Chromatography Solutions

# Selective Detection HPLC Assays via In-Column Derivatisation

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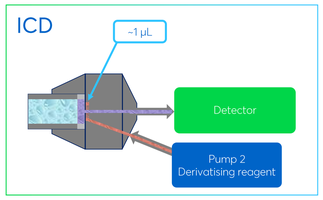
## Background

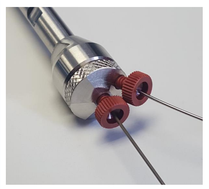
- Selective detection assays via HPLC post-column derivatization (PCD) have many applications: amino acid, antioxidant and phenolic analysis.
- Advantages over pre-column derivatization include:
  - Reduced sample manipulation.
  - Ability to work with less stable derivatization products.
- For amino acid analysis using OPA, PCD requires an in-line reactor which adds significant dead volume:
  - This significantly reduces the chromatographic performance.
- This poster investigates an alternative approach, In-column derivatisation (ICD) to overcome this for amino acid analyses.
  - In ICD, reagent/eluent mixing is initiated at the outlet frit, providing enhanced mixing.
  - Use of reactor eliminated, improving chromatographic performance.
- Application of ICD is demonstrated for authentication of coffee via the antioxidant fingerprint, which is complex and difficult to imitate.

## ICD Approach

- **PCD Setup:**
- More complex
- Reactor, tee and tubing introduce excessive dead column
- Degrades peak efficiency


- **ICD Setup:**
- Simplified setup
- Reaction occurs at column frit
- Dead volume significantly reduced



## Interlaboratory feedback

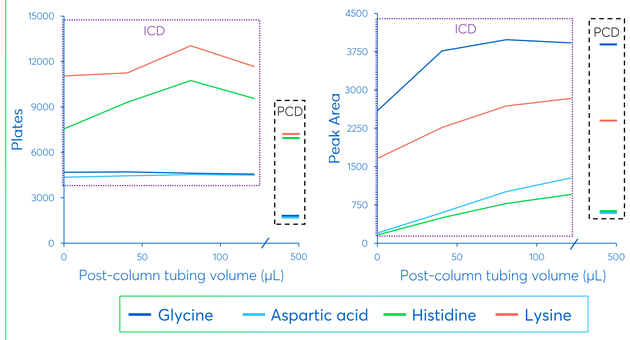
*ICD OPA for amino acid analysis:*

- *Using OPA reagent, the ICD process gave an average of 67% gain in efficiency compared to PCD.*
- *Due to the gain in efficiency, it is probable that the laboratory productivity, in relation to the amino acid analysis, could be substantially increased with further improvements in sensitivity.*
- *The flows were optimised for the PCD process (using existing methodology) rather than the ICD process. This was done to ensure we have a direct comparison between the 2 techniques.*
- *ICD yields reliable operation, negating the need for expensive re-runs.*
- *There is actually 2 connection less (mixing tee) in the ICD setup compared to PCD. If we can teach an analyst to use PCD, I can't see any issues with ICD.*
- *No issues converting our existing PCD methods to ICD.*

*In collaboration and with permission on behalf of Dr. Andrew Jones, Chemika.*

## ICD Amino Acid Assays with *O*-phthalaldehyde (OPA)

- For the OPA reagent, a small amount of post column dead volume was found to be beneficial for ICD:




**Pump 1**
Mobile phase A: 0.3 % Heptafluorobutyric acid
Mobile phase B: MeOH
Flow rate: 1 mL/min
Gradient:

| t (min) | %B |
|---|---|
| 0 | 5 |
| 3.5 | 5 |
| 11 | 38 |
| 13 | 60 |
| 13.5 | 60 |

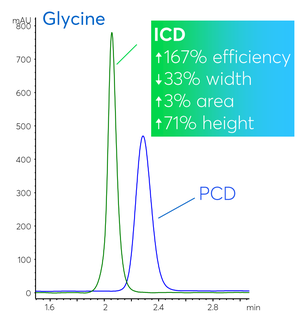
**Pump 2**
Mobile phase: OPA derivatisation mix
Flow rate: 0.4 mL/min

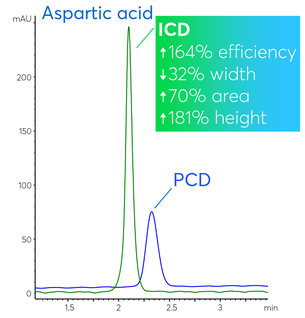
Column: Avantor® ACE® 5 C18
Dimensions: 100 x 4.6 mm
Temperature: 60 °C
Injection volume: 5 µL
Detector: UV, 338 nm
Sampling: 10 Hz

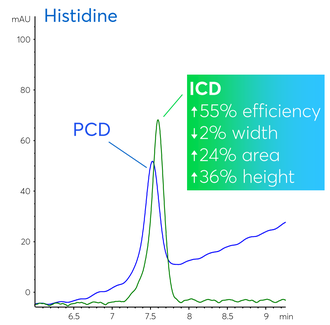
Post-column tubing: 3 x 800 x 0.25 mm capillaries (~40 µL each )

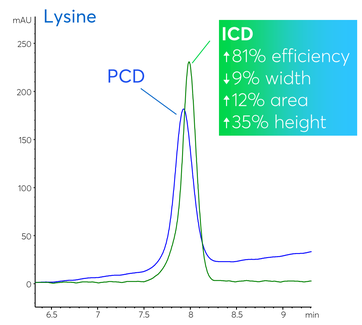
ICD (+80 µL) provided significant improvements vs. PCD (500 µL):

| | Glycine | | Aspartic acid | | Histidine | | Lysine | |
|---|---|---|---|---|---|---|---|---|
| **Plates** | 4620 | 1731 | 4534 | 1719 | 10746 | 6953 | 13045 | 7216 |
| **Peak Width (10%) (min)** | 0.16 | 0.24 | 0.17 | 0.25 | 0.45 | 0.44 | 0.40 | 0.44 |
| **Area (mAU)** | 3986 | 3886 | 1011 | 594 | 780 | 628 | 2689 | 2408 |
| **Height (mAU)** | 795 | 465 | 194 | 69 | 60 | 44 | 222 | 165 |



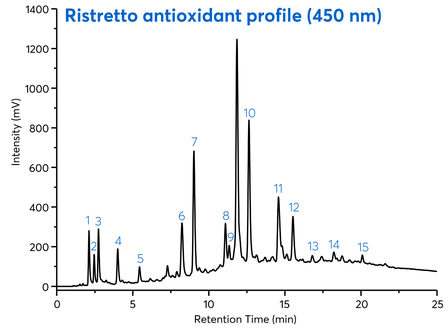

## References

1. Using HPLC with In-Column Derivatization to Authenticate Coffee Samples. *Molecules* 2023 **28** 1651.
2. Determination of antioxidants by a novel on-line HPLC-cupric reducing antioxidant capacity (CUPRAC) assay with post-column detection. *Anal. Chim. Acta* 2010 **674** 79–88.

## Authentication via ICD: Antioxidant Profiling[1]

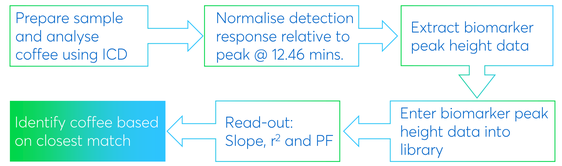
An ICD selective detection assay for antioxidants using a CUPRAC reagent[2] was used to authenticate coffee:

- Antioxidant profiles are complex and unique for different coffees.
- 15 antioxidant indicators varied in detection response.




| Biomarker | Retention Time (minutes) |
|---|---|
| 1 | 2.08 |
| 2 | 2.41 |
| 3 | 2.67 |
| 4 | 3.95 |
| 5 | 5.37 |
| 6 | 8.16 |
| 7 | 8.85 |
| 8 | 10.88 |
| 9 | 11.12 |
| 10 | 12.46 |
| 11 | 14.44 |
| 12 | 15.45 |
| 13 | 16.76 |
| 14 | 18.19 |
| 15 | 20.12 |

- Authentication workflow:




- 5 samples matched to a library of 32 coffees.
- Compilation of a larger library for improved classification.

| | Coffee | | | | |
|---|---|---|---|---|---|
| | Unknown 1 | Unknown 2 | Unknown 3 | Unknown 4 | Unknown 5 |
| **Highest match** | Starbucks Columbia | Guatemala | Vittoria Espresso | Profondo | Or Absolu |
| Slope | 0.9765 | 0.9999 | 0.9782 | 0.9903 | 0.9711 |
| $R^2$ | 0.9748 | 0.9887 | 0.9956 | 0.9914 | 0.9879 |
| P.F. | 14.3692 | 1.2538 | 1.5741 | 1.8529 | 4.0843 |
| **2nd Highest Match** | No Match | Inca Peru | Arabica Catuai | Long Black | No Match |
| Slope | | 1.0043 | 1.0022 | 1.0007 | |
| $R^2$ | | 0.9958 | 0.9831 | 0.9899 | |
| P.F. | | 1.1441 | 1.5687 | 1.8319 | |

## Conclusions

- ICD provides a simple solution to complex/challenging separations requiring PCD.
- Since the application of ICD requires minimal addition of post-column dead volume, post-column dispersion is reduced, conserving the columns theoretical separation efficiency (55 to 167% efficiency improvement and up to 1/3 reduction in peak width compared to PCD).
- Due to the high efficiency, complex samples can be chemically fingerprinted using compounds that are only visible through chemical reactions.
- As the chromatographic efficiency is higher than in PCD, sensitivity is often improved.
- High efficiency also means that shorter columns can be employed, and this increases the analytical throughput.