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# Genotoxicity assessment of some cosmetic and food additives

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ABSTRACT

α-Hexylcinnamaldehyde (HCA) and *p*-tert-butyl-alpha-methylhydrocinnamic aldehyde (BMHCA) are synthetic aldehydes, characterized by a typical floral scent, which makes them suitable to be used as fragrances in personal care (perfumes, creams, shampoos, etc.) and household products, and as flavouring additives in food and pharmaceutical industry. The aldehydic structure suggests the need for a safety assessment for these compounds. Here, HCA and BMHCA were evaluated for their potential genotoxic risk, both at gene level (frameshift or base-substitution mutations) by the bacterial reverse mutation assay (Ames test), and at chromosomal level (clastogenicity and aneuploidy) by the micronucleus test. In order to evaluate a primary and repairable DNA damage, the comet assay has been also included. In spite of their potential hazardous chemical structure, a lack of mutagenicity was observed for both compounds in all bacterial strains tested, also in presence of the exogenous metabolic activator, showing that no genotoxic derivatives were produced by CYP450-mediated biotransformations. Neither genotoxicity at chromosomal level (i.e. clastogenicity or aneuploidy) nor single-strand breaks were observed. These findings will be useful in further assessing the safety of HCA and BMHCA as either flavour or fragrance chemicals.



## 1. Introduction

Flavours and fragrances are defined as agents able to directly stimulate the gustatory and olfactory receptors in mouth and nose, leading to taste and aroma responses. They are included in many pharmaceutical and personal care products (PPCPs) (viz. perfumes, creams, lotions, detergents, and various other personal and household products), and in food, in order to improve or modify their odour and/or taste, and can be released as pollutants in the environment, as a result of human activities, especially by sewage and domestic wastes (Bickers et al., 2003; Daughton, 2004). So the true human exposure to them is widespread and often difficult to predict. The number of flavouring compounds employed in daily products is very large. The list of Scientific Committee on Cosmetic and non-Food Products (SCCNFP) contains 2750 entries as perfume and aromatic raw materials (SCCNFP, 2000). In order to identify the fragrances which may be safely used in foodstuffs, the European Food Safety Authority (EFSA) reported on the CEF (Food Contact Materials, Enzymes, Flavourings and Processing Aids) Panel a list of compounds for which additional toxicity data have to be provided (EFSA, 2012). Among these, aldehydes are a group of potentially reactive compounds, due to the presence of a polarized carbon–oxygen double bond in their structure (Feron et al., 1991). Because of their reactivity, a number of aldehydes are able to interact with electron-rich biological macromolecules and to produce adverse health effects, including general toxicity, allergenic reactions, genotoxicity, and carcinogenicity (Langton et al., 2006). α-Hexylcinnamaldehyde (HCA; FL No. 05.041; Flavouring Group Evaluation, FGE.19, sub-group 3.1) is a synthetic α,β-unsaturated aldehyde (Fig. 1), not occurring in nature and considered as a structural alert for genotoxicity (EFSA, 2008). Likewise, *p*-tert-butyl-alpha-methylhydrocinnamic aldehyde (BMHCA), also known as Lilial®, BPMP, and 2–4-tert-butylphenylpropionaldehyde contains an aldehydic group (Fig. 2), whose oxidation into the corresponding α,β-unsaturated intermediate can be responsible for cell injury and DNA damage (Usta et al., 2013). Both chemicals possess a characteristic floral scent and are used as ingredients in many personal care (perfumes, creams, shampoos, etc.) and household products, and as additives in food and pharmaceutical industry (Schnuch et al., 2007).

Although the use of HCA and BMHCA is restricted due to their sensitizing potential (SCCNFP 0017/98), these compounds are enclosed among the six most frequently used fragrances in UK, being found in about 40% of cosmetic and household products (Buckley, 2007).

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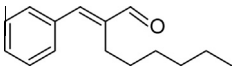

**Fig. 1.** Chemical structure of α-hexylcinnamaldehyde (HCA).

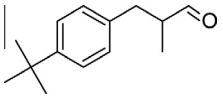

**Fig. 2.** Chemical structure of *p*-tert-butyl-alpha-methylhydrocinnamic aldehyde (BMHCA).

Given the ubiquitous distribution of these flavourings and their potentially reactive structure, it must be assumed that, when used as intended, they do not represent a toxicity hazard for the human health and for the environment. To outline the toxicological profile of a test compound, a broad range of features (acute, sub-chronic and chronic toxicity, genotoxicity, dermal irritation, skin sensitization, photoirritation, photoallergy, developmental and reproductive toxicity, and carcinogenicity) must be evaluated. Among them, genotoxicity is relevant as it relates to carcinogenicity. In fact, it is known that the majority of recognized human carcinogens are also genotoxic (Ennever et al., 1987; Bartsch and Malaveille, 1989). Generally, to evaluate a potential genotoxic risk due to a chemical exposure, an *in vitro* point mutation assay (Ames test) and an *in vitro* mammalian cell chromosomal aberration test (e.g. micronucleus assay) are used in the first instance (Kirkland et al., 2011). Nevertheless, SCCNFP still recommends a three-test battery to assess the mutagenic/genotoxic properties of a cosmetic ingredient. Recently, the comet assay has been validated as a visual method especially sensitive to detect, in interphase cells, DNA double- and single-strand breaks, alkaline labile and transient repair sites, DNA crosslink and oxidative damage (Collins, 2013).

In this context, the present paper aimed at evaluating the potential genotoxic effects of HCA and BMHCA in bacteria, by the bacterial reverse mutation assay, and in mammalian cells, by both the micronucleus and the single cell gel electrophoresis assay (comet assay), in order to highlight different potential genotoxic endpoints. In particular, we applied the cytokinesis-block micronucleus technique on human primary lymphocytes, with a long treatment time (24-h), which represents a well validated system for detecting many clastogenic and aneugenic compounds (Kirsch-Volders et al., 2011). Moreover, the alkaline protocol was used for the comet assay (Tice et al., 2000), and the human colonic epithelial cells (HCEC) were chosen in order to highlight a potential DNA damage due to the use of HCA and BMHCA as additives in food. For both tests, the long treatment duration precludes the inclusion of the exogenous metabolic activator, due to the cytotoxicity of S9 mix and to the short half-life of the enzymatic system (Kirsch-Volders et al., 2011).

## 2. Materials and methods

### 2.1. Chemicals

The test substances α-hexylcinnamaldehyde (HCA; purity > 95%) and 2–4-tert-butylbenzylpropionaldehyde (BMHCA; purity > 90%), the mutagens 2-nitrofluorene (2NF; 98% purity), 2-aminoanthracene (2AA; 96% purity), sodium azide (SA; >99.5% purity), methyl methanesulfonate (MMS; 99% purity), 9-aminoacridine (9AA; >99.5% purity), benzo[a]pyrene (BaP; >96% purity), ethyl methanesulfonate (EMS; ⩾98% purity), and demecolchicine or colcemid (COL; ⩾98% purity), the aminoacids histidine (⩾99% purity), tryptophan (⩾98% purity) and biotin (⩾99% purity), the solvents dimethyl sulphoxide (DMSO; ⩾99.9% purity), methanol (⩾99.9% purity) and ethanol (EtOH; ⩾99.8% purity), the salts sodium chloride (NaCl; ⩾99.5% purity), potassium chloride (KCl; ⩾99.0% purity), magnesium chloride ($MgCl_2$; ⩾98% purity), magnesium sulphate ($MgSO_4$; ⩾99.5% purity), potassium phosphate dibasic ($K_2HPO_4$; ⩾99% purity), ammonium sodium phosphate dibasic ($NH_4NaHPO_4$; ⩾99.9% purity), the media bacteriological agar, nutrient broth, nutrient agar, and RPMI 1640, the stains May-Greünwald and Giemsa, and the chemicals 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (Neutral Red solution; ⩾90% purity), trypan-blue (60% purity), glucose (⩾99.5% purity), cytochalasin-B (⩾98% purity), glucose-6-phosphate (G6P; ⩾98% purity), nicotinamide adenine dinucleotide phosphate (NADP; ⩾98% purity), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA; ⩾99% purity), trizma® base (⩾99% purity), phytohemagglutinin PHA-P, penicillin, streptomycin, and histopaque®-1077 were supplied by Sigma–Aldrich (St. Louis, MO, USA). Ethidium bromide solution was purchased from Invitrogen, Life Technologies (Monza, Italy). All the other reagents used for the comet assay were obtained from Microtech Srl (Naples, Italy). The S9 fraction (the liver postmitochondrial supernatant of rats treated with the mixture phenobarbital/β-naphthoflavone to induce the hepatic microsomal enzymes) was supplied and certified by Moltox (Molecular Toxicology, Boone, NC, USA).

To perform the bacterial reverse mutation assay, HCA, BMHCA, 9AA, 2NF, 2AA, and BaP were dissolved in DMSO, while SA and MMS were prepared in deionized water. For the micronucleus assay, the compounds were dissolved in EtOH (50% v/v), hence diluted in RPMI 1640 medium, in order to avoid their precipitation in the medium. EMS was dissolved in DMSO, while COL was purchased as a Hank's Buffered Salt Solution (10 mg/ml) and added to cultures as it was. For the neutral red and the comet assays, HCA and BMHCA were dissolved in DMSO (0.1% v/v). The S9 metabolic activator was prepared just before use by adding: phosphate buffer (0.2 M) 500 μl, deionised water 130 μl, KCl (0.33 M) 100 μl, $MgCl_2$ (0.1 M) 80 μl, S9 fraction 100 μl, G6P (0.1 M), 50 μl and NADP (0.1 M) 40 μl. The mixture was kept on ice during testing.

### 2.2. Bacterial strains

A set of five strains, *Salmonella typhimurium* TA1535 (*hisG46 chl1005 rfa1001*), TA1537 (*hisC3076 chl1007 rfa1003*), TA98 (*hisD3052 chl1008 rfa1004* pKM101), and TA100 (*hisG46 chl1005 rfa1001* pKM101), and *Escherichia coli* WP2*uvrA* (*trpE65 uvrA155*) was used. The strains TA1535 and TA1537 were supplied by Department of Pharmacology, University of Bologna (Italy), while TA98, TA100 and WP2*uvrA* were provided by the Research Toxicology Centre (Pomezia, Rome, Italy). After confirmation of the genotypes by the Strain Check Assay (Ames et al., 1975), the permanent cultures of each strain were prepared and then frozen. The working cultures, prepared from the permanent ones, were incubated overnight (16 h) at 37° C, to reach a concentration of approximately $1 \times 10^9$ bacteria/ml. In each experiment the number of viable cells for each strain was determined according to OECD (1997). Viability of the tested strains TA1535, TA1537, TA98, TA100 and WP2*uvrA*, expressed as viable cells/plate, was of 275.1 ± 8.7, 343.7 ± 14.2, 240.0 ± 13.1, 209.0 ± 9.5 and 371.5 ± 12.9, respectively.

### 2.3. Cell cultures

Peripheral blood lymphocytes were obtained from two healthy, no-smoker males who were less than 40 years-old and supplied by AVIS (Italian Association of Voluntary Blood donors). The donors provided written, informed consent for use of their samples.

Lymphocytes were separated from whole blood by using a density gradient (Histopaque 1077), hence were cultured in RPMI 1640 medium ($2 \times 10^6$ cells in 5 ml) supplemented with 15% v/v foetal calf serum (FCS), 0.5% v/v phytohemagglutinin (2 mg/ml in sterile deionised water), 1% v/v penicillin–streptomycin solution (5000 UI-5000 g/ml) and 1% v/v L-glutamine (29 mg/ml in sterile deionised water). The cultures were incubated at 37 °C in a wet, 5% $CO_2$ atmosphere for 72 h (Di Sotto et al., 2010).

HCEC were obtained from Fondazione Callerio Onlus (Trieste, Italy). The cells were routinely maintained at 37 °C in a 5% $CO_2$ atmosphere in 75 $cm^2$ polystyrene flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 2 mM L-glutamine and 1 mM sodium pyruvate. The medium was changed every 48 h.

### 2.4. Bacterial reverse mutation assay

Preliminarily, the solubility of the test substances in the final mixture was evaluated according to OECD Guidelines (1997). The cytotoxicity was evaluated as a reduction in the number of revertant colonies and as a change of the auxotrophic background growth (background lawn) in comparison with the control plates (Di Sotto et al., 2008). Each strain was treated with HCA and BMHCA at concentrations of 25, 5, 2.5, 0.5, 0.25 and 0.05 μmol/plate, both in the absence and presence of S9. The mutagenicity was assayed by the pre-incubation method, starting from the highest non-toxic concentration (Di Sotto et al., 2008). The vehicle DMSO (2% v/v) was used as negative control. The mutagens SA (1 μg/plate for TA1535 and TA100), 9AA (50 μg/plate for TA1537), 2NF (2 μg/plate for TA98), MMS (500 μg/plate for WP2*uvrA*), 2AA (1 μg/plate for TA98 and TA100 and 10 μg/plate for TA1535, TA1537 and WP2*uvrA*), and BaP (50 μg/plate for TA98, TA100 and WP2*uvrA*, 100 μg/plate for TA1535, and 50, 100 and 500 μg/plate for TA1537) were used as positive controls, in order to verify the bacteria susceptibility to a known genotoxic damage. The experiments were repeated at least twice and each concentration was tested in triplicate. A positive response in the mutagenicity assay was defined as an increase (at least twofold above the vehicle) in the histidine- or tryptophan-independent revertant colonies.

### 2.5. Micronucleus assay

Preliminarily, the cytotoxicity of test compounds on the peripheral blood lymphocytes was evaluated by scoring at least 1000 cells per each treatment for the presence of one, two, three or more nuclei and determining the nuclear division index (NDI), according to Di Sotto et al. (2010). The cells that did not undergo mitosis, as judged by their size and the density of DNA-positive material, were not included in the count. Genotoxicity was assayed starting from the highest concentration at which neither necrosis nor cytotoxic or cytostatic effects were observed (Di Sotto et al., 2010). The cultured lymphocytes, supplemented with cytochalasin-B (6.25 μM final concentration), were treated for 24 h at 37 °C with HCA and BMHCA at the concentrations of 5, 10, 25, 35, 50, 100, 250 and 500 μM. Each treatment was carried out on the cells obtained from two donors and each in two separate cultures (i.e. four cultures were set up for each treatment). The vehicle DMSO was used as negative control, while EMS (1.93 mM) (an alkylanting agent), and COL (0.054 μM) (a microtubule-disrupting agent binding tubuline) as positive controls. At the end of the incubation time, the lymphocytes were collected, treated for 2 min with a mild hypotonic solution (1:2 RPMI 1640 medium/$H_2O$, supplemented with 2% FCS), and then fixed in ice-cold acetic acid:methanol (1:1). After fixation, the cells were put directly onto slides, distributed by a cytospin centrifuge, air-dried, and stained with conventional May-Grünwald–Giemsa stain. All slides were coded and analyzed by a Zeiss Axioplan light microscope at 1000× magnification under oil immersion. For each treatment at least 1000 lymphocyte were scored to determine the NDI value, and at least 2000 binucleated cells (BNCs) were examined for the presence of micronuclei. A positive response was defined as a statistically significant increase of MN frequencies in the treated cultures respect to the vehicle.

### 2.6. Comet assay

Preliminarily, the cytotoxicity of the test compounds on HCEC cells was evaluated by the neutral red uptake assay (Aviello et al., 2010). The cells were seeded in 96-well plates ($1 \times 10^4$ cells per well) and allowed to adhere for 48 h; after this period, they were incubated with serial dilutions of HCA or BMHCA (starting from 300 μM; dilution factor of about 1:3) for 24 h and subsequently with the neutral red dye solution (50 μg/ml) for 3 h. Cells were lysed with 1% acetic acid, and the absorbance was read at 532 nm (iMarkTM microplate absorbance reader, BioRad). Dimethyl sulphoxide (DMSO) (20%) was used as a positive control. The results were expressed as percentage of cell viability ($n = 3$ experiments including 8–10 replicates for each treatment).

DNA damage was evaluated by the alkaline (pH > 13) comet assay (Aviello et al., 2010). Human colonic epithelial cells (passage between 19 and 23) were seeded in 6 well-plate ($2 \times 10^5$ cells per well). After 48 h, the cells were incubated with the highest not toxic concentration of HCA or BMHCA for 24 h and subsequently they were trypsinized to obtain a suspension of $1 \times 10^4$ cells/ml. Aliquots of cell suspension were centrifuged at 1000*g* for 5 min. The pellets were collected, mixed with 0.85% low melting point agarose and laid on pre-treated glass slides (Trevigen, TEMA ricerca S.r.l., Bologna, Italy). The slides were then suspended, at 4 °C for 1 h (pH = 10), in NaCl (2.5 M), $Na_2EDTA \cdot 2H_2O$ (100 mM), Tris (10 mM) and Triton X-100 (1% v/v), and electrophoresed in alkaline buffer (300 mM NaOH, 1 mM $Na_2EDTA$, pH > 12–13) at 26 V, and 300 mA for 20 min. After neutralisation in Tris–HCl (0.4 M, pH 7.5), the gels were stained with ethidium bromide (20 μg/ml). Images were analyzed using a Leica microscope equipped with Comet Assay™ software (Perceptive Instruments, UK).

### 2.7. Statistical analysis

All values are expressed as mean ± SEM. The one-way analysis of variance (one-way ANOVA), followed by Dunnett's Multiple Comparison Post Test, was used for analysis of multiple treatment means, and Student's *t* test was used for comparing a single treatment mean with a control mean. A $P$ value < 0.05 was considered statistically significant. Statistical analysis was performed with GraphPad Prism™ (Version 4.00) software (GraphPad Software, San Diego, California, USA).

## 3. Results

### 3.1. Bacterial reverse mutation assay

HCA and BMHCA, when assessed on the strains TA1535 and TA1537 in absence of S9, were cytotoxic at the concentration of 0.25 μmol/plate (corresponding to 95 μM) (Table A1). In presence of S9, they exerted cytotoxicity respectively at 0.25 and 0.5 μmol/plate in TA1535, and at 2.5 and 0.25 μmol/plate in TA1537 (Table A2). Moreover, HCA and BMHCA resulted cytotoxic, both in the absence and presence of S9, at the concentrations of 25

and 5 μmol/plate in TA98, TA100 and WP2*uvrA* (Tables A1 and A2). In the mutagenicity assay, HCA and BMHCA, at not-toxic concentrations, did not increase the number of revertant colonies in all strains tested, both in the absence and presence of the metabolic activator S9 (Tables 1 and 2). Conversely, the mutagens 2NF, SA, 9AA, MMS, 2AA and BaP increased the number of revertant colonies (from 2 to 11 times) with respect to the vehicle (Tables 1 and 2), showing that the system was suitable to detect different mechanisms of mutagenicity (i.e. frameshift mutations, base substitutions, oxidative damages). According to Wood et al. (1975), only TA1535 strain was not sensitive to the BaP-mutagenicity: no increase in the number of revertant colonies was highlighted up to the concentration of 500 μmol/plate, while cytotoxic effects were produced at higher concentrations (Table 2).

### 3.2. Micronucleus assay

When tested on the human lymphocyte cultures, neither HCA nor BMHCA produced cytotoxic effect up to 50 μM, being the Nuclear Division Index (NDI) similar to that of the vehicle. Conversely, at the concentration of 100 μM, the compounds reduced the cell proliferation, inducing a less than 70% value of NDI and early signs of cytotoxicity. At 250 and 500 μM, the NDI was not appreciable because of the advanced necrosis (Table A3). On the basis of these results, the mutagenicity test was carried out at concentrations of 50, 35, 25, 10 and 5 μM. In our experimental conditions, no increase in the MN frequency was observed in comparison with the vehicle; in contrast, EMS and COL significantly increased (about three and six fold, respectively) the micronuclei frequency with respect to the vehicle, showing that the lymphocytes were suitable to detect both a clastogenic and an aneuploidogenic damage (Table 3).

### 3.3. Comet assay

HCA and BMHCA, at the concentrations ranging from 1 to 300 μM (about 1:3 dilution factor), did not affect HCEC cell viability after 24 h exposure. The vehicle DMSO (0.1% v/v) did not modify the response; conversely, DMSO (20% v/v), used as positive control, significantly ($P < 0.001$) reduced HCEC viability (Table A4). At the concentration of 100 μM, neither HCA nor BMHCA induced DNA damage, after 24 h exposure, compared to the vehicle, suggesting the lack of a genotoxic effect (Fig. 3). Conversely, $H_2O_2$ (75 μM) significantly ($P < 0.001$) increased the DNA tail, so indicating the induction of single-strand breaks (Fig. 3).

## 4. Discussion

HCA and BMHCA are flavouring compounds widely used as additives in medical and consumer products, in foods, beverages, and sweetmeat. From a chemical point of view, they are synthetic aldehydes, characterized by the presence of a carbonyl group containing a polarized carbon–oxygen double bond. The marked difference in the electronegativity between the oxygen and the carbon atoms makes this group able to react with electron-rich biological macromolecules (i.e. DNA and proteins), and to induce adverse health effects, including general toxicity, allergenic reactions, mutagenicity, and carcinogenicity (Feron et al., 1991; Patlewicz et al., 2002; Garaycoechea et al., 2012). HCA is potentially more reactive than a simple aldehyde, as it also possesses a double bond between carbons 2 and 3 (α and β respectively). The conjugation of the unsaturated function with the carbonyl group makes the α-carbon positively polarized and consequently the preferred site for a nucleophilic attack (Feron et al., 1991). Because of its

**Table 1**
Effect of α-hexylcinnamaldehyde (HCA) and 2–4-tert-butylbenzyl propionaldehyde (BMHCA) on the number of spontaneous revertant colonies of *Salmonella typhimurium* TA1535, TA1537, TA98 and TA100, and of *Escherichia coli* WP2*uvrA*, in absence of the metabolic activator S9. Values are expressed as means ± SEM ($n = 6$ plates).

| Substance | μmol/plate | Number of revertant colonies | | | | |
|---|---|---|---|---|---|---|
| | | TA1535 | TA1537 | TA98 | TA100 | WP2*uvrA* |
| HCA | 0.01 | 144.5 ± 3.5 | 40.3 ± 2.5 | – | – | – |
| | 0.02 | 136.0 ± 20.0 | 34.7 ± 5.8 | – | – | – |
| | 0.05 | 139.0 ± 8.2 | 38.5 ± 3.5 | – | – | – |
| | 0.07 | 129.0 ± 18.3 | 36.0 ± 6.3 | – | – | – |
| | 0.1 | 65.0 ± 7.5[t] | 41.3 ± 5.8[t] | 34.7 ± 0.9 | 79.3 ± 9.4 | 44.0 ± 4.0 |
| | 0.2 | – | – | 38.3 ± 5.6 | 87.0 ± 2.4 | 41.7 ± 2.3 |
| | 1.0 | – | – | 33.3 ± 2.3 | 86.0 ± 5.2 | 53.3 ± 8.1 |
| | 2.0 | – | – | 38.7 ± 2.4 | 86.7 ± 4.8 | 60.0 ± 6.9 |
| | 10.0 | – | – | 37.3 ± 2.2 | 63.0 ± 2.0[t] | 49.5 ± 5.5 |
| BMHCA | 0.01 | 139.1 ± 8.4 | 35.0 ± 2.1 | – | – | – |
| | 0.02 | 130.9 ± 3.6 | 40.0 ± 8.0 | – | – | – |
| | 0.05 | 127.5 ± 7.4 | 38.0 ± 1.8 | – | – | – |
| | 0.07 | 134.3 ± 6.4 | 39.5 ± 2.4 | 48.0 ± 8.3 | 84.7 ± 5.8 | 44.0 ± 6.1 |
| | 0.1 | 129.1 ± 1.8 | 36.0 ± 2.0 | 42.7 ± 4.8 | 73.3 ± 3.5 | 50.7 ± 5.8 |
| | 0.2 | – | – | 37.5 ± 3.2 | 83.3 ± 3.5 | 49.5 ± 5.5 |
| | 1.0 | – | – | 45.3 ± 9.6 | 88.7 ± 5.5 | 57.3 ± 3.5 |
| | 2.0 | – | – | 44.0 ± 2.3 | 77.3 ± 2.7 | 45.7 ± 4.8 |
| | 10.0 | – | – | – | – | – |
| 2-NF[a] | 2.0 | – | – | 117.3 ± 9.4[**] | – | – |
| SA[b] | 1.0 | 1477.0 ± 65.3[**] | – | – | 197.7 ± 7.2[**] | – |
| 9-AA[c] | 50.0 | – | 173.0 ± 22.0[**] | – | – | – |
| MMS[d] | 500.0 | – | – | – | – | 132.7 ± 11.4[**] |
| Vehicle[e] | | 137.5 ± 3.3 | 39.8 ± 3.4 | 40.7 ± 2.5 | 86.5 ± 7.2 | 52.2 ± 2.2 |

–, not tested.
[a] 2-Nitrofluorene.
[b] Sodium azide.
[c] Methyl methanesulfonate.
[d] 9-aminoacridine.
[e] DMSO 50 μl/plate.
[**] $P < 0.01$ *vs.* vehicle.
[t] Toxicity.

**Table 2**
Effect of α-hexylcinnamaldehyde (HCA) and 2–4-tert-butylbenzyl propionaldehyde (BMHCA) on the number of spontaneous revertant colonies of *Salmonella typhimurium* TA1535, TA1537, TA98 and TA100, and of *Escherichia coli* WP2*uvrA*, in presence of the metabolic activator S9. Values are expressed as means ± SEM ($n = 6$ plates).

| Substance | μmol/plate | Number of revertant colonies | | | | |
|---|---|---|---|---|---|---|
| | | TA1535 | TA1537 | TA98 | TA100 | WP2*uvrA* |
| HCA | 0.01 | 111.0 ± 8.0 | 117.0 ± 9.0 | – | – | – |
| | 0.02 | 116.3 ± 5.2 | 110.5 ± 5.5 | – | – | – |
| | 0.05 | 121.3 ± 6.9 | 126.0 ± 8.5 | – | – | – |
| | 0.07 | 123.2 ± 7.3 | 119.0 ± 5.3 | | | |
| | 0.1 | 120.3 ± 7.7 | 114.0 ± 6.0 | 53.3 ± 7.4 | 107.3 ± 8.7 | 50.7 ± 1.3 |
| | 0.2 | – | 116.0 ± 5.3 | 42.5 ± 1.3 | 116.0 ± 6.3 | 51.2 ± 4.4 |
| | 1.0 | – | – | 48.5 ± 3.8 | 115.0 ± 5.3 | 42.7 ± 3.5 |
| | 2.0 | – | – | 45.1 ± 2.7 | 120.6 ± 3.2 | 58.3 ± 6.1 |
| | 10.0 | – | – | 39.6 ± 1.3 | 108.0 ± 2.0[t] | 55.7 ± 3.5 |
| BMHCA | 0.01 | 112.0 ± 7.7 | 129.0 ± 3.8 | – | – | – |
| | 0.02 | 118.0 ± 8.0 | 115.0 ± 5.0 | – | – | – |
| | 0.05 | 113.0 ± 7.3 | 127.0 ± 5.3 | – | – | – |
| | 0.07 | 111.0 ± 8.0 | 121.0 ± 8.3 | 53.2 ± 3.9 | 123.7 ± 8.3 | 54.1 ± 4.2 |
| | 0.1 | 117.0 ± 21.0 | 114.2 ± 4.1 | 58.3 ± 5.8 | 118.0 ± 5.8 | 48.0 ± 6.1 |
| | 0.2 | – | – | 44.0 ± 6.9 | 122.7 ± 7.4 | 52.5 ± 2.9 |
| | 1.0 | – | – | 50.7 ± 8.5 | 124.0 ± 5.1 | 50.7 ± 6.7 |
| | 2.0 | – | – | 41.3 ± 3.5 | 134.7 ± 6.8 | 57.3 ± 4.7 |
| | 10.0 | – | – | – | – | – |
| 2-AA[a] | 1.0 | – | – | 170.7 ± 15.7** | 250.7 ± 10.2** | – |
| | 10.0 | 804.0 ± 20.0** | 446.0 ± 26.0** | – | – | 198.7 ± 21.8** |
| BaP[b] | 50.0 | 126.9 ± 1.9 | – | 430 ± 11.0** | 693 ± 49.6** | 132.5 ± 6.0** |
| | 100.0 | 116.1 ± 2.7 | 354.0 ± 42.0** | – | – | – |
| | 500.0 | 135.0 ± 2.2 | – | – | – | – |
| Vehicle[c] | | 117.9 ± 8.6 | 113.3 ± 6.2 | 43.7 ± 3.3 | 128.0 ± 4.7 | 51.9 ± 2.8 |

–, not tested.
[a] 2-Aminoanthracene.
[b] Benzo[a]pyrene.
[c] DMSO 50 μl/plate.
** $P < 0.01$ *vs.* vehicle.
[t] Toxicity.

**Table 3**
Mean frequency of micronuclei (MN) in binucleated cells (BNCs) and nuclear division index (NDI) in human lymphocytes treated with α-hexylcinnamaldehyde (HCA) and 2–4-tert-butylbenzyl propionaldehyde (BMHCA). Value represents the mean ± SEM ($n = 6$).

| Treatment | [μM] | MN/1000 BNCs[a] (mean ± SE) | NDI (mean ± SE) |
|---|---|---|---|
| HCA | 5 | 2.9 ± 0.3 | 1.53 ± 0.09 |
| | 10 | 3.4 ± 0.2 | 1.48 ± 0.06 |
| | 25 | 3.2 ± 0.4 | 1.45 ± 0.06 |
| | 35 | 3.3 ± 0.3 | 1.44 ± 0.03 |
| | 50 | 3.1 ± 0.2 | 1.41 ± 0.09 |
| BMHCA | 5 | 3.1 ± 0.4 | 1.41 ± 0.03 |
| | 10 | 2.7 ± 0.5 | 1.47 ± 0.01 |
| | 25 | 3.6 ± 0.4 | 1.43 ± 0.03 |
| | 35 | 3.7 ± 0.3 | 1.42 ± 0.04 |
| | 50 | 3.9 ± 0.2 | 1.38 ± 0.06 |
| Vehicle[b] | | 3.7 ± 0.9 | 1.46 ± 0.09 |
| EMS[c] | 120 | 11.2 ± 0.9** | 1.56 ± 0.03 |
| COL[d] | 0.02 | 23.2 ± 1.2** | 1.58 ± 0.03 |

[a] For each treatment, MN frequency was determined by scoring at least 1000 binucleated lymphocyte cells (BNC). For untreated cultures, the value of MN/1000 BNC was of 4.88 ± 0.13, while that of NDI of 1.54 ± 0.03.
[b] DMSO 1%.
[c] EMS, ethyl methanesulfonate.
[d] COL, colcemid.
** Denote a significant difference from the vehicle ($P < 0.01$).

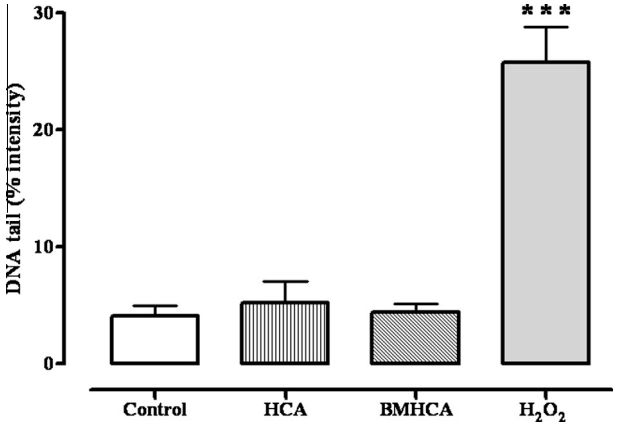


**Fig. 3.** Effect of aldehydes HCA and BMHCA (100 μM for 24 h) on HCEC cells DNA integrity evaluated by the comet assay. $H_2O_2$ (75 μM) was used as positive control. Values represent the mean ± SEM ($n = 3$). ***$P < 0.001$ *vs.* control.

potentially reactive α,β-unsaturated structure, HCA has been listed on the EFSA CEF Panel, in order to collect additional genotoxicity data for safety assessment (EFSA, 2008). BMHCA also represents a potentially health hazard as it can be metabolized into a reactive α,β-unsaturated intermediate (Usta et al., 2013).

Several aldehydes are found to be released as pollutants in the environment during combustion process, such as automobile exhausts, tobacco smoke, and flue gases, so increasing the carcinogenic hazard for human (Seaman et al., 2007; Abraham et al., 2011; Kabir and Kim, 2011). For instance, acetaldehyde, a typical indoor air pollutant, and crotonaldehyde, a component of the cigarette smoke, are potential genotoxic carcinogens (Stein et al., 2006), while the flavouring agent isobutyraldehyde resulted inactive as carcinogens (Benigni et al., 2005). Among α-β unsaturated aldehydes, although cinnamaldehyde and citral were found to be not cancerogenic (Benigni et al., 2005), acrolein and its alkyl derivatives, *p*-nitro cinnalmaldehyde and *o*-methoxycinnamaldehyde are classified as potentially genotoxic/mutagenic compounds (Eder et al., 1991; Eder and Deininger, 2001; Adams et al., 2004).

Due to the widespread and often involuntary human exposure to HCA and BMHCA, it is very important to verify that when used as intended, these aldehydes do not represent a health hazard for humans and environment. In the present study, the potential genotoxic effects of the two aldehydes have been evaluated at different levels of the genome, by an integrated experimental approach: the bacterial reverse mutation assay (to study point mutations in bacteria), the micronucleus test (to evaluate clastogenicity and aneuploidogenicity in human lymphocytes), and the comet assay (to highlight primary DNA damage, including repair-effects and repairable DNA damage). The combination of the bacterial reverse mutation assay and the micronucleus test has been shown to be very suitable for revealing potential genotoxic carcinogens, so that, when results of both tests are negative, a further *in vivo* test may not be necessary (Kirkland et al., 2011). Moreover, including TA100 bacterial strain increases the sensitivity of the test to the aldehydes mutagenicity (Dillon et al., 1998). Nevertheless, the micronucleus assay highlights only a little amount of DNA damage, that occurs in the interphase, and that will lead to fixed chromosome abnormalities after a passage through mitosis (Tafazoli and Volders, 1996). In this context, including the comet assay increases the sensitivity of the experimental system, because it also reveals very early damages (i.e. DNA double- and single-strand breaks, alkaline labile and transient repair sites, DNA crosslink and oxidative damage) (Collins, 2013).

Our results showed that both HCA and BMHCA do not induce genotoxic effects. In particular, a lack of point mutations (i.e. frameshift and base-substitution mutations, and oxidative damages) was observed in bacteria, also in presence of the exogenous metabolic activation system, showing that no genotoxic derivatives were produced by the CYP450-mediated biotransformations. The presence of the metabolic activator reduced in some cases the toxicity of the tested substances: a partial detoxification of the aldehydes by reaction with the nucleophilic components of the S9 could occur. Furthermore, HCA and BMHCA did not induce any kind of damage in mammalian cells, at chromosomal level (i.e. clastogenic or aneuploidic damage, and single-strand breaks). The lack of genotoxicity of HCA is in agreement with the negative results reported by Wild et al. (1983). This fact can be explained also on the basis of its chemical structure. It is known that the presence of bulky substituents at carbon 2 reduces the reactivity of aldehydes toward nucleobases, so their genotoxicity and mutagenicity, and increases their toxicity, probably because it makes the compounds more lipophilic, and allows them to better penetrate into the cells (Eder and Deininger, 2001; Benigni et al., 2005). HCA is characterized by a bulky lateral chain at carbon 2, which makes the compound not genotoxic and high cytotoxic.

In conclusion, the aldehydes HCA and BMHCA appear not genotoxic compounds, as they induced neither point mutations nor primary DNA damages, nor chromosome abnormalities. Our findings improve the knowledge on the genotoxicity of these compounds, which has been scantily investigated up to now, and will be critical to their safety assessment as flavouring/fragrance ingredients.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.yrtph.2013.11.003.

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