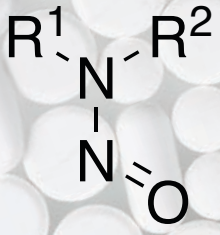
ISSUE 11 | 2022

Analytix Reporter

Determination of N-Nitrosamines in Valsartan



Supelco®
Analytical Products

ISSUE 11 | 2021

Analytix Reporter

For questions & comments contact us at

Analytix@milliporesigma.com

Dear Reader,

Today's pharmaceutical industry is in a constant state of change, proactively and reactively adjusting pipelines and production while optimizing efficiency and bottom line in a global market being buffeted by the pandemic, supply chain, regulatory, and demand uncertainties. Given the rapidly evolving pharma value chain in this environment, there is a constant challenge and demand in analysis & quality control (QC) to keep abreast of changes taking place in the industry. Some of these changes could come from unsuspected impurities or changes in drug delivery formats. Furthermore, with the rise of biologics including proteins, nucleic acids and antibody drug conjugates, the methods for characterizing active pharmaceutical ingredients must be adapted.

In this issue, we highlight two recent applications that have been developed in our laboratories to address these challenges. First, we explore a gas chromatography method for detection of nitrosamines in Valsartan. The issue of nitrosamines as an unexpected impurity rose to international attention and has also culminated in a recall of a range of products due to safety concerns. Our second application of interest is an optimized protocol for peptide mapping of therapeutic monoclonal antibodies (mAbs) with minimum deamidation and oxidation artifacts. While peptide mapping is a central technique for characterizing biotherapeutics, it is important to minimize artifacts in the analysis that could potentially be introduced during sample preparation.

I hope that you will find value in these analytical solutions that we offer and look forward to our continued dialogue with you in the pharmaceutical analysis & quality control community. The insights that you provide us on current trends and bottlenecks in your workflows help us to deliver meaningful solutions that can meet your expectations. For more applications see also our special edition of the Analytix Reporter on Pharma Analysis & QC at **SigmaAldrich.com/Analytix**.

Be on the lookout for future products and applications that address your expressed needs. In particular, we continue to focus on reference materials and chromatography tools & applications that support both small molecule drugs as well as biologics.

Sincerely yours,

Wayne Way
Pharma QC Strategic Marketing Manager

SigmaAldrich.com/HPLC

PHARMA & BIOPHARMA

Determination of N-Nitrosamines in Valsartan

Frank Michel, Analytical & Chromatography Scientific Advisor
Sanjay Poman, Application Expert, Mumbai Analytical Laboratory
Sundaram Palaniswamy, Workflow Solutions Manager Advanced Analytical, Analytix@milliporesigma.com

Introduction

In July 2018, regulatory authorities for medicines were informed about the occurrence of a nitrosamine impurity (N-nitrosodimethylamine, NDMA, **Figure 1**) in valsartan-based products.[1] The active pharmaceutical ingredient (API) valsartan is an angiotensin-II-receptor blocker. It is used to treat hypertension, heart failure, and heart attack in patients intolerant to ACE inhibitor therapy.[2] Valsartan belongs to a group of structurally related compounds known as sartans, having a tetrazole group (a ring with four nitrogen and one carbon) in common. Later, other nitrosamine impurities such as N-nitrosodiethylamine (NDEA), N-nitrosodiisopropylamine (NDIPA), N-nitrosoethylisopropylamine (NEIPA), and N-nitrosodibutylamine (NDBA) were found to be present in other medicines belonging to the sartan family, and in ranitidine drugs. The subsequent issue of worldwide recall on pharmaceutical products using bulk valsartan drug substances lead to an interim shortage of valsartan-based drugs in the market.

N-nitrosamines contain the nitroso functional group (-N=O). According to the International Agency for Research on Cancer (IARC) from the World Health Organization (WHO), most of the nitrosamines are carcinogenic and genotoxic to animals and probable carcinogens to humans. The authorities and API manufacturers discovered the formation of nitrosamines in the reaction of secondary or tertiary amines and nitrites under acidic conditions during the manufacturing process. Sartans require sodium nitrite for the formation of a tetrazole ring which can then result in the formation of N-nitrosamines. Or the nitrosamines might be added with contaminated solvents, reagents, or manufacturing equipment.[3] This led the European Pharmacopoeia (Ph. Eur.) Commission to set up an interim limit of less than 1 ppm for nitrosamines in APIs, and the limit was reduced to 30 ppb at end of 2020.[4]

The measurement at these limits requires the use of highly sensitive and highly selective analytical techniques. Most methods either use GC-MS or HPLC-MS.[5-7] Another challenge to their analysis is the variety of nitrosamines, APIs, and formulations, which necessitate specifically tailored methods for impurity testing.

This work presents a procedure for the determination of 5 nitrosamine impurities (NDMA, NDEA, NEIPA, NDIPA, and NDBA) in a valsartan drug product at trace levels by GC-MS/MS in EI MRM mode, according to US FDA guidelines. One of the FDA's Office of Testing and Research (OTR) published methods was used as base for method development.[8] Method validation was conducted according to the requirements of USP.[9]

Experimental

The GC-MS/MS method used liquid injection to cover a broad range of nitrosamines. Contrary to the OTR method, a wax column with a thinner film thickness (0.5 µm instead of 1 µm) was chosen. But this complied with the USP general chapter <621> on chromatography.[10] The chromatographic conditions, as well as the MS/MS conditions are shown in **Tables 1** to **3**.

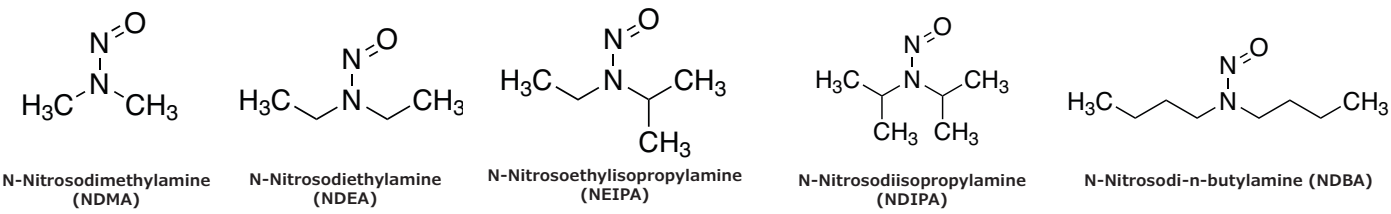


Figure 1. Molecular structure of the N-nitrosamines investigated in this study

Table 1. Chromatographic conditions

Experimental Conditions	
Column:	SUPELCOWAX® 10, 30 m x 0.25 mm I.D., 0.5 µm (**24284**)
Oven:	40 °C (0.5min), 20 °C/min to 200 °C, 60 °C/min to 250 °C (3 min)
Inj. temp.:	250 °C
Carrier gas:	Helium, 1.0 mL/min
Detector:	MS/MS (see **Table 2 & 3**)
Injection:	2 µL – Splitless pulsed injection
Liner:	4 mm single taper liner with glass wool
Sample diluent:	Dichloromethane
Sample preparation:	Using a pill cutter, each tablet was quartered, and the pieces were placed in a 15 mL centrifuge tube followed by addition of 5 mL dichloromethane. Sample was vortexed for 1 min and then centrifuged at 4000 rpm for 2.5 min. Using a disposable pipet, approximately 2 mL of dichloromethane layer was transferred to a 5 mL syringe fitted with a 0.45 µm PVDF filter. Approximately 0.5 mL of sample was filtered into a 2 mL vial and capped.
Standard solutions:	2.5 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 40 ng/mL, 80 ng/mL, and 100 ng/mL each prepared in dichloromethane (NDMA/NDEA/NEIPA/NDIPA/NDBA)

Table 2. MS/MS Conditions

MS/MS Conditions	
Tuning:	Auto-tuning
Acquisition:	MRM (EI mode)
Collision gas:	Nitrogen @ 1.5 mL/min
Quench gas:	Helium @ 4.0 mL/min
Solvent delay:	7 min
MS source temperature:	230 °C
Quad temperature:	150 °C
Electron energy:	70 eV
Dwell time:	50 ms

Table 3. MRMs for the five different nitrosamines

Peaks	Compound	Transition	Retention Time
1	NDMA MRM1	74->44	6.952
	NDMA MRM2	74->42	6.952
2	NDEA MRM1	102->85	7.533
	NDEA MRM2	102->56	7.528
3	NEIPA MRM1	116->99	7.784
	NEIPA MRM2	71->56	7.787
4	NDIPA MRM1	130->42	7.971
	NDIPA MRM2	130->88	7.976
5	NDBA MRM1	158->99	9.497
	NDBA MRM2	84->56	9.494

The five nitrosamines were sufficiently separated in less than ten minutes and the target peaks were well resolved from the solvent and matrix peaks (**Figure 2**). The observed retention times which were shorter compared to the FDA OTR method, could be attributed to the lower film thickness of the GC column.

Low limits of detection were achieved by multiple reaction monitoring (MRM) for two transitions. Two

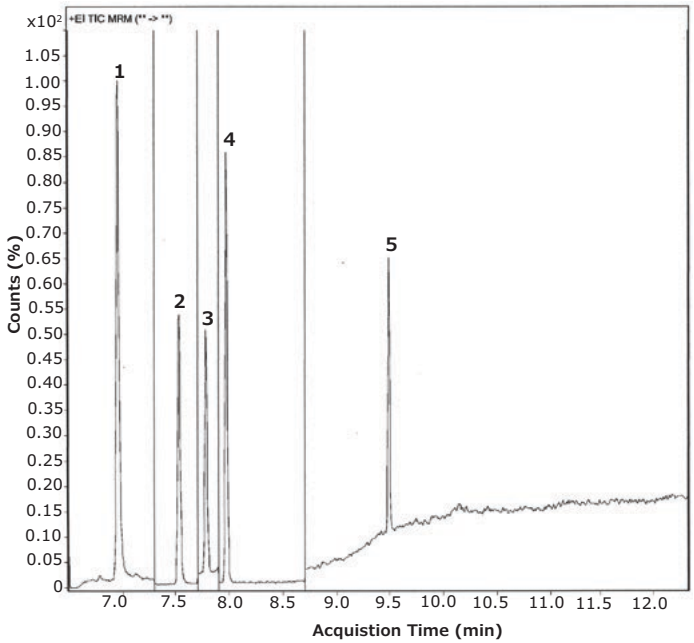


Figure 2. Exemplary chromatogram of the system suitability solution with a concentration of 40 ng/mL. For peak labeling please see **Table 3**.

examples are shown in **Figure 3** by the chromatograms of NDEA and NDIPA at the lowest concentration of 2.5 ppb with a signal to noise ratio of more than 10.

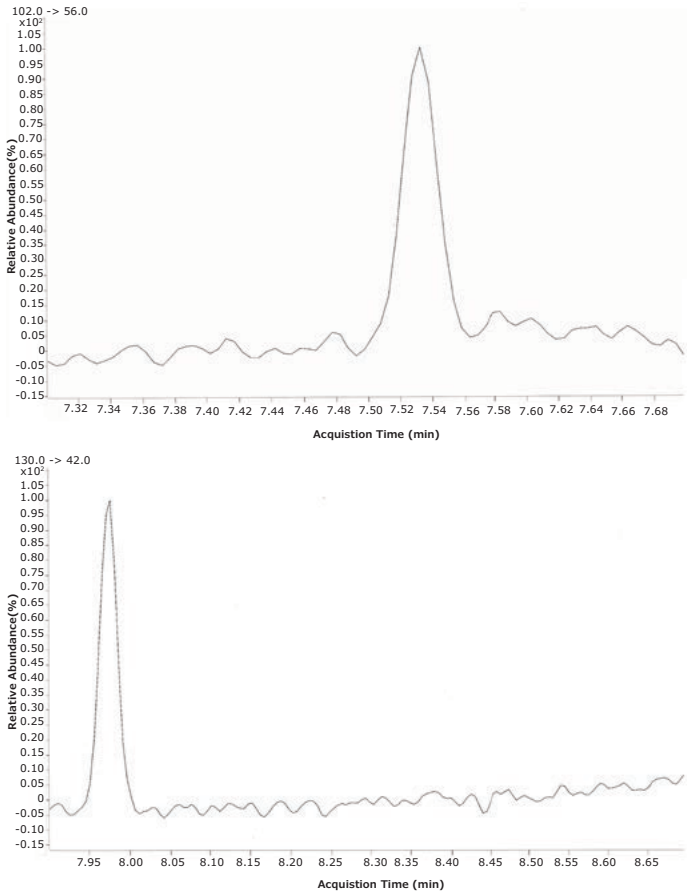


Figure 3. Chromatograms of NDEA (top) and NDIPA (bottom) at the lowest concentration of 2.5 ppb.

Method Suitability

The validated FDA-OTR method requires the % RSD for six replicate injections of a 40 ng/mL standard to be ≤ 5%. Using our method, the % RSD for six consecutive injections of the 40 ng/mL standard was less than 5% for all the impurities at both MRMs, as shown in **Table 4**.

Table 4. Precision of six consecutive injections of the 40 ng/mL nitrosamine standard

Compound	% RSD for MRM1	% RSD for MRM2
NDMA	1.8	1.3
NDEA	1.1	1.1
NEIPA	4.2	1.5
NDIPA	0.9	2.2
NDBA	4.3	3.0

Furthermore, the correlation coefficient (r^2) of the linear calibration curves should be ≥0.998. Our method exceeded this for all five nitrosamines at both MRMs (**Table 5**).

Table 5. Correlation coefficient (r^2) of the nitrosamines at both MRMs

Impurity	MRM1	MRM2
NDMA	0.9994	0.9995
NDEA	0.9991	0.9995
NEIPA	0.9995	0.9995
NDIPA	0.9996	0.9994
NDBA	0.9983	0.9981

Application on Valsartan drug product

A commercial valsartan product purchased at a local pharmacy was spiked with nitrosamine impurities to a concentration of 10 ppb (40 ppb for NDBA) in the drug product. The recovery rates of the nitrosamines were measured and found in the range of 94.5 to 105.7% respectively. (**Table 6**).

Table 6. Recovery rates of nitrosamines in spiked drug product

Impurity	Recovery of 10 ppb in drug product
NDMA	99.0%
NDEA	103.5%
NEIPA	94.5%
NDIPA	103.9%
NDBA	105.7%

The FDA-OTR method reported the limits of quantification (LOQs) for the determination of the nitrosamines in valsartan products to be in the range of 8 – 40 ppb. Our method achieved even lower LOQs in a valsartan drug product (**Table 7**). The LOQs were calculated from the calibration curves based on a signal/noise (S/N) ratio of 10 for each of the compounds and validated by standard addition experiments to valsartan tablets. Limits of detections (LOD) were calculated using a signal/noise (S/N) ratio of 3.

Table 7. LOQs in drug product for FDA-OTR method and our method

Impurity	FDA-OTR LOQ in drug product [ppb]	LOQ in drug product obtained in this study [ppb]
NDMA	13	3
NDEA	8	5
NEIPA	8	3
NDIPA	8	5
NDBA	40	32

Summary

The determination of nitrosamine impurities can be easily achieved by GC-MS/MS in MRM mode using the SUPELCOWAX® column based on the suggested method by FDA-OTR. All nitrosamines were well separated from each other as well as from the solvent and matrix peaks, meeting the system suitability requirements. The method was successfully applied for the analysis of a valsartan drug product spiked with nitrosamine impurities.

References

1. WHO. Information Note Nitrosamine impurities. www.who.int/medicines/publications/drugalerts/InformationNote_Nitrosamine-impurities/en/ (accessed Nov. 2021)
2. European Medicines Agency (EMA). Notification to the CHMP/EMA Secretariat of a Referral under Article 31 of Directive 2001/83/EC. www.ema.europa.eu/en/documents/referral/angiotensin-ii-receptor-antagonists-sartans-article-31-referral-notification_en.pdf (accessed Nov. 2021)
3. EMA. Referral under Article 31 of Directive 2001/83/EC angiotensin-II-receptor antagonists (sartans) containing a tetrazole group. www.ema.europa.eu/en/documents/variation-report/angiotensin-ii-receptor-antagonists-sartans-article-31-referral-chmp-assessment-report_en.pdf (accessed Nov. 2021)
4. European Directorate for the Quality of Medicines and HealthCare (EDQM). Ph. Eur. Commission adopts a new general chapter for the analysis of N-nitrosamine impurities. 07.12.2020, www.edqm.eu/en/news/ph-eur-commission-adopts-new-general-chapter-analysis-n-nitrosamine-impurities (accessed Nov. 2021)
5. FDA. FDA Updates and Press Announcements on Angiotensin II Receptor Blocker (ARB) Recalls (Valsartan, Losartan, and Irbesartan). https://www.fda.gov/drugs/drug-safety-and-availability/fda-updates-and-press-announcements-angiotensin-ii-receptor-blocker-arb-recalls-valsartan-losartan (accessed Nov. 2021)
6. EDQM. Ad-hoc projects of the OMCL Network / Methods for determination of nitrosamines. www.edqm.eu/en/ad-hoc-projects-omcl-network (accessed Nov. 2021)
7. Taiwan Food and Drug Administration (TFDA). Analytical Methods. www.fda.gov.tw/ENG/siteList.aspx?sid=10360 (accessed Nov. 2021)
8. US FDA. Combined Direct Injection N-Nitrosodimethylamine (NDMA), N-Nitrosodiethylamine (NDEA), N-Nitrosoethylisopropylamine (NEIPA), N-Nitrosodiisopropylamine (NDIPA), and N-Nitrosodibutylamine (NDBA) Impurity Assay by GC-MS/MS, www.fda.gov/media/123409/download (accessed Nov. 2021)
9. USP General Chapter <1225> Validation of Compendial Procedures, USP43-NF38
10. USP General Chapter <621> Chromatography USP43-NF38

Featured Products

Description	Cat. No.
GC Column	
SUPELCOWAX® 10 GC Capillary Column 30 m × 0.25 mm, df 0.50 µm	**24284**
Solvents	
Dichloromethane for GC-MS SupraSolv®	**1.00668**
Reference Materials	
N-Nitrosodimethylamine Certified Reference Material 5000 µg/mL in methanol	**CRM40059**
N-Nitrosodimethylamine USP Reference Standard	**1466674**
N-Nitrosodimethylamine Pharmaceutical Secondary Standard; Certified Reference Material	**PHR2407**
N-Nitrosodiethylamine Certified Reference Material 5000 µg/mL in methanol	**40334**
N-Nitrosodiethylamine USP Reference Standard	**1466652**
N-Nitrosodiethylamine Pharmaceutical Secondary Standard; Certified Reference Material	**PHR2408**
N-Nitroso-ethyl-isopropylamine EP Reference Standard	**Y0002262**
N-Nitroso-ethyl-isopropylamine USP Reference Standard	**1466685**
N-Nitroso-ethyl-isopropylamine Pharmaceutical Secondary Standard; Certified Reference Material	**PHR3609**
N-Nitroso-diisopropylamine EP Reference Standard	**Y0002263**
N-Nitroso-diisopropylamine USP Reference Standard	**1466663**
N-Nitroso-diisopropylamine Pharmaceutical Secondary Standard; Certified Reference Material	**PHR3607**
N-Nitrosodi-n-butylamine Certified Reference Material, 2000 µg/mL in methylene chloride	**48320-U**
N-Nitrosodi-n-butylamine USP Reference Standard	**1466641**
N-Nitrosodi-n-butylamine Pharmaceutical Secondary Standard; Certified Reference Material	**PHR3608**
Accessories	
Millex® HV Durapore and (PFDV) 0.45µm syringe filter	**SLHV033NS**
Inlet Liner, Split/Splitless Type, Single Taper FocusLiner™ Design (wool packed), pkg of 5 ea (also available as pkg of 1 ea (2879901-U) and pkg of 25 ea (2879925-U))	**2879905-U**
Supelco® Helium Purifier SS fittings, 1/4 in. Swagelok (nuts and ferrules included)	**27601-U**
Supelco® Helium Purifier SS fittings, 1/8 in. Swagelok (nuts and ferrules included)	**27600-U**
OMI® Tube Holder for use with OMI-2 purifier tubes	**23921**
OMI®-2 Purifier Tube	**23906**
Super Clean (Base-Plate Design) Gas Purifier triple trap (hydrocarbon, moisture, oxygen)	**SU861026**
Super Clean (Base-Plate Design) Base Plate single position, size 1/8 in.	**SU861011**
Super Clean (Base-Plate Design) Kit carrier gas kit (includes **SU861026** + **SU861011**)	**28878-U**

Related Products

Description	Cat. No.
Valsartan Pharmaceutical Secondary Standard; CRM	**PHR1315**
Valsartan EP Reference Standard	**Y0001132**
Valsartan BP Reference Standard	**BP1161**
Valsartan USP Reference Standard	**1708762**
D-Valsartan (Valsartan Related Compound A), Pharmaceutical Secondary Standard; CRM	**PHR1875**
D-Valsartan (Valsartan Related Compound A), USP Reference Standard	**1708773**
Valsartan Related Compound B USP Reference Standard	**1708784**
Valsartan Related Compound B Pharmaceutical Secondary Standard; CRM	**PHR1876**
Valsartan Related Compound C USP Reference Standard	**1708795**
Valsartan Related Compound C Pharmaceutical Secondary Standard; CRM	**PHR1877**
Valsartan Related Compound E USP Reference Standard	**1708810**
Valsartan Related Compound E Pharmaceutical Secondary Standard; CRM	**PHR1878**
Valsartan Chiral System Suitability Mixture solution	**V-073**
Valsartan for peak identification EP Reference Standard	**Y0001131**
Valsartan for system suitability EP Reference Standard	**Y0001145**

To learn more about our complete offer for gas chromatography visit us at **SigmaAldrich.com/GC**

To learn more about our complete offer on Pharmaceutical Reference Materials visit us at **SigmaAldrich.com/pharmastandards**

PHARMA & BIOPHARMA

An optimized Protocol for Peptide Mapping of Therapeutic Monoclonal Antibodies with Minimum Deamidation and Oxidation Artifacts

Pegah Jalili, Principal Scientist, Analytical R&D
Kevin Ray, PhD, Senior Manager, Analytical R&D
Jeffrey Turner, PhD, Head of Protein Assay Development
Maricar Dube, PhD, Analytical Sciences Liaison, Analytix@milliporesigma.com

Abstract

Post-translation modifications (PTM), such as oxidation and deamidation, can have serious consequences on therapeutic monoclonal antibodies. Peptide mapping is a widely used method for the identification of site-specific PTMs, but typical protein digestion workflows often end up introducing significant amounts of artifacts. Hence, to obtain an accurate assessment of the modifications, it is critical to reduce the artifacts that occur during sample preparation steps. This study used NISTmAb as a model monoclonal antibody to demonstrate an optimized peptide mapping protocol resulting in minimal artificial asparagine deamidation and methionine oxidation. The protocol utilizes shorter incubation times and an improved digestion buffer, allowing for complete sample preparation in less than six hours.

Introduction

The development, production, and storage of therapeutic mAbs must be monitored for post-translational modifications (PTMs), to assure consistent quality and safety. PTMs such as deamidation and oxidation are known to influence the efficacy, safety, and stability of therapeutic monoclonal antibodies (mAb).[1,2] Deamidation of asparagine (ASN or D) and the oxidation of methionine (Met or M) are major chemical degradation pathways for protein therapeutics and have been studied extensively.[3,4,5] Asparagine residues can form a succinimide intermediate that subsequently hydrolyzes into isoaspartic or aspartic acid (**Figure 1A**).[6,7] Whereas, hydroxyl radicals can oxidize methionine residues to form methionine sulfoxide (**Figure 1B**).[8,9]

LC-MS based peptide mapping is the method of choice for measuring the relative abundance of PTMs. The sample preparation prior to the LC-MS analysis involves three steps of denaturation/reduction, alkylation, and digestion. The digestion of different mAbs produces different peptide fragments having a wide range of sizes — from single amino acids to longer polypeptides. Since these peptides vary widely in their hydrophobicity, reversed-phase (C18) is the preferred mode of chromatography for peptide mapping.

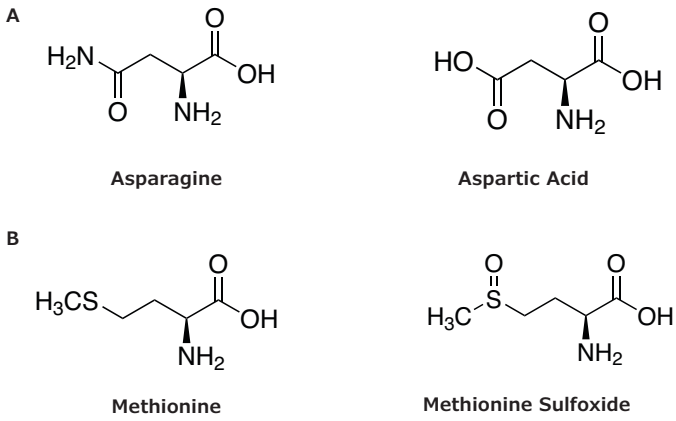


Figure 1. (A) Asparagine residues can undergo deamidation to form aspartic acid, and (B) methionine can undergo oxidation to form methionine sulfoxide.

The conventional trypsin digestion of monoclonal antibodies is lengthy, involving an overnight digestion step. The conditions and reagents used in this step are known to induce artifactual deamidation and oxidation of the mAb sample, leading to inaccurate measurement of PTMs.[10] The first part of this paper compares methionine and asparagine deamidation between conventional trypsin digestion and an optimized digestion protocol that takes less than six hours to complete. The second part compares the optimized protocol with the protocol published by NIST. All LC-MS analyses were carried out using C18 columns with superficially porous particles (BIOshell™ A160 Peptide C18).

Experimental

Sample: NIST Monoclonal Antibody Reference Material 8671 (NISTmAb)

Digestion: **Figure 2** outlines the digestion protocols.

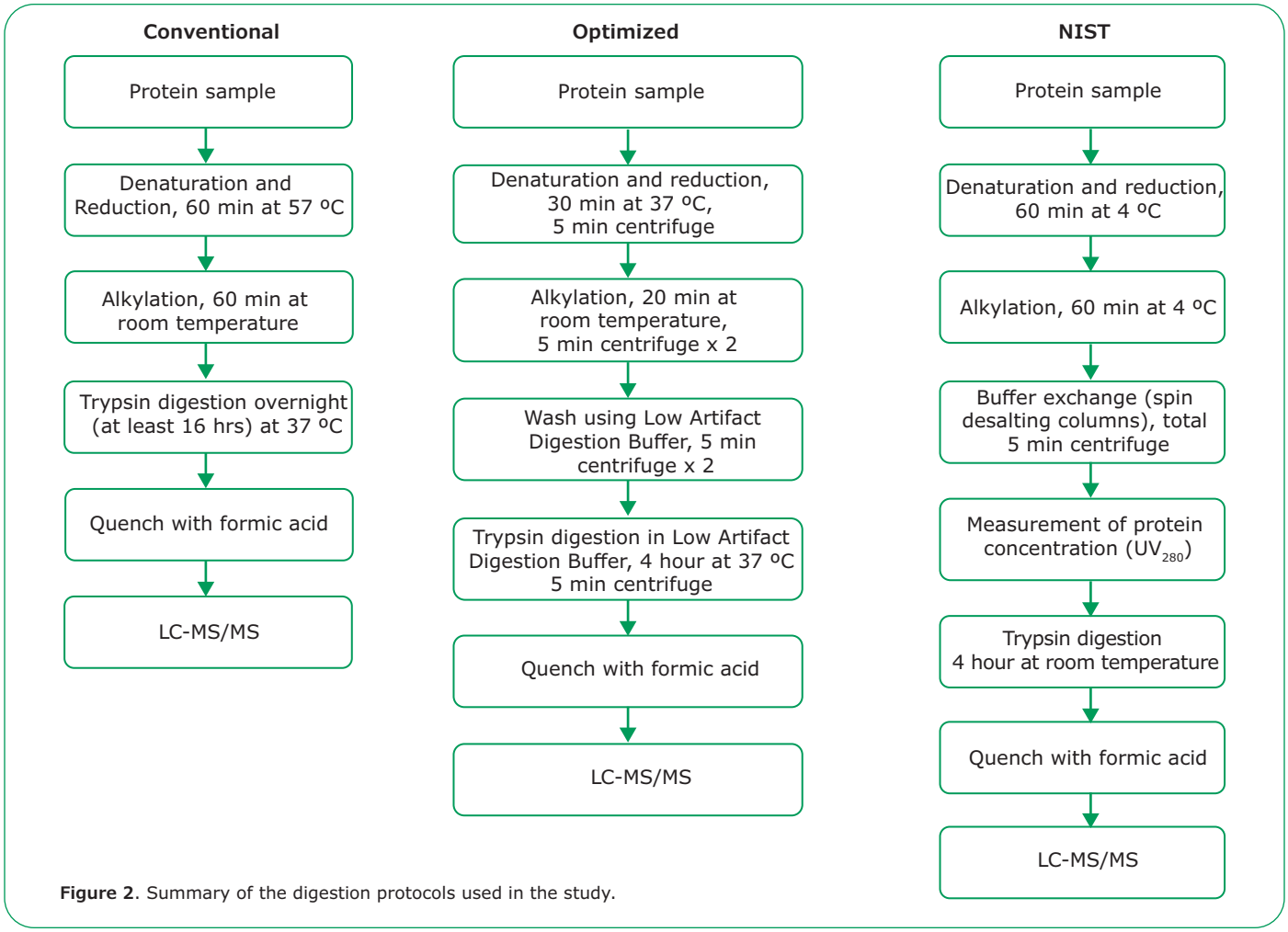


Figure 2. Summary of the digestion protocols used in the study.

The conventional protocol uses sodium deoxycholate (60 mg) in methanol (1 mL) as the denaturation solution. 20 mM TCEP (tris(2-carboxyethyl)phosphine) and the denaturation solution were mixed in 1:1 (v/v), to which 20 µL of dried down sample was added and incubated at 57 ºC for one hour. The sample was brought back to room temperature and centrifuged at 14,000 x g per gram for 30 seconds. This was followed by the alkylation step using 5 µL of 200 mM iodoacetamide (in 50 mM ammonium bicarbonate) and subsequent incubation for one hour in the dark at room temperature. Digestion was carried out by adding enough trypsin solution (trypsin in 50 mM ammonium bicarbonate) to have an enzyme:protein ratio of 1:20 and incubating at 37 ºC overnight (at least 16 hours) on a thermo-shaker. 2 µL neat formic acid was used to quench the digestion.

Detailed procedure for the optimized protocol is described in the technical bulletin for Low Artifact Digestion Buffer.[11] A NIST[12] paper describes the protocol provided by NIST. The reagents used in each protocol are shown in **Table 1**.

Table 1. Reagents used in the protocols of the study.

Reagent	Conventional	Optimized	NIST
Denaturing solution/buffer	Sodium deoxycholate	Urea	Guanidine HCl
Reduction	TCEP	TCEP	DTT
Alkylation	Iodoacetamide	Iodoacetamide	Iodoacetamide
Digestion buffer	Ammonium bicarbonate	Low Artifact Digestion Buffer	Urea
Trypsin	SOLu-Trypsin	SOLu-Trypsin	Recombinant, proteomics grade, expressed in Pichia pastoris

LC-MS Conditions HPLC:

HPLC Conditions	
Instrument:	Waters Acquity UPLC
Column:	BIOshell™ A160 Peptide C18, 15 cm x 1.0 mm, 2.7 µm particles (**67099-U**), two columns in series
Mobile phase	[A] 0.1% formic acid in water [B] 0.1% formic acid in acetonitrile
Gradient:	**Time (min) / %B** 0 / 1 120 / 35 121 / 97 136 / 97 137 / 1 162 / 1
Flow rate:	80 µL/min
Column temp.:	room temperature
Injection:	10 µL (3-4 µg MAb digest)

Mass Spectrometry:

MS Conditions	
Instrument:	Thermo QE Plus
Polarity:	Positive
Spray voltage:	4.0 kV
Capillary temp:	320 ºC
Sheath gas:	10
Aux gas:	5
S-Lens:	50 V
m/z range:	300-4000

Data analysis

The raw MS files were subjected to BioPharma Finder™ 3.0 (Thermo Fisher Scientific) for peptide mapping. The peptide identifications were performed by searching the processed data against the NISTmAb sequence-based accurate mass of a full mass scan and assignments of product ions in MS/MS spectra. The data was filtered to report only the peptides with a mass tolerance of ±10 ppm. The % deamidation and oxidation were calculated by BioPharma Finder software using the mapping tab. Also, the result was manually checked by creating the extracted ion chromatograms (XICs) for unmodified and modified peptide within 10 ppm mass error. Equations 1 and 2 were used to calculate the % modification (oxidation, deamidation) and % missed cleavage (% MC), respectively.

Equation 1:

$$\% \text{ Modification} = \frac{\text{area under the peak of XIC of modified peptide}}{\text{area under the peak of XIC of modified peptide + area under the peak of XIC of un-modified peptide}} \times 100$$

Equation 2:

$$\% \text{ MC} = \frac{\text{area under the peak of XIC of MC peptide}}{\text{area under the peak of XIC of standard peptide + area under the peak of XIC of MC peptide}} \times 100$$

Results and Discussions

Peptide mapping using LC-MS has become a routine analysis in the development and manufacture of therapeutic mAbs. Traditional sample preparation procedures used prior to LC-MS are often cumbersome. These procedures generally involve chemical denaturation, reduction and alkylation, buffer exchange, and overnight protease digestion of the protein sample at elevated pH and temperature. Asparagine deamidation and methionine oxidation take place during these various steps, the extent of which depends on the conditions such as reagents used, ionic strength, temperature, pH, incubation time, digestion buffer, and presence of trace metals (in the case of methionine oxidation).[7] A simpler, shorter method with minimal artifacts is certainly desired to obtain accurate endogenous levels of deamidation and oxidation.

Figure 3A is the base peak chromatogram of tryptic digested NISTmAb, showing examples of typical tryptic peptides used to measure the levels of Met (M) oxidation and Asn (D) deamination in this work. **Figure 3B** is the extracted ion chromatogram and MS spectrum of the peptide DTLMISR (t_R = 51 min) and the peptide with an oxidized methionine residue (position HC:M255), t_R = 44 min. The oxidation of methionine rendered the molecule less hydrophobic, thus less retentive on the BIOshell™ A160 Peptide C18 column. **Figure 3C** is the XIC and MS spectrum of the peptide GFYPSDIAVEWESNGQPENNYK (t_R 88.90 min) and the deamidated peptide (position HC:N387). The deamidated forms (isoASP and ASP) elute before and after the unmodified peak at ~87.91 and 91.92 min, respectively.

Optimized versus Conventional Protocol

The optimized and conventional protocols use the same reduction (TCEP) and alkylation (iodoacetamide) reagents, but they differ in the denaturing solution used, incubation times, and temperature (see **Figure 2** and **Table 1**). The conventional protocol has much longer incubation times and uses higher temperature for the denaturation/reduction step. The digestion step happened overnight with the conventional protocol at a higher pH of 8.5

With the optimized protocol, digestion took only four hours. The digestion buffer used was specifically developed to minimize deamidation and oxidation during the digestion step without sacrificing the digestion efficiency. The buffer was formulated at an optimal pH and contained a proprietary antioxidant.

In both protocols, the protease used was SOLu-Trypsin, a proprietary formulation of recombinant Trypsin (porcine sequence expressed in *Pichia pastori*) and stable in solution when refrigerated.

The deamidation levels between the protocols were extremely different at the two sites (**Figure 4A**). The biggest difference was observed at site HC:N387, where deamidation was 41.1% for the conventional

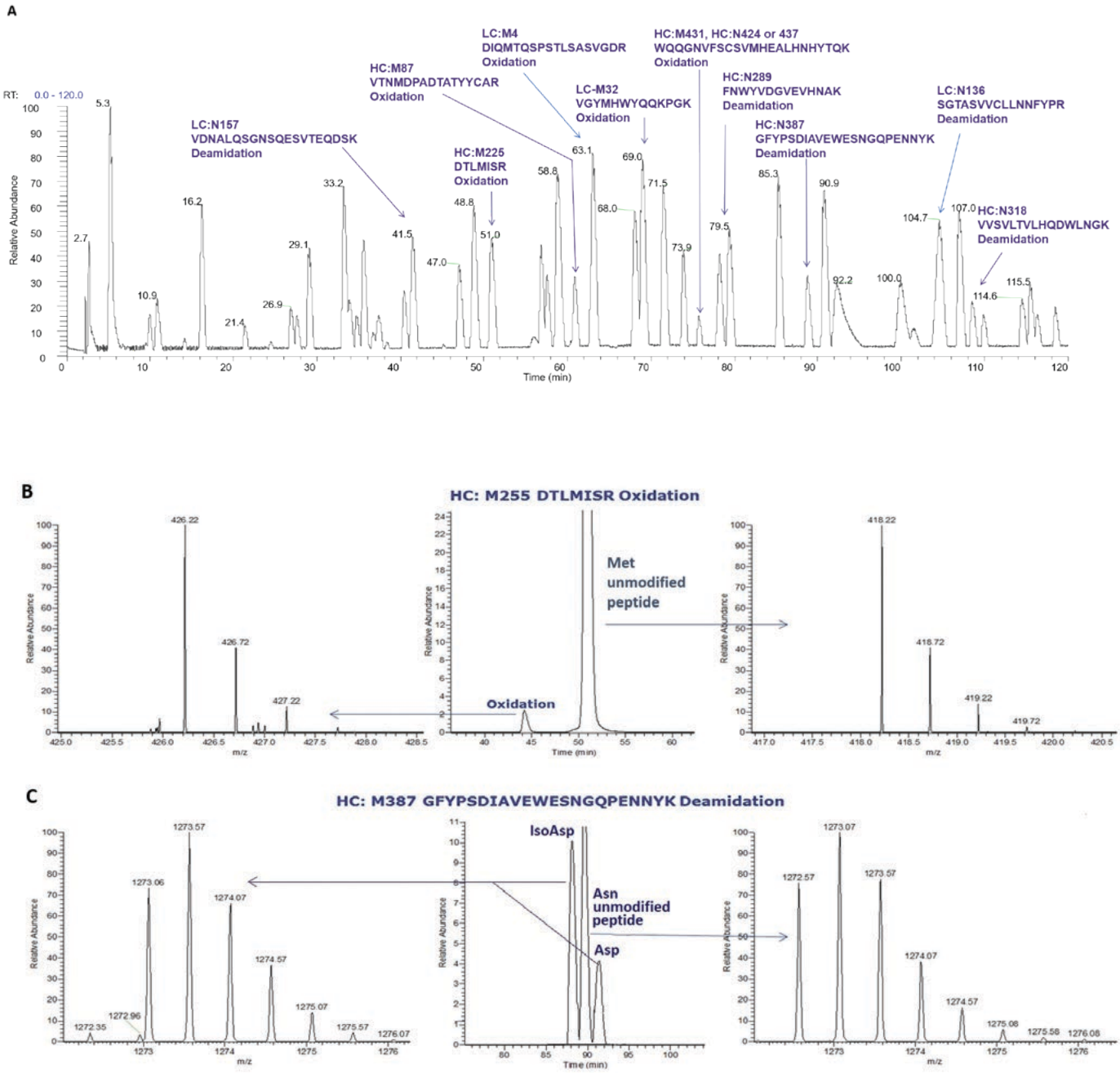


Figure 3. Analysis of tryptic digested NISTmAb (A) Base peak chromatogram of tryptic digested NISTmAb. Labeled peptides are examples of typical tryptic peptides used for determining Met oxidation and Asn deamination levels. (B) Extracted ion chromatogram and spectra of unmodified DTLMISR peptide and the peptide with oxidized Met. (C) Extracted ion chromatogram and spectra of unmodified GFYPSDIAVEWESNGQPENNYK peptide and the peptide with deamidated Asn.

protocol and 0.6% for the optimized one. At site HC:N318, a 21.2% deamidation was observed for the conventional protocol and none was observed for the optimized protocol. These results are not surprising. It is well known that the incubation times of protein samples in the denaturing/reduction and alkylation steps, and to a larger extent, the length of digestion, are directly proportional to the levels of artificial modification.[13] It has been reported that deamidation artifacts are reduced at lower temperatures;[14] in the conventional protocol, the denaturation/reduction step was carried out at an elevated temperature (57 ºC).

The difference in % oxidation was not as high as observed for deamidation. At site LC:M32, the conventional protocol had 2.9% higher oxidation than the optimized protocol, and it was 4.2% higher at site HC:M255 (**Figure 4B**).

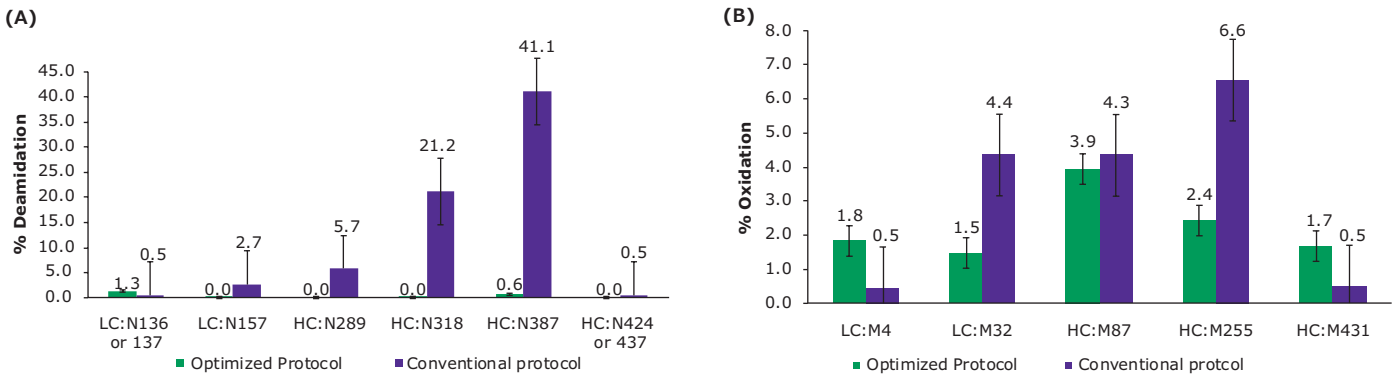


Figure 4. Levels of (A) Asn deamidation and (B) Met oxidation of NISTmAb tryptic peptides using the conventional and optimized protocols.

Optimized Protocol versus NIST Protocol

In 2018, NIST published a paper wherein they described the development of a tryptic digestion protocol used for peptide mapping. Their study focused on parameters such as buffer concentration, digestion time and temperature, and the source and type of trypsin used.[12] (See **Figure 2** for the outline of the protocol and **Table 1** for the reagents used.)

The denaturation/reduction and alkylation steps were carried out at a very conservative temperature (4 ºC) with incubation times much longer than the one for the optimized protocol. In addition, the protocol required a buffer exchange step (into the urea containing digestion buffer) before the tryptic digestion. Overall, the NIST protocol requires more time for reagent and sample preparation compared to the optimized protocol.

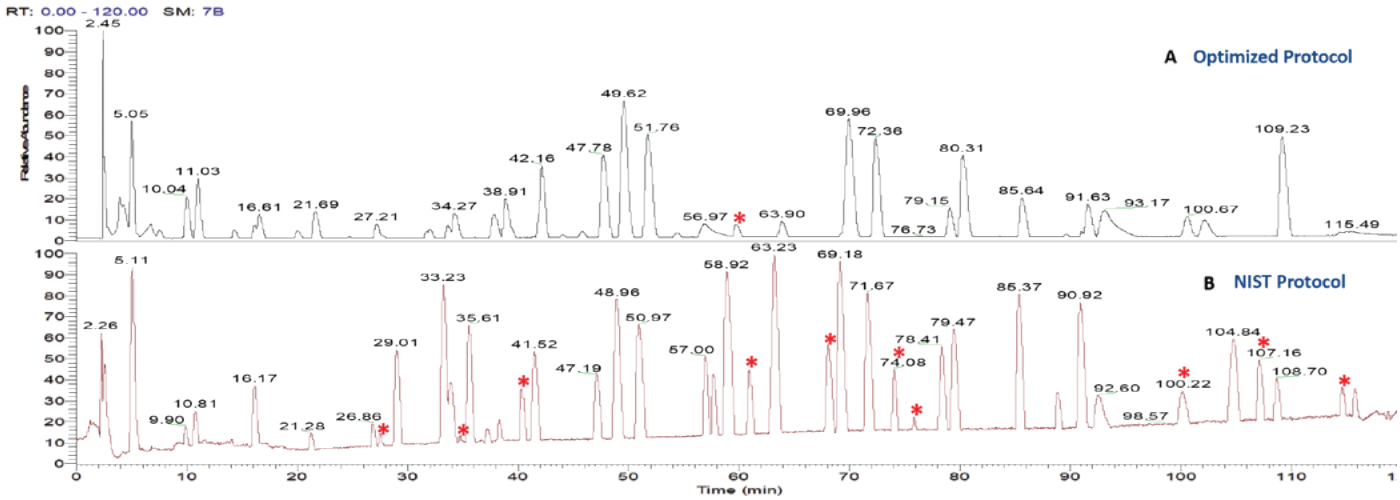


Figure 5. Comparison of base peak chromatograms of tryptic digested NISTmAb using the (A) optimized and (B) NIST protocols. Missed cleavage peptides are labeled with a red asterisk (*).

Figure 5 compares the base peak chromatograms of the digested NISTmAbs using the optimized protocol and the NIST protocol. The profile for the optimized digestion protocol is less complex. The chromatogram from the NIST protocol exhibited many extra peaks which were identified as missed cleavage peptides. The average percent missed cleavage was 16% for the optimized protocol and 35% for the NIST protocol (**Figure 6**).

In terms of Met oxidation and Asn deamidation, the performance of the two protocols is similar. The level of Met oxidation for both the methods is <5% and for deamidation <1.7% (**Figure 7**).

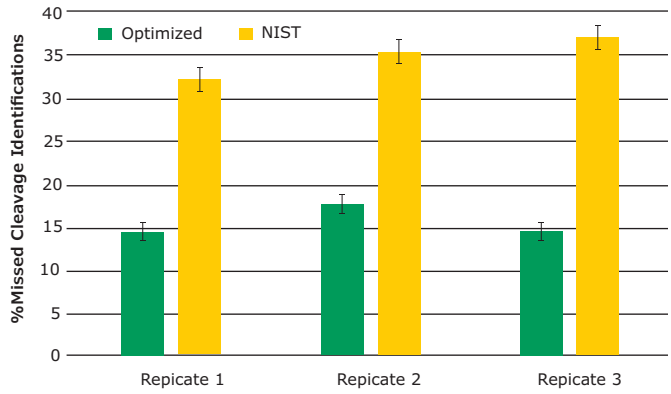


Figure 6. Average percent missed cleavage for the optimized and NIST protocols.

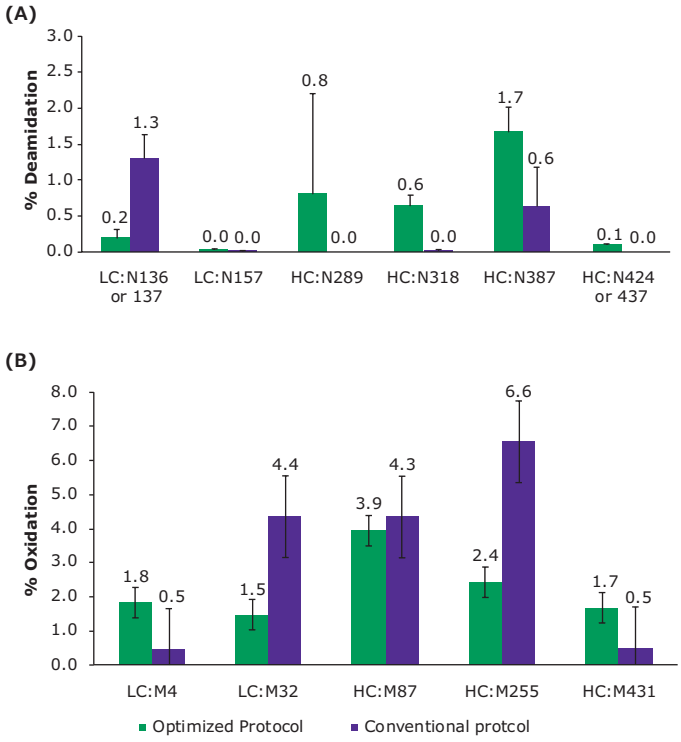


Figure 7. Levels of (A) Asn deamidation and (B) Met oxidation of NISTmAb tryptic peptides using optimized protocol and NIST protocol.

It is important to note that the HPLC conditions are also critical for the study. To enhance resolution and sequence coverage, two BIOshell™ A160 Peptide C18 15 cm x 1.0 mm columns arranged in series were used to provide for a total effective column length of 30 cm. Hydrophilic peptides with less than five amino acids such as VDK, TISK, EYK that typically elute in flow-through, could be retained on the longer column and be subsequently analyzed by mass spectrometry. In addition, the BIOshell™ columns are composed of superficially porous particles (SPPs) containing a solid, nonporous silica core with a porous silica outer layer, providing higher separation efficiency. This particle attribute results in a narrower peak width and improved resolution of the peptide analytes. Together with the optimized gradient conditions shown in the experimental section, a good separation of the unmodified and modified peptides was achieved. This result is well illustrated in **Figure 3B**, where the unmodified DTLMISR peptide and the peptide with oxidized Met were well resolved, allowing for accurate quantitation of each species.

Conclusions

The optimized protocol gave significantly lower levels of Asn deamidation compared to the conventional protocol, particularly at two sites, HC:N387 (over 40% lower) and HC:N318 (over 20% lower). The levels of oxidation (<5%) and deamidation (1.5 %) were comparable with the NIST protocol. In addition, more missed cleavage peptides were observed with the NIST protocol (35%) compared to the optimized protocol (16%). The optimized protocol also offers the advantage of allowing complete digestion in less than 6 hours, with minimal deamidation and oxidation artifacts. The use of two BIOshell™ A160 Peptide C18 (15 cm) columns in series allowed the successful separation of peptides in the tryptic digestion.

Featured Products

Description	Cat.No.
HPLC	
BIOshell™ A160 Peptide C18, 15cm x 1.0 mm, 2.7 µm	**67099-U**
Acetonitrile with 0.1% (v/v) Formic acid, hypergrade for LC-MS LiChrosolv®	**1.59002**
Water with 0.1% (v/v) Formic acid, hypergrade for LC-MS LiChrosolv®	**1.59013**
Formic acid 98% - 100%, for LC-MS LiChropur™	**5.33002**
Standards, Reagents, and Accessories	
NISTmAb, Humanized IgG1k Monoclonal Antibody	**NIST8671**
Low-Artifact Digestion Buffer	**EMS0011**
SOLu-Trypsin	**EMS0004**
Microcon-30kDa Centrifugal Filter Unit with Ultracel-30 membrane	**MRCF0R030**

REFERENCES

1. X. Xu, Y. Huang, H. Pan, R. Molden, H. Qiu, T. J. Daly, and N. Li. PLOS ONE. https://doi.org/10.1371/journal.pone.0223899
2. Y. Wang, X. Li, Y. -H. Liu, D. Richardson, H. Li, M. Shameem, and X. Yang. MAbs. 8, 1477–1486 (2016).
3. I. Schmid, I., Bonnington, L., Gerl, M. et al. Commun Biol. 1, (2018). https://doi.org/10.1038/s42003-018-0032-8.
4. A. L. Pace, R. L. Wong, Y. T. Zhang, Y.-Hs. Kao, Y. J. Wang, Journal of Pharmaceutical Sciences. 102, 1712-1723 (2013).
5. M. E. Alam, et al. Journal of Pharmaceutical Sciences, 109, 656-669 (2020.
6. S. Liu, K. R. Moulton, J. R. Auclair, and Z. S. Zhou. Amino Acids. 48, 1059–1067 (2016).
7. V. Timm, P. Gruber, M. Wasiliu, H. Lindhofer, and D. Chelius. J Chrom B 878, 777-784 (2010).
8. K. Sankar, K. H. et. al. MAbs. 10, 1281–1290 (2018).
9. X. M. Lam, J. Y. Yang, and J. L. Cleland. Journal of Pharmaceutical Sciences, 86, 1250-1255 (1997).
10. D. Wang, C. Nowak, B. Mason, A. Katiyar, and H. Liu. Journal of Pharmaceutical and Biomedical Analysis, 183, 113-131 (2020).
11. Low Artifact Digestion Buffer Technical Bulletin https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/2/ems0011bul.pdf
12. T. Mouchahoir and J. E. Schiel. Analytical and Bioanalytical Chemistry 410, 2111–2126 (2018)
13. D. Ren, et. al. Anal. Biochem. 392, 12-21 (2009).
14. S. D. Stroop. Rapid Commun. Mass Spectrom. 21, 830-836 (2007).

Find more about BIOshell™ U/HPLC columns at

SigmaAldrich.com/HPLC

Optimizing for High Throughput Analysis of Cannabinoids in Cannabis Products

Improved retention time stability and chromatographic performance using Fused-Core® technology

Seamus Riordan-Short, Senior Chemist; Yevgen Kovalenko, Technician;
Matthew Noestheden, Director of Operations, Supra Research and Development
Katherine K. Stenerson, Analytical Sciences Liaison; Jennifer King, Program Marketing Manager, MilliporeSigma, Analytix@milliporesigma.com

With increasing cannabis and hemp legislation, there has been increased demand for development and validation of accurate and precise testing methods for potency quantitation. Cannabinoids present a number of challenges, and there is also the additional burden of dealing with a variety of matrix types. HPLC/UV is the technique most commonly used, and the HPLC parameters must be optimized to maintain good separation and stable retention over many injections and with the various sample types.

Scientists at Supra Research and Development ("SupraRnD") located in Kelowna, British Columbia, Canada (www.suprarnd.ca) have developed a high throughput and reliable method for cannabinoids that is applicable to a variety of matrices. SupraRnD's involvement in cannabis testing began in 2015 when they obtained a license from Health Canada for testing cannabis products. In 2018 they were one of the first laboratories in Canada to obtain their ISO 17025 accreditation for cannabis testing. Their potency method has evolved over time to meet the changing needs of their customers, and is now validated for several different matrices.

Experimental Conditions

Whole flower samples were frozen in hermetically sealed bags at -80 °C for a minimum of 30 minutes and then homogenized immediately.* It is critical that a representative sample is homogenized and subsampled when analyzing cannabis flower, as there can be considerable variance in phytocannabinoid concentrations between and within a given plant. The subsequent workflow involved a simple extraction of a 0.2 g sample size with methanol, followed by sonication and stabilization of the extract at -20 °C for 1 hour. The sample was then centrifuged, and the supernatant diluted 100:1 for HPLC analysis. The small sample size in combination with the pre-analysis dilution minimizes the potential for matrix-related issues (e.g., interferences, column longevity, etc.). The HPLC portion of the analysis has a cycle time of 8 minutes injection to injection. This allows 60 injections per 8-hour interval, which enables more customer samples to be run in a work shift. The cannabinoids analyzed by the method are listed in **Table 1.**

Table 1. 17 Phytocannabinoids separated by HPLC method

1.	CBDVA
2.	CBDV
3.	CBDA
4.	CBGA
5.	CBG
6.	CBD
7.	THCV
8.	THCVA
9.	CBN
10.	CBNA
11.	Δ9-THC
12.	Δ8-THC
13.	CBL
14.	CBC
15.	THCA
16.	CBCA
17.	CBLA

The final, optimized HPLC parameters are summarized in **Table 2.** When developing this method, the following were considerations:

- Chromatographic resolution of all 17 compounds.
- Cycle time (i.e. run time plus equilibration) of less than 10 minutes total.
- A rugged method with consistent performance for >1000 injections with stable retention times, while maintaining good peak shape and response.
- Suitable for use with different matrices such as flower, chocolate, ointment, oil, concentrate, etc.

* After publication of this data SupraRnD later removed the freezing step and homogenized the whole flowers at room temperature. This was done to reduce the possibility of inflating the moisture content through condensation of atmospheric water onto the cold samples.

Table 2. Optimized method HPLC parameters

Column:	Ascentis® Express C18, 15 cm x 2.1 mm I.D., 2 µm
Mobile phase:	(A) 5 mM ammonium formate in water + 0.1% formic acid; (B) 0.1% formic acid in acetonitrile
Gradient:	70 to 90% B in 3 min; held at 90% B for 2 min; to 98% B in 0.1 min; held at 98% B for 0.9 min; to 70% B in 0.1 min; held at 70% B for 0.9 min
Flow rate:	0.4 mL/min
Pressure:	533 bar
Column temp.:	30°C
Detector:	UV, 228 nm
Injection:	25 µL
Sample:	methanolic extract of cannabis derived samples (oil, concentrate, ointment, etc.)

Calibration for the method was from 0.01 µg/mL to 40 µg/mL. This required a high dilution for some samples in order to bring them within this analytical range. For calibration and spiking, Cerilliant® certified reference materials (CRMs) were used. Individual cannabinoid CRMs at 1 mg/mL (with the exception of CBLA at 0.5 mg/mL) were diluted, along with the internal standard solution, directly into HPLC mobile phase component A, to prepare a 17-component stock solution at 40 µg/mL. This stock was then diluted further into a 30:70 mixture of HPLC mobile phases A:B, for the lower concentration calibration standards.

The HPLC column used for the analysis was an Ascentis® Express C18 column, 15 cm x 2.1 mm I.D., 2 µm. Ascentis® Express columns contain Fused-Core® particles with a solid core and porous shell architecture, also referred to as superficially porous. This particle structure provides higher separation efficiency than fully porous particles of the same size, and allows for faster analysis times with lower backpressure than approaches using smaller (<2 µm) fully porous particles. The particle architecture of Ascentis® Express columns allows for the use of larger particles, making them suitable for both conventional and UHPLC systems. For this method, SupraRnD used a UHPLC system, although with proper optimization, a similar result can be achieved on a conventional system using a 15 cm x 3.0 mm, 2.7 µm Ascentis® Express C18 column. Specifically, this would involve minimization of system dispersion. This can be done by reducing tubing length and ID of the column inlet and outlet; and for UV detectors, using a flow cell with a volume of <5 µL.

Method Validation and Performance

Prior to choosing the Ascentis® Express C18 for method validation, SupraRnD screened six other columns of similar chemistry from various manufacturers. They were able to achieve chromatographic resolution and a short run time with several columns, but it was found that the Ascentis® Express C18 was the only column that provided retention time stability – especially for the acidic cannabinoids. This is illustrated in **Figure 1** which shows chromatograms of a check standard at injection #1 and injection #1140, in between which numerous sample extracts were run.

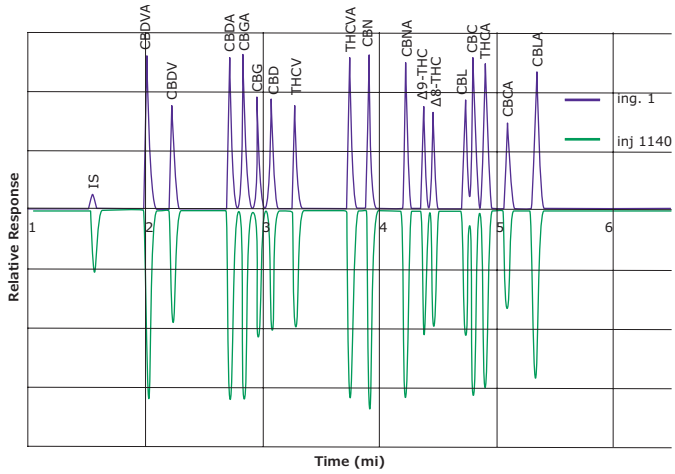


Figure 1. Cannabinoid standard on Ascentis® Express C18 column; comparison of injection #1 and injection #1140.

The method using the Ascentis® Express C18 was validated in several different matrices including hop flowers (as a surrogate matrix to cannabis), hemp seed oil, CBD concentrate, and topical ointments. Recoveries from hops ranged from 85-115% over a spiking range of 0.05 % to 20% by weight. A summary of this validation, as well as the other matrices, is summarized in **Table 3.** The method reporting limits (MRLs) achieved for the cannabinoids (except for CBDV, CBG, CBD and CBC in the concentrate) were all 0.05 wt.%. Repeatability, as %RSD, was <4% for all matrices. Further evaluation was done using proficiency testing in which the method successfully passed for samples of cannabis flower and hemp oil.

Table 3. Summary of method validation data for cannabinoid method in several matrices

	% Recovery range of all 17 cannabinoids spiked into matrix			RSD	MRL (wt%)
Spiking level (wt%)	**0.05%**	**1%**	**20%**		
Hops (surrogate matrix)	86-106	96-115	100.5-116	< 1.5%	0.05
Hemp seed oil	92-118	104-116	101.5-113	< 4%	0.05
Ointment 1					
(CBD isolate)	83-120*	80-122*	--	< 3%	0.05
Ointment 2	79-129**	86-117**	--	< 2.5%	0.05
CBD concentrate	71-123.5*	92-118*	--	< 3.5%	0.05 ***

*CBD recovery not quantitated due to high incurred levels
**Δ9-THC recovery not quantitated due to high incurred levels
***CBDV, CBG, CBD, CBC incurred in matrix led to issues preventing calculation of MRL for these compounds

Figure 2 shows example chromatograms of hop flowers spiked at 1% w/w and at the MRL concentration of 0.05% w/w. At the much lower spiking level, where matrix interference was more apparent, all 17 cannabinoids were discernable from background peaks and could be analyzed. The specific interferences eluting next to THCV and CBL were probably due to certain terpenes present in the hop sample. These peaks were not observed in cannabis flower. In a spiked ointment sample **(Figure 3)**, all cannabinoids were clearly detected at the MRL.

To date, >1,550 injections have been made on a single Ascentis® Express C18 column. SupraRnD has noted that thus far there has been no significant increase in column backpressure, or degradation in performance. Data collected on backpressure over the course of this use, showed a net increase of 2%. They also noted that retention times were stable, allowing them to identify cannabinoid peaks in samples with more confidence. An example is illustrated in **Figure 4** in which two different matrices, dark chocolate and cannabis flower, are compared. Both samples contained measurable amounts of Δ9-THC, and the difference in the retention time between the two matrices were minimal.

Conclusion

After evaluating several HPLC columns, SupraRnD has successfully developed a robust and rugged method using the Ascentis® Express C18 column for the analysis of 17 cannabinoids in a variety of matrices. Thus far, the method has been successfully applied to five different sample types including flower, ointments, chocolate, concentrates and gummies. The Ascentis® Express C18 column was chosen for the final method based on retention time stability over repeated use, and ability to maintain chromatographic performance for the cannabinoids. In addition, the column currently in use has shown minimal increase in backpressure over the course of >1,550 injections.

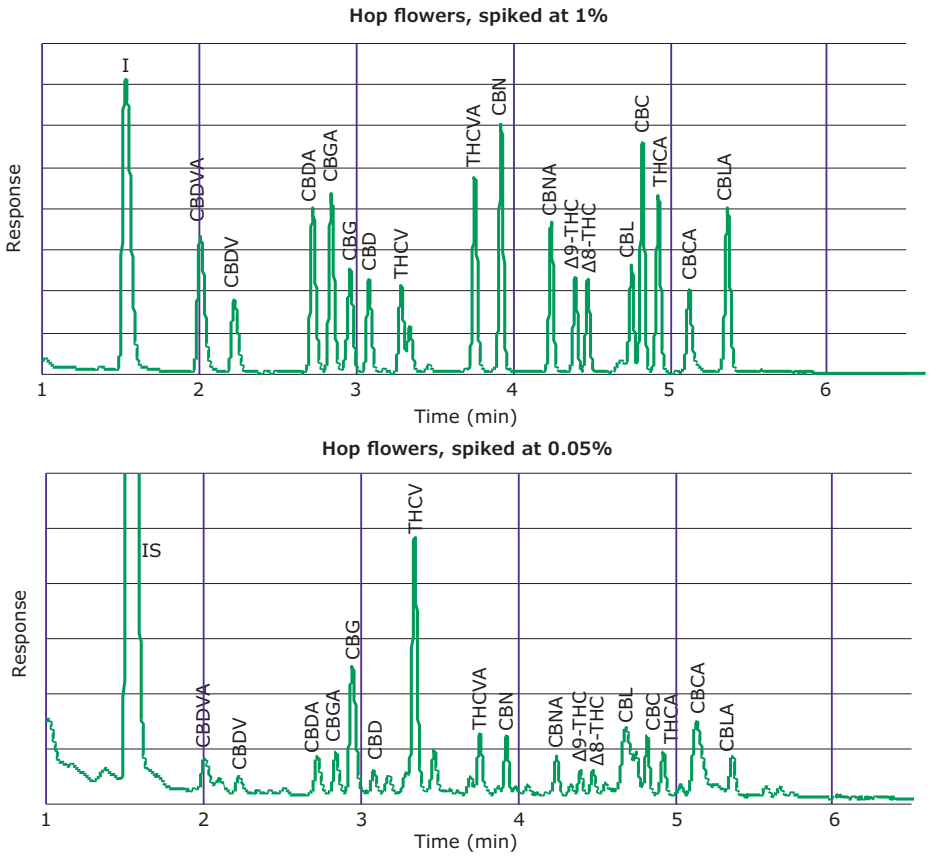


Figure 2. Hop flowers, spiked at 1% and .05% with cannabinoids.

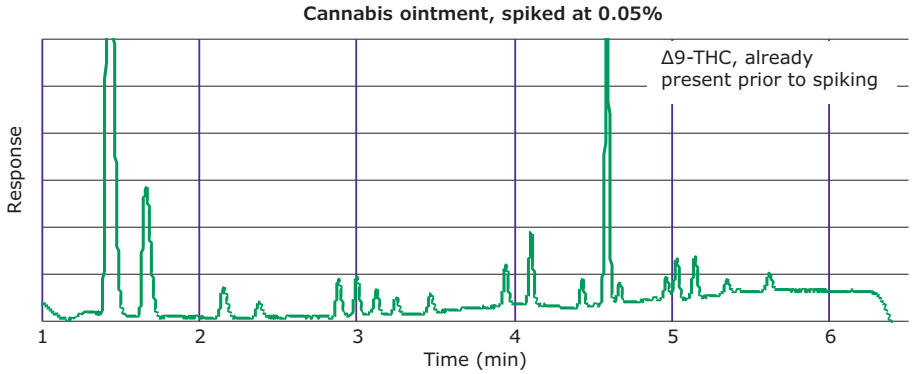


Figure 3. Ointment made from cannabis extract, spiked at 0.05% by weight.

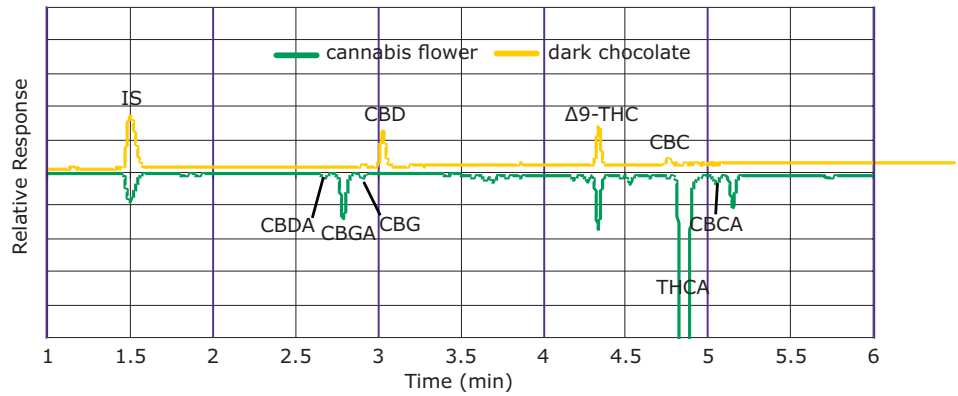


Figure 4. Comparison of elution pattern between dark chocolate and cannabis flower samples (unspiked).

Featured Products

Description	Cat. No.
Ascentis® Express C18, 15 cm x 2.1 mm I.D., 2 µm	**50814-U**
Cerilliant® Certified Reference Materials (all 1.0 mg/ml if not noted otherwise)	
Cannabidivarinic acid (CBDVA), in acetonitrile	**C-152**
Cannabidiolic acid (CBDA), in acetonitrile	**C-144**
Cannabigerolic acid (CBGA), in acetonitrile	**C-142**
Tetrahydrocannabivarinic acid (THCVA), in acetonitrile	**T-111**
Δ9-tetrahydrocannabinolic acid (THCA), in acetonitrile	**T-093**
Cannabinolic acid (CBNA), in acetonitrile	**C-153**
Cannabichromenic acid (CBCA), in acetonitrile	**C-150**
Cannabicyclolic acid (CBLA), 0.5 mg/mL, in acetonitrile	**C-171**
Cannabidivarin (CBDV), in methanol	**C-140**
Cannabigerol (CBG), in methanol	**C-141**
Cannabidiol (CBD), in methanol	**C-045**
Tetrahydrocannabivarin (THCV), in methanol	**T-094**
Cannabinol (CBN), in methanol	**C-046**
Δ9-tetrahydrocannabinol (Δ9-THC), in methanol	**T-005**
Δ8-tetrahydrocannabinol (Δ8-THC), in methanol	**T-032**
Cannabichromene (CBC), in methanol	**C-143**
(±)- Cannabicyclol (CBL), in acetonitrile	**C-154**

Related Products

Description	Cat. No.
Ammonium formate, eluent additive for LC-MS, LiChropur™, ≥99.0%	**70221**
Formic acid, for HPLC LiChropur™	**5.43804**
Acetonitrile, gradient grade LiChrosolv® Reag. Ph Eur	**1.00030**

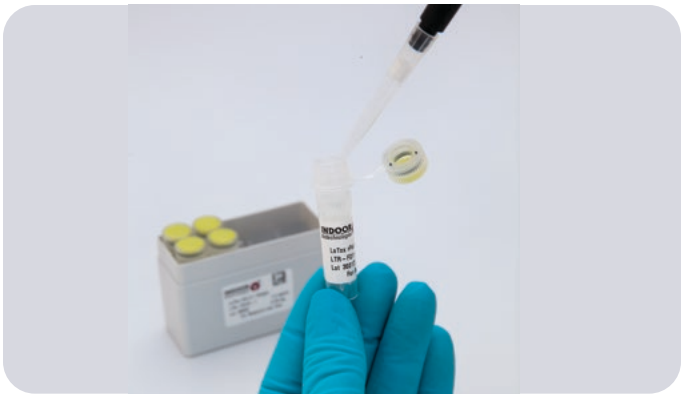
Find more information on Cannabis analysis at **SigmaAldrich.com/cannabis**

FOOD & BEVERAGES

Effective Detection of Food Allergens

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

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



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

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

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

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

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

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

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

Table 1: List of Food Allergen Proteins

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

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

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

References

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

FOOD & BEVERAGES

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

Markus Burholt, Scientist, Instrumental Analytics R&D
Michaela Oberle, Technical Project Manager, Instrumental Analytics R&D
Michael Schulz, Head of HPLC & Instrumental Analytics R&D
Frank Michel, Analytical & Chromatography Scientific Advisor
Monika Bäumle, Global Product Manager Thin-Layer Chromatography, Analytix@milliporesigma.com

Introduction

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

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

Experimental

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

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

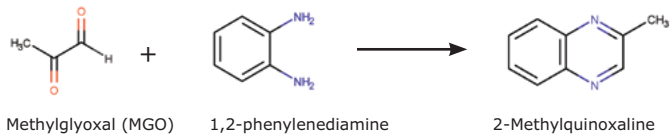


Figure 1. Reaction scheme of MGO with 1,2-phenylenediamine

Table 1. Calibration Curve

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

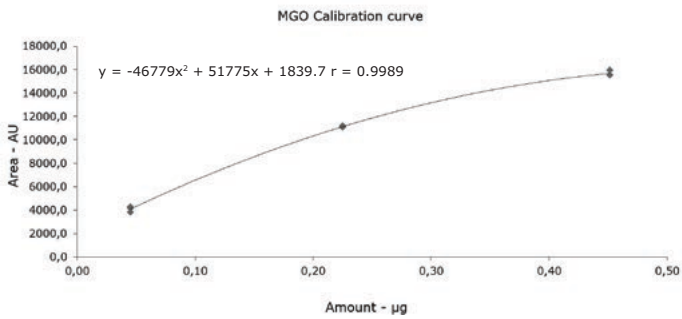


Figure 2. Calibration plot with corresponding calibration function.

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

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

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

Table 3. Quantification of methylglyoxal in the seven honey samples

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

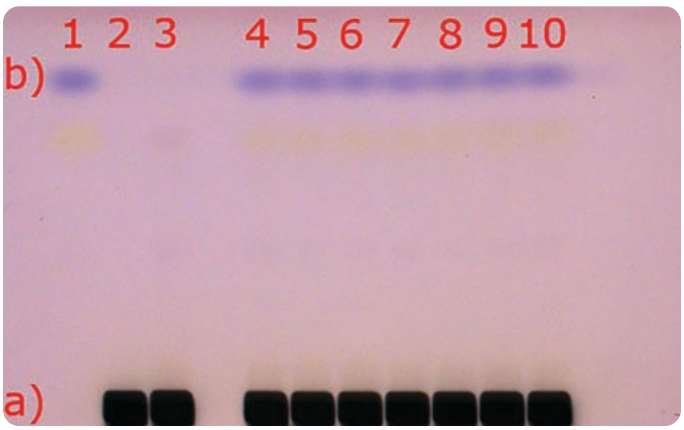


Figure 3. Visualization of the plate under visible light (white light); a) matrix compounds after staining with anisaldehyde sulfuric acid (black areas); b) 2-Methylquinoxaline (blue spot at hR_f 80), (reaction product of Methylglyoxal with 1,2-phenylenediamine)

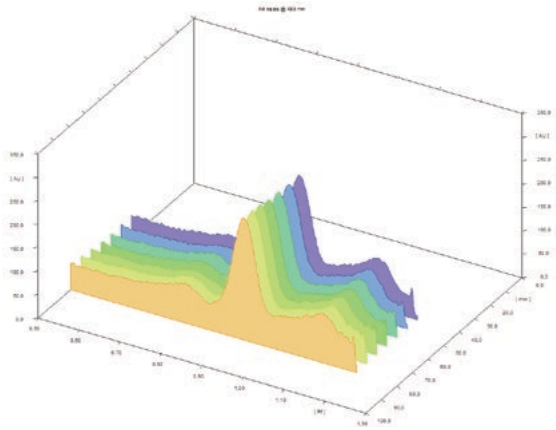


Figure 4. Scan of spiked honey tracks (sample 4 – 10) at 480 nm with CAMAG TLC Scanner 3.

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

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

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

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

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

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

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

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

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

Experimental Conditions

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

Results and Discussion

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

Conclusion

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

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

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

References

1. Mavric, E.; Wittmann, S.; Barth, G.; Henle, T. Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (Leptospermum scoparium) honeys from New Zealand Mol. Nutr. Food Res. 2008, 52, 483– 489
2. https://newzealandhoneyco.com/pages/mgo-vs-umf-calculator-manuka-honey
3. Analytix Reporter Issue 5, (p.3-7) https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/marketing/global/documents/730/477/analytix-reporter-5-2019-mk.pdf#page=3
4. https://magritek.com/2021/01/29/identification-and-quantification-of-methylglyoxal-in-manuka-honey/
5. E. Dimitrova (2013), Master thesis, TU University of Graz, Austria, https://diglib.tugraz.at/download.php?id=576a76ef4c784&location=browse (Accessed Nov. 2021)
6. unpublished results

Featured Products

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

See our portfolio of TLC products at
SigmaAldrich.com/TLC

FOOD & BEVERAGES

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

Emanuela Gionfriddo[a,b] Dominika Gruszecka[a] Xiujuan Li[a,c] Janusz Pawliszyn[a]

[a]Department of Chemistry, University of Waterloo, Waterloo, Ontario, Canada
[b]Department of Chemistry and Biochemistry, The University of Toledo, Toledo OH, 43606, USA
[c]Key Laboratory of Environment Correlative Dietology (Ministry of Education), College of Food Science & Technology, Huazhong Agricultural University, Wuhan, China, Analytix@milliporesigma.com

Abstract

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

Introduction

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

Experimental

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

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

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

Result and Discussion

Optimization of the DI-SPME Procedure

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

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

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

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

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

Method validation

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

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

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

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

Analysis of Real Samples

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

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

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

N.D.=not detected

Conclusions

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

References

1. Council of the E.U. European Parliament, Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on Maximum Residue Levels of Pesticides in or on Food and Feed of Plant and Animal Origin and Amending Council Directive 91/414/EEC, Official Journal of the European Union, Brussels, 2008.
2. Office of the Federal Register, Part 180—Tolerances and Exemptions for Pesticide Chemical Residues in Food, 36 FR 22540, United Stated of America, 2019.
3. Gionfriddo E., Gruszecka D., Li X., Pawliszyn J., Direct-immersion SPME in soy milk for pesticide analysis at trace levels by means of a matrix-compatible coating, *Talanta*. 211 (**2020**). https://doi.org/10.1016/j.talanta.2020.120746.
4. Souza-Silva É.A.; Pawliszyn J, Direct immersion solid-phase microextraction with matrix-compatible fiber coating for multiresidue pesticide analysis of grapes by gas chromatography-time-of-flight mass spectrometry (DI-SPME-GC-ToFMS), *J. Agric. Food Chem*. 63 (**2015**) 4464–4477. https://doi.org/10.1021/jf506212j.
5. De Grazia S.; Gionfriddo E.; Pawliszyn J, A new and efficient Solid Phase Microextraction approach for analysis of high fat content food samples using a matrix-compatible coating, Talanta. 167 (**2017**) 754–760. https://doi.org/10.1016/j.talanta.2017.01.064.
6. Souza-Silva E.A. ; Pawliszyn J., Optimization of Fiber Coating Structure Enables Direct Immersion Solid Phase Microextraction and High-Throughput Determination of Complex Samples, *Anal. Chem.* 84 (**2012**) 6933–6938. https://doi.org/dx.doi.org/10.1021/ac301305u.
7. Souza-Silva É.A.; Lopez-Avila V.; Pawliszyn J., Fast and robust direct immersion solid phase microextraction coupled with gas chromatography-time-of-flight mass spectrometry method employing a matrix compatible fiber for determination of triazole fungicides in fruits., *J. Chromatogr. A.* 1313 (**2013**) 139–46. https://doi.org/10.1016/j.chroma.2013.07.071.

Featured Products

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

Related Products

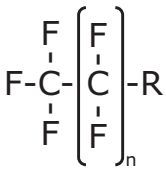
Description	Cat.No.
GC	
SLB®-5ms, 30 m × 0.25 mm, df= 0.25 µm	**28471-U**
Solvents	
Acetone, for gas chromatography MS SupraSolv®	**1.00658**
Water for gas chromatography SupraSolv® (or high purity from Milli-Q® system)	**1.02699**
Reference Materials	
Trifluralin, PESTANAL®, analytical standard, 250 mg	**32061**
Dimethoate, reference material, 100 mg	**52994**
Diazinon, certified reference material, TraceCERT®, 50 mg	**68486**
Malathion, certified reference material, TraceCERT®, 50 mg	**91481**
Chlorpyrifos, certified reference material, TraceCERT®, 100 mg	**94114**
Thiabendazole, certified reference material, TraceCERT®, 50 mg	**67554**
Phosalone, reference material, 100 mg	**44988**
λ-Cyhalothrin, certified reference material, TraceCERT®, 50 mg	**72765**
β-Cyfluthrin, certified reference material, TraceCERT®, 50 mg	**93223**
Esfenvalerate, reference material, 100 mg	**67115**

For our complete SPME portfolio or to download the guide "**SPME for GC - Getting Started with Solid Phase Microextraction**" visit us at **SigmaAldrich.com/SPME**

ENVIRONMENTAL

Fast and High-Resolution LC-MS Separation of PFAS

Petra Lewits, Cory Muraco, Product Manager Liquid Separations, Analytix@milliporesigma.com
Johanna Simon, Scientist Central Analytics, Analytix@milliporesigma.com



PFAS (Per- and poly-fluoroalkyl substances) are persistent, man-made organic compounds, widely found in the environment. Recent awareness about their toxicity has led the U.S. Food and Drug Administration (FDA) and the U.S. Environmental Protection Agency (EPA) to initiate actions against PFAS. Hence reliable and fast methods for their determination are needed.

PFASs are commonly measured using liquid chromatography–mass spectrometry (LC-MS). The column of choice for PFAS analysis by LC-MS(/MS) is a C18 column. The Ascentis® Express PFAS columns are based on superficially porous silica particles (SPP) with C18 modification and are specifically tested using a PFAS compound mixture. This ensures the suitability and reliable performance of these columns for an efficient PFAS analysis.

PFAS compounds originating from the HPLC system and materials used for the analysis are a concern. Therefore, it is recommended to place a delay column before the injection port in the system (**Figure 1**). The Ascentis® Express PFAS Delay column provides exceptionally high retention of PFAS compounds across the various mobile phase conditions. It efficiently delays the PFAS background contamination that originates from the instrument and therefore prevents co-elution with the PFAS compound present in the sample (**Figure 1**).

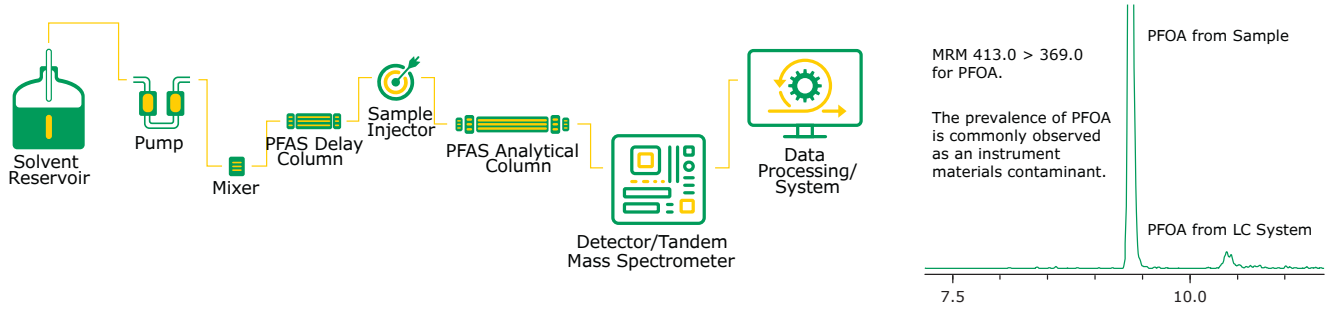


Figure 1. LC-MS instrumental set-up for PFAS analysis & results of perfluorooctanoic acid (PFOA) (MRM 413.0>369.0) using a delay column.

LC-MS Analysis of PFAS - 33 Compounds in 5 minutes

The rapid separation of 33 PFAS compounds found in EPA 537.1, EPA 533, and EPA 8327 demonstrates that the Fused-Core® technology of Ascentis® Express PFAS HPLC columns benefits the PFAS analysis for fast, efficient, and rugged separations—paramount to environmental analysis (**Figure 2**).

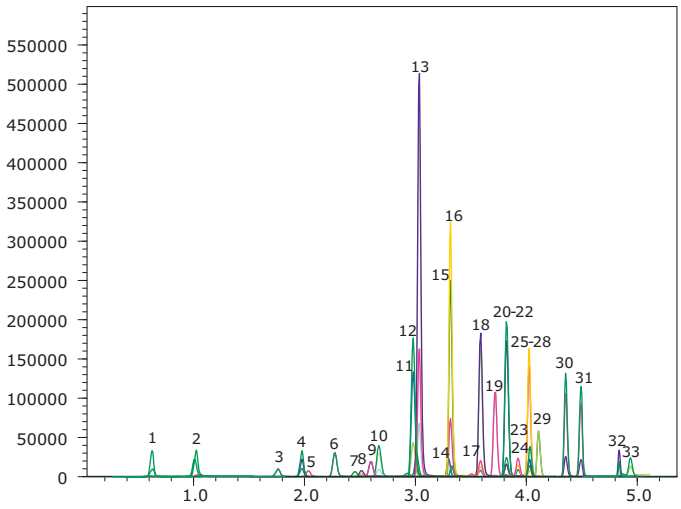


Figure 2. 33 PFAS Compounds in 5 min

LC Conditions

Analytical col.:	Ascentis® Express 90Å PFAS, 10 cm x 2.1 mm, 2.7 µm (**53559-U**)
Delay col.:	Ascentis® Express 90Å PFAS Delay, 5 cm x 3 mm, 2.7 µm (**53572-U**)
Mobile phase :	[A]10 mM Ammonium acetate; [B] Methanol
Gradient:	

Time (min)	%B
0.0	33.0
4.0	98.0
4.1	100.0
6.0	100.0
6.1	33.0
7.5	End

Flow rate:	0.4 mL/min
Pressure:	479 bar (6947 psi)
Temperature:	35 °C
Detection:	ESI (-) MS/MS; ESI LCMS system: Shimadzu LCMS-8040; Spray voltage: -2.0 kV; Nebulizing gas: 2 L/min; Drying gas: 15 L/min; DL temp: 250 °C; Heat block: 400 °C
Inj. vol.:	2.0 µL
Sample solvent:	methanol (96%) water (4%)

Find an overview of all HPLC columns at:
SigmaAldrich.com/HPLC

See our portfolio of PFAS standards under Organic Pollutant Standards at
SigmaAldrich.com/standards

Sample Compounds

Peak	Compound	Transition	Retention Time (min)
1	PFBA	213.0>169.0	0.755
2	4:2FTS	229.0>85.0	1.031
3	PFPeA	263.0>219.0	1.762
4	PFBS	299.0>80.0	1.979
5	PFHpS	279.0>85.0	2.035
6	PFPeS	315.0>135.0	2.273
7	PFMPA	327.0>307.0	2.454
8	PFHxA	313.0>269.0	2.514
9	PFEESA	349.0>80.0	2.599
10	HFPO-DA	285.0>169.0	2.670
11	PFHxS	399.0>80.0	3.013
12	NaDONA	377.0>251.0	3.033
13	ADONA	377.0>250.9	3.034
14	FOSA	427.0>407.0	3.299
15	PFOA	413.0>369.0	3.316
16	PFMBA	449.0>80.0	3.328
17	PFHpA	363.0>319.0	3.388
18	PFOS	499.0>80.0	3.588
19	9Cl-PF3ONS	530.9>351.0	3.719
20	8:2FTS	549.0>80.0	3.816
21	PFNS	527.0>507.0	3.820
22	PFDA	513.0>469.0	3.822
23	N-MeFOSAA	570.0>419.0	3.925
24	PFNA	463.0>419.0	3.942
25	NFDHA	599.0>80.0	4.015
26	PFUnA	563.0>519.0	4.025
27	N-EtFOSAA	584.0>419.0	4.029
28	6:2FTS	498.0>78.0	4.033
29	11Cl-PF3OUdS	630.7>451.0	4.110
30	PFTrDA	663.0>619.0	4.355
31	PFDoA	613.0>569.0	4.496
32	PFTeDA	713.0>669.0	4.745
33	PFDS	295.0>201.0	4.921

Featured Products

Description	Cat. No.
Ascentis® Express 90Å PFAS, 10 cm x 2.1 mm, 2.7 µm,	**53559-U**
Ascentis® Express 90Å PFAS Delay, 5 cm x 3 mm, 2.7 µm	**53572-U**
Solvents & Reagents	
Methanol for chromatography (LC-MS grade) LiChrosolv®	**1.06035**
Water for chromatography (LC-MS grade) LiChrosolv® or tap fresh from an appropriate Milli-Q® system	**1.15333**
Ammonium acetate suitable for mass spectrometry (MS), LiChropur™, eluent additive for LC-MS	**73594**

Related products

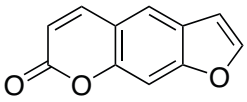
Description	Cat. No
Analytical Standards	
Perfluorobutanoic acid, neat	**68808**
Perfluoropentanoic acid, neat	**68542**
Perfluorohexanoic acid, neat	**43809**
Perfluorooctanoic acid, neat	**33824**
Perfluorononanoic acid, neat	**91977**
Perfluorodecanoic acid, neat	**43929**
Perfluorododecanoic acid, neat	**92291**
Perfluorotetradecanoic acid, neat	**80312**
Pentadecafluorooctanoic acid, 100 µg/mL in methanol	**33603**
Heptadecafluorooctanoic acid, 100 µg/mL in methanol	**33607**
Perfluorooctane sulfonic acid, neat	**33829**

Worried about the Safety of Sunscreens?

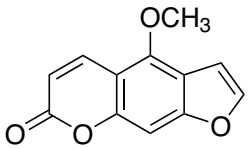
Introducing a new Certified Reference Material Mix for Furocoumarins

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

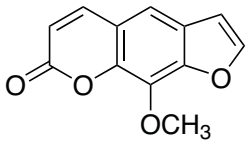
Furocoumarins are a class of chemical compounds produced by a wide range of plants, but mostly by the families *Apiaceae* and *Rutaceae*. For example, they could be found in citrus oil extracts. Generally, the chemical structure of furocoumarins includes a furan ring, fused with coumarin as shown in **Figure 1**.



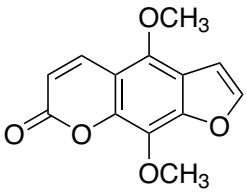
Psoralen



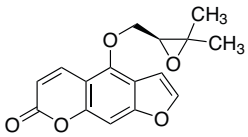
Bergapten



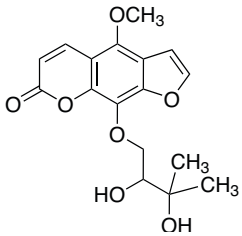
Xanthotoxin



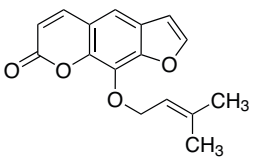
Isopimpinellin hydrate



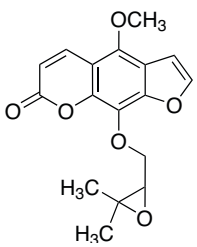
Oxypeucedanin



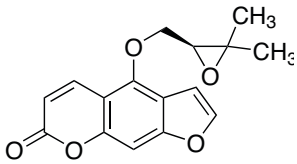
Oxypeucedanin



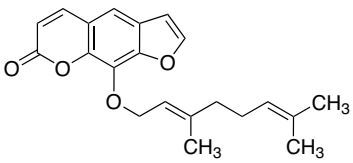
Byakangelicol



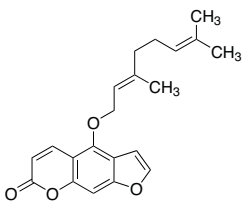
Byakangelicin



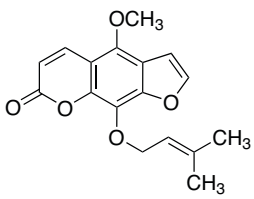
Heraclenin



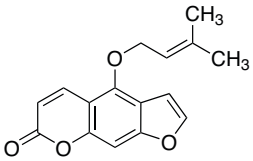
8-Geranyloxypsoralen



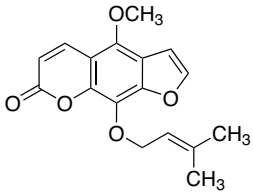
Bergamottin



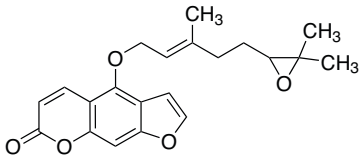
Imperatorin



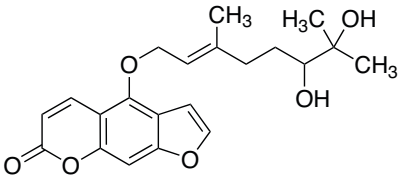
Isoimperatorin



Phellopterin



Epoxybergamottin



6´,-7´-Dihydroxybergamottin

Figure 1. Chemical Structures of Furocoumarins

Furocoumarins undergo activation by UV light and can have toxic effects.

Therefore, in the EU citrus-derived ingredients of sun-protection and bronzing products must have a furocoumarin content of below 1 mg/kg

The International Fragrance Association (IFRA) has developed an official analytical method to test for furocoumarins by HPLC-DAD. We offer a certified reference material mix, compliant with the IFRA method.

Key Features

- *Trace***CERT®** certified reference material traceable to NIST SRM
- Manufactured following an established workflow according to ISO 17034
- qNMR certification of each component of the mix (following ISO/IEC 17025 accreditation)
- Tested for homogeneity and long-term stability using LC method
- Supplied with a comprehensive certificate including the overall uncertainty

Product Number	**93102**
Product Name	**Furocoumarin Mix**
Quality	certified reference material, *Trace***CERT®**
Solvent	Acetonitrile
Concentrations	250 mg/kg per component
Package Size	1 mL

Components:

Compound	CAS No.	Cat. No.
Psoralen	66-97-7	**55738***
Bergapten	484-20-8	**55991***
Xanthotoxin	298-81-7	**55992***
Isopimpinellin	482-27-9	**56182***
Oxypeucedanin	737-52-0	**63346***
Oxypeucedanin hydrate	24724-52-5	**63354***
Byakangelicol	61046-59-1	**63399***
Byakangelicin	19573-01-4	**63503***
Heraclenin	35740-18-2	**63353***
8-Geranyloxypsoralen	71612-25-4	**56349***
Bergamottin	7380-40-7	**56393***
Imperatorin	482-44-0	**55996***
Isoimperatorin	482-45-1	**56148***
Phellopterin	2543-94-4	**56149***
Epoxybergamottin	206978-14-5	**56394***
6´,-7´-Dihydroxybergamottin	145414-76-2	**56446***

*coming soon also as single component reference material

Note: The components of the mix will also be made available as neat certified reference materials under the product numbers listed above.

Stable Isotope-Labeled Amino Acid Mixes

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

Amino acids play a central role in biochemistry being the building blocks of proteins or as precursors in the biosynthesis of secondary metabolites. From being used as food additives (as artificial sweeteners and flavor enhancers) to the synthesis of drugs, biodegradable plastics, and chiral catalysts - amino acids have a range of uses. Amino acid analysis generally involves hydrolysis of the peptide bonds and analysis of the released amino acids by appropriate analytical methods.[1]

Isotopically labeled amino acids can function as internal standards for an amino acid analysis (AAA) using Isobaric-tagged isotope dilution mass spectrometry (IT-IDMS) methods. The IT-IDMS methods offer better accuracy, precision, and sensitivity in comparison to the traditional AAA methods.[2]

We have always strived to provide you with a wide range of high-quality reference materials. Our reference materials portfolio offers more than 20,000 products. View **SigmaAldrich.com/standards** for the complete range. Recently, two new certified solution mixtures of labeled amino acids have been added to the portfolio. These mixes are suitable for use as an internal standard during a LC/MS or GC/MS quantitation (after derivatisation) of amino acids by isotope dilution methods and other research applications.

Key Features

- *Trace***CERT®** certified reference material, traceable to primary material from NIST
- Isotopically Labeled Amino Acid Mix produced by an established workflow following ISO 17034
- qNMR certified components in the mix (following ISO 17025 accreditation)
- Tested for homogeneity and long-term stability using LC method
- Supplied with a comprehensive certificate including the overall uncertainty

Product Number:	**96378**
Product Name:	Stable Isotope Labeled Amino Acid Mix Solution 1
Grade:	certified reference material, *Trace***CERT®**
Solvent:	HCl (0.1 M)
Concentrations:	1250-2500 nmol/mL
Package Size:	1 mL

Component	CAS No.	Nominal Concentration
L-Alanine-$^{13}C_3$, ^{15}N	202407-38-3	2.5 mmol/L
L-Arginine-$^{13}C_6$	55443-58-8	2.5 mmol/L
L-Aspartic acid-$^{13}C_4$	55443-54-4	2.5 mmol/L
L-Cystine-$^{13}C_6$, $^{15}N_2$	1252803-65-8	1.25 mmol/L
L-Glutamic acid-$^{13}C_5$	55443-55-5	2.5 mmol/L
Glycine-$^{13}C_2$,^{15}N	211057-02-2	2.5 mmol/L
L-Histidine-$^{13}C_6$	55443-59-9	2.5 mmol/L
L-Isoleucine-$^{13}C_6$, ^{15}N	202468-35-7	2.5 mmol/L
L-Leucine-$^{13}C_6$, ^{15}N	202406-52-8	2.5 mmol/L
L-Lysine$^{13}C_6$	55443-57-7	2.5 mmol/L
L-Methionine$^{13}C_5$, ^{15}N	202468-47-1	2.5 mmol/L
L-Phenyl-$^{13}C_6$-alanine	180268-82-0	2.5 mmol/L
L-Proline-$^{13}C_5$	201740-83-2	2.5 mmol/L
L-Serine-$^{13}C_3$, ^{15}N	202407-34-9	2.5 mmol/L
L-Threonine-$^{13}C_4$	55443-53-3	2.5 mmol/L
L-Tyrosine-(phenyl-$^{13}C_6$)	201595-63-3	2.5 mmol/L
L-Valine-$^{13}C_5$	55443-52-2	2.5 mmol/L

Product Number:	**01428**
Product Name:	Stable Isotope Labeled Amino Acid Mix Solution 2
Suffix:	certified reference material, *Trace***CERT®**
Solvent:	HCl (0.1M)
Concentrations:	500-2500 nmol/mL
Package Size:	1 mL

Component	CAS No.	Nominal Concentration
L-Alanine-$^{13}C_3$, ^{15}N	202407-38-3	0.5 mmol/L
L-Arginine-$^{13}C_6$	55443-58-8	0.5 mmol/L
L-Aspartic acid-$^{13}C_4$	55443-54-4	0.5 mmol/L
L-Glutamic acid-$^{13}C_5$	55443-55-5	0.5 mmol/L
Glycine-$^{13}C_2$,^{15}N	211057-02-2	2.5 mmol/L
L-Leucine-$^{13}C_6$, ^{15}N	202406-52-8	0.5 mmol/L
L-Methionine$^{13}C_5$, ^{15}N	202468-47-1	0.5 mmol/L
L-Phenyl-$^{13}C_6$-alanine	180268-82-0	0.5 mmol/L
L-Proline-$^{13}C_5$	201740-83-2	0.5 mmol/L
L-Tyrosine-(phenyl-$^{13}C_6$)	201595-63-3	0.5 mmol/L
L-Valine-$^{13}C_5$	55443-52-2	0.5 mmol/L

Find a complete list of amino acid reference material on **SigmaAldrich.com/aminoacidstandards**

References

1. Odia A.; Zekeri Esezobor O. Therapeutic Uses of Amino Acids. Amino Acid - New Insights and Roles in Plant and Animal, Asao T., Asaduzzaman M., Eds; IntechOpen, 2017 DOI: 10.5772/intechopen.68932
2. Woolfitt A.R., Solano M.I., Williams T.L., James L. Pirkle J. L., Barr J.R., Amino Acid Analysis of Peptides Using Isobaric-Tagged Isotope Dilution LC–MS/MS, Anal. Chem. 2009, 81, 10, 3979–3985, https://pubs.acs.org/doi/10.1021/ac900367q

HPLC Products: The Best Choice for Any LC Instrument

A Suitability and Installation Review

Cory E. Muraco, Global Product Manager, Liquid Chromatography, Analytix@milliporesigma.com

Since the 1970's, Supelco® has been a trusted name in high performance liquid chromatography (HPLC) consumables. From a diverse array of columns packed with different stationary phases to a complete portfolio of HPLC accessories, the Supelco® portfolio of HPLC consumables caters to the needs of all analysts. At its most basic level, one major need of the analyst is for the HPLC consumable to be compatible with the instrument. All Supelco™ U/HPLC columns are compatible with instruments from all major vendors and do not require any additional accessories for proper installation.

Supelco® HPLC columns are manufactured with end fittings outfitted with standard 10-32 ports, enabling easy connection to any LC instrument. This trait is demonstrated in **Figures 1** and **2** which show the column connected to an Agilent® 1290 U/HPLC instrument as well as a zoomed-in picture of the column connected to a column nut from the injector side of the instrument, respectively. It is crucial for your HPLC column to be fully in the column oven of your instrument as temperature fluctuations in the laboratory can, in some instances, drastically affect the chromatographic results if a column is only partially in a column oven or if the column oven cannot properly close. Supelco® HPLC columns of all geometries fit comfortably in column ovens of all HPLC, UHPLC, and UPLC® instruments.

In addition to the column fitting into a column oven correctly, it is just as important that the column connecting nuts be seated properly in both the inlet and outlet ports of the column. All Supelco columns use Parker fittings, nuts and ferrules, which is the industry standard in HPLC/UHPLC. By not seating these nuts properly in the column, a mixing chamber (dead volume) can be formed at the interface between the column and the fitting. This mixing chamber can impart additional band broadening (an increase in peak width) in the chromatographic results, which will lead to an overall decrease in efficiency and resolution. Also, when connecting an HPLC column, it is crucial that the shortest length possible be used in plumbing the instrument going from injector to column and column to detector. Excessive tubing length can lead to a decrease in efficiency due to the added system dead volume. Along with tubing length, tubing inner diameter (I.D.) should be considered, and the smallest, practical I.D. should be utilized (typically 0.010 in I.D. and 0.005 in I.D. tubing is used for HPLC and UHPLC instruments, respectively). Utilizing the narrowest I.D. tubing will lead to lower system dead volume and higher chromatographic efficiency. Fortunately, the Supelco® HPLC portfolio has a comprehensive collection of accessories to optimize the performance of your instrument and ensure the most efficient results are generated from your HPLC column.

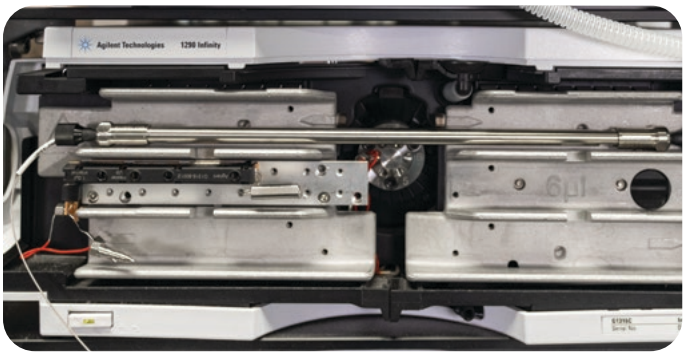


Figure 1. Supelco® analytical HPLC column installed onto an Agilent® 1290 U/HPLC instrument. Notice how the column comfortably fits into the column oven.

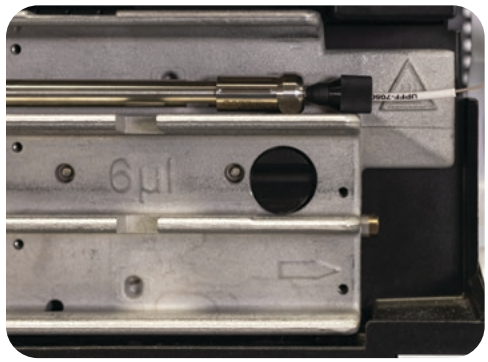


Figure 2. Zoomed-in picture of the column connected properly to the inlet tubing.

Beyond what was mentioned above, the end fittings of some HPLC columns (e.g., Chromolith® columns) consist of PEEK, while others are made out of stainless steel. Mounting metal capillaries with 1/16'' outer diameter and a metal cutting ring fixed to a 3 mm drill hole length can damage the PEEK hardware (both column housing and end fitting) and the silica bed of the aforementioned columns. To avoid any damage, use either flexible metal capillaries (0.25 mm outer diameter) with a polyvinylidene fluoride (PVDF) cone or PEEK capillaries with PEEK screws and adjustable plastic ferrules.

Prior to injecting the sample onto the HPLC column, it is highly recommended to perform sample preparation techniques (filtration, extraction, solid phase extraction (SPE), etc.) on the sample if it contains large, particulate matter, or other undesirable components. Failure to perform sample prep on the sample can lead to a drastic decrease in the column lifetime as particulate matter begins to accumulate on the column frit and/or the stationary phase particles begin to adsorb undesirable sample components (note: Chromolith® monolithic HPLC columns do not have frits; this is one reason for the extended lifetime for these columns). Even if sample prep is performed on a sample, sometimes, not all impurities are removed. In these instances, a guard column should be employed to protect the analytical column from being compromised. There are two common types of guard columns manufactured for use with Supelco® U/HPLC columns: a stand-alone guard column (example in **Figure 3**) that has to be connected to the main column by a capillary and a direct connect guard cartridge (example in **Figure 4**). The direct connect guard cartridge is composed of three pieces (2 fitting parts and the guard cartridge) as depicted in **Figure 5**; **Figure 6** shows the direct connect guard cartridge connected to a 25 cm long HPLC column. As can be seen in the figure, the main and guard column together still fit comfortably in the column oven and all metal parts of the set up do have contact to the heating elements ensuring reliable thermal conductivity. All stand-alone guard columns and direct connect guard cartridges are compatible with all HPLC, UHPLC, and UPLC® instruments.

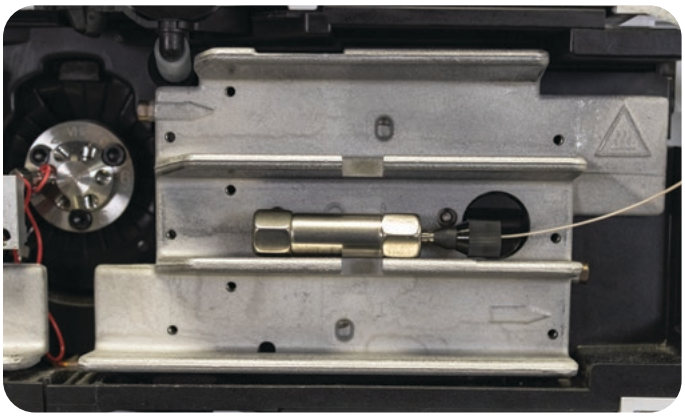


Figure 3. Stand-alone guard cartridge connected to inlet tubing. A second piece of tubing with a connecting nut is required to connect to the analytical column.

Figure 4. Direct connect guard cartridge. This type of guard column does not require any connecting tubing to connect to the analytical column; it screws right into the column inlet (see also **Figure 6**).

Figure 5. Components of the direct connect guard cartridge.

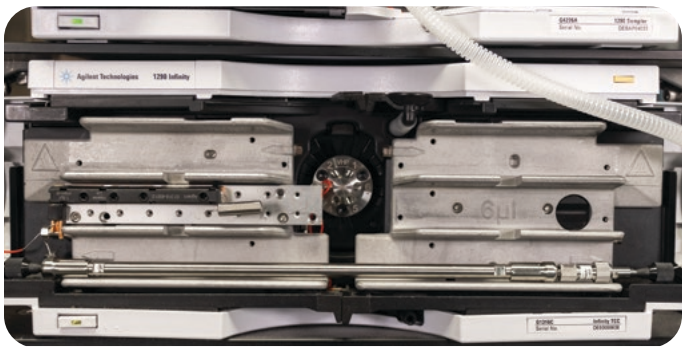


Figure 6. Direct connect guard cartridge connected to a 25 cm length analytical column. Even with this long geometry, the direct connect guard cartridge can still be connected to the column and fit inside a column oven ensuring thermal consistency.

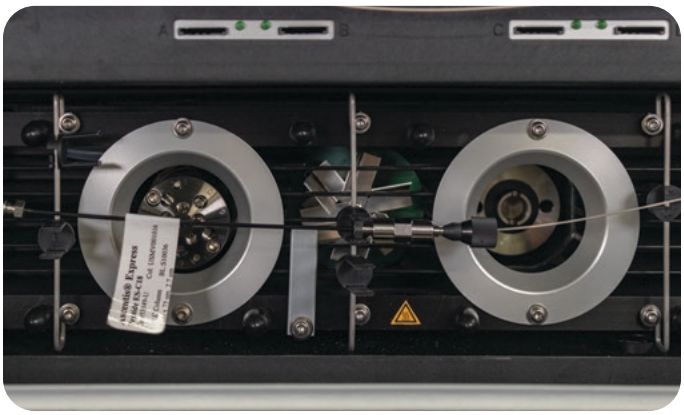


Figure 7. Capillary U/HPLC column connected to a Thermo Fisher Scientific® Ultimate 3000 capillary U/HPLC system. Complete system compatibility is achieved.

In addition to analytical-scale columns, the Supelco® HPLC portfolio also contains capillary and preparative columns for selected stationary phases. Just as with the analytical-scale columns, these capillary and prep columns are compatible with all vendors' capillary and prep LC systems, respectively. As an example, **Figure 7** displays a Supelco® capillary U/HPLC column connected to a Thermo Fisher Scientific® Ultimate 3000 capillary U/HPLC instrument.

The Supelco® HPLC portfolio is a comprehensive column and accessories portfolio that is 100% compatible with any HPLC, UHPLC, or UPLC® system. All columns fit within all column ovens from various instrument vendors, and our HPLC accessories can easily be installed onto any instrument without fear of damaging the instrument. Products from the Supelco® HPLC portfolio are a valuable and reliable choice for your U/HPLC or LC-MS workflow.

See more at **SigmaAldrich.com/HPLC**

Find more information on LC-MS workflow tool on **SigmaAldrich.com/lcms**

SCIENCE & TECHNOLOGY INNOVATIONS

HPLC Tips & Tricks

Mobile Phase Preparation Part 2 - Buffers

Dr. Egidijus Machtejevas, Lead Expert, Analytical Science Liaison, Analytix@milliporesigma.com

When analysing samples containing ionizable compounds, the buffer can be one of the most important variables controlling the retention in an HPLC separation. The pH of the mobile phase determines the presence of ionizable compounds (analytes and matrix) to be in either an ionized or non-ionized state. The ionized species in reverse phase (RP) chromatography always elute from the column earlier than the non-ionized species. Changing the pH can also increase the selectivity for effective separation of closely eluting or overlapping peaks. Run-to-run variability in pH results in a separation inconsistency. Buffers prevent pH variations. Therefore, the proper buffer choice, in terms of buffering species, ionic strength, and pH, is the most critical step in HPLC method development when ionizable substances are analysed.

Tips for choosing an LC buffer

Buffer selection. The choice of the appropriate buffer for an application is governed by the buffer characteristics such as pK_a, pH range, and UV cut-off. As a rule, buffers should be used for a pH within +/- 1 unit of their pK_a value. Within this range, buffers resist any deliberate attempts of change in pH. The buffer's capacity is at its maximum when its pH is equal to its pK_a. The UV cut-off value also needs to be considered, as the detection wavelength should not interfere with the buffer absorbance (significant absorbance: trifluoroacetic acid <220 nm; formic acid, acetic acid <240 nm). For the best results with an ionizable analyte of interest, use a buffer with a pH at least 2 units away from the analyte's pK_a. If the pH of the mobile phase is too close to the analyte's pK_a, split peaks or shoulders might be observed due to the presence of both species in the sample. For several ionizable analytes of interest, it is preferable to choose a pH value wherein all the analytes exist in the same form, either ionized or non-ionized.

Measuring buffer pH. pH of the buffer is the pH of the aqueous portion before the organic mobile phase part is added. The addition of an organic solvent can shift the pH either up or down (pH shift should be consistent for the same buffer). It is not so important to know the exact pH value of the buffer in an organic medium, but it is important to have a consistent pH value (because pK_a of your analytes is also determined in aqueous phase, and we do not know the individual pK_a shifts either).

Chemical Purity. The quality/purity of mobile phase additives (buffers, salts, acids, and bases) along with organic solvents utilized in an HPLC experiment must be adapted to the detector sensitivity and elution protocol.

Chemical Compatibility. Buffer composition, along with mobile phase pH, must be chosen in agreement with column housing material and nature of the stationary as well as different parts of LC instrument (pumps, tubing's, etc.) phase to prevent corrosion or degradation of either.

MS compatibility. Introducing inorganic buffer salts into a mass spectrometer soon fouls the system. Examples of suitable volatile buffers are ammonium acetate, ammonium formate, and ammonium citrate. pH modifiers like formic acid and acetic acid should be used to control pH and help ionization for LC-MS.

Buffer Solubility. Ideally, the buffer should be completely water-soluble (RP methods) and should not precipitate during the analysis when mixed with a chosen organic solvent. Buffer concentration must therefore be carefully chosen to avoid precipitation at higher concentrations in the organic solvent. If neglected, this can create operational problems with the pumps and instigate HPLC column blockage or backpressure rise.

Buffer Ionic Strength. In case of ionic interactions between analytes and stationary phase, the ionic strength of the buffer must be chosen in a way that compounds are eluted. The required ionic strength of the buffer depends on the stationary phase. Besides elution strength, the viscosity of the buffer plays an important role in terms of its suitability for use in HPLC analyses.

Buffer Concentration. Ideally, the lowest concentration that gives reproducible results should be chosen. Higher concentrations lead to a faster elution of polar molecules. Generally, the buffer concentration should not be lower than 5 mM. Below this concentration, the solution may not perform as a buffer (depending on analyte concentration and buffering capability). Raising the buffer concentration can increase viscosity and the risk of buffer precipitation, which in turn can increase column back pressure. Commonly, the concentration should be kept in the 5 to 100 mM range. A concentration higher than 100 mM of mineral salt buffers wear out the pump's movable parts faster, therefore a back-seal wash is recommended to be installed.

It can be observed that buffers play a crucial role in a majority of HPLC separations. Method development often requires careful selection of buffers and adequate care in their preparation. So, the general rules to be kept in mind are— buffer solutions must be homogeneous, clear, and free from any particles. If stored, please keep in mind that buffers have a limited lifetime, so consider their preparation daily.

In case of any chromatography challenges, please contact me at **Analytix@milliporesigma.com**

Minimize complexity. Magnify your focus.

Milli-Q® IQ 7003/05/10/15

Integrated Ultrapure and Pure Water System

- Simple and intuitive Q-POD® and E-POD® dispensers
- Touchscreen display for unparalleled ease of use
- Effortless data management and ensured traceability
- Compact and versatile to optimize lab space

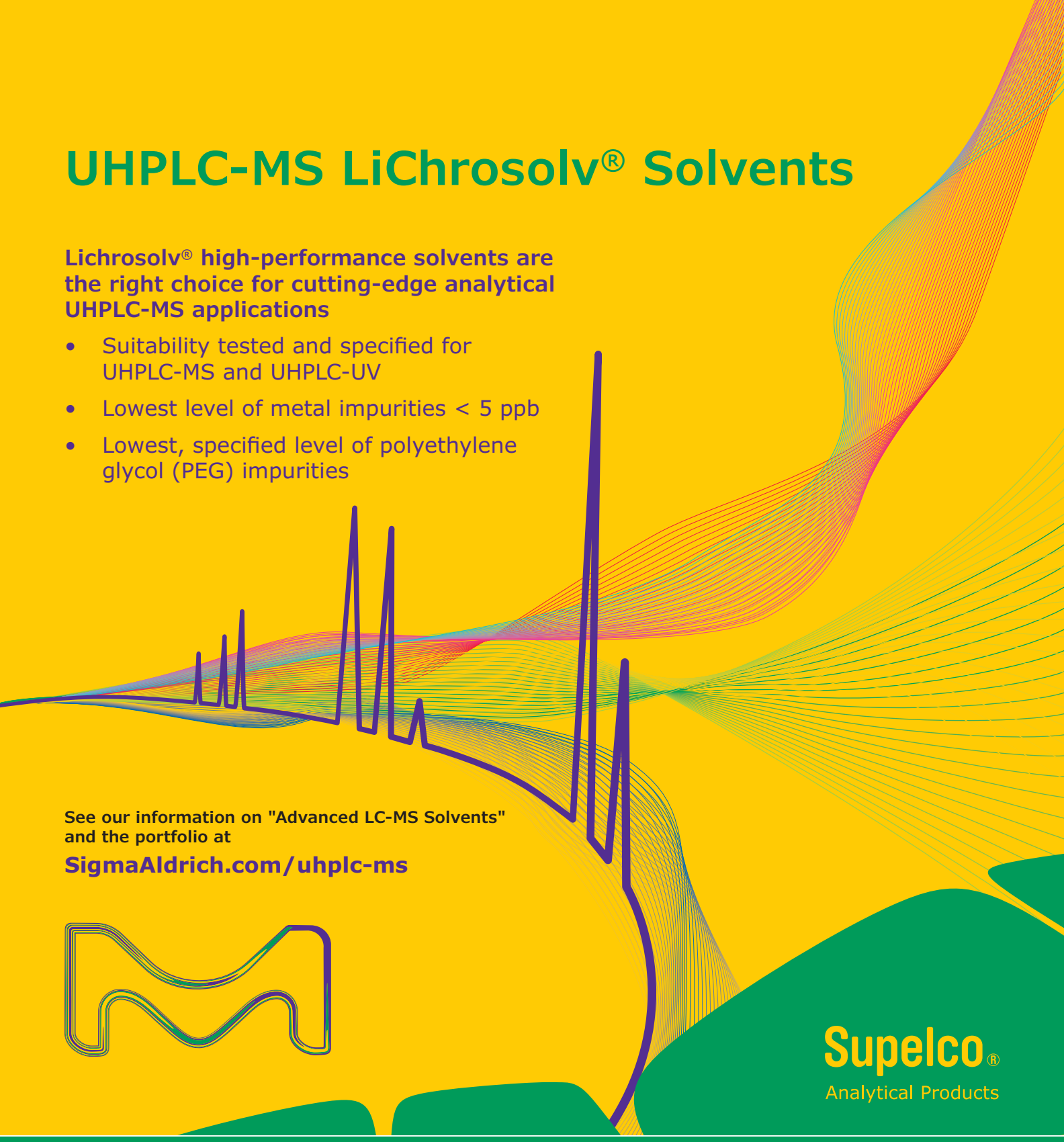
To find out more, visit:
SigmaAldrich.com/labwater

Milli-Q®
Lab Water Solutions

UHPLC-MS LiChrosolv® Solvents

Lichrosolv® high-performance solvents are the right choice for cutting-edge analytical UHPLC-MS applications

- Suitability tested and specified for UHPLC-MS and UHPLC-UV
- Lowest level of metal impurities < 5 ppb
- Lowest, specified level of polyethylene glycol (PEG) impurities



See our information on "Advanced LC-MS Solvents" and the portfolio at

SigmaAldrich.com/uhplc-ms

Supelco®
Analytical Products

To place an order or receive technical assistance

Order/Customer Service: **SigmaAldrich.com/order**
Technical Service: **SigmaAldrich.com/techservice**
Safety-related Information: **SigmaAldrich.com/safetycenter**

SigmaAldrich.com

Lit. No. MS_NL8375EN Ver. 1.0
38223
12/2021