Estimation of mouldiness of paprika powder by near infrared spectroscopy

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Introduction

Detection of mould content in food is important because of economic losses due to fungal spoilage and potential public health hazards (many mould species are able to produce mycotoxins). Testing for moulds in processed foods provides an index of quality, indicating whether food products have been prepared from uncontaminated, high-quality materials or not. Methods able to recognise moulds in foods under all circumstances would be useful for evaluating the possible risks related to fungal contamination.Different methods are available for detecting moulds in food. They include cultural methods,[1] electrical measurements using conductance and other changes in electrical properties of culture media,[2] detection of heat-stable mould components such as chitin,[3] microscopic methods for detecting mycelia[4] etc. These methods have been summarised by Jarvis and co-workers.[5] Of these methods the mould colony count and Howard Mould Count (HMC) are used in many countries as a standard method for quality control purposes. Most of the current methods have certain disadvantages.

Other alternative techniques have been developed to replace HMC. Such methods are the quantification of ergosterol[6] and the direct epifluorescent filter technique.[7] Recently some immunological methods, (ELISA)[8–11], latex agglutination,[12–13] were described for detection of moulds in foods.

Asher and co-workers[14] achieved good results in determining spores of yellow and brown rust in barley, as well as brown rust in wheat, for each fungal species, using near infrared (NIR) spectroscopy . Preliminary studies on screening tomato puree for excessive mould content by NIR spectroscopy were reported by Davies and co-workers.[15] Roberts and co-workers reported results on chemical and spectral quantification of mould in hay[16] and in contaminated barley[17] by NIR spectroscopy. Aramaki and co-workers[18] estimated mycelial weight in rice koji by NIR spectroscopy.

The aim of our investigation was to develop a rapid instrumental method to detect both viable and dead mould biomasses in spice paprika powder.

Materials and methods

Samples

Sixteen commercial paprika powders, collected from different stores in The Netherlands, as well as twelve samples processed individually from Hungarian red paprika, were analysed. Three different sample sets of paprika mixture were prepared. The samples of paprika powder mixtures were obtained by blending in different percentage (w/w) ratios of powder made from practically mould-free paprika pods with (i) powder of mouldy paprika spoiled after artificial inoculation with *Aspergillus niger* (F 1286/17) isolated from a spoiled paprika pod, (ii) powder of naturally totally moulded paprika and (iii) powder of dried biomass of *Aspergillus niger.*

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Practically mould-free samples can be defined as samples without any visible mould contamination. Very mouldy means that paprika pods were completely moulded by *Aspergillus niger* intentionally grown.

Procedure for growing *Aspergillus niger* on paprika

The strain of Aspergillus niger was grown on malt extract agar (OXOID L 39) for one week at 25°C. The cells were harvested by using a small wooden stick to thoroughly rub the surface of the agar into a tube containing 10 cm^3 of sterile peptone water (containing 0.05% Tween 20). 10 cm^3 of these suspensions were used for inoculation of one Petridish containing approximately 5 g dried sliced paprika pods. The Petridishes were incubated at 25°C for five days. After incubation, the moulded paprika was dried for 48 hours at 50°C. The dry material was powdered in a coffee grinder and stored until required for analysis.

Plating technique

Viable mould counts from sample homogenates and their dilution series were determined on agar plates containing 15 cm^3 of RBCA medium (Rose-Bengal Chloramphenicol Agar, OXOID CM 549). 0.1 cm^3-aliquots of the samples were pipetted onto solid RBCA agar in duplicate. Plates were incubated at 24°C for four to five days.

ELISA for detecting moulds

ELISA procedure for detecting moulds of the Penicillium/Aspergillus group was performed according to Notermans and Heuvelman.[19] The ELISA is based on detection of the immunologically-active, extracellular, heat-stable polysaccharides (EPS) released by moulds and are not present in non-moulded food. RIDASCREEN® Moulds A/P commercial ELISA kits (obtained from R-Biopharm GmbH, Darmstadt, Germany) were used according to the manufacturer's instructions.

Ergosterol assay

For determining ergosterol content of paprika powders, a modified method of Seitz and Paukstelis[6] was used. 25 cm^3 methanol (99.8%, REANAL) was added to 2.5 g of the samples and homogenised for five minutes, then filtered (falten filter, ∅9 cm, Macherey Nagel). The procedure was repeated twice. After adding 10 g of KOH and 25 cm^3 of ethanol (96%, REANAL) the solution was saponificated for 45 minutes by boiling. After cooling, the suspension was shaken out twice with hexane (95%, REANAL) then concentrated with a Rotadest evaporator at 40°C.

Quantitative determination of ergosterol was performed by an HPLC method.[20]

NIR measurement

Spectralyzer (PMC type 10-25, Switzerland) scanning NIR spectrophotometer and NIR spectrophotometer (NIRSystems type 6500, USA) were used for recording the log(1/*R*) spectra in the 1000–2500 nm wavelength region. Qualitative NIR measurements were performed according to Kaffka and Gyarmati[21] using the PQS program. "Quality points" of the samples were determined in the "quality plane". The quality point is defined as the centre of gravity of the spectrum (of the spectral points) represented in the polar coordinate system. For calculating *x* and *y* coordinates the following equation was used:

$$x = \frac{1}{k}\sum_{i=0}^{k-1} Vi \cos i\alpha \qquad y = \frac{1}{k}\sum_{i=0}^{k-1} Vi \sin i\alpha$$

where: Vi is the value of the log (1/*R*) spectrum at the *i*-th wavelength, k = (λmax–λmin) s^{-1}, α = 360 / k, k is the number of spectral data, s is the wavelength step.

For quantitative investigation Multiple Linear Regression (MLR) and Principal Component Analysis (PCR) were used.

Results and discussion

Qualitative NIR measurements

In order to get the spectral ranges and bands of interest, absorbance data were transformed. Multiplicative scatter correction (MSC) and normalisation, respectively, were used for this purpose. The MS-corrected log(1/*R*) clearly show the wavelength regions where differences can be noticed in the spectral curves of the samples with different mould content (Figure 1).

The changes in the MS-corrected log(1/*R*) spectral curves are caused, partly by the changes in the mould content itself and partly by the changes in the paprika constituents which are, again, partly reduced, partly increased as a result of the mould growth.

If the selected wavelength regions are chosen (according to the MS-corrected spectra) for calculating x and y coordinates of the quality points, a good relationship can be seen between the location of the quality points and the mould content (Figure 2).

In the second series of our investigations a sample set, contaminated with different mould species as natural contamination, were studied. The presence and identity of these moulds was determined by isolating and identifying mould species on paprika pods. Natural contamination was obtained by leaving the samples to become mouldy by their original mould flora. After colonisation, the samples were treated by heating (during drying and grinding). Therefore, after sample preparation, only a part of the mycoflora was viable.

Similarly to the sample set containing Aspergillus niger biomass, a good relationship can be seen between the location of the quality points and the mould content (Figure 3). The slope of the line connecting the quality points depends partly on the chosen wavelength region and partly on the species of moulds existing in the mould biomass. Looking at the quality points of the first mixture sample set (marked +), together with the second sample set (marked •), a slight difference can be noticed in their slope as the composition of the mould biomass is different. Similar differences can be noticed looking at the quality points of the first and third mixture sample sets, although in both the main mould species is *Aspergillus niger*. In the third mixture sample set (marked *), the shifts of the quality points are only caused by changes in the biomass concentration, whilst in the first mixture sample set both the changes of the biomass concentration and chemical decomposition of the paprika tissue cause the shifts of the quality points.

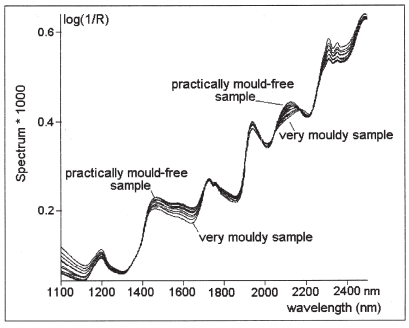

Figure 1. MSC spectrum of the sample set obtained by blending mould-free paprika powder with *Aspergillus niger* contaminated paprika powder in the wavelength range 1100–2500 nm.

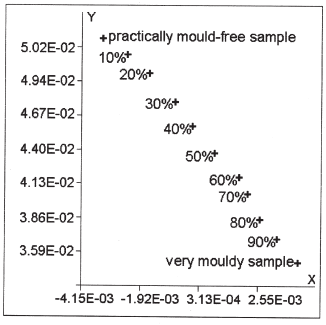

Figure 2. Quality points in the 1100–1700 nm wavelength range of the sample set obtained by blending mould-free paprika powder with *Aspergillus niger* contaminated paprika powder.

A good relationship can be seen between the location of the quality points and the mould content even if the mould content is expressed in ergosterol content (Figure 4).

Quantitative NIR measurements

Due to the indirect nature of NIR spectroscopy, reference values are needed to make calculations. In this work three reference methods were chosen for determination of mould content in the samples. First, the traditional cultural method was used as a traditional standard. An *Aspergillus/Penicillium* ELISA kit was examined as a commercially-available immunological method. Ergosterol assay was chosen because the ergosterol is a mould-specific component and it can be satisfactorily determined.

The log(1/*R*) spectra of the first mixture sample set can be seen in Figure 5. Due to the above-mentioned problems, not much information can be drawn directly from the original log(1/*R*) spectra of the paprika samples with different mould content. The absorbtion bands are not sharp enough and many chemical compounds show overlapping and combination in the NIR region. To reduce spectral variations due to scattering, to get sharper absorbtion bands and to find the wavelength range or wavelength of interest, the original absorbtion data were transformed into second, or higher, derivative spectra. The best results were obtained by multiple linear regression (MLR). This gave the highest correlation coefficient (*R*) and the lowest standard error of calibration (*SEC*). The results presented in Table 1 demonstrated the importance of a reliable and accurate reference method.

These data show clearly that the plating technique is not suited for NIR calibration of total biomass (low correlation coefficient and the highest *SEC* value). This is because only reference values for living moulds were obtained by the traditional cultural method (the range of colony-forming units was $< 10^1$ CFU $g^{-1} - 2.3 \times 10^6$ CFU $g^{-1;}$ CV% = 24%).

Using ELISA titer values, a more reliable calibration was obtained. Using a two-term equation (model), satisfactory results can be achieved taking in account that the standard error of the ELISA titer determination is in the same order of magnitude (range of ELISA titer: 1–5; CV% = 19%). Because of the high cost, we could not use enough kits for a robust calibration.

With the ergosterol method, a larger sample set (80 samples) could be examined and the best results were obtained using it as the reference.

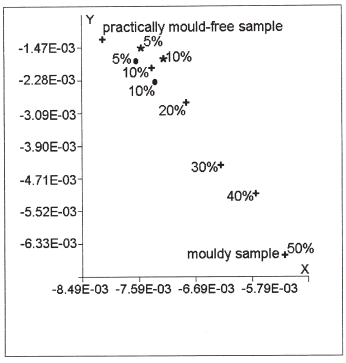

Figure 3. Quality points in the 1100–1700 nm wavelength range of the sample set obtained by blending mould-free paprika powder with *Aspergillus niger* contaminated paprika powder (+) together with the sample set obtained by blending mould-free paprika powder with naturally contaminated paprika powder (●) and the sample set obtained by blending mould-free paprika powder with dried *Aspergillus niger* biomass (*).

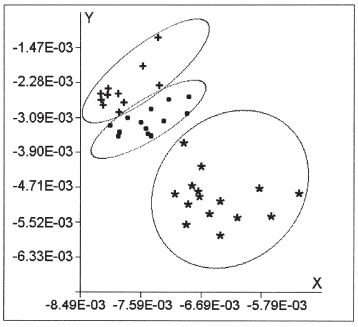

Figure 4. Quality points of paprika samples containing different ergosterol content (samples with ergosterol content under 10 mg^{-1} sample are marked +, between 12 and 16 mg^{-1} are marked ●, more than 30 mg^{-1} are marked *).

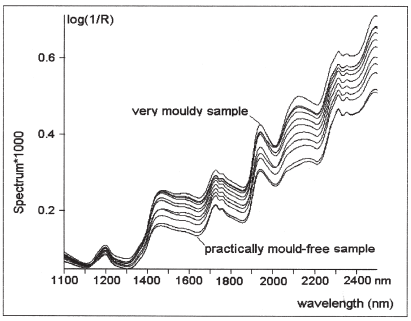

Figure 5. The log(1/*R*) spectra of first sample set.

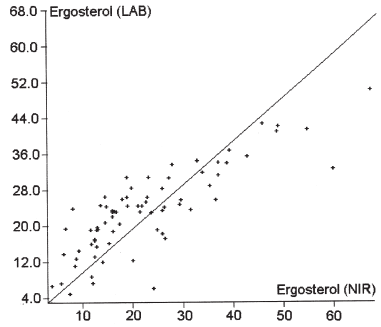

Figure. 6. Relationship between mould biomass as measured by ergosterol method (mg ergosterol 100 g^{-1} sample) and calculated on the bases of NIR technique using MLR.

The lowest *SEC* with highest correlation coefficient was obtained at triangular smoothing with a 4 nm gap and with second derivation with a 16 nm gap (*SEC*: 0.061 mg ergosterol g^{-1} sample; *R*: 0.89 (range of ergosterol content : 0.037 mg g^{-1} – 0.674 mg g^{-1}; CV% = 0.3% – 17%).

A good relationship using ergosterol content determination is demonstrated in Figure 6. The three characteristic wavelengths—determined by MLR—are: λ_1 = 1016 nm, λ_2 = 1466 nm and λ_3 = 1964 nm. The absorption at 1018 and 1960–1970 nm are connected to proteins, amino acids and amines and at 1960–1970 nm are connected with carbohydrates and water. The absorption at 1950–1960 nm can be connected with chitin.[15] The reason for these changes can be summarised as follows: because of the mould growth activity, the mould-consumable constituents of the paprika tissue decrease. Moulds utilise the carbohydrates and lipids of the paprika tissue as a carbon source, protein of the paprika tissue as a nitrogen source. Parallel with these changes, the development of the mould biomass contributes to the protein and carbohydrate content. The changes perceived by NIR spectroscopy are the global outcome of these mechanisms.

Similar observations were made by Gordon and co-workers,[23] who reported on increasing protein content and decreasing carbohydrate content of mouldy corn.

To test whether the model describes new data well, cross-validation was carried out with 80 samples. Statistical parameters of the validation were almost the same as at calibration (*SEP* = 0.062).

Table 1. Constants, wavelength and statistics of equations for estimating mould content of paprika powders.

Reference method	*n*	k_0	k_1	k_2	k_3	λ_1	λ_2	λ_3	*R*	*SEC*
[a]Plating technique[b]	27	5.74	–298	–183	—	2072	1340	—	0.68	1.14
ELISA[c]	28	8.25	173	225	—	1146	1264	—	0.87	0.38
Ergosterol measurement[d]	80	140	–22000	–129000	1350000	1016	1466	1964	0.89	0.06

[a]Parameters from Frankhuisen and Kiskó, 1997
[b]Mould content = $k_0 + k_1(A\lambda_1) + k_2(A\lambda_2)$ where: $A\lambda_1$ and $A\lambda_2$ is the 4th derivative log(1/*R*) spectrum at λ_1 and λ_2 wavelength
[c]Mould content = $k_0 + (A\lambda_1) + k_2(A\lambda_2)$ where: $A\lambda_2$ is the value of the 2nd derivative log(1/*R*) spectrum at λ_1 and λ_2 wavelength
[d]Mould content = $k_0 + k_1(A\lambda_1) + k_2(A\lambda_2) + k_3(A\lambda_3)$ where: $A\lambda_1$, $A\lambda_2$ and $A\lambda_3$ is the value of the 2nd derivative log(1/*R*) spectrum at λ_1, λ_2 and λ_3 wavelength

Calibrations have been done dividing the sample set into two groups according to their origin as well. Thirty samples formed a Hungarian sub-sample set and 50 samples formed a Bulgarian sub-sample set. Similar, or better, results were obtained using MLR than those obtained from the whole sample population. By separating the cultivars according to countries, problems arising from the origin could be eliminated. The problems result from the changing composition caused by different agrotechniques, climate and species. At the Hungarian sub-sample set two wavelengths were selected: 1614 and 2158 nm (R = 0.85; SEC = 0.059). The first and third overtones of an N–H bend can be found in the 1600–1630 region.[24] There is a C–O and O–H combination strech at 2060–2150 nm and a C–O stretch in the 2150–2180 nm region, which is characteristic of alcohols and present in starch- and cellulose-containing substances.[24] At the Bulgarian sub-sample set three wavelengths were selected: 1150, 1466 and 1964 nm. The second and third are almost the same as those of the whole sample set. The region of 1150–1170 has a third overtone of an N–H stretch, relating to amino acids.[24]

The large number of samples makes PCR possible. The advantage of the PCR is that the mathematical transformations cover the whole wavelength range and, in this way, there is no information loss. Using PCR, a slightly lower correlation coefficient (R = 0.87) and higher SEC value (0.065) were obtained.

Using the original log(1/R), spectrum 9 was obtained for the optimum number of principal components. With four principal components, 99% of the information in the data matrix was described by the model (R = 0,87; SEP = 0.065). The latter PCs are likely to describe noise. Using the second derivative spectra, three PCs were optimal. Three PCs contain 94.4% information about the model (R = 87; SEP = 0.084).

Conclusion

One important microbial quality parameter of spices and other food products is their contamination with moulds. At mould propagation, various toxic metabolites can not be excluded, which are not eliminated or inactivated by the processing technology, while colony-forming units of contaminating microorganisms are drastically reduced by those technologies. Therefore, it is important to detect both viable and non-viable mould biomasses together in spices. The available immunological and chemical methods are slow, expensive and inaccurate.

In this work we aimed at estimating excessive mould contamination by NIR spectroscopy compared with determination of colony-forming units, ELISA titer or ergosterol (a common component of fungi) in paprika powders. The results showed that traditional plating techniques cannot be applied for NIR calibration of total mould biomass. ELISA titer gave satisfactory results when it was used as the reference for NIR measurement. The best correlation between the level of mould contamination of paprika powder and calculated NIR values were obtained using ergosterol measurement. Therefore ergosterol content can be used satisfactorily for the NIR calibration.

The NIR technique proved to be a very promising, rapid, non-destructive, reagentless screening method to detect excessive mould biomass content in paprika powder and, therefore, it provides a simple means for monitoring the mycological quality assurance of paprika powder products. However, the work presented is minimal in terms of establishing NIR spectroscopy as a method to estimate mouldiness of paprika. Other moulds, in addition to *A. niger*, need to be studied.

Further systematic studies are also needed to explore all the influencing, disturbing effects (particle size, temperature, seed–skin ratio, climate, agrotechnique etc.) and the possibility of their elimination by proper transformation of the spectra (derivation, normalisation etc.) and by optimising the wavelength range.

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