Analysis of Free and Esterified Ergosterol in Tomato Products

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A new extraction and chromatographic procedure to quantify free and esterified ergosterol in tomato products was devised. The extraction solution was composed of a dichloromethane/methanol mixture in a 2:1 (v/v) ratio. This extraction solvent allowed for higher ergosterol recovery from tomato products (an average of 25% more) compared to hexane, which is frequently employed for ergosterol extraction. Both free and esterified ergosterol were determined by HPLC reverse-phase chromatography employing a Nova-Pak C-18 column (300 × 3.9 mm), filled with 4 mm average particle size and a guard column of the same material. The elution was performed at a flow rate of 1 mL·min^{-1} with a linear gradient of solvent A (methanol/water, 80:20, v/v) and solvent B (dichloromethane). The gradient, starting at sample injection, was from 0 to 50% B for 20 min for the free ergosterol analysis and additional 15 min at 50% B to analyze the ergosterol esters. This technique has proven to be more sensitive for ergosterol determination than other reported chromatographic procedures. Moreover, ergosterol esters, extracted from various fungal sources, separated well and were easily quantified.

Keywords: *Ergosterol; tomato; fungal contamination; microbiological quality*

INTRODUCTION

Fungal contamination of food, generally derived from poor manufacturing practices utilizing molded plant material, is usually determined by microbial counting techniques, such as the Howard mold count method utilized for tomato products (Howard, 1911). These methods suffer from wide subjectivity and require special training and experienced microscopists. Moreover, during industrial operations of product transformation, such as grinding or homogenization, modifications of microbial population occur that may alter the analytical results (Blander et al., 1987). Many substances have been proposed as markers of fungal contamination. One of these, for example, is chitin (Ride and Drysdale, 1972; Mislivec et al., 1987; Ekblad et al., 1998), a component of the fungal cell wall, which therefore can be used as an indicator of mold contamination. When subjected to acid hydrolysis, chitin releases glucosamine, which can be assayed with various methods. However, the results show low analytical sensitivity and reproducibility along with low specificity for fungal contamination. Recently, many papers have been published about the use of ergosterol as a general indicator of fungal contamination (Richardson and Logendra, 1997; Miller and Young, 1997; Gessner and Schmitt, 1996). In fact, ergosterol is a constituent of the fungal cell membrane and therefore can be a useful marker of mold contamination in fresh or transformed food. It has been observed that there is a poor correlation between the total ergosterol content and the Howard value (Grasselli et al., 1993; Battilani et al., 1996). Instead, the correlation between the content of rotten tomatoes with total ergosterol is much higher than with the Howard value, and a limiting content of 15 mg of total ergosterol/g of dry sample has been proposed as an acceptability limit of tomato products (Bertoni et al., 1994). Because part of the ergosterol is released from the fungal cell membrane, it may be of interest to carry out a chromatographic method to separate and quantify esterified and free ergosterol, which could be diagnostic of fungal species contamination. Free egosterol is easily analyzed by an isocratic chromatographic procedure proposed by Schwardof and Muller (1989). Unfortunately, this method, although fast, is unable to separate and quantify free and esterified ergosterol. Here we report a procedure for an efficient extraction of the ergosterol and its esters from tomato products. Moreover, a chromatographic analysis that allows separation of ergosterol and its esters with high sensitivity is described.

MATERIALS AND METHODS

Microbial Growth. Because ergosterol esters are not commercially available, three species of fungi, that is, *Saccharomyces cerevisiae*, *Aspergillus niger*, and *Penicillum solitum*, from the collection of the Stazione Sperimentale Industria Conserve Alimentari of Angri were chosen as sources of ergosterol esters. The microorganisms were grown in malt extract agar at pH 4.2 at 25 °C for 4 days. The cellular suspensions, containing 10^5-10^6 cells/mL, were centrifuged at 10000*g* for 15 min at 4 °C and then lyophilized for 24 h.

Tomato Samples. Three lots of tomato puree from three different firms were purchased in a local supermarket for the

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determination of free and esterified ergosterol in tomato products. Each lot was made up of 12 bottles. The product concentrations in the three lots, expressed as optical residue (OR), were very similar and of ~8 °Brix.

Total Ergosterol Assay. Total ergosterol was determined according to the Schwardorf and Muller (1989) chromatographic method in the alkaline-hydrolyzed tomato purees prepared as described by Grasselli et al. (1993).

Simultaneous HPLC Assay for Free and Esterified Ergosterol. Twenty to fifty grams of tomato pulp, after centrifugation at 10000*g* for 15 min, was quantitatively transferred into a separatory funnel to which was added 100 mL of dichloromethane/methanol (2:1, v/v). After 10 min of shaking and phase separation, the lower phase was collected and vacuum concentrated to 1−5 mL, which was determined on the basis of ergosterol content. The HPLC analyses were conducted on a Waters 600 E chromatograph equipped with autosampler and UV detector set at 282 nm, at which ergosterol shows an absorption maximum. The data were collected by a computer and processed by Waters Millenium software. Ergosterol was quantified by an external standard method by using an ergosterol solution at known concentration. The analyses were made on a reversed phase Nova-Pak C18 column (300 × 3.9 mm i.d.) filled with 4 μm average particle size, with a guard column of the same material. The elution was performed at a flow rate of 1 mL·min^{-1} with a linear gradient of solvent A (methanol/water, 80:20, v/v) and solvent B (dichloromethane). The gradient, starting at sample injection, was linear from 0 to 50% B in 20 min; the elution was protracted for an additional 15 min at 50% B when esterified ergosterol was to be determined. To demonstrate the presence of esterified ergosterol in the peaks eluted after that corresponding to free ergosterol, 1 mL fractions were collected and vacuum concentrated. The residues were hydrolyzed with 200 μL of 1.5 M KOH in absolute methanol in sealed ampules at 50 °C for 30 min. After cooling, the samples were neutralized with 200 μL of 1.5 M acetic acid in absolute methanol. The precipitate, when present, was eliminated by centrifugation in Eppendorf vials. The neutralized solutions were analyzed for free ergosterol content.

Table 1. Comparison of Ergosterol Extracted from Fungal Cells with Hexane and with a Dichloromethane/Methanol (2:1, v/v) Mixture[a]

cell type	hexane	dichloromethane/methanol
S. cerevisiae	0.61 ± 0.04	0.74 ± 0.04
A. niger	1.39 ± 0.04	1.80 ± 0.05
P. solitum	2.08 ± 0.06	2.85 ± 0.08

[a] Values are in g·kg^{-1} of cell dry weight. The results represent the mean of four replications.

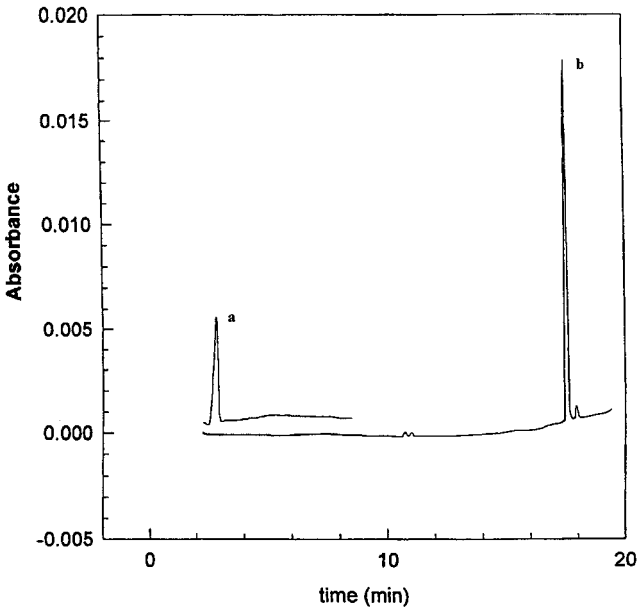

Figure 1. Ergosterol elution profiles obtained by HPLC on the silica Lichrosorb Si 60 column (chromatogram a) and on the reverse-phase Nova-Pak C18 column (chromatogram b) as described under Materials and Methods. The two analyses were conducted by injecting 20 μL of a 5 ppm solution of ergosterol in hexane.

RESULTS AND DISCUSSION

The efficacy of ergosterol extraction methods depends mainly on the ergosterol source to which they are applied (Gessner and Schmitt, 1996; Eash et al., 1996; Young and Games, 1993; Young, 1995). The extraction efficiency of ergosterol from a complex vegetable matrix, such as a tomato product, is of prime importance in establishing the threshold value for product acceptability. In the case of tomato, Grasselli et al. (1993) utilized hexane as an extraction solvent and reported a recovery of ~90% when ergosterol was added as an internal standard. However, this approach may be incorrect, considering that ergosterol is a component of the fungal cell membrane to which it is strongly bound. Therefore, the recovery of ergosterol, added to the product, could be much easier than that of the endogenous ergosterol. For this reason, we compared the extraction procedure that utilized hexane to extract ergosterol from tomato products with an extraction solvent utilizing a mixture made up of dichloromethane/methanol (2:1, v/v). The solvent extraction procedures were compared by subjecting the same cellular dry mass of three species of fungi, grown and harvested as reported under Materials and Methods. Equal volumes of the extraction solvents were then analyzed for ergosterol content by HPLC chromatography according to the Schwardof and Muller method (1989). The analyses were conducted by using ergosterol as an external standard. From the ratio of the ergosterol peaks obtained by analyzing the samples from the two procedures, a higher free ergosterol recovery, of ~25%, was observed with the dichloromethane/methanol extraction, for each of the three fungal species (Table 1).

To achieve ergosterol separation from its esters, reversed phase HPLC was used as outlined under Materials and Methods. In fact, when attempts were made to separate ergosterol from its esters using the Lichrosorb Si 60 column with the isocratic elution conditions reported by Schwardorf and Muller (1989), no separation was observed. As a result, we chose to employ an RP-HPLC column, using an elution gradient of methanol/H_2O (80:20, v/v) and dichloromethane as described by Servillo et al. (1997). A linear gradient was found to give the best result, with a retention time for free ergosterol of ~17 min. The peak elution pattern was very different in these conditions with respect to that obtained with the method of Schwardorf and Muller (1989). The retention times of the various substances were higher. However, because they were not eluted isocratically, but with a rather fast gradient, they emerged from the column as well-separated and sharp peaks with a noticeable increase in analytical sensitivity. As an example, ergosterol standards were analyzed in both conditions. As reported in Figure 1, the peak height obtained with the elution gradient is 3 times higher with respect to the isocratic elution. Because ergosterol esters are not commercially available, three fungal species were employed to obtain ergosterol esters

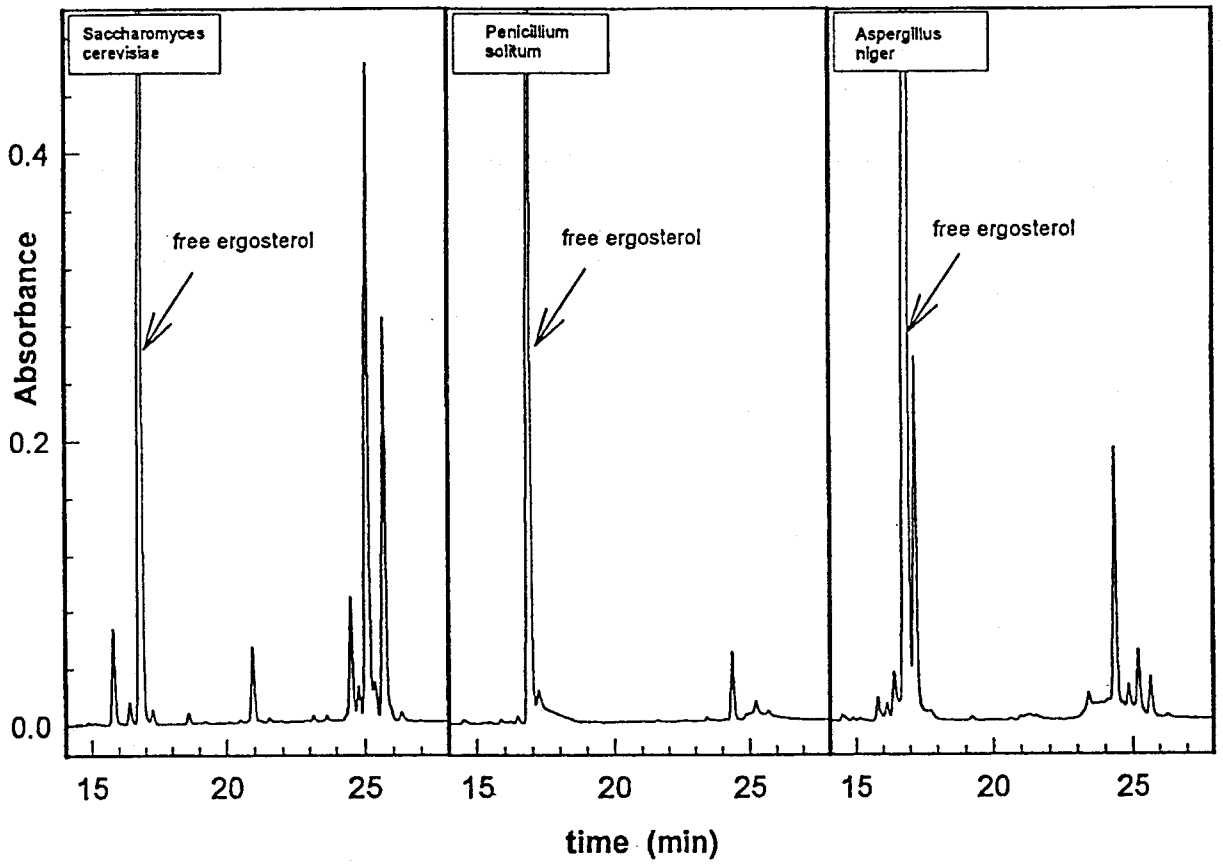

Figure 2. Free ergosterol determination in the cell wall of three fungal species. The chromatographic analysis was performed with a Nova-Pak C18 column.

to use as standards to find the chromatogram region where they are eluted. Cells of *S. cerevisiae*, *A. niger*, and *P. solitum* were extracted with dichloromethane/methanol (2:1, v/v). The solutions were dried, resuspended in hexane, and chromatographed. In Figure 2 the chromatograms from the three species are reported. All of the samples show a peak eluted at ~17 min, corresponding to free ergosterol, and two groups of peaks at ~21 min and between 23 and 26 min. The ergosterol presence was confirmed by collecting the putative ergosterol peak and taking the absorption UV spectrum in the region 260−330 nm, where it shows the characteristic absorption bands. To detect the ergosterol esters, fractions were collected every minute from 20 to 30 min. The UV-absorbing fractions also showed the characteristic spectrum of ergosterol in the 260−330 nm region. These fractions were dried, hydrolyzed, and chromatographed again. Figure 3 reports the chromatograms obtained from the pooled fractions of a sample from *S. cerevisiae*. The relative abundances of free to esterified ergosterol, which were calculated from the peak area by assuming the same extinction coefficient at 282 nm for ergosterol and its esters, were different among the various species. As reported in Table 2, free ergosterol was higher for *Penicillium* compared to *Aspergillus* and *Saccharomyces* species.

On the basis of the above results, three lots of tomato puree were analyzed. The results, which represent the mean of the values obtained from each of the 12 bottles constituting the single lot, are reported in Table 3. Column 5 of Table 2 reports the values obtained by extracting the alkaline-hydrolyzed tomato purees with hexane and analyzing according to the Schwardorf and Muller (1989) method.

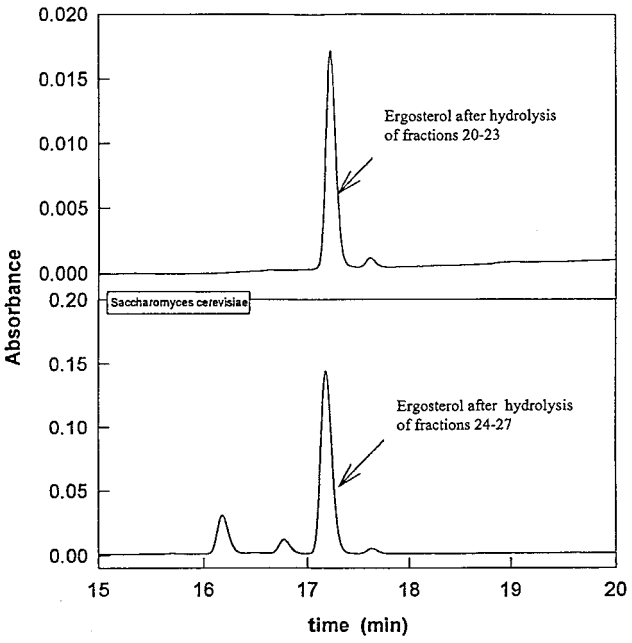

Figure 3. Determination of lipid-bound ergosterol in *S. cerevisiae* after alkaline hydrolysis. Fractions eluted between 20 and 23 min and between 23 and 27 min (see chromatogram of *S. cerevisiae* in Figure 2) were hydrolyzed and subjected to HPLC analysis as described under Materials and Methods.

Table 2. Free and Esterified Ergosterol Extracted from Fungal Cells with a Dichloromethane/Methanol (2:1, v/v) Mixture[a]

cell type	free ergosterol	esterified ergosterol
S. cerevisiae	0.73	0.103
A. niger	1.77	0.010
P. solitum	2.83	0.017

[a] Values are in $g \cdot kg^{-1}$ cell dry weight.

Table 3. Content of Free and Esterified Ergosterol in Tomato Samples[a]

	optical residue (°Brix)	free ergosterol	ergosterol esters	ergosterol total (S&M)
lot 1	8.1	1.11 ± 0.21	0.14 ± 0.05	0.96 ± 0.04
lot 2	7.8	0.32 ± 0.12	nd	0.22 ± 0.06
lot 3	7.9	1.60 ± 0.30	0.27 ± 0.06	1.31 ± 0.32

[a] Values are in ppm. Column 5 reports the total ergosterol content obtained by extracting the alkaline-hydrolyzed tomato samples with hexane and analyzing according to the Schwardorf and Muller method (S&M). nd, not determined.

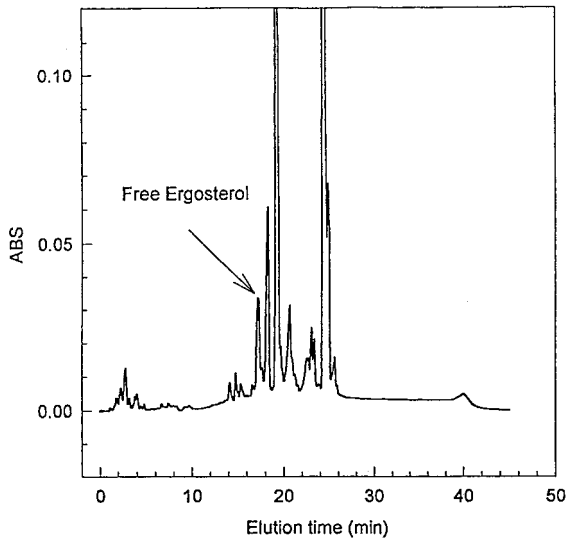

Figure 4. Free ergosterol determination in commercial tomato puree.

A typical chromatogram obtained by analyzing a sample of tomato puree is reported in Figure 4. Besides the ergosterol peak, it shows various peaks with retention times above 20 min. To demonstrate the presence of ergosterol esters, four successive injections of 100 μL were made and, after 20 min of elution, 1 mL fractions were collected every minute. The four fractions corresponding to the same elution time were pooled, dried, subjected to alkaline hydrolysis to release ergosterol from its esters, and finally analyzed again. The results are reported in Figure 5. As one can see, only some fractions contained ergosterol esters. Particularly, only the pooled fractions at 21−22, 23−24, and 24−27 min showed the presence of free ergosterol after alkaline hydrolysis. From the analyses of the three pools, the esterified ergosterol content constituted ∼15% of the total ergosterol.

From the above results, the proposed analytical procedure appears to improve the reliability of ergosterol determination as an index of fungal contamination of tomato products. The chromatographic analysis, although more time-consuming than other methods, shows a higher sensitivity than previous methods. In addition, both ergosterol and its esters can be analyzed

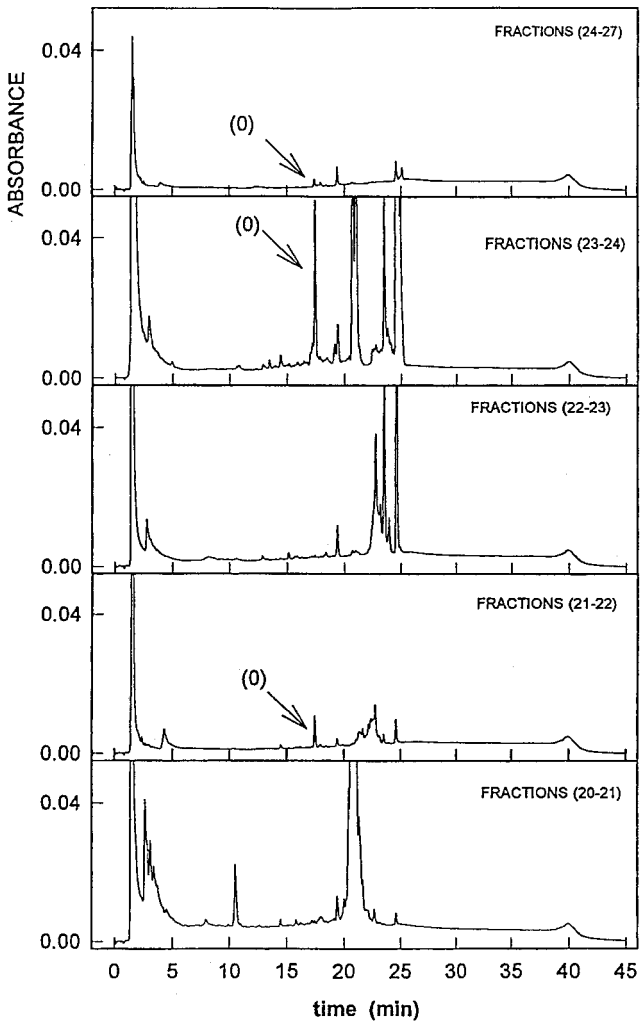

Figure 5. Determination of lipid-bound ergosterol in tomato puree after alkaline hydrolysis. The indicated fractions, obtained according to the chromatogram of Figure 4, were hydrolyzed and subjected to HPLC analysis as described under Materials and Methods. The symbol (0) represents the ergosterol peak.

simultaneously. Moreover, the elution pattern and the identification of the ergosterol esters could give indications about the microorganisms contaminating the product.

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