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Letter to the Editor

Reply to comment on "Isolation and detection of steroids from human urine by molecularly imprinted solid-phase extraction and liquid chromatography" by Tse Sum Bui and Haupt [J. Chromatogr. B 877 (2009) 4180–4181]

The authors appreciate the efforts of Tse Sum Bui and Haupt [1] for attentive reading our paper [2].

The steroidal hormones do occur in several forms in human urine (i.e., free forms, steroid glucuronides, steroid sulphates, etc.). Hence, an analysis of these hormones in urine requires complete hydrolysis of the known conjugates and sulphates. Complete hydrolysis requires a combination of enzymatic treatment, acid hydrolysis and solvolysis (i.e., mild acid hydrolysis for sulphates). The problem with this treatment is in its effectiveness, because many steroid forms are resistant to treatment or prefer only one type of hydrolysis (e.g., sulphates prefer only solvolysis). Hydrolysis is strongly influenced by several parameters including the type of acid employed (i.e., hydrochloric acid, sulphuric acid), acid molarity, temperature, and the time of reaction [3,4]. For these reasons, we did not utilize such methods for preparation of human urine samples in this work. With regard to this issue, there is a mistake in Table 6. The results in this table refer to spiked urine samples.

The urinary excretion noted for total and free testosterone, progesterone, and estradiol was of the same order as reported by other authors [5–14]. The variation within an individual for the urinary excretion of testosterone, progesterone and estradiol was appreciably greater than for the variation in serum. The results of this study do not support the suggestion that the variation in urine values, reflecting the integrated secretion of hormones, might show variation in concentrations of free hormones. (*Note*: These hormones are produced by several organs in human body and levels vary between individuals in humans.) This discrepancy might be due to additional factors, such as changes in the volume of collected urine, levels of physical and sympathetic nervous activity, and dietary or nutritional factors that affect the urinary excretion of hormones [15–18]. It can be concluded from this that there is an appreciable variation in the urinary excretion of testosterone progesterone, and estradiol (both total and free) in men and women. A single determination of testosterone, progesterone and estradiol is probably adequate for clinical and epidemiological studies, but replicate determinations would seem to be necessary for the more complicated measurement of urinary sex hormone excretion. Levels of unconjugated testosterone, estradiol and progesterone in human urine can also change before and after storage at various temperatures [19]. Examples of variations that can occur in the concentrations of urinary steroids are shown in Table 1.

The preparation of molecularly imprinted polymers (MIPs) based on non-covalent interactions has become a widely used technique for creating highly specific sorbent materials and has predominantly been used in separation chemistry. A crucial factor in creating a successful imprinting protocol is the template–functional monomer interaction in the pre-polymerization mixture, which should eventually lead to a maximum of high-affinity binding sites in the resulting polymer matrix. Most approaches to non-covalent imprinting have utilized the formation of hydrogen bonds between the functional monomer and template molecule as the basis for an imprinting effect. This has the limitation of excluding polar solvents from the polymer synthesis step, since they are liable to rupture the hydrogen bonds of the pre-polymerization complex [20]. However, other non-covalent interactions, such as hydrophobic interactions, electrostatic (ionic) interactions and stacking effects, are exploited in polar media, as demonstrated by the work in the research groups of Haupt et al. [21] and Mizaikoff [22]. The MIPs in this study were synthesized in aprotic non-polar solvents such as chloroform, acetonitrile and mixture isooctane–toluene (1:99, v/v) and template was extracted by acetonitrile and methanol containing acetic acid, as chosen to fit with the elution step of molecular imprinted solid-phase extraction (MISPE) and the properties of steroids to be examined. ^{1}H NMR spectroscopy has been routinely used to provide evidence of bleeding of residual template – steroid, which in this case remained in the polymer after processing (template extraction) of the MIP. In aprotic solvents (e.g., chloroform, acetonitrile, toluene, and isooctane) contact hydrogen bonded assemblies are formed where the association strength for a given acid increases with the basicity of the base [23]. However, this factor is important in the preparation and synthesis of MIPs and not in the use of MIPs as sorbents for solid-phase extraction.

In an MISPE procedure, it is important to consider the physical, chemical and biological properties of the analytes (e.g., steroids) that will be determined with this method. With regard to this fact, we selected to use acetonitrile, chloroform (dipolar aprotic), methanol (polar protic) and dichloromethane–methanol (80:20) as non-polar/polar protic combinations. Other factors were also considered before HPLC was used, such as the inertness of the material (packings encapped – DB-C18 column) to the reaction conditions, the solubility of the analytes, the use of a combination with an appropriate boiling point, miscibility with water, and the ability to be removed at the end of the MISPE process. The choice of eluents for the extraction of the steroids was based on information from literature and based on the properties of steroids. Acetonitrile, methanol, chloroform, and dichloromethane are usually used for the extraction of steroids due to their ability to disrupt steroid–protein binding. Despite the successful use of halogenated solvents, such as dichloromethane and chloroform in several studies, such solvents should be avoided when new methods are developed since these solvents are banned from use in

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Table 1
Analytical and intraindividual variations for several types of sex hormones in serum (S) and urine (U), as obtained from repeated measurements at an interval of 3 weeks.

Type of sex hormone	*N*	$C.V._{.a}$%	$C.V._{.i}$ %
S-testosterone	46	4.1	9.3
S-free testosterone	46	4.1	9.3
S-estradiol	46	6.6	23.4
S-free estradiol	46	6.4	22.8
U-total testosterone	46	7.2	25.0
U-free testosterone	46	5.5	51.7
U-total estradiol	46	4.9	30.4
U-free estradiol	46	7.1	38.5

Abbreviations: *N*, number of subjects; $C.V._{.a}$%, analytical coefficient of variation (from duplicate assays); $C.V._{.i}$%, intraindividual variation expressed as coefficient of variation (from duplicate assays) [13].

a number of countries because of their negative effects on the environment [24]. Urinary non-conjugated testosterone was determined by extracting urine samples with dichloromethane without enzyme hydrolysis [25]. Steroids were eluted with methanol and methanol/chloroform at room temperature. Essentially quantitative recoveries were obtained with both adsorbents [26].

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