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Malondialdehyde and 4-Hydroxy-2-Nonenal in Plant Tissue Cultures: LC-MS Determination of 2,4-Dinitrophenylhydrazone Derivatives

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The cytologically active secondary lipid peroxidation products, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) have been detected as their 2,4-dinitrophenylhydrazone (DNP) derivatives in plant tissue cultures using LC-MS. This paper reports, for the first time, the use of LC-MS methodology to definitively identify 4-hydroxy-2-nonenal in plants. Limits of detection for the two derivatives are approximately 5pmol (1.2×10^{-9}g; 1μM) and 0.1pmol (3×10^{-11}g; 20nM) respectively. Mass spectrometer response was linear in the range from 2–200μM DNP-MDA and 0.02–10μM DNP-HNE.

This methodology has been used to assess the formation of aldehydic secondary lipid peroxidation products in dedifferentiated callus cultures of *Daucus carota*. The finding that profiles of MDA and HNE can be correlated with embryogenic competence is of considerable interest as oxidative status has already been implicated as a regulatory factor in animal development.

Keywords: Lipid peroxidation, oxidative stress, malondialdehyde, 4-hydroxy-2-nonenal, tissue culture, free radicals

INTRODUCTION

Free radical production is a normal component of aerobic metabolism. However, due to their high reactivity, it is essential that radical formation and annihilation is strictly controlled. In the absence of metabolic control, free radicals can attack key biomolecules such as DNA, RNA, enzymes, proteins and lipids.[1] For this reason, all aerobic organisms share a common need for antioxidant protection. Metabolic control can often be lost when aerobic organisms are challenged by biotic and abiotic stresses and as a result damaging free radical-mediated oxidative stress occurs. This is an ever-present hazard of aerobic plant life and indeed, almost all types of stressful challenge have been associated with free radical damage.[2] Increasingly, evidence sug-

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Paper dedicated to the memory of Professor Hermann Esterbauer.

gests that plant tissue cultures are predisposed to oxidative stress; however, the concept that free radicals are causal agents of genetic instability and loss of regenerative function in *in vitro* grown plant cells has rarely received attention.[3] Furthermore, as free radical-mediated reactions have been implicated in animal signalling and development,[4] it is also important to consider that free radicals and their reaction products may have an active role in normal plant development as well as their recognised role as mediators of damage during stress.

Oxidative stress can arise due to the action of free radicals directly, and also as a result of their interaction with essential macromolecules. Importantly, free radical reaction products resulting from lipid peroxidation have been identified as potential carcinogens, mutagens and DNA-damaging agents in microbial and animal cells.[5,6] If plant cells generate these secondary oxidation products *in vitro*, it is highly possible that some may be the causal agents which promote tissue culture instability and loss of morphogenetic potential.

One of the major limitations to the study of free radical mediated lipid peroxidation processes in plants is the lack of discerning analytical methodology though several methods have been developed for the determination of aldehydic products of lipid peroxidation in animal systems.[7] Thus, HPLC of non-derivatised aldehydes, GC-MS of the pentafluorobenzyl oximes, HPLC of decahydroacridine derivatives (from reaction with 1,3-cyclohexanedione) and HPLC of 2,4-dinitrophenylhydrazone derivatives have all been utilised. The most often employed technique for the characterisation of aldehydes involves the derivatisation with 2,4-dinitrophenylhydrazine, followed by analysis by either HPLC or TLC.[7] The present method has utilised DNP derivatives and HPLC with on-line NICI to confirm peak identity of the MDA and HNE derivatives. Limits of detection for the method are comparable to those reported for GC-NICI-MS of PFB-oxime-TMS derivatives.[8]

In all cases to date, investigations of this type have been confined to animal studies. In the present work we report for the first time the use of HPLC with negative ion chemical ionisation mass spectrometry to detect and quantitatively measure 2,4-dinitrophenylhydrazone derivatives of aldehydic lipid peroxidation products in methanolic extracts of plant tissues.

The objective of the present study was to develop analytical methods for the extraction, separation and definitive identification of MDA and HNE in plant tissues. This is a prerequisite for our future research which aims to evaluate, in greater detail, the role of free radical-mediated reactions in plant stress and *in vitro* morphogenetic development.

MATERIALS AND METHODS

Materials

HPLC grade acetonitrile was purchased from Rathburn Chemicals (Walkerburn, UK). 2,4-Dinitrophenylhydrazine (2,4-DNP, 30% (w/v) water) and 1,1,3,3-tetramethoxypropane was obtained from Aldrich Chemical Company (Poole, UK).

Tissue Culture

Clonal callus cultures of *Daucus carota* (carrot) were initiated from aseptically produced seedling stem sections plated onto semi-solid Murashige and Skoog medium (MS medium, pH5.7) containing 1mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D), 7.5g/l agar and 30g/l sucrose.[9] Callus colonies were subcultured at 10–14 day intervals and kept in the dark at 25–28°C. Somatic embryogenesis was initiated by transfer onto MS medium, as above but with the exclusion of the hormone 2,4-D.[10] These cultures were maintained at 25°C under fluorescent lighting applied during a 16 hour continuous photoperiod.

This study was performed on four different clonal lines of carrot (designated as lines I, II, III, IV). Morphogenetic potential in these lines was assessed as somatic embryogenesis as defined by the developmental stages conventionally described as: globular, heart, torpedo, advanced torpedo and plantlet.[10] Carrot lines exhibiting none of these stages during the assessment period (5–6 weeks) were termed non-embryogenic (lines I and II). Lines showing any of these developmental stages were categorised as embryogenic (lines III and IV). The embryogenic lines referred to in the present study all showed progression through to the plantlet stage. Line III was particularly morphogenetic as it displayed a particularly rapid and prolific embryogenic response when placed on hormone-free medium. This line was thus termed "highly" embryogenic.

Derivatisation of Aldehydes

2,4-Dinitrophenylhydrazine (30% (w/v) water) was dried and purified by butanol recrystallisation. Aldehyde-DNPH standards were prepared as described by Esterbauer and Cheeseman[11] using malondialdehyde (MDA), obtained by hydrolysis of 1,1,3,3-tetramethoxypropane, and 4-hydroxy-2-nonenal (HNE) from the corresponding diethylacetal. Thus, 1,1,3,3-tetramethoxypropane (0.5g) was dissolved in ethanol (5ml) and 2M sulphuric acid (1ml) was added. The solution was stirred at room temperature for 15 minutes and then treated with a solution of recrystalised 2,4-DNP (5 ml 0.035% w/v in 1M HCl). After allowing to stand for 5 minutes the crystals were removed by filtration, washed with water and dried. The 2,4-DNP derivative of 4-hydroxy-2-nonenal (4-HNE) was prepared in a similar manner by hydrolysis of the diethylacetal. Derivatives of the standard aldehydes prepared according to the standard method[12] were dissolved in acetonitrile prior to LC-MS analysis.

Extraction and Preparation of Plant Tissues

To ensure parity between sampling, callus cultures were analysed at the mid-stage of a routine callus sub-culture cycle (10 days). Accuracy of fresh weight determinations was assured by first blotting carrot callus tissues on Whatman filter papers for 2 minutes to remove excess surface moisture carried over from the culture plates. Samples of callus (250 mg) were subsequently placed into Eppendorf tubes (2.5ml) and transferred directly to liquid nitrogen where they were stored until required for analysis. *D. carota* callus is extremely friable and tissues were readily disrupted following exposure to liquid nitrogen. For preparation of DNP derivatives the callus was removed from liquid nitrogen and thawed at room temperature for 5 minutes. Aldehydes were extracted into methanol (1 ml) assisted by vortex mixing for 1 minute. To aid complete extraction of the tissues the mixture was vortexed at room temperature using several repeated bursts (*ca* 1 minute) over a period of 10 minutes. Cellular debris was removed by microfugation (13,000 rpm for 10 minutes). A sample of the supernatant (850µl) was removed and added to an equal volume of 0.035% w/v 2,4-dinitrophenylhydrazine reagent in HCl and the derivatives were subsequently treated as described previously.[11] After evaporation to dryness the samples were redissolved in 250µl acetonitrile for subsequent LC-MS analyses. Solvent blanks were prepared using neat methanol (850µl) in place of the methanolic tissue extract.

LC-MS Analyses

All analyses (using 5µl samples) were carried out in duplicate on a Finnigan MAT SSQ710C (Hemel Hempstead, UK) single quadrupole instrument with an APCI interface. A Hypersil C_{18} column (250 × 2.0 mm) from Phenomenex (Macclesfield, UK) with a Constrametric 4100MS HPLC pump, AS3000 autosampler and UV 1000

detector (all Thermo Separation Products, Stone, UK) were used throughout. Effluent from the UV detector was passed via a 1 metre length of PEEK tubing (60μm internal diameter) to the APCI interface. The solvent flow was 0.25ml/min and UV detection was at 360nm. Separation was achieved with a gradient elution using, initially, 40:60 water:acetonitrile rising to 25:75 in 5 minutes and held for a further 30 minutes at this ratio. The mass spectrometer was configured for negative ion chemical ionisation (NICI) with coronal discharge at ~4.5kV. Nitrogen, at 80psi was used as the sheath gas. MS parameters comprised a full scan mode: *m/z* 230–340 in 1.5 seconds; selected ion monitoring *m/z* 230, 234, 317, 335 (±0.15 amu) with a dwell time of 0.25 second/window.

RESULTS

Figure 1 shows the UV (A_{360}) and MS elution profile (obtained in full scan mode) of a standard mixture of 2,4-dinitrophenylhydrazone derivatives of a mixture of *n*-aldehydes, spiked with the MDA and HNE derivatives. The peak eluted at approximately 5.8 mins (at *m/z* = 234) is that of the MDA derivative, whilst the peak at approximately 27 mins, is due to a fragment ion of the hexanal derivative. DNP-HNE elutes at approximately 19 mins (*m/z* = 335) and shows limited fragmentation through loss of water (*m/z* = 317, spectrum not shown). In the present study it should be noted that fragmentation of all of the 2,4-dinitrophenylhydrazones is limited and that the base peak is that of the pseudomolecular ion (M-1) on all occasions.

Serial dilutions of a mixture of MDA and HNE derivatives were used to assess the mass spectrometer response (as peak area, in selected ion monitoring mode) at different concentrations (Fig. 2a and 2b). Instrumental response was linear from 2–200μM of the MDA derivative (Figure 2a) and from 0.02–10μM for the HNE derivative (Fig. 2b). The absolute limits of detection for these derivatives, from a 5μl injection, were found to be 5pmol (1.2×10^{-9}g) and 0.1 pmol (3×10^{-11}g) respectively (results not shown).

A typical elution profile from a derivatised extract of an embryogenic culture of *D. carota* (in this case, line IV) is shown in Figure 3. The UV trace reveals multiple components and clearly demonstrates the drawbacks of relying on retention time for the identification and quantification of DNP-MDA and DNP-HNE derivatives using UV detection alone. The ion traces obtained at *m/z* = 234 and 335 readily allow for the rapid quantification of both aldehydes, although we recognise the need for spiking unknown samples with known quantities of standard aldehyde derivatives. This was addressed by the injection of known amounts of aldehyde standards (20μM DNP-MDA and 2μM DNP-HNE) at intervals throughout batches of analyses. Replicated solvent blanks were always incorporated for each batch of tissue analyses thus taking into account possible solvent contamination. This approach led to a day-to-day variability of <10%. All blanks had low levels of aldehydes compared to the tissue samples and contamination from the solvent systems used in the extraction and analysis procedures was thus considered to be minimal.

The above analytical methodology was subsequently applied to a series of clonally propagated callus cultures of *D. carota* which exhibited differing embryogenic potentials. Figure 4 shows the MDA levels in duplicate tissue extracts of the four clonal lines and accompanying solvent blanks. The embryogenic lines, III and IV had lower levels of MDA than those determined in the non-embryogenic lines I and II. The highly embryogenic line III was found to contain 2–3μM MDA compared to the non-embryogenic lines, I and II which contained approximately 30 and 10μM MDA, respectively. The second embryogenic line, IV, had levels higher than those of the highly embryogenic III (approximately 8μM). Levels detected in the blanks were typically 2μM.

HNE profiles (Fig. 5) showed opposite trends to those of MDA. Thus, non-embryogenic lines I

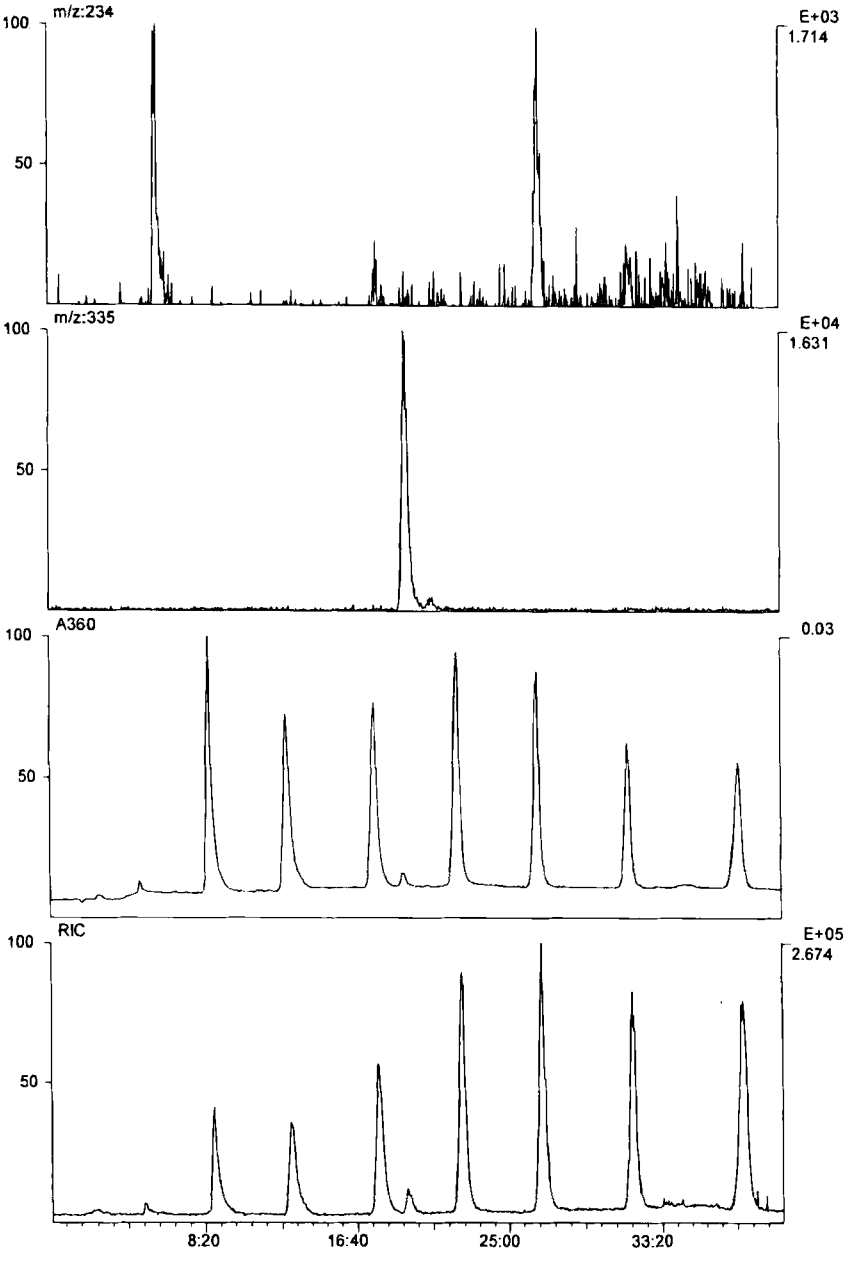

FIGURE 1 LC-UV-MS chromatograms of DNP-MDA & DNP-HNE spiked 2,4-dinitriphenylhydrazone (0.001% w/v) standards. Chromatograms are (from top to bottom): ion current (m/z = 234), ion current (m/z = 335), UV absorption (360nm) and total (reconstructed) ion current. The peaks corresponding to the DNP-MDA and DNP-HNE derivatives elute at approximately 5.8 mins (m/z = 234) and 19 mins (m/z = 335) respectively.

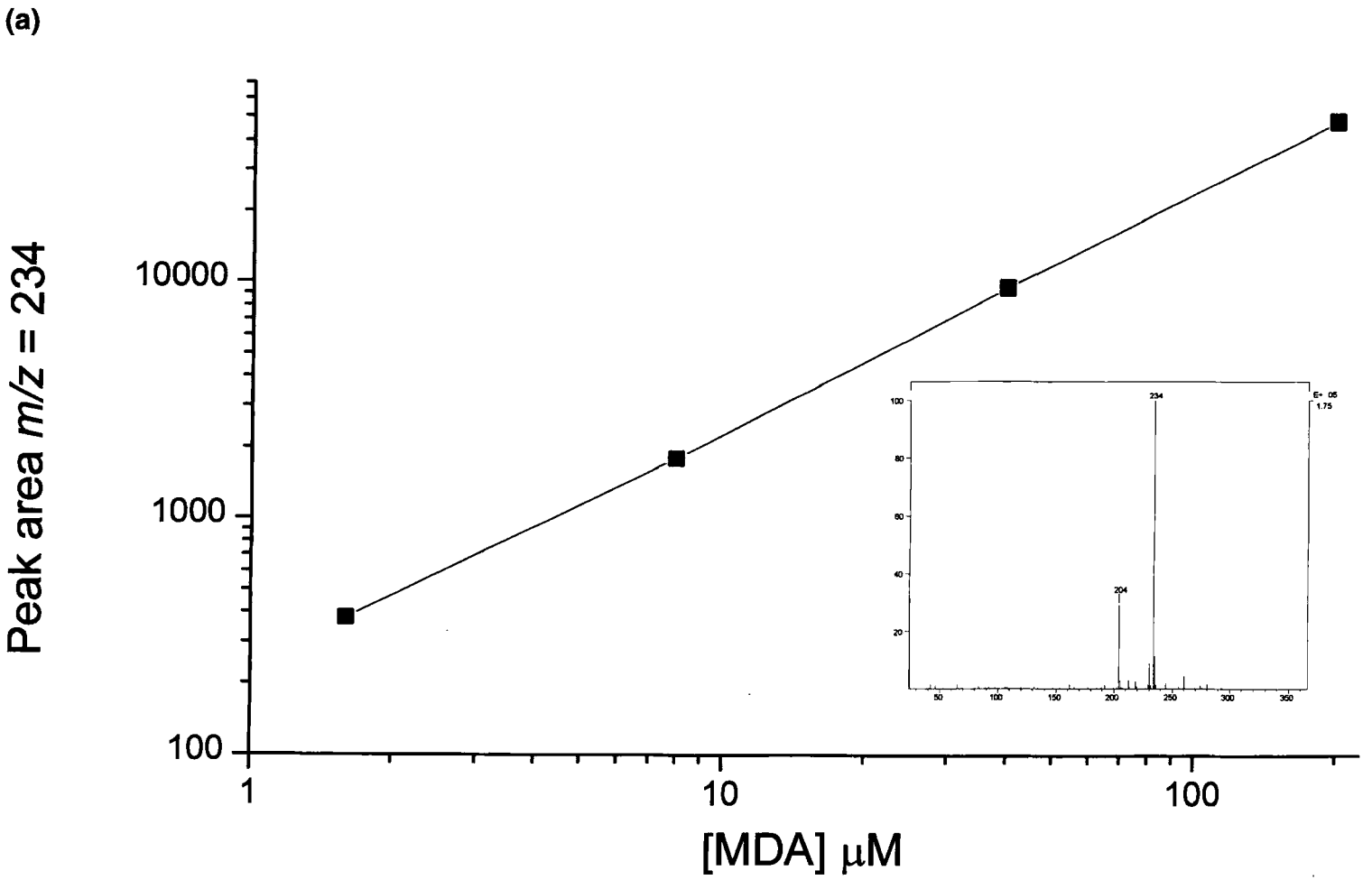

FIGURE 2 Standard curves of (**a**) DNP-MDA and (**b**) DNP-HNE obtained with selected ion monitoring (*m/z* = 234, MDA; *m/z* = 335, HNE). Insets: mass spectra.

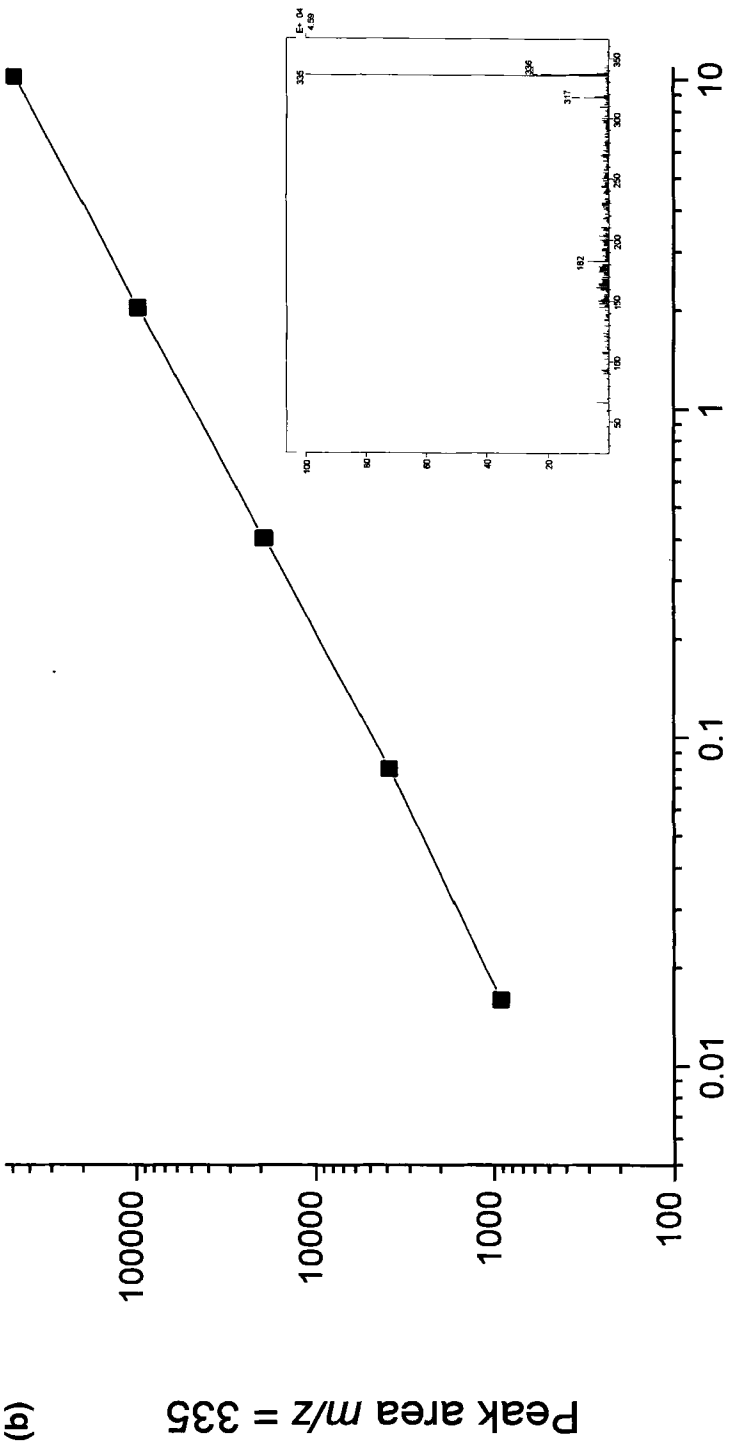

FIGURE 2 *(Continued)*

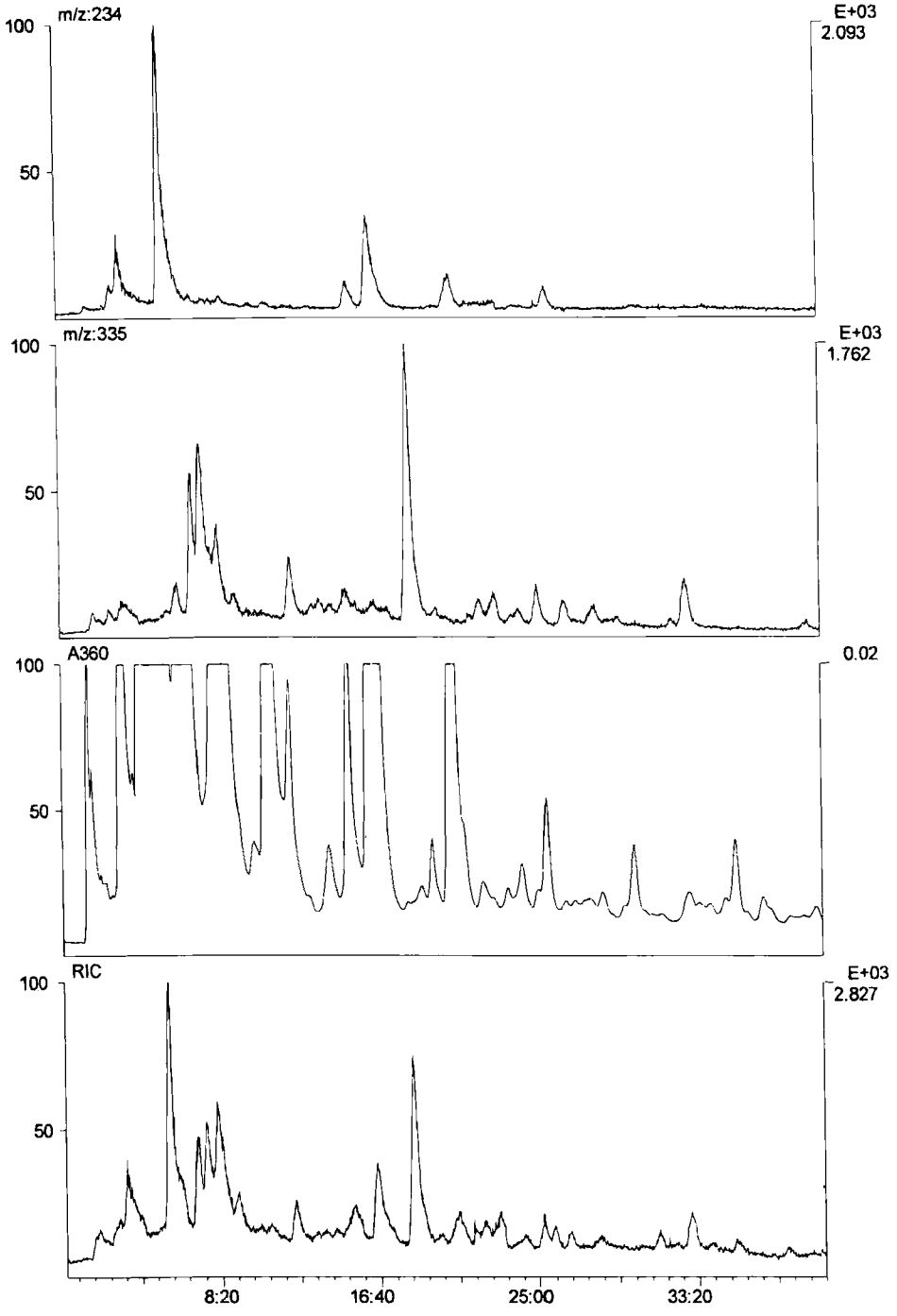

FIGURE 3 Typical HPLC-UV-MS traces from a derivatised extract of the embryogenic *D. carota* line IV. Chromatograms are (from top to bottom): ion curent ($m/z = 234$), ion current ($m/z = 335$), UV absorption (360nm) and total (reconstructed) ion current. The peaks corresponding to the DNP-MDA and DNP-HNE derivatives elute at approximately 5.8 mins ($m/z = 234$) and 19 mins ($m/z = 335$) respectively.

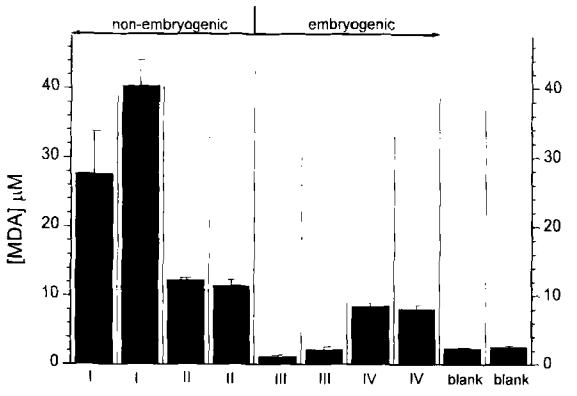

FIGURE 4 MDA levels in derivatised extracts of embryogenic and non-embryogenic callus cultures of *D. carota*. Error bars depict standard error of the mean of duplicate LC-MS analyses. Data values represent the amount of DNP-aldehyde in a 250µl concentrated extract derived from 250 mg of callus tissue (fresh weight basis).

and II had lower levels than those from the embryogenic lines III and IV. The latter had the highest levels of HNE (around 200nM), in contrast to the non-embryogenic lines which contained 30–130nM. The inverse relationship between MDA and HNE contents, relative to embryogenic potential, is more clearly evident when the HNE:MDA ratio is considered (Fig. 6). Both non-embryogenic lines have an HNE:MDA

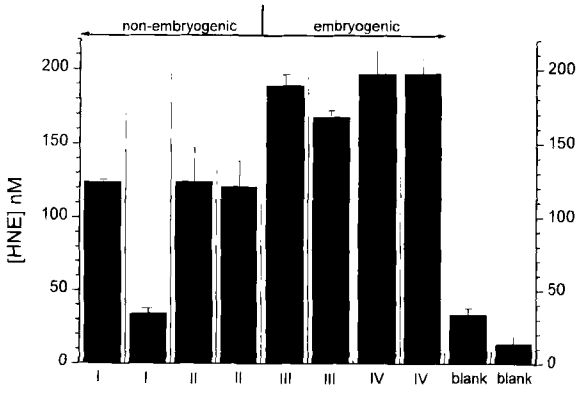

FIGURE 5 HNE levels in derivatised extracts of embryogenic and non-embryogenic callus culture of *D. carota*. Error bars depict standard error of the mean of duplicate LC-MS analyses. Data values represent the amount of DNP-aldehyde in a 250 µl concentrated extract derived from 250 mg of callus tissue (fresh weight basis).

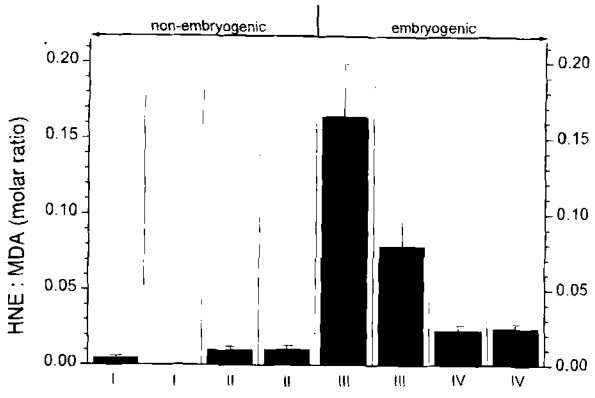

FIGURE 6 Molar ratios of HNE: MDA in embryogenic and non-embryogenic callus cultures of *D. carota*.

molar ratio of less than 0.015; IV has a molar ratio of 0.025 and the highly embryogenic line III, has a ratio in excess of 0.08.

DISCUSSION

For the first time we report the definitive identification and detection of 4-hydroxy-2-nonenal (HNE) in plants by using LC-MS to determine, quantitatively, DNP-MDA and DNP-HNE derivatives. The methodology presented in this paper has been developed to assist in the study of free radical mediated mechanisms in *in vitro* plant development. It is thus essential that the aldehydic breakdown products of lipid peroxidation can be definitively identified as the mechanisms associated with lipid peroxidation are complex and the composition of the resulting breakdown products is largely dependent upon the fatty acid content of the lipids.[13] Our earlier investigations[14] have explored the use of other analytical approaches to assess the production of aldehydic lipid peroxidation products in *D. carota* callus. Thus, thiobarbituric acid reactive substances (TBARS) have been detected[14] in 7 different clonal lines of carrot callus using a fluorimetric method to detect TBARS. Similarly, 4-hydroxy-alkenals were also detected in these cultures using a commercial kit (LPO-586 Bioxytech S.A. Bonneuil/Marne, France).[14] However, the

LC-MS approach presented here has considerable advantages over these other methods which cannot be used to characterise specific aldehydes. The TBARS assay can also be at risk from assay interference and recently, Cherif and colleagues[15] caution the use of non-specific methods to determine MDA in plant cultures. Indeed, the use of thiobarbituric acid assays to evaluate lipid peroxidation has been a subject of debate for several years[16]. Because of its convenience, the TBARS assay has proved useful when applied to plant cultures, however, it is advisable to support the TBARS assay with additional methods which can confirm changes in lipid peroxidation profiles.[14–17]

The sensitivity, selectivity and reproducibility of the LC-MS method now enables the study of individual aldehydes produced via lipid peroxidation in plant systems. For example, levels of detection of HNE can be at or <1 pmol. It is highly significant that HNE was detected in cultures of *D. carota* as numerous studies have quantified HNE in animal tissues and correlated its production with various pathologies.[18] Preliminary investigations arising from this study have demonstrated that both HNE and MDA are present in plant cultures. Interestingly, levels of MDA could be correlated with loss of embryogenic potential in the four clonal lines investigated. The same phenomenon has previously been reported using the TBARS method applied to cell suspension cultures of rice (*Oryza sativa* L.) exhibiting different embryogenic potentials.[17] Conversely, HNE levels appeared to be correlated with the embryogenic potentials of the four clonal lines of *D. carota*. The embryogenic lines, III and IV had the highest levels of HNE, at approximately 200nM. It is interesting to note that this level of HNE is significantly lower than the 2–300μM range that has been reported as having an effect on growth capability of mammalian cell lines.[18] The long term cytotoxic effects of HNE (determined as LD_{50}) are typically in the 5–1000μM range, again well above the range observed in the present study.

We have demonstrated that two potentially cytotoxic products of lipid peroxidation, namely MDA and HNE, are formed in plant tissue cultures. Relatively high levels of MDA are associated with a decline in morphogenetic competence, whereas, HNE is correlated with an increase in morphogenetic capability. These preliminary findings thus implicate lipid peroxidation pathways in the *in vitro* development of plant tissue cultures and indeed, they may be putative "markers" of morphogenesis. Our future studies will be directed towards consolidating these preliminary findings on a wider range of genotypes and morphogenetic systems. The development of the stringent LC-MS method of aldehyde analysis will greatly assist this aim.

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