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Liquid chromatography/electrospray ionisation mass spectrometric tracking of 4-hydroxy-2(*E*)-nonenal biotransformations by mouse colon epithelial cells using [1,2-$^{13}C_2$]-4-hydroxy-2(*E*)-nonenal as stable isotope tracer†

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4-Hydroxy-2(*E*)-nonenal (HNE), a product of lipid peroxidation, has been extensively studied in several areas, including metabolism with radio-isotopes and quantification in various matrices with deuterium-labelled HNE as standard. The aim of this work was to evaluate the relevance of ^{13}C-labelled HNE in biotransformation studies to discriminate metabolites from endogens by liquid chromatography/electrospray ionisation mass spectrometry (LC/ESI-MS). ^{13}C-Labelled HNE was synthesised in improved overall yield (20%), with the incorporation of two labels in the molecule. Immortalised mouse colon epithelial cells were incubated with 2:3 molar amounts of HNE/^{13}C-HNE in order to gain information on the detection of metabolites in complex media. Our results demonstrated that the stable isotope *m/z* values determined by mass spectrometry were relevant in distinguishing metabolites from endogens, and that metabolite structures could be deduced. Six conjugate metabolites and 4-hydroxy-2(*E*)-nonenoic acid were identified, together with an incompletely identified metabolite. Stable-isotope-labelled HNE has already been used for quantification purposes. However, this is the first report on the use of ^{13}C-labelled HNE as a tracer for *in vitro* metabolism. ^{13}C-Labelled HNE could also be of benefit for *in vivo* studies. Copyright © 2011 John Wiley & Sons, Ltd.

The γ-hydroxy-α,β-unsaturated aldehyde 4-hydroxy-2(*E*)-nonenal (HNE) is one of the major aldehydes among the breakdown products formed during the peroxidation of ω6 fatty acids. These polyunsaturated fatty acids (PUFAs) are part of the cell membrane constituents and their peroxidation to reactive aldehydes can alter cell integrity. The cytotoxicity of HNE has been shown and its possible involvement in the development of pathologies related to oxidative stress and aging has been investigated.[1,2] HNE has also been detected in food.[3–5] Therefore, HNE can have both endogenous and exogenous origin.

An enhanced consumption of haem-rich food, red and processed meat, is associated with an increased risk of colon cancer.[6,7] Haem is able to induce the peroxidation of PUFAs *in vitro*. Haemin, a model molecule for haem, increases precarcinogenic lesions in the colon of rats fed with haemin-rich and calcium-poor diet.[8] Therefore, there could be a link between the toxic aldehydes formed by lipid peroxidation and colon cancer.

The mutation of the *Apc* gene is known to be involved in early stages of colon cancer. We have used immortalised mouse colon epithelial cells bearing or not a mutation on the *Apc* gene, as a model to study and compare the biological effects of food components and HNE in terms of cytotoxicity.[9] We have also used this model to compare the biotransformation capacities of the two cell lines in a quantitative way by monitoring HNE consumption and metabolite formation, using a radio-isotope (manuscript in preparation).

In metabolism studies, the identification of metabolites by mass spectrometry (MS) can be hindered by interfering endogens coming from the matrices and co-eluting with the metabolites of interest. In this case, the use of radio-isotopes is the method of choice for the selective and efficient detection and quantification of metabolites by liquid chromatography (LC)/radio-isotope counting. However, since the radio-isotope is used in small amount, identification in LC/MS is not possible. Stable isotopes can overcome this problem since they can be used in quantitative amounts compared with the naturally abundant isotope, thus providing ions of characteristic *m/z* values for selective detection by MS.

^{13}C/^{12}C isotopic ratio analyses by isotope ratio mass spectrometry (IRMS) and conventional MS are used in various fields.[10] The applications of stable-isotope-labelled drugs and toxic compounds in metabolism studies have been recently reviewed.[11] In disposition studies, deuterium-labelled and ^{13}C-labelled glucose have been used simultaneously to study kinetics after ingestion, using gas chromatography (GC)/MS after derivatisation, and gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS).[12] Fatty acid fluxes have been traced after the ingestion of a ^{13}C-triglyceride using GC/C/IRMS.[13] Jian *et al.* reported on the LC/MS

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quantification of a glutathione conjugate of 4-oxo-2(*E*)-nonenal obtained from cells.[14] To our knowledge, the metabolism of lipid peroxidation products has not been studied with a ^{13}C stable isotope tracer by LC/MS. Nevertheless, syntheses of stable-isotope-labelled HNE have been reported. HNE with one ^{13}C atom has been synthesized to study its *in vitro* interaction with proteins by ^{13}C-nuclear magnetic resonance (^{13}C-NMR).[15] Deuterated HNE has been validated as internal standard for quantification by MS.[16–18] However, we reasoned that this molecule was not relevant for metabolism studies because the deuterium could be cleaved by biotransformations occurring at the methyl-deuterated terminus.[19] We recently reported on the synthesis of HNE with two ^{13}C labels.[20]

The aim of this work was to assess the use of a ^{13}C stable isotope tracer included into HNE to distinguish HNE-specific metabolites from endogens produced by mouse colon epithelial cells, using LC/electrospray ionisation (ESI)-MS. We will first discuss the ^{13}C-HNE synthesis, and then the mass spectrometry results obtained from cell incubation media.

EXPERIMENTAL

Chemicals

Ethyl 2-bromoacetate was purchased from Aldrich (Saint Quentin Fallavier, France). Solvents, 4-chlorothiophenol, hydrogen peroxide (30% in water), *n*-heptanal and diisobutylaluminium hydride (DIBAL: 1 M in *n*-hexane), were purchased from Acros Organics (Geel, Belgium). *n*-Heptanal was distilled before use. Ethyl [1,2-$^{13}C_2$]-2-bromoacetate (99% atom ^{13}C) was purchased from Eurisotop (Saint Aubin, France).

Caution: HNE is cytotoxic and ethyl 2-bromoacetate is carcinogenic. They should be handled with gloves in a well-ventilated fume cupboard.

[1,2-$^{13}C_2$]-4-Hydroxy-2(*E*)-nonenal (^{13}C-HNE)

^{13}C-HNE was prepared according to our published method,[20] with modifications. Ethyl [1,2-$^{13}C_2$]-2-bromoacetate was converted into ethyl [1,2-$^{13}C_2$]-4-hydroxy-2(*E*)-nonenoate by reacting consecutively with 4-chlorothiophenol, hydrogen peroxide and *n*-heptanal as described.[21] The remaining steps to ^{13}C-HNE were unchanged and the overall yield was improved to 20% (Fig. 1).

4-Hydroxy-2(*E*)-nonenal (HNE)

HNE was prepared from ethyl 2-bromoacetate by the same method as for ^{13}C-HNE.

Mixtures containing 2:3 molar amounts of HNE/^{13}C-HNE were prepared for cell experimentation and were checked by NMR (^1H, $CDCl_3$).

Mouse colon epithelial cells experimentation

Mouse colon epithelial cells (*Apc*+/+) were established as previously described.[22] Briefly, the cell line was established from the colon of F1 progeny obtained after pairing a heterozygous female Immortomouse from Charles River Laboratories (Wilmington, MA, USA) with a male C57BL/6 J mouse from Charles River Laboratories.

The use of an Immortomouse makes it possible to obtain cell lines C57BL/6 J-*Apc*+/+ that express the heat-labile SV40 large T antigen (AgT tsA58) under the control of an interferon γ (INFγ)-inducible promoter. At 33 °C and with INFγ (Becton Dickinson, Le Pont de Claix, France) in the medium, the temperature-sensitive SV40 large T antigen is active and drives cell proliferation. At 37 °C the temperature-sensitive mutation yields an inactive protein, and cells behave as non-proliferating colonic epithelial cells. The cells were cultured at 33 °C in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Cergy-Pontoise, France) supplemented with 10%

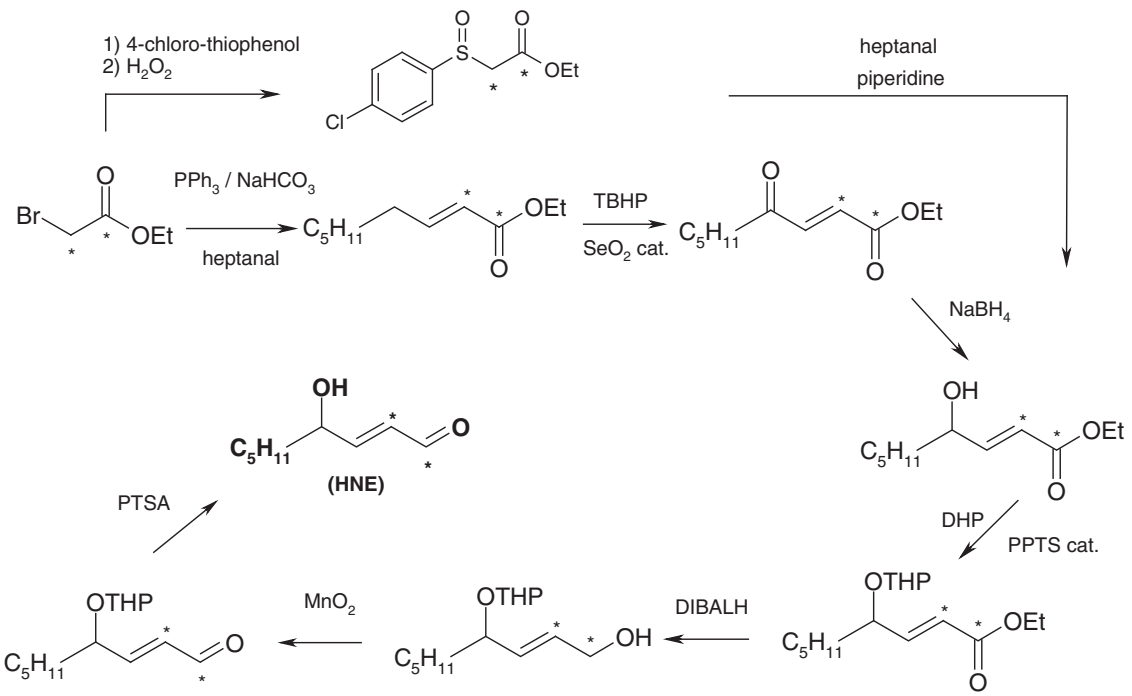

Figure 1. Synthetic scheme for ^{13}C-HNE preparation. Asterisks indicate ^{13}C labels.

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Table 1. LC/MS identified *Apc* +/+ metabolites of HNE

LC	ESI-MS	ESI-MS^2	ESI-MS^2	Metabolites
Retention time (min)	*m/z*	product ions *m/z* (precursor ion *m/z*)	product ions *m/z* (precursor ion *m/z*)	
16	276/278	189, 120 (**276**)	191, 120 (**278**)	HNE-cysteine
19.1/21.1	464/466	446, 272, 254, 335 (**464**)	448, 272, 254, 337 (**466**)	DHN-glutathione
19.9/22.0/24.4	462/464	306, 444, 272, 254, (**462**)	306, 446, 272, 254, (**464**)	HNE-glutathione
32.2	318/320	162, 171, 189 (**318**)	162, 173, 191 (**320**)	HNE-MA
33.5	171/173	153, 127, 71 (**171**)	155, 128, 72 (**173**)	HNA
33.9/34.5	460/462	306, 442, 272, 254, 288 (**460**)	306, 444, 272, 254, 288 (**462**)	HNA-lactone-glutathione
37.2/38.1/39.0	476/478	254, 272, 458 (**476**)	254, 272, 460 (**478**)	?
39.8	316/318	162, 187 (**316**)	162, 189 (**318**)	HNA-lactone-MA

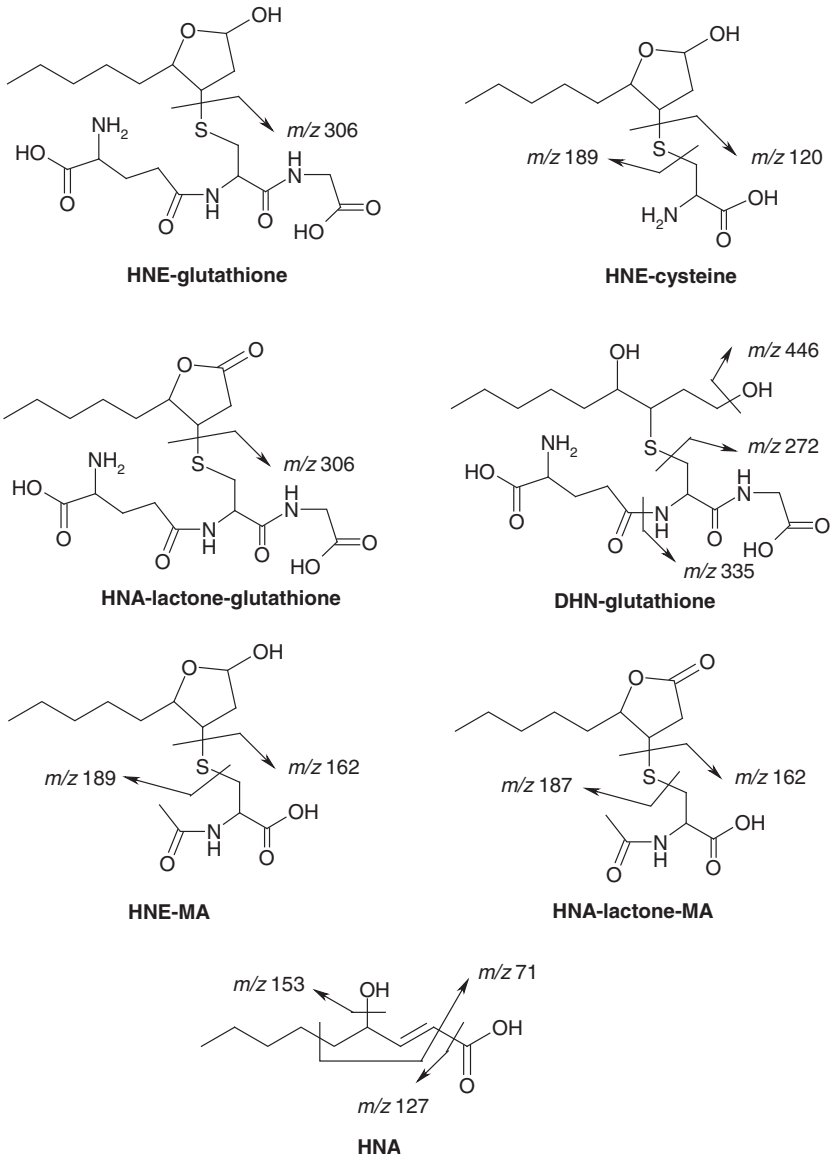

Figure 2. Structures of HNE metabolites by mouse colonocytes. Arrows indicate MS^2 fragments.

foetal calf sera (Sigma-Aldrich, Saint Quentin Fallavier, France), 2% penicillin/streptomycin, 2% glutamine (Invitrogen), and 10U/mL INFγ. The experiments were performed at 37 °C, and without INFγ, to inhibit the SV40 transgene and limit proliferation. For treatment, the *Apc* +/+ colonocytes were incubated with 40 µM ^{13}C-HNE/HNE (3:2 molar amount) in fresh DMEM supplemented with 2% glutamine, at +37 °C for 60 min. The supernatants were then removed, concentrated on a C18 Sep-pak cartridge (200 mg, Waters, Guyancourt, France), stored with 1% 2,6-di-*tert*-butyl-4-hydroxytoluene (BHT) or acetic acid, and analysed.

LC/ESI-MS

The cell culture supernatants were analysed by LC/ESI-MS using a Thermo Separation Products P1500 LC pump (Thermo Fisher, Les Ulis, France) fitted with an ODS Spherisorb column (5 µm, 250 × 4.6 mm; Waters, Guyancourt, France) with the following mobile phases: A: MeCN/water/AcOH (2.5:97.5:0.1); B: MeCN/water/AcOH (60:40:0.1) and gradient:0 min (15%B); 10 min (25%B); 20 min (26%B); 40–45 min (65%B). The flow rate was 1 mL/min with 1/5 post-column splitting.

The liquid chromatograph was coupled to a Finnigan LCQ quadrupole ion trap mass spectrometer (Thermo Fisher) fitted with an ESI source operating in the negative ion mode. Typical ionisation and ion transfer conditions were as follows: electrospray needle voltage, 4.5 kV; heated transfer capillary temperature, 220 °C; heated transfer capillary voltage, –20 V; tube lens offset voltage, 10 V. The MS^2 experiments were carried out under automatic gain control using helium as the collision gas. The tandem mass spectrometric (MS/MS) ion excitation conditions (isolation width, excitation voltage, excitation time) were adjusted to obtain maximum sensitivity and structural information for each compound of interest.

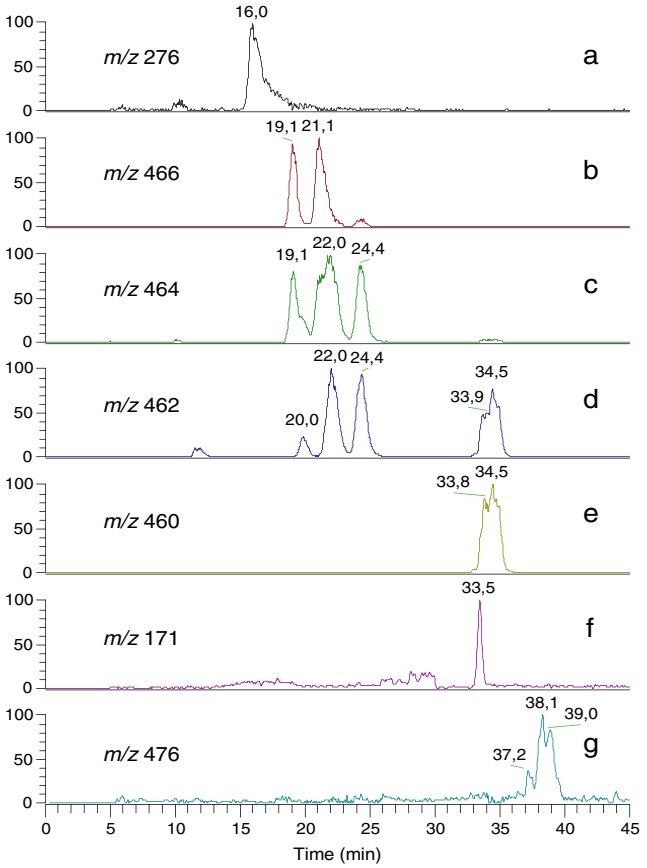

Figure 3. Extracted mass chromatograms for ions at (a) *m/z* 276, (b) *m/z* 466, (c) *m/z* 464, (d) *m/z* 462, (e) *m/z* 460, (f) *m/z* 171, and (g) *m/z* 476, corresponding, respectively, to HNE-cysteine, to glutathione-conjugated metabolites of HNE, to 4-hydroxy-2-nonenoic acid, and to an unidentified metabolite.

RESULTS AND DISCUSSION

Synthesis

In previous work, we reported on the synthesis of ^{13}C-HNE with full characterisation (IR, NMR, HRMS) of the intermediates and the final product.[20] The synthesis was first tested with unlabelled HNE, then with the stable-isotope-labelled compound. However, the overall yield was rather low (4%). To improve this yield we used a synthetic method[21] which enabled the introduction of the three functionalities of HNE (alcohol, alkene, aldehyde) in a more efficient way. We thus prepared directly the ^{13}C-labelled 4-hydroxynonenoate ester starting with the same stable-isotope-labelled precursor (ethyl [1,2-$^{13}C_2$]-2-bromoacetate) (Fig. 1). The yield was thus improved to 20%.

LC/ESI-MS

After incubation of the cells with HNE/^{13}C-HNE (2:3 molar mixture), the supernatants were analysed by LC/ESI-MS in the negative ion mode.

The total ion current reflected the complexity of the supernatants. Along the LC elution, the ions corresponding to the metabolites were tracked using their characteristic ^{12}C/^{13}C patterns. The results are summarised in Table 1, indicating the LC retention times, and the *m/z* values of the HNE metabolites detected by ESI-MS and analysed by MS/MS in the ion trap. The structures of the various metabolites identified together with their corresponding proposed fragmentation pathways are reported in Fig. 2. Typical LC/MS mass chromatograms are reported in Fig. 3.

For a compound eluting at 16 min (Fig. 3(a)), the corresponding negative ESI-MS spectrum (Fig. 4(a)) shows two ions at *m/z* 276/278 displaying the characteristic isotopic pattern of the incubated ^{12}C/^{13}C-HNE mixture, which could correspond to HNE-cysteine.[23] The occurrence of product ions at *m/z* 189 and 120 corresponding to $[HNE–S]^-$ and $[cysteine–H]^-$, respectively, in the MS^2 spectrum acquired from the *m/z* 276 precursor ion (Fig. 4(b)) was also consistent with this compound being the HNE-cysteine conjugate. This assignment was further confirmed by the fragmentation of the *m/z* 278 ion, which yielded the $[cysteine–H]^-$ ion at *m/z* 120 whereas the $[HNE–S]^-$ ion was shifted to *m/z* 191 (Fig. 4(c)). Based on these data, the compound eluted at 16 min was identified as HNE-cysteine.[19] The absence of HNE-cysteine from HNE incubation with DMEM without the cells was checked.

Other metabolites eluted in the 19–34 min retention time range and displaying the characteristic M/M + 2 pattern were detected as smaller peaks (see Table 1). Peaks at 19.1 and 21.1 min (Figs. 3(b) and 3(c)) both exhibited $[M–H]^-$ ions at *m/z* 464/466, consistent with DHN-glutathione conjugate isomers. The $[M–H]^-$ ions of peaks at 19.9, 22.0 and 24.4 min

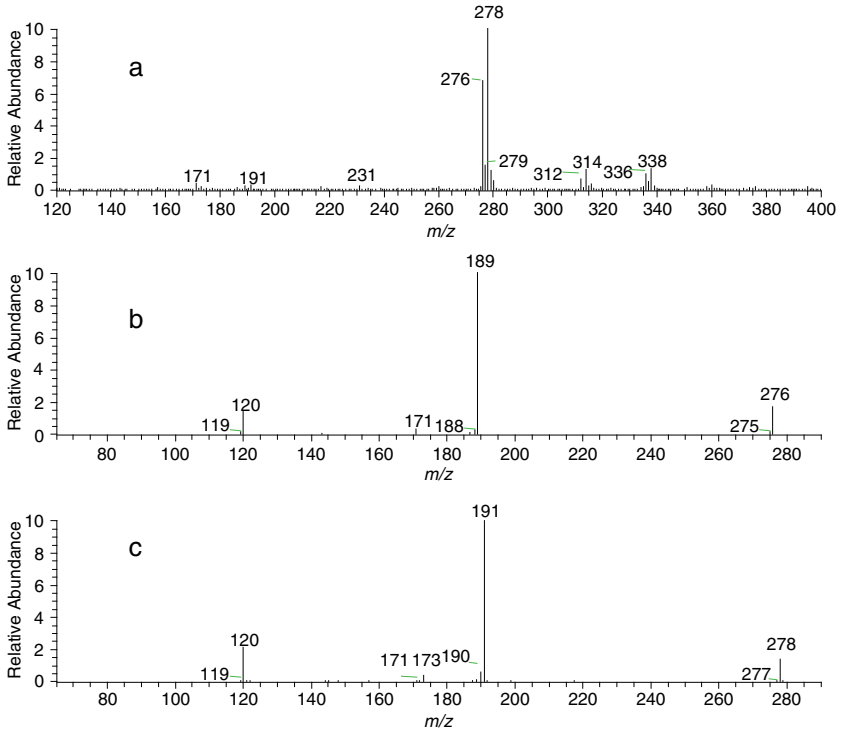

Figure 4. Quadrupole ion trap mass spectra obtained from HNE-cysteine: (a) negative ion ESI mass spectrum, (b) MS/MS spectrum of the *m/z* 276 precursor ion (HNE-cysteine), and (c) MS/MS spectrum of the *m/z* 278 precursor ion ([$^{13}C_2$]-HNE-cysteine).

were all detected at *m/z* 462/464 (Figs. 3(c) and 3(d)), matching HNE-glutathione conjugate isomers.[19] The $[M–H]^-$ ions of peaks at 33.9 and 34.5 min (Figs. 3(d) and 3(e)) at *m/z* 460/462 were characteristic of the HNA-lactone-glutathione conjugate.[23]

The MS/MS spectra acquired from the $[M–H]^-$ ions were in agreement with the proposed structures, as indicated in Table 1, showing the occurrence of the *m/z* 306 and /or 272 product ions, diagnostic of a glutathione moiety (Fig. 2). These assignments were further confirmed by MS/MS experiments performed on the $[M–H]^-$ ions selected from the ^{13}C-labelled HNE metabolites, as reported in Table 1, showing that none of the *m/z* values of the product ions were shifted except for those of the $[M–H–H_2O]^-$ ions. Note that the DHN-glutathione conjugate did not lead to the formation of the *m/z* 306 product ion (cleavage of the thioether bond on the DHN side of the conjugated metabolite, see Fig. 2) but to the *m/z* 272 ion corresponding to cleavage of the glutathione thioether bond. In the case of DHN-glutathione, cleavage of the glutamyl residue of glutathione leading to the formation of the *m/z* 335 product ion (shifted to *m/z* 337 for the ^{13}C-labelled metabolite) was also observed whereas this fragmentation process did not occur for the two other glutathione conjugates (Table 1).

Those glutathione conjugated metabolites are known as major biotransformation products of HNE *in vivo* and *in vitro*[24–30] and have already been identified by comparison with authentic synthesised standards. They result from Michael-type reaction of the glutathione and cysteine thiol group on the electrophilic carbon C3 of HNE. The aldehyde function of the resulting HNE-glutathione can be reduced to the corresponding alcohol (DHN-glutathione) or oxidised to the carboxylic acid form. The carboxylic acid moiety easily undergoes cyclisation to the lactone (HNA-lactone-glutathione).

An additional small peak, partially coeluting with HNA-lactone-glutathione, was detected thanks to the use of the stable isotope labelling. This peak eluted at 33.5 min (Fig. 3(f)) and yielded $[M–H]^-$ ions at *m/z* 171/173. The MS/MS spectra acquired from both the ^{12}C- and the ^{13}C-labelled metabolites (Table 1) displayed product ions corresponding to $[M–H–H_2O]^-$ (*m/z* 153 shifted to *m/z* 155) and $[M–H–CO_2]^-$ (*m/z* 127 shifted to *m/z* 128), with the latter indicating that one labelled carbon atom was involved in the CO_2 elimination. A weaker product ion is also present at *m/z* 71 (shifted to *m/z* 72), resulting from cleavage of the C5–C6 bond of the hydrocarbon chain, giving rise to a $C_4H_7O^-$ ($^{12}C_3{}^{13}CH_7O$) ion (Fig. 2). This characteristic fragmentation allowed us to identify this metabolite as 4-hydroxy-2-nonenoic acid (HNA).

Additional metabolites eluting at 32.2 and 39.8 min showed $[M–H]^-$ ions which were characteristic of the mercapturic acid (MA) conjugates of HNE (*m/z* 318/320) and HNA-lactone (*m/z* 316/318),[31,32] respectively, although their retention times had been expected to be shorter on the basis of previous work.[23] The corresponding MS^2 analyses confirmed these identifications, with the occurrence of the diagnostic *m/z* 162 product ion, corresponding to $[MA–H]^-$, as presented in Fig. 2. In spite of reports demonstrating the occurrence of HNE-CysGly as a HNE-glutathione metabolite detected by LC/ESI-MS (*m/z* 335 in the MS and a MS^2 product ion at *m/z* 177 in positive ion mode),[33] this compound was not detected in our experiments.

In addition to these metabolites formed by classical xenobiotic metabolism processes, minor unidentified peaks were detected later in the gradient elution, at retention times

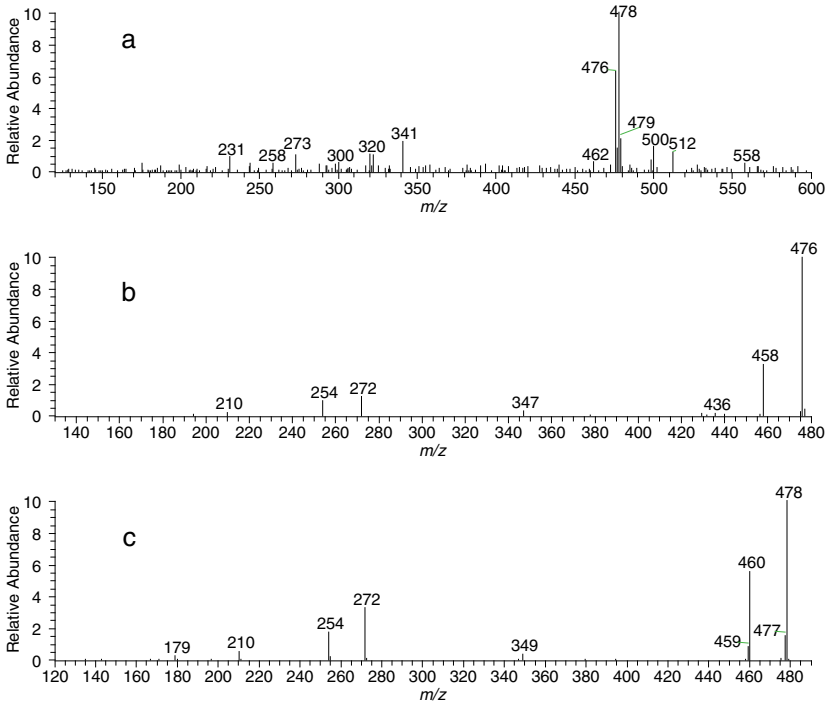

Figure 5. Quadrupole ion trap mass spectra obtained from the unknown metabolite: (a) negative ion ESI mass spectrum, (b) MS/MS spectrum of the *m/z* 476 precursor ion (HNE metabolite), and (c) MS/MS spectrum of the *m/z* 478 precursor ion ([$^{13}C_2$]-HNE metabolite).

36 and 39 min (Fig. 3(g)). These peaks exhibited $[M–H]^-$ ions at *m/z* 476/478 displaying the characteristic isotopic ratio of the incubated HNE/$^{13}C_2$HNE mixture (Fig. 5(a)). The corresponding MS/MS spectra of the two peaks were identical and showed a major product ion at *m/z* 458 (shifted to *m/z* 460 in the MS/MS spectrum of the *m/z* 478 precursor ion), together with product ions at *m/z* 272 and 254 (Figs. 5(b) and 5(c)). The occurrence of these two latter ions was indicative of glutathione conjugates as discussed before. The occurrence of the minor $[M–H–129]^-$ product ion corresponding to the loss of a glutamyl residue (*m/z* 347/349) reinforced this conclusion. Thus, for these metabolites, we first hypothesised isomers of a glutathione conjugate of 4-oxo-2(*E*)-nonenoic acid (ONA-SG), although a shorter retention time would be expected from such a compound. We successfully synthesised ONA-SG but the resulting MS^2 spectrum was different from that of the metabolite (data not shown). The oxidation of the glutathione sulphur atom of HNA-lactone-glutathione into a sulfoxide group may also lead to $[M–H]^-$ ions at *m/z* 476/478[34] but the occurrence of the *m/z* 458/460 (loss of water) product ions in the MS^2 spectra rather suggests the presence of an hydroxyl group. Thus, this metabolite could correspond to a hydroxylated form of HNA-lactone-glutathione. Hydroxylation at C9 has been reported in the metabolism of HNE by rat as urinary metabolites. However, these structures are expected to be more polar.[35] The lactone ring may also constitute a hydroxylation site. However, there was not sufficient information available in the MS/MS spectra to allow the precise location of the hydroxylation position of this metabolite.

CONCLUSIONS

Understanding how HNE, a toxic lipid peroxidation product, is biotransformed by colonocytes is of crucial importance for mechanistic insight on early stages of colon carcinogenesis.

In this work, our aim was to test a chemical tool, namely [1,2-$^{13}C_2$]-4-hydroxy-2(*E*)-nonenal, in order to track metabolites in mouse colonocytes culture media by revealing characteristic $^{13}C/^{12}C$ *m/z* patterns in LC/ESI-MS analyses. ^{13}C was chosen as stable isotope tracer because the labels could not be lost during metabolism. LC/MS analyses and MS^2 experiments proved to be relevant for tracking seven metabolites and an additional incompletely identified compound. Those metabolites, resulting from Michael-type additions and oxidation/reduction reactions, demonstrate the involvement of detoxification pathways in colonocytes.

To our knowledge, this is the first report on the use of ^{13}C-labelled HNE as a tracer for LC/MS analyses in the field of metabolism studies, and this provides an interesting alternative to the use of radioactive compounds. ^{13}C-HNE should be especially well suited for the LC/MS analyses of *in vivo* experimentation samples, by providing identification of metabolites from complex mixtures.

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