Application Note

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# Quantitation of Gut Microbiota-Derived Indole Metabolites by Ion Pairing dMRM LC/QQQ


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## Abstract

The surge of interest in the role of the human gut microbiota in health and disease research has created a demand for quantifying community-derived metabolites representing diverse chemical classes. Agilent has developed a highly reproducible and robust ion-pair reversed-phase (IP-RP) dynamic multiple reaction monitoring (dMRM) method that provides efficient separation of over 200 central carbon metabolites. This Application Note focuses on extending the IP-RP dMRM method to the measurement of tryptophan and indole metabolites that have been associated with effects on host metabolic and immunologic pathways. The extended IP-RP dMRM method enables sensitive detection of these compounds across a wide dynamic range in relevant biological samples.

## Introduction

The gut microbiota, composed of tens of trillions of microbes belonging to all three domains of life, but dominated by members of bacteria, produces many classes of metabolites that have wide-ranging effects on host biology.[1–4] The indole catabolites of the essential amino acid tryptophan constitute an exemplary class of microbial metabolites that confer beneficial effects upon host physiology.

Dietary tryptophan has multiple fates when ingested:

- Incorporation into peptides and proteins
- Catabolism by the host to kynurenine and serotonin through the action of the rate-limiting enzymes tryptophan 2,3-dioxygenase and tryptophan hydroxylase, respectively
- Transformation by members of the gut microbiota to various catabolites, including tryptamine, indole-3-acetic acid, indole-3-propionic acid, indole-3-lactic acid, and indole[5,6]

The latter metabolite is further sulfated in the liver to indoxyl sulfate to enhance its eventual clearance.

Indole metabolites modulate host biology though diverse mechanisms of action. For example, *Clostridium sporogenes* metabolizes tryptophan to indole-3-propionic acid, which is an endogenous ligand for the pregnane X receptor (PXR), which promotes gut barrier integrity by inducing the expression of tight junction proteins such as Occludin, Zonulin-1, E-cadherin, and Claudin-7.[7] Similarly, indole acrylic acid produced by some *Peptostreptococcus* species has anti-inflammatory effects; treatment of human peripheral blood mononuclear cells with this compound leads to reduced IL-6 and IL-1 secretion in response to LPS stimulation.[8]

Some indole metabolites are endogenous ligands of the aryl hydrocarbon receptor (AhR), which mediates anti-inflammatory and anti-microbial effects through effects on intra-epithelial γδ T cells, regulatory T cells, and cytokine production, including IL-22.[9,10] As a case in point, microglial cells use tryptophan-derived indoxyl-3-sulfate in an Ahr-dependent manner to ameliorate the symptoms of experimental autoimmune encephalomyelitis.[11] A recent study demonstrated that *Lactobacillus reuteri* is a producer of indole-3-lactic acid, which activates AhR, and leads to reprogramming of CD4+ intraepithelial lymphocytes into CD8aα cells.[12] Interestingly, patients with Crohn's disease and ulcerative colitis also have reduced fecal levels of tryptophan and indole-3-acetic acid, and increased levels of kynurenine.[13,14] Tryptamine activates the epithelial GPCR 5-HT4 receptor and increases colonic anion flux and secretion in a cAMP-dependent manner.[15]

While our understanding of gut microbiota-derived metabolites is accruing, assays for accurate quantification of metabolites across diverse chemical classes are not widely available. Recently, Agilent developed an IP-RP dMRM method that provides efficient separation of over 200 metabolites representing diverse chemical classes, many a part of central carbon metabolism across organisms. This Application Note describes steps for extending the IP-RP dMRM method to cover a panel of indole metabolites of general interest, including a microbial-derived subset.

## Experimental

### Sample preparation

Indole-3-acetamide, indole-3-acetic acid, indole-3-butyric acid, indole-3-carboxaldehyde, indole-3-carboxylic acid, indole-3-lactic acid, indole-3-propionic acid, indole-3-pyruvic acid, indoxyl sulfate, tryptophol, kynurenic acid, kynurenine, quinolinic acid, xanthurenic acid, and 5-hydroxytryptophan were purchased from Sigma-Aldrich and prepared at a concentration of 1 mg/mL in methanol. Standards were combined into three mixtures where each mixture contained no compounds whose molecular weights were within two daltons of each other. The final concentration of each standard in the mixture was 100 µg/mL mixture.

### Instrumentation

- Agilent 1290 Infinity II LC
- Agilent 6470A triple quadrupole LC/MS

MS parameters, chromatographic conditions, and gradient were taken from a metabolomics dMRM method (described in the Agilent Quick Start Guide, "MassHunter Metabolomics Dynamic MRM Database and Method", G6412-90006 Revision A, 5991-6467EN)

### Adding new compounds to the Agilent metabolomics dMRM method

Agilent's dynamic multiple reaction monitoring (dMRM) uses retention time scheduling of MRM transitions to allow more metabolites to be analyzed in a single run without sacrificing data quality. Therefore, to add new compounds to the metabolomics dMRM method, the retention time of new compounds must first be determined, then compound-specific acquisition parameters can be optimized.

- **Step 1.** To enable us to find the retention time of the new indole metabolites to be added to the dMRM method, we modified the dMRM method to a scan method.
- **Step 2.** We optimized the MRM transition, fragmentor, and collision energy parameters of the indole metabolites using Agilent MassHunter Optimizer. We used Optimizer's injection mode for all new compounds.
- **Step 3.** When Optimizer was finished optimizing the MRM acquisition parameters, we imported the retention time, MRM transitions, fragmentor, and collision energy parameters into the original metabolomics dMRM method, and saved it to a new file name.

## Results and discussion

The microbiota-derived indole metabolites examined were well resolved using this modified IP-RP dMRM method (Figure 1). There was excellent reproducibility (RSD <5% from triplicate determinations) of both retention time and peak area response including at the limit of quantitation, which was below 100 nM for all metabolites tested.

**Table 1.** Tryptophan and indole compounds added to dMRM method.

| Compound | Retention Time | Fragmentor | MRM | Collision Energy (V) |
|---|---|---|---|---|
| Indole-3-acetamide | 12.1 | 110 | 173.1 → 130.0 | 17 |
| Indole-3-acetic acid | 16.8 | 70 | 174.1 → 130.0 | 5 |
| Indole-3-butyric acid | 17.9 | 140 | 202.1 → 158.1 | 17 |
| Indole-3-carboxaldehyde | 14.9 | 140 | 144.0 → 115.0 | 33 |
| Indole-3-carboxylic acid | 15.3 | 100 | 160.0 → 116.0 | 17 |
| Indole-3-lactic acid | 16.5 | 140 | 204.1 → 158.0 | 17 |
| Indole-3-propionic acid | 16.7 | 130 | 188.1 → 59.1 | 13 |
| Indole-3-pyruvic acid | 17.3 | 90 | 202.1 → 174.0 | 5 |
| Indoxyl sulphate | 16.4 | 100 | 212.0 → 80.0 | 25 |
| Tryptophol | 15.9 | 80 | 160.1 → 116.0 | 17 |
| Kynurenic acid | 15.5 | 80 | 188.0 → 144.0 | 13 |

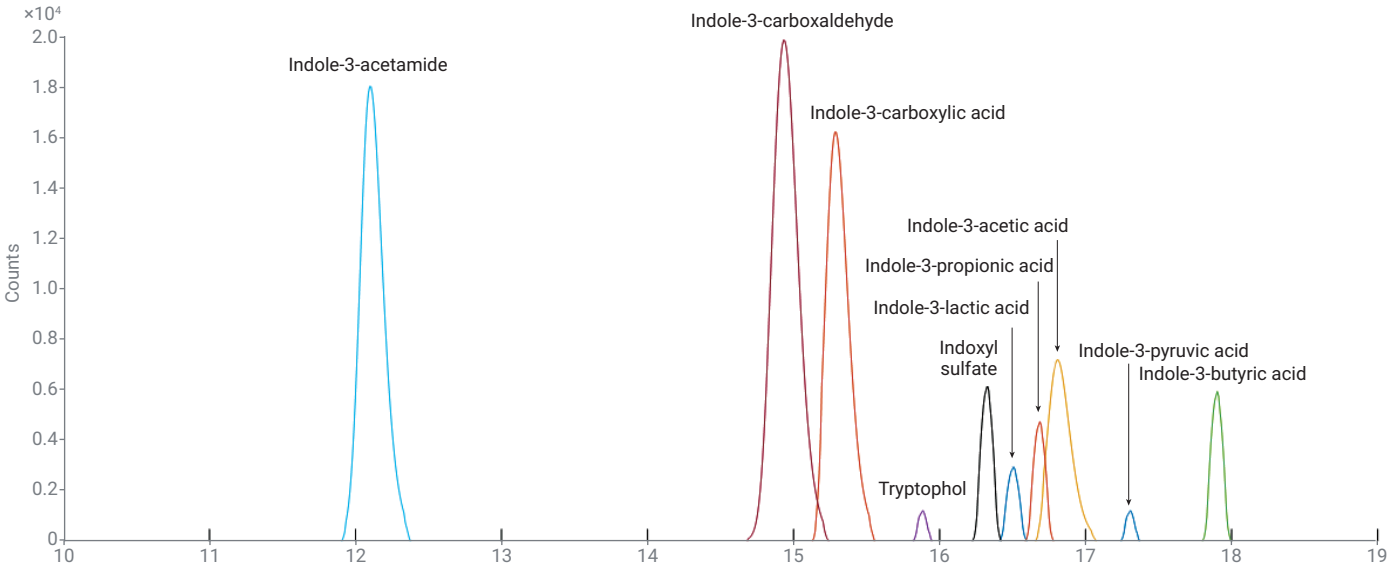


**Figure 1.** Composite dMRM chromatogram of microbial-derived indole metabolites.

The calibration curves for all the standards showed excellent linearity ($R^2$ >0.999) and wide dynamic range (>three orders of magnitude). Figure 2 shows examples of calibration curves for six representative metabolites.

## Conclusion

This Application Note describes a modification to extend an existing IP-RP dMRM metabolomics method to detect and measure a wider panel of important microbial metabolites. Reliable quantitation of the added tryptophan and indole metabolites was achieved using the modified Agilent IP-RP dMRM method. The analytical sensitivity, linearity, and dynamic range of measuring tryptophan and indole metabolites show that this method is applicable for microbiome applications in biological samples.

See the following recently published study that uses this method to measure the levels of indole metabolites in biologic samples:

Gehrig, J. L. *et al.* Effects of Microbiota-Directed Foods in Gnotobiotic Animals and Undernourished Children. *Science* **2019**, *365*, eaau4732.

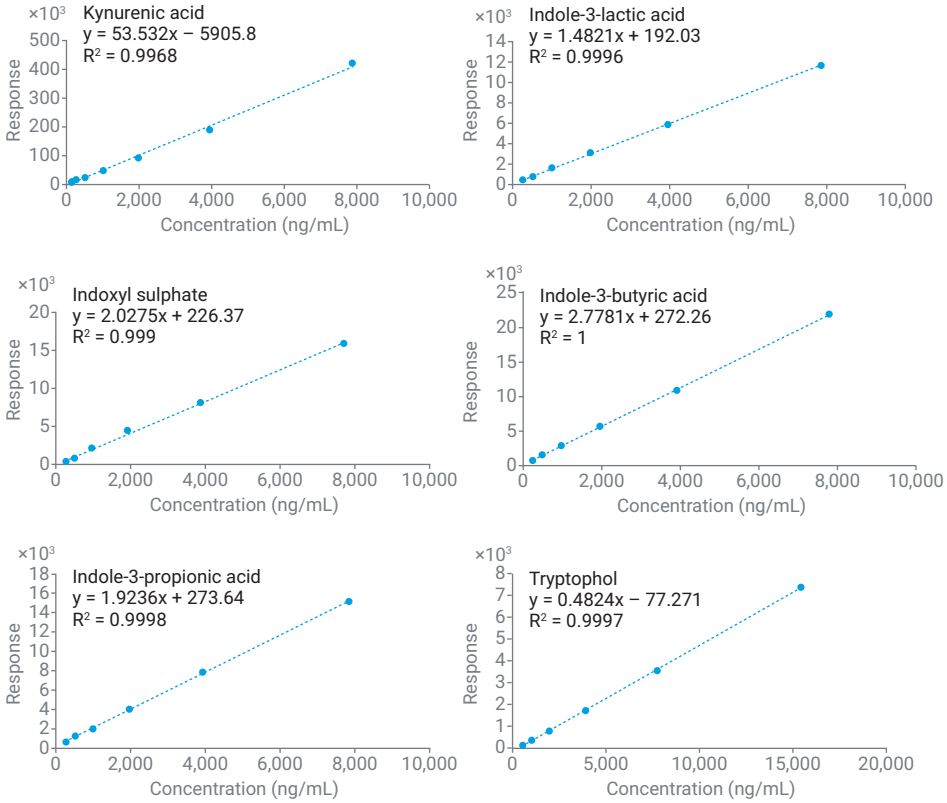


**Figure 2.** Calibration curves of for selected compounds.

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