

A Dual-Standard Reversed-Phase HPLC-UV Method for the Simultaneous Identification, Assay, and Impurities Analysis of Phenylboronic Acid: Using 4-Methoxyphenyl Boronic Acid as a Model Compound

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Abstract

Boronic acids (BAs) are versatile building blocks in organic synthesis and the pharmaceutical industry. A dual-standard reverse phase (RP) HPLC-UV method was developed for the simultaneous identification (as monomer), assay (as monomer), and impurities analysis of phenylboronic acid (PBA), using 4-methoxyphenyl boronic acid (4-MPBA) as a model compound. The method utilizes an Ascentis® Express 90 Å ES-Cyano column with 0.025% TFA in both mobile phases (A: water, B: methanol). It employs a simple gradient with an injection volume of 10 µL and a flow rate of 0.8 mL/min. This QC-friendly, single-sequence method achieves baseline separation of the analyte and its impurities. By utilizing a stable, readily available bromo-analog surrogate for quantitation, it bypasses the need for a single-component primary BA reference standard.

Keywords

Identification, Assay, Impurities, RP-HPLC-UV Analysis of Phenylboronic Acid, Dual-Standard

1. Introduction

Boronic acids (BA) are versatile building blocks in organic synthesis [1] [2]. Through scalable and efficient Suzuki-Miyaura coupling reactions [2], BAs are the

intermediates of Active Pharmaceutical Ingredients (APIs) in pharmaceutical industries [3], drugs [4]-[10], or prodrugs [11]. BAs are also used as analytical probes [12], as well as drug delivery materials [13] [14]. BA typically exists as a mixture of monomer and various oligomers [15]-[20]. As shown in **Figure 1**, these oligomers include dimers, trimers, and tetramers, as well as their partially hydrolyzed forms. The monomer undergoes dehydration to form oligomers when exposed to heat, vacuum, or even ambient conditions [17]-[20]. Conversely, these interchangeable oligomers revert to the monomer via rapid hydrolysis upon exposure to moisture, including atmospheric humidity. Consequently, the exact composition of BA may fluctuate during preparation (from batch to batch), transportation, and storage.

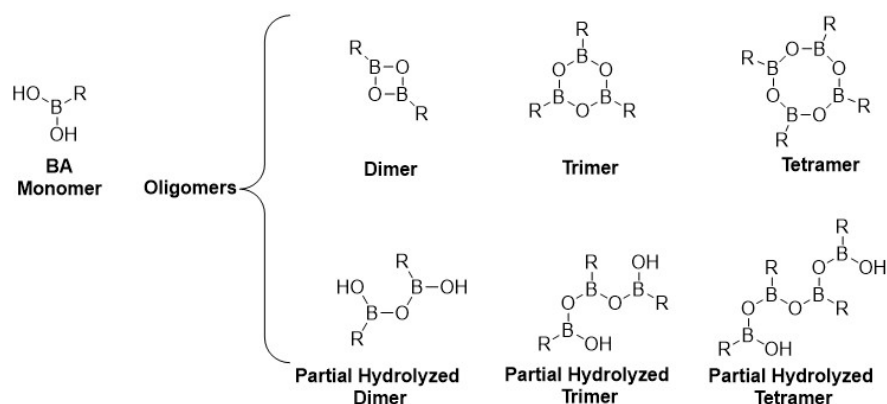


Figure 1. Structure of boronic acid, monomer and oligomers.

Different analytical methods have been applied to boronic acid analysis, including both non-chromatographic and chromatographic techniques. Non-chromatographic methods are primarily qualitative and used for characterization, such as melting point [21], thermogravimetric analysis (TGA) [15], differential scanning calorimetry (DSC) [15], X-ray powder diffraction (XRPD) [15], infrared (IR) [22], Raman spectroscopy [23], and nuclear magnetic resonance (NMR) [15] [16] [24]. Conversely, chromatographic methods, such as gas chromatography (GC) and liquid chromatography (LC), are used for quantitation. GC analyses require derivatization [25] [26], a process that involves extra sample preparation steps which may inevitably introduce additional variation. LC techniques include thin-layer chromatography (TLC) [27], reversed-phase high-performance liquid chromatography (RP-HPLC) [28]-[30], and hydrophilic interaction liquid chromatography (HILIC-HPLC) [31]. Several mass spectrometry (MS)-based HPLC methods have also been reported [30] [32]-[36].

During the manufacture of a PBA-type intermediate for an API in clinical studies, an urgent need arose to develop a QC-friendly HPLC method for identification (ID as monomer), assay (%w/w as monomer), and impurity analysis within a two-week timeframe. Procuring a high-purity, single-component PBA reference standard (monomer or oligomer) from domestic or international sources, such as

those in India, was not feasible within this period.

To resolve these issues, a dual-standard approach was implemented: the PBA was used as a retention time marker for identification, while a readily available bromo-analogue¹ was employed for quantitation (assay and impurity). In this paper, we present a simple, QC-friendly RP-HPLC-UV method using 4-methoxyphenylboronic acid (4-MPBA) as a model compound.

2. Experimental Method

2.1. Chemicals and Abbreviations

4-Methoxyphenylboronic acid (4-MPBA) was purchased from Thermo Fisher Scientific (Cat. No. 30948, $\geq 96\%$). 4-Methoxyphenol (Phenol, Cat. No. M18655, 99.8% by GC), Anisole (Des-Br, Cat. No. 296295, 99.7% by GC), 4-Bromoanisole (Bromo, Cat. No. A11824, 98.8% by GC), and 4, 4'-Dimethoxybiphenyl (BisPh, Cat. No. 148539, 99.5% by GC), were purchased from Sigma-Aldrich. The potential impurities of compound 4-MPBA are illustrated in **Figure 2**, where Bromo is the leftover starting material, Phenol is the oxidation impurity, and Des-Br and BisPh are typical processing impurities.

1,3,5-Trimethoxybenzene (74599-1G, Lot BCCG8790, Potency 0.9999, qNMR reference standard), Dimethyl sulfoxide-d₆ (DMSO-d₆, Cat. No. 320760075, 100.0 atom %D, for NMR solvent), and Deuterium oxide (D₂O, 99.9 atom % D) were purchased from Sigma-Aldrich.

Mobile phase components were HPLC grade and purchased from Fisher Chemical or Sigma-Aldrich: Acetonitrile (ACN, HPLC grade, Fisher Scientific A998-4), Methanol (MeOH, HPLC grade, Fisher Scientific A452SK-4), Trifluoroacetic acid (TFA, HPLC grade, Sigma Aldrich 302031), O-Phosphoric Acid (PA, 85% HPLC Grade, Fisher A260), Formic Acid (FA, LC-MS grade, Thermo Scientific 28905), Ammonium Formate (AF, Optima LCMS grade, Fisher Chemical A115-50), Ammonium Acetate (AAc, $\geq 99.99\%$ trace metals basis, Sigma Aldrich 431311-50G), Acetic acid (AA, glacial (HPLC) grade, Fisher Chemical A35-500), and Ammonium hydroxide (NH₄OH, $\geq 99.99\%$ trace metals basis, Sigma Aldrich 338818-1L).

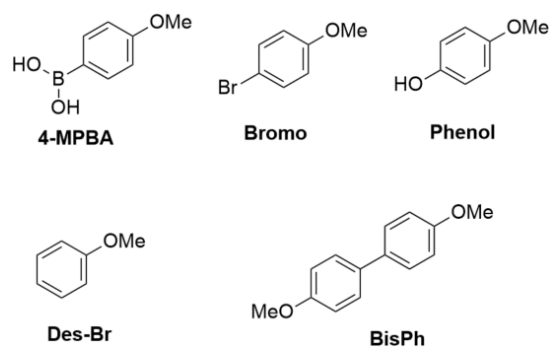


Figure 2. Structure of 4-MPBA and its potential impurities.

¹The bromo-analogue was chosen due to its ready availability, high purity, solution stability, compatible UV response, and distinct chromatographic separation from 4-MPBA and its impurities.

2.2. RP-HPLC-UV Method

The analyses are performed on a Agilent Infinity II 1260/1290 system with a UV detector (wavelength 223 nm), using a reverse phase (RP) HPLC column (Ascen-tis® Express 90 Å ES-Cyano, 2.7 μm, 150 mm × 4.6 mm) at 35 °C, with 0.025% TFA in both mobile phases A (water) and B (methanol), applying a simple gradient (0 – 1 min, 14%B; 1 – 22 min, 14% - 80%B; 22 – 25 min, 80%B; 25 - 25.1 min, 80% - 14%B; 25.1 - 30min, 14%B), with an injection volume of 10 μL, and at a flow rate of 0.8 mL/min. Refer to **Table S1** in the supplementary material for detailed HPLC conditions. A working standard solution (WSS) was a mixture of 0.1 mg/mL of each of two standards, 4-MPBA and Bromo. The purity sample was prepared at 1.0 mg/mL in diluent (50% aqueous acetonitrile). The assay sample was prepared by 10-fold dilution from the purity sample. The resolution solution was prepared by spiking 2% w/w of each potential impurity into 1.0 mg/mL of 4-MPBA. Limit of quantitation (LOQ) solution was prepared by serial dilution (200-fold) from the WSS. To ensure consistent results, all HPLC samples were allowed to equilibrate in the aqueous diluent for approximately 30 seconds prior to injection.

The assay (% w/w) of sample can be calculated from Equation (1). Similarly individual impurity (% w/w) of sample can be calculated from Equation (2).

$$Assay(\%) = \left(\frac{Asam}{Astd} \right) * \left(\frac{Cstd}{Cassay} \right) * \left(\frac{P}{RRF} \right) * 100 \quad (1)$$

$$Impurity(\%) = \left(\frac{Asam(i)}{Astd} \right) * \left(\frac{Cstd}{Cpurity} \right) * \left(\frac{P}{RRF(i)} \right) * 100 \quad (2)$$

Where: *Asam* is the peak area of 4-MPBA in the assay sample. *Astd* is mean peak area ($n = 6$) of the Bromo in the first 6 injections of the WSS. *Cstd* is nominal concentration of the Bromo in the WSS (e.g., 0.1 mg/mL). *Cassay* is nominal concentration of the assay sample solution (e.g., 0.1 mg/mL). *P* is the potency of the Bromo reference standard (e.g., 0.988). *RRF* is the Relative response factor of 4-MPBA relative to the Bromo standard, calculated by dividing the slope of the 4-MPBA linear curve (0.05 - 0.15 mg/mL; 50% - 150% of 0.1 mg/mL) by the slope of the Bromo linear curve over the same range. *Asam (i)* is the peak area of individual impurity in the purity sample. *Cpurity* is nominal concentration of the purity sample solution (e.g., 1.0 mg/mL). *RRF (i)* is the relative response factor of each impurity relative to the Bromo standard, calculated by dividing the slope of the linear curve for each impurity (0.0005 - 0.02 mg/mL; 0.05% - 2.0% of 1.0 mg/mL) by the slope of the Bromo linear curve (0.05 - 0.15 mg/mL; 50% - 150% of 0.1 mg/mL).

3. HPLC Method Development

The HPLC method development involves systematically testing various parameters: diluents, columns, mobile phase buffers, mobile phase B (organic solvent), flow rate, different UV absorption wavelengths, column temperature, and gradient.

3.1. Mobile Phases (MP)

It was challenging to separate the phenol impurity peak from the 4-MPBA peak. Initially, a Waters XBridge Shield RP18 column (4.6×150 mm, $3.5 \mu\text{m}$)—selected for its embedded polar group (carbamate) to assist with phenolic compounds—and various acid/base modifiers (TFA, PA, FA, NH_4OH) were used to analyze 1.0 mg/mL 4-MPBA resolution solutions, both with and without a 2% w/w phenol spike. Although baseline separation between phenol and 4-MPBA was not fully achieved, TFA and phosphoric acid (0.05% v/v) in both mobile phases A (water) and B (ACN) performed better than formic acid; see **Figure 3**.

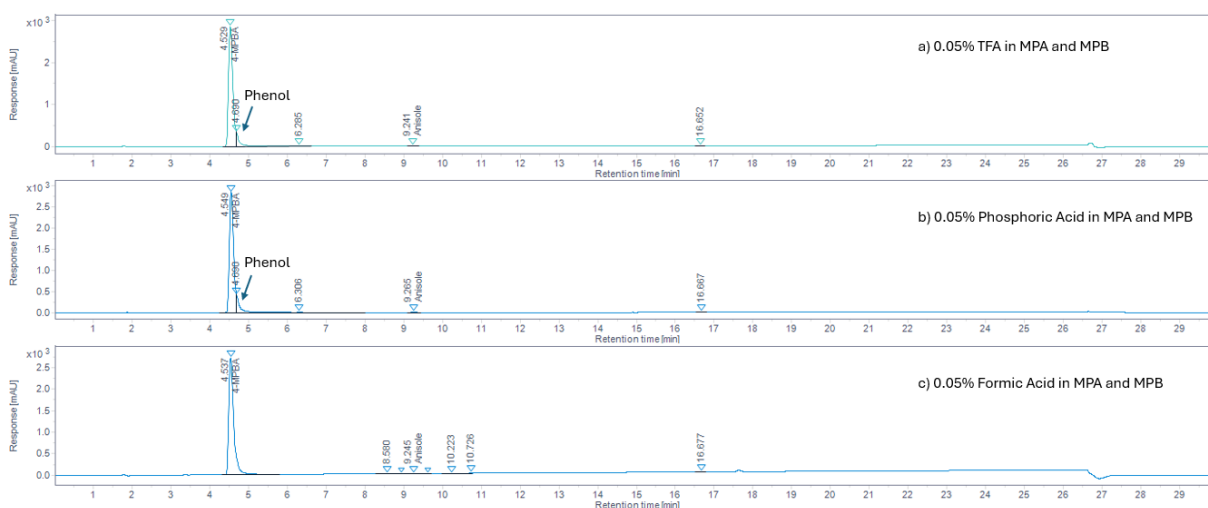


Figure 3. Different acid (0.05%) in mobile phases A and B.

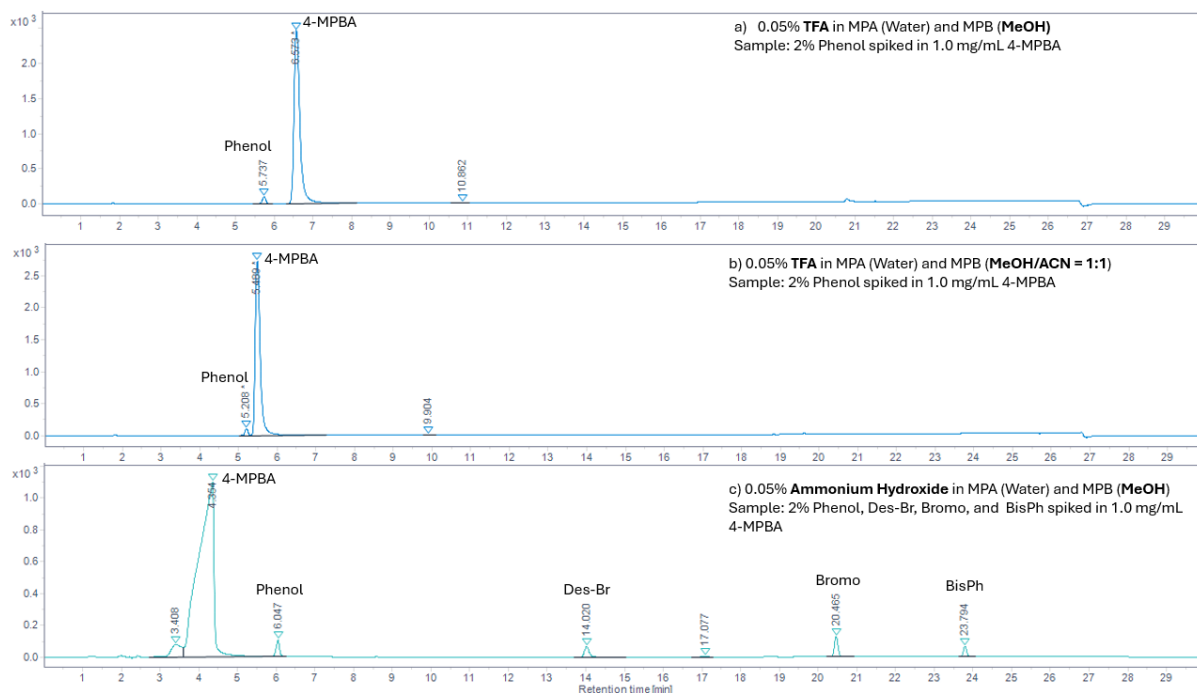


Figure 4. Chromatograms of two resolution solutions.

Baseline separation between the Phenol impurity and 4-MPBA was achieved using 0.05% v/v TFA in methanol as mobile phase B (MPB), as shown in **Figure 4(a)**. However, resolution was compromised upon the addition of acetonitrile to MPB (0.05% TFA in a 1:1 methanol/acetonitrile mixture; **Figure 4(b)**). Furthermore, the 4-MPBA peak exhibited significant broadening and a decrease in retention when 0.05% ammonium hydroxide was utilized in both MPA and MPB (**Figure 4(c)**).

3.2. Column Screening

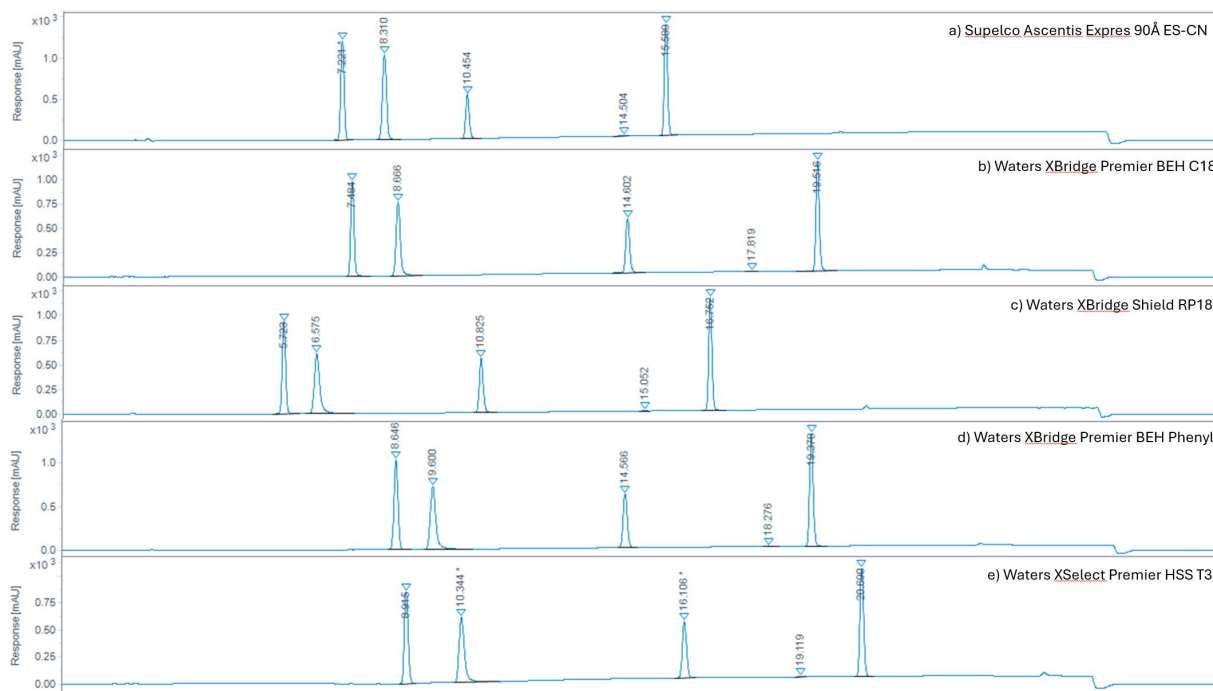


Figure 5. Chromatograms with different columns (PDA wavelength 225 nm, peaks from left to right are Phenol, 4-MPBA, Des-Br, Bromo).

Table 1. Peak tailing with different HPLC columns.

Item	Column	Catalog No.	Tailing
a	Supelco Ascentis Expres 90Å ES-CN (4.6 mm × 150 mm, 2.7 μm)	53492-U	1.0
b	Waters XBridge Premier BEH C18 (4.6 × 150 mm, 3.5 μm)	186010661	1.2
c	Waters XBridge Shield RP18 (4.6 × 150 mm, 3.5 μm)	186003045	1.3
d	Waters XBridge Premier BEH Phenyl (4.6 × 150 mm, 3.5 μm)	186010677	1.3
e	Waters XSelect Premier HSS T3 (4.6 × 150 mm, 3.5 μm)	186010936	1.4
f	Supelco Ascentis Expres 90Å RP-Amide (4.6 mm × 150 mm, 2.7 μm)	53931-U	1.5
g	Waters XSelect Premier CSH C18 (4.6 × 150 mm, 3.5 μm)	186010644	1.6
h	Phenomenex Kinetex Polar C18 (150 × 4.6 mm, 2.6 μm)	00F-4759-E0	1.9
i	Phenomenex Luna Omega Polar C18 (150 × 4.6 mm, 3 μm)	004-4760-E0	2.5
j	Waters Atlantis Premier BEH AX (4.6 × 150 mm, 3.5 μm)	186009398	2.6

Inspired by Waters application notes [37] [38], ten columns were screened by analyzing a mixture containing 0.2 mg/mL each of phenol, 4-MPBA, Des-Br, and Bromo, while maintaining consistent mobile phase components (0.05% TFA in water as MPA and 0.05% TFA in MeOH as MPB). All analytes were not only well retained but also well resolved (as shown in the chromatograms in **Figure 5**). Based on the peak tailing results listed in **Table 1**, the Supelco Ascentis Express 90 Å ES-CN provided the best peak shape for 4-MPBA. The cyano groups on the ES-CN column offer unique interactions that resolve tailing issues with polar compounds, such as boronic acids, more effectively than standard RP phases.

3.3. Wavelength

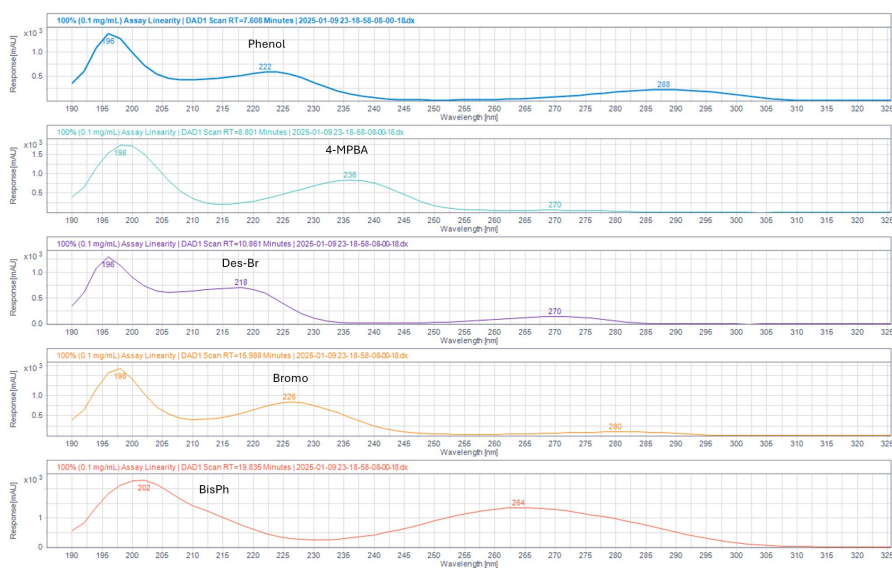


Figure 6. UV Spectra of Phenol, 4-MPBA, Des-Br, Bromo, and BisPh.

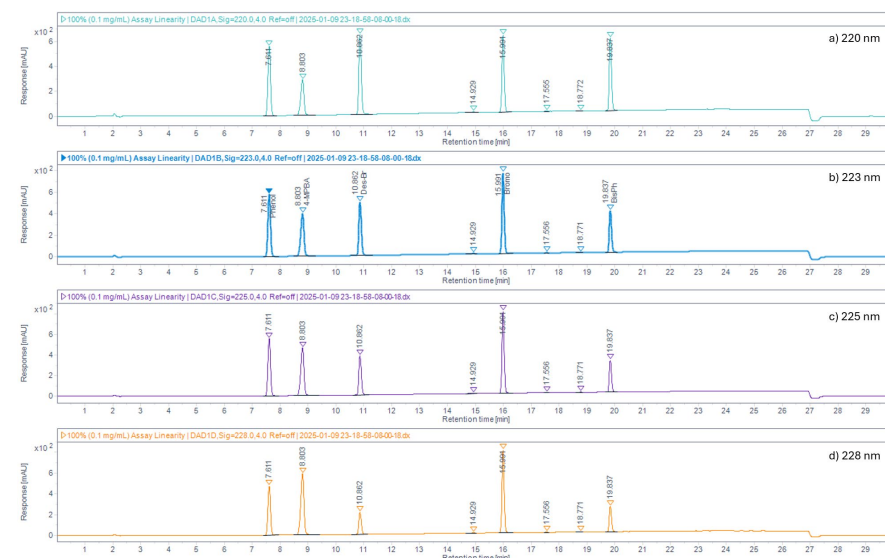


Figure 7. Chromatograms of 0.1 mg/mL mixture of Phenol, 4-MPBA, Des-Br, Bromo, and BisPh at different wavelengths.

Based on the UV spectra of Phenol, 4-MPBA, Des-Br, Bromo, and BisPh (**Figure 6**), several wavelengths (220 nm, 223 nm, 225 nm, and 228 nm) were evaluated. The wavelength 223 nm (**Figure 7(b)**) was selected, because all the resulting peak heights are comparable to that of 4-MPBA, see **Figure 7** for the chromatograms at each tested wavelength.

3.4. Diluent

Since BisPh is insoluble in methanol but soluble in acetonitrile, 50%-100% acetonitrile in water (with or without 0.05% TFA) were used as diluents. **Figure 8** presents the chromatograms of the resolution solution (1.0 mg/mL of 4-MPBA spiked with 2% of Phenol, Des-Br, Bromo, and BisPh). While 100% Acetonitrile resulted in poor peak shapes (**Figure 8(a)**), both 50% acetonitrile in water (**Figure 8(b)**) and 0.05% TFA in 50% acetonitrile (**Figure 8(c)**) yielded excellent peak shapes.

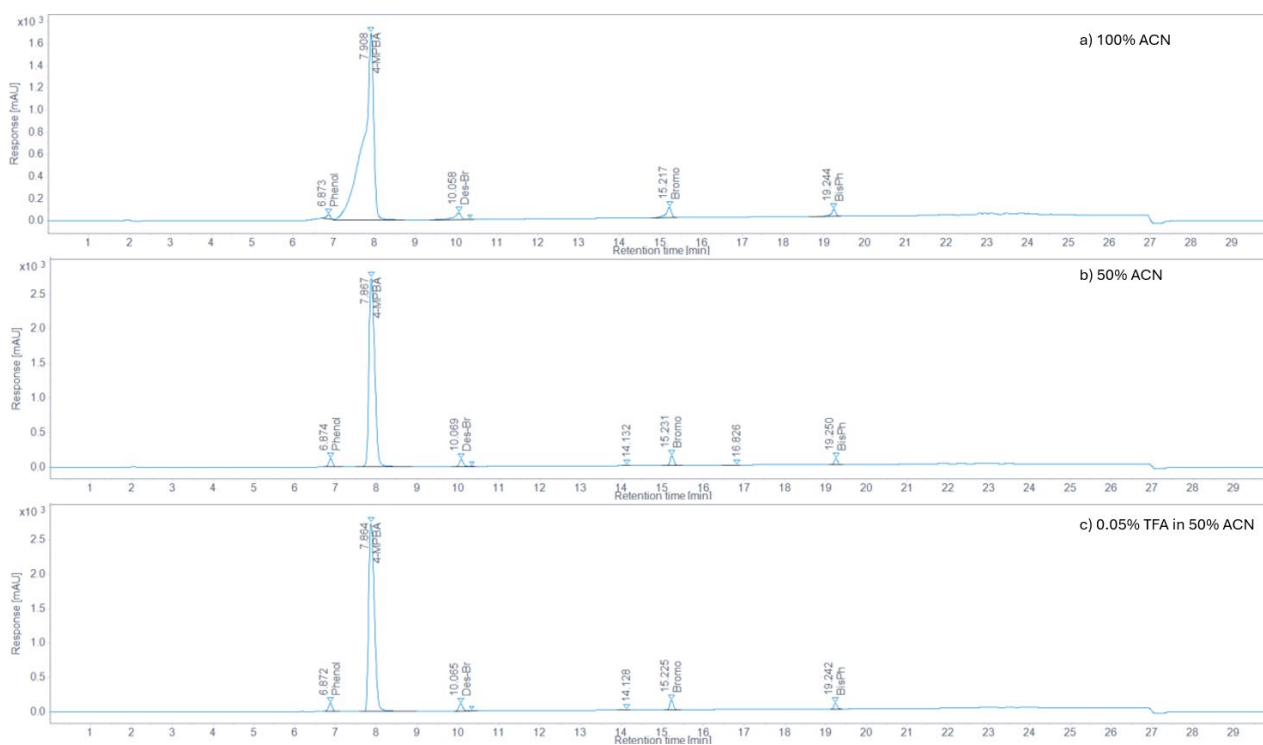


Figure 8. Chromatograms of resolution solution (2% of Phenol, Des-Br, Bromo, and BisPh in 1.0 mg/mL 4-MPBA with different diluents).

3.5. Robustness

The robustness of the method was assessed by varying several parameters, including flow rate, injection volume, initial %B, column temperature, and %TFA in mobile phases (see **Table 2**). Under all tested conditions, the peaks remained well-resolved with comparable peak shape. Chromatograms of the resolution solution and the 0.1 mg/mL mixture solution of five compounds are provided in the supplementary material (see **Figures S6-S15**).

Table 2. Robustness.

Flow Rate (mL/minute)	Injection Volume (μ L)	Initial %B	Column Temperature ($^{\circ}$ C)	%TFA in MPs	MP pH
0.7	9	12	30	0.025	2.4 - 2.5
0.8	10	14	35	0.05	3.5
0.9	11	16	40	0.1	4.5

4. HPLC Method Qualification

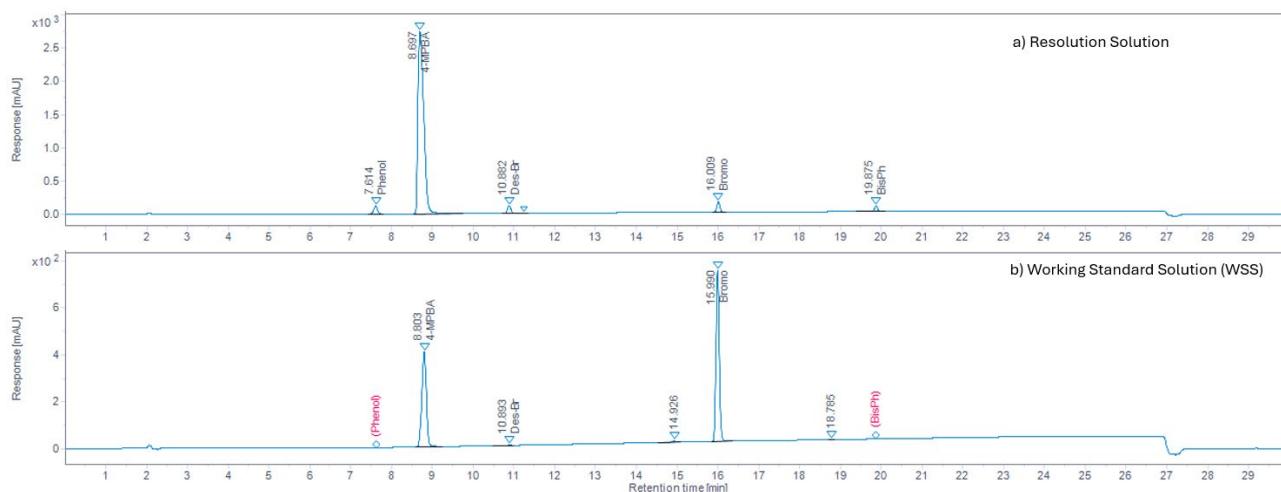
The HPLC method was qualified in terms of specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy, precision, intermediate precision, range, and solution stability. Detailed method validation for the GMP manufacturing of the actual MPA-type intermediate are omitted from this report due to intellectual property considerations.

4.1. Specificity and Carryover

Table 3. USP resolution of peaks in resolution solution.

Compound	RT (min)	USP Resolution
Phenol	7.622	NA
4-MPBA	8.709	5.0
Des-Br	10.890	10.1
RRT 1.29 ^a	11.258	2.1
Bromo	16.011	28.8
BisPh	19.870	27.2

Note: ^a: An unknown/unspecified impurity.

**Figure 9.** Chromatograms of resolution solution (a) and WSS (b).

No interference peaks were observed at the retention time of Phenol, 4-MPBA, Des-Br, Bromo, and BisPh in the diluent injections. The USP resolution of each

peak in resolution solution (containing a 2% spike of Phenol, 4-MPBA, Des-Br, Bromo, and BisPh in 1.0 mg/mL 4-MPBA) was greater than 2.0 (see **Table 3**). A typical chromatogram of resolution solution is shown in **Figure 9(a)**, and a typical chromatogram of WSS is shown in **Figure 9(b)**. No carryover was detected in the blank injections.

4.2. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD was determined to be 0.00025 mg/mL for each of the 5 components, representing 0.025% of nominal concentration of the purity sample (1.0 mg/mL); LOQ was 0.0005 mg/mL for each component, or 0.05% of nominal purity sample concentration. The signal-to-noise ratio (S/N) of each component across three LOD injections was more than 3 (see Table S-2 in the supplementary material). For the six LOQ injections, the S/N of each component was greater than 10 (see Table S-3 in the supplementary material), and the %RSD of each component was less than 6.0% (Table S-3). **Figure 10** presents representative chromatograms of the diluent, LOD, and LOQ.

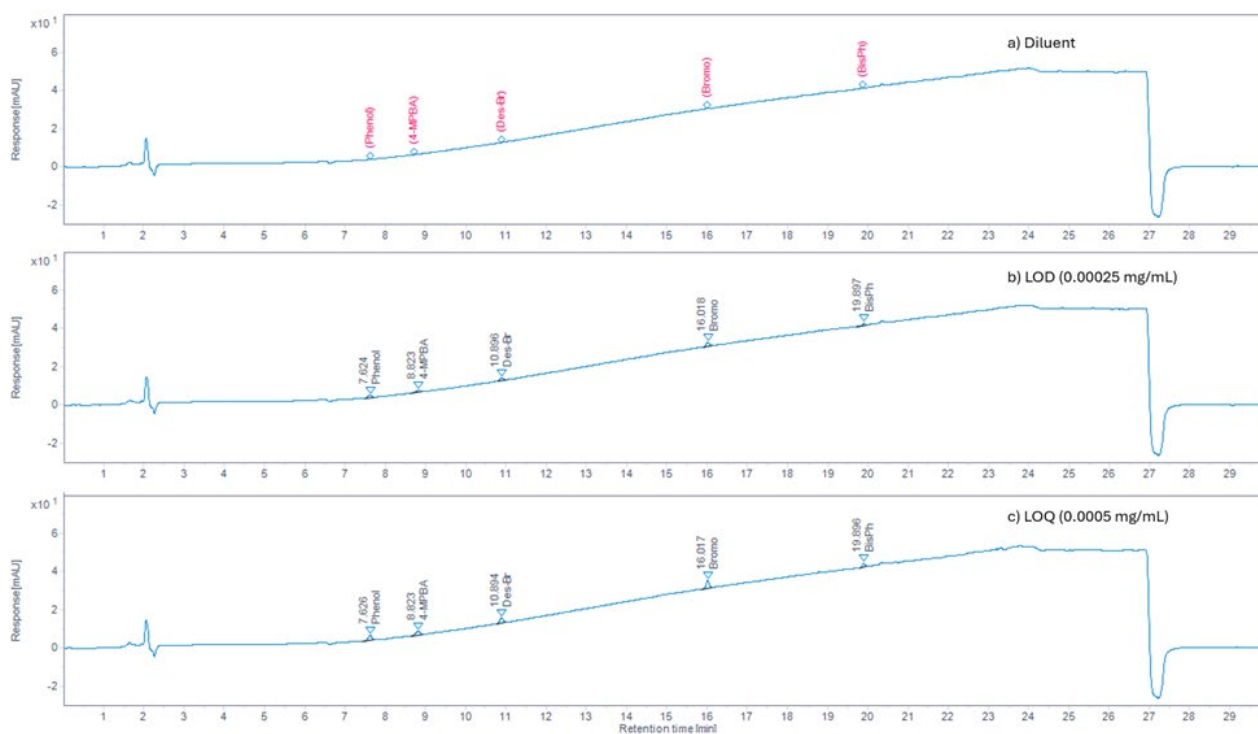


Figure 10. Chromatograms of diluent (a), LOD (b), and LOQ (c).

4.3. Identification as Monomer

The Identification (ID) was verified by the relative retention time (RRT), calculated by dividing the retention time (RT) of 4-MPBA peak in the assay samples by the mean RT ($n = 6$) of 4-MPBA peak in the WSS. The RRT of 4-MPBA cross all assay sample injections was within the acceptance criteria (0.98 - 1.02, see **Table 4**).

Table 4. Identification.

Assay Sample	RT (min)	Mean RT in WSS (min)	RRT	ID Criteria
1	8.791	8.814	1.00	$0.98 < RRT < 1.02$

4.4. Linearity and Relative Response Factor (RRF)

The linearity of all the potential impurities (Phenol, Des-Br, Bromo, and BisPh) was verified over the low concentration range (0.05% (LOQ) to 2.0% of 1.0 mg/mL), with correlation coefficient (R^2) better than 0.995 (see **Table 5**). Linearity of 4-MPBA and Bromo was also confirmed over the assay concentration range (50% to 150% of 0.1 mg/mL), with R^2 better than 0.998 (**Table 6**). The relative response factor (RRF) of each impurity was calculated by dividing its slope (low concentration range) by the slope of the Bromo (assay concentration range). The RRF of 4-MPBA is calculated by dividing its assay-range slope by the Bromo assay-range slope (see **Table 5** and **Table 6**). The RRF of Bromo is defined as 1.00.

Table 5. Linearity of impurities (0.05% (LOQ) – 2.0% of 1.0 mg/mL).

compound	Linear equation	R^2	RRF
Phenol	$y = 37725.718x - 1.163$	1.0000	0.8920
Des-Br	$y = 31727.405x - 0.837$	1.0000	0.7501
Bromo	$y = 41003.840x - 0.906$	1.0000	1.00
BisPh	$y = 21975.097x - 0.414$	0.9999	0.5196

Table 6. Linearity of 4-MPBA and Bromo (50% – 150% of 0.1 mg/mL).

compound	Linear equation	R^2	RRF
4-MPBA	$y = 32564.622x - 72.850$	0.9998	0.7699
Bromo	$y = 42295.187x - 103.303$	0.9998	1.00

4.5. Accuracy, Precision, and Intermediate Precision of Assay and Impurity

The accuracy and precision for assay quantitation of 4-MPBA were verified at three levels (50%, 100%, 150%). Recovery of each replicate at all three levels was within 98.0% - 102.0%, with averages of 99.2%, 98.6%, 98.2%, respectively; the %RSD was less than 1.0% at all levels (see **Table S5** in the supplementary material). Intermediate precision for the assay was verified by two analysts using two HPLC systems (Agilent Infinity II 1290 and 1260) over multiple days, yielding a % RSD of 1.1% ($n = 12$, see **Table S6** in the supplementary material). Similarly, the accuracy and precision of the impurities (Phenol, Des-Br, Bromo, and BisPh) were verified at three levels (0.05% [LOQ], 1.0%, 2.0% of 1.0 mg/mL, see **Table S7** in the supplementary material). Recovery of each replicate at these levels ranged between 93% - 114%, with a %RSD of less than 4.0%. Intermediate precision of all impurities was also confirmed, with a %RSD value less than 6.5% ($n = 12$, see

Table S-8 in the supplementary material).

4.6. Solution Stability

Solution stability was assessed by testing all the solutions (LOQ, Resolution, WSS, Assay sample, and Purity sample) stored at ambient temperature for 24 hours. The peak area of each component in the LOQ, Resolution, and WSS solutions was compared with the corresponding initial value. Assay (% w/w) and impurity (%w/w) were determined against a freshly prepared WSS at 24 h using Equations (1) and (2), respectively. Based on the results (see **Table S9** in the supplementary material), the LOQ, resolution, WSS, Assay, and purity sample solutions are stable for 24 h at ambient temperature.

5. Discussion

5.1. Diluent and Monomer

Using a diluent with approximately 50% water improves peak shape for 4-MPBA and facilitates the *in situ* conversion of anhydrides or oligomers into the monomer, as shown in **Figure 11**. A ^1H NMR study confirms that 4-MPBX (boroxine trimer, a representative anhydride/oligomer) rapidly converts to 4-MPBA upon quenching with deuterated water (**Figure S3** and **Figure S4** in the supplementary material). This conversion allows both the identification and assay value to be reported in the monomeric form, as all oligomers are converted *in situ* and subsequently utilized in the downstream aqueous reaction.

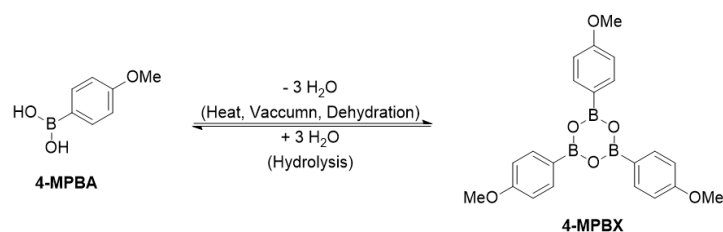


Figure 11. Inter-conversion of 4-MPBA and 4-MPBX.

5.2. MeOH vs ACN as Mobile Phase B

The choice of organic solvent in mobile phase B is critical to achieving baseline separation of Phenol and 4-MPBA. While the aprotic solvent acetonitrile failed to resolve the phenol and 4-MPBA peaks, methanol (a polar protic solvent) achieved baseline resolution (**Figures 3-4**). This improvement is due to methanol's ability to act as both a hydrogen-bond donor and acceptor, allowing it to effectively compete for hydrogen-bonding sites between the analytes and the cyano stationary phase. In contrast, acetonitrile lacks the protic hydrogen necessary to disrupt these interactions.

5.3. Impurity Profile

During in-process control (IPC) testing of the reaction producing the actual GMP

PBA-type intermediate, the formation of two process-related impurities (Phenol and Des-Br types) was observed at levels below the LOQ (0.05%w/w), while the Bromo-type starting material and the BisPh-type impurity were below the LOD (0.025% w/w).

5.4. Range

The established low-level linearity range (0.05% - 2.0% w/w of 1.0 mg/mL) enables the quantification of trace amounts of impurities, while the high-level range (50% - 150% w/w of 0.1 mg/mL) enables accurate quantification of the assay as the monomer of 4-MPBA. The established range meets QC requirements and ICH guidelines.

5.5. RRF Determination

The RRF for 4-MPBA is critical to the RP-HPLC-UV assay method, as the actual concentration of 4-MPBA in the linearity solutions directly affects the RRF calculation. Therefore, determining an accurate potency (as monomer) of 4-MPBA is key. Using an HPLC mass balance calculation (see Equation (3)), a potency of 0.877 (as 4-MPBX) was obtained. Since one molecule of 4-MPBX yields three molecules of 4-MPBA, the potency was calculated as 0.995 (as monomer) according to Equation (4). This potency value is consistent with the assay results (99.4%) obtained via an orthogonal qNMR method (see **Table 7**).

$$\begin{aligned} \text{Potency}(as4MPBX) &= (100 - \text{Imp}(\%area)) * (100 - TV(\%w)) * (1/100) \\ &= (100 - 12.230) * (100 - 0.0724) * 0.01 = 0.87707 \end{aligned} \quad (3)$$

where: *Imp* is the total HPLC impurities, and *TV* is % total volatiles obtained from duplicate measurements of loss of drying. This is supported by thermogravimetric analyses TGA and differential scanning calorimetry (DSC) data (see **Figure S1** and **Figure S2** in the supplementary material).

$$\begin{aligned} \text{Potency}(as4MPBA) &= \text{Potency}(asMPBX) * n * \left(\frac{MW1}{MWn} \right) \\ &= 0.87707 * n * 151.956 / (n * 133.941) \\ &= 0.87707 * 151.956 / 133.941 = 0.995 \end{aligned} \quad (4)$$

where: $n = 3$, *MW1* is molecular weight (151.956) of the monomer (4-MPBA), and *MWn* is molecular weight (3 x 133.941) of the trimer (4-MPBX).

5.6. Verification by an Orthogonal Method

The method was further verified via an orthogonal quantitative NMR (qNMR) method. The mean assay as monomer obtained by qNMR was 99.4%, while the mean assay as monomer by HPLC ($n = 12$) was 99.2%, representing a 0.2% difference (see **Table 7**). A difference of less than 2.0% between an HPLC method and another orthogonal method is considered acceptable. Refer to **Figure S5** and **Tables S10-S14** in the supplementary material for the qNMR experimental data.

Table 7. Assay by HPLC and qNMR methods.

HPLC ($n = 12$)	qNMR ($n = 2$)	Difference
99.2	99.4	-0.2

6. Conclusion

In conclusion, using 4-methoxyphenylboronic acid (4-MPBA) as a model compound, a dual-standard RP-HPLC-UV method suitable for the simultaneous identification, assay, and impurity analysis of phenylboronic acid (PBA) has been developed and qualified. This simple method provides a quality-control-friendly, single-sequence protocol that achieves baseline separation of 4-MPBA and its related impurities. Furthermore, it bypasses the requirement for a single-component primary BA reference standard, which can be difficult to obtain, by utilizing a readily available Bromo-surrogate for quantitation. Indeed, a similar method has been implemented for the analysis (ID, assay, and impurities) of a PBA-type intermediate used in an API for clinical trials.

Supplementary Online Material

Supplementary material is available free of charge via <https://figshare.com> (DIO: 10.6084/m9.figshare.31931391).

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Conflicts of Interest

The authors declare no conflicts of interest, financial or otherwise.

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Appendix

Table S1. HPLC conditions.

HPLC column	Ascentis® Express 90 Å ES-Cyano, 2.7 µm, 150 x 4.6mm, Sigma-Aldrich P/N 53492-U		
HPLC System	Agilent Technologies Infinity II 1260/1290 with PDA or equivalent		
MPA	0.025% TFA in Water		
MPB	0.025% TFA in MeOH		
PDA Wavelength	223 nm		
Column Temperature	35°C		
Sampler Temperature	ambient		
Injection Volume	10 µL		
Flow rate	0.8 mL/min		
Diluent	ACN/Water = 1:1 (v/v)		
Needle Wash	ACN/Water = 1:1 (v/v)		
Gradient	Time (min)	A%	B%
	0	86	14
	1.0	86	14
	22.0	20	80
	25.0	20	80
	25.1	86	14
	30.0	86	14
WSS	0.1 mg/mL of 4-MPBA and Bromo		
Nominal Assay Concentration	0.1 mg/mL		
Nominal Purity Concentration	1.0 mg/mL		

Table S2. S/N of three LOD injections.

LOD (0.00025 mg/mL)	S/N ¹			Minimum
	Inj-1	Inj-2	Inj-3	
Phenol	16	22	19	16
4-MPBA	13	16	12	12
Des-Br	13	19	16	13
Bromo	23	31	29	23
BisPh	14	16	15	14

Note: ¹Criteria: S/N ≥ 3 in all LOD injections (*n* = 3).

Table S3. Peak area and S/N of six LOQ injections.

LOQ (0.0005 mg/mL)	Phenol		4-MPBA		Des-Br		Bromo		BisPh	
	Area	S/N ¹	Area	S/N ¹	Area	S/N ¹	Area	S/N ¹	Area	S/N ¹
1	16.789	49	15.113	35	14.607	45	21.150	69	9.207	38

Continued

2	19.240	64	15.612	45	13.788	52	20.748	86	9.836	48
3	17.383	40	15.268	28	16.209	37	20.896	57	9.746	32
4	18.343	25	17.013	18	14.339	22	22.247	34	10.345	20
5	17.299	21*	14.931	16*	14.310	20*	20.143	31*	9.236	17*
6	16.505	48	14.592	35	14.586	45	20.355	70	9.288	37
Mean	17.593	NA	15.422	NA	14.640	NA	20.923	NA	9.610	NA
%RSD ²	5.8	NA	5.5	NA	5.6	NA	3.6	NA	4.7	NA

Note: *minimal S/N; ¹Criteria: S/N \geq 10 in all LOQ injections ($n = 6$); ²Criteria: %RSD \leq 20% ($n = 6$).

Table S4. System suitability (Resolution \geq 1.5, Peak tailing 0.8 - 1.2).

WSS Injection #	4-MPBA RT (min)	4-MPBA Peak Area	Bromo RT (min)	4-Bromo Peak Area
1	8.816	3155.918	15.994	3980.226
2	8.813	3156.236	15.993	3985.799
3	8.812	3156.074	15.996	3982.341
4	8.813	3160.781	15.995	3988.001
5	8.815	3152.564	15.994	3977.131
6	8.812	3155.138	15.995	3984.942
Bracket #1	8.817	3147.566	15.996	3982.826
Bracket #2	8.812	3162.410	15.994	3951.284
Average ($n = 6$)	8.814	3156.119	15.995	3983.073
Std Dev ($n = 6$)	0.0	2.7	0.0	4.0
%RSD ¹ ($n = 6$)	0.02	0.08	0.01	0.10
Average (all)	8.814	3155.836	15.995	3979.069
Std Dev (all)	0.0	4.6	0.0	11.7
%RSD ¹ (all)	0.02	0.15	0.01	0.29

Note: ¹Criteria: %RSD \leq 2.0% ($n = 6$ or ≥ 7).

Table S5. Accuracy and precision of assay.

Level	Nominal Concentration (mg/mL)	Replicates	Recovery ¹ (%)	Mean Recovery (%)	%RSD ²
50%	0.05	1	99.45	99.2	0.4
		2	98.82		
		3	99.40		
100%	0.10	1	98.02	98.6	0.6
		2	99.16		
		3	98.24		
		4	98.20		
		5	99.45		
		6	98.74		

Continued

			1	98.34		
150%	0.15		2	98.05	98.2	0.2
			3	98.30		

Note: ¹Criteria: 98.0-102.0% for each individual preparation. ²Criteria: %RSD \leq 2.0% ($n = 3$ or 6).

Table S6. Intermediate precision of assay (at 100% level, 0.1 mg/mL).

Replicates	Recovery ¹ (%) Agilent 1290	%RSD ² (n = 6)	Recovery ¹ (%) Agilent 1260	%RSD (n = 6)	Mean Assay (%, n = 12)	%RSD ² (n = 12)
1	101.26		98.02			
2	98.91		99.16			
3	98.07	1.2	98.24	0.6	99.2	1.1
4	100.03		98.20			
5	100.70		99.45			
6	99.73		98.74			

Note: ¹Criteria: 98.0-102.0%. ²Criteria: %RSD \leq 2.0% ($n = 6$ or 12).

Table S7. Accuracy¹ and precision² of impurities.

Compound	Replicates	0.05%	1%	2%
Phenol	1	104.16	101.70	102.17
	2	101.81	104.10	100.25
	3	100.60	103.52	103.53
	4	NA	102.31	NA
	5	NA	106.34	NA
	6	NA	101.68	NA
	Average (%)	102.2	103.3	102.0
	%RSD	1.8	1.7	1.6
Des-Br	1	108.65	108.39	109.30
	2	106.99	111.61	106.47
	3	101.35	110.22	110.49
	4	NA	109.14	NA
	5	NA	113.97	NA
	6	NA	108.79	NA
	Average (%)	105.7	110.4	108.8
	%RSD	3.6	1.9	1.9
Bromo	1	102.98	102.12	103.34
	2	107.49	104.91	100.95
	3	108.98	104.24	104.30

Continued

	4	NA	102.71	NA
	5	NA	107.59	NA
	6	NA	102.70	NA
	Average (%)	106.5	104.0	102.9
	%RSD	2.9	2.0	1.7
BisPh	1	96.67	106.51	107.61
	2	98.75	108.88	104.81
	3	93.14	108.51	108.18
	4	NA	107.63	NA
	5	NA	111.43	NA
	6	NA	107.41	NA
	Average (%)	96.2	108.4	106.9
	%RSD	2.9	1.6	1.7

Note: ¹Criteria: 80-120% for each individual impurity \geq LOQ and \leq 0.15%; 85-115% for each individual impurity $>$ 0.15%. ²Criteria: %RSD \leq 20% ($n = 6$) for each individual impurity \geq LOQ and \leq 0.15%; %RSD \leq 15% ($n = 6$) for each individual impurity $>$ 0.15%.

Table S8. Intermediate precision of impurities (at 1% level).

Compound	Replicates	Recovery ¹ (%) Agilent 1290	%RSD ² (n = 6)	Recovery ¹ (%) Agilent 1260	%RSD ² (n = 6)	%RSD ² (n = 12)
Phenol	1	101.70	1.7	95.43	1.2	5.0
	2	104.10		92.61		
	3	103.52		94.68		
	4	102.31		94.47		
	5	106.34		94.83		
	6	101.68		93.05		
Des-Br	1	108.39	1.9	102.47	0.9	4.6
	2	111.61		99.91		
	3	110.22		102.01		
	4	109.14		102.01		
	5	113.97		101.85		
	6	108.79		101.11		
Bromo	1	102.12	2.0	95.73	1.1	5.3
	2	104.91		92.94		
	3	104.24		95.21		
	4	102.71		94.70		
	5	107.59		95.01		
	6	102.70		93.45		

Continued

BisPh	1	106.51		97.54		
	2	108.88		94.81		
	3	108.51		96.79		
	4	107.63	1.6	96.47	1.2	6.3
	5	111.43		97.08		
	6	107.41		95.06		

Note: ¹Criteria: 85%-115% for each individual impurity for each individual impurity > 0.15%. ²Criteria: %RSD ≤ 15% ($n = 6$ or 12) for each individual impurity > 0.15%.

Table S9. Solutions stability at ambient temperature.

Solution	Compound	T = 0	24 h	Recovery (%), 24 h	Criteria (%)
LOQ	4-MPBA	15.475	14.117	91.2	80 - 120
	Bromo	21.616	20.823	96.3	
Resolution	Phenol	744.921	746.119	100.2	85 - 115
	4-MPBA	26703.948	26708.741	100.0	98.0 - 102.0
	Des-Br	689.547	685.976	99.5	
	Bromo	818.321	801.003	97.9	85 - 115
	BisPh	452.57	451.468	99.8	
WSS	4-MPBA	3136.906	3189.6865	101.7	
	Bromo	4094.605	4123.16	100.7	98.0 - 102.0
Assay	4-MPBA	99.7	99.7	100.0	
Impurity	Des-Br	0.0740	0.0716	96.7	80 - 120
	RRT1.29*	<0.05%	<0.05%	NA	N/A

Note: *An unknown/unspecified impurity.

***q*NMR in duplicates.** Briefly, ~20 - 40 mg of each sample (4-MPBA) and internal standard (IS, 1,3,5-trimethoxybenzene) were weighed accurately into a 4 mL vial, to this vial ~0.75 - 1.5 mL of DMSO-d₆ was added. The vial was capped and vortex for ~30 seconds until complete dissolution, then 20 μL of D₂O was added and vortex for ~30 seconds. The resulting solution was transferred into an NMR tube. The ¹H NMR data was collected on a Bruker Ascend™ 400 and analyzed with ACD/Spectrus Processor 2020.2.1. The mean assay obtained by *q*NMR method is 99.4 (% w/w), see **Tables S10-S14** for weights and peak integrals of sample and IS, and assay calculation. See **Figure S5** for ¹H NMR Spectra of *q*NMR.

Table S10. Peaks integrals of sample and IS (*q*-NMR1).

No.	(ppm)	Non-Negative Value (Integral)	Hydrogen Numbers	Normalized Integral
1	[7.57 - 7.84]	1.9920	2	0.9960
2	[6.75 - 7.05]	2.0000	2	1.0000

Continued

			I (sample, average)	0.9980
IS	[5.92 - 6.25]	3.0418	3	1.0139
			I (IS, average)	1.0139

Table S11. Weights of samples and IS, potency (q NMR1).

Sample Molecular Weight (g/mol)	4-MPBA	151.956
Internal Standard (IS) Molecular Weight (g/mol)	1,3,5-trimethoxybenzene	168.19
Weight (mg)	Sample	20.02
	IS	22.42
*Potency (%)		99.58%

Note: *Potency = $(1.0139/0.9980) \times (151.956/168.19) \times (22.42/20.02) = 99.58\% = 0.9958$.

Table S12. Peaks integrals of sample and IS (q NMR2).

No.	(ppm)	Non-Negative Value	Hydrogen Numbers	Normalized Integral
1	[7.59 - 7.83]	1.9945	2	0.9973
2	[6.72 - 7.04]	2.0000	2	1.0000
			I (sample, average)	0.9986
IS	[5.91 - 6.25]	2.5039	3	0.8346
			I (IS, average)	0.8346

Table S13. Weights of samples and IS, potency (q NMR2).

Sample Molecular Weight (g/mol)	4-MPBA	151.956
Internal Standard (IS) Molecular Weight (g/mol)	1,3,5-trimethoxybenzene	168.19
Weight (mg)	Sample	38.09
	IS	34.99
*Potency (%)		99.29%

Note: *Potency = $(0.9986/0.8346) \times (151.956/168.19) \times (34.99/38.09) = 99.29\% = 0.9929$.

Table S14. Assay by q -NMR.

Assay by q -NMR	q NMR1	q NMR2	Mean
% w/w	99.58	99.29	99.4

DSC, TGA, and 1 H-NMR experiments:

Differential scanning calorimetry (DSC) analyses were performed on a TA instrument DSC Q2000. Thermogravimetric analyses (TGA) were performed on a TA instrument TGA 5500. 1 H NMR analyses were performed on a Bruker Ascend 400.

Based on the DSC analysis (See **Figure S1**), 4-MPBA monomer turns into n-

oligomer (s), through de-solvation and dehydration $\sim 75^\circ\text{C} - 140^\circ\text{C}$. Meanwhile, n-oligomers will turn into a more thermodynamic stable trimer. The crystalline trimer is then melted above 200°C . The de-solvation and dehydration processes are also observed in the TGA analyses (see **Figure S2**).

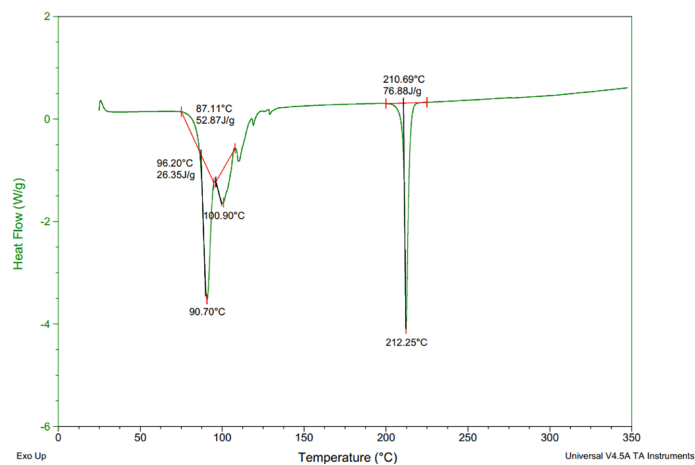


Figure S1. DSC of 4-MPBA.

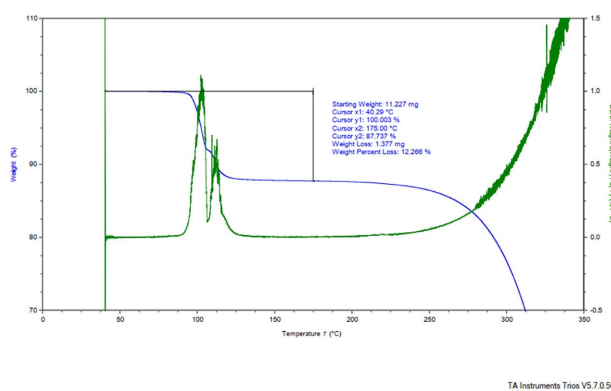
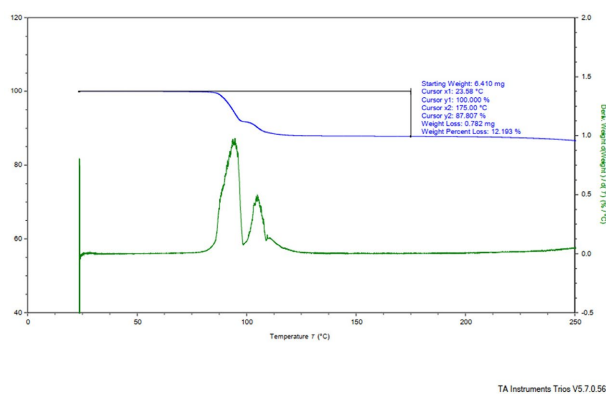


Figure S2. TGA of 4-MPBA (Duplicates).

$^1\text{H-NMR}$ spectrum of the commercially available 4-MPBA in anhydrous DMSO- d_6 (See **Figure S3(a)** and **Figure S4(a)**) shows the major component of commercially available 4-methoxyphenyl boronic acid is the monomer ($\sim 90\%$), with about 10% of n-oligomers. The same sample was quenched with 20 μL of D_2O , all the n-oligomers turn into the monomer, see **Figure S3(b)** and **Figure S4(b)**. The oligomer peak always on the left of the corresponding peak of the monomer.

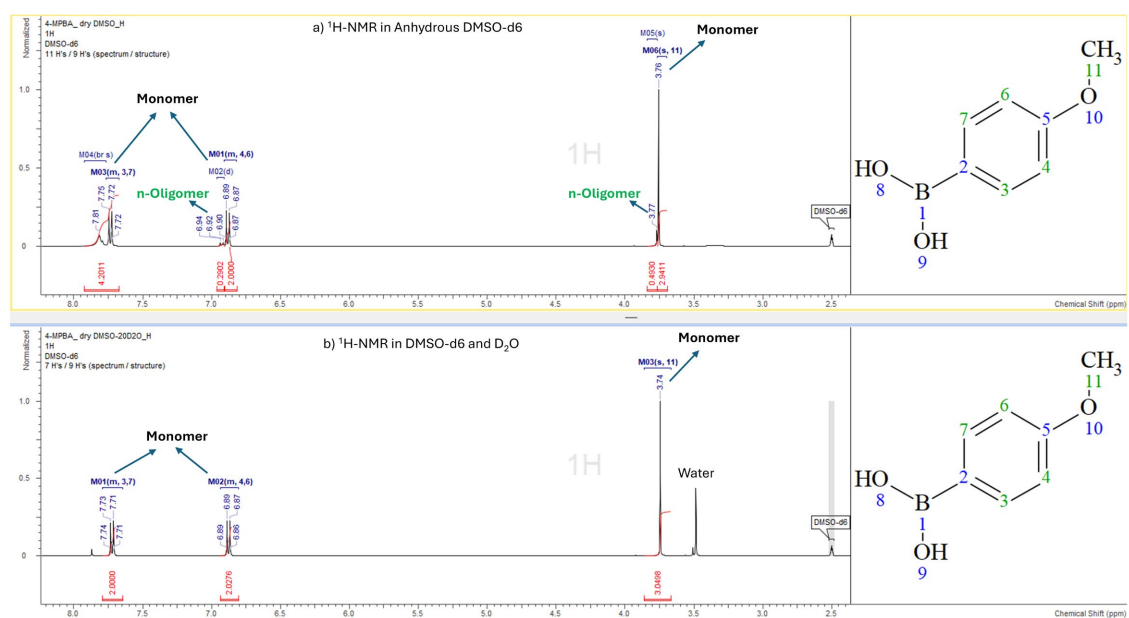


Figure S3. $^1\text{H-NMR}$ spectra of 4-MPBA in anhydrous DMSO- d_6 (a) and in DMSO- d_6 and D_2O (b).

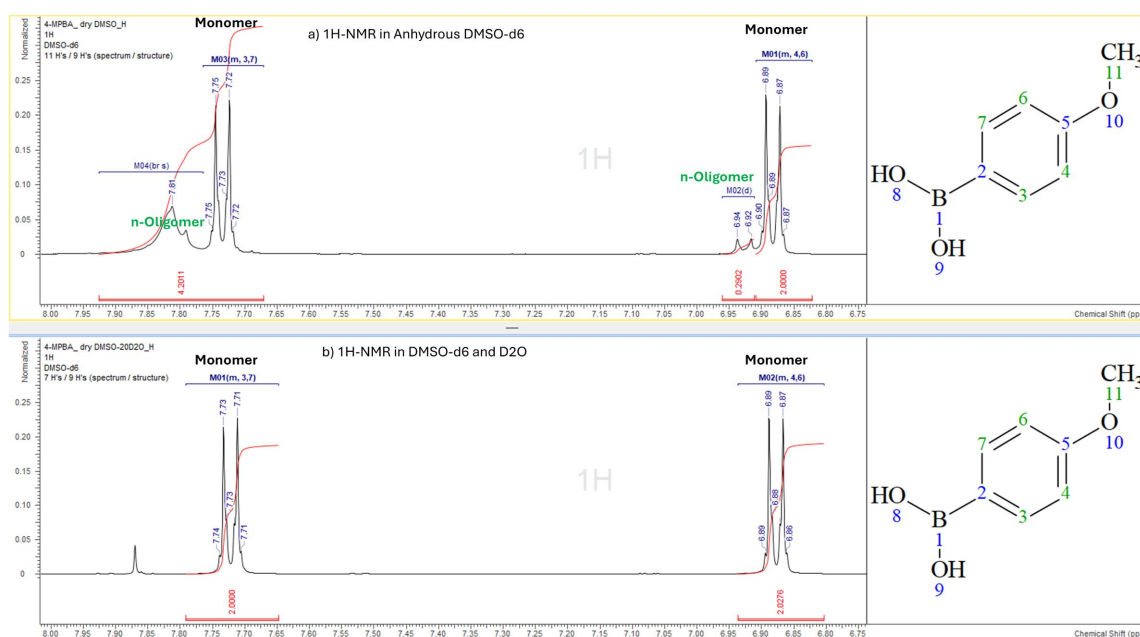


Figure S4. Expanded $^1\text{H-NMR}$ spectra of 4-MPBA in anhydrous DMSO- d_6 (a) and in DMSO- d_6 and D_2O (b).

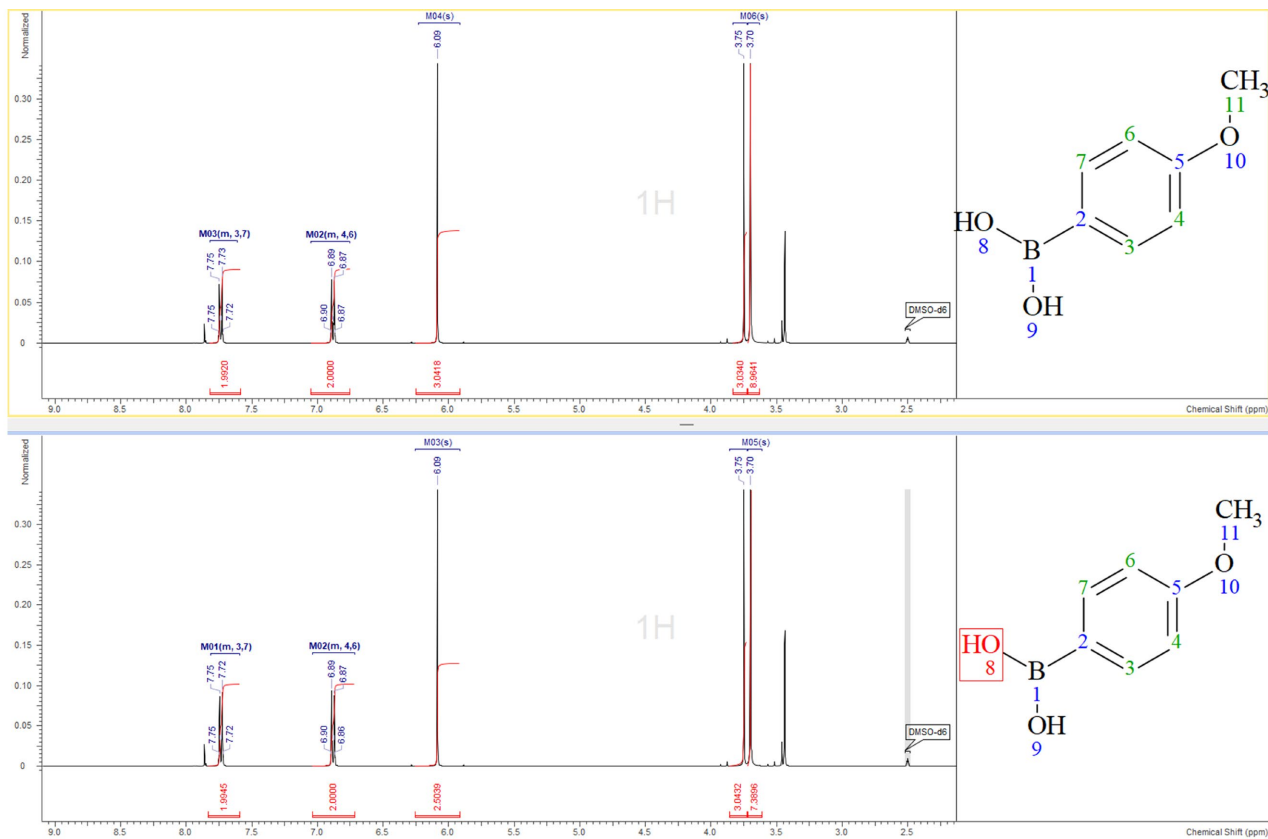


Figure S5. ¹H NMR spectra of q-NMR (Duplicates).

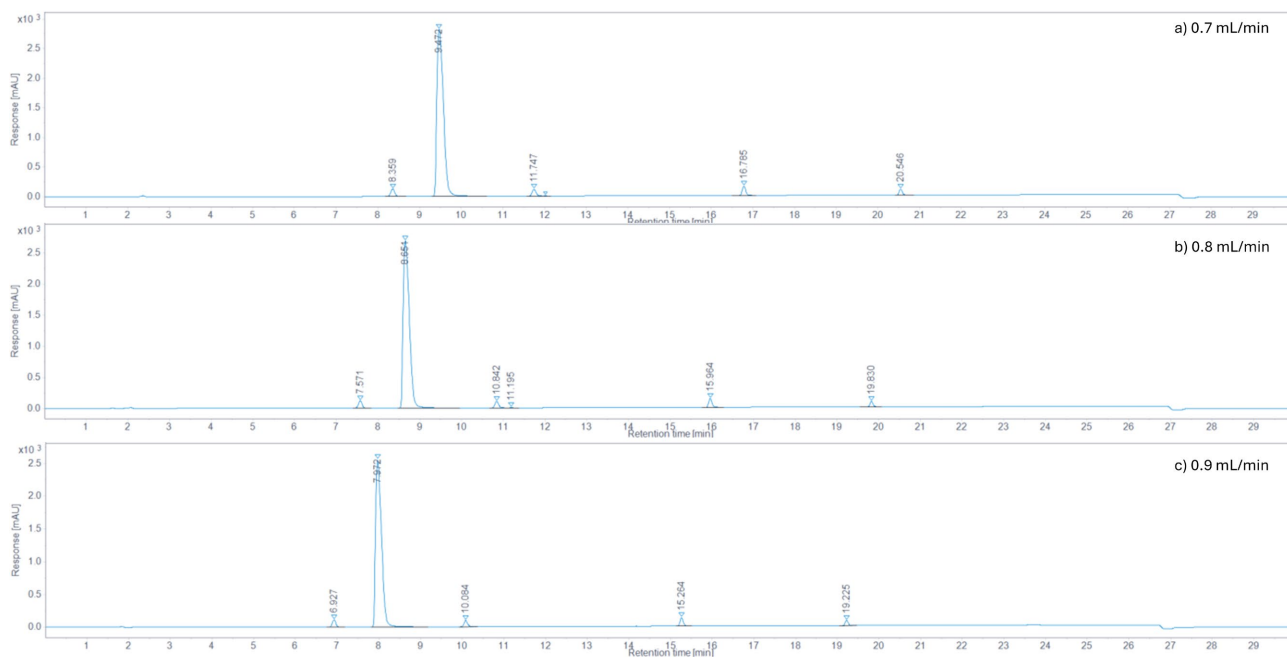


Figure S6. Chromatograms of resolution solution at different flow rates.

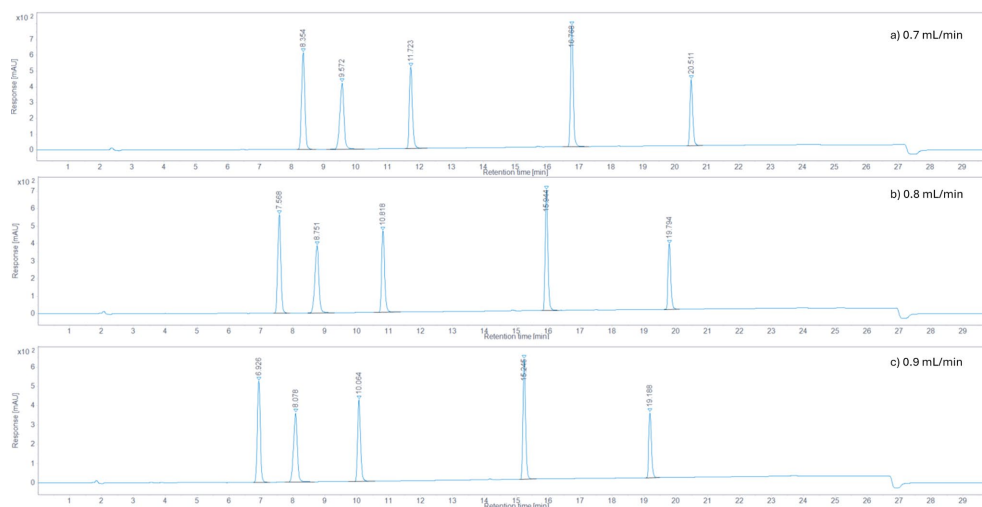


Figure S7. Chromatograms of 0.1 mg/mL mixture at different flow rates.

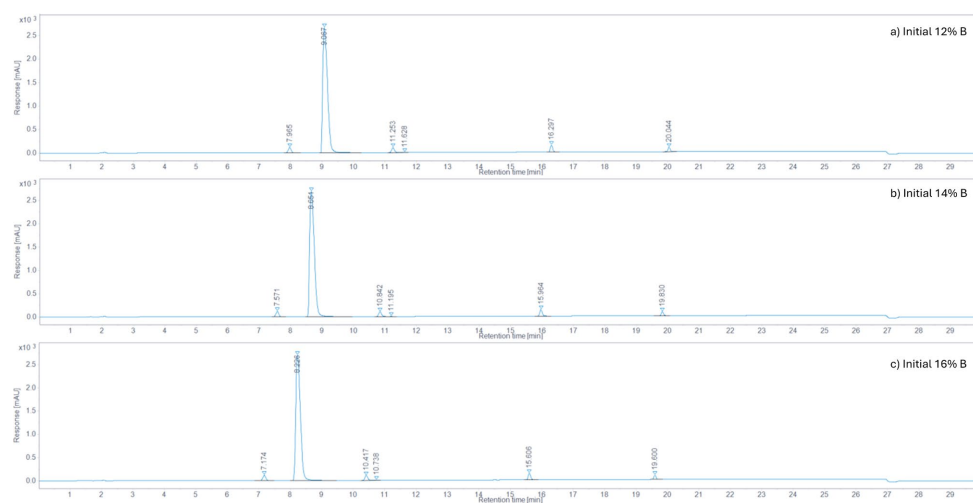


Figure S8. Chromatograms of resolution solution with different initial %MPB (12%, 14%, 16%).

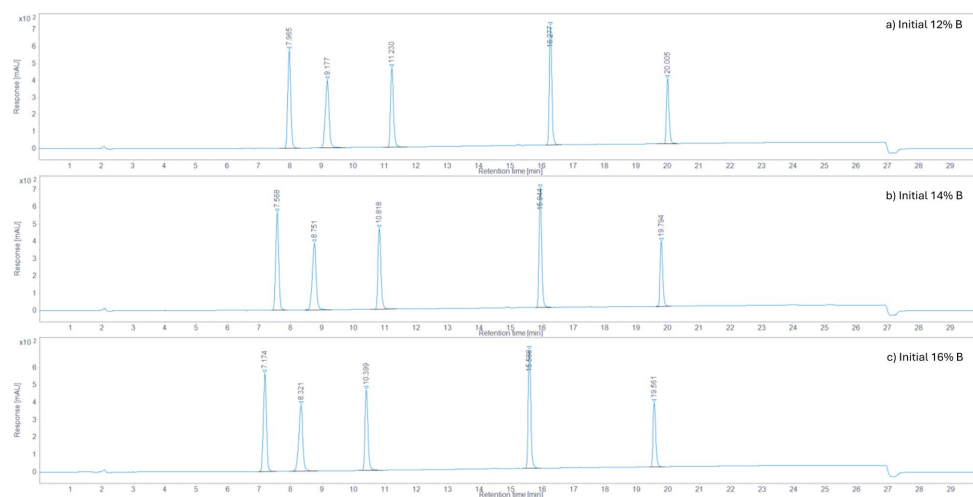


Figure S9. Chromatograms of 0.1 mg/mL mixture with different initial %MPB (12%, 14%, 16%).

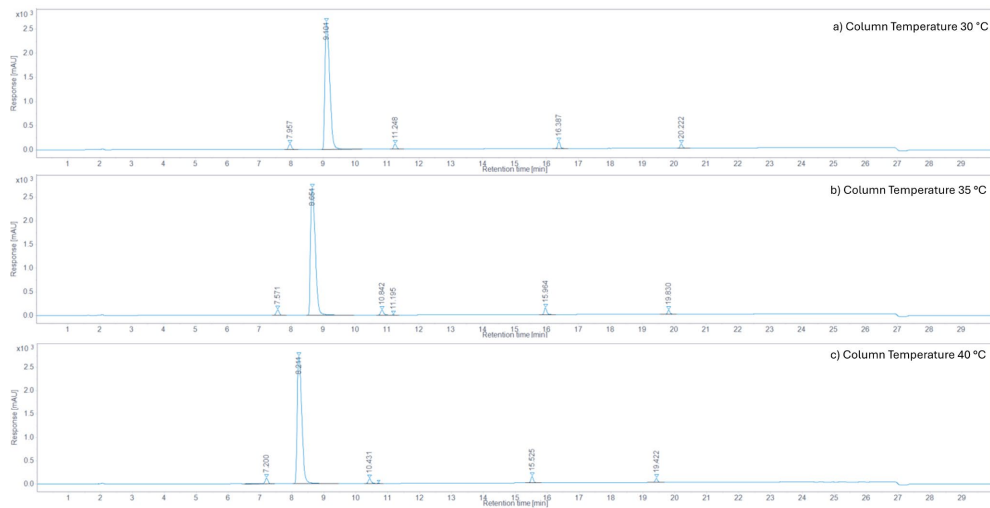


Figure S10. Chromatograms of resolution solution at different temperatures.

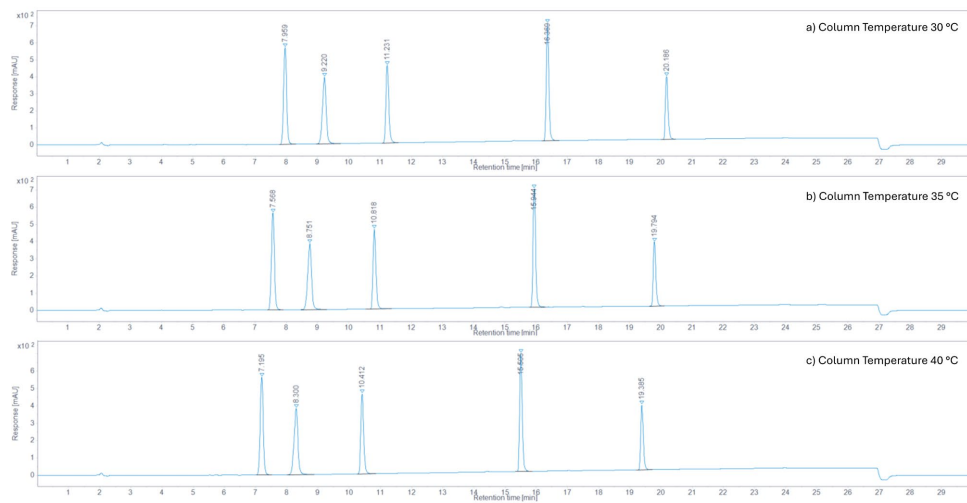


Figure S11. Chromatograms of 0.1 mg/mL mixture at different temperatures.

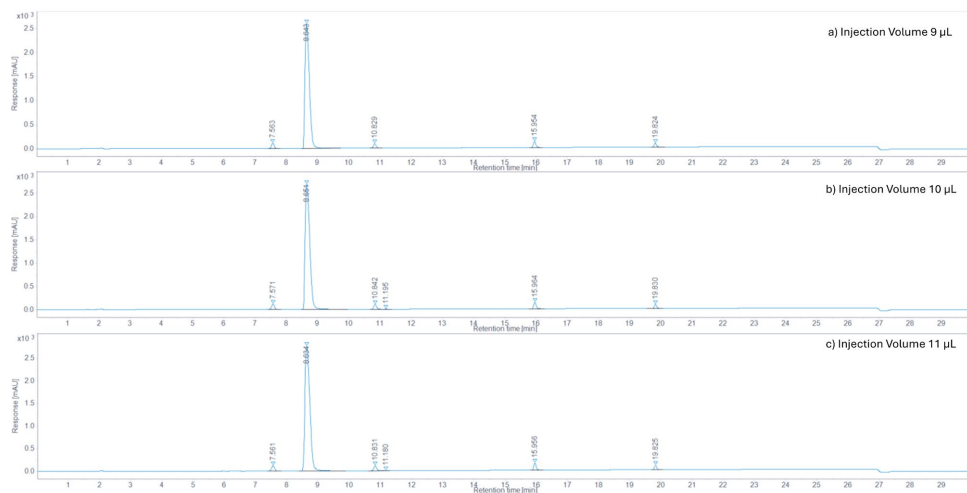


Figure S12. Chromatograms of resolution solution with different injection volumes (9, 10, 11 µL).

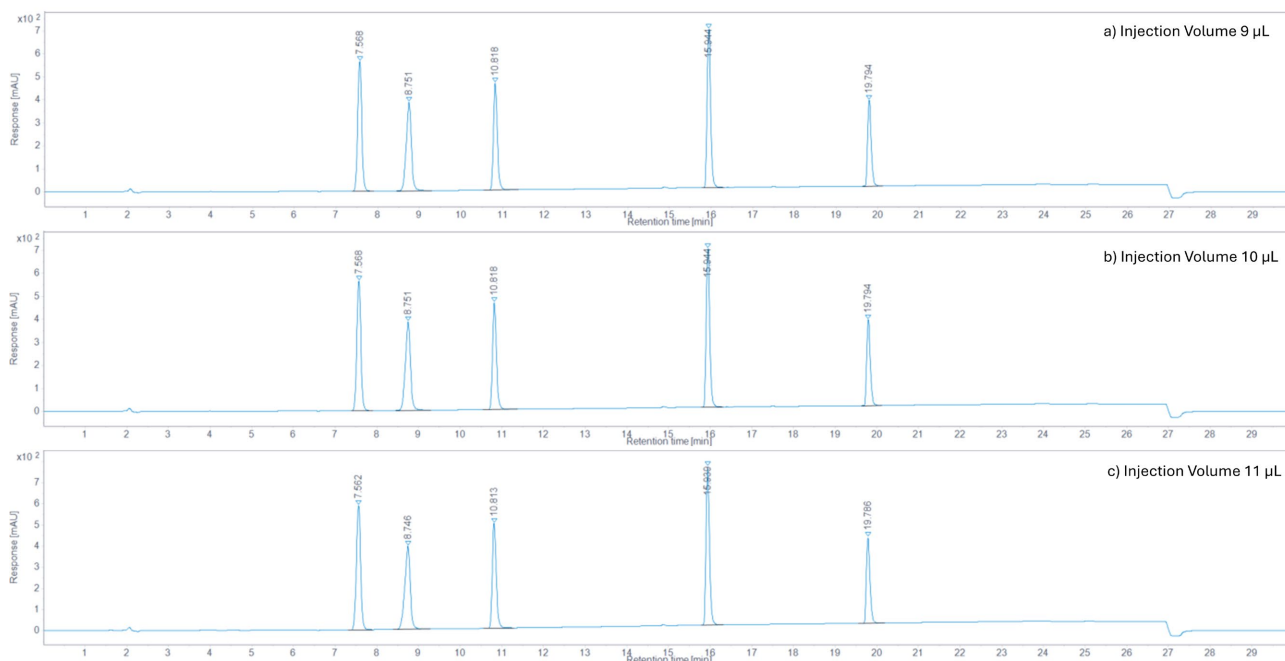


Figure S13. Chromatograms of 0.1 mg/mL mixture with different injection volumes (9, 10, 11 µL).

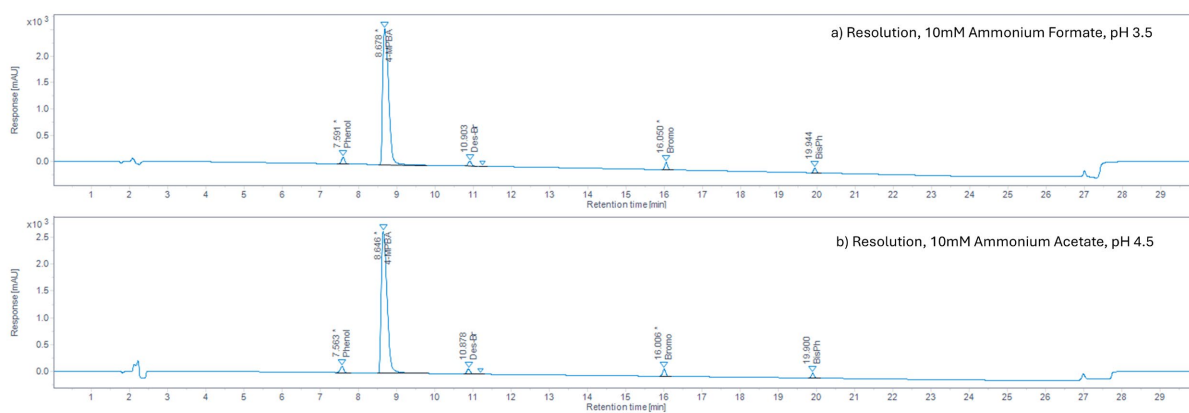


Figure S14. Chromatograms of resolution solution using ammonium formate/acetate as MPA buffer.

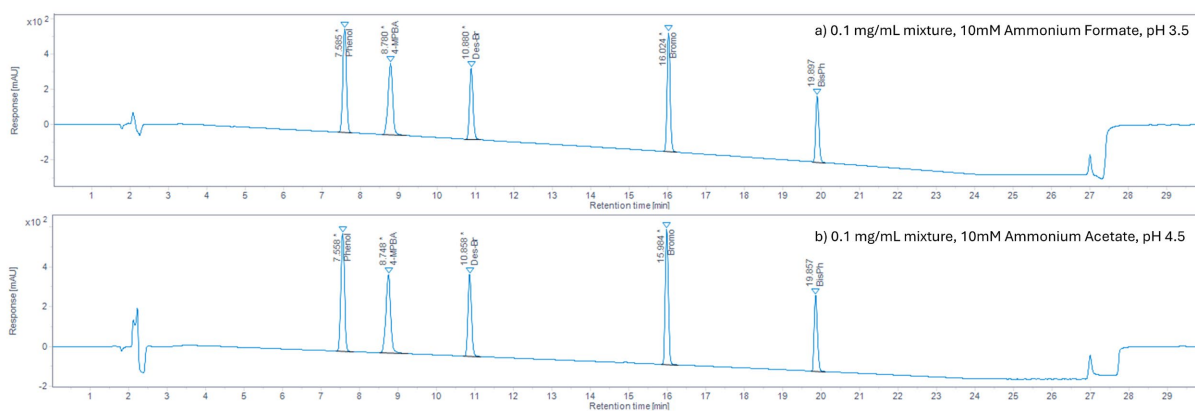


Figure S15. Chromatograms of 0.1 mg/mL mixture using ammonium formate/acetate as MPA buffer.