

**In vitro metabolism of NO-donating aspirin: The effect of positional isomerism**

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**Abbreviations:** NSAIDs, nonsteroidal anti-inflammatory drugs; NO, nitric oxide; ASA, aspirin; GSH, glutathione; HMP, (hydroxymethyl)phenol; NMP, (nitroxymethyl)phenol; HMP-GSH, conjugate of HMP with GSH

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## ABSTRACT

NO-donating aspirin (NO-ASA) is a potentially important chemopreventive agent against cancer. Since positional isomerism affects strongly its potency in inhibiting colon cancer cell growth, we studied the metabolic transformations of its *ortho*, *meta* and *para* isomers in rat liver and colon cytosolic, microsomal, and mitochondrial fractions as well as in intact HT-29 human colon cancer cells. NO-ASA and metabolites were determined by HPLC and products identified by mass spectroscopy, as required. For all three isomers, the acetyl group on the ASA moiety was hydrolyzed rapidly. This was followed by hydrolysis of the ester bond linking the salicylate anion to the spacer. The *ortho* and *para* isomers produced salicylic acid and a putative intermediate consisting of the remainder of the molecule, which via a rapid step generated nitrate, (hydroxymethyl)phenol and a conjugate of spacer with glutathione. The *meta* isomer, in contrast, generated salicylic acid and (nitroxymethyl)phenol, the latter leading to (hydroxymethyl)phenol and the glutathione-spacer. This metabolic pathway takes place in its entirety only in the cytosolic fraction of the tissues tested and in intact human colon cancer cells, perhaps reflecting exposure to the cytosolic glutathione-S-transferase, which catalyzes the formation of the spacer-glutathione conjugate. Thus, the three positional isomers of NO-ASA differ in their metabolism and these differences correlate with their differential effects on cancer cell growth, underscoring the importance of positional isomerism in modulating drug effects.

## INTRODUCTION

NO-donating non-steroidal anti-inflammatory drugs (NO-NSAIDs) are emerging as an important novel pharmacological class that has already entered the phase of clinical testing. NO-NSAIDs consist of a traditional NSAID to which the  $-\text{ONO}_2$  group is covalently bound via a spacer (Fig. 1). Their three main features appear to be enhanced potency, efficacy and greater safety compared to their NSAID counterparts (reviewed in (Rigas et al., 2003; Rigas and Kashfi, 2004). A large body of basic work in recent years indicates that they display novel properties that set them apart from their parent traditional NSAIDs. In view of their envisioned clinical applications, it is becoming increasingly important to know their metabolic transformations by various tissues, especially those that may be targets of their pharmacological action.

We have been interested in the use of NO-NSAIDs, in particularly NO-aspirin (NO-ASA), as a safe and effective chemopreventive agent against colon cancer (Williams et al., 2001; Kashfi et al., 2002). In vitro studies using cultured colon and other cell lines indicate that NO-ASA is the most potent among several NO-NSAIDs in inhibiting cell growth (preceding paper), which is taken to portend its efficacy in intact organisms. Using an animal model of colon cancer, we and others have demonstrated that NO-NSAIDs, including NO-ASA, are effective in inhibiting aberrant crypt foci, which represent the earliest known premalignant lesion of colon cancer (Bak et al., 1998). An interesting feature of NO-ASA is the effect of positional isomerism on its biological effects. Changing the position of the  $-\text{CH}_2\text{ONO}_2$  group in the benzene ring generates *o*-, *m*- and *p*- isomers, with respect to the ester bond between the two rings (Fig 1). We have noted that the biological behavior of these three isomers is quite different. Compared to the *m*- isomer, the *o*- and *p*- positional isomers are between 90 and 264 times more potent in inhibiting colon cancer cell growth (accompanying paper).

Based on the above considerations, we undertook a systematic investigation of the metabolism of the three positional isomers of NO-ASA by rat liver and colon cytosol, microsomes and mitochondria. In this paper, we present the results of our work and propose a scheme for their metabolic transformations.

## MATERIALS AND METHODS

**Reagents.** Three positional isomers of NO-ASA and their de-acetylated and de-nitrated analogs were a gift of NicOx S.A. (Sophia Antipolis, France) as described (preceding paper). Phosphoric acid, aspirin (ASA), salicylic acid (SA), NADP, NADPH, MgCl<sub>2</sub>, 2-hydroxybenzyl alcohol, 3-hydroxybenzyl alcohol, 4-hydroxybenzyl alcohol and N-ethylmaleimide were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All general solvents and reagents were of HPLC-grade or the highest grade commercially available.

**Subcellular fractionation.** Male, Sprague-Dawley rats weighing 180-240g were obtained from Harlan Industries (Indianapolis, IN) and were fed on Purina Rat Chow (Ralston Purina Co., Richmond, IN) and water *ad libitum*. On the day of the experiment, rats were killed by exposure to carbon dioxide and their livers and small intestine were removed rapidly for preparation of mitochondria, microsomes and cytosol. Hepatic mitochondria were isolated by the method of Johnson and Lardy with modifications as described previously (Gamble and Cook, 1985). The post-mitochondrial supernatant was centrifuged at 20,000 *g* for 20 min to remove light mitochondria and peroxisomes and the resulting supernatant was centrifuged at 100 000 *g* for 60 min to collect microsomes and cytosol. Mitochondria and microsomes were further purified and characterized as previously described (Park et al., 1995). Subcellular fractionation of the small intestinal mucosa was carried out as previously described (Kashfi et al., 1995).

**HPLC Analysis.** Our HPLC system consisted of two Waters 501 pumps with an automated gradient solvent delivery controller, a Waters 990 UV/VIS detector, and a

LiChrospher C<sub>8</sub> reverse-phase column (250 X 4.6 mm; particle size, 5 μ; Alltech Inc., Beerfield, IL) with a manual sample injector. The column was maintained at room temperature. Buffer A consisted of water-acetonitrile-phosphoric acid (9:1:0.01, v/v/v); Buffer B consisted of acetonitrile. The flow rate was 1 ml/min. We applied gradient elution from 100% buffer A to 60% buffer B from 0 to 30 min; it was maintained at 60% buffer B until 43 min.

**Isolation and Mass Spectra analysis of HPLC peaks.** The HPLC peaks corresponding to NMP or the glutathione conjugates with HMP were collected, concentrated under vacuum and submitted to MS analyses.

**Metabolism of NO-ASAs by rat liver or colon subcellular fractions.** A volume of 0.05 ml aliquots of NO-ASA (20 mg/ml in CH<sub>3</sub>CN) was added to 4.45 ml rat liver/colon subcellular fractions (protein concentration: 2 mg/ml for liver; 1 mg/ml for colon). After 5 min preincubation at 37° C, 0.5 ml of a [3 mM MgCl<sub>2</sub>, 15 mM NADPH, 15 mM NADP] solution was added to the incubation mixture. The mixture was incubated in a metabolic shaker at 37° C for various time periods. At the end of each of the incubations, 0.5 ml aliquots were mixed with 1.0 ml of CH<sub>3</sub>CN containing 1% phosphoric acid (2M), vortexed and then centrifuged for 10 min at 5000xg. Following this, the supernatants were submitted to HPLC analyses.

**Cell culture:** HT-29 human colon adenocarcinoma cells were grown as described (preceding paper). Cells were seeded in 6-well plates at a density of 1.1x10<sup>5</sup>/cm<sup>2</sup> in 4 ml culture medium and allowed to attach for 24 h. NO-ASA was added to the medium (final concentration 100 μM). At the indicated times, 0.5 ml of medium was removed, extracted with CH<sub>3</sub>CN and fractionated by HPLC as above. To extract NO-ASA and its metabolites

from cells, they were washed three times with cold PBS and lysed with 0.5 ml of cold 5% metaphosphoric acid. The cell lysate was kept at  $-20^{\circ}$  C until analysis; 50  $\mu$ l samples were injected to HPLC and fractionated exactly as above.

**Statistical analyses:** Where indicated, data are presented as *mean*  $\pm$  *SEM* for at least three different sets of plates, three to four different animal preparations, and treatment groups.



## RESULTS

### Metabolism of NO-ASA by rat liver cytosol, microsomes and mitochondria.

HPLC study of the metabolism of NO-ASA by the cytosolic fraction of rat liver revealed the formation of several metabolic products (Fig 2, 3). Two products for which authentic standards were not available were identified by mass spectroscopy (Fig 4). First, the identity of the peak at 13 min was confirmed on the basis of its quasi molecular ion peaks at  $m/z$  414  $[M+H]^+$  and 436  $[M+Na]^+$  as the conjugate of 4-(hydroxymethyl)phenol (4-HMP) with glutathione (GSH; 4-HMP-GSH). Second, the peak at 26 min was confirmed on the basis of the quasi molecular ion peak at  $m/z$  168  $[M-H]^+$  as 3-(nitoxymethyl)phenol (3-NMP). The corresponding UV spectra were consistent with these assignments.

#### **Cytosol**

As shown in Figs 2 and 3, the formation of NO-ASA metabolic products is both time- and isomer-dependent. Fig. 3 summarizes the quantification of the main metabolites of the *m*- and *p*- isomers of NO-ASA over a 5-hour period of observation. For both, *m*- and *p*-, the parent compound becomes undetectable by 10 min, having been reduced to nearly 5% of its original amount by 5 min. The second prominent change is the rapid loss of the acetyl group from NO-ASA. In the first 5 min, most of NO-ASA (both isomers) is in its deacetylated form, becoming undetectable within the first 30 min. By 5 min, salicylic acid is the second prominent species, common to both isomers. The formation of salicylic acid peaks at 30 minutes, being essentially maximal and stoichiometric to the input drug.

There are, however, three important differences in the metabolism of these two isomers by the cytosolic fraction. First, the *m*- isomer generates 3-NMP, whereas the *p*- isomer does not generate detectable amounts of the corresponding 4-NMP. 3-NMP

forms rapidly, reaching its maximal level at 30 min (equal mol for mol to the input drug), gradually declining over the next 270 min to about 40% of the maximum. Second, the *p*-isomer forms 4-HMP slowly. 4-HMP becomes evident only at 10 min, reaching a plateau at 30 min; at its maximum, it represents, on a molar basis, about 20% of the input drug. Third, the *p*-isomer forms rapidly a conjugate with glutathione (4-HMP-GSH), the levels of which are maximal by 15 min, when they also reach their plateau. The amount of this conjugate was calculated in relationship to its peak area at 15 min (% of this area); thus, unlike other metabolites, it cannot be expressed as a percentage of input drug and its quantitative significance remains at present uncertain.

We have also studied the metabolism of the *o*-isomer of NO-ASA in liver cytosol. As can be seen in Fig 2, and based on additional data not shown here, the metabolism of the *o*-isomer of NO-ASA is similar to that of *p*-isomer. Their difference is only quantitative in that the *o*-isomer is converted to 2-HMP and the *p*-isomer to 4-HMP but at a slower rate. That is, the metabolic profiles have the same pattern, all-be-it different final products. For example, at 30 min, only some of the *o*-isomer is converted to salicylic acid and 44% of the input drug is in the deacetylated form. In contrast, no intact or deacetylated *p*-isomer is detectable at this time point (Fig 2, a and c). By 3 hrs, both are completely converted to the same metabolites, as shown in Fig 2 (e and g).

To assess the role of –SH groups in the metabolism of NO-ASA, we pretreated the cytosolic fraction with N-ethylmaleimide 1 mM for 30 min, which reacts avidly with –SH (Shimada and Mitamura, 1994). When –SH groups were unavailable due to their reaction with maleimide, no HMP-GSH conjugate formed, but HMP did form (data not shown). We also used a denitrated derivative of the *p*-isomer (-OH instead of –ONO<sub>2</sub>), to determine the importance of the –ONO<sub>2</sub> group in the formation of the HMP-GSH conjugate. Whereas 4-HMP formed stoichiometrically, no 4-HMP-GSH conjugate

formed, indicating the critical role of  $-\text{ONO}_2$  in this biotransformation, being perhaps a much better leaving group than  $-\text{OH}$ .

### ***Microsomes and mitochondria***

We studied the metabolism of the three NO-ASA isomers by liver microsomes and mitochondria. As shown in Fig 5, microsomes and mitochondria metabolize NO-ASA in a way similar to the cytosol, with one notable difference: only the cytosolic fraction can form the HMP-GSH conjugate. This is expected, as GSH transferase is found mainly in the cytosol and not in microsomes or mitochondria (Strange et al., 2001; Rinaldi et al., 2002). The metabolism of the *o*- isomer is similar to that of the *p*- isomer (data not shown). A quantitative difference between the *m*- and *p*- isomers is that the formation of the corresponding HMP metabolite by microsomes and mitochondria is significantly greater for the latter, even if we consider that the 228 nm UV absorbance of 4-HMP is 3.5 times greater than that of 3-HMP (data not shown).

### **Metabolism of NO-ASA by rat colon**

Given the promising results of NO-ASA in inhibiting colon cancer cell growth ((Williams et al., 2001) and preceding paper) and in preventing colon cancer development in animal tumor models (Bak et al., 1998; Williams et al., 2004), we studied the metabolism of NO-ASA by rat colon tissue. The metabolism of the three isomers by the cytosolic fraction, microsomes and mitochondria of colon tissue mucosa is qualitatively similar to that observed in the corresponding fractions of the liver (Figs 6, 7).

However, there are two important differences. First, compared to the liver, the rate of conversion of NO-ASA to its metabolites by the colon fractions is much slower. For example, at 5 min, less than 5% of either *m*- or *p*- NO-ASA is detectable in liver cytosolic fractions. In contrast, the corresponding values for the colon are 23% and 38%.

As a result, the rate of appearance of the other metabolites is correspondingly slower. Second, there is very limited formation of HMP, and HMP-GSH from either the *m*- or *p*- isomer by colon, in contrast to the liver. In fact, it was technically impossible to quantify either metabolite in the colon; the amplitude or the width or both of these peaks were so small that they precluded determination of their “area-under-the-peak”. Consequently, no values for these two metabolites are shown on Fig 7. The results obtained for the *o*- isomer were similar to those for the *p*-, except that the deacetylated derivative of the *o*- isomer is more stable. For example, after 1 hr of incubation, >40% of its deacetylated derivative is still present, whereas the deacetylated derivatives of both the *p*- and *m*- isomers have undergone complete conversion to salicylic acid (Fig. 6).

Figure 8 summarizes the differences in metabolism of the three positional isomers of NO-ASA by the rat colon cytosolic fraction. At 30 min, the *o*- isomer was predominantly deacetylated whereas about one fourth of it was converted to salicylic acid. No 2-NMP was detected. By this time, the *m*- isomer is converted to salicylic acid (over 80%) and there is maximal formation of 3-NMP, while only residual amounts of its deacetylated form persist. The *p*- isomer follows the same metabolic fate as the *o*-, their only difference being that its transformation proceeds at a faster rate. These two features, differential metabolism of *m*- versus the other two and faster metabolism of *p*- versus *o*-, are highlighted by our findings at 2 hrs, when the *m*- isomer is the only one with the NMP derivative and the *o*- shows a trace of the deacetylated derivative whereas the *p*- is converted entirely to salicylic acid.

### **Metabolism of NO-ASA by HT-29 colon cancer cells**

To assess, even though somewhat indirectly, the relevance of our findings to colon cancer, we studied the metabolism of these three isomers by colon cancer cells. HT-29 human colon cancer cells were incubated with 100  $\mu$ M of the *o*-, *m*- or *p*- isomer

of NO-ASA for 4 hrs. We determined the profile of NO-ASA metabolites in both the culture medium and in cells.

As shown in Fig 9 (top panel), HT-29 cells metabolize all three isomers. Two prominent species extracted from the cells at this time point are salicylic acid and HMP-GSH. Regarding the apparent differences in amplitude between the HMP-GSH peaks in the three chromatograms, we have determined that the absorbance of HMP-GSH is generated almost exclusively by the HMP moiety and that the absorbance at 228 nm of *o*- and *m*- derivatives is virtually identical, whereas that of the *p*- derivative is 3.5 times higher (data not shown). Consequently, the amount of 4-HMP-GSH (derived from *p*- NO-ASA) is 4.4 times that of 3-HMP-GSH (derived from *m*- NO-ASA), whereas the amount of 2-HMP-GSH is about 6 times that of 3-HMP-GSH. Thus, *o*- NO-ASA generates the greatest amount of the HMP-GSH conjugate followed by the *p*-, whereas the lowest amount is generated by the *m*- isomer.

In agreement with our observations in rat liver and colon, only the *m*- isomer generates 3-NMP; no detectable amounts of this metabolite are seen with the other two isomers.

The culture medium of the *o*- and *p*- treated cells contains salicylic acid and deacetylated NO-ASA, whereas that of the *m*- treated cells contains, in addition, NMP. In contrast to the cells, no HMP-GSH is present in any of these culture media. The three isomers of NO-ASA incubated in culture medium in the absence of cells give a profile similar to that obtained here (data not shown), suggesting the possibility that, at least in part, the metabolites we observed in the medium are not secreted from the cells but are generated before NO-ASA enters the cells.

## DISCUSSION

Our work details the metabolic transformations of the three positional isomers of NO-ASA by fractions of the rat liver and colon and by cultured human colon cancer cells. These data provide the basis to propose the schema of biotransformations of NO-ASA presented in Fig 10.

As illustrated in this figure, the three isomers share a common overall metabolic pathway, but there are also important differences, which can potentially affect their pharmacological effects. All three positional isomers initially undergo a rapid deacetylation that is essentially complete within five minutes. Although this is not immediately apparent from the liver data, it is, nevertheless, very clear from our study of the rat colon, where these processes are slower than in the liver. This step is the same as the deacetylation of traditional aspirin, which is also rapid (Insel, 1996). Following deacetylation, the ester bond between the two benzene rings is cleaved but at different rates among the three isomers, being slower for the *o*- isomer than for the other two; the rates for *m*- and *p*- are roughly equal.

The next step differs dramatically among the three isomers. For the *m*- isomer, the deacetylated derivative of NO-ASA is hydrolyzed to salicylic acid and 3-NMP, both of which are detectable for prolonged periods of time, exceeding 5 hrs. For the *o*- and *p*- isomer, no such intermediate, 2-NMP and 4-NMP, respectively, was detectable under our experimental conditions. However, salicylic acid did form stoichiometrically from the corresponding deacetylated derivatives of NO-ASA. We postulate that 2-NMP and 4-NMP form from *o*- and *p*- isomers of NO-ASA, respectively, but that their half-life is so brief as to be undetectable under our experimental conditions. In all three instances, the respective NMP intermediates either react with GSH (or, conceivably, with –SH bearing molecules such as various proteins) while they undergo hydrolysis, releasing NO<sub>3</sub><sup>-</sup>. This

pathway indicates that the mechanism of formation of this intermediate differs among the three isomers.

The rate of formation of these end products differs among the isomers. Their formation is slowest for *m*-, being still incomplete at 5 hrs. Next is *o*-, being complete within 2 hrs. We estimate that this reflects the slow rate of the preceding step, i.e. the cleavage of the ester bond between the two benzene rings. Finally, the fastest of all is the *p*- isomer, its metabolism by liver cytosol being complete within 30 min.

This metabolic pathway takes place in its entirety only in the cytosolic fraction of the tissues tested and in intact human colon cancer cells. The simplest explanation for this observation is that glutathione-S-transferase, the enzyme catalyzing the formation of HMP-GSH, is present only in the cytosolic fraction. Furthermore, glutathione-S-transferase and GSH are present in abundance only in the cytosol, in minimal amounts in the mitochondria and not at all in the microsomal fraction (Strange et al., 2001; Rinaldi et al., 2002). Aside from this difference, it is important to note that the rates of the other metabolic steps parallel those observed in the cytosol.

It is worth mentioning that our data indicate that the –SH groups play a quantitatively important role in these transformations. Their absence, however, does not preclude the metabolism of NO-ASA via any of the other pathways. It is also of interest that –ONO<sub>2</sub> appears to be a critical moiety for the formation of the HMP-GSH conjugate, although its exact mechanistic participation in this reaction remains at present unclear. The denitrated analog of *p*- NO-ASA that we studied failed to form any 4-HMP-GSH.

Rat colon tissue metabolizes the three positional isomers of NO-ASA in a fashion similar to rat liver, generating an identical profile of metabolic products. The only difference lies in the rate of metabolism, which is somewhat slower in the colon. For example, at 5 min 23% and 38% of intact *m*- and *p*- NO-ASA, respectively, is detectable in colon cytosolic fractions, but less than 5% is detectable in the liver cytosolic fraction. A

likely explanation for this difference is the lower protein concentration in the colon samples that were used in our assays (half that of liver).

The metabolism of NO-ASA by intact human colon cancer cells followed the same pattern as for the cell fractions of the two rat organs. These observations suggest that the pattern of metabolic transformations we have detected is not species- or tissue-restricted and likely represents the general mode of biotransformation of these interesting compounds.

Our findings, taken together with our data on the reduced potency of denitrated analogs of NO-ASA, underscore the significance of the  $-\text{ONO}_2$  group for the pharmacological effects of NO-ASA. Such effects are probably not restricted to cancer cell growth but also to other important areas as well as to their safety, at least in the gastrointestinal system (Fiorucci et al., 2003).

Carini *et al* reported recently the *in vitro* metabolism of the *m*- isomer of NO-ASA by rat liver cytosol and microsomes (Carini et al., 2002). In general the pathway they described is similar to the one we observed. Differences in details, such as their reported lack of formation of HMP are probably explained by methodological differences, including differences in cell fractionation and protein and GSH content per assayed sample. Indeed, when we repeated our experiment following their method we obtained virtually identical results (data not shown).

As documented in the accompanying paper, the three positional isomers of NO-ASA differ in their ability to inhibit colon cancer cell growth. The  $\text{IC}_{50}$  for the *o*- , *m*- and *p*- isomers at 24 hrs are  $4.8 \pm 0.8 \mu\text{M}$ ,  $500 \pm 62 \mu\text{M}$  and  $5.0 \pm 1 \mu\text{M}$ , respectively. There is an apparent correlation between the features of their metabolic transformations that we describe in this paper and their effects on colon cancer cell growth. It is tempting to speculate that there is a true mechanistic connection underlying this apparent correlation. For example, as shown in Fig. 10, the rates of release of  $\text{NO}_3^-$  (which



ultimately is converted to NO) by these compounds differ among them, a finding corroborated by work reported in the preceding manuscript. Thus the differences in metabolic transformations of these isomers may account for differences in biological potency; ongoing work by us is attempting to address this issue.

In summary, our data establish the metabolic transformations of three positional isomers of NO-ASA by rat liver and colon tissue fractions and intact human colon cancer cells; demonstrate important differences among them that likely correlate with their effects on cancer cell growth; and provide the basis for further mechanistic studies involving such important cell constituents like GSH. Finally our data underscore the role of positional isomerism in modulating the pharmacological effects of drugs.

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## FOOTNOTES

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## LEGENDS TO FIGURES

**Figure 1. The chemical structure of NO-ASA.** Traditional aspirin (acetylsalicylic acid, shaded area) is covalently bound via a spacer molecule to  $-\text{ONO}_2$ , the NO-donating moiety.

**Figure 2. Metabolism of NO-ASA by rat liver cytosol.** *o*-, *m*- and *p*- isomers of NO-ASA were incubated for either 30 min (a-c) or 3 hrs (e-g) with rat liver cytosolic fraction, as in Methods. NO-ASA and its metabolites were extracted and fractionated by HPLC. As a control, the *p*- isomer was incubated in parallel in PBS for 30 min (d). Only 3% of NO-ASA was converted to its denitrated derivative, the rest of it remaining intact. Similar data were obtained with the other two isomers.

SA, salicylic acid; HMP, (hydroxymethyl)phenol; NMP, (nitroxymethyl)phenol; HMP-GSH, conjugate of HMP with glutathione (GSH).

**Figure 3. Metabolism of NO-ASA by rat liver cytosol.** The *m*- and *p*- isomers of NO-ASA (each 600  $\mu\text{M}$ ) were incubated for up to 300 min with rat liver cytosolic fraction, extracted and fractionated by HPLC, as in Methods. Values were calculated based on area under the peak determinations and data were expressed as % of input drug. HMP-GSH is expressed as % of the maximal peak area, first occurring at 15 min of incubation, and 3-NMP is expressed as % of the maximal peak area occurring at 30 min of incubation.

SA, salicylic acid; HMP, (hydroxymethyl)phenol; NMP, (nitroxymethyl)phenol; HMP-GSH, conjugate of HMP with glutathione (GSH).

**Figure 4. Mass spectrometry of metabolic products of NO-ASA.** The HPLC elution fractions corresponding to the 13 min (A) and 26 min (B) peaks of the extract of cytosol treated with the *p*- and *m*- isomers of NO-ASA, respectively, were processed as in Methods and analyzed by MS. Insets: UV spectra of each fraction. A, 4-(hydroxymethyl)phenol - glutathione conjugate; B, 3-(nitroxymethyl)phenol.

**Fig 5. NO-ASA metabolism by liver cell fractions.** The *m*- and *p*- isomers of NO-ASA (each 600  $\mu$ M) were incubated for 60 min with rat liver cytosolic or microsomal fraction, or mitochondria extracted and fractionated by HPLC, as in Methods. Prominent features are the absence of *HMP-GSH* [(hydroxymethyl)phenol-glutathione conjugate] from the microsomal fraction and the mitochondria and the formation of 3-*NMP* [(nitroxymethyl)phenol] by *m*- NO-ASA. SA, salicylic acid

**Fig 6. Metabolism of NO-ASA by rat colon cytosol.** *o*-, *m*- and *p*- isomers of NO-ASA or solvent (control panel) were incubated for 60 min with rat colon cytosolic fraction as in Methods. NO-ASA and its metabolites were extracted and fractionated by HPLC.

SA, salicylic acid; *HMP*, (hydroxymethyl)phenol; *NMP*, (nitroxymethyl)phenol; *HMP-GSH*, conjugate of HMP with glutathione (GSH).

**Figure 7. Metabolism of NO-ASA by rat colon cytosol.** The *m*- and *p*- isomers of NO-ASA (each 600  $\mu$ M) were incubated for up to 120 min with rat colon cytosolic fraction, extracted and fractionated by HPLC, as in Methods. Values were calculated based on area under the peak determinations and data were expressed as % of input drug. 3-*HMP* is expressed as % of the maximal peak area occurring at 30 min of incubation.

SA, salicylic acid; *HMP*, (hydroxymethyl)phenol; *NMP*, (nitroxymethyl)phenol; *HMP-GSH*, conjugate of HMP with glutathione (GSH).

**Figure 8. Metabolism of the three positional isomers of NO-ASA by rat colon cytosol.** The *o*-, *m*- and *p*- isomers of NO-ASA (each 600  $\mu$ M) were incubated for 30 and 120 min with rat colon cytosolic fraction, extracted and fractionated by HPLC, as in Methods. Values were calculated based on area under the peak determinations and data were expressed as % of input drug. 3-HMP is expressed as % of the maximal concentration, which occurred at 30 min.

SA, salicylic acid; HMP, (hydroxymethyl)phenol; NMP, (nitroxymethyl)phenol.

**Figure 9. Metabolism of the three positional isomers of NO-ASA by human colon cancer cells.** The *o*-, *m*- and *p*- isomers of NO-ASA (each 100  $\mu$ M) were incubated for 4 hrs with HT-29 human colon cancer cells and metabolites were extracted from either the culture medium or the cells and fractionated by HPLC, as in Methods.

SA, salicylic acid; NMP, (nitroxymethyl)phenol; HMP-GSH, conjugate of (hydroxymethyl)phenol (HMP) with glutathione (GSH).

**Fig 10. Proposed metabolism of NO-ASA positional isomers.** All three isomers of NO-ASA start with a common deacetylation step and end up with similar end products. However, following cleavage of the ester bond between the two benzene rings, the *o*- and *p*- isomers release  $\text{NO}_3^-$  and the form end products rapidly via a putative short-lived intermediate, whereas the *m*- isomer forms the stable (nitromethoxy)phenol, which releases  $\text{NO}_3^-$  and forms end products at a much slower rate.

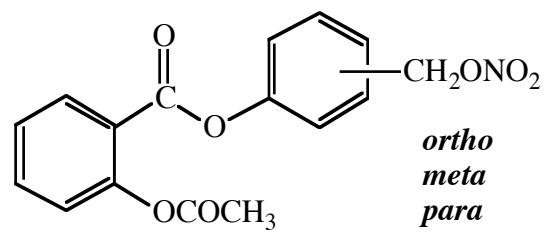


Fig 1

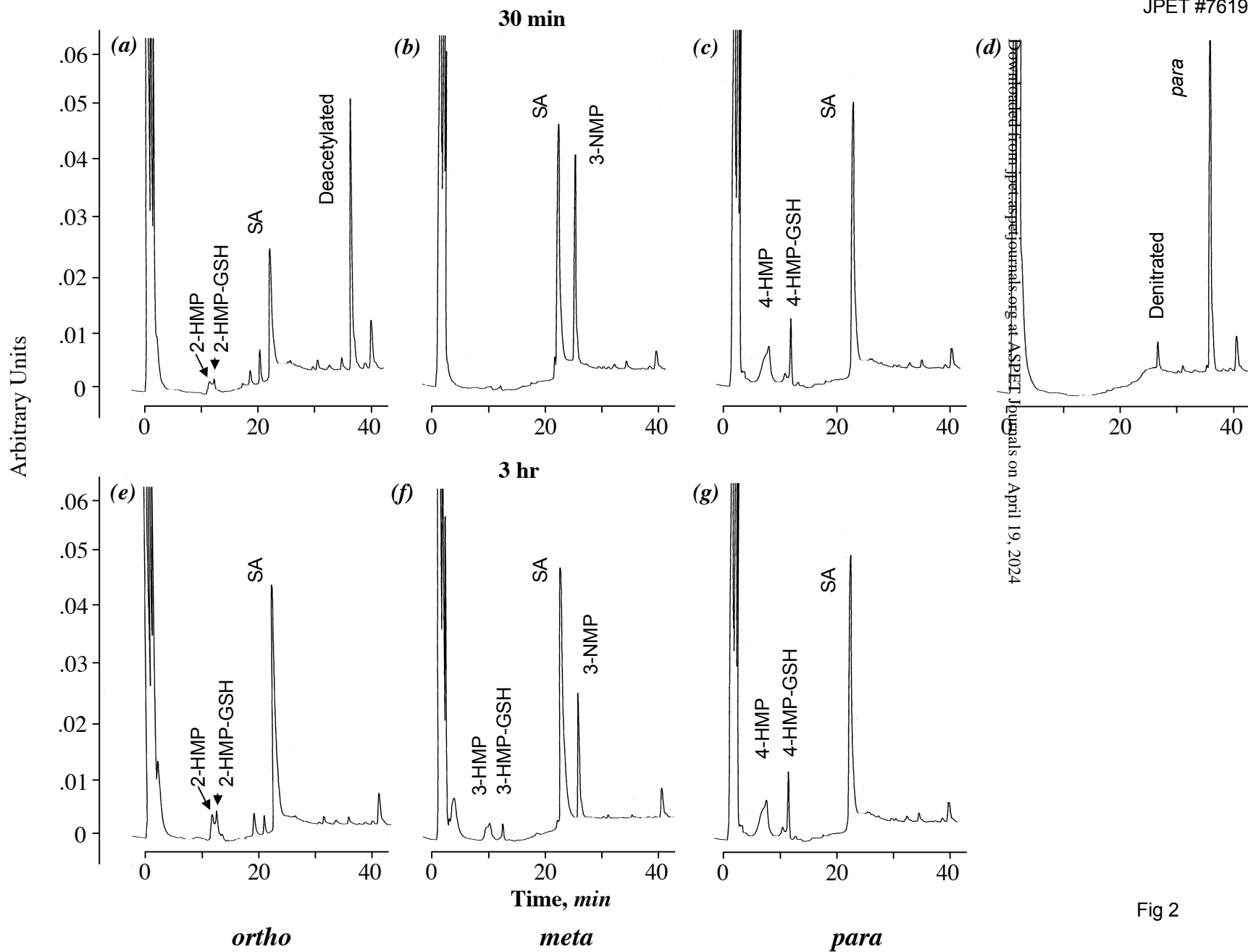


Fig 2



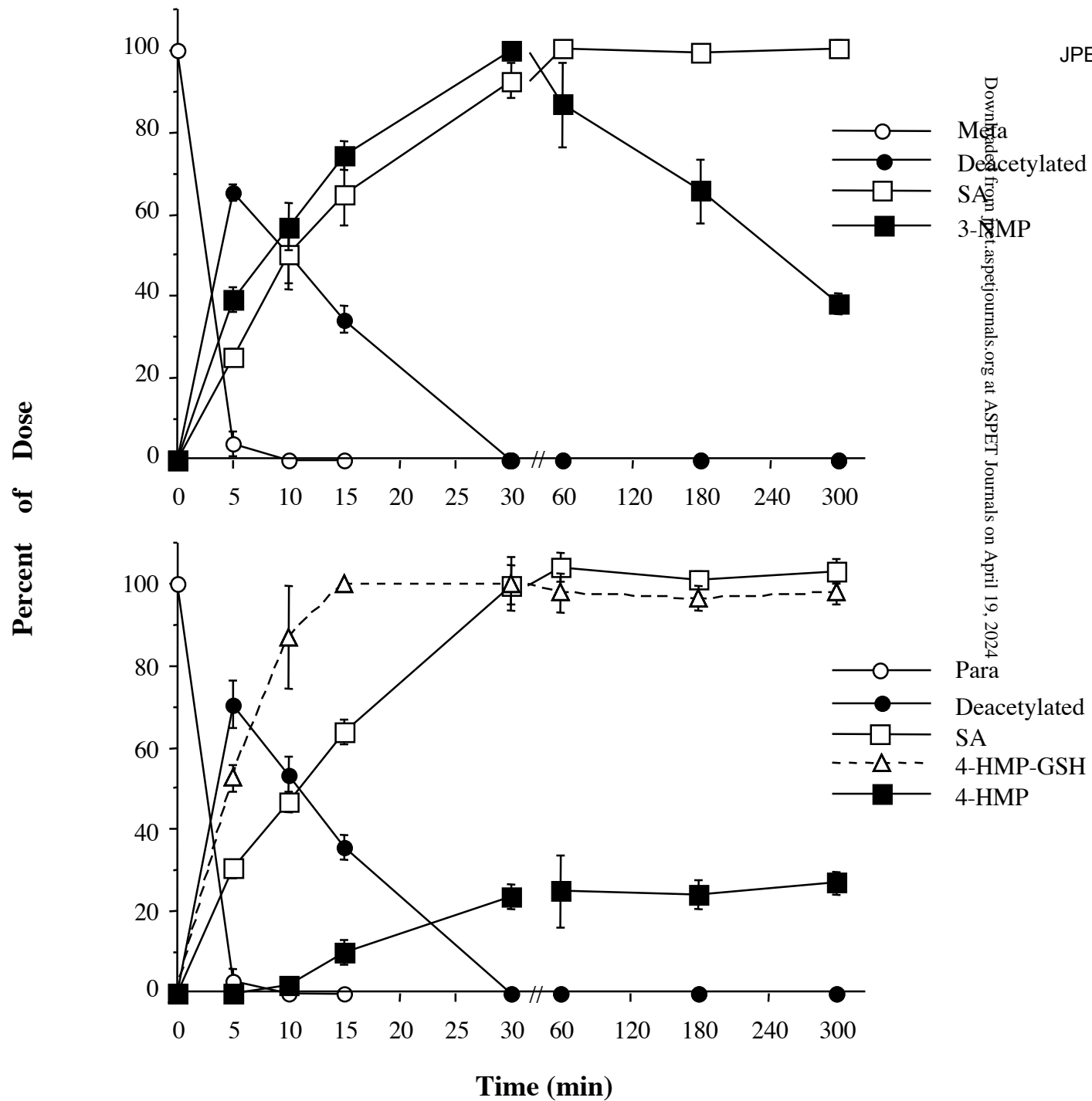


Fig 3

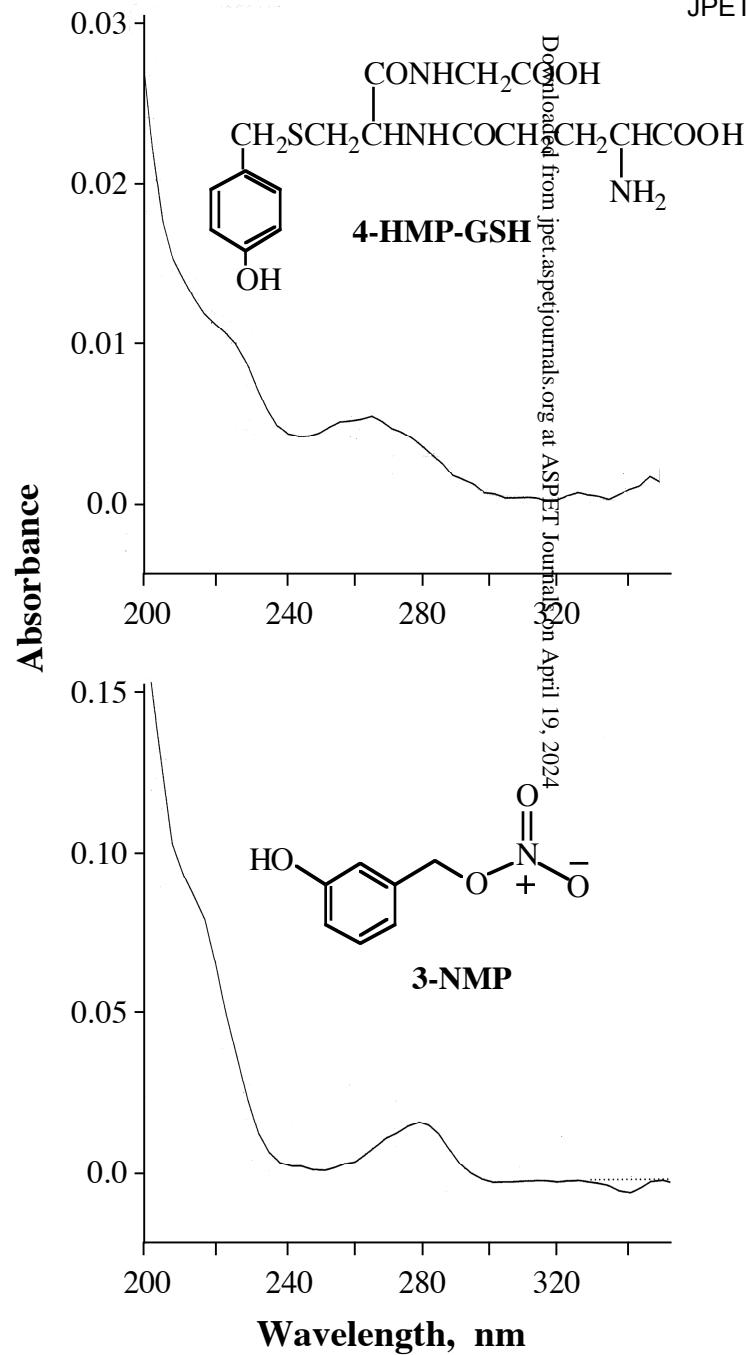
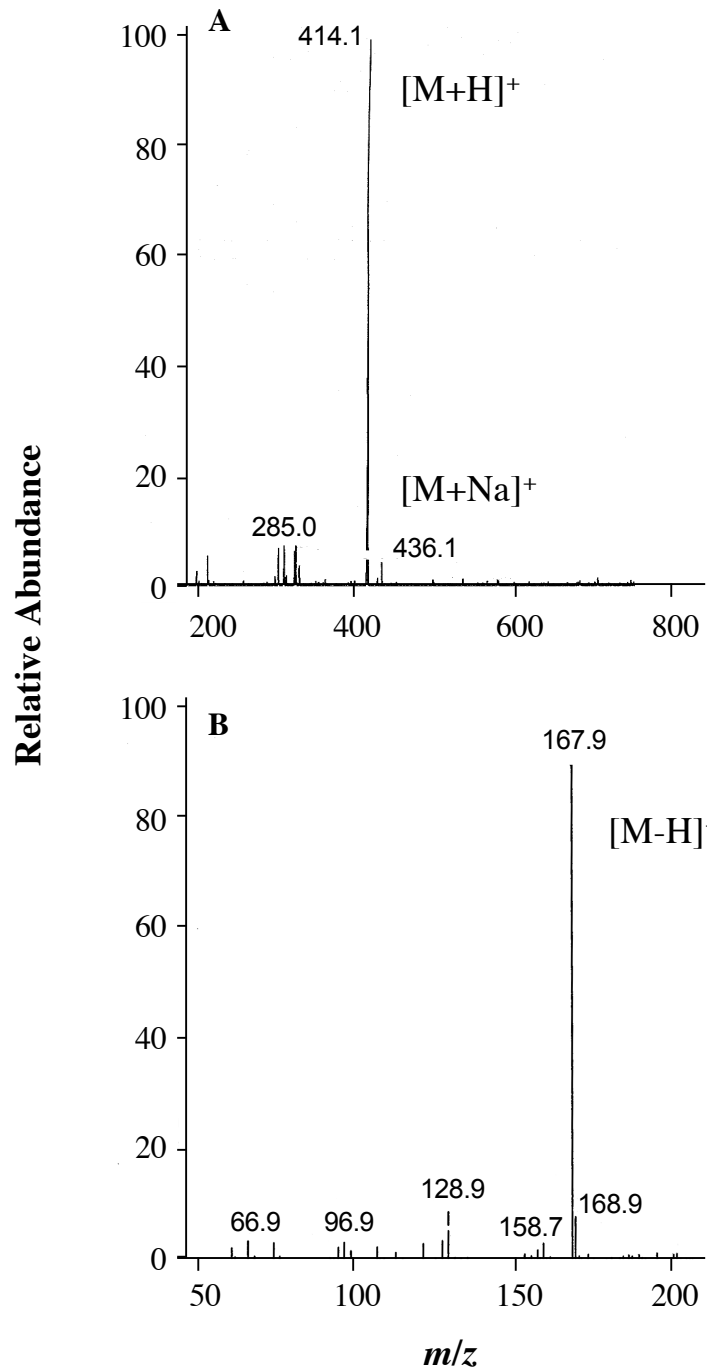
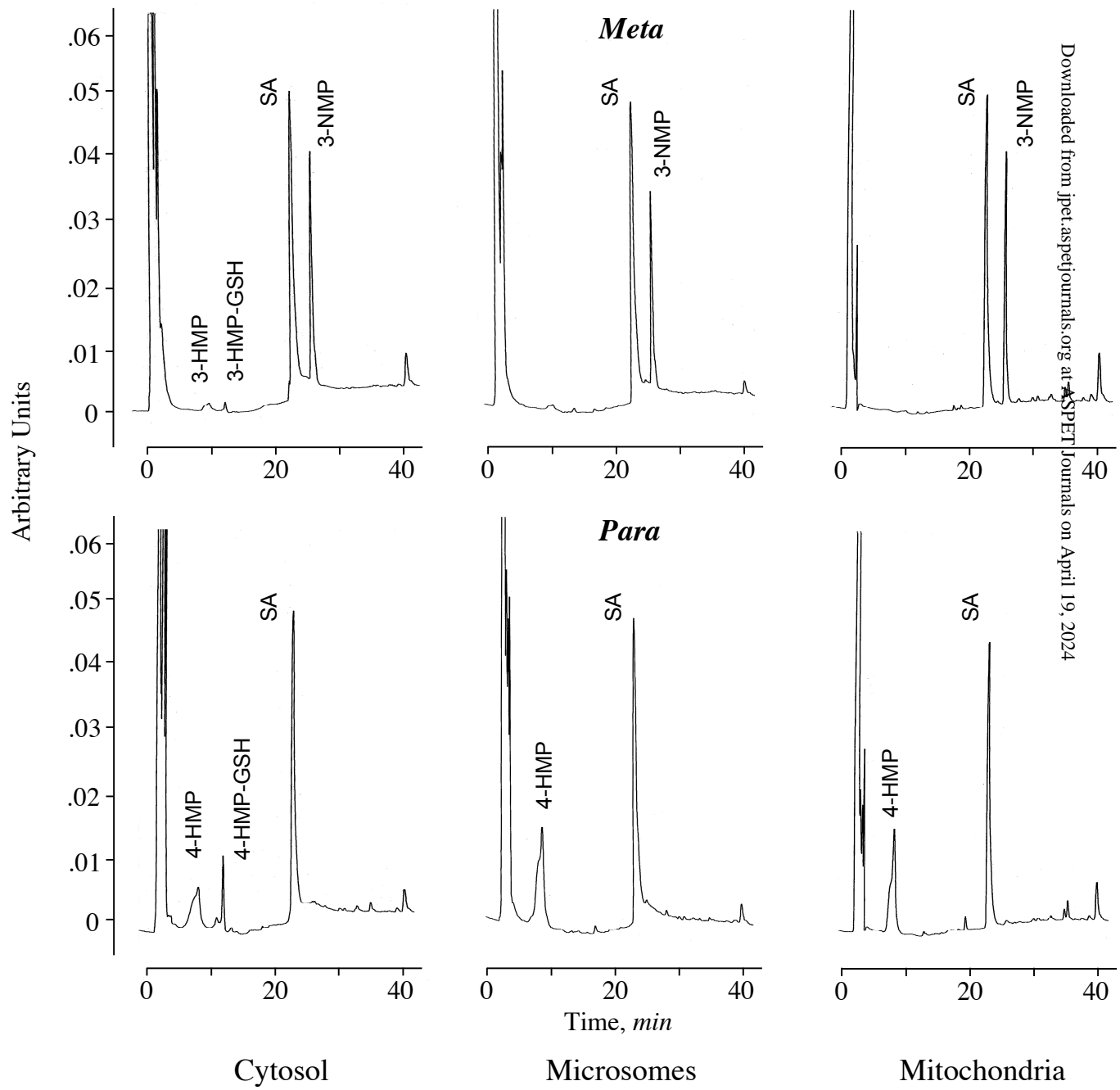


Fig 4



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Fig 5

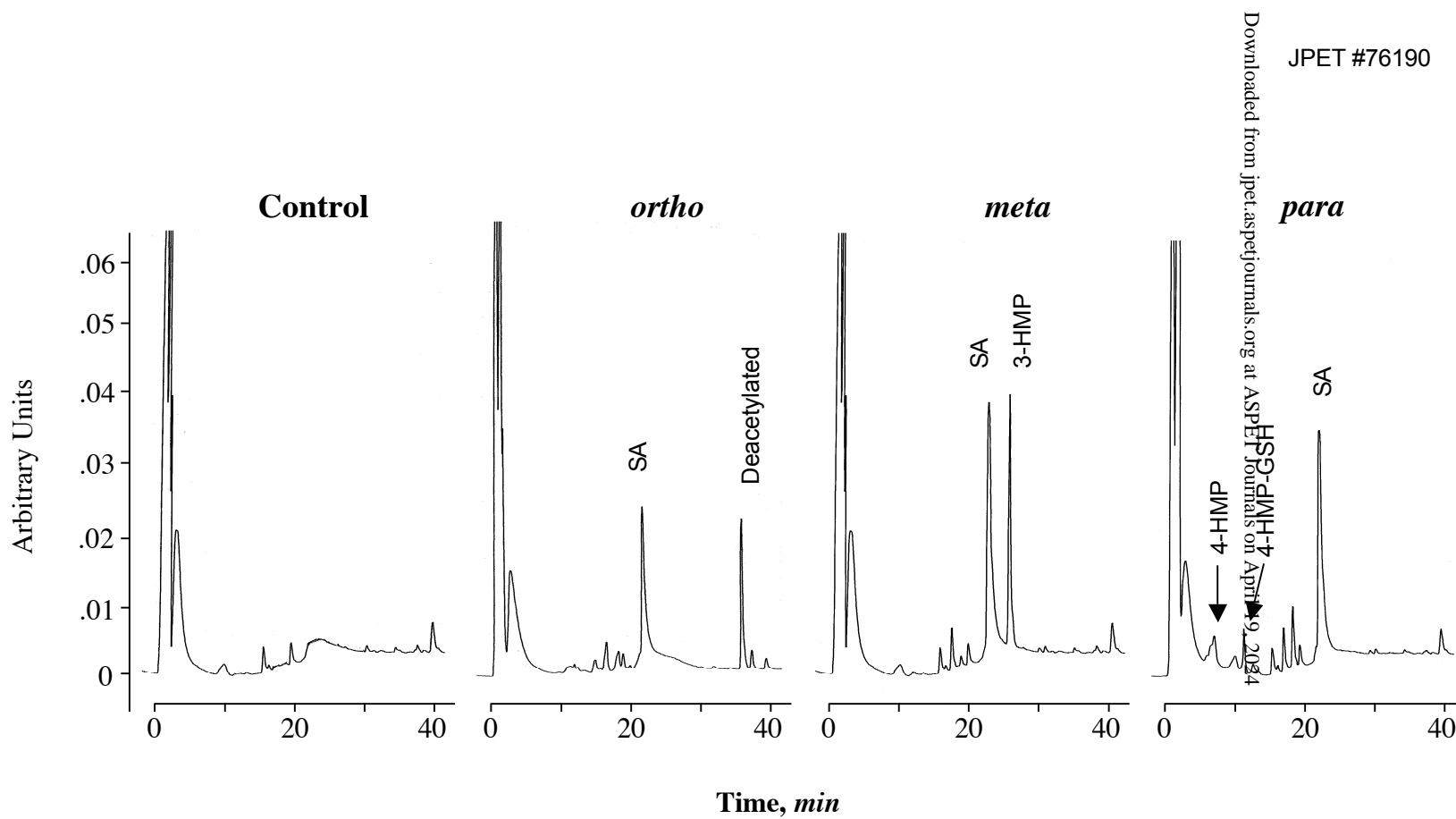
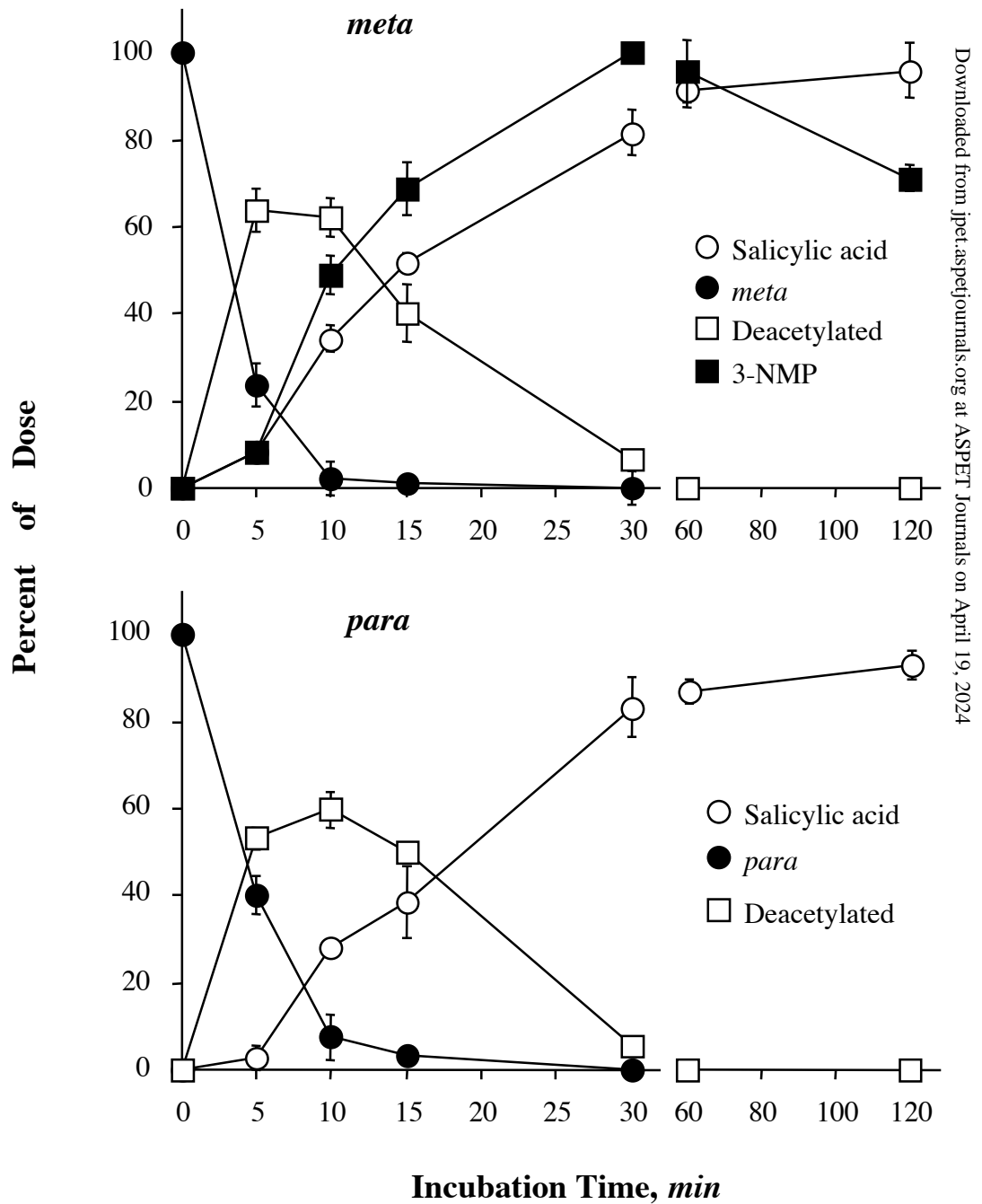
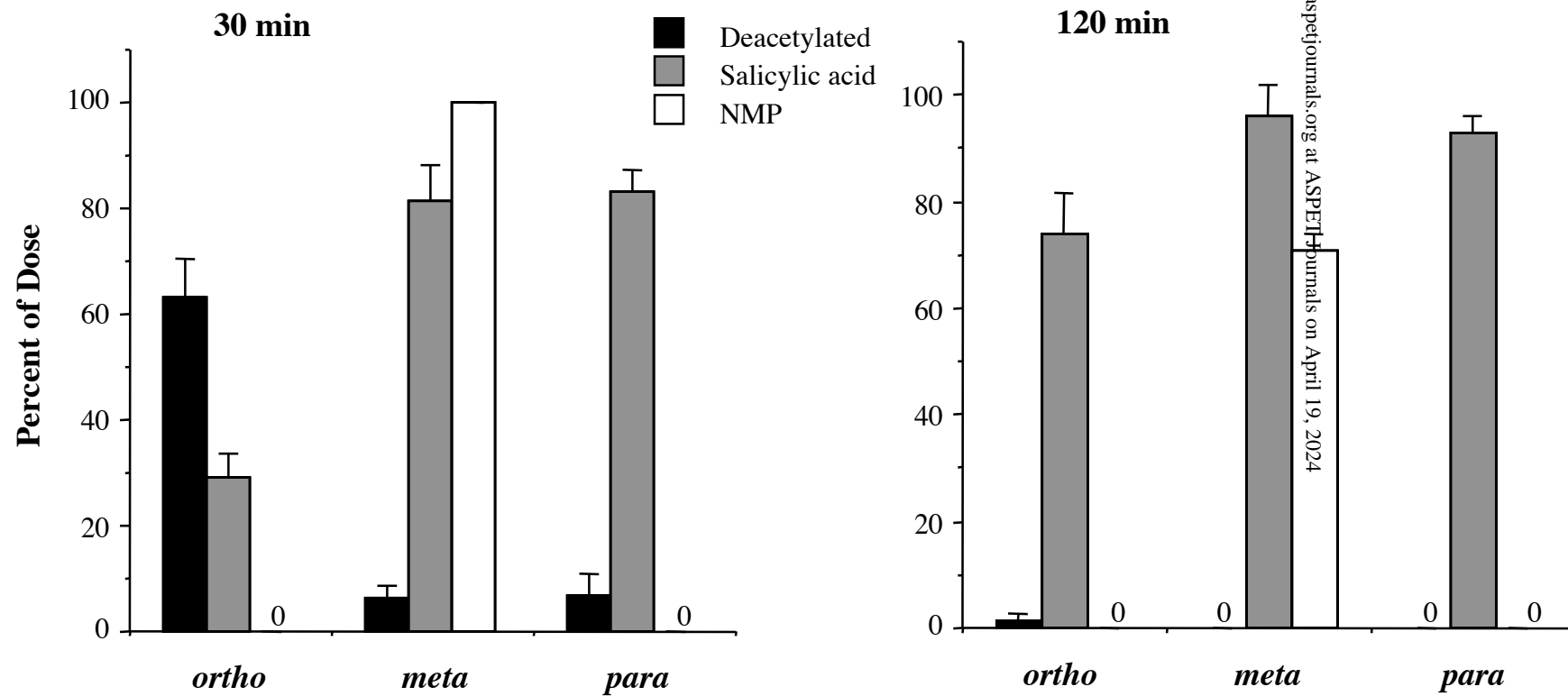


Fig 6



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Fig 7



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Fig 8

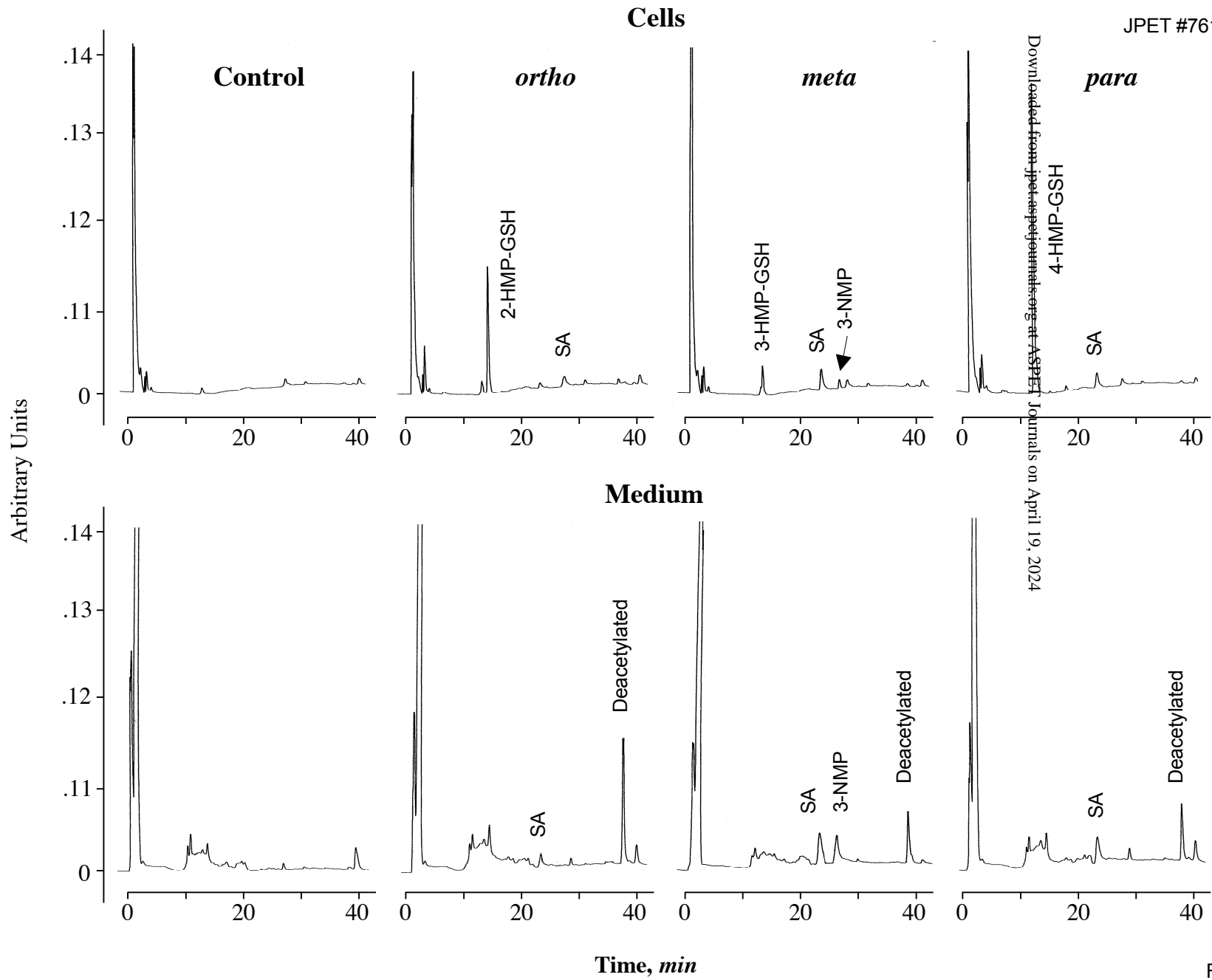


Fig 9

