Article

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High-Performance Thin-Layer Chromatography Method for Assessment of the Quality of Combinatorial Libraries, and Comparison with Liquid Chromatography−Ultraviolet−Mass Spectrometry

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A high-performance thin-layer chromatography (HPTLC) method was developed for fast evaluation of the purity of solid-phase synthesis products. The results obtained were in good agreement with results obtained by the LC−MS method ($r^2 = 0.8404$) or by the LC−UV method ($r^2 = 0.8053$), confirming the suitability of HPTLC for purity analysis of combinatorial syntheses. The synthesis products can be quantified and identified by measuring UV densitograms or in situ UV spectra or by ESI-MS after isolation of the zone of interest. A new, simple, and fast method for transferring the zone of the analyte from the plate to the ESI-MS equipment is described. The new HPTLC method enables rapid and efficient analysis of ∼40 samples in parallel. As such, it offers a cheaper and easier way to analyze the purity of synthesis products than the commonly used LC−UV−MS.

Introduction

The development of combinatorial chemistry has changed the whole strategy of drug discovery during the past decade, providing fast synthesis and high-throughput screening of new compounds at reasonable cost.[1−5] Combinatorial libraries can be created by split synthesis, which produces mixtures of thousands of compounds, or by automated parallel synthesis, in which different compounds are synthesized in separate vessels. The present trend in drug discovery is toward carefully designed and well-characterized libraries produced by solid-phase parallel synthesis.[1,6,7] The assessment of the quality of the synthesis is highly important, since false (negative or positive) results, for instance, in tests of biological activity or determination of early-ADME parameters in vitro (the pharmacokinetic study of drug absorption, distribution, metabolism and excretion), must be avoided.[2,5,8] The development of fast and reliable analytical methods for quality control of libraries, including the identification of synthesis products and purity tests, is a great challenge.

Several IR techniques, such as the KBr pellet method and FT-IR, near-infrared (NIR)-FT Raman,[9−13] matrix-assisted laser desorption mass spectrometry (MALDI),[14,15] and NMR[16] techniques, have been developed for monitoring the synthesis process directly in situ on support material. However, the specificity, reproducibility, and sensitivity of the in situ techniques do not fulfill the requirements for unambiguous detection of the product and possible impurities. Thus, "cleave and analyze" methods with mass spectrometry (MS) are routinely used instead in the quality control of synthesis. The MS methods mostly are either flow injection analysis (FIA-MS)[5,8,17] or liquid chromatography−MS (LC−MS) combined with UV detection.[5,6] FIA-MS is a highly efficient method for rapid qualitative assessment of the synthesis library. However, the purity assessment is normally done by LC−MS, since impurities may suppress the ionization process in FIA-MS, especially electrospray ionization, reducing the reliability of the analysis. Other methods commonly used in purity assessment are LC with UV, diode array (DAD), evaporative light scattering (ELSD), or chemiluminescent nitrogen (CLND) detection.[5,18−22] Capillary electrophoresis has also been mentioned in the literature.[23,24] All of these methods are based on the analysis of one sample per run. The methods for assessment of large libraries must be automated, and multiple probe autosamplers must be used to achieve fast enough analysis. This leads to expensive systems, especially where MS is employed.

Thin-layer chromatography (TLC), in contrast to the methods mentioned above, offers separation of multiple

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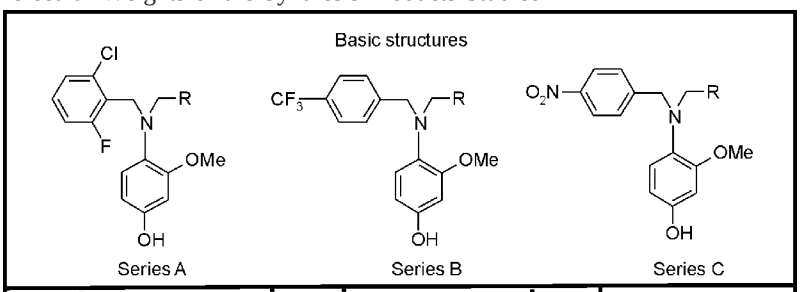
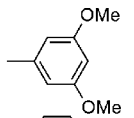
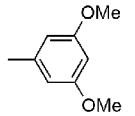
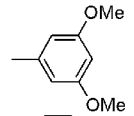
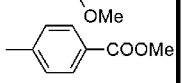
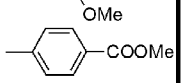
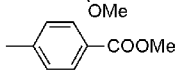
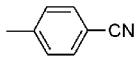
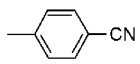
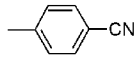
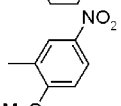
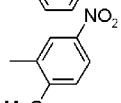
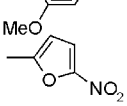
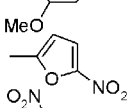
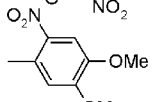
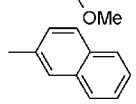
Table 1. Structures and Molecular Weights of the Synthesis Products Studied



Comp. (MW)	R	Comp. (MW)	R	Comp. (MW)	R
A1 (431)		B1 (447)		C1 (424)	
A2 (429)		B2 (445)		C2 (422)	
A3 (396)		B3 (412)		C3 (389)	
A4 (371)		B4 (387)		C4 (364)	
A5 (446)		B5 (311)		C5 (439)	
A6 (406)				C6 (399)	
				C7 (469)	
				C8 (414)	

samples in parallel. The modern HPTLC technique, combined with automated sample application and densitometric scanning, is sensitive and completely reliable (equal to HPLC),[25] suitable for use in qualitative and quantitative analysis of pharmaceutical, environmental, toxicological, forensic, and food chemical applications.[26−30] Although TLC is a routine tool in the monitoring of synthesis processes, HPTLC has not been evaluated in detail in the quality control of combinatorial libraries. For this purpose, HPTLC offers several advantages over the present methods: (1) fast, simple and inexpensive analysis of many samples simultaneously; (2) a disposable stationary phase that avoids memory effects; and (3) the possibility to use a number of nondestructive detection methods and reagents in sequence, so improving the reliability of the detection.

Several methods, such as UV/vis, fluorescence, MS, FT-IR, and Raman spectroscopy have been applied for the in situ analysis of zones on TLC plate,[31−35] the most common of these being UV and fluorescence. Unfortunately, high background associated with the matrix or silica adsorbent and relatively poor sensitivity reduces the suitability of the in situ MS methods. Another approach is to carry out the MS detection after scraping the interested zone from the plate, for example by, FIA-MS (or LC−MS), using atmospheric pressure ionization mass spectrometry (electrospray, atmospheric pressure chemical ionization). The zone of the analyte can be isolated not only by the methods normally used (extraction after scraping with a razor blade, scissors, or spatula) but also with a suction apparatus[36,37] or a microcapillary array. [38]

In this article, we introduce and evaluate the suitability of modern HPTLC in the assessment of the quality of a small and focused combinatorial library obtained by solid-phase synthesis and compare the method with the LC−UV−MS method currently in use. The 19 compounds studied (Table 1) form part of the library of protein kinase C inhibitors. Here, we describe the development of a HPTLC separation method for the synthesis products, the capability of the UV densitometric scanner to identify synthesis products, and a new method of isolating HPTLC sample zones for FIA-MS. In addition, the LC−UV−MS method based on electrospray ionization was applied for purity analysis. The methods are tested and compared in the semiquantitative determination of the purity of the synthesis products. For this work, we have chosen a library with relatively low purity to test the performance of the HPTLC method in a complex case, and therefore, the results obtained here are usable also for more pure libraries. The further purification of the studied library was not included in this work.

Results and Discussion

HPTLC. Separation. The primary aim in the development of the TLC method for the assessment of the quality of a combinatorial library (produced by solid-phase parallel

Table 2. R_F values of Synthesis Products and Impurities[a]

sample	synthesis product	R_F value	impurities: R_F values 1	2	3	4	5	6
1	A1	0.25	0.05	0.09	**0.30**	0.42	0.48	
2	A2	0.22	0.05	0.12	0.18	0.38	0.44	
3	A3	0.25	0.05	**0.20**	0.36	0.42		
4	A4	0.26	0.06	0.11	**0.21**	0.41	0.45	
5	A5	0.31	0.05	0.13	0.19	**0.25**	0.36	0.41
6	A6	0.22	0.05	0.12	0.18	0.38	0.44	
7	B1	0.36	0.05	0.15	0.23	**0.34**	**0.39**	
8	B2	0.43	0.05	0.18	0.26	0.31	**0.49**	
9	B3	0.36	0.05	0.15	0.19	0.28	**0.40**	**0.44**
10	B4	0.47	0.05	0.15	0.29	0.50		
11	B5	0.37	0.05	0.16	0.20	**0.41**		
12	C1	0.38	0.05	0.11	**0.30**	**0.43**		
13	C2	0.38	0.05	0.13	**0.32**	0.48		
14	C3	0.31	0.05	0.12	**0.25**	**0.45**		
15	C4	0.42	0.05	0.11	**0.24**	**0.29**	**0.46**	
16	C5	0.25	0.05	0.12	**0.29**	**0.46**		
17	C6	0.28	0.05	**0.35**	**0.44**	**0.47**		
18	C7	0.24	0.05	0.48				
19	C8	0.45	0.05	0.12	0.19	**0.28**	0.49	

[a] The impurities in each series that were near to or within the range of R_F values of the synthesis products ($R_F = \pm 0.02$) are marked in bold.

synthesis) was to separate unequivocally the targeted synthesis product from the impurities. For this purpose, HPTLC offers better resolution than conventional TLC owing to the smaller particle size and the narrower particle size distribution of the adsorbent. The samples were sprayed as a thin rectangular band onto the adsorbent. The resolution with this application technique is reported to be significantly better than that with the conventional spot injection technique and to preserve a good resolution in the system.[39,40] The normal elution technique from one side allowed analysis of ~20 samples on one 10 × 20 cm plate; however, the number of samples can be doubled ($n = 40$) by carrying out the elution from both sides using the horizontal elution mode instead of the vertical mode.

In the case of small libraries, including homologous series of compounds, such as ours, the use of gradient elution is often not necessary. This is because the retentions of the homologous series of compounds are not expected to differ significantly. The only requirement in the rapid purity analysis of the libraries is to separate synthesis products from the impurities, and there is no need to separate impurities from each other. Optimization of the mobile phase is necessary, however. This was done with the help of the PRISMA model, which is widely used in TLC.[41−46] The optimization process with PRISMA is easy and relatively fast to perform: the overall time required for the optimization is ~5−6 h. The optimum solvent composition for our experiments was identified as 2-propanol/hexane (1:8), with which the R_F values of the synthesis products and impurities in the tested libraries were between 0.05 and 0.50 (Table 2). The values were fairly low, but on the other hand, the diffusion is less for compounds with low R_F values (based on the shorter elution distance) than for those with high R_F values.[27,47] The eluent system selected also offers fast analysis of all 19 samples simultaneously, with elution time of 10 min.

Table 3. Resolution of the HPTLC method, Calculated as Resolution between the Synthesis Product and the Impurities before and after the Synthesis Product.

synthesis product	R_s before	after
A1	1.00	1.00
A2	2.20	0.70
A3	0.73	0.80
A4	1.00	2.40
A5	0.88	1.00
A6	2.75	1.00
B1	0.71	0.50
B2	1.17	1.44
B3	0.67	1.20
B4	0.50	2.57
B5	1.00	3.75
C1	0.71	1.00
C2	1.29	0.91
C3	2.00	0.75
C4	0.60	1.71
C5	0.50	1.40
C6	0.80	1.85
C7	2.63	1.20
C8	0.50	1.06

The capability of our HPTLC method to separate a synthesis product from its impurities was tested by determining the resolution between the synthesis product and the impurity eluted before and after the synthesis product (Table 3). The baseline separation was achieved in 60% of the cases ($n = 38$), and adequate resolution for reliable integration, in 92% of the cases ($n = 38$). Only in 3 cases of 38 was the resolution for accurate quantitative analysis not sufficient. However, in these three cases, the impurity appeared as a shoulder peak, and it was possible to determine the relative purity by using peak heights. In conclusion, acceptable resolution for semiquantitative determination of purity of the synthesis product can easily be achieved by using the PRISMA model.

Detection. The HPTLC detection can be simply done with UV lamp, by densitometric scanning with one wavelength, or by recording the whole UV spectrum; or the spot can be analyzed by an off-line method, such as mass spectrometry. Choice of the detection method depends on the information desired. Very rapid screening, the first tentative assessment, of the quality of the synthesis can be done by visual observation of the zones under a UV lamp, the method routinely used to monitor the synthesis process. One zone may indicate successful synthesis while additional zones indicate impurities. (In our experiments, from three to seven separated zones in every sample were observed under a UV lamp.) High concentrations of impurities are also easily estimated visually to indicate low quality of the library, meaning that the synthesis has to be optimized or the product has to be purified. Often, this visual information is sufficient to show the quality of the library. Other detection techniques, for example, UV densitometry, must be used to identify the synthesis product for purification. It is also worth of mention that the visual observation is really not necessary before densitometric analysis.

UV densitometry offers a method for identification of the synthesis products and a fast and reliable quantitative method

Table 4. (A) Main and Additional Wavelength Maxima and Intensities for the Synthesis Products in Series C,[a] (B) Main and Additional Wavelength Maxima and Intensities for the Impurities in Series C Marked in Bold in Table 2 (Those within the Range of the R_F Values of the Synthesis Products)[b]

	A			B	
				all impurities within the range of R_F values of synthesis products	
sample	synthesis product	λ_{max} (intensity)	additional λ_{max} (intensity)	λ_{max} (intensity)	additional λ_{max} (intensity)
12	C1	280 (100)	202 (75)	265 (100)	
13	C2	284 (100)	248 (93), 187 (91)	198 (100)	277 (80), 240 (70)
14	C3	280 (100)	237 (90), 195 (90)	261 (100)	195 (65)
15	C4	285 (100)	199 (80)	259 (100)	195 (30)
16	C5	292 (100)	199 (68)	277 (100)	198 (63)
17	C6	293 (100)	201 (60)	260 (100)	290 (63), 195 (53)
18	C7	290 (100)	198 (50)	260 (100)	
19	C8	283 (94)	225 (100)	198 (100)	
				260 (100)	
				198 (100)	274 (56)
				274 (100)	
				272 (100)	194 (45)
				198 (100)	
				265 (100)	224 (70)

[a] Highest wavelength maximum of the spectrum is 100. [b] Highest wavelength maximum of the spectrum is 100.

for determination of the quality of libraries. With densitometric measurements, the synthesis products can be identified by their R_F values and by in situ UV spectra**.** In small libraries, including homologous series, the R_F values and UV spectra are not expected to differ significantly. The differences may also be predicted from the structures of the compounds. This information can be utilized in the identification of the synthesis products. On the other hand, in the case of libraries with high diversity, the identification of the synthesis products may not be as unambiguous.

There is no need to record UV spectra from all of the zones in order to identify the synthesis product, since at least some zones can be excluded on the basis of their R_F values. It is important to limit the recording of UV spectra, because the UV measurements are more time-consuming than the measurement of densitograms with one wavelength (254 nm in our experiments). Three guidelines are relevant for the recording of UV spectra. First, exactly the same R_F value for several compounds in a homologous synthesis series suggests an unreacted starting material or other impurity, and these zones can be excluded. Second, the range of R_F values, within which the synthesis products will elute, can be identified by determining the R_F value for the most polar and most nonpolar synthesis products within a homologous series. Thus, any zones eluted outside the range of these R_F values must represent impurities and can be excluded. The range of the R_F values of the synthesis products in our experiments was between 0.22 and 0.31 for series A, 0.36 and 0.47 for series B, and 0.24 and 0.45 for series C (Table 2), indicating only slight variation of the R_F values within the series. Third, the synthesis product will normally be one of the main peaks within the range, which offers additional confidence for the identification of the synthesis products. Unambiguous identification of the synthesis products may be impossible if more than one compound falls inside the range of the R_F values of the synthesis products. The identification must then be confirmed by recording in situ UV spectra for the preselected zones.

The UV spectra of compounds within small homologous combinatorial series are expected to be very similar, while those of the impurities are expected to differ noticeably from those of the synthesis products. The differences in UV spectra in our work were demonstrated by recording the UV spectra for all compounds in series C (see Table 4 and Figure 1). The UV spectra of the synthesis products clearly exhibited one main absorption maximum in the wavelength range 280−293 nm. An additional maximum was recorded at ∼200 nm. The absorption maxima of most of the impurities differed significantly from those of the synthesis products. The results show that combining the information of R_F values and UV spectra allows the synthesis products to be distinguished from the impurities with good reliability.

If unambiguous identification with the recorded UV spectra is still impossible, the identification can be carried out by "cleave and analyze" methods with use of mass spectrometry, for example. In practice, off-line MS analysis is necessary relatively seldom. The conventional isolation and sample cleanup method from the plate was satisfactory but time-consuming. A method presented by Amorese et al.[37] was also tested, but it proved unsuitable for FIA-ESI-MS analysis, because the residues of the silica adsorbent caused significant background in the ESI-MS analysis. In view of these unsatisfactory results, we developed a new scraping method, combined directly with a sample cleanup procedure (Figure 2).

In our apparatus (Figure 2), the cut end of the Finntip allowed the isolation of very narrow zones of the synthesis products without co-isolation of the impurities, even in cases in which the synthesis products were only partially separated from the impurities. The adsorbent particles were sucked onto the filter, from which the particles were extracted with 0.5 mL of methanol for ESI-MS analysis (see the Experimental Section). The whole procedure, from assembling of the device to scraping, sample extraction, and filtration, can be done within 2−3 min using the same device. Memory effects were not a problem, since all parts of the device in contact

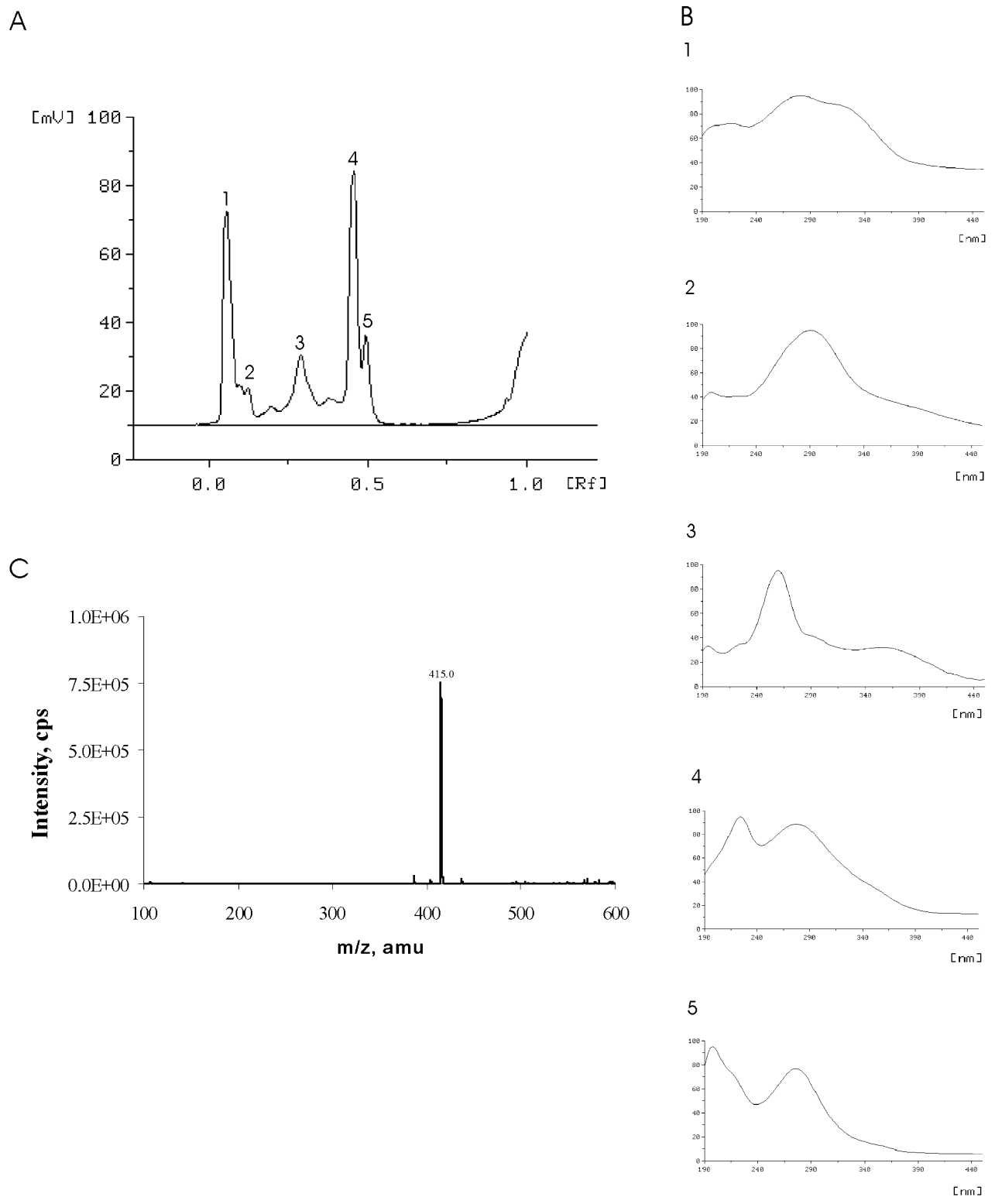


Figure 1. Identification of the synthesis product of C8 (*m*/*z* 414) by the HPTLC method: (A) typical densitogram of the synthesis product (C8); (B) in situ UV spectra of the peaks 1−5 (presented in A); (C) ESI−MS spectrum of the zone of the synthesis product (peak 4).

with the sample are disposable. In addition, the device is simple and easy to set up in any laboratory.

The isolated compounds of a sample can be rapidly identified simply by flow injection analysis (FIA) using ESI-MS. Because ESI offers very soft ionization, protonated or deprotonated molecules are normally the main peaks in ESI spectra, allowing reliable identification of the synthesis product. Approximately 50−100 samples can be analyzed by FIA-ESI-MS within 1 h. Figure 1 shows as an example the ESI-MS spectrum of C8 obtained by using the developed scraping and sample cleanup procedure. The spectrum shows only a very abundant protonated molecule (*m*/*z* 415) with minimal fragmentation. The spectrum does not contain any background ions, indicating efficient cleanup of the sample.

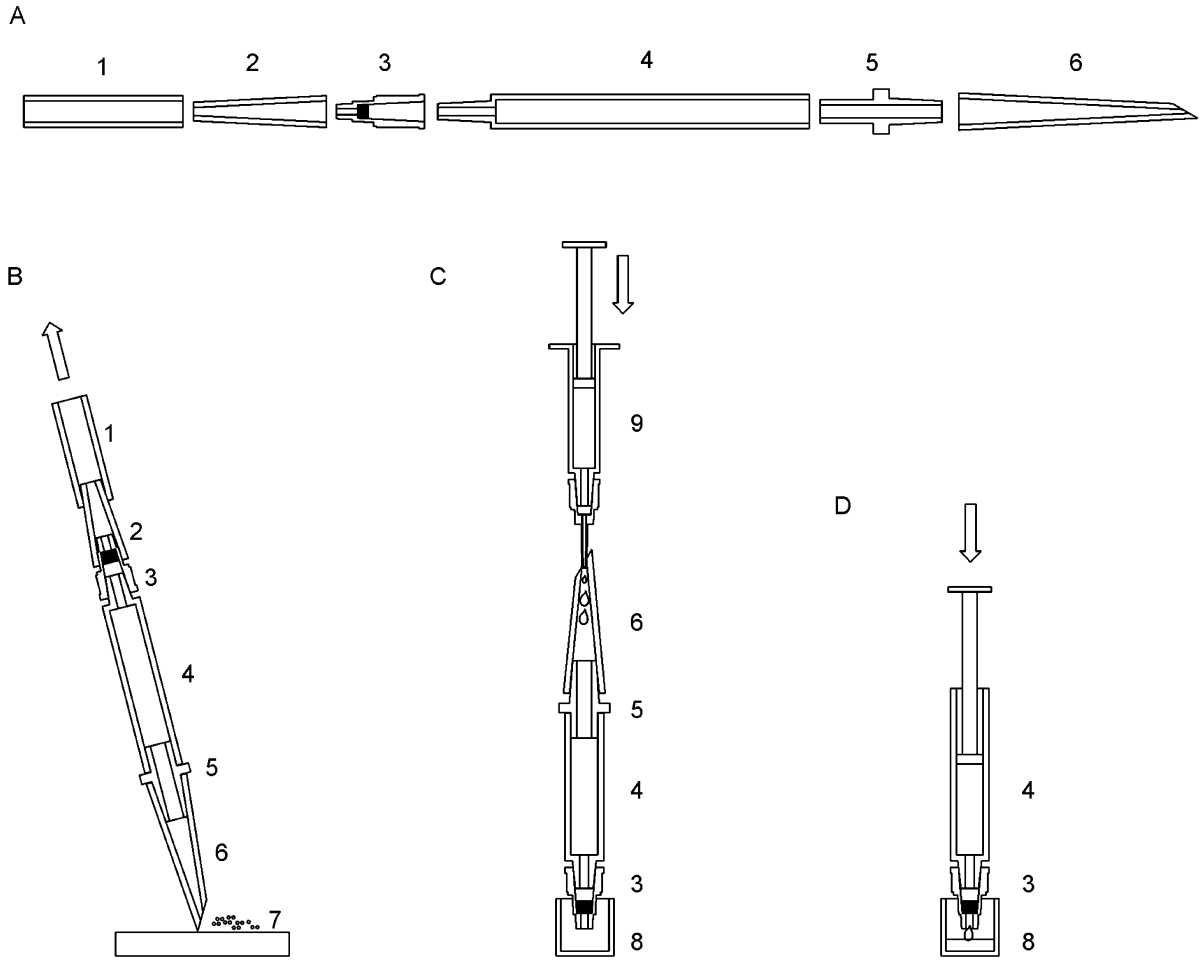


Figure 2. Injection syringe apparatus for transferring HPTLC sample from the plate to MS analysis: (1) tube to vacuum, (2) Finntip FT 300, (3) filter unit, (4) injection syringe, (5) a hollow binding piece, (6) Finntip FT 250 Universal, (7) HPTLC plate, (8) sample vial, and (9) second injection syringe and needle. (A) Unassembled pieces of the injection syringe apparatus. (B) With the help of the vacuum, the scraped sorbent is sucked through the Finntip (6), the binding piece (5), and the injection syringe (4) to the filter unit, on which the sample is retained according to its particle size. (C) Apparatus is turned vertically, and the injection syringe is removed from the vacuum. About 0.5 mL of methanol is introduced to the Finntip (6). (D) The compound of interest is eluted by pushing the solvent with the piston through the filter unit to the sample vial. The sample solution is now ready for MS analysis.

Additional structural information is easily obtained with use of tandem mass spectrometry. This may be necessary if the impurities must be characterized.

LC−UV−MS Method. In addition to the HPTLC method, an LC−UV−ESI-MS method was developed for the quality analysis of the combinatorial libraries. The pH, a suitable buffer, and wavelength used were selected according to the chemical properties of the synthesis products and the expected impurities. In our experiments, owing to the basic characters of the products, the use of a water/methanol gradient elution system with ammonium acetate as buffer at pH 4.5 (adjusted with acetic acid) allowed good ionization efficiency for the synthesis products in positive ion mode. All of the MS spectra showed a very abundant protonated molecule with minimal fragmentation. The ion chromatograms of the protonated molecules showed good chromatographic behavior of the analytes, and all of the synthetic products were separated from the impurities. However, chromatographic separation is not necessary in LC−MS, since the compounds can be separated by MS.

The synthesis product can easily be identified from the protonated molecule. After this, all the peak areas of the total ion chromatogram and UV chromatogram are integrated. The peak area of the synthesis product divided by the sum of the peak areas of all compounds in the total ion chromatogram or in UV chromatogram gives directly the percentage purity of the sample. The response in ESI is dependent on several factors, the most important being the acidic or basic character of the compounds, the surface activity, and the eluent composition employed in LC. Since the chemical and physical properties may vary significantly between synthesized compounds and impurities, the results with LC−MS are always only indicative. Furthermore, a relatively high background in ESI may cause some uncertainty in the analysis. However, the use of special software, such as base peak total ion chromatogram (TIC) included with the Bruker ion trap software, allows automatic subtraction of background ions and provides a useful tool for the extraction of the synthesis products and impurities from the background. The total ion chromatogram, base peak TIC, and extracted ion chromatogram (XIC) of sample 11 (synthesis product B5) are presented in Figure 3. The response of UV detection is dependent on the chromophores of a molecule, and therefore, the response may vary significantly between synthesis

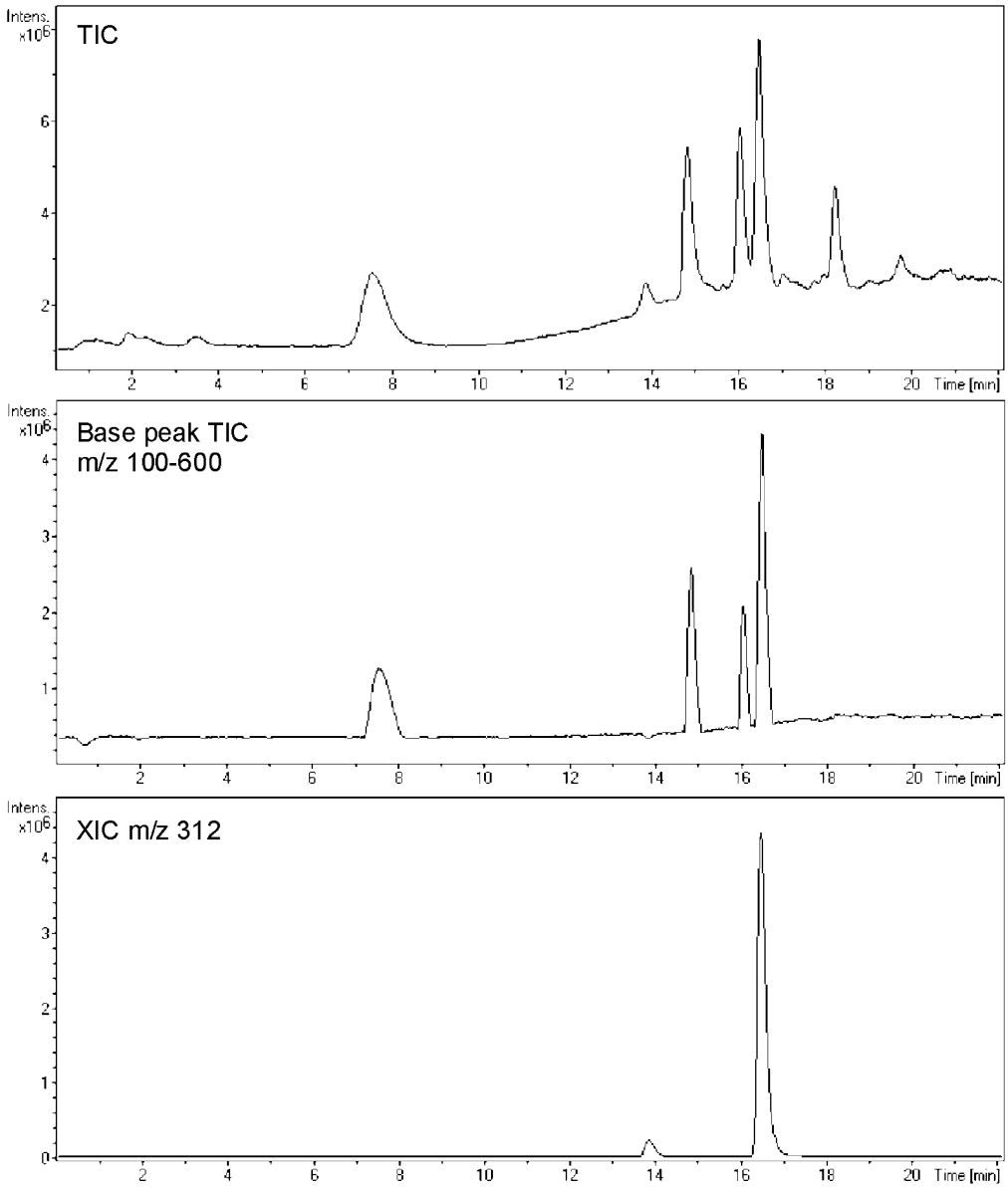


Figure 3. Gradient elution LC−MS of sample 11 in which the synthesis product was B5, *m*/*z* 311: (A) the TIC; (B) the base peak TIC *m*/*z* 100−600; (C) extracted ion chromatogram of the protonated molecule of synthesis product B5.

product and impurities. However, the variation between responses is often more significant in LC−MS than in LC−UV, and therefore, UV detection is commonly used for quantitation, and MS, for the identification of a synthesis product.

Comparison of HPTLC and LC−UV−MS Methods. The percentage of the purity of the synthesis products was measured for all samples by HPTLC and LC−UV−ESI-MS. There was no significant difference between the results when the purity of the product was calculated either by HPTLC−UV or LC−UV−MS (Figure 4). The results obtained by these methods were in reasonable agreement: LC−MS (TIC) and HPTLC−UV ($r^2 = 0.8404$), LC−UV and HPTLC−UV ($r^2 = 0.8053$), and LC−MS (TIC) and LC−UV ($r^2 = 0.8310$). However, when the purity of the product was high, LC−MS (TIC) gave a higher percentage than HPTLC. This may mean that some of the impurities give only low response with LC−ESI-MS. Despite these uncertainties, it can be concluded that HPTLC is as reliable a method as LC−UV−MS in assessment of the purity of combinatorial libraries. It must be noted, nevertheless, that the UV and ESI-MS responses may vary significantly between synthetic product and impurities. In the worst case, some of the compounds in the sample will not include the chromophore or cannot be ionized by ESI.

The advantages and disadvantages of the HPTLC and LC−MS methods are summarized in Table 5. HPTLC has several advantages over LC−MS. Clearly the most important advantage is that HPTLC offers a significantly cheaper and simpler method than LC−MS. Furthermore, the operation of HPTLC does not require such extensive training as LC−MS. The plates are disposable, and therefore, the memory effect is not a problem in HPTLC, as it may be in LC−MS. ESI is suitable only for medium-polar and polar compounds, and HPTLC is suitable for all compounds. In addition, only volatile buffers and medium-polar or polar solvents can be used in ESI, but a significantly wider selection of solvents and buffers can be used in HPTLC.

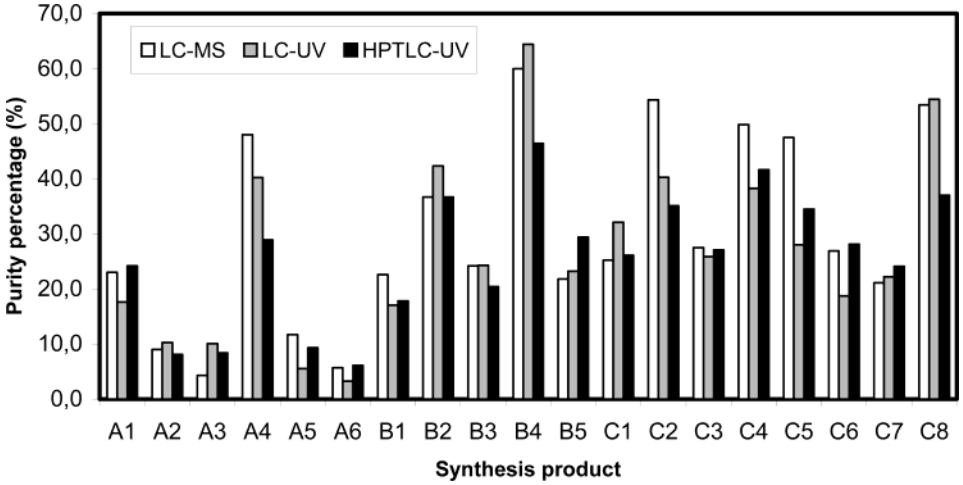


Figure 4. Comparison of the purities of the synthesis products measured by HPTLC and LC−UV−MS methods: LC−MS (TIC) and HPTLC−UV ($r^2 = 0.8404$), LC−UV and HPTLC−UV ($r^2 = 0.8053$), and LC−MS (TIC) and LC−UV ($r^2 = 0.8310$).

Table 5. Comparison of Advantages and Disadvantages of HPTLC and LC−MS Methods in Purity Analysis

	LC−MS	HPTLC
sample	memory effects are possible	disposable plates, no memory effects
solvent used	limited mobile phases and buffers can be used high solvent consumption	various mobile phases and buffers can be used the solvent consumption is reduced by horizontal mode
chromatography	parallel analysis possible, if several columns are used good separation power stabilization of the column prolongs analysis time	parallel analysis possible in one TLC plate limited separation power rapid, no stabilization time
detection	highly selective on-line spectrophotometric detection possible	in situ spectrophotometric detection is possible a number of nondestructive detection methods can be used after isolation of the analyte zone
other	easy automate complicated method expensive	not fully automated simple method inexpensive
method development	relatively fast	optimization of eluent composition prolongs analysis time if sample contains several impurities
time used for analysis	normally 5 to 20 min/sample; 3−10 samples/h	recording of UV spectra prolongs the analysis time 20−40 samples per hour with densitometric scanning

Rapidity is an important factor in the selection of a method for purity analysis. HPTLC with densitometric detection allows the analysis of ∼20−40 samples in one plate within 1 h. Furthermore, the chromatographic step with several plates can be performed parallel in different TLC chambers. In conventional LC−MS, only one sample can be analyzed per run, the analysis time being typically 5−20 min; thus, only 3−10 samples can be analyzed in 1 h. The sample throughput in LC−MS can be improved by using automated methods, special multiple probe autosamplers, and several LC columns in parallel;[20] however, all this leads to an expensive, complicated, and less robust analysis. The time required for the development of the method is normally shorter with LC−MS, since often gradient elution with buffered water/methanol or water/acetonitrile offers acceptable chromatographic behavior and ionization efficiency with ESI. In HPTLC, in which isocratic conditions are used, the development of a sufficient separation may take several hours, increasing the overall analysis time. The data processing can be automated with both methods. The specificity of LC−MS is superior to that of HPTLC with UV detection, and therefore, the identification of the synthesis product with LC−MS is fast and reliable, even if the sample includes several impurities. LC−MS also allows determination of the structures of impurities.

Conclusions

HPTLC has been shown to be an easy and economical method for high-throughput purity analysis in solid-phase combinatorial chemistry. HPTLC was compared with the LC−MS method in the assessment of a combinatorial library containing 19 samples aimed at PKC inhibitors. The percentage purities of the synthesis products obtained by HPTLC and LC−MS ($r^2 = 0.8404$) or by HPTLC and LC−UV ($r^2 = 0.8053$) were in good agreement. HPTLC offers highly rapid tentative screening of the quality of libraries by visual detection under a UV lamp. More detailed information can easily be obtained by HPTLC densitograms and in situ UV spectra or by "cleave and analyze" methods. The new pretreatment method developed to transfer the HPTLC sample zone for the mass analysis is simple and low-cost and takes only 2−3 min per sample.

The results obtained by HPTLC indicate that the continuous reservation of expensive LC−MS apparatus is unnecessary for such semiquantitative purity analysis, as demonstrated in this work. Although LC−MS offers abundant structural information, which is impossible to obtain by HPTLC, our sensitive, economical, rapid timesaving, and simple HPTLC method offers a powerful tool for the purity analysis of small homologous combinatorial libraries. Use of the HPTLC method could be further improved by

development of special software for the identification of synthesis products from the impurities.

Experimental Section

Materials. The 19 compounds investigated (Table 1) were selected synthesis products in a combinatorial library produced by solid-phase method and aimed at protein kinase C inhibitors. The compounds, 3-methoxy-4-*N*,*N*-substituted phenols, were synthesized in our laboratory by the method described by Tois et al.,[48] except that Bromo−Wang resin was used instead of Wang resin. All of the compounds were identified by ESI-MS. All organic solvents were of analytical or chromatographic grade. Methanol and *n*-hexane were purchased from J. T. Baker (Deventer, Holland), and acetic acid, from Rathburn (Walkerburn, Scotland). 2-Propanol was from Riedel-de-Haën (Seelze, Germany), and water was Milli-Q purified (Millipore, Molsheim, France). Ammonium acetate was purchased from Merck (Darmstadt, Germany).

For planar chromatography, silica gel 60 F_{254} HPTLC plates of 10 × 20 cm (Art. 5642) or 10 × 10 cm (Art. 5629) (Merck, Darmstadt, Germany) were used. In the development of a new sample pretreatment method for introducing the HPTLC sample zone for FIA-MS analysis, the following materials were used: two disposable injection syringes of 1.0 mL (BD Plastikpak, Becton Dickinson S. A., Madrid, Spain), a binding piece (not commercially available), a syringe-driven filter unit (nonsterile Millex-HV, 4 mm and 0.45 μm, Millipore, Japan), Finntip FT 250 Universal and Finntip FT 300 (Thermo Labsystems Oy, Helsinki, Finland), and an injection needle (Microlance 25 GA, Becton Dickinson & Co. Ltd., Drogheda, Republic of Ireland).

HPTLC Method. HPTLC plates were prewashed once with methanol before sample application. Sample solutions (20 mM in MeOH) were sprayed in amounts of 3−10 μL on both sides of the plate with a Linomat IV (Camag, Muttenz, Switzerland) at a rate of 1 μL/15 s as 5-mm-long bands with 5 mm spaces. The first band was applied 10 mm from the left edge of the plate. The mobile phase composition was optimized with help of the PRISMA model.[49−52] 2-Propanol/hexane (1:8) was used as final mobile phase, and the elution time was ∼10 min. After elution, the plates were first observed visually under a UV lamp (Desaga, Heidelberg, Germany) and, finally, with a Camag TLC Scanner II (Muttenz, Switzerland) controlled by the CATS 3.17 program at $\lambda = 254$ nm (D_2 lamp). Measurements were performed in absorption and reflection modes. In situ UV spectra of the compounds were measured at wavelength range of 190−450 nm. The final purity, as a percentage, was calculated from the ratio of the peak area of the synthesis product to the total area of all peaks.

Pretreatment of HPTLC Sample for MS Analysis: Injection Syringe Apparatus. The piston of an injection syringe (4) is removed and replaced with a hollow binding piece (5) (see Figure 2A). About 2 mm of the thinner part of a Finntip FT 250 Universal (6) is diagonally cut away and attached to the binding piece. A filter unit (3) is inserted into the other, open end of the injection syringe. A Finntip FT 300 (2) shortened by 1 cm at the wide part is inserted into the filter. The thinner part of the tip is connected to suction with a rubber tube (1).

The cut end of the Finntip (6) is used to scrape the sample zone from the plate (7) (see Figure 2B). With the help of the vacuum, the scraped sorbent is sucked through the Finntip (6), the binding piece (5), and the injection syringe (4) to the filter unit, on which the sample is retained according to its particle size. The whole apparatus is turned vertically, the vacuum is shut down, and the injection syringe is removed from the rubber tube.

The Finntip used as binding piece to the suction (2) is removed and, with another injection syringe and a needle (9), ∼0.5 mL of methanol is introduced to the mouthpiece of the Finntip (6) (see Figure 2C).

The Finntip (6) and the hollow binding piece (5) are removed and the piston is returned to the syringe. With the piston, methanol is pushed from the syringe through the filter unit to a sample vial (8) (see Figure 2D). The sample solution is now ready for MS analysis.

Identification of Separated Compounds by FIA-ESI-MS. The isolated and pretreated sample was identified by FIA-MS using a Sciex API3000 triple quadrupole mass spectrometer (Sciex, Concord, Ontario,Canada) with an electrospray ion source (ESI). Sample solutions were introduced to the mass spectrometer by direct injection (10 μL) using a Rheodyne injector (Cotati, CA). The mobile phase was water/methanol 1:1. The pH was adjusted to 4.5 with acetic acid, allowing the identification of synthesis products as protonated molecules. The eluent was delivered at a flow rate of 12 μL/min with a microsyringe pump (Harvard Apparatus, USA). Synthetic air (CD2, Atlas Copco, Belgium) was used as a nebulizing gas (flow rate, 1.23 L/min), and nitrogen produced by a Whatman generator (Balston, Inc., Tewksbury, MA), as a curtain gas (flow rate 0.95 L/min). The instrument was operated in positive ion mode. The ionspray voltage was 5000 V, and the declustering potential was 30 V. The scan range was *m*/*z* 100−600. Analyst 1.1 software was used for data processing.

LC−UV−MS Method. The LC−UV−MS studies were performed using an HP 1100 series liquid chromatograph (Hewlett-Packard GmbH; Waldbronn, Germany) with UV detector at a wavelength of 254 nm and an Esquire LC ion-trap LC/MS^n system (Bruker-Franzen Analytik GmbH; Bremen, Germany) with an ESI source. Eluent A was 20 mM ammonium acetate in water (pH adjusted to 4.5 with acetic acid), and eluent B was MeOH. Sample solutions were prepared by diluting the standard solution (20 mM in MeOH) to concentration 1:10 with the eluent A (i.e., the buffer). A 2-μL portion of the diluted sample was injected to a 50 × 1 mm Genesis C18 column with particle size 4 μm (Jones Chromatography Ltd.; Hengoed, U.K.). The flow rate of the mobile phase was 0.1 mL/min. The gradient program was 0−10 min 30−90% MeOH and 10−22 min 90% MeOH. The column was stabilized for 15 min between each analysis. LC separations were performed at room temperature. The eluent flow was directed to the electrospray ion source of the mass spectrometer without splitting. The end plate voltage, −534 V; capillary voltage, 4500 V; capillary exit voltage, 91.9 V; and skimmer voltage, 20.6 V, were tuned with the optimization program. Nitrogen was used as the drying (300 °C; 5 L/min) and nebulizing gas (30.14 psi).

The scan range was *m*/*z* 100−600. The Data Analysis 2.0 program (Bruker, Daltonics) was used for data processing. The synthesis products were identified from LC−MS ion chromatograms of the protonated molecules. The purities were determined from total ion chromatograms and UV chromatograms.

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