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ARTICLES

Prediction of *Penicillium expansum* Spoilage and Patulin Concentration in Apples Used for Apple Juice Production by Electronic Nose Analysis

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Classification models for *Penicillium expansum* spoilage of apples and prediction models for patulin concentration in apples usable for apple juice production were made on the basis of electronic nose (e-nose) analysis correlated to HPLC quantification of patulin. A total of 15 Golden Delicious and 4 Jonagold apples were surface sterilized and divided into three groups per variety. The Golden Delicious group consisted of five apples each. Group 1 was untreated control, group 2 was surface inoculated with *P. expansum*, and group 3 was inoculated in the core with *P. expansum*. The apples were incubated at 25 °C for 10 days. E-nose analysis was performed daily. At day 10 the Golden Delicious apples were individually processed for apple juice production. During apple juice production the mash and juice were analyzed by e-nose, and samples were taken for patulin analysis by HPLC. The volatile metabolite profile was obtained by collection of volatile metabolites, on tubes containing Tenax TA, overnight between the 9th and 10th days of incubation and subsequent analysis of the collected compounds by GC-MS. Prediction models using partial least-squares, with high correlation, for prediction of patulin concentration in shredded apples as well as apple juice were successfully created. It was also shown that it is possible to classify *P. expansum* spoilage in apples correctly on the basis of soft independent modeling of class analogy classification of e-nose analysis data. To the authors' knowledge this is the first report of a regression model between e-nose data and mycotoxin content in which actual concentrations are reported. This implies that it is possible to predict mycotoxin production and concentration by e-nose analysis.

KEYWORDS: Patulin; electronic nose; prediction models; *Penicillium expansum*; apple juice; volatile organic compounds; volatile metabolite profiling

INTRODUCTION

In the food industry quality control typically relies on random sampling of the product, description of flavor and aroma by sensory panels, volatile and/or headspace analysis by GC-MS, and mycotoxin analysis by HPLC (*1*). These methods are both time-consuming and expensive; therefore, it is desirable to improve quality control measures, for instance, by combining rapid analysis of volatiles with correlations to mycotoxin production.

The electronic nose (e-nose) has already shown its potential as a replacement for sensory panel analysis, for instance, in quality control of fruit ripeness (*2*), grain odor (*3*), packaged poultry meat (*4*), meat end-products (*5*), and Danish blue cheese (*6*−*8*) and even as a replacement for GC-MS analysis (*9*, *10*). Remaining to be done is the analysis of mycotoxin content, which is still performed by HPLC or LC-MS analysis. Only a limited number of studies (*11*, *12*) have tried to make predictions of mycotoxin contents based on e-nose response. Olsson et al. (*12*), in their combined GC-MS and e-nose analysis of grain samples, did show the possibility of predicting the mycotoxin level in spoiled grain by partial least-squares (PLS) modeling. The model could not, however, differentiate with sufficient accuracy between samples containing more or less than 5 μg/kg ochratoxin A, the threshold level established by the Swedish National Food Administration. Cheli et al. (*11*) looked at identifying mycotoxins in durum grain by e-nose analysis but did not identify the spoilage organism or go beyond principal component analysis (PCA) investigation to make a correlation model between e-nose data and mycotoxin concentrations.

To investigate the potential for mycotoxin prediction by e-nose analysis we chose to work with apples, as the associated

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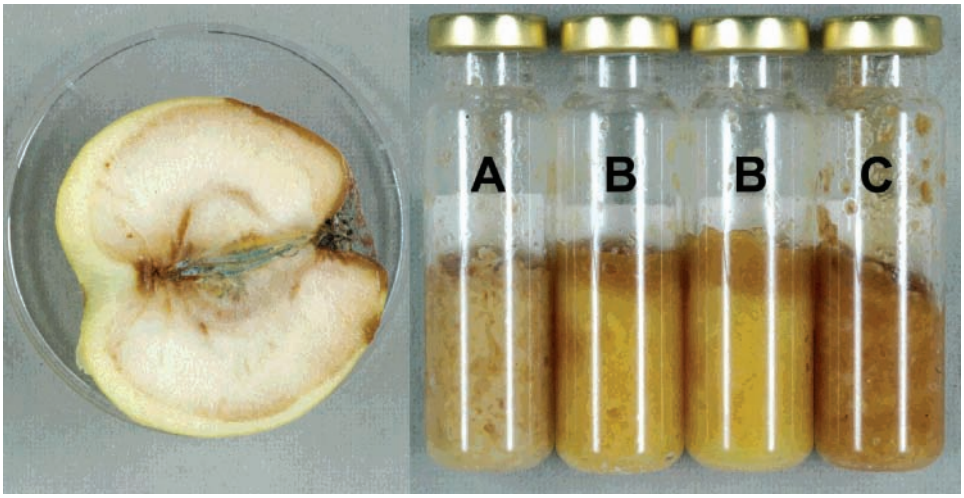

Figure 1. Discoloring of apple flesh by *P. expansum* in Jonagold apples (left) and the resulting apple mash (right). The four flasks with apple mash are from (**A**) a core-infected apple, (**B**, **B**) two control apples (mash showing browning at the surface), and (**C**) a surface-infected apple.

funga of apples is fairly limited (*13*), especially in storage contamination, where *Penicillium expansum* by far is the dominant species. It is known that the incidence of *P. expansum* spoilage of apples is increased by damage to the apples; hence, *P. expansum* spoilage mainly occurs on windfall apples. Storage of windfall apples prior to industrial processing is therefore likely to be the main cause of *P. expansum* spoilage and thus mycotoxin content. *P. expansum* is a known patulin producer (*14*), and food safety authorities, such as the U.S. Food and Drug Administration (FDA) and the Commission of the European Communities (EC), have introduced a threshold level of acceptance, 50 ppb, regarding patulin content in apple juice and other apple products (*15*, *16*).

P. expansum spoilage of and hence patulin content in apples is a well-known problem for the apple juice industry as apples are commonly stored prior to production due to processing capacity limitations; furthermore, apples used for juice production are frequently of a lesser quality than apples sold for consumption in, for instance, supermarkets. Because of the spoilage and mycotoxin problem several HPLC analysis methods have been developed for patulin quantification in apple juice samples (*17*−*21*). Several different sample preparation methods have also been developed in order to have a more robust analysis with increased sensitivity (*22*, *23*).

Because apple juice production is an industrial process it is not feasible or for that matter possible to inspect individual apple quality visually. It is therefore desirable to use screening methods that are rapid and can be used in large scale; e-nose analysis has a potential to fulfill these requirements.

The first aim of this study was to use e-nose analysis to classify both spoiled and nonspoiled samples, thus creating a classification model. Second, it was aimed to correlate the e-nose response with the mycotoxin level in a given sample, thus creating a prediction model for mycotoxin content based on e-nose analysis. It was not the aim of this study to predict biomass. The choice of model system in this study was apples used for apple juice production and the major problem in apple juice production, that is, storage spoilage by *P. expansum* and hence patulin production by *P. expansum*. The study was conducted as a "worst case" study by analysis of spoiled or nonspoiled samples. In this study the choice of apple variety for the major part of the study was Golden Delicious, whereas Jonagold apples were chosen for investigating apple variety influence on the e-nose analysis and prediction modeling.

MATERIALS AND METHODS

Fungi and Media. The *P. expansum* isolate, IBT 3487, the type culture used in this study, was obtained from the Fungal Culture Collection at BioCentrum-DTU (IBT collection), Technical University of Denmark, Kgs. Lyngby, Denmark. The apples, Golden Delicious and Jonagold, used in the experiment were obtained from the local supermarket and were surface sterilized by immersion in 96% ethanol for 1 min, then immersion in 3% hypochlorite solution for 1 min, and re-immersion in the 96% ethanol bath for 1 min, after which the apples were rinsed with autoclaved Milli-Q water and dried in a sterilized laminar flow bench.

A total of 15 Golden Delicious apples were surface sterilized and divided into three groups of 5 apples (replicates) each. Group 1 was untreated control, group 2 was surface-inoculated with *P. expansum*, and group 3 was inoculated in the core with *P. expansum*. Four Jonagold apples were surface sterilized, of which two apples were used as untreated control apples, one apple was surface-inoculated with *P. expansum*, and one apple was core-inoculated with *P. expansum*.

The surface-inoculated apples were stab wounded with a sterilized needle (7 mm deep wound), and 20 μL of spore suspension ($>10^6$ spores/mL) was put on the wound.

Core-inoculated apples were inoculated through the flower opening via a syringe, and 0.2 mL of spore suspension ($>10^6$ spores/mL) was injected. The apples were individually incubated in ethanol-sterilized 600 mL beaker glasses covered with Parafilm M (Alcan Packaging, Menasha, WI) at 25 °C in the dark for 10 days.

Apple Juice Production. Juice was only made from Golden Delicious apples. The processing steps were performed by imitating industrial apple juice production methods. After the 10 day incubation, the apples were individually shredded. Between 15 and 15.3 g was put in headspace flasks for electronic nose analysis (both Golden Delicious and Jonagold samples), and between 2 and 2.4 g was stored in headspace flasks for HPLC analysis (Golden Delicious samples only). The e-nose samples (Golden Delicious samples only) were treated with 12 μL of 10× diluted pectinase solution (Pectinex Smash) (Novozymes, Bagsværd, Denmark) and the samples left for 30 min at room temperature. The juice was then pressed using a funnel and Whatman filters (Whatman International, Brentford, U.K.) by pressing the mash with a metal spatula. The juice was collected in 20 mL headspace flasks (75.5 × 22.5 mm), and the flasks were sealed with crimp caps (3.0 mm membrane thickness silicone/PTFE caps). The juice was then pasteurized by immersion of the bottle in a 98 °C water bath for 60 s. After pasteurization, 10 μL of 3× diluted pectinase solution was added, and the samples were heated in a 53 °C cabinet for 60 min. Clearing of the juice was performed by adding 2−3 grains of Clarit WG Bentonite (Süd-Chemie AG, Moosburg, Germany), after which the sample was shaken for 5 min; then 10 μL of Gammasol Kieselsol (Gamma Chemie GmbH, Darmstadt, Germany) solution was added,

Figure 2. Discoloring of apple flesh by *P. expansum* in Golden Delicious apples (left) and the resulting apple mash (right). The three flasks with apple mash are from (**A**) a control apple (mash already showing more browning at the surface), (**B**) a surface-infected apple, and (**C**) a core-infected apple.

and the sample was shaken for another 5 min. Finally, approximately 10 μg of Rousselot gelatin 75 PS 30 was added, and the sample was shaken for another 5 min and then left overnight in the 53 °C cabinet. The cleared samples were centrifuged (15000*g*) for 5 min, and the supernatant was transferred to fresh 20 mL headspace flasks (75.5 × 22.5 mm), which were sealed with crimp caps (3.0 mm membrane thickness silicone/PTFE caps).

Collection and Analysis of Volatile Metabolites. Volatile metabolites were collected overnight from all 15 whole Golden Delicious apples between days 9 and 10. The volatiles were collected by diffusive sampling onto Tenax TA adsorption material placed in Perkin-Elmer tubes, and the tubes were placed inside glass beakers. Volatiles collected were thermally desorbed on a Perkin-Elmer ATD 400 coupled to a Hewlett-Packard 5890 gas chromatograph further coupled to a HP 5972 mass selective detector. Separation of the volatiles was done on a 60 m, 0.25 mm i.d., 1.0 μm VB-5 ValcoBond capillary column (SIS, Ringoes, NJ) using He as carrier gas. Initial pressure was 13 psi, and the He flow was 1 mL/min. The system was run at a 75:1 split, and the injection temperature was set to 250 °C. Chromatographic conditions were as follows: initial temperature, 35 °C for 1 min, raised at 4 °C/min to 175 °C and then at 10 °C/min to 260 °C. Separated compounds were characterized (tentatively identified) by their mass spectra generated by electron ionization (EI) at 70 eV at a scan range from *m*/*z* 33 to 330.

Electronic Nose Measurements. The headspace of the all 15 Golden Delicious whole apples was analyzed daily on an e-nose from days 1 through 10 by manual injection of 1500 μL of the headspace from the 600 mL beakers containing each apple using a gastight Hamilton syringe. For all of the shredded apple samples (both Golden Delicious and Jonagold) and all of the juice samples (only Golden Delicious) an HS-100 autosampler (CTC Analytics AG, Zwingen, Switzerland) was used; these samples were incubated for 1 min at 35 °C, after which 1500 μL of the sample was withdrawn from the headspace and injected in the sensor chamber and flushed over the sensors at a rate of 150 mL/min. All e-nose samples were analyzed on an αFox-3000 (Alpha M.O.S., Toulouse, France) e-nose. The sensor array system consisted of 12 metal oxide semiconductor (MOS) sensors. MOS sensors are known to be among the least moisture sensitive sensors available (*24*).

Data collection was performed every half second for 2 min, after which the sensors were flushed with dry air (maximum air humidity = 0.5%) for 2 min; furthermore, there was a 2 min delay before the next sample was analyzed.

Extraction and Analysis of Patulin. *Extraction.* A modified version of the method used by Eisele and Gibson (*23*) was used. Ten milliliters of Milli-Q water was added to each headspace flask containing 2.0−2.4 g of apple mash and left for extraction on an ultrasound bath for 30 min; 2.5 mL of supernatant from each sample was transferred to 2.5 mL Eppendorf tubes and centrifuged at 15000*g* for 5 min. Strata-X (30 mg/1 mL, 33 μm) polymeric reversed phase SPE columns (Phenomenex, Torrance, CA) were prepared for sample purification by rinsing the columns with 2 × 1 mL of methanol (Sigma-Aldrich, St. Louis, MO) and then with 2 × 1 mL of Milli-Q water. Two

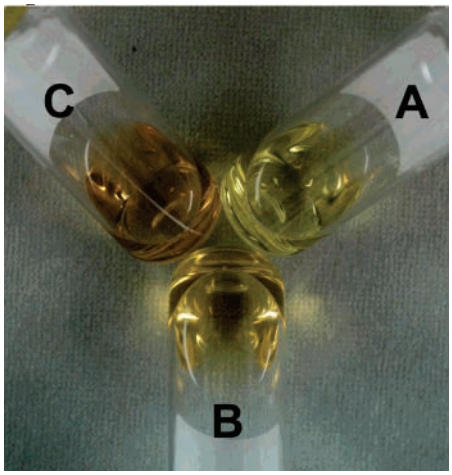

Figure 3. Apple juice from Golden Delicious apples with various amounts of *P. expansum* spoilage: (**A**) juice from control apples; (**B**) juice from surface-infected apples; (**C**) juice from core-infected apples.

milliliters of sample was loaded on each column, after which the columns were washed with 2 × 1 mL of 1% sodium bicarbonate and then 2 × 1 mL of 1% acetic acid solution. The columns were left to dry for 1 min, after which the samples were eluted into HPLC vials with 1.5 mL of 30% acetonitrile.

Quantification of Patulin. Patulin standards were made at concentrations of 10, 25, 50, 100, and 205 μg/L by diluting a standard (100 μg/mL in chloroform) (Supelco, Bellefonte, PA) in acetonitrile and analyzed by HPLC.

Patulin Recovery from Apples. Two grams of apple mash was spiked with patulin standard (100 μg/mL in chloroform) at the following levels: 2, 4, 7.5, 12.5, and 20 mg/kg (by adding 40, 80, 150, 250, and 400 μL of patulin standard to the apples, respectively). The chloroform was removed by nitrogen evaporation while the samples were kept at 40 °C on a heating block. After this, the samples were treated as the other apple samples by SPE and analyzed by HPLC.

Chemicals. Chemicals used in HPLC analysis were as follows: acetonitrile, gradient grade, and trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO); water, Milli-Q grade.

HPLC-DAD Instrument. HPLC-DAD analysis was done on an Agilent HP 1100 with a photodiode array detector (DAD) (Agilent, Böblingen, Germany). Ten microliters of sample was injected, although for the patulin standards only 3 μL was injected. The column used was a 150 × 2.0 mm i.d., 3 μm CuroSil-PFP column (Phenomenex) with a 4 × 2 mm i.d. Phenyl (phenylpropyl) guard column (Phenomenex). The flow rate was 0.3 mL/min with a linear water/acetonitrile gradient starting at 96:4 (water/acetonitrile) going to 25% acetonitrile in 5 min, and then returning to starting conditions (96:4) in 3 min and equilibrating for 8 min. Trifluoroacetic acid (50 ppm) was added to

Table 1. Fifty Volatile Compounds Detected by Headspace Analysis of Whole Golden Delicious Apples[a]

compd no.	RI	compound	log av area			signif diff in log av area[b]
			control	surface	core	
1	443	acetaldehyde	2.00	1.80	1.20	
2	472	ethanol	6.54	8.10	7.53	
3	498	propanal	6.28	7.00	4.68	
4	525	methyl acetate	6.23	7.70	7.74	
5	548	1-propanol	7.23	7.37	6.99	
6	582	butanal	6.23	3.28	3.26	
7	605	ethyl acetate	8.32	8.59	8.56	
8	617	2-methyl-1-propanol	7.19	7.61	7.30	
9	622	methyl propanoate	nd[c]	4.84	3.89	surface > control core > control
10	655	1-butanol	7.85	7.86	7.68	
11	687	unknown 1	6.14	4.86	3.31	
12	707	ethyl propanoate	5.39	7.56	7.22	
13	710	propyl acetate	7.70	7.76	7.40	
14	731	3-methyl-1-butanol	1.94	6.45	7.10	surface > control core > control
15	736	2-methyl-1-butanol	7.71	7.75	7.58	
16	765	unknown 2	4.56	4.83	3.31	
17	772	2-methylpropyl acetate	7.15	7.35	7.22	
18	799	ethyl butanoate	6.44	7.59	7.25	
19	807	propyl propanoate	6.25	5.65	4.82	
20	812	butyl acetate	8.38	8.15	6.97	
21	848	ethyl 2-methylbutanoate	6.40	7.50	7.18	
22	864	1-hexanol	7.42	7.44	7.25	
23	871	3-methyl-1-butyl acetate	0.60	6.40	5.55	surface > control core > control
24	874	2-methyl-1-butyl acetate	8.38	8.20	7.99	control > core
25	891	propyl butanoate	6.35	5.63	3.95	
26	897	styrene	nd	8.36	8.27	surface > control core > control
27	900	butyl propanoate	7.39	7.31	7.07	
28	904	pentyl acetate	7.51	7.44	7.12	
29	937	unknown 3	7.09	5.70	3.40	
30	960	3-methyl-1-butyl propanoate	4.85	4.90	1.20	
31	981	butyl butanoate	7.64	7.48	7.33	
32	984	ethyl hexanoate	6.32	7.51	7.30	
33	991	unknown 4	4.52	4.90	3.99	
34	997	hexyl acetate	8.27	8.01	7.73	
35	1011	1-methoxy-3-methylbenzene	nd	7.35	7.12	surface > control core > control
36	1028	butyl 2-methylbutanoate	7.73	7.51	7.35	
37	1044	pentyl butanoate	5.59	4.96	3.98	
38	1063	α-farnesene	2.83	4.41	4.29	
39	1078	butyl hexanoate	7.22	7.29	6.32	
40	1089	hexyl propanoate	6.48	6.58	5.63	
41	1094	2-methylbutyl 2-methylbutanoate	6.37	5.75	5.54	
42	1129	unknown 5	7.19	5.74	5.49	
43	1137	butyl 2-methylpropanoate	5.56	5.75	4.75	
44	1184	hexyl butanoate	7.94	7.85	7.62	
45	1188	ethyl octanoate	2.89	5.76	3.18	
46	1208	1-allyl-4-methoxybenzene	6.54	7.41	6.42	
47	1233	hexyl 2-methylbutanoate	8.14	7.88	7.73	
48	1254	unknown 6	3.60	4.06	6.28	
49	1282	pentyl hexanoate	5.72	5.89	6.46	
50	1375	hexyl hexanoate	7.50	7.93	7.46	

[a] Compounds are listed by the average log area for the three sample groups (sample size per group was five) control apples, surface-inoculated apples, and core-inoculated apples. [b] Designating significant difference in log average area between sample groups as per Duncan's multiple-range test. [c] Not detected.

both the water and acetonitrile. UV spectra were collected by a DAD from 200 to 700 nm at 2.0 nm resolution with focus on the signal at 276 nm.

In all analysis sequences a standard of 5-hydroxymethylfurfural was included to ascertain that the retention times of this compound and patulin were different in this analysis method.

Data Analysis. *Volatile Metabolites.* All peaks that were significant enough to be integrated, using default parameters, with the ChemStation software (Agilent Technologies) were selected. Mass spectra from compounds with identical retention indices were compared to account for similarity. The identity of the compounds was tentatively established by comparison of mass spectra and volatile metabolite profiles with data from the NIST mass spectra library (NIST92 library). The significance of difference in the amount of each volatile metabolite between the three sample groups (control and core- and surface-inoculated Golden Delicious apples) was determined by using Duncan's multiple-range test (*25*) keeping the areas logarithmized.

Electronic Nose Data. The features, maximum value for resistance change, extracted from the e-nose system responses were evaluated by PCA as well as by classification using soft independent modeling of class analogy (SIMCA) with each apple condition, that is, control, surface-inoculated, and core-inoculated (class), analyzed as a PCA group (*26*). Prediction models for patulin levels were made by PLS regression analysis between the e-nose data and patulin concentrations

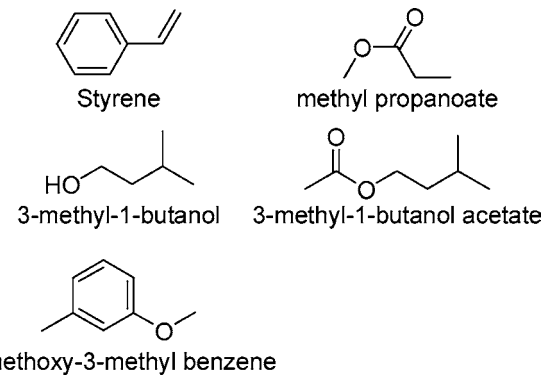

Figure 4. Chemical structures of the five volatile biomarkers for *P. expansum* contamination of Golden Delicious apples.

of the given samples by quantitative HPLC analysis (*26*). The data were standardized by multiplication with the inverse of the standard deviation and analyzed using full cross validation. For the analysis the software package The Unscrambler version 9.1 (CAMO, Oslo, Norway) was used.

Nonvolatile Metabolites. Patulin was identified by comparison of UV spectrum and retention time with HPLC analysis of a patulin standard (100 μg/mL in chloroform). A standard curve was made from the standards analyzed by HPLC. Patulin recovery (R%) from apple samples was calculated from the spiked samples (Sp) run by comparison of the attained HPLC response to the standard curve (Std) and hence expected response level, that is, R% = Sp/Std × 100. The recovery percent was used to correct the patulin levels detected from the apple sample analyses. Patulin concentration in the apples was also corrected for the sample mass extracted. Retention time of 5-hydroxymethylfurfural was checked and compared to the retention time of patulin as 5-hydroxymethylfurfural is also considered to be a quality parameter in fruit juices, and its presence is regarded an indication of quality deterioration (*20*).

RESULTS AND DISCUSSION

Golden Delicious and Jonagold apples were chosen for this study, and the apples were surface- or core-inoculated to emulate the two types of *P. expansum* spoilage commonly found in apples. The inoculation methods were deliberately strong as absolute chance of infection was desired.

It was noted that, depending on apple variety, the color of the apple flesh was either more pale with a brownish edge in the infected part of apples (Jonagold) (**Figure 1**) or dark brown (Golden Delicious) (**Figure 2**). This discoloring could be due to destruction of apple enzymes by *P. expansum*. Apple juice from Golden Delicious apples also differed in color depending on whether the juice came from control apples (light yellow), surface-infected apples (light brown), and core-infected apples (dark brown) (**Figure 3**); hence, visual identification of spoiled samples by spectrophotometric methods is variety dependent.

From the GC-MS analysis of the headspace from the whole Golden Delicious apples after 10 days of incubation 50 volatile organic compounds (VOCs) were detected (**Table 1**). The five compounds shown in **Figure 4** were both unique for apples infected by *P. expansum* and statistically significantly different from control apple samples according to Duncan's multiple-range test. Of these five compounds, four of them, namely, styrene, 3-methyl-1-butanol, 3-methyl-1-butyl acetate, and 1-methoxy-3-methylbenzene, are all well-known fungal volatile metabolites (*27*), and methyl propanoate is a well-known apple volatile metabolite (*28*, *29*). These five compounds are therefore good GC-MS analysis biomarkers for *P. expansum* infection in Golden Delicious apples, although styrene, being produced in the largest quantity, seems the best of the five. From PCA of e-nose data combined with GC-MS results and mycotoxin analysis, styrene by far had the highest positive correlation with patulin (not shown). Styrene is therefore also a relevant biomarker in e-nose analysis of potential apple spoilage by *P. expansum*. The finding of large quantities of a known apple VOC is likely due to *P. expansum* spoilage inducing large-scale release of this compound when, for instance, apple cells or peel is ruptured.

For the remaining 45 apple-related volatiles, by combined analysis of GC-MS and e-nose data, we have found that the level of the majority of the volatile compounds (30 VOCs or 60%) was relatively unchanged between surface- and core-infected apples (no statistically significant difference in VOC levels). Eighteen VOCs or 36% were detected in slightly higher levels (GC-MS), although this was not statistically significant, from surface-infected apples than from core-infected apples and were positively correlated to surface-infected apples (e-nose, PCA). Two VOCs, α-farnesene and unknown 6, corresponding to 4%, were detected at a higher level in core-infected apples compared to surface-infected apples (GC-MS), although this was not statistically significant, and were positively correlated to core-infected apples (e-nose, PCA). The profiles of volatile metabolites of the surface- and core-inoculated Golden Delicious apples, respectively, were thus quite similar and different from those of the control apples (**Figure 5**). This indicates that the differentiation between the two infection types on day 9 could be due to the fungal growth being at different stages. It could also derive from a lesser amount of volatile compounds being released from apples core inoculated with *P. expansum* compared to surface-inoculated apples, as the fungal atmospheric surface area per gram to hyphae ratio is smaller for core-inoculated apples than for surface-inoculated apples. Because of the location of the core infection, the volatile compounds might be bound in the apple tissue as long as the apple maintains structural integrity. Other studies have also indicated that the VOC profiles from apples differ according to whether the apple is whole or damaged (*30*−*32*).

SIMCA analysis of e-nose signals of the whole Golden Delicious apples showed that from days 1 through 8 the volatile metabolite background from the apple was overwhelming the detection of fungal growth by the e-nose even if growth was highly visible. At day 6 it was possible to correctly classify two of five surface-inoculated apples, whereas it was not possible to distinguish core-inoculated apples from the controls until day 7, and then only one of five samples was correctly classified. E-nose signal SIMCA analysis of the whole apples on the 9th and 10th days showed that apples at this stage of infection can be classified into spoiled and nonspoiled apples. On day 9 it was even possible to distinguish the two infection types (surface vs core), whereas it was possible to distinguish between spoiled and control apples only on day 10. In terms of classification of spoiled from control samples, it was possible to classify 40% of the spoiled samples correctly on day 6, rising to 100% correctly classified samples from day 9 onward. The early classification of spoiled apples might improve if the apples are shredded as this will induce an increased release of VOCs from both apple and mold, which could, for instance, potentially reveal core-infected apples at an earlier stage.

By analysis of e-nose data of shredded apples from both Jonagold and Golden Delicious varieties it was evident that the two varieties give rise to two different aroma profiles and hence lead to two different e-nose models (**Figure 6**). This was expected as it is known that GC-MS analysis of apple varieties yields clustering of apples according to whether they belong to

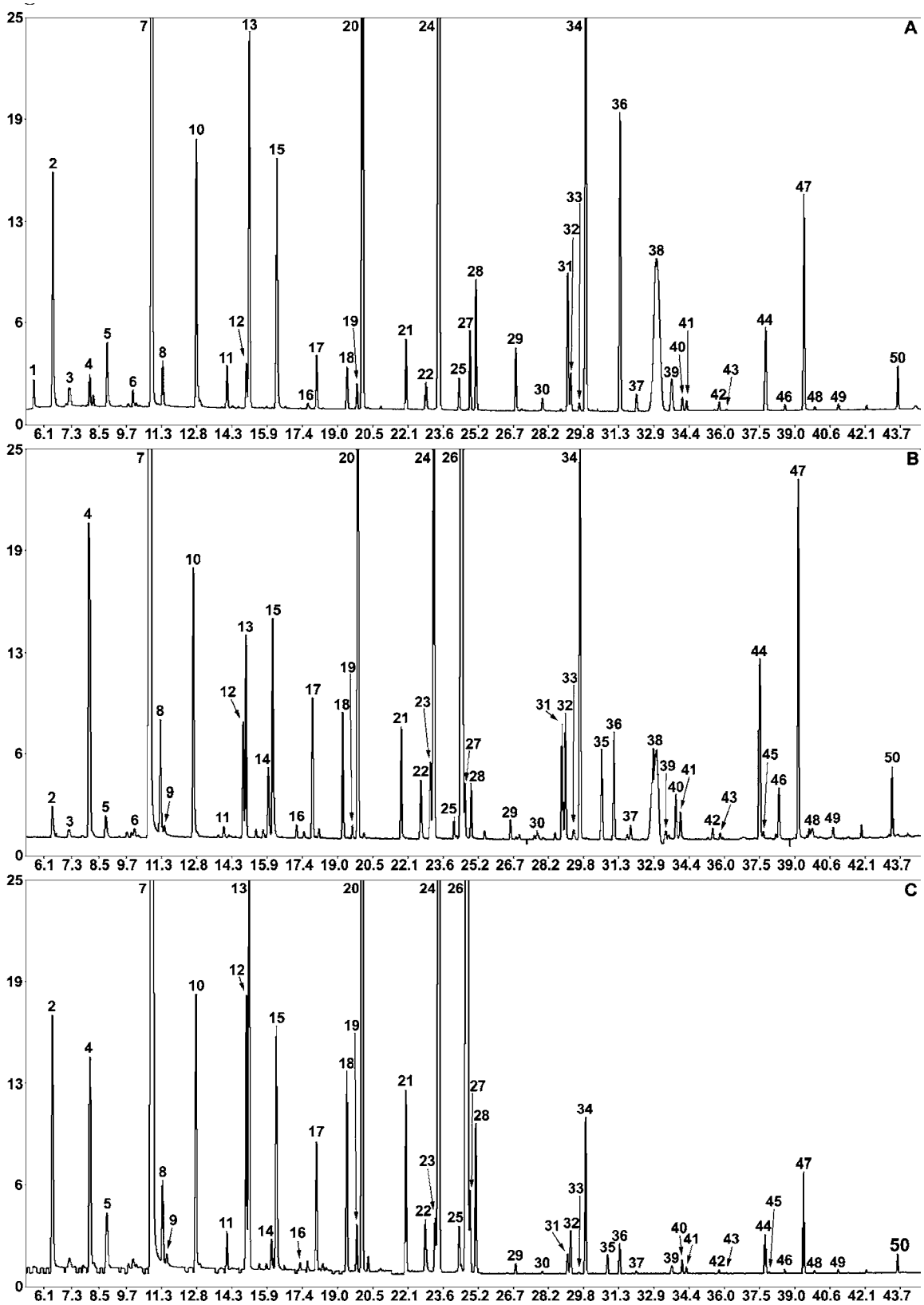

Figure 5. GC-MS chromatogram of the headspace from Golden Delicious apples: (**A**) control apple; (**B**) core-infected apple; (**C**) surface-infected apple. The abundance scale is in percentage of the abundance given in the top left corner of each chromatogram. The noteworthy compounds are numbered corresponding to the numbering of compounds in **Table 1**.

the red skin, green skin (Golden Delicious), or red and green skin (Jonagold) category (*33*).

The HPLC analysis method developed proved sufficient to distinguish between 5-hydroxymethylfurfural and patulin with retention times of 4.77 and 5.79 min, respectively (**Figure 7**). Patulin analysis revealed that patulin, in Golden Delicious apples, had been produced at quite high levels by *P. expansum* in both core and surface inoculations. The patulin concentrations were 3−4 times higher in core-inoculated apples compared to the surface-inoculated apples, probably due to the higher degree

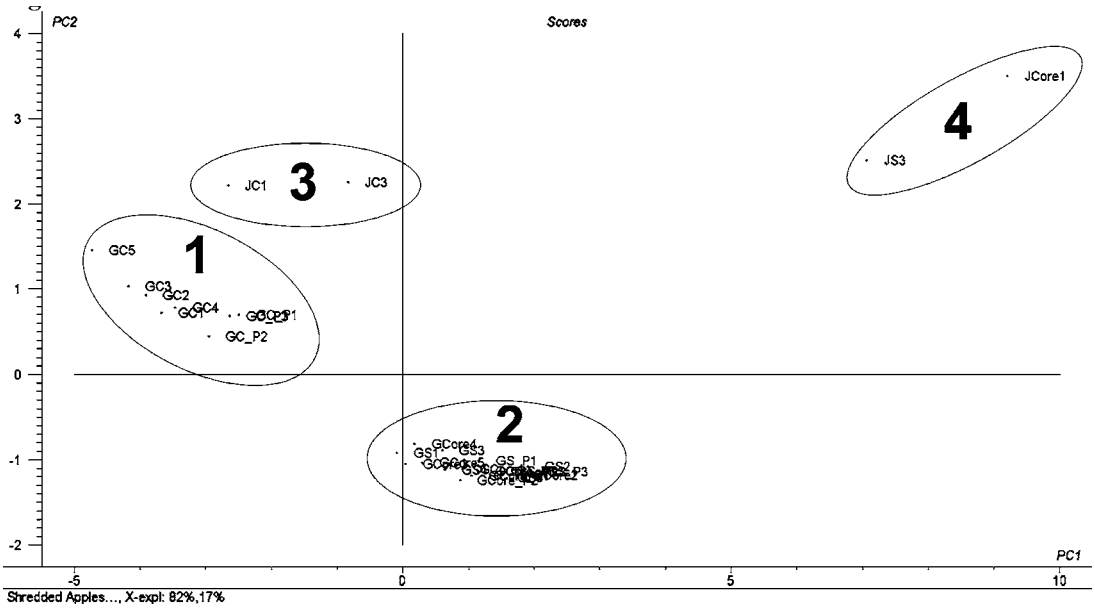

Figure 6. PCA scores plot of e-nose analysis results of shredded Jonagold and Golden Delicious apples: 1, GC1–5, Golden Delicious control apples, and GC_P1–3, pooled samples of Golden Delicious control apples; 2, GCore1–5, Golden Delicious core-inoculated apples, and GS1–5, Golden Delicious surface-inoculated apples; 3, JC1 and 3, Jonagold control apples; 4, JCore1, core-inoculated Jonagold apple, and JS3, surface-inoculated Jonagold apple.

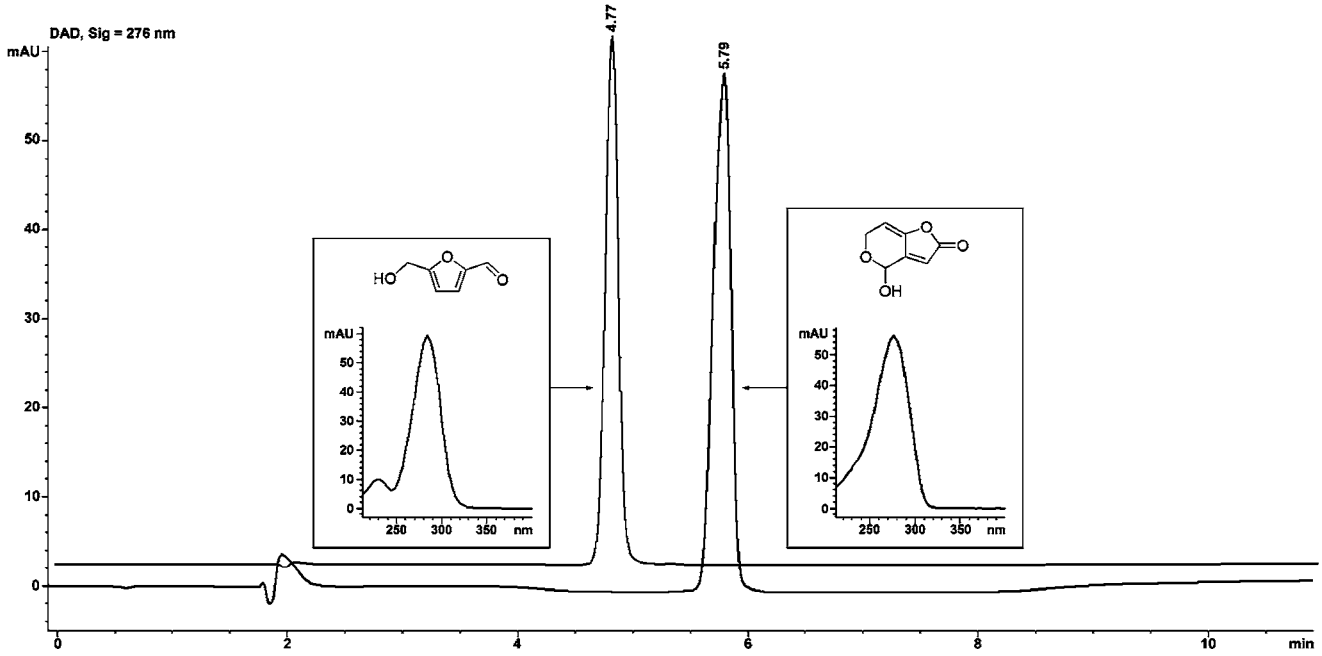

Figure 7. Overlay of HPLC chromatograms with 5-hydroxymethylfurfural, RT 4.77 min, and patulin, RT 5.79 min. To the left of the 5-hydroxymethylfurfural peak the UV spectrum and chemical structure are inserted and the UV spectrum and chemical structure for patulin are inserted to the right of the patulin peak.

of spoilage in these apples as the fungus was able to grow spherically in all directions from the core, whereas the surface-inoculated colony was limited to hemispherical growth; that is, the patulin to hyphae ratio between the two types of infection is expected to be the same. It is also evident that patulin was concentrated in the Golden Delicious apple juice through the apple juice production process, because a 3−4 times higher patulin concentration was detected in the juice compared to the mash from which it came. This concentration could be due to patulin being a very polar compound, hence being highly water soluble and therefore present in a higher concentration in the juice (*34*, *35*). Because the samples taken for analysis were approximately 2 g whether it was mash or juice, more patulin would then be sampled from a pure juice sample than from a mash sample. The resulting juice from the surface- and core-inoculated apples had patulin contents of, respectively, approximately 200 and 600 times above the allowed threshold (*15*, *16*). This indicates that the allowed level of patulin, 50 ppb, will be exceeded by producing apple juice from apples containing as little as two to five spoiled apples per thousand.

As seen in **Figure 8A**, the PLS regression analysis of e-nose data and patulin concentration for Golden Delicious apple mash gave rise to a regression model with a high fit (high correlation value, RMSEP = 14%). From the dispersement of the surface samples in the plot it is evident that it is not a perfect model for these samples. Separate PLS modeling of the data from core-

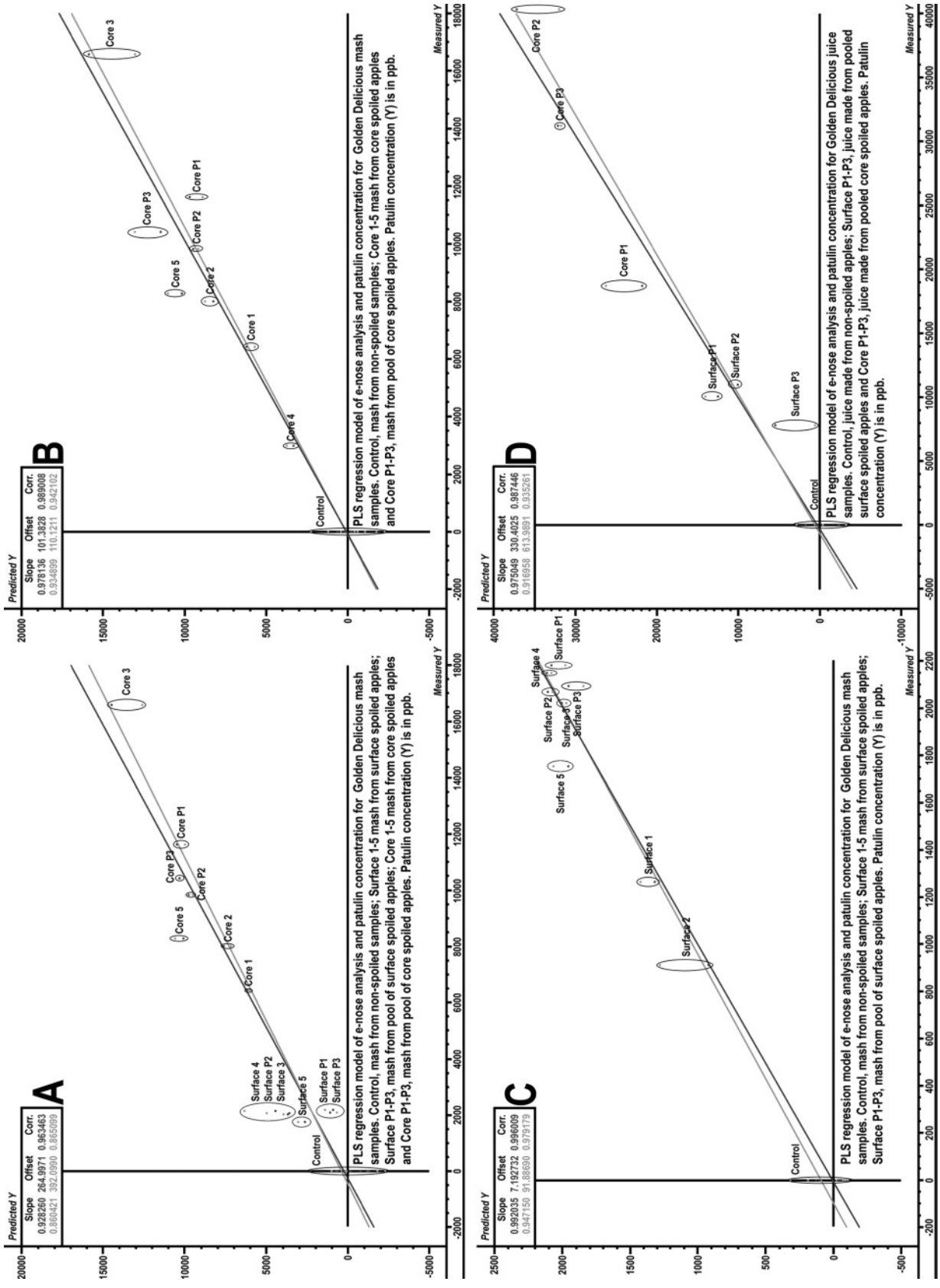

Figure 8. PLS regression models of (**A**) Golden Delicious mash samples from both core- and surface-inoculated apples, (**B**) Golden Delicious mash samples from core-inoculated apples, (**C**) Golden Delicious mash samples from surface-inoculated apples, and (**D**) Golden Delicious juice samples from both core- and surface-inoculated apples.

and surface-inoculated Golden Delicious apples show even better fits, with RMSEP values of 11 and 9%, respectively (**Figure 8B**,**C**), which leads to the conclusion that the two systems, surface- and core-inoculated apples, are indeed two separate entities which cannot easily be covered by one model. This is not too surprising because there is a big difference in the surface to hyphae ratio between the two types of spoilage. PLS regression modeling of e-nose data and patulin concentration for the Golden Delicious juice samples also gave a high-fit regression model with a RMSEP of 12% (**Figure 8D**). For the juice samples it seems that one model covers both the surface inoculation and core inoculation samples.

To our knowledge this is the first report of a regression model between e-nose data and mycotoxin content in which actual concentrations are reported. In a previous study (*11*) the investigation was limited to looking at levels (high, low) of mycotoxins, whereas the regression modeling in another study (*12*) was less accurate and less reliable.

From the results of this "worst case" study it seems very promising to use an e-nose for quality control in the apple juice industry, although additional work has to be carried out to produce prediction models for the various apple varieties, and probably also mixes of apple varieties, used in production. Because this study was a worst case scenario, additional experiments will have to be performed to determine the cutoff concentration for patulin prediction by e-nose analysis. Other factors, such as the influence of apple shredding on e-nose prediction of spoilage and patulin concentration, could also be investigated. The results indicate that it would be feasible to analyze at one of two points during production. E-nose analysis should be carried out either right after shredding of the apples or on the finished juice. The advantage of early, in terms of production, analysis is that there are several process steps in which it is possible to rectify any patulin problem, whereas analysis of the finished juice will yield information on the quality of the finished product. In either case patulin can be, and industrially is, removed by treatment with charcoal (*36*), ascorbic acid (*37*), or irradiation (*38*).

Altogether this study has proven it possible to classify spoiled from nonspoiled apples during the apple juice production steps and at some points even to distinguish between spoilage type (surface and core) by e-nose analysis. It has also been proven possible to create regression models that can be used for quite exact prediction of patulin content in Golden Delicious apples. These results of course are valid for only the chosen cultivar, Golden Delicious, but suggest that it ought to be possible to achieve similar prediction models with any given apple cultivar. To make industrially relevant prediction models it is required to measure many more samples, to use mixed samples of various types of spoilage, to make measurements on various degrees of spoilage, and of course to expand the model with different varieties of apples and mixes of apple varieties. We believe that this study is the first study to show correlation between e-nose analysis data and mycotoxin concentration. The results of this study show a great potential for e-nose prediction of mycotoxin levels even in systems with a high level of volatiles from the matrix such as silage, grain, and fruit juices.

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