# Online Data Processing Software in High-Throughput Screening Applications

## Enantiomeric Excess Determination of Chiral Compounds Using Mass Spectrometry

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***Abstract*—Measurement methods for the determination of the enantiomeric excess of chiral organic substrates usually are a bottleneck in high-throughput applications. Common analytical techniques such as liquid and gas chromatography, capillary electrophoresis, and spectroscopic techniques often require relative long measurement times and a high consumption of solvents and chiral materials. Hence, their application in high-throughput screening procedures is only possible to a limited extent. This requires the development of suitable measurement techniques, which enable the handling of high numbers of samples in a short time frame and which deliver relevant structural information. Parallel kinetic resolution in combination with mass spectrometry is a procedure for a fast and universally applicable enantiomeric excess determination. In previous studies high-throughput screening methods based on parallel kinetic resolution were developed for substance classes such as amino acids, amino alcohols, amino acid esters or natural chiral compounds. In such high-throughput measurements a high number of data points will be generated. Moreover, the data evaluation in enantiomeric excess determination includes some different steps compared to classical analytical tasks including a previous calibration based on the enantiomeric ratio. To provide additional functionalities, a software module based on Visual Basic was developed, which works in connection to the instruments workstation software. The processed mass spectrometric data of single substances or compound mixtures will be online evaluated synchronous to the measurements. Furthermore, the software module enables a final visualization and export of the results achieved. The software was applied in a high-throughput screening system, which includes a fully automated sample preparation, mass spectrometric measurements and data processing.**

***Index Terms*—Laboratory automation, automated sample preparation, software development, data processing, ESI-MS, chiral compounds, human health, environmental monitoring**

## I. Introduction

The biotechnology and the pharmaceutical industry have a great demand for high-throughput screening technologies and are driving forces for the development and optimization of automation processes [1]–[3]. In addition to classical high-throughput applications there is a growing need for automation solutions in the areas of quality control, food technology processes, medical chemistry [4], [5], forensics [6], [7], and increasingly in environmental applications [8]. An outstanding task is the determination of enantiomers. Chiral compounds are consisted of two molecular forms (enantiomers), which have the same physical properties with exception of their optical activity. Enantiomers also have the same chemical characteristics, but they may differ in their biological effects [9]. The influence of amino acid enantiomers related to plants [10], [11], animals [12], [13], and humans [14], [15] has been reported and shows their great potential in the field of drug development [16], [17]. In general, proteins are mainly built from L-amino acid enantiomers, but D-amino acids and their derivatives can also be found in living organisms. D-amino acids are distributed in food such as dairy products, sourdough, fruits and vegetables, and in a multitude of processed foods [18]. A further application for the determination of the enantiomeric excess of amino acids is the investigation in organic chemical evolution before the origin of life [19]. The monitoring of D- and L-amino acids can be used as a tool for dating of fossils [20] and as a tracer for biogeochemical processes in the environment [21]. This wide spectrum of applications shows the requirement of fast analysis methods for enantiomeric excess determination, in many cases in the scope of high throughput screenings.

## II. Analysis Techniques for Determination of Chiral Compounds

In qualitative and quantitative analysis of chiral compounds a multitude of well-established measurement techniques is available. This includes liquid and gas chromatography, capillary electrophoresis, and spectroscopic techniques [22]. A number of these conventional analytical techniques often involve relative time-consuming separation processes and a high consumption of solvents, chiral columns or additives. As a result, the high-throughput requirements can not be fulfilled and the analytical part is often a bottleneck in high-throughput applications [23]. In the past decades, a number of innovative mass spectrometry (MS) based methods was developed for chiral analysis, which enable short analysis times and show great potential in their application in fast screening procedures [24]. One of these promising methods are the parallel kinetic resolution [25], [26]. This process involves the derivatization of the chiral substrates, whereby each enantiomer reacts with two pseudoenantiomeric mass tagged auxiliaries. This results in four reaction products with two of them having the same molecule mass. The ratio of the measured amounts of these characteristic molecule masses corresponds to the enantiomeric composition of the sample. In previous studies the authors have adapted and optimized this

technique for various chiral substrates such as amino alcohols, amino acid esters and natural chiral compounds [27] as well as amino acids [28], [29]. The methods were applied in a system for automated sample preparation, analysis and data processing [30].

## III. System Overview

The complete high-throughput screening system includes the automated sample preparation using a liquid handler Biomek 2000 (Beckman Coulter) and a transport element, an ORCA lab robot (Beckman Coulter), for labware handling between the individual stations. The sample preparation comprises the following steps: dilution of samples and standards, creating standard solutions in various concentration levels, pipetting all solutions into microtiter plates, and the derivatization using the parallel kinetic resolution principle. The latter can be performed using a thermo shaker (Eppendorf) or with the multi parallel reactor HPMR 50-96 [31]. Reaction quenching and sample dilution for the subsequent analysis are tasks in sample post processing. After this, the mass spectrometric analysis will automatically be performed using an LC-MS system (Agilent Technologies) with the following components: G1379B vacuum degasser, G1312B binary pump, G1367C high-performance automated liquid sampler, and G1969A time-of-flight mass spectrometer (TOF-MS) with an electrospray ion source (ESI). This chromatography free mass spectrometric method enables analysis times lower than two minutes per sample [29]. The device control software (Agilent Technologies) and the data evaluation software (in-house development based on Visual Basic) are running on a PC inside of the automation system. Figure 1 shows the HTS system and the respective components.

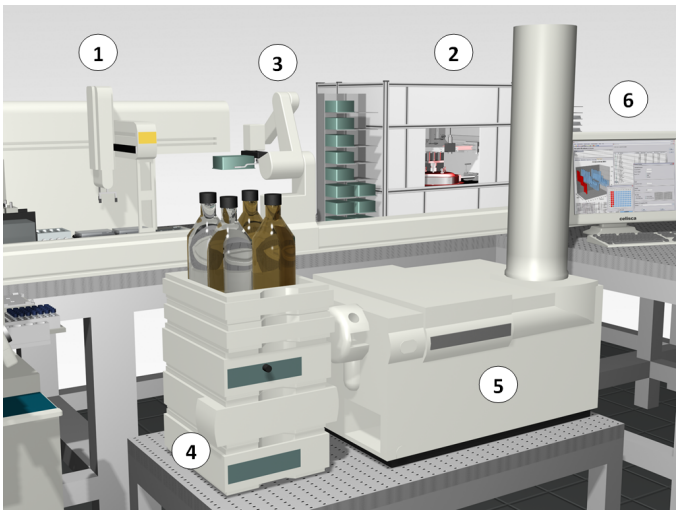


Fig. 1. HTS system - 1: Liquid handler, 2: Multi parallel reactor, 3: Transport element, 4: HPLC system, 5: Time-of-flight MS, 6: PC

## IV. Software Module for Data Evaluation

High-throughput methods for sample preparation and analysis enable measurements of a high number of samples in a relative short time frame. This generates a high amount of data points, which requires a suitable software solution for effective data processing. In the common way in the determination of the enantiomeric excess two calibrations will be generated - one for each enantiomer. Then the concentration of each enantiomer will be determined and the enantiomeric ratio is calculated. The latter step is often manually calculated. The software module presented in this study uses only one calibration based on the enantiomeric excess of the standard solutions (-100, -50, 0, 50, 100ee%). Using this calibration the enantiomeric excess of samples with an unknown chiral composition can be determined. The developed software module ChiralMS is an extension to the instruments software. The mass spectrometer is controlled and the data are acquired using the software MassHunter Acquisition (Agilent Technologies). For each sample one raw data file is stored. A second software MassHunter Qualification (Agilent Technologies) extracts the desired masses from the total ion current and integrates the peak areas of these masses. These values can be stored in several desired file formats such as Excel or PDF. The software module ChiralMS uses Excel files with these processed data as basic input. ChiralMS works either in online mode, synchronously to the measurements or in offline mode, if older data are to evaluate. Figure 2 visualizes the general data flow from the MS measurements to the data evaluation.

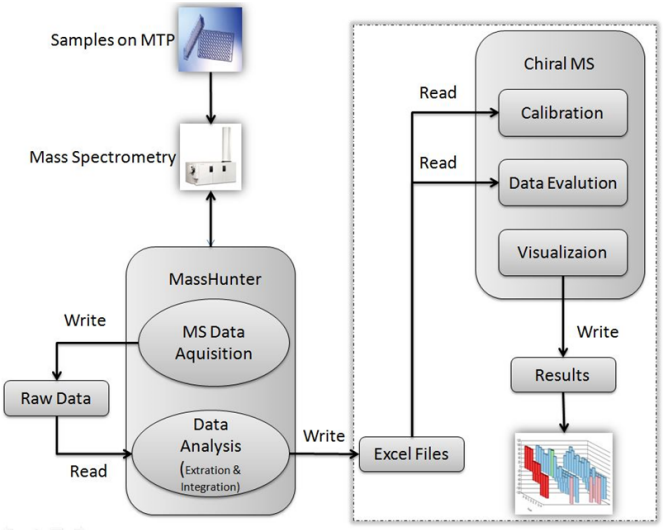


Fig. 2. General data flow between the device software and the in-house software module ChiralMS

## V. Implementation

### A. Graphical User Interface

The graphical user interface (GUI) is an important part in software design and implementation. The software GUI should be clear to users or researchers without or with low specific knowledge in computer science and programming. The basic menu bar provides the following submenus: File, Edit, Measurement, Print and Help. The submenu File includes functionalities concerning data files such as creating new

projects, open and export the measured data. After finishing the data evaluation, the results need to be stored and can be loaded whenever needed. The measured data are exported in an Excel file with the required data along with two output graphs. In the New Project submenu the parameter for the calibration such as the number of data points, replicates and blanks can be defined. The analyte expected in the sample solution must be selected previous to the data evaluation. Information about the analyte compounds are stored in a XML file, which can be edited and extended with new compounds in the submenu Compounds. This enables the addition, deletion and updating of chemical compounds or analyte classes. The recent compound table contains amino acids, amino alcohols, carboxylic acids, alcohols, amino acid esters and natural substances. The most important parameter is the Excel report files location (path) containing the processed data files. Furthermore, it is possible to load and use a previously saved calibration in current measurements. Other information such as operator, sample, date, and experiment specific notes can be entered. The Edit menu provides the sub menus Sample Arrangement, Cell Position Adjustment and ee% Range. Sample arrangement enables viewing the sample position on the microtiter plate or GC vial tray. In the sub menu Measurement the analysis mode (online or offline) can be selected, and the Save Calibration menu provides functionalities of saving calibration data for future use. In the lower part of the GUI the calibration and sample data are visualized using one 2D and two 3D charts. Figure 3 shows the GUI of the software module ChiralMS.

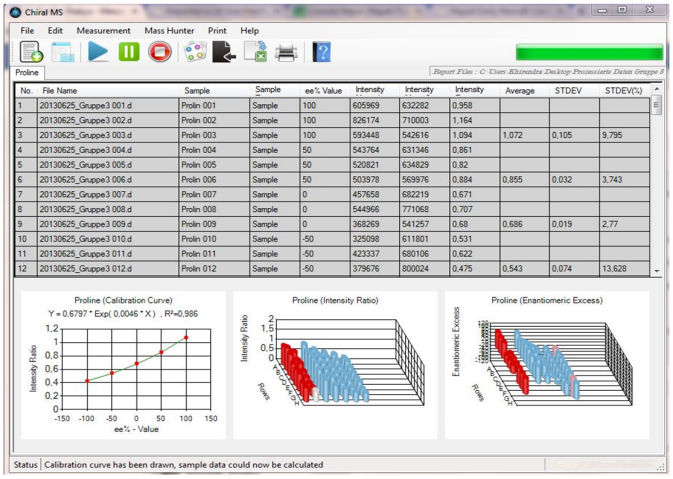


Fig. 3. GUI of the software module ChiralMS

## B. Analysis of Compound Mixtures

In case of analyzing a couple of compounds in one sample, the device software generates an Excel report file containing the values of the integrated peak areas of each compound derivative sorted according to the corresponding m/z value. Based on the information in the compound table (XML file) a search algorithm allocates the measurement values (peak areas) to the compounds. Figure 4 illustrates the data evaluation and enantiomeric excess calculation for a sample containing a mixture of the amino acids proline and valine.

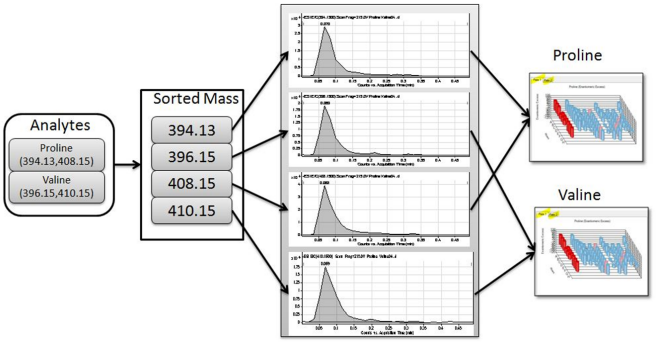


Fig. 4. Workflow of data evaluation and enantiomeric excess determination for sample mixtures (analytes: amino acids proline and valine)

## C. Handling of Variable Labware

The workflow of the data evaluation software module ChiralMS is able to determine the enantiomeric excess of samples on multiple numbers of microtiter plates (MTP) with 96 wells or GC vial plates with 54 vials. If a new project in ChiralMS is started, the user enters all required parameters such as number of data points, replicates and blanks for creating the calibration and drawing the corresponding chart. In addition, the user defines the number of samples with an unknown enantiomeric excess for the analysis. Further microtiter plates or GC vial plates can be added, which use the same calibration curve for the calculation of the enantiomeric excess. The system automatically updates the number of unknown samples to the measurement parameters. For example, samples on a 96-well microtiter plate: if the system detects more than 96 samples, a new microtiter plate will be added to the projects sample list. The number of data points per experiment is only limited by available memory in the used Excel version, because the results will be stored in Excel data format. For instance, Excel 2007/2010 provides maximum 1,048,576 rows. Hence, over ten thousand 96-well microtiter plates can be proceeded in one experiment if necessary. Further limitations are concerned to the process itself such as the re-filling of solvents and reagents, providing the sample solutions as well as storage issues.

## D. Chemical Compound Management

The chemical compound class is referred to a group of analytes contained in the sample solution with similar properties and behavior in parallel kinetic resolution. For example, compound classes are amino acids, amino alcohols, carboxylic acids, alcohols, amino acid esters and natural substances. Information on the compounds of each substance class is provided in a XML file. This includes the compound name and group, the molecular mass and the two ion masses (m/z value) of the reaction products (derivatives). For using the chemical compound features, a compound XML file should be created, if it does not exist. The software module ChiralMS reads this

XML file and displays the content in form of a table in the GUI. In Figure 5 the basic workflow is shown for this feature.

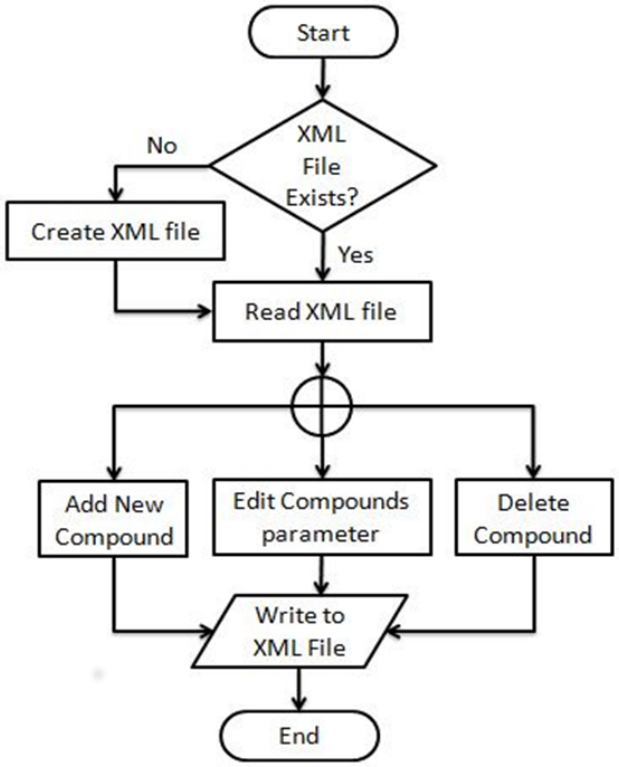


Fig. 5. Schematic workflow in compound management

A clearly designed data entry window enables the addition of new compounds, deleting compounds and the modification of a data set, if required. By creating a new ChiralMS project, the compounds contained in the XML file can be selected and added to the measurement properties list. Figure 6 shows the compound table.

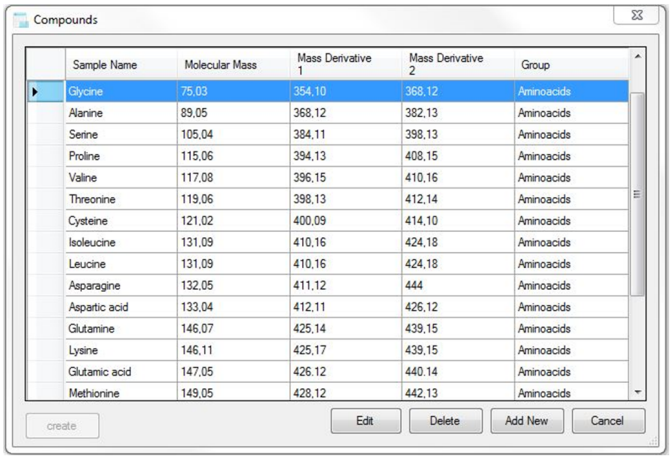


Fig. 6. Compound table with mass information (m/z values)

### *E. Online and Offline Operation Mode*

The software operation mode for automatic data input, creating the calibration data and calculation of the enantiomeric excess can be done in two modes: automatic or online operation mode and manual or offline operation mode. In case of the online operation mode, the data input is performed by reading the Excel report file with the processed data from the desired location whenever a new Excel report file is generated - usually after each sample measurement synchronous to the MS analysis. To avoid collisions and prevent errors - if two processes such as MassHunter Acquisition and ChiralMS access the same report file - the ChiralMS module pauses for thousand milliseconds, when it detects the new report file. This is sufficient time for the first process to finish. The basic workflow in the online operation mode includes the generation of the calibration data, and the results calculation and output sample by sample. The output data also involve the visualization of the measurement values, whereby the charts will be updated after each measurement: one 2D chart for drawing the calibration curve and two 3D charts for the intensity ratio and the enantiomeric excess. Thus the user will receive real-time information on the measurement results. In the offline operation mode, the system does not need a connection to the device software. The data input and processing is performed using previously created Excel report files. In this operation mode ChiralMS firstly reads all report files. After this, the calibration data and the corresponding chart are created and the enantiomeric excess of the samples is calculated. Finally, the two results charts are drawn. These operation modes enable the user to work in a flexible way in data evaluation of previous acquired data points.

### *F. Dynamic Sample Arrangement*

The dynamic sample arrangement assists the user in monitoring the sample arrangement on the MTP or GC vial plate in both online and offline operation mode. In case of the online operation mode, the sample arrangement is updated after each successful import of an Excel report file. In this file information are included regarding the sample position and sample type in the MTP or GC vial plate. Different sample types are coded with different colors for an easier distinction: calibration sample (red), blank (white) and unknown sample (green) (see Figure 7). The dynamic sample arrangement is based on 96 entries on a MTP. In case of a lower sample number unused wells are left without sample position information and filled with a default color (grey). To see the sample arrangement in offline operation mode, the input of all data from the Excel report files has to be finished.

## VI. High-Throughput Applications

The first version of the software module was implemented using Excel and Visual Basic for Applications (VBA). It enables an automated operation mode, which extracts the data and calculates the calibration as well as the enantiomeric excess after the measurement of all samples [27], [28]. This software also was connected to the instruments software and

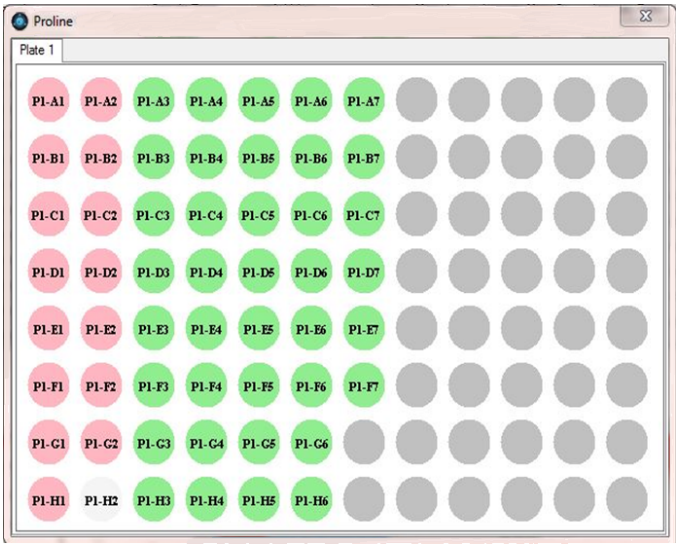


Fig. 7. Sample arrangement on a 96-well microtiter plate

starts the operations with a signal from the mass spectrometer, if the measurement run (worklist) was finished. To take the advantages of a stand alone software module without a host application such as Excel a second version was implemented using Visual Basic with the same functionalities but an improved graphical design [30]. The current version was extended to further functionalities as described above and updated with a new GUI design.

The software module ChiralMS enables a rapid enantiomeric excess determination and a fast data evaluation for a multitude of chiral compounds using parallel kinetic resolution and the mass spectrometry based method. About 80% up to 96% of processing time compared to classical analytical techniques can be saved and the sample throughput significantly increased [29]. Table 1 gives a comparison of the analysis times of common measurement techniques such as liquid (HPLC-MS, HPLC-DAD) and gas (GC-FID) chromatography, and the mass spectrometric method using parallel kinetic resolution and ESI-MS.

| **Measurement technique** | **Compound** | **Measurement time per sample [min]** | **Total time per 96-well MTP incl. sample preparation [h]** | **Time saving [%]** |
|---|---|---|---|---|
| 5 MS | 19 AA | 1.38 | 3.21 | |
| HPLC-MS | Pro | 10.00 | 18.00 | 82.18 |
| GC-FID | Ser, Glu | 48.60 | 81.26 | 96.05 |
| HPLC-DAD | Trp | 25.00 | 40.00 | 91.98 |
| | His | 10.00 | 16.00 | 79.95 |

TABLE I
COMPARISON OF MEASUREMENT TIMES FOR DETERMINATION OF CHIRAL COMPOUNDS USING COMMON MEASUREMENT TECHNIQUES AND THE MASS SPECTROMETRY BASED METHOD (AA: AMINO ACIDS) [29]

In summary, the software module implemented enables a fast data evaluation in the determination of the chiral composition. This advantage is particularly important when a new method for a new compound class is developed and validation experiments with a high number of samples are carried out. Also in routine screening applications this software significantly reduces the overall process times. Up to now a multitude of compound classes was investigated: carboxylic acids, alcohols, amino acid esters, amino alcohols, natural compounds [27] as well as amino acids [28], [29].

## VII. CONCLUDING REMARKS

The software module ChiralMS presented in this work for enantiomeric excess determination provides a promising approach in data evaluation in the scope of high-throughput screening applications. The principle of parallel kinetic resolution in combination with the analysis using ESI-MS enables a rapid analysis of a multitude of different compound classes. In addition, the software module ChiralMS connected to the instruments software allows fast data processing and a maximum of automation. Only one calibration curve for determination the chiral composition in form of the enantiomeric excess is required. The data processing can be performed in online operation mode synchronous to the measurements and additionally in manual operation mode. The fully automated sample preparation, the mass spectrometric analysis system, and the additional software module ChiralMS enable the determination of the chiral composition in a short time frame. Using 96-well microtiter plates analysis times lower than 2 min per sample are possible. The software ChiralMS enables a rapid online data processing, which is suitable for the application in high-throughput screenings.

## VIII. ACKNOWLEDGMENT

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## REFERENCES

[1] J. J. Burbaum, *Miniaturization technologies in HTS: How fast, how small, how soon?,* Drug Discov. Today, vol. 3, no. 7, pp. 313–322, 1998
[2] J. Hüser, High-Throughput Screening in Drug Discovery. Weinheim: Wiley-VCH, 2006
[3] J. N. Kyranos, High throughput analysis for early drug discovery. San Diego, CA: Elsevier Academic Press, 2004
[4] G. L. Horowitz, Z. Zaman, Blanckaert, Norbert J. C, D. W. Chan, J. A. DuBois, O. Golaz, N. Mensi, F. Keller, H. Stolz, K. Klingler, A. Marocchi, L. Prencipe, R. W. McLawhon, and O. Nilsen, *Modular Analytics: A New Approach to Automation in the Clinical Laboratory,* J. Autom. Method. Manag., vol. 2005, pp. 8–25, 2005
[5] K. Thurow and H. Fleischer, *Automatisierung für das Klinische Labor - Aktuelle Herausforderungen und Tendenzen,* GIT Labor-Fachzeitschrift, vol. 55, no. 7, pp. 466–467, 2011
[6] R. A. Middleberg, *Forensic Toxicology: Meeting the Demands of Two Slaves - Science and the Law,* Am. Lab., vol. 45, no. 1, pp. 41–43, 2014
[7] S. Usenko, B. Subedi, L. Aguilar, and E. Robinson, *High-throughput analysis of PPCPs, PCDD/Fs, and PCBs in biological matrices using GC-MS/MS,* Compr. Anal. Chem., vol. 61, pp. 143–158, 2013
[8] T. Chapman, *Lab automation and robotics: Automation on the move,* Nature, vol. 421, no. 6923, pp. 661, 663, 665-666, 2003
[9] S. W. Smith, *Chiral Toxicology: It's the Same Thing...Only Different,* Toxicol. Sci., vol. 110, no. 1, pp. 4–30, 2009

[10] T. Robinson, *D-amino acids in higher plants,* Life Sci., vol. 19, no. 8, pp. 1097–1102, 1976
[11] O. Forsum, H. Svennerstam, U. Ganeteg, and T. Nasholm, *Capacities and constraints of amino acid utilization in Arabidopsis,* New Phytol., vol. 179, no. 4, pp. 1058–1069, 2008
[12] T. Sasamura, A. Matsuda, and Y. Kokuba, *Tumor growth inhibition and nutritional effect of D-amino acid solution in AH109A hepatoma-bearing rats,* J. Nutr. Sci. Vitaminol., vol. 44, no. 1, pp. 79–87, 1998
[13] S. Esteban, C. Garau, S. Aparicio, D. Moranta, P. Barcelo, M. A. Fiol, and R. Rial, *Chronic melatonin treatment and its precursor L-tryptophan improve the monoaminergic neurotransmission and related behavior in the aged rat brain,* J. Pineal Res., vol. 48, no. 2, pp. 170–177, 2010
[14] G. C. Tsai, P. C. Yang, L. C. Chung, N. Lange, and J. T. Coyle, *D-serine added to antipsychotics for the treatment of schizophrenia,* Biol. Psychiat., vol. 44, no. 11, pp. 1081–1089, 1998
[15] R. Bhushan and R. Kumar, *Enantioresolution of DL-penicillamine,* Biomed. Chromatogr., vol. 24, no. 1, pp. 66–82, 2010
[16] S. C. Stinson, *Chiral pharmaceuticals,* Chem. Eng. News, vol. 79, no. 40, pp. 79–97, 2001
[17] A. M. Rouhi, *Chiral roundup - As pharmaceutical companies face bleak prospects, their suppliers diligently tend the fertile fields of chiral chemistry in varied ways,* Chem. Eng. News, vol. 80, no. 23, pp. 43–50, 2002
[18] M. Friedman, *Chemistry, nutrition, and microbiology of D-amino acids,* J. Agr. Food Chem., vol. 47, no. 9, pp. 3457–3479, 1999
[19] J. R. Cronin and S. Pizzarello, *Enantiomeric excesses in meteoritic amino acids,* Science, vol. 275, no. 5302, pp. 951–955, 1997
[20] V. R. Meyer, *Amino-Acid Racemization - A Tool for Dating?,* in Chiral Separations by Liquid Chromatography, ACS Symposium Series 471, pp. 217–227, 1991
[21] T. Dittmar, H. P. Fitznar, and G. Kattner, *Origin and biogeochemical cycling of organic nitrogen in the eastern Arctic Ocean as evident from D- and L-amino acids,* Geochim. Cosmochim. Ac., vol. 65, no. 22, pp. 4103–4114, 2001
[22] N. M. Maier, P. Franco, and W. Lindner, *Separation of enantiomers: needs, challenges, perspectives,* J Chromatogr A, vol. 906, no. 1-2, pp. 3–33, 2001.
[23] M. G. Finn, *Emerging methods for the rapid determination of enantiomeric excess,* Chirality, vol. 14, no. 7, pp. 534–540, 2002.
[24] K. A. Schug and W. Lindner, *Chiral molecular recognition for the detection and analysis of enantiomers by mass spectrometric methods,* J. Sep. Sci., vol. 28, no. 15, pp. 1932–1955, 2005.
[25] E. Vedejs and X. H. Chen, *Parallel kinetic resolution,* J. Am. Chem. Soc., vol. 119, no. 10, pp. 2584–2585, 1997.
[26] J. H. Guo, J. Y. Wu, G. Siuzdak, and M. G. Finn, *Measurement of Enantiomeric Excess by Kinetic Resolution and Mass Spectrometry,* Angew. Chem. Int. Edit., vol. 38, no. 12, pp. 1755–1758, 1999.
[27] H. Fleischer, D. Gördes, and K. Thurow, *High-Throughput Screening Application For Enantiomeric Excess Determination Using ESI-MS,* Am. Lab., vol. 41, no. 7, pp. 21–24, 2009.
[28] H. Fleischer and K. Thurow, *Rapid Enantiomeric Excess Determination of D- and L-Proline Using ESI-MS,* Am. Lab., vol. 43, no. 9, pp. 32–36, 2011.
[29] H. Fleischer and K. Thurow, *Fast Mass Spectrometry-based Enantiomeric Excess Determination of Proteinogenic Amino Acids,* Amino Acids, vol. 44, no. 3, pp. 1039–1051, 2013.
[30] H. Fleischer and K. Thurow, *Innovative software solution for special data evaluation in mass spectrometry,* Conference Record - IEEE Instrumentation and Measurement Technology Conference I²MTC, pp. 1624–1629, 2013.
[31] A. Allwardt, U. Dingerdissen, N. Stoll, K. Thurow, *An 8fold Parallel Reactor System for Combinatorial Catalysis Research,* J. Autom. Method. Manag., vol. 2006, art. no. 56329, pp. 1–9, 2006.