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# Biological Monitoring of Environment Exposure to Safrole and the Taiwanese Betel Quid Chewing

M. J. W. Chang, C. Y. Ko, R. F. Lin, L. L. Hsieh

Department of Public Health, Chang Gung University, 259 Wen-Hwa 1st Rd., Tao-Yuan, 333, Taiwan

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**Abstract.** A rapid and sensitive biological monitoring (BM) method for assessing exposure to the environmental carcinogen safrole has been developed. The method is an isocratic high-performance liquid chromatographic (HPLC) analysis of urinary dihydroxychavicol (DHAB) and eugenol, the urinary metabolites of safrole. Good linearity, precision, and accuracy were demonstrated. A recovery of 98.8 ± 5.4% (SD, n = 3) was found for DHAB and 84.1 ± 3.4% (n = 3) for eugenol. The quantitation limits of the method were 8 ng for DHAB and 10 ng for eugenol. The validity of the method was demonstrated by a linear dose-response relationship observed in rats given oral doses of safrole at 30, 75, and 150 mg/kg body weight. The method was also used to monitor the environmental exposure to the Taiwanese betel quid (TBQ) chewing, because TBQ used in Taiwan not only contains areca (betel) nut, slaked lime, and catechu but also *Piper betle* inflorescence or its leaves. Both of the latter have a high content of safrole. The feasibility of the method to monitor TBQ chewing was demonstrated by an analysis of 153 spot human urine samples. The results showed that the p value of the nonparametric group comparison was $< 0.001$ for DHAB and 0.832 for eugenol. The TBQ chewers also exhibited a significantly higher rate of urinary DHAB (but not eugenol) than the nonchewers with an odd ratio of 3.47 (95% CI, 1.61–7.51). However, when only the eugenol-positive subjects were taken into analysis, the ratio rose to 24.38 (95% CI, 3.00–197.90).

Safrole (4-allyl-1,2-methylenedioxybenzene) is a known animal liver carcinogen. Its carcinogenicity correlates well with its DNA adducts formed in the rat liver (Phillips *et al.* 1981). Safrole exists in many plant species, such as sassafras root bark, star anise, cumin, black pepper, ginger, as well as *Piper betle* inflorescence (Fischer and Dengler 1990; Hwang *et al.* 1992). The latter is frequently included in the Taiwanese betel quid (TBQ) chewing for its aromatic flavor. Other ingredients of the TBQ include areca (betel) nut, slaked lime, and catechu (Hsieh *et al.* 2001). Fresh *P. betle* inflorescence contains 15.35 mg safrole, 9.37 mg dihydroxychavicol (DHAB), and 2.53 mg eugenol per gram (Hwang *et al.* 1992).

It has been shown that DHAB is the major urinary metabolite of safrole in humans and rats, and eugenol is its minor metabolite (Benedetti *et al.* 1977; Klungsoyr and Scheline 1983). Both DHAB and eugenol are excreted in conjugated forms. Structurally, eugenol is the methylenedioxy-ring-opened safrole, and DHAB is the demethylated eugenol (Figure 1). Both metabolites are phenolic compounds found in plants (Shenoy and Choughuley 1989; Fischer and Dengler 1990). DHAB itself is genotoxic, as shown in the induction of oxidative DNA damages in Chinese hamster ovary cells (Lee-Chen *et al.* 1996). Eugenol, a constituent of essential oils, has been used as an analgesic in dentistry (Fischer and Dengler 1990). Both compounds possess an antinitrosating activity, as demonstrated by Shenoy and Choughuley (1989).

Based on a case-control study, Ko *et al.* (1995) demonstrated a statistically significant association between oral cancer and TBQ chewing alone in the Taiwanese population. The cigarette-smoking and alcohol-drinking adjusted odd ratio was 6.9 with 95% CI of 3.1–15.2 (Ko *et al.* 1995). Recently, safrole-DNA adducts were demonstrated, with $^{32}$P-postlabeling technique, in 77% of the oral squamous cancer specimens collected from Taiwanese patients who were TBQ chewers, as compared to 0% of the nonchewers (Chen *et al.* 1999). An etiology of the involvement of safrole-DNA adducts in the induction of the Taiwanese oral squamous cell carcinoma (OSCC) was strongly suggested.

To assess the contribution of safrole in the induction of oral and possibly liver cancer among the TBQ chewers, it is desirable not only to further delineate the association between the safrole-DNA adducts and the cancer incidence in a larger cohort but also to have some assessable biomarkers to serve as a measure of the internal dose of TBQ exposure. This study was initiated to establish a suitable biological monitoring method for this purpose.

## Materials and Methods

### *Chemicals*

Safrole (purity 97%) was purchased from Aldrich (Milwaukee, WI). Eugenol (4-hydroxy-3-methoxyallybenzene, 99%) and glucuronidase

*Correspondence to:* M. J. W. Chang; *email:* mjwc@mail.cgu.edu.tw

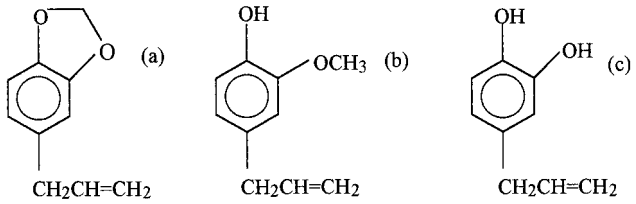


**Fig. 1.** The molecular structure of safrole (a), eugenol (b), and DHAB (c)

(type H-2 from *Helix pomatia*) were from Sigma (St. Louis, MO). Aluminum iodide, acetonitrile, and hydrochloric acid were from Merck (Munchen, FRG). Glacial acetic acid was from Tedia (Fairfield, OH). The maker of Mazola corn oil was CPC International (Englewood Cliffs, NJ).

## *Synthesis of DHAB*

DHAB (also known as 1,2-dihydroxy-4-allybenzene) was not commercially available. It was synthesized via a demethylation of eugenol with freshly prepared aluminum iodide in dry acetonitrile and cleaned up with preparative thin-layer chromatography (TLC; Shenoy and Choughuley 1989). Its structure was confirmed by nuclear magnetic resonance (NMR) (AC-300, Bruker, Rheinstetten, FRG), and a purity of $\geq$ 95% was estimated by the results of NMR, TLC, and HPLC analyses. The molecular structures of safrole, eugenol, and DHAB are depicted in Figure 1.

## *Animal Study*

For validity study, adult male Wistar rats (obtained from the animal center of the National Science Council, ROC) were acclimated individually in metabolic cages (Nalge, Rochester, NY) for a few days before dosing. Four animals per dose group were fasted overnight, from 5 PM to 9 AM of next morning and administered orally with safrole dissolved in Mazola corn oil at 0, 30, 75, 150, and 300 mg/kg body weight. Individual urine sample was collected in the dark for 0–24 h, and stored at −30°C until analysis (Chang *et al.* 1997).

## *Samples of Human Urine*

For feasibility study, a portion of each of the 153 spot urine samples of male subjects, with and without habitual TBQ chewing, were generously shared by a public health study from the south of Taiwan. Chewing habit was documented by questionnaire. The starting age of TBQ chewing and year of habitual chewing ranged from 14 to 61 years and less than 1 to 61 years, respectively. The number of TBQ chewed per day ranged from 2 to 100, most typically 10/day (33.3%), then 20/day (19.4%), and 5/day (13.9%). Individual spot urine samples were collected in a wide-mouth polyethylene bottle, transported to the lab in a container packed with blue ice, and stored at −70°C until analysis.

## *Sample Preparation*

The preparation involved a quick thaw of the frozen specimen at 37°C and a brief centrifugation at 10,000 *g* for 20 min to remove particulate matters. A small aliquot of the supernatant was saved for the determination of urinary creatinine by Jaffe's reaction (Chang *et al.* 1993).

Initially, glucuronidase was used to cleave urinary conjugates as described in the reports of Benedetti *et al.* (1977) and Fischer and Dengler (1990). The incubation time at 37°C was 16 h. The enzymatic hydrolysate was then extracted two times with ethyl acetate. The extracts were combined, dried under nitrogen gas, and dissolved in methanol for HPLC analysis.

Later, a dilute hydrochloric acid (HCl, 1 N) hydrolysis under nitrogen gas was used to accomplish the deconjugation of metabolites (Chang *et al.* 1993). Essentially, 0.9 ml of a supernatant was mixed with 80 μl of the cold concentrated HCl (12.4 N) in a 2-ml brown microcentrifuge tube, flushed with nitrogen gas, capped, and incubated in an 85°C water bath for 2 h. At the conclusion of the acid hydrolysis, the mixture was cooled in an ice-water bath for 20 min. Another centrifugation and then a syringe-filtration with Millex HV 13 mm filter (0.45 μm, Millipore, Bedford, MA) were used to further clean up the sample.

## *Chromatographic Equipment and Conditions*

The quantitative analysis of urinary DHAB and eugenol was done with a reverse-phased isocratic HPLC system (an Eldex 9600 coupled with an Agilent 1100 autosampler, and a Linear UVIS 204 set at 280 nm) as described previously (Chang and Lin 1995; Chang *et al.* 1996, 1997). The column used was a Hypersil Elite ODC column (250 × 4.6 mm, 5 μ, Shandon HPLC, Cheshire, UK). The mobile phase consisted of acetonitrile mixed with 1% acetic acid in water at a 25:75 v/v ratio. A flow rate of 1.0 ml/min was used and the injection volume was 50 μl. An HPLC chromatogram of the DHAB and eugenol standards and a representative human urinary chromatogram are presented in Figure 2.

## *Recovery Studies*

The recovery studies were carried out separately on DHAB- and eugenol-spiked control human urine (nonchewers). Each recovery study was done with two series of parallel, spiked standards in mobile phase or pooled control urine. Both series were analyzed simultaneously. A linear regression equation was computed separately for each series. The recovery was calculated as the slope ratio of the urine standards over mobile phase standards (Chang *et al.* 1993, 1996, 1997; Chang and Lin 1995). No correction for the recovery was made for animal or human urines.

## *Quality Control*

A working standard curve, consisting of five-pair of mobile phase standards of DHAB and eugenol, was included in every batch of the analysis. The percentage of relative error for each standard was kept at no greater than ± 10%. Additionally, the linear correlation coefficient was kept at no less than 0.995.

## *Data Analysis*

The linearity and accuracy of each of the standard curves were evaluated by the correlation coefficient of the least-squares method and percentage of relative error, respectively. The precision of all interday determinations was evaluated by the percent coefficient of variation (%CV). Detection limit was calculated by the three times standard

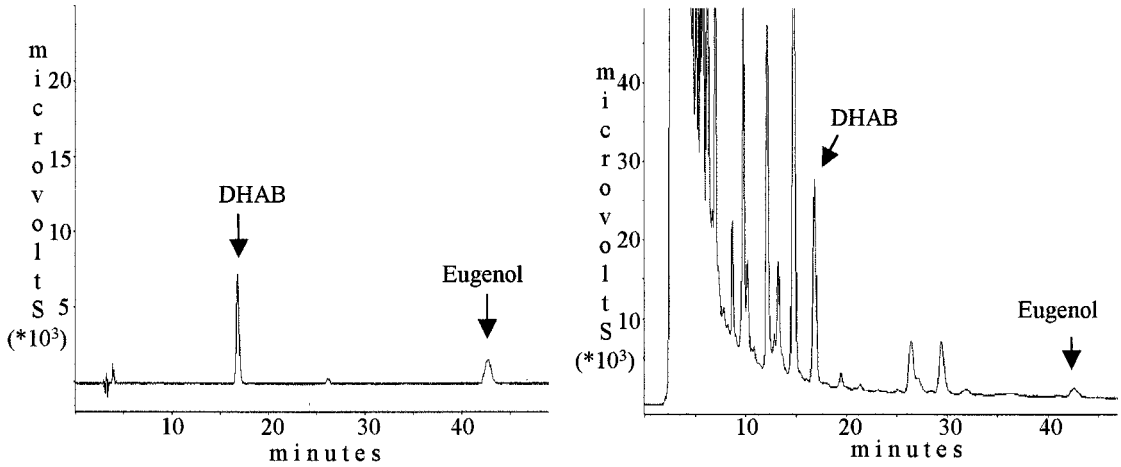


**Fig. 2.** HPLC chromatograms of standards in mobile phase and a representative human urine sample

deviation of a mean from seven samples of a very low concentration of spiked standard, with all samples going through the acid hydrolysis. The association between TBQ chewing and the detection of urinary DHAB and/or eugenol was examined by the chi-squared test. The group comparison was done with the nonparametric Mann-Whitney analysis.

## Results

### *Enzymatic Versus Acid Hydrolysis*

It has been shown that the major urinary metabolite of safrole in both humans and rats is DHAB, which is excreted in conjugated forms (Benedetti *et al.* 1977; Klungsoyr and Scheline 1983). Benedetti *et al.* (1977) also detected mall quantity of eugenol in the rat urine. Initially, our focus was centered on the analysis of urinary DHAB only. To free DHAB from its conjugated forms, enzyme glucuronidase of Sigma type H-2 was used. At the end of the enzymatic hydrolysis, protein was removed either by solvent (ethyl acetate) extractions or by an acid precipitation (cold hydrochloric acid to a final concentration of 0.5 N). When these two approaches of protein removal were compared using samples of safrole-dosed rat urine, a significantly lower recovery of DHAB was obtained by the solvent extraction, *i.e.*, 80.7 ± 8.4% (n = 4) of the acid precipitation.

The higher recovery of DHAB by acid precipitation suggested that DHAB was stable in the diluted hydrochloric acid. Therefore, an attempt was made to evaluate the possibility of using acid hydrolysis (Chang *et al.* 1993) to replace the enzymatic hydrolysis (Benedetti *et al.* 1977; Fischer and Dengler 1990). Again, safrole-dosed rat urine samples were used to evaluate the efficacy of glucuronidase versus HCl hydrolysis. Results showed that the recovery of the enzymatic hydrolysis under optimal conditions (30 μl enzyme per ml urine at pH 5.0, 37°C, for 16 h) was only 83.3 ± 11.2% (n = 4) of the acid hydrolysis. It was, therefore, decided to use the 1 N HCl hydrolysis to release DHAB and eugenol from their conjugated forms.

### *Recovery Studies*

After the initial condition settings for sample preparation were accomplished, the evaluation of recovery as well as linearity of both standard curves, DHAB and eugenol, were ready. A full-range recovery study of DHAB and eugenol was performed on DHAB- and eugenol-spiked, pooled TBQ nonchewer urine. The DHAB standard curve covered a range of 3.5–28 μg/ml and that of eugenol 1.5–12 μg/ml. All standard curves had a linear correlation coefficient greater than 0.995 (Tables 1 and 2). The recovery, calculated as the slope ratio of the spiked urine standards over mobile phase standards, was found to be 98.8 ± 5.4% (n = 3) for DHAB, and 84.1 ± 3.4% (n = 3) for eugenol. All interday %CVs were about or less than 10% for DHAB and less than 5% for eugenol. All percent relative errors were less than 5% for both metabolites (Tables 1 and 2).

### *Validity Study*

An animal model was used to evaluate any relevance between the measured urinary metabolites and an exposure to safrole. As shown in Table 3, a linear dose-response relationship was observed both for the urinary DHAB and eugenol for the dose groups of 30, 75, and 150 mg/kg, $r = 0.992$ and 0.998, respectively. However, when the dose range extended to 300 mg/kg, $r$ dropped to 0.955 and 0.985. This strongly suggested that a metabolic saturation of safrole occurred between 150 and 300 mg/kg in rats.

### *Feasibility Study*

The feasibility of the developed HPLC method for the biological monitoring of an environmental exposure to safrole was verified with analysis of spot urine samples collected from 153 TBQ chewers and nonchewers. The level of DHAB and eugenol in these human subjects was found to be much lower than

**Table 1.** Recovery and linearity studies of the DHAB-spiked control human urine

| | Mean Peak Height ± SD | %CV, n = 3 | % Relative Error |
|---|---|---|---|
| Mobile phase std DHAB (μg/ml) | | | |
| 3.5 | 19,121 ± 1,992 | 10.4 | +2.4 |
| 7.0 | 39,044 ± 3,524 | 9.0 | +0.5 |
| 14 | 78,033 ± 3,614 | 4.6 | −1.5 |
| 21 | 119,864 ± 6,477 | 5.4 | +0.1 |
| 28 | 160,475 ± 4,950 | 3.1 | +0.2 |
| Mean slope ± SD | 5,774 ± 201 | 3.5 | $r$ = 0.99993 |
| Spiked urine std DHAB (μg/ml) | | | |
| 3.5 | 15,786 ± 1,399 | 8.9 | +1.8 |
| 7.0 | 35,220 ± 2,378 | 6.8 | −0.4 |
| 14 | 75,220 ± 6,227 | 8.3 | −0.2 |
| 21 | 114,778 ± 8,877 | 7.7 | −0.4 |
| 28 | 155,695 ± 12,310 | 7.9 | +0.3 |
| Mean slope ± SD | 5,707 ± 452 | 7.9 | $r$ = 0.99998 |

All spiked urine samples have been through the 1.0 N HCl hydrolysis. The recovery, by slope ratio, was 98.8 ± 5.3% (n = 3).

**Table 2.** Recovery and linearity studies of the eugenol-spiked control human urine

| | Mean Peak Height ± SD | %CV, n = 3 | % Relative Error |
|---|---|---|---|
| Mobile phase std eugenol, μg/mL | | | |
| 1.5 | 667 ± 16 | 2.3 | −0.4 |
| 3.0 | 1,579 ± 57 | 3.6 | +1.6 |
| 6.0 | 3,254 ± 85 | 2.6 | −1.7 |
| 9.0 | 5,128 ± 185 | 3.6 | +1.0 |
| 12 | 6,827 ± 201 | 2.9 | −0.2 |
| Mean slope ± SD | 588 ± 19 | 3.2 | $r$ = 0.99986 |
| Spiked urine std eugenol, μg/mL | | | |
| 1.5 | 950 ± 38 | 4.0 | −1.3 |
| 3.0 | 1,713 ± 21 | 1.2 | +0.8 |
| 6.0 | 3,192 ± 35 | 1.1 | +0.3 |
| 9.0 | 4,646 ± 45 | 1.0 | −0.4 |
| 12 | 6,157 ± 237 | 3.9 | +0.2 |
| Mean slope ± SD | 494 ± 19 | 3.9 | $r$ = 0.99998 |

As in Table 1 and the recovery was 84.1 ± 3.4% (n = 3).

**Table 3.** Dose-response relationship of safrole versus acid-released urinary DHAB and eugenol in rat

| Safrole (mg/kg) | DHAB in Day 1 Urine | Eugenol in Day 1 Urine |
|---|---|---|
| 30 | 386.9 ± 97.6 | 12.8 ± 3.6 |
| 75 | 962.9 ± 139.0 | 22.5 ± 2.6 |
| 150 | 1,568.5 ± 214.2 | 35.2 ± 13.6 |
| 300 | 2,063.1 ± 197.8 | 50.3 ± 12.0 |
| Linear $r$, n = 3 (30–150 mg/kg) | 0.992 | 0.998 |
| n = 4 (30–300 mg/kg) | 0.955 | 0.985 |

All values were expressed as mean ± SD in μg/mg creatinine, n = 4. No correction was made for the recovery of either DHAB or eugenol.

those seen in the rat study. Two new standard curves, ranging from 0.2 to 2.0 μg/ml for DHAB and 0.6–5 μg/ml for eugnol, had to be reestablished. These low standard curves also possessed good linearity ($r$ = 0.9996 for DHAB and 0.9981 for eugenol), precision (all interassay %CV < 10% for DHAB and < 15% for eugenol, n = 3), and accuracy (all % relative errors ≃ ± 10%). The quantitation limits of the HPLC method, established with these new low standards, were 8 ng for DHAB and 10 ng for eugenol.

The group means and ranges of the 153 human samples are summarized in Table 4, and the group population distributions are presented as box-plots in Figure 3. Because the distribution was not normal, nonparametric Mann-Whitney analysis was used for group comparison. A significant difference was found for DHAB ($p < 0.001$) but not eugenol ($p = 0.832$). The TBQ chewers also exhibited a significantly higher rate of urinary DHAB (66% versus nonchewers, 36%) but not eugenol. The chi squared analysis gave an odd ratio of 3.47 (95% CI, 1.61–7.51) for TBQ chewing associated with DHAB and only 0.95 (95% CI, 0.45–1.98) for eugenol. However, when the chi squared analysis was performed on the eugenol-positive subjects only (a total of 70), the ratio rose to 24.38, with a 95% CI of 3.00–197.90.

## Discussion

Both gas chromatography (Klungsoyr and Scheline 1983) and HPLC (Fischer and Dengler 1990; Hwang *et al.* 1992) have

**Table 4.** Data of urinary DHAB and eugenol of the 153 human subjects

| | DHAB, Group Mean, Range (% Positive) | Eugenol, Group Mean, Range (% Positive) |
|---|---|---|
| TBQ nonchewer (n = 115) | 2.47<br>ND–5.4 (41/115 = 36%) | 0.31<br>ND–5.8 (53/115 = 46%) |
| TBQ chewer (n = 38) | 2.18<br>ND–37.2 (25/38 = 66%) | 0.55<br>ND–10.9 (17/38 = 45%) |

Unit in μg/mg creatinine.
ND: below detection limit, which was 0.16 μg/ml for DHAB and 0.20 μg/ml for eugenol.

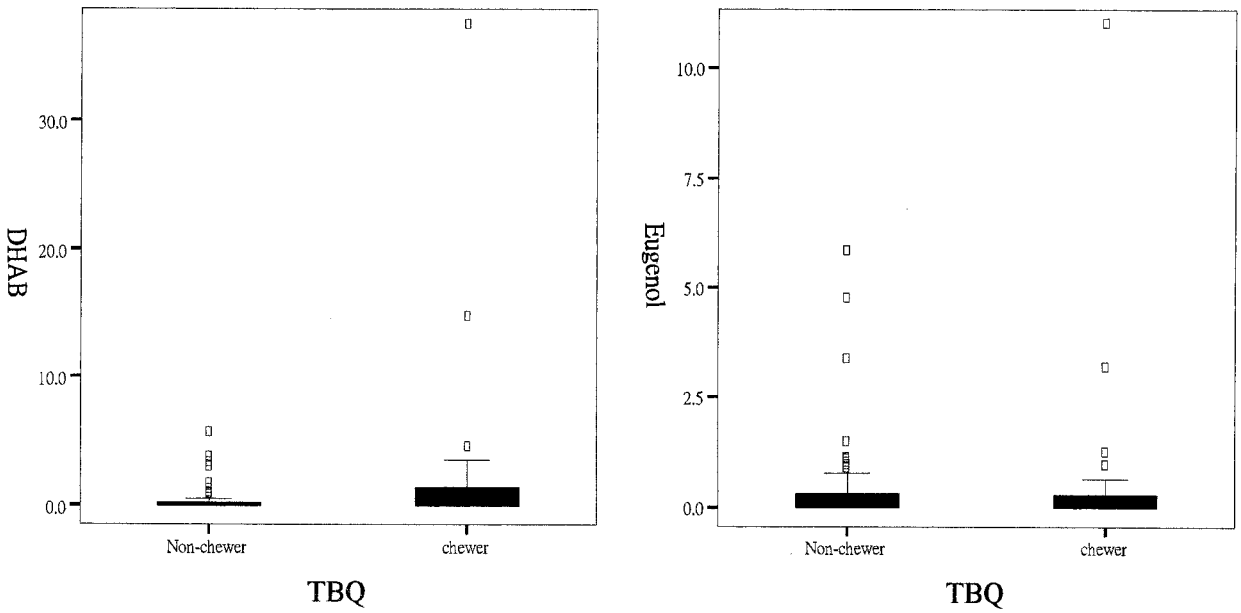


**Fig. 3.** Box-plots of TBQ chewing versus urinary DHAB ($p < 0.001$) and eugenol ($p = 0.832$), units in μg/mg creatinine

been used to analyze DHAB or eugenol. Neither Klungsoyr and Scheline (1983) nor Hwang *et al.* (1992) gave data on the recovery, accuracy, and precision. In the latter report, gradient elution was used.

The final standardized sample preparation described herein involved a rapid and simple acid hydrolysis (1 N HCl at 85°C for 2 h) without solvent extraction. This method, when compared with the sample preparation of urine of the eugenol-dosed human volunteer involving an enzymatic hydrolysis and clean-up with a Sep-Pak cartridge, is much simpler and suitable for an analysis of large sample size.

Initially, two comparisons were done to reach this final version of sample preparation. One was solvent extraction versus acid precipitation in the removal of unwanted macromolecules, and the second was enzymatic hydrolysis (Fischer and Dengler 1990) versus acid hydrolysis (Chang *et al.* 1993) to free DHAB from its conjugates. As shown in the results, the acid precipitation was better than the solvent extraction in recovery as well as in simplicity, and the acid hydrolysis is better than the enzymatic hydrolysis in recovery and in time efficiency (2 h versus 16 h).

Once our sample preparation was finalized, the overall recovery from the acid hydrolysis of the DHAB- and eugenol-spiked urines, ranging from 3.5 to 28 μg/ml and 1.5 to 12 μg/ml, respectively, was evaluated. The overall recovery of DHAB was 98.8 ± 5.3% and eugenol, 84.1 ± 3.4% (n = 3) (Tables 1 and 2). The recovery of eugenol reported by Fischer and Dengler (1990) was 76.8 ± 0.3% to 98.4 ± 2.3% (n = 3) and was accomplished by simple solvent extraction on the eugenol-spiked body fluids without involving hydrolysis. Their intra-assay %CVs were less than 4%, and percent relative errors were less than 5%. By comparison, our interday assays, as presented in Tables 1 and 2, found that the overall %CVs including acid hydrolysis were of ≤ 10% and percent relative errors of less than 3%.

Our quantitation limits were 8 ng (or 0.16 μg/ml) for DHAB and 10 ng (0.20 μg/ml) for eugenol. The latter was 100-fold higher than what was reported by Fischer and Dengler (1990). Again, only the eugenol-spiked urine was used, and no hydrolysis was involved in their evaluation. Another contributing factor was 220 nm, the maximum UV absorption of eugenol, was used in the report for detection, whereas we used 280 nm to maximize the response of DHAB.

The validity of our method for the biological monitoring of an exposure to safrole was clearly demonstrated by our rat study. As presented in Table 3, all safrole-dosed rat urines showed measurable DHAB and eugenol. The eugenol measured was only about 2.3 ± 0.1% of the DHAB. No detectable

DHAB or eugenol was seen in the control rat urines collected from animals given Mazola corn oil only (n = 4, data not shown). The metabolic saturation observed in our rat study was compatible with observations made by others. Previously when safrole was given orally to rats at 1 mmol/kg (*i.e.*, 162.2 mg/kg), about 77.5% of the dose was eliminated in urine within 24 h (Klungsoyr and Scheline 1983). Another study reported that rats orally administered with safrole at 750 mg/kg resulted in an elimination of about 25% of the dose in urine in 24 h (Benedetti *et al.* 1977).

The feasibility study of using urinary DHAB and eugenol as the exposure biomarker for TBQ chewing was evaluated with 153 spot human urines. As stated before, the reason for proposing to use urinary DHAB and/or eugenol as potential exposure biomarker for TBQ chewing was that one of the TBQ ingredients, the *P. betle* inflorescence, has a high content of safrole (Hwang *et al.* 1992). However as shown in Table 4, positive detection of DHAB and eugenol were also observed in urine of the nonchewers (DHAB 36%, eugenol 46%). This was not totally surprising due to the fact that safrole exists in many other common spices, such as ginger and black pepper, which are frequently used in the Taiwanese cooking. A daily consumption of eugenol at 0.6 mg per capita, established by the World Health Organization (Fischer and Dengler 1990), also suggests that seeing eugenol in some of the nonchewers was not unexpected.

As shown in Figure 3, the level of DHAB found in the nonchewers was significantly lower than in the chewers, but not the level of eugenol. This strongly suggested that the measured DHAB in the TBQ chewers was mainly derived from the *P. betle* inflorescence. Based on the analyses of the odds ratio on the entire population as well as the eugenol-positive subjects only, we conclude that DHAB alone and/or simultaneous detection of urinary DHAB and eugenol can be the exposure biomarker for TBQ chewing.

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