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Commentary

Hydroxylation of Salicylate and Phenylalanine as Assays for Hydroxyl Radicals: a Cautionary Note Visited for the Third Time

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Hydroxylation of salicylate to 2,3- and 2,5-dihydroxybenzoates (DHBs) is widely used as an index of hydroxyl radical ($OH^{\bullet}$) formation *in vivo* and *in vitro*. Several recent studies indicate that peroxynitrite can lead to generation of DHBs from salicylate and it is uncertain as to whether or not $OH^{\bullet}$ is involved. A similar problem may occur in the use of phenylalanine as an $OH^{\bullet}$ detector. Hence formation of hydroxylation products from salicylate (or phenylalanine) may not in itself be a definitive index of $OH^{\bullet}$ generation, especially in cases where such generation in physiological systems is decreased by inhibitors of nitric oxide synthase. Determination of salicylate (or phenylalanine) nitration products can allow distinction between peroxynitrite-dependent aromatic hydroxylation and that involving "real" $OH^{\bullet}$.

INTRODUCTION

Highly-reactive hydroxyl radical ($OH^{\bullet}$) is often generated in biological systems[1] and numerous assays have been described to measure it. Of the methods available, probably the most specific are electron spin resonance-spin trapping, and aromatic hydroxylation, although both suffer problems when they are used in biological systems.[2–5] The technique of aromatic hydroxylation is based upon a wealth of chemical literature, some of it over 80 years old, showing the ability of $OH^{\bullet}$ to add on to aromatic rings (reviewed in[2]). The resulting radicals have a number of fates, depending on pH and on what else is present in the reaction system (e.g. O_2, metal ions). Under physiologically-relevant conditions (pH 7.4, metal ions and oxygen present), formation of hydroxylated aromatic products seems a favoured reaction pathway and so the formation of such products is often used as an index of $OH^{\bullet}$ generation, although the isomeric distribution of products observed can vary under different reaction conditions.[2,5–8]

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Several different aromatic "targets" have been used for the detection of $OH^{\bullet}$. Currently the most popular is salicylate (2-hydroxybenzoate): attack of $OH^{\bullet}$ upon salicylate generates 2,3- and 2,5-dihydroxybenzoates (DHBs) and some catechol (Fig. 1). Formation of these two DHBs has been used as an assay for $OH^{\bullet}$ formation both *in vitro* and *in vivo* (examples are given in references[9–18]). In some cases the $OH^{\bullet}$ formation has been decreased by inhibitors of nitric oxide synthase, or other evidence has been obtained suggesting that nitric oxide ($NO^{\bullet}$) is involved in $OH^{\bullet}$ formation (for examples see[9,19,20]).

In 1991, we published a "cautionary note" about the use of aromatic hydroxylation of salicylate, emphasising that it is necessary to measure both 2,3- and 2,5-DHBs as indices of $OH^{\bullet}$ trapping; measuring 2,5-DHB alone is insufficient.[21] This cautionary note was "revisited" in 1995 when a possible artefactual $OH^{\bullet}$ formation involving metal ion release from microdialysis equipment used to infuse salicylate for measurement of cerebral free radical generation was identified.[22]

A THIRD CAUTIONARY NOTE: PEROXYNITRITE-DEPENDENT SALICYLATE HYDROXYLATION

Several authors have shown that addition of peroxynitrite (a species formed, among other mechanisms, by the rapid combination of $O_2^{\bullet-}$ and $NO^{\bullet}$[23,24]) to salicylate causes formation of 2,3- and 2,5-DHBs.[25–27] Salicylate hydroxylation on addition of $ONOO^-$ is inhibited by several $OH^{\bullet}$ scavengers[26] and a simple explanation is that salicylate is trapping $OH^{\bullet}$ produced when $ONOO^-$ protonates and then breaks down.[28–31] However, evidence supporting $OH^{\bullet}$ production from $ONOO^-$ is matched by considerable evidence against it.[32–37] Peroxynitrite chemistry is complex.[23,32,38–40]; if it is true that no $OH^{\bullet}$ is formed during $ONOO^-$ breakdown, it follows that the salicylate hydroxylation is due to other $ONOO^-$-derived species.[25,26] If so, it further follows that formation of 2,3- and 2,5-DHBs from salicylate is *not* in itself diagnostic of $OH^{\bullet}$ production.

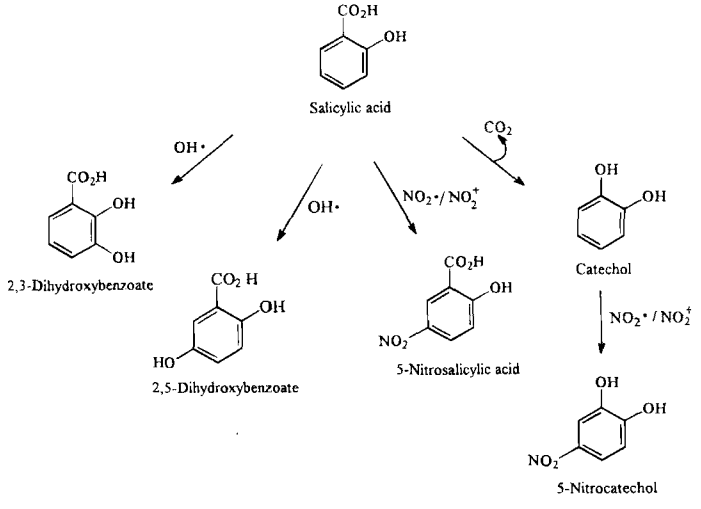

FIGURE 1 Hydroxylated and nitrated products of salicylic acid detected by our HPLC system. Catechol and hence 5-nitrocatechol are minor products.

CAN PHENYLALANINE HELP?

In principle, almost any aromatic compound can be used[2] as a detector for $OH^{\bullet}$; one alternative to salicylate is phenylalanine. Both L-and D-phenylalanine are hydroxylated to give three isomeric tyrosines, *ortho-*, *meta-* and *para-* tyrosines (Figure 2).[41–45] Although currently less popular than salicylate, phenylalanine has some advantages for detection of $OH^{\bullet}$ *in vivo* (reviewed in[46]) and its use is increasing.[41–47] For example, L-phenylalanine enters cells through an amino acid carrier that will not transport D-phenylalanine, so a comparison of results using the two isomers should help to distinguish intracellular and extracellular $OH^{\bullet}$ generation.[46]

However, addition of $ONOO^-$ to solutions of phenylalanine gives *ortho-*, *meta-* and *para-* tyrosines and their formation is inhibited by $OH^{\bullet}$ scavengers.[26,29] Again, it is possible to interpret this observation as evidence[26] for $OH^{\bullet}$ formation from $ONOO^-$. It is also possible to argue, however, that $ONOO^-$-derived species different from $OH^{\bullet}$ can hydroxylate phenylalanine. If this is so, it follows that formation of three isomeric tyrosines from phenylalanine cannot in itself be held to be diagnostic of $OH^{\bullet}$ production.

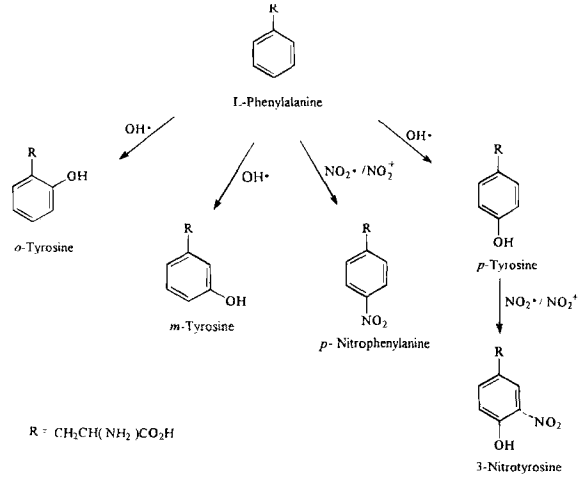

FIGURE 2 Hydroxylated and nitrated products of phenylalanine. Only products identified in our system are included.

A SOLUTION TO THE CONUNDRUM: MEASURE NITRATION PRODUCTS

Given the arguments, confusion and evidence on both sides about whether or not some $OH^{\bullet}$ is formed when $ONOO^-$ breaks down, we cannot be fully confident that formation of specific hydroxylation products from salicylate and phenylalanine (Figs. 1, 2) is in itself diagnostic of $OH^{\bullet}$ formation. The doubt is greatest in cases where $NO^{\cdot}$ appears to be involved in the "$OH^{\bullet}$ generation" (e.g. references[9,19,20]). Peroxynitrite also leads to generation of hydroxylation products (perhaps via $OH^{\bullet}$, but perhaps not). Hydroxyl radical scavengers could inhibit damage in both cases.[26,29,48]

There is a solution, however. "Real" $OH^{\bullet}$ hydroxylates aromatic compounds as well as leading to other reactions such as decarboxylation and dimerization, to extents depending on reaction conditions.[2] It can never *nitrate* aromatic compounds. However, addition of $ONOO^-$ to salicylate or phenylalanine leads to generation not only of hydroxylated products but also of nitrated ones, e.g. 5-nitrosalicylate (and trace amounts of 5-nitrocatechol) have been identified from salicylate, or 3-nitrotyrosine and *p*-nitrophenylalanine from phenylalanine.[26] Nitrated aromatic compounds can be clearly separated from hydroxylation products by HPLC (Figs. 3, 4, 5, 6). If such nitration products are observed as well as 2,3- and 2,5-DHBs, or *o-*, *m-* and *p-* tyrosines, one should worry about $ONOO^-$. If they are not observed, it suggests that the hydroxylation is due to "real" $OH^{\bullet}$, especially if the use of nitric oxide synthase inhibitors in physiological systems does not decrease formation of hydroxylated products. Of course, the identity of peaks on HPLC should always be validated, e.g. by diode array (Figs. 3–6).

Acknowledgements

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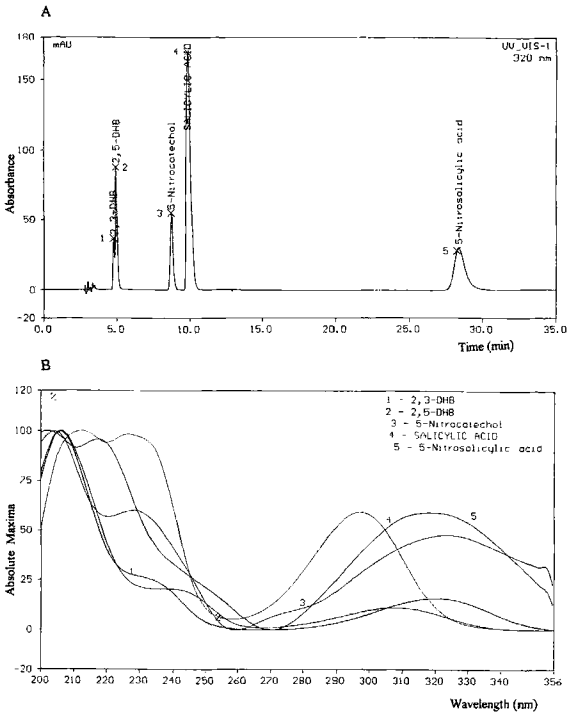

FIGURE 3 **A**. HPLC separation of a standard mixture of salicylate and its hydroxylation and nitration products following our previously-published procedure[26] (2,3-DHB and 2,5-DHB each at 25 μM, 5-nitrocatechol at 25 μM, 5-nitrosalicylic acid at 25 μM, and salicylic acid at 1.25 mM). Essentially 100 μl of the standard mixture was injected onto a nucleocil 5 μm C_{18} column (30 × 4.6 mm) with a Hibar guard column and 500 mM KH_2PO_4-KOH (pH 6.6) plus methanol (80:20, v/v) at a flow rate of 1 ml min^{-1} as the eluent. Detection was on a photo-diode array detector (Gynkotek—UVD 320, HPLC Technology Ltd) set at 320 nm. Peaks before 4 min are due to the solvent front. **B**. UV absorbance spectrum of each peak on the photo-diode array detector. (See Color Plate I at the back of this issue.)

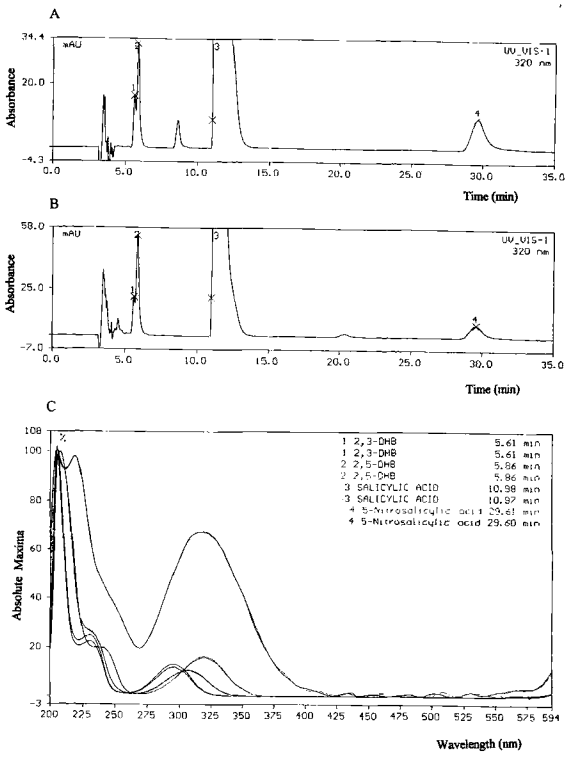

FIGURE 4 **A**. HPLC separation of a standard mixture as in Fig. 3A with 5-nitrocatechol at 5 μM, 5-nitrosalicylic acid at 10 μM and salicylic acid at 5 mM instead of the amounts stated above. (The scale is expanded). **B**. Separation of the above products from a reaction mixture resulting from addition of peroxynitrite (1.0 mM) to salicylate (5 mM) in 100 mM phosphate buffer pH 7.4. Nitrocatechol (peak at ~ 8.3 mins on Fig. 4A) is presumably produced in amounts too small to detect, but the nitrosalicylate peak is clear. Peaks before 4 min are due to the solvent front. **C**. UV absorbance spectral match of each peak detected with that of authentic standards. At this pH nitrocompounds have little absorbance at higher wavelength. (See Color Plate II at the back of this issue.)

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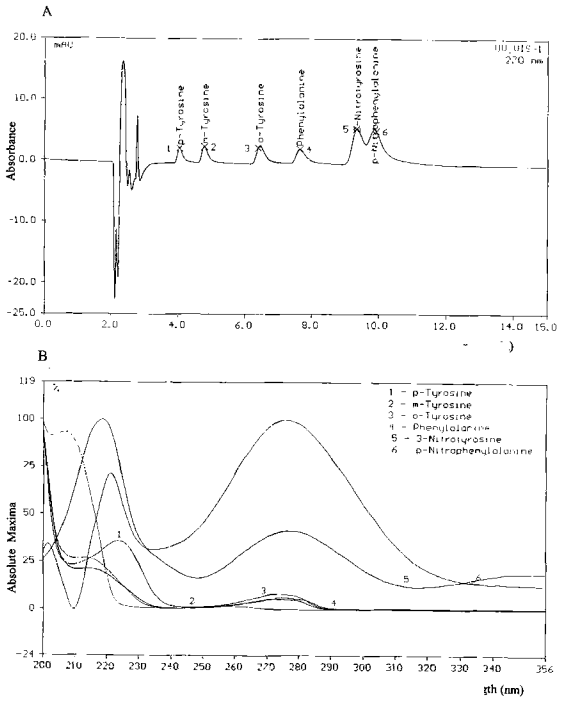

FIGURE 5 **A**. HPLC separation of a standard mixture of phenylalanine and its hydroxylation and nitration products (*para-*, *meta-*, *ortho-*, and 3-nitro-tyrosines, *p*-nitrophenylalanine, 5 μM each; and phenylalanine, 200 μM) following our previously-published procedure.[26] Essentially 100 μl of the standard mixture was injected onto a nucleocil 5 μm C_{18} column (25 × 4.6 mm) with a Hibar guard column and 500 mM KH_2PO_4-H_3PO_4 (pH 3.01) plus methanol (90:10, v/v) as eluent at a flow rate of 1 ml min^{-1}. Detection was on a photo-diode array detector (Gynkotek—UVD-320, HPLC Technology Ltd) set at 270 nm. Peaks before 4 min are due to the solvent front.**B**. UV absorbance spectrum of each peaks on the photo-diode array detector. (See Color Plate III at the back of this issue.)

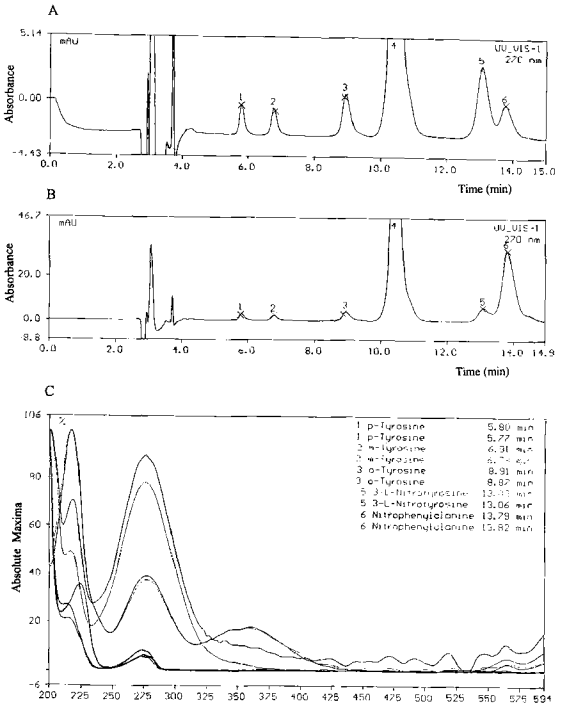

FIGURE 6 **A**. HPLC separation of a standard mixture as in 5A with 5 mM phenylalanine instead of 200 μM. (The scale is expanded). **B**. Separation of the above products from a reaction mixture resulting from addition of peroxynitrite (1.0 mM) to phenylalanine (5 mM) in 100 mM phosphate buffer pH 7.4. Peaks before 4 min are due to the solvent front. **C**. UV absorbance spectral match of each the peak detected with those of authentic standards. (See Color Plate IV at the back of this issue.)

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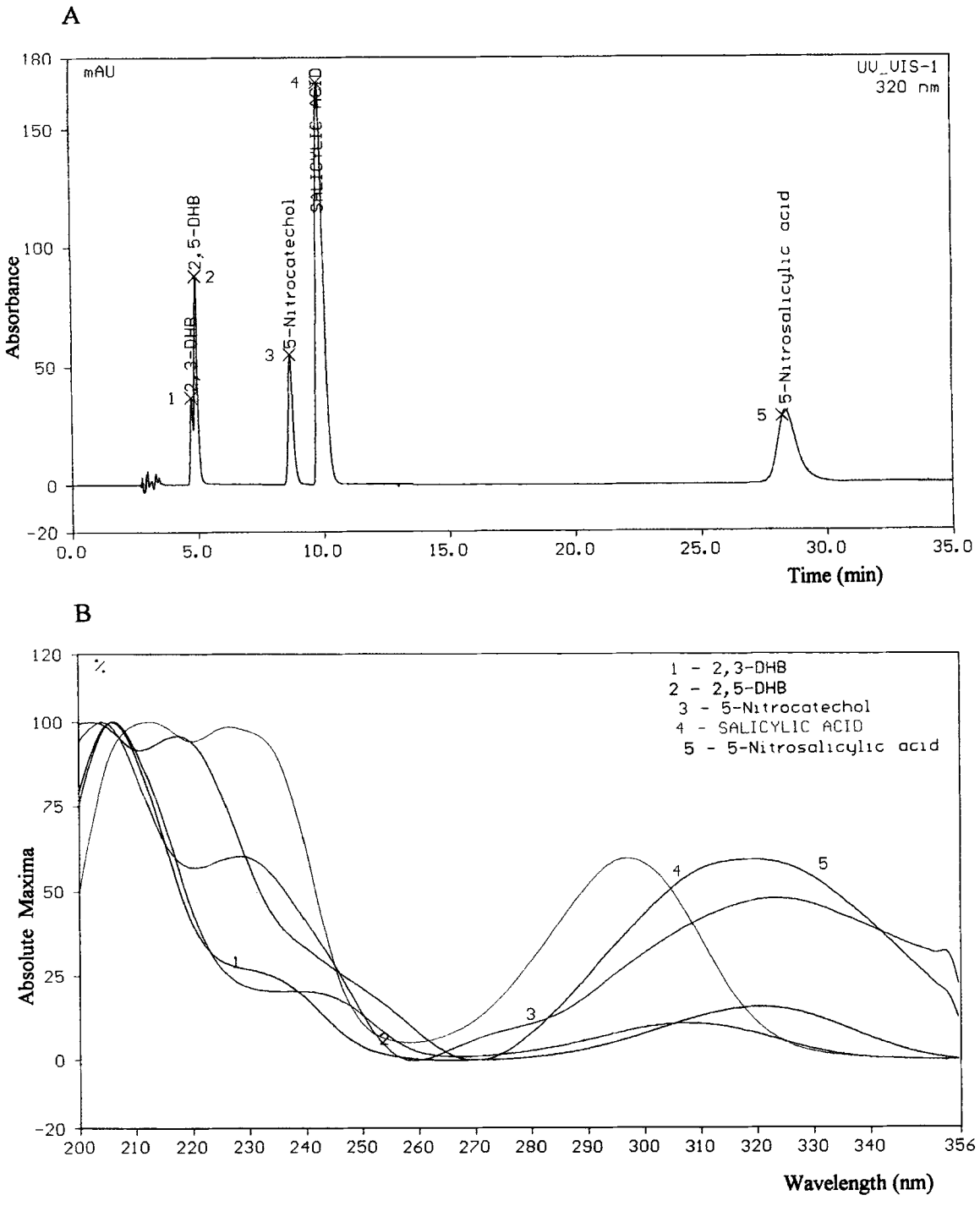
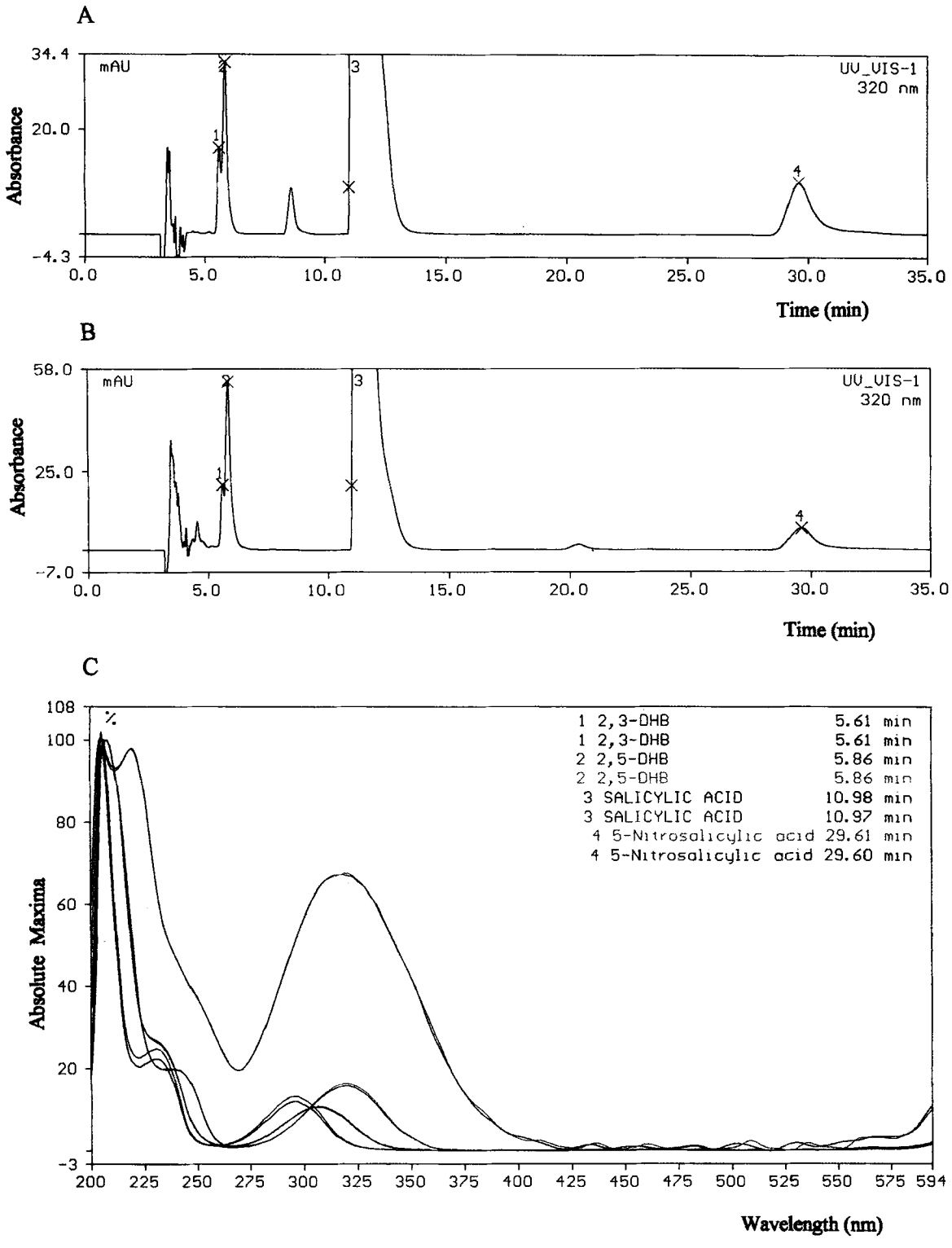
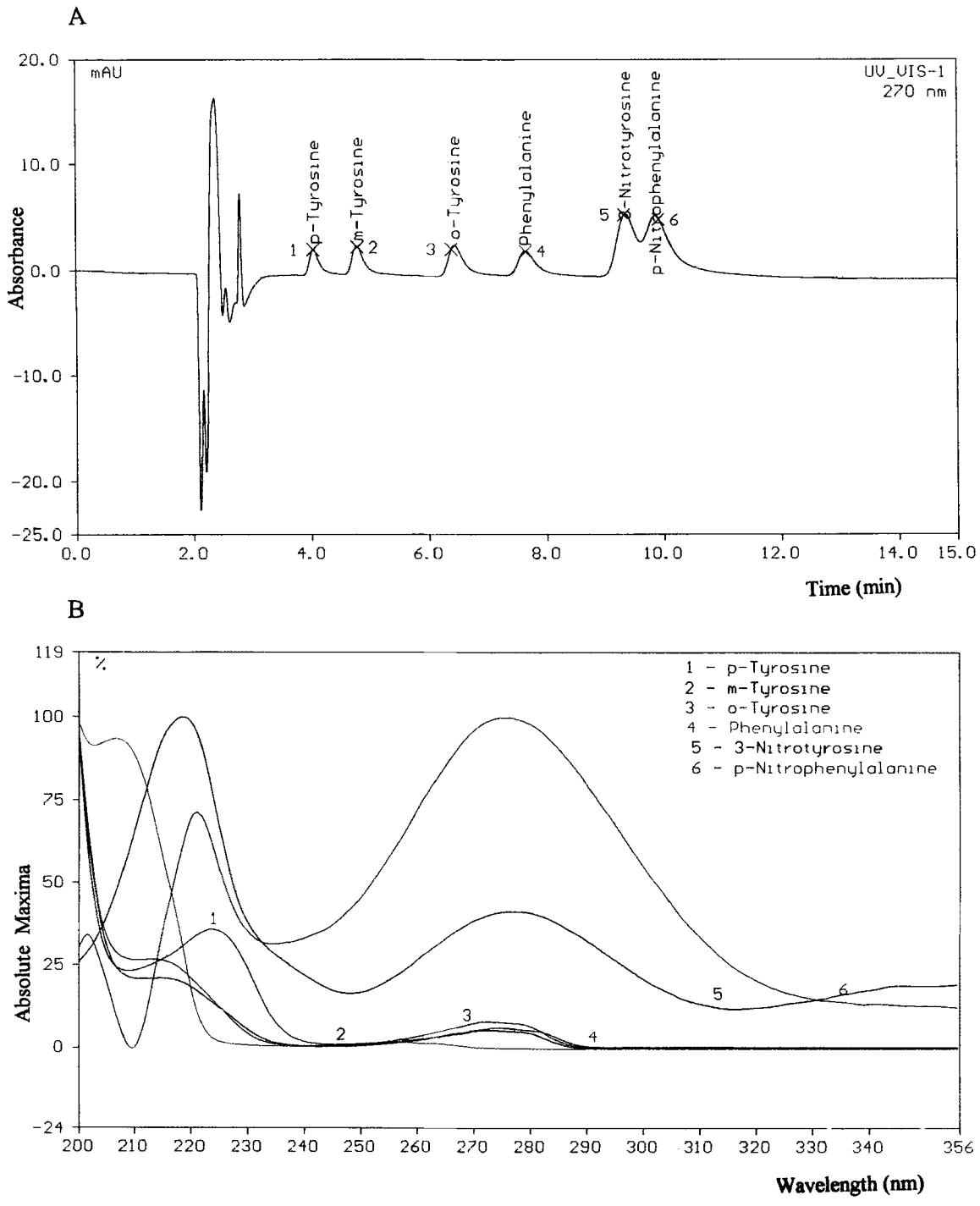


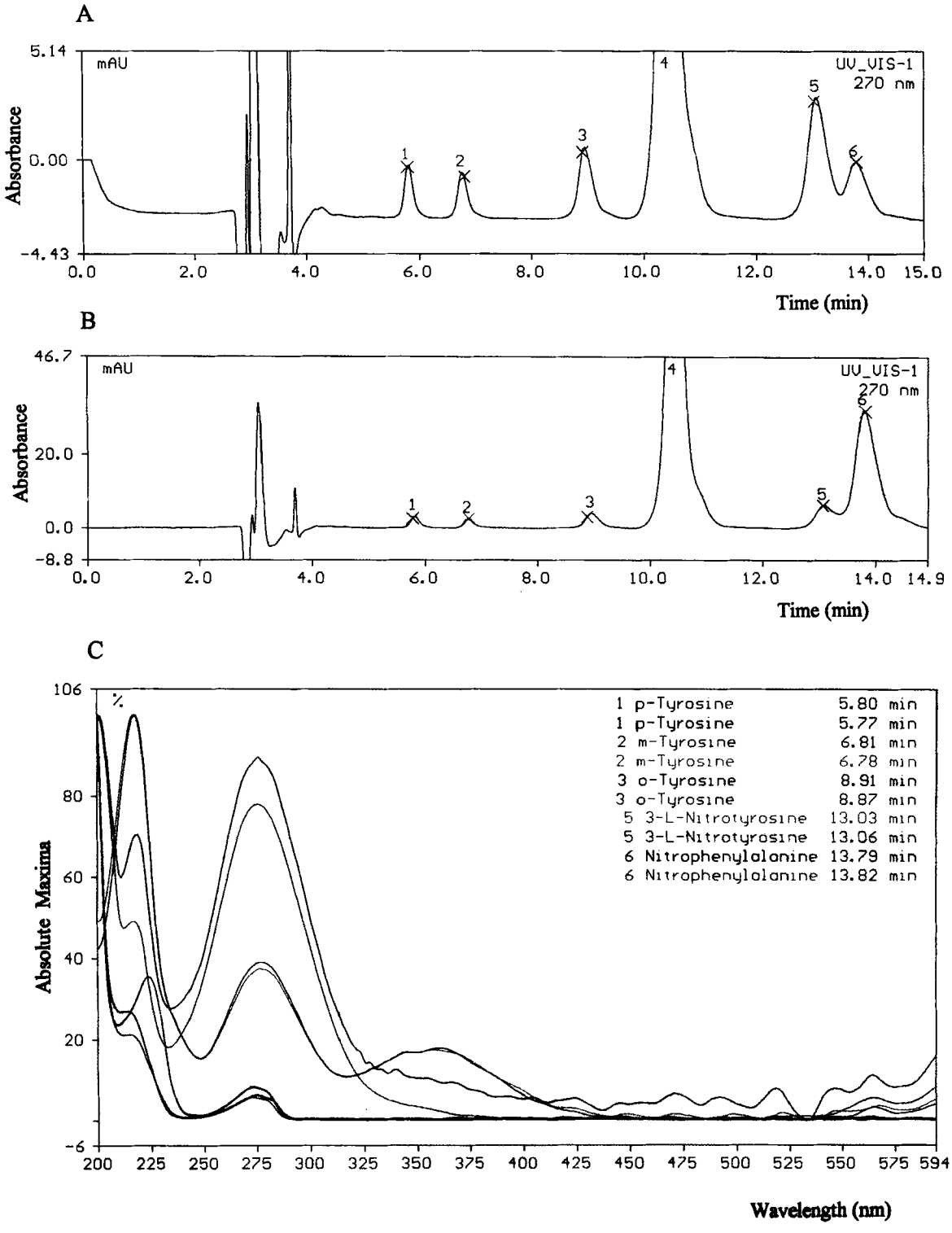
Color Plate I (See page 242 Figure 3) **A**. HPLC separation of a standard mixture of salicylate and its hydroxylation and nitration products following our previously-published procedure[26] (2,3-DHB and 2,5-DHB each at 25 µM, 5-nitrocatechol at 25 µM, 5-nitrosalicylic acid at 25 µM, and salicylic acid at 1.25 mM). Essentially 100 µl of the standard mixture was injected onto a nucleocil 5 µM C_{18} column (30 x 4.6 mm) with a Hibar guard column and 500 mM KH_2PO_4-KOH (pH 6.6) plus methanol (80:20, v/v) at a flow rate of 1 ml min^{-1} as the eluent. Detection was on a photo-diode array detector (Gynkotek—UVD 320, HPLC Technology Ltd) set at 320 nm. Peaks before 4 min are due to the solvent front. **B**. UV absorbance spectrum of each peak on the photo-diode array detector.



Color plate II (See page 242 Figure 4) **A**. HPLC separation of a standard mixture as in Fig. 3A with 5-nitrocatechol at 5 μM, 5-nitrosalicylic acid at 10 μM and salicylic acic at 5 mM instead of the amounts stated above. (The scale is expanded). **B**. Separation of the above products from a reaction mixture resulting from addition of peroxynitrite (1.0 mM) to salicylate (5 mM) in 100 mM phosphate buffer pH 7.4. Nitrocatechol (peak at ~ 8.3 mins on Fig. 4A) is presumably produced in amounts too small to detect, but the nitrosalicylate peak is clear. Peaks before 4 min are due to the solvent front. **C**. UV absorbance spectral match of each peak detected with that of authentic standards. At this pH nitrocompounds have little absorbance at higher wavelenght.



Color Plate III (See page 243 Figure 5) **A**. HPLC separation of a standard mixture of phenylalanine and its hydroxylation and nitration products (*para*-, *meta*-, *ortho*-, and 3-nitro-tyrosines, *p*-nitrophenylalanine, 5 μM each; and phenylalanine, 200 μM) following our previously-published procedure.[26] Essentially 100 μl of the standard mixture was injected onto a nucleocil 5 μm C_{18} column (25 x 4.6 mm) with a Hibar guard column and 500 mM KH_2PO_4-H_3PO_4 (pH 3.01) plus methanol (90:10, v/v) as eluent at a flow rate of 1 ml min^{-1}. Detection was on a photo-diode array detector (Gynkotek—UVD-320, HPLC Technology Ltd) set at 270 nm. Peaks before 4 min are due to the solvent front. **B**. UV absorbance spectrum of each peaks on the photo-diode array detector.



Color Plate IV (See page 243 Figure 6) **A**. HPLC separation of a standard mixture as in 5A with 5 mM phenylalanine instead of 200 µM. (The scale is expanded). **B**. Separation of the above products from a reaction mixture resulting from addition of peroxynitrite (1.0 mM) to phenylalanine (5 mM) in 100 mM phosphate buffer pH 7.4. Peaks before 4 min are due to the solvent front. **C**. UV absorbance spectral match of each the peak detected with those of authentic standards.

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