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Solid-phase extraction method for the determination of free and conjugated phenol compounds in human urine

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Abstract

A rapid flow system for automatic sample conditioning for the determination of phenol compounds in human urine has been developed and optimised. Free phenols are detected directly in urine samples while total phenols require acid hydrolysis to convert their conjugate fraction into free phenols, all compounds then being cleaned up and preconcentrated by solid-phase extraction. Separation and determination are done by gas chromatography, using mass spectrometry operating in the selective ion monitoring mode for quantitation. The linear range was 1–160 ng/ml of urine for most of the phenols. Limits of detection for phenol compounds (phenol, alkylphenols and chlorophenols) in the nanogram-per-millilitre range (0.3–0.6 ng/ml) are thus achieved by using 1 ml of urine; also, the repeatability, as RSD, is less than 6.5%. Based on the results for urine samples from unexposed individuals, 2-methylphenol, 2-chlorophenol and 2,4-dichlorophenol are largely detected in hydrolysed urine samples, whereas phenol and 4-methylphenol are detected in hydrolysed and unhydrolysed urine. Other chlorophenols such as trichlorophenols and pentachlorophenol are not detected. The results obtained in the analysis of urine from an individual before and after dietary intake reveal that the levels of phenol compounds in urine look related to food intake. © 2002 Published by Elsevier Science B.V.

Keywords: Phenols; Chlorophenols; Alkylphenols

1. Introduction

Phenols are widely used as preservative agents, pesticides, antiseptics, disinfectants and in a variety of industrial applications; naturally polyphenols are extensively distributed in plant kingdom. They can be absorbed into the human body, whether dermally, orally or via the airways [1,2]. Phenol compounds are released in urine, both as such and as sulfate or glucoronide conjugates, the amount of conjugation depending on the particular phenols and their concentrations in the urine [3]. Of special importance are chlorophenols, which are also usually present in urine by effect of the intake of food and water containing them or the metabolization of other chlorinated substances present in the environment [4]. Phenol compounds (particularly chlorophenols) are frequently monitored in human urine and other biological samples to obtain an indication of occupational exposure or exposure to environmental contamination [2,5]. The most frequently employed analytical procedures for measuring chlorophenols in human urine involve solvent extraction [3,6–8], solid-phase extraction (SPE) [4,6,9–11] and, more recently, solid-phase microextraction [2] and super-

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critical fluid extraction [12]. Liquid–liquid extraction is the most frequently used method for this purpose but has the disadvantages that it produces emulsions and that the extraction efficiency varies among different compounds. In addition, it uses large amounts of solvents and is labour-intensive and time-consuming. The SPE technique is today extensively used for the trace enrichment from environmental and biological samples. Chlorophenols in urine can be determined in a simultaneous manner by gas chromatography (GC) with electron-capture detection (ECD) [4,6,10] or mass spectrometry (MS) [2–4,8,9] and by capillary electrophoresis [11,12]; on the other hand, phenols in urine are usually determined by liquid chromatography [7,13–15]. Our group has developed on-line SPE methods for the determination of phenol compounds in water [16,17] and agricultural soils [18,19] by GC.

Conjugation with D-glucuronic acid is one of the major detoxification pathways for phenol compounds; this process makes glucuronide metabolites highly water-soluble and facilitates release through urine. A rapid, accurate, sensitive method for the simultaneous determination of phenol compounds and their glucuronide conjugates in human urine is therefore greatly needed.

In this work, we have assessed the potential of continuous SPE for reducing human intervention in the isolation of alkylphenols and chlorophenols from human urine. Free phenol compounds in dilute urine can be easily cleaned-up and preconcentrated in a simple SPE system. In alternative, free and conjugated phenol compounds (total phenols) are hydrolysed under drastic conditions in the batch mode for direct introduction into the SPE unit. The eluate from the sorbent is injected into a GC–MS instrument for separation and identification of free or total phenols.

2. Experimental

2.1. Chemicals and standards

All reagents were analytical grade or better. Phenols [phenol, 2-methylphenol (2-MP), 4-methylphenol (4-MP), 2-chlorophenol (2-CP), 4-chlorophenol (4-CP), 2,4-dichlorophenol (2,4-DCP), 2,5-dichlorophenol (2,5-DCP), 2,6-dichlorophenol (2,6-DCP), 2,4,5-trichlorophenol (2,4,5-TCP), 2,4,6-trichlorophenol (2,4,6-TCP) and pentachlorophenol (PCP)], non-ionic Amberlite XAD-2 and methyl nonanoate (internal standard) were supplied by Sigma–Aldrich (Madrid, Spain); all other reagents (ethanol, ethyl acetate, acetone, hydrochloric acid, and sodium hydroxide) were purchased from Merck (Darmstadt, Germany).

Stock standard solutions containing 1000 μg/ml of the individual phenols were prepared in acetone. All solutions were stored in glass stoppered bottles at 4 °C in the dark. More dilute solutions were prepared as needed by diluting the stock standard solutions in 0.1 *M* HNO_3 (pH 1).

2.2. Apparatus

A gas chromatograph equipped with an 8000/MD800 mass spectrometer (ThermoQuest, Madrid, Spain) was used to separate and determine phenols in urine. Analytes were separated on a TRB-5 capillary column (30 m×0.25 mm I.D., 0.25 μm film) with a stationary phase of 5% phenyl–methylpolysiloxane (Teknokroma, Barcelona, Spain). The carrier gas, helium (6.0 grade, Air Liquide, Seville, Spain), was held at a flow-rate of 1 ml/min by using electronic pressure control. The GC injection port, GC–MS interface and MS source temperatures were maintained at 250, 250 and 200 °C, respectively. The oven temperature programme was as follows: 60 °C, hold 2 min, 10 °C/min to 110 °C with a 3 min hold, then 30 °C/min to 260 °C and hold for 1 min. The MS instrument was operated in the electron impact ionization mode, using an energy of 70 eV. The quadrupole MS was set in the selected ion recording mode (SIR or SIM) and sample injections were done in the split mode (split ratio 1:20). The optimal GC–MS conditions were established by using a mixture containing 10 μg/ml of each phenol and the internal standard in ethyl acetate; the injected volume was 1 μl. For identification, three m/z values per analyte were selected; for quantitation, the peak for each individual ion was used as the molecular or base peak (Table 1).

The SPE system consisted of a Minipuls-3 (Gilson, Villiers-le-Bel, France) peristaltic pump fitted with poly(vinyl chloride) and Solvaflex pumping tubes for aqueous and organic solutions, respectively.

Table 1
Analytical figures of merit for the GC–MS determination of phenols

Analyte	m/z[a]	Sensitivity ($\cdot 10^{-4}$)	Linear range (ng/ml)	Limit of detection (ng/ml)	RSD (%)
Phenol	65, 66, ***94***	260	0.8–160	0.3	5.5
2-MP	77, **107**, *108*	180	1–160	0.4	5.8
4-MP	77, **107**, *108*	220	0.8–160	0.3	6.0
2-CP	64, ***128***, **130**	230	0.8–160	0.3	5.3
4-CP	65, ***128***, 130	175	1–160	0.4	6.7
2,4-DCP	98, ***162***, 164	140	1–160	0.4	5.5
2,5-DCP	98, ***162***, 164	125	1–160	0.4	5.9
2,6-DCP	98, ***162***, 164	160	1–160	0.4	6.1
2,4,5-TCP	132, ***196***, 198	160	1–160	0.4	6.7
2,4,6-TCP	132, ***196***, 198	70	2–160	0.6	5.4
PCP	165, 230, ***266***	20	2–160	0.6	6.4

[a] m/z values in italics are M^+ values; those in boldface correspond to base peaks and those underlined are SIM quantitation values.

Two Model 5041 injection valves (Rheodyne, Cotati, CA, USA), PTFE tubing of 0.5 mm I.D. for coils and standard connectors were also used. The sorbent column was constructed by packing a commercially available glass column of 50 mm×2 mm I.D. (Omnifit, Cambridge, UK) with 50 mg of non-ionic Amberlite XAD-2; small glass wool plugs were used on both ends to prevent packing losses. A laboratory-made PTFE filter furnished with a paper disk (3.8 cm^2 filtration area) was also used for filtration purposes. An oil bath (Selecta, Barcelona, Spain) was also employed.

2.3. Sampling and hydrolysis of urine samples

Urine samples (100 ml) from unexposed individuals were collected in sterilized containers and frozen immediately (−20 °C) until analysis. For the recovery experiments, samples were spiked with a few microlitres of standard solutions containing 10 and 50 ng of each phenol, in acetone, per millilitre of urine.

Phenol compounds were hydrolysed by heating 1 ml of urine sample plus 1 ml of 6 *M* HCl in a screw-capped glass centrifuge tube that was immersed in an oil bath at 100 °C for 30 min. After cooling to room temperature, the hydrolysed urine was adjusted to pH 1 with 2 *M* NaOH (up to 5 ml). For the determination of free phenol compounds, 1 ml of urine sample was diluted in a glass tube to 5 ml with 0.2 *M* HNO_3 to pH 1. Then, the whole sample volume (5 ml), either for free or total phenols determination, was continuously aspirated into the solid-phase extraction system of Fig. 1. The line for

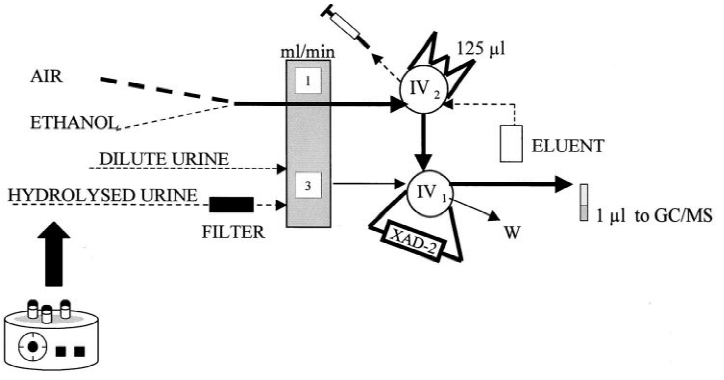

Fig. 1. Solid-phase extraction system for the preconcentration and clean-up of phenol compounds in unhydrolysed and hydrolysed urine samples. IV, Injection valve; W, waste; GC–MS, gas chromatography–mass spectrometry system.

hydrolysed urine was fitted with an in-line filtering device.

2.4. Analytical procedure

The continuous SPE unit used to extract free and total (free plus conjugated) phenols from unhydrolysed and hydrolysed urine samples, respectively, is depicted in Fig. 1. A volume of 5 ml of standard solution or dilute (unhydrolysed or hydrolysed) urine containing 0.8–160 ng of analytes at pH 1 was aspirated through the sorbent column (located in the loop of IV_1) at 3 ml/min; in this step, phenols were retained and the sample matrix sent to waste (W). Then, valve IV_1 was switched and the sorbent column dried for 3 min with a stream of air introduced via the carrier line of the second valve (IV_2); simultaneously, the loop of IV_2 (125 μl) was filled with eluent (10 μg/ml internal standard in ethyl acetate) with a syringe. In the elution step, valve IV_2 was switched and 125 μl of eluent, carried by the same air stream at a flow-rate of 1 ml/min, was passed upstream through the column to elute adsorbed phenols. The eluate was collected in 1-ml glass vials containing anhydrous sodium sulfate and a 1 μl aliquot was injected into the gas chromatograph for analysis. Between samples, the sorbent column was flushed with 1 ml of ethanol (from IV_2) to remove potentially retained interferents and conditioned with 1 ml of 0.1 *M* HCl. Under these conditions, the sorbent column remained active for 2 weeks (ca. 300 extractions).

3. Results and discussion

3.1. Optimization of the preconcentration/elution process

In previous work, we developed an SPE system for the separation of phenols in waters by GC–flame ionization detection (FID) [17]. XAD-2 resin and ethyl acetate were found to be the most efficient sorbent and eluent, respectively. The previously reported system for water samples was initially used to examine the effect of the sample pH and select the sorbent and eluent for urine samples. For this purpose, urine samples with no free phenols were taken as urine blanks. A volume of 1 ml of urine was spiked with 50 ng of phenol, 2-MP, 4-MP, 2-CP, 4-CP, 2,4-DCP, 2,5-DCP, 2,6-DCP, 2,4,5-TCP, 2,4,6-TCP and PCP each, diluted to 5 ml and processed with the SPE system, using XAD-2 as sorbent and ethyl acetate as eluent.

The first variable studied was the sample pH, the effect of which was examined over the range 1–12. Phenols are uncharged at a low pH because their pK_a values are generally high; chlorophenols, however, have lower pK_a values than methylphenols and can therefore be ionized even at low pH values. Other urine components may also be retained on the column. The peak areas for pentachlorophenol and trichlorophenol were smaller at low pH values (≈5); on the other hand, the signals for the other phenols remained constant over wider ranges (up to about pH 8) because their pK_a values were higher than 7. Because reproducibility and selectivity also increased upon acidification, sample solutions for all subsequent experiments were prepared in a 0.1 *M* HNO_3 medium (pH 1).

Three common non-polar sorbent materials (XAD-2, RP-C_{18} and LiChrolut-EN) for conventional SPE of organic compounds were assayed for the clean-up and preconcentration of spiked urine blanks. Several columns were packed with 50 mg of each sorbent. The sorption efficiency was assessed by comparing the amount of each compound recovered from the eluate (unsorbed) with its concentration in the sample (taken to be 100%). XAD-2 exhibited the best sorption properties (ca. 100% recovery for all phenols except phenol), whereas RP-C_{18} and LiChrolut-EN provided average recoveries of only 20% and 75%, respectively. In all instances, phenol was the least efficiently extracted compound (25% with XAD-2). Various eluents (ethyl acetate, *n*-hexane and methanol) were evaluated with the XAD-2 column, using a fixed volume of 200 μl. Complete elution was only achieved with ethyl acetate; the other solvents provided lower efficiency (1% *n*-hexane and 50% for methanol). The effect of the amount of sorbent material used was examined over the range 20–80 mg XAD-2; chromatographic signals increased with increasing amount of sorbent up to 40 mg, beyond which they remained virtually constant. On the basis of these experiments, a working column packed with 50 mg of XAD-2, and ethyl acetate (125

μl) as eluent were adopted for further experiments. The flow-rate of the sample solution had very little effect on recovery between 1 and 4 ml/min. Retained phenol compounds were eluted with no carry-over at flow-rates between 0.5 and 1.5 ml/min. A sample flow-rate of 3 ml/min and an air flow-rate (eluent carrier) of 1 ml/min were thus chosen.

3.2. Acid hydrolysis of phenol compounds

In mammals, the major fraction of phenol compounds is released with urine, partially as free phenols but mainly as sulfate or glucuronide conjugates [3,4]. Chlorophenols have been determined in urine samples following acid hydrolysis [3,4,6,7,9–11,14] of their conjugates in preference over alkaline or enzyme-based alternatives [6]. In this work, we used acid hydrolysis to determine total phenols in urine. However, there is no general consensus as regards the optimal conditions for acid hydrolysis of phenols [6,7,10,11,14]. In view of the situation, we established the optimal conditions in the present method. The variables studied were the hydrolysis time (15–60 min), temperature (60–120 °C) and the HCl concentration (2–8 *M*). For this purpose, 1 ml of a urine that had previously tested positive (containing free phenol and 4-MP) was spiked with 50 ng of 2-MP, 2-CP, 4-CP, 2,4-DCP, 2,5-DCP, 2,6-DCP, 2,4,5-TCP, 2,4,6-TCP and PCP each, and mixed with 1 ml of the hydrolysis reagent (HCl). After hydrolysis, the phenol compounds solution, adjusted to pH 1, was introduced into the SPE system. The highest hydrolysis efficiency was obtained under the following conditions: 1 ml of urine diluted with 1 ml of 6 *M* HCl, with heating in an oil bath at 100 °C for 30 min; after cooling, the mixture was diluted to 5 ml and adjusted to pH 1 before introduction into the SPE system. Finally, the hydrolysis reaction was repeated with an aqueous standard solution containing 10 ng/ml of each of the eleven phenols; the results obtained showed that acid hydrolysis did not degrade the phenols.

3.3. Calibration and statistical study

Phenol compounds were separated underivatized by GC, and discrimination between free and total phenols in urine samples was accomplished by using unhydrolysed and hydrolysed samples, respectively. The performance and reliability of the proposed method were assessed by determining the regression equation, linear range, limit of detection and precision for the phenols assayed. For this purpose, volumes of a few microlitres of standard solutions, containing all phenols at variable concentrations in acetone were spiked to individual urine blank volumes of 1 ml, over the range 0.5–160 ng, diluted and adjusted to pH 1 (final volume, 5 ml). Methyl nonanoate was used as internal standard (I.S.), added to the eluent at a concentration of 10 μg/ml. Calibration curves were constructed by plotting the analyte-to-I.S. peak area ratio against the analyte concentration. The figures of merit of the proposed method are listed in Table 1, which also shows the limits of detection (based on a signal-to-noise ratio of 3) and relative standard deviations (obtained by measuring eleven samples containing 10 ng/ml of each phenol). The linear range was 1–160 ng/ml of urine for most of the analytes. No interferences from the matrix of urine blank was observed, which testifies the high selectivity of the proposed method. By way of example, Fig. 2 shows the gas chromatogram for an urine fortified with the phenols listed in Table 1.

3.4. Determination of free and total phenols in human urine

The proposed SPE method for the screening and determination of phenol compounds in urine was validated by conducting a recovery study. Three urine samples from healthy individuals were taken as sample blanks, phenols being determined in free form (unhydrolysed urine) and total (hydrolysed urine). Next, the samples were spiked with undetected phenols at two concentration levels (10 and 50 ng/ml urine) and recoveries evaluated in replicate analyses. The average recoveries ($n=4$) ranged from 95 to 99% for all phenols, which testifies to the high accuracy of the proposed method.

The method was then applied to the screening of 10 urine samples from unexposed individuals. The results obtained are listed in Table 2. As can be seen, two samples (Nos. 5 and 9) contained no detectable amounts of any of the eleven phenols studied; their hydrolysis, however, released phenols conjugates and

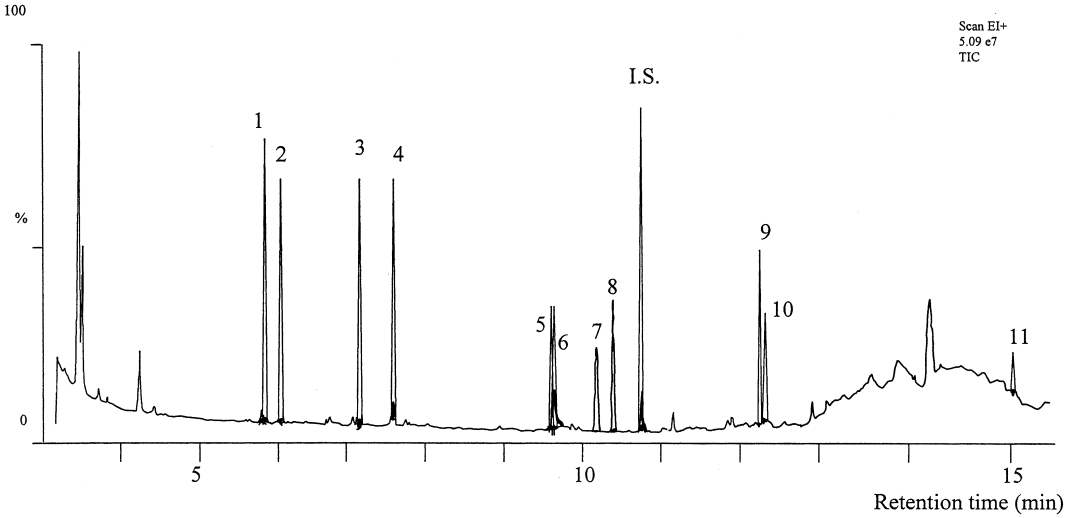

Fig. 2. Chromatogram for a urine sample fortified with 40 ng/ml of each phenol. Chromatographic peaks of interest: (1) phenol; (2) 2-CP; (3) 2-MP; (4) 4-MP; (5) 2,4-DCP; (6) 2,5-DCP; (7) 4-CP; (8) 2,6-DCP; (9) 2,4,6-TCP; (10) 2,4,5-TCP; (11) PCP; and I.S.: internal standard.

yielded detectable concentrations of phenol, 2-MP and 4-MP (lower than 3 ng/ml in any case). Of the phenols studied, only phenol (50% of samples) 2,4-DCP (30%) and, especially, 4-MP (80%) were detected in unhydrolysed samples; all other compounds were either absent or present as conjugates (i.e., 2-aminophenyl-β-D-glucuronide, 2-chlorophenyl-β-D-glucuronide, etc.). Consequently, hydrolysing the urine is essential with a view to determining the total amount of phenols present and discriminating physiologically and non-physiologically released concentrations in order to assess environmental contamination. Thus, the hydrolysis exposed the permanent presence of 4-MP and phenol over very broad concentration ranges (2–900 and 2–530 ng/ml, respectively). Also, 2-MP and 2-CP were only detected in hydrolysed urine (albeit at very low concentrations). Only 2,4-DCP among the higher chlorophenols was detected (both in unhydrolysed and in hydrolysed urine). None of the urine samples was found to contain 4-CP, 2,5-DCP, 2,6-DCP, 2,4,5-TCP, 2,4,6-TCP or PCP, probably because they were

Table 2
Determination of phenol compounds[a] in unhydrolysed (A) and hydrolysed (B) urine samples

Sample	Phenol		2-MP		4-MP		2-CP		2,4-DCP	
	A	B	A	B	A	B	A	B	A	B
1	93±6	530±40	n.d.	5.4±0.4	245±20	900±50	n.d.	1.0±0.1	1.9±0.1	7.8±0.6
2	n.d.	2.7±0.2	n.d.	n.d.	195±15	890±50	n.d.	n.d.	n.d.	3.5±0.2
3	n.d.	4.1±0.3	n.d.	1.6±0.1	150±10	650±40	n.d.	1.2±0.1	n.d.	n.d.
4	64±5	310±25	n.d.	n.d.	180±15	755±45	n.d.	n.d.	12±1	50±3
5	n.d.	3.1±0.2	n.d.	1.8±0.1	n.d.	3.2±0.2	n.d.	n.d.	n.d.	n.d.
6	54±4	200±15	n.d.	5.1±0.3	160±10	570±40	n.d.	0.9±0.1	n.d.	n.d.
7	46±3	160±10	n.d.	2.2±0.2	110±8	430±30	n.d.	n.d.	2.1±0.1	7.5±0.6
8	n.d.	5.5±0.4	n.d.	n.d.	135±10	625±40	n.d.	1.5±0.1	n.d.	n.d.
9	n.d.	2.2±0.1	n.d.	n.d.	n.d.	2.5±0.2	n.d.	n.d.	n.d.	n.d.
10	65±4	320±25	n.d.	1.5±0.1	72±5	345±25	n.d.	n.d.	n.d.	n.d.

All concentrations (±SD) are expressed in ng/ml (n=3).
[a] 4-CP, 2,5-DCP, 2,6-DCP, 2,4,5-TCP, 2,4,6-TCP and PCP were not detected (n.d.).

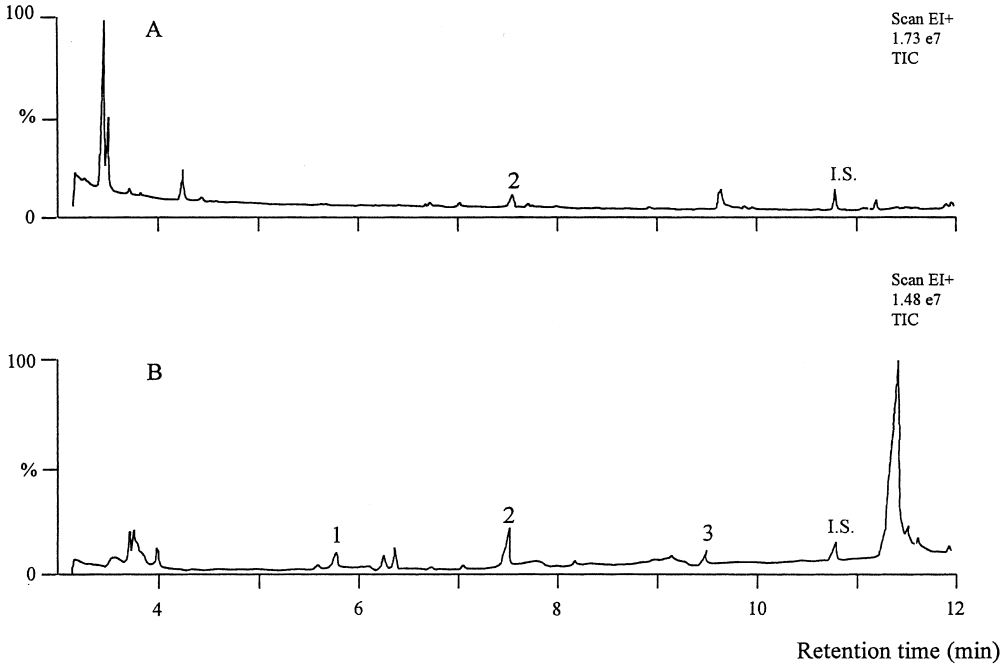

Fig. 3. Total ion current chromatograms for an unhydrolysed (A) and a hydrolysed (B) urine sample. Peaks: (1) phenol; (2) 4-MP; (3) 2,4-DCP; and I.S.: internal standard.

obtained from individuals that were exposed neither to these compounds or to other chlorinated substances commonly present in the environment that are metabolized into chlorophenols [3,4]. Fig. 3 shows the total ion current chromatograms for an unhydrolysed (A) and a hydrolysed (B) urine sample, where phenol, 4-MP and 2,4-DCP were detected.

Taking into account the heterogeneous, and extensive distribution of polyphenols in the plant kingdom, this type of substance becomes a substantial

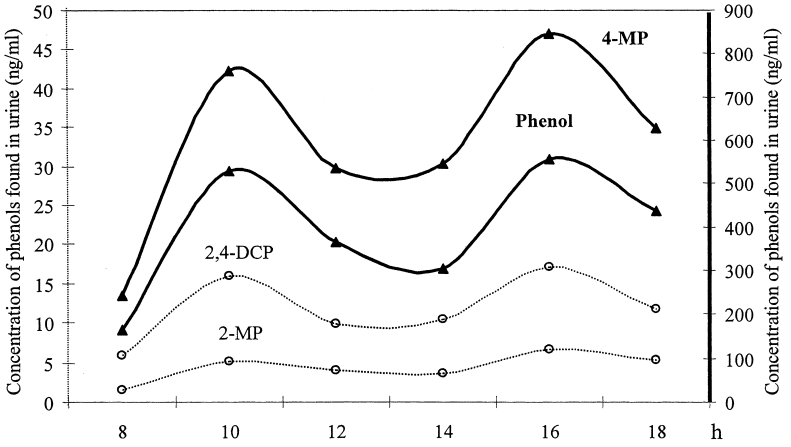

Fig. 4. Influence of dietary intake on the amount of phenol compounds released in urine by a healthy person. 8 and 14 h correspond to urine samples taken before breakfast and lunch, respectively. For details, see text.

part of our daily diet through the intake of vegetables. The proportion of alkylphenols and, especially, those of phenol and 4-MP (*p*-cresol) is related to food intake [20]. The proposed method was used to determine phenol, alkylphenols and chlorophenols in urine from a healthy individual prior to intake food and during digestion. Samples were collected at different times of day, namely: 8 (before breakfast), 10, 12, 14 (before lunch), 16 and 18 (before dinner). Each urine sample was hydrolysed and analysed using the proposed method. The results obtained are shown in Fig. 4. As can be seen, only phenol, 4-MP, 2,4-DCP and 2-MP were detected. The curves are similar for these four compounds. Thus, the lowest levels were found during fasting and after digestion; by contrast, the highest levels were observed 2 h after food intake, whether it was breakfast (coffee, milk, butter, fruit juice and toasts) or lunch (legumes, meat, fruit, etc.). Consequently, the levels of these compounds in urine look related to food intake. A larger population should be studied, however, in order to confirm these results.

Acknowledgements

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