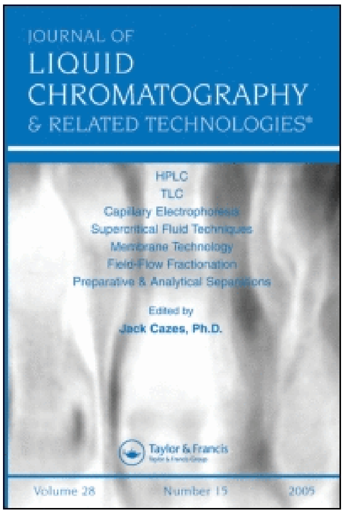

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# High Temperature High Performance Liquid Chromatography of Substituted Anilines using a $C_{18}$ Hybrid Stationary Phase

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**Abstract:** Separation of twelve substituted anilines in less than ten minutes is reported. A $C_{18}$ hybrid stationary phase and a mobile phase consisting of pure water was used for separation. To separate the analytes a temperature program between 160°C and 200°C with 5°C/min was used. Under isothermal conditions van't Hoff plots were constructed between 150°C and 200°C. All the plots were straight lines indicating only one type of interaction between the analytes and the stationary phase. The compounds were detected using either UV or simultaneous UV and FID detection.

**Keywords:** Hybrid stationary phase, van't Hoff plots, UV detection, FID detection

## INTRODUCTION

Temperature is a very important parameter for the optimization of selectivity, the analyte's mass transfer rates, and resolution. Increasing the temperature decreases solvent viscosity, allowing for high flow rates with low column back pressures and short analysis times.[1–4] It has been shown that increasing the temperature can decrease the analyte retention time by 5%/°C.[5] The acceptance of chromatography at high temperature has been hindered

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due to a lack of stationary phases that can sustain high temperatures.[6–9] Furthermore, temperature mismatch between the mobile phase and the column should be considered. Such mismatches can produce band broadening, which becomes very significant at higher flow rates. At the same time, some compounds are labile at higher temperatures; as a consequence the time spent by an analyte in the column under high temperature conditions has to be minimized.[3]

The use of high temperature water (HTW), at temperatures above 100°C as a mobile phase, has led to new applications for chromatographic separation. The solvation properties of pressurized hot water changes, when compared to ambient liquid water. Changes in temperature have dramatic effects on the polarity of water, measured by the dielectric constant. The consequence of such phenomena is that water behaves as a moderately polar solvent. For instance, at 50 bar the dielectric constant of water at 200°C is 35, similar to that of methanol.[10,11] Because of that, a gradual increase in temperature (when using pure water as a mobile phase) will produce effects similar to those achieved when using an elution gradient.[12]

Silica based reversed phase stationary phase columns remain the most commonly used adsorbent for liquid chromatography separations. Significant technological improvements have been made in stabilizing silica columns under acidic or basic conditions. Shielding the silica backbone from underlying agents (acids or bases) by applying multiple attachment points of the hydrocarbon ligand to the silica surface results in a highly crosslinked bonded phase.[13] High temperatures make these reversed phases extremely unstable, especially under mobile phase conditions of HTW. That is why silica based reversed-phase columns cannot be used above 150°C when using pure water as the mobile phase.

Introduction of hybrid stationary phases brought a new dimension to chromatographic separations.[14] They are the product of the synthesis between an organosilane, which is the organic moiety, and tetraalkoxysilane (the inorganic moiety). Compared to silica gel phases, these phases are characterized by less acidic silanols and longer life time under alkaline mobile phase conditions. These hybrid packings are designed to incorporate the mechanical strength and selectivity of silica particles and the pH stability of polymeric particles.[14]

The use of a flame ionization detector (FID) in conjunction with HTW gives an additional advantage for the analytes that have no chromophores. Since water does not respond to flame ionization, detection of such analytes can be facilitated using such a detector.[15]

Substituted anilines are important intermediates in organic synthesis. They have, however, high toxicity in low concentration and, due to their high solubility in water, can be found as contaminants in rivers, soils, and drinking water. They also have low biodegradability, which, in turn, increases the possibility of diffusing into the environment.[16] Therefore, their analysis is very important. The separation of substituted anilines is documented in the literature. Chang et al. reports the separation of substituted anilines using a $\beta$-cyclodextrin bonded stationary phase, along with normal phase and reversed phase mobile phases.[17] Genaro et al. described the separation of anilines using ion pair chromatography.[18] In a more recent paper, Gennaro et al. reports separation of 13 aniline derivatives in ~80 minutes using reversed-phase chromatography along with an elution gradient.

In this paper, we report the separation of substituted anilines using a hybrid $C_{18}$ stationary phase, with HTW as a mobile phase, and UV and flame ionization detection. Separation of twelve substituted anilines is reported in less than 10 minutes.

## EXPERIMENTAL

### Reagents

HPLC grade water was purchased from EMD chemicals, Inc. (Gibbstown, NJ, USA). 3,5-Dichloro aniline, 2,4-dichloro aniline, N,N-diethyl aniline, 2-nitro aniline, 3-nitro aniline, 4-nitro aniline, 3-chloro aniline, N-methyl aniline, 2-bromo-4-chloro aniline, 3-ethyl aniline, and N,N-dimethyl aniline were purchased from Aldrich (Milwaukee, WI, USA); 4-chloro-2,6-dimethyl aniline was prepared in house by the process research chemists.

### Chromatography and Chromatographic Conditions

The set up used for our experiments is presented in Figure 1 and consisted of an Agilent 1100 HPLC system to which a Series 8000 high temperature oven (Selerity Technologies, Salt Lake City, UT, USA) was interfaced.

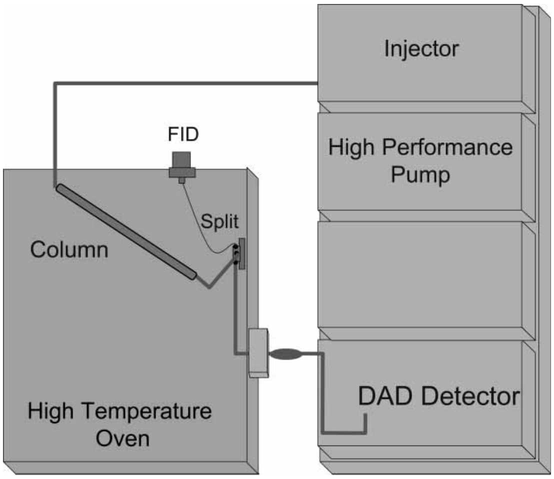


***Figure 1.*** Set up for high temperature chromatography.

The flow of mobile phase emerging from the column was split such that a portion of the eluent entered into the photodiode array detector (PDA), with the other portion directed to the FID. A flow restrictor consisting of metal beads sintered inside a 30 cm long stainless steel tubing, with an inner diameter of 0.010 inches. (Selerity Technologies, Salt Lake City, UT, USA), was used to control the amount of mobile phase introduced into the detector. The temperature of the FID detector was 400°C. A back pressure device was used to increase or decrease the split ratio between the mobile phase entering the FID and that entering the PDA.

The stationary phase consisted of an XBridge HPLC column 2.1 × 150 mm packed with a $C_{18}$ hybrid organic-inorganic stationary phase, with a particle size of 3.5 μm. The mobile phase consisted of pure water, which was pumped at a flow rate of 1 mL/min. The temperature of the column was kept isothermal at 150°C and 200°C, and temperature programming was performed at an initial temperature of 165°C to 200°C at 5°C/min.

## RESULTS AND DISCUSSIONS

The introduction of the hybrid stationary phases further expanded the possibilities for chromatographic separations. The synthesis of such phases leads to a less active surface concerning the amount of free silanol groups. This insures first, that the polarity of such phase is diminished, and second, that there is higher stability towards extreme pHs, as well as high temperatures.[19] In our experiments, we studied the behavior of aniline derivatives using the hybrid stationary phase along with a pure water mobile phase, at temperatures varying from 150°C to 200°C.

### Influence of Temperature on the Separation

To ascertain the behavior of the aniline derivatives on the XBridge column, the initial experiments were conducted under isothermal conditions at temperatures varying between 150°C and 200°C. The relationship between the retention factor, k′, and the temperature is given by the van't Hoff equation:

$$\ln k' = -\frac{\Delta H^o}{RT} + \frac{\Delta S^o}{R} + \ln \Phi \qquad (1)$$

Equation (1) predicts that a plot of ln k′ versus 1/T will be a straight line provided that ln Φ (phase ratio defined as the ratio between the volume of the stationary phase and the volume of the mobile phase) is independent of the temperature. $\Delta H^o$ and $\Delta S^o$ are the standard enthalpy and the standard entropy, respectively, of the transfer of the solute from mobile phase into the stationary phase. If the stationary phase or the analyte undergoes a change in conformation, the enthalpy and the entropy of the retention process will show a change in slope and intercept at the transition temperature.[20]

In our study, the temperature was varied for all halo anilines between 150°C and 200°C. All plots are linear with an $R^2 > 0.999$ (Figure 2), indicating that for the temperature interval studied, there is not a change in the interaction between the analytes and the stationary phase.

While the phase ratio could not be determined, the results show the dependence of the enthalpies of the transfer of the analytes from mobile phase to the stationary phase. The results are presented in Table 1.

For chloro anilines the increase in the number of chlorine atoms present on the aniline molecule enhances the interaction with the stationary phase, leading to a more negative $\Delta H^o$. The position of the chlorine atoms on the aromatic ring also influences the interaction with the stationary phase. Thus, 3,5-dichloroaniline has stronger interaction with the hybrid stationary phase

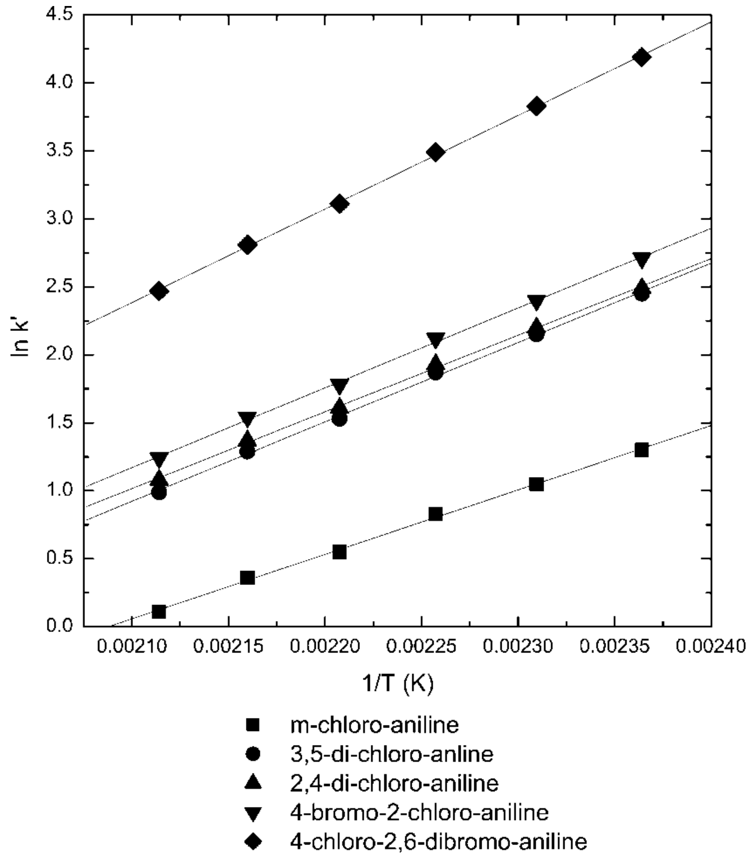


***Figure 2.*** Influence of temperature on k′ of halo anilines.

*Table 1.* $\Delta H^o$ values for halo anilines

| Analyte | $\Delta H^o$ (kcal/mol) |
|---|---|
| Meta-chloroaniline | −9.43 |
| 2,4-Di-chloroaniline | −11.20 |
| 3,5-Di-chloroaniline | −11.61 |
| 2-Chloro-3-bromoaniline | −11.67 |
| 4-Chloro-2,6-di-bromoaniline | −13.68 |

(more negative $\Delta H^o$) than 2,4-dichloroaniline. The presence of a bromine atom in the molecule further enhances the interactions between the analyte and the stationary phase, leading to longer retention.

The influence of temperature on the resolution and selectivity between the two pairs of chloro anilines, (3,5-dichloro- and 2,4-dichloro aniline) was also investigated. The results are shown in Figures 3a and b.

Upon increasing the temperature, the resolution between the two compounds increased up to 190°C (Figure 3a) and remained constant for the temperatures above 190°C in our experiments. At the same time, the selectivity factor, $\alpha$, remained constant for the experimental range of temperatures (Figure 3b).

The resolution equation between two peaks is given by:

$$R_s = \frac{1}{4} * \frac{\alpha - 1}{\alpha} * \frac{k'_2}{1 + k'_2} * (N_2)^{1/2} \tag{2}$$

The second and the third term in Eq. (2) represent the thermodynamic contribution to the resolution, while the last term represents the kinetic contribution. Our data suggests that the increase in resolution is due to the kinetic term in the resolution equation and not to the thermodynamic term of the equation.

## Separation of Aniline Derivatives

We further attempted the separation of other aniline derivatives such as nitro anilines (Figures 4a and b).

The initial separation was performed under isothermal conditions at 200°C. Strong overlapping between the nitro aniline peaks was observed (Figure 4a). To improve the resolution between the nitro aniline derivatives, the separation was performed using a temperature program from 165°C to 200°C at a rate of 5°C/min. Complete separation was obtained between 4-nitro, 3-nitro, and 2-nitro aniline (Figure 4b). The order of elution of the three nitro anilines is somewhat difficult to explain due to the fact that the interaction between the analytes and the hybrid stationary phase cannot be explained entirely through the concept of hydrophobic interaction. Hydrophobic interactions occur under aqueous conditions when the three

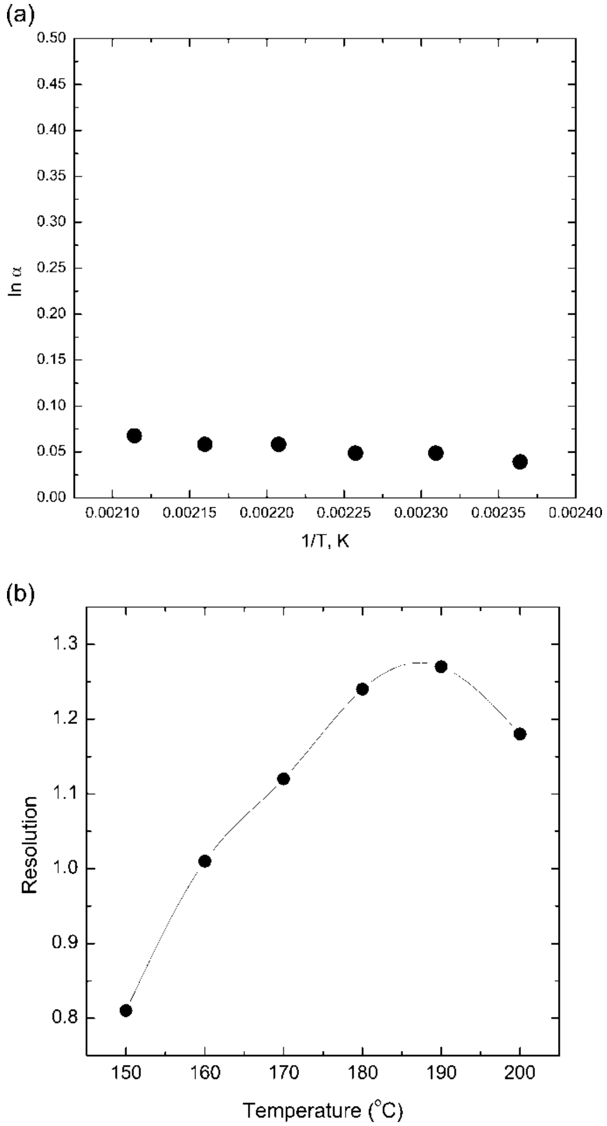


***Figure 3.*** Influence of temperature on the selectivity factor (a) and resolution (b) for 3,5-dichloroaniline and 2,4-dichloroaniline.

dimensional structure of water is disturbed only by the cage formation of the analyte. This is followed by the subsequent interaction of the analytes with the hydrocarbon stationary phase. At the temperatures used for this experiment, the structure of water was already disturbed and the weakened hydrogen bonds were unable to sustain the percolated structure of water, leading to

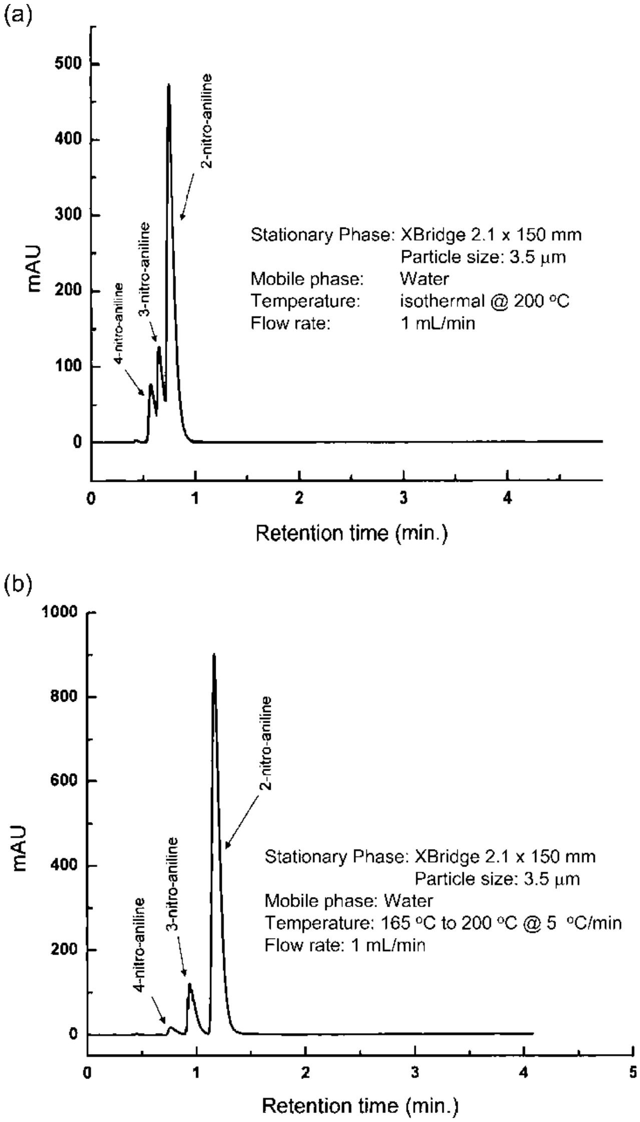


***Figure 4.*** Chromatogram of nitro anilines isothermal (a) and with temperature program (b).

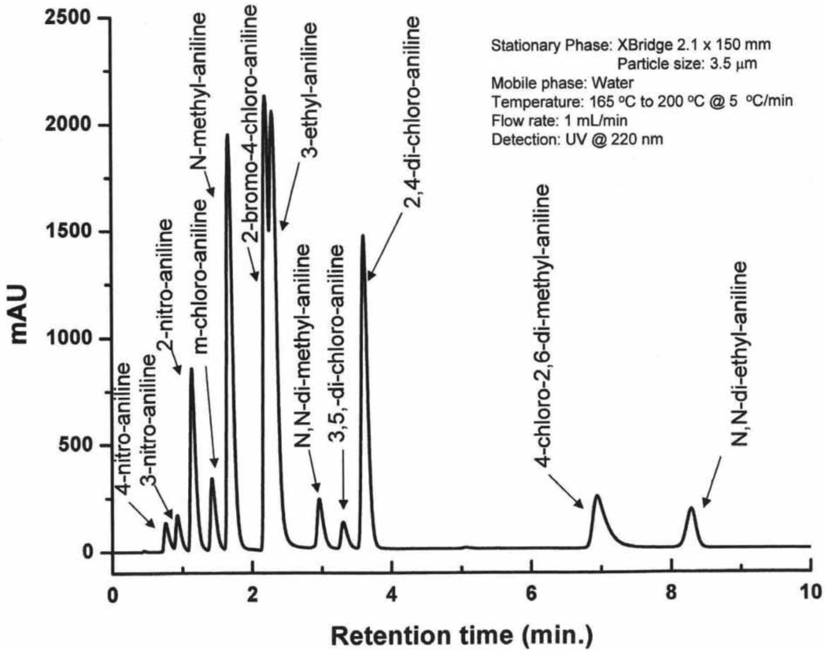
clusters which approached the dielectric constant of an organic solvent. However, we can speculate that the order of elution can be explained in terms of the steric hindrance and distortion from planarity, which is caused by the position of the nitro group in reference to the amino group on the

*Table 2.* The hydration energy of nitro anilines calculated with hyperchem V. 7.5

| Order of elution | Hydration energy (Kcal/mol) |
|---|---|
| 2-Nitro aniline | −9.28 |
| 3-Nitro aniline | −10.25 |
| 4-Nitro aniline | −10.54 |

aniline structure.[21] The position of these nitro groups will impair the way the molecules approach the stationary phase. In fact, the behavior can also be associated with the energy of hydration, which was calculated using Hyper Chem V.7.5. The energy of hydration becomes more negative the further the nitro group is in the aromatic ring relative to the amino group. Table 2 shows a good correlation was obtained between the order of elution and the values for the hydration energy.

We further broaden the classes of substituted anilines using the same stationary phase along with aqueous mobile phase and temperature programming. A chromatogram of twelve aniline derivatives is presented in Figure 5. All show separation in less than ten minutes.



Stationary Phase: XBridge 2.1 x 150 mm
Particle size: 3.5 µm
Mobile phase: Water
Temperature: 165 °C to 200 °C @ 5 °C/min
Flow rate: 1 mL/min
Detection: UV @ 220 nm

*Figure 5.* Chromatogram of aniline derivatives.

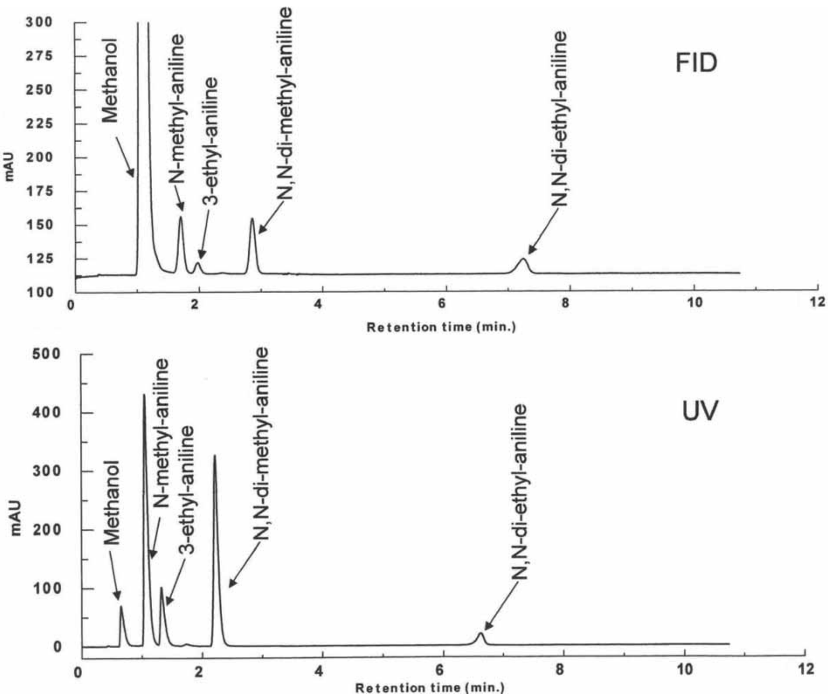


***Figure 6.*** Chromatogram of alkyl anilines using UV detection (lower panel) and FID detection. (For chromatographic conditions see the panel in Figure 5.)

## Separation of Anilines Derivatives Using Dual Detection: UV and FID

In order to detect the separated anilines we proceeded in two ways: first, the detection was performed using UV, and second, the mobile phase eluent exiting the column was split. Part of the eluent was directed toward the UV detector and the other part was directed into the FID. The separation of the substituted anilines using dual detection is presented in Figure 6.

In order to increase the detection in the FID detector, a restrictor was added between the split point and the UV detector forcing more mass into the FID. This led in turn, to a small delay between the peaks appearing in UV and FID.

## CONCLUSION

Separation of substituted anilines was demonstrated using HTW. The position and the number of halogen substituents on the aromatic ring influence the interaction of the analytes with the stationary phase. Using HTW, dual detection was achieved by UV and FID. Separation of twelve substituted anilines was accomplished in under ten minutes.

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