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ARTICLE

Enantiomeric Analysis of Chiral Compounds in Irradiated Foods Using Multidimensional Gas Chromatography

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The usefulness of both solid phase microextraction (SPME) and multidimensional gas chromatography (MDGC) coupled to mass spectrometry (MS) to detect chiral compounds in irradiated cheese was evaluated. The enantiomeric resolution of relevant chiral aroma compound was achieved by analyzing the extracts obtained from SPME by means of a permethylated β-CD stationary phase as the main column of the multidimensional system to separate specific selected cuts containing components unresolved in the first dimension. The proposed procedure allowed to determine, in less than 90 min, that no significant variations had been produced in the stereoisomeric distribution of limonene, 3-hydroxybutanone, and 2,3-butanediol in cheese when applying irradiation doses ranging from 0 to 8 kGy.

KEYWORDS: Chiral; enantiomers; irradiation; multidimensional gas chromatography; solid phase microextraction; stereoisomeric distribution

INTRODUCTION

As previously reported by different authors, exposing food to ionizing radiations damages the DNA of specific microorganisms so effectively that living cells become inactivated and, as a result, the microbiological and parasitological safety of the irradiated products is eventually improved. Moreover, irradiation can also extend the shelf life of certain foods and reduce storage losses from spoilage and pests (*1*−*7*).

In recent years, increased awareness about the benefits of food irradiation has resulted in a growing demand of reliable analytical methods suitable to detect directly in a product whether or not it has been irradiated (or if a nonirradiated foodstuff contains irradiated ingredients). Obviously, advanced technologies also offer new and powerful tools to assess if the irradiation process might occasionally produce adverse effects for human health or if irradiation-induced changes might result in quality degradation and eventually in modifying the characteristic organoleptic attributes of a specific product.

Particularly, as the detection of non-natural enantiomers may be relevant for assessing food quality and, on the other hand, chiral food components may racemize as consequence of technological processes (*8*−*11*), the evaluation of the effect of irradiation on the enantiomeric composition of chiral markers is of special interest. However, this aspect is not usually considered as the similar properties of the enantiomers of a chiral compound make it difficult adequate enantioresolutions of chiral compounds occurring in complex substrates to be obtained (*12*−*14*). Thus, single column chromatographic techniques cannot always provide acceptable separations of all components occurring in complex mixtures and, particularly, may be inadequate to detect the presence of enantiomers in irradiated foods, especially at trace levels.

In this respect, the use of multidimensional gas chromatography (MDGC) was initially proposed to enhance peak capacity by combining two or more separation steps. Concretely, the heart-cutting MDGC technique (*15*, *16*), which involves the transfer of one or more unresolved fraction from the first to the second dimension, has already proven its utility for those separations requiring very high efficiencies (*17*−*20*).

Concerning the sample preparation step which usually must be performed prior to the chromatographic separation to achieve the sensitivity required in the overall analysis, the effectiveness of applying the solid phase microextraction methodology (*21*, *22*) to very different complex matrices has already been widely reported in the literature (*23*−*26*). Interestingly, SPME is a rapid, inexpensive, easily accessible, and easy-to-handle procedure to isolate minor compounds from complex matrices.

The possibility of using efficient techniques for both the sample preparation step and the chromatographic separation itself is of special relevance when determining stereoisomeric distribution of chiral compounds in complex matrices because of the high analytical requirements that must be fulfilled. Moreover, in those cases in which high enantiomeric excesses are observed, the large differences existing between the concentrations of both enantiomers involves an additional difficulty due to the chromatographic overlapping of the minor and the major enantiomers. However, as many enantiomers exhibit different physiological behaviors, it is mandatory to have efficient methods to ensure not only the enantioresolution of the target chiral compounds but also to

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minimize the risk of racemization, which obviously would avoid the reliable determination of the enantiomeric purity.

With a view to provide additional information about the effect of the irradiation process on foods, the objective of this work was to evaluate the usefulness of both solid phase micro-extraction (SPME) and multidimensional gas chromatography coupled to mass spectrometry (MDGC-MS) to study the variation of the stereoisomeric composition of relevant chiral markers occurring in a cheese sample submitted to irradiation at doses ranging from 0 to 8 kGy.

MATERIALS AND METHODS

Samples and Materials. Manchego-type cheese was acquired in a local market and cut into slices of about 2 -mm thickness using an electric machine. Slices were vacuum packaged in 10 cm × 10 cm laminated film bags of low gas permeability (35 cm^3/24 h m^2 bar to oxygen and 150 cm^3/24 h m^2 bar to CO_2) and submitted to ionizing radiations as described below. Standards used for identification purposes were obtained either from Fluka, Buchs, Switzerland (limonene and 2,3-butanediol) or from Merck, Hohenbrunn, Germany (3-hydroxybutanone). In all cases, cheese samples were analyzed in triplicate.

Irradiation and Dosimetry. An electron-beam radiation operated at 10 MeV (IONMED Sterilization SA, Tarancón, Cuenca, Spain) was used as the source of ionizing radiation. Samples were irradiated at different doses (i.e.; 0.5, 1, 2, 4, and 8 kGy) while maintaining the temperature at 19 ± 1 °C. During the irradiation treatment, the product temperature increased less than 2 °C. To confirm the dose received, there was also determined the absorbance of cellulose triacetate dosimeters which had been simultaneously irradiated with the investigated samples (*27*). A sample (labeled as 0 kGy) was left unirradiated to be used as reference control. Irradiated and nonirradiated samples were maintained at −20 °C until analysis.

Solid-Phase Microextraction. A Supelco (Bellefonte, PA) SPME fiber assembly holder and an 85 μm carboxen/polydimethylsiloxane (CAR/PDMS) coated fused-silica fiber were employed. Following the recommendations of the manufacturer, prior to its use, the fiber was conditioned for 60 min at 300 °C in the GC injector port.

After grating the samples to enable volatiles to be released, a 1 g weight of the grated cheese was directly placed into a 5 -mL vial that was sealed with plastic film. The extraction was carried out on a SPME manual holder in the headspace mode (i.e., HS-SPME) during 30 min at 80 °C using a thermostatic silicone bath to control sample temperature. After finishing the extraction step, the target compounds were thermally desorbed into the GC injector (splitless mode) at 250 °C for 2 min and then analyzed by MDGC as explained below.

MDGC-MS Analysis of the Extracts Obtained by HS-SPME. The MDGC equipment included two independent Varian (Palo Alto, CA) gas chromatographs (model CP-3800) housing two columns, namely a precolumn and a main column, which were serially coupled through a Deans based switching system (*15*, *16*) and a transfer line kept at 180 °C throughout the experimentation. The chromatographic analysis was started by thermal desorption of the solutes previously retained onto the SPME fiber as above-mentioned. The preseparation was performed using a 30 m × 0.25 mm i.d. fused-silica capillary column coated with a 0.25 μm layer of ZB-Wax (Micron Analítica, SA, Madrid, Spain). The oven program temperature was started at 40 °C and increased at a rate of 4 °C/min up to 95 °C, then up to 120 °C (2 °C/min) and to 230 °C (4 °C/min). The final temperature was maintained for 10 min. The selected cuts were then transferred into the main column (i.e., to the second dimension) and analyzed on a 25 m × 0.25 mm i.d. fused-silica capillary column coated with a 0.25 μm film thickness of permethylated β-CD (Chirasil-β-Dex, Varian, Middelburg, The Netherlands). In this case, the oven temperature was initially set at 50 °C (15 min) and then successively raised to 70 °C (1 °C/min), to 140 °C (2 °C/min), and finally to 200 °C (4 °C/min). In both dimensions, helium served as the carrier gas at an approximate head pressure of 30 psig in the precolumn and 24 psig in the main column.

Separations resulting from the first and the second dimensions were monitorized using an FID detector operated at 250 °C and a Saturn 2000 ion-trap mass spectrometer Varian, respectively. Data acquisition was performed using a Star Toolbar system acquired from Varian. Identification of the target compounds were performed by comparing the GC retention times observed in both dimensions with those resulting from standards analyzed under identical conditions. Additionally, mass spectra recorded from the standard compounds were also compared with those obtained from the U.S. National Institute of Standards and Technology (NIST) library. For the MS, the electron multiplier was set to 1850 V and ionization was accomplished by electron impact (EI). The temperatures of the transfer line, the manifold, and the trap were fixed at 180, 120, and 220 °C, respectively. The recorded spectra covered the range from 40 to 650 m/z.

Satisfactory blanks between consecutive runs were obtained for the complete procedure when applying the experimental conditions above-described.

RESULTS AND DISCUSSION

Chiral Aroma Compounds. To exemplify the potential of the proposed procedure based on the use of SPME and MDGC-MS to perform the stereoisomeric analysis of chiral compounds occurring in cheese irradiated at different doses, we focused our work on to optimize the separation of three chiral compounds representative of key odorants occurring in various cheese types. Specifically, we aimed to design a rapid and reliable method to detect the stereoisomeric composition of limonene, 3-hydroxybutanone, and 2,3-butanediol due to their contribution in cheese aroma (*28*−*30*). The former has been reported to be a relevant terpenoid in different types of cheese, while the two latter can result from the transformation, caused by the enzymatic activities of different microorganisms, of diacetyl (considered as a potent odorant in cheese) into more reduced compounds, namely 3-hydroxybutanone, 2,3-butanediol, butanone, and finally 2-butanol.

Obviously, the fingerprint pattern of flavor compounds depends on each particular cheese, but it is widely recognized that a high number of important odor-active compounds, occurring in many types of cheese, belong to terpenes, alcohols, or ketones among other chemical classes. Thus, as many enantiomers show different biological activities, the chirospecific analysis of terpenes, alcohols, and ketones having chiral centers must be necessarily accomplished when assessing different aspects related to food safety and quality. Nevertheless, at present, no data are available concerning the effect of electron-beam irradiation on the stereoisomeric distribution of the above-mentioned compounds in irradiated foods. Precisely for that reason, we considered the opportunity of the present study to specifically evaluate new aspects regarding the highly demanded stringent control of irradiated foods.

SPME-MDGC-MS Analysis. The experimental conditions used for the SPME procedure, concretely the CAR/PMDS fiber, temperature, and sorption exposure times were initially established as proposed by other authors (*29*) for the analysis of achiral volatile compounds in unirradiated cheese although some modifications were introduced (mainly related to the desorption conditions) according to the specific requirements of the intended direct stereodifferentiation of the target chiral compounds.

Figure 1 shows the GC separations obtained in the precolumn of the MDGC system from the SPME extracts of cheese left unirradiated and irradiated at the highest dose applied (i.e., 8 kGy). In both chromatograms, it is evident that the complexity of the sample makes it difficult for the resolution of various groups of compounds to be attained in the first dimension. In fact, in most cases, the peak shape suggests the coelution of various components and, consequently, the low probability of eventually achieving an acceptable enantioresolution of chiral compounds by using a unidimensional GC system.

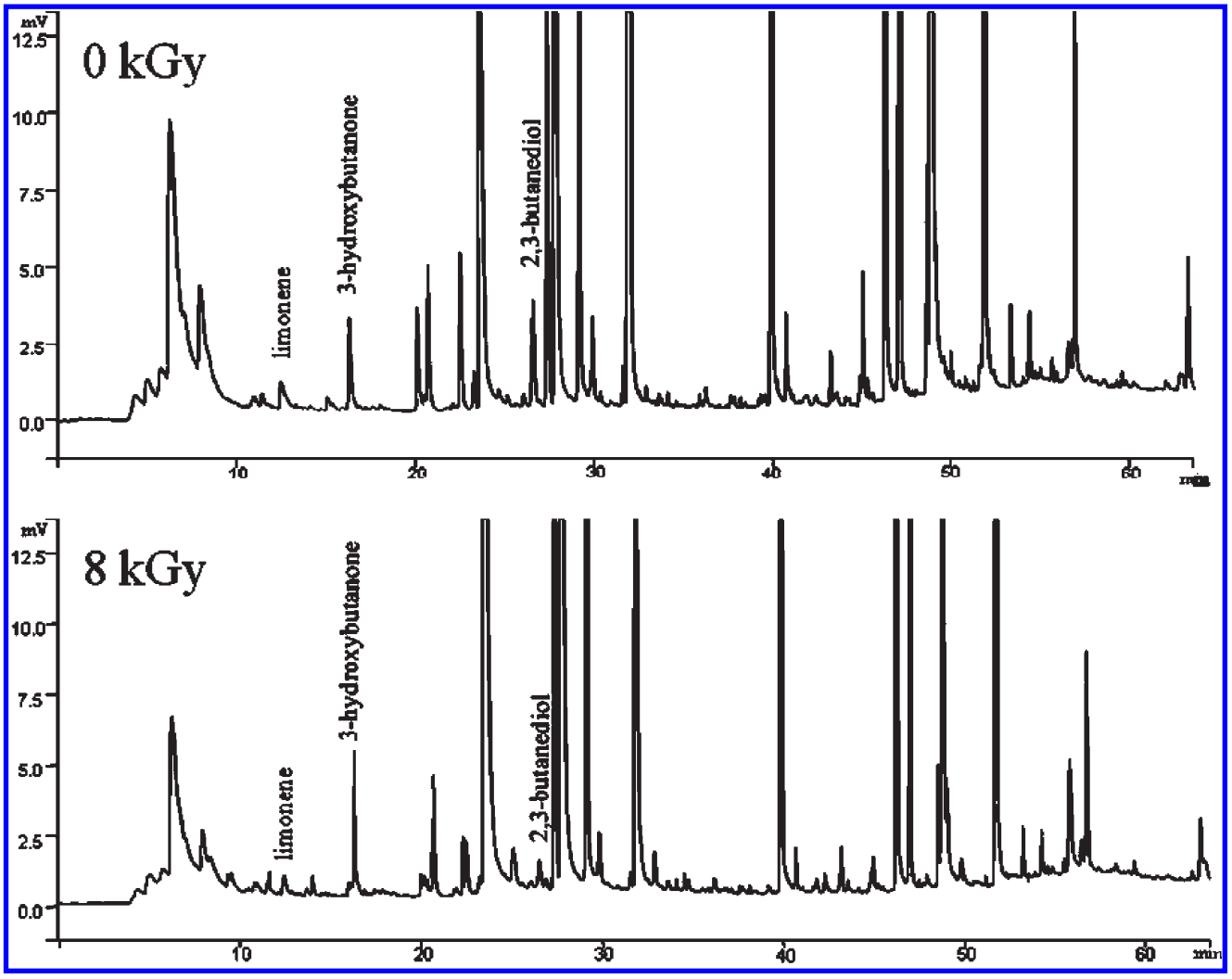

Figure 1. Gas chromatograms achieved in the precolumn of the MDGC system from the SPME extracts of cheese either left unirradiated or irradiated at 8 kGy. Compounds were tentatively identified by comparison of their retention times with those of the corresponding standards.

However, as shown in **Figure 2**, the optimization of the experimental variables involved in the chromatographic separation in both the precolumn and the main column of the MDGC system allowed us satisfactory resolutions to be finally obtained for the *R* and *S* enantiomeric forms of limonene (see GC trace in **Figure 2a** to prove the resolution of both standards) and 3-hydroxybutanone as well as for the three stereoisomers of 2,3-butanediol, namely *R*,*R*, *S*,*S*, and *R*,*S* (*meso*) forms. Interestingly, the working conditions detailed in the Materials and Methods were established to allow the transfer of three specific cuts of limited broadness eluting from the precolumn between 12.7 and 13.1 min, 15.9 and 16.4 min, and 26.9 and 29.4 min. From the chromatograms showing the separation achieved in the main column (**Figure 2c**), it can not only be recognized the tremendous increase in resolution that can be achieved when transferring the selected cuts from the precolumn (**Figure 2b**) into the main column but also the possibility of significantly reducing the overall analysis time. Actually, as demonstrated in **Figure 3**, the three selected cuts can be successively transferred from the precolumn to the main column of the multidimensional system in such a way that in a single analysis, which takes less than 90 min, the intended stereoisomeric analysis of the three target chiral odorants can be simultaneously performed. Furthermore, the separation obtained in the second dimension also allowed us to detect other compounds (see **Figures 2** and **3**) whose occurrence in the analyzed sample could not be suspected from results obtained in the first dimension as the transferred cuts apparently contained exclusively the target compounds. The mentioned **Figure 3** also includes the stereodifferentiation observed for the target compounds in all samples irradiated in the dose interval under study (i.e., 0−8 kGy).

Effect of Irradiation on Enantiomeric Excesses of Chiral Compounds. Table 1 gives the enantiomeric excesses (i.e., the excess of the major enantiomer over the minor enantiomer quoted as a percentage) obtained for limonene and 3-hydroxybutanone as well as the percentage values calculated for 2,3-butanediol stereosiomers from the SPME-MDGC-MS analysis of the irradiated samples included in this study. As can be seen, the chirospecific analysis indicates that limonene always occurs exclusively as the *R*-form irrespective of the irradiation dose applied to the sample while both *R*- and *S*- enantiomers of 3-hydroxybutanone were detected in all instances. From the results reported in **Table 1**, it also arises that concerning the stereoisomeric distribution of 2,3-butanediol, the *R*,*S* form is the prevalent, followed by the *R*,*R* and minor amounts of the *S*,*S* stereoisomer. In any case, it is clear that significant alterations of the stereoisomeric distribution of the target chiral compounds do not occur during irradiation of the cheese sample studied. In this respect, it is interesting to point out that RSD values (calculated from three replicates) obtained for the enantiomeric excesses of 3-hydroxybutanone was less than 2.5%, while 9.6%, 11.1%, and 7.7% were observed for the

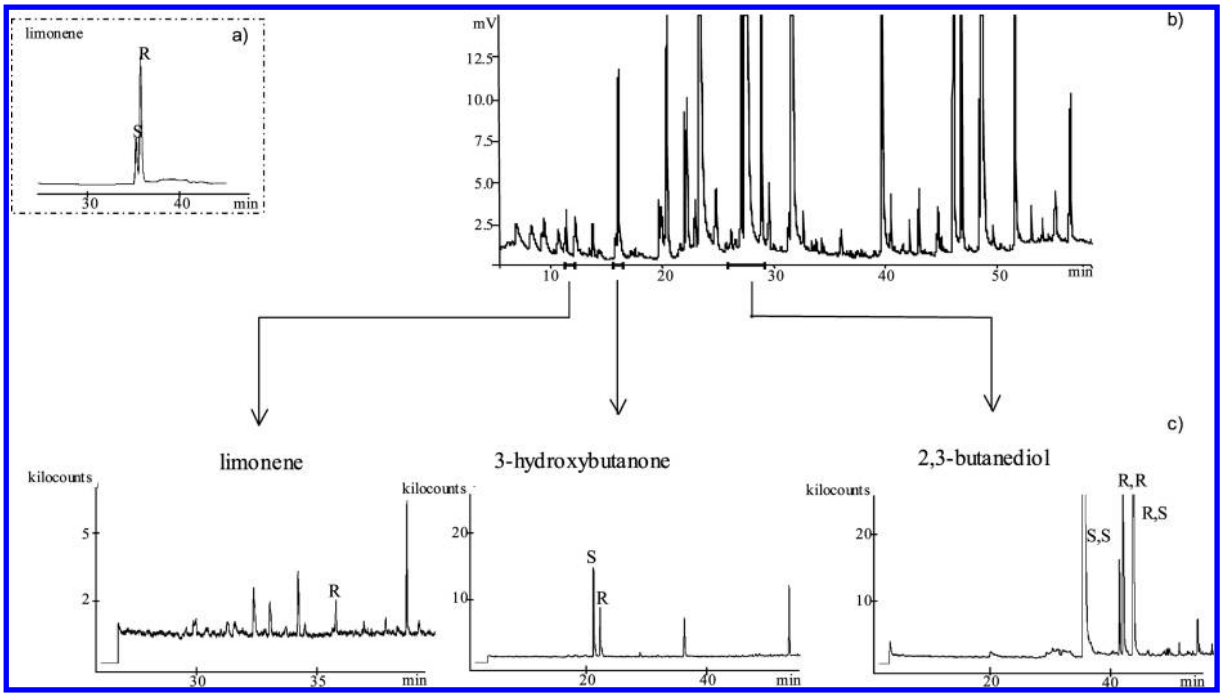

Figure 2. GC trace obtained in the main column for *R*- and *S*-limonene standards (**a**), GC separation resulting from the precolumn of the MDGC system from the SPME extract of cheese irradiated at 2 kGy (**b**), and stereodifferentiation of limonene, 3-hydroxybutanone, and 2,3-butanediol by transferring the selected cuts to the main column (**c**).

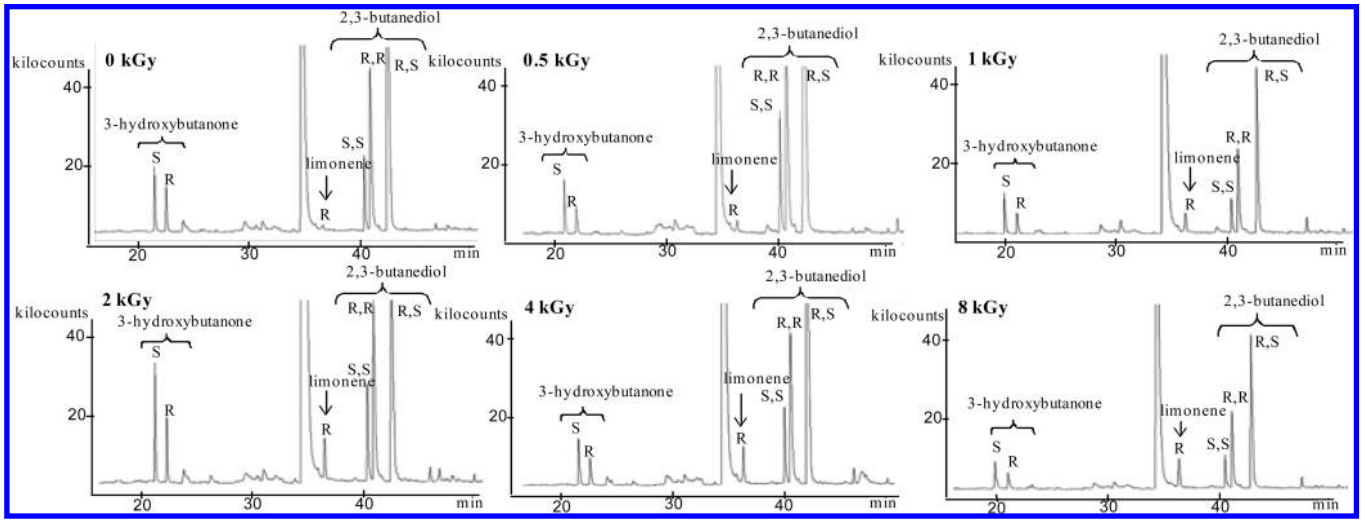

Figure 3. Chiral resolution by SPME-MDGC-MS analysis of limonene, 3-hydroxybutanone, and 2,3-butanediol in cheese samples irradiated at 0, 0.5, 1, 2, 4, and 8 kGy. The chromatograms show the separation obtained in the second dimension of the system.

Table 1. Stereoisomeric Distribution of Limonene, 3-Hydroxybutanone, and 2,3-Butanediol in Irradiated Cheese by SPME-MDGC-MS Analysis

irradiation dose (kGy)	*R*-limonene (ee, %)[a]	*S*-3-hydroxybutanone (ee, %)[a]	2,3-butanediol		
			(% *R*,*R*)	(% *S*,*S*)	(%*R*,*S*)
0	100	87	29	8	63
0.5	100	66	26	9	65
1	100	67	27	10	63
2	100	71	34	12	54
4	100	78	27	9	64
8	100	81	25	10	65

[a] ee: enantiomeric excess = ((predominant enantiomer − minor enantiomer)/(predominant enantiomer + minor enantiomer)) × 100.

determination of percentage values of *S*,*S*, *R*,*R*, and *R*,*S* forms of 2,3-butanediol, respectively.

Summarizing, the fact that the irradiation treatment itself does not seem to alter the stereoisomeric composition of the investigated compounds is interesting because data obtained precludes the occurrence of non-natural enantiomers in the irradiated samples.

As specific cheese flavor profiles result for the synergistic effect of a number of compounds occurring at levels that arises from the balance between its production and its degradation and, on the other hand, irradiation treatment involves a microbiological modification that may damage specific microorganism patterns, the use of the proposed SPME-MDGC-MS analysis to control possible changes (e.g., those causing racemization of chiral compounds) in the naturally prevalent stereoisomers occurring in unirradiated samples may be relevant when studying irradiated foods.

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