Evaluation of the Colorimetric Tetrazolium Assay for the Cytotoxicity Testing a Commercial Vanilla Flavouring

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Received 4 March 2002, accepted 20 March 2002

Introduction

The cytotoxicity assays on aroma chemicals used in flavouring formulations are very useful in respect of the current work concerning the updating of the Register of flavouring substances used in or on foodstuffs and adopted by the European Commission Decision 1999/217/EC (23.02.1999).

The current toxicity data regarding single aroma chemicals arise from toxicological assays performed on commercial flavouring formulations containing one or more unusual chemicals. In this sense our researches have been focused for many years on R-substituted-1,3-dioxolanes-4-methyl, derived from induced or spontaneous reactions between molecules with carbonylic groups (aldehydes and ketones) and 1,2-propylene glycol used as flavouring diluent (1–10). Several studies on the kinetics of dioxolanes formation from vanillin and their identification by HRGC/MS analyses were conducted by the same authors (7) and by *Woelfel* et al. (11).

The low toxicity of 1,2-propylene glycol is supported by the literature (12, 13); on the contrary, data about many dioxolanes safety are not available.

The colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, also called "metabolic assay", is an in vitro toxicological method with good reproducibility, sensitivity and rapid response as compared with the in vivo tests. These features have promoted its spread and use in different fields.

This in vitro short-term test is well accepted and suggested to evaluate the cytotoxic potential of chemicals by evaluation of the cellular vitality of Chinese hamster lung cells (V79).

In this paper we present the application of the colorimetric assay for the cytotoxic evaluation of a vanilla flavouring found on the market. The rapid method developed by *Mosmann* (14) was here adopted with some modifications.

Materials and methods

Methods

This colorimetric assay is measuring the activity of the mitochondrial enzyme succinic dehydrogenase which occurs only in living cells. Succinic dehydrogenase metabolises the pale yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to brick red water-insoluble formazan crystals. Formazan crystals are then dissolved in sodium dodecyl sulfate (SDS) and formazan is determined by optical density measurement at a wavelength of 570 nm.

Since the assay detects only living cells, the optical density is strictly dependent on the degree of cytotoxicity of the agent tested.

Samples

Assays were performed on a commercial vanilla flavouring preparation used for bakery products. This flavour was chosen for analysis since GC/MS analyses revealed 1,2-propylene glycol and glycerol as diluents and the presence of the following major constituents: vanillin, heliotropine, the corresponding 1,2-propylene glycol and glycerol acetals, lactones (see fig. 1 and 2). The assay was also performed on 1,2-propylene glycol (code 346703, Carlo Erba, Milan, Italy).

Reagents and cell lines

Tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (code M2128, Sigma-Aldrich S.r.l., Milan, Italy), DME-HIGH (Dulbecco's modified eagles medium – High glucose) growth medium (code LCB7501L50, Euroclone, Celbio s.r.l., Milan, Italy), phosphate buffer saline (PBS) at pH 7.5, sodium dodecyl sulfate (SDS) (code L 4509, Sigma-Aldrich S.r.l., Milan, Italy).

The cell lines used in this study were Chinese male hamster V79 lung cells (code FTV79, Amplimedical S.p.A. Diagnostic Group – Bioline Division, Turin, Italy).

Apparatus

A Multiwell spectrophotometer Ultramark Microplate Reader model 550 (Bio-Rad Laboratories, Segrate, Milan, Italy) was used.

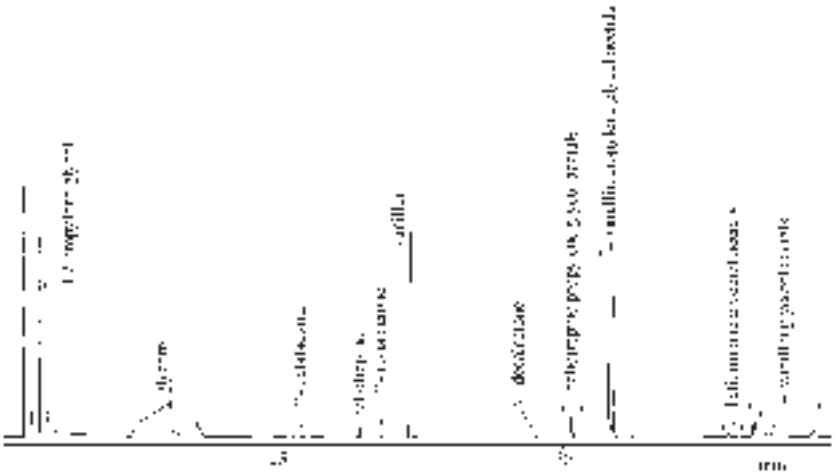

Figure 1 **GC chromatogram of the vanilla flavouring tested**
Operating condition: SPB-5 column 30 m × 0.32 mm i.d., 0.25 µm film (Supelco, Bellefonte, PA); column temperature 60°C (1 min) 2°C/min to 220°C; injector temperature 200°C; detector temperature (FID) 220°C; volume injected 1.0 µl (split 1:5)

Colorimetric MTT (tetrazolium) assay based on Mosmann method (Mosman 14)

The experimental design was the following.

Individual wells of the 96-well trays were inoculated with 90 µl DME-HIGH medium containing 5000 cells. The plates were incubated at 37°C for 12 hours.

DME-HIGH mediums containing different amounts (0.5–1.0–2.0% v/v) of flavour were prepared and a blank medium not containing the flavour.

After the incubation the growth medium was removed and the cells were re-fed with unmodified medium (control) or with 100 µl medium at various concentrations of the test agent (flavour). Thereafter, the plates were incubated at 37°C for 48 hours.

Tetrazolium salt (MTT) was dissolved in PBS (phosphate buffer saline, pH 7.5) at 5 mg ml^{-1} and the mixture filtered (0.45 µm filter) before use. 10 µl MTT solution were added to the wells and the plates were incubated at 37°C for 5.5 hours.

After the incubation 100 µl SDS (sodium dodecyl sulfate) solution at 10% in 0.01 mol/l HCl were added to the wells and the plates were incubated at 37°C for 12 hours.

The plates were then transferred to a microplate reader and the optical density was measured at a wavelength of 570 nm.

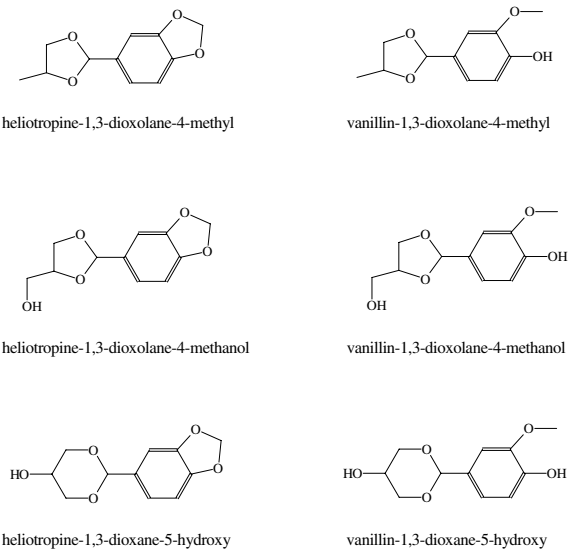

Figure 2 **Structures of 1,2-propylene glycol and glycerol acetals derived from heliotropine and vanillin.** Each formula summarise the corresponding stereo- and geometric isomers

MTT assay was also performed on the diluent 1,2-propylene glycol in the same way described above.

Statistics

All experiments were performed in quadruplicate and each assay was performed in triplicate using three V79 cell lines. Data for the dose-response cytotoxicity curves were presented as the arithmetic mean ±SD (standard deviation).

Results

In table 1 the results obtained with the vanilla flavouring are presented. Figure 3, derived from data of table 1, shows the corresponding cytotoxicity curve.

The data derived from all the experiments clearly show a dose-effect relation between the reduction of the optical density and increasing of the flavouring concentrations. The percentage of viable cells is significantly lower than 50 % in mediums dosed with flavouring at 1.0 % v/v (24 % for the cell line 2 and 30 % for the cell line 3) and is equal to 34 % in mediums dosed with flavouring at 2.0 % v/v for the cell line 1.

Table 1
Results of the cytotoxicity assay for the vanilla flavouring preparation. Each value is the average of four measures of optical density. Lines 1, 2, 3 correspond to the three assays performed with three groups of Chinese hamster lung cells (V79)

	Line 1		*Line 2*		*Line 3*	
Concentration (% v/v)	*Mean ± SD**	*Percent viable cells*	*Mean ± SD*	*Percent viable cells*	*Mean ± SD*	*Percent viable cells*
Control	1.66 ± 0.12	100	1.21 ± 0.04	100	1.38 ± 0.05	100
0.5	1.03 ± 0.11	62	0.60 ± 0.07	50	0.75 ± 0.05	54
1.0	0.94 ± 0.06	57	0.29 ± 0.01	24	0.42 ± 0.07	30
2.0	0.56 ± 0.06	34	0.11 ± 0.04	9	0.21 ± 0.05	15

* SD = Standard deviation

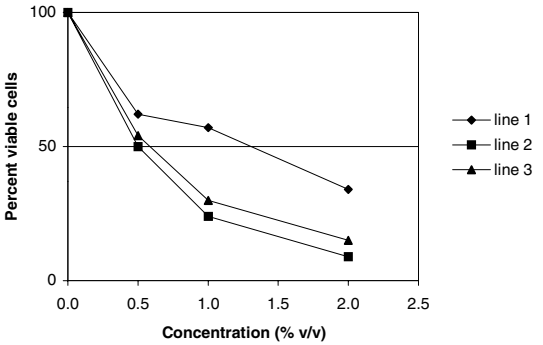

Figure 3 **Results of the cytotoxicity assay for the vanilla flavouring preparation.** Lines 1, 2, 3 correspond to the three assays performed with three groups of Chinese hamster lung cells (V79)

Table 2 reports the results of the assays performed with 1,2-propylene glycol; figure 4 shows the corresponding cytotoxicity curve. The percentage of viable cells in this case was higher than 50 % in all the three assays realised with mediums at 0.5 and 1.0 % v/v diluent. This percentage decreases (<50 %) only in cell lines 2 and 3 where mediums at 2.0 % v/v 1,2-propylene glycol were tested.

Table 2
Results of the cytotoxicity assay for 1,2-propylene glycol. Each value is the average of four measures of optical density. Lines 1, 2, 3 correspond to the three assays performed with three groups of Chinese hamster lung cells (V79)

	Line 1		*Line 2*		*Line 3*	
Concentration (% v/v)	*Mean ± SD**	*Percent viable cells*	*Mean ± SD*	*Percent viable cells*	*Mean ± SD*	*Percent viable cells*
Control	1.66 ± 0.12	100	0.92 ± 0.04	100	0.67 ± 0.04	100
0.5	1.41 ± 0.16	85	0.75 ± 0.21	82	0.48 ± 0.04	72
1.0	1.32 ± 0.16	80	0.66 ± 0.13	72	0.45 ± 0.03	67
2.0	0.97 ± 0.15	58	0.40 ± 0.07	43	0.22 ± 0.02	33

* SD = Standard deviation

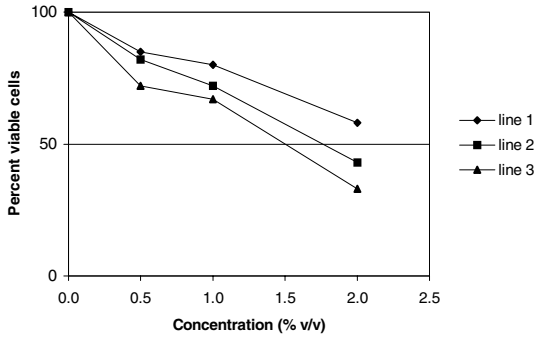

Figure 4 **Results of the cytotoxicity assay for 1,2-propylene glycol.** Lines 1, 2, 3 correspond to the three assays performed with three groups of Chinese hamster lung cells (V79)

Conclusions

The results showed that the colorimetric tetrazolium assay is applicable to preliminary screening useful of the toxicity of flavouring preparations.

Moreover, the cytotoxicity methodologies applied to flavouring preparations allow the investigation of possible synergies of toxic effects of the different constituents in flavourings. These are not always detectable through an assay performed on single compounds.

The composition of the flavouring tested here rises once again doubts on the safety and lawfulness connected with the use of 1,3-dioxolanes in flavouring formulations. On this subject, the conclusions of a previous work published by *Tateo* et al. (10) are supported about the suggestion of deleting 1,2-propylene glycol from the list of solvents/diluents allowed in flavourings.

Acknowledgement

Thanks are due to *E. Lubian* for her assistance in the editorial work.

Summary

The tetrazolium colorimetric assay (MTT-assay) has been used to evaluate the cytotoxicity of a vanilla flavouring found on the market. It was characterised by the use of 1,2-propylene glycol and glycerol as diluents and by the presence of vanillin, heliotropine and the corresponding 1,2-propylene and glycerol acetals. The method developed by *Mosmann* (14) was used with some modifications. The same assay was applied to the diluent 1,2-propylene glycol in order to verify its possible influence on the toxic effect.

This technique proved useful for preliminary screening useful to evidence doubts relating to the safety of flavouring preparations.

Zusammenfassung

Der Tetrazol-Kolorimeter-Test (MTT-assay) wurde verwendet, um die Zytotoxizität eines auf dem Markt erhältlichen Vanillearomas zu bestimmen, in dem 1,2-Propylenglycol and Glycerol als Verdünnungsmittel und Vanillin, Heliotropin und die entsprechenden 1,2-Propylen- und Glycerolacetale enthalten sind. Die von *Mosmann* (14) entwickelte Methode wurde mit einigen Abwandlungen angewendet. Der gleiche Test wurde auch mit dem Verdünnungsmittel 1,2-Propylenglycol vorgenommen, um dessen eventuellen Einfluss auf die toxische Wirkung zu überprüfen.

Die Testergebnisse zeigen die Möglichkeiten und die Zweckmässigkeit des Einsatzes dieser Technik für Vorfelduntersuchungen auf, die dazu dient, Zweifel bezüglich der Unschädlichkeit von Aromapräparaten aufzuzeigen.

Résumé

Le test colorimétrique du tétrazolium (MTT-assay) a été pratiqué pour évaluer la cytotoxicité de l'arôme de vanille qui se trouve sur le marché. Il se caractérise par le recours au 1,2-propylène glycol et au glycérol en tant que diluants ainsi que par la présence de vanilline, d'héliotropine et des acétals de glycérols et de 1,2-propylène correspondants. La méthode mise en place par *Mosmann* (14) a été utilisée en y apportant certaines modifications. Ce même test a été appliqué au diluant 1,2-propylène glycol dans le but de vérifier son influence possible sur les effets toxiques.

Les résultats ont révélé la possibilité et l'utilité de recourir à cette technique dans les essais préliminaires servant à mettre en évidence les doutes quand à la sécurité des préparations aromatisées.

Key words

Colorimetric tetrazolium assay, Vanilla flavouring, 1,2-propylene glycol

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