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In vitro mutagenicity assessment of fried meat-based food from mass catering companies

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ABSTRACT

The current article aimed to evaluate the *in vitro* mutagenicity of ten fried meat-based food extracts obtained from different catering companies from Navarra (Spain). A miniaturized 6-well version of the Ames test in *Salmonella typhimurium* TA98, and the *in vitro* micronucleus test (OECD TG 487) in TK6 cells were performed. None of the ten extracts of fried meat-based food induced gene mutations in *S. typhimurium* TA98 with or without metabolic activation, but five induced chromosomal aberrations after 24 h treatment of TK6 without metabolic activation. More studies are needed to check the biological relevance of these *in vitro* studies.

1. Introduction

Meat is an essential part in every diet, and one of the most important sources of macro and micronutrients. Nonetheless, processed meat and red meat have been recently classified as 'carcinogenic to humans' (Group 1) and 'probably carcinogenic to humans' (Group 2A), respectively (IARC, 2018). This classification was mainly based on epidemiological studies showing a positive association between high consumption of processed and red meat, and colorectal cancer and other types of cancer. However, the specific biological mechanisms underlying carcinogenicity in meat remain unclear (Cascella et al., 2018). In fact, the classification of the IARC has renewed the interest in providing information on the molecular mechanisms that may sustain the epidemiological evidence. Genotoxicity of meat is one of the potential mechanisms.

In fact, several genotoxic compounds are found in raw red meat (*e.g.,* heme group, or N-glycolylneuraminic acid), while some others are formed by meat processing or cooking (*e.g.,* N-nitroso compounds (NOCs), polycyclic aromatic hydrocarbons (PAHs), or heterocyclic aromatic amines (HAAs)) (Cascella et al., 2018; Demeyer et al., 2016; IARC, 2018). Moreover, other environmental contaminants that could be present in raw meat have been proposed as potential carcinogenic agents in cooked meat, although it is assumed that their concentration would probably be lower after cooking (Domingo and Nadal, 2016).

Among the different meat cooking procedures, frying is commonly used in daily household cooking and mass catering companies. It has been reported that frying leads to the production of appreciable levels of mutagenic compounds in food, commonly HAAs (IARC, 2018).

However, it is important to consider meat as a complex matrix containing different genotoxic compounds that might interact with each other. The evaluation of meat as a whole, instead of individual genotoxic compounds separately, allows to study the food in the most comparable conditions to human exposure. In this regard, the current European Food and Safety Authority (EFSA) guideline on the "Genotoxicity assessment of chemical mixtures" indicates that if the complete chemical characterization of the mixture is not possible, testing of the whole mixture is recommended (EFSA Scientific Committee, 2019).

The Food and Agriculture Organization of the United Nations in conjunction with World Health Organization (FAO/WHO) and the EFSA include the evaluation of the genotoxicity as an essential part for the

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Table 1
List of fried meat-based food samples. Each sample was obtained from a different mass catering company from Navarra (Spain).

Sample	Meat	Weight (g)[a]	Cooking temperature (°C)	Oil	Sector
1	Veal escalope	74.2	160 °C	High oleic sunflower	Sanitary
2	Sausages	94.4	N/D	Sunflower	Education
3	Veal escalope	39.9	180 °C	High oleic sunflower	Education
4	Veal escalope	47.7	160 °C	High oleic sunflower	Social
5	Meatballs	90.7	180 °C	High oleic sunflower	Social
6	Sausages	93.0	180 °C	Sunflower	Social
7	Sausages	99.5	170-180 °C	Sunflower	Education
8	San Jacobo[b]	84.1	180 °C	Sunflower	Social
9	Veal escalope	75.4	N/D	N/D	Social
10	Meatballs[c]	85.0	N/D	N/D	Social

Abbreviations. N/D: no data.
[a] Weighted just before extraction (unthawed).
[b] Breaded ham and cheese.
[c] Meatballs were also stewed after being fried.

safety assessment of chemicals in food, and a stepwise battery approach from *in vitro* to *in vivo* tests is proposed (EFSA Scientific Committee, 2011; FAO/WHO, 2009). The basic *in vitro* phase is commonly composed by two assays that cover different genetic endpoints: gene mutations, structural chromosome aberrations and aneuploidy. In the case of the EFSA approach, these endpoints should be assessed by the bacterial reverse mutation (Ames) assay and the *in vitro* micronucleus assay (EFSA Scientific Committee, 2011). Moreover, it is suggested that these assays are carried out following their corresponding Organization for Economic Co-operation and Development (OECD) guidelines (EFSA Scientific Committee, 2011; OECD, 2020, 2016). Negative results in the *in vitro* studies are usually sufficient to discard genotoxic potential (EFSA Scientific Committee, 2011). The EFSA guideline for testing the genotoxicity of chemical mixtures also recommends following the aforementioned strategy (EFSA Scientific Committee, 2019); also in this case, if the testing is performed in an adequate set of *in vitro* assays, clearly negative results do not raise concern for genotoxicity of the mixture.

Very recently, a systematic review about the genotoxicity of fried meat was conducted (Sanz-Serrano et al., 2020). The majority of studies used the Ames test to assess the mutagenicity of meat extracts and very few followed the OECD guidelines. Consistent positive results were observed in strains TA98 and TA1538 with metabolic activation. Only five studies evaluated meat samples prepared in food businesses and some positive results were detected in all of them.

The objective of the current study was to evaluate the genotoxic potential of fried meat-based food samples obtained from ten different catering companies from Navarra (Spain). For this matter, two *in vitro* assays were carried out, one capable of detecting gene mutations (Ames test) and another one detecting chromosomal aberrations (micronucleus test). The Ames test was performed applying a miniaturized 6-well version and using the *S. typhimurium* TA98 strain, and the micronucleus test was carried out in TK6 cells, following the OECD guideline 487 (OECD, 2016).

2. Material and methods

2.1. Chemicals and reagents

All the chemicals were purchased from Sigma Aldrich (Steinheim, Germany) unless otherwise stated hereunder. RPMI 1640 medium 'American Type Culture Collection (ATCC) modified', and heat-inactivated fetal bovine serum (FBS) were obtained from Gibco (NY, USA); Dulbecco's Phosphate Buffered Saline (DPBS) was obtained from Gibco (Paisley, UK); Phosphate Buffered Saline (PBS) tablets were obtained from Oxoid (Hampshire, UK); Penicillin-streptomycin was acquired from Lonza (Cologne, Germany); *Mutazime* S9 preparation from livers of Aroclor 1254-induced rats, S9 fraction from livers of Aroclor 1254-induced rats and mitomycin C (MitC) were purchased from Moltox (NC, USA); Cell sorting set-up *beads* for blue lasers (beads) and Sytox dye were obtained from Invitrogen (OR, USA); RNAase was obtained from Invitrogen (CA, USA).

2.2. Meat-based food samples

Ten fried meat-based food samples were kindly provided by ten different mass catering companies from different sectors in Navarra (Spain) (Table 1). Samples were stored at −40 °C for no more than five months until extraction was conducted as explained below.

In order to set up the extraction method and to serve as preliminary mutagenicity analysis, hamburger samples were prepared and deep-fried under controlled conditions in the laboratory as described hereunder. Minced meat (50/50 pork/beef) was purchased from a local store. Each hamburger patty (1 cm height × 10 cm diameter) was prepared using 100 g of minced meat and stored at −40 °C until cooked. Unthawed hamburgers were deep-fried in high oleic sunflower oil at 165 ± 5 °C for 2, 5, 10, 20, and 30 min. Two cooked patties per condition were grinded and stored in a flask at −40 °C until extraction was performed as explained below.

2.3. Extraction procedure

The extraction procedure was conducted as previously described (Bjeldanes et al., 1982a) with some modifications. Each sample, either from catering companies or cooked in the laboratory, was homogenized with an Ultra turrax in 2–3 mL of distilled water per g of sample. The mixture was brought to pH ~ 2 adding 1 N aqueous HCl, and remained without agitation for 5 min before it was centrifuged at 5251 × *g* for 15 min. The supernatant was then filtered through glass wool, and the process was repeated twice with the resulting sediments. The combined three supernatants were basified to pH 12 with 1 N NaOH and passed through a column of preconditioned Amberlite XAD-2 resin (0.2 bed volumes/min). Glass wool was placed above and below the resin. Column dimensions were 1.5 cm × 10 cm and fresh resin was used for each sample. Once the sample was passed and before the column dried, 150 mL of acetone were added to elute the compounds adsorbed in the resin (1 drop/sec). The acetone fraction was evaporated in a rotary evaporator, and the resultant viscous solid was dissolved in 2 mL DMSO and stored at −40 °C.

2.4. Miniaturized Ames test

The Ames test following the previously described 6-well-plate miniaturized version methodology (Burke et al., 1996) was used with some modifications. The assay was performed in *S. typhimurium* TA98, and conducted according to the recommendations of the OECD

guideline 471 for the Ames test (OECD, 2020).

Extracts from hamburgers deep-fried for 2-, 5-, 10-, 20- and 30-min and consecutive 1/2 serial dilutions were tested in *S. typhimurium* TA98 (Moltox, NC, USA). Similarly, extracts of meat-based food samples from catering companies and consecutive 1/2 serial dilutions were also tested in TA98. In all cases, the Ames test was performed in the presence and absence of metabolic activation (*i.e.*, Mutazime S9).

Twenty μg/well of 4-nitro-o-phenylenediamine (NPD) and 10 μg/well 2-aminofluorene (AF) were used as positive controls in the absence and presence of S9, respectively. Both AF and NPD were dissolved in DMSO. DMSO was also used as negative control.

Briefly, tubes placed in a PHMT-PSC24 thermo shaker (Grant Instruments, UK) at 47 °C and 0.2 × *g* were filled in the following order: 20 μL of extract, positive control or solvent (*i.e.*, DMSO), 500 μL of histidine-biotin top agar (9.5 μg/mL histidine, 11.1 μg/mL biotin, 4.5 mg/mL sodium chloride, and 5.5 mg/mL Bacto agar, in distilled water), 25 μL of 2×10^9 bacteria/mL, and 100 μL of PBS or S9. Then, each tube was poured onto minimal-medium-agar-filled wells in 6-well plates. Each condition was studied per triplicate using three wells. Plates were incubated at 37 °C and saturated humidity for 48–72 h until revertant colonies were counted by visual examination.

The following criteria were used to consider an extract to induce point mutations: a) a two-fold increase at one or more concentrations in the number of revertant colonies per well either with or without metabolic activation system, b) a concentration-related increase over the range tested.

2.5. In vitro MN test

The MN test was performed in TK6 cells following the recommendations of OECD guideline 487 (OECD, 2016).

TK6 cells (human-derived lymphoblastoid cells) were obtained from the ATCC (VA, USA). Cells were maintained in RPMI 1640 medium (supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin) as a suspension culture (0.2–1 x 10^6 cells/mL) in continuous agitation in a humidified incubator at 37 °C and 5% CO_2 for no longer than 60 days. Cell doubling time was approximately 15 h during the experimental period.

An extract derived from hamburgers deep-fried for 30 min and consecutive 1/2 serial dilutions were tested for 4 h, with and without S9 (S9-/S9+), and 24 h (S9-). Extracts of meat-based food samples from catering companies and consecutive 1/2 serial dilutions were also assessed for 4 h (S9-/S9+) and 24 h (S9-).

For the metabolic activation of the compounds, 1% S9 fraction with 1.5 mg/mL β-nicotinamide adenine dinucleotide phosphate sodium salt hydrate (NADP) and 2.7 mg/mL DL-isocitric acid, as cofactors, was used. Positive controls were included in each experiment: 1 μg/mL of cyclophosphamide (CP) for 4 h (S9+), and 10 ng/mL of colchicine (COL) for 24 h (S9-). DMSO (1%) was used as negative control.

In the short treatment, 6×10^5 TK6 cells/1 mL in a 12-well plate were treated with 10 μL of extract dilutions, positive controls or solvent (i.e., DMSO) for 4 h (S9-/S9+), and incubated for additional 20 h (i.e., until 1.5–2 cell cycles). In the long treatment, 3×10^5 TK6 cells/1 mL in a 12-well plate were treated for 24 h (S9-) (i.e., until 1.5–2 cell cycles). One replicate was treated per dilution of the extracts. In both cases, cells were then centrifuged (141 × *g*, 8 min, 4 °C), resuspended in 120 μL of cold 0.025 mg/mL EMA solution (in 2% FBS/DPBS) and incubated for 20 min on ice under 60 W direct light (30 cm). After the incubation, cells were washed with 3 mL of 2% FBS/DPBS by centrifugation (141 × *g*, 8 min, 4 °C) and incubated for 1 h in 250 μL of lysis solution 1 (0.2 μM Sytox dye, 1 mg/mL RNAase, 0.584 mg/mL sodium chloride, 1 mg/mL trisodium citrate dihydrate, 0.3 μL/mL IGEPAL). Then, 250 μL of lysis solution 2 (0.2 μM Sytox dye, 1.5 μL/mL beads, 85.6 mg/mL sucrose, 15 mg/mL citric acid) were added and cells were incubated for additional 30 min. Both lysis incubations were performed at room temperature in darkness. Samples were stored at 4 °C for no more than 24 h until the analysis in a cytometer FACSCanto™ II Six Colors (BD, NJ, USA) was performed. A fixed number of healthy nucleated cells (20,000 ± 1000) were scored per condition and MN were determined using FlowJo™ V10.2 software from BD (NJ, USA) by following the MicroFlow Instructions Manual (Litron Laboratories, NY, USA).

The cytotoxicity was evaluated by calculating the relative survival value (RS) for each condition. A fixed number of beads were added to each condition (included in lysis solution 2) and were counted during the cytometry analysis until 20,000 healthy nuclei were reached. Then, healthy-nuclei-to-bead ratios were determined for each condition and divided by the same ratio of the negative control, and showed as a percentage (×100).

$$RS = \left(\frac{No.\ of\ healthy\ cells}{No.\ of\ beads} \Big/ \frac{No.\ of\ healthy\ cells\ in\ negative\ control}{No.\ of\ beads\ in\ negative\ control} \right) x100$$

A compound was considered to induce MN if the following criteria was met in the short treatment (S9-/S9+) or long treatment (S9-): a) MN induction is statistically significant in at least one of the non-cytotoxic (*i.e.*, RS > 40%) conditions tested compared to control values, b) a three-fold increase in the level of MN is observed in at least one of the non-cytotoxic conditions, c) the MN induction follows a concentration-related increase over the non-cytotoxic range tested.

2.6. Statistical analysis

Miniaturized Ames test results are shown as the mean and SD of the technical triplicates at each condition. The concentration-response trend was studied by simple linear regression analysis. In the MN test, data was analysed using the chi-squared test and the trend was studied by simple linear regression analysis. In all cases, the Statistics and Data Analysis (STATA) software v12.1 (TX, USA) was used and statistical significance was set at $p \leq 0.05$.

3. Results

The *in vitro* mutagenicity of extracts from ten meat-based food samples obtained from different mass catering companies was assessed by using two complementary assays, one detecting gene mutations and another detecting chromosome aberrations. Preliminary studies were performed using hamburgers fried under controlled conditions.

3.1. Hamburger samples cooked in the laboratory

Before analyzing the samples obtained from the catering services, preliminary tests were carried out in extracts from hamburgers deep-fried in the laboratory using the mini-Ames in *S. typhimurium* TA98 and the *in vitro* micronucleus test.

The mini-Ames test was performed in *S. typhimurium* TA98 (S9-/S9+) by testing consecutive 1/2 dilutions of extracts from hamburger deep-fried for 2, 5, 10, 20, and 30 min (Fig. 1).

Dilutions 1/1 and 1/2 are not shown in Fig. 1 due to toxicity. The absence of data in dilutions 1/4 and 1/8 at some of the frying times is also due to toxicity. Induction of His^+ revertant colonies was not observed in the absence of S9. In the presence of S9, a two-fold increase in His^+ reverted was observed in several conditions (Fig. 1). Moreover, a clear dose-response induction of His^+ revertant colonies was observed after 30 min, though it was not significant. Moreover, in 1/32, 1/16 and 1/8 dilutions the effect was frying-time dependent.

Regarding edibility, hamburger samples were rare (cooked for 2 min), medium (cooked for 5 min), dry/overcooked (cooked for 10 min), and inedible (cooked for 20 and 30 min).

The induction of MN was studied in cells treated with an extract derived from hamburgers deep-fried for 30 min and its 1/2 serial dilutions (Table 2). Statistically significant concentration-response trend and a more than three-time MN increase compared to the control were

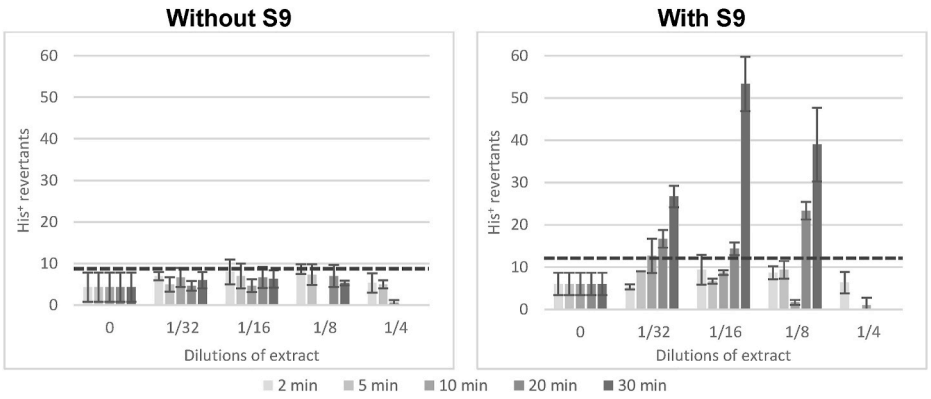

Fig. 1. Ames test results of extracts derived from hamburger samples deep-fried for 2, 5, 10, 20, and 30 min in the laboratory. Mean ± SD His $^{+}$ revertants in *S. typhimurium* strain TA98 (S9-/S9+) of the three technical triplicates are shown. Dashed line indicates the two-fold value of each negative control (referred as 0 in abscissa axis). Dilutions 1/8 and 1/4 were toxic in some conditions and are showed as absence of data. Positive controls: 20 μg/well NPD without S9 (560 ± 56 revertants/plate) and 10 μg/well AF with S9 (635 ± 134 revertants/plate).

Table 2
Results of the MN test after TK6 exposure to serial dilutions of an extract derived from hamburgers deep-fried for 30 min in the laboratory. The experiment was conducted for 4 h with and without metabolic activation (S9+/S9-), and for 24 h without metabolic activation (S9-). MN have been analysed by flow cytometry in 20,000 cells. MN per 10^3 nucleated cells and relative survival rate (RS) are shown for each condition.

Dilutions	4 h (S9-)		4 h (S9+)		24 h (S9-)	
	MN	RS	MN	RS	MN	RS
0	7.5	100	10.8	100	9.1	100
1/16	nt	nt	nt	nt	19.5***	72
1/8	7.8	107	9.7	127	18.6***	80
1/4	6.1	101	8.2	193	**35.5*** **	74
1/2	7.1	76	7.2	100	**214.9*** **	22
1/1	12.1	6	22.1***	48	–	0
trend	ns		ns		*	

Abbreviations. nt: not tested under the specified condition.
Statistical significance is set as: ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ (compared to negative controls, *i.e.*, 0). Bold text indicates three-fold increase compared to the control value. Positive controls (C+). 4 h: CP (S9+) 88.3 MN/10^3 cells. 24 h: COL (S9-) 151.7 MN/10^3 cells.

observed when cells were treated for 24 h (S9-).

3.2. Meat-based food samples from mass catering companies

In view of these preliminary results obtained with hamburger extracts, the mutagenicity of extracts derived from meat-based food samples fried in mass catering companies was then assessed using the miniaturized Ames test in the TA98 *S. typhimurium* strain and the MN test in TK6 cells.

Six serial 1/2 dilutions of each extract, starting from 1/2 due to toxicity, were tested in TA98 (S9-/S9+) (Table 3). Although a different pattern of toxicity was observed through the different extracts, none of them showed a two-fold increase in the number of His^{+} revertants with respect to the control values.

The results in the MN test after 4 h treatment (S9+/S9-) and 24 h (S9-) are shown in Table 4. None of the extracts met the criteria for a positive result after 4 h of treatment. After 24 h, extracts 1, 5, 6, 7, and 8, showed a three-fold statistically significant MN induction together with a concentration-response trend at non-cytotoxic concentrations.

Table 3
Results of the miniaturized Ames test after TA98 strain exposure to extracts derived from meat-based food samples fried in mass-catering companies. The experiments were conducted with and without external metabolic activation (S9+/S9-). Mean ± SD revertants of the technical triplicates are shown.

Dilutions	Extract 1		Extract 2		Extract 3		Extract 4		Extract 5	
	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+
0	8 ± 2	8 ± 3	8 ± 2	8 ± 3	3 ± 2	4 ± 2	3 ± 2	4 ± 2	8 ± 2	8 ± 3
1/64	5 ± 2	5 ± 2	6 ± 3	9 ± 7	6 ± 1	5 ± 2	4 ± 2	3 ± 3	6 ± 3	6 ± 2
1/32	5 ± 3	6 ± 2	5 ± 3	6 ± 3	3 ± 2	5 ± 1	2 ± 1	7 ± 2	6 ± 2	7 ± 3
1/16	3 ± 1	5 ± 2	4 ± 2	4 ± 1	3 ± 2	4 ± 2	3 ± 1	1 ± 1	2 ± 0	3 ± 1
1/8	1 ± 1	1 ± 2	4 ± 2	4 ± 3	1 ± 1	1 ± 1	tox	tox	tox	tox
1/4	tox	tox	3 ± 1	3 ± 2	tox	tox	tox	tox	tox	tox
1/2	tox	tox	tox	tox	tox	tox	tox	tox	tox	tox
C+	93 ± 9	84 ± 24	93 ± 9	84 ± 24	125 ± 53	361 ± 22	125 ± 53	361 ± 22	93 ± 9	84 ± 24

	Extract 6		**Extract 7**		**Extract 8**		**Extract 9**		**Extract 10**	
	S9-	**S9+**	**S9-**	**S9+**	**S9-**	**S9+**	**S9-**	**S9+**	**S9-**	**S9+**
0	3 ± 2	3 ± 2	3 ± 2	3 ± 2	3 ± 2	3 ± 2	3 ± 2	4 ± 2	3 ± 2	4 ± 2
1/64	3 ± 3	4 ± 2	3 ± 1	4 ± 1	3 ± 1	3 ± 2	6 ± 2	6 ± 2	2 ± 1	7 ± 4
1/32	3 ± 1	5 ± 2	4 ± 1	5 ± 1	4 ± 0	2 ± 2	3 ± 2	4 ± 0	4 ± 4	4 ± 2
1/16	4 ± 2	5 ± 2	3 ± 2	4 ± 3	4 ± 5	3 ± 1	3 ± 2	5 ± 1	1 ± 0	5 ± 1
1/8	3 ± 3	4 ± 2	4 ± 2	4 ± 2	4 ± 3	5 ± 3	4 ± 2	3 ± 3	2 ± 0	4 ± 1
1/4	1 ± 0	1 ± 1	1 ± 1	1 ± 1	5 ± 2	4 ± 2	1 ± 2	1 ± 1	3 ± 3	5 ± 2
1/2	1 ± 1	1 ± 0	tox	tox	5 ± 5	4 ± 4	tox	tox	1 ± 0	1 ± 2
C+	89 ± 11	80 ± 11	89 ± 11	80 ± 11	89 ± 11	80 ± 11	125 ± 53	361 ± 22	125 ± 53	361 ± 22

Abbreviations. tox: toxicity observed.
Positive controls (C+): 20 μg/well NPD without S9 (80–361 His^{+} revertants) and 10 μg/well AF with S9 (89–125 His^{+} revertants).

Table 4
Results of the MN test after TK6 exposure to serial dilutions of extracts derived from meat-based food samples fried in mass-catering companies. The experiments were conducted for 4 h with and without metabolic activation (S9+/S9-) and for 24 h without metabolic activation (S9-). MN have been analysed by flow cytometry in 20,000 cells and MN per 10^3 nucleated cells and relative survival rate (RS, in brackets) are shown for each condition.

4 h (S9-/S9+)

Dil.	Extract 1		Extract 2		Extract 3		Extract 4		Extract 5	
	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+	S9-	S9+
0	6.8 (100)	6.9 (100)	6.8 (100)	6.9 (100)	11.3 (100)	10.5 (100)	8.7 (100)	12.3 (100)	10.3 (100)	8.1 (100)
1/8	4.8 (85)	6.9 (86)	14.2*** (61)	10.7*** (85)	10.0 (136)	9.8 (102)	8.9 (119)	8.0 (115)	11.5 (68)	13.4*** (97)
1/4	6.5 (74)	7.6 (84)	12.5*** (48)	13.4*** (89)	13.5* (107)	13.8** (90)	6.9 (83)	7.3 (94)	29.7*** (60)	15.4*** (84)
1/2	7.8 (48)	6.0 (77)	14.8*** (37)	13.2*** (48)	14.9** (81)	12.1 (55)	12.6*** (71)	9.4 (99)	**72.9** ***(33)	14.1*** (49)
1/1	8.7* (34)	9.4* (41)	17.7*** (21)	16.3*** (33)	25.8*** (45)	18.3*** (67)	13.3*** (50)	21.3*** (53)	- (0)	- (0)
trend	ns	ns	ns	ns	**	*	ns	ns	ns	ns
	Extract 6		**Extract 7**		**Extract 8**		**Extract 9**		**Extract 10**	
	S9-	**S9+**	**S9-**	**S9+**	**S9-**	**S9+**	**S9-**	**S9+**	**S9-**	**S9+**
0	8.7 (100)	9.5 (100)	9.9 (100)	6.1 (100)	7.2 (100)	5.6 (100)	8.0 (100)	12.6 (100)	7.6 (100)	7.2 (100)
1/8	9.2 (110)	8.5 (104)	12.8** (70)	11.6*** (64)	10.5*** (63)	13.6*** (66)	9.2 (80)	5.3 (183)	6.3 (125)	10.7*** (90)
1/4	12.7*** (94)	8.8 (85)	17.9*** (64)	9.9*** (56)	15.5*** (41)	10.6*** (67)	8.9 (92)	12.3 (101)	7.2 (108)	9.8** (91)
1/2	19.4*** (64)	8.4 (100)	**57.2***** (35)	14.1*** (44)	20.2*** (32)	**24.7***** (51)	15.3*** (49)	8.7 (114)	15.4*** (51)	11.7*** (69)
1/1	**81.1***** (30)	12.1* (71)	**58.1***** (20)	**27.4***** (19)	**31.6***** (30)	**35.7***** (24)	**54.3***** (17)	18.9*** (56)	**35.2***** (37)	18.3*** (39)
trend	*	ns	ns	ns	ns	ns	ns	ns	ns	**

24 h (S9-)

Dil.	**Extract 1**		**Extract 2**		**Extract 3**		**Extract 4**		**Extract 5**	
	MN	**RS**	**MN**	**RS**	**MN**	**RS**	**MN**	**RS**	**MN**	**RS**
0	6.5	(100)	6.5	(100)	4.6	(100)	4.6	(100)	3.9	(100)
1/128	nt	nt	nt	nt	nt	nt	nt	nt	5.5*	(79)
1/64	nt	nt	nt	nt	nt	nt	nt	nt	6.6***	(88)
1/32	nt	nt	nt	nt	nt	nt	nt	nt	5.7**	(86)
1/16	8.5*	(65)	5.4	(80)	5	(82)	6.2***	(88)	**12.2*****	(55)
1/8	8.5*	(58)	10.2***	(52)	6.8**	(70)	5.9***	(86)	**27.5*****	(42)
1/4	12.7***	(50)	18.0***	(47)	5.8	(44)	7.8***	(59)	–	(0)
1/2	**21.1*****	(74)	**36.3*****	(10)	9.9***	(40)	7.9***	(50)	–	(0)
1/1	–	(0)	–	(0)	**81.8*****	(6)	**80.5*****	(15)	–	(0)
trend	**		ns		*		ns		***	
	Extract 6		**Extract 7**		**Extract 8**		**Extract 9**		**Extract 10**	
	MN	**RS**	**MN**	**RS**	**MN**	**RS**	**MN**	**RS**	**MN**	**RS**
0	3.9	(100)	4.0	(100)	4.0	(100)	9.4	(100)	9.4	(100)
1/128	nt	nt	6.5***	(87)	nt	nt	nt	nt	nt	nt
1/64	5.2	(95)	7.3***	(89)	7.9***	(88)	nt	nt	nt	nt
1/32	6.0**	(90)	8.8***	(80)	10.0***	(82)	nt	nt	nt	nt
1/16	9.6***	(86)	**20.4*****	(63)	5.3	(68)	8.4	(101)	10.3	(107)
1/8	**19.7*****	(67)	**70.2*****	(38)	**13.6*****	(62)	15.1***	(97)	17.9***	(92)
1/4	**85.0*****	(38)	–	(0)	**55.1*****	(41)	**51.2*****	(63)	**94.4*****	(52)
1/2	–	(0)	–	(0)	–	(0)	**144.0*****	(5)	**57.7*****	(6)
1/1	–	(0)	–	(0)	–	(0)	–	(0)	–	(0)
trend	**		**		**		ns		ns	

Abbreviations. nt: not tested under the specified condition.
[#] <20,000 cells were analysed due to toxicity. Statistical significance set as: ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ (compared to negative controls, *i.e.*, 0). Bold text indicates a three-fold increase compared to the control value. Positive controls. 4 h: CP (S9+) 55.5–194.35 MN/10^3 cells. 24 h: COL (S9-) 78.1–365.7 MN/10^3 cells.

4. Discussion

The mutagenicity of fried meat has been studied by many authors (for a review see Sanz-Serrano et al., 2020) and it is known that frying can produce an appreciable level of genotoxic compounds (Cascella et al., 2018; Demeyer et al., 2016; IARC, 2018). According to the mentioned review, extracts from fried meat have given consistent positive results in *S. typhimurium* TA98 strains in combination with external metabolic activation (S9+). Most of these studies were carried out with beef and pork samples but meat of other origins (mutton, horse, goat, chicken, lamb) gave also positive results in the *S. typhimurium* TA98/TA1538 reversion test in the presence of S9. Very few studies have used other assays different than the Ames test and most of the meat samples evaluated were cooked under controlled conditions in a laboratory. The scarce information regarding fried meat that may be consumed by the general population in restaurants or at home, was one of the reasons for performing this study, using a more realistic approach.

In this context, the current article evaluated the mutagenicity of extracts derived from fried meat-based food samples obtained from ten different mass catering companies. Reported frying temperatures were over 160 °C (Table 1), so it can be assumed that HAAs were formed (Trafialek and Kolanowski, 2014). Information on the duration of frying could not be retrieved, but it is also assumed that it was the adequate to prepare good edible products. The mutagenicity of the samples was studied *in vitro*, using the Ames test, in a miniaturized 6-well version, and the MN test. The XAD-2 extraction method was used to obtain the alkaline fraction of the samples (Bjeldanes et al., 1982a).

The miniaturized version of the Ames test was performed using *S. typhimurium* TA98, due to the fact that extracts from fried meat are consistently positive in this strain in combination with S9 (Sanz-Serrano et al., 2020). This was also confirmed in a preliminary assay; a meat extract prepared in the laboratory gave clear positive results in TA98 (S9+), when 5 strains (TA97, TA98, TA100, TA1535 and TA1537) were used (data not shown). The MN test was performed according to its corresponding OECD guideline (OECD, 2016). In summary, preliminary assays performed by using deep-fried hamburgers obtained in the

laboratory under controlled conditions showed induction of point mutations in *S. typhimurium* TA98 in the presence of metabolic activation and MN increase after 24 h of exposure in the absence of metabolic activation.

None of the extracts derived from fried meat-based food samples obtained from catering companies were mutagenic in TA98 (S9-/S9+), in contrast to that found in the available literature with samples obtained in restaurants. Five articles assessed the mutagenicity of extracts derived from commercial fried meat in *S. typhimurium* TA98/TA1538 strains: grilled hamburgers were evaluated in TA1538 (Bjeldanes et al., 1982b; Pariza et al., 1979); hot-plate and pan-fried hamburgers were evaluated in TA98 (Stavric et al., 1995; Spingarn and Weisburger, 1979) and sausages and hot-plate hot dogs were tested in TA98 (Gocke et al., 1982; Stavric et al., 1995). The number of samples evaluated, the assay conditions (*e.g.,* extraction method; concentrations tested; controls used; criteria to consider a positive result) and even the number of positive samples obtained are difficult to retrieve from these studies. Nevertheless, a wide range of mutagenic activity was observed in the commercially cooked meat samples in all the aforementioned approaches, always depending on metabolic activation (S9+). Bjeldanes et al. (1982b) tested twenty commercially grilled hamburgers from seven different vendors in TA1538 strain, and a wide range of values of revertants were observed in all the samples except two. Pariza et al. (1979) studied the mutagenic activity in eight hamburger samples obtained in four restaurants at two time points (0 and 1 week after). Results were generally reproducible when sampling one week after, probably due to standardized cooking conditions. Nonetheless, results were normalized with negative controls and thus its interpretation is complex; authors stated that mutagenic activity ranged from virtually undetectable to moderately high. Spingarn and Weisburger (1979) stated that there was statistically significant ($p < 0.01$) mutagenic activity in hamburger samples from two fast-food chains compared to control plates, but the number of positive samples with respect to the total number of samples analysed is not provided in the article. Nonetheless, using the available data presented, it can be gathered that around six samples out of twenty-six produced two-fold more revertants/plate than in the control plates. No data about different dilutions of the extracts were reported. Another author stated that fried sausages from a local stand produced similar number of revertants per sausage than those obtained with 'edible' sausages cooked under controlled conditions in TA98 strain (Gocke et al., 1982). Finally, Stavric et al. (1995) showed positive results in all seven pan-fried hamburgers obtained from the same commercial establishment, and two out of three pan-fried hot-dogs from different establishments. In summary, the five studies found positive results in some samples obtained from restaurants.

Very recently, Chamlal et al. (2021) observed that polar and non-polar extracts of some industrially processed meat products were not mutagenic in the Ames test (*S. typhimurium* TA1537, TA98, TA100 and TA102). Although some significant increases in the number of colonies were observed with some polar extracts, they were concluded not relevant in terms of mutagenicity. The authors attributed the absence of mutagenicity, in contrast with published results in similar samples, to the difference in ingredients, the extraction method, the range of concentrations tested, and the sample processing methods. Moreover, no chromosomal aberration was induced in the *in vitro* micronucleus assay in micromethod in TK6 cells, except for a weak genotoxic effect in a non-polar extract of corned-beef, in which no concentration-response trend was observed.

In the present study, all the samples showed negative results in TA98 strain. There are some factors that could partially explain the differences between the results in the current article and the abovementioned studies. The 6-well miniaturized Ames test is not currently OECD 471 compliant although its principles have been followed in the current study. Therefore, as in most scale down processes, a possible loss in sensitivity could be considered as 5×10^7 bacteria are used compared to 2×10^8 bacteria in the standard version (4 times less). It is also important to consider that different extraction methods may influence the recovery of the genotoxic compounds. In this regard, most of the studies (Bjeldanes et al., 1982b; Gocke et al., 1982; Pariza et al., 1979; Spingarn and Weisburger, 1979), including the current, applied extraction methods to obtain the alkaline fraction of meat and show substantially similar thin-layer chromatography and high-pressure liquid chromatography profiles (Bjeldanes et al., 1982a). This means that same mixtures of compounds were isolated but varying in the quantitative recovery of each method. Indeed, the procedure used in the current study, the XAD-2 extraction method, was found to be the most efficient method for recovering the mutagens present in meat (Bjeldanes et al., 1982a). A discussion about the different extraction methods can be found in Sanz-Serrano et al. (2020). It is also worth to mention that the awareness of food businesses about food quality and hygienic conditions has increased, decreasing the potential toxicity of food samples during the last years. Moreover, legislation related to residues and contaminants in meat has also evolved in this century.

Commonly, the evidence about the genotoxicity of fried meat extracts in the literature is obtained by the Ames test, and information about the mutagenic effects in other systems is limited or insufficient (Sanz-Serrano et al., 2020). To our knowledge, this is the first time that fried meat extracts have been analysed by the *in vitro* MN test. Extracts derived from meat-based food 1, 5, 6, 7, and 8 induced a statistically significant three-fold increase in the number of MN after 24 h at non-cytotoxic concentrations (RS > 40%), and a statistically significant dose-response trend (Table 4). Extracts 3 and 6 exerted a statistically significant three-fold increase and a statistically significant dose-response after 24 and 4 h (S9+), respectively. However, cell toxicity was observed at the highest concentration tested. Cytotoxicity could be related to MN induction by mechanisms not relevant to genotoxicity (Honma, 2011); the corresponding OECD guideline therefore states that the highest concentration should aim to achieve 55 ± 5% cytotoxicity but care should be taken in interpreting positive results only found in the higher end of this range (OECD, 2016). Extracts 9 and 10 also showed a statistically significant three-fold increase in MN at non-cytotoxic concentrations, but the dose-response was not statistically significant, although a positive trend was observed.

Each sample in its entirety was used for the preparation of the extract in order to maximize the possibility to find a positive result (Table 1). All samples weighed 90 ± 15 g except for samples 1, 3 and 4 that weighed less (Table 1). However, different concentrations of the extracts have always been prepared and checked to assess a dose-response effect and toxicity. Indeed, extracts obtained from these samples showed toxicity at the highest concentrations tested in *S. typhimurium* TA98 in the Ames test (Table 3). Moreover, sample 1 was positive in the MN test, and samples 3 and 4, although being negative, they also induced cytotoxicity in TK6 cells at the highest concentrations tested. In principle, there was no relation between the different types of meat-based foods, the temperature or type of oil used and the MN induction, though it is important to mention that the number of analysed samples was limited to assess these relationships.

The link between red consumption and cancer has been established by epidemiological studies (IARC, 2018). In the current article, deep-fried meat-based samples obtained from mass catering companies have been evaluated for chromosomal aberrations for the first time, finding positive results. The *in vivo* follow-up testing should be considered to assess the relevance of these findings (EFSA Scientific Committee, 2019). Nonetheless, analysing samples from mass catering companies would be hardly feasible due to the number of samples to be tested. Indeed, this approach would go against the Reduction principle of the 3 R's. There are few approaches assessing fried meat mainly in rodents (Sanz-Serrano et al., 2020) which only two checked MN induction of fried meat cooked in the laboratory with inconclusive results (Gocke et al., 1982, Taj and Nagarajan, 1994). More relevant *in vitro* studies could also be performed by subjecting food to physiological conditions simulating the gastrointestinal tract and testing the

genotoxicity of the bioavailable fraction and imitating human metabolism using different sources of S9.

5. Conclusion

The fried meat-based samples obtained from mass catering companies did not induce gene mutations in the mini-Ames using only TA98 strain, but half of them (5/10) induced MN in TK6 cells thus indicating their capability to produce chromosomal aberrations. More studies are needed to check the biological relevance of these *in vitro* findings.

CRediT authorship contribution statement

Julen Sanz-Serrano: Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Roncesvalles Garayoa:** Methodology, Investigation. **Ana Isabel Vitas:** Methodology, Investigation. **Amaya Azqueta:** Writing – review & editing, Conceptualization, Visualization, Supervision, Project administration, Funding acquisition. **Adela López de Cerain:** Writing – review & editing, Conceptualization, Visualization, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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