Free Radical Research

ISSN: 1071-5762 (Print) 1029-2470 (Online) Journal homepage: http://www.tandfonline.com/loi/ifra20

Isotopic labelling for the characterisation of HNE-sequestering agents in plant-based extracts and its application for the identification of anthocyanidins in black rice with giant embryo

Mara Colzani, Luca Regazzoni, Angela Criscuolo, Giovanna Baron, Marina Carini, Giulio Vistoli, Yoon-Mi Lee, Sang-Ik Han, Giancarlo Aldini & Kyung-Jin Yeum

To cite this article: Mara Colzani, Luca Regazzoni, Angela Criscuolo, Giovanna Baron, Marina Carini, Giulio Vistoli, Yoon-Mi Lee, Sang-Ik Han, Giancarlo Aldini & Kyung-Jin Yeum (2018): Isotopic labelling for the characterisation of HNE-sequestering agents in plant-based extracts and its application for the identification of anthocyanidins in black rice with giant embryo, Free Radical Research, DOI: 10.1080/10715762.2018.1490735

To link to this article: https://doi.org/10.1080/10715762.2018.1490735

Published online: 23 Jul 2018.

FREE RADICAL RESEARCH
https://doi.org/10.1080/10715762.2018.1490735

Taylor & Francis
Taylor & Francis Group

ARTICLE

Isotopic labelling for the characterisation of HNE-sequestering agents in plant-based extracts and its application for the identification of anthocyanidins in black rice with giant embryo

Mara Colzani[a], Luca Regazzoni[a], Angela Criscuolo[a], Giovanna Baron[a], Marina Carini[a], Giulio Vistoli[a], Yoon-Mi Lee[b], Sang-Ik Han[c], Giancarlo Aldini[a]* and Kyung-Jin Yeum[b]*

[a]Department of Pharmaceutical Sciences, University of Milan, Milan, Italy; [b]Division of Food Bioscience, College of Biomedical and Health Sciences, Konkuk University, Chungju-si, South Korea; [c]National Institute of Crop Science, Rural Development Administration, Suwon-si, South Korea

ABSTRACT

Reactive carbonyl species (RCS) are cytotoxic molecules that originate from lipid peroxidation and sugar oxidation. Natural derivatives can be an attractive source of potential RCS scavenger. However, the lack of analytical methods to screen and identify bioactive compounds contained in complex matrices has hindered their identification. The sequestering actions of various rice extracts on RCS have been determined using ubiquitin and 4-hydroxy-2-nonenal (HNE) as a protein and RCS model, respectively. Black rice with giant embryo extract was found to be the most effective among various rice varieties. The identification of bioactive compounds was then carried out by an isotopic signature profile method using the characteristic isotopic ion cluster generated by the mixture of HNE: $^{2}H_{5}$-HNE mixed at a 1:1 stoichiometric ratio. An in-house database was used to obtain the structures of the possible bioactive components. The identified compounds were further confirmed as HNE sequestering agents through HPLC-UV analysis.

ARTICLE HISTORY
Received 7 April 2018
Revised 13 June 2018
Accepted 14 June 2018

KEYWORDS
Black rice with giant embryo; HNE sequestering agents; mass spectrometry

Introduction

Reactive carbonyl species (RCS) are a class of breakdown products arising from the oxidation of sugars and lipids which can be formed either exogenously, for instance during food processing or cooking, and endogenously, via different pathophysiological conditions [1]. RCS are chemically quite heterogeneous, belonging to different classes, including di-aldehydes, keto-aldehydes, and α,β-unsaturated aldehydes [2]. RCS have the common property of covalently reacting with nucleophilic substrates such as proteins. Covalent adducts originating from lipids are named advanced lipoxidation end-products (ALEs), whereas RCS originating from sugars are known as advanced glycoxidation end-products (AGEs) [1]. Since the 1980s, RCS and their corresponding reaction products have been widely used as markers of oxidative stress and several analytical methods for their measurements have been developed and widely applied [3]. More recently, due to a growing body of evidence reporting the involvement of AGEs and ALEs in the onset and propagation of some human diseases [4,5], RCS have been considered not only as biomarkers but also as potential drug targets. Among the damaging RCS, 4-hydroxy-2-nonenal (HNE) is one of the most studied since its discovery by Hermann Esterbauer in 1964, due to its abundance, reactivity, and biological effects [6,7]. The strict link between elevated HNE tissue/blood levels and some human diseases suggests that HNE contributes towards pathophysiology of these diseases [8]. Several studies based on cell signalling and protein covalent modification have since reported the molecular mechanisms involved in the cytotoxic effect of HNE [9,10].

Different molecular approaches for reducing the overproduction of HNE and in general of RCS have been reported and summarised in some recent reviews [4,11,12]. Among the proposed approaches, the most promising is based on small nucleophilic molecules (RCS sequestering agents) which covalently react with RCS, forming unreactive adducts which are then metabolised and excreted. Several RCS sequestering agents have been proposed such as aminoguanidine, pyridoxamine, hydralazine, and carnosine [13,14]. Their

CONTACT Giancarlo Aldini giancarlo.aldini@unimi.it Department of Pharmaceutical Sciences, University of Milan, Milan, Italy; Kyung-Jin Yeum kyeum@kku.ac.kr Division of Food Bioscience, College of Biomedical and Health Sciences, Konkuk University, Chungju-si, South Korea
*These authors contributed equally to this work.

protective effects have been confirmed in animal models by several independent labs, further supporting the promising therapeutic efficacy of targeting HNE [8,13,15]. Carnosine has recently been reported as the most efficient and selective sequestering agent of HNE and its promising activity has been demonstrated in animal models and most recently in obese subjects [16–18]. However, the application of carnosine as an HNE sequestering agent in humans is limited because of its poor bioavailability, due to the presence of serum carnosinases, which catalyse the hydrolytic cleavage of the dipeptide [19]. Nowadays there is great interest in the discovery of novel HNE sequestering agents such as carnosine derivatives resistant to carnosinases [20,21].

Among others, an interesting discovery approach to identify novel HNE sequestering agents is based on searching for bioactive compounds in plant extracts [22]. Plants are also subject to RCS stress, in particular those mediated by α,β-unsaturated aldehydes with HNE being the most abundant and toxic carbonyl compound formed in stress conditions [23]. Because plants are subject to RCS and protein carbonylation damage [24], they have developed efficient enzymatic and non-enzymatic detoxification pathways. Hence, besides detoxifying enzymes, it is reasonable to consider the presence of secondary metabolites acting as RCS-sequestering agents. Such a hypothesis has been partially confirmed by previous studies which have reported that EGCG and other polyphenols are effective as sequestering agents of HNE [25] of other RCS, including acrolein and methylglyoxal [26]. These results prompted scientists to screen the sequestering activity of different plant metabolites and, in particular, the polyphenol class. As an example, Zhu et al. [27] compared 21 natural polyphenols with diverse structural characteristics for their ACR-/HNE-trapping capacities under simulated physiological conditions.

To the best of our knowledge, the search for a potential RCS natural sequestering agent has been carried out by using isolated molecules or highly purified extracts and not by applying off-target methods involving crude extracts. One reason is the lack of analytical methods able to test the sequestering activity of complex matrices and the inherent difficulties associated with fishing out compounds that are bioactive. As an example, one of the most used methods to evaluate HNE sequestering efficacy is based on a HPLC-UV method for measuring the residual amount of HNE. Such an approach is clearly suitable for testing single molecules and not mixtures, which would interfere with HNE analysis.

We have recently reported an *in vitro* high-resolution MS method to test the ability of compounds, mixtures, and extracts to trap RCS by measuring their efficacy in inhibiting ubiquitin carbonylation induced by RCSs including HNE [28]. Such a method was found suitable to evaluate the overall quenching activity of extracts, however it does not permit the identification of the active components.

In this article, we report an LC-ESI-MS method based on an isotopic labelling procedure which permits the identification of the HNE sequestering agents contained in a crude mixture. Together with the ubiquitin method, it was then applied to search for HNE sequestering agents contained in extracts prepared from different varieties of rice.

Materials and methods

Chemicals

Formic acid (HCOOH), sodium dihydrogen phosphate ($NaH_2PO_4{\cdot}H_2O$), sodium hydrogen phosphate ($Na_2HPO_4{\cdot}2H_2O$), phenylalanine, gamma-aminobutyric acid, histidine, tricin-5-O-glucoside, lyophilised ubiquitin from bovine erythrocytes (BioUltra, ≥98%), and LC–MS grade solvents were purchased from Sigma–Aldrich (Milan, Italy). Peonidin-3-O-glucoside, malvidin, peonidin, and pelargonidin were from Extrasynthese (Genay, France). Carnosine (β-alanyl-L-histidine) was a generous gift from Flamma SpA (Chignolo d'Isola, Bergamo, Italy). LC-grade H_2O (18 MΩ cm) was prepared with a Milli-Q H_2O purification system (Millipore, Bedford, MA). All other reagents were of analytical grade. 4-Hydroxy-2-nonenal diethylacetal (HNE-DEA) and $^2H_5-$4-hydroxy-2-nonenal diethylacetal ($^2H_5$-HNE-DEA) were synthesised according to the literature [29] and stored at −20 °C. For each experiment, fresh HNE was prepared starting from stored HNE-DEA, which was evaporated under nitrogen stream and hydrolysed with 1 mM HCl, pH 3 for 1 h at room temperature to obtain HNE. The concentration of HNE was estimated by measuring the absorbance at λ = 224 nm (molar extinction coefficient = 13,750 $M^{-1} \times cm^{-1}$) [30].

Plant material

Black rice with giant embryo and white rice were provided by the National Institute of Crop Science, Rural Development Administration, Republic of Korea. A detailed method for the development and culture of black rice with giant embryo has been previously reported [31].

Rice extract preparation: the seeds were manually dehulled with a wooden rice dehuller and ground to a powder by using a mortar and pestle. The milled rice powders were stored at −80 °C until further use. Water extract of black rice with giant embryo was provided by National Institute of Crop Science, Rural Development Administration, Republic of Korea. Two hundred grammes of black rice with giant embryo extract (BRGE) was extracted with 200 mL of water for 5 days at room temperature, and the extracts were filtered and evaporated under vacuum, followed by drying. The yield of extraction was 4.32%.

Rice extract filtration: for the ubiquitin assay as well as for the isotopic labelling procedure, rice powder extracts were then dissolved in water and ultrafiltered by using a 3 kDa ultrafilters so to remove proteins and high MW components. The yield of the low molecular fraction after 3 kDa ultrafiltration was ≈10% of the starting sample for all the rice varieties tested.

Ubiquitin carbonylation assay

The effect of rice extracts on HNE-induced protein carbonylation was tested by using the ubiquitin assay as previously reported [28]. Briefly, ubiquitin (10 μM final concentration) was chosen as the protein target of HNE-induced protein adduction. Ubiquitin was incubated for 24 h at 37 °C in 10 mM phosphate buffer in the presence of 500 μM HNE together with rice extracts of increasing concentrations (10, 25, and 50 mg/mL). To remove high molecular components, which could interfere with the assay, the rice extracts were filtered by using Amicon YM3 filters (Millipore, Milan, Italy) and the filtered fractions were then used for the assay. After an incubation time of 24 h, the reactions were stopped by centrifugation using Amicon YM3 filters and the extent of ubiquitin carbonylation was determined by MS intact protein analysis using the microflow automated loop injection procedure as already described [28].

Identifying the HNE sequestering agents by an isotopic labelling procedure

After filtration by using Amicon YM3 filters, the black rice giant embryo extract (BRGE) was dissolved in PBS at a final concentration of 50 mg/mL and was then incubated for 24 hours in presence of HNE or deuterium labelled HNE ($^2H_5$-HNE). Control samples were incubated neither with HNE nor with deuterium labelled HNE. After 24 h, the samples were analysed individually by LC-MS together with a sample prepared by mixing in a 1:1 v/v ratio the samples incubated with HNE and $^2H_5$-HNE. LC-HRMS were run in positive and negative ion mode.

Chromatographic conditions

Chromatographic separation was performed on a reversed-phase Agilent Zorbax SB-C18 column (150 × 2.1 mm, i.d. 3.5 μm, CPS analitica, Milan, Italy), protected by an Agilent Zorbax guard column, by an UltiMate 3000 system (Dionex) equipped with an autosampler kept at 4 °C working at a constant flow rate (200 μL/min). Ten microlitres of sample was injected into the column and the analytes were eluted with a 45-min multistep gradient of phase A H_2O-HCOOH (100:0.1, v/v) and phase B CH_3CN-HCOOH (100:0.1, v/v): 0–5 min, isocratic of 1% B; 5–15 min, from 1% B to 25% B; 15–30 min, from 25% B to 65% B; 30–32 min, from 65% B to 80% B; 32–36 min, isocratic of 80% B; 36–36.1 min, from 80% B to 1% B, and then 36.1–45 min of isocratic 1% B.

MS conditions

The acquisitions were performed on a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose) using an ESI source, acquiring in positive and in negative ion mode. A real-time mass calibration was obtained by using a list of 20 background ions [32]. The source parameters used for the positive mode are: spray voltage 3 kV, capillary temperature 275 °C, capillary voltage 49 V, sheath gas flow 15 units, auxiliary gas flow 5 units, tube lens offset 130 V; for the negative ion mode: spray voltage 3.5 kV, capillary temperature 275 °C, capillary voltage −17 V, sheath gas flow 15 units, auxiliary gas flow 5 units, tube lens offset −100 V. Full MS spectra were acquired in profile mode by the FT analyser in a scan range of 100–1000 *m/z*, using AGC scan target 5×10^5 and resolution 100,000 FWHM at *m/z* 400. Tandem mass spectra were acquired by the linear ion trap (LTQ) that was set up to fragment the three most intense ions exceeding 1×10^3 counts. Mass acquisition settings were: centroid mode, AGC scan target 1×10^4, precursor ion isolation width of *m/z* 2.5, and collision energy (CID) of 30 eV. Dynamic exclusion was enabled to reduce redundant spectra acquisition: two repeat counts, 20 s repeat duration, 30 s of exclusion duration. Instrument control and spectra analysis were provided by the software Xcalibur 2.0.7 and Chromeleon Xpress 6.80.

HPLC-UV HNE sequestering assay

The compounds identified as putative HNE binders with the isotopic labelling procedure were purchased as pure standards and tested individually for their HNE sequestering ability. The assay was a 3-hour time course experiment at pH 7.4 and 37 °C, in the presence of 50 μM HNE and 1 mM of the tested compound. The residual HNE was measured hourly within a 3-hour reaction time by using the HPLC-UV assay described by Vistoli et al. [33] with some minor modifications. Briefly, HNE concentration was determined by a Surveyor HPLC platform (Thermo Scientific, Milan, Italy) setting the UV detector at 224 nm. HNE elution was performed in 6 minutes by reverse phase chromatography at 37 °C and flow rate of 0.3 mL/min A Kinetex column (75 × 2.1 mm i.d., 2.6-μm particle size, 100-Å porosity, Phenomenex, Castel Maggiore, Italy) was used as stationary phase, while mobile phase consisted of H_2O-CH_3CN-HCOOH, 80:20:0.1 (v/v/v).

The amount of reacted HNE was calculated by the following formula:

$$reacted\ HNE\ (\%) = \frac{[HNE]_{t0} - [HNE]_{t3}}{[HNE]_{t0}} * 100$$

$[HNE]_{t0}$ being the concentration of HNE at the beginning of the incubation and $[HNE]_{t3}$ being the concentration of HNE at the end of the incubation. Carnosine, which is a HNE sequestering agent not containing a thiol moiety, was used as positive control to assess the assay reproducibility. To demonstrate that HNE consumption over time is not caused by auto-oxidation, an incubation in pure phosphate buffer was performed as negative control.

The activity of each compound was also given as carnosine units (i.e. the ratio between the % of HNE reacted with the compound at a given time and the % of HNE reacted with carnosine in the same span of time).

Computational studies

For simplicity, attention was focussed on two anthocyanidins chosen as representative of active (pelargonidin) and inactive (malvidin) compounds. Given the tested physiological pH, the compounds were simulated in their quinoidal neutral form by considering three major tautomers. In order to investigate their stereo-electronic properties, these structures were minimised at their ground state and at gas phase by density functional theory (DFT) using the Becke three-parameter hybrid function with LYP correlation (DFT/B3LYP) and with the 6–31 G basis set as implemented by the GAMESS software [34].

Results and discussion

Overview of the approach

RCS are well-known pathogenetic factors of various oxidative stress associated diseases [5]. Recently, molecular approaches based on detoxification of RCS have been found to be effective in preventing the onset and progression of some diseases in different animal models [11]. Among such efforts, those based on small nucleophilic compounds capable of trapping and detoxifying RCS are of particular interest. Several synthetic and natural RCS sequestering agents have so far been reported to be effective in several animal models, but for many of these compounds, clinical application is limited due to their ill-defined activity, lack of selectivity, and of suitable bioavailability. Hence, a discovery approach aimed at identifying novel RCS sequestering agents is necessary to overcome this gap. In this study, we report a discovery approach aimed at identifying RCS sequestering agents in crude mixtures such as natural extracts. Natural sources and, in particular, plant extracts, represent an attractive source of RCS sequestering agents due to the fact that plants are also subject to RCS stress, in particular mediated by α,β-unsaturated aldehydes, and that they have developed efficient enzymatic and nonenzymatic detoxification pathways [23,35]. Many nonenzymatic pathways and in particular those based on small nucleophilic agents are still unexplored and they would represent an interesting potential source of novel bioactive compounds. However, searching for RCS sequestering agents in a crude mixture is quite a challenging process because they are usually present as minor components with respect to the complex matrix, making their identification and characterisation analytically challenging. Moreover, a robust method capable of measuring the overall RCS sequestering activity is needed in order to select and identify the most effective plant extracts.

Here we report a new method to overcome these challenges that is based on the following steps: (i) the RCS sequestering activities of the tested extracts are first characterised by using the ubiquitin assay; (ii) the most active extracts are then selected in order to identify sequestering agents by using an innovative isotopic labelling procedure, and (iii) the RCS sequestering activities of the identified compounds are then validated by using standard compounds and a HPLC-UV method.

The method proposed has been applied by using HNE as a target aldehyde and eight rice extracts as

potential sources of RCS sequestering agents. HNE is an α,β-unsaturated aldehyde generated endogenously and exogenously (it is present in food) by the radical-mediated peroxidation of ω-6 polyunsaturated fatty acids [36]. Since its identification, 4-HNE, an autoxidation product of unsaturated fats and oils (at first erroneously described as 4-hydroxy-octenal), has attracted great scientific interest, as demonstrated by the publication of more than 4200 papers since 1980. Compared to other 4-hydroxyalkenals, HNE is the most extensively studied and reviewed as it was the first one discovered [37], it represents the main 4-hydroxyalkenal formed during the autoxidation of unsaturated fatty acids, it is highly reactive and numerous biological effects have been demonstrated [6,38], leading HNE being considered as a promising drug target [8].

Rice (*Oryza sativa*) is one of the five different cereal grains, which are the most commonly consumed throughout the world and are functionally reported to have antioxidant activity. Bioactive components of rice, have demonstrated antioxidant and anti-inflammatory activities in cells and animals [39,40].

However, although the direct and indirect antioxidant activity of rice has been demonstrated in different *in vitro* and also *in vivo* models, no data to our knowledge have so far been reported regarding the efficacy of rice towards protein carbonylation and, in particular, its ability to sequester RCS. We then decided to apply the proposed method to test the RCS sequestering capability of rice and so we evaluated the water extracts of white, brown, and black rice.

HNE sequestering activity of rice extracts

The overall HNE sequestering activity of eight rice extracts was studied using the ubiquitin assay. The method consists of incubating ubiquitin as protein target with HNE at a specific molar ratio and incubation time, thus inducing ubiquitin covalent modification of almost 50%. The protein carbonylation was monitored by measuring the ratio of the areas of two peaks: one at *m/z* 779.61239 relative to the 11-charged peak (named z11) of native ubiquitin and the second at *m/z* 793.80415 corresponding to the 11-charged peak of ubiquitin covalently modified by HNE. Figure 1 shows the MS spectra of ubiquitin incubated in the absence and in the presence of HNE. When ubiquitin incubated with HNE was spiked with rice extracts, a dose-dependent reduction of the peak area relative at *m/z* 793.80415 was observed. The IC_{50} value, which is the rice extract concentration able to decrease by 50% the level of protein carbonylation, was determined. Table 1 summarises the IC_{50} of the tested rice extracts. White rice extract was found less effective in respect to coloured rice and of these, black rice with giant embryo was the most effective and for this reason it was

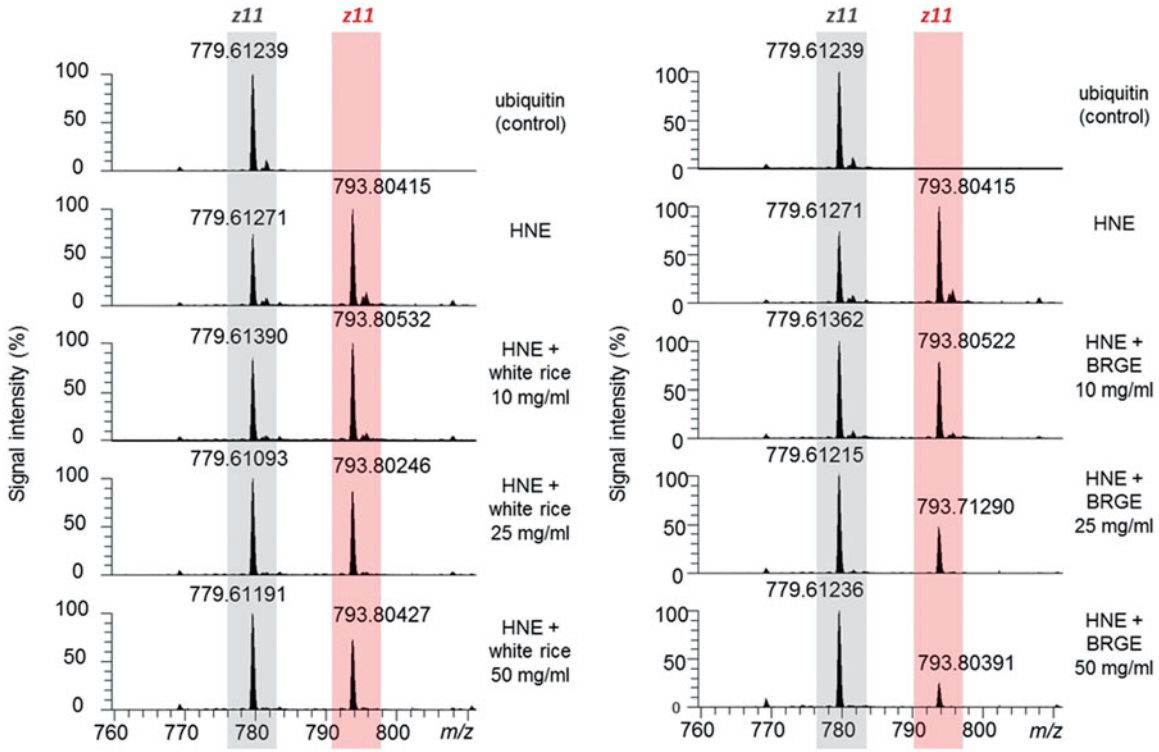

Figure 1. 11-charged MS peaks of ubiquitin incubated in the absence and in the presence of HNE and increasing concentration of two extract: white rice extract (panel on the left) and BRGE (panel on the right).

Table 1. HNE Sequestering activity of rice extracts.

Rice extract	Average IC_{50} (mg/mL)	SD	CV%
Black rice giant embryo (BRGE)	29.10	0.79	2.7
Black rice giant embryo bran	30.63	3.70	12.4
Miryang282	39.32	2.48	6.3
White rice (brown)	45.46	2.29	5
Black rice	52.04	8.34	16
Black rice giant embryo 2 (BRGE2)	65.92	2.13	3.2
Red rice	71.32	4.04	5.7
White rice (brown)	151.00	17.70	11.7

Activities are reported as IC_{50} which represents the concentration of the tested compound able to reduce by 50% HNE-induced ubiquitin carbonylation.

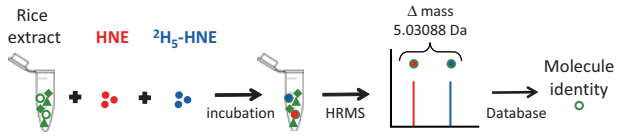

Figure 2. Graphic representation of the method. The first step consists of spiking the plant extract with HNE (156.11502 Da) and $^2H_5$-HNE (161.07590 Da) at the same molar ratio. After incubation, the HNE adducts are identified based on the typical isotopic pattern and the identity of the HNE scavenger determined by matching the experimental accurate masses and MS/MS fragment ions with those contained in a database.

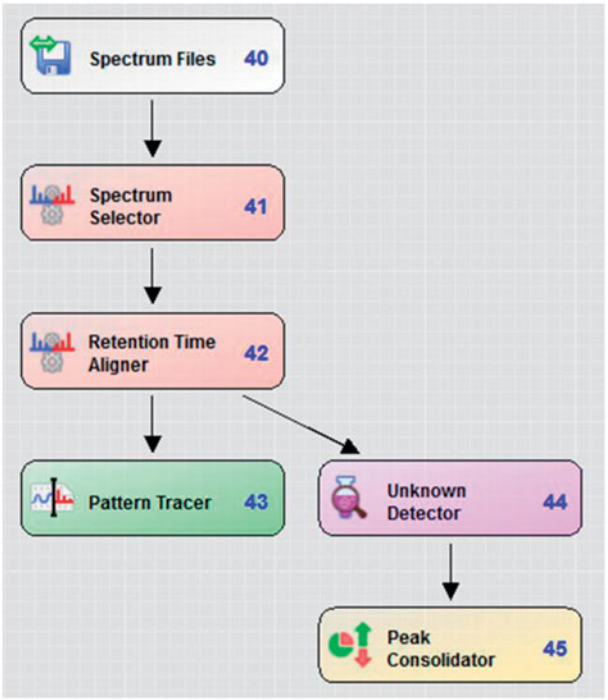

Figure 3. Workflow's nodes used in Compound Discoverer.

selected for the next step aimed at identifying the compounds responsible for the sequestering effect of the extract.

Set-up of an isotopic labelling procedure for the identification of HNE-sequestering agents

An off-target approach based on isotopic signature was then set-up to identify HNE adducted compounds when present in complex matrices such as plant extracts. As summarised in Figure 2, the method consists of the following steps: first the plant extract is spiked with HNE (156.11502 Da) and $^2H_5$-HNE (161.07590 Da) mixed at the same molar ratio. Compounds which are reactive towards HNE, react with both the isotopes at the same rate forming reaction products which have a specific isotopic pattern. A pair of peaks with a similar intensity spaced by the delta mass corresponding to the incremental mass of $^2H_5$-HNE with respect to HNE (5.03088 Da ±10 ppm) is generated. The second step consists of extracting the *m/z* values relative to the ions of the HNE adducts. This is automatically carried out by Compound Discoverer™ on the basis of the specific isotopic pattern characterised by the two peaks with the same intensity and with a delta mass corresponding to five deuterium atoms. Figure 3 shows in more detail the specific workflow generated to extract the peak lists from the mass spectra. By setting the "Pattern Tracer" node with a delta mass of 5.03088 Da, a chromatogram of the peak pairs characterised by the set delta mass was obtained. Elution times of the most abundant peaks were then extracted and the *m/z* values of each peak pair were then obtained by the total ion chromatogram (TIC). HNE adducts were then confirmed by manually inspecting the characteristic relative abundance and mass shift of the isotopic pattern.

Further confirmation of the identity of the HNE adducts was given by the *Unknown detector node* which cheques for the absence of the detected peaks as above reported in nonspiked samples.

As a final adduct confirmation, the MS/MS spectra of each ion of the isotopic pattern (nondeuterated and deuterated ions) were checked in order to verify the overlap between the spectra, thus confirming the same structures and avoiding false positives. The MW of the bioactive compounds were then calculated by subtracting the MW of the HNE molecule (≈156 Da) from the masses of the identified compounds, and assuming that the detected ions are attributed to the Michael adduct between HNE and the bioactive molecule, as reported for a series of bioactive molecules able to quench HNE [41].

The method was firstly validated by using glutathione (GSH) and carnosine as known sequestering agents. Figure 4 shows the Full MS and MS/MS spectra of the GSH spiked with HNE and $^2H_5$-HNE mixed at the same molar ratio. The GSH-HNE and

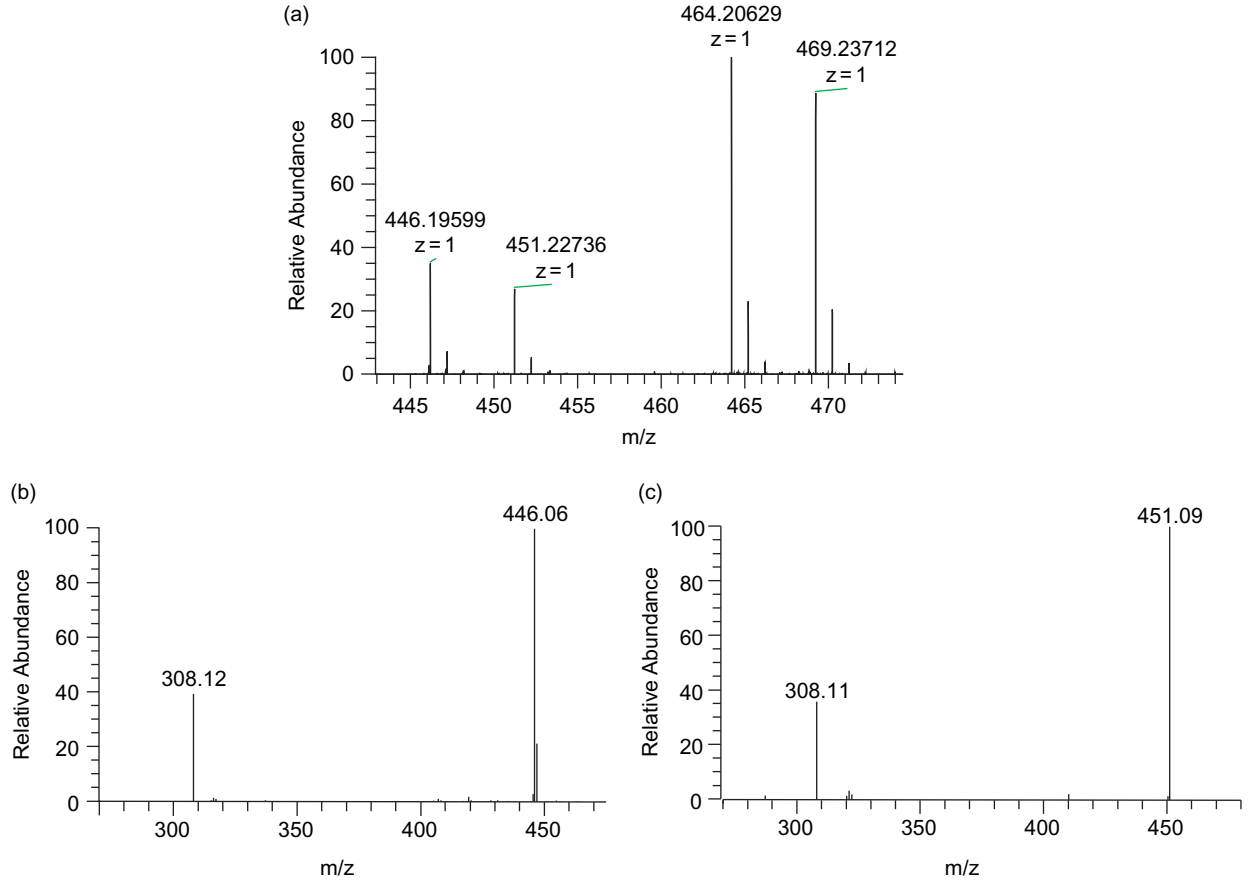

Figure 4. Full MS and MS/MS spectra of GSH spiked with HNE and $^{2}H_5$-HNE mixed at the same molar ratio. (a) Full MS spectrum showing the ions at *m/z* 464.20629 and at *m/z* 446.19599 relative to the $[M+H]^+$ and the $[M+H\text{-}H_2O]^+$ of GSH-HNE, and the ions at *m/z* 469.23712 and *m/z* 451.22736 referred to the $[M+H]^+$ and the $[M+H\text{-}H_2O]^+$ of GSH-$^{2}H_5$-HNE. (b) MS/MS spectrum of GSH-HNE adduct: the ion at *m/z* 446.06 derives from the loss of H_2O and that at *m/z* 308.12 from the neutral loss of HNE. (c) MS/MS spectrum of GSH-$^{2}H_5$-HNE adduct: the ion at *m/z* 451.09 derives from the loss of H_2O and the ion at *m/z* 308.11 which is the GSH moiety deriving from the neutral loss of $^{2}H_5$-HNE.

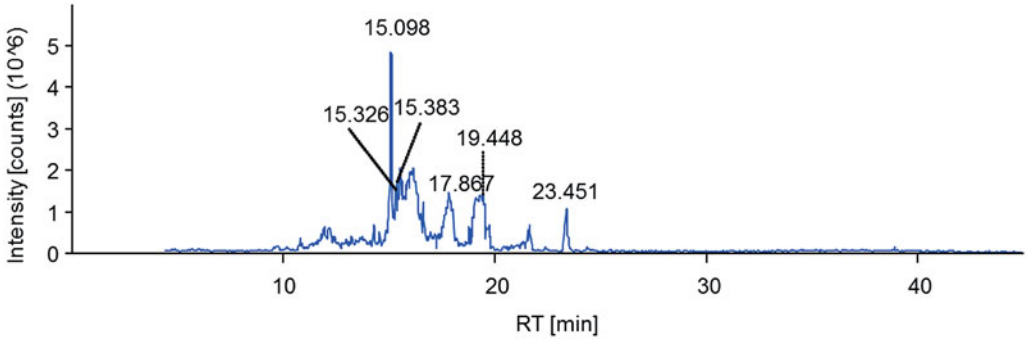

Figure 5. The "pattern tracer" chromatogram relative to BRGE incubated in the presence of HNE and 2H5-HNE mixed at the same molar ratio. The chromatogram reports only peak pairs characterised by the delta mass of 5.03088 Da.

GSH-$^{2}H_5$-HNE adducts are present as single charged ions at *m/z* 464.20629 and *m/z* 469.23712. They are also present as $[M+H\text{-}H_2O]^+$ ions as shown in Figure 4(a). The fragmentation patterns are characterised by the H_2O loss and the HNE and $^{2}H_5$-HNE neutral loss (Figure 4(b,c)), giving the ion at *m/z* 308.1 that is the GSH moiety.

Identification of sequestering components in black rice with giant embryo

Figure 5 shows the pattern trace of BRGE incubated in the presence of HNE and $^{2}H_5$-HNE mixed at the same molar ratio. Eleven peaks over the threshold intensity were easily identified and their HNE adducted nature

Table 2. HNE Quenchers identified in rice extract BRGE by using the isotopic labelling procedure.

Quencher monoisotopic MW	Calculated MW	Δppm	Possible structure
103.06336	103.06333	0.304	γ-Aminobutyric acid
155.06950	155.06948	0.151	Histidine
165.07922	165.07898	1.462	Phenylalanine
271.06133	271.06064	2.514	Pelargonidin
301.07236	301.07121	3.809	Peonidin
317.06864	317.06612	7.923	Petunidin
317.12164	317.12229	−2.082	Asp–Ser–Pro
330.07634	330.07395	7.232	Tricine
331.08361	331.08177	5.533	Malvidin

was confirmed by manual inspection taking into account the mass shift between the ion pairs, their relative abundances and the overlapping MS/MS fragmentation pattern. The MW of the sequestering agents were then determined by subtracting the adduct MW from that of HNE. The identity of the parent compounds was then assigned by matching the experimental accurate masses and MS/MS fragment ions with those contained in a database of compounds compiled by including the chemicals contained in rice [42–44]. The database contained 112 entries (32 anthocyanins, 14 phenolic acids, 22 between flavones and flavonols, nine tocopherols, 20 ferulates (γ-oryzanol), four carotenoids, seven sterols, and four triterpenoids). A list of nine compounds with a MW lower than 350 Da was then obtained and listed in Table 2. Five out of nine compounds were flavonoids, four of them belonging to the anthocyanidins class, three compounds were amino acids, two of them proteogenic, while one a peptide.

HNE sequestering activity

The commercially available compounds identified by the isotopic labelling procedure were then tested as HNE sequestering agent by using an HPLC-UV test. The test is based on measuring free-HNE after incubating the tested compound with HNE. Table 3 reports the percentage of HNE sequestered by the active compounds identified in BRGE extract. Results are reported either as % of HNE consumption at 1 and 3 hours and as carnosine units at 1 and 3 hours, setting as 1 the value of carnosine activity for both the time points. Histidine, phenylalanine, and aminobutyric acid were the amino acids identified as having HNE sequestering activity. The reactivity for histidine is due to the imidazole ring as has already been reported, together with the full elucidation of the reaction adduct by nuclear magnetic resonance [45]. The activity of histidine is increased when bonded to specific amino acids such as β-alanine, as in the case of carnosine, or analogues, which are histidine peptides found in mammals [46].

Table 3. HNE Sequestering activity of standard compounds identified in rice extract BRGE. Activity was determined by measuring free-HNE by HPLC-UV analysis.

	Activity (carnosine units)		HNE consumption (%)	
	1 h	3 h	1 h	3 h
Reference compounds				
Buffer	<0.10	<0.10	0.17 ± 0.31	0.71 ± 0.67
Carnosine	1.00	1.00	13.78 ± 2.34	36.06 ± 4.98
Polyphenols				
Peonidin-3-O-glucoside	0.29	0.20	4.00 ± 1.39	7.27 ± 2.42
Malvidin	<0.10	<0.10	1.15 ± 1.10	1.87 ± 1.35
Peonidin	<0.10	0.12	1.02 ± 0.47	4.31 ± 0.92
Pelargonidin	0.57	0.35	7.81 ± 0.49	12.60 ± 1.99
Tricin-5-O-glucoside	0.30	0.20	5.03 ± 2.58	8.31 ± 2.91
Amino acids				
Phenylalanine	<0.10	<0.10	0.84 ± 0.78	1.28 ± 0.47
GABA	<0.10	<0.10	0.34 ± 0.59	0.35 ± 0.62
Histidine	0.12	0.17	1.77 ± 1.82	6.26 ± 2.69

However, no histidine peptides were detected as HNE adducts in the rice extract. The HNE sequestering activity of GABA and phenylalanine, which is more likely due to their primary amino group, is very weak and much lower in respect to that of carnosine (the HNE sequestering activity was under the detection limit as determined by using the HPLC-UV assay). However, even if these two compounds are characterised by a very low potency as HNE sequestering agents in comparison to that of carnosine, they act as HNE sequestering agents in the crude mixture. This apparent contradiction could be explained by considering that they are probably contained in high concentrations in the extract or could be explained by the specific milieu which catalyses their reactions. In other words, it should be considered that the overall sequestering efficacy of compounds in plant extracts is not only due to their intrinsic nucleophilic reactivity but also to their relative concentration as well as to the matrix which could affect the intrinsic activity itself.

A set of polyphenols were then identified as HNE sequestering agents, four anthocyanidins, and one flavone (tricine). All of the identified compounds were found to be less effective than carnosine but characterised in the HPLC-UV assay by a well detectable activity. Among these polyphenols, pelargonidin was the most potent, followed by tricine, peonidin, and malvidin. The activity of anthocyanindins as HNE sequestering agents which are characteristic components of black rice can also explain the higher overall activity of black rice in respect to white rice and to the other rice varieties.

Anthocyanidins as HNE sequestering agents: molecular modelling studies

Several studies investigating various adducts on anthocyanidins discovered that the C8 atom is the most

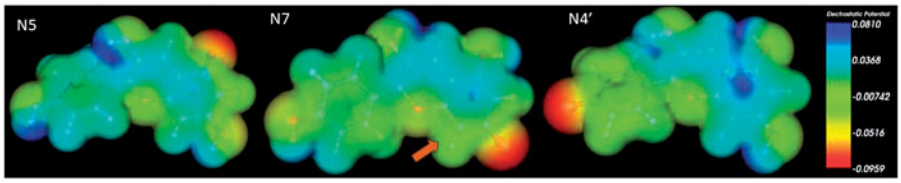

Figure 6. DFT-based MEP as projected on solvent accessible surface for the three major pelargonidin tautomers. The brown arrow indicates the C8 electrophilic attack centre in the most nucleophilic tautomer.

Table 4. DFT-based properties of the three major tautomer of malvidin and pelargonidin (ΔE values are expressed in kJ/mol; nucleophilicity descriptors are based on the parr's indices as nucleo $=1/\omega$ [44].

Compound	Nucleo_N5	Nucleo _N7	Nucleo _N4′	ΔE_N5	ΔE_N7	ΔE_N4′
Malvidin	5.18	5.08	4.86	0.00	+9.59	+15.63
Pelargonidin	5.15	5.29	4.60	+7.78	0.00	+18.27

prone centre for the electrophilic attack [47]. As exemplified by the pelargonidine tautomers in Figure 6, DFT calculations showed that the electron density at C8 vastly differs in the three simulated major tautomers. These differences, which are similarly detected in both considered compounds, revealed that the N7 tautomers are the most electron rich one at C8 due to the effect of the vicinal deprotonation in seven, while the N4′ tautomers are the least electron rich one at C8 due to the electron-drawing effect exerted by the C4′ deprotonation. While showing comparable stereo-electronic features, Table 4 reveals that the tautomers of pelargonidin and malvidin greatly differ in their relative stability. In detail, Table 4 shows that the N7 tautomer is the most stable for pelargonidin, while the N5 tautomer is the preferred one for malvidin and the C4′ tautomer is the least stable form for both anthocyanidins. Table 4 also evidences that the nucleophilicity differences, despite being very limited, parallel the tautomer stability, the pelargonidin N7 tautomer being the most nucleophilic species.

These results are in agreement with similar calculations performed by Estevez et al. [44] and suggest that the observed differences in the quenching activity between pelargonidin and malvidin are ascribable to different relative abundance of the reactive N7 tautomer. Differences in the stereo-electronic properties, despite the high nucleophilicity of the pelargonidin N7 tautomer, appear to be too limited to explain alone the difference in reactivity towards HNE. Taken together, these results confirm that the relative tautomer stability is markedly influenced by the substituents on the B ring and suggest that the stability of the reactive N7 tautomer is lowered by electron-donating groups on the B ring, a finding which may explain the intermediate reactivity of peonidin.

In conclusion, we report a novel approach, which permits the screening of plant extracts and in general complex matrices for their overall activity as HNE sequestering agents and the identification of small molecules responsible for the sequestering effects. This approach could be applied to screen a variety of plant extracts with the final aim of identifying novel and potent HNE sequestering agents. These methods can also be easily adapted to other cytotoxic RCS compounds such as MDA, glyoxal, and methylglyoxal for which potent sequestering agents are yet to be found.

By using this assay, we also found that rice, and in particular black rice with giant embryo is a valuable source of detoxifying agents of HNE which is known to be formed during food digestion and has a role in the promotion of colon cancer [48]. HNE derived from food is estimated to be 16 μg day^{-1} as daily exposure in Korean diet for a 60-kg Korean adult [49]. Based on the fact that 29.1 mg, which is corresponding to 673-mg black rice, inhibits 50% of protein carbonylation induced by 78.0 μg mL^{-1} of HNE, we can conclude that the HNE daily exposure of 16 μg day^{-1} should be easily detoxified in the gastro-intestinal lumen by a serving portion of 75 g. However, we do not know whether the g.i. microbiota changes the black rice components thus affecting their HNE sequestering activity (an increase or decrease of activity are both plausible), an aspect which should be further investigated for better understanding the in vivo effect.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported in part by Rural Development Administration, Republic of Korea [grant number: PJ010059].

References

[1] Vistoli G, De Maddis D, Cipak A, et al. Advanced glycoxidation and lipoxidation end products (AGEs and ALEs): an overview of their mechanisms of formation. Free Radic Res. 2013;47:3–27.

[2] Sousa BC, Pitt AR, Spickett CM. Chemistry and analysis of HNE and other prominent carbonyl-containing lipid oxidation compounds. Free Radic Biol Med. 2017;111:294–308.

[3] Mano J, Biswas MS. Analysis of reactive carbonyl species generated under oxidative stress. Methods Mol Biol. 2018;1743:117–124.

[4] Aldini G, Dalle-Donne I, Facino RM, et al. Intervention strategies to inhibit protein carbonylation by lipoxidation-derived reactive carbonyls. Med Res Rev. 2007;27:817–868.

[5] Dalle-Donne I, Aldini G, Carini M, et al. Protein carbonylation, cellular dysfunction, and disease progression. J Cell Mol Med. 2006;10:389–406.

[6] Poli G, Schaur RJ, Siems WG, Leonarduzzi G. 4-hydroxynonenal: a membrane lipid oxidation product of medicinal interest. Med Res Rev. 2008;28:569–631.

[7] Poli G, Schaur RJ. 4-hydroxynonenal in the pathomechanisms of oxidative stress. Tbmb. 2001;50:315–321.

[8] Mol M, Regazzoni L, Altomare A, et al. Enzymatic and non-enzymatic detoxification of 4-hydroxynonenal: methodological aspects and biological consequences. Free Radic Biol Med. 2017;111:328–344.

[9] Nègre-Salvayre A, Garoby-Salom S, Swiader A, et al. Proatherogenic effects of 4-hydroxynonenal. Free Radic Biol Med. 2017;111:127–139.

[10] Xiao M, Zhong H, Xia L, et al. Pathophysiology of mitochondrial lipid oxidation: role of 4-hydroxynonenal (4-HNE) and other bioactive lipids in mitochondria. Free Radic Biol Med. 2017;111:316–327.

[11] Aldini G, Vistoli G, Stefek M, et al. Molecular strategies to prevent, inhibit, and degrade advanced glycoxidation and advanced lipoxidation end products. Free Radic Res. 2013;47:93–137.

[12] Davies SS, Zhang LS. Reactive carbonyl species scavengers-novel therapeutic approaches for chronic diseases. Curr Pharmacol Rep. 2017;3:51–67.

[13] Colzani M, De Maddis D, Casali G, et al. Reactivity, selectivity, and reaction mechanisms of aminoguanidine, hydralazine, pyridoxamine, and carnosine as sequestering agents of reactive carbonyl species: a comparative study. ChemMedChem 2016;11:1778–1789.

[14] Aldini G, Dalle-Donne I, Colombo R, et al. Lipoxidation-derived reactive carbonyl species as potential drug targets in preventing protein carbonylation and related cellular dysfunction. ChemMedChem 2006;1:1045–1058.

[15] Aldini G, Carini M, Yeum KJ, et al. Novel molecular approaches for improving enzymatic and nonenzymatic detoxification of 4-hydroxynonenal: toward the discovery of a novel class of bioactive compounds. Free Radic Biol Med. 2014;69:145–156.

[16] Aldini G, Orioli M, Rossoni G, et al. The carbonyl scavenger carnosine ameliorates dyslipidaemia and renal function in Zucker obese rats. J Cell Mol Med. 2011;15:1339–1354.

[17] Albrecht T, Schilperoort M, Zhang S, et al. Carnosine attenuates the development of both type 2 diabetes and diabetic nephropathy in BTBR ob/ob mice. Sci Rep. 2017;7:44492.

[18] Regazzoni L, de Courten B, Garzon D, et al. A carnosine intervention study in overweight human volunteers: bioavailability and reactive carbonyl species sequestering effect. Sci Rep. 2016;6:27224.

[19] Yeum K-J, Orioli M, Regazzoni L, et al. Profiling histidine dipeptides in plasma and urine after ingesting beef, chicken or chicken broth in humans. Amino Acids. 2010;38:847–858.

[20] Vistoli G, Colzani M, Mazzolari A, et al. Computational approaches in the rational design of improved carbonyl quenchers: focus on histidine containing dipeptides. Future Med Chem. 2016;8:1721–1737.

[21] Vistoli G, De Maddis D, Straniero V, et al. Exploring the space of histidine containing dipeptides in search of novel efficient RCS sequestering agents. Eur J Med Chem. 2013;66:153–160.

[22] Hwang S, Lee Y-M, Aldini G, Yeum K-J. Targeting reactive carbonyl species with natural sequestering agents. Molecules. 2016;21:280.

[23] Mano J, Nagata M, Okamura S, et al. Identification of oxidatively modified proteins in salt-stressed Arabidopsis: a carbonyl-targeted proteomics approach. Plant Cell Physiol. 2014;55:1233–1244.

[24] Biswas MS, Mano J. Reactive carbonyl species activate caspase-3-like protease to initiate programmed cell death in plants. Plant Cell Physiol. 2016;57:1432–1442.

[25] Beretta G, Furlanetto S, Regazzoni L, et al.. Quenching of alpha, beta-unsaturated aldehydes by green tea polyphenols: HPLC-ESI-MS/MS studies. J Pharm Biomed Anal. 2008;448:606–611.

[26] Yeh W-J, Hsia S-M, Lee W-H, Wu C-H. Polyphenols with antiglycation activity and mechanisms of action: a review of recent findings. J Food Drug Anal. 2017;25:84–92.

[27] Zhu Q, Zheng Z-P, Cheng K-W, et al. Natural polyphenols as direct trapping agents of lipid peroxidation-derived acrolein and 4-hydroxy-trans-2-nonenal. Chem Res Toxicol. 2009;22:1721–1727.

[28] Colzani M, Criscuolo A, De Maddis D, et al. A novel high resolution MS approach for the screening of 4-hydroxy-trans-2-nonenal sequestering agents. J Pharm Biomed Anal. 2014;91:108–118.

[29] Rees MS, van Kuijk FJGM, Siakotos AN, Mundy BP. Improved synthesis of various isotope labeled 4-hydroxyalkenals and peroxidation intermediates. Synth Commun. 1995;25:3225–3236.

[30] Aldini G, Granata P, Marinello C, et al. Effects of UVB radiation on 4-hydroxy-2-trans-nonenal metabolism and toxicity in human keratinocytes. Chem Res Toxicol. 2007;20:416–423.

[31] Dasuri K, Ebenezer P, Fernandez-Kim SO, et al. Role of physiological levels of 4-hydroxynonenal on adipocyte biology: implications for obesity and metabolic syndrome. Free Radic Res. 2013;47:8–19.

[32] Keller BO, Sui J, Young AB, Whittal RM. Interferences and contaminants encountered in modern mass spectrometry. Anal Chim Acta. 2008;627:71–81.
[33] Vistoli G, Orioli M, Pedretti A, et al. Design, synthesis, and evaluation of carnosine derivatives as selective and efficient sequestering agents of cytotoxic reactive carbonyl species. ChemMedChem. 2009;4:967–975.
[34] Schmidt MW, Baldridge KK, Boatz JA, et al. General atomic and molecular electronic structure system. J Comput Chem. 1993;14:1347–1363..
[35] Mano J. Reactive carbonyl species: their production from lipid peroxides, action in environmental stress, and the detoxification mechanism. Plant Physiol Biochem. 2012;59:90–97.
[36] Schaur RJ. Basic aspects of the biochemical reactivity of 4-hydroxynonenal. Mol Aspects Med. 2003;24: 149–159.
[37] Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med. 1991;11: 81–128.
[38] Schaur R, Siems W, Bresgen N, Eckl P. 4-hydroxy-nonenal-A bioactive lipid peroxidation product. Biomolecules. 2015;5:2247–2337.
[39] Masisi K, Beta T, Moghadasian MH. Antioxidant properties of diverse cereal grains: a review on in vitro and in vivo studies. Food Chem. 2016;1;196:90–97.
[40] Lee Y-M, Han S-I, Song BC, Yeum K-J. Bioactives in commonly consumed cereal grains: implications for oxidative stress and inflammation. J Med Food. 2015;18:1179–1186.
[41] Aldini G, Facino RM, Beretta G, Carini M. Carnosine and related dipeptides as quenchers of reactive carbonyl species: from structural studies to therapeutic perspectives. BioFactors 2005;24:77–87.
[42] Abdel-Aal e-SM, Young JC, Rabalski I. IAnthocyanin. composition in black, blue, pink, purple, and red cereal grains. J Agric Food Chem. 2006;54:4696–4704.
[43] Pereira-Caro G, Cros G, Yokota T, Crozier A. Phytochemical profiles of black, red, brown, and white rice from the Camargue region of France. J Agric Food Chem. 2013;61:7976–7986.
[44] Suttiarporn P, Chumpolsri W, Mahatheeranont S, et al. Structures of phytosterols and triterpenoids with potential anti-cancer activity in bran of black non-glutinous rice. Nutrients. 2015;7:1672–1687.
[45] Hashimoto M, Sibata T, Wasada H, et al. Structural basis of protein-bound endogenous aldehydes. Chemical and immunochemical characterizations of configurational isomers of a 4-hydroxy-2-nonenal-histidine adduct. J Biol Chem. 2003;278:5044–5051.
[46] Aldini G, Carini M, Beretta G, et al. Carnosine is a quencher of 4-hydroxy-nonenal: through what mechanism of reaction? Biochem Biophys Res Commun. 2002;298:699–706.
[47] He F, Pan Q-H, Shi Y, Duan C-Q. Chemical synthesis of proanthocyanidins in vitro and their reactions in aging wines. Molecules. 2008;13:3007–3032.
[48] Bastide NM, Chenni F, Audebert M, et al. A central role for heme iron in colon carcinogenesis associated with red meat intake. Cancer Res. 2015;75:870–879.
[49] Surh J, Kwon H. Estimation of daily exposure to 4-hydroxy-2-alkenals in Korean foods containing n-3 and n-6 polyunsaturated fatty acids. Food Addit Contam. 2005;22:701–708.