

## Background

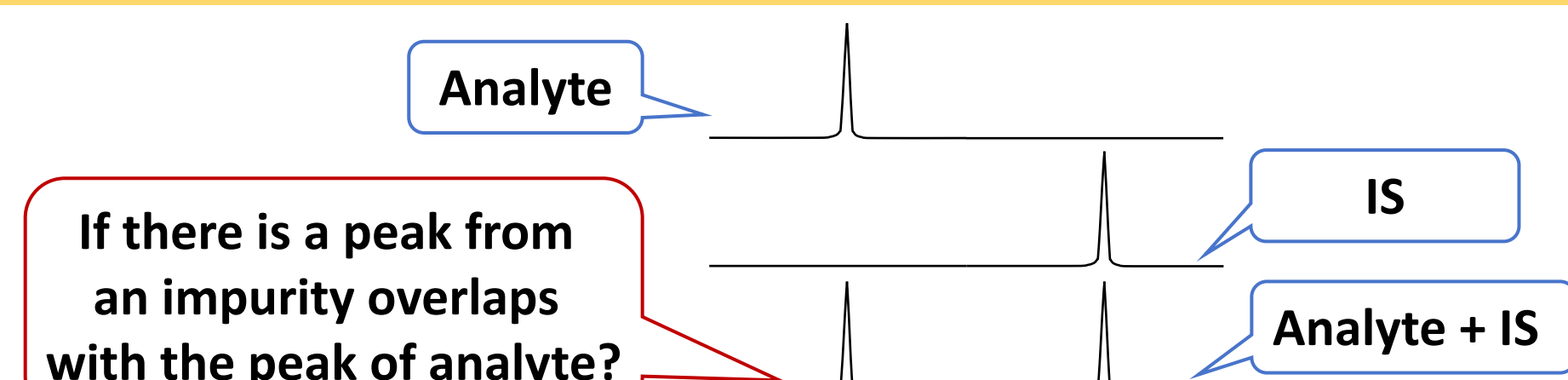
### Principle and Challenge of qNMR

**QNMR is a universal direct method for organic compound purity.**

$$P_x = \frac{I_x}{I_{std}} \frac{N_{std}}{N_x} \frac{M_x}{M_{std}} \frac{m_{std}}{m} P_{std}$$

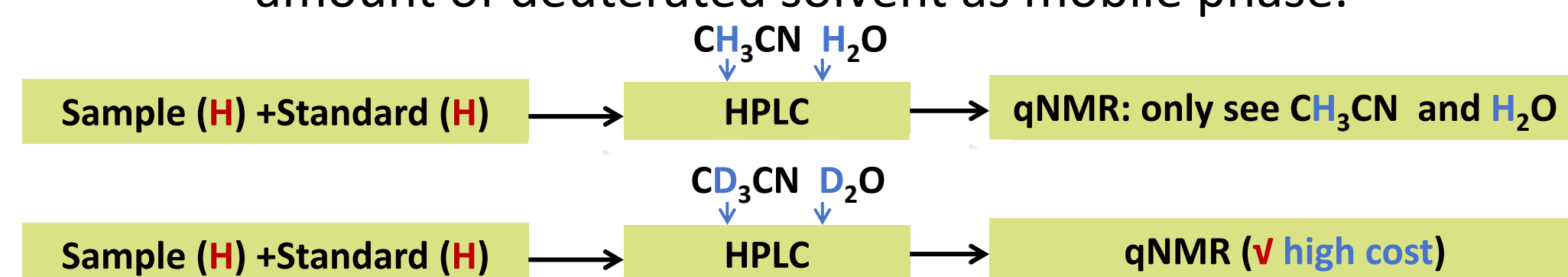
X = Analyte; Std = Internal Standard (IS); I = Integral area; M = molar mass; m = mass; P = purity in %, g/g  
N = Number of spins belonging to the respective molecular unit

**Require: The peaks for quantitation isolate from that of impurities**



**Major challenge for low-purity compound analysis**  
Especially for larger molecules (peptides/proteins)

- Direct HPLC-qNMR can remove impurities, but needs a large amount of deuterated solvent as mobile phase.



**Aim : Solve the problem of qNMR to improve its accuracy.**  
**Extend application range of qNMR to large molecules**

## Objectives

A series of novel qNMR approaches have been developed by NIM to mitigate biases associated with chemical interferences.

| Method                                             | Application Scope                                                     | Challenge                     | Sample                       |
|----------------------------------------------------|-----------------------------------------------------------------------|-------------------------------|------------------------------|
| Direct qNMR <sup>1</sup>                           | Identifiable impurities                                               | Qualitative capability        | Valine                       |
| H-D exchange qNMR <sup>1</sup>                     | Peptides or proteins containing H, F, Y, W                            | Structural related impurities | hCG-T5                       |
| HPLC-qNMR with two signal suppression <sup>2</sup> |                                                                       | Signal distortion             | Avermectin B1a               |
| HPLC-qNMR-HPLC <sup>3,4</sup>                      | The NMR signals of impurities highly overlap with the main components | Purification capability       | C-peptide Insulin            |
| ISRC-HPLC-qNMR <sup>5</sup>                        |                                                                       |                               | Avermectins                  |
| ISC-HPLC-qNMR <sup>6,7</sup>                       |                                                                       | HPLC separation               | Pyributicarb Oxytetracycline |

### SI Units

**Primary method**  
(Coulometry or Freezing point depression method...)

**Internal Standard CRMs**  
(DMSO<sub>2</sub>, BA, KHP, MA, AK...)

**Novel qNMR methods**

**Almost all organic compounds**

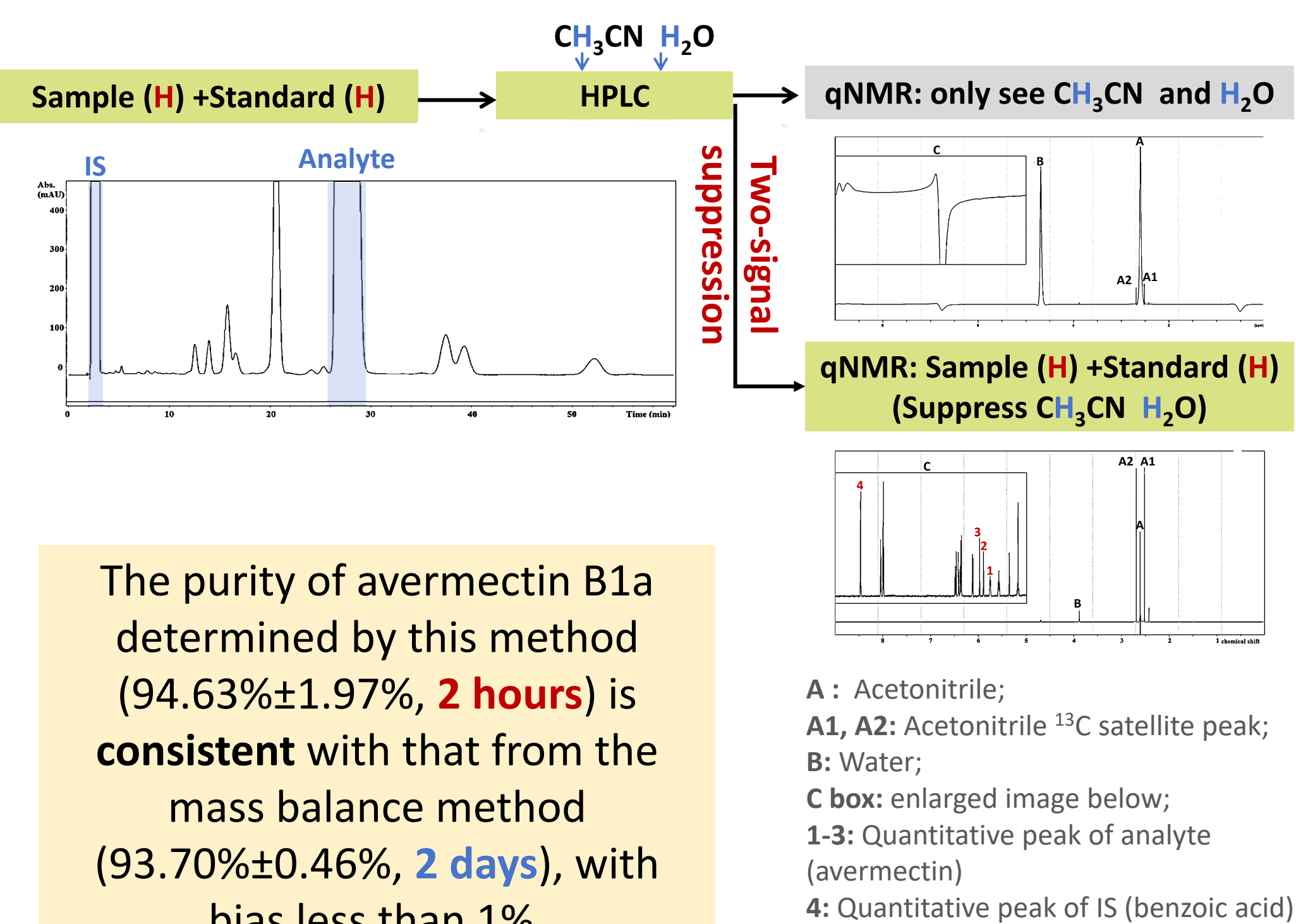
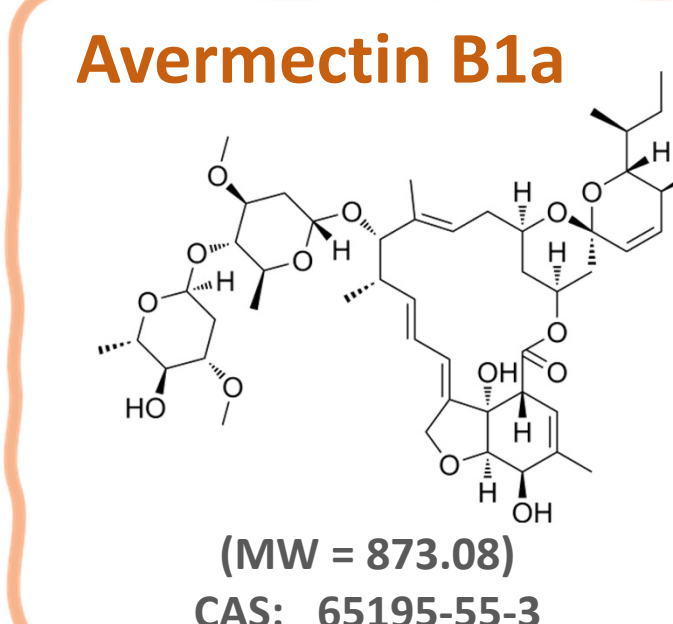
Internal Standard CRMs for <sup>1</sup>H qNMR developed by NIM

| No.       | Name                                  | δ (×10 <sup>-6</sup> ) |
|-----------|---------------------------------------|------------------------|
| GBW06119  | Dimethyl sulfone (DMSO <sub>2</sub> ) | 3.0                    |
| GBW06120  | Ethylparaben (EP)                     | 8.0, 6.9, 4.4, 1.4     |
| GBW 06148 | Benzoic acid (BA)                     | 8.0-8.1, 7.6, 7.4-7.5  |
| GBW06149  | Potassium hydrogen phthalate (KHP)    | 7.0-8.3                |
| GBW06150  | Maleic acid (MA)                      | 6.3                    |
| GBW06151  | Acesulfame potassium (AK)             | 5.3, 1.9               |

<https://www.ncrm.org.cn>

## HPLC-qNMR with two-signal suppression

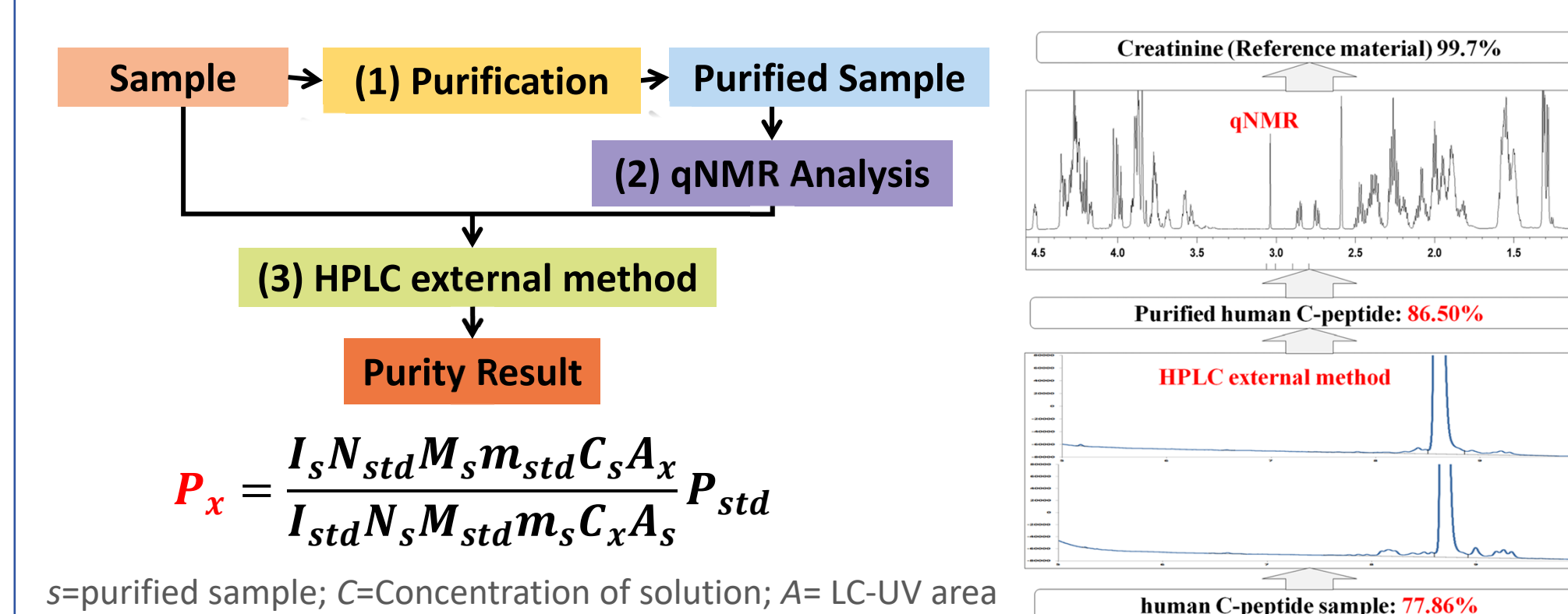
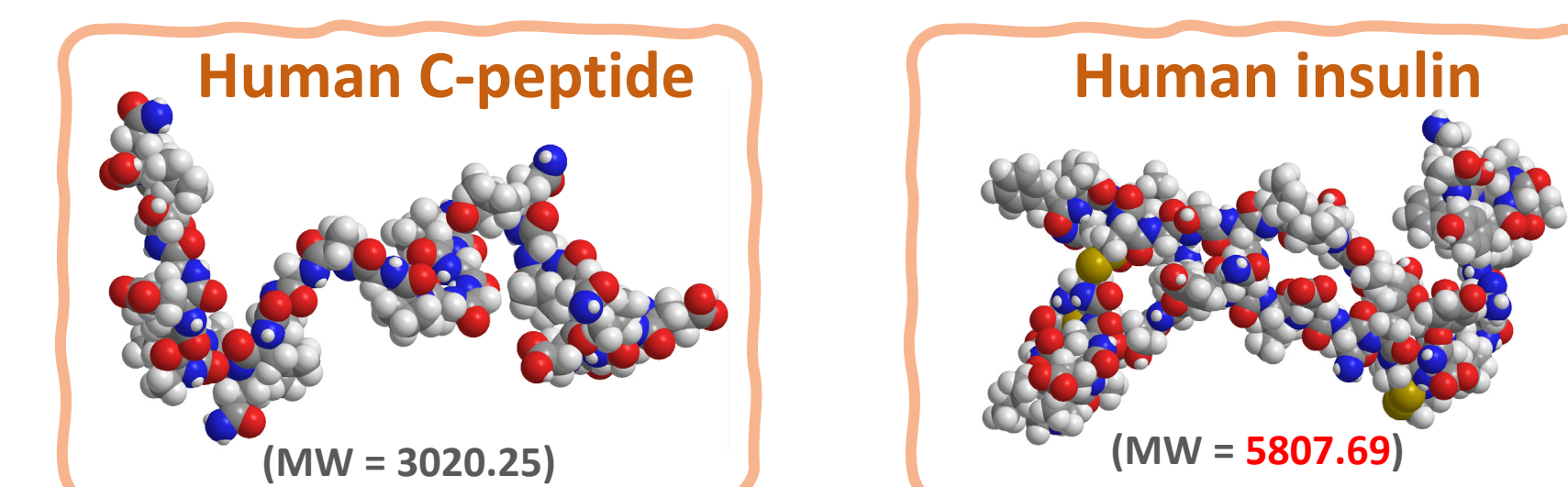
Avermectins is a natural complex pesticide. Non-deuterated solvents were used as the HPLC mobile phase, and only the eluents containing **avermectin B1a** and IS were gathered and scanned by qNMR, avoiding the interference of other avermectins.



**Challenge: Signal distortion**

## HPLC-qNMR-HPLC

Peptides are key biomarkers and pharmaceuticals. The HPLC-qNMR-HPLC was proposed for **peptide purity determination**, removing the overlapped peaks from impurities by generating a high purity calibration standard from the original sample.

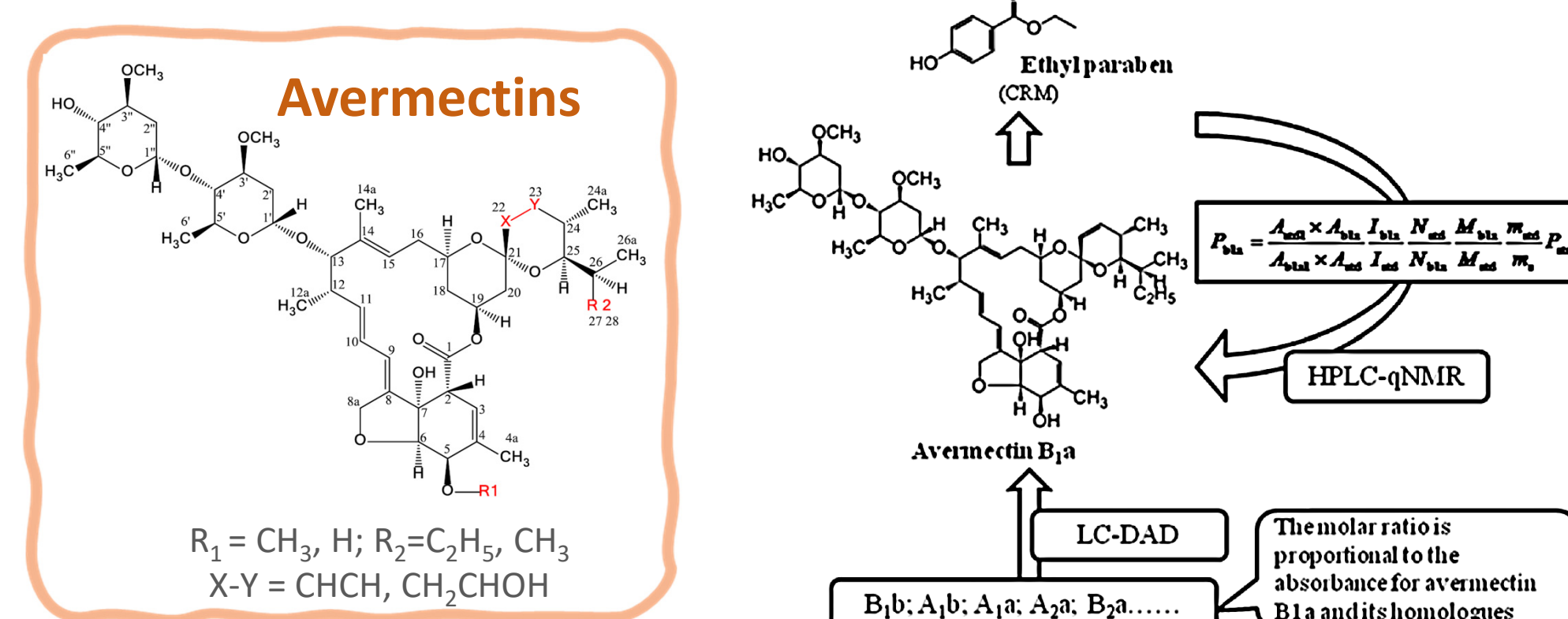
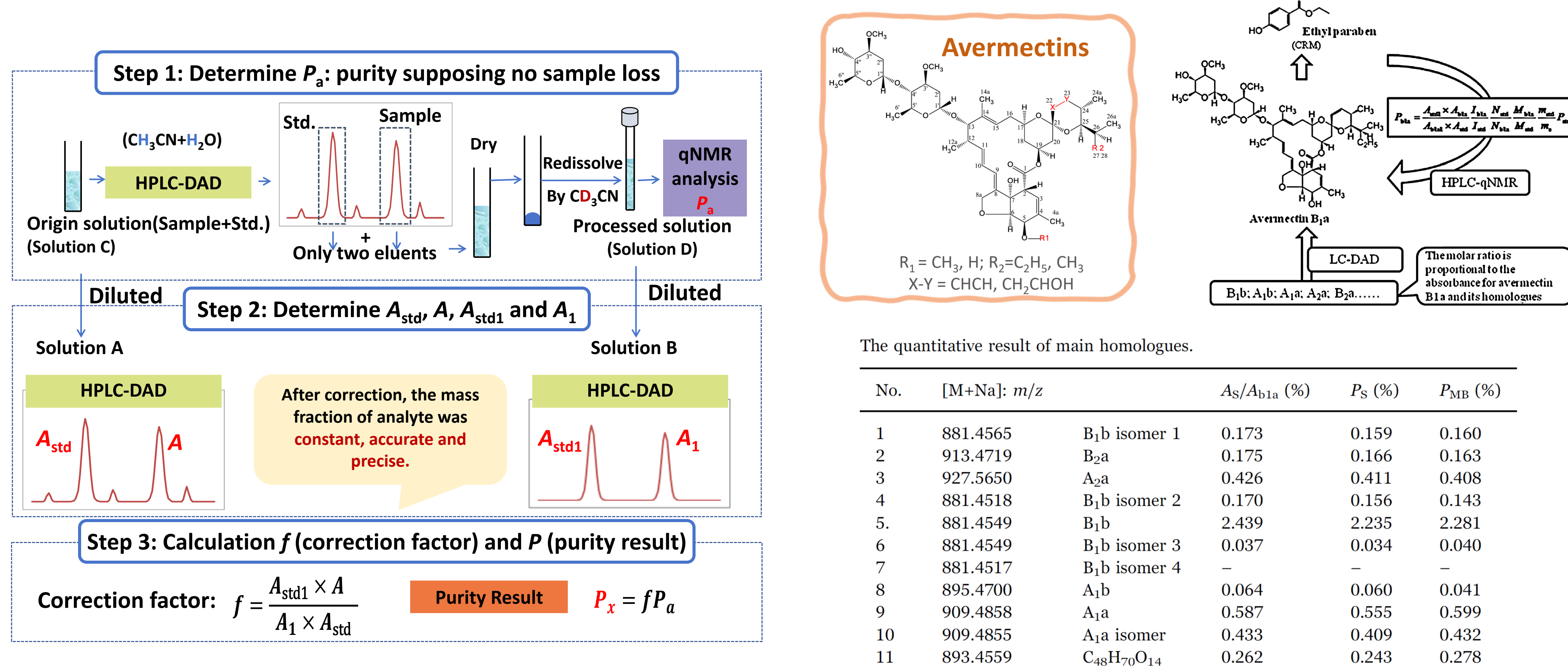


The results agreed with isotope dilution mass spectrometry (**IDMS**), overcoming **qNMR** measurement challenges of peptides.

**Challenge: Purification capability**

## ISRC-HPLC-qNMR

The **ISRC** (internal standard recovery correction)-**HPLC-NMR** method introduced a **recovery correction factor (f)** based on the HPLC area ratios between the analyte and IS before and after the process, ensuring the result accuracy **despite the sample loss** during the process or poor resolution happened in the HPLC separation stage.

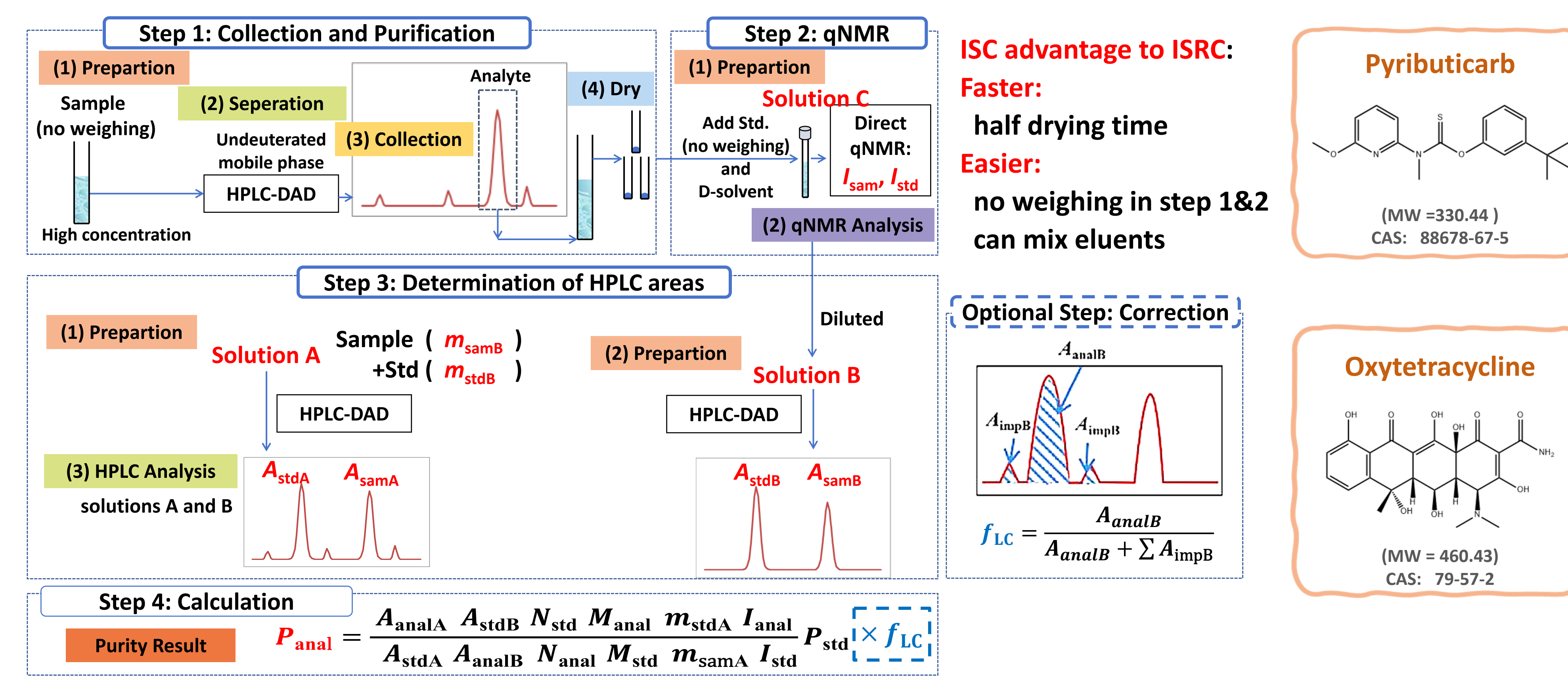


The quantitative result of main homologues.

| No. | [M+Na] <sup>+</sup> m/z |                                                 | $A_s/A_{B1a}$ (%) | $P_s$ (%) | $P_{MB}$ (%) |
|-----|-------------------------|-------------------------------------------------|-------------------|-----------|--------------|
| 1   | 881.4565                | B <sub>1</sub> b isomer 1                       | 0.173             | 0.159     | 0.160        |
| 2   | 913.4719                | B <sub>2</sub> a                                | 0.175             | 0.166     | 0.163        |
| 3   | 927.5650                | A <sub>2</sub> a                                | 0.426             | 0.411     | 0.408        |
| 4   | 881.4518                | B <sub>1</sub> b isomer 2                       | 0.170             | 0.156     | 0.143        |
| 5   | 881.4549                | B <sub>1</sub> b                                | 2.439             | 2.235     | 2.281        |
| 6   | 881.4549                | B <sub>1</sub> b isomer 3                       | 0.037             | 0.034     | 0.040        |
| 7   | 881.4517                | B <sub>1</sub> b isomer 4                       | —                 | —         | —            |
| 8   | 895.4700                | A <sub>1</sub> b                                | 0.064             | 0.060     | 0.041        |
| 9   | 909.4858                | A <sub>1</sub> a                                | 0.587             | 0.555     | 0.599        |
| 10  | 909.4855                | A <sub>1</sub> a isomer                         | 0.433             | 0.409     | 0.432        |
| 11  | 893.4559                | C <sub>48</sub> H <sub>70</sub> O <sub>14</sub> | 0.262             | 0.243     | 0.278        |

## ISC-HPLC-qNMR

The internal standard correction (ISC)-HPLC-qNMR method was proposed for **time-saving and more controllable sample preparation**. The **post-collection purity correction factor ( $f_{LC}$ )** for ISC-HPLC-qNMR was recently proposed to extend the applicability to the samples where HPLC cannot purify low-purity complex compounds to 100%.



**Accurate:** Even NMR peaks are unresolved, or HPLC peaks are partial resolved; **Low cost:** Only common solvent as mobile phase  
**Improve qNMR method:** Overcome the impact by highly similar impurity; **Simple operation:** Only eluate of analyte collected.