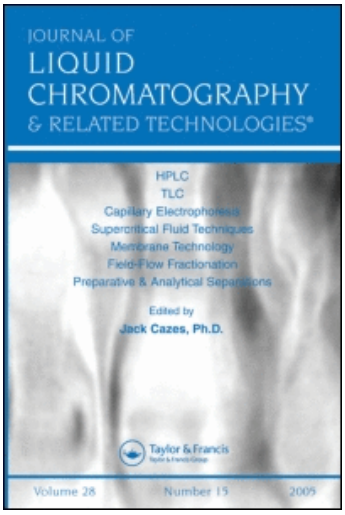

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DEVELOPMENT OF A FACILE CARBON DIOXIDE DERIVATIZATION PROCEDURE FOR THE CHROMATOGRAPHIC ANALYSIS OF ARYL GRIGNARD REAGENTS

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ABSTRACT

A rapid and facile carbon dioxide derivatization procedure was developed to stabilize Grignard reagents for reversed-phase liquid chromatographic analysis. Derivatization of the neat Grignard reagent was performed in a septum-sealed vial at 0°C under 10 psi carbon dioxide. Complete conversion of active Grignard reagents to carboxylic acid derivatives was achieved in less than 1 min. Active titer determinations for ~1 *M* 4-fluorophenylmagnesium bromide were demonstrated to be precise (<2% relative standard deviation) and accurate (5% difference) compared with the results of *sec*-butanol titrations. Additionally, sensitive quantification (limit of quantification <0.1%) of both active and inactive impurities was demonstrated, which is not possible via nonspecific conventional titrimetric methods. Such information may prove invaluable

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in the optimization of subsequent organic reactions using Grignard reagents.

INTRODUCTION

Numerous literature reports cite the use of Grignards as derivatization reagents to increase analyte volatility before gas chromatographic analysis. Grignard pentylation has been used to determine germanium compounds (1) and alkylation of organotin compounds has been used for the analysis of biological tissue (2) and environmental samples (3,4). Derivatization with 4-fluorophenylmagnesium bromide allowed determination of tellurium in urine by gas chromatography (GC)/isotope dilution inductively coupled plasma-mass spectrometry (5). Aryl Grignards have also been used to volatilize antimony in drinking water before GC speciation (6).

Grignards are versatile reagents used extensively in organic synthesis to produce alcohols, carboxylic acids, and ketones from reactions with aldehydes, esters, ketones, carbon dioxide, and nitriles (7). In synthetic organic Grignard reactions, there is a need to assess Grignard concentration to determine appropriate reaction charge as well as Grignard quality. Historically, titration methods have allowed indirect determination of active titer using the double titration of Gilman and Cartledge (8) or direct determination of active titer with *sec*-butanol (9) or diphenylacetic acid (10).

Although facile, these titration methods lack specificity and are, therefore, unable to discriminate among active and inactive components that may be present in Grignard samples. This limitation prevents the analytical chemist from identifying the potential sources of downstream impurities. One approach to overcome this limitation used the reaction of anthraldehyde with an alkyl Grignard before high-performance liquid chromatography (HPLC) analysis (11): In this way, active Grignard components were converted to the corresponding anthraldehyde analogues, providing both added specificity and introducing an ultraviolet (UV) chromophore for direct UV detection. However, this work required numerous liquid-liquid extractions before HPLC analysis and anthraldehyde resulted in a significant derivatization blank.

In this work, a novel carbon dioxide Grignard derivatization procedure is presented, which allows for determination of the aryl Grignard titer, as well as quantification of trace impurities. This is of particular interest in the synthesis of active pharmaceuticals in which trace impurities (0.1%) can be of concern. Carbon dioxide serves both to make the reaction vessel inert and derivatize active Grignard components into stable carboxylic acids (Eq. 1).

$$\text{R-MgBr} \xrightarrow[2)\,H_2O]{1)\,CO_2} \text{R-COOH} + \text{MgBrOH} \qquad (1)$$

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Inactive components in the Grignard sample are not derivatized. Resulting carboxylic acid derivatives are speciated by HPLC to assess Grignard quality. The derivatization has been extensively characterized for 4-fluorophenylmagnesium bromide, which is of interest because of its use in the synthesis of a new drug candidate. The derivatization is also applied to the analysis of benzylmagnesium chloride and mesitylmagnesium bromide to demonstrate method versatility. For all examples considered, reaction with CO_2 has been found to be quantitative, rapid, and reproducible. Standards of aryl carboxylic acids are often commercially available, which facilitates accurate Grignard quantification. Additionally, excess CO_2 does not contribute a significant derivatization blank to the HPLC chromatogram, simplifying data analysis.

EXPERIMENTAL

Materials Used

4-Fluorophenylmagnesium bromide was purchased from Aldrich (Milwaukee, WI, USA), Fluka (Milwaukee, WI, USA), and Boulder Scientific (Mead, CO, USA). Benzylmagnesium chloride, mesitylmagnesium bromide, 4-fluorobenzoic acid, 4-fluorophenol, benzoic acid, 3-fluorobenzoic acid, 4-fluorobenzene, 4-fluorobiphenyl, and 4,4′-difluorobiphenyl were all obtained from Aldrich.

Biphenyl Grignard was made in house from 4-bromo-4′-fluorobiphenyl. Carbon dioxide cylinders were purchased from Aldrich. Headspace GC vials were obtained from Perkin-Elmer Corp. (Norwalk, CT, USA). Phosphoric acid, HPLC grade acetonitrile, HPLC grade methanol, *sec*-butanol, and xylenes were purchased from Fisher Scientific (Pittsburgh, PA, USA).

HPLC Separation Development

All HPLC separations were performed using a Hewlett-Packard (Wilmington, DE, USA) HP1100 diode array HPLC system with UV detection at 210 nm. Separations were performed using a Waters (Milford, MA, USA) Symmetry Shield C8 phase (250 × 4.6 mm; 5-μm particles) or a Zorbax RX C8 column (250 × 4.6 mm; 5-μm particles) from Hewlett-Packard.

Carbon Dioxide Derivatization

Working in an appropriate hood, a small magnetic stir bar was added to a gas chromatographic headspace vial that was capped and crimped to provide an

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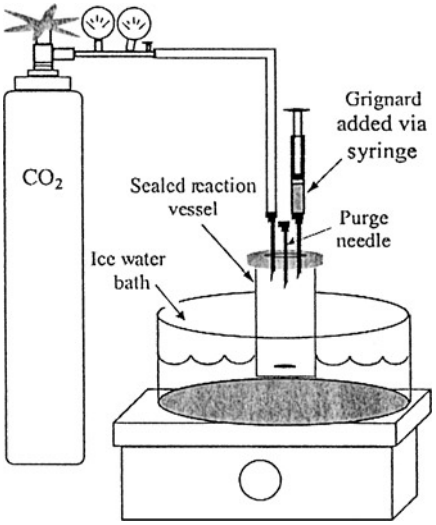

Figure 1. Schematic diagram of carbon dioxide derivatization procedure.

airtight seal for the reaction vessel. A disposable 22-gauge needle was inserted through the septum of the crimped vial to serve as a gas exit. The vial was placed in an ice bath and stirred continuously. A small (laboratory) cylinder of carbon dioxide was equipped with a pressure regulator, and the needle outlet was inserted into the headspace vial. The vial was flushed with 2 psi CO_2 for approximately 30 s. While flushing with CO_2 was continued, 0.50 mL of 4-fluorophenylmagnesium bromide was accurately added to the reaction vessel via a nitrogen-purged 1.0-mL disposable syringe (Fig. 1).

The CO_2 regulator pressure was increased to 10 psi, and the exit needle was removed to pressurize the vial. After approximately 10 s, the carbon dioxide needle line was removed (CO_2 flow was shut off only after the needle line was removed) and the stirring solution was allowed to react in the ice bath for approximately 15 min to produce a 4-fluorobenzoic acid solution. The crimped cap was carefully removed, and the contents of the vial were quantitatively transferred to a volumetric flask and diluted 250-fold with 50% methanol/50% water (v/v). The resulting sample solution was analyzed directly by HPLC on a Waters Symmetry Shield column. HPLC conditions are described in the legend to Figure 3D.

sec-Butanol Titrations

Approximately 1 *M sec*-butanol titrant solution was prepared in xylenes using sieve-dried *sec*-butanol and sieve-dried xylenes. A 4.0-mL aliquot of Grignard

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was removed with a nitrogen-purged syringe and accurately delivered to a side-arm flask which was held under nitrogen. Approximately 5 mg of *N*-phenyl-1-naphthylamine (indicator) was added to the Grignard and the resulting yellow solution was titrated with 0.942 *M sec*-butanol to a colorless end point.

Liquid Chromatography-Mass Spectrometry (LC-MS) Conditions for Impurity Identification

A Hewlett-Packard model 1100 high-performance liquid chromatograph equipped with a photodiode array detector was used for LC-MS analysis of 4-fluorophenylmagnesium bromide. The mass spectrometer was a Finnigan TSQ 7000 equipped with an electrospray interface. The LC conditions included a Waters Symmetry Shield C8 column, 250 × 4.6 mm, operated at a flow rate of 1 mL/min with a 20-μL injection volume. The column temperature was 30°C. The aqueous mobile phase component was 0.05% formic acid, and the organic component was 0.05% formic acid in acetonitrile. A gradient from 25% organic to 80% organic in 45 min was used. UV detection was monitored at 210 nm. 4-Fluorophenylmagnesium bromide was derivatized with CO_2 using the procedure described above and the derivatization mixture was diluted 100× with 50:50 methanol/water before analysis.

Full-scan MS measurements in the negative ion mode were performed using a scan range from 130 to 300 amu at a dwell time of 5.9 ms. The sheath and auxiliary gases were 60 psi and 30 units (rotameter) of nitrogen, respectively. The interface capillary was 250°C, the spray voltage was 3.9 kV, and the photomultiplier tube was operated at 1.5 kV. A 10% solution of ammonium hydroxide was introduced as a sheath flow liquid within the electrospray needle at a flow rate of 3 μL/min to form the anion of the CO_2 derivatives.

RESULTS AND DISCUSSION

HPLC Method Development

To evaluate Grignard quality, it was necessary, first, to identify and separate both active and inactive components. Potential active impurities of note in 4-fluorophenylmagnesium bromide (4F Grignard) include 3-fluorophenylmagnesium bromide, phenylmagnesium bromide, 4′-fluorobiphenyl-3-magnesium bromide, and 4′-fluorobiphenyl-4-magnesium bromide. Reaction with carbon dioxide transforms these Grignards to stable carboxylic acids (Fig. 2) before HPLC speciation.

Inactive impurities, which are unchanged by CO_2, can also be present in Grignard samples. In 4F Grignard, inactive impurities can include 4-fluorobenzene

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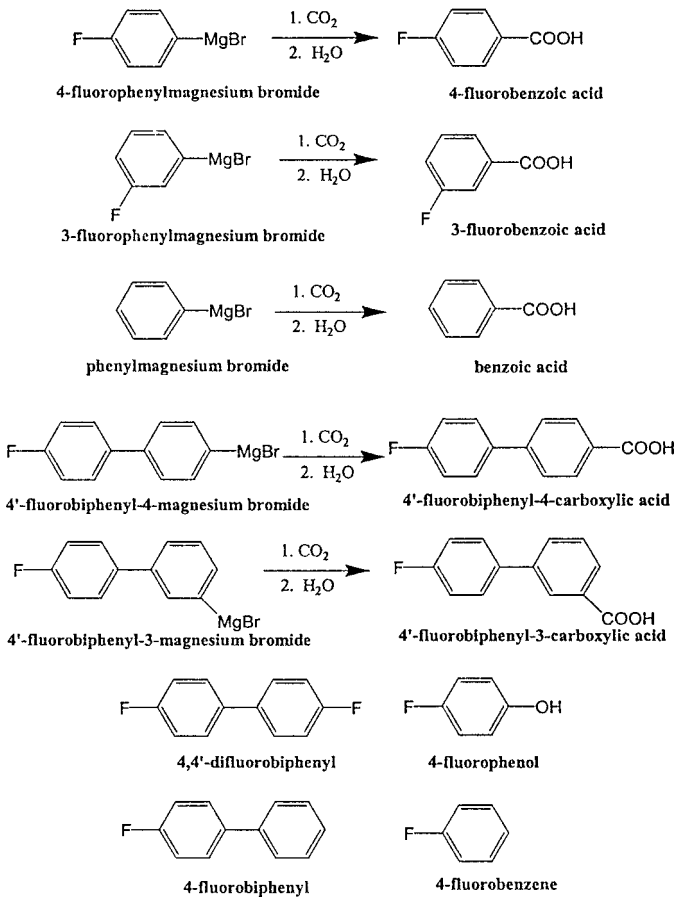

Figure 2. Components present in 4-fluorophenylmagnesium bromide.

(quench of 4F Grignard with water), 4-fluorophenol (reaction with oxygen), 4-fluorobiphenyl, and 4,4′-difluorobiphenyl. Commercially available authentic samples of 4-fluorophenol, benzoic acid, 4-fluorobenzoic acid, 3-fluorobenzoic acid, fluorobenzene, 4-fluorobiphenyl, and 4,4′-difluorobiphenyl were used to optimize the HPLC separation.

Low eluent pH was essential to achieve retention in the reversed phase for the carboxylic acid derivatives. Using a 0.1 v-% phosphoric acid/acetonitrile mobile phase, good peak shape was observed on a Waters Symmetry Shield C8 column. However, 4-fluorobenzoic acid and 4-fluorophenol co-eluted (Fig. 3A). Resolution of 4-fluorobenzoic acid and 4-fluorophenol improved on a Zorbax RX-C8 phase, as shown in Figure 3B, with a concomitant decrease in resolution of 4-fluorobenzoic

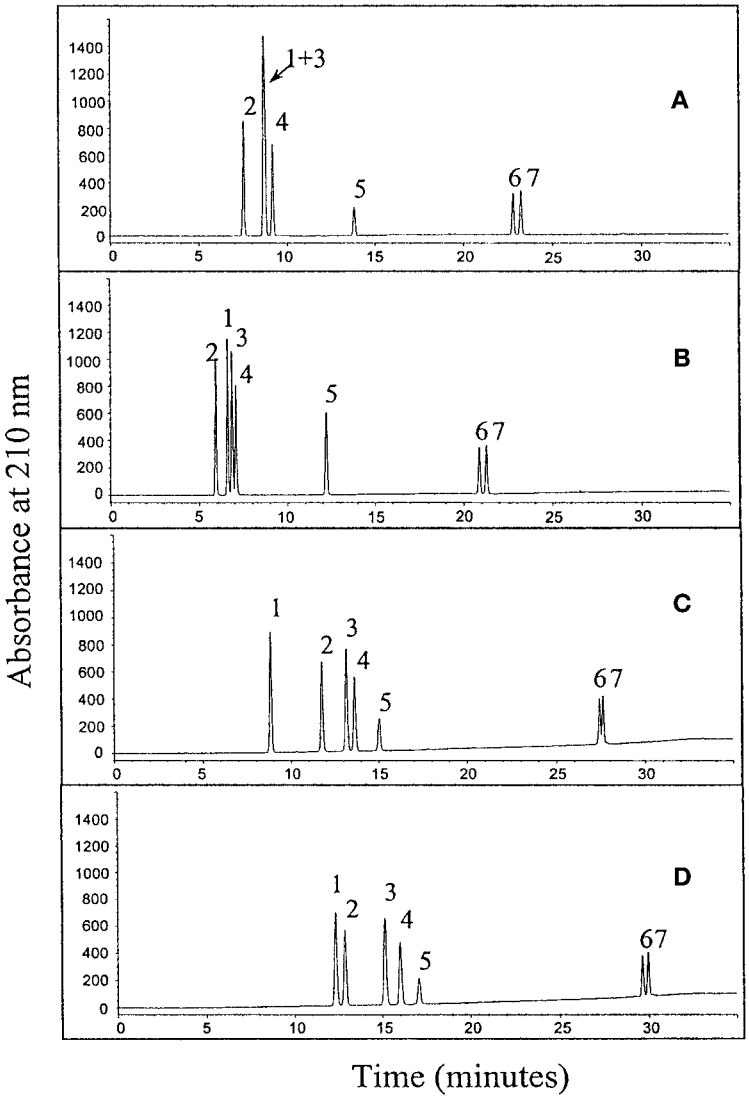

Figure 3. Mix of seven Grignard derivatives and impurities: 1) 4-fluorophenol; 2) benzoic acid; 3) 4-fluorobenzoic acid; 4) 3-fluorobenzoic acid; 5) 4-fluorobenzene; 6) 4-fluorobiphenyl; 7) 4,4′-difluorobiphenyl. A) Waters Symmetry Shield C8 250 × 4.6 mm, 25% acetonitrile/75% 0.1 v-% phosphoric acid in deionized water to 80% acetonitrile/20% 0.1 v-% phosphoric acid in deionized water over 30 min. Flow rate was 1.5 mL/min and column temperature was 30°C. B) similar to (A), but column was Zorbax RX-C8 250 × 4.6 mm. C) similar to (B), but organic modifier was methanol. D) similar to (A) with methanol as organic modifier.

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acid and 3-fluorobenzoic acid. Substituting methanol for acetonitrile increased retention and resolution of early eluting components at the expense of decreased resolution between 4-fluorobiphenyl and 4,4′-difluorobiphenyl.

A Waters Symmetry Shield C8 column with methanol/phosphoric acid mobile phase resulted in the best separation of all impurities of interest and was, therefore, chosen for the analysis of 4F Grignard samples following CO_2 treatment (Fig. 3D).

Assay Development and Evaluation

Dry ice and laboratory CO_2 cylinders were both evaluated as sources of carbon dioxide to react with 4F Grignard. When the Grignard was added directly to dry ice, 11 mol% 4-fluorobenzene (quenched 4F Grignard) was observed. Purging the reaction flask with CO_2 from dry ice boil-off resulted in 5 mol% of 4-fluorobenzene. Presumably, condensed water on the dry ice quenched 4F Grignard to produced 4-fluorobenzene as shown below.

$$\text{R-MgBr} + \text{H}_2\text{O} \rightarrow \text{R-H} + \text{MgBrOH} \quad (2)$$

As little as 2 mol% 4-fluorobenzene was detected when laboratory cylinders of CO_2 were used, suggesting that Grignard concentrations may be underestimated by max 2% using CO_2 via laboratory cylinders. Therefore, laboratory cylinders were used as a CO_2 source to evaluate this derivatization technique for aryl Grignards. A typical HPLC chromatogram for 4F Grignard after CO_2 treatment is shown in Figure 4. 4F Grignard was also quenched with water and then analyzed by HPLC to discriminate between active and inactive components. As shown in Figure 4, CO_2 derivatives of active components such as B, D, and E are no longer present while inactive impurities remain.

In synthetic reactions with 4F Grignard, 4′-fluorobiphenyl-4-magnesium bromide and 4′-fluorobiphenyl-3-magnesium bromide resulted in downstream impurities. Therefore 4′-fluorobiphenyl-4-carboxylic acid was synthesized in house and both 4′-fluorobiphenyl-3-carboxylic acid and 4′-fluorobiphenyl-4-carboxylic acid were identified by LC-MS analysis in negative ion mode as $(\text{M-H})^{\cdot}$, m/z 215.

The carbon dioxide derivatization was found to be linear ($r^2 = 0.99952$) with respect to the volume of Grignard added to the reaction vessel. The reaction consistently reached completion despite variable amounts (0.3–0.7 mL) of Grignard present. To minimize assay variability, a sufficient amount of sample should be added, so that it can be accurately measured. In this study, 0.5 mL delivered via a 1-mL disposable syringe was found to meet these needs. Derivatization precision was determined for three preparations (0.5 mL each) of two Grignard samples. Lot A was found to be 1.07 ± 0.01 *M* and Lot B was 0.85 ± 0.01 *M*. Relative standard deviations were less than 2%, indicating that the carbon dioxide derivatization

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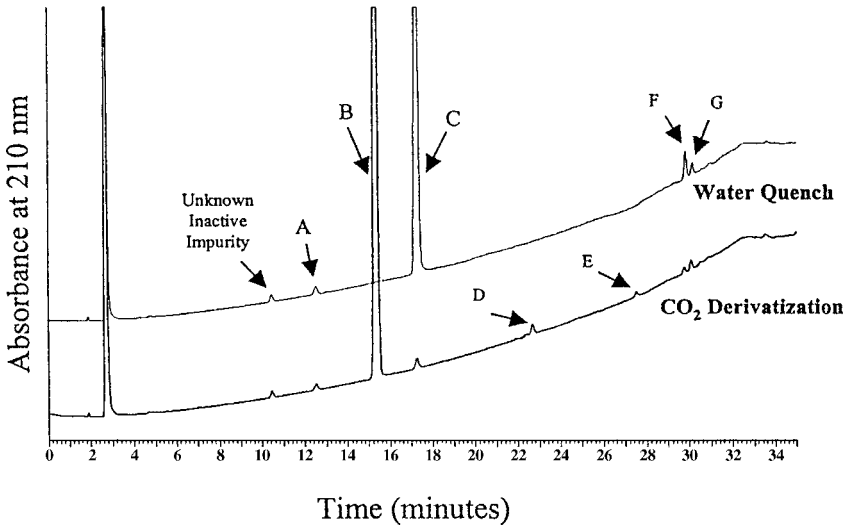

Figure 4. Typical chromatogram of 4-fluorophenylmagnesium bromide following CO_2 derivatization and after water quench. HPLC conditions as in the legend to Figure 3D. A) 4′-fluorophenol; B) 4-fluorobenzoic acid; C) 4-fluorobenzene; D) 4′-fluorobiphenyl-3-carboyxlic acid; E) 4′-fluorobiphenyl-4-carboxylic acid; F) 4-fluorobiphenyl; G) 4,4′-difluorobiphenyl.

procedure described in this study is a precise method to determine active Grignard concentrations.

Several Grignard samples were titrated with *sec*-butanol in xylenes to determine active Grignard concentrations. These samples were also analyzed by carbon dioxide derivatization followed by HPLC analysis to determine the concentration of active Grignard. As shown in Table 1, relatively good agreement (on average <5% difference) was found between the two methods. Therefore, derivatization with carbon dioxide followed by HPLC is an accurate alternative to *sec*-butanol titrations for the Grignard assay.

Table 1. Comparison of CO_2 Derivatization with *sec*-Butanol Titration of 4-Fluorophenylmagnesium Bromide

4F Grignard Sample	CO_2 Derivatization (*M*)	*sec*-Butanol Titration (*M*)
Lot A	1.07	1.11
Lot B	0.85	0.86
Lot C	0.93	0.86
Lot D	0.96	0.91

Table 2. Effect of CO_2 Pressurization Time on 4F Grignard Assay and Impurity Levels

Reaction Time at 10 psi (min)	Grignard	4-Fluorobiphenol-4-Carboxylic Acid (area %)[a]
0	1.06	0.32
1	1.06	0.46
2.5	1.08	0.44

[a](100) (peak area 4-fluorobiphenol-4-caboxylic acid)/(peak area 4-fluorobenzoic acid).

During Grignard addition, the reaction vessel was purged with CO_2 gas at ~2 psi and then pressurized to ~10 psi. Reaction time at 10 psi was varied from 0 to 15 min to determine minimum time for complete derivatization. As shown in Table 2, reaction of CO_2 with 4F Grignard reached completion even when the reaction vessel was not pressurized. However, the level of derivatized 4-fluorobiphenyl Grignard (4′-fluorobiphenyl-4-carboxylic acid) was slightly depressed when the vial was not pressurized. To ensure complete reaction of major Grignard components, as well as trace impurities, pressurization at 10 psi for a minimum of 5 min would be recommended.

HPLC separation following reaction with carbon dioxide allows Grignard quality to be assessed. Four lots of 4F Grignard were evaluated from three different manufacturers. Levels of known impurities varied significantly including 4′-fluorobiphenyl-4-magnesium bromide (detected as 4′-fluorobiphenyl-4-carboxylic acid), which varied by more than 1 order of magnitude (Table 3). The amount of quenched 4F Grignard and other inactive impurities also differed among the sources and lots, demonstrating the power of this technique to evaluate Grignard purity.

Table 3. Comparison of 4-Fluorophenylmagnesium Bromide Quality

Sample	4-Fluoro-biphenyl-3-carboxylic Acid (area %)[a]	4-Fluoro-biphenyl-4-carboxylic Acid (area %)[b]	4F Benzene (mol-%)	4-Fluoro-phenol (mol-%)	4-Fluoro-biphenyl (mol-%)	4,4′-Difluoro-biphenyl (mol-%)
Lot A	0.83	0.39	1.70	0.54	0.09	0.14
Lot B	1.23	1.47	5.83	0.7	0.07	1.22
Lot C	1.02	1.22	2.84	0.51	0.05	0.61
Lot D	0.28	0.10	2.24	0.98	0.14	0.08

[a](100) (peak area 4′-fluorobiphenol-3-carboxylic acid)/(peak area 4-fluorobenzoic acid).
[b](100) (peak area 4′-fluorobiphenol-4-carboxylic acid)/(peak area 4-fluorobenzoic acid).

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Spike Recovery and Method LOQ

Des-F Grignard (phenylmagnesium bromide) was spiked in 4F Grignard to determine the accuracy of this CO_2 derivatization procedure for low-level active impurities. Recoveries were 121 and 125% for a 1 and 0.1% spike, respectively (Fig. 5). Precision was excellent for three CO_2 derivatizations of the 1% spike, such that Des-F Grignard was determined with 0.8% relative standard deviation (RSD). Precision decreased for the 0.1% spike with 10% RSD for Des-F Grignard (detected as benzoic acid following CO_2 treatment). Based on precision and spike recovery data, low level (0.1%) active Grignard impurities can be accurately quantified by this CO_2 derivatization procedure.

Method precision decreases at concentrations near the limit of quantification (LOQ). Using the Eurachem approach (12), the LOQ can be estimated by comparing observed RSD for a given sample concentration versus an RSD limit. This technique was used to estimate the method LOQ for this derivatization and HPLC procedure, assuming a 15% RSD limit. Serial dilutions of Aldrich lot 11707DU 4F Grignard following CO_2 derivatization were analyzed by HPLC to determine the method LOQ for 4F biphenyl Grignard. At 0.05%, 4′-fluorobiphenyl-4-carboxylic acid was quantified with 13% RSD (three injections) and was therefore estimated as

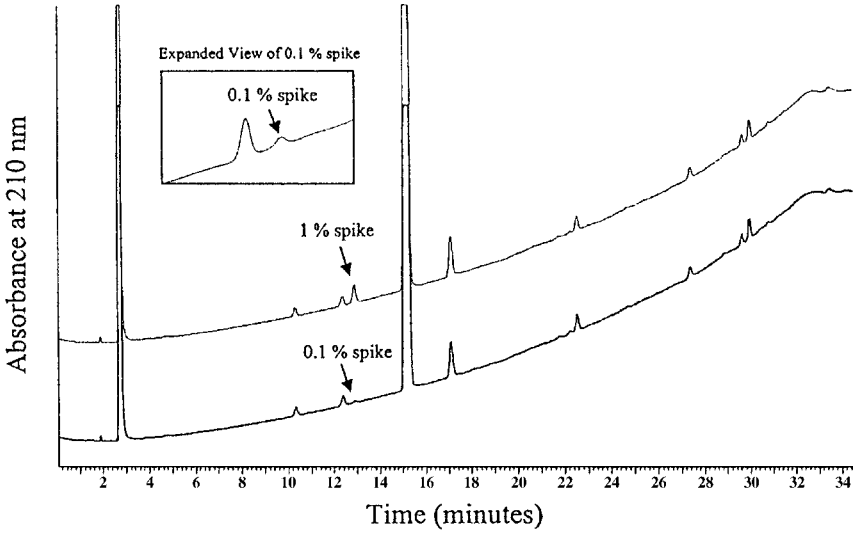

Figure 5. Spike recovery of 0.1 and 1.0% phenylmagnesium bromide in 4-fluorophenylmagnesium bromide. Analysis by CO_2 derivatization with HPLC method conditions as in the legend to Figure 3D.

the LOQ. Similarly, the LOQ was estimated to be <0.05 wt-% for 3-fluorobenzoic acid, benzoic acid, 4-fluorophenol, 4-fluorobiphenyl, and 4,4′-difluorobiphenyl based on serial dilutions of a mixed standard.

Applications of Carbon Dioxide Derivatization to Other Aryl Grignard Samples

This carbon dioxide derivatization procedure was applied to the analysis of other aryl Grignard samples including benzylmagnesium chloride and mesitylmagnesium bromide. As shown in Figure 6, the HPLC conditions used for analysis

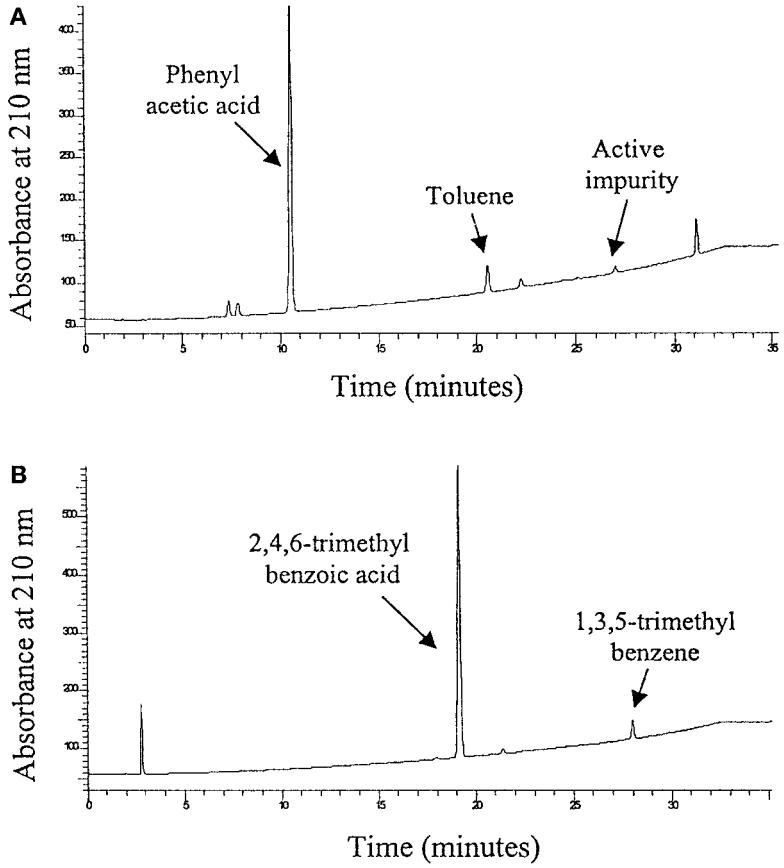

Figure 6. Analysis of other Grignard samples. HPLC conditions as in the legend to Figure 3D with both samples diluted 1250-fold. A) Benzylmagnesium chloride after CO_2 derivatization. B) Mesitylmagnesium bromide after CO_2 derivatizaiton.

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Table 4. Comparison of CO_2 Derivatization with *sec*-Butanol Titration for Benzylmagnesium Chloride and Mesitylmagnesium Bromide

Aryl Grignard	CO_2 Derivatization (M)	*sec*-Butanol Titration (M)	Certificate of Analysis Assay by *sec*-Butanol Titration (M)
Benzylmagnesium chloride lot E	1.75	1.82	2.02
Mesitylmagnesium bromide lot F	0.93	0.95	1.05

of 4F Grignard allow benzylmagnesium chloride and mesitylmagnesium bromide assay and quality to be evaluated. Benzylmagnesium chloride and mesitylmagnesium bromide were also quenched with water and then analyzed by HPLC to discriminate between active and inactive components. Both carbon dioxide derivatization and *sec*-butanol titrations showed similar Grignard concentrations for benzylmagnesium chloride and mesitylmagnesium bromide (2.1–3.8% difference between two methods), supporting the accuracy of this CO_2 derivatization procedure (Table 4). The Grignard assay decreased by approximately 10% since release by Aldrich (Certificate of Analysis via *sec*-butanol titration), indicating a need for assay determination before Grignard use.

CONCLUSIONS

The carbon dioxide derivatization procedure described in this study is an accurate alternative to *sec*-butanol titrations for the aryl Grignard assay. Reactions with carbon dioxide are quantitative, rapid, and reproducible. Additionally, HPLC separation of the derivatized Grignard reagents allows speciation of active and inactive components, which is not possible by titration methods. Comparison of this carbon dioxide derivatization with a water quench can distinguish active Grignard impurities from inactive components.

Sensitivity to trace Grignard impurities has been demonstrated by spiking experiments with low-level (1 and 0.1%) active impurities. In addition, the HPLC chromatogram of Grignard samples derivatized with carbon dioxide can serve as a fingerprint and allow comparisons of different Grignard samples, providing a useful tool for synthetic reactions with Grignard reagents.

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