated solution of magnesium chloride.
Developing distance	70 mm (from the lower edge)
Plate drying	5 min in the ADC 2
Documentation	With the TLC Visualizer under UV 366 nm and white light after derivatization.
Derivatization	Reagent name: 2-Naphthol
Reagent preparation (dipping):	2 g of 2-naphthol in 180 mL of ethanol and 12 mL of 50% sulfuric acid.
Reagent use:	The plate was immersed into 200 mL of 2-naphthol reagent using the Chromatogram Immersion Device (immersion time 0 s and immersion speed 3 cm/s) and then heated at 120°C for 5 minutes.

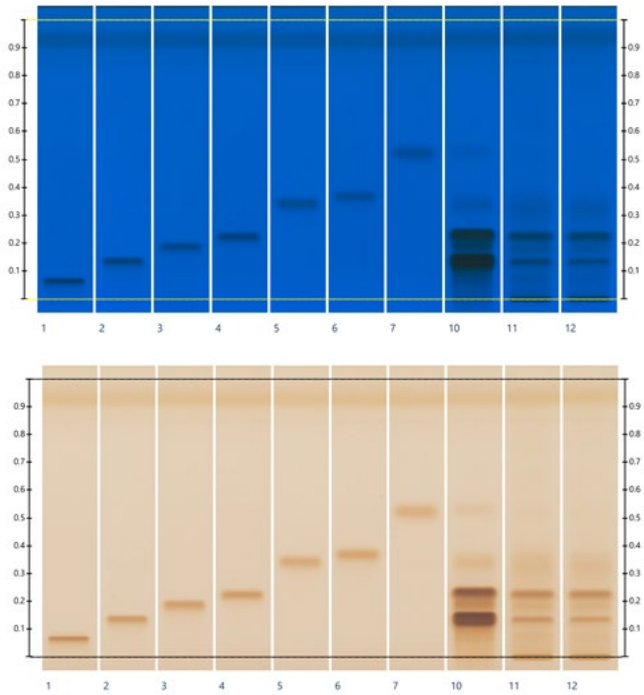
Results

The derivatized plates were viewed under UV light at 366 nm and white light (**Figure 1**.) In the fingerprint of the HWI extract (track 8), zones corresponding in color and position to those of the standards Rebaudioside A, C, Stevioside, Rebaudioside B and Dulcoside A (which are co-eluting), and Steviolbioside are seen. The fingerprint is similar to those of *S. rebaudiana* leaf (tracks 9 and 10). Rebaudioside D is only seen in the fingerprint of the leaf, particularly under UV 366 nm (very faint zone).

References:

1. Analytix 5 (2016): HPTLC Fingerprint Applications for Ginkgo Biloba
2. Analytix 1 (2017): Fingerprint Applications for *Hypericum perforatum*
3. Analytix Reporter 2 (2018): Fingerprinting of Medicinal Plants with TLC
4. Analytix Reporter 3 (2018): HPTLC Application for *Passiflora incarnata*
5. Wald JP, Morlock G. Quantification of steviol glycosides in food products, Stevia leaves and formulations by planar chromatography, including proof of absence for steviol and isosteviol. Journal of Chromatography A, 2017.
6. WHO Food Additives Series 54; 2006; page 117

Figure 1. HPTLC chromatograms after derivatization under UV 366 nm (top) and white light (bottom). **Track 1**: Rebaudioside D; **2:** Rebaudioside A; **3:** Rebaudioside C; **4:** Stevioside; **5:** Rebaudioside B; **6:** Dulcoside A; **7:** Steviolbioside; **8:** *Stevia rebaudiana* leaf dry extract reference material (HWI); **9:** *Stevia rebaudiana* leaf 1; **10:** *Stevia rebaudiana* leaf 2



Featured Products

Description	Package Size	Cat. No.
HPTLC Silica gel 60 F_{254}, 20 x 10 cm	50 ea	**1.05642**
Stevia Extract	500 mg	**06295001**
Analytical Standards for Stevia rebaudiana constituents		
Dulcoside A	10 mg	**90378**
Isosteviol	10 mg	**92273**
Rebaudioside A	10 mg	**38462**
Rebaudioside B	10 mg	**49747**
Rebaudioside C	10 mg	**30987**
Rebaudioside D	10 mg	**19189**
Rubusoside	10 mg	**62933**
Steviol	10 mg	**19345**
Steviolbioside	10 mg	**59754**
Stevioside	10 mg	**50956**

For a complete listing of your Stevia reference materials visit us at SigmaAldrich.com/stevia

Related Products

Description	Cat. No.
Solvents & Reagents	
Methanol gradient grade for liquid chromatography LiChrosolv®	**1.06007**
Ethyl acetate for liquid chromatography LiChrosolv®.	**1.00868**
Formic acid 98% - 100% for LC-MS LiChropur®	**5.33002**

For more information on our complete TLC offer, please see SigmaAldrich.com/TLC

Are You Made of Sugar?

Examples of Ion Chromatography applications for sugar testing in food and environmental analysis

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Daniel Weibel, Product Manager Trace Organic Analysis & Sensorics, Analytix@merckgroup.com

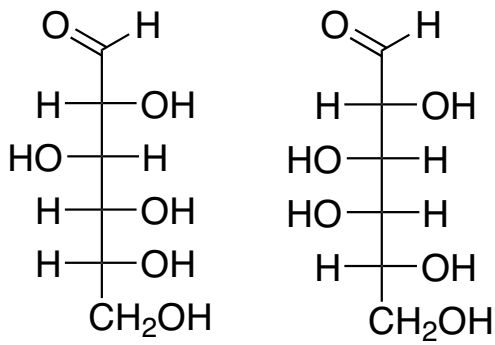
Carbohydrates constitute the biggest part of the biomass on Earth. They are produced by photosynthesis and are present in all plants and plant-based materials. The amount and composition of carbohydrates in a sample can reveal a wide range of information, depending on the context. As a result, they are subject to analysis in various industries.

In this article we demonstrate how ion chromatography is well-suited as an analytical technique for sugar analysis. All the eluents and standard solutions used for these applications are available from **SigmaAldrich.com/ic**

Carbohydrates are everywhere

In the food industry, carbohydrate and sugar content are notable for being key factors in determining the nutritional value of food and drink. In environmental analysis—to mention but one example—the anhydrosugar levoglucosan, which is produced by the pyrolysis of cellulose and acts as a tracer for biomass combustion, is determined in aerosols. These are just two of the many applications of carbohydrate analysis. Carbohydrates are composed of one or more monosaccharide units, each of which has a carbonyl group (aldehyde or ketone group) and several hydroxyl groups.[1] Because mono-, di-, and oligosaccharides are water-soluble, ion chromatography, which is performed in the aqueous phase, is particularly suitable for their analysis. It does not require extraction to the organic phase; thus, determination can be performed directly. However, a high-capacity column is necessary because sugars are relatively large molecules which are in many cases similar in structure (e.g., glucose and galactose; see **Figure 1**).

Figure 1. Structural formulae of glucose and galactose. The molecules differ only in the position of the OH group at the C4 atom (highlighted with an asterisk).



Sugars in Foods

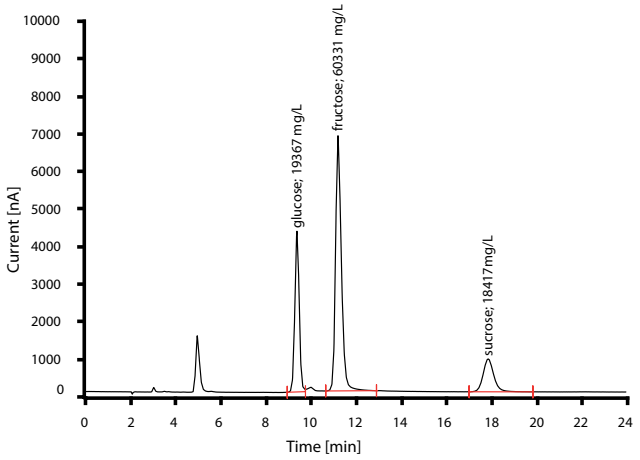
Since December 2016, the European Union (EU) requires that nutritional values are indicated on all foodstuffs, with the exception of unprocessed products and products sold loose (regulation no. 1924/2006). What is already established practice, i.e., indicating the calorific value and certain nutrients, including sugar and carbohydrates, is set to become mandatory.

Along with starch, which is a polymer of glucose, the usable carbohydrates found in foodstuffs are largely in the form of sugars. According to the EU definition, this includes all mono- and disaccharides with the exception of polyvalent alcohols. The majority of sugars in foodstuffs are made up of the monosaccharides glucose, fructose, galactose, and the disaccharides sucrose, lactose, and maltose.

Apple Juice Analysis

The chromatogram in **Figure 2** was taken after the injection of apple juice, which was diluted (1:1000) with ultra-pure water. Apart from that, no sample preparation is necessary. The alkaline eluent (100 mM sodium hydroxide/10 mM sodium acetate, Cat. No. **78348**) ensures that the sugars are present in dissociated form (as anions) and can therefore be separated in the column using the ion exchanger.

Figure 2. Determination of glucose, fructose, and sucrose in apple juice. Except for simple dilution, no sample preparation is required.[2]

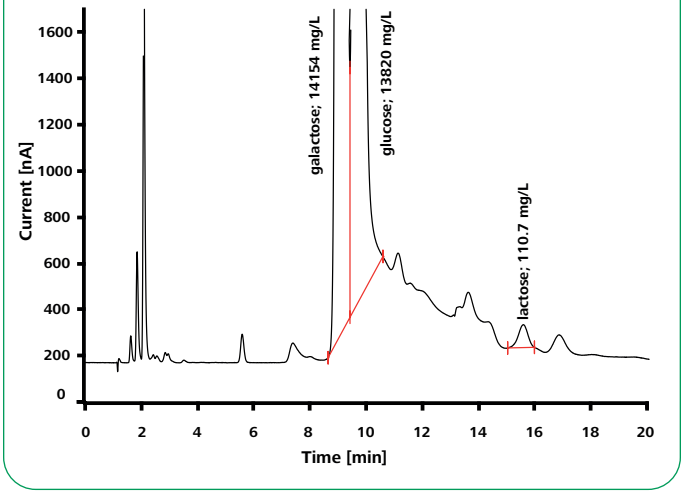


Because carbohydrates are electrochemically active, they can be detected amperometrically. During amperometric detection, the analytes are oxidized to a working electrode by applying a potential to the latter. This results in an electrical current that reveals the concentration. Over time, however, carbohydrates form deposits on the working electrode when a continuous potential is applied. The amperometric detector is therefore operated in PAD mode (pulsed amperometric detection). Here, a three-stage cyclic potential ensures that after measuring the current, i.e., after the determination stage, the electrode is cleaned from the adsorbed molecules and eventually conditioned.

Residual Lactose in 'lactose-free' Products

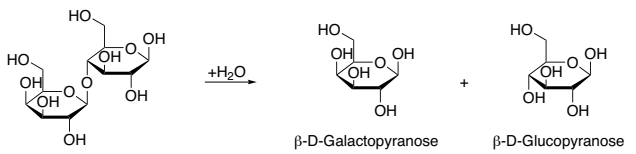
A key part of the quality control of products declared lactose-free is the determination of residual lactose. The ion chromatogram in **Figure 3** illustrates the determination of lactose in 'lactose-free' milk to which 100 mg/L lactose was added. Again, the separation takes place under strongly alkaline conditions (eluent of 5 mM sodium hydroxide/2 mM sodium acetate) and the analyte is detected by pulsed amperometry.

Figure 3. Determination of lactose traces in milk declared lactose-free, spiked with 100 mg/L lactose.[3]



The high concentrations of galactose and glucose illustrated in the chromatogram are a result of the enzymatic breakdown of lactose into these very monosaccharide constituents (**Figure 4**). Because of its protein-rich matrix milk must undergo dialysis before being analyzed, with the Metrohm Inline Sample Preparation. This is a fully automated process, and therefore does not involve any additional effort.

Figure 4. Lactose is composed of the monosaccharides galactose and glucose. The hydrolysis of lactose illustrated here is catalyzed by the enzyme lactase.



Carbohydrates as Tracers in Environmental Analysis

Fine dust limit values, which are used as health protection measures, are regularly being violated in many places. When looking for the culprit, the usual suspects are traffic and industry, but residential wood burning used for heating has also been linked to high fine dust values.[4] The tracer levoglucosan (**Figure 5**) is often determined in order to detect wood combustion.

Figure 5. Levoglucosan (1,6-Anhydro-β-D-glucopyranose) is produced in the pyrolysis of cellulose and is therefore commonly used as an indicator for biomass combustion.

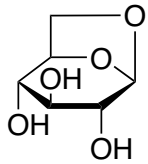
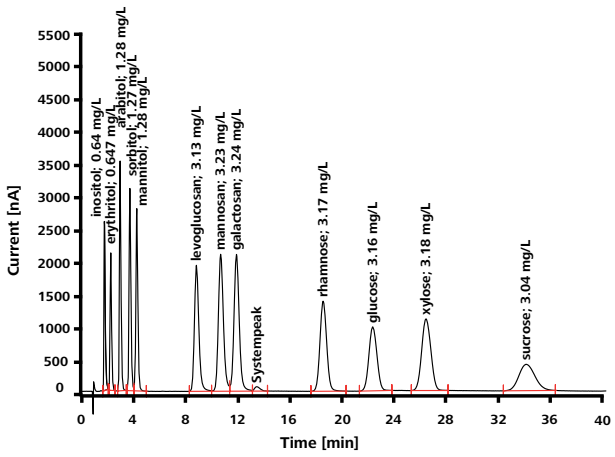


Figure 6 shows the determination of a standard solution in which levoglucosan, mannosan, and galactosan—all products of wood combustion—were analyzed, as were several biological sugars, alcohols, etc., which are typically found on aerosol particles. The high-capacity column achieves good separation of all substances, which then can be determined in a single analysis.

Figure 6. Determination of indicators for wood combustion (levoglucosan, mannosan, and galactosan) and biological sugars and alcohols, which are found in aerosols such as pollen.[5]



The new 'Carbohydrate Column'

The Metrosep Carb 2 chromatography column excels with its high ion exchange capacity, i.e., with the high number of ion exchange groups contained in its carrier material. This allows clean separation of the various sugars. Applications are found in a wide range of industries: water and environmental analysis, the pharmaceutical and food industry, forensics, the cosmetic industry, and the quality control of biofuels. In addition to carbohydrate analysis, the Metrosep Carb 2 is also suitable for determinations in samples with high salt content where lower-capacity columns fail, e.g., seawater.

For best performance, we have developed the alkaline IC eluent (Cat. No.**78348**) for the Metrosep Carb 2 column. We also offer a representative range of carbohydrate certified reference materials (CRM) solutions for IC.

References

1. Ebermann, R., Elmadfa, I. (2008): Lehrbuch Lebensmittelchemie und Ernährung. Vienna: Springer Verlag.
2. Metrohm Homepage Application Finder: «IC-amperometric sugar analysis in apple juice pursuant to EU regulation 1169/2011». Retrieved on January 17, 2019 from: https://www.metrohm.com/en/applications/AN-P-070
3. Metrohm Homepage Application Finder: «Lactose residues in "lactose-free" milk». Retrieved on January 17, 2019 from: https://www.metrohm.com/en/applications/AN-P-055
4. German Federal Institute for Risk Assessment (2015): «Analyse des quantitativen Einflusses der Holzverbrennung auf die Feinstaubkonzentration in Berlin und Brandenburg anhand des Tracers Levoglucosan (Levoglucosan)». Retrieved on May 18, 2015 from: http://www.bfr.bund.de/de/analyse_des_quantitativen_einflusses_der_holzverbrennung_auf_die_feinstaubkonzentration_in_berlin_und_brandenburg_anhand_des_tracers_levoglucosan_levoglucosan_-193056.html.
5. Metrohm Homepage Application Finder: «Anhydrosugars in addition to sugar alcohols and sugars from air filter sampling». Retrieved on January 17, 2019 from: https://www.metrohm.com/en/applications/AN-P-054

Featured Products

Description	Package Size	Cat. No.
Sodium acetate/Sodium hydroxide eluent for Metrosep Carb 2	1 L, 2.5 L	**78348**
Glucose Standard for IC	50 mL	**69222**
Fructose Standard for IC	50 mL	**72669**
Lactose Standard for IC	50 mL	**72622**
Sucrose Standard for IC	50 mL	**69631**
Galactose Standard for IC	50 mL	**72637**
Glycerol Standard for IC	50 mL	**72619**
Levoglucosan (1,6-Anhydro-β-D-glucose)	25 mg	**06724**

For a comprehensive overview of our product range for ion chromatography, please visit

SigmaAldrich.com/ic

Is your food healthy?

Measuring Total Sugar (Glucose and Fructose) in Potatoes using Mobile Reflectometry

Saskia Schröter, Product Manager Mobile and Analytical Workflows, Analytix@merckgroup.com

Who doesn't love golden, hot French fries or a steaming baked potato? In fried or baked goods, much of the savory taste and aroma can be attributed to the Maillard reaction. It is what creates the brown compounds that give many cooked foods this flavor. Unfortunately, the reaction between asparagine and reducing sugars (e.g., fructose or glucose) can also produce acrylamide, which is considered toxic and potentially carcinogenic: In the body, acrylamide is converted into glycidamide, which can bind to DNA and cause mutations. High levels of acrylamide can be found in starchy foods, such as potatoes and bread, when cooked at temperatures over 120 °C. This potentially dangerous acrylamide formation can be minimized by ensuring that the levels of reducing sugars are within safe limits, thereby ensuring the quality and nutritional value in processed food.

Precise in-process results—directly on-site

Reflectometry, or remission photometry, is a rapid, sensitive method for quantitating a wide variety of organic and inorganic parameters in water, food, beverages, and environmental samples. Using test strips in combination with a reflectometer, readings can be taken in the laboratory, on the production line, or in the field.

The total free sugar in potatoes can be determined in minutes with the simple Reflectoquant® Total Sugar Test and the RQflex® 20 reflectometer:

Reflectometric determination after enzymatic reaction with glucose-6-phosphate dehydrogenase and diaphorase

Sample preparation

Homogenize the potato in a blender (e.g. Ultraturrax). Weigh 10-20 g of the mashed sample into a 50 mL volumetric flask, and note down the exact sample weight. Add approx. 40 mL distilled water and stir for 10 minutes. Afterwards, make up to the mark with distilled water. Filter through a folded filter.

Analysis

Place 10 mL distilled water, 5 drops of reagent TS-1 and 1 mL filtrated sample in the test vessel and swirl. Press the reflectometer START key and simultaneously dip the test strip into the measurement sample (23 ± 3 °C) for ca. 2 s, ensuring that both reaction zones are immersed. Allow excess liquid to run off via the long edge of the strip on to an absorbent paper towel. Insert the strip immediately into the strip adapter.

After 60 s the strip is measured in the reflectometer. The value [mg/L] will be stored automatically.

The total sugar content can then be calculated as [g/kg] = Measured value [mg/L] x 50 mL / Sample weight [g] x 100

Results and comparison with classic enzymatic method

Sample	Total Sugar [g/kg] Reflectoquant® method	Total Sugar [g/kg] Enzymatic method
1	15.4	17.3
2	5.7	6.4
3	0.4	0.4
4	<0.2	0.1

From Potatoes with 0.2–1.0 g/kg reducing sugar, roasted products of optimum culinary quality can be prepared and, if prepared under conditions minimizing acrylamide formation, acrylamide contents remain below 500 µg/kg.[1]

Featured Products

Description	Cat. No.
Reflectoquant® Total Sugar Test, 50 Tests	**116136**
Reflectometer RQflex® 20	**117246**

Reference:

1. How much reducing sugar may potatoes contain to avoid excessive acrylamide formation during roasting and baking? Biedermann-Brem, S., Noti, A., Grob, K. et al. Eur Food Res Technol (2003) 217: 369. https://doi.org/10.1007/s00217-003-0779-z

RQflex® 20 Reflectometer

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Simple and easy to use menu system to access all your methods, results and quality assurance options, along with the ability to tailor your settings to your needs.

Portable Analysis

A light and compact system that acts like a mobile laboratory to perform critical analyses and obtain quantitative results directly on-site. Get results in minutes for easy monitoring of your materials at any stage of your process.

Barcoded test strips with batch specific calibration

For every test condition, each batch of test strips is calibrated and equipped with a barcode for accurate and reproducible results. Obtain consistent results quickly with an average accuracy within ± 10 %.

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For analytical quality assurance (AQA), our comprehensive RQcheck and total system monitoring process ensures accurate and precise measurement results. Calibration is straightforward, so your measurements will always be reliable.

Countdown timer for streamlined analysis

For maximum reproducibility, a countdown timer with an advanced warning alert before the end of the test strip preparation time can be tailored to fit in with your workflow.

Broad range of test kits and application notes

From ammonium through to nitrate and urea, the Reflectoquant product line offers a comprehensive range of test kits for fast instrumental analysis, e.g., for testing the nitrate content in vegetables or water, determining the freshness of honey, or finding out how much ammonium is in the soil.

For more information and an application overview visit **SigmaAldrich.com/reflectoquant**

Rapid, Flexible and Reliable Analysis for Water, Food and Beverage Industries - Take your Lab to the Sample

Quantification of Methylglyoxal in Manuka Honey – A simple HPTLC Based Approach

Markus Burholt, Scientist, Instrumental Analytics R&D
Michaela Oberle, Technical Project Manager, Instrumental Analytics R&D
Michael Schulz, Head of HPLC & Instrumental Analytics R&D
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Introduction

Honey — a natural product, is one of the most frequently tested food products. In recent years, manuka honey has gained popularity because of its high antibacterial activity.[1] Methylglyoxal (MGO) has been identified as one of the major contributors to its antibacterial activity. MGO is present in high concentrations in manuka honey and is directly responsible for its potency. This makes the manuka honey exclusive and high-priced as compared to the other traditional kinds of honey. Manuka honey from New Zealand usually contains 40 to 800 mg/kg of MGO but can even contain up to 1900 mg/kg.[2] To avoid adulteration of manuka honey products, a strict quality regulation regarding its origin, purity, and quality need to be fulfilled and is a prerequisite for the UMF™ (Unique Manuka Factor) grading.[2] It mostly reflects the MGO amount in the honey but also considers other authenticity markers.

In the following application, we focus on the MGO quantification using High-Performance Thin-Layer chromatography (HPTLC) with subsequent substance confirmation by MS measurement. The high viscosity and high sugar content of honey makes it a very complex and matrix-rich sample for an analysis. Thin-layer chromatography (TLC) and High-Performance Thin-Layer chromatography (HPTLC) are convenient, fast, and efficient separation techniques that enable the development of analytical methods without the need for complicated sample preparations or high investments.[3] Low cost and short analysis time per sample are given by the parallel analysis of many samples on one plate. Furthermore, the high matrix tolerance of TLC offers additional opportunities to existing routine methods.

Experimental

Six different commercially available manuka honey samples were analyzed. MGO shows a mesomeric effect and reacts immediately with water to form either methylglyoxal monohydrate or methylglyoxal dihydrate in aqueous environments.[4] Only a small amount of around 1% MGO remains unreacted. Direct detection of MGO in manuka honey is found to be difficult using conventional methods. In this application, MGO is converted to stable 2-methylquinoxaline by derivatizing it with 1,2-phenylenediamine (see **Figure 1**).[5] The stable form is then used as the reference. For confirmation of the method and determination of the recovery rate regular honey samples have been spiked with MGO and 1,2-phenylenediamine. Other derivatization options were tested but the reaction with 1,2-phenylenediamine performed best. Water and honey matrix were tested to confirm, that the optimzed reaction conditions provide reproducible results for both matrices.

A calibration curve of 2-methylquinoxaline was calculated based on 3 different standard volumes (**Table 1** and and **Figure 2**).

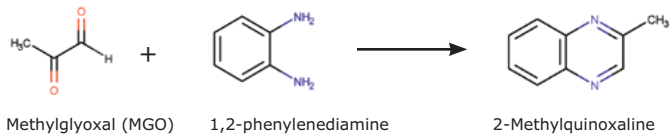

Figure 1. Reaction scheme of MGO with 1,2-phenylenediamine

Table 1. Calibration Curve

Spots	Application volume µL	Amount (µg)	Mean Area
1, 10, 19	0.3	0.045	4080.52
2, 11, 20	1.5	0.225	11120.91
3, 12, 21	3.0	0.451	15677.39

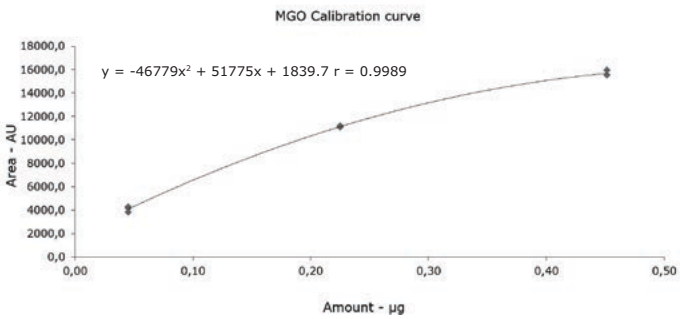

Figure 2. Calibration plot with corresponding calibration function.

A recovery study was performed using regular honey to simulate honey matrix. It was spiked with a known amount of MGO standard solution, followed by the addition of 1,2-phenylenediamine. The measured (and calculated) MGO amount allowed for the correlation of the actual amount of MGO in the Manuka honey samples. The experimental details of the recovery rate study can be found in **Table 2**, **Table 3**, **Figure 3** and **Figure 4**.

Table 2. TLC data of recovery rate: In total, nine regular honey samples were applied and one MGO standard sample. Seven honey sample (4-10) were spiked with MGO and 1,2-phenylenediamine.

Spots	Application volume µL	Description
1	1.0	Methylglyoxal standard 0.15 mg/mL (water) with 0.2% 1,2-diphenylenediamine
2	5.0	Regular honey, 100 mg/mL in water/ethanol 3:2
3	5.0	Regular honey, 100 mg/mL in water/ethanol 3:2 + 0.2% 1,2-phenylenediamine
4 - 10	5.0	Regular honey, 100 mg/mL in water/ethanol 3:2 + 0.2% 1,2-phenylenediamine spiked with methylglyoxal 0.024 mg/mL

Table 3. Quantification of methylglyoxal in the seven honey samples

Honey Sample #	Area AU
1	6759.37
2	6665.00
3	6911.29
4	6756.10
5	7055.36
6	7059.58
7	7014.80
Mean Area	**6888.79**
RSD %	2.35
Amount (µg)	0.108
Spiked Amount (µg)	0.12
Recovery rate (%)	90.05

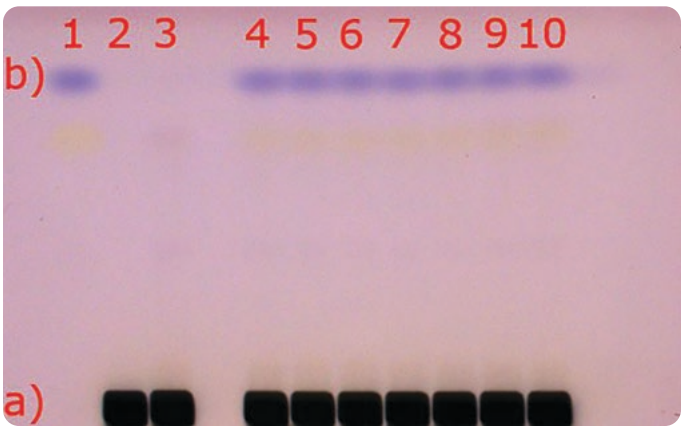

Figure 3. Visualization of the plate under visible light (white light); a) matrix compounds after staining with anisaldehyde sulfuric acid (black areas); b) 2-Methylquinoxaline (blue spot at hR_f 80), (reaction product of Methylglyoxal with 1,2-phenylenediamine)

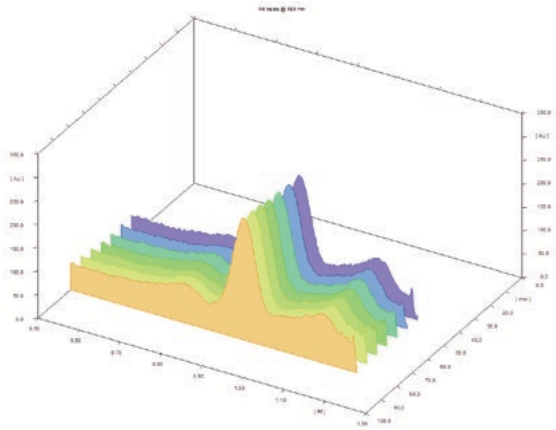

Figure 4. Scan of spiked honey tracks (sample 4 – 10) at 480 nm with CAMAG TLC Scanner 3.

All TLC analyses were performed using HPTLC Silica gel 60 F_{254}. The plates were pre-washed with the mobile phase (up to 7 cm) and dried before use.

The standards were prepared by dissolving 100 µL of ~40% aq. MGO solution (exact content known) diluted in 20.0 mL water. 800 µL of this stock solution was further diluted with water to 10.00 mL volume and 0.2% (20 mg) of the reactant 1,2-phenylenediamine was added. All standard solutions were stored at 8°C for two days before use to achieve reproducible reaction of MGO with 1,2-phenylenediamine. Longer storage times (>3 days) lead to partly degradation of 2-methylquinoxaline.

Honey sample solutions of 100 mg/mL in case of sample numbers 1, 3, 5, and 150 mg/mL in case of honey samples 2, 4 and 6 were applied with a higher volume due to the expected lower amount of MGO. To each sample 0.2% of 1,2-phenylenediamine was added, e.g., sample 1, 4.0 g honey diluted in 40 mL solution of water / ethanol in 3:2. To the solution 0.2% (80 mg) of the reactant 1,2-phenylenediamine was added. Before using the samples, they needed to be stored at 8 °C for two days to complete the reaction.

The samples and standards were applied as spots in an area of 5 x 3 mm^2. This step is necessary because of the high matrix and high application volumes of the honey samples. The plate was developed, dried, and then derivatized by dipping in an anisaldehyde-sulfuric acid reagent. Blue spots of 2-methylquinoxaline (product of the reaction of MGO with 1,2-phenylenediamine) appeared at hR_f 80. Daylight examination and scanning of the plate at 480 nm were carried out for quantification. Experimental results are shown in **Figure 5** and **Table 4**.

Figure 5. Visualization of the plate under visible light (white light); a) matrix compounds after staining with anisaldehyde sulfuric acid (black areas); b) 2-methylquinoxaline (blue spot at hR_f 80), (reaction product of methylglyoxal with 1,2-phenylenediamine)

Table 4. TLC data: In total 27 samples were applied. Track numbers with applied samples and volumes and obtained hR_f values are summarized here (details of tracks 1-3, 10-12 and 19-21 for calibration are given in **Table 1**):

Manuka Samples	Application position	Conc. Sample (mg/mL)	Application volume (µL)	Mean Area (AU)	Mean Amount (µg)	%RSD	MGO in Honey (mg/kg)	Expected Amount MGO in Honey according to information on product label (mg/kg)
1	4, 13, 22	100.0	5.0	11225.00	0.228	2.68	507.4	600.0
2	5, 14, 23	150.0	9.0	4548.80	0.055	2.84	45.3	nd
3	6, 15, 24	100.0	5.0	8002.84	0.136	3.10	301.4	300.0
4	7, 16, 25	150.0	8.0	6031.58	0.088	2.48	81.4	80.0
5	8, 17, 26	100.0	5.0	9674.57	0.181	3.06	401.8	400.0
6	9, 18, 27	150.0	8.0	7578.22	0.125	3.14	115.6	nd

* The expected MGO concentrations in sample 2 and 6 were not specified by the supplier

A separate plate without staining was used for MS measurement. The coupling to MS was performed on an elution-based approach, that utilized a TLC-MS interface. This enabled the dissolution of the analyte from the silica plate at the zone of hR_f 80 by a solvent and a transfer to the mass spectrometer in the liquid phase. This additionally confirmed the spot identification of the MGO derivative 1-methylquinoxaline.[6]

Experimental Conditions

Plate:	HPTLC Silica Gel 60 F_{254} 20 x 10 cm (**1.05642**)
Application volume:	0.3 – 9.0 µL, area application 5 x 3 mm with CAMAG ATS 4
Detection:	480 nm
Chamber:	20 x 10 chamber without filter paper
Mobile phase:	Ethyl acetate/Acetonitrile 85:15 (v/v)
Staining:	Anisaldehyde-sulfuric acid reagent (0.5 mL p-anisaldehyde, 85 mL methanol, 10 mL glacial acetic acid, 5 mL sulfuric acid 98%)
Migration distance:	5 cm
hR_f:	80
Drying:	60 °C
Standard preparation:	100 µL of ~40 % aq. methylglyoxal solution (exact content known) diluted in a 20.0 mL volumetric flask and filled up with water. 800 µL of this stock solution is diluted again in a 10.0 mL volumetric flask and made up to the mark with water. Addition of 0.2 % (20 mg) of the reactant 1,2-phenylenediamine. Before the standard is ready for use it is refrigerated at 8 °C for two days to complete the derivatization reaction.
Sample:	Solutions of 100 mg/mL of sample nos. 1, 3, 5, and 150 mg/mL of sample nos. 2, 4, 6 were prepared. To every sample 0.2 % of 1,2-phenylenediamine was added. e.g., sample 1: 4.0 g honey diluted in 40 mL solution of water/ethanol in 6:4. To the solution 0.2 % (80 mg) of the reactant 1,2-phenylenediamine was added. Before the samples are ready to use, they are refrigerated at 8 °C for two days to complete the derivatization reaction.
MS measurement:	The samples are extracted with the Plate Express and measured with the single-quadrupole expression compact mass spectrometer (CMS) from Advion.
Extraction solvent:	Acetonitrile/Water 95:5 (v/v) + 0.1% formic acid

Results and Discussion

As demonstrated, MGO can be identified and quantified in different honey samples within the concentration range of 50 mg to 600 mg/kg. The conversion of MGO into the more stable compound 2-methylquinoxaline allows for an easy evaluation of the MGO content. The recovery study showed a detectable MGO amount of around 90%. The correlated MGO amount in manuka samples was calculated accordingly. One of the samples (sample 1) showed a lower MGO content than indicated by the supplier. This might be because of the degradation of the MGO during storage. Sample 2 and sample 6 only showed MGO concentrations of 50 and 100 mg/kg. These manuka honey samples are considered of lower quality. Although no indication of MGO concentration was provided by the supplier.

Conclusion

The analysis of MGO in a complex and challenging food matrix like honey was described. Target analyte could be easily separated and detected without time-consuming and labor-intensive sample preparation. The flexible set-up enabled a combination with MS measurements.

Screening and method development capabilities were shown by the application of 27 tracks on one plate (honey samples and standard solutions). The study clearly differentiated various honey qualities (referring to MGO content) on the market. Though the analysis of MGO is challenging, MGO content could be well quantified in the expected range.

To summarize, a fast, cheap, and simple quantification of methylglyoxal can be accomplished with HPTLC. This application demonstrates the main advantages of the method, such as quick sample preparation, high matrix tolerance, and high-throughput.

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6. unpublished results

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Description	Cat.No.
HPTLC Silica gel 60 F_{254} 20 x 10 cm	**1.05642**
Methylglyoxal solution ~40% in H_2O	**M0252**
1,2-Phenylenediamine ≥99%	**694975**
Ethyl acetate, for liquid chromatography LiChrosolv®	**1.00868**
Acetonitrile, gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur	**1.00030**
Ethanol, gradient grade for liquid chromatography LiChrosolv®	**1.11727**
Methanol, gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur	**1.06007**
Sulfuric acid, ACS reagent, 95.0-98.0%	**258105**
Acetic acid, glacial, ACS reagent, ≥99.7%	**695092**
p-Anisaldehyde 98%	**A88107**
TLC-MS	
Acetonitrile, for UHPLC-MS LiChrosolv®	**1.03725**
Water, for UHPLC-MS LiChrosolv®	**1.03702**
Formic acid 98% - 100% for LC-MS LiChropur™	**5.33002**
Millex LCR PTFE Syringe filter	**SLCRBZ5NZ**

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