Development of an Impregnated Reagent and Automation of Solid-Phase Analytical Derivatization for Carbonyls: Proof of Principle

J. Rosenfeld[1], M. Kim[2], and **A. Rullo[2]**

[1]Department of Pathology and Molecular Medicine and [2]Central Analytical Laboratory, Faculty of Health Sciences, McMaster University, Hamilton Ontario, Canada

Abstract

This study undertakes reduction of scale and automation of a solid-phase analytical derivatization of carbonyls with 2,4-dinitrophenylhyrazine on a styrene-divinylbenzene resin (XAD-2). Three processes are tested. In the batch process, an aqueous phase consisting of 50 µL of sample and 150 µL of reagent solution is contacted with 6 mg XAD-2 by shaking. An impregnated reagent consisting of 2,4-dinitrophenylhydrazine hydrochloride (DNPH) deposited on XAD-2 enables two additional processes. In-vial derivatization with an impregnated reagent requires shaking 50 µL of sample with 6 mg of the impregnated reagent and reduced the reaction time from 10 to 5 min. The third process involves packing impregnated reagent a mini-column and flowing 50 µL of sample through under positive pressure supplied by a Harvard Pump. This reduces sample preparation time to 1 min. Studies are conducted with worst-case model analytes: butanone, 2-pentanone, and malonyldialdehyde. The carbonyl of the two ketones is hindered, and, thus, these two compounds react very slowly with DNPH in aqueous solution. Malonyldialdehyde is highly water soluble, and it does not react in aqueous phase but also would not sorb onto XAD-2 for reaction. Nevertheless, derivatization/extraction of all model compounds any of the three procedures result in reproducible and high yields.

Introduction

Determination of carbonyls is a requirement in diverse fields of investigation. Such analyses support research on oxidative stress in disease or formation and toxicity of advanced glycation end products (AGEs) (1–5). In a recent multilaboratory study, malonyldialdyde was found to be one of the significant markers of oxidative stress (5), a process that is involved in conditions such as renal disease (5,6) or rheumatoid arthritis (7). The AGEs have been implicated as toxins that are involved in the pathophysiology of neurodegenerative diseases such as Alzheimer's (4,8), complications during haemodialysis for management of renal failure (9–11), and in periodontitis (12). In environmental samples, carbonyls are present either as anthropogenic compounds or a products of photo-oxidation of organics in the atmosphere (13,14), as well as being formed in oxidative water remediation technologies (15). Carbonyls measured in such biomedical or environmental investigations share the characteristics of hydrophilicity and volatility. Research in physiology or pharmacology usually involves more complex carbonyls with higher molecular weight such as steroids or prostaglandins, which are important mediators of biological function (16). Given their prevalence in so many processes, considerable effort has been focused on the development of analytical methods that combine high resolution and high sensitivity with speed and facility of procedure.

Several challenges arise in determining this class of compounds. First, in general, there is little in the structural features of carbonyls that make them inherently detectable at the high sensitivity required in many applications. Even the relatively high molar absorptivity of the α-β unsaturated ketones in some progestagens and androgens is insufficient to permit detection at physiological concentrations. Second, lower molecular weight carbonyls are also volatile or very hydrophilic (or both) and need to be trapped prior to analysis. Third, some of the more complex carbonyl containing molecules are relatively unstable. For instance, prostaglandins of the E and D series readily dehydrate during sample preparation. Finally, the matrices in which these compounds are found can vary from those that are relatively simple, such as polished water, to the considerably more complex, such as tissue extract, plasma, or urine.

For these reasons, analytical derivatization (17,18) plays a major role in the analysis of carbonyls, with the primary function being to isolate carbonyls as stable derivatives detectable at high sensitivity. Consequently, there is substantial literature on such trapping of volatiles from air with much recent work being reported (19–23). Because of the instability of the labile α-ketol functionality in the cyclopentane ring of the prostaglandins, sample preparation for the determination of these analytes often involves derivatization to prepare stable derivatives also suitable

* Author to whom correspondence should be addressed: email rosenfel@mcmaster.ca.

for gas chromatography (GC) (24,25). These reactions, however, are not yet fully optimized. Ketones react at a much slower rate than aldehydes, meaning that a determination of a complete profile of carbonyls can require 12 to 24 h (or longer) reaction times (15,26–32) and may require elevated temperatures (33,34) for complete derivatization.

Stashenko et al. provided an excellent example of the flexibility derived from analytical derivatization (35) by permitting use of a wide range of detection techniques. These authors reacted aldehydes present in aqueous matrices (water extract of oxidized oil and urine) with pentafluoro-phenylhydrazine ($PFPNH_2NH_2$), forming lipophilic pentafluorophenylhydrazones. After extraction the derivatives were detected by high resolution GC at concentrations ranging from nmol/mL to fmol/mL, depending on instrumentation used for detection [i.e., flame ionization detector, phosphorus-nitrogen detector, electron capture detector (ECD), or mass spectrometry (MS)].

An analogous approach was described by Stalikas and Konidari (36) for the analysis of malonyldialdehyde (MDA) in a biological matrix by derivatization with 2,4,6-trichlorophenylhydrazine. These authors also took advantage of the electrophoric properties of the trichlorophenylhydrazone and based their measurements on GC with either ECD or with detection by MS functioning as a mass selective detector.

A number of issues in the determination of carbonyls can be addressed by solid-phase analytical derivatization (SPAD) (17,37). This combination of adsorption and reaction has been effective in developing methods for use in environmental (38) and biomedical studies (39–41). Shanguan's group (39), working with amines, and that of Kuklenyik (38), studying phenols, first sorbed analytes onto a solid phase and then derivatized in situ. Breckenridge et al. (26) and Rodriquez et al. (41) simultaneously extracted and derivatized carbonyls on solid-phase particles dispersed in the aqueous reaction mixture.

Microextraction fibers loaded with reagent have also been used extensively for simultaneous sorption/derivatization (37). A frequent technique is to coat the solid-phase microextraction (SPME) fibers with pentafluorobenzyl–hydroxylamine or $PFPNH_2NH_2$ as the reagent to derivatize volatile carbonyls in the headspace above the sample (30–32). This combined process effectively traps volatile, highly water-soluble carbonyls as lipophilic and stable derivatives that are not volatile at ambient temperature but are readily desorbed at the elevated temperature of an injector port.

Though this process was not used for carbonyls, an alternative to SPME was reported by Keough (40). Derivatization of tryptic peptides for proteomic analysis occured on small columns of sorbent phase. Two features of this SPAD reaction were pertinent to the present work. First, the extraction and derivatization proceeded from solutions of analytes in the aqueous phase. Second, the reaction rate was very fast (40), providing a reaction time that was of the order of seconds.

In a previous work, it was demonstrated that SPAD for carbonyls can be carried out directly from plasma to form pentafluorobenzyloximes or 2,4-dinitrophenyl hydrazones (DNPHone) (26,41). Determination of analytes from a full 1 mL of plasma showed that a large sample volume consisting of a protein and lipid-rich aqueous matrix was not an impediment to SPAD of carbonyls (26,41). Because the complexity of matrices can affect any analytical technique, this was a useful observation. The technique was subsequently applied to the determination of MDA plasma concentrations consequent to oxidative stress induced by forearm ischemia in humans (41). Nevertheless, the reaction conditions required, depending on the application, several hundreds milligrams of XAD-2, 5 mL of aqueous phase consisting of 1 mL plasma, and 4 mL reagent solution. Sample preparation was carried out in a 16- × 100-mm screw-cap vial. All these characteristics mitigated against the development of the technique.

In the current practice of sample preparation and analysis, there is considerable impetus towards the use of smaller volumes, smaller apparatus, and automation. From the point of view of analysis, reduction of scale often enables shorter sample preparation time, reduction in reagent, and, consequently, savings in cost. It is also a requirement in many applications, particularly those in the biological sciences, in which the size of sample can be very restrictive. These applications put a premium on all aspects of the analytical process. The present work has its genesis in the general challenge of reducing the scale and automation of methods, but also in the needs of biomedical studies.

In this paper, a substantial reduction in scale of the sample preparation relative to that previously described (26,41) is reported. Subsequently, the development of reactions on an impregnated reagent that reduced reaction time by half compared with reported SPAD techniques is described. The latter reaction evolved into a flow-through process that had a reaction time of 1 min and could be automated.

Experimental

Reagents, analytes, and derivatives

Carbonyls, 1,1',3,3'-tetramethoxypropane, and 2,4-dinitrophenylhydrazine hydrochloride (DNPH) were from from Sigma (St. Louis, MO) (note that TMP is a precursor to MDA). Solvents and acids were obtained from the usual suppliers (Caledon, Georgetown, Ontario, Canada, and Fisher Scientific, Markham, Ontario, Canada). The DNPHone derivatives of carbonyls were synthesized in-house using standard procedures. The reagent was 1.6mM DNPH in 2M HCl. Again standard conditions were use to prepare this material.

Preparation of stock and working solutions

Stock solution of methyl ethyl ketone (MEK) was prepared by dissolving 10 µL of this analyte in 240 µL of acetonitrile. An intermediate solution for MEK was prepared by adding 10 µL of working solution to 1.990 mL of 2M HCl. The working solution of MEK was prepared by dissolving 100 µL of the intermediate solution in 900 µL of 2M HCl. Concentration of analyte in the MEK working solution was 16 µg/mL. Stock solution for 2-pentanone (2-Ptn) was prepared by dissolving 20 µL of this ketone in 9.980 mL acetonitrile. The working solution was prepared by diluting 10 µL stock solution into 290 µL of 2M HCl. The concentration of analyte in the 2-Ptn working solution was 53 µg/mL. The stock solution of MDA was prepared by hydrolysis of TMP as follows. One hundred microliters of TMP was added into 9.9 mL of 0.25M

acetic acid, and this solution was stored in the dark at room temperature for 48 h. The working solution for the dialdehyde was prepared by dissolving 100 µL of the hydrolyzed stock solution in 9.90 mL of 2M HCl. The concentration of analyte in the MDA working solution was 44 µg/mL.

Sizing of the XAD-2

A purified form of the polystyrene–divinylbenzene cross-linked copolymeric macroreticular resin, XAD-2, was purchased from Rhom and Haas (Spring House, PA). It was sieved to isolate the 50–80-mesh fraction. The native material and larger sized fractions contained too many large beads, resulting in irreproducible sampling of the resin. Smaller sized fractions were difficult to work with, particularly with the simple sample handling techniques used.

Instrumentation

Chromatography was performed on a Waters system (Ontario, Canada) consisting of a 712 W.I.S.P. autoinjector, E-600 pump, Supleco 18 ODS 5-µm column (Bellefonte, PA), and a 428 photodiode array detector. Data was acquired and analyzed using Millenium software (Waters). Chromatograms were extracted at 360 nm, which corresponded to the maximum absorption of all DNPones.

Standard reactions conditions for in-vial reactions

A volume of 50–80-mesh XAD-2 was transferred to reaction vials using a ruby scoopula that delivered 6 ± 0.2 mg ($N = 5$) of the resin. The reaction vessels were 8- × 25-mm glass screw-cap vials (vials designed for the W.I.S.P. injector) fitted with Teflon-lined caps. Following transfer to reaction vials, the solid phase was treated with 15 µL of acetonitrile to facilitate wetting of the resin by water. One hundred and fifty microliters of reagent was added to the reaction vial. This was followed by 50 µL of analytical solution, which consisted of 35 µL of MEK working solution and 15 µL 2-Ptn working solution. The reaction mixture was shaken for 10 min on a wrist action shaker. Supernatant was removed and the solid phase was then washed three times with 50 µL of distilled water. Derivatives were eluted with three times 50 µL acetonitrile, and all the eulates were combined. Fifteen microliters of this solution was injected onto the high-performance liquid chromatography column for analysis.

Impregnated reagent in the in-vial reaction

The impregnated reagent for in-vial extraction/derivatization was prepared in situ. The resin was transferred to the reaction vial and was treated with 15 µL of acetonitrile (described earlier) followed by 150 µL of reagent. This mixture was shaken for 10 min, and the supernatant was removed by aspiration. At this stage, the resin was loaded with both adsorbed DNPH and reagent solution in the pores. Three washings with 50 µL of distilled water removed the pore water. The third washing was apparently colorless, but analysis revealed that it did contain a small amount of DNPH, corresponding to approximately 0.5% of the loaded reagent. The resin with the sorbed DNPH but without reagent solution in the pores was termed the "impregnated reagent". Fifty microliters analytical solution was added, and this consisted of 35 µL of MEK working solution and 15 µL 2-Ptn working solution. The reaction mixture was shaken on the wrist action shaker. Reaction times of 10, 5, and 1 min were studied. Washing and elution conditions were as described previously.

Impregnated reagent with flow-through conditions

The impregnated reagent for the flow-through process was prepared in larger batches as follows. One hundred and twenty milligrams of 50–80 mesh XAD-2 was transferred to a reaction vial and wetted with 300 µL of acetonitrile. Three milliliters of reagent solution was added, and the reaction mixture was shaken for 10 min. The supernatant and pore water were removed as described previously. The impregnated reagent was then removed and in-vacuo on a rotary evaporator. Such drying was necessary because wet reagent could not be practically transferred either by weighing or transferring a volume of the XAD-2, as described earlier. A scoopula of this dried reagent was transferred to a 200-µL Eppendorf pipette tip (color coded yellow) that was fitted with a small plug of glass wool. An additional plug of glass wool was then placed on top to keep the impregnated reagent from floating. Fifty microliters of the analytical solutions was added to the loaded pipette tip. The analytical solutions consisted of 35 µL of analyte working solutions (either MEK or MDA) or serial dilutions thereof and 15 µL of the 2-Ptn working solution. The pipette tip was then fitted to a 200 µL Eppendorf pipette clamped in a Harvard pump (Holliston, MA). The pump was set to deliver the 50-µL sample through the column of impregnated reagent in 1 min. Once the sample had been pushed through the column, it was washed with three times 50 µL distilled water, and the analytes were then eluted directly into the injection vial with three times 50 µL acetonitrile.

Application

The test of biological applicability was the determination of MDA in oxidatively stressed tissue. In this condition, the capacity of the cell to reduce reactive oxygen species (ROS) was exceeded, and this produced an excees of these free radicals. Polyunsaturated fatty acids, which contain methylene interrupted double bonds, were oxidized by ROS to form aldehydes, with the predominant one being malonyldialdehyde. This compound is one of several recognized markers of oxidative stress (5). Samples of mouse trapezius muscle were obtained as the supernatants of centrifuged tissue homogenates in phosphate buffer at pH = 7.4 and prepared as 4% weight wet tissue/weight buffer.

The laboratory was blinded to the oxidative stress status of the samples when they were received. These homogenates were treated with an equal volume of 4M HCl for 2 h at ambient temperature to hydrolyze Schiff bases formed between MDA and proteins. Hydrolysates were then subjected to SPAD with DNPH under standard batch reaction conditions, and the isolates were analyzed by HPLC.

Results and Discussion

Selection of model analytes

Three model compounds were selected for this study: MEK, 2-Ptn, and MDA. The two ketones belong to a class of compounds

Table I. Water–Organic Carbon Partition Coefficient (Koc) of Low-Molecular-Weight Carbonyls and Ethanol*

Sorbate	EtOH	AcA	Actn	PrpA	MDA	MEK	2-Ptn
Koc†	18.8‡	19.6‡	19.6‡	38‡	14.6§	38.1‡	74‡

* Abbreviations: EtOH, ethanol; AcA, acetaldehyde; Actn, acetone; PrpA, propionaldehyde; MDA, malonyldialdehyde; MEK, methylethyl ketone; and 2-Ptn, 2-pentanone.
† Koc data obtained from SciFinder Scholar.
‡ Independent of pH.
§ At pH = 1.

that is inherently slow reacting. In addition, as experimentally determined, both are differentially sorbed from water. When an aqeous solution of MEK at a concentration of 20 µg/mL was equilibrated with the solid phase, the final concentration of this analyte in the liquid phase (or the equilibrium concentration) was Ceq of 15 µg/mL. In the case of 2-Ptn at an initial concentration of 53 µg/mL in the aqueous phase at equilibrium the Ceq was 29 µg/mL. This pattern is consistent with the calculated water organic carbon partition constant (Koc) values shown in Table I. Thus, a substantial portion of MEK, in particular, partitioned into the aqueous phase and would be expected to react slower than a highly sorbed analyte (26). In contrast, Ptn was moderately sorbed and would be expected to react faster than MEK. Additionally, unlike acetone, neither of these ketones was sporadically present at high concentrations in the laboratory background, and this simplified the work at lower concentrations. Finally, MEK and 2-Ptn have been identified as possible markers of oxidative stress (42), and the latter has been identified as an environmental contaminant (43). Malonyldialde-hyde, on the other hand, is quite hydrophilic, and the data in Table I shows it to be more water soluble than most other carbonyls. From these data it is apparent that MDA would partition predominantly to the aqueous phase and so should be another "worst case" for SPAD. This dialdehyde is also the most common marker of oxidative stress (3,5,11,33) and (as described later) allowed comparison of results from this work with those obtained by standard measurement techniques.

Reduction of scale

Reducing the scale of the standard, batch process SPAD reaction was relatively straightforward. An advantage, however, rapidly became evident. Scaling down produced a disproportionate decrease of solid phase required: the volume of sample was reduced 20-fold (from 1 mL to 50 µL of sample), whereas weight of solid phase required was reduced by 50-fold (from 300 to 6 mg of XAD-2). As a result, less volume was required for elution than might otherwise have been necessary. Reduced requirement for stationary phase was attributed to more efficient mixing when compared with the larger scale reaction. In the latter conditions, the XAD-2 deposited at the bottom of a 16- × 100-mL tube in a reaction bed that was several millimeters thick. On the smaller scale, for instance, the 6 mg of XAD-2 beads dispersed well in a single layer on the bottom of the reaction vessel. These reaction conditions maximized contact between the water and surface and, in turn, enhanced the efficiency of the process. The scaled down technique was tested for the determination of malonyldialdehyde in muscle homogenate from a mouse model of oxidative stress (44).

Results obtained for the analysis of MDA in 50 µL of muscle homogenate were compared with a more standard colorimetric technique for determining carbonyls as measures of oxidative stress, but one that was not specific (44). Both the colorimetric technique and SPAD–HPLC identified the oxidatively stressed mice by the increase in total carbonyls in the former case and increased MDA in the latter. The SPAD–HPLC technique, however, used 1/5 the amount of sample. Data from this experiment showed that the muscle of mice that had undergone oxidative stress produced elevated amounts of MDA (0.85 ± 0.2 µg/mg tissue, $N = 6$) compared with the controls (0.2 ± 0.1 µg/mg tissue, $N = 5$). A direct comparison between the two techniques was not possible because the colorimetric technique determined all aldehydes, including those on protein, whereas the chromatographic conditions were set only for the determination of MDA.

This data demonstrated a technical advance, as well as application, but there was still considerable room for improvement in the technique. First, analysis of a 50-µL sample required an additional 150 µL of an aqueous reagent. This constituted a relatively large volume burden of a non-volatile liquid. Second, the reaction vessel was a screw-cap vial. The reaction conditions required shaking and, thus, involved repeated placement and removal of a screw cap as well as aspiration of a small volume of liquid in the presence of a small sample of solid phase. This complicated considerations of automation. We studied methods to overcome these constraints.

Knowledge of whether the reaction occurred in the aqueous phase or on the solid phase would assist in the optimization of this reaction. This issue, however, was difficult to establish. The reactions between oximating reagents and carbonyls in aqueous solution have been widely studied. It is known that the reaction rate for derivatization of ketones is an order of magnitude or more lower than that of the aldehydes (26,35,45). This difference was attributed to steric hindrance. Reaction mediated by XAD-2, however, enhanced reaction yields for all ketones tested so that in a 10–20-min reaction time, the yield was suitable for analytical work. The same result was found for MDA, which reacted very slowly with DNPH in the aqueous phase, but was derivatized within 10 min in the heterogeneous reaction with XAD-2. This implied that, at least in a substantial proportion, the reaction occurred on the solid phase. For purposes of further method development, the argument that the reaction predominantly occurs on the solid phase was taken.

Impregnated reagent as an in-vial reaction

The possibility that the reaction occurred on the solid phase suggested that an impregnated reagent could be used to some advantage. These can be prepared by depositing reagent on a solid support that can also adsorb the analytes. Impregnated reagents have considerable utility. For instance, their use can minimize dilution of sample with reagents in solution and may increase the reaction rate.

In this regard, XAD-2 was a highly suitable material for use as a sorbent for DNPH. It is stable to acidic conditions and equilibrates quickly with material in the aqueous phase. Concentrations of DNPH in solution contacted with XAD-2 reached equilibrium

within 5 min. In addition, there was a high loading of DNPH onto the solid phase. Equilibration of 6 mg of XAD-2 with 150 µL of 1.6mM DNPH solution deposited 200 nmol of reagent on the surface of the resin, providing an impregnated reagent with a loading of 33 nmol DNPH/mg. This rapid and efficient sorption showed that the impregnated reagent could be rapidly prepared.

The selected reaction conditions minimized desorption by using 50 µL of analyte solution in 2M HCl. This volume was in contact with an impregnated reagent prepared with 150 µL of 1.6mM DNPH because a smaller volume of solution would elute a smaller proportion of the DNPH from the phase, which would then retain a substantial proportion of the reagent during contact with the 50 µL of analyte solution. This hypothesis was proven by equilibration of the impregnated reagent with 50 µL of 2M HCl, which produced a loading of 32 nmol DNPH/mg. During the desorption stage, the time to equilibrium was also 5 min. Accordingly, during the course of a 10-min reaction, the impregnated reagent retained approximately the same amount of DNPH as was present on the surface during the batch process.

A comparison of the yields for derivatization of MEK and 2-Ptn by standard conditions and with impregnated reagent in a 10-min reaction time shows no statistical difference, although the impregnated reagent may be slightly lower (Table II). At 5 min, there was a slight, further reduction, but yields were still acceptable. At 1 min, there was a 50% reduction in yield. The relative standard deviations ranged from 8–10%, which is reasonable for a measurement without an internal standard. Small particle size and small amounts of beads coupled with aspiration of small, low volumes complicated the manipulations and probably contributed to the variance. Although the variance could be explained, analytical techniques and applications required better precision. This was obtained by use of an internal standard. Reproducibility for isolation/derivatization of MEK was only 4% for both the standard conditions and impregnated reagent when using 2-Ptn as an internal standard and determining response ratio (area MEK peak/area 2-Ptn peak)

The 1-min data were informative. They demonstrated that, even with the short reaction time, derivatization of analytes proceeded and suggested that optimization could further improve the yield. This also provided some insight as to mechanism. In the case of MEK, the fraction remaining in the aqueous phase and that did not react was removed upon aspiration of the liquid and could not affect final yields. Only the product remained on the XAD-2. In the case of 2-Ptn, a higher proportion of the analyte would have been sorbed. Upon recovery of the DNPHone in the acetonitrile eluate from the solid phase, the unreacted 2-Ptn would have coeluted with the derivative along with excess DNPH. It could then have reacted during storage in the eluting solvent, which contained the reagent. However, the yields for both analytes were the same. This demonstrated that the reaction does not occur in the acetonitrile used for elution because both unreacted analyte and DNPH were present in the eluate. Such data further suggested that derivatization requires contact time with the reagent on the resin.

Results from the in-vial reaction, however, did not resolve the issue of whether the reaction takes place completely on the resin or in the aqueous phase. When the 150 µL of reagent solution (1.6mM DNPH in 2M HCl) was contacted with 6 mg native XAD-2 by shaking for 5 min, the corresponding Ceq was then 0.272 molar DNPH. When 50 µL of 2M HCl was contacted under the same conditions with 6 mg of impregnated reagent, sufficient DNPH desorbed to give a Ceq of 0.192 molar DNPH, and this may be sufficient for derivatization in the aqueous phase. It may be that the impregnated reagent served as a reservoir of DNPH. But again, this hypothesis is contradicted by the obligatory presence of XAD-2 for rapid reaction of ketones and MDA.

Impregnated reagent with a flow-through process

The feasibility and efficacy of the flow-through technique were tested with methodology analogous to the Aziptip approach used in proteomic analyses (40). Keough et al. (40) used octadecylsilica as a solid phase, packed as a small column inside an Eppendorf tip, and the sample was drawn through the sorbent by centrifugation. In the process described herein, a small column of impregnated reagent is similarly packed into an Eppendorf tip, but the sample is pushed through the bed with a Harvard pump.

In migrating to the flow-through technology, it was, however, necessary to deal with the lower density of the impregnated reagent. Transferring a constant weight of XAD-2, whether in native form or as impregnated reagent, is best achieved with the ruby scoopula. For this procedure, the solid phase had to be dry. But in such an instance, the density of the impregnated reagent (as is the case for XAD-2) is less than that of water, and it is poorly wetted. As a result, the solid phase floated to the top of the 50 µL aqueous sample and, not surprisingly, yields, were consequently both low and highly variable. It was not practical to pre-wet the resin with acetonitrile in the usual manner because this would elute the DNPH. Sandwiching the impregnated reagent between two plugs of glass wool produced a stable column, and the sample flowed evenly through the packed bed.

A comparison of the yield for a reaction time 10 min under standard conditions with that obtained for a reaction time of 1 min on the flow-through system demonstrated the efficiency of the packed column. With the flow-through technique, the yield for 2-Ptn was 95% ± 8% (n = 5), which was comparable with the results obtained in the standard or in-vial reaction. Table III compares the results for 1-min reaction times for in-vial reaction and the flow-through methods.

The coefficient of correlation on a five-point calibration curve for MEK in the range of 20–1.25 µg/mL was 0.99993, and it was 0.9990 on a five-

Table II. Comparison of Standard SPAD with Derivatization on Impregnated Reagent

Condition	Standard			Impregnated reagent in-vial					
Reaction time	10 min			10 min		5 min		1 min	
Analyte	MEK	2-Ptn	MDA	MEK	2-Ptn	MEK	2-Ptn	MEK	2-Ptn
Percent yield	93	95	72	77	89	70	80	46	50
RSD* (N = 5)	11	11	6	11	11	9	8	10	13

* Relative standard deviation.

Table III. Comparison of Yields at 1-Min Reaction Time for the In-Vial and Flow-Through Techniques.

Condition	In-vial		Flow-through		
Analyte	MEK	2-Ptn	MEK	2-Ptn	MDA
Percent yield	46	50	81	97	78
RSD (n = 5)	10	13	13	9	7

point curve for MDA in the range of 44–5.5 µg/mL. A high background value for MDA required the use of a higher concentration range in these experiments. Such a high background can arise because MDA is a naturally occurring compound that is present in breath, and preparation of the dried impregnated reagent required additional handling.

The flow-through technique requires a shorter reaction time than most methods currently available (31,33,46). Equally important, the same procedure is used to extract a highly water-soluble, poorly-sorbed aldehyde, as well slow-reacting and moderately-sorbed ketones. In contrast, for instance, headspace techniques show a distinct difference in reaction rates of C-2 to C-7 aldehydes as a function of temperature (33). There is also a difference in maximum yields as a function of molecular weight. This is to be expected for a headspace analysis because within a homologous series molecular weight and volatility are inversely related.

Conclusion

We have demonstrated a facile and rapid sample preparation technique for analysis carbonyls from an aqueous matrix. The process culminated in the development and utilization of an impregnated reagent capable of high-yield derivatization of highly water soluble and lipophilic carbonyls from the same sample. As a result, it was possible to migrate from a technique requiring shaking in a sealed reaction vial to one using a flow-through system, with all reactions being carried out at ambient temperature and with a relatively simple apparatus.

References

1. U. Karst. Aldehydes 2001—an unusual conference. *Anal. Bioanal. Chem.* **372:** 605 (2002).
2. N. Zarkovic. 4-Hydroxynonenal as a bioactive marker of pathophysiological processes. *Mol. Aspects Med.* **24:** 281–91 (2003).
3. A.M. Vincent, J.W. Russell, P. Low, and E.L. Feldman. Oxidative stress in the pathogenesis of diabetic neuropathy. *Endocr. Rev.* **25:** 612–28 (2004).
4. M. Dib, C. Garrel, A. Favier, V. Robin, and C. Desnuelle. Can malondialdehyde be used as a biological marker of progression in neurodegenerative disease? *J. Neurol.* **249:** 367–74 (2002).
5. M.B. Kadiiska, B.C. Gladen, D.D. Baird, D. Germolec, L.B. Graham, C.E. Parker, A. Nyska, J.T. Wachsman, B.N. Ames, S. Basu, N. Brot, G.A. Fitzgerald, R.A. Floyd, M. George, J.W. Heinecke, G.E. Hatch, K. Hensley, J.A. Lawson, L.J. Marnett, J.D. Morrow, D.M. Murray, J. Plastaras, L.J. Roberts, J. Rokach, M.K. Shigenaga, R.S. Sohal, J. Sun, R.R. Tice, D.H. Van Thiel, D. Wellner, P.B. Walter, K.B. Tomer, R.P. Mason, and J.C. Barrett. Biomarkers of oxidative stress study II: are oxidation products of lipids, proteins, and DNA markers of CCl4 poisoning? *Free Radic. Biol. Med.* **38:** 698–710 (2005).
6. L.B. Pupim, J. Himmelfarb, E. McMonagle, Y. Shyr, and T.A. Ikizler. Influence of initiation of maintenance hemodialysis on biomarkers of inflammation and oxidative stress. *Kidney Int.* **65:** 2371–79 (2004).
7. G. Baskol, H. Demir, M. Baskol, E. Kilic, F. Ates, D. Kocer, and S. Muhtaroglu. Assessment of paraoxonase 1 activity and malondialdehyde levels in patients with rheumatoid arthritis. *Clin. Biochem.* **38(10):** 951–55 (2005).
8. J. Gasic-Milenkovic, C. Loske, and G. Munch. Advanced glycation endproducts cause lipid peroxidation in the human neuronal cell line SH-SY5Y. *J. Alzheimers. Dis.* **5:** 25–30 (2003).
9. J. Uribarri, M. Peppa, W. Cai, T. Goldberg, M. Lu, S. Baliga, J.A. Vassalotti, and H. Vlassara. Dietary glycotoxins correlate with circulating advanced glycation end product levels in renal failure patients. *Am. J. Kidney Dis.* **42:** 532–38 (2003).
10. J. Uribarri, M. Peppa, W. Cai, T. Goldberg, M. Lu, C. He, and H. Vlassara. Restriction of dietary glycotoxins reduces excessive advanced glycation end products in renal failure patients. *J. Am. Soc. Nephrol.* **14:** 728–31 (2003).
11. F. Antolini, F. Valente, D. Ricciardi, and R.M. Fagugli. Normalization of oxidative stress parameters after kidney transplant is secondary to full recovery of renal function. *Clin. Nephrol.* **62:** 131–37 (2004).
12. S. Kashket, M.F. Maiden, A.D. Haffajee, and E.R. Kashket. Accumulation of methylglyoxal in the gingival crevicular fluid of chronic periodontitis patients. *J. Clin. Periodontol.* **30:** 364–67 (2003).
13. D.B. Van, V.H. Coeckelberghs, H. Vankerckhoven, F. Compernolle, and C. Vinckier. Study of the carbonyl products of terpene/OH radical reactions: detection of the 2,4-DNPH derivatives by HPLC–MS. *Anal. Bioanal. Chem.* **379:** 484–94 (2004).
14. R.J. Griffin. Modeling the oxidative capacity of the atmosphere of the south coast air basin of California. 2. HOx radical production. *Environ. Sci. Technol.* **38:** 753–57 (2004).
15. C. Zwiener, T. Glauner, and F.H. Frimmel. Method optimization for the determination of carbonyl compounds in disinfected water by DNPH derivatization and LC–ESI-MS–MS. *Anal. Bioanal. Chem.* **372:** 615–21 (2002).
16. G.J. Tortora and S.R. Grabowski. *Principles of Anatomy and Physiology*. John Wiley & Sons, Hoboken, NJ, 2002, pp. 1028–92.
17. J.M. Rosenfeld. Derivatization in the current practice of analytical chemistry. *Trends Anal. Chem.* **22:** 785–98 (2003).
18. J.M. Rosenfeld. *Recent Developments in the Chemistry and Application of Analytical Derivatizations*. Elsevier Science B.V., Amsterdam, the Netherlands, 2002, pp. 609–68
19. H. Destaillats, R.S. Spaulding, and M.J. Charles. Ambient air measurement of acrolein and other carbonyls at the Oakland-San Francisco Bay Bridge toll plaza. *Environ. Sci. Technol.* **36:** 2227–35 (2002).
20. W.A. McClenny, K.D. Oliver, H.H. Jacumin, Jr., and E.H. Daughtrey, Jr. Ambient level volatile organic compound (VOC) monitoring using solid adsorbents—recent US EPA studies. *J. Environ. Monit.* **4:** 695–705 (2002).
21. M. Possanzini and P. Di, V. Simultaneous determination of HCHO, CH3CHO and O(x) in ambient air by hydrazine reagent and HPLC. *Ann. Chim.* **93:** 149–56 (2003).
22. S.S. Ho and J.Z. Yu. Determination of airborne carbonyls: comparison of a thermal desorption/GC method with the standard DNPH/HPLC method. *Environ. Sci. Technol.* **38:** 862–70 (2004).
23. S. Uchiyama, E. Matsushima, S. Aoyagi, and M. Ando. Simultaneous determination of C1-C4 carboxylic acids and aldehydes using 2,4-dinitrophenylhydrazine-impregnated silica gel and high-performance liquid chromatography. *Anal. Chem.* **76:** 5849–54 (2004).
24. H. Tsukamoto, T. Hishinuma, T. Mikkaichi, H. Nakamura, T. Yamazaki, Y. Tomioka, and M. Mizugaki. Simultaneous quantification of prostaglandins, isoprostane and thromboxane in cell-cultured medium using gas chromatography-mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **774:** 205–14 (2002).

25. J.M. Rosenfeld, Y. Moharir, and R. Hill. Direct solid-phase isolation and oximation of prostaglandin E2 from plasma and quantitation by gas chromatography with mass spectrometric detection in the negative-ion chemical ionization mode. *Anal. Chem.* **63:** 1536–41 (1991).
26. S.M. Breckenridge, X. Yin, J.M. Rosenfeld, and Y.H. Yu. Analytical derivatizations of volatile and hydrophilic carbonyls from aqueous matrix onto a solid phase of a polystyrene-divinylbenzene macroreticular resin. *J. Chromatogr. B Biomed. Sci. Appl.* **694:** 289–96 (1997).
27. P.M. Scott, G.A. Lawrence, and B.P.Y. Lau. Determination of thujone by derivatization with dansylhydrazine and liquid chromatography. *J. Liq. Chromatogr. Rel. Technol.* **27(6):** 1071–81 (2004).
28. R. Andreoli, P. Manini, M. Corradi, A. Mutti, and W.M. Niessen. Determination of patterns of biologically relevant aldehydes in exhaled breath condensate of healthy subjects by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **17:** 637–45 (2003).
29. E.E. Stashenko, M.A. Puertas, and J.R. Martinez. SPME determination of volatile aldehydes for evaluation of in-vitro antioxidant activity. *Anal. Bioanal. Chem.* **373:** 70–74 (2002).
30. E.E. Stashenko, M.A. Puertas, W. Salgar, W. Delgado, and J.R. Martinez. Solid-phase microextraction with on-fibre derivatisation applied to the analysis of volatile carbonyl compounds. *J. Chromatogr. A* **886:** 175–82 (2000).
31. C. Deng, W. Zhang, J. Zhang, and X. Zhang. Rapid determination of acetone in human plasma by gas chromatography–mass spectrometry and solid-phase microextraction with on-fiber derivatization. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **805:** 235–40 (2004).
32. C. Deng, J. Zhang, X. Yu, W. Zhang, and X. Zhang. Determination of acetone in human breath by gas chromatography–mass spectrometry and solid-phase microextraction with on-fiber derivatization. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **810:** 269–75 (2004).
33. C. Deng and X. Zhang. A simple, rapid and sensitive method for determination of aldehydes in human blood by gas chromatography/mass spectrometry and solid-phase microextraction with on-fiber derivatization. *Rapid Commun. Mass Spectrom.* **18:** 1715–20 (2004).
34. C. Deng, X. Zhang, and N. Li. Investigation of volatile biomarkers in lung cancer blood using solid-phase microextraction and capillary gas chromatography-mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **808:** 269–77 (2004).
35. E.E. Stashenko, M.C. Ferreira, L.G. Sequeda, J.R. Martinez, and J.W. Wong. Comparison of extraction methods and detection systems in the gas chromatographic analysis of volatile carbonyl compounds. *J. Chromatogr. A* **779:** 360–69 (1997).
36. C.D. Stalikas and C.N. Konidari. Analysis of malondialdehyde in biological matrices by capillary gas chromatography with electron-capture detection and mass spectrometry. *Anal. Biochem.* **290:** 108–15 (2001).
37. E.E. Stashenko and J.R. Martinez. Derivatization and solid-phase microextraction. *Trends Anal. Chem.* **23:** 553–61 (2004).
38. Z. Kuklenyik, J. Ekong, C.D. Cutchins, L.L. Needham, and A.M. Calafat. Simultaneous measurement of urinary bisphenol a and alkylphenols by automated solid-phase extractive derivatization gas chromatography/mass spectrometry. *Anal. Chem.* **75:** 6820–25 (2003).
39. D. Shangguan, Y. Zhao, H. Han, R. Zhao, and G. Liu. Derivatization and fluorescence detection of amino acids and peptides with 9-fluorenylmethyl chloroformate on the surface of a solid adsorbent. *Anal. Chem.* **73:** 2054–57 (2001).
40. T. Keough, R.S. Youngquist, and M.P. Lacey. Sulfonic acid derivatives for peptide sequencing by MALDI MS. *Anal. Chem.* **75:** 156A–165A (2003).
41. M.C. Rodriguez, J. Rosenfeld, and M.A. Tarnopolsky. Plasma malondialdehyde increases transiently after ischemic forearm exercise. *Med. Sci. Sports Exerc.* **35:** 1859–65 (2003).
42. S.S. Kim, D.D. Gallaher, and A.S. Csallany. Lipophilic aldehydes and related carbonyl compounds in rat and human urine. *Lipids* **34:** 489–96 (1999).
43. D. Grosjean, E. Grosjean, and A.W. Gertler. On-road emissions of carbonyls from light-duty and heavy-duty vehicles. *Environ. Sci. Technol.* **35:** 45–53 (2001).
44. G. Parise, S.M. Phillips, J.J. Kaczor, and M.A. Tarnopolsky. Antioxidant enzyme activity is up-regulated after unilateral resistance exercise training in older adults. *Free Radic. Biol. Med* **39:** 289–95 (2005).
45. J. March. *Advanced Organic Chemistry*. John Wiley & Sons, New York, NY, 1985, pp. 805–806.
46. E. Schulte. Determination of higher carbonyl compounds in used frying fats by HPLC of DNPH derivatives. *Anal. Bioanal. Chem.* **372:** 644–48 (2002).

Manuscript received September 18, 2005;
revision received December 4, 2005.