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Review

Analytical methods for the monitoring of solid phase organic synthesis

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Abstract

Solid phase synthesis (SPS) is a powerful technique to assemble compound libraries in high-throughput parallel and combinatorial synthesis. The widespread applications of these techniques required the development of analytical methods for both structural elucidation and reaction monitoring. This review covers some recently developed techniques for on-bead analyses together with solution-state ones. Particular emphasis is devoted to software and hardware improvements for automated high-throughput analysis.

Keywords: Combinatorial chemistry; Mass spectrometry; Gel-phase NMR; Magic-angle spinning NMR; Infrared spectroscopy

1. Introduction

Basic principles of combinatorial chemistry were first applied in the simultaneous synthesis of large arrays of peptides, with a solid phase methodology initiated in 1963 by Barany and Merrifield [1]. During the 80s the procedure, applied also to non-peptidic structures, experienced an unparalleled growing, finding applications in biomedical, agricultural and fine chemical fields [2,3].

A combinatorial synthesis approach can be applied during different steps of the drug discovery process, where the molecular composition of a library depends on the targeted problem. Libraries can be roughly grouped into two main classes: pool libraries and parallel synthesis. Pool libraries are mainly produced on solid phase with the 'mix and split' technology to generate large number of compounds with multiple compounds present in every vessel (one bead-one compound). In parallel synthesis both solution and solid phase techniques are applied, with typical size of a library far less than 10 000 compounds as discrete (one well-one compound).

Whatever is the application or the approach of the synthesised library, the primary goal of combinatorial chemistry is to produce more compounds and a wider variety of compounds, in a shorter period of time. Recent developments in solid phase synthesis (SPS), automation, information sciences and high-throughput screening have all contributed to speeding up the entire drug discovery process so that the chemical analysis of the resulting samples may be a rate-limiting step. Analytical methods must be able to follow organic transformations rapidly, reliably and, ideally, in an automated manner, so the analytical control of a combinatorial library is a challenging issue. The difficulty of the analysis depends on the size of the library, if its a pool library or a collection of pure compounds, and on the chemical nature of the compounds. In the case of solution-phase synthesis, problems are mainly related to the large number of samples and the requirement of fully automated systems with minimum sample handling and efficient data reporting. If SPS resins are used, analytical difficulties are related to both the use of solid support and the small quantity of sample available for the analysis and specific monitoring tools had to be developed [4–11].

For the analysis of SPS, two possible strategies are used: conventional analytical liquid phase techniques, after cleavage of the compound from the solid support, or non-destructive techniques for samples still bound to the solid phase. The cleavage process is time-consum-

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ing, irreversible, potentially alters the product, and by definition lowers the yield of product. Moreover, some synthetic intermediates are not stable enough in the cleavage conditions. This can be a significant limitation especially if the issue is to monitor a reaction in progress or to characterise an intermediate in a multistep synthesis. Nowadays, generally solution-state techniques are used to characterise the final products (compounds must be cleaved from the resin prior to running the bioassays), while on-bead analysis are performed during the assessment of the chemistry and screening of the best reaction conditions.

2. Destructive techniques

2.1. Colorimetric tests

The use of coloured reagents to follow the appearance or disappearance of a functional group, a technique widely used in classical organic chemistry, is successfully applied also in SPS during the library assessment phase. These 'on bead' assays are destructive techniques but due to their simple and rapid use they are routinely employed in the laboratory. The most widely used are the qualitative ninhydrin [12] or bromophenol blue [13] based tests for amines and the Ellman's reagent for the thiols [14].

2.2. Spectrophotometric tests

Some 'off bead' assays can also be used as methods to quantify the outcome of SPS and to determine the yield estimation. They are usually more sophisticated approaches and require the use of analytical instruments such as spectrophotometers. An example of this approach is the classical fluorenylmethoxycarbonyl (Fmoc) group determination (FMOC reading test). The Fmoc group is commonly used for protecting primary and secondary amines during SPS and can easily be removed by treating the beads with a base (generally a 20% DMF solution of piperidine at room temperature for 20 min). The solution is brought to a constant volume (typically 10 ml) by addition of DMF, and the quantitation is carried out by reading the UV absorbance of the piperidine–dibenzofulvene adduct in solution at 301 nm against a blank solution of piperidine in DMF. The loading (quantity of material on resin) can be determined from the equation:

$$L = \frac{AV}{7.8W}$$

where L is the Fmoc loading (mmol/g), A is the absorbance value, V is the solution volume in litres, 7.8 is a constant and W is the weight of the resin in milligrams. The Fmoc loading compared with the known initial resin loading give the yield of deprotection.

2.3. Mass spectrometry

In the last 5 years mass spectrometry techniques found a wide application in the characterisation of combinatorial libraries. The development of soft ionization techniques, such as electrospray (ESI) and matrix-assisted laser desorption (MALDI) has considerably increased the speed, sensitivity, and specificity of the analysis. Fully automated instruments equipped with autosamplers and LC systems are now commercially available, and all instruments are controlled by software able to manage all steps of the process: sample preparation, injection and analysis. These software control systems are able to process many data files in a few minutes and give a readily visible response on the computer screen in a microtiter plate scheme format with different colour scheme (i.e. green and red wells).

Different mass spectrometry based approaches can be adopted depending upon the library type. The quicker technique is the flow injection analysis: after cleavage from the solid support an aliquot of the compound in solution (usually in the range 5–20 μl) is directly injected in the mass spectrometer obtaining a mass spectrum in a very short time ranging between 1 and 3 min. The automatic data processing phase provides a qualitative response as YES/NO (or green/red) based on the presence of the molecular weight of the target compound. This analytical approach is useful for a rapid quality control of parallel synthesis or for pool libraries in which more than one target compound is present in the same well. In the latter case, a limitation of the technique is related to the possible presence of compounds with the same molecular weight, which are not distinguishable. As an example, a mass spectrum obtained from a flow injection analysis of a pool library well is reported in Fig. 1.

To circumvent this problem mass spectrometry has to be coupled with a separation technique: the most popular one is the liquid chromatography. LC/MS is the most used analytical technique in combinatorial chemistry to analyse libraries for many reasons. First of all the short column available (3 cm length, with i.d. from 4.6 to 1 mm) allows the possibility to develop very fast LC gradients able to analyse a sample in less than 10 min. Another potential advantage is the possibility to compare signals coming from more detectors, if present. Standard LC/MS instruments are equipped with both mass spectrometer and UV detector, usually a diode array (DAD), but other detectors, such as evaporative light scattering (ELSD) or chemiluminescence nitrogen detector (CLND), can be used simultaneously. With this analytical approach a microplate containing 96 wells can be analysed in less than 20 h. All raw data obtained are processed by dedicated software able to extract chromatograms and spectra and to summarise all information, such as percentage area/

area (% a/a) based on the available detectors, in a computer file, that can be read directly by the chemist.

This technique is currently used for libraries produced by parallel synthesis but finds limited application in the analysis of large primary libraries, due to its limited throughput. In the latter case, a 5–10% sampling of the wells is analysed and forms the basis of the overall quality control of the entire library.

Another approach, involving the use of mass spectrometry to monitor solid phase reactions, is related to MALDI/ionization-time of flight (MALDI–TOF) spectrometry. Some applications of MALDI–TOF in this field were reported in literature [15–17] and even if the compound is cleaved from the resin some authors consider this method 'on bead'. Mass spectra are acquired after in situ cleavage of a small number of resin beads and addition of a matrix and a calibrant. The resulting mixture is allowed to crystallise and MS analysis is performed by irradiation of the sample with a N_2 laser at 337 nm, producing ions due to a desorption process. The MALDI technique has a high sensitivity but at the moment has big limitations for molecular weight < 600 Da, due to the presence of intense matrix signals.

2.4. Encoding techniques

Combinatorial chemistry is a very productive process in terms of number of compound synthesised and screened, especially when pool libraries (mixture of compounds in the same well) are used. The main problem related to pool libraries is the deconvolution of the mixture in case of a positive signal in a biological test. The more obvious answer to this problem is to synthesise each component of the mixture and test them separately in order to identify the active ones directly [18,19]. Repeat synthesis is a very time-consuming process and sometime no active component can be individuated as the biological activity can be due to an additive effect of moderately active compounds (called a 'false positive').

A more rational strategy is the use of an encoding tag, something like a bar code present in each bead and representative of the single compound synthesised in that bead. If biological assays reveal an active signal from the mixture tested, the 'positive' can be 'decoded' by means of analytical methodologies in order to identify the structure of the active molecule. Two different classes of encoding procedures are possible: chemical and non-chemical encoding. Different procedure of each class is reported in literature, such as secondary amine tags and isotope encoding (using ^{13}C and ^{15}N) for the chemical class and radio frequency encoding for the other one [20–23]. Tags decoding is performed with different analytical methodologies like HPLC/MS, HPLC/fluorescence detection, FIA MS, or appropriate equipment for the radio frequency reading.

As first example of chemical encoding method, the secondary amine tags procedure, depicted in the schematic diagram in Fig. 2, is discussed [24].

To obtain a double functionalisation, the use of orthogonally protected resin sites is necessary, so that 90% of the sites are functionalised with a photolinker (cleavable using UV), then protected with Fmoc, while the tag sites (10%) are protected with an orthogonal group (Boc or Alloc). For each synthetic step a different 'tag' is linked to the resin so that at the end of the synthetic path each 'code', that is a specific sequence of tags, is unequivocally associated to a specific product.

A first biological assay is performed directly on the pool of compounds, and if a positive signal is observed a secondary screening is performed on single beads to identify the positive one (that means the active

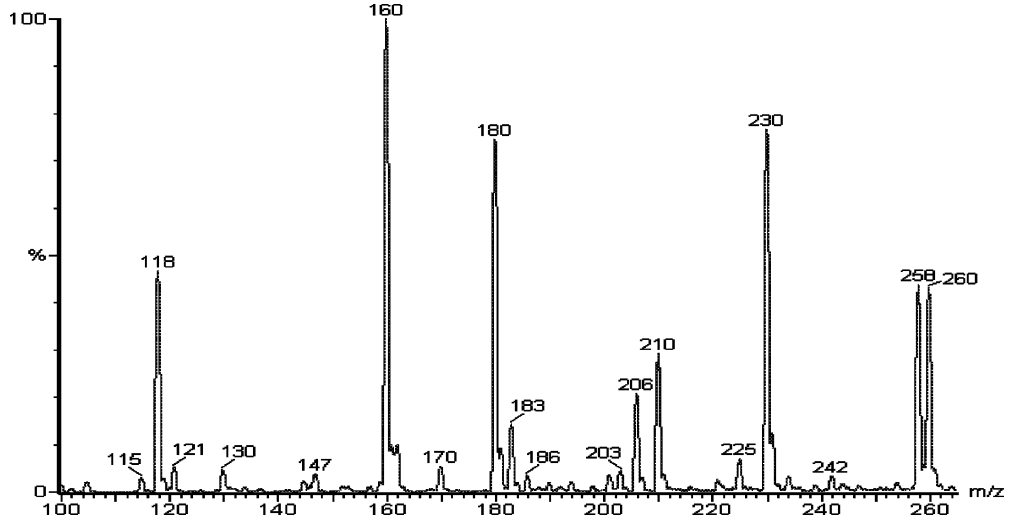

Fig. 1. Flow injection analysis of a pool library well (14 samples/well). Mass spectrum acquired using Micromass Platform II mass spectrometer coupled with a LC system HP1100. Injection volume 10 ul, flow rate 15 ul/min, mobile phase water + 0.1% formic acid/acetonitrile 1/1 v/v.

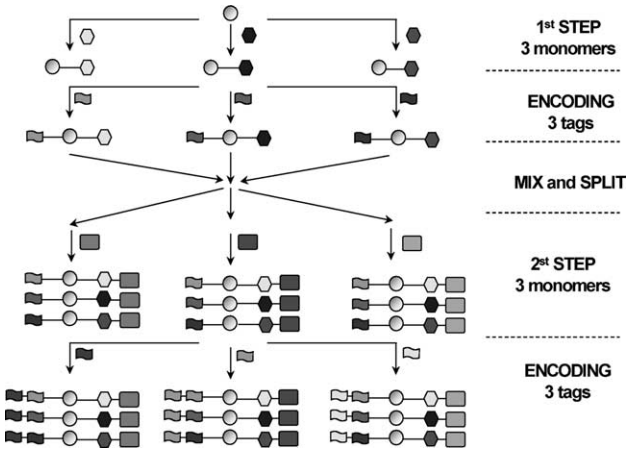

Fig. 2. Chemical encoding scheme using secondary amine tags [13]: each sample is encoded by a different mixture of 10 secondary amines.

compound). Automated instruments, called *bead pickers*, are used to automate the distribution process [25]. They use microcapillaries mounted on a manifold (array of eight or more) that is switchable from vacuum, for the aspiration of a bead from a pool, to pressure, for the release of the single bead to a plate well. They can pick and deliver up to 96 single bead in 5 min. A statistically meaningful number of beads are chosen in order to ensure that they are representative of all the compounds eventually present in the pool. At the same time the 'tag code' of a positive bead is cleaved from the resin and submitted to the analysis for identification (each sequence of tags can be directly correlated to the structure of a single compound). The entire process from primary screening to decoding is described in Fig. 3.

Decoding procedure can be performed using LC/MS or LC equipped with a fluorescence detector. In the last case all tags have to be treated with dansyl chloride to obtain a fluorescent derivative.

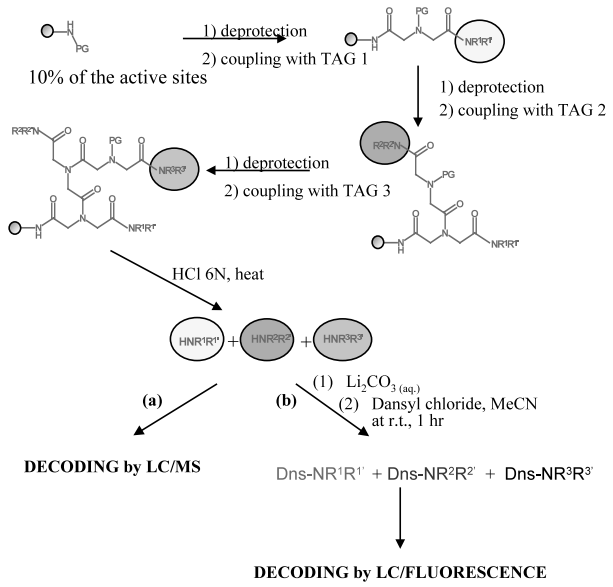

Fig. 4. Chemical encoding scheme using secondary amine tags [13]: the chemical process.

In Fig. 4, a schematic way chemical encoding with secondary amine tags is represented: for each synthetic step another reaction is performed to couple a secondary amines with the resin, after deprotection.

At the end of the synthetic procedure, the 'code' is cleaved from the resin and the tags are analysed by means of HPLC–MS analysis (Fig. 5) operating in single ion monitoring (SIM) mode, to monitor only the m/z of interest. Using these procedure impurities, which can be present in the mixture, do not affect the result of the analysis. A standard mixture, containing all tags, is injected every 10 compounds to evaluate the response of the instrument.

An alternative decoding approach is by means of LC–fluorescence: after the cleavage amines are derivatized by reaction with dansyl chloride. In this case a

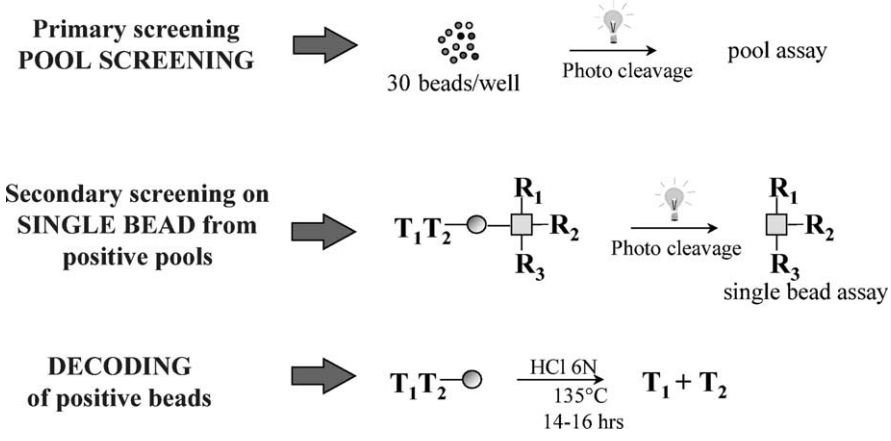

Fig. 3. Chemical encoding from primary screening to decoding step. Main steps are screening of pool library, screening of single beads from positive pools and then decoding of positive beads.

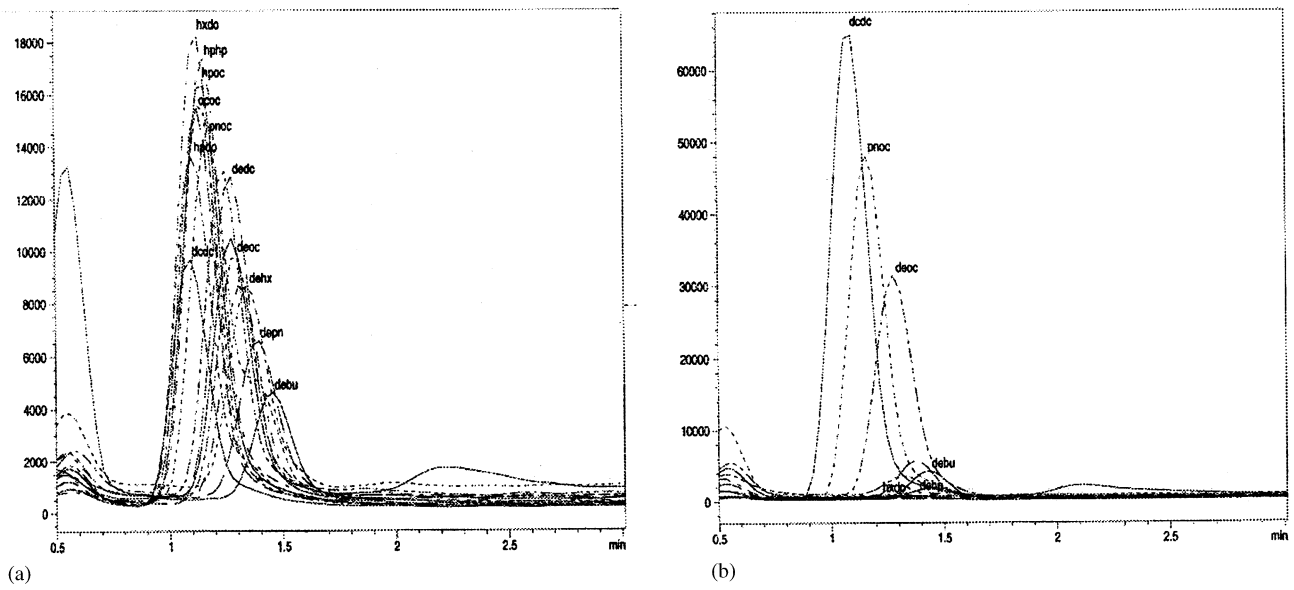

Fig. 5. Decoding using LC/MS. (a) Standard mixture of 10 secondary amine tags. (b) Decoding of a positive bead. Column: Supelco LC-SCX 50 × 1 mm, ion exchange. Column temperature 40 °C. Mobile phase: isocratic 85/15 v/v acetonitrile/water + 0.4% TFA, flow rate 0.2 ml/min, injection volume 20 ul. Analysis time 3 min.

real separation of the different peaks is required, so the analysis time is longer. The characterisation is performed by comparison of the retention time with that of a standard mixture of all possible amines. The LC/fluorescence process is described in Fig. 6.

An alternative 'coding procedure' involves the use of isotopically labelled tags. The principle underlying is the same as in the use of secondary amines, although in this case codes are generated using labelled glycines and alanines [26]. The amino acid mixtures with various numbers of isotopically labelled carbons and nitrogens give a series of peaks in the MS spectrum. This technique has the advantage of a fast decoding procedure that can be completely automated by means of mass spectrometry and a specific software package (developed at GSK-RTP, US) that can read codes and give the ligand molecular structure. Other advantages of this approach are that it does not require a chemical tag, is amenable for most chemistry and is particularly suited to determine side reactions. It is generally used in the ratio screening process (that means in the screening for best reaction conditions): the codes are used as an internal standard in order to evaluate the reaction yield by mass spectrometry.

From this idea presented by Geysen et al. [26], a new approach called 'analytical construct' was developed, based on the scheme reported in Fig. 7. Several ideas based on this approach were developed using other analytical detectors such as UV [27–29].

The last possibility is the use of radiofrequency (RF) encoding. In this case the RF tag is 'inside' the microreactor along with the solid phase resin. A single component is synthesised in each well and each one contains a unique miniature RF label. This procedure is used for reactions with a common step, like a common building

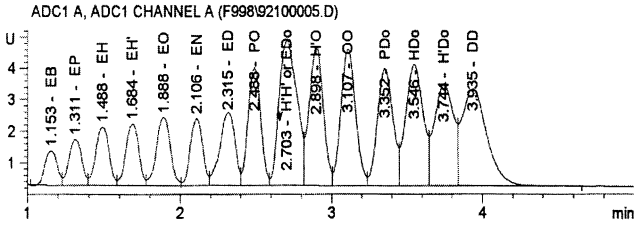

Fig. 6. Decoding using LC/fluorescence, standard mixture of 14 secondary amine tags. Column: Zorbax SB-CN 50 × 4.6. Column temperature 40 °C. Mobile phase: gradient from 50 to 70% acetonitrile/water in 3 min and 70 to 75% in 1.5 min, 1.5 min to recover conditions, flow rate 2 ml/min, injection volume 20 ul. Fluorescence detection: excitation at 352 nm, emission at 510 nm. Analysis time 6 min.

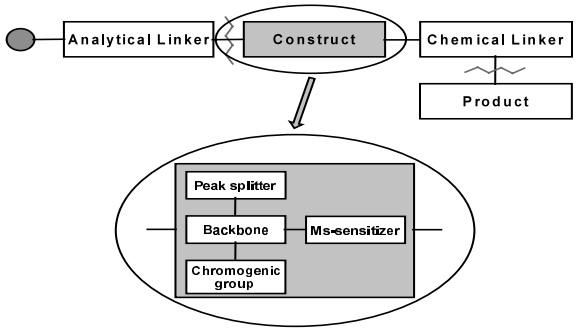

Fig. 7. Analytical construct scheme using a chromogenic group as internal reference to quantify product coming from SPS.

block reagent or a common purification. In each reaction step all microreactors that share a common building block reagent are pooled together into a single reaction flask.

2.5. Solution-phase NMR

At the final stage of a library, even if SPS resins were used, compounds must be cleaved from the resin for the bioassays and that should allow in principle the use of conventional solution-state NMR techniques for final product characterisation. Unfortunately, for many years the acquisition of NMR data at this stage was assumed to be difficult because of several factors. First of all, products are normally dissolved in protonated solvents with solution volumes often too small for conventional NMR samples to be taken. Then samples are stored in containers like 96-well microtiter wells not suitable for the NMR analysis and the throughput is not fast enough (due to the large number of samples to be analysed). These problems have been solved by some recent applications of LC–NMR, allowing the development of high-throughput techniques applied in combinatorial chemistry library analysis.

Nowadays presaturation techniques provide high quality suppression even for fully protonated solvents and can be used to suppress multiple solvent resonances [30,31], so that high resolution NMR spectra can be acquired also in non-deuterated solvents. To address the problem of small sample volume, flow-probes with high sensitivity have been developed. These probes are directly connected to HPLC columns so that the sample flows through narrow bore tubing into the NMR coil without sample tubes. The sample geometry is fixed and approximates an infinite cylinder thus avoiding the necessity of reshimming each sample while PFG coils allow fast automatic ^{2}H gradient shimming of B_0 field.

The LC–NMR system was simplified, creating an [autosampler]–[injector]–[NMR probe] system. In this case the autosampler syringe transfers a sample aliquot (typically 150–300 μl) from a microtiter directly into the NMR detector cell without the use on any mobile phase and therefore without dilution. After acquisition of the NMR spectrum, the sample can be returned to the microtiter plate or can be flushed to a waste container. This system is considered a 'high-throughput spectroscopy' (HTS), as the rate of analysis can be less than 3 min per sample. That means that the acquisition of 1D spectra of a 96-well microtiter plate takes about 5 h. Another advantage of this system is that samples can be loaded on to autosampler in the same plate in which they are delivered, so significantly reducing the sample handling.

An example of proton, gCOSY and gHSQC spectra acquired with the versatile automated sample transport (VAST) system, are reported in Fig. 8.

The dramatic increase in the speed of acquisition would potentially transform NMR into a routine technique for the analysis of libraries, but unfortunately the interpretation of the resulting spectra is still a bottleneck. Recently, some NMR techniques have been developed for accurate expert NMR spectral prediction. All of these programs use chemical shift-structure databases or sets of rules as the basis for their predictions and allow some customisation by addition of data for class of compounds or solvents used. The development of software for automated spectral analysis like that used in MS spectrometry will allow a fully automated, real-time microtiter plate NMR analysis system for combinatorial chemistry libraries. This tool will become even more important in the future if, as it seems now, solution methods will overcome SPS ones in the synthesis of libraries. But in any case there are two important reasons to develop methods for the analysis of solution-state samples. First, most bioassays are performed in solution, on cleaved samples, and second automated manipulation of liquid samples is much easier than that of heterogeneous resin slurries. The role of NMR would become crucial if it would be possible to analyse the actual samples sent to biological tests, regardless if they have been synthesised by solid or solution-phase methods.

3. Non-destructive techniques

3.1. On-bead analysis by NMR

3.1.1. Gel-phase NMR

Gel-phase NMR is obtained on a slurry, generally heterogeneous, of the resin in a standard NMR tube: it is non-destructive and the sample can be readily recovered.

NMR analysis of samples still bound to solid-phase resins present many difficulties mainly due to line broadening and to the small amount of sample available for the analysis. One of the main reasons for the line-broadening effect is the limited internal motional freedom. To increase the motional freedom and consequently narrow the signal's linewidth, all SPS resins are swollen in a solvent as much as possible before acquiring NMR data. The resulting solvent swollen slurries are neither fully solid nor fully liquid: the technique of acquiring NMR data of these samples is called 'gel-phase NMR'.

Gel-phase spectra typically exhibit very broad resonances, due to magnetic susceptibility variations into the sample. For ^{1}H NMR linewidths are often 100–300 Hz, so proton spectra are generally too broad to be used for structure elucidation. Since the line-broadening effects of susceptibility discontinuities scale up with the NMR frequency, lower frequency nuclei like ^{13}C may

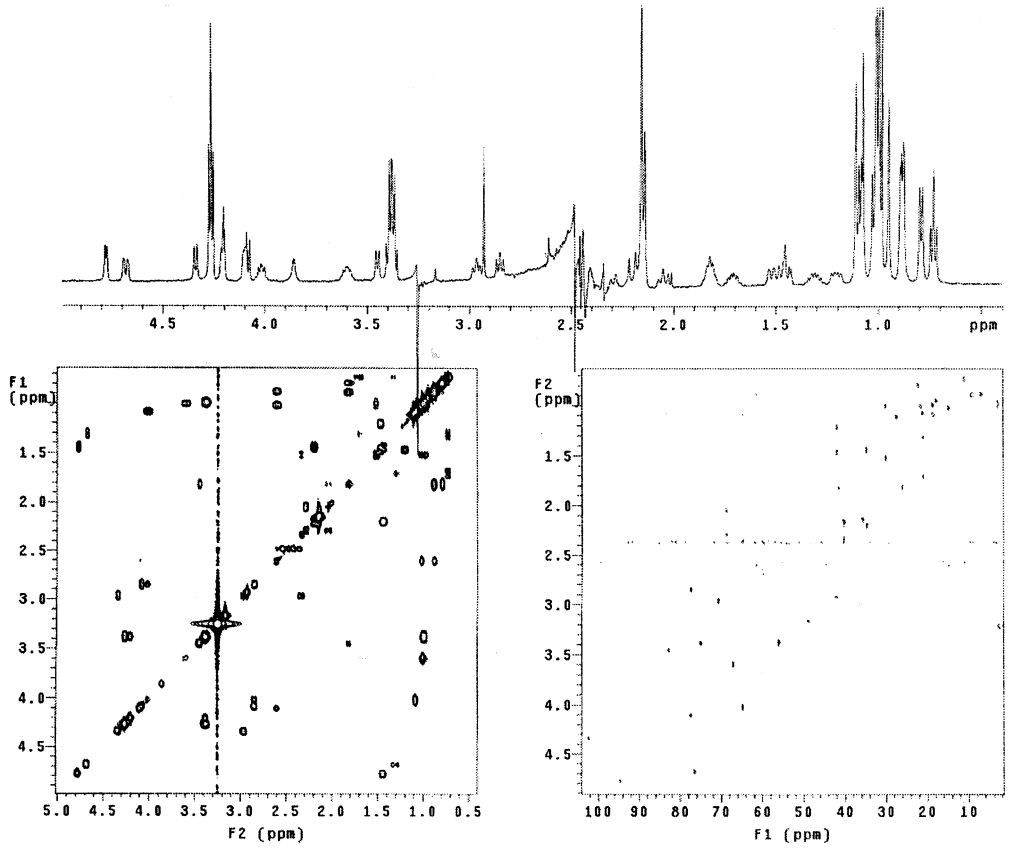

Fig. 8. Five hundred megahertz proton, gCOSY and gHSQC spectra acquired with the VAST system.

produce linewidths of only 25–75 Hz. If the observed nucleus also have a significant chemical shift dispersion (such as ^{13}C or ^{19}F) the resulting spectra may be resolved well enough to allow reasonably easy structural assignment. Unfortunately, spectra of natural abundance ^{13}C exhibit poor sensitivity and so the method is impractical for rapid reaction monitoring. To overcome this problem is possible to use building blocks labelled with ^{13}C near the reaction site of interest: this method is called 'FAST' ^{13}C NMR, and is quite useful in monitoring the progress of reactions [32].

Manatt reported the first application of gel-phase NMR to organic SPS in 1980 and since then the use of ^{13}C gel-phase NMR proved to be invaluable. Many applications are reported both in structure elucidation and product quantitation, the main advantage being the possibility to analyse reactions without cleavage of products from solid support [33,34]. In our example, reported in Fig. 9, a benzaldehyde labelled with ^{13}C at the carbonyl was used in order to follow the formation of a protected cyanohydrin. The reaction was run in the NMR tube and stopped when there was evidence that the cyanohydrin peak was not increasing any longer.

Although the technique is used primarily with ^{13}C, gel-phase spectra of other nuclei such as ^{19}F and ^{31}P are also used.

Together with the good sensitivity, ^{19}F has wide chemical shift dispersion, so reducing the chance of coincidental signals overlapping and structural modifications remote from the fluorine often give raise to sufficiently large chemical shift changes in the NMR spectrum. A large variety of fluorine containing

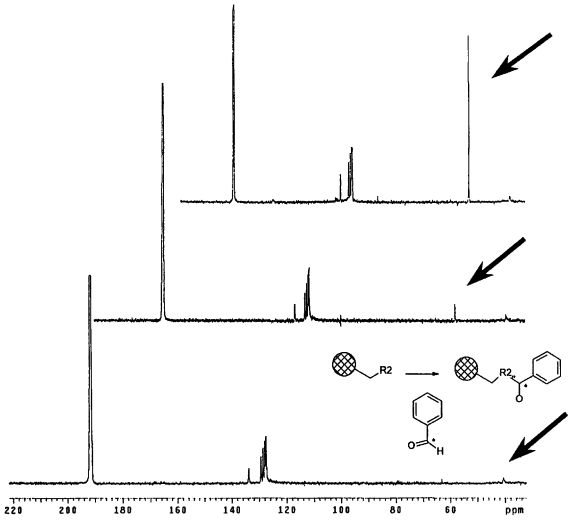

Fig. 9. ^{13}C gel-phase NMR application in reaction monitoring.

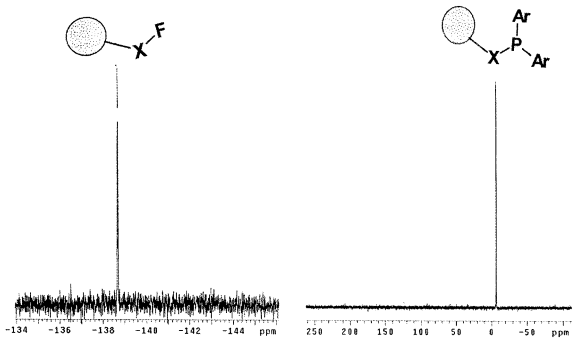

Fig. 10. ^{19}F gel-phase NMR application (left) and ^{31}P gel-phase NMR application (right).

building blocks are commercially available making its incorporation into the synthesis quite easily. As a consequence ^{19}F NMR is an excellent probe for reaction monitoring as can be seen from the spectrum reported in Fig. 10 and from many examples reported in the literature [35–38].

^{31}P gel-phase NMR is also successfully used for reaction monitoring, an example being reported in Fig. 10. As with ^{19}F NMR, the resins currently used do not interfere with ^{31}P signals and spectra can be acquired with short acquisition time [39].

3.2. Magic-angle spinning (MAS) NMR

The ideal situation for ^{1}H NMR in the SPS library analysis would be to obtain the same spectral resolution for compounds still bound to swollen polymer beads as is possible in solution-state. Many different parameters influence the spectral quality: (1) line broadening due to different mechanisms: B_0 homogeneity, difference in magnetic susceptibility, chemical shift anisotropy and dipolar broadening; (2) solvation; (3) mobility; (4) spin diffusion.

Magnetic susceptibility is a physical property of every substance that measures its effect on a surrounding magnetic field. A homogeneous substance will cause a uniform change of the static magnetic field, an effect that can be easily corrected, especially if the sample can be approximated to a cylinder infinitely long relative to the detection coil. A heterogeneous sample, like a slurry of a resin, will contain regions with differing magnetic susceptibility: the resulting distortions in the applied magnetic field cannot be corrected by shimming or rotating the sample around the z-axis and will create line-broadening effects.

It has been known for almost 30 years that the magnetic susceptibility-induced line broadening and dipole–dipole coupling observed in the NMR spectra of heterogeneous samples can be removed by using a MAS probe. Dipole–dipole coupling depends on the angle between the nuclear dipole and the static field direction with the correlation: $(3\cos^2\theta - 1)$. Spinning the sample about an axis 54.7° relative to the static magnetic field (the so called 'magic angle') averages the bulk magnetic susceptibility while dipolar couplings average to zero $(3\cos^2\theta - 1 = 0)$.

The use of MAS should also provide substantial line narrowing in one-dimensional ^{13}C and ^{1}H spectra of solvent swollen polymer gels. Unfortunately traditional MAS probes were made to provide, by solution-state standards, only moderate resolution. Only in the last years MAS spinning and proper probe construction, using magnetic susceptibility matching materials, were combined and high resolution MAS spectra (HR-MAS), especially high-field ^{1}H spectra, were obtained [40–43]. Now ^{1}H linewidth as narrow as 4 Hz and even 1 Hz are obtainable for resin-bound samples.

Another advantage of HR-MAS probe is the high sensitivity, achieved by placing the entire sample within the active region of the receiver coil. This sample geometry would generate magnetic susceptibility discontinuities at the edges of the sample (the sample/air and the sample/container interfaces near the receiver coil) that in turn produce bad lineshapes. The use of a moderate speed (1–3 kHz) MAS spinning of the sample, however, causes the magnetic susceptibility terms in the line broadening equation to go to zero thus eliminating the effects of all discontinuities both around and within the sample. These effects are of increasing importance at higher field strengths and with higher frequency nuclei, since in those cases the line broadening becomes more severe (susceptibility broadening is proportional to both the observed frequency and the field strength) and the chemical shift dispersion is typically small.

In summary, the use of MAS removes magnetic susceptibility variation within the resin sample itself and discontinuities around the edges of the sample. However, the narrowest NMR linewidths can only be obtained if magnetic susceptibility variations within the probe are reduced by proper materials, construction and design of the probe hardware (this is the case of the so-called HR-MAS probe).

High spin rate is not generally a critical issue in SPS resin spectra and rates of 2–3 kHz are often used. The intensity of spinning sidebands should be low ($< 1\%$) but if the side bands still complicate the ^{1}H NMR spectrum, the spin rate can be adjusted to move spinning sidebands of a large peak away from any small peaks of interest. This problem is less critical in ^{13}C spectra as spinning sidebands are not normally visible.

Spectral appearance depends on molecular motions that are in turn influenced by the resin's structure and rigidity and by the properties of the solvent used to swell the resin. The quality of the acquired spectra can be quite variable depending upon the resin and solvent used so that the achievable linewidths are primarily

determined by the nature of the resin, although this choice is actually driven by chemical considerations rather than NMR properties. Resins which offer the bound compounds the greatest mobility, particularly those resins which contain long PEG tethers, have been shown to produce the narrowest ^{1}H NMR linewidths. The choice of the solvent is then another critical issue and this choice depends not only on the nature of the resin but also on the structure of compound bound to the resin. Narrow NMR resonances will be obtained only if both the compound and its supporting structures (the cross-linked polymer and any tethers) are well solvated [44]. Swelling properties of resins have been studied over the years and the correlations of swelling abilities with solvent properties, such as polarities, solubility, and hydrogen-bonding abilities have been proposed, although the correlation between quality of the spectrum and solvent does not always hold.

Traditional polystyrene resins (PS) do not swell very well when compared with PEG-polystyrene resins such as Tentagel and this will be reflected in the resolution of the NMR spectrum (this was the main limitation of the use of NMR in this field). Tentagel-type resins, which have the longest tethers and the most mobile moieties, provided the narrowest linewidths and the best overall data in the widest variety of solvents. WANG, PA-500 and to a lesser extent Rink MBHA resins, which have only short tether/linker structures and hence more restricted mobility, sometimes produced suitable spectra but clearly required a proper choice of solvent. The more conventional polystyrene resins have no tethers and little regional mobility, and never generated high-quality spectra.

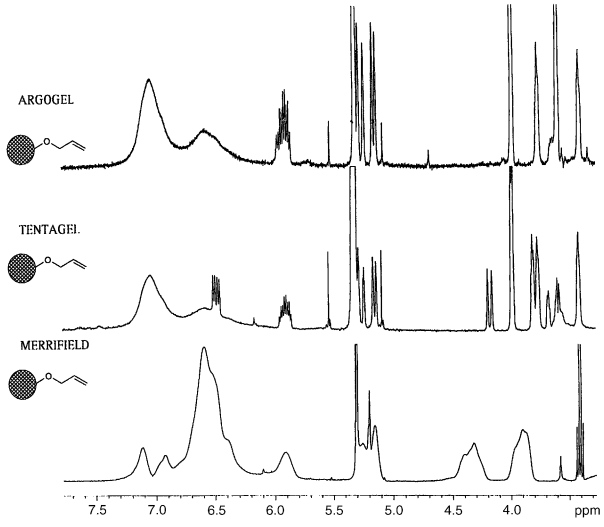

Fig. 11. MAS HR-NMR comparison of substituted ArgoGel®, Tentagel® and Merrifield resins with the same organic moiety (an allylic group).

Fig. 11 illustrates the NMR spectra of the same organic moiety (an allylic group) attached to three different resins. In the case of Merrifield the peaks of different hydrogens are not well resolved, while with Argogel® and TentaGel® resins the quality of the spectra are comparable with classical solution ones.

Dichloromethane-d_2 is a good first choice solvent due to the narrow resin linewidths it actually produces, its swelling properties, the unobtrusive location of the residual solvent resonances (^{1}H triplet at 5.32 ppm, ^{13}C quintuplet at 53.8 ppm) and for its ease of evaporative removal. DMF or $CDCl_3$ are good second choices, while DMSO produces variable results.

The resin in MAS conditions gives rise to a number of intense signals, which can complicate the interpretation of the proton spectrum. Different techniques are currently used to reduce the intensity of such signals, depending mainly upon the nature of the polymeric support. Selective presaturation is particularly effective because it also removes spinning-sideband artefacts of the irradiated lines. Presaturation of a resonance from the tether can sometimes reduce the signal intensities of other polymer resonances at totally different chemical shifts, as can be easily seen in Fig. 12. On the other hand the effectiveness of this method depends strongly upon the resin's structure; with a PEG tether it works quite well while if no tether exists it is much less useful. A spin diffusion process probably spreads the signal saturation, and the effectiveness of this process is undoubtedly affected by the motional differences between the two resins. Spin echo [45] has also been used to suppress broad polymer signals in resins that do not contain tethers, as well as combination of both spin echo and presaturation [46]. The spin echo, however, causes distortions in both the lineshapes and amplitudes of the resulting signals giving rise to problems in quantitation of the resulting spectra. Some other different approaches are reported in literature, like the use of untilted projections of homonuclear J-spectra [47], chemical shift scaled E-cosy [48], backwards linear prediction processing and others.

2D experiments, like HMQC, TOCSY, COSY are of great use in the spectral assignment because in 1D spectra coupling constants are generally lost due to the linewidth and only chemical shift information can be used. Recently, introduction of HR-MAS probes equipped with pulsed field gradients along the spinning axis [49] have extended even further the utility of this technique (an example of gradient 2D experiment is reported in Fig. 13). Gradients in MAS NMR are employed for coherence selection and for removing artefacts, like in solution-phase NMR. The latter application is particularly useful as spinning introduces additional sources of modulation.

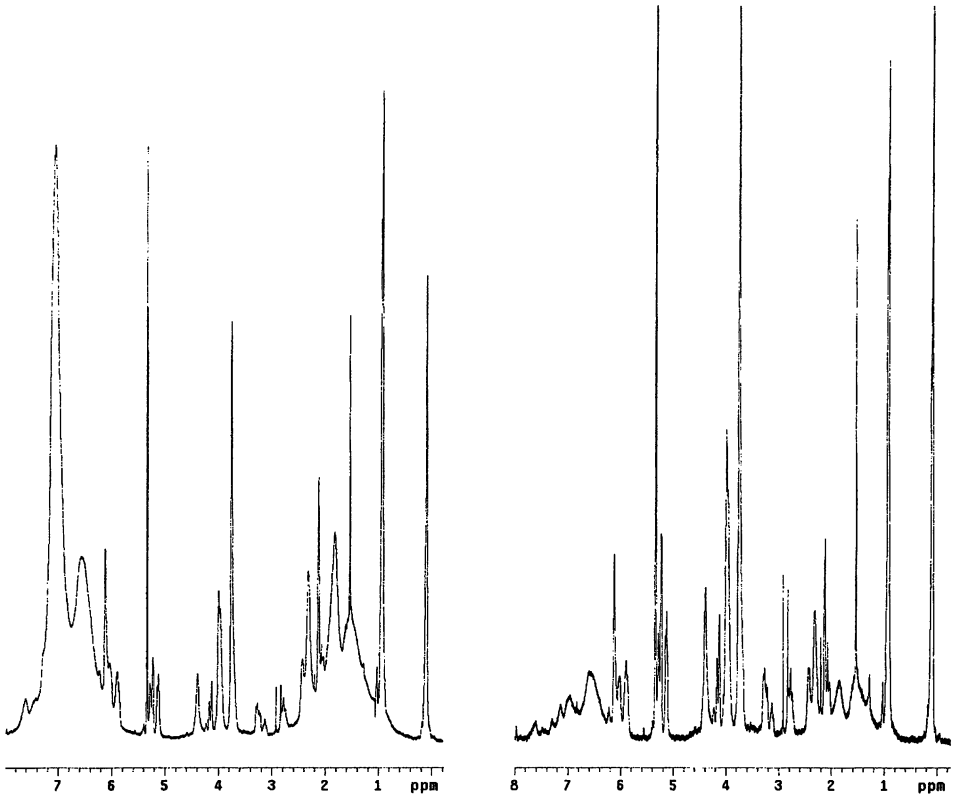

Fig. 12. ^{1}H NMR spectrum of a substituted Wang resin without presaturation (left) and with presaturation (right).

4. Infrared (IR) and Raman spectroscopy

IR and Raman spectroscopy are powerful techniques for detection and characterisation of reaction products directly on a single bead [50–53]. Structural determination of resin-bound, totally unknown compounds is rarely required, and generally the analytical task is to confirm the desired chemistry rather than a full structural elucidation. Since FTIR is a technique sensitive to organic functional group changes, it is well suited for the confirmation of organic transformation. The principle of monitoring reactions by IR is based on the functional group interconversions via chemical reaction or by appearance or disappearance of functional groups carried by building blocks or protecting group introduced or removed during the reaction. The direct functional group analysis permits qualitative analysis of each reaction step throughout the synthesis.

Transmission spectroscopy on KBr pellet is the simplest sampling technique and, because FTIR is one of the most popular instruments in organic chemistry laboratories, is widely used for routine spectral measurements on solid samples. It presents some disadvantages of which the main one is light scattering due to the fact that resin cannot be ground to the required dimension. This method requires a large amount of sample (5–10 mg of beads), is destructive, and time-consuming (for pellets preparation), so it is suited for analysis of resin-bound compound but not for reaction monitoring.

Attenuated total reflection (ATR) [54] is a versatile, non-destructive technique for acquiring the IR spectrum from the surface of a material or for materials that are either too thick or too strongly absorbing to be analysed by standard transmission spectroscopy. Under certain conditions, IR radiation passing through a prism made of a high refractive index IR transmitting material (ATR crystal) will be totally internally reflected. If a sample is brought in contact with the surface of the ATR crystal, an evanescent wave extends beyond the reflecting surface and will be attenuated in regions of the IR spectrum where the sample adsorbs energy. This technique is very sensitive (only enough beads to cover the area of the crystal are necessary) and requires no sample preparation. The speed of analysis is very high although no automation is so far reported. This approach can be used for real time reaction monitoring and is the method of choice for the analysis of surface grafted solid supports such as multi-pins and 'Micro Tubes' (like IRORI).

The FTIR microspectroscopy method [54] records spectrum from a small sample (such as a single bead) either in transmission mode or with use of an ATR objective. A microscope accessory is required for the measurement and a liquid nitrogen cooled mercury–cadmium–telluride (MCT) detector is used to enhance the sensitivity. Several beads are spread on a diamond

window and the IR beam is focused on a single bead using the view mode of the microscope. This is the most sensitive methodology today available: the amount of sample required is so small that real time reaction monitoring (without interrupting the reaction) can be performed as in the case reported in Fig. 14. Although it is the most expensive IR technique, FTIR microspectroscopy offers several advantages: high quality spectra can be recorded in a few minutes, no sample preparation is required, qualitative, quantitative (as percentage of conversion) and kinetics information can be obtained [55,56].

Fig. 15 illustrates some examples of the use of FTIR microspectroscopy in different reactions: the formation of an azide group (the typical band of the azide group appears at 2135/cm), of a cyano and an isocyanate group.

An issue of this technique is the quantitation for samples with inherently differing diameters (resulting in variant transmission pathlengths) and inhomogeneous transmission cross-sections. To date only one method is reported for absolute functional group quantitation, using deuterium labelling and internal pathlength referencing [57].

Diffuse reflectance IR Fourier transform spectroscopy (DRIFT) [54] enables the analysis of many solid samples for which traditional techniques fail. When IR radiation is directed onto the surface of a solid sample, two types of energy reflectance can occur: specular and diffuse reflectance. The specular component is the radiation, which reflects directly off the sample surface (energy not absorbed by the sample) while diffuse reflectance is the radiation, which penetrates into the sample and then emerges. A diffuse reflectance accessory is designed so that the diffuse reflected energy is optimised and the specular component is minimised. The optics collects the scattered radiation and directs it to the IR detector. The

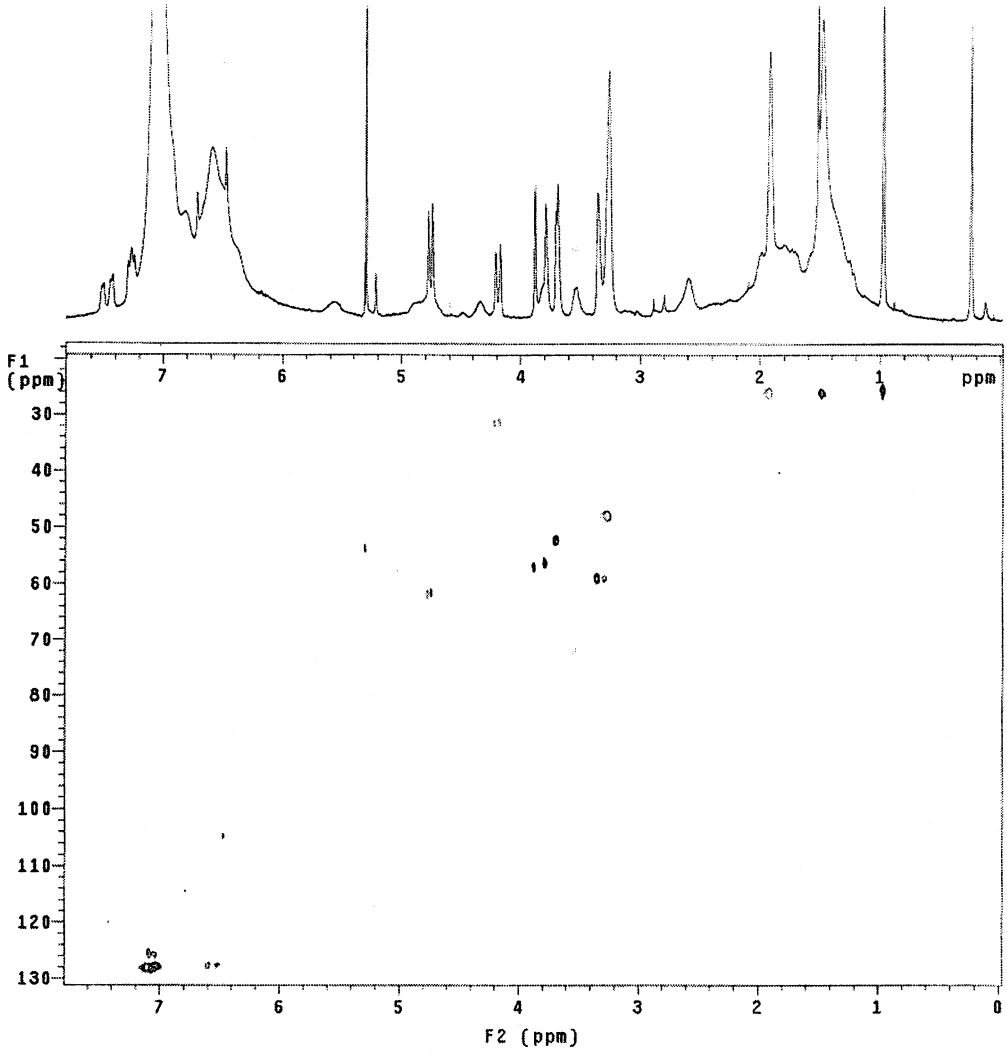

Fig. 13. Example gHSQC spectrum of a Wang resin acquired with a gradient HR-MAS probe.

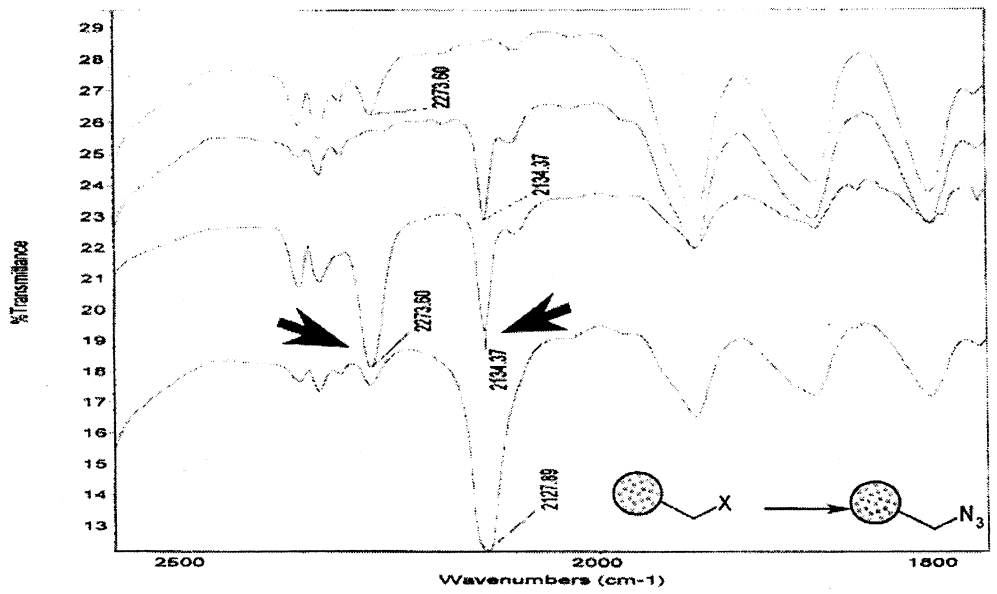

Fig. 14. Example of reaction monitoring by microinfrared spectroscopy.

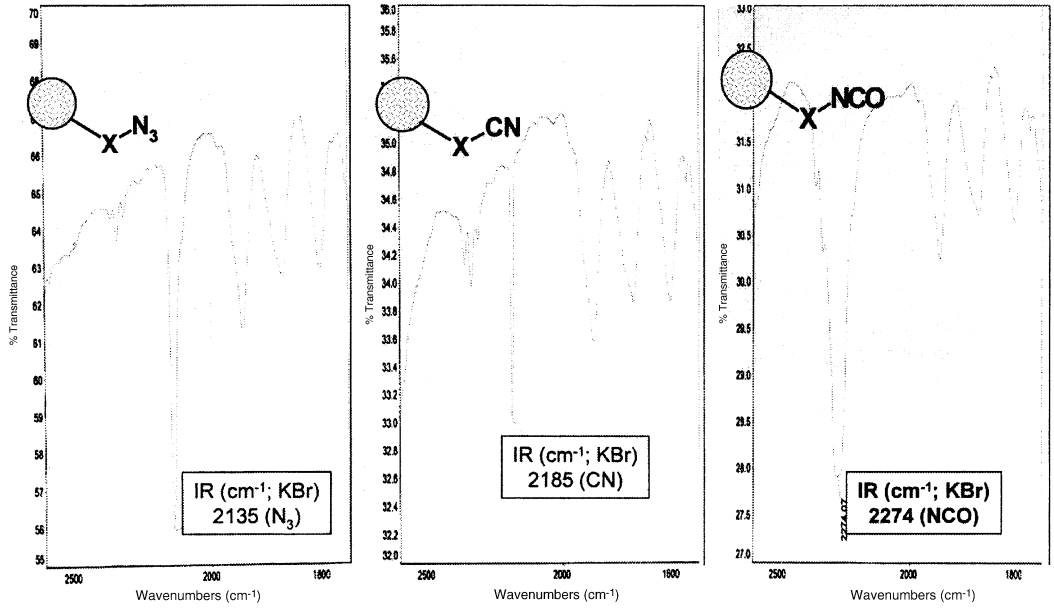

Fig. 15. IR monitoring of the formation of an azide group (left), of a cyano group (centre) and of an isocyanate group (right).

technique is non-destructive and the sample can be recovered, the only limitation being the sample size (5–10 mg of beads). Because of the high speed of analysis, the absence of sample preparation and the availability of an automated DRIFT accessory, this approach can be considered a high-throughput method for on-bead reaction monitoring.

Photoacustic spectra of resin samples have also been reported [58]. The photoacustic effect arises from the fact that IR radiation absorbed by the sample converts into heats. This heating is converted into a pressure wave that can be communicated to a surrounding gas. The thermal expansion of this gas produces a photoacustic signal detected by microphone, which replaces the IR detector of the spectrometer. The method is fast, non-destructive, quite sensitive, requires no sample preparation and the spectral quality is similar to DRIFT and ATR spectra. Photoacustic spectroscopy offers a convenient alternative to other IR techniques.

In recent years also Macro and Single Bead Raman Spectroscopy also are becoming more popular for the analysis of SPS resins [59]h. Raman is based on inelastic light scattering, in which scattered photons exchange energy with the sample. The intensity of the Raman

signals depends on changes in polarizability during the vibration. Strong Raman signals can be obtained for functional groups with low polarity and high polarizability such as c–c triple and double bonds, N=N, S–S, C–H, S–H, CtripleN, C=N, C=S, C–S, and asymmetric vibrations of NO_2, SO_2, CO_2. This approach is also useful for confirming the presence of conjugated groups. Many important vibrations are not detectable by the Raman method (such as OH, NH, C=N), however some IR-inactive vibrational modes can be detected by this method like S–S. Because of the high speed of analysis and sensitivity (comparable with other methods), Raman spectra can be used for real time reaction monitoring. This technique provides complementary information to FTIR, especially for resin-bound compounds containing symmetric vibrations of organic functional groups like NO_2, and bands below 600/cm such as S–S and C–Cl.

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