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Evaluation of the migration of mutagens/carcinogens from PET bottles into mineral water by *Tradescantia*/micronuclei test, Comet assay on leukocytes and GC/MS

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Abstract

This study monitored the release of mutagenic/carcinogenic compounds into mineral water (natural and carbonated) from polyethylene terephthalate (PET) bottles, using a plant mutagenicity test which reveals micronuclei formation in *Tradescantia* pollen cells (Trad/MCN test), a DNA damage assay (Comet assay) on human leukocytes and gas chromatography/mass spectrometry (GC/MS) for the characterisation of migrants. The water samples were collected at a bottling plant and stored in PET bottles for a period ranging from 1 to 12 months. Every month some samples were randomly collected and lyophilised, the residual powders were extracted with organic solvents and then analysed by GC/MS and tested for DNA damage in human leukocytes, or reconstituted with distilled water to obtain concentrates for the exposure of *Tradescantia* inflorescences. Micronuclei increase in pollen was found only in natural mineral water stored for 2 months. DNA-damaging activity was found in many of the natural and carbonated water samples. Spring water was negative in the plant micronuclei test and the Comet assay, whereas distributed spring water showed DNA-damaging effects, suggesting a possible introduction of genotoxins through the distribution pipelines. GC/MS analysis showed the presence in mineral water of di(2-ethylhexyl)phthalate, a nongenotoxic hepatocarcinogenic plasticizer, after 9 months of storage in PET bottles.

Keywords: Mineral water; PET bottles; Mutagenicity; *Tradescantia*/MCN test; Comet assay; Di(2-ethylhexyl)phthalate

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1. Introduction

Mineral waters and soft drinks are very often placed in plastic bottles where they may be stored for several months. However, very little attention has been paid to the potential changes in quality of these beverages after prolonged storage in plastic containers. It is well known that food and water may become contaminated with components of plastic containers (Tice and McGuinness, 1987; Ashby, 1988; Gilbert et al., 1988; Begley et al., 1990; Kim et al., 1990) by a diffusion process known as migration. Some of these migrants are known mutagenic or carcinogenic agents, e.g. acetaldehyde (CASRN 70-07-0) (Eberhartinger et al., 1990; Linssen et al., 1995), formaldehyde (CASRN 50-00-0) (Wegelin et al., 2001) and vinyl chloride (CASRN 75-01-4) (Benfenati et al., 1991).

Polyethylene terephthalate (PET) bottles are the most widely used beverage containers. The migration of acetaldehyde from PET under various conditions (different temperatures and times) was analysed by head-space gas chromatography and flame ionization detection and in some cases high levels of this compound (11–7447 ng/ml) were found in carbonated mineral water and lemonade (Eberhartinger et al., 1990; Linssen et al., 1995). Wegelin et al. (2001) have found up to 44 μg/l of formaldehyde in mineral water stored in PET bottles and exposed for 2 months to sunlight.

However, complete chemical characterisation of the migrants is very difficult. For this reason the application of short-term tests for the evaluation of the overall mutagenicity of migrants seems to be a promising tool for the screening of beverage quality. De Fusco et al. (1990) have shown the appearance of mutagenicity with the Ames test in mineral water stored in PET bottles after 3 months of storage at daylight. Monarca et al. (1994) did not find any water mutagenicity in another similar experiment, but they did identify several migrants by means of gas chromatography/mass spectrometry (GC/MS), some of them with potential genotoxic properties (acetaldehyde and dimethyl terephtalate).

Plant bioassays, especially the *Allium cepa* root test and *Tradescantia*/micronuclei test, have been used for the detection of mutagenicity in drinking water (Al-Sabti and Kurelec, 1985; Monarca et al., 1998; Steinkellner et al., 1999), since they can be used for in situ experiments, by exposing these bioindicators directly to the unconcentrated water samples.

The Single Cell Gel Electrophoresis test (SCGE or Comet assay) is widely used for DNA damage detection in individual cells and has been used for the study of surface water (Zhong et al., 2001) and drinking water disinfection by-products (Muller-Pillet et al., 2000), but not for the study of drinking water concentrates.

The aims of this research were to study the potential migration of genotoxic compounds in mineral water samples stored for different periods of time in PET bottles and to evaluate the potential human health hazards. The release of mutagenic/carcinogenic substances was studied using a combined approach with a short-term plant genotoxicity test (*Tradescantia*/micronuclei test), a DNA damage test on human leukocytes (Comet assay) and an analytical method, the GC/MS.

2. Materials and methods

2.1. Mineral water samples

The mineral water samples have been collected at a commercial bottling plant. The main chemical characteristics of the mineral water were: pH: 6.2; residue at 180 °C: 998 mg/l; conductivity at 18 °C: 1353 μs/cm; oxydizability as permanganate value: 0.7 mg/l; alkalinity HCO_3: 158 mg/l. At the mineral water plant 24 PET bottles from the same batch were concurrently filled with 2 l of mineral water per bottle, which was distributed from the spring source to the bottling apparatus: 12 bottles were filled with natural mineral water and 12 with carbonated mineral water. Each PET bottle was enclosed in a sealed and signed envelope and all the bottles were stored in the dark for up to 12 months in locked cupboards. Two negative controls, the natural mineral water at the spring and the same water after distribution to the bottling plant, were concurrently collected in glass bottles and analysed immediately.

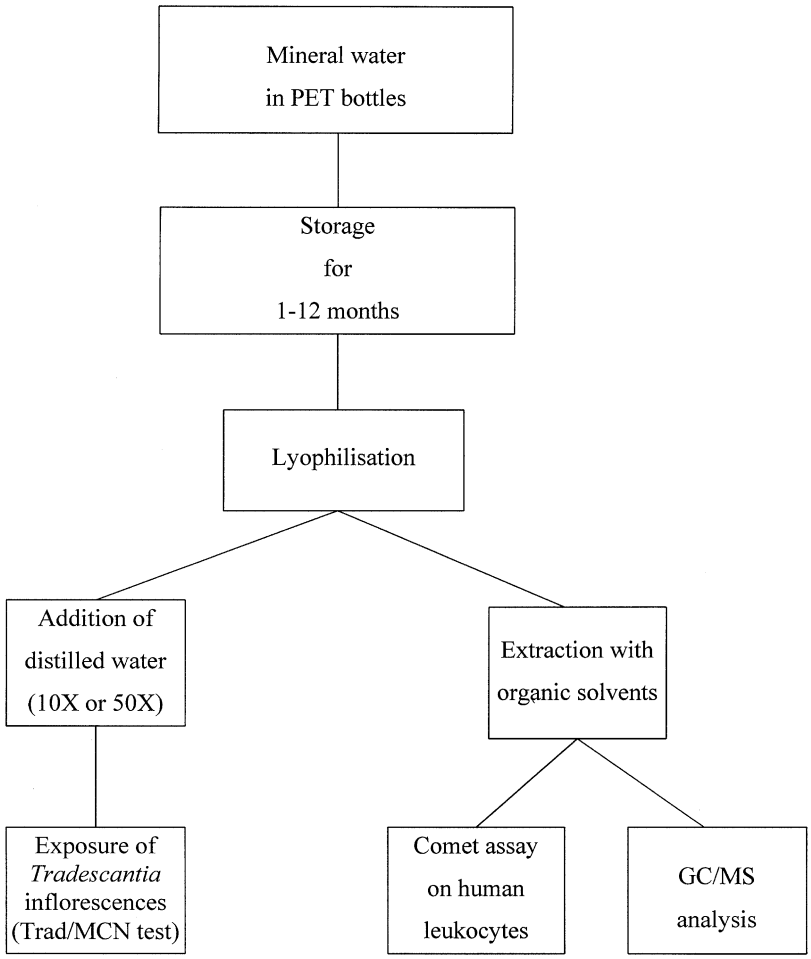

Fig. 1. Scheme of the study carried out on mineral water stored in PET bottles.

Every month one PET bottle of natural mineral water and one PET bottle of carbonated water were randomly sampled, the water was lyophilised, the powders were homogenised, divided in different portions and processed in three different ways (Fig. 1):

1. small amounts of distilled water were added to the powders and the concentrates were analysed using the *Tradescantia*/micronuclei test;
2. the powders were sequentially extracted with cyclohexane, dichloromethane and acetone, and the pooled extracts were studied using the Comet assay; and
3. other portions of the powders were extracted by shaking with acetone, then the acetone extracts were dried, weighed and analysed using GC/MS to detect the presence of phtalates and similar compounds.

2.2. Analysis of aqueous concentrates by Tradescantia/micronuclei test

Small amounts of distilled water were added to the powders equivalent to 1000 ml of mineral water in order to obtain aqueous concentrates (10× or 50×) which were tested by means of the *Tradescantia*/micronuclei test (Trad/MCN test)

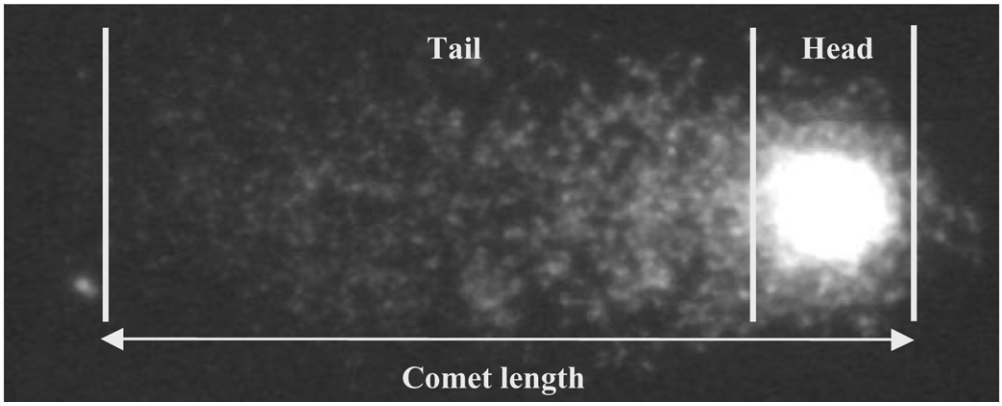

Fig. 2. Comet image with the visualisation of the total Comet length as measured by the automatic image analysis system.

using a hybrid of *Tradescantia hirsutiflora* and *Tradescantia subacaulis* (clone #4430) (Ma et al., 1994). Distilled water was used as negative control and maleic hydrazide (0.2 mmol/l) was used as positive control. The inflorescences were placed in the water samples (approx. 20 ml of concentrates) for 24 h (exposure time) and then maintained in distilled water for 42 h (recovery time). After that the inflorescences were fixed in aceto-ethanol and then stored in 70% ethanol. Normal tetrads and tetrads containing one or more micronuclei were counted from 5 slides in each experimental group. The frequencies were calculated by dividing the total number of MCN by the total number of tetrads scored and expressed as MCN/100 tetrads. The data were analysed using the nonparametric Kruskal–Wallis and Mann–Whitney tests at a 0.05 level of significance between the mineral water samples stored in PET bottles and the distilled water or the spring mineral water, used as negative controls.

2.3. Analysis of organic extracts by Comet assay on leukocytes

For each monthly sampling different sub-aliquots of powders equivalent to 250 ml mineral water were sequentially extracted by sonication for 60 min with 100 ml of each of the following solvents: cyclohexane, dichloromethane and acetone. The extracts were pooled, dried in a rotating evaporator, and dimethyl sulfoxide (DMSO) was added. The treatment of isolated leukocytes from whole blood from a non-smoking healthy donor was performed for 1 h at 37 °C with an equivalent dose of 0.5 l for all the samples due to the limited amount of extract, according to the procedure of Poli et al. (1999). Negative (DMSO, 60 μl/ml) and positive (Melphalan, 10 μg/ml) controls were also performed. Cell viability was checked using the Trypan blue exclusion method. The Comet assay was basically performed according to the method described by Singh et al. (1988). After cell lysis, the DNA was allowed to unwind for 20 min in an electrophoretic alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13) and subjected to electrophoresis in the same buffer for 20 min at 0.78 V/cm and 300 mA. After DNA staining with ethidium bromide, the samples were examined under a fluorescent microscope (Leitz Dialux 20) equipped with a BP 515–560 nm excitation filter and an LP 580 nm barrier filter, using an automatic image analysis system (Cometa Release 2,1-Sarin, Florence, Italy). One hundred cells per sample, selected at random, were analysed at constant sensitivity. All the steps were performed under a yellow light to prevent additional DNA damage. The distributions of the total Comet length (Fig. 2) and the number of cells giving values of > 95th percentile of the DMSO control distribution were used as the most relevant indexes of DNA damage. The comparison between the grade of DNA damage was analysed using the Mann–

Table 1
Frequency of micronuclei in pollen of *Tradescantia* inflorescences placed for 24 h in aqueous concentrates (50× and 10×) of lyophilised mineral water

Months of storage in PET bottles	Micronuclei/100 tetrads (mean ± S.D.)			
	Natural mineral water concentrates		Carbonated mineral water concentrates	
	50×	10×	50×	10×
1	4.0 ± 1.2	4.5 ± 4.0	3.6 ± 1.2	4.5 ± 2.3*
2	20.0 ± 11.3**	9.3 ± 4.8**	2.7 ± 1.6	1.3 ± 0.4
3	5.0 ± 3.6	6.0 ± 3.9*	4.9 ± 1.1*	3.3 ± 1.2
4	5.6 ± 5.1	2.9 ± 1.2	3.5 ± 3.6	2.6 ± 2.6
5	4.4 ± 3.6	1.6 ± 0.7	2.1 ± 1.3	3.5 ± 2.0
6	7.6 ± 7.5	2.9 ± 1.9	2.3 ± 0.6	0.4 ± 0.3
7	1.2 ± 0.7	1.6 ± 0.6	1.4 ± 0.6	3.2 ± 1.9
8	4.9 ± 3.8	4.1 ± 4.2	6.0 ± 3.1	6.9 ± 10.5
9	2.4 ± 1.6	2.1 ± 0.9	2.7 ± 1.2	6.7 ± 4.1*
10	3.5 ± 2.5	3.3 ± 1.0	1.7 ± 0.7	7.3 ± 5.6
11	6.3 ± 5.5	2.1 ± 0.9	2.6 ± 1.5	7.8 ± 6.2
12	4.0 ± 4.9	4.3 ± 2.9	2.6 ± 1.2	2.3 ± 0.8
Controls				
Natural mineral water at the spring[a]	2.8 ± 0.7	3.2 ± 1.2		
Natural mineral water at the bottling plant[b]	3.5 ± 0.9	2.8 ± 0.8		
Distilled water			2.5 ± 0.9	
Maleic hydrazide (0.2 mmol/l)			13.7 ± 2.5***	

*$P<0.05$; **$P<0.01$; ***$P<0.001$, vs. natural mineral water at the spring or vs. distilled water, according to nonparametric tests (Kruskal–Wallis and Mann–Whitney *U* tests).
[a] Natural mineral water before distribution to bottling apparatus.
[b] Natural mineral water after distribution to bottling apparatus.

Whitney *U* test for length distributions and χ^2-test for the number of cells giving values of >95th percentile of the DMSO control distribution.

2.4. Analysis of acetone extracts by GC/MS

The powders of lyophilised samples equivalent to 250 ml of mineral water were shaken with acetone and extracts were concentrated in vacuo, dried under nitrogen and weighed at constant weight. This process was performed in triplicates. These extracts suspended again in acetone were then tested by GC/MS using a Hewlett-Packard 5890 A apparatus equipped with an HP 1 fused-silica capillary column (30 m×0.25 mm i.d., film thickness 0.33 μm), linked on line with an HP Mass Selective Detector MSD 5970 (ionization voltage 70 eV, multiplier energy 2000 V). Gas chromatographic conditions were as follows: injector and transfer line 250 and 290 °C, respectively; the oven was set to 40 °C for 5 min and then raised to 260 °C at a rate of 2 °C/min. Compounds were identified by comparison of their GC retention indexes, and MS spectra with those reported in literature and by computer matching with the NIST98 and Wiley-5 libraries.

3. Results

Tests on *Tradescantia* pollen showed no significant increase of micronuclei in tetrads for the spring water and the same water collected at the bottling plant in comparison with distilled water. A highly significant increase of micronuclei was found in the sample of natural mineral water stored for 2 months (20 micronuclei/100 tetrads in 50× concentrated water and 9.3 in 10× concentrated water vs. 2.5 in distilled water). A light increase of micronuclei was also found in natural water stored for 3 month and in carbonated mineral

Table 2
Comet assay: DNA damage induced in human leukocytes by natural and carbonated water samples (0.5 l equivalent)

Natural mineral water			Carbonated mineral water		
Sample (months of storage)	Comet length median[a] (μm)	>95th percentile (cell number)	Sample (months of storage)	Comet length median[a] (μm)	>95th percentile (cell number)
1***	15.3	5	1***	22.0	58^{+++}
2	14.4	9	2**	15.2	12
3	13.9	19^{++}	3***	15.8	32^{+++}
4	14.6	18^{++}	4***	15.2	13^{+}
5	14.1	30^{+++}	5	14.6	5
6***	16.5	42^{+++}	6	14.6	4
7	14.0	5	7	14.6	7
8	14.2	11	8	14.4	6
9	14.0	15^{+}	9*	13.0	21^{+++}
10	14.6	6	10	14.2	5
11	13.7	7	11***	13.0	13^{+}
12*	16.2	13^{++}	12	13.8	12
Controls					
Natural mineral water at the spring[b]	13.8	6			
Natural mineral water at the bottling plant[c]*	28.1	75^{+++}			
DMSO (50 μl/ml)	14.3	5			
Melphalan (10 μg/ml)***	17.8	89^{+++}			

*$P<0.05$; **$P<0.01$; ***$P<0.001$, vs. natural mineral water at the spring according to Mann–Whitney U test. $^{+}P<0.05$; $^{++}P<0.01$; $^{+++}P<0.001$, vs. natural mineral water at the spring according to χ^2-test.

[a] The damage was reported as median of Comet length (100 cell scored for each sample) and number of cells exceeding the 95th percentile of the reference distribution (>95th percentile) of Comet length.

[b] Natural mineral water before distribution to bottling apparatus.

[c] Natural mineral water after distribution to bottling apparatus.

water stored for 1, 3 and 9 months whereas the other samples were always negative (Table 1).

The median Comet length values out of 100 cells scored and the number of cells exceeding the 95th percentile of the reference distribution (negative control) are presented in Table 2. The results for Comet length showed significant differences (Mann–Whitney U test) for the natural mineral water samples stored for 1, 6 and 12 months and carbonated mineral water samples stored for 1, 2, 3, 4, 9 and 11 months. The analysis of the number of cells giving values of >95th percentile of the DMSO control, one of the most relevant indexes of highly damaged cells, resulted in significant differences (χ^2-test) for natural mineral water samples after 3, 4, 5, 6, 9 and 12 months and for carbonated mineral water samples after 1, 3, 4, 9 and 11 months.

No correlation between storage period and DNA damage was found. The spring water sample did not show any DNA damage whereas the same water before bottling showed the most significative damaging effects.

Storage time of mineral water in PET influenced the weight and composition of the acetone extracts of lyophilised samples (Table 3). GC/MS analysis of these extracts identified a plasticizer, di(2-ethylhexyl)phthalate (DEHP) (CASRN 117-81-7), known to be hepatocarcinogenic (Huber et al., 1996; James et al., 1998) and teratogenic (Yagi et al., 1980; Shiota and Nishimura, 1982; Melnick et al., 1987), although this compound is not genotoxic. DEHP was found in the same samples in which increase of extract weights begins. After 9 months of storage for natural water and after 10 months for carbonated water samples the extract weights increased from 0.4 to 3.2 mg/l in all the samples.

4. Discussion and conclusion

The aim of this study was to perform a preliminary research about the release of potential haz-

Table 3
Analysis of acetone extracts of lyophilised mineral water by GC/MS

Months of storage in PET bottles	GC/MS identification		Acetone extracts[a] (mg/l)	
	Natural water	Carbonated water	Natural water	Carbonated water
1	–	–	0.40 ± 0.01	0.39 ± 0.0006
2	–	–	0.40 ± 0.01	0.40 ± 0.01
3	–	–	0.39 ± 0.01	0.40 ± 0.004
4	–	–	0.40 ± 0.004	0.40 ± 0.01
5	–	–	0.40 ± 0.02	0.39 ± 0.01
6	–	–	0.41 ± 0.006	0.39 ± 0.004
7	–	–	0.40 ± 0.01	0.40 ± 0.01
8	–	–	0.40 ± 0.01	0.40 ± 0.006
9	DEHP[b]	–	3.21 ± 0.10	0.40 ± 0.01
10	DEHP[b]	DEHP[b]	3.19 ± 0.10	3.20 ± 0.30
11	DEHP[b]	DEHP[b]	3.20 ± 0.0	3.19 ± 0.10
12	DEHP[b]	DEHP[b]	3.22 ± 0.10	3.21 ± 0.20
Controls				
Natural mineral water at the spring[c]	–		0.0	
Natural mineral water at the bottling plant[d]	–		0.0	

–, No migrants identified.
[a] Mean ± S.D. of triplicates.
[b] Di(2-ethylhexyl)phthalate.
[c] Natural mineral water before distribution to bottling apparatus.
[d] Natural mineral water after distribution to bottling apparatus.

ardous compounds from PET bottles using a biological/chemical approach.

The bioassays chosen for this study seem to be successful in detecting both genotoxic and carcinogenic compounds present in mineral water. *Tradescantia*/MCN test and Comet assay showed the leaching of unknown mutagens in some samples without correlation with the storage time. The distribution system is probably responsible for the leaching of genotoxins, since the Comet assay revealed a very significant DNA damage in the water samples collected at the bottling plant, but not at the spring.

GC/MS enabled us to identify DEHP, a compound with carcinogenic and teratogenic activity, especially after 9–10 months of storage in PET bottles suggesting a possible correlation with storage time. On the contrary, as DEHP is not genotoxic the presence of this compound is not related to the genotoxicity found in many samples.

In conclusion, our biological and chemical analyses showed mineral water contamination with genotoxic/carcinogenic compounds not only during mineral water storage in PET bottles but also from the pipes supplying the water to the bottling process. This research seems to indicate the need for more in-depth analysis of the leaching of toxins from PET bottles. The study could be enriched with other short-term mutagenicity tests showing different genetic end-points and with chemical analysis to obtain more information about the chemical nature of mineral water contaminants. This approach could be useful for periodic monitoring of the quality of mineral water from the spring to the shelf in order to prevent human exposure to genotoxic or carcinogenic water contaminants.

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