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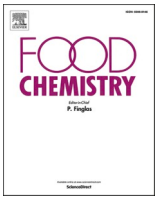

Early prediction of ochratoxigenic *Aspergillus westerdijkiae* on traditional Italian caciocavallo during ripening process by MS-based electronic nose

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ABSTRACT

A rapid and non-invasive mass spectrometry-based electronic nose (MS-eNose) method, combined with chemometric analysis, was developed for the early detection of *Aspergillus westerdijkiae* on caciocavallo cheeses during ripening process. MS-eNose analyses were carried out on caciocavallo inoculated with ochratoxin A (OTA) non-producing species and artificially contaminated with *A. westerdijkiae*, an OTA producing species. Two classification models, i.e. PLS-DA and PC-LDA, were used to discriminate cheese samples in two classes, based on their contamination with toxigenic or non-toxigenic fungal species. Accuracy values were between 87 and 100 % and 86–100 %, in calibration and validation, respectively, with best results obtained at 15-ripening days with 98 % (PLS-DA) and 100 % (PC-LDA) of accuracy in validation. Moreover, eighteen potential volatile markers of the presence of *A. westerdijkiae* were identified by GC–MS analysis. Results show that MS-eNose represents a useful tool for a rapid screening in preventing *A. westerdijkiae* and related OTA contamination in caciocavallo cheese during ripening process.

1. Introduction

In recent years, an increase of demand for artisanal cheeses has been observed worldwide. Local cheesemakers respect traditional practices, in the production on a small scale of those products, which are considered as knowledge of inestimable social and cultural value. Among traditional practices, the ripening in natural environments and the consequent spontaneous colonization of cheese by native molds are considered valuable procedures both by cheesemakers and consumers. The firsts believe that they generate a distinctive link between the environment and their products, while consumers believe that an added value is given to the taste, due to the ability of fungi to contribute to desirable sensory qualities, attributing specific rheological and sensorial characteristics.

Among several artisanal Italian cheeses, caciocavallo is a *Pasta Filata* cheese representing one of the most important traditionally manufactured in southern part of the country, that can be ripened in caves by artisanal dairies (Uzun et al., 2020). This cheese is commonly produced exploiting fungal population naturally occurring on cave walls, without adding any fungal starter culture, which indeed attributes both rheological and sensorial characteristics, highly appreciated by consumers. Mycobiota occurring in caves is composed mainly by *Aspergillus* and *Penicillium* species, including toxigenic species (Anelli et al., 2024; Garnier et al., 2017). However, in some cases the presence of filamentous fungi can be a detrimental effect to cheese quality and/or safety, due to their ability to cause appearance defects and to produce toxic secondary metabolites, including mycotoxins (Anelli et al., 2019). Several studies reported the occurrence of mycotoxins in cheese, including the presence of ochratoxin A, citrinin, penitrem A, roquefortine C, sterigmatocystin and aflatoxin M1 (Sengun et al., 2008; Decontardi et al., 2017; Kalinina et al., 2018; Anelli et al., 2019; Camardo Leggieri, Pietri, & Battilani, 2020). Ochratoxin A (OTA) has potent toxicity due to its nephrotoxic, hepatotoxic, teratogenic, carcinogenic, and immunosuppressive effects demonstrated in several

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mammalian species (Malir et al., 2016). Furthermore, OTA was classified by the International Agency for Research on Cancer as a possible human carcinogen (Group 2B), based on evidence of carcinogenicity in experimental animal studies (IARC, 1993). Although, to protect consumers from exposure to OTA through the consumption of food products, several countries have established maximum levels in several foodstuffs, although no limits are still available for cheese.

Recently, the presence of *A. westerdijkiae* on cheese has been reported as associated to OTA contamination of artisanal cheese produced both in Italy and Brazil (Anelli et al., 2019; Marcelao et al., 2024). Due to its ability, *A. westerdijkiae* is able to grow on cheese surface and possibly to produce OTA, a liposoluble molecule which can migrate insight the cheese, contaminating the whole product, not only the surface, which is often considered an edible part, where fungi grow during ripening (Anelli et al., 2019; Yang et al., 2019). Therefore, the accidental presence of this toxigenic species is an undesirable event, because of potential risk for consumer's health, and should be avoided or limited.

Furthermore, in case of contaminated samples there are still limitations of mycotoxin degradation or detoxifications in food (Ding et al., 2023; Jouany, 2007; Kolossova et al., 2009; Lee et al., 2024), therefore prevention of mould contamination is still the best solution to eliminate or reduce the mycotoxin levels: cold storage, reduction or absence of oxygen, plant extracts and microbial control could be efficient solutions for cheesemakers.

Early detection of ochratoxigenic molds growth in cheese is a necessary step for managing the toxigenic fungi contamination with one of physical, chemical, and biological methods potentially able to prevent fungal growth and indirectly eliminate or reduce mycotoxin levels in cheese and is also highly recommend to prevent human exposure to OTA. Although, morphological identification and molecular detection represent techniques which are highly specific and reliable for fungal detection in food, they are time consuming, expensive and require specialist expertise.

The availability of rapid methods for monitoring these fungal species is crucial and constitutes a key stage to produce safe ripened foods, mainly when carried out under not-controlled environmental conditions. Fungal monitoring methods ideally should be non-invasive and should allow early detection of toxigenic fungal species and related mycotoxin risk.

Characterization and comparison of patterns of volatile organic compounds (VOCs) have been often used for early detection of fungal growth and to distinguish samples contaminated by toxigenic and non-toxigenic strains (Barkat et al., 2017; Josselin et al., 2024; Magan & Evans, 2000; Vinaixa et al., 2004). Given the complexity of the volatile headspaces of the real matrices, they should be evaluated as a whole pattern using untargeted approaches, including electronic noses, which represent convenient tools thanks to their speed, simplicity and low costs. For this reason, electronic nose approaches have been often applied for the discrimination of mycotoxigenic and non-mycotoxigenic fungal species (Cabañes et al., 2009; Falasconi et al., 2005; Keshri & Magan, 2000; Camardo Leggieri, Pont, Battilani, & Magan, 2011; Lippolis et al., 2016; Sahgal et al., 2007).

The mass spectrometry-based electronic nose (MS-eNose) technique is one of the most innovative approaches that can be used to analyse VOCs of complex matrices. This technique is based on the use of headspace solid-phase microextraction directly coupled to MS where the extraction of the sample headspace generates VOCs which are immediately introduced and fragmented into a mass spectrometer, that can usually be a single quadrupole or time-of-flight. Each measured ion with a specific mass-to-charge (m/z) ratio represent a "sensor" with the relevant intensity determining its contribution which is subsequently submitted to multivariate statistical analysis. This technique has been also classified as an electronic nose and it can analyse samples in very short times (few minutes) and with minimal sample pretreatment (Peris & Escuder-Gilabert, 2009). Although this technique is less frequently used than conventional sensor-based systems due to its high cost, it has several advantages in terms of sensitivity, range of application and possibility to provide chemical information (Gliszczyńska-Świgło & Chmielewski, 2017).

The aim of this study was to develop a rapid and non-invasive MS-eNose method for the early detection of ochratoxigenic *A. westerdijkiae* on surface of traditional Italian caciocavallo during ripening process. In addition, a pattern of volatile compounds specifically related to the presence of *A. westerdijkiae* on the surface of cheese samples was identified and characterized by headspace solid-phase microextraction combined to gas chromatography–mass spectrometry (GC–MS) analysis.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile, methanol (HPLC-grade), glacial acetic acid and phosphate buffered saline (PBS) tablet were purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Glass microfiber filters (Whatman GF/A) were obtained from Whatman (Maidstone, UK). Standards of ochratoxin A (OTA) were purchased from Sigma-Aldrich (Milan, Italy). A mixture of normal alkanes (C5-C29) was purchased from o2si Smart Solutions (Charleston, SC, USA). Chemical standards (Acetic acid, Nonanoic acid, 1-Ethanol, 1-Pentanol, 3-Methyl-1-butanol, 1-Hexanol, 1-Octen-3-ol, 2-Ethyl-1-hexanol, 1-Octanol, 1-Nonanol, Phenylethyl Alcohol, 2-Methylbutanal, 3-Methylbutanal, Nonanal, Decanal, Benzaldehyde, Methyl butanoate, Methyl hexanoate, Methyl octanoate, Methyl hexadecanoate, γ-Caprolactone, 2-Methyldecane, Acetone, 3-Octanone, Acetoin and 6-Methyl-5-hepten-2-one) were purchased from Ultra Scientific Italia S.r.l. (Bologna, Italy). The 2-Methylpentanal (≥98 %) and Dichloromethane (≥99.9 %) were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Helium at a purity of 99.9995 % was obtained from Sapio S.r.l. (Bari, Italy).

2.2. Fungal strains

Penicillium charlesii ITEM 19059, *P. copticola* ITEM 19068, *P. solitum* ITEM 19072, *P. canescens* ITEM 19074 and *Aspergillus westerdijkiae* ITEM 18008 were selected as secondary starter for cheese ripening. They were isolated from cheese aged in natural environments and are maintained from Agri-Food Toxigenic Fungi Culture Collection at Institute of Sciences of Food Production (CNR-ISPA, Bari, IT, http://server.ispa.cnr.it/ITEM/Collection/). All strains were revitalized on Potato Dextrose Agar (PDA), then each of them was inoculated on sterile rice UR 40 % (p/v) and incubated for 5 days at 25 °C, in the darkness and occasionally mixed. From each fungal culture conidia were washed with sterile 1× PBS, drained gently from rice and filtered through a sterile Miracloth filter paper (Millipore®) to remove fungal hyphal debris. The concentration of the mycelium-free conidial suspension was estimated using a Thoma counting chamber and finally adjusted by dilution to 2×10^6 spores/mL per each strain. Strains were mixed at equal ratio to constitute two different inocula, namely ST and STw, as reported in Table 1.

Table 1
Fungal species used in equal ratio for inoculum preparation of secondary starters. ST and STw differs for the presence of ochratoxigenic species, *A. westerdijkiae*.

INOCULUM ID	SPECIES mixtures
ST	*P. charlesii* ITEM 19059, *P. copticola* ITEM 19068, *P. solitum* ITEM 19072, *P. canescens* ITEM 19074
STw	*P. charlesii* ITEM 19059, *P. copticola* ITEM 19068, *P. solitum* ITEM 19072, *P. canescens* ITEM 19074, *A. westerdijkiae* ITEM 18008

2.3. Inoculation and ripening process

Ten caciocavallo cheese wheels per each inoculum were used for the microbiological and chemical analyses. After a drying phase at 15 °C for 15 days, cheeses were inoculated with the respective inoculum by dipping (T0). Samples were dried at 22 °C and relative humidity (RH) 85 % for 24 h in separate laboratory-scale seasoners, one per each inoculum. After 24 h (T1), the inoculated cheeses were ripened at 18 °C and RH 75 % for 90 days. In order to simulate at laboratory scale the ripening process and according the traditional ripening process, after initial 15 days of ripening the surface of each caciocavallo cheese was washed and gently brushed with a saturated sterile solution of NaCl (300 g/L). The procedure was repeated after 15 days for 5 times. After each washing/brushing procedure the caciocavallo cheeses were transferred back in the seasoning chambers in order to continue the ripening process. The load of inoculated mycoflora was verified after the first 24 h (T1), and successively at 15 (T15), 30 (T30) and 45 (T45) days of ripening process. The total surface of inoculated cheese was washed with sterile 1× PBS at a ratio of 2:1 *w/v* and adequate decimal dilutions were plated on Dichloran Rose Bengal Chloramphenicol Agar (DRBC, Conda) medium, and incubated in the dark at 25 °C for 72 h. The mycoflora load was expressed as CFU/cm^2 of cheese surface. This analysis was not carried out after 60 (T60) and 90 (T90) days of ripening due to the extremely rarefaction of mycelium on the cheese surface after continuous brushing and washing operations. The persistence of inoculated fungal strains on cheese was assessed by re-isolation of fungi from cheese washing solutions, followed by identification of single-spore cultures based on both morphological identification (Peterson, 2003) and DNA analysis by beta-tubulin gene sequencing, according to Anelli et al. (2019).

2.4. Specific detection of A. Westerdijkiae

A targeted PCR molecular assay was applied to specifically detect the presence of *A. westerdijkiae* ITEM 18008, by amplifying the *otaR1* gene, coding for the bZIP transcription, essential for transcription regulation of OTA cluster genes (Susca et al., 2021). Specific primers were designed using Prime3Plus (Untergasser et al., 2012) on the sequence of *otaR1* gene from *A. westerdijkiae* ITEM18008 (MW526250.1). Sequences of primer set were AwotaR1_F CGAAACCCAATTCGGCTGA and AwotaR1_R CCGGATCTCGTCCTCTAGG. The PCR reaction was carried out in 10 μL reaction mixture containing Platinum Hot Start SuperFi PCR 1× Master Mix (Invitrogen, Carlsbad, CA, USA), 500 nM of each primer and 20 ng of crude DNA extract obtained from the washing solution after 1, 15, 30 and 45 days of ripening according to Ferrara et al. (2020). PCR amplification conditions were 98 °C for 30 s for initial denaturation, 30 cycles of 98 °C for 10 s for denaturation, 58 °C for 15 s for annealing, and 72 °C for 30 s for extension, followed by a final extension at 72 °C for 5 min. PCR amplification products were separated by 2 % (*w/v*) agarose gel electrophoresis and stained with GelRed (Biotium, USA). PCR products were sequenced with an 3730xl DNA Analyzer sequencer (Applied Biosystems) and sequence identity was verified by BLASTN analysis against NCBI nucleotide sequence database to confirm the nucleotide sequence of targeted gene.

2.5. Sample preparation

A slice of about 1/8 caciocavallo cheese was vertically cut and 4–5 mm of the rind was sampled, chopped with a knife in a 2–3 mm pieces and homogenized by mixing manually. For OTA analysis, 5 g rind were placed in a blender jar, whereas for VOCs analysis 1.5 g rind were placed in a 10 mL vial for headspace analysis with a screw seal (Agilent Technologies).

Caciocavallo cheese rind after washing steps was sampled and analyzed at the beginning of ripening process (T0) for OTA determination by HPLC with fluorescence detection and after 1 day of ripening (T1) for both HS-SPME/MS-eNose and HS-SPME/GC–MS analyses. Cheeses were subsequently sampled and analyzed by the three methods (HPLC, MS-eNose and GC–MS) after 15 (T15), 30 (T30), 45 (T45), 60 (T60) and 90 (T90) days of ripening.

The Dichloromethane was used as internal standards for HS-SPME/MS-eNose analyses by adding 5 μL of 10 μg/μL solution diluted with methanol (internal standard/sample ratios of 33.33 μg/g). The 2-Methylpentanal was used as internal standards for HS-SPME/GC–MS analyses by adding 4 μL of 0.1 μg/μL solution diluted with methanol (internal standard/sample ratios of 0.27 μg/g).

2.6. HS-SPME/MS-eNose analysis

2.6.1. Spectral acquisition

HS-SPME/MS-eNose analysis were performed by using the mass spectrometry-based electronic nose (MS-eNose) GERSTEL Headspace ChemSensor System (GERSTEL, Mülheim, Germany). A total of 15 analysis were performed per day of ripening on caciocavallo wheels with the two different inoculums (ST and STw), resulting in a total of 180 analyses. MS-eNose consisting of a headspace multi-purpose sampler MPS 2 (Gerstel, Mulheim an der Ruhr, Germany) and the Agilent 7890 A GC System (Agilent Technologies, Palo Alto, CA, USA), modified for non-separative analysis with a deactivated fused-silica tubing (transfer column,10 m × 0.18 mm i.d., 0 μm film thickness, Agilent Technologies), coupled to the Agilent 5975C inert MSD mass spectrometer. Moreover, MPS 2 sampler was equipped with headspace incubation chamber and SPME sampling unit. An in-house optimized protocol was used to carry out the HS-SPME/MS-eNose analyisis. In particular, the headspace vial was kept at temperature of 50 °C for 10 min in the incubator-agitator of the MPS 2 autosampler to generate the headspace. The extraction from the headspace was performed by exposing a divinylbenzene/carboxen/polydimethylsiloxane (SPME-Fast Fit Fiber Assembly-FFA-DVB/CAR/PDMS, 50/30 μm film thickness, 1 cm fiber length; Chromline, Prato, Italy) fiber at 50 °C for 30 min. After extraction, volatile compounds were thermally desorbed exposing the fiber in the split/splitless injection port (Agilent Technologies) of the MS-eNose at 250 °C for 5 min. The injection port was fitted with 0.75-mm i.d. Ultra Inert liner Straight (Agilent Technologies) and was maintained at 250 °C in splitless mode. The oven, transfer line, ion source and quadrupole temperatures were 180, 280, 230 and 150 °C, respectively; the helium flow rate was held constant at 1 mL/min; electron impact Ionization (EI+) mode with an electron energy of 70 eV was used; the mass spectrometer acquired data in Total Ion Current mode (*m/z* range: 40–300 u).

2.6.2. Spectral processing

For each analysis a Fingerprint Mass Spectrum (FMS) was obtained by the software Chemsensor 6.912 (Gerstel, Mülheim and der Ruhr, Germany) corresponding to the sum of mass spectra obtained in the time range 0.22–2.0 min. A matrix of 260 columns (ions intensity) and 180 rows (samples) was used for chemometric analysis. Internal standardization was performed for each FMS dividing each mass intensity by the intensity of *m/z* 49 u fragment of Dichloromethane added as internal standard (Pérez Pavón et al., 2003; Pérez Pavón et al., 2006).

2.7. HS-SPME/GC–MS analysis

2.7.1. Spectral acquisition

For each ripening period and per type of inoculum, 3 analyses (replicates) were carried out, on caciocavallo cheese wheel, resulting in a total of 36 analyses. The SPME protocol is the same used for the HS-SPME/MS-eNose analysis. GC–MS analyses were carried out by an Agilent 6890 Series GC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a VF-WAXms (60 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies) fused-silica capillary column and coupled to an Agilent 5973 Network Mass Selective Detector mass spectrometer. The injection port, fitted with a 0.75 mm i.d. liner, was

maintained at 250 °C for 5 min in splitless mode. The analyses were performed with programmed temperature: initial temperature 40 °C is maintained for 5 min, then raised from 40 to 140 °C at 2 °C/min, then from 140 to 210 °C at 5 °C/min, then from 210 to 230 °C at 20 °C/min, and the final temperature being maintained for 10 min. The helium flow rate was held constant at 1 mL/min. The transfer line, ion source and quadrupole temperatures were 280, 290 and 150 °C, respectively. Electron impact Ionization (EI+) mode with an electron energy of 70 eV was used. The mass spectrometer acquired data in full scan mode (*m/z* range: 40–300 u).

2.7.2. Spectral processing

The volatile organic compounds were identified by comparison of experimental mass spectra with ones present in the NIST/EPA/NIH Mass Spectral Database library (National Institute of Standards and Technology, Version 2.4, 2020, USA), using a MS match factor greater than or equal to 80 %. In addition, the identification was also verified by comparison of their linear retention indices (LRI) determined in relation to the retention times of C5–C29 n-alkanes series, with those reported in literature (Zellner et al., 2008; NIST, 2023). Chemical standards were also used to confirm the identification of a set of 26 volatile organic compounds. Agilent Technologies (Santa Clara, USA) provided the MSD Chemstation, which was used to calculate the compounds' total ion peak area. Quantification of compounds was performed by the same method of internal standardization used for HS-SPME/MS-eNose analysis. The amount of each identified compound was estimated by comparing the total ion current (TIC) peak area with 2-methyl-pentanal peak area and expressed as area ratio (Cefola et al., 2023; Souza & Bragagnolo, 2014; Zhang et al., 2022).

2.8. Ochratoxin A determination in cheese by HPLC/FLD

OTA was analyzed, in triplicate, using an internal method by extraction 5 g rind with 25 mL acetonitrile:acetic acid:water (74:1:25 v:v:v) for 3 min by blending. The extract was centrifuged at 3900*g* for 10 min, then 5 mL were diluted with 20 mL PBS (pH:7.4) and filtered through glass microfiber filter. A volume of diluted extract (12.5 mL) was cleaned up through AOF MS-PREP® (R-Biopharm Rhone, Glasgow, UK) immunoaffinity column. The column was washed with 10 mL PBS and the toxin was eluted with 1 mL of methanol. The final extract was diluted with water 1 mL and quantified using (HPLC/FLD). In particular 100 µL volume extract was injected into the HPLC apparatus (technology series 1100, Agilent, Santa Clara, CA). The fluorometric detector was set at wavelengths of 340 nm (excitation) and 460 nm (emission). The analytical column was a Zorbax SB-C18 (4.6 by 150 mm by 5 mm; Agilent). The mobile phase consisted of a mixture of acetonitrile/water/glacial acetic acid (99:99:2, vol/vol/vol) at a flow rate of 1 mL/min. OTA was quantified by measuring peak areas at the retention time of OTA standard solutions and comparing them with the calibration curve from 0.5 to 200 ng/mL. Calibration curves were prepared by drying different aliquots of the OTA stock solution (1 mg/mL) and successively reconstituted in the HPLC mobile phase acetonitrile:water:acetic acid (99:99:2, v:v:v). The detection limit was 0.5 µg/kg, based on a signal to noise ratio of 3:1.

2.9. Statistical analysis

The chemometric analysis was conducted with Chemometric Agile Tool (Leardi et al., 2024), for principal component analysis (PCA), and with Classification Toolbox (Ballabio & Consonni, 2013) in Matlab (Mathworks Inc., Natick, Massachusetts, USA) for the development and validation of the supervised pattern recognition methods, i.e. Partial Least Squares Discriminant Analysis (PLS-DA) and Principal Components Linear Discriminant Analysis (PC-LDA)., In detail, performances of the PLS-DA and PC-LDA models were compared by using both accuracy values in calibration to evaluate the ability to correctly classify samples used for the building of the models, and accuracy values in prediction by cross-validation (Monte Carlo Cross Validation) to evaluate the ability to correctly classify samples of a test set generated in leave-20 %-out cross validation. Permutational multivariate analysis of variance (PERMANOVA) and *t*-test was performed using Real Statistics Resource Pack software (Release 7.6; Copyright 2013–2021 Charles Zaiontz; www.real-statistics.com) and Statistica 10.0 (StatSoft Italia srl, Padova, Italy), respectively.

3. Results and discussion

3.1. Analysis of inoculated mycobiota

The persistence of fungal species included in the 2 fungal mixtures ST and STw (without and with ochratoxigenic species, respectively) on the surface of caciocavallo samples, was assessed by plate counting at T1, T15, T30, and T45. Related total counts were $3\bullet10^3$, $1\bullet10^4$, $3\bullet10^5$, and $9\bullet10^4$ CFU/cm^2 in ST samples and $6\bullet10^3$, $2\bullet10^4$, $4\bullet10^5$, and $2\bullet10^4$ CFU/cm^2 in STw samples respectively. The *A. westerdijkiae* counts reached at T1, T15, T30 and T45 were $2\bullet10^3$, $9\bullet10^4$, $3\bullet10^5$, and $8\bullet10^2$ CFU/cm^2 respectively. DNA-sequences of beta-tubulin gene and observation of morphological characters of pure fungal colonies confirmed the occurrence of the same species previously inoculated, and no other fungal species were detected (data not shown).

3.2. Detection of A. Westerdijkiae

Primers set AwotaR1_F CGAAACCCAATTCGGCTGA and AwotaR1_R CCGGATCTCGTCCTCTAGG was designed within the *otaR1* target region. The rapid procedure to obtain a crude DNA extract from the mycelium grown on the surface of artificially contaminated caciocavallo cheeses was effective to detect *A. westerdijkiae* with the PCR assay. Agarose gel electrophoresis demonstrated the presence of the target DNA only on caciocavallo cheeses inoculated with the ochratoxigenic strain *ITEM 18008* (inoculum STw), confirming the presence of the *A. westerdijkiae* from the first day after inoculation, up to 45 days of ripening (Fig. S1).

3.3. Presence of OTA in caciocavallo cheese samples

As shown in the Fig. 1 a superficial proliferation of fungi during ripening process at day 90 of the caciocavallo cheese was observed. Results obtained showed that OTA was not detectable in all caciocavallo ST samples inoculated with OTA non-producing species while in the case of STw samples containing also *A. westerdijkiae*, a production of OTA was observed with a trend initially increasing up to a final concentration of 540 mg/kg (Table 2). The presence of OTA in these samples indicated

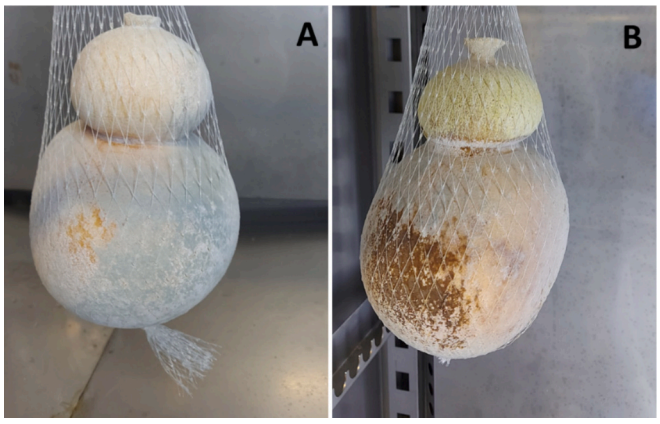

Fig. 1. Caciocavallo cheese inoculated with non-toxigenic (ST, A) or toxigenic (STw, B) fungal strains after 90 days of ripening process.

Table 2
OTA concentration in STw samples rind during cheese ripening process.

Sampling days	[OTA] mg/kg
T0	< LOD
T15	3 ± 1
T30	4 ± 2
T45	45 ± 7
T60	460 ± 40
T90	540 ± 48

that the *A. westerdijkiae* produced OTA up to high contamination levels despite the common technological processing operations, such as washing and brushing, applied during the ripening process.

These results confirmed the potential risk of contamination for caciocavallo cheese samples contaminated by *A. westerdijkiae* and the relevance to develop a rapid tool to predict the presence of these species on cheese samples during ripening processes.

3.4. Prediction of ochratoxigenic A. Westerdijkiae by MS-eNose

Principal Component Analysis (PCA) was carried out with the purpose of getting general information on the ability of the MS-eNose data to cluster caciocavallo cheese samples based on their contamination by toxigenic (STw) and non-toxigenic (ST) fungal species. Specifically, the PCA was initially applied on all data obtained for the different sampling days, but no noteworthy clustering of samples was observed in the PC1 vs. PC2 score plot (Fig. S2) based on the different fungal contaminations, i.e. STw and ST. Otherwise, by applying PCA on data obtained at beginning and after 30, 45, 60 and 90 days, the score plots of PC1 vs PC2 (Fig. S3) showed appreciable clusters of the samples. In particular, PCA obtained for samples after 15 days of ripening showed a more evident separation between the STw and ST cheeses mainly along the PC2 (Fig. 2).

Subsequently, aiming to get a rapid method for an early detection of *A. westerdijkiae* during ripening process, supervised pattern recognition techniques were used to discriminate cheese samples in STw and ST classes containing different fungal species. In detail, two different classification models were used, i.e. Partial Least Squares Discriminant Analysis (PLS-DA) and Principal Components Linear Discriminant Analysis (PC-LDA) for a total of fourteen statistical models as reported in Table 3. Specifically, the two statistical approaches PC-LDA and PLS-DA were applied either on each dataset of ripening times (i.e. 6, from T1 to T90) and on the entire dataset including all ripening times. In Table 3 are reported the performance of the 14 models in terms of accuracy (%), both in calibration and validation, together with the total variance explained using the number of latent variables (for PLS-DA) and principal components (for PC-LDA) providing the lowest error in cross validation and used for their construction. In addition, the percentage of false ST is reported, i.e. the rate of samples belonging to the STw class but identified as ST ones. As reported, all models showed good discrimination abilities between ST and STw samples as the accuracy values of the models were between 87 and 100 % and between 86 and 100 % in calibration and validation, respectively. In particular, the models built on caciocavallo samples ripened for 15, 45 and 90 days, showed accuracy values up to 100 % in validation. Furthermore, no false ST were observed at 15 ripening days for both models thus indicating that this ripening period could be use as sampling point to get an early prediction of presence of *A. westerdijkiae* in caciocavallo cheese.

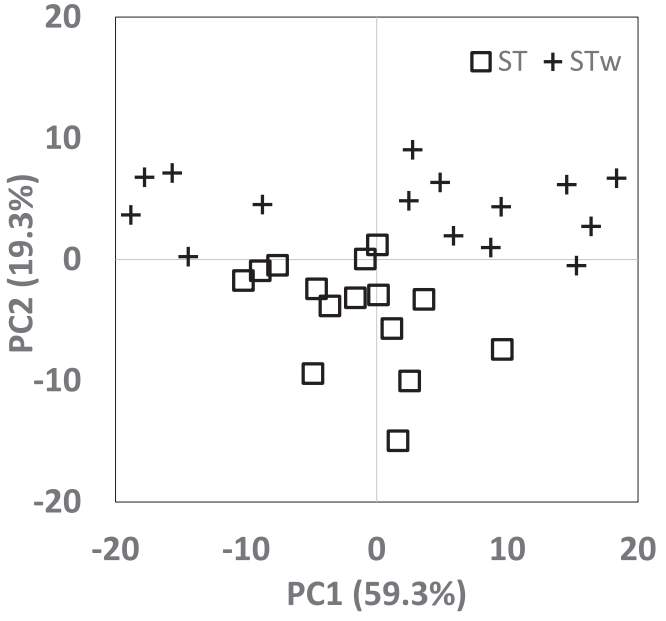

Fig. 2. Score plot of HS-SPME/MS-eNose data for caciocavallo cheese samples inoculated with toxigenic (STw) and non-toxigenic (ST) fungal strains at 15 days of ripening.

3.5. Characterization of the pattern of volatile compounds by HS-SPME/GC–MS

A subset of cheeses belonging to the STw and ST classes was further analyzed by HS-SPME/GC–MS for each ripening time to get information about the chemical identity of the volatile organic compounds (VOCs) composition emitted by caciocavallo cheese samples. A total of 118 VOCs were detected: 9 aldehydes, 15 carboxylic acids, 14 ketones, 23 alcohols, 16 esters, 7 lactones, 2 alkanes, 6 aromatic compounds, 1 sulfides, 1 sulfone, 1 terpene and 23 unknown compounds. In Table 4 are listed the VOCs detected with the relative Linear Retention Indices and MS match factor as percentage. Then, statistical analyses were applied on the GC–MS data to highlight changes in the VOCs profile between STw and ST classes. A permutational multivariate analysis of variance (PERMANOVA) revealed that significant differences ($p \leq 0.05$, Table 5) between STw and ST sample were observed at 15, 45, 60 and 90 days. Moreover, increasing the significance level of PERMANOVA ($p \leq 0.01$) only at 15 days a significant difference was still observed. To identify compounds having a role at the 15th ripening day in the discrimination

Table 3
Performance parameters in terms of accuracy obtained for the tested models (PC-LDA and PLS-DA) in calibration and validation for cheese sample classifications during ripening.

Ripening day	Statistical models	Explained variance (components)	Model accuracy (%)		False ST samples (%)
			Calibration	Validation	
T1	PLS-DA	100 % (7)	100	90	6
	PC-LDA	100 % (10)	100	87	9
T15	PLS-DA	84 % (3)	100	98	0
	PC-LDA	98 % (4)	100	100	0
T30	PLS-DA	100 % (10)	100	90	18
	PC-LDA	100 % (7)	97	89	14
T45	PLS-DA	89 % (2)	100	97	5
	PC-LDA	99 % (6)	100	100	1
T60	PLS-DA	91 % (2)	97	91	11
	PC-LDA	100 % (7)	97	92	12
T90	PLS-DA	98 % (3)	100	100	0
	PC-LDA	100 % (5)	100	98	3
T1, T15, T30, T45, T60 and T90	PLS-DA	100 % (10)	92	90	10
	PC-LDA	100 % (8)	87	86	19

Table 4
Volatile compounds ($n = 118$) identified by HS-SPME/GC–MS analysis of cheese samples.

Volatile compounds	LRI_{lt} / LRI_{exp} [a] (MS match factor %)	Volatile compounds	LRI_{lt} / LRI_{exp} [a] (MS match factor %)
Aldehydes		***Ketones***	
Acetaldehyde	694/693 (97)	Acetone [b,d]	808/806 (95)
2-Methylbutanal [b]	906/906 (87)	2-Pentanone	968/968 (97)
3-Methylbutanal [b]	912/910 (86)	2-Hexanone [d]	1074/1074 (92)
2-Methylpentanal [c]	−/1001 (93)	2-Heptanone [d]	1178/1178 (90)
Nonanal [b]	1396/1390 (89)	3-Octanone [b,d]	1249/1249 (90)
Furfural	1469/1464 (81)	2-Octanone	1279/1279 (91)
Decanal [b]	1494/1494 (88)	Acetoin [b]	1285/1282 (87)
Benzaldehyde [b]	1521/1521 (80)	6-Methyl-5-hepten-2-one [b]	1333/1333 (80)
2-phenyl-2-Butenal	1932/1930 (85)	2-Nonanone	1387/1387 (94)
Carboxylic acids		8-Nonen-2-one	1473/1439 (91)
Acetic acid [b]	1451/1451 (95)	2-Decanone	1489/1487 (95)
Propanoic acid	1535/1537 (93)	2-Undecanone	1592/1592 (95)
2-Methylpropanoic acid	1565/1565 (86)	Acetophenone	1647/1647 (88)
Butanoic acid	1623/1623 (91)	2-Tridecanone	1801/1801 (90)
3-Methylbutanoic acid [d]	1665/1665 (82)	***Alcohols***	
Pentanoic acid	1738/1737 (88)	Ethanol [b]	926/926 (87)
2-Methylpentanoic acid [d]	1764/1765 (90)	2-Pentanol	1112/1117 (90)
Hexanoic acid	1841/1841 (97)	1-Methoxy-2-propanol	1131/1125 (84)
3-Methylhexanoic acid	1869/1879 (83)	1-Butanol	1141/1141 (92)
2-Ethylhexanoic acid	1954/1944 (90)	2-Hexanol	1216/1216 (83)
Heptanoic acid	1950/1948 (87)	1-Pentanol [b]	1244/1246 (92)
Octanoic acid	2054/2053 (92)	3-Methyl-1-butanol [b]	1203/1203 (85)
Nonanoic acid [b]	2163/2163 (90)	2-Heptanol	1315/1315 (93)
n-Decanoic acid	2261/2260 (95)	1-Hexanol [b]	1348/1349 (95)
Dodecanoic acid	2470/2470 (93)	2-Butoxy-1-ethanol	1393/1396 (80)
Lactones		2-Octanol [d]	1416/1415 (85)
Butyrolactone	1602/1599 (81)	1-Octen-3-ol [b,d]	1446/1446 (96)
γ-Caprolactone [b]	1699/1698 (92)	1-Heptanol [d]	1449/1450 (86)
δ-Caprolactone	1792/1791 (80)	2-Ethyl-1-hexanol [b]	1481/1484 (91)
γ-Octalactone	1916/1916 (90)	2-Nonanol [d]	1514/1514 (80)
δ-Octalactone	1970/1971 (82)	1-Octanol [b]	1552/1553 (85)
γ-Decalactone	2155/2155 (89)	(E)-2-Octen-1-ol [d]	1611/1610 (84)
δ-Decalactone	2200/2199 (86)	1-Nonanol [b]	1654/1654 (82)
Aromatic compounds		2 -Furanmethanol	1658/1658 (83)
Toluene	1034/1034 (92)	2-Undecanol	1712/1713 (84)

Table 4 (*continued*)

Volatile compounds	LRI_{lt} / LRI_{exp} [a] (MS match factor %)	Volatile compounds	LRI_{lt} / LRI_{exp} [a] (MS match factor %)
2,6-Dimethylpyrazine [d]	1328/1325 (92)	2-Methoxy phenol	1860/1860 (81)
Trimethylpyrazine	1400/1400 (80)	Benzyl alcohol	1875/1875 (86)
1,3-Dimethoxybenzene	1740/1748 (98)	Phenylethyl Alcohol [b,d]	1908/1908 (89)
Benzothiazole	1958/1958 (80)	***Esters***	
Phenol	2000/2000 (86)	Methyl butanoate [b]	983/983 (87)
Unknown compounds		Methyl pentanoate	1081/1081 (80)
Unknown 1	−/1054	Pentyl acetate	1169/1169 (80)
Unknown 2	−/1146	Methyl hexanoate [b]	1183/1183 (90)
Unknown 3	−/1148	Butyl butanoate	1213/1213 (87)
Unknown 4 [d]	−/1221	Methyl octanoate [b]	1383/1384 (85)
Unknown 5	−/1406	Hexyl butanoate	1407/1409 (93)
Unknown 6	−/1419	Methyl nonanoate	1481/1486 (85)
Unknown 7	−/1444	Methyl decanoate	1586/1588 (95)
Unknown 8	−/1480	Methyl dodecanoate	1793/1795 (94)
Unknown 9	−/1555	Methyl tridecanoate	1911/1898 (83)
Unknown 10	−/1567	Methyl tetradecanoate	1998/1998 (92)
Unknown 11	−/1637	Methyl pentadecanoate [d]	2108/2110 (80)
Unknown 12	−/1642	Methyl hexadecanoate [b,d]	2205/2205 (81)
Unknown 13	−/1645	Isopropyl hexadecanoate	2232/2226 (85)
Unknown 14	−/1690	Triethyl citrate	2461/2460 (84)
Unknown 15	−/1850	***Alkanes***	
Unknown 16	−/1869	2-Methyldecane [b]	1053/1047 (89)
Unknown 17	−/1902	Undecane	1100/1093 (90)
Unknown 18	−/2030	***Sulfide***	
Unknown 19	−/2041	Carbon disulfide [d]	735/725 (97)
Unknown 20	−/2075	***Sulfone***	
Unknown 21	−/2368	Dimethyl sulfone [d]	1906/1904 (86)
Unknown 22	−/2484	***Terpene***	
Unknown 23	−/2497	Menthol	1631/1633 (80)

[a] LRI_{lt}: Linear Retention Indices reported in literature by www.nist.gov; LRI_{exp}: Linear Retention Indices calculated against n-alkanes (C5–C29) on VF-WAXms column.
[b] VOCs identified by chemical standards.
[c] Internal standard (I.S.).
[d] VOCs selected by the *t*-test statistically different ($p \leq 0.05$) between NT and WD samples ripened for 15 d.

of cheese samples based on the toxigenic and non-toxigenic fungal strains presence, a *t*-test was carried out highlighting that the median values of 18 molecules were significantly different ($p \leq 0.05$, Tables 4, S1 and Fig. S4). Specifically, apart from one unknown VOC (Unknown 4) the others were Carbon disulfide; Acetone; 2-Hexanone; 2-Heptanone; 3-Octanone; 2,6-Dimethylpyrazine; 2-Octanol; 1-Octen-3-ol; 1-Heptanol; 2-Nonanol; (E)-2-Octen-1-ol; 3-Methylbutanoic acid; 2-Methylpentanoic acid; Dimethyl sulfone; Phenylethyl Alcohol; Methyl pentadecanoate and Methyl hexadecanoate. In particular, all 18 VOCs had median values

Table 5
P-values obtained by PERMANOVA analysis of HS-SPME/GC–MS data performed between samples belonging to STw and ST classes during ripening process.

Ripening day	p-value
T1	0.061
T15	0.007
T30	0.064
T45	0.037
T60	0.018
T90	0.025

in STw samples statistically higher than those measured in ST samples. These VOCs were potential markers of the presence of *A. westerdijkiae* even though experiments should be further carried out to confirm this statement.

The above results suggest that the 15th day of ripening may be chosen as critical control point to check the contamination by *A. westerdijkiae* on caciocavallo surface, and subsequently to apply adeguate measures of control, aimed at limiting potential risks to the health of consumers.

4. Conclusions

A mass spectrometry-based electronic nose was successfully applied for early and rapid prediction of presence of *Aspergillus westerdijkiae* ochratoxin A-producing species on traditional Italian caciocavallo during ripening process. The developed MS-eNose method was able to detect the presence of *A. westerdijkiae* species among ochratoxin A-non producing species, commonly used as starters. The highest prediction rates were obtained after 15 days of ripening period of caciocavallo cheese with recognition values close to 100 % for both PLS-DA and PC-LDA classification approaches. In addition, a pattern of eighteen volatile compounds related to the discrimination of samples inoculated with the two different starters was characterized by HS-SPME/GC–MS analysis. To the best of our knowledge, this is the first study describing the application of the MS-eNose technique to the early prediction of ochratoxigenic species in cheese products during ripening process.

CRediT authorship contribution statement

Salvatore Cervellieri: Writing – review & editing, Writing – original draft, Validation, Formal analysis. **Francesco Longobardi:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **Antonia Susca:** Writing – review & editing, Conceptualization. **Pamela Anelli:** Writing – review & editing, Formal analysis. **Massimo Ferrara:** Writing – review & editing, Conceptualization. **Thomas Netti:** Writing – review & editing, Formal analysis. **Miriam Haidukowski:** Writing – review & editing, Formal analysis. **Antonio Moretti:** Writing – review & editing, Supervision, Conceptualization. **Vincenzo Lippolis:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.142470.

Data availability

No data was used for the research described in the article.

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