Lebanese pita extracts with presence of trace elements: hazard assessment

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Introduction

Among Trace Elements (TE) that can be present in food, some are known to be toxic and must be monitored. Regarding food safety, it is relevant to study the case of the pita, since it represents a large part of the daily diet of the Lebanese population. In a previous study, the Lebanese Agricultural Research Institute (LARI) analyzed the presence of several TE (arsenic, cadmium, cobalt, chromium, mercury, nickel and lead) in white Lebanese pita. Although the toxicity of each TE is known individually, it is important to test the toxicity of the mixture of TE, present in the bread, in order to check the presence of "cocktail" effects between TE, especially synergism.

Materials & Methods

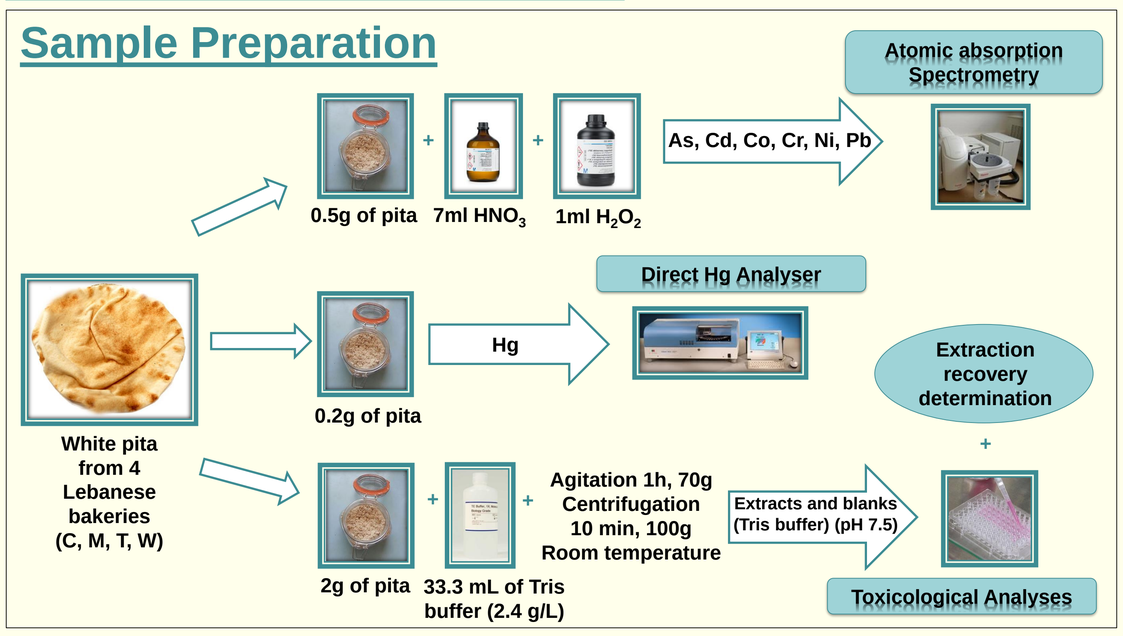


Figure 1: Sample preparation for chemical and toxicological analyses of trace elements (As, Cd, Co, Cr, Ni, Pb and Hg) in white Lebanese pita.

Cell Model

HepG2 human hepatoma cell line, with enzymatic activities (phase I and II metabolism) and a functional protein P53.[1]

Cytotoxicity

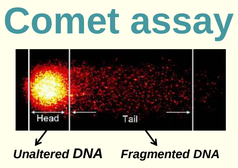
Resazurin assay measurement of cell viability after 2h of incubation with the dye and reduction by the mitochondrial enzymes, from blue resazurin (weakly fluorescent) to red resorufin (highly fluorescent).[2]

Oxidative Stress

Cellular Reactive Oxygene Species (ROS) detection by the oxidation of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) into a highly fluorescent dye, 2',7'- dichlorofluorescein (DCF).[3]

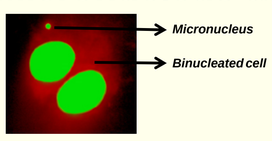
Genotoxicity

- Comet assay



Measurement of DNA single and double strand breaks and alkali-labile lesions, after an electrophoresis step in alcaline conditions (pH 13) and DNA staining with a flurosecent dye, propidium iodide.[4]

- Micronucleus assay



Micronuclei counting in the cytotplasm of interphase cells (performed according to the OECD 487 guideline). Micronuclei are formed from an entire chromosome or an acentric fragment of a chromosome. Reading is performed by fluorescence microscopy after staining with acridine orange.[5]

- Ames test

Bacterial reverse mutation measurement (performed according to the OECD 471 guideline), on *Salmonella typhimurium* TA 98 strain, detecting the presence of gene mutations[6].

Results/Discussion

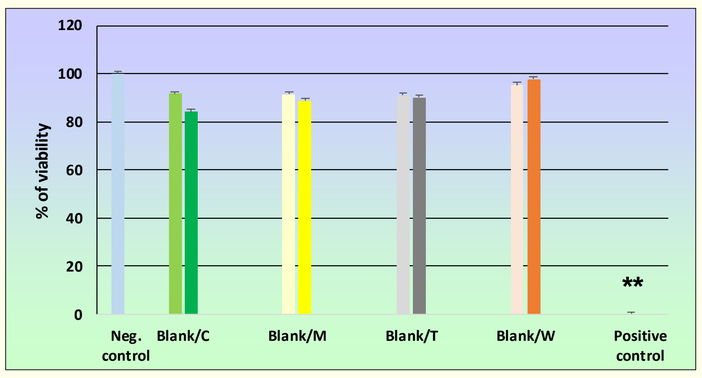


Figure 2: Cell viability determined with the resazurin assay after 24h exposure of HepG2 cells with 4 pita extracts. ** P < 0.01, significantly different from negative control. Positive control: Sodium Dodecyl Sulfate 3% (m/v), 3 independent experiments. Negative control raw data: 3 923 571.

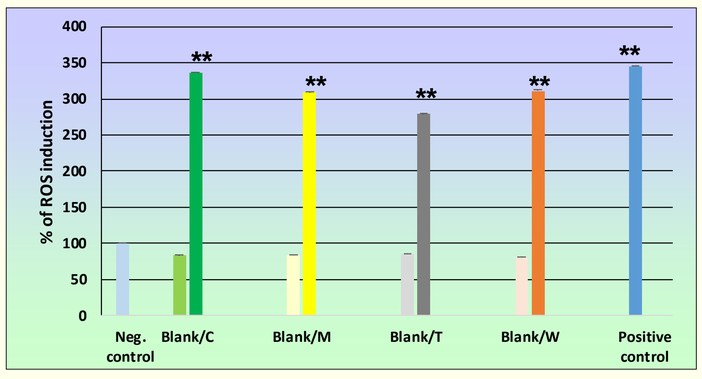


Figure 3: Oxidative stress determined with the DCFDA assay after 24h exposure of HepG2 cells with 4 pita extracts. ** P < 0.01, significantly different from negative control. Positive control: Tert-butyl hydroperoxide (15 µM), 3 independent experiments. Negative control raw data: 33 400.

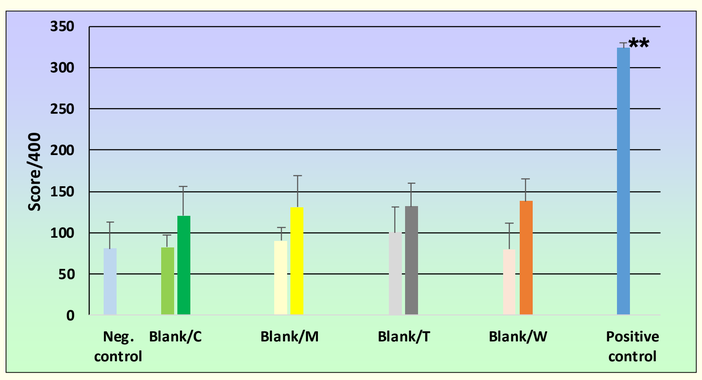


Figure 4: DNA damage determined with the comet assay after 24h exposure of HepG2 cells with 4 pita extracts. ** P < 0.01, significantly different from negative control. Positive control: Methylmethanesulfonate (50 µM), 3 independent experiments. Negative control score: 80/400 (maximal score).

Score = (0x% of cells in class 0) + (1x% of cells in class 1) + (2x% of cells in class 2) + (3x% of cells in class 3) + (4x% of cells in class 4)

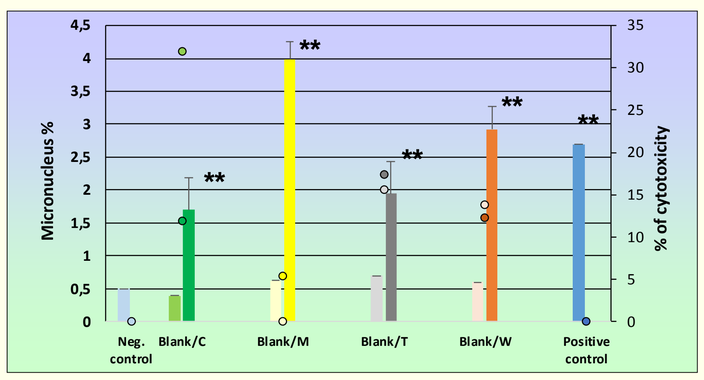


Figure 5: Chromosomal (clastogenic and/or aneugenic) damage determined with the micronucleus assay after 44h exposure of HepG2 cells with 4 pita extracts. ** P < 0,01, significantly different from negative control. Positive control: Vinblastine sulfate (0.625 ng/ml), 1 independent experiment. Negative control raw data: 10 micronuclei/2000 binucleated cells. Colored dots corresponds to % of cytotoxicity of extracts, respectively.

Table 1: Trace Elements chemical composition of different pita (C, M, T, W).
Concentrations are expressed in weight of the dry matter. N.D. = not determined

TE	Concentrations of MTE in the pita C (µg/kg)	Extraction recovery for pita C	Concentrations of MTE in the pita M (µg/kg)	Extraction recovery for pita M	Concentrations of MTE in the pita T (µg/kg)	Extraction recovery for pita T	Concentrations of MTE in the pita W (µg/kg)	Extraction recovery for pita W	Maximum level in bread (Lebanese norm) (µg/kg) [7]
Ni	N.D.	-	**1292.14 ± 0.17**	46	**1539.6 ± 0.07**	N.D.	364.82 ± 0.02	49	-
As	321.10 ± 19.81	79	234.64 ± 21.96	78	204.3 ± 2.38	N.D.	399.5 ± 7.13	74	-
Hg	0.89 ± 0.06	58	0.73 ± 0.10	64	0.51 ± 1.66	N.D.	0.70 ± 0.15	56	-
Cd	5.77 ± 1.77	70	6.47 ± 0.27	61	7.33 ± 0.01	N.D.	8.69 ± 1.79	65	200
Co	84.24 ± 2.04	66	91.09 ± 2.93	62	87.2 ± 0.05	N.D.	86.70 ± 4.62	63	-
Cr	**362.76 ± 10.18**	73	40.37 ± 2.17	81	46.84 ± 13.6	N.D.	15.53 ± 1.58	76	-
Pb	**259.10 ± 6.97**	61	73.98 ± 4.60	60	**214.99 ± 7.18**	N.D.	**203.13 ± 30.38**	59	200

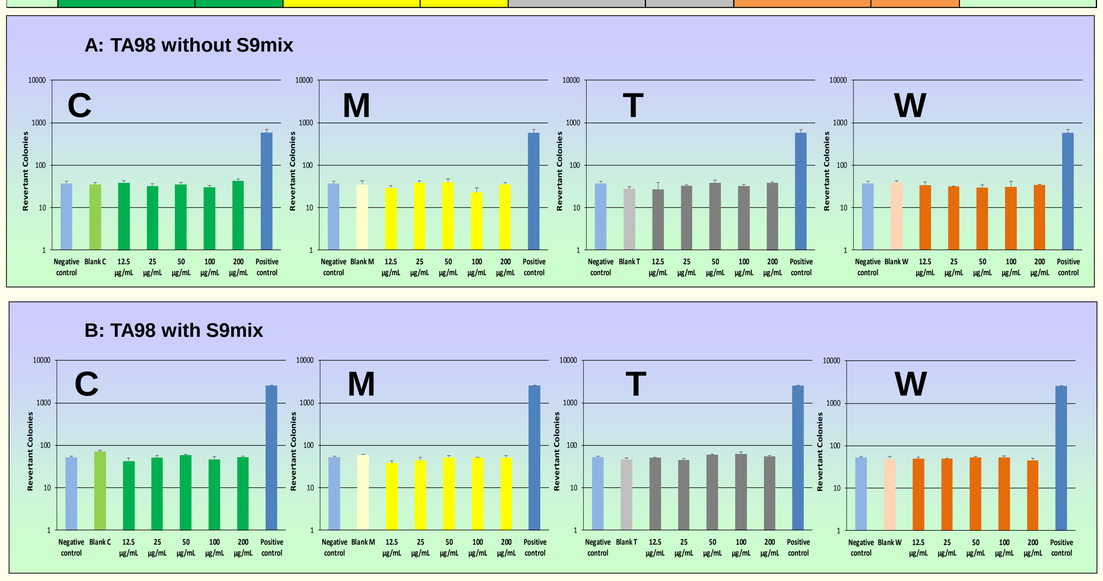


Figure 6: Number of revertant colonies measured in the Ames test on the TA98 strain in absence (A) and in presence (B) of S9 mix with 5 different concentrations (12.5 to 200 µg/mL) of Lebanese pita extracts (one independent experiment). Positive controls: 2-Nitrofluorene (2 µg/plate) without S9 mix, 2-Aminoanthracen (2.5 µg/plate) with S9 mix.

- The chemical analysis (Table 1) indicates a different TE content of pita according to the bakeries:

- a very high level of nickel (1292 µg/kg) in pita made by bakeries M and T, despite the fact that the extraction efficiency is lower than 50%.

- a higher chromium level (362 µg/kg) for pita C, with an extraction yield of 73%

- presence of lead in pita C, T and W at concentrations of 259, 215 and 203 µg/kg respectively, close to the limit allowed in Lebanon.

- cadmium at levels between 5.77 and 8.69 µg/kg, more than 20 times lower than the Lebanese standard with an average extraction recovery of 65%.

- None of the extracts, from 4 different bakeries, were cytotoxic (Fig. 2) but they all induced a positive response in the DCFDA assay (x 4.5 on average), indicating the presence of ROS (Fig. 3) after 24h exposure.

- This effect did not lead to an increase in DNA breaks since the comet test is negative (Fig. 4), even in the presence of the fpg enzyme detecting specifically oxidative damage (data not shown).

- The Ames test used to detect gene mutations was negative with TA 98 (Fig. 6) and TA 100 (data not shown), in the absence or presence of an exogenous metabolism system (S9 mix) whatever the extract.

- On the other hand, the micronucleus test showed a significant increase (x 3.4 to 8) in the number of breaks and/or chromosome losses in the 4 extracts (Fig. 5), especially for the M extract, which has a high nickel content (1.29 mg/ kg), without strong cytotoxic effects for all the extracts (less than 20%).

Conclusion

Pita extracts from 4 different Lebanese bakeries induced oxidative stress leading to chromosomal damage (results to be confirmed). Each extract will be tested at different concentrations to demonstrate dose effects. The chemically quantified TE will be evaluated individually at the representative concentrations found in each extract to check the impact of TE alone, in order to highlight any interactions.

Références : **1) Gerets *et al.* (2012)** Cell Biol Toxicol 28, 69-87 **2) Binderup *et al.* (2002)** Food Addit. Contam. 19, 13-28, **3) Souton, (2017)** PhD**, 4) Riquet *et al.* (2016)** Food Chem. 199, 59-69, **5) OCDE 487, 6) OCDE 471, 7) Lebanese Norm NL 240 (2010).** 13 pp.