Ultra high performance liquid chromatography coupled to quadruple time-of-flight with MS^E technology used for qualitative analysis of non-volatile oxidation markers in sliced packed mushrooms (*Agaricus Bisporus*)

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ABSTRACT

61 different non-volatile compounds were determined in *Agaricus Bisporus* sliced mushrooms using UHPLC/Q-TOF with MS^E technology. Both positive and negative electrospray ionization were applied. Chemical profile of three parts of mushroom was created: cap, gills and stipe. The analysed mushrooms were oxidized to identify the non-volatile markers in their parts. MarkerLynx® was proposed as a powerful tool to distinguish mushrooms purchased in different countries (Spain and Portugal) by determining their non-volatile markers. Some metabolites were identified. Surprisingly a mix of polyethylene glycols (PEGs) was detected in cap and gills of mushrooms. Whole mushrooms were considered as vegetable resistant to migration from packaging compounds. Additionally migration tests were performed to determine the source of migrating compounds.

1. Introduction

Fresh, sliced, white mushrooms are one of the most sensitive food products to postharvest treatment and storage, what unfortunately is revealed by their quick decay [1–3]. A discoloration of mushrooms is attributed to its enzymatic activity. The polyphenol oxidases (PPO) cause oxidation of phenolic compounds in presence of oxygen. A result of the oxidation reactions is the formation of *o*-quinones that are further irreversibly changed into brown products, the melanins [4–6]. Therefore, there is a high interest in extending the shelf life of fresh white mushrooms. An interesting approach is creating a new active packaging that would extend the freshness of mushrooms without compromising their quality [7–9]. However, the determination and characterization of oxidation markers of fresh mushrooms is especially noteworthy if we want to demonstrate a real influence of new active packaging into the mushrooms.

According to the literature there are several methods for determining the individual groups of compounds. A lot of methods for determining free amino acids [10–17] and phenolic compounds [18,19] in mushrooms have been proposed in scientific publications. Some authors have successfully used UHPLC/Q-TOF for getting the metabolic profile of fruits and vegetables. For instance, Iswaldi et al. determined phenolic and other polar compounds in two varieties of zucchini [20] and Rodríguez-Pérez et al. did it in three different varieties of Spanish melon [21]. In turn, Di Lecce et al. presented phenolic profile of the different parts (skin, pulp and seeds) of the Albariño grape [22].

Detection by ultra performance liquid chromatography coupled to quadruple time-of-flight (UHPLC/Q-TOF) with novel technology called mass spectrometry^{ElevatedEnergy} (MS^E) was proposed to identify the non-volatile compounds of fresh and oxidized mushrooms. MS^E is the ultimate technology for comprehensive, reproducible profiling and characterization. It is one of the most powerful technics used for determination of non-volatile compounds. Simultaneous acquisition of low (first function) and high-energy data (second function) with energy ramp between 15 eV and 30 eV was applied. MS^E allows a generation of an exact mass precursor ion

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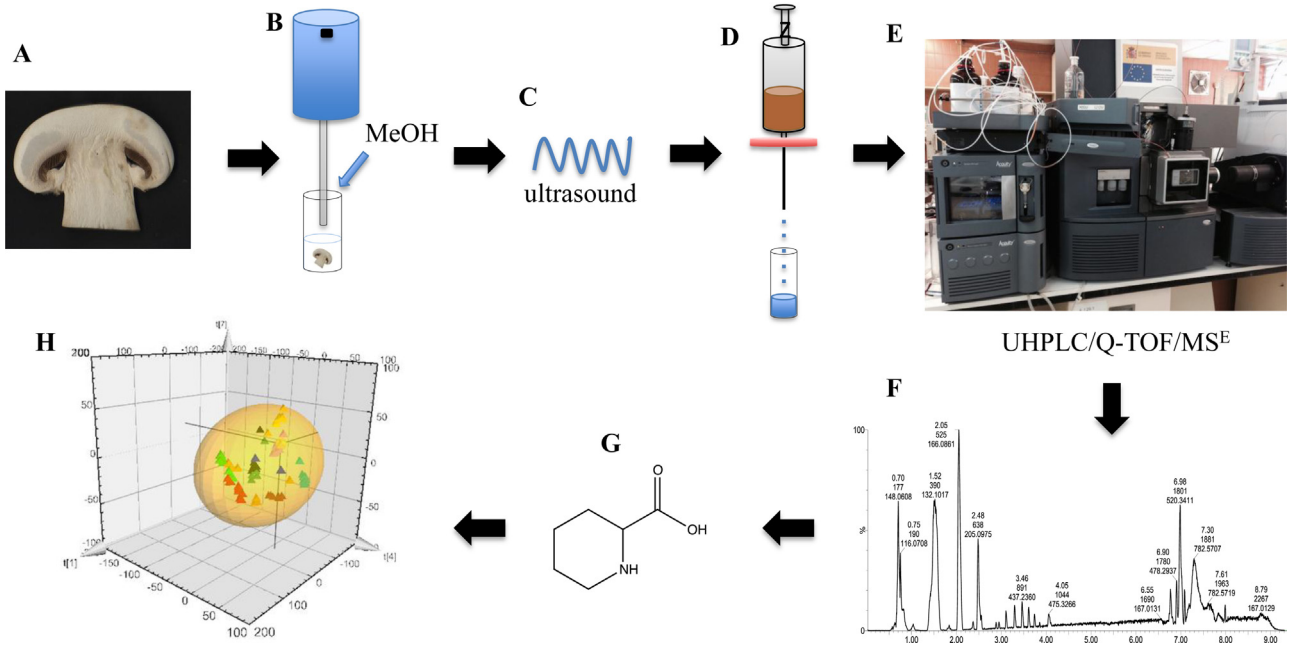

Fig. 1. Schematic procedure for determination of non-volatile compounds and migrants in mushrooms. Where A—separation of mushrooms' parts; B—trituration of mushrooms with MeOH; C—sonication of samples; D —filtration; E—sample analysis by UHPLC/Q-TOF/MS^E; F—analysis of chromatograms; G—identification of compounds by MassLynx based on 1st and 2nd function mass spectrum; H—principal component analysis by MarkerLynx®.

and the exact mass fragments ions in a single analytical run. Mass precursors and mass fragments are collected for each detectable compound in the sample. MS^E detection is quicker then standard MS or tandem mass spectrometry. An additional advantage is that the proposed method does not require time-consuming sample preparation and the solvent consumption is low. The main objective of the present investigation was the qualitative analysis of oxidation markers in sliced packed mushrooms. To the best of our knowledge, the profile of non-volatile compound of *Agaricus Bisporus* mushrooms using UHPLC/Q-TOF/MS^E has not been presented yet in scientific literature. On top of that, in this paper the three different parts of mushrooms have been analysed and the profile obtained. The results obtained are shown and discussed.

2. Materials and methods

2.1. Reagents

Methanol used for sample preparation was of HPLC grade supplied by Scharlau Chemie (Sentmenat, Spain). Methanol and ultrapure water for Q-TOF/MS^E were purchased from Baker (Deventer, The Netherlands) and were used as a mobile phase. Poly(ethylene glycol) average M_n 300 and poly(ethylene glycol) average M_n 400 (CAS 25322-68-3) were from Sigma–Aldrich (Madrid, Spain). Reference standard containing: acetaminophen, caffeine, sulfaguanidine, sulfadimethoxine, val-tyr-val, verapamil, terfenadine, leucine-enkephalin, reserpine, erythromycin were supplied by Waters (Cerdanyola del Vallès, Barcelona, Spain).

2.2. Apparatus

Ultra-Turrax T18 from IKA (Staufen, Germany) was used to homogenize samples of mushrooms. An ultrasonic bath Branson 3510 (Branson Ultrasonic Corporation, USA) was used for the extraction of non-volatile compounds and also a CENTROMIX centrifuge model S-549 was utilized.

2.3. Samples

Samples of *A. Bisporus* mushrooms were obtained from a local company (placed in Spain) specialized in production and distribution of vegetables. Two types of mushrooms were supplied: whole and sliced. Also sliced mushrooms from supermarket in Portugal were bought. All types of mushrooms were packed in the PP trays overwrapped with PVC extensible micro-perforated film to ensure change of gases with environment. Moreover, whole sylvan mushrooms were bought loose. All samples were stored in a refrigerator at 4 °C.

2.4. Sample preparation

Mushrooms in trays were mixed and then they were randomly chosen for the experiment. Three parts of fresh white mushrooms were manually separated: cap (pileus), gills and stipe.

1 g of each part was mixed with methanol (1:10, w:v) and was homogenized using Ultra-Turrax at 18,000 rpm. Then the samples were extracted in the ultrasonic bath during 15 min and centrifuged at 3600 during 10 min. Supernatant was taken and diluted with ultrapure water (1:1, v:v). The extract was filtrated using 2 mL syringe filter with CHROMAFIL®Xtra PET-20/25, 0.20 um pore size. Trays of mushrooms were overwrapped with the same film and stored in the refrigerator at 4 °C during one month to allow their total deterioration by oxidation. Such conditions were chosen to simulate the reactions occurring in the product after the consumer purchase. Again three parts of oxidized brown mushrooms were manually separated: cap, gills and stipe were prepared according to the recipe described above. Finally 5 μL of aliquot were injected into UHPLC/Q-TOF/MS^E.

Blank sample was prepared by filtering some amount of methanol using CHROMAFIL®Xtra PET-20/25, 0.20 μm pore size filter. Also mix sample was prepared by mixing 10 uL of one replicate of each sample in one vial. A volume of 5 μL of the mix sample was injected.

To confirm the origin of non-volatile compounds that naturally do not occur in the fresh mushrooms, the analysis of the whole

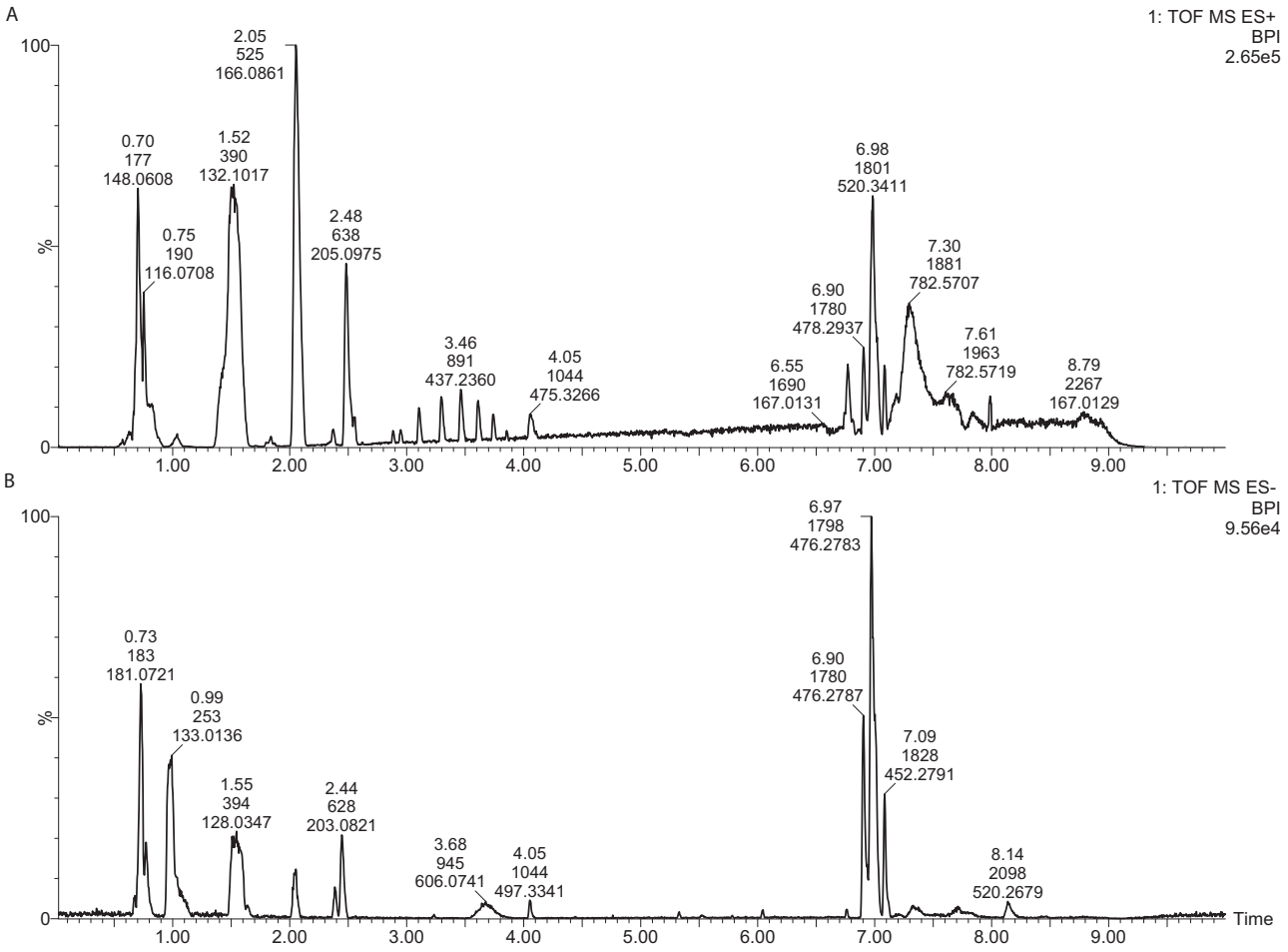

Fig. 2. Chromatograms of fresh white cap analysed in A—positive mode; B—negative mode.

mushrooms was also carried out. Two types of whole mushrooms were acquired: whole mushrooms packed in the same packaging as sliced mushrooms and whole sylvan mushrooms bought loose. The recipe of sample treatment applied was described above. Here the skin covering the mushroom cup was separated and treated as an additional part of mushroom. The skin was analysed to check the migration capability of polyethylene glycols (PEGs).

The extract dilution and methanol:water ratio were optimized to allow the detection of analysed compounds and to obtain the best resolution of chromatographic peaks. Two types of dilution of the extract were tested: firstly, the non-diluted extract (1 g of mushrooms per 10 g of methanol) was injected and secondly, the extract diluted 10 times with methanol was injected. In the case of more diluted sample no signal was detected. Then also three methanol:water ratios were tested: 1:0, 1:1 and 1:3. The best peak resolution and selectivity were obtained in the case of the ratio 1:1. Methanol was used as a principal solvent to extract polar and semi-polar compounds. Water was used to facilitate the chromatographic elution of the analyte, as in the case of 1:0 ratio the overlapping peaks were observed. Fig. 1 shows a scheme of performed experiments.

2.5. Packaging materials

Samples of materials were obtained from the same local company that produces and distributes mushrooms. Two types of materials were obtained: plastic tray made of PP and PVC transparent colorless film.

2.6. Migration tests

Migration assays for fresh mushrooms were carried out in accordance with the parameters contained in the legislation for food contact materials EU/11/2011 [23]. 10% ethanol was used as a simulant. Migration assay was carried out during 10 days at 20 °C as mushrooms usually do not stay longer than 30 days at a temperature range between 5 °C and 20 °C. Total immersion was used in case of plastic tray and PVC transparent film. Each material (2 cm × 3 cm) was accurately weight and placed in a 20 mL vial with 18 g of simulant. Three replicates of each sample and blank sample consisting of pure simulant were prepared. Samples were analysed by UHPLC/Q-TOF/MS^E.

2.7. Calibration and analytical parameters

Two solutions of PEGs (PEG300 and PEG400) at a concentration 1 μg/g were injected into UHPLC/Q-TOF/MS^E to select the best standard for quantification. External standard calibration was used. PEGs solutions were prepared in methanol and then were diluted in ultrapure water with a ratio 1:1. The calibration curve for each polyethylene glycol in the range from 0.08 μg/g to 4.60 μg/g was prepared. The concentration of each type of PEG (from $n = 6$ to $n = 13$) in PVC material was expressed as milligrams of compound per kilogram of simulant solution. The ratio 6:1 (6 dm^2 of material per 1 Kg of food or food simulant) was applied in accordance with the recommendations contained in the legislation for food contact materials EU/11/2011 [1]. Also the amount of each

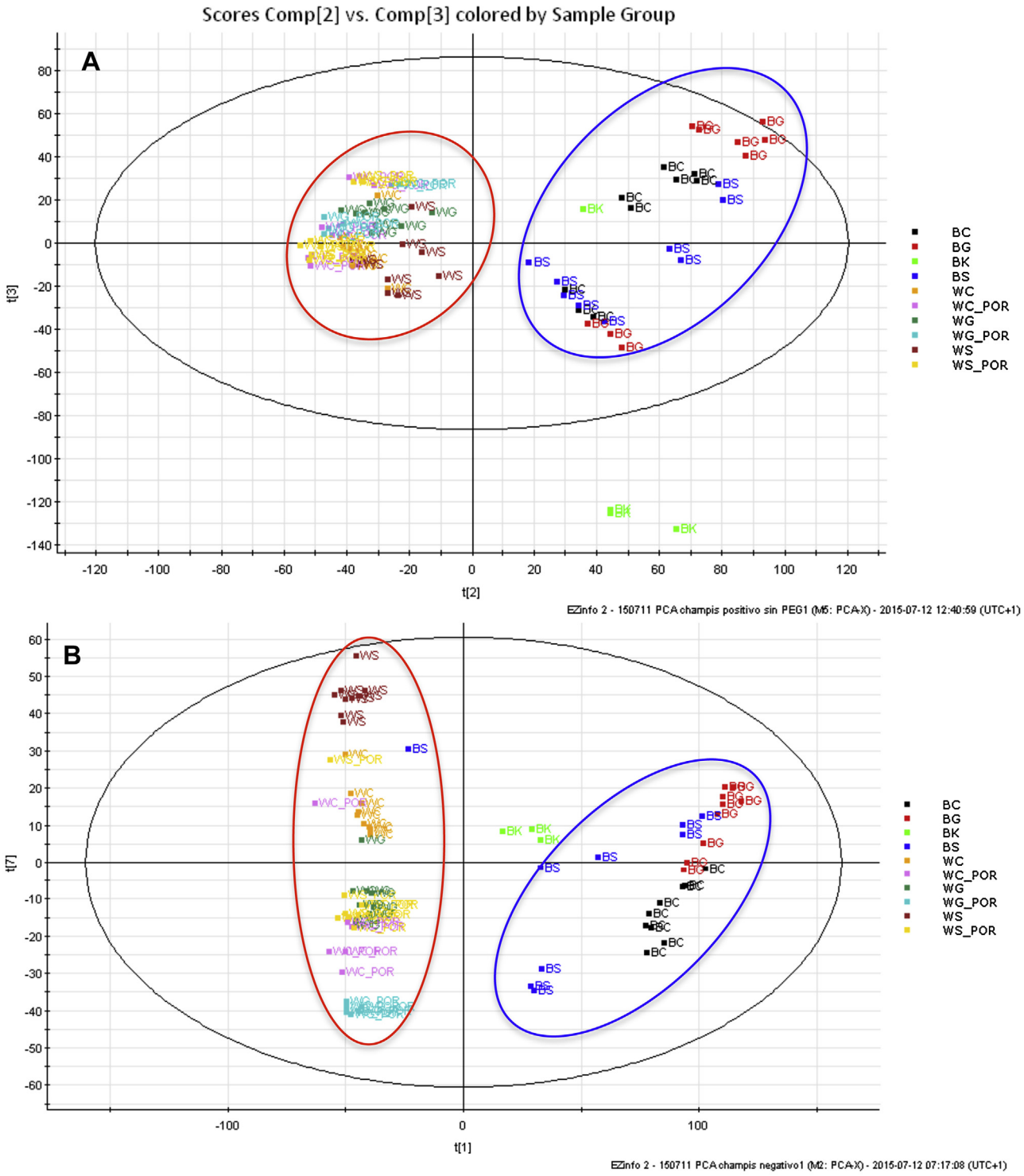

Fig. 3. Scores plot for A—samples acquired in positive mode (component 2 vs. 3); B—samples acquired in negative mode (component 1 vs. 7). Red circle—white samples; Blue circle—oxidised samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

type of PEG (from $n = 6$ to $n = 13$) in sliced mushrooms (cap and gills) was calculated and expressed as milligrams of analyte per kilogram of specific part of mushroom. Moreover, the total concentration of all detected PEGs was calculated and expressed as milligrams of analyte per kilogram of specific part of mushroom in the case of quantification of analyte in mushrooms and as 6 dm^2 of material per 1 Kg of food simulant (10% ethanol) in the case of migration tests. Then, the total standard deviation of sum was calculated by the square root of the sum of the squares of the standard deviations of the numbers used in the calculation of sum [24].

2.8. *UHPLC/Q-TOF/MS^E conditions and analysis*

XEVO^{TM} G2 QTof MS from Waters (Milford, MA, USA) an Acquity UPLC BEH C18 separation column of 2.1 × 100 mm and 1.7 μm particle size from Waters were used. The following UHPLC conditions were applied: flow rate was 0.3 mL min^{-1}, column temperature was

Table 1
Parameters of UHPLC/Q-TOF/MS^E.

Instrument parameters		
Polarity	ES+	ES-
Analyser	Sensitivity Mode	Sensitivity Mode
Capillary (kV)	3.0	2.0
Source temperature (°C)	120	100
Desolvation temperature (°C)	450	250
Cone gas flow (Lh^{-1})	20	0.0
Function 1–TOF PARENT FUNCTION [ACQUSITION]		
Survey start time (min)	0	0
Survey end time (min)	10	10
Survey cone voltage (V)	30	–
Function 2 – Tof parent function [Collision energy]		
MS collision energy low (eV)	15	15
MS collision energy high (eV) [Expression]	30	30
Collision energy ramp start (eV)	15	15
Collision energy ramp end (eV)	30	30

35 °C, and injection volume of sample was 5 μL. The autosampler was maintained at 10 °C. The mobile phase was water with 0.1% formic acid (phase A) and methanol with 0.1% formic acid (phase B). The optimized UPLC elution conditions were initial time—95% A and 5% B; 2 min—95% A and 5% B; 6 min—5% A and 95% B; 8 min—5% A and 95% B; 8.10 min—95% A and 5% B; 10 min—95% A and 5% B. MS detector consisted of an hexapole, a quadrupole, a collition cell and time of flight analyser. All analyses were acquired using a LockSpray™ providing authenticated exact mass measurement, ensuring accuracy and reproducibility. Two modes of electrospray ionization were applied: positive (ESI+) and negative (ESI-). Chromatograms were acquired in the mass range between 50 and 1200 *m*/*z*. Table 1 shows in detail the parameters of detection.

Acquisition of all data and qualitative analysis were carried out using MassLynx software, version 4.1 from Waters.

Reference standard was injected three times into UHPLC/Q-TOF/MS^E at the beginning of the work to check the retention time shift, mass accuracy and reproducibility. Then, the mix sample was injected to check if ranges of analysis were correct and the peaks could be characterized by high chromatographic resolution. Thirdly, the blank sample was injected. Also blank sample was applied after the same group of samples. It needs to be highlighted that filtration using PTFE filters cause extraction of compounds from the filter that appear in the chromatogram. Because of that fact pure methanol was also filtrated using the same type of filter. Comparison of blank sample chromatogram with chromatograms of analysed samples let to assigned peaks belonging to each analysed sample.

Qualitative analysis of compounds was carried out firstly by determination of elemental composition of spectrum obtained from the first function chromatogram and secondly by the molecule mass fragment matching to the spectrum obtained from the second function chromatogram. TOF transform function was applied for spectrums derived from the second function chromatograms (only in case of ESI+ mode). This tool booth the isotopic masses and realigns to a single charge state mass axis. The ChemSpider® and SciFinder® databases, sorted by the number of references, were used to determine the chemical structure of molecules corresponding to their chemical formula. In each database, five of the most referenced molecules were chosen. All qualitative results were compared with the data contained in the literature.

2.9. Optimization of chromatographic conditions

UHPLC conditions were optimized in order to achieve a good chromatographic resolution and sensitivity. First the elution time was set to 10 min. After this time no more elution of analytes was observed. Different values of survey cone voltage and MS collision energy range were optimized. The following survey cone voltages were tested: 10 V, 30 V and 40 V. The best peak resolution was obtained in case of 30 V. Then applying 30 V of survey cone voltage the following collision energy ranges were tested: 15–30 eV and 20– 45 eV. The optimal collision energy ramp was 15–30 eV.

A gradient of two solvents applied as a mobile phase was optimized. Gradient 1 had the following UPLC elution conditions: time—95% A and 5% B; 2 min—95% A and 5% B; 6 min—5% A and 95% B; 8 min—5% A and 95% B; 8.10 min—95% A and 5% B; 10 min—95% A and 5% B. While, gradient 2 had the following UPLC elution conditions: time—100% A and 0% B; 2 min—100% A and 0% B; 6 min—5% A and 95% B; 8 min—5% A and 95% B; 8.10 min—100% A and 0% B; 10 min—100% A and 0%. The best peak resolution was obtained in the case of gradient 1.

2.10. MarkerLynx XS® analysis

The collected mass data was imported to MarkerLynx XS® from Waters (Milford, MA, USA) within the MassLynx software. Three replicates of each sample were injected three times ($n = 9$) to ensure sufficient statistical information. The injected samples were randomized in such a way that the same sample was not injected three times in a raw. MarkerLynx® is a powerful software allowing conduction of principal component analysis (PCA) on the good quality data. The data were statistically analysed in the built-in XS platform using EZinfo® software.

MarkerLynx® method parameters were optimised. The peak detection was chosen as analysis type. Low mass was set to 50 and high mass was set to 800 for positive ionization mode and 1200 for negative mode. The tolerance of mass values was 0.02 Da. The collection parameters were adjusted to decrease the number of markers detected. Intensity threshold was set at 3000 counts, mass window was 0.05 Da and retention time window was 0.10 min.

In case of positive ionization mode a mass exclusion list was created and imported for compounds considered as migrants.

The variables were scaling using pareto type. The number of components was automatic.

The trend view of the markers in all samples was used to determine the presence of specific markers and to estimate their amount. Also loading plots and bi-plot were analysed and compared with the trend view to determine the oxidation markers.

3. Results and discussion

3.1. Spectral analysis and migration tests

In the case of positive electrospray ionization various peaks (retention times: 4.06; 6.80; 5.45; 6.64; 6.77; 7.87; 7.98; 8.52) placed at the end of chromatogram were qualified as filter origin peaks and were not taken into account. While in the case of negative electrospray ionization peak at 4.06 min was qualified as filter origin peak and was not taken into account.

Fig. 2A shows the obtained chromatogram of the white cap using positive ionization mode. Table 2 lists the identified compounds using ESI+. The analysis of data from Table 3, that presents a chemical composition of each sample by ESI+, emphasizes that different analytes were determined in each part of mushroom. This is true for both fresh white mushrooms and also oxidized, brown mushrooms. The following abbreviations were applied in Tables 2 and 5:

Table 2
Characterisation of determined compounds in mushrooms using ES+ mode.

Retention time[min]	Molecular formula	Name	CAS	*m/z*	Adduct	Fragments
0.55	C2H4 N6 O	N-2H-tetrazol-5-yl-urea	0006973-21-3	151.0349	¬+(+23Na)	110.0085; 86.9922; 84.0794; 82.0141; 68.9826; 66.0220
0.57	C5H12 N2 O2	Ornithine	000070-26-8	133.0958	¬+(+1H)	116.0546; 110.0083
0.66	C7H14 N2 O3	Acetylornithine	006205-08-9	104.0697	¬+(+1H)	116.0726; 95.0591; 87.0424
0.69	C5H11 N2 O3	Glutamine	000056-85-9	147.0762	¬+(+1H)	130.0504; 116.0698; 113.0661; 102.0556; 88.0394; 84.0442
0.70	C5H9 N O4	Glutamic acid isomer 1	000617-65-2	148.0606	¬+(+1H)	258.1098; 184.0773; 130.0497; 124.9999; 104.1069; 102.0551; 88.0388; 84.0445; 74.0241
0.71	C5H9 N O4	Glutamic acid isomer 2	000617-65-2	148.0606	¬+(+1H)	258.1098; 184.0773; 130.0497; 124.9999; 104.1069; 102.0551; 88.0388; 84.0445; 74.0241
0.75	C5H9 N O2	Proline isomer 1	000344-25-2 (R)000147-85-3 (S)	116.0707	¬+(+1H)	236.1481; 162.0753; 148.0602; 130.0496; 126.0906; 124.0393; 98.0602; 84.0442; 70.0651
0.80	C5H9 N O2	Proline isomer 2	000344-25-2 (R)000147-85-3 (S)	116.0707	¬+(+1H)	236.1481; 162.0753; 148.0602; 130.0496; 126.0906; 124.0393; 98.0602; 84.0442; 70.0651
0.90	C8H8 N2 O7	Glycol aldehyde	000141-46-8	98.9838	¬+(+39 K)	196.9595; 60.9369
1.32	C6H13 N O2	Leucine isomer 1	000061-90-5	132.1017	¬+(+1H)	86.0963
1.55	C6H13 N O2	Leucine isomer 2	000061-90-5	132.1017	¬+(+1H)	86.0963
1.63	C5H7 N O3	Pyroglutamic acid	000098-79-3	239.1024	¬+(+1H)	130.0503; 113.0340; 110.0598
1.85	C10H13 N5 O5	Guanosine	000118-00-3	284.0989	¬+(+1H)	152.0555; 137.0446; 135.0295; 110.0363; 85.0278
1.88	C7H18 N4	Diethylaminoethyl guanidine	068758-73-6	181.1429	¬+(+23Na)	152.0574; 112.0952; 84.0866; 70.0659
2.05	C9H11 N O2	Phenylalanine	000063-91-2	166.0859	¬+(+1H)	204.0325; 166.0829; 131.0480; 120.0799; 118.0633; 107.0489; 103.0532; 93.0690; 91.0533; 77.0380
2.18	C7H6 O3	Protocatechuic acid	000539-47-9	155.0649	¬+(+1H)	141.0655; 137. 0455; 89.9854; 90.0218
2.25	C10H11 N O3	Citrulline	002637-76-4	194.0804	¬+(+23Na)	176.0707; 158.1340; 115.0124
2.32	C6H11 N O2	Pipecolic acid	003105-95-1	130.1584	¬+(+1H)	105.1135; 91.1655; 87.1635; 83.9994; 72.7090
2.38	C9H17 N O5	Pantothenic acid	000079-83-4	220.1176	¬+(+1H)	233.0592; 202.0966; 156.0527; 123.0211; 111.5275; 98.0228
2.47	C11H12 N2 O2	Tryptophan	000073-22-3	205.0971	¬+(+1H)	188.0702; 170.0607; 159.0914; 146.0595; 142.0655; 132.0806; 118.0647; 115.0538
2.57	C11H15 N5 O3 S	Thiomethyladenosine	002457-80-9	298.0918	¬+(+1H)	146.0634; 136.0618
2.83	C5H12 N4 O3	Canavanine	000543-28-4	215.1494	¬+(+39 K)	177.2315; 118.0012; 86.0245; 76.1203; 72.0798
2.88	C12H26 O7	Hexaethylene glycol	002615-15-8	305.1571	¬+(+23Na)	321.1236; 149.0293; 136.0710; 133.0852; 125.9870; 117.0369; 89.0574; 84.9601; 66.0209
3.11	C14H30 O8	Heptaethylene glycol	005617-32-3	349.2012	¬+(+23Na)	161.0637; 133.0856; 89.0600
3.30	C16H34 O9	Octaethylene glycol	005117-19-1	393.2091	¬+(+23Na)	409.1707; 89.0597
3.46	C18H38 O10	Nonaethylene glycol	003386-18-3	437.2362	¬+(+23Na)	453.2069; 415.2688; 89.0605
3.61	C20H42 O11	Decaethylene glycol	005579- 66-8	481.2613	¬+(+23Na)	497.2366; 249.1109; 89.0602
3.74	C22H46 O12	Undecaethylene glycol	not available	525.2891	¬+(+23Na)	525.2881; 133.0851; 89.0604; 84.9599
3.85	C24H50 O13	Dodecaethylene glycol	006790-09-6	569.3163	¬+(+23Na)	585.2825; 475.3248
3.96	C26H54 O 14	Tridecaethylene glycol	not available	613.3417	¬+(+23Na)	591.3622; 315.2710; 133.0701
6.60	C29H45 N5 O8	Leu-tyr-pro-leu-ser (uknown order)	not available	591.8327	¬+(+1H)	535.2832; 284.2241; 256.2264; 86.0963
6.86	C21H42 N O7 P	Lysophosphatidylethanolamine 16:1	not available	452.2778	¬+(+1H)	434.2655; 429.1704; 104.1074
6.91	C22H40 N O8 P	Phosphatidylethanolamine 34:3 isomer 1	not available	478.2939	¬+(+1H)	714.3302; 520.3412; 460.4952; 337.2731; 187.0729;
6.98	C22H40 N O8 P	Phosphatidylethanolamine 34:3 isomer 2	not available	478.2939	¬+(+1H)	714.3302; 520.3412; 460.4952; 337.2731; 187.0729;
7.02	C22H40 N O8 P	Phosphatidylethanolamine 34:3 isomer 3	not available	478.2939	¬+(+1H)	714.3302; 520.3412; 460.4952; 337.2731; 187.0729;
7.09	C21H44 N O7 P	Lysophosphatidylethanolamine 16:0	not available	454.2936	¬+(+1H)	599.5043; 313.2737; 177.1128; 133.0862; 89.0593
7.19	C16H18 O9	Chlorogenic acid	000327-97-9	355.0167	¬+(+1H)	191.2341
7.79	C17H34 O2	Methyl palmitate	000112-39-0	269.2468	¬+(+1H)	154.1004; 95.0886; 81.0693
7.99	C19H34 O2	Methyl linolenate isomer 1	000112-63-0	295.2645	¬+(+1H)	184.0736; 167.1086; 133.0873
8.07	C19H34 O2	Methyl linolenate isomer 2	000112-63-0	295.2645	¬+(+1H)	184.0736; 167.1086; 133.0873

Table 3
List of determined compounds in the samples using ES positive mode.

Retentiontime	Name	Sample								
		WC	WS	WG	BC	BS	BG	WC_POR	WS_POR	WG_POR
0.55	N-2H-tetrazol-5-yl-urea	+	–	+	–	–	–	+	+	+
0.57	Ornithine	+	+	+	–	–	–	+	+	+
0.66	Aacetylornithine	+	+	+	–	–	–	+	+	+
0.69	Glutamine	+	+	+	–	–	–	+	+	+
0.70	Glutamic acid isomer 1	+	+	+	+	+	+	+	+	+
0.71	Glutamic acid isomer 2	+	+	+	+	+	+	+	+	+
0.75	Proline isomer 1	+	+	+	+	+	+	+	+	+
0.80	Proline isomer 2	+	+	+	–	–	–	+	+	+
0.90	Glycol aldehyde	–	–	–	+	+	+	–	–	–
1.32	Leucine isomer 1	–	–	–	–	–	–	+	+	+
1.55	Leucine isomer 2	+	+	+	+	+	+	+	+	+
1.63	Pyroglutamic acid	+	+	+	–	–	–	+	+	+
1.85	Guanosine	+	+	+	–	–	–	–	–	–
1.88	Diethylaminoethyl guanidine	+	–	–	–	–	–	–	–	–
2.05	Phenylalanine	+	+	+	+	+	+	+	+	+
2.18	Protocatechuic acid	–	–	–	–	–	+	–	–	–
2.25	Citrulline	–	–	+	–	–	–	–	–	+
2.32	Pipecolic acid	–	–	–	+	+	+	–	–	–
2.38	Pantothenic acid	+	+	+	–	–	–	+	+	+
2.47	Tryptophan	+	+	+	+	+	+	+	+	+
2.57	Thiomethyladenosine	+	+	+	+	+	+	+	+	+
2.83	Canavanine	+	–	–	–	–	–	–	–	–
2.88	Hexaethylene glycol	+	–	+	–	–	–	+	–	+
3.11	Heptaethylene glycol	+	–	+	–	–	–	+	–	+
3.30	Octaethylene glycol	+	–	+	–	–	–	+	–	+
3.46	Nonaethylene glycol	+	–	+	–	–	–	+	–	+
3.61	Decaethylene glycol	+	–	+	–	–	–	+	–	+
3.74	Undecaethylene glycol	+	–	+	–	–	–	+	–	+
3.85	Dodecaethylene glycol	+	–	+	–	–	–	+	–	+
3.96	Tridecaethylene glycol	+	–	+	–	–	–	+	–	+
6.60	Leu-tyr-pro-leu-ser (uknown order)	–	–	–	+	+	+	–	–	–
6.86	Lysophosphatidylethanolamine 16:1	–	–	–	+	+	+	+	–	–
6.91	Phosphatidylethanolamine 34:3 isomer 1	+	+	+	+	–	+	+	+	+
6.98	Phosphatidylethanolamine 34:3 isomer 2	+	+	+	+	+	+	+	+	+
7.02	Phosphatidylethanolamine 34:3 isomer 3	+	+	+	+	–	+	+	+	+
7.09	Lysophosphatidylethanolamine 16:0	+	+	+	+	–	+	+	+	+
7.19	Chlorogenic acid	+	+	+	+	–	+	+	+	+
7.79	Methyl palmitate	–	–	–	–	–	+	–	–	–
7.99	Methyl linolenate isomer 1	+	+	+	+	+	+	–	–	+
8.07	Methyl linolenate isomer 2	+	+	+	+	+	+	–	–	+

white cap (WC), white stipe (WS), white gills (WG), brown cap (BC), brown stipe (BS), brown gills (BG). Moreover, the samples acquired in Portugal contain íPORí in their name.

Fig. 2B shows the obtained chromatogram of white cap using negative ionization mode. Again the analysis of data from Table 4, that presents a chemical composition of each sample, highlights that different analytes were determined in each part of mushroom. Moreover there are common peaks with those obtained in positive mode. Substances such as: glutamine, pyroglutamic acid, phenylalanine, protocatechuic acid, pantothenic acid, tryptophan, three isomers of phosphatidylethanolamine 34:3 and lysophosphatidylethanolamine 16:0 were detected in both modes. Table 5 shows the chemical composition of each sample injected using ES-.

The potential good oxidation markers could be those compounds that are either only present in the oxidised parts of mushrooms or compounds that disappear (or decrease) during the oxidation process. The main group of functionally essential compounds found in analysed mushrooms were amino acids. As was above mentioned, other authors studied the composition of free amino acids in mushrooms. However, the purpose of this research is to identify the best oxidation markers and in this context some amino acids showed clear differences before and after the oxidation process. When analysing the results from positive mode, ornithine and glutamic acid that take part in urea formation [25] and glutamine and acetylornithine, that play a role in nitrogen metabolism [26] were found in all parts of fresh mushrooms both from Spain and Portugal. Samples from Portugal had higher amount of ornithine, especially in caps and stipes while the highest amount of glutamine and glutamic acid were found in gills and caps of Spanish mushrooms, respectively. Other compounds that are also present in urea pathway are proline and citrulline. Proline isomer 1 was found in all samples but the oxidised samples contained very small amount of this amino acid, what makes it good oxidation marker. Moreover, proline isomer 2 was found only in white samples. Citrulline was detected only in gills of white mushrooms. Spanish mushrooms had more citrulline than the Portuguese ones. Valine was only detected in caps and stipes of mushrooms from Portugal, probably because the concentration of this amino acid in the Spanish ones was too low. Then pyroglutamic acid, also related to nitrogen formation, was found in the oxidised mushrooms.

Mushrooms are able to produce phenylalanine by shikimic acid pathway [27]. Phenylalanine was found in all samples and its amount was drastically decreased during oxidation. Then leucine was detected before and after oxidation in all samples. Both isomers of leucine were detected only in Portuguese mushrooms while tryptophan was present in all samples. Canavanine was detected only in white caps of Spanish mushrooms. N-2H-tetrazol-5-yl-urea was found in the white parts such as caps and gills of Spanish mushrooms and in all white parts of Portuguese mushrooms. In turn, n-[(4-methoxy-3-nitrophenyl) sulfonyl]-β-alanine disappeared after oxidation. Two peaks corresponding to amino

Table 4
Characterisation of determined compounds in mushrooms using ES- mode.

Retention time [min]	Molecular formula	Name	CAS	*m*/*z*	Fragments
0.67	C 15H10 O2	Isoflavone	000574-12-9	221.0558	131.0435; 104.0362
0.68	C4H8 N2 O3	Asparagine	000070-47-3	131.0461	118.0524; 104.0346; 113.0368; 70.0317; 72.0106; 74.0231
0.69	C5H11 N2 O3	Glutamine	000056-85-9	145.0620	131.0472; 104.0363
0.73	C5H15 N2 O3 P	1,5-diaminopentyl) phosphonic acid isomer 1	018865-33-3	181.0716	314.1069 146.0452; 133.0370; 128.0354;
0.77	C5H15 N2 O3 P	(1,5-diaminopentyl) phosphonic acid isomer 2	018865-33-3	181.0716	314.1069 146.0452; 133.0370; 128.0354;
0.82	C5H15 N2 O3 P	(1,5-diaminopentyl) phosphonic acid isomer 3	018865-33-3	195.0504	314.1069 146.0452; 133.0370; 128.0354;
0.97	C4H6 O5	Malic acid	006915-17-7	133.0141	292.9236; 194.9467; 114.9968; 99.0044; 96.9691; 89.0229; 85.5915; 78.9595; 72.9913
1.53	C5H7 N O3	Pyroglutamic acid isomer 1	000098-79-3	128.0347	96.9649; 91.0022; 89.0219; 86.0230; 84.0443; 82.0322; 78.9594; 73.0279; 71.0106
1.64	C4H6 O4	Succinic acid	000110-15-6	117.0192	73.0334
1.64	C5H7 N O3	Pyroglutamic acid isomer 2	000098-79-3	237.0880	128.0341; 96.9649; 91.0022; 89.0219; 86.0230; 84.0443; 82.0322; 78.9594; 73.0279; 71.0106
2.05	C9H11 N O2	Phenylalanine	000063-91-2	164.0710	147.0454; 103.0559; 72.0086
2.38	C9H17 N O5	Pantothenic acid	000079-83-4	218.1032	146.0816; 88.0460; 71.0506
2.44	C11H12 N2 O2	Tryptophan	000073-22-3	203.0822	186.0524; 168.0015; 161.0788; 159.0899; 142.0664; 116.0508; 74.0248
2.63	C14H17 N5 O8	Succinyladenosine	004542-23-8	382.0979	266.1084; 207.0692; 206.0736; 188.0625; 134.0477; 115.0054; 113.0238
3.00	C7H6 O3	Protocatechuic acid	000539-47-9	153.0193	137.0234; 94.0382; 93.0342
3.68	C10H13 N O4 S	n-[(4-methoxy-3-nitrophenyl) sulfonyl]-β-alanine	042908-33-8	303.0741	606. 0613; 402.9947; 384.9808; 362.0034; 323.0206; 282.0452
4.11	C12H24 N2 O3	Leucyl-isoleucine	036077-41-5	243.1234	265.1004; 263.0636; 247.0975; 243.1416; 181.1176; 153.0915; 71.0156; 61.9884
4.55	C5H10 N O2	Valine	000072-18-4	116.9068	–
4.70	C8H10 N6 O2	3-(adenin-9-yl)-(RS)-alanine	023513-76-0	221.0794	209.4297; 208.9427; 177.0846; 155.1073
5.53	C11H20 N2 O5	α-glutamylleucine	111320-19-5	259.9536	213.1191; 170.1030; 167.0969; 165.0884; 165.0884; 155.0995; 148.0282; 144.0485; 138.0620; 125.0642; 120.0461; 113.0252
5.97	C13H12 O9	Caftaric acid	067879-58-7	311.2225	179.0119; 135.2435;
6.61	–	Not identified	–	1142.6923	–
6.78	C30H60 O2	Tetradecyl palmitate isomer 1	004536-26-9	451.2404	391.3879; 253.2167; 139.1109; 57.0352
6.86	C30H60 O2	Tetradecyl palmitate isomer 2	004536-26-9	451.2404	391.3879; 253.2167; 139.1109; 57.0352
6.90	C22H40 N O8 P	Phosphatidylethanolamine 34:3 isomer 1	not available	476.2775	712.3643; 450.2711; 279.2326; 253.2114
6.97	C22H40 N O8 P	Phosphatidylethanolamine 34:3 isomer 2	not available	476.2775	712.3643; 450.2711; 279.2326; 253.2114
7.01	C22H40 N O8 P	Phosphatidylethanolamine 34:3 isomer 3	not available	476.2775	712.3643; 450.2711; 279.2326; 253.2114
7.09	C21H44 N O7 P	Lysophosphatidylethanolamine 16:0	not available	452.2782	255.2336; 196.0388
7.15	C23H46 N O7 P	Lysophosphatidylethanolamine 18:1	not available	478.2930	281.2483; 196.0366;
7.23	C16H32 O3	16-hydroxypalmitic acid	000506-13-8	271.2270	253.2167; 225.2251; 130.9490
8.13	C25H35 N9 O6	His-his-lys-thr (uknown order)	not available	520.2657	433.2343; 409.3055; 255.2321; 152.9954

Table 5
List of determined compounds in the samples using ES negative mode.

Retentiontime	Name	Sample								
		WC	WS	WG	BC	BS	BG	WC_POR	WS_POR	WG_POR
0.67	Isoflavone	–	–	–	–	–	–	–	–	+
0.68	Asparagine	+	+	–	–	+	–	–	+	–
0.69	Glutamine	–	–	+	–	–	+	–	–	+
0.73	(1,5-diaminopentyl) phosphonic acid isomer 1	+	+	+	+	+	+	+	+	+
0.77	(1,5-diaminopentyl) phosphonic acid isomer 2	+	+	+	+	+	+	+	+	+
0.82	(1,5-diaminopentyl) phosphonic acid isomer 3	–	–	–	–	–	–	–	+	+
0.97	Malic acid	+	+	+	+	+	+	+	+	+
1.53	Pyroglutamic acid isomer 1	+	+	+	–	–	–	+	+	+
1.64	Succinic acid	+	+	–	+	+	+	+	+	+
1.64	Pyroglutamic acid isomer 2	–	–	+	–	–	–	–	–	–
2.05	Phenylalanine	+	+	+	–	–	–	+	+	+
2.38	Pantothenic acid	+	+	+	+	–	–	+	+	+
2.44	Tryptophan	+	+	+	+	–	–	+	+	+
2.63	Succinyladenosine	–	–	–	–	–	–	+	–	+
3.00	Protocatechuic acid	–	–	–	–	–	–	–	+	+
3.68	n-[(4-Methoxy-3-nitrophenyl) sulfonyl]-β-alanine	+	+	+	–	–	–	+	+	+
4.11	Leucyl-isoleucine	–	–	–	+	+	–	–	–	+
4.55	Valine	–	–	–	–	–	–	+	+	–
4.70	3-(denine-9-yl)-(RS)-alanine	–	–	+	+	–	–	–	–	–
5.53	α-glutamylleucine	–	–	–	–	–	–	–	–	+
5.97	Caftaric acid	–	–	–	–	–	–	–	–	+
6.61	Not identified	–	–	–	+	+	+	–	–	–
6.78	Tetradecyl palmitate isomer 1	–	–	–	+	+	+	–	–	–
6.86	Tetradecyl palmitate isomer 2	–	–	–	+	+	+	–	+	–
6.90	Phosphatidylethanolamine 34:3 isomer 1	+	+	+	+	+	+	+	+	+
6.97	Phosphatidylethanolamine 34:3 isomer 2	+	+	+	+	+	+	+	+	+
7.01	Phosphatidylethanolamine 34:3 isomer 3	+	+	+	+	+	+	+	+	+
7.09	Lysophosphatidylethanolamine 16:0	+	+	+	+	+	+	+	+	+
7.15	Lysophosphatidylethanolamine 18:1	–	–	–	+	+	+	–	–	–
7.23	16-hydroxypalmitic acid	–	–	–	–	–	–	+	+	–
8.13	His-his-lys-thr (uknown order)	–	–	+	+	+	–	–	–	–

acid chains such as leu-tyr-pro-leu-ser and his-his-lys-thr were detected only in oxidised samples.

The second big group of identified compounds were phosphatidylethanolamines (PE) that are a class of phospholipids found in biological membranes [28]. Lysophosphatidylethanolamine 16:1 and lysophosphatidylethanolamine 18:1 were found only in oxidised samples. Then the three isomers of phosphatidylethanolamine 34:3 and lysophosphatidylethanolamine 16:0 were present in all samples.

Moreover, 16-hydroxypalmitic acid and palmitic acid esters were detected in the analysed samples. Two isomers of tetradecyl palmitate were identified in all oxidised parts of mushrooms.

Compounds such as protocatechuic acid was found only in brown gills and caftaric acid was found in white gills of Portuguese mushrooms as well as isoflavone. Whereas, pipecolic acid was only detected in oxidised mushrooms. Panthotenic acid was not only present in oxidised samples. Nucleoside guanosine was found only in white parts of Spanish mushrooms and diethylaminoethyl guanidine was identified only in white caps of Spanish mushrooms. Succinyladenosine was found in white caps and gills of Portuguese mushrooms, whereas 3-(adenin-9-yl)-(RS)-alanine was detected in white gills and brown caps of Spanish mushrooms. Chlorogenic and succinic acids were present in all samples with the exception of brown stipes and two isomers of linoleic acid methyl ester were found also in all samples but with exception of caps and stipes of Portuguese mushrooms. Two isomers of (1,5-diaminopentyl)phosphonic acid, malic acid and thiomethyladenosine were found in all samples. A good oxidation marker that was detected in oxidised mushrooms can be glycolaldehyde. It is a sugar related molecule formed during oxidative degradation processes [29]. Only one compound characterised by mass 1142.6893 m/z could not be identified. Unfortunately it did not have any fragmentation in the second function. Finally it can be concluded that oxidation markers for both Spanish and Portuguese white mushrooms were: 2H-tetrazol-5-yl-urea, ornithine, acetylornithine, glutamine, proline isomers 1 and 2, leucine isomer 2, pyroglutamic acid, phenylalanine, pantothenic acid and n-[(4-methoxy-3-nitrophenyl) sulfonyl]-β-alanine. The marker present only in white Spanish mushrooms was guanosine. Markers of oxidised mushrooms were glycol aldehyde, pipecolic acid, lysophosphatidylethanolamine 16:1, leucyl-isoleucine, lysophosphatidylethanolamine 18:1, his-his-lys-thr, and two isomers of tetradecyl palmitate. Obviously, loading plots also showed markers of Portuguese mushrooms, distinguishing these samples from the Spanish ones. However, these results are not shown, as they are out of scope of this work.

One unexpected result was obtained in the range of retention times: 2.88–3.96 min using positive electrospray ionization. A characteristic set of peaks appearing with an interval of 44 was observed. The peaks belong to polyethylene glycols, which molecular mass is $C_{2n+2}H_{4n+6}O_{n+2}$. Eight ethylene glycols were determined with íní from the range 6–13. It was determined that polyethylene glycols belonged to a mixture with average mass 400 (PEG400). PVC could be plasticized with polyethylene glycol by solution mixing. PEG enriching PVC matrix changes its physical properties [30].

Polyethylene glycols were only qualified in samples of fresh caps and gills of both types of mushrooms from Spain and Portugal. When samples were oxidized PEGs were no longer present. Analysis of oxidized mushrooms took place only in case of Spanish samples. Different kind of mushrooms (sliced, whole and whole sylvan) were analysed and migration test of several types of materials were performed to investigate the origin of PEGs migration. It was confirmed that PEGs were present only in cap and gills of sliced mushrooms. Analysis of different parts of whole and whole sylvan mushrooms did not show the presence of polyethylene glycols. Table 6 shows the quantitative results of cap (WC) and gills (WG) and also the results of migration tests carried out for the lid

Table 6
Results of quantitative analysis of PEG and analytical features of UHPLC/Q-TOF/MS^E method.

Compound	Average concentration			RSD [%]			Linear range[c] [mg/Kg]		R^2	LOD[c] [mg/Kg]	LOQ[d] [mg/Kg]
	WC[a] [mg/Kg]	WG[a] [mg/Kg]	Ratio 6:1[b] [mg/Kg]	WC	WG	Ratio 6:1	Min	Max			
Hexaethylene glycol	15.03 ± 2.66	9.52 ± 1.21	0.73 ± 0.20	18%	13%	27%	0.08	4.60	0.997	0.024	0.080
Heptaethylene glycol	7.00 ± 0.90	12.30 ± 1.81	1.31 ± 0.34	13%	15%	26%	0.08	4.60	0.993	0.016	0.053
Octaethylene glycol	5.51 ± 0.53	11.98 ± 0.51	1.60 ± 0.28	10%	4%	18%	0.08	4.60	0.997	0.015	0.049
Nonaethylene glycol	4.97 ± 0.43	12.07 ± 3.41	1.60 ± 0.36	9%	28%	23%	0.08	4.60	0.993	0.023	0.077
Decaethylene glycol	4.46 ± 1.18	11.99 ± 1.97	1.22 ± 0.27	26%	16%	22%	0.08	4.60	0.994	0.022	0.074
Undecaethylene glycol	3.14 ± 0.64	14.27 ± 1.34	0.66 ± 0.15	20%	9%	23%	0.08	4.60	0.995	0.018	0.060
Dodecaethylene glycol	4.63 ± 1.03	9.44 ± 0.74	0.26 ± 0.07	22%	8%	28%	0.08	4.60	0.995	0.024	0.079
Total concentration[mg/Kg]	49.43 ± 3.39	88.32 ± 4.95	7.47 ± 0.68[c]								

[a] Results expressed as mg of analyte per Kg of mushrooms.
[b] Results expressed as mg of analyte per Kg of food simulant (10% ethanol).
[c] Results expressed as mg of analyte per Kg of food simulant (10% ethanol).
[d] Results expressed as mg of analyte per Kg of solvent.

film (Ratio 6:1) using 10% ethanol as food simulant. The analytical features of the method such as linear range, limits of detection (LOD) and quantification (LOQ) were determined for 8 polyethylene glycols and they are also listed in Table 6. LOD and LOQ were determined using signal-to-noise method. The quantitative results of PEGs in mushrooms showed that the total concentration of PEGs was 49.43 ± 3.39 mg/Kg in the case of white caps and 88.32 ± 4.95 mg/Kg in the case of white gills. When analysing PEGs individually it can be seen that almost always there was a higher content of each PEG in gills compared to caps (with the exception of hexaethylene glycol, $n = 6$). The highest concentration of individual PEG (15.03 ± 2.66 mg/Kg) was found for hexaethylene glycol in the cap and undecaethylene glycol (14.27 ± 1.34 mg/Kg) in gills.

Migration assay confirmed the origin of PEGs. Positive results gave PVC film used for overwrapping mushrooms tray be the responsible for that. The results of migration using 10% ethanol as food simulant showed low level of migration of individual analytes (maximum concentration of nonaethylene glycol 1.60 ± 0.36 mg/Kg). The total migration of PEGs was 7.47 ± 0.68 mg/Kg. All components of plastics used in contact with food must fulfil European legislation. According to the legislation for food contact materials EU/11/2011 [23] polyethylene glycol (CAS 0025322-68-3) is indicated as food additive without specific value of migration limit (SML). Investigation has shown that the whole mushrooms are resistant to migration of any compound coming from the packaging, probably because of the presence of protective skin. RSD% of all replicates of the samples does not exceed 28% both in case of PEGs quantification in parts of mushrooms and migration assay. In the case of analytical features linear concentration of PEGs was obtained in the range 0.08 mg/Kg to 4.60 mg/Kg. All calibration curves showed good linear regression ($r > 0.99$).

The chromatograms obtained from migration assays were also compared to the chromatograms of analysed samples. Fortunately, no more migrants were detected.

3.2. *MarkerLynx XS® analysis*

Principal component analysis is a descriptive analysis that let to perform an overview of data. Here the individual parts of mushrooms are observations and the masses are the variables. The score plot of the PCA displays part of the mushrooms, those near each are similar and those opposite or far away are dissimilar. In these data, the similarities and dissimilarities of these samples are determined by masses of their compounds.

Analysing the results of PCA it can be seen that there are 3 principal components for the data collected in positive ionization mode and 9 principal components for the data collected in negative mode. Each combination of two components was analysed. Fig. 3 shows that each type of observations representing different kind of samples is grouped. First of all the samples were grouped in two main groups of dissimilar observations: fresh white mushrooms (both Spanish and Portuguese) and brown oxidised mushrooms. Then, between theses two main groups, each part of mushrooms was grouped together. The worst aggrupation can be observed in the case of brown gills and brown stipes that are practically divided in two groups that can indicate not homogenous oxidation. Another interesting issue is that the same parts of mushrooms collected in different countries were not near each other. Abnormal values (outliers) were the blank samples (MeOH), as expected. Good aggrupation of different types of samples proved that MarkerLynx® can successfully distinguish the same species of mushroom acquired in different countries. Moreover, fresh and oxidised samples can be easily identified.

4. Conclusion

UHPLC/Q-TOF method allowed us to identify the naturally occurring compounds in mushrooms and to define oxidation markers. A total number of 61 different compounds were determined in sliced mushrooms. Potential oxidation markers were: 2H-tetrazol-5-yl-urea, ornithine, acetylornithine, glutamine, proline isomers 1 and 2, glycol aldehyde, leucine isomer 2, pyroglutamic acid, phenylalanine, guanosine, pipecolic acid, pantothenic acid, n-[(4-Methoxy-3-nitrophenyl) sulfonyl]-β-alanine, leucyl-isoleucine, his-his-lys-thr, two isomers of tetradecyl palmitate. Among phospholipids they could be lysophosphatidylethanolamine 16:1 and lysophosphatidylethanolamine 18:1. Eight compounds found in mushrooms were classified as migrants from PVC film that were overwrapping the mushrooms tray. As a result, migrants were qualified, as polyethylene glycols with 'n' number between six and thirteen. Fresh sliced mushrooms showed susceptibility to migrant compounds more than entire mushrooms. No more presence of polyethylene glycols was detected in the case of totally oxidized samples. Then, MarkerLynx® was applied as a powerful tool to distinguish the same species of mushroom acquired in different countries and also differences between fresh and oxidised samples were found. Thus, it was possible to determine some oxidation markers.

The proposed procedure is an interesting approach for further investigation that could be focused on the demonstration of positive influence of active packaging on extending the shelf-life of fresh sliced mushrooms. Composition of mushrooms depends on many cultivation conditions and deeper studies could be performed to show and compare composition of different samples of mushrooms from different parts of particular countries. Moreover,

investigation of the influence of undesired plastic migrants into melanin biosynthesis pathway in *A. Bisporus* mushrooms could be done.

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