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An *In-vitro* Assessment of the Genotoxic Potential of In-Shell Nut Ingredients According to OECD Genotoxicity Guidelines 471 and 487

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Authors' contributions

This work was carried out in collaboration among all authors. Authors TA, ST and HV contributed to the conceptualization of the study. Authors PCB, GH, RH, YK, GK, AV and CZ developed the methodology and Author CZ was responsible for producing and selecting the study products. PCB, Authors GH, RH, YK, GK and AV did investigation. Authors TA and SP prepared the original draft of the manuscript. Authors RL, TA, PCB, RH, TH, YK, SP, ST, AV and HV reviewed and edited the manuscript. Author RL was responsible for funding acquisition. All authors read and approved the final manuscript.

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Original Research Article

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Abstract

Aims: *In vitro* genotoxicity of three shell-comprising nut ingredients, i.e., Almond Solids from roasted in-shell Almonds (Almond Solids), Almond Liquid Extract from roasted in-shell Almonds (Almond Liquid Extract) and Peanut Paste from roasted in-shell Peanuts (Peanut Paste) was assessed.
Study Design: Genotoxic safety was evaluated by performing two standard genotoxicity tests according to OECD 471 (the bacterial reverse mutation assay) and OECD 487 (the *in vitro* mammalian cell micronucleus test)
Place and Duration of Study: OECD 471 assays were performed in 2025 at Toxi-Coop Zrt., Balatonfüred, Hungary and at Charles River Laboratories, DD's-Hertogenbosch, The Netherlands. OECD 487 assays were performed in 2025 at Eurofins Munich, Planegg, Germany.
Methodology: All assays were conducted in accordance with internationally recognized OECD Test Guidelines 471 and 487 and complied with the principles of Good Laboratory Practice (GLP).
Results: The test results showed that neither Almond Solids nor Peanut Paste at 1.6-5000 *µ*g/plate nor Almond Liquid Extract at 52-5000 *µ*g/plate induced significant bacterial reverse mutations in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and in *Escherichia coli* strain WP2uvrA, both in the presence and absence of a metabolic activation system.
The *in vitro* micronucleus assays using human lymphocytes, performed on Almond Solids at 250-1500 *µ*g/mL, Almond Liquid Extract at 78.15-1250 *µ*g/mL and Peanut Paste at 50-200 *µ*g/mL were all negative for genotoxicity, with no increases in micronucleated cells frequency, either with or without metabolic activation.
Conclusion: Under the experimental conditions used, negative results in the genotoxicity assays support the good safety profile of Almond Solids, Almond Liquid Extract and Peanut Paste. These three shell-comprising nut ingredients can thus be considered not to be of genotoxic concern. These studies lay the foundation for future toxicological evaluations of ingredients based on nutshells.

Keywords: Almond solids; almond liquid extract; peanut paste; nutshell; mutagenicity; genotoxicity; OECD guideline 471; OECD guideline 487.

1. Introduction

Recently there has been an increased interest in the preparation of plant-based alternatives to the animal-based products (meat, dairy, milk …) to address health-related problems linked to its consumption as well as to contribute to the reduction of the environmental impact that comes with the production of animal-based products. As a result, the food industry faces the dual challenge of developing nutritionally improved products while minimizing environmental impacts along the value chain.

For that purpose, extracts from culinary nuts such as almonds, peanuts, and hazelnuts are widely used as raw materials to produce such alternative products. However, the nut production is in general highly resource-intensive, though still far more environmentally friendly than meat production. For example, the water footprint of Californian almonds is calculated at 10240 L of water per kg of kernels over the period 2004-2015, making them one of the most water-demanding crops (Fulton, 2019; Marvinney & Kendall, 2021). Peanut cultivation, while less water-intensive due to its nitrogen-fixing properties, still contribute to notable fresh-water use and ecotoxicity in production systems (Deepa et al., 2022). Hazelnut farming, on the other hand, imposes considerable land, fertilizer, and energy demands, with conventional hazelnut systems having higher global warming potential and eutrophication potentials than organic systems, largely driven by fertilizer use and orchard maintenance (Biagetti, 2023; International Conference on Harmonisation 2011; U.S. Food and Drug Administration 2016; United States Environmental Protection Agency 1998).

Besides their intensive resource use, nut processing also generates substantial by-product streams, mainly from shelling and hulling operations. Traditionally, the kernel (with or

without skin) has been considered the only valuable and edible component, leaving other fractions, such as shells, underutilized. However, this particular by-product represents a promising source of fibers and functional ingredients that could be incorporated into food formulations, contributing to a more circular and sustainable nut industry.

RE-NUT AG (Switzerland) developed and patented an innovative process that allow the processing of in-shell nuts (unshelled, containing the outer hard shell): Almond Solids from Roasted in-shell Almonds (Almond Solids), Almond Liquid Extract from Roasted in-shell Almonds (Almond Liquid Extract) (Laux & Hühn, 2022) and Peanut Paste from Roasted in-shell Peanuts (Peanut Paste). This patent-protected technology is now ready to be licensed by nut processors and food companies. Such food ingredients fall under the EU Novel Food Regulation (EU) 2015/2283 (Ververis, 2020).

Given that nut shells lack a documented history of dietary consumption and considering the limited toxicological data available for shell-comprising ingredients, it is necessary to evaluate their safety through established genotoxicity and other testing. Almond and peanut shells consist largely of a lignocellulosic structure and are known to contain phenolic constituents, including flavonoids and condensed tannins like proanthocyanidins (Li et al., 2018; Queirós, 2020). Polyphenols and tannins are redox-active compounds that can exhibit both antioxidant and pro-oxidant behavior depending on their chemical structure, concentration, and environmental conditions. Under certain circumstances, including metal-catalyzed oxidation, these compounds may form electrophilic quinones and contribute to the generation of reactive oxygen species (ROS). Quinones and ROS have been shown in experimental systems to interact with DNA, leading to strand breaks, oxidative DNA damage, or adduct formation. In addition, lignin-rich plant materials may contain phenolic degradation products that warrant evaluation from a genotoxicity perspective (Bolton & Dunlap, 2017; Chedea et al., 2012; Stoeva et al., 2025).

From a regulatory standpoint, incorporating shell-comprising nut into food ingredients differs from traditional nut consumption and introduces compositional elements with potential biological activity. As such, and consistent with internationally accepted safety-assessment frameworks, the evaluation of genotoxic potential is a critical component of the safety assessment of in-shell nut derived food ingredients prior to market introduction. Conducting standard *in vitro* genotoxicity assays provides assurance that the combined shell, skin, and kernel matrix does not pose a genotoxic hazard under the intended conditions of use, thereby supporting the overall safety determination for these novel food ingredients (EFSA, 2024; Crebelli, 2025).

Accordingly, the genotoxic potential of these RE-NUT ingredients was assessed in this manuscript in compliance with OECD Test Guidelines 471 (Bacterial Reverse Mutation Test) (Ames, 1975) and 487 (*in vitro* Mammalian Cell Micronucleus Test) (Schmid, 1975). These two *in vitro* tests are aligned with “Tier 1” of the European Food Safety Authority’s approach to genotoxicity testing for novel food ingredients (EFSA, 2011).

These genotoxicity studies were conducted as part of a comprehensive safety assessment package supporting the use of shell-comprising nut ingredients in food applications, thereby establishing a scientific basis for their inclusion in sustainable, plant-based product formulations.

2. Material and Methods

2.1 Test Materials

The ingredients evaluated in this study were manufactured in Germany by three different partners: Almond Solids from Roasted in-shell Almonds (Almond Solids) by Jäckering Research GmbH, Almond Liquid Extract from Roasted in-shell Almonds (Almond Liquid Extract) by SIG Combibloc Systems GmbH and Peanut Paste from Roasted in-shell Peanuts (Peanut Paste) by ProXES Technology GmbH.

The production was carried out on behalf of RE-NUT and supervised by RE-NUT to ensure full compliance with the patents held by RE-NUT (Laux & Hühn, 2022). The ingredients generated and used for the study are RE-NUT property.

Dietary fibers, protein, ash, and fatty acids have been identified as major constituents by compositional analyses along with minor constituents including minerals, polyphenols and tannins. All three ingredients are soluble in water. The detailed analyses of the ingredients Almond Solids, Almond Liquid Extract and Peanut Paste are shown in Table 1.

Table 1. Specifications of almond solids, almond liquid extract and peanut paste and analytical methods

	Almond Solids		Almond Liquid Extract		Peanut Paste	
Compositional parameters	***Specification***	***Method***	***Specification***	***Method***	***Specification***	***Method***
Dietary fibers(%)	60-78	VIM (ASU L00.00-18,1997-01, [Ber.2017-10])	<4	VIM (ASU L00.00-18,1997-01, [Ber.2017-10])	14 to 35	AOAC 2011.25-M
Fat (%)	10-20	VIM	<12	VIM	40 to 70	VIM ; Gravimetry [Weibull-Stoldt]
Protein (%)	≤13	VIM (§64 LFGB L 06.00-7 [Stand 2014-08])	<4	VIM (§64 LFGB L 06.00-7 [Stand 2014-08])	<15	VIM (§64 LFGB L 06.00-7 [Stand 2014-08])
Water (%)	≤8	VIM (§64 LFGB L 06.00-3 [Stand 2014-08])	80-99	VIM (§64 LFGB L 06.00-3 [Stand 2014-08])	<2.0	VIM (§64 LFGB L 06.00-3 [Stand 2014-08])
Ash (%)	≤4	VIM (§64 LFGB L 06.00-4 [Stand 2014-10])	<1	VIM (§64 LFGB L 06.00-4 [Stand 2014-10])	<3.0	VIM (§64 LFGB L 06.00-4 [Stand 2014-10])
Heavy Metals						
Arsenic (mg/kg)	<0.1		<0.1		<0.1	
Lead (mg(kg)	<0.1		<0.1		<0.01	
Cadmium (mg/kg)	<0.05		<0.05		<0.05	
Mercure (mg/kg)	<0.01	DIN EN 15763:2010 (2010-04), modified	<0.01	DIN EN 15763:2010 (2010-04), modified	<0.01	DIN EN 15763:2010 (2010-04), modified
Microbiological Parameters						
Aerobic plate count (CFU/g)	<10 000	ISO 4833-1	<10 000	ISO 4833-1	<10 000	ISO 4833-1
Yeasts (CFU/g)	<10	ISO 21527-1-modified	<100	ISO 21527-1-modified	<100	ISO 21527-1-modified
Molds (CFU/g)	<100	ISO 21527-1-modified	<100	ISO 21527-1-modified	<100	ISO 21527-1-modified
Escherichia coli (CFU/g)	<10	ISO 16649-2-modified	<10	ISO 16649-2-modified	<10	ISO 16649-2-modified
Salmonella (CFU/25g)	Negative	AFNOR EGS 38/01-03/15	Negative	AFNOR EGS 38/01-03/15	Negative	AFNOR EGS 38/01-03/15
Listeria monocytogenes (CFU/25g)	Negative	AFNOR EGS 38/05-03/17	Negative	AFNOR EGS 38/05-03/17	Negative	AFNOR EGS 38/05-03/17
Aflatoxins (µg/kg)						
Aflatoxin B1	<0.1		<0.1		<0.1	
Aflatoxin B2	<0.1		<0.1		<0.1	
Aflatoxin G1	<0.1		<0.1		<0.1	
Aflatoxin G2	<0.1	DIN EN 14123 (2008-03), modified	<0.1	DIN EN 14123 (2008-03), modified	<0.1	DIN EN 14123 (2008-03), modified

AOAC = Association of Official Analytical Collaboration; AFNOR = Association Française de Normalisation; CFU = colony-forming units; DIN = Deutsches Institut für Normung (German Institute for Standardization); ISO = International Organization for Standardization; LFGB = Lebensmittel-, Bedarfsgegenstände- und Futtermittelgesetzbuch (the German Food and Feed Code); VIM = Validated Internal Method

2.2 Assays

The *in vitro* genotoxicity assays reported below were conducted under Good Laboratory Practice and in compliance with the internationally accepted guidelines. Almond Solids and Peanut Paste studies (OECD 471) were performed at Toxi- Coop Zrt., Arácsi út 97 and Ady E. utca 12, 8230 Balatonfüred, Hungary. The Almond Liquid Extract study (OECD 471) was performed at Charles River Laboratories, Hambakenwetering 7, 5231 DD 's-Hertogenbosch, The Netherlands. All OECD 487 studies, for Almond Solids, Almond Liquid Extract and Peanut Paste, were performed at Eurofins Medical Device Testing Munich GmbH, Robert-Koch-Strasse 3a, 82152 Planegg, Germany. All studies were notified to EFSA to support novel food application as per Regulation (EC) No 178/2002, Article 32b.

2.2.1 Bacterial Reverse Mutation Test (OECD TG 471)

The bacterial reverse mutation assays were conducted, employing a range of *Salmonella typhimurium* and *Escherichia coli* strains to evaluate potential mutagenic effects according to the OECD Test Guideline No 471, adopted 21st July 1997/ corrected 26th June 2020 (OECD, 2020); the ICH Guideline S2 (R1): "Genotoxicity testing and data interpretation for pharmaceuticals intended for human use", dated November 2011 and EPA Health Effects Test Guidelines, OPTTS 870.5100, EPA 712-C-98-247, August 1998.

The test concentrations of the ingredients for the bacterial reverse mutation test were based on solubility tests plus concentration range finding tests. No inhibitory effect of the three test ingredients was seen in the concentration range finding tests. For Almond Solids, triplicate concentrations of 5000, 1600, 500, 160, 50, 16, 5 and 1.6 µg/plate were freshly prepared in ultrapure water (ASTM Type 1). For Peanut Paste, the concentrations were from 5000 to 5 µg/plate and they were prepared using dimethyl sulfoxide (DMSO). To prepare triplicates of Almond Liquid Extract, concentrations of 5000, 1600, 512,164 and 52 µg/plate and 5000, 2800, 1568, 878 and 492 µg/plate were prepared in Milli-Q water in the initial and confirmatory mutation tests. Known mutagens (4-Nitro-o-phenylenediamine (NDP), Sodium azide (SAZ), 2-Aminoanthracene (2AA), Methyl methanesulfonate (MMS), 9-Aminoacridine (9AA), 4-nitroquinoline N-oxide (4-NQO), 2-nitrofluorene (2NF) were included in the assays as positive controls, specifically according to the different bacterial strains. All samples were tested with *S. typhimurium* tester strains TA98, TA100, TA1535, and TA1537, and with *E. coli* tester strain WP2 uvrA, both in the absence and presence of S9 liver microsomal fraction prepared from phenobarbital/5,6-benzoflavone-induced rats according to Matsushima. The colony numbers on the untreated, vehicle control, positive control and the test item treated plates were counted manually by unaided eye and when necessary, with a microscope at 40X magnification.

For each of the Almond Solids and Peanut Paste studies (Toxi-Coop), the initial experiment was conducted according to the standard plate incorporation method, with confirmatory assays conducted according to the pre-incubation method. For Almond Liquid Extract (Charles River), the initial experiment was conducted using the plate incorporation method, including a "treat and wash" procedure to limit potential interference from the possible presence of amino acids such as histidine and tryptophan in the test item. The confirmatory experiment used the plate incorporation method as well, including a "treat and wash" phase, but included a higher percent (10% vs 5%) v/v S9 mix. The two different confirmatory assays (the specifics of which vary by testing laboratory) are both in accordance with OECD Test Guideline 471.

The "Mutation Rate" was calculated by dividing the mean number of the revertants at the test item (or control) treatments by the mean number of revertants of the corresponding vehicle control. The biological relevance of the resulting data was assessed according to the criterion of the OECD guideline (OECD 471).

2.2.2 *In-vitro* Micronucleus Test (OECD TG 487)

The mammalian cell micronucleus test *in vitro* was performed using human peripheral blood lymphocytes from healthy donors to assess chromosomal damage according to the OECD Guideline for Testing of Chemicals Section 4, No 487 – "*In Vitro* Mammalian Cell Micronucleus Test, adopted 29 July 2016, corrected 4 July 2023" (OECD, 2023). For these assays, concentrations of in-shell nut ingredients were based on the results of preliminary dose selection tests. The concentration of 5000 µg/mL is the highest test concentration to be used in this test system following the recommendation of the corresponding OECD testing guideline 487.

As the ingredients demonstrate limited solubility and precipitates at concentrations below 5,000 µg/mL, the selection of the concentrations for evaluation was based on precipitation in each case. The highest concentration selected for micronucleus analysis was the lowest concentration at which precipitation occurs, provided that the precipitate does not interfere with cell scoring. The two immediately lower concentrations that did not result in precipitation were also evaluated.

For Almond Solids duplicate concentrations of 250, 500, 1000 *µ*g/mL for Almond Liquid Extract, duplicate concentrations of 312.5, 625 and 1250 *µ*g/mL, and for Peanut Paste duplicate concentrations of 50, 100 and 200 *µ*g/mL were incubated for 4 hours without and with metabolic activation with 48 h precultured lymphocytes. The metabolic activation was provided by the addition of phenobarbital/benzoflavone-induced Sprague Dawley rat liver S9 prepared with appropriate cofactors at a final concentration of 5% S9 in cultures tested with metabolic activation. Cells were incubated and harvested after 40 hours.

Similarly, in the long-term treatment assay 250, 500 and 1500 *µ*g/mL of Almond Solids, 78.15. 156.3, and 312.5 *µ*g/mL of Almond Liquid Extract, 50, 100 and 200 *µ*g/mL Peanut Paste were incubated, without metabolic activation, in duplicate for 44 hours up to harvest.

Methylmethanesulfonate (MMS, 50 µg/mL and 65 µg/mL) and Colchicine (0.015 *µ*g/mL and 0.4 *µ*g/mL) were used in experiments without metabolic activation (-S9) respectively as clastogenic and aneugenic positive controls. Cyclophosphamide (CPA, 12.5 and 15 *µ*g/mL) was used as clastogenic control in tests that included S9 metabolic activation (+S9). All are known to induce statistically significant increases in micronucleus frequency in this assay.

Once harvested, the cells were treated for fixation, and cell suspensions were dropped onto glass slides to be dried and then stained before micronuclei analysis, according to the criteria of Fenech (Fenech 2000). Micronuclei were scored blind in at least 2000 cells per concentration, either manually by trained technicians or using the semi-automated scoring Metafer System (Neon-Version: 1.3.8; Metafer-Version: 4.3.6) from Metasystems, Germany.

The Cytokinesis Block Proliferation Index (CBPI) was calculated to estimate cytoxicity. This index was determined from 500 cells of each culture, by counting mononucleate (c1), binucleate (c2) and multinucleate (c3), according to the following formula CBPI= (c1 x 1)+(c2 x 2)+ (c3x3) / n (total cell number).

The CBPI from treated and control cells were subsequently used to assess the % of cytotoxicity (cytostasis) which indicates the inhibition of cells growth in treated cultures in comparison to control cultures. The calculation $100 – 100 \times ((CBPI_T – 1) / (CBPI_C – 1))$ gives the % cytotoxicity, $CBPI_T$ being the Cytokinesis Block Proliferation Index of treated cultures and $CBPI_C$ being Cytokinesis Block Proliferation Index of control cultures.

2.3 Statistical Analysis

Statistical analyses were deemed unnecessary for the OECD 471 bacterial reverse mutation test, as indicated in the evaluation and interpretation of results chapter of the eponym guideline (OECD 471).

Regarding the OECD 487 *in vitro* mammalian cell micronucleus assays in human lymphocytes performed to assess chromosomal damage, the nonparametric χ^2 test was used to compare the number of micronucleated cells of each test group with the concurrent vehicle control group with statistical significance set at $p < 0.05$. The χ^2 Cochran-Armitage test for trend was used to examine concentration-related increases at a statistical significance level of 5% ($p < 0.05$, two-sided). Statistical methods were performed using the software GraphPad Prism version 6.

3. Results

3.1 The Bacterial Reverse Mutation Assays

The Bacterial Reverse mutation assay detects genotoxic compounds by evaluating the mutation rate occurring in bacterial genomes upon exposure to a test substance. For all three ingredients tested, validity of the performed experiments and controls were checked. The *S. typhimurium* and *E. coli* tester strains demonstrated the specific phenotype characteristics and were in conformity with the corresponding historical control data values, as were the negative (ultrapure water and Milli-Q, and DMSO for Peanut Paste, no increase) and positive (diagnostic mutagens, more than 3- fold increase) control conditions. Each S9 fraction used showed the appropriate biological activity.

In the Almond Solids experiment, the spontaneous revertant counts of the vehicle control (ultrapure water, ASTM Type I) were within the historical control ranges for all tester strains and experimental phases. The positive control mutagens produced the expected, biologically relevant (>3-fold) increases in revertant colonies, confirming assay validity. A slight deviation was noted for the sodium azide (SAZ) control in *S. typhimurium* TA1535 without metabolic activation (–S9), with a mean value of 462 revertant colonies per plate while the laboratory's (Toxi-Coop) historical lower limit is 467. This response is considered acceptable due to a 46-fold increase over the vehicle control. Revertant counts for untreated and DMSO controls were comparable to those of ultrapure water and within historical limits.

In the Almond Liquid Extract assays, the spontaneous revertant counts of the vehicle control (Milli-Q water) fell within the corresponding historical control ranges for all tester strains. Positive controls were consistent with historical control ranges, except for slight deviations in TA98 and TA1535 (–S9) in the initial test, which had no impact on study validity.

Similarly, in the Peanut Paste experiment, the spontaneous revertant counts of the vehicle control (DMSO) were within the historical control ranges for all tester strains and experimental phases. Positive control mutagens produced the expected, biologically relevant (>3-fold) increases in revertant colonies, confirming the validity of the assay. Revertant numbers for untreated and ultrapure water (ASTM Type I) controls were consistent with those of DMSO and remained within the historical control ranges.

In summary, for the three tested ingredients, the validity criteria of the Ames test were fulfilled. Vehicle, untreated, and positive control values were within the corresponding historical control data ranges, confirming the reliability of the test system and the adequate performance of the metabolic activation system (S9 mix).

It is noteworthy that none of the assays showed evidence of cytotoxicity, bacterial growth inhibition, or precipitation that may disturb the scoring, at any dose tested up to the maximum concentration identified for this test system (5000 µg/plate).

The results of the initial and confirmatory mutation assays with Almond Solids from Roasted in-shell Almonds (Almond Solids) are summarized in Table 2. No biologically relevant increases in revertant colony numbers were observed in any of the four *Salmonella typhimurium* and the *E. coli* tester strains at any tested concentration, neither in the presence nor absence of metabolic activation (S9 mix). Occasional slight increases were noted, but they lacked dose-response relationships and remained within the expected biological variability of the test system. The highest mean revertant count occurred in strain TA100 at 5000 µg/plate (–S9) during the confirmatory test, slightly exceeding the historical control range but remaining well below the threshold for a positive response (mutation rate = 1.72; threshold 2.00)). This isolated increase was attributed to natural variation.

In both assays testing the mutagenic activity of Almond Liquid Extract from Roasted in-shell Almonds (Almond Liquid Extract), no increases in revertant colonies were observed in any of the four *S. typhimurium* strains or in *Escherichia coli* at any concentration, neither with nor without metabolic activation (Table 3).

No biologically significant increases in revertant colony numbers were detected in any of the various *S. typhimurium* and *E. coli* strains treated with Peanut Paste from Roasted in-shell Peanuts (Peanut Paste), with or without metabolic activation (Table 4). Minor variations observed during testing were random, showed no concentration-related trend, and remained within the normal biological range. The highest mean revertant count was recorded in strain TA1535 at 16 µg/plate (–S9) during the initial assay (mutation rate= 1.95; threshold= 3.00), which remained within historical limits and far below mutagenic thresholds.

As none of the three test ingredients produced a concentration-dependent rise in revertant colony numbers, nor did they generate any reproducible or biologically meaningful increases at any concentration tested, no statistical analysis had to be carried out.

In conclusion, under the conditions of these studies, Almond Solids, Almond Liquid Extract and Peanut Paste ingredients showed no evidence of mutagenic activity in *S. typhimurium* or *E. coli* tester strains, indicating an absence of genotoxic potential in the bacterial reverse mutation assay according to OECD Guideline 471.

Table 2. Summary results of the genotoxicity tests with Almond Solids according to OECD Guideline 471. Mean values (Mean) and mutation rates (MR) of controls and Almond Solids at various concentrations in the bacterial reverse mutation assay with (+S9) and without (-S9) metabolic activation using various strains of *S. typhimurium* and *E. coli*

	***Salmonella typhimurium* tester strain**																***E coli* WP2*uvrA***			
	TA98				**TA100**				**TA1535**				**TA 1537**							
	-S9		**+S9**		**-S9**		**+S9**		**-S9**		**+S9**		**-S9**		**+S9**		**-S9**		**+S9**	
	Mean	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**
Initial Mutation Test																				
Negative controls																				
Untreated	16.7	0.93	25.3	0.93	82.0	0.76	80.7	0.96	12.3	1.23	9.3	0.78	7.0	1.11	11.7	1.35	52.0	1.26	47.3	1.15
DMSO	14.3	1.00	32.7	1.00	-	-	86.3	1.00	-	-	9.00	1.00	6.3	1.00	11.3	1.00	-	-	40.3	1.00
Ultrapure water	18.0	1.00	27.3	1.00	107.7	1.00	83.7	1.00	10.0	1.00	12.0	1.00	6.3	1.00	8.7	1.00	41.3	1.00	41.3	1.00
Positive controls	*183.0*	*12.77*	*1286.7*	*48.57*	*523.3*	*4.86*	*881.7*	*10.21*	*462.0*	*46.20*	*150.0*	*16.67*	*206.0*	*32.53*	*120.0*	*10.59*	*489.3*	*11.83*	*165.0*	*4.09*
Almond Solids (µg/plate)																				
5000	24.0	1.33	20.0	0.73	99.0	0.92	104.0	1.24	11.7	1.17	6.7	0.56	4.7	0.74	6.7	0.77	41.7	1.01	46.7	1.13
1600	16.0	0.89	17.7	0.65	107.3	1.00	88.0	1.05	10.3	1.03	10.3	0.86	7.0	1.11	6.7	0.77	49.3	1.19	41.0	0.99
500	17.0	0.94	21.0	0.77	76.6	0.71	91.3	1.09	7.7	0.77	10.0	0.83	7.0	1.11	7.3	0.85	49.7	1.20	45.7	1.10
160	25.0	1.39	19.0	0.70	86.0	0.80	86.0	1.03	8.3	0.83	13.7	1.14	5.0	0.79	8.0	0.92	47.0	1.14	49.7	1.20
50	20.0	1.11	20.3	0.74	88.0	0.82	80.0	0.96	9.3	0.93	13.0	1.08	7.3	1.16	8.0	0.92	45.3	1.10	51.3	1.24
16	20.7	1.15	19.3	0.71	82.0	0.76	76.0	0.91	13.7	1.37	9.7	0.81	8.0	1.26	9.3	1.08	45.3	1.10	48.7	1.18
5	24.7	1.37	20.0	0.73	86.0	0.80	78.3	0.94	10.0	1.00	10.0	0.83	5.3	0.84	9.0	1.04	34.0	0.82	48.7	1.18
1.6	15.7	0.87	20.7	0.76	83.7	0.78	84.7	1.01	14.3	1.43	13.0	1.08	8.0	1.26	9.3	1.08	43.7	1.06	50.0	1.21
Confirmatory Mutation Test																				
Negative controls																				
Untreated	11.7	0.74	16.3	1.17	69.3	0.81	70.7	0.91	8.7	0.76	11.3	0.89	6.0	0.72	9.7	0.85	30.3	0.71	43.7	1.00
DMSO	13.7	1.00	10.7	1.00	-	-	66.7	1.00	-	-	11.0	1.00	7.0	1.00	7.7	1.00	-	-	40.7	1.00
Ultrapure water	15.7	1.00	14.0	1.00	86.0	1.00	78.0	1.00	11.3	1.00	12.7	1.00	8.3	1.00	11.3	1.00	42,7	1.00	43.7	1.00
Positive controls	*207.0*	*15.15*	*1037.3*	*97.25*	*644.0*	*7.49*	*1005.3*	*15.08*	*686.0*	*60.53*	*98.3*	*8.94*	*437.3*	*62.48*	*158.3*	*20.65*	*845.3*	*19.81*	*207.3*	*5.10*
Almond Solids (µg/plate)																				
5000	17.0	1.09	20.3	1.45	147.7	1.72	103.3	1.32	14.7	1.29	12.3	0.97	7.0	0.84	11.3	1.00	43.7	1.02	48.3	1.11
1600	10.0	0.64	19.3	1.38	86.3	1.00	80.3	1.03	11.7	1.03	12.3	0.97	6.0	0.72	10.3	0.91	43.7	1.02	59.0	1.35
500	13.7	0.87	18.0	1.29	78.3	0.91	76.3	1.03	9.0	0.79	13.0	1.03	6.3	0.76	10.7	0.94	48.3	1.13	49.0	1.12
160	15.7	1.00	18.0	1.29	73.7	0.86	78.3	1.00	10.7	0.94	12.0	0.95	9.3	1.12	8.0	0.71	44.7	1.05	52.7	1.21
50	13.7	0.87	19.0	1.36	80.0	0.93	86.3	1.11	11.0	0.97	10.3	0.82	10.7	1.28	10.7	0.94	38.7	0.91	39.7	0.91
16	15.0	0.96	16.0	1.14	75.7	0.88	79.3	1.03	8.7	0.76	10.0	0.79	7.7	0.92	8.7	0.76	42.0	0.98	38.3	0.88
5	15.7	1.00	20.0	1.43	77.0	0.90	79.3	1.02	10.0	0.88	14.7	1.16	10.3	1.24	11.3	1.00	39.0	0.91	45.0	1.03
1.6	15.3	0.98	20.3	1.45	67.3	0.78	81.3	1.04	10.0	0.88	12.7	1.00	9.7	1.16	12.3	1.09	41.0	0.96	47.3	1.08

MR: Mutation Rate; NPD: 4-Nitro-o-phenylenediamine; SAZ: Sodium azide; 9AA: 9-Aminoacridine; MMS: Methyl methanesulfonate; 2AA: 2-Aminoanthracene; - : Not Applicable. Ultrapure water was applied as vehicle of the test item and the positive control substances SAZ and MMS. The DMSO was applied as solvent of the positive control substances NPD, 9AA and 2AA. The MR obtained at the test item, at the untreated control; furthermore, at SAZ and MMS refers to the ultrapure water. The MR obtained at NDP, 9AA and 2AA refers to DMSO. Positive controls were distributed as follow: NDP (-S9) and 2AA (+S9) for S. typhimurium TA98; SAZ(-S9) and 2AA (+S9) for S. typhimurium TA100 and TA1535; 9AA(-S9) and 2AA (+S9) for S. typhimurium TA1537; MMS (-S9) and 2AA(+S9) for E. coli WP2 uvrA

Table 3. Summary results of the genotoxicity tests with Almond Liquid Extract according to OECD Guideline 471. Mean values (Mean) and mutation rates (MR) of controls and Almond Liquid Extract at various concentrations in the bacterial reverse mutation assay with (+S9) and without (-S9) metabolic activation using various strains of *S. typhimurium* and *E. coli*

	***Salmonella typhimurium* tester strain**																***E coli* WP2*uvrA***			
	TA98				**TA100**				**TA1535**				**TA 1537**							
	-S9		**+S9**		**-S9**		**+S9**		**-S9**		**+S9**		**-S9**		**+S9**		**-S9**		**+S9**	
	Mean	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**
Initial Mutation Test																				
Negative controls																				
Milli-Q water	14.3	-	18.3	-	93.0	-	80.3	-	4.7	-	7.7	-	2.7	-	9.7	-	147.0	-	62.3	-
Positive controls	*1146.3*	*80.0*	*712.3*	*38.9*	*192.0*	*2.1*	*723.7*	*9.0*	*20.3*	*4.4*	*141.0*	*18.4*	*38.3*	*14.4*	*75.0*	*7.8*	*1735.3*	*11.8*	*123.3*	*2.0*
Almond Liquid Extract (µg/plate)																				
5000	23.0	1.6	26.7	1.5	115.0	1.2	97.7	1.2	9.0	1.9	9.0	1.2	3.7	1.4	10.3	1.1	155.3	1.1	68.7	1.1
1600	20.3	1.4	29.0	1.6	112.0	1.2	89.3	1.1	3.7	0.8	8.0	1.0	2.3	0.9	14.3	1.5	145.0	1.0	59.3	1.0
512	19.7	1.4	21.0	1.1	95.7	1.0	91.3	1.1	7.0	1.5	7.7	1.0	3.3	1.3	12.0	1.2	167.0	1.1	58.0	0.9
164	17.0	1.2	24.7	1.3	106.7	1.1	102.0	1.3	6.0	1.3	10.7	1.4	3.3	1.3	12.0	1.2	164.3	1.1	52.3	0.8
52	21.7	1.5	25.3	1.4	98.0	1.1	90.3	1.1	6.7	1.4	12.0	1.6	1.0	0.4	11.0	1.1	156.7	1.1	66.0	1.1
Confirmatory Mutation Test																				
Negative controls																				
Milli-Q water	18.0	-	22.3	-	109.0	-	80.0	-	8.3	-	17.0	-	4.3	-	9.0	-	215.7	-	58.3	-
Positive controls	*1002.0*	*55.7*	*292.7*	*13.1*	*240.3*	*2.2*	*705.0*	*8.8*	*38.0*	*4.6*	*105.3*	*6.2*	*35.0*	*8.1*	*41.0*	*4.6*	*1699.0*	*7.9*	*144.0*	*2.5*
Almond Liquid Extract (µg/plate)																				
5000	23.3	1.3	18.7	0.8	105.7	1.0	87.7	1.1	5.0	0.6	12.7	0.7	6.0	1.4	6.3	0.7	192.0	0.9	72.3	1.2
2800	24.7	1.4	29.7	1.3	105.7	1.0	105.7	1.3	15.3	1.8	17.3	1.0	3.0	0.7	5.0	0.6	146.7	0.7	70.7	1.2
1568	21.0	1.2	28.3	1.3	105.0	1.0	100.0	1.3	13.3	1.6	21.3	1.3	5.0	1.2	8.7	1.0	187.7	0.9	72.0	1.2
878	21.7	1.2	21.7	1.0	107.7	1.0	87.3	1.1	10.0	1.2	17.0	1.0	0.7	0.7	5.7	0.6	174.3	0.8	61.3	1.1
492	23.7	1.3	27.7	1.2	109.3	1.0	101.3	1.3	10.3	1.2	11.7	0.7	2.0	0.5	7.0	0.8	178.3	0.8	56.0	1.0

MR: Mutation Rate; SAZ: Sodium azide; 2-NF:2-nitrofluorene; MMS: Methyl methanesulfonate; 4-NQO: 4-nitroquinoline N-oxide; 2AA: 2-Aminoanthracene. The DMSO (not presented) was applied as solvent of the test item and the positive control substances SAZ, 2-NF, MMS; 4-NQO and 2AA. Positive controls were distributed as follow: 2NF (-S9) and 2AA (+S9) for S. typhimurium TA98; MMS(-S9) and 2AA (+S9) for S. typhimurium TA100, SAZ(-S9) and 2AA (+S9) for S. typhimurium TA1535; 2NF(-S9) and 2AA (+S9) for S. typhimurium TA1537 and 4-NQO (-S9) and 2AA(+S9) for E. coli WP2 uvrA

Table 4. Summary results of the genotoxicity tests with Peanut Paste according to OECD Guideline 471. Mean values (Mean) and mutation rates (MR) of controls and Peanut Paste at various concentrations in the bacterial reverse mutation assay with (+S9) and without (-S9) metabolic activation using various strains of *S. typhimurium* and *E. coli*

	***Salmonella typhimurium* tester strain**																			
	TA98				**TA100**				**TA1535**				**TA 1537**				***E coli* WP2*uvrA***			
	-S9		**+S9**		**-S9**		**+S9**		**-S9**		**+S9**		**-S9**		**+S9**		**-S9**		**+S9**	
	Mean	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**	**Mean**	**MR**
Initial Mutation Test																				
Negative controls																				
Untreated	14.0	1.17	22.3	1.08	71.3	1.16	75.7	1.15	7.0	1.11	8.7	0.84	8.7	1.04	6.3	0.83	30.7	1.00	35.0	1.04
DMSO	12.0	1.00	20.7	1.00	61.7	1.00	66.0	1.00	6.3	1.00	10.3	1.00	8.3	1.00	7.7	1.00	30.7	1.00	33.7	1.00
Ultrapure water	-	-	-	-	64.3	1.00	-	-	8.00	1.00	-	-	-	-	-	-	35.7	1.00	-	-
Positive controls	*352.7*	*29.39*	*1223.3*	*59.19*	*499.3*	*7.76*	*667.3*	*10.11*	*754.7*	*94.33*	*149.0*	*14.42*	*182.0*	*21.84*	*119.0*	*15.52*	*615.3*	*17.25*	*170.3*	*5.06*
Peanut Paste (µg/plate)																				
5000	11.7	0.97	19.0	0.92	74.0	1.20	63.7	0.96	7.3	1.16	10.0	0.97	6.7	0.80	9.0	1.17	32.3	1.05	34.0	1.01
1600	18.7	1.56	17.7	0.85	62.7	1.02	68.3	1.04	7.7	1.21	8.0	0.77	7.0	0.84	10.0	1.30	29.0	0.95	35.7	1.06
500	14.7	1.22	23.7	1.15	67.0	1.09	71.7	1.09	8.7	1.37	9.7	0.94	9.3	1.12	9.7	1.26	30.3	0.99	37.7	1.12
160	13.7	1.14	27.7	1.34	66.0	1.07	73.7	1.12	8.3	1.32	9.3	0.90	8.7	1.04	10.3	1.35	34.7	1.13	38.3	1.14
50	19.0	1.58	22.7	1.10	66.3	1.08	89.0	1.35	9.3	1.47	12.0	1.16	6.7	0.80	8.0	1.04	33.7	1.10	42.0	1.25
16	12.7	1.06	15.0	0.73	62.0	1.01	73.7	1.12	12.3	1.95	8.3	0.81	8.0	0.96	8.0	1.04	35.7	1.16	42.0	1.25
5	20.0	1.67	20.0	0.97	59.0	0.96	66.0	1.00	8.0	1.26	10.0	0.97	7.7	0.92	8.3	1.09	32.0	1.04	31.3	0.93
Confirmatory Mutation Test																				
Negative controls																				
Untreated	17.0	1.04	23.3	1.17	103.0	1.76	99.0	1.26	10.0	0.88	12.0	1.00	7.7	1.21	9.3	1.17	29.3	1.09	39.3	0.94
DMSO	16.3	1.00	20.0	1.00	58.7	1.00	78.3	1.00	11.3	1.00	12.0	1.00	6.3	1.00	8.0	1.00	27.0	1.00	42.0	1.00
Ultrapure water	-	-	-	-	75.3	1.00	-	-	11.3	1.00	-	-	-	-	-	-	35.0	1.00	-	-
Positive controls	*579.3*	*35.47*	*1262.7*	*63.13*	*632.7*	*8.40*	*641.3*	*8.19*	*795.3*	*70.18*	*171.0*	*14.25*	*430.7*	*68.00*	*104.3*	*13.04*	*1132.0*	*32.34*	*161.3*	*3.84*
Peanut Paste (µg/plate)																				
5000	15.7	0.96	18.0	0.90	71.7	1.22	59.0	0.75	10.7	0.94	9.7	0.81	5.7	0.89	6.0	0.75	39.3	1.46	40.3	0.96
1600	16.7	1.02	16.3	0.82	64.7	1.10	60.3	0.77	9.3	0.82	12.3	1.03	6.7	1.05	6.7	0.83	29.7	1.10	43.7	1.04
500	17.7	1.08	24.7	1.23	68.0	1.16	87.3	1.11	8.3	0.74	13.0	1.08	7.3	1.16	6.3	0.79	29.7	1.10	43.7	1.04
160	16.3	1.00	21.7	1.08	64.7	1.10	79.3	1.01	10.7	0.94	13.3	1.11	8.0	1.26	6.7	0.83	30.7	1.14	39.0	0.93
50	15.0	0.92	22.7	1.13	50.3	0.86	73.3	0.94	11.3	1.00	10.3	0.86	7.3	1.16	7.0	0.88	24.0	0.89	39.0	0.93
16	15.0	0.92	19.0	0.95	60.7	1.03	71.7	0.91	10.7	0.94	11.7	0.97	7.0	1.11	10.0	1.25	24.3	0.90	45.0	1.07
5	17.3	1.06	22.0	1.10	62.0	1.06	69.3	0.89	8.0	0.71	15.7	1.31	7.3	1.16	8.7	1.08	24.0	0.89	40.3	0.96

MR: Mutation Rate; DMSO. Dimethyl sulfoxide; NPD: 4-Nitro-o-phenylenediamine; SAZ: Sodium azide; 9AA: 9-Aminoacridine); MMS: Methyl methanesulfonate); 2AA: 2-Aminoanthracene; -: Not Applicable. DMSO was applied as vehicle of the test item and the positive control substances NPD, 9AA and 2AA. The Ultrapure water was applied as vehicle of the positive control substances SAZ and MMS. The mutation rate obtained at the test item, at the untreated control; furthermore, at NDP, 9AA and 2AA refers to the DMSO. The mutation rate obtained at SAZ and MMS refers to ultrapure water. Positive controls were distributed as follow: NDP (-S9) and 2AA (+S9) for S. typhimurium TA98; SAZ(-S9) and 2AA (+S9) for S. typhimurium TA100 and TA1535; 9AA(-S9) and 2AA (+S9) for S. typhimurium TA1537; MMS (-S9) and 2AA(+S9) for E. coli WP2 uvrA

3.2 The *In-vitro* Mammalian Cell Micronucleus Test Using Human Lymphocytes

The test ingredients, Almond Solids, Almond Liquid Extract, and Peanut Paste were investigated in the OECD 487 test for a possible potential to induce micronuclei in human lymphocytes in the absence and presence of metabolic activation.

Within all three test ingredients, the negative control cultures gave expected results and were within the historical control ranges for the testing facility. For Almond Solids, the micronucleated cell frequencies of the negative controls were within historical limits in the 4-h and 44-h without metabolic activation (-S9) treatments, whereas one value (1.10%) in the 4-h with metabolic activation treatment (+S9) was slightly above the upper limit defined as 1.04%, but it was still considered acceptable for inclusion (Table 5). For Almond Liquid Extract, all negative control frequencies in 4h (+/-S9) and 44-h in absence of S9 fell within their respective historical control ranges (Table 6). Similarly, for Peanut Paste, the negative control frequencies were consistently within the historical ranges across the 4-h (±S9) and the 44-h (-S9) treatments (Table 7).

The positive controls gave the expected results in all assays. MMS (50 and 65 µg/mL) and CPA (12.5–15 µg/mL) produced clear and statistically significant increases in micronucleus frequency, respectively (2.10 - 4.90%) and (2.20 - 2.50%) respectively for MMS and CPA. demonstrating their clastogenic activity. Colchicine, used at 0.015 and 0.4 µg/mL as an aneugenic control, also induced robust and statistically significant MN responses, from 1.65 to 4.65%. The magnitude and consistency of these increases confirm the responsiveness of the test systems and the proper functioning of the metabolic activation system where applicable.

As such, the results of both negative and positive controls validate the reliability of the *in vitro* micronucleus assays conducted for all three test ingredients.

Across all experimental conditions, Almond Solids did not induce cytotoxicity at levels that would compromise the interpretation of chromosomal damage (Table 5). In the 4-h treatment in absence of metabolic activation (-S9), cytostasis remained below the 30% threshold established by the testing laboratory up to 250 µg/mL. It should be noted that the OECD 487 guideline does not define a threshold for cytotoxicity. At higher concentrations (500–1000 µg/mL), moderate cytostasis was observed, with values ranging from 35% to 47%. In the 44-h without S9, a similar pattern was seen, with cytostasis remaining below or only moderately above the 30% at the 500 and 1500 µg/mL doses. In presence of S9, in the 4-h treatment, cytostasis clearly stayed below 30%. The higher levels observed were considered to remain within the acceptable range defined by OECD 487 for evaluating genotoxic potential (maximum of 55 +/- 5% cytotoxicity /cytostasis).

In any experiment testing Almond Solids, no treatment-related increase in micronucleated cells was detected. In the 4-h without S9, micronucleus frequencies ranged from 0.40% to 0.55% through the tested concentrations, all within historical negative-control limits. With S9 activation and 4 h exposure, micronucleus frequencies were 0.45–0.60%, again consistent with expected background variation. The 44-h without S9 treatment confirmed the absence of clastogenic or aneugenic effects, with values between 0.50% and 0.90%, comparable to the concurrent control.

Almond Liquid Extract produced no evidence of cytotoxicity exceeding the threshold compatible with reliable genotoxicity assessment (Table 6). Cytostasis remained below 30% in the 4- and 44-h treatment, regardless of metabolic activation, indicating that all evaluated concentrations were appropriate for interpreting micronucleus formation.

Micronucleus frequencies following Almond Liquid Extract exposure did not deviate from historical controls. In 4-h without S9 exposure, frequencies varied from 0.55% to 0.90%, all within the established historical interval. In 4-h with S9 activation, frequencies were ranged from 0.55 to 1.05%; only the highest concentration, 1250 µg/mL showed a slight elevation, 1.05%, above the upper historical bound set at 1.04%. However, this increase lacked statistical significance and was therefore considered biologically irrelevant. The 44-h exposure in absence of S9 further supported the absence of genotoxic activity, with micronucleus frequencies (0.40–0.80%) falling well within historical variation.

No excessive cytotoxicity was observed with Peanut Paste in any experimental condition

(Table 7). Cytostasis remained below the 30% threshold in the 4- and 44-h, both with and without S9, indicating that all concentrations tested were suitable for assessing genotoxic potential under OECD 487.

Peanut Paste exposure did not induce micronucleus formation in the tested conditions. In 4-h without S9 treatment, micronucleus frequencies ranged from 0.20% to 0.45%, values that were within historical negative-control ranges. In presence of S9, frequencies of 0.45 – 0.65% were observed, again consistent with normal background variability. 44-h treatment without S9 results mirrored these findings: micronucleus frequencies following treatment (0.45 –0.65%) remained within historical limits.

In summary, across all three test ingredients and experimental conditions, statistical analyses supported the descriptive interpretation of the micronucleus data. For Almond Solids, Almond Liquid Extract, and Peanut Paste, the non-parametric χ^2 test revealed no statistically significant elevation ($p < 0.05$) in the frequency of micronucleated cells at any concentration, neither in the presence nor absence of metabolic activation.

An additional statistical analysis set was performed for testing the hypothesis of concentration dependencies, through the χ^2 Cochran-Armitage test, on each series of doses, in each experiment, and for each of the three ingredients. A positive trend, i.e. statistically significant concentration-related increase in micronucleated cells frequency, was defined as $p < 0.05$.

This specific χ^2 test for trend demonstrated no evidence of a dose-related increase in micronucleus formation for any of the three ingredients (Table 8). These findings confirm that the slight fluctuations observed among individual dose groups reflected normal biological variability of the test system rather than treatment-related effects.

Table 5. Summary results of the genotoxicity test with Almond Solids according to OECD Guideline 487. Cytotoxicity (Cytostasis) Index, Relative Cell Growth, Micronuclei (MN) frequencies of controls and various Almond Solids concentrations

Treatment	Concentration (µg/mL)	Metabolic activation S9 or +S9	Number of cells evaluated	Cytotoxicity (Cytostasis) (%)	Relative cell growth (%)	MN frequency (%)	Historical control limits Negative control
4-hour treatment							
Culture medium	0		2000	0	100	0.90	
Almond Solids	250	-S9	2000	1	99	0.40	
Almond Solids	500	-S9	2000	47	53	0.55	C: 0.07% - 1.00 %
Almond Solids	1000	-S9	2000	35	65	0.40	
MMS	65	-S9	2000	17	83	2.10*	
Colchicine	0.4	-S9	2000	42	58	3.30*	
Culture medium	0	+S9	2000	0	101	1.10	
Almond Solids	250	+S9	2000	0[a]	148	0.45	
Almond Solids	500	+S9	2000	9	91	0.60	C: 0.06% - 1.04%
Almond Solids	1000	+S9	2000	6	94	0.45	
CPA	15	+S9	2000	0[a]	110	2.20*	
44-hour treatment							
Culture medium	0	-S9	2000	0	100	0.90	
Almond Solids	250	-S9	2000	23	77	0.90	
Almond Solids	500	-S9	2000	38	62	0.90	C: 0.1% - 1.03%
MMS	50	-S9	2000	35	65	3.80*	
Colchicine	0.015	-S9	2000	64	36	2.15*	

*Culture medium, RPMI1640, was applied as solvent of the test item and the positive control substances: methylmethanesulfonate (MMS), colchicine and cyclophosphamide (CPA). Relative Cell Growth : 100 x ((CBPI test conc-1)/(CBPI control-1)), Cytotoxicity (Cytostasis) = 100 - Relative Cell Growth (%), *: significant increase compared to negative control (χ^2 test, $p<0.05$), [a]: the cytotoxicity (cytostasis) is defined as 0, when the relative cell growth exceeds 100%*

Table 6. Summary results of the genotoxicity test with Almond Liquid Extract according to OECD Guideline 487. Cytotoxicity (Cytostasis) Index, Relative Cell Growth, Micronuclei (MN) frequencies of controls and various Almond Liquid Extract concentrations

Treatment	Concentration (µg/mL)	Metabolic activation S9 or +S9	Number of cells evaluated	Cytotoxicity (Cytostasis) (%)	Relative cell growth (%)	MN frequency (%)	Historical control limits Negative control
4-hour treatment							
Culture medium	0		2000	0	100	0.75	
Almond Liquid Extract	312.5	-S9	2000	0[a]	109	0.90	
Almond Liquid Extract	625	-S9	2000	0[a]	104	0.75	C: 0.07% -
Almond Liquid Extract	1250	-S9	2000	0[a]	108	0.55	1.00 %
MMS	65	-S9	2000	0[a]	106	3.85*	
Colchicine	0.4	-S9	2000	67	33	2.65*	
Culture medium	0	+S9	2000	0	100	0.55	
Almond Liquid Extract	312.5	+S9	2000	0[a]	115	0.70	
Almond Liquid Extract	625	+S9	2000	5	95	0.55	C: 0.06% -
Almond Liquid Extract	1250	+S9	2000	0[a]	104	1.05	1.04%
CPA	12.5	+S9	2000	36	64	2.20*	
44-hour treatment							
Culture medium	0	-S9	2000	0	100	0.75	
Almond Liquid Extract	78.15	-S9	2000	0[a]	123	0.60	
Almond Liquid Extract	156.3	-S9	2000	11	89	0.80	C: 0.10% -
Almond Liquid Extract	312.5	-S9	2000	0[a]	132	0.40	1.03%
MMS	50	-S9	1274	37	63	3.76*	
Colchicine	0.015	-S9	2000	61	39	1.65*	

*Culture medium, RPMI1640, was applied as solvent of the test item and the positive control substances: methylmethanesulfonate (MMS), colchicine and cyclophosphamide (CPA). Relative Cell Growth: 100 x ((CBPI test conc-1)/(CBPI control-1)), Cytotoxicity (Cytostasis) = 100 - Relative Cell Growth (%), *: significant increase compared to negative control (χ^2 test, $p<0.05$), [a]: the cytotoxicity (cytostasis) is defined as 0, when the relative cell growth exceeds 100%*

Table 7. Summary results of the genotoxicity test with Peanut Paste according to OECD Guideline 487. Cytotoxicity (Cytostasis) Index, Relative Cell Growth, Micronuclei (MN) frequencies of controls and various Peanut Paste concentrations

Treatment	Concentration (µg/mL)	Metabolic activation S9 or +S9	Number of cells evaluated	Cytotoxicity (Cytostasis) (%)	Relative cell growth (%)	MN frequency (%)	Historical control limits Negative control
4-hour treatment							
Culture medium	0		2000	0	100	0.65	
Peanut Paste	50	-S9	2000	0[a]	102	0.45	
Peanut Paste	100	-S9	2000	0[a]	118	0.25	C: 0.07% -
Peanut Paste	200	-S9	2000	0[a]	104	0.20	1.00 %
MMS	65	-S9	2000	0[a]	121	4.90*	
Colchicine	0.4	-S9	2000	62	38	4.65*	
Culture medium	0	+S9	2000	0	100	0.30	
Peanut Paste	50	+S9	2000	5	95	0.65	
Peanut Paste	100	+S9	2000	0[a]	110	0.45	C: 0.06% -
Peanut Paste	200	+S9	2000	0[a]	104	0.50	1.04%
CPA	12.5	+S9	2000	13	87	2.50*	
44-hour treatment							
Culture medium	0	-S9	2000	0	100	0.25	
Peanut Paste	50	-S9	2000	0[a]	121	0.65	
Peanut Paste	100	-S9	2000	12	88	0.45	C: 0.10% -
Peanut Paste	200	-S9	2000	0[a]	101	0.55	1.03%
MMS	50	-S9	2000	37	63	4.70*	
Colchicine	0.015	-S9	2000	57	43	1.90*	

*Culture medium, RPMI1640, was applied as solvent of the test item and the positive control substances: methylmethanesulfonate (MMS), colchicine and cyclophosphamide (CPA). Relative Cell Growth: 100 x ((CBPI test conc-1)/(CBPI control-1)), Cytotoxicity (Cytostasis) = 100 - Relative Cell Growth (%), *: significant increase compared to negative control (χ^2 test, $p<0.05$), [a]: the cytotoxicity (cytostasis) is defined as 0, when the relative cell growth exceeds 100%*

Table 8. Summary results of the χ² Cochran-Armitage testing concentration-related increase in micronucleated cells frequency

Genotoxicity OECD 487 test	Treatment Time [h]	Almond Solids P value	Almond Liquid Extract P value	Peanut Paste P value
Without metabolic activation (- S9)	4	0.7155	0.4286	0.3103
With metabolic activation (+ S9)	4	0.7397	0.1772	0.6647
Without metabolic activation (- S9)	44	0.2461	0.2615	0.6937

Collectively, the statistical evaluations corroborate the absence of clastogenic or aneugenic activity for all three shell comprising nut ingredients, Almond Solids, Almond Liquid Extract and Peanut Paste, under the experimental conditions of the *in vitro* micronucleus assay according to OECD Guideline 487.

4. Discussion

The present studies were conducted to evaluate the genotoxic potential of three shell comprising nut ingredients—Almond Solids, Almond Liquid Extract, and Peanut Paste—produced using the patented RE-NUT in-shell processing technologies. These ingredients represent a new category of shell-containing nut fractions with no established history of dietary use. The studies presented are the first *in vitro* genotoxic studies reported on in-shell nut ingredients.

The results of the bacterial reverse mutation tests (OECD 471) provide clear evidence that none of the three products induce gene mutations in *S. typhimurium* or *E. coli* tester strains. All assay validity criteria were satisfied, including expected performance of the positive and negative controls, adequate strain functionality, and proper metabolic activation. In all the tested concentrations, revertant colony numbers showed no biologically meaningful increases, whereas an occasional minor fluctuation was consistent with natural assay variability. The bacterial reverse mutation assay was conducted following recognized international standards, employing a range of *S. typhimurium* and *E. coli* strains well documented to evaluate potential mutagenic effects. This test showed no induction of gene mutations neither in the presence nor in the absence of metabolic activation.

These findings demonstrate the absence of mutagenic activity in a highly sensitive screening system and contribute to the evidence that supports the genotoxic safety of these three shell-comprising nut ingredients for potential use in food applications.

The complementary evaluations using the *in vitro* micronucleus assay in human lymphocytes (OECD 487) further support the lack of genotoxicity. For all three ingredients, micronucleated cell frequencies remained within historical control intervals through all exposure conditions. No statistically significant increases were observed, and no concentration-related trends emerged. Cytostasis values remained within the recommended ranges for reliable interpretation, confirming that the doses tested were appropriate and not confounded by excessive cytotoxicity. The expected robust responses of clastogenic and aneugenic controls demonstrated the sensitivity of the test system. Collectively, these results show that Almond Solids, Almond Liquid Extract, and Peanut Paste do not induce chromosomal damage or aneugenicity under the conditions of the assays.

Together, the outcomes of the two independent genotoxicity assays form a consistent and coherent body of evidence indicating that the three studied shell-comprising nut ingredients lack mutagenic, clastogenic, or aneugenic potential. This integrated assessment aligns with the toxicological profiles broadly recognized for edible nut components and provides the essential safety data needed for shell-comprising fractions, which have only a limited, if any, history of consumption. These findings therefore contribute meaningfully to the scientific basis required for a GRAS determination and/or an EFSA Novel Food assessment, supporting the conclusion that the tested ingredients do not present a genotoxic hazard under their intended conditions of use.

To our knowledge, this work represents the first systematic assessment of genotoxic potential for shell-comprising nut ingredients. The consistently negative results obtained across the *in vitro* genotoxicity assays indicate no genotoxic activity for the tested ingredients under the conditions evaluated. In line with internationally accepted tiered testing strategies, these findings support a Tier 1 conclusion of no genotoxic concern, with no further genotoxicity testing warranted. This outcome provides important support for the regulatory safety assessment of in-shell nut

ingredients. It is worth to note that for a close but different product, Pecan shell fiber, these same tests have also demonstrated an absence of genotoxicity in another type of shell nut (U.S. FDA, 2016).

5. Conclusion

The genotoxic potential of Almond Solids, Almond Liquid Extract, and Peanut Paste produced using the patented RE-NUT process was systematically evaluated using two internationally recognized *in vitro* systems, adhering to OECD Test Guidelines 471 and 487. Under the experimental conditions applied, none of the tested ingredients induced gene mutations in bacteria or chromosomal damage in human lymphocytes, either in the presence or absence of metabolic activation. These results provide consistent and complementary evidence supporting the absence of genotoxic concern for the tested ingredients. In conclusion, the results reinforce the assumption that the three nut ingredients containing shells are non-genotoxic *in vitro*. Since ingredients that come from nut fractions have no history of consumption in human diets, the current studies offer crucial Tier 1 genotoxicity information according to EFSA guidelines for assessing the safety of novel foods. The information provided here creates a strong scientific foundation for the ongoing toxicological assessment of in-shell nut components and endorses their safe application in food products when used as intended.

Disclaimer (Artificial Intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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Competing Interests

Roland Laux and Christian Zimmermann are employees of RE-Nut AG; Tatiana Avellaneda was an employee of Re-Nut AG at the time of the study. Tilo Hühn is a named co-inventor on patents related to the in-shell nut processing technologies evaluated in this study and is affiliated with a university institution. The university affiliation did not influence the design, conduct, interpretation, or reporting of the studies. The other authors declare no conflict of interest.

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