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PAIRED-ION REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PHENOL SULFATES IN SYNTHETIC MIXTURES, ALGAL EXTRACTS AND URINE*

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SUMMARY

Phenol sulfate esters have been analyzed by paired-ion reversed-phase high-performance liquid chromatography. The method provided direct, rapid chromatophy of phenol sulfates in crude extracts of the red alga *Polysiphonia lanosa* (2,3-dibromo-4,5-dihydroxybenzyl alcohol 1′,4-disulfate), of the brown alga *Ascophyllum nodosum* (1,2,3,5-tetrahydroxybenzene 2,5-disulfate), and in rat urine (resorcinol mono- and disulfates). Detector response (254 nm) was linear within the approximate range from 30–125 ng to 5–10 μg. Semipreparative scale chromatography provided sufficient amounts of purified phenol sulfates for further analysis by paper electrophoresis.

INTRODUCTION

A century ago, Baumann[1] isolated potassium phenyl sulfate from urine. His studies led to the recognition that many higher animals, including man, detoxify ingested phenols by converting them to sulfate ester salts[2]. More recently, phenol sulfates have been demonstrated in red algae[3], brown algae[4], and higher plants[5].

Naturally occurring phenol sulfates are often difficult to isolate and purify, particularly when the source also contains large quantities of salts and polyols[6]. Under these circumstances, previously investigated techniques such as paper chromatography[7,8] and paper electrophoresis[5,8,9] usually are inadequate not only for preparative procedures, but also for analytical scale separation of isomeric phenol sulfates. Analyses are further complicated by the relative instability of many phenol sulfates in aqueous solution. These difficulties have led most investigators to study only the phenols released by acid hydrolysis of the sulfate esters[10,11], thereby usually sacrificing structural information on the parent molecule.

High-performance liquid chromatography (HPLC) offers a potentially direct,

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rapid and sensitive approach to both qualitative and quantitative analysis of phenol sulfates; however, normal (polar) phase adsorption HPLC usually leads to poor retention characteristics and resolution of such strongly ionized compounds (refs. 12, 13; but *cf.* ref. 14). We now describe a specific and rapid analysis of individual phenol sulfates in synthetic mixtures, crude algal extracts, and urine, using paired-ion reversed-phase HPLC[12,13].

EXPERIMENTAL

Materials

Potassium salts of phenol sulfate esters were synthesized as described [6,8]; dihydroxybenzene monosulfates were prepared by controlled acid hydrolysis of the corresponding disulfates. Lanosol disulfate (2,3-dibromo-4,5-dihydroxybenzyl alcohol 1',4-disulfate) was a generous gift from Dr. J. S. Craigie.

Reversed-phase columns of μBondapak C_{18} (10 μm; 30 cm × 4.0 mm I.D.; Waters Assoc., Milford, Mass., U.S.A.) and RP-8 (10 μm; 25 cm × 4.6 mm I.D.; Brownlee Labs., Berkeley, Calif., U.S.A.) were used. A precolumn of Corasil C_{18} (37–50 μm; 4 cm × 4.0 mm I.D.; Waters Assoc.) was employed during chromatography of crude extracts; the precolumn improved analytical reproducibility and increased column life, without producing obvious band spreading. The paired-ion chromatography (PIC) reagent preparation "PIC-A" (phosphate-buffered tetra-*n*-butylammonium phosphate) was purchased from Waters Assoc., $(n\text{-Bu})_4NHSO_4$ from Aldrich (Milwaukee, Wisc., U.S.A.), and HPLC grade solvents from Fisher Scientific (Fair Lawn, N.J., U.S.A.) and Caledon Labs. (Georgetown, Ont., Canada). Water was prefiltered through a Millipore "Q" system.

Algal extracts

Polysiphonia lanosa (L.) Tandy (Rhodophyceae) and *Ascophyllum nodosum* (L.) Le Jol. (Phaeophyceae) were collected at Morris Point, Halifax County. Fresh *P. lanosa* (*ca.* 1 g) was ground in a mortar with two 2-ml portions of ethanol–water (7:3, v/v). The extract was evaporated *in vacuo*, resuspended in water, clarified by centrifugation, and injected either directly (1–10 μl) or after concentration under nitrogen. Fresh *A. nodosum* (*ca.* 1 g) was extracted with aqueous acetone[15], and the extract was treated as above.

Urine

Resorcinol (20 mg) was thoroughly mixed with cheddar cheese (*ca.* 2 g) and was fed to a 244 g female Wistar rat. Urine was collected daily, clarified by centrifugation and stored at −15°. Samples (*ca.* 1 ml) were thawed and extracted with ethyl acetate just prior to use. The organic phase was discarded, and an aliquot (0.3 ml) of aqueous $(n\text{-Bu})_4NHSO_4$ (20 mg/ml) was added to the remaining aqueous phase. The tetrabutylammonium phenol sulfates were then extracted twice into 1 ml portions of chloroform. The combined chloroform extracts were evaporated under nitrogen, redissolved in water (100 μl), and centrifuged before injection.

HPLC methods

All HPLC solvents were filtered through precombusted (450°) glass fibre filters (Whatman GF/C) before use. HPLC columns were pre-equilibrated by flushing

with at least 100 ml of the mobile phase. A flow-rate of 1.5 ml/min was used throughout the study. Chromatography was carried out using a Spectro-Physics SP 8000 instrument (10 μl sample loop; oven temperature 40°), or with a component system (Altex model 110A single-piston pump; Rheodyne type 50 injector) at room temperature. An SP 8300 fixed wavelength (254 nm) detector was used with both systems.

RESULTS AND DISCUSSION

Purified phenol sulfates and synthetic mixtures

The sulfated phenols examined were: phenyl sulfate, pyrocatechol disulfate, resorcinol mono- and disulfates, hydroquinone disulfate, phloroglucinol mono-, di- and trisulfates, 1,2,3,5-tetrahydroxybenzene 2,5-disulfate, and lanosol disulfate. All of these compounds were readily chromatographed as the corresponding tetrabutylammonium salts using reversed phase HPLC with mobile phases (methanol–water) containing PIC-A (Table I). For a given polyphenol, the relative order of elution was: free phenol > monosulfate > disulfate > trisulfate (Fig. 1). Isomeric polyphenol sulfates could be resolved under certain isocratic conditions (Fig. 2); however, improved separations were produced by gradient elution with samples containing strongly retained compounds such as lanosol disulfate (Fig. 3). The relatively large retention volume observed for lanosol disulfate (Table I) may be due in part to the sulfated hydroxymethyl group as well as the bromine substituents (compare tyrosine, mono- and diiodotyrosines, ref. 16).

Under our conditions the corresponding free phenols were eluted shortly after the solvent front, in the relative order: phloroglucinol > hydroquinone > resorcinol ≫ phenol (Table I). The *o*-dihydroxyphenols examined (pyrocatechol and pyrogallol) could not be detected when PIC-A was present in the mobile phase.

The maximum sample load was limited to *ca.* 10 μg (depending on the sample) when the PIC-A reagent was prepared according to the manufacturer's recommendations[17]. Overloading the ion-pairing system produced asymmetric peaks with faster-moving shoulders. Much larger samples could be accommodated by adding a tetrabutylammonium salt (*e.g.*, $(n\text{-Bu})_4NHSO_4$) to the PIC-A-buffered solvent system. For example, addition of 0.75 mg/ml of $(n\text{-Bu})_4NHSO_4$ increased the carrying capacity of the mobile phase by as much as 20-fold; values of k' were altered somewhat (for a theoretical treatment, see ref. 12). No pH adjustments were required.

The precise composition of mobile phase required to optimize separation of these phenol sulfates depended both on the compounds under investigation, and on the column used. Absolute retention volumes (at a specified mobile phase composition) tended to decrease with prolonged column use. However, column aging did not adversely affect relative retention characteristics.

Phenol sulfates chromatographed in the absence of tetrabutylammonium ions showed very little retention on either of the reversed-phase columns investigated, and exhibited considerable tailing.

Algal extracts

Direct analysis of a crude extract from the red alga *Polysiphonia lanosa* revealed a major component with retention volume identical to that of lanosol disulfate (Fig. 3). This component was collected from repeated injections of extract by evaporating

TABLE I

DEPENDENCE OF CAPACITY FACTOR [$k' = (V_1 - V_0)/V_0$] OF FREE PHENOLS AND PHENOL SULFATE ESTERS ON MOBILE PHASE COMPOSITION (VOLUME % METHANOL IN WATER, WITH PIC-A) WITH REVERSED-PHASE COLUMNS

Compound	*Waters μBondapak C_{18} column*						*Brownlee RP-8 column (LiChrosorb RP-8)*									
	21%	*24%*	*27%*	*30%*	*32%*	*35%*	*23%*	*26%*	*29%*	*32%*	*36%*	*40%*	*44%*	*47%*	*50%*	*53%*
Phenol	2.56	2.25	1.90	1.64	—	—	5.16	4.22	3.63	2.98	2.33	1.74	0.89	—	—	—
Phenyl sulfate	3.31	2.40	1.64	1.18	—	—	—	19.9	11.5	5.46	3.18	2.11	1.13	—	—	—
Catechol disulfate	3.14	1.93	1.13	0.32	—	—	—	—	14.4	7.85	3.86	2.10	1.32	—	—	—
Resorcinol	1.15	0.97	0.78	0.64	—	—	1.51	1.24	1.03	0.84	0.66	0.53	0.39	—	—	—
Resorcinol disulfate	4.25	2.48	1.35	0.82	—	—	—	—	15.2	8.33	4.15	2.09	1.15	—	—	—
Hydroquinone	0.60	0.51	0 39	0.34	—	—	0.67	0.60	0.51	0.43	0.36	0.30	0.22	—	—	—
Hydroquinone disulfate	2.88	1.61	0 91	0.53	—	—	—	14.2	8.42	5.02	2.73	1.58	0.82	—	—	—
Phloroglucinol	0.51	0.42	0.32	0.25	—	—	0.58	0.51	0.39	0.34	0.21	0.16	0.13	—	—	—
Phloroglucinol monosulfate	3.13	1.70	1.07	0.59	—	—	3.52	2.49	1.74	1.27	0.83	0.55	0.31	—	—	—
Phloroglucinol disulfate	—	2.69	1.74	1.17	—	—	—	19.8	10.8	6.38	3.24	1.71	0.76	—	—	—
Phloroglucinol trisulfate	—	5.72	3.59	2.08	—	—	—	—	—	—	9.41	4.00	2.20	1.34	—	—
1,2,3,5-Tetrahydroxybenzene 2,5-disulfate	3.95	2.38	1.26	—	—	—	18.9	11.7	8.22	5.91	3.30	1.83	1.13	—	—	—
Lanosol disulfate	—	—	*ca.* 18	—	3.70	2.13	—	—	—	—	—	9.14	4.09	2.15	0.74	0.32

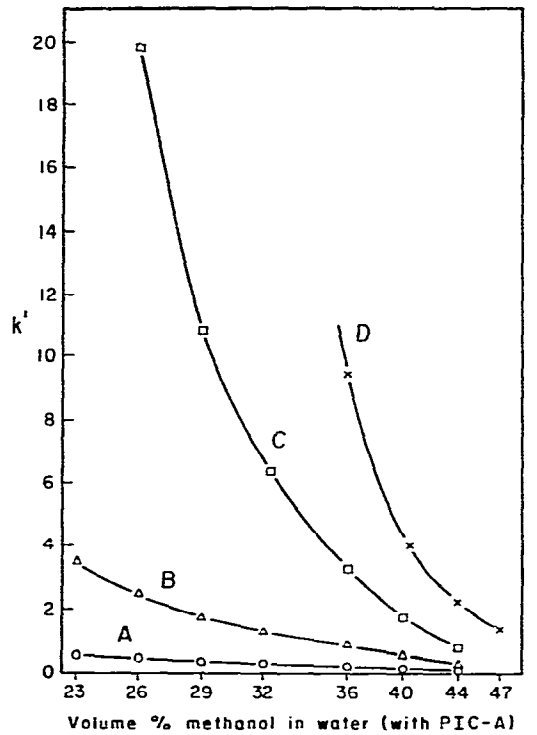

Fig. 1. Capacity factor k' as a function of mobile phase composition for phloroglucinol (A), phloroglucinol monosulfate (B), phloroglucinol disulfate (C), and phloroglucinol trisulfate (D). Column: Brownlee RP-8 (LiChrosorb RP-8).

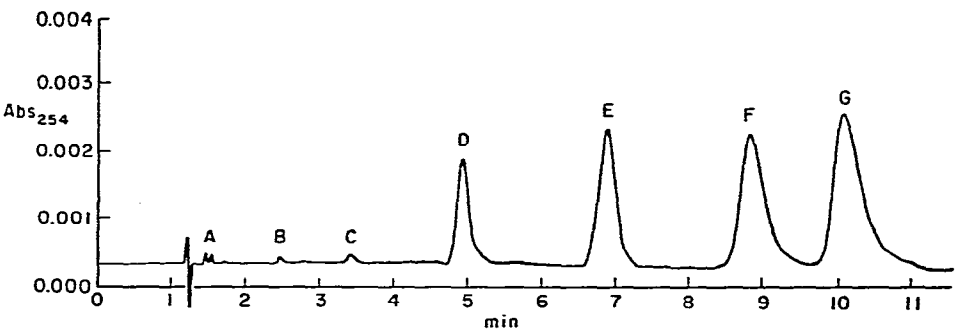

Fig. 2. Isocratic separation of isomeric dihydroxybenzene disulfates on μBondapak C_{18} column, mobile phase 22% methanol in water (+PIC-A). A = Free phenols; B = hydroquinone monosulfate; C = resorcinol monosulfate; D = pyrocatechol monosulfate; E = hydroquinone disulfate; F = pyrocatechol disulfate; G = resorcinol disulfate.

the appropriate fraction to dryness (35°, *in vacuo*), redissolving the eluted compound in *ca.* 1 ml of aqueous $(n\text{-Bu})_4NHSO_4$ (10 mg/ml), and extracting it as the tetrabutylammonium salt into chloroform. Electrophoresis (Whatman No. 1 paper; potassium acetate buffer (0.1 *M*, pH = 4.0); 30 min at 10 V/cm followed by 90 min at 25 V/cm; 5°) and color reactions[11] confirmed its identity with authentic lanosol disulfate. Further

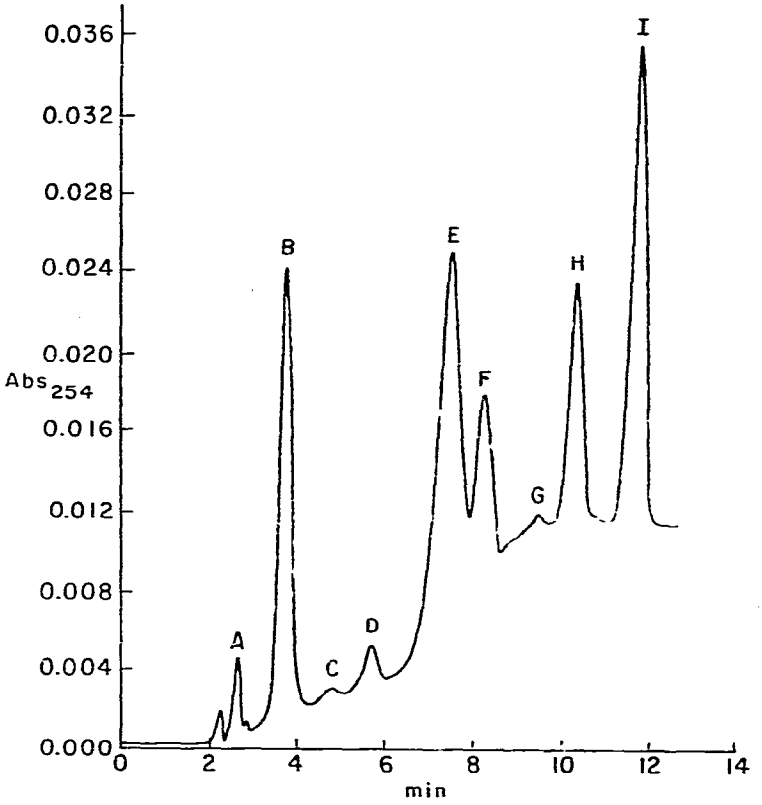

Fig. 3. Separation of phenol sulfates by gradient elution using a Brownlee RP-8 column. Linear gradient from 32% to 50% methanol in water (+PIC-A) at 3.6%/min, where it was held. A = Phloroglucinol; B = phloroglucinol monosulfate; C = decomposition product (monosulfate?) contained in sample of F; D and G = minor contaminants from sample of H; E = phloroglucinol disulfate; F = 1,2,3,5-tetrahydroxybenzene 2,5-disulfate; H = phloroglucinol trisulfate; I = lanosol disulfate.

identification was provided by co-chromatography of the compound with lanosol disulfate during HPLC using a variety of methanol–water (+PIC-A) mobile phases.

Hydrolyzed extracts from *P. lanosa* and related red algae often contain, in addition to lanosol, the corresponding aldehyde 2,3-dibromo-4,5-dihydroxybenzaldehyde and brominated benzyl alkyl ethers[18]. Although reference sulfate esters of the latter compounds were not available, we could find no evidence for the presence of any sulfated phenols, other than lanosol disulfate, in extracts of *P. lanosa* (Fig. 4). This result supports the suggestions (see, *e.g.*, ref. 19) that the aldehydes and alkyl ethers are artifacts of the isolation procedure normally used.

In direct injections of the crude extract from the brown alga *Ascophyllum nodosum* (Fig. 5), a component with retention volume very similar to that of 1,2,3,5-tetrahydroxybenzene 2,5-disulfate was observed. This fraction was collected and worked up as above. Paper electrophoresis, color reactions, and co-chromatography during HPLC were used to demonstrate that the compound in this fraction was identical to synthetic 1,2,3,5-tetrahydroxybenzene 2,5-disulfate.

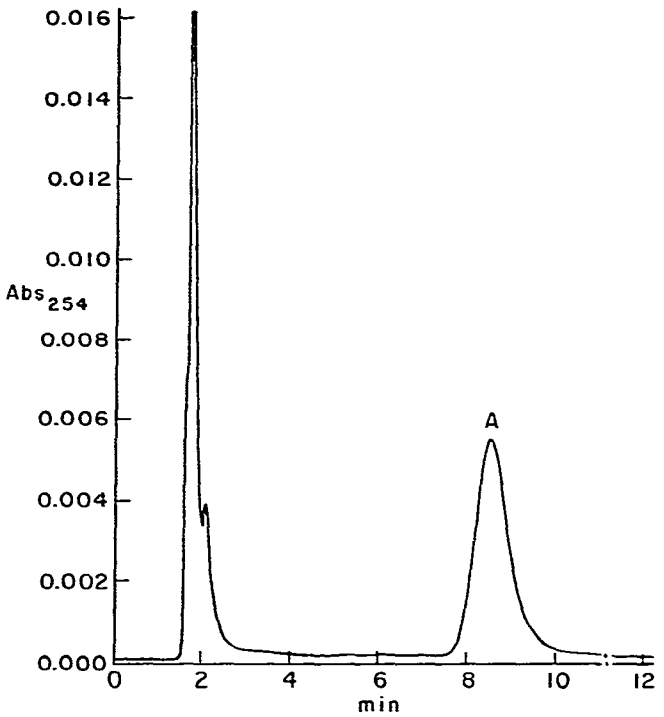

Fig. 4. Isocratic HPLC analysis of crude extract of *Polysiphonia lanosa* on μBondapak C_{18}, with Corasil C_{18} precolumn. Solvent: 32% methanol in water (+PIC-A). A = Lanosol disulfate.

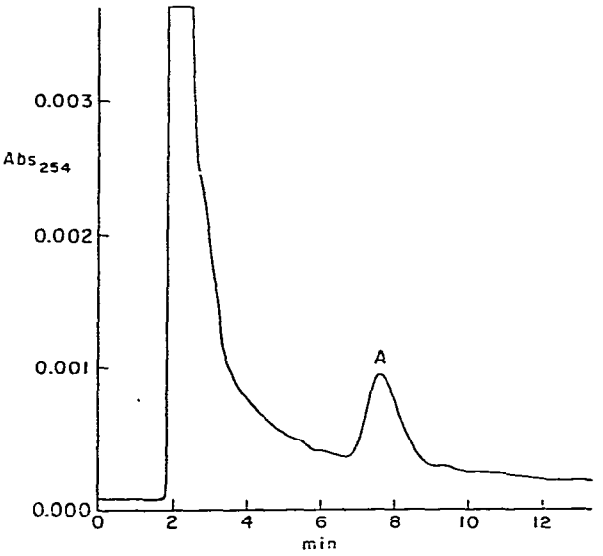

Fig. 5. Isocratic HPLC analysis of crude extract of *Ascophyllum nodosum* on μBondapak C_{18}, with Corasil C_{18} precolumn. Solvent: 19% methanol in water (+PIC-A). A = 1,2,3,5-Tetrahydroxybenzene 2,5-disulfate.

Urine

Chromatograms of crude rat urine were too complex to permit direct identification of resorcinol sulfates. Pre-extraction with ethyl acetate (see Experimental) removed copious quantities of an easily crystalline material, but little resorcinol and no resorcinol sulfates. To the remaining aqueous phase was added an excess of $(n\text{-Bu}_4)NHSO_4$; chloroform extraction then removed (*inter alia*) two compounds with k' values approximately equal to those of resorcinol mono- and disulfates, respectively (Fig. 6). These two fractions were collected from repeated injections of the extract (representing *ca.* 0.8 ml of the urine collected during the first 24 h after administration of resorcinol), and were evaporated to dryness, redissolved in aqueous $(n\text{-Bu})_4NHSO_4$, and once again extracted as the tetrabutylammonium salts into chloroform. Electrophoresis, color reactions, and HPLC (see above) demonstrated the identity of these compounds with resorcinol mono- and disulfates.

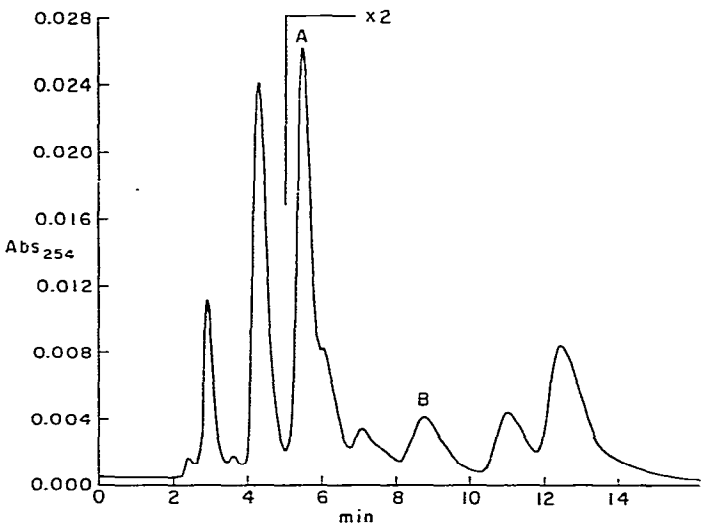

Fig. 6. Isocratic HPLC analysis of the chloroform-extractable fraction from rat urine on μBondapak C_{18} (hand-packed column), with Corasil C_{18} precolumn. Solvent: 20% methanol in water (+PIC-A). A = Resorcinol monosulfate; B = resorcinol disulfate.

These results demonstrate that sulfate conjugation is a mechanism of resorcinol metabolism in the rat. As with pyrocatechol[20] and hydroquinone[21], the monosulfate is a major product, while the disulfate is produced in smaller amounts. The possible occurrence of glycosyl ethers and hydroxylated metabolites of resorcinol[2] was not investigated.

Quantitation and limits of detection

We could readily detect 30 ng of lanosol disulfate, 125 ng of 1,2,3,5-tetrahydroxybenzene 2,5-disulfate, and 50 ng of resorcinol disulfate in relatively pure samples using the fixed wavelength (254 nm) detector. With those compounds investigated, detector response (peak height) was a linear function of concentration up to 5–10 μg per injection (at a given mobile phase composition). It is likely that the

upper limit of linearity could be extended by further addition of tetrabutylammonium pairing ions to the mobile phase. Optimization of detection wavelength[6,8] should further improve sensitivity.

CONCLUSIONS

The paired-ion reversed-phase HPLC method described is rapid, sensitive, and capable of distinguishing between isomeric phenol sulfates. It thus represents a considerable improvement over paper chromatographic and paper electrophoretic analyses of phenol sulfates, particularly those occurring in crude extracts and in urine. Previously required hydrolytic steps are bypassed, thereby significantly reducing problems of artifact formation. Detector response was linear within the approximate range from 30–125 ng to 5–10 μg, depending on the compound. Much greater concentrations of phenol sulfates could be separated by further addition of tetrabutylammonium ions to the mobile phase. Extraction of the eluted tetrabutylammonium phenol sulfates into chloroform provided sufficient quantities of purified material for further analysis by paper electrophoresis. The preparative procedure probably can be scaled up further if required.

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