NEW TECHNIQUE FOR HIGH-THROUGHPUT SYNTHESIS

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Abstract. A new technique for high throughput solid phase synthesis using the centrifuge based liquid removal from readily available standard microtiterplates is described. This technique eliminates the filtration step and is therefore applicable to simultaneous processing of an unlimited number of reaction compartments. Its application is illustrated on the synthesis of an array of 380 tetrahydroisoquinolinones. © 1999 Elsevier Science Ltd. All rights reserved.

The arrival of combinatorial techniques (for reviews see ref. 1) stimulated the interest in new methods for the automation of synthetic processes. The scientific community is waiting for an instrument that would be rather simple, therefore inexpensive, and would allow each chemist to synthesize 100 - 1000 compounds in a batch. This instrument would be used for the deconvolution of active compound from biologically active mixtures, or for compound optimization, so called "lead explosion".

One of the basic problems of solid-phase synthesis automation is the separation of liquid and solid phases. All known automatic solid-phase synthesizers utilize filtration as the principle for separation of solid and liquid phase (for reviews see ref. 2). However, filtration always brings the danger of the filter clogging, which is especially harmful in the case of multiple synthesizers. We have tested the principle of "surface suction" for removal of supernatant from the sedimented suspension of solid-phase particles.[3,4] We have successfully designed and built the separation station for 96-well plates and applied this principle to our robotic synthesizer in which up to 72 plates can be processed simultaneously.[3] Even though powerful, the surface suction does not allow processing an unlimited number of reaction vessels simultaneously - the number of processed vessels depends on the number of needles performing the suction. The simpler way for the simultaneous processing of hundreds of reaction vessels is based on a different technique, which we call "tilted centrifugation".[5] The principle of tilted centrifugation is illustrated in Figure 1. When the resin is suspended in the flask, the flask is tilted, placed at the perimeter of the centrifugal plate and spun, the surface of liquid supernatant moves. If the speed of rotation is increased, the centrifugal force created by rotation (which depends on the radius of rotation and the speed) combines with gravitation and the resulting force causes the liquid surface to stabilize at the angle perpendicular to the resulting force vector. At the ratio of relative centrifugal force (RCF) to G of 3, the angle of the liquid surface will be about 61 degrees. If we increase the
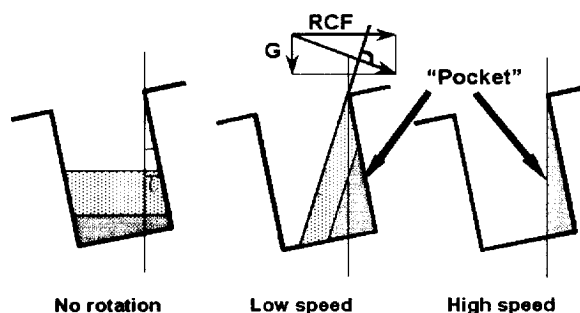
Figure 1 Principle of tilted centrifugation.

speed so that the ratio of these forces is more than 50, we will be getting close to the situation where RCF is infinity - therefore the liquid angle will be close to 90 degrees. Designed properly the pocket created by the tilt will allow only solid phase to remain in the pocket and all of the liquid will be expelled. The pocket can be created in the vessel of basically any shape - flat bottom, U bottom, or V bottom vessel - and it can be applied on the array of vessels (e.g. the commonly used microtiterplates).

The wells in microtiterplates are equal geometrically, but after being placed on the perimeter of the centrifuge they are subjected to different RCF depending on their distance from the axis of rotation. Therefore the volume of the "pocket" created by centrifugation in the so-called inner wells is bigger than the volume of the pocket in the outer wells. The absolute volume of the pocket is not as important as the ratio of volumes of pockets on opposite sides of the microtiterplate. This ratio depends on the dimension of the centrifugal plate, speed of the rotation, and the tilt of a plate. A very large plate will have insignificant difference between forces exerted onto inside and outside wells. Very fast rotation also diminishes the relative differences between pockets, as well as a larger degree of the tilt. For our example, we were working with the tilt of 9 degrees and 350 rpm and the diameter of centrifugal plate of 50 cm. Under these conditions, inner and outer wells differed by 8%, which we found to be an acceptable difference.

If we would work with an array of wells created by drilling holes into inert material, the liquid expelled from one well would inadvertently enter another well placed closer to the perimeter of the centrifuge. However, a 96-well shallow microtiterplate is actually 96 small cylinders attached to a flat piece of polypropylene and connected by a thin "rib", creating thus an array of 96 round wells plus 117 interwell spaces. When the liquid is expelled by centrifugal force from one well, it comes into the interwell space, accelerates across this space and ends up on the outer wall of the adjacent well. Then it flows along the well until it detaches and flies across another interwell space, eventually ending at the edge of the plate from where it flies onto the well of the centrifuge drum or it is collected in retaining reservoirs placed on the perimeter of the centrifuge rotor. Also, at
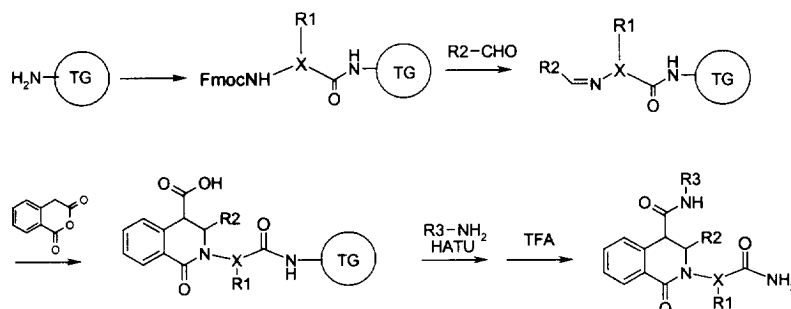
Scheme 1

the time of liquid removal, the reaction on the solid phase in all of the wells should already be complete and possible contamination would not result in any additional reaction.

The first experiments using tilted plate centrifugation were performed in the Savant centrifuge, which we have equipped with a custom-built rotor. Later, we built the dedicated centrifuge with 8 positions for microtiter plates. This centrifuge is driven from the computer and all centrifugation parameters can be flexibly changed. The delivery of washing solvents and common reagents is performed by the 96-channel distributor connected to a 6 port selector valve. We have integrated the centrifuge with the Packard Multiprobe 104 liquid distribution system, which allows us to perform the whole synthesis in a completely automatic regimen. The advantage of this compact system is that it can be easily enclosed in an inert atmosphere.

We have prepared an array of 380 substituted tetrahydroisoquinolinones to demonstrate the simultaneous processing power of the tilted centrifugation technique. The synthetic protocol for preparation of these heterocyclic molecules (see Scheme 1) was developed earlier[6] for synthesis in "tea-bags" (a technique invented by Houghten[7]). S-RAM Tentagel resin (0.24 mmol/g, Rapp Polymere, Tubingen, Germany) was distributed into the wells of four polypropylene shallow well microplates (3 mg of resin per well). The microtiterplates were placed on the centrifugal plate in a tilted position (9 degree tilt) and solvent was removed by centrifugation at 350 rpm. Solutions of Fmoc protected amino acids[8] in DMF (50 μL of 0.2 M solution) containing N-hydroxybenzotriazole (0.2 M) and DIC (0.2 M, added just prior to distribution) were delivered into the individual wells of the microtiterplate by 8 channel pipettor. Progress of the reaction was monitored by bromophenol blue method.[9] After 3 h the solutions were removed by centrifugation and washing solvent (DMF, 75 μL) was added by multichannel pipettor. Washing step was repeated four times with DMF and the solution of 50% piperidine in DMF was added (50 μL). After 15 min of incubation the plates were centrifuged and washing cycle with DMF was repeated four times, followed by washing with 0.05 M (50 μL) triethylorthoformiate (2x) and appropriate aldehyde solutions[8] (50 μL, 0.5 M in DMF) were added by multichannel pipetting. After overnight incubation plates were placed onto the centrifuge, liquid was removed

Table 1. Analyses of crude products. Purities were evaluated by HPLC on the reversed phase (Vydac C-18, 10 x 0.2 cm, gradient 0 to 70% acetonitrile in water/0.05% trifluoroacetic acid, HP 1060) with total ion current trace generated by mass spectrometer (Finnigan Mat LCQ) as detector.

Product quality category	Number of cases	Percentage of total (%)
Single peak (>95%)	201	52.9
Major peak (85-95%)	129	33.9
Product present (50-85%)	14	3.7
Minor peak (<50%)	21	5.6
Not present	15	3.9

and two washes with DMF were performed. A solution of homophthalic anhydride (1 M in DMF, 50 µL) was added to each well and closed multititerplates were shaken for 5 h. The liquid was removed and four washes with DMF were performed on the centrifuge. A solution of HATU (0.3 M in DMF, 50 µL) was added and removed by centrifugation after 30 min incubation and solution of an aminoethylpyrrolidine (0.3 M in DMF, 50 µL) was added. After overnight incubation of closed multititerplates on the shaker, the solution was removed by centrifugation and preincubation with HATU and incubation with amine solution was repeated once more for 4 h. The solution was removed by centrifugation and multititerplates were washed with DMF five times and with tert.butyl methyl ether twice. Trifluoroacetic acid was added to the plates by a multichannel pipettor (75 µL to each well) and closed plates were shaken for 2 h. Multititerplates were then opened, placed into the SpeedVac (Savant), and TFA was evaporated in vacuo. The plates were placed onto the table of the Multiprobe 104 and solid support was extracted by repeated (four times) addition and removal of 165 µL of DMF into the individual wells of multititerplate. The extracts were transferred to deep well polypropylene microtiterplates and evaporated in the SpeedVac. All wells were analysed by LCMS. The purities of prepared compounds were ranked into four categories and the results are given in Table 1. The results were compared with analogous synthesis performed in "tea-bags" and centrifuge results were found superior in purity. HPLC traces of four compounds synthesized in adjacent wells are shown in Figure 2. HPLC traces document the fact that the neighboring wells are not contaminated by products from other wells (either by direct transfer of reagents or solid support, or by contamination through vapors) and at the same time they show that the yield of products in individual wells are comparable. We have determined the "individual" yields of the crude product by pooling compounds from twelve randomly selected wells from one microtiterplate and we have found a good correlation between the yield determined in this way (78.2%) and the average yield determined by weighing the plates before the extraction and after evaporation of extracting solvent (76.4%, 77.6%, 82.4%, and 86.1%). Yield determination confirm also the fact that the resin is not lost during the synthesis by repeated cycles of centrifugation (described synthesis employed 28 cycles of centrifugation). In comparison with "tea-bag"
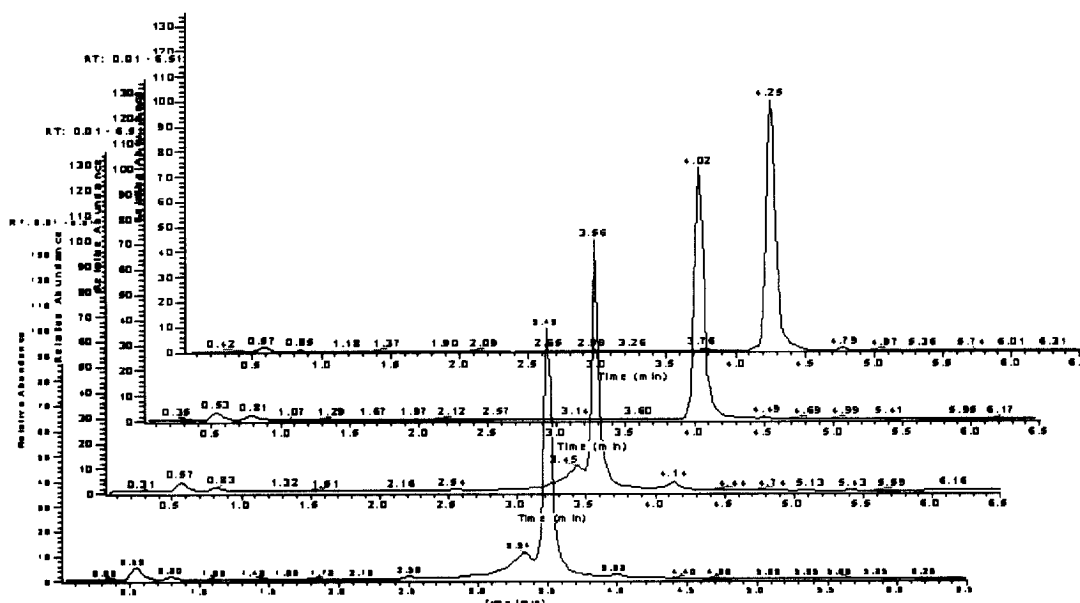
Figure 2 HPLC traces of four tetrahydroisoquinolinones synthesized in neighboring wells

technique the application of centrifugation resulted in significant time saving; washing and resorting of comparable number of "tea-bags" would require at least 5 h (in centrifuge resorting is not needed and all washing operations were completed in less then an hour).

Conclusions: Tilted centrifugation is the most effective and simplest method for liquid removal from a multiplicity of vessels of any geometry and size. Polypropylene microtiterplates are ideal reaction vessels for tilted centrifugation based synthesis. The fact that tilted centrifugation is the only way for removal of liquids from an unlimited number of reaction vessels simultaneously is suggesting its application in ultraminiaturized synthesizers.

References and Notes

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8. List of building blocks used in the first and the second step of the synthesis of 380 tetrahydroisoquinolinones. Amino acids: glycine, 3-aminopropionic acid, 5-aminopentanoic acid, 7-aminoheptanoic acid, (S)2,3-diaminopropionic acid, (S)2,6-diaminohexanoic acid, (S/R)-3-amino-2-methylpropionic acid, 2-(2-aminoethoxy)acetic acid, trans-4-(aminomethyl)cyclohexanecarboxylic acid, 4-(aminomethyl)benzoic acid; Aldehydes: benzaldehyde, 1,4-benzodioxan-6-carboxaldehyde,1-methylindole-3-carboxaldehyde, 2,3-difluorobenzaldehyde, 2-bromobenzaldehyde, 2-chloro-5-nitrobenzaldehyde, 2-furaldehyde, 2-imidazolecarboxaldehyde, 2-naphthaldehyde, 2-pyridinecarboxaldehyde, 2-thiophenecarboxaldehyde, 3,4-dichlorobenzaldehyde, 3,5-bis(trifluoromethyl)benzaldehyde, 3,5-dihydroxybenzaldehyde, 3,5-dimethoxybenzaldehyde, 3,5-dimethyl-4-hydroxybenzaldehyde, 3-(4-methoxyphenoxy)benzaldehyde, 3-furaldehyde, 3-hydroxybenzaldehyde, 3-methyl-4-methoxybenzaldehyde, 3-methylbenzaldehyde, 3-nitrobenzaldehyde, 3-pyridinecarboxaldehyde, 3-thiophenecarboxaldehyde, 4-(3-dimethylaminopropoxy)benzaldehyde, 4-(dimethylamino)benzaldehyde, 4-(methylthio)benzaldehyde, 4-(trifluoromethyl)benzaldehyde, 4-biphenylcarboxaldehyde, 4-bromo-2-thiophenecarboxaldehyde, 4-cyanobenzaldehyde, 4-methoxy-1-naphthaldehyde, 4-nitrobenzaldehyde, 4-pyridinecarboxaldehyde, 5-(hydroxymethyl)-2-furaldehyde, 5-bromo-4-hydroxy-3-methoxybenzaldehyde, 5-nitro-2-furaldehyde, 6-methyl-2-pyridinecarboxaldehyde.

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