

An Executive  
Summary

# Chromatographic Testing of Dietary Supplements following United States Pharmacopeia Monograph Methods

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**W**hat actually are dietary supplements? They are consumed in large quantities, but they are often referred to as vitamins, minerals or even botanicals derived from plants? To get a better understanding of what a dietary supplement is, we have chosen three examples to discuss in this paper, which are being tested according to the USP (United States Pharmacopeia) guidelines for dietary supplement. The examples we have chosen are extracts of black pepper, turmeric and ginger. So even though they are common spices used for cooking, they are also used as dietary supplements.

## Regulated Analysis Using USP Methodology

Let's talk about the regulated analysis of dietary supplements. We know that pharmaceutical analysis is regulated through monograph methodologies developed by different global pharmacopeial bodies, including the United States, British, European, Chinese and Japanese Pharmacopeias. Usually when an analytical method is developed, the sample is the focal point of the analysis. From this, we try to select the best instrumental technique, the consumables or accessories needed, and the optimum sample preparation method to analyze the sample... which finally produces an answer in the form of data. In other words, every decision we make is dictated by the sample.

However, with a regulated method we have to follow the monograph instead of putting the sample at the center of our decision making. So it's more like following a recipe in a cookbook. We can specify what type and quantity of ingredients to use and how to use them, which produces the finished dish. If you relate that to a regulated method, the finished product is an answer, a value, which has also to include the analytical merits of the generated data following the instructions in the monograph.

In the case of using a regulated method to characterize black pepper, ginger, or turmeric, this means that to understand the requirement of a regulated instrumental analysis, you need to follow the specifications defined in the method. You have to know how to apply the dictated product specification according to the methods being used to get to the point where the value of interest can be reported. We provide the reliable and traceable products, an up-to-date knowledge base about the regulations and an advanced technical support team to deliver a solution with the same kind of quality today, tomorrow, next week... or even next year.

But understanding the method and how different products will be used is important, particularly when working in the complex area of regulatory analysis. There is clearly defined information about what can be changed and how the method should be validated. We at Merck see ourselves as...a responsive, collaborative partner with a good understanding of the regulatory aspects of the analysis. For a number of years, we have been focused on understanding the different pharmacopeial methods and how best to put them into practice and to clearly communicate how our products are used according to these guidelines.

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## Monograph for Black Pepper

Moving into practical examples, we start with the monograph for powdered black pepper. The monograph from USP39-NF34 has a section dedicated to its identification characterized as a botanical, and another section which outlines its composition analysis. Under the identification test, it refers to a specific method related to its botanical characteristics, which requires high performance thin layer chromatography (HPTLC). However in the section on compositional analysis it refers to the use of high performance liquid chromatography (HPLC). So let's discuss the differences in the testing protocol and in particular why the current monograph for powdered black pepper was updated in the last USP38 to include HPTLC.

## Identification Using HPTLC

The monograph itself specifies the adsorbent as an HPTLC glass plate Si 60 F<sub>254</sub><sup>1</sup> using a plate size of 20 x 10 cm, and the mobile phase as a mixture of hexane and ethyl acetate with a volume ratio of 5:3. The solutions are applied as bands with a bandwidth of 8 mm, using a migration distance of 6 cm. The first track is the 3 µL application of standard solution A, which is a USP reference solution of 0.9 mg/mL piperine. Track 2 is the same volume but using solution B which is a 2 mg/mL borneol standard. Track 3 is a reference standard solution C, which is a powdered black pepper extract of 5 mg/mL, sonicated, centrifuged, and the supernatant extracted. Tracks 4 and 5 represent two different commercial pepper samples, of the same concentration, taken through the same sample preparation steps. The resulting HPTLC is shown in **Figure 1**.

The acceptance criteria, i.e. requirements for characterization, for black pepper extracts (the numbers on the chromatogram are identified below):

1. Under 254 nm, the chromatogram of the sample solution exhibits an intense quenching band at a RF (retention factor) of about 0.15 corresponding to the Piperine band in the chromatogram of standard solution A
2. A quenching band at RF of about 0.02
3. Three quenching bands of similar intensity equally spaced located between RF of about 0.3 and 0.5
4. Under white light, the derivatized chromatogram of the sample solution exhibits main bands similar in position and color to the main bands in the chromatogram of standard solution C
5. These bands include a dark green band of the same color and RF as the Piperine band in standard solution A (RF of about 0.15)
6. A weak violet band at RF of about 0.47 below the position of the band due to Borneol in Standard solution B
7. A greenish band in the lower part of the chromatogram at RF of about 0.07

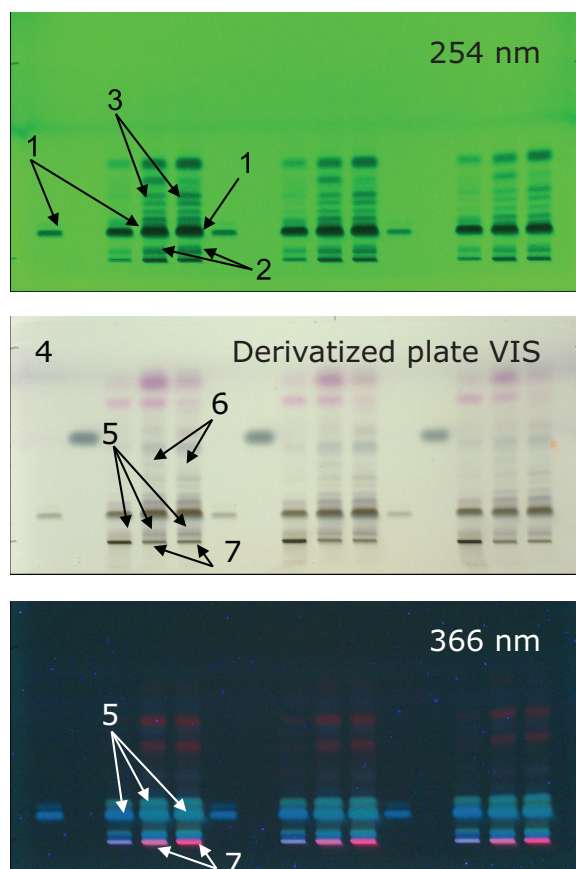
It's also worth pointing out that minor bands may be observed in the sample solution and standard solution C

chromatograms. No blue bands are detected in the chromatogram of the sample solution at RF of about 0.10 and 0.58 (distinctive from longer pepper). So using these plates and following the prescribed recipe in the USP monograph, we can meet the acceptance criteria for black pepper under the identification test by HPTLC.

## Compositional Analysis by HPLC

The HPLC compositional analysis method described in the monograph, which is a gradient method, is followed by applying a Purospher® STAR, 250 x 4.6 mm ID RP-18 endcapped column and two mobile phases as described in **Table 1**. Solution A is a mix of anhydrous potassium dihydrogen phosphate in water and titrated with phosphoric acid, while solution B is gradient grade acetonitrile. It's important that the column is appropriately re-equilibrated in gradient methods to get good reproducibility. This means to sufficiently exchange the volume inside the column. With a flow rate of about 1.5 mL/min, a tube volume of about 4.2 mL using a 250 x 4.6 mm column geometry, re-equilibrating the column for about 20 minutes means that you're flushing through 30 mL of solvent (corresponds to seven complete column tube volumes), which is sufficient to ensure reproducibility for the method.

**Figure 1:** Identification of powdered black pepper by HPTLC as described in USP39-NF34.



The sample preparation is a bit complex. The black pepper powder is put into a flask with a condenser and refluxed with methanol. Because, the HPLC analysis is carried out with a gradient method, we use a high grade of solvent in order to prevent the spikes in the chromatogram. We do an extraction of the sample, and finally, we filter through a 0.45 micron Millex® PVDF membrane filter, before it is introduced into the HPLC system.

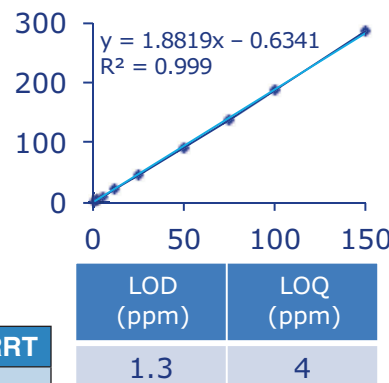
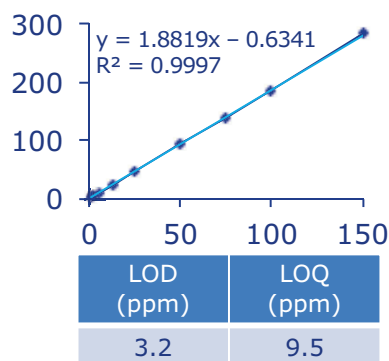
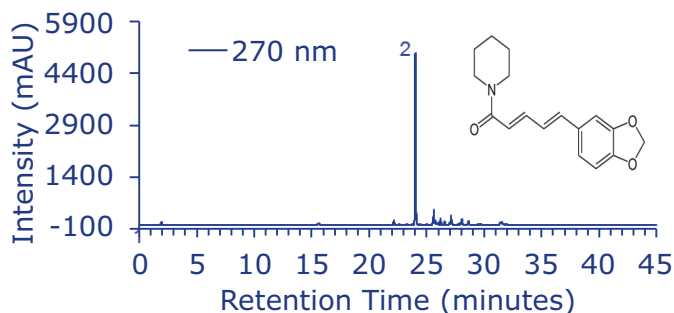
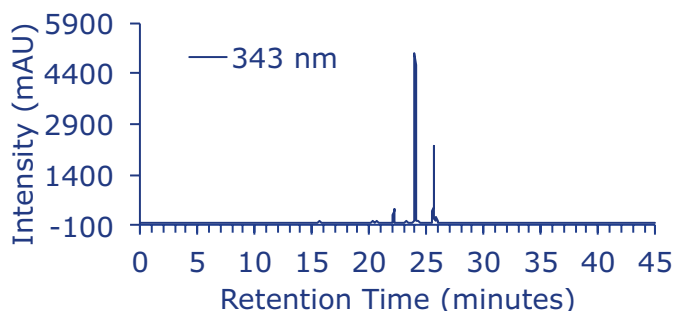
The resulting chromatogram with detection at two different wavelengths (343 nm and 270 nm), is shown in **Figure 2**. The chromatogram of the sample solution obtained at 343 nm exhibits a major peak at the retention time corresponding to piperine. The identification of other piperamide peaks in the sample solution was confirmed by comparison with the chromatogram of standard solution B and the reference chromatogram obtained from a lot of USP powdered black pepper extract.

The sample solution chromatogram at the 343 nm wavelength shows an additional peak corresponding to piperlyline. The chromatogram of the sample solution obtained at 270 nm does not exhibit that peak due to the compound, 2E, 4E-N-isobutyldecadienamide at a relative retention time (RRT) of 1.1 to the piperine peak (distinctive from long pepper). As a result, by comparing calibration curves for Piperine at the two different wavelengths,

**Table 1:** Two mobile phases used for the compositional analysis of black pepper by HPLC.

Time (min)	A (%)	B (%)
0-8	95->55	5->45
18-25	55->20	45->80
25-28	20	80
28-35	20->55	80->45
35-40	55->95	45->5
40-45	95	5

**Figure 2:** Resulting chromatogram of the compositional analysis of powdered black pepper by HPLC.



Compound	RT (min)	Tailing Factor (USP)	RRT
to (void volume)	2.5		
Piperine	24.0	0.9	1.0
(2E,4E)-N-Isobutyldecadienamide	25.6	1.0	1.1

it can be seen that the 343 nm gives slightly poorer limits of detection (LOD) and quantitation (LOQ) compared to the 270 nm wavelength.

### USP Monograph for Turmeric Powder

The example of turmeric powder extract is similar to the black pepper; first there is a description of an identification test using classical TLC, or HPTLC but with a composition test using an isocratic HPLC method. The HPTLC separating conditions are shown in **Figure 3**, together with the resulting chromatogram. As can be seen, the adsorbent is silica gel 60 F<sub>254</sub> 20x20 cm plate size. The development solvent is a mixture of chloroform, methanol, formic acid, at a volume ratio of 96/4/1. The sample and standard solutions are applied as bands, with a migration distance of 14 cm and a migration time of 30 minutes. Track 1 is a standard solution with a USP curcuminoids reference solution, at a concentration of 0.2 mg/mL and track 2 is the sample, which is a 10 mg/mL powdered turmeric extract, prepared from the pulverized rhizomes of *Curcuma longa*. So how is this analysis carried out? The developed plates are first examined in daylight and then under UV light at 365 nm. According to the acceptance criteria, the sample solution must show yellowish brown bands due to the compounds bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin at RF values of 0.4, 0.6, and 0.7 respectively, corresponding

in position and color to those obtained from the standard solution, as demonstrated on the left hand plate.

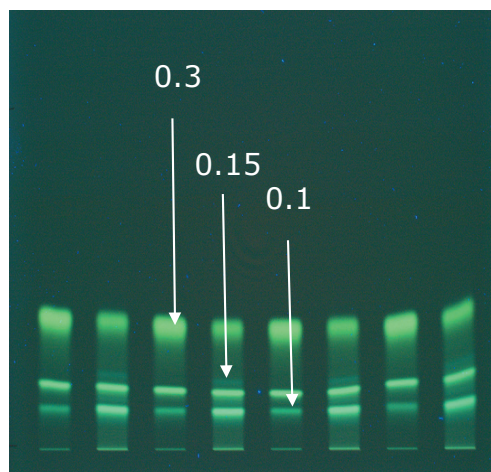
However, on the right-hand side, you can see the same plate at 365 nm, which are clearly showing different results, compared to the acceptance criteria. Instead of 0.4, 0.6, and 0.7, we have RF values of 0.1, 0.15, and 0.3. We have altered the method within allowed changes to see if it's possible the meet these acceptance criteria, but with very little success. We believe the issue is related to the experimental description, where there is no clear definition of what type of particle was used to make the TLC. One of the conclusions could be that the pore size of the particles were different, which would make it very difficult to comply with the acceptance criteria. If that is the case, this is a good example of a monograph which needs to be updated because the analytical methodology makes it very difficult to achieve a successful analysis.

### Compositional Analysis by HPLC

This is an isocratic method, where the chromatographic separation conditions and sample preparation steps are shown in **Table 2**. By following the USP general Chapter 621 (chromatography techniques) it is possible to make specific changes to the method. Two different HPLC columns were used, with the same geometry - 150x4.6 mm, but with different particle sizes (5 and 3µm). An example of method transfer from a

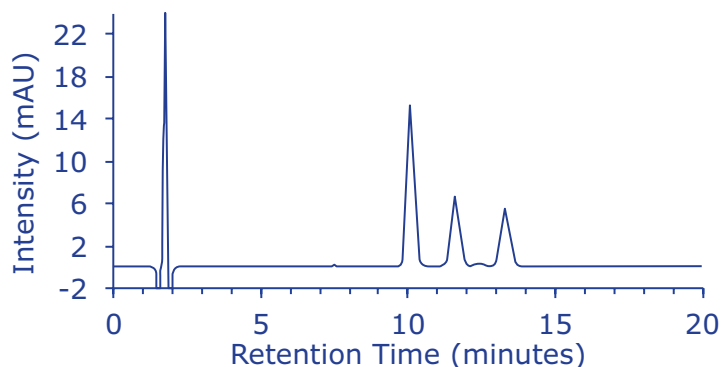
**Figure 3:** Separating conditions and chromatogram of of tumeric powder by TLC.

<b>Adsorbent</b>	<b>TLC Silica gel 60 F254 20 x 20 cm (1.05715.0001)</b>	
<b>Mobile phase</b>	Chloroform/Methanol/formic acid 96/4/1 (v/v/v) 1.02444/1.06018/1.00264	
<b>Solutions applied as bands</b>	X = 20.0 mm Y = 15.0 mm	
<b>Migration distance 14.0 cm (migration time 30 min)</b>		
<b>Track 1</b>	10 µL	Standard solution: 0.2 mg/ml of USP Curcuminoids RS
<b>Track 2</b>	10 µL	Sample: 10 mg/ml of powdered Turmeric extract



**Table 2:** HPLC operating conditions for the compositional analysis of turmeric powder using two different columns.

<b>Column 1:</b>	Purospher® STAR RP-18 endcapped (5 µm) 150x4.6 mm (1.51455.0001)
<b>Column 2:</b>	Purospher® STAR RP-18 endcapped (3 µm) 150x4.6 mm (1.50470.0001)
<b>Injection:</b>	20 µL
<b>Detection:</b>	420 nm
<b>Cell:</b>	11 µL
<b>Flow Rate:</b>	1 mL/min
<b>Mobile Phase A:</b>	1 mg/mL Citric acid in water
<b>Mobile Phase B:</b>	Tetrahydrofuran LiChrosolv® (1.08101)
<b>Isocratic:</b>	A/B 60/40 (v/v)
<b>Temperature:</b>	Ambient
<b>Diluent:</b>	A/B 60/40 (v/v)
<b>Sample Prep:</b>	Powdered Turmeric extract. Transfer about 100 mg of powdered Turmeric extract, accurately weighed, to a 50 mL volumetric flask, add 30 mL of acetone, and sonicate for 30 min. Dilute with acetone to volume and centrifuge. Transfer 5 mL to a 50 mL volumetric flask, dilute with diluent to volume and mix.
<b>Pressure Drop:</b>	Column 1: 145 bar (2103 psi) Column 2: 276 bar (4002 psi)

**Figure 4:** HPLC chromatogram of turmeric powder using a 5µm particle size column.

Compound	RT (min)	Tailing factor (USP)	Rs	RRT
<i>t</i> 0 (void volume)	1.8			
Curcumin	10.1	1.0		1.0
Desmethoxycurcumin	11.6	1.0	3.1	1.2
Bisdesmethoxycurcumin	13.3	1.1	3.0	1.3

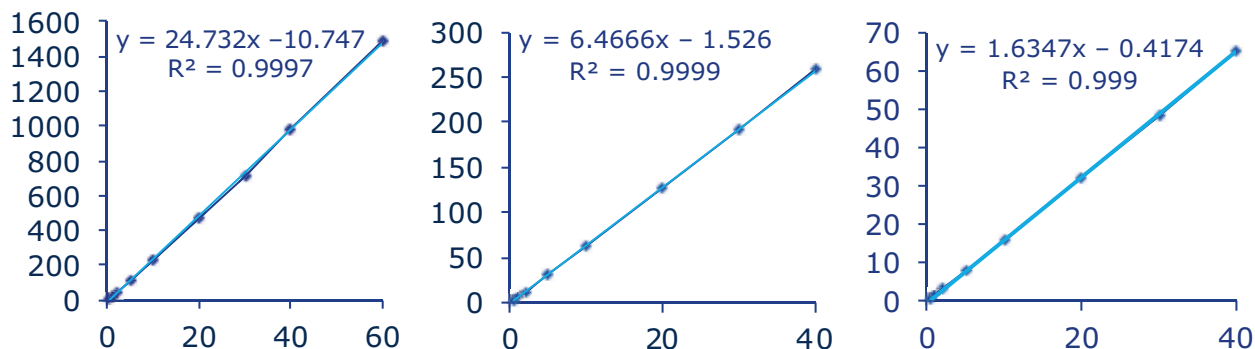
large particle to smaller particle column, where you gain in separation efficiency but paying with a higher back pressure.

In **Figure 4** it is shown that the three main compounds; curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin have been separated with good resolution and retention time in about 14 minutes. According to the system suitability criteria the requirements for this method is that the relative retention time for these three molecules should be 1.2, 1.3, and 1.4. It can be seen

that they are all meeting these specifications within experimental error. The resolution should be > 2.0 between the curcumin and desmethoxycurcumin and between the bisdesmethoxycurcumin and desmethoxycurcumin. And as can be seen the resolution in both cases is higher. In addition, tailing factors for all peaks are well below the specification of 1.5. If we take a look at the calibration plots for this analysis shown in **Figure 5**, it can be seen that the correlation coefficient ( $R^2$ ) for curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin

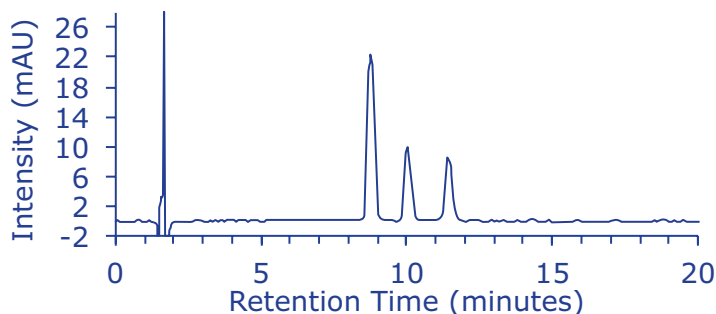
are all 0.999 or better, with both LOD and LOQ in the low ppm range. So clearly this meets the criteria of the method for the compositional testing of the turmeric powder. In **Figure 6** the same separation is shown but with a column with 3µm particles. Using smaller particles higher back pressure is observed but also higher sensitivity and improved resolution. The relative retention times and peak tailing are similar to the 5 µm column.

**Figure 5:** Calibration graphs and achievable detection limits of compositional analysis of turmeric powder using a 5µm column.



Analyte	LOD (ppm)	LOQ (ppm)
Curcumin	1.3	4.0
Desmethoxycurcumin	1.0	3.1
Bisdesmethoxycurcumin	1.1	3.4

**Figure 6:** HPLC chromatogram of turmeric powder using a 3µm particle size column.



Compound	RT (min)	Tailing factor (USP)	Rs	RRT
t0 (void volume)	1.6			
Curcumin	8.8	1.1		1.0
Desmethoxycurcumin	10.0	1.1	4.4	1.2
Bisdesmethoxycurcumin	11.4	1.1	3.2	1.3

The resolution improved between the curcumin and desmethoxycurcumin while remaining in the same for the bisdesmethoxycurcumin and desmethoxycurcumin. So the result is that we have a slightly more sensitive and efficient method if we go to a smaller particle size. If we look at the calibration plots shown in **Figure 7**, it can be seen that we have the same excellent linearity but with somewhat lower LOD and LOQ values using 3 µm.

### Ground Ginger

The final example is ground ginger. If we look at this monograph, we actually have three identification tests - two of them are

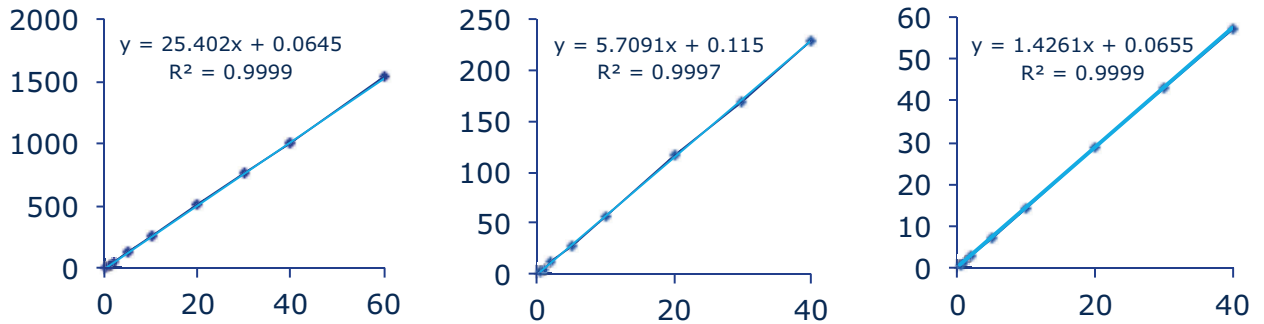
based on turbidity and a colorimetric test, while the third is using TLC. We won't go into the first two procedures as they are non-chromatographic techniques. The TLC for ginger test is very similar to the turmeric and black pepper examples discussed earlier, where the plates are prepared and standard solutions and typical samples are compared for the RF values. Details of the operating conditions and different tracks are shown in the table on the top of **Figure 8**, while the resulting TLC chromatogram is shown

underneath. In this case, the acceptance criteria is that the sample solution exhibit a spot due to gingerols that occurs at a RF value of 0.2 (shown by spot #1), whereas a spot of 6-shogaol may occur at a value of 0.4 (shown by spot #2), corresponding to those shown in the chromatogram of the standard solution. This is exemplified on the left-hand side plate using UV light and on the right-hand side at 254 nm.

### Compositional Analysis by HPLC

A gradient HPLC method using a 5 µm 250x4.6 RP-18 column and a gradient that's optimized at 1 ml/min. The mobile phase A solution was acetonitrile, Milli-Q water with 0.1% phosphoric

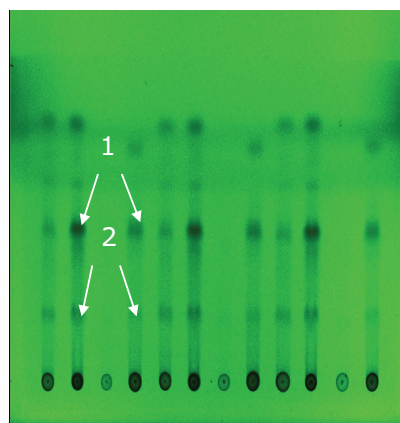
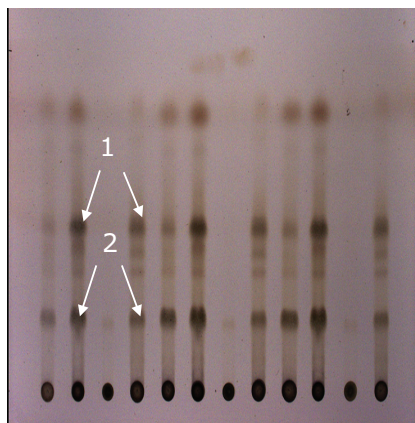
**Figure 7:** Calibration graphs and achievable detection limits of compositional analysis of turmeric powder using a 3µm column.



Analyte	LOD (ppm)	LOQ (ppm)
Curcumin	0.7	2.0
Desmethoxycurcumin	0.9	2.6
Bisdesmethoxycurcumin	0.5	1.5

**Figure 8:** Standard conditions and chromatogram for identification of ginger extract using TLC.

Adsorbent	PLC Silica gel 60 F254 0.5 mm layer 20x20 cm (1.05744)	
Mobile phase	n-Hexane/diethyl ether 7/3 (v/v) 1.04391 / 1.00930	
Solutions applied as spots	X = 15.0 mm Y = 20.0 mm	
Migration distance 10.0 cm (migration time 14 min and 30 sec)		
Track 1	20 µL	Standard solution A: USP Powdered Ginger RS 40 mg/mL
Track 2	40 µL	Standard solution B: 1.0 mL of USP Powdered Ginger RS 40 mg/mL added to one vial of USP Ginger. Constituent Mixture RS
Track 3	20 µL	Sample 1: Sheng Chang Ginger 40 mg/mL
Track 4	20 µL	Sample 2: Ginger Sample 40 mg/mL



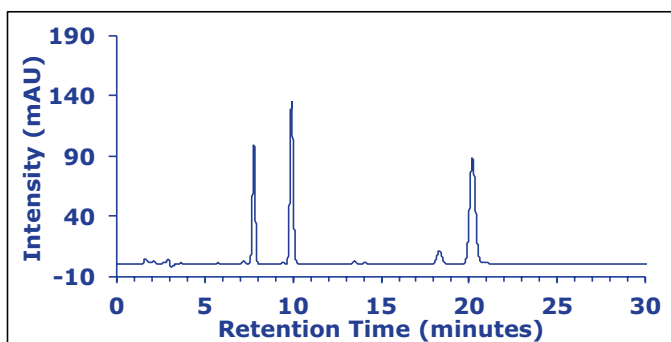
acid and methanol, while the B solution was neat acetonitrile. Specific gradient times and % concentration changes of the two mobile phases, together with the details about the sample preparation steps are shown in **Table 3**. The actual

chromatogram, retention times with resolution and peak tailing data is shown in **Figure 9**. It can be seen that the 6-gingerol, capsaicin and 6-shogaol are all well separated. We have a resolution between the first two compounds of about 7 and

**Table 3:** Sample prep and gradient HPLC conditions for compositional analysis of ginger extract (USP39-NF34).

Column:	Purospher® STAR RP-18 endcapped (5•m) 250x4.6 mm (1.51456.0001)		
Injection:	15 µL		
Detection:	UV = 282 nm		
Cell:	11 µL		
Flow Rate:	1 mL/min		
Gradient:	Time (min)	A (%)	B (%)
	0-2	100->0	0->100
	2-12	0	100
	12-24	0->100	100->0
	14-29	100	0
Mobile Phase A:	Acetonitrile, Milli-Q® water with 0.1% phosphoric acid, methanol 55/44/1 (v/v/v)		
Mobile Phase B:	Acetonitrile gradient grade (1.00030)		
Temperature:	Ambient		
Diluent:	Methanol gradient grade (1.06007)		
Sample Prep:	Powdered Ginger. Transfer about 4 g of air dried, coarsely powdered material, accurately weighed, to a glass-stoppered conical flask. Add 100 mL of alcohol, insert a stopper into the flask and macerate for 24 hours. Shake frequently during the first 8 hours, and then allow to stand. Filter rapidly, taking precautions against loss of alcohol (retained filtrate from the test for articles of Botanical Origin, Alcohol-Soluble Extractives, Method_2).		
Pressure Drop:	102 bar (1479 psi)		

**Figure 9:** HPLC chromatogram and peak retention times for ginger extract.



Compound	RT (min)	Tailing factor (USP)	Rs	RRT
t <sub>0</sub> (void volume)	1.8			
Curcumin	10.1	1.0		1.0
Desmethoxycurcumin	11.6	1.0	3.1	1.2
Bisdsmethoxycurcumin	13.3	1.1	3.0	1.3

21 between the latter two components with a relative retention times of 0.8, 1.0, and 2.0 for the three peaks, with a tailing factor of 1.0. If we examine the system suitability criteria of the USP monograph, it states that the relative retention time for 6-gingerol, capsaicin, and 6-shogaol should be about 0.8, 1.0, and 1.9 respectively. And with regard to resolution, it states that it should be not be less than 3 between 6-gingerol and capsaicin peaks, and not less than 10 between capsaicin and the 6-shogaol peaks, with tailing factors not more than 2 for any of the peaks. Thus all criteria for the compositional analysis of ginger extract are met. The calibration plots produced LODs from 1.9-5.4 ppm and LOQs of 5.8-16.1.

### **Summary**

If you have an interest in testing your dietary supplement products using regulated chromatographic methods, it's important to know the details of the analytical methods involved. Being compliant with the monograph is essential, and understanding how different products can be used and operated based on global directives and guidelines. However, what's also important is being able to optimize and fine tune methods, when a monograph is in clear need of improvement or modification. Merck is positioned and committed to supporting its many products that are being used in this type of regulated environment.