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Measurement of phenol and *p*-cresol in urine and feces using vacuum microdistillation and high-performance liquid chromatography

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ABSTRACT

In this article, we describe a simple, sensitive, accurate, and repeatable method for the measurement of phenol and *p*-cresol (4-methylphenol) in human urine and feces. We examined a number of parameters to identify an optimal extraction protocol. Purification of sample extracts was achieved by low-temperature vacuum microdistillation. Separation was achieved in approximately 15 min by high-performance liquid chromatography (HPLC) with quantification by fluorescence at 284/310 nm. Limits of detection for phenol were 2 ng/ml for urine and 20 ng/g for feces, and those for *p*-cresol were 10 ng/ml for urine and 100 ng/g for feces. For comparison, approximate mean values for urine are 3 μg/ml for phenol and 30 μg/ml for *p*-cresol, and those for feces are 1 μg/g for phenol and 50 μg/g for *p*-cresol. An experienced analyst can process 60 samples each day using this method.

Measurement of phenols and cresols in feces and urine is of interest for two main reasons. First, these substances serve as markers for monitoring environmental exposure to aromatic hydrocarbons such as benzene and toluene [1,2]. Second, phenol and *p*-cresol (4-methylphenol) are putative biomarkers of large bowel health both in humans [3–5] and in animal models [6]. Diets that promote the generation of these potentially toxic metabolites are associated with enhanced rates of colonic DNA damage and greater risk of large bowel cancer [6,7]. Although environmental exposure can lead to the generation of a large number of phenols and cresols [1,8], only phenol and *p*-cresol, which arise from bacterial action on dietary aromatic amino acids [9], are present in normal samples.

A number of high-performance liquid chromatography (HPLC)[1] and gas chromatography (GC) methods for the measurement of phenols and cresols in urine have been published [1,8–14]. In contrast to urine, we are aware of only two methods for feces [9,11]. In urine, phenols and cresols are present largely as glucuronide and sulfate conjugates [15,16], and before chromatography these must be hydrolyzed using acid [2,9–13] or enzymatic [1,8,13,15] methods. Hydrolysis is not required for fecal samples [9] because, as is the case for bile acids [17], endogenous bacterial hydrolases generate the free forms of phenols and cresols. Initial extracts have most commonly been obtained by simple extraction from acidified samples using a variety of solvents, including dichloromethane [1], isopropyl ether [2,14,15], *t*-butyl ether [8], hexane [10], and diethyl ether [11]. Other methods have used distillation [9,13] and solid phase extraction [12]. Most methods [1,11–15,18,19], but not all methods [2,9], use an internal standard, and these have included 4-ethylphenol [14,19], 4-chlorophenol [1,11], 2,6-dimethylphenol [13,15], and *o*-cresol [18]. For HPLC methods, analytes are detected and quantified with ultraviolet (UV) [1,2,11], fluorescence [9,14,15], electrochemical [13], or chemiluminescence [12] methods.

Measurement of phenol and *p*-cresol in urine and feces presents a challenge because phenol is usually present at concentrations at least one to two orders of magnitude less than *p*-cresol and between-individual differences in concentrations can also be of a similar magnitude. In the current study, and as expanded on below, we examined a number of elements of the assay of phenols and cresols in urine and feces with a view to optimizing and validating their measurement.

With regard to choice of internal standards, we have examined a number of substances to identify the best candidate. For sample cleanup, distillation is inherently superior to solvent extraction because it removes all nonvolatile substances and provides a purer extract [9,13]. This is particularly important for fecal samples. However, previously reported distillation methods for phenols and cresols have involved collection of up to 50 ml of distillate [9,13,20] and process only one sample at a time, thereby limiting throughput. For the measurement of short-chain fatty acids in feces, our group has used a simple multisample low-temperature vacuum microdistillation apparatus [21] based on a single-sample apparatus described by others

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[1] *Abbreviations used:* HPLC, high-performance liquid chromatography; GC, gas chromatography; UV, ultraviolet.

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[22]. In the current article, we examined its application for the measurement of phenols and cresols.

With regard to detection methods, especially in feces, the lowest concentrations of phenol are close to or below the limits of detection of UV methods [11]; therefore, fluorescence detection methods are preferable because they are much more sensitive. They have the additional advantage that the detector sensitivity can be changed by at least an order of magnitude during an HPLC run to accommodate on-scale the (low) phenol concentrations as well as the (high) *p*-cresol concentrations.

In this article, we describe a sensitive repeatable method for the measurement of phenol and *p*-cresol in urine and feces using *o*-cresol as internal standard, a multisample vacuum microdistillation apparatus for sample cleanup and HPLC with fluorescence detection for quantification. The method is capable of a throughput of approximately 60 samples per day, which is comparable to solvent extraction methods that produce less clean extracts.

Materials and methods

Reagents

Phenol, *o*-cresol (3-methylphenol), *p*-cresol (4-methylphenol), indole, 2,6-dimethylphenol, and skatole (3-methylindole) (all $\geqslant$98% pure) were obtained from Sigma–Aldrich (Castle Hill, New South Wales, Australia), and 4-ethylphenol (>98% pure) was obtained from Merck (Kilsyth, Victoria, Australia). Acetonitrile was HPLC grade (Merck). All other reagents were analytical grade. MilliQ water was used throughout.

Collection of urine and feces

Collection of feces and urine samples was approved by the human research ethics committee of CSIRO Human Nutrition (Adelaide, South Australia, Australia). Urine from six volunteers was collected into a suitable container without preservative and stored in aliquots at −20 °C in capped plastic tubes. Feces from six volunteers were voided into a large plastic bag placed over the toilet seat. The sample from each volunteer was made homogeneous by hand kneading in the plastic bag, and subsamples of approximately 70 g were transferred to plastic vials and stored at −20 °C. Pools of samples were subsequently made as appropriate.

Preparation of standards and other analytes

Stock 1-mg/ml solutions of all analytes were prepared in MilliQ water and stored for up to 1 month at 4 °C. Working solutions were prepared by making appropriate dilutions in MilliQ water or, for some tests, $NaPO_4$ (0.1 M, pH 5.5) or HCl (0.1, 1, or 4 M).

Choice of internal standard and effect of extractant volume on recoveries for feces

In other studies we have conducted, the concentration of phenol in approximately 60% of fecal samples was less than 1 μg/g. This is close to or below the limit of detection of UV methods [11]. In an attempt to increase the concentrations of analytes in extracts from feces in the current study, we examined the effect of reducing the volume of extractant on recoveries of phenol and *p*-cresol as well as the internal standard candidates *o*-cresol and 4-ethylphenol. Duplicate 0.5-g aliquots of a fecal sample were extracted with 1.5, 4.5, and 10 ml of water or the same volumes of a solution containing 10, 20, 20, and 20 μg/ml phenol, *p*-cresol, *o*-cresol, and 4-ethylphenol, respectively. Concentrations were calculated by comparison to external standards, and recoveries were calculated by subtracting the nonspiked value from the spiked value.

Effect of extractant composition and hydrolysis on extraction efficiency from fecal samples

The two published methods for the measurement of phenol and *p*-cresol in feces used 0.1 M phosphate buffer (pH 5.5) for extraction [9,11]. One appeared not to hydrolyze the extract [9], whereas the other hydrolyzed with HCl for 60 min at 100 °C. Therefore, we examined the effect of extractant composition and acid hydrolysis on the efficiency of extraction from feces as follows. Duplicate 0.5-g fecal sample aliquots were extracted by thorough mixing with 10 ml of water, 10 ml of 0.1 M $NaPO_4$ buffer (pH 5.5), 10 ml of 0.1 M HCl, 10 ml of 1 M HCl, or 10 ml of 4 M HCl containing 10 μg/ml *o*-cresol internal standard. Extracts were centrifuged, and 150 μl was distilled and applied to the HPLC. We examined the effect of hydrolysis as follows. After removal of the aliquot for distillation as described above, the samples that had been extracted with 1 M HCl at room temperature were heated for 60 min at 100 °C. Extracts from these samples were also centrifuged and distilled as described above, and the values were compared with corresponding samples that had not been hydrolyzed.

Extraction of fecal samples: Final method

Based on the results of the studies of the effects of extractant composition and volume on recoveries, the following method was adopted. Duplicate 0.45- to 0.55-g fecal samples were weighed into 15-ml plastic centrifuge tubes, and 10 ml of internal standard (10 μg/ml *o*-cresol in water) was added. Samples were mixed thoroughly by vortex and hand and then were centrifuged for 15 min at 2000*g* and 10 °C. An aliquot of 150 μl was taken from each tube into a 5-ml Quickfit flask for distillation.

Extraction of urine samples: Final method

Because measurement of phenol and *p*-cresol in urine has been well studied, we did not examine all elements of this assay. However, we did confirm that 60 min at 100 °C led to complete hydrolysis of glucuronide and sulfate conjugates (data not shown). The final method we employed was as follows. An aliquot of frozen urine was thawed, mixed, and then centrifuged for 15 min at 1000*g* and 10 °C, and duplicate 1-ml subaliquots of supernatant were transferred to 15 × 60-mm flat-bottomed glass vials with Teflon-lined caps and containing 100 μg of internal standard (100 μl of *o*-cresol, 1 mg/ml in water). MilliQ water (400 μl) and 500 μl of 4 M HCl were added, and the vials were capped, mixed, and heated for 60 min at 100 °C in a dry block heater. Tubes were cooled to room temperature, and 150 μl was taken from each vial into a 5-ml Quickfit flask for distillation.

Recovery of analytes from urine and feces

Recoveries of spikes of phenol and *p*-cresol added to a urine sample with low endogenous levels of these metabolites were determined at two concentrations of analytes as follows. To obtain nonspiked concentrations, duplicate 1-ml aliquots of urine were extracted using the method for urine described above except that 100 μl of water replaced 100 μl of internal standard solution. Duplicate urine samples for each spike concentration also were extracted using the method for urine described above except that 100 μl of appropriate spike solution mix containing phenol, *p*-cresol, and *o*-cresol replaced 100 μl of internal standard solution. Concentrations of phenol, *p*-cresol, and *o*-cresol in the 100 μl added were 20, 100, and 100 μg/ml for the low-level spike and 100,