

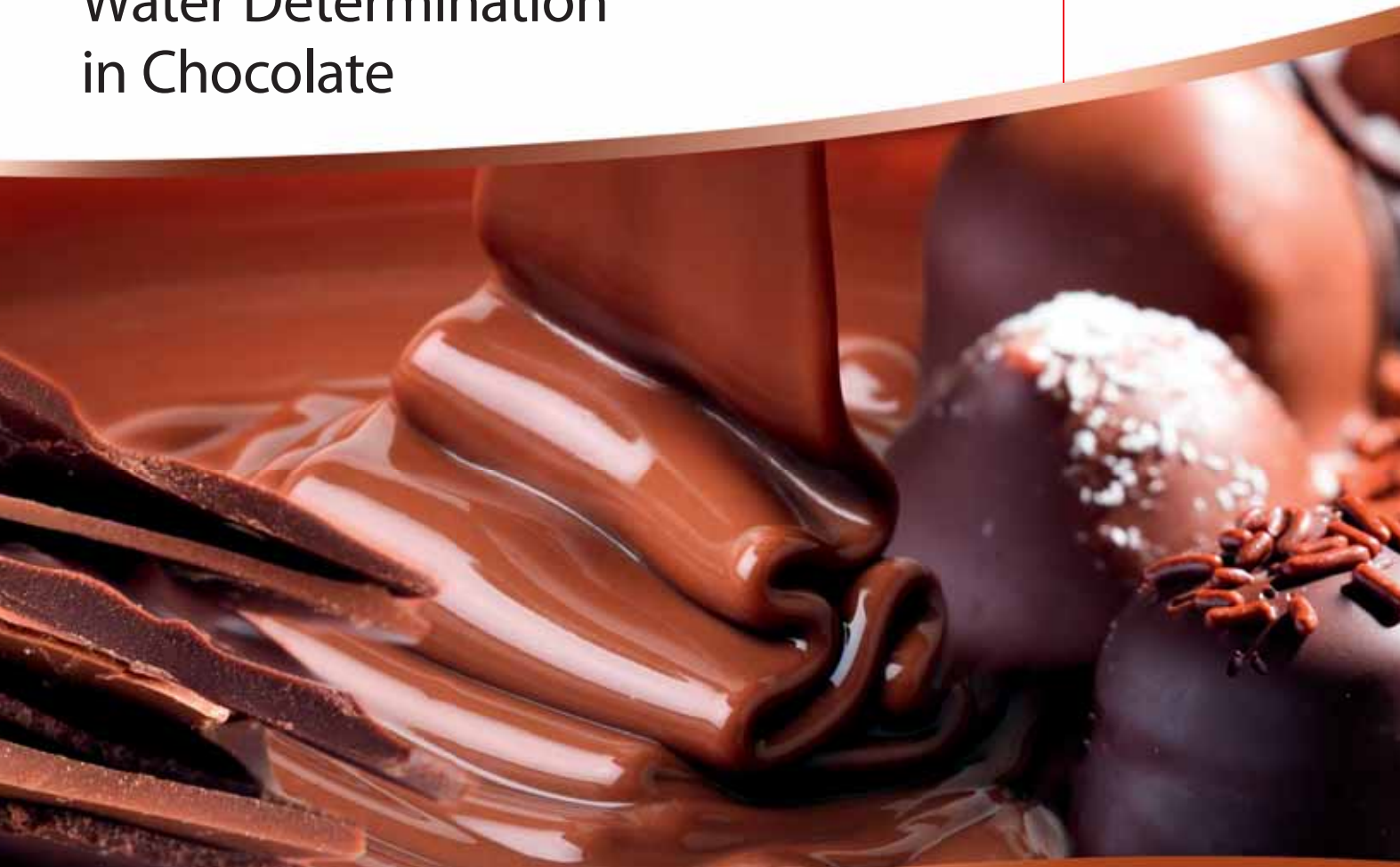
# Analytix

Issue 2 • 2010

 **Fluka**  
Analytical

Riedel-deHaën®

## Water Determination in Chocolate



- Antibiotic-resistant bacteria
- CRMs for qNMR
- Enantiomeric Purities of Amino Acid
- Activated Silylating Reagents
- Volumetric Titration Reagents
- Packaging Services for RMs

## Celebrating 30 years HYDRANAL® reagents for Karl Fischer titration



Helga Hoffmann  
Manager Technical Service  
HYDRANAL

### Dear Colleague,

The significance of water extends far beyond what is suggested by its simple formula: H<sub>2</sub>O. It is central to sustaining life and essential for human existence. However, in many industrial and technical applications, water must be eliminated or regulated to very specific levels, for example in chemicals used in the electronics industry. In other fields, such as pharmacology, optimum water content is desired as specified in pharmacopoeias. The ingenuity of the chemist in refining various applications results in a broad range of water specifications.

With similar ingenuity, petrochemist Karl Fischer devised a successful measure of water content based on the Bunsen equation, publishing his discovery in 1935. The acceptance of the Karl Fischer method experienced a slow but extensive expansion throughout the scientific community around the world. In spite of this growth, the wide base of users looked for improvements to the slow reaction rate and elimination or replacement of pyridine due to its undesirable properties.

As we at former Riedel-de Haën actively began to pursue solutions to these issues, Dr. Eugen Scholz, my former superior, assigned me to develop together with him a procedure that did not involve pyridine. The quest began to create pyridine-free reagents.

In 1980, the HYDRANAL family of pyridine-free reagents was introduced and gained wide popularity as its users enjoyed benefits such as increased speed of water content determination and the elimination of the unpleasant pyridine. These developments have consequently extended the applicability of the HYDRANAL product line to an increasingly diverse range. The advantages of HYDRANAL reagents continue to be leveraged as Sigma-Aldrich® expands relevant formulations of this product line.

Presently, the HYDRANAL product line consists of more than 50 reagents, specialities and standards – expertly designed to provide effective determination of water content in a wide array of samples. The range of detection extends from 100% to just a few ppm in combination with suitable titrators.

In 2010, the goal of the HYDRANAL Technical Service group is the further development of advanced HYDRANAL reagents that are subjected to rigorous quality assurance measures and are supported by an applications and technology knowledge base that gives value-added benefit to our customers.

With kind regards,

*Helga Hoffmann*

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## Water Determination in Chocolate and Cocoa

Karl Fischer titration with HYDRANAL® reagents

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Cocoa and chocolate have a fascinating history. Pre-Columbian cultures in Latin America cultivated cacao trees over 3,000 years ago. The main use was a chocolate beverage called xocolatl. In Mayan and Aztec cultures the cacao beans were not only used for xocolatl, but also as a very valuable currency. In the early 16th century, Spanish conquerors brought the first cacao beans back in their ships, and chocolate began to conquer Europe.

The largest consumers of chocolate today are the western world countries, while the largest cacao-producing countries are Ivory Coast, Ghana and Indonesia. Nearly 75% of the world's cocoa crop comes from Africa [1]. **Figure 1** shows the chocolate confectionery consumption per country in kg per head (2007).

The tropical cacao tree, *Theobroma cacao*, probably originated in the northern part of South America, where it grows in a warm, moist climate. Evergreen, it flowers all year round, and it yields 20 to 50 ripe fruits per tree. The cacao beans are the seeds of these fruits. [3]

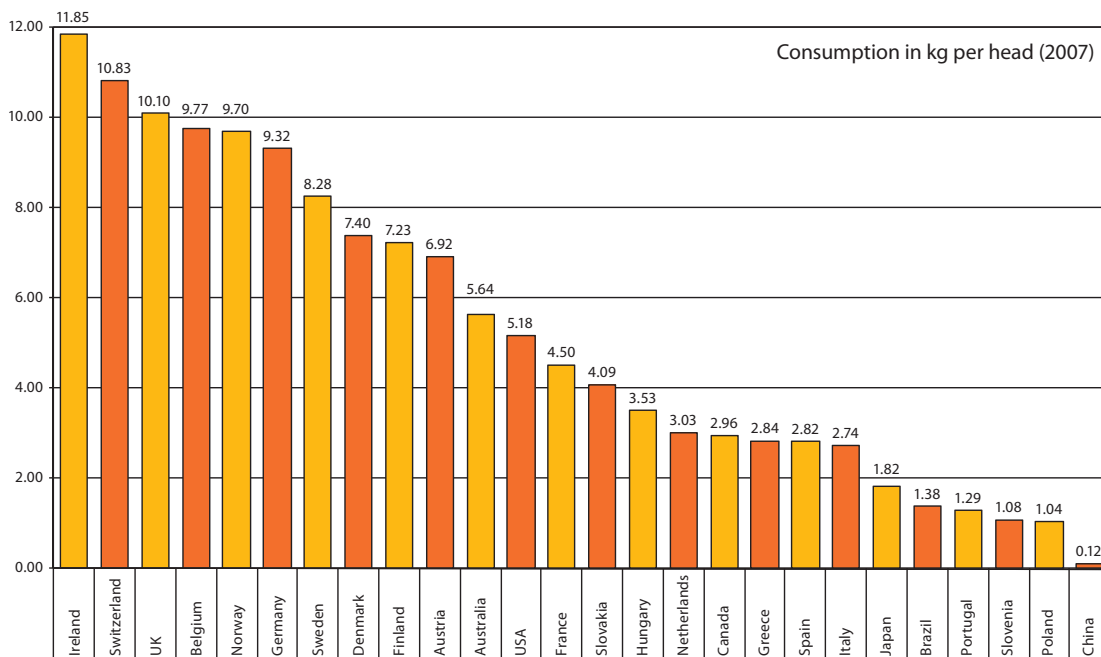
After the ripe cacao pods have been harvested, the cacao beans are removed from the pods and left to ferment for five to seven days. The fermentation process naturally removes all

remaining fruit pulp from the beans, while the beans change their colour and develop many aroma compounds. After fermentation, the beans are dried to stop the fermentation process and reduce the moisture content for storage.

The further processing steps leading to cocoa powder and chocolate are described in **Figure 2**. The beans' water content is a very important factor and should be 6 to 8%. If the water content is too high, microorganisms can infest the beans and their quality will suffer. After the beans have been roasted, the water content is further decreased to approximately 3%.

### Regulations

Water content is also an important parameter for chocolate and chocolate products and must be closely monitored. The Swiss Food Register states in method "1010.1 Determination of Water content of Cocoa and Cocoa Products, acc. to Karl Fischer" that a homogenous sample, if necessary finely grated or melted for two hours at  $40 \pm 2$  °C, can be titrated in a 1:1-mixture of methanol and chloroform as a Karl Fischer working medium. Also the AOAC International gives an official method for "Moisture in Cacao Products, by Karl Fischer method, 977.10".



**Figure 1** Ranking of consumption: chocolate confectionery 2007 [2]

Our HYDRANAL® laboratory has developed applications for chocolate and chocolate products, using volumetric KF titration techniques, which are described below.

#### Application: Water determination in chocolate

Chocolate and milk chocolate samples with high fat content need pre-treatment before the water determination can be carried out. Directly before the titration, the chocolate sample should be ground or grated. It should not be left exposed to ambient air once it is grated, otherwise its water content will change according to the room conditions. In order to dissolve the fat and finely disperse chocolate samples in the Karl Fischer working medium, the addition of chloroform to the working medium is recommended. Alternatively, the titration can be carried out at 50 °C. Recommended sample size is approximately 1 g. Before starting the titration and after sample addition, a stirring time of two to three minutes should be applied. Titration duration is about three minutes, following these procedures:

	Alternative 1	Alternative 2
Reagent	One-component technique	One-component technique
Titration agent	HYDRANAL-Composite 5	HYDRANAL-Composite 5
Working medium	HYDRANAL- Methanol Rapid/HYDRANAL- Methanol dry and HYDRANAL-Chloroform 1:1	HYDRANAL- LipoSolver CM
	Two-component technique	Two-component technique
Titration agent	HYDRANAL-Titrant 5	HYDRANAL-Titrant 5
Working medium	HYDRANAL-Solvent: HYDRANAL-Chloroform 1:1	HYDRANAL-Solvent CM

(Application reports L071 Chocolate, and L079 Milk chocolate)

#### Application: Water determination in chocolate truffles and pralines

Chocolate truffles and pralines are usually composed of different layers that also have different water content. It is therefore crucial to create a homogenous mass and take a representative sample. For example, a single praline can be quickly homogenised, using a mortar; afterwards, the sample should be stored in a tightly sealed container. Larger sample amounts can also be homogenised, using a blender.

Reagent	One-component technique
Titration agent	HYDRANAL-Composite 5
Working medium	HYDRANAL-Methanol dry : HYDRANAL-Chloroform : HYDRANAL-Formamide dry 2:1:1
	Two-component technique
Titration agent	HYDRANAL-Titrant 5
Working medium	HYDRANAL-Solvent : HYDRANAL-Chloroform : HYDRANAL-Formamide dry 2:1:1

(Application report L028 Chocolate truffles and pralines)

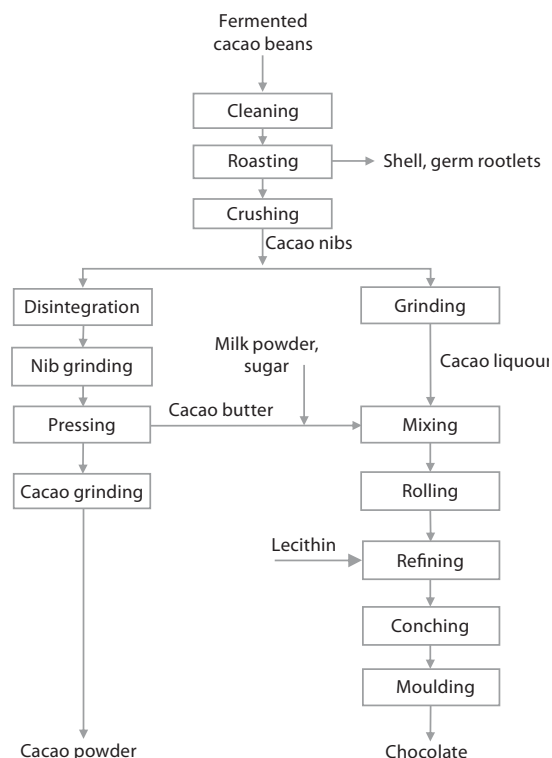


Figure 2 Production of chocolate and cocoa powder [3]

When methanol is used as the sole component of the working medium, the chocolate mass disperses very slowly. The addition of formamide for dissolving contained sugars, as well as chloroform for fat content, is highly recommended. Carrying out the titration at an elevated temperature of 50 °C also has a positive effect on the dissolving of the chocolate samples. The duration of the titration may vary approximately from 3 to 6 minutes, depending on the composition of the sample. In this application, a sample size of approximately 400 mg was used.

Cat. No.	Brand	Description	Pack Size
34805	Fluka	HYDRANAL-Composite 5	500 mL, 1 L, 2.5 L
37855	Fluka	HYDRANAL-LipoSolver CM	1 L
34741	Fluka	HYDRANAL-Methanol dry	1 L, 2.5 L
37817	Fluka	HYDRANAL-Methanol Rapid	1 L, 2.5 L
34724	Fluka	HYDRANAL-Formamide dry	1 L
37863	Fluka	HYDRANAL-Chloroform	1 L
34801	Fluka	HYDRANAL-Titrant 5	500 mL, 1 L, 2.5 L
34800	Fluka	HYDRANAL-Solvent	1 L, 2.5 L
34812	Fluka	HYDRANAL-Solvent CM	1 L, 2.5 L

Table 1 Selected HYDRANAL Karl Fischer reagents

#### References

- [1] ICCO – International Cocoa Organisation, [www.icco.org](http://www.icco.org)
- [2] CAOBISCO – Association of chocolate, biscuit and confectionery industries of the European Union, [www.caobisco.com](http://www.caobisco.com)
- [3] Belitz, Grosch, Schieberle - Food Chemistry, 4<sup>th</sup> rev. and ext. Edition, Springer-Verlag Berlin Heidelberg, 2009.

(continued on page 6)

Sigma-Aldrich® offers over 600 application reports. A full list can be found on our website [sigma-aldrich.com/hydranal](http://sigma-aldrich.com/hydranal)

To obtain an application report, please contact one of our HYDRANAL® laboratories:

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## HYDRANAL Karl Fischer Seminars 2010

Date		Location
March	9	Warsaw, Poland
	10	Budapest, Hungary
	18	Zofingen, Switzerland
April	12	Porto Alegre, Brazil
	13	Belo Horizonte, Brazil
	14	Rio de Janeiro, Brazil
	15	Rio de Janeiro, Brazil
	16	Sao Paulo, Brazil
	19	Monterrey, Mexico
	20	Monterrey, Mexico
May	20	Haan, Germany
	21	Guadalajara, Mexico
	22	Mexico City, Mexico
	23	Altamira, Mexico
	4	Bratislava, Slovakia
	5	Brno, Czech Republic
	6	Prague, Czech Republic

Date		Location	
June	1	Grobbendonk, Belgium	
	2	Gembloux, Belgium	
July	6	Ulm, Germany	
September	6	Helsinki, Finland	
	7	Turku, Finland	
	8	Stockholm, Sweden	
	9	Oslo, Norway	
	28	Firenze, Italy	
October	29	Roma, Italy	
	4	Göteborg, Sweden	
	5	Aarhus, Denmark	
	6	Kobenhavn, Denmark	
	7	Lund, Sweden	
	18	Guangzhou, China	
	20	Shanghai, China	
	22	Beijing, China	
	November	23/24	Seelze, Germany



## Celebrating 30 years of HYDRANAL reagents

Outstanding performance and excellent quality for Karl Fischer titration

- Improved reagents' safety, simplified use and handling
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- Extensive storage stability and extensive shelf life, no crystallisation of reagents (patented formulation)

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34734	Fluka	HYDRANAL-CompoSolver E
34732	Fluka	HYDRANAL-Titrant 5 E
34723	Fluka	HYDRANAL-Titrant 2 E
34730	Fluka	HYDRANAL-Solvent E
34726	Fluka	HYDRANAL-Coulomat E

## Staphylococcus aureus: a Spreading Bacteria

*Staphylococcus aureus* is frequently a part of our skin flora but is also a cause of a broad range of illnesses. Current studies report a remarkable increase of Methicillin Resistant *Staphylococcus aureus* (MRSA) over recent years.

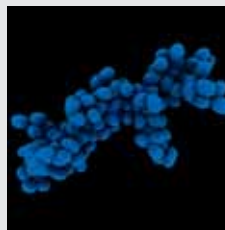
Jvo Siegrist, Product Manager Microbiology [ivo.siegrist@sial.com](mailto:ivo.siegrist@sial.com)

Staphylococci may be airborne and can occur in both animals and humans, in sewage, water, milk or food, and on environmental surfaces or food equipment. It is still one of the five most common causes of nosocomial infections, often causing postsurgical wound infections. Consequently, it poses a major concern in hospitals, especially in regard to MRSA, methicillin-resistant *Staphylococcus aureus*.

*Staphylococcus aureus* is an invasive pathogen that can cause disease in almost any tissue or organ in the human body, primarily in compromised individuals. Staphylococcal infections were treated using penicillin, but over the years this pathogen developed resistance to penicillin by building penicillinase. Methicillin was the next drug of choice as it is not cleaved by the penicillinase. While methicillin is very effective in treating most *Staphylococcus* infections, some strains have developed resistance to methicillin by production of penicillin-binding protein, and can no longer be killed by this antibiotic. These resistant bacteria are called Methicillin Resistant *Staphylococcus aureus* (MRSA) [1]. Patients with breaks in their skin due to wounds, indwelling catheters or burns are at high risk of developing MRSA infection [2]. Spread of MRSA infections can be controlled to a great extent by maintaining personal hygiene after interaction with an MRSA-infected person [1].

Today there are many innovative solutions to detect MRSA. Sigma-Aldrich® strongly supports the microbiologist with a selective chromogenic HiCrome MeReSa Agar for detection of MRSA from clinical isolates and other samples. The proprietary chromogenic mixture incorporated in the medium is specifically cleaved by *S. aureus* to give bluish-green colonies on this medium and can be clearly differentiated from other species. The medium is made selective for MRSA by the addition of methicillin.

### Did you know?



*Staphylococcus aureus* comes from the Greek, meaning golden (aureus) grapes (staphylé) ball (coccus).

Figure 1 *S. aureus*

Foods that are frequently associated with staphylococcal food poisoning include meat and egg products, milk and dairy products, and various other products that may contain these food ingredients. Processes in the food industry that are kept at slightly elevated temperatures must guard against staphylococcal food poisoning, one of the leading causes of gastroenteritis. The food poisoning is due to the presence of staphylococcal enterotoxins produced by *Staphylococcus aureus* in the food.

Staphylococci are facultative anaerobes Gram-positive bacteria that grow by aerobic respiration or by fermentation that yields principally lactic acid. The bacteria are catalase-positive and oxidase-negative. *S. aureus* produce diverse enzymes such as staphylokinase (coagulase), proteases, phosphatase, a lipase, a deoxyribonuclease (DNase) and a fatty acid modifying enzyme (FAME). The majority of clinical isolates of *S. aureus* express special surface polysaccharide and protein A. Differentiation and identification of *S. aureus* can be made based on these biochemical characteristics. More details may be found in the ID flow chart (Figure 2), the table of Fluka's kits and tests (Table 1), and the table listing Fluka's media for the differentiation of *Staphylococcus aureus* (Table 2).

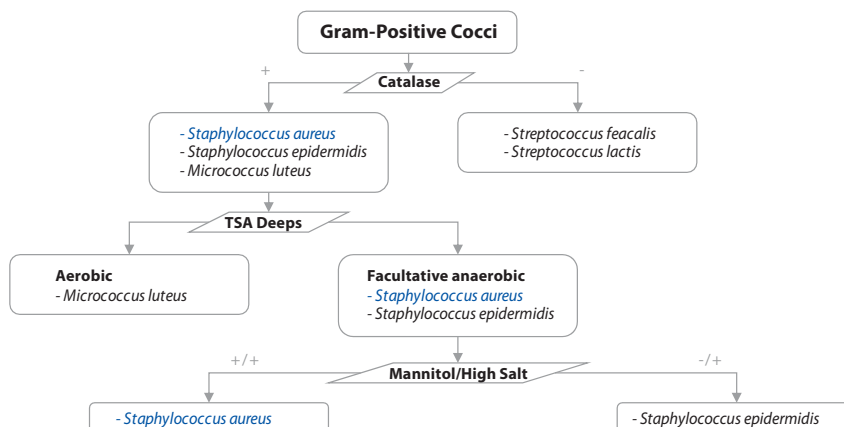


Figure 2 ID flow chart for *Staphylococcus aureus*

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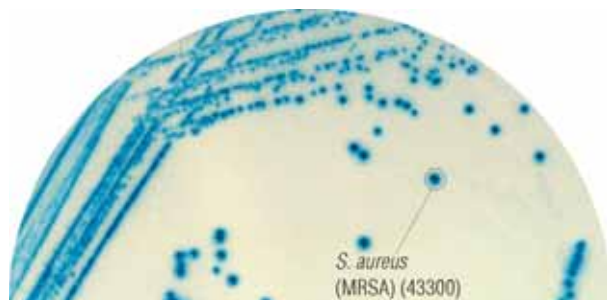
**Table 1** and **Table 2** specify characteristics which Fluka®'s kits and tests utilise for the identification of *S. aureus*.

Kit or Test	Cat. No.	Testing features
Catalase Test (Hydrogen peroxide 3%)	88597	Testing of catalase production
Coagulase Test (Slide)	75832	Detection of coagulase
Coagulase Test (Tubes)	74226	"
Oxidase Reagent acc. Gaby-Hadley A + Oxidase Reagent acc. Gaby-Hadley B	07345 + 07817	Checking presence of Oxidase
Oxidase Reagent acc. Gordon-McLeod	18502	"
Oxidase Strips	40560	"
Oxidase Test	70439	"
Staphylo Monotec test kit Plus	50448	Coagulase and protein A can be detected in one step (increased sensitivity and specificity compared to the previous Staphylo Monotec test kit, resulting in increased detection of MRSA)

**Table 1** Test for detection and identification of *Staphylococcus aureus*

Identification Media	Cat. No.	Testing features
<b>Baird Parker Agar</b> Supplements: Egg-Yolk Tellurite Emulsion (Fluka 75208) or RPF Supplement (Fluka 05939)	11705	Detection of lipolytic and proteolytic activity, ability to reduce tellurite to metallic tellurium (EN-ISO 6888-1: 1999); with RPF Supplement the coagulase activity and the ability to reduce tellurite is detected (EN-ISO 6888-2:2000)
<b>Blood Agar</b> Supplement: defibrinated blood	70133	Detection of $\beta$ -hemolysis
<b>Blood Agar No. 2</b> Supplement: defibrinated blood	B1676	"
<b>Bromo Thymol Blue (B.T.B.) Lactose Agar</b>	B3676	Differentiated by their ability to grow at a high pH and in the presence of bromo thymol blue (golden yellow colonies)
<b>CLED Agar</b>	55420	Detection of lactose fermentation
<b>Deoxyribonuclease Test Agar</b>	30787 70136	Detection of deoxyribonuclease activity
<b>DNase Test Agar with Toluidine Blue</b>	D2560	"
<b>Giolitti Cantoni Broth</b>	48905	Ability to reduce tellurite to tellurium and selective conditions
<b>HiCrome™ Aureus Agar Base</b> Supplement: Egg-Yolk Tellurite Emulsion (Fluka 75208)	05662	Testing for ability to reduce tellurite to metallic tellurium and detection of lipase and protease by chromogenic substrate; brown-black colonies
<b>HiCrome™ MeReSa Agar Base</b> Supplement: MRSA Selective Supplement (Fluka 51387)	90923	Detection by chromogenic substrate mixture specifically cleaved by <i>S. aureus</i> ; selective to MRSA; MRSA give bluish-green colonies
<b>China Blue Lactose Agar</b>	22520	Detection of lactose fermentation
<b>Mannitol Salt Agar</b>	63567 09166 (plattes)	Detection of mannitol fermentation in high sodium chloride concentration
<b>Nutrient Gelatin</b>	70151	Detection of gelatin-liquefying (proteolytic enzymes)
<b>Phenolphthalein Phosphate Agar</b>	68879	Phosphatase detection; pink-red colonies
<b>Spirit Blue Agar</b> Supplement: Lipase Substrate (see data sheet)	54306	Detection and enumeration of lipolytic activity
<b>Staphylococcus Agar</b>	70193	Detection of salt tolerance, pigmentation, D-mannitol utilisation and gelatin liquefaction
<b>Tributyryn Agar</b> Supplement: Neutral Tributyrin (Fluka 91010)	91015	Detection and enumeration of lipolytic activity
<b>Vogel-Johnson Agar</b> Supplements: Potassium Tellurite 1% (Fluka 17774)	70195	Checking for ability to reduce tellurite to tellurium and ability to ferment mannitol

**Table 2** Media for detection and identification of *Staphylococcus aureus*



**Figure 3** Chromogenic HiCrome™ MeReSa Agar Base



**Figure 4** Coagulase Test (Tubes)

#### References:

[1] Methicillin Resistant Staphylococcus aureus Copyright © 1997–2005 Canadian Centre for Occupational Health and Safety, Sept 19<sup>th</sup> (2005).

[2] Dr. Alan Johnson, methicillin resistant Staphylococcus aureus (MRSA) infection. The Support group for MSRA sufferers and Dependents, Aug 1<sup>st</sup> (2005).

## Certified Standards for Quantitative $^1\text{H-NMR}$ (qNMR)

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*This is the first of a series of articles related to the launch of new organic CRMs certified by quantitative NMR under double accreditation. In this issue, we focus on CRMs intended for use as internal standards in qNMR, while in the next issue of Analytix we will present a first series of certified organic standards for chromatography.*

The quantification of content or purity of organic substances has up to now often been performed with chromatographic techniques such as HPLC or GC. Over the last years, quantitative NMR (qNMR) has evolved not only in the pharmaceutical industry but also in many other fields.

In the R&D lab of Sigma-Aldrich® Buchs (Switzerland) a new Bruker Avance III 600 MHz NMR spectrometer was installed recently for high-resolution qNMR measurements of organic substances. By the end of 2009, the new NMR lab was fully accredited under both ISO/IEC 17025 [1] and also ISO Guide 34 [2] for the certification of organic reference materials using  $^1\text{H-qNMR}$ . The combination of both accreditations represents the highest achievable reliability level and earns the label of “gold standard” among CRM producers.

A set of calibration standards for use as internal standards in qNMR is being launched as a first series of new CRMs. These CRMs are comprehensively characterised by qNMR and other techniques, leading to the highest accuracy and very low uncertainties of the certified values. All of these qNMR reference materials are traceable to internationally accepted references from NIST and a detailed certificate is delivered with each CRM.

### Performance and advantages of qNMR measurements

Quantitative NMR shows many advantages over other analytical techniques with regard to quantification or purity determination of organic substances. The most outstanding attribute of qNMR is that it is a relative primary method: the signal intensity is in direct proportionality with the number of protons contributing to the resonance. The structures of the chemical substances are therefore irrelevant. In addition, no significant empirical factors or unknown biases contribute to the ratio of signals. The signal ratio of two different protons can therefore

be measured with tremendous precision and the only significant contribution to the measurement uncertainty is the integration of the signals. In other words, the direct response of a qNMR experiment is of the greatest accuracy.

Of course, a few basic requirements must be met in order to obtain good qNMR results. To begin with, the weighing of the reference and the sample prior to the qNMR measurements must be done with utmost accuracy, since this is the essential sample preparation step in qNMR analysis. It is self-evident that the availability of a suitable reference material for internal calibration is mandatory (see next section). Furthermore, the reference substance and the sample must not react with each other or with the solvent. The relevant signals which are selected for the measurement must be clearly separated from each other and also from other signals. Appropriate instrument settings are required so that no intensity is lost through incomplete relaxation.

### Requirements to qNMR calibration standards

Since NMR signal intensity is fully independent of the nature of the substance, many organic substances could potentially serve as an internal reference for qNMR. In reality, the number of suitable candidates for qNMR references is rather limited, since they must conform to a series of the following specific requirements: only substances with a very limited number of signals and of highest organic purity are suitable as a qNMR reference. The candidates must be stable in solution and should not be chemically reactive. They should not contain residual water, since the presence of water can lead to line broadening or baseline distortion in many cases. Of course, to ensure proper weighing results, a solid qNMR reference should not be hygroscopic and a liquid qNMR reference should not be volatile. The number of isochronic proton nuclei be optimum, that is, neither too high nor too low in relation to the molecular mass of the qNMR reference substance, since a 1:1 signal intensity ratio of sample and internal standard is targeted. Otherwise, the amount of substance needed for one measurement would be either very low (leading to bad weighing result) or too high (causing solubility problems). The signals of a qNMR reference should not cover areas in the spectrum where most often the analyte signals are expected to be. Finally, a qNMR reference substance should be neither extremely toxic nor carcinogenic or mutagenic.

It is obvious that many different qNMR standards must be available to cover the broad variety of analytes and deuterated solvents. We have developed a series of qNMR reference

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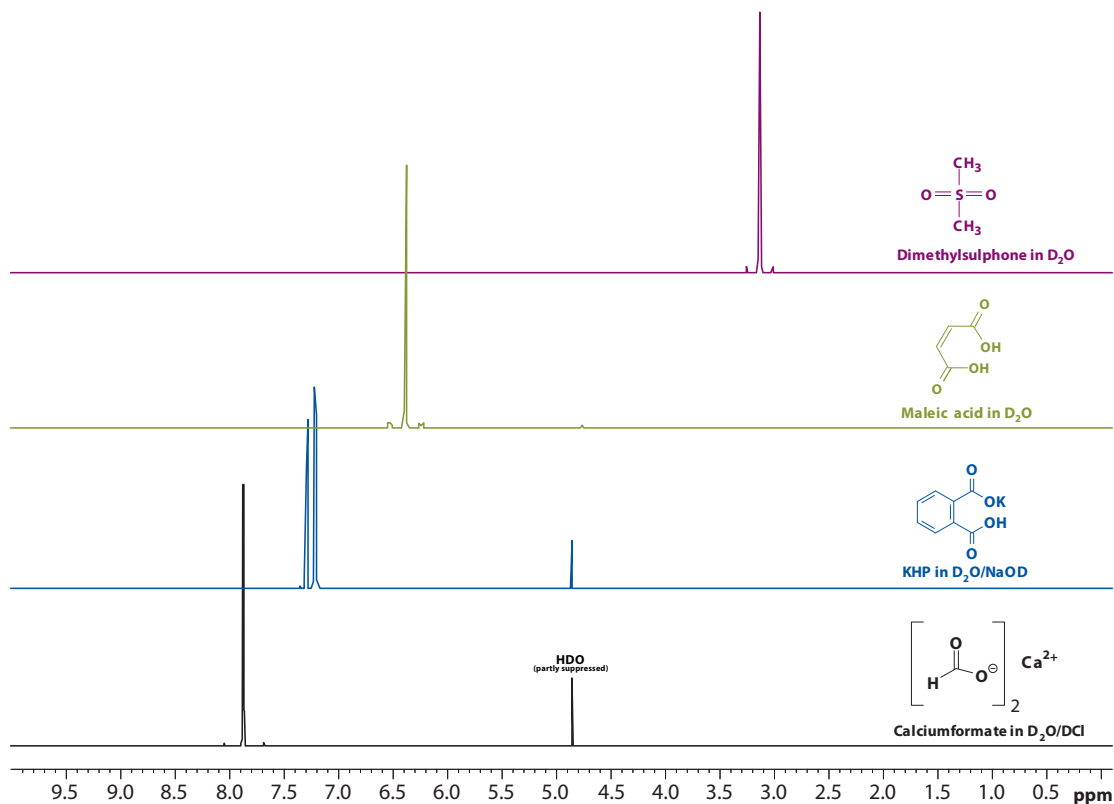


Figure 1 <sup>1</sup>H NMR spectra of water soluble qNMR standards. The residual signal of HDO is partly suppressed

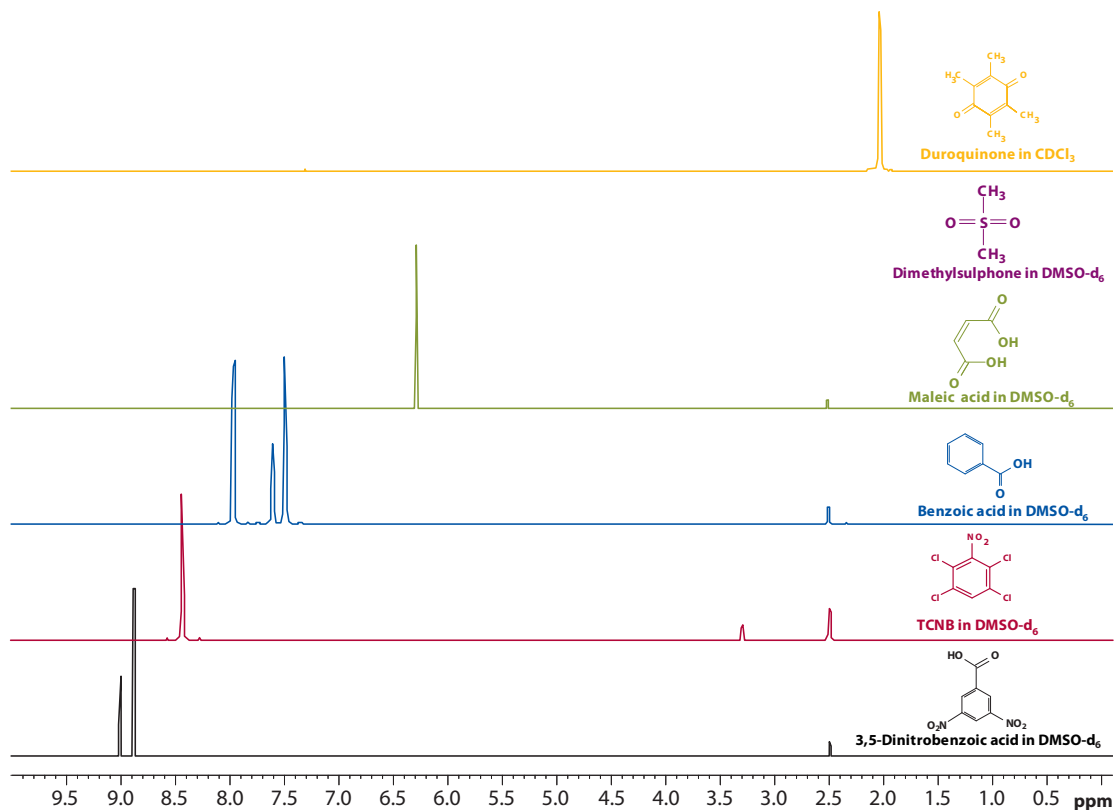


Figure 2 <sup>1</sup>H NMR spectra of qNMR standards which are soluble in organic solvents

substances whereby every reference substance has its individual solubility behaviour and chemical shift. The selection of new qNMR calibration standards is given in **Figure 1** (water soluble standards) and **Figure 2** (standards soluble in apolar organic solvents).

### Certification of calibration standards for qNMR

To achieve all the above-mentioned requirements, the new class of certified qNMR reference substances undergo a series of pre-tests and extensive certification measurements in order to provide compliance with ISO/IEC 17025 and ISO Guide 34 requirements. Only high-purity materials are carefully sourced or purified and extensively characterised by chromatography, LC-MS and other standard techniques before they undergo the qNMR certification procedure.

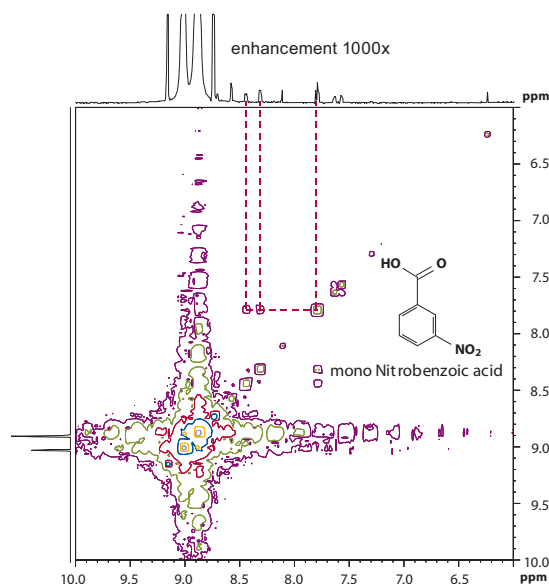
- Pre-testing: the substance is tested for hygroscopy or volatility, stability in different deuterated solvents, and reactivity against NIST certified reference substances (>24 hour period).
- Stability testing: Replicate tests after storage at higher temperature are performed to ensure that even extreme transportation conditions do not affect the certified value (>1 month period). Data on ageing studies at the recommended storage temperature are used to define the shelf life of the products (>3 year period).
- Signal suitability test: Since only "pure" signals are allowed for NMR quantification (no overlapping and no underlying impurity signals), extensive 2D experiments are required (H-H COSY and C-H correlated experiments in some cases). With H-H COSY experiments, hidden signals from impurities can easily be detected with a limit of detection below 0.1% (see example in **Figure 3**). This suitability check is performed before the certification measurements are started.
- Homogeneity and traceability: 10 samples are taken from different places within the batch. The NMR samples are then prepared by weighing equivalents of the qNMR substance and the NIST reference together in one vial, using an ultramicro balance. The samples are dissolved, measured and analysed further under standardised measurement settings on a Bruker Avance III 600 MHz NMR instrument. Following the analysis of variances, a detailed uncertainty budget is calculated and all the data is summarised in a comprehensive certificate.

By this quality procedure, most qNMR reference substances are obtained with purities  $\geq 99.5\%$  and have typical uncertainties  $\leq 0.2\%$ . All qNMR standards are packed in brown-glass bottles and closed with a sealed cap. A detailed certificate in accordance with ISO Guide 31 is delivered with each CRM. The certificate contains a comprehensive documentation tabulating lot-specific values, traceability, uncertainty calculation, expiration date and storage information.

Since qNMR offers several advantages over other analytical techniques, Sigma-Aldrich® will launch a new series of certified organic standards for chromatography whereby the certified value of these CRM will not be measured by HPLC or GC only but also by qNMR.

### References:

- [1] ISO/IEC 17025, General requirements for the competence of testing and calibration laboratories.
- [2] ISO Guide 34, General requirements for the competence of reference material producers.



**Figure 3** 2D COSY of 3,5-dinitrobenzoic acid: the cross peaks of the impurity 3-nitrobenzoic acid can be identified although the relative content of this impurity is below 0.1%.

Cat. No.	Substance	Chemical Shifts*	Solubility				Pack Size
			D <sub>2</sub> O	MeOD	CDCl <sub>3</sub>	DMSO-d <sub>6</sub>	
40384 <sup>1</sup>	1,2,4,5-Tetrachloro-3-nitro-benzene	8.5 ppm			●	●	5 g
74658	1,2,4,5-Tetramethylbenzene	6.9/2.2 ppm			●		5 g
06185	Benzoic acid	8.2 – 7.4 ppm		●		●	5 g
06856 <sup>2</sup>	Duroquinone	2.0 ppm			●		5 g
15639	3,5,-Dinitrobenzoic acid	9.2 ppm				●	5 g
41867	Dimethylsulphone	3.2 ppm	●		●	●	5 g
14659	Potassium phthalate monobasic	8.2/7.5 ppm	●	●			5 g
03826	Calcium formate	8.3 ppm	●				5 g
92816	Maleic acid	6.2 ppm	●	●		●	5 g

\* chemical shifts may slightly vary depending on the experimental conditions 1; 2 will be available soon

**Table 1** List of Fluka branded certified qNMR standards

Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	Ge	As
Zr	Nb	Mo	Tc	Ru	Rh	Pd	Ag	Cd	In	Sn	Sb
Hf	Ta										

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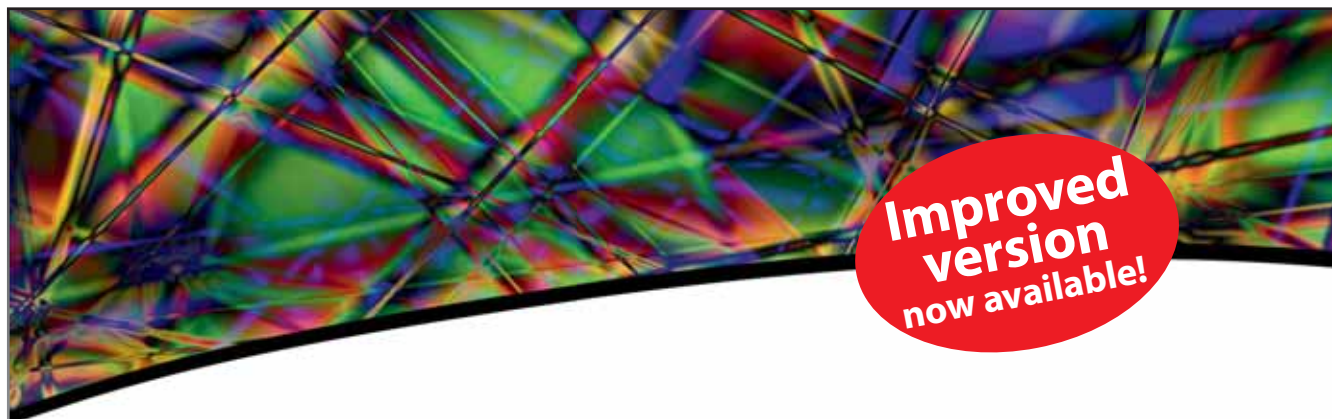
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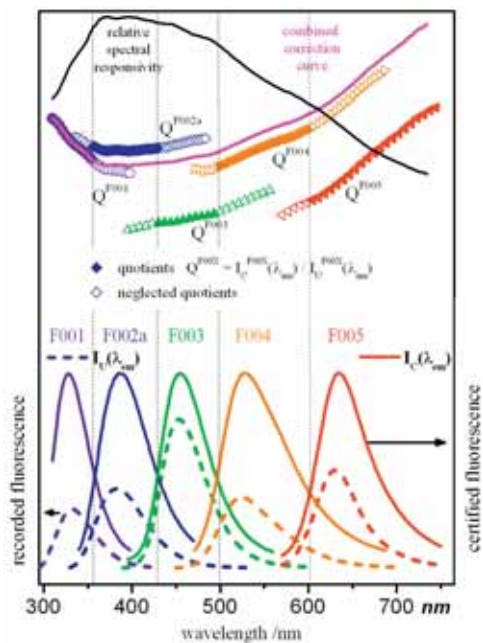


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## Enantiomeric Purities of Amino Acids Using Carbohydrate-Based Isothiocyanates

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### Introduction

In the first paper of this series (Analytix Nr. 1/2010), an efficient enantiomeric analysis of a series of alkyl oxiranes was described, using inexpensive reversed-phase columns as an alternative to high-cost so-called “chiral columns”. The analysis utilised isothiocyanate derivatisation reagents based on monosaccharide such as BGITC (Figure 1). The oxiranes were first converted by reaction with isopropyl amine into the corresponding  $\beta$ -amino alcohols. In a second step, these alcohols were derivatised with BGITC into the corresponding diastereomeric thioureas. Baseline separations were observed in all cases, thus establishing a highly efficient and general method for the analysis of this class of molecule.

### Amino acids

The above reagents (Figure 1) have been shown to be also highly suitable for the enantiomeric analysis of amino acids of widely varying structures such as proteinogenic-, non-proteinogenic (non-coded)-, and non-natural- (1,2) amino acids as well as  $\alpha,\alpha'$ -disubstituted (3) and  $\beta$ -amino acids (4).

To their advantage, native (underivatised) amino acids (“straight out of the bottle” or the reaction medium) react under mild conditions and at a rapid rate (at room temperature) with these reagents, leading to the corresponding diastereomeric thioureas (Figure 2). These can then be injected directly into the HPLC without the need for further purification. In the majority of cases, baseline separations are achieved.

As derivatives of natural monosaccharides, all of the above reagents are enantiomerically pure by definition, and the ratios of the subsequent diastereomers directly reflect the enantiomeric composition of the amino acid in question. This requires, of course, that both enantiomers react rapidly and quantitatively and with the same rate in order to avoid a diastereoselectivity during the derivatisation process. For new target molecules, this must be ascertained in every case by calibration with the corresponding racemate. The approach described in this article frequently has distinct advantages over the so-called *direct* method employing chiral stationary phases in that a) the separation of diastereomers

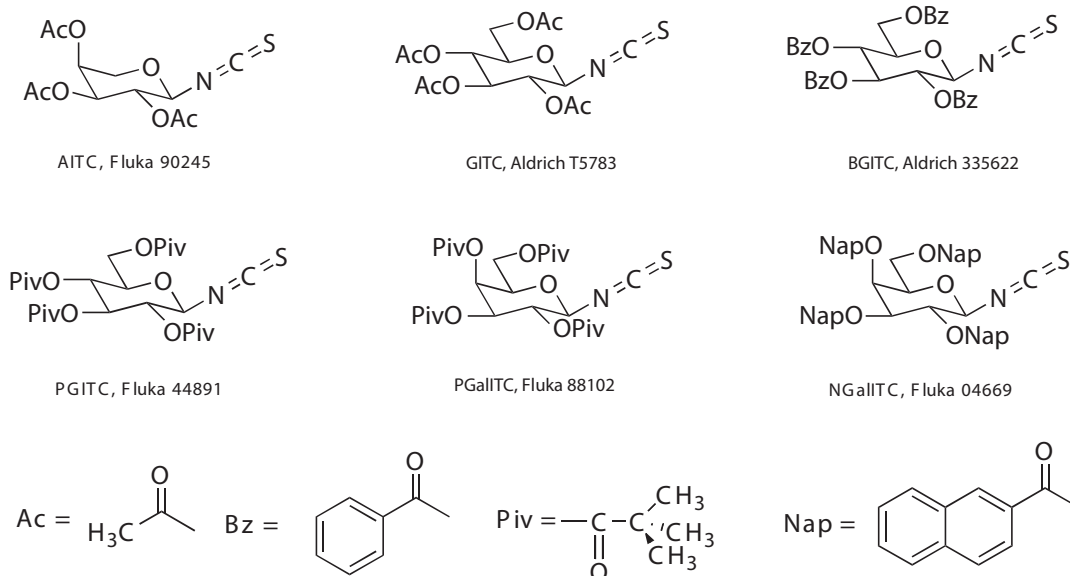


Figure 1 Structure formulas of isothiocyanate derivatisation reagents based on monosaccharide

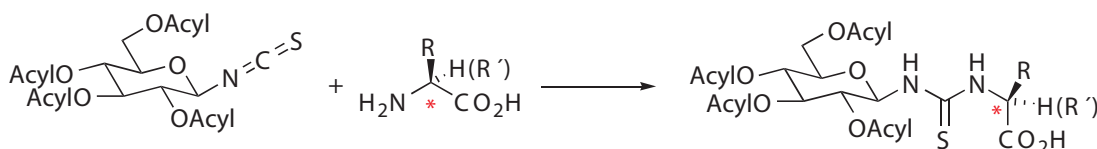


Figure 2 Amino acids: formation of diastereomeric thioureas (schematic) [\* denotes centre of chirality]

(continued on page 16)

is usually simpler to perform and often provides better resolutions, b) the choice of chromatographic conditions is much greater and these can be more easily optimised, and c) the reagents already contain chromophores (fluorophores) for convenient UV-detection and no pre-column derivatisations (OPA, PITC) are required as is frequently the case with chiral columns. In view of the range of novel derivatisation reagents which recently became available (PGITC, PGallTC, NGallTC) (5), the method is an interesting alternative and can also substitute for the frequently employed Marfay's reagent (6,7).

In principle, all of the above reagents can be employed for the analysis of amino acids. Thus, Kinoshita et al. [1] achieved baseline separations in the analysis of various  $\alpha$ -amino acids employing GITC (Aldrich T8783) and AITC (Fluka 90245). The introduction of benzoyl groups (BGITC Aldrich 335622) [2] and naphthoyl groups (NGallTC Fluka 04669) [5] considerably enhanced the UV- and fluorescence detectability of the corresponding thioureas by factors ranging from 6 (BIGTC) to 40 (NGallTC). Furthermore, the introduction of these residues often provides considerable improvement to the separation of these diastereomers, as did the incorporation of extremely bulky pivaloyl groups, such as in PGITC (Fluka 44891) and PGallTC (Fluka 88102).

While simple RP-18 columns are usually employed, the separation conditions can be varied widely in order to achieve the best separating conditions. Various mobile phases have been used in order to optimise the separation conditions. These include MeOH : phosphate buffer (pH 2.8) [1] over MeOH : H<sub>2</sub>O : phosphate buffer (pH 7) to acetonitrile : water/0.1% trifluoroacetic acid (3,5). In certain cases, the reagent may interfere with the separation if its retention time is very near that of the analyte. The addition of small amounts of ethanolamine is sufficient to destroy excess reagent by formation of the corresponding thiourea which elutes at widely different retention times. The method – after further optimisation of the separation conditions – allows the separation of all racemic, proteinogenic amino acids in one go [1].

In **Tables 1** and **2** the results obtained with the above reagents in the analysis of a variety of structurally different amino acids are summarised by listing capacity factors  $k$ , separation factors  $\alpha$  and resolutions  $R$ , or simply  $\Delta t_{R1/2}$ . The listed data is representative; however, the absolute values may change, depending on the actual separation conditions (type of column, mobile phase, flow rate etc.).

	AITC					GITC				
	$k_L$	$k_D$	$\alpha$	$R_S$	mobile phase	$k_L$	$k_D$	$\alpha$	$R_S$	mobile phase
Ala	1,60	2,45	1,53	3,78	A	3,85	5,50	1,43	4,40	B
Asp	1,40	1,65	1,18	1,43	A	3,80	4,35	1,14	1,69	B
Glu	1,20	1,40	1,17	1,14	A	2,70	3,15	1,17	1,80	B
Ile	4,05	7,25	1,79	6,94	C	3,55	5,50	1,55	5,01	D
Leu	4,25	7,35	1,73	7,75	C	3,75	5,65	1,51	5,43	D
Phe	6,25	11,1	1,78	8,43	C	5,00	8,10	1,62	6,89	D
Pro	1,55	2,15	1,39	3,00	A	2,80	3,70	1,32	3,00	B
Trp	6,55	9,85	1,5	6,00	C	4,95	7,25	1,46	5,11	D
Tyr	1,65	2,45	1,48	3,56	C	1,95	2,55	1,46	3,20	D
Val	1,95	3,45	1,77	4,96	C	2,00	3,10	1,55	4,00	D

	BGITC					NGallTC			
	$k_L$	$k_D$	$\alpha$	$R_S$	mobile phase	$t_1$	$t_2$	$D t_{1/2}$	mobile phase
Ala	18,00	20,85	1,16	2,72	E				
Ile	9,27	12,35	1,33	3,08	F	22,37	25,20	2,83	H
Leu	9,51	12,65	1,33	3,74	F	22,00	24,69	2,69	H
Lys	13,48	15,32	1,14	2,28	G				
Met	8,08	10,24	1,27	4,55	F	19,41	21,28	1,87	H
Phe	10,54	13,81	1,31	4,84	F	19,47	21,81	2,40	H
Pro	6,41	5,19	1,23	2,50	F				
Thr	5,35	6,24	1,17	2,28	F	22,45	25,80	3,35	H
Trp	9,43	12,03	1,27	4,36	F	20,96	22,37	1,41	H
Tyr	6,22	7,41	1,19	2,00	F				
Val	7,22	9,16	1,27	3,00	F				

MeOH/10mM phosphate buffer pH 2.8: A (40:60); B (45:55); C (50:50); D (55:45)

MeOH /H<sub>2</sub>O: E (70:30); F (80:20); G (85:15)

Acetonitrile/H<sub>2</sub>O (0.1% TFA): H (80:20)

**Table 1** Separations of proteinogenic amino acids

	BGITC				mobile phase
	$k_L$	$k_D$	$\alpha$	$R_S$	
2-ABuA	6,24	7,57	1,21	2,97	B
3-ABuA	16,88	18,95	1,12	2,74	A
2-AHeA	12,89	16,81	1,3	5,92	B
ornithine	11,94	13,9	1,16	2,54	C
phenylglycine	6,38	7,86	1,23	2,2	B

2-ABuA: 2-aminobutyric acid; 3-ABuA:3-aminobutyric acid; 2-AHeA : 2-aminoheptanoic acid  
MeOH /H<sub>2</sub>O: A (70:30); B (80:20);C (85:15)

**Table 2** Separations of non-proteinogenic amino acids

The detection limits are already very low in the case of GITC (ca. 5 ng) and can be further reduced by the introduction of aromatic chromophores or fluorophoric groups. This is particularly important in cases where body fluids are analysed directly.

### Summary

The method described above allows the rapid, efficient and inexpensive determination of enantiomeric purities in a wide variety of structurally varied amino acids. By using the suitable derivatisation reagent, baseline separations are observed in nearly all cases. The method is quite general and applicable to a) detection of trace amounts of amino acids in biological samples; b) check for racemisations, and c) monitoring asymmetric syntheses of amino acids. The method is clearly adaptable to automation using reaction batteries and auto-samplers. The method is thus applicable both on a laboratory scale and in online quality control. It is thus highly suitable for monitoring asymmetric syntheses of amino acids including enzyme-catalysed transformations.

### Experimental

5 mg of the corresponding amino acid is dissolved in 50% (v/v) aqueous acetonitrile containing 0.55%(v/v) triethyl amine to give a final volume of 10 mL. To 50  $\mu$ L of this stock solution 50  $\mu$ L of 0.66% (w/v) BGITC in acetonitrile is added. The resulting solution is shaken on a laboratory shaker for 30 min, after which 10  $\mu$ L of 0.26% (v/v) ethanolamine in acetonitrile is added and shaking is continued for another 10 min. Ethanolamine reacts with any excess of BGITC and the resulting thiourea derivative is eluted well behind any of the amino acid derivatives. The mixture is then diluted to a final volume of 1 mL and a 10  $\mu$ L aliquot is injected into the

HPLC. (RP-18, mobile phase MeOH :H<sub>2</sub>O [67 mM phosphate buffer ( pH 7) = 65 : 27 : 8 up to 70:25:5 and 80:15:5], depending on the case, flow rate 0.5 mL/min, compare tables).

### References

- [1] Nimura, N., Ogura, H., Kinoshita, T. Reversed-phase liquid chromatographic resolution of amino acid enantiomers by derivatization with 2,3,4,6-Tetra-O-acetyl- $\beta$ -D-glucopyranosyl isothiocyanate. *J. Chromatogr.* 202 (1980), 375–9; and other papers in this series: Kinoshita, T., Kasahara, Y., Nimura, N. *ibid.* 210 (1981) 77–81; Nimura, N., Kasahara, Y., Kinoshita, T. *ibid.* 213 (1981), 327–30; Nimura, N., Toyoma, A., Kinoshita, T. *ibid.* 213 (1984), 547–52.
- [2] Lobell, M., Schneider, M. 2,3,4,6-Tetra-O-benzoyl- $\beta$ -D-glucopyranosyl isothiocyanate: an efficient reagent for the determination of enantiomeric purities of amino acids,  $\beta$ -adrenergic blockers and alkyloxiranes by high performance liquid chromatography using standard reversed phase columns. *J. Chromatogr.* 633 (1993), 287–94.
- [3] Peter, A., Olajos, E., Casimir, R., Tourwe, D., Broxterman, Q.B., Kaptein, B., Armstrong, D.W. High performance liquid chromatographic separation of the enantiomers of unusual  $\alpha$ -amino acid. *J. Chromatogr.* 871 (2000) 105–13; for an industrial application, see: German Patent Application: DE 198 33853 A1, Degussa-Hüls AG, 28 July 1998.
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- [6] Review: Görög, S., Gazdag, M. Enantiomeric derivatization for biomedical chromatography. *J. Chromatogr. B* 659 (1994), 51–84.
- [7] Bushan, R., Bruckner, H. Marfey's reagent for chiral amino acid analysis: A review; *Amino Acids* 27 (2004), 231–47. Bushan, R., Kumar, V., Tanwar, S. Chromatographic separation of enantiomers of non-protein  $\alpha$ -amino acids after derivatization with Marfey's reagent and its four variants, *Amino Acids* 36 (2009), 571–9.

### List of reagents:

2,3,4-Tri-O-acetyl- $\alpha$ -D-arabinopyranosyl-isothiocyanate	[AITC, Fluka 90245]
2,3,4,6-Tetra-O-acetyl- $\beta$ -D-glucopyranosyl-isothiocyanate	[GITC, Aldrich T5783]
2,3,4,6-Tetra-O-benzoyl- $\beta$ -D-glucopyranosyl-isothiocyanate	[BGITC, Aldrich 335622]
2,3,4,6-Tetra-O-pivaloyl- $\beta$ -D-glucopyranosyl-isothiocyanate	[PGITC, Fluka 44891]
2,3,4,6-Tetra-O-pivaloyl- $\beta$ -D-galactopyranosyl-isothiocyanate	[PGalITC, Fluka 88102]
2,3,4,6-Tetra-O-(2-naphthoyl)- $\beta$ -D-galactopyranosyl-isothiocyanate	[NGalITC, Fluka 04669]

## Derivatisation of Non-Steroidal Anti-Inflammatory Drugs with Different Activated Silylating Reagents

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### Introduction

Human and veterinary pharmaceuticals are a group of “emerging” contaminants [1, 2], some of which are produced in increasingly large volumes every year. The amounts produced are reaching quantities similar to those of pesticides and other organic pollutants. Residues of these biologically active compounds can enter the environment via different transport pathways – emissions during manufacture, disposal of unused or expired medicines, human and animal excretion, or direct discharge of aquaculture products [2].

Sewage treatment plants (STP) are not able to completely remove these drugs and their excretion metabolites; consequently they are discharged to different environmental compartments at concentrations ranging from ng/L to µg/L. Recent studies have shown that, in addition to contaminating river and sea water, they may also enter drinking water produced from ground water [3, 4].

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the pharmaceuticals most widely used in human health care. Therefore, they and their metabolites constitute some of the most frequently found pharmaceutical contaminants in the aquatic environment.

### GC Derivatisation

NSAID residue analysis in water samples has usually been performed by a gas chromatograph coupled to a mass spectrometer.

Silylation is the most widely used technique for the derivatisation of functional groups present in these drugs. Among the various silylating reagents, MSTFA is one of the most important, because the by-products of MSTFA silylation, primarily N-methyltrifluoroacetamide, are more volatile than BSA and BSTFA. This characteristic enables MSTFA to be valuable in identifying compounds that would otherwise go undetected or obscured in the GC analysis. In addition, MSTFA reactions do not produce by-products that can damage the capillary GC column.

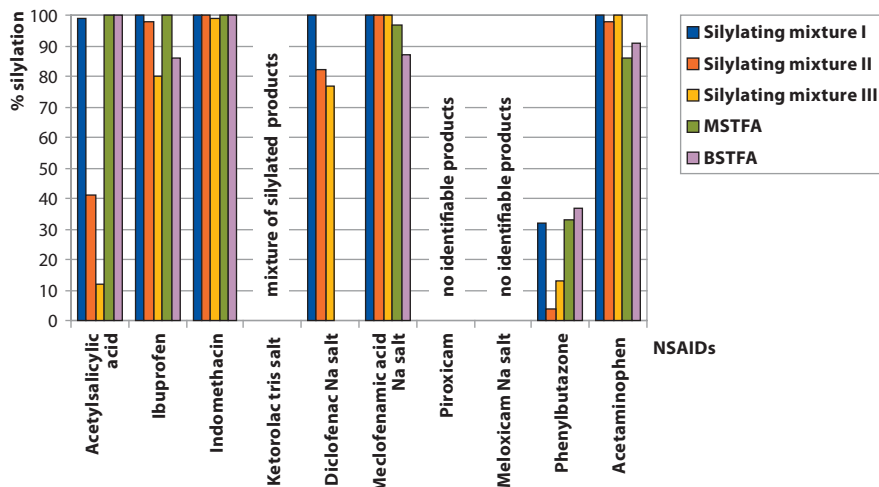


Figure 1 Results of derivatisation experiments

GC instrument	Trace GC Ultra Finnigan
MS instrument	Finnigan PolarQ, ion trap
Detector	MS and FID
Injection volume	4 µl of the silylating reaction solution
Column	Zebtron 2B-5 ms, 30 m x 0.25 mm ID, 0.25 µm d <sub>i</sub>
Carrier gas and flow rate	Helium, 1 ml/min, constant
Ionisation	EI at ~70eV (filament)
Injection temperature	50 °C
Temperature program oven	50 °C, 10 °C/min to 280 °C, 12 min at 280 °C
Detector temperature	FID, 350 °C
Sample	each analyte between 16 and 20 ppm in acetonitrile
Derivatisation	4–5 mg NSAID + 0.5 mL acetonitrile + 0.5 mL silylating reagent, crimped vial heated for 30 min to 70 °C

Table 1 Operating conditions for GC and GC-MS

The silylation power of MSTFA can be enhanced by the use of catalysts or additives that scavenge reaction by-products. MSTFA reacts *in situ* with ammonium iodide (NH<sub>4</sub>I) to produce trimethylsilylchlorine (TMSI), a powerful trimethylsilyl donor [5].

In order to prevent iodine incorporation into the product, ethanethiol is added to reduce the formed iodine to hydrogen iodide. As a result, diethyl disulphide is produced during the derivatisation reaction. Imidazole acts as a base catalyst in the MSTFA silylation reaction.

In order to gain more silylating power, mixtures of silylation reagents are applied. Trimethylsilylchlorine, a powerful trimethylsilyl donor, is mixed together with a trimethylsilylimidazole compound and either a silylated trifluoroacetamide or a silylated acetamide.

### Experimental

The chemical classification of NSAIDs includes two broad groups – non-selective COX inhibitors and selective COX inhibitors [6]. COX, cyclooxygenase, is an enzyme necessary for the prostaglandin synthesis which causes swelling and pain. In this study, we investigated the silylating power and performance of five silylating agents – three ready-to-use mixtures and two single reagents

Cat. No.	Product	Details	Pack Size
85431	Silylating mixture I according to Sweeley	Hexamethyl-disilazane/trimethylchlorosilane (TMCS) 2:1 (v/v)	10 ml
85432	Silylating mixture II according to Horning	N,O-Bis(trimethyl-silyl)acetamide/1-Trimethylsilylimida-zole/TMCS 3:3:2 (v/v/v)	10 ml
85433	Silylating mixture III	Trimethylsilylimdia-zole/BSTFA/TMCS 3:3:2 (v/v/v)	10 ml
15222	N,O-Bis(trimethylsilyl)trifluoroacetamide, BSTFA	puriss. p.a. for GC	1 ml, 5 ml, 10x1 ml, 25 ml
69479	N-Methyl-N-(trimethylsilyl)trifluoroacetamide, MSTFA	puriss. p.a. for GC	5 ml, 10x1 ml, 25 ml

**Table 2** Silylating reagents

(**Table 2**) – on ten different NSAIDs classified as non-selective COX inhibitors, (**Table 3**) with GC-MS. This group is the most frequently detected in the aquatic environment. **Table 3** shows a classification of the tested substances. All derivatisation reagents and NSAID substances are available from Sigma-Aldrich. The GC-MS operating conditions are presented in **Table 1**. **Figure 1** provides a graphic summary of the results.

## Results of silylation of the ten NSAIDs with the three silylating mixtures, MSTFA and BSTFA

### I. Carboxylic acids

Within this group, the two representatives acetylsalicylic acid and ibuprofen react very differently with the five derivatisation reagents. Acetylsalicylic acid reacts with Silylating mixture I, MSTFA and BSTFA to produce complete silylation, giving one peak by GC-MS. The mass spectrum confirms the complete silylation of the OH-group. With the Silylating mixture II and III, besides the monosilylated compound (~46%) the chromatogram and MS show a double-silylated compound (~39%, M-H+ peak at 269 m/z), can be defined as 2-Trimethylsiloxybenzoic acid trimethylsilylester.

Ibuprofen reacts with the Silylating mixture I and MSTFA to result in a 100% conversion to the monosilylated product. With Silylating mixture II, III and BSTFA the conversion is 98%, 80% and 86% respectively. The 2-20% by-product is, according to MS, a reaction product formed by two ibuprofen molecules – 5,8,9,10-Tetrahydrobenz[a]anthracen-11(6H)-one.

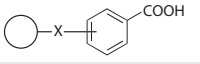
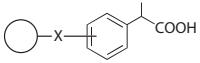
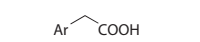
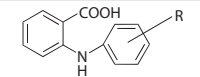
The three aryl acetic acids, indomethacin, ketorolac tris salt and diclofenac sodium salt – behave very differently too. Indomethacin reacts with all five silylation reagents (98–100% conversion) to the single silylated trimethylsilylester. The ketorolac tris salt could give several varieties of silylation:

- Complete silylation of the ketorolac and the tris salt part
- Monosilylation of the ketorolac part
- Mixture of silylated products with partly silylated tris salt

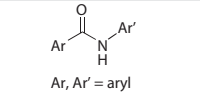
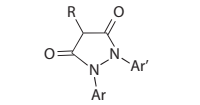
The chromatograms of all five silylating substances show the third variety. Up to seven different silylated compounds are formed – all of them give a peak at m/z 73, demonstrating that there are silylating groups involved. None of the compounds can be identified as a complete silylated ketorolac tris salt. Two by-products can be identified by MS: Trimethylsilylbenzoic acid – formed possibly by thermal decomposition of the ketorolac salt at the GC injector – and a four-fold silylated “tris salt”. In contrast, the diclofenac sodium salt reacts with the Silylating mixture I and MSTFA to produce the single silylated compound with 100% conversion. Silylating

### Non-selective COX inhibitors

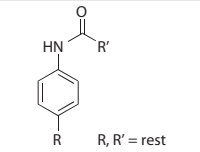
#### I. carboxylic acids

salicylates		Acetylsalicylic acid
propionates		Ibuprofen
acetic acids		Indomethacin Ketorolac tris salt Diclofenac sodium salt
	Ar = indoleacetic acid Ar = pyroacetic acid Ar = phenylacetic acid	
anthranilates		Meclofenamic acid sodium salt

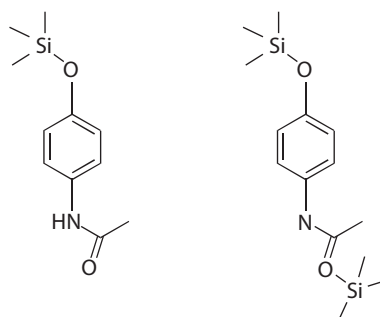
#### II. enolic acids

oxicams		Piroxicam Meloxicam
	Ar, Ar' = aryl	
pyrazolones		Phenylbutazone
	Ar, Ar' = aryl R = rest	

#### III. anilides

anilides		Acetaminophen
	R, R' = rest	

**Table 3** Classification of the tested substances



**Figure 3a and b** Main product and by-product of the acetaminophen silylation

(continued on page 20)

mixtures II and III produce the monosilylated compound with 82% and 77% conversion, respectively; with BSTFA no silylation is observed. One of the two main by-products is formed by ring-closure of the diclofenac (see **Figure 2a**); the other one could not be verified completely by an MS-library, but based upon the MS-spectra it could be a silylated molecule derived from the other main by-product (see **Figure 2b**).

The fourth subgroup of the carboxylic acids, the anthranilates, with the representative – meclofenamic acid – reacts with all three silylating mixtures to form a complete silylated derivative, showing a single, sharp peak in the GC chromatogram. With MSTFA, the conversion is only 93%. The derivatisation with BSTFA produces, along with five by-products, the monosilylated compound to 87% conversion.

## II. Enolic acids

The enolic acids can be split into two subgroups – the oxicams and the pyrazolones. Piroxicam and meloxicam sodium salt, as representatives of the first subgroup, provide no identifiable silylation products with all five reagents. The GC chromatograms show some broad but non-evaluable peaks. This could be ascribed to some crystal water in the acids being released during the heating of the sample, which then hydrolyses the silylating reagent.

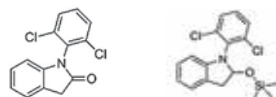
For phenylbutazone – representative of the pyrazolone subgroup – the five chosen silylation reagents are not really applicable. The derivatisation reactions provide a maximum of 40% silylation, with the rest being educt. This is especially true with the silylating mixtures II and III, where a yield of only 4% and 13% of the monosilylated product is achieved. The highest silylation conversion to the monosilylated product – 37% – is provided by the reaction with BSTFA.

## III. Anilides

Although the anilides do not properly belong to the NSAIDs – they do not have an acidic group – they are normally included in the group of the non-selective COX inhibitors. Interestingly, acetaminophen reacts with the activated silylation reagents – Silylating mixture I, II and III – to produce only the monosilylated product with 100% yield. In contrast, the silylation with MSTFA and BSTFA also produces a by-product, double silylated acetaminophen, (see **Figure 3b**) but in very low yields, 14% and 9% respectively.

## Summary

In this article, we report the results of the ability of five silylating reagents (Silylating mixture I, II, III, MSTFA and BSTFA) to form GC-MS-compatible trimethylsilylmethyl derivatives of ten important non-steroidal anti-inflammatory drugs (NSAIDs). Although some drugs do not show any silylation with any of the reagents, it can be concluded that, for these targeted compound classes, the activated Silylating mixture I appears to be the most effective choice.



**Figure 2a and b** Two main by-products of the diclofenac reaction with Silylating mixtures II, III and BSTFA

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- [6] Brunton, L.L., Lazo, J.S., and Parker, K.L. (eds) (2005) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*. McGraw-Hill.

Cat. No.	Drug	Pack. Size	Structure
I4883	Ibuprofen	1 g, 5 g, 10 g	
A5376	Acetylsalicylic acid	100 g, 250 g, 500 g	
I7378	Indomethacin	5 g, 10 g, 25 g, 100 g	
K1136	Keterolac tris salt	1 g, 5 g	
D6899	Diclofenac sodium salt	10 g, 25 g, 100 g	
M4531	Meclofenamic acid sodium salt	1 g, 5 g	
P5654	Piroxicam	1 g, 5 g, 10 g	
M3935	Meloxicam sodium salt	100 mg	
P8386	Phenylbutazone	25 g, 100 g, 500 g	
A7085	Acetaminophen	100 g, 500 g	

**Table 4** NSAID substances evaluated

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60738-1KG	Fluka	Silica gel 60 Å	for column chromatography	0.035–0.070 mm
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