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Analyses of compound libraries obtained by high-throughput parallel synthesis: strategy of quality control by high-performance liquid chromatography, mass spectrometry and nuclear magnetic resonance techniques

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Abstract

The growing interest in combinatorial chemistry has led to a new source of compounds from which a large number of leads has emerged over recent years. Parallel synthesis, in particular, allows a quick production of a wide number of individual compounds. A rapid analytical control is needed to determine their quality. A strategy using automated, fast reversed-phase C18 high-performance liquid chromatography with diode-array detection (LC–DAD–MS) followed by atmospheric pressure chemical ionisation mass spectrometry (APCI–MS) and NMR has been developed for their characterisation and purity control. Complementary NMR analyses are done on selected compounds to provide a better structural characterisation of the expected compounds and their potential side-products. Validated libraries are then registered in ISIS databases using automated procedures. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Parallel synthesis; Compound libraries

1. Introduction

In the traditional concept (one compound–one test), the time of development was a long-term process with a small number of compounds under study. With the increased availability of high-throughput screening (screening of thousands of compounds against tens of targets), time of research needed to discover and develop more rapidly potential lead compounds can be reduced significantly. In order to be able to cope with the throughput in screening, scientists have been interested by the promise of combinatorial chemistry to allow rapid access to a large number of new chemicals. Pharmaceutical, biotechnology and agrochemical companies have developed in a first attempt large peptide combinatorial libraries (10^5–10^6 compounds) by split and mix synthesis, liquid synthesis or tea bag synthesis [1–6]. More and more pharmaceutical companies focus on small molecule libraries with lower numbers of products obtained by parallel synthesis based either on solid or liquid systems [7–9]. Many manual to semi-automated systems and automated robots are now available for liquid or solid-phase synthesis. To obtain as much structural information in the shortest time possible on large peptide libraries and small-molecules libraries, the first analytical methods have been developed with

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FIA–MS by electrospray (ESI) [10–20] and matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI–TOF) [21–26] with direct analysis of beads or LC–MS analysis.

In our laboratory parallel high-throughput synthesis of discrete compounds has been selected to produce libraries of hundreds to thousands of molecules. To assess the structure and purity of such large sample batches, traditional analytical approaches are evidently too time consuming to be applied. Consequently, fast analytical methods had to be developed based on automated reversed-phase C18 HPLC using short columns and fast gradient elution, coupled on-line with a diode-array detector (DAD) and a mass spectrometer (LC–DAD–MS), thus providing rapid characterisation by MS and purity control by UV absorbance. Atmospheric pressure ionisation (API) either electrospray (ESI) or atmospheric pressure chemical ionisation (APCI) used with LC–MS systems have considerably improved quantitative and qualitative trace analysis. In addition, the high sensitivity and selectivity of single and tandem mass spectrometric methods have decreased the time needed for method development and libraries analysis. MALDI–TOF analyses provide information on peptides libraries or on mixtures with a high sensitivity. To complete the LC–DAD–MS analysis, selected compounds are analysed by NMR providing additional structural information.

Traditional solution analytical techniques are used to follow the solution phase synthesis and various techniques have been reported to deal with the samples on solid supports [27]: FT-IR, Magic Angle Spinning NMR, gel-phase NMR, elemental analysis and MS. In this article, we describe our approach based on a combination of automation, generic methods, various analytical techniques and data management to assure the quality and to validate chemical libraries.

2. Experimental

2.1. HPLC instrumentation

Analytical HPLC analyses were performed using a Hewlett-Packard HP 1050 quaternary gradient system (Hewlett-Packard, Les Ulis, France) and controlled through the MassLynx software of the Micromass spectrometer (Micromass, Manchester, UK). A Waters DAD-996 was used to characterise and quantify expected compounds and side products. The DAD-996 was controlled by a Millennium work station (Waters, Saint-Quentin en Yvelines, France).

2.2. Atmospheric pressure chemical ionisation mass spectrometry

2.2.1. Instrumentation

All analyses were performed using a Micromass Platform2 for production libraries and either Micromass Platform2 or a Finnigan TSQ7000 (Finnigan, Les Ulis, France) for model libraries. Both mass spectrometers were equipped with an atmospheric pressure chemical ionisation source. The mass spectrometers were operated at unit resolution and mass spectra were acquired by scanning from 100 amu to 800 amu in 1.4 s.

2.2.2. Flow-injection analyses

For rapid analysis of library compounds, 10 μl of a 50 μ*M* analyte solution (70% acetonitrile in water) was injected at 2-min intervals by an autosampler Gilson 232XL (Gilson, Villiers-le-Bel, France). The carrier flow consisted of acetonitrile (70%) in water (30%) delivered by an HP 1050 system at a flow-rate of 0.15 ml min^{-1}. No split was used for the FIA–MS analyses.

2.2.3. LC–DAD–MS analyses

Analytical HPLC analyses were performed using either Kromasil C18 50–150 mm long×3 or 4 mm I. D. columns (AIT, Saint-Nom La Bretèche, France). For LC–DAD–MS analyses the mobile phase consisted of eluent A (water containing 0.02% TFA) and eluent B (acetonitrile containing 0.02% TFA). Compounds were separated using a gradient of 10% B to 100% B in 7–12 min depending on the column with a hold at 100% B from 0 to 5 min. Then, the column was equilibrated for 3–5 min at initial conditions between two runs. Typical flow-rate used was comprised between 0.6 ml min^{-1} and 1.5 ml min^{-1}.

For the Micromass Platform2 mass spectrometer, ion source temperature was set at 150°C, the vaporiser at 350°C and the voltage of the sample cone was fixed at a value comprised between 20 and 60 V

depending on the library. For the Finnigan TSQ7000 mass spectrometer, the heated capillary temperature was set to 240°C and the vaporiser to 270°C.

2.3. Nuclear magnetic resonance

All spectra were acquired on a AM-360 spectrometer from Bruker (Wissembourg, France). The frequencies were 360 MHz for proton and 338.8 MHz for fluorine. The temperature was set to 297 K. Usually, 1–3 mg of a compound selection were diluted in 0.5 ml of DMSO-d6.

2.4. Data management

The software package Chem-X from Oxford Molecular (Oxford, UK) is used by the chemists as a tool to design the libraries, to chose the reagents and to enumerate the chemical structures of the libraries. These structures are then imported via SD-files in an IsisBase corporate database (MDL, Basel, Switzerland), dedicated to combinatorial chemistry compounds, and general information is (semi)automatically added, like chemist name, chemical family, library name, compound reference and unique code. Lists of compounds with formulae and molecular masses are then exported as sample lists for the Micromass or the Finnigan mass spectrometer using the Isis SAR table functionality and Excel macros. The analytical results are automatically entered into this Isis database and then Excel spreadsheets are created to generate automatically electronic reports of the results for each library.

3. Results and discussion

3.1. Strategy

In combinatorial chemistry projects, the analytical part rapidly becomes a major bottleneck in the information process. Therefore, the choice of analytical techniques required for an optimal quality assurance is very important. In the past few years, different strategies of analysis and compound selection have been reported in a number of publications [20,27–29] and symposia. Some general items hold the attention but each strategy depends on the size of libraries (a few hundred compounds to millions of compounds), on the type of libraries (peptidic or non-peptidic), on the type of synthesis (one compound/well or mixtures). Apparently, the best strategy would be to apply all techniques for all compounds, but this has proven to be impossible in view of the high numbers of new chemicals generated. In our strategy, automated LC–DAD–MS and NMR are the technologies of choice to provide the chemists with all necessary information to allow an optimal fine-tuning of the reaction conditions.

In our laboratories, libraries are based on discrete, non-peptidic compounds, one per well, in a 96 microtiter plate format, with a size of about a few thousand compounds per library. The libraries are prepared in the context of lead discovery. To avoid as much as possible false positive results in the HTS process, priority has been given to the purity of each library or sub-library and the design of analytical tools has been optimised to reach these criteria. Great attention is paid to a good interaction between organic chemists and analytical chemists, in order to optimise the reaction conditions using so-called model libraries. Chemists design their libraries with the Chem-X software, linked to a database of commercially available reagents. The monomers for the final library are chosen based on a certain number of filters as dissimilarity, price, availability, etc. All monomers are then tested in model libraries in order to apply the reactivity of the monomers as final filter. It is of utmost importance to characterise as well as possible expected compounds and side products in the model library, necessitating a thorough analysis at this stage. All compounds in the model library undergo LC–DAD–MS and NMR analysis, to provide indications to the chemists where further optimisations may be needed, as reaction times and numbers of washing cycles in case of solid-phase synthesis. LC–DAD–MS conditions have been optimised to minimise side-products eluted at dose to the void volume (V_0). Indeed, side products have often been observed at V_0 when very short columns and very fast analysis were employed. Nevertheless, non-retained compounds are sometimes observed even when longer columns are used. In that case, the complementary NMR studies are really necessary, often allowing the ability to attribute concrete structures to observed m/z of major

impurities, to readjust the response factors of the compounds detected by UV absorbance, and especially to enable quantification of impurities too polar to be retained on reversed-phase columns or possessing insufficient chromophores to be detected by UV absorbance.

Once the model-library is validated, the production phase is started. In this phase, the analytical techniques serve only as a quality control, in order to ascertain that no problems with the synthesis, the robots, a change in reactivity by changing to another batch of reagents, etc. have occurred. Until recently, for production libraries, all our samples were submitted to FIA–MS, 25% were analysed by LC–DAD–MS to determine the purity and to identify major impurities, and 10–15% were characterised by NMR. To face the growing number of samples, and based on our experience, we have realised that optimal information about the quality of a production library may be obtained by LC–DAD–MS run on a statistical sample of 25% of the compounds (see Section 3.5), indicating both a purity level and the presence of the expected molecular ion. NMR analysis is still used at the beginning of the production of a new library to complement the analytical information.

Fig. 1 shows the role of the quality control in the validation of model library and production library. MS and NMR analyses afford complementary information for both model and production libraries.

After the analysis itself, the main bottleneck is the import and exploitation of raw data, transfer and printing of calculated data from the database and writing final analytical reports. A considerable effort has been dedicated to the automation of this process, and a number of companies, in collaboration with scientists of pharmaceutical companies, have developed softwares (Fig. 2) to reduce the time from the analysis to the reporting. Links and macros have been designed and built to increase the input and the output of information and to increase the quality control. After the ISIS-Base registration of libraries, a batch of information concerning each compound is directly downloaded in a selected sample list, which contains all the parameters needed to run the analysis and the post-acquisition process. A few standard procedures and acquisition files have been selected and saved to address different types of analysis (i.e. FIA–MS in the positive or negative mode with or without ion source collision or LC–DAD–MS with different LC gradients or wavelengths, or scanning modes, etc.). These predefined procedures reduce considerably errors or mistakes during the preparation of each batch of analysis, and under these conditions, the set-up of the analysis of a library takes only a few minutes. From the data selected for

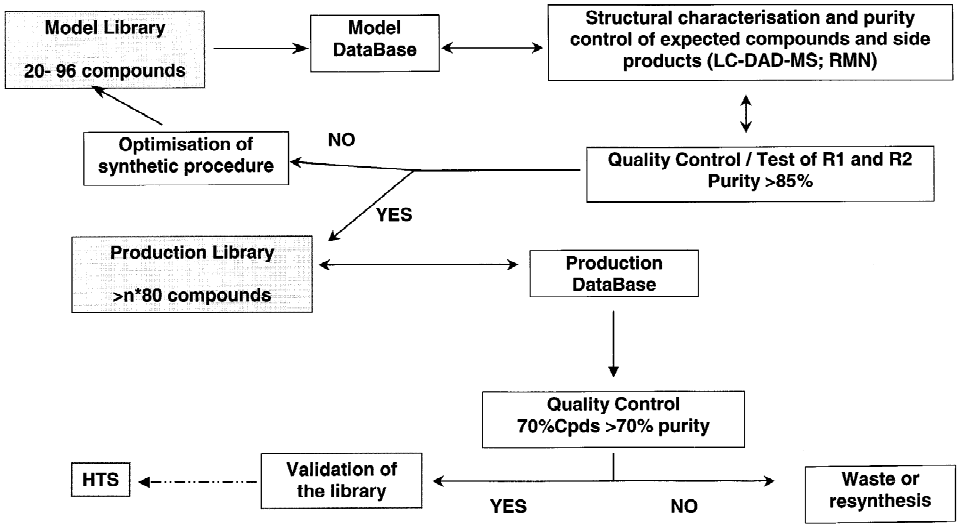

Fig. 1. Strategy of analysis of combinatorial projects.

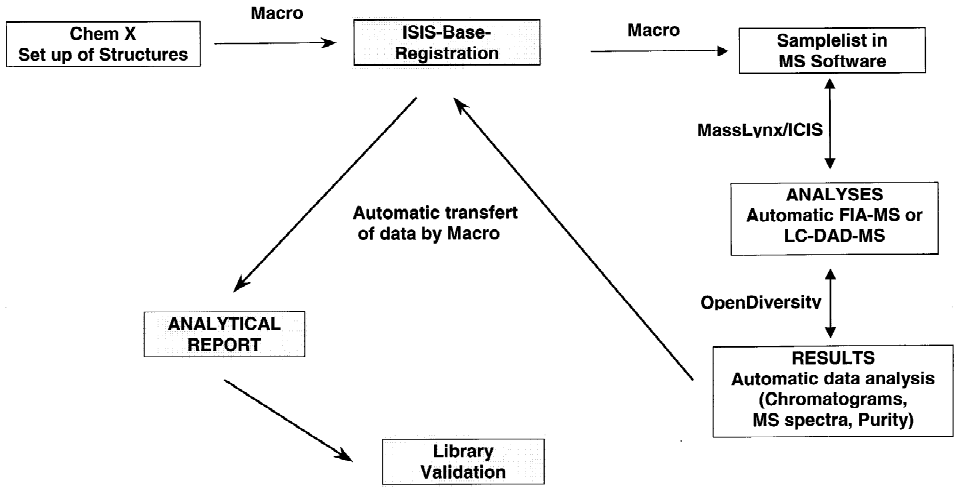

Fig. 2. Flow of information for model or production library.

each library in the database, electronic reports are automatically created and prepared by macros written in Excel. The automatic reporting allows us to reduce significantly the time of the analytical process and allows each scientist to focus primarily his work on data interpretation.

3.2. FIA–MS

The APCI mass spectra contain the protonated molecules with no or little fragmentation. In contrast to the reported privileged use of ES as the ionisation methodology, we have observed that the APCI methodology, in our hands, does provide a higher detection percentage. This may of course be due to the nature of our libraries, and it is clear that both methodologies should receive equal attention at the start of the analysis of novel chemical series. FIA–MS analyses done on production libraries allow us to determine the presence of the expected compounds in each well and in a lot of cases the presence of major known side products already described in the model library. Unfortunately, for some poorly ionised compounds or contaminated samples, the expected $[M+H]^+$ or $[M\text{-}H]^-$ is not observed and thus these samples need to be analysed by LC–DAD–MS to confirm the presence or the absence of the expected compound.

The major advantage of FIA–MS is speed of analysis but the method does not give any quantitative information on the purity of the sample in the absence of a standard and it often does not give the molecular ion for the above mentioned reasons. Therefore, priority was given to LC–DAD–MS as a more complete analytical tool.

3.3. LC–DAD–MS

The major reasons for the occurrence of side products in the solid-phase synthesis are often incomplete reactions, as coupling and deprotection steps, and incomplete washing cycles. Another form of side products may arise from partial destruction of the compounds during the often quite aggressive cleavage conditions. Reactions which may be observed are, for example, double coupling, internal cyclisation due to proximity of complementary reactive centres or partial removal of side chains of building blocks. It is thus of utmost importance to understand perfectly the nature of these side products, in order to be able to circumvent them by changing the reaction conditions. In view of the large number of compounds, LC–DAD–MS analyses have proven to be very instructive, since they allow us to calculate the purity of each well and determine the potential side products (identification, percentage).

Typical LC–DAD–MS analyses of single compounds with a total run time of 10 min are shown in Figs. 3 and 4. Fig. 3 illustrates the LC–DAD–MS of an expected compound with a purity >85%, no

significant side product has been detected. In Fig. 4, the expected compounds represent the main compound with two major side products corresponding to the desired derivative without the last alkylation and a derivative of R2. The characterisation of these side products has been confirmed by NMR experiments.

Software like OpenLynx Diversity from Micromass are very helpful for result evaluation but a quick check of the spectra by the analyst is still needed.

3.4. NMR

Although we used standard NMR probes with 5 mm tubes which is a slow throughput process, we still analysed a selection of compounds because of the valuable structural information generated by NMR. The time needed for the interpretation of ^{1}H-NMR spectra is clearly a bottleneck but this technique allows us to refine the purity obtained by HPLC with UV absorbance detection. Synthesis reagents, cleavage reagents and also residual solvents

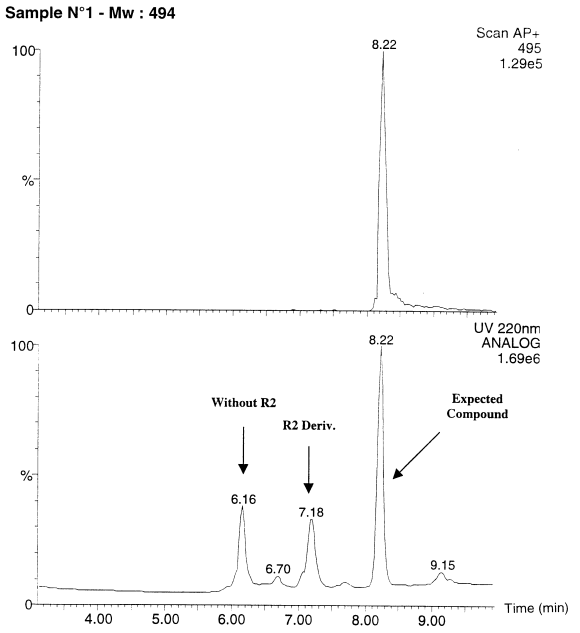

Fig. 4. LC–MS of a compound with side products obtained by parallel synthesis (purity <70%).

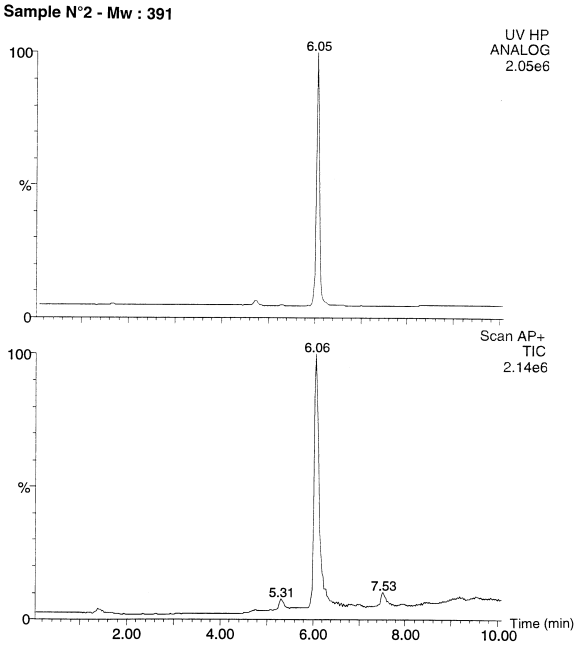

Fig. 3. LC–MS of a compound without a major side product obtained by parallel synthesis (purity >85%).

can be quantified by NMR. TFA which is often used for cleavage from the resin is easily observed by ^{19}F-NMR. Fig. 5 shows the NMR quantification of a sample containing residual solvents, reagents and by-products.

3.5. Quality control

To validate a library, LC–DAD–MS, NMR and sporadically FIA–MS data are automatically registered in ISIS databases and an analytical report in an Excel sheet is created. The validity of a library is based on the presence of the expected compound at a specified purity. As mentioned before, a retrospective analysis of data obtained from complete libraries, comparing this with the same analysis on a randomly chosen set of 25% and 50%, has allowed us to observe the incidence of a partial analysis on the final result. Table 1 summarises these data and shows that there is no or little incidence of a statistical trial of 50% or of 25% of a library. The

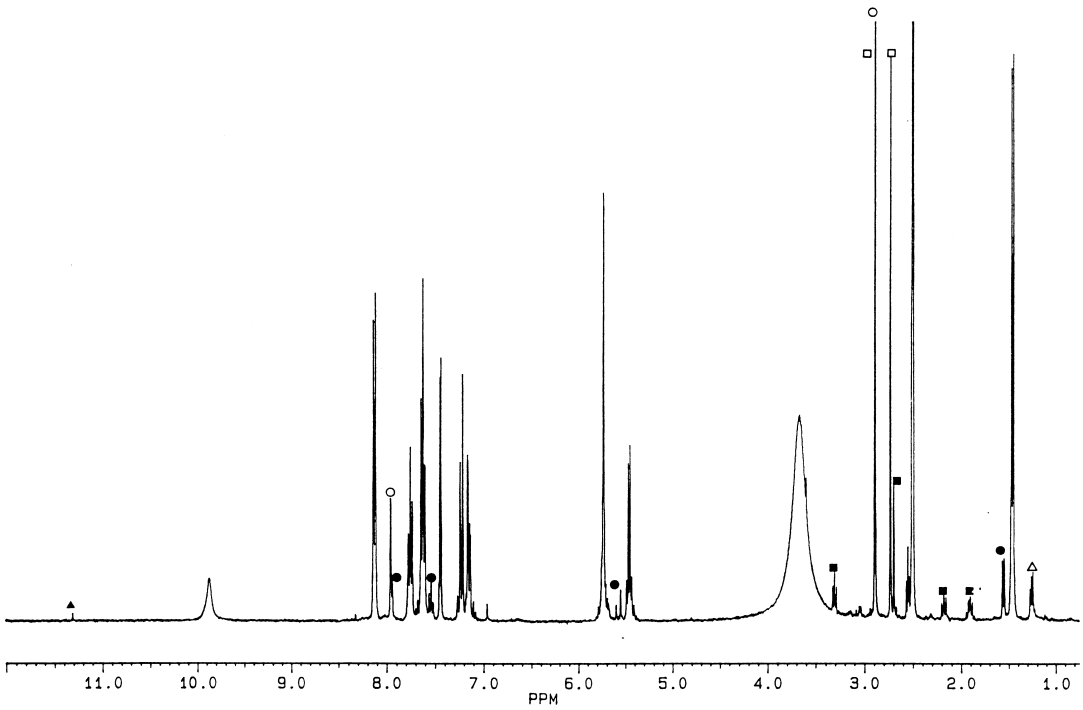

Fig. 5. 360 MHz ^{1}H-NMR of a compound obtained by combinatorial chemistry; *c*, ~10^{-2} *M*; solvent, DMSO-d^{6}; ●, compound+R2, ~10%; ▲, compound−R2, traces; ■, NMP, ~7%; ○, TMG, ~8%; □, DMF, ~15%; △, other, ~5%; NMR purity~55% (LC–MS purity ~85%).

purity calculated on a statistical set of 25% of a library is very close to those of the entire library.

When looking at the purity ranges of a given plate, one always observes from very pure samples to very impure compounds, and even some compounds may not be formed. This phenomenon may be attributed to different reactivities of the 'diverse' reagents, and even by different cross-reactivities. Our criteria for library validation for lead generation libraries is that 70% of the compounds show a purity superior to 70% by UV absorbance and that no major discrepancy is observed by NMR for the selected compounds. This purity level has allowed a successful lead discovery in a number of research programs, after confirmation of the biological activity carried out on a resynthesized and >95% pure sample. On the other hand, a false positive has been observed only in one case, due to a still uncharacterised side-product.

3.6. Stability

To ensure that the interpretation of the high-throughput screening data is properly done and correlated to the presence of a well-defined compound, stability control of the compounds is done regularly. Effectively, the stability of the compounds is a critical issue since all the libraries are stored for months or years as DMSO solutions at −20°C. From the few analyses that have been conducted we have found, as expected, that degradation is very structure dependent. For given libraries little or no degradation (<10%) was detected after one year, whereas for other ones up to 30% of the compounds were almost fully degraded. This reinforces the importance of structural confirmation of a biological hit before entering re-synthesis or SAR studies. Nevertheless, we re-analyse by LC–DAD–MS randomly selected compounds to evaluate the lifetime expectancy of the

Table 1
Range of LC–UV–MS purity

	% Analysed	0	0–50	50–70	70–85	85–100
Library 1	100	0.0	24.7	5.2	44.0	26.1
Library 1	50	0.0	18.7	4.2	50.0	27.1
Library 1	25	0.0	13.0	13.0	39.1	34.9
Mean		0.0	18.8	7.5	44.4	29.4
S.D.		0.0	5.9	4.8	5.5	4.8
Library 2	100	0.0	3.1	12.5	18.8	65.6
Library 2	50	0.0	4.2	6.2	8.3	81.3
Library 2	25	0.0	4.0	4.0	12.0	80.0
Mean		0.0	3.8	7.6	13.0	75.6
S.D.		0.0	0.6	4.4	5.3	8.7
Library 3	100	0.0	0.0	1.0	17.7	81.3
Library 3	50	0.0	0.0	0.0	18.8	81.2
Library 3	25	0.0	0.0	0.0	20.8	79.2
Mean		0.0	0.0	0.3	19.1	80.6
S.D.		0.0	0.0	0.6	1.6	1.2
Library 4	100	0.0	4.0	6.0	40.0	50.0
Library 4	50	0.0	6.2	8.3	43.5	42.0
Library 4	25	0.0	4.3	13.0	34.8	47.9
Mean		0.0	4.8	9.1	39.4	46.6
S.D.		0.0	1.2	3.6	4.4	4.1
Library 5	100	3.1	2.1	6.2	32.3	56.3
Library 5	50	4.2	0.0	2.1	35.4	58.3
Library 5	25	4.2	0.0	4.2	25.0	66.6
Mean		3.8	0.7	4.2	30.9	60.4
S.D.		0.6	1.2	2.1	5.3	5.5
Library 6	100	1.0	0.0	6.3	46.9	45.8
Library 6	50	2.1	0	8.3	45.8	43.8
Library 6	25	0	0	8.3	54.2	37.5
Mean		1.0	0.0	7.6	49.0	42.4
S.D.		1.1	0.0	1.2	4.6	4.3

libraries. If an interesting library must be screened and its purity becomes too low, a decision can be taken to synthesise a new batch or to eliminate this library from the screening process.

4. Conclusions

The continuously increasing speed of synthesis has had a big impact on the way analytical chemists have to plan their analyses. As with the synthesis itself, there is a continuous debate on how to deal with higher numbers without neglecting the quality aspects. Parts of the speeding up of the analytical process may find their origin in extensive automation of the data-management or cheminformatics flows. Another way to cope with the higher numbers during the production is to rely on a very thorough analysis of small partial libraries, so-called model libraries, before initiating the production of the actual libraries. By applying this strategy, quality control of the production libraries necessitates only statistical analysis, without compromising the quality of this analysis.

The strategy reported here describes our development of high-throughput analysis tools for model and production libraries in the context of lead discovery programs, and is based on a perfect integration of different softwares, and an optimal use of NMR and LC–DAD–MS analysis. Each step of the analytical process has been studied to reduce the bottlenecks and to increase the quality control of each library.

The reduction of manual sample handling and data entry reduce the delay and improve the quality by avoiding mistakes and errors, and allows us to validate carefully and rapidly the prepared libraries. The comparison of LC–DAD–MS and NMR data enables satisfying structure determination of potential side products and reagents present in the sample besides the expected compound, and gives the chemists the means to fine tune the reaction conditions in order to minimise problems during the synthesis of thousands of compounds in the production process. Fast reanalysis procedures ascertain that the results of a screening are directly correlated to a well-defined compound in a known sample.

The computer and analytical systems described here have proved to be reliable over two years of extensive use with thousands of chemicals. Notwithstanding the diverse structures of non-peptidic samples and the concentration of expected compounds and side products, quality control analyses have been made in good conditions using both Micromass Platform2 and Finnigan TSQ-7000 mass spectrometers.

The analytical strategy described shows that it is possible to obtain optimal information by mainly LC–DAD–MS and NMR analyses, linked to a strategy employing model and production libraries, to characterise and validate libraries of thousands of derivatives with no major bottlenecks in the analytical and the data management process. Continuous developments in the field, such as miniaturization, more automated instruments, faster sample preparations, etc., will hopefully enable analytical chemists to cope with the ever increasing number of new chemicals produced by combinatorial chemistry without losing track of the quality.

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