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Determination of Water in Cannabis & Hemp by Karl Fischer Titration

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Analytix Reporter

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Dear Reader,

Exact water determination of samples is crucial in many industries to ensure continuous product quality, stability, and properties. It is relevant for agricultural products like cannabis and hemp as well. For example water content in the dry weight of cannabis and hemp affects the delta-9 tetrahydrocannabinol (THC) concentration calculation and in turn their potency. Hence correct water content should be determined to ensure its legal use. This sets the need for a fast and reliable method to determine the water content. The Karl Fischer oven method is a precise and accurate way for such kind of samples as presented in the featured article of this edition.

The Karl Fischer titration in itself has a long history and it is still used for exact water content determinations in the range of 0,001 % to 100% in a variety of applications. The method developed 87 years ago and since then has undergone an evolution from manual titration to an automated and digital approach.

Here is a short overview of the history of Karl Fischer titration: In 1935, Karl Fischer started with pyridine containing reagents and a visual endpoint detection. In 1943, Wernimont and Hopkinson developed the first dead-stop indication, that provided more sharp and reproducible endpoints. In 1947, Johansson proposed using the titrant and solvent separately for more stability. From 1950 onwards, separated Karl Fischer reagents and dead-stop titration apparatus were commercially available. Later in 1955, Peters and Jungnickel succeeded in the use of stabilized single-component reagents. The coulometric method for low water content samples was developed in 1958, but was not commercially available until 1970. Automatic volumetric titrators with piston burettes launched in 1960 leading to an enormous increase of Karl Fischer applications. Further investigations in 1980, improved the reagents to pyridine free reagents. This was followed by exploring different bases and in 1984 imidazole as a base was tested. These bases ensured a better performance and were less toxic. In the 1990s, the above-mentioned Karl Fischer oven method was introduced for samples that were either solids or not soluble. The oven method is preferred for herbal samples, oils, polymers, and salts because it extracts the water out of a sample and transfers it directly into a Karl Fischer titrator cell. One of the latest innovations, Merck KGaA, Darmstadt Germany introduced in 2019, were the SmartChemicals. A digital approach of transferring the reagent data, seamlessly from an integrated RFID tag, and saving in the titrator software.

Karl Fischer titration continuously evolved over its long history. It secured its place in the analytical labs today, and also as the reliable and efficient water determination method for hemp and cannabis samples.

Sincerely yours,

Bettina Straub-Jubb
Global Product Manager

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CANNABIS

Determination of Water in Cannabis & Hemp by Karl Fischer Titration

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Introduction

Hemp and cannabis are becoming important agricultural products, and are being increasingly used in medicinal products, cosmetics, foods, oils, and textile fibers around the world. The cannabis market is growing rapidly, mainly due to the use as therapeutics for medicial treatments by the pharmaceutical industry.

Water in cannabis and hemp impacts the determination of the potency and must be determined accurately to calculate the correct delta-9 tetrahydrocannabinol (THC) content of the plant. Although both plant types share similar characteristics, cannabis contains a higher amount of THC compared to hemp. However, to be legally classified as hemp, the United States Department of Agriculture (USDA) set a limit for the total delta-9 tetrahydrocannabinol (THC) concentration. The limit is set to contain not more than 0.3 % THC on a dry-weight basis (see definition 7 CFR Part 990 Oct 2019).[1] European Union guidlines currently define a limit ≤ 0.2% for the total THC concentration on a dry-weight basis for industrial hemp.(status Jan. 2022).[2]

To calculate the dry-weight delta-9 THC concentration, an accurate analytical method must be employed for determining the exact water content. Currently most laboratories use loss on drying methods (LOD) which measure all volatile components by heating. This, however, can overstate the water content of the sample, which in turn would lead to an incorrect delta-9 THC concentration in the dry weight, resulting in with wrong classification as hemp. This could potentially lead to penalties for a farmer or processor, or the forced destruction of their product.

The purpose of this application is to demonstrate a moisture determination method for hemp and cannabis flower which is selective for water, and will also provide rapid and accurate test results.

Methods to Determine the Water Content

Three methods for determining water in hemp and cannabis were evaluated:

- Loss on drying (LOD)
- Karl Fischer oven method with coulometry
- Direct volumetric Karl Fischer titration with external extraction

Loss on drying is a simple weighing-based technique that removes water by heating. The equipment needed is reasonably priced, but the method can be time consuming. Nowadays, this method is commonly used in several different industries. However, the loss on drying method is not specific for water and the test results obtained can include amounts of other volatile compounds too. Depending on the conditions chosen, this could lead to an incorrect water value, which in turn would affect the accuracy of the reported dry weight THC concentration result.

Karl Fischer titration based methods are simple to run, but the equipment is a bit more expensive than that used for LOD determination. The advantage of the Karl Fischer titration method is its specificity for water. As a result, the reported water value does not include amounts of other volatile compounds. Two methods based on the Karl Fischer titration were evaluated — coulometry with a Karl Fischer oven and a direct volumetric titration with an external extraction. The coulometric method is best suitable for samples with low water content in the range of 10 ppm to 10,000 ppm (1%) or when only little sample material is available. In contrast, the volumetric titration is used for solid and liquid samples with water contents from 0,01% to 100%. Samples for direct volumetric Karl Fischer titration must be soluble in the Karl Fischer solvent. Organic plant materials like hemp and cannabis are not suitable for direct measurement, so either an external extraction with a suitable solvent or a Karl Fischer oven method is employed. The Karl

Fischer oven method is ideal for this measurement as it completely evaporates the water from the sample and directly transfers it to the titrator. Both approaches of direct volumetric titration after external extraction and the Karl Fischer oven method have been examined. Water content of finely ground hemp flower samples was determined using the methods described below. For any hemp or cannabis analysis, proper preparation is important to provide a representative and well homogenized sample. A method of sample homogenization that is frequently applied in the industry is cryogenic ball milling. This thoroughly homogenizes the sample and leads to a particle size of 100 µm or smaller.

Experimental

Karl Fischer instrumentation used

- Karl Fischer coulometer
- Karl Fischer oven with sample processor
- Karl Fischer volumeter with 5 mL burette

Experimental conditions— Karl Fischer oven method with coulometry

Table 1. Reagents used in the determination of water by Karl Fischer oven method with coulometry

Ground hemp flower sample Reagents & Sample	
Standard:	Water standard oven 1%, solid water standard for Karl Fischer oven method Aquastar® (**1.88054**)
Cell type:	Cell without diaphragm
Reagent type: (Anolyte)	CombiCoulomat fritless; Karl Fischer reagent for coulometric water determination for cells with and without diaphragm Aquastar® (**1.09257**) or Anolyte; Karl Fischer reagent for coulometric water determination for cells without diaphragm Aquastar® (**1.88079**)

Table 2. Titration parameters for water determination by Karl Fischer oven method with coulometry

Coulometer settings for cell without diaphragm, e.g.:	
I(pol):	10 µA
Generator current:	400 mV
Endpoint:	50 mV
Drift stop:	Relative < 10 µg/min
Stirring time:	5 s
Sample size:	20 – 50 mg

Table 3. Oven settings for water determination by Karl Fischer oven method with coulometry

Oven settings	
Temperature:	150 °C
Extraction time:	5 min
Gas flow:	60 – 70 mL/min

Experimental conditions — Direct Karl Fischer volumetric titration with external extraction

Table 4. Reagents used for the water determination by Karl Fischer volumetric titration with external extraction

Reagents & Sample	
Sample:	Hemp methanol extract (from external extraction), 0.5 - 1.0 g (depending on expected water content)
Standard:	Water standard 1%, standard for volumetric Karl Fischer titration 1 g ≙ 10 mg H_2O Aquastar® (**1.88052**)
Titrant:	CombiTitrant 2 Aquastar® (**1.88002**)
Solvent:	CombiMethanol Aquastar® (**1.88009**)

Results and Discussion

Experiment 1 – Water determination by Karl Fischer oven method with coulometry

The water content of a hemp sample was determined by coulometric Karl Fischer titration combined with a Karl Fischer oven. A temperature ramp was run prior to the analysis for evaluating the optimum temperature at which the water is completely and efficiently released without decomposition of the sample. The optimal temperature for the sample used was determined to be 150 °C. Samples were weighed into sealed vials for use in the Karl Fischer oven. An empty vial was used as a blank to determine any water which may have adhered to the vial. The value obtained for the blank vial was subtracted from each sample's value as determined by the instrument.

The sample was analyzed in quintuplicate, and the measured values were averaged to obtain the result (see results with 2 different coulometric reagents, CombiCoulomat and Anolyte, in **Tables 5** and **6**).

Table 5. Karl Fischer oven titration results with Aquastar® CombiCoulomat fritless

Sample	Weight (g)	Start drift (µg/min)	Time (min)	Water content (%)
1	0.0255	6.8	16	7.59
2	0.0222	6.2	14	7.51
3	0.0271	6.1	19	7.69
4	0.0250	5.4	19	7.79
5	0.0303	5.7	21	7.78
Mean				**7.67**
Standard Deviation				**0.12**
(%) RSD				**1.60**

Table 6. Karl Fischer oven titration results with Aquastar® Anolyte

Sample	Weight (g)	Start drift (µg/min)	Time (min)	Water content (%)
1	0.0239	5.4	15	7.42
2	0.0265	5.1	19	7.67
3	0.0237	5.2	18	7.67
4	0.0244	4.7	19	7.75
5	0.0333	4.9	24	7.59
Mean				**7.62**
Standard Deviation				**0.13**
RSD				**1.65**

Experiment 2 – Water determination by volumetric titration with external extraction

1 g hemp was extracted with 25 g of methanol by stirring in septum sealed vials. Different extraction conditions (extraction times and temperature) were applied. The water value of the methanol determined was used as a blank value for use in the final calculations. The solid hemp was allowed to settle, and an exact weight aliquot of the mixture (methanol/extracted water) was taken using a syringe, and injected into the titration cell of a volumetric Karl Fischer titrator. The exact sample weight was determined by back weighing. The titrator then measured the water content of the injected sample. The exact value of water content of the hemp sample was then calculated using the following equation:

$$W_1 = [W_3 \times (m_1+m_2) - W_2 \times m_2]/m_1$$

Where:
- W_1 is the result in %
- W_2 is the % water of the methanol used for extraction
- W_3 is the % water determined for the extracted methanol aliquot
- m_1 is the mass of the sample extracted
- m_2 is the mass of the extraction methanol

The results of the analysis were found to be insufficiently reproducible and highly dependent on the chosen extraction conditions. And hence are not presented here in detail. The external extraction technique was found to be disadvantageous in comparison to the KF oven technique and thereby cannot be recommended for water determination in hemp.

Experiment 3 - Water determination by loss on drying (LOD)

In this experiment, the water content of the sample was determined by loss on drying until a constant mass was reached. The sample was heated for 2 h at a temperature of 150 °C. The weight of the sample was determined before and after heating, to calculate the weight lost during the experiment — the loss on drying. The sample was analyzed in duplicate and the values were averaged to obtain the result (see results in **Table 7**).

Table 7. Loss on drying results

Sample	Starting weight (g)	Weight after 2 h at 150 °C (g)	Weight loss (%)
1	1.7093	1.5307	10.45
2	2.0872	1.8748	10.18
Mean			**10.31**

Comparison of Karl Fischer oven method and loss on drying

The Karl Fischer oven method with coulometry was compared to the loss on drying method (see **Table 8**). Results for the latter (LOD) were about 35% higher than for the Karl Fischer oven method. This requires to consider the measument of other volatile compounds by the loss on drying method to avoid wrong water content results.

Table 8. Comparison between the water content measured by Karl Fischer coulometry method with oven and loss on drying

Samples	Water content (%) Karl Fischer oven + Coulometry CombiCoulomat fritless	Water content (%) Karl Fischer oven + Coulometry Anolyte	Water content (%) Loss on drying (LOD)
1	7.59	7.42	10.45
2	7.51	7.67	10.18
3	7.69	7.67	
4	7.79	7.75	
5	7.78	7.59	
Mean	7.67	7.62	10.31

Conclusion

Water content of hemp samples was determined using Karl Fischer titration techniques (coulometry with oven; volumetric titration with external extraction) and compared to loss on drying.

The Karl Fischer coulometric titration in combination with a Karl Fischer oven provides reproducible results. It prevents an overestimation of water content caused by volatile compounds, as to be considered for the loss on drying method. In addition it requires only a small amount of sample and reagent. The volumetric Karl Fischer titration with external extraction did not produce reproducible results and is therefore not recommended. However, the volumetric Karl Fischer method in combination with a Karl Fischer oven can be employed, but since volumetry is not as sensitive as coulometry, the sample size needs to be increased to get reliable results.

Therefore, it is recommended to use the Karl Fischer oven method with coulometry for water determination in hemp and cannabis to achieve the most accurate results. This enables the exact and precise calculation of the dry weight delta-9 THC concentration.

(continued on page 6)

References

1. Establishment of a Domestic Hemp Production Program Department of Agriculture, Agricultural Marketing Service, 7 CFR Part 990 (Oct 2019) 84 FR 58522 https://www.federalregister.gov/d/2019-23749 (accessed 14.01.2022)
2. European Commission - Hemp: https://ec.europa.eu/info/food-farming-fisheries/plants-and-plant-products/plant-products/hemp_en (accessed 14.01.2022)

Featured Products

Description	Cat. No.
Reagents	
CombiCoulomat fritless, Karl Fischer reagent for the coulometric water determination for cells with and without diaphragm, Aquastar®	**109257**
Anolyte, Karl Fischer reagent for the coulometric water determination for cells without diaphragm, Aquastar®	**188079**
CombiTitrant 2, one component reagent for volumetric Karl Fischer titration 1 ml @ ca.2 mg H_2O, Aquastar®	**188002**
CombiMethanol Solvent for volumetric Karl Fischer titration with one component reagents max. 0.01% H_2O, Aquastar®	**188009**
Reference Materials	
Water standard 0.1%, Standard for coulometric Karl Fischer Titration 1 g = 1 mg H_2O, Aquastar®	**188051**
Water standard oven 1%, solid standard for Karl Fischer oven, Aquastar®	**188054**

Did you know ...

…..that High-Performance Thin-Layer Chromatography (HPTLC) coupled to MS enables both, qualitative and quantitative analyses of CBN, CBD and Δ9-THC in CBD oils of different matrices such as hempseed oil, olive oil or sunflower oil?

Reference: Schmidt T, Stommel J, Kohlmann T, Kramell AE, Csuk R, Separating the true from the false: A rapid HPTLC-ESI-MS method for the determination of cannabinoids in different oils, Results in Chemistry 3 (2021) 100234 https://doi.org/10.1016/j.rechem.2021.100234

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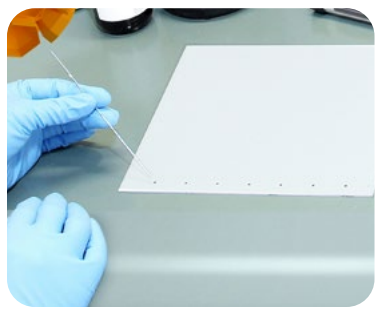

CANNABIS

Cannabinoid Certified Reference Materials (CRMs) for Improved Testing Accuracy and Traceability

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Abstract

Reference materials play a critical role in cannabis workflows. Your results are only as accurate as your reference material. We have developed a portfolio of cannabinoid Certified Reference Materials (CRMs) for use in calibration & quantitation, system suitability studies, and qualitative screening.

Introduction

The interest in cannabinoid quantitation or potency testing of marijuana and hemp continues to grow with the expanding commercialization of cannabis dietary supplements and recreational products. While most of the U.S. states have legalized marijuana for medical use and several for recreational use, it still remains federally illegal and is classified as a schedule 1 substance. The growing of hemp crops in the U.S. was federally legalized by the U.S. Agricultural Improvement Act of 2018, also known as the 2018 Farm Bill. The U.S. Department of Agriculture (USDA) final rule for hemp production published on January 19, 2021 requires the total Tetrahydrocannabinol (THC) content of plant material on a dried weight basis to be less than 0.3% for it to be legally defined as hemp. Total THC content is taken as the sum of Δ-9 THC and its biosynthetic precursor, Tetrahydrocannabinolic acid (THCA), as THC is the degradation product of THCA after its decarboxylation. A hemp producer must discard the entire lot if the sampled plant material contains more than 0.3% of THC resulting in potentially dire financial repercussions. Production of plant material containing more than 1% of total THC is defined as a negligent violation and could result in suspension or revocation of the producer's USDA license to grow hemp. These implications underscore the importance of accurate cannabinoid analytical testing. However, the final rule does acknowledge the importance of analytical variability and requires testing laboratories to report the measurement of uncertainty (MU) associated with the THC test results, in order to allow the "acceptable hemp THC level" to account for the MU.[1]

In the interim final rule (IFR), the Agricultural Marketing Service (AMS) requested inputs on the potential requirements for hemp testing laboratories to obtain ISO/IEC 17025 accreditation. However, the requirement was not included in the final rule due to the comments citing insufficient laboratory capacity currently available to accommodate all the needed hemp testing. Labs are required to meet certain standards of performance and use test methods that are fit-for-purpose, however currently there is no federal laboratory approval process in place and the state requirements vary widely. The allowed analytical test methodologies are GC-FID or HPLC with either UV or MS detection, and the methods must meet the AOAC Standard Method Performance Requirements 2019.003.[2] AOAC has published official method 2018.11 for the quantification of cannabinoids in plant materials, that has undergone a rigorous approval process and is accepted as a highly credible method.[3] The United States Pharmacopeia (USP) has noted the need for increased quality control and harmonized practices in cannabis testing for medical purposes and has published important quality attribute considerations to aid in addressing these gaps.[4] NIST offers a cannabis quality assurance program similar to a proficiency test to help laboratories evaluate their testing comparability and competence.[5] Even with these guidelines and resources in place, the industry has acknowledged the continued problem of inconsistent results obtained from different laboratories.

The accuracy of a testing laboratory's calibration standards is a critical factor that directly impacts the accuracy of the test results. The labs must manufacture or purchase a suitable raw material or solution-based reference material for use as a calibrator. Availability of cannabinoid Certified Reference Materials (CRMs) allows testing laboratories to make cost-effective in-house calibrators, traceable to the CRMs, thereby contributing to a higher level of test accuracy and reproducibility. ISO 17034 - specific to reference material producers and ISO/IEC 17025 - specific to testing laboratories provide standardization to the manufacturing and testing of CRMs. CRMs are considered to have the highest level of accuracy and traceability to the SI unit of measurement for of all the materials manufactured by ISO accredited producers.[6,7] There is a selection of cannabis related reference standards offered by USP

for medical use. But there are no cannabinoid reference standards manufactured by any national metrology institute. However, hemp plant and oil reference materials are currently being developed by NIST.[5]

We have designed and manufactured a portfolio of cannabinoid CRMs listed in **Table 1** to support the cannabis testing industry. These products are offered as single or multiple analytes dissolved in an appropriate diluent and packaged in amber flame-sealed ampoules. Appropriate handling and process controls were put in place to ensure the analyte's stability in storage and transit. The concentration of each analyte is certified in accordance with ISO 17034 and ISO/IEC 17025. The concentration uncertainty is calculated and reported along with the certified concentration in the accompanying certificate of analysis (CoA). The cannabis testing laboratories should propagate this value of unexpanded uncertainty in their own method's uncertainty calculations.

Table 1. Cerilliant® portfolio of cannabinoid CRMs

Description	Cat. No.
Single compound solutions	
Cannabidiol (CBD), 1 mg/mL in methanol	**C-045**
Cannabinol (CBN), 1 mg/mL in methanol	**C-046**
Cannabidivarin (CBDV), 1 mg/mL in methanol	**C-140**
Cannabigerol (CBG), 1 mg/mL in methanol	**C-141**
Cannabigerolic acid (CBGA), 1 mg/mL in acetonitrile	**C-142**
Cannabichromene (CBC), 1 mg/mL in methanol	**C-143**
Cannabidiolic acid (CBDA), 1 mg/mL in acetonitrile	**C-144**
Cannabichromenic acid (CBCA), 1 mg/mL in acetonitrile	**C-150**
Cannabidivarinic acid (CBDVA), 1 mg/mL in acetonitrile	**C-152**
Cannabinolic acid (CBNA), 1 mg/mL in acetonitrile	**C-153**
Cannabicyclol (CBL), 1 mg/mL in acetonitrile	**C-154**
Cannabicyclolic acid (CBLA), 0.5 mg/mL in acetonitrile	**C-171**
Δ9-tetrahydrocannabinol (Δ9-THC), 1 mg/mL in methanol	**T-005**
Δ8-tetrahydrocannabinol (Δ8-THC), 1 mg/mL in methanol	**T-032**
Exo-THC, 1 mg/mL	**T-033**
Δ9-tetrahydrocannabinolic acid (THCA), 1 mg/mL in acetonitrile	**T-093**
Tetrahydrocannabivarin (THCV), 1 mg/mL in methanol	**T-094**
Tetrahydrocannabivarinic acid (THCVA), 1 mg/mL in acetonitrile	**T-111**
THC-O-Acetate Solution, 1 mg/mL	**T-151**
Mixes	
Cannabinoid Mixture (Acids), 6 Component, 500 µg/mL each	**C-218**
Cannabinoid Mixture (Neutrals), 8 Component, 500 µg/mL each	**C-219**
Hemp Compliance Mix, 1 mg/mL CBD, 3 µg/mL THC	**C-217**
THC Cannabinoids Mixture-3, 1 mg/mL	**T-108**

Manufacturing and Certification of Cannabinoid CRMs

The cannabinoid raw materials used to prepare Cerilliant® CRMs were all synthesized in-house and certified by a predetermined test plan designed in accordance with ISO 17034 and ISO/IEC 17025. The identity of each cannabinoid was verified by ^{1}H-NMR and high-resolution liquid chromatography-mass spectrometry (LC-MS). The potency or mass fraction of each raw material was assigned as a mass balance purity factor (MBPF), calculated by subtraction of the mass of impurities from the mass of the analyte using **Equation 1**. Impurities were determined through a range of techniques accounting for those that are volatile, inorganic, and organic. The residual water content was determined by Karl Fischer coulometry. While other residual volatile content was determined by headspace gas chromatography with flame ionization detection (HS-GC-FID), the residual inorganic content was determined by residue on ignition (sulfated ash).

Organic impurities were determined by high performance liquid chromatography with ultraviolet detection (HPLC-UV) and reported as the average of two orthogonal methods. Orthogonal selectivity of the two methods was established through different chromatographic stationary and mobile phases. GC-FID was used as a confirmatory technique for analysis of organic impurities wherever appropriate. In some cases, quantitative ^{1}H-NMR was also used as a confirmatory technique to MBPF.

Formulation studies were performed to determine appropriate handling techniques, storage conditions, suitable concentrations and diluents for the proposed products. The final CRM designs were based on the results of the formulation studies. Each solution was prepared by gravimetric measurement of the analyte(s) and diluent

Equation 1. Mass balance purity factor calculation.

$$\text{Mass Balance Purity Factor} = \left[100 - (\text{wt\% Solvents}) - (\text{wt\% } H_2O) - (\text{wt\% Inorganics}) \right] * \frac{\text{ChromPurity}}{100}$$

Where:

wt% Solvents = residual solvent content with uncertainty, u_{OVI}

wt% Inorganics = residual inorganic content with uncertainty, u_{ROI}

wt% H_2O = residual water content with uncertainty, u_{KF}

ChromPurity = Chromatographic purity with uncertainty, u_{cp}

with the concentration determined by **Equation 2**, using the actual measured mass, purity adjustment of the analyte(s), measured mass of the solution, and density of the pure diluent at 20 °C. For viscous, glassy, or hard-to-handle raw materials, a stock solution was made and analytically verified prior to the final dilution. All mass measurements are traceable to the International System of Units (SI) through qualified and calibrated analytical balances and were reported on the conventional basis for weighing in air. The mass of each solution was converted to volume by dividing the mass by the density of the solution. Density measurements were made on a density meter and are traceable to higher order standards through calibration. The prepared concentration of each analyte is reported in units of mass per volume, with expanded uncertainty and specified confidence interval. The solutions were dispensed into amber ampoules with a fill volume of not less than 1 mL and flame sealed under argon.

Equation 2. Certified concentration calculation for gravimetrically prepared CRM.

$$C = \frac{(m_{v+a}-m_v)d}{(m_{f+s}-m_f)p} \pm U$$

Where:

- C = Certified Property Value, concentration of analyte in solution in units of mass/volume
- m_{v+a} = mass of analyte + vial
- m_v = mass of empty vial
- m_{f+s} = mass of flask + solvent
- m_f = mass of empty flask
- d = density of solution
- p = purity adjustment, 100/mass balance purity factor (for the analyte)
- U = the assigned combined expanded measurement uncertainty

The concentration, homogeneity, and purity of each CRM was verified through HPLC-UV analytical testing. Sealed ampoules were selected for testing from across the batch based on a random weighted stratified sampling plan, with a higher percentage of samples taken at critical process points. The concentration was verified by comparison to an independently prepared calibration solution and calculated as the average of replicate analyses of samples. Within and between ampoules homogeneity was verified through relative standard deviation of the replicate analyses of samples. The accuracy and homogeneity acceptance criteria included allowances for uncertainty contributions from the analytical measurement, and variability from transfer and evaporative loss during preparation of samples for analysis.

Short-term transit stability was established through temperature stress studies performed at freezer (-25 to -10 °C), refrigerator (2-8 °C), room temperatures (15-30 °C) and a stressed temperature (40 °C +/-2 °C). One ampoule of each solution standard was removed from each storage condition at specified time points and moved to sub-freezer storage until analysis. The ampoules stored at stressed temperatures were evaluated for purity and/or concentration by HPLC-UV.

Long-term stability of the CRMs was assessed for 14 months following their manufacture. This was done using HPLC-UV analysis for real-time studies of solution purity and concentration. These studies are subsequently carried out for the entire shelf life of the product.

Results and Discussion

The ISO defines a reference material (RM) as a material that is homogenous, stable, and fit for its intended measurement use. A CRM must meet additional requirements to those for RMs. CRM characterization methods must be metrologically valid and traceable to the measurement unit of the certified property value.[8] The ISO guides give some flexibility to the CRM manufacturers as to how they meet these requirements. As a result, the certification process can vary widely among the manufacturers, from assigning a potency from a simple chromatographic purity to a comprehensive MBPF approach. Cannabis testing laboratories must be mindful of this and review the CRM's certificate of analysis (CoA) to ensure it is fit for their intended use. **Table 2** shows possible pitfalls associated with using only a chromatographic purity. The chromatographic purity of cannabinol is 99.5% but the potency assigned by MBPF to it is 96.0%— due to the presence of residual solvent and water.

Table 2. Calculated potency and impurity contributions for representative cannabinoid raw materials.

Compound	Chrom. Purity (%)	Residual solvent content (%)	Residual water content (%)	Trace inorganic content (%)	Content/Potency (%)
Cannabidiol (CBD)	99.3	0.85	< LOD	< LOQ	98.4
Cannabinol (CBN)	99.5	3.39	0.11	NA	96.0
(-)-Δ^9-THC	98.1	1.47	NA	NA	96.7
Cannabigerol (CBG)	99.0	< LOD	< LOQ	< LOQ	99.0
Cannabichromene (CBC)	99.0	< LOD	< LOQ	NA	99.0
Cannabidiolic acid (CBDA)	99.0	1.40	< LOQ	< LOQ	97.6
Cannabigerolic acid (CBGA)	99.3	0.16	< LOD	< LOQ	99.1
Δ^9-Tetrahydrocannabinolic acid (THCA-A)	98.4	0.41	< LOD	< LOQ	98.0
Tetrahydrocannabivarin (THCV)	98.8	1.68	< LOQ	NA	97.2
Cannabidivarin (CBDV)	98.8	0.91	< LOQ	< LOQ	97.9

In addition to the certification method, every CoA should include data to support stability during transit and long-term over the shelf life of the product. **Figure 1A** shows an example of a temperature stress study for an unstable prototype formulation, where we see a decrease of concentration with an increase in storage temperature and time. A final stable formulation of product **C-218** was developed through optimized diluent selection, material handling, and formulation process controls. **Figure 1B** shows that the concentrations of all analytes in **C-218** remain stable at multiple storage temperatures up to four weeks, with degradation only observed in the samples kept at 40 C°. **Figure 1C** shows the same accelerated stability data plotted as a line graph for two representative analytes, CBDA and THCA-A, with additional real-time stability shown up to 6 months. Continued real-time stability is assessed throughout the shelf life of the product.

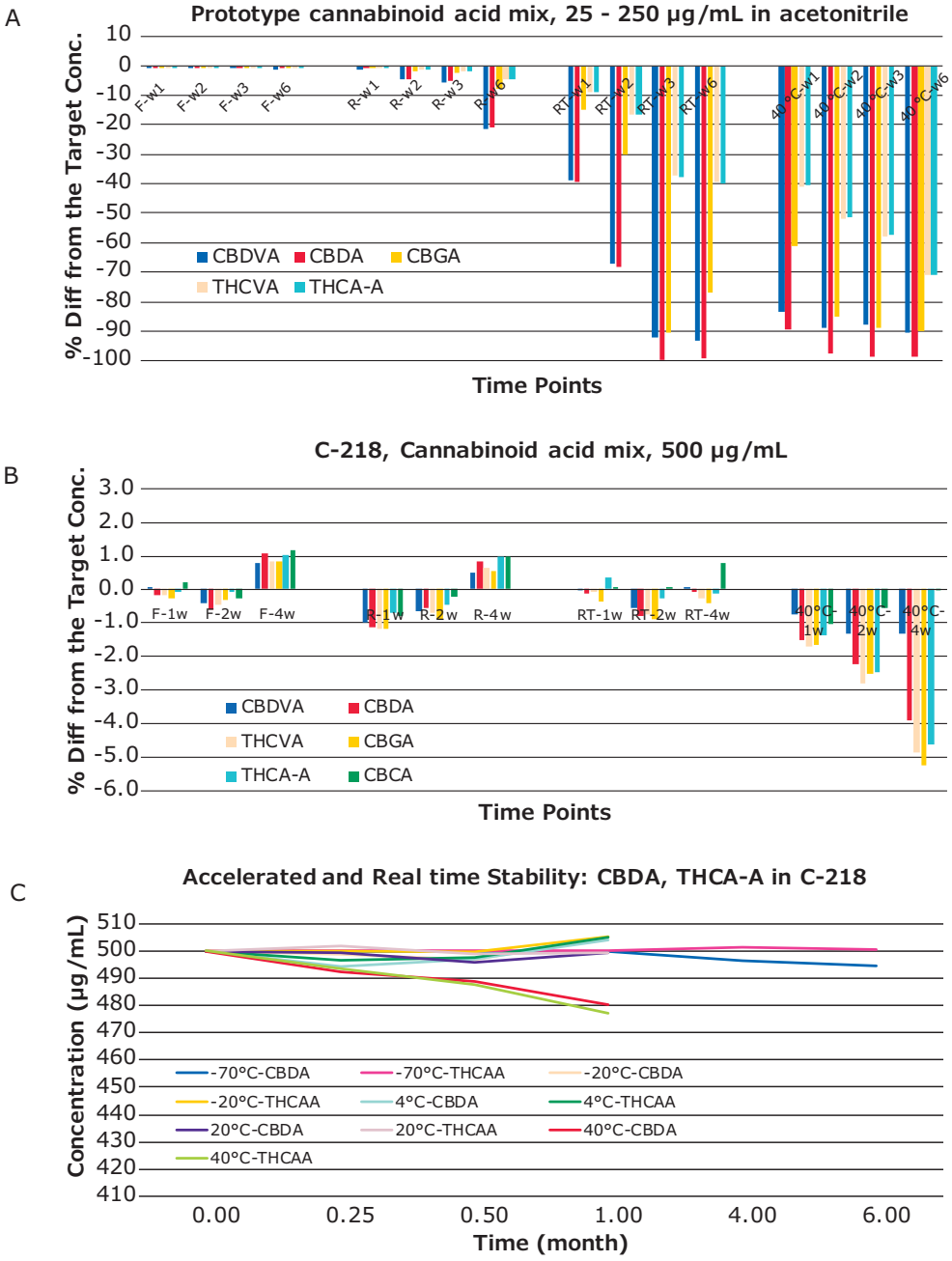

Figure 1. Bar graphs depicting the temperature stress studies of a prototype **(A)** and finalized **(B)** acidic cannabinoid mixture with bars showing the change in concentration related to time stored at varied temperatures: F: freezer (-25 to -10 °C), R: refrigerate (2-8 °C), RT: room temperature (15-30 °C) and 40 (±2) °C. The bottom graph **(C)** plots the change in concentration over six months of CBDA and THCA-A in cat. no. **C-218** stored at varied temperatures.

A CRM CoA should report the method used to determine the measurement uncertainty. **Figure 2** shows an example fishbone diagram for the sources of uncertainty associated with the certified concentration of a gravimetrically prepared solution standard. **Equation 3** shows the calculation for propagation of uncertainty. The method used to calculate the uncertainty was established in accordance with ISO 17034 and ISO Guide 35 through identification of the production process variables and risks coupled with statistical analysis. Uncertainty is expressed as expanded uncertainty at the approximate 95% confidence interval using a coverage factor of k=2. It incorporates uncertainty of the purity factor, material density, balance, weighing procedure, solution standard homogeneity, and stability. The uncertainty of the certified concentration is stated in the CoA in terms of mass per volume.

Equation 3. Uncertainty calculation for the certified concentration of a CRM including homogeneity and stability terms.

$$u_{cert} = \sqrt{(u_{char}^2 + u_{bb}^2 + u_{stab}^2)}$$

Where:

u_{cert} = Standard uncertainty of the Certified Property Value

u_{char} = Standard uncertainty of the solution standard preparation and includes u_{pf} for characterization of the analyte mass balance purity factor(pf), u_m for mass measurements, and u_d for the solvent density.

$$u_{char} = \sqrt{(2u_{pf}^2 + 4u_m^2 + u_d^2)}$$

u_{bb} = Standard uncertainty of between bottle homogeneity

u_{stab} = Standard uncertainty of stability

Determining the uncertainty value for a CRM incorporates technical studies on all aspects of a solution standard preparation process, including mass measurement, density measurement, verification, homogeneity, and stability. The studies incorporate replicate measurements under different process conditions and establish standard uncertainties for the density and mass measurements. The studies also provide validated process controls for weighing and dispensing. Homogeneity uncertainty contributions are assessed through statistical analysis of concentration accuracy data for samples pulled at critical timepoints during the dispensing of a given lot. Stability uncertainty contributions are assessed from temperature stressed or real-time stability data.

CRMs may be used for identification, quantitation, system suitability and method control. **Figure 3** shows an example of a CRM dilution scheme to achieve a standard containing seventeen cannabinoids. This can initially be used as a screening tool to evaluate the presence or absence of cannabinoids in samples. Once the cannabinoids of interest are identified, the mixture can be diluted into a calibration curve spanning the expected concentration range of the constituents and used for quantitation. Alternatively, single analyte CRMs can be used to prepare the calibration curves. Example HPLC-UV chromatograms of the 17-cannabinoid mix prepared from 5 CRMs (cat. nos. **C-218**, C-219, C-153, C-154 and C-171) and hemp bud extract are compared in **Figure 3**. **Figure 4** illustrates the use of our Hemp Compliance Mix (C-217) as a system suitability test to ensure method performance for detection of THC at the maximum allowed level of 0.3%. A separate solution containing 5 µg/mL of cannabidiolquinone (CBDQ) was run using the same method. CBDQ is

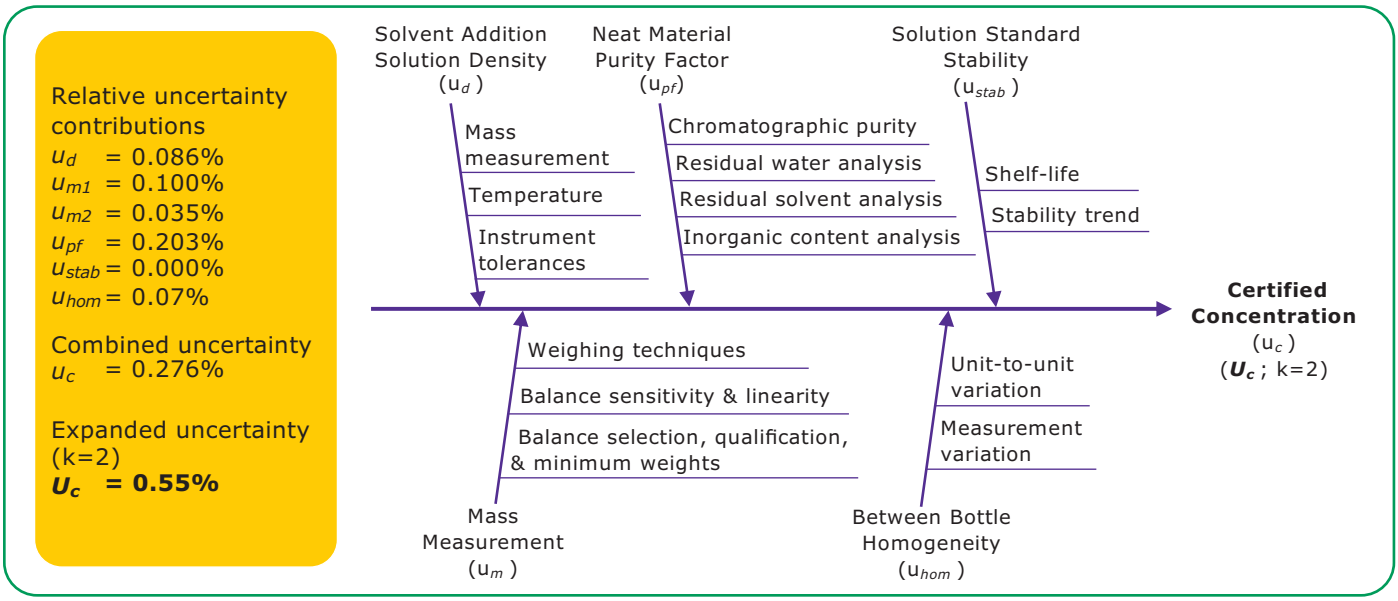

Figure 2. Example fishbone diagram of measurement uncertainty contributions for a gravimetrically prepared CRM.

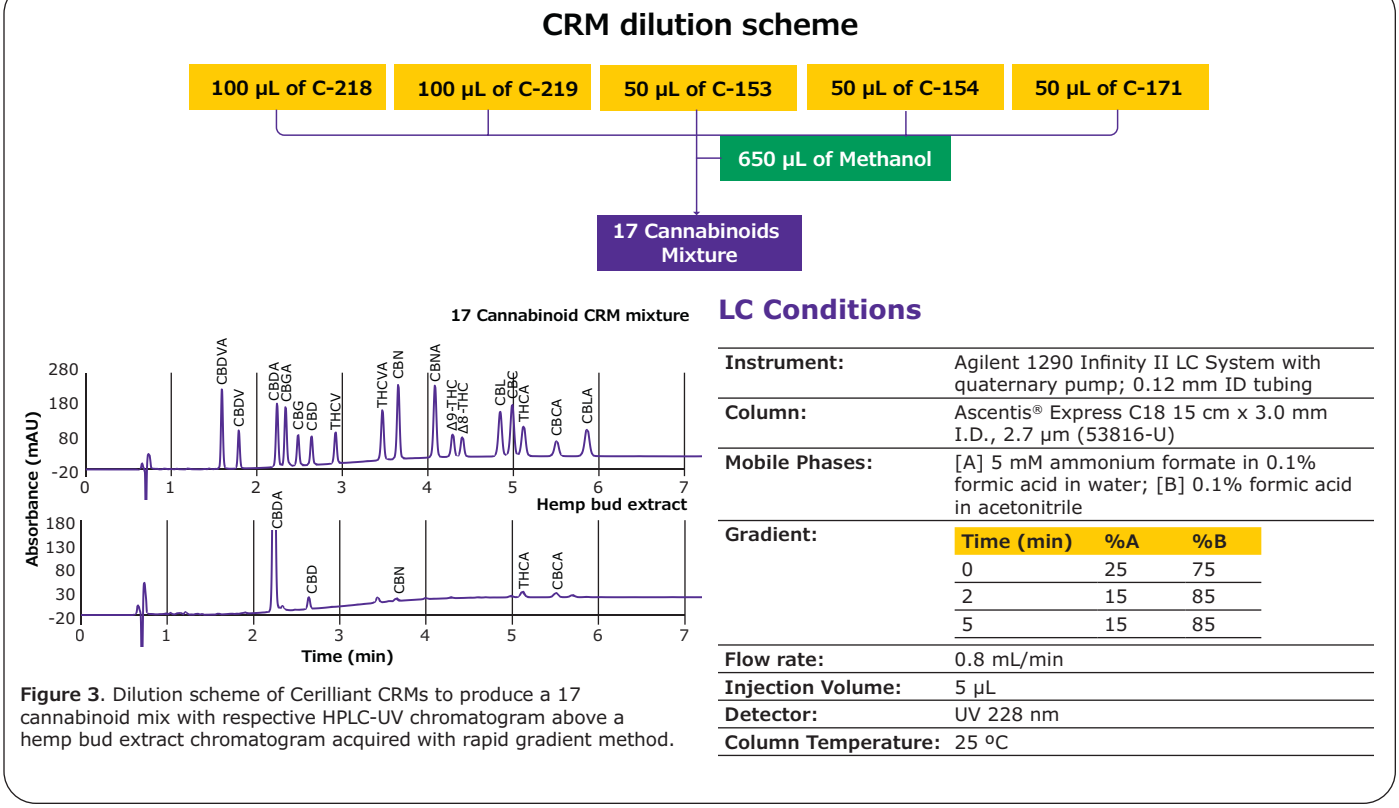

Figure 3. Dilution scheme of Cerilliant CRMs to produce a 17 cannabinoid mix with respective HPLC-UV chromatogram above a hemp bud extract chromatogram acquired with rapid gradient method.

LC Conditions

Instrument:	Agilent 1290 Infinity II LC System with quaternary pump; 0.12 mm ID tubing
Column:	Ascentis® Express C18 15 cm x 3.0 mm I.D., 2.7 µm (53816-U)
Mobile Phases:	[A] 5 mM ammonium formate in 0.1% formic acid in water; [B] 0.1% formic acid in acetonitrile
Gradient:	Time (min) / %A / %B: 0 / 25 / 75; 2 / 15 / 85; 5 / 15 / 85
Flow rate:	0.8 mL/min
Injection Volume:	5 µL
Detector:	UV 228 nm
Column Temperature:	25 ºC

one of the oxidation degradants of CBD. The overlaid chromatograms show baseline separation of THC and CBDQ by this method. If other cannabinoids or impurities coelute with THC, errors in quantitation will result." It is thus important to routinely check method performance using a mix formulated specifically for this purpose, such as illstrated in **Figure 4**.

Conclusion

We have developed CRMs of individual cannabinoids as well as mixes (**Table 1**). These can be used for potency profiling of both hemp and cannabis. Specifically, our Hemp Compliance Mix was formulated to simplify standards preparation for analysis of THC content in hemp. Using rigorous process controls and formulation studies as described here, we formulated stable cannabinoid mixtures. Our optimized raw material and packaging processes protect cannabinoids from oxidation, thus producing CRMs with long term stabilities. With the variability in testing methods and accreditation across laboratories, the use of accurate and traceable and properly certified CRMs is critical.

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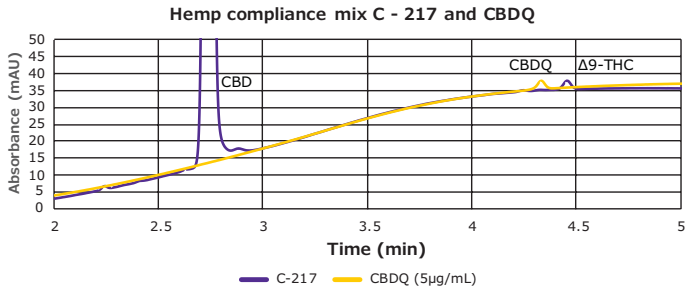

Figure 4: Overlay of HPLC-UV chromatograms of C-217 hemp compliance mix and a solution containing CBDQ.

References

1. Establishment of a Domestic Hemp Production Program. Fed. Regist., December 2021, 86 FR 5596, 5596-5691. https://www.govinfo.gov/content/pkg/FR-2021-01-19/pdf/2021-00967.pdf
2. AOAC SMPR 2019.003. Standard Method Performance Requirements (SMPRs) for Quantitation of Cannabinoids in Plant Materials of Hemp (Low THC Varieties Cannabis sp.).
3. AOAC Official Method of Analysis 2018.11. Cannabinoids in Cannabis plant materials, concentrates, and oils. www.aoac.org/
4. Sarma ND, Waye A, ElSohly MA, Brown PN, Elzinga S, Johnson HE, Marles RJ, Melanson JE, Russo E, Deyton L, Hudalla C, Vrdoljak GA, Wurzer JH, Khan IA, Kim NC, Giancaspro GI. Cannabis Inflorescence for Medical Purposes: USP Considerations for Quality Attributes. J Nat Prod. 2020;83(4):1334-1351. doi: 10.1021/acs.jnatprod.9b01200.
5. NIST Tools for Cannabis Laboratory Quality Assurance: https://www.nist.gov/programs-projects/nist-tools-cannabis-laboratory-quality-assurance (accessed 11.10.2021).
6. ISO 17034:2016, General requirements for the competence of testing and calibration laboratories, International Organization for Standardization, Geneva, Switzerland, 2016. www.iso.org
7. ISO/IEC 17025:2017, General requirements for the competence of reference material producers, International Organization for Standardization, Geneva, Switzerland, 2017. www.iso.org
8. ISO GUIDE 30:2015, Reference materials – Selected terms and definitions, International Organization for Standardization, Geneva, Switzerland, 2017. www.iso.org

PHARMA & BIOPHARMA

Three Methods for Critical Quality Attribute Determination of Monoclonal Antibodies

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Abstract

Three step-by-step protocols for the monitoring of critical quality attributes (CQAs) of monoclonal antibodies were developed using adalimumab as an example. Monoclonal antibodies are glycoproteins used as drugs in the treatment of a variety of cancers and immune system disorders. These large biomolecules have complex structures making thorough characterization essential to ensure the safety and efficacy of drugs. Here, we provide an overview of the different workflows that can be used in these molcules intact and reduced mass analysis, primary structure determination (i.e. peptide map), and N-linked glycan analysis to enable an accurate characterization of their CQAs.

Introduction

Increasing importance of monoclonal antibody (mAb) therapeutics has fundamentally changed the pharmaceutical market in recent years. These macromolecules are used in cancer therapies and in combating autoimmune and other diseases. Biomolecules are produced using recombinant techniques in mammalian cell lines, expressing the mAbs in large bioreactors. mAbs are glycoproteins with a molecular weight of approximately 150 kDa. Antibodies are composed of two light chains and two heavy chains (LC and HC, respectively) linked to one another through covalent inter- and intra-chain disulfide bonds. The individual chains are composed of amino acids and can be post-translationally modified (PTM). The different modifications include glycosylation, phosphorylation, methylation, oxidation, and nitrosylation.

A reliable characterization of mAbs is essential to ensure the safety and efficacy of both the innovator and biosimilar drugs.[1] Therefore, the critical quality attributes (CQAs) are defined for each protein along with their acceptable ranges. For example, the extent of deamidation, pyroglutamination, oxidation, or formation of lysine variants in mAbs is quantified in relation to their respective native forms.[2] Common analytical techniques employed to verify CQAs being within the set limits include capillary zone electrophoresis, isoelectric focusing, ion exchange, size exclusion (SEC), reversed phase (RP) or hydrophilic interaction liquid chromatography (HILIC), coupled with ultraviolet (UV), fluorescence, or mass spectrometric (MS) detection.[3,4] Strategies used to aid the analysis include chemical or enzymatic fragmentation of the protein into subunits,[5] removal and analysis of glycans, and proteolytic digestion of the protein into smaller peptides followed by their sequencing. A combination of these techniques can also be applied. For the analysis of mAbs from cell culture supernatants, an affinity purification step is often used to prepare samples prior to their analysis.[6]

Recently, we prepared step-by-step workflows for three approaches commonly used in mAb characterization.[7-9] These approaches determine:

1. the mass of the intact molecule and of the heavy and light chains (intact and middle-up mass analysis),
2. the amino acid sequence (peptide mapping), and
3. the glycosylation state (glycan analysis).

Intact mass analysis relates to the measurement of the mass of an intact mAb without its dissociation into subunits. Middle-up experiments are performed after cleaving the mAbs into several large fragments, or subunits, via chemical reduction or proteolytic digestion. This can provide information on the sites of modification.

In peptide mapping, the protein is cleaved into fragments with a protease enzyme, most commonly trypsin. Prior to the enzymatic digestion, it is common to denature the protein to expose internal amino acids. As in middle-up analysis, the protein may be treated chemically to break disulfide bonds linking different portions of the protein. This reduction step is typically followed by alkylation of the exposed free thiols to prevent reformation of disulfide bonds. Minimizing the introduction of modifications to individual amino acids during the analytical stages of peptide mapping is also important. For example, under appropriate conditions, asparagine can be deamidated and methionine can be oxidized, both of which alter the mass of the peptide.

Glycan analysis is a third means of characterizing CQAs of mAb therapeutics. Glycans are polysaccharide chains that are attached to proteins (glycoprotein) or lipids (glycolipid). They exist as an array of different sugar units attached through different linkages and sometimes existing in complex di-, tri-, and tetra-

antennary structures. Glycans are typically described as "N-linked," when attached at the side chain nitrogen of asparagine, or "O-linked", when linked to the side chain oxygen of serine or threonine. Glycan profiles can affect the stability and bioavailability of mAb therapeutics and can vary with manufacturing conditions. Characterization of glycans is essential to ensure the equivalence of newly released mAb lots to the approved drug.

Glycan analysis is achieved by releasing glycans from the protein by using chemical or enzymatic methods followed by derivatization and fluorescence or mass spectral analysis. Common derivatization agents include 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), and procainamide. The latter has properties suited to both fluorescence and MS detection. Fluorescent derivatization allows relative quantification of glycan species while mass spectral analysis provides structural confirmation.

Figure 1 illustrates how the three approaches fit into the overall characterization of a mAb, along with a technique for purification of mAbs when necessary. Next, we look at each of the three workflows in a bit more detail.

Intact Mass Analysis

Non-reducing (intact) and reducing (middle-up) SEC-MS workflows for mAb analysis provide a high-level characterization of the protein structure and subunits.[10] Intact analysis yields mass measurement of the entire mAb, without dissociation of the subunits. Use of high resolution, high mass accuracy mass spectrometers to verify molecular weight can be utilized to reveal information about stoichiometry and proteoforms that may exist. Multiple charge state spectra are deconvoluted to allow correlation of observed masses with expected or theoretical masses.

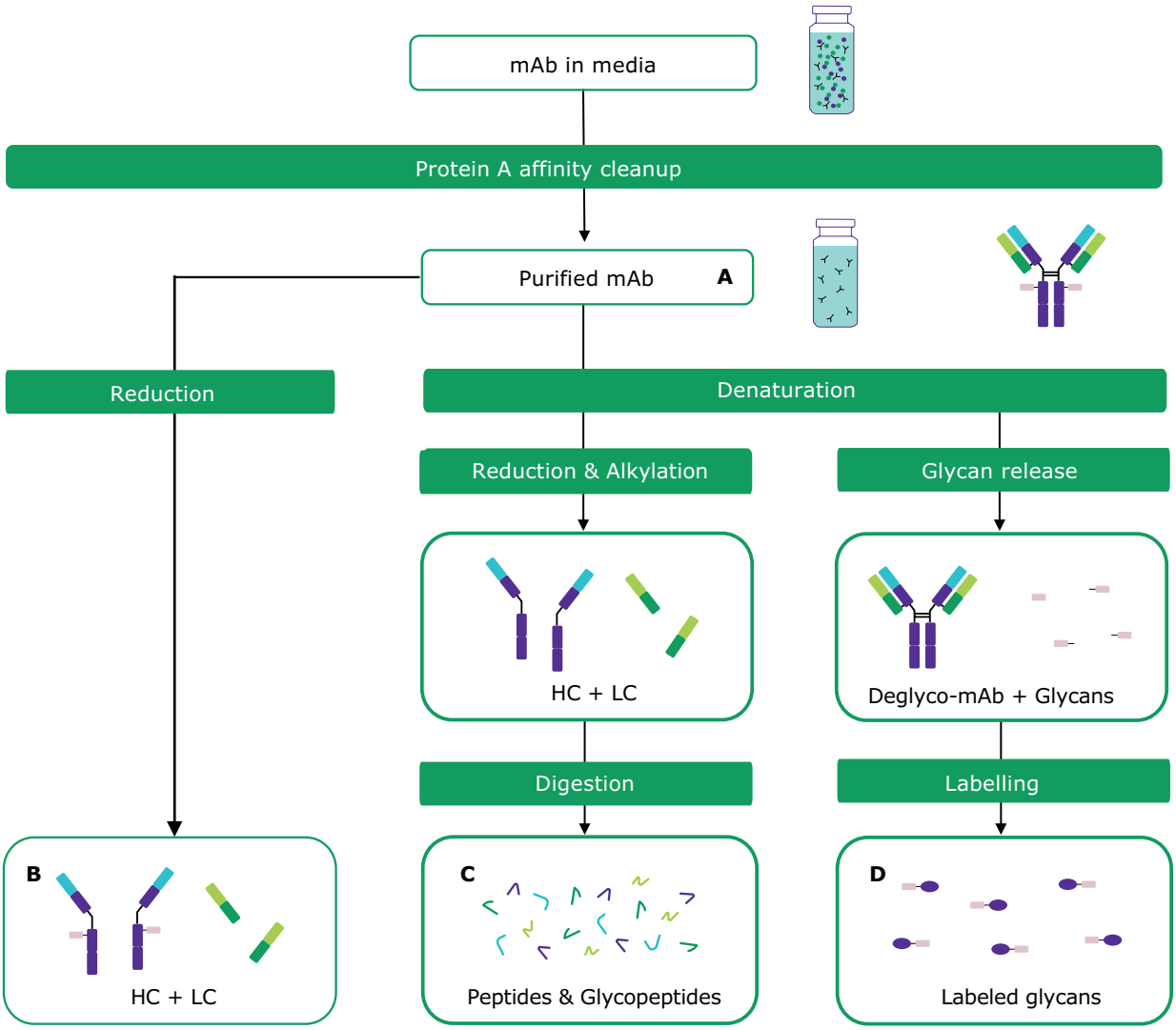

Figure 1. Overview of analytical techniques for antibody sample preparation and analysis showcased in this work. **A:** Intact mass analysis. **B:** Middle-up analysis of mAb after reduction. **C:** Peptide mapping after denaturation, reduction, alkylation, and tryptic digestion. **D:** Glycan analysis after denaturation, glycan release and labelling with procainamide.

In SEC, analytes enter, or are excluded from, the pores of the chromatographic support based on size. In a mixture of mAb, mAb aggregates, and antibody fragments, large aggregates elute first, followed by the antibody and then the fragments. In a middle-up analysis, HCs can be separated from LCs in this same fashion to identify the glycosylation sites on one or the other chains.

A photodiode array (280 nm) trace, total ion chromatogram, MS spectrum, and deconvoluted MS spectrum of adalimumab are shown in **Figures 2** and **3** (see conditions in **Table 1**). Observed masses of the non-reduced mAb are found to correlate well with the theoretical masses and observed mass error was found to be 0.010% or less. Deconvolution of the spectrum revealed several different glycoforms.

Table 1. SEC method conditions for intact mass analysis

SEC Method Conditions	
Column:	Tosoh TSK Gel SW3000, 30 cm x 2.0 mm I.D., 4 µm (**821485**)
Mobile phase:	Acetonitrile/water 30/70 (v/v) containing 0.1% trifluoroacetic acid (TFA) (v/v)
Isocratic elution:	0.1 mL/min
Column temp.:	Ambient
Detection:	UV 280 nm and MS
Injection:	20 µL

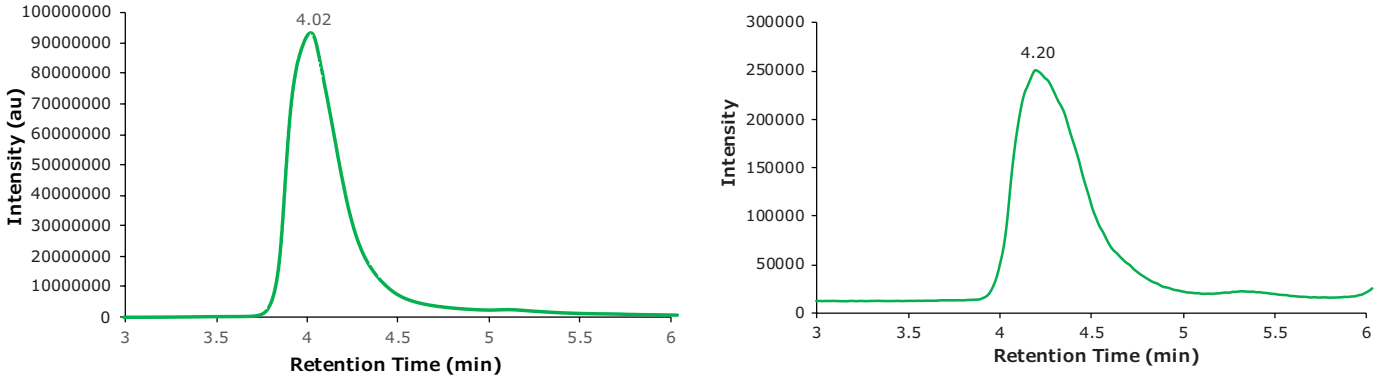

Figure 2. UV photodiode array (280 nm, left) and TIC traces (right) of intact adalimumab.

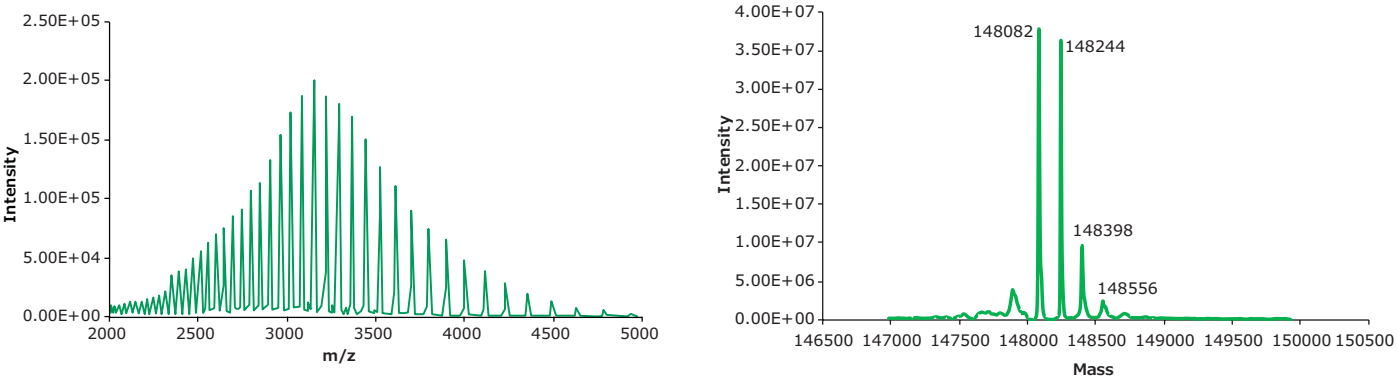

Figure 3. MS spectra for intact adalimumab. Left: summed spectrum; Right: deconvoluted spectrum showing several different glycoforms. (MS spectra generated on a QTOF instrument)

Peptide Mapping

Peptide mapping attempts to characterize the exact sequence of amino acids in a protein along with any modifications to those amino acids. In this work, sample proteins were digested with trypsin in a low-artifact digestion buffer[11] and utilizing filter-assisted sample preparation (FASP).[12] The latter makes use of a molecular weight cut-off filter, in microcentrifuge tube format, to separate peptide fragments from whole protein. The low-artifact digestion buffer minimizes amino acid modification through the digestion process to provide a more accurate depiction of the protein being produced.

The digest is injected onto an HPLC column capable of resolving as many of the peptide fragments as possible, including those bearing glycans, prior to MS detection. HPLC columns based on superficially porous particles (SPP) like Ascentis® Express and BIOshell™ columns are suitable to obtain high efficiencies and resolution. **Figure 4** shows the chromatographic profile obtained for the model mAb, adalimumab while **Table 2** displays the method conditions. Coupling two 15 cm columns provided greater separation of peptides across the run as well as slightly better retention of hydrophilic peptides.

The depth of information provided by peptide mapping has led to the development of multi-attribute methods (MAM) that are used to ensure quality and consistency of protein therapeutics with a single method.[13] With MAMs, a list of PQAs (product quality attributes) is generated from an in-depth characterization of the protein-of-interest using high-resolution, high-mass accuracy MS/MS, and peptide mapping experiments. These analyses include characterization of PTMs, particularly glycosylation. Results of the in-depth characterization are used to create a library of features expected from the therapeutic products during their manufacture. A simpler, MS only instrument

Table 2: Peptide mapping method conditions

Columns:	Two coupled Ascentis® Express Peptide ES-C18 columns, 15 cm x 1.0 mm, I.D. 2.7 µm (**53561-U**)
Mobile phase:	[A] 0.1% formic acid (FA) in water (v/v); [B] 0.1% FA in acetonitrile (v/v)
Gradient:	

Time (min)	A (%)	B (%)
0.00	99	1
120	65	35
121	3	97
136	3	97
137	99	1
162	99	1

Flow:	0.08 mL/min
Column Temp.:	Ambient
Detection:	MS/MS
Injection:	10 µL

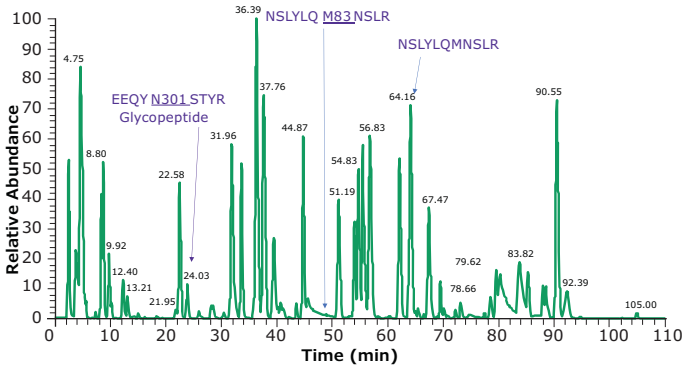

Figure 4. Base peak chromatogram of adalimumab tryptic peptides. Heavy chain glycosylated peptide (EEQYN301STYR) is observed at 24.03 min. Using a dual column set up, we observe hydrophilic peptides such as PGK, SCDK, VDK eluting at under 4 min. An example peptide and its oxidized form are NSLYLQM83NSLR and NSLYLQMNSLR eluting at 49.30 and 64.16 min, respectively.

can then be used to monitor the essential PQAs, or CQAs, during on-going monitoring of drug manufacture. The occurrence of newly identified, missing, or changed peaks during analysis triggers further investigation. The mild, rapid digestion provided by FASP and the low artifact digestion buffer, along with the excellent sequence coverage here, lends itself to MAM quality control of protein therapeutics.

N-linked Glycan Analysis

A prerequisite for accurate quantification of glycans is their complete release and isolation from the protein. Enzymatic cleavage with PNGase F is remarkably effective for achieving this. PNGase F is characterized by its high activity, broad substrate specificity, and its property of cutting most asparagine linked (N-linked) glycans. The glycan residue remains intact and can be subjected to further analysis. Here, a PNGase Fast Kit was used which, in combination with the enzyme, provided drastically reduced reaction time but with an accuracy equivalent to traditional methods.

Among glycan derivatization reagents, procainamide offers better fluorescence and electrospray ionization, and therefore better sensitivity than the more traditional 2-AB and 2-AA labeling. Here, released N-glycans were analyzed by a UHPLC-FLR-MS method that takes advantage of the quantitation provided by fluorescence detection and mass spectrometric identification. In total, 16 glycan features of adalimumab were observed. **Figure 5** illustrates the fluorescence chromatogram of adalimumab glycans. while **Table 3** provides the chromatographic conditions. Glycan peak areas were integrated, and the MS spectra were used to confirm glycan identities (**Table 4**). In total, twelve different glycans were quantified using this approach. The glycan profile, including qualitative and quantitative aspects, is comparable to the results obtained elsewhere.[14,15]

Table 3: Glycan analysis method conditions

Column:	BIOshell™ Glycan 15 cm x 2.1 mm I.D., 2.7 µm (**50994-U**)
Mobile phase:	[A] 75 mM ammonium formate, pH 4.4 in water; [B] acetonitrile
Gradient:	

Time (min)	A (%)	B (%)
0.00	75	25
75	59	41
76	75	25

Flow rate:	0.3 mL/min
Column Temp.:	58 °C
Detection:	1:1 split to both fluorescence detector, Ex: 308 nm, Em: 359 nm, and MS
Injection:	10 µL

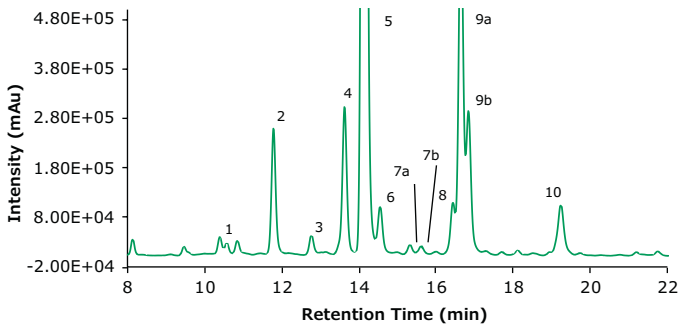

Figure 5. Fluorescence chromatogram of adalimumab after PNGase release and derivatization with procainamide. Peak annotations correspond to glycans shown in **Table 4**.

Table 4. N-Glycans, monosaccharide composition, and content in adalimumab

Peak #	Glycan	Monosaccaride Composition	Content (%)
1	G0-N	Man3 GlcNAc3	0.6
2	G0F-N	Fuc1 Man3 GlcNAc3	4.1
3	G0	Man3 GlcNAc4	0.9
4	Man5	Man5 GlcNAc2	5.3
5	G0F	Fuc1 Man3 GlcNAc4	65.0
6	G1F-N	Fuc1 Man3 Gal1 GlcNAc3	1.7
7a	G1(1,6)	Man3 Gal1 GlcNAc4	0.4
7b	G1(1,3)	Man3 Gal1 GlcNAc4	0.4
8	Man6	Man6 GlcNAc2	1.8
9a	G1F(1,6)	Fuc1 Man3 Gal1 GlcNAc4	12.1
9b	G1F(1,3)	Fuc1 Man3 Gal1 GlcNAc4	5.1
10	G2F	Fuc1 Man3 Gal2 GlcNAc4	2.7

Conclusion

Characterization and monitoring of therapeutic mAbs is required by regulatory authorities to ensure efficacy and safety of these drugs. Workflows for three important approaches to mAb analysis were developed and presented elsewhere as detailed step-by-step procedures.[7-9]

References

1. Berkowitz SA, Engen JR, Mazzeo JR, Jones GB. Analytical tools for characterizing biopharmaceuticals and the implications for biosimilars. Nat Rev Drug Discov. 2012,11(7):527-40. doi: 10.1038/nrd3746.
2. Song YE, Dubois H, Hoffmann M, D Eri S, Fromentin Y, Wiesner J, Pfenninger A, Clavier S, Pieper A, Duhau L, Roth U. Automated mass spectrometry multi-attribute method analyses for process development and characterization of mAbs. J Chromatogr B Analyt Technol Biomed Life Sci. 2021,1166:122540. doi: 10.1016/j.jchromb.2021.122540
3. J. H. Knox, High Performance Liquid Chromatography, Edinburgh University Press, 1978.
4. D'Atri V, Fekete S, Beck A, Lauber M, Guillarme D. Hydrophilic Interaction Chromatography Hyphenated with Mass Spectrometry: A Powerful Analytical Tool for the Comparison of Originator and Biosimilar Therapeutic Monoclonal Antibodies at the Middle-up Level of Analysis. Anal Chem. 2017,89(3):2086-2092. doi: 10.1021/acs.analchem.6b04726.
5. Sjögren J, Olsson F, Beck A. Rapid and improved characterization of therapeutic antibodies and antibody related products using IdeS digestion and subunit analysis. Analyst. 2016,141(11):3114-25. doi: 10.1039/c6an00071a.
6. Beck A, Wagner-Rousset E, Ayoub D, Van Dorsselaer A, Sanglier-Cianférani S. Characterization of therapeutic antibodies and related products. Anal Chem. 2013, 85(2):715-36. doi: 10.1021/ac3032355.
7. Altmaier S, Jalili P, Sreenivasan U, Ray K, Protocol for Purification, Optional Reduction, and SEC-MS Analysis of a Monoclonal Antibody, https://www.sigmaaldrich.com/technical-documents/protocol/pharmaceutical-and-biopharmaceutical-manufacturing/biologics-biosimilars-characterization/protocol-for-analysis-of-a-monoclonal-antibody (accessed March 2022)
8. Rule G, Jalili P, Sreenivasan U, Ray K, Peptide Mapping LC-MS/MS Analysis Workflow for Adalimumab, https://www.sigmaaldrich.com/technical-documents/protocol/pharmaceutical-and-biopharmaceutical-manufacturing/biologics-biosimilars-characterization/peptide-mapping-lc-ms-ms-analysis-workflow-for-adalimumab (accessed March 2022)
9. Rigger R, Jalili P, Sreenivasan U, Ray K, Step-by-Step Protocol for Procainamide Labelled Glycan Profiling of a Monoclonal Antibody, https://www.sigmaaldrich.com/technical-documents/protocol/pharmaceutical-and-biopharmaceutical-manufacturing/biologics-biosimilars-characterization/released-n-linked-glycan-analysis-workflow (accessed March 2022)
10. Lermyte F, Tsybin YO, O'Connor PB, Loo JA. Top or Middle? Up or Down? Toward a Standard Lexicon for Protein Top-Down and Allied Mass Spectrometry Approaches. J Am Soc Mass Spectrom. 2019, 30(7):1149-1157. doi: 10.1007/s13361-019-02201-x
11. P. Jalili, K. Ray, J. Turner, M. Dube, LCGC Europe ebook, 3-12, March 2021.
12. Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009, 6(5):359-62. doi: 10.1038/nmeth.1322
13. Rogers RS, Nightlinger NS, Livingston B, Campbell P, Bailey R, Balland A. Development of a quantitative mass spectrometry multi-attribute method for characterization, quality control testing and disposition of biologics. MAbs. 2015,7(5):881-90. doi: 10.1080/19420862.2015.1069454.
14. Lee N, Lee JJ, Yang H, Baek S, Kim S, Kim S, Lee T, Song D, Park G. Evaluation of similar quality attribute characteristics in SB5 and reference product of adalimumab. MAbs. 2019, 11(1):129-144. doi: 10.1080/19420862.2018.1530920
15. Tebbey PW, Varga A, Naill M, Clewell J, Venema J. Consistency of quality attributes for the glycosylated monoclonal antibody Humira® (adalimumab). MAbs. 2015, 7(5):805-11. doi: 10.1080/19420862.2015.1073429.

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Product	Cat. No.
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BIOshell™ Glycan 15 cm x 2.1 mm, 2.7 µm	**50994-U**
Tosoh TSKgel® SW3000, 30 cm x 2.0 mm, 4 µm	**821485**
System Suitability Standards and Samples	
SILu™Lite SigmaMAb Universal Antibody Standard human	**MSQC4**
SILu™Lite SigmaMAb Adalimumab Monoclonal Antibody	**MSQC16**
Sample Preparation	
SOLu-Trypsin	**EMS0004**
PNGase Fast Kit	**EMS0001**
Low Artifact Digestion Buffer	**EMS0011**
Tris(2-carboxyethyl) phosphine BioUltra	**68957**
Dithiothreitol BioXtra	**D5545**
Procainamide hydrochloride	**PHR1252**
Protein A–Agarose Fast Flow 50%, aqueous suspension	**P3476**
Other Solvents and Reagents	
Water, LiChrosolv® for LC-MS	**1.15333**
Acetonitrile for UHPLC-MS LiChrosolv®	**1.03725**
Trifluoroacetic acid, LiChropur™ for LC-MS	**80457**
Formic acid, LiChropur™ for LC-MS	**5.33002**

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Difluoroacetic Acid as an Effective Mobile Phase Modifier for the LC-UV/MS Analysis of Proteins

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Abstract

Trifluoroacetic acid (TFA) is typically the mobile phase modifier used for protein analysis by reversed-phase high performance liquid chromatography (RP HPLC), especially when combined with UV detection. TFA improves retention, peak shape, and due to the latter the sensitivity. However, TFA also causes ion suppression in mass spectrometry (MS), thereby significantly reducing the sensitivity. Formic acid (FA) is generally the modifier of choice with MS detection as it provides efficient ionization. However, the use of formic acid results in less efficient chromatographic separation. Difluoroacetic acid (DFA) is a suitable alternative to TFA and FA in the LC-UV/MS analysis of proteins, allowing adequate separation efficiency when compared to FA, and better MS compatibility when compared to TFA.

Introduction

The growth of protein-based therapies and the field of proteomics have propelled technical advancements in the analysis of proteins. Characterization of these large and complex molecules utilize a range of techniques - chromatography being one of them. The analysis of intact proteins and protein digests by reversed-phase high performance liquid chromatography (RP-HPLC) provides researchers with valuable information not only about a protein's identity, but also the post-translational modifications that affect its properties.

During the analysis of proteins by RP-HPLC, trifluoroacetic acid (TFA) is added to the mobile phase to obtain sharp and symmetrical peaks with adequate retention,[1] particularly when used in conjunction with UV detection. The added TFA.

(1) lowers the pH of the mobile phase to well below the pK_a of side-chain carboxyls, thereby facilitating maximum retention of acidic moieties, and

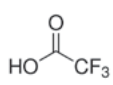
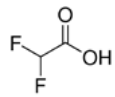
(2) acts as an effective ion-pairing agent[2] for the basic moieties of the protein. In the absence of ion pairing reagents, the residual silanols in the HPLC column interact with the protein analytes, causing band broadening, which in turn leads to reduced efficiency and sensitivity.

HPLC coupled to a mass spectrometer (specifically electrospray ionization (ESI)) has become a widely used technique in protein analysis. The mass spectrometer offers mass selectivity and higher sensitivity compared to UV, giving researchers more valuable information about analyzed protein(s). However, TFA is not compatible with MS; it suppresses signal intensity because of ion-pair formation, high conductivity and surface tension.[1] TFA also leaves background in the mass spectrometer that is difficult to remove.[3] This signal or ion suppression in turn compromises the sensitivity of the technique.

Because of TFA's incompatibility with LC-MS, investigators often use formic acid (FA) when conducting protein separations with MS detection.[4] Unfortunately, separations with FA as a modifier often result in poor peak shape and less efficient separations.[3] This result is because FA is a poorer ion-pairing agent and also a weaker acid compared to TFA.

This article explores the use of difluoroacetic acid (DFA) as an attractive acid modifier alternative in the LC-UV/MS analysis of proteins. DFA is a strong ion-pairing agent and provides the desired low pH (it has a lower pK_a compared to FA).[5] **Table 1** shows the structures and pK_a's of TFA, FA and DFA.

Table 1. Structure and pK_a of additives used in HPLC-MS

Additive	Trifluoroacetic acid	Formic acid	Difluoroacetic acid
Abbreviation	TFA	FA	DFA
Structure			
pKa	0.43	3.75	1.34

Experimental Conditions

The HPLC analyses were carried out using a Shimadzu Nexera LC system equipped with UV and MS detectors. The flow was split between detectors to minimize band broadening from the sample plug having to pass through multiple detectors in line. The conditions are listed in **Table 2**.

Table 2. HPLC conditions for the separation of proteins

Column:	BIOshell™ A400 Protein C4; 10 cm x 2.1 mm I.D., 3.4 µm (**67463-U**)
Column temp.:	50 °C
Mobile phase:	[A]: water with various acid modifiers (**Table 3**)
	[B]: acetonitrile (with various acid modifiers)
Flow rate:	0.35 mL/min
Gradient:	15 – 55% acetonitrile in 30 min
Injection:	10 µL
UV detection:	280 nm
MS parameter:	Single quad (ESI+); Acquisition range: 400 – 2000 m/z; Cone voltage: 3.8 kV
Cone voltage:	3.8 kV
Sample:	25 pmol each of the proteins in **Table 4**

Table 3. Acids used as modifier/additive

	Experiment 1	Experiment 2
Acid	**%**	**mM**
Trifluoroacetic acid (TFA)	0.1	10
Formic acid (FA)	0.25	10
Difluoroacetic acid (DFA)	0.1	10

Table 4. Proteins analyzed

Protein	Molecular Weight (kDa)
Ribonuclease (R)	13.7
Ubiquitin (U)	10.7
Lysozyme (L)	14.4
apo-Myoglobin (M)	17
Enolase (E)	46

Results and Discussion

The combination of high performance liquid chromatography with mass spectrometry (LC-MS) has become a critical tool in protein analysis. Traditionally, TFA is the mobile phase additive used as it provides adequate retention of proteins, and also gives sharp and symmetrical peaks with UV detection (**Figure 1A**). However, it is not recommended for MS detection as it suppresses the analyte signal (**Figure 1B**).

To get better MS signals, investigators use FA because it gives better ionization efficiency of the analytes (**Figure 2B**). Unfortunately, FA as a mobile phase modifier often results in poor peak shape and inefficient separations (**Figure 2A**).

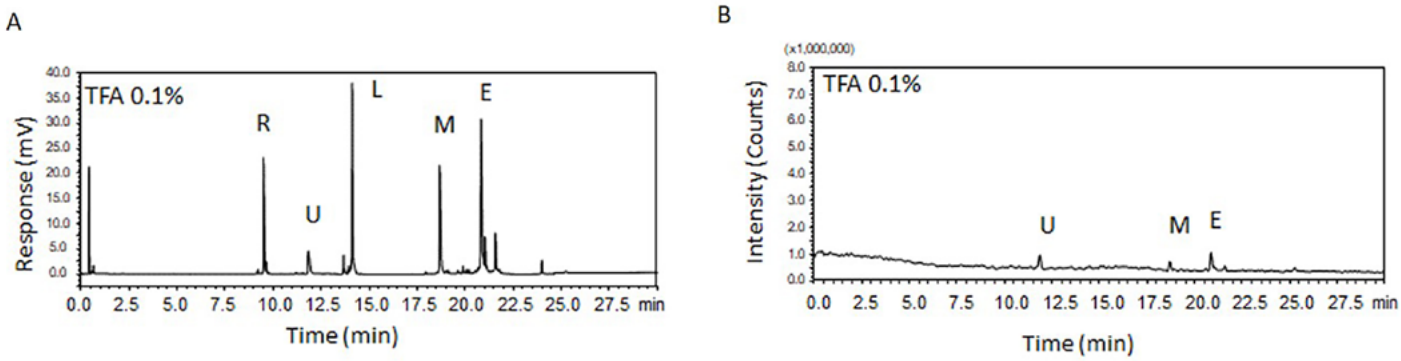


Figure 1. (A) UV and **(B)** MS chromatograms of five proteins when using TFA as modifier. R – Ribonuclease, U – Ubiquitin, L – Lysozyme, M – apo-Myoglobin, E – Enolase

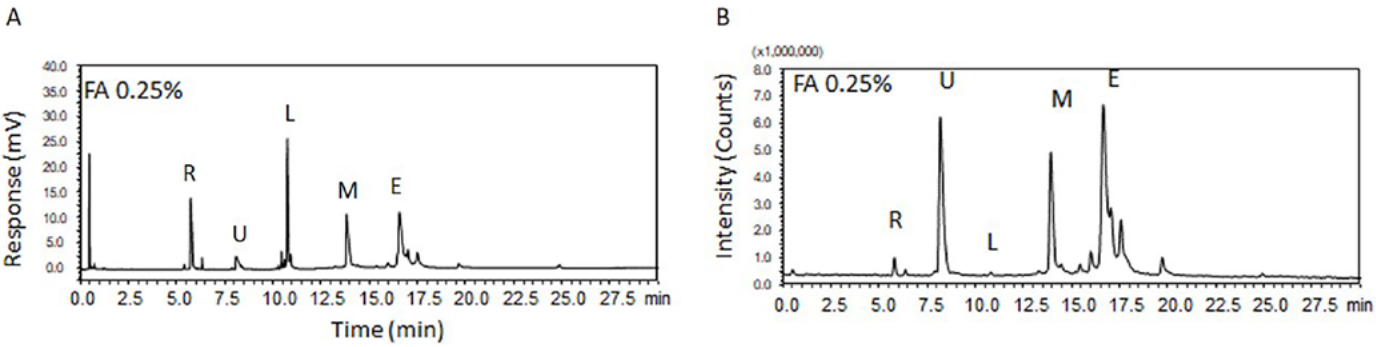


Figure 2. (A) UV and **(B)** MS chromatograms of five proteins with FA as modifier. R – Ribonuclease, U – Ubiquitin, L – Lysozyme, M – apo-Myoglobin, E – Enolase

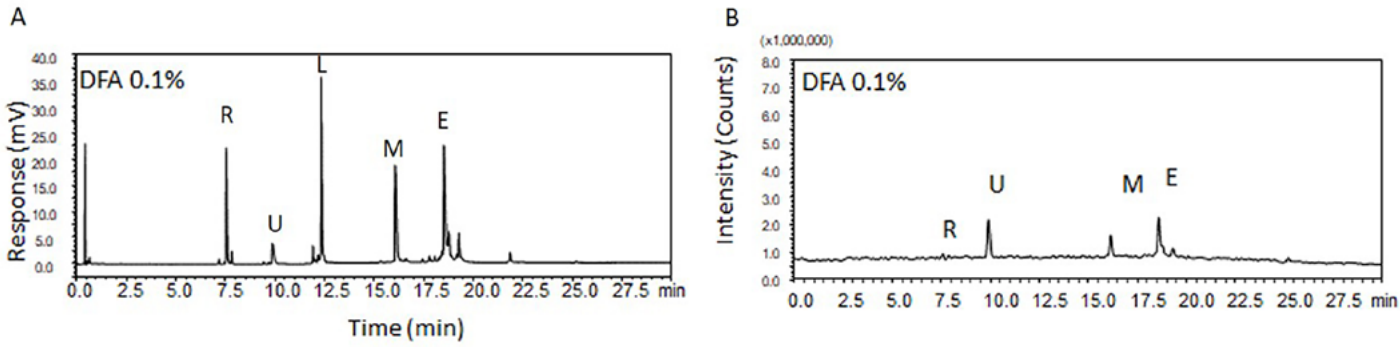


Figure 3. (A) UV and (B) MS chromatograms of five proteins with DFA as modifier. R – Ribonuclease, U – Ubiquitin, L – Lysozyme, M – apo-Myoglobin, E – Enolase

A combination of UV and MS detectors provides investigators with much richer information than just a UV detector, although using both the detectors in line will contribute to band broadening in the MS and must be considered. Using TFA to benefit UV detection does not provide any useful MS data. Using FA to benefit MS detection sacrifices separation efficiency. Therefore, there is a need to find a suitable, MS-friendly solvent additive for the HPLC analysis of proteins that will provide efficient separation without sacrificing on MS signal because of ion suppression. DFA is a viable candidate. This reagent is a stronger acid than FA and does not suppress ionization as much as TFA. **Figure 3** shows the UV and MS chromatograms of five proteins with DFA as the mobile phase modifier.

Comparing the UV chromatograms in **Figures 1A, 2A,** and **3A** illustrates the ion pairing strength of TFA, FA and DFA. TFA provides the strongest ion pairing strength, affording the strongest retention of the proteins, followed by DFA. FA is the poorest ion pairing reagent of the three.

To further explore how the different modifiers influence separation efficiency, a separate experiment was carried out where the concentrations of TFA, FA and DFA were kept the same at 10 mM. Peak widths at half height ($W_{1/2}$) were measured for each of the analyte peaks, as peak width at half height can be used as a measure of efficiency during gradient LC assays. Smaller values correspond to narrower peak width, indicating better efficiency. The results are shown in **Figure 4**.

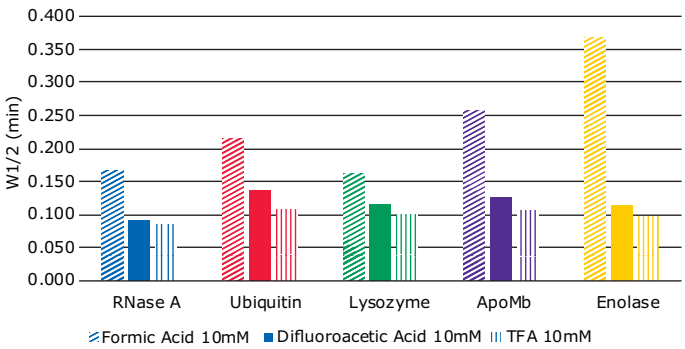


Figure 4. Peak width at half height (W1/2) of five proteins, using three different mobile phase modifiers – formic acid (FA), difluoroacetic acid (DFA), and trifluoroacetic acid (TFA)

TFA gave the narrowest peak widths, indicating better efficiency, followed closely by DFA. FA as the modifier gave the least efficient separation.

The influence of the three modifiers on the MS response can be seen by comparing **Figures 1B**, **2B**, and **3B**. Clearly, the addition of FA as modifier resulted in strong MS signals, but at the cost of separation efficiency. DFA gave better signals than TFA. This observation is also illustrated in **Figure 5A** where signals from each analyte for each of the modifiers are plotted against the total ion current (TIC). **Figure 5B** is an exploded view of just DFA and TFA MS signals, for better comparison.

As seen in **Figure 5B**, as well as **Figure 1B**, MS signals from TFA are strongly suppressed. In fact, the proteins RNase A and Lysozyme were not detected in MS. The use of DFA as modifier gave detectable MS signals as shown in **Figure 3B**.

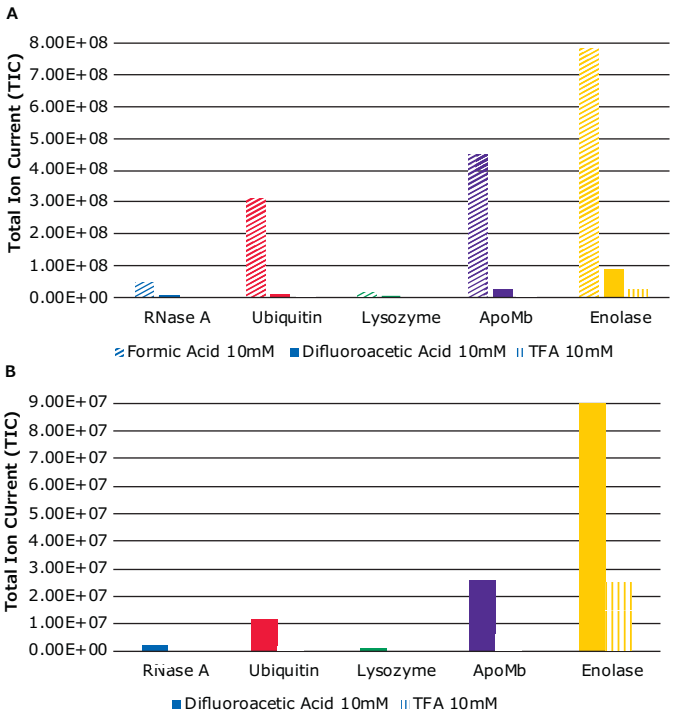


Figure 5. (A) MS signals when using three different modifiers – FA, DFA, and TFA. (B) Exploded view of MS signals when using DFA and TFA as modifiers.

Conclusion

In the HPLC analysis of proteins where combining UV and MS detectors is sometimes necessary, DFA is an attractive alternative to TFA and FA as mobile phase modifier. This reagent provides better separation efficiency than FA, and it does not cause strong ion suppression like TFA. However, DFA causes reduced MS ion yield compared to FA and slightly broader peaks than obtained with TFA. Therefore, users need to do an assessment regarding a possible and acceptable compromise for their application.

References

1. Bobály B, Beck A, Fekete J, Guillarme D, Fekete S. Systematic evaluation of mobile phase additives for the LC-MS characterization of therapeutic proteins. Talanta. 2015, 136:60-7. doi: 10.1016/j.talanta.2014.12.006
2. Corradini D, Kalghatgi K, Horváth C. Effect of mobile phase additives on peptide retention in reversed-phase chromatography with pellicular and totally porous sorbents. J Chromatogr A. 1996, 728(1-2):225-33. doi: 10.1016/0021-9673(95)01019-x
3. Wagner, B.M..; et. al. Tools to Improve Protein Separations, LCGC, 2015, 33, 856-865
4. Bobály B, D'Atri V, Lauber M, Beck A, Guillarme D, Fekete S. Characterizing various monoclonal antibodies with milder reversed phase chromatography conditions. J Chromatogr B Analyt Technol Biomed Life Sci. 2018, 1096:1-10. doi: 10.1016/j.jchromb.2018.07.039
5. Yamamoto E, Ishihama Y, Asakawa N. Application of partially fluorinated carboxylic acids as ion-pairing reagents in LC/ESI-MS. Talanta. 2014, 127:219-24. doi: 10.1016/j.talanta.2014.03.060.

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Water, for chromatography (LC-MS Grade) LiChrosolv®	**1.15333**
Difluoroacetic acid (DFA), for LC-MS LiChropur™, ≥97.5% (GC)	**00922**
Trifluoroacetic acid (TFA), eluent additive for LC-MS, LiChropur™, ≥99.0% (GC)	**80457**
Formic acid, for LC-MS LiChropur™	**5.33002**

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Extractables Study with LC-UV/MS

Efficient identification and quantification of unknown extractables of a single-use system using the certified reference material (CRM) mix for extractables and leachables

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Single-Use Equipment

In issue 8 of the Analytix Reporter magazine, we presented a GC/MS method to detect extractables and leachables (E&L) in a single-use equipment. In addition, we also demonstrated the convenience of using a TraceCERT® extractables and leachables certified reference material (CRM) mix for GC to efficiently detect and quantify the most common extractables. Here we present the use of a corresponding CRM mix for liquid chromatography methods by means of an LC-UV/MS based E&L study.

Single-use systems (SUS) made of polymers are commonly used components in the manufacturing or handling of drugs. This direct contact can lead to the contamination of the drug by leaching of the polymeric material components into the product.

To manage this risk, it is crucial to understand the compounds that might potentially migrate from a material (extractable study) and also the quantities at which such a migration is occurring under certain conditions (leachable study).

As described in the BPOG (BioPhorum Operations Group) guidelines[1] and USP <665> guidelines for polymeric components and systems[2] (draft version), investigations regarding extractables should be performed using various solvents and incubation times with the analysis done using a variety of analytical methods applied to the extracts.

A well-suited method to analyze non-volatile extractables such as additives, impurities, polymer components, or degradation products is liquid chromatography-ultraviolet spectroscopy/mass spectrometry (LC-UV/MS). UV and MS are chosen to detect a wide range of extractables. As generally the exact composition of the polymeric material is unknown, a non-targeted analysis is required that involves the detection and identification of any potential extractable.

To facilitate this type of analysis, we have developed a CRM mixture for 21 extractables typically found in LC-UV/MS studies. This CRM mix is not only helpful for a quick identification of unknown extractables but can also be used for quantification with traceability to a NIST SRM. Since the mix contains a wide variety of substance classes, it is also suitable to check the analytical method to reduce the risk of overlooking potential extractables. The 21 compounds in the mix are listed in **Table 2**. The corresponding single component reference materials are shown under “Related Products” below.

Leachables: Chemical compounds that migrate into a drug formulation from any product contact material (e.g., single-use systems) because of direct contact under a typical process or storage conditions; Leachables may affect the toxicity or efficiency of the drug product

Extractables: Chemical compounds that are extracted from any product contact material usually under extreme conditions (harsh solvents, exaggerated time, and temperature); an Extractables profile represents a worst-case Leachables profile

Single-use Systems (SUS): Usually polymeric, disposable equipment for bioprocessing used in the manufacturing of pharmaceuticals

- ***Advantages:*** Flexibility, no need for cleaning validation, low investment, no cross contamination
- ***Examples of SUS:*** Bioreactors, disposable filters, or tubing

In the following, an application is described using the Extractables and Leachables Screening Standard for LC mix, for the identification and quantification of the main extractables within an extractable study of a filter.

LC Method for Extractables Testing

The applied instrument parameters for an extractable study on single-use equipment (filter) are summarized in **Table 1**. According to the BPOG protocol[1], the separation was performed on a C18 column (Ascentis® C18 Column: 15 cm x 2.1 mm, 3 µm). A representative sample was taken after 24 hours of extraction at 40 °C under orbital rotation with 50% ethanol. The sample and the standard mix were run in one sequence.

Table 1. Experimental conditions

Instrument:	Agilent Infinity II, QToF 6546
Column:	Ascentis® C18, 15 cm x 2.1 mm, 3 µm (**581302-U**)
Mobile Phases:	[A] water; [B] methanol
Gradient:	**Time (min) / %A / %B** 0 / 100 / 0 1 / 100 / 0 15 / 0 / 100 25 / 0 / 100 25.1 / 100 / 0 30 / 100 / 0
Flow:	0.5 mL/min
Columns Temp.:	40 °C
Detector:	DAD, 191 – 400 nm; MSD, full scan, m/z 75-1500
Injection:	5 µL
Samples:	1. Extract of a single use filter (24 h extraction with 50% ethanol) 2. Extractables and Leachables Screening Standard for LC (**95636**), 10 mg/L in acetonitrile

Results & Discussion

The chromatograms of the Extractables and Leachables Screening Standard for LC are shown in **Figure 1-3** (UV, ESI pos, ESI neg, peak IDs in **Table 2**). All 21 reference compounds were detected by the combination of UV-MS detector with almost complete separation. Sixteen reference compounds could be detected with UV (220 nm), 13 reference compounds with ESI positive, and 14 reference compounds with ESI negative ionization. By matching of retention time and m/z ratio, Pentaerythritol tetrakis(3,5-di-tert-butyl-4-hydroxyhydrocinnamate) (Irganox 1010) was identified as the main extractable during the extraction

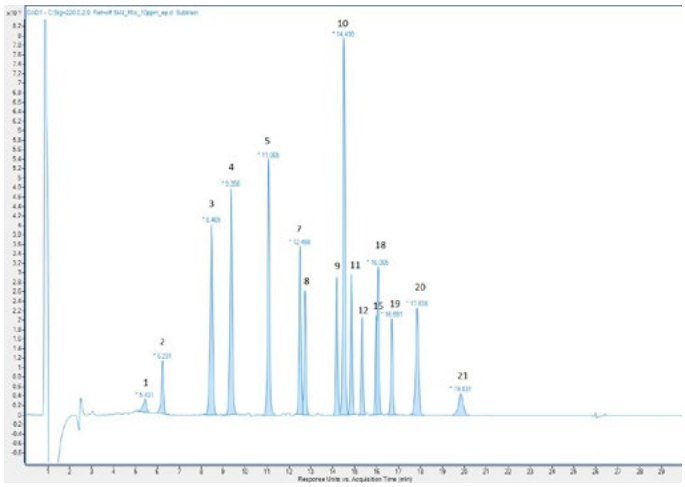

Figure 1. Extractables and Leachables Screening Standard for LC, UV (220 nm), 10 mg/L in acetonitrile.

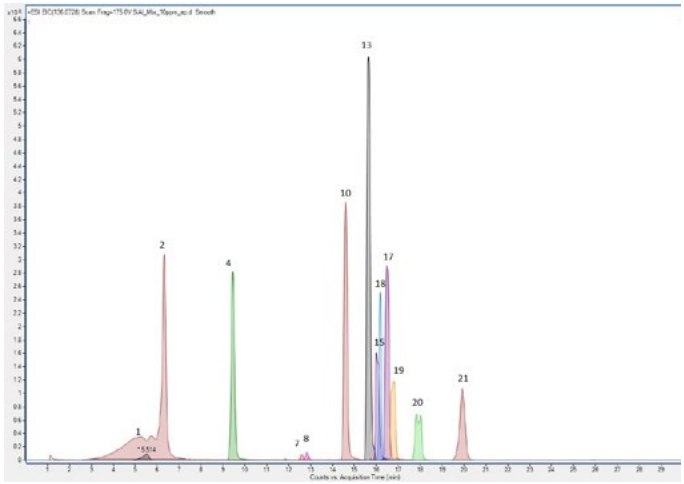

Figure 2. Extractables and Leachables Screening Standard for LC, ESI positive, 10 mg/L in acetonitrile.

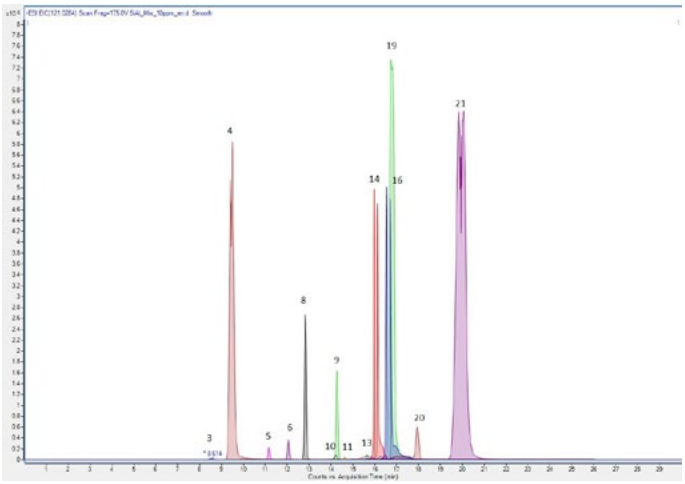

Figure 3. Extractables and Leachables Screening Standard for LC, ESI negative, 10 mg/L in acetonitrile.

of the single-use filter (**Figure 4**). A quantitative analysis against the Pentaerythritol tetrakis(3,5-di-tert-butyl-4-hydroxyhydrocinnamate) (Irganox 1010) peak within the Extractables and Leachables Screening Standard for LC could be performed based on UV (220 nm), ESI positive or ESI negative chromatograms.

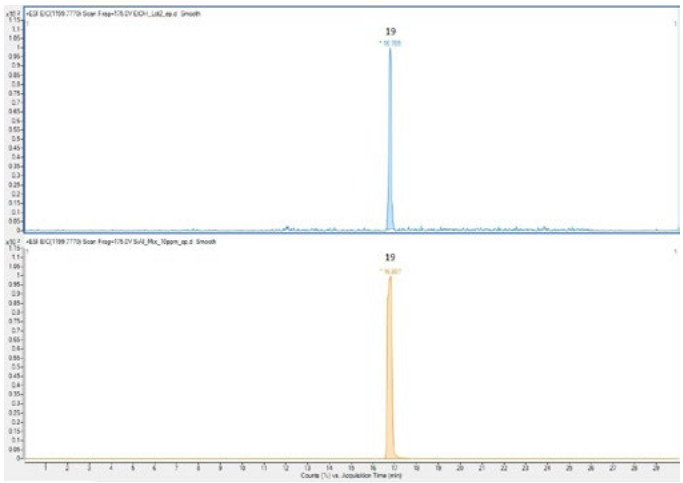

Figure 4. Representative sample of a single-use equipment extraction. Top: Extract Sample of Single Use Filter with 50% Ethanol, bottom: Extractables and Leachables Screening Standard for LC

Table 2. Peak IDs, selected ions, and retention times of compounds

Peak	Compound	CAS	Molecular Formula	m/z	UV absorption at 220 nm	Most prominent adducts		RT [min]
						ESI pos	ESI neg	
1	ε-Caprolactam	105-60-2	$C_6H_{11}NO$	136.0728	very weak	[M+H]+	-	5.43
2	Dibenzylamine	103-49-1	$C_{14}H_{15}N$	198.1279	yes	[M+H]+	-	6.23
3	Benzoic acid	65-85-0	$C_7H_6O_2$	121.0284	yes	-	[M-H]-	8.48
4	2-Mercaptobenzothiazole (2-MBT)	149-30-4	$C_7H_5NS_2$	167.9931 165.9788	yes	[M+H]+	[M-H]-	9.36
5	Bisphenol A (BPA)	80-05-7	$C_{15}H_{16}O_2$	227.1077	yes	-	[M-H]-	11.1
6	2-Ethylhexanoic acid	149-57-5	$C_8H_{16}O_2$	143.1082	no	-	[M-H]-	12.1
7	Bis(4-chlorophenyl) sulfone	80-07-9	$C_{12}H_8Cl_2O_2S$	308.9519	yes	[M+H]+	-	12.5
8	3,5-Di-tert-butyl-4-hydroxybenzyl alcohol	88-26-6	$C_{15}H_{24}O_2$	259.1672 235.1705	yes	[M+Na]+	[M-H]-	12.7
9	2,4-Di-tert-butylphenol	96-76-4	$C_{14}H_{22}O$	205.1597	yes	-	[M-H]-	14.2
10	2-(2-Hydroxy-5-methylphenyl) benzotriazole	2440-22-4	$C_{13}H_{11}N_3O$	226.0972 224.0826	yes	[M+H]+	[M-H]-	14.5
11	Butylhydroxytoluene (BHT)	128-37-0	$C_{15}H_{24}O$	219.1754	yes	-	[M-H]-	14.9
12	1,3-Di-tert-butylbenzene	1014-60-4	$C_{14}H_{22}$	-	yes	-	-	15.3
13	Oleamide	301-02-0	$C_{18}H_{35}NO$	282.2795 280.2643	very weak	[M+H]+	- [M-H]-	15.6
14	Palmitic acid	57-10-3	$C_{16}H_{32}O_2$	255.2331	no	-	[M-H]-	16.1
15	Bis(2-ethylhexyl) phthalate (DEHP)	117-81-7	$C_{24}H_{38}O_4$	391.2839	yes	[M+H]+	-	16.0
16	Stearic acid	57-11-4	$C_{18}H_{36}O_2$	283.2644	no	-	[M-H]-	16.5
17	Erucamide	112-84-5	$C_{22}H_{43}NO$	338.3417	very weak	[M+H]+	-	16.5
18	Tris(3,5-di-tert-butyl-4-hydroxybenzyl) isocyanurate	27676-62-6	$C_{48}H_{69}N_3O_6$	806.5084	yes	[M+Na]+	-	16.1
19	Pentaerythritol tetrakis(3,5-di-tert-butyl-4-hydroxyhydrocinnamate)	6683-19-8	$C_{73}H_{108}O_{12}$	1199.7770 1175.7726	yes	[M+Na]+	[M-H]-	16.7
20	Tris(2,4-di-tert-butylphenyl) phosphate	95906-11-9	$C_{42}H_{63}O_4P$	685.4365 661.4378	yes	[M+Na]+	[M-H]-	17.8
21	Octadecyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate	2082-79-3	$C_{35}H_{62}O_3$	553.4597 529.4624	yes	[M+Na]+	[M-H]-	19.8

Conclusion

The example shown demonstrates the applicability and value of the Extractables and Leachables Screening Standard in LC analysis. Analysis of the most common extractables resulting from single-use equipment. The shown LC-UV/MS method using an Ascentis® C18 column provided a reliable identification and quantification of the 21 components in the mix.

References

1. Ding et al. Standardized Extractables Testing Protocol for Single-Use Systems in Biomanufacturing, Pharmaceutical Engineering, (2017). https://ispe.org/pharmaceutical-engineering/ispeak/standardized-extractables-testing-protocol-single-use-systems-0
2. USP <665> Plastic Materials, Components and Systems Used in the Manufacturing of Pharmaceutical Drug Products and Biopharmaceutical Drug Substances and Products, third draft, published on March 1, 2019 by the USP.

Featured Products

Description	Cat. No.
Extractables and Leachables Screening Standard for LC, certified reference material, 50 µg/mL per component, 1 mL or 5 mL	**95636**
Ascentis® C18, 15 cm x 2.1 mm, 3 µm	**581302-U**

Related Products

Description	Pack Size	Cat. No.
Extractables and Leachables Screening Standard for GC, certified reference material, 50 µg/mL per compound in tert-butyl methyl ether	1 mL or 5 mL	**01829**

Description	Pack Size	Cat. No.
Single Component Certified Reference Materials		
Benzoic acid	1 g	**PHR1050**
Bis(2-ethylhexyl) phthalate	100 mg	**67261**
Bis(4-chlorophenyl)sulfone	100 mg	**CRM96153**
Bisphenol A	100 mg	**42088**
ε-Caprolactam	100 mg	**CRM01483**
Dibenzylamine (DBA)	100 mg	**CRM95728**
3,5-Di-tert-4-butyl-hydroxytoluene (BHT)	100 mg	**CRM96857**
1,3-Di-tert-butylbenzene	100 mg	**CRM96659**
2,4-Di-tert-butylphenol	100 mg	**CRM00437**
cis-13-Docosenoamide (Erucamide)	100 mg	**CRM01374**
2-Ethylhexanoic acid	3 X 1.2 ML	**PHR1914**
2-(2-Hydroxy-5-methylphenyl) benzotriazole (Drometrizole)	100 mg	**CRM96697**
2-Mercaptobenzothiazol	100 mg	**CRM96051**
Octadecyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate (Irganox 1076)	100 mg	**CRM00318**
Oleamide	100 mg	**CRM96709**
Palmitic acid	1 g	**PHR1120**
Pentaerythritol tetrakis(3,5-di-tert-butyl-4-hydroxyhydrocinnamate) (Irganox 1010)	100 mg	**CRM96656**
Stearic acid	1 g	**PHR1114**
Tris(2,4-di-tert-butylphenyl)phosphate (Irgafos 168-oxide)	100 mg	**CRM96839**
Tris(3,5-di-tert-butyl-4-hydroxybenzyl) isocyanurate (Irganox 3114)	100 mg	**CRM96737**
Solvents		
Water for Chromatography (LC-MS Grade) Lichrosolv® (or water tab fresh from a Milli-Q® IQ ultrapure water system)		**1.15333**
Methanol, gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur		**1.06007**

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

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

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

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

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

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

SigmaAldrich.com/Medicinalplants

References

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

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

FOOD & BEVERAGE

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

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Abstract

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

Introduction

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

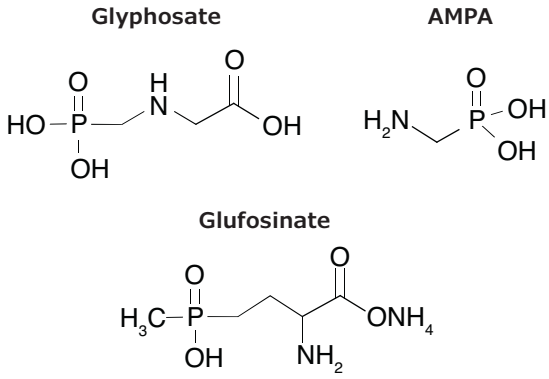


Figure 1. Structures of Glyphosate, AMPA and Glufosinate

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

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

Experimental

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

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

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

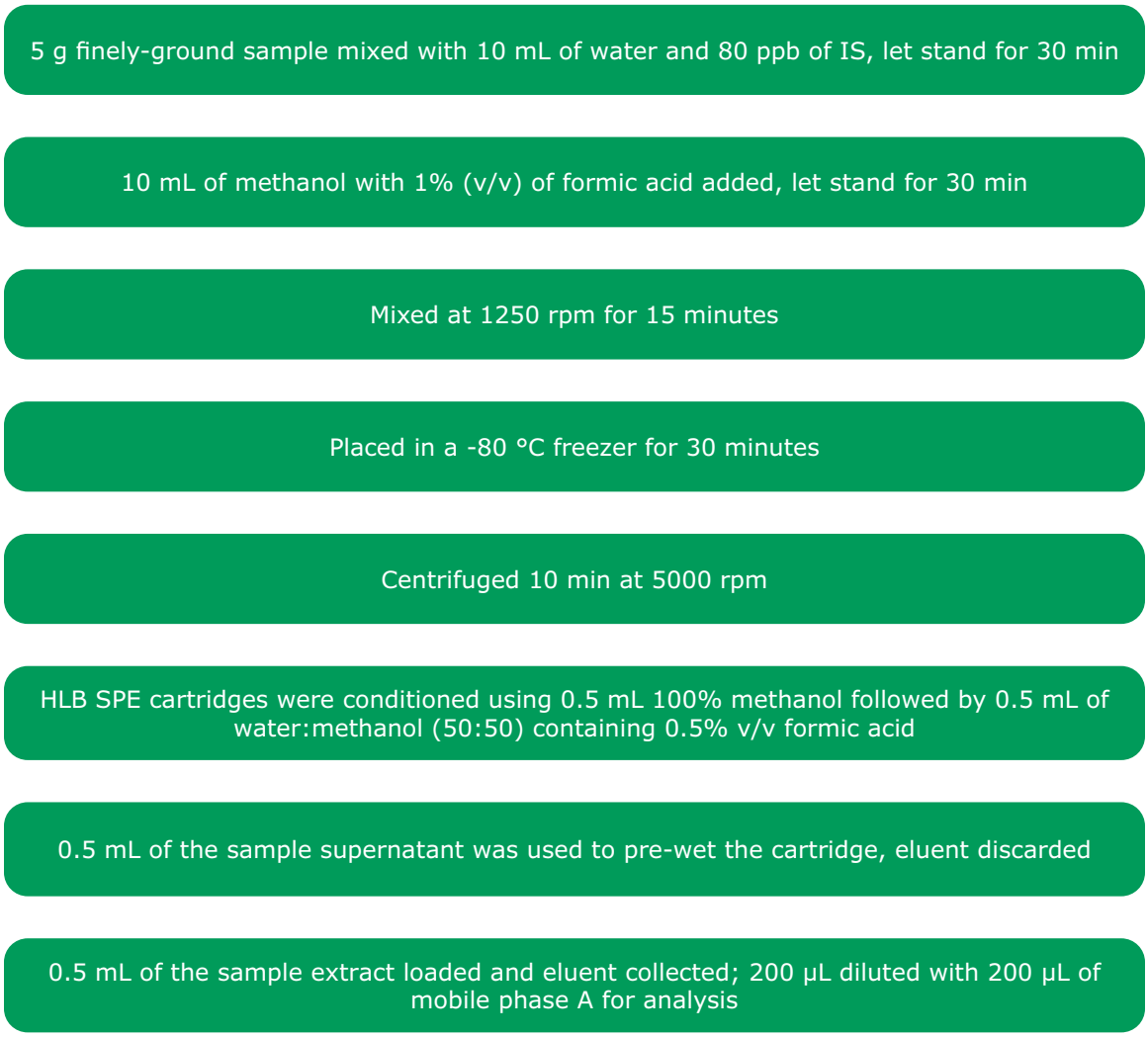


Figure 2. Sample preparation method

Sample preparation

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

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

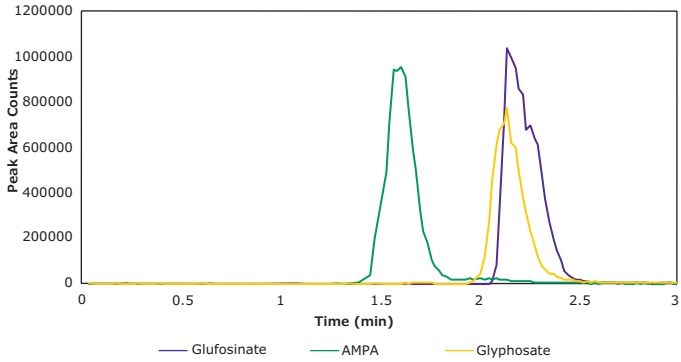
LC-MS/MS method

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

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

Table 1. MS parameters for analytes

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



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

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

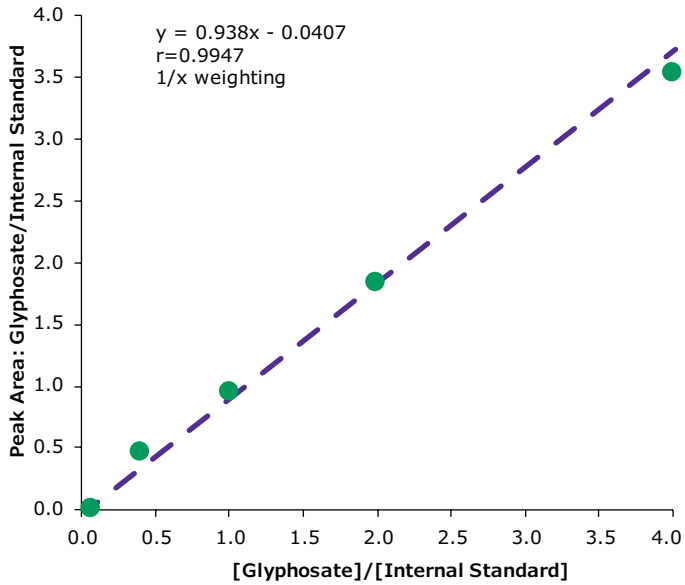


Figure 4. Representative calibration curve for glyphosate. Calibrators for glyphosate range from 3 to 200 ng/mL with an internal standard of 50 ng/mL.

Results

Method development

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

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

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

Table 2. Background analysis results for organic oat cereals

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

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

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

Sample N was spiked with the three analytes at both 80 ppb and 800 ppb levels. Glyphosate and AMPA chromatograms for the 800 ppb spiked sample are shown in **Figure 5**. The quantitation results are shown in **Table 3**. All three analytes were detected and quantified at both spiking levels. Accuracy of the method was measured as the percent recovery of the known spiked amounts. For 80 ppb and 800 ppb spikes, the recovery values for glyphosate were 124% and 96%, for glufosinate, they were 132% and 104%, and for AMPA the recoveries were at 80% and 88% respectively on day one. Similar recoveries were achieved on the second day of testing when the samples were extracted and analyzed again. Reproducibility of the method was excellent with below 10% RSD for 800 ppb spiked samples and below 20% RSD for 80 ppb spiked samples.

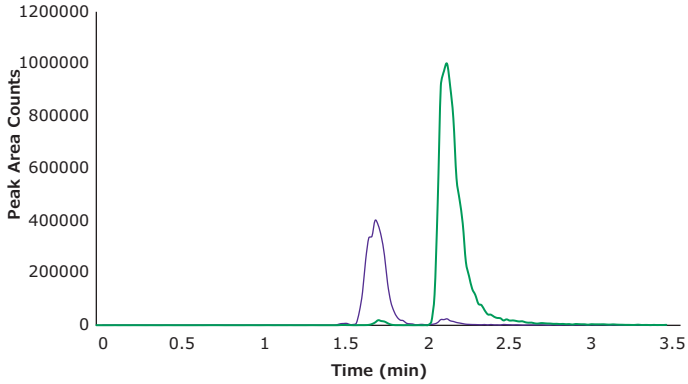


Figure 5. Glyphosate (green trace) and AMPA (purple trace) spiked into oatmeal at 800 ppb.

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

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

Identification and quantitation of glyphosate in cereals

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

Table 4. Results of analysis for oat-containing cereals

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

Conclusion

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

References

1. Grossmann E., What Do We Really Know About Roundup Weed Killer?; National Geographic Apr 23, 2015, http://news.nationalgeographic.com/2015/04/150422-glyphosate-roundup-herbicide-weeds/ (accessed December 17, 2021).
2. Glyphosate; tolerances for residues. §180.364 https://www.govinfo.gov/app/details/CFR-2020-title40-vol26/CFR-2020-title40-vol26-sec180-364 (accessed January 14, 2022).
3. European commission EU pesticide database, pesticide residue(s) and maximum residue levels (mg/kg) https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/mrls/?event=details&pest_res_ids=120,320&product_ids=236,237&v=1&e=search.pr (assessed January 12, 2022)
4. Methods for the Determination of Organic Compounds in Drinking Water- Supplement I (EPA/600/4-90-020). This document is available through NTIS (http://www.ntis.gov). https://www.nemi.gov/methods/method_summary/4806/ (accessed January 25, 2022).
5. Ehling, S.; Reddy, T.M. Total Residue Analysis of Glyphosate and Three Metabolites in Soy and Milk-based Nutritional Ingredients by Derivatization with Fluorenylmethyloxycarbonyl Chloride and Liquid Chromatography/Mass Spectrometry. Poster in Proceedings of ASMS, San Antonio, TX, 2016EURL-SRM
6. Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement Quick Polar Pesticides Method, I. Food of Plant Origin (QuPPe-PO-Method), Version 12, https://www.eurl-pesticides.eu/userfiles/file/EurlSRM/EurlSrm_meth_QuPPe_PO_V12.pdf (accessed December 17, 2021)
7. Nagatomi, Y., et al. Simultaneous LC-MS/MS Analysis of Glyphosate, Glufosinate, and Their Metabolic Products in Beer, Barley Tea, and Their Ingredients, BioSci. Biotechnol. Biochem., 2013, 77 (11), 2218–2221. DOI: 10.1271/bbb.130433
8. Shimelis O., LC-MS/MS Method for Determination of Glyphosate, AMPA, and Glufosinate in Cereals; Chromatography Today, Jun 12 2019, https://www.chromatographytoday.com/article/lc-ms/48/milliporesigma/lc-msms-method-for-determination-of-glyphosate-ampa-and-glufosinate-in-cereals/2556 (accessed Dec 22, 2021)
9. Chamkasem., N.; Harmon, T. Direct determination of glyphosate, glufosinate, and AMPA in soybean and corn by liquid chromatography/tandem mass spectrometry. Anal. Bioanal. Chem. 2016, 408(18), 4995-5004. DOI: 10.1007/s00216-016-9597-6

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Glufosinate-ammonium, analytical standard PESTANAL®, 100 mg	**45520**
Glyphosate	**89432**
Glufosinate-ammonium	**49677**
Glyphosate-2-^{13}C,^{15}N, analytical standard PESTANAL®, 5 mg	**90479**
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Ammonium hydroxide, OmniTrace® Ultra	**AX1308-7***
Formic acid, puriss. p.a., ACS reagent, reag. Ph. Eur., ≥98%	**33015**
Brand® Centrifuge tubes, PP, w/caps, 50 mL, Pk. 300	**BR114820**
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* US only product, see below for alternatives

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Formic acid for LC-MS LiChropur™, 97.5-98.5% (T)	**00940**
Reference Materials	
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Glyphosate-2-13C, PESTANAL®, analytical standard, 5 mg	**90007**
Glufosinate-ammonium, certified reference material, TraceCERT®, 100 mg	**49677**

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SCIENCE & TECHNOLOGY INNOVATIONS

Tips & Tricks: Karl Fischer Titration

Measuring water content of samples that do not easily release water

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Karl Fischer titration for water determination is probably the best known and most widely used titration method. The reaction mechanism is well explored and there is a broad offering of suitable reagents and instruments to be used. The method is described in numerous regulations and guidelines with thousands of available applications. As part of method development, the type of sample should be considered. Specifically, how to best release water from it so as to make it completely accessible for titration.

The Karl Fischer method determines only water and no other compounds because of the selective and stoichiometric reaction between the water in the sample and the iodine in the reagent. There are multiple different ways to release the water from the samples and determine the correct water content. The following preconditions and questions are important to consider before getting started:

- The water in the sample needs to be completely released. What is the best solvent or method to do this?
- Any contamination with ambient moisture must be avoided. Is the sample hygroscopic? Is the instrument tight? With hygroscopic samples fast working procedures are needed and the samples have to be kept protected in closed containers.
- Water or iodine generating and consuming side reactions need to be suppressed or avoided. Is the sample reacting with the components in the Karl Fischer reagents and how can this be prevented?

How water is present in a sample and how to completely release it?

One of the first things to consider is whether the sample can be dissolved fast and completely in the Karl Fischer solvent without any additional treatments or if addition of co-solvents (solubilizers) or any sample preparation is needed to extract the water from the sample.

Solid samples can bind water in different forms:

- Enclosed water
- Crystal water
- Surface water

To measure the total water content of a solid sample, either it must be totally dissolved, or the water completely extracted.

If only surface water should be determined it must be ensured that the sample is not getting dissolved, which usually can be achieved by adding chloroform and by a fast procedure.

If the sample is soluble in the Karl Fischer solvent, it can be added directly to the titration cell, without requiring any additional sample preparation. Some samples might need additional preparations to dissolve fast and completely in the titration cell, such as, heating, homogenizing, extended stirring or addition of co-solvents. For samples that do not dissolve in the Karl Fischer solvent or cause side reactions, sample preparation is of particular importance. In coulometry, the direct addition of a solid sample to the titration cell is not recommended. This would require opening of the coulometric titration cell, and depending on the ambient humidity and handling, up to 10 µg of water can enter alongside the sample. In coulometry samples with low water content (eg. in the range of 100 ppm) are measured, which can then result in an error e.g of up to 10% in water content for a 1 g sample. With liquid samples, a septum is used to add the sample to the titration cell and prevent the simultaneous entry of ambient moisture. For a volumetric titration, a higher amount of sample is added, and therefore the relative error due to addition of ambient moisture while adding solid samples directly, is very low and can be neglected.

Many liquid and solid samples do not release their water completely and rapidly in the Karl Fischer solvent. They either do not dissolve or dissolve very slowly, and are often of non-homogeneous nature. This can result in a continuous release of water during the titration of such samples, which leads to incorrect and non-reproducible results. Other samples can cause side reactions with the Karl Fischer reagents which leads as well to incorrect water content values.

Methods to dissolve or extract the water from the samples

After homogenizing a sample by grinding, mixing or dispersing, there are different options available to completely release water for titration:

- Internal extraction / dissolution by direct titration – the sample is added directly into the titration cell to dissolve; the samples that do not release water fast, can be supported by heating, adding co-solvents, homogenisation, or extended stirring
- External extraction / dissolution - the sample is added in an appropriate solvent outside of the titration cell to release the water, and an exact weighted aliquot is then added into the titration cell
- Gas phase extraction (Karl Fischer oven method) - heating the sample in a Karl Fischer oven to extract the water and transferring the evaporated water into a Karl Fischer titration cell

Solubilizers to dissolve the sample in the Karl Fischer solvent

Many samples do not dissolve in the Karl Fischer solvent, usually methanol. They need addition of a solubilizer to the methanol containing Karl Fischer solvent or there are specific Karl Fischer solvents available already containing co-solvents. Ethanol based solvents can only be used for samples that dissolve in ethanol, and in such cases, the addition of a co-solvent is not recommended.

To find the right solubilizer, first add the different possible solubilizers in separate Erlenmeyer flasks followed then by the sample. After mixing, check in which solubilizer the sample is completely dissolved and then use this solvent as a co-solvent in the Karl Fischer titration. A co-solvent overview is shown in **Table 1**.

Table 1. Examples for recommended co-solvents/ solubilizers by sample type

Sample	Solubilizer/Co-Solvent
Heavy oils (transformer oil, motor oil, crude oil, gasoline) and long chain hydrocarbons	Toluene, Decanol, Hexanol, 1-Propanol or (Chloroform)*
Light oils (vegetable oils & fats, castor oils)	Decanol, Xylene or (Chloroform)*
Inorganic salts, carbohydrates (wheat, flour, noodles), coffee, proteins	Formamide

*because of its toxicity and health hazard to humans, animals and the environment, chloroform should be avoided if possible. For most applications, other solubilizers are suitable.

It should be kept in mind, that the addition of co-solvents as mentioned above, influences the conductivity and other conditions of the system.

Add not more than 40-50 % of solubilizer to volumetric solvents, and not more than 20-30 % to the coulometric reagents to avoid indication problems. If endpoint indication or over-titration problems occur, reduce the amount of your co-solvent.

Internal extraction/dissolution

In direct titration, the sample dissolves in the titration vessel before the titration starts. For some samples that dissolve slowly in the solvent, an extended stirring time is necessary.

Internal extraction is performed in a specific heated cell at 50 °C for samples that are not soluble and release water slowly. Examples are the applications for coffee and starchy products such as wheat, flour or rice. The addition of a co-solvent like formamide can also increase the solubility. For some samples a combination of the mentioned sample preparations is needed.

External extraction/ dissolution

Some samples need an external extraction or dissolution either because of the possible side reactions taking place, or their low water content or insolubility in the Karl Fischer solvent. With such samples, first determine the water content of the used solvent, then weigh the exact amount of the solvent in a closed flask, add the weighted sample and mix them well. Certain samples will have a slow water release, therefore they require an extented extraction or mixing time. Titration at higher temperature up to 50 °C can accelerate slow water release as well. These approaches can be combined and should be evaluated during method development to achieve reproducible results. After complete water release, add an exactly weighted aliquot into your Karl Fischer titration cell and start the titration. Once finished, subtract the water content of the solvent from your result.

As a solvent methanol can be used or one of the previously mentioned solubilizers (in **Table 1**) or a mixture, depending on the sample.

Gas phase extraction / Karl Fischer oven method

A more convenient method to be used for critical samples that are not soluble, cause side reactions, or are solid with low water content is the Karl Fischer oven in combination with a volumetric or coulometric titrator. However, the samples need to be thermally stable and should not decompose during heating. A temperature ramp should be done first with each sample to determine the exact heating temperature needed. The samples are then heated to this specific temperature, causing the water in them to evaporate and to be

transported into the titration vessel by a constant flow of dry air or nitrogen.

If you have further questions, please contact us at **aquastar@merckgroup.com**

Related Aquastar® Products

Description	Cat. No.
Volumetric Reagents	
CombiTitrant 5, one component titrant for volumetric Karl Fischer titration, approx. 5 mg H_2O/mL	**1.88005**
CombiTitrant 2, one component titrant for volumetric Karl Fischer titration, approx. 2 mg H_2O/mL	**1.88002**
CombiTitrant 1, one component titrant for volumetric Karl Fischer titration, approx. 1 mg H_2O/mL	**1.88001**
CombiMethanol, solvent for volumetric Karl Fischer titration with one component reagents max. 0.01% H_2O	**1.88009**
Titrant 5, two component titrant for volumetric Karl Fischer titration, approx. 5 mg H_2O/mL	**1.88010**
Titrant 2, two component titrant for volumetric Karl Fischer titration, approx. 2 mg H_2O/mL	**1.88011**
Solvent for volumetric Karl Fischer titration with two component reagents	**1.88015**
CombiTitrant 5 Keto, one component titrant for volumetric Karl Fischer titration, approx. 5 mg H_2O/mL	**1.88006**
CombiSolvent Keto, suitable for Karl Fischer titration for aldehydes and ketones	**1.88007**
CombiSolvent, methanol-free solvent for volumetric Karl Fischer titration with one component reagents	**1.88008**
CombiSolvent Oils, solvent for volumetric Karl Fischer titration with one component reagents for mineral oils	**1.88020**
CombiSolvent Fats, solvent for volumetric Karl Fischer titration with one component reagents for fats in food	**1.88021**
Solvent Oils & Fats, solvent for volumetric Karl Fischer titration with two component reagents for long-chain hydrocarbons and non-polar substances	**1.88016**
Buffer	
Buffer solution for strong acids, for Karl Fischer titration	**1.88035**
Buffer solution for strong bases, for Karl Fischer titration	**1.88036**
Coulometric Reagents	
CombiCoulomat frit, for coulometric Karl Fischer titration for cells with diaphragm	**1.09257**
CombiCoulomat fritless, for coulometric Karl Fischer titration for cells without diaphragm	**1.09255**
Anolyte for coulometric Karl Fischer titration, for cells without diaphragm	**1.88079**
Certified Reference Materials	
Water standard 0.01%, 0.1 g 1 mg H_2O	**1.88050**
Water standard 0.1%, 1 g 1 mg H_2O	**1.88051**
Water Standard 1%, 1 g contains 10 mg H_2O	**1.88052**
Water Standard oven 1 %, solid standard for Karl Fischer oven method	**1.88054**

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