# Ames Test to Detect Mutagenicity of 2-Alkylcyclobutanones: A Review

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**Abstract:** Food irradiation is an effective and safe method for preservation and long-term storage, and it is approved for use in over 60 countries for various applications in a wide variety of food products. This process is performed by use of accelerated electron beams, X-rays, or gamma radiation ($^{60}$Co or $^{137}$Cs). 2-Alkylcyclobutanones (2-ACBs) are the only known radiolytic products generated from foods that have fatty acids (triglycerides) and are subjected to irradiation. Since the 1990s toxicological safety studies of 2-ACBs have been conducted extensively through synthetic compounds, then and tests to determine if the compounds have any mutagenic activity are strictly necessary. The Ames test was chosen by many researchers to assess the mutagenicity of 2-ACBs. The test uses distinct bacterial cell lines *Salmonella typhimurium* to detect point mutations at sites guanine–cytosine (G–C) and *Escherichia coli* to detect point mutations at sites adenine–thymine (A–T). This bibliographic research aims to bring together all the results obtained and a comparison and cell lines used, type of plates, and solvents. This research showed that no mutagenic activity was observed in any of the cell lines and concentrations evaluated by the works of authors, so the 2-ACBs compounds showed no mutagenic substance in concentrations detectable by the Ames test.

**Keywords:** 2-ACB, 2-alkylcyclobutanone, Ames test, food irradiation, mutagenicity

## Introduction

### Food irradiation

When we start discussing about food preservation and storage, we can find some methodologies to do it. Physical treatments can be performed by conservation in cold (refrigeration and freezing) and heat (cooking, blanching, pasteurization, and sterilization), but may induce adverse effects on the taste and texture of food (Agencia Embrapa de Informação Tecnológica; Chen and others 2016). Chemical treatments made with antioxidants and preservatives that act directly to protect against spoilage of foodstuffs but present health-damaging toxicity and/or mutagenicity (Shibamoto and Bjeldanes 2014). It has been shown that most of these chemicals used in the preservation and storage of food products have shown toxicity and can be carcinogenic or environmentally harmful. Many of these chemicals have been banned and many countries have had to limit or stop exports, resulting in economic losses, trade imbalances, thus making food irradiation the best choice to store safely and effectively (Ihsanullah and Rashid 2016).

Food irradiation presents itself as a secure method for preservation and long-term storage. The benefits of food irradiation have resulted in a number of practical advantages that include versatility, broad spectrum effective against bacteria and pests, is pervasive (foods are treated in their final packaging, target organisms are not protected by the fact that the product is packed, And the treatment of the products in pallets is possible), solid and raw foods can be treated, the treatment does not involve products or chemical residues, the process is relatively easy to control (usually only depends on the transport speed and the feeding/activity of the source of radiation), and the food can be readily distributed in the food chain after treatment (Calucci and others 2003; Roberts 2014).

Since the 1980s, food irradiation has been extensively studied and increasingly being used as an effective method to improve quality and maintain a fundamental and crucial point, which is to maintain food safety integrity and nutritional standards, thus maintaining collective health. The technology is being used in more than 60 kinds of food in more than 40 countries worldwide, and is perhaps the most studied food processing on the toxicological safety in the history of food preservation (Delincée 2002; Ehlermann 2009; Song and others 2014).

The food irradiation processing is performed by using accelerated electron beams, X-rays, or gamma radiation ($^{60}$Co or $^{137}$Cs). Ionizing radiation induces the formation of a variety of hydrocarbons, and 2-Alkylcyclobutanones (2-ACBs) (Kim and others 2004). Treatment performed by the radiation when used for a purpose of reducing the microbial burden inhibit cell division in microorganisms, thereby promoting molecular structural change, then once absorbed by the biological material, the gamma radiation develops a direct and indirect impact on the material that received this treatment, thus performing disinfection function (Fanaro and others 2014).

The main concern regarding the consumption of irradiated food is if 2-ACB may promote cancer. There are reports of toxicity by the preliminary evidence to the possible genotoxic effects of 2-ACBs (Delincée and Pool-Zobel 1998). Complete studies of the possible effects of 2-ACBs in irradiated foods and its mechanism (s) of action to promote some tumor effects are extremely needed to respond public health concerns. The in-depth investigation of the effect of 2-ACB at levels consumed by the human population and models (*in vitro* and *in vivo*) of various types of cancers in different tissues is of fundamental importance before proposing that irradiated foods may or may not promote cancer colon or any other type of cancer (Chinthalapally 2003). This survey data related to the results of studies conducted by Ames test to check possible mutagenic effect are very relevant, as these results serve to clarify any questions concerning this effect and also contributes to

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**Table 1–Types 2-ACBs generated during irradiation.**

| | Fatty acid | 2-ACB generated | Initials | Food |
|---|---|---|---|---|
| C 10:0 | Capric acid | 2-hexyl-cyclobutanone | 2-HCB | Beef; butter; coconut |
| C 12:0 | Lauric acid | 2-octyl-cyclobutanone | 2-OCB | Coconut oil |
| C 14:0 | Myristic acid | 2-decyl-cyclobutanone | 2-DCB | Animal fats; coconut oil; milk |
| C 16:0 | Palmic acid | 2-dodecyl-cyclobutanone | 2-dDCB | Animal fats; palm oil |
| C 16:1 | Palmitoleic acid | 2-(dodec-5′-enyl)-cyclobutanone | 2-dDeCB | Macadamia oil |
| C 18:0 | Stearic acid | 2-tetradecyl-cyclobutanone | 2-tDCB | Animal fats and vegetable |
| C 18:1 | Oleic acid | 2-(tetradec-5′-enyl)-cyclobutanone | 2-tDeCB | Olive oil; olives; avocados |
| C 18:2 | Linoleic acid | 2-(tetradeca-5′,8′-dienyl)-cyclobutanone | 2-tD2eCB | Vegetable oils |
| C 18:2 | Linolenic acid | 2-(tetradeca-5′8′11′-trienyl)-cyclobutanone | 2-tD3eCB | Fish oil |

Adapted from Sommers and others (2007).

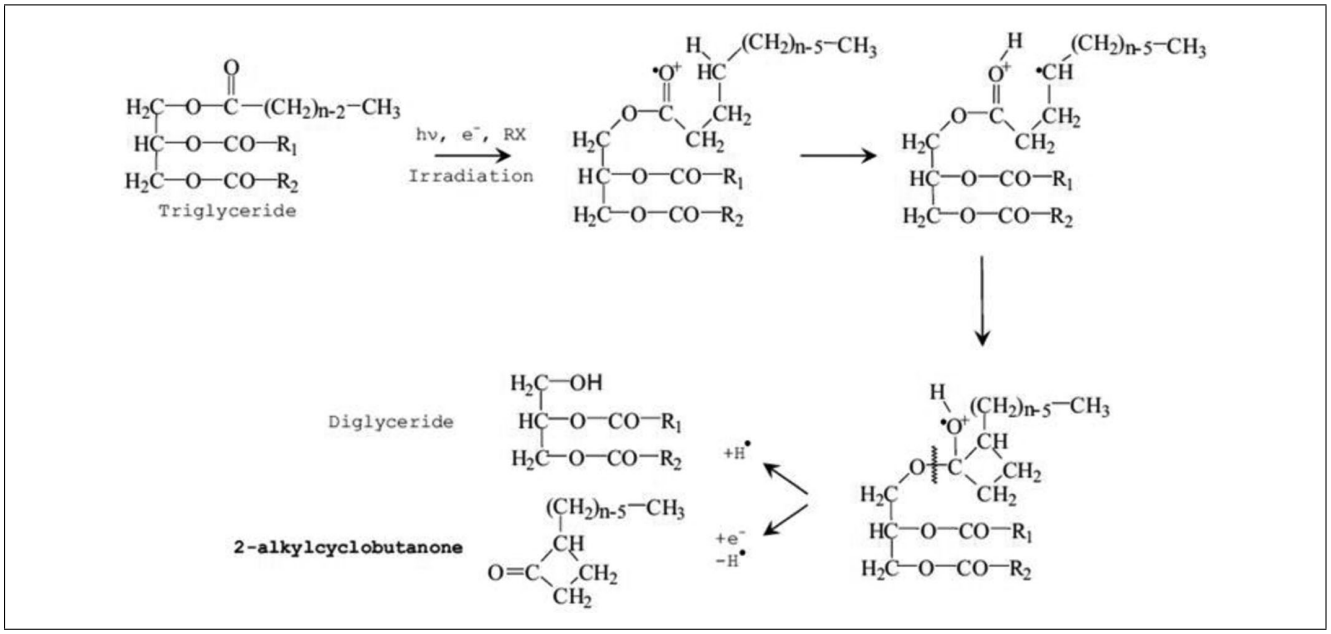


Figure 1–Formation of 2-alkylcyclobutanone from a triglyceride by irradiation. From: (LeTellier and Nawar, 1972).

the necessary studies required by regulatory agencies to regulate norms and standards for processed foods for radiation.

## Formation of 2-ACBs

The processed food with ionizing radiation induces the formation of various by-products such as free radicals, hydrocarbons, and 2-ACBs that are generated as a result of radiolysis triglycerides, phospholipids, and fatty acids present in the food (Kim and others 2004; Sommers and others 2004). It is reported the 1st time by Letellier and Nawar (1972). The formation of 2-ACBs was related to the lipid content contained in the food, as well as the dose that this food receives, the higher the radiation dose the greater the formation of hydrocarbons and 2-ACBs. The Table 1 summarizes the different types of 2-ACBs that can be formed and fatty acids from which they are derived.

2-ACBs are cyclic compounds formed by loss of 1 electron of oxygen over a carbonyl of fatty acid or triglycerides, followed by a rearrangement process that consequently produces specific 2-ACBs for each irradiated fatty acid (Figure 1). The resulting compounds have the same number of carbon atoms of the fatty acid precursor with an alkyl group linked to ring in the position 2 (Letellier and Nawar 1972; Gadgil and others 2002; Song and others 2014).

The 4 major fatty acids contained in most foods are: palmitic, stearic, oleic, and linoleic acid. When exposed to radiation, these acids are converted into their corresponding alkylcyclobutanones (Gadgil and others 2002).

## Ames Test

The Ames test was developed by Dr. Bruce Ames and colleagues in the 1970s and reviewed by Maron and Ames (1983). It is a test performed *in vitro* in short term to evaluate possible mutagenic effects caused by chemicals (Tagliari and others 1999).

Mutagenicity test of chemical compounds with *Salmonella typhimurium* and/or *Escherichia coli* cells are based on the knowledge that a substance that is mutagenic in the bacterium may subsequently be carcinogenic in laboratory animals and thus presents a risk of cancer for humans. The ease and speed (usually results in 3 to 4 wk) and low cost of the test make it an important tool for the screening of substances with possible carcinogenicity potential (Maron and Ames 1983).

Several cell lines of *S. typhimurium* bacteria can be used for testing. Each one is genetically different, so using multiple strains in a test raises the chance of detecting a chemical product mutagen. The most frequently used cell lines are TA97, TA98, TA100, TA102, TA104, TA1535, TA1537, and TA1538. In addition to

**Table 2–Studies performed to detect possible changes in the compounds of 2-ACBs using the Ames test.**

| Author/year | 2-Alcylcyclobutanone | Doses used | Cell line | Solvent | Results |
|---|---|---|---|---|---|
| Burnouf and others (2002) | 2-DCB<br>2-dDCB<br>2-tDCB | 0.4 $\mu$M to 1 $\mu$M to 2 $\mu$M to 4 $\mu$M to 40 $\mu$M<br>4 $\mu$M to 40 $\mu$M<br>4 $\mu$M to 40 $\mu$M | TA 97, TA 98, and TA 100. | Ethanol | Negative |
| Sommers and Schiestl (2003) | 2-DCB | 0.0 to 0.5 mg to 0.1 mg 2 to DCB 0.5 mg, 1.0 mg 130 $\mu$g MMS | WP2 (pKM101), WP2 uvra (pKM101) | NI | Negative |
| Sommers and Schiestl (2004) | 2-DCB | 0.05 mg to 0.10 mg to 0.50 mg to 1.0 mg/well | TA98, TA100, TA1535, TA1537 | DMSO | Negative |
| Gadgil and Smith (2004) | 2-DCB | 0.25 $\mu$M to 0.5 $\mu$M to 0.75 $\mu$M to 0.1 $\mu$M | TA97, TA98, TA100, TA102, TA1535 | DMSO | Negative |
| Hartwig and others (2007) | 2-DCB; 2-dDCB; 2-tDCB | (2-DCB 0.4 $\mu$M to 1 $\mu$M to 2 $\mu$M to 4 $\mu$M)<br>(2-dDCB 4 $\mu$M to 40 $\mu$M) (2-tDCB 40 $\mu$M to 400 $\mu$M) | TA97, TA98, TA100 | Ethanol | Negative |
| Yamakage and others (2014) | 2-dDCB | 19.5, 39.1, 78.1, 156, 313, 625, 1250, 2500, and 5000 $\mu$L | TA98, TA100, TA1535, TA1537, WP2 *uvra* | Ethanol | Negative |
| | 2-tDCB | 78.1, 156, 313, 625, 1250, 2500, and 5000 $\mu$L | | | |

DMSO, dimethyl sulfoxide; NI, nonidentified.

the *Salmonella* cell lines, *E. coli,* WP2 *uvr*A pKM101 cells are also routinely used. (Mortelmans and Zeiger 2000; Sommers 2003).

All bacterial cell lines *S. typhimurium* used in the Ames test carries a defective gene (mutant) that prevents them from synthesizing amino acid called histidine essential from ingredients using standard bacterial culture. The *E. coli* cell line is bearer of a mutant gene that prevents the synthesis of the essential amino acid tryptophan. Hence, these cell lines can only survive and grow in medium containing excess histidine (or the cell lines of *E. coli* tryptophan). However, in the presence of a product/mutagenic chemical compound, defective genes can be mutated back to the functional state, allowing the bacteria to grow in standard medium that contains only traces of histidine or tryptophan additional (Mortelmans and Zeiger 2000; Mortelmans and Riccio 2000). These mutations that lead to a recovery of activity or normal function, are called "reverse" and the process is referred to as "reversion." The mutant colonies that begin to synthesize histidine and tryptophan are called "revertant." The number of colonies revertant spontaneously is relatively constant for each cell line. However, when a mutagenic agent is added to the plate, the number of revertant colonies per plate is increased, generally in a dose-related manner of the carcinogenic chemical substances, such as aromatic amines or polycyclic aromatic hydrocarbons, are biologically inactive unless are metabolized to active forms (Maron and others 1981; Levin and others 1982).

Many chemicals are not mutagenic (or carcinogenic) in their native forms, but they are converted to mutagenic substances by metabolism in the liver. In human beings and mammals, the metabolic system of cytochrome P450-based rust that is present mainly in the liver is able to metabolize a large number of these chemicals reactive electrophiles to form DNA (Malling 1971; Ames and others 1973). Bacteria does not have the metabolic capacity, an exogenous activation system of a mammal has to be added to the plate of petri in conjunction with the test substance. The Ames test protocols using rat liver enzymes or hamster (S9 microsomal fraction) to promote metabolic conversion of the test substance. This allows determining whether a chemical compound needs to be metabolized to express mutagenic activity. The metabolic activation system usually consists of the supernatant fraction of rat liver homogenate that is incorporated into the test system in the presence of NADP (Nicotinamide adenine dinucleotide phosphate) and cofactors for oxidation (S-9 mix). To increase the level of metabolizing enzymes, mixed function oxidase inducer Aroclor 1254 is added. Some chemical mutagenic compounds are active with and others without metabolism (Shimada and Okuda 1988; Mortelmans and Zeiger 2000; Tejs 2008; LeMieux 2016).

Over the years its value as such, has been recognized by the scientific community, government, agencies, and corporations. The test is used worldwide an initial screen to determine the mutagenic potential of new chemicals and drugs, because there is a high predictive value for carcinogenicity. In addition, the test data are submitted to regulatory agencies in support of the registration or acceptance of many chemicals, including drugs and biocides (CETESB 1993; Mortelmans and Zeiger 2000; Gadgil and others 2002; Tejs 2008).

## Solvent

Usually the solvent of 1st choice is sterile distilled water. The compounds/chemicals that do not dissolve in water can be dissolved in dimethyl sulfoxide. There are also other solvents that can be considered such as: acetone, ethyl, ethanol (95%), tetrahydrofuran, dimethylformamide, and methyl ethyl ketone. Care is

required on the choice of solvent since these others may be toxic to the bacteria at higher concentrations (Maron and Ames 1983).

### Mutagenicity studies carried out of 2-CBA using the Ames test

Observing the Table 2 mutagenicity studies already undertaken as well as information contained in the experiments, we can compare authors and tests. Small changes such as solvent used, such great differences in regard the dosage of the compound and cell lines used were noted. All these analyses are an important factor since it combines all information providing an analysis of test reliability and the results herein to be useful for future research on the topic.

## Methods

Articles researched to accomplish this work were collected from digital collections available in the research platforms: Sciencedirect, Scielo, PubMed, Web of Science, and USP Digital Library, where national and international materials were consulted from 1971 to 2016.

## Discussion and Conclusion

This analysis aims to locate the mutagenic tendency present in chemical compounds, in this case a specific survey regarding the work carried out was given by a single rule; the use of compounds derived from the 2-ACB group, more specifically 2-dodecylcyclobutanone (2-DCB) and 2-tetradecylcyclobutanone (2-tDCB) as they are found more readily distributed in the food chain. All tests performed for the mutagenicity of 2-DCB and 2-tDCB compounds both showed negative results. In the inevitable comparison between the authors and the protocol provided by Ames (Ames and Others 1973) and important information available in the OECD (2015), we can observe the discrepancies caused by the absence of data, they begin with the choices of the cell lines used from one experiment to another. The OECD suggests that 5 strains of *S. typhimurium* and one of *E. coli* be used, as well as the use of plate of petri dish, these suggestions corroborate so that there is no compromise in the reliability and veracity of the results, however, this is not what we can verify in the experiments performed.

We can illustrate all these differences in the various existing changes in Table 2 citing as an example Burnouf and others (2002), which concluded that the test compound was not mutagenic, but in their experiment used only *Typhimurium* cell lines and did not add as indicated in the protocol, cell lines of *E. Coli*, the same applies to the work done by Gadgil and Smith (2004) and thus in their experiments were not analyzed all the chain sites that could be affected by a possible mutation. On the other hand, Sommers (2003) tested in 2 first lines *E. coli* and then in a later article Sommers and Schiestl (2004) has completed its experiment by using *S. typhimurium* cell lines. We can mention Yamakage and others (2014) as a reference of how to perform correctly all the suggestions for a complete and reliable study, the group used all necessary lines, most suitable board type, and a wide range of dosages, and as other studies also showed negative for mutagenicity of 2-DCB compounds and 2-tDCB.

Protocols are required to validate the standardization of the result and competent regulatory bodies recognize it, to not follow the standardizations determined experiments run the risk of getting a false positive, compromising the accuracy of the above result.

New methodologies based on colorimetric methods using microplates have been developed by several manufacturers and research institutions. An example is the mutagenicity assay developed by Xenometrix®, which consists of exposing bacterial lines to the test substance for 90 min in a medium with a small amount of histidine, then the cultures are diluted in a medium lacking histidine and bookmark pH and distributed in a 384 well plate. After 48-h incubation, the reading is performed and compared with the positive and negative controls. If the substance is mutagenic, bacteria will have their mutation reversed and will grow in greater quantities, thus causing the decrease of pH and change its color. The assay may be performed with or without metabolic activation system. Unlike existing methods, this method only uses the TA-98 strains, TA-100, TA-1535, and TA 1537 of *S. typhimurium* (Xenometrix 2008). Therefore, it can be said that this assay detects a smaller range of mutations than other methods because does not use any strain that detects mutations in places with presence of thymidine (-TAA-) (MT-102, MT-104, and WP2 *uvr*A). This test and methodology still require validation and greater acceptance by health authorities (Bispo 2014).

The Ames test is not only financially viable, FDA (Food and Drugs Administration)-recognized, reproducible, and nonfaulty when used for most chemical classes, it has been able to detect about 90% of known human carcinogens. Therefore, it has been chosen as the 1st choice method for a preliminary assessment of mutagenicity and carcinogenicity. It is considered the best autonomous assay for mutagenicity identification, being widely used in several segments (Mahadevan and others 2011). Thus, in this way, it can safely be concluded that the 2-ACBs at the concentrations tested do not show any mutagenicity detected by the Ames test.

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