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Discrimination of defective dry-cured Iberian ham determining volatile compounds by non-destructive sampling and gas chromatography

Andrés Martín-Gómez [a,c], M. Pilar Segura-Borrego [b], Rocío Ríos-Reina [b], M. José Cardador [a], Raquel M. Callejón [b], M. Lourdes Morales [b], Vicente Rodríguez-Estévez [d], Lourdes Arce [a,*]

[a] Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry, Marie Curie Annex Building, University of Córdoba, Campus de Rabanales, E-14071, Córdoba, Spain
[b] Dpto. de Nutrición y Bromatología, Toxicología y Medicina Legal, Facultad de Farmacia, Universidad de Sevilla, C/P. García González n 2, E-41012, Sevilla, Spain
[c] Research, Development and Innovation Department, Sociedad Cooperativa Andaluza Ganadera del Valle de los Pedroches (COVAP), Polígono Industrial Dehesa Boyal Parcela 10-13, E-14400, Pozoblanco, Córdoba, Spain
[d] Department of Animal Production, University of Córdoba, Campus de Rabanales, E-14071, Córdoba, Spain

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ABSTRACT

Evaluation of dry-cured Iberian ham quality is carried out by means of the sense of smell of experts and a percentage of these hams are discarded due to odour defects. However, hams in cellars cannot be altered by sampling because they would be devaluated, which makes instrumental analysis difficult. Thus, the aim of this work is to assess the potential of headspace gas chromatography coupled to ion mobility spectrometry (HS-GC-IMS) or mass spectrometry (HS-GC-MS) to discriminate defective Iberian hams using a non-destructive sampling. Fifty hams from pigs fed with acorns were sampled in a real industry setting and were classified according to their condition (defective or non-defective). Validated classification rates of 80% and 100% using partial least squares discriminant analysis were obtained with HS-GC-IMS and HS-GC-MS, respectively, demonstrating the potential of the instrumental methods tested, which can be seen as complementary to the traditional olfactory technique. Furthermore, the volatile profile of spoiled Iberian hams was also determined; on average, spoiled pieces showed higher values of nonanal and decanal. Besides that, formic acid was only detected in spoiled pieces. These results might lead to an easier discrimination of spoilage to guarantee the quality of Iberian hams on the market.

1. Introduction

A traditional dry-cured 100% Iberian acorn-fed ham takes 5 years or more on average to be produced (from mating of sows to ham sale). This laborious process is reflected in its high price, which in the year 2021 started at ≈65 €/kg for pieces >7 kg. Currently, producers do not have any instrumental method to evaluate defects in individual hams before putting them on the market. Slice analysis is ruled out among producers because it requires opening the piece, thereby reducing its value and accelerating its expiration. Iberian ham processing consists of five stages: salting, post-salting, drying, maturing and ageing (Ventanas, 2001). Curing conditions are controlled based on a system to prevent the growth of microorganisms involving the monitoring of temperature, water activity (a_w), salt, and pH values. The cured pieces must present a_w values < 0.96 and at least 5% saline concentration (Ventanas, 2001). Under these conditions undesirable microbial activity is inhibited. The aroma of a ripe Iberian ham should be pleasant, yet in some pieces sensory defects may appear, such as foul-smelling notes near the bone (e.g. hip joint, knuckle and femur) or rancidness due to excessive oxidation (Ventanas, Ventanas, Ruiz, & Estévez, 2005). Moreover, amine odours may arise due to microbial alteration (Ventanas, 2001). The disposal of flawed pieces implies major costs to producers due to their high value, as well as defects leading to consumer complaints and loss of brand loyalty. Thus, a trustworthy method for the detection of defects of Iberian hams at the final stage (their ripening in a cellar) before commercialisation is essential. The most common approach for the detection of sensory defects in Iberian ham is the *cala* (meaning olfactory punching test), a traditional method involving expert personnel punching the ham with a bone punch and sniffing it, but it is difficult to detach their subjective perception from the final verdict. What is more, sensory exhaustion and

* Corresponding author.
E-mail address: qa1arjil@uco.es (L. Arce).

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subjectivity promote errors in classification, resulting in notable costs for the food industry. Therefore, several authors have tried to discriminate defective hams through objective instrumental methods.

The first attempted approach with this aim was based on optode membranes and fluorescence quenching (Choi & Hawkins, 1997). This sensor determined dimethyl disulphide (DMDS) in spoiled ham vapours, allowing the detection of spoilage. Almost a decade later, García, Aleixandre, and Horrillo (2005) employed an electronic nose (e-nose) with a similar purpose, with a multisensor based on semiconductor doped films in which electrical resistance data and neural networks discriminated spoiled hams. Likewise, impedance spectroscopy and potentiometry were employed with a similar aim (De Jesús et al., 2014; Girón et al., 2015). Physico-chemical parameters are different in bone-tainted hams because this defect might be influenced by poor salting and higher a_w at early stages (Blanco et al., 1997). Temperatures not low enough (>25 °C) may promote spoilage (Losantos, Sanabria, Cornejo, & Carrascosa, 2000; Paarup, Nieto, Peláez, & Reguera, 1999), and electrophoretic methods can determine microorganisms responsible for deep-spoilage (Martín et al., 2008). Still, according to the number of published papers, the most common technique for ham analysis is gas chromatography-mass spectrometry (GC-MS), a technique that has been combined with preconcentration techniques and olfactometry (Carrapiso, Martín, Jurado, & García, 2010). The first characterisation attempts with GC-MS associated Enterobacteriaceae with defects, and a list of possible volatile indicators was described (García, Martín, Timón, & Córdoba, 2000). Later GC-MS approaches revealed VOCs related to bone-taint and deep-spoilage (Martín et al., 2010), in which 30 spoiled samples and 3 control samples were analysed with a destructive approach. Furthermore, the contribution of odour-active compounds in defective pieces has also been determined using olfactometry (Carrapiso et al., 2010). More recent studies characterised defective dry-cured ham using GC-MS and concluded that volatile acids might be the main reason for the defect (Zhou et al., 2020). Likewise, the level of degradation of a dry-cured ham can be determined by its proteolysis index (PI). A high PI has been inversely correlated to VOCs originated by lipid oxidation, mainly hydrocarbons, alcohols, aldehydes, ketones and acids (Pérez-Santaescolástica et al., 2018). It is important to note that the application of a destructive sampling method for defect discrimination is not possible in industrial conditions and during quality control for sale purposes. Amongst the aforementioned studies to characterise and detect sensory defects in ham, only one (Andrés, Cava, & Ruiz, 2002) was based on non-destructive sampling. The methodology described in this work employed a direct-extraction device based on SPME fibre, which was inserted into the ham without damaging the piece. However, the equilibration time of the SPME fibre was 45 min, which is incompatible with the rate of sampling required in the industry.

Currently, headspace (HS) coupled to GC-MS is still the first option for Iberian ham analysis, due to it being a well-known technique that is very useful for VOC determination in food matrices (Wang, Chen, & Sun, 2020). One of its strongest assets is its identification capability through comprehensive spectral libraries. Headspace-gas chromatography-ion mobility spectrometry (HS-GC-IMS) represents a possible alternative; this innovative technique integrates gas chromatography (GC) separation and ion mobility (IMS) ultra-high sensitivity. HS-GC-IMS has already been employed for the discrimination of breed and feeding regime of Iberian pigs (Arroyo-Manzanares et al., 2018; Martín-Gómez, Arroyo-Manzanares, Rodríguez-Estévez, & Arce, 2019), although IMS radioactive sources have trouble ionising acids or alcohols and IMS compound libraries are lacking (Jurado-Campos, Martín-Gómez, Saavedra, & Arce, 2021).

Thus, the main goal of the present work is to evaluate the potential of HS-GC-IMS and HS-GC-MS to discriminate defective dry-cured Iberian hams using a non-destructive sampling based on the traditional olfactory punching test. A high number of samples has been studied in the present work, in contrast with previous papers, e.g. 4 samples (Carrapiso et al., 2010) or 14 samples (García et al., 2000). In addition, most of the previous works (Carrapiso et al., 2010; García et al., 2000) used a sampling method that was destructive with the ham piece, e.g. slices, so this time a sampling method was used that did not damage the ham and allowed its subsequent sale as a whole piece, without any loss of value. Moreover, previous discrimination attempts employed sample groups that were not equilibrated (e.g. 3 unspoiled hams vs 30 spoiled (Martín et al., 2010)), whereas the present approach used two classes for the chemometric models that were well balanced (25 samples each). The second aim of the present work is to determine characteristic compounds of the volatile profile of spoiled Iberian hams that allow the rapid and objective discrimination of defective samples. Therefore, defect detection in dry-cured ham will be attempted with HS-GC-IMS for the first time.

2. Materials and methods

2.1. Samples and standards

Fifty subcutaneous fat samples were extracted by needle punching by duplicate from the rump of 50 dry-cured hams from pure Iberian breed acorn-fed pigs. The sampling method was non-destructive and already described in a previous work (Martín-Gómez et al., 2019) using 2.1 × 60 mm sterile disposable stainless-steel needles (Bovivet-Kruuse, Langeskov, Denmark). Sampling was carried out in the industrial facilities of Sociedad Cooperativa Andaluza Ganadera del Valle de los Pedroches (COVAP, Pozoblanco, Córdoba, Spain). All samples had full traceability and heterogeneous maturing times (from 185 to 242 weeks). Out of the 50 samples, 25 belonged to pieces with defects that ruled them out for consumption and the other 25 were in good condition. Classification criteria were the experience and sensory capabilities of a trained expert (*maestro jamonero*, meaning expert dry-cured ham artisan). This expert is responsible for the selection of dry-cured ham pieces before shipping.

In addition, 65 individual standards at 1 mg L^{-1} were prepared in refined oil supplied by *Sovena S.A.* (Brenes, Spain). The selection was based on lists of VOCs of dry-cured ham described by previous papers (León-Camacho, Narváez-Rivas, & Gallardo, 2012). The complete list of sources is shown in Supplementary Table 1. The selected standards were alcohols; (E)-hex-2-en-1-ol, (Z)-pent-2-en-1-ol, 1-penten-3-ol, 2-methylbutan-1-ol, 2-phenylethan-1-ol, 3,3-dimethylbutan-1-ol, 3-methylbutan-1-ol, butan-1-ol, butane-1,3-diol, ethanol, heptan-1-ol, heptan-2-ol, hexan-1-ol, oct-1-en-3-ol, octan-1-ol, pentan-1-ol, phenylmethanol and propan-2-ol; aldehydes; (E)-decen-2-al, (2E)-hepten-2-al, (E)-hexen-1-al, (E)-nonen-2-al, (E)-octen-2-al, 2-methylbutyraldehyde, 2-methylpropanal, 3-methylbutyraldehyde, benzaldehyde, butyraldehyde, decanal, heptanal, hexanal, nonanal, octanal and pentanal; ketones; 6-methyl-5-hepten-2-one, butan-2-one, cyclohexanone, heptan-2-one, hexan-2-one, nonan-2-one, oct-1-en-3-one and pentan-2-one; acids; 3-methylbutanoic, acetic, butanoic, pentanoic and propionic; ethyl esters; 2-methylbutyrate, 2-methylpropanoate, 3-methylbutyrate, acetate, heptanoate, hexanoate, octanoate, pentanoate and propanoate: aromatics; p-xylene, m-xylene, 2,6-dimethylpyrazine and 2-methylpyrazine; terpenes; (R)-limonene and α-pinene; and others; dimethyl disulfide, octane and γ-caprolactone.

An alkane standard mixture C_{10}–C_{40} purchased by *Fluka* (Madrid, Spain) was used for calculating the Linear Retention index (LRI) for GC-MS analysis. A ketone mix was prepared dissolving six high purity (≥99%) ketones (nonan-2-one, octan-2-one, heptan-2-one, hexan-2-one, pentan-2-one and butan-2-one) at 0.5 mg L^{-1} in ultrapure water (Milli-Q Plus, *Millipore Bedford*, MA, USA) and used for calculating the retention index (RI) for GC-IMS analysis.

2.2. Instrumentation and methods

The samples extracted by means of the impregnated punched needles were analysed in parallel with both HS-GC-IMS and HS-GC-MS optimised methods. The 65 individual standards were analysed with the HS-

Table 1
List of VOCs detected and identified in defective and non-defective samples of subcutaneous fat of dry-cured hams from pure Iberian breed acorn-fed pigs sampled with needles.

GC-MS						GC-IMS			
VOC	LRI	id.	Signal (D)	Signal (ND)	p (0.95)	RI	Signal (D)	Signal (ND)	p (0.95)
ALCOHOLS									
Ethanol	–	–	–	–	–	344**	525.7 ± 97.2	501.9 ± 120.7	>0.05
Propan-2-ol	–	–	–	–	–	358**	636.4 ± 109.6	666.0 ± 112.3	>0.05
3-Methylbutan-1-ol	–	–	–	–	–	553	409.7 ± 115.9	398.2 ± 80.9	>0.05
Pentan-1-ol	903*	c	12314 ± 9908	17365 ± 11723	>0.05	582	869.1 ± 256.4	804.2 ± 219.7	>0.05
Heptan-1-ol	972	a	**37603 ± 25077**	**17000 ± 8095**	**<0.01**	782	577.1 ± 183.3	496.0 ± 136.7	>0.05
Octan-1-ol	1076	b	**20556 ± 15647**	**12494 ± 5738**	**<0.05**	881	524.0 ± 149.3	492.1 ± 134.2	>0.05
Oct-1-en-3-ol	–	–	–	–	–	790	293.4 ± 38.6	297.9 ± 46.4	>0.05
ALDEHYDES									
2-Methylpropanal	713*	a	28842 ± 16874	30031 ± 20857	>0.05	386**	210.0 ± 51.7	215.2 ± 39.9	>0.05
Butyraldehyde	–	–	–	–	–	402	58.7 ± 18.2	50.5 ± 13.2	>0.05
3-Methylbutyraldehyde	728*	a	84311 ± 33129	96085 ± 98554	>0.05	468	1996.0 ± 561.0	1793 ± 360.6	>0.05
2-Methylbutyraldehyde	731*	a	34860 ± 14340	55209 ± 49020	>0.05	481	1788.0 ± 555.0	1567.7 ± 345.1	>0.05
Pentanal	741*	a	154935 ± 69654	154632 ± 54126	>0.05	511	**810.8 ± 327.1**	**629.8 ± 248.8**	**<0.05**
Hexanal	800	a	682780 ± 355690	750748 ± 351094	>0.05	611	2349.0 ± 582.0	2124.9 ± 430.5	>0.05
(E)-Hexen-2-al	–	–	–	–	–	667	112.29 ± 48.7	116.6 ± 56.7	>0.05
Heptanal	895	a	119694 ± 59392	102571 ± 36390	>0.05	711	**2077.0 ± 572.0**	**1748.3 ± 449.8**	**<0.05**
(E)-Hepten-2-al	953	b	46118 ± 28341	46795 ± 23223	>0.05	769	861.7 ± 351.6	925.2 ± 388.9	>0.05
Benzaldehyde	–	–	–	–	–	770	291.0 ± 61.3	270.3 ± 59.4	>0.05
Octanal	1003	a	137367 ± 78938	125131 ± 53309	>0.05	813	**2374 ± 766**	**1978 ± 579**	**<0.05**
(E)-Octen-2-al	1059	b	32910 ± 25138	37844 ± 23559	>0.05	868	887.6 ± 362.9	766.5 ± 276.2	>0.05
Nonanal	1106	a	**276284 ± 117362**	**137462 ± 59675**	**<0.01**	915**	**4193.0 ± 1617.0**	**3357.0 ± 1019.0**	**<0.05**
(E)-Nonen-2-al	1160	b	12936 ± 9556	11928 ± 5654	>0.05	958**	454.0 ± 127.6	427.8 ± 119.8	>0.05
Decanal	1206	b	**10197 ± 10398**	**2816 ± 1013**	**<0.001**	987**	**2451.0 ± 1250.0**	**1802.0 ± 758.0**	**<0.05**
(E)-Decen-2-al	1263	b	31036 ± 28417	27742 ± 15771	>0.05	1025**	902.4 ± 339.9	860.5 ± 309.4	>0.05
(E)-Undecen-2-al	1364	b	43709 ± 23782	33382 ± 16595	>0.05	–	–	–	–
ACIDS									
Formic acid	709*	c	**25672 ± 22835**	**0 ± 0**	**<0.001**	–	–	–	–
Acetic acid	716*	a	**43808 ± 20372**	**82236 ± 40976**	**<0.001**	401**	1205.4 ± 234.3	1275.3 ± 247.2	>0.05
KETONES									
Pentan-2-one	–	–	–	–	–	501	**824.0 ± 703.0**	**338.8 ± 175.8**	**<0.01**
Hexan-2-one	–	–	–	–	–	599	**204.5 ± 74.5**	**154.4 ± 29.2**	**<0.01**
Heptan-2-one	–	–	–	–	–	699	**1307.0 ± 526**	**1058.9 ± 226.7**	**<0.05**
Oct-1-en-3-one	–	–	–	–	–	789	99.1 ± 15.4	98.4 ± 20.9	>0.05
Octan-2-one	–	–	–	–	–	799	**332.0 ± 178.7**	**238.1 ± 69.0**	**<0.02**
Nonan-2-one	–	–	–	–	–	898	**280.6 ± 76.6**	**243.0 ± 48.2**	**<0.05**
ESTERS									
Ethyl 2-methylbutyrate	–	–	–	–	–	666	**30.1 ± 12.0**	**23.0 ± 4.5**	**<0.01**
Ethyl hexanoate	–	–	–	–	–	812	**238.6 ± 57.8**	**185.0 ± 27.8**	**<0.01**
Ethyl octanoate	–	–	–	–	–	980**	**229.4 ± 46.0**	**191.6 ± 25.0**	**<0.01**
OTHERS									
γ-Caprolactone	–	–	–	–	–	864	240.8 ± 57.0	223.9 ± 30.4	>0.05
2-Pentylfuran	989	b	30731 ± 11898	33727 ± 12184	>0.05	–	–	–	–
Unknown	1096	–	11658 ± 8417	15205 ± 9746	>0.05				

LRI: Linear Retention Index, ***LRI** values estimated by lineal regression.; **id.:** Reliability of GC-MS identification (a: Identified by mass spectrum and LRI agreed with standards, b: Identified by mass spectra of database and LRI agreed with PubChem data, c: Identified by mass spectra of database); **D:** Defective samples. **ND:** Non-defective samples. All VOCs identified by GC-IMS were compared with standards. IMS signal was calculated as the sum of the signal of all the identified ions of each compound. ** HS-GC-IMS RI values calculated by lineal regression of a ketone mix.

GC-IMS device.

The HS-GC-IMS device integrated an Agilent 7697A headspace sampler connected by a transfer line to an Agilent 8860 gas chromatograph (*Agilent*, Santa Clara, CA, US) and a standalone ion mobility spectrometer (*G.A.S. Gesellschaft für analytische Sensorsysteme mbH*, Dortmund, Germany) with a ^{3}H ionisation source and a 10 cm drift tube. For HS-GC-IMS the sample incubation consisted of 15 min at 60 °C. Subsequently, 1 mL of headspace volume was injected in split mode 1:5. The transfer line was heated at 110 °C. GC separation was performed on a 30 m HP-5 (5%-phenyl)-methylpolysiloxane non-polar column with an internal diameter of 0.32 mm and a 0.5 μm film (*Agilent*, Santa Clara, CA, US). Helium (*Abelló Linde*, Sevilla, Spain) was used as the carrier gas at a constant flow rate of 1 mL/min. The ramp temperature consisted of 3 min at 40 °C from the start of the analysis, an increase to 100 °C at a rate of 5 °C/min, an increase to 130 °C at a rate of 15 °C/min, and a 130 °C plateau until the end of the analysis at 27 min. After separation in the GC column, VOCs entered the ionisation chamber of the IMS module, whose detector was working at positive polarity. Nitrogen was used as drift gas at a flow of 150 mL/min. IMS parameters were 150 μs of injection pulse width, 45 °C drift tube temperature, signal averaging each 32 spectra, a repetition rate of 30 ms, and drift, blocking and injection voltages of 237 V, 40 V and 2500 V, respectively.

The HS-GC-MS device was comprised of an Agilent 6890 GC system coupled to an Agilent 5975 inert quadrupole mass spectrometer (*Agilent*, Santa Clara, CA, US) equipped with a MPS2 multipurpose autosampler headspace (*Gerstel*, Müllheim an der Ruhr, Germany). For HS-GC-MS, the sample incubation was performed at 100 °C and 250 rpm for 40 min. The injection of 300 μL of headspace was carried out using a 2.5 mL syringe in splitless mode. The inlet temperature was fixed at 200 °C. Separation was performed on a 30 m HP-5MS (5%-phenyl)-methylpolysiloxane nonpolar column with an internal diameter of 0.25 mm and a 0.25 μm film (*Agilent*, Santa Clara, CA, US). Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The initial GC oven temperature was 35 °C for 5 min, then ramped at 2.50 °C/min to 130 °C and held for 1 min for a total runtime of 44 min. The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 250 °C,

respectively. Electron ionisation mass spectra in the full-scan mode were recorded at 70 eV with the electron energy in the range of 29–350 m/z.

2.3. Data processing and statistical analysis

HS-GC-IMS data was extracted with VOCal 1.0.0. software (*G.A.S. Gesellschaft für analytische Sensorsysteme mbH*, Dortmund, Germany) and individual peak volumes were obtained. The HS-GC-IMS peaks that belonged to ions of the same VOC (e.g. protonated monomer and proton-bound dimer) were added to obtain a single intensity value for each VOC. HS-GC-MS data was extracted by Agilent MSD Chemstation software and total ion current (TIC) peak areas were obtained with PARADISe 5.8 software (Johnsen, Skou, Khakimov, & Bro, 2017). This software allows the obtaining of data with good resolution and enables chemical information to be extracted directly from the raw data. Furthermore, it allows the simultaneous deconvolution of the pure mass spectra of peaks and the integration of areas of deconvoluted peaks for all samples; resolved peaks are identified using their deconvoluted pure mass spectra. The identification of compounds was performed by the NIST MS Search program (version 2.0) and this was confirmed by LRI data. Subsequently, data was processed in Matlab R2016a software (*Mathworks*) with PLS Toolbox plug-in (*Eigenvector*). ANOVA tests were performed by SPSS software. Then, prior to modelling, data pre-treatment consisted of autoscaling of the volume of GC-IMS features and the area of GC-MS peaks. Principal component analysis (PCA) was carried out to explore the data and detect possible outliers. Subsequently, partial least squares discriminant analysis (PLS-DA) models were built to classify dry-cured Iberian ham samples according to the presence of defects. These models were built using a set with 80% of the samples (n = 40) for calibration and cross-validation. Cross-validation was performed employing randomly selected samples with 5 data splits and 20 iterations. Prediction (external validation) was performed with the remaining samples (20%, n = 10). The external validation set consisted of 5 defective and 5 non-defective samples and the selectivity ratio and variable importance for the projection (VIP) score of each variable were calculated.

3. Results and discussion

The sampled hams belonged to two quality classes (defective or non-defective) with 25 samples in each class to avoid biased results; they shared processing conditions but had different curing times (185–242 weeks). Therefore, if differences were to be found between defective and non-defective classes these would be caused by defects, not by the curing period; the latter might influence the volatile composition (Andrés et al., 2002; Martín, Timón, Petrón, Ventanas, & Antequera, 2000; Narváez-Rivas, Gallardo, & León-Camacho, 2014). This study was limited to 25 samples in each group because it is difficult to obtain a high number of defective pieces, in accordance to the real availability of defective samples in the production of one year.

3.1. Characterisation of spoiled or not spoiled dry-cured Iberian hams according to their VOCs profile

Firstly, the area of 21 HS-GC-MS identified peaks and the volume of 59 HS-GC-IMS identified plot features was extracted. HS-GC-IMS selection criteria was based on the volume above the baseline and resolution. As described in section 2.3., the features in HS-GC-IMS plots that belong to ions of the same VOC were added to obtain a single intensity value for each VOC. HS-GC-MS peak selection was done automatically by PARADISe software.

As described in section 2.2., different incubation methods were used for GC-IMS and GC-MS. Each technique employed its optimal one. The use of different incubation conditions was justified, when GC-MS is used at the same incubation temperature and time as employed with GC-IMS (60 °C for 15 min) no peaks were detected or these were almost indifferentiable from signal noise. Thus, in the HS-GC-MS method, 40 min at 100 °C were selected for sample incubation because at a lower temperature and time no suitable signals appeared in the chromatogram. With HS-GC-IMS, incubation temperature and time higher than 60 °C and 15 min, respectively, caused overlapping of signals. For this reason, both methods did not have the same experimental conditions.

Despite the more aggressive incubation conditions for the HS-GC-MS method (40 min at 100 °C vs 15 min at 60 °C), many more features were visible in HS-GC-IMS plots than in GC-MS chromatograms. This fact is explained by the higher sensitivity of HS-GC-IMS, which detects analytes at very low amounts. Nevertheless, the objective of the present work was not to compare both techniques, but to demonstrate that both are useful to detect defects in dry-cured ham if methods are optimised accordingly.

In the present work, a different chromatographic column or different extraction techniques (e.g. solid-phase microextraction, SPME) might have improved the sensitivity of GC-MS. However, the use of SPME fibres would have increased the cost of analysis per sample. As previously stated, the objective of this work is to assess if GC-IMS and GC-MS are suitable for an application in the industry. Therefore, SPME was discarded because the added cost to the product per analysis would have been too high to be practical. In addition, more sophisticated devices not available in an industrial setting would have been required.

HS-GC-MS analysis of the subcutaneous fat of 100% Iberian acorn-fed ham samples was able to detect 21 VOCs, as shown in Table 1. Of these, the average area of 5 of those VOCs was higher in the sampled fat of defective dry-cured Iberian hams than in non-defective pieces ($p < 0.05$): formic acid, heptan-1-ol, octan-1-ol, nonanal and decanal, while the average area of acetic acid was significantly higher in non-defective hams. This is the first time that formic acid, heptan-1-ol and octan-1-ol have been described in spoiled dry-cured ham. Remarkably, in the present work formic acid was only detected in defective samples, and therefore, its presence might be directly associated to spoilage in dry-cured Iberian ham, which may open the door to targeted approaches for very fast discrimination. Formic acid is described as having a penetrating and pungent odour that matches the smell emanating from bone-tainted hams (Ruth, 1986). Likewise, heptan-1-ol is one of the most common alcohols found in spoiled meat (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015). Nonanal and decanal may derive from hydrolysis of triglycerides, β-oxidation of unsaturated fatty acids and lipid auto-oxidation (Calkins & Hodgen, 2007). In addition, nonanal is produced by Gram-positive and Gram-negative bacteria. When aldehydes concentration exceeds a certain threshold, their perception is unpleasant (Calkins & Hodgen, 2007); while nonanal is an aldehyde commonly found in spoiled meat, to find its correlation with spoilage is unusual (Casaburi et al., 2015).

The identification of HS-GC-IMS plot features required the analysis of standards because current IMS libraries are lacking. Standard analysis allowed their comparison with sample features to achieve a tentative identification. 65 standards in total were analysed with the HS-GC-IMS method. The HS-GC-IMS analysis of the subcutaneous fat from needles impregnated by punching the dry-cured hams was able to detect 217 features, of which 59 were identified through comparison with the features of the standards. The 59 identified features corresponded to protonated monomers and/or proton-bound dimers of 34 compounds, shown in Table 1. Of these 34 compounds, the average signal of 13 was higher ($p < 0.05$) in the fat of defective hams: pentan-2-one, pentanal, hexan-2-one, ethyl 2-methylbutyrate, heptan-2-one, heptanal, octan-2-one, octanal, ethyl hexanoate, nonan-2-one, nonanal, ethyl octanoate and decanal. As can be seen, these VOCs are mainly ketones, ethyl esters or aldehydes, which have also been described in spoiled fresh meat (Casaburi et al., 2015).

Nonanal and decanal were found at higher amounts in the subcutaneous fat of defective ham samples with both HS-GC-MS and HS-GC-IMS methods (see Table 1). While the analysis results of both techniques do not completely agree (since the VOCs extraction temperature selected was not the same in both methods), they are coherent. So far,

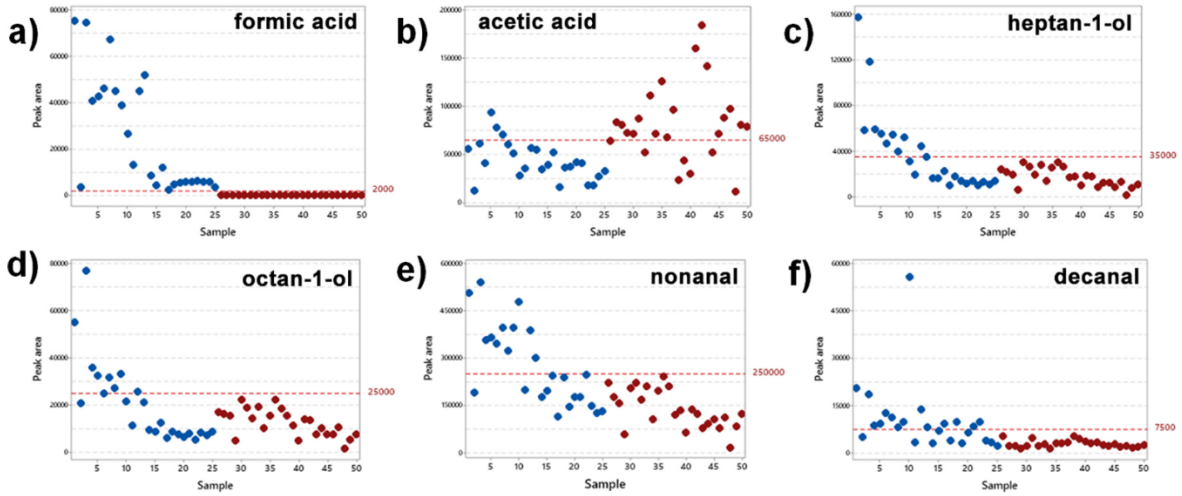

Fig. 1. HS-GC-MS peak areas of a) formic acid, b) acetic acid c) heptan-1-ol, d) octan-1-ol, e) nonanal and f) decanal. The average amount of these VOCs was different ($p < 0.05$) in samples of defective and non-defective Iberian ham. The dotted line represents the possible limits between classes. Blue: Defective, Red: Non-defective. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

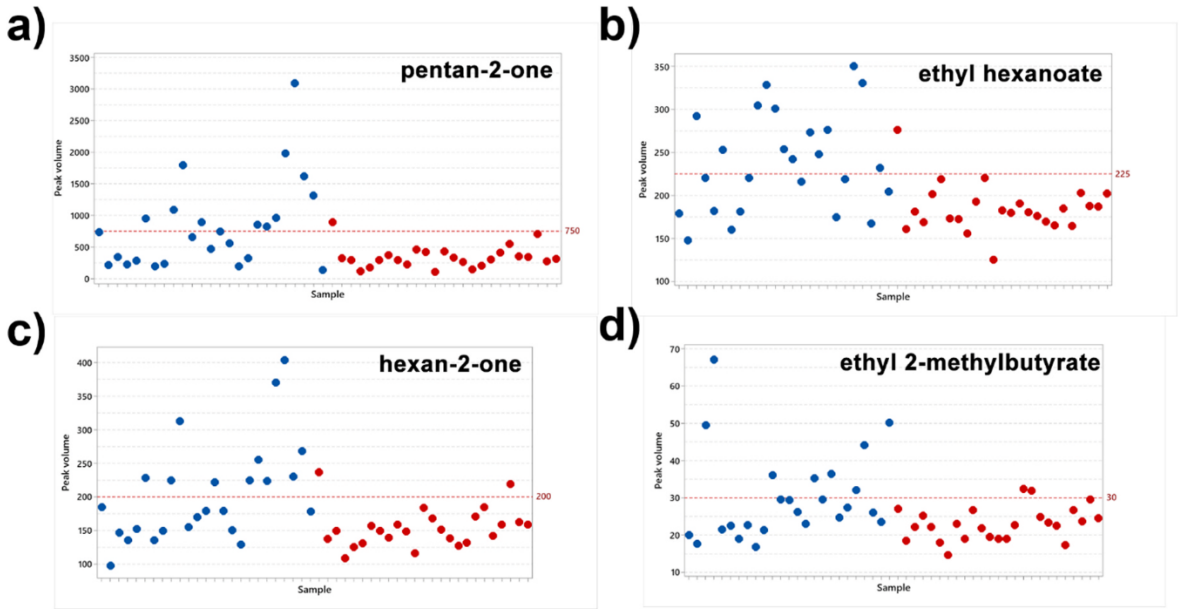

Fig. 2. HS-GC-IMS peak volumes of a) pentan-2-one, b) ethyl hexanoate, c) hexan-2-one and d) ethyl 2-methylbutyrate. The average amount of these VOCs was different ($p < 0.02$) in samples of defective and non-defective Iberian ham. The dotted line represents the possible limits between classes. Blue: Defective, Red: Non-defective. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the list of compounds correlated with dry-cured ham bone-taint or deep-spoilage in the bibliography has been ambiguous. As an example, benzene acetaldehyde, pentanoic acid, 1-octen-3-ol, 6-methyl-5-hepten-2-one, toluene, butan-2-one, 3-hydroxy-2-butanone and tetradecane have been previously described as both spoilage and positive markers, as shown in Supplementary Table 1.

Likewise, the most relevant HS-GC-MS markers (shown in Table 1) were studied individually in the 50 samples analysed to corroborate if a targeted approach with only a few compounds can be used for faster discrimination. For this, Fig. 1 represents the peak areas of the 6 individual markers in the samples analysed. As can be seen, while a correlation was found with HS-GC-MS between defects and the average amount of these VOCs (compounds with significant difference, $p < 0.05$, which are shown in Table 1), not all samples can be discriminated by individual values of VOCs in them (see Fig. 1). Only formic acid might be employed directly as a marker of defect. The remaining compounds only showed high values in a few samples, which can cause confusion when the average of their areas is used to differentiate samples with defects. The same approach was attempted with HS-GC-IMS data: No individual compound showed a clear difference between the two sample groups, as can be seen in Fig. 2. As previously stated, this is partially due to the heterogeneity of Iberian hams from free range pigs with a fully natural foraged diet. This fact makes it difficult to perform a differentiation by analysing a single VOC, although formic acid seemed to be useful for this univariate differentiation with HS-GC-MS. This conclusion will need to be verified with more samples in future studies. Subsequently, multivariate analysis was tested to achieve a clear classification of all samples.

3.2. Classification of spoiled or not spoiled dry-cured Iberian hams using PLS-DA chemometric models

With the area of 21 VOCs detected by HS-GC-MS and the volume of

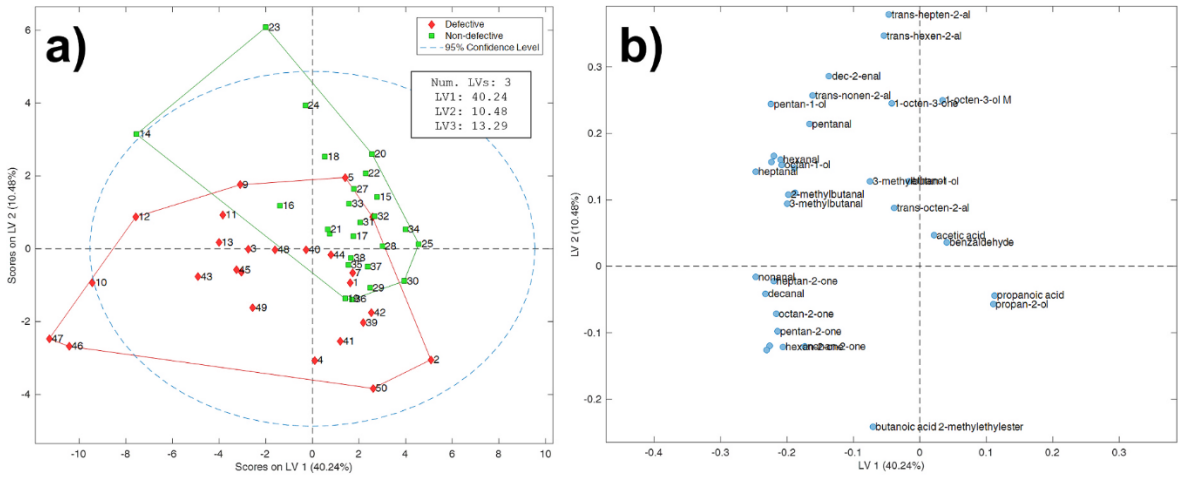

Fig. 3. PLS-DA score plots of Iberian ham samples obtained with the total intensity of 34 VOCs detected by HS-GC-IMS.

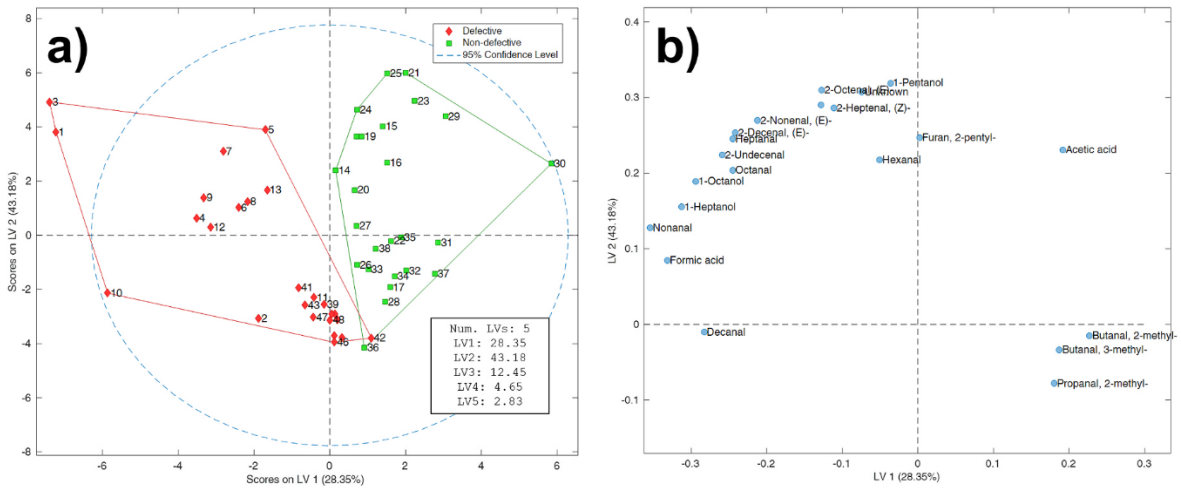

Fig. 4. PLS-DA score plots of Iberian ham samples obtained with the total intensity of 22 VOCs detected by HS-GC-MS.

34 VOCs identified by HS-GC-IMS, PCA were performed in order to explore the data and detect possible outliers. As can be seen in Supplementary Fig. 1, PCA using 34 VOCs detected by HS-GC-IMS showed three possible outliers (samples 23, 46 and 47). Likewise, PCA using the area extracted from 21 peaks detected by HS-GC-MS showed three samples as possible outliers (samples 1, 3 and 30). Presumably, these "outliers" were not actual outliers, for several reasons. Firstly, their identities were different depending on the analysis method used (HS-GC-IMS or HS-GC-MS); therefore, they were not really different to the rest of the samples. Besides, their presence is justified by the high VOCs heterogeneity in dry-cured Iberian ham (Jurado, Carrapiso, Ventanas, & García, 2009; León-Camacho et al., 2012). In fact, part of its charm is the uniqueness of each piece, and these "outliers" might not be intrinsically different to the other samples, which could be explained because there are differences of diet intake for free range foraging pigs (Rodríguez-Estévez, García, Peña, & Gómez, 2009). In a real industry setting, a method for routine analysis must provide high classification success regardless of the natural heterogeneity of dry-cured ham samples. This could be achieved by the increment of samples to study in the future. For this reason, no sample was discarded for the calibration and validation of PLS-DA models.

The score plot of the first two latent variables (LVs) from the PLS-DA models for HS-GC-IMS showed overlapping of some samples (see Fig. 3a) while a clear separation between classes was observed in the score plot of the first two LVs obtained by PLS-DA performed with HS-GC-MS data (see Fig. 4a). These figures include the scores of both calibration and validation sets. Classification rates of the PLS-DA models are shown in Table 2. As can be seen, calibration was performed flawlessly (100% of success) with HS-GC-MS and with a lower than 85% success rate using HS-GC-IMS. In addition, the cross-validation success rate with both techniques was $\geq$70%. Still, while HS-GC-MS was able to correctly predict the whole validation set, HS-GC-IMS had a prediction success of around 70%.

In order to locate which VOCs were the most relevant to discriminate the samples, their VIP scores were considered, and they had to be ≥ 1. Subsequently, additional PLS-DA models were built with the intensity of these VOCs as variables. For HS-GC-IMS these variables were the volume of 11 VOCs: Ethyl 2-methylbutyrate, ethyl hexanoate, propanoic acid, ethyl octanoate, pentan-2-one, (E)-hepten-2-al, benzaldehyde, hexan-2-one, pentan-1-ol, acetic acid and 2-methylpropanal. Likewise, the 5 most relevant variables for HS-GC-MS sample group differentiation were the peak area of nonanal, formic acid, acetic acid, decanal and heptan-1-ol. These results are in accordance with the differences in individual VOCs found between defective and non-defective samples, shown in Table 1. In addition, they are in accordance with the distribution of variables in the loadings plots of HS-GC-IMS and HS-GC-MS PLS-DA models (see Figs. 3b and 4b).

The classification potential of these relevant markers with high VIP was assessed. The prediction success rate of HS-GC-MS PLS-DA models using just 5 markers with VIP $\geq$1 (nonanal, formic acid, acetic acid,

Table 2
Classification results of calibration and validation sets with HS-GC-IMS and HS-GC-MS using PLS-DA models. D: Defective, ND: Non-defective dry-cured Iberian hams sampled with needles.

HS-GC-IMS				HS-GC-MS			
CALIBRATION							
Actual \ Predicted	D	ND	% correct	Actual \ Predicted	D	ND	% correct
D	17	3	85	D	20	0	100
ND	3	17	85	ND	0	20	100
% TOTAL			**85**	% TOTAL			**100**
CROSS-VALIDATION							
Actual \ Predicted	D	ND	% correct	Actual \ Predicted	D	ND	% correct
D	12	8	60	D	20	0	100
ND	4	16	80	ND	1	19	95
% TOTAL			**70**	% TOTAL			**97.5**
PREDICTION							
Actual \ Predicted	D	ND	% correct	Actual \ Predicted	D	ND	% correct
D	4	1	80	D	5	0	100
ND	2	3	60	ND	0	5	100
% TOTAL			**70**	% TOTAL			**100**

decanal and heptan-1-ol) was 100%, as shown in Supplementary Table 2. Therefore, considering some additional VOCs for discrimination of defects with HS-GC-MS might be unnecessary. Similarly, the HS-GC-IMS prediction rate improved from 70% using 34 variables to 80% using just the 11 most relevant variables with a VIP $\geq$1 (see Supplementary Table 2). Thus, a careful selection of HS-GC-IMS VOCs' features for the calibration of chemometric models is recommended.

This assessment of the classification potential of HS-GC-MS and HS-GC-IMS can be considered reliable because it was evaluated with a significant number of samples (50 samples) and chemometrics models have been validated. Compared with previous studies, the results obtained were like those of Girón et al. (2015), which were obtained with 28 samples. Other authors obtained similar success rates but analysing fewer samples, using destructive methods and/or without validation (De Jesús et al., 2014; García et al., 2005).

4. Conclusions

The developed PLS-DA models for HS-GC-MS and HS-GC-IMS data were able to successfully discriminate defective dry-cured Iberian ham. In addition, good results were achieved using a selection of only 5 markers detected by HS-GC-MS: nonanal, formic acid, acetic acid, decanal and heptan-1-ol. This is the first time that formic acid, heptan-1-ol and octan-1-ol are described in spoiled dry-cured ham. However, these results should be verified with more samples. Likewise, the average amount of acetic acid was higher in non-defective pieces and formic acid in defective pieces.

While both techniques have shown potential, HS-GC-MS showed better classification results and may be the most appropriate for discrimination of defective dry-cured Iberian ham. Currently, a targeted approach based on the determination of a single analyte might be

unreliable to discriminate between defective and non-defective hams. Only formic acid determined by HS-GC-MS was able to discriminate samples by itself. Therefore, a discrimination method based on multivariate analysis and a careful selection of markers is recommended. In the future, after instrumental analysis, hams that not having a clear quality classification may be opened for tasting and thus avoid consumer claims; thus, if these are not defective, they can be sold sliced. The non-destructive sampling method employed in the present work ensures the viability in an industrial setting.

CRediT authorship contribution statement

Andrés Martín-Gómez: sampling, GC-IMS data treatment, Methodology, Writing – original draft. **M. Pilar Segura-Borrego:** analysis of the samples by GC-MS, Data curation, Methodology, Writing – original draft. **Rocío Ríos-Reina:** GC-MS data treatment, writing and reviewing. **M. José Cardador:** GC-IMS analysis (optimization), Writing – original draft, Writing – review & editing. **Raquel M. Callejón:** Methodology, Supervision, Writing – review & editing. **M. Lourdes Morales:** Methodology, Supervision, review & editing. **Vicente Rodríguez-Estévez:** sampling design, Funding acquisition, writing and review. **Lourdes Arce:** Conceptualization, Methodology, Funding acquisition, Writing – review & editing.

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.lwt.2021.112785.

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