

Analytix

Issue 4 • 2007



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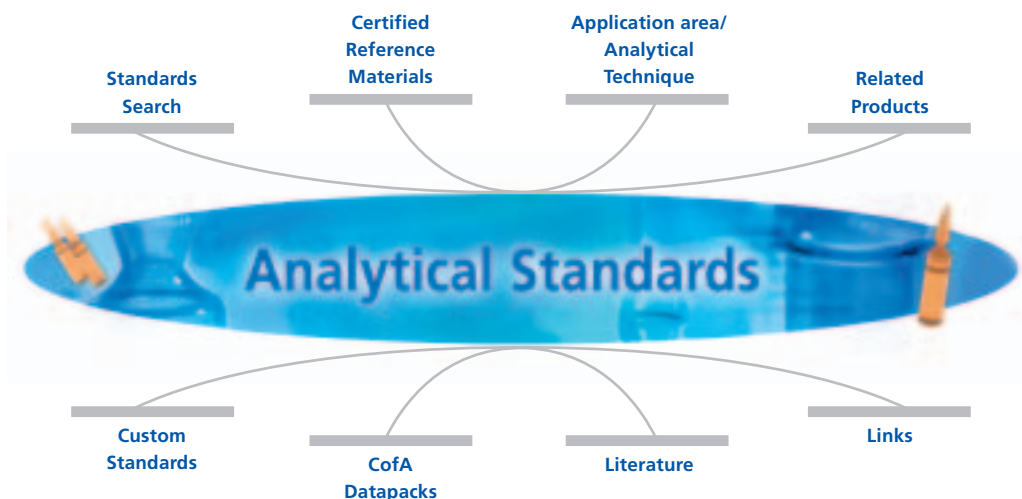
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Feature Article

- 04** Biodiesel Analysis
Biodiesel standards and **HYDRANAL**[®] reagents for the determination of water in biodiesel

Food Analysis

- 16** Enzymatic Kits for Nutrients
Quantitative enzymatic determination of sucrose, glucose, fructose, starch and total dietary fiber in food and other materials

Standards

- 06** Analysis of Alkylphenols and Alkylphenol Ethoxylates Using new ¹³C-Labeled and Deuterated Standards According to ISO 18857-2
Sigma-Aldrich offers these useful isotopically labeled compounds for GC-MS and LC-MS applications
- 08** *TraceCERT*[™] – Traceable Certified Reference Materials. Part 5 – Reliability is a matter of proper uncertainty calculation
This is the fifth and final installment of a series of articles on Certified Reference Materials

Titration

- 19** **HYDRANAL**[®]-Water Standard
Sigma-Aldrich is proud to present a new solid standard and a new oven
- 21** Laboratory Chemicals for Titration
Convenient, accurate, ready-to-use volumetric solutions

New Product Corner

- 23** New DNPH and DAIH Standards for Air Monitoring
- 23** IDRANAL[®] VII, HEDTA-Na₃

Chromatography

- 11** Chiral HPLC Separations
Comparing normal and reversed-phase modes with various ion sources

Spectroscopy

- 14** NMR Reference Standards
Standards suitable for instrument qualification and performance verification of modern NMR instruments

Biodiesel Analysis

Part 1 – Supelco Biodiesel Standards for ASTM D6584 and EN 14105

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Rising prices and unstable supply along with environmental issues surrounding petroleum-based fuels are driving research into alternate energy sources. Currently, the spotlight is on biodiesel, the renewable, alternative diesel fuel produced from vegetable oils, animal fats and recycled restaurant grease. Residual glycerin, both free glycerin and its fatty acid esters, affects the quality of biodiesel and causes clogged fuel systems, injector deposits, plugged filters and build-up in the vehicle's fuel tanks. The biodiesel assay for free and total glycerin is outlined in DIN Method EN 14105 and ASTM D6584. Sigma-Aldrich offers a comprehensive line of glycerin and glycerin esters as standards for the characterization of biodiesel according to both methods (Table 1), including six new standards specifically for EN 14105 (Table 2). As your one source for many analytical products, we also offer the N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) derivatization reagents and HT-5 capillary GC columns used to analyze the compounds as their FAMES (Table 3).

Table 1 Biodiesel Standards

Cat. No.	Brand	Description	Package Size
44892-U	Supelco	Glycerin (CAS#: 56-81-5), 500 µg/mL pyridine	1 x 1 mL
44893-U	Supelco	Monoolein (CAS#: 111-03-5), 5000 µg/mL pyridine	1 x 3 mL
44894-U	Supelco	Dioline (CAS#: 2465-32-9), 5000 µg/mL pyridine	1 x 2 mL
44895-U	Supelco	Triolein (CAS#: 122-32-7), 5000 µg/mL pyridine	1 x 2 mL
44896-U	Supelco	Butanetriol (CAS#: 42890-76-6) Internal Standard #1, 1000 µg/mL pyridine	1 x 5 mL
44897-U	Supelco	Tricaprin (CAS#: 621-71-6), Internal Standard #2, 8000 µg/mL pyridine	1 x 5 mL
44898-U	Supelco	ASTM D6584 Individual Stock and Internal Stds Mix Kit Kit contains 1 ea: 44892-U, 44893-U, 44894-U, 44895-U, 44896-U, and 44897-U	1 kit
44899-U	Supelco	ASTM D6584 Standard Solution 1, varied concentration, pyridine Glycerin 5 µg/mL, Monoolein 100 µg/mL, Dioline 50 µg/mL, Triolein 50 µg/mL	1 x 1 mL
44914-U	Supelco	ASTM D6584 Standard Solution 2, varied concentration, pyridine Glycerin 15 µg/mL, Monoolein 250 µg/mL, Dioline 100 µg/mL, Triolein 100 µg/mL	1 x 1 mL
44915-U	Supelco	ASTM D6584 Standard Solution 3, varied concentration, pyridine Glycerin 25 µg/mL, Monoolein 500 µg/mL, Dioline 200 µg/mL, Triolein 200 µg/mL	1 x 1 mL
44916-U	Supelco	ASTM D6584 Standard Solution 4, varied concentration, pyridine Glycerin 35 µg/mL, Monoolein 750 µg/mL, Dioline 350 µg/mL, Triolein 350 µg/mL	1 x 1 mL
44917-U	Supelco	ASTM D6584 Standard Solution 5, varied concentration, pyridine Glycerin 50 µg/mL, Monoolein 1000 µg/mL, Dioline 500 µg/mL, Triolein 500 µg/mL	1 x 1 mL
44918-U	Supelco	ASTM D6584 Standard Solution Kit with Internal Standards, Kit contains 1 ea: 44899-U, 44914-U, 44915-U, 44916-U, & 44917-U	5 x 1 mL

For more information, please visit www.sigma-aldrich.com/biodiesel

Table 2 New Glycerin Standards for Biodiesel Analysis by EN14105:2003

Cat. No.	Brand	Description	Package Size
49446-U	Supelco	EN 14105:2003 Monoglyceride Stock Solution, each compound 10 mg/mL in pyridine, Monoolein, Monopalmitin, Monostearin	1 mL
49441-U	Supelco	EN 14105:2003 Standard Solution 1, varied concentration, pyridine Butanetriol 80 µg/mL, 1,3-Diolein 50 µg/mL, Glycerol 5 µg/mL Monoolein 250 µg/mL, Tricaprin 800 µg/mL, Triolein 50 µg/mL	1 mL
49442-U	Supelco	EN 14105:2003 Standard Solution 2, varied concentration, pyridine, Butanetriol 80 µg/mL, 1,3-Diolein 200 µg/mL, Glycerol 20 µg/mL, Monoolein 600 µg/mL, Tricaprin 800 µg/mL, Triolein 150 µg/mL	1 mL
49443-U	Supelco	EN 14105:2003 Standard Solution 3, varied concentration, pyridine Butanetriol 80 µg/mL, 1,3-Diolein 350 µg/mL, Glycerol 35 µg/mL, Monoolein 950 µg/mL, Tricaprin 800 µg/mL, Triolein 300 µg/mL	1 mL
49444-U	Supelco	EN 14105:2003 Standard Solution 4, varied concentration, pyridine Butanetriol 80 µg/mL, 1,3-Diolein 500 µg/mL, Glycerol 50 µg/mL Monoolein 1250 µg/mL, Tricaprin 800 µg/mL, Triolein 400 µg/mL	1 mL
49445-U	Supelco	EN 14105:2003 Standard Solution Kit 1 mL of each of 49441-U, 49442-U, 49443-U, 49444-U	1 Kit

Table 3 Derivatization Reagents and Columns for GC Analysis of Biodiesel

Cat. No.	Brand	Description	Package Size
394866-5 mL	Aldrich	MSTFA Derivatization Reagent	5 mL
394866-10X1 mL	Aldrich	MSTFA Derivatization Reagent	10 x 1 mL
394866-25 mL	Aldrich	MSTFA Derivatization Reagent	25 mL
25002	Supelco	HT-5 Capillary GC Column, 12 m x 0.32 mm I.D. x 0.1 µm df	Each
25004	Supelco	HT-5 precolumn, 6 m x 0.53 mm I.D. x 0.1 µm df	Each

Part 2 – HYDRANAL® Reagents for Determination of Water in Biodiesel by Karl Fischer Titration

Andrea Felgner, Product Manager Analytical Reagents andrea.felgner@sial.com

Water, whether from the processing operation, food residue, or as a result of leakage or condensation in storage tanks, is an unwanted contaminant in biodiesel. Among other effects, water can cause instability of the biodiesel and reduce its efficacy.

To help assure the stability and functionality of biodiesel fuels, EN 14214 specifies a maximum water content of 500 ppm to be determined accurately and reproducibly by Karl Fischer Titration (EN ISO 12937). Various Karl Fischer procedures, including volumetric, coulometric and Karl Fischer oven methods, can be used to measure the moisture content of biodiesel. The well-established

HYDRANAL® products from Sigma-Aldrich include pyridine-free reagents for all types of Karl Fischer determinations, including solubilizing agents for effective dispersion of biodiesel samples that contain hydrocarbon components of different alkyl chain lengths.

Table 1 lists just a few **HYDRANAL®** products for coulometric Karl Fischer determination in biodiesel. All **HYDRANAL®** products and applications for water determination in biodiesel can be found on our web site www.sigma-aldrich.com/biodiesel. Specifically, we have created **HYDRANAL®** Application for Biodiesel (L 546) that contains detailed information and instructions on this analysis.

Table 1 HYDRANAL® Products for Coulometric Karl Fischer Determination in Biodiesel

Cat. No.	Brand	Description	Use
34868	Riedel-de Haën	HYDRANAL® Coulomat Oil (Analyte)	Analyte for coulometric Karl Fischer titration in oils (for cells with diaphragm).
34840	Riedel-de Haën	HYDRANAL® Coulomat CG (Catholyte)	Catholyte for coulometric Karl Fischer titration. Free of halogenated hydrocarbons.
34828	Riedel-de Haën	HYDRANAL® -Water Standard 1.00 (Control)	Standard for coulometric Karl Fischer titration (1 g contains 1 mg = 0.1% H ₂ O). Tested against NIST SRM 2890).
34847	Riedel-de Haën	HYDRANAL® -Water Standard 0.10 (Control)	Standard for coulometric Karl Fischer titration (1 g contains 0.10 mg = 0.01% H ₂ O). Tested against NIST SRM 2890).
34748	Riedel-de Haën	HYDRANAL® -Water Standard KF-Oven 230 °C	Solid standard for control of KF ovens, 5.55% ± 0.05% as H ₂ O.

Analysis of Alkylphenols and Alkylphenol Ethoxylates Using new ¹³C-Labeled and Deuterated Internal Standards According to ISO 18857-2

Sigma-Aldrich offers these useful isotopically labeled compounds for GC-MS and LC-MS applications

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Alkylphenols are starting materials for the synthesis of alkylphenol ethoxylates, which are widely used as non ionic tensides, dispersive agents in paper and leather manufacturing, emulsifiers for pesticide formulations and as auxiliary agents for drilling and flotation. The most important members are ethoxylates of nonylphenol (NP) and octylphenol (OP). The number of ethoxylate units can be as high as 100. Alkylphenol ethoxylates are produced in huge quantities; the annual worldwide usage is estimated to be around 600,000 tons [1].

As a consequence of the wide-spread use for more than forty years, alkylphenols and alkylphenol ethoxylates have become ubiquitous environmental contaminants [2, 3] and have even been found in foodstuffs [4]. Under environmental conditions, the long-chain alkylphenol ethoxylates degrade quickly to the corresponding alkylphenols. The physical-chemical properties of alkylphenols are the reason for the special relevance to aquatic habitats. First, they are highly toxic to many aquatic organisms. Second, compared to alkylphenol ethoxylates OP and NP are highly persistent and show a high potential for bioaccumulation because of their low water solubility. The environmental consequences of this bioaccumulation are of serious concern: Alkylphenols exhibit estrogen-like activity and disrupt male fertility in fish and aquatic mammals [5]. The same mechanism operates for short-chain ethoxylates of OP and NP with one to two ethoxy units.

The environmental impact of OP and NP and their mono- and diethoxylates have prompted their inclusion in national and international legislation with corresponding monitoring programs. One of the most important agreements is the OSPAR Convention for the Protection of the Marine Environment. Since 1998 OP, NP and NP ethoxylates are listed by OSPAR as Chemicals for Priority Action. Another fundamental regulation is the European Water Framework Directive 2000/60/EC. Annex X of this directive lists NP and OP as priority

hazardous substances with OP still in the testing process. The aim of these regulations is to ensure, that emissions and losses of these substances to surface waters must be reduced to zero within the next two decades. For an efficient monitoring of these compounds in the environment and verification of the compliance with regulations, a reliable and rugged analytical method is crucial. However, the analysis of alkylphenols and their ethoxylates is challenging, primarily because of the lack of availability of labeled internal standards. Instead, often the n-isomers of OP and NP and their ethoxylates are used as internal standards, which usually leads to erroneous results because the n-isomers exhibit different adsorption and elution properties compared with the branched isomers.

Another particular analytical challenge is caused by NP itself. Due to the production process, technical grade NP comprises a complex mixture of isomers. The GC-MS analysis in **Figure 1** shows that the mixture consists mainly of branched para-isomers (>90%) with branched ortho-isomers (<10%) and traces of decylphenol. The para-isomers are further shown to comprise approximately ten distinct compounds. The NP ethoxylates normally display the same pattern of isomers as the technical grade NP they are derived from. One of the most prevalent isomers in technical grade NP is 4-(3,6-dimethyl-3-heptyl)phenol (363-NP), the structure and MS spectrum of which appear in **Figure 1**.

Table 1 Labeled octyl- and nonylphenol and associated ethoxylates from Sigma-Aldrich, solutions in acetone

Compound	Cat. No.	Cat. No.
¹³C₆-labelled:	1 mL, 10 µg/mL	10 mL, 1 µg/mL
4-tert-Octylphenol (Ring ¹³ C ₆)	33565	33566
4-tert-Octylphenol monoethoxylate (Ring ¹³ C ₆)	33563	33564
4-tert-Octylphenol diethoxylate (Ring ¹³ C ₆)	33229	33244
4-(3,6-Dimethyl-3-heptyl)-phenol (Ring ¹³ C ₆)	33574	33575
4-(3,6-Dimethyl-3-heptyl)-phenol monoethoxylate (Ring ¹³ C ₆)	33572	33573
4-(3,6-Dimethyl-3-heptyl)-phenol diethoxylate (Ring ¹³ C ₆)	33207	33222
Deuterated:		
4-tert-Octylphenol (Ring D ₂)	33557	33559
4-tert-Octylphenol monoethoxylate (Ring D ₂)	33523	33525
4-tert-Octylphenol diethoxylate (Ring D ₂)	33254	33257
4-(3,6-Dimethyl-3-heptyl)-phenol (Ring D ₂)	33569	33571
4-(3,6-Dimethyl-3-heptyl)-phenol monoethoxylate (Ring D ₂)	33567	33568
4-(3,6-Dimethyl-3-heptyl)-phenol diethoxylate (Ring D ₂)	33249	33252

Cat. No.	Brand	Description	Package Size
33623	Riedel	Alkylphenol Target Analyte Mix in Acetone	10 mL
33627	Riedel	Alkylphenol Internal Standard Mix in Acetone	10 mL
33629	Riedel	Alkylphenol Kit for DIN EN ISO 18857-2	1 Kit

Other products for alkylphenol and alkylphenol ethoxylate analysis

Cat. No.	Brand	Description	Package Size
442876	Supelco	Bisphenol A-d16	50 mg
394866	Aldrich	N-Methyl-N-(trimethylsilyl)trifluoroacetamide, derivatization grade (MSTFA)	10 x 1 mL, 5 mL, 25 mL

The mono- and diethoxylates of NP behave similarly, with 4-(3,6-dimethyl-3-heptyl)phenol-monoethoxylate (363-NP1EO) and 4-(3,6-dimethyl-3-heptyl)phenol-diethoxylate (363-NP2EO) being the major isomers. The high percentage and the favorable fragmentation pattern of these isomers make them ideal internal standards for the analysis of technical grade NP and its ethoxylate derivatives. ^{13}C -ring-labeled and deuterated 363-isomers of NP and its mono- and diethoxylates have been synthesized for this purpose.

The analysis of OP differs from the analysis of NP because it possesses only one significant isomer, 4-(1,1,3,3-tetramethylbutyl)phenol, often called 4-tert-octylphenol, and the resulting ethoxylates. Other isomers do not contribute so the synthesis of internal standards for OP and ethoxylates focuses only on the exact these compounds. **Figure 2** is a summary of all isotopically-labeled high-purity internal standards that have been synthesized for the analysis of NP and OP and their ethoxylates described above. The mass difference of 6 amu is ideal for GC-MS. For LC-MS/MS applications, twice-deuterated compounds are available.

Actually, in Germany the ISO 18857-2 for the analysis of OP, NP and their mono- and diethoxylates, including bisphenol A, in surface water is going to be worked out. The method utilizes solid phase extraction (SPE) and quantification by GC-MS after derivatization with MSTFA. Comprehensive studies, which included

interlaboratory proficiency testings, proved the suitability of the internal standards listed in **Figure 2** and resulted in precise and accurate measurements of the target analytes. As a result, all internal standards so listed were included in ISO 18857-2. For a final evaluation there will be an international interlaboratory proficiency testing in October/ November 2007 in which all interested laboratories are encouraged to participate (for information on participation please contact the authors).

Sigma-Aldrich is pleased to offer the complete range of labeled compounds listed in ISO 18857-2 for the analysis of OP, NP and their mono- and diethoxylates. The corresponding product numbers and concentrations are listed in **Table 1**.

Figure 1 GC-MS of technical grade nonylphenol and detailed mass spectrum of isomer 363-NP

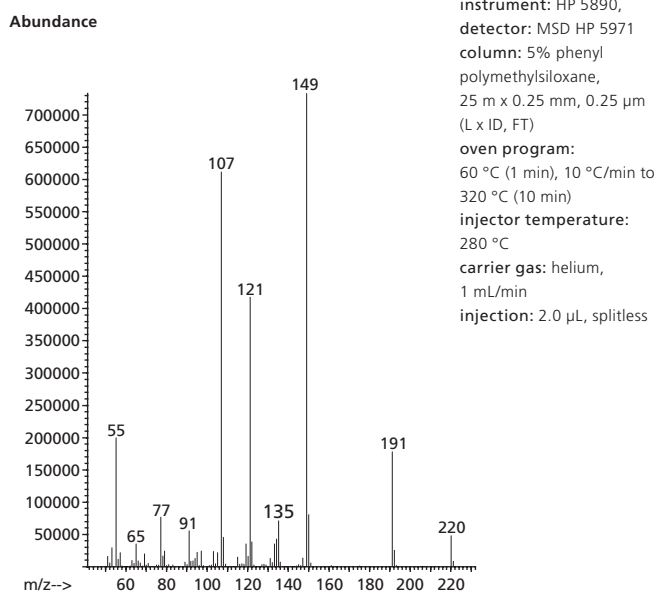
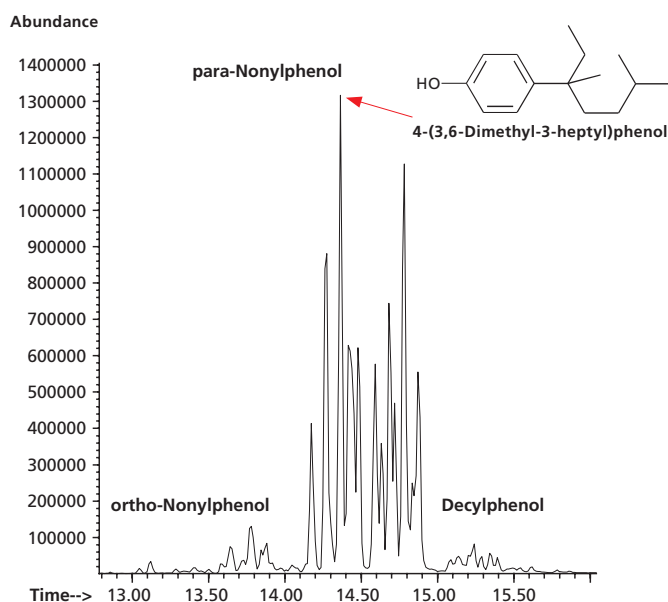
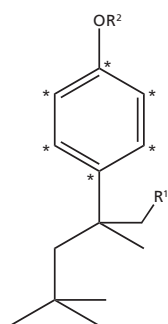


Figure 2 Molecular Structures of ^{13}C -labelled octyl- and nonylphenol and associated ethoxylates. * = ^{13}C



	R ¹	R ²
[$^{13}\text{C}_6$]-4-tert-OP	H	H
[$^{13}\text{C}_6$]-4-tert-OP1EO	H	$\text{CH}_2\text{H}_2\text{OH}$
[$^{13}\text{C}_6$]-4-tert-OP2EO	H	$\text{CH}_2\text{H}_2\text{OCH}_2\text{CH}_2\text{OH}$
[$^{13}\text{C}_6$]-363 NP	CH_3	H
[$^{13}\text{C}_6$]-363 NP1EO	CH_3	$\text{CH}_2\text{H}_2\text{OH}$
[$^{13}\text{C}_6$]-363 NP2EO	CH_3	$\text{CH}_2\text{H}_2\text{OCH}_2\text{CH}_2\text{OH}$

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TraceCERT™ – Traceable Certified Reference Materials. Reliability is a matter of proper uncertainty calculation

Part 5 – This is the fifth and final article in the series on Certified Reference Materials to appear in Analytix.

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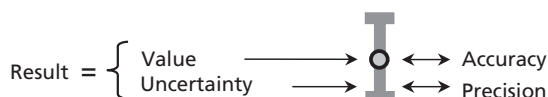


In addition to the concept of traceability as it applies to analytical chemistry, the concept of uncertainty is also poorly understood. It is not uncommon for chemists to hesitate when they are asked for the uncertainty budgets of their measurement values. Although there are published guidelines on how to deal with this issue [1–2], admittedly the calculation of uncertainty can be a real challenge. As is true for makers of fine watches, who must know how even the smallest component contributes to the overall mechanism, it is crucial for analysts to have a detailed understanding of the entire measurement process in order to calculate a true and reliable uncertainty budget. It is not possible to present in this short article a comprehensive discourse on the subject. However, we will use this space to provide some basic understanding and show how we calculate and report the uncertainty values for our TraceCERT™ reference materials.

Uncertainty vs. error

Confusion often arises because of the incorrect use of the terms uncertainty and error. Whereas error is not quantifiable, being a blunder or a mistake, uncertainty can be estimated or calculated and therefore expressed as an actual number. The uncertainty characterizes the variability that can reasonably be attributed to the measurand, the physical parameter being quantified by measurement. Consequently, each measurement result consists of two components: the *value* (predominantly the average of replicate measurements) and the *uncertainty* (the attributed variability), as shown in Figure 1.

Figure 1 A measurement value without an uncertainty budget is not a measurement result

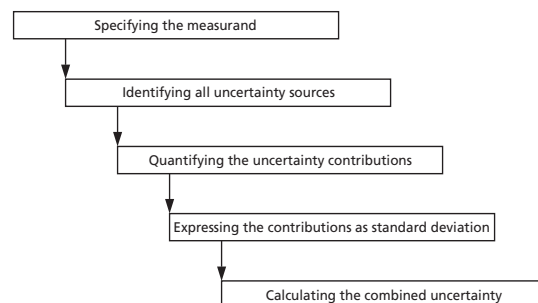


Calculating the uncertainty budget: Empiric vs. bottom-up

Generally, there are two approaches to obtain an uncertainty budget for a measurement: the empiric approach and the so-called “bottom-up” approach. The empiric approach is based on historical data, such as proficiency test performance (round robins), control charts or validation data. Hence, uncertainties calculated by the empiric approach are based on actual experience. The empiric approach is also called the top-down estimation. Typically it is not necessary to know in detail all of the influence parameters since the uncertainty estimation is based on the analysis of the whole process. In some cases the empiric approach may lead to meaningful results. However, because it is based on a snapshot in time, it cannot be assumed to be representative of future measurements.

A more sophisticated approach is the so-called “bottom-up” approach. Here, the uncertainty of a process or a measurement is calculated by summing all contributing influence parameters. Hence, all the details of the process must be identified and quantified individually (Figure 2). Obviously, the bottom-up approach is much more challenging and, in some cases, is not even possible due to lack of information. However, there is greater confidence in uncertainty calculations that use the bottom-up approach since by definition all sub-processes at the actual time of measurement are fully known and understood.

Figure 2 Step-by-step procedure of an uncertainty evaluation by the bottom-up approach



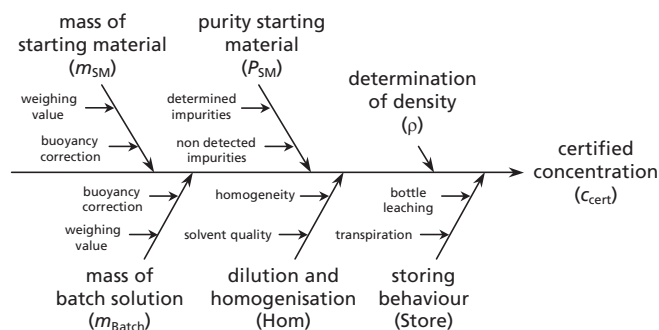
Combined uncertainty, u_c

After specification of the measurand and identification of the influence parameters, the individual influence parameters (contributors to the uncertainty budget) must be quantified. There are many different ways input data on the individual influence parameters of a process can be obtained. They might include min/max tolerance values from instrument specifications, nonlinearity data, any repeatabilities, and so forth. However, for these various data to be summed they must be first converted to standard deviation values (SD). Only SDs can be added by applying mathematical rules to get the combined uncertainty. Besides triangular or rectangular distributions, there are other methods to convert data into SDs [1]. After all influence parameters are converted to SDs, the combined uncertainty u_c can be calculated by applying the mathematical rules of uncertainty propagation. **Figure 3** shows the two basic algorithms. With the help of a cause-effect (Ishikawa or fishbone) diagram, like that shown in **Figure 4**, the influence parameters can be visualized to give an overview of the uncertainty determination. This tool is very helpful, especially when analyzing highly-complex processes.

Figure 3 Uncertainty propagation rules. In the case of additive parameters the absolute uncertainties are combined (square root of the sum of the squared contributions). In the case of multiplicative contributions the relative uncertainty contributions are combined

Additive parameters:	
$M = a + b$	$u_c(M) = \sqrt{u^2(a) + u^2(b)}$
Multiplicative parameters:	
$M = a \cdot b$	$\frac{u_c(M)}{M} = \sqrt{\left(\frac{u(a)}{a}\right)^2 + \left(\frac{u(b)}{b}\right)^2}$

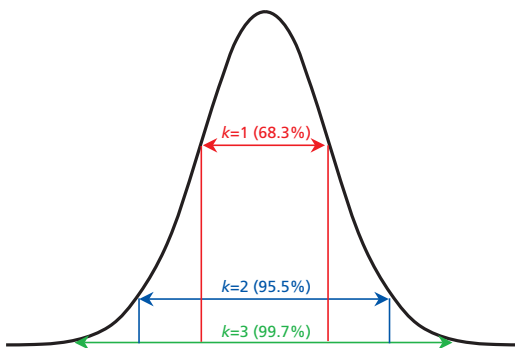
Figure 4 Cause effect diagram of the preparation procedure of TraceCERT™ reference materials. Only first and second order influence parameters are shown



Expanded uncertainty, U

Since the combined uncertainty, u_c , is a single standard deviation, the associated confidence level is only 68% (based on a Gaussian distribution, **Figure 5**). However, because a reported uncertainty value is meaningful only when the underlying level of confidence is adequate, it is quite common to expand u_c with an expansion factor, k , equaling 2. Assuming that a Gaussian distribution is fulfilled, the reporting of a double SD ($k=2$) leads to a confidence level of 95%. It is therefore important avoid at all costs citing an uncer-

Figure 5 Gaussian distribution showing the increase in confidence level with increasing expansion factor, k (number of standard deviation units)



tainty value based on a single SD since it can lead to misunderstandings and overly-optimistic uncertainty values. Instead, it is recommended to report the expanded uncertainty, U , which is equal to $k \cdot u_c$, where k is the number of standard deviation units (expansion factor).

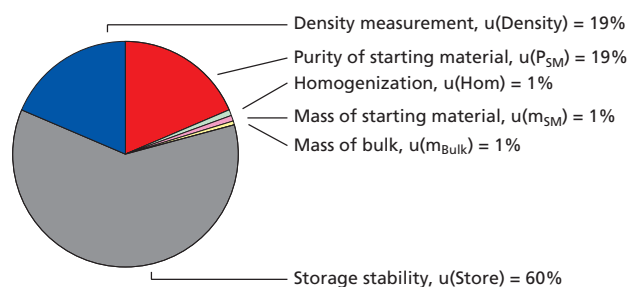
Evaluation of TraceCERT™ CRM uncertainties

In the previous four installments of this series of technical articles on TraceCERT™ reference materials, we discussed the manufacturing, analysis and storage effects [3]. Without going into mathematical details, in this article we want to give an overview of the key influence parameters to the uncertainty calculation of TraceCERT™ CRMs and discuss their relevance to the overall uncertainty budget. In **Figure 4**, the identified key influence parameters are shown as a cause-effect diagram. Each of the primary parameters (vertical arrows) is composed of a set of secondary parameters (horizontal arrows). For clarity, we have shown only the major effects. It should be noted that most of the secondary influence parameters comprise multiple minor influence parameters (tertiary parameters). For example, the secondary influence parameter “weighing value” (left upper corner in **Figure 4**) is made up of the following contributions: repeatability of weighing, readability, nonlinearity and eccentric load of balance, uncertainties of the calibration weights and temperature coefficient. This is a good example of how the bottom-up approach for uncertainty calculation is only possible with a deep understanding of the details of the measurement process.

Of course not all of the influence parameters are of equal importance in terms of their contribution to the overall uncertainty budget. Only when the whole uncertainty calculation is done can the relevance of the individual parameters be analyzed. **Figure 6** shows the primary influence parameters of the uncertainty budget of a TraceCERT™ Ultra, a CRM for ICP calibration, including the storage effects. By including the storage

effects into the uncertainty budgets of all *TraceCERT™* products, we can guarantee that the certified value is not only within specification at the time of production, but also when the bottle is first opened by the end-user. Two major contributions will be explained in more detail in the next section: the uncertainty calculation of the starting material purity statement and the storage effect contribution.

Figure 6 Partitioning of the uncertainty budget of *TraceCERT™* Ultra reference materials relative to the total uncertainty (expanded uncertainty usually is 0.2% relative to the certified value)



Uncertainties of starting material purity and storage effects

We discussed in a previous article of this series how the “100% minus sum of impurities” approach is the best method for the characterization of materials with high purity (>99.8%). In this case, the found trace impurities and an estimated contribution from the non-found impurities (below detection limit, DL) are subtracted from 100%. These two different impurities (real found and below DL) are also treated in separate ways in terms of their contribution to the uncertainty budget. For the real found impurities, an individual uncertainty contribution is considered for each element. For trace analysis with ICP-OES or ICP-MS, these contributions are typically in the range of several percent relative to the found value. For all the unfound impurities (below DL) a contribution of half of the DL is applied to the uncertainty budget.

The storage effects arise primarily from the loss of solvent by transpiration through the container wall [3]. Hence, the associated uncertainty contribution of this effect depends to a great extent on the container and the packaging material (aluminized bag vs. “naked” bottle). Long-term studies have yielded maximum transpiration rates for each individual type of bottle/packaging under various storage conditions and solvent systems. These data are considered in the uncertainty budget, and also are used to calculate the expiry date and the maximum storage temperature for the product.

We hope that this series of technical articles gave you an inside look into the production, testing and certification of our *TraceCERT™* reference materials. Since buying an analytical standard is a matter of trust, it is our intention to bring as much transparency as possible to our customers. As a consequence of the continuous improvement in our production of certified reference materials and our extensive experience in the CRM field, our laboratories will soon be accredited by the Swiss Accreditation Service according to both EN-ISO/IEC 17025 (general requirements for the competence of testing and calibration laboratories) and also ISO Guide 34 (general requirements for the competence of reference material producers). We look forward to supplying your need for CRMs, in terms of application, composition and quality, long into the future.

To view the entire *TraceCERT™* line, please visit our web site www.sigmaaldrich.com/tracecert

References

- 1] Quantifying uncertainty in analytical measurement, Eurachem/CITAC Guide, second edition, 2000.
- 2] Guide to the expression of uncertainty in measurement (GUM), ISO, Geneva, corrected edition, 1995.
- 3] *TraceCERT™* – Traceable Certified Reference Materials. Part 1: Analytix, Vol. 5, 2006 and Part 2–4: Analytix, Vol. 1–3, 2007, available at www.sigmaaldrich.com/analytix

Chiral HPLC Separations

Comparing normal and reversed-phase modes with various ion sources

Rudolf Köhling, R&D Chemist, LC/MS Applications rudolf.koehling@sial.com

Chiral chromatography requires chiral stationary phases (CSPs) with the power to resolve enantiomers, molecules that are non-superimposable mirror images that differ only in their molecular symmetry and, in most cases, their bioactivity. The powerful combination of chiral HPLC, both normal and reversed-phase modes, with MS detection offers both specificity and sensitivity. Sigma-Aldrich's analytical brands, Supelco and Fluka, offer chiral HPLC columns¹ and high-purity LC-MS **CHROMASOLV**[®] solvents and additives suitable for the most sensitive chiral LC-MS methods.

Normal phase with MS detection

Although reversed-phase is the dominant separation mode in HPLC, normal phase has three primary application areas: chiral HPLC separations, analysis of poorly water soluble compounds and preparative LC, the latter because normal phase solvents are easily removed from the purified fractions. Conventional normal phase mobile phases comprise binary or ternary mixtures of non-polar solvents with polar modifiers; hexane or heptane with aliphatic alcohols or ethylacetate are common examples.

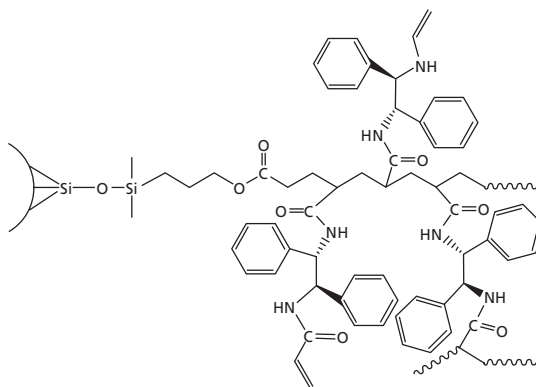
A significant drawback to using normal phase HPLC with MS detection is that normal phase solvents are typically not compatible with electrospray ion sources (ESI). High organic solvent flows prevent the ionization of dissolved solute molecules, as the solvent itself does not contain charge carriers like protons. However, other ion sources, like APCI (atmospheric pressure chemical ionization) and APPI (atmospheric pressure photoionization), do overcome this problem and produce detectable amounts of analyte ions under normal phase conditions. Importantly, APPI exhibits higher sensitivity when compared with all other MS sources.

Normal phase CSPs for LC-MS

The separation of warfarin enantiomers is an example of a normal phase chiral HPLC application with MS detection. For this analysis, we employed an Astec P-CAP-DPTM column with APPI detection. P-CAP-DP, based on the unique polycyclic amine polymer shown in **Figure 1**, was designed to separate a wide range of analytes under normal phase conditions.

A very versatile column, P-CAP-DP permits a wider choice of eluants, additives, flow rates and temperatures compared to many other CSPs. It is also available in two configurations, R,R and S,S, providing a predictable means of easily reversing elution order. Exceptional stationary phase stability, which is a result of covalent bonding of the chiral selector, makes it ideal for use with MS detection.

Figure 1 Molecular structure of Astec P-CAP-DP CSP, with covalent attachment to the underlying silica surface



Choosing the ion source

Figure 2 shows the chromatograms of a racemic warfarin solution analyzed under normal phase conditions and two different ion sources: APCI and APPI. The APPI source (Syagen PhotoMate APPI source for Bruker/Agilent MS) gave the highest sensitivity, followed by APCI. Warfarin gave no ESI signal under these conditions. The APPI source outperforms the other sources since it uses photoionization that provides the benefit of selective analyte ionization. However, it is important to realize that very small amounts of impurities like DMSO or TEA in solvents, additives or dopants can dramatically suppress ionization. Solvents and additives in LC-MS **CHROMASOLV**[®] quality guarantee the high purity necessary for this application.

¹ Sigma-Aldrich products for chiral chromatography include Astec CSPs for HPLC, and chiral GC phases under both Supelco and Astec brands.

Optimizing the normal phase LC-MS separation

The use of additives in the mobile phase, like formic acid, acetic acid, or ammonium acetate, can positively influence peak shape and retention. In the case of the

warfarin separation on the P-CAP-DP column, **Figure 3** shows that by adding formic acid to the heptane and replacing the ethanol with 2-propanol, resolution is significantly improved compared to the conditions in **Figure 2**.

Figure 2 Normal phase chiral HPLC separation of warfarin enantiomers on an Astec P-CAP DP column with APCI and APPI detection

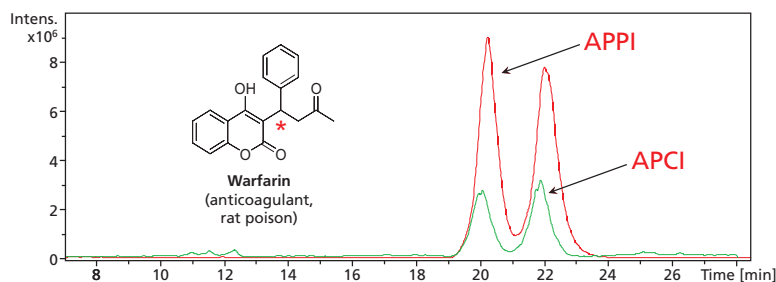
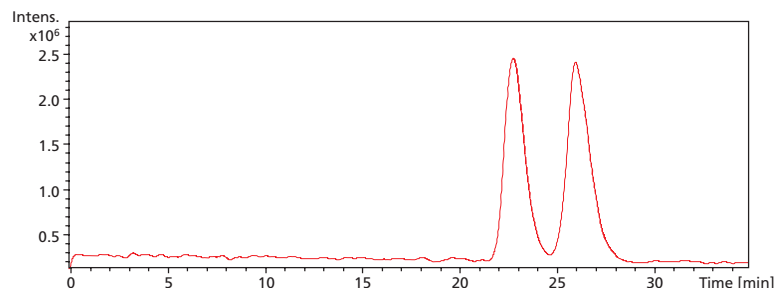


Figure 3 Chiral separation of warfarin enantiomers on an Astec P-CAP DP column with optimized conditions for APPI



HPLC Conditions:

column: Astec (R,R) P-CAP DP, 25 cm x 4.6 mm I.D., 5 μ m particles (35024AST)
mobile phase: 0.1% acetic acid in 95:5, n-heptane:ethanol
flow rate: 0.8 mL/min.
temp.: 50 $^{\circ}$ C
det.: APPI (dry gas flow: 4 L/min; dry gas temp.: 350 $^{\circ}$ C; nebulizer press.: 50 psi; vaporizer temp.: 450 $^{\circ}$ C), APCI (same as APPI), ESI (nebulizer press.: 30 psi; dry gas: 8 L/min; dry gas temp.: 350 $^{\circ}$ C)
(trace not shown) injection: 5 μ L
sample: racemic warfarin (Sigma A2250), 1 mg/mL in mobile phase

HPLC Conditions:

column: Astec (R,R) P-CAP DP, 25 cm x 4.6 mm I.D., 5 μ m particles (35024AST)
mobile phase: 92:8, n-heptane with 0.1% formic acid:2-propanol
flow rate: 1.0 mL/min.
temp.: 55 $^{\circ}$ C
det.: APPI (dry gas flow: 4 L/min; dry gas temp.: 350 $^{\circ}$ C; nebulizer press.: 50 psi, vaporizer temp.: 450 $^{\circ}$ C)
injection: 5 μ L
sample: racemic warfarin (Sigma A2250), 1 mg/mL in mobile phase

Reversed-phase CSPs for LC-MS

Macrocyclic antibiotics, like vancomycin, are used as CSPs for HPLC in reversed-phase mode, in addition to polar and normal phase modes. The Astec **CHIROBIOTIC™ V** phase is one of three macrocyclic CSPs that feature a large number of side chains with differing chemistries and can therefore provide multiple types of interactions, including strong ionic interactions (**Figure 4**). The mobile phase solvents, pH and

ionic strength all strongly affect retention by changing the interactions between the analyte and the various functional groups on the vancomycin molecule. The other retention modes that are possible with the macrocyclic CSPs depend on the solvent combination used. For example, polar ionic mode (PIM) comes into play with mobile phases of methanol containing triethylamine and acetic acid, or volatile salts, such as ammonium acetate.

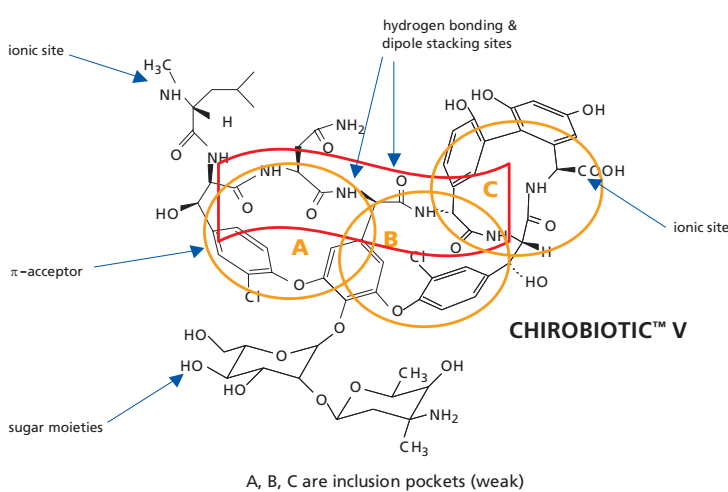


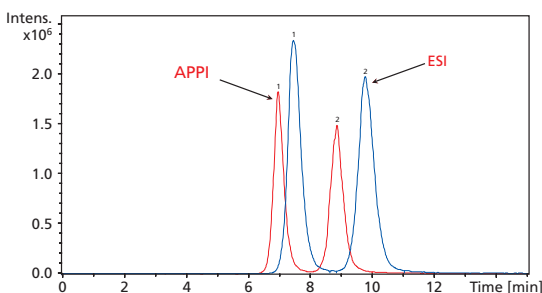
Figure 4 Molecular structure of vancomycin, the chiral selector of CHIROBIOTIC™ V, showing the different types of interactions that are possible

The aqueous and polar organic solvent systems with ionic additives used for reversed-phase HPLC are also suitable for ESI detection. **Figure 5** compares APPI and ESI for the warfarin separation, this time under reversed-phase conditions. As expected, ESI gave

greater sensitivity than APPI. However, the difference between these two ion sources is not as great as was observed under normal phase conditions.

Comparing the two approaches, the reversed-phase method gave the best separation of the warfarin

Figure 5 Chromatograms of the chiral separation of warfarin with APPI and ESI on an Astec CHIROBIOTIC™ V column



HPLC Conditions:

column: CHIROBIOTIC V, 15 cm x 2.1 mm I.D., 5 µm particles (11019AST)
 mobile phase: 80:20, water with 5 mM ammonium acetate (pH 4): acetonitrile
 flow rate: 0.4 mL/min.
 temp.: 15 °C
 det.: or ESI (MS conditions same as in Fig.2)
 injection: 5 µL
 sample: racemic warfarin (Sigma A2250), 1 mg/mL in mobile phase

enantiomers, with baseline resolution and symmetrical peaks. The shorter run time is also amenable to processing more samples per unit time; a key consideration in clinical and forensic toxicology, and other high-throughput applications. Both ion sources (APPI and ESI) are sensitive enough to reach LLODs in the ppb range down to 1 ng/mL in blood plasma (ESI, negative ion mode, after SPE) [1]. To maximize sensitivity, it

is strongly recommended to use solvents and additives specially tested for LC-MS, like the **CHROMASOLV®** and puriss p.a. LC-MS product line from Sigma-Aldrich. For a complete listing of our chiral HPLC columns, please visit the web site www.sigma-aldrich.com/astec. All LC-MS **CHROMASOLV®** solvents, additives and blends can be accessed at www.sigma-aldrich.com/chromasolv

Table 1 Selection of chiral HPLC columns featured in this article (see www.sigma-aldrich.com/astec for the complete offering. Other column sizes and guard columns also available)

Cat. No.	Brand	Description
35024AST	Supelco	Astec (R,R) P-CAP™ DP column (25 cm x 4.6 mm, 5 µm particles)
37024AST	Supelco	Astec (S,S) P-CAP™ DP column (25 cm x 4.6 mm, 5 µm particles)
11019AST	Supelco	Astec CHIROBIOTIC™ V column (15 cm x 2.1 mm, 5 µm particles)

Table 2 **CHROMASOLV®** mobile phase solvents and additives used in this article (see www.sigma-aldrich.com/chromasolv for the entire list)

Cat. No.	Brand	Description	Package Size
34972	Riedel-de Haën	Ethyl acetate, LC-MS CHROMASOLV®	1 L, 2.5 L
34859	Riedel-de Haën	n-Hexane CHROMASOLV®	1 L, 2.5 L, 7 L, 45 L
34873	Riedel-de Haën	n-Heptane CHROMASOLV®	1 L, 2.5 L, 7 L, 45 L
39253	Riedel-de Haën	Water, LC-MS CHROMASOLV®	1 L
34967	Riedel-de Haën	Acetonitrile, LC-MS CHROMASOLV®	1 L, 2.5 L
34966	Riedel-de Haën	Methanol, LC-MS CHROMASOLV®	1 L, 2.5 L
34965	Riedel-de Haën	2-Propanol, LC-MS CHROMASOLV®	1 L, 2.5 L
56302	Fluka	Formic acid, puriss p.a., eluent additive for LC-MS	10 x 1 mL, 50 mL
49199	Fluka	Acetic acid, puriss p.a., eluent additive for LC-MS	50 mL
49638	Fluka	Ammonium acetate, puriss p.a., eluent additive for LC-MS	50 g

Reference

1] Čápková, V.; Viccarone, S.; Carter, S.; Nguyen, N.; Merkle, S. *JASMS* 2007, 18 (5), S26–S49.

NMR Reference Standards

Suitable for instrument qualification and performance verification of modern NMR instruments

Lisa Roth, Technical Marketing Manager lisa.roth@sial.com

Over the last decade, the utility of Nuclear Magnetic Resonance (NMR) spectroscopy has expanded. Traditionally, NMR spectroscopy was primarily a qualitative method used for structural identification. With the introduction of high field NMR instruments (600 MHz and higher), NMR is increasingly used in quantitative applications with sensitivities into the parts per billion range. These applications include binding studies for drug screening, metabolite identification, cGMP release testing and trace level analysis of impurities, such as residual solvents. At the same time, analytical laboratories have been required to demonstrate tighter instrument controls as customer expectations related to quality systems have increased. ISO-9001 standards and cGMP equipment validation require the spectroscopist to demonstrate consistent and reliable instrument performance. This has resulted in a need to use NMR reference standards to ensure proper instrument performance in quantitative applications.

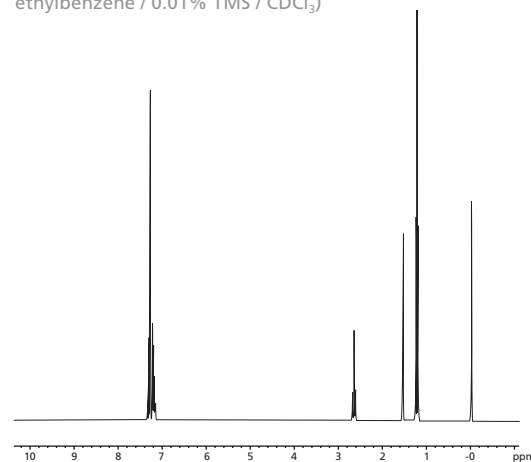
Quality assurance of NMR instrument performance typically includes methods for Installation Qualification (IQ) and Operational Qualification (OQ), which Aldrich has supported through the sale of its NMR reagents and standards. However, Performance Qualification (PQ), which allows the user to routinely verify their instrument performance, is essential for meaningful quantitative results. All three methods ensure instrument performance at every stage, from initial installation and operational functionality in the selected environment to reliable performance during routine use. Aldrich is a well-established supplier of standards and reagents to the NMR community. We also offer a wide range of NMR reference standards needed to verify instrument performance. These standards allow the user to monitor key operational parameters such as pw90, sensitivity, resolution and line shape (see **Table 1**).

Sensitivity and pw90

The ^1H sensitivity standard (0.1% ethylbenzene / 0.01% TMS / CDCl_3 , **Table 1**) is widely used by the NMR community to evaluate the signal-to-noise ratio (SNR) in the 3 to 7 ppm range for a variety of NMR instruments (see **Figure 1**). To accurately assess the SNR and, therefore, instrument performance, spurious peaks from impurities must be avoided. Hence, the use of high purity reagents and solvents is important. This standard is also recommended to determine the 90-degree pulse width (pw90), an essential parameter for any NMR experiment. For ^{13}C sensitivity, the preferred Aldrich

standard among spectroscopists is 40% p-dioxane/benzene- d_6 (see **Table 1**). It is also used for ^{13}C line shape verification. Keep in mind that certain aspects of system suitability such as line shape and sensitivity are also affected by precision. This is directly related to sample preparation and accuracy, which is associated with self-consistency among the integrated signals within the molecule¹. Using an Aldrich NMR reference standard eliminates the sample preparation uncertainty when evaluating instrument performance. The Stable Isotope Group of Aldrich has a long history of supplying NMR reference standards for instrument qualification. We have the high quality materials, meticulous preparation and QC procedures in place to provide the user with reliable, high quality standards.

Figure 1 Aldrich Proton (^1H) Sensitivity Standard (0.1% ethylbenzene / 0.01% TMS / CDCl_3)

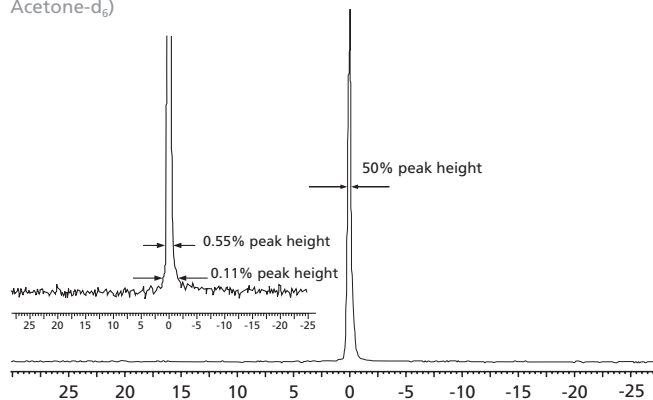


Line Shape

Another essential parameter for the NMR spectroscopist is magnetic field homogeneity, which is indicated by the NMR signal line shape. The ^1H Line Shape Standard (1% or 5% Chloroform / Acetone- d_6 , **Table 1**) is used to demonstrate that the NMR instrument meets the manufacturer's line shape specifications. Optimized shimming of the NMR instrument is required to achieve the designated values. The line width is measured at three peak heights: 50%, 0.55%, and 0.11% (see **Figure 2**) and the measured peak widths are compared to the manufacturer's specifications to either initially qualify the instrument or verify its performance. As mentioned earlier, Aldrich also provides NMR reference standards to evaluate the ^{13}C signal line shape (40% p-Dioxane / Benzene- d_6 , **Table 1**). It is used in a manner similar to the proton line shape standard to verify instru-

ment performance. In routine use, when a line shape does not meet the user's expectations, this may be due to field inhomogeneity or a resonance from a possible impurity overlapping with the analyte. Verifying the performance with a reusable NMR reference standard removes these variables and allows the spectroscopist to compare the results with historical instrument performance.

Figure 2 Proton (^1H) Line Shape Standard (1% Chloroform / Acetone- d_6)



Resolution:

Resolution directly affects the peak separation and hence is associated with sensitivity and line broadening issues. For ^1H NMR, resolution is checked using 1% 1,2-Dichlorobenzene / Acetone- d_6 (see Table 1). Peak sharpness and resolution are important for low-level analyses especially when a mixture is being tested.

Aldrich is committed to providing the highest quality NMR standards and reagents, and maintaining its leadership position as a trusted name in the NMR community. We have the combined

knowledge and expertise to supply the standards required to ensure proper instrumental qualification and performance verification. Looking forward, we have the passion and resources to continually expand our product offering. Although quantitative NMR is usually associated with ^1H NMR, applications continue to grow in ^{19}F NMR and ^{31}P NMR. The Stable Isotope Group of Aldrich also provides NMR reference standards for Fluorine Sensitivity and Phosphorus Sensitivity verification and other applications (see Table 2). The NMR Reference Standards are also available in several tube sizes from 3 mm x 8" to 10mm x 8". NMR continues to find new and exciting applications to solve today's analytical challenges. Count on Aldrich to provide the relevant and high-quality reagents necessary to ensure reliable NMR analyses. Besides reference standards, Aldrich offers many other product lines for NMR, including:

- LC-NMR **CHROMASOLV**[®] solvents
- Deuterated NMR solvents (Chloroform- d , Methanol- d_4 , Deuteriumoxide, Dimethyl sulfoxide- d_6)
- Labeled products for synthesis
- Derivatization reagents
- NMR tubes, cleaners and accessories (Aldrich, Wilmad, Norell, Shigemi brands)
- ^{13}C and ^1H FT-NMR Libraries with Spectral Viewer in Text and CD-ROM versions
- Reference books and software

For more information, visit our Web site: www.sigma-aldrich.com/isotec. To request a quotation or technical information, please email us at isosales@sial.com

Table 1 NMR Reference Standards for monitoring key operational parameters (pw90, sensitivity, resolution and line shape)

Cat. No.	Brand	Description	Standard Type
487104	ALDRICH	NMR Std. 0.1% Ethylbenzene / 0.01% TMS / CDCl_3	^1H Sensitivity Standard
487163	ALDRICH	NMR Std. 1% Chloroform / Acetone- d_6	^1H Line Shape Standard
487759	ALDRICH	NMR Std. 5% Chloroform / Acetone- d_6	^1H Line Shape Standard
611905	ALDRICH	NMR Std. 40% p-Dioxane / 5mg/ml $\text{Cr}(\text{acac})_3$ / Benzene- d_6	^{13}C pw90, Sensitivity Standard
551368	ALDRICH	NMR Std. 40% p-Dioxane / Benzene- d_6	^{13}C pw90, Sensitivity Standard
487147	ALDRICH	1% 1,2-Dichlorobenzene / Acetone- d_6	^1H Resolution Standard

Table 2 NMR Reference Standards for fluorine and phosphorus sensitivity verification

Cat. No.	Brand	Description	Standard Type
612685	ALDRICH	NMR Std. 0.05% Trifluorotoluene / Benzene- d_6	^{19}F Sensitivity Standard
551392	ALDRICH	NMR Std. 0.0485M Triphenyl phosphate / CDCl_3	^{31}P Sensitivity Standard
611891	ALDRICH	NMR Std. 45% Formamide / $\text{DMSO-}d_6$	^{15}N Sensitivity Standard
551384	ALDRICH	NMR Std. 90% Formamide / $\text{DMSO-}d_6$	^{15}N Sensitivity Standard
487139	ALDRICH	NMR Std. 0.1 mg/ml Gadolinium (III) Chloride / D_2O	Auto Test
551406	ALDRICH	NMR Std. 0.05% α,α,α - Trifluorotoluene / Benzene- d_6	^{19}F Sensitivity Standard

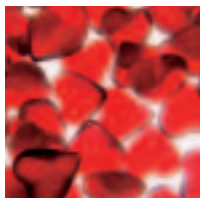
Reference

- 1] Maniara, G.; Rajamoorthi, K.; Rajan, S.; Stockton, G. W. Method performance and Validation for Quantitative Analysis by ^1H and ^{31}P NMR Spectroscopy. Applications to Analytical Standards and Agricultural Chemicals. *Anal. Chem.*, 1998, 70(23), 4921–49280.

Enzymatic Assay Kits for Nutrients

Quantitative enzymatic determination of sucrose, glucose, fructose, starch and total dietary fiber in food and other materials

Andrea Felgner, Product Manager Analytical Reagents andrea.felgner@sial.com



Enzymes have found widespread use as analytical tools in food, biochemical and pharmaceutical industries. Because they provide specific, reproducible, sensitive and rapid measurements, they are ideal for analytical purposes. As a result, diverse enzymatic methods have been implemented in national food guidelines and standard procedures in many countries. A representative list appears at the end of this article. The high specificity and sensitivity of enzyme assays permit quantitative analysis on crude materials with little or no sample preparation.

The Sigma-Aldrich range of food-related enzymatic test kits allows for quantitative determination of sucrose, glucose, fructose, starch and total dietary fiber in food and other materials (Table 1). These kits utilize enzymatic and/or gravimetric methods in combination with photometric detection. Their ease-of-use belies their high sensitivity and specificity.

A wide variety of substances can be synthesized or broken down by enzyme-catalyzed reactions. There are three major properties that influence the catalytic activity of enzymes: temperature, pH-value and substrate concentration.

Influence of temperature, pH-value and substrate concentration

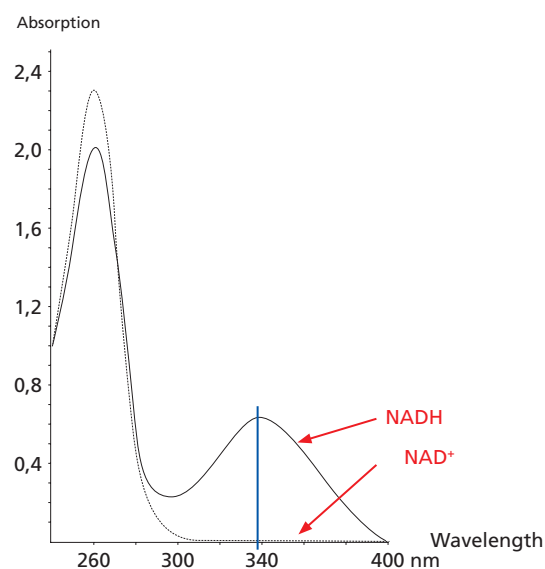
Every enzyme has specific optimum temperature, pH range and substrate concentration where the enzyme has the highest catalytic activity. Decreasing temperature from the optimum leads to slower reaction rates. An increase in temperature can lead to an increase in the enzymatic reaction rates, but at the same time may enhance denaturation since enzymes are proteins. Working at pH values outside the enzyme's optimum range reduces its substrate binding ability due to changes in its ionic state. The substrate concentration is also specific for each enzyme since the enzyme may be inhibited by excess substrate concentration.

Detection of enzymatic reactions

The fundamental basis for the use of enzymes as analytical tools is measuring the concentration changes that occur during the enzymatic reaction. Reactants (substrates) are consumed, products are generated and their concentration changes can be measured, typically via photometric detection, and related stoichiometrically to calculate the initial concentration of the compound of interest in the reaction.

A commonly-used substrate-product system for this purpose is nicotinamide adenine dinucleotide (NAD⁺), an important coenzyme in humans, and its reduced form, NADH. As shown in Figure 1, these substances show different absorption curves. NADH has an absorption maximum at 340 nm, while the oxidized form NAD⁺ does not absorb at this wavelength.

Figure 1 Absorption spectra of NAD⁺ and NADH [1]



Activity of enzymes that use the NAD⁺/NADH system can be measured by monitoring changes in absorbance at 340 nm. Correspondingly, using the stoichiometry of the reaction, the concentration of substrate (analyte) in this system can be measured using the Beer-Lambert relationship:

$$\log_{10} (I_0/I) = \epsilon d c$$

where	I_0	incident radiation
	I	transmitted radiation
	ϵ	molar absorption coefficient
	d	distance of light path
	c	concentration

With constant d and ϵ , the concentration of the substance can thus be calculated by the change in transmission at the target wavelength. For accurate results when analyzing complex samples, it is important to run blanks to account for interference from matrix constituents, and even solvents and the cuvette.

Equipment and sample preparation

Most food samples must be diluted with deionized water in order to obtain a quantifiable concentration of analyte prior to analysis. Additional sample pretreatment that may be required includes filtration, centrifugation, deproteinization, Carrez clarification, decolorization, degassing and pH adjustment. Detailed descriptions of these procedures are included in the instructions that accompany each Enzymatic Assay Kit.

The kits include the necessary enzyme reagents and standards, when appropriate. For some kits, additional reagents may be required as listed in **Table 2**. Optimum storage temperature for all kits listed here is 2–8 °C. It is important to follow the accompanying instructions carefully regarding pH, temperature and sample dilution; these parameters are critical to ensure the proper functioning of the kits and reliable results.

Almost all of the Enzymatic Assay kits require the use of UV or Vis photometers. Kits utilizing the NADH/NAD⁺ system require measuring at 340 nm using a UV photometer, while those using the o-dianisidine system require a wavelength of 540 nm and therefore a Vis photometer. For glucose and starch determinations, we offer kits for both UV and Vis detection.

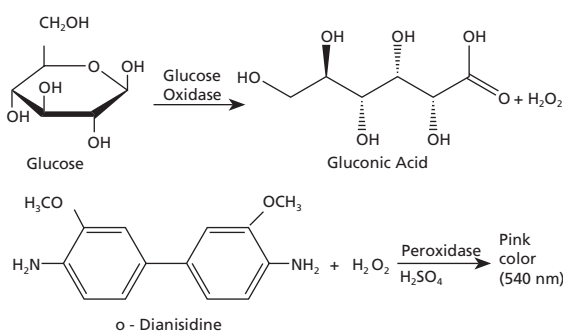
Glucose Assay Kits

The kits for the enzymatic assay of sucrose, fructose and starch are based on the enzymatic determination of glucose. The first step is the catabolism of the carbohydrate to glucose. The second step is the measurement of the glucose (according to the two different methods described for the Glucose GO and HK Kits) and stoichiometric back calculation of the concentration of the target carbohydrate in the original sample.

Glucose (GO) Assay Kit (via glucose oxidase)

Glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with o-dianisidine in the presence of peroxidase to form a colored product. Oxidized o-dianisidine reacts with sulfuric acid to form a more stable colored product. The absorption of the mixture measured at 540 nm is proportional to the original glucose concentration (**Figure 2**).

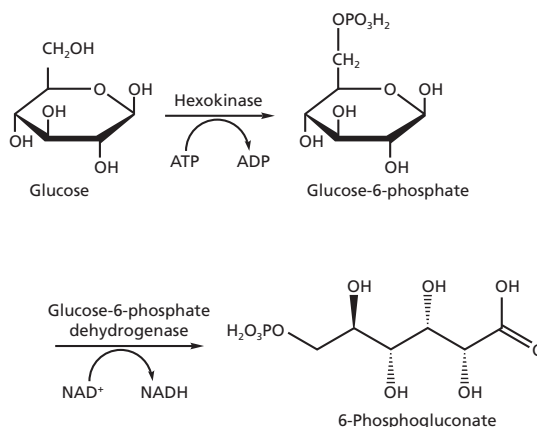
Figure 2 Oxidation of Glucose via Glucose Oxidase



Glucose (HK) Assay Kit (via hexokinase)

Glucose is phosphorylated by ATP in a reaction catalyzed by hexokinase. The glucose-6-phosphate is then oxidized to 6-phosphogluconate in the presence of oxidized NAD⁺ in a reaction catalyzed by glucose-6-phosphate dehydrogenase. During this oxidation, an equimolar amount of NAD⁺ is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to the original glucose concentration in the sample (**Figure 3**).

Figure 3 Oxidation of Glucose via Glucose Oxidase



The different catabolic routes of our two Glucose Kits lead to products with different detection wavelengths, one in the UV wavelength region (Sigma GAHK20: analyte NADH/NAD⁺, wavelength 340 nm), the other in visible wavelength region (Sigma GAGO20: analyte o-dianisidine, wavelength 540 nm).

Sucrose Assay Kit

Sucrose is hydrolyzed to glucose and fructose by invertase. Glucose and fructose are phosphorylated by ATP in a reaction catalyzed by hexokinase. Glucose-6-phosphate is then converted to 6-phosphogluconate via glucose-6-phosphate dehydrogenase with corresponding reduction of NAD⁺ to NADH. The increase in absorbance at 340 nm from NADH formation is proportional to the original sucrose concentration.

Fructose Assay Kit

Fructose is phosphorylated by ATP in a reaction catalyzed by hexokinase. The resulting fructose-6-phosphate is then converted to glucose-6-phosphate by phosphoglucose isomerase. Glucose-6-phosphate is converted to 6-phosphogluconate (**Figure 3**) and the consequent increase in absorbance at 340 nm through NADH formation is proportional to the original fructose concentration.



Starch Assay Kits: Starch (GO/P) Assay Kit

The hydrolysis of starch to glucose is catalyzed by α -amylase and amyloglucosidase. Glucose is then converted to gluconic acid by glucose oxidase (Figure 2) and the intensity of the pink color measured at 540 nm is proportional to the original starch concentration.

Starch (HK) Assay Kit

The hydrolysis of starch to glucose is catalyzed by amyloglucosidase. Glucose is phosphorylated by ATP in a reaction catalyzed by hexokinase. Glucose-6-phosphate is converted to 6-phosphogluconate (Figure 3) and the subsequent increase in absorbance at 340 nm through NADH formation is proportional to the original starch concentration.

Total Dietary Fiber Assay Kit

Dietary fiber is a mixture of complex organic substances and was initially defined as remnants of plant cells resistant to hydrolysis by the alimentary enzymes of man [1]. This definition was later modified to include hemicelluloses, celluloses, lignins, pectins, gums, nondigestible oligosaccharides, and waxes [2, 3]. This broader

definition acknowledges the significance of fiber as a chemical and physiological component of the diet.

The total dietary fiber content of food is determined with our Dietary Fiber Kit by a combination of enzymatic and gravimetric methods. Samples of dried, fat-free foods are gelatinized with heat-stable-amylase and then enzymatically digested with protease and amyloglucosidase to remove protein and starch in the sample. Ethanol is added to precipitate the soluble dietary fiber. The residue is then filtered and washed with ethanol and acetone. After drying, the residue is weighed. Half of the sample is analyzed for protein and the other half is ashed. Total dietary fiber equals weight of residue minus weight of protein and ash.

Our Assay Kit for total dietary fiber (Sigma TDF100A) contains reagents sufficient to perform 100 assays; the additional control kit (Sigma TDFC10) contains the necessary standards for internal control, sufficient for approximately ten assays.

Additional information and instruction bulletins to all our enzymatic assay kits are available on our web site www.sigma-aldrich.com



Official Methods (examples)

ICUMSA GS 2-4 (2007): Glucose and Fructose in White Sugar

ICUMSA GS 8/4/6-4 (2007): Determination of Glucose and Fructose in Beet Juices/Processing Products

AOAC 969.39: Glucose in Corn Syrups and Dextrose Products

AOAC 985.09: Glucose and Fructose in Wine

AOAC 985.29: Total Dietary Fiber in Foods

SLMB 305: Different sugars in ice cream

SLMB 467: Starch and starch decomposition products in special foods

SLMB 468: Dietary fibers in special foods

Table 1 Sigma Enzymatic Assay Kits for determination of sucrose, glucose, fructose, starch and dietary fiber

Cat. No.	Brand	Description	Standard Type
SCA20	Sigma	Sucrose Assay Kit	Sufficient for ~20 assays
GAGO20	Sigma	Glucose (GO) Assay Kit	Sufficient for ~20 assays
GAHK20	Sigma	Glucose (HK) Assay Kit	Sufficient for ~20 assays
FA20	Sigma	Fructose Assay Kit	Sufficient for ~20 assays
STA20	Sigma	Starch (GO/P) Assay Kit	Sufficient for ~20 assays
SA20	Sigma	Starch (HK) Assay Kit	Sufficient for ~20 assays
TDF100A	Sigma	Total Dietary Fiber Assay Kit	Sufficient for ~100 assays
TDFC10	Sigma	Total Dietary Fiber Assay Control Kit	Sufficient for ≥ 10 assays

Table 2 Additional required reagent

Cat. No.	Brand	Product	Required for Kit Number
258105	Sigma	Sulfuric acid, ACS reagent	GAGO20, STA20
154938	Sigma	Dimethyl sulfoxide, ACS reagent	STA20
459844	Sigma	Ethyl alcohol, ACS reagent	STA20, TDF100A
184519	Sigma	Petroleum ether, ACS reagent	TDF100A
320110	Sigma	Acetone, ACS reagent	TDF100A
S0876	Sigma	Sodium phosphate dibasic, anhydrous	TDF100A
S0751	Sigma	Sodium phosphate monobasic, anhydrous	TDF100A
S2567	Sigma	Sodium hydroxide, 1.0 M	TDF100A
H3162	Sigma	Hydrochloric acid, 1.0 M	TDF100A

References

- 1] Trowell, H. Definitions of Fibre. *The Lancet* **1974**, 303 (7856), 503.
- 2] Trowell, H., Southgate, D. A.; Wolever, T. M. S.; Leeds, A. R., Gassull, M. A.; Jenkins, D. A. Dietary Fibre Redefined. *The Lancet*, **1976**, 307 (7966), 967.
- 3] Van Soest, P.J. and McQueen, R.W. The chemistry and estimation of fiber. *Proc. Nutr. Soc.* **1973**, 32, 123–130.
- 4] Official Methods of Analysis of AOAC International, 16th Edition, Volume II, Section 45.4.07, Method 985.29 (1997).
- 5] Matissek, R.; Schnepel, F.; Steiner, G. *Lebensmittelanalytik*. 2nd Edition (1992), p. 397.

HYDRANAL®-Water Standard

New **HYDRANAL®**-Standard sodium tartrate dihydrate, tested against NIST SRM 2890, and New **HYDRANAL®**-Water Standard KF Oven 140–160°C

Helga Hoffmann, Technical Service **HYDRANAL®** Manager helga.hoffmann@sial.com
Andrea Felgner, Product Manager Analytical Reagents andrea.felgner@sial.com



Calibration, validation and control of analytical instruments and reagents by using suitable standards are essential requirements in order to meet ISO, GMP, GLP and FDA guidelines. Traceability to a national standard or to a SI unit is often required in these guidelines. In the case of Karl Fischer titrations, the most widely used method for water determination, a known amount of water is required. Pure water can be used, but the amounts needed (10 mg for volumetry and 100 µg - 1 mg for coulometry) are difficult to weigh.

We recommend **HYDRANAL®** standards, which have precisely confirmed water content, for the following applications:

- Titer determination
- Monitoring precision and accuracy
- Validation and inspection of KF titrators according to ISO 9000, GMP, GLP and FDA guidelines

Figure 1 **HYDRANAL®**-Water Standards



Three of our liquid **HYDRANAL®**-Water Standards (**Figure 1**), one for volumetry and two for coulometry, as well as one of our solid **HYDRANAL®**-Standards are tested against the NIST standard reference material SRM 2890, Water Saturated Octanol. NIST stands for the National Institute of Standards and Technology of the USA.

New! **HYDRANAL®**-Standard sodium tartrate dihydrate, tested against NIST SRM 2890

The new **HYDRANAL®**-Standard sodium tartrate dihydrate is a primary standard for volumetric Karl Fischer titration. It has a water content of $15.66 \pm 0.05\%$, is tested against NIST SRM 2890 and is presented with a Certificate of Analysis. This standard remains stable under normal laboratory conditions and does not lose or adsorb moisture. It comes in a finely powdered form that dissolves relatively quickly in methanol, although its solubility is limited. This is the primary standard for Karl Fischer applications.

New! **HYDRANAL®**-Water Standard KF Oven 140–160°C

This is a solid standard for the control of Karl Fischer ovens using coulometric and volumetric water determination. It contains 5.0% chemically combined water. The exact water content of each lot is given in the enclosed Certificate of Analysis. This standard may be used to verify the results of a Karl Fischer oven system between 140 and 160°C.

Liquid **HYDRANAL®**-Water Standards

Liquid **HYDRANAL®**-Water Standards can be used for the titer determination of volumetric Karl Fischer reagents, to control coulometric Karl Fischer instruments and, most importantly, for the quality control of Karl Fischer instruments in general as it relates to ISO 9001 and other standard procedures.

HYDRANAL®-Water Standards consist of solvent mixtures with specific composition and precisely determined water content. The exact amount of water can be found on the Certificate of Analysis that is enclosed in each product package. **HYDRANAL®**-Water Standards are filled under Argon in 4 mL or 8 mL glass ampuls. The humidity-proof packaging allows for a minimum storage of five years. Each box contains 10 ampuls.

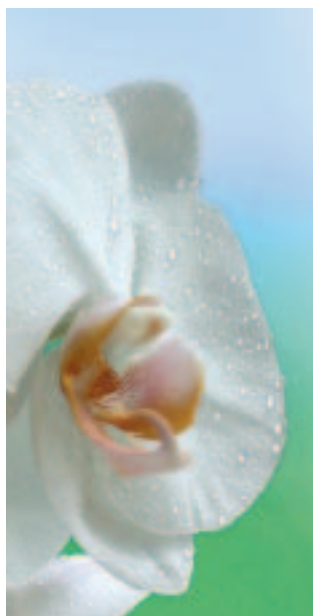
Table 1 HYDRANAL®-Water Standards

Cat. No.	Brand	Product	Description	Package Size
Solid Standards				
34696 NEW	Riedel-de Haën	HYDRANAL ®-Standard Sodium tartrate dihydrate	Primary standard for volumetric KF titration Water content indicated on Certificate of Analysis Tested against NIST SRM 2890	25 g
34803	Riedel-de Haën	HYDRANAL ®-Standard Sodium tartrate dihydrate	Primary standard for volumetric KF titration Water content: 15.66%	100 g
34693 NEW	Riedel-de Haën	HYDRANAL ®-Water Standard KF Oven 140–160°C	Solid standard for control of KF Ovens Water content: 5.0%	10 g
34748	Riedel-de Haën	HYDRANAL ®-Water Standard KF Oven 230°C	Solid standard for control of KF Ovens Water content: 5.55%	10 g
Liquid Standards				
34849	Riedel-de Haën	HYDRANAL ®-Water Standard 10.0	Standard for volumetric KF titration 1 g (1ml) contains 10.0 mg = 1.00% water at 20 °C, Contains 10 glass ampuls of 8 mL, Tested against NIST SRM 2890	80 mL
34828	Riedel-de Haën	HYDRANAL ®-Water Standard 1.00	Standard for coulometric KF titration 1 g (1ml) contains 1.00 mg = 0.10% water at 20 °C, Contains 10 glass ampuls of 4 mL, Tested against NIST SRM 2890	40 mL
34847	Riedel-de Haën	HYDRANAL ®-Water Standard 0.10	Standard for coulometric KF titration 1 g contains 0.10 mg = 0.01% water Contains 10 glass ampuls of 4 mL Tested against NIST SRM 2890	40 mL
34813	Riedel-de Haën	HYDRANAL ®-Standard 5.00	Standard for titer determination of volumetric reagents, Water content: 5.00 ± 0.02 mg/mL at 20 °C	100 mL 500 mL
34802	Riedel-de Haën	HYDRANAL ®-Water-in-methanol-Standard 5.00	Standard for volumetric back titration according to Karl Fischer Water content: 5.00 ± 0.02 mg/mL	1 L

Technical Service

We are happy to provide you with support for the analysis of your samples using our twenty years of experience with Karl Fischer titration. We can suggest a solution to your analytical problem and, if necessary,

develop an individual analytical method for you. Our comprehensive application collection makes daily work easier for **HYDRANAL**® users.



Please find more information about water determination according to Karl Fischer at www.sigma-aldrich.com/hydranal. For answers to all your KF titration questions, please contact our **HYDRANAL**® specialists:

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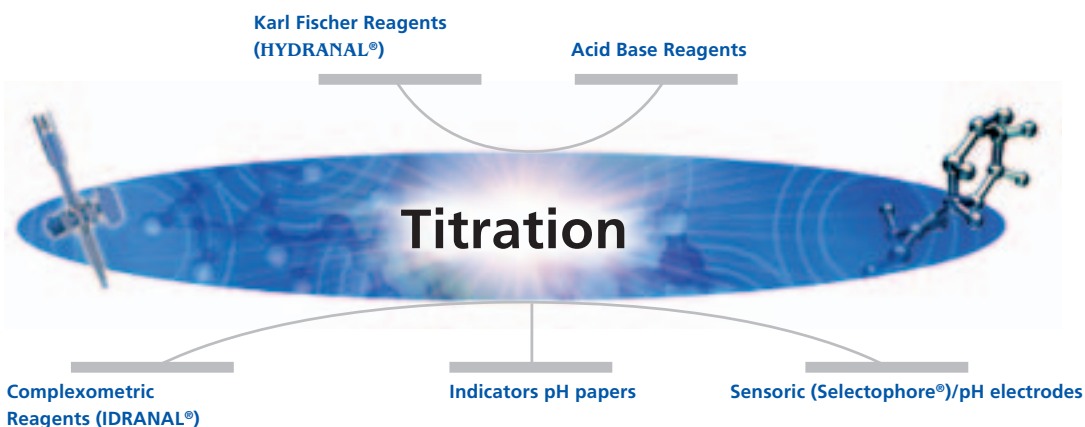
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Today's analytical laboratories typically face a six-prong challenge of increasing sample load, demand for accuracy, pressure for quick turn-around on results, compliance with evolving regulations, lower and lower analyte levels, and constraints on budget and staffing. Our product design and development are aimed at helping analysts cope with and successfully face these challenges.

The Sigma-Aldrich analytical product range includes a wide array of ready-to-use solutions for the many different types for titration, including: acidimetric/alkalimetric, redox, argentometric and complexometric titrations, as well as acid, base, buffer and salt solutions.

Figure 2 Ready-to-use solutions



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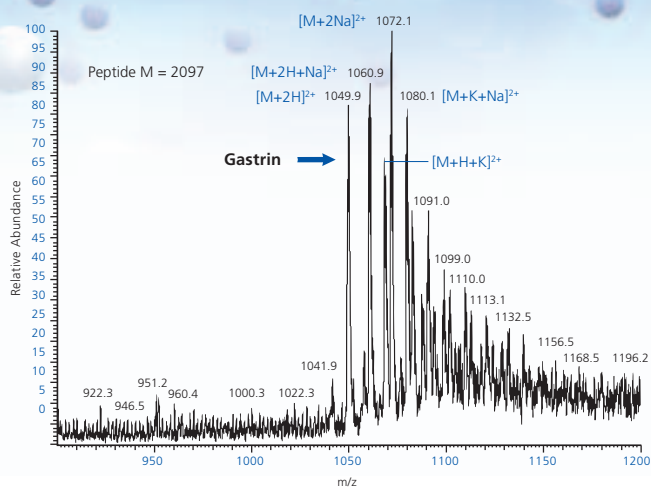
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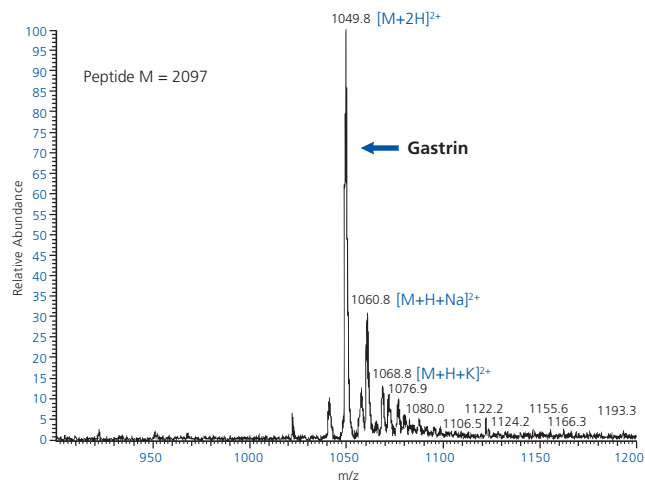
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New Product Corner

New DNPH and DAIH Standards for Air Monitoring

Nicole Amann, Product Manager Analytical Standards nicole.amann@sial.com

Sigma-Aldrich is pleased to offer new standards for air monitoring. The four DNPH derivatives of long-chain aldehydes complete the comprehensive Supelco brand portfolio of air monitoring standards. With DAIH derivatives of seven aldehydes and ketones routinely monitored in work environment safety Fluka offers a new group of versatile standards for LC [1].

Table 1 NEW Fluka brand 2,4-dinitrophenylhydrazone standards of long-chain aldehydes

Part No.	Brand	Description	Package Size
33848	Fluka	Heptanal 2,4-dinitrophenylhydrazone	100 mg
33849	Fluka	Octanal 2,4-dinitrophenylhydrazone	100 mg
33851	Fluka	Nonanal 2,4-dinitrophenylhydrazone	100 mg
33852	Fluka	Decanal 2,4-dinitrophenylhydrazone	100 mg

Table 2 NEW Fluka brand 2-diphenylacetyl-1,3-indandion-1-hydrazone standards of aldehydes and ketones routinely monitored in work environment safety

Part No.	Brand	Description	Package Size
02819	Fluka	Acetone, DAIH derivative	50 mg
06947	Fluka	Formaldehyde, DAIH derivative	50 mg
13173	Fluka	Acrolein, DAIH derivative	50 mg
14423	Fluka	Acetaldehyde, DAIH derivative	50 mg
51299	Fluka	Propionaldehyde, DAIH derivative	50 mg
55556	Fluka	Crotonaldehyde, DAIH derivative	50 mg
91547	Fluka	Cyclohexanone, DAIH derivative	50 mg

Reference

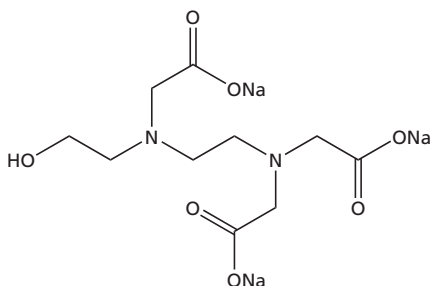
1] Possanzini, M.; di Palo, V. (1997): Determination of Formaldehyde and Acetaldehyde in Air by HPLC with Fluorescence Detection, *Chromatographia*, 46, 235.

IDRANAL® VII, HEDTA-Na₃

Andrea Felgner, Product Manager Analytical Reagents andrea.felgner@sial.com

In complexometric titrations, metals react with complexing agents (chelating agents) to form soluble complexes. A large group of complexing agents is represented by the aminopolycarboxylic acids, which Sigma-Aldrich markets for different applications under the IDRANAL® brand.

Figure 1 IDRANAL® VII, HEDTA-Na₃



New IDRANAL® VII, hydroxyethylethylenediaminetriacetic acid trisodium salt (HEDTA-Na₃), is a chelating agent particularly useful for controlling Fe³⁺ ions and well-suited for use in acidic formulations.

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Find more information about our IDRANAL® product range, please visit www.sigma-aldrich.com/idranal

Cat. No	Brand	Description	Package Size
34461 NEW	Riedel-de Haën	IDRANAL® VII, ≥99.0% (calc. on anhydrous substance)	100 g, 6x100 g

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IYU 9.2007/64