

# NATURAL PRODUCTS



## APPLICATION NOTEBOOK

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# NATURAL PRODUCTS



## RESEARCH

- Drug discovery
- Mechanism studies



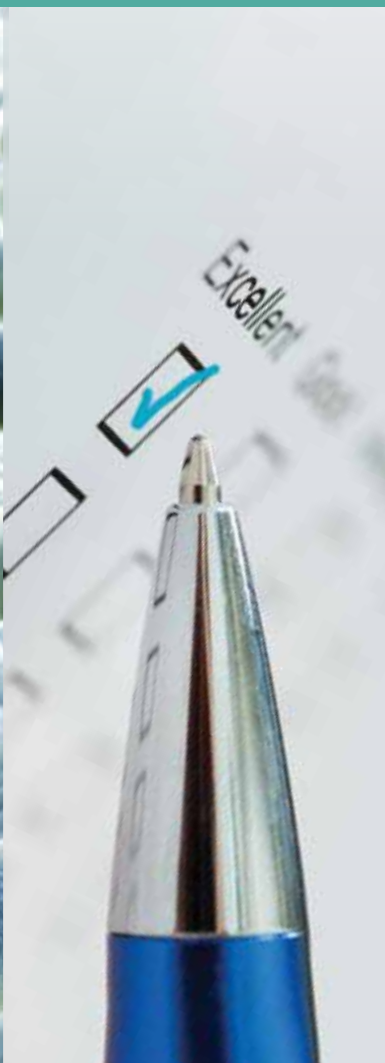
## REGULATORY

- Regulatory surveillance
- Standards setting and guidance



## MANUFACTURING

- Product development
- QC testing



## It all starts with R&D

Natural Products begins with research for either novel drug discovery or a mechanism study. Drug discovery is the process of screening samples from naturally occurring sources -- botanicals, microorganisms, and marine life – to uncover new chemical scaffolds with therapeutic benefit. Mechanism study scientists seek to improve understanding of herbal medicine and uncover the link of plants to disease targets and biomarkers. Scientists from both groups face many challenges that require rigorous analytical tools. One of the top needs is to deliver meaningful results more quickly.



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## Regulatory: the protector and safety net

Global regulatory agencies, such as the U.S. Food and Drug Administration (FDA), the Chinese FDA, and the European Medicines Agency, maintain an important role in ensuring all dietary supplements and herbal remedies are safe and effective. Among their chief concerns are adulterated or contaminated products, traceability and inconsistencies in quality.



## QC and Manufacturing: a critical and final gate

The key challenges of QC and Manufacturing are to support a healthy product development pipeline, verify ingredient and finished product quality and to facilitate continuous improvement in the manufacturing process. New products and global supply chain issues introduce additional challenges making the twin goals of productivity and compliance no easy task.

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# CHAPTER 1



## Drug Discovery and Mechanism Study



## UPLC-QTOF-MS and UNIFI

## Using Natural Products Application Solution with UNIFI to Identify Chemical Ingredients and Deduce Possible Herbal Composition from Unknown Traditional Medicine Tablets

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### APPLICATION BENEFITS

UltraPerformance LC® (UPLC®) is combined with orthogonal quadrupole time-of-flight mass spectrometry within the Natural Products Application Solution with UNIFI to identify the unknown chemical ingredients and deduce the potential herbal composition of Traditional Medicine tablets. This comprehensive workflow enables researchers to determine the chemical and herbal composition of completely unknown samples, while significantly enhancing accuracy and efficiency.

### WATERS SOLUTIONS

Natural Products Application Solution with UNIFI®

ACQUITY UPLC® I-Class System

Xevo® G2-S QToF Mass Spectrometer

UNIFI Scientific Information System

Waters Analytical Standards and Reagents

### KEY WORDS

Traditional Medicine Library, ingredient analysis of unknown natural products, UPLC/QToF MS<sup>E</sup>, identification of herbal materials, DanShen, (*Salvia miltiorrhiza*), SanQi, (*Panax Notoginseng*)

### INTRODUCTION

Traditional Medicines are known for being comprised of extremely complex elements that can include a variety of plants, extracts, minerals, and animal parts. The critical foundation for their effectiveness originates from the chemical ingredients of their raw herbal materials. In a related application note,<sup>1</sup> we have described how to efficiently identify chemical ingredients from samples with known plants by utilizing the Natural Products Application Solution with UNIFI and its Traditional Medicine Library. For this type of analysis, a researcher only needs to import the compounds associated with the known plants from the Traditional Medicine Library, and use them as search targets to compare with the acquired data. The result is a list of identified components that can be used for further verification. The workflow has a straightforward strategy and its analytical procedure contains simple steps.

However, the reality is that researchers often need to identify chemical ingredients and deduce possible herbal composition for a completely unknown Traditional Medicine product. This type of work is extremely difficult; many times one may not even know where to start. Because the available sample background information is close to none, even with the large amount of data that can be generated using popular approaches such as LC/MS, researchers are still challenged to narrow down their scope and to obtain meaningful information quickly.

The classic workflow for profiling the components of unknown Traditional Medicine products is this: to manually extract each individual chromatographic peak, propose possible molecular formula based on the exact mass of intact protonated or deprotonated ions, and that result is used to search online libraries to obtain potential hits. Afterwards, fragmentation pathways are deduced based on MS/MS fragment ions so that the proposed chemical structure of a target component is confirmed. This process not only requires manual intervention by researchers in almost every single step throughout the entire process, but also has very high demands for expertise levels (both in natural products and in chemistry), as the researcher must be able to find answers in oceans of information.

## EXPERIMENTAL

### Sample preparation

Two tablets of a TCM product were used for the analysis. After removing the coatings, they were ground into powder. 500 mg of the powder was dissolved in 50 mL MeOH/H<sub>2</sub>O (3:1) by ultrasonic the solution for 5 minutes. The final solution was filtered through a 45 µm membrane prior to injection.

### LC conditions

LC system: ACQUITY UPLC I-Class  
with FTN Sample Manager

Column: ACQUITY UPLC HSS T3  
2.1 x 100 mm, 1.8 µm

Column temp.: 40 °C

Sample temp.: 15 °C

Mobile phase: A: water  
(0.1% formic acid);  
B: acetonitrile

Gradient:

Time	Flow rate	Solvent A	Solvent B	Curves
(mL/min)	(%)	(%)		
0	0.6	90	10	Starting
1	0.6	90	10	6
12	0.6	5	95	6
14	0.6	0	100	1
17	0.6	90	10	1

### MS conditions

MS system: Xevo G2-S QTof

Acquisition range: 100-1500 Da

Scan time: 0.1 s

Acquisition mode: MS<sup>2</sup> ESI- and ESI+ in  
resolution mode

Lock mass: Leucine Enkephalin (LE)  
1 ppm (scan for 0.3 s,  
interval: 15 s)

Capillary voltage: 3 kV (ESI+)/2.5 kV (ESI-)

Cone voltage: 100 V

Collision energy (eV): low CE: 6/High CE:  
20-50

Source temp.: 120 °C

Desolvation temp.: 500 °C

Cone gas flow: 30 L/h

Desolvation gas flow: 1000 L/h

Acquisition time: 17 min

### Data acquisition, processing, and reporting

UNIFI Scientific Information System with  
Traditional Medicine Library

The Natural Products Application Solution with UNIFI provides a completely new and comprehensive strategy for solving such a problem. It utilizes the ACQUITY UPLC I-Class System and Xevo G2-S QToF MS to acquire data-independent MS<sup>E</sup> data. These data are then searched against the integrated Traditional Medicine Library. The structures of the matched components are verified by MassFragment™ using their corresponding fragment ions. Finally, detailed information of the identified components are displayed automatically in UNIFI using preset workflow templates.

This application note describes how to use the Natural Products Application Solution with UNIFI to identify chemical ingredients and deduce possible herbal content from unknown samples using a Traditional Chinese Medicine (TCM) tablet product as an application example.

## RESULTS AND DISCUSSION

UPLC and QToF MS were used for the ingredient separation and MS data acquisition of the unknown TCM tablet sample. The Natural Products Application Solution with UNIFI along with the Traditional Medicine Library was used for the data processing, which resulted in 288 components identified by having a match from the library. Among them, 37 high-level ingredients were initially verified and labeled as “confirmed” based on fragment analysis by MassFragment.

By associating the confirmed components with potential plants, it was deduced that the tablets may contain DanShen (*Salvia miltiorrhiza*) and SanQi (*Panax notoginseng*). By searching the Internet to find known TCM recipes that contain these two herbs, the chemical ingredients (listed from the Traditional Medicine Library) of related herbs from matched recipes can then be used to compare with components found from experiment data. As a result, for this example, 59 major chemical ingredients from the tablets were verified, all from DanShen and SanQi. Hence, the final conclusion was that herbal composition of this TCM product is DanShen and SanQi, which leads us to believe that this product was possibly to be the Sanqi Danshen Tablet or the Compound DanShen Tablet.

The workflow of chemical ingredient analysis with known plants using the Natural Products Application Solution with UNIFI has been described previously in detail.<sup>1</sup> For samples that are complete unknown, additional steps would be deducing possible herbal identities, searching online for potential known TCM recipes that contain these herbs, and, from the UNIFI Traditional Medicine Scientific Library, re-importing corresponding compounds related to potential herbs listed in the matched recipes to verify the existence of these herbs. Figure 1 shows the complete workflow of chemical and herbal ingredient identification for unknown samples.

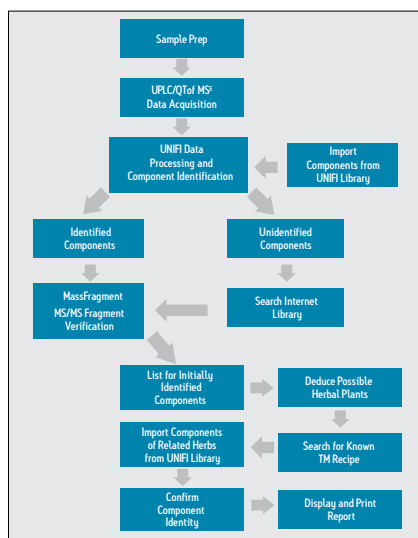


Figure 1. Complete workflow for identifying chemical and herbal ingredient for unknown samples.

Figure 2A shows the UPLC/QToF MS base peak ion (BPI) chromatogram for the unknown TCM tablet. With the UNIFI Scientific Information System, the same results can also be displayed in a 3D format, shown in Figure 2B. Compared with 2D plot (Figure 2A), the information displayed from the 3D plot is closer to the true representative of the components within the sample. It provides a directive visual profile that is much more intuitive for observing the entire chemical component distribution of the sample. For example, from Figure 2B, one can quickly conclude that the range of molecular weights of the chemical ingredients from this sample is mainly between 400 and 1000 Da. In addition, it also allows chemists to have a quick observation on the compounds' coeluting status within the entire run.

The plot displayed in Figure 2B is generated from Apex 3D image scan mode, which is unique to UNIFI. Apart from providing a direct visual effect, it helps to enhance the accuracy of the qualitative and quantitative work for future steps, and it provides major advantages in identifying and eliminating background peaks.

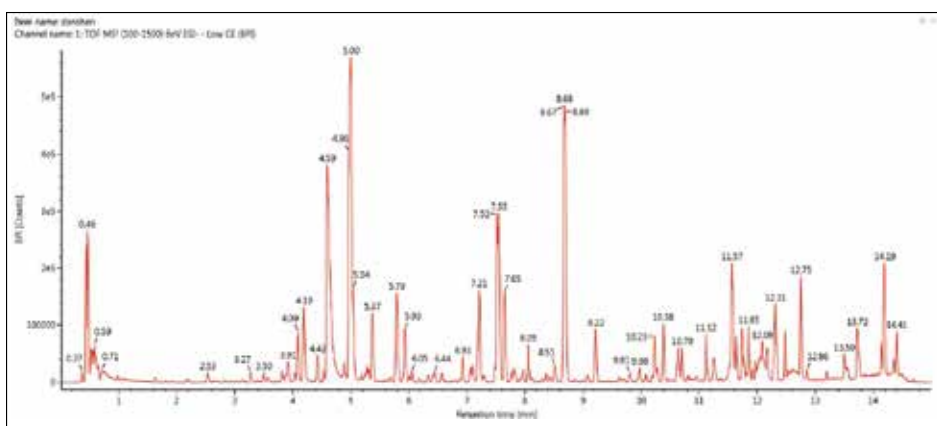


Figure 2A. UPLC/QToF MS base peak ion (BPI) chromatogram of the unknown TCM tablet.

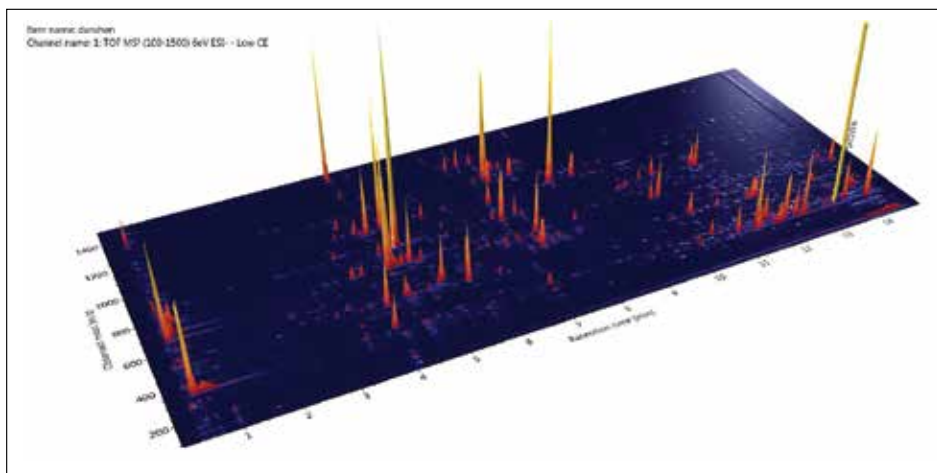



Figure 2B. 3D LC/MS plot of the unknown TCM tablet.

With the Natural Products Application Solution with UNIFI:

1. All steps are completed in automated fashion without the need for operator's intervention. These steps include chromatographic peak extraction, elemental composition determination, Traditional Medicine Library searching, fragment ion structural elucidation, and component identification.
2. Researchers only need to verify whether the fragment ion structural elucidation that was automatically provided by MassFragment is reasonable or not.
3. If a false positive is suspected, or any component that is not matched from the Traditional Medicine Library, the researcher can then initiate a manual process for further identification.

Compared with conventional research protocols, the Natural Products Application Solution with UNIFI converts a manual process of seeking meaningful targets from oceans of information into an automated workflow. This significantly reduces the blindness of the work and enhances productivity. Meanwhile, the demands for the researcher's expertise level is greatly reduced as well.

Figure 3 shows the UNIFI's results for the chemical ingredients identification of the unknown TCM tablet after data processing. The ingredient table shown in Figure 3B lists the components initially identified from the library match. It is possible to have multiple isomers corresponding to the same chromatographic peak at the same retention time. This is when researchers need to verify whether a match is reasonable by looking at the adduct ions as well as the structural elucidation of the fragmentation ions (blue icon  ).

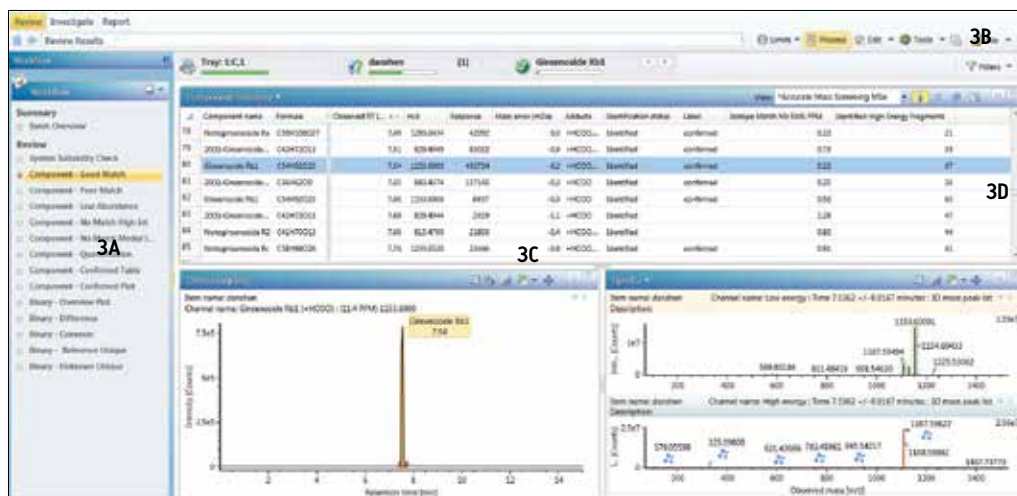


Figure 3. Chemical ingredient identification results in UNIFI for the unknown TCM tablet. 3A shows the template workflows; 3B is the component identification list; 3C is the selected ion chromatogram of single component corresponding to 3B; and 3D is the respective mass spectrum of 3C.



For example, the chromatographic peak at 7.54 minutes is automatically identified by UNIFI as ginsenoside Rb1 or Yesanichinoside E. By clicking the window represented by Figure 3D, an enlarged figure is obtained (Figure 4). Since all fragment ions have been automatically elucidated by the MassFragment, researchers can easily verify whether the fragmentation pathway is reasonable or not. In this example, the compound's cleavage started from the glycosidic bond, and ended at the formation of protopanaxadiol aglycone fragments, indicating the reasonable structure should be the ginsenoside Rb1, which was then labeled as confirmed.

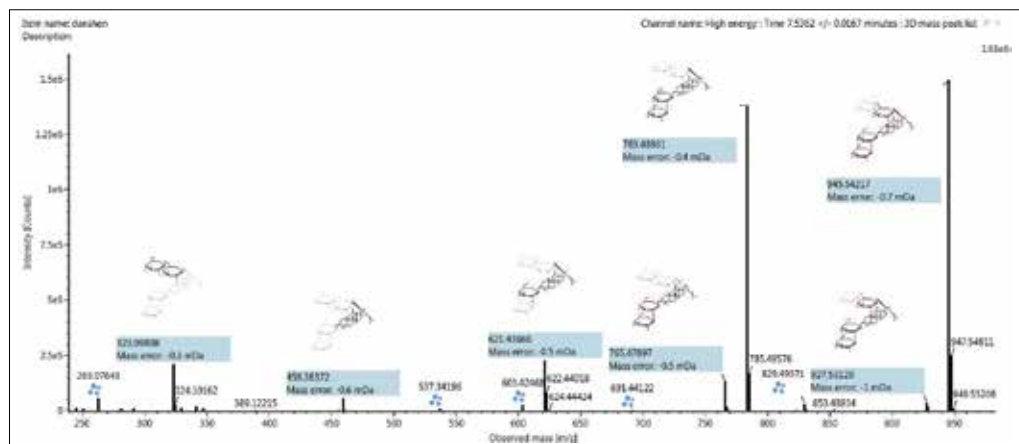


Figure 4. Structural elucidation of the fragmentation ions of ginsenoside Rb1 by MassFragment.

After component verification and confirmation as shown above, it can be observed that this unknown tablet contains chemical classes such as ginsenosides, salvia phenol, tanshinone, notoginsenoside, etc. These components are clearly associated with the herbal materials of DanShen and SanQi. Further online search indicated that some known TCM recipes that contain these two herbal ingredients could be Sanqi Danshen tablet, and Compound DanShen tablet. Of course, other recipes were also discovered, such as recipes containing American ginseng (*Panax quinquefolius*) or ShanZha (*Crataegi fructus*), etc.

Now, this research project has progressed from a non-targeted screening at the initial stage to a targeted screening, which is the chemical ingredient identification of known plants. This part of the workflow has already been well defined,<sup>1</sup> which is to import chemical ingredients of known herbal materials (*Salvia miltiorrhiza*, *Panax notoginseng*, *Panax quinquefolius*, and *Crataegi fructus*) from the Traditional Medicine library into the target list of the UNIFI analysis method, and compare them with the experimental data.

The result was that no component was matched with the ingredients listed for the American ginseng and ShanZha (such as American ginseng saponin, gynostemma saponin, ShanZha saponin, etc., which are characteristic to these two herbs). This provides further confirmation to the initial conclusion that this tablet doesn't contain American ginseng and ShanZha. Meanwhile, all major chromatographic peaks obtained from the sample matched well with the major ingredients of DanShen and SanQi (59 key components confirmed), which is listed in Table 1.

Thus our final conclusion is this tablet was mainly composed of DanShen (*Salvia miltiorrhiza*) and SanQi (*Panax notoginseng*). The commercial product could be the Sanqi Danshen Tablet, or the Compound DanShen Tablet.

-	Component name	Formula	RT (min)	Response	m/z	Error (mDa)	Error (ppm)	Adducts	Label
1	Salvianic acid A	C9H10O5	0.99	7002	197.0451	-0.4148	-2.10	-H	confirmed
2	Protocatechuic aldehyde	C7H6O3	1.63	4283	137.0242	-0.1994	-1.41	-H	confirmed
3	Uthospermic acid	C27H22O12	3.50	19188	537.1027	-1.1279	-2.10	-H	confirmed
4	Salvianolic acid D	C20H18O10	3.57	7772	417.0820	-0.6713	-1.61	-H	confirmed
5	20-O-Glucopyranosyl ginsenoside Rf	C48H82O19	4.04	24616	1007.5421	-1.1674	-1.16	+HCOO, -H	confirmed
6	Rosmarinic acid	C18H16O8	4.09	60848	359.0770	-0.2202	-0.61	-H	confirmed
7	Salvianolic acid A	C26H22O10	4.19	129863	493.1138	-0.1842	-0.37	-H	confirmed
8	20-O-Glucopyranosyl ginsenoside Rf	C48H82O19	4.43	62970	1007.5423	-0.9179	-0.91	+HCOO, -H	confirmed
9	Notoginsenoside Fc	C58H98O26	4.51	1010	1255.6329	0.0889	0.07	+HCOO	confirmed
10	Uthospermic acid B	C36H30O16	4.60	724974	717.1453	-0.8088	-1.13	-H	confirmed
11	Notoginsenoside R1	C47H80O18	4.64	204023	977.5314	-1.2607	-1.29	+HCOO, -H	confirmed
12	Baicalin	C21H18O11	4.69	5640	445.0780	0.3632	0.82	-H	confirmed
13	Ginsenoside Rd	C48H82O18	4.97	368264	991.5475	-0.8503	-0.86	+HCOO, -H	confirmed
14	Ginsenoside Rg1	C42H72O14	4.99	1102946	845.4903	-0.0922	-0.11	+HCOO, -H	confirmed
15	Ginsenoside Rg1	C42H72O14	5.25	12929	845.4902	-0.2179	-0.26	+HCOO	confirmed
16	Monomethyl lithospermate	C28H24O12	5.37	114604	551.1197	0.2131	0.39	-H	confirmed
17	Salvianolic acid A	C26H22O10	5.60	1545	493.1141	0.0894	0.18	-H	confirmed
18	Salvianolic acid C	C26H20O10	5.79	175372	491.0990	0.6454	1.31	-H	confirmed
19	Dimethyl lithospermate	C29H26O12	5.81	10725	565.1354	0.2655	0.47	-H	confirmed
20	Dimethyl lithospermate	C29H26O12	5.93	92868	565.1357	0.5784	1.02	-H	confirmed
21	20-O-Glucopyranosyl ginsenoside Rf	C48H82O19	6.33	19364	1007.5433	0.0201	0.02	+HCOO, -H	confirmed
22	Notoginsenoside T	C64H108O31	6.62	8889	1417.6854	-0.2913	-1.21	+HCOO, -H	confirmed
23	Ginsenoside Fa	C59H100O27	6.93	44213	1285.6447	1.2916	0.00	+HCOO, -H	confirmed
24	Ginsenoside Rg1	C42H72O14	7.06	34689	845.4906	0.2163	0.26	+HCOO	confirmed
25	Cryptocetate	C18H22O3	7.10	9539	285.1497	0.0427	0.15	-H	confirmed
26	Notoginsenoside T	C64H108O31	7.16	7914	1417.6854	-0.2771	-0.20	+HCOO, -H	confirmed
27	Notoginsenoside Fa	C59H100O27	7.20	83126	1285.6443	0.8786	0.68	+HCOO, -H	confirmed
28	Notoginsenoside R2	C41H70O13	7.21	219884	815.4801	0.3041	0.37	+HCOO, -H	confirmed
29	Ginsenoside Rb3	C53H90O22	7.28	20796	1137.6065	0.3184	0.28	+CH3COO	confirmed
30	Notoginsenoside S	C63H106O30	7.43	871	1387.6732	-1.9177	-1.38	+HCOO, -H	confirmed
31	Notoginsenoside Fa	C59H100O27	7.49	42092	1285.6434	-0.0362	-0.03	+HCOO, -H	confirmed
32	20(S)-Ginsenoside Rg3 (Ginsenoside Rg3)	C42H72O13	7.51	83032	829.4949	-0.5657	-0.68	+HCOO, -H	confirmed
33	Ginsenoside Rb1	C54H92O23	7.54	492734	1153.6009	-0.2281	-0.20	+HCOO, -H	confirmed
34	20(S)-Ginsenoside Rb1 (Ginsenoside Rb1)	C36H62O9	7.65	157145	683.4374	-0.2032	-0.30	+HCOO	confirmed
35	Ginsenoside Rb1	C54H92O23	7.66	8457	1153.6006	-0.5535	-0.48	+HCOO	confirmed
36	Notoginsenoside Fc	C58H98O26	7.76	23444	1255.6320	-0.8412	-0.67	+HCOO, -H	confirmed
37	Ginsenoside Rb2	C53H90O22	8.06	97093	1123.5891	-1.4610	-1.30	+HCOO, -H	confirmed
38	20(S)-Ginsenoside Rb1 (Ginsenoside Rb1)	C36H62O9	8.52	37225	683.4371	-0.5177	-0.76	+HCOO	confirmed
39	Notoginsenoside Fe	C47H80O17	8.54	15952	975.5528	-0.6039	-0.62	+CH3COO	confirmed
40	Ginsenoside Rd	C48H82O18	8.68	880119	991.5489	0.5661	0.57	+HCOO, -H	confirmed
41	Ginsenoside Rb3	C53H90O22	9.11	1715	1123.5896	-0.9565	-0.85	+HCOO	confirmed
42	Ginsenoside Rd	C48H82O18	9.21	105207	991.5476	-0.7623	-0.77	+HCOO, -H	confirmed
43	20-O-Glucopyranosyl ginsenoside Rf	C48H82O19	9.39	3311	961.5371	-0.6979	-0.73	-H	confirmed
44	Ginsenoside Rb4	C36H60O8	10.23	65829	665.4266	-0.4154	-0.62	+HCOO	confirmed
45	Danshenxinin A	C18H16O4	10.27	22334	295.0972	-0.4093	-1.39	-H	confirmed
46	Ginsenoside Rb4	C36H60O8	10.39	97784	665.4261	-0.9420	-1.42	+HCOO	confirmed
47	20(S)-Ginsenoside Rg3 (Ginsenoside Rg3)	C42H72O13	10.70	68724	829.4949	-0.6142	-0.74	+HCOO, -H	confirmed
48	Ginsenoside F2	C42H72O13	10.79	8835	829.4944	-1.1291	-1.36	+HCOO	confirmed
49	Methylenedithyrotan-shinquinone	C18H16O3	11.12	54015	279.1022	-0.5008	-1.79	-H, +CH3COO	confirmed
50	Salviolone	C18H20O2	11.25	28599	313.1442	-0.3307	-1.06	+HCOO	confirmed
51	Dihydrotanshinone I	C18H14O3	11.57	171319	277.0868	-0.2240	-0.81	-H	confirmed
52	Sugiol	C20H28O2	11.85	53468	299.2016	-0.0323	-0.11	-H	confirmed
53	Tanshinone II B	C19H20O3	12.31	95703	295.1340	0.0329	0.11	-H	confirmed
54	Miltirone	C19H22O2	12.48	47717	281.1547	-0.0260	-0.09	-H	confirmed
55	Salvianen	C21H21NO2	12.66	3930	378.1703	-0.7997	-2.11	+CH3COO	confirmed
56	Miltiradiol	C19H22O3	13.37	5256	297.1492	-0.4174	-1.40	-H	confirmed
57	Ursolic acid	C30H48O3	13.52	53957	455.3529	-0.1933	-0.42	-H	confirmed
58	Linolic acid	C18H32O2	13.73	80398	279.2330	0.0428	0.15	-H	confirmed
59	Hexadecanoic acid	C16H32O2	14.19	145228	255.2332	0.2836	1.11	-H	confirmed

Table 1. Summary table of identified components for the unknown tablet. The table was automatically obtained by importing the Component Summary Reporting Template in UNIFI.

## CONCLUSIONS

This application note has described the overall workflow obtained by applying the Natural Products Application Solution with UNIFI to identify and deduce chemical and herbal composition for unknown samples. The workflow progressed from an initial non-targeted screening into a targeted screening process.

Sample analysis by UPLC/QToF MS required just 14 minutes. The initial non-targeted screening identified 37 major chemical ingredients, which clearly showed association with DanShen (*Salvia miltiorrhiza*) and SanQi (*Panax notoginseng*). By searching the known TCM recipes from the Internet, ingredients related to relevant herbs (*Salvia miltiorrhiza*, *Panax notoginseng*, *Panax quinquefolius*, and *Crataegi fructus*) were matched against components detected from experiment data for the second time. As a result, among the 103 chemical ingredients associated with *Salvia miltiorrhiza* and *Panax notoginseng* within the Traditional Medicine Library, 59 were identified and confirmed. No match was found to match any of the major chemical ingredients related to the other two potential herbs, *Panax quinquefolius* and *Crataegi fructus*. This led to our final conclusion that the unknown product could be either a Sanqi Danshen or Compound DanShen tablet.

The Natural Products Application Solution with UNIFI is based on UPLC/QToF MS<sup>E</sup> data acquisition, accompanied by the Traditional Medicine Scientific Library, which are integrated with an automatic identification process. This is a novel approach for ingredient analysis of total unknown samples. The result is the reduction of the blindness of such a research and significant enhancement of productivity.

## Reference

1. Using Natural Products Application Solution with UNIFI for the Identification of Chemical Ingredients of Green Tea Extract. Waters Application Note, November 2013; 720004837en.

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## UPLC-QTOF-MS and UNIFI

## Using Natural Products Application Solution with UNIFI for the Comparison of the Chemical Ingredients of Shuanghuanglian Oral Drink from Two Different Manufacturers

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### APPLICATION BENEFITS

Using the binary comparison workflow available with the Natural Products Application Solution with UNIFI, this application demonstrates the fast and accurate detection of the differences and similarities of chemical ingredients in Shuanghuanglian oral drink from two different manufacturers. The solution improves researchers' ability to understand ingredient differences of the same product manufactured by different companies, and it enables more accurate quality control of a product between batches made by a single manufacturer. UNIFI Software provides a simple and effective approach to all binary comparisons among similar samples.

### WATERS SOLUTIONS

Natural Products Application Solution with UNIFI®

ACQUITY UPLC® I-Class

Xevo® G2-S QToF MS

UNIFI Scientific Information System

Waters Analytical Standards and Reagents

### KEY WORDS

Traditional Medicine Library, binary comparison of natural products, UPLC®/QToF MS, Shuanghuanglian oral drink, natural product quality control, product batch comparison

### INTRODUCTION

Traditional Medicine is as complex as it is amazing, having gone through development for thousands of years. However, there has not been a well accepted, systematic, and scientific method to evaluate the authenticity and quality of traditional medicine products. For example, there has not been a consistent and effective method for the control of the active ingredients of raw materials in the Traditional Chinese Medicine (TCM). The same is true for methods to evaluate raw materials with different planting techniques and location, or different harvest times, or different processing methods.

In recent years, pharmaceutical researchers have carried out many studies focused on improving Traditional Medicine quality standards, and have already accumulated good amount of valuable experience and methods. Still, generally speaking, binary comparison of Traditional Medicine is time-consuming and expensive. In addition, some methods lack scientific support and can be not very objective. This application note provides a novel approach by incorporating advanced separation and detection technologies with the Natural Products Application Solution with UNIFI.

Similarities and differences in chemical ingredients of raw material from different locations, differences in the same product produced by different manufacturers, and product quality from different batches by one manufacturer all require a comparison of similar samples. The goal is to provide a clear direction for further R&D by identifying any differences via observing the chemical ingredients of each sample. With the constant evolution in analytical technology, the application of high-resolution LC/MS instrumentation such as UPLC/QToF MS has been gaining steadily in acceptance. Application of these technologies can help shorten analysis times and increase separation efficiency. Meanwhile, UPLC/QToF MS offers advantages in wide adaptability, high specificity, low detection limits, small injection volumes, low solvent consumption, highly automated workflows, strong capability for identification, etc. As a result, analytical technologies are playing a more important role for quality control of traditional medicines.

### Sample preparation

200  $\mu$ L Shuanghuanglian oral drink was dissolved in 2 mL H<sub>2</sub>O (effectively 10X dilution) for later use. Injection volume was 1  $\mu$ L.

### LC conditions

LC system: ACQUITY UPLC I-Class  
with FTN Sample Manager

Column: ACQUITY UPLC HSS T3  
2.1 x 100 mm, 1.8  $\mu$ m

Column temp.: 40 °C

Sample temp.: 15 °C

Mobile phase: A: water (0.1% formic acid);  
B: acetonitrile

Gradient:

Time	Flow rate (mL/min)	Solvent A (%)	Solvent B (%)	Curves
0	0.5	95	5	Starting
1	0.5	90	10	6
8	0.5	55	45	6
12	0.5	45	55	6
13	0.5	5	95	6
15	0.5	95	5	1

### MS conditions

MS system: Xevo G2-S QTof MS

Acquisition range: 100-1500 Da

Scan time: 0.1 s

Acquisition mode: ESI+, ESI-;  
resolution mode; MS<sup>E</sup>

Lock mass: Leucine Enkephalin (LE)  
1 ppm (scan for 0.3 s,  
interval: 15 s)

Capillary voltage: 3 KV (ESI+)/2.5 KV (ESI-)

Cone voltage: 100 V

Collision energy (eV): Low CE: 6/High CE:  
20-45

Source temp.: 120 °C

Desolvation temp.: 500 °C

Cone gas flow: 60 L/h

Desolvation gas flow: 1000 L/h

Acquisition time: 15 min

### Data acquisition, processing, and reporting

UNIFI Scientific Information System



In this application note, we use the comparison of Shuanghuanglian oral drink from two different manufacturers as an example to illustrate the use of the Natural Products Application Solution with UNIFI. The solution combines the ACQUITY UPLC I-Class System, the Xevo G2-S QToF MS, as well as a Traditional Medicine Library, and uses the UNIFI's binary comparison workflow to compare similar samples for the purpose of quickly identify differences between the two samples; thus providing the scientific basis for better quality control and generating quality standards at later stage. As a result, productivity is greatly improved and the requirements for the operator's expertise level is significantly reduced.

The Shuanghuanglian oral drink contains three herbs: JinYinHua (*lonicerae japonicae flos*), known as honeysuckle; HuangQin (*scutellariae radix*), known as scutellaria; and LianQiao (*forsythiae fructus*), known as forsythia. The product is used to alleviate symptoms caused by upper respiratory tract infections, tonsillitis, pharyngitis, and bacterial and viral infectious diseases such as viral pneumonia, etc. Within this oral drink, known active ingredients include compounds such as Baicalin, forsythin, and wogonin. Being popular on the market, this product is being made by numerous manufacturers in China. Therefore, we decided to use this representative application example to illustrate the binary comparison workflow provided within the Natural Products Application Solution with UNIFI (Figure 1).

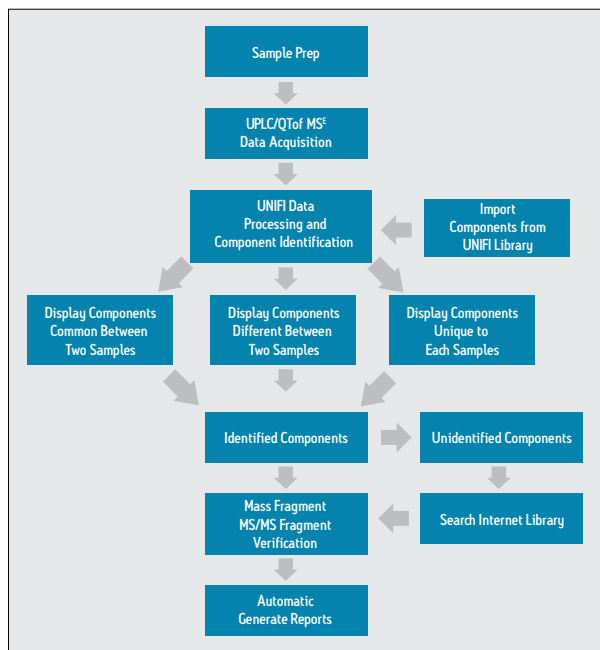


Figure 1. The binary comparison workflow within the Natural Products Application Solution with UNIFI.

## RESULTS AND DISCUSSION

UPLC and quadrupole time-of-flight mass spectrometry were used to acquire analytical data of the chemical ingredients in Shuanghuanglian oral drink from two different manufacturers. The binary comparison workflow in Natural Products Application Solution with UNIFI combining with its Traditional Medicine Library was used for data mining. The results obtained have the following information clearly displayed: summary plot of components that are common, summary plot of components that are different, as well as the tables for components that are unique to each sample. Final results indicated that ingredient contents such as forsythiaside A, forsythiaside B, dextrorotation hydroxyl forsythiaside A, arctin, and rutin were higher in manufacturer 1, while ingredient contents such as baicalin was higher in manufacturer 2.

The Natural Products Application Solution with UNIFI includes five preset binary comparison workflow templates, and one report template. As a result, the entire analytical process can be automated from data collection, to peak picking, to library search and structural elucidation (of fragment ions by MassFragment), to producing the final report.

The UPLC/QToF MS base peak ion (BPI) chromatograms of the two Shuanghuanglian oral drink products were compared with the mirror (or, binary) plot, shown in Figure 2. This plot clearly demonstrates the advantages of using UPLC for the analysis of complex natural products. UPLC provides not only a shorter run time (i.e., an effective separation time of 12 min), but also higher separation efficiency and peak capacity. Meanwhile, QToF MS also provides exact mass MS data. In addition, as shown in Figure 2, the chemical ingredients of the two samples are not only complicated, but also very similar. Without the help of an informatics platform to quickly and accurately identify similarities and differences from samples, this is a complex task that is nearly impossible to accomplish.

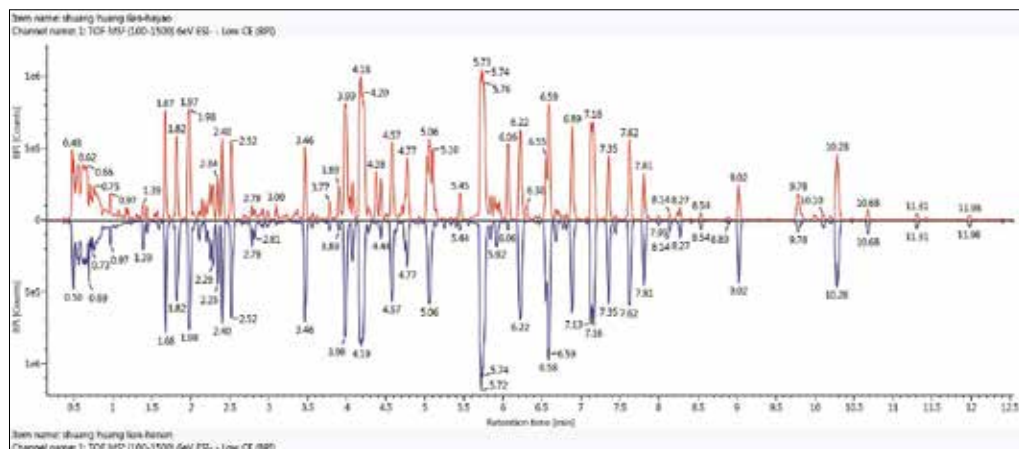


Figure 2. The mirror plot of the UPLC/QToF MS base peak BPI ion chromatograms for the two Shuanghuanglian oral drinks (Figure 2A. product of manufacturer 1; Figure 2B. product of manufacturer 2).

In the natural products field, having a clear understanding of the ingredients in detail is the foundation for all future steps for any research, whether for qualitative identification of chemical ingredients from a single sample, or for component comparison of two different samples. In a previous application note,<sup>1</sup> we have described the analytical workflow of ingredient identification for samples with known plants. Following this workflow, all chemical ingredients related to the three herbal plants (honeysuckle, scutellaria, and forsythia) were imported into the component list from the Traditional Medicine Library for data processing. This was then followed by verifying the identified components in a manner described in previous application note.<sup>1</sup> Respective results can be easily viewed from the Review window of UNIFI by clicking any of the five preset binary comparison templates. For example, Figure 3 shows the result displayed after clicking the Binary-OverviewPlot template.

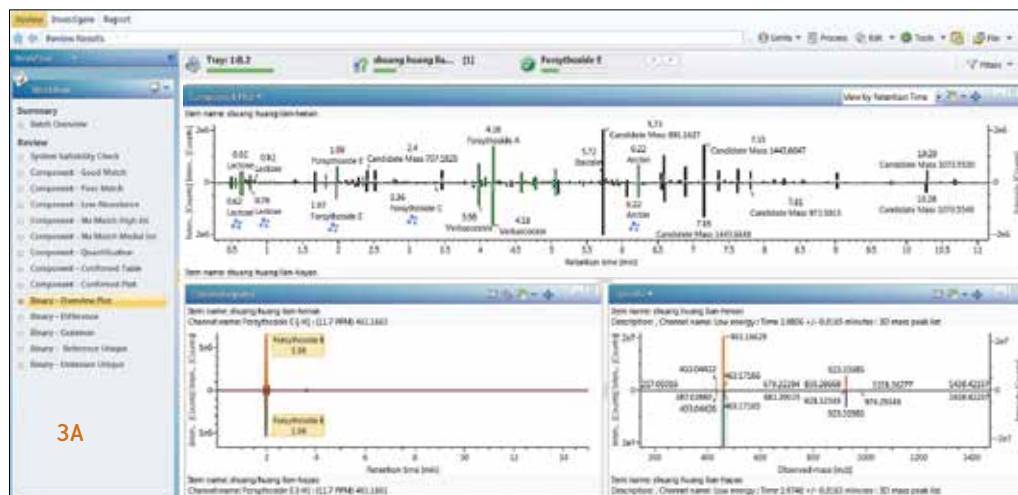


Figure 3. The binary comparison result obtained after data processed by UNIFI for Shuanghuanglian oral drinks from two different manufacturers.

Figure 3A shows the 14 preset workflow templates in UNIFI. Figure 3B is the comparison of the UPLC/QToF MS component summary plots for samples from two manufacturers. Here, green bars represent ingredients that were matched and automatically identified with the Traditional Medicine Library; the black bars represent ingredients that did not have a match and thus cannot be automatically identified. Figure 3C shows the mirror plot of the selected ion chromatograms (XIC) corresponding to any component that user had clicked from. Figure 3B (here, it displayed the XIC mirror plot of forsythoside B at retention time 4.03 minutes). Figure 3D is the mirror plot of the low-energy MS spectra of the ingredient (shown in 3C) from the two samples.

Further understanding of the similarities and differences between these two samples can be obtained by clicking either the Binary-Common workflow or the Binary-Difference workflow. Ingredients that are common or different between the two samples can be directly observed. For example, Figure 4 shows the results provided by the Binary-Common workflow. Figure 4A lists the components that are common in two samples (total of 1978 from preliminary result). Figure 4B shows levels of MS responses in these two samples for the each of the components listed in Figure 4A, which is a reflection of the relative amount of this ingredient in each sample (wogonin in this case). Similar information is presented if the Binary-Difference workflow is clicked, and the results will show ingredients that are different between these two samples (total of 408 from preliminary result).

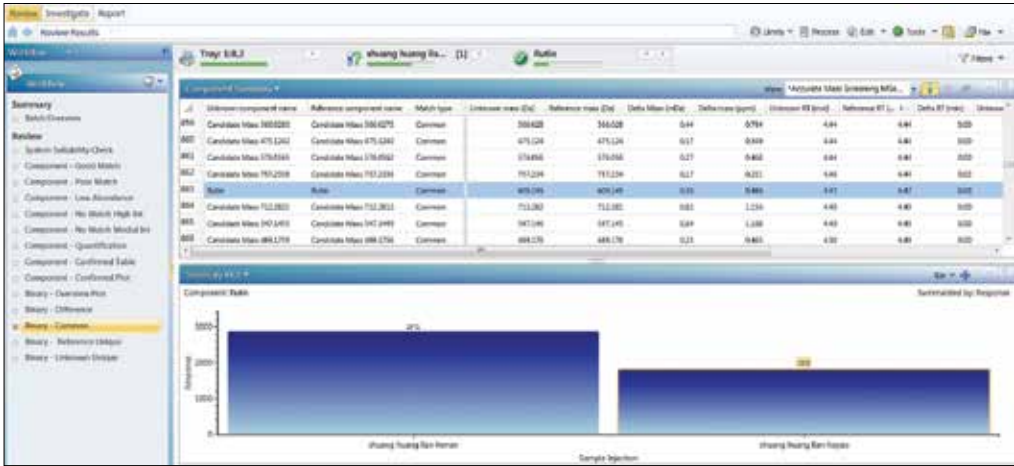


Figure 4. Result obtained after clicking the Binary-Common template.

Using the Binary-Common and Binary-Difference workflow templates, the ingredients that are common and different for these two samples can also be shown in bar chart format, which can be more intuitive. For example, the ingredient difference of these two samples obtained by clicking Binary-Difference can be easily viewed by clicking Component Summary Plot from the Component Summary list (Figure 5). This plot allows researchers to observe the differences between two samples clearly and intuitively.

The results of analyzing these two traditional medicine drinks suggested that the baicalin content from manufacturer 2 is significantly higher than that of manufacturer 1, while the content of forsythiaside A, forsythiaside B, dextrorotation hydroxyl forsythiaside A, etc. are much higher in manufacturer 1 than that of manufacturer 2. Figure 5 also clearly shows that there are components that were not identified, being not matched with the Traditional Medicine Library. In this case, we can use UNIFI's Structural Elucidation Tool for further identification should it be necessary.

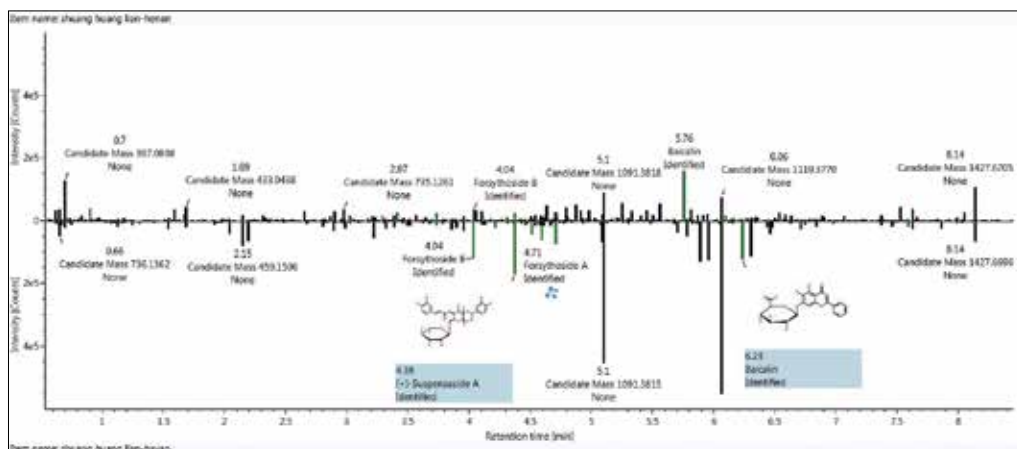


Figure 5. Summary plot showing ingredient differences after binary comparison of the two traditional medicine drinks.



## CONCLUSION

This application note systematically describes how to apply the binary comparison workflow of the Natural Products Application Solution with UNIFI to compare the ingredient similarities and differences between Shuanghuanglian oral drink from two different manufacturers. This workflow is based on collecting data by UPLC/QToF MS, and using the Traditional Medicine Library as well as automated structure elucidation features within UNIFI. The solution provides a novel and comprehensive approach for the natural products analysis.

This solution is applicable for solving a series of problems, such as comparison of raw materials from different locations, or from the same location but different harvesting times or processing methods; it is also useful for quality control of the same product from different manufacturers, or the same products from the same manufacturer but different batches, etc.

This binary comparison solution contains five preset binary comparison workflow templates and one report template. Used together, UNIFI enables researchers to compare samples effectively and quickly to understand the similarities and differences among samples, providing a foundation for future product research steps. The final result is a significant enhancement of effectiveness and productivity for the research and quality control of traditional medicines.

## Reference

1. Using Natural Products Application Solution with UNIFI for the Identification of Chemical Ingredients of Green Tea Extract. Waters Application Note, November 2013; 720004837en.

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# Using the Elucidation Tool in UNIFI Scientific Information System to Identify Unknown Compounds in Natural Products

Lirui Qiao, Kate Yu, Waters China, Waters Milford

## UPLC-QTOF-MS and UNIFI

### GOAL

To identify components from a sample that either were not matched with scientific libraries within the UNIFI® Scientific Information System, or were matched but were suspected to be false positive, the Elucidation Tool within UNIFI can be used. An example is used to demonstrate the process using the Natural Product Application Solution with UNIFI.

### BACKGROUND

Several scientific libraries are integrated within workflows in the UNIFI Scientific Information System, providing convenience and support for component identification of unknown samples. For components successfully matched with a library, researchers only need to verify the rationality of the fragments that have been classified by MassFragment™ Software to confirm the target components.

However, for components that cannot be matched with a UNIFI library, or that can be matched but false positives are suspected, UNIFI's Elucidation Tool can be used to manually identify the target compounds of interest through searching online libraries. Here, using the Natural Products Application Solution with UNIFI, we illustrate this process by investigating the identification of an unknown component in a natural product extract as an example.

The Elucidation Tool is a standard feature within the UNIFI Scientific Information System that facilitates the identification of unknown compounds. This feature combines compound identification, by searching online libraries based on elemental composition, with structural elucidation using MassFragment and its MS fragmentation data.

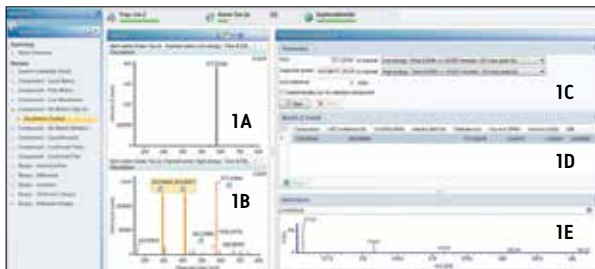


Figure 1. The process of an unknown component's elemental composition determination. 1A: Low collision energy mass spectrum of the unknown component. 1B: High collision energy mass spectrum of the unknown component. 1C: Settings for elemental composition search. 1D: Search results of the unknown component's elemental composition. 1E: Isotope distribution plot for the unknown sample.

## THE SOLUTION

The processes and steps for unknown compound identification using the Elucidation Tool are shown in Figures 1 and 2.

The first step is to determine the elemental composition of the unknown component. In UNIFI, the elemental composition of an unknown compound is determined by three combined factors: exact mass of the intact precursor ion, exact mass and abundance ratio of isotopic peaks, as well as confirming elemental composition of the secondary fragment ions that are correspond to precursor ions.

As shown in Figure 1, for the unknown component with an accurate mass of 577.1550, we can obtain the only possible elemental composition combining the above three factors:  $C_{27}H_{30}O_{14}$  (assuming this natural product is only composed of C, H, and O).

If the elemental composition corresponding to the secondary fragments is not taken into consideration, three possible molecular formulas could be obtained when searching the elemental composition of this unknown component, and manual evaluation is then required. However, because the fragmentation ions are taken into consideration, the false positives were excluded, leading to a single elemental composition as the accurate and reliable result.

The second step in this identification process is to search possible names and associated structures of chemical ingredients through online libraries. UNIFI Software links directly to ChemSpider, enabling researchers to search online and obtain possible structures in a variety of ways. For example, one can select all 558 UNIFI default libraries, or simply select some associated libraries. Alternatively, one can conduct the online search by either elemental composition or by accurate mass. Figure 2 shows the process of searching the online libraries.

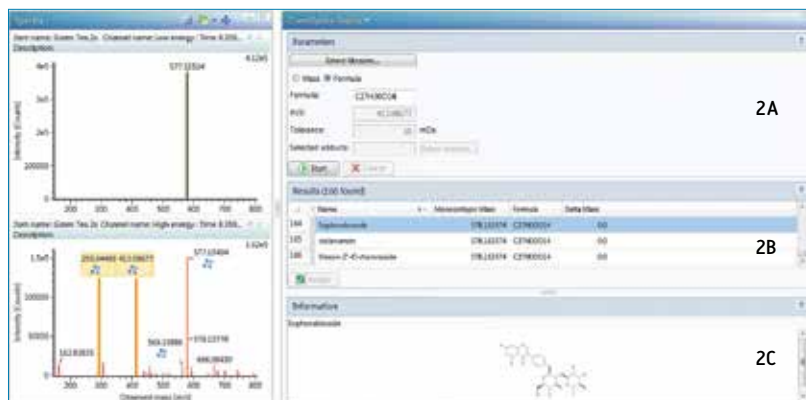


Figure 2. UNIFI directly links to ChemSpider for online library searches. 2A: Parameter settings for ChemSpider online search; 2B: Matching list as the result of the online search; 2C: Chemical structures corresponding to the results list. This figure shows the preliminarily confirmed compound structures after screening.



## SUMMARY

This work describes how to use the Elucidation Tool within the UNIFI Scientific Information System to manually identify unmatched or matched but suspected false-positive components. This process has a rational design, the elemental composition search is reliable and accurate, and the ability to use online library searches is simple and intuitive. The target compound can be further confirmed utilizing MassFragment Software. In this example, by using the Natural Product Application Solution with UNIFI, the unknown compound that elutes at 8.35 min with [M-H]<sup>-</sup> of 577.1550 is confirmed to be Sophrabioside.

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## SFC-SQD

## Qualitative and Quantitative Analysis of Enantiomers (Epigoitrin/Goitrin) from *Isatis Indigotica* Fort Root Extract Using SFC-MS

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1. Waters Corporation, New Castle, DE, U.S.A.

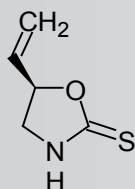
2. Shanghai Traditional Chinese Medicine University, Shanghai, P. R. China;

3. Waters Corporation, Milford, MA, USA

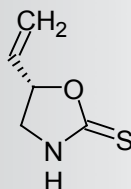
4. Roche R&D Center (China) Ltd., Shanghai, P. R. China.

### APPLICATION BENEFITS

This application note showcases an optimized SFC system solution for separating enantiomers possessing vastly different bioactivities extracted from Traditional Chinese Medicine (TCM) in six minutes.



(R)-Goitrin



(S)-Goitrin

### WATERS SOLUTIONS

SFC-MS Resolution™ System

MassLynx™ Software

### KEY WORDS

SFC, chiral separation, Traditional Chinese Medicine, TCM, R/S Goitrin, Ban Lan Gen

### INTRODUCTION

In recent years, much research has been conducted in the chirality of active ingredients in Traditional Chinese Medicine (TCM). For example, the enantiomers (R, S)-goitrin are found in the dried root of *Isatis indigotica* Fort (known as Bei Ban Lan Gen (板蓝根) in China). Pharmacokinetic studies indicate that only the R-goitrin (epigoitrin) displays the desired anti-viral activity. It is therefore important to separate the R- and S-goitrin to better assess the potency of the Ban Lan Gen on the market, as well as for better quality control in the manufacturing process.

Existing reverse phase liquid chromatography (RPLC)-based methodology does not resolve R- and S-goitrin enantiomers, and therefore cannot accurately quantify the bioactive R-goitrin. Lin et al. have demonstrated a normal-phase liquid chromatography (NPLC)/UV-based chiral separation and quantitation of R- and S-goitrin<sup>1</sup>. The run time was 50 min.

In this application note we report an SFC-MS based quantitation method for R- and S-goitrin with a run time of 6 min. The optimized method was used to quantify the amounts of R- and S-goitrin in three Ban Lan Gen products manufactured in China, as well as the dried root extract from different geographic region, as shown in Figure 1.



Figure 1. Ban Lan Gen products from three different manufacturers purchased from TCM drug store.

## EXPERIMENTAL

### LC conditions

Co-solvent:	methanol
Flow rate:	3 mL/min
Temp.:	40 °C
Backpressure:	120 bar
Injection volume:	10 µL
PDA scan range:	220 to 300 nm.

The gradient conditions are listed in the respective figure captions.

### MS conditions

Mode:	APCI (+)
Corona:	10 uA
Cone:	20 V
Source temp.:	150 °C
Probe temp.:	400 °C
Desolvation gas:	400 L/hr

### Extraction

100 mg of the Ban Lan Gen powder was soaked in 5 mL water, sonicated for 1 hr, and sat for 1 hr. The sample was centrifuged and the supernatant was filtered through a 0.45 µm filter. Next, 5 mL of diethyl ether was added to the filtered extract and the ether portion was collected. This process was repeated three times. The combined diethyl ether extract was dried down and reconstituted in 5-mL methanol.

## Instruments

All experiments were performed on a Waters® SFC-MS Resolution System, shown in Figure 2 and controlled by MassLynx Software.

SFC-MS system consists of the following: FDM, CTC autosampler, 10-port column oven, photodiode array (PDA) detector, and a single quadrupole mass detector.



Figure 2. SFC-MS Resolution System.

## Chromatography

A racemic mixture of (R, S)-goitrin (Purchased from Thermo Fisher Scientific) was used for method development. Six chiral columns (4.6 x 250 mm): AD-H (5 µm), OD-H (5 µm), (S,S)-Whelk-O1 (10 µm), AS-H (5 µm), OJ-H (5 µm), and IC (5 µm) purchased from commercial sources were used for screening.

## RESULTS AND DISCUSSION

The initial step was to find optimal chiral separations by screening multiple chiral stationary phases. Figure 3 shows the result of the chiral screening for the (R,S)-goitrin standard on six chiral stationary phases. The AD-H, OD-H, and (S,S)-Whelk-O1 columns were all capable of separating the enantiomers; the (S,S)-Whelk-O1 clearly demonstrated the best resolution.

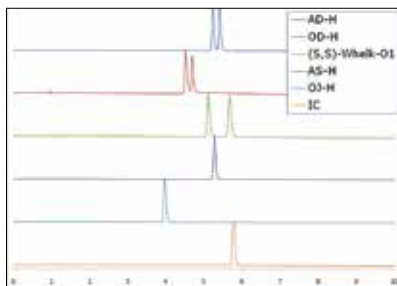


Figure 3. SFC chromatograms of the (R, S)-goitrin standard run on six different stationary phases. Generic gradient was as follows: 5% to 40% for 10 min, hold at 40% for 2 min, 40% to 5% for 2 min, hold at 5% for 2 min.

The chromatographic parameters were further optimized to shorten the run time. As shown in Figure 4, the R- and S-goitrin were separated from the extract matrix while preserving the enantiomeric resolution of R- and S-goitrin in 6 min.

In China, there are two types of herbs, both called Ban Lan Gen. The authentic herb is called Bei Ban Lan Gen, and the second herb, known as Nan Ban Lan Gen, is not considered authentic Ban Lan Gen. This was clearly stated in the 2010 Chinese Pharmacopeia.

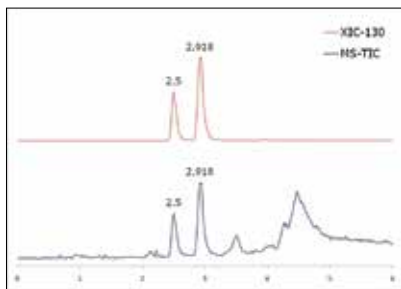


Figure 4. MS-TIC and XIC at 130  $m/z$  of the Ban Lan Gen extract. Optimized mobile phase conditions were as follows: hold at 20% for 2.0 min, 20% to 40% in 0.5 min, hold at 40% for 2 min, 40% to 20% in 0.5 min. Peak 1 (2.500 min) is S-goitrin, Peak 2 (2.918 min) is R-goitrin.

Figure 5 shows the SFC-MS chromatograms (SIR,  $m/z$  130) of the Bei Ban lan Gen extract vs. the Nan Ban Lan Gen extract. Here it clearly showed that there is no goitrin detected from the Nan Ban lan Gen extract. This finding is in agreement with previous reports. Clearly, goitrin can be a potential key marker that distinguishes Bei Ban Lan Gen from Nan Ban Lan Gen.

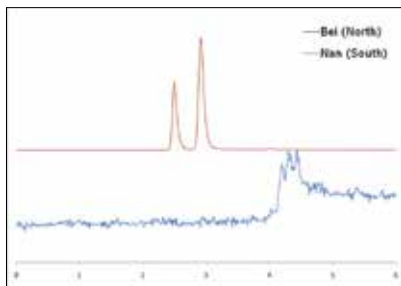


Figure 5. Selected ion chromatograms ( $m/z$  130) of Bei Ban Lan Gen extract vs Nan Ban Lan Gen extract.



Calibration curves were constructed using the 6-min method, and the results are shown in Figure 6. Excellent linearity was achieved for both R- and S-goitrin. The limit of detection (LOD) and limit of quantification (LOQ) were 2 ng/mL and 10 ng/mL, respectively.

Figure 7 shows the SFC-MS chromatograms of three Ban Lan Gen teas. It is evident that there are substantial differences amongs three Ban Lan Gen products: Tea 1 contains no Goitrin while Tea 3 contains the most Goitrin. The amount of goitrin as well as the ratio of R/S goitrin can be calculated using the calibration curves shown in Figure 6 (results not shown).

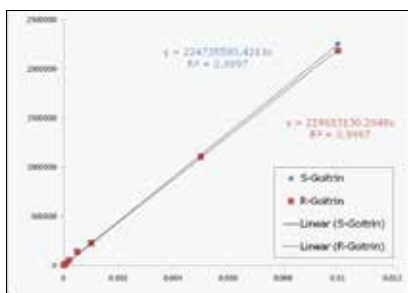


Figure 6. Calibration curves for R- and S-goitrin using SFC-MS.

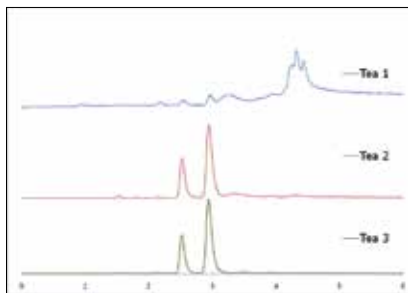


Figure 7. SFC-MS chromatograms of the three Ban Lan Gen tea extracts.

## CONCLUSIONS:

The enantiomeric resolution of R- and S-goitrin were achieved in 6 minutes using the SFC-MS Resolution System. The capability of SFC-MS in fast qualitative and quantitative analyses of goitrin in a complex TCM formulation has been demonstrated. SFC is the technique of choice for chiral separation and it holds great promise for replacing NPLC. SFC should be considered a valuable addition to the overall analytical technology platform for TCM research.

## References:

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# Waters

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July 2011 720004049en AG-PDF

## Cytotoxic and Anti-Inflammatory Prenylated Benzoylphloroglucinols and Xanthenes from the Twigs of *Garcinia esculenta*.

Five new prenylated benzoylphloroglucinol derivatives, garciesculentones A–E (1–5), a new xanthone, garciesculenxanthone A (6), and 15 known compounds were isolated from the petroleum ether extract and the EtOAc-soluble fraction of a 80% (v/v) EtOH extract of *Garcinia esculenta*. The structures of the new compounds were elucidated by 1D- and 2D-NMR spectroscopic analysis and mass spectrometry. Experimental and calculated ECD and a convenient modified Mosher's method were used to determine the absolute configurations. The cytotoxicity of these compounds were evaluated by MTT assay against three human cancer cell lines (HepG2, MCF-7, and MDA-MB-231) and against normal hepatic cells (HL-7702). In addition, these isolates were evaluated for their inhibitory effects on interferon- $\gamma$  plus lipopolysaccharide-induced nitric oxide production in RAW264.7 cells.

Hong Zhang, Dan-Dan Zhang, Yuan-Zhi Lao, Wen-Wei Fu, Shuang Liang, Qing-Hong Yuan, Ling Yang, and Hong-Xi Xu

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## Prenylated Benzoylphloroglucinols and Xanthenes from the Leaves of *Garcinia oblongifolia* with Antienteroviral Activity.

An acetone extract of the leaves of *Garcinia oblongifolia* showed antiviral activity against enterovirus 71 (EV71) using a cytopathic effect inhibition assay. Bioassay-guided fractionation yielded 12 new prenylated benzoylphloroglucinols, oblongifolins J–U (1–12), and five known compounds. The structures of 1–12 were elucidated by spectroscopic analysis including 1D- and 2D-NMR and mass spectrometry methods. The absolute configurations were determined by a combination of a Mosher ester procedure carried out in NMR tubes and ECD calculations. Compared to ribavirin (IC<sub>50</sub> 253.1  $\mu$ M), compounds 1, 4, and 13 exhibited significant anti-EV71 activity in vitro, with IC<sub>50</sub> values of 31.1, 16.1, and 12.2  $\mu$ M, respectively. In addition, the selectivity indices of these compounds were 1.5, 2.4, and 3.0 in African green monkey kidney (Vero) cells, respectively.

Hong Zhang, Ling Tao, Wen-Wei Fu, Shuang Liang, Yi-Fu Yang, Qing-Hong Yuan, Da-Jian Yang, Ai-Ping Lu, and Hong-Xi Xu

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**UPLC-ESI-Q-TOF- high definition mass spectrometry (HDMS)****Ultra-performance liquid chromatography tandem mass spectrometry combined with automated MetaboLynx analysis approach to screen the bioactive components and their metabolites in Wen-Xin-Formula.**

Wen-Xin-Formula (WXF), a famous traditional prescription, has been widely used to treat myocardial ischemia syndrome for thousands of years. However, the constituents absorbed into blood after oral administration of WXF remain unknown. Here, an integrative ultra performance liquid chromatography coupled with electrospray ionization/quadrupole-time-of-flight mass spectrometry (UPLC-ESI-Q-TOF-MS) combined with the MetaboLynx approach was established to investigate the absorbed constituents in rats after oral administration of WXF. A hyphenated electrospray ionization and quadrupole-time-of-flight analyzer was used for the determination of accurate mass of the molecule and fragment ions. With this rapid and automated analysis method, a total of 32 peaks were tentatively characterized in vivo based on MS and MS/MS data and comparison with available databasess, 26 of which were parent components and six metabolites. These components mainly were ginsenosides, paeoniflorin, galloyl glucose, berberis alkaloids, phenolic, phenolic glycosides and unsaturated fatty acids, glucuronide products of original berberis alkaloids. The present study demonstrates that integrative UPLC-ESI-Q-TOF-MS technique and MetaboLynx data processing method were successfully applied for the rapid discovery of potentially bioactive components and metabolites from WXF, and proved that the established method could help to explore the effective substances for further research into WXF. Copyright © 2014 John Wiley & Sons, Ltd.

**Hongxin Cao, Aihua Zhang, Fang-mei Zhang, Qin-qin Wang, He Zhang, Yan-hua Song, Ying Zhou, Hui Sun, Guang-li Yan, Ying Han, and Xijun Wang**

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## Identification and characterization of the chemical constituents of Simiao Wan by ultra high performance liquid chromatography with mass spectrometry coupled to an automated multiple data processing method.

The chemical constituents of Simiao Wan (SW), a traditional Chinese medicine preparation, are difficult to determine and remain unclear. To more efficiently detect ions, a multiple data processing approach has been used in the characterization of the compounds. In this study, a rapid and sensitive method based on ultra high performance liquid chromatography with mass spectrometry and the multiple data processing approach was established to characterize the chemical constituents of SW. Ultra high performance liquid chromatography with mass spectrometry coupled with the multiple data processing approach could efficiently remove nonrelated ion signals from accurate mass data. We report the application of the multiple data processing approach for comprehensive detection and rapid identification of chemical constituents of SW. Of note, the total analysis time for separation was less than 20 min without losing any resolution. In the variable, importance in projection plot of orthogonal projection to latent structure-discriminant analysis, a total of 72 ions of interest (37 ions in positive mode, 38 ions in negative mode and three ions in both mode) were extracted or tentatively characterized based on their retention times, exact mass measurement for each molecular ion and subsequent fragment ions. In summary, the methodology proposed in this study could be valuable for the structural characterization and identification of the multiple constituents in the traditional Chinese medicine formula SW.

Aihua Zhang, Di Zou, Guangli Yan, Yunlong Tan, Hui Sun, and Xijun Wang

*Journal of Separation Science*, Volume 37, Issue 14, pages 1742–1747, July 2014

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## A high performance liquid chromatography fingerprinting and ultra high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry chemical profiling approach to rapidly find characteristic chemical markers for quality evaluation of dispensing granules, a case study on Chuanxiong Rhizoma.

A high performance liquid chromatography-photodiode array detector (HPLC-PDA) fingerprinting and ultra high performance liquid chromatography-photodiode array detector coupled with quadrupole time-of-flight mass spectrometry (UHPLC-PDA-QTOF-MS/MS) based chemical profiling approach was developed to rapidly find characteristic chemical markers for quality control of dispensing granules, taking Chuanxiong Rhizoma (CR) as a model herb. Firstly, CR crude drugs, their traditional decoctions and CR dispensing granules were analyzed by HPLC-PDA to rapidly establish the fingerprints and thereby generate the simulative median chromatograms of CR crude drugs, decoctions and dispensing granules, and by comparing the simulative median chromatograms, major characteristic peaks of CR decoctions and dispensing granules could be determined. Secondly, UHPLC-PDA-QTOF-MS/MS was used to identify the major characteristic peaks of CR decoctions and dispensing granules. The identities of three major peaks were elucidated and confirmed to be ferulic acid (1), senkyunolide I (2) and senkyunolide H (3) by comparing the mass/UV spectra and retention times with that of the reference compounds. Thirdly, an HPLC-PDA method was validated to quantify the three characteristic components in commercial CR dispensing granules. The average contents of ferulic acid and senkyunolide H were found to be less than 1.0mg/g, whereas that of senkyunolide I was 4.40mg/g in CR dispensing granules, which indicated that senkyunolide I might be chosen as a suitable quantitative marker, while ferulic acid and senkyunolide H as qualitative markers for the quality evaluation of CR dispensing granules. It is suggested that this newly established approach could be used to practically and rapidly find suitable marker compounds for quality control of dispensing granules derived from other medicinal herbs.

Xiao-Lin Zhang, Li-Fang Liu, Ling-Ying Zhu, Ying-Jia Bai, Qian Mao, and Song-Lin Li

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## UPLC-Q/TOF MS standardized Chinese formula Xin-Ke-Shu for the treatment of atherosclerosis in a rabbit model.

Xin-Ke-Shu (XKS), a patent traditional Chinese medicine (TCM) preparation, has been commonly used for the treatment of coronary heart disease in China. In order to understand its mechanism of action, a metabonomic approach based on ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q/TOF MS) was utilized to profile the plasma metabolic fingerprints of atherosclerosis (AS) rabbits with and without XKS treatment. The metabolic profile of model group clearly separated from normal, and that of XKS group was closer to the control group. Metabolites with significant changes during atherosclerosis were characterized as potential biomarkers related to the development of atherosclerosis by using orthogonal partial least-squares-discriminate analysis (OPLS-DA). Twenty potential biomarkers, including L-acetylcarnitine (1), propionylcarnitine (2), unknown (3), phytosphingosine (4), glyoursodeoxycholic acid (5), LPC(14:0) (6), sphinganine (7), LPC(20:5) (8), LPC(16:1) (9), LPC(18:2) (10), LPC(18:3) (11), LPC(22:5) (12), LPC(16:0) (13), LPC(18:1) (14), LPC(22:4) (15), LPC(17:0) (16), LPC(20:2) (17), elaidic carnitine (18), LPC(18:0) (19) and LPC(20:1) (20), were identified by their accurate mass and MS(E) spectra. The derivations of those biomarkers can be regulated by administration of XKS, which suggested that the intervention effect of XKS against AS may involve in regulating the lipid perturbation including fatty acid  $\beta$ -oxidation pathway, sphingolipid metabolism, glycerophospholipid metabolism and bile acid biosynthesis. This study indicated that the UPLC-Q/TOF MS-based metabonomics not only gave a systematic view of the pathomechanism of AS, but also provided a powerful tool to study the efficacy and mechanism of complex TCM prescriptions.

**Yue-Tao Liu, Jing-Bo Peng, Hong-Mei Jia, Da-Yong Cai, Hong-Wu Zhang, Chang-Yuan Yu, and Zhong-Mei Zou**

*Phytomedicine*. 2014 Sep 25;21(11):1364-72.

DOI: 10.1016/j.phymed.2014.05.009. Epub 2014 Jun 7.

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## Chemical comparison of two dosage forms of Hemp Seed Pills by UHPLC-Q-ToF-MS/MS and multivariate statistical techniques.

Hemp seed soft gel capsule (HSSGC) is a modernised dosage form that is derived from a traditional Chinese patent medicine, Hemp Seed Pills (HSP). Two dosage forms claim the same therapeutic effects; however, their chemical components and chemical equivalency are unclear. In the present study, an ultra performance liquid chromatography coupled with time-of-flight mass spectrometry (UHPLC-ToF-MS)-based chemical profiling approach was proposed to rapidly evaluate the chemical differences between HSP and HSSGC as model dosage forms. Samples of the two dosage forms were subjected to UHPLC-ToF-MS analysis. The datasets of retention time (TR) and mass-to-charge ratio ( $m/z$ ) pairs, ion intensities and sample codes were processed with principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) to holistically compare the difference between these two dosage form samples. A clear classification trend was observed in the score plot, and a loading bi-plot was generated in which the variables are correlated with the group and the samples that were observed. The important chemical components that caused differences among the samples were explored with a Variables Importance Projection (VIP) index. Using the proposed approach, global chemical differences were found between the two dosage forms and among samples of the same dosage form. The most important components that are related to the differences were identified and most of them were attributed to *Fructus Aurantii Immaturus*. It is suggested that this newly established approach could be used for pre-clinical trial chemical equivalence study or the quality evaluation of the traditional medicinal products with large variations in quality.

Wen-Jun Zhou, Jing-Zheng Song, Wen-Wei Fu, Hong-Sheng Tan, Zhao-Xiang Bian, and Hong-Xi Xu

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## Hippocampus and serum metabolomic studies to explore the regulation of Chaihu-Shu-Gan-San on metabolic network disturbances of rats exposed to chronic variable stress.

Chaihu-Shu-Gan-San (CSGS), a traditional Chinese medicine formula, has been effectively used for the treatment of depression. However, studies of its anti-depressive mechanism are challenging, due to the complex pathophysiology of depression, and complexity of CSGS with multiple constituents acting on different receptors. In the present work, metabolomic studies of biochemical changes in the hippocampus and serum of chronic variable stress (CVS)-induced depression rats after treatment with CSGS were performed using ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS). Partial least squares-discriminate analysis indicated that the metabolic perturbation induced by CVS was reduced by treatment with CSGS. A total of twenty-six metabolites (16 from the hippocampus and 10 from serum) were considered as potential biomarkers involved in the development of depression. Among them, 11 were first reported to have potential relevance in the pathogenesis of depression, and 25 may correlate to the regulation of CSGS treatment on depression. The results combined with a previous study indicated that CSGS mediated synergistically abnormalities of the metabolic network, composed of energy metabolism, synthesis of neurotransmitters, tryptophan, phospholipids, fatty acid and bile acid metabolism, bone loss and liver detoxification, which may be helpful for understanding its mechanism of action. Furthermore, the extracellular signal-regulated kinase (ERK) signal pathway, involved in the neuronal protective mechanism of depression related to energy metabolism, was investigated by western blot analysis. The results showed that CSGS reversed disruptions of BDNF, ERK1/2 and pERK1/2 in CVS rats, which provides the first evidence that the ERK signal system may be one of the targets related to the antidepressant action of CSGS.

Zhi-heng Su, Hong-mei Jia, Hong-wu Zhang, Yu-fei Feng, Lei An, and Zhong-mei Zou

*Mol Biosyst.* 2014 Mar 4;10(3):549-61.

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PMID: 24398477 [PubMed - indexed for MEDLINE]

## UPLC-TQD

## Pharmacokinetics difference of multiple active constituents from decoction and maceration of Fuzi Xiexin Tang after oral administration in rat by UPLC-MS/MS.

Fuzi Xiexin Tang (FXT) is a classic traditional Chinese medicine formula which has been employed in clinical for more than 1800 years. The distinctive preparation method (maceration) recorded in ancient time is different from one in modern clinical practice (decoction). Aim of this study is to investigate the pharmacokinetic difference of alkaloids, flavones and anthraquinones in rats after oral administration of decoction of FXT (DFXT, 30gkg(-1)), maceration of FXT (MFXT, 30gkg(-1)) and decoction of Aconiti Lateralis Radix Preparata (DAR, 6gkg(-1)) by a validated UPLC-MS/MS method. Plasma concentrations and pharmacokinetic parameters of 16 active constituents (aconitine, hypaconitine, mesaconitine, benzoylaconine, benzoylhypaconine, benzoylmesaconine, berberine, palmatine, jatrorrhizine, coptisine, baicalin, wogonin, wogonoside, emodin, aloe-emodin, rhein) in rat were quantified and compared. Different preparative methods resulted in significant difference on exposure and pharmacokinetic characteristics of alkaloids, flavones and anthraquinones from FXT, especially protoberberine alkaloids. Concentrations of monoester-diterpenoid alkaloids were below the LOD in rat plasma after administration of DFXT and MFXT because of the existence of other three herbs from FXT. Maceration could decrease the absorption of flavones while increased the absorption of anthraquinones. Cmax of emodin and rhein were 3.1 and 10.3 times increased, while eliminations of these two constituents were 8.0 and 19.0 times slower after administration of MFXT. Bioavailability of both flavones and anthraquinones increased after administration of MFXT, especially emodin and rhein increasing as much as 13.5 and 20.7 times. Herb-herb interaction between DAR and other three herbs from FXT significantly influenced the exposure of aconitum alkaloids.

Qian Zhang, Yue-ming Ma, Zheng-tao Wang, and Chang-hong Wang

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## Metabolic pathways involved in Xin-Ke-Shu protecting against myocardial infarction in rats using ultra high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry.

Xin-Ke-Shu (XKS) is a patent drug used for coronary heart diseases in China. This study evaluated the protective effect of XKS against isoproterenol (ISO)-induced myocardial infarction (MI). For its underlying mechanism in rats with MI, a metabonomic approach was developed using ultra high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC/QTOF-MS). Plasma metabolites were profiled in MI rats, pretreated orally with or without XKS. Two genres of metabolic biomarkers were used to elucidate the pharmacological action of XKS: pathological biomarkers and pharmaco biomarkers. Fifteen metabolites significantly varying between MI rats and normal rats were characterized as potential pathological biomarkers related to MI, including L-acetylcarnitine (1), L-isoleucyl-L-proline (2), tyramine (3), isobutyryl-L-carnitine (4), phytosphingosine (5), sphinganine (6), L-palmitoylcarnitine (7), lysoPC(18:0) (8), uric acid (9), L-tryptophan (10), lysoPC(18:2) (11), lysoPC(16:0) (12), docosahexaenoic acid (13), arachidonic acid (14) and linoleic acid (15). Among them, eight (1-6, 9 and 10) were first reported as pathological biomarkers related to ISO-induced MI, which mainly involved into fatty acid  $\beta$ -oxidation pathway, sphingolipid metabolism, proteolysis, tryptophan metabolism and purine metabolism. The metabolites significantly varying between MI rats with and without XKS pretreatment were considered as pharmaco biomarkers. A total of 17 pharmaco biomarkers were recognized, including 15 pathological biomarkers (1-15), hexanoylcarnitine (16) and tetradecanoylcarnitine (17). The results suggested that pretreatment of XKS protected metabolic perturbations in rats with MI, major via lipid pathways, amino acid metabolism and purine metabolism, which also provided a promising approach for evaluating the pharmacodynamics and mechanism of traditional Chinese medicines (TCM) formulas.

Yue-tao Liu, Hong-mei Jia, Xing Chang, Wei-hua Cheng, Xin Zhao, Gang Ding, Hong-wu Zhang, Da-yong Cai, and Zhong-Mei Zou

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DOI: 10.1016/j.jpba.2013.11.008. Epub 2013 Nov 15.

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## Combination of UHPLC/Q-TOF-MS, NMR spectroscopy, and ECD calculation for screening and identification of reactive metabolites of gentiopicroside in humans.

The metabolic investigation of natural products is a great challenge because of unpredictable metabolic pathways, little knowledge on metabolic effects, and lack of recommended analytical methodology. Herein, a combined strategy based on ultrahigh-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (UHPLC/Q-TOF-MS), nuclear magnetic resonance (NMR) spectroscopy, and electronic circular dichroism (ECD) calculation was developed and employed for the human metabolism study of gentiopicroside (GPS), a naturally hepato-protective iridoid glycoside. The whole metabolic study consisted of three major procedures. First, an improved UHPLC/Q-TOF-MS method was used to separate and detect a total of 15 GPS metabolites that were obtained from urine samples (0 to 72 h) of 12 healthy male participants after a single 50-mg oral dose of GPS. Second, a developed “MS-NMR-MS” method was applied to accurately identify molecular structures of the observed metabolites. Finally, given that the associated stereochemistry may be a crucial factor of the metabolic activation, the absolute configuration of the reactive metabolites was revealed through chemical calculations. Based on the combined use, a pair of diastereoisomers (G05 and G06) were experimentally addressed as the bioreactive metabolites of GPS, and the stereochemical determination was completed. Whereas several novel metabolic transformations, occurring via oxidation, N-heterocyclization and glucuronidation after deglycosylation, were also observed. The results indicated that GPS has to undergo in vivo metabolism-based activation to generate reactive molecules capable of processing its hepato-protective activity.

Han Han, Ai-Zhen Xiong, Chun-Yong He, Qing Liu, Li Yang, and Cui Tao

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DOI: 10.1007/s00216-013-7572-z. Epub 2014 Jan 10.

PMID: 24408300 [PubMed - indexed for MEDLINE]

## An optimized ultrasound-assisted extraction and simultaneous quantification of 26 characteristic components with four structure types in functional foods from ginkgo seeds.

An optimized method of ultrasound-assisted extraction followed by ultra-high-performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry (UAE-UHPLC-TQ/MS(2)) was proposed for the simultaneous extraction and determination of 26 characteristic components covering four structure types (flavonoids, terpene lactones, ginkgolic acids and phenylpropanols) in ginkgo seeds (GSs). The UAE parameters (ultrasound power, time and solvent-to-material ratio) were optimized using a response surface methodology. This is the first report of the simultaneous analysis of 26 compounds in Ginkgo biloba using UHPLC-TQ/MS(2); this analysis afforded good linearity, precision, repeatability and accuracy. UAE-UHPLC-TQ/MS(2) was successfully applied to ginkgo seed samples, and the analysis showed that GSs are rich in terpene lactones and could be selected as a healthy food resource. The results suggest that UAE-UHPLC-TQ/MS(2) might be able to be utilized as a tool for the quality assessment of samples from GSs or other related products using flavonoids, terpene lactones, ginkgolic acids and phenylpropanols as markers.

**Guisheng Zhou, Xin Yao, Yuping Tang, Dawei Qian, Dawei Qian, Shulan Su, and Li Zhang**

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## Microbial Biotransformation of Gentiopicroside by the Endophytic Fungus *Penicillium crustosum* 2T01Y01.

Endophytic fungi are symbiotic with plants and possess multienzyme systems showing promising metabolite potency with region selectivity and stereoselectivity. The aim of this study was to use these special microorganisms as an *in vitro* model to mimic the potential mammalian metabolites of a natural iridoid gentiopicroside (GPS, compound 1). The fungi isolated from a medicinal plant, *Dendrobium candidum* Wall. ex Lindl., were screened for their biotransformation abilities with GPS as the substrate, and one strain with high converting potency was identified as *Penicillium crustosum* 2T01Y01 on the basis of the sequence of the internal transcribed spacer of the ribosomal DNA region. Upon the optimized incubation of *P. crustosum* 2T01Y01 with the substrate, seven deglycosylated metabolites were detected by ultraperformance liquid chromatography/quadrupole time of flight mass spectrometry (UPLC/Q-TOF MS). Preparative-scale biotransformation with whole cells of the endophytic fungus resulted in the production of five metabolites, including three novel ones, 5 $\alpha$ -(hydroxymethyl)-6 $\beta$ -methyl-3,4,5,6-tetrahydropyrano[3,4-c]pyran-1(8H)-one (compound 2), (Z)-4-(1-hydroxybut-3-en-2-yl)-5,6-dihydropyran-2-one (compound 3), and (E)-4-(1-hydroxybut-3-en-2-yl)-5,6-dihydropyran-2-one (compound 4), along with two known ones, 5 $\alpha$ -(hydroxymethyl)-6 $\beta$ -methyl-1H,3H-5,6-dihydropyrano[3,4-c]pyran-1(3H)-one (compound 5) and 5 $\alpha$ -(hydroxymethyl)-6 $\alpha$ -methyl-5,6-dihydropyrano[3,4-c]pyran-1(3H)-one (compound 6), aided by nuclear magnetic resonance and high-resolution mass spectral analyses. The other two metabolites were tentatively identified by online UPLC/Q-TOF MS as 5-hydroxymethyl-5,6-dihydroisochromen-1-one (compound 7) and 5-hydroxymethyl-3,4,5,6-tetrahydroisochromen-1-one (compound 8), and compound 8 is a new metabolite. To test the metabolic mechanism, the  $\beta$ -glucosidase activity of the fungus *P. crustosum* 2T01Y01 was assayed with  $\beta$ -D-nitrophenyl- $\beta$ -D-glucopyranoside as a probe substrate, and the pathway of GPS biotransformation by strain 2T01Y01 is proposed. In addition, the hepatoprotective activities of GPS and metabolite compounds 2, 5, and 6 against human hepatocyte line HL-7702 injury induced by hydrogen peroxide were evaluated.

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*Appl Environ Microbiol.* 2014 Jan;80(1):184-92.

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## Discrimination of leaves of *Panax ginseng* and *P. quinquefolius* by ultra high performance liquid chromatography quadrupole/time-of-flight mass spectrometry based metabolomics approach.

In present study, an ultra high performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS/MS) based metabolomics approach was established to investigate the metabolic profiles and characteristic chemical markers for distinguishing between leaves of *Panax ginseng* (LPG) and *Panax quinquefolius* (LPQ). The UHPLC-QTOF-MS/MS data were subjected to principal component analysis (PCA) and orthogonal partial least squared discrimination analysis (OPLS-DA) to rapidly find the potential characteristic components of LPG and LPQ, and the identities of detected peaks including the potential characteristic components were elucidated. Totally, 86 components were identified from these 2 kinds of leaf samples, in which 9 ginsenosides could be regarded as the characteristic chemical markers for the discrimination of LPG from LPQ. These results suggested that UHPLC-QTOF-MS/MS based metabolomics approach is a powerful tool to rapidly find characteristic markers for the quality control of LPG.

**Qian Mao, Min Bai, Jin-Di Xu, Jin-Di Xu, Ming Kong, Lin-Yin Zhu, and He Zhu**

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## UPLC-PDA-TOF/MS coupled with multivariate statistical analysis to rapidly analyze and evaluate Ginkgo biloba leaves from different origin.

In the present study, an ultra performance liquid chromatography coupled with photodiode array detector and time-of-flight mass spectrometry (UPLC-PDA-TOF/MS) was proposed and validated for rapidly analyzing and evaluating Ginkgo biloba leaves from different origins by using multivariate statistical analysis. Batches of these kinds of G. biloba leaves were subjected to UPLC-PDA-TOF/MS analysis, the datasets of retention time (RT)-m/z pairs, ion intensities and sample codes were further processed with orthogonal partial least squared discriminant analysis (OPLS-DA) to holistically compare the difference between these G. biloba leaves, and to generate an S-plot. Those compounds correlated to the points at the two ends of S were regarded as the most differentiating components. By comparing the mass/UV spectra and retention times with those of reference compounds and/or tentatively assigned by matching empirical molecular formulae with those of the known compounds published in the literatures, these differentiating components were finally characterized as kaempferol 3-O-[2-O-(6-O-p-hydroxy-trans-cinnamoyl)- $\beta$ -D-glucosyl]- $\alpha$ -L-rhamnoside], kaempferol 3-O-[2-O,6-O-bis( $\alpha$ -L-rhamnosyl)- $\beta$ -D-glucoside], ginkgolide C, kaempferol 3-O-[2-O-( $\beta$ -D-glucosyl)- $\alpha$ -L-rhamnoside], and bilobetin. These compounds would be the potential chemical markers for the two kinds of leaves. The results suggested that this newly established approach could be used to rapidly evaluate the quality of herbs from different origin, and to provide good strategy for further rectify and standardize the herb market

Xin Yao, Guisheng Zhou, Yuping Tang, Sheng Guo, Yefei Qian, and Chun Jin

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## Comparative study on intestinal metabolism and absorption in vivo of ginsenosides in sulphur-fumigated and non-fumigated ginseng by ultra performance liquid chromatography quadruple time-of-flight mass spectrometry based chemical profiling approach.

Our previous study indicated that sulphur-fumigation of ginseng in post-harvest handling processes could induce chemical transformation of ginsenosides to generate multiple ginsenoside sulphur derivatives. In this study, the influence of sulphur-fumigation on intestinal metabolism and absorption in vivo of ginsenosides in ginseng was sequentially studied. The intestinal metabolic and absorbed profiles of ginsenosides in rats after intra-gastric (i.g.) administration of sulphur-fumigated ginseng (SFG) and non-fumigated ginseng (NFG) were comparatively characterized by a newly established ultra performance liquid chromatography quadruple time-of-flight mass spectrometry (UPLC-QTOF-MS/MS) with electrospray ionization negative (ESI-) mode. A novel strategy based on the characteristic product ions and fragmentation pathways of different types of aglycones (saponin skeletons) and glycosyl moieties was proposed and successfully applied to rapid structural identification of ginsenoside sulphur derivatives and relevant metabolites. In total, 18 ginsenoside sulphur derivatives and 26 ginsenoside sulphur derivative metabolites in the faeces together with six ginsenoside sulphur derivatives in the plasma were identified in the SFG-administrated group but not in the NFG-administrated group. The results clearly demonstrated that the intestinal metabolic and absorbed profiles of ginsenosides in sulphur-fumigated and non-fumigated ginseng were quite different, which inspired that sulphur-fumigation of ginseng should not be recommended before the bioactivity and toxicity of the ginsenoside sulphur derivatives were systematically evaluated.

He Zhu, Hong Shen, Jun Xu, Jin-Di Xu, Ling-Ying Zhu, Jie Wu, Hu-Biao Chen, and Song-Lin Li

*Drug Test Anal.* 2015 Apr;7(4):320-30.

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## Simultaneous determination of original, degraded ginsenosides and aglycones by ultra high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry for quantitative evaluation of Du-Shen-Tang, the decoction of ginseng.

In the present study, an ultra-high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) method for simultaneous determination of eleven original, fourteen degraded ginsenosides and five aglycones was developed and validated to quantitatively evaluate the transformation of ginsenosides during preparation of Du-Shen-Tang, the decoction of ginseng. Both positive and negative modes as well as the step wave ion transfer optics technology were used to increase the detection sensitivity of QTOF-MS. The extracting ion mode based on the quasi-molecular ions, molecular ions and fragment ions characteristic to each analyte was used to increase the selectivity for quantitative analysis. Under the optimized UHPLC and QTOF-MS conditions, the 30 analytes with different polarities were separated (except for Re and Rg1) within 26 min. The developed method was applied for the quantitative comparison of Du-Shen-Tang and its raw materials derived from Asian ginseng (ASG) and American ginseng (AMG), respectively. It was found that the contents of the original ginsenosides decreased from 26,053.09 to 19,393.29 µg/g or 45,027.72 to 41,865.39 µg/g, whereas the degraded ginsenosides and aglycones increased from 159.72 to 685.37 µg/g or 676.54 to 1,502.26 µg/g in Du-Shen-Tang samples of ASG or AMG when compared with their raw materials, indicating that decocting could dramatically increase the proportion of the less polar degraded ginsenosides in Du-Shen-Tang. Whether these changed proportions of different polar ginsenosides could affect the bioactivities of the decoctions and their raw materials derived from ASG and AMG deserves further investigation.

Shan-Shan Zhou, Jin-Di Xu, He Zhu, Hong Shen, Jun Xu, and Qian Mao

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DOI: 10.3390/molecules19044083.

PMID: 24699150 [PubMed - indexed for MEDLINE]

## Neuroprotective effects of scutellarin and scutellarein on repeatedly cerebral ischemia-reperfusion in rats.

Scutellarin had protective effects against neuronal injury, however, there are few studies on the protective effect of scutellarein, which is the main metabolite of scutellarin in vivo. This study investigated whether the neural injury by ischemia/reperfusion would be influenced by different doses of scutellarin and scutellarein. Male Wistar rats were orally administered with scutellarin and scutellarein at the doses of 0.09, 0.17, 0.35, 0.70, 1.40 mmol/kg, respectively; then after six consecutive days, they were subjected to global ischemia by occlusion of the bilateral common carotid arteries (BCCAO). After reperfusion for about 21 h, neurological and histological examinations were performed. The present results showed that scutellarein attenuated neuronal cell damage, reduced cerebral water content, regulated the expression of glutamic acid (Glu), aspartic acid (Asp), glycine (Gly),  $\gamma$ -aminobutyric acid (GABA) and taurine (Tau), and improved the  $\text{Ca}^{2+}$ -ATPase and  $\text{Na}^{+}$ ,  $\text{K}^{+}$ -ATPase activity. Meanwhile, significant difference was found among various doses of scutellarin and scutellarein. Our studies indicated that scutellarin and scutellarein could improve neuronal injury, and scutellarein had better protective effect than scutellarin in rat cerebral ischemia.

Hao Tang, Yuping Tang, Nianguang Li, Qianping Shi, Jianming Guo, and Erxin Shang

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## Effect of different drying methods on the quality of *Angelicae Sinensis Radix* evaluated through simultaneously determining four types of major bioactive components by high performance liquid chromatography photodiode array detector and ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry.

In the present study, the effect of drying methods on the quality of *Angelicae Sinensis Radix* (DG), was evaluated by newly developed high performance liquid chromatography photodiode array detector (HPLC-DAD) and ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS/MS). Ten major bioactive components including two phenolic acids, two hydroxyl phthalides, four alkyl phthalides and two phthalide dimers were selected as evaluation chemical markers and the newly-established method was qualitatively and quantitatively validated. DG slices and whole roots dried in shade, sun light, hot air, vacuum, microwave, far infrared ray and combination of microwave and far infrared ray as well as the fresh DG samples were determined by the established methods. DG slices dried in hot air kept the similar chemical composition to that of fresh DG, while DG whole roots dried in vacuum retained highest contents of the major components. Coniferyl ferulate and ligustilide degraded significantly in DG slices dried by microwave, far infrared ray and their combination. The influence of such chemical changes induced by different drying methods on the bioactivities of DG warrants further investigation, so that the optimal drying method can be obtained for the standardization of DG herb.

Ying-Jia Bai, Ming Kong, Jin-Di Xu, Xiao-Lin Zhang, Shan-Shan Zhou, and Xiao-Ning Wan

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## Comparative metabolomics analysis on hematopoietic functions of herb pair Gui-Xiong by ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry and pattern recognition approach.

The compatibility of *Angelicae Sinensis Radix* (Danggui, DG) and *Chuanxiong Rhizoma* (Chuanxiong, CX), a famous herb pair Gui-Xiong (GX), can produce synergistic and complementary hematopoiesis. In present study, global metabolic profiling with ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QTOF/MS) combined with pattern recognition method was performed to discover the underlying hematopoietic regulation mechanisms of DG, CX and GX on hemolytic and aplastic anemia rats (HAA) induced by acetyl phenylhydrazine (APH) and cyclophosphamide (CP). Thirteen endogenous metabolites contributing to the separation of model group and control group were tentatively identified. The levels of LPCs including lysoPC (18:0), lysoPC (20:4), lysoPC (16:0) and lysoPC (18:2), sphinganine, nicotinic acid, thiamine pyrophosphate, phytosphingosine, and glycerophosphocholine increased significantly ( $p < 0.05$ ) in HAA, while the levels of oleic acid, 8,11,14-eicosatrienoic acid, ceramides (d18:1/14:0), and 17 $\alpha$ -hydroxypregnenolone decreased significantly ( $p < 0.05$ ) in comparison with control rats. Those endogenous metabolites were chiefly involved in thiamine metabolism and sphingolipid metabolism. The metabolic deviations could be regulated closer to normal level after DG, CX and GX intervention. In term of hematopoietic function, GX was the most effective as shown by the relative distance in PLS-DA score plots and relative intensity of metabolomic strategy, reflecting the synergic action between DG and CX. The relative distance calculation was firstly used in metabolomics for semi-quantization.

**Weixia Li, Yuping Tang, Jianming Guo, Erxin Shang, Yefei Qian, and Linyan Wang**

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## Metabolites profile of Xian-Ling-Gu-Bao capsule, a traditional Chinese medicine prescription, in rats by ultra performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry analysis.

Xian-Ling-Gu-Bao capsule (XLGB), a well-known traditional Chinese medicine prescription (TCMP), is widely used for the treatment of osteoporosis. However, due to lack of metabolism research, the effective material of XLGB is still unknown. It entails a huge obstacle for the clinical-safe medication administration and quality control of XLGB. To explore the metabolic fate of multiple components of XLGB, herein, we proposed a “representative structure based homologous xenobiotics identification” (RSBHXI) strategy based on ultra performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry (UPLC/Q-TOF-MS) and mass defect filter (MDF) technique. A total of 147 XLGB-related xenobiotics were identified or tentatively characterized in rat biofluids after oral administration of XLGB, including 134 (57 prototype components and 77 metabolites) in plasma, 93 (37 prototype components and 56 metabolites) in urine and 118 (46 prototype components and 72 metabolites) in bile. Our results indicated that prenylated flavonol glycosides from *Herba epimedii*, prenylated flavonoids from *Fructus psoraleae*, saponins from *Radix dipsaci* and *Rhizoma anemarrahenae*, as well as tanshinones from *Radix Salviae Miltiorrhizae* were major absorbed chemical components of XLGB. Hydrolysis, glucuronidation and sulfation were major metabolic reactions of XLGB. As more xenobiotics were detected in bile than those in urine, it demonstrated that multiple components of XLGB underwent comprehensive hepatobiliary excretion. The present study expands our knowledge about the metabolism of XLGB which will be conducive to revealing its *in vivo* pharmacological material basis. In addition, the application of RSBHXI strategy provides a new approach for metabolite identification of TCMPs and other complex mixture.

Jian-Liang Geng, Yi Dai, Zhi-Hong Yao, Zi-Fei Qin, Xin-Luan Wang, and Ling Qin

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**Preparative-LC–UV**

## Analytical HPLC to Preparative HPLC: Scale-Up Techniques using a Natural Product Extract

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### INTRODUCTION

Chromatographic separation methods can be developed on any scale. To minimize the consumption of sample and solvents there is a benefit in developing separation methods on a small scale and transferring them to a larger scale. Taking into account the important parameters and applying appropriate scaling factors, in a logical manner, enables users to scale up from analytical chromatography to larger-scale preparative separations easily and successfully. In this application note the analytical-scale separation of Kudzu (*Pueraria lobata*) root extract is used to demonstrate the calculations and techniques used to move from a 4.6 mm I.D. analytical column separation through 10, 19, and 30 mm I.D. preparatory column separations.

Kudzu is a climbing, woody or semi-woody, perennial vine with a tuberous root. The roots of Kudzu contain a number of potentially useful isoflavones, including daidzein, daidzin, genistein, genistin, and quercetin. Kudzu is also a unique source of the isoflavone puerarin. Kudzu root extracts are thought to reduce alcohol intake and reduce alcohol withdrawal symptoms. Antibacterial, anti-cancer, anti-inflammatory, and antioxidant effects have also been noted.<sup>1</sup>



Figure 1. AutoPurification System.

### EXPERIMENTAL

#### Extraction

Kudzu root pieces (20 g) were added to 100 mL of water/methanol 9:1 and shaken for 1 hour, allowed to stand overnight, and shaken for an additional 1 hour. This extract was centrifuged at 3000 RPM for 20 minutes and used without further treatment.

**Separations** Chromatographic separations, at all scales, were carried out using the Waters® AutoPurification™ System (Figure 1), which consisted of the following components:

Pump	Waters 2545 Binary Gradient Module
Detectors	Waters 2998 Photodiode Array Waters 3100 Mass Detector
Injector/collector	Waters 2767 Sample Manager
Column management	Waters System Fluidics Organizer

An initial analytical-scale separation was developed on a 4.6 x 50 mm Waters SunFire™ C18, 5- $\mu$ m column, using the conditions described below.

Column temp.:	Ambient
Flow rate:	1.5 mL/min
Mobile phase A:	Water + 0.1% Formic acid
Mobile phase B:	Methanol
Gradient:	5 to 70% B over 7 minutes
Injection vol.:	20 $\mu$ L
Detection:	UV (200 to 400 nm) and MS Full Scan 150 to 700 $m/z$

The resulting chromatogram (Figure 2) showed a number of resolved compounds and was considered an acceptable candidate for scale-up.

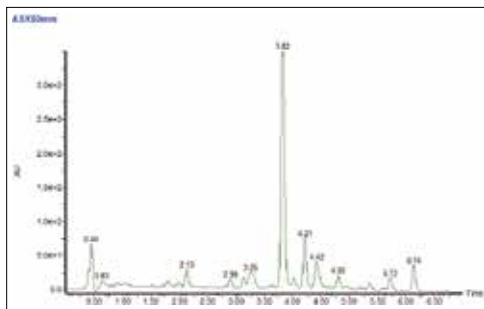


Figure 2. Analytical separation (4.6 mm I.D.) of Kudzu root extract.

## RESULTS AND DISCUSSION

### Scale-up method

A systematic approach to scale up will provide the best possible result. The ultimate goal is to maintain chromatographic resolution between key components and enable users to better predict chromatographic performance between analytical and preparative chromatography.

There are a number of key factors to consider when approaching this scale-up process.

### Column chemistry

The heart of the separation is the column. Ideally you should choose column chemistries that are identical. If the analytical and preparative columns are of different chemistry, it becomes very difficult to predict the preparative separation based on the analytical results.

Waters offers a wide range of column chemistry choices available in analytical- and preparative-scale dimensions. As well as the chemistry itself, particle size should also be considered. Columns of the same particle size will provide similar resolution of critical pairs at both separation scales. Column length also influences the separation efficiency; columns of identical length, when scaled, give similar separation power. It is possible to scale to shorter or longer columns, but keep in mind that the separation will change.

### Injection volume

To maintain peak shape and loading capacity, the injection volume needs to be suitably scaled using the following equation:

$$\text{Vol}_{\text{PREP}} = \text{Vol}_{\text{ANALYTICAL}} \cdot \frac{D_{\text{PREP}}^2}{D_{\text{ANALYTICAL}}^2} \cdot \frac{L_{\text{PREP}}}{L_{\text{ANALYTICAL}}}$$

where Vol is the injection volume ( $\mu\text{L}$ ), D is the inner diameter of the column (mm), and L is the column length (mm). For example, a 20  $\mu\text{L}$  injection on a 4.6 x 50 mm column corresponds to a 341  $\mu\text{L}$  injection on a 19 x 50 mm preparative column.

### Flow rate

To maintain separation quality the flow rate must be scaled based on column dimensions. With columns of identical particle size, the following equation is used to geometrically scale flow rate:

$$F_{\text{PREP}} = F_{\text{ANALYTICAL}} \cdot \frac{D_{\text{PREP}}^2}{D_{\text{ANALYTICAL}}^2}$$

where F is flow rate (mL/min) and D is the inner diameter of the column (mm). For example, a 1.5 mL/min flow rate on a 4.6 mm I.D. column equates to a 25.6 mL/min flow rate on a 19 mm I.D. column.

## Gradient scaling

When columns are of identical length, no changes to the gradient profile are required. If scaling to longer or shorter columns, the gradient segment volume must be maintained to preserve the separation profile.

The Waters Optimum Bed Density (OBD™) Prep Calculator, a free download, (Figure 3) is an easy-to-use tool that aids in these analytical-to-preparative scaling calculations (search on [www.waters.com](http://www.waters.com) for “Prep Calculator” or go to [www.waters.com/prepcalculator](http://www.waters.com/prepcalculator)). The Waters OBD Prep Calculator was used to convert the analytical separation method to the preparatory separation methods described in this application note.



Figure 3. Waters OBD Prep Calculator.

## Using the Waters OBD Prep Calculator

To calculate injection volume and flow rates, select the mass load scaling calculation (Figure 4) from the opening screen. Input your analytical and preparative column dimensions, analytical flow rate, and injection volume and the calculator returns the correct preparative values.



Figure 4. Waters OBD Prep Calculator mass load scaling calculation.

If your column lengths are identical, you can simply input the preparative flow rates into your gradient table using the same gradient segment times as your analytical method. Alternatively, for gradient methods, choose the basic gradient scalar calculation (Figure 5) from the opening screen, select your analytical and preparative column dimensions, input your analytical gradient table, and click the Calculate button. The preparative gradient table is automatically calculated and shown on the bottom half of the page. The Waters OBD Prep Calculator User Guide gives detailed instructions on use of all calculator functions.



Figure 5. Waters OBD Prep Calculator basic gradient scalar calculation.

## RESULTS

To demonstrate the previously described techniques, the analytical separation method described in the experimental section was scaled to three different preparative dimension columns (10.0, 19.0, and 30.0 mm I.D.). The scaled flow rates and injection volumes (all calculated using the Waters OBD Prep Calculator) are shown in Table 1.

Inside diameter (mm)	Flow rate (mL/min)	Injection volume (L)
4.6	1.5	20
10.0	7.1	95
19.0	25.6	341
30.0	63.8	851

Table 1. Waters OBD Prep Calculator scaled flow rates and injection volumes.

All of the preparative columns are SunFire Prep C18 OBD, 5  $\mu$ m, 50 mm in length, and all of the separations were performed on the same system as the analytical-scale chromatography. As can be seen in Figure 6, regardless of the scale, the chromatography (UV TIC) is very similar. When compared to the original 4.6-mm I.D. scale (Figure 2), it can be seen that in terms of resolution and retention time the chromatography is again very similar.

This simple experiment demonstrates that a systematic approach to scale up meets the goal of maintaining chromatographic resolution between key components, and enables users to better predict chromatographic performance between analytical and preparative chromatography. This exercise also demonstrates the unique capability of the Waters AutoPurification System, which allows users to perform both analytical and preparatory chromatography on the same system with no performance compromise.

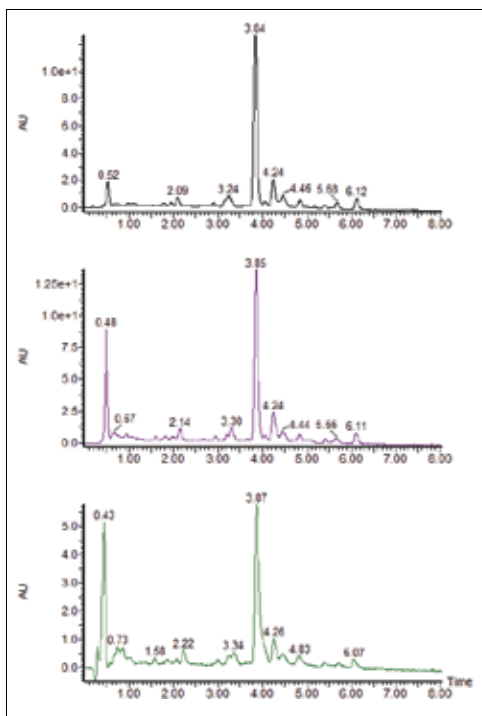


Figure 6. Scaled preparative separations, 10 mm I.D. (top), 19 mm I.D. (middle), 30 I.D. mm (bottom).

## CONCLUSION

Analytical chromatography can be successfully scaled to preparatory chromatography easily by using a systematic approach.

- The use of identical column chemistry and identical column lengths maintains separation quality.
- Waters' proprietary Optimum Bed Density (OBD) column design offers excellent sample loading and column stability in an extensive array of chemistries and configurations.
- The Waters Prep OBD Calculator aids in the scaling calculations.
- Using the Waters AutoPurification System, separation methods can be developed on an analytical scale and transferred to preparatory scale on the same system, reducing a laboratory's overall capital investment.
- Developing methods on the analytical scale and transferring them to preparatory scale reduces solvent and sample consumption, while reducing waste disposal cost, compared to developing separation methods only at the preparatory scale.

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# Waters

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## Prep 150 LC

## Isolation of a Natural Product from Echinacea Extract Using the Prep 150 LC

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### APPLICATION BENEFITS

- The Prep 150 LC System – an affordable, highly reliable system for preparative chromatography – is suitable for compound isolation from natural product extracts.
- The Prep 150 LC System is controlled by ChromScope™ Software, an intuitive and easy-to-use software that enables users to quickly purify compounds, reducing the amount of time required for training.
- The Prep 150 LC System, with its straightforward design and uncomplicated software control, facilitates users to more efficiently process samples and thereby increase productivity.

### WATERS SOLUTIONS

[Prep 150 LC System](#)

[ChromScope Software](#)

[Atlantis® T3 Prep OBD Column](#)

[Atlantis T3 Analytical Column](#)

### KEY WORDS

Isolation, purification, preparative chromatography, Prep 150 LC, Echinacea, natural product, extract, ChromScope, cichoric acid

### INTRODUCTION

Echinacea, or purple coneflower, an herbaceous flowering plant in the daisy family, is a perennial that can withstand dry climates.<sup>1,2</sup> One of the major compounds in Echinacea purpurea flowers is cichoric acid, a phenylpropanoid and a caffeic acid derivative.<sup>3,4</sup> A recent literature search on cichoric acid indicated that over 50% of the published research on this compound is related to its medicinal uses, including the treatment of upper respiratory infections, cancer, and the improvement of immune responses to different stimuli.<sup>5,6,7</sup> Many factors, including location, growing conditions, and sample handling, contribute to how much cichoric acid is found in different natural product extracts. Isolating enough of a target compound to effectively perform other experimental studies often requires several injections of the crude mixture. In this study, we focus on the feasibility of using the Prep 150 LC System as a tool for isolating cichoric acid from Echinacea purpurea extract. The Prep 150 LC System is controlled by ChromScope Software, intuitive software that enables rapid user success in isolating compounds. The principles outlined here are applicable to any isolation where the compounds have a UV chromophore.

## EXPERIMENTAL

## LC conditions

Preparative LC system: Prep 150 LC with 2545 Binary Gradient Module, Prep Inject manual injector module, 2489 UV/Visible Detector, and Waters® Fraction Collector III

Analytical column: Atlantis T3, 5  $\mu$ m, 4.6 x 50 mm  
([p/n 186003744](#))

Preparative column: Atlantis T3 OBD Prep 5  $\mu$ m, 19 x 50 mm,  
([p/n 186003696](#))

Mobile phase A: Water with 0.1% formic acid

Mobile phase B: Acetonitrile with 0.1% formic acid

Gradient: Reported in figures

Column temp.: Room

Sample temp.: Room

Injection vol.: Reported in figures

Flow rate: Reported in figures

## UV conditions for Preparative System

Detector: 2489 UV/Visible

Wavelength mode: Single

Wavelength: 330 nm

Sampling rate: 5 points/sec

Filter time constant: Normal

## Data management

ChromScope Software

## Sample description

A total of 5.3 g of *Echinacea purpurea* powdered organic root (StarWest Botanicals, Sacramento, CA 95838) was extracted with 20 mL of 70:30 methanol/water with shaking for approximately 3 hours in each of two 50 mL centrifuge tubes (containing 2.66 g and 2.64 g powdered *Echinacea*, respectively). The tubes were then centrifuged and the supernatant was filtered with several 13 mm GHP Acrodisc syringe filters. The methanol was evaporated from the extract to an orange-brown gum. The gum was dissolved in 14 mL of 95:5 water/acetonitrile.

## RESULTS AND DISCUSSION

Although there are at least four caffeic acid derivatives in *Echinacea* extracts, cichoric acid is one of the most abundant, and its usefulness in medicinal herb products arises from its immunostimulating properties.<sup>8</sup> Analytical chromatography on the crude *Echinacea* extract before methanol evaporation indicated that cichoric acid was the most abundant compound present (Figure 1A). Cichoric acid is well resolved from its neighboring peaks in the crude extract using the fast screening gradient. To increase peak resolution even more, the gradient was focused<sup>9</sup> and a loading study was performed on the analytical column. As shown in Figure 1B, an injection volume of 35  $\mu$ L of crude extract was an acceptable load on the analytical column.

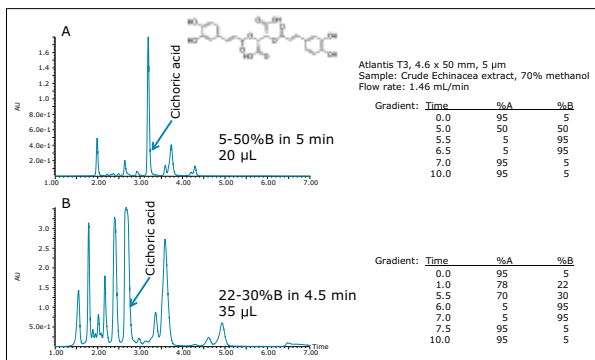


Figure 1. A: Cichoric acid in the crude echinacea extract. B: Focusing and loading on the 4.6 x 50 mm column.



Because large volume injections of strong solvent can distort preparative chromatography, the methanol in the crude extract was evaporated to a gum with a rotary evaporator. The residue was then dissolved in 14 mL 95:5 water/acetonitrile. Using the integrated Prep Calculator tool in ChromScope, geometric scaling of the flow rate and injection volume from the analytical column to the preparative column resulted in the preparative chromatography shown in Figure 2. The gradient method is conveniently displayed with the chromatogram in the injection information tab. Alternatively, fraction collection information can be viewed, eliminating the need to search for tube collection volumes (Figure 3).

Multiple injections on the Prep 150 LC System showed very good reproducibility. A total of six preparative injections of the reconstituted echinacea extract were performed. Fraction analysis on the pool showed excellent purity for the isolated product (Figure 4).



Figure 2. Preparative isolation of cichoric acid (4) and a minor impurity (3), an isomer of cichoric acid, from the crude echinacea extract.

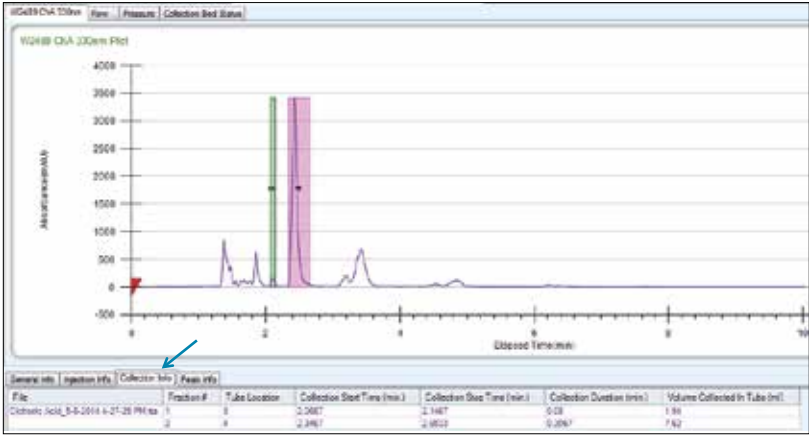


Figure 3. Fraction collection information is conveniently accessible for viewing with the chromatogram.

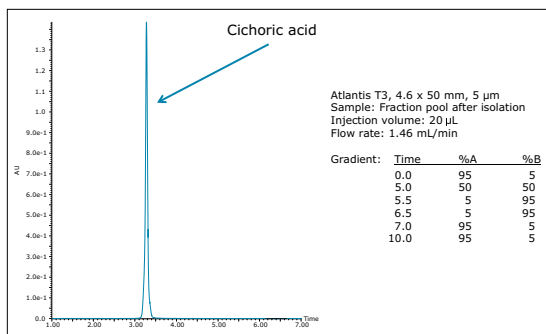


Figure 4. Analysis of the fraction pool after isolation.

## CONCLUSIONS

- The affordable, robust Prep 150 LC System is ideal for synthetic chemists who require their own conveniently accessible purification instrumentation, improving sample turnaround time.
- ChromScope, the intuitive Prep 150 LC System control software, is easy for users to learn and use, reducing the investment in time required for training.
- The integrated Prep Calculator Tool simplifies scaling and saves time by eliminating the need to manually calculate prep gradients.
- Highly-visible, colored fraction collection bars on the chromatogram inform the user of the location of the isolated product, reducing errors in sample handling and workup.
- The Prep 150 LC System enables users to rapidly isolate and purify compounds, improving productivity.
- The Prep 150 LC System is suitable for compound isolation from natural product extracts and from any sample mixture that has UV-absorbing compounds.

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## Metabolomics driven analysis of six *Nigella* species seeds via UPLC-qTOF-MS and GC-MS coupled to chemometrics.

*Nigella sativa*, commonly known as black cumin seed, is a popular herbal supplement that contains numerous phytochemicals including terpenoids, saponins, flavonoids, alkaloids. Only a few of the ca. 15 species in the genus *Nigella* have been characterized in terms of phytochemical or pharmacological properties. Here, large scale metabolic profiling including UPLC-PDA-MS and GC-MS with further multivariate analysis was utilized to classify 6 *Nigella* species. Under optimized conditions, we were able to annotate 52 metabolites including 8 saponins, 10 flavonoids, 6 phenolics, 10 alkaloids, and 18 fatty acids. Major peaks in UPLC-MS spectra contributing to the discrimination among species were assigned as kaempferol glycosidic conjugates, with kaempferol-3-O-[glucopyranosyl-(1 $\rightarrow$ 2)-galactopyranosyl-(1 $\rightarrow$ 2)-glucopyranoside, identified as potential taxonomic marker for *N. sativa*. Compared with GC-MS, UPLC-MS was found much more efficient in *Nigella* sample classification based on genetic and geographical origin. Nevertheless, both GC-MS and UPLC-MS support the remote position of *Nigella nigellastrum* in relation to the other taxa.

**Mohamed A. Farag, Haidy A. Gad, Andreas G. Heiss, and Ludger A. Wessjohann**

*Food Chem.* 2014 May 15;151:333-42.

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## Distribution of toxic alkaloids in tissues from three herbal medicine *Aconitum* species using laser micro-dissection, UHPLC-QTOF MS and LC-MS/MS techniques.

Aconite poisoning continues to be a major type of poisoning caused by herbal drugs in many countries. Nevertheless, despite its toxic characteristics, aconite is used because of its valuable therapeutic benefits. The aim of the present study was to determine the distribution of toxic alkaloids in tissues of aconite roots through chemical profiling. Three species were studied, all being used in traditional Chinese Medicine (TCM) and traditional Indian medicine (Ayurveda), namely: *Aconitum carmichaelii*, *Aconitum kusnezoffii* and *Aconitum heterophyllum*. Laser micro-dissection was used for isolation of target microscopic tissues, such as the metaderm, cortex, xylem, pith, and phloem, with ultra-high performance liquid chromatography equipped with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF MS) employed for detection of metabolites. Using a multi-targeted approach through auto and targeted LC-MS/MS, 48 known compounds were identified and the presence of aconitine, mesaconitine and hypaconitine that are the biomarkers of this plant was confirmed in the tissues. These results suggest that the three selected toxic alkaloids were exclusively found in *A. carmichaelii* and *A. kusnezoffii*. The most toxic components were found in large *A. carmichaelii* roots with more lateral root projections, and specifically in the metaderm, cork and vascular bundle tissues. The results from metabolite profiling were correlated with morphological features to predict the tissue specific distribution of toxic components and toxicity differences among the selected species. By careful exclusion of tissues having toxic diester diterpenoid alkaloids, the beneficial effects of aconite can still be retained and the frequency of toxicity occurrences can be greatly reduced. Knowledge of tissue-specific metabolite distribution can guide users and herbal drug manufacturers in prudent selection of relatively safer and therapeutically more effective parts of the root. The information provided from this study can contribute towards improved and effective management of therapeutically important, nonetheless, toxic drug such as Aconite.

**Yogini Jaiswal, Zhitao Liang, Alan Ho, LaiLai Wong, Peng Yong, Hubiao Chen, and Zhongzhen Zhao**

*Phytochemistry*. 2014 Nov;107:155-74

DOI: 10.1016/j.phytochem.2014.07.026. Epub 2014 Aug 26.

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## Metabolomics evaluation of the effects of green tea extract on acetaminophen-induced hepatotoxicity in mice.

Green tea has been purported to have beneficial health effects including protective effects against oxidative stress. Acetaminophen (APAP) is a widely used analgesic drug that can cause acute liver injury in overdose situations. These studies explored the effects of green tea extract (GTE) on APAP-induced hepatotoxicity in liver tissue extracts using ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry and nuclear magnetic resonance spectroscopy. Mice were orally administered GTE, APAP or GTE and APAP under three scenarios. APAP alone caused a high degree of hepatocyte necrosis associated with increases in serum transaminases and alterations in multiple metabolic pathways. The time of GTE oral administration relative to APAP either protected against or potentiated the APAP-induced hepatotoxicity. Dose dependent decreases in histopathology scores and serum transaminases were noted when GTE was administered prior to APAP; whereas, the opposite occurred when GTE was administered after APAP. Similarly, metabolites altered by APAP alone were less changed when GTE was given prior to APAP. Significantly altered pathways included fatty acid metabolism, glycerophospholipid metabolism, glutathione metabolism, and energy pathways. These studies demonstrate the complex interaction between GTE and APAP and the need to employ novel analytical strategies to understand the effects of dietary supplements on pharmaceutical compounds.

Yihong Lu, Jinchun Sun, Katya Petrova, Xi Yang, James Greenhaw, William F. Salminen, Richard D. Beger, and Laura K. Schnackenberg

*Food Chem Toxicol.* 2013 Dec;62:707-21.

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## Profiling of phenolic and other polar constituents from hydro-methanolic extract of watermelon (*Citrullus lanatus*) by means of accurate-mass spectrometry (HPLC-ESI-QTOF-MS).

Watermelon, *Citrullus lanatus* (formerly *Citrullus vulgaris*), is a natural and rich source of the phytochemical compounds. In this regard, the use of high-performance liquid chromatography coupled with electrospray-quadropole-time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS) has shown to be a powerful technique for the characterization of phenolic and other polar compounds from a hydro-methanolic extract of watermelon. Thus, in the present work, 71 polar compounds such as phenolic acids, flavonoids, iridoids, coumarins, lignan, and other phenolic derivatives have been detected and characterized by using MS and MS/MS data provided by the QTOF-MS, in addition to using the relevant literature on the same botanical family. Watermelon flesh was found to contain an array of diverse phytochemical components. These results indicate that watermelon offers a good source of natural phyto-components.

**Ibrahim M. Abu-Reidah, David Arráez-Román, Antonio Segura-Carretero, and Alberto Fernández-Gutiérrez**

*Food research international* 2013 v.51 no.1 pp. 354-362

DOI: 10.1016/j.foodres.2012.12.033

## A chemometric approach to the quality control of *Sutherlandia* (cancer bush).

*Sutherlandia frutescens* (Fabaceae) commonly known as cancer-bush, is a well-known traditional phytomedicine in South Africa used to treat a range of ailments. There is limited information available on the phytochemistry and chemical variation within and between the *S. frutescens* and *Sutherlandia microphylla* species complex. This paper aims to elucidate the chemical variation of phytoconstituents (other than the non-protein amino acids) between the two species *S. frutescens* and *S. microphylla* and also between the wild and cultivated varieties of *S. frutescens*. An UPLC–MS analysis in tandem with chemometric analysis has been performed to assess the metabolite content of aerial plant parts obtained from different populations. Principal component analysis (PCA) was performed to observe groupings and trends in the data matrix. An orthogonal partial least square discriminant analysis (OPLS-DA) was performed which resulted in clear groups between the two taxa. Several flavonoid and triterpenoid glycoside derivatives contribute to the quantitative chemotypic variation within and between the species as observed. The identification of these compounds using advanced chromatographic techniques (UPLC–MS) and chemometric analysis leads to a better understanding of the phytochemical variation of *Sutherlandia* which can aid in quality control of raw material, phytomedicines and commercial herbal products.

Debabrata Acharya, Gill Enslin, Weiyang Chen, Maxleene Sandasi, Thandazile Mavimbela, and Alvaro Viljoen

*Biochemical Systematics and Ecology*, Volume 56, October 2014, Pages 221–230

DOI: 10.1016/j.bse.2014.06.009

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## 20 (R)-Ginsenoside Rf: A new ginsenoside from red ginseng extract.

In spite of the general concept that herbal supplements are safe, there is a lack of appropriate quality control measures and regulations that often culminates in serious undesirable effects such as allergic reactions and renal and liver damage. Thus, there is a growing need to establish a suitable methodology that enables authentication and quality assurance of herbal products. The root of *Panax ginseng* C. A. Meyer (Araliaceae), commonly called ginseng, is traditionally recognized as a prominent herbal medicine in Far East Asia. There are two types of processed ginseng, white and red ginseng, based on processing methods, and these play a significant role in modifying ginsenosides, which are the major bioactive metabolites in these products. Herein we purify and characterize a new ginsenoside, 20(R)-ginsenoside Rf, utilizing NMR, UPLC-ESI-Q-TOF-MS and validate the metabolite is generated from its epimer, 20(S)-ginsenoside Rf during the steaming process to manufacture red ginseng. We further propose a relevant mechanism for the chemical conversion. This finding updates chemical profiling of ginseng products that can be employed in quality assurance and authentication.

Sang Myung Lee, Seok Chang Kim, Joonseok Oh, Jin Hee Kim, and MinKyun Na

*Phytochemistry Letters* (Impact Factor: 1.45). 11/2013; 6(4):620-624.

DOI: 10.1016/j.phytol.2013.08.002



## Updating chemical profiling of red ginseng via the elucidation of two geometric isomers of ginsenosides Rg9 and Rg10.

With the increasing popularity of dietary supplements, the quantitative analysis and quality control of their constituents have emerged as a significant regulatory and safety challenge. Ginseng, the root of *Panax ginseng*, has been used as a folk medicine to improve immunity, provide nutrition and diminish fatigue. Steam-processed ginseng, commonly called “red ginseng” in Korea and China, is prevalent as a dietary supplement. The different processing methods for the production of ginseng products could lead to quantitative and qualitative variations in biologically active compounds, such as the ginsenosides, present in the products. Herein, we have verified that ginsenoside Rf was transformed into two geometric isomers of ginsenoside Rg9, e.g., (20E)- and (20Z)-ginsenosides Rg9, and another ginsenoside here designated Rg10, which was inappropriately reported as ginsenoside Rg8 in a previous study. This study could be of practical use in the establishment of a comprehensive chemical profile of red ginseng for the quality control and standardization of commercial dietary supplements.

**Sang Myung Lee, Hyun Kyu Seo, Joonseok Oh, and MinKyun Na**

*Food Chem.* 2013 Dec 15;141(4):3920-4.

DOI: 10.1016/j.foodchem.2013.07.012. Epub 2013 Jul 10.

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## Investigating sub-2 $\mu\text{m}$ particle stationary phase supercritical fluid chromatography coupled to mass spectrometry for chemical profiling of chamomile extracts.

Roman and German chamomile are widely used throughout the world. Chamomiles contain a wide variety of active constituents including sesquiterpene lactones. Various extraction techniques were performed on these two types of chamomile. A packed-column supercritical fluid chromatography-mass spectrometry method was designed for the identification of sesquiterpenes and other constituents from chamomile extracts with no derivatization step prior to analysis. Mass spectrometry detection was achieved by using electrospray ionization. All of the compounds of interest were separated within 15 min. The chamomile extracts were analyzed and compared for similarities and distinct differences. Multivariate statistical analysis including principal component analysis and orthogonal partial least squares-discriminant analysis (OPLS-DA) were used to differentiate between the chamomile samples. German chamomile samples confirmed the presence of cis- and trans-tonghaosu, chrysosplenols, apigenin diglucoside whereas Roman chamomile samples confirmed the presence of apigenin, nobilin, 1,10-epioxynobilin, and hydroxyisonobilin.

**Michael D. Jones, Bharathi Avula, Yan-Hong Wang, Lu Lu, Jianping Zhao, Cristina Avonto, Giorgis Isaac, Larry Meeker, Kate Yu, Cristina Legido-Quigley, Norman Smith, and Ikhlas A. Khan**

*Anal Chim Acta*. 2014 Oct 17;847:61-72.

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## Analysis of Chemical Constituents in Jackfruit Peel by UPC<sup>2</sup>/Q-TOF-MS Method

A new method for the analysis chemical constituents in jackfruit peel by ultra performance convergence chromatography (UPC<sup>2</sup>) was developed for the first time. The extraction of jackfruit peel was carried out under classical heating with ultrasonic wave and extracted with ethyl ether. The analytes were separated on an ACQUITY UPC<sup>2</sup> HSS C18 SB column with a gradient elution (80:20 to 99.8:0.2) of mobile phase consisting of CO<sub>2</sub> and methanol. The result indicated that UPC<sup>2</sup>/Q-TOF-MS is a simple, rapid, reliable and effective method to analyze the biochemical compounds in jackfruit peel. A total of 65 compounds were identified, including acids, esters, alcohols, and pyrazine, etc.

Xiao Gong Hai Long Yu, Ning Li Qi, Shao Dan Peng, and Li Jing Lin

*Applied Mechanics and Materials* (Volumes 556-562)

DOI: 10.4028/www.scientific.net/AMM.556-562.607

## A rapid and highly specific method to evaluate the presence of 2-(2-phenylethyl)chromones in agarwood by supercritical fluid chromatography-mass spectrometry.

The detection and structural characterization of the major constituents of agarwood, 2-(2-phenylethyl)chromones, are important to quality control and the establishment of the authenticity of agarwood samples. However, a highly specific and rapid method for the evaluation of 2-(2-phenylethyl)chromones in agarwood has not been reported to date. In this study, we developed a method using supercritical fluid chromatography in combination with mass spectrometry (SFC-MS). Tropylium ions, the characteristic product ions of 2-(2-phenylethyl)chromones in tandem mass spectrometric experiments, were selected for the targeted detection of 2-(2-phenylethyl)chromones. This method used precursor ion scans for tropylium ions with different possible substitutions on a triple-quadrupole mass spectrometer. To evaluate the usefulness of the developed method, a diethyl ether extract from a Chinese agarwood "Qi-Nan" sample was first separated using SFC, and the elutes were later subjected to precursor ion scans, which searched for 15 common substituted tropylium ions to evaluate the 2-(2-phenylethyl)chromones. In the precursor ion scans, a total of 29 2-(2-phenylethyl)chromones were detected and investigated further using tandem mass spectrometry (MS/MS) to obtain more detailed structural information. By comparing the retention times and  $m/z$  values of the precursor ions with reference standards, nine of the detected compounds were unequivocally identified. The remaining compounds were tentatively identified by analyzing the MS/MS spectra. This method provides a rapid and efficient method for evaluating the 2-(2-phenylethyl)chromones present in a sample, which aids in quality control and the establishment of the authenticity of the agarwood sample.

Bing Xia, Jinrong Li, Delan Yang, Wenli Mei, Lisheng Ding and Yan Zhou

*European Journal of Mass Spectrometry*, Volume 20 Issue 5, Pages 395–402 (2014)

DOI: <http://dx.doi.org/10.1255/ejms.1289>

## Rapid and simultaneous analysis of sesquiterpene pyridine alkaloids from *Tripterygium wilfordii* Hook. f. Using supercritical fluid chromatography-diode array detector-tandem mass spectrometry.

Sesquiterpene pyridine alkaloids are considered to be the active components of *Tripterygium wilfordii* Hook. f. A rapid method was developed for comprehensive profiling of sesquiterpene pyridine alkaloids from an extract of root bark of *T. wilfordii* using supercritical fluid chromatography-diode array detector-tandem mass spectrometry (SFC-DAD-MS/MS). Alkaloids were separated on a BEH 2EP column within 10 min, eluted by CO<sub>2</sub>-methanol as mobile phase with a back pressure of 13.8 Mpa and a column temperature of 45 °C. MS/MS analysis of [M + H]<sup>+</sup> ion of each alkaloid standard showed that all the pyridine alkaloids produced very similar fragmentation patterns. The product-ions at m/z 206 and 204 were identified as the diagnostic fragments while mass region of 200–500 Da was assigned as the characteristic region. As a result, 71 components in the extract were identified as sesquiterpene pyridine alkaloids, including 40 wilfordate/evoninate type alkaloids, 13 iso-wilfordate/evoninate type alkaloids and 19 hydroxyl-wilfordate/evoninate type alkaloids. The results proved the feasibility of SFC-DAD-MS/MS method for the rapid and high-throughput analysis of sesquiterpene pyridine alkaloids in complex samples.

Qing Fu, Zhenyu Li, Cuicui Sun, Huaxia Xin, Yanxiong Ke, Yu Jin, and Xinmiao Liang

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**UPLC-QTOF-MS and UNIFI**

## Using Natural Products Application Solution with UNIFI for the Identification of Chemical Ingredients of Green Tea Extract

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### APPLICATION BENEFITS

This application note describes how to analyze and identify the chemical ingredients of green tea using the Natural Products Application Solution with UNIFI®, which combines UltraPerformance LC® (UPLC®), orthogonal quadrupole time-of-flight mass spectrometry (QToF MS), and a Traditional Medicine Library. The integration of data acquisition and processing with the Traditional Medicine Library in this solution provides a simple, efficient process to effectively facilitate the identification of chemical ingredients from complex natural product samples. As a result, it greatly improves productivity and reduced the demands for operator's technical expertise level.

### WATERS SOLUTIONS

Natural Products Application Solution with UNIFI

ACQUITY UPLC® I-Class System

Xevo® G2-S QToF MS

UNIFI Scientific Information System

Waters Analytical Standards and Reagents

### KEY WORDS

Traditional Medicine Library, ingredient analysis of natural products, UPLC/QToF MS<sup>2</sup>, natural products analytical workflow, identification of chemical ingredients, green tea extract

### INTRODUCTION

The practice of using natural products as Traditional Medicines for health benefits and for therapeutic effects is common in many countries worldwide. The efficacy of Traditional Medicines has been affirmed by thousands of years of history as well as by modern clinical practice. However, many challenges still remain for research and development in this area; for example, to reveal the material base and the mechanism of efficacy. The core of these challenges is to gain understanding on all of the chemical ingredients from a Traditional Medicines.

All research for natural products starts from ingredient analysis. However, classical methods used for ingredient analysis are complicated, time-consuming, and inefficient. To summarize, they typically follow one of these approaches:

1. Purchase of standards and comparing them with the components from samples. The cost of this approach is very high and not all compounds have relevant standards available.
2. Using various separation and preparation methods to purify the components. The problem of this approach is its blindness; it is also very time-consuming and prone to a lot of repeated work.
3. Searching for answers from literature. However, the data from previous literature could be dated, and may have been acquired by low-resolution instruments, which will lead to many false positives.

Regardless of the approach, an additional requirement is that the analyst must have a very strong technical background, both in terms of chemistry and natural product knowledge.

The inefficiency in the material base research has always been the bottleneck that limits the modernization of Traditional Medicines. In recent years, the rising popularity of liquid chromatography coupled with mass spectrometry (LC/MS) has helped to improve this situation; yet, no significant breakthrough has been made.

## EXPERIMENTAL

### Sample preparation

The powder (33 mg) of the green tea extract (Waters, No. 186006962) was dissolved in 2 mL MeOH/H<sub>2</sub>O 1/3 solution and then diluted 2X to 8.25 mg/mL final concentration for later use. The catechin standard mixture (Cerrilant No. G-016) was diluted 2X with methanol to 50 µg/mL as final concentration. Injection volume was 1 µL.

### LC conditions

LC system:	ACQUITY UPLC I-Class with FTN Sample Manager
Column:	ACQUITY UPLC HSS T3 2.1 x 100 mm, 1.8 µm
Column temp.:	40 °C
Sample temp.	15 °C
Mobile phase:	A: water (0.1% formic acid); B: acetonitrile

### Gradient:

Time	Flow Rate (mL/min)	Solvent A (%)	Solvent B (%)	Curves
0	0.6	99	1	Starting
0.5	0.6	99	1	6
16	0.6	65	35	6
18	0.6	0	100	1
20	0.6	99	1	1

### MS conditions

MS system:	Xevo G2-S QToF MS
Acquisition range:	100-1500 Da
Scan time:	0.1 s
Acquisition mode:	ESI+, ESI-; resolution mode; MS <sup>F</sup>
Lock mass:	Leucine Enkephalin (LE) 1 ppm (scan for 0.3 s, interval: 15 s)
Capillary voltage:	3 kV (ESI+)/2.5 kV (ESI-)
Cone voltage:	100 V
Collision energy:	low CE: 6 eV; high CE: 15-40 eV
Source temp.:	120 °C
Desolvation temp.:	500 °C
Cone gas flow:	30 L/h Desolvation
gas flow:	1000 L/h
Acquisition time:	20 min

Currently, the mostly commonly used LC/MS solution still includes manually observing the chromatographic peaks one by one, searching possible structural information from various Internet libraries, and checking literature to match the identities of fragments and to rationalize fragmentation pathways for the purpose of determining the chemical structure of the target component. The limitations of such an approach are still the same: time-consuming, inefficient, and high expertise requirement for the operators.

In this application note, the new Waters Natural Products Application Solution with UNIFI is introduced using the analysis of the green tea extract as an example. This solution combines the ACQUITY UPLC I-Class System, the Xevo G2-S QToF MS, and a Traditional Medicine Library that is integrated within the UNIFI Scientific Information System. The application-based solution unites data acquisition and processing within a streamlined workflow that incorporates the Traditional Medicine Library to provide a simple and efficient process for the identification of the chemical ingredients from complex natural product samples. As a result, productivity can be greatly improved and the demand for operator expertise is significantly reduced. The workflow of the ingredient analysis using the Natural Product Application Solution with UNIFI is outlined in Figure 1.

Green tea is a natural product drink favored by many. Because it is not fermented, many ingredients found from fresh leaves still remain in their original forms; for example, polyphenols, catechins, pyrocatechins, caffeine, amino acids, vitamins, etc. This provides a good foundation for chemical ingredient analysis, hence it allows us to use green tea as an example to demonstrate use of the Natural Product Application Solution with UNIFI to verify the analytical results with the available standard substances. The entire analytical process, from sample injection to report generation, required merely two hours to complete.

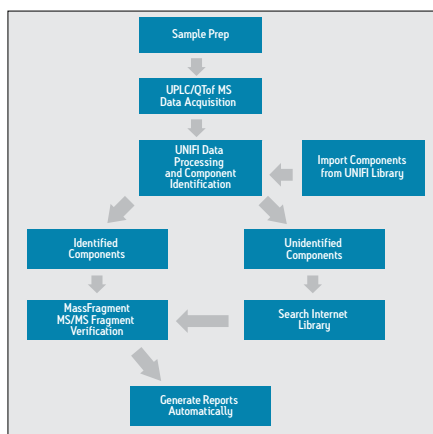


Figure 1. The workflow of ingredient analysis using the Natural Product Application Solution with UNIFI.

## RESULTS AND DISCUSSION

UPLC and QToF MS with MS<sup>E</sup> were used to analyze the chemical ingredients in green tea extract. The Natural Products Application Solution with UNIFI featuring the Traditional Medicine Library was used to process the data. 28 components were initially identified with 16 being confirmed upon verification by MassFragment.<sup>TM</sup> The complete analysis took two hours from sample injection to report generation.

The Natural Product Application Solution with UNIFI includes the ACQUITY UPLC I-Class, Xevo G2-S QToF MS, and the UNIFI Scientific Information System containing the Traditional Medicine Library, along with a Waters Green Tea Extract and the Catechin Standard Mixture. This integrated solution also includes 14 preset component and binary analysis workflow templates, and three report templates, which result in a fully automated procedure from data acquisition and processing, to library search and structure verification, to report generation.

Figure 2 shows the UPLC/QToF MS base peak ion (BPI) chromatogram of the green tea extract. The advantages of using UPLC for the analysis of complex samples are fully demonstrated here. Not only is the run time shortened (effective separation time of 15 min), but also it enhanced the separation efficiency and peak capacity. In the meantime, MS information with accurate mass measurements was also provided by the QToF MS. Additionally, with the MS<sup>E</sup> data acquisition strategy, chemists can obtain the molecular weight information of the compounds (from low-collision-energy MS scan) and the respective fragmentation ion information (from high-collision-energy MS scan) from a single injection. All provides a solid foundation for in-depth ingredient analysis and structure identification in future steps.

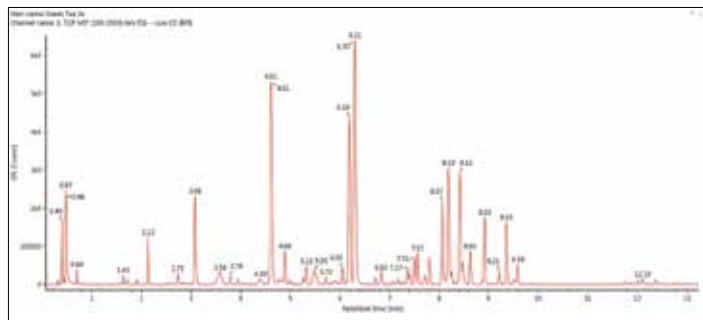









Figure 4. The list of identified components from green tea after processing by UNIFI.

All ingredient analysis solutions offered before UNIFI Software required researchers to manually extract each individual chromatographic peak, check its corresponding mass spectrum, and deduce the possible molecular formula based on the exact mass. Afterwards, researchers need to search online libraries based on molecular formula, deduce fragmentation pathways based on the fragment ions, then finally determine the chemical structure of the target component. Now with the Natural Products Application Solution with UNIFI, after all relevant components are imported from the Traditional Medicine Library, data processing and library search are performed automatically as a single step. The identification result is presented directly (Figure 5), while the rationality of the structure is automatically verified based on fragment ions via MassFragment.

As shown in Figure 4D, each fragment ion that corresponds to the compound precursor ion can be examined by simply clicking on the blue icon . Researchers only need to determine if the verified fragment structure is sensible. If yes, then it can be potentially concluded that the proposed compound structure is correct, and this component can be defined as confirmed. If a false positive is suspected, further identification and structural elucidation can be carried out by using the UNIFI Structural Elucidation Tool to search more online libraries and again utilizing MassFragment. By the same token, all unidentified components can be investigated in this manner. Final results of all confirmed components can be shown as a table by clicking the “Component – Confirmed Table” (Figure 4A), or as a plot by clicking “Component - Confirmed Plot” (Figure 5), or as a list that can be generated using a report template (Figure 6).

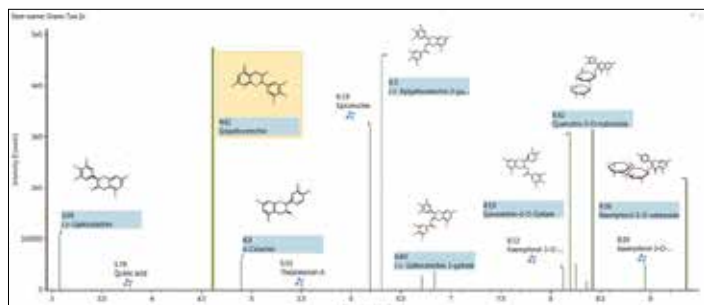
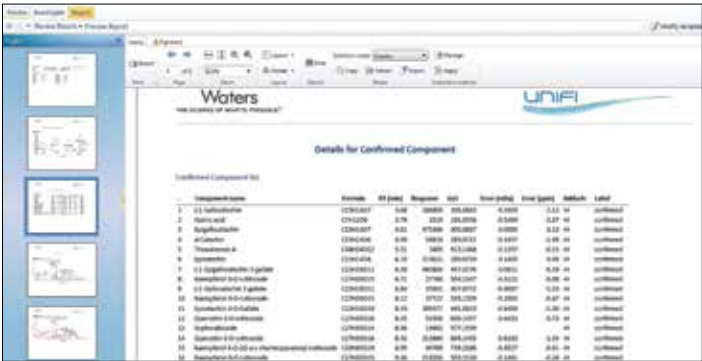


Figure 5. Summary Plot of the identified components of green tea extract after processing by UNIFI.

The Natural Products Application Solution with UNIFI contains three report templates. The report templates related to ingredient analysis are: NP Component Summary Template and NP Component Details Template. For example, a summary report of the status of the components identification can be easily obtained by importing the NP Component Summary Template (Figure 6). This report contains sample information, data acquisition and processing methods, as well as related data information.



Component Name	Retention	MS peak	Mass	MS/MS	MS/MS	MS/MS	MS/MS	MS/MS	MS/MS
1. 1,1-Dichloroethane	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11
2. Ethyl acetate	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11
3. Propyl acetate	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11
4. n-Butyl acetate	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11
5. n-Pentyl acetate	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11
6. n-Hexyl acetate	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11
7. n-Heptyl acetate	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11
8. n-Octyl acetate	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11
9. n-Nonyl acetate	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11
10. n-Decyl acetate	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11
11. n-Undecyl acetate	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11
12. n-Dodecyl acetate	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11
13. n-Tridecyl acetate	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11
14. n-Tetradecyl acetate	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11	1.11

Figure 6. Summary report for the status of component identification, which is easily obtained by importing the NP Component Summary Template.

CONCLUSIONS

Green tea ingredient analysis has been used as an example to introduce the Natural Products Application Solution with UNIFI. UPLC overcomes the shortcomings of traditional HPLC separations, such as long separation time, limited resolution, and low peak capacity. QToF MS provides molecular weight and fragmentation information in exact mass, and sufficient dynamic range, laying a solid foundation for natural product component identification and quantification.

The Natural Products Application Solution with UNIFI is accompanied by the Traditional Medicine Library, with the ability to automatically identify component structures. It is a new solution for ingredient analysis of complex natural product samples.

The UNIFI informatics platform enables this entire process to be completed all at once, from injecting sample to processing data to printing report. The entire green tea ingredient analysis was completed in only two hours. This solution also contains preset workflow templates and various report templates, provide further time saving for method re-editing.

In effect, we provide a simple and efficient process for the identification of chemical ingredients in complex natural product samples. Productivity is greatly improved and demands for an operator’s technical expertise is greatly reduced, so that these analyses can be performed much more routinely, and experienced operators can now apply their knowledge to more advanced laboratory challenges. As a result, this provides a breakthrough for alleviating the bottleneck of natural product research.

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## UPLC/QTOF/MarketLynx

## Identification and Characterization of Key Chemical Constituents for The Authentication of *Hoodia Gordonii* Using UPLC/QTOF MS<sup>E</sup>

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### APPLICATION BENEFITS

Allows for rapid characterization of the key chemical constituents from the authentic *H. gordonii*, provides easy generic workflow to authenticate dietary supplements that claim to contain *H. gordonii*.

### WATERS SOLUTIONS

ACQUITY UPLC® System

SYNAPT® G2 MS System

MarkerLynx XS™ Application Manager

MassFragment™ Application Manager

### KEY WORDS

Authentication, *Hoodia*, weight loss, dietary supplement, UPLC, TOF MS<sup>E</sup>, Multivariate statistical analysis (MSA), plant metabolomics, functional food

### GOAL

To develop a practical workflow that allows quick authentication of any botanical or commercially packaged product claiming to contain *Hoodia gordonii*.

### INTRODUCTION

*Hoodia gordonii*, from the family *Asclepiadaceae*, is a slow growing succulent plant that is traditionally used in South Africa as an appetite suppressant.<sup>1,2</sup> Over the past 5 to 10 years, the *Hoodia* plant and the preparations of *Hoodia* have become increasingly popular. These dietary supplements are promoted for weight reduction. Previously published *Hoodia*-related phytochemical papers focused on two species: *H. gordonii* and *H. pilifera*. These species had about 40 different pregnane glycosides reported as being isolated and structurally elucidated. They were comprised of a few aglycones, such as hoodigogenin A, calogenin, hoodistanal, and dehydrohoodistanal. The aglycon hoodigogenin A is unique for *Hoodia*.<sup>3</sup>

The limited availability of the *Hoodia* plant material and its increasing popularity has opened the possibility of adulteration by other botanicals. One possible adulterant may be the *Opuntia species*, which grows quickly. There have been no appetite-suppressing properties associated with these species. Consequently, analytical methods have been developed based on the acquired knowledge of the plants composition in order to detect adulteration.<sup>3</sup> Previous screening results have prompted serious concerns about the safety of commercial products that claim to be *Hoodia*, as a considerable amount of these products seem to lack *Hoodia*.

In this application note, we present a novel UPLC®/QTOF-MS<sup>E</sup>/multivariate statistical analysis (MSA) workflow for the holistic characterization of the chemical constituents from *H. gordonii* and to authenticate the dietary supplements that claim to contain *H. gordonii*. This workflow provides a new method for the fast generation, and the automated analysis of information-rich data.

EXPERIMENTAL

For each commercial *Hoodia* sample, five capsules were weighed and opened, and the contents were emptied. The content of the capsules were mixed and triturated using a mortar and pestle.

Dry plant sample (0.3 g) and an adequate amount of powdered capsule content (average wt of five capsules) were sonicated in 2.5 mL of methanol for 25 min, respectively followed by centrifugation for 15 min at 3300 rpm. The supernatant was transferred to a 10 mL volumetric flask. The procedure was repeated three times, and then the respective supernatants were combined. The final volume was adjusted to 10.0 mL with methanol and mixed thoroughly. Prior to injection, an adequate volume (ca. 2.0 mL) of the extracted solution was passed through a 0.45 µm nylon membrane filter. The first 1.0 mL was discarded, and the remaining volume was collected in an LC sample vial. Each sample solution was injected in triplicate.

UPLC conditions

LC system: ACQUITY UPLC  
Column: ACQUITY UPLC HSS T3 Column  
2.1 x 100 mm, 1.8 µm  
Column temp.: 60°C  
Flow rate: 500 µL/min.  
Mobile phase: A: Water + 0.1% formic acid  
B. Methanol  
Injection volume: 5 mL  
Gradient: 98% A to 80% A in 1 min;  
then to 5% A linear to 25 min.  
At 25 min, step to 0% A and  
hold at 0% A till 30 min.  
Equilibrate for 4 min.  
Total run time: 34 min.

MS conditions

MS System: SYNAPT G2 Mass Spectrometer  
Ionization mode: ESI- and ESI+  
Acquisition range: 50 to 1600 m/z  
Capillary V: 3 kV  
Cone V: 40 V  
Desolvation temp.: 500 °C  
Desolvation gas: 900 L/Hr  
Source temp.: 120 °C  
CE: Low: 4 eV  
High: 20 to 50 eV

RESULTS AND DISCUSSION

The UPLC/oaTOF MS<sup>E</sup> analysis was performed for multiple samples, as shown in Table 1, in both positive and negative ionization modes. Here, we mainly focus on results obtained in negative ion mode. To ensure data integrity, all samples were pooled into a single vial and used as the QC run. For each individual sample, six replicates of injection were performed with the sequence of the injections randomized.

NCNPR Accession #	Name	Place
2821	<i>Hoodia gordonii</i>	Missouri Botanical Garden, Missouri, USA
2925	<i>Hoodia gordonii</i>	Universiteit Van Vry staat, South Africa
3165	<i>Hoodia gordonii</i>	American Herbal Pharmacopoeia (AHP), USA
3229	<i>Hoodia gordonii</i>	TRISH, USA
2799	<i>Hoodia gordonii</i>	Commercial Source
2888	<i>Opuntia ficus-indica</i>	Commercial, USA
2915	<i>Opuntia leptocaulis</i>	Commercial, USA
3558	Product-1	Dosage Form: Capsules
HG-1	Product-2	Dosage Form: Capsules
HG-4	Product-3	Dosage Form: Capsules
HG-5	Product-4	Dosage Form: Capsules
HG-6	Product-5	Dosage Form: Capsules

Table 1. Samples analyzed for this project. Sample 2925 is *H. gordonii* obtained from South Africa. It was used as the benchmark to authenticate samples throughout this project.

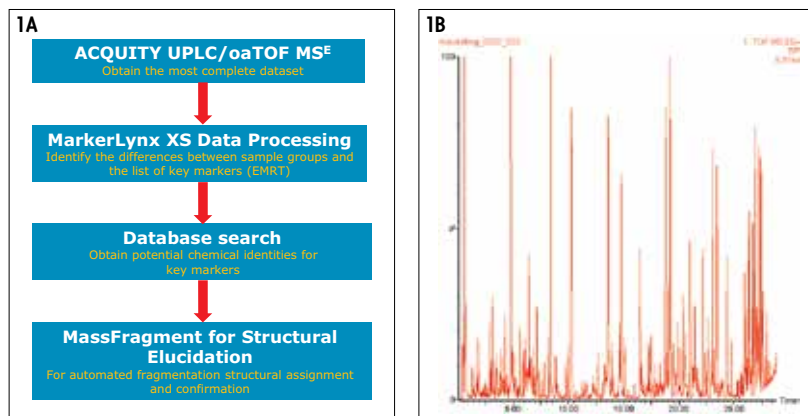


Figure 1. Metabolomics workflow and example of the UPLC/oaTOF base peak ion chromatogram (BPI). 1A. The UPLC/oaTOF MS<sup>E</sup>/MSA analysis workflow. 1B. Example BPI for an authentic *H. gordonii* extract (Sample 2925).

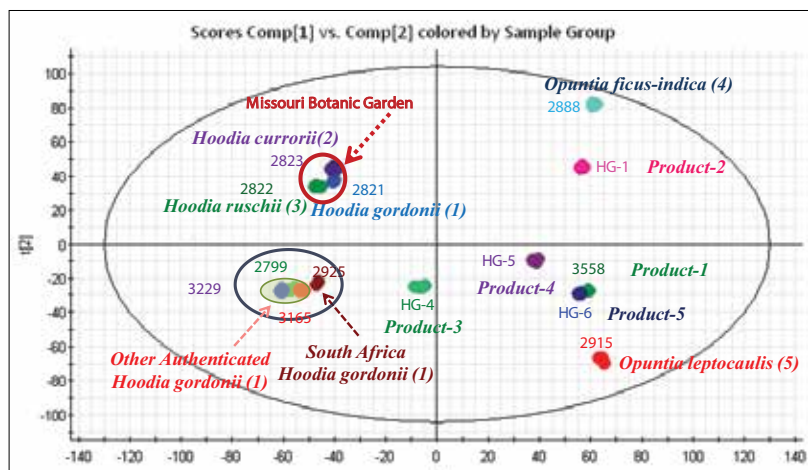


Figure 2. PCA Scores Plot of the entire data set obtained from negative ion mode.

Figure 1A shows the UPLC/oaTOF MS<sup>E</sup>/MSA workflow used for this project. Figure 1B shows the Base Peak Ion (BPI) chromatogram that was obtained for the authentic *H. gordonii* extract (Sample 2925). For these types of complex samples, the priority is to maximize the separation resolution with sufficient peak capacity. This resulted in a 100 mm column with a 30 min runtime being adapted for the analysis.

The initial stage of the data mining was to perform the Principal Component Analysis (PCA) for the entire dataset. The Scores Plot of the PCA analysis is shown in Figure 2. Of the five *H. gordonii* samples analyzed, four were grouped closely together. However, Sample 2821 from the Missouri Botanical Garden clearly grouped closer to the other two Missouri Botanical Garden *Hoodia* spp samples. This indicated that the plant location has a profound influence on the chemical content. Further chemical investigation about plant location is not within the scope of this application note, and will be presented elsewhere.

The data mining strategy steps were as follows:

1. Obtain the total PCA based on the entire dataset.
  2. Perform OPLS-SA for five groups of samples.
  3. Obtain lists of key markers from the S-Plot (five lists obtained).
  4. Obtain a master list of key markers that consistently showed as key markers for *H. gordonii* based on the five comparisons.
  5. Obtain chemical identities of the key markers.
  6. Use MS<sup>E</sup> high energy fragment ions to perform structural elucidation and confirm the chemical identities.
  7. Reprocess the dataset using only the key markers to obtain the PCA S-Plot of key markers.
- The key marker PCA S-Plot provides a visual display of the authentication of the commercial samples.

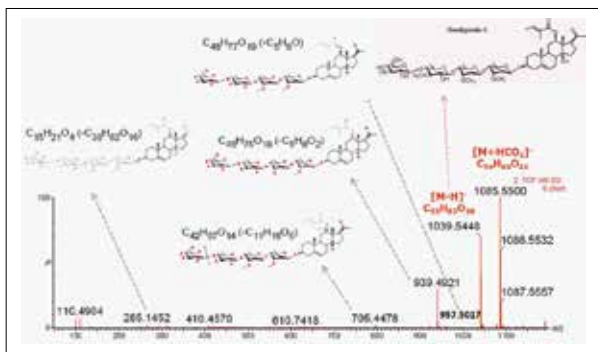
For the OPLS-DA analysis, we used all five *H. gordonii* samples collectively as Group 1, and used the commercial *Hoodia* product as Group 2. As there are five *Hoodia* products, the comparison was performed five times, each with a different *Hoodia* product as Group 2. The S-Plot generated by OPLS-DA for each of the group comparisons allowed leading markers to be obtained. Five S-Plots were obtained; hence five lists of leading markers were obtained, each showing the key markers that have major contributions of differentiating the two sample groups. From the five key marker lists, a group of **twelve (12) markers** were commonly shown in all lists indicating their positive existence in the *H. gordonii* samples.

The identification of the key markers was obtained by an elemental composition search, that matched the results with components reported from *H. gordonii* in literature references.<sup>4</sup> This was further confirmed with fragment analysis from the high-energy MS<sup>E</sup> data obtained from the same injection.

*H. gordonii* is a rich source of pregnane glycosides. In each of these steroidal glycosides there is always a sugar moiety linked to position C-3, and often there is a second moiety attached to C-20. The steroidal glycosides in *Hoodia gordonii* have been classified into two major core groups: hoodigogenin A and calogenin. The hoodigosides E, K, D, C, Unknown-2, Unknown-3, and gordonoside D have structures similar to hoodigogenin A. The hoodigosides M, O, and Unknown-1 have a structure similar to calogenin. In hoodigogenin-type compounds, the sugar moiety is substituted on the C-3 of aglycone for hoodigogenin compounds (the aglycone was characterized as 12-O- $\beta$ -tigloyl-3 $\beta$ ,14 $\beta$ -dihydroxy-pregn-5-ene-20-one) and tigloyl moiety attached to C-20. In calogenin-type compounds, a tigloyl moiety and the sugar linkage were present at C-3, and an attachment of (1 to 6) linked  $\beta$ -D-glucopyranoside units to C-20 of calogenin.

With the TOF MS<sup>E</sup> data acquisition strategy, the proposed structure of the key marker was confirmed by checking the fragment ions obtained at the high energy scan (obtained within the same LC injection). MassFragment Application Manager (imbedded in MassLynx Software) was used for automatic structural elucidation. In Figure 3, the the MS<sup>E</sup> spectrum provides the structural characterization of hoodigoside E, one of the key markers listed in Table 2. The structures of the fragments were automatically assigned if the intact parent structure was available as a mol file.

Table 2 provides a summary of the twelve (12) key markers that were identified using the workflow specified above. Figure 4 shows the key marker generated by PCA for the dataset using only the key markers as the inclusion list.



*Figure 3. Structural characterization for Hoodigaside E automatically performed by MassFragment using the high energy spectrum.*

Compounds	Formula	RT (min)	[M-H] <sup>-</sup>	2799	2821	2925	3165	3229	2888	2915	P-1	P-2	P-3	P-4	P-5
Flavonoid glycoside	C <sub>21</sub> H <sub>19</sub> O <sub>11</sub>	4.81	447.0928	+	+	+	+	+	-	-	-	-	+	+	-
Flavonoid glycoside	C <sub>21</sub> H <sub>19</sub> O <sub>11</sub>	7.15	447.0924	+	+	+	+	+	-	-	-	-	+	+	-
Hoodigside M	C <sub>54</sub> H <sub>89</sub> O <sub>27</sub>	13.64	1123.5513	+	+	+	+	+	-	-	-	-	+	+	-
Unknown-1	C <sub>54</sub> H <sub>89</sub> O <sub>27</sub>	13.97	1123.5509	+	+	+	+	+	-	-	-	-	+	+	-
Unknown-2	C <sub>47</sub> H <sub>77</sub> O <sub>20</sub>	14.83	961.5007	+	+	+	+	+	-	-	-	-	+	+	-
Hoodigside K	C <sub>45</sub> H <sub>70</sub> O <sub>18</sub>	15.14	897.4423	+	+	+	+	+	-	-	-	-	+	+	-
Hoodigside O	C <sub>53</sub> H <sub>85</sub> O <sub>23</sub>	18.86	1043.5442	+	+	+	+	+	-	-	-	-	+	+	-
Hoodigside E	C <sub>55</sub> H <sub>84</sub> O <sub>20</sub>	19.27	1039.5499	+	+	+	+	+	-	-	-	-	+	+	-
Hoodigside D	C <sub>55</sub> H <sub>87</sub> O <sub>21</sub>	20.6	1037.5646	+	+	+	+	+	-	-	-	-	+	+	-
Hoodigside C	C <sub>54</sub> H <sub>86</sub> O <sub>18</sub>	21.51	1021.5662	+	+	+	+	+	-	-	-	-	+	+	-
Unknown-3	C <sub>54</sub> H <sub>86</sub> O <sub>18</sub>	21.67	1021.5782	+	+	+	+	+	-	-	-	-	+	+	-
Gordonoside D	C <sub>47</sub> H <sub>89</sub> O <sub>24</sub>	22.22	991.563	+	+	+	+	+	-	-	-	-	+	+	-

Table 2. Key markers identified for *H. gordonii* as a result of the MSA analysis using the UPLC/oaTOF MSE analysis workflow.

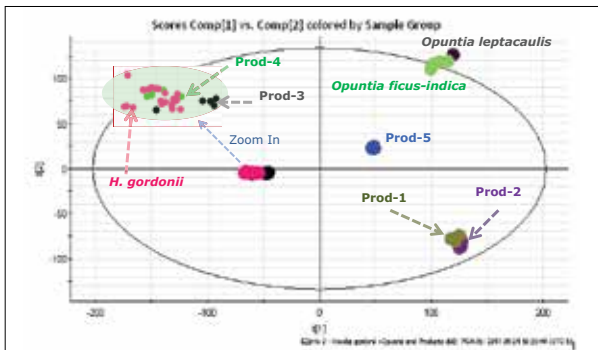


Figure 4. The Scores Plot of the key marker PCA analysis result, which was obtained by processing the dataset with only the markers identified.



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## CONCLUSIONS

This application note demonstrated a simple UPLC/*oa*TOF MS<sup>E</sup> coupled with Multi Variate Statistical Analysis workflow to allow a list of key markers for *H. gordonii* to be identified. As a result, specific data analysis method was created. It contains identified key markers for *H. gordonii* so that authentication of commercial products, or their herbal extracts, can be easily accomplished with confidence.

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July 2011 720004038en AG-PDF

## UPLC/SYNAPT G2 (ion mobility)

## Flavonoids Identification in Complex Plant Extracts using Ion Mobility TOF MS and MS<sup>E</sup>

Melvin Gay, Evelyn Goh, Mark Ritchie  
Waters Pacific Pte Ltd., Singapore

### APPLICATION BENEFITS

- Screening of flavonoids in complex plant extract (*Ficus* sp.) using ACQUITY UPLC®/SYNAPT® G2 HDMS.™
- Identification of isomers using ion mobility and MS<sup>E</sup> acquisition functionality.
- HDMS<sup>E</sup> provides another dimension of orthogonal separation, delivering unprecedented peak capacity for increased confidence in isomers identification of complex mixtures.

### WATERS SOLUTIONS

ACQUITY UPLC System

SYNAPT G2 High Definition Mass Spectrometry™ (HDMS)

DriftScope™

MS<sup>E</sup> Data Viewer

ACQUITY UPLC HSS  
(High Strength Silica) Technology

### KEY WORDS

Flavonoids, isomers, ion mobility, TOF, HDMS

### INTRODUCTION

Flavonoids are a remarkable group of plant metabolites that ubiquitously exist in natural products that have been considered as an active ingredient of many medicinal plants.<sup>1</sup> Generally, the backbone of flavonoids consists of two phenyl rings and a heterocyclic ring, but they are often conjugated to a carbohydrate moiety with individual differences arising from various chemical processes, such as hydroxylation, methoxylation, glycosylation, and acylation.<sup>2</sup>

Plants containing flavonoids have been used for thousands of years in traditional Eastern medicine. In recent years, plant flavonoids have been shown to be of vital significance to humans. They have been linked as active contributors of health benefits, including its antioxidant properties in beverages such tea and wine, and in foods such as fruits and vegetables.

Waters® SYNAPT G2 High Definition Mass Spectrometry (HDMS), a combination of high-efficiency ion mobility separation (IMS) and time-of-flight (TOF) mass spectrometry, has been used to effectively separate and identify natural product structural isomers.<sup>3</sup> The rapid orthogonal gas separation technique in the IMS T-Wave™ allows another dimension of separation via their mass and shape without compromising MS data quality or sensitivity.

MS<sup>E</sup> is an acquisition technique that provides a simple, unbiased, and parallel route to deliver exact mass, low energy precursor (MS) and high energy fragment ion (MS<sup>E</sup>) information from every detectable component, without the need for multiple injections.

This application note describes the analysis of *Ficus* sp. extract using Waters ACQUITY UPLC System combined with the SYNAPT G2 HDMS System with IMS and MS<sup>E</sup> functionality to provide chromatographic and isobaric separation for a more comprehensive structural characterization of flavonoids.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY HSS T3 2.1 x 100 mm, 1.8 μm
Column temp.:	40 °C
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid
Injection volume:	5.0 μL PLNO
Total run time:	10.0 min

MS conditions

MS System:	SYNAPT G2 HDMS
Ionization:	ESI negative
Capillary voltage:	1.7 kV
Sampling cone:	30 V
Extraction cone:	4.0 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Desolvation gas:	1000 L/hr
Cone gas:	50 L/hr
Trap CE:	4 V
Transfer CE:	0 V
Trap/transfer gas:	Ar
IMS gas:	N <sub>2</sub> (~3.4 mbar)
IMS T-Wave speed:	650 m/sec
IMS T-Wave height:	40 V
Mass range:	50 to 1200 m/z

Sample preparation

Plant samples (*Ficus* sp.) were extracted in 50% methanol/water solution. The extract was then centrifuged and the supernatant was collected for further analysis.

Mobile phase gradient is detailed in Table 1.

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.4	99	1	–
1.0	0.4	95	5	6
6.5	0.4	50	50	6
7.5	0.4	5	95	6
8.0	0.4	99	1	6
10.0	0.4	99	1	6

Table 1. ACQUITY UPLC System mobile phase gradient.

RESULTS AND DISCUSSION

In this profiling study, the base peak ion chromatograms of the extracted *Ficus* sp. samples showed a high degree of complexity, with numerous co-eluting components and also the presence of isomers. The advantages of ACQUITY UPLC not only produces highly reproducible chromatograms between injections, as shown in Figure 1, but also high throughput with a rapid analysis time of 10 mins. When coupled together with IMS and MS<sup>E</sup> functionality of the SYNAPT G2 HDMS, another dimension for the separation of isomers/isobaric compounds is attained. With this system, comprehensive structural information can be acquired without compromising sensitivity and analysis time.

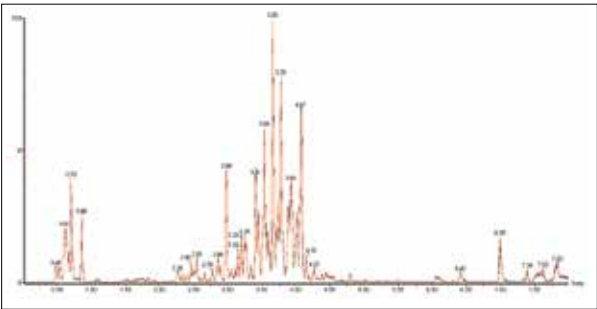


Figure 1. Overlay BPI chromatograms of extracted *Ficus* sample (six injections).

The flavone C-glycosides are an important subclass of the flavonoids family. Flavone C-glycosides are present in foodstuffs and nutraceuticals and they include orientin (luteolin-8-C-glucoside), isoorientin (luteolin-6-C-glucoside), vitexin (apigenin-8-C-glucoside), and isovitexin (apigenin-6-C-glucoside). They are also reported to exhibit anti-inflammatory and anti-nociceptive properties.<sup>4,5</sup>

Both vitexin and isovitexin have the same chemical formula of  $C_{21}H_{20}O_{10}$  with an exact mass of  $m/z$  431.0978 [M-H]<sup>-</sup>. Using the above UPLC® method, the extracted ion chromatogram showed two peaks with a baseline chromatographic separation at 4.07 min and 4.66 min, as shown in Figure 2A. As both compounds are isobaric, the assignment of vitexin and isovitexin to these peaks (4.07 min and 4.66 min) are not possible with only high resolution spectra alone. The identities of these two peaks were further confirmed using MS<sup>E</sup> and IMS.

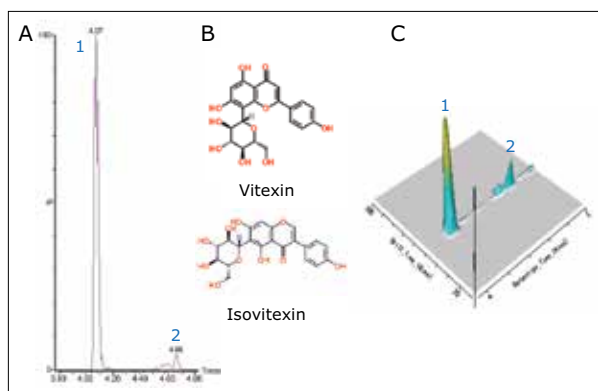


Figure 2. UPLC/SYNAPT G2 HDMS of *Ficus* sp. extract. 2A. XIC of *Ficus* sp. extract at 431.0978  $m/z$ . 2B. Molecular structure of vitexin and isovitexin. 2C. 3D illustration of *Ficus* sp. extract from 4 to 5 min. The 3D plot shows the components were separated by chromatographic retention time. Vitexin and isovitexin are labeled as 1 and 2 respectively.

Baseline chromatographic separation of vitexin and isovitexin via retention time was achieved. However the fragmentation patterns observed in the MS<sup>E</sup> spectra for both vitexin and isovitexin were identical. The predicted product ions of vitexin and isovitexin, were then cross-checked against the MS<sup>E</sup> spectra of the *Ficus sp.* extract samples using MassFragment™ Software to provide added confidence. The MS and MS<sup>E</sup> spectra of isovitexin are shown in Figure 3.

Using HDMS, both compounds were further separated via ion mobility based on their structural configuration and a 3D plot was generated, as shown in Figure 2C. From Figure 2C, it can be observed that vitexin and isovitexin have drift times of 81.78 bins (4.45 ms) and 83.44 bins (4.53 ms) respectively. Thus using retention times, MSE and HDMS, the identity of vitexin and isovitexin can be determined.

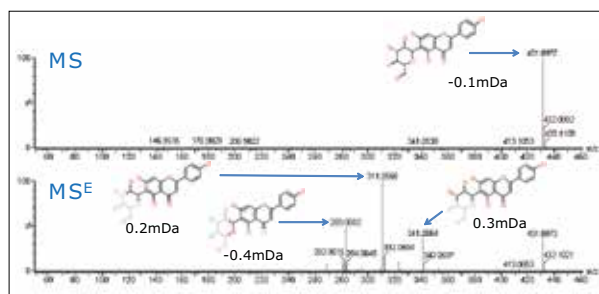


Figure 3. MS and MS<sup>E</sup> spectra of isovitexin (with mass error) at 4.66 min.

Two additional important C-glycoside flavonoids are orientin and isoorientin. They have a chemical formula of C<sub>21</sub>H<sub>20</sub>O<sub>11</sub> with an exact mass of  $m/z$  447.0927 [M-H]<sup>-</sup>. The extracted ion chromatogram in Figure 4 shows two major peaks at 3.72 min and 3.83 min.

Baseline chromatographic separation of isoorientin and orientin via retention time was achieved. However upon further interrogation of these peaks using ion mobility and DriftScope Data Viewer, when a 3D plot was generated, shown in Figure 4C, it was observed that an unknown peak ( $m/z$  635.1767) co-eluted with the orientin peak (marked with an asterisk in Figure 4C).

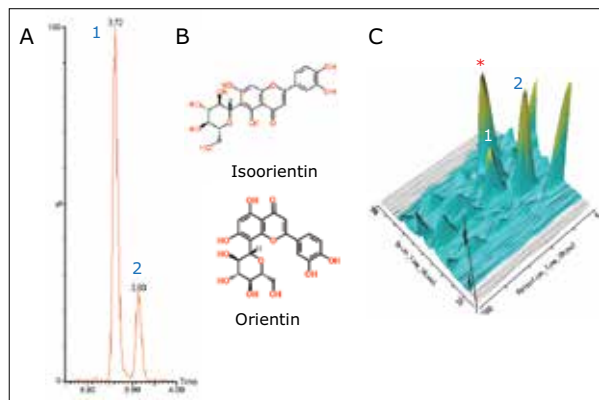


Figure 4: UPLC/SYNAPT G2 HDMS of *Ficus sp.* extract. 4A. XIC of *Ficus sp.* extract at 447.0927  $m/z$ . 4B. Molecular structure of isoorientin and orientin. 4C. 3D illustration of *Ficus sp.* extract from 3.5 to 4.0 min. The 3D model shows the components were separated by chromatographic retention time and ion mobility drift time. Isoorientin and orientin are labeled as 1 and 2 respectively. An unknown co-eluting compound is marked with an asterisk.

The 2D DriftScope plot in Figure 5 illustrates the IMS separation of isobaric orientin and isoorientin (447.0927  $m/z$ ), showing two isomers with drift times of 76.83 bins (4.16 ms) and 84.33 bins (4.54 ms) respectively. Thus by estimating the cross-sectional structure of orientin and isoorientin, shown in Figure 4B, it can be proposed that the more compact orientin is the species with the drift time of 4.16 ms and the more extended structure of isoorientin has a longer drift time of 4.54 ms. Using the HDMS<sup>E</sup> available on the SYNAPT G2 HDMS, the product ions of orientin and isoorientin were easily visualized by their drift times and mass-to-charge ratios, as shown in the insert in Figure 5.

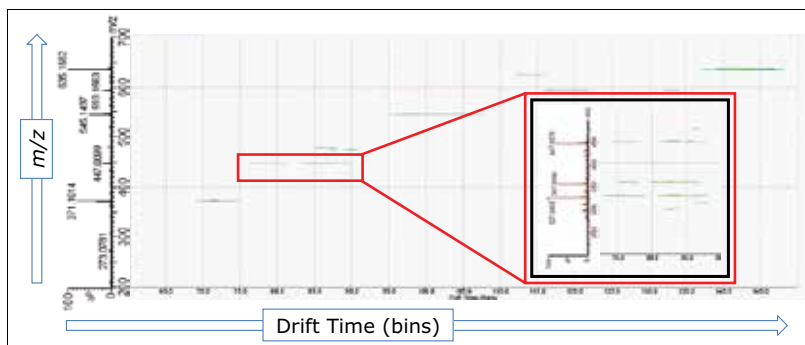


Figure 5. Visualization (drift time versus  $m/z$ ) of compounds eluting between 3.7 and 3.9 min. Note the separation of orientin and isoorientin with drift times of 4.16 and 4.54 ms respectively. Inset: Product ions of orientin and isoorientin via post-IMS collision-induced dissociation.

Using the MS<sup>E</sup> Data Viewer, with the selection of  $m/z$  447.0927, the BPI chromatogram window showed two prominent peaks at 3.73 and 3.84 min, as shown in Figure 6A. However upon further data interrogation of the peak at 3.84 min, it was observed that there were several co-eluting compounds, which were of higher intensity than the peak of interest, as shown in Figure 6B. Thus due to the high complexity of the sample and the vast amount of product ions present in the MSE spectra. It is impossible to accurately determine the fragmentation pattern of orientin, see Figures 6C and 6D.

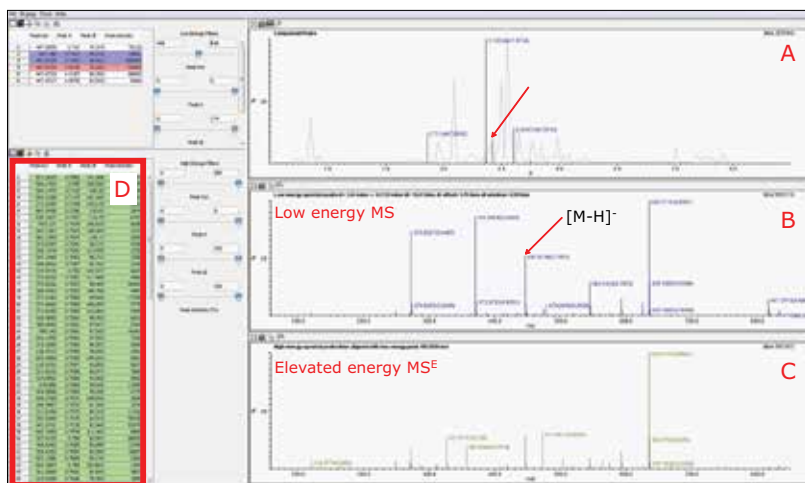


Figure 6. Orientin peak at 3.84 min using MS<sup>E</sup> Data Viewer software. 6A. Chromatogram of peaks with  $m/z$  447.0927. Orientin peak with retention time of 3.84 min was selected (highlighted in red with an arrow). 6B. Low energy mass spectrum (MS) of peak at 3.84 min. Molecular mass of orientin [M-H]<sup>-</sup> is indicated (arrow). 6C. High-energy mass spectrum (MSE) of peak at 3.84 min. 6D. List of product ions present in the MSE spectrum. HDMS function is not activated.

However, by activating the HDMS function of the MS<sup>E</sup> Data Viewer, the co-eluting components in the same peak at 3.84 min could be mobility resolved. As shown in Figure 7B, the precursor ion of orientin has a drift time of 4.16 ms. With the HDMS functionality activated, the product ions were easily resolved and the list of product ions were also greatly reduced, as shown in Figures 7C and 7D, thus increasing the confidence level of identifying orientin. HDMS<sup>E</sup> is an essential tool for separating compounds in complex mixtures containing numerous co-eluting compounds as it provides another dimension of orthogonal separation for increased confidence in isomer identification.

For isoorientin with a retention time at 3.73 min, a drift time of 4.54 ms with similar HDMS<sup>E</sup> fragmentation pattern as orientin was observed, as shown in Figure 8.

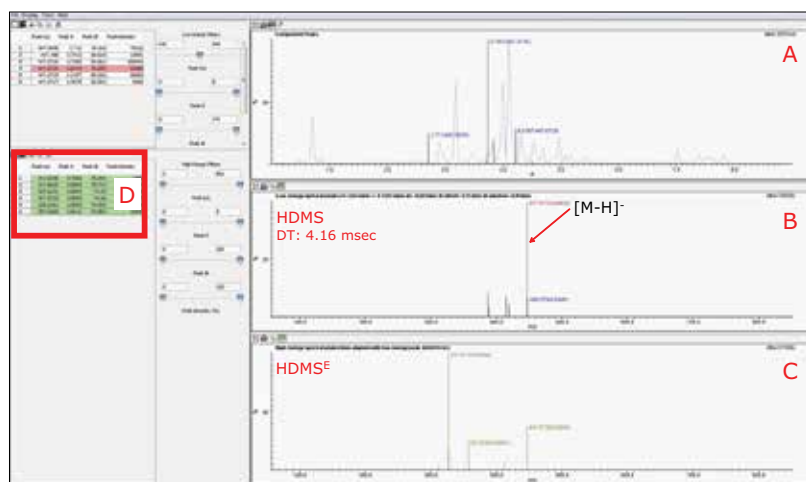


Figure 7. Orientin peak at 3.84 min with HDMS function activated. 7A. Chromatogram of peaks with 447.0927 m/z. 7B. Low energy mass spectrum (HDMS) with orientin peak selected (highlighted in red). 7C. High-energy mass spectrum (HDMS<sup>E</sup>) of orientin peak. 7D. List of product ions present in the HDMS<sup>E</sup> spectrum.

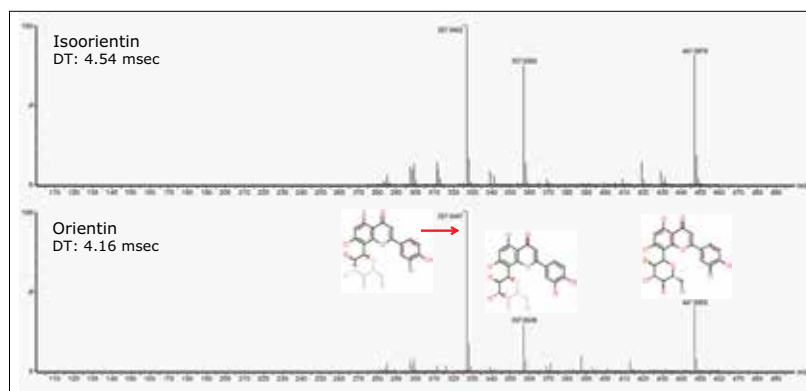


Figure 8. HDMS fragmentation pattern of orientin and isoorientin. Inset: Product ions of orientin generated using MassFragment Software.

## CONCLUSIONS

- The ACQUITY UPLC System combined with SYNAPT G2 HDMS is an effective system solution for rapid screening and identification of flavonoids in *Ficus* sp. extract.
- Plant extracts are complex matrices that contain many co-eluting compounds and isomers. HDMS provides an extra dimension of separation via ion mobility, which allows the separation of orientin from other co-eluting compounds. Together with the MS<sup>E</sup> functionality, where both low energy (precursor) and high energy (product ion) data can be acquired within a single analysis, provides greater confidence in the identification of flavonoids in *Ficus* sp. extract.
- The co-eluting interference peak with orientin are easily visualized using 3D models and drift-time plots generated using DriftScope. While further spectra cleanup for accurate product ions fingerprint for qualitative analysis is achieved using MS<sup>E</sup> Data Viewer.

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## SFC-SQD

## A Metabolomics Approach to Profile Novel Chemical Markers for Identification and Authentication of *Terminalia* Species

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### APPLICATION BENEFITS

First metabolomics approach for a comprehensive chemical profiling of the *Terminalia* species using UPLC®/TOF MS<sup>E</sup>, coupled with Multivariate statistical analysis (MSA), and applying the same approach to authenticate a popular Ayurveda medicine, Triphala.

### WATERS SOLUTIONS

ACQUITY UPLC® System

Xevo® G2 QTof Mass Spectrometer

MarkerLynx XS™ Application Manager

MassFragment™ Application Manager

### KEY WORDS

UPLC, QTof MS<sup>E</sup>, Multivariate Statistical Analysis, plant metabolomics, plant species authentication, *Terminalia*, Traditional Medicine, Ayurveda medicine, triphala

### INTRODUCTION

*Terminalia* is a genus of large trees of the flowering plant family Combretaceae, which is comprised of around 100 species that are distributed in tropical regions around the world.<sup>1</sup> Figure 1 shows the different *Terminalia* species that were included in this experiment. The fruits and bark from various *Terminalia* species have been used in Traditional Indian Medicine since ancient times for the treatment of various ailments. Some species have been used in Ayurvedic formulations, in either a single herb formulation, or in multiple herbal formulations.<sup>2</sup>

*Terminalia* species provide rich sources of secondary metabolites, including cyclic triterpenes and their derivatives, flavonoids, tannins, and phenolic acids.<sup>3</sup> The exact chemical classes and levels may vary in different *Terminalia* species, which may contribute to the different biological activities observed. Yet mixtures of multiple species of *Terminalia* show other health benefits versus their individual effects. The Ayurveda medicine triphala is a perfect example of such a case.

To date, Thin Layer Chromatography (TLC) is the common QC method used for analysis of triphala, and gallic acid and ellagic acid are used as the marker standards. However, gallic acid and ellagic acids are present in a majority of the *Terminalia* species, including the three fruits used in triphala, making them less than ideal for use as markers for authentication of triphala. It is important to have method to distinguish individual herbs that will allow adequate quality control (QC) of raw materials and standardization of finished products.

## EXPERIMENTAL

## UPLC conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY UPLC HSS T3 2.1 x 100 mm, 1.8 $\mu$ m
Column temp.:	60 °C
Flow rate:	500 $\mu$ L/min.
Mobile phase:	A: water + 0.1% formic acid B: methanol
Injection volume:	5 mL
Gradient:	80% A to 40% A for 29 min., then step to 0% A and hold for 3 min. before re-equilibration
Total cycle time:	35 min.

## MS conditions

MS system:	Xevo G2 QTof Mass Spectrometer
Ionization mode:	ESI <sup>-</sup> and ESI <sup>+</sup>
Acquisition range:	50 to 1200 <i>m/z</i>
Capillary voltage:	3 kV
Cone voltage:	30 V
Desolvation temp.:	500 °C
Desolvation gas:	900 L/Hr
Source temp.:	120 °C
CE:	Low: 4 eV High: 45 to 60 eV

The UPLC/oaTOF MS<sup>E</sup> analysis was performed for multiple *Terminalia* samples, shown in Table 1 in both positive and negative ionization modes. In this study, the results obtained in negative ionization mode were the main focus. To ensure data integrity, all samples were pooled into a single vial and used in the QC run. For each individual sample, six replicates of injection were performed with the sequence of the injections randomized.

## Sample preparation

In order to perform the determinations for commercial products, five capsules were weighed and opened, and the contents were emptied. The content of capsules were mixed and triturated using a mortar and pestle. Dry plant samples (0.5 g), or an adequate amount of capsule/powder content, were weighed (about 500 mg) and sonicated in 2.5 mL of ethanol for 30 min, followed by centrifugation for 15 min at 4000 rpm. The supernatant was transferred to a 10-mL volumetric flask. The procedure was repeated three times, and the respective supernatants were combined. The final volume was adjusted to 10 mL with ethanol and mixed thoroughly. Prior to injection, an adequate volume (ca. 2 mL) was passed through a 0.2  $\mu$ m nylon membrane filter. The first 1.0 mL was discarded and the remaining volume was collected in an LC sample vial. Each sample solution was injected in triplicate.

## RESULTS AND DISCUSSION

The UPLC/oaTOF MS<sup>E</sup>/MSA was performed for a total of 15 samples, as shown in Table 1. The key separation factors were the resolution and the peak capacity. Methanol was chosen over acetonitrile as it appeared to better retain peaks. Figure 2 shows the Base Peak Ion (BPI) chromatogram comparison for the five different herbal extracts, plus the two commercial products. The differences of these samples are shown in Figure 1.

NCNPR Accession #	Name	Place
7523	<i>Terminalia chebula</i>	CRISM, INDIA
7786	<i>Terminalia chebula</i>	CHINA
7787	<i>Terminalia chebula</i>	CHINA
8207	<i>Terminalia arjuna</i>	CRISM, INDIA
4995	<i>Terminalia arjuna</i>	INDIA
1229	<i>Terminalia arjuna</i>	INDIA
7504	<i>Terminalia bellerica</i>	CRISM, INDIA
4992	<i>Terminalia bellerica</i>	CRISM, INDIA
4993	<i>Terminalia bellerica</i>	CRISM, INDIA
2518	<i>Emblca officinalis</i>	HAMDARD UNIVERSITY, PAKISTAN
7795	<i>Emblca officinalis</i>	COMMERCIAL
4923	<i>Emblca officinalis</i>	CRISM, INDIA
1356	<i>Terminalia species</i>	COMMERCIAL
	Product-1	Dosage Form: Capsules
	Product-2	Dosage Form: Powders

Table 1. List of the 15 samples analyzed for this experiment.



Figure 1. *E. officinalis* and *Terminalia* species that were included in this work.

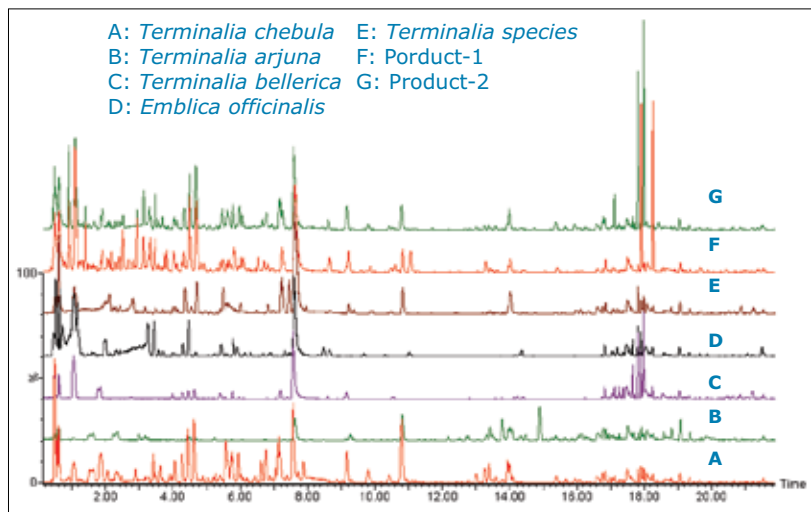


Figure 2. BPI comparison of the different species, plus two commercial *Terminalia* products.

A multivariate statistical analysis tool was used to obtain the comprehensive chemical profile of the samples. For multi-sample group analysis, Principle Component Analysis (PCA) is an effective tool. When performing MSA analysis, each sample group needs to be injected no less than three times to ensure the statistical validity ( $N=6$  for this analysis); and a QC sample must be prepared for the system suitability test. In this study, all 15 samples were pooled into a single sample and used as the QC sample.

Figure 3 shows the PCA scores plot for the entire dataset. There is a clear grouping pattern among different species of the herb. All "old" samples (collected and stored for more than two years), showed clear differences from the fresh samples. These differences seem to be more significant than the differences caused by various plant locations. The *Terminalia* species showed a closer similarity to *Terminalia chebula* than to *Terminalia bellerica* and *Terminalia arjuna*. *Embelica officinalis* was significantly different from all of the *Terminalia* spp, which was somewhat expected. The tightness of all of the QC injections provided a good sign of the system stability.

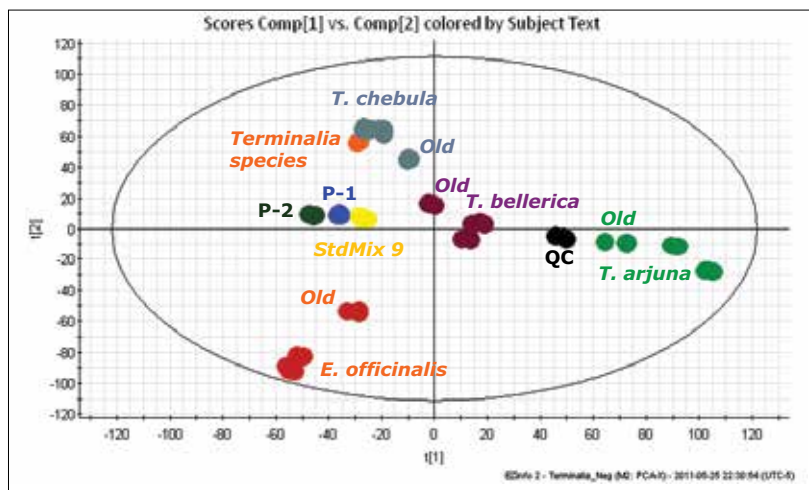


Figure 3. The PCA scores plot for the entire sample set including the QC.

Figure 4 shows the loadings plot that correlates to the scores plot from the same PCA analysis, which highlights the key markers – exact mass retention time (RT) pairs – that have the most significant contributions for the sample grouping. The geographic distribution of the markers from the loadings plot correlated to the geographic distribution of the sample groups on the scores plot shown in Figure 3. For example, the two markers highlighted in Figure 4 are closely related to the sample groups *T. arjuna* and the StdMix-9. Hence, the markers in this region were expected to be higher in content in these two groups of samples. And the markers in the similar location were expected to have similar trending plots.

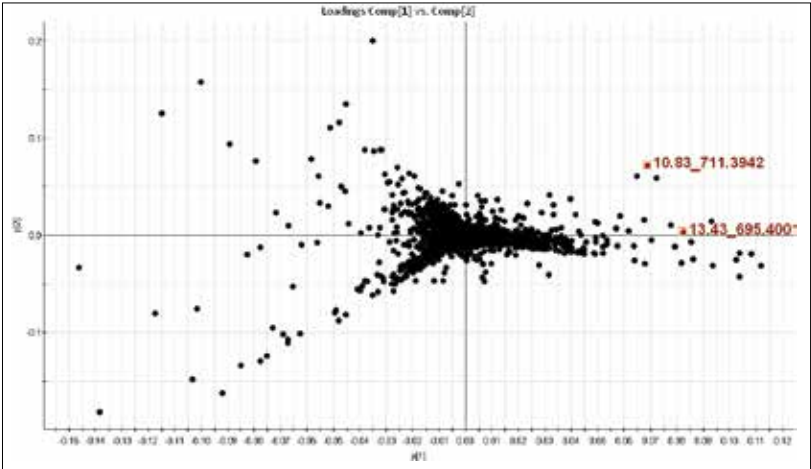


Figure 4. PCA loadings plot of the entire *Terminalia* data set.

Figure 5 shows the trending plot for the two markers shown in Figure 4. The trends of these two markers are identical from sample to sample. As expected, they were high in *T. arjuna* and in StdMix9. The StdMix-9 is a mixture of nine markers that were known from previous studies. As it is standard in pure solvents, it is not surprising to see these two markers were very high in content. These two markers were also high in *T. chebula*. However, there are other markers more dominating in *T. chebula*. This finding provides an explanation as to why the markers were relatively distant from the *T. arjuna* group from the PCA scores plot.

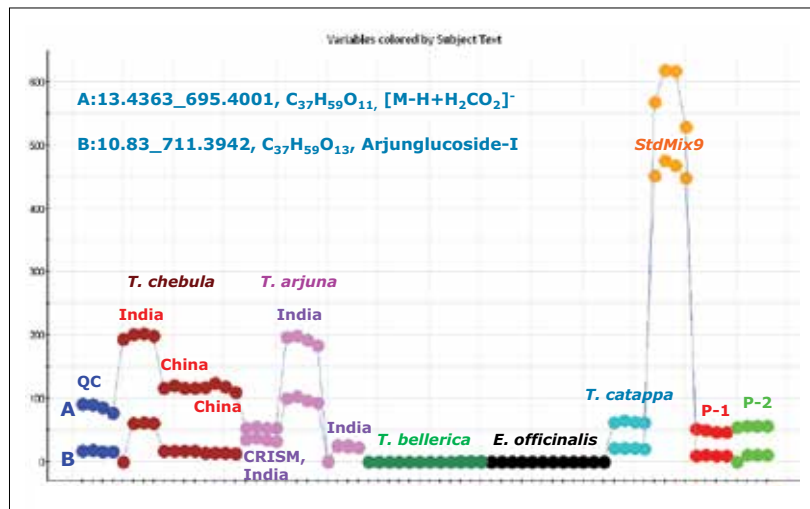


Figure 5. Trend plot of the two key markers labeled from the PCA loadings plot shown in Figure 4.

Also observed from the Trend plot was the fact that these two markers were significantly higher in content in the samples that were harvested in India. Using *T. chebula* as an example, the contents of these two markers were significantly higher in the India sample than the two samples from China, which suggests that plant location has a more profound influence in chemical content.

*Terminalia* is also a popular herb in Traditional Indian Medicine. For example, the Ayurveda medicine, triphala is a remedy that calls for the mixing of *T. chebula*, *T. bellerica*, and *Emblica officinalis* in equal portions. The authentication of triphala can become a challenge. The two commercial products (P-1 and P-2) in our sample set for this project are indeed the triphala extract. Figure 6 shows the PCA scores plot of the two products, plus the other herbs that the formula calls for: *T. chebula*, *T. bellerica*, and *Emblica officinalis*.

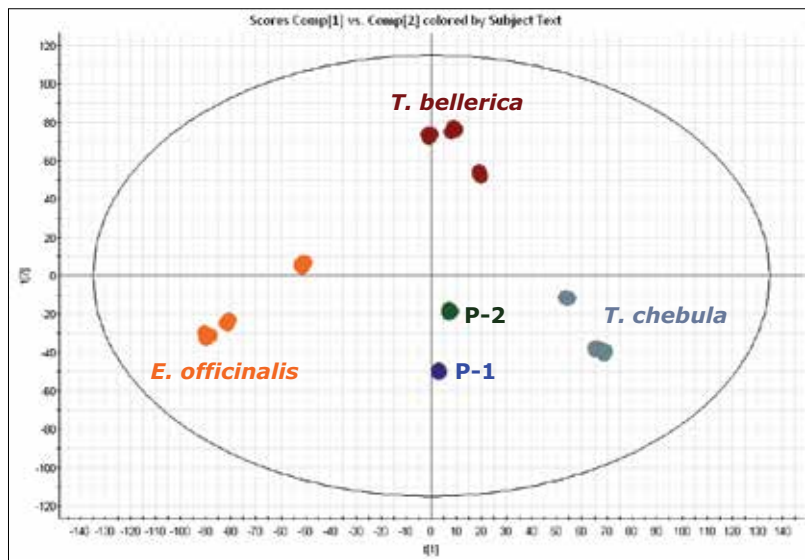


Figure 6. PCA scores plot of the two commercial Triphala products and the three herbs called for by the Triphala formula.

The two products are positioned close to the center of the triangle seemingly formed by the three herbs, *T. bellerica*, *T. chebula*, and *Emblica officinalis*. This suggests that the two commercial products are highly likely to be an equally portioned mix of these three herbs.

In future experiments we plan to prepare a solution by mixing the three herbs in equal portion, and performing the UPLC/oaTOF MS<sup>2</sup>/MSA, analysis workflow in the hopes that the PCA scores plot will show a correlation of this mixture with the P-1 and P-2 commercial products.

## CONCLUSIONS

- The UPLC/oaTOF MS<sup>E</sup>/multivariate statistical analysis workflow offers an effective approach to obtain answers for complex samples in a complicated sample set.
- PCA analysis shows that the chemical content of *Terminalia* differs from species to species. Within the same species, plant location and collection time also affects the chemical content. Chemical identities of key markers can be obtained from PCA loadings plot.
- The two commercial products of triphala appeared to be the results of mixing *T. chebula*, *T. bellerica*, and *E. officinalis*. Further experimental analysis and data mining are required to specifically understand the mixing ratio.

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## SFE 500/H-Class/WFM

## Small Scale Purification of Constituents from Complex Natural Product Extracts Using ACQUITY H-Class and Waters Fraction Manager-Analytical Systems

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Waters Corporation, Milford, MA, USA

### APPLICATION BENEFITS

The Waters® Fraction Manager – Analytical (WFM-A) is a fraction collector designed specifically to collect the narrow and closely eluting compounds found with UPLC® separations.

### WATERS SOLUTIONS

SFE 500 System

ACQUITY UPLC® H-Class System

ACQUITY UPLC PDA Detector

Waters Fraction Manager – Analytical

Empower® 3 Software

ACQUITY® BEH C<sub>18</sub>,  
1.7 µm, 2.1 x 100 mm Column

### KEY WORDS

Fraction collection, WFM-A, rosemary, schisandra, angelica, SFE, Waters Fraction Manager – Analytical, Waters Fraction Manager

### INTRODUCTION

Extracts from natural product samples can be complex, often containing a large number of diverse compounds. Increased separation performance of the ACQUITY UPLC H-Class System combined with ACQUITY BEH C<sub>18</sub> sub-2-µm Column technology provides a tool that produces sharp, narrow, and more concentrated peaks. When there is a need to collect narrow peaks from these complex mixtures, traditional preparative HPLC fraction collection instrumentation is not suitable for the collection of these narrow peaks. Collection valves and tubing designed for HPLC fraction collection introduce excessive peak broadening, making target isolation difficult. This application note will demonstrate several collection features of the WFM-A, a fraction collector designed to collect peaks generated from sub-2-µm chromatography using 3 natural product extracts.



Analytical Purification System with ACQUITY UPLC H-Class System, ACQUITY UPLC PDA Detector, and WFM-A.

## EXPERIMENTAL

Extracts from three natural products (rosemary, schisandra berry, and angelica root) were analyzed using an ACQUITY UPLC H-Class System combined with the ACQUITY BEH C<sub>18</sub> Column. Potential peaks of interest were identified and isolated using the WFM-A. Collected fractions were then analyzed to determine purity.

### Extractions

Samples (~25 g) of each of the three plant materials were first extracted using supercritical fluid extraction (SFE) using a Waters SFE 500 System equipped with a 100-mL extraction vessel. The extractions were performed using 99% CO<sub>2</sub> and 1% isopropanol at 50 g/min, 40 °C, and 200 bar (2901 psi) for 60 minutes.

### Separations

All separations were done using an ACQUITY UPLC H-Class System equipped with an ACQUITY UPLC PDA Detector along with a Waters Fraction Manager – Analytical controlled by Empower 3 Software. Details of the separation conditions are listed within the figure captions.

## RESULTS AND DISCUSSION

The Waters Fraction Manager – Analytical is a versatile analytical fraction collector for UPLC systems that minimizes fraction loss and carryover to better manage low-volume peaks and allows for efficient collection of small amounts of material for further assays. The WFM-A under Empower 3 control allows for several modes of collection.

### Fraction collection based on time

For the rosemary extract, a simple time-based collection mode was employed (Figure 1). Time based collections are simple and efficient but require precise, reproducible chromatography to be successful. In this example, the 2 largest peaks were collected and pooled over eight injections (Figure 2). Over the eight injections, 558 µL and 479 µL were collected for each of the two peaks, respectively, and analyzed (Figure 3) with peaks showing purities of 94.0% and 97.8%. Cumulative, real-time collection volume and location information can be viewed in the ACQUITY console (Figure 4).

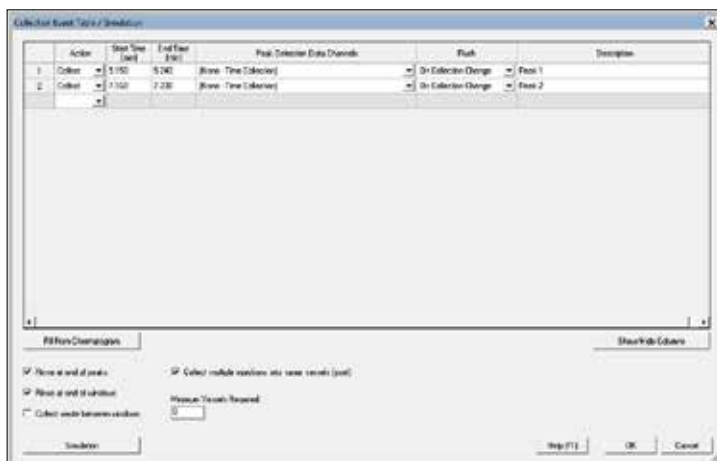


Figure 1. Empower 3–WFM-A method editor showing a simple time-based fraction collection.

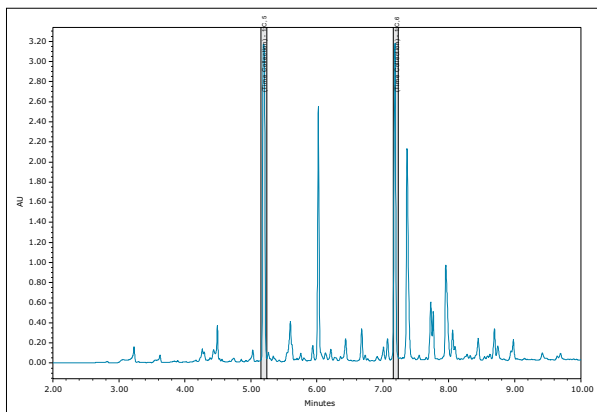


Figure 2. Separation of a rosemary extract with time-based fraction collection. Mobile phase – 0.1% formic acid in water, 0.1% formic acid in ACN gradient, 99:1 to 1:99 over 10 min, temperature – 50 °C, flow – 0.50 mL/min, UV at 254 nm, Column – ACQUITY BEH  $C_{18}$ , 1.7  $\mu$ m, 2.1 x 100 mm.

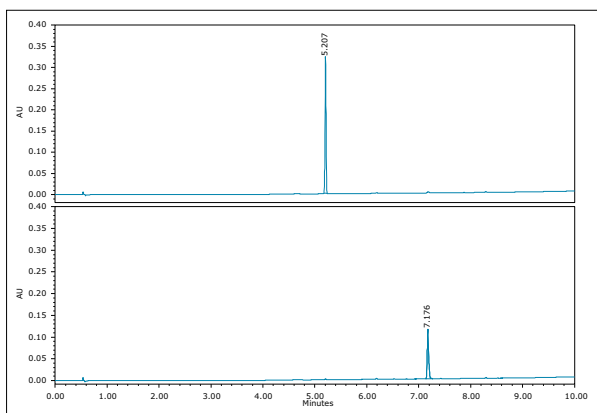


Figure 3. Analysis of pooled, collected fractions from a rosemary SFE extract. Mobile phase – 0.1% formic acid in water, 0.1% formic acid in ACN gradient, 99:1 to 1:99 over 10 min, Temperature – 50 °C, Flow – 0.50 mL/min, UV at 254 nm, Column – ACQUITY UPLC BEH  $C_{18}$ , 1.7  $\mu$ m, 2.1 x 100 mm.

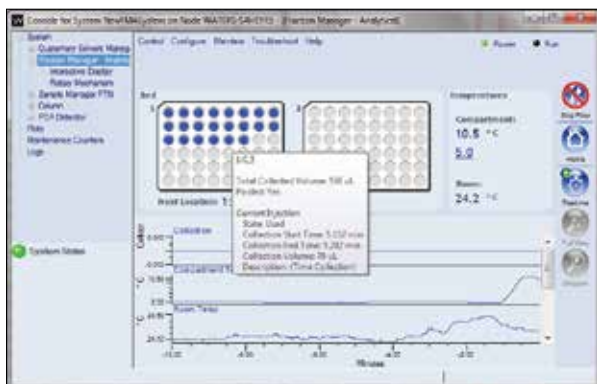


Figure 4. ACQUITY Console showing real-time fraction collector bed status.

### Fraction collection based on threshold

Fraction collection for the angelica root (Figure 5) was achieved through peak detection using threshold. In this mode, fraction collection is triggered based on peak intensity. When you specify a start threshold value, peak collection starts when the UV detector data channel level rises above the preset value. When you specify an end threshold, collection continues until the data channel level falls below the end threshold value. This mode is useful when chromatography is unstable or there is a desire to collect multiple peaks within a single time window. Using this collection mode requires the use of an optimized, low-dispersion delay coil which allows the software time to make the correct collection decision. Despite this delay coil, it is still possible to accurately collect closely eluting peaks (inset, Figure 5). Analysis of the collected fraction indicated purity (based on UV) of 89.4% and 94.1% for peaks 1 and 2, respectively (Figure 6).

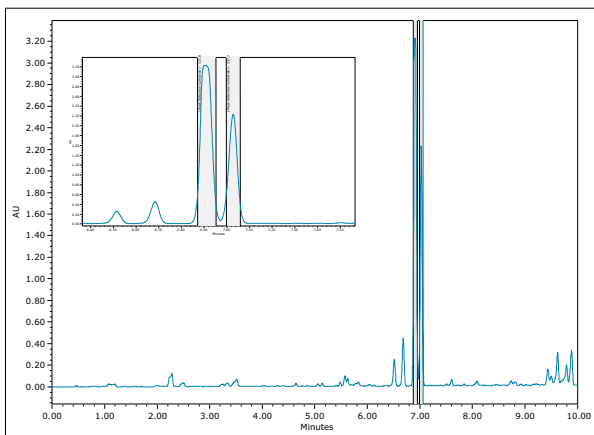


Figure 5. Separation of an angelica root extract with peak detection (threshold) based fraction collection. Mobile Phase – 0.1% formic acid in water, 0.1% formic acid in ACN, gradient – 80:20 to 30:70 over 10 min, temperature – 50 °C, flow – 0.50 mL/min, UV @ 254 nm, column – ACQUITY UPLC BEH  $C_{18}$ , 1.7  $\mu$ m, 2.1 x 100 mm.

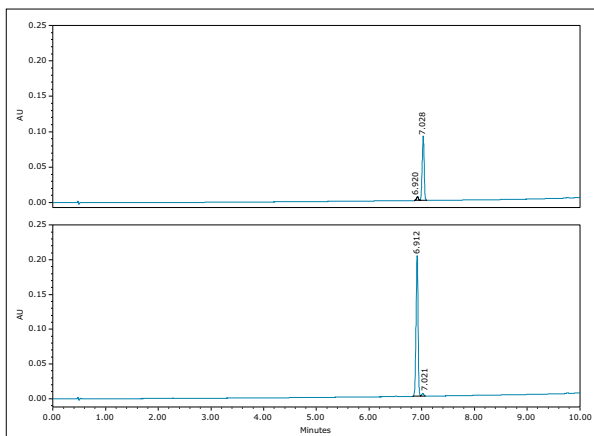


Figure 6. Analysis of collected fractions from angelica root SFE extract. Mobile phase – 0.1% formic acid in water, 0.1% formic acid in ACN, gradient – 80:20 to 30:70 over 10 min, temperature – 50 °C, flow – 0.50 mL/min, UV at 254 nm, column – ACQUITY UPLC BEH  $C_{18}$ , 1.7  $\mu$ m, 2.1 x 100 mm.

### Mixed mode fraction collection

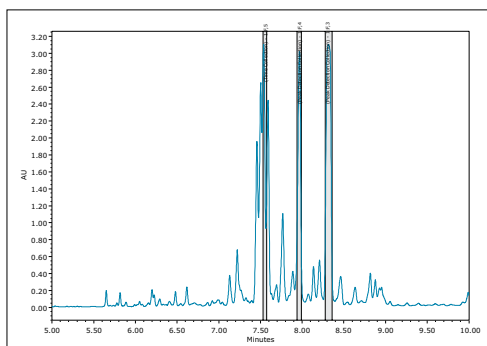
As a demonstration, fraction collection for the schisandra berry was achieved through a mix of timed and threshold collection (Figure 7).

The WFM-A method editor software allows for the use of different collection modes within the same chromatographic run. Washing functions are also available to reduce sample to sample collection carryover.

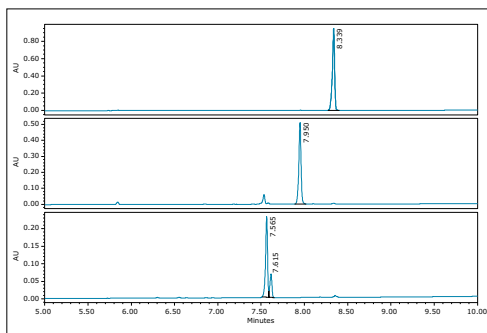
For the schisandra berry extract, a peak was cut out of a co-eluting group four peaks using a time collection (Figure 8), two subsequent peaks were collected using threshold collection in a time window. Analysis of the three fractions (Figure 9) showed purity (by UV) for the three peaks of 70.6%, 85.9%, and 98.9% respectively.



Figure 7. Empower 3 WFM – A method editor showing a time based fraction collection for peak 1 along with a threshold collection within a time window.



**Figure 8.** Separation of a schisandra berry SFE extract with time based fraction collection for peak 1 and threshold triggering for peaks 2 and 3. Mobile phase – 0.1% formic acid in water 0.1% formic acid in ACN gradient – 99:1 to 1:99 over 10 min, temperature – 50 °C, Flow – 0.50 mL/min, UV @ 254 nm, column – ACQUITY UPLC BEH C<sub>18</sub>, 1.7 µm, 2.1 x 100 mm



**Figure 9.** Analysis of collected fractions from schisandra berry SFE extract. Mobile phase – 0.1% formic acid in Water 0.1% formic acid in ACN gradient – 99:1 to 1:99 over 10 minutes, temperature – 50 °C, flow – 0.50 mL/min, UV at 254 nm, column – ACQUITY BEH C<sub>18</sub>, 1.7 µm, 2.1 x 100 mm.

Along with collection mode flexibility, the software also provides a method simulator (Figure 10). The simulator can take any previously run chromatogram and apply the collection method parameters to it. This allows users to make edits to the collection method and instantly see how the modification will affect subsequent collections.

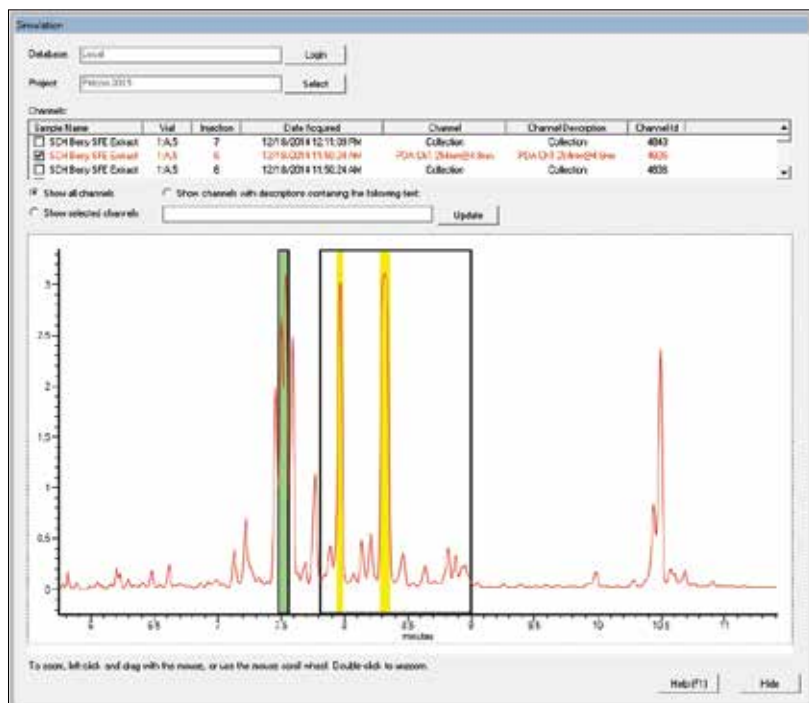


Figure 10. WFM-A method editor collection simulation.

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## CONCLUSIONS

- Using the Waters Fraction Manager – Analytical (WFM-A) peaks of interest were isolated from three complex natural product extracts.
- Multiple modes of collection were demonstrated (time, threshold, and mixed mode).
- Real time collection and collection bed information can quickly be viewed from the ACQUITY console.
- Collections can be simulated using the WFM-A method editor collection simulation which helps the user optimize and understand collection conditions.
- Analysis of collected fractions showed purities of greater than 85% up to 98%, with the exception of schisandra berry peak 1 at 70.6%, which was expected as this was known to be a co-elution.
- The WFM-A is capable of collecting very narrow fractions from sub-2- $\mu$ m columns without distortion from band broadening making this a useful tool for challenging separations and collections.

# Waters

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Prep 100q SFC/ACQUITY UPC<sup>2</sup>

## Improving the Productivity in Isolating a Naturally Occurring Bioactive Compound Using Supercritical Fluid Extraction and Preparative Supercritical Fluid Chromatography

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Waters Corporation, Milford, MA, USA

### APPLICATION BENEFITS

- SFE alleviates the sample complexity in natural product extracts prior to chromatographic analysis and purification, enabling a more efficient purification downstream.
- SFC offers complementary separation to RPLC. In addition, there is a wide range of column chemistries available in SFC with vastly different separation mechanisms. The combinations of SFC/RPLC and SFC/SFC provide unmatched resolving power to meet the challenges, primarily arising from sample complexity, in natural product isolation.
- Both SFE and SFC reduce the use of organic solvents and provide an easy sample recovery under mild conditions, thereby increasing the overall purification productivity and cost-effectiveness.

### WATERS SOLUTIONS

[ACQUITY UPC<sup>2</sup>@ System](#)

[ACQUITY UPLC® H-Class System](#)

[AutoPurification™ LC System](#)

[Prep 100q SFC MSD Directed System](#)

[ACQUITY UPC<sup>2</sup> BEH 2-EP Column](#)

[Viridis® Silica 2-EP Column](#)

[ChromScope™ Software](#)

### KEY WORDS

Natural product, purification, prep chromatography, SFE, SFC, UPC<sup>2</sup>, Selectivity, Productivity, Orthogonality

### INTRODUCTION

Natural products are a productive source of leads for new drugs due to their high chemical diversity, biochemical specificity, and many “drug-likeness” molecular properties.<sup>1-4</sup> A large portion of today's existing drugs on the market are either directly derived from naturally occurring compounds or inspired by a natural product. In addition, natural products are also used in the forms of food supplements, nutraceuticals, and alternative medicines.<sup>5</sup>

Isolation and purification of bioactive compounds play an important role in natural product research. The most commonly used process often involves extraction of target compounds from the cellular matrix, pre-purification by various chromatographic techniques including flash chromatography (FC), low pressure liquid chromatography (LPLC), and medium pressure liquid chromatography (MPLC), followed by preparative high pressure liquid chromatography (prep HPLC).<sup>6</sup> However, this process is not without its challenges. For example, conventional extraction methods for natural products include Soxhlet extraction, maceration, percolation, and sonication. These methods are often time- and labor-intensive, consume large amounts of organic solvents, and can lead to the degradation of thermally labile compounds. Furthermore, prep chromatography is largely dominated by reversed-phase liquid chromatography (RPLC), whereby the separation is driven by the differentiating polarity of the analytes. While a generally applicable chromatographic technique for a variety of compound classes, RPLC does not necessarily guarantee an adequate resolution for all analytes, especially for the structural analogs and isomers of similar polarities often found in natural products. As a result, the purification step is perceived by many as a rate-limiting step and a major bottleneck for natural product drug discovery.<sup>7</sup>



supercritical fluid chromatography (SFC), can offer viable additions to the natural product isolation toolbox by leveraging the unique properties of supercritical CO<sub>2</sub>: high diffusivity, low viscosity, and superb solvation power. SFE has been successfully applied to the extraction of many bioactive compounds from medicinal plants, including steroids, terpenes, alkaloids, and phenolic compounds.<sup>6</sup> Preparative SFC has been widely adopted by the pharmaceutical industry for active pharmaceutical ingredient (API) purification. Its applications in natural product isolation, however, remain scarce.<sup>8</sup>

We describe herein a systematic effort to holistically improve the productivity in isolating a naturally occurring terpene derivative with proven anti-cancer bioactivity from a raw plant sample. The process involves an extraction by SFE followed by three different, two-step purification routes, including MPLC+HPLC, MPLC+SFC, and SFC+SFC. The overall productivity and solvent consumption for each purification route are compared.

## EXPERIMENTAL

### Materials and reagents

HPLC-grade methanol and isopropanol (IPA) were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Denatured ethanol (reagent grade) was purchased from Sigma (St. Louis, MO, USA). The fine ground plant material was used as received.

### Sample preparation

#### Solvent extraction

A total of 0.3 g of ground plant material and 6 mL methanol were placed into a 10 mL test tube. After sonication at 40 °C for 1 hour, the suspension was centrifuged for 5 min. The supernatant was transferred to a clean vial for further analysis.

#### Supercritical fluid extraction

The extraction experiments were performed on a Waters MV-10 ASFE® System controlled by ChromScope Sample Prep Software. A total of 3 g of ground plant material was weighed into a 5 mL extraction vessel. The extraction was performed for 60 minutes with 8 mL/min CO<sub>2</sub>. The effluent was carried into a 100 mL collection vessel with a makeup flow of 1 min/mL of methanol/isopropanol/hexane (1:1:1).

### Chromatography

Analytical LC-MS experiments were performed on a Waters ACQUITY UPLC H-Class System/SQ Detector 2 MS and a Waters AutoPurification LC System. The analytical UPC<sup>2</sup>-MS experiments were performed on a Waters ACQUITY UPC<sup>2</sup>-MS System. All systems were controlled by MassLynx software. The MS-directed SFC preparative experiments were performed on a Waters Prep 100q SFC MS-Directed System controlled by MassLynx/FractionLynx Software. All UV-directed preparative experiments were performed on a Waters SFC 80 Preparative System controlled by ChromScope software. Detailed experimental parameters are summarized in Tables 1-3.

	Figure 2A	Figure 2B	Figure 6A			
Instrument	ACQUITY UPLC H-Class System/SQD2 MS	AutoPurification LC MS System	ACQUITY UPLC H-Class System/SQD2 MS			
Flow rate (mL/min)	0.60	1.46	0.75			
Mobile phase A	Water	Water	Water			
Mobile phase B	Methanol	Methanol	Methanol			
Backpressure (psi)	N/A	N/A	N/A			
MS detection	ESI+	ESI+	ESI+			
Column	ACQUITY HSS T3 (1.8 μm, 3.0 x 150 mm)	Atlantis T3 (5 μm, 4.6 x 150 mm)	ACQUITY BEH C <sub>18</sub> (1.7 μm, 2.1 x 50 mm)			
Temperature (°C)	60	Ambient	60			
Injection volume (μL)	1	Varying	0.5			
Gradient	Time (min)	%B	Time (min)	%B	Time (min)	%B
	0	92	0	88	0	80
	5	96	3.08	88	4	80
	5.25	92	8.21	94		
	6	92	8.61	100		
			9.22	88		
			20.90	88		

Table 1. Key experimental parameters for analytical LC.

	Figure 1	Figure 3A	Figure 4A	Figure 4B	Figure 6B	
Instrument	ACQUITY UPC <sup>2</sup> System/TQD MS					
Flow rate (mL/min)	1.5					
Backpressure (psi)	1740					
MS Detection	APCI+					
Temperature (°C)	45					
Injection volume (µL)	1					
Mobile phase A	CO <sub>2</sub>					
Mobile phase B	Methanol	Isopropanol	Isopropanol	Isopropanol	Isopropanol	
Column	ACQUITY UPC <sup>2</sup> 2-EP (1.7 µm, 3.0 x 100 mm)	ACQUITY UPC <sup>2</sup> 2-EP (1.7 µm, 3.0 x 100 mm)	ACQUITY UPC <sup>2</sup> 2-EP (1.7 µm, 3.0 x 100 mm)	GreenSep Nitro (1.8 µm, 3.0 x 100 mm)	ACQUITY UPC <sup>2</sup> 2-EP (1.7 µm, 3.0 x 100 mm)	
Gradient	Time (min)	%B	Time (min)	%B	Time (min)	%B
	0	5	0	5	0	5
	4.5	45	2.50	25	2.5	50
	5	45	2.75	40	3	50
	5.25	5	3.25	40	3.25	20
	6	5	3.50	5	4	20
			4	5		4

Table 2. Key experimental parameters for UPC<sup>2</sup>.

	Figure 3B	Figure 5A	Figure 5B			
Instrument	Prep 100q SFC MS-Directed System	SFC 80 Preparative System	SFC 80 Preparative System			
Flow rate (mL/min)	80	80	80			
Mobile phase A	CO <sub>2</sub>	CO <sub>2</sub>	CO <sub>2</sub>			
Mobile phase B	Isopropanol	Isopropanol	Ethanol			
Backpressure (psi)	1740	1740	1740			
Column	Viridis Silica 2-EP (5 µm, 19 x 150 mm)	Viridis Silica 2-EP (5 µm, 19 x 150 mm)	Nitro (5 µm, 21 x 150 mm)			
Temperature (°C)	40	40	40			
Sample diluent	Isopropanol	Isopropanol	Ethanol			
Injection volume (mL)	0.6	3	1			
Collection trigger	MS	UV	UV			
Gradient	Time (min)	%B	Time (min)	%B	Time (min)	%B
	0	5	0	5	0	8
	1	5	5	5	9	8
	6.5	9	7	30		
	7	9	10	30		
	7.25	5	11	5		
	8	5	12	5		

Table 3. Key experimental parameters for preparative chromatography.

## RESULTS AND DISCUSSION

### Target Compound Extraction Using SFE

Any solid-liquid extraction process, such as solvent extraction and SFE, is predominantly a solubility driven process. The process involves diffusion of the extracting solvent into the matrix, solubilization of the target analytes in the extracting solvent, diffusion of the target analytes in the extraction solvent, and transport of the extracted analytes into a collection vessel.<sup>6</sup> Conventional polar extraction solvents, such as alcohols, often produce extracts comprised of mixtures of many polar and non-polar compounds. Supercritical CO<sub>2</sub>, on the other hand, is a highly lipophilic solvent. As a result, only relatively non-polar compounds are typically extracted by SFE using neat CO<sub>2</sub>. In the current study, the target compound is a terpene derivative with a nominal mass of 390.28 Da and a LogP of 3.0. The low molecular weight and the relatively low polarity make it an ideal candidate for extraction by SFE.

Figure 1 shows the UPC<sup>2</sup>-MS chromatograms of two extracts obtained by SFE (Figure 1A) and methanol extraction (Figure 1B) using a BEH 2-EP column. Since 2-EP is a polar stationary phase, the elution order of the compounds generally tracks their polarities; the later the elution, the more polar the compounds. While both extracts contain similar amount of the target compound, it is evident that SFE yielded a much simpler extract compared to methanol extraction. For the SFE extract, the peaks immediately after the target compound (1.20-1.75 min, blue rectangle) are much lower in intensity than those in the methanol extract. The peaks between 1.75-3.50 min (red rectangle) are only present in the methanol extract. Overall, the SFE extract is a much simpler mixture consisting of fewer polar components. The target compound was therefore enriched by SFE prior to chromatography. This makes the SFE extract ideal for large mass loading in prep chromatography and requires relatively low organic co-solvent (mobile phase B) composition to completely elute off the components in the extract; thereby shortening the total run time, reducing the solvent consumption, and increasing purification productivity. Detailed prep SFC experiments are described in a later section.

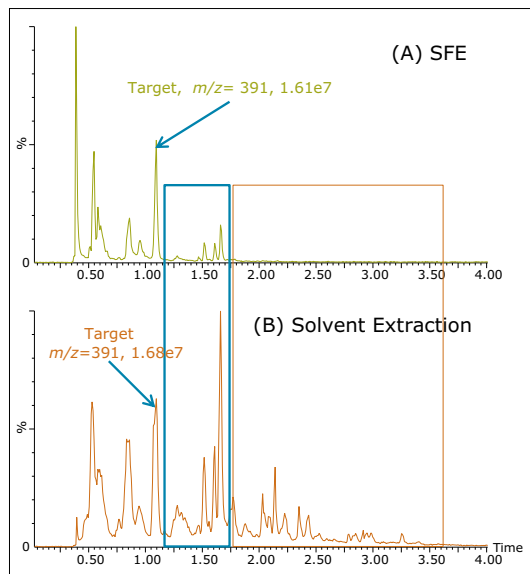


Figure 1. UPC<sup>2</sup>-MS chromatograms of the mixtures obtained by (A) SFE and (B) methanol extraction, using an ACQUITY UPC<sup>2</sup> 2-EP Column.

### Conventional purification approach: MPLC + HPLC

One of most commonly used approaches in natural product isolation involves MPLC followed by HPLC. In the current case, the SFE extract first underwent a purification step by MPLC (results not shown), attaining the target compound of >97% purity (referred to as the MPLC fraction hereafter). The main remaining impurity has a nominal mass of 360.27 Da, and results from the demethoxylation of the target compound. The structural similarity between the target and impurity presented a challenge in RPLC purification. Figure 2A shows the UPLC<sup>®</sup>-MS chromatogram of the MPLC fraction. A baseline resolution between the target and the impurity was achieved using a 3.0 x 150 mm UPLC column, where the impurity was present as a sodium adduct with an  $m/z$ =383. The close elution of the two peaks, however, severely hampered the sample loadability in the ensuing RPLC purification. Figure 2B summarizes a loading study of the MPLC fraction on an analytical column (5  $\mu$ m, 4.6 x 150 mm). The baseline resolution was only preserved with a 10- $\mu$ L injection. With an 80- $\mu$ L injection, the impurity peak completely merged into the target peak. In addition to the limited resolution, the elution order of the compounds also contributed to the low purification productivity. With RPLC, the impurity elutes before the target compound. In the case where target and impurity are partially separated, such as the one with 40- $\mu$ L injection in Figure 2B, though it is still possible to obtain pure target compound by excluding the front of the target peak where the impurity co-elutes, such practice is generally inadvisable in prep chromatography as the front of a peak often accounts for a high percentage of the total peak. Based on the loading study performed on the analytical column, the maximum loading on a 19 x 150 mm semi-prep column without compromising yield or purity was projected to be 170  $\mu$ L. At ~20 mg/mL, this translates into a maximum loading of 3.4 mg/injection.

### Leveraging the orthogonality between RPLC and SFC for improved loading capacity: an MPLC + SFC approach

SFC offers an attractive alternative. SFC is generally considered a normal-phase chromatographic technique when a polar stationary phase, such as 2-EP, is used. As a result, the elution order often reverses that in RPLC using a non-polar  $C_{18}$  column. Figure 3A shows a UPC<sup>2</sup>-MS chromatogram of the MPLC fraction using a BEH 2-EP column. Compared to Figure 2A, not only did the UPC<sup>2</sup> method provide a better resolution, the elution order of the target and the impurity also reversed. The chromatography was then scaled up to a 19 x 100 mm semi-prep column, and the resulting chromatogram is shown in Figure 3B. The resolution was well maintained with a 600- $\mu$ L injection at 20 mg/mL. The total run time using SFC was 8 min compared to the 20-min run time using RPLC. By using prep SFC to replace prep RPLC, the overall productivity was increased by 9-fold: 2.5-fold from the reduced run time and 3.5-fold from the increased sample loading.

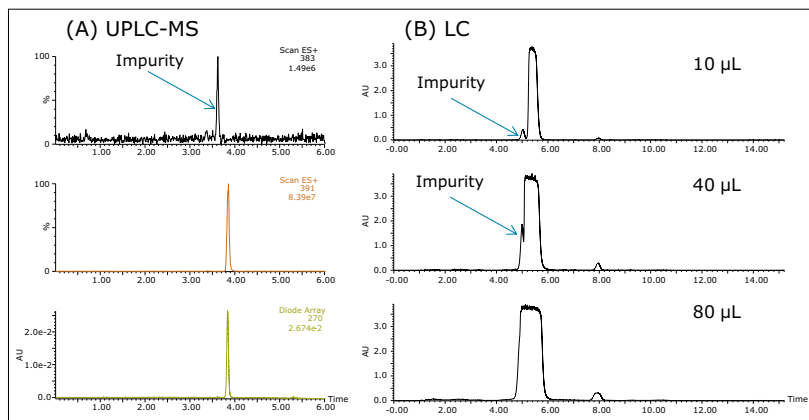


Figure 2. (A) UPLC-MS chromatograms of the MPLC fraction at 1 mg/mL; and (B) LC/UV chromatograms of the MPLC fraction at 20 mg/mL.

### Leveraging the orthogonality between different column chemistries in SFC for improved purification productivity: an SFC + SFC approach

Though the approach demonstrated in Figure 3 led to a notable improvement in productivity, the overall process still suffers from large solvent consumption, mainly due to the initial MPLC step. The target compound in the current study has a relatively low polarity. For this sample, a high percentage of organic solvent is required to elute the target compound in LC; hence, the large solvent consumption. In SFC, however, the lipophilic CO<sub>2</sub> is the main mobile phase that elutes the target compound, thus minimizing the use of organic solvents (mobile phase B). Moreover, the raw sample was extracted with neat CO<sub>2</sub> and is, therefore, inherently compatible with SFC.

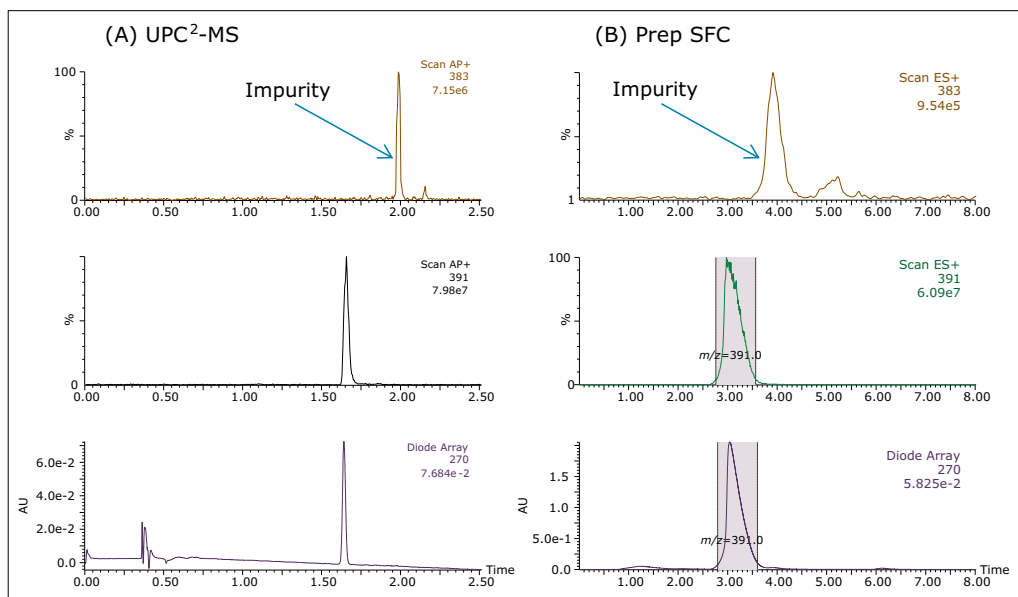


Figure 3. (A) UPC<sup>2</sup>-MS chromatograms of the MPLC fraction at 1 mg/mL; and (B) prep SFC-MS chromatogram of the MPLC fraction at 20 mg/mL.

There is a wide range of column chemistries available in SFC, with retention mechanisms encompassing polar interactions, hydrophobic interactions,  $\pi$ - $\pi$  interactions, and steric recognitions. With proper selection of column chemistries, SFC can offer orthogonal selectivity necessitated by the sample complexity intrinsic to natural product isolation. Figure 4 shows the UPC<sup>2</sup>-MS chromatograms of the SFE extract using a BEH 2-EP (Figure 4A) and a nitro column (Figure 4B), respectively. While 2-EP columns typically render polar interactions between analytes and stationary phase, nitro columns often retain and separate analytes based on  $\pi$ - $\pi$  interactions. This kind of combination provides complementary separation around the target compound. As can be seen in Figure 4, using a 2-EP column, the target compound at  $m/z=391$  is well separated from the impurity at  $m/z=361$ , but less separated from another later eluting impurity at  $m/z=239$ . In contrast, using a nitro column, the impurity at  $m/z=239$  became an earlier eluting peak and was well separated from the target compound, but the impurity at  $m/z=361$  co-eluted with the target compound.

### Leveraging the orthogonality between different column chemistries in SFC for improved purification productivity: an SFC + SFC approach

Though the approach demonstrated in Figure 3 led to a notable improvement in productivity, the overall process still suffers from large solvent consumption, mainly due to the initial MPLC step. The target compound in the current study has a relatively low polarity. For this sample, a high percentage of organic solvent is required to elute the target compound in LC; hence, the large solvent consumption. In SFC, however, the lipophilic CO<sub>2</sub> is the main mobile phase that elutes the target compound, thus minimizing the use of organic solvents (mobile phase B). Moreover, the raw sample was extracted with neat CO<sub>2</sub> and is, therefore, inherently compatible with SFC.

Based on the retention behavior illustrated in Figure 4, a two-step SFC purification strategy was implemented: using a 2-EP column to remove the main impurity with an  $m/z=361$  followed by using a nitro column to remove any remaining impurities after the first step, such as the one with an  $m/z=239$ . The resulting chromatograms are shown in Figure 5. The overall yield, defined as the weight of the purified pure target compound/the total weight of SFE extract taken for purification, was similar to those from the other two approaches: MPLC+HPLC and MPLC+SFC.

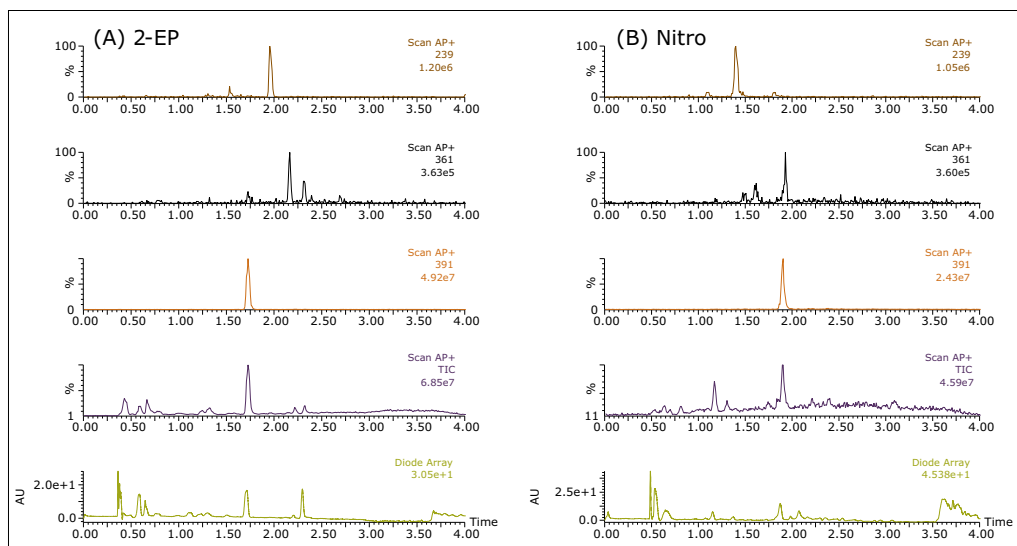


Figure 4. UPC<sup>2</sup>-MS chromatograms of the SFE extract using (A) a 2-EP; and (B) a nitro column.

Aliquots of the purified final product were analyzed by both UPC<sup>2</sup>-MS and UPLC-MS to ensure a true representation of the sample profile. The resulting chromatograms are shown Figure 6. Both impurities at  $m/z=361$  and  $m/z=239$  illustrated in Figure 4 were successfully removed. The results indicate that the final product has a purity >99% by UV.

The SFC purification process resulted in smaller fraction volumes compared to MPLC and HPLC. The SFC fractions were quickly dried under mild conditions, minimizing the possible compound loss due to thermal degradation associated with the post-purification dry-down process. Compared to LC, SFC offered an easier and faster compound recovery.

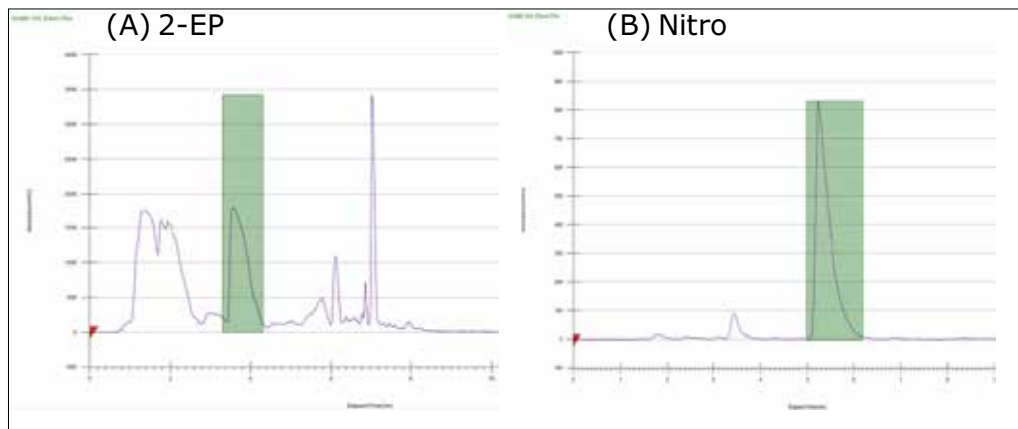


Figure 5. (A) SFC/UV chromatogram of the SFE extract at 133 mg/mL using a Viridis 2-EP column; and (B) SFC/UV chromatogram of the collected fraction from the Viridis 2-EP step on a nitro column.

The SFC purification process resulted in smaller fraction volumes compared to MPLC and HPLC. The SFC fractions were quickly dried under mild conditions, minimizing the possible compound loss due to thermal degradation associated with the post-purification dry-down process. Compared to LC, SFC offered an easier and faster compound recovery.

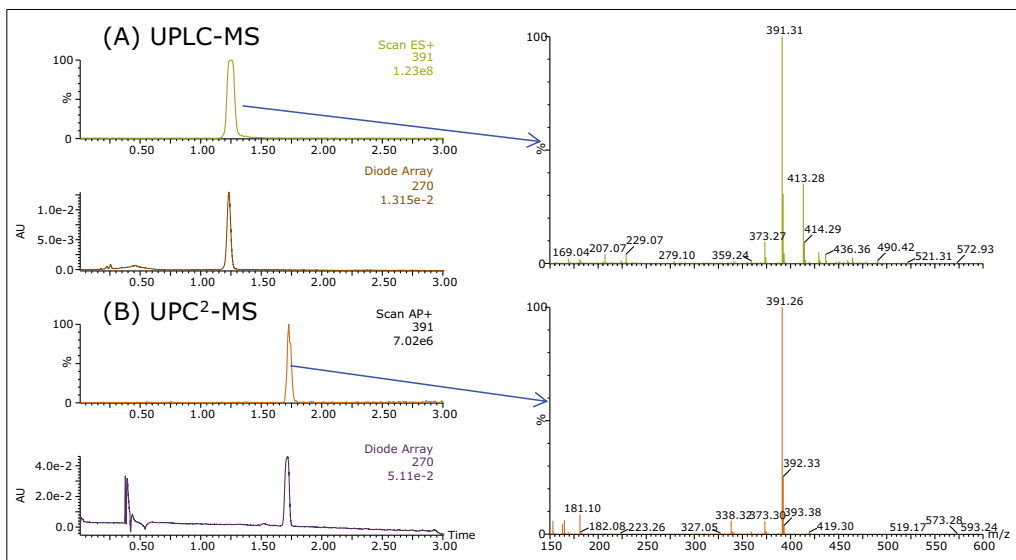


Figure 6. Purity analysis of the final product by (A) UPLC-MS and (B) UPC<sup>2</sup>-MS.

Process	Rate-limiting step	Productivity (g/24 hr)	Solvent	Solvent consumption (L/24 hr)	CO <sub>2</sub> use (kg/24hr)
MPLC+ HPLC*	HPLC	0.25	MeOH	95	N/A
MPLC+SFC**	SFC	2.25	MeOH/IPA	75	105
SFC+SFC	First step SFC	3.50	IPA/Ethanol	11	105

\*HPLC calculations were based on a 19 x 150 mm column.

\*\*SFC calculations were based on 19 x 150 mm columns.

Table 4. Comparison on productivity and solvent consumption of different purification processes.



## CONCLUSIONS

In this application note, we have demonstrated employing SFE and prep SFC to holistically improve the productivity in isolating a low-polarity, bioactive compound from a complex natural product extract. The SFE alleviated the sample complexity prior to analysis and purification, thereby improving sample loading and reducing solvent use in the ensuing chromatography. The SFE extract also lends itself well for SFC analysis and purification.

For the MPLC+HPLC purification route, the target compound and its demethoxylated derivative formed a critical pair in HPLC that limited the column loading and overall purification productivity. The same critical pair was better separated on a 2-EP column using SFC. The elution order of the pair was also altered, enabling an increased column loading. Overall, the MPLC+SFC route offered a 9-fold improvement in productivity. However, both routes still suffered from large solvent consumption because of the MPLC step. Finally, an SFC+SFC purification process was developed, leveraging the orthogonal selectivity between different column chemistries available in SFC. The SFC+SFC route not only led to a 16-fold improvement in productivity, but also a 90% reduction in solvent consumption. In addition, both SFE and SFC also provided an easy sample recovery under mild conditions that minimized potential compound loss due to thermal degradation associated with post-purification dry-down.

The supercritical fluid-based techniques, SFE and SFC, augment the conventional toolbox for natural product research by offering unique selectivity in both extraction and chromatography; and empower laboratories and manufacturers in pharmaceutical, traditional medicine, nutraceutical, and dietary supplement industries for more efficient and more cost-effective natural product isolation and purification.

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Prep 100q SFC/ACQUITY UPC<sup>2</sup>/QDa

## Extraction and Isolation of a Natural Product from Schisandra Berry Extract Using SFE and SFC

Andrew J. Aubin and Jo-Ann M. Jablonski  
Waters Corporation, Milford, MA, USA

### APPLICATION BENEFITS

- The Prep 100q SFC System is a highly reliable mass-directed preparative supercritical fluid chromatography instrument, suitable for compound isolation from natural product extracts.

### WATERS SOLUTIONS

Prep 100q SFC system

SFE 500 System

ACQUITY UPC<sup>2</sup>® System

ChromScope™ Software

MassLynx®/FractionLynx™ Software

Empower® 3 Software

Viridis® BEH OBD™ Prep Column

ACQUITY UPC<sup>2</sup> BEH Column

ACQUITY® QDa® Detector

### KEY WORDS

Isolation, purification, preparative chromatography, Prep 100q SFC, Schisandra, schisandrin A, natural product, extract, SFE, UPC<sup>2</sup>

### INTRODUCTION

Berries of schisandra (*Schisandra chinensis*) have been widely used for medicinal purposes in traditional Chinese medicines for centuries. The berries contain a wide variety of organic compounds with dibenzo[a,c]cyclooctadiene lignans being of primary interest to many researchers. In this study, we focus on the feasibility of using the Prep 100q SFC System as a tool for isolating schisandrin a (CAS 61281-38-7, also known as deoxyschisandrin or wuweizisu A, Figure 1) from dry schisandra berries extracted using supercritical fluid extraction (SFE). Principles, techniques, and tools outlined here are applicable to the isolation of any compound from natural product samples.

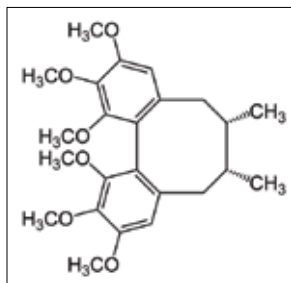


Figure 1. Chemical structure of Schisandrin A (Formula Wt. 416.5).

### Sample description and extraction

50 grams of dry, coarsely chopped Schisandra berries (StarWest Botanicals, Sacramento, CA, USA) were extracted for 60 minutes using an SFE 500 supercritical fluid extraction system fitted with a 100 mL extraction vessel. Extractions were performed dynamically at a total flow of 50 g/minute with an extraction pressure of 200 bar using a mixture of 99% CO<sub>2</sub> and 1% isopropyl alcohol. Extraction temperature was set to 40 °C. This extraction liberated approximately 30 mL of a dark yellow solution which was used in all subsequent experiments. For this kind of qualitative work, SFE provides a concentrated extract that contains no particulate matter. This removes the need for filtration, evaporation or other post extraction preparation which can greatly improve the overall workflow speed. No attempt was made to optimize the extraction conditions as it had been previously shown that with Schisandra, there appears to be little effect on extraction yield with changes of temperature or pressure.<sup>1</sup> The same authors also compared SFE of Schisandra to conventional solvent extractions (methanol, chloroform/methanol, hexane, and petroleum ether) and showed that there was little benefit to traditional solvent extractions compared to SFE.

### SFC conditions

Preparative SFC system:	Prep 100q SFC System with an ACQUITY QDa Mass Detector
Analytical SFC system:	ACQUITY UPC <sup>2</sup> System with an ACQUITY QDa Mass Detector
Analytical column:	ACQUITY UPC <sup>2</sup> BEH Column, 130Å, 3.5 µm, 3 mm x 100 mm, Part Number 186006640
Preparative column:	Viridis BEH OBD Prep Column, 130Å, 5 µm, 19 mm x 150 mm, Part Number 186005733
Mobile phase A:	CO <sub>2</sub>
Mobile phase B:	Acetonitrile/methanol 1:1
Gradient:	1 to 10% B over 5 min
Column temp.:	40 °C
Injection vol.:	2 µL analytical 120 µL prep
Flow rate:	2.5 mL/min analytical, 100 mL/min prep
ABPR:	1600 psi (110 bar) analytical, 1450 psi (100 bar) prep
Detection:	UV at 220 nm ACQUITY QDa Detector conditions
Scan range:	150 – 600 <i>m/z</i>
Ionization:	ESI+
Data type:	Centroid
Cone voltage:	10 V
Sampling rate:	2 Hz
Probe temp.:	600 °C
ESI capillary voltage:	1.5 kV

### Data management

Preparative SFC (Prep 100q SFC System) – MassLynx/  
FractionLynx

Analytical SFC (ACQUITY UPC<sup>2</sup> System) – Empower 3  
SFE 500 – ChromScope v1.5

## RESULTS AND DISCUSSION

Although there are at least forty lignans of *Schisandra chinensis*,<sup>2</sup> Schisandrin A is abundant and its usefulness in medicinal extracts arises from its antiviral and anti-inflammatory effects.<sup>3</sup> Analytical chromatography was developed using the ACQUITY UPC<sup>2</sup> System on the crude Schisandra SFE extract (Figures 2 and 3) and showed a large peak at approximately 1.9 minutes. ACQUITY UPC<sup>2</sup>-QDa data confirmed that this peak had an  $m/z$  of 417.3, consistent with that of Schisandrin A (M+H) and was the most abundant compound present. Schisandrin A is well resolved from neighboring peaks in the crude extract using a simple 1 to 10% gradient. The analytical separation was scaled to the Prep 100q SFC System using techniques previously described.<sup>4</sup>

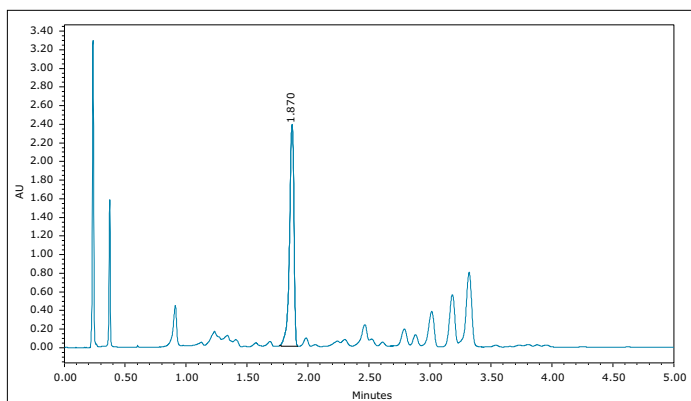


Figure 2. ACQUITY UPC<sup>2</sup> separation of Schisandra SFE extract. 2.5 mL/minute, 1%–10% gradient over 5 minutes, 1600 psi, 40 °C, 2  $\mu$ L injection, UV at 220 nm.

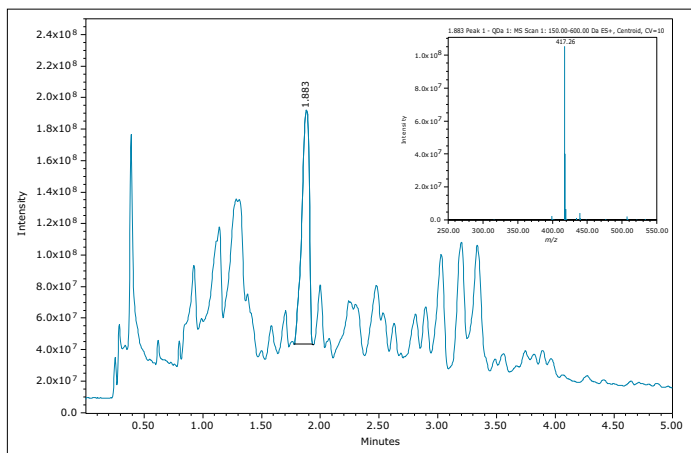


Figure 3. ACQUITY UPC<sup>2</sup> separation of Schisandra SFE extract. 2.5 mL/minute, 1%–10% gradient over 5 min, 1600 psi, 40 °C, 2  $\mu$ L injection, total ion chromatogram 150–600  $m/z$ . Inset, mass spectrum of peak at 1.9 min.

Schisandrin A was collected from the preparative runs by mass triggering, in this case  $m/z$  417 (Figure 4). The collected fraction was removed, concentrated and analyzed using the previously described ACQUITY UPC<sup>2</sup> method. When using SFC as the purification tool, dry down or concentration of sample is faster than traditional HPLC as collections only contain organic solvent (residual co-solvent and make-up solvent, usually methanol). Analysis of the collected fraction showed significant improvement in purity moving from approximately 29% purity in the crude to just over 92% based on the UV 220 nm area counts (Figure 5).

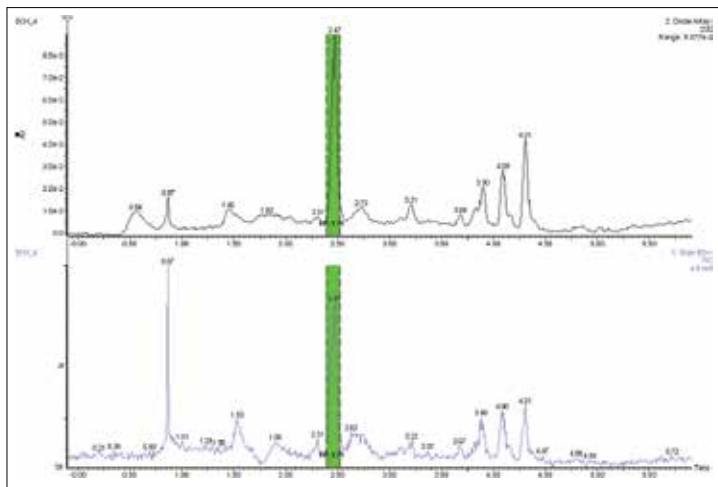


Figure 4. Prep 100q SFC System separation of Schisandra SFE extract. 100 mL/min, 1%–10% gradient over 5 min, 100 bar, 40 °C, 120  $\mu$ L injection, Top – PDA at 220 nm, Bottom – ACQUITY QDa total ion chromatogram 150–600  $m/z$ . Green bar indicates the area where collection occurred.

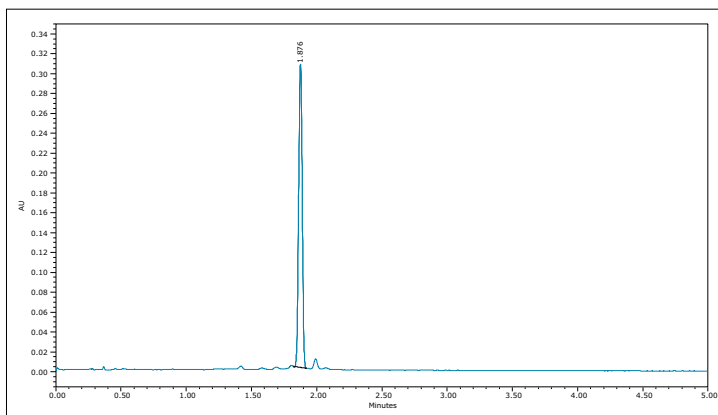


Figure 5. ACQUITY UPC<sup>2</sup> separation of collected fraction. 2.5 mL/min, 1%–10% gradient over 5 min, 1600 PSI, 40 °C, 0.5  $\mu$ L injection, UV at 220 nm.

## CONCLUSIONS

- Schisandra berries were successfully extracted using supercritical fluid extraction
- SFE extracts were analyzed using ACQUITY UPC<sup>2</sup> and a target compound (Schisandrin A) was selected for purification
- Purification of the SFE extract was achieved using a Prep 100q SFC System with collections triggered by mass
- A purified fraction was analyzed using ACQUITY UPC<sup>2</sup> and showed a significant increase in purity from 29% to 92%
- An extraction, analysis, purification, and reanalysis workflow using only supercritical fluid techniques was successfully demonstrated

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## Autopurification System

## Improving Resolution and Column Loading Systematically in Preparative Liquid Chromatography for Isolating a Minor Component from Peppermint Extract

Jo-Ann M. Jablonski and Rui Chen  
Waters Corporation, Milford, MA, USA

### APPLICATION BENEFITS

- Focused gradients improve the resolution of closely eluting components, thereby increasing the column loading for more efficient target compound purification.
- At-column dilution alleviates the peak distortion and loss of resolution attributed to the injection of large volumes of strong solvent, leading to improved resolution, column loading, and overall productivity in natural product isolation.

### WATERS SOLUTIONS

AutoPurification™ System

XSelect® CSH™ Column

XSelect C<sub>18</sub> Prep OBD™ Column

### KEY WORDS

Preparative liquid chromatography, natural product isolation, focused gradients, at-column dilution

### INTRODUCTION

Natural products are widely used in the pharmaceutical, food supplement, nutraceutical, and alternative medicine industries.<sup>1-4</sup> Chromatography has long been an integral part of natural product research, including chemical fingerprinting, structural elucidation, and isolation of bioactive compounds on the preparative scale. Since natural product extracts are usually complex mixtures comprised of many different compound classes with a variety of functional groups, acid-base properties, and molecular sizes, reversed-phase liquid chromatography (RPLC) often lends itself as the technique of choice for the analysis and purification of natural products, largely due to its general applicability.

The use of preparative high performance liquid chromatography (prep HPLC) has become a mainstay in the isolation of most classes of natural products over the last ten years.<sup>4</sup> In target compound purification, adequate resolution between target analytes and their adjacent interference peaks is a prerequisite for successful preparative chromatography. Typical approaches for improving resolution include the following: evaluating different stationary phases, mobile phases, and modifiers; changing the temperature of the separation; and varying the gradient slope. However, the ultimate objective for prep chromatography is to efficiently collect target compounds of desired purity. Consequently, experimental parameters such as sample diluents and injection techniques and their impact on solvent consumption and productivity should also be considered in the overall method development strategy.<sup>5</sup> This is particularly important for natural product isolation, since the desired compounds often exist at low concentrations within very complex matrices. To that end, at-column dilution (ACD) has proven to be a viable alternative to conventional injection techniques. ACD allows for injections of large volumes of sample in strong solvents while preserving chromatographic integrity and resolution, thereby improving overall purification productivity.<sup>6</sup>

This application note uses peppermint extract<sup>7</sup> to demonstrate a typical prep HPLC method development workflow, systematically improving resolution and column loading for the isolation of a minor component in a natural product.

## EXPERIMENTAL

### Sample description

A total of 3.3 g dried peppermint was extracted with a 20 mL 80:20 methanol/water mixture for six hours at room temperature.<sup>7</sup> The supernatant was filtered with an Acrodisc Syringe Filter with GHP Membrane, 25 mm, 0.45 µm

### LC conditions

System: AutoPurification

Columns: XSelect CSH<sub>C18</sub>  
4.6 x 100 mm, 5 µm;  
XSelect CSH  
Phenyl-Hexyl  
4.6 x 100 mm, 5 µm;  
XSelect CSH  
Fluoro-Phenyl  
4.6 x 100 mm, 5 µm;  
XSelect C<sub>18</sub> Prep OBD  
19 x 100 mm, 5 µm

Mobile phase A: 0.1% trifluoroacetic acid (TFA) in water

Mobile phase B: 0.1% TFA in acetonitrile

UV wavelength: 220 nm

Flow rate: 1.46 mL/min  
for analytical  
and 25.0 mL/min  
for preparative  
experiments

The analytical and preparative gradients used in this study are summarized in Table 1. For the ACD injections, a separate ACD pump delivered a constant 1.3 mL/min acetonitrile (5% of the total flow rate) directly to the injection valve while the gradient pump delivered the gradient at a flow of 23.7 mL/min. The two flow streams were combined at the head of the column. The number of column volumes (CV) per gradient segment was constant for all three methods, ensuring that the chromatography at the prep scale was identical to the chromatography at the analytical scale. Other key experimental parameters are listed in the respective figure captions.

Analytical		Prep *conventional injection		Prep **ACD	
Time (min)	%B	Time (min)	%B	Time (min)	%B
0.0	5.0	0.0	5.0	0.0	0.0
1.0	17.4	0.4	5.0	4.3	0.0
11.7	25.4	1.4	17.4	5.3	12.4
12.2	95.0	12.2	25.4	16.1	20.4
17.2	95.0	12.6	95.0	16.5	90.0
17.4	5.0	17.6	95.0	21.5	90.0
25.4	5.0	17.8	5.0	21.7	0.0
		25.8	5.0	29.7	0.0

\* 2-mL loop, system volume = 6.3 mL

\*\* 5-mL loop, system volume = 9.3 mL; ACD pump flows at 1.3 mL/min, total flow rate was 25.0 mL/min

Table 1. Gradients used in the study. The analytical flow rate was 1.46 mL/min and the preparative flow rate was 25.0 mL/min.



Since the analyte of interest eluted at  $\sim 22\%$  B in the initial generic gradient, shown in Figure 2A, focused gradients ranging from 17% to 25% B were employed to further improve the resolution, as shown in Figures 2B and 2C. Focused gradients increase the residence time of closely eluting compounds on the column for better partition, improving the selectivity ( $\alpha$ ) between compounds with minute polarity differences.<sup>8</sup> However, decreased gradient slope also increases retentivity ( $k'$ ), which in turn leads to broader peaks, reduced peak heights, prolonged run time, and greater solvent cost for prep chromatography. Therefore, caution should be exercised when using focused gradients to ensure the balance between resolution and run time. In the current study, at 0.72 %B/CV, shown in Figure 2C, the target peak was clearly baseline resolved from all adjacent peaks with a total run time of 25 minutes. For a shorter run time, the method could be terminated immediately after target peak collection with column washing steps to follow.

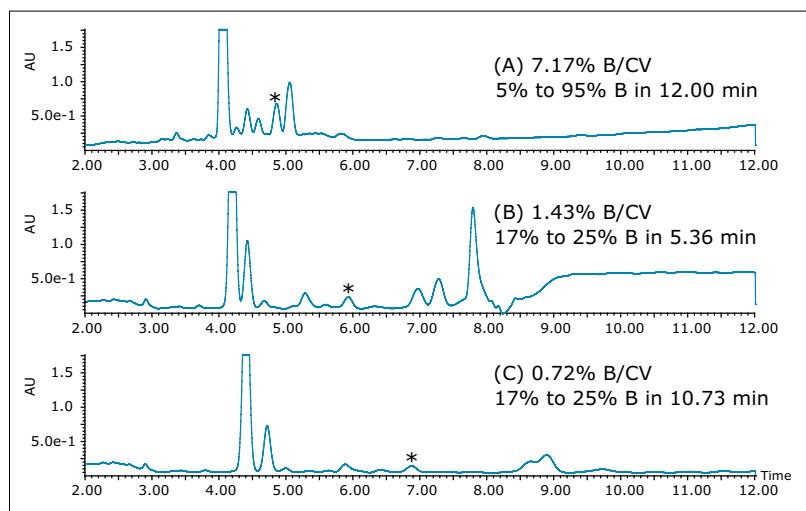


Figure 2. LC/UV chromatograms of the peppermint extract on an XSelect CSH C<sub>18</sub> 4.6 x 100 mm, 5  $\mu$ m Column using three different gradients.

## Scale-up

Proper scaling from analytical to prep requires the gradient slope (change in %B/CV) to be maintained for each step of the chromatography.<sup>9</sup> The flow rate and the injection volume are scaled geometrically. Table 2 summarizes the flow rate and injection volumes used when the chromatography was scaled from the analytical column to the preparative column. The properly scaled focused gradient methods are shown in Table 1.

	Analytical	Prep
Column dimension	4.6 x 100 mm	19 x 100 mm
Column volume	1.4 mL	23.8 mL
Flow rate	1.46 mL/min	25.0 mL/min
Injection volume	30 $\mu$ L	512 $\mu$ L
Injection volume	40 $\mu$ L	682 $\mu$ L

Table 2. Summary of scale-up parameters.

A loading study (not shown) performed on the XSelect CSH C18 4.6 x 100 mm Column showed that 30  $\mu$ L was the maximum volume that could be injected without losing resolution between the target compound and the impurity. Geometrically, an injection volume of 30  $\mu$ L on the analytical column scales to 512  $\mu$ L on a 19 x 100 mm prep column. In the conventional injection technique, the target peak is resolved from its closely eluting neighbors, as shown in Figure 3A. Further increasing the injection volume, such as the 682- $\mu$ L injection in Table 2, shown in Figure 3B, resulted in decreased resolution between the peak of interest and its closely eluting neighbors. For the peppermint extract in this study, a sensible injection volume for a 19 x 100 mm column was, therefore, limited to 512  $\mu$ L using the conventional injection technique. The observed resolution loss was partially due to the strong solvent used as the sample diluent. Natural products sometimes require the use of strong organic solvents, such as methanol, ethanol, and acetone, to extract them from the sample matrix. However, large injection volumes of strong solvents often distort chromatography and result in a loss of chromatographic resolution. Sample molecules entering the column in a strong solvent do not retain. Instead, they move through the column until the strong solvent is diluted sufficiently by the initial-strength mobile phase to promote retention. As a result, the samples retain on the column as wide bands. The samples elute from the column as broad, poorly resolved peaks, thereby limiting the chromatographic efficiency and overall productivity.

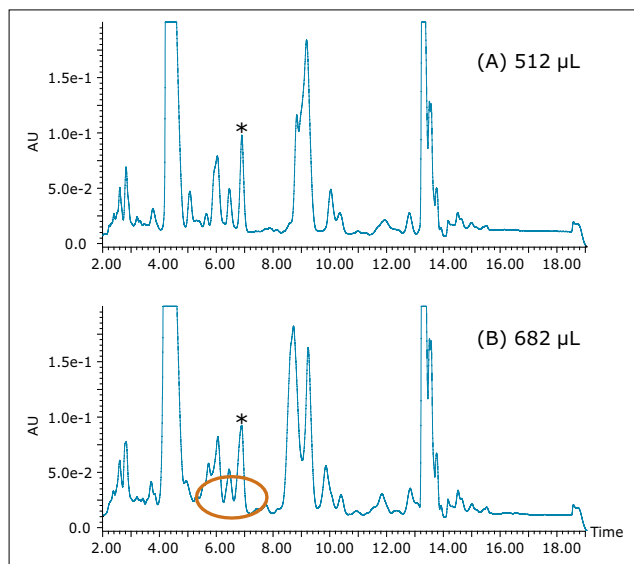


Figure 3. 512- $\mu$ L and 682- $\mu$ L injections of peppermint extract on an XSelect C<sub>18</sub> Prep OBD 19 x 100, 5  $\mu$ m Column with the Waters® AutoPurification System plumbed in the conventional mode.

### At-column dilution

ACD, an alternative injection technique, permits the injection of large volumes of strong solvents and concurrently improves sample solubility, column loading, and resolution.<sup>6</sup> With ACD, the chromatographic system is plumbed so that the sample in strong solvent is diluted at the head of the column with aqueous mobile phase. The sample is deposited on the column and the strong solvent flushes from the column before sample elution begins. Once the gradient is initiated, the sample components elute as narrow, sharply resolved bands, as shown in Figure 4. The strong solvent effect is effectively alleviated and the resolution is preserved. Furthermore, because the sample is continually surrounded by organic solvent until the point of dilution at the head of the column, no sample precipitation occurs.

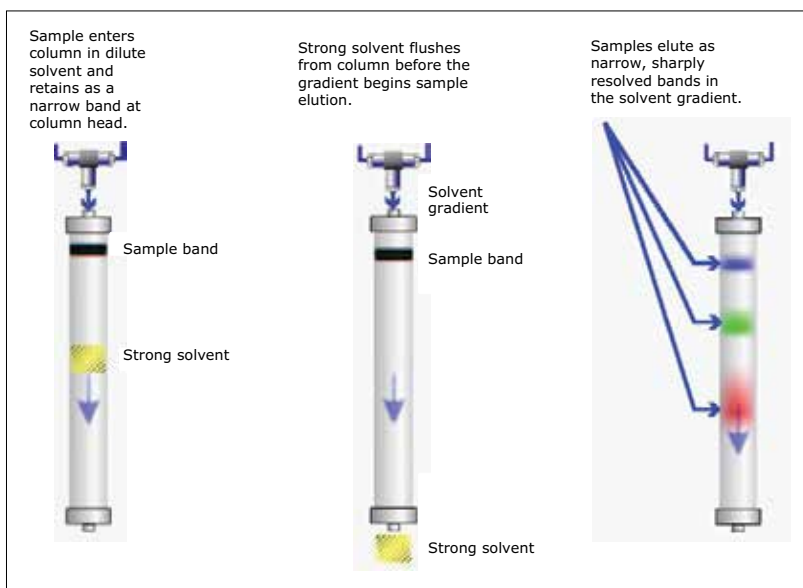
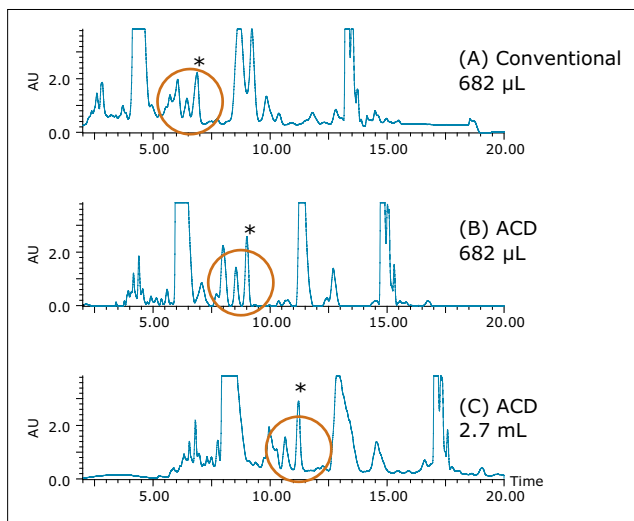


Figure 4. Schematic of at-column dilution.

Figures 5A and 5B show the chromatograms using the conventional injection technique and ACD with the same 682- $\mu$ L injection volume. Clearly, the one with the ACD, as shown in Figure 5B, provides improved resolution of the target compound from the closely eluting neighboring peaks. With ACD, a maximum injection volume of 2.7 mL was possible without the loss of resolution, as shown in Figure 5C. This represents a five-fold increase in column loading compared to the 512- $\mu$ L injection volume using the conventional injection technique.

It is important to note that the initial hold at the beginning of the ACD method ensures a complete sweeping of the sample loop. For example, a 5-mL loop was used for the 2.7-mL sample injection in Figure 5C, so an extra four minutes was added to the initial hold at 1.3 mL/min, as shown in Table 1.



The target minor component was successfully isolated from the 2.7-mL sample load in a fraction with a purity of 94% by UV analysis, as shown in Figure 6.

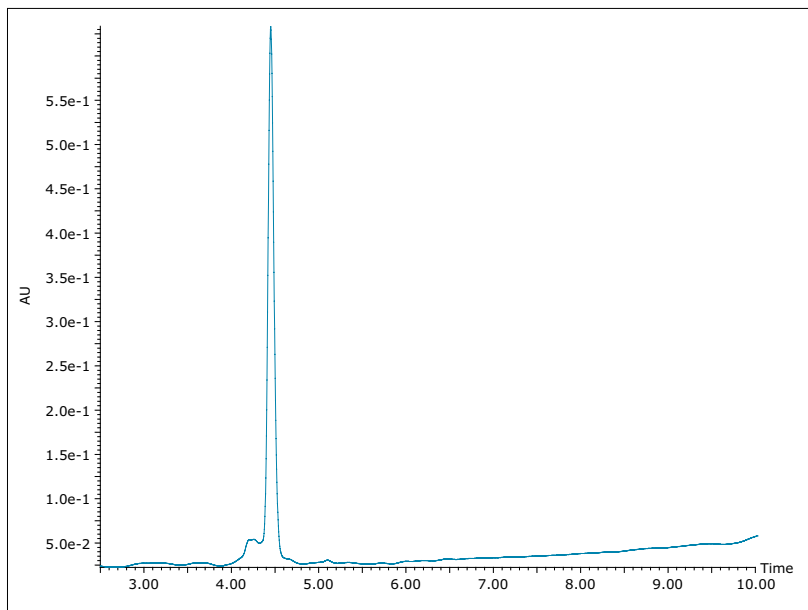


Figure 6. Purity analysis by UV of the fraction from a 2.7-mL injection with ACD.

## CONCLUSIONS

This application note illustrated a systematic preparative HPLC method development to isolate a minor component from peppermint extract using an AutoPurification System. The overall workflow included screening different column chemistries, applying focused gradients, scaling up, and employing an ACD injection scheme. With proper scale-up from an optimized analytical chromatographic condition, employing ACD increased the sample loading by five-fold while maintaining the resolution on the preparative scale. The techniques demonstrated in this case study have general applicability for laboratories routinely performing natural product isolation using preparative HPLC.

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## Chemical profiling and chemometric analysis of South African propolis.

UPLC-ESI-MS analysis of 39 South African propolis samples was undertaken to report on the chemical composition and variability of South African propolis and to compare the chemical profiles to Brazilian samples ( $n = 3$ ). Chemo-geographical patterns within South African propolis were further analysed by chemometrics. South African propolis samples displayed typical UPLC-ESI-MS fingerprints, which were different from their Brazilian counterparts. UPLC-PDA-qTOE-MS/MS was used to identify marker compounds from representative groups and 15 major phenolic acids and flavonols from common South African propolis were identified. Chemometric analysis of the UPLC-ESI-MS data revealed two distinct clusters among the South African samples and also confirmed that the South African propolis was chemically distinct from the Brazilian propolis. The majority of the samples were phytochemically congruent with propolis from the temperate regions.

**Deepak Kasote, Tasneem Suleman, Weiyang Chen, Maxleene Sandasi, Alvaro Viljoen, and Sandy van Vuure**

*Biochemical Systematics and Ecology* (Impact Factor: 0.97). 08/2014; 55:156–163.

DOI: 10.1016/j.bse.2014.03.012

## RIKEN tandem mass spectral database (ReSpect) for phytochemicals: a plant-specific MS/MS-based data resource and database.

The fragment pattern analysis of tandem mass spectrometry (MS/MS) has long been used for the structural characterization of metabolites. The construction of a plant-specific MS/MS data resource and database will enable complex phytochemical structures to be narrowed down to candidate structures. Therefore, a web-based database of MS/MS data pertaining to phytochemicals was developed and named ReSpect (RIKEN tandem mass spectral database). Of the 3595 metabolites in ReSpect, 76% were derived from 163 literature reports, whereas the rest was obtained from authentic standards. As a main web application of ReSpect, a fragment search was established based on only the  $m/z$  values of query data and records. The confidence levels of the annotations were managed using the MS/MS fragmentation association rule, which is an algorithm for discovering common fragmentations in MS/MS data. Using this data resource and database, a case study was conducted for the annotation of untargeted MS/MS data that were selected after quantitative trait locus analysis of the accessions (Gifu and Miyakojima) of a model legume *Lotus japonicus*. In the case study, unknown metabolites were successfully narrowed down to putative structures in the website.

Yuji Sawada, Ryo Nakabayashi, Yutaka Yamada, Makoto Suzuki, Munee Sato, Akane Sakata, Kenji Akiyama, Tetsuya Sakurai, Fumio Matsuda, Toshio Aoki, Masami Yokota Hirai, and Kazuki Saito

*Phytochemistry*. 2012 Oct;82:38-45.

DOI: 10.1016/j.phytochem.2012.07.007. Epub 2012 Aug 4.

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## Biosynthesis and localization of parthenolide in glandular trichomes of feverfew (*Tanacetum parthenium* L. Schulz Bip.).

Feverfew (*Tanacetum parthenium*) is a perennial medicinal herb and is a rich source of sesquiterpene lactones. Parthenolide is the main sesquiterpene lactone in feverfew and has attracted attention because of its medicinal potential for treatment of migraine and cancer. In the present work the parthenolide content in different tissues and developmental stages of feverfew was analyzed to study the timing and localization of parthenolide biosynthesis. The strongest accumulating tissue was subsequently used to isolate sesquiterpene synthases with the goal to isolate the gene encoding the first dedicated step in parthenolide biosynthesis. This led to the isolation and characterization of a germacrene A synthase (TpGAS) and an (E)- $\beta$ -caryophyllene synthase (TpCarS). Transcript level patterns of both sesquiterpene synthases were analyzed in different tissues and glandular trichomes. Although TpGAS was expressed in all aerial tissues, the highest expression was observed in tissues that contain high concentrations of parthenolide and in flowers the highest expression was observed in the biosynthetically most active stages of flower development. The high expression of TpGAS in glandular trichomes which also contain the highest concentration of parthenolide, suggests that glandular trichomes are the secretory tissues where parthenolide biosynthesis and accumulation occur.

**Mohammad Majdi, Qing Liu, Ghasem Karimzadeh, Mohammad Ali Malboobi, Jules Beekwilder, Katarina Cankar, Ric de Vos, Sladjana Todorovi, Ana Simonovi, and Harro Bouwmeester**

*Phytochemistry*. 2011 Oct;72(14-15):1739-50

DOI: 10.1016/j.phytochem.2011.04.021. Epub 2011 May 26.

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## Methods for the analysis of oxylipins in plants.

Plant oxylipins comprise a highly diverse and complex class of molecules that are derived from lipid oxidation. The initial oxidation of unsaturated fatty acids may either occur by enzymatic or chemical reactions. A large variety of oxylipin classes are generated by an array of alternative reactions further converting hydroperoxy fatty acids. The structural diversity of oxylipins is further increased by their occurrence either as free fatty acid derivatives or as esters in complex lipids. Lipid peroxidation is common to all biological systems, appearing in developmentally regulated processes and as a response to environmental changes. The oxylipins formed may perform various biological roles; some of them have signaling functions. In order to elucidate the roles of oxylipins in a given biological context, comprehensive analytical assays are available for determining the oxylipin profiles of plant tissues. This review summarizes indirect methods to estimate the general peroxidation state of a sample and more sophisticated techniques for the identification, structure determination and quantification of oxylipins.

**Cornelia Göbel and Ivo Feussne**

*Phytochemistry*. 2009 Sep;70(13-14):1485-503.

DOI: 10.1016/j.phytochem.2009.07.040. Epub 2009 Sep 6.

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## Alkaloids analysis using off-line two-dimensional supercritical fluid chromatography X ultra-high performance liquid chromatography.

In this study, an off-line two-dimensional (2-D) supercritical fluid chromatography (SFC) ultra-high performance liquid chromatography (UHPLC) method with high orthogonality was developed for the analysis of the practical amide alkaloids fraction from *P. longum* L. The effects of SFC parameters such as column type, organic modifier, temperature and back-pressure on separation were systematically evaluated. Different selectivity was observed for different columns (BEH, BEH 2-EP, XAmide and CSH FP). An investigation was then carried out of the orthogonality of different columns and systems following a geometric approach with a set of amide alkaloid samples. The orthogonality between a CSH FP column and a BEH column reached 50.79%, which was much higher than that for the other columns. While the orthogonality between SFC and UHPLC based on an XAmide column and an HSS T3 column reached 69.84%, which was the highest of all the combinations. At last, the practical amide alkaloids fraction was analyzed with an off-line 2-D chromatography SFC UHPLC system. In total, at least 340 peaks were detected by this method. Rapid separation in these two dimensions and easy post treatment of SFC facilitated this 2-D system for the separation of complex samples.

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*Analyst*, 2014,139, 3577-3587

DOI: 10.1039/C4AN00438H

## Modern analytical supercritical fluid chromatography using columns packed with sub-2 $\mu\text{m}$ particles: a tutorial.

This tutorial provides an overview of the possibilities, limitations and analytical conditions of modern analytical supercritical fluid chromatography (SFC) using columns packed with sub-2  $\mu\text{m}$  particles. In particular, it gives a detailed overview of commercially available modern SFC instrumentation and the detectors that can be employed (UV, MS, ELSD, FID, etc.). Some advice on the choice of the stationary phase dimensions and chemistries, the nature of the mobile phase (choice of organic modifier and additives) and its flow rate as well as the backpressure and temperature are also provided. Finally, several groups of potentially problematic compounds, including lipophilic compounds, hydrophilic substances and basic drugs, are discussed in detail. All these families of analytes can be resolved with SFC but require specific analytical conditions.

**Novakova, Lucie ; Grand-Guillaume Perrenoud, Alexandre ; Francois, Isabelle ; West, Caroline ; Lesellier, Eric ; and Guillarme, Davy**

*Anal Chim Acta.* 2014 May 8;824:18-35.

DOI: 10.1016/j.aca.2014.03.034. Epub 2014 Mar 28.

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## Supercritical fluid chromatography as a tool for enantioselective separation; a review.

Supercritical fluid chromatography (SFC) has become popular in the field of enantioselective separations. Many works have been reported during the last years. This review covers the period from 2000 till August 2013. The article is divided into three main chapters. The first one comprises a basic introduction to SFC. The authors provide a brief explanation of general principles and possibilities of this method. The advantages and drawbacks are also listed. Next part deals with chiral separation systems available in SFC, namely with the commonly used chiral stationary phases. Properties and interaction possibilities of the chiral separation systems are described. Recent theoretical papers are emphasized in this chapter. The last part of the paper gives an overview of applications of enantioselective SFC in analytical chemistry, in both analytical and preparative scales. Separation systems and conditions are summed up in tables so that they provide a helpful tool for analysts who search for a particular method of analysis.

**Kalíková, Květa ; Šlechtová, Tereza ; Vozka, Jiří ; and Tesařová, Eva**

*Anal Chim Acta.* 2014 Apr 22;821:1-33.

DOI: 10.1016/j.aca.2014.02.036. Epub 2014 Feb 28.

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## Potential and limitations of on-line comprehensive reversed phase liquid chromatography supercritical fluid chromatography for the separation of neutral compounds: An approach to separate an aqueous extract of bio-oil

On-line comprehensive Reversed Phase Liquid Chromatography Supercritical Fluid Chromatography (RPLC<sub>x</sub>SFC) was investigated for the separation of complex samples of neutral compounds. The presented approach aimed at overcoming the constraints involved by such a coupling. The search for suitable conditions (stationary phases, injection solvent, injection volume, design of interface) are discussed with a view of ensuring a good transfer of the compounds between both dimensions, thereby allowing high effective peak capacity in the second dimension. Instrumental aspects that are of prime importance in on-line 2D separations, were also tackled (dwell volume, extra column volume and detection). After extensive preliminary studies, an on-line RPLC<sub>x</sub>SFC separation of a bio-oil aqueous extract was carried out and compared to an on-line RPLC<sub>x</sub>RPLC separation of the same sample in terms of orthogonality, peak capacity and sensitivity. Both separations were achieved in 100min. For this sample and in these optimized conditions, it is shown that RPLC<sub>x</sub>SFC (with Hypercarb and Acquity BEH-2EP as stationary phases in first and second dimension respectively) can generate a slightly higher peak capacity than RPLC<sub>x</sub>RPLC (with Hypercarb and Acquity CSH phenyl-hexyl as stationary phases in first and second dimension respectively) (620 vs 560). Such a result is essentially due to the high degree of orthogonality between RPLC and SFC which may balance for lesser peak efficiency obtained with SFC as second dimension. Finally, even though current limitations in SFC instrumentation (i.e. large extra-column volume, large dwell volume, no ultra-high pressure) can be critical at the moment for on-line 2D-separations, RPLC<sub>x</sub>SFC appears to be a promising alternative to RPLC<sub>x</sub>RPLC for the separation of complex samples of neutral compounds.

Sarrut, Morgan ; Corgier, Amélie ; Crétier, Gérard ; Le Masle, Agnès ; Dubant, Stéphane ; and ; Heinisch, Sabine

*J Chromatogr A*. 2015 Jul 10;1402:124-33.

DOI: 10.1016/j.chroma.2015.05.005. Epub 2015 May 13.

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## Supercritical fluid chromatography for the separation of isoflavones.

The first protocol for the analysis of isoflavones by supercritical fluid chromatography is reported. Optimum results were obtained on an Acquity UPC(2) BEH 1.7  $\mu\text{m}$  column, using a solvent gradient of supercritical carbon dioxide and methanol (with phosphoric acid as additive) for elution. The method enables the baseline separation of nine isoflavones (aglyca and glycosides) in 8 min, and is suitable for their quantitative determination in dietary supplements containing soy (Glycine max), red clover (*Trifolium pratense*) and kudzu (*Pueraria lobata*). Method validation confirmed that the assay is selective, linear ( $R(2) > 0.9994$ ), accurate (recovery rates from 97.6 to 102.4%), as well as precise on the short- and long-term level (intra-day precision  $\leq 2.1\%$ ), and shows an on-column detection limit of 0.2 ng and below. This, together with an excellent performance shown in the analysis of real samples, indicates that SFC is well suited for the fast and accurate determination of isoflavones in complex matrices. Disadvantages compared to the established approaches were not observed, so that SFC has to be considered in this case as an (at least) equivalent analytical alternative.

**Markus Ganzera**

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## UPLC-QToF MS AND UNIFI

## Analysis of Plant Alkaloids Through Accurate Mass Screening and Discovery

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### APPLICATION BENEFITS

Analyze plant alkaloids using the Forensic Toxicology Application Screening Solution with UNIFI<sup>1</sup> to demonstrate the simplicity of library creation and expansion. This application note also showcases the power of the latest suite of discovery tools within the UNIFI Scientific Information System v1.8.

### WATERS SOLUTIONS

[Forensic Toxicology Application Solution with UNIFI®](#)

[ACQUITY UPLC® I-Class System \(FTN\)](#)

[ACQUITY UPLC HSS Column](#)

[Xevo® G2-S QToF](#)

[Maximum Recovery Vials](#)

### KEY WORDS

Plant alkaloids, forensic toxicology,  
UPLC-QToF-MS<sup>2</sup>, UNIFI,  
identification, discovery

### INTRODUCTION

Over the last decade there has been a significant increase in the popularity of time-of-flight mass spectrometry (ToF-MS) for multi-residue analysis. Accurate mass imparts high specificity for substance identification and, together with the isotopic data, can provide the user with the opportunity to propose likely elemental compositions. The proposal of elemental formulae is often the starting point for a complex multi-stage process to elucidate chemical structures.

For screening, accurate mass instrumentation presents a significant, and key, advantage over its nominal mass counterpart, i.e., an ability to implement screening methodologies without the requirement of reference material. In this particular workflow the theoretical (expected) exact mass can be determined empirically from the elemental formula. In a toxicological setting this can provide a valuable means with which the analyst may 'prospectively' target novel psychoactive drugs, or new substances and metabolites where reference material may not yet, be available.

An on-going initiative to expand the UNIFI Toxicology Scientific Library led to the analysis of a series of plant alkaloids. These nitrogen-containing compounds are derived from plants and plant material. They are pharmacologically active and have been used for many centuries for both medicinal and recreational purposes. Consequently, their analysis is of forensic importance. Analysis of these substances provided an opportunity to evaluate the tools within the UNIFI Scientific Information System for both target assignment and structural elucidation.

## EXPERIMENTAL

## Materials

The following plant alkaloids were obtained from Sigma-Aldrich (Poole, UK) as solid material: amygdalin, berberine chloride, bufalin, coumarin, digitoxin, gitoxin, lanatocide C, neriifolin, and  $\alpha$ -solanine.

## Sample preparation

Individual stock solutions of the plant alkaloids were initially prepared, by dilution with methanol, to a concentration of 10  $\mu\text{g/mL}$ ; these solutions were stored at  $-20^\circ\text{C}$  until further use. Prior to ToF-MS analysis, the stock solutions were further diluted with mobile phase A to yield samples for injection at a concentration of 1  $\mu\text{g/mL}$ .

## LC-MS method conditions

## ACQUITY UPLC conditions

System:	ACQUITY UPLC I-Class (FTN)
Column:	ACQUITY HSS C <sub>18</sub> , 2.1 x 150 mm, 1.8 $\mu\text{m}$
Run time:	15 min
Vials:	Waters Maximum Recovery Vials
Column temp.:	50 $^\circ\text{C}$
Sample temp.:	10 $^\circ\text{C}$
Injection vol.:	10 $\mu\text{L}$
Flow rate:	0.4 mL/min
Mobile phase A:	5 mM aqueous ammonium formate, adjusted to pH 3.0
Mobile phase B:	Acetonitrile containing 0.1% formic acid
Gradient:	

Time	%A	%B
0.00	87	13
0.50	87	13
10.00	50	50
10.75	5	95
12.25	5	95
12.50	87	13
15.00	87	13

MS<sup>E</sup> conditions

MS system:	Xevo G2-S QTof
Ionization mode:	ESI+
Source temp.:	150 $^\circ\text{C}$
Desolvation temp.:	400 $^\circ\text{C}$
Desolvation gas:	800 L/h
Reference mass:	Leucine enkephalin $[\text{M}+\text{H}]^+ = m/z$ 556.2766
Acquisition range	$m/z$ 50–1000
Scan time:	0.1 s
Capillary voltage:	0.8 kV
Cone voltage:	25 V
Collision energy:	Function 1: 6 eV Function 2: Ramped 10 to 40 eV

## Data management

Forensic Toxicology Screening Application Solution with UNIFI v1.8



RESULTS AND DISCUSSION

Prior to analysis, a new UNIFI Scientific Library was created specifically for plant alkaloids, by simply entering the names of the nine alkaloids. A MOL file describing the structure of each substance was added to each entry in the library (Figure 1). Individual solutions of the plant alkaloids were injected and data were acquired using the standard screening conditions supplied with the Forensic Toxicology Screening Application Solution with UNIFI.<sup>1</sup> These data were subsequently processed using the UNIFI Scientific Information System and screened against the new plant alkaloid library.

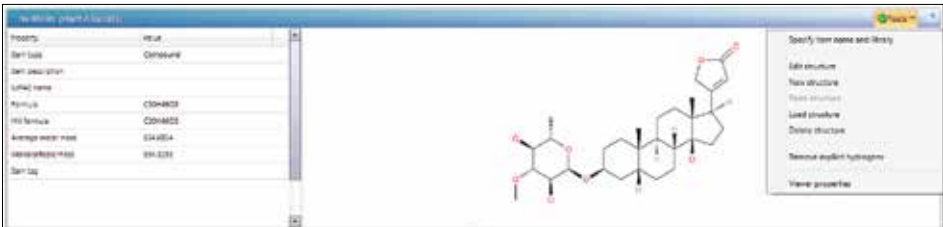


Figure 1. Creating a library entry for neriifolin. Existing MOL file structures can be appended (Load structure) or created by standard chemical drawing packages and subsequently appended (New structure).

Identification through the application of *in-silico* fragmentation techniques

The presence of each plant alkaloid was confirmed through the mass accuracy of the protonated precursor ion in combination with theoretical fragment ions that were automatically generated from the structure of each substance and matched to ions in the high-energy spectrum.

Figure 2 shows the identification of  $\alpha$ -solanine as presented in UNIFI. The Component Summary table presents the information related to the identification of this alkaloid and includes; the observed  $m/z$  value together with the deviation from the expected  $m/z$  value, the difference between measured and theoretical isotope patterns in terms of both  $m/z$  and intensity distributions, the observed retention time, the number of theoretical fragment ions found, and the detector counts, which represents the abundance of all the low-energy ions associated with the detected compound.



Figure 2. Identification of  $\alpha$ -solanine in the UNIFI Scientific Information System.

## Updating library entries

All of the alkaloids were identified on the basis of the mass accuracy of the precursor ion and theoretical fragment ions generated during processing. Upon identification, a retention time was associated with each substance. With UNIFI, the library entries can be updated directly from the analysis such that they contain the expected retention time and the expected  $m/z$  value for each assigned adduct and fragment ion. Following the update, a typical library entry has information similar to that shown for neriifolin in Figure 3. This additional information can be used to target the substance in subsequent analyses.

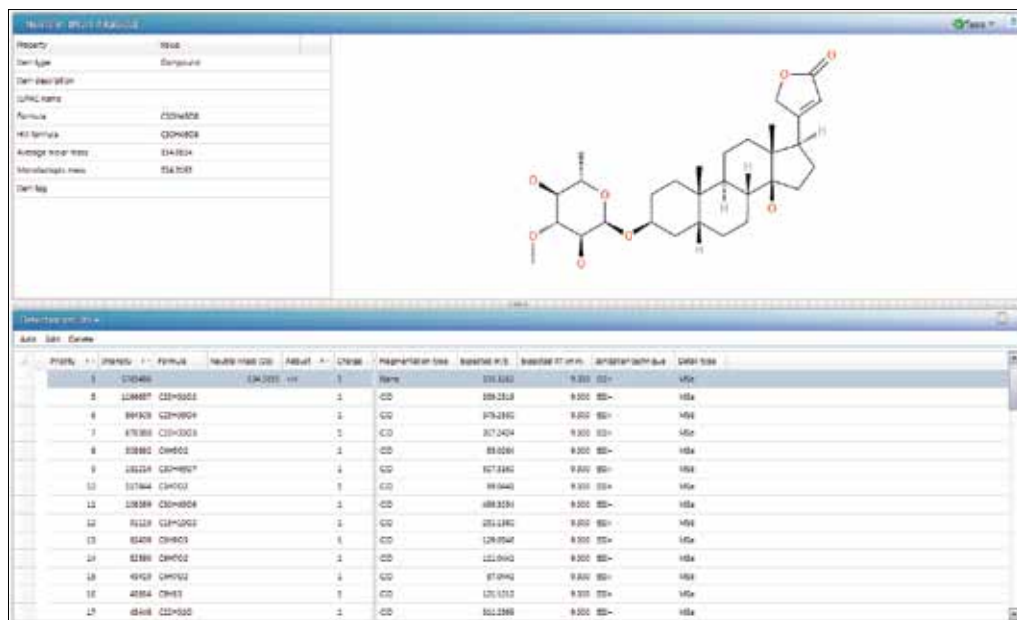


Figure 3. Library entry for neriifolin. The lower section of the composite is now populated with expected retention time and the expected  $m/z$  values of precursor and fragment ions.

Multiple adducts

Data for gitoxin, one of the other alkaloids investigated in this study, is shown in Figure 4. The low-energy ions assigned to this substance are highlighted in green within the spectrum and correspond to the protonated isotope cluster. The detector counts determined for the protonated isotope cluster of gitoxin is 568. The high-energy spectrum is annotated with sub-structures of gitoxin, as determined automatically by UNIFI and associated to the high-energy spectral peaks as fragment ions.

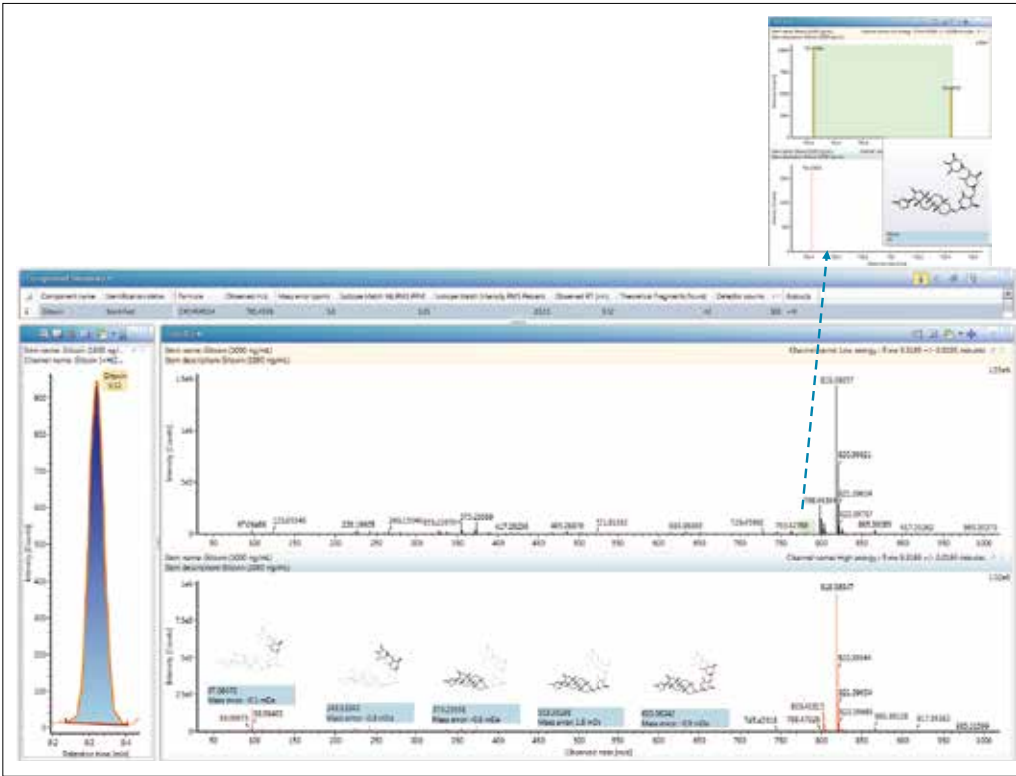


Figure 4. Identification of gitoxin in the UNIFI Scientific Information System.

Further examination of the low-energy spectrum for this substance revealed that some of the ions may correspond to other adducts of gitoxin. Consequently the data was reprocessed to target the  $[\text{NH}_4]^+$ ,  $[\text{Na}]^+$ , and  $[\text{K}]^+$  adducts in addition to the protonated species. Figure 5 details the isotope clusters in the low-energy data assigned to each adduct following reprocessing. The assignment of the additional adducts to gitoxin has been reflected in the detector counts which has increased from 568, determined from the isotope cluster of the protonated adduct, to 118680. Similar results were obtained for the other substances in this analysis.

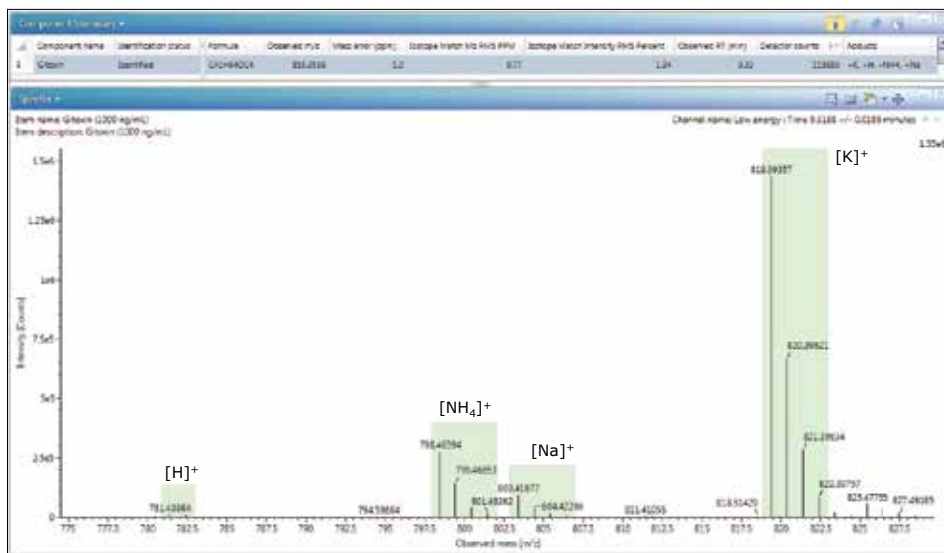


Figure 5. Multiple adduct assignment for gitoxin.

### The discovery tool

Another new feature in the UNIFI Scientific Information System v1.8 is the discovery tool, which chains together elemental composition, library searching and fragment match functionality into a single step process making it easier to obtain the identity of unexpected substances within a sample. The parameters used to run the discovery tool are detailed in Figure 6A–D.

The first set of parameters, displayed in Figure 6A, control the maximum number of elemental compositions returned for each component, and the number of library hits returned for each elemental composition. For each component selected, the measured  $m/z$  value is submitted to the elemental composition application, the parameters of which are displayed in Figure 6B. Each scientific formula returned by the elemental composition application is then automatically submitted to the list of selected libraries. The libraries can either belong to the UNIFI Scientific Library or, if connected to the internet, ChemSpider. The dialog showing the selection of ChemSpider libraries is presented in Figure 6C.

Every hit for each scientific formula that is returned from the library search is then automatically submitted to the fragment match application, provided the library hit has an associated structure in the form of a MOL file.

The fragment match application performs a systematic bond disconnection of each structure, applying the parameters selected through the dialog displayed in Figure 6D, and matches the  $m/z$  values of theoretical sub-structures to measured high-energy fragment ions. The number of fragment ions matched and the percentage of the intensity of the high-energy spectrum accounted for by those matches are both determined.

For the purposes of illustration, the candidate component identified as amygdalin in the targeted analysis was submitted to the discovery tool. The results, upon running the application with respect to the parameters shown in Figure 6A–D, are presented in Figure 7.

The component submitted to the discovery tool was Candidate Mass  $m/z$  458.1649. The results show that one elemental composition,  $C_{20}H_{27}NO_{11}$ , with an i-FIT™ confidence of 89% was determined for  $m/z$  458.1649. This elemental composition, was automatically submitted to the FDA UNII – NLM library within ChemSpider and a hit for amygdalin was returned with a list of synonyms, a structure and the number of citations. The structure was used automatically in conjunction with fragment match and appropriate sub-structures were assigned to the high-energy spectrum associated with Candidate Mass  $m/z$  458.1649, as shown in Figure 7. The number of high energy fragments matched by sub-structures and the percentage of the intensity of the high energy spectrum accounted for by those fragment matches are displayed for the library hit.

Access to this information for a range of components, elemental compositions, and library hits enables the analyst to make an informed decision with respect to the identity of unexpected substances in their samples.

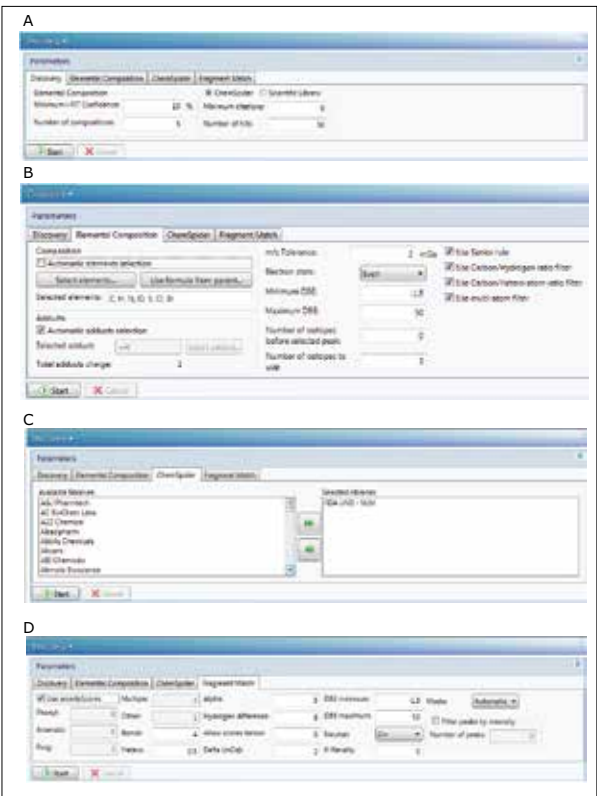


Figure 6. Discovery tool in UNIFI. A) General discovery tool parameters. B) Elemental composition parameters. C) ChemSpider parameters. D) Fragment match parameters.

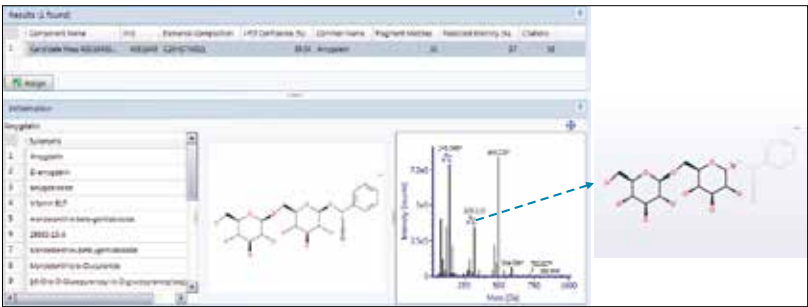


Figure 7. A typical result in the discovery tool.

## CONCLUSIONS

In this study, the Forensic Toxicology Application Screening Solution with UNIFI<sup>1</sup> was applied to a selection of plant alkaloids. The ease by which the scientific library items can be created and updated has been clearly demonstrated. The UNIFI Scientific Information System v1.8 was used to process the MS<sup>E</sup> data and for these plant alkaloids multiple adducts were detected. The fragment match functionality was also able to assign sub-structures to high-energy ions. Additionally, the new discovery tool has been shown to enhance the elucidation of unknown components.

## Reference

1. Forensic Toxicology Screening Application Solution. Waters Brochure (P/N [720004830EN](#)).

# Waters

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**UPLC-TQ-S**

# UPLC/MS/MS Method for the Routine Quantification of Regulated and Non-Regulated Lipophilic Marine Biotoxins in Shellfish

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## APPLICATION BENEFITS

A rapid, robust method for the analysis of both regulated and non-regulated lipophilic marine biotoxins in shellfish. Compared to the conventional HPLC/MS/MS method, the speed of analysis increased 4-fold: (from 20 min to 5 min per analysis). This method is also able to meet the regulated levels of detection that can be used instead of the mouse bioassay.

## WATERS SOLUTIONS

ACQUITY UPLC® System

 ACQUITY UPLC BEH C<sub>18</sub> column

Xevo® TQ-S Mass Spectrometer

TargetLynx™ Application Manager

QUANPEDIA™ Database

## KEY WORDS

Shellfish, biotoxins, lipophilic, phycotoxins, mussels, clams, oyster, diarrhetic shellfish poisoning, DSP

## INTRODUCTION

Consumption of shellfish (mussels, oysters, clams, etc.) contaminated with biotoxins can cause severe intoxications in humans, such as diarrhetic shellfish poisoning (DSP). Due to their lipophilic properties, DSP toxins are often classified as lipophilic marine biotoxins. Marine biotoxins are naturally produced by different types of phytoplankton and are, therefore, also named phycotoxins. The complexity of the lipophilic marine biotoxins lies in the variety of physiochemical properties, such as carboxylic acids, sulfonic acids, and amino and imino functionalities.

In the European Union (EU) legislation, various toxin groups are regulated and describe how these toxins should be monitored in official control programs. The lipophilic biotoxins that should be monitored, shown in Figure 1A, are okadaic acid (OA), dinophysistoxin-1, -2, -3 (DTX1, -2, -3), where DTX3 are the ester forms of OA, DTX1 and -2, respectively, pectenotoxin-1, -2 (PTX1, -2), yessotoxin (YTX), 45OH yessotoxin (45OH YTX), homo yessotoxin (homoYTX), 45OH homo yessotoxin (45OH homoYTX), azaspiracid-1, -2 and -3 (AZA1, -2, -3).<sup>1</sup>

Before July 2011, the official method was a bioassay based on the oral administration of shellfish meat to a rat or the intraperitoneal injection of a shellfish extract in mice. There were two main issues with this method: first, it was perceived as unethical; and second, the method was not scientifically robust

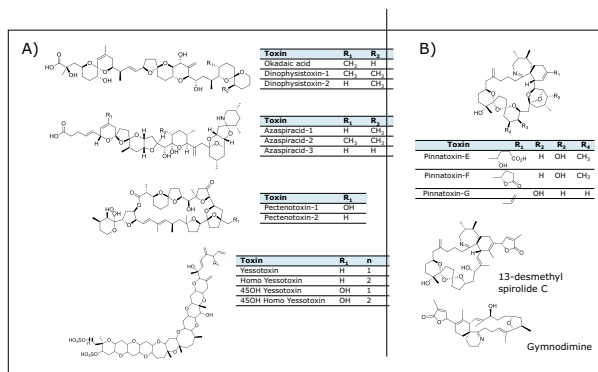


Figure 1. Chemical structure of a) regulated toxins and b) non-regulated cyclic imines.

enough to determine trace amounts of specific toxins. The presence of cyclic imines would cause a physical reaction using the bioassay – the result often fatal for the animal. Cyclic imines, shown in Figure 1B, are also classified as lipophilic marine toxins based on their physiochemical properties. Although these toxins are not currently regulated, the European Food Safety Authority (EFSA) has indicated that more data on toxicity, as well as their occurrence in shellfish, should be collected.

Since July 2011, the official method for control of shellfish on the presence of lipophilic marine biotoxins has been LC/MS/MS.<sup>2</sup> The EU reference LC/MS/MS method is based on a fixed extraction procedure followed by separation using conventional LC separation, with either an acidic mobile phase or alkaline mobile phase and detection by tandem quadrupole MS.

The aims of this study were to produce a much faster routine analysis than the conventional LC method under alkaline conditions, and include additional non-regulated compounds that are of interest to EFSA. In this application note, various classes of lipophilic marine biotoxins including some of the non-regulated cyclic imines were analyzed in a five-minute analysis using a Waters® ACQUITY UPLC System coupled with a Xevo TQ-S. UPLC® technology allows for analytical run times to be reduced without compromising peak resolution and quality, while the Xevo TQ-S (tandem quadrupole mass spectrometer) will provide ultra-high sensitivity for the detection, and allows the simultaneous acquisition of multiple reaction monitoring (MRM) transitions in alternating electrospray positive (ESI<sup>+</sup>) and negative ionization (ESI<sup>-</sup>) modes, which is required for the analysis of these lipophilic marine biotoxins.

## Standard preparation

Certified standards OA, DTX1, -2, PTX2, YTX, AZA1, -2, -3, gymnodimine (GYM), and 13-desmethyl spirolide C (SPX1) were purchased from the National Research Council, Institute for Marine Biosciences (NRC-CNRC), Halifax, Canada. Semi-purified standards for pinnatoxin-E (PnTX-E), -F (PnTX-F) and -G (PnTX-G) were obtained from Cawthron Institute, Nelson, New Zealand. For each toxin, a standard stock solution was prepared in methanol. From the stock solutions, the Matrix Matched Standards (MMS) calibration curves were prepared in blank mussel extract. Blank extract was prepared according to the sample extraction procedure. The levels of the matrix matched standard were 0, 0.125, 0.25, 0.5, 1.0, and 1.5 times the validation level (which for the regulated toxins is similar to the permitted levels, as shown in Table 1).

Lipophilic marine biotoxin	EU permitted level	(MMS) (µg/kg)					
		0	0.125	0.25	0.5	1	1.5
OA, DTX1, DTX2, PTX2	160 µg OA-equivalents/kg*	0	20	40	80	160	240
AZA1, -2, -3	160 µg AZA1 equivalents/kg*	0	20	40	80	160	240
Yessotoxins	1000 µg YTX equivalents/kg*	0	125	250	500	1000	1500
GYM, PnTX-E, PnTX-F	200 µg/kg*	0	25	50	100	200	300
SPX1	100 µg/kg*	0	12.5	25	50	100	150
PnTX-G	50 µg/kg*	0	6.25	12.5	25	50	75
OA, DTX1, DTX2, PTX2	160 µg OA-equivalents/kg*	0	20	40	80	160	240
AZA1, -2, -3	160 µg AZA1 equivalents/kg*	0	20	40	80	160	240
Yessotoxins	1,000 µg YTX equivalents/kg*	0	125	250	500	1000	1500
GYM, PnTX-E, PnTX-F	200 µg/kg*	0	25	50	100	200	300
SPX1	100 µg/kg*	0	12.5	25	50	100	150
PnTX-G	50 µg/kg*	0	6.25	12.5	25	50	75

Table 1. The EU permitted levels and recommended reporting levels for the lipophilic marine biotoxins. \* Edible shellfish



## EXPERIMENTAL

### UPLC conditions

System:	ACQUITY UPLC
Run time:	5.0 min
Column:	ACQUITY UPLC BEH C <sub>18</sub> 2.1 X 100 mm, 1.7 µm
Column temp.:	40 °C
Sample temp.:	10 °C
Mobile phase A:	Water containing 6.7 mM ammonium hydroxide
Mobile phase B:	9:1 acetonitrile/water containing 6.7 mM ammonium hydroxide
Weak wash:	9:1 water/acetonitrile
Strong wash:	9:1 acetonitrile/water
Flow rate:	0.6 mL/min
Injection volume:	5 µL

Time (min)	Flow rate (mL/min)	%A	%B	Curve
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Initial	0.6	70	30	N/A
0.5	0.6	70	30	6
3.5	0.6	10	90	6
4.0	0.6	10	90	6
4.1	0.6	70	30	6
5.0	0.6	70	30	6

### MS conditions

Mass spectrometer:	Xevo TQ-S
Ionization mode:	ESI- / +
Capillary voltage:	3.0 kV
Source temp.:	150 °C
Desolvation temp.:	500 °C
Desolvation gas:	800 L/h
MRM method:	See Table 2.

### Sample extraction

Homogenized whole flesh shellfish tissue (1 g) was extracted in triplicate with 3-mL methanol. After each addition of methanol, the extract was vortex-mixed. After vortex-mixing, the extract was centrifuged for 5 min at 2000 g, and the supernatant was transferred to a 10-mL volumetric flask. After the third extraction (9 mL in total), the volume was made up to 10 mL with methanol. The crude shellfish extract was then filtered through a 0.2-µm membrane filter, prior to spiking or analysis.

In order to determine the amount of DTX3 (ester forms of OA, DTX1, and -2), the extracts were also subjected to alkaline hydrolysis using 2.5-M sodium hydroxide. After heating the alkaline mixture for 40 min at 76 °C, the mixture was cooled to room temperature, and subsequently neutralized using 2.5 M hydrochloric acid.

Compound name	Parent (m/z)	Daughter (m/z)	Ionisation	Dwell (s)	Cone (V)	Collision (eV)	Standard available
trinoYTX	550.4	396.4	Negative	0.003	75	30	No
	570.4	467.4	Negative	0.003	75	30	
YTX	570.4	467.4	Negative	0.003	75	30	Yes
	577.4	403.4	Negative	0.003	75	30	
homoYTX	577.4	474.4	Negative	0.003	75	30	No
	578.4	396.4	Negative	0.003	75	30	
45OH YTX	578.4	467.4	Negative	0.003	75	30	No
	585.4	403.4	Negative	0.003	75	30	
45OH Homo YTX	585.4	474.4	Negative	0.003	75	30	No
	586.4	396.4	Negative	0.003	75	30	
COOH YTX	586.4	467.4	Negative	0.003	75	30	No
	593.4	396.4	Negative	0.003	75	30	
COOH OH YTX	593.4	403.4	Negative	0.003	75	30	No
	593.4	467.4	Negative	0.003	75	30	
COOH Homo YTX	593.4	474.4	Negative	0.003	75	30	No
	803.5	113.1	Negative	0.003	80	60	
OA/DTX2	803.5	255.2	Negative	0.003	80	45	Yes
	817.5	113.1	Negative	0.003	80	60	
DTX1	817.5	255.2	Negative	0.003	80	45	Yes
	508.2	162.2	Positive	0.003	60	55	
GYM	508.2	490.2	Positive	0.003	60	40	Yes
	692.5	164.3	Positive	0.003	60	55	
SPX1	692.5	444.2	Positive	0.003	60	40	Yes
	694.5	164.3	Positive	0.003	60	55	
PnTX-G	694.5	676.5	Positive	0.003	60	40	Yes
	706.5	164.3	Positive	0.003	60	55	
20-Me SPX G	706.5	346.2	Positive	0.003	60	40	No
	766.5	164.3	Positive	0.003	60	55	
PnTX-F	766.5	748.5	Positive	0.003	60	40	Yes
	784.5	164.3	Positive	0.003	60	55	
PnTX-E	784.5	766.5	Positive	0.003	60	40	Yes
	828.5	658.4	Positive	0.003	35	40	
AZA3	828.5	792.5	Positive	0.003	35	30	Yes
	842.5	658.4	Positive	0.003	35	40	
AZA1	842.5	672.4	Positive	0.003	35	40	Yes
	842.5	824.5	Positive	0.003	35	30	
AZA1/6	844.5	658.4	Positive	0.003	35	40	Yes/No
	844.5	674.4	Positive	0.003	35	40	
AZA5	844.5	826.5	Positive	0.003	35	30	No
	856.5	672.4	Positive	0.003	35	40	
AZA4/5	856.5	838.5	Positive	0.003	35	30	Yes
	874.5	213.1	Positive	0.003	40	30	
PTX12	874.5	821.5	Positive	0.003	40	30	No
	876.5	213.1	Positive	0.003	40	30	
PTX2	876.5	823.5	Positive	0.003	40	30	Yes
	892.5	213.1	Positive	0.003	40	30	
PTX11	892.5	839.5	Positive	0.003	40	30	No
	894.5	213.1	Positive	0.003	40	30	
PTX2sa	894.5	805.2	Positive	0.003	40	30	No

Table 2. MRM transitions of the various lipophilic marine biotoxins.

## Single-day validation

A single lab single-day validation study was performed in order to assess the performance of the developed UPLC/MS/MS method. The following characteristics criteria were assessed: linearity, recovery, repeatability, within-laboratory reproducibility (empirically calculated from the repeatability), selectivity, and decision criteria (CC $\alpha$ ). Blank shellfish from the Dutch national monitoring program were used for validation. The shellfish species that were included in the validation were mussels (*Mytilus edulis*), oysters (*Crassostrea gigas*), cockles (*Cerastoderma edule*), and ensis (*Ensis directus*). In order to ensure that the shellfish species used for the validation were blank, they were subject to analysis for the toxins shown in Table 2 prior to the beginning of the study. Seven different shellfish samples (four mussels, one oyster, one cockle, and one ensis) were extracted and spiked at 0.0, 0.5, 1.0, and 1.5 times the validation level.

## RESULTS AND DISCUSSION

The individual stock solutions of the cyclic imines were diluted in methanol to obtain tuning parameters with IntelliStart™ Technology. IntelliStart greatly simplifies the use of LC/MS systems by automating instrument setup, compound tuning, and performing system suitability checks. The  $m/z$  of the various toxins, as well as cone voltages and collision energies, are shown in Table 2. In order to save valuable toxin standards, extracts were spiked instead of raw shellfish homogenate, which saves a factor 10 in standards. Spiking to extracts is justified by the fact that the extraction efficiency is very high (>90%) for all relevant toxins. Therefore, the spiking of extracts is very unlikely to lead to false negative results. The developed UPLC/MS/MS method is based on the chromatography earlier described by Gerssen *et al.*<sup>3</sup> The UPLC separation provided good results for all different toxins classes; only peak shapes for azaspiracids were somewhat negatively affected by the alkaline conditions. Due to the high selectivity and sensitivity of the Xevo TQ-S, this is not problematic for routine applications, as the peaks can still be easily detected at the sensitivity levels required by regulation. The resulting MRM chromatograms from the five-minute UPLC separation of various toxins are shown in Figure 2.

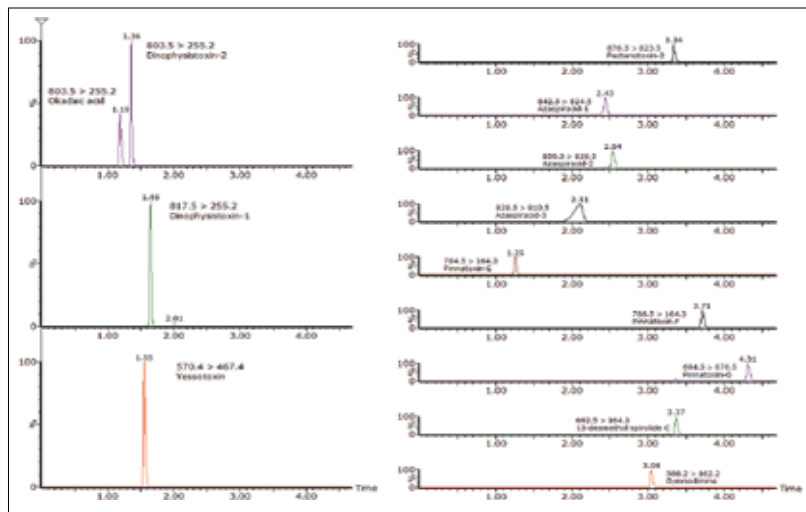


Figure 2. MRM chromatograms of matrix matched standard in mussel matrix at 1.0x validation level.

For each compound, two optimized MRMs are shown: one for confirmation and the other for quantification. The ion ratio between the results of two MRMs is one of the analytical criteria that will determine whether or not a biotoxin has been positively identified. Another regulatory criterion is the maximum residue limit (MRL) of the compound. Within the processing software, TargetLynx, it is possible to automatically flag parameters that are set within the regulatory framework so that the analyst can very easily see when these criteria have not met the permitted levels.

Sensitivity for the various toxins was good, even at levels of 0.125 times the validation level (regulatory level for regulated toxins), a signal-to-noise above 3 could be obtained. Currently for some of the regulated toxins, standards are not yet available (PTX1, 45OH YTX, homoYTX, and 45OH homoYTX). In order to determine these toxins, MRM transitions are included in the method based on the structural relation with the toxin from which a standard is available, respectively PTX2 and YTX. Furthermore, MRM transitions of other non-regulated lipophilic toxins are included, which could be screened for using this confirmatory method, as described in Table 2. The method parameters that have been determined in these experiments are now integrated into the Quanpedia Database, allowing any user of this system to have access to this method protocol.

### Single-day validation

With respect to single-day validation, different method performance parameters were assessed, such as linearity, recovery, repeatability, within-laboratory reproducibility, selectivity and CC $\alpha$ , shown in Table 3.

Compound name	Validation level ( $\mu\text{g/kg}$ )	Recovery (%)	RSDr (%)	RSDrl (%)	CC $\alpha$ ( $\mu\text{g/kg}$ )
OA	160	99	2.7	4.1	171
DTX1	160	99	7.6	12.2	192
DTX2	160	102	2.6	4.1	171
YTX	1000	100	2.5	4.0	1070
AZA1	160	98	1.3	2.1	166
AZA2	160	98	1.9	3.0	168
AZA3	160	99	1.9	3.0	168
PTX2	160	103	8.7	13.9	197
GYM	200	99	3.9	6.3	221
SPX1 <sup>1</sup>	100	108	14.6	23.4*	141
SPX1 <sup>2</sup>	100	104	12.8	20.4	135
PinE	200	122	23.1	36.9*	347
PinF	200	91	5.1	8.1	224
PinG	50	102	3.9	4.8	54

Table 3. Validation results of the single-day validation. <sup>1</sup>including the ensis matrix <sup>2</sup>without the ensis matrix, and \*poor reproducibility.

For all toxins, the linearity of the matrix matches standard calibration curve was excellent ( $R^2 > 0.990$ ) for the concentration range used (0.125 - 1.5 x PL). The recoveries obtained for the various toxins were good and ranged from 91% to 104%. The only exception to this was PnTx-E, where a recovery of 122% was obtained. This early eluting (tr 1.2 min) compound tends to form methyl esters when extracts are prepared in methanol, and this behavior can be used to explain the higher than expected recovery, as well as the poor repeatability (RSDr 23.1%), when compared to the other analytes. For all regulated compounds, the repeatability observed was good. For the non-regulated toxins, only PnTx-E and SPX1 were somewhat higher than expected. For SPX1, this was caused by the ensis matrix by removing the results of the ensis and re-calculating the repeatability results were acceptable, respectively 14.6% and 12.8% with and without ensis matrix. In order to decide if a sample is non-compliant, the toxin level should be at or above the decision limit (CC $\alpha$ ). The decision limit is a concentration at which it can be concluded with a probability of 1- $\alpha$  or 95% ( $\alpha = 5\%$ ) that the sample is above the permitted level and thus non-compliant.

## CONCLUSIONS

By using a UPLC system coupled with a tandem quadrupole MS (Xevo TQ-S), a robust, rapid method has been developed for the analysis of both regulated and non-regulated lipophilic marine biotoxins in shellfish. Compared to the conventional HPLC/MS/MS method, the speed of analysis increased four-fold, from 20 min to 5 min per analysis. The method is able to meet the regulated levels of detection that can be used instead of the mouse bioassay.

The use of IntelliStart allows the user to quickly and automatically determine the optimal settings and extend this method when more toxin standards become available. As this method (and all the instrument settings) has now been included into the Quanpedia Database, it is easy to implement this approach into other laboratories.

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# Waters

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February 2013 720004601EN AG-PDF

## Two-dimensional ultra high pressure liquid chromatography quadrupole/time-of-flight mass spectrometry for semi-targeted natural compounds identification.

Isolation of minor compounds from natural extracts remains a complicated task and development of new protocols to facilitate the access to new structure is still needed. Application of a highly efficient 2D LC hyphenated to high resolution mass spectroscopy on the crude extract of the Mediterranean marine sponge *Crambe crambe* led to the identification of seven new compounds. The structure elucidation has been performed using 2D LC-MS/MS and NMR. The structure elucidation of these minor metabolites encouraged us to speculate on the metabolic pathway leading to this important family of sponge alkaloids.

**Grégory Genta-Jouve, Julie Croué, Lionel Weinberg, Vincent Cocandeau, Serge Holderith, Nataly Bontemps, Marcelino Suzuki, and Olivier P. Thomas**

*Phytochemistry Letters* (Impact Factor: 1.45). 11/2014;

DOI: 10.1016/j.phytol.2014.10.029

## Ion mobility mass spectrometry enables the efficient detection and identification of halogenated natural products from cyanobacteria with minimal sample preparation.

Direct observation of halogenated natural products produced by different strains of marine cyanobacteria was accomplished by electrospray ionization and matrix assisted laser desorption ionization and gas phase separation via ion mobility mass spectrometry of extracts as well as intact organisms.

**Eduardo Esquenazi, Michael Daly, Tasneem Bahrainwala, William H. Gerwick and Pieter C. Dorrestein**

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Acquity UPC<sup>2</sup>/Xevo TQD  
Acquity UPLC/Q-ToF Micro

## Chiral separation of a diketopiperazine pheromone from marine diatoms using supercritical fluid chromatography.

The proline derived diketopiperazine has been identified in plants, insects and fungi with unknown function and was recently also reported as the first pheromone from a diatom. Nevertheless the stereochemistry and enantiomeric excess of this natural product remained inaccessible using direct analytical methods. Here we introduce a chiral separation of this metabolite using supercritical fluid chromatography/mass spectrometry. Several chromatographic methods for chiral analysis of the diketopiperazine from the diatom *Seminavis robusta* and synthetic enantiomers have been evaluated but neither gas chromatography nor high performance liquid chromatography on different chiral cyclodextrin phases were successful in separating the enantiomers. In contrast, supercritical fluid chromatography achieved baseline separation within four minutes of run time using amylose tris(3,5-dimethylphenylcarbamate) as stationary phase and 2-propanol/CO<sub>2</sub> as mobile phase. This very rapid chromatographic method in combination with ESI mass spectrometry allowed the direct analysis of the cyclic dipeptide out of the complex sea water matrix after SPE enrichment. The method could be used to determine the enantiomeric excess of freshly released pheromone and to follow the rapid degradation observed in diatom cultures. Initially only trace amounts of c(d-Pro–d-Pro) were found besides the dominant c(l-Pro–l-Pro) in the medium. However the enantiomeric excess decreased upon pheromone degradation within few hours indicating that a preferential conversion and thus inactivation of the l-proline derived natural product takes place.

Johannes Frenkel, Carsten Wess, Wim Vyverman, Georg Pohnert

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## Environmental neurotoxins $\beta$ -N-methylamino-L-alanine (BMAA) and mercury in shark cartilage dietary supplements.

Shark cartilage products are marketed as dietary supplements with claimed health benefits for animal and human use. Shark fin and cartilage products sold as extracts, dry powders and in capsules are marketed based on traditional Chinese medicine claims that it nourishes the blood, enhances appetite, and energizes multiple internal organs. Shark cartilage contains a mixture of chondroitin and glucosamine, a popular nutritional supplement ingested to improve cartilage function. Sharks are long-lived apex predators, that bioaccumulate environmental marine toxins and methylmercury from dietary exposures. We recently reported detection of the cyanobacterial toxin  $\beta$ -N-methylamino-L-alanine (BMAA) in the fins of seven different species of sharks from South Florida coastal waters. Since BMAA has been linked to degenerative brain diseases, the consumption of shark products may pose a human risk for BMAA exposures. In this report, we tested sixteen commercial shark cartilage supplements for BMAA by high performance liquid chromatography (HPLC-FD) with fluorescence detection and ultra performance liquid chromatography/mass spectrometry/mass spectrometry (UPLC-MS/MS). Total mercury (Hg) levels were measured in the same shark cartilage products by cold vapor atomic fluorescence spectrometry (CVAFS). We report here that BMAA was detected in fifteen out of sixteen products with concentrations ranging from 86 to 265  $\mu\text{g/g}$  (dry weight). All of the shark fin products contained low concentrations of Hg. While Hg contamination is a known risk, the results of the present study demonstrate that shark cartilage products also may contain the neurotoxin BMAA. Although the neurotoxic potential of dietary exposure to BMAA is currently unknown, the results demonstrate that shark cartilage products may contain two environmental neurotoxins that have synergistic toxicities.

**Eduardo Esquenazi, Michael Daly, Tasneem Bahrainwala, William H. Gerwick and Pieter C. Dorrestein**

*Food and Chemical Toxicology*, Volume 70, August 2014, Pages 26–32

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## Stereoselective Determination of Tebuconazole in Water and Zebrafish by Supercritical Fluid Chromatography Tandem Mass Spectrometry

A simple and sensitive method for the enantioselective determination of tebuconazole enantiomers in water and zebrafish has been established using supercritical fluid chromatography (SFC)-MS/MS. The effects of the chiral stationary phases, mobile phase, auto back pressure regulator (ABPR) pressure, column temperature, flow rate of the mobile phase, and compensation pump solvent were evaluated. Finally, the optimal SFC-MS/MS working conditions were determined to include a CO<sub>2</sub>/MeOH mobile phase (87:13, v/v), 2.0 mL/min flow rate, 2200 psi ABPR, and 30 °C column temperature using a Chiralpak IA-3 chiral column under electrospray ionization positive mode. The modified QuEChERS method was applied to water and zebrafish samples. The mean recoveries for the tebuconazole enantiomers were 79.8–108.4% with RSDs ≤ 7.0% in both matrices. The LOQs ranged from 0.24 to 1.20 µg/kg. The developed analytical method was further validated by application to the analysis of authentic samples.

Liu, Na ; Dong, Fengshou ; Xu, Jun ; Liu, Xingang ; Chen, Zenglong ; Tao, Yan ; Pan, Xinglu ; Chen, Xixi ; and; Zheng, Yongquan

*J. Agric. Food Chem.*, 2015, 63 (28), pp 6297–6303

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UPC<sup>2</sup> SQD

# Improving Productivity in Purifying Antroquinonol Using UltraPerformance Convergence Chromatography (UPC<sup>2</sup>) and Preparative Supercritical Fluid Chromatography (Prep SFC)

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## APPLICATION BENEFITS

- For antroquinonol and its derivative, UPC<sup>2</sup> results in improved resolution, compared to RPLC, allowing for increased mass loading in the ensuing prep SFC method.
- UPC<sup>2</sup> and prep SFC methods yielded a more favorable elution order compared to RPLC, further facilitating the purification step due to increased mass loading.
- Purification via prep SFC offered a nine-fold improvement in overall productivity and reduced the organic solvent use by 77% compared to the prep HPLC approach.

## WATERS SOLUTIONS

ACQUITY UPC<sup>2</sup>™ System with ACQUITY TQD

ACQUITY UPLC® H-Class System  
with SQ Detector 2

AutoPurification™ LC System with  
3100 Mass Spectrometer

Prep 100q SFC System with  
3100 Mass Spectrometer

ACQUITY UPC<sup>2</sup> 2-EP and BEH 2-EP Columns

Viridis® Silica 2-EP Column

HSS T# Columns

## KEY WORDS

Natural products, purification, prep, UPC<sup>2</sup>, SFC, convergence chromatography, CC, antroquinonol

## INTRODUCTION

Natural products are a productive source of leads for new drugs due to their high chemical diversity, biochemical specificity, and many “drug-likeness” molecular properties.<sup>1-4</sup> A large portion of today's existing drugs on the market are either directly derived from naturally occurring compounds or inspired by a natural product. In addition, natural products are used directly in the forms of food supplements, nutraceuticals, and alternative medicines.<sup>5</sup> Isolation and purification of bioactive compounds play an important role in natural product research. The most commonly used process involves extraction of target compounds from the cellular matrix and pre-purification by various low to medium pressure liquid chromatographic techniques, predominantly reversed-phase liquid chromatography (RPLC).<sup>6</sup> While being a generally applicable chromatographic technique for a variety of compound classes, RPLC does not guarantee adequate resolutions for all analytes, especially for those structural analogs and isomers of similar polarities often found in natural products. As a result, the purification step is perceived by many as a rate-limiting step and a major bottleneck for natural product drug discovery.<sup>7</sup> To that end, supercritical fluid based chromatographic techniques, including UltraPerformance Convergence Chromatography™ (UPC<sup>2</sup>®), a novel analytical chromatographic technique that applies the performance advantages of UPLC to supercritical fluid chromatography (SFC), and preparative supercritical fluid chromatography (prep SFC) have brought viable new additions to the natural product research toolbox by offering a wide range of selectivity complementary to RPLC.

Antroquinonol, with its structure shown in Figure 1, is a ubiquinone derivative recently isolated from the mycelium of *Antrodia camphorata*, a

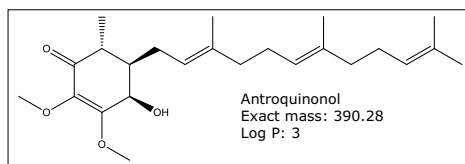


Figure 1. The chemical structure, molecular mass, and Log P of antroquinonol.

## EXPERIMENTAL

### Materials and Reagents

HPLC grade methanol and isopropanol (IPA) were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Denatured ethanol (reagent grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The antroquinonol raw product (98% purity) was from a commercial source and dissolved in methanol at 1 mg/mL for analytical experiments and 20 mg/mL for preparative experiments.

### Chromatography

The UPLC<sup>®</sup>/MS experiments were performed on a Waters<sup>®</sup> ACQUITY UPLC H-Class with SQ Detector 2. The UPLC<sup>2</sup>/MS experiments were performed on a Waters ACQUITY UPC<sup>2</sup> with ACQUITY<sup>®</sup> TQD Mass Spectrometer. Both systems were controlled by MassLynx<sup>®</sup> Software. The LC/MS experiments were performed on a Waters AutoPurification LC System with 3100 Mass Detector. The preparative SFC experiments were performed on a Waters Prep 100q SFC System with 3100 Mass Detector. Both systems were controlled by MassLynx Software and FractionLynx<sup>™</sup> Application Manager. Detailed experimental parameters are summarized in Tables 1 and 2.

unique to Taiwan.<sup>8</sup> Antroquinonol has proven cytotoxic activities against multiple tumor cell lines.<sup>9-11</sup> Pre-purification of antroquinonol from the mycelium extract involves two RPLC steps of using silica gel and size exclusion gel, respectively, resulting in a raw product of 98% purity.<sup>9</sup> In order to support medicinal research where a higher purity (>99%) product is generally required, it is imperative to develop an efficient and cost-effective purification strategy to further purify the raw product. Described herein is a comparative study on using prep LC and prep SFC to purify the antroquinonol raw product to achieve >99% purity. Chromatographic behavior of the analytes, including resolution and elution order, using each technique and their implications on downstream preparative chromatography is discussed. The productivity and solvent consumption for each purification technique are also compared.

	Figure 2A		Figure 2B		Figure 4A	
Instrument	ACQUITY UPLC H-Class/ SQD 2 System		AutoPurification LC MS System		ACQUITY UPLC H-Class/ SQD 2 System	
Flow rate (mL/min)	0.60		1.46		0.75	
Mobile phase A	Water		Water		Water	
Mobile phase B	Methanol		Methanol		Methanol	
Backpressure (psi)	N/A		N/A		N/A	
MS detection	ESI+		ESI+		ESI+	
Column	ACQUITY UPLC HSS T3 (3.0 x 150 mm, 1.8 $\mu$ m)		Atlantis T3 (4.6 x 150 mm, 5 $\mu$ m)		ACQUITY UPLC BEH C <sub>18</sub> (2.1 x 50 mm, 1.7 $\mu$ m)	
Temperature (°C)	60		Ambient		60	
Injection volume ( $\mu$ L)	1		Varying		0.5	
Gradient	Time (min)	%B	Time (min)	%B	Time (min)	%B
	0	92	0	88	0	80
	5	96	3.08	88	4	80
	5.25	92	8.21	94		
	6	92	8.61	100		
			9.22	88		
			20.90	88		

Table 1. Key experimental parameters for LC.

	Figure 3A		Figure 3B	
Instrument	ACQUITY UPC <sup>2</sup> /ACQUITY TQD		Prep 100q SFC System with 3100 Mass Detector	
Flow rate (mL/min)	1.5		80	
Mobile phase A	CO <sub>2</sub>		CO <sub>2</sub>	
Mobile phase B	Isopropanol		Isopropanol	
Backpressure (psi)	1740		1740	
MS detection	APCI+		ESI+	
Temperature (°C)	45		40	
Injection volume (μL)	1		600	
Column	ACQUITY UPC <sup>2</sup> 2-EP (3.0 x 100 mm, 1.7 μm)		Viridis Silica 2-EP (19 x 150 mm, 5 μm)	
Gradient	Time (min)	%B	Time (min)	%B
	0	5	0	5
	2.50	25	1	5
	2.75	40	6.5	9
	3.25	40	7	9
	3.50	5	7.25	5
	4	5	8	5

Table 2. Key experimental parameters for UPC<sup>2</sup> and Prep SFC.

RESULTS AND DISCUSSION

Figure 2A shows the UPLC/MS chromatogram of the antroquinonol raw product. The peak at *m/z* 391 is the sodium adduct of antroquinonol and the impurity peak at *m/z* 383 is the sodium adduct of the demethoxylated antroquinonol. Although baseline resolved, the structural similarity between antroquinonol and its derivative resulted in a rather limited resolution, which severely hampered the sample loadability in the prep LC. Figure 2B summarizes a loading study of the raw product on an analytical column (4.6 x 150 mm, 5 μm). The baseline resolution was only preserved with a 10-μL injection. The resolution deteriorated as the injection volume increased, and completely diminished with an 80-μL injection. If geometrically scaled up to a 19 x 150 mm semi-prep column, the maximum loading is projected to be 170 μL. At 20 mg/mL, this translates into a maximum loading of 3.4 mg/injection.

UPC<sup>2</sup> offers an attractive alternative. Figure 3A shows the UPC<sup>2</sup>/MS chromatogram of the antroquinonol raw product. Compared to UPLC (Figure 2A), the UPC<sup>2</sup> method provided a better resolution between antroquinonol and its derivative, allowing for an increased mass loading in the ensuing prep chromatography. It is also noted that the elution order of antroquinonol and its derivative is the opposite of that in RPLC. When a polar stationary phase, such as 2-EP, is used, UPC<sup>2</sup> resembles normal phase chromatography (NPLC) and offers orthogonal selectivity to RPLC. As a result, the elution order of the analytes is often the reverse of that in RPLC. Elution order could play an important role in the overall productivity of prep chromatography, especially for those closely eluting target/impurity pairs. Since the peak front generally accounts for a higher weight percentage of the total peak than the peak tail of the same time interval, it is highly desirable to have the target compound elute before the impurity, so that when the target and impurity are less than baseline resolved, only a small portion of the target peak is excluded during collection. In the current study using RPLC, the impurity elutes before the target. With a 40- $\mu$ L injection (Figure 2B), high purity antroquinonol can only be obtained at the expense of target recovery and total productivity. In contrast, the prep SFC chromatograms depict a much more favorable scenario for prep chromatography (Figure 3B). With impurity eluting after the target, high purity antroquinonol can be collected with negligible loss in productivity, even at the loading level where antroquinonol and the impurity slightly overlap, as shown in Figure 3B.

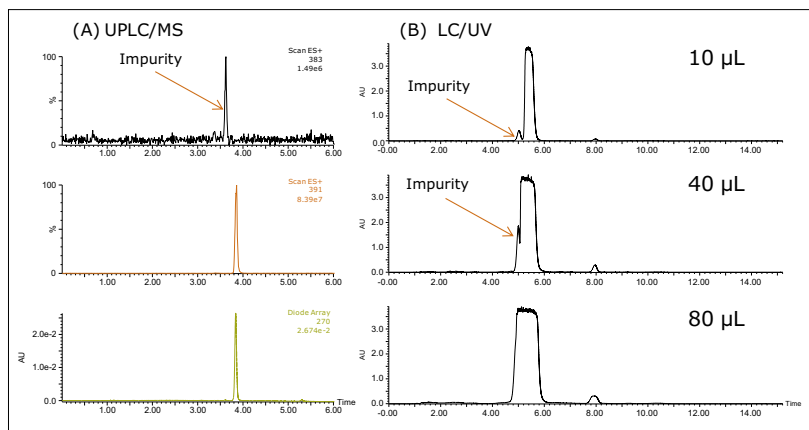


Figure 2. (A) UPLC/MS of the raw antroquinonol product at 1 mg/mL and (B) LC/UV chromatograms of the raw antroquinonol product at 20 mg/mL.

The UPC<sup>2</sup> method was scaled up to a 19 x 100 mm semi-prep column. Based on the chromatographic behavior shown in Figure 3A, a focused gradient ranging from 5 to 9 B% was used. The resulting chromatogram is shown in Figure 3B. The total run time was 8 min, compared to the 20-min run time using RPLC. The maximum loading was empirically determined to be 600  $\mu$ L. At 20 mg/mL, this represents a 12 mg/injection.

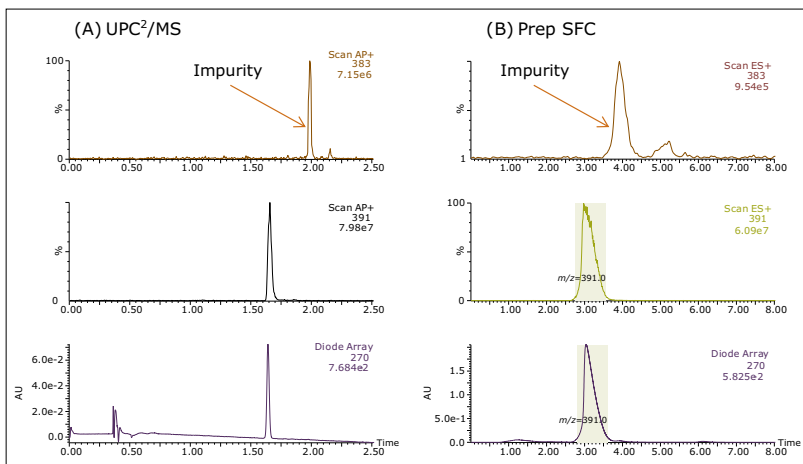


Figure 3. (A) UPC<sup>2</sup>/MS of the raw antroquinonol product at 1 mg/mL and (B) prep SFC/MS chromatogram of the raw antroquinonol product at 20 mg/mL.

Aliquots of the purified antroquinonol product were analyzed by UPC<sup>2</sup>/PDA/MS and the results are shown in Figure 4. The main impurity at *m/z* 361 was successfully removed, as shown in the corresponding mass spectrum. The final antroquinonol product has a >99% purity by UV at 270 nm.

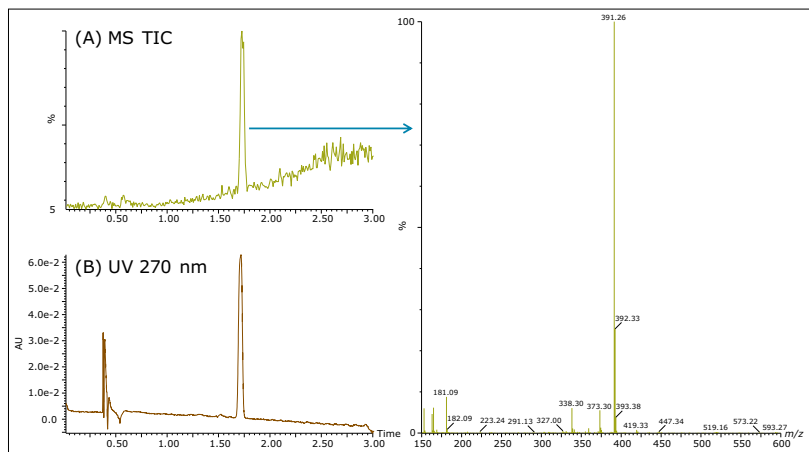


Figure 4. Purity analysis of the final antroquinonol product by UPC<sup>2</sup> with UV and MS detection.

A comparison on the productivity and solvent consumption was summarized in Table 3. Overall, by using prep SFC to replace prep RPLC, the purification productivity was increased by nine-fold with the following breakdown: 2.5-fold from the reduced run time and 3.5-fold from the increased sample loading. The organic solvent use was also reduced by 77%.

Prep chromatographic technique	Productivity (g/24 hr)	Solvent	Organic solvent consumption (L/24 hr)	CO <sub>2</sub> use (kg/24 hr)
HPLC	0.25	MeOH	33.52	N/A
SFC	2.25	MeOH/IPA	7.70	105

Table 3. Comparison on productivity and solvent consumption of two purification approaches.



## CONCLUSIONS

Two different chromatographic approaches to purify a raw antroquinonol product to the desired 99% purity have been demonstrated. In the HPLC approach, the critical pair of antroquinonol and its demethoxylated derivative resulted in a limited resolution; hence, limited mass loading in prep chromatography and limited purification productivity. The same critical pair was better separated, and had a more favorable elution order versus RPLC, using Waters UPC<sup>2</sup> and Prep 100q SFC technologies, allowing for an increased mass loading in prep SFC when the analytical UPC<sup>2</sup> method was scaled up. Overall, the prep SFC approach offered a nine-fold improvement in productivity and reduced the organic solvent use by 77% compared to the prep HPLC approach. The supercritical fluid-based techniques, UPC<sup>2</sup> and prep SFC, augment the conventional toolbox for natural product research by offering complementary selectivity to RPLC, and enable laboratories and manufacturers in pharmaceutical, traditional medicine, nutraceutical, and dietary supplement industries with more efficient and more cost-effective natural product purification.

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# Illustrating Benefits Other than Increased Sensitivity for Mycotoxin Analysis Using Xevo TQ-S

## UPLC-QTOF-MS and UNIFI

### GOAL

To show that Waters® Xevo™ TQ-S reduces ion suppression and makes peak integration easier at sub-legislation concentrations for mycotoxin analysis.

### BACKGROUND

Mycotoxins are a class of chemical compounds produced by fungi growing on food. These chemicals are subject to legislation around the world as they are a concern to human health. They are known to cause problems associated with the digestive system and liver.

Using the most sensitive mass spectrometers, such as Xevo TQ-S for analysis of these compounds makes compliance with legislative limits easier, but there are other benefits to be attained as well.

**Ion suppression is lowered with smaller injection volumes, but this can only be achieved with the sensitivity increases obtained with Xevo TQ-S.**

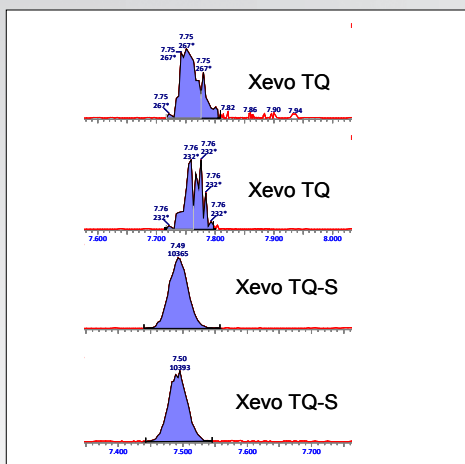


Figure 1. The same barley sample analyzed with Xevo TQ and Xevo TQ-S at a concentration of 0.1 µg/kg, 20 times lower than EU legislation levels. The mycotoxin shown is Aflatoxin B1.

### THE SOLUTION

The increased sensitivity achieved by Xevo TQ-S improves peak shapes at sub-legislation concentration, levels already achieved by Xevo TQ MS. The relative intensities of any imperfections in peak shape created by poor chromatography are reduced because the peak is larger. The start and finish of the peaks are more clearly defined, so processing software such as TargetLynx™ can achieve baseline to baseline integration more routinely. This is shown in Figure 1; the injection is spiked barley

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matrix at a pre-extraction concentration of 0.1 µg/kg. This concentration is 20 times below EU legislation levels for aflatoxin B1. The Xevo TQ chromatography has been manually changed but the Xevo TQ-S chromatography was automatically integrated. The increased sensitivity at low levels helps to reduce the number of manual integrations in a sample batch. This in turn reduces operator variability and the results from processed data sets become more consistent.

A further advantage of Xevo TQ-S is that the response for the secondary (confirmation) ion is large in size. This is also shown in Figure 1. The consistency of integration, even at low concentrations, helps to stabilize any fluctuations in the ion ratio produced by the two MRM transitions. This increased peak area gives a more accurate ion ratio at lower concentrations, and thus increases user confidence by clarifying the identity of the compound analyzed.

Extracted animal feed samples were also injected; animal feed is considered to be a complex matrix that can cause significant problems with regards to ion suppression. The results of 10 µL, 5 µL, and 2.5 µL injections are shown in Table 1. The ion suppression was calculated in the normal way of comparing the response of a solvent standard to that of an equivalent concentration in matrix.

The ability to inject a smaller amount on column can help reduce the effects of ion suppression because a smaller amount of matrix is also injected onto the column. The increased sensitivity of Xevo TQ-S makes detection at lower injection volumes possible. The reduction in ion suppression is clear at a 2.5 µL injection volume, with the majority of results between 70% and 120% (shown in light blue).

	10 µL injection	5 µL injection	2.5 µL injection
Nivalenol	88%	89%	86%
DON	81%	83%	88%
3-ac-DON	75%	78%	84%
Aflatoxin G2	64%	71%	112%
Aflatoxin G1	60%	68%	103%
Aflatoxin B2	52%	57%	89%
Aflatoxin B1	47%	50%	77%
Fumonisin B1	92%	93%	89%
HT2 toxin	44%	41%	62%
Beta zea	67%	71%	80%
Alpha zea	76%	81%	93%
T2 toxin	44%	49%	66%
T2-C13	76%	86%	104%
Zearealone	20%	24%	37%
Ochratoxin A	76%	80%	82%
Cytochalasin E	108%	102%	90%
Key	70% to 120%	50% to 69%	0% to 49%

Table 1. Ion suppression data using different injection volumes.

## SUMMARY

Improved peak shapes have been achieved due to the increase in sensitivity provided by Xevo TQ-S.

Data processing becomes quicker as baseline to baseline integrations are more common, reducing the time that analysts require when reviewing and editing processed data.

Ion suppression is lowered with smaller injection volumes, but this can only be achieved with the sensitivity increases obtained by Xevo TQ-S.

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## Rapid Analysis of Aflatoxins with the ACQUITY UPLC H-Class System

### UPLC-QTOF-MS and UNIFI

Decrease analysis times for aflatoxins from 12 to 4.5 minutes with the ACQUITY UPLC H-Class System and automated ternary solvent blending – using an instrument that provides the operational familiarity of HPLC and the chromatographic performance of UPLC.

#### GOAL

To demonstrate improved resolution and rapid analysis times achieved for the UPLC separation of aflatoxins M1, G2, G1, B2, and B1, without derivatization, using the ternary mixing capabilities of the ACQUITY UPLC® H-Class System with the Waters Aflatoxin Analysis Kit.

#### BACKGROUND

Aflatoxins are a group of mycotoxins produced as metabolites by the fungi *Aspergillus Flavus* and *Aspergillus Parasiticus*. They can be found in various foodstuffs such as grains, nuts, spices, and dairy products. There are four naturally occurring aflatoxins: B1, B2, G1, and G2. The third subset, M1, arise as a metabolic by-product when dairy cattle eat B1-contaminated grains. This can result in contaminated dairy products such as milk.

These compounds are toxic and can be carcinogenic to humans and animals. B1 and G1 are the more potent of the four naturally-occurring aflatoxins. Due to their toxicity, government regulatory agencies impose strict limits on aflatoxins in foodstuffs. For this reason, the food industry needs sensitive, accurate, and reproducible analytical methods to measure these compounds. The methods are usually based on reversed-phase HPLC with fluorescence detection. However, since reversed-phase eluents quench the fluorescence of aflatoxins B1 and G1, derivatization is commonly used to enhance the response of these analytes. Typical choices are pre-column derivatization with trifluoroacetic acid (TFA) or post-column derivatization with iodine, electrochemically-generated bromine, or photochemical UV.

These approaches are time consuming and require the purchase of costly post-column reactors, or, in the case of electrochemically-generated bromine, an electrochemical cell. In particular, the use of TFA raises concerns of handling safety by technicians.

## THE SOLUTION

Samples were prepared using the Waters Aflatoxin Analysis Kit featuring the VICAM AflaTest® P methodology prior to analysis. Using the ACQUITY UPLC H-Class System with its UPLC®-optimized fluorescence (FLR) Detector, a baseline separation of aflatoxins M1, G2, G1, B2, and B1 was achieved with a run time of 4.5 minutes. No derivatization was necessary. The ACQUITY UPLC H-Class System features a Quaternary Solvent Manager (QSM) and Auto•Blend™ Technology enabling dynamic, programmable blending of solvents. Here, a simple ternary mixture of water, methanol, and acetonitrile was applied in conjunction with an ACQUITY UPLC BEH C18 Column to perform the separation.

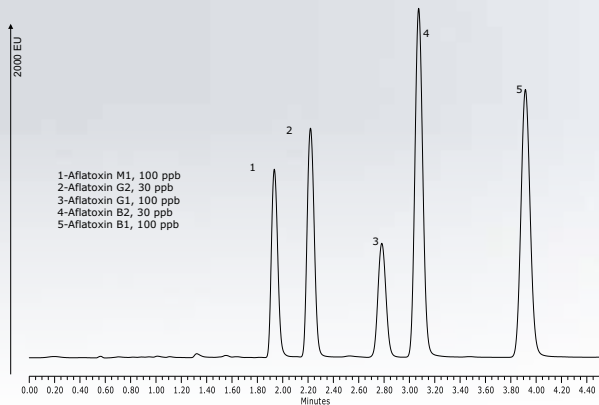


Figure 1. Aflatoxin separation using ACQUITY UPLC H-Class System with FLR Detector and Large Volume Flow Cell.

## SUMMARY

Two immediate benefits of using the ACQUITY UPLC H-Class System are its familiar operation compared to a traditional HPLC instrument, and, with UPLC capabilities, the system's ability to decrease analysis time from 12 minutes to 4.5 minutes.

Eliminating a post-column derivatization system and post-column flow reduces band broadening, providing sharper peaks with higher signal-to-noise ratios. In turn, this enables more accurate integration and quantitation. Removing the need for derivatiza-

tion also simplifies the system and reduces training, troubleshooting, and upkeep requirements. Additionally, using UPLC instead of HPLC reduced solvent consumption by 85 percent.

The ACQUITY UPLC H-Class System with the FLR Detector and the Aflatoxin Analysis Kit provides laboratories with the ability to reach the required level of sensitivity for quantifying aflatoxins without derivatization. The reduction in analytical run time offers faster sample turnaround times and improved efficiency.

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## Identification of fungal microorganisms by MALDI-TOF mass spectrometry.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a reliable tool for fast identification and classification of microorganisms. In this regard, it represents a strong challenge to microscopic and molecular biology methods. Nowadays, commercial MALDI systems are accessible for biological research work as well as for diagnostic applications in clinical medicine, biotechnology and industry. They are employed namely in bacterial biotyping but numerous experimental strategies have also been developed for the analysis of fungi, which is the topic of the present review. Members of many fungal genera such as *Aspergillus*, *Fusarium*, *Penicillium* or *Trichoderma* and also various yeasts from clinical samples (e.g. *Candida albicans*) have been successfully identified by MALDI-TOF MS. However, there is no versatile method for fungi currently available even though the use of only a limited number of matrix compounds has been reported. Either intact cell/spore MALDI-TOF MS is chosen or an extraction of surface proteins is performed and then the resulting extract is measured. Biotrophic fungal phytopathogens can be identified via a direct acquisition of MALDI-TOF mass spectra e.g. from infected plant organs contaminated by fungal spores. Mass spectrometric peptide/protein profiles of fungi display peaks in the  $m/z$  region of 1000–20 000, where a unique set of biomarker ions may appear facilitating a differentiation of samples at the level of genus, species or strain. This is done with the help of a processing software and spectral database of reference strains, which should preferably be constructed under the same standardized experimental conditions.

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## Phylogeny, identification and nomenclature of the genus *Aspergillus*.

*Aspergillus* comprises a diverse group of species based on morphological, physiological and phylogenetic characters, which significantly impact biotechnology, food production, indoor environments and human health. *Aspergillus* was traditionally associated with nine teleomorph genera, but phylogenetic data suggest that together with genera such as *Polypaecilum*, *Phialosimplex*, *Dichotomomyces* and *Cristaspora*, *Aspergillus* forms a monophyletic clade closely related to *Penicillium*. Changes in the International Code of Nomenclature for algae, fungi and plants resulted in the move to one name per species, meaning that a decision had to be made whether to keep *Aspergillus* as one big genus or to split it into several smaller genera. The International Commission of *Penicillium* and *Aspergillus* decided to keep *Aspergillus* instead of using smaller genera. In this paper, we present the arguments for this decision. We introduce new combinations for accepted species presently lacking an *Aspergillus* name and provide an updated accepted species list for the genus, now containing 339 species. To add to the scientific value of the list, we include information about living ex-type culture collection numbers and GenBank accession numbers for available representative ITS, calmodulin,  $\beta$ -tubulin and RPB2 sequences. In addition, we recommend a standard working technique for *Aspergillus* and propose calmodulin as a secondary identification marker.

R.A. Samson, C.M. Visagie, J. Houbraken, S.-B. Hong, V. Hubka, C.H.W. Klaassen, G. Perrone, K.A. Seifert, A. Susca, J.B. Tanney, J. Varga, S. Kocsubé, G. Szigeti, T. Yaguchi, Jand .C. Frisvad

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## Genome Rearrangements of *Streptomyces albus* J1074 Lead to the Carotenoid Gene Cluster Activation

*Streptomyces albus* J1074 is a derivative of the *S. albus* G1 strain defective in SalG1 restriction–modification system. Genome sequencing of *S. albus* J1074 revealed that the size of its chromosome is 6.8 Mb with unusually short terminal arms of only 0.3 and 0.4 Mb. Here we present our attempts to evaluate the dispensability of subtelomeric regions of the *S. albus* J1074 chromosome. A number of large site-directed genomic deletions led to circularization of the *S. albus* J1074 chromosome and to the overall genome reduction by 307 kb. Two spontaneous mutants with an activated carotenoid cluster were obtained. Genome sequencing and transcriptome analysis indicated that phenotypes of these mutants resulted from the right terminal 0.42 Mb chromosomal region deletion, followed by the carotenoid cluster amplification. Our results indicate that the right terminal 0.42 Mb fragment is dispensable under laboratory conditions. In contrast, the left terminal arm of the *S. albus* J1074 chromosome contains essential genes and only 42 kb terminal region is proved to be dispensable. We identified overexpressed carotenoid compounds and determined fitness costs of the large genomic rearrangements.

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and Luzhetskyy, Andriy**

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## CHAPTER 2



### QC and Manufacturing of Herbal and Dietary Supplements



## UPLC-QDA

## Selective Quantitative Determination of Water Soluble Vitamins in Various Food Matrices Using the ACQUITY UPLC H-Class System and ACQUITY QDa Detector

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Waters Corporation, Milford, MA, USA

### APPLICATION BENEFITS

- Selectivity of mass detection ensures unambiguous detection of vitamins at low levels, enabling simpler sample preparation protocols with dilution of sample extracts.
- The ACQUITY QDa™ Detector has been designed for integration with UPLC® and HPLC systems to provide robust reliable orthogonal detection to UV spectroscopy and enables new users to quickly take advantage of the most selective ACQUITY detector.
- The ACQUITY QDa Detector can be incorporated into existing liquid chromatography workflows in order to vastly increase selectivity over other LC detectors.

### WATERS SOLUTIONS

ACQUITY UPLC® H-Class System

ACQUITY QDa Detector

### KEY WORDS

WSV, vitamins, QDa, mass detection

### INTRODUCTION

Many food and beverage products are routinely fortified with vitamins to enhance their nutritional value and to help address any deficiencies in dietary requirements. In order to meet legal requirements, food and beverage manufacturers must label products according to the regulations of the country in which the product is consumed. Examples of these regulations include European Commission (EC) 1925/2006 on the addition of vitamins and minerals, and Title 21 Code of Federal Regulations (C.F.R.), Part 101 on food labeling in the United States.

Rapid, reliable, and cost-effective methods are required by food manufacturers and ingredient suppliers in order to verify product consistency and ensure that label claims are met. This can be a challenging task with the combination of complex matrices and low fortification levels of some vitamins. In addition, many of the methods currently employed stipulate that the vitamins are either analyzed separately, or in small groups. Established techniques include microbiological assays, colorimetric and fluorimetric analysis, titrimetric procedures and HPLC methodologies.<sup>1</sup> LC-MS offers the opportunity to consolidate methods along with the ability to improve detector selectivity and reduce limits of quantification. In order to offer laboratories the opportunity to capture the benefits of mass detection without the challenges associated with the adoption of mass spectrometers, recent advances in technology have focused on improving instrument usability and robustness. These motivations have resulted in the introduction of the ACQUITY QDa Detector.

In this application note, 12 water soluble vitamins (WSVs) were analyzed in dietary supplements and beverage samples using the ACQUITY UPLC H-Class System with the ACQUITY QDa Detector.

EXPERIMENTAL

include microbiological assays, colorimetric and fluorimetric analysis, titrimetric procedures and HPLC methodologies.<sup>1</sup> LC-MS offers the opportunity to consolidate methods along with the ability to improve detector selectivity

Analyte	RT (Min)	SIR m/z	Cone voltage (V)
Ascorbic Acid (C)	0.91	177	2
Thiamine (B1)	1.01	265	5
Nicotinic Acid (B3)	1.27	124	15
Pyridoxal (B6)	1.75	168	5
Nicotinamide (B3)	2.48	123	15
Pyridoxine (B6)	2.50	170	10
Ca_Pantothenate (B5)	5.88	242	15
Cyanocobalamin (B12)	7.17	678	2
Folic Acid (B9)	7.22	442	5
B2-5-Phosphate	7.35	457	5
Biotin (B7)	7.50	245	10
Riboflavin (B2)	7.74	377	15

Table 1. Retention times, SIR channels, and cone voltages for the water soluble vitamins.

Standard	Individual B complex vitamins (mg/L)	Vitamin C (mg/L)
1	1.00	50.0
2	0.75	37.5
3	0.50	25.0
4	0.25	12.5
5	0.10	5.0
6	0.075	3.75
7	0.050	2.5
8	0.025	1.25
9	0.010	0.5
10	0.005	0.25
11	0.001	0.05

Table 2. Standards concentrations for B vitamins and vitamin C.

Standard preparation

Individual 1mg/mL WSV stocks were prepared in water. In the case of vitamins B2, B7, and B9, 200 µL of 1 N NaOH were added to effect dissolution. Vitamin C was dissolved in a low pH acetate buffer to enhance stability. From these individual stocks, a mixed stock was prepared by adding 1.25 mL of the vitamin C stock and 0.025 mL of the other stocks and diluting to 25 mL with water. This mixed stock (50 ppm vitamin C, 1 ppm other analytes) was further diluted to provide 11 individual calibration standards, listed in Table 2.

Sample preparation

A packet (8.50 g) of a powdered vitamin beverage was dissolved in 100 mL water and filtered through a 0.2-µm PVDF filter. This sample was then prepared at two additional dilution levels: 1:250 and 1:10. These three dilution levels were injected to cover the different concentrations of vitamins in this sample.

A multi-vitamin supplement tablet was crushed using a mortar and pestle. The powder (1.34 g) was quantitatively transferred to a beaker to which 100 mL water was added. This mixture was sonicated for 15 minutes then stirred and filtered through a 0.2-µm PVDF filter. Three additional dilutions of this sample were prepared in water: 1:1000, 1:100, and 1:20. These dilutions and the initial dissolved tablet solution (undiluted) were analyzed in order to cover the different concentrations of vitamins in this sample.

Two different vitamin water samples were prepared by diluting 1:20 with water and filtering through a 0.2-µm PVDF filter.

## EXPERIMENTAL

## UPLC conditions

LC system:	ACQUITY UPLC H-Class
Run time:	17.5 min
Column:	ACQUITY UPLC HSS T3 1.8 $\mu$ m, 2.1 x 100 mm
Column temp.	30 °C
Mobile phase A:	10 mM ammonium formate, 0.1% formic acid in water
Mobile phase B:	10 mM ammonium formate, 0.1% formic acid in methanol
Injection volume:	5 $\mu$ L

	Time (min)	Flow rate (mL/min)	%A	%B
1.	Initial	0.45	99	1
2.	3.0	0.45	99	1
3.	3.1	0.45	95	5
4.	5.1	0.45	80	20
5.	7.1	0.45	2	98
6.	9.0	0.45	2	98
7.	9.1	0.45	99	1
8	17.5	0.45	99	1

Table 3. UPLC gradient for the separation of water soluble vitamins.

## Detector conditions

Detector 1:	ACQUITY UPLC PDA
Wavelength:	Scanning 210 to 400 nm;  Analog channel at 270 nm
Scan rate:	10 pts/sec
Detector 2:	ACQUITY QDa
Ionization mode:	ESI+
Run time:	8.0 min
Probe:	600 °C
Capillary voltage:	0.8 kV
Mass range:	$m/z$ 50 to 800 (centroid) and select SIRs*
Sampling freq.:	5 Hz
Cone voltage:	Full scan data: 15 V

\*See Table 1 for cone voltage of individual SIR channels.

SIR  $m/z$  were assigned based on previous work.<sup>2</sup>

## RESULTS AND DISCUSSION

A chromatogram showing an overlay of all 12 water soluble vitamins used in this study is shown in Figure 1, where all compounds eluted within eight minutes. Using this method, there were two co-eluting pairs (nicotinamide and pyridoxine at ~2.5 minutes and cyanocobalamin and folic acid at ~7.25 minutes). The use of mass detection means that it is no longer necessary to ensure baseline separation of all the analytes. The discrimination offered with mass detection means that these compounds can be accurately measured using their mass-to-charge ratio ( $m/z$ ). This is demonstrated in Figure 2 where the linearity of selected vitamins are shown, including vitamins that co-eluted. Figure 2D and 2F show the calibration curves of folic acid ( $m/z$  442) and cyanocobalamin ( $m/z$  678), respectively. The selectivity offered with mass detection means that these compounds can be determined quantitatively, even though they co-elute. Figure 2 also shows example calibration curves of vitamins that can be challenging to analyze by UV. For example, biotin (Figure 2A) and calcium pantothenate (Figure 2H) are vitamins that show low responses using UV detection. Those compounds are often analyzed at low wavelengths to obtain a sufficiently sensitive response.<sup>3</sup> At such low wavelengths, the specificity of the analysis may be compromised. Mass detection ensures that the analysis is both specific and sensitive.

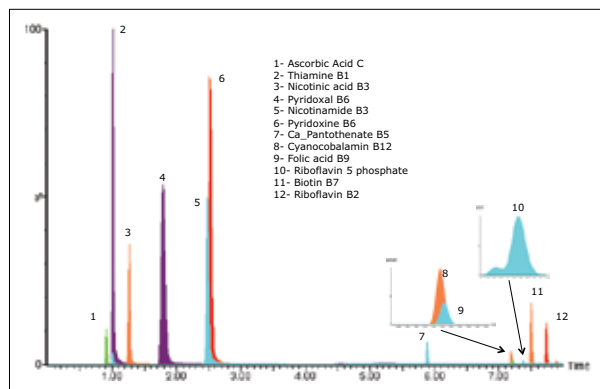


Figure 1. Overlay of SIR chromatograms of 12 water soluble vitamins separated in eight minutes.

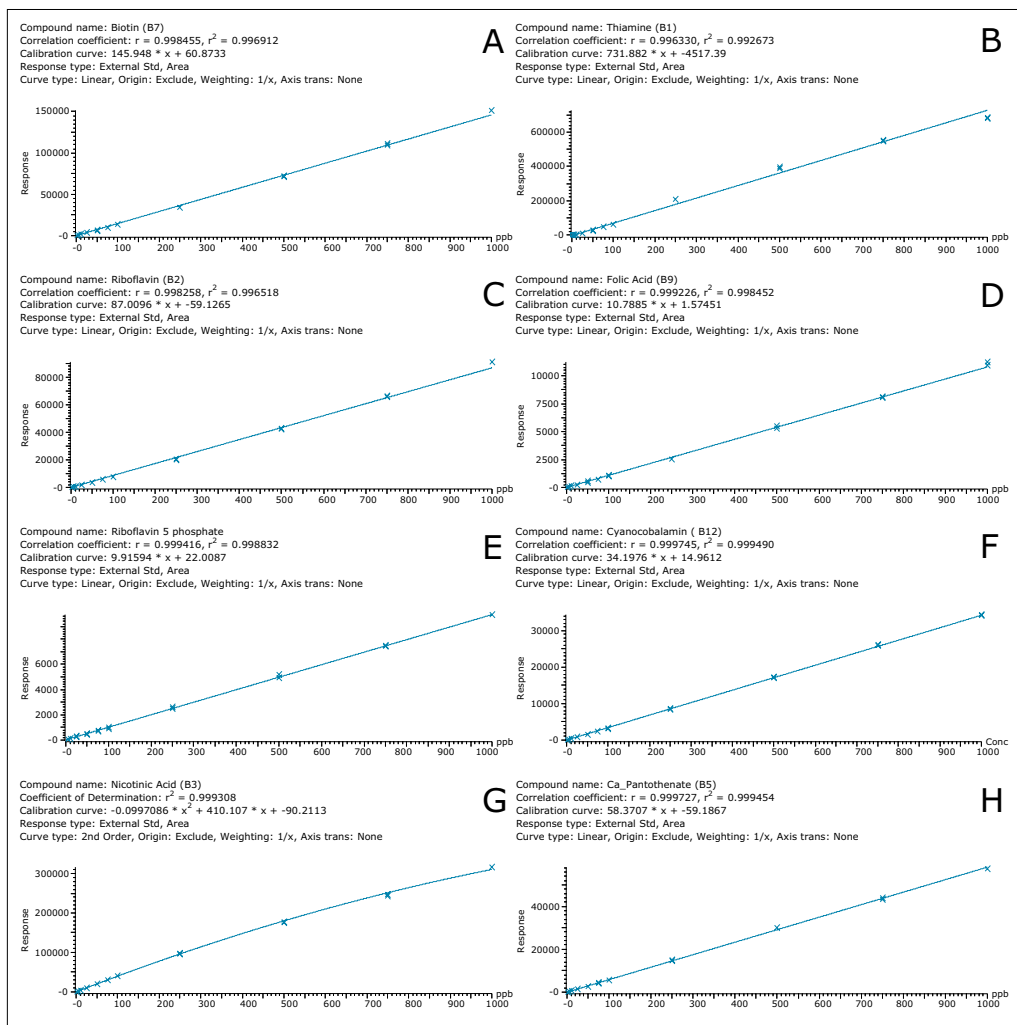


Figure 2. Calibration curves for selected water soluble vitamins.

Mass detection offers the opportunity to detect vitamins at lower levels than can be achieved with UV detection. In Figure 3, the SIR chromatograms of vitamins pyridoxine, pyridoxal, nicotinic acid, and nicotinamide at 5 ppb (5  $\mu\text{g/L}$ ) are shown, along with the UV chromatogram (Figure 3A, 270 nm). As shown in Figure 3A, the vitamins could not be detected by UV at this level. The lower limits of quantification that can be achieved with mass detection is important for the quantification of vitamins at low levels. Improved sensitivity also helps to deal with the wide variety of matrices that are encountered by allowing sample extracts to be diluted. In this work, vitamin supplements and drinks were analyzed simply by diluting the sample (in the case of a tablet an initial step to crush the tablet was required).

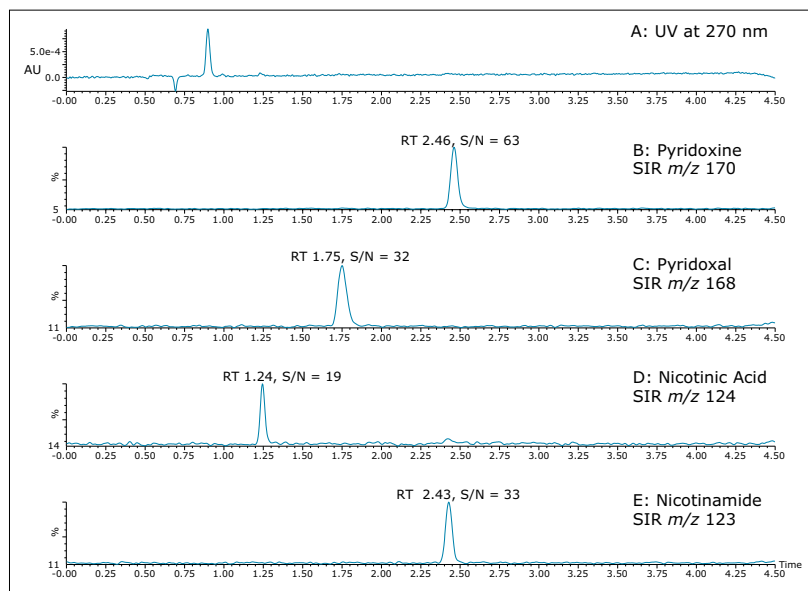


Figure 3. UV chromatogram at 270 nm and SIR chromatograms of a standard mix of vitamins at 5  $\mu\text{g/L}$ . SIR channels for four vitamins are shown. B: pyridoxine, C: pyridoxal; D: nicotinic acid; and E: nicotinamide.

Figure 4 shows the detection of vitamin B5 (calcium pantothenate) in two vitamin water samples.

As seen from the UV chromatogram, this vitamin could not be detected by UV without additional sample preparation. Vitamin B1 (thiamine) is another vitamin that is difficult to detect using UV. Figure 5 shows an example of the detection of vitamin B1 and vitamin C (ascorbic acid) in a diluted powdered vitamin beverage. Although vitamin C could be detected in the UV chromatogram, vitamin B1 was not detected. Vitamin B1 however, was clearly detected using SIR with the ACQUITY QDa Detector, as shown in Figure 5A.

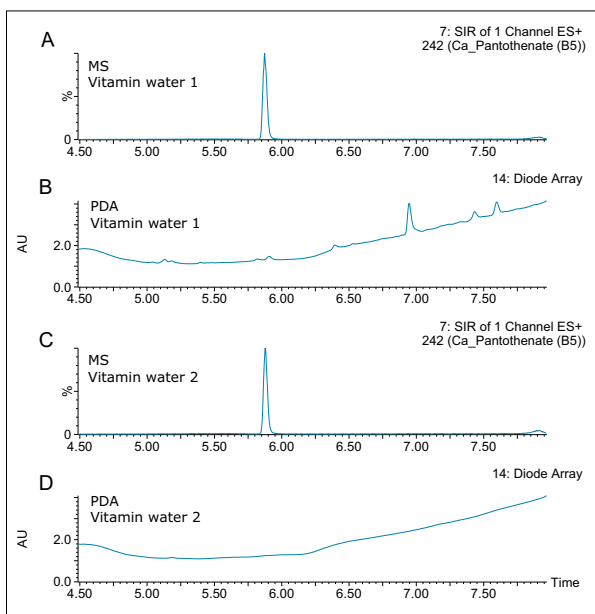


Figure 4. Detection of vitamin B5 in two different vitamin water samples. The peak at 5.9 minutes shows excellent signal-to-noise using mass detection (A and C) but cannot be detected using UV (B and D).

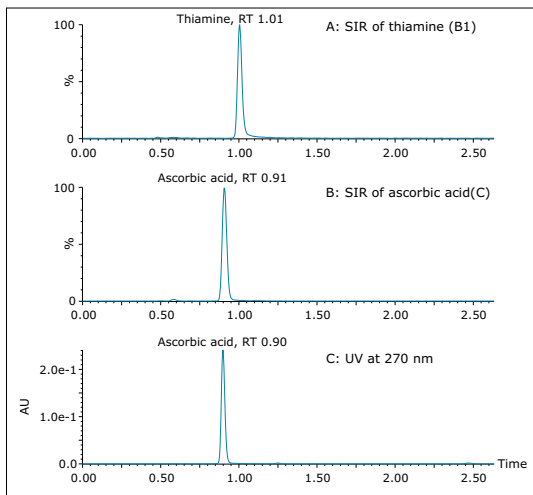


Figure 5. Chromatograms from a 1:250 dilution of a powdered vitamin beverage. A: SIR of vitamin B1 (thiamine), B: SIR of vitamin C (ascorbic acid), C: UV at 270nm; vitamin C was detected in the UV trace while vitamin B1 was not detected by UV.



Cyanocobalamin is a WSV that is fortified at very low levels in supplements and foods and it traditionally requires separate methodologies for its quantification. Two-dimensional chromatography is a routine strategy for the detection of this vitamin.<sup>4</sup> Figure 6 shows an example of cyanocobalamin detected in the multi-vitamin supplement tablet using the UPLC-MS method presented here. At this level, no peak was apparent in the UV chromatogram (Figure 6B). Mass detection offers the ability to detect vitamin B12 using the same method used to detect vitamins that are fortified at much higher levels. The ACQUITY QDa Detector, which can easily be incorporated into existing LC workflows, offers an easier-to-use method than existing multi-dimensional methods.

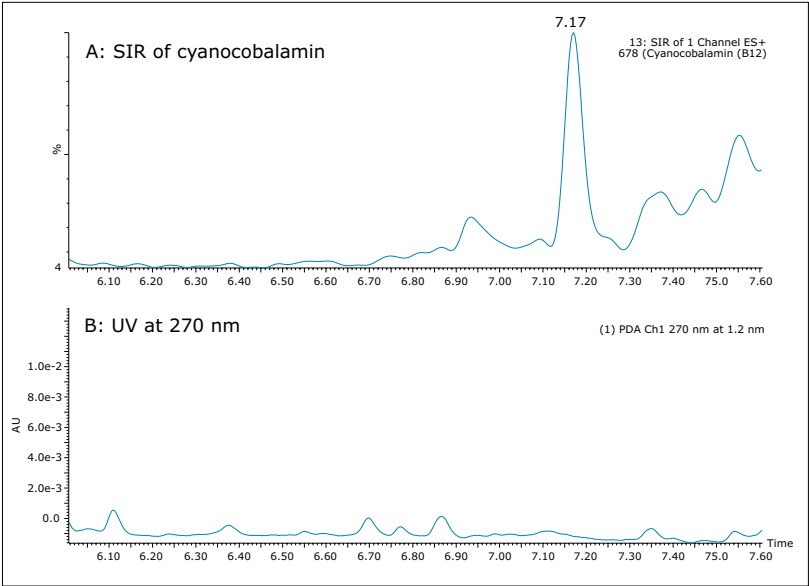


Figure 6. Detection of Vitamin B12 in a vitamin supplement tablet using mass detection (A). The level of the vitamin is below the UV detection limit (B).

One challenge that is encountered in vitamin analysis is the wide range of concentrations at which the vitamins are fortified. For the example of the multi-vitamin supplement in tablet form that was used in this study, the label stated that the B vitamins ranged from 6 µg for vitamin B12 (cyanocobalamin) to 16 mg for B3 (labeled as niacin (nicotinic acid)), with other B vitamins within that range. In this work, the same LC-MS method was used for the analysis of all the vitamins, with different dilution factors of the initial extraction in order to account for the different vitamin levels. Figure 7 shows chromatograms from the analysis of the multi-vitamin tablet. Figures 7A and 7B show the SIR channels of riboflavin (B2) in the 1:100 dilution of the sample and the undiluted sample, respectively. Figure 7C shows the SIR channel of cyanocobalamin (B12) in the undiluted sample. No peak was detected in the diluted sample (data not shown). The UV trace of the undiluted sample at 270 nm is shown in Figure 7D, and the riboflavin peak showed a good response for this sample. The quantified amount for riboflavin and cyanocobalamin were 12.5 ppm and 41 ppb, respectively. These amounts corresponded to 96% and 68% of the label claim of the supplement. Although the label claims were not verified for this work, nor did we undertake a recovery study, this short study demonstrated the feasibility of using the multiple dilution strategy within the calibration range specific in Table 2.

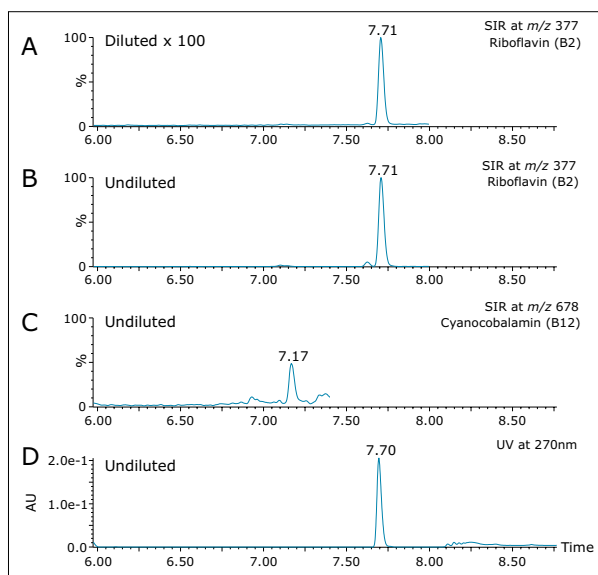


Figure 7. Detection of two B vitamins of very different concentrations in a vitamin supplement tablet. Riboflavin can clearly be detected in a 1:100 dilution of the sample extract (A), whereas B12 is only apparent in the undiluted extract (C). The response of riboflavin in the undiluted extract (B) is outside the calibrated range. At this level it can easily be detected using UV (D).

In order to assess the repeatability of the method for the B vitamins, multiple injections at different vitamin levels were assessed. Results for retention time repeatability, and peak area repeatability are shown in Tables 4 and 5, respectively. In Table 4, 10 injections of two different standards had been combined to give a total of 20 injections. Retention time stability was excellent, even for the early eluting water soluble vitamins, with all RSDs at or below 0.6%. Peak area repeatability was assessed with 10 injections at 0.025 mg/L (Table 5). For the majority of vitamins, %RSDs were well below 10%, with the exception of folic acid and riboflavin 5 phosphate, which were the lower responding analytes mentioned above. Vitamin C was excluded from this study as it is known to degrade over time.

Analyte	%RSD for retention time
Thiamine (B1)	0.6
Nicotinic Acid (B3)	0.19
Pyridoxal (B6)	0.23
Nicotinamide (B3)	0.22
Pyridoxine (B6)	0.26
Ca_Pantothenate (B5)	0.04
Cyanocobalamin (B12)	0.03
Folic Acid (B9)	0.03
Riboflavin 5 phosphate	0.03
Biotin (B7)	0.02
Riboflavin (B2)	0.03

Table 4. Repeatability of retention times for 20 injections using two different standards 0.75 mg/L (10 injections) and 0.025 mg/L (10 injections).

Analyte	%RSD for area
Thiamine (B1)	6.78
Nicotinic Acid (B3)	2.35
Pyridoxal (B6)	2.62
Nicotinamide (B3)	2.24
Pyridoxine (B6)	2.65
Ca_Pantothenate (B5)	4.60
Cyanocobalamin (B12)	7.00
Folic Acid (B9)	11.53
Riboflavin 5 phosphate	14.29
Biotin (B7)	2.77
Riboflavin (B2)	2.28

Table 5. Repeatability of B vitamins peak areas for 10 injections of a standard mix at 0.025 mg/L.

## CONCLUSIONS

This work shows the capability of the Waters ACQUITY QDa Detector to accurately quantify water soluble vitamins at levels that cannot be achieved with UV. The acquisition of SIR channels allows for sensitive and selective quantification of analytes, even when co-elution occurs. This helps to remove the burden of ensuring all analytes are baseline separated and enables the detection of lower levels of vitamins.

The ACQUITY QDa Detector allows new users to:

- Quantify analytes which have little or no UV response.
- Selectively quantify compounds that co-elute but have different masses.
- Consolidate water soluble vitamin methods into a single LC-MS method.
- Reduce limits of quantification in order to assess easier sample preparation strategies.
- Easily integrate into existing LC workflows, with the choice of Empower® 3 or MassLynx® software control.
- Quickly take advantage of the ACQUITY QDa's mass detection capabilities – no special mass spectrometry knowledge required.

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ACQUITY UPC<sup>2</sup>/PDA

## Rapid Separation of Vitamin K<sub>1</sub> Isomers and Vitamin K<sub>2</sub> in Dietary Supplements Using UltraPerformance Convergence Chromatography with a C<sub>18</sub> Column

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Waters Corporation, Milford, MA, USA

### APPLICATION BENEFITS

- Fast and reliable separation of vitamin K<sub>1</sub> *trans* and *cis* isomers and MK-4 in less than three minutes.
- Separation is achieved on a C<sub>18</sub> column; no special C<sub>30</sub> column is needed.
- The use of carbon dioxide as the primary mobile phase minimizes organic solvent waste.

### WATERS SOLUTIONS

ACQUITY UPC<sup>2</sup>® System with the  
ACQUITY UPC<sup>2</sup> PDA DetectorACQUITY UPC<sup>2</sup> HSS C<sub>18</sub> SB Column

Empower® 3 CDS Software

### KEY WORDS

Vitamin K<sub>1</sub> isomers, phyloquinone,  
menaquinone, menatetrenone, MK-4,  
UPC<sup>2</sup>

### INTRODUCTION

Vitamin K<sub>1</sub> (phyloquinone) is an essential human nutrient produced in plants, especially green leafy vegetables. The vitamin K<sub>1</sub> in natural products exists mainly as the *trans* form, while the vitamin K<sub>1</sub> used in food supplementation is often synthetic K<sub>1</sub>, which may contain appreciable amounts of the *cis* form. The *trans*-vitamin K<sub>1</sub> is bioactive, while the *cis*-K<sub>1</sub> is not. It is highly desirable to separate the *trans*- and the *cis*-vitamin K<sub>1</sub> isomers to truly evaluate the nutritional value of the supplement ingredient. Available HPLC methods for the separation of vitamin K<sub>1</sub> isomers require C<sub>30</sub> columns. Their typical run time is about 20 minutes, and chlorinated solvents are used in some of the methods.<sup>1-3</sup>

UltraPerformance Convergence Chromatography™ (UPC<sup>2</sup>) is a separation technique that leverages the unique properties (*i.e.*, low viscosity and high diffusivity) of compressed CO<sub>2</sub> at or near its supercritical state, as well as sub-2 micron particle packed columns to significantly improve the separation efficiency, speed, and selectivity.<sup>4</sup> This application note demonstrates a fast separation of vitamin K<sub>1</sub> *trans* and *cis* isomers and menatetrenone (MK-4), a common form of vitamin K<sub>2</sub>, by UPC<sup>2</sup> in less than three minutes on an ACQUITY UPC<sup>2</sup> HSS C<sub>18</sub> SB Column. Figure 1 shows the structures of vitamin K<sub>1</sub> isomers and MK-4. Comparing to current LC-based vitamin K<sub>1</sub> *trans* and *cis* isomers analysis methods, this UPC<sup>2</sup> method is faster, simpler (no need to use a C<sub>30</sub> column), and it uses less organic solvent.

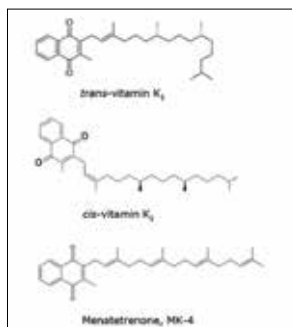


Figure 1. Structures of *trans*- and *cis*-vitamin K<sub>1</sub> and menatetrenone.

## EXPERIMENTAL

### Sample preparation

Vitamin K<sub>1</sub> (Sigma-Aldrich) and MK-4 (Sigma-Aldrich) were weighed and dissolved in iso-octane (ReagentPlus, Sigma-Aldrich) to obtain a stock solution at 1 mg/mL. Intermediate and working standard solutions were obtained by serial dilution of the stock solution with iso-octane. Vitamin K<sub>1</sub> supplement tablets were purchased from a local store and were ground into a powder and extracted with iso-octane. The supernatant was filtered with a 0.45- $\mu$ m PTFE syringe filter and diluted before injection.

### Conditions

#### UPC<sup>2</sup> conditions

System:	ACQUITY UPC <sup>2</sup> with ACQUITY UPC <sup>2</sup> PDA Detector
Software:	Empower 3
Detection:	UV at 243 nm  (compensation reference 400 to 500 nm, res. 6 nm)
Column:	ACQUITY UPC <sup>2</sup> HSS C <sub>18</sub> SB  3.0 x 100 mm, 1.8 $\mu$ m
Column temp.:	50 °C
Sample temp.:	10 °C
Injection volume:	20 $\mu$ L (Full loop)
Flow rate:	3.00 mL/min
Mobile phase A:	Compressed CO <sub>2</sub>
Mobile phase B:	Acetonitrile/methanol mixture (50/50 v/v)
Run time:	4 min
ABPR pressure:	1500 psi
Gradient:	0.5% B for 2 min, ramp to 20% B in 1.5 min, hold at 20% B for 0.5 min

## RESULTS AND DISCUSSION

Vitamin K<sub>1</sub> *cis* and *trans* isomers and MK-4 were baseline separated in less than three minutes by UPC<sup>2</sup> using a single UPC<sup>2</sup> HSS C<sub>18</sub> SB Column (3.0 x 100 mm, 1.8  $\mu$ m). The *cis* form eluted first, followed by the *trans* form, then the MK-4, as shown in Figure 2. The USP resolution between the critical pair, the *cis*- and the *trans*-K<sub>1</sub>, was 1.7 (Table 1). In the gradient program, the initial two-minute isocratic elution at 0.5% B was necessary for the baseline separation of the *cis*- and the *trans*-vitamin K<sub>1</sub>. Precise control of the mobile phase B delivery volume at 0.5% is critical for the critical pair separation. The ACQUITY UPC<sup>2</sup> System is the only SFC system on the market that can provide this level of precision control. Following the isocratic hold, a generic gradient from 0.5% to 20% B was used in the study. This gradient range could be modified in applications depending on the retention of the actual vitamin K<sub>2</sub> homologues of interest. MK-4 was included in this study because it is a common form of vitamin K<sub>2</sub>, and it is structurally the closest vitamin K<sub>2</sub> to K<sub>1</sub>. Other forms of vitamin K<sub>2</sub>, such as MK-7, have longer side chains, and tend to be retained longer at column. They can therefore be easily separated from vitamin K<sub>1</sub>. The total run time was four minutes, which was at least five times faster than the typical run time for HPLC methods using C<sub>30</sub> columns. The organic solvent consumption was less than 1 mL per injection, which is only a fraction of the typical 15 to 30 mL of solvent used in LC methods.

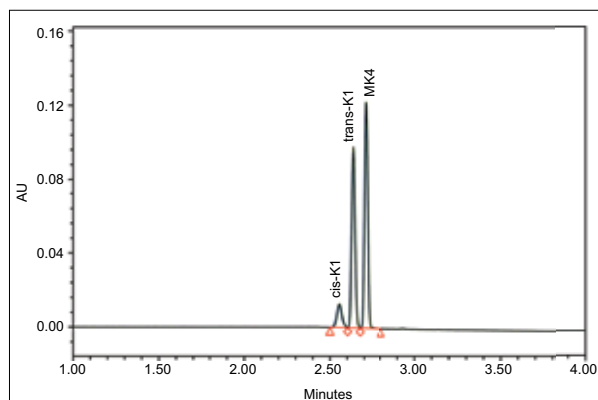


Figure 2. Chromatogram overlay of vitamin K<sub>1</sub> isomers and MK-4 standard mixture ( $n=10$ ).

	RT (min)	RTRSD	Peak area RSD	Resolution	Resolution RSD
<i>cis</i> -vitamin K1	2.553	0.08%	0.6%	—	—
<i>trans</i> -vitamin K1	2.636	0.05%	0.2%	1.7	1.1%
MK-4	2.710	0.05%	0.2%	2.0	0.9%

Table 1. Results of replicate analysis of vitamin K standard mixture ( $n=10$ ).

Ten replicate analyses of a standard mixture demonstrated excellent repeatability (Table 1). The limits of quantitation (LOQ), estimated at a signal-to-noise ratio at 10, were 0.06, 0.06, and 0.04  $\mu\text{g/mL}$  for the *cis*-vitamin K<sub>1</sub>, the *trans*-vitamin K<sub>1</sub> and the MK-4, respectively (Table 2). Excellent linearity ( $R^2 > 0.998$ ) was obtained for these compounds (Table 2). Analysis of a commercial vitamin K supplement product also showed excellent repeatability and resolution (Figure 3). In this product, the *cis*-K<sub>1</sub> was found to account for 11.2% of the total vitamin K<sub>1</sub> (Table 3).

Parameters	<i>cis</i> -vitamin K <sub>1</sub>	<i>trans</i> -vitamin K <sub>1</sub>	MK-4
Range ( $\mu\text{g/mL}$ )	0.03 to 1.5	0.02 to 8.5	0.02 to 10
Regression ( $R^2$ )	0.9980	0.9997	0.9999
Slopes (mV sec mL/ $\mu\text{g}$ )	17.7	16.3	16.0
LOQ ( $\mu\text{g/mL}$ )	0.06	0.06	0.04

Table 2. LOQ and linearity.

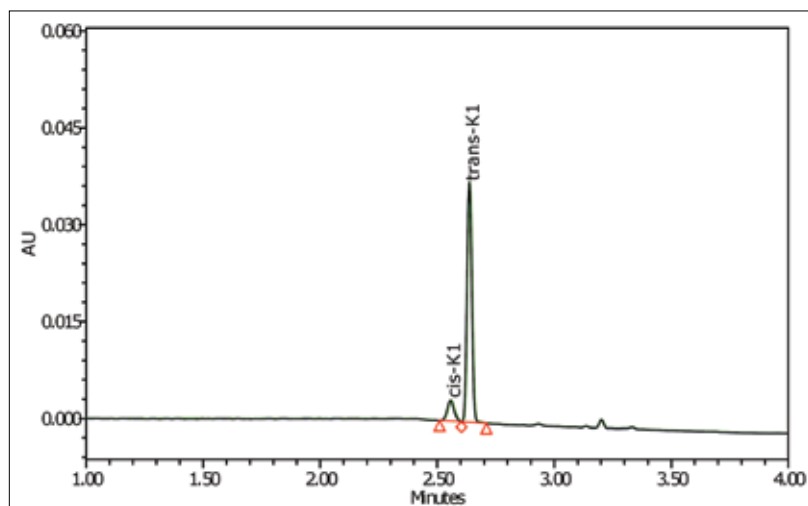


Figure 3. Chromatogram overlay of replicate analysis of vitamin K tablet ( $n=3$ ).

	RT		Conc.		% of total K <sub>1</sub> Conc.
	Mean (Min)	RSD (%)	Mean ( $\mu\text{g/mL}$ )	RSD (%)	
<i>cis</i> -vitamin K <sub>1</sub>	2.558	0.09	0.38	2.1	11.2
<i>trans</i> -vitamin K <sub>1</sub>	2.638	0.06	3.20	0.3	88.8

Table 3. Results of replicate analysis of vitamin K supplement tablet ( $n=3$ ).

## CONCLUSIONS

UPC<sup>2</sup> Technology enables a rapid separation of the *cis*- and the *trans*-vitamin K<sub>1</sub> isomers and MK-4 on an ACQUITY UPC<sup>2</sup> HSS C<sub>18</sub> SB Column in less than three minutes. The analysis time is at least five times faster than the current available HPLC methods, and no special C<sub>30</sub> column is needed. This UPC<sup>2</sup> method has excellent separation selectivity, resolution, sensitivity, repeatability, and it uses much less solvent than HPLC methods. UPC<sup>2</sup> can potentially be used by food ingredient testing labs for routine vitamin K analysis with significant increases in throughput and decreases in operating cost.

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## GCT-Premier (GC-ToF/MS)

## Rapid Marker Identification and Characterization of Essential Oils Using a Chemometric Approach

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### AIM

To provide a solution for the identification of potential markers of essential oils for the characterization of fragrances, to aid in quality control and authentication within the flavor and fragrance market.

### INTRODUCTION

The flavor, fragrance, and aroma chemical market is said to be worth around \$15 billion worldwide, with the essential oils market seeing significant growth backed largely by the increasing preference among consumers for more natural products.

As commodities in the fragrance and flavor market are generally high value items, the ability to characterize and compare raw materials, blends, and final goods has important financial implications.

As prices of natural products fluctuate widely depending on the raw material, determining factors such as country of origin, climate, batch, concentration, extraction method, and shelf life become paramount for quality control and to prevent product counterfeiting.

With reports of 5 to 7% of world trade being counterfeit products, amounting to a mammoth \$500 billion annually, validation of factors in natural products such as essential oils become even more vital.

Currently identification and characterization of fragrances is carried out manually, a very time-consuming process that can take a trained analytical chemist many hours, with a high risk of human error.

By implementing a GC-ToF exact mass-MS solution, a powerful; information rich data set can be generated. Automated, chemometricsbased software, using a statistical interpretation of patterns in multivariate data can be used for further analysis. This results in a faster and more precise assessment of composition.

The advantage of using a chemometrics with GC-TOF-MS approach is the production of a unique "fingerprint" for each essential oil. This allows a good statistical separation and facilitates the identification of differences or similarities between groups.

The following method describes essential oil characterization for a range of samples from the citrus family using a GC-TOF-MS solution complete with chemometric data analysis.

Examples of essential oil separation, marker identification, interand intra-class differentiation will be illustrated as well as database building and fragrance characterization.



*Four types of essential oils from the citrus family.*

## EXPERIMENTAL

## Samples

Four types of essential oils from the citrus family were studied (lemon, mandarin, orange, and bergamot). The essential oils analyzed are listed in Table 1. The samples were diluted 1000:1 in ethanol prior to analysis by GCT Premier™.

	Essential oils	Origin
1	Bergamot	Ivory Coast
2	Bergamot	Italy
3	Lemon	Argentina
4	Lemon	Argentina
5	Lemon	Ivory Coast
6	Lemon	Spain
7	Lemon	Italy
8	Lemon	Italy
9	Lemon	Italy
10	Mandarin Yellow	Italy
11	Mandarin Yellow	Italy
12	Mandarin Red	Italy
13	Mandarin Red	Italy
14	Mandarin Red	Italy
15	Mandarin Red	Italy
16	Mandarin Green	Italy
17	Mandarin Green	Italy
18	Mandarin Green	Italy
19	Orange	South Africa
20	Orange	Florida
21	Orange	Florida
22	Orange	Italy
23	Orange bitter	Ivory Coast
24	Orange pera	Brazil
25	Orange	Brazil

Table 1. List of the pure essential oils.



GCT Premier  
Mass Spectrometer.

## GC conditions

GC system:	Agilent 6890N
Column:	Restek Rtx -5 ms, 30 m x 0.25 mm, 0.25 µm
Flow rate:	1.0 ml/min Helium constant flow
Injection method:	Split (split ratio of 10:1), 1 µL
Temperature ramp:	60 to 180 °C at 7.5 °C/min and to 250 °C at 50 °C/min (hold for 1.6 min)
Total run time:	20 min

## MS conditions

MS system:	Waters GCT Premier
Ionization mode:	Electron ionization (EI+)
Electron energy:	70 eV
Source temp.:	200 °C
Trap current:	200 µA
Interface temp.:	280 °C
Detector voltage:	2850 V
Acquisition range:	$m/z$ 50–500
Acquisition time:	0.19 s with 0.01 s delay (2.5 spectra/s)

Exact mass spectra were obtained using a single-point lock mass (perfluorotributylamine,  $m/z = 218.9856$ ) infused into the source continuously during the run. The instrument was tuned so that resolution was greater than 7000 FWHM (Full Width Half Maximum).

## Acquisition and processing methods

The data were acquired using Waters® MassLynx® Software v4.1 and processed using MarkerLynx™ and ChromaLynx™ Application Managers.

- MarkerLynx is used for comprehensive data processing for chemometric applications, including multivariate statistical analysis and visualization.
- ChromaLynx facilitates rapid detection, identification and semi quantitative determination of components in complex matrices.

## RESULTS AND DISCUSSION

Figure 1 shows that differences between citrus essential oils are not easily discerned and a manual chromatogram-by-chromatogram inspection is very labor-intensive. MarkerLynx automates this process by rapidly characterizing essential oils, based on a PCA data set and will generate a list consisting of  $t_R$  -  $m/z$  pairs ranked in order of statistical significance.

### Rapid inter-class citrus oil differentiation

Figure 2 illustrates the MarkerLynx scores plot after processing 25 essential oils. The results show that the three types of mandarin oils are statistically similar, while divergent from orange, lemon and bergamot. The high analytical reproducibility of the system is demonstrated through the tight sample clustering of each class of oil.

The results show that the oils can be successfully separated. However, in order to determine the reasons for this differentiation, closer inspection of these results are required.

Figure 3 shows one of the  $t_R$  -  $m/z$  pairs that contributed most significantly to the separation of lemon from the rest of the oils.

This trend plot showing sample number versus significance of marker to class, illustrates a potential marker for lemon oils.

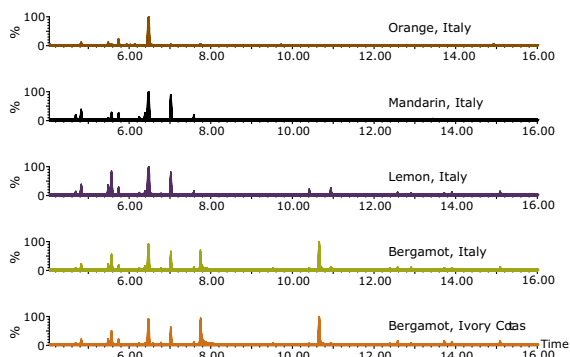


Figure 1. Total ion chromatograms of five citrus essential oils obtained using GC-TOF-MS.

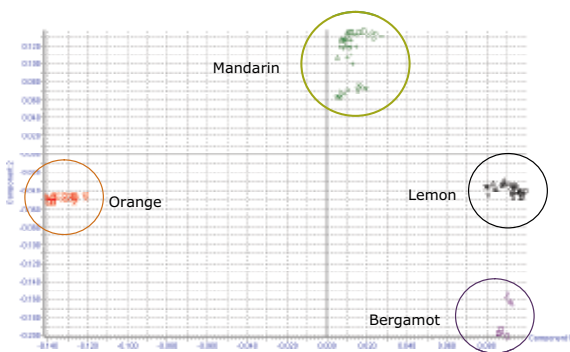


Figure 2. Scores plot obtained from MarkerLynx, illustrating group similarities between 25 essential oils.

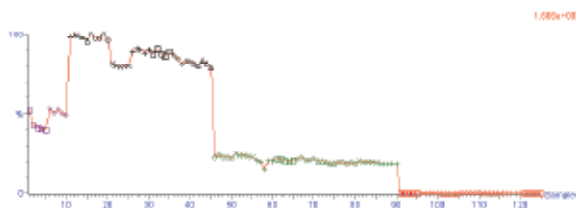


Figure 3. Trend plot of the  $t_R$  -  $m/z$  pair 12.89–93.0700, across 25 essential oils.

## Identification of potential essential oil markers

After automated processing of the data sets using ChromaLynx, the potential marker shown in Figure 4 was identified by library searching and exact mass. The marker was identified as  $\beta$ -pinene with an exact mass of 136.1256, 0.4 mDa from the calculated mass of  $C_{10}H_{16}$ .

If the potential marker does not exist in the library, exact mass will enable the elemental composition to be determined as a useful start point in establishing structural identification.

After potential markers have been identified, MarkerLynx can be used to create a database of compounds to help search for markers in subsequent analyses.

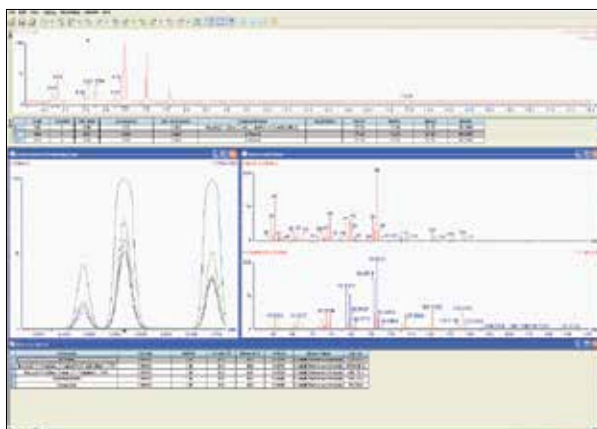


Figure 4. Identification of  $\beta$ -pinene in a lemon oil using ChromaLynx.

## Rapid intra-class essential oil differentiation

It is also possible to find similarities within each class of oil using multivariate analysis. These intra-class differences include country of origin, age, quality, etc.

For example, Figure 5 illustrates the intra-class separation of mandarin red oils obtained using MarkerLynx. Differences between sample clusters relate to the year of harvest and quality of the olfactory citrus notes.

Additionally, a supplementary software package, SIMCA-P from Umetrics, can be used to search for further differences within each class. SIMCA-P identifies differences between two samples, using advanced statistical tools such as partial least squares discriminate analysis (PLSDA) and orthogonal partial least squares (OPLS), allowing researchers to visualize their data and extract more meaningful information from their experimental results.

Figure 6 shows enhanced differentiation between two of the lemon oils. The points above 80% significance (highlighted in red) were selected and used to create an additional list of potential markers, which can be imported back into MarkerLynx.

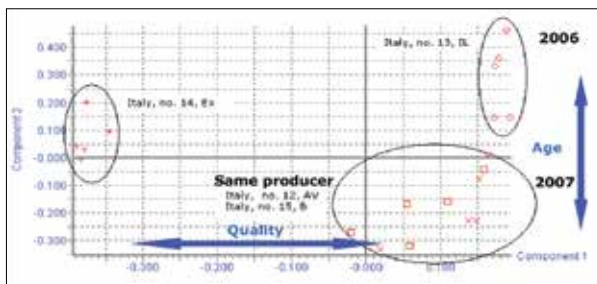


Figure 5. Scores plot obtained from MarkerLynx illustrating group similarities between four Italian mandarin red oils.

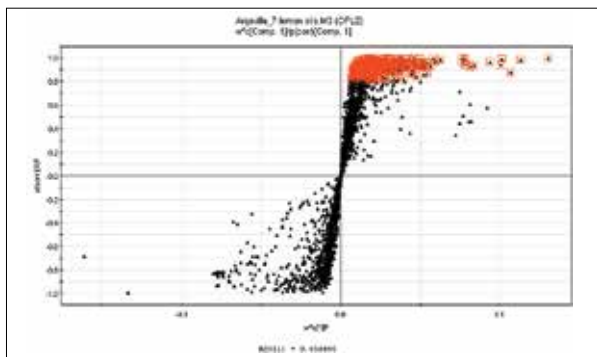


Figure 6. Scatter plot of lemon oil no. 7 vs. lemon oil no. 9, obtained from an OPLS model using SIMCA-P.

## Identifying essential oils within a fragrance

ChromaLynx Compare offers complementary functionality that is able to evaluate multiple samples to identify either common or unique components between them.

Figure 7 shows the comparison between a fragrance and one lemon oil. The results show unique components by retention time and mass (flagged with the dark triangles). The common components suggest that this variety of lemon oil is part of the composition of the fragrance analyzed.

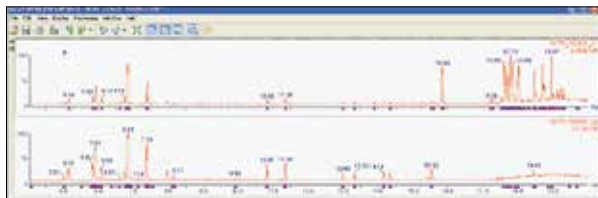


Figure 7. Comparison of a fragrance (top chromatogram) with one lemon oil (bottom chromatogram), using ChromaLynx Compare.

## CONCLUSIONS

As the prices of raw materials vary greatly and product counterfeiting is a rising problem, the GCTPremier with MarkerLynx and ChromaLynx Application Managers is the ideal solution for product authentication and quality control in the flavor and fragrance market.

With an unmatched combination of capabilities, as well as high full scan sensitivity and rapid spectral acquisition rates, this total Water's solution is ideal for full characterization and profiling of components in a wide range of sample types.

Using GC-exact mass TOF-MS on the GCTPremier enabled separation and identification of potential markers in essential oil samples from the citrus family.

MarkerLynx with a chemometric approach to the interpretation of data, allowed valuable detection and identification of potential markers in these essential oils. Inter- and intra-class differentiation between samples was also achieved using SIMCA-P software with multivariate PLS or OPLS statistical methods.

ChromaLynx Compare is also a useful tool in the characterization of fragrances. It enables the comparison of unique (or common) components that have previously undergone automated library search for identification.

The GCTPremier with MarkerLynx and ChromaLynx Application Managers enables powerful data acquisition and analysis capabilities for fragrance characterization. Plus, the automation of manually intensive processes will result in increased productivity and efficiency within the lab.

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## UPLC- SQD

## Simple Differentiation of Black Tea Samples Using a Chemometric Approach with MarkerLynx XS Application Manager

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### AIM

To identify intra- and inter-class differences between black tea samples using UPLC®/MS and a multivariate approach (MVA) with chemometrics-based software.

### INTRODUCTION

Extensive testing of food and beverages is required in manufacturing, ranging from testing for contaminants (accidental or deliberate), monitoring and developing the composition, to testing end product for taste and flavor to ensure quality and consistency. Recent food safety scares have highlighted the importance of effective QC for both protecting consumers and minimizing the business risks associated with a product recall or failure.

Today's food manufacturers must meet the challenges presented by growing consumer demands within the food and beverage industry. This competitive environment adds to the already increasing need for simple, repeatable, and reliable testing techniques that can be used consistently, routinely, and with confidence in results.

One issue is the sourcing of raw materials that can include products from diverse geographies. Ensuring the consistency of supply and product composition is essential in maintaining product safety and quality. Many manufacturers are seeking to profile, or 'fingerprint' raw materials by using rapid and powerful analytical techniques capable of detecting contaminants and impurities before materials are processed.

As the world's black tea production is projected to increase to 2.4 million tons in 2010, issues with tea manufacturing processes that could lead to a poor quality product and negatively impact the consumer's experience need to be addressed.

Traditional approaches to the analysis of tea samples include monitoring differences within key compounds, or training people to recognize differences in specific ratios of compounds; however both processes can be extremely time-consuming and sometimes inaccurate.

The use of chemometrics can provide the analyst with more information than traditional techniques. The automation of this process will also relieve some of the demands placed on a trained analyst and can help reduce the occurrence of manual errors.

The following method describes the application of UPLC and single quadrupole mass detection for the analysis of commercially available black tea samples; one sample from an economy brand and three samples from premium brands. Overall differences were assessed via chemometric analysis using MarkerLynx™ XS Application Manager.



## EXPERIMENTAL

### Sample preparation

- Remove the tea leaves from their bags
- Weigh 100 mg of leaves and add 100 mL of hot water
- Allow to infuse for at least three minutes
- Decant 1 mL of water and 0.45  $\mu\text{m}$  filter

### LC conditions

LC system: ACQUITY UPLC®  
Column: ACQUITY UPLC BEH C<sub>18</sub> Column  
2.1 x 100 mm, 1.7  $\mu\text{m}$

A simple gradient of increasing percentage of acidified acetonitrile was used for the analysis. The run time was 7.5 minutes.

### MS conditions

MS system: ACQUITY® SQD (SQ Detector)  
Ionization mode: ESI negative and positive

### Acquisition and processing methods

Data were acquired using Waters MassLynx® Software, v4.1. These data were processed using MarkerLynx XS Application Manager. This software package is designed to interpret multivariate data, such as the complex data sets obtained from mass spectrometers.



ACQUITY SQD.

## RESULTS AND DISCUSSION

The method was developed using the ACQUITY UPLC System to enable rapid run times, as samples were looked at in duplicate to ensure reproducible retention times.

The SQ Detector simultaneously analyzed each sample in ESI positive and negative modes to better investigate the compounds present in the tea samples. The mass spectral information was then processed within MarkerLynx XS.

A generic gradient was used for the chromatographic conditions as the primary objective was to use retention time and the associated  $m/z$  value within MarkerLynx XS and allow the software to interpret the data and determine any trends observed.

MarkerLynx XS Software takes the  $m/z$  and retention time associated with it and populates them into a table. This produces a fingerprint for each sample analyzed that is represented in relation to the other samples by principal component analysis (PCA).

MarkerLynx XS provides users with a full suite of advanced statistical tools, including partial least squares discriminate analysis (PLS-DA) and orthogonal partial least squares (OPLS), transforming data into information.

- PLS-DA is performed in order to sharpen the separation between groups of observations. It allows groups to be predefined and therefore does not allow for other response variables – all measured variables play the same role with respect to the class assignment.
- OPLS is a modification of the usual PLS model that allows the user to compare two groups of samples, for example, good products versus non-conforming products.

These advanced statistical tools, along with a complete graphical display suite as shown in Figure 1, allow researchers to visualize their data and extract more meaningful information from their experimental results.

This additional functionality allows sample differentiation, including batch-to-batch comparisons, to be performed quickly and easily.



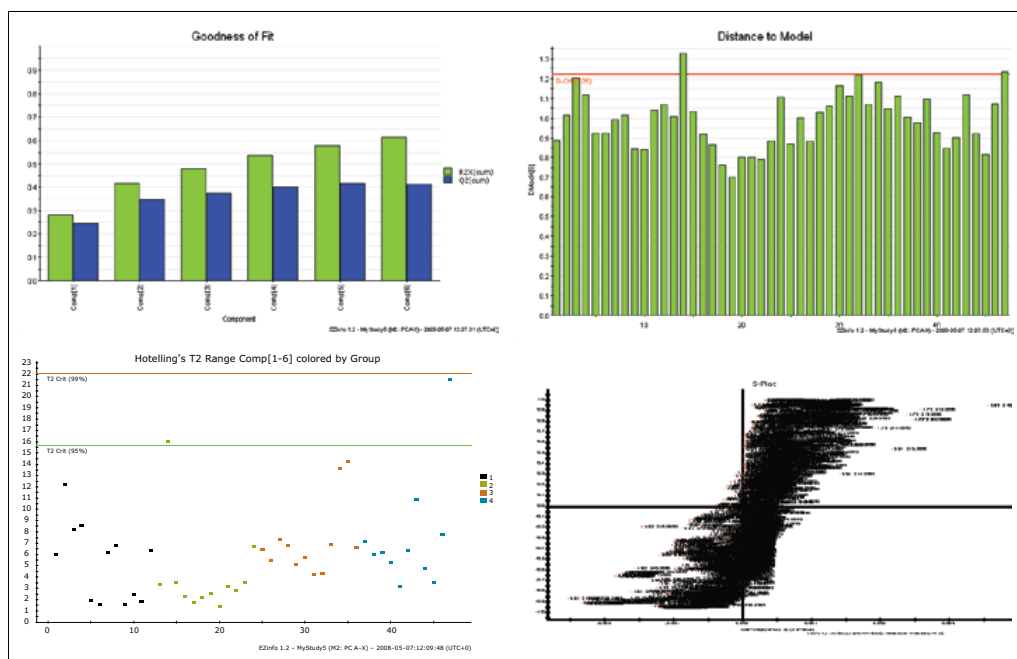


Figure 1. A suite of statistical plots that is available within MarkerLynx XS Application Manager.

Two models were selected to view the tea sample data: the first was the PCA model and the second the PLS-DA model.

Figure 2 shows the scores plot – differences between the samples – using the PCA model. Three of the tea groups cluster together (the premium samples), suggesting they are similar, and the other tea group (the economy sample) is separated from the main cluster.

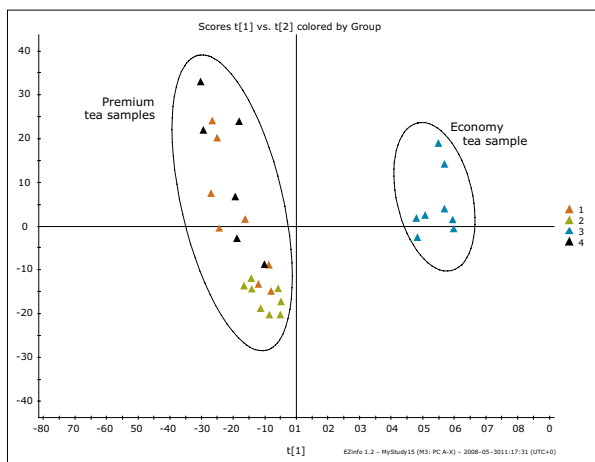


Figure 2. Scores plot using a PCA model.



This difference can also be seen in Figure 3, the three-dimensional (3-D) viewer of the PCA model. In this example, Tea 3 (economy brand) is grouped away from the other three teas. Rotating this model does not separate premium brand Teas 1, 2, and 4 away from one another.

Using the 3-D viewer from the PCA and PLS-DA models, it is possible to see trends and patterns that are not as apparent in a two-dimensional view. The 3-D view becomes an especially powerful tool when the history of samples is known, for example if the manufacturing process is known to have been successful.

As seen with the PLS-DA data, Figure 5 not only shows the main clustering seen using the PCA model, but a slight difference can now be seen between Teas 1 and 2 (same premium brand but different batches) and Tea 4 (different premium brand).

The 3-D PLS-DA model, as shown in Figure 5, accentuates the degree of separation seen for all four tea samples. The main factor to be considered when interpreting the data from a PLS-DA model is that the results need to be calibrated, by running samples not used in the model and seeing whether they fit correctly into the clusters created for that sample group.

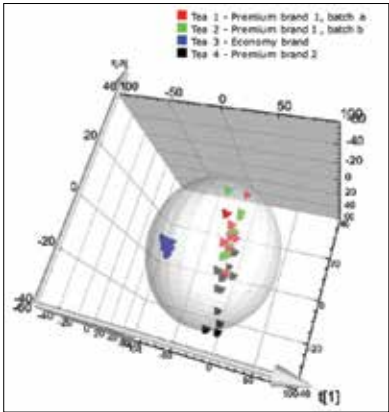


Figure 3. 3-D viewer from MarkerLynx XS using the PCA model.

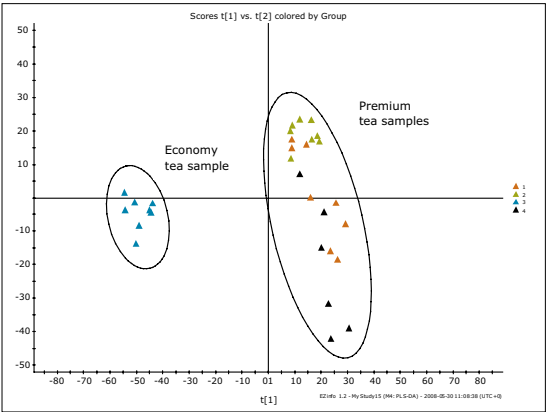


Figure 4. PLS-DA model of the four teas.

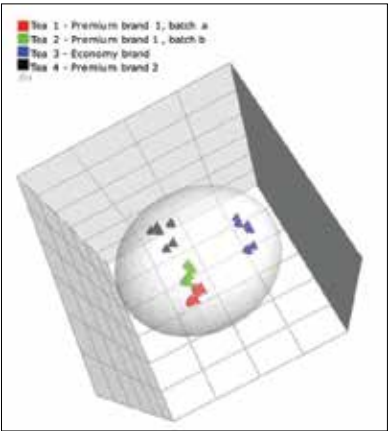


Figure 5. 3-D scatter plot of PLS-DA data for the four tea samples.

## CONCLUSIONS

The analysis of four different tea samples was performed using ACQUITY UPLC with the SQ Detector and MarkerLynx XS Application Manager to interpret the data.

Using this UPLC/MS system solution, it was possible to identify the intra- and inter-class differences between all tea samples; one economy brand and two premium brands, where two of the samples were the same brand, but from different manufacturing batches. Clear cluster grouping was achieved, accentuated by the 3-D view of the PLS-DA model data.

The power of UPLC enables rapid analysis times, which allows many samples to be compared in a very short amount of time. In addition, retention times are reproducible from sample to sample which is critical when looking at this type of comparison technique, as it is sensitive to changes between chromatograms.

The SQ Detector's rapid polarity switching enables information from both positive and negative electrospray to be acquired and interrogated for comprehensive results.

This information-rich dataset was then processed using MarkerLynx XS Application Manager. This software is a multivariate analytical tool that identifies differences between samples using PCA; and differences between sample groups using PLS-DA. This fully automates the data analysis process, producing accurate, consistent results in a short time period.

The Waters UPLC/MS solution provides:

- ACQUITY UPLC System for high productivity
  - Rapid throughput with improved resolution and sensitivity for enhanced sample differentiation capabilities
  - Flexible solution for analysis of multiple compounds in complex matrices
  - Improves laboratory efficiency
  - Delivers excellent return on investment for QC laboratory
- ACQUITY SQD for simplified mass detection
  - Rapid separation and detection of multiple compounds
  - Provides an easy-adoption route for MS to speed up method development and improve method performance in a QC environment
  - IntelliStart™ Technology reduces the burden of complicated instrument operation
  - Small laboratory bench footprint
- MarkerLynx XS Application Manager for automated data analysis
  - Extracts relevant marker information and processes complex multivariate data from LC/MS analyses
  - Via an interactive browser, performs data reduction and statistical analyses to identify characteristic markers of specific compounds within similar products, that can then be used to distinguish differences in the properties of final commodities

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**ACQUITY UPC<sup>2</sup>**

## Qualitative and Quantitative Analysis of $\beta$ -carotene Using UPC<sup>2</sup>

Jacquelyn Runco, Lakshmi Subbarao, and Rui Chen  
 Waters Corporation, Milford, MA, USA

### APPLICATION BENEFITS

- A fast UPC<sup>2</sup>™ method to separate the three most common carotenoids that minimizes the risk of degradation.
- The UPC<sup>2</sup> method is four times faster than the traditional methods of analysis, thereby, reducing organic solvent consumption by 85%.
- For the targeted analysis, the  $\beta$ -carotene extract in MTBE can be directly injected onto an ACQUITY UPC<sup>2</sup>™ System for analysis without the need for time-consuming evaporation and reconstitution steps.

### WATERS SOLUTIONS

ACQUITY UPC<sup>2</sup> System with a photodiode array (PDA) detector

MassLynx® Software

ACQUITY UPC<sup>2</sup> HSS C<sub>18</sub> SB Column

### KEY WORDS

Carotenoids, lutein,  $\beta$ -carotene, lycopene, fat-soluble, vitamins, convergence chromatography, UPC<sup>2</sup>

### INTRODUCTION

Carotenoids are natural pigments synthesized by plants and some microorganisms. For animals and humans, carotenoids play an important role in vision. Carotenoids also act as important antioxidants with a preventative effect for various diseases.<sup>1-2</sup> Since carotenoids cannot be synthesized *de novo* in the human body, humans need to acquire them through diet and supplements. In 2010, the market value of commercially used carotenoids was estimated to be \$1.2 billion and projected to grow to \$1.4 billion by 2018.<sup>3</sup> As more stringent legislation for regulatory compliance of micronutrients in fortified food products and dietary supplements is being enacted or contemplated, there is an increasing demand for rapid and reliable analytical methods for the analysis and quantification of carotenoids in a variety of matrices.<sup>4</sup> The speed of analysis is of particular importance because regulatory compliance monitoring often requires a large number of assays. In addition, many carotenoids are thermal- or photo-sensitive and highly susceptible to isomerization and chemical degradation. Prolonged analysis time could lead to inaccurate quantification results.

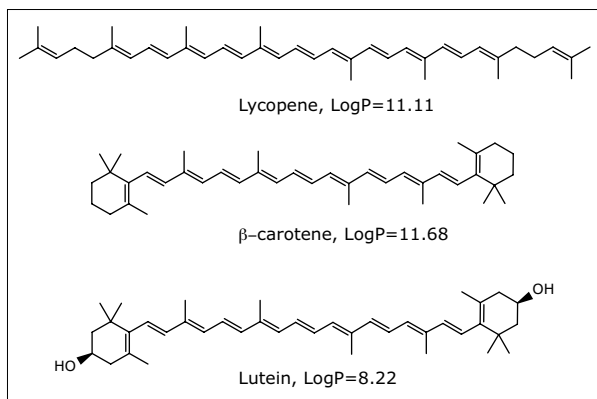


Figure 1. Chemical structures and LogP values of three carotenoids used in this study.

## EXPERIMENTAL

UPC<sup>2</sup> conditions for column screening

System:	ACQUITY UPC <sup>2</sup>
Detection:	PDA detector
Flow rate:	1.5 mL/min
Mobile phase A:	CO <sub>2</sub>
Mobile phase B:	Ethanol
Column:	ACQUITY UPC <sup>2</sup> BEH, CSH™ Fluoro-Phenyl, BEH 2-EP (3.0 x 100 mm, 1.7 µm), and HSS C <sub>18</sub> SB (3.0 x 100 mm, 1.8 µm)
Back pressure:	2190 psi
SM temp.:	5 °C
Temp.:	40 °C
Sample diluent:	MTBE
Injection volume:	1 µL
Vials:	Waters® Amber Glass 12 x 32 mm Screw Neck Vial, 2 mL
PDA scan range:	220 to 600 nm
Data management:	MassLynx Software
Gradient:	

Time (min)	B%
0	5
5	20
7	20
8	5
10	5

The central part of the carotenoid structure is the long polyene chain of alternating double and single bonds, as shown in Figure 1. Consequently, the carotenoids often possess high hydrophobicity, especially those that do not contain any hetero-atoms, such as lycopene and  $\beta$ -carotene. High-performance liquid chromatography (HPLC) with various absorbance detectors is the most commonly used analytical technique for determining carotenoids qualitatively and quantitatively.<sup>1-2, 6-10</sup> Due to their high hydrophobicity, separation of carotenoids by RPLC often results in lengthy analysis times. Furthermore, all RPLC-based methodologies generally suffer from the low solubility of carotenoids in the mobile phase. Non-aqueous reversed phase (NARP) LC has been employed to reduce the run time by using semi-aqueous or non-aqueous mobile phases. However, the NARP approach often involves the use of complex mixtures of organic solvents as the mobile phase. For example, in the official AOAC method for  $\beta$ -carotene in supplements and raw material,<sup>11</sup> a mixture of butylated hydroxytoluene (BHT), isopropanol, *N*-ethyl-diisopropylamine, ammonium acetate, acetonitrile, and methanol is used as the mobile phase.

The separation of carotenoids has long been the subject of supercritical fluid chromatography (SFC)<sup>12-18</sup> studies since its inception.<sup>12</sup> The primary component of the mobile phase in SFC, CO<sub>2</sub>, offers superior solubility for carotenoids and promotes non-polar interactions between carotenoids and the mobile phase, thereby reducing the retention time.<sup>17</sup> In addition to high chromatographic efficiency rendered by the high diffusivity of CO<sub>2</sub>, the mild temperatures used in SFC are advantageous by avoiding thermal degradation of carotenoids.

UltraPerformance Convergence Chromatography™ (UPC<sup>2</sup>) is a new category of separation science that marries the merits of both SFC and UPLC. While adhering to the basic principles of SFC, UPC<sup>2</sup> leverages the reduced system volume of UPLC, and more importantly, the exceptional separation power of sub-2-µm particle packed columns, thereby, resulting in a greatly reduced run time, improved resolution, and increased detection sensitivity.

In this application note, we describe fast separations of three common carotenoids by UPC<sup>2</sup> in less than 2 minutes. A quantitative analysis of  $\beta$ -carotene dietary supplement capsules is also demonstrated.

## EXPERIMENTAL

### Optimized UPC<sup>2</sup> conditions for $\beta$ -carotene extract analysis

Flow rate:	1.5 mL/min
Mobile phase:	75:25 CO <sub>2</sub> /ethanol, isocratic
Column:	ACQUITY UPC <sup>2</sup> HSS C <sub>18</sub> SB 3.0 x 100 mm, 1.8 $\mu$ m
Back pressure:	2190 psi
SM temp.:	5 °C
Temp.:	40 °C
Sample diluent:	MTBE
Injection volume:	1 $\mu$ L
Vials:	Waters Amber Glass 12 x 32 mm Screw Neck Vial, 2 mL
PDA scan range:	350 to 600 nm
Wavelength compensation:	440 nm with a reference wavelength 550 to 600 nm

### Sample description

All sample preparation was performed in an environment with subdued lighting. For the column screening and subsequent optimization, 1 mg each of lycopene,  $\beta$ -carotene, and lutein was dissolved in 10 mL of methyl tert-butyl ether (MTBE) to make a 0.1 mg/mL (each) stock solution.

Calibration curve: A serial dilution of a stock solution of  $\beta$ -carotene (0.1 mg/mL in MTBE) was performed. The average peak area of three replicate injections at each concentration was used for each data point.

Capsule analysis: Three  $\beta$ -carotene capsules with a label claim of 15 mg/capsule were prepared by cutting them open and dissolving the contents in 250 mL of MTBE with slight perturbation. For each assay, six replicate injections were performed, and the average peak area was used for calculating  $\beta$ -carotene content in the capsules.

## RESULTS AND DISCUSSION

Lutein, lycopene, and  $\beta$ -carotene are the three most common carotenoids found in the North American diet. Preliminary screening work revealed that methanol as mobile phase B (co-solvent) resulted in poor peak shape due to the low solubility of carotenoids in methanol, while isopropanol as a co-solvent led to broader peaks. Ethanol was, therefore, chosen as the co-solvent in all experiments. Figure 2 shows the UPC<sup>2</sup>/UV chromatograms of the carotenoids mixture from the column screening. The peak identities were confirmed by injecting individual standard using the same condition. While the ACQUITY UPC<sup>2</sup> C<sub>18</sub> SB Column yielded baseline resolution of all three carotenoids, another relatively non-polar column, CSH Fluoro-Phenyl, also provided partial separation between lycopene and  $\beta$ -carotene. No separation between lycopene and  $\beta$ -carotene was observed with either BEH or BEH 2-Ethyl Pyridine columns. Despite the similarities in structure and polarity between lycopene and  $\beta$ -carotene, the octadecyl carbon chains on the ACQUITY UPC<sup>2</sup> C<sub>18</sub> SB stationary phase offered sufficient resolution to differentiate the two analytes in UPC<sup>2</sup>.

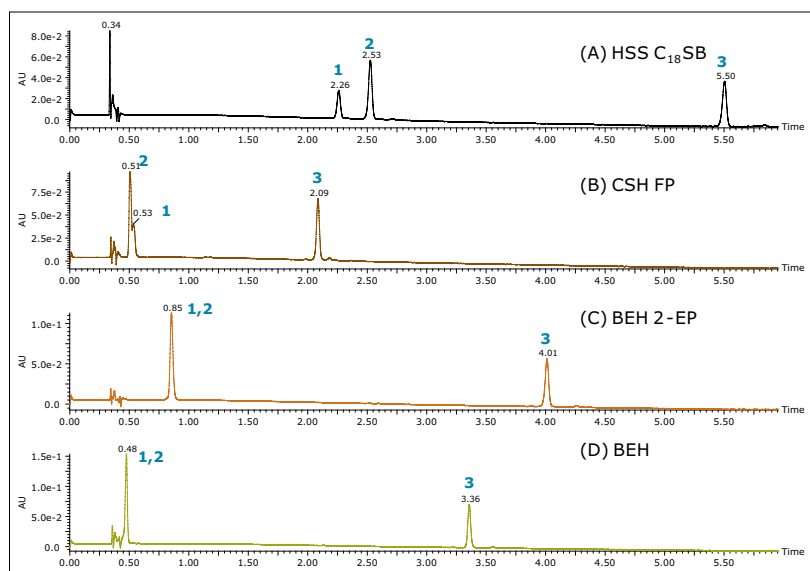


Figure 2. UPC<sup>2</sup>/UV chromatograms of a mixture of lycopene,  $\beta$ -carotene, and lutein using the following different columns: (A) HSS C<sub>18</sub> SB, (B) CSH Fluoro-Phenyl, (C) BEH 2-EP, and (D) BEH. The identities of the peaks are as follows: 1. Lycopene, 2.  $\beta$ -carotene, and 3. Lutein.

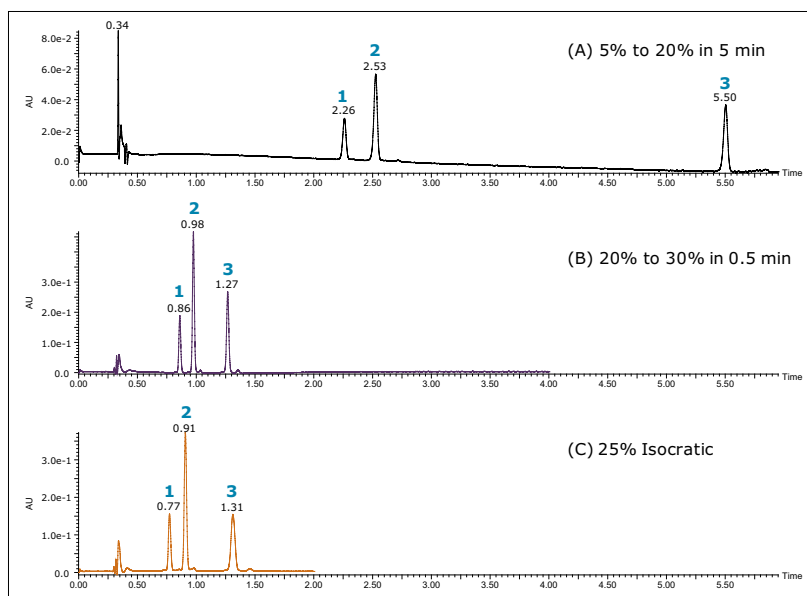


Figure 3. UPC<sup>2</sup>/UV chromatograms obtained using an ACQUITY UPC<sup>2</sup> HSS C<sub>18</sub> SB Column under different gradient/isocratic conditions including: (A) the initial screening condition: 5% to 20% in 5 min, (B) 20% to 30% in 0.5 min, and (C) 25% isocratic. The identities of the peaks are: 1. Lycopene, 2.  $\beta$ -carotene, and 3. Lutein.

Next, an optimization step was performed to shorten the run time. A ballistic gradient of 20% B/min, shown in Figure 3B, and an isocratic method at 25% B, as seen in Figure 3C, both offered sufficient resolution for all three carotenoids with a run time of less than 2 min. The late-eluting peak (lutein) from the isocratic method has a slightly wider peak width than that from the gradient method, but the isocratic method generated a smoother baseline that can be beneficial for low level detection. The isocratic method was, therefore, chosen for ensuing quantitative analyses. The optimized method is four times faster than traditional methods of analysis.<sup>5</sup> As a result, the organic solvent consumption was reduced by ~85%. It is also important to note that for SFC using C18 columns, retention of non-polar analytes, such as carotenoids, decreases with the analytes' solubility in the mobile phase.<sup>17</sup> Since compressed CO<sub>2</sub> offers superior solubility for non-polar analytes, UPC<sup>2</sup> is inherently more compatible and faster for carotenoids analyses than RPLC.

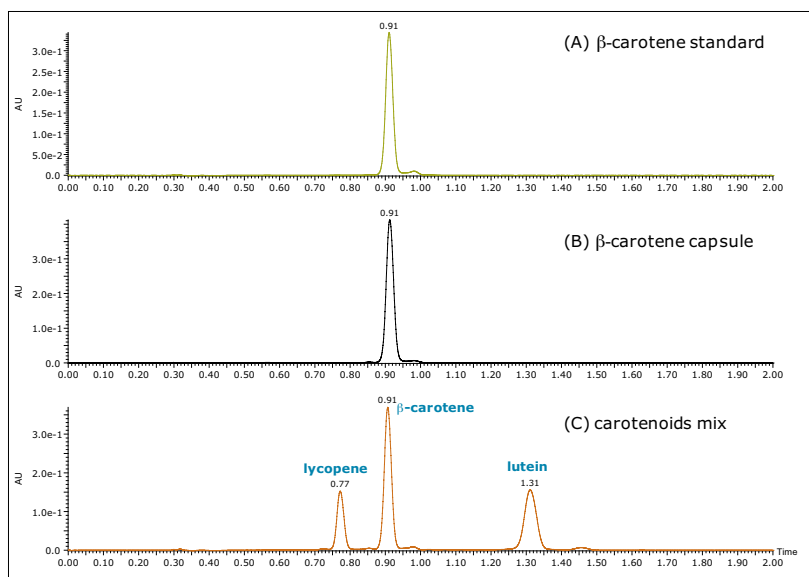


Figure 4. UPC<sup>2</sup> chromatograms of (A)  $\beta$ -carotene standard, (B)  $\beta$ -carotene extract from capsules, and (C) three carotenoids mixture under optimal chromatographic conditions.

For quantification, the  $\beta$ -carotene content of a commercially available capsule formulation was simply dissolved in MTBE, and the resulting extract was directly injected onto an ACQUITY UPC<sup>2</sup> System for analysis using the optimized method, shown in Figure 3C. A representative chromatogram of the resulting  $\beta$ -carotene extract is shown in Figure 4B. The simple sample preparation exemplifies another advantage of using UPC<sup>2</sup> for low polarity sample analysis. Dissolving low polarity samples often requires the use of low polarity solvents, such as MTBE and hexane, which are inherently compatible with UPC<sup>2</sup>. In contrast, RPLC requires that samples dissolved in low polarity organic solvents be evaporated and reconstituted into suitable diluents prior to analysis.



Figure 5 shows a calibration curve for  $\beta$ -carotene in MTBE with concentrations ranging from 0.0001 to 0.1 mg/mL. The linearity range spans three orders of magnitude with  $R^2 > 0.99$ . The limit of detection (LOD, defined as  $S/N > 3$ ) and the limit of quantitation (LOQ, defined as  $S/N > 10$ ) are 50 and 100 ng/mL, respectively. These values are equivalent or better than those reported using HPLC.6-8 The high detection sensitivity can be attributed to, in part, the inherent compatibility between carotene analysis and UPC2. The non-polar interaction between CO<sub>2</sub> and  $\beta$ -carotene greatly reduces its retention, thus results in an early eluting sharp peak for improved detection sensitivity.

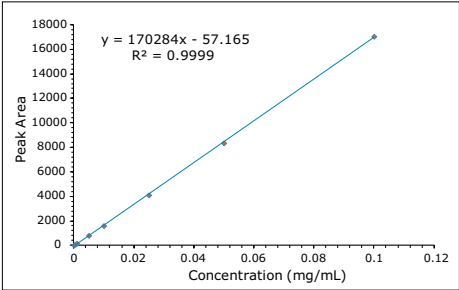


Figure 5. Calibration curve for  $\beta$ -carotene in MTBE with a concentration range of 0.0001 to 0.1 mg/mL in MTBE.

Tables 1 and 2 summarize the  $\beta$ -carotene capsule analyses. Excellent inter- and intra-assay reproducibility in both retention time and peak area was achieved. Overall assays also yielded good accuracy against the label claim. The sample preparation was simple and straightforward, and the chromatographic analyses using UPC2 were fast and reproducible.

Injection	Peak area	Retention time (min)
1	10348	0.91
2	10291	0.91
3	10382	0.91
4	10330	0.91
5	10313	0.91
6	10293	0.91
Average	10326.17	0.91
RSD%	0.34	0

Table 1. Reproducibility of a  $\beta$ -carotene capsule assay with six replicate injections.

Label Claim: 15 mg/capsule				
Assay #1	Assay #2	Assay #3	Average	RSD%
mg/capsule				
15.13	15.39	15.24	15.25	0.84%

Table 2. Quantification of  $\beta$ -carotene in three capsules.

## CONCLUSIONS

In summary, a UPC<sup>2</sup> method was successfully developed to separate the three most common carotenoids in less than two minutes. The method is four times faster than traditional methods of analysis, thereby reducing organic solvent consumption by 85%. The short analysis time also minimizes the risk of on-column degradation of the analytes. The improved speed of analysis is attributed to the inherent compatibility between UPC<sup>2</sup> and low polarity analytes. The UPC<sup>2</sup> method uses ethanol as the co-solvent instead of mixtures of organic solvents often used in HPLC methods. Thus, the UPC<sup>2</sup> method is a much more environmentally sustainable method.

A targeted 1.5-minute UPC<sup>2</sup> method was developed for the quantitative analysis of  $\beta$ -carotene in dietary supplement capsules. The dynamic range spans three orders of magnitude, with an LOD and an LOQ of 50 ng/mL and 100 ng/mL, respectively. Using MTBE as the extraction solvent, the resulting  $\beta$ -carotene extract can be directly injected onto an ACQUITY UPC<sup>2</sup> System for analysis without the need for time-consuming evaporation and reconstitution steps often associated with RPLC-based methodology. Excellent reproducibility and accuracy were also demonstrated for dietary supplement capsule analysis. The high-throughput UPC<sup>2</sup> method is ideally suited for laboratories routinely performing quality control and regulatory compliance monitoring where a large number of assays are required.

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ACQUITY UPC<sup>2</sup>

## Quantitative Analysis of Astaxanthin in Dietary Supplements by UltraPerformance Convergence Chromatography (UPC<sup>2</sup>)

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Waters Corporation, Milford, MA, USA

### APPLICATION BENEFITS

- Due to the non-polar nature of astaxanthin, the UPC<sup>2</sup>® method offers reduced analysis time due to superior solubility in the supercritical CO<sub>2</sub> mobile phase.
- UPC<sup>2</sup> employs sub-2-μm particle packed columns resulting in a higher efficiency separation.
- The UPC<sup>2</sup> method uses a simple CO<sub>2</sub>/methanol mobile phase and gradient for astaxanthin analysis, in comparison to the complex solvent scheme currently in use in HPLC, reducing solvent costs and improving safety.
- The excellent precision (RSD <1.5%) and the experimentally determined label claim agreement (within 5%) proves the UPC<sup>2</sup> astaxanthin analysis can be easily adapted to the current workflow.
- The proposed 5-min UPC<sup>2</sup> method can improve productivity for laboratories routinely performing quality control and regulatory compliance monitoring where a large number of assays are required.

### WATERS SOLUTIONS

[ACQUITY UPC<sup>2</sup>® System](#)

ACQUITY UPC<sup>2</sup> PDA Detector

[ACQUITY UPLC® HSS C<sub>18</sub> column](#)  
(3 × 150mm, 1.8 μm)

[MassLynx® v4.1](#)

### KEY WORDS

Astaxanthin, carotenoid, dietary supplement, ACQUITY UPC<sup>2</sup> SFC, label claim, quantitative analysis, food, natural product, nutraceuticals

### INTRODUCTION

In recent years, carotenoids have received considerable attention for their antioxidant activity and potential clinical uses.<sup>1</sup> They are widely used in various industries including food, dietary supplements, aquaculture, pharmaceutical, and cosmetics.<sup>2</sup> In particular, astaxanthin (Figure 1) is a carotenoid known for its anti-inflammatory effects and strong antioxidant activity (superior to β-carotene and Vitamin C).<sup>3</sup> Found in large quantities primarily in *Haematococcus pluvialis* algae, astaxanthin is responsible for the familiar red color of salmon, shrimp, and lobster.<sup>4</sup>

Astaxanthin supplements are produced commercially by many manufacturers.<sup>4</sup> As regulatory compliance monitoring of nutraceuticals becomes more stringent, rapid and reliable analytical methods for quantitation become increasingly necessary. Currently, astaxanthin quantitation is done by two methods: spectrophotometrically, and chromatographically by HPLC. The spectrophotometric method suffers from a lack of specificity between the astaxanthin and other carotenoids, resulting in an overestimation of the astaxanthin content, sometimes by as much as 20%.<sup>4</sup> The standard HPLC method has long analysis times, and involves unfriendly, complex (three component normal phase) solvent schemes due to the non-polar properties of the analytes.<sup>5</sup>

Naturally-derived astaxanthin is present primarily as a mixture of fatty acid esters. Many commercial supplements are kept in this form for stability reasons. Therefore, the esterified astaxanthin must first be hydrolyzed (de-esterified) to yield free astaxanthin prior to analysis. An internal standard, *trans*-β-apo-8'-carotenol (Figure 1) is used for quantitation in order to account for any variation in the assays.<sup>5</sup>

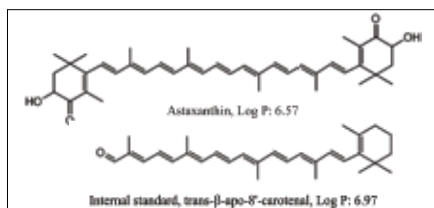


Figure 1. Chemical structures and LogP values of astaxanthin and the internal standard, *trans*-β-apo-8'-carotenol.

## EXPERIMENTAL

### UPC<sup>2</sup> conditions

All experiments were performed on a Waters ACQUITY UPC<sup>2</sup> system, equipped with an ACQUITY UPC<sup>2</sup> PDA Detector, and controlled by MassLynx software. Following an initial screen of five columns, the ACQUITY UPLC HSS C<sub>18</sub> (1.8  $\mu$ m, 3 x 150 mm) Column was selected for method optimization and all quantitative experiments. Table 1 contains the optimized UPC<sup>2</sup> method parameters.

Mobile phase A:	CO <sub>2</sub>
Mobile phase B:	Methanol
Flow rate:	1.0 mL/min
Backpressure:	200 Bar
Temperature:	30 °C
Injection volume:	2 $\mu$ L
Column:	ACQUITY UPLC HSS C <sub>18</sub>
PDA detector:	Compensated: 457 nm Reference: 530–600 nm

Gradient:	Time (min)	%B
	0	5
	2	15
	3	15
	4	5
	5	5

Table 1. UPC<sup>2</sup> method parameters for astaxanthin analysis.

In UltraPerformance Convergence Chromatography™ (UPC<sup>2</sup>) the primary component of the mobile phase, CO<sub>2</sub>, has lower viscosity, allowing for faster flow rates and the use of smaller particle sizes, which increases separation efficiency. The efficiency combined with the higher solubility of the non-polar analytes in CO<sub>2</sub> results in faster run times. Here, a fast 5-minute method was developed for astaxanthin quantitation. The method was applied to confirm the label claim for three commercially available astaxanthin supplements.

### Standards

For Standard A (Std A) 2.50 mg of *trans*-astaxanthin (Alexis Biochemicals, Farmingdale, NY, USA) was dissolved in 100 mL acetone and then diluted 1:10 for a final concentration of 2.50  $\mu$ g/mL. The internal standard (I.S.) was prepared by dissolving ~ 3.75 mg 20% oil suspension of *trans*- $\beta$ -apo-8-carotenal (Sigma-Aldrich, Allentown, PA, USA) in 100 mL acetone for a final concentration of ~7.50  $\mu$ g/mL. Standard B (Std B) is a mixture of 7.50  $\mu$ g/mL astaxanthin and ~7.50  $\mu$ g/mL I.S. The samples were kept in the refrigerator, protected from light, to minimize acetone evaporation and possible photo-degradation of the analytes.

### Supplement assay solutions

Three astaxanthin supplement formulations were obtained from commercial sources. The content of one capsule from each supplement was dissolved in 100 mL acetone. The aliquots were further diluted by 1:10 (v/v) in acetone to make the Assay A solution. For each brand of supplement, samples were prepared in triplicate using 2 mL of Assay A solution and 1 mL of I.S. solution. The samples were hydrolyzed by enzymatic de-esterification using cholesterol esterase (following the Fuji methodology).<sup>6</sup> The resulting solution was extracted with 2 mL hexane and centrifuged. The top hexane layer was transferred to another test tube, dried down by nitrogen and reconstituted in 1 mL acetone (Assay B solution). For method development purposes, a hydrolyzed sample (Assay B solution) was spiked with the non-hydrolyzed esters (Assay A solution) to ensure the sample contained both free astaxanthin and its fatty acid esters.

## Calculations

The calculations used to determine astaxanthin concentration and % label claim are displayed in Figure 2. All injections were done in triplicate and average areas were used. Due to the unavailability of 9-*cis* and 13-*cis* standards, previously established response factors of 1.1 and 1.3 were used respectively in the peak ratio calculation. Std B injections were performed and the Std B peak ratio (RstdB) was calculated for each supplement. Using the concentration of astaxanthin in Std B, the RstdB value, and the peak ratio for the Assay B solution (RAstx), the astaxanthin concentration in Assay B could be determined. A simple back calculation was done to account for dilution, and compared against the label claim (%Label claim).

**Peak ratio (R) of total astaxanthin to I.S:**

$$R = \frac{A_{trans} + 1.1A_{9-cis} + 1.3A_{13-cis}}{A_{I.S.}}$$

$A$  = Peak Area

**Astaxanthin concentration (CAstx) in Assay B:**

$$C_{Astx} = \frac{C_{stdB} \times R_{stdB}}{R_{Astx}}$$

$C_{stdB}$  = Concentration of *trans*-astaxanthin in Std B (7.5 µg/mL)

$R_{stdB}$  = Peak ratio for Std B

$R_{Astx}$  = Peak ratio for Assay B

**% Label claim:**

$$= \frac{C_{Astx}(mg/mL) \times \frac{1mL \text{ Assay B}}{2mL \text{ Assay A}} \times 1000 \text{ mL dilution}}{\text{Label claim (mg/capsule)}} \times 100$$

Figure 2. Calculations used to determine astaxanthin concentration and % label claim.

## RESULTS AND DISCUSSION

### HPLC vs. UPC<sup>2</sup> methods

There are multiple chromatographic challenges associated with astaxanthin quantitation. Free astaxanthin is a mixture of geometric *trans*, 9-*cis* and 13-*cis* isomers; *trans* being the most dominant form. Due to the difference in UV absorption coefficients, an accurate quantitation requires the separation of all three isomers and the internal standard. Also, in the event of incomplete hydrolysis, the astaxanthin esters must be resolved from the rest of the analytes to avoid interfering with the peak areas.

The HPLC method is currently accepted as the standard for astaxanthin quantitation (Figure 3). The method exhibits good separation, but suffers challenges mostly due to the non-polar nature of the analytes. A complex three component mobile phase is required, employing methanol, t-butylmethylether, and an aqueous phosphoric acid solution in a relatively lengthy 35-minute gradient method.

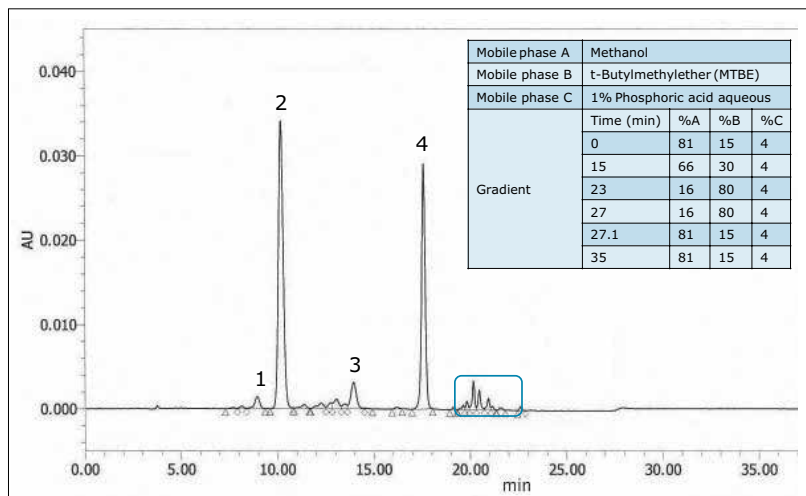


Figure 3. Astaxanthin separation under the standard HPLC gradient conditions. The peaks are: (1) 13-*cis*-astaxanthin; (2) *trans*-astaxanthin; (3) 9-*cis*-astaxanthin; and (4) I.S. The esters are indicated by the blue rectangle.

In contrast to HPLC, UPC<sup>2</sup> employs supercritical CO<sub>2</sub> as the main component of the mobile phase, offering superior solubility for non-polar analytes. The UPC<sup>2</sup> method uses a simple CO<sub>2</sub>/methanol mobile phase and 5-minute gradient method to achieve separation in a little over 2 minutes (a 10-fold improvement over the HPLC method).

In figure 4, three chromatograms are shown. The first (A) displays a sample containing unhydrolyzed esters used for method development to ensure resolution of the esters from the analytes. Figure 4(B) shows Std B (*trans*-astaxanthin standard and I.S.) used to determine the peak ratio ( $R_{std}$ ). Lastly, a fully hydrolyzed supplement (Assay B) is shown in Figure 4(C). The internal standard and astaxanthin peaks were confirmed by MS (not shown), and the geometric isomers were distinguished by their UV spectra, where the 13-*cis* isomer has a characteristic dual maximum.<sup>1</sup>

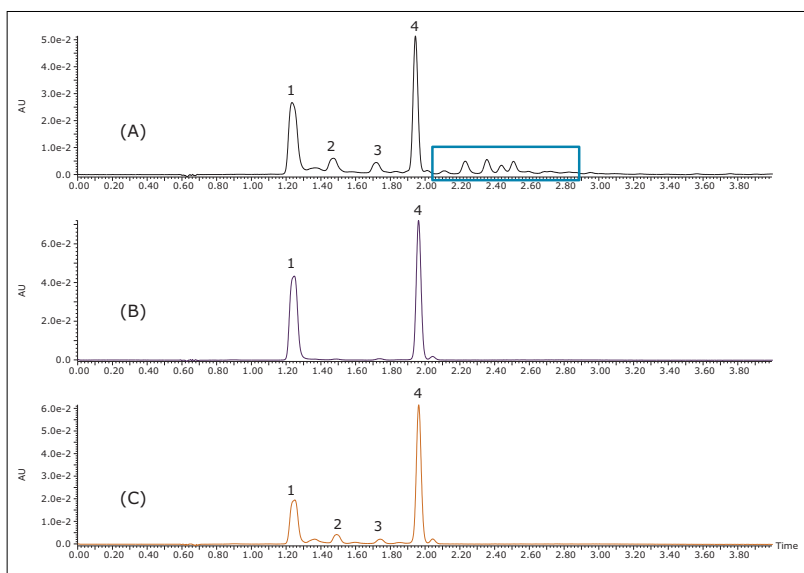


Figure 4. UPC<sup>2</sup>-UV chromatograms of (A) sample containing esters, (B) Std B, (C) hydrolyzed supplement (Assay B). The peaks are: (1) *trans*-astaxanthin; (2) 9-*cis*-astaxanthin; (3) 13-*cis*-astaxanthin; and (4) I.S. The esters are indicated by the blue rectangle.

## Repeatability

Intra- and inter-day experiments were performed using one of the Assay B solutions and the corresponding %RSD was calculated. Six replicate injections were done for the intra-day experiment while the inter-day experiments were carried out over 3 days (6 replicate injections each day). The results are summarized in Table 2.

For intra-day repeatability, the 13-*cis*-astaxanthin exhibits the highest %RSD, possibly due to its relatively small peak area. For inter-day assays, the %RSD values are slightly elevated. This can be ascribed to the propensity of these analytes for degradation in the presence of light and oxygen, and the tendency for isomeric conversion between the *cis* and *trans* isomers. Nevertheless, satisfactory RSDs (<5%) were obtained for both inter- and intra-day assays.

Inj #	Intra-day				Day	Inter-day			
	<i>Trans</i>	9- <i>cis</i>	13- <i>cis</i>	I.S.		<i>Trans</i>	9- <i>cis</i>	13- <i>cis</i>	I.S.
1	1997	320	133	2199	1	1957	320.7	126.7	2156
2	1982	330	131	2155	2	1872	325.8	139.3	2233
3	1963	323	128	2154	3	1813	315.3	133.7	2249
4	1943	318	123	2146					
5	1937	318	122	2143					
6	1921	315	123	2139					
%RSD	1.47	1.65	3.69	1.02	%RSD	3.86	1.64	4.76	2.25

Table 2. Calculated % RSDs for intraday and inter-day area results.

## Supplement analysis

The described UPC<sup>2</sup> method for quantitative analysis of astaxanthin was utilized to confirm the label claim for three commercially available supplements. Example chromatograms for the three dietary supplement assays are displayed in Figure 5 and exhibit similar profiles. No astaxanthin esters were detected, indicating complete hydrolysis.

Each supplement was assayed in triplicate and injected in triplicate. Average areas were used to calculate the % label claims presented. For each supplement, excellent repeatability (%RSD <1.5) was attained, and the experimentally determined content agreed well with the label claim.

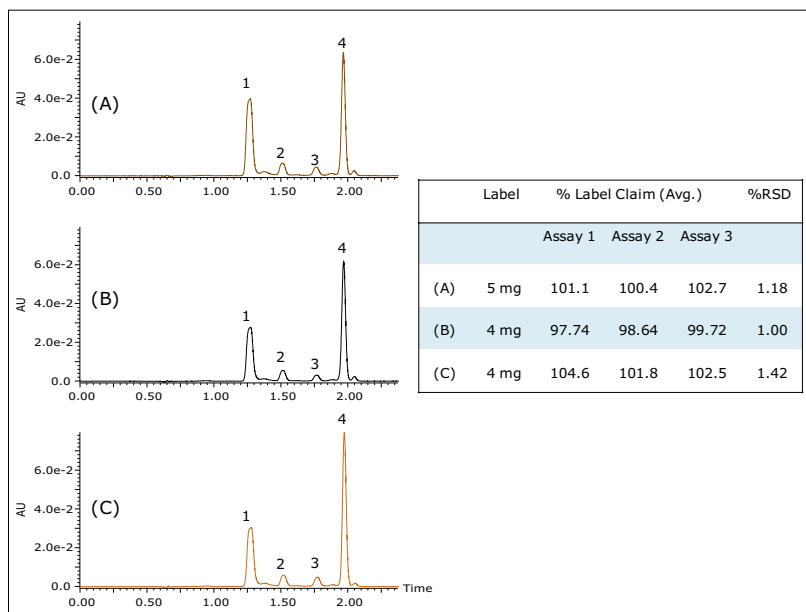


Figure 5. UPC<sup>2</sup>-UV chromatograms of the hydrolyzed astaxanthin supplements and the calculated %label claim results. The peaks are: (1) trans-astaxanthin, (2) 9-cis-astaxanthin, (3) 13-cis-astaxanthin, and (4) I.S.



## CONCLUSIONS

- In UPC<sup>2</sup>, supercritical CO<sub>2</sub> offers superior solubility for astaxanthin, resulting in a 10-fold reduction in analysis time when compared to HPLC.
- The optimized UPC<sup>2</sup> method is superior to the standard HPLC method, achieving good resolution with a simpler and faster gradient and mobile phase.
- The method was repeatable, which meant it could be successfully applied to the quantitation of three commercially available astaxanthin dietary supplements.
- Excellent precision was attained for the assays, and the experimentally determined content agreed well with the label claims proving it could be easily adapted into the currently accepted process.
- The UPC<sup>2</sup> method was demonstrated to be rapid and reliable, meeting the requirements necessary in an increasingly regulated and growing market.

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## UPLC SQD

## Determination of Soy Isoflavones in Foods and Dietary Supplements by UPLC

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### APPLICATION BENEFITS

- Accurate determination of soy isoflavone content in foods and dietary supplements
- Alternate selectivity to traditional  $C_{18}$  methods
- Significant time savings relative to currently accepted methods

### WATERS SOLUTIONS

ACQUITY UPLC® System

ACQUITY® SQD System

XSelect™ HSS Cyano, XSelect HSS Cyano **XP**, and ACQUITY UPLC HSS Cyano Columns

ACQUITY UPLC Columns Calculator

GHP Acrodisc® Minispike Filters

### KEY WORDS

Soy, isoflavone, UPLC, XP, infant formula, method development, method transfer

### INTRODUCTION

Isoflavones are a class of plant-derived compounds, produced almost exclusively by members of the *Fabaceae family*, that have been shown to have estrogenic activity in mammals. The major source of isoflavones in the human diet comes from soybeans, in which genistein and daidzein are the predominant components. Isoflavones remain the subject of many scientific studies with accepted methods established by organizations such as AOAC<sup>1</sup> and USP<sup>2</sup>, utilizing traditional reversed-phase  $C_{18}$  columns. Recently, the National Institute of Standards and Technology (NIST) has developed a suite of soy-based candidate Standard Reference Materials (cSRMs)

for the certification of soy isoflavones in foods and dietary supplements. For the analysis of those standards, they have developed a method utilizing a 60-minute gradient on a 5  $\mu$ m cyano column which enables the separation of the soy isoflavones from other components not resolved using the standard  $C_{18}$  methods.<sup>3</sup> Using these conditions, they have demonstrated resolution of the three main soy isoflavones and their glycosides (structures shown in Figure 1), as well as the acetyl and malonyl conjugates of the glycosides. Since the conjugates are somewhat unstable in solution, they have added an additional sample preparation step to hydrolyze these conjugates to their associated glycoside, providing a more accurate, reproducible determination of total isoflavone content. Optimization of this method utilizing the XSelect HSS Cyano **XP** 2.5  $\mu$ m Column provides a significant reduction in analysis time by traditional HPLC. Further optimization of this method to UPLC® with the ACQUITY UPLC HSS Cyano 1.8  $\mu$ m Column provides additional savings in time, while maintaining the resolution for accurate determination of soy isoflavones.

## EXPERIMENTAL

### UPLC Conditions

System:	ACQUITY SQD with PDA detector
Column:	ACQUITY UPLC HSS C <sub>18</sub> , 2.1 x 50 mm, 1.8 µm, part number 186005986
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	0.1% formic acid in acetonitrile
Column Temp.:	30 °C
Gradient:	10% (B) for 0.36 min, 10-30% (B) in 3.6 min, hold at 30% (B) for 0.36 min, re-equilibrate at 10% (B) for 1.8 min between injections
Flow Rate:	0.58 mL/min
Detection:	UV at 260 nm
Injection Volume:	3 µL
Strong Needle Wash:	50/50 acetonitrile/water
Weak Needle Wash:	10/90 acetonitrile/water

These UPLC conditions were scaled directly from the 5 µm HPLC method using the ACQUITY UPLC Columns Calculator. The calculator can be used to scale these conditions back to the HPLC conditions, for both the 5 µm and 2.5 µm materials.

### MS Conditions

MS System:	Waters SQD
Ionization Mode:	ESI positive
Acquisition Range:	Single Ion Recording (SIR)
Capillary Voltage:	3.19 kV
Cone Voltage:	50 V
Desolvation Gas:	600 L/hr
Cone Gas:	0 L/hr
Source Temp.:	100 °C
Desolvation Temp.:	350 °C

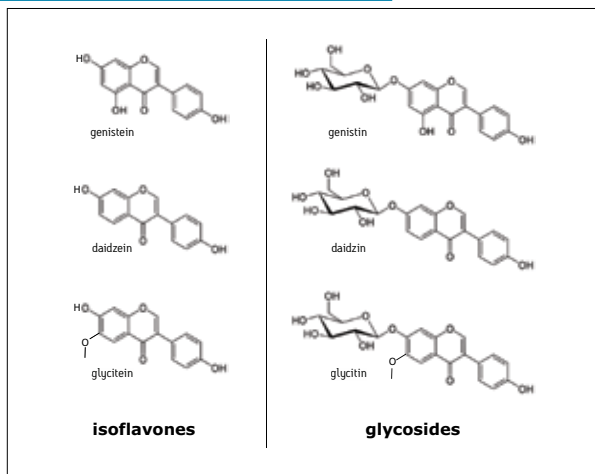


Figure 1. Structures of the three main soy isoflavones (genistein, daidzein, and glycitein) and their corresponding glycosides (genistin, daidzin, and glycitin).

### SAMPLE PREPARATION

**Standard Solution:** Prepared from daidzin (25 ppm), glycitin (25 ppm), genistin (15 ppm), daidzein (25 ppm), glycitein (25 ppm), and genistein (15 ppm) using 10/90 acetonitrile/water diluent.

### Samples:

Candidate Standard Reference Materials were obtained from the National Institute of Standards and Technology. Each sample was weighed into 12 mL centrifuge tubes (Table 1). For each tube, 4 mL of 80/20 methanol/water was added followed by sonication for 1 hour. Tubes were centrifuged for 2 minutes at 3000 rpm. A 2 mL aliquot of supernatant was collected from each tube and filtered using a 0.45 µm GHP syringe filter prior to analysis. The remainder of the sample in each tube was hydrolyzed using 150 µL of 2N sodium hydroxide. After mixing for 10 minutes, the solutions were neutralized with 50 µL of glacial acetic acid. The sample was again centrifuged for 5 minutes at 3000 rpm, with the collected supernatant filtered using a 0.45 µm GHP syringe filter prior to analysis.

Table 1. Samples used for analysis

	Weight
Soy flour (cSRM)	104.2 mg
Soy tablet (cSRM)	111.9 mg
Soy protein isolate (cSRM)	365.6 mg
Soy protein concentrate (cSRM)	973.7 mg
Soy-based infant formula (commercially-available)	1119.2 mg

## RESULTS AND DISCUSSION

Based on the method presented by NIST, we developed a method using the soy standard solution with an XSelect HSS Cyano, 4.6 x 150 mm, 5  $\mu$ m Column, resulting in chromatography with excellent resolution of the soy isoflavones and glycosides. To maximize productivity of the HPLC system, this method was transferred, using the ACQUITY UPLC Columns Calculator, to an XSelect HSS Cyano **XP**, 4.6 x 75 mm, 2.5  $\mu$ m Column. This resulted in an HPLC method with a 76% reduction in run time, relative to the 5  $\mu$ m column. To achieve the greatest benefit, the method was then transferred to UPLC using an ACQUITY UPLC HSS Cyano, 2.1 x 50 mm, 1.8  $\mu$ m Column. Figure 2 shows the chromatography of the isoflavone standards under HPLC and UPLC conditions. These chromatograms demonstrate the ability to scale between different column configurations by maintaining the same ratio of column length to particle size ( $L/d_p$ ), resulting in similar chromatography but with a significant decrease in analysis time (~88% decrease in run time for the UPLC column relative to the 5  $\mu$ m HPLC column).

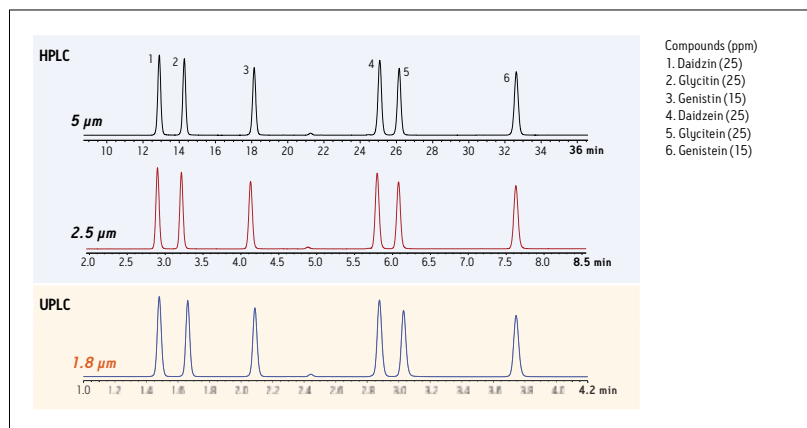


Figure 2. HPLC and UPLC separations (UV) of soy isoflavone standards on cyano columns: XSelect HSS Cyano, 4.6 x 150 mm, 5  $\mu$ m (top); XSelect HSS Cyano XP, 4.6 x 75 mm, 2.5  $\mu$ m (middle); and ACQUITY UPLC HSS Cyano, 2.1 x 50 mm, 1.8  $\mu$ m (bottom). The gradient profiles, flow rates, and injection volumes were scaled for each method using the ACQUITY UPLC Columns Calculator. The flow rates were 1.0 mL/min, 2.0 mL/min, and 0.58 mL/min, respectively.

The UPLC method was used to analyze each of the candidate SRM extracts, both before and after hydrolysis of the glycoside conjugates (Figure 3). The identity of the peaks in the UV chromatograms were assigned based on retention times and  $m/z$  values from the LC/MS analyses (Soy Flour cSRM example shown in Figure 4). The disappearance of the glycoside conjugate peaks after sample treatment with sodium hydroxide confirms the hydrolysis of these conjugates was successful and complete, which allows for simplified determination of the total isoflavone content.

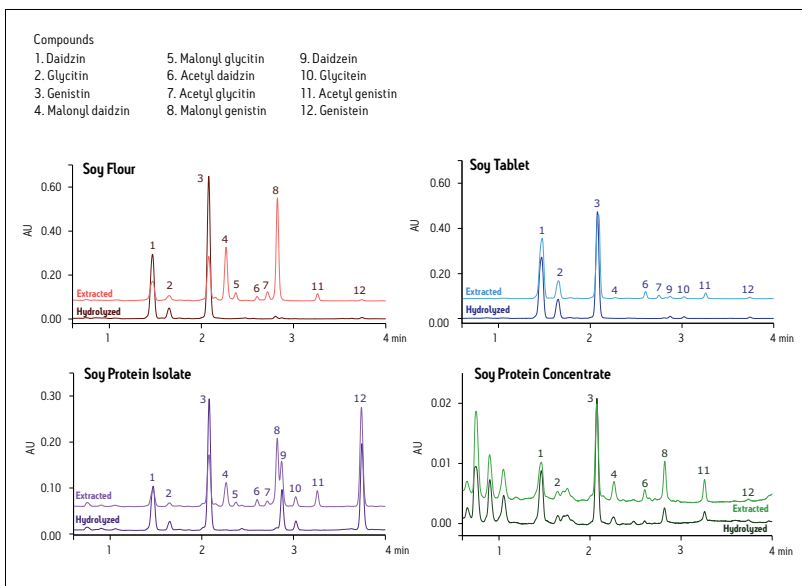


Figure 3. UPLC analysis of extracted (top chromatograms) and hydrolyzed (bottom chromatograms) candidate Standard Reference Materials (cSRMs).

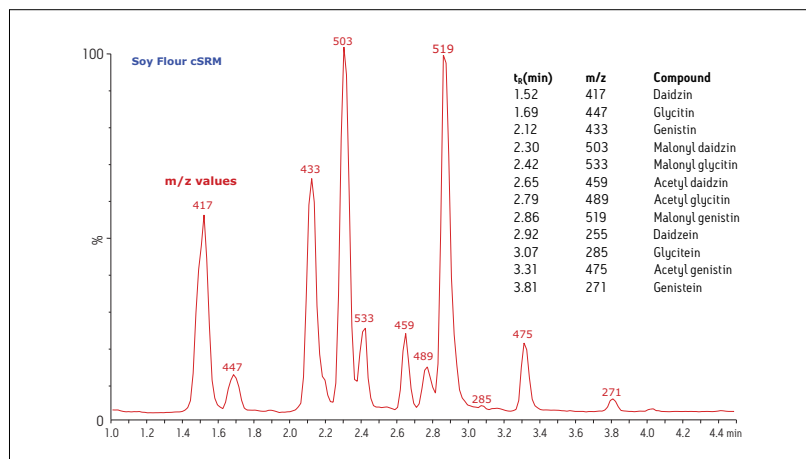


Figure 4. ESI+ LC/MS confirmation of peak identity for the Soy Flour cSRM, using single ion recording (SIR).

## CONCLUSIONS

Analysis of soy isoflavones using the HSS Cyano stationary phase offers complimentary selectivity to traditional C<sub>18</sub> based methods. Hydrolysis of the glycoside conjugates simplifies the resulting chromatography and the determination of total isoflavone content of a sample. The availability of the HSS Cyano phase in 5-, 3.5-, 2.5-, and 1.7- $\mu$ m particle sizes facilitates scaling between different instrument platforms (HPLC and UPLC). Method conditions can be easily scaled for different column configurations using the ACQUITY UPLC Columns Calculator. Scaling methods to utilize the XSelect HSS Cyano **XP** 2.5  $\mu$ m Columns maximizes productivity with existing HPLC instrumentation. Additional method transfer to UPLC conditions offers the greatest savings in time and resources.

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## UPLC PDA

## ACQUITY UPLC for the Rapid Analysis of Anthocyanidins in Berries

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### INTRODUCTION

Anthocyanins are plant pigments that provide vibrant blues and reds in fruits, fruit juices, wine, and vegetables. Konczak et al. has reported that anthocyanins are rich in antioxidant compounds and provide great health benefits to the consumer.<sup>1</sup>

There are reports of more than 600 anthocyanins identified in food samples, including berries, thus making direct analysis of these compounds very challenging. However, the analysis of these compounds can be simplified by converting them into their aglycon form (anthocyanidin) by performing an acid hydrolysis on the sample.

Acid hydrolysis will convert any of the 600+ anthocyanins into one of the six common anthocyanidins: delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin. Estimation of the relative abundance of anthocyanins through the analysis of anthocyanidins then becomes a simpler task.

Previously, methods for analyzing anthocyanidins have been shown to take anywhere from 34 minutes on a SuperPac Pep-S-RP C<sub>18</sub> Column (4 x 250 mm, 5 µm), to 55 minutes on a C<sub>18</sub> Inertsil ODS-3 Column (4.0 x 150 mm, 3 µm).<sup>2,3</sup>

A breakthrough method developed on the Waters® ACQUITY UPLC® System completes this analysis in just 2.1 minutes with an ACQUITY UPLC BEH C<sub>18</sub> Column (2.1 x 50 mm, 1.7 µm) – 16 to 26 times faster than the above-described HPLC methods, respectively – while still maintaining baseline resolution of all six anthocyanidins (Figure 1).

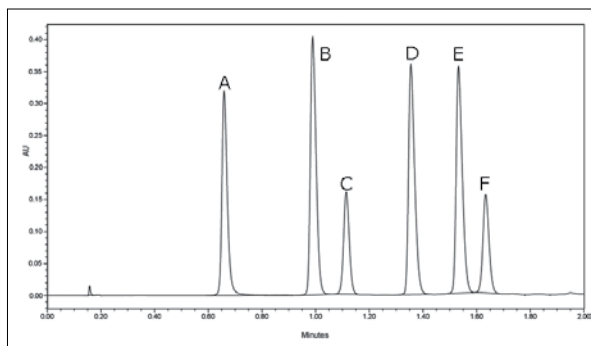


Figure 1. UPLC® separation of the six anthocyanidins, where:

- a. Delphinidin = 0.67 min
- b. Cyanidin = 0.99 min
- c. Petunidin = 1.11 min
- d. Pelargonidin = 1.34 min
- e. Peonidin = 1.53 min
- f. Malvidin = 1.63 min

## EXPERIMENTAL

### Materials

A variety of fresh, frozen, and dried berry samples were purchased from a local store. All samples were then stored in a freezer. Anthocyanidin standards were purchased from ChromaDex (Santa Ana, CA). Hydrochloric Acid Gold, Phosphoric Acid, and Acetonitrile Optima were purchased from Fisher Scientific (Agawam, MA). Water was purified with a MilliQ system (Millipore, Billerica, MA).

### UPLC conditions

The ACQUITY UPLC System consisted of the ACQUITY UPLC Binary Solvent Manager (BSM), the ACQUITY UPLC Sample Manager (SM) fitted with a 10  $\mu$ L loop, and the ACQUITY UPLC Tunable UV (TUV) Detector. The system was controlled and data was collected and analyzed using Empower® 2 Software.

Separations were performed on a 2.1 x 50 mm, 1.7  $\mu$ m ACQUITY UPLC BEH C<sub>18</sub> Column at a flow rate of 1.00 mL/minute. Column temperature was set at 40 °C and injection volumes for all samples and standards were 2  $\mu$ L.

Water/Acetonitrile (95:5) was used as the weak wash solvent and a mixture of Acetonitrile/Isopropyl Alcohol/Water (7:2:1) was used as the strong wash solvent. Mobile phase components and gradient conditions are outlined in Table 1.

Detection was set at 525 nm, which is common to all anthocyanidins, using a sampling rate of 20 points per second and a filtering constant of 0.1 seconds.

Time (min)	%A	%B	Curve
Initial	90	10	—
2	80	20	6
2.1	90	10	6

Table 1. Gradient separation conditions, where Solvent A = 0.3% phosphoric acid in water, and Solvent B = 100% acetonitrile.

### Calibration

A standard solution of cyanidin, petunidin, pelargonidin, and peonidin (0.100 mg/mL) was prepared from crystalline material in methanol. A seven-point standard curve was created from 0.100 mg/mL to 0.001 mg/mL.

A second standard solution was created for delphinidin from a pre-dissolved purchased sample, as the crystalline material was not available. The concentrations of delphinidin were the same as those of the previously mentioned standard solution.

A third standard solution for malvidin (0.300 mg/mL) was prepared from crystalline material in methanol. A five-point standard curve was created covering a range of 0.300 mg/mL to 0.001 mg/mL. Calibration curves were created for each standard and fit to linear equations with R<sup>2</sup> values shown in Table 2.

A UPLC chromatogram of all six anthocyanidin standards is shown in Figure 1.

Standard	R <sup>2</sup> value
Delphinidin	0.9988
Cyanidin	0.9992
Petunidin	0.9992
Pelargonidin	0.9992
Peonidin	0.9992
Malvidin	0.9980

Table 2. R<sup>2</sup> values for the standard calibration curves.

### Sample preparation extraction procedure

Anthocyanidins were extracted from the samples by weighing out 25 0.3 g of berries into a weighing boat. The berries were then transferred into a 100 mL graduated cylinder and 30 mL of extraction solution (80/20 acetonitrile/0.3% phosphoric acid in water) was added. The berries were then homogenized using a Janke & Kunkel homogenizer for 1.5 minutes. This duration ensured that all 25 grams of sample would be completely homogenized. The homogenized liquid was then transferred into a graduated centrifuge tube. The graduated centrifuge tube was centrifuged for 10 minutes at 2,500 rpm.



## Hydrolysis procedure

A 2 mL aliquot of the supernatant was pipetted into a 4 mL vial along with 200  $\mu\text{L}$  of concentrated hydrochloric acid. The vial was then capped with a cap that contained a self-sealing septum to minimize loss of liquid to evaporation during hydrolysis. The vial was then placed on a vortex mixer for 5 seconds, and then placed into a chemical oven at  $150 \pm 2^\circ\text{C}$  for 30 minutes.

After 30 minutes, the vial was removed and placed into a freezer for 10 minutes at  $0^\circ\text{C}$  to stop the hydrolysis. Samples were removed from the freezer and allowed to come to room temperature. Once the vial reached room temperature, the berry sample was filtered through a  $0.45\ \mu\text{m}$  filter into a 2 mL vial. The vial was capped and then analyzed by the ACQUITY UPLC System.

## RESULTS AND DISCUSSION

Area counts from injections of hydrolyzed berry extracts, Figures 3 to 8, were compared to the standard calibration curves, and the amounts (mg/mL) of each component were calculated using Empower 2 Software. These amounts were then used to backcalculate to the original concentration of anthocyanidins in the berries as dry weight, Tables 3 and 4. Dry weight was determined by taking a representative sample of each berry ( $\sim 15\ \text{g}$ ), accurately weighing it, and then placing it into a chemical oven at  $40^\circ\text{C}$  overnight to remove the water content. The change in weight was considered to be the water content of the berry.

The results for this experiment were consistent with those of Wu et al.<sup>4</sup> As shown in Figure 2, low-bush blueberries contained the highest total concentration ( $12.84 \pm 0.59\ \text{mg/g}$ ) of anthocyanidins per gram of dry berry, while purchased dried blueberries contained the lowest ( $0.26 \pm 0.06\ \text{mg/g}$ ).

Tables 3 and 4 outline the concentration of each anthocyanidin and the total anthocyanidin concentration for each berry sample tested. Each berry sample was tested in triplicate. The results reported in Table 3 and 4 are the mean of the three trials with standard deviations.

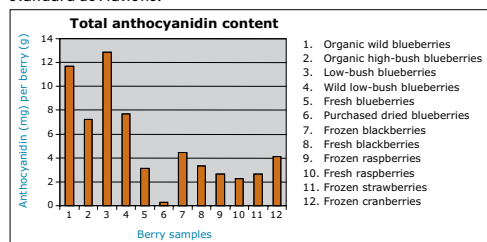


Figure 2. Comparison of total anthocyanidin content in berry samples.

Blueberries contain the highest concentration of anthocyanidin, as can be seen in Figures 3 to 8. Blueberries contain either five or all six anthocyanidins, while other berries contain anywhere from one to four. Notably, each berry contained one of the six anthocyanidins in a much higher concentration than the rest of the berries. In the case of blueberries, malvidin is present at almost twice the concentration of any other anthocyanidin. In addition, blueberries were the only berry to contain malvidin.

Fresh blackberries contained primarily cyanidin, while frozen blackberries contained both cyanidin and a slight trace of pelargonidin. Cyanidin in blackberries was two to three times higher than in any other berry.

Strawberries gave a distinct pelargonidin peak, while other berries only contained trace amounts of it.

The concentration of peonidin in cranberries was three to four times higher than in blueberries, while most other berries contained no peonidin at all.

Cyanidin was the major anthocyanidin in raspberries, but was contained at concentrations approximately half of those found in blackberries.

Even though these differences gave each berry a unique chromatogram, cyanidin was one common anthocyanidin present in each berry sample.

Each berry tested contained one of the six anthocyanidins at a higher concentration than the rest of the berries. However, blueberries had the highest total anthocyanidin content compared to the other berries. Among the blueberries tested, low-bush blueberries contained the highest concentrations of total anthocyanidins per gram of dry berry. Following blueberries, blackberries contain the highest concentrations, followed by cranberries, raspberries, and strawberries, respectively.

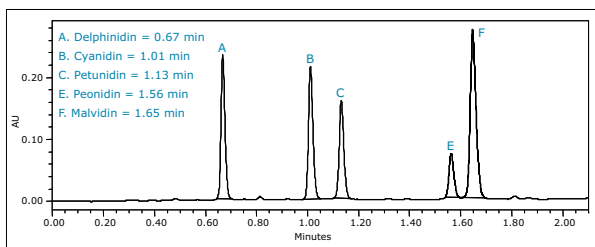


Figure 3. UPLC separation of low-bush blueberries.

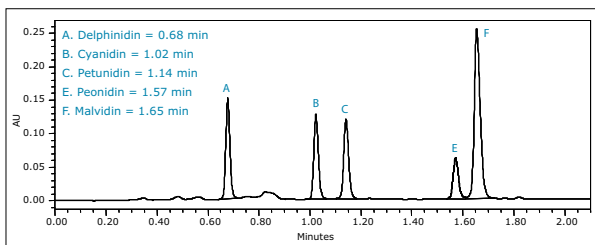


Figure 4. UPLC separation of wild low-bush blueberries.

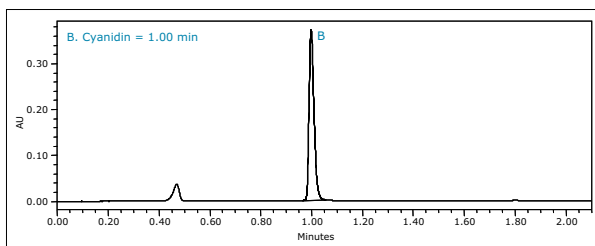


Figure 5. UPLC separation of frozen blackberries.

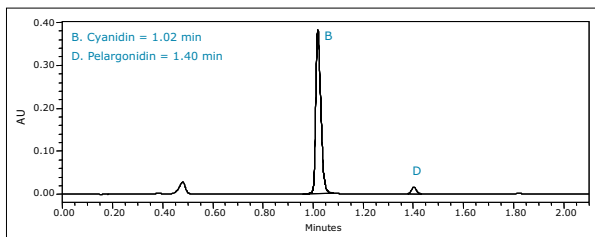


Figure 6. UPLC separation of frozen raspberries.

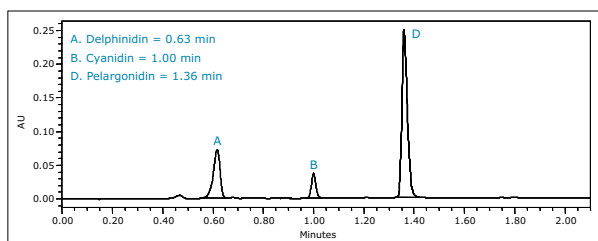


Figure 7. UPLC separation of frozen strawberries.

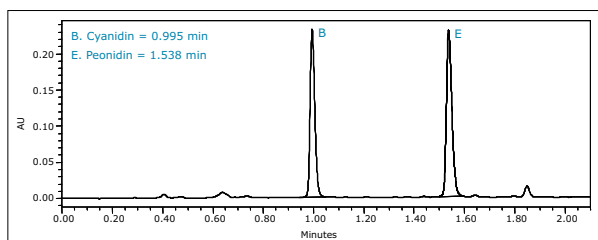


Figure 8. UPLC separation of frozen cranberries.

Anthocyanidin	Organic wild blueberries	Organic high-bush blueberries	Low-bush blueberries	Wild low-bush blueberries	Fresh blueberries	Purchased dried blueberries
Delphinidin	0.59 ± 0.09	0.22 ± 0.07	0.89 ± 0.13	0.34 ± 0.02	0.09 ± 0.01	–
Cyanidin	1.59 ± 0.09	0.25 ± 0.02	1.75 ± 0.06	0.82 ± 0.08	0.14 ± 0.00	0.01 ± 0.00
Petunidin	3.14 ± 0.33	1.52 ± 0.20	3.91 ± 0.25	2.03 ± 0.21	0.79 ± 0.05	0.04 ± 0.01
Pelargonidin	0.03 ± 0.00	–	0.03 ± 0.00	0.03 ± 0.00	–	–
Peonidin	0.67 ± 0.02	0.14 ± 0.02	0.61 ± 0.02	0.47 ± 0.09	0.09 ± 0.00	0.01 ± 0.00
Malvidin	5.63 ± 0.57	5.07 ± 0.11	5.66 ± 0.22	4.01 ± 0.68	2.00 ± 0.08	0.21 ± 0.05
Total (mg/g)	11.66 ± 1.09	7.21 ± 0.33	12.84 ± 0.59	7.70 ± 1.05	3.11 ± 0.09	0.26 ± 0.06

Table 3. Quantitative comparison of blueberry samples.

Anthocyanidin	Frozen blackberry	Fresh blackberry	Frozen raspberry	Fresh raspberry	Frozen strawberry	Frozen cranberry
Delphinidin	–	–	–	–	0.27 ± 0.04	–
Cyanidin	4.18 ± 0.26	3.14 ± 0.43	2.40 ± 0.14	2.03 ± 0.12	0.25 ± 0.05	1.73 ± 0.30
Petunidin	–	–	–	–	0.03 ± 0.04	0.04 ± 0.00
Pelargonidin	0.02 ± 0.02	–	0.12 ± 0.02	0.10 ± 0.02	1.96 ± 0.62	0.04 ± 0.00
Peonidin	–	–	0.03 ± 0.00	–	–	2.13 ± 0.43
Malvidin	–	–	–	–	–	–
Total (mg/g)	4.20 ± 0.23	3.14 ± 0.43	2.54 ± 0.15	2.13 ± 0.13	2.51 ± 0.68	3.94 ± 0.73

Table 4. Quantitative comparison of other berry samples.

## CONCLUSIONS

The ACQUITY UPLC System combined with ACQUITY UPLC BEH Column technology provides rapid analysis of anthocyanidins and baseline resolution of each of the six standard anthocyanidin components. The ability to dramatically decrease analysis times while maintaining resolution creates more efficient and productive laboratories. UPLC Technology, when applied to the burgeoning field of natural products research, opens possibilities for more rapid and extensive analysis of natural compounds than ever before.

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UPC<sup>2</sup>/PDA

## Analysis of Fat-Soluble Vitamin Capsules using UltraPerformance Convergence Chromatography (UPC<sup>2</sup>)

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### APPLICATION BENEFITS

- Fast analysis of a wide range of fat-soluble vitamin formulations.
- Waters® ACQUITY UPC<sup>2</sup>™ System was able to successfully analyze six different formulations of fat-soluble vitamins. A single technique was able to quickly analyze these different formulations.
- This system can greatly streamline fat-soluble vitamin analysis by allowing labs to use a single technique on a single system to analyze a wide range of FSV formulations.
- Each of the fat-soluble vitamin formulations were analyzed rapidly, and components of interest resolved well from excipient materials.
- Isomers of vitamins A, E, and K1 were successfully resolved from each other.

### WATERS SOLUTIONS

ACQUITY UPC<sup>2</sup> System

ACQUITY UPC<sup>2</sup> Column Kit

Empower™ 3 Software

ACQUITY UPC<sup>2</sup> PDA Detector

### KEY WORDS

UPC<sup>2</sup>, fat-soluble vitamins, vitamin E, vitamin A, vitamin D3, vitamin K1, vitamin K2

### INTRODUCTION

The analysis of fat-soluble vitamins (FSV) formulations, often from oil-filled and powder-filled capsules, or pressed tablets, can be a challenging task. Most often, analysis of these formulations employs a normal phase chromatographic method using traditional normal phase solvents (hexane, tertiary butyl alcohol, ethyl acetate, dichloromethane, and others) that can be expensive to procure and dispose. Other analytical chromatographic techniques for these analyses include reversed phase liquid chromatography, gas chromatography, thin layer chromatography, and colorimetric techniques. The use of UltraPerformance Convergence Chromatography™ (UPC<sup>2</sup>™) in fat-soluble vitamin analysis provides a single viable technique that is cost-effective, sustainable, and a green technology alternative that lowers the use of organic solvents, provides fast analysis times, and maintains chromatographic data quality. A series of FSV formulations were analyzed using the ACQUITY UPC<sup>2</sup> System. The examined formulations contained vitamin A only, vitamins A + D3, vitamin E, vitamin D3 only, vitamin K1 only, and vitamin K2 only, as shown in Table 1. Results from these experiments show that UPC<sup>2</sup> has the potential to replace many of the separation methods in use today as the sole technique with no compromises.

Active ingredient(s)	Amount per capsule/tablet	Inactive ingredients
Vitamin A	10,000 IU A	Soy oil, gelatin, glycerin, water
Vitamin A & D3	10,000 IU A 2000 IU D3	Soy oil, gelatin, glycerin, water
Vitamin D3	2000 IU D3	Sunflower oil, gelatin, glycerin, water
Vitamin E	400 IU E	Soy oil, gelatin, glycerin, water, FD&C yellow #6 lake, FD&C blue #1 lake, titanium dioxide
Vitamin K1	100 µg	Cellulose, CaHPO <sub>4</sub> , stearic acid, Mg stearate, croscarmellose sodium
Vitamin K2	50 µg	Cellulose, Mg stearate, silica

Table 1. Fat-soluble vitamin formulations.

## EXPERIMENTAL

System:	ACQUITY UPC <sup>2</sup> consisting of ACQUITY UPC <sup>2</sup> Binary Solvent Manager, Sample Manager, Convergence Manager, Column Manager, and PDA Detector
Columns:	ACQUITY UPC <sup>2</sup> BEH, 3.0 x 100 mm, 1.7 $\mu$ m ACQUITY UPC <sup>2</sup> HSS C <sub>18</sub> SB, 3.0 x 100 mm, 1.8 $\mu$ m ACQUITY UPC <sup>2</sup> HSS C <sub>18</sub> SB, 2.1 x 150 mm, 1.8 $\mu$ m
Data system:	Empower 3 Software
Separation methods:	Details of each separation method are included in the individual results and discussion sections of this application note

## Sample Preparation

Oil-filled capsules (vitamins A, A + D3, D3) – contents of four individual capsules were removed and dissolved in 10 mL of iso-octane. No further pre-treatment was used. The contents of one individual vitamin E capsule was removed and dissolved in 10 mL of iso-octane. No further pre-treatment was used.

Eight crushed tablets of vitamin K1 were sonicated with iso-octane for 30 minutes. Following settling, an aliquot of the extract was filtered directly into a sample vial through a 1.0- $\mu$ m glass fiber filter.

Contents of eight powder-filled vitamin K2 capsules were removed and sonicated for 30 minutes with iso-octane. Following settling, an aliquot of the extract was filtered directly into a sample vial through a 1.0- $\mu$ m glass fiber filter.

## RESULTS AND DISCUSSION

## Vitamin A

This formulation of vitamin A was labeled as derived from fish liver oil and contained soy oil, gelatin, glycerin, and water as inactive ingredients. Two primary forms of vitamin A palmitate (*cis* and *trans* isomers, 1.325 and 1.394 minutes, respectively) were noted and resolved well from the small excipient peaks, as shown in Figure 1, which elute in the range of 2.0 to 2.5 minutes. This separation was accomplished using a gradient of carbon dioxide and methanol (containing 0.2% formic acid) 97:3 to 90:10 over 3 minutes with an Active Back Pressure Regulator (ABPR) setting of 2176 psi. Further details are contained in Table 2. Using this separation method, vitamin A acetate, palmitate, and retinol were easily resolved, as seen in Figure 2.

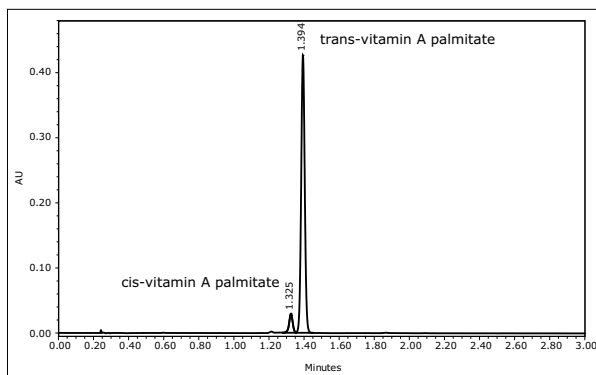


Figure 1. UPC<sup>2</sup> separation of the components of a vitamin A capsule.

Column	ACQUITY UPC <sup>2</sup> HSS C <sub>18</sub> SB, 3.0 x 100 mm, 1.8 μm
Flow rate	2.0 mL/min
Gradient	97:3 to 90:10 over 3 minutes
Mobile phase A/B	CO <sub>2</sub> and methanol containing 0.2% formic acid
Detection	UV at 320 nm, compensated (500 to 600 nm)
Injection volume	1 μL
ABPR pressure	2176 psi
Column temp.	50 °C

Table 2. Separation method details of vitamin A.

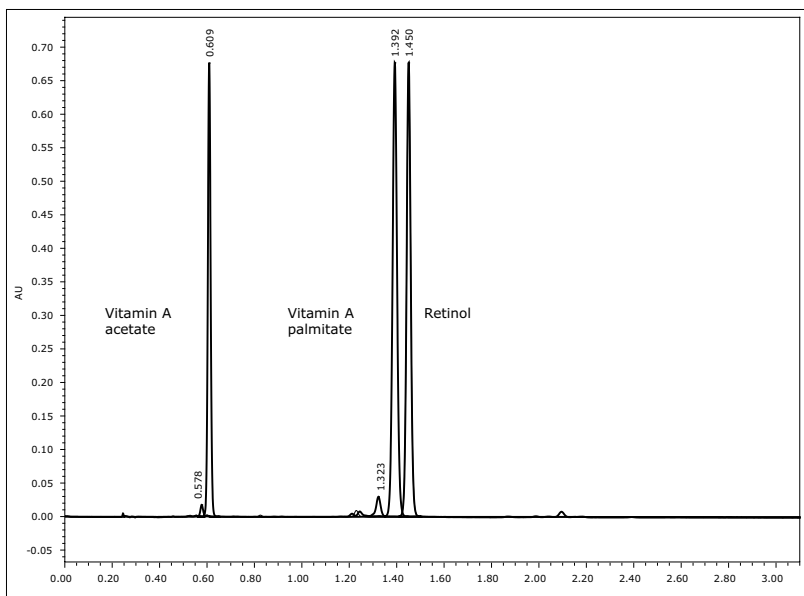


Figure 2. Separation of vitamin A acetate, vitamin A palmitate, and retinol.

## Vitamin A + D3

Similar to the previous example, this formulation of vitamins A + D was also labeled as derived from fish liver oil and contained soy oil, gelatin, glycerin, and water as inactive ingredients. Again, two forms of vitamin A palmitate (*cis* and *trans* isomers, 2.626 and 2.851 minutes, respectively) were noted before the bulk of excipient peaks. To fully resolve vitamin D3 (cholecalciferol, 6.862 minutes) from the major excipient materials and a number of other compounds contained in the formulation, shown in Figure 3, it was necessary to use a longer column that provided enough separation efficiency to accomplish this goal. The system provided enough sensitivity to easily detect the vitamin D3 peak, as shown in Figure 3 inset.

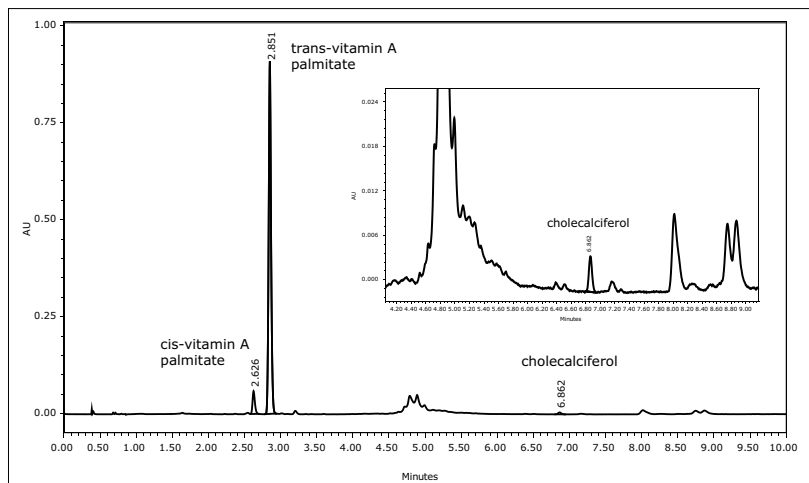


Figure 3. UPC<sup>2</sup> separation of the components of a vitamin A + D3 capsule.



This separation was accomplished using a gradient of carbon dioxide and methanol (containing 0.2% formic acid), 99:1 to 90:10 over 10 minutes. Further details are outlined in Table 3.

Column	ACQUITY UPC <sup>2</sup> HSS C <sub>18</sub> SB, 2.1 x 150 mm, 1.8 μm
Flow rate	1.0 mL/min
Gradient	99:1 to 90:10 over 10 minutes
Mobile phase A/B	CO <sub>2</sub> and methanol containing 0.2% formic acid
Detection	UV at 263 nm, compensated (500 to 600 nm)
Injection volume	1 μL
ABPR pressure	2176 psi
Column temp.	50 °C

Table 3. Separation method details of vitamin A + D3 and D3 only.

Vitamin D3

Using identical separation conditions as those used for vitamins A + D3, as shown in Table 3, vitamin D3 (cholecalciferol, 6.867 minutes) was easily resolved from the capsule excipient material, which was labeled as primarily sunflower oil, shown in Figure 4 and Table 3.

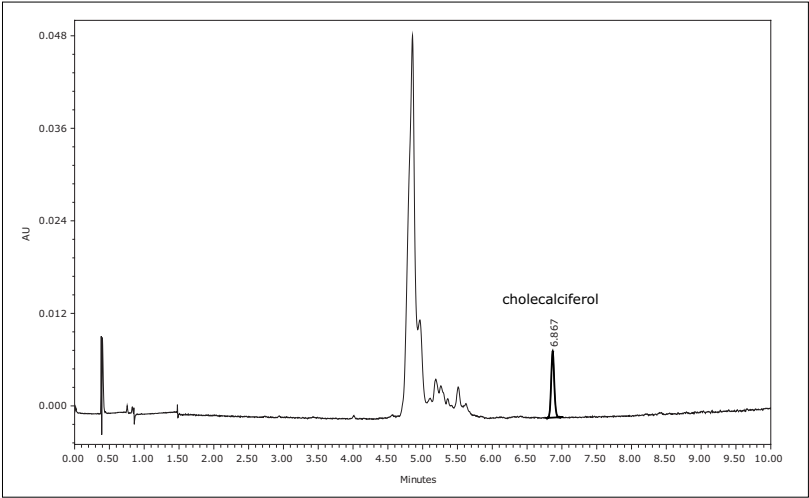


Figure 4. UPC<sup>2</sup> separation of the components of a vitamin D3 capsule..

## Vitamin E

A very rapid gradient analysis (~ 90 second run time) that easily provided baseline resolution of the four tocopherol isomers (d-alpha, d-beta, d-gamma, d-delta) was developed for the vitamin E capsule, shown in Figure 5. This separation was accomplished using a gradient of carbon dioxide and methanol, 98:2 to 95:5 over 1.5 minutes. Further details are shown in Table 4.

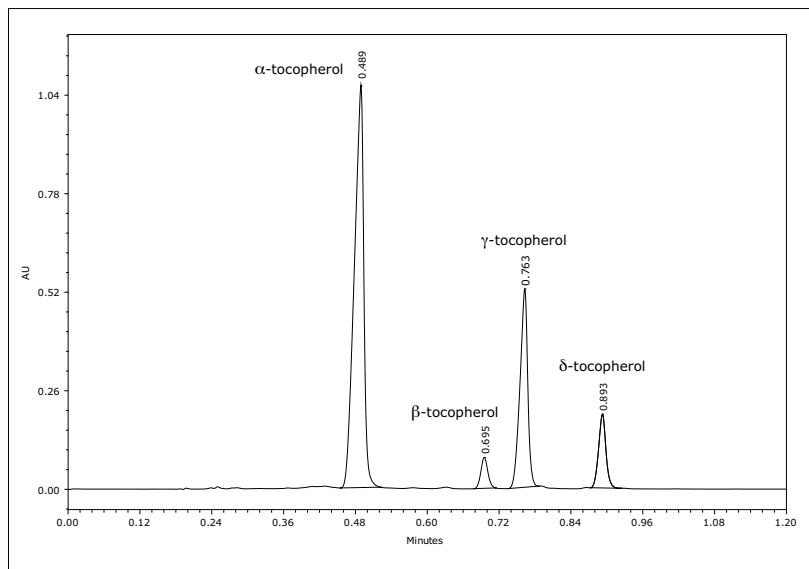


Figure 5. UPC<sup>2</sup> separation of the components of a vitamin E capsule.

Column	ACQUITY UPC <sup>2</sup> BEH, 3.0 x 100 mm, 1.7 μm
Flow rate	2.5 mL/min
Detection	UV at 293 nm, compensated (500 to 600 nm)
Gradient	98:2 to 95:5 over 1.5 minutes
Mobile phase A/B	CO <sub>2</sub> and methanol
Injection volume	1 μL
ABPR pressure	1885 psi
Column temp.	50 °C

Table 4. Separation method details of vitamin E.

Vitamin K1

The vitamin K1 tablets generated two fully resolved ( $R_s > 2.0$ ), distinct peaks with a simple isocratic method consisting of 99% CO<sub>2</sub> and 1% methanol/acetonitrile 1:1, shown in Figure 6. UV spectra (collected simultaneously as the UV at 246 nm channel) of both peaks were similar, indicating that the peaks were related, as displayed in Figure 7. Although not confirmed (individual standards of each of the isomers were not available at time of analysis), it is likely that the two peaks are stereoisomers of phylloquinone (vitamin K1). Further details are shown in Table 5.

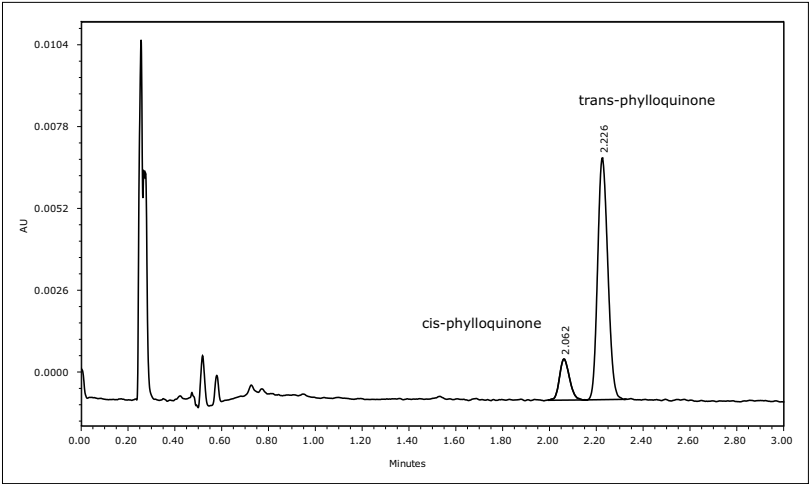


Figure 6. UPC<sup>2</sup> separation of the components of a vitamin K1 tablet.

Column	ACQUITY UPC <sup>2</sup> HSS C <sub>18</sub> SB, 2.1 x 150 mm, 1.8 μm
Flow rate	1.5 mL/min
Isocratic	99% A and 1% B
Mobile phase A/B	CO <sub>2</sub> and methanol/acetonitrile 1:1
Detection	UV at 248 nm, compensated (300 to 400 nm)
Injection volume	2 μL
ABPR pressure	1885 psi
Column temp.	50 °C

Table 5. Separation method details of vitamin K1.

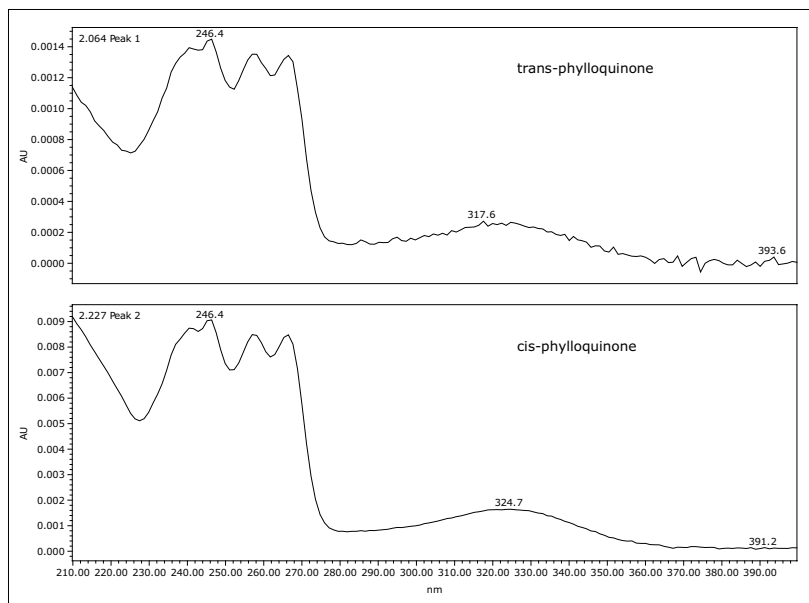


Figure 7. UV spectra of vitamin K peaks observed at 2.064 and 2.227 minutes.

Vitamin K2

Vitamin K2 consists of menaquinone (MK) forms MK-3 through MK-14. The various forms of vitamin K2 have side chain lengths comprised of a variable number of unsaturated isoprenoid units. This tablet formulation showed one predominant peak and several smaller ones, as seen in Figure 8, using an isocratic separation of 95:5 CO<sub>2</sub> /methanol, and was identified as MK-7 (data not shown). This result is consistent with the capsule label claim, which indicated that this formulation should have contained predominantly MK-7. Further method details are shown in Table 6.

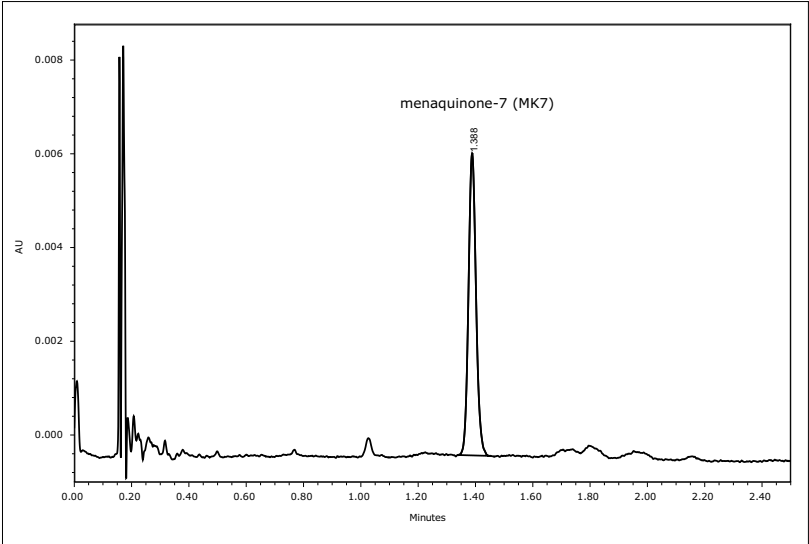


Figure 8. UPC<sup>2</sup> separation of the components of a vitamin K2 capsule.

Column	ACQUITY UPC <sup>2</sup> HSS C <sub>18</sub> SB, 3.0 x 100 mm, 1.8 μm
Flow rate	3.0 mL/min
Isocratic	95% A and 5% B
Mobile phase A/B	CO <sub>2</sub> and methanol
Detection	UV at 248 nm, compensated (500 to 600 nm)
Injection volume	1 μL
ABPR pressure	1885 psi
Column temp.	50 °C

Table 6. Separation method details of vitamin K2.

## CONCLUSIONS

- Waters' ACQUITY UPC<sup>2</sup> System was able to successfully analyze six different formulations of fat-soluble vitamins.
- Each of the FSV formulations were analyzed rapidly with components of interest resolved from excipient materials.
- Isomers of vitamins A, E, and K1 were successfully resolved from each other.
- This system can greatly streamline FSV analysis by enabling laboratories to use a single technique on a single system to analyze a wide range of FSV formulations.

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## UPLC/TQS

## Dramatic Improvements in Assay Reproducibility for Water-Soluble Vitamins Using ACQUITY UPLC and the Ultra-Sensitive Xevo TQ-S Mass Spectrometer

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### APPLICATION BENEFITS

- Quantification of seven water-soluble vitamins in a single method using UPLC®/MS/MS
- Improvement in precision to obtain RSDs below 3% for all vitamins

### WATERS SOLUTIONS

ACQUITY UPLC® System

Xevo® TQ-S Mass Spectrometer

ACQUITY UPLC HSS T3 column

MassLynx® Software

### KEY WORDS

Water-soluble vitamins, infant formula, Xevo TQ-S

### INTRODUCTION

Fortification of infant formula and adult nutritionals with vitamins that are essential for health and well being is widely accepted as necessary to address the nutritional needs of those who consume these products. Much research has been conducted to ensure the delivery of the appropriate level of these vitamins from both a health benefit and safety perspective.<sup>1</sup>

In order to ensure that the appropriate levels of vitamins are available throughout the shelf life of a product, manufacturers must take into account any degradation over time of the vitamins and make up for this with an increase in the initial amount of the fortified vitamin. With both maximum and minimum levels required for these vitamins, a delicate balance must be reached between overages and degradation. Precise and accurate measurements of vitamin concentrations becomes critical. When measurements from different laboratories are added to an already complicated analytical challenge, the task can appear insurmountable. Variation must be reduced to ensure intra- and inter-lab reproducibility can meet the analytical requirements.

LC/MS/MS technology has begun to be more widely accepted for the quantitative analysis of fortified vitamins in food products.<sup>2-5</sup> The advantages in selectivity and sensitivity, along with the ability to analyze multiple analytes in a single injection make this technology highly suitable for this application. Recent advances in LC/MS/MS have further decreased the limits of detection that can be attained. In this work, however, the latest advances in LC/MS/MS technology have been used to specifically attain much lower RSDs than have been previously attainable with this type of multi-analyte method. This high level of reproducibility is required in order to address label claim disputes, minimize overage amounts, and maintain profitability while ensuring the health and safety of all consumers of these products.

## EXPERIMENTAL

## UPLC conditions

System:	ACQUITY UPLC
Column:	ACQUITY HSS T3 C <sub>18</sub> 1.0 X 100 mm, 1.8 µm
Column temp.:	60 °C
Injection volume:	10 µL
Flow rate:	0.15 mL/min
Mobile phase A:	Water + 0.05% HCOOH and 0.01% HFBA
Mobile phase B:	Methanol with 10 mM NH <sub>4</sub> OH
Strong wash:	Methanol
Weak wash:	Water

## MS conditions

Mass spectrometer:	Xevo TQ-S
Ionization mode:	ESI +
Capillary voltage:	2.5 kV
Desolvation temp.:	500 °C
Desolvation gas flow:	750 L/h
Source temp.:	150 °C
Cone gas:	300 L/h

## MRM transitions

The MRM transitions, cone voltage, and collision energy selected for each of the water-soluble vitamins and their internal standards are shown in Table 2, along with the expected compound retention time.

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.15	99	1	6
0.4	0.15	99	1	6
6.0	0.15	40	60	6
6.5	0.15	1	99	2
7.5	0.15	1	99	6
7.6	0.15	99	1	6
9.0	0.15	99	1	6

Table 1. UPLC method for water-soluble vitamins analyses.

Compound name	MRM Transition	Rt	Cone (V)	Collision (V)
Niacinamide	123.0 > 80.0	1.32	30	35
<sup>2</sup> H <sub>4</sub> -Niacinamide	127.0 > 84.0	1.30	30	35
Nicotinic acid	123.9 > 80.0	1.22	30	25
<sup>2</sup> H <sub>4</sub> -Nicotinic acid	128.0 > 84.1	1.22	30	25
Pantothenic Acid	220.1 > 90.1	2.58	12	25
<sup>13</sup> C <sub>3</sub> , <sup>15</sup> N-Pantothenic acid	224.3 > 93.9	2.58	12	25
Thiamine	265.3 > 122.0	2.53	24	40
<sup>13</sup> C <sub>3</sub> -Thiamine	268.3 > 122.0	2.53	24	40
Pyridoxine	170.2 > 151.7	2.32	30	25
<sup>13</sup> C <sub>4</sub> -Pyridoxine	174.2 > 155.7	2.32	30	25
Biotin	245.3 > 97.0	3.82	22	24
<sup>13</sup> C <sub>5</sub> -Biotin	250.3 > 232.0	3.82	22	24
Riboflavin	377.2 > 242.8	4.29	40	30
<sup>13</sup> C <sub>4</sub> , <sup>15</sup> N <sub>2</sub> -Riboflavin	383.2 > 248.6	4.29	40	30
Folic acid	442.3 > 295.0	3.64	20	25
<sup>13</sup> C <sub>5</sub> -Folic acid	447.3 > 295.0	3.64	20	25

Table 2. MRM transitions, retention times, and tuning parameters for the water-soluble vitamins and their internal standards.

## Standard preparation

Seven working standards containing a mix of all the analyzed vitamins and isotopically labeled standards were prepared in 1% ascorbic acid solution, then pH adjusted with ammonium hydroxide.



Sample preparation

Precisely weighed amounts of sample were made up according to the (proprietary) standard operating procedure (SOP) for the method. The amount depended upon the specific product to be analyzed. Products included both ready-to-feed and powdered formulations. Isotopically labeled standards for each of the vitamins were added. Following thorough mixing of the samples, 25 mL of 1% ascorbic acid was added to the samples. Following another thorough mixing, 80 µL of 30% ammonium hydroxide was added. The samples were mixed again and allowed stand for 10 minutes. An aliquot of the supernatant from the settled samples was filtered through 0.45 µm PTFE directly into autosampler vials.

The SOP for the analysis resulted in working standards and samples that were far too concentrated for analysis on the Xevo TQ-S. In order to meet the SOP, tune parameters were optimized to bring the response into the linear range of the instrument.

RESULTS AND DISCUSSION

Example MRM chromatograms of each of the vitamins in the NIST SRM 1849a are shown in Figure 1. The calculated levels in SRM 1849a for each of the vitamins is given in Table 3, along with the NIST reported levels and expected range. As can be seen in Table 3, there was good agreement with the published values and the precision (RSD) was excellent.

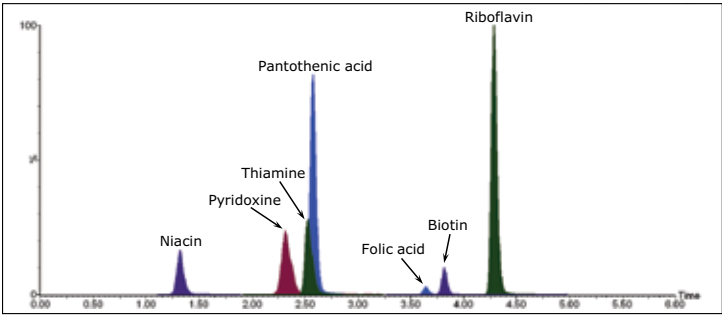


Figure 1. MRM chromatograms for each of the vitamins for the analysis of the NIST SRM 1849a.

	NIST SRM 1849a Amount ± range		Mean	RSD	Accuracy	n
Biotin (µg/kg)	1990.0	± 130.0	2140.0	3.0%	108%	11
Folic acid (µg/kg)	2290.0	± 60.0	2320.0	2.2%	101%	19
Niacin (mg/kg)	109.0	± 10.0	109.0	1.9%	100%	19
Pantothenic acid (mg/kg)	68.2	± 1.9	69.8	2.0%	102%	19
Pyridoxine (mg/kg)	13.5	± 0.9	13.7	1.9%	101%	19
Riboflavin (mg/kg)	20.4	± 0.5	20.7	2.8%	101%	19
Thiamine (mg/kg)	12.6	± 1.0	13.2	2.3%	105%	19

Table 3. Expected amount and acceptable range for the NIST SRM 1849a along with the calculated mean values, RSD, and accuracy for 19 separate analyses over an eight-month period. The first eight preparations used a different internal standard RSD for biotin, therefore only analyses with the final internal standard for biotin were included (n=11).

Examples of MRM chromatograms for the lowest level standard of each vitamin along with their calibration curves are shown in Figure 2. Each of the seven calibration curves showed  $r^2$  values  $<0.999$ . Intra-day variability on four separate days is shown in Table 4 and ranged between 0% and 2.8%. The concentration of the lowest level working standard for each vitamin is shown in Table 5.

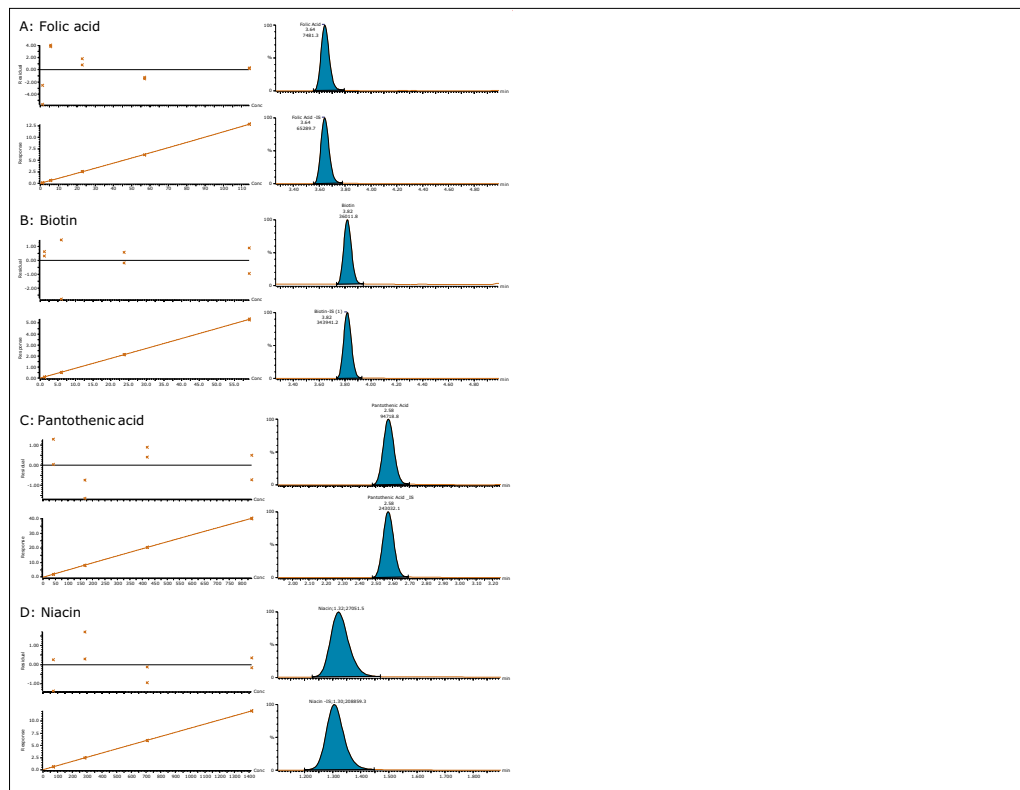


Figure 2. Calibration curves and residuals plots along with the MRM chromatograms of the lowest level standard and internal standard of each vitamin. Concentrations of the lowest level standard are listed below.

A: Folic acid, 1.14 ng/mL

B: Biotin, 1.18 ng/mL

C: Pantothenic acid, 8.40 ng/mL

D: Niacin, 14.17 ng/mL

E: Pyridoxine, 1.78 ng/mL

F: Riboflavin, 1.40 ng/mL

G: Thiamine, 2.10 ng/mL

	Day 1 (n=4)	Day 2 (n=4)	Day 3 (n=4)	Day 4 (n=8)
Biotin (µg/kg)	1.1	1.4	2.7	0.7
Folic acid (µg/kg)	1.8	2.1	2.8	1.6
Niacin (mg/kg)	0.7	1.3	1.9	0.5
Pantothenic acid (mg/kg)	0.9	1.8	2.1	0.4
Pyridoxine (mg/kg)	0.8	1.7	1.7	0.4
Riboflavin (mg/kg)	1.7	1.4	2.3	1.2
Thiamine (mg/kg)	0.0	2.0	1.2	1.3

Table 4. Intra-day precision for four separate days. Values are the percentage relative standard deviation (% RSD) for the NIST SRM 1849a. For days 1 through 3, n=4; for day 4, n=8.

Vitamin	Concentration of lowest level standard (ng/mL)
Biotin	1.18
Folic acid	1.14
Niacin	14.17
Pantothenic acid	8.40
Pyridoxine	1.78
Riboflavin	1.40
Thiamine	2.01

Table 5. Concentration levels for the lowest level working standard.

Reports of the intra-day and inter-day variability from the analysis of vitamins in fortified products are available in the literature. Goldschmidt and Wolf<sup>2</sup> published a method using HPLC with MS detection with RSDs below 3% for niacinamide, pyridoxine, and pantothenic acid. For riboflavin and biotin, the RSDs were approximately 5% but for thiamine and folic acid the RSDs were typically reported to be above 5%. Huang *et al.*<sup>3</sup> found reproducibility for eight replicates to be below 5% for a commercial infant formula. Zhang *et al.*<sup>4</sup> reported intra-day variability ranging from 1.17% to 7.81% for 14 vitamins and vitamin-like compounds. The inter-day variability was reported to range between 2.61% and 8.42%. As can be seen in Table 4, the intra-day variability for this method was vastly improved compared to the literature for each of the compounds analyzed. To assess the reproducibility of the method, 19 independent preparations on 19 different days over an eight-month period were performed. During this period of time, the internal standard for biotin was changed, therefore only the measurements with the final biotin internal standard are included in Table 3 (i.e. from the final 11 analyses performed). For the measurements with the former biotin internal standard, the RSD was 2.0% and the accuracy compared to the NIST value was 102% (n=8). Therefore, either of the biotin internal standards were deemed suitable for the method. Overall, the variability for this study was typically below 2.5%, as shown in Table 3. Only biotin and riboflavin showed slightly higher values than this. The accuracy was between 100% and 108% for all analytes.

## CONCLUSIONS

- LC/MS/MS offers the opportunity to combine single-analyte water-soluble vitamin methods into a multi-analyte method, saving time and improving laboratory efficiency.
- A single UPLC/MS/MS method for the analysis of biotin, folic acid, pantothenic acid, niacin, pyridoxine, riboflavin, and thiamine has been presented.
- With the employment of an ultra-sensitive mass spectrometer, the variability in measurements could be vastly improved to ensure RSDs better than 3% for the vitamins tested.
- This reduction in variation is important to ensure that intra- and inter-lab reproducibility can meet the analytical requirements to guarantee that label claims are met at the end of shelf life.
- Improvements in precision also enable reductions in the overages required during product formulation in order to improve product profitability.

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**UPLC/TQD**

## Rapid Analysis of Water-Soluble Vitamins in Infant Formula by Standard-Addition

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### APPLICATION BENEFITS

This method allows for the simultaneous analysis of 12 water-soluble vitamin compounds:

- Replaces time-consuming microbiological assays of single compounds
- Detects target compounds at low concentrations (particularly cyanocobalamin), in a very complex matrix, such as infant formula powder
- Simultaneous acquisition of MRM and full scan data in a single analysis run

### WATERS SOLUTIONS

ACQUITY UPLC® System

Xevo® TQ MS

MassLynx™ Software

TargetLynx™ Application Manager

IntelliStart™ Technology

RADAR™

### KEYWORDS

Water-soluble vitamins,  
Vitamin C, B3, B6, B12, B1,  
B9, B2, B7

### INTRODUCTION

Vitamins are minor constituents that have to be introduced, via our food, in small quantities because they are not synthesized by the human body. The vitamin composition of infant formula is critical for correct infant development, particularly if the mother is unable to breast-feed and formula is the primary source of nutrition. Official analytical methods for the determination of water-soluble vitamins are based on procedures, mainly microbiological assays, which have been established for decades<sup>1,2</sup>. Each vitamin is analyzed separately in order to apply extraction conditions, which permit the determination of its total content in a food. Vitamin analysis in food is generally a time-consuming process.

The development of a single method for their simultaneous determination of vitamins in fortified infant formula is difficult for several reasons:

- Diverse structures and chemical properties of the vitamin compounds
- Trace levels of vitamins present
- Matrix complexity
- Instability to light and heat
- Solubility issues
- Huge range of concentrations in infant formulas

In this application note, we describe a rapid, five-minute UPLC®/MS/MS method using positive ESI ionization for the simultaneous analysis of 12 water-soluble vitamin compounds in infant formula.

### EXPERIMENTAL

Throughout the sample preparation and analyses, all solutions were protected from exposure to light and stored at <5 °C.

Standard solutions of the vitamin compounds were prepared fresh daily.

**LC conditions**

LC system:	ACQUITY UPLC System
Column:	ACQUITY UPLC HSS T3, 2.1 x 50 mm, 1.8 µm
Column temp:	40 °C
Sample temp:	4 °C
Flow rate:	0.6 mL/min
Mobile phase A:	10 mM Ammonium formate in water + 0.1% formic acid
Mobile phase B:	10 mM Ammonium formate in methanol + 0.1% formic acid

**Gradient:**

Time (min)	%A	%B
0.0	99	1
2.0	99	1
3.0	45	55
3.1	1	99
4.0	99	1
5.0	99	1

Total runtime:	5.0 min
Injection volume:	10 µL, full loop

**MS conditions**

MS system:	Xevo TQ MS
Ionization:	ESI positive
Capillary voltage:	1.0 kV
Source temp:	150 °C
Desolvation temp:	600 °C
Desolvation gas:	1200 L/hr
Acquisition:	Multiple Reaction Monitoring (MRM) with RADAR full scan
Collision gas:	Argon at 3.5 x 10 <sup>-3</sup> mbar

**Standard-addition method**

Since blank samples were not available, quantitative analysis of water soluble vitamins in infant formula powders was performed by the standard-addition method (except for nicotinamide)\*. An analyte solution of known concentration (standard solution) was added to the sample so any matrix effects were accounted for in the calibration. The analyst did not know the amount of analyte in the sample initially, but tracked how much standard solution was added, and how the instrument response changed after adding the standard solution. Thus, by extrapolation of the calibration curve, the concentration of analyte in the sample could be determined. In practice, the volume of standard solution added is kept small to avoid dilution of the sample matrix.

\* Using the current dilution factor (see experimental steps below) for the sample matrix without addition of standards, the instrument response of nicotinamide (B3) initially present in the infant formula is close to reaching the saturation of the detector. If standard-addition method is used for nicotinamide, detector saturation will be reached.

**Sample preparation, extraction, and standard-addition**

Prepare the infant formula to three times the concentration described on the packaging.

Place 10 mL of infant formula solution into a PP tube covered with foil; add 20 mL of 100% ethanol.

Shake vigorously for 2 min, and centrifuge for 15 min at 3500 RPM.

Filter supernatant using a 0.45 PVDF filter.

Transfer 20 µL of the supernatant into an amber autosampler vial, add 10 µL of known concentrations of standards containing 11 analytes, and top off the autosampler vial to 1 mL with water (the analyte in sample matrix was diluted 50 times; analyte in standard was diluted 100 times in final volume).

A separate standard curve of nicotinamide in solution was prepared.

Analyze by LC/MS/MS.

**Acquisition and processing methods**

Data were acquired using MassLynx Software, v.4.1, and processed using TargetLynx Application Manager.

IntelliStart Technology was used to automatically develop fully optimized MRM acquisition methods for the 12 vitamin compounds targeted in this analysis. IntelliStart requires only the entry of basic compound information, and automatically locates the precursor ion, optimizes cone voltage, locates product ions, and optimizes collision energy.

Two MRM transitions were optimized for each vitamin compound; the first transition for quantitation and the second transition for confirmation. The dwell times for the transitions were automatically optimized to give a minimum of 12 points across each chromatographic peak for reproducible quantitation. The MRM transitions, cone voltages, and collision energies for the analyzed compounds, along with expected retention times, are shown in Table 1.

Analyte		Parent (m/z)	Dau 1/ Dau 2 (m/z)	CV (V)	CE 1/ CE 2 (eV)	RT (min)
Ascorbic acid	C	177.0	141.0 95.0	16	8 12	0.37
Thiamine	B1	265.2	122.0 144.0	18	16 12	0.41
Nicotinic acid	B3	124.0	80.2 53.0	34	20 22	0.51
Pyridoxal	B6	168.0	150.0 94.0	14	14 24	0.64
Pyridoxine	B6	170.0	152.0 134.0	20	12 20	0.86
Nicotinamide	B3	123.0	80.0 106.0	32	18 12	0.93
Pantothenic acid	B5	220.1	90.0 202.1	20	14 12	2.73
Cyanocobalamin	B12	678.6	147.1 359.2	30	36 24	2.98
Folic acid	B9	442.2	295.1 176.0	18	16 36	2.99
Riboflavin-5'-phosphate	B2	457.2	439.2 359.2	30	16 20	3.04
Biotin	B7	245.1	227.0 97.0	20	14 30	3.10
Riboflavin	B2	377.2	243.1 172.1	36	24 42	3.15

Table 1. LC/MS/MS parameters for the identification of water-soluble vitamin compounds.

In addition to MRM data, full scan data were acquired using the RADARTM mode of the Xevo TQ MS. RADAR is an information-rich acquisition approach that enables real time acquisition of spectral information on background components in the sample matrix, while simultaneously collecting MRM data. The use of RADAR does not compromise the quality of the MRM data.

## RESULTS AND DISCUSSION

12 water-soluble vitamin compounds were successfully analyzed using the ACQUITY UPLC System, coupled with Xevo TQ MS under ESI positive ionization. The use of ACQUITY UPLC enabled rapid separation of all analytes in <5 min, including 1 min for equilibration, as shown in Figure 1.

In the same analysis, full scan spectra to assess the background components in the infant formula matrix were also monitored using the RADAR mode of the Xevo TQ MS. RADAR utilizes the fast acquisition rates of the Xevo TQ MS, allowing full scan MS data to be acquired, while still collecting a sufficient number of points across the analyte peak, in MRM mode, for accurate quantification and confirmation.

With RADAR, the analyst can observe untargeted contaminants in the sample matrix, and get an idea of the level and type of compounds causing possible matrix effects. This provides insight in the development of matrix reduction strategies during LC-MS/MS methods development. For example, the separation method could be modified to move the peaks of interest away from where areas of potential matrix effects or ion suppression are likely to be present. An illustration of a MS spectrum extracted from a section of the full scan data of the infant formula sample matrix is shown in the insert of Figure 1.

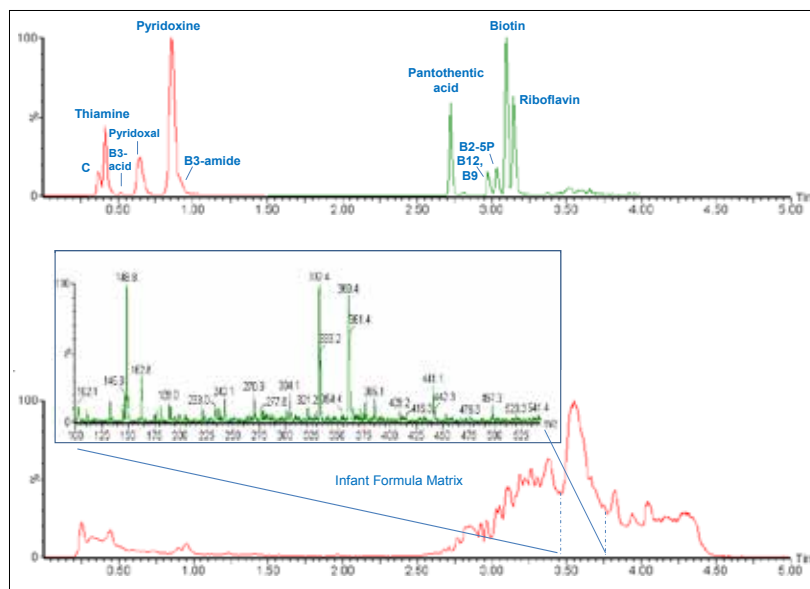


Figure 1. RADAR technology allows for simultaneous acquisition of MRM and full scan data in a single analysis run. Spectrum identifying untargeted contaminants in sample matrix are shown in the insert of the full scan data.



Analyte		Spiked level (ng/g)	Formula A		Formula B	
			Average Recovery (%)	%RSD (n=6)	Average Recovery (%)	%RSD (n=6)
Ascorbic acid	C	1000	100.4	1.1	99.4	1.1
Thiamine	B1	10	98.7	1.6	118.6	2.2
Nicotinic acid	B3	10	90.3	4.0	96.4	4.2
Pyridoxal	B6	10	83.5	1.2	88.0	1.1
Pyridoxine	B6	10	98.9	0.7	100.1	0.7
Nicotinamide	B3	10	101.6	0.5	98.7	0.6
Pantothenic acid	B5	10	104.2	1.9	112.6	0.8
Cyanocobalamin	B12	10	111.4	1.0	106.2	0.9
Folic acid	B9	100	86.6	3.7	82.0	2.4
Riboflavin-5'-phosphate	B2	100	77.6	2.2	75.1	2.8
Biotin	B7	10	95.4	2.2	98.7	2.4
Riboflavin	B2	10	102.7	1.8	103.3	2.2

Table 2. Recoveries and % RSDs of water-soluble vitamin compounds in pre-extracted spiked samples from two infant formula products.

Linearity and quantitation

Linear dynamic range, sensitivity, and suitability of the linear model were evaluated by applying the standard-addition method. An advantage of the standard-addition method is the avoidance of the evaluation of the matrix effect, responsible for signal ion suppression.

Excellent linearities were observed with correlation coefficients  $\geq 0.99$ , as shown in Table 3, for all of the analytes in infant formula tested, over wide concentration ranges: 10 to 10000 ng/mL for ascorbic acid (C); 0.1 to 10.0 ng/mL for cyanocobalamin (B12); and 1 to 100 ng/mL for the rest of the analytes. Calibration curves of ascorbic acid (C), and cyanocobalamin (B12), based on standard-addition in one brand of infant formula powder are shown in Figure 3.

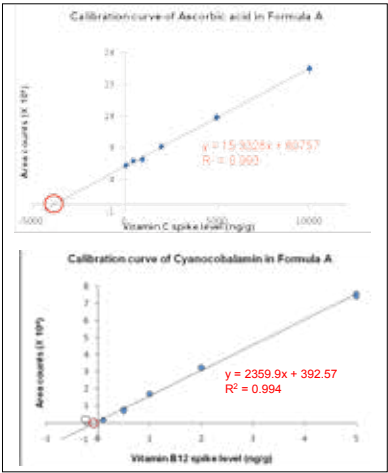


Figure 3. Calibration curves of ascorbic acid and vitamin B12 in Formula A using standard-addition.

The calibration curve was extrapolated, and the absolute value of the x-intercept showed the concentration of the analyte in the infant formula, after taking into account the dilution factor (50 x) that was used. The sensitivity of the Xevo TQ MS allowed us to dilute the sample to reduce matrix effects, while still detecting the target compounds with confidence.

Analyte		R <sup>2</sup>	Calculated concentration (ng/g)
Ascorbic acid	C	0.993	198130
Thiamine	B1	0.990	1778
Nicotinic acid	B3	0.999	Not present
Pyridoxal	B6	0.996	205
Pyridoxine	B6	0.998	2354
Nicotinamide	B3	0.992	14201
Pantothenic acid	B5	0.994	8113
Cyanocobalamin	B12	0.994	8.3
Folic acid	B9	0.993	152
Riboflavin-5'-phosphate	B2	0.997	Not present
Biotin	B7	0.997	65
Riboflavin	B2	0.992	1143

Table 3. Correlation coefficients (R2) for calibration curves constructed, based on the quantifier transitions, and calculated concentrations of water-soluble vitamins in Formula A.

## CONCLUSIONS

A rapid 5-minute method using ACQUITY UPLC with Xevo TQ MS in positive ESI ionization mode was developed for the simultaneous analysis of 12 water-soluble vitamin compounds. This method replaces individual, lengthy methods for vitamin analysis. By combining separate vitamin analyses into a single run, laboratories can increase sample analysis throughput, reduce solvent consumption, and decrease their operational costs.

With the sensitivity of the Xevo TQ MS, it is possible to detect target compounds at low concentrations (particularly cyanocobalamin), in a very complex matrix, such as infant formula powder. With the low limit of quantification achievable on the Xevo TQ MS, samples can be diluted to reduce matrix effects.

RADAR Technology allows for monitoring of matrix interferences, impurities, and degradants in samples, while accurately quantifying target compounds. This allows analysts to make informed decisions when assessing matrix effects, and enables a true assessment of whether matrix effects are likely to be present.

IntelliStart Technology simplifies system setup and MRM methods development, ensuring scientists of all levels can operate the instrument quickly and confidently, and start generating reproducible UPLC/MS/MS data of the highest quality.

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2. AOAC Official Method 992.07, Microbiological Turbidimetric Method of Pantothenic Acid in Milk-based Infant Formula.

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## Extraction of Fat-Soluble Vitamins from Fortified Foods Using Oasis HLB

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### APPLICATION BENEFITS

- Increase the efficiency of Food QC labs with a simple, single solid phase extraction procedure to simultaneously extract all fat-soluble vitamin compounds.
- Reduce sample processing time from hours (saponification/solvent extraction) to less than 30 minutes using this SPE method.
- Eliminate the use of harmful chemicals that are potential threats to individuals and the environment.

### WATERS SOLUTIONS

ACQUITY UPLC® System

Xevo® TQ-S

Oasis® HLB

RADAR™

MassLynx™ Software

### KEY WORDS

FSV, vitamins, fat-soluble vitamins, fortification, SPE, solid phase extraction, APCI

### INTRODUCTION

Micronutrient malnutrition is a global threat affecting more than one third of the world population.<sup>1</sup> Numerous strategies have been proposed to provide a solution to this problem. Among them, food fortification has been a major approach aimed at improving the nutritional quality of food in developing nations. Of special significance is the fortification of vitamins in various food supplies. Food and drinks are the major source of vitamins and with the growing awareness for a balanced diet, consumption of fortified food has substantially increased.

The presence of fat-soluble vitamins (A, D, E, and K) in humans is of vital importance to metabolism although they are not required in the everyday diet. Reliable and sensitive determination of fat-soluble vitamins in fortified foods is essential for both nutritional and economic reasons.

Official analytical methods for the determination of fat-soluble vitamins (FSVs) mainly use HPLC with ultra-violet (UV), fluorescence (FLR), or electrochemical detection (ECD). Though these detection techniques are selective and sensitive, they become problematic with the increasing complexity of the matrix. More recently the industry trend has been towards adoption of multi-analyte methods to improve lab efficiency.<sup>2</sup> In addition, the use of Waters® ACQUITY UPLC System allows for faster analyses compared to traditional HPLC methods, which typically require 15 to 60 minutes.<sup>3</sup>

Sample preparation is often the rate-limiting step of the analysis and the most challenging for labs that routinely test for the presence of vitamins in fortified food products. Vitamins are unstable in the presence of light, oxidizing reagents, temperature, etc., and this can further complicate sample pretreatment. The common methods employed for the extraction of FSVs are described in Table 1. In recent years, solid phase extraction (SPE) has attracted increasing interest in the analysis of FSVs due to their short preparation time and gentle extraction procedure.

EXPERIMENTAL

LC conditions

LC system: ACQUITY UPLC  
Column: ACQUITY UPLC BEH C<sub>18</sub>, 2.1 X 100 mm, 1.7 µm  
Column temp.: 40 °C  
Sample temp.: 24 °C  
Flow rate: 0.6 mL/min  
Mobile phase A: 90:10 acetonitrile: water  
Mobile phase B: Methanol  
Gradient:  

Time (min)	%A	%B
0.0	100	0
0.5	100	0
2.5	0	100
4.5	0	100
5.0	100	0
6.0	100	0

  
Total run time: 6 min  
Injection volume: 5 µL, PLNO

MS conditions

MS system: Xevo TQ-S  
Ionization: APCI positive  
Corona current: 15 µA  
Extractor: 3.0 V  
Source temp.: 150 °C  
Probe temp.: 550 °C  
Desolvation gas: 1000 L/Hr  
Acquisition: RADAR [multiple reaction monitoring (MRM) with full scan]  
Collision gas: Argon at 3.5 x 10<sup>-3</sup> mbar

Method	Extraction process	Advantage	Disadvantage
Saponification	Alkaline hydrolysis followed by extraction with hexane/petroleum ether.	■ Good for the determination of Vitamins A, D, and E.  ■ Removes fats and lipids effectively.	■ Analyte degradation, especially Vitamin K (decomposes in alkaline medium).  ■ Emulsion formation.  ■ Requires addition of antioxidant to prevent oxidation of FSVs.  ■ Health and environmental issues with the use of toxic solvents such as hexane, ether <i>etc.</i>
Enzymatic digestion	Use of enzymes such as lipase, takadiastase, savinase, <i>etc.</i> to digest the interfering proteins and lipids.	■ Suitable for Vitamin E analysis.	■ An appreciable amount of vitamins may be present in commercially available enzyme. Thus blank must be run in parallel to allow correction.
Non-hydrolytic extraction	Extraction using suitable solvent. Rose Gottlieb method was more popular.	■ Good for Vitamin A and E esters determination.	■ Not suitable for Vitamins E and K because of the alkali used that decomposes the vitamins.
Solid phase extraction	Use of cartridge for enrichment and generating clean extract.	■ Could be applied for all vitamins.	■ Lack of information on the suitability of the approach.

Table 1. Methods used for the extraction of FSVs from various matrices.4-7

In this application note, an SPE method for the simultaneous extraction of fat-soluble vitamins from fortified food products was developed. The proposed method was applied for the extraction of FSVs from breakfast cereal, infant formula, and chocolate. A rapid six-minute UPLC®/MS/MS method using positive atmospheric pressure chemical ionization (APCI) was utilized for the analysis of the fat-soluble vitamin compounds.

Individual stocks of Vitamin A palmitate (retinyl palmitate), D2 (ergocalciferol), D3 (cholecalciferol), E (α-tocophenol), and E acetate (α-tocophenol acetate) were prepared by dissolving 1 mg of the respective FSVs in 1 mL of methanol. Stocks of Vitamin K1 (phyllquinone) and K2 (menaquinone) were prepared by dissolving 1 mg in 1 mL of 50:50 acetonitrile:methanol. Working standards were prepared by further dilution in methanol. The solutions were protected from exposure to light throughout sample preparation and analysis.

Breakfast cereal, infant formula, and chocolate were obtained from a local supermarket for use in recovery experiments.

The SPE protocol for the extraction of fat-soluble vitamins is illustrated in Figure 1.

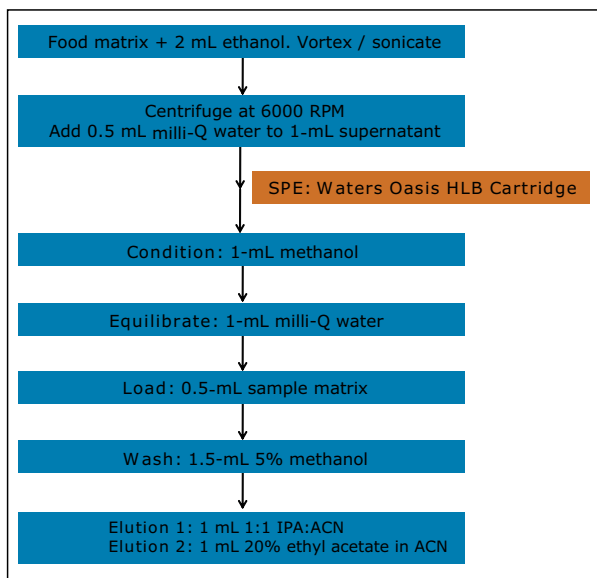


Figure 1. Illustration of extraction protocol.

Data were acquired using MassLynx Software, v. 4.1 and processed using TargetLynx™ Application Manager.

Two MRM transitions were monitored for each fat-soluble vitamin compound: the more intense transition was used for quantitation and the other for confirmation. Details of the optimized parameters for the FSVs, along with respective retention times, are summarized in Table 2.

Vitamin	Precursor ( <i>m/z</i> )	Fragment 1 / Fragment 2 ( <i>m/z</i> )	CV (V)	CE1/CE2 (eV)	RT (min)
A-palmitate	269.2	81.0 93.0	20	24 22	4.48
D2	397.5	107.0 379.4	20	24 14	2.53
D3	385.5	107.0 367.4	20	20 12	2.59
E	431.5	137.0 165.0	18	40 26	2.91
E-Ac	473.6	165.1 207.1	28	40 18	3.12
K1	451.5	128.0 187.1	34	74 24	3.34
K2	445.5	81.0 187.1	24	46 22	2.45

Table 2. APCI positive MRM conditions for FSVs.

## RESULTS AND DISCUSSION

### Sample preparation optimization

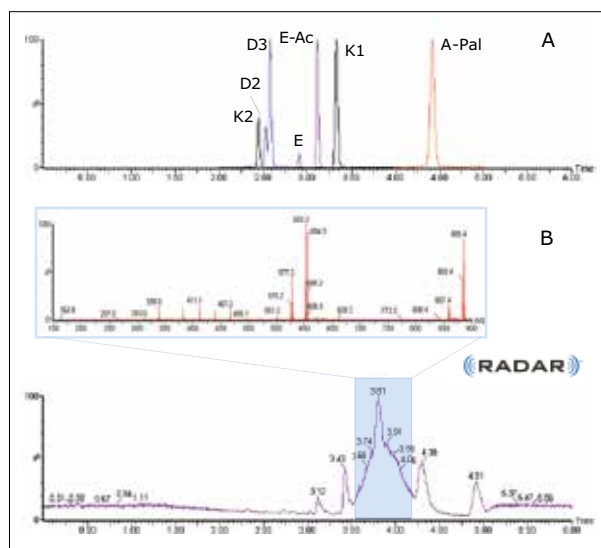
The use of the Oasis HLB Cartridge to extract seven different FSVs (vitamin A-palmitate, D2, D3, E, E-acetate, K1, and K2) was evaluated. During the initial experiments, it was found that if the extraction solution contained > 90% ethanol as the loading solvent, the SPE cartridge failed to retain FSVs. In order to successfully retain the FSVs on the SPE cartridge, it was necessary to add a small amount of water after ethanol extraction.

The retained analytes were successfully eluted using a two-step elution approach, which proved to be effective for all FSVs. With the inclusion of ethyl acetate as a second-step elution, recoveries with vitamins K1 and K2 improved significantly. Extraction efficiency for the method was evaluated by comparing the pre- and post-fortified samples. Figure 2 shows the MRM along with the RADAR chromatogram of FSVs in infant formula analyzed after SPE.

RADAR mode is a full scan function used to assess background components during a standard MRM analysis. For these experiments, the RADAR functionality on the Xevo TQ-S Mass Spectrometer provided useful information during the method development phase. Having both full scan and MRM data acquired simultaneously, allowed the analyst to: a) easily monitor the matrix changes that occurred during the sample preparation trials; and b) review the peak characteristics of the vitamins.

### Recovery data

The experiments were repeated over time to evaluate the variation in recoveries and response. The average percentage recoveries and RSD values of the FSVs from their respective matrices are detailed in Tables 3, 4, and 5. The results demonstrated good recoveries with all the FSVs analyzed in the different matrices tested (breakfast cereal, infant formula, and chocolate). Recoveries of FSVs analyzed in breakfast cereal, infant formula, and chocolate were in range of 93% to 108%, 89% to 100% and 83% to 104%, respectively.



BREAKFAST CEREAL			
Vitamin	Spike concentration (ng/mL)	Intra-day variation (n=6)	Inter-day variation (n=3)
		Average %recovery ± %RSD	Average %recovery ± %RSD
A-palmitate	100	90.8 ± 2.2	93.5 ± 5.1
D2	100	100.6 ± 2.0	106.9 ± 5.1
D3	100	103.0 ± 2.6	104.7 ± 9.9
E	10	98.5 ± 2.1	102.3 ± 4.1
E-acetate	10	103.1 ± 1.4	107.4 ± 7.0
K1	10	100.7 ± 1.4	99.0 ± 0.6
K2	10	99.1 ± 3.0	99.6 ± 3.6

Table 3. FSVs spiked %recovery in breakfast cereal.

INFANT FORMULA			
Vitamin	Spike concentration (ng/mL)	Intra-day variation (n=6)	Inter-day variation (n=3)
		Average %recovery ± %RSD	Average %recovery ± %RSD
A-palmitate	100	101.8 ± 4.8	106.4 ± 4.1
D2	100	93.2 ± 3.0	99.0 ± 5.2
D3	100	91.5 ± 6.2	94.9 ± 5.0
E	10	105.1 ± 2.0	104.2 ± 6.8
E-acetate	10	94.1 ± 7.6	101.0 ± 7.4
K1	10	85.3 ± 6.0	97.3 ± 10.0
K2	10	81.8 ± 7.3	89.6 ± 8.0

Table 4. FSVs spiked %recovery in infant formula.

CHOCOLATE			
Vitamin	Spike concentration (ng/mL)	Intra-day variation (n=6)	Inter-day variation (n=3)
		Average %recovery ± %RSD	Average %recovery ± %RSD
A-palmitate	100	83.7 ± 6.1	83.8 ± 5.8
D2	100	82.9 ± 3.0	91.2 ± 8.1
D3	100	94.8 ± 6.6	95.9 ± 2.5
E	10	112.9 ± 6.0	103.5 ± 8.0
E-acetate	10	107.6 ± 5.0	99.0 ± 8.8
K1	10	84.6 ± 3.7	85.8 ± 8.1
K2	10	84.0 ± 1.2	85.2 ± 1.3

Table 5. FSVs spiked %recovery in chocolate.



## CONCLUSIONS

The Waters Oasis HLB Cartridge (60 mg, 3 cc; P/N WAT094226) proved to be effective in extracting seven different FSVs from fortified food products with good recoveries. A two-step elution method was utilized to attain maximum recoveries for all vitamins from various food matrices. As compared to hours of saponification or liquid-liquid extraction, this SPE method reduces sample preparation time to less than 30 minutes. The result is a simple, rapid, and selective method for extracting FSVs from fortified food products. Good recoveries suggest the acceptable analysis of all the FSVs even in the presence of oils and other interferences present in food products. This method provides an effective approach that could be applied as a universal technique and a potential alternative for the solvent extraction.

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January 2012 720004193en AG-PDF

## UPLC/TQ-S

## Analysis of Fat-Soluble Vitamins in Fortified Infant Formula and Standard Reference Material 1849a by Standard Addition

Tarang Nema and Evelyn Goh  
Waters Pacific Pte Ltd., Singapore

### APPLICATION BENEFITS

- Provides a selective, reliable, and sensitive method for successful determination of FSVs in complex infant formulas.
- Potentially replace time-consuming conventional assays for FSVs.
- Improve efficiency of Food QC labs with a single extraction method for all fat-soluble vitamins.
- Detect target compounds at trace concentrations (particularly Vitamin D3) in complex infant formula matrices. RADAR™ Technology facilitates simultaneous MRM and full scan data acquisition, which enables quick decisions pertaining to sample matrices during method development.
- Increases sample analysis throughput, while avoiding the use of toxic organic solvents.

### WATERS SOLUTIONS

ACQUITY UPLC® System

Xevo® TQ-S Mass Spectrometer

MassLynx™ Software

Oasis® HLB Cartridge

### KEY WORDS

Fat-soluble vitamins, Standard Reference Material, SRM 1849a, Retinol palmitate (A-Palm), Ergocalciferol (D2), Cholecalciferol (D3),  $\alpha$  tocopherol (E),  $\alpha$  tocopherol acetate (E-Ac) Phylloquinone (K1), Menaquinone (K2), SPE

### INTRODUCTION

Infant formulas provide the best possible solution to meet the nutritional requirements of infants when mothers are unable, or choose not to breastfeed. The manufacturing of infant formula is primarily based on the composition of human milk, which serves as a gold standard.<sup>1</sup> Since infant formula is the only source of nutrients for infants, it is a highly regulated food product in the market.<sup>2</sup> In addition, surveillance by regulatory bodies helps to control and consolidate the level of fortification among manufacturers to provide homogeneity in formulation. Generally, these regulatory bodies govern the manufacturing process, composition, and labeling of infant formula. Besides some regional variations, there is considerable equality among the major regulatory bodies<sup>3-5</sup> (World Health Organization, U.S. Food and Drug Administration, etc). Fat soluble vitamins (FSVs) therefore need to be fortified in infant formulas, as these nutritional components are essential for the growth and development of infants. For infants that rely solely on infant formula for their dietary requirements, any deficiency or over dosage from the product can lead to various health problems in infants. Therefore, it is mandatory to accurately optimize the level of fortification to meet the regulatory requirements. This requires a selective, reliable, and sensitive method for successful determination of FSVs in a complex infant formula.

With the advancements in analytical technology the recent trend within the industry has been the adoption of multi-analyte methods. As more laboratories migrate towards the use of Waters® ACQUITY UPLC, analyses that previously took over 30 minutes can now be accomplished in few minutes. When UPLC® is coupled with tandem quadrupole mass spectrometry (MS), additional sensitivity and selectivity are realized.

Sample preparation is often the rate-limiting step of the analysis and it is also the most challenging step for labs that routinely test for the presence of vitamins in infant formulas. Due to the complexity of infant formula, solid phase extraction (SPE) has attracted increased interest in the analysis of FSVs due to their short and gentle extraction procedure.

This application note describes an SPE method for the simultaneous extraction of fat-soluble vitamins from infant formula and Standard Reference Material (SRM) 1849a, and quantitation by standard addition.

EXPERIMENTAL

UPLC conditions

LC system:	ACQUITY UPLC	
Column:	ACQUITY UPLC BEH C <sub>18</sub> , 2.1 x 100 mm, 1.7 µm	
Column temp.:	40 °C	
Sample temp.:	24 °C	
Flow rate:	0.6 mL/min	
Mobile phase A:	90:10 Acetonitrile:water	
Mobile phase B:	Methanol	
Gradient:		
<u>Time (min)</u>	<u>%A</u>	<u>%B</u>
0.0	100	0
0.5	100	0
2.5	0	100
4.5	0	100
5.0	100	0
6.0	100	0
Total run time:	6 min	
Injection volume:	5 µL, PLNO	

MS conditions

MS system:	Xevo TQ-S
Ionization:	APCI positive
Corona current:	15 µA
Extractor:	3.0 V
Source temp.:	150 °C
Probe temp.:	550 °C
Desolvation gas:	1000 L/Hr
Acquisition:	Multiple Reaction Monitoring (MRM) with RADAR full scan
Collision gas:	Argon at 3.5 X 10 <sup>-3</sup> mbar

Individual stock solutions (1 mg/mL) were prepared by dissolving the respective FSVs in methanol, except for Vitamins K1 (phyloquinone) and K2 (menaquinone), which were prepared by dissolving in 50:50 acetonitrile:methanol. Working standards were prepared by further dilution in methanol. The solutions were protected from exposure to light throughout sample preparation and analysis.

Infant formula was obtained from a local supermarket for use in experiments. SRM 1849a was procured from the National Institute of Standards and Technology (NIST), Gaithersburg, MD, U.S.A.

Standard-addition method

Due to complexity of the matrix and non-availability of blank samples, quantitation of FSVs in infant formula was performed by standard addition method. A series of known concentrations of the analyte was added to the infant formula to generate a matrix-matched calibration. This calibration tracked the variation in the instrument response after standard addition, and also accounted for any matrix effects. The concentration of the vitamins present in infant formula was determined by extrapolation of the calibration curve.

Sample preparation, extraction, and standard addition

The extraction protocol of FSVs from infant formula is described in full detail in Waters application note, no. 720004193en<sup>6</sup>; a summarized protocol is illustrated in Figure 1.

The collected eluent from the extraction steps after drying and reconstitution was further used for the standard addition method. 50 µL of the reconstituted eluent was transferred to an autosampler vial and 1 µL of a standard mixture containing known concentrations of FSVs was added. Finally, the volume was made up to 100 µL with ethanol. This resulted in diluting the matrix 2x, and the standard 100x in the final volume.

For Vitamins E and E-Acetate, retinol palmitate (A), and phyloquinone (K1), which are present in higher amounts in infant formula, the sample was further diluted to a final dilution factor of 200. This reduced the matrix effect while accurately quantifying the amount of FSVs present in infant formula and SRM 1849a.

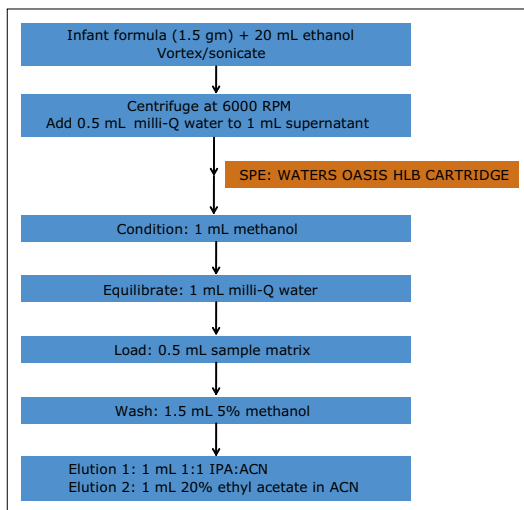


Figure 1. Illustration of extraction protocol.

### Acquisition and processing methods

Data were acquired using MassLynx Software, v. 4.1, and processed using TargetLynx™ Application Manager.

IntelliStart™ Technology was used to automatically develop optimized MRM methods for all FSVs using basic analyte information (molecular mass or empirical formula). With this small amount of information, IntelliStart can automatically locate precursor and fragment ions, and optimize compound specific parameters, such as cone voltage and collision energy. Details of the optimized parameters for the FSVs, along with respective retention times, are summarized in Table 1.

Vitamin	Precursor ( <i>m/z</i> )	Fragment 1 / Fragment 2 ( <i>m/z</i> )	CV (V)	CE1/CE2 (eV)	RT (min)
A-palmitate	269.2	81.0 93.0	20	24 22	4.48
D2	397.5	107.0 379.4	20	24 14	2.53
D3	385.5	107.0 367.4	20	20 12	2.59
E	431.5	137.0 165.0	18	40 26	2.91
E-Ac	473.6	165.1 207.1	28	40 18	3.12
K1	451.5	128.0 187.1	34	74 24	3.34
K2	445.5	81.0 187.1	24	46 22	2.45

Table 1. APCI positive MRM conditions for FSVs.

Full scan data were also acquired using RADAR mode of the Xevo TQ-S. RADAR is an information-rich acquisition that allows the analyst to monitor matrix complexity. It enables users to have a better understanding of matrix complexity, which allows them to make instant and intelligent decisions while assessing matrix effects and developing a method.

## RESULTS AND DISCUSSION

The extracted FSVs were analyzed using the ACQUITY UPLC System coupled with the Xevo TQ-S Mass Spectrometer under APCI positive ionization. The use of ACQUITY UPLC enabled rapid separation of all FSVs within 6 min, as shown in Figure 2A.

Waters Oasis HLB Cartridge was used in the extraction of seven different forms of FSVs (Vitamins A-palmitate, D2, D3, E, E-Acetate, K1, and K2) from infant formula and SRM 1849a. The retained analytes were successfully eluted using a two-step elution approach, which proved to be effective for all FSVs. Table 2 shows the percent recoveries for FSVs from infant formula and SRM 1849a. The results demonstrated good recoveries in the range of 85% to 100% for all the vitamins in both matrices with %RSD well below 8% except for Vitamin K2 (~11%) in SRM 1849a. The results demonstrated an acceptable level of repeatability and robustness of the proposed solution for commercial infant formula and SRM 1849a.

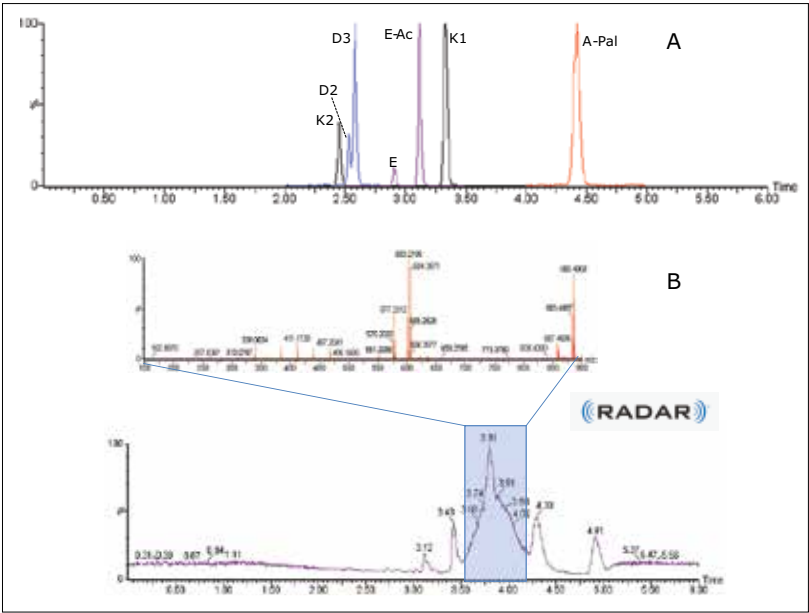


Figure 2. (2A) MRM acquisition demonstrating the separation of all FSVs; (2B) Full scan data of the matrix combined in the range of 3.50 to 4.25 min.

## RADAR Technology

RADAR Technology is a full scan function used to assess background components during a standard MRM analysis. It benefits from fast acquisition rates while maintaining duty cycle in MRM mode. With RADAR, untargeted contaminants can be observed, and at the same time, samples can be evaluated for matrix effects. This provides analysts with valuable information during method development. An MS spectrum extracted from the full scan data of the infant formula sample matrix is shown in the inset of Figure 2B.

This method was tested on a commercially available infant formula and further verified using SRM 1849a. Figure 3 shows the extracted quantifier ion (primary MRM transition) chromatograms for the FSVs detected in infant formula. The high signal-to-noise ratio obtained for each of the vitamins at the fortified levels showed the excellent selectivity and sensitivity achieved using UPLC-MS/MS for this application.

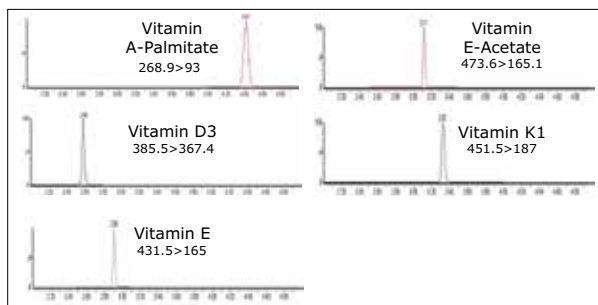


Figure 3. Quantifier MRM transition for the analysis of FSVs in infant formula.

Infant Formula/SRM 1849a			
Vitamin	Spike concentration (ng/mL)	Average % recovery $\pm$ %RSD (n=3)	
		Infant formula	SRM 1849a
A-Pal	100	101.8 $\pm$ 4.8	107.2 $\pm$ 4.0
D2	100	93.2 $\pm$ 3.0	101.7 $\pm$ 6.8
D3	100	91.5 $\pm$ 6.2	97.7 $\pm$ 6.9
E	10	105.1 $\pm$ 2.0	96.5 $\pm$ 6.4
E-Ac	10	94.1 $\pm$ 7.6	98.7 $\pm$ 0.3
K1	10	85.3 $\pm$ 6.0	86.3 $\pm$ 2.3
K2	10	81.8 $\pm$ 7.3	91.9 $\pm$ 11.2

Table 2. FSVs spiked % recovery in infant formula.

Other parameters such as linearity and sensitivity were also evaluated using the standard addition method. With the application of the standard addition method, evaluation of matrix effects that could potentially be responsible for ion suppression or enhancement can be calculated.

Excellent linearity was obtained (with correlation coefficients more than 0.99) for all the FSVs in infant formula and SRM 1849a tested over the concentration ranges listed in Table 3. Calibration curves of Vitamin D3 and Vitamin E acetate based on standard addition in SRM 1849a are shown in Figure 4.

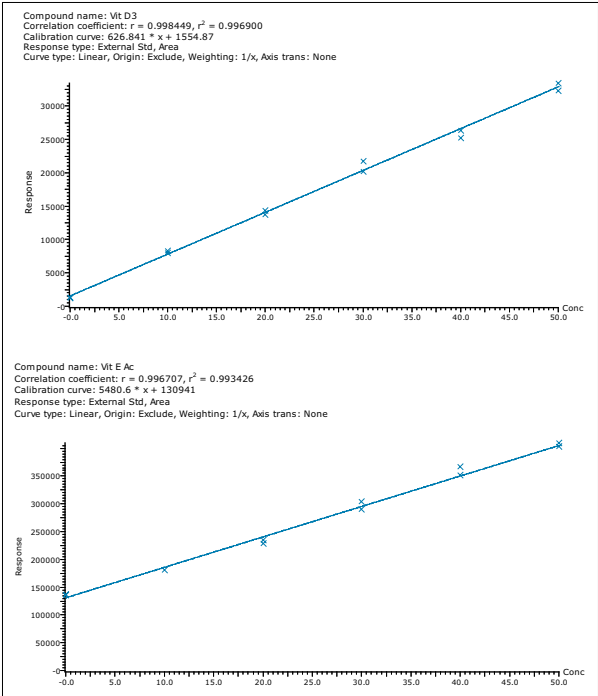


Figure 4. Calibration curves of Vitamin D3 and Vitamin E-Acetate in SRM 1849a using standard addition.

The concentration of FSVs in infant formula was calculated by extrapolating the calibration curve and recording the absolute value of the x-intercept after taking the dilution factor into consideration. The sensitivity of the Xevo TQ-S permitted dilution of the sample to reduce matrix effects, while detecting the targeted FSVs with confidence. Vitamin D, which is present in very low amounts, can be quantified using The Xevo TQ-S even after diluting the sample matrix. The calculated amount of FSVs in SRM 1849a was found to be within the acceptable limits of the labeled amount. The variation between the amounts reported here and the NIST reported amount could be attributed to the fact that the data (reported here) is single laboratory data, whereas the SRM 1849a assigned values were based on the combination of the results obtained by NIST and collaborating labs.<sup>7-8</sup> Also, the sample preparation method adopted in analyzing FSVs in SRM 1849a is different from the method described in this application note.

Infant formula				
Vitamin	Concentration range (ng/mL)	Correlation coefficient	Amount (mg/kg)	
			Calculated	Labeled
A-Pal	10-50	0.997	14.060	9.510
D2	10-50	0.999	Not present	Not present
D3	10-50	0.996	0.055	0.077
E	10-50	0.998	169.080	180.180
K1	10-50	0.999	0.560	0.480
K2	10-50	0.997	Not present	Not present
SRM 1849a				
A-Pal	10-50	0.993	8.210 ± 0.520	7.680 ± 0.230
D2	10-50	0.993	Not present	Not present
D3	10-50	0.997	0.108 ± 0.010	0.111 ± 0.017
E	10-50	0.993	150.050 ± 17.650	177.000 ± 47.000
K1	10-50	0.992	0.930 ± 0.120	1.060 ± 0.170
K2	10-50	0.991	Not present	Not present

Table 3. Concentration range, correlation coefficient for calibration curves constructed, and calculated concentrations of FSVs in Infant formula and SRM 1849a.



## CONCLUSIONS

Simultaneous extraction of each of the FSVs was achieved using an Oasis HLB Cartridge, followed by rapid analysis of all FSVs in infant formulas using ACQUITY UPLC with Xevo TQ-S in positive APCI ionization mode. RADAR mode was applied, which allows for MRM and full scan data to be acquired simultaneously, without affecting quantification of target compounds. This provided valuable information to the analyst in the development of the sample preparation and LC method development, and ensured a robust final method.

The standard addition method was found useful to quantify the amount of FSVs in infant formula, which was further verified using SRM 1849a. The standard addition method was able to combat the matrix effect issues, when blank matrix was not available.

The sensitivity of the Xevo TQ-S made it possible to detect targeted FSVs at low concentrations in infant formula, which is a highly complex matrix. The high sensitivity of the ACQUITY UPLC System, coupled to the Xevo TQ-S allowed for the dilution of samples to reduce matrix effects.

This method has the potential to replace time-consuming conventional assays for FSVs and it can enhance sample analysis throughput, avoid the use of toxic organic solvents, and increase the lab efficiency.

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April 2012 720004314en AG-PDF

## Characterizing of the Natural Product Goldenseal Using CORTECS 2.7 $\mu$ m Columns and ACQUITY QDa Detection

Kenneth D. Berthelette, Thomas Swann, and Kenneth J. Fountain  
Waters Corporation, Milford, MA, USA

### APPLICATION BENEFITS

- Separation of complex sample matrices using CORTECS® 2.7  $\mu$ m Columns, allowing for accurate characterization of the sample
- Rapidly identify compounds by mass using the ACQUITY® QDa® Detector in 5 minutes

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[Alliance® HPLC](#)

[Empower® 3 CDS](#)

[LCMS Certified Max Recovery Vials](#)

[ACQUITY QDa Detector](#)

### KEY WORDS

Goldenseal, cosmetics, personal care products, natural products

### INTRODUCTION

Natural product herbal supplements are widely used as remedies for physical ailments. Depending on sample origin and processing, product composition can range widely in the number, type, and amount of natural product compounds present. As consumers are growing in concern to the side effects of chemicals used in cosmetics and personal care products, the use of herbal plants in cosmetics,<sup>1</sup> and personal care products,<sup>1,2</sup> is growing in demand. Accurate sample characterization from numerous sources is useful to control supplement quality. However, the sample complexity and/or variability require highly efficient columns that do not sacrifice analysis speed. CORTECS 2.7  $\mu$ m Columns contain solid-core particles which produce high peak capacity separations with reduced back pressure, making them ideal for use on traditional HPLC instruments. Using CORTECS 2.7  $\mu$ m Columns, therefore, allows easier analysis of natural product mixtures.

Goldenseal is a plant native to southeastern Canada and the northeastern United States. Traditionally, it has been used to support digestion, mucous membranes, bile secretions, and many other bodily functions, as well as a topical treatment. In this application note, five different sources of Goldenseal which span four different manufacturers, two different parts of the plant, and two different formulation matrices are analyzed. This variety of sources was tested to determine the differences between each sample and if there were any compounds common to all the tested sources. Each sample was analyzed using a CORTECS C<sub>18</sub>+, 2.7  $\mu$ m, 3.0 x 50 mm Column on an Alliance HPLC System with both UV and an ACQUITY QDa Detector in order to get fast and reliable mass data for peaks present in the sample, providing additional and crucial information for the characterization of the natural products.

EXPERIMENTAL

LC conditions

System: Alliance HPLC

Column: CORTECS C<sub>18</sub>+,  
2.7 µm, 3.0 x 50 mm  
([p/n 186007400](#))

Mobile phase A: 0.1% formic acid in water

Mobile phase B: 0.1% formic acid  
in acetonitrile

Gradient: 7–30% B in 5.0 minutes,  
return to 7% B  
in 0.1 minutes,  
hold for 1.0 minutes

Flow rate: 1.0 mL/min

Column temp.: 30 °C

Detection (UV): 300 nm

ACQUITY QDa setting: ESI+ mode, full scan from  
150–1250 amu

Injection volume: 1.0 µL

Sample vials: LCMS Certified Max  
Recovery Vials  
([p/n 600000670CV](#))

Data management: Empower 3 CDS

Sample preparation

Capsule samples<sup>3</sup>

20 mg of powdered sample was removed and placed into a 10 mL centrifuge tube. 2.5 mL of 90:10 methanol:water with 0.1% acetic acid was added. Samples were sonicated for 15 minutes, and centrifuged at 4000 rpm for 5 minutes. The supernatant was then removed and placed into a separate vial. Extraction was performed three additional times. Extracted liquid filtered through a 0.1 µm nylon filter prior to injection.

Liquid sample

Two drops of liquid Goldenseal was added to 10 mL of 90:10 methanol:water with 0.1% acetic acid. Sample filtered through a 0.1 µm nylon filter prior to injection.

RESULTS AND DISCUSSION

Five commercial sources of Goldenseal were acquired for characterization. Table 1 outlines the source of each sample as well as details regarding the part of the plant used and the sample format.

Name	Source	Format
Goldenseal liquid	Root	Liquid
Goldenseal herb	Stem, flower, leaf	Capsule
Goldenseal root	Root	Capsule
Goldenseal extract	Root	Capsule
Goldenseal	Rhizome/root	Capsule

Table 1. Summary of the five sources of Goldenseal obtained.

After sample preparation, the samples were injected and both UV and mass data were collected.

Figure 1 shows the full scale separation of the five samples.

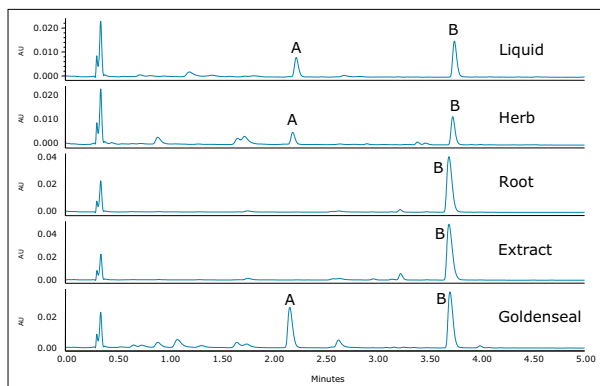


Figure 1. Separation of five different sources of Goldenseal on a CORTECS  $C_{18}$ +, 2.7  $\mu$ m, 3.0 x 50 mm Column at 300 nm. Main components A and B are indicated. Maximum pressure for the separation was 2600 psi.

The chromatograms were then scaled to show the low level constituents that exist in the samples.

Figure 2 shows the zoomed in chromatograms of the five samples.

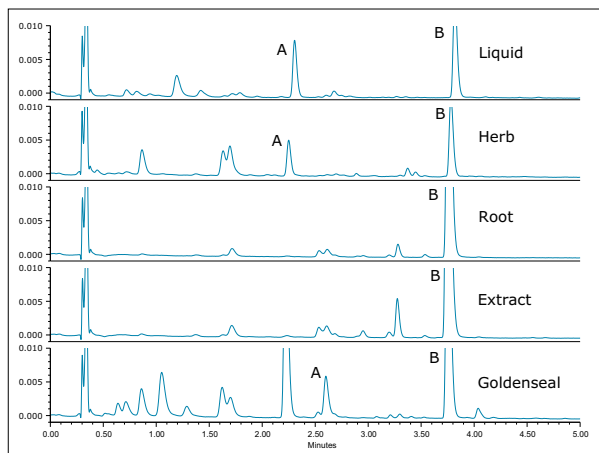


Figure 2. Zoomed in UV chromatograms of the five samples from Figure 1.

The distinct differences in the UV chromatograms of the five different samples were evident due to the high efficiency of the CORTECS Column. The intensity of peak B varied from 0.04 to 0.02 AU, indicating that different samples had different amounts of that particular compound. Further sample characterization required additional information. To obtain this, ACQUITY QDa mass spectral data was examined and compared with Goldenseal data from the literature.<sup>4</sup> A total of seven alkaloids were identified (see Table 2 and Figure 3).

Compound	Mass (M+H)
Dihydro Berberine	338.1
Canadine	340.3
Berberine	336.1
Isocorypalmine	370.1
Methyl Hydrastine	398.4
Hydrastine	384.1
Palmatine	352.2

Table 2. Summary of alkaloids identified and their masses as described in the literature.<sup>4</sup>

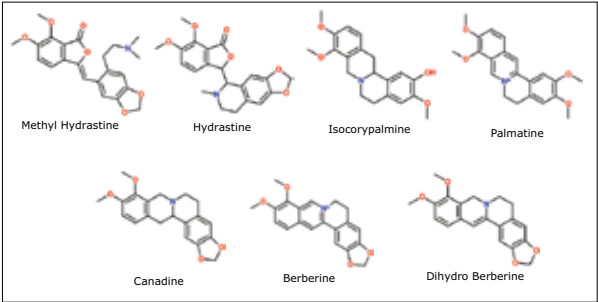


Figure 3. Structures of identified alkaloids.

Using the ACQUITY QDa Detector, some of the peaks observed in the UV chromatograms were identified. The liquid sample had the most identifiable peaks, showing the presence of all seven compounds listed in Table 2. The remaining samples each exhibited two or more identifiable peaks. Berberine was the most abundant component and the only compound present in all samples. Figure 4 shows the identification of peaks in the liquid sample using extracted ion chromatograms (EIC).

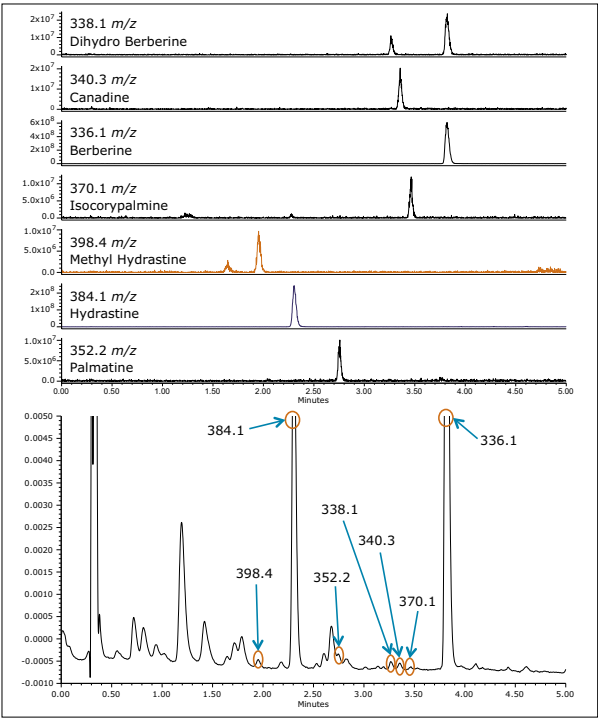


Figure 4. Identification of UV peaks in the liquid sample of Goldenseal by m/z value.

By using the ACQUITY QDa Detector, the two main component peaks (A and B in Figures 1 and 2) were identified as hydrastine and berberine, respectively. An additional five compounds were also identified and linked to peaks in the UV chromatogram. No two samples show exactly the same compounds at the same concentrations. These traces represent a type of “fingerprint” that is characteristic of each Goldenseal sample. Such fingerprints can be useful in comparing Goldenseal from different manufacturers as well as different sources of the plant. The rapid separation of a complex sample such as Goldenseal is possible due to high efficiency of CORTECS 2.7  $\mu\text{m}$  Columns. By combining UV data with the mass data obtained with an ACQUITY QDa Detector, a full characterization of each sample can be made giving an analyst valuable information with minimal effort.

## CONCLUSIONS

Natural product analysis and characterization can be a difficult process due to the complex nature of the sample. Gathering fast and reliable data is essential for characterization of complex samples such as Goldenseal in this application. Using a CORTECS C<sub>18</sub>+ 2.7  $\mu\text{m}$  Column, a complex separation can be performed more easily. CORTECS 2.7  $\mu\text{m}$  Columns offer high efficiency while operating within the pressure limits of an HPLC system. By combining the newest column technology with the newest technology in mass detection (ACQUITY QDa), a simple separation of five sources of Goldenseal was performed in five minutes and seven compounds were identified.

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SFC-UPC<sup>2</sup>-PDA

## Purification of Vanillin from Vanilla Beans Using an SFE-SFC Workflow

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### APPLICATION BENEFITS

- The MV-10 ASFE® is a supercritical fluid extraction system that extracts small amounts of sample and is capable of screening extraction parameters to optimize yield and minimize extract complexity
- Extraction and subsequent purification are achieved using non-toxic CO<sub>2</sub> and ethanol as opposed to toxic organic solvents
- A total supercritical fluid solution is demonstrated to purify vanillin from vanilla beans, a process that can be adapted to other applications

### WATERS SOLUTIONS

MV-10 ASFE® System

Prep 80q SFC System

2489 UV/Vis Detector

ChromScope™ Software

ACQUITY UPC<sup>2</sup>® SystemACQUITY UPC<sup>2</sup> PDA Detector

MassLynx® Software

ACQUITY UPC<sup>2</sup> BEH 2-Ethylpyridine Column, 130Å, 1.7 µm, 3 x 100 mm

Viridis® BEH 2-Ethylpyridine OBD Prep Column, 130Å, 5 µm, 19 x 150 mm

### KEY WORDS

Flavors, purification, SFE, SFC, UPC<sup>2</sup>, extraction, vanilla, vanillin, natural products

### INTRODUCTION

Nature is full of target compounds that are used in a variety of consumer products from pharmaceuticals and nutraceuticals to flavors and fragrances. Currently, natural flavors are in high demand, a trend that ties in with increased consumer awareness and preference for traditional or organic food.<sup>1</sup> Vanillin is the world's most popular flavor, used in the production of ice-cream, yogurt, beverages, baked goods, cereals, and even chocolate. Vanillin is the primary flavor component that lends that signature vanilla flavor and is present in varying amounts depending on the origin and treatment of the beans.<sup>2</sup> Natural vanilla extracts, based on their concentration level, cost around \$1800 per kg (~\$800 per lb), while synthetic vanillin costs \$25 per kg (~\$10 per lb).<sup>1</sup>

Currently, the two most widely used techniques for vanilla bean extraction are percolation (with ethanol and water) which can take 2–3 days, and the oleoresin method that uses ethanol and requires 8–9 days. Extractions using supercritical fluid CO<sub>2</sub> have been utilized as well; this process produces better quality extracts in less time that have a higher vanillin concentration and therefore demand higher prices.<sup>3,4</sup>

In a supercritical fluid workflow, CO<sub>2</sub> with or without the addition of an organic modifier is used to extract (SFE) and purify (SFC) target compounds. The CO<sub>2</sub> used as a solvent is safe and the extracts produced by this process are free from biological contaminants, have longer shelf life, high potency, and address major international concerns regarding residual solvent concentration.<sup>3</sup> Here we demonstrate a complete SFE-SFC workflow solution using an MV-10 ASFE System (MV-10) and a Prep 80q SFC System (Figure 1) for the extraction and purification of vanillin from whole vanilla beans. This process can be adapted to purify target compounds from a variety of natural products and matrices.

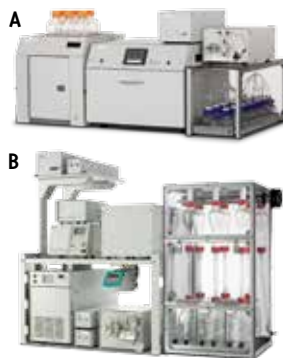


Figure 1. MV-10 ASFE (A) and Prep 80q SFC (B) systems used to extract and purify the vanillin.

## EXPERIMENTAL

## Sample description and extraction

Whole Mexican vanilla beans were obtained from a local supermarket. Initially, three SFE solvent conditions were evaluated using three 5 mL extraction vessels each containing approximately 2 grams of diced Mexican vanilla beans. By adjusting the make-up flow accordingly, the total ethanol flow rate was kept at 1.5 mL/min into the collection bottles. These conditions are shown in Table 1. The extracts were diluted to 100 mL for evaluation. Only relative yields were determined, no attempt was made to exhaustively extract the vanillin. To obtain pure vanillin, the Prep 80q SFC System was utilized to separate and collect the vanillin from the extract.

## Extract and fraction analysis

All supporting analysis was performed on an ACQUITY UPC<sup>2</sup> System. To determine extract and fraction yields, a linear calibration curve ( $R^2=0.9999$ ) was developed on the ACQUITY UPC<sup>2</sup> System using a vanillin standard at concentrations from 0.01 to 0.5 mg/mL. To determine fraction recovery on the Prep 80q SFC, an injection standard was prepared by doing five 2 mL injections and collecting the flow directly off of the injector. Both the standard and the fraction were diluted to 50 mL for analysis.

## Method conditions

## SFE conditions

System:	MV-10 ASFE System
Software:	ChromScope v1.5
Mobile phase A:	CO <sub>2</sub>
Mobile phase B:	Ethanol (200 Proof HPLC Grade)
Flow rate:	10 mL/min
Make-up solvent:	Ethanol (200 Proof HPLC Grade)
Pressure:	300 Bar
Temp.:	40 °C

## Method steps:

Vessel #	Mobile phase B (%)	Mobile phase B (mL/min)	Make-up flow (mL/min)
1	0	0	1.5
2	5	0.5	1
3	10	1	0.5

Dynamic 1: 3 min  
 Static: 60 min  
 Dynamic 2: 30 min

Table 1. MV-10 SFE method screening conditions.

## SFC 80q preparative SFC conditions

System:	Prep 80q SFC System with a 2489 UV/Vis Detector
Software:	ChromScope v1.2.1
Mobile phase A:	CO <sub>2</sub>
Mobile phase B:	Ethanol (Reagent HPLC Grade)
Flow rate:	72 mL/min
Gradient:	5 to 15% B in 4.5min
Pressure:	220 Bar
Temp.:	40 °C
UV:	267nm
Preparative column:	Viridis 2-EP Column (19 x 150mm, 5 µm)
Injection volume:	2 mL

## Extract and Fraction Analysis

System:	ACQUITY UPC <sup>2</sup> System
Software:	MassLynx v4.1
Mobile phase A:	CO <sub>2</sub>
Mobile phase B:	Ethanol (200 proof HPLC grade)
Flow rate:	1.5 mL/min
Gradient:	2 to 20% in 5 min
Pressure:	150 bar
Temp.:	40 °C
Analytical column:	ACQUITY UPC <sup>2</sup> BEH 2-EP Column (3 x 100 mm, 1.7 µm)
Injection volume:	2 µL

## PDA

Scan:	220–400nm
Absorbance compensated:	267 nm
Reference:	320–400nm



## RESULTS AND DISCUSSION

There are two main factors to consider in SFE method development – yield and extract complexity. Depending on the application, overall yield is important, but the complexity of the extract can also be important to the purification process. If an extract has a higher percentage of the compound of interest (even if the yield is relatively lower) and fewer impurities, it simplifies and improves efficiency in the collection process. If possible, CO<sub>2</sub>-only conditions are preferred for food related applications because of improved consumer safety and a lack of organic solvent waste.

Using the Mexican vanilla beans, SFE method development was performed using the automated capabilities of the MV-10 ASFE System. The software controlled automation allows the user to program various extraction conditions for multiple samples and run them unattended. Extractions of three samples were performed at 0%, 5%, and 10% ethanol. Chromatographic analysis of the resulting extracts and their yields are shown in Figure 2. The average yield was 24.2 mg, which represents 1.2% of the approximately 2 g of initial sample. There was little statistical difference in the extraction yields and complexities. Since a CO<sub>2</sub>-only method is preferred, the 0% extract was selected for purification.

Generally when purification is the goal, it is more practical for method development to be performed at the analytical scale to save solvent, time, and sample. The initial gradient on the ACQUITY UPC<sup>2</sup> System was 2–20% ethanol in 5 minutes (1.44%/cv), and the vanillin eluted at about 6%. In order to optimize the separation for purification, the gradient was modified and a loading study was performed (Figure 3). The modified gradient was 5–15% ethanol in 3 minutes (1.33%/cv). Under these conditions, separation was maintained up to 10  $\mu$ L while reducing the overall run time.

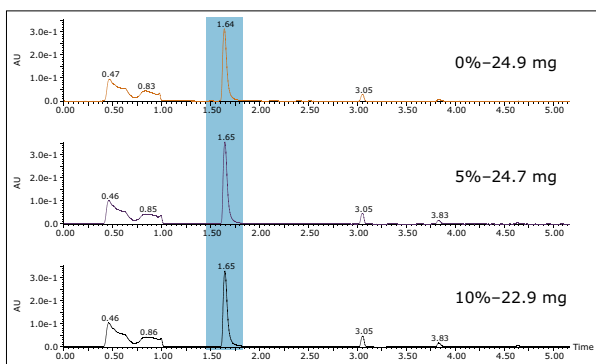


Figure 2. UPC<sup>2</sup> separations of the Mexican vanilla bean extracts and vanillin yields obtained using 0%, 5%, and 10% ethanol extraction conditions. 1.5 mL/min, 2–20% gradient in 5 min, 150 bar, 40°C, 2  $\mu$ L injection, PDA absorbance compensated at 267 nm. The vanillin peak is highlighted in blue.

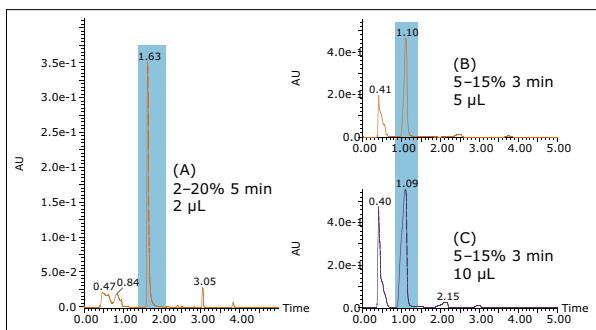


Figure 3. Scale-up method modification (A and B) and loading study (B and C) on the ACQUITY UPC<sup>2</sup> System. 1.5 mL/min, 150 bar, 40 °C, PDA absorbance compensated at 267 nm. The vanillin peak is highlighted in blue.

The optimized UPC<sup>2</sup> method parameters were scaled for the 19 x 150 mm Viridis 2-EP Prep Column, resulting in a 5–15% 4.5 min gradient. Due to the small particle size of the column and tubing I.D. on the ACQUITY UPC<sup>2</sup> System, the front pressure was 260 bar (110 bar pressure drop across the system). In order to ensure consistent separation, the two systems needed to be operated at similar pressures. To maintain a 260 bar front pressure on the Prep 80q SFC System, the BPR pressure was set to 220 bar (40 bar pressure drop). The calculated geometric scale-up was 600 µL, but the experimental separation allowed for much higher loading, so 2 mL injections were used (Figure 4).

The Prep 80q SFC System is designed for bulk purification, making it useful for applications where large amounts of a single sample need to be purified. While various collection methods can be used on the Prep 80q SFC System, in this case fractions were collected by time. The SFC 80 vanillin fraction was evaluated for purity and recovery on the ACQUITY UPC<sup>2</sup> System. Figure 5 shows collection on the SFC 80 along with analysis of the collected fraction on the UPC<sup>2</sup>. The initial extract was only 36% vanillin based on the peak area counts in the UV chromatogram, after purification on the SFC 80 the fraction was close to 100% pure vanillin (no impurities were detected). Recovery of the vanillin in the extract was greater than 90%.

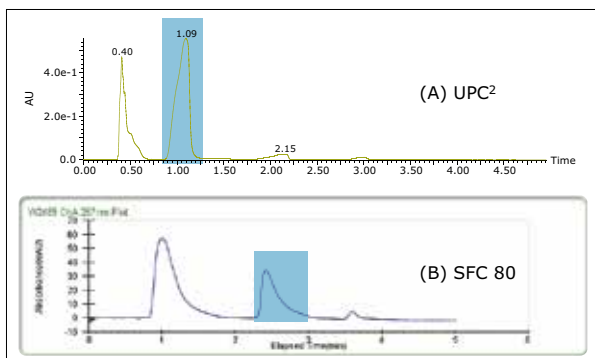


Figure 4. Chromatograms showing the method scale-up from the ACQUITY UPC<sup>2</sup> System (A) to the Prep 80q SFC system (B). Conditions: (A) 1.5 mL/min, 5–15% gradient over 3 min, 150 bar, 40 °C, 10 µL injection, PDA absorbance compensated at 267 nm (B) 72 mL/min, 5–15% gradient in 4.5 min, 220 bar, 40 °C, 2 mL injection, UV/Vis at 267nm. The vanillin peak is highlighted in blue.

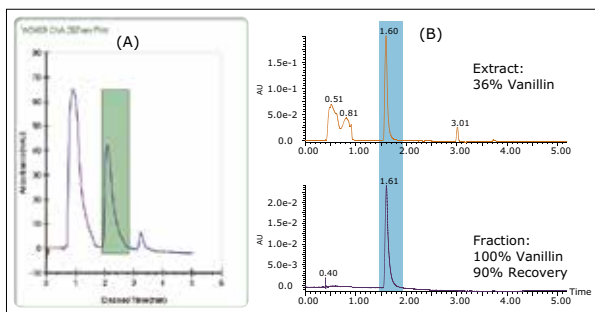


Figure 5. (A) Collection on the Prep 80q SFC System and (B) fraction analysis on the ACQUITY UPC<sup>2</sup> System compared to the extract. Conditions: (A) 72 mL/min, 5–15% gradient in 3 min, 220 bar, 40 °C, 2 mL injection, UV/Vis at 267nm, collection marks in green. (B) 1.5 mL/min, 2–20% gradient over 5 min, 150 Bar, 40 °C, 2 µL injection, PDA absorbance compensated at 267 nm. The vanillin peak is highlighted in blue.

## CONCLUSIONS

- A total supercritical fluid workflow solution is presented for the extraction, purification and analysis of vanillin in vanilla beans, utilizing less solvent and processing time than other traditional methods.
- The MV-10 ASFE System allowed for simple method development of the vanilla extraction by screening three solvent conditions using software controlled automated processes.
- Successful scale-up of the ACQUITY UPC<sup>2</sup> separation to the Prep 80q SFC was demonstrated which makes it possible to take advantage of fast method development on the ACQUITY UPC<sup>2</sup> System.
- Vanillin was successfully isolated from the raw extract using the Prep 80q SFC System.
- The entire workflow employed non-toxic CO<sub>2</sub> and ethanol as the mobile phase or extraction solvent which is ideal for food related applications.
- The process presented can be adapted to isolate target compounds in many natural product applications.

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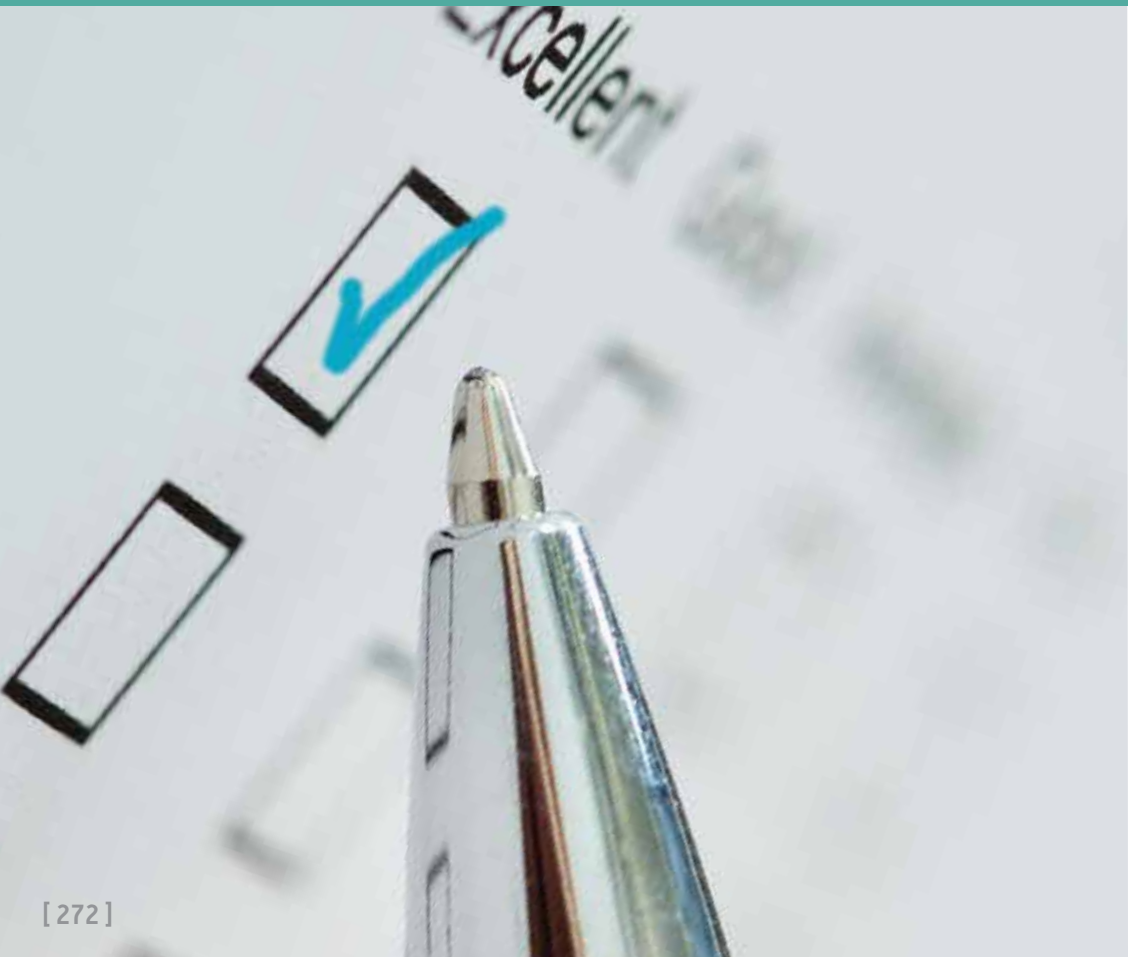
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## CHAPTER 3



### Adulteration and Counterfeit Products



## UPLC/PDA/Xevo TQ

## Detection and Identification of Synthetic Phosphodiesterase Type-5 Inhibitors in Adulterated Herbal Supplements using UPLC and Data-Directed Analysis by Mass Spectrometry

Marian Twohig, Andrew Aubin, Michael Jones, and Robert S. Plumb  
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### INTRODUCTION

There are currently three synthetic phosphodiesterase type 5 (PDE5) inhibitors that have been approved by the U.S. Food and Drug Administration for the treatment of erectile dysfunction (ED): sildenafil citrate (brand name Viagra), vardenafil hydrochloride (brand name Levitra), and tadalafil (brand name Cialis). Their chemical structures are shown in Figure 3. These medications can be obtained legally by prescription from a licensed physician and they should be used under medical supervision.

It has been extensively reported that there have been adulterations to herbal dietary supplements (HDS) with synthetic drugs. 1-4 Natural aphrodisiacs are heavily advertised on the Internet. Some claim to enhance sexual function as natural alternatives to the three approved, synthetic PDE-5 inhibitors. Recently, there have been reports that these supposed natural alternatives have actually been illicitly adulterated with one of the pharmaceutical ED drugs or their structurally-modified analogues.<sup>5-16</sup>

When an HDS product is labelled as natural, there is also a sense of security that it is safe to use. Given that an HDS could contain undeclared synthetic drugs and that it can easily be obtained over the Internet, without prescription, there is the potential for a threat to public health.

It is therefore very important that analytical methods be able to rapidly detect both known PDE5 adulterants and potentially new analogues that have not yet been reported.

In this paper, we evaluate the use of the Waters® Xevo™ TQ MS tandem quadrupole mass spectrometer, equipped with a novel collision cell design, for the detection and identification of PDE5 adulterants in herbal supplements.



Figure 1. Xevo TQ MS with the ACQUITY UPLC System.

### Survey scanning with the Xevo TQ MS

On a conventional tandem quadrupole mass spectrometer, the search for unknowns generally requires multiple injections: one injection in full-scan LC/MS mode, followed by a second injection for targeted LC/MS/MS experiments. This increases the time required to obtain necessary data, in addition to the time the analyst needs to construct MS/MS methods.

The Survey Scan feature of the Xevo TQ MS allows intelligent switching from LC/MS to LC/MS/MS data modes in a single run, thus improving productivity. Conventional MS or ScanWave™ MS scanning experiments can be used to trigger MS/MS experiments in real time as the peaks are eluting from the LC column. Conventional product ion or enhanced product ion spectra (ScanWave) data can be generated for all the components present in these complex samples.

In ScanWave mode, duty cycle improvements result in signal enhancement in scanning acquisition modes, which facilitates the detection of low-level adulterants.<sup>17</sup> A more targeted screen can also be performed, using parent ion or neutral loss spectral acquisition, to screen for compounds that have common structural features.

## EXPERIMENTAL

## LC conditions

LC system:	Waters ACQUITY UPLC® System
Solvent delivery:	ACQUITY UPLC Binary Solvent Manager
Sample delivery:	ACQUITY UPLC Sample Manager
Column:	ACQUITY UPLC BEH C18 (P/N 186002352) 2.1 x 100 mm, 1.7 µm
Column temp.:	60 °C
Sample temp.:	4 °C
Injection volume:	5 µL
Flow rate:	550 µL/min
Mobile phase A:	10 mM Ammonium acetate in water, pH 6.7
Mobile phase B:	50:50 Methanol/acetonitrile
Gradient:	0 to 9 min, 20 to 52% B, hold at 52% B until 10.5 min, 10.5 to 12.5 min 85% B, hold at 85% B until 15 min, then return to initial conditions

## MS conditions

MS system:	Waters Xevo TQ MS
Ionization mode:	ESI Positive
Capillary voltage:	3.0 kV
Cone voltage:	35 V
Collision energy:	20 to 40 eV
Desolvation temp.:	400 °C
Desolvation gas:	800 L/Hr
Collision gas:	0.15 mL/min
Source temp.:	150 °C
Acquisition range:	100 to 700 amu
Scan speed:	5000 amu/sec

## PDA conditions

Range:	210 to 400 nm
Sampling rate:	20 points/sec

## Sample extraction

The crushed tablet sample or powdered capsule contents were transferred quantitatively to a 100 mL volumetric flask, and sonicated in 50:50 methanol/water for 20 minutes. A representative aliquot was transferred to a centrifuge tube and centrifuged at 3000 RPM for 10 minutes. A portion of the supernatant was placed in a vial and used for analysis by LC/MS.

## A summary of the samples

Five products were obtained over the Internet (Figure 2) and analyzed by UPLC/MS/MS using data-directed analysis on the Xevo TQ MS. Four capsules and one tablet supplement were purchased. All five were found to be adulterated, containing sildenafil and/or tadalafil, or analogues of these drugs.

- Sample 1, a capsule, was found to be adulterated with tadalafil only.
- Sample 2, a capsule, contained sildenafil, although it shared the same product name as Sample 1 but was shipped from a different geographical location.
- Sample 3, the tablet, contained sildenafil, tadalafil, and other compounds we believe are related to the API.
- Sample 4, a capsule, contained sildenafil and tadalafil.
- Sample 5, a capsule, contained what we suspect to be analogues of the known synthetic PDE5 inhibitors.



Figure 2. Some of the adulterated herbal supplement products obtained over the Internet.

## Survey scan of sildenafil, vardenafil and tadalafil standards

A survey scan of a standard solution of sildenafil, vardenafil, and tadalafil is shown in Figure 4. The primary function is ScanWave MS, which switches to ScanWave DS when a peak is detected to acquire a full MS/MS spectrum from 50 to 700 amu at a collision energy of 30 eV.

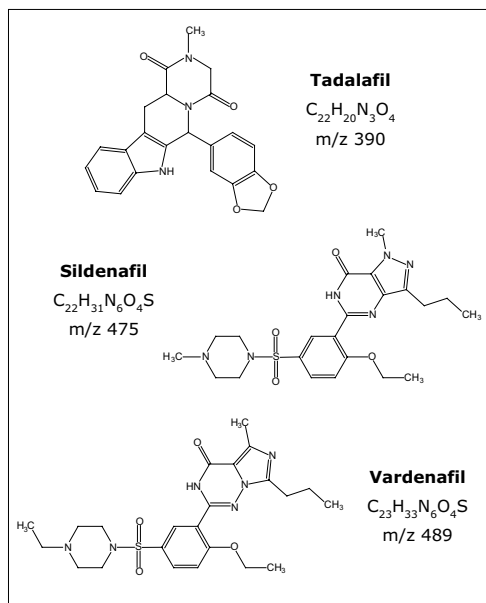


Figure 3. Structures of tadalafil, sildenafil, and vardenafil.

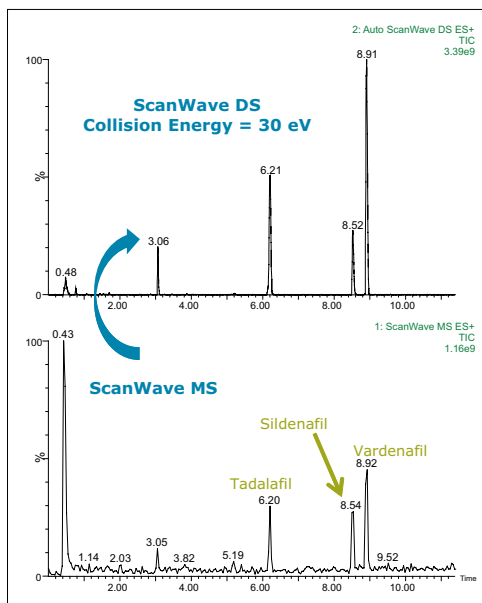


Figure 4. Survey ScanWave MS (lower trace) switching to ScanWave DS for a standard mix of tadalafil, RT 6.21 min, sildenafil, RT 8.52 min, and vardenafil, RT 8.91 min.

In the spectrum for tadalafil (Figure 5) from the peak eluting at retention time (RT) 6.21 min,  $m/z$  390, several diagnostic fragments are seen:  $m/z$  268,  $m/z$  262,  $m/z$  169, and  $m/z$  135.

In the spectrum for sildenafil, RT 8.52 min, fragments  $m/z$  377,  $m/z$  311,  $m/z$  283, and  $m/z$  99 are seen.

The spectrum for vardenafil, at RT 8.91 min, shows MS/MS fragments of  $m/z$  312,  $m/z$  299,  $m/z$  169, and  $m/z$  99. These ions can be used to confirm peak identity.

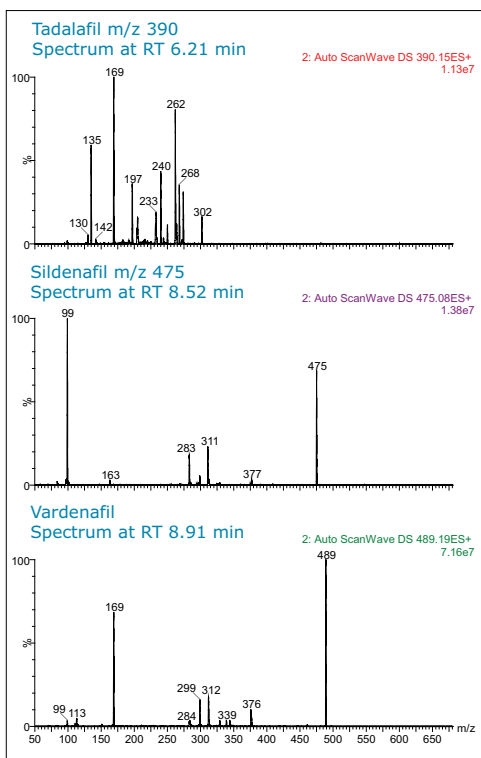


Figure 5. Spectra in ScanWave DS mode for tadalafil, sildenafil, and vardenafil, of the chromatographic peaks shown in Figure 3 (top).



## Tadalafil adulteration

Sample 1 was found to be adulterated with tadalafil, as can be seen in Figures 6 and 7. The spectrum of a tadalafil standard at a collision energy of 35 eV (Figure 7 inset) exactly matches the spectrum taken at 6.18 minutes in the Sample 1 chromatogram. The survey scan was set up to collect two MS/MS functions,

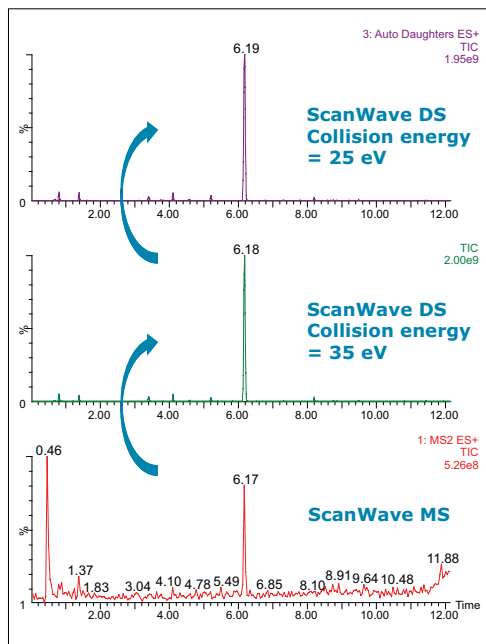


Figure 6. Survey ScanWave MS (lower trace) switching to ScanWave DS at collision energies of 25 and 35 eV for Sample 1.

one at a collision energy of 25 eV and a second at 35 eV. This is advantageous in the analysis of complex samples, where the range of collision energies required to obtain useful structural information can vary.

The signature fragments of tadalafil,  $m/z$  268,  $m/z$  169, and  $m/z$  135, can be seen in the spectra.

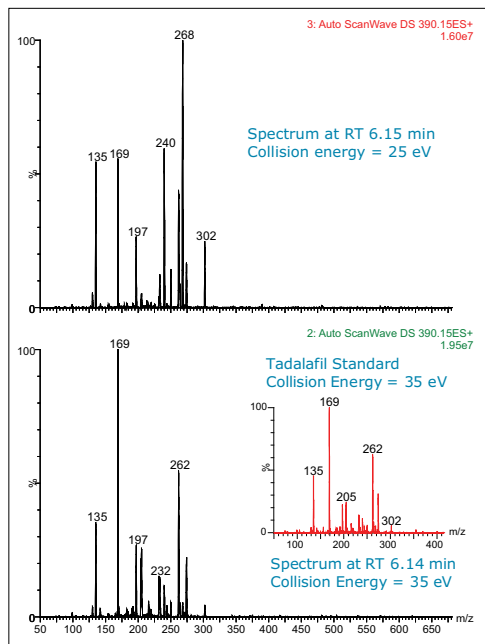


Figure 7. MS/MS spectra in ScanWave DS mode from the chromatographic peaks at RT 6.18 min at collision energies of 25 and 35 eV.

This sample indicated the presence of many natural ingredients, including *Dioscorea spinosina* (wolfberry fruit), *Glycyrrhiza glabra* (licorice root), as well as others. Neither the patient information nor the packaging declared the presence of tadalafil in the product.

### Tadalafil and sildenafil adulteration

Sample 3, in tablet form, was found to be adulterated with tadalafil and sildenafil. A survey function is shown in Figure 8 and selected spectra are shown in Figure 9. The spectra taken at RT 6.18 min and RT 8.5 min match with the spectra from a standard mix of tadalafil and sildenafil (see Figure 4). The survey scan was set up to collect two collision energy functions, one at 25 eV and a second at 35 eV. The expected diagnostic fragments were apparent in the sildenafil spectrum  $m/z$  311,  $m/z$  283, and  $m/z$  99.

In addition to finding known compounds, it can be seen from the MS/MS fragmentation patterns in Figure 9 that there are other

potentially related impurities in the sample. Many of them are above the ICH guideline's reporting threshold of 0.05% of the active pharmaceutical ingredient (API) peak (measured at 230 nm). In spectrum 1 shown in Figure 9, the precursor mass of  $m/z$  503 gives rise to MS/MS fragments  $m/z$  99,  $m/z$  283, and  $m/z$  311.

In spectra numbers 2, 4, 5, 7, and 8, one or more of these fragments are present. This information was obtained from one survey experiment without the need for extra confirmatory MS/MS analyses. This allows the analyst to acquire important structural information in a single run.

The patient information leaflet for this tablet sample said it contained 10 rare animal and plant extracts. It also stated on the packaging and in the patient information that it was acceptable for persons with heart disease and hypertension to take it. However, use of synthetic PDE5 inhibitors is contraindicated when a patient is currently taking nitrate medications (i.e., nitroglycerin) as it can cause low blood pressure –

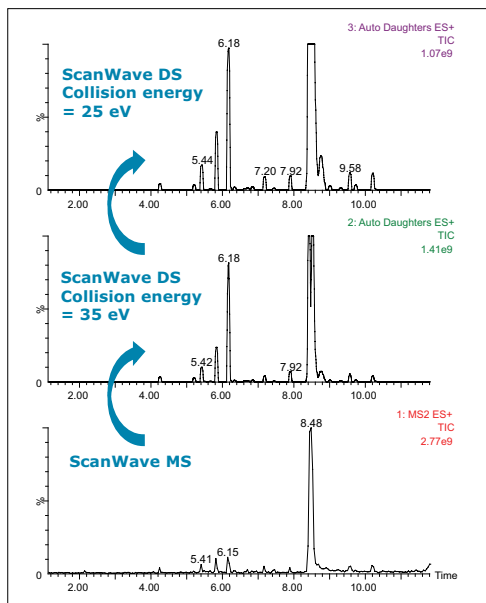


Figure 8. Survey ScanWave MS (lower trace) switching to ScanWave DS at a collision of 25 and 35 eV for Sample 3.

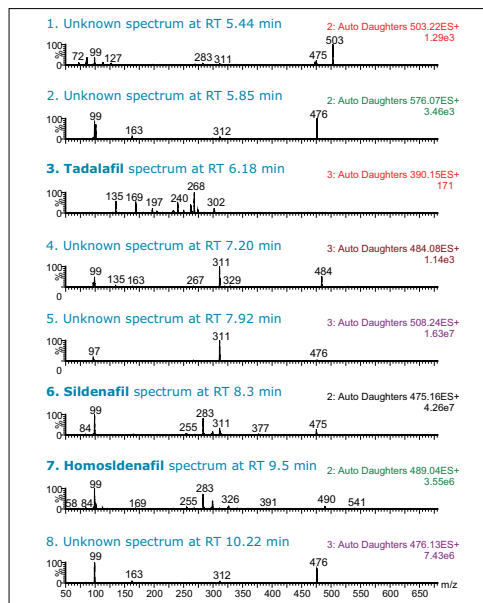


Figure 9. MS/MS spectra in ScanWave DS mode from selected chromatographic peaks shown in Figure 8.

there can be a synergistic effect of these drugs in relaxing vascular smooth muscle and drastically lowering blood pressure.<sup>18</sup> Consequently, men in this group who suffer from ED may resort to seeking out this kind of herbal alternative – not knowing the remedy's actual risk to their health.

## Adulteration with analogues

Sample 5 was analyzed using a more targeted approach. A precursor scan of  $m/z$  99 was used to trigger a ScanWave DS MS/MS function. Sildenafil, tadalafil, and vardenafil were not found in this sample. A chromatographic peak with a RT of 8.38 min had precursor mass of  $m/z$  489. The retention time of this component did not match that of vardenafil,  $m/z$  489 at RT 9.5 min, or homosildenafil,  $m/z$  489 at RT 9.5 min.

However, when Sample 5 was subjected to MS/MS fragmentation, the characteristic fragments of sildenafil were seen:  $m/z$  99,  $m/z$  283, and  $m/z$  311. There was also another fragment observed,  $m/z$  113. The presence of this ion in certain analogues has been reported previously in literature.<sup>13</sup> The other chromatographic peaks, at retention times 10.70, 12.06, and 12.71 min, showed common fragments for the  $m/z$  99 and  $m/z$  113. These peaks are likely to arise from structural analogues of sildenafil.

This sample also declared on its packaging that it is all-natural, stating that it has helped support male performance for centuries. The supplement's ingredients were supposed to contain wild yam extract, Siberian Ginseng extract, jujube extract, and cayenne extract, as well as others

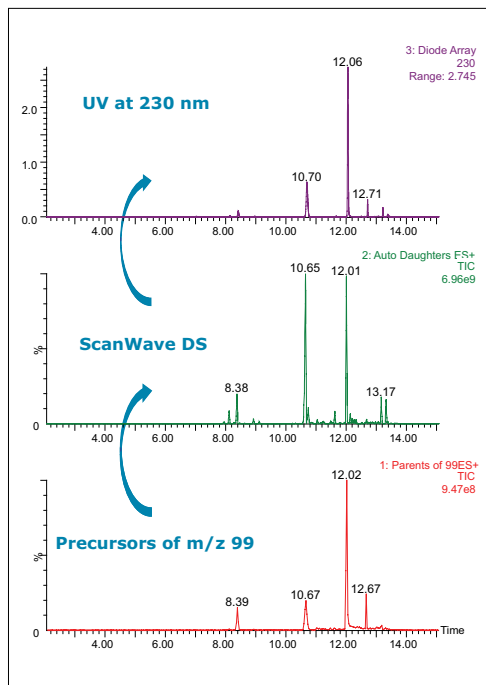


Figure 10. Survey precursors of  $m/z$  99 are observed (lower trace) when switching to ScanWave DS for Sample 5. UV data was collected simultaneously.

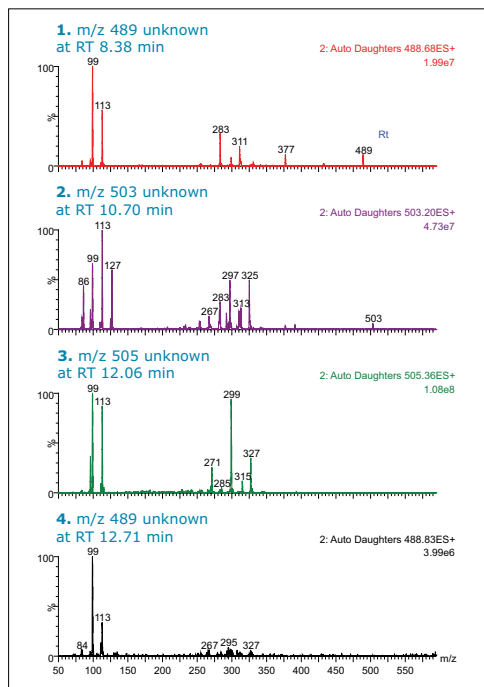


Figure 11. MS/MS Spectra in ScanWave DS mode from selected chromatographic peaks shown in Figure 9.

## Quantitation of tadalafil and sildenafil in HDS samples

In an effort to understand the levels of adulteration to the samples, sildenafil and tadalafil were quantified. The drug substances for tadalafil and sildenafil citrate were purchased. Stock solutions of sildenafil and tadalafil were prepared at a concentration of 1.0 mg/mL in methanol. Working standard solutions were prepared at concentrations of 0.1 ng/mL to

500 ng/mL in a simulated matrix. This was performed using extraction of a mixture of some of the ingredients listed on the patient information of the HDS samples and subsequently spiking the resulting solution with the appropriate levels. Quantitation curves were injected in triplicate and were linear over the calibration range for both tadalafil and sildenafil (Figures 12 and 13). QC samples were within acceptable limits (< 15%).

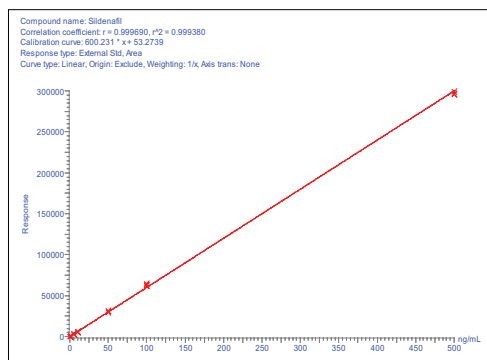


Figure 12. Quantitation curve for sildenafil 0.1 to 500 ng/mL.

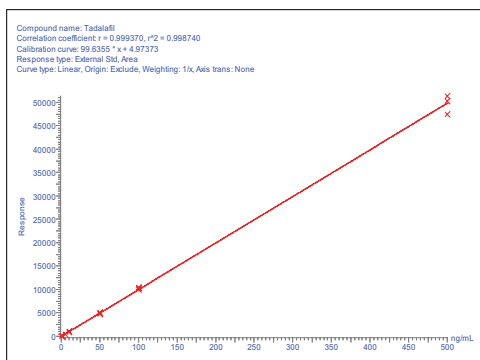


Figure 13. Quantitation curve for tadalafil, 0.1 to 500 ng/mL.

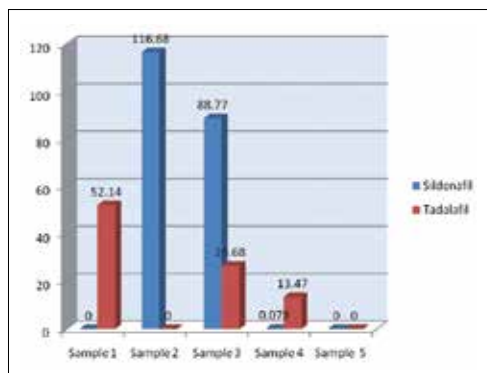


Figure 14. Summary of the calculated concentrations of sildenafil and tadalafil for the HDS Samples 1 through 5.

The range of sildenafil measurements quantified in the HDS samples that were purchased via the Internet was 0.078 to 116.68 mg/dose. The range of tadalafil measurements was 13.47 to 52.14 mg/dose.

For samples 1 through 4, these concentrations would be at therapeutic levels.

- In Sample 2, the sildenafil was calculated to be 116.7 mg/dose.
- Sample 3 contained both sildenafil and tadalafil at levels of 88.7 and 26.7 mg/dose.
- Sample 5 could not be accurately quantified as it contained suspected analogues for which there was no available standard.

It should be noted that the analytical method used for quantitation of the samples had not been validated at the time when these results were reported.

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## CONCLUSIONS

- Adulteration of herbal dietary supplements with synthetic pharmaceuticals is a growing problem. There have been reports in published literature that show many of the supposed natural alternatives to approved synthetic PDE5 inhibitors used to treat ED are actually adulterated with them. Many reports also indicate that this adulteration of herbal additives is a growing trend. Due to the threat to public health posed by unknowingly ingesting both the known PDE5 inhibitors and their analogues, it is vital that analysts have tools that allow them to characterize these complex samples adequately.
- The Waters Xevo TQ Mass Spectrometer, with its unique collision cell design, facilitates the simultaneous acquisition of MS and MS/MS data in one LC/MS run. Its high scan speed of up to 10,000 amu/sec allows for these experiments to be performed with sufficient points across the peak to accurately define the narrow peaks produced by UPLC. This capability facilitates data-dependant experiments where real-time switching between MS and MS/MS allows more information to be acquired from a single injection. This reduces the need for separate experiments and accelerates the process of structural identification and unknown compound determination.
- The value of using the Waters Xevo TQ MS has been demonstrated for the analysis of adulterated herbal dietary supplements. Furthermore, use of the Xevo TQ MS for quantitation of the samples revealed that the doses of the sildenafil and tadalafil are sufficiently high to be therapeutic.

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## ASAP / LCT PREMIER XE (QToF) MS

## Rapid Detection and Identification of Synthetic Phosphodiesterase Type-5 Inhibitors in Counterfeit and Adulterated Products Using the Atmospheric Solids Analysis Probe

Marian Twhig, Gordon Fujimoto, Nicholas Ellor, and Robert S. Plumb  
Waters Corporation, Milford, MA, USA

### INTRODUCTION

When hyphenated methods such as LC-MS and GC-MS are used in situations that require high-throughput sample screening, sample extraction and chromatographic separation can create a processing bottleneck. In recent years, novel ambient desorption ionization techniques for surface analysis of solid and liquid samples with subsequent MS detection have been reported. Techniques include desorption electrospray ionization<sup>1</sup> (DESI), direct analysis in real time<sup>2</sup> (DART), and use of an atmospheric solids analysis probe<sup>3</sup> (ASAP).

The advantage of using these direct ionization methods is that sample preparation is often minimal or absent altogether. Total analysis time can be decreased significantly due to the elimination of the chromatographic separation. Additionally, direct ionization techniques are a good first step to determine the necessity of engaging in chromatographic separation.

ASAP was invented by McEwen et al.<sup>3</sup> and can be used to analyze volatile or semi-volatile solid or liquid samples using atmospheric pressure ionization (API). The sample is applied to a glass melting point capillary and vaporizes when inserted into a heated stream of gas (100 to 500 °C). A corona discharge is used for ionization (Figure 1). The probe is readily fitted to an API source by replacing the electrospray (ESI) or atmospheric pressure chemical ionization (APCI) probe and ensuring that a corona pin is installed. The probe consists of two parts: an outer assembly and an inner probe (Figure 2) that holds the melting point capillary securely in place.

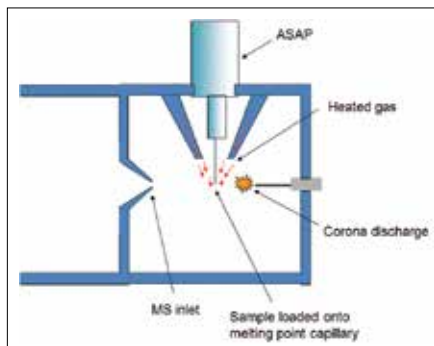


Figure 1. Illustration of ionization using ASAP. Used with permission.<sup>19</sup>



Figure 2. The ASAP Probe. A shows outer assembly and inner probe. B shows the assembled device.

The probe inserts into the source through a lock. When removed, the source is closed to atmosphere. Once the probe is installed the sample is completely enclosed by the source. With other direct ionization methods, the sample is exposed to the environment. This leads to safety concerns about sample vapors reaching the surrounding air, and analytical concerns about the impact that ambient conditions may have on the sample itself.

In this application note we examine the utility of the Waters® Atmospheric Pressure Solids Analysis Probe for the rapid determination of synthetic phosphodiesterase type-5 inhibitors in counterfeit tablet samples (Figure 3) and adulterated herbal supplements (Figure 4).

### Phosphodiesterase type-5 counterfeiting and adulteration

Pharmaceutical counterfeiting is a global phenomenon and the number of detected cases continues to grow.<sup>4-7</sup> The Center for Medicine in the Public Interest predicts that counterfeit medicine sales will reach approx. €55.5 billion globally by 2010.<sup>8</sup>

The pervasive success of the three approved synthetic phosphodiesterase type-5 (PDE-5) inhibitors for the treatment of erectile dysfunction (ED) has led to an explosion in the number of detected cases of counterfeit sildenafil citrate (brand name Viagra), vardenafil hydrochloride (brand name Levitra), and tadalafil (brand name Cialis) (Figure 5). In addition to the reported detection of counterfeit tablet forms of these products, herbal dietary supplements (HDS) that claim to be all-natural alternatives to the PDE-5 inhibitors are being heavily advertised on the Internet.

Recently, there have been reports that these supposed natural alternatives to the drugs used to treat ED have been illicitly adulterated with the pharmaceutical products or their structurally-modified analogues.<sup>9-17</sup> When a HDS product is labelled as natural, there is also an implicit assumption that it is safe. Given that the HDS could contain undeclared synthetic drugs and can be obtained without prescription easily over the Internet, there is the potential for a threat to public health.



Figure 3. Shown is a picture of some of the imitation brand and generic products for treatment of ED.



Figure 4. Shown is a picture of some of the adulterated products obtained over the Internet.

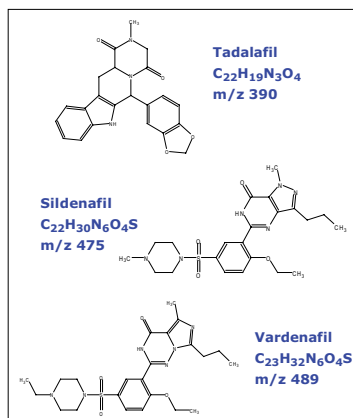


Figure 5. Structures of tadalafil, sildenafil, and vardenafil.



## EXPERIMENTAL

### Sample procurement

Counterfeit tablet samples: Imitation “brand” and “generic” samples of these drugs (there are currently no approved generic equivalents for the three approved synthetic PDE-5 inhibitors) were obtained from Internet pharmacies.

Herbal dietary supplements (HDS): Five products were obtained over the Internet and analyzed by ASAP with high-resolution mass spectrometry. Four capsules and one tablet were purchased. All five were found to be adulterated, containing sildenafil, and/or tadalafil, or analogues of these drugs – none of which were declared on the box or enclosed information.

### MS conditions

MS system:	LCT Premier™ XE Mass Spectrometer
Ionization mode:	ESCI positive
Capillary voltage:	3.0 kV (ESI)
Corona current:	5 $\mu$ A (APCI)
Cone voltage:	40 V
Aperture 1:	15 V
Desolvation temp.:	100 to 450 °C
Desolvation gas:	500 L/Hr
Source temp.:	120 °C
Acquisition range:	100 to 1000 amu
Scan time:	0.5 sec
Lock Mass reference:	Leucine Enkephalin (ESI)

The instrument was operated in combined ESI/APCI mode (ESCI®). This enabled the acquisition of analyte data in APCI mode and reference data in ESI mode.

### Sample loading

Tablet samples were loaded onto the glass capillary by first exposing the inside of the tablet and making contact between the glass capillary and the inner pill.

The herbal supplement samples were predominantly capsules containing fine powder. Once the sample was applied to the glass capillary, excess was then removed using a stream of nitrogen.

### Sildenafil citrate, vardenafil hydrochloride, and tadalafil tablet samples

Authentic brand sildenafil citrate, vardenafil hydrochloride, and tadalafil were obtained from reputable pharmaceutical wholesalers.

Thirteen sample profiles from legitimate products and Internet pharmacy samples are shown in Figure 6. Tablet samples purchased from one Internet pharmacy were manufactured to look like authentic sildenafil citrate, vardenafil hydrochloride, and tadalafil tablets. While the appearance of these tablets conformed to the appearance of the genuine medicines, results from using ASAP with time-of-flight (TOF) MS detection showed that the vardenafil and tadalafil pills contained the wrong active pharmaceutical ingredient (API). Data showed that the sildenafil citrate tablet did contain the correct API ( $m/z$  475); however the vardenafil ( $m/z$  489) and tadalafil ( $m/z$  390) samples did not (Figures 7 and 8).

This case alone highlights the level of risk a consumer is taking when purchasing drugs from random and uncertified Internet pharmacies.

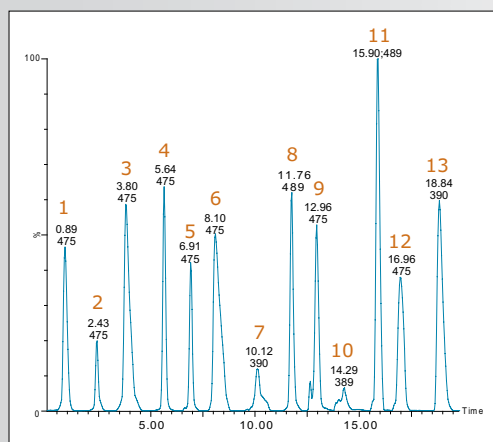


Figure 6. ASAP sample vaporization profiles (desolvation temperature is ramped from 100 to 450 °C) for 13 tablet samples obtained over the internet and authentic products obtained from reputable sources.

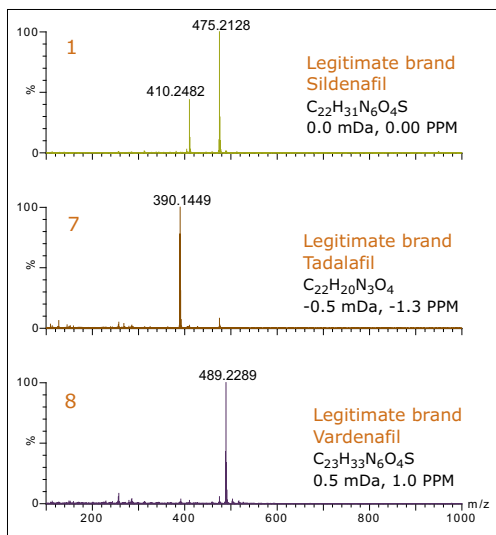


Figure 7. Elemental composition results for legitimate sildenafil, tadalafil, and vardenafil.

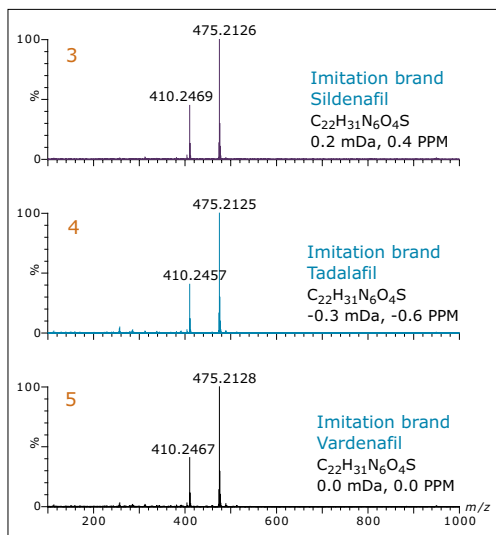


Figure 8. Elemental composition results for imitation-brand sildenafil, tadalafil, and vardenafil. See also Figure 9.

The principal active component in 13 tablet samples was identified by ASAP in less than 20 minutes. Sample extraction was not necessary and chromatographic separation was not required to determine if the sample contained what it claimed.

In certain analyses, it is sometimes sufficient to get a yes/no answer before the sample is passed for further characterization (Figure 9). ASAP used in conjunction with TOF-MS detection provides a simple way to obtain this answer rapidly.

Detailed results obtained from subsequent analysis by UPLC® coupled to TOF-MS or MS/MS data and PDA spectral comparisons agree with the results obtained by ASAP for all of the samples analyzed.

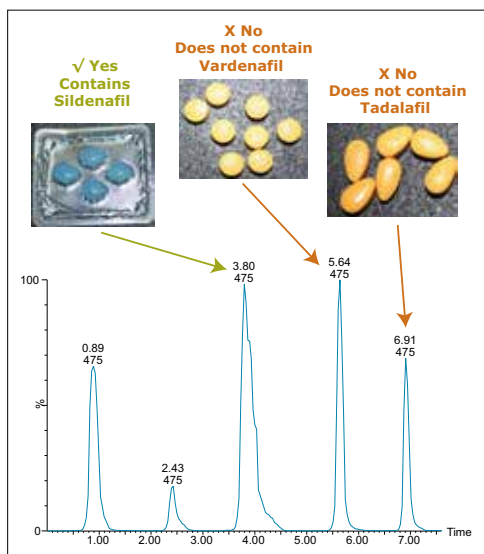


Figure 9. Profiles for the counterfeit tablet samples. ASAP with TOF-MS detection identified the vardenafil and tadalafil to be fraudulent, counterfeit tablets.

## Adulteration of HDS samples with synthetic PDE-5 inhibitors

Five herbal products purchased on the Internet were analyzed using ASAP with TOF-MS detection.

From the analyses, it was possible to show that all five were adulterated with tadalafil, and/or sildenafil, or suspected analogues.

Sample 1 was found to be adulterated with tadalafil ( $m/z$  390) as can be seen from spectrum 1 in

Figure 11. Package information for this sample declared the presence of many natural ingredients, including *dioscorea spinosina* (wolfberry fruit) and *glycyrrhiza glabra* (licorice root). Neither the patient information nor its packaging declared the presence of tadalafil.

In each case, the principle component identified in the sample agreed with that determined when the same HDS sample was analyzed by UPLC-MS/MS utilizing a Waters Xevo® TQ MS with simultaneous UV detection.<sup>20</sup> Sample 2 showed that it was adulterated with sildenafil; interestingly this sample shared the same product name as sample 1 but was shipped from a different geographical location (Europe versus China).

In samples 3 and 4, mixed adulteration was identified using ASAP. Sample 3 was adulterated with tadalafil and a higher level of sildenafil. Sample 4 had tadalafil as the major adulterant with less sildenafil. Quantitation of the samples using LC/MRM-MS revealed that the doses of the sildenafil and tadalafil are sufficiently high to be therapeutic.

The use of ASAP in combination with accurate mass MS detection identified the presence of a suspected analogue, with suggested elemental composition of  $C_{23}H_{33}N_6O_3S_2$  (-0.3 mDa, -0.6 PPM). It is suspected that this analogue is thiohomosildenafil ( $m/z$  505), where one oxygen is substituted with a sulphur atom and an ethyl group replaces the methyl group attached to the piperazinyll nitrogen.<sup>17</sup>

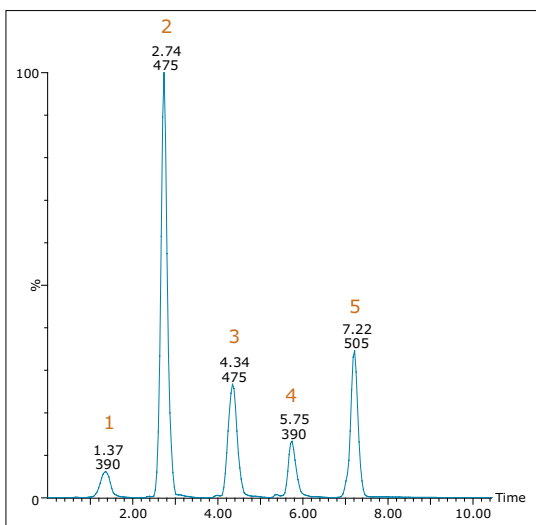


Figure 10. Vaporization profiles of the five HDS samples.

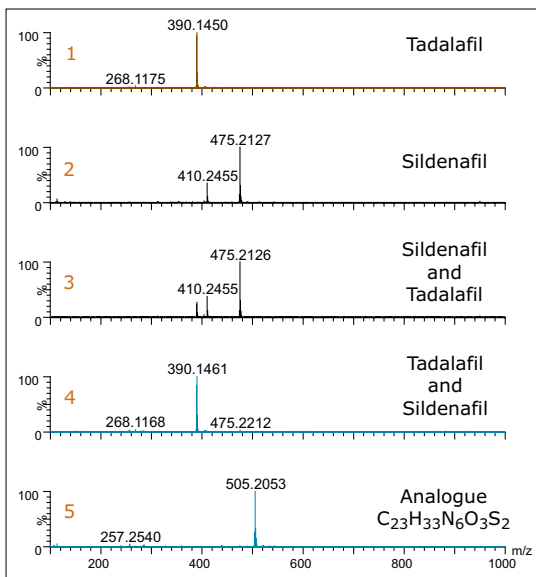


Figure 11. Spectra from the direct analysis of five HDS samples.

When MS/MS fragmentation of this sample was carried out in previous work (data not shown), a characteristic fragment of sildenafil,  $m/z$  99, and related compounds were observed. Other fragments observed included  $m/z$  113, 299, 327, and 393. These fragments have been reported in certain analogues including thiohomosildenafil in the literature.<sup>13,16,17,18</sup>

Sample 5 was also declared to be all-natural. Packaging stated its ingredients had helped to “support male performance” for centuries. It was said to contain wild yam extract, Siberian Ginseng extract, jujube extract, and cayenne extract, as well as others.

## CONCLUSIONS

The illegal counterfeiting of pharmaceuticals and adulteration of herbal dietary supplements with synthetic pharmaceuticals has been recognized as a growing global problem, with the number of detected cases rising every year. This has created a demand for high-throughput analytical techniques. When hyphenated methods such as LC-MS and GC-MS are used to analyse these samples, the need for chromatographic separation and sample extraction often creates a sample processing bottleneck.

The ASAP probe enables direct sample analysis by mass spectrometry, therefore providing a very useful tool for rapidly screening large amounts of solid or liquid samples, providing that they are sufficiently volatile.

The probe can be fitted very quickly to a mass spectrometer's API source by simply replacing the electrospray (ESI) or atmospheric pressure chemical ionization (APCI) probes and ensuring that a corona pin is installed.

When paired with TOF-MS, the ASAP probe enables analysts to rapidly identify unknown compounds using exact mass measurement and elemental composition determination with isotope ratio comparison (iFIT™). Using ASAP, the turnaround time from sample receipt to structural identification and unknown compound determination is greatly accelerated.

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## Screening Synthetic Adulterants from Herbal and Dietary Supplements (HDS) Using the Natural Products Application Solution with UNIFI

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### APPLICATION BENEFITS

- The Adulterant Screening Application within the Natural Products Application Solution with UNIFI® consists of a set of dedicated workflows
- The Natural Products Application Solution with UNIFI offers a turn-key solution from sample to report that utilizes ultra performance liquid chromatography (UPLC®), coupled with a quadrupole time of flight mass spectrometer (Q-ToF™ MS) for data acquisition
- Significant enhancement in efficiency and ease of use for the screening and identification of synthetic adulterants with minimal requirement for an operator's technical expertise

### WATERS SOLUTIONS

[ACQUITY UPLC® I-Class System](#)

[Xevo® G2-XS QToF Mass Spectrometer](#)

[Natural Products Application Solution with UNIFI](#)

### KEY WORDS

Identification of synthetic adulterants, adulteration, UNIFI Synthetic Adulterants Library, UPLC/Q-ToF MS, data independent acquisition, herbal supplements, dietary supplements, LC/MS workflow

### INTRODUCTION

In recent years, there has been a tremendous increase in the use of natural products in the form of herbal supplements for health benefits and therapeutic effects. Unfortunately, this rise in usage has also led to the increased chance of products being adulterated with synthetic compounds by unscrupulous manufacturers. There have been reports of severe to even fatal cases<sup>1</sup> related to adulteration in herbal products. This has raised global concerns within the natural products community and with various regulatory authorities since it poses a significant risk to consumers.

Liquid chromatography coupled with mass spectrometry (LC–MS) is widely-used for screening herbal supplement products due to their high sensitivity and selectivity, which is critical for analyzing complex matrices typical of natural product samples. However, a major challenge for LC-MS screening has been the lack of robust streamlined workflows that allow quick and easy processing from sample to report.

Here, we demonstrate the utility of the Natural Products Application Solution with UNIFI Adulterant Screening Application and the Synthetic Adulterant Library with the analysis of a commercial herbal supplement product. This product is a herbal capsule sold by street vendors to alleviate back and joint pain (in rheumatoid arthritis). The label claim indicates that it contains various herbs such as *Picrorhiza kurroa* and *Strychnos nux-vomica* Linn. Using the Natural Products Application Solution with UNIFI Adulterant Screening Application, we were able to automatically detect the existence of any synthetic compounds, and then confirm the identified adulterants through an easy review process, to quickly draw a conclusion.

We showcase a turn-key solution with a streamlined process from sample to report using the Adulterant Screening Application within the Natural Products Application Solution with UNIFI. Figure 1 displays the entire analytical process, which integrates all steps (data acquisition, peak picking, library searching, fragment ion confirmation, and report generation) into a streamlined process. Additionally, structure elucidation tools are available to allow further investigation on unknown peaks that did not show matches from the Synthetic Adulterants Library.

EXPERIMENTAL

Sample preparation

1 g of powdered sample (Figure 2) in 10 mL of LC-MS grade methanol solution was sonicated for 20 minutes, followed by centrifugation at RCF of 4472 g for 5 minutes. The supernatant was diluted 250 times with methanol prior to injection (1 µL).

Catechin standard mixture (p/n 186007465) was used for the system suitability test. The purchased stock solution was diluted 10x with methanol with a final concentration of 10 µg/mL. Injection volume was 1 µL.

LC conditions

LC system:	ACQUITY UPLC I-Class with Flow Through Needle (FTN) Sample Manager
Column:	ACQUITY UPLC HSS T3, 1.8 µm, 2.1 x 100 mm, 40 °C
Sample temp.:	15 °C
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid
Gradient table:	

Flow rateSolvent ASolvent B				
Time(mL/min)	(%)	(%)	Curves	
0	0.6	98	2	Initial
0.5	0.6	98	2	6
1.0	0.6	95	5	6
5.0	0.6	70	30	6
9.0	0.6	40	60	6
11.0	0.6	40	60	6
12.0	0.6	30	70	6
12.5	0.6	30	70	6
14.5	0.6	0	100	1
15	0.6	98	2	1

17 0.6 98 2 1

MS conditions

MS system:	Xevo G2-XS QTof
Acquisition range:	50–1500 Da
Scan time:	0.1 s
Acquisition mode:	ESI+ and ESI-; MS <sup>E</sup> ; resolution mode
Lock mass:	Leucine enkephalin (LE) 0.2 ppm (scan for 0.3 s, interval: 30 s)
Capillary voltage:	1.5 KV (ESI+) 1.5 KV (ESI-)
Cone voltage:	40 V
Collision energy (eV):	Low CE: 6 High CE: 10–40
Source temp.:	120 °C
Desolvation temp.:	500 °C
Cone gas flow:	50 L/h
Desolvation gas flow:	1000 L/h
Acquisition time:	17 min

Data management

Natural Products Application Solution with UNIFI for instrument control, data mining, and report generation.

## RESULTS AND DISCUSSION

The Natural Products Application Solution with UNIFI provides turn-key solutions for two types of applications: ingredient profiling for natural product extracts, and adulterants screening for herbal supplements. Each application comes with its own set of workflows. For the Ingredient Profiling Application, a set of 14 preset templates (data filters linked to specified views) are provided in a workflow which incorporates the utility of the Traditional Medicine Library. Detailed utility of this ingredient profiling application had been elaborated in previous applications notes.<sup>2-4</sup>

For the Adulterant Screening Application, a dedicated set of acquisition method templates with generic conditions and parameters is provided for data acquisition. A dedicated workflow containing a set of 7 templates is also provided, which incorporates the utility of the Synthetic Adulterants Library for adulterant screening, reviewing, and confirmation. Finally, a specific report template is provided for automatic report generation. Similarly as with the Ingredient Profiling Application, catechin standard mixture and green tea extract are provided for system suitability testing, and to facilitate familiarization with the utility of the Natural Products Application Solution with UNIFI.

When performing the screening and identification of synthetic adulterants in herbal supplements by LC-MS, one of the most difficult challenges comes from the fact that a fit for purpose library is not available. To solve this challenge, and to help enhance the speed of the entire analytical process, the Synthetic Adulterants Library was created and incorporated into the Adulterants Screening Application as a critical component.

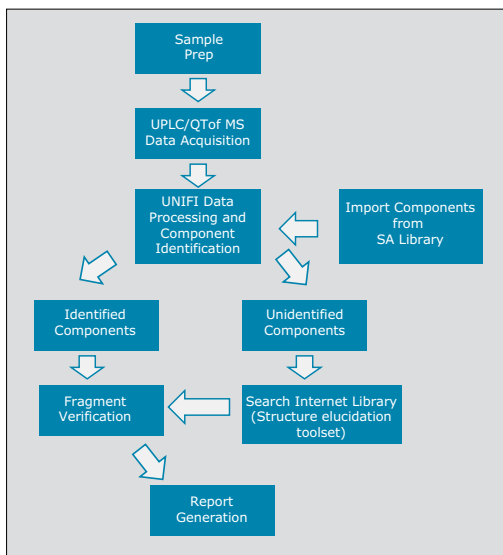


Figure 1. The step by step procedure of the Natural Products Application Solution with UNIFI Adulterant Screening Application.



Figure 2. Powdered anti-inflammatory herbal supplement product obtained from a street vendor.

Figure 3 shows the detailed screen capture of the Synthetic Adulterants Library. The library contains a list of synthetic compounds that are frequently found in herbal supplement products as adulterants. These compound names are listed both in English and in simplified Mandarin, and grouped according to their known pharmacological actions. Other relevant information such as chemical structure, molecular formula, average molecular and mono-isotopic exact molecular mass, CAS number, Chempidder ID, and PubChem ID are also included for each compound. In addition, for each of the compound entry, key diagnostic fragment ions are included.

Figure 3A shows the 'Properties' tab for a compound, displaying its name, molecular formula, and other identifiers. Figure 3B shows the 'Pharmacological Actions' tab, listing various actions such as 'Anti-inflammatory', 'Analgesic', and 'Antispasmodic'.

Figure 3. This screen shows the infra-structure of the Synthetic Adulterants Library. A) Example screen capture for each of the compound entries. B) List of the pharmacological actions from the item tags.

For UPLC/Q-ToF MS experiments, MS<sup>E</sup> data acquisition mode was used for sample analysis, which in essence is a data independent acquisition strategy. From a single LC injection, the mass spectrometer is collecting data in two independent functions, one with collision energy set to low so that the MS full scan precursor ion information is collected, and another with collision energy set to high, so that corresponding MS/MS fragment ion information is collected. The two sets of data are connected and correlated by the LC retention time, as a result for each of the analytes in the sample, both MS and MS/MS information is obtained. Figure 4 shows the two UPLC/Q-ToF MS base peak ion (BPI) chromatograms obtained from the single injection on the test sample. Figure 4A is the BPI from the low CE scan, and Figure 4B is the BPI from the high CE scan.

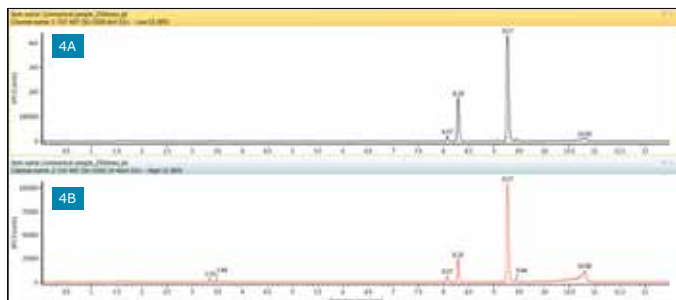


Figure 4. The UPLC/Q-ToF MS BPI chromatograms of an anti-inflammatory capsule. 4A) BPI from a low CE scan; 4B) BPI from a high CE scan.



Data processing is automatic and requires minor parameter setting as templates are provided in the Natural Products Application Solution with UNIFI Adulterant Screening Application. The only user intervention required is to enable sample specific processing, to ensure that the correct analysis method is chosen, the peak processing time window matches the specific analysis, and to ensure that the Synthetic Adulterants Library is connected to the analysis method. The next step is for the user to go to the review page, click the Process tab, and wait for the result to display while all process steps take place automatically.

The steps involved after the Process button is clicked are as follows: 1. Peak picking based on 3D Apex Peak Detection, so a complete peak list is obtained for this sample. The peak picking happens simultaneously for both low CE MS full scan and high CE MS full scan mode. 2. Matching of the component list obtained from the Low CE MS full scan with the parent ion information that was obtained from the library. 3. Fragment ion confirmation by either matching with known key diagnostic fragments ions from the library, or matching with predicted fragment ions generated with MassFragment™ in high CE MS<sup>2</sup> full scan. MassFragment is an *in-silico* informatics tool which utilizes mol file structure of a given compound to predict possible fragmentation patterns based on the soft spot of that molecule. This is significantly helpful to confirm the correct match and to eliminate false positives.

Figure 5 shows a screen capture of the review window that displays results obtained at the end of process. This review window is consistent across UNIFI Scientific Information System. Results are organized in multiple segments within this window and discussed in detail below.

Figure 5A shows the available preset templates in the Adulterant Screening Application. As mentioned previously, for the Natural Products Application Solution with UNIFI, two complete sets of workflows are provided: the Ingredient Profiling Application<sup>2,4</sup> and the Adulterant Screening Application.

A set of 7 workflow templates is provided for the Adulterant Screening Application with the purpose of facilitating easy result review and confirmation. The System Suitability ensures a routine system check prior to analysis to ensure data quality. The Good Match lists all components (ion count above 5,000) that matched with the Synthetic Adulterants Library. Both parent ion (mass error <2mDa) and minimum of one diagnostic fragment ion must be present. The Tentative Match lists all components (ion count above 5,000) that match with the Synthetic Adulterants Library, parent ion only. The No Match High Intensity lists all components (ion count above 20,000) that did not match with the Synthetic Adulterants Library. The All Identified lists all components that matched with the Synthetic Adulterants Library with ion count above 5,000. The Confirmed Table and Confirmed Plot are provided for quick review of all components that have been reviewed and confirmed by the user.



Figure 5. Results displayed in the Review tab within UNIFI Scientific Information System after processing. 5A) List of the available workflows. The Adulterant Screening Application provides a total of seven preset templates. Here, ADL refers to adulterant. 5B) Shows a list of components associated with the Good Match filter. 5C) XICs of  $m/z$  309.1599 of the adulterant. 5D) MS spectra correlate to the XICs, both in low energy MS full scan and high energy MS<sup>2</sup> scan.

Figure 5B displays the list of components that correlate to each of the designated preset workflow templates. In this section, all critical information of the detected compound is listed such as expected neutral mass (Da), observed neutral mass (Da), mass error (ppm), observed retention time (minutes), response factor, and adduct ions. In this application example, for the herbal supplement we were analyzing, phenylbutazone is the only component listed under Good Match. Phenylbutazone is a nonsteroidal anti-inflammatory drug (NSAID) and has been banned for human use in US and UK as it can cause severe adverse effects such as suppression of white blood cell production and aplastic anemia.<sup>5</sup>

Figure 5C displays the extracted ion chromatogram (XIC) that correlates to the component displayed in Figure 5B. In this case, phenylbutazone from the Good Match template is selected in Figure 5B, therefore, the XIC associated with this component is displayed.

Figure 5D shows the MS spectra that correlate to the component listed in Figure 5B and the XIC displayed in Figure 5C. Here, the top spectrum is the MS full scan spectrum, and the bottom spectrum is the MS<sup>E</sup> spectrum, both correlate to the 9.27 min peak from the XIC of  $m/z$  309.1599. In the MS<sup>E</sup> spectrum, the blue mark indicates the fragment ion that matches the expected fragment list in the Synthetic Adulteration Library. For easy review, users can also choose to have all matched fragment ions listed as a

table below the high energy spectrum, as shown in Figure 5D. For phenylbutazone, all expected fragments were observed in the high energy spectrum.

With the increasing trend in adulteration in herbal supplements, new or analogues of common synthetic adulterants are continually being created, as such, regardless of the size of the library, there will always be components that will not match. Any unmatched peaks that are in high abundance warrant further investigation. These peaks will be listed under the No Match High Intensity (Figure 6A and 6B). To further investigate unmatched high intensity components, the Structural Elucidation Tool<sup>6</sup> can be used. The structural elucidation tool is easily accessible by right clicking and selecting Elucidate from the drop down list (Figure 6E). For this specific sample analysis, one component was listed under the No Match High Intensity at the retention time of 8.29 minutes with high intensity (Figure 6C).

The key steps of elucidation are displayed in sequence in Figure 7. Step 1 is to determine the elemental composition of the component based on exact mass measurement. A unique feature in the UNIFI Scientific Information System for determining elemental composition is the consideration of the MS/MS fragment ions, in addition to the precursor ion exact mass and isotope distribution in the parameter settings (as displayed in Figure 7 Step 1).

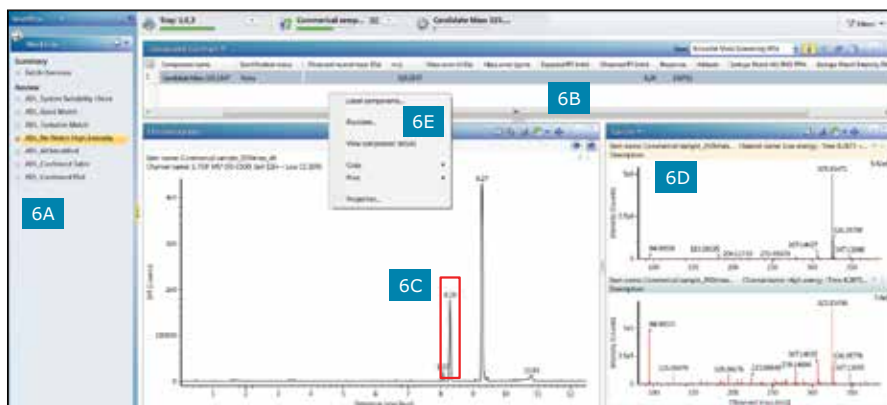


Figure 6. High intensity component (Red Box in Figure 6C) from the No Match High Intensity preset template (Figure 6A) that did not match with the Synthetic Adulteration Library. The structure elucidation tools can be used by selecting the Elucidate option (Figure 6E). 6B) Reveals the list of the components associated with each of the workflows. 6D) The low and high energy scan of the component selected from Figure 6B.

This significantly eliminates a large number of false positives. In addition, an i-FIT™ confidence (%) is generated to show which formulas have a higher probability of being correct. In our sample analysis, we obtained the elemental composition match of  $C_{19}H_{20}N_2O_3$  with the highest i-FIT score of 99.86%.

Step 2 is to perform a library search from the on-line public database ChemSpider, which has compound entries from over 500 libraries. UNIFI Scientific Information System allows users to select searching parameters, and provides a direct link into ChemSpider. In the analysis, the ChemSpider search resulted in an initial match of oxyphenbutazone, which is the metabolite of phenylbutazone and is also an NSAID.

Step 3 is to validate and confirm the initial match with structural elucidation of fragment ions. This takes the advantage of the availability of the MS/MS fragment ions from the  $MS^E$  scan and utilizes MassFragment. MassFragment is one of the structural elucidation tools that conducts calculations on any mol file structure *in silico* and predicts possible fragmentation patterns based on the weak bonds of the molecule. The predicted fragment ion is then compared with the fragment ions obtained from the  $MS^E$  scan. This significantly helps to confirm the right match, and to eliminate false positives. Shown in Figure 7 Step 3, the possible fragments of oxyphenbutazone predicted by MassFragment were checked and matched with fragment ions obtained from the high collision energy scan of the same sample. The results show that key predicted fragments of oxyphenbutazone were found in a high CE  $MS^E$  spectrum, which confirms the identified unknown peak as oxyphenbutazone.

As a result, two synthetic adulterants, phenylbutazone and oxyphenbutazone, were detected and identified in the herbal supplement sample. They both have pharmacological actions as NSAIDs. The final report of the analysis was then automatically generated using the report template, and is shown in Figure 8.

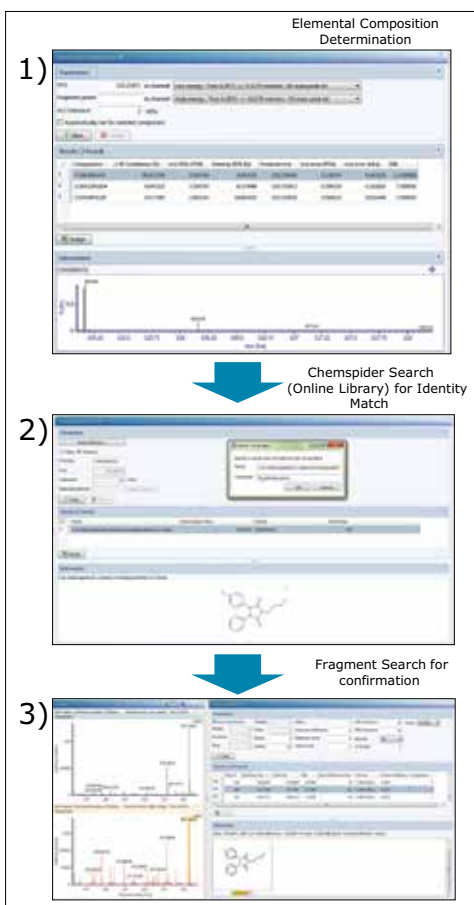


Figure 7. Step by step process of the structure elucidation tool for the identification and confirmation of the unknown component eluted at 8.29 min.

Figure 8 shows a summary report for the status of component identification. The report is titled "Table for Confirmed Adulterants" and lists the identified adulterants, their chemical structures, and their status.

Compound Name	Chemical Structure	Status	Adulterant	Adulterant	Adulterant	Adulterant	Adulterant	Adulterant	Adulterant
Oxyphenbutazone	<chem>O=C1C=CC(=C(C=C1)C2=CC=CC=C2)C3=CC=CC=C3</chem>	Confirmed	Adulterant	Adulterant	Adulterant	Adulterant	Adulterant	Adulterant	Adulterant

Figure 8. Summary report for the status of component identification, which is easily obtained by importing the natural products adulteration summary template.

## CONCLUSION

The utility of the Natural Products Application Solution with UNIFI Adulterant Screening Application is demonstrated through the analysis of a herbal supplement product obtained from a street vendor. Two synthetic adulterants, phenylbutazone and oxyphenbutazone (both NSAIDS), were identified and confirmed from a single LC injection. The entire process from sample to report was completed in a couple of hours.

The Natural Products Application Solution with UNIFI Adulterant Screening Application contains a set of acquisition method templates, processing method template, Synthetic Adulterants Library, a set of dedicated workflows, and a report template, that allow a streamlined process from sample to report. Reliable answers about commercial products can be obtained quickly, without demands for a chemist's expertise or extensive previous related experience. This results in significantly enhanced efficiency and productivity for contract testing labs and regulatory agencies.

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## Screening of synthetic PDE-5 inhibitors and their analogues as adulterants: Analytical techniques and challenges.

The popularity of phosphodiesterase type 5 (PDE-5) enzyme inhibitors for the treatment of erectile dysfunction has led to the increase in prevalence of illicit sexual performance enhancement products. PDE-5 inhibitors, namely sildenafil, tadalafil and vardenafil, and their unapproved designer analogues are being increasingly used as adulterants in the herbal products and health supplements marketed for sexual performance enhancement. To date, more than 50 unapproved analogues of prescription PDE-5 inhibitors were found as adulterants in the literature. To avoid detection of such adulteration by standard screening protocols, the perpetrators of such illegal products are investing time and resources to synthesize exotic analogues and devise novel means for adulteration. A comprehensive review of conventional and advance analytical techniques to detect and characterize the adulterants is presented. The rapid identification and structural elucidation of unknown analogues as adulterants is greatly enhanced by the wide myriad of analytical techniques employed, including high performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS), liquid chromatography mass-spectrometry (LC–MS), nuclear magnetic resonance (NMR) spectroscopy, vibrational spectroscopy, liquid chromatography–Fourier transform ion cyclotron resonance–mass spectrometry (LC–FT-ICR–MS), liquid chromatograph–hybrid triple quadrupole linear ion trap mass spectrometer with information dependent acquisition, ultra high performance liquid chromatography–time of flight–mass spectrometry (UHPLC–TOF–MS), ion mobility spectroscopy (IMS) and immunoassay methods. The many challenges in detecting and characterizing such adulterants, and the need for concerted effort to curb adulteration in order to safe guard public safety and interest are discussed.

**Dhaval Kumar Narendrabhai Patel, Lin Li, Chee-Leong Kee, Xiaowei Ge, Min-Yong Low, Hwee-Ling Koh**

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