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Versatile Monitoring Tools in Parallel Solid-Phase Synthesis

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Abstract: Parallel synthesis on solid-phase is routinely applied in our combinatorial chemistry efforts for lead finding and lead optimization in drug discovery. The synthetic products are released in solution, isolated individually, and purified, but most of the synthetic process is carried out on solid-phase. This facilitates lab automation and enhances throughput. The vastly increased synthetic productivity poses the question of how to best characterize the quality of compound libraries in an efficient and accurate way. In order to provide maximal information with minimal sample consumption, we have integrated a combination of analytical techniques in our workflow. The techniques, comprising IR, NMR, LC-MS, and HPLC online nitrogen detection (CLND), are illustrated on simple examples, but are usually applied to hundreds of molecules, and recurrently, within the timeframe of a library production.

Keywords: Chemiluminescent nitrogen detector · Combinatorial chemistry · High-throughput analytics · Magic angle spinning NMR · Solid-phase analytics

1. Introduction

Solid-phase synthesis is a key methodology in the industrial context of high-throughput processes and laboratory automation. The number of synthetic methods adapted to the solid-phase format has increased significantly over recent years, [1] thus creating new opportunities for useful applications. For instance, the combinatorial derivatization of solid-supported heterocyclic scaffolds represents nowadays a rich source of novel drug-like compounds. This burgeoning synthetic activity poses the question of how to characterize the quality of compound libraries and the validity of their preparation process in an efficient and accurate way. A full characterization of each library component by the ordinary routines of analytical chemistry, including elemental analysis, may not be feasible (with reasonable efficiency) for large arrays of compounds obtained in non-crystalline form. While liquid chromatography in conjunction with mass spectrometry (LC-MS) is the most widespread methodology used for high-throughput characterization of combinatorial chemistry samples, its information content is evidently incomplete.

Here we briefly discuss how a variety of analytical tools are used in microscale formats, also for direct detection on solid-phase, so as to integrate well with the overall workflow of combinatorial chemistry.

2. Monitoring 'Tool Box'

In order to monitor effectively the quality of combinatorial chemistry processes and products, it is advantageous to maintain a set of carefully selected analytical techniques. Key qualitative and quantitative information must be obtained from (typically) amorphous samples in a timely fashion, with minimal sample consumption. The main selection of analytical tools applied in our parallel synthesis workflow is summarized in the Table.

The individual peculiarities of each technique ensure that all stages of a solid-phase synthesis process, from synthesis optimization to compound library production, are covered with an appropriate monitoring tool. None of the methods is, by itself, indispensable. In principle, all traditional methodologies maintain their validity, as there is no intrinsic difference between compounds synthesized individually, and compounds obtained in a parallel

Table. Principal analytical methods ('Tool Box') in combinatorial chemistry

Technique	Applicable directly on solid phase	Quantitative[a] (routinely)	Non-destructive	Measuring format (typical)
IR on resin	yes	-	-[b]	Solid resin beads (IR-ATR) or KBr pellet
Magic Angle Spinning NMR	yes	-	yes	1-3 mg resin in 40 µl CD_2Cl_2
NMR in solution: DMSO-d_6	-	yes	yes	2 µMol as 3.33 mM DMSO-d_6 sol.
Direct-injection NMR: DMSO-H_6	-	-	yes	400 µl 4 mM sol. in DMSO-H_6
LC-MS	-	-	-	HPLC online (inj. 10 µl 1 mM sol. in DMSO)
Nitrogen Detection (CLND)[c]	-	yes	-	HPLC online (inj. 10 µl 1 mM sol. in DMSO)

[a] Routinely used for quantitation of samples; [b] Recovery of material is usually not worth pursuing; [c] Chemiluminescent Nitrogen Detection

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process. The outlined selection of modern methods is solely based on the fact that combinatorial chemistry compounds are frequently obtained in large numbers, in small amounts, in non-crystalline form, and often *via* intermediates grafted on a solid support.

In this communication our commonly deployed methods are discussed on a scholastic case, which is representative of more complex situations. While our focus is on highlighting the role of various analytical techniques, also the synthetic steps illustrated here have some general meaning. They are initial steps, which could extend and diversify in a variety of different pathways.

3. Application Case: Reductive Amination of a Formyl Resin for Subsequent Derivatization

Support-bound aldehyde linkers are useful starting points for combinatorial syntheses on solid-phase [2]. In our example, a first site of diversity is introduced *via* reductive amination with a primary amine, generating a supported secondary amine, ready for subsequent derivatization steps [3]. If a whole set of different amines is reacted in parallel, a variety of intermediates can be prepared (Scheme). The potential use of such resin-bound secondary amines is very broad.

Analytical tools have an important role not only as quality control elements of a library production, but also in the investigational phase, before inception of production. The combinatorial synthesis scheme needs to be validated, *i.e.* the envisaged preparation process must be assessed for its broad applicability and robustness. The applied emphasis and significance of individual analytical technologies vary as the process evolves from an exploratory study to the actual production. The techniques, which we frequently use in our laboratories, are listed in the Table and exemplified in the following sections (see also the Scheme).

The preliminary evaluation of step 1 involved the reaction of a series of diverse primary amines with the commercially available (4-formyl-3-methoxyphenoxy)-butyrylaminomethyl resin (AMEBA) [2] in parallel, in a systematic comparison of different reaction conditions. The various tested conditions influence the product yield and purity. The analytical characterization provides information on the key factors affecting the reaction. A full report of the systematic study is beyond the scope of this article. The experiments reported here were chosen for their value in illustrating a method's role and are not a documentation of optimized conditions.

The second step was carried out, as an example, with 48 compounds in parallel: carboxamido, sulfonamido, and urea derivatives were prepared [4].

3.1. *Infrared Spectroscopy (IR)*

IR spectroscopy is a straightforward, simple monitoring tool for the evaluation of reaction steps directly on solid-phase [5]. The development of diagnostic absorption bands of starting materials or products can be followed with comparative measurements. The decrease of an absorption band is a measure of the starting material conversion. Fig. 1 illustrates the disappearance of the aldehyde stretching band at 2765 cm^{-1} upon completion of the reductive amination [6].

For absolute quantitation, the amount loaded on the resin must be determined first by other methods (*e.g.* post-cleavage with UV spectrophotometry, CLND or quantitative NMR). If IR spectra are recorded from KBr pellets, 1–3 mg of resin are needed and the sample is not recovered.

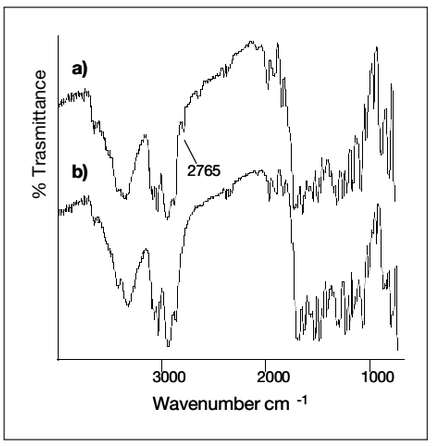
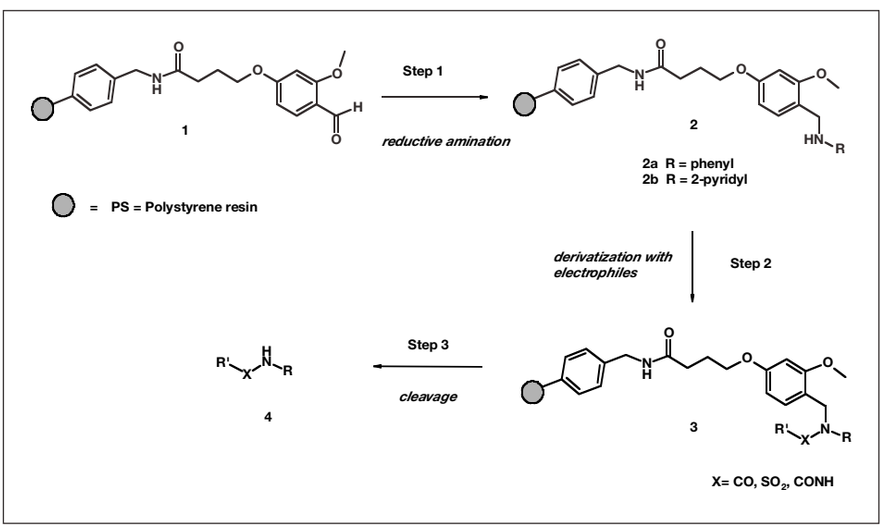


Fig. 1. Monitoring the reaction progress of step 1 with FT IR (KBr pellet method).

Internal reflection spectroscopy, also known as attenuated total reflectance (ATR), is a non-destructive technique, which measures on the surface of the analyte [7]. In combinatorial chemistry, internal reflection spectroscopy can be applied to both spherical beads [8], as well as to polymer substrates such as pins or crowns [5].

3.2. *Nuclear Magnetic Resonance (NMR)*

NMR is a most powerful tool, which could potentially answer all major analytical questions a combinatorial chemist would ask. In practice, its use is limited to some extent for reasons of efficiency, considering the lack of (sufficiently robust) fully automated spectra interpretation soft-



Scheme. Loading of primary amines on (4-formyl-3-methoxyphenoxy)-butyrylaminomethyl resin (AMEBA) resin, and subsequent derivatization and cleavage

ware. Other limiting factors to consider are the investment costs required for enhancing sensitivity and automation. The versatility of NMR is outstanding, ranging from analyses on swollen resin beads to precise quantitation of final products (not to mention the multitude of applications beyond the combinatorial chemistry area).

3.2.1. *NMR on Solid-supported Intermediates*

Historically, high-resolution ^{1}H-NMR spectra of resin-bound compounds were usually acquired on special supports [9], bearing highly flexible spacers, such as polyoxyethylene chains (*e.g.* TentaGel™ from Rapp-Polymere GmbH), in order to minimize line-broadening effects. The so called 'magic angle spinning' (MAS) technique [10] was soon applied more often also on more common, all-polystyrene resins. Fig. 2 gives an impression of the MAS experiment types run in our laboratories [11].

The superpositioned 1D traces indicate how simple 1D experiments may be tuned to provide reasonably well-resolved spectra from common polystyrene. The method leverages on the use of a spin-echo pulse sequence with appropriate delay times, leading to the suppression of signals from the rotationally restricted polymer atoms. The spin-echo series of spectra is produced by the CPMG-T2 pulse sequence [12], where a variable delay (the spin-echo delay) discriminates the signals on the basis of their mobility. As these delays increase, the signals of the bulk resin are the first to disappear, followed by the signals of the linker, and then by those of the bound moieties. In this way, it is possible to 'extract' the signals of the bound compounds that are obscured by the broad (polystyrene) hump, *e.g.* the signal around 6.5 ppm. The signals of residual solvents (*e.g.* DCM, DMF, *etc.*) are not much affected by this method and are easily identified.

The gradient-enhanced heteronuclear single-quantum correlation [13] (gHSQCe) 2D spectrum is one of the many indirect detection heteronuclear (^{1}H-^{13}C) correlation experiments available in the NMR literature. We use the edited version (the 'e' in the acronym), because it allows the discrimination of CH_2 *vs.* CH and CH_3 on the basis of the opposite phase of their signals produced by the experiments. CH_2 groups are generally displayed as negative, while the CH and CH_3 groups are positive. A further discrimination of CH *vs.* CH_3 is possible by means of the ^{1}H and ^{13}C chemical shifts. The signals around 3.5 ppm in the ^{1}H dimension (F2) and at about 50 ppm in the ^{13}C dimension (F1) are clearly derived from the Ar-O-CH_3 moieties.

NMR analysis can provide invaluable information about the quality of solid-supported intermediates. Side-products or undesirable functionalities can be detected even if they cannot be cleaved and released in solution. Fig. 3 and Fig. 4 illustrate analyses of the reductive amination of AMEBA resin [2] with 2-aminopyridine [3]. Fig. 3 provides evidence that the reaction did not proceed optimally.

The residual presence of unreacted linker (CHO function) is visible, but also the undesired appearance of reduced linker (-CH_2OH function) can be seen.

We also acquired homonuclear 2D double-quantum (DQ) MAS NMR spectra [14] to confirm the presence of the pyridine moiety [15] (see Fig. 4).

Depending on the chemistry envisaged for subsequent synthesis steps, the presence of free aldehyde and alcohol functions could be harmful. With only a conventional HPLC analysis of cleaved intermediates in solution, this kind of problem could not be brought to the chemist's attention. The fact that commercial resins are, unfortunately, rarely characterized by NMR in the manufacturer's lab, prompted us to control the quality of derivatized and pre-loaded resins routinely by NMR. Indeed we have been confronted with sporadic cases of faulty resin batches.

Occasionally, we also use NMR for semi-quantitative assessments of incomplete chemical transformations on solid-phase (see Fig. 5).

It is possible to calculate the conversion of a reaction or the relative content of a side-product, assuming each signal is assigned. The example of Fig. 5 shows overlapped signals originating from the desired product (H(9) with X = NH-Py), two common groups (H(8), H(18)) and a side-product (H(9) with X = OH). After a computational deconvolution of the spectral region, a list of individual signals and their relative integrals (indicative of the relative amount of the two compounds) is produced [16].

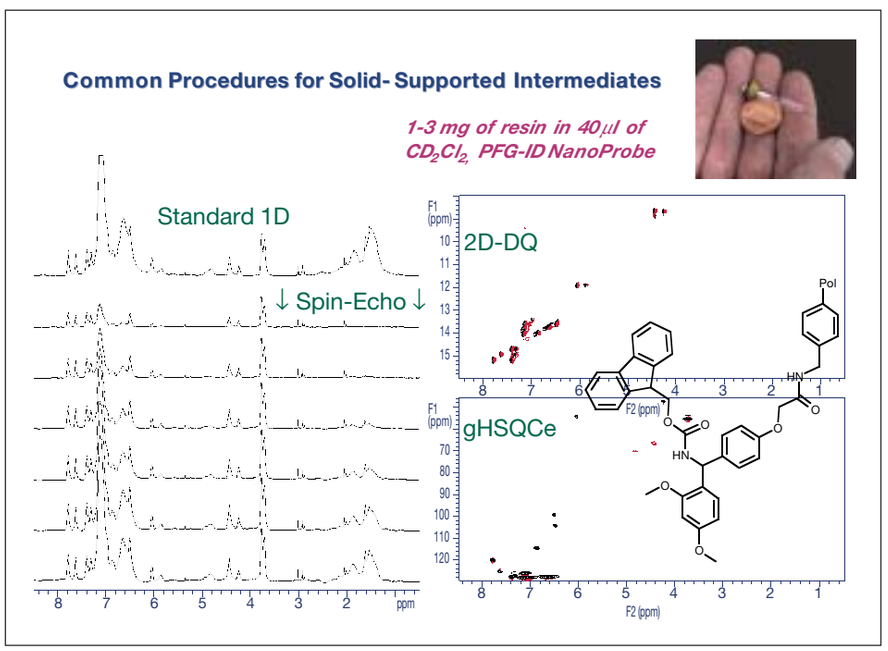


Fig. 2. Simple 1D spectra (acquired in ~1 min) are placed side-by-side with more demanding 2D experiments (10–20 min for 2D-DQ, 40 min to 2 h for gHSQCe). In the spin-echo series of 1D spectra the delay (see text) is increased from bottom to top. In the gHSQCe spectrum the black cross-peaks derive from CH and CH_3, and the red peaks from CH_2.

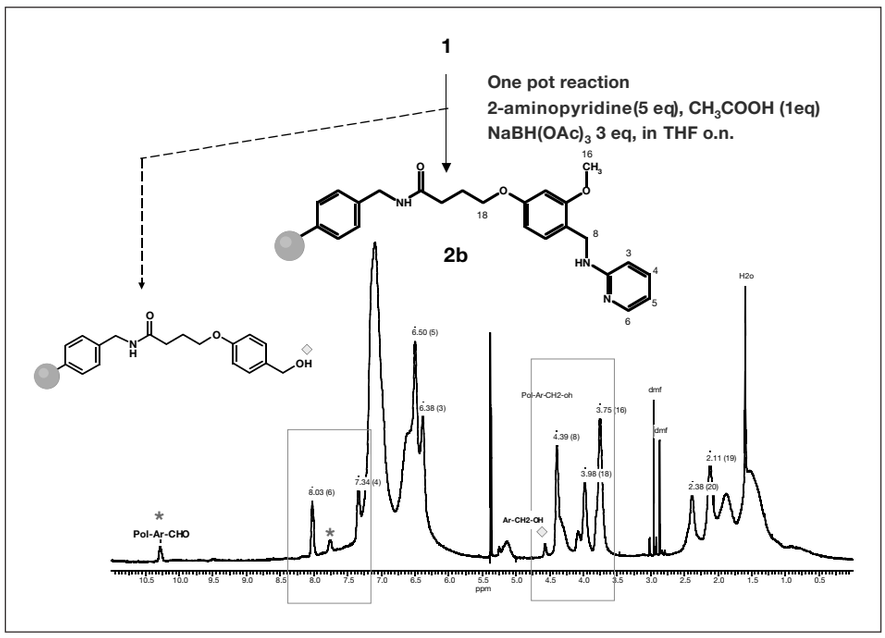


Fig. 3. The spectrum shows a 10.30 ppm signal from the presence of residual –CHO. A new signal at 4.39 ppm indicates the newly formed methylene group (ortho with respect to $-OCH_3$). Furthermore, two new aromatic signals are clearly distinct from the broad aromatic region of the polymeric matrix.

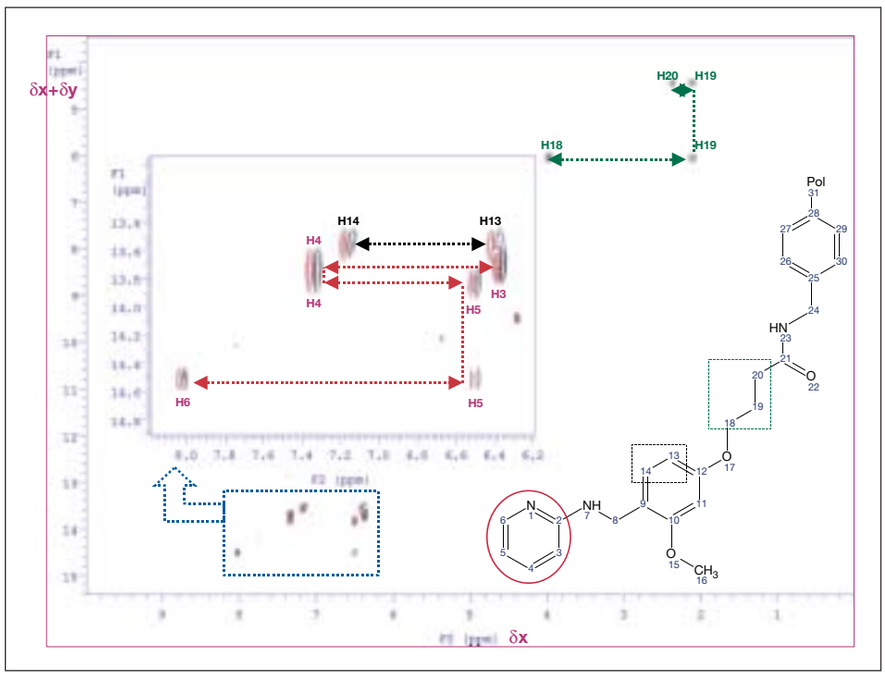


Fig. 4. 2D-DQ MAS NMR spectrum of the 2-aminopyridyl derivatization of the polystyrene based AMEBA resin. Note that in 2D-DQ NMR spectra the two dimensions have a different meaning: F2 shows the chemical shift of the coupled protons, *e.g.* H(6) and H(5), while F1 represents the sum of the two chemical shifts, *e.g.* δH(6)+δH(5) and δH(5)+δH(6). The 2-pyridyl group derived spin system is highlighted in the central expansion. Red arrows connect the double quantum cross-peaks. The other aromatic ring is indicated by the black arrows. The green arrows show the spin system of the aliphatic spacer.

3.2.2. *NMR in Solution*

3.2.2.1. *Accurate NMR Quantitation with BTMSB Standard*

A robust, validated NMR procedure for the generic quantitation of small organic molecules in DMSO-d_6 solution was developed in the analytical laboratories, which (in our organization) are responsible for the final quality control of the newly synthesized research compounds [17]. The method is based on the comparison of a molecule's diagnostic signals with the internal reference signal of a novel silane standard, 1,4-bis-trimethylsilylbenzene (BTMSB). This is an easily weighable solid with low volatility, which is stable for at least one month in DMSO solution, and possesses a strong singlet in a region usually free of signals (Fig. 6).

After synthesis, work-up and purification, combinatorial chemistry compounds are usually dissolved in DMSO to form a stock solution. The concentration is estimated either on assumptions or on sample weights. The latter are usually an inaccurate measure, when small amounts of amorphous residues (~1–15 mg) are handled, and contaminants (*e.g.* solvent inclusions, salts) may be present. Spectral interpretation is mandatory for the application of this quantitation method. Keeping in mind this limitation, the NMR measurements are an excellent measure for the determination of compound purity (1–2% accuracy) on multiple samples, using a single reference compound. Under the defined experimental conditions of the validated protocol, we can use NMR as a universal quantitation detector, because the response factor is proportional to the number of nuclei associated with a given signal [18]. Fig. 7 represents the normal, favorable situation, where a distinct diagnostic signal can be assigned, measured and compared with the internal reference signal upfield. Usually this kind of experiments is run in deuterated DMSO as described [18], but also measurements in non-deuterated DMSO-H_6 (with direct-injection in a high-throughput mode) are possible, if an appropriate solvent suppression pulse sequence is applied (see next Section). As indicated in Fig. 7, the 70% strength values determined in these two different formats coincide. They also match very well with the 72% peak area integration value of the HPLC-UV trace (at 220 nm wavelength).

In Fig. 8 a more problematic situation occurs, because the UV trace is not able to reveal the presence of important amounts of contaminating cycloaliphatic material, which had not been effectively washed off from the resin. The NMR spectrum resolves this problem by giving a more complete,

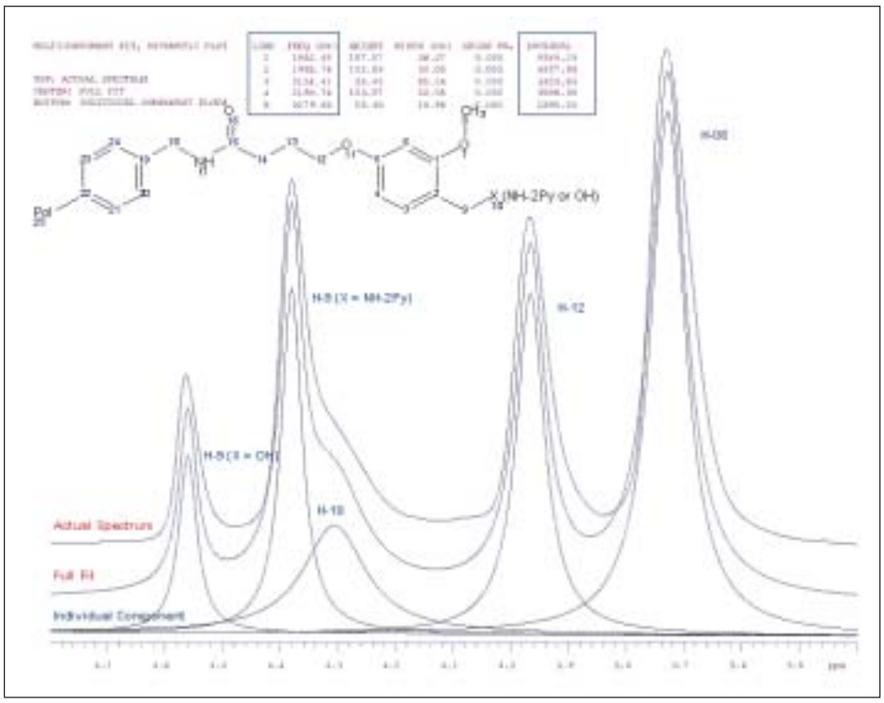


Fig. 5. Deconvolution of the region from 3.5 to 4.7 ppm of a ^{1}H HR-MAS NMR spectrum containing overlapped signals. The boxes highlight the numerical results of individual lines, their frequencies and their integrals, as produced by the method. The actually measured spectrum is the trace at the top. The calculated (Full Fit) and the individual component signals are shown underneath. The quality of the fit can be judged easily by visual comparison of the actual and the 'Full Fit' spectrum.

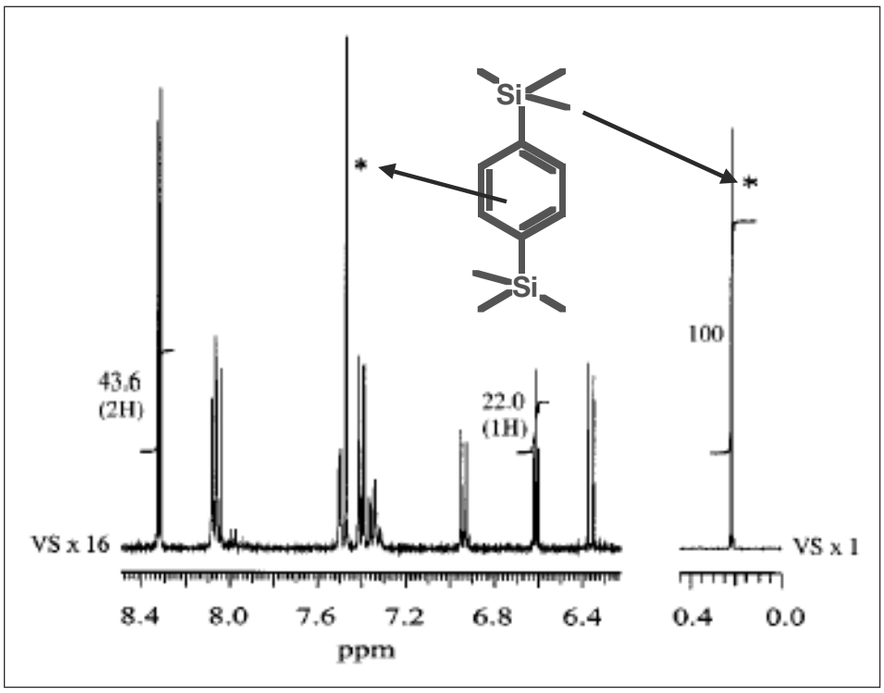


Fig. 6. Two areas of the quantitative ^{1}H-NMR spectrum of a library compound. The aromatic and the upfield signals of BTMSB (7.48 and 0.22 ppm respectively) are marked with an asterisk. The vertical scale expansion is indicated (factor 16 and 1 respectively). The two resonances used for the sample quantitation are marked with the integral values.

realistic picture of the actual sample composition (21% rather than 72% desired product). As explained in Section 3.4. (Fig. 10), an HPLC method including chemiluminescent nitrogen detection (CLND) leads to a similar result with a much smaller sample consumption than the NMR experiment (<100 nMol, as opposed to 2000 nMol).

3.2.2.2. *Direct-Injection NMR in DMSO-H_6*

New flow probes in conjunction with liquid-handling automation and the application of solvent-suppression algorithms enable NMR analyses in a high-throughput mode. Without reaching the 2% accuracy of the standardized method described in Section 3.2.2.1, the same quantitation principle (with the BTMSB standard) can also be used in a flow probe mode, in non-deuterated solvent. As an example, a 48-membered library was characterized by direct-injection NMR (DI-NMR) in an automated overnight process [19]. The total recycle time for each sample, including sample injection, acquisition, sample recovery and probe rinsing, was about 10 min per sample. A representative spectrum is shown in Fig. 9.

The direct-injection NMR technology is useful for the acquisition of analytical data on a large number of samples, which may be heterogeneous in terms of purity and material recovery (obtained amount) after a library production. The produced data, which is complementary to the routinely acquired LC-MS data, (see Section 3.3.), helps to weed out failed samples and identify the nature of main contaminants.

3.3. *Liquid Chromatography – Mass Spectrometry (LC-MS)*

High-pressure liquid chromatography coupled to both UV and MS detection [20] is probably the most widespread methodology used for the characterization of combinatorial chemistry processes [21]. The extraordinary utility of LC-MS remains undisputed. Its versatility and throughput capacity are often exploited also for the indirect characterization of solid-phase synthesis intermediates. Small resin samples are subjected to premature cleavage with appropriate reagents and the material is released in solution for subsequent analysis. While qualitative and semi-quantitative information is readily accessible, an exact quantitation of solutes is cumbersome (for lack of appropriate reference compounds), whenever a large number of new compounds must be analyzed. The variability of UV and MS response factors make LC-MS a questionable choice for accurate purity and concentration measurements. Coupling

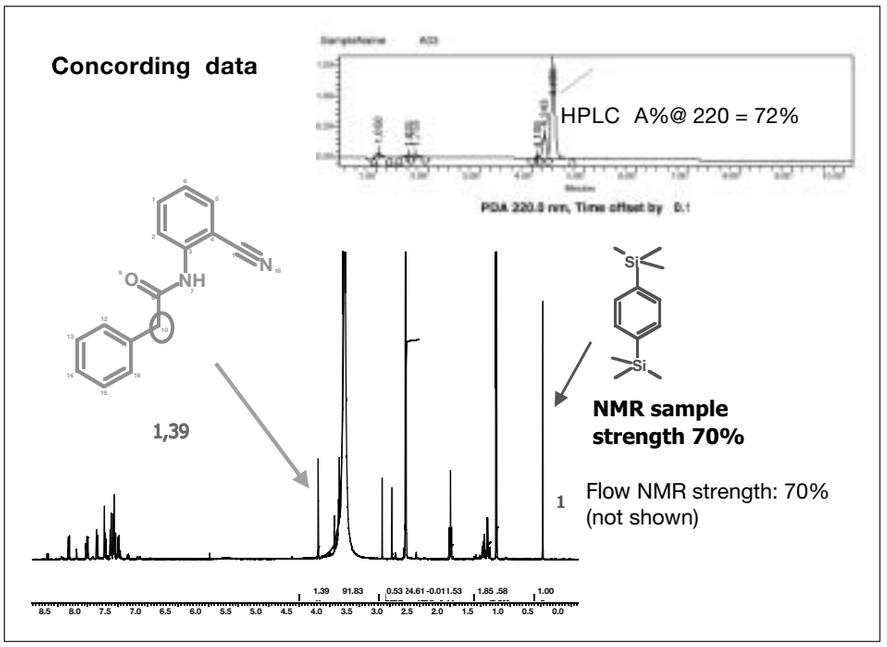


Fig. 7. Good agreement between LC-UV assessment and the two NMR quantitation methods carried out in different formats (deuterated and non-deuterated solvent in tube and flow probe respectively).

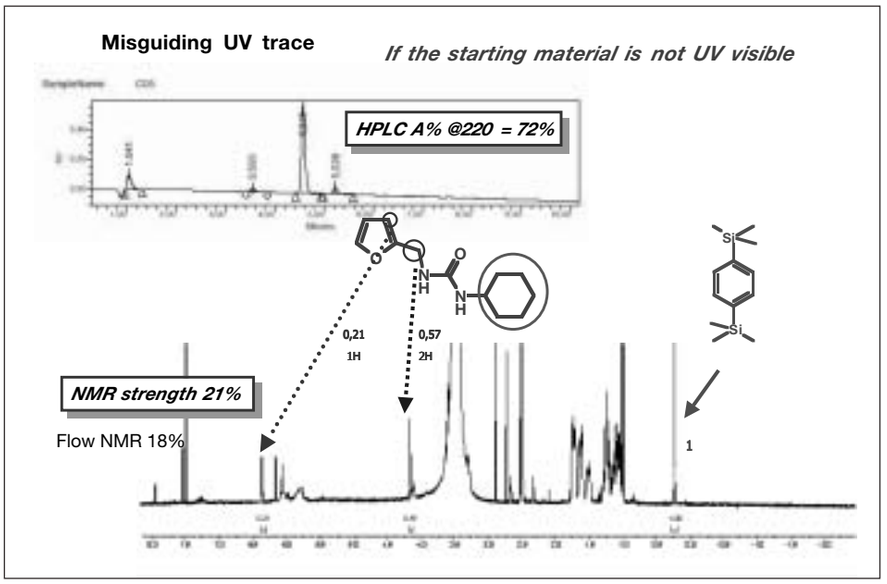


Fig. 8. Misguiding UV trace, overestimating the sample purity. Instead, the NMR data clearly reveals the presence of a contamination.

with a chemiluminescent nitrogen detector (CLND) has helped to broaden the method's scope in that respect.

3.4. *Chemiluminescent Nitrogen Detection (CLND)*

A new method development area, which we have adopted with good results, is the application of chemiluminescent nitrogen detection (CLND) [22] for routine, 'material-saving' quantitation of compounds obtained in small amounts [23]. CLND is a destructive method but requires minimal amounts of sample (0.1 μMol in our standard protocol). Nitrogen-containing compounds are transformed into NO, which is converted to excited NO_2 by reaction with ozone. Upon reassuming the ground state, a photon is released and measured by a photomultiplier. Most initial limitations of HPLC online CLND have been overcome (aside from the obvious requirement of the presence of nitrogen in the analyte). Usually, an equimolar response is observed from all nitrogen atoms in a molecule. This generally allows for the use of a single reference compound for calibration [24], without the need for purified reference samples of each analyte. Significant exceptions may occur, but these cases are usually related to the presence of N–N bonds, and they are fairly predictable. In such cases it is advisable to run calibrations with a representative molecule of the same chemical class, bearing the same nitrogen atom distribution (*e.g.* the core scaffold of a library). This minimizes the risk of an underestimation of the nitrogen content related to some formation of stable molecular nitrogen, which would escape the detection mechanism. We have noticed that if one of the vicinal nitrogen atoms is fully substituted, the detection response is mostly unaffected by this problem.

We routinely run CLND analyses on newly produced combinatorial libraries (see example in Fig. 10) and store the determined strength factors in a database. Normally, this data is complemented with analogous information obtained from quantitative NMR measurements, as long as sufficient compound material has been isolated. These correction factors are used for the calculation of the actual concentration of our liquid samples.

4. Conclusions

We have incorporated a selection of analytical tools into the standard workflow of our combinatorial chemistry operations. The use of LC-MS is ubiquitous, as it allows to monitor the qualitative progress of new synthesis protocols, but enabling also routine in-process controls and a quality assessment of produced libraries. NMR complements this area of application, but provides more in-depth analyses and extends its use to purity and exact quantity determination of final compounds. In general, the consumption of material for a full analytical characterization, including NMR, is low (3.5 μMol/sample). HPLC online quantitation with CLND is optimal for minimizing material loss of precious samples, as 0.1 μMol are more than sufficient for an LC-MS-CLND analysis (excluding NMR).

Some general considerations are worth mentioning:

(a) In our experience, NMR quantitation *vs.* standard is the most accurate quantitative method with a throughput capable of processing '*parallel chemistry compounds*' with sufficient efficiency. The

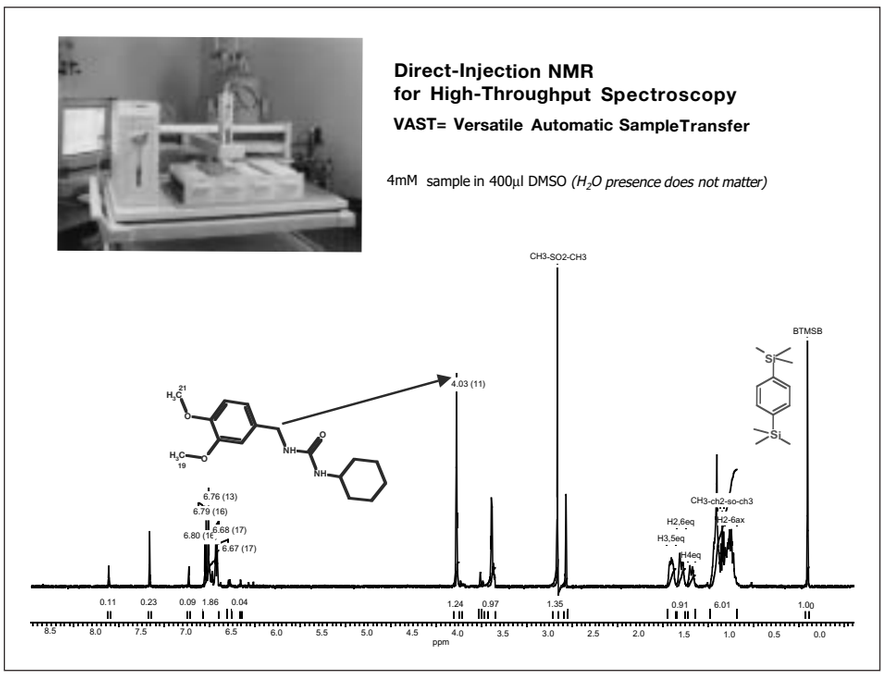


Fig. 9. The quality of the multiple solvent suppression method is evident in this typical NMR spectrum obtained by DI-NMR. There is a small residual DMSO-H_6 signal and virtually no signal from H_2O. The silylated BTMSB standard is added in a known amount to the solvent used for dissolving the samples. The strength calculation is based on the observed integral ratio of the standard's methyl groups and an assigned signal of the product, *e.g.* the indicated methylene around 4 ppm.

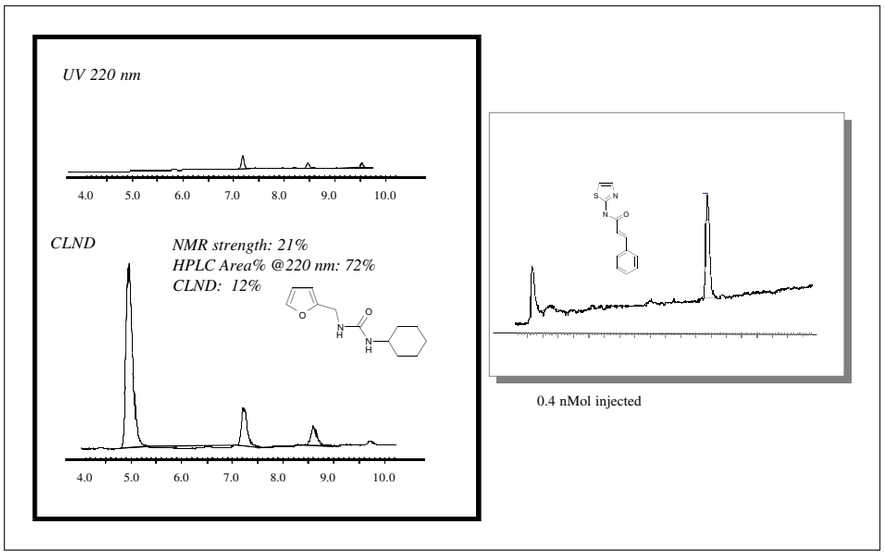


Fig. 10. CLND of crude samples. The left panel indicates an example of superior information content compared to short wave UV: Residual non-aromatic starting material is detected (4.95 min) in a quantifiable manner. The right panel illustrates a typical trace obtained with minimal sample consumption.

method becomes impractical if all compounds in a sample series are available in <1 mg amounts, and if a series comprises many hundreds of samples delivered at once.

(b) We have learned to beware of quantitation by weighing of amorphous samples <5 mg (films, evaporation residues), as unpredictable weight inconsistencies may occur.

(c) Nitrogen detection (HPLC online with CLND) has excellent throughput and is our method of choice for the quantitation of small samples of large libraries.

(d) Initial concerns on analytical limitations of solid-phase chemistry have been overcome. On one hand it is common practice to rapidly cleave an aliquot from resin into solution, whenever an intermediate needs to be characterized. On the other hand, IR and especially MAS NMR on solid-phase give good qualitative and semi-quantitative information, although the interpretation of spectra (computational deconvolution) is more demanding.

The implemented combination of technologies ('Tool Box') has been tuned to provide maximal information with minimal sample consumption, in an efficient manner. Thorough analytical characterization does not significantly delay the availability for biological testing of newly synthesized compounds, even if hundreds of molecules are generated simultaneously, and recurrently, within the time frame of a library production.

Acknowledgements

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[2] M.A. Fivush, T.M. Willson, *Tetrahedron Lett.* **1997**, 38, 7151.

[3] **General procedure for the synthesis of resin 2:** To 1 g of 4-(4-formyl-3-methoxyphenoxy)butyryl aminomethyl resin (0.94 mmol/g) swollen in 12 ml of anhydrous THF, 5 equiv. of amine (*e.g.*

aniline or 2-aminopyridine), 3 equiv. of reducing agent (sodium triacetoxyborohydride or sodium cyanoborohydride) and a catalytic amount of acetic acid were added. Stirring was maintained for 18 h at rt. The resin was washed with DMF (3 × 10 ml), DMF/H_2O 1:1 (3 × 10 ml), THF (3 × 10 ml), and DCM (3 × 10 ml). The resin was dried under vacuum to constant weight.

[4] **General procedure for the synthesis of crude products of type 4 (Scheme):** To 100 mg portions of a suspension of resin **2**, 2 equiv. of acyl chloride or sulfonyl chloride, or 3 equiv. of isocyanate were added. The suspensions were shaken overnight at rt, then filtered and the resins washed with DMF (3 × 10 ml), DMF/H_2O 1:1 (3 × 10 ml), THF (3 × 10 ml), and DCM (3 × 10 ml). A mixture of TFA/DCM 30% was added to the resins, and the suspensions were shaken for 2 h at rt. The resins were filtered and rinsed with appropriate solvents to a combined filtrate. The obtained crude product solutions were evaporated in a vacuum centrifuge to form amorphous residues. This being a test library, variable yields and product purities were obtained.

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[6] FTIR: KBr pellet method: The resin was finely mixed with pure, dry (spectroscopic grade) KBr (resin contents of formulation: ~1%), and then pressed to a clear disk. The sample measurement was performed on a Perkin Elmer Spectrum 1000 FTIR spectrometer.

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[11] **Experimental:** All HR-MAS NMR spectra were recorded at rt on a Varian Inova 500 MHz spectrometer equipped with a 4 mm gHX Nanoprobe. Samples of ~2 mg resin were swollen in 40 µl of deuterated solvent (*e.g.* CD_2Cl_2) and placed into the nanoprobe tube; the spinning rate was approximately 2.5 kHz for all samples.

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[14] M. Rance, O.W. Sorensen, G. Bodenhausen, G. Wagner, R.R. Ernst, K. Wüthrich, *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479.

[15] In 2D-DQ spectra the singlets (*e.g.* isolated CH_n) are eliminated due to the DQ coherence selection, *i.e.* only the scalarly coupled spin systems are visible. Furthermore, because of two intrinsic characteristics of the DQ methodology (fast relaxation of the double quantum signals and cancellation of the broad antiphase multiplet in ω2), the broad signals of the resin are almost completely cancelled out. Only the 'mobile' spin systems (with sharper resonances) are observed. Fig. 4 is the spectrum of resin 2b. It can be noted that: a) only the three indicated spin systems are visible b) no signals arise from the isolated CH_2/CH_3 functions, nor from the aromatic and aliphatic protons of the resin (*e.g.* compare the region from 6.5 to 7.5 ppm of Fig. 3 *vs.* Fig. 4).

[16] The Vnmr Varian software allows the deconvolution of observed spectra into individual Lorentzian and/or Gaussian lines using the Levenberg-Marquart method for curve fitting; see 'Modeling of Data' (Chapter 14, p. 542) in W.H. Press, B.P. Flannery, S.A. Teukolsky, W.T. Vetterling, 'Numerical Recipes in C, The Art of Scientific Computing', Cambridge University Press, **1988**. Up to 2048 data points from an expansion of an experimental spectrum can be deconvoluted at one time, and up to 25 lines can be fit to this section of the observed spectrum. For each line it is possible to have a quite precise value of the relative integral.

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[18] The NMR samples are prepared by mixing 200 µl of an (estimated) 10 mM stock soln. with 200 µl of a 5/9th mM (0.556 mM) BTMSB soln. and 200 µl of DMSO-d_6 directly in the NMR tube. The concentration ratio of silane to analyte species is 1:18, which exactly compensates for the 18 protons of the trimethylsilyl groups present in the reference signal.

[19] **Experimental:** The 48 individual members of a test library were dissolved in DMSO-H_6: 400 µl of a 4 mM soln. were transferred to a 96-well plate with a 2-ml capacity per well. The NMR spectra were acquired on a standard Varian Unity-Inova 500 MHz spectrometer, equipped with a standard Z-axis-PFG triple-resonance (H{C,N}) flow probe (maintained at 25 °C), and using Vnmr 6.1B software. The flow probe has an active volume of 60 µl. A standard VAST liquids-handler accessory (see P.A. Keifer *et al., J. Comb. Chem.* **2000**, *2*, 151) fills the system, returns the entire sample to its original well after the acquisition, and washes the entire tube line and the cell with DMSO-H_6 after each sample injection. All the NMR spectra were run with a multiple-frequency solvent suppression method (see C. Dalvit *et al., Magn. Reson. Chem.* **1999**, *37*, 7) combined with a SCOUT-scan automation, to cancel the strong signals of DMSO-H_6 and H_2O (P.A. Keifer *et al.*, see above). 64 scans + 8 dummy scans, and a repetition rate of 6 s were used for each spectrum, all adding up to an NMR measurement time of 7.2 min per sample (total recycle time ~10 min). Exponential line broadening (1 Hz) zero filling was applied to all spectra.

[20] Our standard LC-MS equipment consists of a Waters 2790 HPLC system equipped with a 996 Waters PDA detector and a Micromass mod. ZQ, single quadrupole mass spectrometer equipped with an electrospray (ESI) ion source. The separations are carried out at 25 °C at a flow rate of 1 ml/min using a RP18 Waters X Terra (4,6 × 50 mm, 3.5 µm) column. Mobile phase A is ammonium acetate 5 mM buffer (pH 5.5 with acetic acid/acetonitrile 95:5). Mobile phase B is H_2O/acetonitrile 5:95; the gradient runs from 10 to 90% B in 8 min, then holds 90% B for 2 min. The injection volume is 10 µl. The mass spectrometer is operated in positive and in negative ion mode, with the capillary voltage set to 2.5 KV; the source temperature is 120 °C; the cone is 10 V; full scan, mass range set from 100 to 800 amu.

[21] J.N. Kyranos, J.C. Hogan, *Anal. Chem.* **1998**, *70*, 389A.

[22] a) E.W. Taylor, M.G. Qian, G.D. Dollinger, *Anal. Chem.* **1998**, *70*, 3339; b) N. Shah, M. Gao, K. Tsutsui, A. Lu, J. Davis, R. Scheuerman, W. Fitch, R. Wilgus, *J. Comb. Chem.* **2000**, *2*, 453; c) D. Yurek, D.L. Branch, M.-S. Kuo, *J. Comb. Chem.* **2002**, *4*, 138.

[23] Our HPLC-CLND system consists of a Waters 2790 Alliance Separation Module interfaced with a UV detector with dual wavelength (set to 220 nm) and with a Micromass ZQ single quadrupole mass detector with ESI interface. The flow rate is set to 1ml/min and split after the column in order to have a flow of 100 µl/min into the nitrogen detector and 100 µl/min into the mass detector. The chemiluminescent nitrogen detector is an Antek 8060. The furnace temperature is 1050 °C, the argon flow is 65 ml/min with an oxygen flow of 273 ml/min. The reaction chamber pressure is maintained at 25 Torr by the vacuum pump and the ozone flow is 30ml/min. Zorbax SB C8 (4.6 × 50 mm, 5 µm) analytical columns are used and the injection volume is 10 µl. The column is eluted with a linear gradient from 5 to 95% buffer B in 10 min. Buffer A is 0.01% (v/v) formic acid (FA) in water; buffer B is 0.01% (v/v) FA in MeOH.

[24] The CLND was calibrated using a caffeine standard (99% from Aldrich) at different concentrations (100, 200, 400 and 800 µM). Each standard soln. was run at least in triplicates.