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Ergosterol as a new quality parameter together with patulin in raw apple juice produced from decayed apples

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Abstract

The raw apple juice samples produced from surface-decayed apples as sound, 30%, 60% and 100%, were tested for pH, Brix, patulin and ergosterol concentrations. The patulin concentrations in juice samples produced from golden delicious apples, that are sound, 30%, 60% and 100% decayed, ranged from 0.3–2.0, 139–23, 504–630, and 889.6–940.2 $\mu g l^{-1}$, respectively. The ergosterol concentrations in the same juice samples ranged from 0.1–1.8, 14.4–34.3, 27.2–75.8 and 95.7–131 $mg l^{-1}$. When juice samples were analyzed, decay proportion was linearly correlated to patulin ($r = 0.99$) and ergosterol ($r = 0.99$) but not to pH and Brix. However, a linear correlation ($r = 0.98$) between patulin and ergosterol was determined in juice samples with all the decay proportions.

Keywords: Decayed apple; Ergosterol; HPLC; Juice; Patulin

1. Introduction

When a fungal biomass cannot be separated from a solid substrate, a growth of the fungus can be monitored by measuring a chemical component. Ergosterol has not been widely used to monitor fungal growth, even though it is the predominant sterol component of most fungi and is either absent or a minor constituent in most higher plants. Ergosterol, a major fungal sterol, was reported by Seitz, Sauer, Burroughs, Mohr, and Hubbard (1979) as a relatively specific product of fungi, using a straightforward method, suggesting that ergosterol is a measure of fungal growth in plant materials.

In bacteria, ergosterol is present only in traces; the amounts of sterols found in some bacteria are never higher than 0.01% of cell dry weight and only a very small part of this percentage is represented by ergosterol. So, ergosterol is only a minor component of the sterol mixture of several plants and animals; therefore its occurrence, if any, in tomato products is to be ascribed almost exclusively to the presence of moulds (Bocchi, Ghiretti, Sandei, Spotti, & Leoni, 1995; Ghiretti et al., 1995). For that reason, ergosterol, a constituent of the cell wall of some important parasites, such as molds, has been recently recognized as a potential objective parameter, useful for the characterization of the quality of processing tomatoes (Grasselli, Leoni, Sandei, & Mori, 1993). However, no study on ergosterol as a potential objective parameter for characterization of the quality of apple products has been attempted. Major molds isolated from rot areas of apples are *Penicillium expansum*, *Monilla* spp., *Mucor* spp., *Cladosporium* spp., *Byssochlamys* spp., *Aspergillus* spp., and *Penicillium* spp. (Swanson, 1989). On the basis of the different specific biosyntheses and growth kinetics, ergosterol total production/day of mycelium growth was significantly higher in some of the most important tomato-contaminating moulds (*Rhizopus oryzae*, *Penicillium chrysogenum*, *Aspergillus oryzae*, *Mucor spinescens*, *Botrytis cinerea* and *Alternaria alternate*) (Ghiretti et al., 1995) and can be reliably and rapidly determined by HPLC (Seitz, Mohr, Burroughs, & Sauer, 1977).

Patulin [4-hydroxy-4 H-furo (3,2c)-pyran-2-(6H) one], a mycotoxin produced by several species of *Aspergillus*, *Penicillium* and *Byssochlamys* fungi, is often detected in apples. Studies with fungi or animals have

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demonstrated that patulin is mutagenic, carcinogenic and teratogenic. Consequently, patulin is an important quality parameter in apple and apple products for human health (Lai, Fuh, & Shih, 2000). The World Health Organization (WHO) and many countries such as Turkey, Sweden, Belgium, Russia and Norway, have established 50 $\mu g\,l^{-1}$ patulin as the upper allowable-concentration in apple juice (Prieta, Moreno, Blanco, Suarez, & Dominguez, 1992).

Apple juice plants start buying apples from the middle of September and apple juice processing continues until the end of January. Some of the apples bought during this period are processed. The rest are stored in open piles. The storage condition of the raw material is typical of juice production for the low capacity plants in Turkey. As a result of this storage period, apple juice yield decreases and the concentration of patulin in apple juice increases due to microbiological load (Kadakal & Nas, 2003).

No study has been carried out to determine the effects of decay proportion on ergosterol concentration. Therefore, the effect of the decay proportion on patulin and ergosterol concentration of apples needs to be determined. For other reasons, the poor microbiological quality of raw material may have a significant influence on the ergosterol and patulin concentrations of raw apple juice. It is thought that statistical examination of these values would be useful in order to understand any relationship. So, in this study, we tried to ascertain whether there is any correlation between patulin and ergosterol concentration or, at least, whether it is possible to find a correlation between ergosterol concentration and apple decay proportion. The other aim of this study was to determine whether measurement of ergosterol concentration in the product could be useful for the assessment of apple products as related to the decay proportion and hence the quality of the raw material (e.g., patulin or not).

2. Materials and methods

2.1. Material

2.1.1. Sampling and preparation procedures

In this research the apples (*Malus domestica* cv "Golden delicious") used for the production of raw apple juice were obtained from a well-established local factory (Çal town in Denizli, Turkey). On each sampling day, 20 kg of apples were obtained randomly for every decay group when the contents of each truck container of the day were transferred to the receiving pool (each day approximately 50 containers in the factory yard). Each apple group was transferred to the laboratory and processed for raw apple juice. Naturally decayed apples (colonized visibly by mold) were sorted as sound, 30%, 60%, and 100%, based on the surface ratio of mold growth and decay to apple whole surface. In order to estimate 30% and 60% of decayed ones, apples were classified by marking on the fruit surface of the decay proportion after dividing them into ten equal parts with a colour pen. Each individual apple was examined closely enough to state that its surface was 0%, 30%, 60% or 100% decayed. The sound and 100% decayed apples were separated visually. Eight different samplings for each decay group were carried out during 8 days to obtain sound and 30%, 60% and 100% decayed apples.

2.1.2. Production of raw apple juice

The apples were cut into quarters with stainless steel knives, crushed (Beko model BKK 1146, İstanbul, Turkey) and then pressed in a cloth bag using a hydraulic press (R Gulch Products model H, Mokelumne Hill, CA, USA) to get raw apple juice. The raw apple juice samples were analysed for their pH, Brix, patulin and ergosterol concentrations, and were frozen at −20 °C until analysis.

2.2. Methods

2.2.1. Ergosterol determination

The HPLC method of Schwadorf and Muller (1989), modified by Ghiretti et al. (1995), was used for the determination of ergosterol in the samples. Ergosterol in the crystalline form (analytical reagent grade) was obtained from Sigma (Sigma–Aldrich Chemie GmbH, Deisenhofen–Germany). Twenty millilitres of raw apple juice were saponified with 75 ml of methanol, 50 ml of ethanol and 10 g of potassium hydroxide. The mixture was boiled for 1 h and the resultant reflux was filtered, followed by separation in a separatory funnel, with water and *n*-hexane (water/hexane; 1:2) and shaking for 1 min. After that, the lower layer was collected into an Erlenmeyer flask and the upper one was filtered over anhydrous Na_2SO_4. The lower layer was re-transferred to the separatory funnel and shaken for 2 min after adding 50 ml of *n*-hexane, followed by filtration over Na_2SO_4. The combined organic extracts were evaporated using a rotary evaporator to approximately 1 ml and transferred into a 10 ml test tube, and the contents of the tubes were evaporated to dryness at 40 °C (heating block) under a gentle stream of nitrogen. The residue was dissolved in 5 ml *n*-hexane and a 20 μl portion of the solution was injected into the HPLC for the analysis. The mobile phase was *n*-hexane and isoamyl alcohol (95/5; v/v) with a flow rate of 2 $ml\,min^{-1}$. For the analysis, a Nucleosil 100-7 C18 (250X4.6 ID mm) column, a photodiode array detector (Shimadzu, model SPD-M10 Avp) set at 282 nm, a LC-10AT-VP Shimadzu HPLC pump, a column oven (Shimadzu, CTO-10AS) set at 25 °C and a software programme (Shimadzu) were used. The sample (20 μl) was injected with a syringe (Hamilton Co., Reno, NV,

USA) into the HPLC system. Coefficient of determination (r^2) was found to be 99.5% for ergosterol. The detection limits ($S/N = 3$) (Hua-Bin & Feng, 2000) ranged from 0.1 to 0.5 mg l^{-1}.

2.2.2. Recovery of ergosterol

In the recovery experiment, juice samples, for which ergosterol concentrations were predetermined, were spiked with the different concentrations of ergosterol, using aliquots at 1, 5, 10, 25, 50 mg l^{-1}, to determine the recovery of the extraction procedure in the initial step. Three determinations were carried out for each addition level.

2.2.3. Patulin determination

The determination of patulin in the samples was carried out by using a Shimadzu Class-VP V5.01 high pressure liquid chromatography apparatus (Shimadzu Corp., Kyoto, Japan), as suggested by ISO (1993). Five millilitres of raw apple juice were extracted with 5 ml of ethyl acetate for at least 1 min. The extraction was repeated twice more using 5.0 ml portions of ethyl acetate. Then, three ethyl acetate phases were combined and extracted with 2.0 ml of sodium carbonate (14 g l^{-1}) solution. The carbonate phase was extracted with another 5.0 ml portion of ethyl acetate which was combined with the preceding portions. After adding five drops of acetic acid (glacial), the ethyl acetate extracts were mixed evaporated using a vacuum evaporator until 1–2 ml remained. The solution was transferred quantitatively to a vial of about 5 ml capacity, using several portions (about 1 ml each) of ethyl acetate, and evaporated to dryness under a stream of nitrogen at about 40 °C. The residue was dissolved in 500 μl of mobile phase. Patulin in the crystalline form (analytical reagent grade) was obtained from Sigma (Sigma–Aldrich Chemie GmbH, Deisenhofen–Germany). Patulin was extracted from samples of the juice with three times the equivalent volume of ethyl acetate. The mobile phase employed was a 10% aqueous acetonitrile solution with a flow rate of 1.0 ml min^{-1}. For the analysis, a 250×4.6 mm C-18 column (Macherey Nagel, Düren, Germany), a column oven (Shimadzu, Model CTO-10ASVP), a photodiode array detector (Shimadzu, Model SPD-M10AVP-UV-VIS) set at 276 nm, a degasser (Shimadzu, Model DGU 14A), liquid chromatography pump (Shimadzu, Model LC-10AT-VP), and a software programme (Shimadzu) were used. The sample (20 μl) was injected with a syringe (Hamilton Co., Reno, NV, USA) into the HPLC system. Coefficient of determination (r^2) was found to be 99.1% for patulin. The detection limits ($S/N = 3$) (Hua-Bin & Feng, 2000) ranged from 0.1 to 0.5 μg l^{-1}.

2.2.4. Recovery of patulin

Juice samples containing known amounts of patulin were spiked with the different concentrations (25, 50, 75, 100 and 200 μg l^{-1}) of patulin to determine the recovery of the extraction procedure. Three determinations were carried out for each addition level.

2.2.5. Brix and pH

Brix (the soluble solids) from raw apple juice were determined by refractive index measurements using a digital refractometer (RFM, Model 340, İstanbul–Turkey). The pH was measured with a pH meter (WTW GmbH & Co, Model 537, Weilheim-Germany). Results were expressed as the average of duplicate samples. Analyses for Brix and pH were performed according to AOAC methods (AOAC, 1990). All raw apple juice samples were diluted to 11.2 Brix for the analysis of pH, ergosterol and patulin. Each experiment was done in duplicate with two replicates.

2.2.6. Statistical analysis

Statistical analysis of the data was performed using SAS® software (SAS, 1985). When analysis of variance (ANOVA) revealed a significant effect ($P < 0.05$), data means were compared by the least significant difference (LSD) test.

3. Results and discussion

Ergosterol and patulin determinations in raw apple juice samples was done using HPLC methods and expressed as mg l^{-1} μg l^{-1}, respectively. Typical chromatograms of ergosterol and patulin are given in Figs. 1 and 2, respectively. The analytical methods proved reliable, with detection limits of 0.1 and 0.1 μg l^{-1} for ergosterol and patulin, respectively. The recovery rates of patulin and ergosterol in the juice samples, for five different concentrations added to samples, ranged from 95.6% to 103% and 96.7% to 101.16% with average percent recoveries of 98.9 (±3.12) and 97.5 (±2.04),

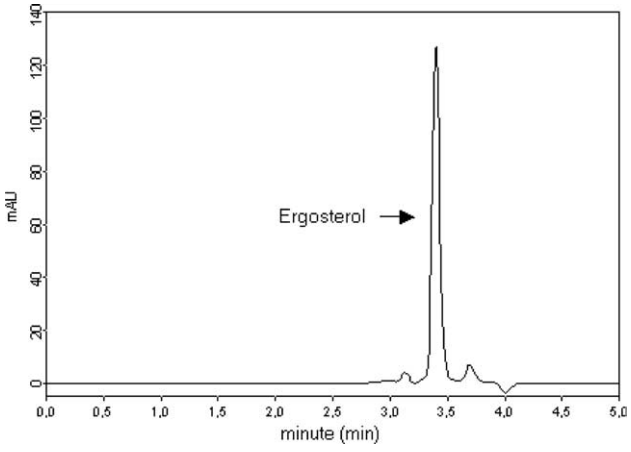

Fig. 1. HPLC chromatogram of ergosterol for a sample of 100% decayed apple.

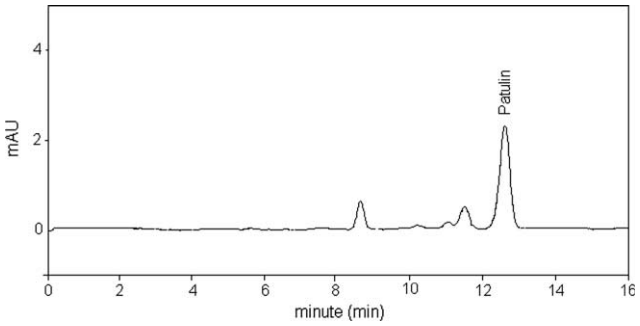

Fig. 2. HPLC chromatogram of patulin for a sample of 100% decayed apple.

respectively. Therefore, the concentrations of patulin and ergosterol in juice samples were corrected for the average recovery rates.

The pH, Brix, patulin and ergosterol concentrations of the raw apple juice samples produced from sound and 30%, 60% and 100% decayed apples are given in Table 1. Changes in patulin and ergosterol concentrations of the raw apple juice samples produced from Golden delicious apples are shown in Fig. 3.

The statistical analysis of the data showed that there were very highly significant differences between the patulin and ergosterol concentrations of raw apple juice samples and the decay proportions ($P < 0.05$). As can be clearly seen from Fig. 3, both patulin and ergosterol concentrations of raw apple juice increased with the increase of the apple decay proportion. The most significant increases in patulin and ergosterol were observed in raw apple juice samples produced with the 100% decayed apples. The patulin concentrations in juice samples, produced from sound, 30%, 60% and 100% decayed ranged from 0.3 to 2.0 $\mu g\,l^{-1}$, 139 to 23 $\mu g\,l^{-1}$, 504 to 630 $\mu g\,l^{-1}$, 890 to 940 $\mu g\,l^{-1}$, respectively. The ergosterol concentrations in the same juice samples ranged respectively from 0.1 to 1.8 $mg\,l^{-1}$, 14.4 to 34.3 $mg\,l^{-1}$, 27.2 to 75.8 $mg\,l^{-1}$ and 95.7 to 131 $mg\,l^{-1}$. The statistical analysis of the data showed that there were significant differences between the patulin and ergosterol concentrations of raw apple juice samples and the decay proportions ($P < 0.05$). Decay proportion significantly affected patulin and ergosterol concentrations of the juice samples. As seen in Table 1, as decay proportion

Table 1
The pH, Brix, patulin ($\mu g\,l^{-1}$) and ergosterol ($mg\,l^{-1}$) concentrations of the raw apple juice samples produced from sound and 30%, 60% and 100% decayed apples

Decay proportion (%)	pH[B]	Brix[B]	Patulin[B]	Ergosterol[B]
Sound (0)	3.88 ± 0.03a[A]	15.75 ± 1.3a[A]	1.9 ± 0.6a[A]	0.7 ± 0.6a[A]
30	3.86 ± 0.05a	15.42 ± 1.8a	179 ± 32.4b	23.9 ± 7.0b
60	3.82 ± 0.08a	13.86 ± 1.4b	599 ± 42.8c	63.0 ± 9.3c
100	3.49 ± 0.07b	11.90 ± 2.1c	861 ± 20.6d	111 ± 12.8d

[A] Values within a column followed by different letters are significant ($P < 0.05$).
[B] Values are the means of eight determinations with two replicates.

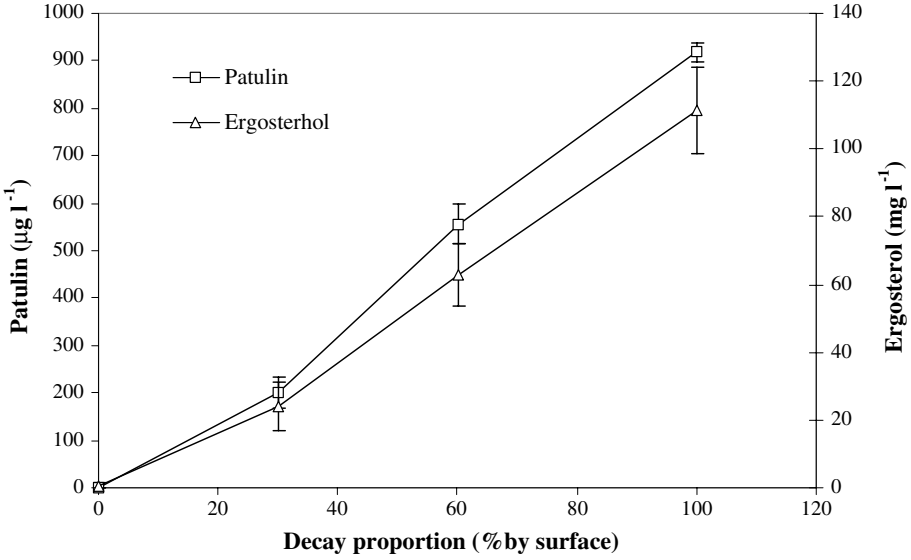

Fig. 3. Changes in patulin and ergosterol concentrations of the raw apple juice samples produced from sound and 30%, 60% and 100% decayed apples.

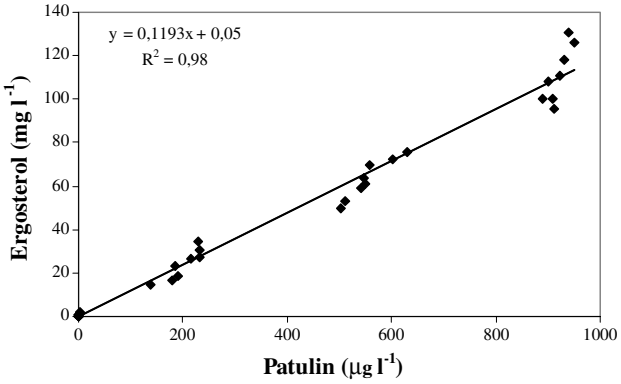

Fig. 4. Correlation of patulin and ergosterol in raw apple juice samples produced from sound and 30%, 60% and 100% decayed apples.

increased, patulin and ergosterol concentrations of juice samples increased ($P < 0.05$). As shown in Fig. 3, good correlation coefficients were found, namely 0.99 ($y = 9.0404x + 0.75$) for patulin, and 0.99 ($y = 1.0712x + 0.5$) for ergosterol in raw apple juice, respectively.

Correlations of patulin and ergosterol in raw apple juice samples produced from sound and 30%, 60% and 100% decayed apples are shown in Fig. 4. The statistical analysis yielded the most significant correlation coefficient of 0.98 ($y = 0.1193x + 0.05$), indicating a linear relationship between the patulin and ergosterol in raw apple juice when all samples were taken into account (Fig. 4). It is well known that the presence of patulin in apple juice is caused by the processing of decayed apples. According to the results obtained, it is thought that a significant correlation coefficient between the patulin and ergosterol concentrations of raw apple juice might be an indication of ergosterol occurrence, resulting from microbiological activity of patulin producing molds. Data for the ergosterol concentrations showed significant correlation ($P < 0.05$) between the decay proportion and patulin concentration.

The pH values of the raw apple juice samples produced from sound, 30% and 60% decayed apples were not affected by the decay proportion and were similar. Only very slight decreases in pH values ($P > 0.05$) of raw apple juice samples produced from 100% decayed apples were observed compared to the initial pH in the juice samples produced from sound apples. The Brix values of the raw apple juice samples produced from sound and 30% decayed apples were not affected by the decay proportion and were similar. The biggest Brix values were determined in the samples produced from sound apples, followed by 30%, 60% and 100% decayed ones. As seen in Table 1, decay proportion had a significant effect on the Brix content of raw apple juice samples produced from 60% and 100% decayed apples. These results for the pH and total soluble solids showed that the effect of the decay proportion on the pH change and Brix values of the juice could not be related to patulin and ergosterol concentration and was not indicative of the raw material decay proportion.

4. Conclusion

As stated earlier, the patulin concentration of apple juice is related to the microbiological quality of the raw material. In this study, the relationship between the patulin and ergosterol concentrations of decayed apples was examined to determine whether ergosterol may also be an indication of the microbiological quality of apples. Our results indicate that measurement of ergosterol concentration in the product has great potential for the assessment of apple products as related to the decay proportion and hence the quality of the raw material, e.g., patulin. The specific production of the ergosterol by mold genera, and the high sensitivity (0.1 $mg l^{-1}$) of the ergosterol determination, make it extremely useful in for the quality measurement of apple products. Ergosterol and patulin concentrations may change during apple juice processing. Depending on the mold genera, different concentrations of ergosterol and patulin can be produced in the same apple sample. Research is now focussed on this matter. Patulin and ergosterol contents, produced by different molds, in decayed apples need to be determined.

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References

AOAC. (1990). *Official methods of analysis* (15th ed.). Arlington, VA: Association of Official Analytical Chemists, Publ. by AOAC.

Bocchi, M., Ghiretti, G. P., Sandei, L., Spotti, E., & Leoni, C. (1995). Ergosterol production by different types of yeast able to colonize tomatoes. *Industria Conserve, 70*, 404–409.

Ghiretti, G. P., Spotti, E., Strina, F., Sandei, L., Mori, G., Attolini, G., & Leoni, C. (1995). Ergosterol production by different types of moulds able to colonize tomatoes. *Industria Conserve, 70*, 3–12.

Grasselli, C., Leoni, C., Sandei, L., & Mori, G. (1993). Contenute di ergosterolo nei derivati industriali del pomodoro come indice di contaminazione microbica della materia prima utilizzata e ricerca di una eventuale correlazione con Il valore Howard. *Industria Conserve, 68*, 1–10.

ISO. (1993). Apple juice, apple juice concentrates and drinks containing apple juice-determination of patulin concentration I. method using high performance liquid chromatography. International

Standard Organisation 8128, I. International Organisation for Standardisation, Geneva.

Kadakal, Ç., & Nas, S. (2003). Effect of heat treatment and evaporation on patulin and some other properties of apple juice. *Journal of the Science of Food and Agriculture, 83*, 987–990.

Lai, C. L., Fuh, Y. M., & Shih, D. Y. C. (2000). Detection of mycotoxin patulin in apple juice. *Journal of Food and Drug Analysis, 8*, 85–88.

Hua-Bin, L., & Feng, C. L. (2000). Determination of silicate in water by ion exclusion chromatography with conductivity detection. *Journal of Chromatography A, 874*, 143–147.

Prieta, J., Moreno, M. A., Blanco, J. L., Suarez, G., & Dominguez, L. (1992). Determination of patulin by diphasic dialysis extraction and thin-layer chromatography. *Journal of Food Protection, 55*, 1001–1002.

SAS® Institute. (1985). SAS user's guide. Statistics, Version 5 Edition. Cary, NC: SAS Institute Inc.

Seitz, L. M., Mohr, H. E., Burroughs, R. M., & Sauer, D. B. (1977). Ergosterol as an indicator of fungal invasion in Grains. *Cereal Chemistry, 54*(6), 1207–1217.

Seitz, L. M., Sauer, D. B., Burroughs, R. M., Mohr, H. E., & Hubbard, J. D. (1979). Ergosterol as a measure of fungal growth. *Phytopathology, 69*, 1202–1203.

Schwadorf, K., & Muller, H. M. (1989). Determination of ergosterol in cereals, mixed feed components and mixed feed by liquid chromatography. *Journal of the Association of Official Analytical Chemists, 72*, 457–462.

Swanson, K. M. J. (1989). Microbiology and preservation. In D. L. Downing (Ed.), *Processed apple products* (pp. 343–359). New York: AVI Publishing Company.