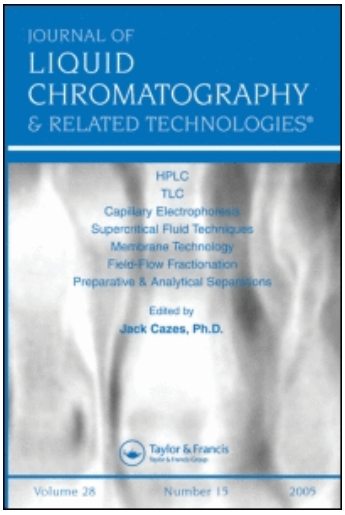

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DEVELOPMENT OF AN INDIRECT REVERSE PHASE METHOD FOR THE QUALITY ASSESSMENT OF AN ACYL HALIDE

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ABSTRACT

An indirect reverse phase HPLC method to monitor low level impurities in an acyl halide, 3-phenylpropionyl chloride, is described. The method involves derivatization with aniline to form a stable *N*-anilide derivative followed by HPLC analysis. The selection of the derivatizing agent aniline was based on achieving similar detector responses for impurities relative to the 3-phenylpropionyl chloride. The method described is rugged, selective, and sensitive and provides an excellent way to assess the quality of acyl halides.

INTRODUCTION

The quantitation of acyl halides is primarily achieved by gas[1] or liquid chromatography[2] following acylation of an alcohol or amine. In general, the resulting ester or amide derivative is pre-treated with acidic aqueous extractions, neutralized and washed with water. This form of sample pre-

treament ultimately affords a stable derivative prior to UV detection and quantitation.[3] Chromatographic procedures have been incorporated to enhance the specifity of the method.[1,2,4-6] The combination of an acylation reaction during sample pre-treament and separation techniques has fostered the quantitation of residual levels of phosgene.[2] In some applications, acylation reactions have similarly been applied to the quantitation of residual thioureas,[4-6] amines, alcohols, and phenols[7] employing various acyl halides as derivatizing agents.

A quantitative methodology to fully resolve and monitor potential low level impurities in an acyl halide, in particular, the corresponding carboxylic acid, was required. The corresponding carboxylic acid of an acyl halide is a common degradation product often present as a result of hydrolysis. In addition, the respective carboxylic acid of an acyl halide is often used as a chemical precursor in the synthetic route to acyl halides. The derivatization of an acyl halide with an amine or alcohol employs an acidic aqueous extraction to remove excess amine or alcohol. As a result, the carboxylic acid impurity is separated from the desired ester or amide derivative and not routinely measured. An additional method is generally utilized to quantitate the corresponding carboxylic acid level present in an acyl halide.[2] Subsequently, various titrimetric methods have been developed in combination with chromatographic methods to quantitate pre-existing carboxylic acid levels.[3] To our knowledge, no single method for the sensitive quantitative assessment of all impurities in an acyl halide has been reported.

For improved quantitative analysis and simplicity, we focused on developing a single, sensitive and selective chromatographic method to monitor all low level impurities in an acyl halide, including the respective carboxylic acid. Thin layer chromatography has been used to evaluate the purity of a labeled hydrocinnamoyl imidazole derivative in a spectroscopic study of acyl-chymotrypsin ligand conformations,[8] however, we did not evaluate this TLC system because of our need for a quantitative method and also our need to be able to easily isolate impurities detected in commercial sources of acyl halides. Preparative HPLC methods have a greater capacity in comparison to preparative TLC methods,[9] making isolation of unknown impurities more readily achieved via HPLC methods.

3-Phenylpropionyl chloride (common usage name is hydrocinnamoyl chloride, HCC) is a key raw material used in the synthetic route to Crixivan®, *Indinavir Sulfate*, an effective HIV protease inhibitor.[10,11] In an effort to maintain high quality and yields in the synthetic steps, we required a rugged method capable of monitoring low level impurities in HCC before release for process use. More specifically, we needed a linear, precise, sensitive, and

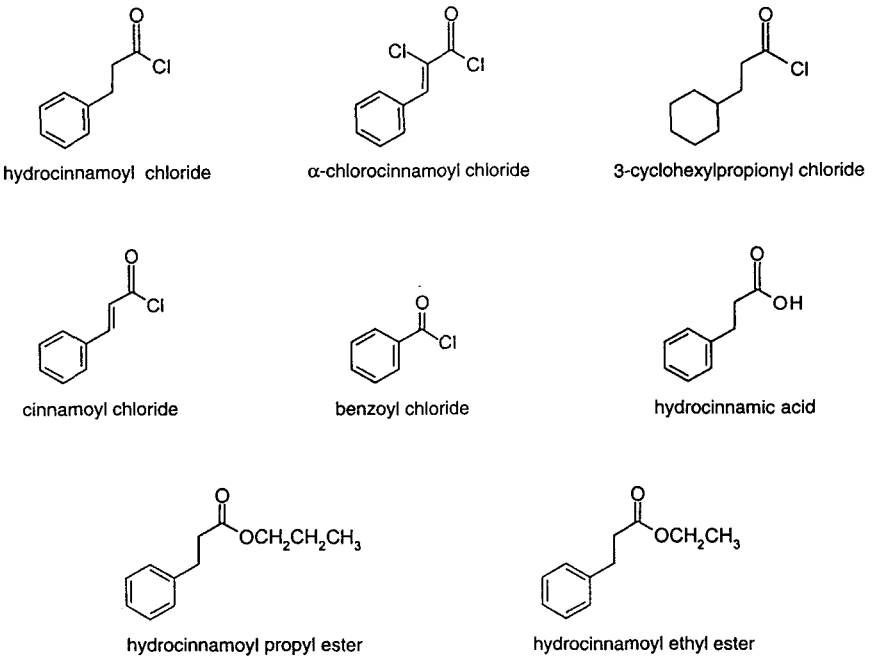

Figure 1. Structures of hydrocinnamoyl chloride and several process related impurities. Also, the structure of hydrocinnamoyl chloride derivatized with propanol and ethanol to yield the propyl ester and ethyl ester, respectively.

selective analytical method which could be used routinely to measure low level impurities. These impurities include hydrocinnamic acid, the corresponding carboxylic acid, and other process related acid chloride impurities shown in Figure 1. All of the acid chloride impurities were previously isolated by prep HPLC from commercial sources of HCC and identified by LC/GC-MS and $^{1}H/^{13}C$ NMR as either their corresponding *N*-benzylamide or *N*-anilide derivatives.

EXPERIMENTAL

Materials

Hydrocinnamoyl chloride was supplied by Ashwood Chemicals and First Chemical Corporation. All solvents used in the derivatization and as mobile

phase components were HPLC reagent grade (Fisher Optima). The acetonitrile and methanol did not exceed 0.01% w/w and 0.07% w/w water content respectively. Benzylamine (99% purity, Janssen Chimica) and aniline (99.5%+ purity, Aldrich Chemical Co.) were A.C.S. reagent grade. Hydrocinnamic acid was obtained from Aldrich Chemical Co.

For UV evaluation, *N*-hydrocinnamoyl benzylamide and *N*-hydrocinnamoyl anilide derivatives were prepared by treating 10 mL of HCC with 2 molar equivalents of the benzylamine and aniline, respectively, in 150 mL of methylene chloride. The excess amine in the resulting amide slurry solution was extracted with an aqueous 3 N HCl solution (2 x 100 mL). The aqueous phase was separated and the organic phase was washed with water (2 x 50 mL). The solution was then neutralized with an aqueous 5% sodium carbonate solution (2 x 50 mL) and washed again with water (2 x 50 mL). The neutralized solution was then concentrated by vacuum distillation, collected by vacuum filtration, and the solids washed with water. The solids were dried at 40°C under vacuum for 14 h prior to UV analysis.

Sample Derivatization

The derivatization was performed as outlined in Figure 2. Methylene chloride (2 mL) or acetonitrile (10 mL) and aniline (0.20 mL, 2.19 mmol) were mixed in a 50 mL round bottom flask on a bed of dry ice. HCC (0.15 mL, 1.01 mmol) was then added to the solution while mixing. A white precipitate formed and the resulting slurry was allowed to mix for an additional 5 to 10 minutes at room temperature to ensure reaction completion. The anilide and the excess aniline were dried under a nitrogen purge to remove methylene chloride. Once dry, acetonitrile (30 mL) was then added to dissolve the anilide derivative and to provide a medium for further mixing. Methanol (3 mL) was then added to dissolve the remaining anilide hydrochloride salt. A 3 mL aliquot of the mixed, clear solution was transferred to a 50 mL volumetric flask and diluted with a 1:1 v/v mixture of methanol and water. This was an approximate 0.4 mg/mL *N*-hydrocinnamoyl anilide solution based on a typical 100% reaction yield.

LC Conditions

The separation was performed on a Rockland Technologies Inc. Zorbax© R_X-C8 packed with diisopropyl C8 chemically bonded silica (5 micron particle size), 250 x 4.6 mm i.d. column at room temperature. A 10 μL injection volume was used. Initial gradient conditions began at 55% methanol and 45%

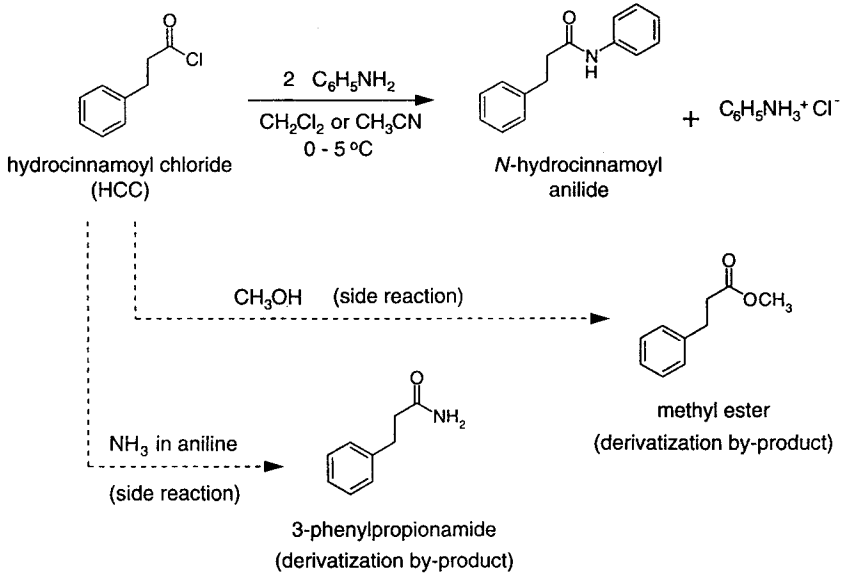

Figure 2. The derivatization of hydrocinnamoyl chloride with aniline and possible reaction by-products resulting from undesirable reaction conditions.

aqueous 0.1% phosphoric acid (pH 2.2) at 1.2 mL/min flow rate. A 10% linear methanol sweep to 65% methanol over 15 minutes was used followed by a linear methanol sweep to 75% methanol over 10 minutes then held for 5 minutes for a total of 30 minutes run time. UV detection was set at 215 nm.

LC Instrumentation

The instrumentation was a Thermo Separation Products SP8700 solvent delivery system, a Thermo Separation Products Model SP8780XR autosampler, and an ABI/Kratos Spectroflow Model 757 variable wavelength UV detector.

A PE-Nelson Access*Chrom chromatography data system was used for peak integration and calculations of system suitability parameters such as tailing factor (T_f) and resolution (R_s).

DISCUSSION

Selection of a Derivatizing Agent

A gas chromatographic assay was initially attempted incorporating a Restex® Rtx-1701 (14% cyanopropylphenyl/86% methyl polysiloxane) 60 m x 0.32 mm i.d. capillary column and FID detection, to evaluate an ester derivative of HCC. The column was chosen because of its unique ability to chromatograph numerous compounds of varying polarity as claimed by the vendor. Primary alcohols were initially chosen to derivatize HCC. HCC was refluxed in propanol for 5 minutes, cooled, and injected neat onto the GC. Adequate peak shape was achieved, but GC-MS confirmed that the resulting hydrocinnamoyl propyl ester shown in Figure 1 and hydrocinnamic acid were not resolved. HCC was then refluxed in ethanol for 5 minutes, cooled, and injected neat onto the GC. Hydrocinnamic acid was well resolved from the HCC ethyl ester derivative. No hydrocinnamic acid was observed initially in the sample preparation. However, following several successive injections of the identical sample preparation spiked with hydrocinnamic acid, the peak area counts of the acid decreased while the peak area counts of the ester increased. This observation suggested conversion of the acid to the ester derivative and left us no means of determining the pre-existing levels of hydrocinnamic acid. The conversion of hydrocinnamic acid to the ethyl ester adduct was attributed to an excess of ethanol in the presence of liberated HCl.[12] An effort was made to quench liberated HCl from the reaction mixture using a non-competitive basic reagent such as pyridine. However, the corresponding pyridinium chloride salt was insoluble in the ethanol solution. Incorporating centrifugation in the final step of the sample preparation for separation of this insoluble solid meant evaluation of the supernatant versus the preferred analysis of the entire solution. Alternatively, employing additional aqueous extractions would ultimately compromise the integrity of the sample by potentially removing water soluble impurities such as hydrocinnamic acid.

An alternative derivatization was assessed using a primary amine in 100% excess. HCC treated with two molar equivalents of a primary amine affords a stable *N*-hydrocinnamoyl amide compound and the excess amine captures liberated HCl from the reaction. In an attempt to by-pass general capillary GC limitations i.e., poor reproducibility of the amide peak attributed to acid/base interactions with a single phase and also dependence on sample thermal volatility and lability,[6] we chose to explore an amide derivative suitable for ultraviolet detection following LC separation. We initially chose benzylamine as the derivatizing agent because of its additional aromatic chromophore.

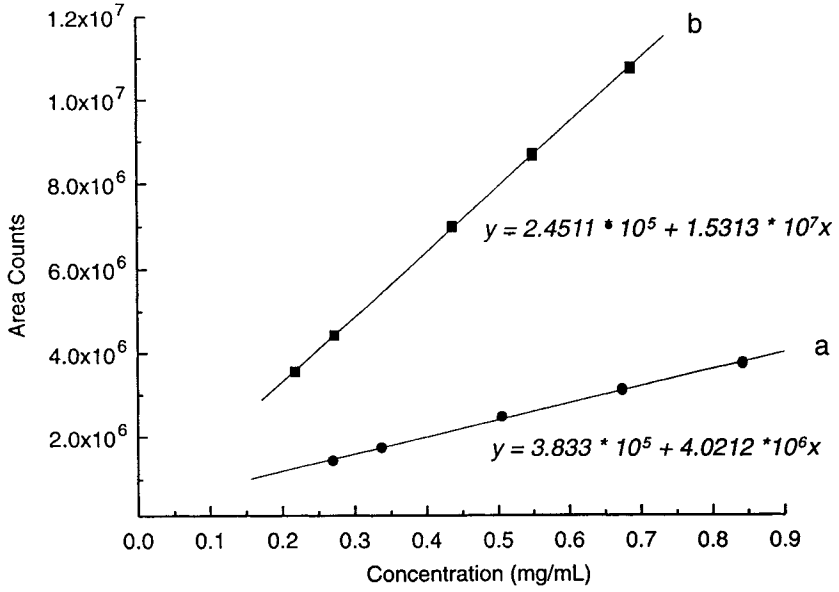

Figure 3. a) Linearity of the *N*-hydrocinnamoyl benzylamide derivative separated by HPLC and detected at 220nm. b) Linearity of the *N*-hydrocinnamoyl anilide derivative separated by HPLC and detected at 215 nm.

A UV spectrum of *N*-hydrocinnamoyl benzylamide derivative in methanol was measured from 350 nm to 200 nm to assess the best detection wavelength. The extinction coefficient of the derivative at 220 nm proved this to be a suitable wavelength for detection in terms of sensitivity. Figure 3 is a plot of the area counts of the main analyte versus concentration from 0.2 mg/mL to 0.7 mg/mL at 220 nm detection. The resulting correlation coefficient of 0.9992 suggests the method is adequate for measuring the main analyte. However, further evaluation of the detector response to other potential impurities demonstrated significant differences in detector response relative to the main analyte. The relative response factors of hydrocinnamic acid and two other potential acyl halide impurities as their derivatized counterparts were determined relative to the main analyte following LC separation at 220 nm detection (see Table 1). The relative response of hydrocinnamic acid was 0.5 while cinnamoyl and benzoyl chloride as their amide derivatives were 5.5 and 7.0 respectively. This was primarily attributed to the extended conjugation of the *N*-cinnamoyl and *N*-benzoyl benzylamide derivative compared to the *N*-hydrocinnamoyl benzylamide derivative and hydrocinnamic acid. Detection in

Table 1

A Comparison of *N*-Benzylamide and *N*-Anilide Derivatives Relative Response Factors (RR_f), Using HPLC Separation and UV Detection

Component	Conc. (mg/mL)	RR_f* of Benzylamide Derivative @220nm	Conc. (mg/mL)	RR_f* of Anilide Derivative @215 nm
Hydrocinnamoyl chloride	1.0	1.0	0.19	1.0
Hydrocinnamic acid	1.0	0.5	0.88	0.8
Cinnamoyl chloride	0.10	5.5	0.20	1.8
Benzoyl chloride	0.07	7.0	0.37	1.8
3-Cyclohexyl-propionyl chloride	----	----	0.72	0.5
α-Chlorocinnamoyl chloride	----	----	0.41	1.2
Toluene	----	----	0.30	1.1

* $RR_f = R_f$ (impurity)/R_f (derivative of hydrocinnamoyl chloride).

the region of the UV spectrum from 220 nm to 208 nm, the *N*-hydrocinnamoyl benzylamide maximum, would further increase the variation in detector response between hydrocinnamic acid and the main analyte and also approaches the UV cutoff for the mobile phase solvent methanol. The UV spectra of both compounds at identical concentrations in methanol demonstrates the increasing variation below 220 nm, see Figure 4.

We investigated the use of another primary amine derivatizing agent which would provide a stronger UV chromophore and greater similarity in response for all potential components. We chose aniline which afforded a stable derivative with the combined properties of aromaticity and conjugation.

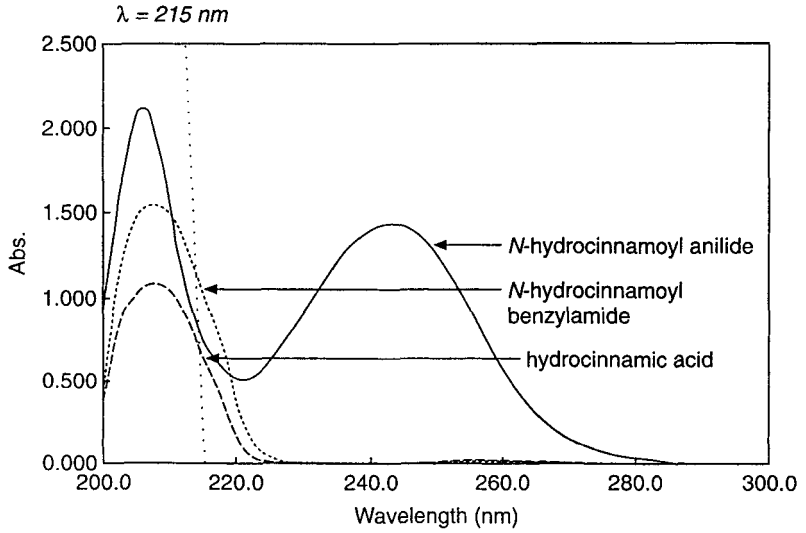

Figure 4. The UV spectrum of the *N*-hydrocinnamoyl anilide and benzylamide derivatives compared to hydrocinnamic acid at identical concentrations in methanol. At 215 nm, the *N*-hydrocinnamoyl anilide derivative is closest in absorption to hydrocinnamic acid.

A UV spectrum of the isolated *N*-hydrocinnamoyl anilide derivative in methanol resulted in maxima at 243 nm and 206 nm. Conjugation greatly improved the response for *N*-hydrocinnamoyl anilide relative to the *N*-hydrocinnamoyl benzylamide derivative, see Figure 3. Hydrocinnamic acid, having the weakest chromophore of all components at wavelengths greater than 220 nm, was evaluated with respect to *N*-hydrocinnamoyl anilide to determine the relative response. At 243 nm, it is apparent the extinction coefficients were largely unfavorable for detection because hydrocinnamic acid has little or no absorption in this region, see Figure 4. The UV spectra suggest minimal variation between the two responses at 215 nm although this is located near a point of inflection on the UV spectrum of *N*-hydrocinnamoyl anilide. To further evaluate the optimum working wavelength, the detector response of hydrocinnamic acid relative to *N*-hydrocinnamoyl anilide was determined using a variable wavelength UV detector following LC separation. The relative response factor of hydrocinnamic acid with respect to *N*-hydrocinnamoyl anilide was calculated at several points between 250 nm and 215 nm (Table 2). The relative response was closest to unity at 215 nm. Since methanol was an organic component of the mobile phase, we chose to work at wavelengths at least 10 nm above the reported UV cutoff of 205 nm.[7]

Table 2

The Response Factor of Hydrocinnamic Acid and *N*-Hydrocinnamoyl Anilide at Several Wavelengths Between 25 nm and 215 nm Using HPLC Separation

Wavelength (nm)	R_f* of Hydrocinnamic Acid	R_f** of *N*-Hydrocinnamoyl Anilide	RR_f*
250	419877	16440111	0.03
248	375644	18096042	0.02
242	239258	20230937	0.01
225	503135	11713221	0.04
220	2588689	9780005	0.26
215	9693308	12288421	0.79

* $RR_f = R_f$ (hydrocinnamic acid) / R_f(*N*-hydrocinnamoyl anilide).
** R_f = Area counts of component / Concentration of component (mg/mL).

Consequently, evaluation of the relative response of hydrocinnamic acid was not extended to shorter wavelengths below 215 nm. The relative response factor of hydrocinnamic acid at 215 nm was 0.79 suggesting this wavelength was optimal for measuring this impurity, see Table 2. Other process related impurities were then assayed and their relative response factors calculated following LC separation, see Table 1. The data in Table 1 indicates 3-cyclohexylpropionyl chloride has exactly half the response of *N*-hydrocinnamoyl anilide while the remaining impurities vary in relative responses between 1.1 and 1.8. The resulting underestimation or overestimation using the *N*-anilide derivatives would be minimized in comparison to using the *N*-benzylamide derivative.

Optimization of the Derivatization

Reactions of acyl halides with primary amines occur more readily than with primary alcohols and water. Kinetic studies of benzoyl flourides with various primary amines of pKa = 3.9 - 11[13] have revealed that the reaction most likely proceeds by a concerted S_N2 type mechanism despite the majority of studies in favor of an addition-elimination sequence.[14] The same conclusion was expanded to include substituted benzoyl chlorides as their reactivity rates are an order of 10^3 times faster than benzoyl fluorides at lower temperatures.[13]

The acylation of aniline (pKa = 4.7) in excess with HCC proceeds readily and in high yields providing a derivatization with minimal formation of by-products. However, acylation reactions feature significant exotherms which may also promote the formation of by-products. Instead of a slow addition rate, the molar quantities of HCC and aniline were reduced to 1.01 mmol and 2.19 mmol, respectively, so that the generated exotherm was negligible.[12] To prevent the formation of the 3-phenylpropionamide by-product, which results from reaction of HCC with free ammonia (see Figure 2) present as a degradate in poor quality aniline, the derivatization employs high quality aniline.

A primary concern during sample handling and pre-treatment is the potential hydrolysis of the acyl chloride to its corresponding carboxylic acid. The use of dried solvents and monitoring the reproducibility of the hydrocinnamic acid levels in duplicate sample preparations ensures the levels of hydrocinnamic acid observed are not by-products of the derivatization. A poor derivatization is also marked by the presence of the methyl ester adduct which is not reproducible from one sample preparation to another. Poor mixing and/or an undercharge of aniline (less than 100% excess) may afford the methyl ester adduct upon the addition of methanol (used to dissolve the anilide salt as shown in Figure 2) when combined with unreacted HCC.

As an alternative to using methylene chloride, the derivatization reaction was performed in an acetonitrile medium ($\leq$ 0.01% water content) and proceeded equally well. The use of acetonitrile minimized sample preparation as the need to remove methylene chloride under a nitrogen purge was eliminated. As an added advantage in eliminating the nitrogen purge, the use of acetonitrile allowed for the detection of volatile impurities such as toluene, a HCC process solvent.

Chromatographic Conditions

Initial HPLC chromatographic conditions were chosen using a Zorbax® R_X-C8 25.0 cm x 4.6 mm i.d. with pH 2.2 mobile phase, methanol gradient elution and 215 nm detection. The Zorbax® R_X-C8 phase offers the separation of basic compounds as well as weakly acidic compounds at pH 2.2. That is, hydrocinnamic acid (apparent pKa = 6.2) and *N*-hydrocinnamoyl anilide were separated as their fully protonated counterparts under the prescribed conditions.

Table 3 lists the known HCC derivatized impurities and their relative retention times. The methyl ester derivatization by-product was not resolved from toluene (rrt 0.92). However, reproducible levels of an impurity observed

Table 3

The Relative Retention Times of Several Derivatized Impurities in Hydrocinnamoyl Chloride Using HPLC Separation

Component	Retention Time (min.)	Relative Retention Time* (rrt)
1. Aniline	2.3	0.19
2. 3-Phenylpropionamide	4.0	0.33
3. Hydrocinnamic acid	6.3	0.53
4. Cinnamic acid	6.8	0.57
5. *N*-Benzoyl anilide	7.8	0.65
6. α-Chlorocinnamic acid	10.3	0.88
7. Toluene	11.0	0.92
8. Methyl ester adduct	11.0	0.92
9. *N*-Hydrocinnamoyl anilide	12.0	1.00
10. *N*-Cinnamoyl anilide	13.6	1.13
11. 3-Cyclohexylpropionic acid	17.9	1.49
12. *N*-α-Chlorocinnamoyl anilide	19.9	1.66
13. *N*-Cyclohexylpropionyl anilide	24.3	2.03

* rrt = Retention time of impurity / Retention time of main analyte

at rrt 0.92 in duplicate preparations suggested the impurity peak was toluene and not the methyl ester by-product. All impurities are resolved from the main component, *N*-hydrocinnamoyl anilide. Figure 5 is a sample chromatogram with several spiked impurities.

System suitability was measured with respect to the resolution (R_s) between the hydrocinnamoyl chloride derivative and the β-unsaturated cinnamoyl chloride derivative. We achieved a minimum resolution of 3.0.

The detector response to *N*-hydrocinnamoyl anilide was linear over the concentration range 0.2 mg/mL to 0.7 mg/mL (R = 0.9999; RSD = 1.4%, n = 18). Figure 3 demonstrates the enhanced sensitivity (slope) of the *N*-hydrocinnamoyl anilide derivative relative to the *N*-hydrocinnamoyl benzylamide. The detector response was also linear at low levels over the concentration range 0.2 mg/mL to 2.1 mg/mL (R = 0.9998). The limit of detection was estimated to be < 0.2 mg/mL or < 2 ng based on a signal to noise ratio of 4.7:1 at this concentration (<0.05% of the recommended 0.4 mg/mL

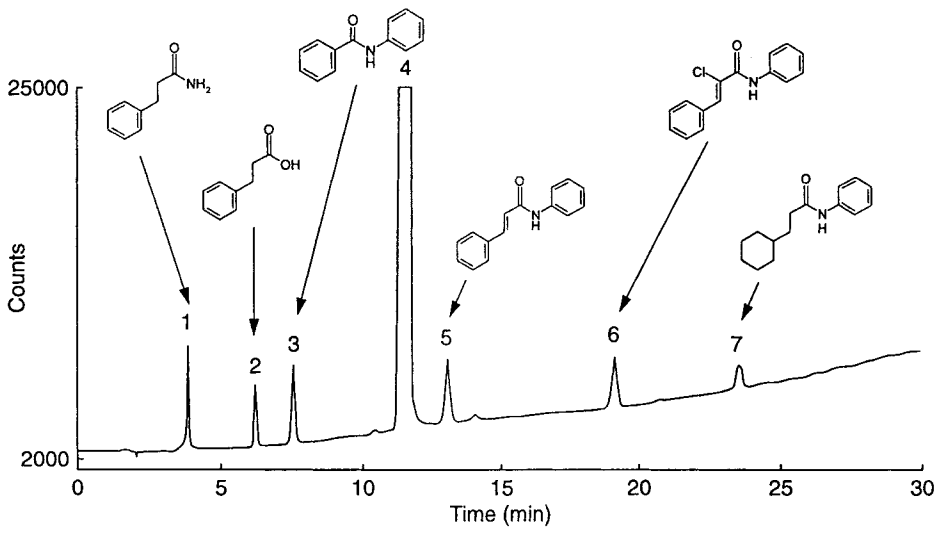

Figure 5. A chromatogram of several process related impurities spiked at ~0.5% w/w into a sample preparation. 1. 3-phenylpropionamide, 2. hydrocinnamic acid, 3. *N*-benzylanilide, 4. *N*-hydrocinnamoyl anilide, 5. *N*-cinnamoyl anilide, 6. *N*-a-chlorocinnamoyl anilide, 7. *N*-3-cyclohexylpropionyl anilide.

sample concentration). The limit of quantitation was estimated to be 0.4 mg/mL or 4 ng based on a signal to noise ratio of 13:1 at this concentration (<0.1% of the recommended sample concentration). Precision was determined on an injection to injection basis (RSD <0.1% for the area% of the main analyte; n = 12) and on a day to day basis (RSD = 0.1% for the area% of the main analyte). Lastly, the ruggedness of the method was evaluated with respect to solution stability. Using high purity aniline during the derivatization, the sample solution was stable up to 5 days at room temperature under ambient light with no apparent degradation (RSD = 0.05% for the area percent of the main analyte).

CONCLUSION

A single, precise, sensitive and selective quantitative method was developed to monitor low level hydrocinnamic acid and undesirable acid chloride impurities in hydrocinnamoyl chloride. The corresponding *N*-anilide derivatives of all the acid chloride components proved to be stable and suitable for our application. This was attributed to the additional chromophore common

to all derivatized acid chloride components producing a tightened range of UV responses ideal for quantitative analysis. The combination of an *N*-anilide derivative and detection at 215 nm with reverse phase liquid chromatography provided an accurate quantitative assessment of hydrocinnamoyl chloride.

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