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Microwave-Assisted Photochemical Reactor for the Online Oxidative Decomposition and Determination of *p*-Hydroxymercurybenzoate and Its Thiolic Complexes by Cold Vapor Generation Atomic Fluorescence Detection

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We developed a photochemical method for the online oxidation of *p*-hydroxymercurybenzoate (PHMB), an organic mercury species widely used for mercaptan and thiolic compound labeling. The method is based on a fully integrated online UV/microwave (MW) photochemical reactor for the digestion of PHMB, followed by cold vapor generation atomic fluorescence spectrometry (CVG-AFS) detection. The MW/UV process led to the quantitative conversion of PHMB and thiol−PHMB complexes to Hg(II), with a yield between 91% and 98%, without using chemical oxidizing reagents and avoiding the use of toxic carcinogenic compounds. This reaction was followed by the reduction of Hg(II) to Hg^0, performed in a knitted reaction coil with $NaBH_4$ solution, and AFS detection in an Ar/H_2 miniaturized flame. The low MW power applied (18 W) allowed us to keep constant the temperature of the photochemical reactor (21 ± 1 °C), using a flowing water bath. This avoided peak widening due to diffusion processes generally occurring at high temperatures and in the additional cooling coil. This method has been applied to the determination of thiols in human plasma, blood, and wine.

Chemical labeling of −SH groups represents a common approach for quantification of thiolic compounds. More than 70% of all known proteins contain sulfhydryl groups.

Thiolic groups play fundamental structural and functional roles in protein chemistry, being located mainly within the active site of many enzymes and directly involved in catalysis.[1] Organic mercurial compounds are very specific and sensitive reagents for reaction with sulfhydryl(s) because of the strong mercury−sulfur affinity in a wide pH range (1−13), both for low molecular weight compounds (such as glutathione and cysteine) and for macromolecules (proteins, humic matter).[2,3] RHg^+ species form complexes of well-defined stoichiometry, −S−Hg−R, and they have been widely used for analytical and diagnostic purposes.[4] In particular, *p*-hydroxymercurybenzoic acid (PHMB) has been used for the first time more than 50 years ago for the determination of "free" sulfhydryl groups in proteins.[5] PHMB is a monofunctional, organic mercurial probe; it interacts with high affinity and specificity at room temperature in less than 90 s with reactive −SH groups at basic and neutral pH[6] and in about 50 min in acid medium,[7] giving stable complexes. The interaction between PHMB and −SH functional groups was studied for analytical and diagnostic purposes for proteins,[4,6,8−10] low molecular weight thiols,[7,11−13] mercaptans,[14] and nitrosothiols[11,15,16] using liquid chromatography coupled to chemical vapor generation and atomic fluorescence spectrometry (CVG-AFS). Other authors used PHMB for protein/thiol labeling coupled to mass

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spectrometric detection techniques (MALDI-MS, ICPMS, HPLC−ESIMS).[17−22]

From late the 1980s, the CVG-AFS technique has become the most popular detector for laboratories working on mercury for its high sensitivity, selectivity, and relatively inexpensiveness,[23−26] but the direct introduction of organic mercury into the detector lowers its performance. The use of online oxidation systems for the conversion of organomercury species to Hg(II) is mandatory to obtain higher sensitivity and reproducible results using atomic spectrometric detectors. Common chemical oxidants include $KBr/KBrO_3$,[6−11,14−16,27−31] $K_2S_2O_8$,[32,33] and $K_2Cr_2O_7$.[34,35]

The $KBr/KBrO_3$ oxidation system has the advantage of being performed at room temperature. However, $KBrO_3$ is a toxic and carcinogenic reagent classified as R 45-9-E25. Furthermore, bromine is a fluorescence quencher and the generated excess has to be reduced into bromide during the subsequent reducing step by hydrazine, a compound classified as R45, R10, R23/24/25, R34, R43, R50/53. Currently, chemical oxidation and UV irradiation techniques have been reported. Falter and co-workers adopted UV irradiation to decompose organic mercury.[36−38] Photo-oxidation and photoreduction of mercury and other elements have been recently reviewed by Bendicho et al. in an excellent work.[39]

Recently, Tang et al. proposed UV/HCOOH-induced Hg CVG instead of $K_2SO_8-KBH_4/NaOH-HCl$ and/or $KBrO_3/KBr-KBH_4/NaOH-HCl$ systems as oxidizing/reducing system for low molecular weight thiols tagged with PHMB.[13]

Other authors proposed acidic $K_2S_2O_8$ solution combined with microwave (MW) digestion[40] or MW digestion in acidic conditions.[41]

To the best of our knowledge, the combination between MW and UV irradiation in a unique photochemical reactor without any further online oxidation has never been used for PHMB digestion.

This paper describes a novel high-performance liquid chromatography (HPLC)−MW/UV combined reactor coupled to a CVG-AFS detection system for the determination of PHMB-tagged thiols. The use of a fully integrated MW/UV photochemical reactor[42,43] allowed us to obtain the online digestion of PHMB and thiol−PHMB complexes, to Hg(II) with a yield between 91% and 98%. Hg(II) was reduced to Hg^0 in a knitted reaction coil with $NaBH_4$ solution and detected. The integrated photochemical reactor is able to measure and control the MW power working on the sample during experiments and overcome the large amount of drawbacks given by reactors placed in a microwave oven, or in a waveguide applicator working at 2450 MHz,[43,44] or by an immersed electrodeless MW/UV lamp.[45]

The method was applied to the analysis of low molecular weight thiols labeled with PHMB in blood, plasma, and wine.

EXPERIMENTAL SECTION

Chemicals. Analytical reagent-grade chemicals were used without further purification. The stock solution of 1000 ± 5 $\mu g\ mL^{-1}$ of inorganic Hg in the form of $Hg(NO_3)_2$ was purchased from Merck Laboratory Supplies (Poole, Dorset, U.K.). PHMB (4-(hydroxymercuric) benzoic acid, sodium salt (CAS no. 138-85-2, $HOHgC_6H_4CO_2Na$) was purchased from Sigma (Sigma-Aldrich, Milan, Italy). A 1×10^{-2} M stock solution of PHMB was prepared by dissolving the sodium salt in 0.01 M NaOH in order to improve its solubility, stored at 4 °C, and diluted freshly, just before use. The precise concentrations of PHMB solutions were determined from the absorbance at 232 nm ($\varepsilon_{232} = 1.69 \times 10^4\ cm^{-1}\ M^{-1}$).

Stock solutions of cysteine (Cys, Merck KGaA, 64271 Darmstadt, Germany), glutathione (GSH, G6529-5G), homocysteine (HCys, H4628-1G), cysteinyl-glycine (CysGly, C0166) (Sigma-Aldrich, Inc., St. Louis, MO, U.S.A.) were prepared in 0.1 mol/L HCl. In order to prevent oxidation, standard solutions of thiols were prepared daily, kept cold (4 °C), and protected from light until used.

Trifluoroacetic acid (TFA), ethanol, and methanol for reversed-phase chromatography were purchased from Carlo Erba (Rodano, Milan, Italy).

Solutions of $NaBH_4$ more concentrated than 0.27 M (1% m/v) were prepared by dissolving the solid reagent (Merck & Co., Inc., N.J., U.S.A., pellets, reagent for AAS, minimum assay >96%) into 0.3% (m/v) NaOH solution. The solutions were

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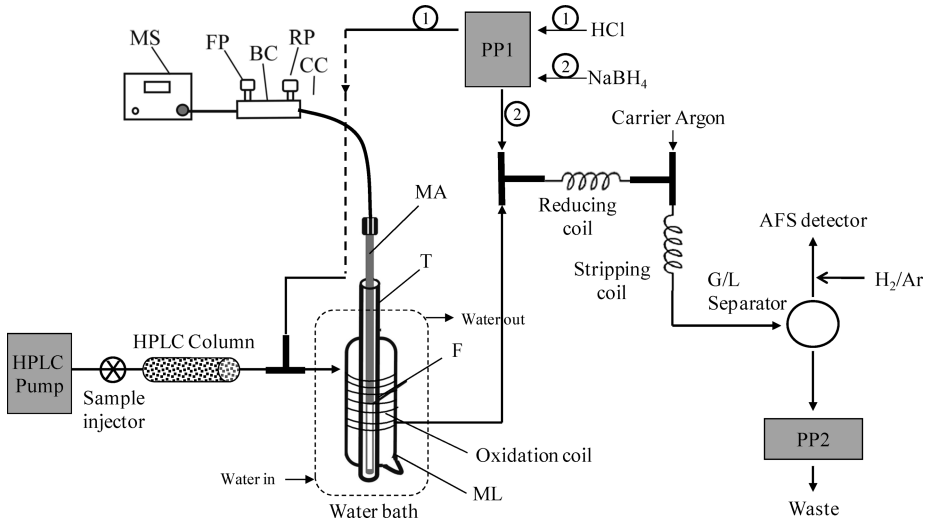

Figure 1. Schematic diagram of the HPLC−MW/UV−CVG-AFS system. MW/UV generator system: MS, 100 W, 2450 MHz, solid-state source; BC, bidirectional coupler; FP, forward power probe-head; RP, reflected power probe-head; CC, flexible coaxial cable. MW/UV quartz photoreactor: ML, bulb of the coaxial electrodeless UV lamp; T, quartz coaxial tubing; MA, microwave antenna; F, point of maximum MW power emission, or feed point; G/L separator, gas/liquid separator; PP, peristaltic pump.

microfiltered through a 0.45 μm membrane and stored in a refrigerator. Diluted solutions of $NaBH_4$ (0.05 M) were prepared by appropriate dilution of the stock solutions, the total NaOH concentration being kept at 0.3% (m/v). HCl diluted solutions were prepared from 37% HCl (Carlo Erba, Rodano, Milan, Italy).

Water deionized with a Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used throughout.

Safety Considerations. PHMB and inorganic Hg are toxic. Inhalation and contact with skin and eyes should be avoided. All work should be performed in a well-ventilated fume hood.

Instrumentation and Chromatographic Conditions. An HPLC−MW/UV combined reactor with CVG-AFS detection system was used for all the measurements and is shown in Figure 1.

An HPLC gradient pump (P4000, ThermoQuest) equipped with a Rheodyne 7125 injector (Rheodyne, Cotati, CA, U.S.A.) and a 50 μL injection loop was used. Separations were carried out with a Phenomenex C12 reversed-phase column (150 mm × 4.6 mm i.d., silica particle size 4 μm). The pump flow rate was 1.0 mL/min. Samples were eluted with a 15 min linear gradient from 100% A (90% TFA 0.01%/10% methanol) to 30% B (methanol 100%). The eluent from the column was mixed in a T with 2 M HCl (2.8 mL/min) and passed through a Teflon coil coiled around a quartz ML bulb (see the next paragraph), immersed in a flowing water bath kept at 21 ± 1 °C. The effluent coming out reacted in a knitted reduction coil with $NaBH_4$ solution introduced by a peristaltic pump at 2.8 mL/min. The mercury vapor generated in the reaction was separated in a gas−liquid separator by Ar/H_2 gas, and the mercury vapor coming out was delivered into the atomizer, which was a miniature Ar/H_2 diffusion flame supported on a simple quartz tube (i.d. 6.5 mm). The gas−liquid separator was in borosilicate glass (60 mm long, 10 mm i.d., inlet and outlet tubing 6 mm o.d. and 2 mm i.d.). A peristaltic pump was used to pump off the waste liquid solutions. A laboratory-assembled nondispersive atomic fluorescence (NDAF) detector[46] equipped with an EDLII mercury lamp (Perkin-Elmer, Monza, Italy) was employed. A description of the NDAF detector has been previously reported.[6,10] More details of the CVG-AFS system can be found in a previous paper.[28] The output data from the lock-in amplifier were collected with a personal computer equipped with a data acquisition card (DAC, National Instruments, Austin, TX) and its acquisition software (LabVIEW version 6, National Instruments). Table 1 summarizes all experimental conditions.

MW Apparatus and Photoreactor. The photochemical reactor is an electrodeless UV lamp excited by MW, surrounded by a Teflon coil, and immersed in tap flowing water for temperature control. The coaxial electrodeless MW/UV lamp was constructed by sealing off a 15 mm external diameter quartz ML bulb, filled with about 1 mg of Hg and 0.66 kPa of argon (Figure 1). A 5 mm × 3 mm fused quartz tubing T, coaxial to ML, was used to accommodate a coaxial dipole antenna, MA, operating at 2450 MHz and producing a plasma discharge inside the bulb. The dipole antenna was made out of a 2.5 mm semirigid coaxial cable, with a maximum power handling capability of about 80 W. About 25% of the MW power was converted in UV radiation, depending on the temperature of the lamp.[43] A 3 mL Teflon coil (length 3.82 m; i.d. 1 mm) was coiled around the quartz ML bulb. In order to perform an isothermal process at room temperature the bulb was partially immersed in a flowing water bath, using cooled tap water from a thermostat and a double-head peristaltic pump (not shown) so that the temperature of the reagents during the process was kept constant within 21 ± 1 °C.

In the MW apparatus a magnetron source MS with a variable output power of up to 100 W in a continuous wave regime at 2450 MHz and provided with a coaxial 50 Ω output port was used for the excitation of the lamp. More details of the microwave apparatus and of the electrodeless lamp (EDL) can be found in previous papers.[42,43]

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Table 1. Summary of Experimental Conditions

Reagents	
HCl conc 2 mol/L	
HCl flow rate 2.8 mL/min	
$NaBH_4$ conc 1% w/v	
$NaBH_4$ flow rate 2.8 mL/min	
HPLC Conditions	
flow rate 1 mL/min	
15 min linear gradient from 100% A (90% TFA 0.01%/10% methanol) to 30% B (methanol 100%)	
MW/Reactor	
optimized power 18 W	
volume of postcolumn reaction coil 3 mL	
length of postcolumn reaction coil 3.82 m	
i.d. of postcolumn reaction coil 1 mm	
temperature of postcolumn reaction coil 21 ± 1 °C	
reaction time 50 s	
NDAFS Detector	
miniature flame atomizer: Ar (stripping) = 140 mL/min, Ar/H_2 (flame) = 130/80 mL/min	
observation height 3 mm above the burner top	
modulation frequency for EDL 500 Hz	
RC time constant 1 s	

A commercial, electrically powered UV lamp (20 W, Helios Italquartz) with a 2.8 mL reaction coil has been used to evaluate the efficiency of the MW/UV system.

Sample Collection, Storage, and Derivatization Procedure. Fresh thiol stock solution (5−7 g/L) was prepared daily by dissolving the powder in 0.1 mol/L HCl. For calibration experiments, thiol stock solutions were diluted in TFA 0.01%, derivatized with a stoichiometric amount or an excess of PHMB, incubated for 50 min at room temperature (21 ± 1 °C),[7] and then injected in the HPLC system. All the thiol solutions derivatized with PHMB were kept at room temperature and were stable during the working day.

Plasma aliquots were derived from routine clinical samples obtained from six different patients. Venous blood was collected by venipuncture using ethylendiaminotetracetic acid (EDTA) as anticoagulant. After low-speed centrifugation (1500*g*, 10 min) at room temperature, plasma samples were stored at −20 °C until analysis.

Before analysis, plasma samples were diluted 1:1 in TFA 0.01%, 0.5 mmol/L EDTA, loaded onto the sample reservoir of an Amicon Microcon YM-3 centrifugal filter units (cutoff 3000 Da; Millipore, Bedford, MA, U.S.A.) and centrifuged at 11 000*g* for 90 min at 4 °C, to remove proteins and high molecular weight compounds. After centrifugation, ultrafiltrated samples were treated with 100 μmol/L PHMB, incubated for 50 min at room temperature, and injected in the chromatographic system.

Blood was obtained from five volunteer donors. Venous blood was collected by venipuncture in resting conditions into evacuated tubes containing EDTA as anticoagulant. For the determination of free reduced GSH 500 μL of the collected blood was mixed with 500 μL of 10% TCA immediately after collection, and the acidified sample was centrifuged at 10 000*g* for 10 min at 21 °C. A volume of 10 μL of the supernatant was diluted in 990 μL of 0.01% TFA, 0.5 mmol/L EDTA, derivatized with 100 μM PHMB, incubated for 50 min at room temperature, and then injected.

Red wine was sampled from original glass bottles ($N = 4$), kept at 4 °C, and analyzed within 24 h from sampling. An amount of 1 mL of all samples was treated with 200 μM PHMB and injected.

Thiols recovery in plasma, blood, and wine was evaluated by spiking these samples with a known concentration of thiol standard solution before any treatment.

RESULTS AND DISCUSSION

MW/UV Online Oxidation of PHMB. MW/UV online oxidation of PHMB was studied in flow injection (FI) mode by injecting PHMB, Cys−PHMB, GS−PHMB complexes and inorganic mercury as reference. Figure 2A shows the oxidation efficiency for each species, calculated on the basis of their FI peak area with respect to FI peak area of an equimolar concentration of Hg(II) analyzed in the same operating conditions. The oxidation efficiency of PHMB, Cys−PHMB, and GS−PHMB complexes (0.25 μmol injected, $N = 10$ injections) was 98.0% ± 0.4%, 91.1% ± 1.9%, and 91.8% ± 1.7%, respectively, when the microwave generator was turned on (MW power = 18 W). The signal was not significantly different from blank when the MW generator was off. The yield of photo-oxidation reaction of PHMB and thiol−PHMB complexes was comparable with that obtained using chemical oxidation by Br^-/BrO_3^-.[6] The MW/UV system did not affect the intensity of Hg(II) signal. The photo-oxidation reaction was performed in acid medium to obtain a higher sensitivity.

We verified that electrically powered UV lamp can oxidize PHMB and RS−PHMB complexes to Hg(II) with a yield of 83% ± 1% and 86% ± 1%, respectively. Temperature in this case was not controlled and ranged between 26 and 30 °C. In the specific case of mercury detection the use of MW/UV leads to a small but significant enhancement of the yield. In perspective this result suggests that the simultaneous application of MW and UV may be useful for the treatment of more recalcitrant compounds.

Although the electrically powered UV lamp is simpler than our apparatus, the UV lamp in the MW-assisted photoreactor is the only apparatus that permits the control of temperature using a thermostatic bath. This is not possible with electrically powered UV lamps, nor with commercial EDLs. To avoid temperature changes in our system, the photochemical reactor was immersed in a flowing water bath and the temperature was kept constant within 21 ± 1 °C.

The control of photoreaction temperature is important. Using the MW/UV system we observed a decrease of the oxidation yield of PHMB from 98% to 93% and 90% in experiments performed at 21, 30, and 40 °C, respectively. This effect is even more evident in the photo-oxidation of other elements such as selenium (work in progress).

Influence of MW Power. The effect of MW power on oxidation yield was studied on two mercurial species, PHMB and GS−PHMB complex, in order to establish the optimal conditions for organic mercury digestion. Figure 2B shows that, in our conditions, a MW power of 18 W gave the best recovery in mercury digestion for both species, and it was adopted for all measurements.

The use of the MW/UV reactor described in this work has several advantages: (i) it was effective also at low MW power

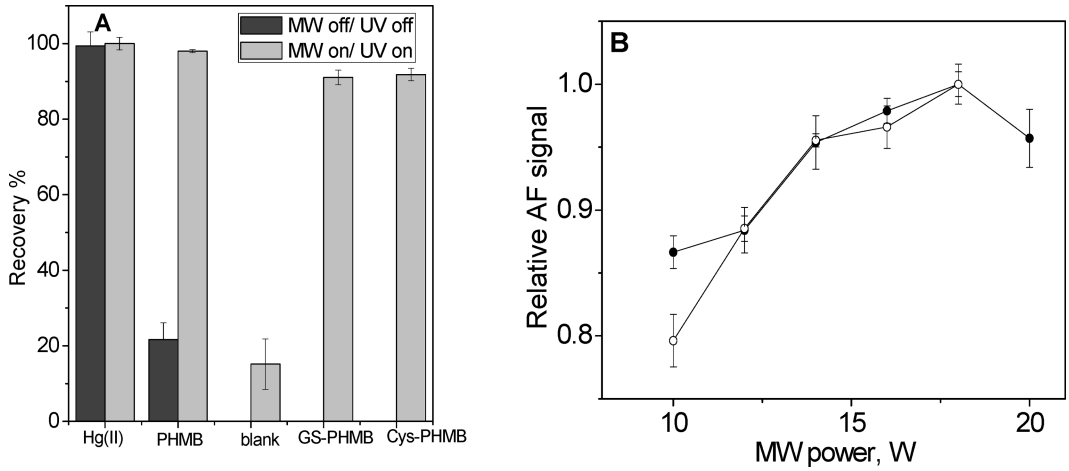

Figure 2. (A) Oxidation yield of PHMB, GS−PHMB, and Cys−PHMB species (2.5 μM concentration injected, 1:1 stoichiometric complexes) digested online in the MW/UV reactor and detected by CVG-AFS. The AFS signal of Hg(II) was taken as reference (diluted in 0.1 mol/L PBS pH 7.4 with 0.5 mmol/L EDTA). MW power = 18 W. (B) Effect of MW power on the oxidation yield of PHMB (filled circles) and GS−PHMB stoichiometric complex (open circles) measured by FIA coupled with CVG-AFS.

Table 2. Calibration Parameters of Mercury Species Analyzed by the HPLC−MW/UV−CVG-AFS System[a]

analyte	t_R (min)	slope ± SD ($10^{-6} \times$ V $\times$ min μM^{-1})	R^2	N	RSD %[b]	recovery %[c]
PHMB	19.78	1.19 ± 0.04	0.9973	5	3	
Cys−PHMB	9.75	1.06 ± 0.07	0.9871	5	1.2	89.1 ± 6.6
GS−PHMB	14.98	1.06 ± 0.03	0.9986	5	0.8	89.1 ± 3.3
GlyCys−PHMB	8.18	1.01 ± 0.06	0.9878	5	1.5	84.9 ± 5.5
HCys−PHMB	12.05	0.93 ± 0.07	0.9799	5	2	78.2 ± 8.0

[a] MW power = 18 W. [b] N = 10 at 2.5 μmol/L. [c] Calculated from the ratio of the slope of calibration curve of each species with respect to the slope of calibration curve of PHMB.

applied (18 W), (ii) it allowed us to control with a simple flowing bath and keep low the temperature of the online photo-oxidation process in the reaction coil, thus avoiding diffusion phenomena that give band widening of chromatographic peaks. In this system the cooling coil is not required, which represents a further advantage with respect to the broadening of chromatographic peaks.

Most important, this reactor allowed us to avoid the use of hydrazine and chemical oxidizing reagents, among these BrO_3^-.

Analytical Figures of Merit. Calibrations of PHMB and thiol−PHMB complexes were performed by injecting standard solutions in the HPLC−MW/UV−CVG-AFS system (0.1−10 μM concentration injected). Sensitivity was the slope value obtained by least-squares regression analysis of calibration curves based on the peak area. Table 2 reports the equations for the calibration curves and the chromatographic retention times (t_R) of PHMB, Cys−PHMB, GS−PHMB, GlyCys−PHMB, and Hcys−PHMB. The limit of detection, equal to about 45 nM for all the species considered in the study, was defined as $3 \times s \times \text{slope}^{-1}$ of the calibration curve, with s being the standard deviation corresponding to 10 blank injections. The limit of quantification (LOQ), defined as $10 \times s \times \text{slope}^{-1}$ of calibration curve, was 150 nM. The RSD %, estimated from 10 standard replicates and calculated at concentrations of 2.5 μM, was between 1% and 3%. Recoveries of thiol−PHMB complexes with respect to PHMB were calculated by the ratio of the slope of the calibration curve obtained for each mercurial complex and the slope of calibration curve of PHMB. Recoveries ranged overall between 78% and 89%, with an RSD of about 3−5%.

Application of the Method: Determination of Low Molecular Weight Thiols as PHMB Species in Blood, Plasma, and Wine. PHMB derivatization coupled with HPLC separation and MW/UV−CVG-AFS was applied to the determination of low molecular weight thiols in blood, plasma, and wine to show its reliability also in complex matrixes.

Accuracy was estimated for GSH in blood and wine and Cys in plasma, on the basis of recoveries obtained on measurements of spiked samples (GSH recoveries in blood and wine, 99.3% and 98.4%, respectively; Cys recovery in plasma, 102.8%).

GSH concentration level in blood ranged between 632 and 864 μM (738 ± 88.6 μM, mean ± SD, N = 5), in agreement with data previously reported.[7] Cys concentration level in plasma ranged between 7.2 and 19.9 μM (14.6 ± 4.4 μM, mean ± SD, N = 6), in agreement with those previously reported.[47] However, GSH in plasma was in all cases undetectable, although in a normal subject this generally ranges between 1 and 10 μM.[47] Instead, low concentrations of CysGly were present (0.2−4 μM). This can be due to the decomposition of GSH by γ-glutamyl transferase, an enzyme of GSH metabolism,[48] and/or its oxidation, which may occur during storing or ultrafiltration. Indeed, GSH determination

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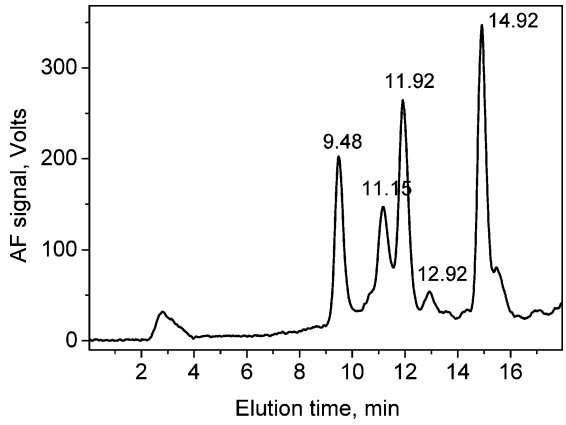

Figure 3. Mercury-specific AF chromatogram of Dolianova red wine (1 mL derivatized with 2×10^{-4} mol/L PHMB). Chromatographic and detection conditions: see the Experimental Section.

in plasma is generally performed adopting a fast deproteinization in 5% TCA, which avoids GSH oxidation.[47] For this work we used plasma samples collected and stored for routine clinical analysis, not specifically treated for GSH determination.

Figure 3 shows, as an example, the AFS chromatogram of PHMB-derivatized red wine (Dolianova).

GSH was first discovered in grapes as early as 1989.[49] Little has been written about assaying this compound in wine.[50−52] Although the function of this peptide in wine is not well-understood, it seems that GSH reacts with certain quinones and its level is important for preventing the oxidation of phenolic compounds in wine, thus contributing to its stability.[51,53]

The values of GSH (the peak at $t_R = 14.9$) found in this work in wine samples ranging between 3.6 and 8.8 μM (6.7 ± 2.3 μM, mean ± SD, $N = 4$) agreed with those reported by other authors.[50,52,53] GSH in wine rapidly and significantly decreases during aging, especially in new barrels, where oxidative phenomena are more prevalent.[53]

Injection of standard solutions of 3-ethylmercaptopropionate allowed also the identification of the peak at 9.48 min (Figure 3). Identification of the other peaks is beyond the aim of this paper.

CONCLUSIONS

We have proposed a photochemical, online UV/MW oxidation method followed by CVG-AFS detection for the online digestion of PHMB and its thiolic complexes.

The combination between MW radiation and MW-powered UV lamp in a unique photochemical reactor, in acidic conditions, at room temperature, allowed us to obtain the digestion of PHMB and its thiolic complexes with a yield significantly higher than that obtained with a commercial UV lamp. The MW/UV system replaced effectively the use of chemicals such as Br^-/BrO_3^- as an oxidizing mixture, and hydrazine, previously used in HPLC−CVG-AFS.[6−11,14,16,27−29] This represents a significant contribution toward the implementation of "green" interfaces between the separative apparatus and the detection system.

Although electrically powered UV lamps used in all the studies published and reviewed by Bendicho et al.[39] are simpler than MW/UV-assisted photoreactor; the latter is the only apparatus that permits the control of temperature using a thermostatic bath. Furthermore, our apparatus permits us to investigate nonthermal effects of MW in chemical reactions in controlled conditions (temperature, MW power, and UV intensity). The temperature control is not possible with electrically powered UV lamps, nor with commercial EDLs. Nonthermal effects are important in many applications and have been recently claimed in a review by Horikoshi and Serpone and references therein.[54]

Our method has been applied to the determination of low molecular weight thiols in three complex matrixes, like blood, plasma, and wine.

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