Application Note # LCMS-73

Interlaboratory Tests Demonstrate the Robustness and Transferability of the Toxtyper™ Workflow

Abstract

There is high demand in clinical and forensic toxicology for comprehensive, specific and transferable techniques that overcome the well-known limitations of current GC-MS, LC-UV/DAD and immunoassay solutions. Liquid chromatography-tandem mass spectrometry (LC-MS) combined with library searching is an emerging screening solution for toxicology.

Here we describe a robust and automated solution for the detection and identification of drugs and drugs of abuse in biological specimens. The workflow was tested with regard to method- and result-transferability from lab to lab.

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Drugs of Abuse	Ion Trap

Introduction

LC-MS/MS is an emerging screening solution for clinical, routine and forensic toxicology. This method is far more specific than routinely used immunoassays and provides more information and higher identification rates than LC-UV/DAD detection (Application Note LCMS-72).

Compared to GC-MS, LC-MS promises to cover a more comprehensive range of analytes. Due to the availability of large spectral libraries (see Ref. 1) and a high degree of transferability of results from lab to lab, GC-MS is currently regarded as the gold standard in toxicology for general unknown screening (GUS). However, the disadvantages of GC-MS – such as the need for derivatization and its incompatibility with thermolabile and polar substances – mean that LC-MS is being increasingly used for GUS.

A central feature of the ion trap LC-MS^n solution described here is the unique, patented SmartFrag™ technology, which provides transferability and reproducibility of screening results from instrument to instrument and from lab to lab. Using SmartFrag provides the highest possible transferability by virtually eliminating variation and tuning from the MS/MS process. This approach identifies substances by retention time and MS^2/MS^3 spectra combined with a library search, and represents a robust and automated solution for the detection and identification of common drugs, drugs of abuse, and metabolites in biological specimens. A fast LC-gradient for separation, the auto-MS^n capability of the amaZon speed™ ion trap for detection of analytes, and a fully automated script for fast and user-friendly data analysis and reporting provide results in the shortest time possible.

To demonstrate the transferability of the Toxtyper workflow and to compare the overall performance of different LC-MS ion trap systems, three spiked serum samples and one blank serum sample were sent to five different labs and analyzed using seven different LC-MS systems.

Experimental

Sample preparation

Three mixtures of toxicologically relevant substances were spiked into blank human serum at different concentrations. Additionally, a blank human serum sample was extracted. Sample preparation was carried out using the following liquid-liquid extraction (LLE) protocol. Serum (1 mL) was spiked with 50 ng of D5-diazepam as an internal standard and then mixed with 0.5 mL borate buffer (pH 9) and 1.5 mL 1-chlorobutane. After a 3 min mixing step, the solution was centrifuged at 4000 × g for 5 min. The organic phase was separated, aliquoted, and evaporated at 40°C with N_2. These aliquots were forwarded to the 5 participating labs, where the residues were redissolved in 25 µL solvent A/B (50:50; v/v; see Table 1).

LC-MS^n conditions

Two microliters of the redissolved samples was separated on an Ultimate3000 RSLC system using the settings described in Table 1. Seven different amaZon speed ion trap systems were used for generation of MS and MS^n spectra in continuous polarity switching mode (for details refer to Table 2). Data were acquired using a data-dependent scheduled precursor list approach.

Table 1: HPLC conditions used for the Toxtyper screening workflow

LC settings	
LC system	Thermo Dionex Ultimate3000 RSLC
Eluent A	H_2O, 0.1% formic acid, 2 mM ammonium formiate, 1% acetonitrile
Eluent B	Acetonitrile, 0.1% formic acid, 2 mM ammonium formiate, 1% H_2O
Analytical column	Acclaim® RSLC 120 C18 2.2 µm, 120A, 2.1 x 100 mm
Flow rate	500 µl/min
Gradient	0.0 to 1.0 min: 1% B
	1.0 to 8.0 min: 1% B to 95% B, linear
	8.0 to 9.0 min: 95% B
	9.0 to 9.06 min: 95% B to 1% B, linear
	9.06 to 11 min: 1% B

Table 2: amaZon speed ion trap MS and MS/MS parameters

MS settings	
Scan mode	UltraScan 32.500 m/z sec-1
Scan range	70 - 800 m/z
Source	Electrospray ionisation (ESI)
Polarity	Zero Delay Alternating polarity
MS^n Acquisition	Data dependent using a Scheduled Precursor List with 830 cpds
	Active exclusion after 1 spectrum, reconsider if intensity increase by factor 5
Target mass	300 m/z
ICC	200.000

Library search and reporting

The data sets were post-processed using DataAnalysis (DA) 4.1 and the processed spectra were submitted to the DA 4.1 library search module. The whole process, up to final report generation and visualization of results in the web-based Compass OpenAccess interface, was driven by a predefined Toxtyper automation script. The automatically generated reports from the different labs were evaluated and used for generation of the final result tables.

Results

The goal of this study was to test the Toxtyper workflow (see Figure 1) with regard to its transferability and the reproducibility of results from lab to lab. The toxicologically relevant substances present in the three mixtures are routinely found in forensic toxicology (personal communication, Forensic Institute, Freiburg) and were chosen without regard to their retention times or molecular masses. The compounds were spiked into blank human serum at different concentrations (see Table 1).

The spectral library, which consists of more than 830 compounds of clinical and forensic interest, was generated in close collaboration with the Forensic Institute in Freiburg, Germany (see Bruker Application Note 72). All samples in the interlaboratory test were processed in a completely automated manner using Compass OpenAccess. After completion of a run, the user received a PDF report of the LC-MS^n results; either by logging onto the web based COA system or by email.

The automatically generated reports from the different labs were evaluated to demonstrate transferability of the Toxtyper solution and compare the overall performance of the different LC-MS ion trap systems. If a substance was not identified, the respective raw data file was inspected manually to find the cause.

All compounds spiked into sample 1 could be identified by all participating labs. Several substances – for example, the benzodiazepines Diazepam and Temazepam in sample 1 – were present in sub-therapeutic concentrations. The identification results demonstrate the high sensitivity of the procedure. The results of the automatic reports are summarized in Table 4. Trimipramine was not identified by one lab. Inspection of the raw data revealed that in this lab, extensive coelution of matrix led to a mixed MS^2 spectrum and therefore to a score value below the cut-off for ID reporting. Metoprolol from sample 3 was not identified by two systems at HUG 1 and HUG 2. This was due to coelution with Mirtazapine, which led to a mixed MS^2 spectrum and subsequently to a score value below the cut-off for ID reporting. It should be noted that Metoprolol

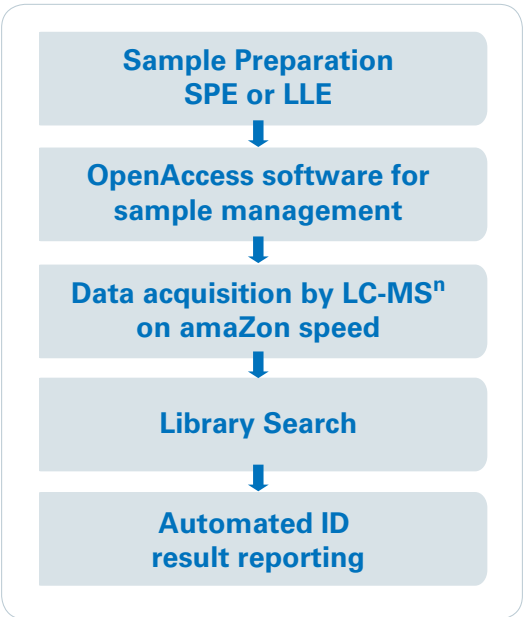


Figure 1: Schematic of the Toxtyper library-based screening workflow.

Table 3: Compounds spiked in human blank serum to test the lab-to-lab transferability of the identification workflow

Sample 1	Sample 2	Sample 3
Methadone (**250**)	Trimipramine (**100**)	Duloxetin (**600**)
EDDP (**50**)	Amitryptiline (**100**)	Nordoxepin (**300**)
Diazepam (**100**)	Zolpidem (**500**)	Mirtazepine (**50**)
Nordazepam (**500**)	Midazolam (**150**)	Metoprolol (**200**)
Oxazepam (**200**)	α-OH-midazolam (**50**)	
Temazepam (**100**)	Fentanyl (**3**)	
	Lidocaine (**200**)	

Given in brackets are the respective spiked concentrations in ng/mL (spiked levels: **no therapeutic level known**, **sub-therapeutic**, **therapeutic**, **toxic**)

and Mirtazapine not only have very similar retention times, but also differ only slightly in mass (2 Da). This problematic combination of characteristics can be regarded as a very rare case.

Common false positives were identified in the blank serum sample and the other samples, but these could be easily excluded after manual inspection of the reports and the respective raw data files. For example, a common false positive was benzododecinium. This compound is used as skin disinfectant during blood withdrawal and is present in the sample as a contaminant.

The first page of the result report is shown in Figure 2. This page shows an overview of the screening results; consisting of base peak chromatograms (positive and negative ionization mode) and a table that summarizes the identification results using purity score, intensity, and mass/retention time shifts. A separate report page – which displays the extracted ion chromatogram of the substance as well as its MS, MS^2, and if acquired, MS^3 spectrum – is generated for each identified compound (see Figure 3). This enables potentially critical IDs – for example false positives – to be ruled out very quickly.

The transferability and robustness of the fragmentation process on different Toxtyper systems is demonstrated by comparing the fragmentation reproducibility of spiked compounds. Figure 4 shows the MS^2 spectra of Amitriptyline recorded from spiked serum extracts of all participants and the respective library spectrum. SmartFrag technology provides reproducible fragmentation results that lead to the highest level of lab-to-lab transferability and reproducibility.

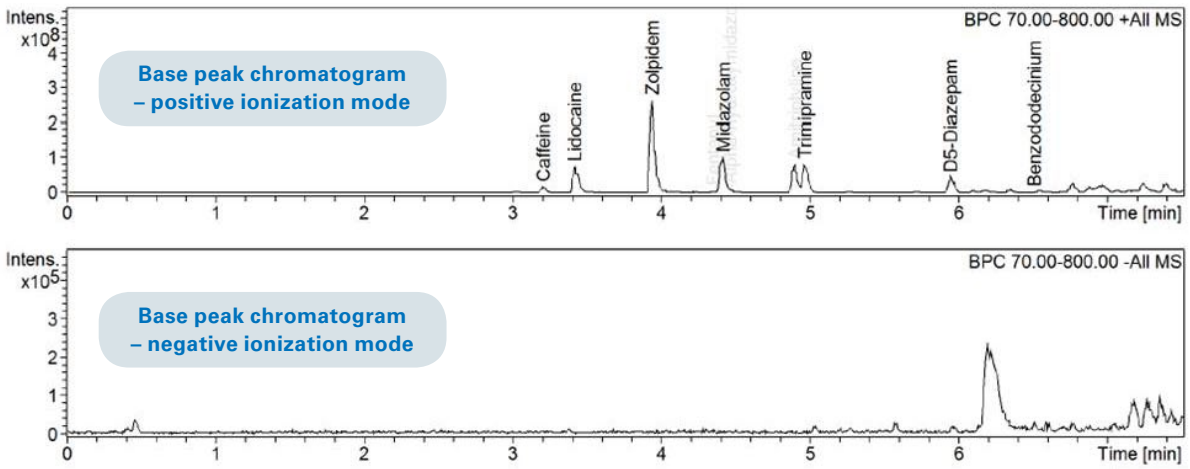
Table 4: Results from the interlaboratory test

Spiked Compounds	**Participants**						
Sample 1	**IKR**	**IRM**	**HUG 1**	**HUG 2**	**UK**	**BDal 1**	**BDal 2**
Methadone	✓	✓	✓	✓	✓	✓	✓
EDDP	✓	✓	✓	✓	✓	✓	✓
Diazepam	✓	✓	✓	✓	✓	✓	✓
Nordazepam	✓	✓	✓	✓	✓	✓	✓
Oxazepam	✓	✓	✓	✓	✓	✓	✓
Temazepam	✓	✓	✓	✓	✓	✓	✓
Sample 2	**IKC**	**IRM**	**HUG 1**	**HUG 2**	**UK**	**BDal 1**	**BDal 2**
Amitryptiline	✓	✓	✓	✓	✓	✓	✓
α-OH-midazolam	✓	✓	✓	✓	✓	✓	✓
Fentanyl	✓	✓	✓	✓	✓	✓	✓
Lidocaine	✓	✓	✓	✓	✓	✓	✓
Midazolam	✓	✓	✓	✓	✓	✓	✓
Trimipramine	✓	✓	✓	✓	–	✓	✓
Zolpidem	✓	✓	✓	✓	✓	✓	✓
D5-diazepam (IS)	✓	✓	✓	✓	✓	✓	✓
Ingredient of Serum							
Caffeine	✓	✓	✓	✓	✓	✓	✓
Theobromine	–	✓	–	✓	–	–	✓
Sample 3	**IKC**	**IRM**	**HUG 1**	**HUG 2**	**UK**	**BDal 1**	**BDal 2**
Duloxetin	✓	✓	✓	✓	✓	✓	✓
Metoprolol	✓	✓	–	–	✓	✓	✓
Mirtazepine	✓	✓	✓	✓	✓	✓	✓
Nordoxepin	✓	✓	✓	✓	✓	✓	✓
D5-diazepam (IS)	✓	✓	✓	✓	✓	✓	✓
Ingredient of Serum							
Caffeine	✓	✓	✓	✓	✓	✓	✓
Theobromine	–	✓	–	–	–	–	–

Result reporting of interlaboratory test serum 2

Sample-ID	RV Se 2	Station	Amazon
Submitter	abderrahim karmime	Supervisor	System Administrator
Analysis Name	RV Se 2_8352_RA8_01_7572.d	Acquisition Date	15.06.2012 16:32:03
Sample Description	RV Se 2		

Base Peak Chromatogram



Library Search Results

Cmp Name	cmp #	Purity'	RT [min]	d RT [min]	m/z [Da]	d m/z [Da]	Intensity
Zolpidem	3	995	3.88	0.05	308.13	-0.05	2.8 E8
Midazolam	5	975	4.36	0.05	326.06	-0.03	1.0 E8
Trimipramine	8	881	4.96	0.00	295.16	-0.06	8.9 E7
Amitriptyline	7	994	4.80	0.09	278.14	-0.05	8.1 E7
Lidocaine	2	999	3.28	0.14	235.12	-0.06	8.1 E7
Caffeine	1	995	3.09	0.12	194.98	-0.11	1.7 E7
Alpha-hydroxymidazolam	6	958	4.37	0.09	342.04	-0.04	1.5 E7
D5-Diazepam	9	986	5.89	0.05	290.06	-0.05	1.0 E7
Fentanyl	4	998	4.28	0.07	337.21	-0.02	4.3 E6
Benzododecinium	10	928	6.59	-0.09	304.36	-0.95	7.5 E5

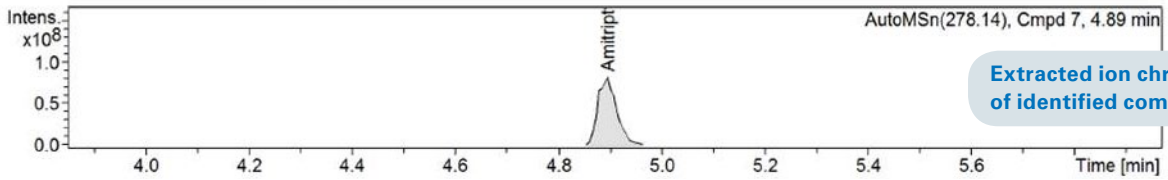
Search result table

Figure 2: Result reports can be accessed by the web or sent by e-mail.

Detailed MS and MS^2 spectra results of the Toxtyper reporting for Amitryptilin

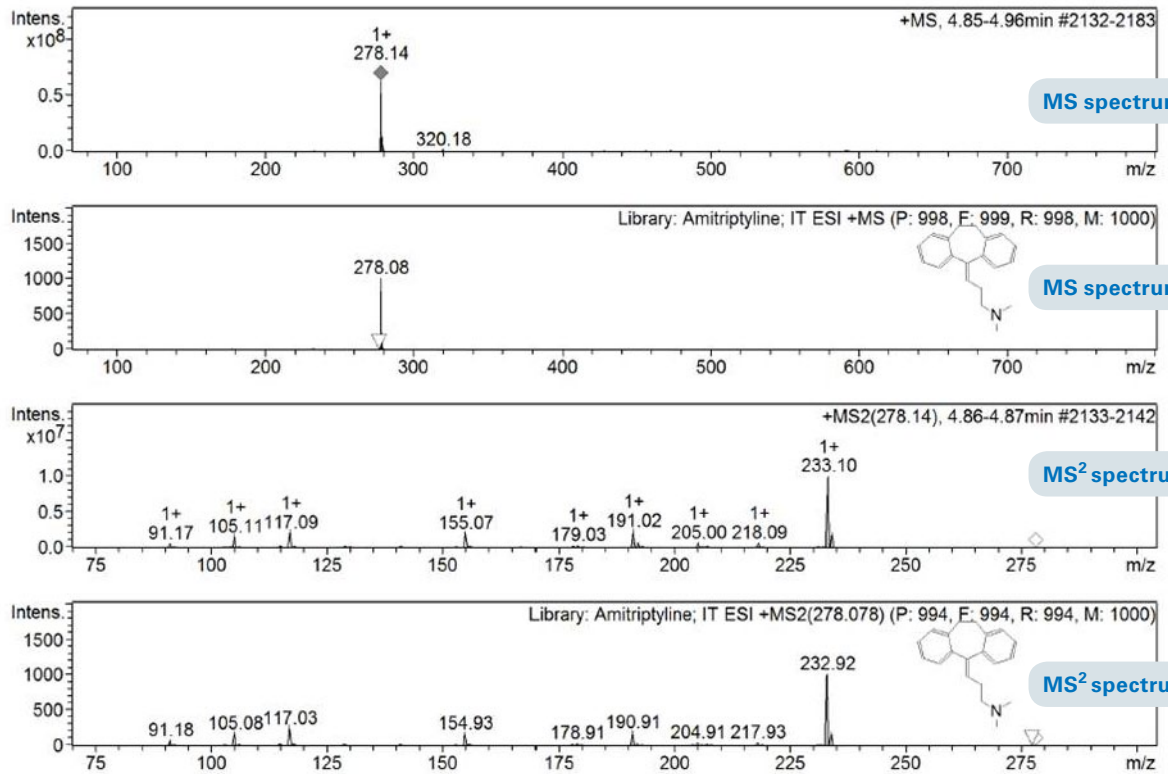
Cmpd 7, AutoMS^n (278.14), 4.89 min, Amitriptyline

Extracted Ion Chromatogram



Extracted ion chromatogram of identified compound

Compound Spectra



MS spectrum measured

MS spectrum library

MS^2 spectrum measured

MS^2 spectrum library

Figure 3: Result reports can be accessed by the web or sent by e-mail.

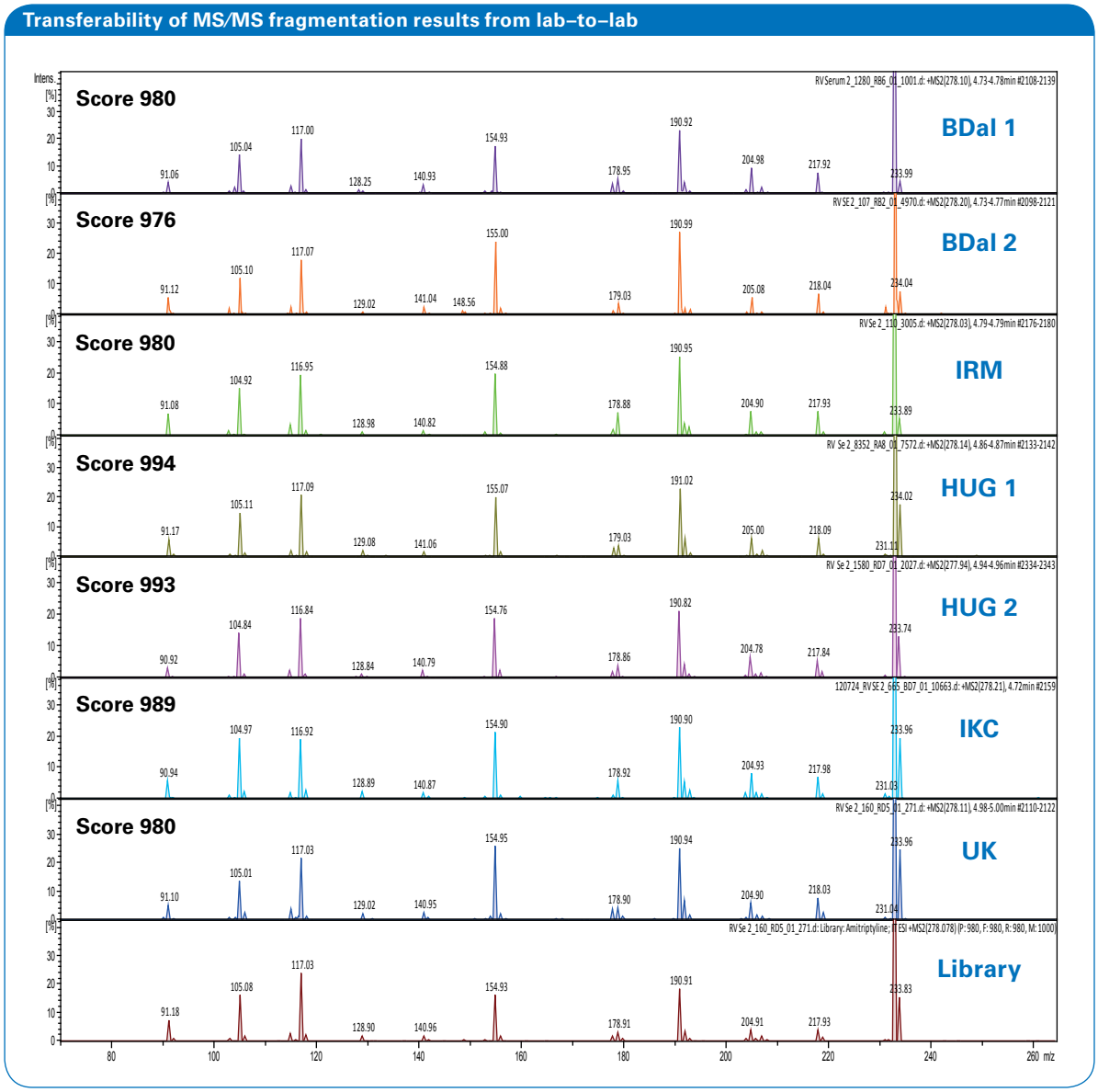


Figure 4: Shown are the MS^2 spectra of Amitryptilin measured on 7 different amaZon speed systems during the interlaboratory test.

Summary and Conclusion

The Toxtyper workflow offers a fast and robust identification tool for clinical and forensic analysis. The combination of MS^2/MS^3 spectral information and the respective retention time meets common criteria for identification of analytes. The results of the interlaboratory test demonstrated the efficiency and transferability of the complete workflow over seven independent systems in different clinical and research labs. The high rate of substances correctly identified in different laboratories reflects the superior performance of this approach. The high degree of automation offered by Compass OpenAccess is ideally suited for the transfer of this solution to routine laboratories. The use of additional libraries to solve specific questions offers further screening possibilities; for example, high-throughput screening of certain substance classes, such as illicit drugs.

References

[1] Hans H. Maurer, Karl Pfleger, Armin A. Weber: Mass Spectral and GC Data of Drugs, Poisons, Pesticides, Pollutants and Their Metabolites (2011)

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[3] Jürgen Kempf, Laura Huppertz, Susanne Vogt (Universitätsklinikum Freiburg, Germany), Markus Meyer, Sebastian Götz, Birgit Schneider (Bruker DaltoniK GmbH, Germany) (2012): Toxtyper - a Comprehensive LC-MS^n Screening Solution for Clinical and Forensic Toxicology; Bruker Application Note LCMS-72 #703614

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